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**Development of transition metal
catalysts for bioorthogonal reactions**

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Jury composition

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*People will believe a lie because they want to believe it is true,
or because they are afraid it might be true.*

Work on the edge of the failure, but never make a step forward.

People stumble on small rocks, never on mountains.

The sky is always blue behind the clouds.

Allons-y pédestrement...

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List of publications

The work of this thesis permitted the publication of two papers:

- [1] *(η^5 -Pentamethylcyclopentadienyl)iridium Complex Catalysed Imine Reductions Utilizing the Biomimetic 1,4-NAD(P)H Cofactor and N-Benzyl-1,4-dihydronicotinamide as the Hydride-Transfer Agent*, M. Soetens, F. Drouet, O. Riant, *ChemCatChem* **2017**, 9, 929.
- [2] *Improving the selectivity to 4-tert-butylresorcinol by adjusting the surface chemistry of heteropolyacid-based alkylation catalysts*, C. Pezzotta, G. Fleury, M. Soetens, S. Van der Perre, J.F.M. Denayer, O. Riant, E.M. Gaigneaux, *J. Catal.* **2018**, 359, 198.

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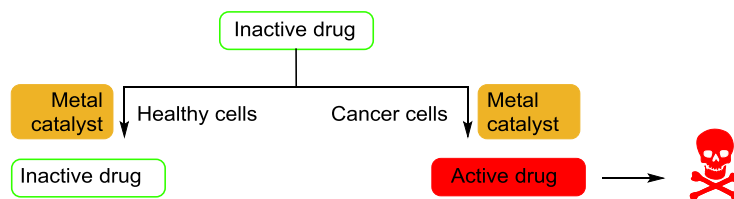
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Abstract

In the context of cancer therapy, where new generations urgently need an improved selectivity, we explored in this work how transition-metal complexes could be used in order to activate prodrugs in cancer cells. It is only a deep understanding of cancer metabolism which enabled to characterize the hallmarks of cancer cells, which can become the key for a selective treatment provided chemists are able to find the right bioorthogonal reaction to make good use of that specific feature of cancer.



In the first part of this work, we were interested in the first part by hydride transfers, as NAD(P)H cofactors are affected by the state of hypoxia of tumours. We developed a transfer hydride reduction of imines using an Ir catalyst and the cellular NADH and a set of fluorogenic probes aiming to get activated within cancer cells.

In the second phase, we kept the same goal in mind, but focused on the allyl carbamates deprotection by palladium complexes in the context of Tsuji-Trost reaction. Several new structures were found, validated *in vitro* and *in cellulo* and applied to the uncaging of a vectorised cytotoxic drug. Finally, the electrophilic properties of the π -allyl palladium complexes were evaluated in the context of targeted covalent inhibitors.

Abbreviation list

[M]	Metal complex
5FU	5-fluorouracile
A2780	Human ovarian carcinoma
A431	Epidermoid carcinoma
A549	Human lung carcinoma
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
Alloc	Allyloxy carbamate
ATP	Adenosine triphosphate
au	Arbitrary units
Boc	Tertbutoxy carbonyl
BODIPY	Boron-dipyrrromethene
BPin	Pinacolato boron
Cat	Catalyst / catalytical
COD	1,5-cyclooctadiene
Cp	Cyclopentadienyl
Cp [*]	Pentamethyl cyclopentadienyl
CTAB	Cetyl trimethylammonium bromide
CuAAC	Copper-catalysed Azide-Alkyne Cycloaddition
Cy	Cyclohexyl
Cy3	Cyanine 3 dye
DAPI	4',6-diamidino-2-phenylindole
DBA	Dibenzylidene acetone
DCM	Dichloromethane

DEAD	Diethyl azadicarboxylate
DIBAL-H	Diisobutylaluminium hydride
DIPEA	Diisopropyl ethyl amine
DMAP	4-dimethylamino pyridine
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic
EGF/EFR-R	Epidermal growth factor (- receptor)
en	Ethylene diamine
EPR	Enhanced permeability and retention
ERK1/2	Extracellular signal-regulated kinases
Fmoc	Fluorenylmethyloxycarbonyl
GC	Gas chromatography
GDH	Glucose dehydrogenase
GFP	Green fluorescent protein
GSH	Glutathione
HATU	1-[Bis(dimethylamino)methylene]-1H ⁻¹ ,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HeLa	Cervix adenocarcinoma
hmb	Hexamethylbenzene
HOMO/LUMO	Highest / Lowest occupied molecular orbital
HPG	Homopropargyl glycine
HPLC	High Performance liquid chromatography
IC ₅₀	Half maximal inhibitory concentration

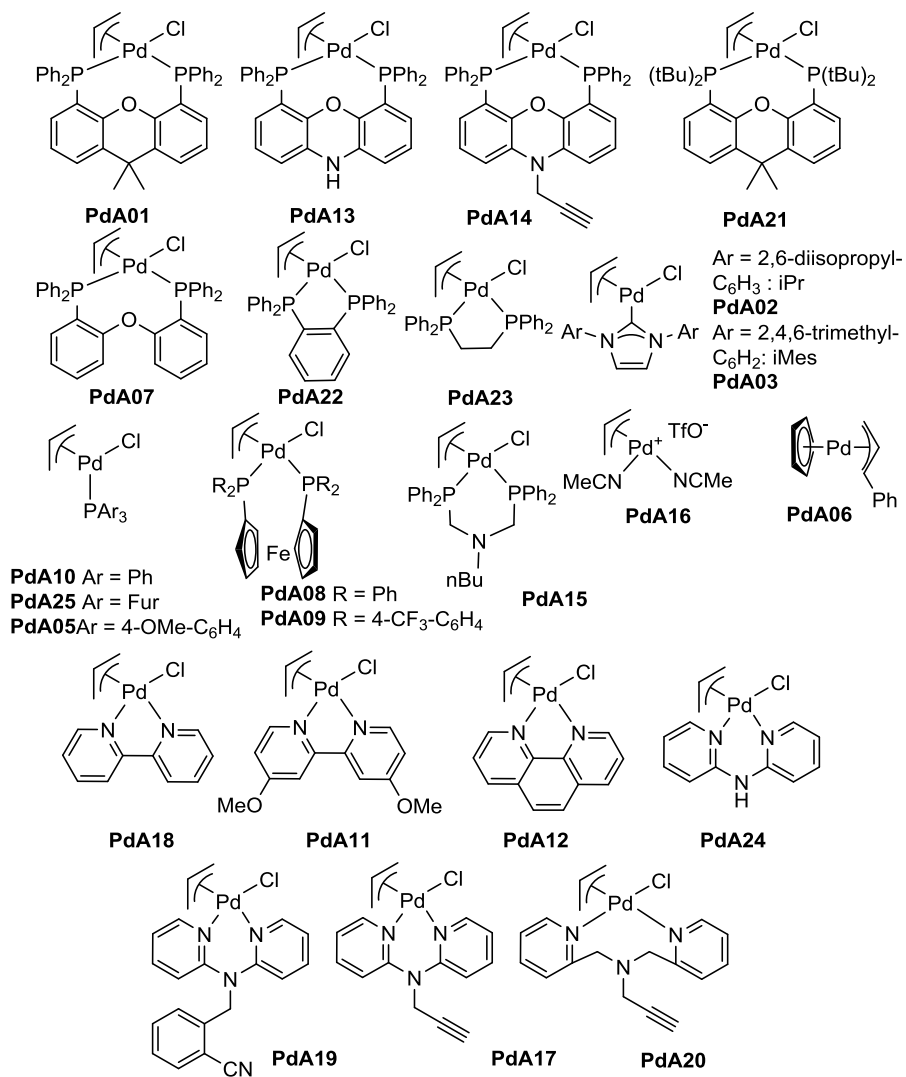
ICP-MS	Inductively coupled plasma mass spectrometry
KB	Subline of HeLa cells
L-BAPA	N α -Benzoyl-L-arginine
	4-nitroanilide hydrochloride
LDH	Lactate dehydrogenase
LG	Leaving group
LOD/LOQ	Limit of detection / quantification
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase
MEM	2-Methoxyethoxymethyl
MMAE	Monomethyl auristatin E
MS	Mass spectrometry
MS-275	Entinostat
Msen	N-Mesyl-ethylenediamine
MTBE	Methyl-tertbutyl ether
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
NaAsc	Sodium ascorbate
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NCS	N-chlorosuccinimide
NHC	N-heterocyclic carbene
NHS	N-hydro succinimide
NMR	Nuclear Magnetic Resonance
Nu	Nucleophile

PBS	Phosphate buffer solution
<i>p</i> -cym	4-isipropyl-1-methylbenzene
PDC	Pyruvate decarboxylase
PEG	Polyethylene glycol
pH, pD	$-\log_{10}[\text{H}^+ \text{ or } \text{D}^+]$
pKa	$-\log_{10}K_a$
PLGA	Poly(lactic-co-glycolic acid)
PNP	4-nitrophenyl
Proc	Propargyloxy carbamate
PVP	Polyvinylpyrrolidone
Py	Pyridine
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SiHa	Uterus carcinoma cells
TBAF	Tetrabutylammonium fluoride
TCI	Targeted covalent inhibitor
TEM	Transmission electron microscopy
TEMPO	(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
Tf	Trifluoromethansulfonyl
TFA	Trifluoroacetic Acid
Tfen	N-triflyl-ethylenediamine
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TMRE	Tetramethyl rhodamine, ethyl ester

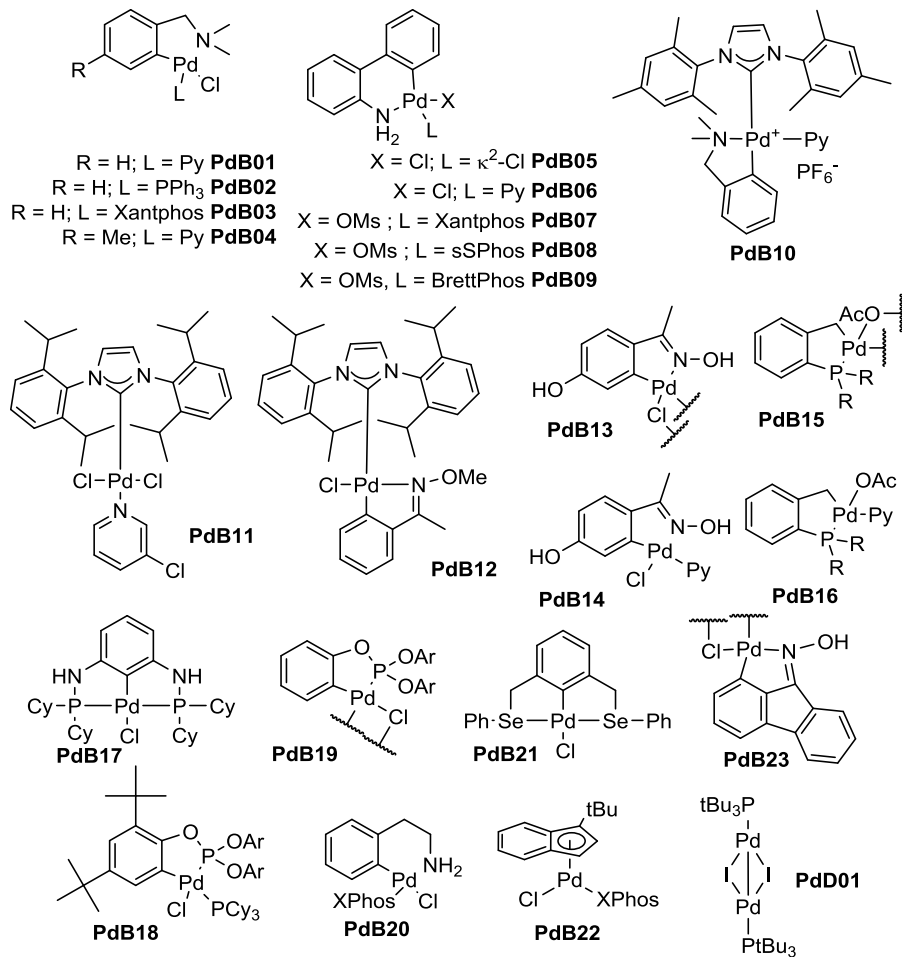
TMS	Trimethylsilyl
TOF	Turnover frequency
TON	Turnover number
TPPTS	3',3''-Phosphanetriyltris(benzenesulfonic acid) trisodium salt
TSA	Thiosalicylic acid
TsDPEN	N-Tosyl-diphenylethylene diamine
Tsen	N-Tosyl-ethylenediamine
U	Enzymatic unit
VEGF/VGF-R	Vascular endothelial growth factor (-receptor)
δ	NMR shift
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength

Complexes and ligands

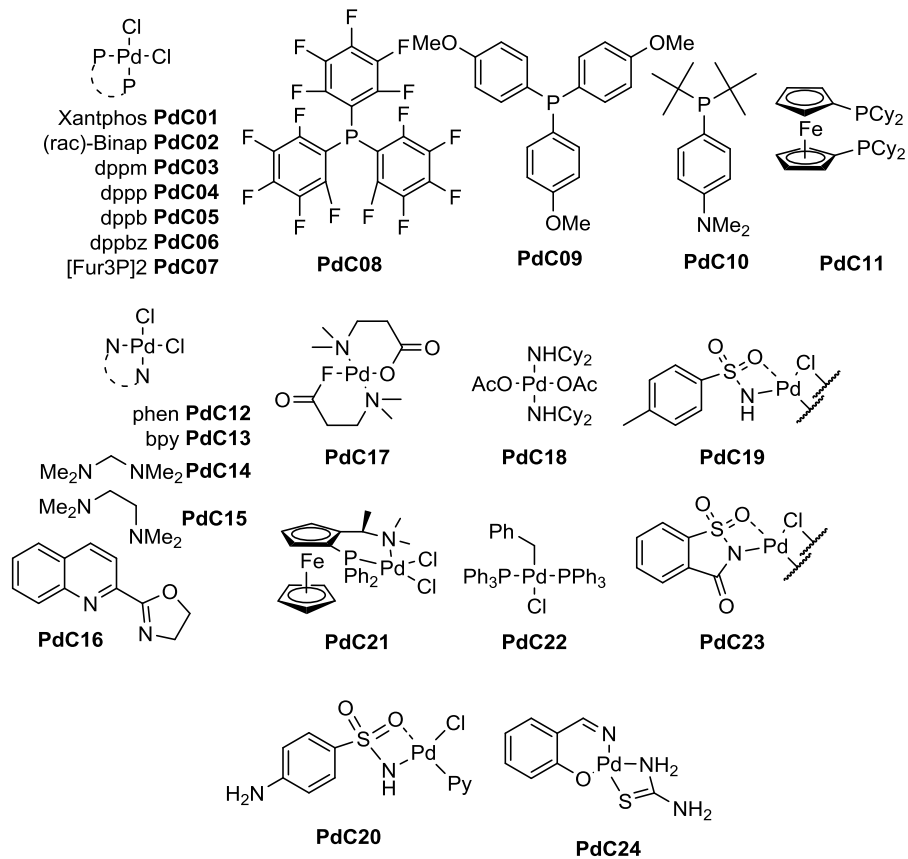
Family PdA: π -allyl palladium

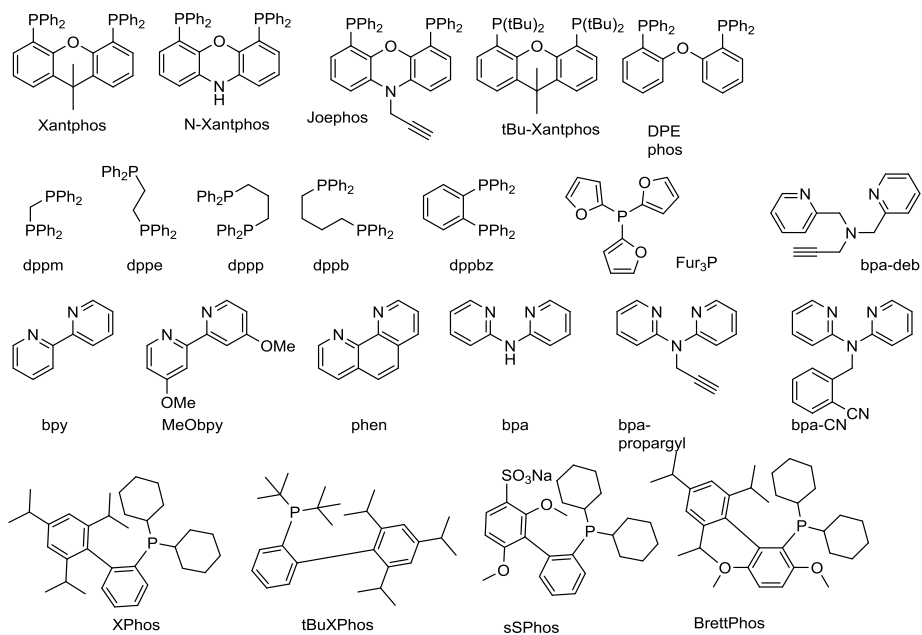


Family **PdB**: Palladacycles



Family **PdC**: Pd halides and others





Introduction

1 General strategy

Cancer is a metabolic disease characterized by immortal cells which develop in a non-controlled and anarchic fashion after undergoing mutations and alterations in their DNA. Those cells tend to grow in masses which are called tumours, which can be benign or malignant depending on their phenotype. During the development of these tumours, the cells start to develop their own phenotype, which starts to become distinct from the healthy cells nearby. The common characteristics of cancer cells have been summarized as the “Hallmarks of Cancer” and draw a clear border between healthy and cancer cells (Figure 1. 1).²

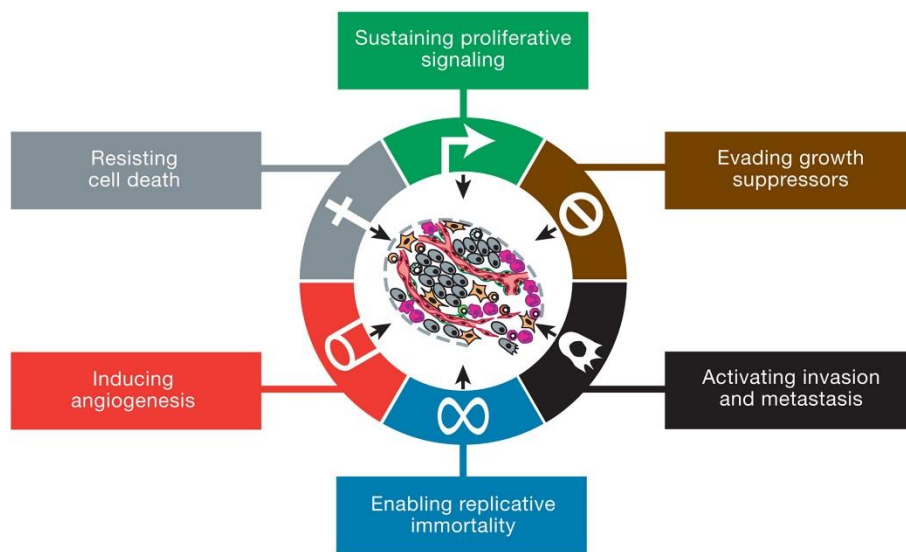


Figure 1. 1 The Hallmarks of cancer, from ²

Curing cancer involves therapies which selectively kill the malignant cells while leaving the healthy cells untouched. Common treatment techniques involve surgery, if a tumour is localized and not embedded in vital tissues, radiotherapy, immunotherapy and chemotherapy. Whereas the first methods are targeted,

chemotherapy's major drawback is the selectivity towards cancer cells. The earliest versions of chemotherapy were responsible for severe side effects such as hair loss, appetite reduction and blood white cells depletion. Indeed, those highly toxic molecules' target was either DNA (*cis*-platinum, Doxorubicin) or microtubules (Taxol) as they are involved in cell division and cancer cells divide faster than healthy cells, exception made for immune system cells, endothelial cells along the digestive tract and hair stem cells (Figure 1. 2). Therefore, the second generation of chemotherapy came to solve the problems of the first generation.

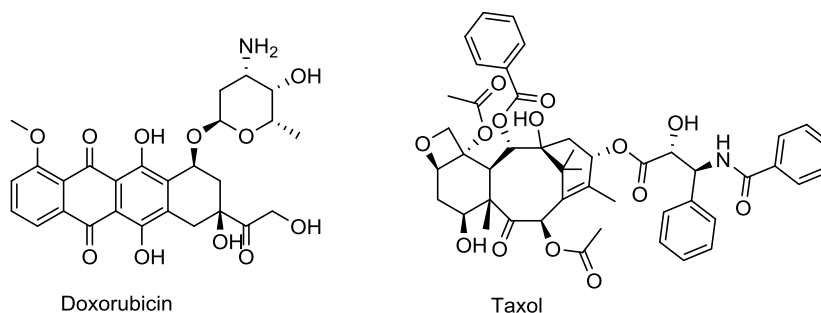


Figure 1. 2 Structure of Doxorubicin and Taxol

The second generation of chemotherapies involves targeting other paths, such as signalling or metabolism of nutrients, which are specifically important to cancer cells or by targeting mutated proteins only found in cancers. Indeed, it is known that cancer cells avidly consume not only glucose but also other metabolites such as amino acids in order to get the required building blocks and energy to support the growth and proliferation. A pertinent example is glutamine, which is consumed by cells when the glucose supply is not sufficient. Inhibitor such as BPTES or CB-835 has been the subject of research in our team as it was shown to be a valuable target for cancer

therapy (Figure 1. 3). Also, due to the anarchic growth, mutations inevitably occur and accumulate within the genome. As a significant example, the compound Vemurafenib (Zelboraf, from Plexxikon) is an FDA-approved drug which recognizes the V600E mutated form of Raf kinase in the context of the treatment against malignant melanoma. Kinases are involved in the proliferation (EGF pathway) or in neovasculature (VEGF pathway) crucial for the tumour development in most cancers.

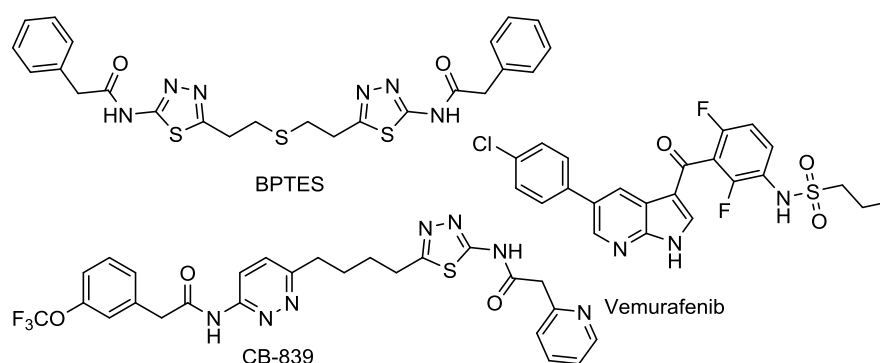
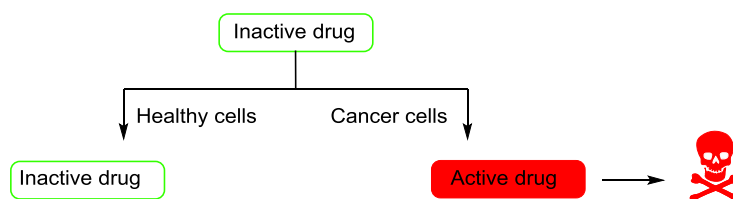


Figure 1. 3 Pertinent examples of second-generation chemotherapy

What will be the third generation of chemotherapy? The keyword associated will be selectivity, as a direct consequence of the drawbacks of the first and second generation (Scheme 1. 1).



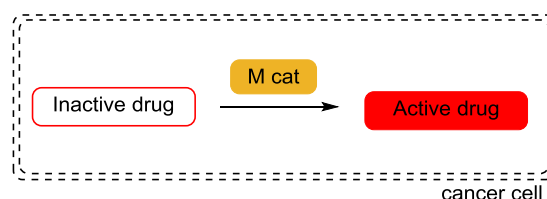
Scheme 1. 1 Concept of targeted chemotherapy

Part of the answer has been already unveiled. Antibody-Drug Conjugates (ADCs) are a perfect example of newly-approved systems bearing a drug (monomethyl auristatin E for instance) bound to an antibody recognizing a cancer-specific surface protein and a linker

which is sensitive towards a specific protein for a space-time-controlled fashion. Born in the early years 2000, immunotherapy aims to use the selectivity of the immune system to selectively recognize cancer cells thanks to highly specific antibodies or small molecules which can reprogram the immune system. However, there are still few FDA-approved examples reported so far. Vectorization has been known for decades but is only recently that significant improvements were seen in the field. This interesting strategy enables to control, thanks to an optimal design of the vector, the transport, localization and the release of the active principle. Commonly used vectors involve nanoparticles, micelles or liposomes for encapsulation. Targeting moieties can be biomolecules such as sugars or vitamins such as folic acid. However, vectorization appears as a chemotherapy adjuvant for the distribution and its full potency has not been exploited so far in therapy.

Our work will aim to use bioorthogonal chemistry and vectorization as new tools to control the space-time distribution and activation of drugs with selectivity for the cancer cells. Bioorthogonal chemistry, defined more precisely in the next paragraph, is a branch of chemistry aiming to carry out reactions with abiotic functions inside living organisms while leaving the host organism untouched. It offers a tremendous advantage in terms of selectivity as most abiotic functions are untouched by metabolism. It can be considered as metabolically inert if it stable towards oxidation and nucleophiles. If the reaction itself is bioorthogonal, it is expected that there will be virtually no chance for the host cell to circumvent it and the protecting group will be thus called bioorthogonal.

The tool we decided to employ is transition metal catalysis (Scheme 1. 2). At first, our team has been specialized in transition metal catalysis, especially copper, for the last 20 years. Expertise in this field was deemed as a prerequisite in order to understand the way the catalyst performs, lead a rational optimization, and turn the metabolic peculiarities into our advantage in the catalysed reaction.



Scheme 1. 2 Core principle of this work.

Organometallic catalysis is nowadays a rich toolbox in synthesis, whose major tools were developed during the XXth century. Nearly all the metals from the most common to the most exotic ones have been employed to catalyse transformations which would never take place with non-metallic catalysts due to the unique redox, coordination and steric properties of organometallic complexes. However, the bioorganometallic chemistry of artificial complexes is a much younger discipline whose hallmarks were set in the early XXIst century. It is worth noticing at this stage that very few reports were published on the subject at the time the work was started.³⁻⁴

Our approach centred on bio organometallic catalysis would gain advantage compared to metallodrugs through the turnover of the catalyst, leading to lower and probably sub-toxic doses, avoiding the problems of detoxification which are crucial in the field of metallodrugs. The third advantage is the high diversity of transition-metal-catalysed reactions. To date, there are very few biological reactions catalysed by organometallic compounds, and all that is

known in the field is purely artificial. Our final argument is that at the time we entered in the field in 2013, very little was known.

The work presented here will aim to develop new biocompatible transition-metal catalysts to activate probes or cytotoxic molecules within cancer cells selectively. In the first project, an optimization-driven screening on a smaller molecular space was performed to discover several new iridium complexes active in transfer hydride reduction. In the second, a diversity-oriented screening (bottom-up approach) was performed to unveil new scaffold of palladium complexes able to deprotect allyl carbamates. Fluorogenic probes have become a key tool to increase the screening throughput for the second project and have been used as an easy-to-implement *in cellulo* validation method. With robust tools in hands, we have moved to therapeutic applications in the field of vectorization and irreversible protein inhibition.

To understand better the need of this work, the introduction will define in a first phase bioorthogonal reactions, with a few examples illustrating the bioorthogonal toolbox. In a second phase, we will show how organometallic catalysis could be applied inside living cells while observing the principles of bioorthogonality, leading to bioorganometallic chemistry.

2 From organometallic chemistry to bioorganometallic chemistry

In this introduction, we will explain how transition metal catalysed reactions were added to the bioorthogonal reaction toolbox and how the advantages of using transition metal catalysis were used to develop novel applications in imaging and drug delivery.

In this section, the principles of bioorthogonal chemistry will be introduced, with several recent examples from the literature. In the second part of this introduction, we will see how several groups combined the principles of transition metal catalysis and bioorthogonality to establish the field of “bioorthogonal transition metal catalysis”, which we are working on during this thesis.

2.1 Bioorthogonal chemistry

2.1.1 Definition

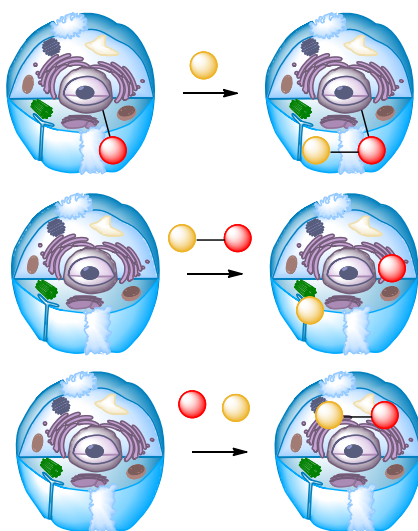
Bioorthogonal chemistry was born at the dawn of the years 2000 in pioneer works of Bertozzi, who was at the team avidly seeking new tools to study the biological function of sugars in glycoproteins. The idea which motivated her was to develop new methods to study biomolecules in their native environment without interfering with the rest of the biological materials. In 2003, she introduced the concept of bioorthogonality to summarize the requirements such a method should meet to be efficient.⁵ By definition, it describes a reaction taking place (i) in a biological medium, (ii) without interfering with it, (iii) without interfering with its homeostasis, and (iv) without inducing toxicity on the biological medium.

It is possible to distinguish three different families of bioorthogonal reactions (Scheme 1. 3).

1- In the first family, the reagent can selectively reach a target and bind to it by a bioconjugation reaction (Scheme 1. 3A). This technique is very helpful to study the location of a target biomolecule or detect its presence specifically.

2- In a second family, a drug or a probe can be selectively activated either by cleaving a protective group by an endogenous action or by carrying out a transformation on the molecule, triggering the transformation into an active moiety (Scheme 1. 3B).

3- In the third family, by far less exploited due to slower kinetics, the two endogenous entities are incubated in order to combine together inside the cells (Scheme 1. 3C).

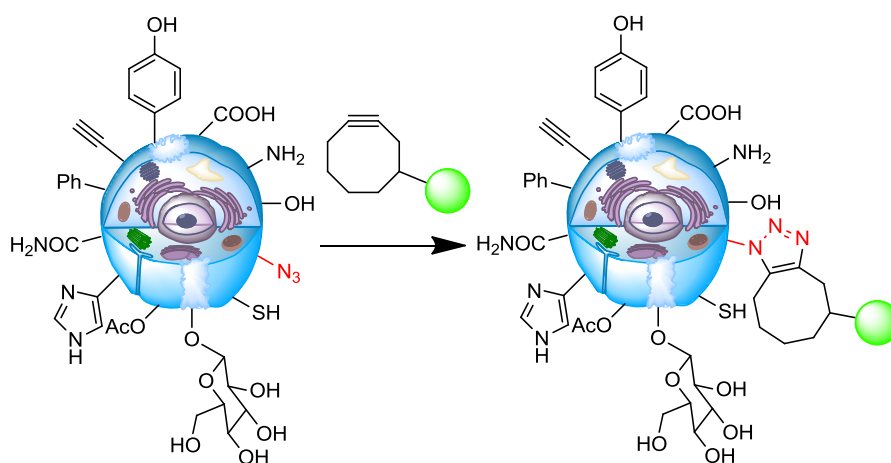


Scheme 1. 3 A) Bioconjugation to an endogenous biomolecule; B) in cellulo cleavage; C) in cellulo cross-coupling

Bioorthogonal chemistry is of particular interest nowadays for tomorrow's medicine as it adds to the toolbox some new ideas, new reactions which create new opportunities for specific treatments.

2.1.2 *In cellulo* conjugation to biological substrates

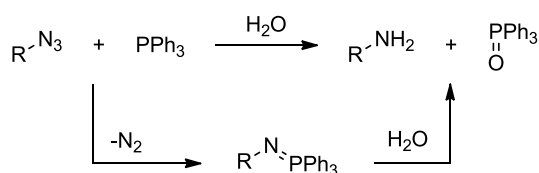
Historically developed by Bertozzi since the years 2000,⁶ this approach aims to selectively tether a reporter to a marked biomolecule for visualization purpose (Scheme 1. 4), but also in few cases for drug delivery.⁷⁻⁹ To date, azides, phosphines and alkynes are the most commonly used functions as they are to date known to be abiotic and they are metabolically stable. Bertozzi's most renowned contributions are the so-called "Staudinger ligation" and the "*in cellulo* copper-free copper-alkyne cycloadditions".



Scheme 1. 4 Bioorthogonal chemistry, "fishing for selectivity in a sea of functionalities"¹⁰

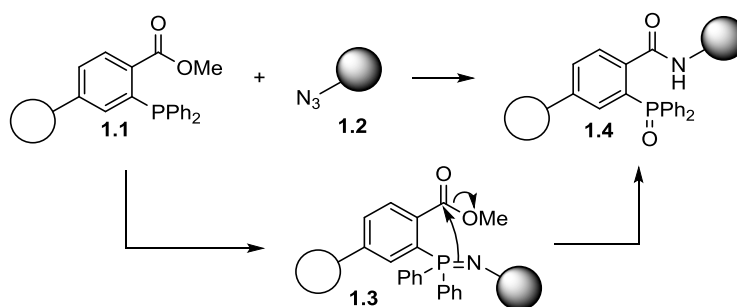
2.1.2.1 Staudinger ligation

Staudinger reaction is a well-known named reaction since 1919 (Scheme 1. 5).¹¹ It consists of the reaction between an azide and a phosphine to give an iminophosphorane. It can be further reacted by simple hydrolysis with water to yield an amine, which is the Staudinger reduction.



Scheme 1. 5 Classical Staudinger azide reduction into amine

Knowing that the iminophosphorane is a good nucleophile, the reaction was turned by Bertozzi in 2000 into a traceless amide bond formation methodology.¹² Since the iminophosphorane **1.3** is a good nucleophile, it can be trapped in an intramolecular fashion by an ester located in *ortho* position relative to the phosphorus group (Scheme 1. 6).¹³ It is nowadays considered as the launch of the field of bioorthogonal chemistry. It found multiple application in such as labelling of azido tagged proteins,¹⁴ or azido-tagged glycoproteins.¹⁵



Scheme 1. 6 Staudinger ligation, adapted from ¹²

2.1.2.2 Click chemistry

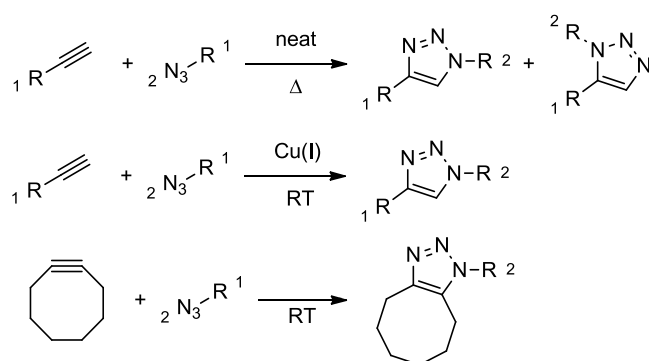
The concept of click chemistry was introduced by Sharpless in 2001 to denote “reactions that are high yielding, wide in scope, that create only by-products that can be removed without chromatography, that are stereospecific, simple to perform, and can be conducted in easily removable or benign solvents”.

A famous example is the 1,3-dipolar cycloaddition on azides onto alkynes. The non-catalyzed reaction was reported by Huigens

and required high temperature to yield the cycloadduct and gave a mixture of regioisomers. As a major improvement, Sharpless reported in 2005 the use of copper (I) salts as catalysts, providing 10^7 to 10^8 acceleration factor.¹⁶⁻¹⁷ The copper (I) salt can be formed *in-situ* from copper (II) salts and sodium ascorbate. This reaction is nowadays mistakenly called “click chemistry”.¹⁸

Click chemistry is a very useful tool. Among them, the CuAAC is a very useful methodology which has been utilized uncountable times in synthesis and chemical biology.¹⁹ However, it turned out to be hard to apply to living systems due to inherent toxicity coming from Cu salts.

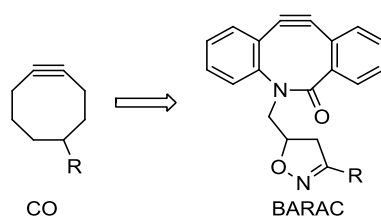
Copper can, however, be avoided thanks to the use of cyclooctynes (Scheme 1. 7). Due to their ring strain ($\cong 18$ kcal/mol), the cycloaddition takes place spontaneously in copper-free conditions. The reaction was first developed by Krebs and Wittig in 1961.²⁰



Scheme 1. 7 A) Thermal azide-alkyne 3+2 cycloaddition (Huisgens); B) Copper(I) catalysed azide-alkyne cycloaddition (Sharpless)¹⁶⁻¹⁷; C) Copper-free cyclooctyne-azide 3+2 cycloaddition (Wittig)²⁰

It was adapted *in cellulo* for the first time by Bertozzi in 2004 to label a recombinant glycoprotein GlyCAM-IgG (Glycosylation-dependent cell adhesion molecule-1) with OCT-Biotin.²¹ Whereas the toxicity was much lower than the analogue Cu- catalysed version, the

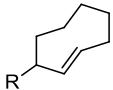
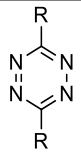
rate was significantly lower, with a low second-order constant of $0.0024 \text{ M}^{-1}\text{s}^{-1}$. Driven by mechanistic studies, they synthesized a wide range of cyclooctynes with improved rate constants, up to $0.96 \text{ M}^{-1}\text{s}^{-1}$ for the “BARAC” compound (Biarylazacyclooctynones, Scheme 1. 8). Some excellent reviews summarize the later progress on the topic.²²



Scheme 1. 8 Last generation cyclooctyne for copper-free 3+2 cycloaddition with alkynes²²

Later, variations to this method were reported using analogues to the cyclooctyne (Table 1. 1) and the azide (Table 1. 2). Similarly, (*E*)-cyclooctenes can be used as analogues, even though they can isomerize over time.⁷ An interesting case comes from 1,2,4,5-tetrazines, which are aromatic compounds which can act as a diene, but the cycloadduct quickly decomposes to release a nitrogen molecule and a 1,2-diazine.²³

Table 1. 1 Example of partners for in cellulo 4+2 cycloaddition²³

Structure	Name
	(<i>E</i>)-cyclooctene
	1,2,4,5-tetrazine

Similar to azides, some other dipoles such as nitrones, nitrile oxides or aliphatic diazo can be used to couple to a dipolarophile

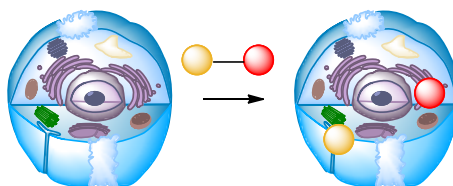
(Table 1. 2). The bioorthogonal toolbox was summarized by Prescher in 2014.²⁴

Table 1. 2 Example of dipoles which are compatible with in cellulo 1,3-dipolar cycloadditions²⁴

Structure	Name
$\text{R}-\text{C}=\text{N}^+\text{O}^-$	Nitrone
$\text{N}^-\text{N}^+=\text{C}-\text{R}$	Diazo
$\text{R}-\text{C}\equiv\text{N}^+\text{O}^-$	Nitrile oxide

2.1.3 In cellulo activation

This section has traditionally been associated with the prodrug uncaging strategy, frequently used to reduce side effects by administering the drug in an inactive form, and which can be activated only once it has reached its target (Scheme 1. 9). The molecule is then activated by the action of either an exogenous cofactor (H_2O_2 , thiols, phosphine, azide ...), an endogenous redox metabolite (NADH, FADH_2), or an enzyme²⁵ (reductase, hydrolase or oxidase). However, in this section, we cannot consider the cases where the prodrug is activated by an enzyme or by an endogenous cofactor, as they are not truly bioorthogonal according to the definition proposed by Bertozzi.



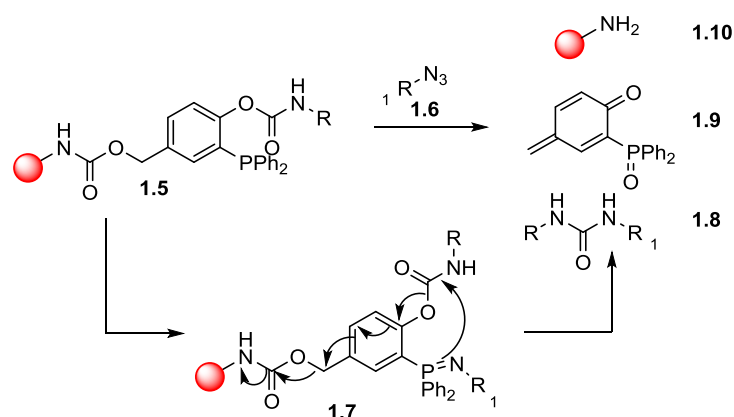
Scheme 1. 9 Principle of bioorthogonal in cellulo activation

Three significant examples are presented below. The first one uses the Staudinger ligation and the last ones use cycloaddition

reactions as a key step to immolation leading to the release of the active drug.

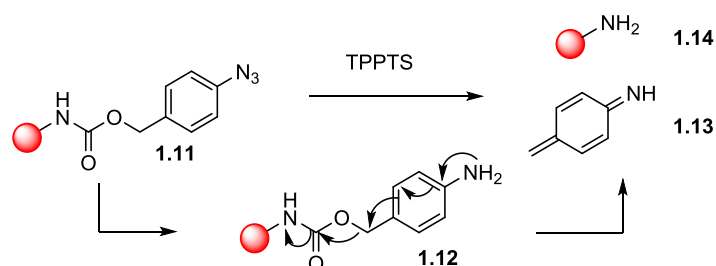
2.1.3.1 Staudinger ligation-triggered release

The first example of bioorthogonal activation of a prodrug comes back to 2006, where Doxorubicin (Dox, **1.10**) could be decaged by reaction of a phosphine trigger **1.5** with an azide **1.6**, leading to an intramolecular nucleophilic attack of the generated phosphoranimine **1.7** on a carbamate (Scheme 1. 10). The generated phenol eliminated in a 1,6 fashion and released the Dox **1.10**.²⁶



Scheme 1. 10 Staudinger-triggered prodrug release mechanism²⁶

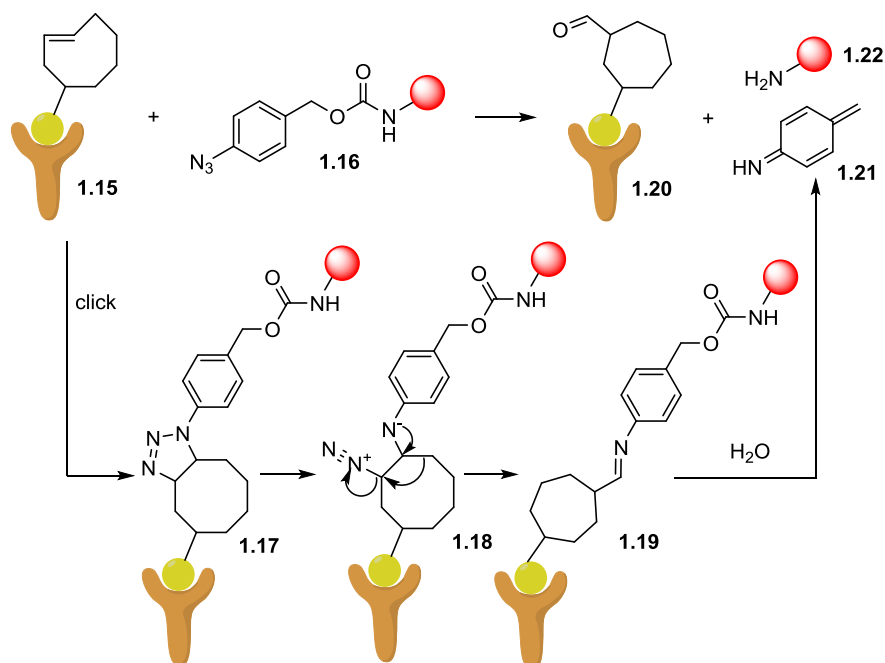
A similar approach reported by Robillard (Scheme 1. 11) focused on the reduction of an azide **1.11** by a water-soluble phosphine (TPPTS). The generated amine **1.12** underwent a 1,6 elimination to release Dox **1.14**, similarly to the previous example. The main drawback of the method was the deactivation of TPPTS by oxidation. The authors could not determine if the reaction was intracellular or extracellular.⁹



Scheme 1. 11 Staudinger azide reduction-triggered prodrug release⁹

2.1.3.2 Click and release

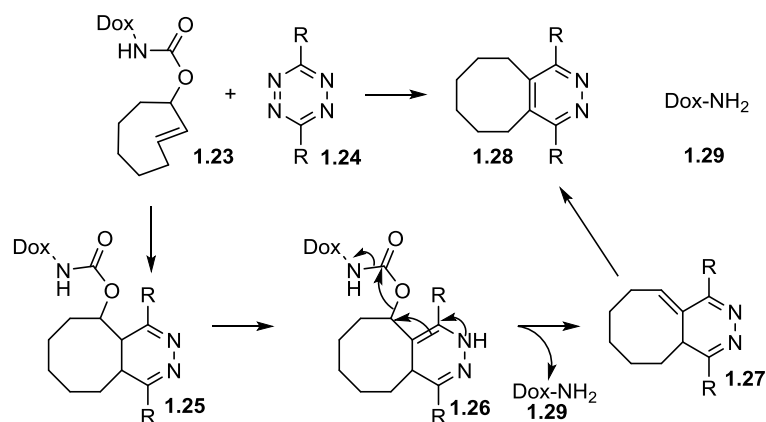
The *click and release* approach is based on two different objects which react together by a click reaction (Scheme 1. 12).²⁷ On one side, a functionalized *E*-cyclooctene **1.15** by a ligand recognizes its target. In the other side, the drug is caged with a 4-azido benzyl carbamate **1.16** of the drug of interest. The click product decomposed in a cycle contraction fashion and yielded an imine **1.19** which readily hydrolysed at acidic pH to yield the 4-aminobenzyl carbamate, which spontaneously decomposed by 1,6-elimination to release the drug **1.22**. Instead of the phosphine, the reduction of the azide was done during the click step.²⁸



Scheme 1. 12 « click and release » with an *E*-cyclooctene and an azide-based prodrug²⁸

2.1.3.3 Diels Alder release

Diels-Alder reaction can be considered as a sister reaction to the copper-free click chemistry. Robillard described the same “click and release” concept with *trans* cyclooctenol –Dox carbamate **1.23** with a tetrazine **1.24** (Scheme 1. 13).⁸ When the 4+2 cycloadduct **1.25** tautomerized, the nitrogen pushed with its lone pair and eject the doxorubicin, which decomposed to release the Dox **1.29** and the trigger part re-aromatized **1.28**.



Scheme 1.13 Diels-Alder bioorthogonal release ⁸

2.2 Bioorganometallic chemistry

This first part has shown that the bioorthogonal toolbox remains limited to cycloadditions and amide bond formations through the Staudinger ligation. By combining the principles of bioorthogonal chemistry to organometallic chemistry, several new bioorthogonal organometallic reactions, or simply bioorganometallic reactions, were developed and reported. It broadly extends the scope of bioorthogonal reactions and enables new transformations which were not possible before to take place in the cellular medium.

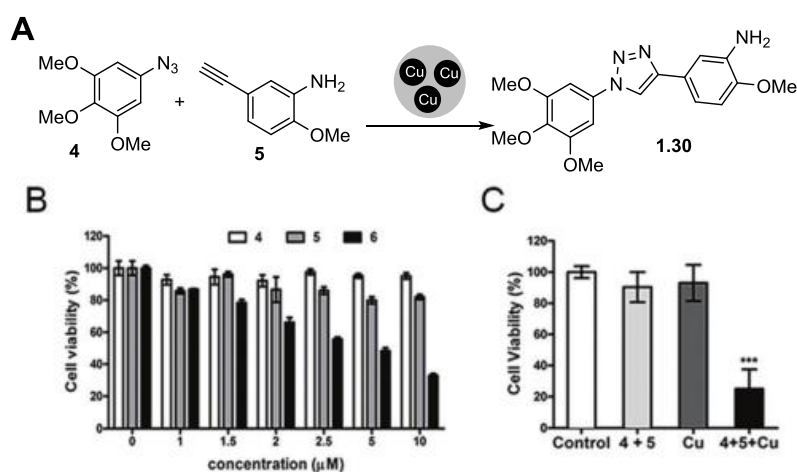
This section of the introduction is divided into section, one for each metal. Before explaining the bioorganometallic reactions, a brief overview of the capabilities of each metal in the context of organometallic catalysis and synthesis is presented. The field has been reviewed recently by Meggers²⁹, Zimmermann⁴ and Mascareñas³.

2.2.1 Click chemistry

The CuAAC is a powerful methodology in synthetic chemistry, but due to the toxicity of the copper salts to living organisms and cells, its implementation was severely hampered *in*

cellulo. Nowadays, most of the *in cellulo* examples make use of the copper-free analogue reactions, as well as a full range of cycloadditions. However, there are still a couple of reports of copper-catalysed click reaction in living cells, featuring various techniques to overcome the toxicity of copper salts.

Bradley used a copper nanoparticle-based system aiming to simultaneously encapsulate copper, avoiding thus the toxicity, and use it in a copper catalysed click reaction to assemble an azide and an alkyne (Scheme 1. 14). The produced molecule is an analogue drug of Combretastatin **1.30** with a bioisostere moiety replaced by the triazole.³⁰ The nanoparticle system was implanted in the vitellus bag of the zebrafish embryo and the drug synthesis took place inside it in an extracellular fashion.



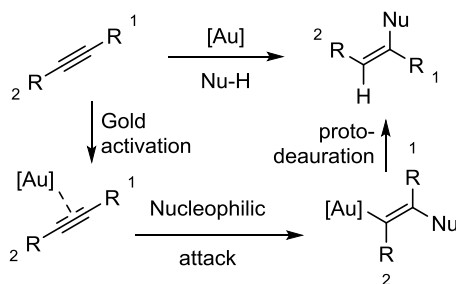
Scheme 1. 14 A) *in cellulo* assembly by *in cellulo* click chemistry with supported copper(0) nanoparticles; B) Cell viability for compounds **4** (azide), **5** (alkyne) and **6** (cycloadduct); C) Cell viability for the control, the coupling partners, the copper catalyst and eventually the combination leading to more toxicity. Adapted from³⁰

2.2.2 Gold chemistry

Gold was at first thought to be an inert metal in catalysis and therefore little or no attention was paid to this metal until the years

1990.³¹ Gold exists in mostly 2 oxidation stages, (+I) and (+III). Gold (+I) tends to disproportionate to gold (0) and gold (+III) when it is not bound to organic ligands. Gold (III) complexes have generally high reduction potential, making them good oxidants.

Gold is known to almost selectively bind to triple bonds, activating them like a *soft proton* (Scheme 1. 15). If there is a nucleophile available for an intramolecular cyclization (typically a 5 or 6-members ring), the cyclization will give rise to a vinyl gold species. In some cases, when there is a positive charge on the α or the γ position, the gold can stabilize the positive charge due to relativistic effects by giving their electrons to form a gold carbene. While the carbene can add on double bonds for cyclopropanation, it can also be attacked by a nucleophile. The catalytic cycle closes often by a proton-gold exchange, called a protodeauration.

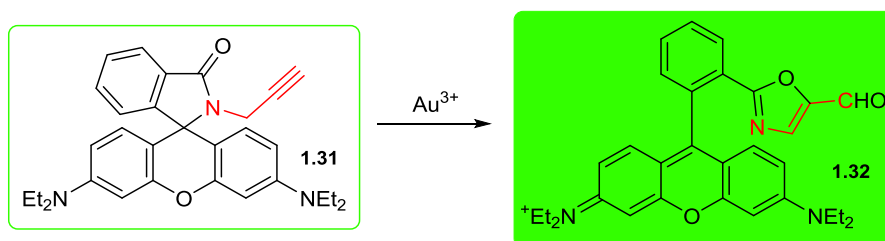


Scheme 1. 15 General reactivity of gold complexes on alkynes

While gold (I) is often employed nowadays, gold (III) can be used as the precatalyst and be reduced *in-situ*. Gold (III) also has the opportunity to close the catalytic cycle *via* a reductive elimination, whereas gold (I) is unable and needs to be re-oxidized prior to its re-entry to the cycle.

Several probes were designed in order to detect gold in trace amounts in aqueous solutions, and few of them have been applied to

in cellulo assay. Most of them feature an alkyne moiety as the trigger. The first example reported was published by Yoon in 2009 (Scheme 1. 16), where he was able to restore the activity of a rhodamine moiety **1.31** whose conjugation was blocked by an *N*-propargyl lactam on the spirocycle of the rhodamine part.³² Treatment with a stoichiometric quantity of Au (+III) restored the conjugation and hence the fluorescence in cells (Figure 1. 4). The product **1.32** was identified as the aldehyde and characterized. This probe **1.31** is highly selective for Au (+III).



Scheme 1. 16 Fluorogenic and Au³⁺ sensitive probe and isolated product after hydrolysis³²

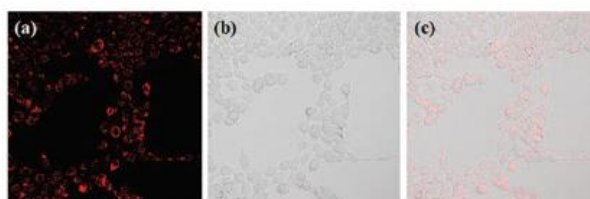
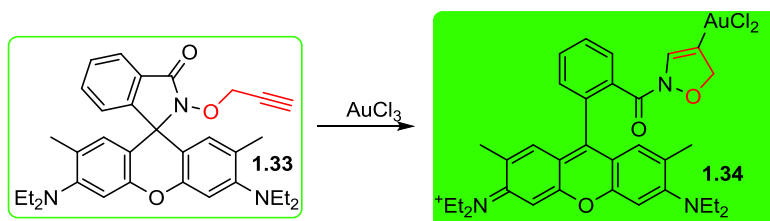


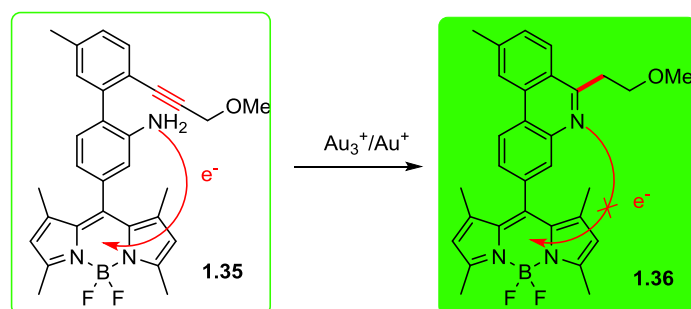
Figure 1. 4 Cellular images of cells treated with **1.31** 20 μ M for 2h, washed by PBS 3 times, then incubated with AuCl₃ 10 μ M for 1h. a) Fluorescence image at 558 nm, b) Brightfield, and c) Merged images. From ³²

Shortly after, Tae reported a very similar example, also working in stoichiometric fashion, but this time with no clear identification of the final product **1.34** (Scheme 1. 17).³³



Scheme 1.17 Au³⁺ sensitive probe developed by Tae.³³

However, the first catalytic example was the Au (+I) or Au (+III) catalysed BODIPY activation by Wang in 2015 (Scheme 1.18).³⁴ In this example, the starting material **1.35** has its fluorescence quenched due to an intramolecular electron transferred from the amine to the BODIPY core. This phenomenon is absent from the product **1.36**. It enabled him to detect intracellular gold (+III) after it was incubated in cells (Figure 1.5).



Scheme 1.18 Fluorogenic probe **1.35** developed by Wang.³⁴

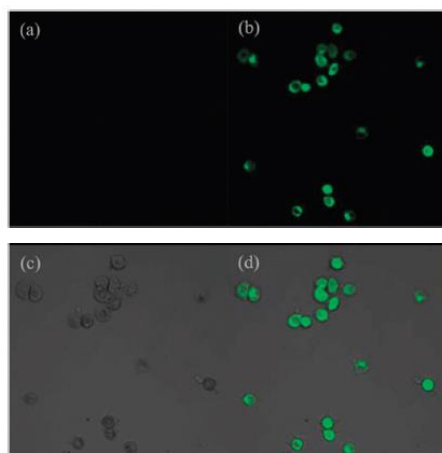
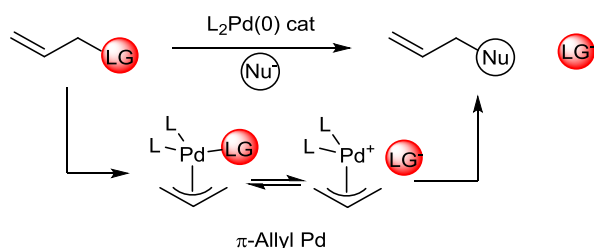


Figure 1. 5 Testing of the fluorogenic probe developed by Wang. Incubation with the **1.35** $10\ \mu\text{M}$ for 1h, 3 items of washing with PBS, $20\ \mu\text{M}$ AuCl_3 for 1h. a) No gold, fluorescence mode, b) no gold, bright field, c) $10\ \mu\text{M}$ AuCl_3 , fluorescence mode, d) merged image with the micrograph. From ³⁴

2.2.3 Tsuji Trost reaction

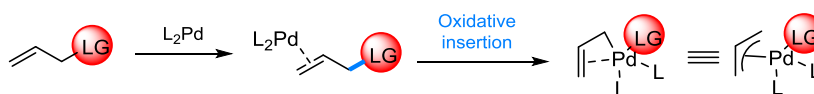
2.2.3.1 General considerations

The Tsuji-Trost reaction was first reported by Tsuji in 1965,³⁵ and later improved by Trost in 1973.³⁶ It consists of a nucleophilic allylic substitution catalysed by palladium (0) complexes. The key intermediate of this reaction is a π -allylpalladium (+II) adopting a pseudo-square planar geometry (Scheme 1. 19). After the introduction of phosphine ligands by Trost, the versatility of this type of ligands enabled to gain enantioselectivity and diastereoselectivity provided chiral substrates or chiral catalysts are used.



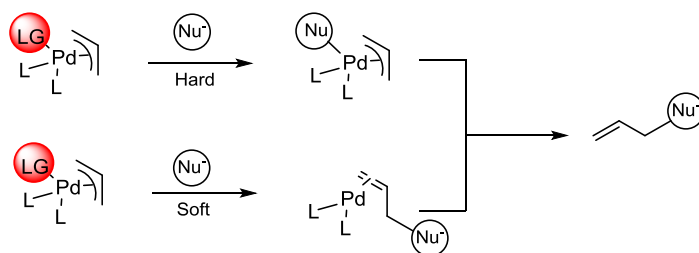
Scheme 1. 19 A general example of a Tsuji-Trost reaction. The key intermediate is the π -allyl palladium complex

One of the particularities of the Tsuji-Trost reaction is that the stereoselectivity depends on the hardness or the softness of the nucleophile (Scheme 1. 20). In the case of a chiral allylic leaving group, the palladium (0) will form on the opposite face of the allyl as the palladium (0) is electron rich and needs to interact with the σ^* orbitals of the C-LG bond in order to break it.



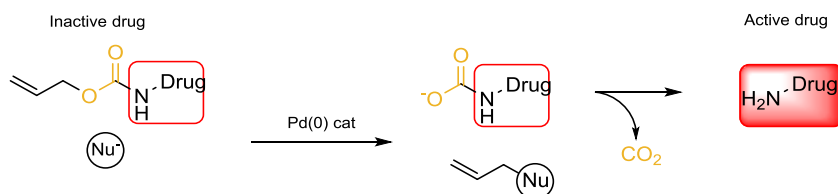
Scheme 1. 20 First steps of the mechanism leading to the formation of the π -allyl palladium

Then, the π -allyl palladium will be attacked differently depending on the hardness or the softness of the nucleophiles (Scheme 1. 21). Charged hard nucleophiles will tend to attack the palladium directly under its cationic form, and after the reductive elimination will give the product with retention for this last step, so in total a net inversion. In the other hand, soft nucleophiles will attack the π -allyl by the other side, leading to the reduction of the palladium and the departure of the leaving group with a net inversion for the last step, so net retention over the global reaction.



Scheme 1. 21 Nucleophilic attack on the π -allyl palladium species depending on the hardness or the softness of the nucleophile

A particular case has been extensively used when the leaving group is a carbamate (Alloc) or a carbonate (Scheme 1. 22).³⁷ In that case, once the π -allyl is attacked, the carbonate or the carbamate decarboxylates in an irreversible fashion. This strategy has been extensively used in the literature as a robust protecting group for amines as carbamates are more stable than carbonates, which hydrolyse readily in aqueous conditions. This case will be extensively used in this work.

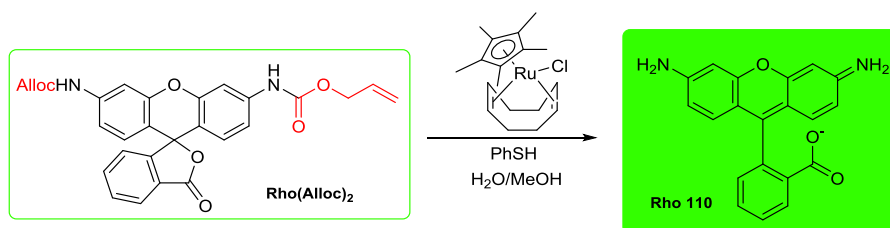


Scheme 1. 22 When the leaving group is an O-allyl carbamate, after cleavage of the allyl group, the decarboxylation leads to the free amine. It is a useful palladium zero-sensitive deprotection method

2.2.3.2 Pioneer examples

The very first of *in cellulo* application of Tsuji-Trost reaction was reported by Meggers in 2006 (Scheme 1. 23).³⁸ Using the Ru (II) catalyst $\text{Cp}^*\text{Ru}(\text{COD})\text{Cl}$ (Cp^* = pentamethylcyclopentadienyl, COD = 1,5-cyclooctadiene), he was able to cleave the Alloc protection of the bis-Alloc Rhodamine 110, called **Rho(Alloc)₂**, and thus restores its fluorescence of the rhodamine 110, called **Rho 110**. This Ru (II)

catalyst, later named the Meggers catalyst, constitutes thus the first generation of biocompatible organometallic catalyst for Tsuji-Trost reaction. During a brief optimisation phase, Meggers reported a medium activity range of his catalyst, as it had low reactivity on his model substrate with endogenous thiol such as glutathione but exhibited 80% conversion while PhSH was used.



Scheme 1. 23 Deprotection of **Rho(Alloc)₂** with $\text{Cp}^*\text{Ru}(\text{COD})\text{Cl}^{38}$

The cells were treated with 50 μM of **Rho(Alloc)₂** for 1 h, followed by washing in order to remove the extracellular prodrug. Then, the cells were treated by the Ru catalyst at 20 μM . The appearance of the green fluorescence could be observed qualitatively by confocal microscopy (Figure 1. 6). To the best of our knowledge, this is the first study of this type. However, no further experimental details, such as quantification, measurement of extracellular fluorescence or even quantification of the ruthenium cell content were provided in this study.

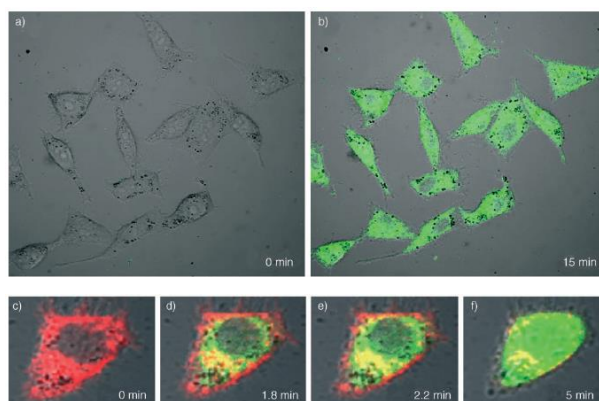
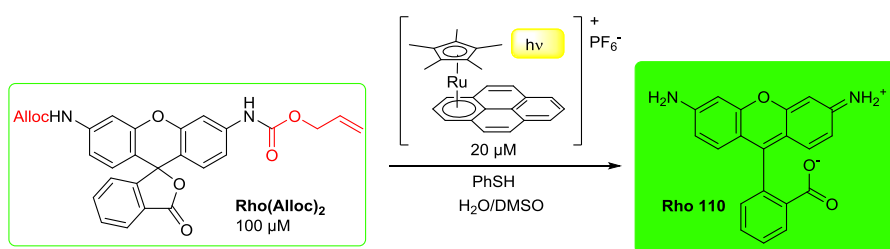


Figure 1. 6 In cellulo evolution of the fluorescence of the **Rho 110** after its cellular release. The membranes were dyed in red. From ³⁸

The catalyst was then improved by Meggers in two new generations. In one hand, a catalyst could be activated by light, and in another hand, another catalyst featured an improved quinoline carboxylic acid instead of the COD ligand for a better activity.

The photoactivable, so-called “second generation” features a pyrene ligand and a PF_6^- counter anion (Scheme 1. 24).³⁹ After irradiation for 5 min at $\lambda = 330$ nm, the catalyst was activated under the form of $\text{Cp}^*\text{Ru}(\text{Solvent})_3^+$. This study does not indicate if thiol was used for the cellular assay on the model **Rho(Alloc)₂** assay *in cellulo*.



Scheme 1. 24 Second generation of Meggers catalyst³⁹

Then, based on previous reports of Kitamura, Meggers transposed the use of the Ru catalyst $\text{Cp}^*\text{Ru}(4\text{-R-quinolinic acid})(\text{Allyl})$ as effective catalysts for the deprotection of a sulfonated coumarin *in cellulo* (Figure 1. 7, Scheme 1. 25).⁴⁰ This family of

catalysts include R = H, R = OMe and R = NMe₂, with a respective TON of 90, 150 and 270. The price to pay for a greater activity is smaller stability while handling those complexes.

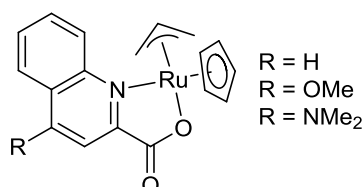
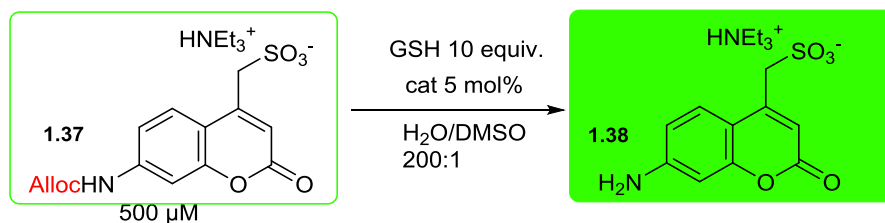
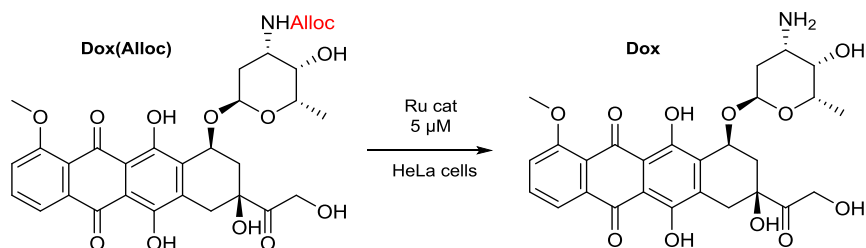


Figure 1. 7 Third generation of Meggers catalyst, first reported by Kitamura⁴⁰

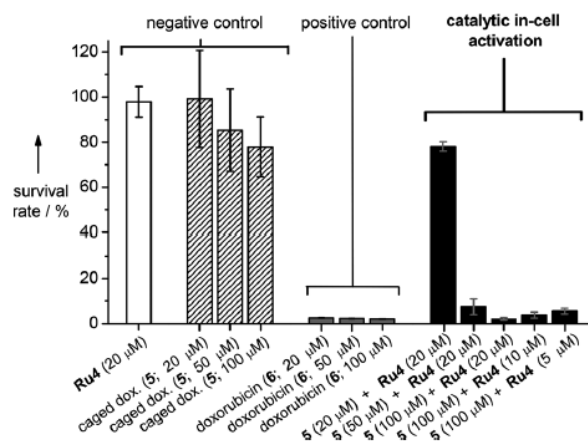


Scheme 1. 25 Catalytic deprotection of a sulfonated coumarin as a test reaction for the second generation of catalyst⁴⁰

The model was later transposed to the decaging of Doxorubicin (**Dox**), with a nearly complete cell death with 100 μM of **Dox(Alloc)** and 5 μM of the -NMe₂ catalyst, whereas the prodrug at the same concentration gave 80% viability (Scheme 1. 26, Graph 1. 1). A postulated mechanism was also reported, involving a Ru (+IV) intermediate reported. A zero-order was noted for glutathione, whereas a first order was noted for the aminocoumarin, indicating that the rate-determinant step was located at the interaction step between the catalyst and the substrate.



Scheme 1. 26 Application of the third-generation catalyst to the in cellulo deprotection of Doxorubicin.⁴⁰

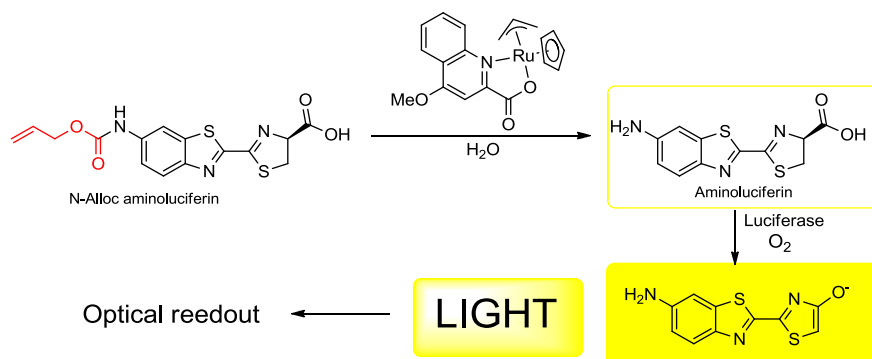


Graph 1. 1 Biological result of the in cellulo decaging of Dox(Alloc). From⁴⁰

2.2.3.3 Intracellular or extracellular reaction?

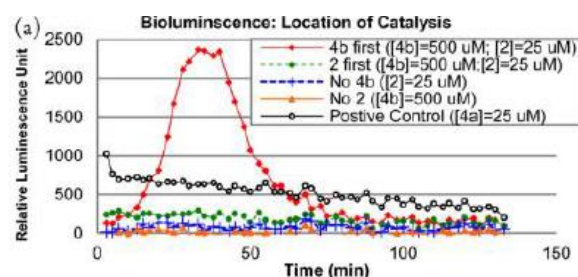
Based on these pioneer studies, Wender wanted to verify whether the activation proceeds *via* an intra or extracellular fashion.⁴¹ In the second case, the reaction would take place either at the outer side of the membrane or in the extracellular medium and the products could be internalized after. He, therefore, developed a luminescent model based on the release of an amino luciferin by a Ru-catalysed studied reaction which is further converted to decarboxylated product by a genetically encoded luciferase (Scheme 1. 27). If the reaction is intracellular, the generated product will be oxidized, and luminescence will be quickly observed. If the reaction is extracellular, no

luminescence will be observed. This system was used to evaluate the third generation of catalysts of Meggers.



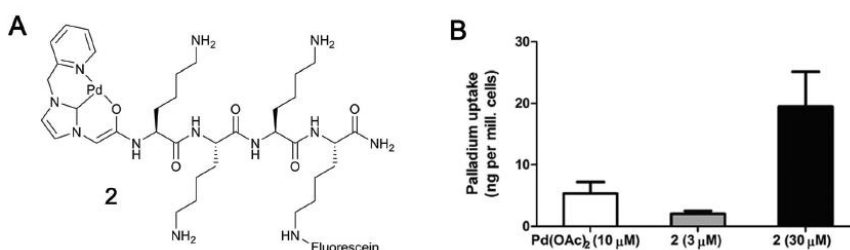
Scheme 1. 27 Luminescent model based on amino luciferin employed by Wender to probe whether the reaction takes place in or out the cellular medium⁴¹

When the cells were treated by the catalyst first, followed by washing, and then treatment by the *N*-Alloc-amino luciferin, no luminescence is observed. However, when the order is reversed, thus incubating the *N*-Alloc-aminoluciferin first, a clear peak of luminescence was observed (Graph 1. 2). This indicated that the catalyst was readily washed out, suggesting that the catalyst is either bound to the external membrane or does not penetrate the cells efficiently. As reminded by Wender, “extreme care must be taken” in order to know if the reaction takes place in or out the cellular medium.



Graph 1. 2 Measure of the bioluminescence due to amino luciferin release and its oxidation by luciferase. In orange, Alloc-amino luciferin; in blue, catalyst; in black, amino luciferin (positive control). In green, incubation of the catalyst, then washing and incubation of the probe. In red, incubation of the probe first, then the catalyst. From ⁴¹

The question of whether the reaction proceeds in an intracellular or extracellular fashion was again addressed by Bradley in 2017.⁴² He developed a new model palladium (+II) catalyst with an NHC ligand (Figure 1. 8A). The cell penetration is ensured by a Lys₄ cell-penetrating peptide to which fluorescein can be optionally tethered for visualization purposes. As a direct consequence, a better cell-accumulation was noted by the authors compared to palladium acetate (Figure 1. 8B). As the palladium (II) is not meant to be reduced into palladium (0), the model was tested on the deprotection (Figure 1. 8C) of the **Rho(Proc)₂** (Proc = propargyloxy carbamate, see Part 2, paragraph 2.1).



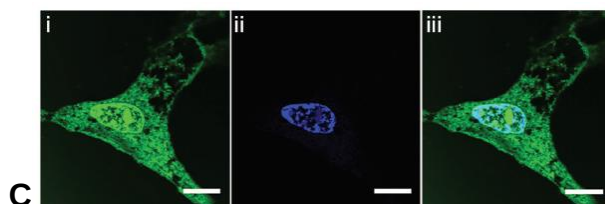
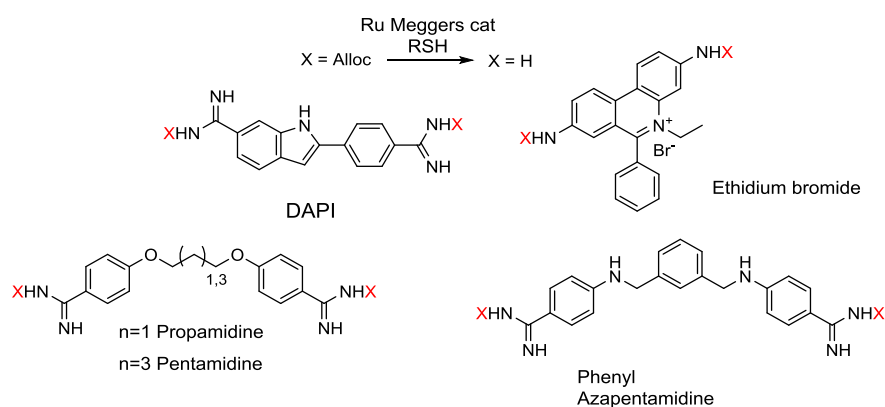


Figure 1.8 A) Cell-penetrating catalyst with a fluorescent tag. B) Improvement of the cellular uptake compared to palladium acetate. C) Green: (i) catalyst, blue: (ii) nucleus stain. Merged images (iii) From ⁴²

2.2.3.4 Use the Meggers catalyst *in cellulo*.

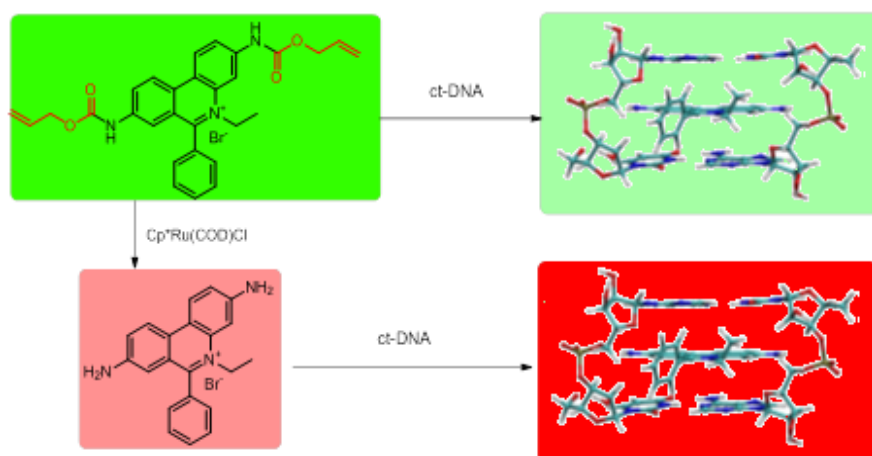
Later, several groups made use of the so-called Meggers catalyst for various applications. The first and third generation of catalysts served as model catalysts to test a home-designed application, or as a template for more specific catalysts.

Mascareñas reported in 2014 the decaging of cationic DNA binders (ethidium bromide, DAPI, propamidine and pentamidine, Scheme 1. 28) using the first generation of the Meggers catalyst in conjunction with PhSH as allyl capture agent.⁴³ This technique would bring the advantage to reduce the amount administrated as those derivatives are toxic. The deprotection was followed by NMR titration with *h*-A3-T3 oligonucleotide and the K_d value decreased as the deprotection occurred.



Scheme 1. 28 Deprotection of DNA binders using the first generation of Meggers catalyst⁴³

Once the concept was validated on oligonucleotides, it was validated on the deprotection of Ethidium Bromide. This DNA binder has a low red fluorescence (610 nm) while in its free form, and the fluorescence increases once bound to *ct*-DNA (Scheme 1. 29). On the other hand, the *bis*-Alloc form is brightly fluorescent at 500 nm and its fluorescence decreases once bound to *ct*-DNA. He then verified that he observed by confocal microscopy a bright green fluorescence while incubating the *bis*-Alloc ethidium bromide in absence of the catalyst, whereas a bright fluorescence was observed upon incubation of the *bis*-Alloc probe, the catalyst and PhSH, sign of the deprotection taking place.



Scheme 1. 29 Impact on the photophysical properties of the presence of the protecting group and the DNA binding. ⁴³

Almost at the same time the report of Wender⁴¹ stating that the reaction is maybe extracellular, Mascareñas independently reported⁴⁴ that he was able to confine the activity to the mitochondria by adding a lipophilic cation⁴⁵ (triphenylphosphonium salt) to the 3rd generation Meggers catalyst (Figure 1. 9). In a first step, he demonstrated that the location of the released **Rho110** from **Rho(Alloc)₂** matched the mitochondria distribution, as they can be stained selectively with TMRE (Figure 1. 10).

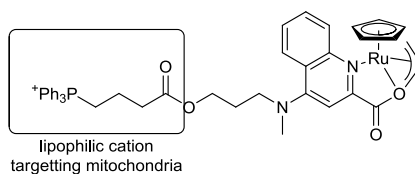


Figure 1. 9 Mascareñas catalyst based on Meggers' 3rd generation catalyst with a mitochondrial targeting moiety⁴⁴

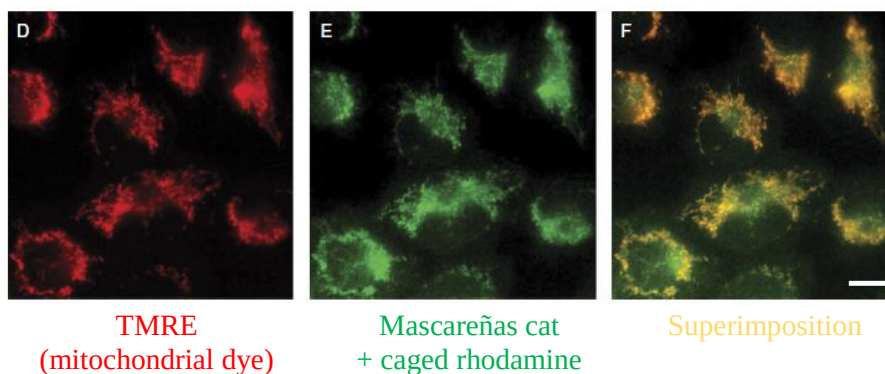


Figure 1. 10 D) Distribution of the mitochondria, E) Distribution of the released rhodamine, F) Superimposition of D and E. From⁴⁴

In a second step, he modified the triphenyl phosphonium by pyrenemethylene-diphenylphosphonium salt in order to take the benefits of the fluorescence of the pyrene to localize the catalysts (Figure 1. 11). Again, a perfect match was observed between the catalyst and the mitochondrial distribution (Figure 1. 12).

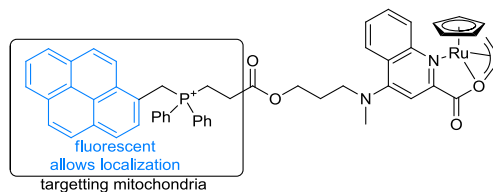


Figure 1. 11 Catalyst designed by Mascareñas in order to target the mitochondria and to localize it due to its fluorescence.⁴⁴

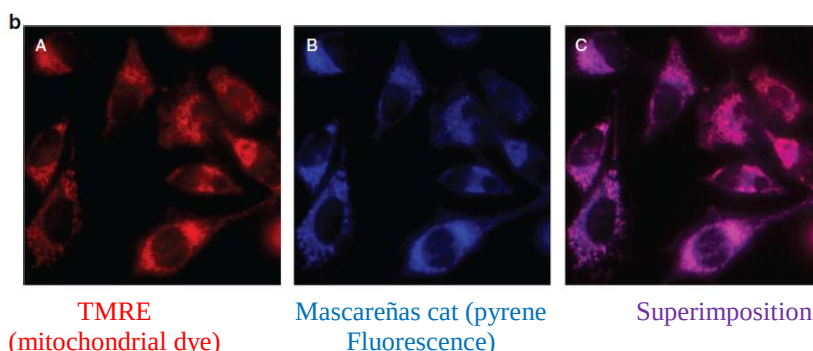
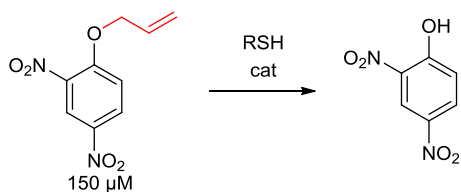
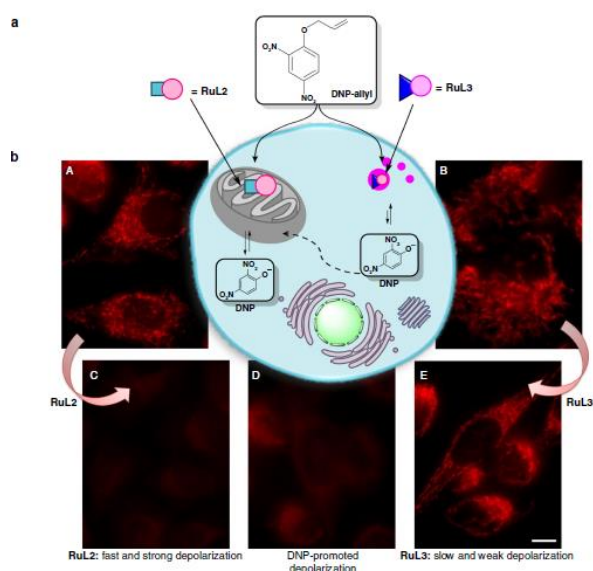
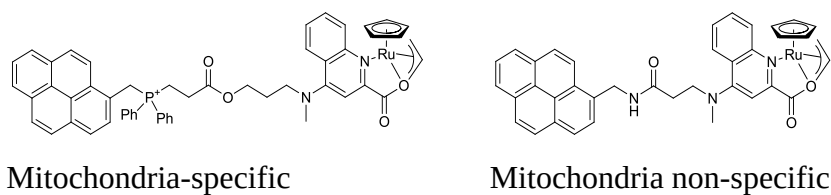


Figure 1. 12 A) Location of the mitochondria, B) Location of the catalyst, C) merged image of A and B. From ⁴⁴

Then, he demonstrated that he could cleave the allyl protection on the phenolic position to release the 2,4-dinitrophenol, which is a membrane de-polarizing agent which decreases the membrane potential and stops the ATP production (Scheme 1. 30). The depolarization of the membrane also reduces the binding affinity of cationic TMRE and the staining becomes less efficient, which can be realized by a darker image. Mascareñas showed that a non-specific catalyst (featuring a pyrene moiety but no cation) was unable to release the nitrophenol at the desired location, thus causing no drop of mitochondrial staining (Scheme 1. 31). However, with the specific catalyst (featuring the pyrene and the lipophilic cation), a net drop of mitochondrial staining was visible, and the effect was similar to direct staining of 2,4-dinitrophenol.



Scheme 1. 30 Deprotection of *o*-dinitrophenol⁴⁴

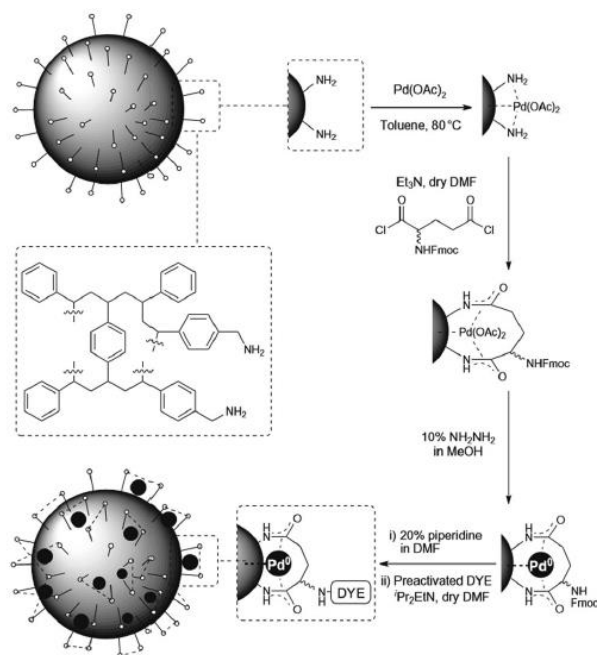


Scheme 1. 31 Intracellular deprotection of *o*-dinitrophenol. Left, the catalyst targets the mitochondria. Right, the catalyst is not specific, with no loss of colouration noted. Centre, positive control made with *o*-dinitrophenol. From ⁴⁴

2.2.3.5 Nanoparticles catalysing Tsuji-Trost reaction

Starting from 2011, Bradley developed a series of supported Pd (0) nanoparticles on a functionalized polymeric matrix.⁴⁶ The Pd(OAc)₂ was deposited onto the surface by coordination to the free amine moieties (Scheme 1. 32). The amines were then bridged together with a bis acyl chloride and the palladium (II) was reduced to

small-sized nanoparticles by hydrazine. The stability was ensured *via* the chemical entrapping. A fluorescent dye could be optionally tethered on the bridge after the removal of the Fmoc protecting group.



Scheme 1.32 Synthesis of Bradley's palladium supported nanoparticles on a reticulated polystyrene resin. From ⁴⁶

That catalyst was then used on the Meggers' model reaction with the **Rho(Alloc)₂** as a proof of concept (Figure 1.13). Fluorescence microscopy images and flow cytometry revealed the internalization of the catalyst and the distribution of the released **Rho110**.

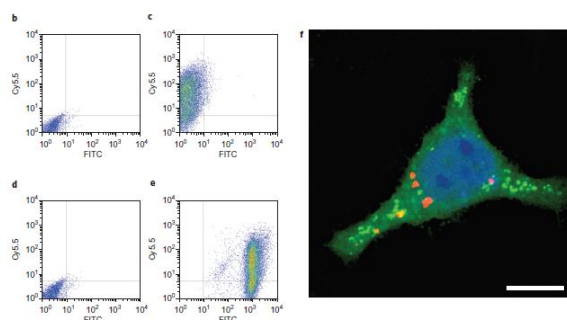


Figure 1.13 A) Flow cytometry determination of the fluorescence; b) negative control; c) Palladium catalyst; d) bis-Alloc rhodamine probe; e) probe + catalyst. From ⁴⁶

Apart from the Tsuji-Trost reaction, this catalyst can be theoretically used for any Pd (0)-catalysed reaction, like Suzuki coupling, but this aspect will be covered in the Paragraph 2.2.4.2 (p 48).

2.2.3.6 *In vivo* application of Tsuji-Trost reaction

The palladium-resin developed by Bradley⁴⁶ was also tested *in vivo* in zebrafish embryo models (Figure 1.14).⁴⁷ This time, the authors selected the **Rho(Proc)₂** model deprotection to **Rho110**. When one was injected into the vitellus bag, they showed good tolerance of the bead, no matters if it was naked or loaded with palladium. As expected, when the culture medium contained the **Rho(Proc)₂**, a green fluorescence appears in the vitellus bag, and also in the guts of the zebrafish injected with a Pd- loaded bead. However, the authors noticed that embryos injected with naked resin underwent a fluorescence increase localized in the guts, probably due to non-specific digestion of the **Rho(Proc)₂** by intestinal enzymes. The same effect was also observed by Meggers with an azide reduction by an iron-based porphyrin (Paragraph 2.2.5, p56).

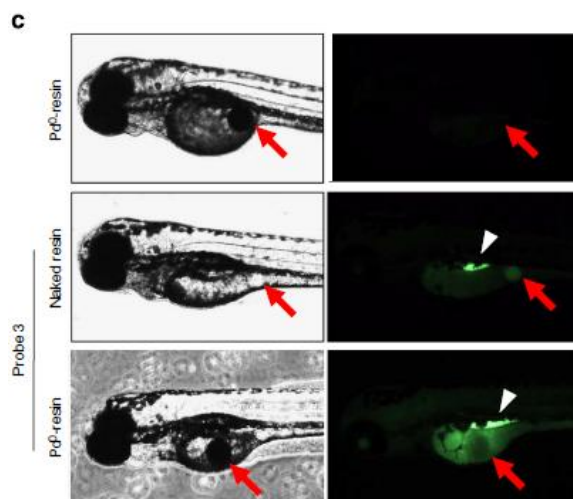
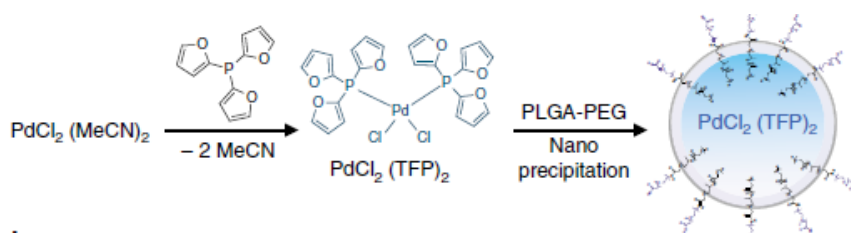


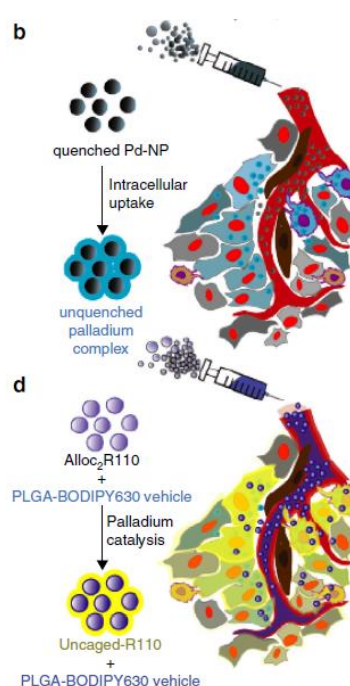
Figure 1. 14 Top: naked resin, no fluorescence is observed. Middle: Naked resin + bis Proc Rhodamine probe. Fluorescence visible in the guts. Bottom: fluorescence visible around vitellus bag and also the guts. From ⁴⁷

However, the most significant example of *in vivo* application was achieved by Weissleder in 2017.¹ After selecting the complex $(\text{Fur}_3\text{P})_2\text{PdCl}_2$ (also called **PdC07**) as a catalyst for the model deprotection of **Rho(Alloc)₂**, he encapsulated the catalyst in a biodegradable polymer matrix, thus ensuring a better uptake and stability (Scheme 1. 33). It is worth mentioning that the authors never reveal how the catalyst is activated although Pd (II) is not able to catalyse Alloc deprotection, whereas it still can deprotect Proc moieties. It remains unclear whether the authors carried out a chemical reduction of the Palladium contained in the micelles before taking TEM images or if the nanoparticles were formed during the TEM micrograph by taking up the electrons of the electron beam.



Scheme 1. 33 Weissleder catalyst obtained by nanoprecipitation of $(\text{Fur}_3\text{P})_2\text{PdCl}_2$ in PLGA-PEG polymer. From ¹

The system was tested by the authors on mice models infected by a tumour above which a window was placed to be able to visualize the release of the **Rho110** after its deprotection from the **Rho(Alloc)₂** (Scheme 1. 34). In the experiment, the cell nuclei were labelled in red. The fluorescence of the catalyst was quenched by the tight packing inside the micelle but was restored once the micelle decomposed and disappeared while the catalyst was internalized in the



Scheme 1. 34 Concept of the in vivo assay of the catalytic system and the **Rho(Alloc)₂** uncaging. From ¹

cells (Figure 1. 15). Afterwards, the **Rho(Alloc)₂** encapsulated within a micelle containing a NIR fluorescent (in order to visualize the distribution of the vehicle) was injected and a gradual appearance of yellow-green fluorescence was noted, a sign of the release of the **Rho110** (Figure 1. 16). The sophisticated system relied on the EPR effect for targeting, as neither the micelles, the catalyst nor the probe has a specific affinity for the tumour and the selectivity may not be optimal.

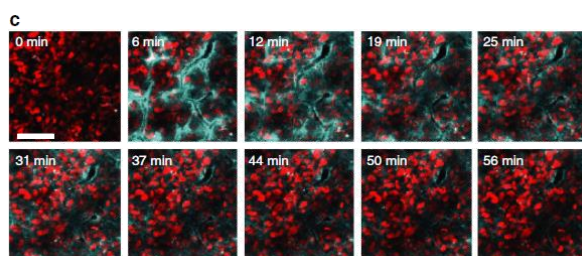


Figure 1. 15 Timelapse of the distribution of the catalyst. Red: nucleus; Blue: residual fluorescence of the catalyst after release from the vehicle. From ¹

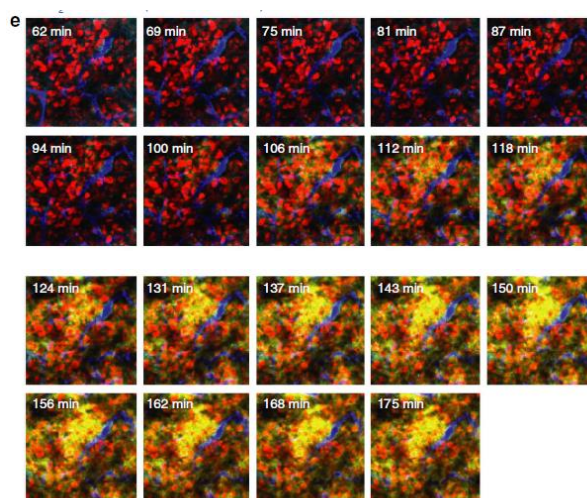
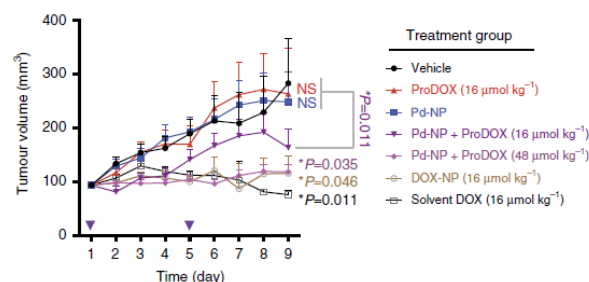


Figure 1. 16 Timelapse distribution of the **Rho(Alloc)₂** (non-fluorescent, its vehicle is dark blue) into **Rho110** (yellow). From ¹

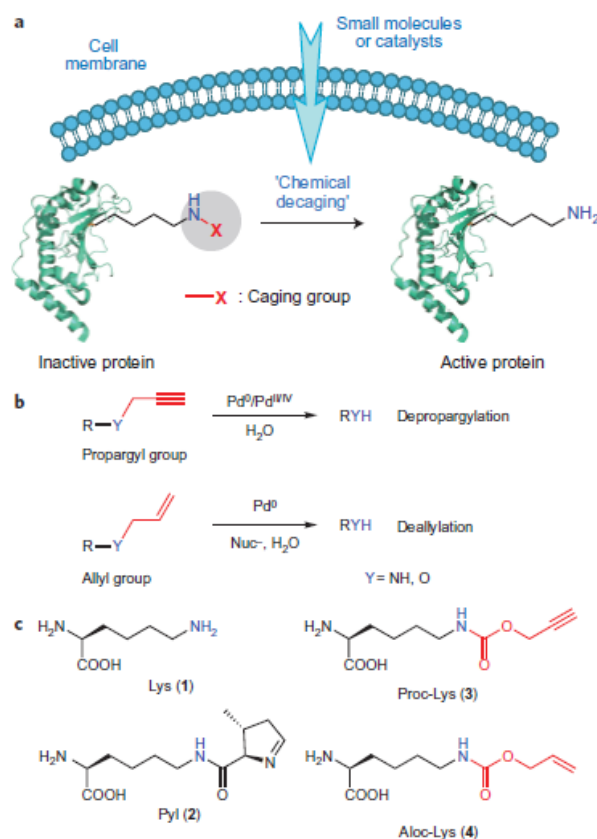
The team later studied the deprotection of the *N*-Alloc Doxorubicin (Graph 1. 3). They reported a good distribution of the Doxorubicin prodrug and even a synergistic effect while the mice were treated with the encapsulated caged and caged palladium complex, which was assessed by increased DNA damage of the cancer cells.



Graph 1. 3 Evolution of the tumoral volume depending on the time and the treatment.
From ¹

2.2.3.7 *In cellulo* protein decaging

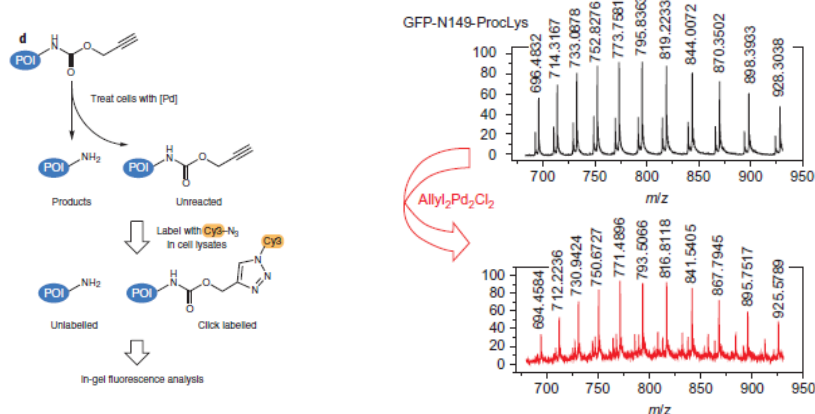
Another approach, reported first by Chen in 2014, is the “protein rescue”, where a genetically encoded protein contains a non-natural protected amino acid until it is deprotected (Scheme 1. 35).⁴⁸ The “protected protein” is thus inactive, provided the protected amino-acid is located at a critical position near the active site. Biological effects can thus be measured due to inactivity of the protein. Later, when the protecting group was cleaved, the activity could be restored and either the deprotected protein could be detected and quantified or a biological effect could be observed, and even quantified. Such protecting groups can involve the classical allyloxy carbamate (Alloc), Propargyloxy carbamate (Proc), Allenyl (Ale), Propargyl (Pro), and Allyl (All). The targeted residues are usually the amine-containing amino acids such as Lysine (Lys) or Pyrrolysine (Pyl, an amino acid encoded by archaea and some bacteria).



Scheme 1.35 Strategies employed in “protein rescue”. a) The general concept, b) possible leaving groups, c) Example of protected amino acids. From ⁴⁸

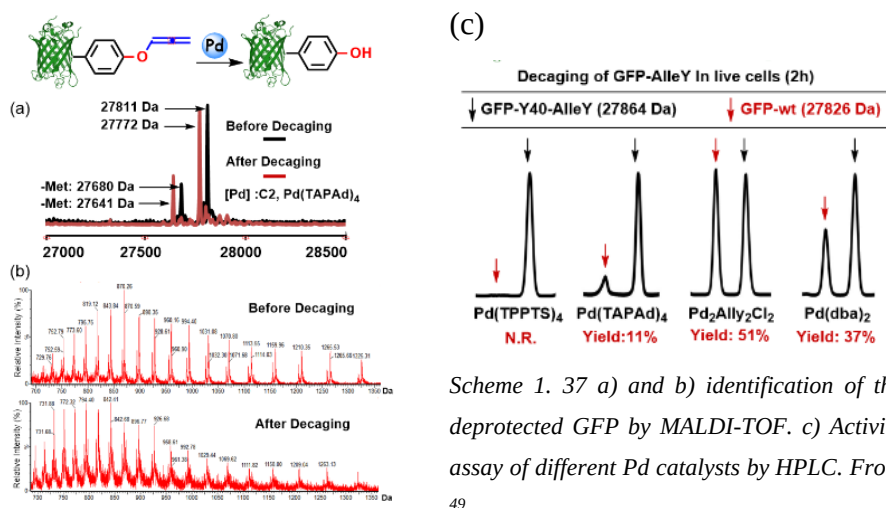
Chen reported that the simple and commercially available $[(\text{Allyl})\text{PdCl}]_2$ was an efficient catalyst to deprotect both **Rho(Alloc)**₂ and **Rho(Proc)**₂. He then developed a series of tools enabling him to qualify and quantify the amount of labelled and non-labelled protein (Scheme 1. 36). In case of a Proc protected amino acid on the protein, the non-deprotected residue still contained a triple bond, which was prone to be clicked to a cyanine-based dye in the cell lysate. By measuring the total amount of protein of interest in the gel and by quantifying the fluorescence of the post labelling of the non-deprotected protein, the yield can be calculated. Additional mass

spectrometry (typically MALDI-TOF) enabled them to see a mass shift in the pattern corresponding to the removed protecting group.



Scheme 1.36 Quantification of the deprotection of the protein of interest (POI): the unreacted Proc can be clicked to a cyanine-based dye to be quantified. B) Mass spectrum of the isolated protein indicates a shift due to the loss of the Proc group. From ⁴⁸

The same group in 2016 used the same technique to uncage Ale protected tyrosine in GFP-based systems (Scheme 1.37).⁴⁹ Ale was reported by the authors as a more reactive moiety than Pro in a deprotection context.

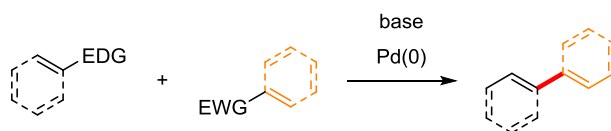


Scheme 1.37 a) and b) identification of the deprotected GFP by MALDI-TOF. c) Activity assay of different Pd catalysts by HPLC. From ⁴⁹

2.2.4 Cross-coupling reactions

2.2.4.1 General considerations

Cross-coupling is a reaction aiming to couple two partners A and B to form the coupling adduct A-B. The cross-coupling reaction distinguishes itself from homocoupling reaction, where A can be dimerized to A-A. Cross-coupling development has faced the problem to minimize homocoupling product to strictly favour hetero couplings.



Depending on, the exact nature of the partners, several cross-coupling reactions have been reported and are summarized in Table 1.3. Nowadays, the most famous and widely used ones are often associated with palladium and were awarded the Nobel Prize in 2010.

Table 1. 3 Summary of the 9 most famous palladium-catalysed cross-coupling reactions

Entry	Nucleophile	Electrophile	Co-catalyst/ additive	Name	Biocompatibility
1	Boronic acid Boronic ester	Vinylic/aromatic halide/triflate	Base	Suzuki- Miyaura	+
2	Stannane	Vinylic/aromatic halide/triflate	Base	Stille	-
3	Zincic	Vinylic/aromatic halide/triflate	base	Neigishi	-
4	Silane	Vinylic/aromatic halide/triflate	Fluoride	Hiyama	-
5	Grignard reagent	Vinylic/aromatic halide/triflate	None	Kumada- Coriu	-
6	Alkene	Vinylic/aromatic halide/triflate	Base	Heck	+
7	Copper	Vinylic/aromatic	Cu (I),	Sonogashira	+

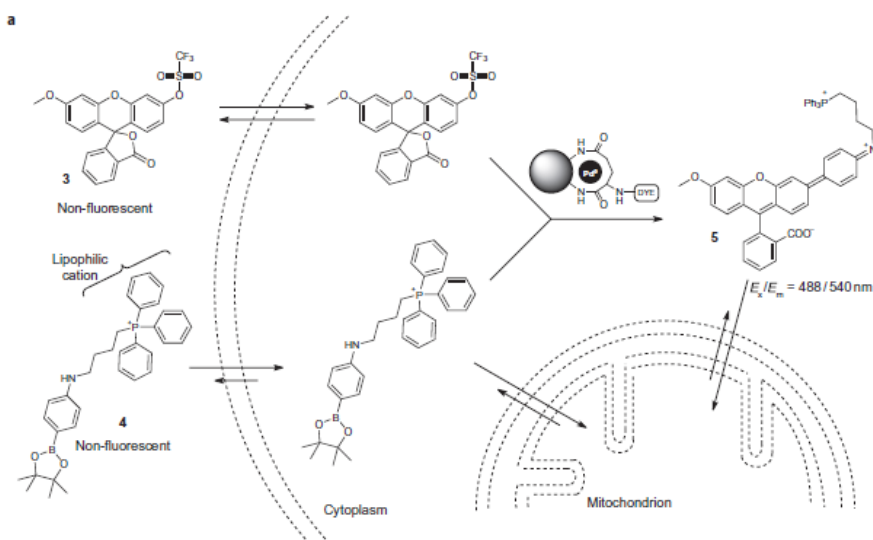
	acetylide	halide/triflate	base		
8	Trifluoroborate	Vinylic/aromatic halide/triflate	Base	Suzuki- Molander	-
9	Amine	Vinylic/aromatic halide/triflate	Base	Buchwald- Hartwig	+

Among the nine presented Palladium-catalysed cross-couplings (Table 1. 3), the Stille coupling and the Hiyama coupling are excluded for biological applications as the tin and silicon derivatives are too. Then, the Kumada coupling and the Negishi coupling are excluded due to the incompatibility of the zinc and magnesium reagents in an aqueous medium. However, the Suzuki coupling, the Buchwald-Hartwig coupling, the Sonogashira coupling, the Heck coupling are theoretically compatible with the biological medium. Indeed, there appear to be several reports of *in cellulo* Suzuki and Sonogashira couplings. Surprisingly, the Heck coupling is poorly documented although more attention should be focused for either simple cyclization or triggering a cyclization cascade to finalize the synthesis of a drug *in cellulo*. Finally, to the best of our knowledge, Suzuki-Molander coupling was never documented *in cellulo*.

2.2.4.2 Suzuki-Miyaura reaction

Compared to the Tsuji-Trost reaction, where classically a first-order for the substrate is observed, this type of coupling needs either a high concentration of both coupling partners or a huge excess of a coupling partner when the other one is a biomolecule such as a protein. The direct consequence is often longer reaction time and lower efficiency.

The first example of *in cellulo* cross coupling was reported by Bradley with the previously described Pd (0) nanoparticles supported on polystyrene resin (See Paragraph 2.2.3.5).⁴⁶ In 2011, he was able to restore the fluorescence of a protected fluorescein with one side a methoxy group and on the other side a triflate, blocking thus the conjugation. By coupling a 4-amino phenylboronic ester, the nitrogen could exert a push-pull effect, opening the spirocycle and restoring the fluorescence (Scheme 1. 38). A triphenylphosphonium cation, which was the same strategy used by Mascareñas used in 2016, enabled to target the mitochondria. Micrograph revealed the nucleus in blue, the mitochondria in red and the released fluorescein in green (Figure 1. 17). Additionally, the location of the catalyst beads was ensured provided they feature a cyanine-based dye tethered to the nanoparticle organic core.



Scheme 1. 38 Concept of *in cellulo* Pd catalysed Suzuki coupling to form a rhodamine with an affinity for mitochondria. From ⁴⁶

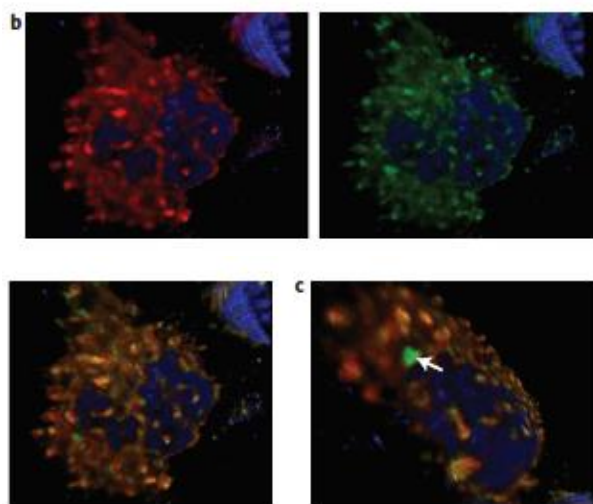
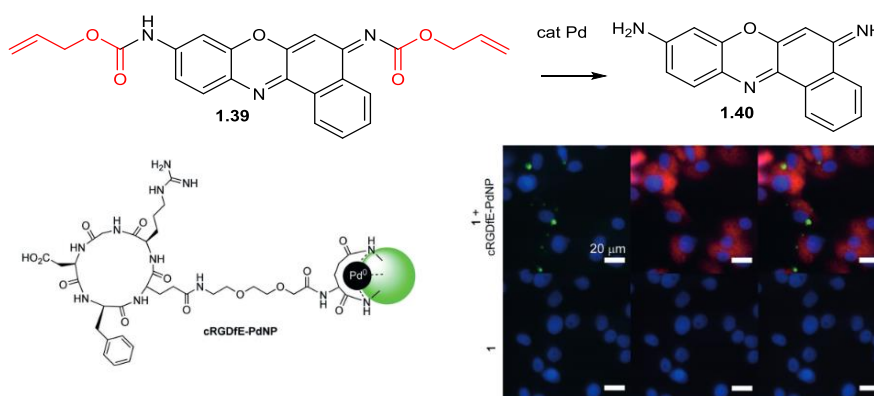


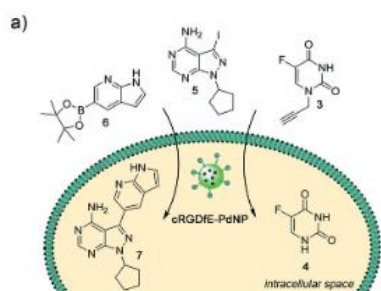
Figure 1. 17 Fluorescence images. Top left: nucleus, blue. Mitochondria green. Bottom left image, merge of top images. Bottom right image: another angle, the catalyst can be seen in bright green. From ⁴⁶

The system was later adapted to trigger glioblastoma cells by tethering a cyclic peptide which is able to cross the haematoma-encephalic barrier (Scheme 1. 39).⁵⁰ The catalyst was first validated on a slightly different phenoxazine-cored fluorogenic **1.39** substrate by cleaving both Alloc groups.

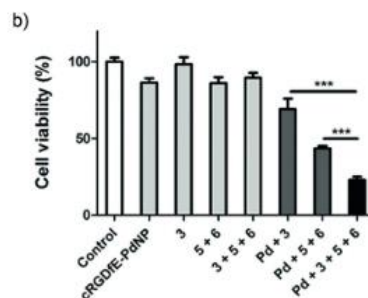


The same catalyst was also employed to assemble *in cellulo* a drug (**7**) by a Suzuki coupling between an aryl iodide (**5**) and a BPin

moiety (6) (Scheme 1. 40).⁵⁰ A synergic effect was noted when the authors carried out the Suzuki coupling and the deprotection of 5-Fluorouracil (4) from its prodrug (4), which is an RNA transcriptase inhibitor (Graph 1. 4).



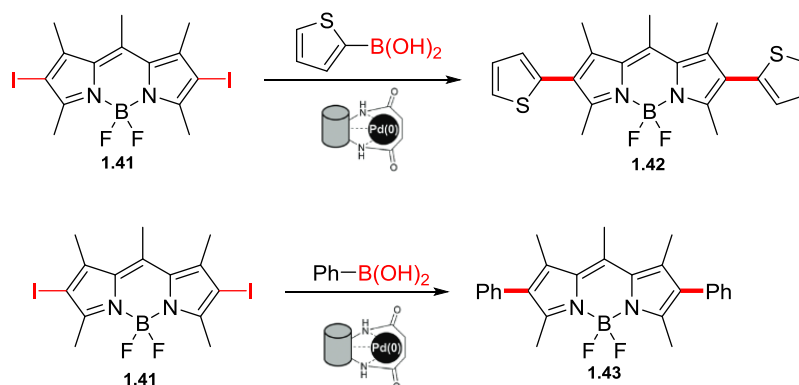
Scheme 1. 40 Principle of the dual targeting method: 5 and 6 are coupled to form 7 and 3 is deprotected to give 4.



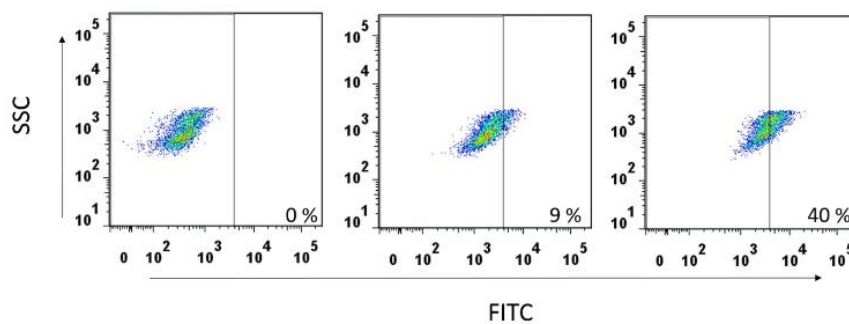
Graph 1. 4 Synergistic effects when the drug 7 is obtained by coupling and the 4-fluorouracil is obtained by deprotection.

From ⁵⁰

The concept was re-used by the same group to restore the fluorescence of a bis-iodo BODIPY **1.41**, whose fluorescence was quenched due to the presence of both heavy atoms (Scheme 1. 41, Graph 1. 5, Figure 1. 18).⁵¹ The fluorescence could be restored by coupling either thiophen-2-ylboronic acid or phenylboronic acid in presence of the same Palladium-supported catalyst. However, the authors faced competition between de-iodination and the coupling. Nevertheless, a difference of 20 nm enabled the authors to discriminate both phenomena in their study.



Scheme 1.41 Suzuki coupling of bis iodo BODIPY **1.41** with thiophenyl boronic acid and phenylboronic acid



Graph 1.5 Assessment of the released fluorescence by flow cytometry. Left: control; Centre: starting material; Right: catalytic system and probe. From ⁵¹

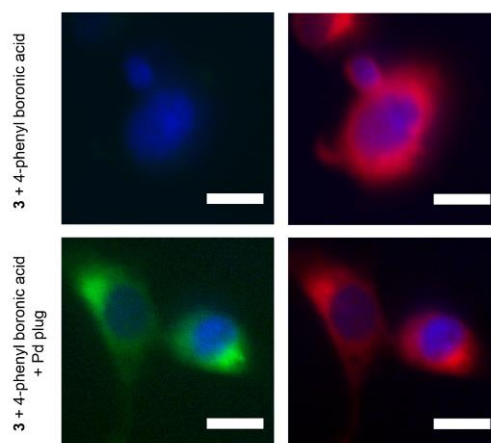
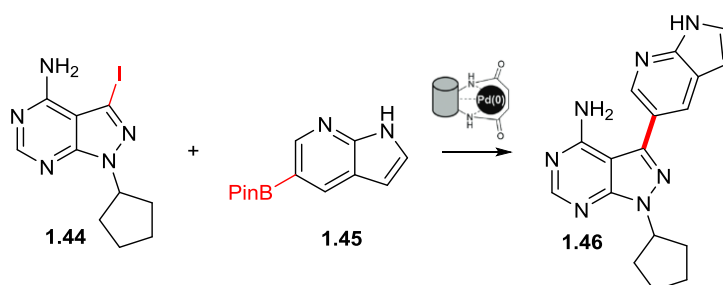
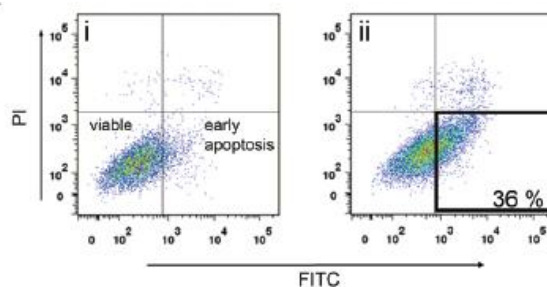


Figure 1.18 Fluorescence assay. Blue: nucleus, Red: mitochondria, Green: released probe. From ⁵¹

The same catalyst was then used to assemble the drug PP121 (**1.46**), which is an mTOR inhibitor, by coupling the heteroaryl pinacol boronate **1.45** with a heterocyclic iodide **1.44** (Scheme 1. 42, Graph 1. 6).⁵¹ The authors verified that the toxicity of the drug was superior to the toxicity of both components taken individually at the same concentration. However, noted a long reaction time, reaching 3-5 days and a moderate conversion of 70% due to the reasons exposed at the beginning of the paragraph.



Scheme 1. 42 Palladium (0) catalysed synthesis of mTOR inhibitor **1.46**.

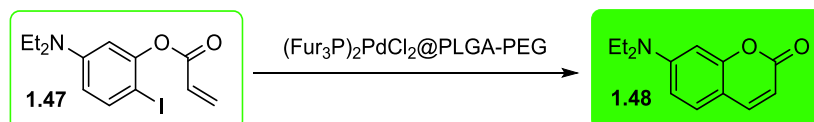


Graph 1. 6 Flow cytometry assay. Left: both precursors in absence of the catalyst Right: In presence of the catalyst. From ⁵¹

2.2.4.3 Heck reaction

Heck reaction is the only cross coupling reaction able to functionalize an unfunctionalized alkene thanks to a carbopalladation step followed by a β -hydride elimination to regenerate the palladium catalyst and restore the double bond.

Although this reaction is on paper totally biocompatible, there is only one example in the literature by Weissleder in 2018.¹ He was able to achieve the last step of the synthesis of an aminocoumarin **1.48** with an intramolecular Heck coupling (Scheme 1. 43).



Scheme 1. 43 In cellulo synthesis of 7-diethylaminocoumarine

It was then validated *in cellulo*, where the green fluorescence was observed by microscopy (Figure 1. 19).

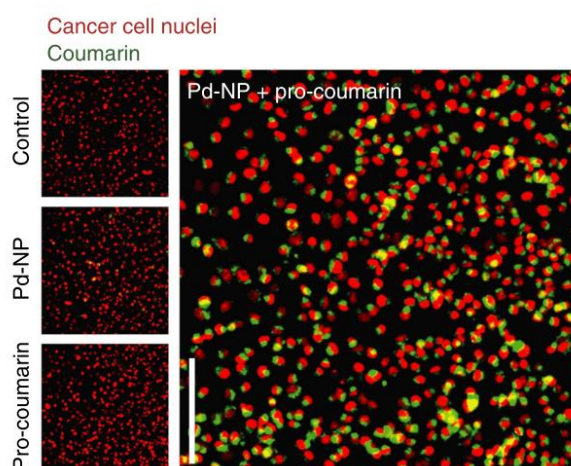


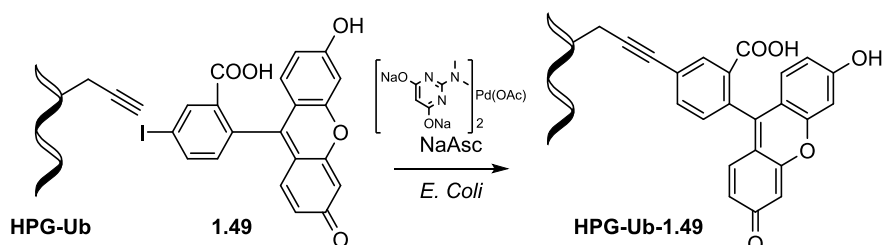
Figure 1. 19 In cellulo synthesis of 7diethylaminocoumarine

2.2.4.4 Sonogashira reaction

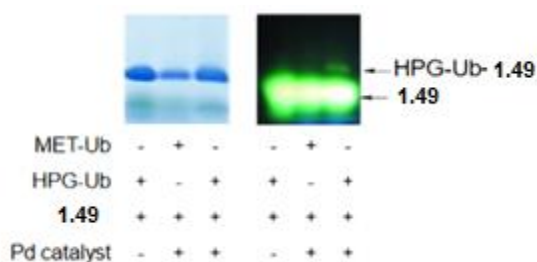
Sonogashira coupling is one of the most classical palladium catalysed coupling between an aryl iodide and an acetylene in presence of palladium (+II) and copper (+I). Whereas most of the time, copper is needed to activate the alkyne to copper acetylide in presence of a base, copper-free Sonogashira reaction features the advantage of avoiding the formation of acetylenic dimers *via* a Glazer-coupling side reaction in aerobic conditions. Moreover, the copper is

generally more toxic than metals like palladium and its use severely hampered broad applications in click chemistry *in cellulo*. The absence of copper also simplifies the catalytic system, which is critical while working in a complex biological medium.

While Bradley was developing Suzuki cross-coupling using nanoparticles as strategy,⁴⁶ Lin independently reported a broad-scope Sonogashira coupling method in the context of non-enzymatic protein post-functionalization (Scheme 1. 44) thanks to water-soluble ligands, such as 2-(dimethylamino)pyrimidine-4,6-diol and ascorbic acid to reduce the palladium.⁵² The reaction was then validated in *E. Coli* expressing a mutated Ubiquitin containing a homopropargyl glycine (HPG). Iodo fluorescein (**1.49**) was coupled via a Sonogashira reaction to the HPG-mutated Ubiquitin (**HPG-Ub**), and the coupling product was detected in the cell lysate (Scheme 1. 45).



Scheme 1. 44 Copper-free Sonogashira coupling between an HPG encoded Ubiquitin and an iodo fluorescein **1.49**.



Scheme 1. 45 Copper-free Sonogashira coupling between an HPG encoded Ubiquitin and an iodo fluorescein. From ⁵²

2.2.5 Metallodrugs and transfer hydride

In cellulo transfer hydride was discovered serendipitously by Sadler, who has been interested in ruthenium and iridium complexes acting as metallodrugs through some unknown mechanisms at the time. Metallodrugs are nowadays an expanding new class of drugs, which contain one or more metal atoms in their structure.⁵³ The most common metallodrug, cis-platinum, is a DNA crosslinking compound. Theoretically, transition metals offer many more modes of action than classical drugs due to the coordination fashion of metals at every stage of oxidation.

Whereas the first strategy of metallodrug design involves adding metal groups to an already existing bioactive organic ligand to improve some binding or pharmacological properties,⁵⁴ some novel structures with no similarity to existing drugs were also found to be active and selective. The most prevalent examples in this category are $(\eta^6\text{-C}_6\text{H}_6)\text{Ru}(\text{metronidazole})\text{Cl}_2$ for its anti-proliferative activity (Figure 1. 20) and $(\eta^6\text{-arene})\text{Ru}(\text{PTA})\text{Cl}_2$, nicknamed *RAPTA*. Its toxicity arises from interactions mostly with proteins rather than DNA.

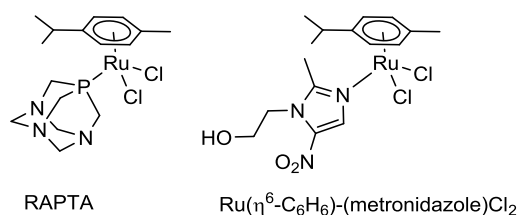


Figure 1. 20 Most famous ruthenium anti-cancer molecules

The idea of bypassing NADH-dependant reductases and mimicking their activity with transition metal complexes has been discovered by Sadler. To understand how it was found, the different steps are detailed below.

2.2.5.1 NADH/NAD⁺ disruption: NAD⁺ reduction

Sadler has been interested in the toxicity of [(arene)RuL₂X] compounds since 2001.⁵⁵⁻⁵⁶ He reported that [(*p*-cym)Ru(en)Cl] (*p*-cym = 4-methyl-1-isopropylbenzene; en = NH₂-CH₂-CH₂-NH₂), Figure 1. 21, was inhibiting the growth of the human ovarian cancer cell line A2780. This type of ruthenium complex shows similarities with Noyori catalysts, widely used in transfer hydrogenation with formic acid or isopropanol. They were used by Sadler in the context of NAD⁺ regioselective 1,4 reduction to NADH (See First part, Paragraph 1.2.2). *In cellulo*, this new scaffold was first assumed to have a mode of action similar to *cis*-platinum by binding to guanine,⁵⁷⁻⁵⁸ but the authors remained open to new putative mechanisms, such as redox activity by interfering with NADH/NAD⁺ balance, which was an unprecedented mode of action for ruthenium (III) complexes. The subject was well reviewed by Sadler in 2005.⁵⁹

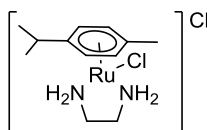
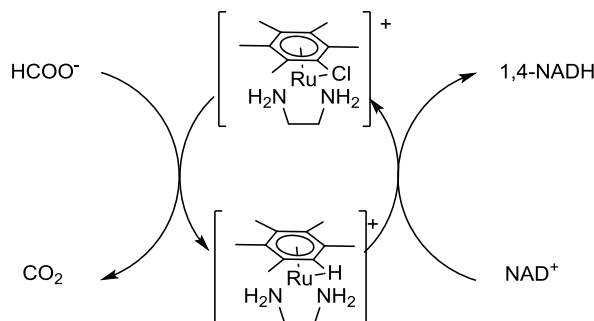


Figure 1. 21 [(p-cym) Ru(en)Cl] Cl

In that same review, Sadler stated “*However, such reactions [NADH 1,4 reduction] are slow and formate is scarce in eukaryotic cells. It seems unlikely therefore that these reactions are of importance for the anticancer activity of Ru (II) arene complexes*”.⁵⁹ However, one year later, Sadler eventually remarked that this family of Ru (II) complexes were actually capable of selective 1,4 reduction of NADH, yet with low TOF of 1 h⁻¹ for all the analogues he tested.⁶⁰ In the same article, the authors already stated that they know that A549 cells are tolerant to 2.5 mM of HCOONa, the highest concentration tried. They presented the idea to reduce cellular NAD⁺ to NADH as a perspective at the end of their paper (Scheme 1. 46).



Scheme 1. 46 Formate driven ruthenium hydride formation followed by reduction of NAD⁺ to 1,4-NADH

In 2012, some improvements were brought by Sadler.⁶¹ By adding a sulphonamide moiety (Tfen, Tsen or Msen) (Figure 1. 22), the TOF was reaching 6.4 h⁻¹ thanks to better stabilization of the excess of electrons coming from the hydride. The proposed mechanism by Sadler, based on kinetic studies, proposed a

coordination of the NH_2 to the oxygen of the amide bond of NADH accounting for the selectivity of the hydride transfer on the 4 position.

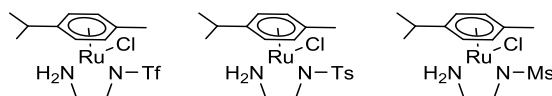
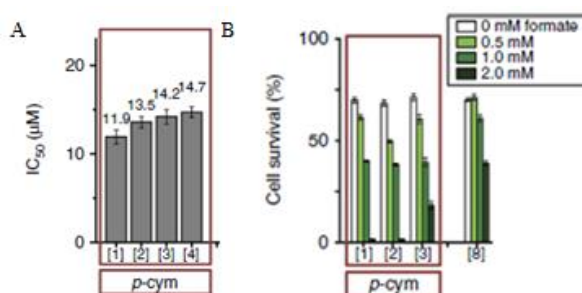
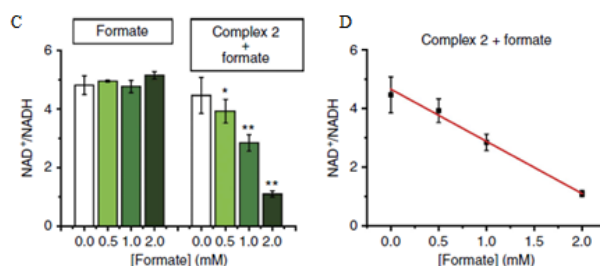


Figure 1. 22 Improved ruthenium anticancer molecules with the ability to reduce NAD^+ to 1,4-NADH

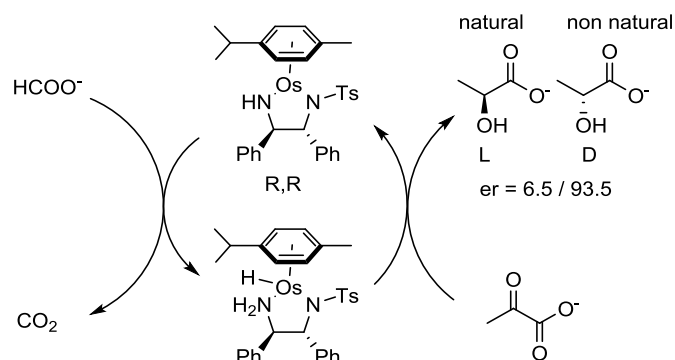
This improvement paved the way for biological applications, now that sufficient turnovers were reached. Sadler reported in 2015 that the toxicity of his leading ruthenium (II) sulfonamido ethylenediamine complexes against human ovarian cancer cells is strongly enhanced by the presence of non-toxic millimolar concentrations of formate (Graph 1. 7).⁶² The combination of the ruthenium catalyst and formate triggered an increase of the NADH/NAD^+ ratio, leading to a novel “reductive stress”. It was rationalized by the authors as the transfer hydride reduction of NAD^+ to NADH. This mechanism does not involve apoptosis or perturbation of mitochondrial potentials. In the study, the bpy analogue was found to be ineffective, and the highest activity was seen for NbEn ligand, a strong electron withdrawing group, was the most active catalyst, despite still low TON of 4.1 h^{-1} in water and high concentrations of formate needed to observe an effect (0.5 to 1 mM as threshold).





Graph 1. 7 **A)** IC_{50} for four complexes in A2780 cells: (1) [(p-cym)Ru(Msen)Cl], (2) [(p-cym)Ru(Tsen)Cl], (3) [(p-cym)Ru(Tfen)Cl], (4) [(p-cym)Ru(NO₂En)Cl. (8) [(bpy)Ru(en)Cl]⁺ **B)** Cell survival when $C = 1/3$ of IC_{50} of each complex is co-administered with increasing concentrations of HCOONa. **C)** Variation of the $NAD^+/NADH$ ratio in A2780 cells for different HCOONa concentration with or without (p-cym)Ru(Tsen)Cl for $C = 1/3$ IC_{50} . **D)** This graph shows the linear correlation between the $NAD^+/NADH$ ratio and the formate concentration

At last, Sadler even showed that they could carry out asymmetric reductions in cells, such as pyruvate to D-lactate, which can be easily differentiated from the natural L-lactate as it is the non-natural enantiomer (Scheme 1. 47).⁶³ This reaction meets the criteria of bioorthogonality. The authors used an improved osmium complex, (p-cym)Os(TsDPEN)Cl and the L or D enantiomer could be obtained by using either (R, R) or (S, S)-TsDPEN ligand. The authors highlighted that the catalyst efflux was low, which tended to show that the catalysis happened in an intracellular fashion, especially because pyruvate is absent from the cellular medium.

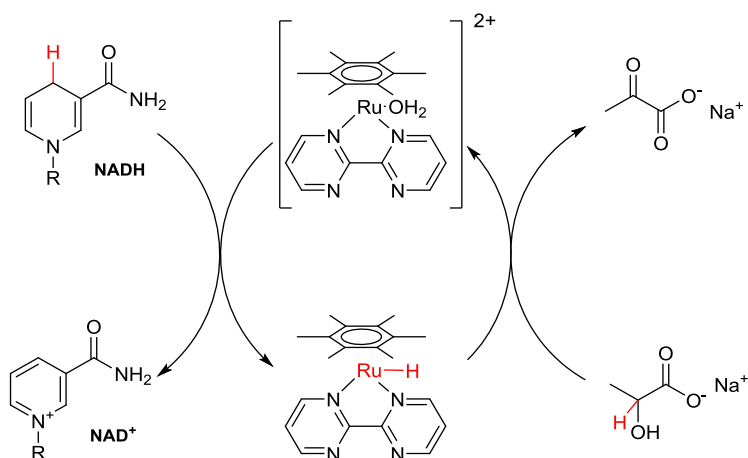


Scheme 1. 47 Osmium-mediated asymmetric reduction of pyruvate to lactate ($\text{er} = 93.5/6.5$ for D-Lactate when (R, R) catalyst is used)

2.2.5.2 NADH/NAD⁺ disruption: NADH oxidation

a) Re-discovery of NADH oxidation

When Sadler reported his first anticancer ruthenium complexes in 2002-2003,⁵⁷⁻⁵⁸ he was convinced that new modes of action will be found for this family of metallodrugs. It is only in 2012 that Sadler re-discovered the reverse reaction.⁶⁴ Consistently with the reports by Fish that the hydride transfer was reversible in 2001⁶⁵, Sadler noted that some complexes featuring a bipyrimidine ligand, such as (hmb)Ru(bpm)Cl, had a tendency to abstract the hydride of NADH to form NAD⁺ and the ruthenium hydride (Scheme 1. 48). It was easily identified by ¹H-NMR with a peak at -7.44 ppm (Figure 1. 23). In NMR experiments, the hydride transfer was seen to be fast (15 min) and was stable enough to be observed in non-degassed solutions for almost 4h 30. The authors demonstrated that the *in-situ* generated hydride could be transferred to a biologically relevant substrate such as pyruvate to reduce it into lactate. Low yields were observed due to the poisoning of the catalyst by carboxylate adducts.



Scheme 1. 48 In-situ hydride transfer from NADH to a ruthenium complex and subsequent reduction of pyruvate to lactate

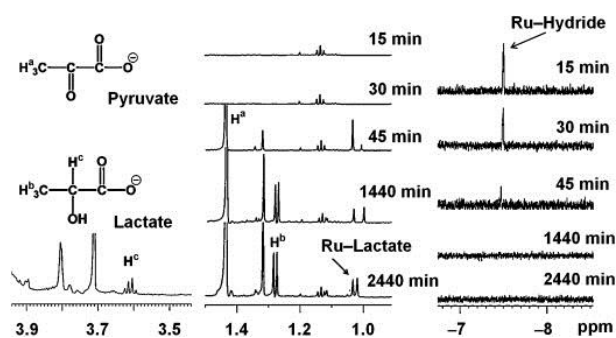
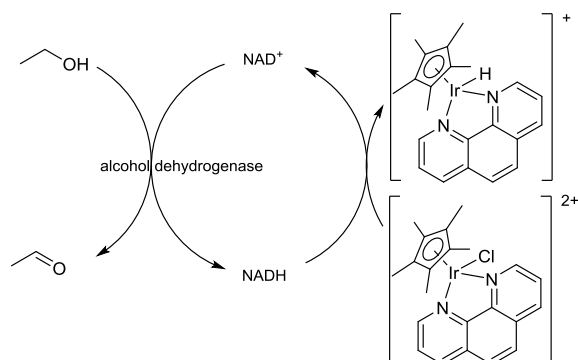


Figure 1. 23 NMR spectrum shows the reduction of pyruvate (H^a) to lactate ($CH_3 = H^b$ and $CH = H^c$). Ru-lactate adducts detected. Right: observation of the Ru-hydride with 1,4-NADH and 1 equivalent of Ru complex in 10% D_2O in H_2O at 310 K.

$[(\eta^5\text{-Cp}^*)\text{Ir(phen)(H}_2\text{O)}]_2$ exhibits the same type of properties and can be reduced by NADH (Scheme 1. 49). Moreover, this complex shows compatibility with enzymes, as it can react with NADH generated by an enzymatic reaction (oxidation of ethanol by ADH) in the context of pyruvate reduction.



Scheme 1. 49 Iridium hydride formation thanks to NADH produced by the action of alcohol dehydrogenase and ethanol as a hydride donor

The present discovery led to two different applications. The first one, followed by Sadler, was to the fact that hydride could be transferred to molecular oxygen, catalytically causing the formation of ROS, for improved cytotoxicity. In the second one, Ariga and Do used the generated hydride in redox-sensitive sensors.

b) Catalytic transfer reduction of oxygen to produce ROS

Starting from the $[\text{Cp}^*\text{Ir}(\text{phen})\text{Cl}]\text{Cl}$, Sadler improved the design of the complex for better cytotoxicity (Figure 1. 24).⁶⁶ The Cp^* was improved by adding a biphenyl in place of a methyl for better DNA intercalation. The replacement of the phenanthroline by a phenyl pyridine protected the complex from a fast aquation, which hampered the cell penetration. The replacement of the chloride by a pyridine protected the complex against GSH poisoning. The new catalyst thus exhibited an unprecedented ability to catalyse the formation of H_2O_2 from NADH and molecular oxygen by a catalytic hydride transfer and insertion of the molecular oxygen in the metal-hydride bond, followed by disproportionation (Graph 1. 8). The direct consequence was oxidative stress.

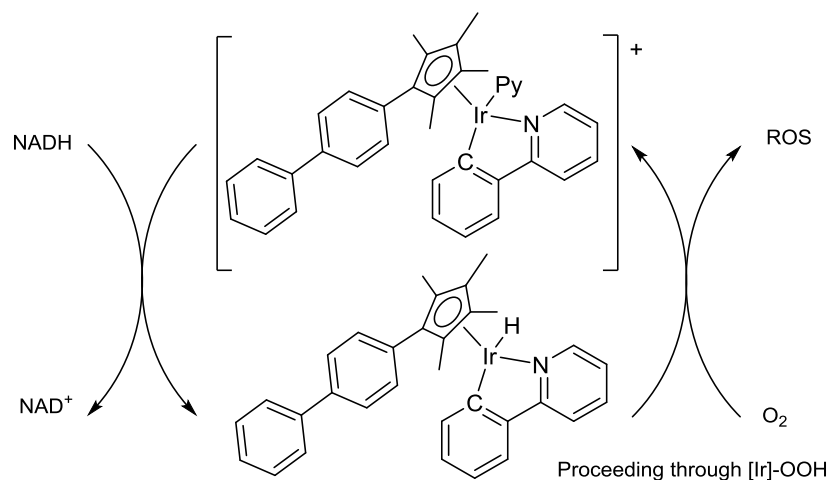
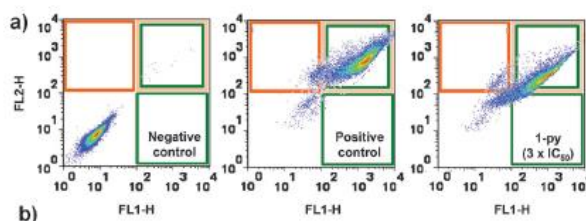


Figure 1. 24 Sadler's best anticancer organoiridium agent

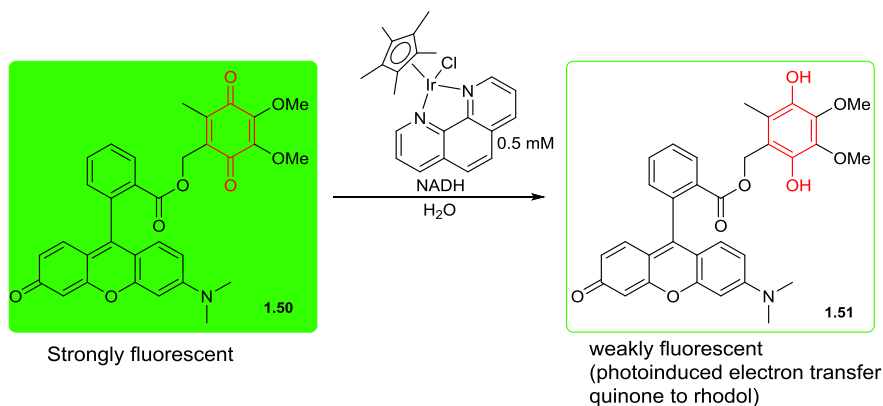


Graph 1. 8 Flow cytometry: the Ir complex induces ROS production (right) while compared to a positive and negative control (respectively centre and left)

c) Redox sensors activated by transfer hydride reductions

Very soon after Sadler's report⁶⁴, Ariga reported a fluorescent on-off sensor based on Ubiquinone – Rhodol **1.50**, whose fluorescence could be quenched by the reduction of the quinone to the hydroquinone **1.51**, which is virtually fluorescent. After identifying the catalyst $[\text{Cp}^*\text{Ir}(\text{phen})(\text{H}_2\text{O})]^{2+}$ as a non-toxic and biocompatible transfer hydride catalyst, he applied the system on HeLa cells stained (10 μM) with his probe (Scheme 1. 50). After treatment with 0.5 mM of Cp^*Ir complex and addition of sodium pyruvate in the medium (in order to activate the tricarboxylic acid cycle and cause an increase of cellular NADH concentrations) the fluorescence was reduced by 90%

within 5 minutes, which is a sign of the reduction of the quinone by the iridium hydride (Figure 1. 25).



Scheme 1. 50 Ubiquinone-Rhodol On-Off probe used by Ariga. Fluorescence is lost upon the reduction of the quinone by the iridium hydride

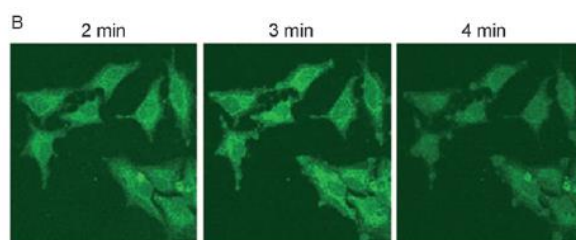
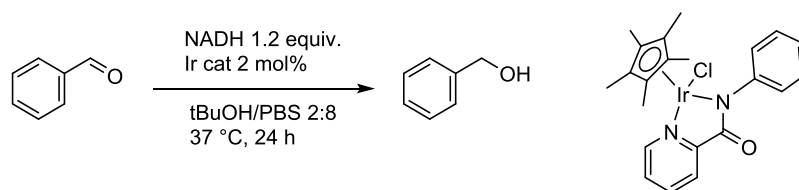
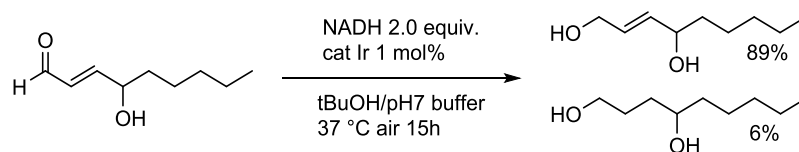


Figure 1. 25 Fluorescence images of HeLa cells incubated with the probe 10 μ M and the Ir complex 0.5 mM. Excitation at 488 nm

In another way, in 2016, Do proposed that the iridium hydride could be useful to carry transfer hydride reduction of instaurations such as aldehydes or ketones (Scheme 1. 51).⁶⁷ By fine-tuning the catalyst, he could reduce aldehydes in water/^tBuOH medium using NADH as hydride donor with up to 2 mol% of Ir catalyst. The authors verified that the postulated iridium hydride was the active species. Although the authors promote their work, stating they can reduce cytotoxic aldehydes (Scheme 1. 52), applications *in cellulo* of the methodology were missing in the paper, which is posterior to our research (2013-2016).

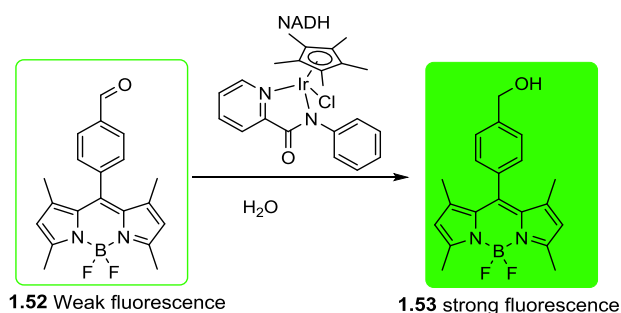


Scheme 1. 51 Catalytic transfer reduction of aldehydes using an Ir catalyst and NADH as a hydride source



Scheme 1. 52 Catalytic transfer reduction of cytotoxic aldehydes employing the newly described method.

In 2017, the same author described the reduction of a BODIPY–aldehyde probe **1.52**, whose fluorescence is diminished after the reduction of the aldehyde by NADH and an iridium catalyst (Scheme 1. 53).⁶⁸ Using their described lead catalyst, cells treated with the probe saw their fluorescence divided by a factor two upon treatment. To test their hypothesis, when NADH levels were depleted by adding pyruvate, no increase of fluorescence was noted by the authors (Figure 1. 26).



Scheme 1. 53 in cellulo reduction of the bodipy-aldehyde (weakly fluorescent) to the alcohol (strongly fluorescent) with the previously described Ir catalyst

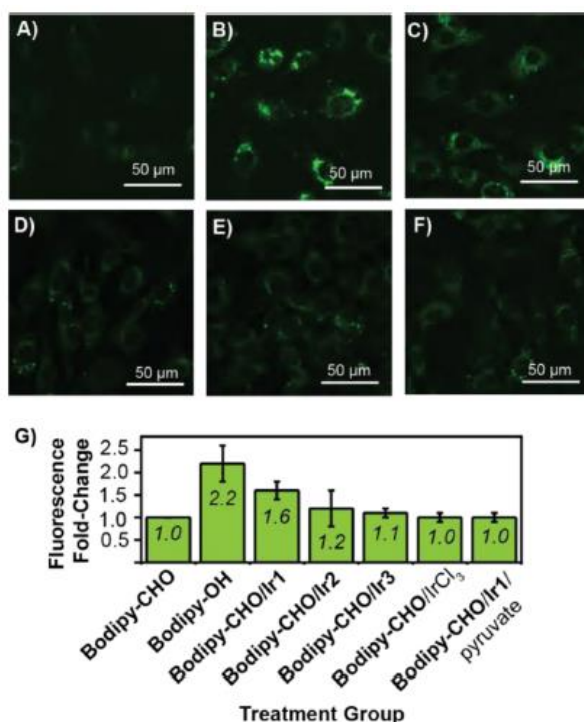
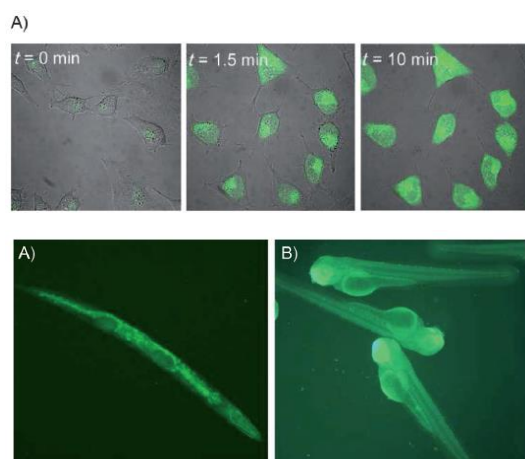
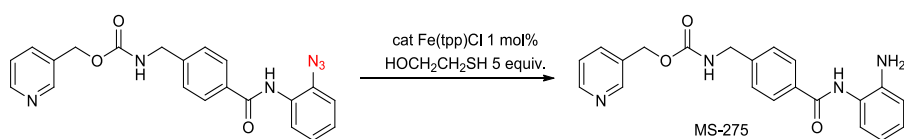
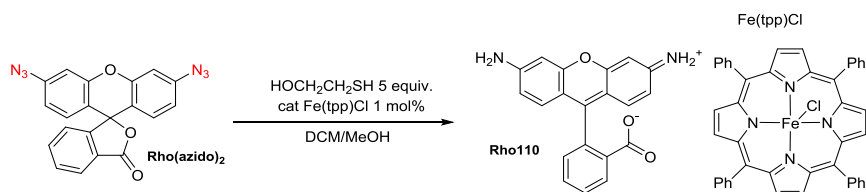


Figure 1. 26 Fluorescence image and intensity of the fluorescence relative to the images above in fibroblast cells.

2.2.6 Iron catalysis

In addition to the previously described examples, one last example does not fall into the previous categories, as it involves iron catalysis. Meggers reported in 2012 the reduction of the **Rho(azido)₂**, whose azides could be reduced to the amine to give the **Rho110** and to restore the fluorescence (Scheme 1. 54).⁶⁹ The reduction was catalysed by the Fe(tpp)Cl (tpp = tetraphenyl porphyrin) with low catalyst loadings and with mercaptoethanol as reducing agent. The model was successfully transposed to the reduction of the azido equivalent of the **MS-275** drug (Scheme 1. 55). The Rhodamine model was validated *in cellulo* but failed while used in nematodes and zebrafish embryo (Figure 1. 27). Similar to Bradley, the team experienced stability issues of their azide probes, which got digested

by the organisms in absence of a catalyst; no improvement was observed.



3 Conclusion

This introduction highlights one possible route which 3rd generation chemotherapies may follow in the future. Bioorthogonal uncaging of vectorized prodrugs offers significant advantages in terms of robustness of the protecting group, controlled time-space distribution and release. Very few reactions meet the strict criteria of bioorthogonality, namely inertness towards the biological materials, selectivity of the reagents for each other and non-toxicity. The only suitable reactions were the Staudinger ligation, taking benefits from the selective reactivity of phosphines and azides and their relative inertness towards biomolecules, and cycloadditions between azides (or various dipoles) and alkynes (or various dipolarophiles). Bioorthogonal prodrug activation offers the advantage that the protection cannot be circumvented by the metabolism and offers better control of the activation.

Bioorthogonal transition-metal catalysis was introduced in 2006 by Meggers who introduced the allyl carbamate deprotection thanks to four generations of ruthenium catalysts.³⁸ During the last twelve years, the bioorganometallic toolbox has been expanded by a significant number of new reactions mostly catalysed by palladium, ruthenium, iridium, gold, copper and osmium, as summarized below.

Table 1. 4 Most significant discoveries in bioorganometallic chemistry

Author (date)	Catalyst	Reaction
Meggers (2006)	Cp*Ru(COD)Cl	First allyl carbamate deprotection
Bradley (2011)	PdNP/PS	First Suzuki coupling, first example with Pd

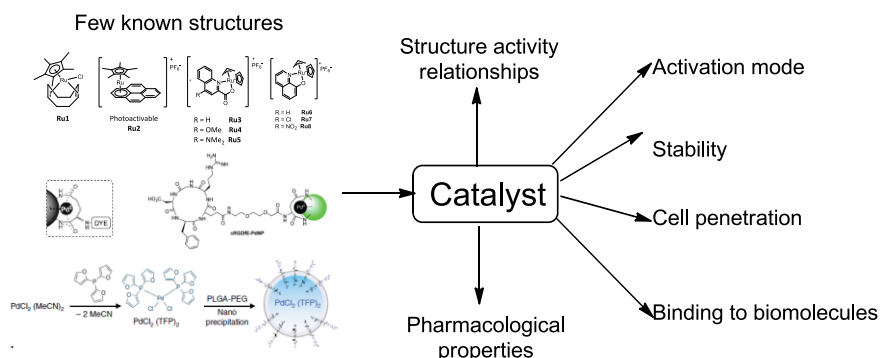
Mascareñas (2016)	Meggers 3 rd generation – phosphonium grafted	Uncaging localized in mitochondria
Weissleder (2017)	PdCl ₂ (Fur ₃ P) ₂ @Polymer	<i>In vivo</i> in mice

Although some new bioorthogonal functions were introduced, such as allyl and propargyl moieties as protecting groups, a few new problems were added, and all of those aspects have been poorly documented.

- Slower kinetics due to an additional molecule, the catalyst, in the global reaction scheme.
- Pharmacological properties of the catalyst (classical medicinal chemistry rules do not apply).
- Cellular distribution of the catalyst and cell permeability.
- Activation of the catalyst (reduction, nucleophilic attack).
- Deactivation and poisoning of the catalyst (nucleophiles, oxygen, water).
- Toxicity of the catalysts or potential intermediates.

Those results illustrate that there are opportunities for therapeutic applications, but to achieve this goal, more performant catalysts are needed, and their behaviour, ranging from distribution, stability, activity to excretion, have to be taken into consideration urgently.

An ideal catalyst candidate needs to be discussed under the light of the criteria explained above and summarized in Scheme 1. 56.



Scheme 1. 56 Questions to be addressed for bioorganometallic catalysis with Pd compounds in the context of Tsuji-Trost reaction

Aiming to develop therapeutic applications of bioorthogonal and transition-metal catalysed prodrug decaging, we saw an opportunity to develop new families of catalysts while addressing the aforementioned problems in a rational fashion. Bearing in mind the difficulties of administering transition metal catalysts in a complete organism, vectorization of discrete-molecule catalysts seems to be an unprecedented challenge which needs to be explored.

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**First part: Hydride transfers: From the
methodology to applications in prodrug decaging**

1 Introduction

The general introduction describes what has been done and gives a clear overview of what should be done to bring the chemotherapies activated by bioorthogonal metal-catalysed therapies to the front of the stage.

From what we presented before, we saw two major opportunities for research in the field. The first one involves transfer hydride reductions and will be the subject of this first part. The second part will be dedicated to Tsuji-Trost decaging of allyl carbamates with discrete palladium complex.

Regarding the hydride transfers, a literature introduction is necessary to understand clearly how the idea emerged from early ages of hydride transfer hydrogenation to biocompatible and biomimetic methodologies in water.

1.1 Transfer hydride reductions

Noyori was awarded in 2001 the Nobel Prize for his contribution to the development of asymmetric ketone reductions.⁷⁰ He developed the use of (*R* or *S*)-BINAP RuX₂ catalysts (Figure 2. 1) along with hydrogen to transfer the hydride with facial selectivity to reduce functionalized ketones to chiral alcohols.⁷¹⁻⁷² For simple ketones, the catalyst (*R* or *S*)-BINAP Ru (diamine) X₂ can be used.⁷³

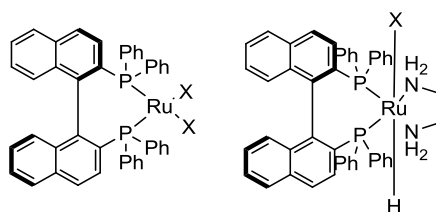


Figure 2. 1 First generation Noyori catalyst

Noyori introduced a new type of complex in 1995 which allowed reducing ketones and imines (Figure 2. 2).⁷⁴ The biphosphine ligand was replaced by *p*-cymene (4-isopropyl toluene). The enantioselectivity is achieved with the chiral diamine (*R, R*) or (*S, S*)-TsDPEN.

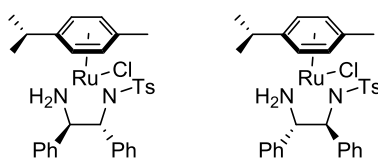


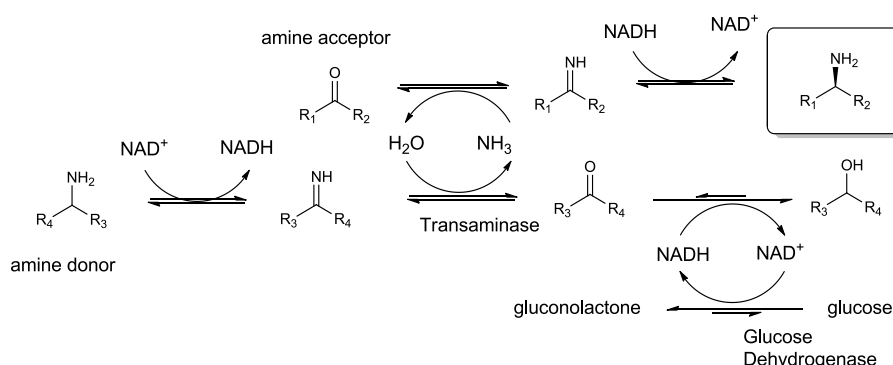
Figure 2. 2 Second generation Noyori catalyst (*p*-cym)Ru((*R,R* or *S,S*)-TsDPEN)Cl

This last type of complex can be used in combination with molecular hydrogen, isopropanol in basic conditions or formic acid to generate the hydride.

1.2 Non-enzymatic NAD⁺ reduction

Non-enzymatic NADH regeneration is a field of biocatalysis explored since the years 1980 in which rhodium catalysis and transfer hydride reduction played a key role. It is a particularly important technique in biocatalysis, as isolated enzymes may have a small driving force for the reaction, they catalyse but in a biological medium. In organisms, the driving force comes from a global process involving several enzymes and various reactions. The subject of non-enzymatic NADH regeneration was well reviewed by Ward and Hollmann.⁷⁵⁻⁷⁶

A state of the art example developed by Turner in 2014 involved a tailor-made transaminase (Scheme 2. 1), which could convert an amine acceptor such as a ketone into a chiral amine with high enantiomeric excess from a cheap amine donor.⁷⁷ The reaction itself lacks a driving force and needs a thermodynamic push in order to reach completion. The strategy employed by Turner involves the reduction of the produced ketone, which is the by-product of the amine donor, into an alcohol by a reductase and NADH. In order to regenerate the NADH, an additional enzyme, glucose dehydrogenase, needs to be used to regenerate NADH, reducing then NAD^+ into NADH. This costly method needs the use of two additional enzymes.

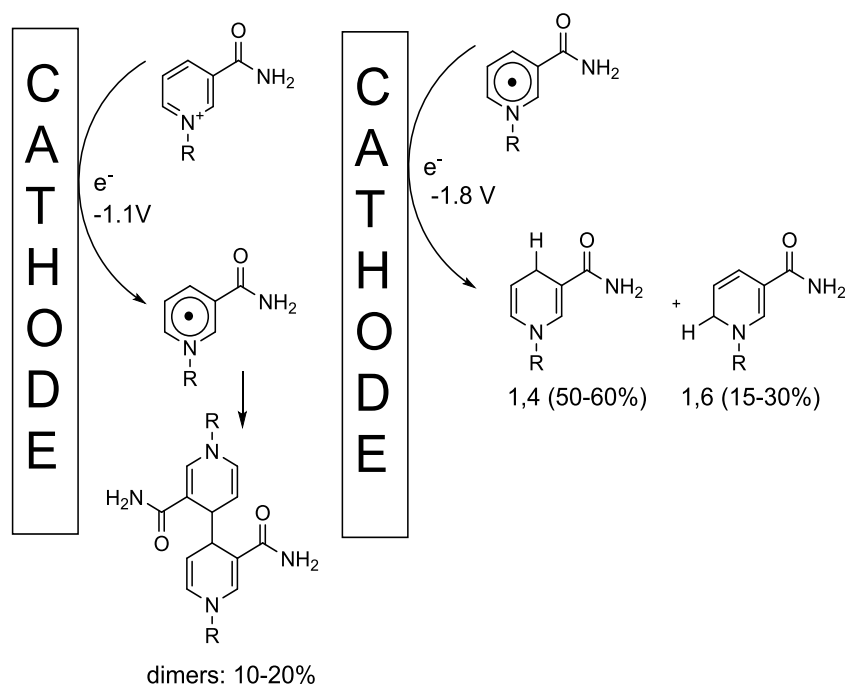


Scheme 2. 1 Importance of the NADH regeneration in a transaminase-integrated system. Here, it is an enzymatic regeneration of NADH with glucose

1.2.1 Electrochemical regeneration of NADH

NADH regeneration is a difficult reaction to carry out due to the possibility to form besides the enzymatically active 1,4- isomer the 1,2 and 1,6 isomers, as well as dimers, which are enzymatically inactive (Scheme 2. 2). If the method does not a selectivity of at least 99.5%, the undesired isomers will start to accumulate after a few hundred cycles. In this paragraph, we will examine the electrochemical reduction of NAD^+ to NADH.

Electrons are often viewed as the cheapest reducing agent. In 1981, Jaegfeldt was able to reduce NAD^+ to NADH using a mercury electrode.⁷⁸ Reduction with an electrode potential of -1.1 V leads to a massive formation of 4,4' dimers (90% of the total mixture) and up to 10% of 4-6' dimers who cannot be further reduced. A lower voltage up to -1.8 V is needed to further reduce the dimers into enzymatically active NADH with a yield of 50%, in a complex mixture with 30% 1,6-NADH and 20% of dimers. The absence of selectivity is explained by the stepwise monoelectronic transfer, leading to the radical, which can rapidly isomerize or dimerize to yield many by-products. A very low voltage of -1.8 V is therefore needed to further reduce the radicals before they further react. Direct reduction by a cathode does not appear as an appealing method due to the large cathodic potential applied for a low selectivity. There are very few reports of efficient electrochemical direct methods.⁷⁹

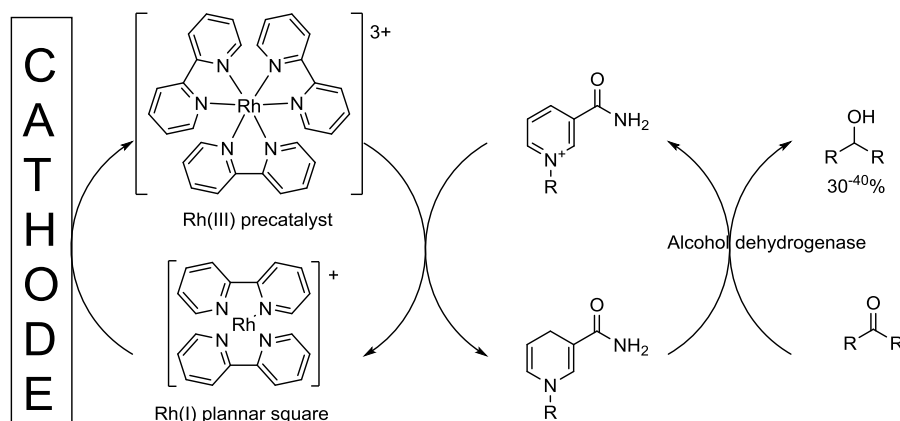


Scheme 2. 2 NADH electrochemical reduction of NAD^+ to NADH.

Rapidly, it appeared vital to use a redox mediator in order to increase the potential to values of -1.1 V or above and avoid the formation of radicals. One more constraint is to be able to carry out the NAD^+ reduction in presence of the enzyme and its substrate without interference.

An improvement came from the implementation of the use of rhodium (+III) tris-bipyridine chloride, $[\text{Rh}(\text{bpy})_3]\text{Cl}_3$, as a valuable mediating agent by Steckhan in 1982 (Scheme 2. 3).⁸⁰ With only -730 mV, the $[\text{Rh}(\text{bpy})_3]\text{Cl}_3$ was reduced to the square-planar and cationic rhodium (+I) bis bipyridine, which then reduced NAD^+ to NADH by a bielectronic transfer, avoiding thus the formation of radicals. With only -730 mV, the electrode was not able to reduce directly NAD^+ to the radical, so no virtually no dimer (0.9% max) was observed. The

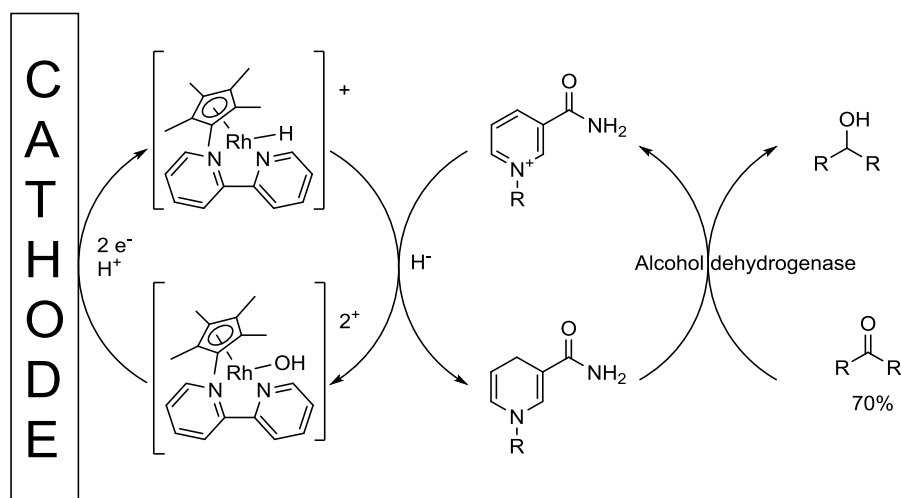
reaction, however, suffered from low a TOF of 0.5 h^{-1} and passivation of the cathode by $[\text{Rh}(\text{bpy})_2(\text{OH})_2]\text{Cl}$.



Scheme 2. 3 Electrochemical regeneration of NADH with $[\text{Rh}(\text{bpy})_3] \text{Cl}_2$ as redox mediating agent.

Further improvement was brought by Steckhan in 1987 by using $[\text{Cp}^*\text{Rh}(\text{bpy})\text{Cl}]\text{Cl}$ as a second-generation redox mediator.⁸¹ Steckhan reported improved compatibility with enzymes, a 10-times enhancement of the TOF and a TON reaching 14. The system did not have the capability to discriminate NADP^+ and NAD^+ as the phosphate moiety is too far away from the metallic centre. The system was able to perform between pH 4 and 9 and to temperatures reaching 70°C . The irreversible cathodic peak of -0.71 V prevented the formation of dimers and the reaction had an exquisite close-to-total selectivity for the 1,4 regioisomer.

A proof of concept of the regioselectivity was made by coupling the NADH generation to an enzymatic reduction of a ketone by an alcohol dehydrogenase, as enzymes only recognize the 1,4 isomer of NADH and leave the other isomers such as 1,6 unreacted (Scheme 2. 4).



Scheme 2. 4 Electrochemical regeneration of NADH with $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$ as redox mediating agent.

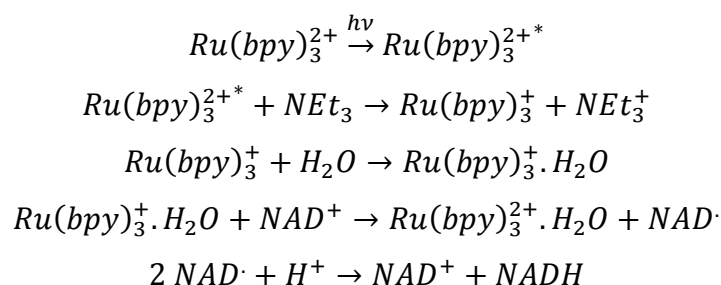
1.2.2 Chemical regeneration of NADH

The previously mentioned reports enlightened the efforts put for the development of methods to efficiently regenerate NADH from NAD^+ by using electrons as the reductant. Despite several successful attempts, the TON still remained limited. Electroenymology is not a standard technique and needs special equipment. It appeared a need for easier to use and more effective methods to regenerate NADH in a selective 1,4 way with fewer side products.

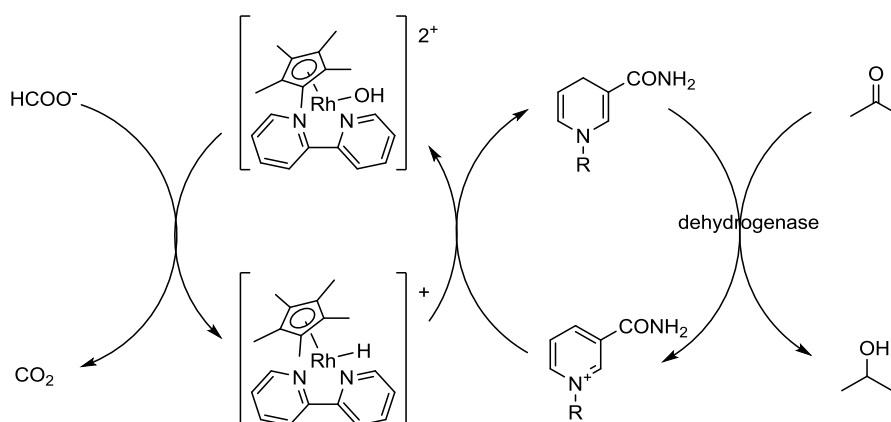
Classical reductants such as NaBH_4 are inefficient at achieving selectivity for 1,4-fashion reduction of pyridine cores and give a mixture of isomers depending on the substituents, as well as over-reduced products.⁸² Sodium dithionite is suitable to reduce NAD^+ to NADH in a selective 1,4 manner in a slightly basic medium with only small traces amounts of 1,2 and dimers. However, it is incompatible with enzymes.

At the same time, Jaegfeldt⁷⁸ laid the foundations of non-enzymatic NADH regeneration, Kiwi came in 1981 with a proposition

of electrochemical method based on the $Ru(bpy)_3^{2+}$ as photosensitizer and Et_3N as a sacrificial electron donor.⁸³ The method relied on the photochemical activation of the ruthenium (+II) tris-bipyridine complex by light, followed by a reduction by Et_3N which transfers an electron to the ruthenium, which becomes ruthenium (+I). After the formation of the aqua adduct, it transfers an electron to NADH to the radical. The problems of the radicals are fully described previously. The disproportionation of the radical yields some valuable 1,4-NADH but many side products as isomers or dimers.



The use $HCOONa$ as a reductant and the cationic $[Cp^*Rh(bpy)]^{2+}$ was unanimously elected as the simplest, most efficient and most tolerant system. It was reported by Steckhan in 1988 (Scheme 2. 5).⁸⁴ Formic acid is a medium-strength acid with a pKa of 3.77. At neutral pH, it is present under its deprotonated form, the formate. With a reduction potential around -0.4 V at pH 7,⁸⁵ it is one of the simplest, cheapest, simplest and cleanest reductants as it only gives carbon dioxide as a by-product, and sodium formate is a crystalline solid which requires virtually no precaution for handling. It has been extensively used to reduce various substrates including ketones, aldehydes, aldimines and ketimines.



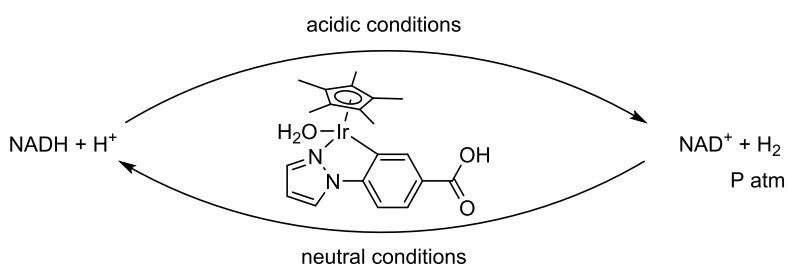
Scheme 2. 5 Formate-driven NADH regeneration involving the $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$ complex as a chemical mediator

The newly described method reported by Steckhan used the same rhodium precatalyst as used for the indirect electrochemical regeneration.⁸¹ However, it distinguishes from the previous reports by a TOF of 82 h^{-1} compared to 5 h^{-1} obtained by electrochemistry. One of the major factors is the avoidance of the diffusion limit around the cathode as formate is fully soluble.

One major key difference with the electrochemical method is the absence of oxygen-sensitivity. It was postulated by the authors that the mechanism should be different, even if both methods involve the same key intermediate, the rhodium hydride. It was suspected at very early stages that the Rhodium (+I) intermediate $[\text{Cp}^*\text{Rh}(\text{bpy})]$ was not an intermediate in the Formate-driven NADH regeneration but was an intermediate of the electrochemical regeneration pathway and ended up protonated to give the rhodium hydride.

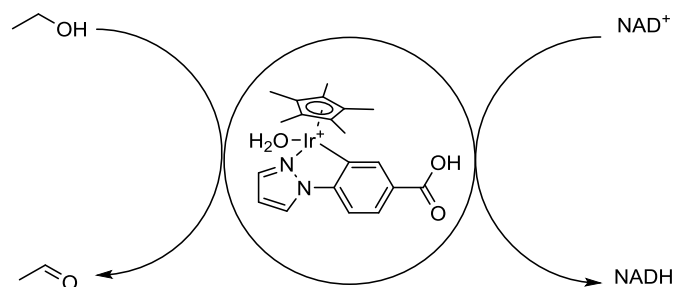
Alternatively, molecular hydrogen can be used to regenerate NADH. Fukuzumi reported the use of a cyclometalated $\text{Cp}^*\text{Ir}(\text{N,C})(\text{H}_2\text{O})$ catalyst, where (N,C) is 1H-pyrazol-1-ylbenzoic acid (Scheme 2. 6).⁸⁶ The reaction was driven towards NADH

regeneration in neutral to slightly alkaline conditions with a TON of 9.3 at pD = 8, whereas it was proceeding in the other was in acidic conditions.



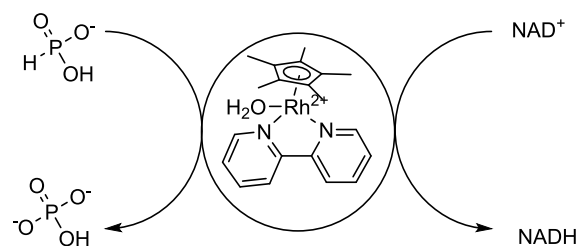
Scheme 2. 6 pH dependency on the NADH regeneration with H_2 and Fukuzumi's catalyst

The same complex was also used in NADH regeneration with alcohols being oxidized to carbonyls while NAD^+ is reduced to NADH, thus mimicking the action of alcohol dehydrogenase (ADH) (Scheme 2. 7).⁸⁷



Scheme 2. 7 EtOH-mediated NADH regeneration

Hollmann relied on phosphite and $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$ as a well-established catalyst to regenerate NADH from NAD^+ ,⁸⁸ with a TOF of 21 h^{-1} , mimicking thus the phosphite dehydrogenase which employs the same substrates (Scheme 2. 8).⁸⁹

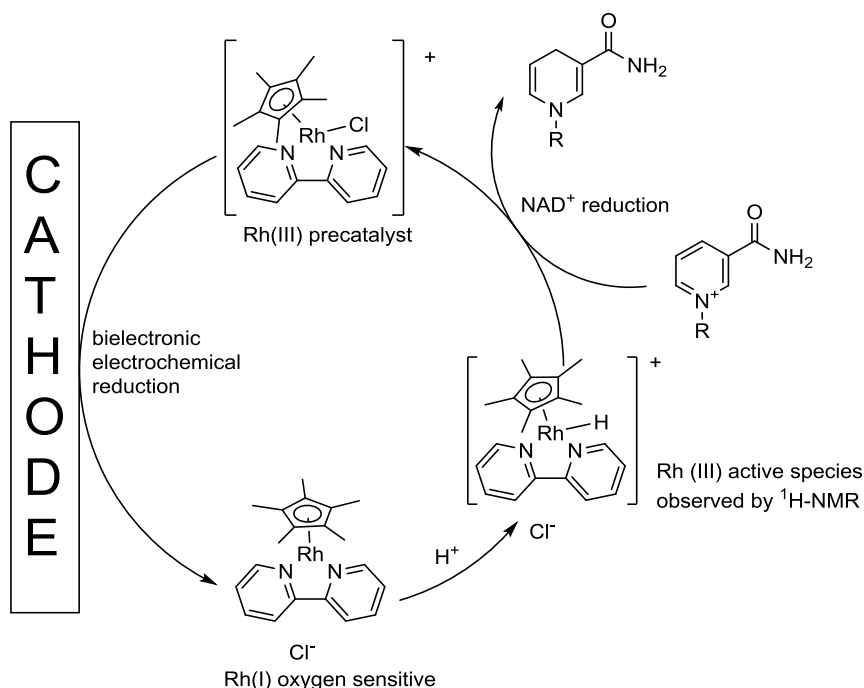


Scheme 2. 8 Phosphite-mediated NADH regeneration

1.2.3 Mechanistic investigations on the chemical NADH regeneration.

1.2.3.1 Electrochemical reduction of the rhodium mediator

Electrochemical studies by cyclic voltammetry carried out by Steckhan in 1991 indicated that the cathodic peak accounted for the bielectronic reduction of the $[\text{Cp}^*\text{Rh}(\text{bpy})\text{Cl}]^+$ to the pseudo-square-planar rhodium (I) complex $[\text{Cp}^*\text{Rh}(\text{bpy})]$. It then reacts with a proton to form the $[\text{Cp}^*\text{RhH}(\text{bpy})]^+$ which effectively reduced NAD^+ to NADH (Scheme 2. 9).⁹⁰ He brought the proof that the rhodium complex acted as a hydride transfer agent by directly observing the hydride by ^1H -NMR with $\delta = -7.2$ ppm. This method had the drawback of being oxygen-sensitive due to the rhodium (I) intermediate which was readily oxidized to the aqua complex, which could re-enter the cycle until full consumption of the dissolved oxygen. The limited TOF to 5 h^{-1} is probably due to the diffusion limit around the electrode.



Scheme 2. 9 Rhodium cycle in the electrochemical NADH regeneration

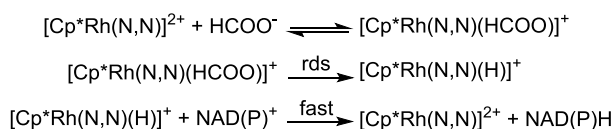
1.2.3.2 Chemical reduction of the rhodium mediator

The reduction of the $[\text{Cp}^*\text{Rh}(\text{bpy})\text{Cl}]^+$ by formate was deeply investigated in the literature in the context of transfer hydride reduction. This section will only deal with complexes of the form $[\text{ArM}(\text{N},\text{N})\text{X}]^{+0}$ as they are the most commonly used candidates. They were selected for several criteria: high turnover number, water solubility, ability to adopt a water stable reduced form in water. It is now a consensus in the literature that the active species is a hydride; it thus replaces the labile X ligand.

In the first report of Steckhan,⁸⁴ the authors already postulated the existence of the hydrido intermediate and that the rate-determining step was the decomposition of the formate complex to release CO_2 and the hydride. It was identified by the authors as the rate determinant step due to a marked isotopic effect with DCOOD instead

of HCOOH. The authors ruled out the formation of the sensitive $\text{Cp}^*\text{Rh}(\text{bpy})$ complex as it showed an absorption band at 512 nm and here, a band at 610 nm was observed. The hydride was later observed clearly by ^1H -NMR.

The reaction rate depends on time in a linear fashion and does not depend on NAD^+ concentration, which is indicative of a pre-equilibrium.⁸⁴ Compared to the formate dehydrogenase, there is no allosteric inhibition with increasing amounts of NADH formed, the reaction proceeds to 99% (Scheme 2. 10).

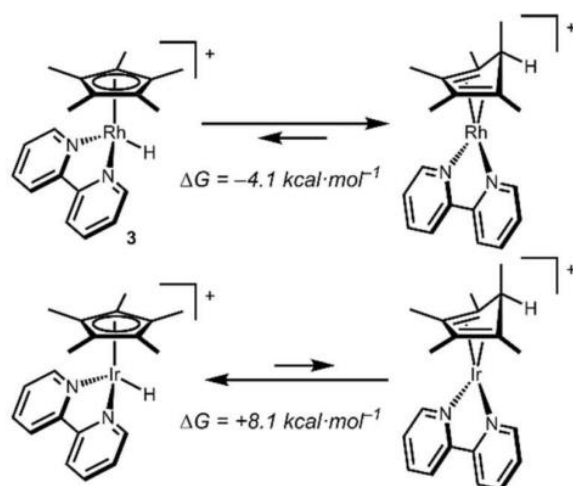


Scheme 2. 10 Formate driven NADH regeneration with Rh(III) catalyst

Whereas the nature of the key intermediate was thought to be the rhodium hydride since 2001, in the detailed mechanistic study of Fish, its true nature was never determined with exactitude.⁶⁵ It remained commonly admitted by the community for nearly 15 years. A decisive mechanistic study by Miller in 2016 shed light on the true nature of the hydride species.⁹¹ The intermediate was also synthesized for the first time by carrying out the reduction of $[\text{Cp}^*\text{Rh}(\text{bpy})\text{Cl}]\text{Cl}$ by sodium formate at pH 5, already described for the iridium analogue. Precipitation by NH_4PF_6 gave a green solid which could be obtained by filtration and was then analysed by NMR. The authors faced an inconsistent NMR spectrum. Whereas the Ir analogue gave a singlet accounting for the 15 Cp^* protons and one singlet peak for the hydride in the downfield region, the rhodium complex had two singlets (6H integration each) and a doublet ($J = 6.2$ Hz, 3H) present in the aliphatic region, and a downfield quartet (δ 2.31, $J = 6.2$ Hz,

1H). Alternative methods failed to generate a different compound, by using NaBH₄ and then HCl for instance.

The proposed structure was the η^4 diene coordination with an H transferred on the C1, in germinal position to the methyl. DFT calculations performed by Miller suggested that the rhodium hydride isomerizes to [(Cp^{*}H)Rh(bpy)]Cl species, whereas the iridium analogue has an unfavourable ΔG for the same rearrangement (Scheme 2. 11).



Scheme 2. 11 Determined structure of the hydride intermediate, from ⁹¹

The proposed structure by Miller was shown to be a viable intermediate in the reduction of NAD⁺ to 1,4-NADH by a similar mechanism as described in the next paragraph.

1.2.3.3 Reduction of the NAD cofactor

Although Steckhan⁸⁴ in 1988 was the first to notice that [Cp^{*}Rh(bpy)Cl]Cl provided a clean and regioselective 1,4 reduction of NAD⁺ to NADH, the reason was not investigated by himself but by Fish 13 years later.⁶⁵ The decisive study aimed to determine the critical interactions between Cp^{*}Ru(N,N) complexes and a more

simple and more flexible analogue, whose ribose-phosphate-adenine moiety was replaced by a more simple benzyl group. It is commonly admitted that the adenine-phosphate-ribose does not have an influence on the chemistry as it is too far away from the reactive centre compared to the size of the Cp^*Rh complex. For a better water solubility, a second model with a phosphoribosyl moiety was used by the authors. The closest NADH analogue (**Bn-NADH**, Figure 2. 3) could be obtained by dithionite reduction of the cheap and easy to make *N*-benzyl nicotinamide with excellent 1,4 selectivity. The diversity can be brought by starting from 3-substituted pyridines.

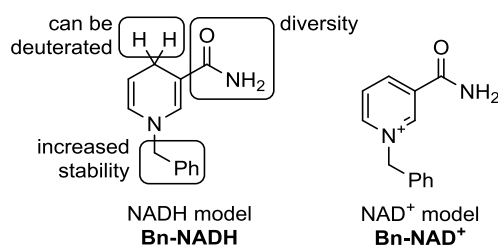
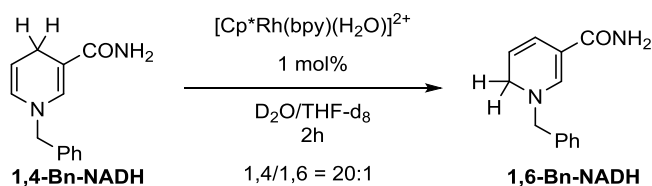
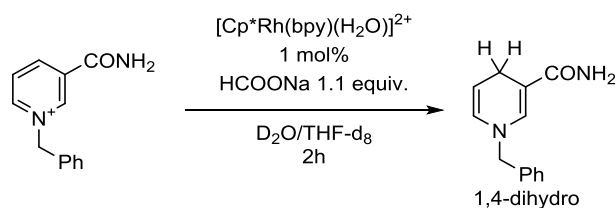


Figure 2. 3 **Bn-NADH** and **Bn-NAD⁺** model

Fish found that the benzyl nicotinium could be reduced to the kinetic 1,4 regioisomer with up to 1 mol% of $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$ complex acting as a catalyst. However small amounts up to 5% of 1,6-regioisomers were clearly identified by NMR (Scheme 2. 12). The key experiment of the study was to identify that the 1,4 regioisomer, in presence of the catalyst and without HCOONa , rearranged to give 5% of 1,6 product. When DCOONa was used, it was incorporated at the 4 position and never to the 6 position. The 1,2 product has never been observed by the authors. This experience indirectly showed that the overall reaction is reversible (Scheme 2. 13). For nearly eleven years, this conclusion will remain unexploited in the literature.

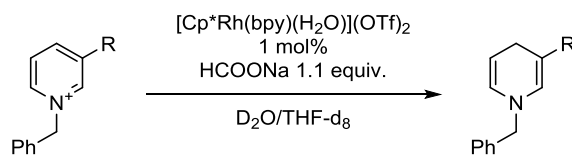


Scheme 2. 12 Cp^*Rh -catalysed isomerization of **1,4-Bn-NADH** et **1,6-BN-NADH** indicates that the hydride transfer is reversible



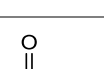
Scheme 2. 13 1,4 reduction of **Bn-NAD⁺** to **Bn-NADH** by HCOONa

Fish showed that the key feature for the 1,4 regioselectivity was the coordination with the amide moiety of native NADH (Scheme 2. 14). By changing the R group in the 3 position of his model, he could demonstrate that the coordination took place with the oxygen of the amide. Remarkably, when a ketone was used instead of the amide, the reaction took place at the same rate. When a thioamide was used, the softer sulphur atom coordinated better to the rhodium and provided acceleration of 30%. Non-coordinative alkyl groups such as methyl gave no reduction. The bulky diethylamide moiety hampered the coordination and was ineffective.



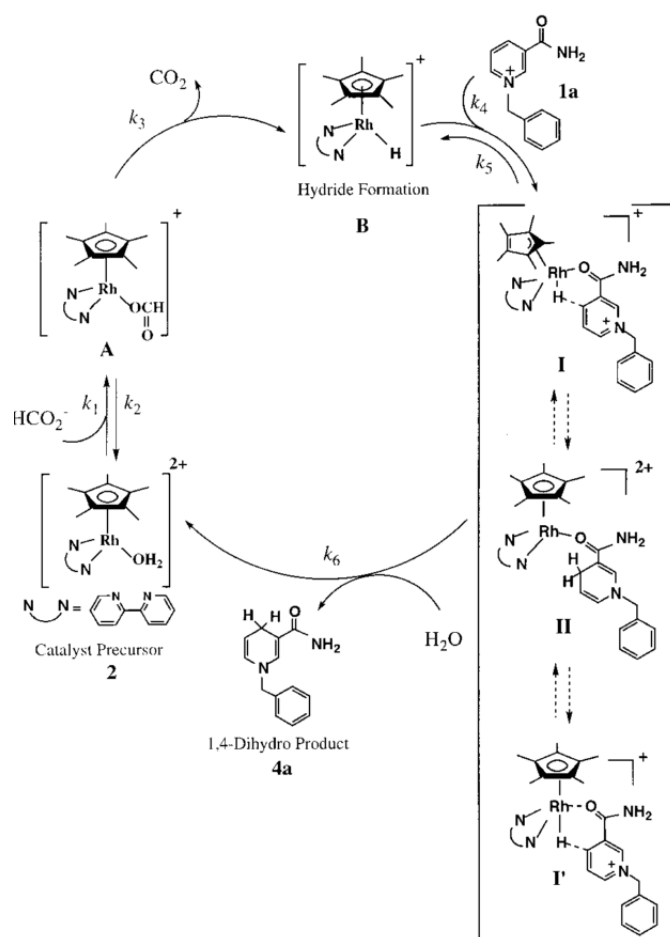
Scheme 2. 14 Reduction of the NAD^+ models by HCOONa and $[\text{Cp}^*\text{Rh}(\text{N,N})(\text{H}_2\text{O})]^{2+}$

Table 2. 1 Relative rate and TOF of the NADH model depending on the R group

Entry	R	Relative rate	Turnover (h ⁻¹)
1		1.0	9
2		0.9	8
3		0.0	0
4		1.3	11
5		1.0	9
6		1.3	11
7		0.9	8
8		0.0	0
9		0.0	0

The whole mechanism is nowadays known as the Fish mechanism (Scheme 2. 15). It is commonly accepted among the community, even though the identity of the rhodium hydride was elucidated 15 years by Miller. The [Cp^{*}Rh(bpy)Cl]⁺ complex works faster than the [Rh(bpy)₃]²⁺ as it does not need the decoordination of a bipyridine to leave a free space for the incoming NADH. Instead, a ring slip from η⁵ to η³ gives the required coordination vacancy on the metal centre to proceed.⁹⁰ The catalytic activity was found to be enhanced by the presence of electron donating groups on the

bipyridine rings, whereas it was completely hampered with electron withdrawing groups.

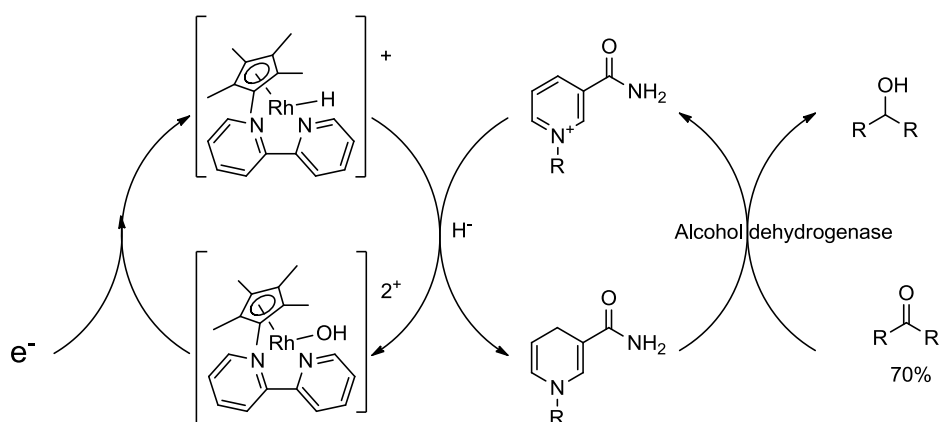


Scheme 2. 15 Fish mechanism, from ⁶⁵

2 Research goals

This specific literature introduction showed that efforts were put to adapt the early-generation of hydride transfers, taking place in organic solvents with sensitive catalysts, to water and using less benign hydride donors such as HCOONa. Detailed mechanistic investigations studied in detail the structure of the intermediate and determined that it was a metal hydride in many cases. By fine-tuning the ligands around the metal, it was possible to get less reactive and more stable hydride of the general structure $[(\eta^6\text{-Ar})\text{M}(\text{N},\text{N})\text{H}]^{n+}$ for arenes of the family of benzene and of the general structure $[(\eta^5\text{-Ar})\text{M}(\text{N},\text{N})\text{H}]^{n+}$ for arenes of the family of cyclopentadiene. Although working in water is highly desirable for the industry, it unexpectedly influenced people in the field of enzymatic biocatalysis such as Steckhan, who found advantageous to work this type of complexes in conjunction with enzymes in the context of non-enzymatic NADH regeneration.

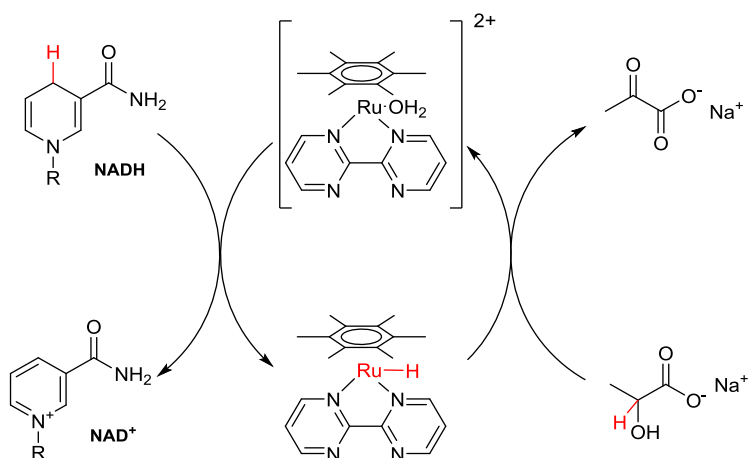
Already in the years 1980, it was demonstrated by Steckhan and Fish that this type of hydride, and especially $[\text{Cp}^*\text{Rh}(\text{bpy})\text{H}]^+$, was easily obtained by treatment of $[\text{Cp}^*\text{Rh}(\text{bpy})\text{H}_2\text{O}]^{2+}$ by HCOONa and could reduce the NAD^+ cofactor with outstanding selectivity to the 1,4 regioisomer of NADH through a transfer hydride mechanism in water in biocompatible conditions and in presence of other biomolecules (Scheme 2. 16). This concept has been heavily exploited by Fish and Hollmann during the next 25 years with various examples. Fish showed briefly that the reaction was reversible, but never moved forward in terms of applications.



Scheme 2. 16 Non-enzymatic regeneration of NADH with $[Cp^*Rh(bpy)(H_2O)]^{2+}$ complex

In 2013, just before we started to work on the subject, a report of Sadler, who was studying the anticancer properties of the family $[(\eta^6\text{-Ar})M(N,N)H]^{n+}$ emitted the hypothesis that the NADH cofactor was transferring its hydride to the metal centre, which was disrupting the NADH/NAD⁺ balance and causing a form of oxidative stress leading to cell death through an unprecedented mechanism (Scheme 2. 17).

Knowing the reducing capability of metal hydrides and the biocompatibility of the ruthenium hydride he was studying, he attempted to reduce pyruvate to lactate, but low yields were obtained due to the poisoning of the catalyst (Scheme 2. 17). At the time, the potency of such a transfer hydride reduction using a biocompatible – yet cytotoxic – catalyst using an endogenous hydride source – namely NADH – was still unexploited and could lead to interesting therapeutical applications.



Scheme 2. 17 In-situ hydride transfer from NADH to a ruthenium complex and subsequent reduction of pyruvate to lactate

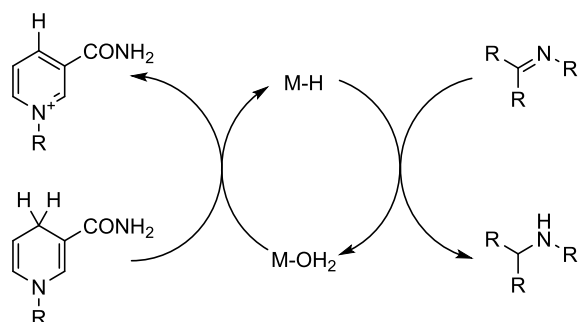
We hypothesized that such a reaction would be a new valuable addition to the bioorthogonal toolbox in the section of transition-metal catalysed reactions. It must be noted here that the reaction is not totally bioorthogonal as it consumes NADH but could be one step closer to bioorthogonality if it is substituted by a non-natural with the same reactive entity, as it was attempted with success *in vitro* but not *in cellulo*. We considered that the reaction could be considered as bioorthogonal if it is used in the context of the activation of a pro-fluorophore if it consumes a non-lethal amount of NADH that the cell can re-synthesize by adjusting its metabolism.

For such a project, we need to divide the work into two parts. At first, the plan is to devise a realistic model reaction to get acquainted with the catalytic system and make it work in biocompatible conditions. The problem of the inhibition by the product must be kept in mind at all stages of the study. The second part consists of applying the reaction inside living cells on fluorogenic

substrates to verify if the reaction can happen efficiently in cells and match our adapted criteria of bioorthogonality.

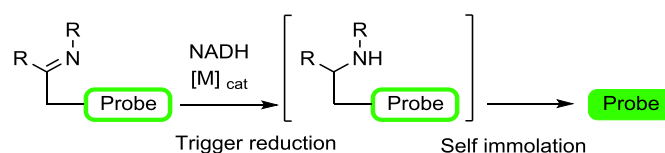
In the first part, our first idea in mind was to carry out the reduction of imines in water using NADH as the hydride source and a metal catalyst (Ru, Rh, Ir). Imines are more challenging than aldehydes and ketones to reduce due to their reduced electrophilicity and their hydrolytic behaviour. However, they benefit from an activation by protonation which happens even in neutral pH buffers.

The first section will aim to find a suitable catalyst and a biocompatible reaction set to carry out an efficient reduction of a model imine, as well as study the scope of the reaction (Scheme 2.18). Due to the high price of NADH, we will try to replace the costly NADH by a simpler analogue in order to validate the concept and increase the atom efficiency. With a proof of concept in hands on our model reaction, we will carry out additional mechanistic studies to better understand the mechanism of our reaction. Then, an exemplification will show the range of applicability and the limits. It will also enable us to select good trigger moieties for the second application of this method.



Scheme 2. 18 Transfer hydride reduction of an imine with NADH as hydride source and a metal catalyst

Finally, the second section of this part will aim to develop reducible prodrugs employing the described method from the design to the synthesis and the *in vitro* evaluation (Scheme 2. 19). Fluorogenic probes were considered as a preliminary step towards prodrugs carrying cytotoxic payloads as the release of a fluorophore is easier to follow during the development of the probes in the early stages. Such a bioorthogonal reaction and appropriate probes could be useful to map the NADH within that the cell in space and time-controlled fashion and to activate cytotoxic molecules specifically within the cytoplasm in a time-controlled fashion.



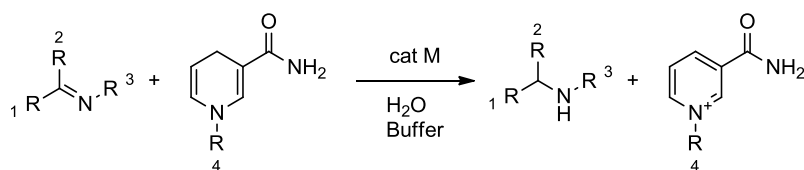
Scheme 2. 19 Principle of NADH-responsive prodrugs

First part: From the methodology to applications
in prodrug decaging
First chapter: Development of an imine reduction
by iridium catalysis and NADH a hydride donor

3 First chapter: Development of an imine reduction by iridium catalysis and NADH a hydride donor

3.1 Choice of a model reaction

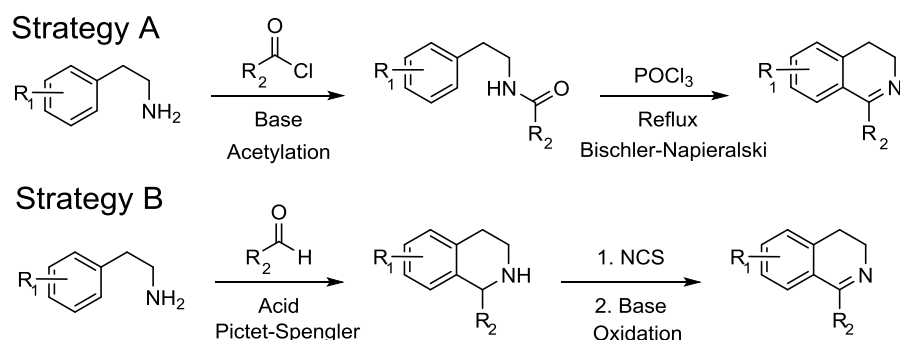
After we identified the need for developing a biocompatible transfer hydride reduction in the context of prodrug activation, we started to devise a model reaction (Scheme 2. 20). We decided to choose imines as a model substrate in order to start our investigation. Despite some known disadvantages, such as their hydrolytic behaviour, we know that it gets protonated in water, which leads to activation of the C=N. Also, the formed amine is expected to be significantly less prone to catalyst poisoning thanks to its protonation in a neutral buffer.



Scheme 2. 20 Global reaction studied in this chapter. R_1 , R_2 and R_3 are alkyl or aryl groups, R_4 = Bn or ribosyl phosphatidyl adenine (NADH)

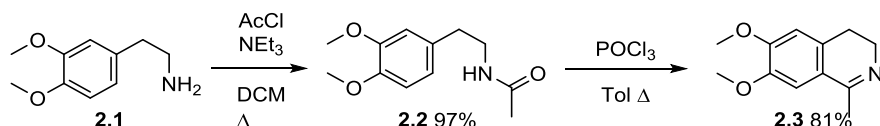
Our attention was drawn to dihydroisoquinolines as model substrates for multiple reasons. As it is a widely used scaffold for natural products, a lot of synthetic routes to dihydro and tetrahydroisoquinolines were developed and reported in reviews.⁹²

Their synthesis was well described in the literature. In this work, we started with phenethylamines as a building block. The strategy consisted of a Bischler-Napieralski reaction (Scheme 2. 21, Strategy A) or a Pictet-Spengler reaction (Scheme 2. 21, Strategy B) followed by oxidation and a basic treatment to gain access to dihydroisoquinolines.



Scheme 2. 21 Two strategies to synthesize dihydroisoquinolines

We first selected the 6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline **2.3** as a model substrate (Scheme 2. 22). It was synthesized in two steps using the Bischler-Napieralski reaction. The product was distilled in a Kugel Rohr under vacuum and white crystals could be easily obtained. The imine **2.3** was already employed by Pikho⁹³ and Vidal in asymmetric transfer hydride reduction using transfer hydride reduction in water.



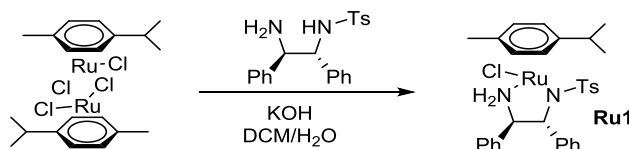
Scheme 2. 22 Synthesis of 6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline **2.3**

3.2 Preliminary choice of a catalyst

We then considered the choice of the catalyst. From the known literature, we had a choice between (i) Noyori-type catalysts, well described for imine and carbonyls reduction and for which interactions with NAD cofactors were still unknown, and (ii) Steckhan & Sadler-type catalysts, known for NAD⁺ reduction, but still not all documented for imine reduction at the time we started our work.

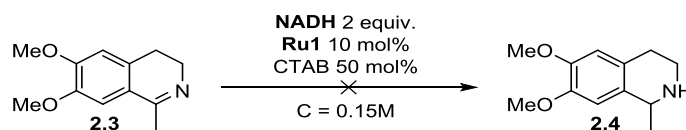
3.2.1 Noyori-type catalysts

The first catalyst which was synthesized is a Noyori catalyst [(*p*-cym) Ru ((*R,R*)-TsDPEN)Cl] (**Ru1**, Scheme 2. 23).⁹⁴ It could be obtained by treatment of (*R,R*)-TsDPEN with [(*p*-cym)RuCl₂]₂ in dry DCM to allow the (*p*-cym)RuCl₂(N) by substitution with a chloride and de-symmetrisation of the starting ruthenium source. Addition of one equivalent of KOH and water enabled the deprotonation of the sulphonamide and yield an air-sensitive bright purple complex. The complex was thought to be the easiest choice as it is known for many types of carbonyl and imine reduction, even though this type of scaffold was not known at the time for its interactions with NADH.



Scheme 2. 23 Synthesis of the (*R, R*)**Ru1**

It was tried in catalysis in the context of imine reduction on our model reaction (Scheme 2. 24). To start, we chose to use 2 equivalents of NADH, and 10 mol% of **Ru1** in degassed water as the solvent. Also, we used 50 mol% of CTAB as a cationic ammonium surfactant, as prescribed by Pikho.⁹³ However, no conversion was observed.



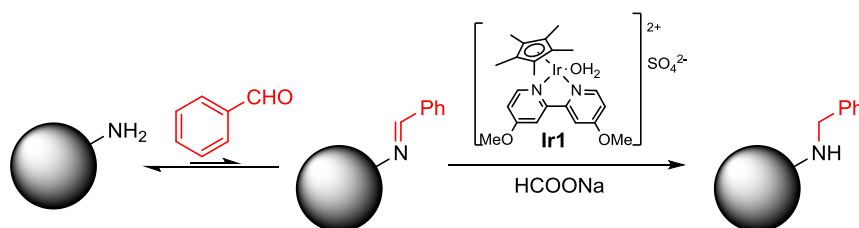
Scheme 2. 24 Reduction of the imine **2.3** by NADH and **Ru1**

At this stage, we had no clue to explain the inactivity of **Ru1**. We blamed the lability of the chloride ligand. Many literature reports use counteranions such as triflates or hexafluorophosphates to allow a water molecule in the vacant coordination site.

The lability of the chloro ligand is also a critical point. It is one of the most frequently discussed characteristics of ruthenium and platinum anti-cancer agents as it is the key activation step to the active aqua adduct. Even though it could be a factor, little attention was paid to that parameter throughout all this work.

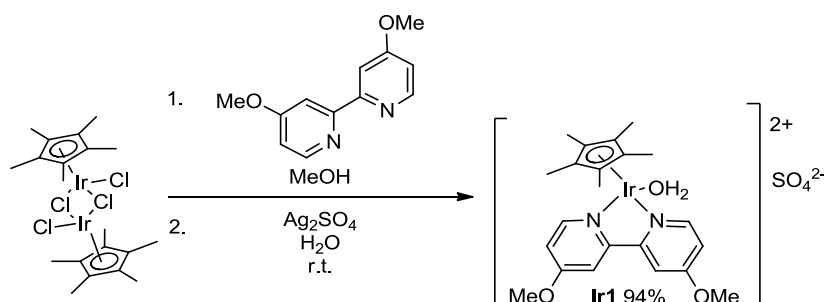
3.2.2 Fish-Sadler type catalyst

Due to the inactivity of the only Noyori-type (**Ru1**) catalyst we tried, we quickly decided to move to another type of catalysts for which more was known about imine reduction and NAD cofactors interactions. Our attention was drawn by the report of Francis in 2005 where he was focusing on protein reductive alkylation benzaldehyde by sodium formate and a Cp*Ir catalyst (**Ir1**, Scheme 2. 25).⁹⁵ The rhodium analogue is the most frequently used for NADH regeneration.



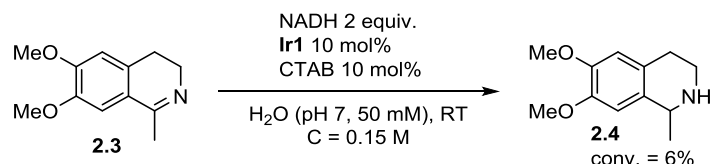
Scheme 2. 25 Francis' reductive alkylation based on imine reduction with **Ir1** catalyst

The catalyst **Ir1** was synthesized by a simple method developed by Dadci (Scheme 2. 26).⁹⁶ The $[\text{Cp}^*\text{IrCl}_2]_2$ was reacted with the (*N,N*) ligand to give a quantitative and clean complexation in 15 min, after which the chloride was abstracted with a silver salt. After filtration of the silver salts, the complex could be isolated by evaporation of the water preferably with a lyophilizer to avoid thermal degradation of the complex. This method was later applied with success to several other catalysts later in this project.



Scheme 2. 26 Synthesis of Francis catalyst

The catalyst **Ir1** was tried on the model reaction (Scheme 2. 27). The quantity of CTAB was reduced as the extraction workup was very difficult. We used a commercial pH 7 phosphate buffer at 50 mM (commercial) as it is more biocompatible. For the first time, a small conversion of 6% was obtained. All the conversions were determined by ^1H -NMR by comparing $1\text{H}_{2.3}$ and $1\text{H}_{2.4}$. The yield was determined with dimethyl terephthalate as the internal standard by comparing the integrals of the aromatic peaks of the **2.4** and **2.3** with the aromatic peaks of the internal standard.



Scheme 2. 27 First catalytic test on **2.3** with the Noyori catalyst

This result showed us that we found a suitable catalyst and that further optimization could lead us to a valuable reaction.

3.3 Imine reduction of NADH

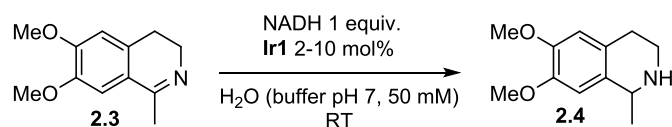
3.3.1 First optimization

Bothered using a surfactant, we rapidly found that in the same conditions, it could be removed with a little impact on the yield, reaching this time 9%. However, the catalytic loading of 10% for a

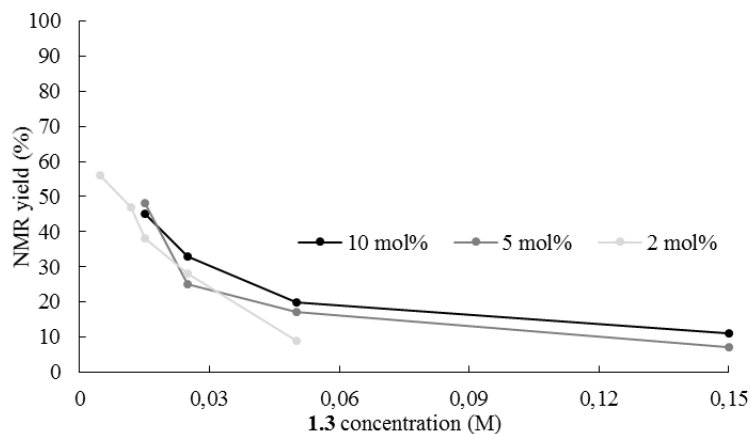
yield of 10% was still unsatisfactory, as it clearly shows that the catalyst can perform one cycle before being deactivated.

We first thought that the produced NAD^+ could bind to **Ir1** and prevent from new NADH to bind to it. However, we ran the reduction of **2.3** to **2.4** with 1 equivalent of NAD^+ as well as 1 equivalent of NADH, leading to 11% of yield, with 9% without NAD^+ . This difference is not significant and ruled out an NAD^+ inhibition. At this stage, we started to believe that the amine **2.4** was responsible for the inhibition.

Surprisingly, we noticed that increasing the volume of phosphate pH 7 buffer (50 mM) had a beneficial effect on the yield, whereas we expected an opposite trend as the reaction may be slower at lower concentrations (Scheme 2. 28). With 10 mol% of **Ir1**, we saw the yield increasing from 10% with a concentration of 150 mM in the phosphate buffer (Graph 2. 1). Lowering the concentration to 15 mM enabled us to see the yield increasing to 48%. We then lowered the catalytic loading to 2 % and at lower concentrations, the yield was not decreasing compared to higher catalytic loadings. However, we did not manage to try higher dilutions as the quantity of solvent became very high compared to the mass of the starting material. We thus decided to stick with 2 mol% of **Ir1** and 15 mM as the concentration of the **2.3** in the reaction medium. A yield of 38-42% was obtained in those conditions.

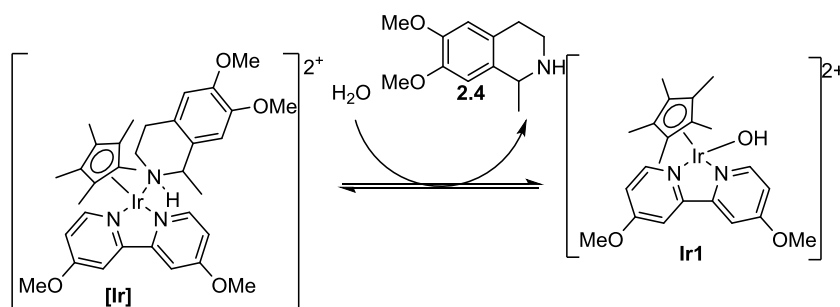


Scheme 2. 28 Model reaction conditions. Study of the impact of the pH buffer volume



Graph 2. 1 Impact of the substrate **2.3** concentration on the yield at various catalytic loadings

At this stage, we brought the hypothesis that the inhibition was due to a coordination between the amine **2.4** and the iridium centre, thus preventing from re-opening the cycle with the attack of NADH on **Ir1** (Scheme 2. 29).



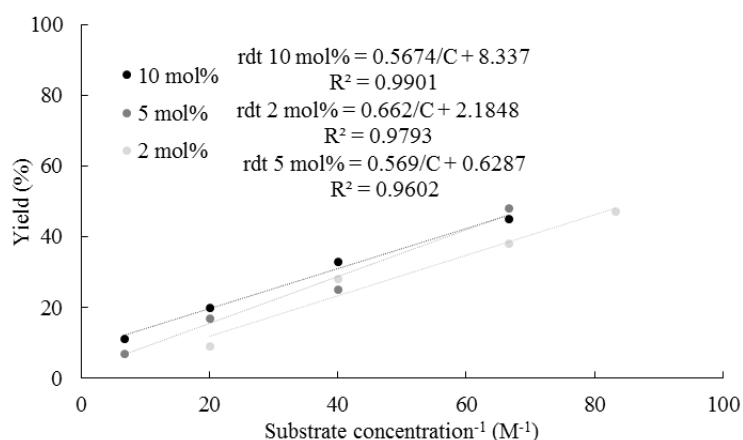
Scheme 2. 29 Hypothesis of catalyst poisoning by the produced amine **2.4**

By writing the equation, and then further re-arrange it, we can write:

$$K_C = \frac{[\text{Ir1}] \cdot [\text{2.3}]}{[\text{Ir}] - \text{2.4}} = \frac{\frac{n_{\text{Ir1}}}{V} \cdot \frac{n_{\text{2.4}}}{V}}{\frac{n_{[\text{Ir}]-\text{2.4}}}{V}} = \frac{n_{\text{Ir1}} \cdot n_{\text{2.3}}}{n_{[\text{Ir}]-\text{2.4}}} \cdot \frac{1}{V}$$

$$n_{\text{2.4}} = \text{cte} \cdot \frac{1}{C}$$

If we plot the yield in function of $1/C$ **2.3**, we should see the points aligned (Graph 2. 2).



Graph 2. 2 Linearization of the yield in function of $1/C$ of the **2.3**

However, although this interpretation enabled us to reach nearly 40-45% of yield, it turned out to be wrong as we, in fact, changed the number of protons available in the solvent for reprotonation of **2.4**. The correct version will be discussed in the paragraph 3.3.3 of this section.

3.3.2 Detailed catalyst study

In order to try to improve the catalytic performances, and convinced it was the key point to improve, we focused on improving the catalyst first. Still unsatisfied by the high price of our iridium source we wanted to turn our attention to a cheaper metal such as ruthenium. Two commercially available sources were then used: $[(\text{hmb})\text{RuCl}_2]_2$ and $[(p\text{-cym})\text{RuCl}_2]_2$ (hmb = hexamethylbenzene, *p*-

cym = 1-Methyl-4-isopropylbenzene). We had four (*N,N*) heteroaromatic ligands readily available: bpy, MeObpy, phen and bpm (Figure 2. 4, Scheme 2. 30).

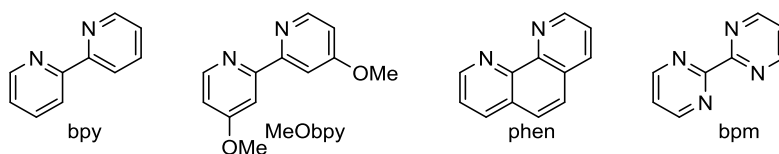
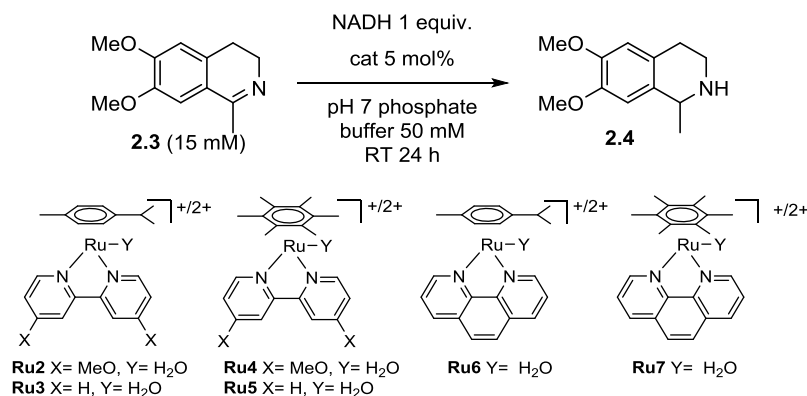
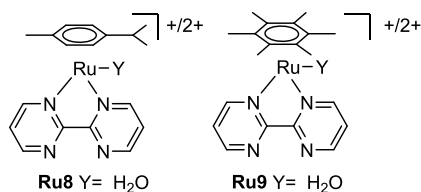


Figure 2. 4 Ligands used for the primary catalyst study

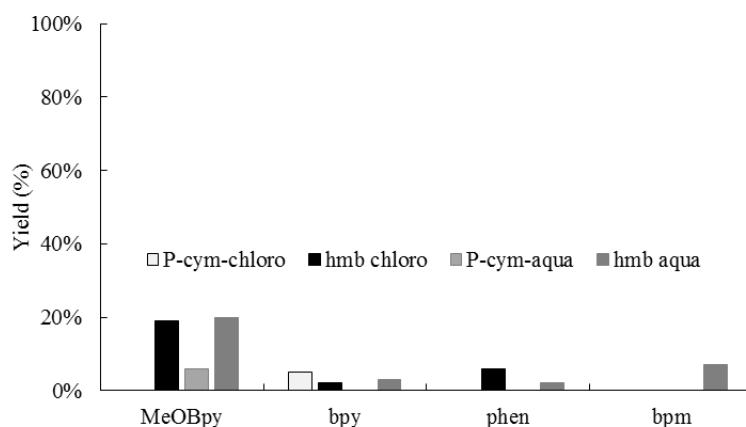
We synthesized both the aqua and the chloro ligand with the method developed by Dadci and used by Francis.⁹⁵⁻⁹⁶ We decided to increase the catalytic loading from 2 mol% to 5 mol% (Graph 2. 3).

From this experiment, we noticed that the yield remained quite low despite a higher catalytic loading, where a TON of 2 could be observed with the ligand 4,4'-dimethoxy-2,2'-bipyridine (MeObpy). We immediately noticed that the bulky hexamethylbenzene (**Ru4**, **Ru5**, **Ru7** and **Ru9**) gave better results than *p*-cymene (**Ru2**, **Ru3**, **Ru6**, **Ru8**) in every single case. Also, the chloro complexes were significantly less active than their aqua analogues, possibly due to slow hydrolysis in solution.



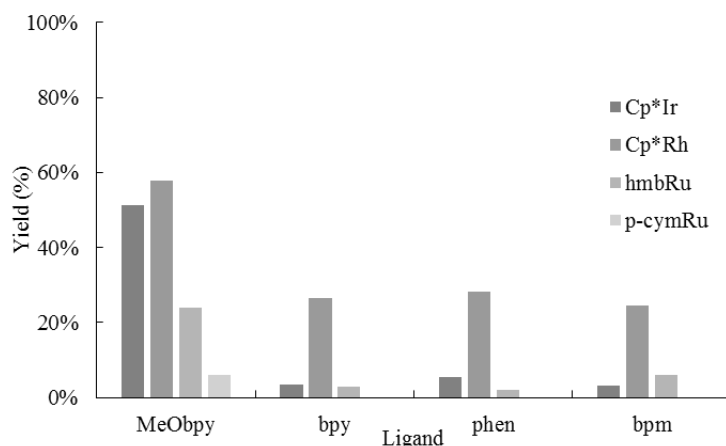


Scheme 2. 30 Ruthenium catalyst screening on the reduction of 2.3



Graph 2. 3 Ru2 to Ru9 screening

With those unsatisfactory catalysts, we decided to synthesize the Cp^*Ir and Cp^*Rh surrogates only in the aqua form, as they are also described in the literature. We immediately noticed a strong ligand effect within this family, an electron rich bipyridine is needed to provide sufficient reactivity, presumably due to the higher electron density on the metal reinforcing the hydridic character (Graph 2. 4). Therefore, the electron-rich MeOBpy outperformed the classical bpy ligand in each case. For comparison, the *p*-cym Ru and hmb Ru analogues were added to the graph. Although the $[\text{Cp}^*\text{Rh}(\text{MeOBpy})(\text{H}_2\text{O})]^{2+}$ (**Rh1**) slightly outperformed its analogue **Ir1**, we decided to stick to **Ir1** to have a better comparison throughout the project.



Graph 2. 4 Ruthenium, rhodium and iridium screening

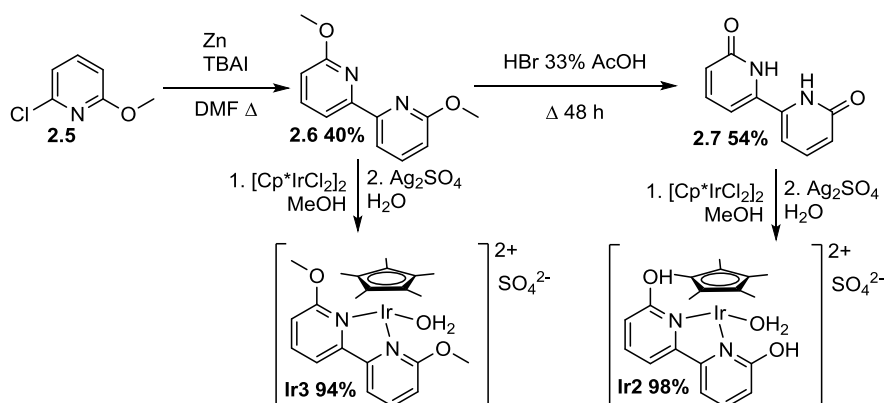
All the aforementioned complexes are present under the form of yellow to orange powders, and those who form the hydride adopt a dark yellow or dark orange colour in solution. An exception was made for $[\text{Cp}^*\text{Rh}(\text{bpm})(\text{H}_2\text{O})]^{2+}$ which has a dark blue hydride; the colour disappeared during the workup upon quenching of the reaction.

These results enabled us to see a ligand effect while comparing different complexes with the same metal. While referring to the literature, bipyridines are a good choice of ligand due to their electron-withdrawing effect as they are π -deficient systems, so they can stabilize the excess of charge on the metal. An electron-rich bipyridine is needed for a good reactivity, as it enhances the hydridic character of the complex and helps the reduction to take place.

To further enhance the reactivity of the catalyst and further push the yield up, we synthesized the Yamaguchi catalyst (**Ir2**) and its methoxy analogue (**Ir3**). It can be deprotonated in neutral buffers and further increase the electronic density on the heteroaromatic rings.

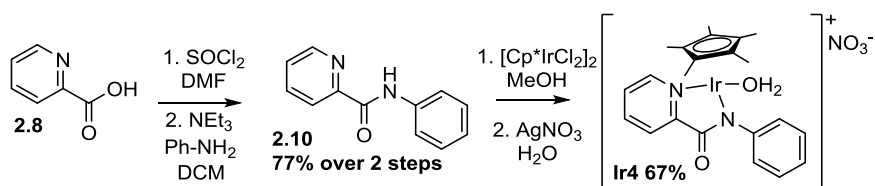
The Yamaguchi ligand was synthesized in two steps, by homodimerizing the pyridine **2.5** with zinc and followed by

demethylation using HBr in AcOH in refluxing conditions (Scheme 2. 31). The highly insoluble dilactam tautomer **2.7** was reacted with $[\text{Cp}^*\text{IrCl}_2]_2$ to form the catalyst **Ir2** after abstraction of the chloride with a silver salt. However, identical results in catalysis were obtained as when **Ir1** was used.



Scheme 2. 31 Synthesis of Yamaguchi catalyst

At the moment we reached the end of our investigations and were writing the paper of the project,⁹⁷ a report came from Do,⁶⁷ where he was able to reduce aldehydes with NADH and an iridium catalyst **Ir4**, we decided to synthesize his catalyst and test it on our model reaction (Scheme 2. 32). The complex was synthesized in two steps by acylating picolinic acid **2.8** with aniline, followed by complexation with the Cp^* iridium chloride. It was tested on the same model reaction, but no higher yield was obtained with this catalyst compared with our model catalyst **Ir1**.

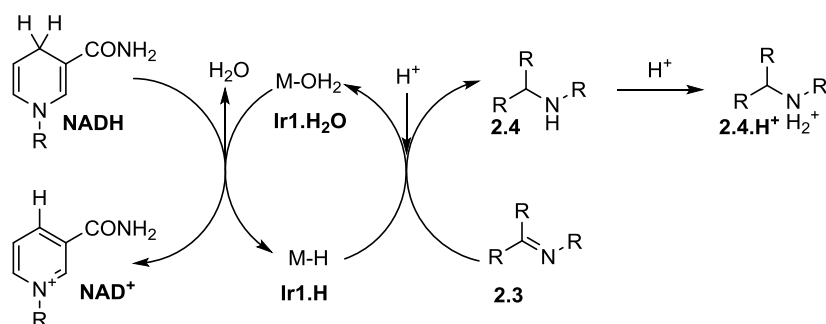


Scheme 2.32 Synthesis of Do catalyst **Ir4**

From that moment, we started to get convinced that the reaction was more on thermodynamic control rather than kinetic control. Increasing the amount of catalyst or changing the catalyst did not bring significant improvement. However, the impact of the acid had been neglected and was a key to understand the equilibrium.

3.3.3 Impact of the acid

In early investigations, we noticed that the reaction was performing poorly in distilled water (10%) and much better in a buffered medium (40%). To get a better insight, we decided to re-examine the global reaction scheme and see where the acid could impact it. It had been neglected that the reaction consumes one equivalent of acid during the reduction itself, and that the formed amine should be protonated in neutral pH buffers due to its pKa expected to be within an accessible range in buffered solutions (Scheme 2.33).⁹⁸



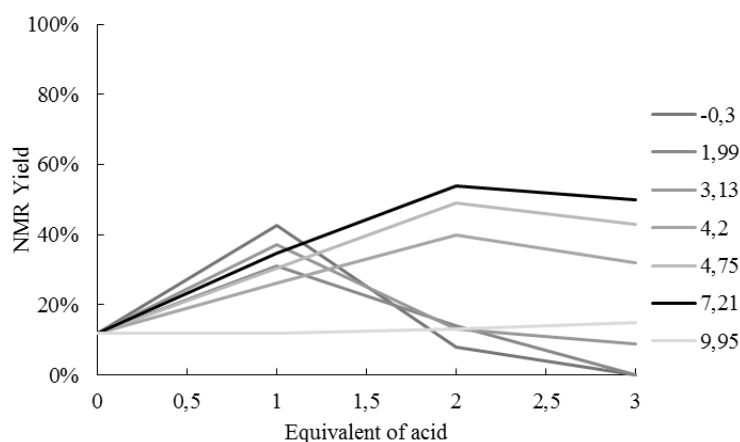
Scheme 2.33 Catalytic cycle and proton balance

Knowing at the same time acidic conditions can activate the substrate, but at the same time can protonate the hydride, we found crucial to test acids with pKa ranging from a pKa of -0.9 for TFA to phenol with 10.0 (Table 2. 2). The results could be presented in Graph 2. 5, featuring one curve per acid used. For KH₂PO₄, as it was giving the best results, we made more points to determine the optimum.

Our study systematically tested acids in stoichiometric quantities ranging from a pKa of -0.9 for TFA to phenol with 10.0 (Table 2. 2). The results could be presented in Graph 2. 5, featuring one curve per acid used. For KH₂PO₄, as it was giving the best results, we made more points to determine the optimum.

Table 2. 2 pKa of the acids tried in the screening

Acid	TFA	NaHSO ₄	Citric acid	PhCOOH	AcOH	KH ₂ PO ₄	PhOH
Ka	-0,30	1,99	3,13	4,20	4,75	7,21	9,95



Graph 2. 5 Impact of the acid on the yield of the reduction of **2.3**

We noted that different acids had very different behaviour depending directly on their pKa.

1. First, for strong acids (pKa until 3), adding one equivalent of acid had beneficial effects on the yield but has detrimental effects above one equivalent.
2. Then, medium acids have a distinctive behaviour, as they do not see their yield decreasing with more than one equivalent. They generally have an optimum between 2 and 3,
3. Eventually, for the weakest acid, phenol, it did neither inhibit nor activate the reaction,

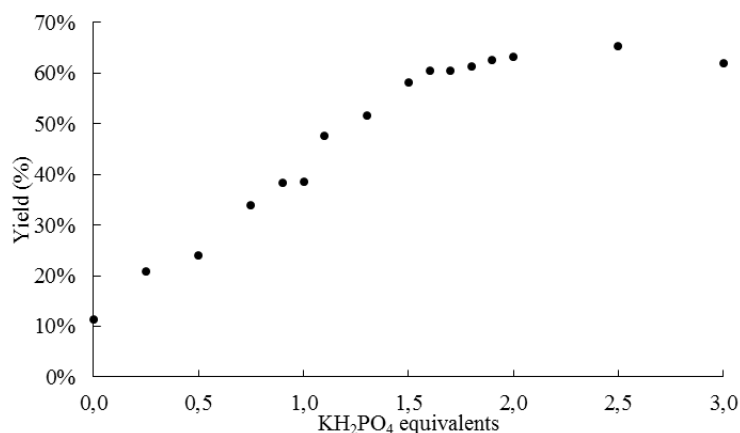
From all those observations, we could gain some valuable information about the mechanism.

1. For strong acids, it is probably due to the acid-sensitivity of the **Ir1.H**. Noticeably, the acid was added before the catalyst. It is thus reasonable to think that the acid is first consumed by **2.3** for its activation, explaining why the strongest acids always give the best yield with 1 equivalent, with a higher yield for stronger acid. While more than one equivalent is used, it is assumed to protonate

the hydride, as studied by Fukuzumi, who showed that a similar iridium hydride was stable until pH 2 without decomposing.⁹⁹

2. For medium acids, we believe that the first equivalent is consumed for the activation of the substrate, similar to above, and that the rest is compatible with the hydride and thus helps the reaction to take place by protonating the formed amine. At the same time, the two equivalents are consumed and the excess buffers the medium in a good pH range for the catalyst. The amine is certainly protonated at pH 7 and thus cannot poison the catalyst anymore.
3. Weak acids are probably not strong enough to activate **2.3** by protonation.

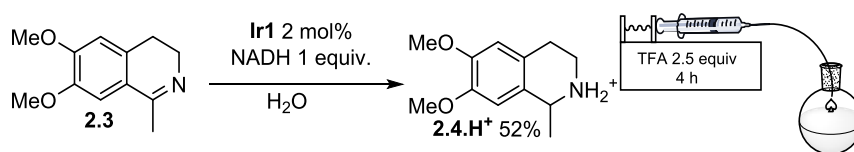
Remarkably, the yield was improved to 63% with KH_2PO_4 used at 2.5 equivalents (Graph 2. 6). It is thus the real optimum for this reaction, taking account of all the parameters and keeping 1 equivalent of NADH. The same yield could be obtained by using 100 mM or above phosphate buffer at pH 7, and not less for the considered reaction at 15 mM of substrate. At this concentration of substrate, the buffer is sufficiently concentrated to give enough protons to drive the reaction.



Graph 2. 6 Impact of the KH₂PO₄ quantity on the yield of the reduction of **2.3**

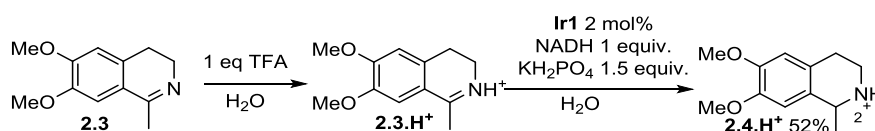
To verify our hypothesis that strong acids are not compatible with the acid and that the acid activates first the imine, we carried out two more experiments.

In the first, **2.3** was set to react with 2.5 equivalents of NADH and 2 mol% of **Ir1** at 15 mM in water. Then, 2.5 equivalents of trifluoroacetic acid were added with slow addition using an automatic syringe plunger (Scheme 2. 34). To make the experimental setup compatible with the small amount of TFA added, the reaction was launched in 2.3 mL of solvent and the TFA was dissolved in 1 mL and added in 4 hours and stirred for 20 more hours thereafter. A yield of 52% was obtained. It is an intermediate value between 0% with 2.5 equivalents and 60% with 2 equivalents of KH₂PO₄ in direct addition. The minimized contact between the strong acid and the hydride is our rational explanation.



Scheme 2.34 Slow addition of TFA

In a second experiment, we added TFA 1 equivalent to maximize the activation of **2.3**, and then we added 1.5 equivalent of KH_2PO_4 (Scheme 2.35). Again, a yield of 52% was observed, and we did not exceed the yield of our model reaction of 63%.



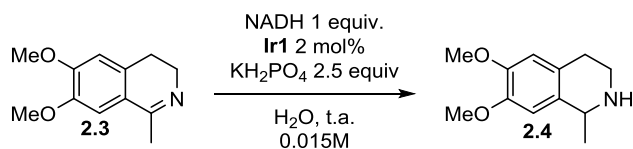
Scheme 2.35 Combination of TFA and KH_2PO_4

3.3.4 Kinetic study

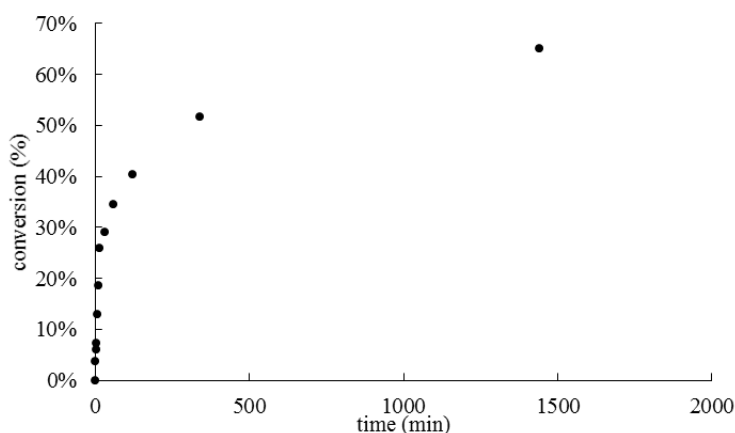
After we realized that 2.5 equivalents of KH_2PO_4 were an optimal condition, we decided to carry out a kinetic study. Since at that time, we did not have any expertise on fluorescence spectroscopy, we decided to run the reaction in a larger batch (Scheme 2.36) and for each point, an aliquot was taken, quenched by NaOH , and the **2.3** and **2.4** were extracted in DCM. The extract was dried and analysed by ^1H -NMR and the conversions were measured. We assumed that the conversion equals the yield as no side products have been observed so far.

We noticed that the reaction was proceeding very fast at first, which was accompanied by a bright yellow colouration attributable to the **Ir1.H** (Graph 2.7). A conversion of 29% was reached in only 30 minutes, during which the catalyst works at its maximal speed v_{max} , and after which the reaction slows down to reach 65% of conversion after 24 h. However, in this present experiment, we did not run the

concentration at a fixed pH as we were using a stoichiometric amount of acid, whereas the reaction consumed some acid.



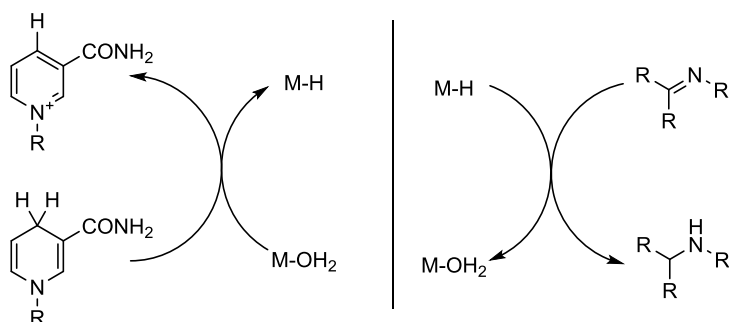
Scheme 2. 36 Reaction conditions for the kinetic experiment



Graph 2. 7 Kinetic studies of the model reduction of **2.3**

3.3.5 Iridium hydride observation

Literature reports suggested clearly that our reaction should proceed through a hydride transfer from the NAD cofactor to the metal and that the metal should be able to transfer its hydride to **2.3**. The process can be separated into two phases, and both have been documented in the literature (Scheme 2. 37).



Scheme 2.37 Left: hydride transfer from the cofactor to the metal centre. Right: hydride transfer from the metal centre to the imine for reduction

- The first step has been discovered by Fish⁶⁵, but studied by Sadler,⁶⁴ in the context of non-enzymatic NADH regeneration. Sadler concluded that it was possible to observe the metal hydride (on a ruthenium complex) provided that the ligand is sufficiently electron-poor to stabilize the electron density on the metal.
- Fukuzumi investigated the hydride structure and on a similar complex as we used (without the methoxy) as shown in Figure 2.5.¹⁰⁰ He was able to reduce it by HCOONa and to crystallize them and gain access to their structure through X-Ray diffraction. He verified that they were stable until pH 2-3.

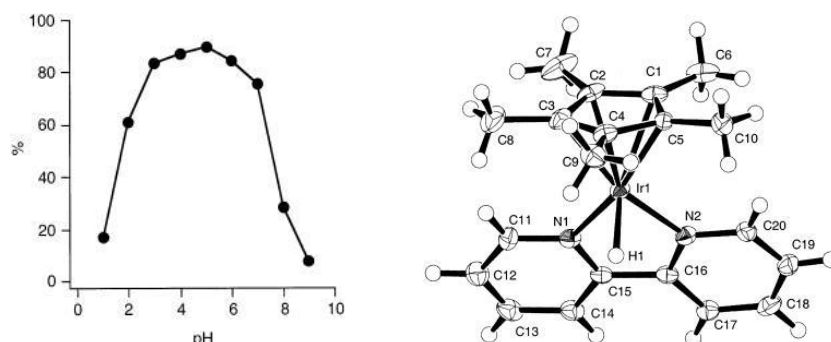


Figure 2.5 Left: stability of the iridium hydride over pH. Right: Crystal structure of $[Cp^*(bpy)Ir(H)]^+$ ¹⁰⁰

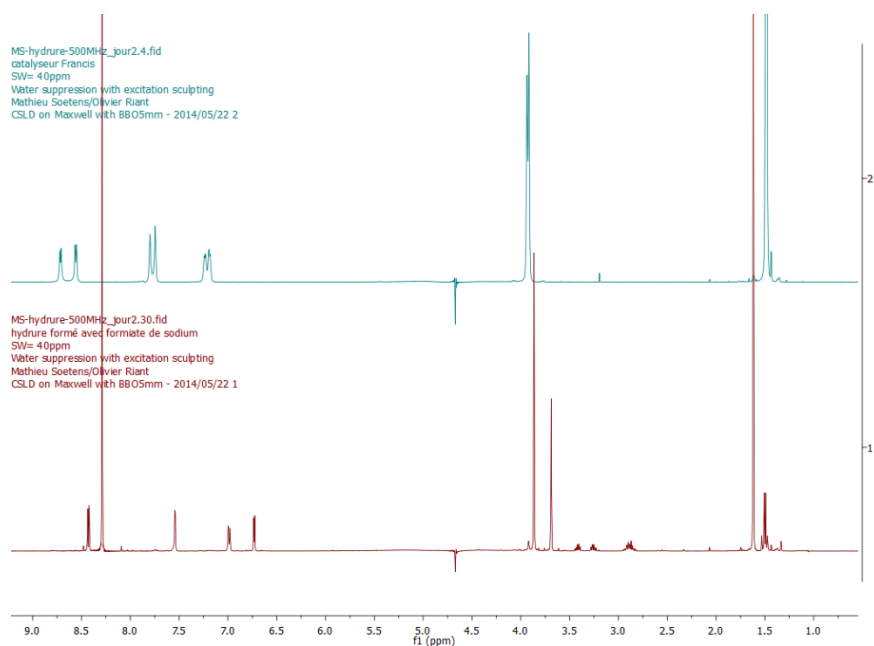
- Do also carried out the same study and was able to isolate the hydrides of his catalysts formed by reduction with NaBH₄ and

showed that they were viable intermediates in aldehyde reduction by using them as a stoichiometric reductant.⁶⁷

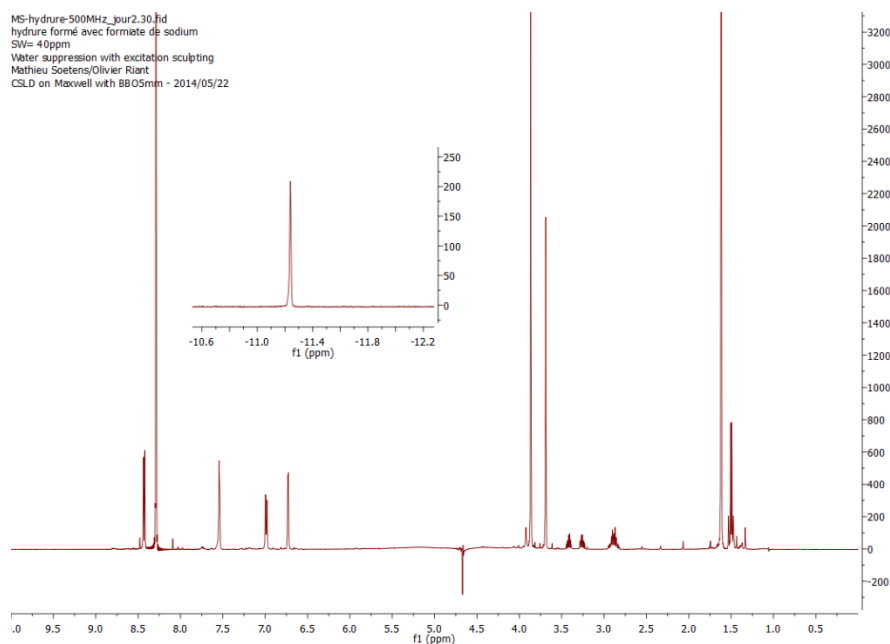
Knowing that this type of complex, treated by HCOONa, is able to reduce imines in water, as demonstrated by Francis, we wanted to verify if we could observe the iridium hydride **Ir1.H** by treating our iridium complex **Ir1** by NADH and see if we could observe the signal of the hydride in the high field part of the spectrum. It is according to the literature at around -11 ppm for this type of complex.¹⁰⁰

Since this type of iridium hydride was reported to exchange with protons due to its ambivalent hydridic and protonic character as it is virtually a pure covalent bond with the iridium. Therefore, we needed to carry out the experiment in water, use 10% of D₂O for the lock, and use a sequence to suppress the water signal, called *excitation sculpting*.

We set up our experiment in two steps. At first, we treated **Ir1** by 10 equivalents of HCOONa and could observe the signal of the hydride at -11.3 ppm (Graph 2. 9). The first obvious observation which we can make is the shift of the aromatic peaks of the bipyridine ligand on the left of the spectrum in average to the right, due to the increase of electron density. The OMe and the Cp* peaks, materialized by single peaks at respectively 4 and 1.5 ppm, underwent a slight change (Graph 2. 8). The spectrum is easily readable due to the simple structure of the catalyst and HCOONa (singlet at 8.3 ppm).

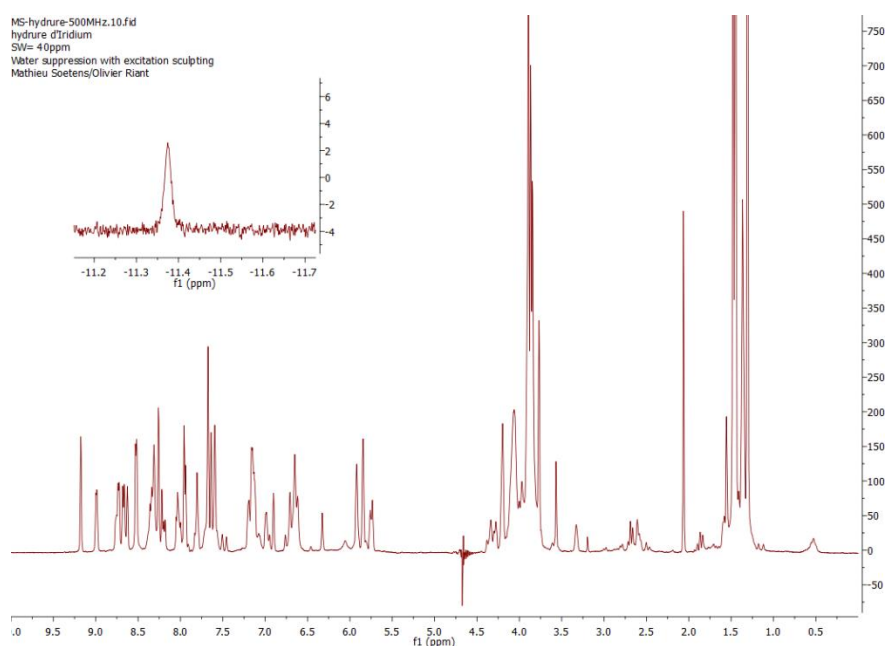


Graph 2. 8 Top: Reference **Ir1**: From left to right: 3 aromatic signals for the bipyridine, 1 signal at 4 ppm for both methoxy, 1 signal for C_5Me_5 (Cp^*). Bottom: After treatment with $HCOONa$, **Ir1.H**. Peak of $HCOONa$ at 8.3 ppm. Other peaks undergo a slight change.



Graph 2. 9 **Ir1** after treatment with $HCOONa$ giving **Ir1.H**: full spectrum. The hydride signal is put in evidence at -11.3 ppm.

Then, when we used NADH and observed a signal at the same shift of -11.3 ppm, but with a weaker intensity for the same quantity of iridium (Graph 2. 10). The smaller intensity can be attributed to the smaller stoichiometric amount used with NADH compared to the 10 equivalents used with HCOONa, but also to the electronic properties of the ligands, which tend to destabilize the iridium hydride. The spectrum is more complex and could not be easily assigned due to the numerous present species, among whose NADH and NAD^+ have complex spectra. However, the presence of the hydride was confirmed.



Graph 2. 10 **Ir1** treated by NADH giving **Ir1.H**. The spectrum is more complex.
The hydride signal is visible at -11.3 ppm.

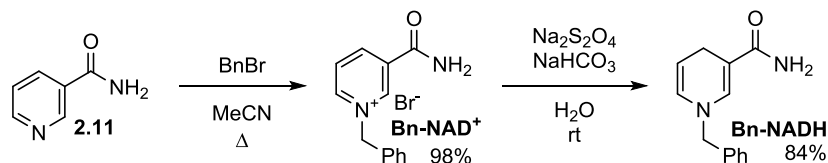
Those results, therefore, conclude that the **Ir1.H** is formed upon mixing of NADH and **Ir1**, and is very probably an intermediate of the reaction. Care must still be taken, as we did not attempt to

visualize the reduction after adding a stoichiometric amount of imine to the hydride, as done by Do.⁶⁷

3.4 Imine reduction with an NADH analogue

3.4.1 Model reaction: new hydride source

Due to the high price of NADH, we attempted to find an analogue for practical use in synthesis of the developed method. We found in the literature an NADH analogue featuring the classical dihydropyridine moiety coming from NADH, but with just a benzyl group, called **Bn-NADH**. The synthesis was easy to perform,¹⁰¹ but the product seemed to be sensitive to at least two factors among light, oxygen or temperature. It was synthesized up to 15 gram-scale by benzylation of nicotinamide **2.11** by benzyl bromide to give **Bn-NAD⁺**, which was reduced by Na₂S₂O₄, which selectively gives the 1,4-reduction product **Bn-NADH** as a yellow solid (Scheme 2. 38).

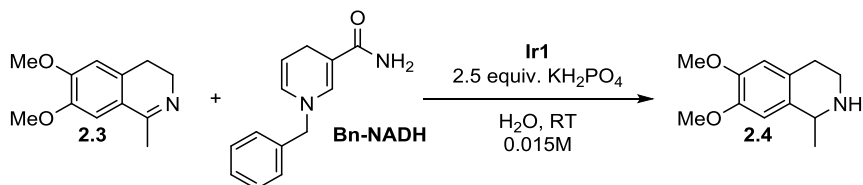


Scheme 2. 38 **Bn-NAD⁺** and **Bn-NADH** synthesis¹⁰¹

3.4.2 Optimisation

We decided to stick to the same model reaction of reduction of **2.3** to **2.4**, with 2.5 equivalents of acid and keeping the substrate concentration of 15 mM (Scheme 2. 39). However, we wanted to know how much **Ir1** and how much **Bn-NADH** could be used. We chose to test 0.5, 1 and 2 mol% and see the result using between 1 and 2 equivalents of **Bn-NADH** (Table 2. 3). The first observation was that the yield was much higher with **Bn-NADH**, with 84% using 1 equivalent, while a yield of 63% was obtained with NADH. This

effect is attributed to a higher reduction potential compared to NADH, thus providing a higher thermodynamic driving force. In summary, we considered the result with 1.5 equivalent of Bn-NADH and 1 mol% of **Ir1** as the best as it was the most atom economic.



Scheme 2.39 Optimization of the reduction of **2.3** by **Bn-NAD⁺**

Table 2.3 Optimization of the reduction of **2.3** by **Bn-NAD⁺**: influence of the catalytic loading and the equivalents of **Bn-NADH**

		Ir1 loading		
		0,5	1	2
		mol%	mol%	mol%
Bn-NADH equivalents	1	77%	79%	84%
	1,25	84%	89%	89%
	1,5	85%	95%	96%
	1,75	91%	97%	95%
	2	95%	99%	98%

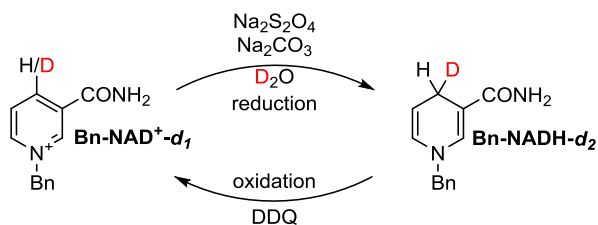
Those aforementioned conditions were considered optimal and were kept as such for an exemplification carried out later

3.4.3 Deuteration

For our understanding of the mechanism, being able to see a hydride by treating **Ir1** with NADH was the first step. However, this was not sufficient as we did not know if the hydride was transferred to the imine. With the use of **Bn-NADH**, we saw the opportunity to make a deuterated analogue on the position 4 to see if that hydride was transferred to the imine.

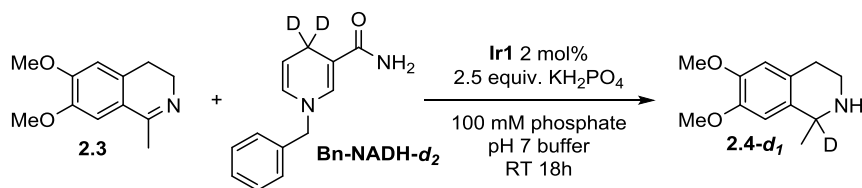
Bn-NADH-d2 was synthesized by cycling the reduction of the **Bn-NADH** with Na₂S₂O₄,¹⁰¹ but with Na₂CO₃ and in D₂O with its re-

oxidation with DDQ (Scheme 2. 40). After 3 cycles, the deuteration reached 80%.

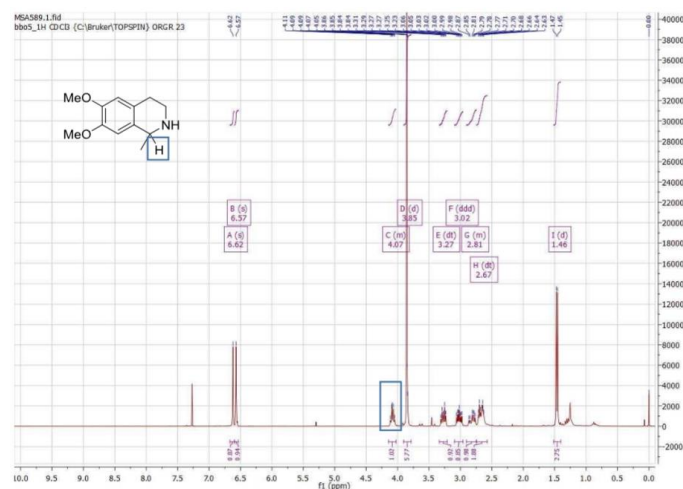


Scheme 2. 40 Synthesis of **Bn-NADH-d₂**

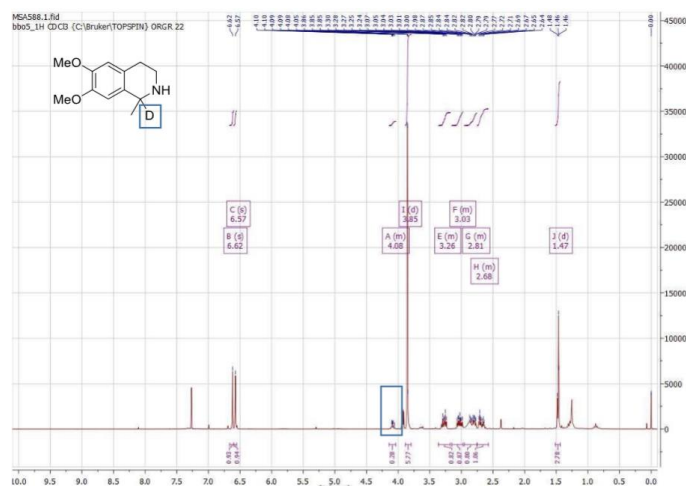
The reaction was performed as usual in a non-deuterated solvent and gave a complete conversion. The product was isolated and subjected to ¹H-NMR, and a decrease of the integral of the proton in α to the methyl from 1.00 (**Bn-NADH**, Scheme 2. 41, Graph 2. 11) to 0.28 (**Bn-NADH-d₂**, Scheme 2. 41, Graph 2. 12) was noted.



Scheme 2. 41 Deuteration experiment of the **2.3** by **Bn-NADH-d₂**



Graph 2. 11 ^1H -NMR spectra of the **2.4** obtained by reduction with **Bn-NADH**



Graph 2. 12 ^1H -NMR spectra of the **2.4-d₁** obtained by reduction with **Bn-NADH-d₂**

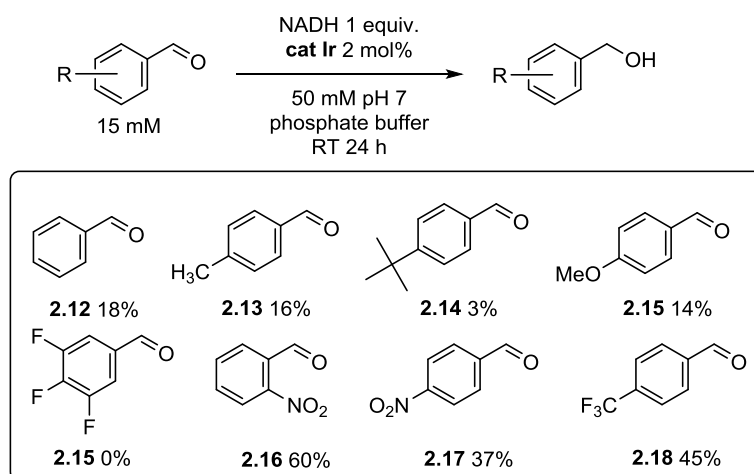
This is the sign that the reaction proceeds through a hydride transfer from the 4 position, and that the deuteride does not have the time to exchange in a large extent with the protons of the reaction medium. Combined with the ^1H -NMR observation of the hydride signal of **Ir1.H** generated with NADH, it now confirms that the reaction takes place through a hydride transfer to the metal centre.

3.5 Exemplification with NADH and Bn-NADH

During the course of this work, several exemplifications were carried out, but in many different condition sets which were thought to be optimal at the time. For each of them, care must be taken while reading the conditions.

3.5.1 NADH

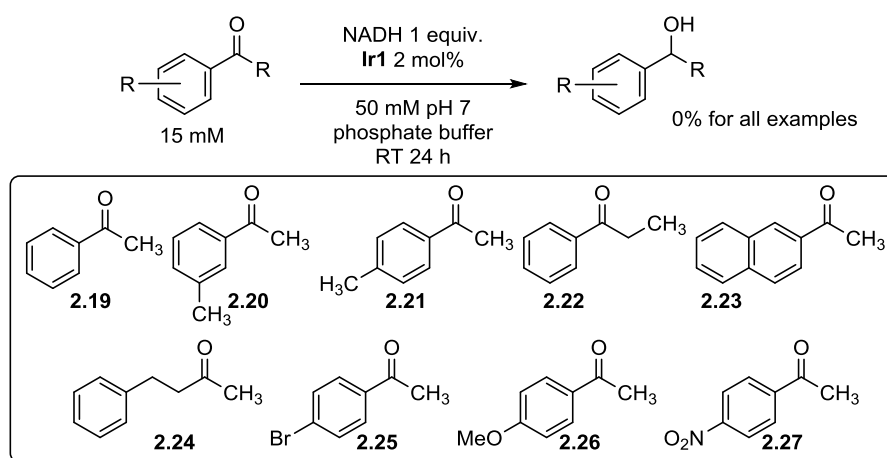
The first screening to be performed was to try to extend the scope of the reaction to aldehydes (Scheme 2. 42). At the time, we were still thinking that using 50 mM phosphate buffer with the substrate at 15 mM was the optimal conditions.



Scheme 2. 42 Screening on aldehydes with NADH. Yields determined by ¹H-NMR with dimethyl terephthalate

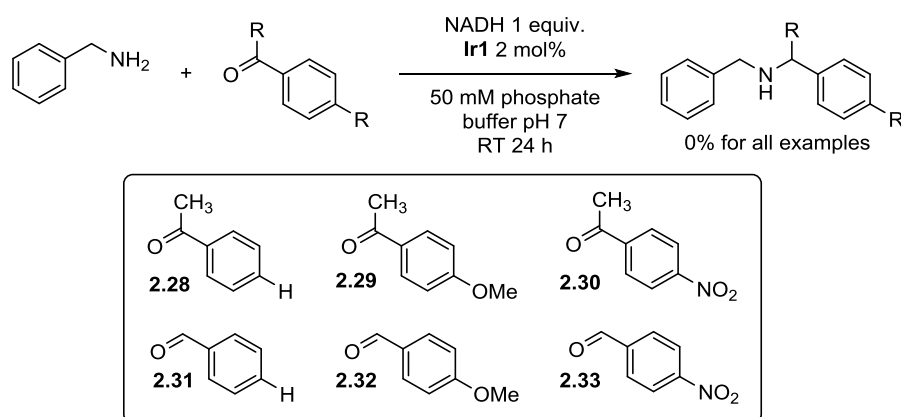
We noticed that the aldehydes could be reduced with low yields except from **2.16**, **2.17** and **2.18**. Although they are more electrophilic than imines, the absence of protonation at pH 7 makes them less reactive than imines in general. Also, as some of them are poorly soluble in water, the yield has certainly been biased and is thus underestimated.

Facing these results with aldehydes, we attempted to test the reaction on more challenging substrates, the ketones (Scheme 2. 43). Whereas in organic solvents, they are more reactive than imines but slightly less reactive than aldehydes, they are by far less reactive in water than imines, as they cannot be activated by protonation. None of them could be reduced to the corresponding alcohol.



Scheme 2. 43 Ketone screening.

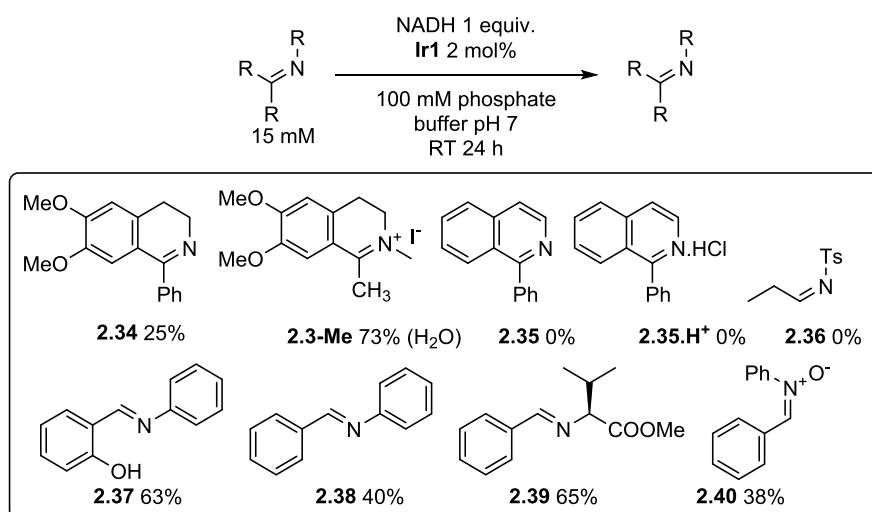
As no success was met with ketones, but more success was reached with aldehydes, we attempted to carry out reductive amination using ketones and amines (Scheme 2. 44). The clear advantage was that we would not suffer from a side reaction consuming the ketone, as it is inert under those reductive conditions. So, keeping benzylamine as the amine partner, we tried several ketones (2.28 to 2.30) with very different electronic properties. Facing an absence of success with ketones, we even tried with aldehydes (2.21 to 2.33), but in each case, the starting material was observed without reduction. It is probable that the reaction needs more acidic conditions, but we did not have enough expertise at that time.



Scheme 2. 44 Attempt of reductive amination on ketones and aldehyde in neutral conditions

From the moment we found the optimal condition after elucidating the role of the acid, we tried a few structurally diverse substrates in those optimized conditions (Scheme 2. 45). At first, we tried the closest analogies we could find relative to **2.3**. The 1-phenyl analogue **2.34** was easily synthesized using a benzoylation followed by a Bischler-Napieralski cyclization, similarly to the **2.3**. When **2.34** was treated in the standard conditions with NADH, a yield of 25% was obtained, which is due to a lower solubility than **2.3**. For problems encountered in the next chapter, we tried to synthesize an imine with reduced nucleophilic properties, by methylating its nitrogen, the compound **2.3.Me**. It would also create a highly activated iminium, with a good water solubility, facilitating the reduction. A yield of 73% was obtained, which is superior to what was obtained for **2.3** with similar conditions (60%). We also got interested in isoquinolines **2.35** and its protonated form **2.35.H**, as we wanted to know if the reduction potential of the **Ir1.H** was sufficient to break the aromaticity, but neither the **2.35** nor its hydrochloride **2.35H** could be reduced. We then turned our attention to non-cyclic systems, for

which the hydrolysis must be slower than the reduction. Otherwise, hydrolysis products would be observed. Our interest went for two simple imines which were easily synthesized by condensation, *N*-Salicylidene aniline **2.37** and *N*-benzylidene aniline **2.38**, for which yields of 63% and 40% were obtained. We tried the *N*-benzylidene valine methyl ester **2.39**, which was already available for other projects, and this trickier substrate still gave us 65% of yield. We ended up this exemplification with phenyl benzyl nitron **2.40**, for which a yield of 38% was obtained by NMR and a low solubility.



Scheme 2. 45 Exemplification of the imine reduction with more structurally diverse imines

This series of examples show that the method is applicable to structurally diverse imines and convinced us that we would have a broad choice of NADH-responsive trigger moieties for the project about prodrugs.

3.5.2 Bn-NADH

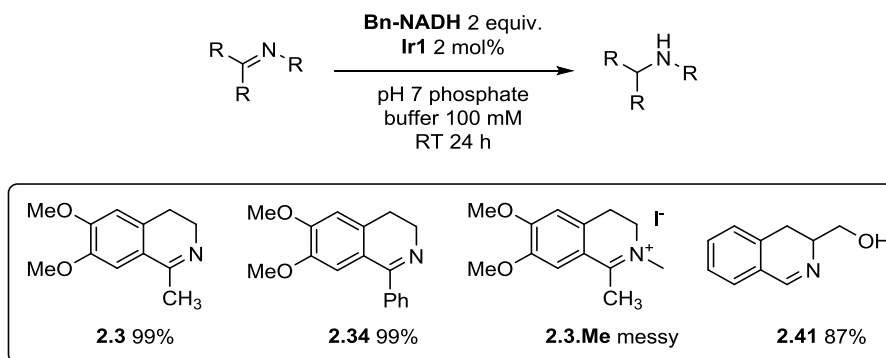
After a successful exemplification of the method with NADH, and as we found that quantitative yields could be easily obtained with

our catalytic system and **Bn-NADH**, we decided to use this hydride donor for an exemplification.

Our screening was performed only using the optimized conditions of either 2.5 equivalents of KH_2PO_4 or 100 mM phosphate buffer. Our screening involved increasingly complex structures starting from tetrahydroisoquinolines, linear imines and some non-linear imines with more special structure.

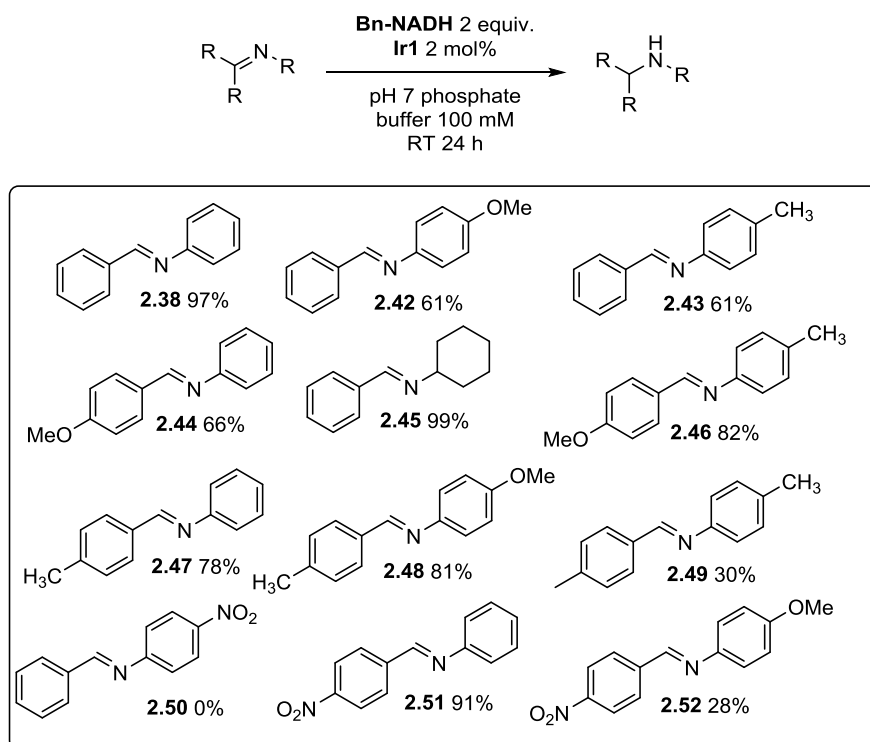
For all the isolated yields, we had to change NaHCO_3 to NaOH in the workup procedure, as the yield was systematically above 100% with NaHCO_3 whereas the conversion was total as seen by $^1\text{H-NMR}$. We attributed to the presence of a counter-anion to **2.4.H⁺**, but we could never identify it precisely.

At first, we remained within the chemical space of dihydroisoquinolines, for which the four substrates had been prepared before (Scheme 2. 46). Excellent yields were obtained with the **2.3** and its 1-Ph analogue **2.34**, although the position is bulkier and harder to reduce. Although the reduction of the *N*-methylated analogue of **2.3** gave a satisfactory reduction yield with NADH , the reaction mixture was too messy to be analysed when **Bn-NADH** was used. Finally, the compound **2.41** was reduced with a satisfactory yield of 87%, similar to the one obtained with NADH .



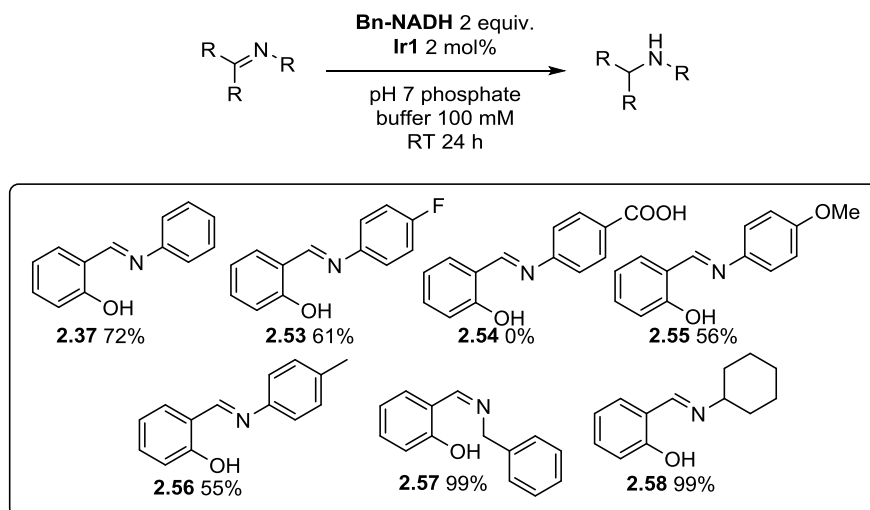
Scheme 2. 46 Imine exemplification. Family 1: tetrahydroisoquinolines

Then, we studied with a bit more details the *N*-benzylidene imines (Scheme 2. 47). They could be easily synthesized by mixing the aldehyde and the imine without solvent, followed by filtration on a Na_2SO_4 pad to dry the compound; the pure products were isolated by Kugel Rohr distillation to give the imines under the form of white powders, except **2.45**, which was the only liquid. Whereas good reduction yields were obtained most of the time, we found that the yields were dependant on the solubility of the compounds in water. On the contrary to similar studies⁶⁷, we did not use any co-solvent as the goal of the project was to make a biocompatible reaction. Whereas electron-withdrawing groups (**2.50**, **2.51** and **2.52**) should activate the substrates regardless if they are placed on the aldehyde or the amine part. However, the examples **2.50** and **2.52** were caused by their low solubility. In the other substrates used, no clear trend could be seen, indicating that the low solubility of several compounds could be to blame. The only liquid compound **2.45** gave the best results, indicating that the contact between the compound and the reaction medium is primordial.



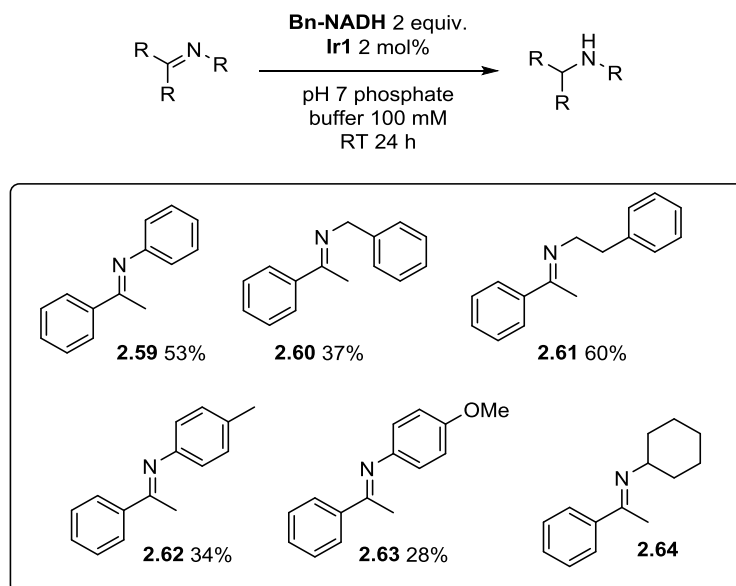
Scheme 2. 47 Imine exemplification. Family 2: *N*-benzylidene series

The *N*-Salicylidene family was the third one to be tested (Scheme 2. 48). All the compounds were synthesized by mixing neat the salicylaldehyde with the amine until all the liquid was consumed with manual stirring using a spatula. The compounds were all solid, except **2.57** and **2.58** which were bright yellow liquids. Again, the same trend was observed, as the liquid compounds (**2.57** and **2.58**) gave always a quantitative yield. In the case of **2.54**, the compound was so insoluble that we could not even dissolve it in DCM for the workup.



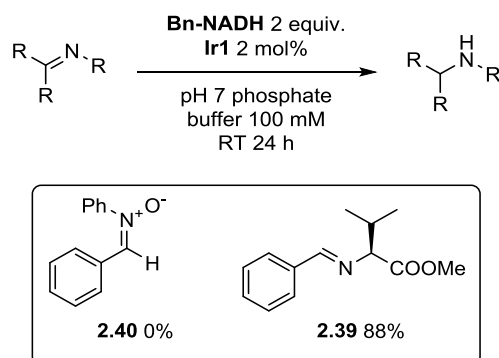
Scheme 2. 48 Imine exemplification. Family 3: N-Salicylidene series

The fourth family, synthesized again using the same method, was even more challenging as those compounds were less reactive due to the steric hindrance caused by the methyl group at the carbonyl centre (Scheme 2. 49). However, as they were all liquids, the contact with the reaction medium was better and thus moderate yields were obtained. It is again difficult to understand the trend in the reactivity.



Scheme 2. 49 Imine exemplification. Family 4: α -methyl-N-Benzylidene series

The last family contained the nitron **2.40**, which turned to be non-reactive under our conditions, as well as a benzylidene derivative of L-valine methyl ester **2.39**, which was successfully reduced with 88% (Scheme 2. 50).

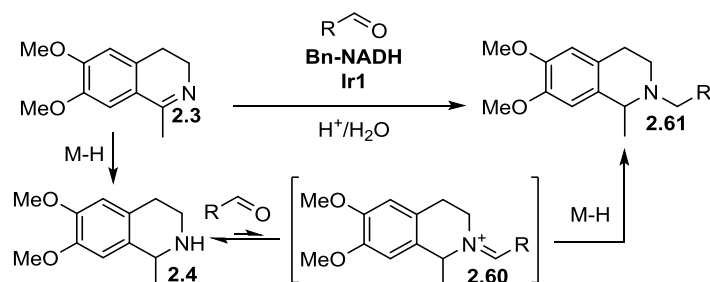


Scheme 2. 50 Imine exemplification. Family 5: various

3.5.3 Reductive alkylation

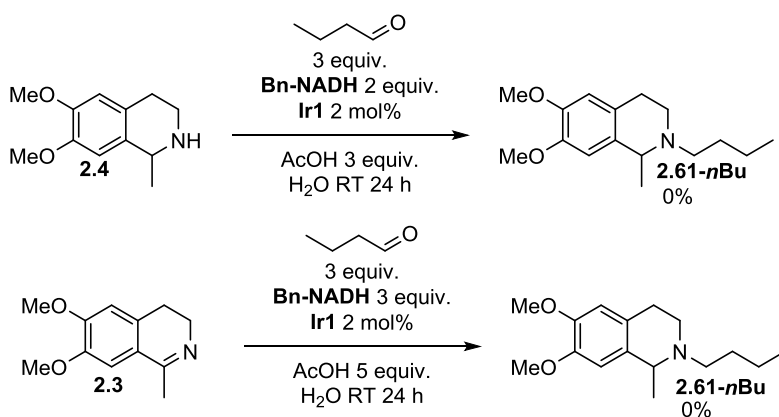
To conclude this substrate scope, we wanted to see if we could carry out the one-pot reduction of **2.3** directly followed by its

reductive alkylation substrate to form **2.61** with an aldehyde and using our catalytic system for the reduction involved in the two steps (Scheme 2. 51).



Scheme 2. 51 General concepts of one-pot reduction and reductive alkylation

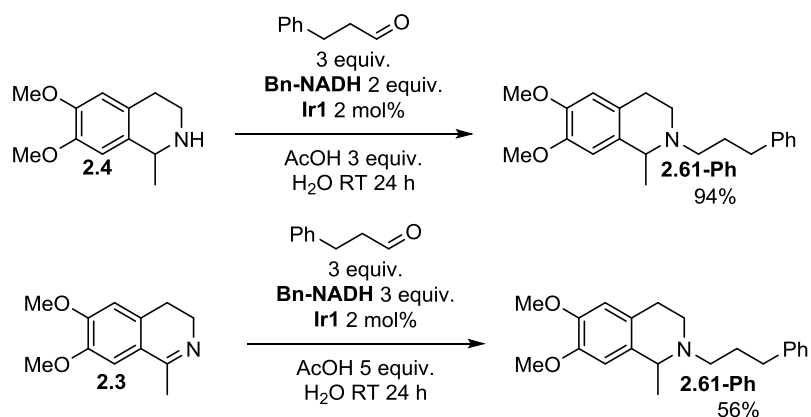
Before testing the whole system, we moved one step backwards and synthesized **2.4** by reduction with NaBH₄ (Scheme 2. 52). It was then reacted with butyraldehyde, **Bn-NADH** and the catalyst in presence of AcOH. However, no product **2.61-nBu** was observed. We tried the complete system starting from **2.3**, but no success was met again since **2.3** and **2.4** were isolated in proportions **2.3/2.4** 5:13, sign that the reduction only took place partially.



Scheme 2. 52 First attempt with butyraldehyde

While replacing the butyraldehyde by dihydro cinnamaldehyde, a yield of 94% in **2.61-Ph** could be obtained starting

from **2.4** (Scheme 2. 53). By using the same conditions as attempted above, the product was obtained with 56% using the complete system starting from **2.3**, with no cinnamyl alcohol isolated but some unreacted **2.3** isolated.

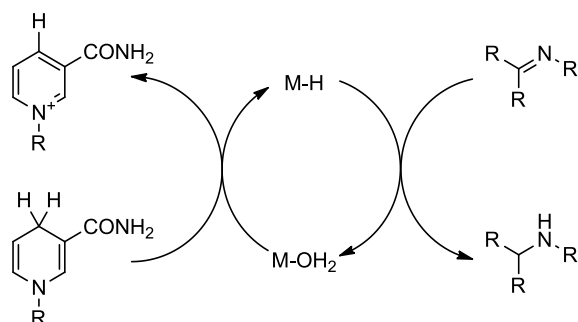


Scheme 2. 53 Second attempt with dihydro cinnamaldehyde

This example concludes the work on the development of the iridium-catalysed imine reduction.

3.6 Discussion

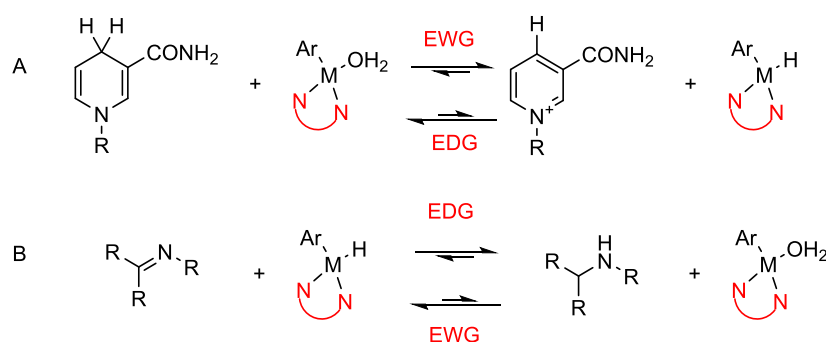
In this first part, our aim was to enrich the bioorthogonal toolbox with transfer hydride reductions using organometallic catalysts. We also aimed to demonstrate the applicability and the biocompatibility of this reaction in a cellular environment in the context of prodrug uncaging (Scheme 2. 54).



Scheme 2. 54 Global concepts of imine reduction by transfer hydride couple to an enzymatic cofactor recycling system

In this first part, we faced only minor difficulties as this project is the most in line with the research interests of the team. The only difficulty to overcome was to find a catalyst able to react with NADH and whose hydride can reduce an imine in water. Although both steps are well documented in the literature, we made the mistake at first to focus exclusively on the literature about imine reduction, which is classically carried out with hydrogen or sodium formate and works with different catalysts. We spent a substantial amount of time at the very beginning of the work screening catalysts without knowing what the requirement was to interact with NAD(P)H cofactors. It became clear once we dug into it that the electronic properties of the ligand are the determinant factor enabling or not the hydride transfer. Our observation did not differ from Sadler⁶⁴ and Francis, who stated that an electron poor ligand was necessary to stabilize through inductive effect the additional electrons on the metal (Scheme 2. 55A). However, such an electron-poor hydride would be unreactive for hydride transfer reduction, blocking the catalyst in the hydride form. Francis reported that to reduce imines in water with HCOONa, he needed an electron-rich bipyridine (Scheme 2. 55B).⁹⁵ In our case, the Francis catalyst **Ir1** was found to be the most efficient. And any

structural variation we made did not lead to any improvement. It seems that the optimal ligand design involves a globally electron poor ligand, such as a bipyridine to lower the activation energy of the hydride transfer towards the catalyst, but still not too poor otherwise it would stabilize too much the hydride intermediate. That may result in too high activation energy for the hydride transfer to the imine, making the reaction very slow.



Scheme 2.55 Effect of the electron properties of the two hydride transfer steps

We ended up thinking that the reaction is limited by an equilibrium, as changing the catalyst could not give us higher conversions. One experiment in which the imine was reacted with NAD⁺ and the catalyst gave us 30% of imine, showing that the reaction is clearly reversible. Adding a second equivalent of NADH increased the yield, which is a logical consequence of Lechatelier Principle. The yield was lower than the opposite reaction as the amine should be lower in energy than the imine for the same putative intermediates in the reverse reaction. As the last argument, changing the hydride donor to **Bn-NADH**, with a higher E⁰ than NADH, gave us a higher yield¹⁰².

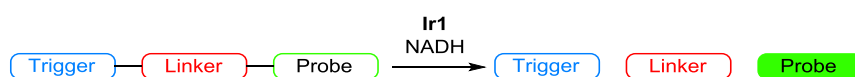
The optimization showed globally a good applicability of the method towards imines, cyclic or not, and had more difficulties to

reduce aldehydes. We believe that all the measured yields after isolation are biased due to the low solubility of several species in water. On contrary to other studies where co-solvents were used (Do⁶⁷: tBuOH, Fish⁶⁵: THF, Sadler⁶⁴: MeOH), we did not use any co-solvent as we were focused on biological application and considered that using a co-solvent was a solution for synthesis but not for bioorthogonal chemistry. Co-solvents can be used in biological applications with max 1% in the culture medium to improve the solubility. DMSO has been the most frequently used due to its excellent compatibility; DMF has been infrequently used with good results as well. This decision could have penalized us by giving lower results, especially while using **Bn-NADH** in the exemplification.

First part: From the methodology to applications
in prodrug decaging
Second chapter: Design, synthesis and testing of
NADH-responsive fluorogenic probes

4 Second chapter: Design, synthesis and testing of NADH-responsive fluorogenic probes

Following the successful development of an easily applicable imine reduction proceeding through a hydride transfer, we wanted to transpose the method to the activation of a fluorogenic probe responding to NADH in presence of our iridium catalyst **Ir1** (Scheme 2. 56).



Scheme 2. 56 Concept of NADH-responsive fluorogenic probes

Such a fluorogenic probe comprises several interesting features. The reaction can only be intracellular as there is no extracellular NADH. Then, since many solid tumour masses tend to have a poor neovasculature resulting in hypoxic areas. Those hypoxic zones are characterized by a low P_{O_2} , an acidic pH between 7.2 and 6.5 and a higher NADH/NAD⁺ ratio.¹⁰³

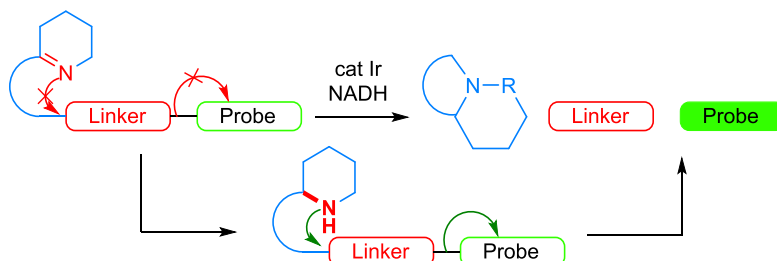
When we examine our imine reduction method, developed in the previous paragraph, it is enhanced by the presence of acid and consumes NADH. We may expect that our method will be able to recognize those conditions, which will result in selectivity.

4.1 First generation of fluorogenic probe

4.1.1 Design of an NADH-responsive fluorogenic probe

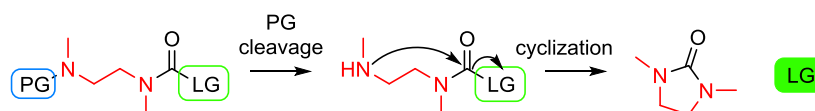
Following the literature on the subject, we decided to devise fluorogenic probes featuring three parts, a *trigger*, a *linker* and the *payload*. As a direct consequence of the previous project, we decided to choose cyclic imines as a trigger. Indeed, they have proven to be water-stable, soluble and reactive (Scheme 2. 57). This first generation

of prodrugs is based on the higher nucleophilicity of the amine compared to the starting imine.



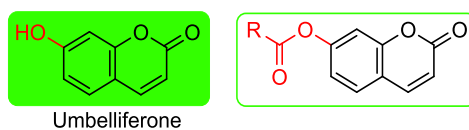
Scheme 2. 57 Principle of cyclization and immolative prodrugs

Then, a suitable self-immolative linker is needed to bring the thermodynamic driving force to the system. Our choice went for the *N,N'*-dimethyl ethylene diamine dicarbamate, described for the first time by Shabbat (Scheme 2. 58).¹⁰⁴⁻¹⁰⁶ Once one of the two amines is released, it attacks the carbamate and forms the cyclic urea and released the O-leaving group. The driving force is both entropic and enthalpic.



Scheme 2. 58 Proposition of self immolative linker: dimethyl ethylenediamine

For the payload, our interest went for umbelliferon, which is a cheap fluorophore whose fluorescence can be quenched easily by functionalizing the 7-OH position by around 96% (Scheme 2. 59). It can easily be followed by fluorimetric methods thanks to its broad excitation and an emission peak at 325 nm and 454 nm respectively.



Scheme 2. 59 Umbelliferon

The final prodrug design ended up with the following structure (Figure 2. 6).

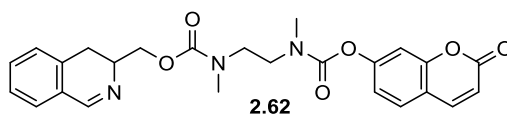
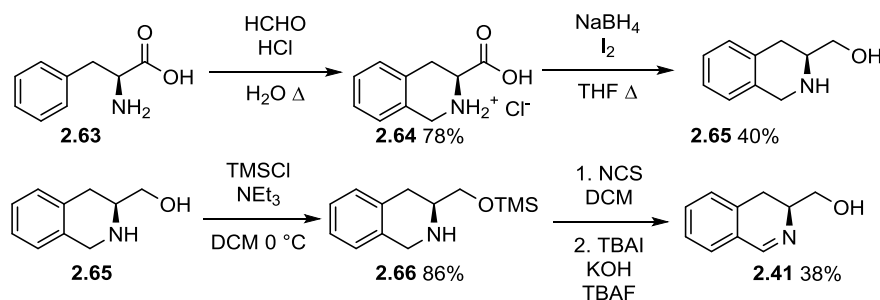


Figure 2. 6 First generation prodrug

4.1.2 Synthesis of an NADH-responsive fluorogenic probe

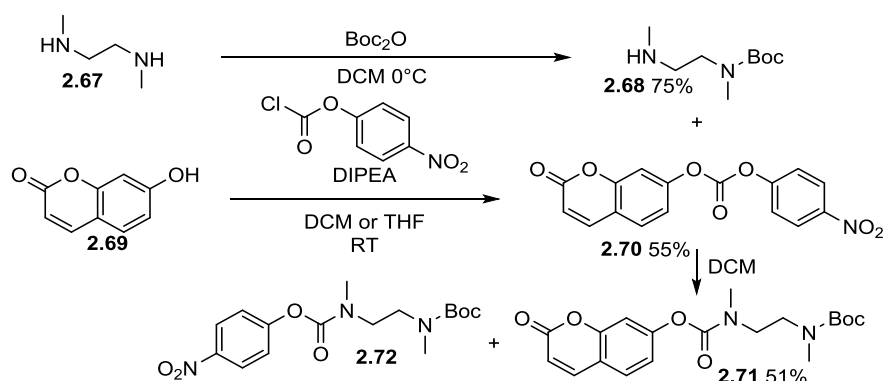
The work of synthesis was partially performed by Liban Abubaker, a trainee from a technical school in Brussels for his Bachelor Degree.¹⁰⁷

We first synthesized the trigger part of the fluorogenic probe (Scheme 2. 60). At first, L-phenylalanine **2.63** was treated by formaldehyde 35% in water in strongly acidic conditions under reflux for 4h to yield the Pictet Spengler adduct **2.64** in the form of a salt.¹⁰⁸ The carboxylic acid **2.64** was reduced to the alcohol **2.65** by the classical $\text{NaBH}_4 + \text{I}_2$ combination, which generates *in-situ* B_2H_6 and which can selectively reduce carboxylic acids.¹⁰⁹ To oxidize the amine **2.65** to the corresponding imine **2.41**, we had to protect first the alcohol **2.65** with TMS to **2.66** before carrying out the electrophilic chlorination of the **2.66** in benzylic position with NCS.¹¹⁰ Upon addition of TBAF, followed by a basic treatment, the deprotonation lead to the formation of the imine **2.41** obtained in the form of pale orange cubic crystals.



Scheme 2. 60 Synthesis of the first generation of the trigger **2.41**

With the trigger **2.41** hands, we started to synthesize the linker part **2.68** (Scheme 2. 61). To a solution of *N,N'*-dimethyl ethylenediamine **2.67** in DCM at 0 °C, a substoichiometric amount of Boc₂O in DCM was added by syringe using a syringe plunger over 4-6 hours to get the monoprotected compound **2.68**.¹¹¹ Separately, umbelliferone **2.69** was treated with PNP-chloroformate with DIPEA to get the PNP-activated umbelliferon **2.70**, obtained as a white powder.¹⁰⁶ It was reacted with mono protected **2.68** in DCM to give the coupling product **2.70**.¹⁰⁶ However, a large amount of by-product **2.73** was obtained (nearly 50%) as the pK_a of umbelliferon and 4-nitrophenol are similar and around 7. The side product **2.72** could be eliminated by flash chromatography. The presence of the two carbamates in **2.71** is responsible for the formation of several rotamers, giving broad signals with variable multiplicity in ¹H-NMR.

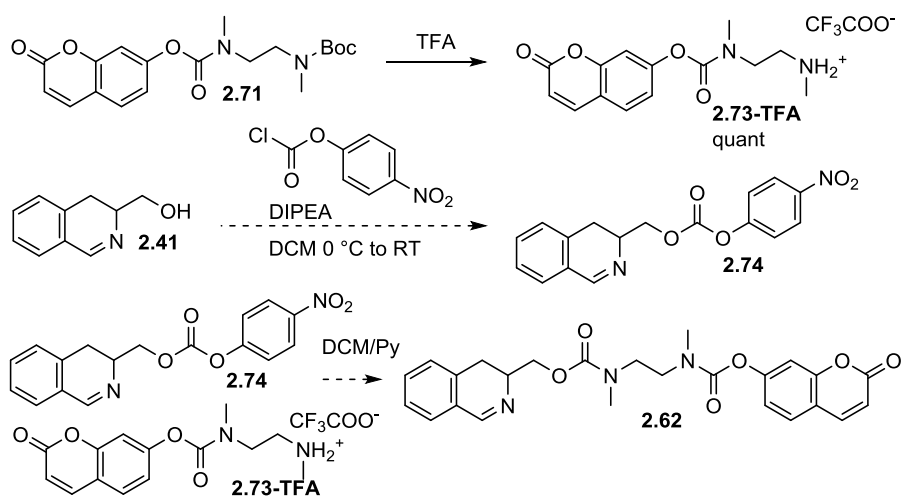


Scheme 2. 61 Coupling of the payload **2.69** to the linker **2.68**

At first, we wanted to obtain the final product **2.62** in three simple steps (Scheme 2. 62):

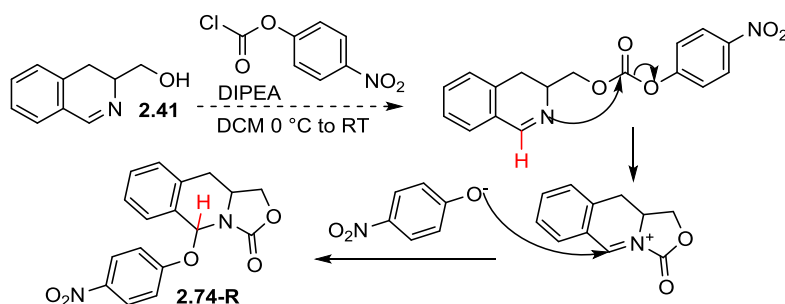
- Boc deprotection of **2.71** to give **2.73-TFA**,
- PNP-activation of the **2.41**
- Coupling between **2.74** and **2.73-TFA**.^{106, 111}

So, the Boc **2.71** was deprotected by treatment in TFA giving **2.73-TFA** in a quantitative yield.¹¹¹ The protonated amine is unable to cyclize on the carbamate and can only be deprotonated if the electrophile partner is present in the reaction medium. In parallel, we tried to couple the **2.41** with PNP chloroformate in DCM with DIPEA as the base.



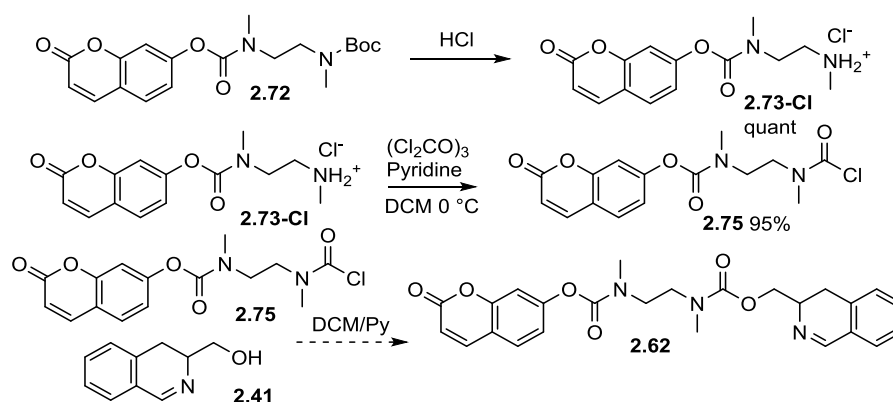
Scheme 2.62 First strategy: linker deprotection – activation and coupling of the trigger

However, the desired product **2.74** has never been isolated, and only a rearrangement by-product **2.74-R** was observed (Scheme 2.63). It was formed following the intramolecular attack of the imine on the carbonate. Upon leaving of the 4-nitrophenol, the iminium carbamate was back-attacked by the 4-nitrophenol to yield a hemiaminal *N*-carbamate **2.74-R**, which was stable and isolable. The NMR identification of the product was possible as the expected imine proton around 8.3 ppm was not visible and was found to be shifted at around 6.5 ppm in the form of a singlet.



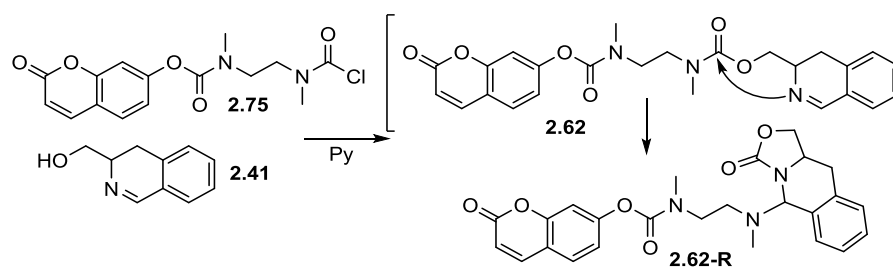
Scheme 2. 63 Rearrangement of the activated trigger **2.74**

So, concluding that the imine was too nucleophilic towards the 4-nitrophenyl carbonate, we believed that proceeding differently would avoid this type of problem (Scheme 2. 64). Hydrochloric acid was chosen to deprotect the Boc. We tried first to use an excess of HCl 4 M in diethyl ether, but without success because the product was not soluble in ether and because the reaction time was not sufficient. So, we opted for an *in-situ* generation of HCl from AcCl in MeOH/Et₂O.¹¹² The salt **2.73-Cl** was obtained as a white powder as it precipitated in ether. The salt **2.73-Cl** could be almost quantitatively converted to the carbamoyl chloride **2.75** by treatment with triphosgene and pyridine (Scheme 2. 64).¹¹¹ The identity was confirmed by HRMS. The compound **2.75** survived the aqueous workup and was used as such as the purity was sufficient in the crude NMR.



Scheme 2. 64 Third strategy: use of HCl instead of TFA

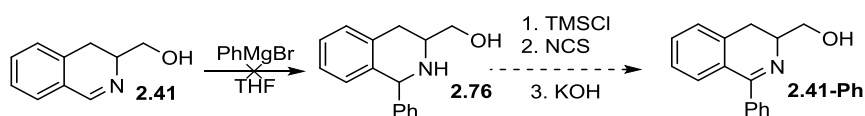
With **2.75** in hands, we attempted to couple it with the trigger **2.41** in pyridine,¹¹¹ and the product **2.62** was never isolated (Scheme 2. 64). Instead, the imine attacked and after elimination of the leaving group, the iminium carbamate was back-attacked to yield the hemiaminal carbamate **2.62-R** (Scheme 2. 65). It was confirmed by HRMS and by ^1H -NMR, where the imine signal around 8.3 ppm was lost and a new singlet appeared around 6.5 ppm accounting for the hemiaminal proton. Again, the imine was found to be too nucleophilic.



Scheme 2. 65 Rearrangement of the coupling product **2.62** to **2.62-R**

As a last attempt, we decided to try in a single test to convert **2.41** into **2.41-Ph** by reacting it with 2.1 equivalents of PhMgBr to increase the steric bulk, after which the **2.76** would need to be re-oxidized to the **2.41-Ph** (Scheme 2. 66). However, in those conditions,

no product **2.76** was observed, and the starting material was recovered.



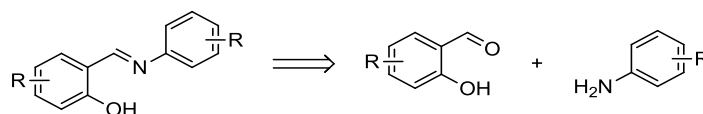
Scheme 2. 66 Attempt to synthesize a bulkier trigger

The problem of the nucleophilicity was judged critical. We were unable to come with a realistic idea in a synthesis which would give us a stable prodrug. We decided to turn our attention to other types of imines to serve as a trigger.

4.2 Second generation of fluorogenic probe

4.2.1 Design of an NADH-responsive fluorogenic probe

For this second generation, we chose to move to a non-cyclic imine. We noticed that in the exemplification section *N*-Salicylidene anilines were interesting as they could be easily synthesized and were efficiently reduced to the corresponding amines with no observation of hydrolysis products (Scheme 2. 67). Additionally, it would be possible to modulate the reactivity of the trigger by using different building blocks at the end of the synthesis, following a divergent synthesis fashion.

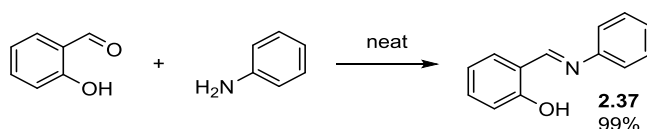


Scheme 2. 67 Interest of *N*-Salicylidene anilines: two commercially available building blocks

4.2.2 Synthesis of an NADH-responsive fluorogenic probe

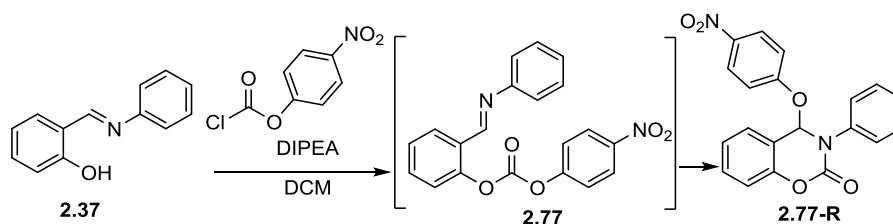
The work of synthesis was partially performed by Liban Abubaker, a trainee from a technical school in Brussels for his master thesis.¹⁰⁷ Similarly to the previous section, we started to synthesize the

trigger **2.37** by condensing salicylaldehyde (Scheme 2. 68).¹¹³ It was already available as it was synthesized and used during the exemplification previously.



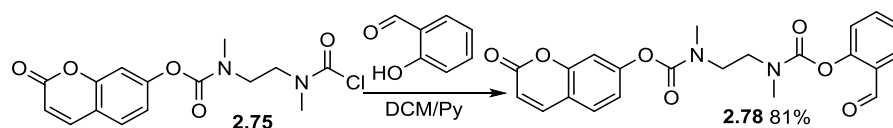
Scheme 2. 68 Synthesis of *N*-Salicylidene aniline **2.37**

The salicylidene **2.37** was then subjected to PNP chloroformate to activate it in the form of a mixed carbonate in the presence of DIPEA (Scheme 2. 69).¹⁰⁵ However, a re-arrangement took place and the desired product could not be isolated, but rather a cyclization product **2.77-R**, which could be identified thanks to ¹H-NMR.



Scheme 2. 69 Attempt to activate **2.37** with PNP-chloroformate

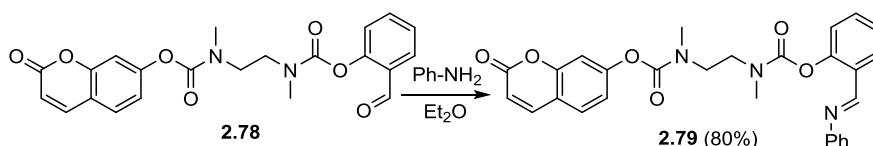
We then decided to proceed the other way. We were able to couple it to the carbamoyl chloride **2.75**, obtained previously to give the compound **2.78** (Scheme 2. 70).¹¹¹ Noticeably, the aldehyde proton was visible on the NMR.



Scheme 2. 70 Coupling of **2.75** with salicylaldehyde

Later, **2.78** was condensed with in diethyl ether and with a molecular sieve (Scheme 2. 71). The corresponding imine **2.79** was

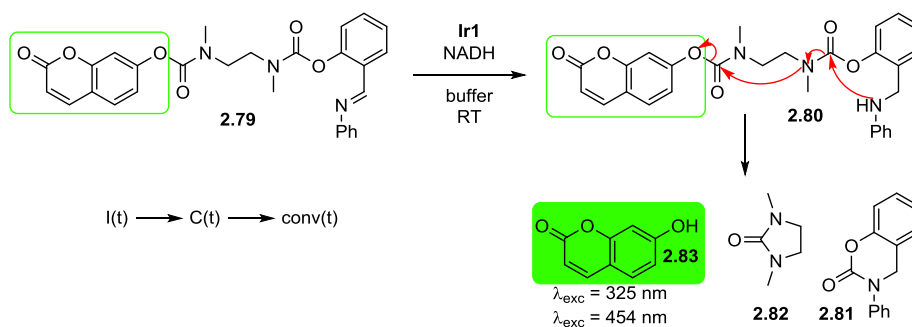
obtained and characterized. The probe **2.79** was purified by vacuum distillation with a Kugel Rohr apparatus to remove solvent traces, as well as the excess of aniline. The idea behind was to be able to modulate the reactivity of the free amine after the reduction of the imine to the amine.



Scheme 2. 71 Second generation of probes **2.79** obtained by condensation of anilines with the aldehyde

4.2.3 Testing of an NADH-responsive fluorogenic probe

The work was performed under supervision by Balázs Pogrányi, an Erasmus student, for a 3-months internship.¹¹⁴ The goal of his project was to see if our catalytic system was able to reduce the trigger, leading to the uncaging of the umbelliferon probe **2.83** (Scheme 2. 72).

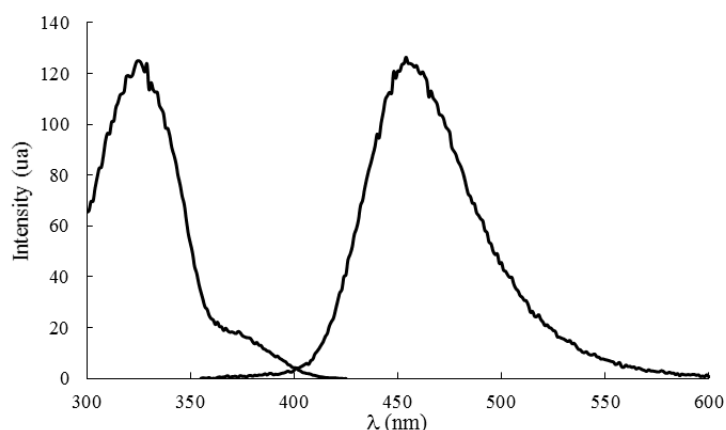


Scheme 2. 72 Fluorogenic decaging of the second-generation prodrug

4.2.3.1 Determination of the photophysical parameters of the prodrug

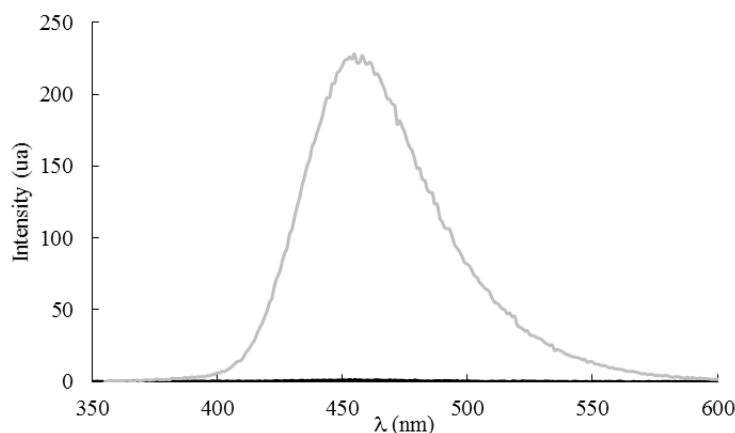
At first, our study aimed to get used to the spectrometer and carry out preliminary tests on the system. The spectrometer we had

available comprises a single quartz cell holder. The first step was to record an excitation-emission spectrum of the umbelliferon **2.83** in the buffer solution. We elected to work at 5 μM with 2% of DMSO. It was measured in our conditions that the excitation maximum was 325 nm and the emission maximum was 454 nm (Graph 2. 13).



Graph 2. 13 Excitation-emission of umbelliferon (5 μM in pH 7 phosphate buffer 50 mM with 2% DMSO), slits 2.5, average on 10 spectra

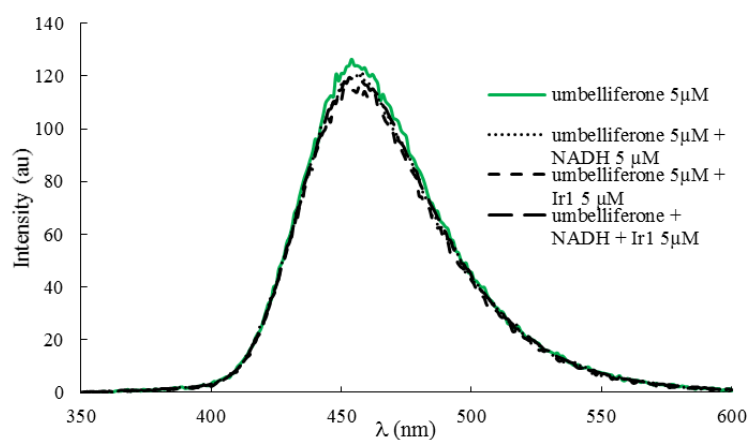
With the excitation and emission wavelengths in hands, we wanted to measure the on-off ratio between the umbelliferon and the probe **2.79**. Indeed, while working at 10 μM , the fluorescence of **2.79** is only 1.5% of the value reached by umbelliferon, while irradiating 325 nm (Graph 2. 14). It is in good agreement in the literature, stating that umbelliferon has its fluorescence quenched once the 7-hydroxy position is acylated.¹¹⁵ The probe can thus be called an *on-off* system.



Graph 2. 14 Excitation-emission of umbelliferon in grey (10 μ M in pH 7 phosphate buffer 50 mM with 2% DMSO) and 2.79 in black in the same conditions

4.2.3.2 Fluorescence quenching study

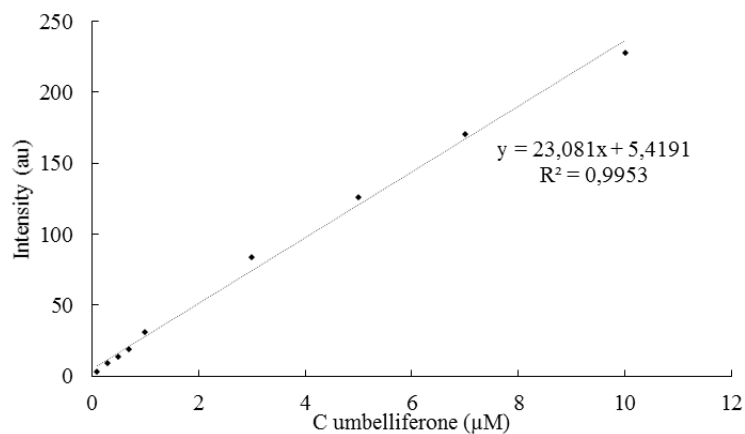
In order to make a quantitative method, we needed to study the interactions between the different components and umbelliferon, in order to see if its fluorescence is quenched or not. NADH can pose a threat to short-wavelength fluorophores (Graph 2. 15). As the LUMO is quite low in energy and that NADH is a reducing agent with a high HOMO, it can result in an electronic transfer between NADH and the excited state of the coumarin. Eventually, NADH is known to be fluorescent at a similar wavelength, $\lambda_{\text{exc}} = 340 \text{ nm}$, $\lambda_{\text{em}} = 460 \text{ nm}$, but at far lower intensities compared to the coumarin. So, with the concentrations that we tried, almost no quenching of fluorescence was observed with the different components of the system. This statement was found to be only true for low concentrations of NADH. The Stern-Volmer equation clearly shows that the quenching depends on the concentration with a sensitivity parameter which varies considerably depending on the pair of fluorophore-quencher, but also the solvent and the presence of oxygen.



Graph 2. 15 Emission spectrum of umbelliferone in presence of various quenching agents.
Phosphate buffer pH 7 50 mM + 2% DMSO

4.2.3.3 Calibration curve

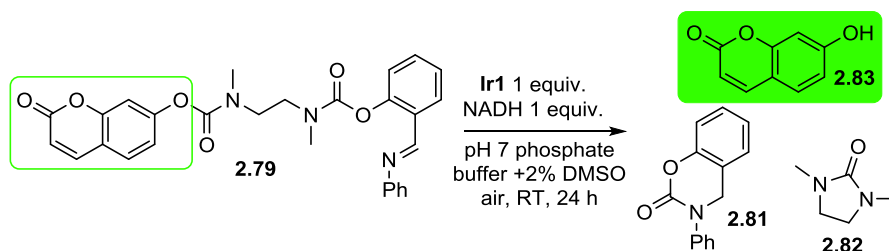
These results indicate that a simple calibration curve taking only account of umbelliferon is sufficient, thus enabling us to plot $I = a.C + b$ (Graph 2. 16).



Graph 2. 16 Umbelliferon calibration curve

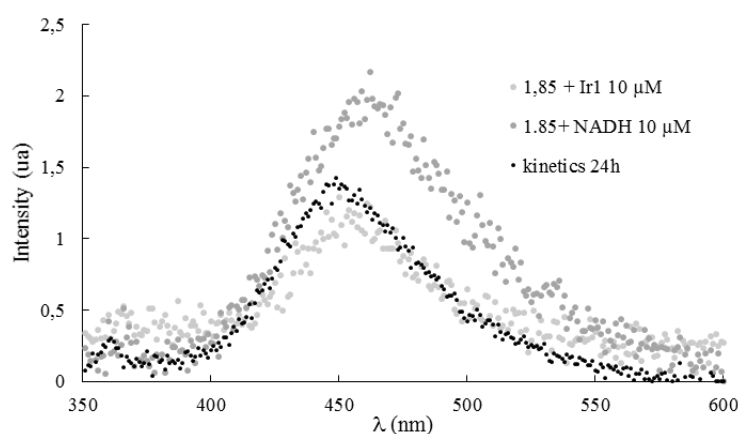
4.2.3.4 Fluorogenic kinetic test

With a calibration curve in hands, we could start testing the fluorogenic probe **2.79**. To start, we set the conditions as follows: 10 μ M of **2.79**, 1 equivalent of **Ir1** and 1 equivalent of NADH in pH 7 phosphate buffer 50 mM + 2% DMSO (Scheme 2. 73).



Scheme 2. 73 Condition of the fluorimetric assay of the second generation of prodrug

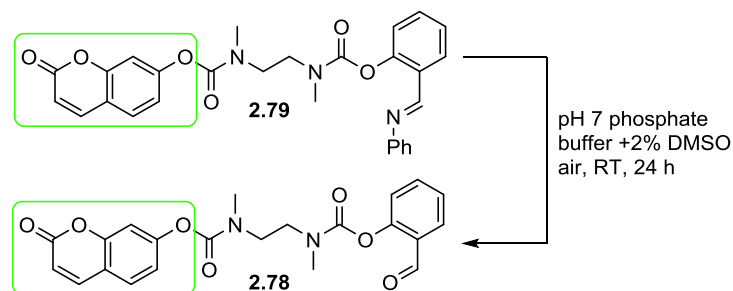
A blank was recorded with **2.79** and **Ir1**, then another one with the **2.79** and NADH (Graph 2. 17). To launch the reaction, NADH, **2.79** and the catalyst were mixed at 10 μM and were left stirring for 24 h, after which a spectrum was taken with excitation at 325 nm. However, the curve after 24 h did not significantly differ from the blank curves. On the blank curve with NADH, we can see a double peak accounting for the fluorescence of the fluorogenic probe and NADH. The NADH peaks seem to have disappeared after 24 h, possibly due to degradation.



Graph 2. 17 First generation fluorogenic probe testing

The experiment was replicated with 10 equivalents of NADH, but again no more success was reached. We proposed three hypotheses.

- One potential explanation is that the non-cyclic imine **2.79** hydrolyses at such a low concentration and becomes present under the form of the aldehyde **2.78**, which is by far less reactive than the imine (Scheme 2. 74).



Scheme 2. 74 Deactivation of the prodrug by hydrolysis

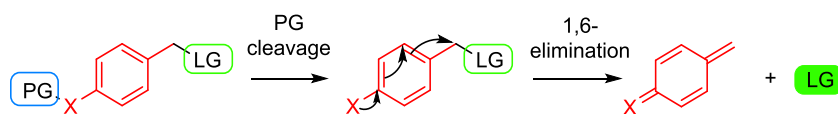
- Another reason would be that the reaction, at this concentration range, proceeds slower than the degradation of NADH.
- The last hypothesis could be that the aniline **2.80** is not nucleophilic enough to attack the carbamate, although it is very unlikely knowing the issues faced during the synthesis of the fluorogenic probe. HPLC-MS is the technique which would give us the answer.

4.3 Third generation of fluorogenic probe

Facing the absence of success with the second generation of imine fluorogenic probe, we decided to move on to a third generation, with a different type of elimination mechanism.

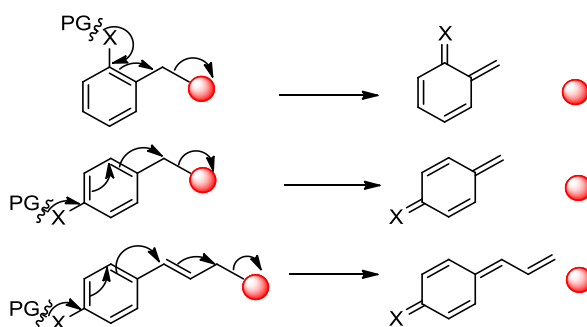
4.3.1 Design of an NADH-responsive fluorogenic probe

In this section, we got interested in 1,6-elimination mechanism instead of cyclization (Scheme 2. 75).¹¹⁶ Since there is only one electron flow after the reduction of the imine, we expect a faster elimination than with the previous system.



Scheme 2. 75 Principle of 1,6-elimination fluorogenic probe

This new linker will rely, on contrary to the previous generation of prodrug on an eliminative linker (Scheme 2. 76). The three most frequent ones rely on 1,4, 1,6 or 1,8 eliminations¹¹⁷⁻¹¹⁸



Scheme 2. 76 Global scheme for 1,4 (top), 1,6 (middle) and 1,8 (bottom) self immolative linkers¹¹⁷⁻¹¹⁸

As linker, we elected to use the 4-aminobenzyl alcohol (Figure 2. 7). The leaving group will be the oxygenated part.

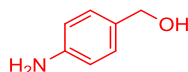
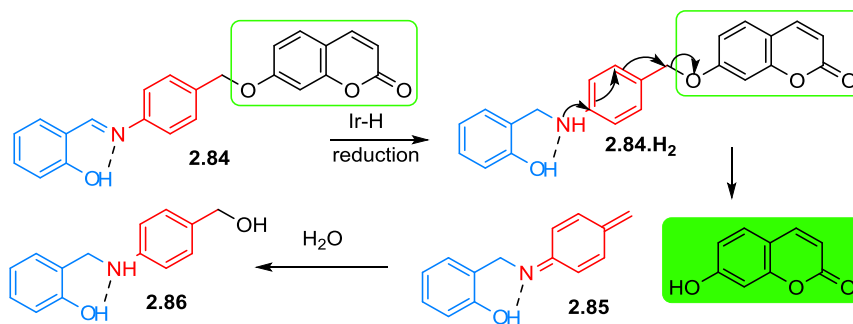


Figure 2. 7 4-aminobenzyl alcohol

By putting all the pieces together – the umbelliferon, the 4-aminobenzyl alcohol and salicylaldehyde – it became possible to draw our third generation of fluorogenic probe **2.84** by connecting the fragments together (Scheme 2. 77). The principle was that the lone pair of the nitrogen of **2.84** would not be available for the 1,6-elimination in the imine form. Once reduced to **2.84.H₂**, it would be available to kick out the umbelliferon, which is the leaving group in the benzylic position. We believed that the intramolecular H bonding

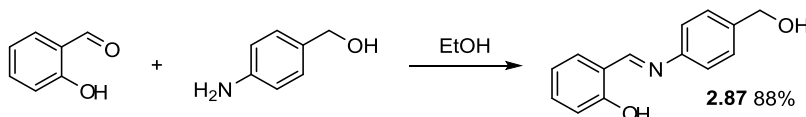
at the *N*-Salicylidene aniline would enhance the stability against the hydrolysis.



Scheme 2. 77 Mechanism of reduction-elimination of the second generation of fluorogenic probe **2.84**

4.3.2 Synthesis of an NADH-responsive fluorogenic probe

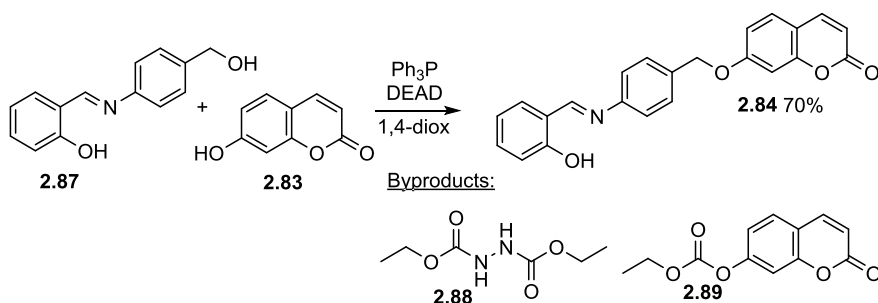
The synthesis was envisioned in 2 steps: (i) construction of the *N*-Salicylidene aniline and (ii) coupling with the umbelliferon. The compound **2.87** was obtained by reacting the salicylaldehyde with 4-aminobenzyl alcohol (Scheme 2. 78).¹¹⁹



Scheme 2. 78 One-step synthesis of the trigger-linker assembly **2.87**

After a few unsuccessful attempts, we employed a Mitsunobu reaction, which is well described for phenols with a low pKa (Scheme 2. 79).¹²⁰ As, we realized that the coupling product **2.84** was obtained with some side products and by-products, such as umbelliferon ethyl carbonate **2.89** and also DEAD.H₂ **2.88**. Whereas **2.89** could be separated by flash chromatography, **2.88** could not be separated by flash chromatography even after 2 runs. Later, we managed to separate **2.88** by vigorously stirring the finely powdered impure **2.84**

into diethyl ether for 4 days, followed by filtration to collect the pure fluorogenic probe **2.84**.



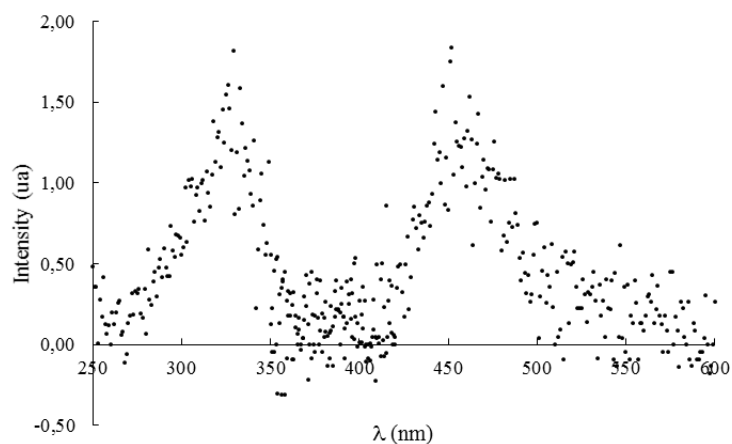
Scheme 2. 79 Fourth strategy: Mitsunobu coupling

4.3.3 Testing of an NADH-responsive fluorogenic probe

With this third generation of the fluorogenic probe (**2.84**) in hands and fully characterized, we decided to carry out a fluorimetric assay.

4.3.3.1 Determination of the photophysical parameters of the system

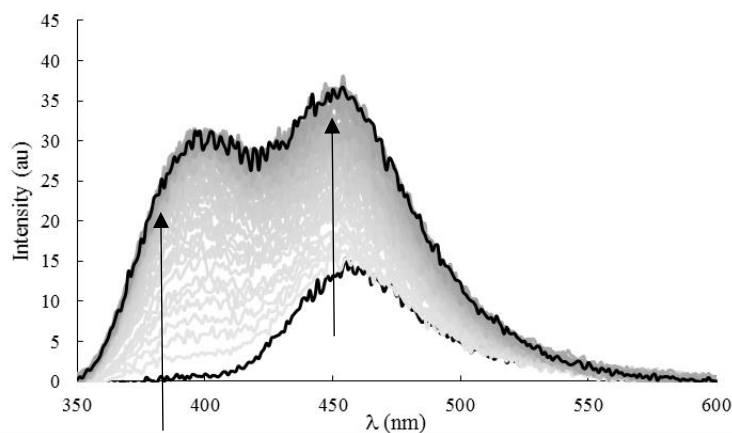
The first parameter that we tried to determine was the on-off ratio between the **2.84** and the umbelliferon (Graph 2. 18). On contrary to the first fluorogenic probe, this one was far less soluble. A solution at 1 μM of **2.84** with 2% of DMSO was prepared (it was not well dissolved) and an excitation-emission spectrum was recorded (Graph 2. 18). An intensity of 1.5 was reached for the probe **2.84**, whereas a value of 25 was reached with umbelliferon. The value of **2.84** is underestimated because the probe was not well dissolved. An on-off ratio of 12 was obtained, but it is certainly over-estimated. Compared to other probes described in the next part of this work, it is a low ratio.



Graph 2. 18 Excitation-emission of **2.84** at 1 μM in 50 mM phosphate pH 7 buffer 50 mM + 2% DMSO

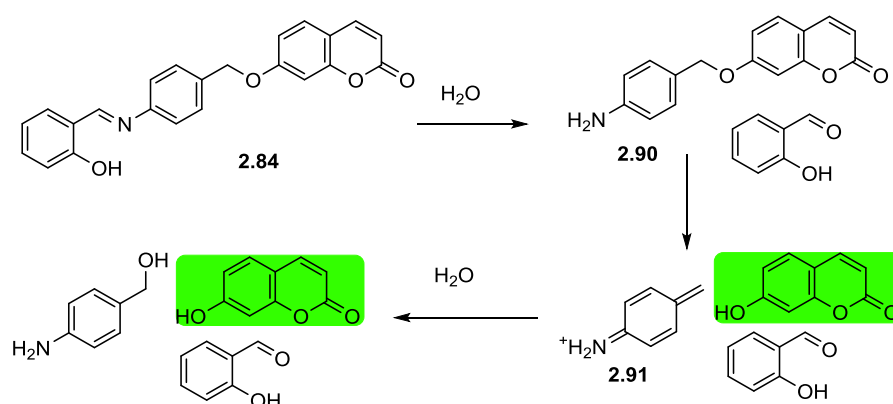
4.3.3.2 Hydrolysis study

Since we feared potential hydrolysis problems, we decided to study the stability of **2.84** in pH 7 phosphate buffer. A 4 μM solution in phosphate buffer was constituted and its fluorescence was followed for 13 h (Graph 2. 19). During the time of the experiment, the peak at 454 nm, attributed to the fluorogenic probe and/or the umbelliferon, increased and reached a plateau. At this stage, we highly suspected fluorogenic probe hydrolysis, but also a long time after we ended the project, the possible gradual dissolution of the fluorogenic probe. However, the band at 400 nm cannot be explained.

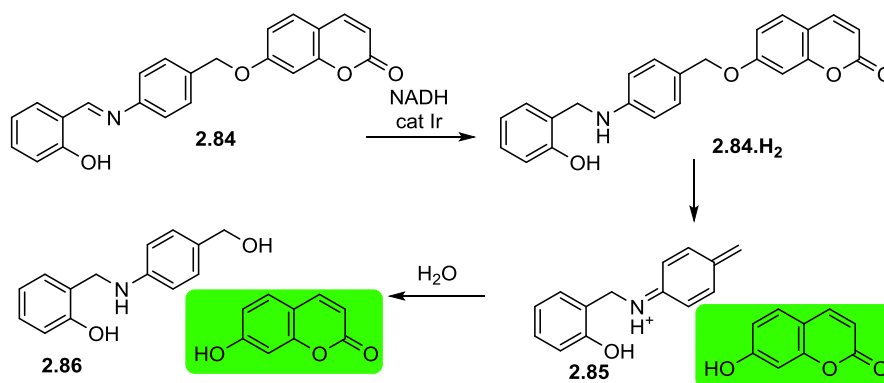


Graph 2. 19 Study of the behaviour of **2.84** in water (irradiation at 325 nm, 4 μ M in 50 mM phosphate pH 7 buffer 50 mM + 2% DMSO). One spectrum every 5 min for 13 h

One of the disadvantages of fluorescence spectroscopy is that it gives scarce information about the identity of the products. Several compounds could emit fluorescence at the same wavelength, and if a new band at an unexpected wavelength is observed, it is difficult to identify it. In this case, we feared that we would not be able to discriminate in a kinetic experiment the origin of the fluorescence as it could come from hydrolysis (Scheme 2. 80), from the catalysed reduction (Scheme 2. 81), or from the fluorogenic probe itself. HPLC-MS would solve the problem by giving the identity of the by-products.



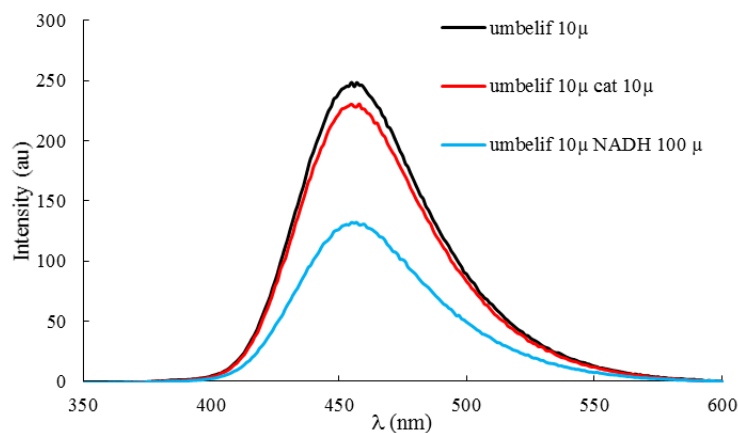
Scheme 2. 80 Hydrolytic paths accounting for umbelliferon release



Scheme 2. 81 Iridium-catalysed paths accounting for umbelliferon release

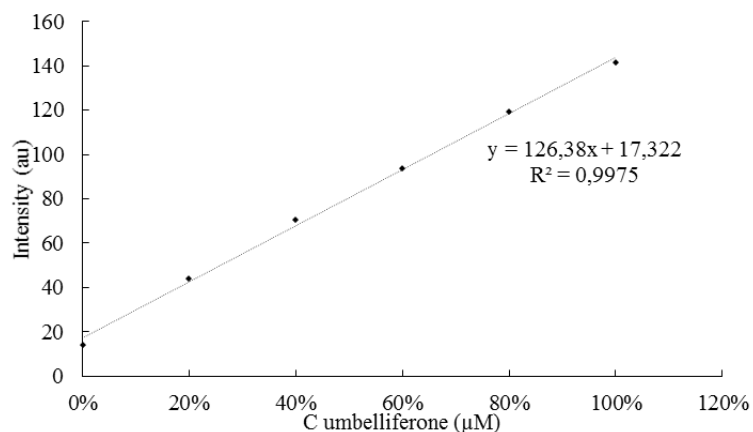
4.3.3.3 Fluorescence quenching and calibration

We elected to work with a concentration of 10 μM of the probe **2.84** to stick to the protocol we developed with the previous one. But as we wanted to use 10 equivalents instead of 1 of NADH, we had to verify if there was a quenching phenomenon. Although the effect of the catalyst was minimal, a quenching of nearly 50% was observed when the spectrum of umbelliferon was recorded with 10 equivalents of NADH, as seen on Graph 2. 20.



Graph 2. 20 Study of the quenching of the fluorescence of umbelliferon by NADH (10 μ M or by the catalyst **Ir1** (10 μ M). Irradiation at 325 nm, 4 μ M in 50 mM phosphate pH 7 buffer 50 mM + 2% DMSO

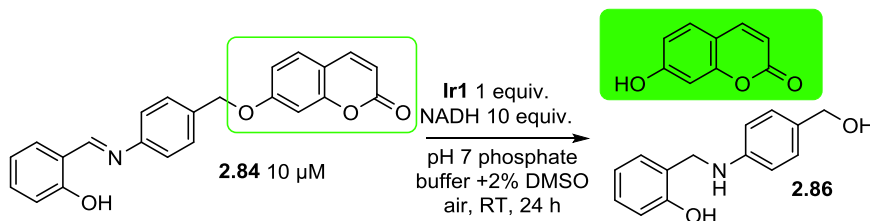
Therefore, as NADH quenches the fluorescence of the umbelliferon and as the probe is fluorescent, we had to make a complex calibration curve. We calculated for every 20% of conversion the stoichiometry of the reaction to prepare standards matching those concentrations (Graph 2. 21).



Graph 2. 21 Calibration curve of umbelliferon (0-10 μ M), **2.84** (10-0 μ M) and NADH (100-90 μ M). Irradiation at 325 nm, in 50 mM phosphate pH 7 buffer 50 mM + 2% DMSO

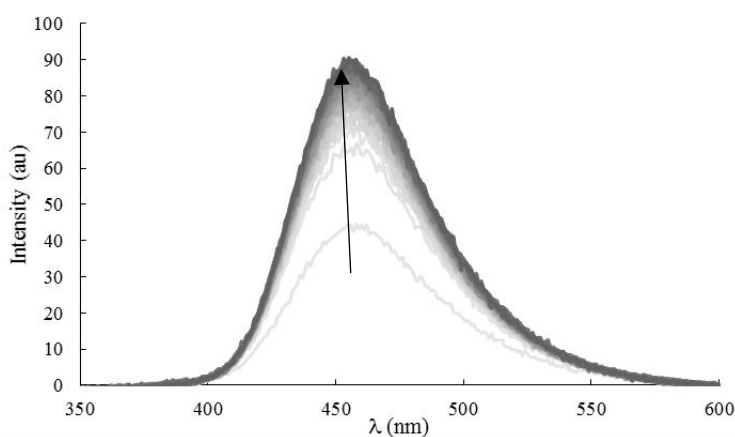
4.3.3.4 Kinetic test

With a good calibration curve in hands, we decided to launch the kinetics with 10 μM of **2.84**, 10 μM of **Ir1** and 100 μM of NADH (Scheme 2. 82).



Scheme 2. 82 Fluorimetric assays of the third generation of prodrug

As expected, the kinetic curve obtained was puzzling, as we could not determine the origin of the fluorescence, either from hydrolysis or by our catalysed method. We would be tempted to say that the reaction gave 65% of conversion, but that would naively mean that we neglect the t_0 curve, which appears too high if we compare our t_0 curve, measured just 10 seconds after the launch of the kinetics (Graph 2. 22).

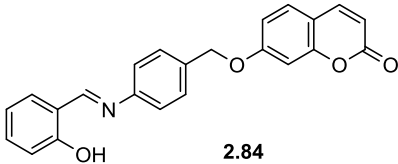
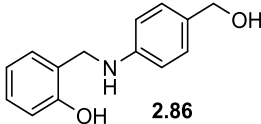
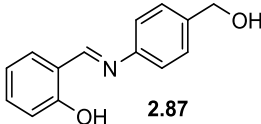
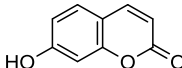
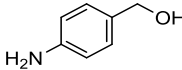
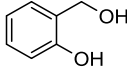
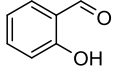


Graph 2. 22 Kinetic experiment with the **2.84** 10 μM , **Ir1** 10 μM and NADH 100 μM . Irradiation at 325 nm, in 50 mM phosphate pH 7 buffer 50 mM + 2% DMSO

4.3.3.5 HPLC following of the uncaging process

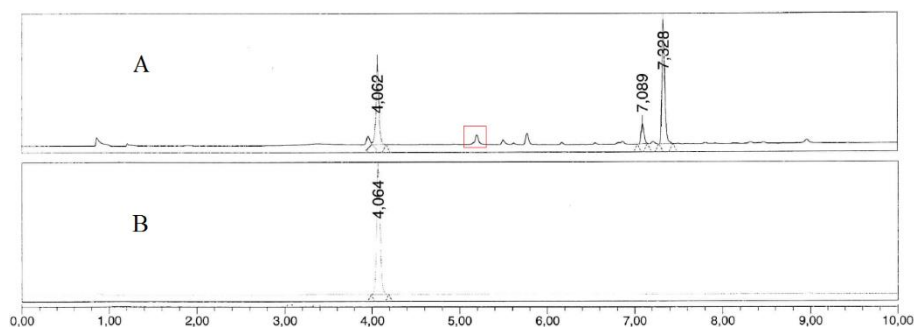
Facing the impossibility to discriminate the fluorescence emanating from the fluorogenic probe or the catalysed and non-catalysed path, we considered using HPLC technique to follow the known compounds during the reaction. The catalyst **Ir1**, the NADH and the NAD^+ were invisible in HPLC as they are polar compounds and came out immediately. The retention time of the other compounds is summarized in the following table.

Table 2. 4 Retention time of the possible compounds of the fluorogenic probe hydrolysis and release. Sunfire column, reverse phase, $\text{H}_2\text{O}/\text{MeCN}$ 90:10 to 10:90.

Entry	Compound	Retention time (min)
1	 2.84	7.328
2	 2.86	4.817
3	 2.87	5.912
4	 4.604	4.604
5	 1.515	1.515
6	 5.168	5.168
7	 3.193	3.193

We immediately noticed on the HPLC chromatogram that **2.84** underwent hydrolysis and decomposed to give umbelliferon during the sample preparation and the analysis (Graph 2. 23). Salicylaldehyde

was also detected in the sample. The product was pure, and no umbelliferon was present in prior to the analysis, we are sure that it appeared during the analysis.



Graph 2. 23 Retention time of **2.84** (A) and umbelliferon (B). Salicylaldehyde is explicitly shown. Sunfire column, reverse phase, H₂O/MeCN 90:10 to 10:90.

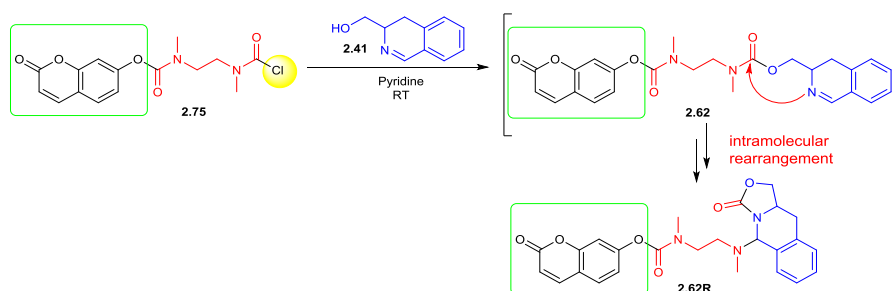
However, following the kinetics turned out to be strictly impossible by HPLC as the fluorogenic probe was too insoluble and was masked by the DMSO peak. Although the HPLC did not bring any clue about the kinetics, the detection of the hydrolysis in the pure sample of the fluorogenic probe during the analysis to simply get the retention time was enough for us to prove that it hydrolyses and releases the fluorophore in the absence of a catalyst. This experiment, combined with the failure in the synthesis of analogues, lead us to decide to stop the project.

4.4 Discussion

The application of the transfer hydride reduction which we developed was to use endogenous NADH to activate fluorogenic probes in an intracellular fashion. At the time we were working on it, no report was available in the literature and the two reports came after we finished working on the subject.⁶⁷⁻⁶⁸

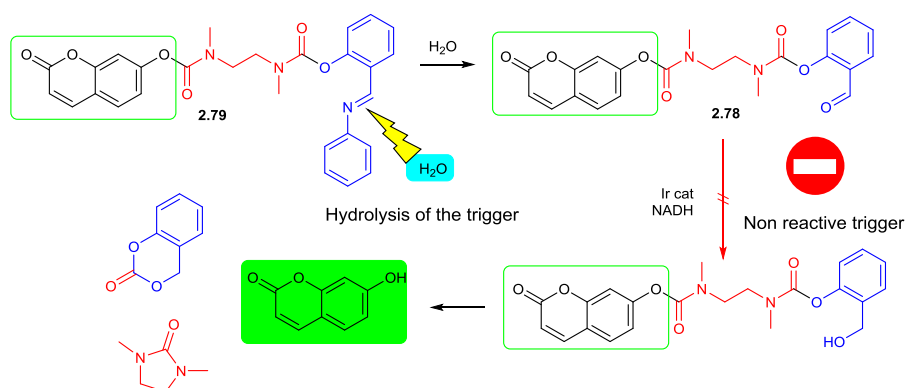
Our first and safe idea was to work on cyclic imines as they are not prone to hydrolysis. However, our mistake was that we assumed

that the nucleophilicity of the imine and of the amine would be significantly different. However, our first generation of fluorogenic probe decomposed during the synthesis and was rearranging through a putative intramolecular cyclization (Scheme 2. 83). We should have spent more time on stereo electronic variations of the trigger structure as there are possibilities to lower the nucleophilicity of the imine just below the threshold, making the reduced form prone to the cyclization. However, such an approach requires a more complex synthesis.



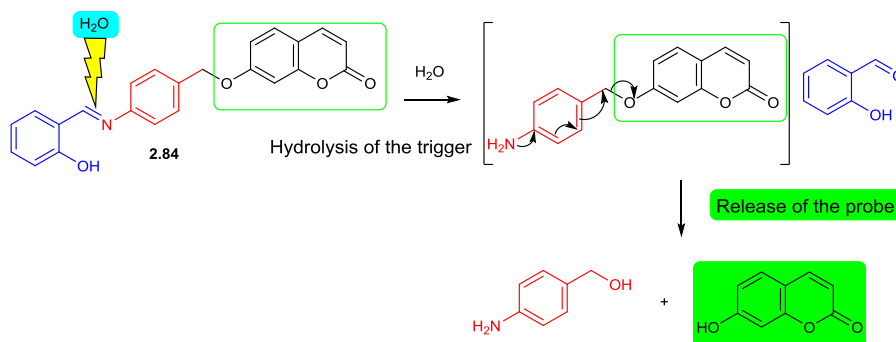
Scheme 2. 83 Rearrangement problems of the first generation of the fluorogenic probe

Then, by attempting to solve one problem, we created another one. We replaced the cyclic imine by a linear one, prone to hydrolysis. To our delight, it solved the problem of the intramolecular attack, as the imine was stale and did not cyclize, as the imine's shift was still visible at around 8.3 pm. However, this fluorogenic probe has never been reduced in our catalytic tests. We believe that it got readily hydrolysed to the aldehyde, thought to be unreactive towards our methodology (Scheme 2. 84). We did not verify this assumption, although it could be made by HPLC. Such a methodology was applied for our last fluorogenic probe.



Scheme 2. 84 Hydrolysis problems of the second generation of the fluorogenic probe

We did not find any design with a cyclic imine, but as we remembered that *N*-Salicylidene anilines were efficiently reduced and contained an aromatic amine in their reduction product, we believed that it could trigger a 1,6 elimination and eliminate a leaving group on the benzylic position. Our hypothesis was that the intramolecular H bonding would stabilize the imine bond. However, the fluorogenic probe was very insoluble and remained prone to hydrolysis, as showed by the HPLC analysis (Scheme 2. 85).



Scheme 2. 85 Hydrolysis problems of the third generation of the fluorogenic probe

To date, we do not have a design of imine-based fluorogenic probe avoiding hydrolysis and avoiding relying on a difference of nucleophilicity. One idea is to synthesize a drug molecule *in cellulo* by carrying out the reduction as a last step inside the cell. It needs to

be a cyclic imine to avoid hydrolysis, and it needs to be significantly less toxic than the target molecule. Perhaps the best short-term solution would be to avoid using hydrolytic functions such as esters or imines unless they have a specific role to play in the whole molecular system. Several other functions should be explored, such as quinones, aldehydes, nitrones or oxazolines. They could become new candidates if more effort is put on their study.

Moreover, even though we found a very active catalyst **Ir1**, we doubt that it could be an efficient catalyst *in cellulo*. The double charge makes it highly soluble, but such a cationic species has very little chances to cross any cellular membrane. The logical idea would be to decrease its cationic character by changing ligands to negatively charged ligands. However, it is risky, as this strategy was employed by Sadler to design catalytic metallodrug with very high toxicity (Figure 2. 8).⁶⁶ Although there are chances to get an active catalyst, the effects on cells could involve oxidative stress, GSH depletion or DNA/protein binding. The best way to achieve this would be to test the catalytic properties of rejected candidates by Sadler in terms of toxicity and see if one meets our criteria and has low toxicity enough.

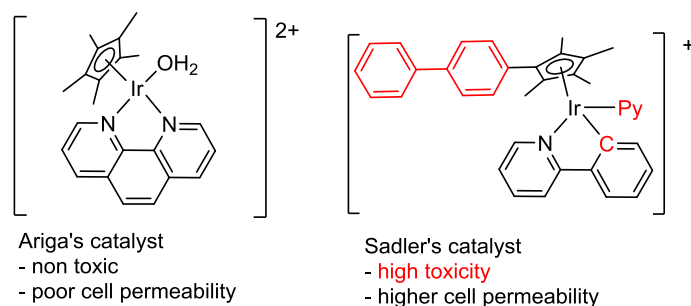
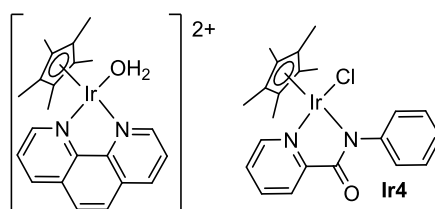


Figure 2. 8 Design difference between a cytotoxic and a non-cytotoxic catalyst

The problem was addressed in two different ways in the literature (Scheme 2. 86). Ariga used millimolar concentration of

$\text{Cp}^*\text{Ir}(\text{phen})(\text{H}_2\text{O})^{2+}$, which is very high, hoping that some would enter in the cells; it was known to have low toxicity.¹²¹ Do used an *N*-phenyl picolinamide ligand which can be deprotonated and behaves like an anionic ligand.⁶⁸ In the form of a chloro complex, it is water soluble enough and probably does not hydrolyse too fast to keep it neutral prior to its entry inside the cells. The cellular hydrolysis may activate the catalyst, but also may enable it to bind to the 9-N of the guanine.

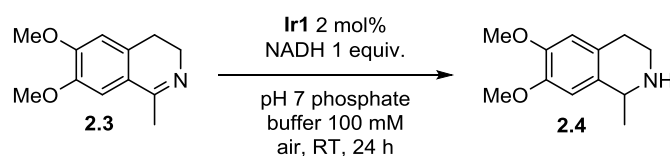


Scheme 2. 86 Left: Ariga's catalyst. Right: Do's catalyst (**Ir4**).

5 Conclusion

The global goal of the present work is to develop new bioorthogonal reactions catalysed by transition metal catalysts in the context of prodrug uncaging. The work of the first section aimed to implement transfer hydrides as a new bioorthogonal reaction in the context of prodrug uncaging. At the time we started the work, in 2014, there were only seminal reports on the subject, by Sadler, mostly. We could rely on the literature on non-enzymatic NADH regeneration of the years 1990-2005 by Fish and Hollmann, but mostly the first report by Sadler of a transfer hydride reduction of lactate to pyruvate with NADH.

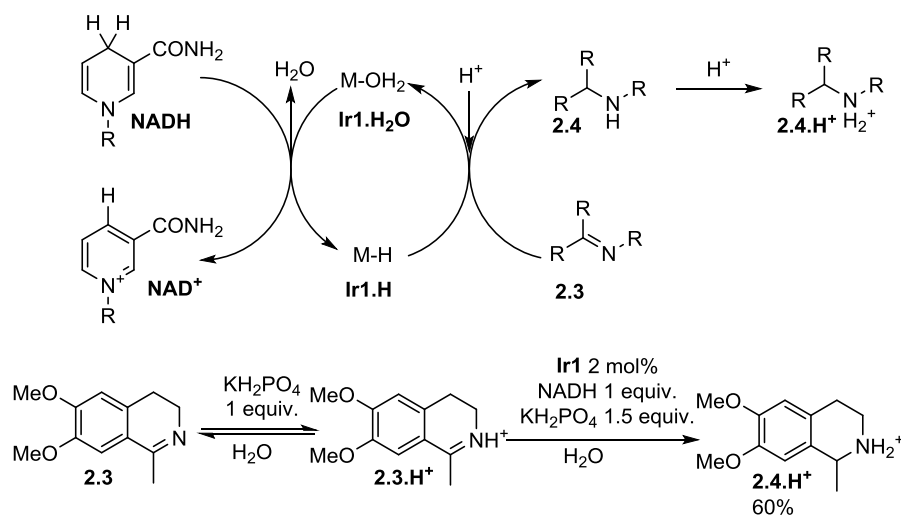
In the first phase of this project, we were able to develop such a reaction meeting the following criteria, namely efficiency at neutral aqueous buffered and capability to use the cofactor NADH or an analogue (Scheme 2. 87). It appears to be the first report of transfer hydride reduction of imines in water with NADH as the hydride source, the concept was published a few months before us on aldehydes by Do.⁶⁷



Scheme 2. 87 Biocompatible imine reduction by transfer hydride with NADH

At first, we developed imine reduction catalysed by a half sandwich Cp* iridium complex **Ir1** and using NADH or an analogue called **Bn-NADH** as a hydride source, thus mimicking the action of an imine reductase (Scheme 2. 88). Whereas we found that ruthenium

complexes of the form $[(\eta^6\text{-arene})\text{Ru}(\text{N},\text{N})\text{X}]$ (where (N,N) is a bipyridine, phenanthroline, bipyrimidine or Tsen ligand) are slightly or not active, complexes of the form Cp^*Ir or Cp^*Rh with a (N,N) ligand (where (N,N) is a bipyridine, phenanthroline or bipyrimidine) are by far more active. In our ligand optimisation, we came across the 4,4'-dimethoxy-2,2'-bipyridine, also called MeObpy, as the best ligand as it is by nature electron poor due to the electron deficient heteroaromatic system, but still a bit enriched by the methoxy ligands. Although we had this ligand in hands, we tried several other (N,N) ligands, but they could only reach the performance of **Ir1**, our lead complex, without outperforming it. Combined with the experiment showing that the reaction was reversible and the one where an increase of NADH lead to an improvement of the yield, we started to believe that the reaction was limited by an equilibrium. Moreover, we gained a lot of valuable information while studying the impact of the acid. At first, we noticed that acidity was needed to activate the substrate by protonation but could also be deleterious as it could quench the hydride by protonating it to form H_2 . An intermediate acid such as KH_2PO_4 was the best in our case as he was compatible with the hydride and capable to activate the substrate. We also learned that the species which is reduced by the iridium hydride is the protonated imine and it is not in its neutral form. Lastly, we were capable to make the reaction work in a buffered medium at pH 7 with a 100 mM phosphate buffer for 15 mM on substrate thanks to strict control of the acidity and pH at the same time.



Scheme 2. 88 Impact of the acid on the model reaction

We also studied the scope of the reaction and realized that the imines are generally well reduced, no matters if they are cyclic or not (Figure 2. 9). The only problem comes from their solubility in water, which biased most of the yields which we measured. We found that ketones were totally non-reactive, whereas we observed some reactivity from aldehydes showed lower yields due to solubility problems but might be equally reactive if compared with imines.

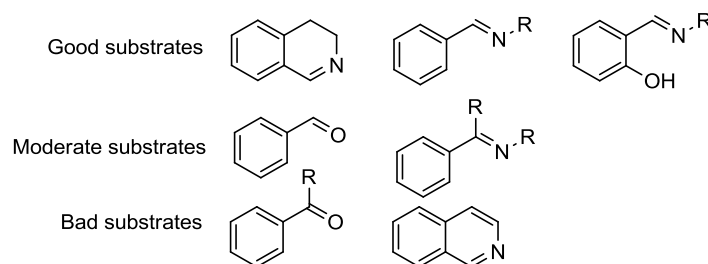
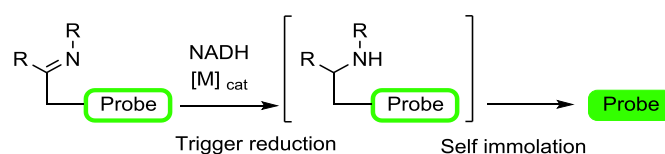


Figure 2. 9 Substrate families for the scope broadening

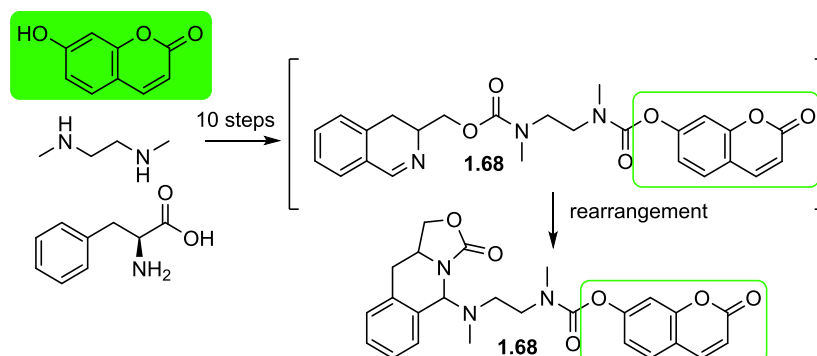
The second part of this work was focused on the development of prodrugs aimed to release their payload after being reduced by our catalytic system (Scheme 2. 89). Such a system would be of particular interest in a therapeutic context as it would be possible to visualize

redox-dependant metabolic responses in which NAD cofactors are involved or deliver a drug in cells which are in a more reductive state, such as hypoxic tumour cells. To date, there has been a significant breakthrough in developing specific prodrugs against this type of cells, but there appears to be no report of prodrug activation by metal-catalysed imine transfer, although it represents a valuable strategy which deserves to be exploited.



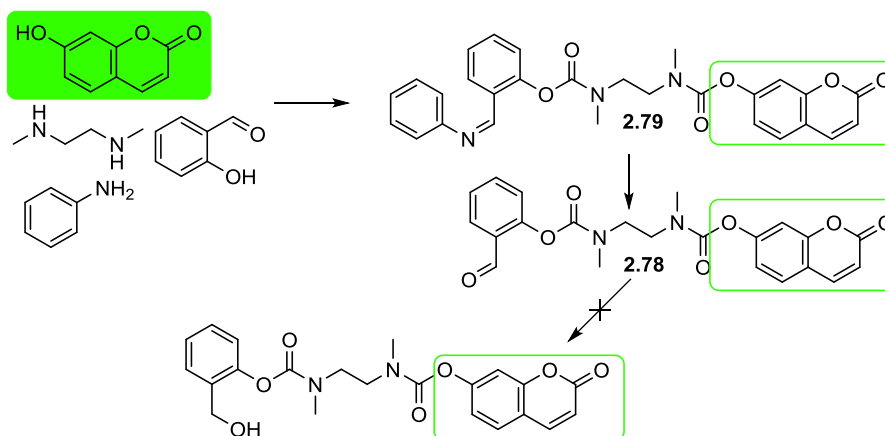
Scheme 2. 89 Principle of NADH-responsive prodrugs

In the last part, we design and synthesized three fluorogenic probes and tested the last two *in vitro*. The first generation of fluorogenic probe comprised a dihydroisoquinoline trigger, a bis-carbamate as a linker and umbelliferon as the fluorescent payload (Scheme 2. 90). For the synthesis, we were able to synthesize the trigger in one piece, then we could couple the fluorogenic probe to the linker and activate it. However, the coupling gave us a re-arrangement product due to the nucleophilic character of the imine.



Scheme 2.90 First generation of prodrug

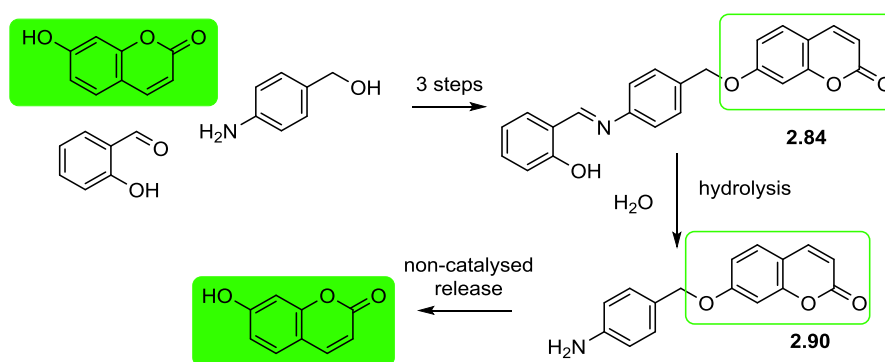
In the second generation fluorogenic probe, we changed the trigger by an N-Salicylidene aniline which could be mounted in two steps on the activated linker-probe moiety previously synthesized (Scheme 2.91). The stable fluorogenic probe was fully characterized and tested. However, even with 100 mol% of catalyst and 10 equivalents of NADH, we could not observe any increase of fluorescence, presumably due to the hydrolysis of the trigger leading to a non-reactive aldehyde.



Scheme 2.91 Second generation of prodrug

The third generation of the fluorogenic probe also comprised an N-salicylidene aniline as trigger, but it was working with a 1,6-elimination mechanism once the imine was reduced by the iridium

hydride (Scheme 2. 92). It was synthesized with good yields *via* an imine condensation and a Mitsunobu reaction to attach the fluorogenic moiety. Although the full system was easily synthesized, it proved to be sensitive towards hydrolysis, as showed by HPLC, but possibly reacting through the iridium-catalysed pathway, thus making the job of finding how much fluorescence comes from each path impossible. We tried to synthesize various analogues, but as we never made it to the end of the synthesis, we simply stopped to work on the project.



Scheme 2. 92 Third generation of prodrug

In this section, we have been able to develop the transfer hydride reduction method applicable to imines in biocompatible conditions. The reaction conditions met our expectations for potential biological application, as it works in presence of nucleophiles in a neutral buffered aqueous medium at room temperature to 37 °C. The only criterium which we have neglected is the biocompatibility of the catalyst, as it may be toxic or have poor cell penetrating properties.

Although we had a tailor-made activation method for the activation, we were unable to design and synthesize prodrugs with all the required characteristics, namely an imine trigger, stability towards hydrolysis and reactivity towards reduction. We have been unable to

come up with a design which meets all the criteria on paper, although there are some ideas which require complex synthesis.

While we were developing the prodrugs and after we finished the practical work, a few paper were published by other authors, thus demonstrating the general interest in intracellular hydride transfer to some extent. Sadler focused on inducing oxidative stress through reaction of the hydride with oxygen to produce ROS. Do and Ariga used the method to reduce quinones or aldehydes to activate or quench a fluorophore in cells in simple systems. However, there is still no application in prodrug activation of this methodology, and it would represent a useful tool if developed.

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**Second part: New applications of Tsuji Trost
reaction in bioorthogonal prodrug decaging**

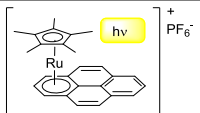
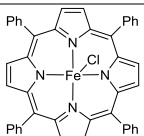
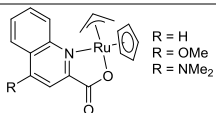
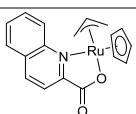
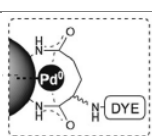
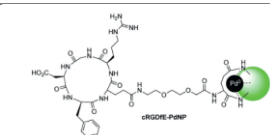
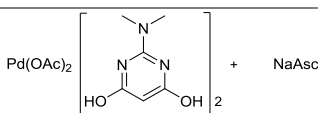
1 Research goals

The Tsuji-Trost reaction, first reported by Tsuji and then improved by Trost, has evolved since 1965 as one of the major palladium-catalysed reaction. It is widely used in synthesis for allylic substitution, but also thanks to the famous allyloxy carbamates, Alloc, which are robust protecting groups which can be easily deprotected with Pd(0) and a nucleophile. The robustness is attributed to the relatively small number of paths leading to deprotection, the leading one being with Pd(0) catalysis. Since it is assumed in biocompatible conditions that only Pd(0) is capable to deprotect it, it was considered as a good model for our studies from a mechanistic point of view.

It is only once Meggers, in 2006, showed that it was a valid bioorthogonal protecting group that it became the lead bioorthogonal protecting group, often sharing the first place with propargyl carbamate (Proc). They can be deprotected in less stringent conditions, by more different metals (Pd, Au, Ru ...) and with less selectivity for low-valent oxidation numbers.

At the time we started to work on the project in September 2016, we were aware of the seminal report of Meggers (Ru complexes), Bradley (supported nanoparticles) and Mascareñas (ruthenium complexes). It is worth noting that the only purely bioorthogonal is the one involving the first generation of Meggers catalyst, as it requires PhSH to work, although the next generations rely on the endogenous GSH, but are more practical. Bioorthogonality shall not be confused with applicability here.

Table 2. 5 Remarkable literature examples before 2016

Author (date)	Catalyst	Application
Meggers (2006)	Cp*Ru(COD)Cl	Uncaging of rhodamine 110 bis Alloc
Meggers (2012)		Uncaging of rhodamine 110 bis Alloc
Meggers (2012)		Azide reduction
Meggers (2014)	 R = H R = OMe R = NMe ₂	Uncaging of Alloc protected Doxorubicin
Mascareñas (2014)		Uncaging of Alloc-protected DNA binding agents
Bradley (2011)		Alloc deprotection and Suzuki coupling
Bradley (2014)		<i>In cellulo</i> drug synthesis by Suzuki coupling
Chen (2014)	(AllylPdCl) ₂	Protein N-Alloc deprotection in bacteria
Lin (2011)		Sonogashira fluorophore labelling in bacteria

In our literature review, we found that few discrete palladium complexes had been used in the context of *in cellulo* deprotection of

allyl carbamate. This quite surprising as palladium is probably the most ubiquitous metal in the context of Tsuji-Trost reaction.

Working with discrete palladium complexes has several key advantages (Figure 3. 1). As there are hundreds of phosphine ligands available, it is possible to study in detail the impact of the stereo electronic parameters of the ligand on the metal centre, which is critical for the global reactivity. Also, and more indirectly, it is possible to modulate its pharmacological properties such as its polarity, its polar area or even the number of heteroatoms to enable a good cell penetration. Eventually, understanding its activation from palladium (II) to palladium (0) paves the way for vectorization and realistic *in vivo* applications.

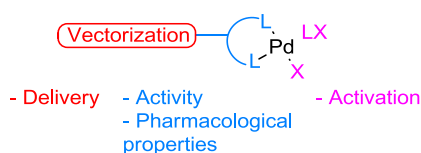
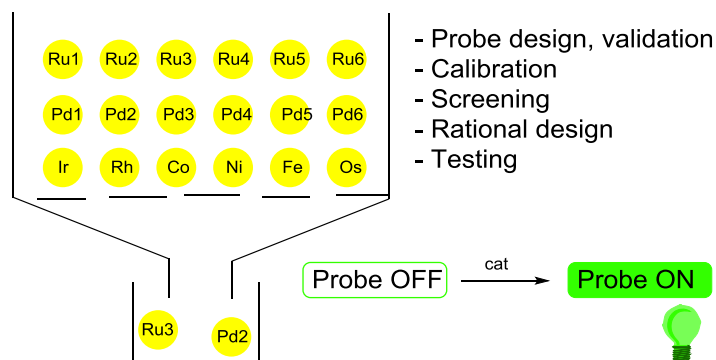


Figure 3. 1 Guidelines of catalyst design

We segmented the work in three phases. In the first phase, we will examine how we were able to find by fluorogenic systematic screening to find new candidates (Scheme 3. 1). As a preliminary to this chapter, all the data concerning the development, the synthesis and the assessment of the quality of the fluorogenic probes was written in a separate chapter at the beginning of the second part for the sake of clarity. With catalysts in hands, a substantial amount of time was spent to make an optimization to gather pertinent mechanistic information.



Scheme 3. 1 Screening and optimization of bio-organometallic catalysts

In the second phase, we applied the same catalytic system inside cells to validate the concept *in cellulo* and demonstrate the viability of intracellular catalysis with our complexes. We were able to follow the fluorescence release by measuring the total fluorescence, but also to visualize it by fluorescence microscopy (Figure 3. 2). To make sure of the absence of the toxicity of our catalysts, we performed viability test with various doses of the catalyst.

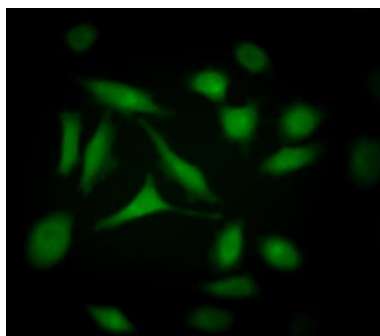
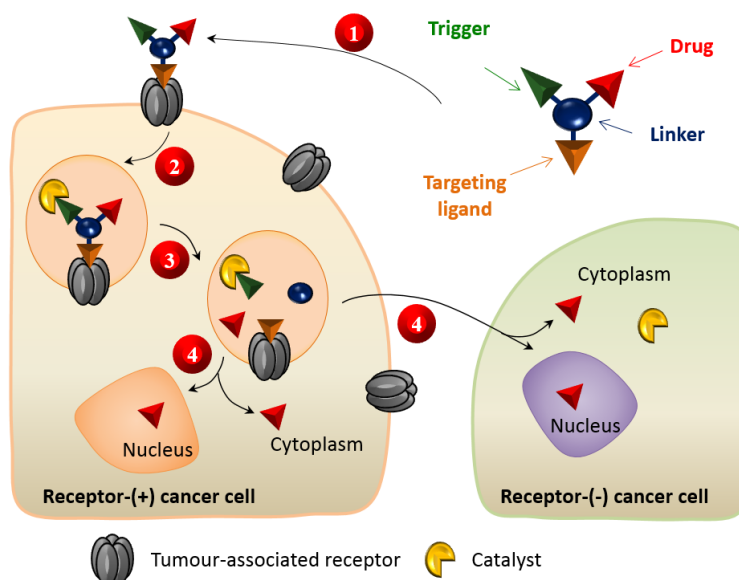


Figure 3. 2 Fluorescence microscopy images of the bioorthogonal decaging of ***Rho(Alloc)₂***

In the last phase, we focused on two applications of those new catalytic systems. We realized that in the literature, very few efforts were put on vectorizing existing catalysts or prodrugs. Regarding the catalyst, Mascareñas was able to tether a lipophilic phosphonium to the catalyst to address it to mitochondria and Bradley targeted an

NHC palladium complex by tethering a cell penetrating peptide. However, no example of using a vectorized drug with a transition-metal catalytic uncaging had been reported. The most frequently used drug was Doxorubicin, and its N-Alloc caged form.

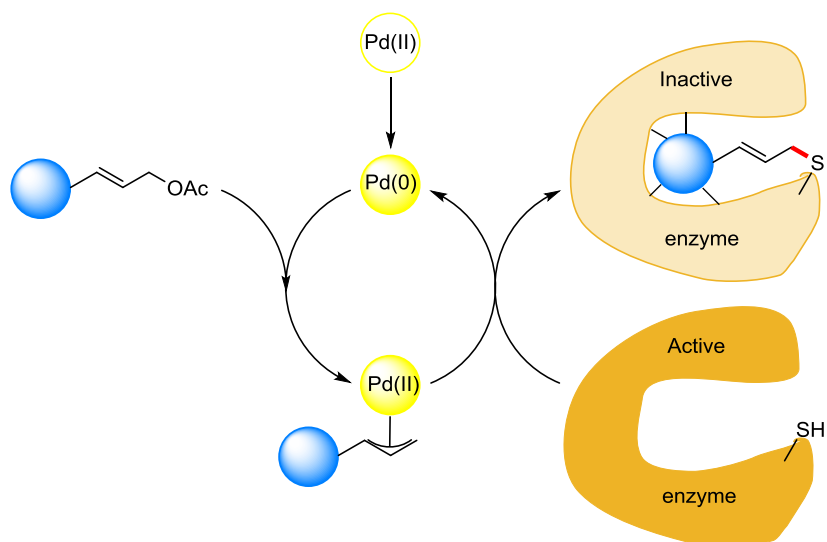
In the first project, we developed in collaboration with Prof. Papot (Université de Poitiers) a “molecular programmed system” aiming to deliver a cytotoxic molecule (monomethyl auristatin E, later called **MMAE**) in cells over-expressing folic acid receptor in order to gain the selectivity with the prodrug (Scheme 3. 2).



Scheme 3. 2 Molecular programmed system targeting cells expressing a cancer-specific receptor and activated by bio-organometallic catalysis

In the last project, we attempted to use the electrophilic properties of palladium π -allyl groups to turn them into an electrophilic warhead to block the activity of an enzyme, such as EGF-R and papain, by creating a covalent bond (Scheme 3. 3). The recognition would come from a recognition part, tethered to the

warhead, based on a known literature inhibitor forming non-covalent bonds with its target.



Scheme 3. 3 Inhibition of a cysteine enzyme by a targeted covalent inhibitor and a π -allyl as an electrophilic warhead

Second part: New applications of Tsuji Trost
reaction in bioorthogonal prodrug decaging
First chapter: Development of fluorogenic probes

2 First chapter: Development of fluorogenic probes

In this project, we aimed to develop several catalytic systems working in the cellular medium. Although in classical organometallic catalysis, where the yield or the conversion rate is usually determined by GC, HPLC or NMR, it is difficult to apply to bio-organometallic chemistry due to the limit of detection of the apparatus.

Instead of choosing a **top-down approach**, as it was done in the previous project, we chose a **bottom-up approach**. Since the subject was far less documented on biocompatible palladium complexes, we thought that emphasizing the structural diversity would enable to report totally new structures. It became clear for us that we needed to use a technique which enables us to screen wider libraries of catalysts, with systems in the low micromolar range, and with higher throughput.

The technique we chose is fluorimetry. It can provide quantitative results, provided that a good calibration curve is taken, and that there is no quenching effect between the constituents of the system. However, it does not provide clear information about the identity of the compounds. There are two types of fluorescence spectrometers (Table 3. 1).

Table 3. 1 Comparison between wavelength-sweep and microplate reader fluorimeters

Type 1	Type 2
Single cell holder	96-well plate
Wavelength sweep	Filters
Measurement of photophysical parameters	Single wavelength measurements
Information about the species in solution	No information about the species in solution
Low throughput	High throughput

Less precise calibration	More precise calibration
Fast sample preparation	Long sample preparation

These two techniques have been used to assess the catalytic performances of every catalyst during this work.

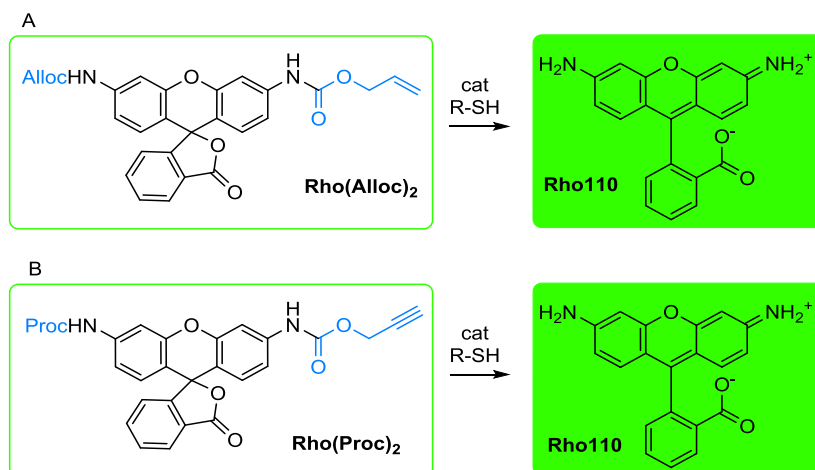
2.1 Fluorogenic models in the literature used with palladium catalysis

Throughout our studies, we realized that fluorogenic reactions were a standard procedure to quantify and visualize the progress of a cellular metallo-catalysed reaction. We noticed that to date few models have been employed for biological applications. However, the fluorogenic models had to be improved first to ensure a good quantification of the fluorescence.

The most common fluorophore is the **Rhodamine 110** (**Rho110**), whose fluorescence can be quenched when the two Ar-NH₂ groups are turned into electron-withdrawing groups such as an Alloc group (**Rho(Alloc)**₂, Scheme 3. 4A). The **Rho(Alloc)**₂ has been used in numerous literature reports, such as by Meggers^{38-39, 122}, by Mascarenas^{44, 123}, by Bradley⁴⁶, by Rotello¹²⁴, by Weissleder¹ and by Chen⁴⁸. With a green fluorescence around 520 nm and an excitation band around 490 nm, it is ideal as it uses visible light and it has a very high on-off ratio, nearing 300. Its bis-Proc analogue (**Rho(Proc)**₂, Scheme 3. 4B) shares many similarities and has been used several times in the literature as well by Bradley^{42, 46-47}, by Chen⁴⁸ and Mascarenas¹²³.

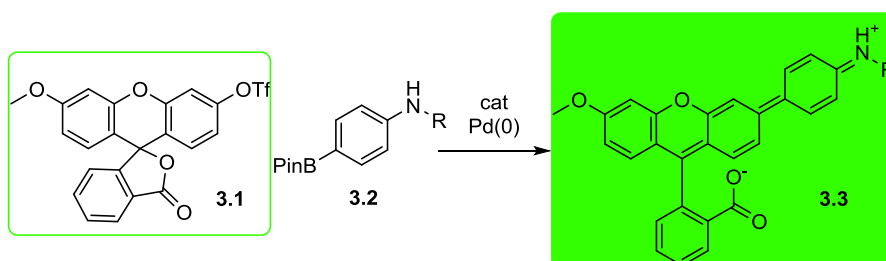
Alloc protecting groups are known to be cleaved by palladium (0) complexes or ruthenium (+II) with a key π -allyl intermediate, whereas palladium (+II) or (+IV) are inefficient, whereas Proc groups can be cleaved by many metals at various oxidation numbers, such as

palladium (0), (+II), (+IV), but also gold and ruthenium complexes following different several possible mechanisms.



Scheme 3. 4 *Rho(Alloc)₂* and *Rho(Proc)₂*

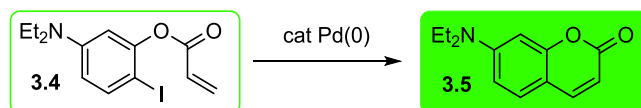
Similar to **Rho110**, the fluorescein family shares many similarities with the rhodamine family about the on-off properties and excitation wavelength (Scheme 3. 5). When the two oxygen are functionalized with groups such as triflate or methyl, Bradley restored the fluorescence by a Suzuki coupling with a 4-BPin aniline **3.2**, whose mesomeric donating properties will push to open the spiro lactone and recover the fluorescence of **3.3**.⁴⁶



Scheme 3. 5 *Fluorescein-templates Suzuki-triggered fluorogenic model*

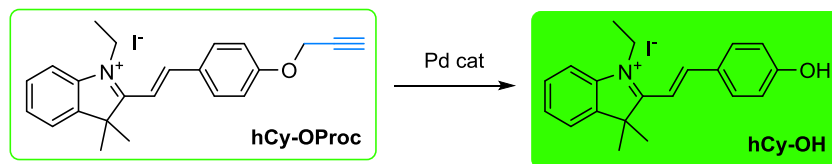
Coumarin models also have proven to be interesting models due to their high fluorescence, easy synthesis and versatility (Scheme 3. 6). As an example, 7-diethylamino coumarin **3.5** can be generated

from the corresponding acrylate ester **3.4** by an intramolecular Heck reaction using Pd nanoparticles.¹



Scheme 3. 6 Coumarin-template Heck-triggered fluorogenic model

The last family of probes we came across are hemicyanines (**hCy**), which are cationic dyes containing conjugated systems of various lengths of chain, with a high tunability of the fluorescence properties. Mascareñas used one (**hCy-OProc**) of them as an on-off probe by cleaving a propargyl ether by palladium catalysis to restore the fluorescence (**hCy-OH**, Scheme 3. 7).¹²³



Scheme 3. 7 Fluorogenic cyanine-based fluorogenic model

2.2 Goals of this chapter

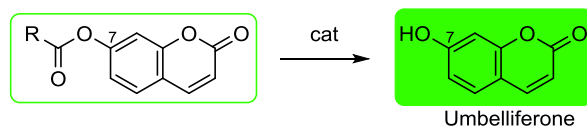
The goal of this chapter is to develop new fluorogenic probes with an easy synthesis, good fluorogenic properties, and an easy mathematical treatment. The best probes will be applied to biological systems. In this case, good cell permeability is a highly desired quality.

2.3 Home-developed fluorogenic probes

2.3.1 Umbelliferon model

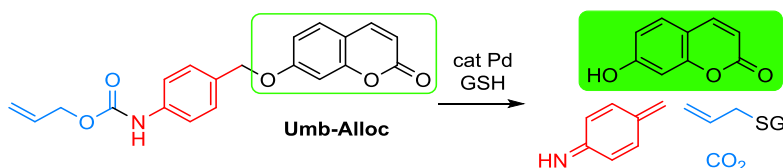
To develop a first palladium(0) sensitive fluorogenic probe, we turned our attention to a well-known fluorophore employed in the previous section, umbelliferon (Scheme 3. 8). We know that its solubility is good enough, we are familiar with its fluorescence

properties and also we know that its fluorescence could be blocked when the 7-OH position was functionalized.¹²⁵



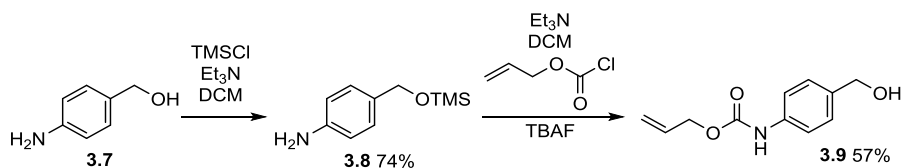
Scheme 3. 8 On-off probes based on umbelliferon

The most logical idea would have been to make attach an Alloc group to the umbelliferon, but the carbonate would be prone to hydrolysis. Therefore, a linker (N,O) such as 4-amminobenzyl alcohol had to be inserted to create a carbamate. By simply linking the elements together, the design was achieved (Scheme 3. 9). The umbelliferon's phenol could be directly attached to the benzylic position of the linker and the carbamate would be directly on the aniline.



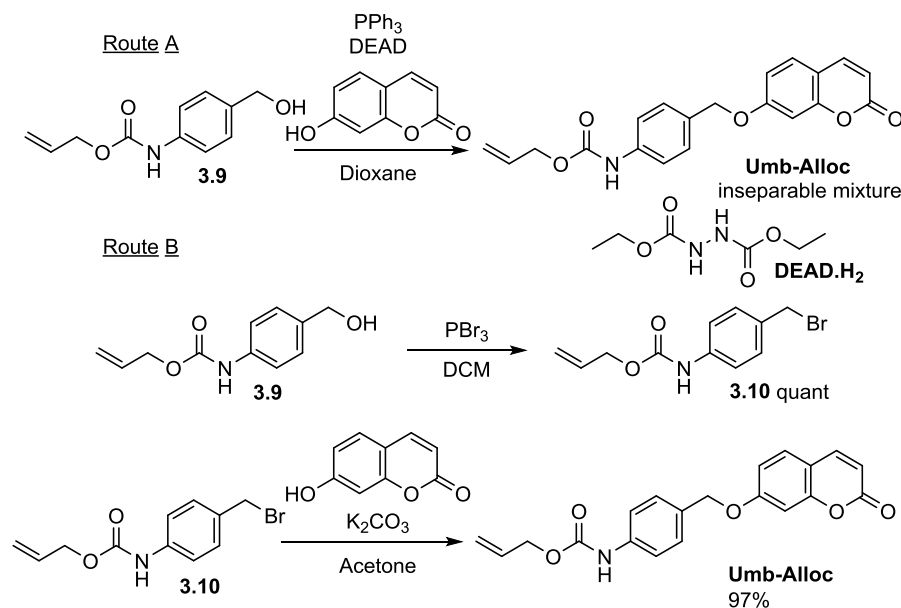
*Scheme 3. 9 Umbelliferon-based probe **Umb-Alloc**. Blue: trigger, Red: linker*

The synthesis was by far easier than the previous probes (Scheme 3. 10). We started by protecting the benzylic alcohol of 4-aminobenzyl alcohol **3.7** by a TMS, before reacting the aniline **3.8** with allyl chloroformate to build the carbamate trigger **3.9**. TBAF treatment was needed to deprotect the alcohol, and the fragment **3.9** was obtained in the form of white needle crystals.



Scheme 3. 10 Synthesis of the key benzylic alcohol intermediate **3.9**

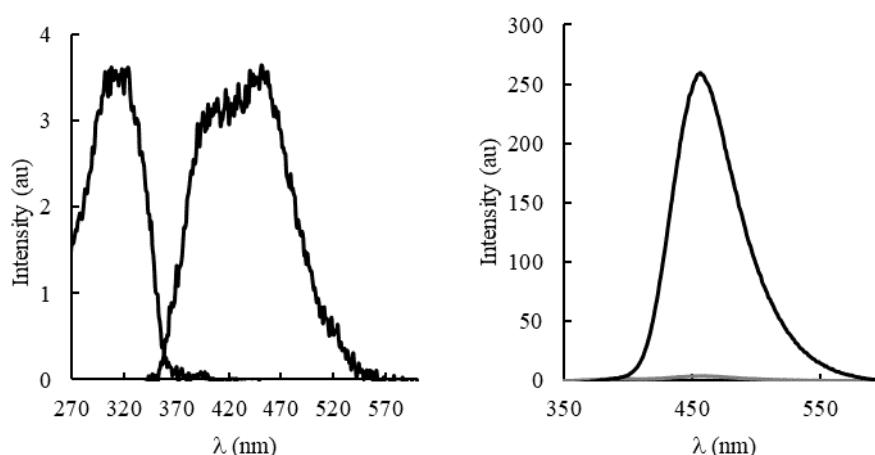
To graft the umbelliferon, our first attempt aimed to use a Mitsunobu reaction, similar to the strategy used in the first part of this work. However, we realized that we were unable to separate the desired probe **Umb-Alloc** from the **DEAD.H₂** (Scheme 3. 11A). So, we opted for a benzylic bromination of **3.9** with PBr_3 followed by a nucleophilic attack of the umbelliferon anion on the benzylic bromide **3.10** (Scheme 3. 11B). Remarkably, the bromide **3.10** was stable and was used immediately after being isolated.



Scheme 3. 11 Umbelliferon probe (**Umb-Alloc**) synthesis

With the new probe in hands, we started to characterize its photophysical properties to verify its suitability as an on-off fluorescence probe. We started to take an excitation-emission

spectrum of the probe at 10 μM to compare it with umbelliferon (Graph 3. 1 left). The probe appeared to have a small residual fluorescence, and a maximum of excitation and emission at respectively 320 nm and 454 nm. Once the emission spectrum was compared with the umbelliferon, it showed an on-off ratio of 70, which is good in comparison to many other probes reported in the literature (Graph 3. 1 right).



Graph 3. 1 Left: Excitation-emission of **Umb-Alloc** at 10 μM . Right: comparison of the emission of the **Umb-Alloc** (grey) and the umbelliferon (black) with irradiation at 325 nm at 10 μM

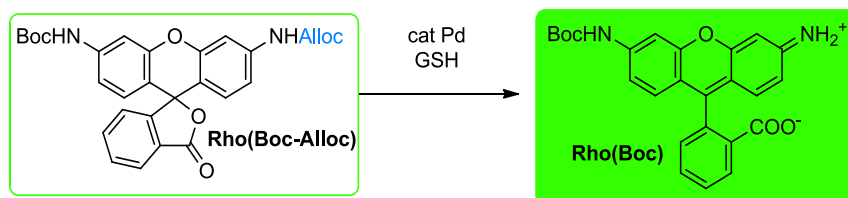
As a compromise between solubility and linearity of the spectrometer, we decided to work at 10 μM of the probe.

2.3.2 Rhodamine model

Although during our investigations, the umbelliferon model **Umb-Alloc** offered an acceptable residual background fluorescence and an acceptable solubility, some new problems emerged. It needs to be irradiated in the UV range, making it impractical for biological applications. Moreover, the cellular uptake of that probe was too low

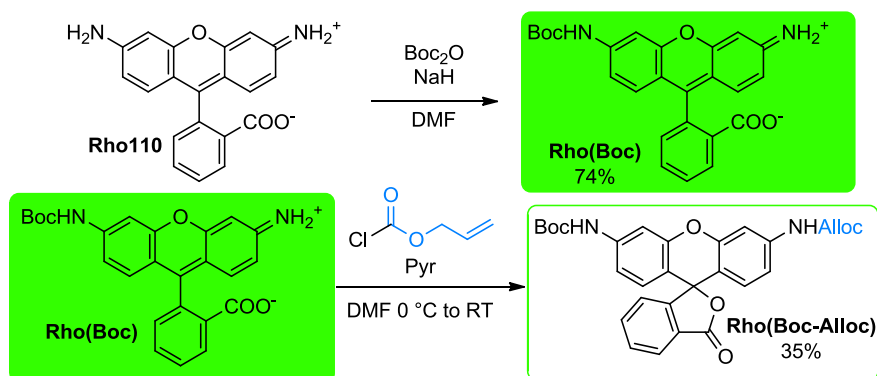
to make it usable in practical conditions. Long incubation time was needed.

To overcome these drawbacks, we turned our attention to fluorogenic models in the visible part of the UV-visible spectrum. As a side effect, it would also solve the long-lasting problems of fluorescence quenching. Our attention was drawn by the model fluorogenic probe employed first by Meggers, the Rhodamine 110 bis Alloc (**RhoAlloc**₂).³⁸ It has been widely used in the literature for *in cellulo* assays. However, we did not use this model as the quantification of the conversion may not be possible due to the presence of the intermediate **Rho(Alloc)**, involving a 2-steps reaction scheme. We chose to covalently modify one amine group by a Boc group, which cannot be deprotected by palladium catalysis, to make our new model **Rho(Boc+Alloc)**, as shown on Scheme 3. 12.



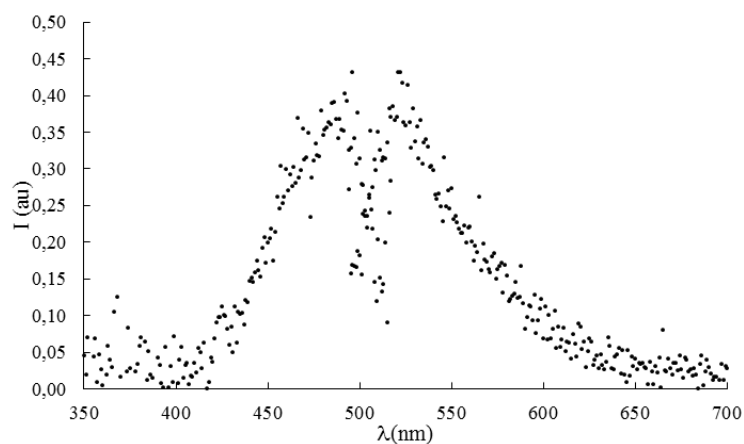
Scheme 3. 12 Rhodamine Boc-Alloc, known as **Rho(Boc-Alloc)** and fluorogenic decaging to **Rho(Boc)**

It was synthesized in two steps according to reported or adapted literature methods (Scheme 3. 13).^{38, 126}

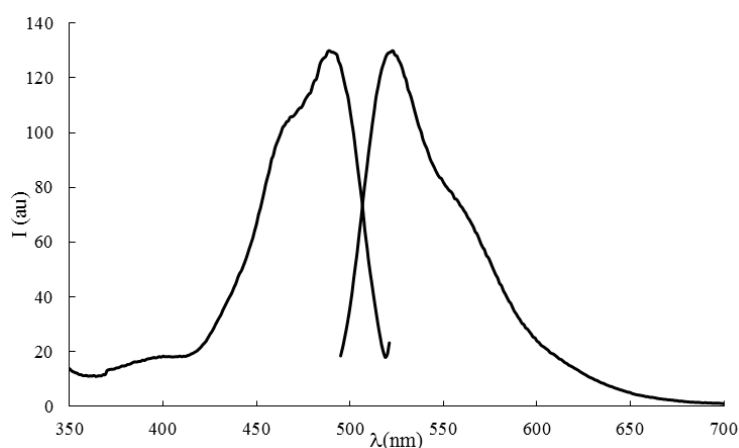


Scheme 3.13 Synthesis of **Rho(Boc)** and **Rho(Boc-Alloc)**

We first measured the photophysical properties of the system to assess its suitability as a fluorogenic probe. The λ_{exc} and λ_{em} were respectively 489 nm and 522 nm (Graph 3. 3) for **Rho(Boc)**, which is consistent with the literature¹²⁶. The probe **Rho(Boc+Alloc)** was found to have similar λ_{max} , but with an on/off ratio of 300 (Graph 3. 2), enabling to make detections at low concentrations of **Rho(Boc)** with very low background noise. We also verified that NADH did not affect the fluorescence intensity in a significant manner, as the difference may come from the preparation of the solutions instead.



Graph 3. 2 Excitation-emission spectra of **Rho(Boc-Alloc)** at 5 μ M (pH7 phosphate buffer +2% DMSO, slits 2.5)

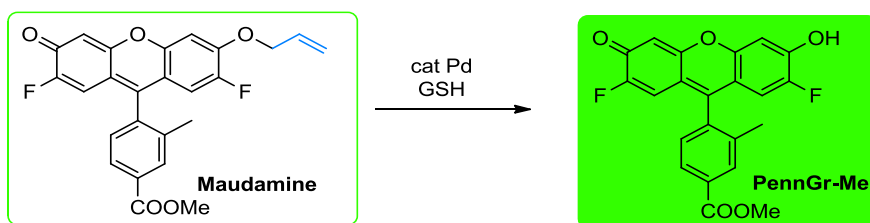


Graph 3. 3 Excitation-emission spectra of **Rho(Boc)** at 5 μ M (pH7 phosphate buffer +2% DMSO, slits 2.5)

2.3.3 Pennsylvania Green and Maudamine

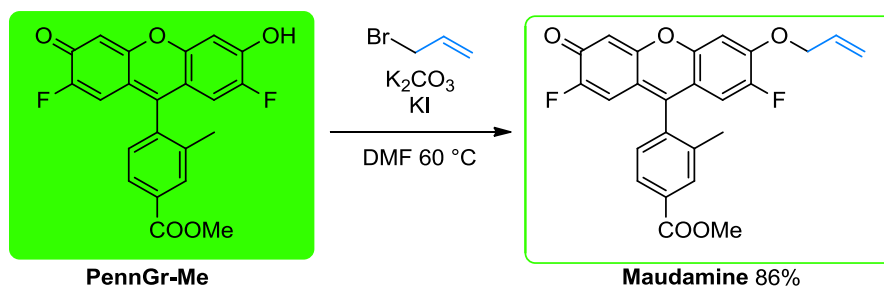
During our investigations, we discovered that several chemical moieties existed to address a specific molecule to a certain compartment. One classical example is to tether a lipophilic cation, such as a triphenylphosphonium salt, to address a certain compound to mitochondria, which are negatively charged due to the proton gradient required for the respiration.

We found the example of Pennsylvania Green methyl ester which accumulates in endoplasmic reticulum due to its lipophilicity (Scheme 3. 14).¹²⁷ Pennsylvania green Methyl ester (**PennGr-Me**) is a red solid which exhibits a strong fluorescence in the green region being irradiated in the visible region, with an excitation and emission of respectively 494 nm and 515 nm (Graph 3. 4), similarly to our rhodamine model. Starting from this scaffold, we made a few attempts before considering the O-allyl Pennsylvania Green methyl ester, called **Maudamine**.



Scheme 3. 14 Pennsylvania Green methyl ester **PennGr-Me** and **Maudamine**

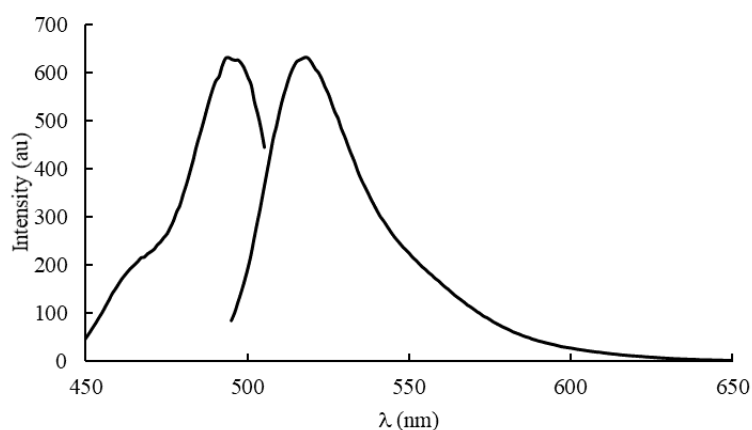
PennGr-Me was synthesized using an optimized literature procedure.¹²⁷ The **Maudamine** was synthesized by a simple allylation of the precursor. **Maudamine** was obtained in quantitative yield with an easy purification (Scheme 3. 15).



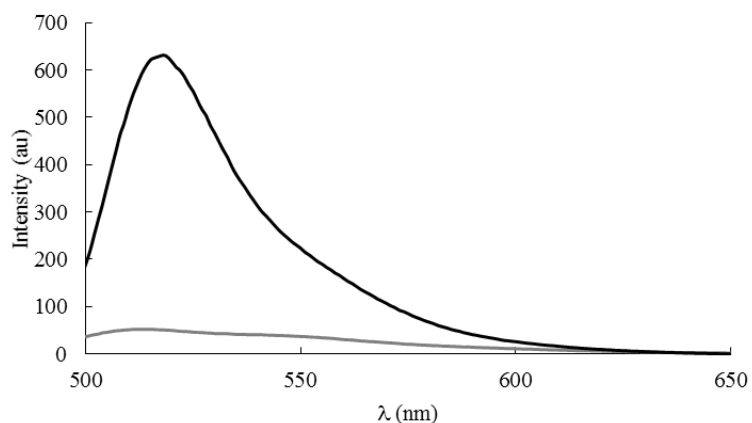
Scheme 3. 15 **Maudamine** synthesis

The **Maudamine**'s fluorescence properties were measured with irradiation at 485 nm for 4 μM , and the intensity was found to be

reduced of 90% compared to PennGr-Me (Graph 3. 5), making this probe a potential fluorogenic model for endoplasmic reticulum-targeted activation.



Graph 3. 4 Excitation-emission spectra of Pennsylvania Green at 4 μ M



Graph 3. 5 Excitation spectra of Pennsylvania Green (Black) and **Maudamine** (Grey) for comparison

2.4 Discussion

What is a good fluorogenic probe? In this project, we quickly realized that a good fluorogenic probe was an advantage for *in vitro* tests as it simplifies the mathematical treatment and gives a quick and

reliable readout. The criteria for good fluorogenic probes *in vitro* and *in cellulo* can be summarized as follows.

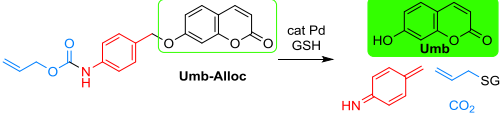
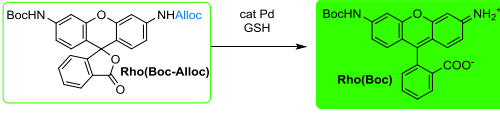
- Excitation in the visible range ideally
- Stokes shift above 20 nm
- No fluorescence quenching (especially by NADH). Not a factor for Visible-range fluorophores.
- No degradation
- An on-off ratio above 20, better above 50
- Solubility at neutral pH with 1-2% of DMSO
- One step deprotection

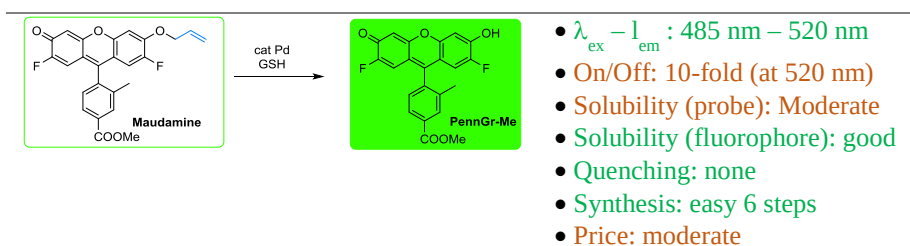
For biology, several additional criteria need to be met.

- Excitation in the visible range mandatory
- Cell permeable
- Lipophilic, in order to remain inside the cell

In this project, we have developed a series of fluorogenic models. Table 3. 2 summarizes our knowledge about them and is helpful to see their advantages and drawbacks.

Table 3. 2 Summary of the three fluorogenic models used in this work

Fluorogenic model	Characteristics
 Umb-Alloc	• $\lambda_{\text{ex}} - \lambda_{\text{em}}$: 325 nm – 454 nm • On/Off: 70-fold (at 454 nm) • Solubility (probe): acceptable • Solubility (fluorophore): good • Quenching: severe by NADH • Synthesis: 4 steps easy • Price: cheap
 Rho(Boc-Alloc)	• $\lambda_{\text{ex}} - \lambda_{\text{em}}$: 489 nm – 522 nm • On/Off: 300-fold (at 522 nm) • Solubility (probe): enough for our applications • Solubility (fluorophore): good • Quenching: none • Synthesis: 2 steps, poor yield, difficult purification • Price: expensive starting material



Among them, we have found one close to ideal, as it meets all the requirements except the synthesis ease and the price. It is the **Rho(Boc-Alloc)** probe. With excitation at 489 nm and emission at 522 nm, it has an on-off ratio of 300, making the calibration easy to carry out. As it has a very high intensity, it allows detection without devices in the low micromolar range. A next step would be to validate it *in cellulo*.

**Second part: New applications of Tsuji Trost
reaction in bioorthogonal prodrug decaging**
**Second chapter: Discovery of new biocompatible
palladium complexes in cellular Tsuji-Trost
reaction**

3 Second chapter: Discovery of new biocompatible palladium complexes in cellular Tsuji-Trost reaction

3.1 Introduction

Although palladium is the most widely used metal to catalyse nucleophilic allylic substitutions in synthesis, there was a big gap to fill in bioorganometallic chemistry (Figure 3. 3). The very first examples use either a limited range of ruthenium complexes.^{38-40, 122} Some supramolecular systems have been reported. Bradley focused his research on the development of supported pre-formed palladium (0) nanoparticles on a polymer matrix.^{46-47, 50-51, 128} Those catalytic beads were assayed *in vitro* and also *in vivo* on zebrafish embryo models. Interesting results were obtained while attaching a targeting moiety to control the intracellular distribution of the complex.^{42, 50} Eventually, Weissleder reported in 2017 the use of (Fur₃P)₂PdCl₂ catalyst entrapped in biodegradable polymeric micelles for better cellular uptake and stability.¹ However, the authors do not describe the activation mechanism of activation, which is thought to be a bioreduction. At the time we were conducting our study, Mascareñas shed light for the first time on bioorthogonal palladium complexes, reporting [π -allyl Pd (PR₃)Cl] as a lead scaffold.¹²³ The scaffold has been modified by tethering lipophilic cations to address the complexes to mitochondria, similarly to another work of the same group.⁴⁴

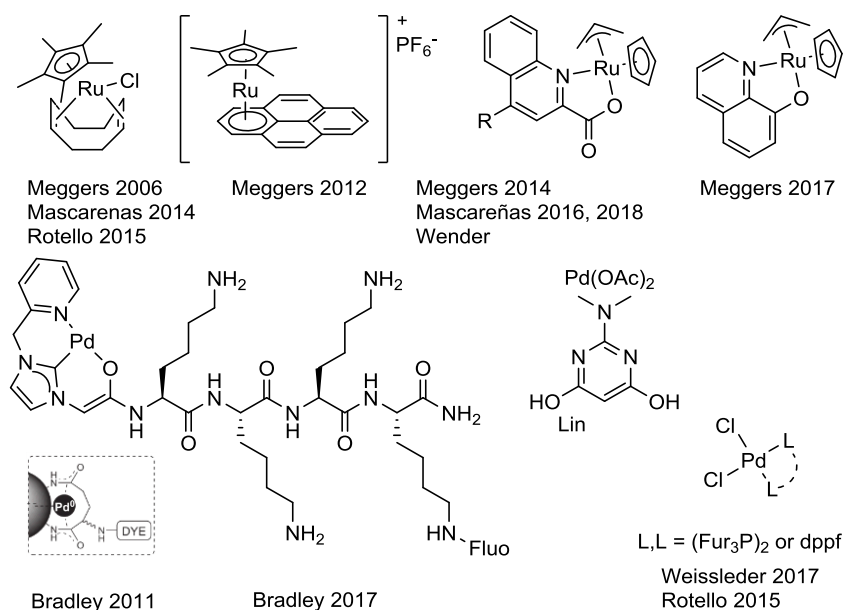
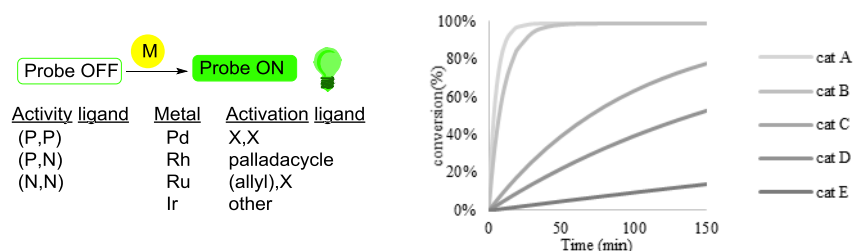


Figure 3.3 Summary of the palladium and ruthenium catalysts for the cellular Tsuji-Trost reduction

The former examples gather remarkable examples of the use of a relatively small number of catalyst structures, although Tsuji-Trost reaction has been applied with a huge range of palladium (0) catalysts in synthesis. Those described studies also do not address the problem of the activation of the precatalyst, which is critical relative to the biological application for which the catalyst is aimed to be used.

3.2 Goals of this chapter

In this section, we developed a medium throughput fluorimetric assay which enabled us to discover new palladium catalysts active in a model Alloc cleavage reaction in biocompatible conditions (Scheme 3. 16). After a successful *in vitro* validation, the system was validated *in cellulo* in SiHa cancer cell lines. Emphasis was put on the study of the activation mode of the catalyst and the impact of the ligands.



Scheme 3.16 General strategy in the catalyst screening

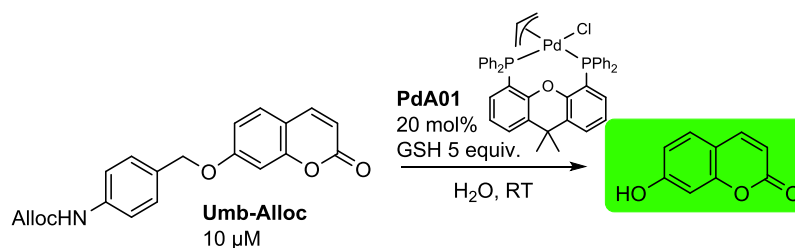
3.3 *In vitro* assays

3.3.1 First screening: Umbelliferon model

As we got acquainted with the Varian Carry Eclipse spectrometer, working with one single fluorescence cell holder, we tried to work on the development of a low throughput screening centred on a library of palladium catalysts, but also some other metals.

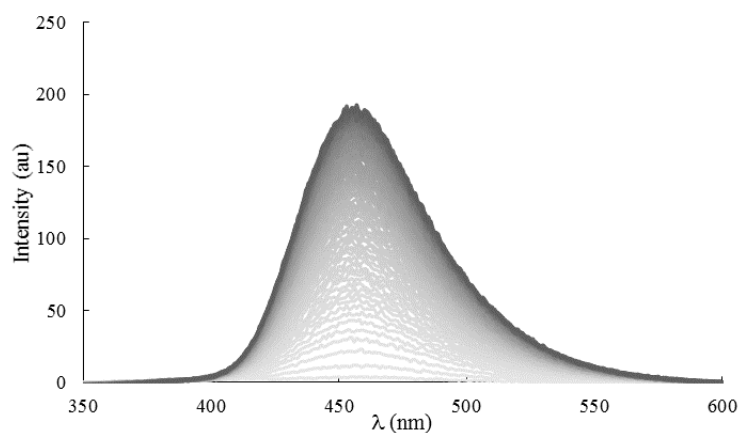
3.3.1.1 Proof of concept: a case study with PdA01

We developed a set of arbitrary conditions for the catalytic loading and the GDH quantity. The concentration of 10 μM of **Umb-Alloc** is a compromise between the response factor, the calibration and the solubility (Scheme 3. 17). Working at such a low concentration will be challenging for the catalyst, so only the fastest, the easiest to activate and the most resistant to deactivation will come out the screening. It will perhaps reject catalysts with some activity for Tsuji-Trost reaction in higher concentration or temperature, as well as other solvents.

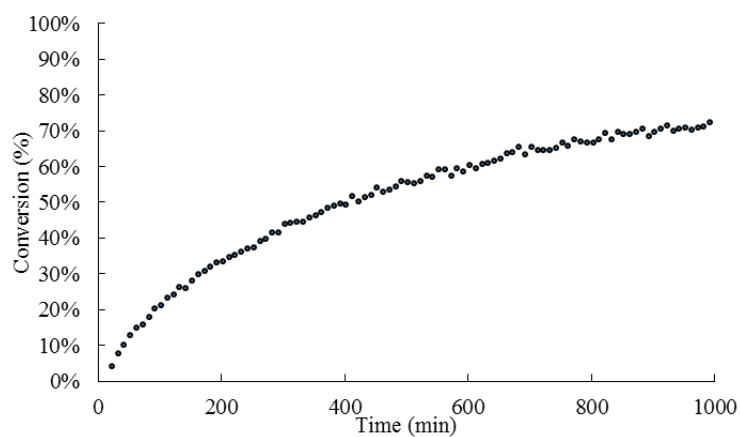


Scheme 3. 17 Model fluorogenic deprotection of **Umb-Alloc** to umbelliferon used for the first catalyst screening

Our interest was to compare easily activable complexes, belonging to the family of π -allyl chloro complexes (**PdA** family), but with different catalytic ligands such as biphosphines or bipyridines to understand the impact of the ligands on the catalysis. These catalysts can be reduced by a nucleophilic attack of a thiol, such as glutathione, to be activated to (P,P)Pd (0).¹²⁹ This work was started by Martin Appelmans and continued by Maud Decock, both of them being master students in chemistry and bioengineering.¹³⁰⁻¹³¹ The first catalyst to be tried on the model reaction (Scheme 3. 17) was **PdA01**. Over 6h40 of reaction, we could see a regular increase of the fluorescence until 200 units of fluorescence, after calibrating the curves with $I_{600\text{ nm}} = 0$ (Graph 3. 6). A simple reading at 454 nm and the use of the calibration curve gave us a plot of the conversion in function of the time (Graph 3. 7). A conversion nearing 80 % was obtained after 6h40 of reaction. We admit that we should have run the reaction longer to see the final conversion.



Graph 3. 6 Deprotection of the **Umb-Alloc** ($10\ \mu\text{M}$) with **PdA01** ($20\ \mu\text{M}$) and GSH (5 equiv) in water: spectra



Graph 3. 7 Deprotection of **Umb-Alloc** ($10\ \mu\text{M}$) with **PdA01** ($20\ \mu\text{M}$) and GSH (5 equiv) in water: conversion plot

3.3.1.2 Screening

Afterwards, we screened 25 catalysts (Figure 3. 4), among which 20 were palladium complexes, 3 were ruthenium complexes and one was a gold complex.¹³⁰

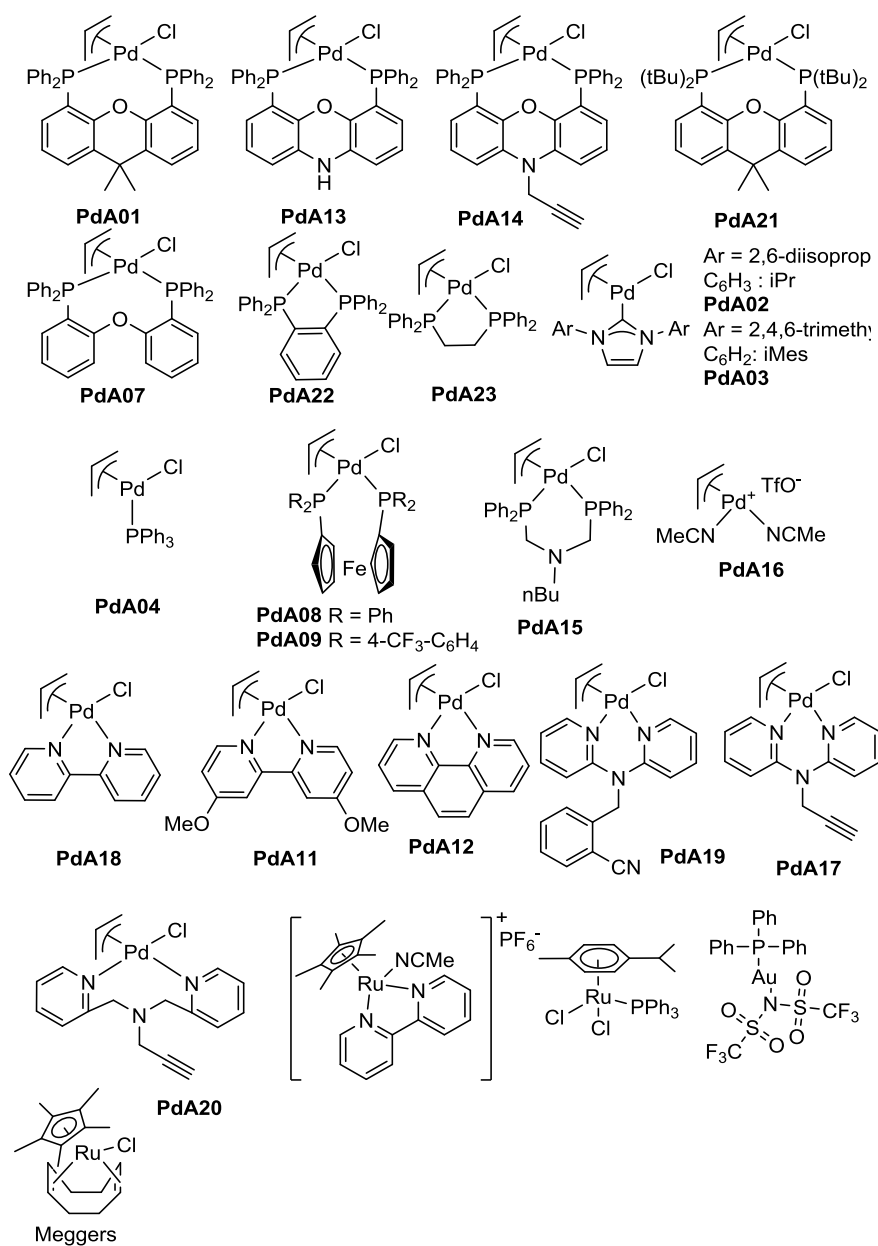
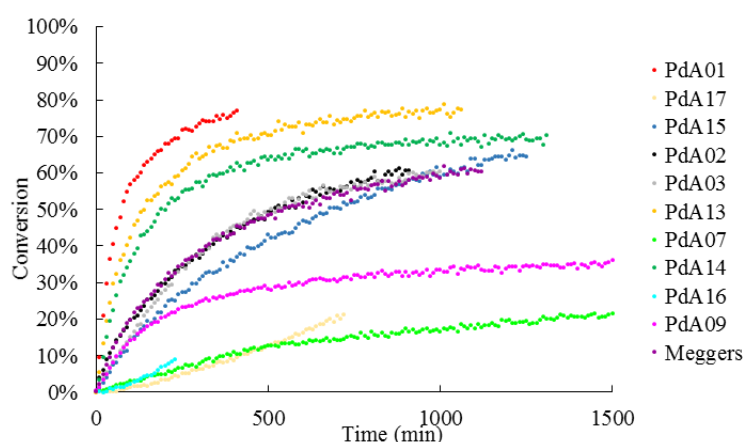


Figure 3. 4 Catalysts tried in vitro with *Umb-Alloc*

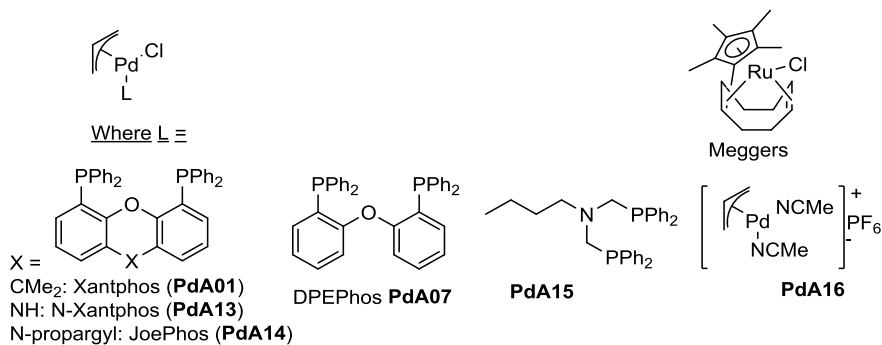
We rapidly noticed that all the complexes featuring chelating nitrogenated ligands were inactive (**PdA18**, **PdA11**, **PdA12**, **PdA19**), except with the bpa-propargyl (**PdA17**, Graph 3. 8). That complex was synthesized but the characterization was impossible for

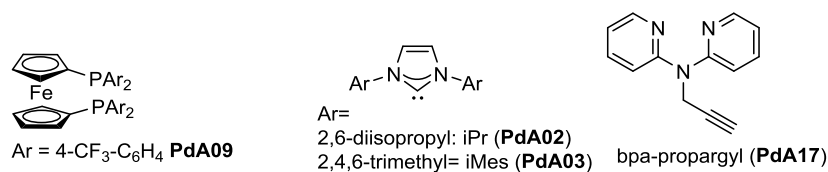
undetermined reasons. For phosphine ligands, not all ligands were suitable for catalysis.¹³² All the Xantphos family complexes (**PdA01**, **PdA13**, **PdA14**) were active, and **PdA01** was our best candidate. The exotic structure of PdA14 was tested as for a project involving vectorising the catalyst. NHC were also good candidates (**PdA02** and **PdA03**), yet with lower activities. Our only gold complex and the new ruthenium complexes were found to be inactive as well.



Graph 3. 8 Catalyst screening on **Umb-Alloc**. 24 catalysts tried, only the active ones were shown for clarity

Active catalysts:





Inactive catalysts

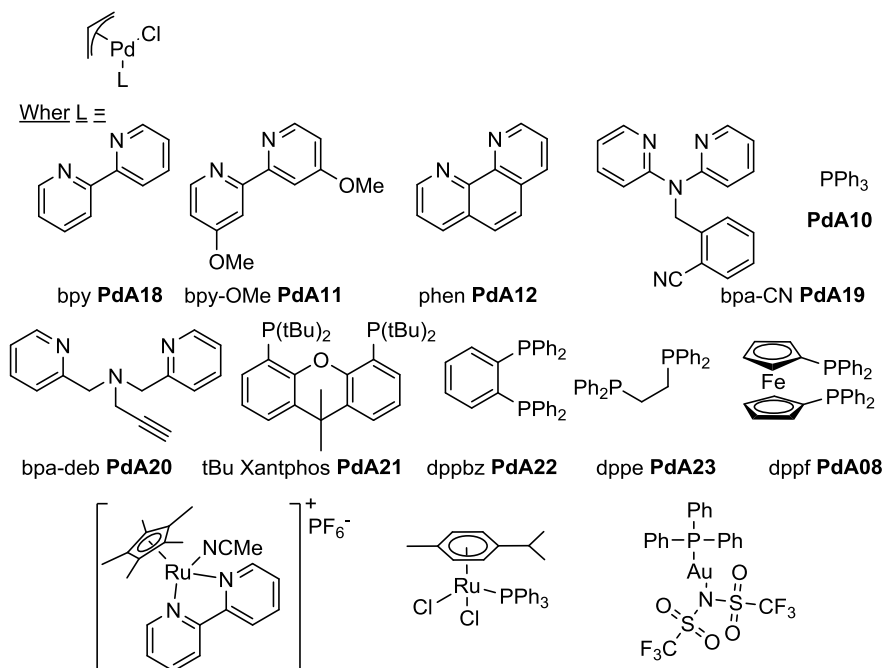
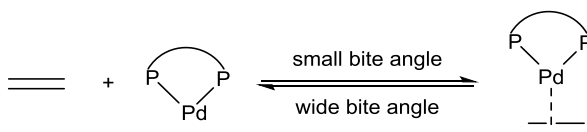


Figure 3. 5 Catalysts used for the screening with **Umb-Alloc**

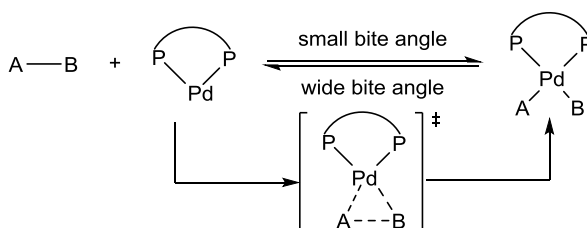
3.3.1.3 Discussion about the screening

At this stage, we found one first important criteria for the design of the catalytic ligand, which is the bite angle. While looking at the catalytic cycle, two steps could be influenced by the bite angle. At first, the Pd (0) catalytic species needs to coordinate to the double bond of the Alloc group (Scheme 3. 18). Therefore, a large bite angle would favour the de-coordination and a smaller bite angle would favour the coordination step. All the steps of the mechanism are reversible.



Scheme 3. 18 Influence of the bite angle on the coordination-decoordination step on the olefin

Additionally, we know that the palladium (0) active species enters by an oxidative addition in the C-O bond and leaves the cycle by a reductive elimination (Scheme 3. 19). Notoriously, a small bite angle favours the oxidative addition and a large one favours the reductive elimination.¹³² This effect leads us to think that this effect is the sign that the reductive elimination is the rate determinant step in the cycle. Further examination in the next chapter brought the second proof while carrying out a detailed optimization. This result is in opposition to what Meggers reported for his case using the third generation of catalysts, where a first order in the coumarin substrate indicated that the rate determinant step was located at the oxidative insertion step.⁴⁰



Scheme 3. 19 Influence of the bite angle on the oxidative insertion and reductive elimination

3.3.2 Second screening: Rhodamine model

3.3.2.1 General principles

After the successful study of the π -allyl palladium family, we wanted to expand the scope of the active catalysts by realizing a higher-throughput screening on a more structurally diversified catalyst

bank. We moved the **Rho(Boc-Alloc)** model, described in the first chapter. The screening was performed by Mathieu Marlière, a master student at a technical school.¹³³

To improve the throughput:

- We opted for a 96-well plate reader using optical filters as monochromators.
- One reaction was performed per well in a 200 μ L scale.
- Multichannel micropipettes were used to add aliquots of the stock solutions

To improve the quality of the quantification:

- We used a fluorophore with simply no residual background signal or quenching, which is **Rho(Boc-Alloc)**.
- The experiments were frequently performed in triplicates and were averaged prior to any mathematical treatment.
- To take account of the light fluctuation, a calibration curve was prepared in the two bottom rows (in duplicate) and was read at every measurement to ensure that the regression parameters a and b are up to date.

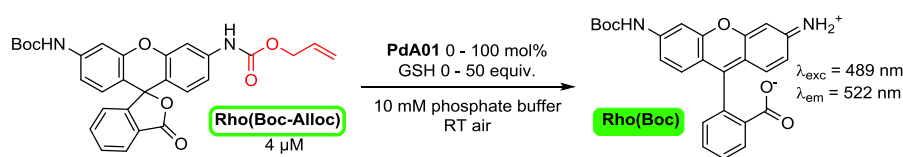
In this study, we considered complexes bearing two types of ligands:

- (i) a catalytic ligand, which remains on the active species, which is typically but not restrictively a biphosphine¹³⁴⁻¹³⁵, a bipyridine or an NHC¹³⁶
- (ii) an activation ligand, which will permit the *in-situ* reduction in response to an exogenous stimulus. Among that family, we will examine π -allyl (Family **PdA**)¹²⁹, which can be activated by a nucleophilic attack of a thiol, palladacycles (Family **PdB**)¹³⁷⁻¹³⁸,

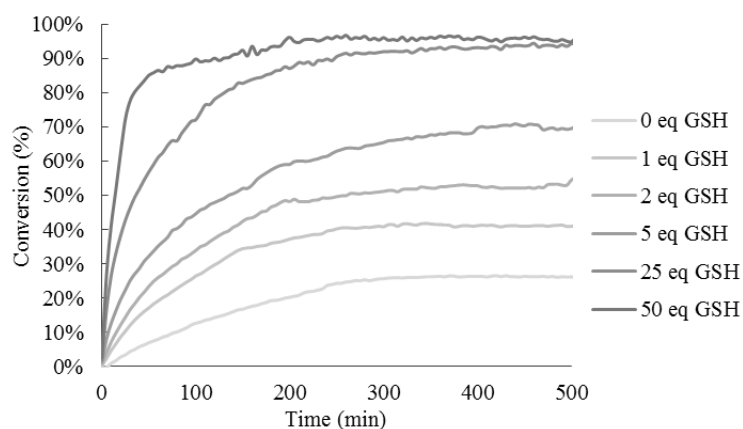
activated generally by a base, palladium dichlorides (**PdAC01-PdC16** and **PdC21**) which can be reduced non-specifically *in-situ*. The palladium complexes list included as well as more exotic structures (**PdD01**, **PdC17** to **PdC24**) for which the potential activation mechanism is less known to give us a chance to find some unexpected compounds catalysing our model reaction.

3.3.2.2 Optimization

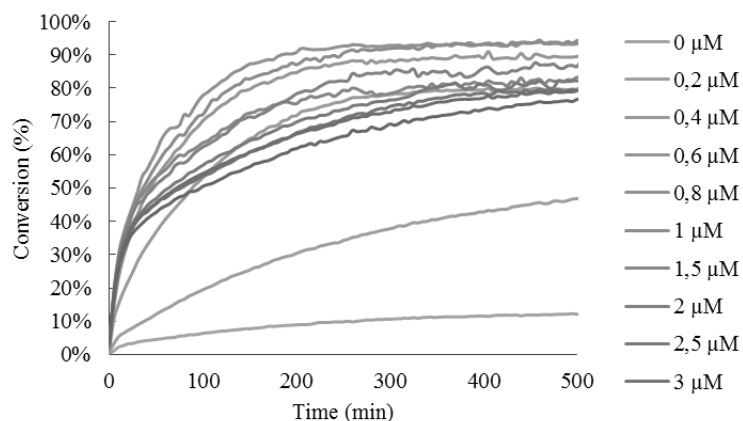
We started our investigation by an optimization which was performed in only one set of 72 simultaneous experiments (Scheme 3. 20). For the catalyst choice went for (Allyl)Pd(Xantphos)Cl (**PdA01**) as a model catalyst as it was our bet hit in the previous screening.¹³⁹ Glutathione remained our nucleophile as it is the most biologically relevant thiol. For technical reasons related to the response factor and the sensitivity of the detector, we had to set the Rho(Boc-Alloc) concentration to 4 μ M. The optimization aimed to determine the optimal concentration of GSH and catalyst between 0 and 50 equiv. and between 0 and 100 mol% respectively. Representative graphs depict the curves for the optimal GSH (Graph 3. 10) and catalyst (Graph 3. 9) concentration.



Scheme 3. 20 Model reaction and conditions used for the optimization



Graph 3. 9 Impact of GSH. Conditions: **PdA01** (25 mol %); GSH 0 – 50 equiv.



Graph 3. 10 Impact of the catalyst. Conditions: **PdA01** 0 – 100 mol%; GSH 25 equiv.

With 15 mol% of **PdA01**, we could delightfully reach nearly 96% of conversion in 3 h with 15 mol% of **PdA01** and 25 equivalents of glutathione.

3.3.2.3 Discussion about the optimization

Such a high number of curves obtained with **PdA01** with the same stock solutions, in the same device at the same time represent a high amount of data from which a lot of information can be extracted. The global shape of the curves, as well as the final yield are the easiest aspects to discuss. With a bit more of mathematical treatment, data

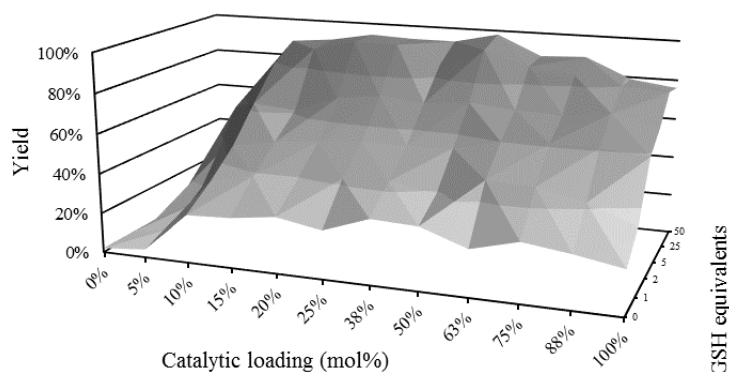
such as TON, can be extracted. Eventually, from the slopes, the TOF or even reaction partial orders can be also extracted. This is not common practice in organometallic chemistry, where reactions are usually performed one at the time without kinetic analysis.

a) Final yield

The most trivial one is simply the final yield calculated by (where a and b are the regression parameters and C^0 is the initial concentration of the probe):

$$\eta_{t_f} = \frac{I_{t_f} - b_{t_f}}{a_{t_f} \cdot C^0}$$

The final yield was then plotted in function of the catalyst **PdA01** and the GSH concentration (expressed in equivalents). So, Graph 3. 11 was obtained.



Graph 3. 11 Yield in function of the catalytic loading (**PdA01**) and the GSH equivalents

- Increasing amounts of GSH gave a steady increase of conversion with saturation between 25 and 50 equivalents.
- An optimum was found between 15 and 50 mol% of **PdA01**.
- The reaction does actually take place in absence of **PdA01** when higher amounts of thiol to reach a conversion of 20% with 50

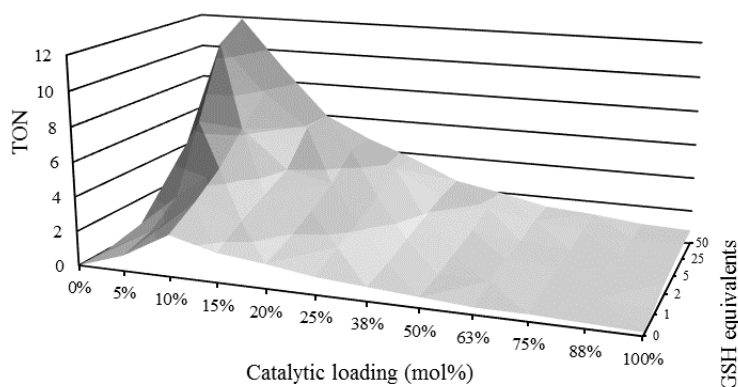
equivalents. The plate in which the reaction was performed was new when used, and the risk of cross-contamination by palladium is low.

- Tiny amounts of GSH brought a remarkable effect, as the yield was multiplied by a factor 2 almost between 0 and 1 equivalent.
- To reach the 90% of conversion, 25 equivalents of GSH were needed, and increasing the GSH to 50 equivalents brought a small improvement was observed. This concentration remains well below the physiologic concentration, usually in the mM range.
- Noticeably, the yield decreased when more catalyst **PdA01** is added, which is probably the sign of a secondary reaction involving the catalyst. This will be discussed in the next paragraph.

b) Turnover Number (TON)

Directly derived from the yield and obtained by calculating the ratio between the yield and the catalytic loading (**PdA01**), it indicates the number of cycles performed by the catalyst (Graph 3. 12).

- The **PdA01** can perform up to 11.9 cycles when a low catalytic loading is used.



Graph 3. 12 TON in function of the catalytic loading (**PdA01**) and the GSH equivalents

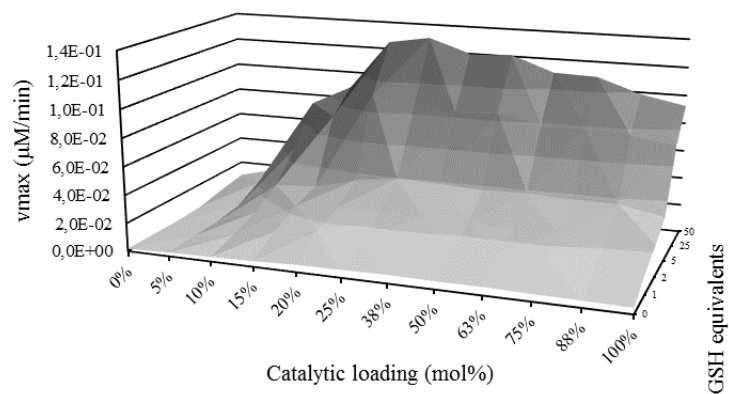
c) V_{max}

It corresponds to the maximum speed of the catalyst calculated at the initial time.

$$v_{max} = \lim_{t \rightarrow 0} \frac{dC(t)}{dt}$$

As we did not go into curve fitting with a software, we simply took the slope over the 3 first points, spaced by 5 minutes. The v_{max} was then plotted in function of the catalyst and GSH concentration expressed in equivalents (Graph 3. 13).

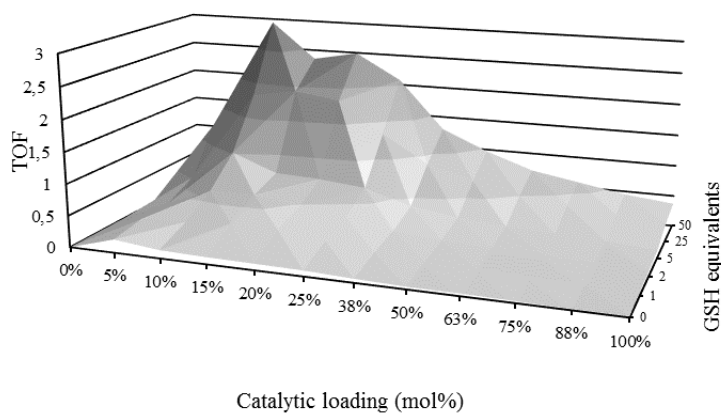
- The maximal observed speed was found to be 0.13 $\mu\text{M}/\text{min}$.
- The v_{max} is directly influenced by GSH as it is directly correlated to the regeneration of the catalyst by capturing the π -allyl, although Colacot reported that those catalysts were activated by a base in organic solvent conditions¹³⁴.
- Above 25 mol% and at high GSH concentration, the v_{max} decreases and does not increase as expected.



Graph 3. 13 $v_{\max}$ in function of the catalytic loading (**PdA01**) and the GSH equivalents

d) The turnover frequency (TOF)

It corresponds to the maximum frequency at which a catalytic cycle takes place (Graph 3. 14). It is calculated by the ratio between the $v_{\max}$ and the catalyst concentration.



Graph 3. 14 TOF in function of the catalytic loading (**PdA01**) and the GSH equivalents

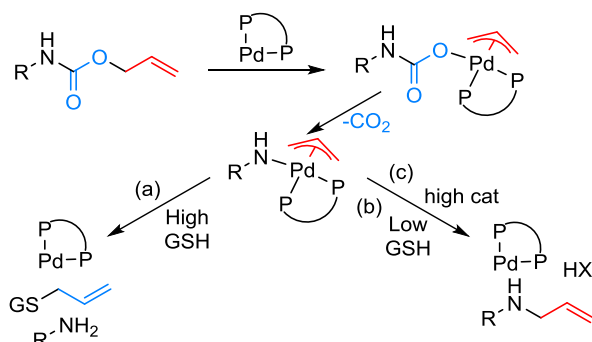
- The catalyst was able to perform at the beginning nearly 3 cycles per minute.

e) Global shape of the curves

When we analysed all the curves for the first time, we were surprised to see the intensity of fluorescence decreasing over the time, especially when a high catalytic loading was combined with a low to moderate GSH concentration.

Combined with the observations around the v_{max} , this indicates that the catalyst is responsible for a side reaction of allylation consuming the released product. In case the glutathione concentration is low or the catalyst loading is high, the formed π -allyl Pd amide may either undergo a reductive elimination or undergo a nucleophilic attack by the product of **Rho(Boc)** either by the amine or the carboxylate, depending on the conversion of the reaction to form either the allyl ester or the *N*-allyl derivative, which are assumed to be less fluorescent than **Rho(Boc)** (Scheme 3. 21). Such a side reaction has been observed by Meggers by HPLC.¹²² Higher GSH concentrations act as an allyl trap and thus hampers the side reaction.

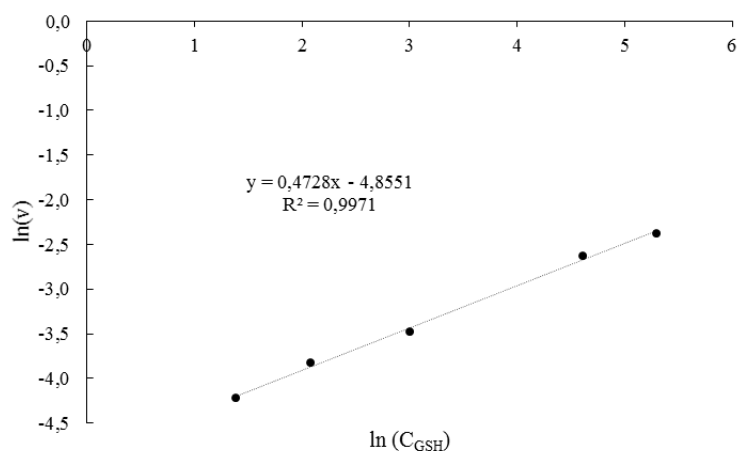
Those results highlighted for the first time that care must be taken while developing fluorogenic bioorthogonal reactions as a single readout is not enough to compare the activity of a catalyst.



Scheme 3. 21 Mechanistic proposition accounting for the influence of GSH and the catalyst

f) Partial order determination

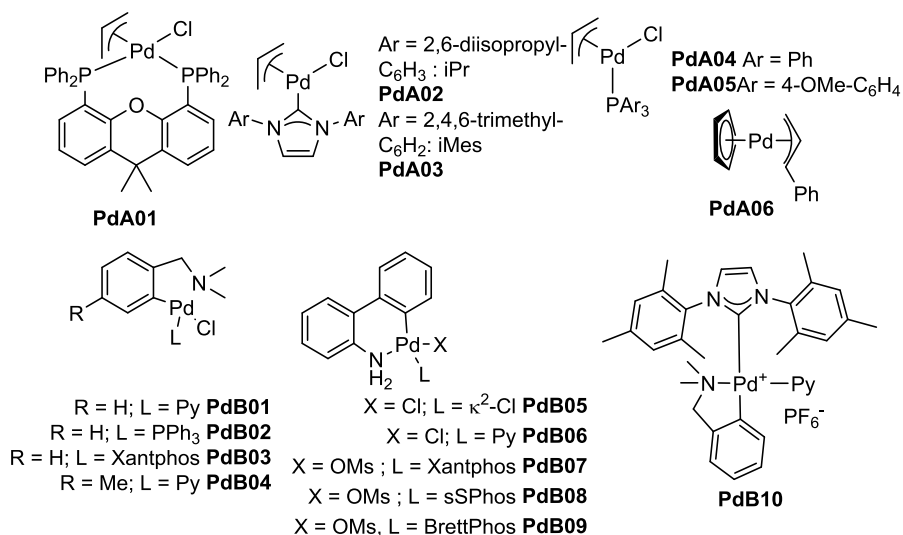
We also attempted to determine the reaction order of the reaction respectively to GSH (Graph 3. 15). We plotted the $\ln$ of the $v_{\max}$ for 15 mol% of catalyst in function of the $\ln$ of the concentration of GSH. A remarkable R^2 of 0.9971 was found for the equation $\ln(v_{\max}) = 0.4278 \cdot \ln(C \text{ GSH}) - 4.8551$, giving us an apparent order 0.43. Although this value is not an entire number, it gives us an idea about the involvement of GSH in the rate-determinant step as the kinetics is complex.

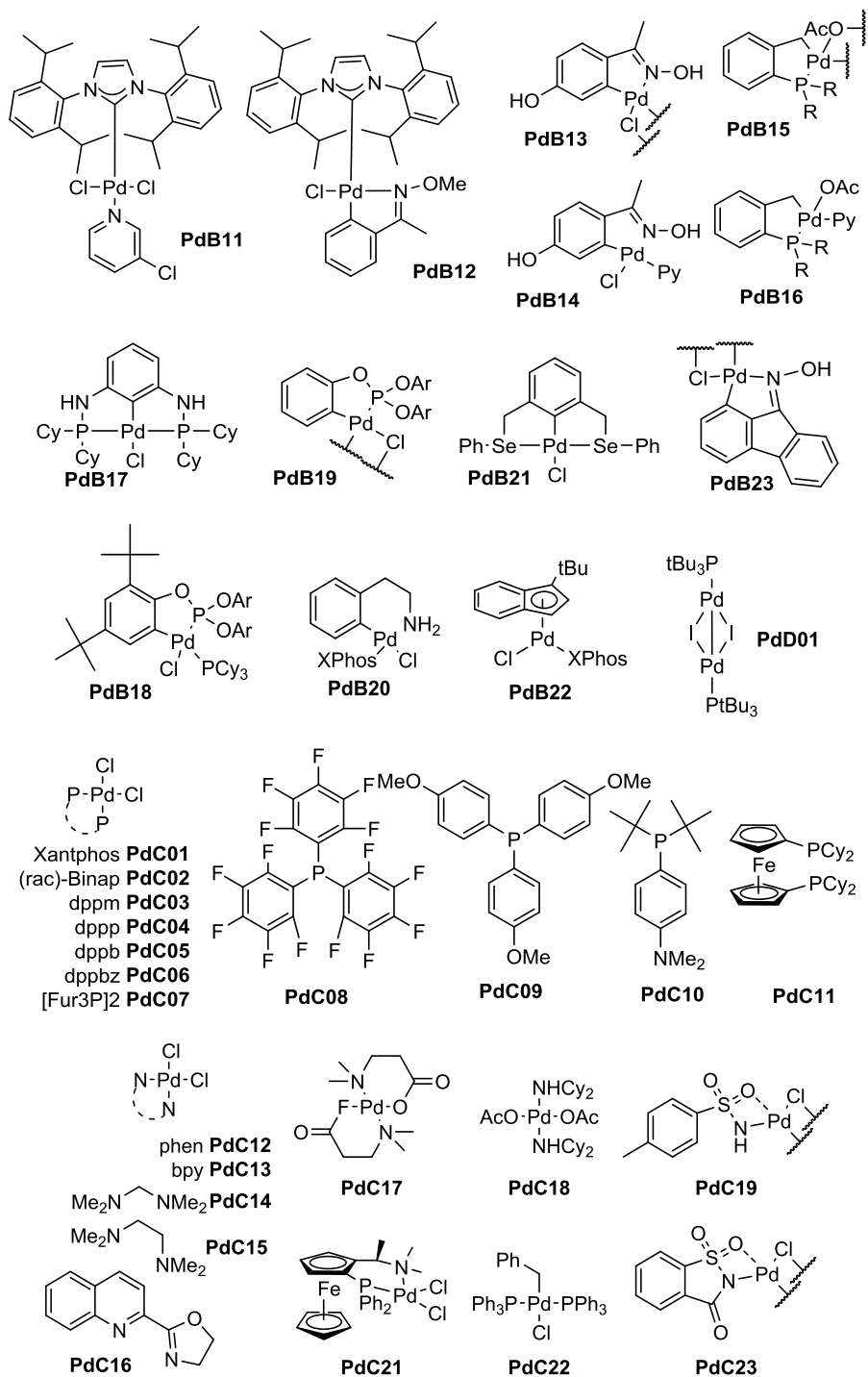


Graph 3.15 Determination of the reaction order of the GSH

3.3.2.4 Screening

Our goal was to broaden the range of possible structures of palladium complex compatible with cellular conditions (Figure 3. 6). This screening included π -allyl Pd complexes bearing NHC, monophosphines and biphosphines (Family **PdA**) which are mostly activated by nucleophiles or bases, palladacycles (family **PdB**) which are mostly activated by bases, and also a broad range of PdX_2L_2 , for which less is known about their activation mechanism.





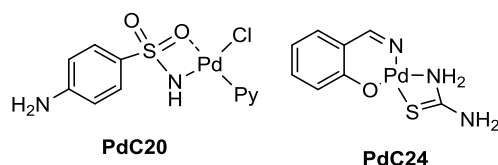
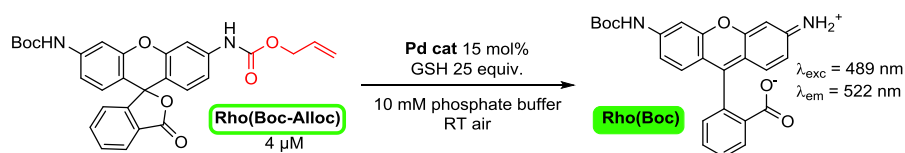


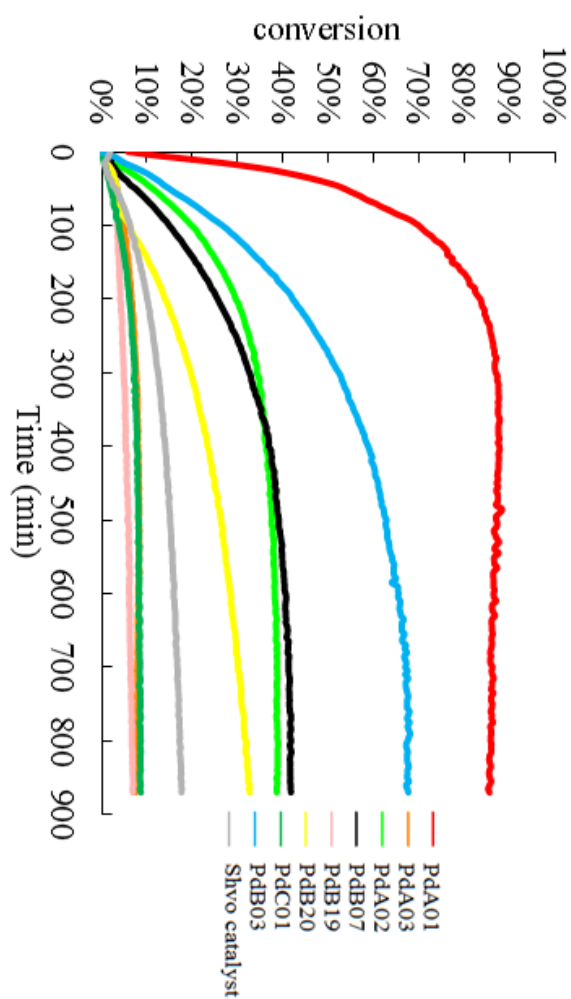
Figure 3. 6 List of all the catalysts tried in the in vitro screening with the rhodamine model

With optimal conditions (15 mol% of catalyst and 25 equivalents of GSH for 4 μ M of the probe) and a data processing method in hands, we decided to proceed to the catalyst screening (Scheme 3. 22). All the solutions were freshly prepared in 10 mM pH 7 phosphate buffer with 2% of DMSO prior to the experiment.



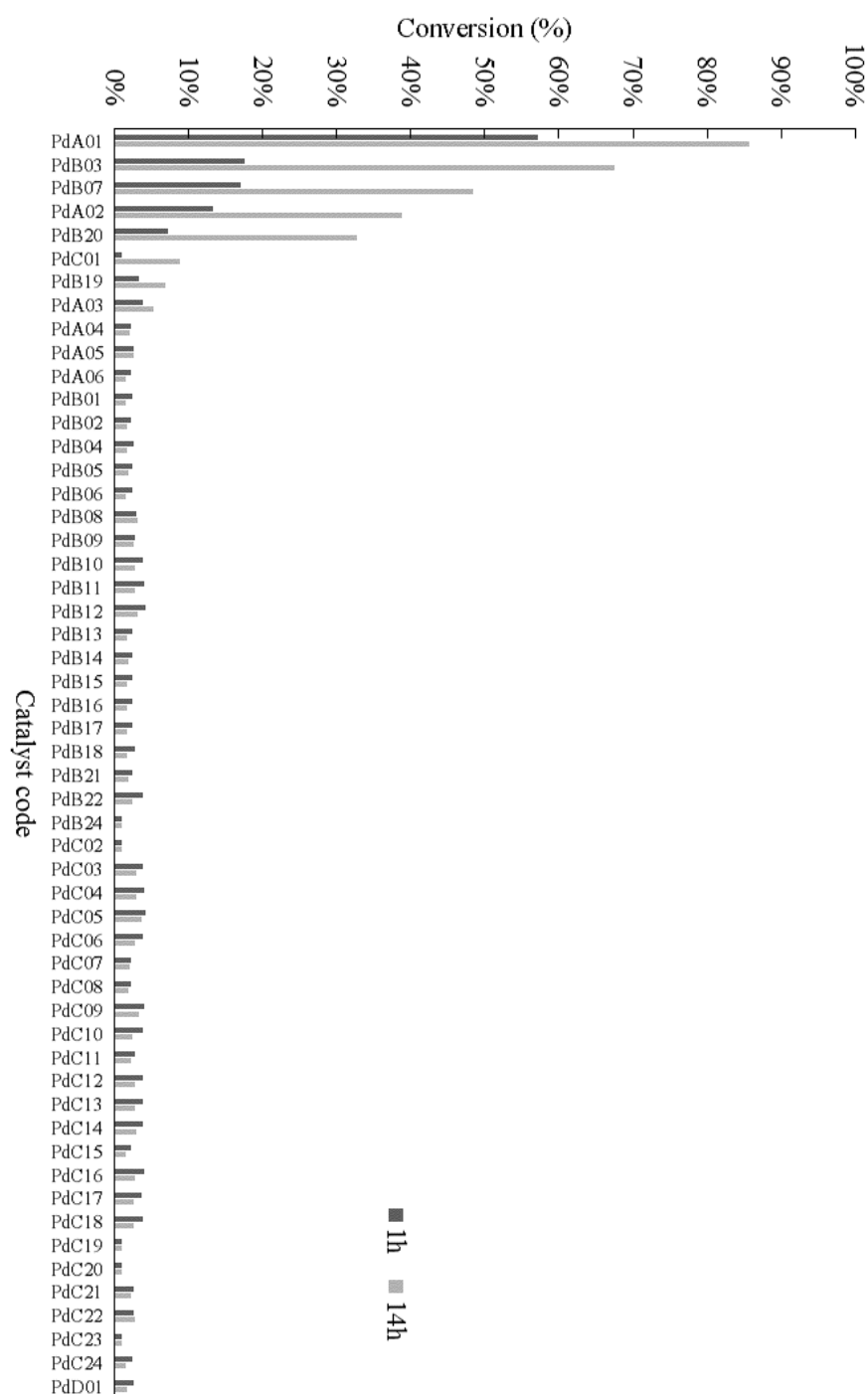
Scheme 3. 22 Conditions used in the screening of the whole catalyst database

All the catalysts were then tested at a fixed concentration in triplicate. An average was performed and thanks to the regression parameters $a(t)$ and $b(t)$, the conversion could be plotted in function of the time. For Graph 3. 16, only the hits were plotted and the inactive catalysts were omitted for the sake of clarity.



Graph 3. 16 Kinetic curves of the active catalysts found in the screening

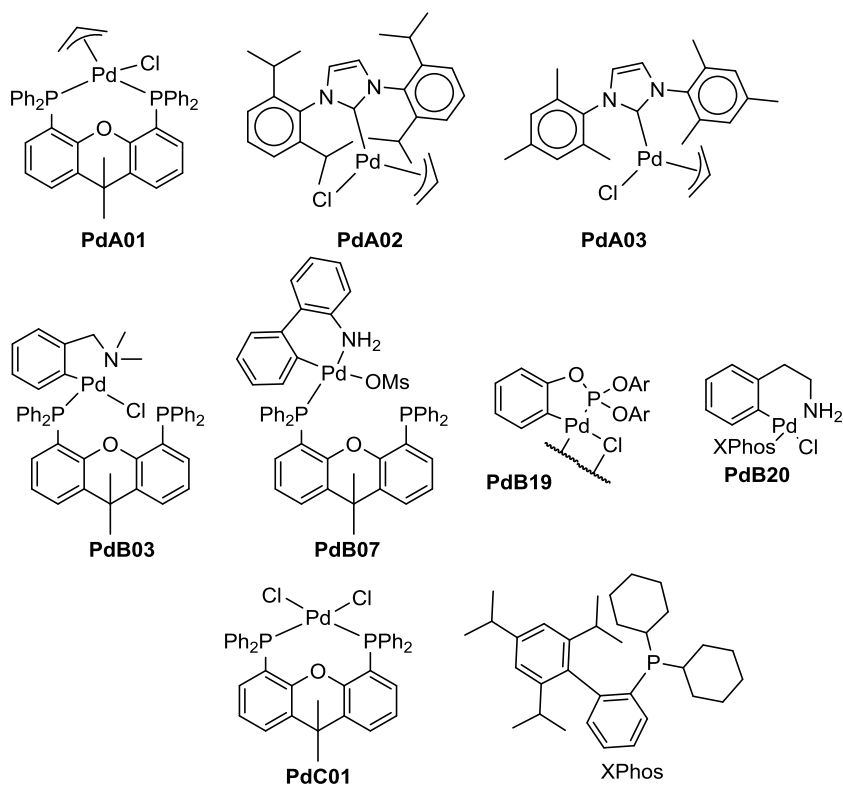
The result for all the database is presented on Graph 3. 17. Although the curves are available in the Experimental Section, the results are presented here as a single readout at 1 h and 14 h.



Graph 3. 17 in vitro catalyst screening. Conditions: **Rho(Boc-Alloc)** 4 μ M, catalyst 15 mol%, GSH 25 equiv., pH 7 phosphate buffer 10 mM, 2% DMSO.

3.3.2.5 Discussion about the screening

Among all the candidates we tried, 9 catalysts were found to be active. The complexes **PdA01**, **PdB03**, **PdB07**, **PdB20** and **PdA02** were found to be clearly active, as well as **PdAC01**, **PdB19** **PdA03** with much less activity (Scheme 3. 23).



Scheme 3. 23 Active catalysts in the screening.

The interesting point is the presence of the ligand Xantphos in **PdA01**, **PdB03**, **PdB07**, but also **PdAC01** (Figure 3. 7). Its catalytic properties are attributed to its large bite angle near 110 °.^{132, 140}

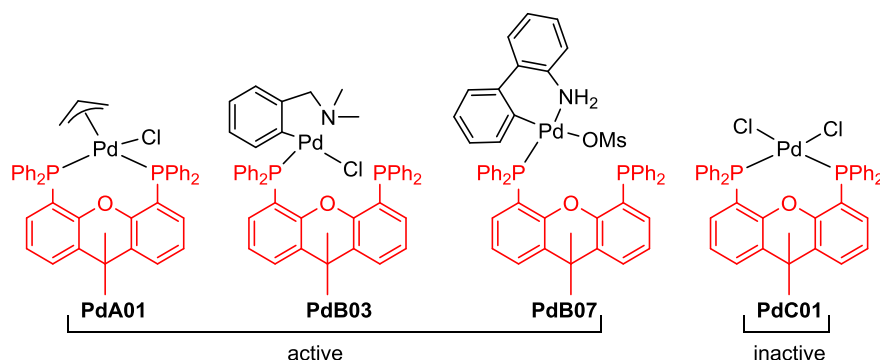


Figure 3. 7 Comparison of the complexes containing Xantphos

This set of active catalyst featuring the same catalytic ligand enables us to draw a comparison between the different modes of activation (Figure 3. 7).

- **PdA01** activates probably by a nucleophilic attack, as shown by our data. It activates very quickly in presence of GSH (gives a quantitative yield) and slowly in absence with simply water or the buffer (only 9% of yield).
- **PdB03** activates through an unknown mechanism.
- **PdB07** activates by a base-dependant mechanism (typically sodium acetate in organic synthesis).¹⁴¹ The NH_2 is deprotonated by the buffer and the palladium (0) is released by reductive elimination giving the carbazole.
- **PdC01** could be reduced thanks to GSH proceeding through nonspecific electron transfers.

In addition to this Xantphos family, we found that **PdA02** (with ^iPr ligand) had good activity, reaching nearly 40%.¹⁴² Remarkably, the palladacycle **PdB20**, reported by Buchwald¹⁴³, reached 32% of conversion in 14 h. This result is surprising as the first generation of palladacycle normally requires a strong base, such as

NaO^tBu, for activation and the active species is reported to have a short life in solution.

3.4 *In cellulo* assays

3.4.1 *In cellulo* validation: Rhodamine model

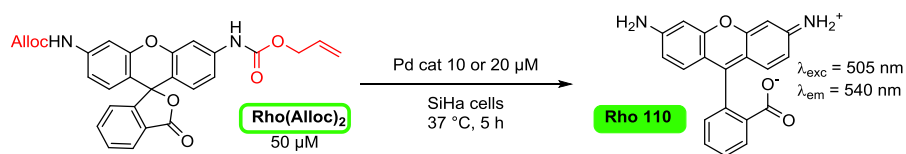
The *in cellulo* evaluation was performed by Maud Decock in the team of Prof. Olivier Feron (UCLouvain) with the technical support of Charline Degavre.¹³⁰

The *in cellulo* screening works in three phases as follows.

- A first fluorimetric assay aims to exclude the catalyst which do not penetrate the cells, and which are catalytically inactive.
- Then, a viability assay verifies that the cells are still alive after the 5 hours of incubation to eliminate the catalysts which are too toxic and active. Alternatively, the concentration of catalyst can be adjusted to work below the toxicity threshold.
- The third phase involves following the kinetics in fluorescence microscopy to make sure that the fluorescence appears inside the cells first.

3.4.1.1 Screening: model reaction and protocol

With our best *in vitro* hits (**PdA01**, **PdB03**, **PdB07**, **PdA02**, **PdAC01**, **PdB19** and **PdA03**) in hands, we moved to their biological evaluation in SiHa cells (Scheme 3. 24). Since the activity *in vitro* and *in cellulo* may differ, we did not restrain the list of catalysts to the hits, as we may miss some structures. A series of 28 catalysts including the hits were selected for the *in cellulo* evaluation (Figure 3. 8). For this experiment, we moved back to the Rhodamine 110 bis Alloc, **RhoAlloc₂** ($\lambda_{\text{exc}} = 505$ nm and $\lambda_{\text{em}} = 540$ nm) as a model substrate.



Scheme 3. 24 in cellulo deprotection of **Rho(Alloc)₂** using palladium catalysis

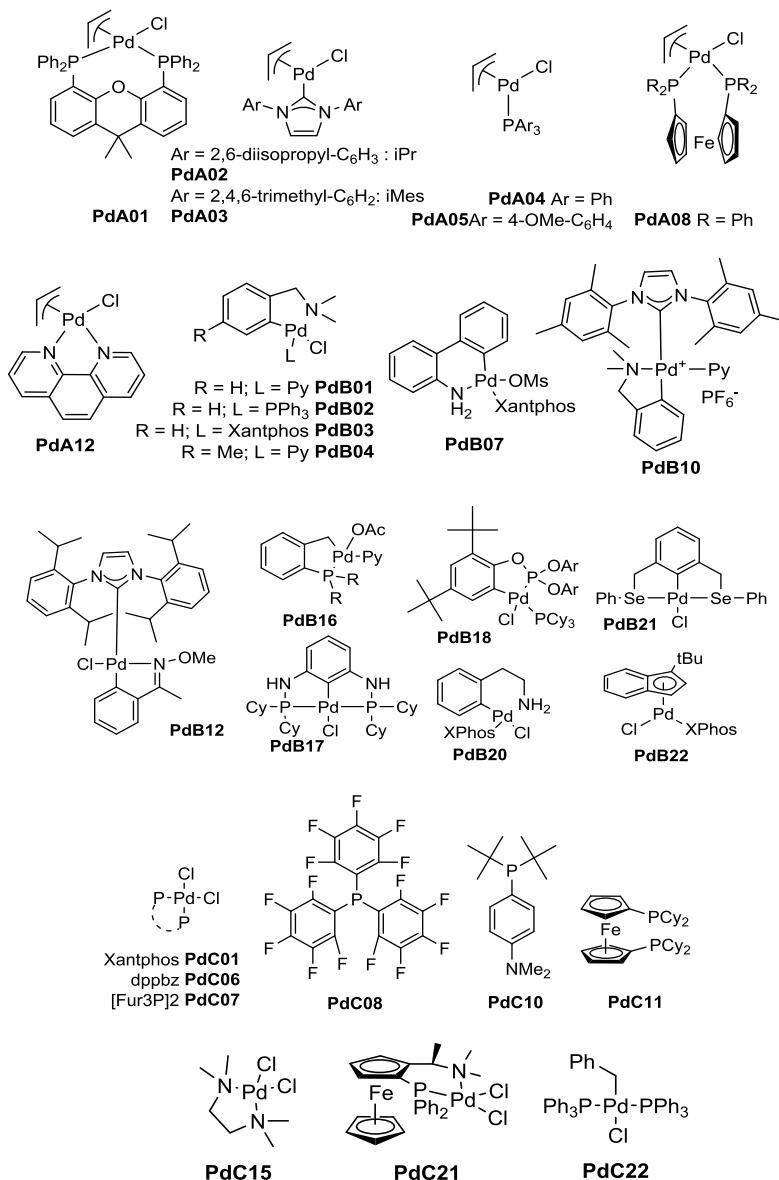


Figure 3. 8 List of the catalysts used in the in cellulo fluorogenic assay

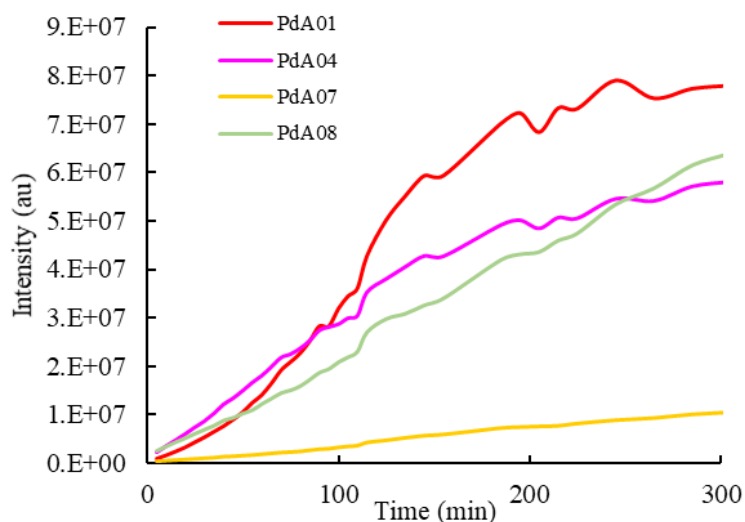
For the fluorimetric assay, the following protocol was used:

- Incubation of the probe **RhoAlloc**₂ at 50 μ M during 18 h.
- Washing 3 times with PBS buffer.
- Addition of the catalyst in the fresh culture medium.
- A measure of the fluorescence during 5 hours in the 96-wel plate.

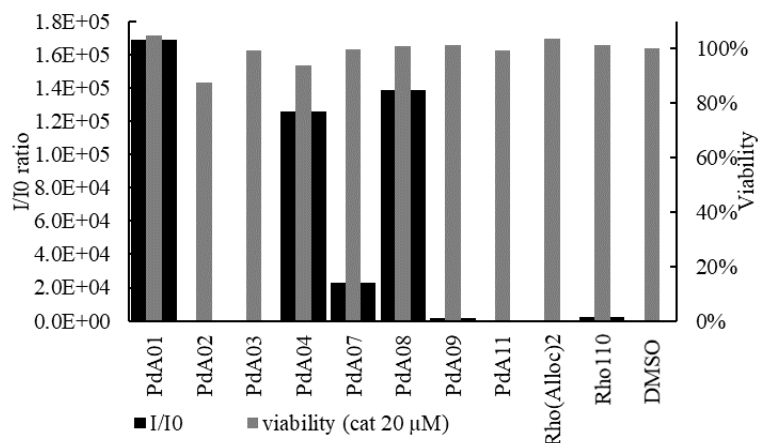
Whereas the conversion could be easily quantified in the *in vitro* test, it could not be quantified *in cellulo* due to the unknown cell volume and disparities in cell endocytosis of the **Rho(Alloc)**₂. It would be possible by HPLC-MS in the cell lysate.

3.4.1.2 First screening

After some work to find suitable conditions, we performed our first screening with 20 μ M of the catalyst. The first screening focused on π -allylpalladium complexes found in the screening with **Umb-Alloc**. In the pool, some were active as we wanted to know if our model was able to discriminate the active and the inactive catalysts. The selected candidates were **PdA01**, **PdA02**, **PdA03**, **PdA04**, **PdA07**, **PdA08**, **PdA09** and **PdA11**. For each catalyst, the intensity in function of the time was recorded. The Graph 3. 18 summarizes the measured I/I_0 and the Graph 3. 19 summarizes the cell viability of the catalysts incubated alone determined by Presto Blue[®].



Graph 3. 18 Conditions: **Rho(Boc-Alloc)** 50 μM overnight, washing with PBS buffer, cat incubation 20 μM 5 h, Kinetics with $\lambda_{\text{exc}} = 505 \text{ nm}$ and $\lambda_{\text{em}} = 540 \text{ nm}$. The inactive complexes **PdA02**, **PdA03**, **PdA09** and **PdA11** were omitted for clarity. The complete graph is given in the experimental section



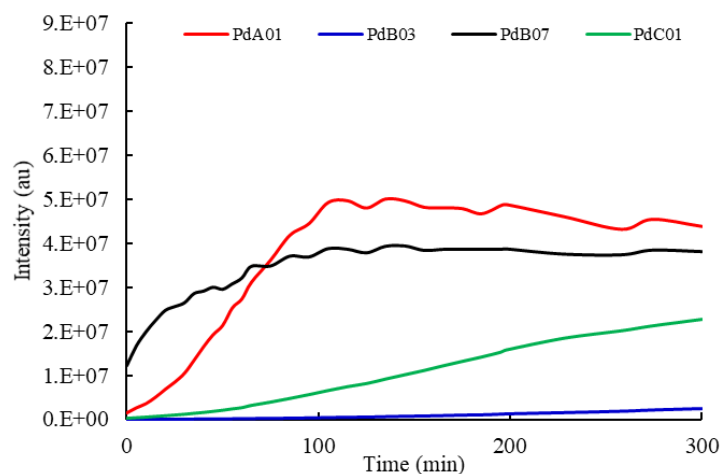
Graph 3. 19 Graph combining the I/I_0 plot of Graph 3. 18 with the Presto Blue obtained for the catalysts alone at 20 μM incubated for 5 hours.

This first screening showed that the catalysts **PdA01**, **PdA04** and **PdA08** showed good activity and **PdA07** was far less active. To compare with the literature,¹²³ **PdA04** exhibited a ratio of 126000 and slightly higher toxicity than **PdA01** (170000). Similarly, **PdA08** (140000) exhibited interesting levels of activity and slightly higher

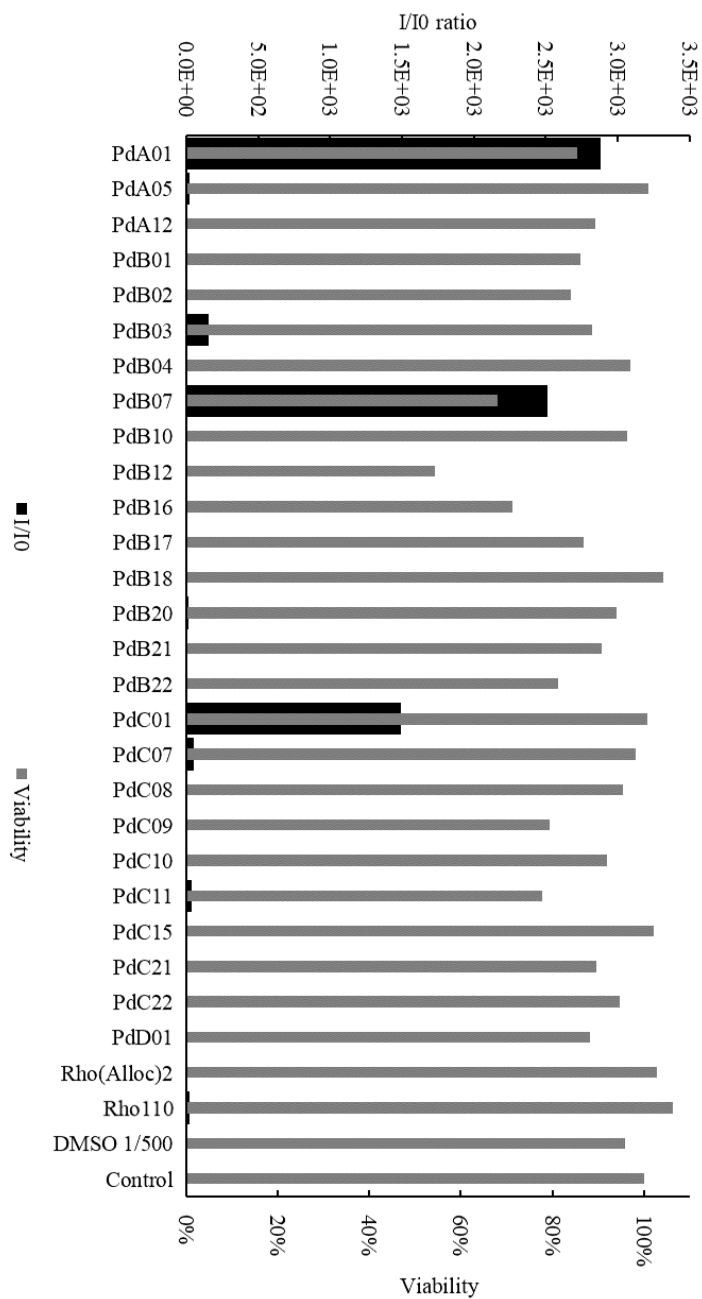
toxicity than our reference. The results showed that we were able to discriminate the activity of the catalysts, but a warning sign was raised due to the toxicity measured by the Presto Blue analysis directly on the plate after reaction in the spectrometer. It is not a good idea as the plate used for the kinetics is not incubated at 37 °C and could be contaminated. The result was taken qualitatively.

3.4.1.3 Second screening

The screening on the rest of the database, composed of the families **PdB**, **PdC** and **PdD**, was performed with 10 μ M to mitigate the toxicity. In this batch, 24 catalysts were tested (**PdA01** was added as a reference) and the measurement of the total intensity in function of the time is plotted on Graph 3. 20.



Graph 3. 20 Conditions: **Rho(Boc-Alloc)** 50 μ M overnight, washing with PBS buffer, cat incubation 10 μ M 5 h, Kinetics with $\lambda_{exc} = 505$ nm and $\lambda_{em} = 540$ nm



Graph 3. 21 Graph combining the I/I_0 plot of Graph 3. 20 with the Presto Blue obtained after the kinetics with the full system.

The second screening aimed to expand the structural range by adding to a few π -allyl palladium complexes (**PdA**, containing **PdA01**

for reference) some palladacycles (**PdB**) and palladium dichlorides (**PdC**) and analogues. The concentration of the complex was lowered to 10 μM to mitigate the toxicity issues.

Two palladacycles, **PdB03** and **PdB03**, showed more modest activities *in vitro* than **PdA01**. Although **PdB07** was moderately active in our *in vitro* test, it was a lot more active *in cellulo*; however, higher toxicity was observed for that catalyst. Although the activity of **PdAC01** was very low *in vitro*, it exhibited an activity of 90% of our best hit, **PdA1**. On contrary, **PdB03** exhibited a very promising activity *in vitro*, it only gave low activity, approximately 1/200 of **PdA01**. Whereas Weissleder showed that $(\text{Fur}_3\text{P})_2\text{PdCl}_2$ (**PdC07**) was an active catalyst in the context of **Rho(Alloc)₂** deprotection while encapsulated in PEG-PGLA polymers,¹ it was found to be inactive in our model system. The two screenings showed us a total of seven hit structures (Figure 3. 9). Among them, the (Allyl)Pd(Xantphos)Cl (**PdA01**) shows the most remarkable activity.

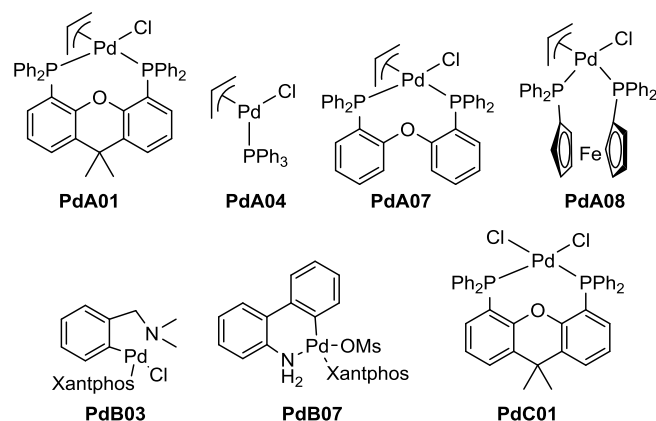
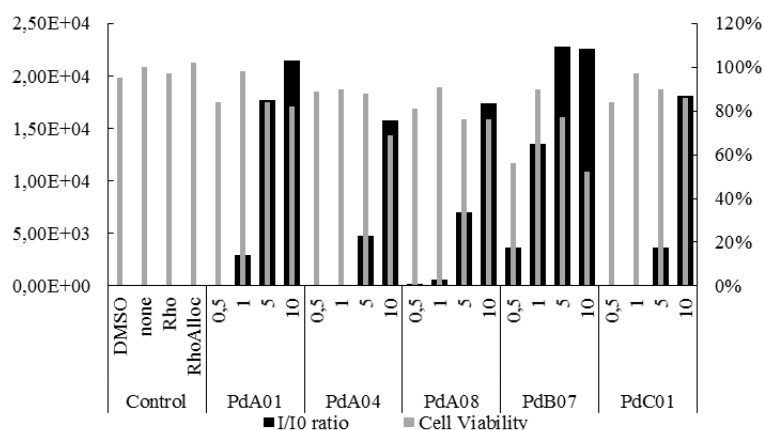


Figure 3. 9 Active catalysts in our *in vitro* screening

3.4.1.4 Optimization

Whereas the previous experiment was a first step towards the discovery of new palladium catalyst structures, further optimization

was needed to mitigate the effects of the toxicity of the catalysts (Graph 3. 22). To maintain enough fluorescence intensity readout, we kept using the same concentration of probe but we reduced the catalyst loading to reduce the toxicity and study the impact on the catalytic performances. The system was applied to our 5 most active catalysts, **PdA01**, **PdA08**, **PdA04**, **PdAC01** and **PdB07**.



Graph 3. 22 Impact on the concentration on the activity and the toxicity for the hit catalysts

Whereas **PdA08**, **PdA04** and **PdAC01** lose all of their activity at or below 1 μM, **PdA01** and also **PdB07** to a larger extent maintain some activity. For each catalyst we studied, we observed a slight drop in viability between 1 and 0.5 μM of catalyst, and we still have no explanation for it. This information brings us some interesting data although we do not have access to the nature of the palladium species present inside the cells, or their oxidation levels. There is certainly a phenomenon of inactivation due to the multiple biological ligands available.

3.4.1.5 Fluorescence microscopy

The last experiment aimed to verify that the fluorescence originated from the inside of the cells by looking at the cells using fluorescence microscopy. Our first guess was that we could use the same protocol as the one used for the fluorimetric assay and simply take a photo at the end of the experiment with the appropriate filters for the rhodamine 110. However, our first experiments showed that the whole image was bright green and the cells were not visible anymore (Figure 3. 10). We were able to wash the cells with PBS buffer and record another image, but we came to the same conclusion, presumably due to the high cellular concentration and strong leaching of **Rho110**.

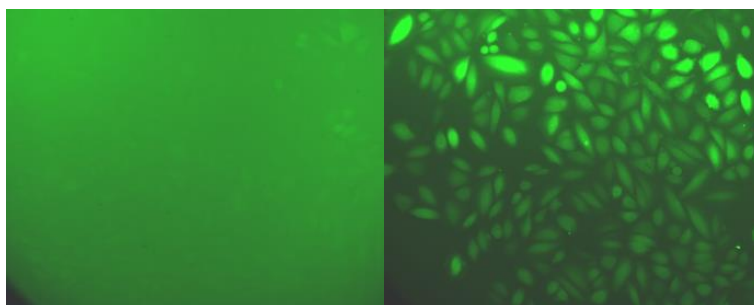


Figure 3. 10 Left: SiHa cells Microscopy (40x) of SiHa cells incubated with **Rho(Alloc)₂** 50 μ M for 18 h, washed with PBS and then incubated with the catalyst **PdA01** 10 μ M for 1 hour. Right: After washing the cells with PBS (total 2 hours)

To avoid those leaching problems was to reduce the quantity of **Rho(Alloc)₂** in the cells by lowering the incubation time from 12 hours previously to 2 hours (Figure 3. 11). After washing the cells with PBS and adding the catalyst **PdA01** at 10 μ M, we were able to see the intense green fluorescence coming from the cells with a very low extracellular background. This result thus confirms that the reaction takes place in an intracellular fashion.

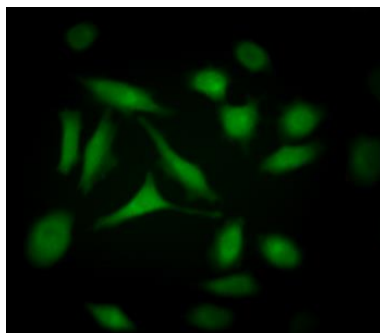
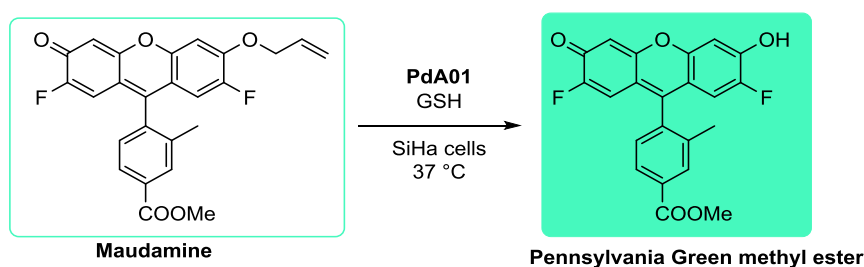


Figure 3. 11 Microscopy (40x) of SiHa cells incubated with **Rho(Alloc)₂** 50 μ M for 2 h, washed with PBS and then incubated with the catalyst **PdA01** 10 μ M for 15 min.

3.4.2 In cellulo fluorogenic assay with Maudamine

In the course of our investigations, we wanted to see if we could localize the fluorescence inside a specific organelle of the cell. We know that it is possible, as Mascareñas^{44, 123} localized the catalyst in the mitochondrion and Bradley⁴⁶ localized the fluorophore in the mitochondrion by appending a triphenylphosphonium cation, with selectivity for the mitochondrion's membranes which are charged.

Our idea was to target the endoplasmic reticulum (ER) taking the template of Pennsylvania Green, which has an affinity for the ER (Scheme 3. 25). **Maudamine** had been previously designed and synthesized (See 2.3.3).



Scheme 3. 25 Fluorogenic decaging of **Maudamine** in SiHa cells

By incubating the SiHa cells with 20 μ M of **Maudamine** for 6 hours, a small fluorescence was observed, and it disappeared rapidly (no image was taken). If the non-internalized fluorophore is washed

with PBS and if 10 μM of catalyst are added, a blue-green fluorescence appears and can be observed inside the cells, a sign of the decaging of the probe inside the cells (Figure 3. 12).

However, it was impossible due to the resolution of the image to determine if the fluorescence emanates from the endoplasmic reticulum or other places. To do so, we would need to co-incubate the cells with an ER tracker, which has a fluorescence in a totally different channel, and superimpose the images taken in both channels on a confocal microscope.

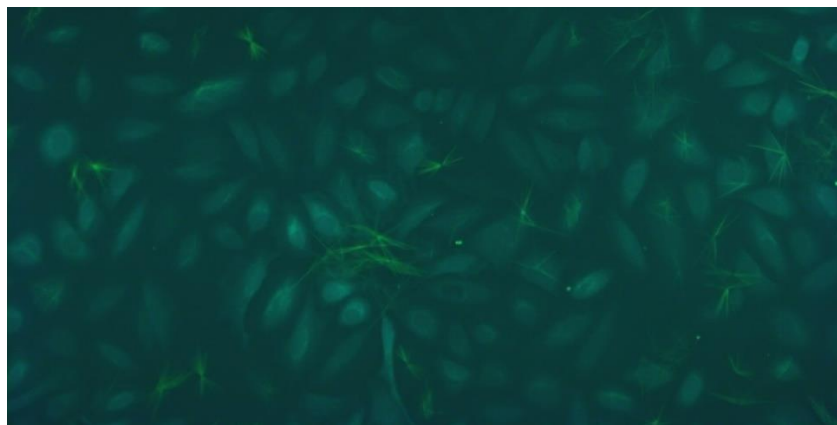


Figure 3. 12 Fluorescence imaging of SiHa cells treated for 6 hours with **Maudamine** (20 μM), washed by PBS and incubated with **PdA01** at 10 μM . Images recorded after 15 minutes.

3.5 Discussion

In this work, we have developed a reliable method to find new metal catalysts active at low concentration in biocompatible conditions and performing bioorthogonal transformations. This method has been successful to discover the second generation of discrete palladium complexes active in the bioorthogonal deprotection of allyl carbamates.

The method was organized in two steps: (i) *in vitro* screening of a large complex library on a fluorogenic model reaction and (ii) *in*

cellulo validation on a fluorogenic model. To verify the bioorthogonality of the method, some additional tests were performed such as a viability assay (Presto Blue) and a microscopy experiment to verify that the release is intracellular. At our level, we considered complexes (i) able to trigger a fluorescence increase, (ii) not inducing significant toxicity after 5 hours of reaction and (iii) inducing a fluorescence evolution from inside the cells as hit structures.

The efficacy of a catalyst on a given transformation is the product of two factors, namely (i) the activation and (ii) the catalytic activity itself.

- For the activity, we determined that the critical factor was the bite angle, for which a compromise must be made to avoid hampering the oxidative addition or the reductive elimination. An optimum was found around 110 °, which is considered as large. It suggests that the reductive elimination is probably the rate determinant step in the whole process.

Furthermore, the detailed mathematical analysis of the curves obtained during the optimization showed that the π -allyl palladium amide reductive elimination and the departure of the leaving group are competitive pathways. Higher glutathione concentrations thus prevent the formation of allylated side-product and favour the release of the probe. Whereas it was not directly observed, the allylated side-product tends to form in high catalyst concentrations and in low glutathione concentrations.

- The second critical point is the activation mode. Unlike ruthenium catalysts used by Meggers, which are bench stable in the form of Ru (II) for the early generation³⁸⁻³⁹, it needs an activation step to

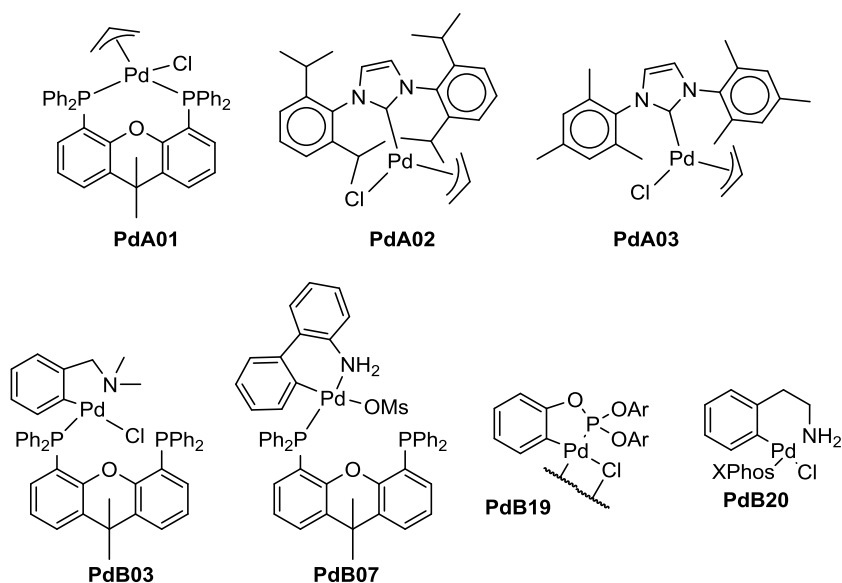
reach the level Pd (0) to start the reaction from a Pd (II) precatalyst. It is a specificity of Tsuji-Trost reaction to require Pd (0) active species for allyl carbamate probes.

From our results and the literature, we found out that π -allyl palladium chlorides (**PdA01**) activate quickly in presence of nucleophiles such as GSH and slowly in absence.

Buchwald palladacycles (**PdB07**) activate promptly in presence of the buffer and deactivate if not immediately mixed with the substrate.

Palladium dichlorides (**PdC01**) do not activate with just GSH and the buffer, leading to poor performances *in vitro*, but performed very well *in cellulo*. This is probably the consequence of an *in cellulo* bioactivation.

For some other active complexes, the activity has not been totally rationalized.



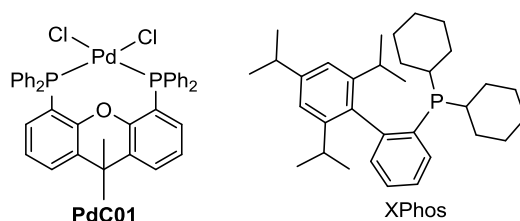


Figure 3. 13 Hit complexes found in the *in vitro* screening

For the biological assays, several other key factors played a role, such as (i) the toxicity, (ii) the cell-permeability and (iii) the solubility. Although we did not study in detail the last two parameters, we measured the toxicity by Presto Blue[®] assay and could bring some rationalization in some cases.

- At first, the marked toxicity of **PdB07** can be rationalized by the carbazole ligand which is released; this scaffold has been employed several times as DNA intercalant for drugs such as Ellipticin.¹⁴⁴
- In the case of **PdA01**, the toxicity can be attributed to the electrophilic character of the π -allyl, which can allylate non-specific nucleophilic residues.
- Then, the complex **PdAC01** does not exhibit noticeable toxicity although it has the same active species as the two former examples. It tends to rule out toxicity coming from the active species itself.

•

**Second part: New applications of Tsuji Trost
reaction in bioorthogonal prodrug decaging**
**Third chapter: Development of a prodrug
targeting cells expressing folate receptor and
activated by biocompatible palladium catalysis**

4 Third chapter: Development of a prodrug targeting cells expressing folate receptor and activated by biocompatible palladium catalysis

This work was performed in the team of Prof Sébastien Papot (Université de Poitiers) with the help of Dr Brigitte Renoux and Dr Mikael Thomas for the chemical part, as well as Dr Jonathan Clarhaut and Anaïs Rougier for the biological assay.

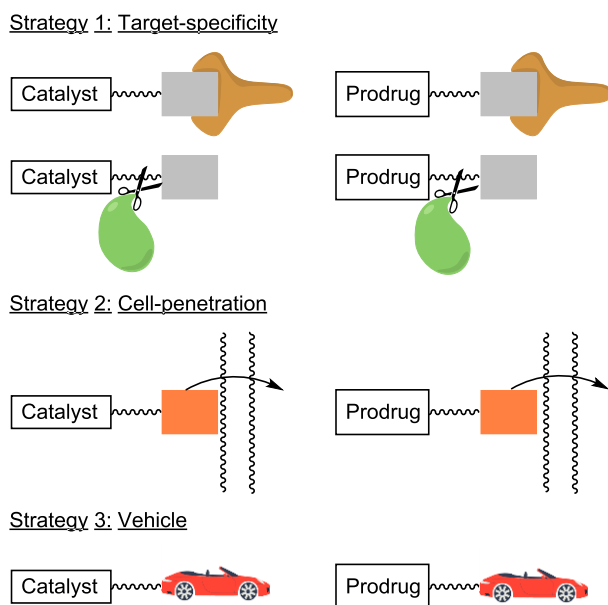
4.1 Introduction

After we found some active catalysts meeting the capable of bioorthogonal decaging of allyl carbamates *in cellulose*, we wanted to get one step closer to our therapeutical goal. Our current systems, prodrug and catalyst, do not have any selectivity and cannot recognize cancer cells in any way.

4.1.1 Strategies in vectorization

Our response to achieve this goal is to use vectorization. There are mostly three approaches in vectorization (Scheme 3. 26), and each of them has been seen in literature reports in bioorganometallic catalysis.

- It can enable a prodrug or a catalyst to reach a specific target, such as a specific receptor, to control the localization of the prodrug.⁴⁴
- Then, the addition of a cell-penetrating moiety can increase the intracellular concentration and increase the efficacy of the system.^{42, 50}
- Finally, a vehicle can help the active principle to its desired location.¹



Scheme 3. 26 Strategies in vectorization

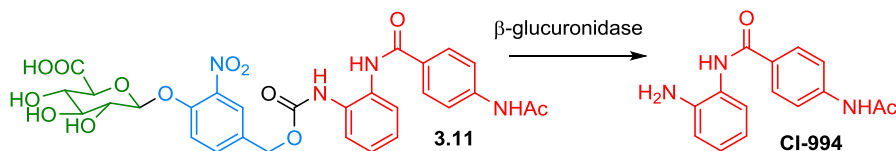
We have focused our efforts to apply this strategy to the prodrug first, although we also attempted to apply it on catalysts.

4.1.2 Previous work on the topic by Papot

The work described in this section is a collaboration work between our team and the team of Pr. Papot from Université de Poitiers and his co-workers. To better understand the design of the prodrug of this chapter, we will review literature examples from Papot describing all the prodrug parts from which we took inspiration to design ours.

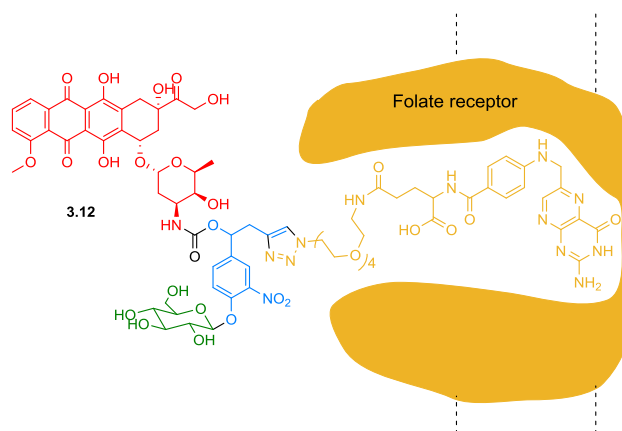
As a key part to all the prodrugs developed in his team, the core unit makes the link between the trigger and the payload. Optionally, an additional unit can be grafted to the core for recognition purpose. In 2008, he reported the use of 3-nitro-4-hydroxybenzyl alcohol unit as an efficient central unit in the activation of a CI-994 prodrug **3.11** (Scheme 3. 27).¹⁴⁵ The key point is the *m*-

nitro group which lowers the pKa of the phenol to 7 because only the phenolate is able to trigger the elimination.



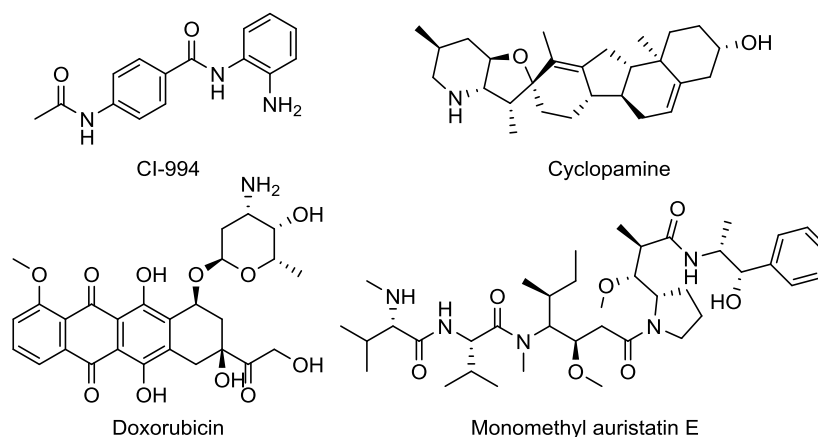
*Scheme 3. 27 Release of **CI-994** by β -glucuronidase-mediated cleavage of a glucuronide trigger. The central unit is represented in blue.*

In 2011, Papot reported that by modifying the benzylic position of the central unit by a propargyl group, it was possible to click a folic acid flanked by a PEG chain terminated by an azide function (Scheme 3. 28).¹⁴⁶ This enables the prodrug to recognize the folic acid receptor, which is known to be expressed at the cellular surface of many cancer types (Scheme 3. 28). Due to the large size of the prodrug **3.12** coupled to a high hydrophilicity coming from the linker, it cannot penetrate by passive diffusion but rather undergoes endocytosis after binding the receptor and follows the endosomal path. Therefore, the prodrug cannot penetrate in cells which do not express the folate receptor.



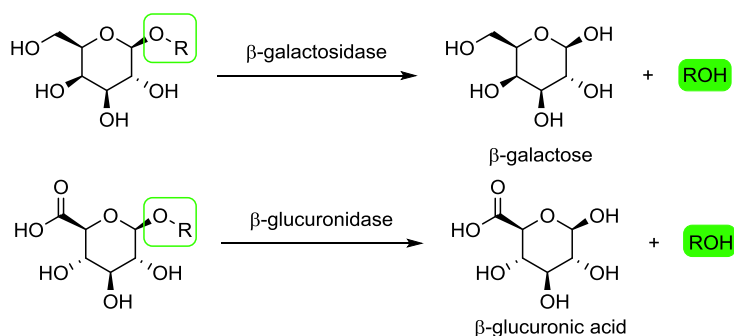
Scheme 3. 28 Folate-receptor targeted prodrug concept

Several drugs have been used by Papot and co-workers (Scheme 3. 29), such as CI-994¹⁴⁵, Cyclopamine¹⁴⁷, Doxorubicin¹⁴⁸⁻¹⁴⁹ (Dox) and Monomethyl Auristatin E (MMAE).¹⁵⁰⁻¹⁵² The last two feature some interesting properties making them desirable such as a nanomolar (Dox) to picomolar (MMAE) IC_{50} and a good solubility of the prodrugs. Moreover, the on-off ratio is quite large as the prodrugs have an IC_{50} several orders of magnitude higher than their parent drugs. Interestingly, Doxorubicin is fluorescent and its release is fluorogenic.



Scheme 3. 29 Drugs employed by Papot

For the trigger part, sugars such as galactose and glucuronic acid have been employed by Papot (Scheme 3. 30). Each sugar can be cleaved by its own enzyme, β -galactosidase or β -glucuronidase.

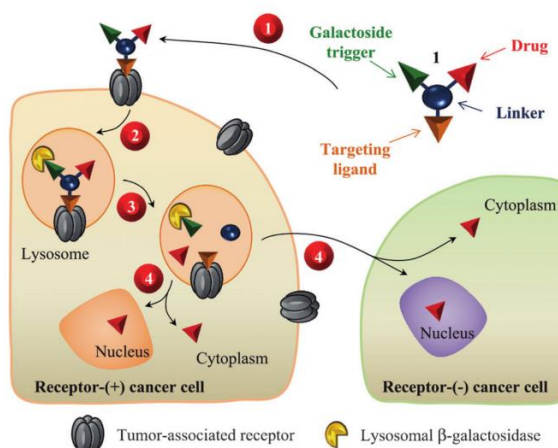


Scheme 3. 30 β -galactoside and β -glucuronide trigger

- In the case of β -glucuronidase,¹⁴⁵ it is known that only cancer cells excrete large amounts of the enzyme in the extracellular medium, whereas healthy cells do not, and the enzyme remains exclusively intracellular. Glucuronide triggers are therefore useful for extracellular activation of non-cell permeable prodrugs and cannot be coupled with folic acid as there is no β -glucuronidase in early endosomes.

- Regarding β -galactosidase, it is ubiquitously present in healthy and cancer cells in early endosomes.¹⁵² It therefore ensures the release of the drug in a strict intracellular fashion. It is compatible with folic acid targeting.

The galactoside prodrug **3.12** with folic acid and a MMAE payload has been validated *in cellulo* and *in vivo* thanks to its excellent selectivity for cells expressing the folate receptor (FR+) (Scheme 3. 31).¹⁵² The prodrug exhibited an IC_{50} of 240 pM, comparable to **MMAE** in cells expressing the folate receptor, whereas an IC_{50} around 200 nM was observed for cells which do not express the folate receptor (**MMAE** has an IC_{50} of 800 pM for the same cell line).

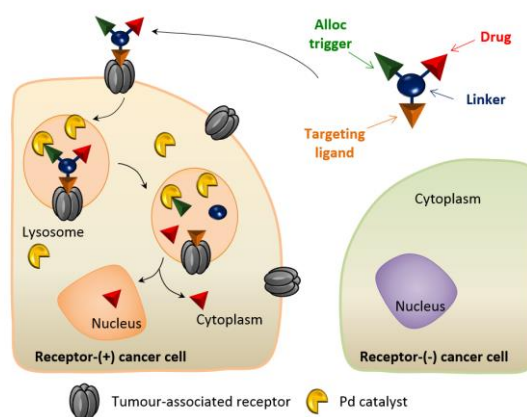


Scheme 3. 31 Principle of the programmed molecular system; 1) binding onto folic acid receptor and 2) entry into the lysosomes after which 3) the trigger is cleaved by β -galactosidase. 4) The cytotoxic drug makes its way to the nucleus and can act on other neighbour cancer cells which do not express the folic acid receptor. From ¹⁵³

4.2 Goals of this chapter

The goal of this chapter is to show that selectivity can be achieved by vectorising the drug and the catalyst (Scheme 3. 32). In a first part, we focused our efforts to design, synthesize and test in

cellulo a molecular programmed system triggered by palladium catalysis and targeted thanks to folic acid. In the second part, we tried to develop a vectorised version of a palladium catalyst.



Scheme 3.32 Goals of this chapter

4.3 Results

4.3.1 Prodrug vectorization

4.3.1.1 First generation of prodrug

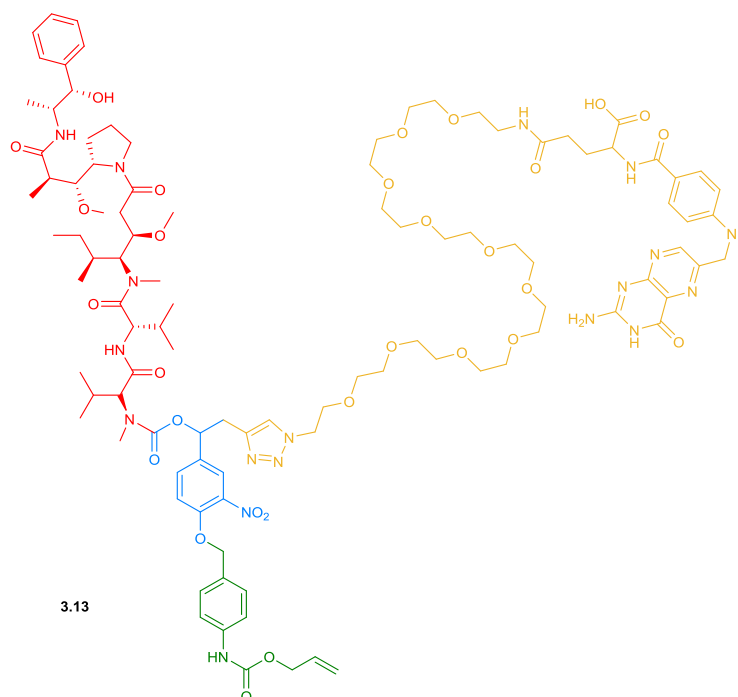
a) Design

Our prodrug aimed to combine the speciality of Papot's group and ours (Scheme 3.33).¹⁵²

- Regarding the North part, we employed the 3-nitro-4-hydroxybenzyl alcohol used by Papot without modification.
- As cytotoxic, we chose the monomethyl auristatin E for its IC_{50} in the nanomolar range. It is important to have a very potent cytotoxic as even small amounts released would trigger an important biological response which we need to be able to measure.
- For the recognition, we used the folic acid bound to the central unit by a PEG. However, due to the extreme insolubility of folic

acid derivatives, we decided to increase the PEG length from 4 units in the previous work of Papot to 10 units.

- For the trigger, we could not simply make an allyl carbonate on the phenolic site of the trigger, as carbonates are prone to nonspecific hydrolysis, on contrary to carbamates. Therefore, we needed to extend that Southern part with an (N,O) linker in order to present a free amine to make the Alloc trigger. As described in Paragraph 2.3.1 of Chapter 1 of this part, we chose 4-aminobenzyl alcohol.



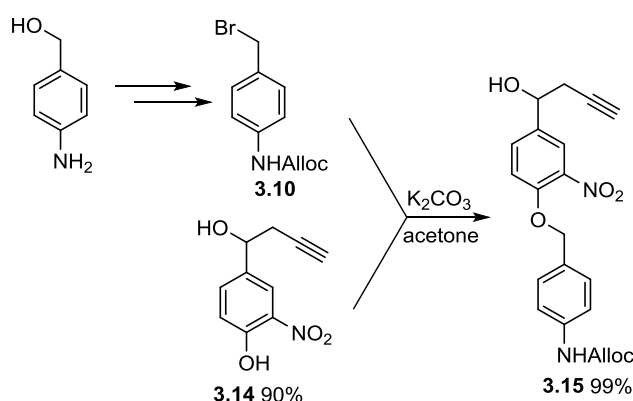
*Scheme 3. 33 Design of the first generation of palladium-activated prodrug **3.13** targeting folate receptor*

b) Synthesis

The synthesis of the South fragment was very straightforward, as it employs the bromo intermediate **3.10** used for the umbelliferon prodrug. The synthesis of the North fragment has been well described

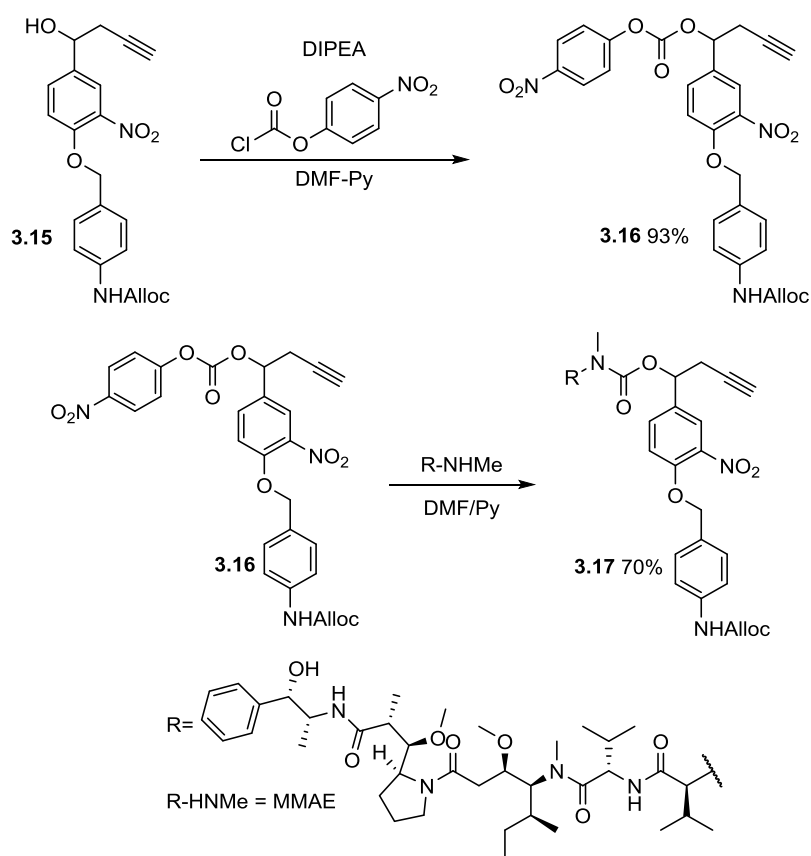
by Papot previously.¹⁵⁴ We repeated the synthesis to get the intermediate **3.14** prior to the coupling with the trigger part **3.10**. Then, the **MMAE** was coupled, followed by the PEG-folic acid **3.18**.

At first, **3.14** was reacted with the bromide **3.10** in acetone to give the compound **3.15** in nearly quantitative yield, with the bromide **3.10** in a slight defect (Scheme 3. 34). The selectivity was complete for the phenolic functionalization.



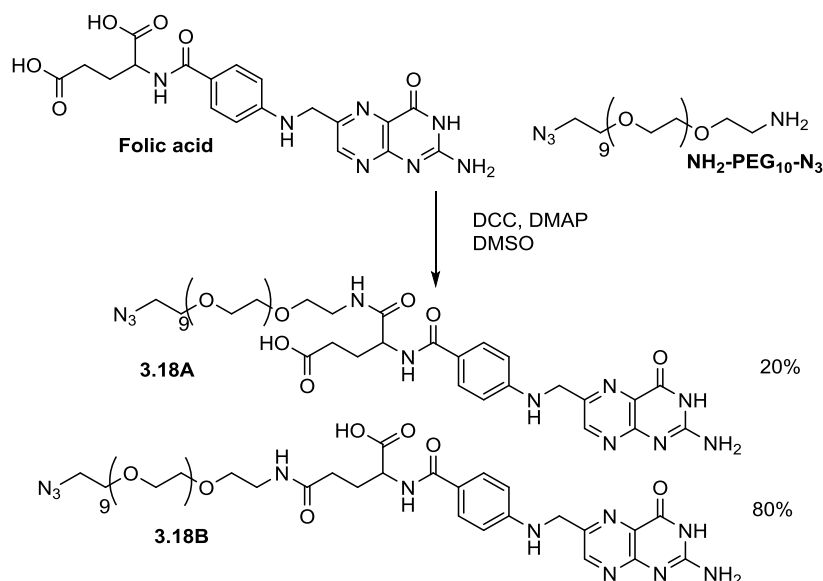
Scheme 3. 34 Mounting of the trigger and central unit. The Trigger part had been synthesized before

The coupling with **MMAE** was carried out in two steps (Scheme 3. 35). At first, the benzylic alcohol **3.15** was treated by 4-nitrophenyl chloroformate in presence of DIPEA to form the mixed carbonate **3.16**. The **MMAE** was coupled in DCM/Py 8:2 with DIPEA. The reaction was monitored by HPLC and upon completion, the solvent was evaporated and the compound **3.17** was purified by flash chromatography. Care must be taken while handling the **MMAE**, but also all the intermediates containing **MMAE**, due to their toxicity.



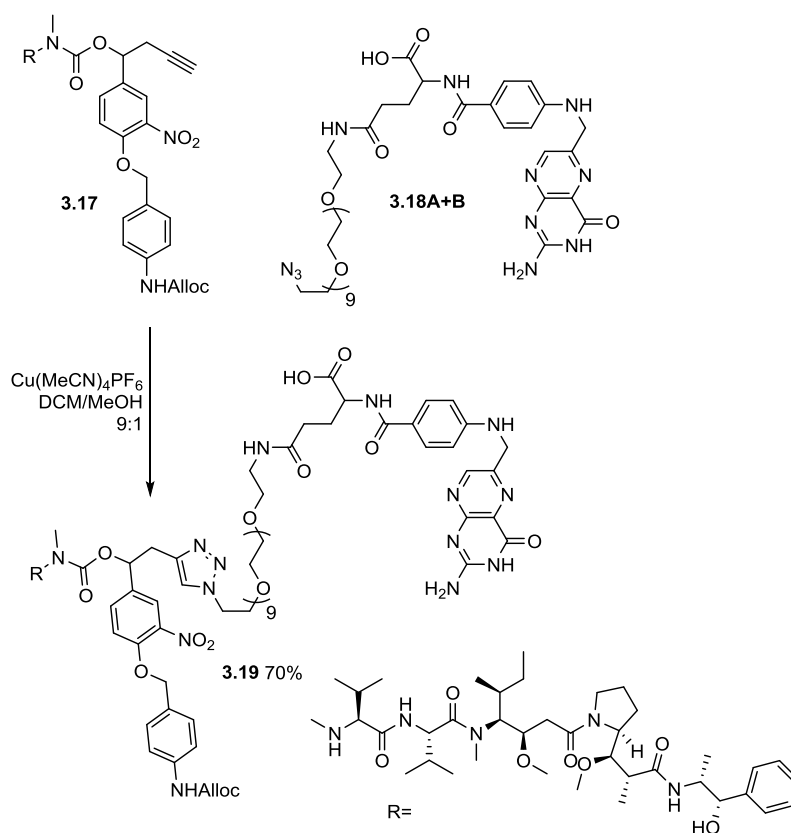
Scheme 3.35 Coupling with the **MMAE** payload

The N₃-PEG-10-Folic-NH₂ **3.18** was prepared by Dr Mikael Thomas by coupling folic acid to the PEG derivative with DCC and DMAP to obtain **3.18B** and **3.18A** in ratio 8:2. Despite the purification by reverse phase column chromatography, the mixture **3.18A+B** could not be separated as was used as such (Scheme 3.36).



Scheme 3.36 Synthesis of the targeting part. Two isomers were obtained

The folic acid arm **3.18** and the central unit **3.17** were coupled together with CuAAC cycloaddition (Scheme 3.37). When a slight defect of **3.18** is used, it ends up fully consumed. Otherwise, it is very difficult to separate it from the prodrug **3.19** by other means than preparative HPLC. The copper was removed by treatment of the reaction mixture with a copper-chelating resin. The prodrug was purified by normal phase chromatography, which was surprising for folic acid derivatives. The pure prodrug **3.19** in the form of a yellow rubber-like solid. Although the ^1H -NMR in DMSO was hard to analyse, the HPLC indicated that the product was pure and the HRMS indicated the expected mass.



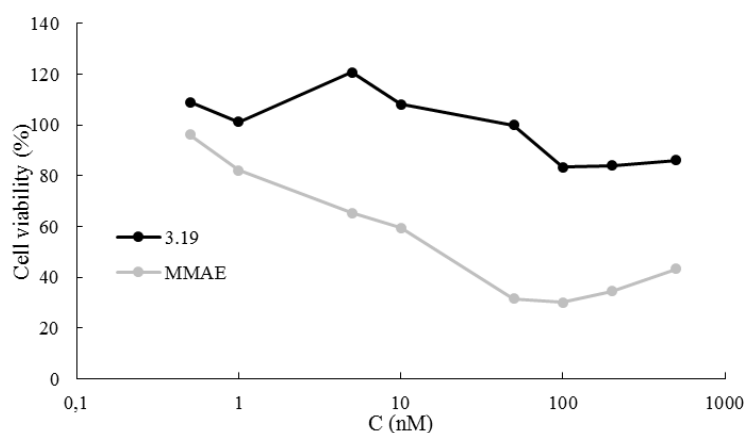
Scheme 3.37 Click of the targeting arm to the central unit by copper catalysis

c) *In cellulo* testing

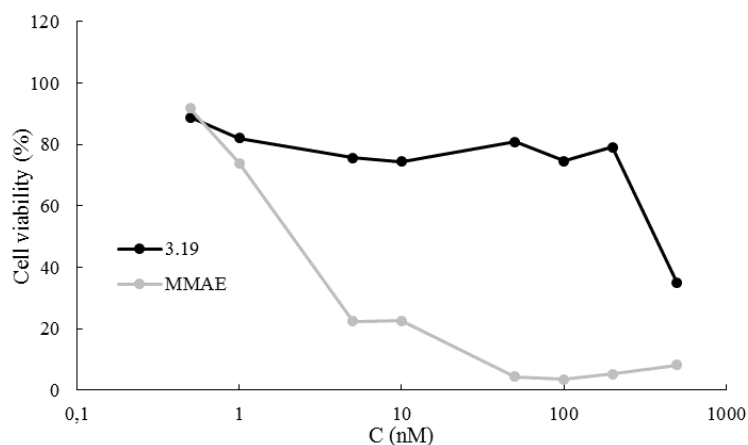
The *in cellulo* evaluation was carried out in Poitiers by Dr Jonathan Clarhaut and Anaïs Rougier in the biology laboratory of IC2MP.

As we noticed that those palladium catalysts were inducing toxicity on the cells while incubated alone, the first step was to measure the toxicity of **PdA01**, which was our chosen catalyst for this application, on the two cell lines (A549 and KB) and for 72 h, which is the time of the uncaging experiment. The maximum concentration we found was 2 μM of **PdA01**.

The second step was to verify if the prodrug **3.19** is significantly less toxic than the **MMAE**. It is particularly important to identify a concentration range at which the drug leads to total cell mortality and the prodrug does not induce cell mortality. Compared to the glucuronide prodrug of Papot¹⁵⁵, our prodrug is by far less toxic. This can be turned to our advantage as higher doses of prodrug **3.19** can be used and more **MMAE** can thus be released for a better efficiency. We noted a higher sensitivity to **MMAE** in KB cells (FR+, Graph 3. 24) rather than A549 cells (FR-, Graph 3. 23). Whereas KB cells show sensitivity to the prodrug 2.28 above 500 nM, A549 cells do not as they cannot internalize the prodrug as they do not express the folate receptor.



Graph 3. 23 Dose-toxicity of **3.19** and **MMAE** on A549 cells after 72 h of incubation

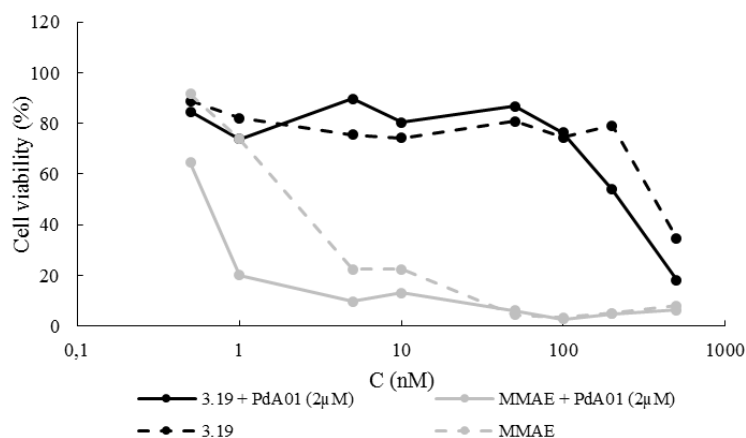


Graph 3. 24 Dose-toxicity of **3.19** and **MMAE** on KB cells after 72 h of incubation

After we found a broad concentration range where **MMAE** leads to total cell death and **3.19** does not show toxicity, we were able to proceed to the catalytic test. The protocol was:

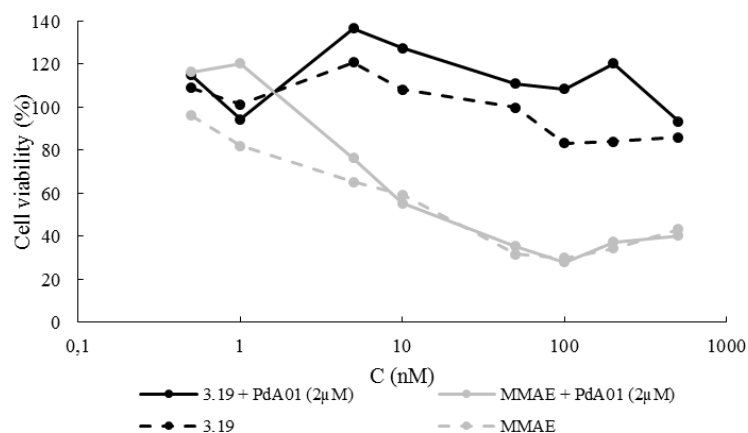
- Incubation of the cells A549 (FR-) and KB cells (FR+) for 1 h with the prodrug **3.19**.
- Washing with PBS.
- Incubation for 72 hours with the catalyst (500 nM, 1 μ M or 2 μ M).
The time of 72 h corresponds to a cell cycle and is mandatory to see the effect of **MMAE**.
- Measure of the cell viability with a Presto Blue analysis.

In KB cells (FR+) (Graph 3. 25), **MMAE** exhibits an IC_{50} around 5 nM whereas the prodrug has an IC_{50} above 200 μ M. A slight increase in toxicity was noted when **PdA01** was added to **MMAE**. However, no increase of toxicity of the prodrug **3.19** was observed in presence of **PdA01**.



Graph 3. 25 Testing of the catalytic system with the prodrug with **PdA01** (2 μ M) on KB cells (FR+) for 72 hours.

In the case of FR- cells (Graph 3. 26), no toxicity of the prodrug **3.19** observed, and the IC_{50} of **MMAE** was a bit higher, reaching 10 μ M. Again, no impact of **PdA01** was observed, and no toxicity was obtained.



Graph 3. 26 Testing of the catalytic system with the prodrug with **PdA01** (2 μ M) on A549 cells (FR-) for 72 hours.

At this stage, we noticed that the **MMAE** release did not take place in concentrations leading to a biological effect. The only phenomenon observed is nonspecific toxicity of the probe at a high

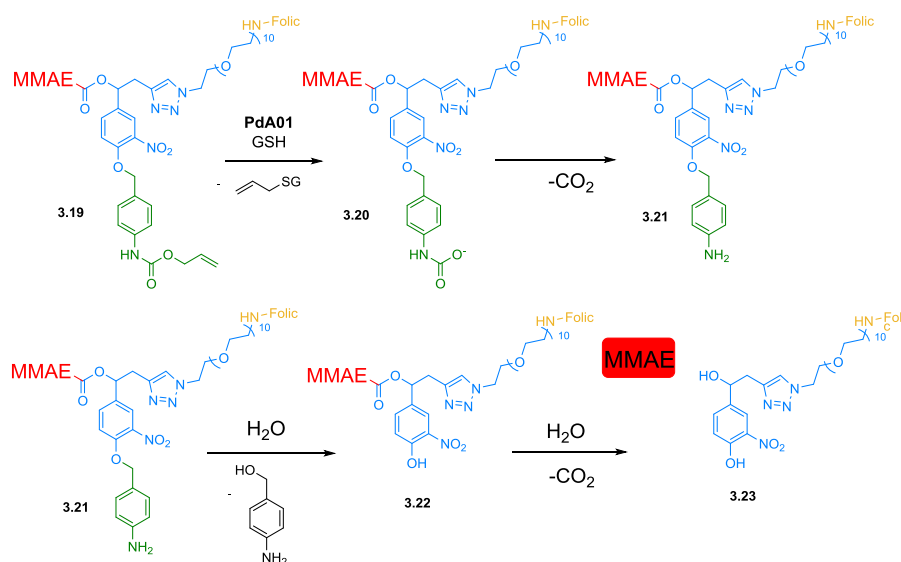
concentration on FR+ cells, whereas no toxicity was seen on FR-cells. At this stage, we considered three potential hypotheses.

- The first hypothesis is that the catalyst concentration was not sufficient. In the light of the optimization carried at the end of the previous chapter, it is possible that 5 to 10 μ M of **PdA01** would have been better. However, increasing the catalyst loading is only possible when the toxicity of the catalyst is low enough, which is not the case for **PdA01**.
- The second hypothesis is about the protocol itself. On contrary to the glucuronide prodrug of Papot¹⁵⁵, the prodrug was incubated for 72 h and the cells were not washed during the experiment. In our case, we first incubated the prodrug for 1 hour, and the wells were washed by PBS prior to the incubation with the catalyst for 72 h. The major problem of this method is that if not all the prodrug penetrates inside the cells and is washed away, it will reduce the amount of prodrug available for the catalysis.
- The third hypothesis was a dysfunction in the elimination of the prodrug. Mechanistically, it is unlikely that one of the intermediates fails to eliminate as either the Northern or the Southern part have been tested separately. However, the most probable cause is an insoluble intermediate which blocks the whole elimination process.

d) *In vitro* testing

To verify at which stage, either the catalysis or the self-immolation, the problem was located, we performed an *in vitro* HPLC-MS study of the release *in vitro*. There is no other choice of method as **MMAE** is neither visible by HPLC with a UV detector nor

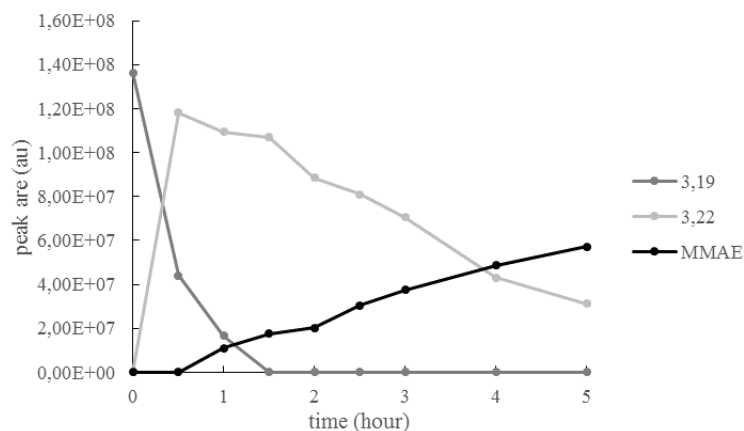
by TLC. We launched the reaction at 10 μM of **3.19**, 10 μM of **PdA01** and 100 μM of GSH and 10% of DMSO for solubility and analysed it in HPLC-MS (Scheme 3. 38).



Scheme 3. 38 Activation and elimination of the prodrug

We noticed that in these conditions, the prodrug **3.19** had disappeared completely after 1 h 30, so very fast compared to our *in vitro* experiments due to the high catalyst and GSH loading. However, a product whose mass corresponds to the phenolic intermediate **3.22** started to accumulate and decomposed slowly, giving **MMAE**. The product **3.23** was never observed. Two theories co-exist.

- The phenol **3.22** precipitates and does not eliminate. It must be reminded that this intermediate had never been seen in Papot's work, as a PEG 4 was always employed.
- The problem could come from the aniline **3.21**, the intermediate before the phenol **3.22**, which precipitates and does not eliminate. In that case, the elimination could take place in the analyser of the mass spectrometer.



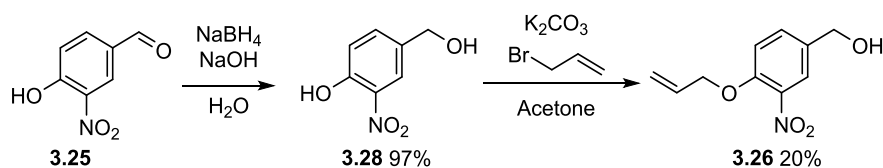
Graph 3. 27 HPLC MS analysis of the decomposition of the prodrug **3.19** after activation by **PdA01** (100 mol%) and GSH (10 equiv.) and 10% DMSO

Therefore, we designed a new generation of prodrugs to try to solve the aforementioned problems.

4.3.1.2 Second generation of prodrug

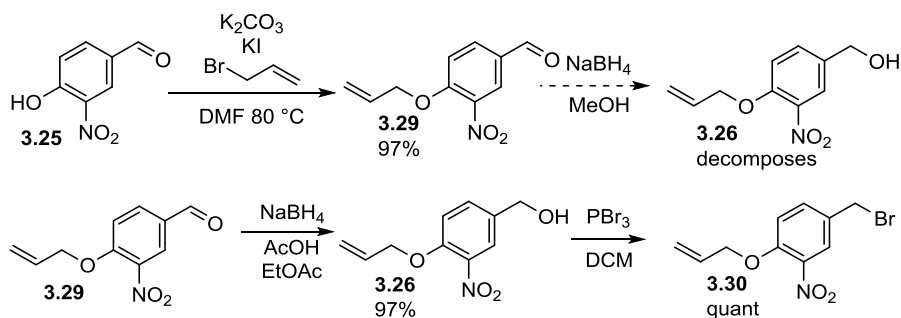
a) Design

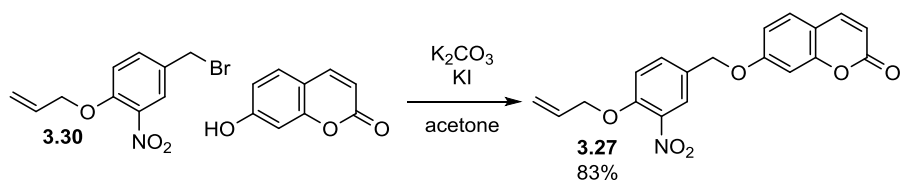
In this second-generation prodrug, the goal was to avoid going through an aniline in the elimination mechanism and use a shorter PEG, such as the PEG-4 used in Papot's previous work.¹⁵² Literature reports indicate that allylic, allenic, propargylic phenols could be deprotected by palladium catalysis.⁴⁹ One direct idea would require to shorten the trigger part and limit it to a simple allyl group attached to the phenol of the Northern part (Scheme 3. 39).



Scheme 3. 41 First route: reduction of the aldehyde 2.22 followed by allylation

Improvement was found by allylating the starting aldehyde **3.25** using K_2CO_3 in DMF at 80 °C and with a catalytic amount of KI as catalyst (Scheme 3. 42). However, the reduction of **3.29** using NaBH_4 in methanol leads to complete degradation of the product due to $\text{S}_{\text{N}}2$ or $\text{S}_{\text{N}}2'$ attack of oxyanions on the allyl group. We, therefore, had to find a way to reduce the aldehyde in acidic conditions. The reagent $\text{NaBH}(\text{OAc})_3$ was generated *in-situ* by treating NaBH_4 by AcOH in ethyl acetate. Once generated, **3.29** was added to the $\text{NaBH}(\text{OAc})_3$ solution, and **3.26** was obtained in these conditions. The rest of the prodrug **3.27** was mounted according to the standard procedure by brominating the benzylic alcohol **3.26** and then reaction with umbelliferon and K_2CO_3 in acetone to give the compound **3.27**.

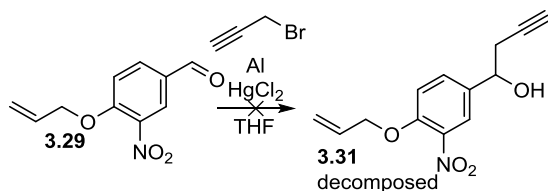




Scheme 3.42 Second route: Alkylation followed by a reduction in acidic conditions.

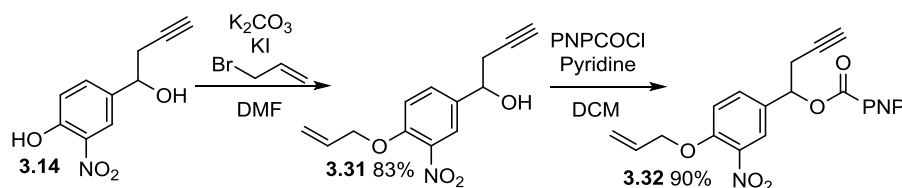
c) Synthesis of the vectorised prodrug

The same strategy as for the first generation of prodrug was applied. At first, we attempted to treat **3.29** with *tris*-propargyl aluminium to give **3.31** in a direct way, but it decomposed (Scheme 3.43).



Scheme 3.43 Direct synthesis of **3.31** by nucleophilic propargylation of **3.29**

Instead, we had to follow the other way and allylate the phenol **3.14** with a stoichiometric amount of allyl bromide and a catalytic amount of KI . The secondary alcohol **3.31** was then activated with PNP-chloroformate to get the activated derivative **3.32**. (Scheme 3.44). The coupling of **MMAE** and the click with folic acid have not been performed yet but are in our plans. The biological assay will be performed according to the same methodology.



Scheme 3. 44 Synthesis of the second-generation prodrug. Trigger, central unit and activation.

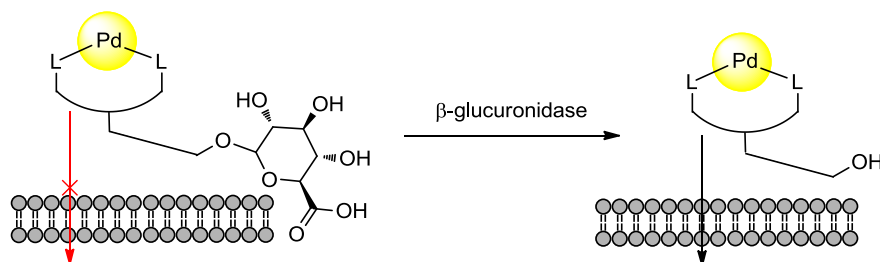
4.3.2 Catalyst vectorization

Keeping in mind the general objective in this chapter, after we focused on the prodrug, we will now focus on the vectorization of the catalyst to ensure a selectivity in the distribution for possible *in cellulo* or *in vivo* applications (Scheme 3. 46).

4.3.2.1 Strategy

A vectorised prodrug and a vectorised catalyst would increase the selectivity one step further (Scheme 3. 35).

- In one hand, the prodrug with folic acid would only recognize cells expressing the folate receptor and would be internalized through the lysosomal path.
- In another hand, a vectorised catalyst with a glucuronic acid would recognize the tumoral microenvironment. Glucuronylated compounds are too polar to be internalized and would remain extracellular unless the glucuronic acid is cleaved. At this stage, it would be able to penetrate by passive diffusion in the cells to activate the prodrug

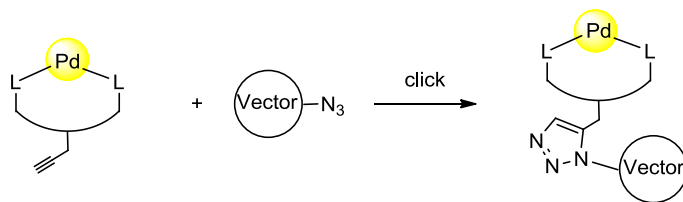


Scheme 3.45 Vectorization of a catalyst by glucuronic acid: strategy

Since both the ligand and the glucuronide part have limited possibilities in terms of variation, we had to focus on a convergent synthesis involving a coupling step at the end. The strategy we chose is CuAAC. However, due to some constraints, we had to consider only one way to design the catalyst.

- At first, phosphines are not compatible with azides, so the ligand cannot be derivatized with an azide, but only by the propargyl.
- Then, as the glucuronic acid is in its protected form, it can only be deprotected in the last step as the product would become insoluble in organic solvents, but only in water. Also, palladium complexes are probably not compatible with the basic treatment to deprotect the glucuronide. Therefore, it needs to be deprotected prior to the click.

As a direct effect, it means that the propargyl needs to be located on the ligand part and that the click has to be performed on the palladium complex and not the ligand only.



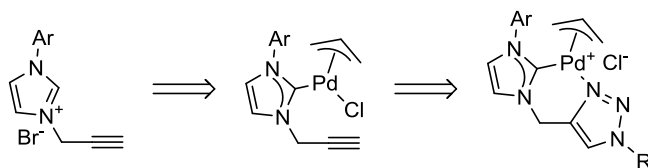
Scheme 3. 46 Concept of a clickable catalyst

To the best of our knowledge, a “ready to click” biphosphine ligand has never been described. One example described by Reeks involve a monophosphine obtained by propargylation of PPh_2Cl .¹⁵⁶ Few examples of clickable nitrogenated bidentate ligands have been released, but the π -allyl palladium complexes were found to be inactive in catalysis.

4.3.2.2 Synthesis of clickable catalysts

a) NHC propargyl

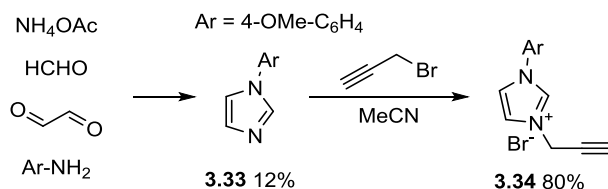
Our first idea, as we knew that NHC were valuable ligands, was to use them for vectorization. NHC have strong binding energy due to the bonding and backbonding. Our idea was based on the design of Elsevier, who reported a family of NHC-allyl palladium featuring a triple bond, which is the easiest anchor for a copper-catalysed cycloaddition with an azide (Scheme 3. 47).¹⁵⁷



Scheme 3. 47 Elsevier's Clickable NHC allyl palladium

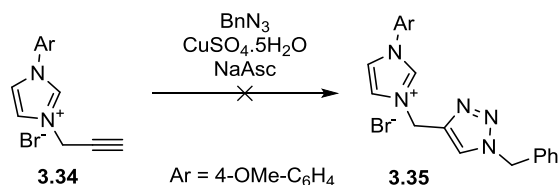
Mounting the imidazole **3.33** from HCHO , NH_4OAc and anisidine was a straightforward synthesis (Scheme 3. 48).¹⁵⁷ The triple bond was mounted in one step by propargylation the second nitrogen of **3.33** with propargyl bromide to give the salt **3.34**. The difficulty in

this synthesis is to mount the palladium complex without affecting the triple bond.



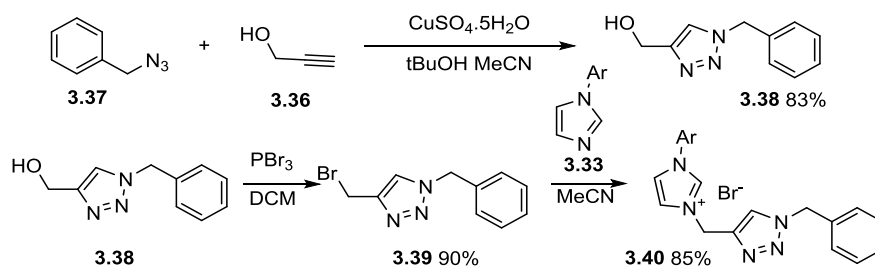
Scheme 3. 48 Elsevier's NHC synthesis

At first, we wanted to test the activity of the final complex without carrying out the click reaction on the complex itself, but rather on the ligand only (Scheme 3. 49). The ligand **3.34** was treated by $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in MeCN and with sodium ascorbate and a model azide, BnN_3 . However, the desired product **3.35** was not detected.¹⁵⁷



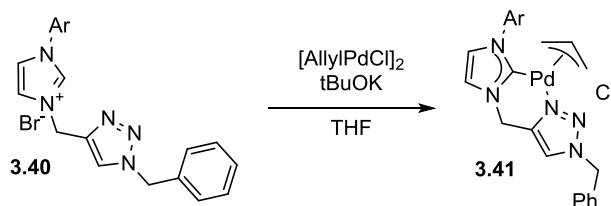
Scheme 3. 49 First strategy: attempt to click BnN_3 on the ligand

We attempted to mount the ligand **3.35** in the other way (Scheme 3. 50). We started to click propargylic alcohol **3.36** and benzyl azide **3.37** with $\text{Cu}(\text{OAc})_2$ in $t\text{BuOH}$, and **3.38** was obtained in the form of a white powder.¹⁵⁸ After bromination with PBr_3 to form **3.39**, it was successfully coupled to the imidazole **3.33** and gave **3.40**.



Scheme 3. 50 Second strategy: Attempt to click first and then attach to the imidazole

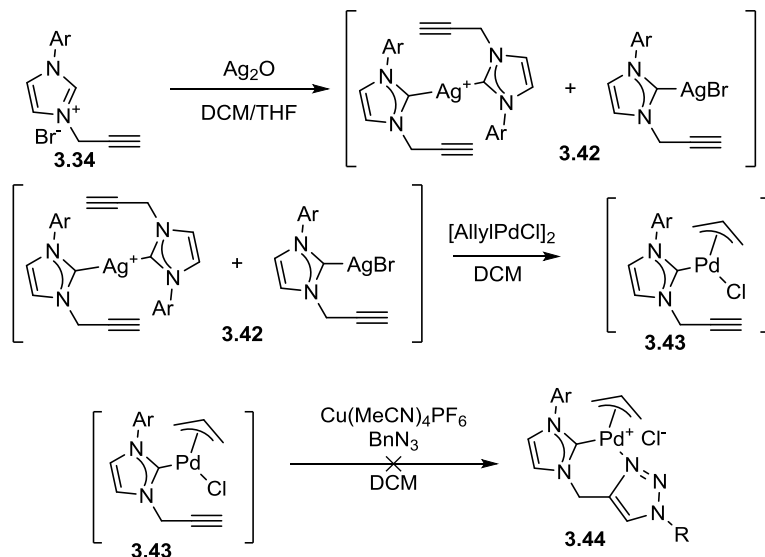
The palladium complex **3.41** was synthesised by classical methods of the literature by deprotonation of the NHC salt **3.40** and treatment by the palladium source (Scheme 3. 51). Although the complex **3.41** was isolated, it decomposed promptly after being isolated.



Scheme 3. 51 Synthesis of the palladium complex **3.41** from the already clicked ligand

Then, the synthesis of **3.41** was attempted in the other order, with the click at the last stage as it is required for our design. The palladium complex **3.43** was synthesized from the propargyl NHC according to classical procedures of the NHC chemistry (Scheme 3. 52) but had to be carried out under Ar as the complexes are unstable.¹⁵⁷ The propargylic NHC **3.34** was treated by Ag₂O to get the silver (I) NHC **3.42**. After treatment with [(Allyl)PdCl]₂ and filtration of the AgCl, a yellow palladium complex **3.43** was formed, but it decomposes and could not be isolated. We attempted to carry out the filtration of silver oxide under Ar and keep the allyl palladium NHC in solution, and we added to the solution the copper and the BnN₃.

However, despite several attempts, we were unable to identify or isolate the desired complex **3.44**.

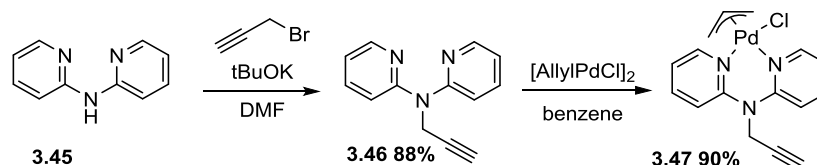


Scheme 3.52 Three-steps synthesis: silver activation, palladation and click

We realized that, although advertised in the article,¹⁵⁷ the protocol of the click was not described. As the intermediates were too sensitive, we decided to abandon this project.

b) *N*-propargyl bipyridylamine

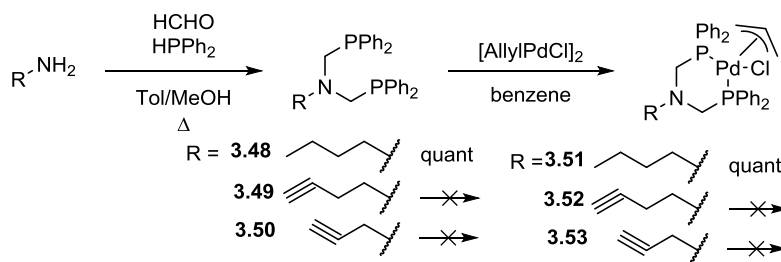
Although complexes such as $\text{AllylPdCl}(\text{bpy})$ are totally inactive in the case of Alloc deprotection we were surprised that the complex **3.47** had a little activity on the model reaction. The ligand was obtained by deprotonation and propargylation of **3.45** to give the ligand **3.46** (Scheme 3.53).¹⁵⁹ It was prone to decomposition if not stored cold under Ar. Upon treatment of the ligand **3.46** with $[(\text{Allyl})\text{PdCl}]_2$ in benzene, a purple powder was isolated after pouring the reaction mixture in diethyl ether. The resulting solid **3.47** was analysed by NMR in CDCl_3 and DMSO, but we always failed to get an analysable spectrum.



Scheme 3. 53 Propargylated bipyridylamine

c) ***N,N*-bis((diphenylphosphino)methyl)prop-2-yn-1-amine**

Another attempt aimed to use the group $(\text{PH}_2\text{P-CH}_2)_2\text{N-R}$ as it should have a large enough bite angle and because we can replace the R group by a triple bond-containing moiety. Although butylamine could be easily reacted with formaldehyde and diphenylphosphine to get the desired ligand **3.48**, the reaction was messy in when propargylamine **3.50** and homopropargyl amine **3.49** were used (Scheme 3. 54).¹⁶⁰ The palladium complex **3.51** was easily synthesized and purified in the case of butylamine. We tried to synthesize the complexes with the two other impure ligands and hoped to precipitate the desired complex from the impure mixture. However, after two unsuccessful attempts, this design was abandoned.



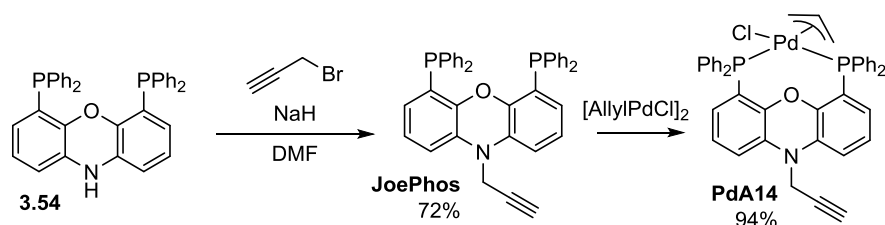
Scheme 3. 54 Bis-diphenylphosphinomethylene amine strategy

d) ***N*-propargyl Xantphos**

Xantphos is an interesting wide-bite angle which has proven to be active in our model reaction. However, there is no method of Xantphos post-functionalization available. Fortunately, the ligand *N*-Xantphos **3.54**, based on the phenoxazine tricycle, offers more

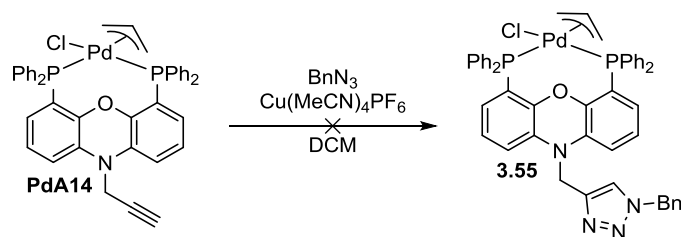
promising perspectives. It is commercially available and although the corresponding *aza* anion is known to be a poorly nucleophile, it has been alkylated in the literature with NaH in DMF, following by the addition of a bromide.¹⁶¹ The idea was to propargylate the aromatic nitrogen of the ligand, synthesize the π -allyl palladium complex and click it on a glucuronylated vector.

N-propargyl Xantphos, called **JoePhos**, could be easily synthesized by treating *N*-Xantphos **3.54** in DMF by NaH, followed by the addition at 0 °C of an excess of propargyl bromide (Scheme 3. 55). The **JoePhos** could be obtained as a white powder after silica filtration. Treatment with [(Allyl)PdCl]₂ lead to the conversion to the (Allyl)Pd(**JoePhos**)Cl (**PdA14**) after filtration of the yellow precipitate. Flash column chromatography could be used to purify it if required.



Scheme 3. 55 *JoePhos* synthesis

We tried to run the click coupling of **PdA14** to a model substrate, BnN₃, in DCM and using [Cu(MeCN)₄]PF₆ as the copper source (Scheme 3. 56). However, the mixture turned brown and we have failed to remove the copper and get an idea of the products of the reaction.



Scheme 3. 56 Attempt to click BnN_3 to allyl $\text{Pd}(\text{JoePhos})\text{Cl}$

4.4 Discussion

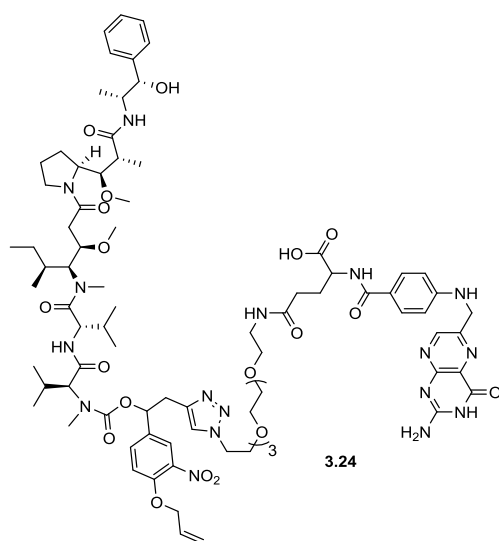
In this work, we developed the first generation of prodrug targeting cells over-expressing the folic acid receptor and activated by palladium catalysis. A collaboration was established with Prof Papot from the University of Poitiers as his expertise is valuable in the prodrug design, and that we wanted to use a safer design of prodrug and avoid the trap of hydrolytic functions.

As the central unit of Papot's prodrugs contains a phenol, and as allyl carbonates are prone to hydrolysis, we had to add an (N,O) extension to be able to make an allyl carbamate as a trigger. It was materialized by 4-aminobenzyl alcohol. The synthesis was performed as planned without major hurdles.

Our activation mechanism was by far more complex than the one reported by Papot, where a single enzymatic cleavage triggered one electron flow for only one 1,4-elimination step to release the **MMAE**. In our case, although we have a thermodynamic advantage thanks to the release of two CO_2 molecules, we have one aniline intermediate for which we don't know how fast it eliminates. Although Papot reported that the phenol with a PEG-4 is soluble enough and eliminates, the PEG-10 phenol should share similar properties, although we have not verified it experimentally. We suspect that the aniline intermediate, due to the other large groups,

Chemical structure of compound **3.19**. The molecule consists of a complex bicyclic core (a pyrrolidine ring fused to a cyclohexane ring) substituted with a hydroxyl group, a phenyl group, and a long polyether chain. The polyether chain is terminated by a pyrimidine ring substituted with an amino group and a carboxylic acid group. The structure is labeled **3.19**.

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Besides the elimination problem, we faced another problem in the biological test. At the time we started to work on it, we did not have any experience in the behaviour or the activity of the catalysts in cells. Chronologically, the experiments of the previous chapter came a few weeks after the ones presented here.

Since **MMAE** is a tubulin binder, a typical experiment needs to be performed for 72 h, which corresponds to a cell cycle, to see the toxicity appearing. So, that means that the catalyst needs to be incubated for the whole experiment. In our first toxicity tests, we noticed that the palladium catalysts exhibited marked toxicity, with IC_{50} in the range 1-10 μM . The toxicity may come from the catalyst itself but could come from degradation species of the catalyst. The main difficulty is to determine which percentage of the toxicity comes from the catalyst and which one from the released **MMAE**. Therefore, we think that for further investigations, a better approach would be to choose a less toxic catalyst, maybe a bit slower to activate, but more stable, such as **PdC01**.

The second problem is more bound to the protocol itself. Papot's procedure includes a 72-hours incubation of the prodrug without washing the cells, as the enzyme is already present in the lysosomes. That means that even if the prodrug penetrates slowly, 72 h are enough to get the required amount of cytotoxic released to trigger the biological response. In our case, the prodrug was incubated for 1 hour and the non-internalized prodrug was washed. There is a risk that only a small portion of the prodrug actually gets internalized and gets activated by the catalyst, yet without knowing how much actually eliminates the **MMAE**.

The risk of working with a cytotoxic prodrug and measuring the cytotoxicity is that the final readout is the sum of the toxicity of the catalyst, the prodrug and the released drug, and the last being weighted by the catalyst's efficiency. At first, it is hard to quantify the conversion of a reaction in cells by other means than HPLC-MS. Then, with a cytotoxic, if the released concentration of cytotoxic does not reach the IC_{50} threshold, no biological effect will be observed even if the uncaging takes place. That leads us to consider another system with the release of a non-cytotoxic probe, but a specific binder of an enzyme whose downstream action can be inhibited specifically. In that case, a simple Western Blot analysis would reveal if the target is inhibited, provided enough data from chemical biology support that the target is known and is bound selectively, and provided that the viability is high enough.

Second part: New applications of Tsuji Trost
reaction in bioorthogonal prodrug decaging
Fourth chapter: Development of a targeted
covalent inhibitor using palladium catalysis

5 Fourth chapter: Development of a targeted covalent inhibitor using palladium catalysis

5.1 Introduction

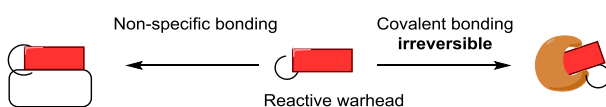
Enzyme inhibitors are small molecules which bind to enzymes and which can modulate the catalytic activity (Scheme 3. 57).

An inhibitor is said to be reversible if the residence time in the target is smaller than the synthesis of the protein by the ribosomal system (Scheme 3. 57a). If the residence time is longer, the inhibitor is considered as irreversible (Scheme 3. 57b). This kinetic notion is not entirely correlated to the nature of the bond, which is thermodynamic. Traditionally, the reversible inhibitors bind *via* non-covalent bonds, such as H bonding, hydrophobic interactions or π -stacking, whereas irreversible tend to bind *via* covalent bonds, although there is no strict limit between them. In this work, the inhibitor type we will be interested in is called a targeted covalent inhibitor, which combines the advantages of covalent and non-covalent inhibitors (Scheme 3. 57c).

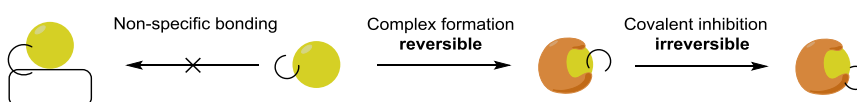
a) Reversible inhibitor



b) Irreversible inhibitor

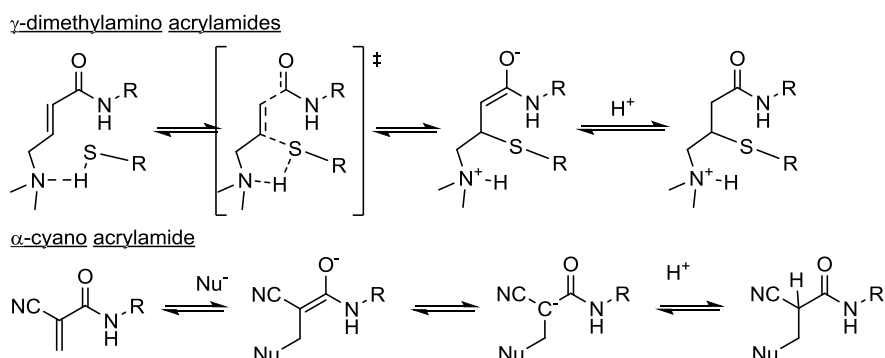


c) Targeted Covalent inhibitor



Scheme 3. 57 Types of protein inhibitors. Adapted from ¹³¹

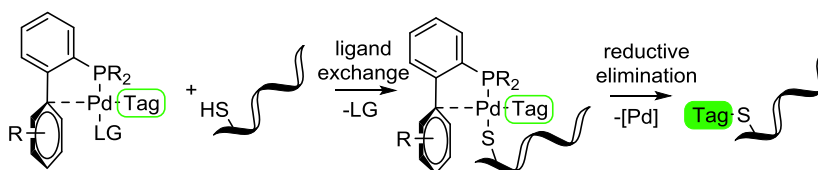
At first, the inhibitor binds to the substrate through non-covalent bonds. Then, a mild electrophilic function appears to be oriented towards a nucleophilic residue of the binding site and reacts with it to create a covalent bond. Normally, the electrophile part must not be too reactive to avoid reacting with the plethora of cellular nucleophiles, such as glutathione, cysteines and lysines. Therefore, some engineered Michael acceptors such as α -cyano acrylamides¹⁶², or γ -dimethylamino acrylamides are good candidates as they form reversible covalent bonds. For the α -cyano case (Scheme 3. 58 top), the reversibility comes from the acidity of the α -proton of the Michael adduct, and the rapid reactivity comes from the presence of 2 electrophilic groups on the double bond. For the γ -dimethylamino case (Scheme 3. 58 bottom), it has the function of creating an H bond with the cysteine to increase the acidity, resulting in an increase of the nucleophilicity of the cysteine towards the acrylamide.



Scheme 3. 58 Mechanism of electrophilic warheads found in TCI

In the literature, there are examples of protein covalent modifications by palladium using genetically encoded non-natural residues^{52, 163-168} or deprotection^{48-49, 169}.

Remarkably, Buchwald prepared some oxidative insertion intermediate complexes, the classical intermediate in most Pd-catalysed cross-coupling reactions, which selectively functionalize cysteines through the reductive elimination with a residue present of the palladium complex (Scheme 3. 59).¹⁷⁰⁻¹⁷¹ However, those residues do not have selectivity for a certain target, and only recognize cysteines.

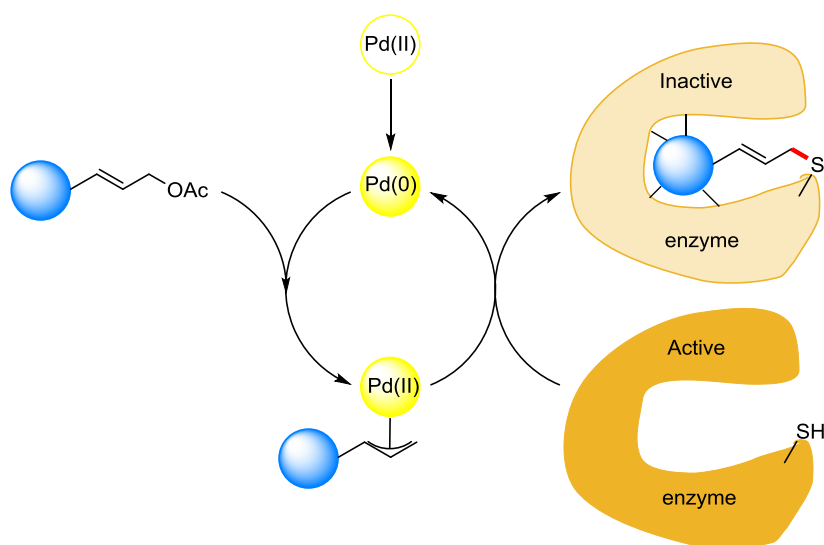


Scheme 3. 59 Oxidative insertion complexes able to conjugate a tag to a cysteine

5.2 Goals of this chapter

In this project, we hypothesized that we could use the classical intermediate of Tsuji-Trost reaction, the π -allyl palladium, as an electrophilic warhead for a TCI. Our contribution will aim to get selectivity by adding a group recognized by the target protein and an

electrophilic metal group to catalyse the formation of the covalent bond between the inhibitor and the target (Scheme 3. 60). In this chapter, two projects were developed. The first one was applied in cells and was targeting EGF-R and the second one was performed on an isolated enzyme, the papain.



Scheme 3. 60 Our approach: π -allyl palladium as electrophilic warheads

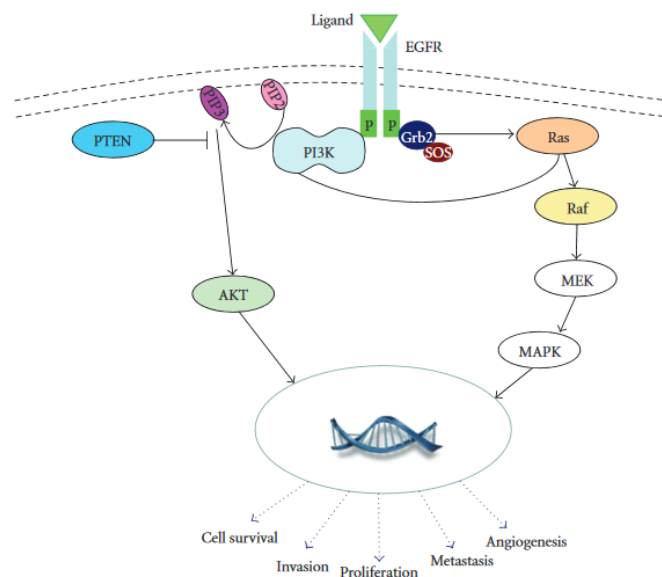
5.3 Development of an EGF-R TCI

This project was carried out by Martin Appelmans, a master student. The biological experiments were performed in the team of Prof Olivier Feron with technical support of Charline Degavre.¹³¹

5.3.1 About EGF-R and inhibitors

As a first model target, we chose EGF-R, which is a transmembrane receptor of the epidermal growth factor (Scheme 3. 61). The binding of EGF (epidermal growth factor) to EGF-R causes dimerization of the receptor and will trigger the intracellular kinase activity. This leads to the signal transduction through other kinases

including RAF, MEK and ERK $\frac{1}{2}$, in the family of MAP kinases, to finally trigger growth by sending signals to the nucleus.¹⁷²



Scheme 3. 61 EGF-R signalling pathway. From¹⁷²

EGF-R has been the subject of multiples studies and is nowadays a target for anticancer drugs.¹⁷³ Several generations of EGF-R inhibitors have been developed, since the first report of thio adenosine forming a disulphide with EGF-R in 1990.¹⁷⁴ The first generation of selective EGF-R inhibitors are based on 4-anilinoquinazoline and are reversible non-covalent inhibitors. The second generation (Afatinib and Neratinib) use the 4-anilinoquinazoline scaffold for targeting and the *gem*-dimethyl amino acrylamide as a Michael acceptor, thus forming a covalent bond with the cysteine in the pocket.

5.3.2 Prodrug design

Our attention was drawn by **Gefitinib**¹⁷⁵, as it is commercially available at a reasonable price, and also because its crystal structure

with EGF-R has been reported. It appears that the 4-anilinoquinazoline is deeply inside the enzymatic pocket and that the tail appears to be outside the pocket and with the morpholine acting as a “pearl” to reduce the mobility of the chain.

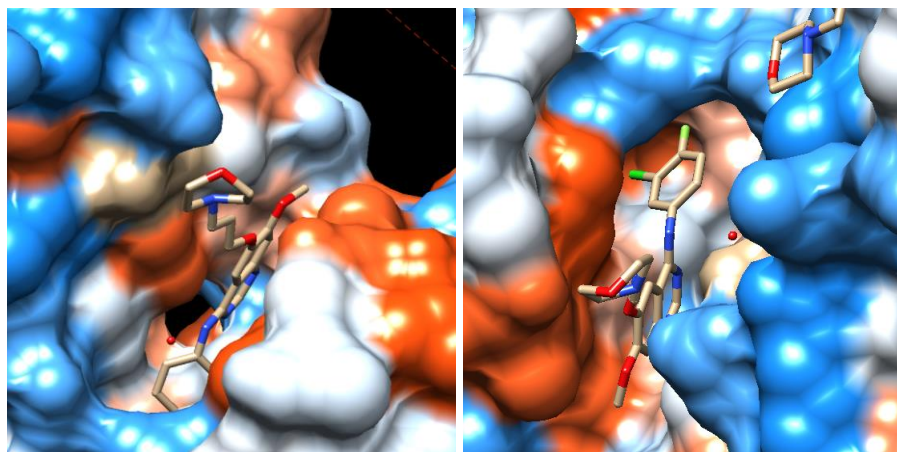
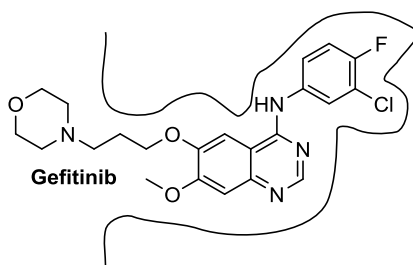
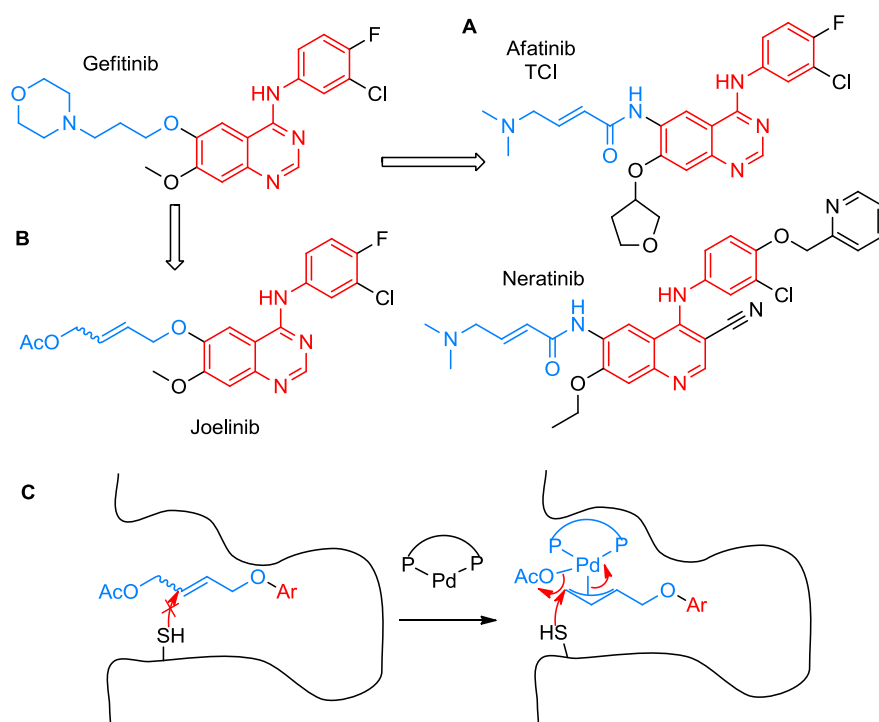


Figure 3. 14 Binding of **Gefitinib** in the pocket of EGF-R (PDB entry: 4WKQ)

The case of **Gefitinib** is interesting as a template for the design of a TCI targeting EGF-R (Scheme 3. 62A). We hypothesized that we could use the 4-anilinoquinazoline part as a targeting moiety, as it is commercially available, and the tail to create the covalent bond between an electrophilic residue and a well-oriented cysteine in the enzymatic pocket. We decided to combine this targeting moiety with an allylic acetate as π -allyl precursor to give our model inhibitor, the **Joelinib** (Scheme 3. 62B). Allylic acetates are not electrophilic unless activated by palladium (Scheme 3. 62C).



Scheme 3.62 A) Design of Neratinib and Afatinib (TCI) on the template of **Gefitinib**. B) **Joelinib** design based on **Gefitinib**. C) Mode of action of **Joelinib**

A docking model was developed by Pr. Raphael Frédéric and shows that the heteroaromatic part of **Joelinib** and **Gefitinib** overlap and shows also the proximity of the cysteine with the allylic acetate (Figure 3.15).

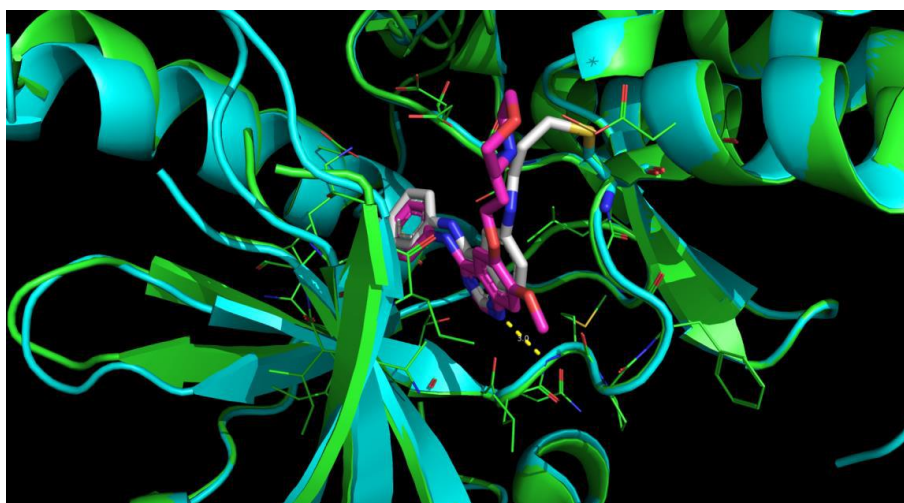
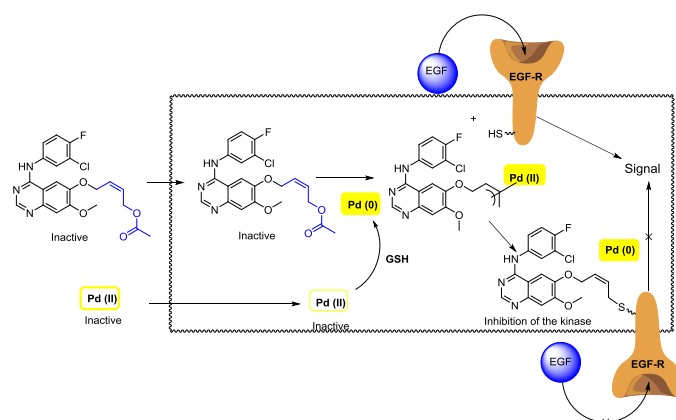


Figure 3. 15 Docking model of **Joelinib** (grey) superimposed with **Gefitinib** (purple). The cysteine of the pocket is visible on the right of the side chain in yellow

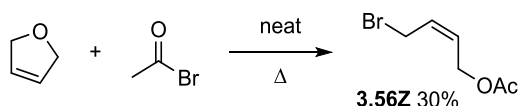
Instead of pre-synthesizing the π -allyl palladium, we decided to perform a biological assay by allowing it to form *in-situ* by co-incubation of the **Joelinib** with the palladium pre-catalyst. The active π -allyl species, which would recognize the EGF-R intracellular kinase domain and would bind to it *via* its electrophilic warhead, causing the inhibition of its activity (Scheme 3. 63).



Scheme 3. 63 Concept of the catalytic inhibition of EGF-R by Tsuji-Trost reaction and **Joelinib**

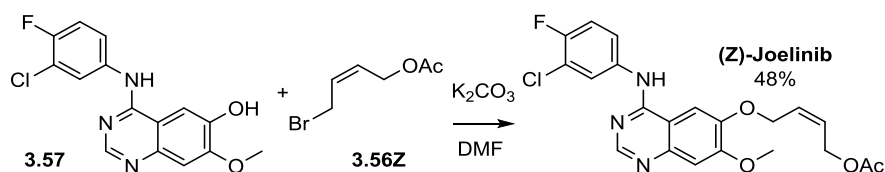
5.3.3 Prodrug synthesis

The **Joelinib** could be obtained in two forms: Z and E.¹³¹ We started to synthesize the E-isomer of the arm prior to its coupling with the core part, but it was harder to make from expensive starting materials. The (Z) was far easier to make in one single step. The compound **3.56Z** was obtained in high purity by treating 2,5-dihydrofuran and acetyl bromide, and **3.56Z** was obtained by Kugel Rohr distillation (Scheme 3. 64).



Scheme 3. 64 Facile synthesis of the bromoacetate (Z) **3.56Z** by an acidic opening of 2,5-dihydropyrane

As the arm in (Z) configuration was the easiest to obtain in higher quantities, we only synthesized the (Z)-**Joelinib**. It was obtained by treating **3.57** with **3.56Z** in DMF with K_2CO_3 and was purified by chromatography (Scheme 3. 65).



Scheme 3. 65 **(Z) Joelinib** synthesis

5.3.4 Biological assay

5.3.4.1 Choice of the biological model

As EGF-R is a transmembrane receptor, it is harder to isolate than cytosolic proteins, so we did not envision to work on a purified form of the protein, and thus preferred to work on A431 cells (epidermal cancer cells) as they overexpress EGF-R. This renders the cells more sensitive to EGF-R inhibition.

Since at the time we did those experiments, we still had little experience in the palladium catalysts, we opted for our “best candidate” **PdA01**.¹³¹

Our strategy for this biological assay was based in a two-step procedure.

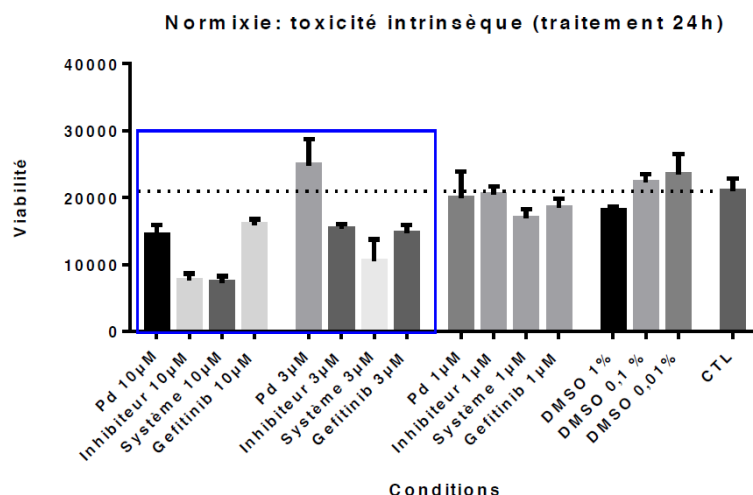
- The first step is a viability assay and aims to determine if there is cell mortality coming from the combination of **Joelinib** and **PdA01** whereas they are both non-toxic. As a positive control, we expect that the toxicity should match the level of **Gefitinib**, a known EGF-R inhibitor on which the design of **Joelinib** is based.
- The second step is a Western Blot and aims to verify that the target EGF-R is effectively reached by the activated **Joelinib**.

5.3.4.2 Viability assay

For the viability test, the A431 cells were put in culture in 96-well plates. Then, the cells were incubated with **PdA01** (200 μ L at 1, 3 or 10 μ M) for 30 min. After this pre-incubation, the cells were

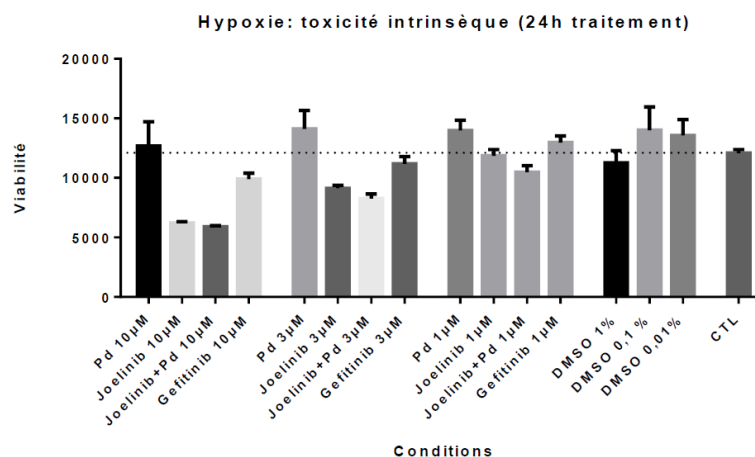
washed with PBS and fresh cell culture medium, to which **Joelinib** in solution in DMSO was added at different concentrations ranging from 1 to 10 μM . After a fixed time the viability was measured by Presto Blue®. Control experiments were made with the catalyst, **Gefitinib** and **Joelinib** taken alone.

We first noticed that the toxicity of the catalyst was not a factor up to 3 μM (Graph 3. 28). Although **Joelinib** and **Gefitinib** exhibited the same level of toxicity above 3 μM , we found that **Joelinib** was more toxic than **Gefitinib** at 1 μM . In each case, the catalyst had no impact on the toxicity and it remained identical to the value obtained with **Joelinib**.



Graph 3. 28 Intrinsic toxicity of the different component (**PdA01**, **Joelinib**, **Gefitinib**) and the catalytic system in normoxia

The test was replicated in hypoxia to see if oxygen had an impact on the palladium species or redox metabolites. The cells were pre-incubated for 2 hours in a hypoxia chamber.



Graph 3. 29 Intrinsic toxicity of the different component (**PdA01**, **Joelinib**, **Gefitinib**) and the catalytic system in hypoxia

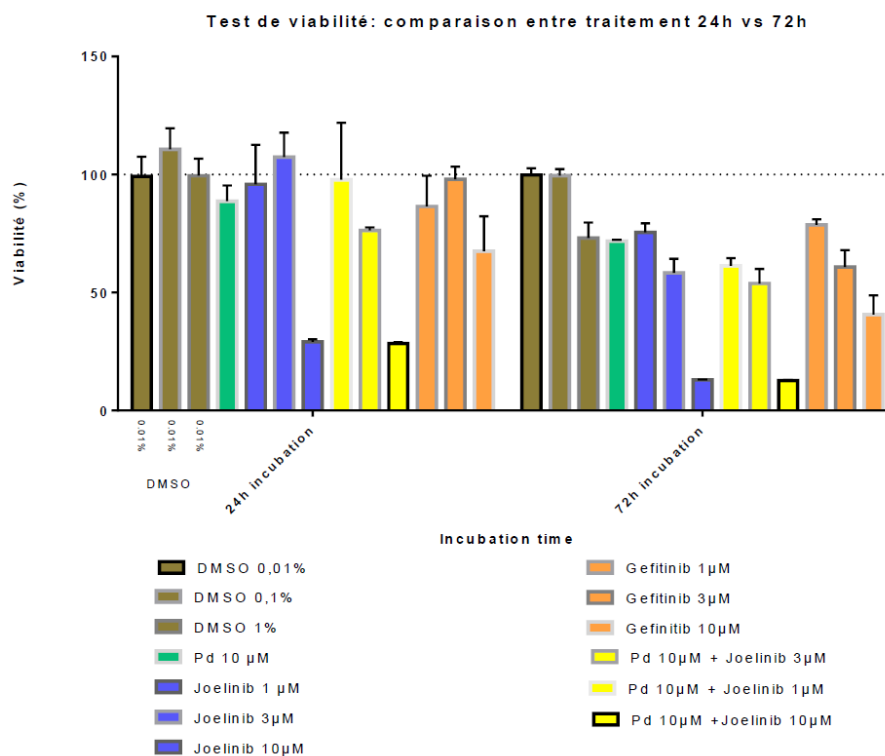
When repeated in hypoxic conditions, the results were similar (Graph 3. 29). Therefore, we know that oxygen does not interfere with our system.

The most obvious conclusion from those two experiments is that we can draw from those results is that the palladium had no effect. Several hypotheses were emitted:

- **Joelinib** and **PdA01** don't meet in the cytoplasm.
- The π -allyl palladium does not form or is too unstable.
- The π -allyl palladium is unable to enter in the pocket due to its size.
- The π -allyl palladium forms, enters in the pocket and due to the strong binding energy, it cannot be differentiated from the **Gefitinib**.

As our knowledge about π -allyl palladium complexes in solution and in cells were lacking, we could test the last hypothesis in a simple way. By increasing the time of incubation, we may see a discrimination between **Gefitinib** and **Joelinib** and **PdA01**, as the

covalent bond is stronger and should not decompose over the time. So, the time was increased to 72 h, and a parallel reaction was carried out for 24 h for the sake of the comparison (Graph 3. 30). However, no difference was observed.



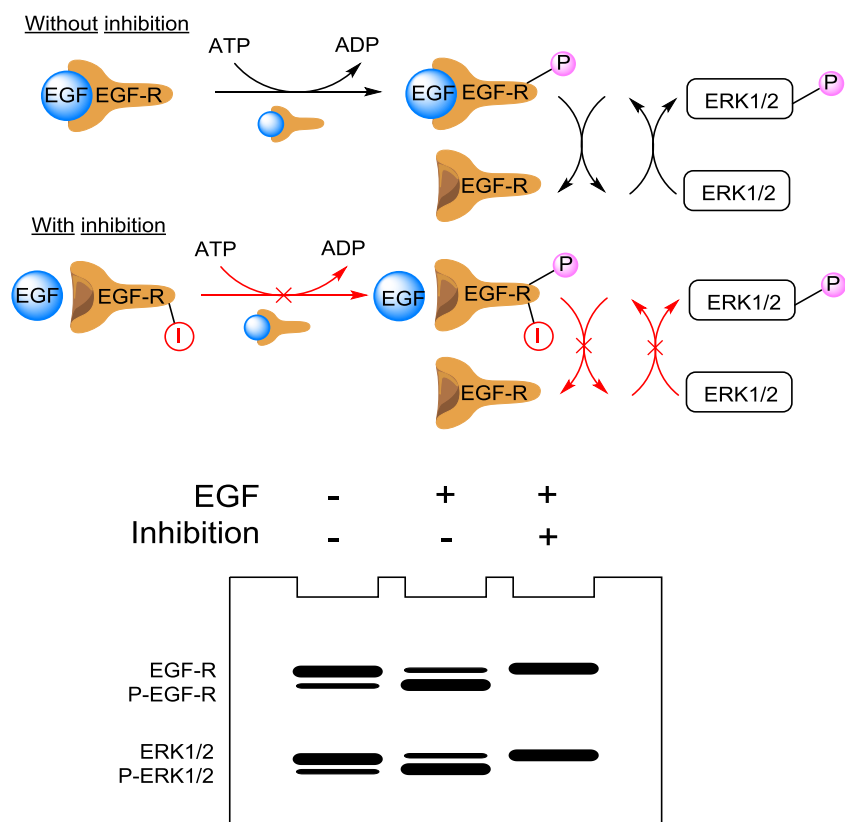
Graph 3. 30 Impact of the time on the catalytic system

5.3.4.3 Western Blot

a) Principle

To verify if the **Joelinib** was targeting EGFR-R, we performed a Western Blot. After the catalytic test described above, the proteinic content of the cells was analysed by electrophoresis gel. It was followed by the detection of EGF-R and its phosphorylated form P-EGF-R, but also and ERK1/2 and its phosphorylated form P-ERK1/2, which are downstream kinases (Scheme 3. 66A). EGF-R is the first

kinase in the series, if the phosphorylation of EGF-R is blocked, the downstream phosphorylation will be also inhibited (Scheme 3. 66B). We performed the Western Blot in this way as we were not sure to detect efficiently EGF-R and the antibody for ERK1/2, phospho-ERK1/2 (P-ERK1/2) were readily available at the biology lab, in addition to EGF-R and P-EGF-R antibodies.



Scheme 3. 66 A: Impact of the inhibition of EGF-R on the (P)-EGF-R and (P)-ERK1/2 levels. B: Prediction of the Western blot with and without inhibition

b) Basal expression

To get familiar with the system, we started by testing the detection of our 4 proteins while the A431 cells were incubated with or without EFG (2 ng/L). Those experiments would serve as control experiments in the next paragraph.

While the cells were grown in a medium containing bovine serum, there appears to be no significant difference with or without EGF in the phosphorylation levels (Figure 3. 16A). It could be explained by the fact that bovine serum contains some EGF at a higher concentration than 2 ng/mL of our solution.

So, we had to repeat the experiment without bovine serum albumin (Figure 3. 16B). In this case, we could see that the intensity of the spots increases for P-EGF-R and P-ERK1/2 when EGF is added.

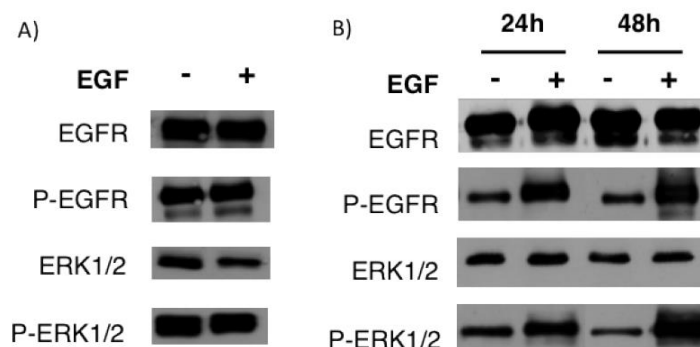


Figure 3. 16 Western Blot aiming to detect the levels of EGF-R and ERK1/2 and their phosphorylated forms with or without the addition of EGF at 10 ng/mL. A) Incubation in presence of bovine serum. B) Without bovine serum

c) Catalytic test

So, knowing the basal expression levels of P-EGF-R and P-ERK1/2, but also EGF-R and P-EGF-R, we will be able to know if EGF-R is inhibited or not with our system.

Therefore, the cells were incubated for 24 h in a serum-free medium in normoxic or in hypoxic conditions (Figure 3. 17). Then, the cells were incubated with the catalyst if needed for 30 min, then washed before adding the appropriate inhibitor system at 10 μ M for

24 h. In the end, the culture medium was washed and EGF (2 ng/L) was added for 10 min before cell lysis and further treatment.

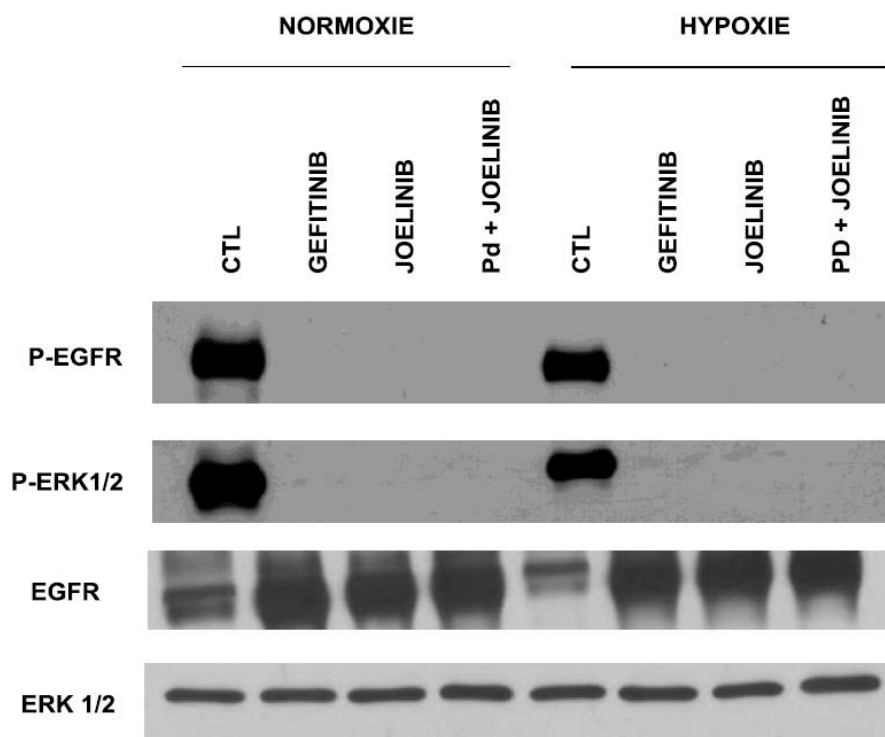


Figure 3. 17 Western Blot assay aiming to determine the impact of **Joelinib**, **Gefitinib** and the catalytic system on the level of phosphorylated and non-phosphorylated kinases EGF-R and ERK1/2

The figure shows clearly a complete disappearance of the phosphorylated forms of EGF-R and ERK1/2 upon treatment with **Gefitinib** and **Joelinib**. However, no difference in phosphorylation was observed between **Gefitinib** and **Joelinib**. This is the sign that we cannot establish the precise role of the palladium. All we can conclude is that **Joelinib** goes to its target and inhibits EGF-R. However, no information can be extracted about the binding mode of **Joelinib** on EGF-R.

5.3.5 Discussion

The goal of this project was to inhibit the activity of an enzyme with a targeted covalent inhibitor whose warhead is a π -allyl palladium complex and bearing a recognition moiety borrowed to a known inhibitor of the enzyme we considered.

In this project, we developed **Joelinib**, aimed to become a TCI targeting EGF-R after activation by a palladium catalyst. The first point of discussion will be the choice of the catalyst, the second will be the design of **Joelinib** and the third will be the experiment.

a) Catalyst choice

At first, we chose **PdA01** as a model catalyst as at the time, we still had limited experience with biocompatible palladium catalysts. We decided to privilege catalysts with a high “catalytic activity” on Alloc uncaging reactions. However, it turns out that it was probably not an optimal choice for two reasons.

- At first, the criteria for a Tsuji-Trost deprotection are not the same as for a Tsuji-Trost bioconjugation. For a Tsuji-Trost, we search for complexes with a fast reductive elimination and for which the oxidative insertion is not the rate determining step. In this case, we need a complex with a fast oxidative insertion and a slow reductive elimination. Therefore, a complex such as **PdA01** will be activated promptly, will possibly form the π -allyl, but this π -allyl has high chances to be attacked by any nucleophile prior to reaching its target. A better idea would be to use a smaller bite angle ligand such as dppbz, as it would slow down the reductive elimination.

- The other problem with the catalyst is the big size of the phosphine ligands. Although the steric bulk tends to protect the palladium and gives air-stable phosphines, it may prevent the π -allyl of **Joelinib** from entering the pocket.

b) **Design of the Joelinib**

Regarding the design of **Joelinib**, the risk is that if we remain too close to **Gefitinib**, we will keep an inhibitor with a low micromolar activity, similar to the parent inhibitor. The difference of activity will be very hard to see in viability tests or in Western Blot.

Also, we cannot exclude that **Joelinib** reacts thanks to a new mode of action with the cysteine of EGF-R, such as a SN2 or a SN2' attack on the allylic acetate, creating a covalent bond.

Eventually, we cannot exclude that **Gefitinib** has been chosen as a drug for its pharmacological parameters rather than its IC₅₀ only. It is possible that the tail interacts stronger thanks to con-covalent bonding, resulting in a lower IC₅₀.

c) **Design of the experiment**

Regarding the experiment, a major difficulty is to make sure that both partners meet and react together in the cell. For the catalyst, we only have an indirect indication that it can penetrate the cells, as shown in Chapter 2. As suggested by Wender, the catalyst could be trapped in the periplasmic space or remain bound to the membrane.

The 72-hours experiment did not give us any about the reversibility. Cells do not have excretion system, like animals, which enable to get rid of chemical compounds, as they just have enzymes which pump out of the cells. As the cells remain in the same medium,

the compounds have the opportunity to enter again. We would need to wash the cells on a daily basis to remove the pumped-out compounds.

To determine whether there is covalent bond, we would need to use another technique, such as surface plasmonic resonance, as covalently bound and non-covalently bound inhibitors would give a totally different signal. Protein X-ray crystallography is a valuable technique, although extremely complicated in the case of co-crystals and membrane-bound proteins.

In the first part, we aimed to inhibit EGF-R with a TCI borrowing the recognition part of **Gefitinib** and an allylic acetate at the tail section aimed to be activated by a palladium (0) complex to bind to the cysteine of the active pocket (Figure 3. 18). The synthesis of the allylic acetate-modified **Gefitinib**, called **Joelinib**, was trivial as the core part of **Gefitinib** without the tail was commercially available.

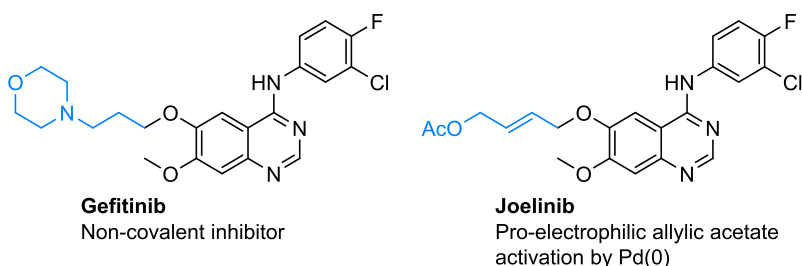


Figure 3. 18 **Gefitinib** and **Joelinib**

We performed rapidly biological trials, although we had very little experience on the catalysts, their activity *in cellulo* and the concentration at which they shall be used. Western blot analysis showed that we could detect a change in phosphorylation of EGF-R, but also other kinases in the downstream pathway. We verified that both **Joelinib** and **Gefitinib** were reaching their target, EGF-R, and

were inhibiting its activity. Cell viability assay showed that **Joelinib** was more potent than **Gefitinib**, and the addition of the catalyst had no effect.

5.4 Development of a papain TCI

This work was partially performed by Céline Ruijs, a master student in chemistry, and Bouazza Es Saadi for the technical support under the supervision of Prof Raphael Frédéric.

The case of **Joelinib** showed us that we tried to work with a too complex system directly. We then decide to simplify the system and work with an isolable and commercially available protein.

5.4.1 About Papain

Papain was therefore chosen. It is a cysteine protease for which many inhibitors are known in the literature. It is commercially available and extracted from papaya latex.

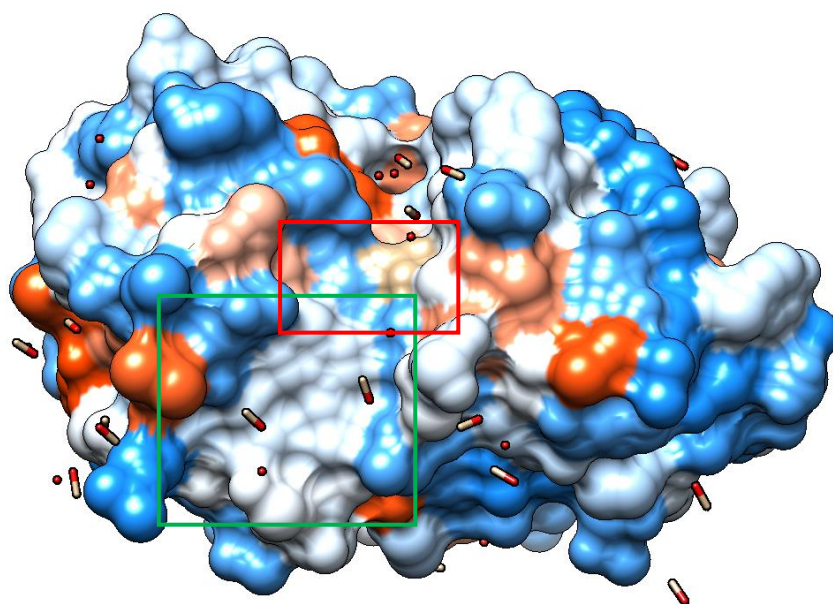


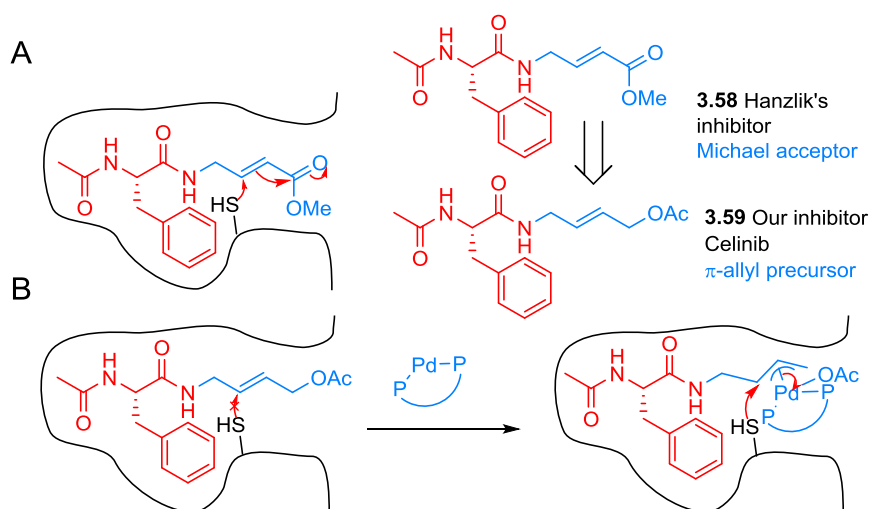
Figure 3. 19 Papain crystal structure centred on the active site (PDB ID: 9PAP). Green: hydrophobic pocket. Red: Active cysteine

Its active side comprises a valley able to accommodate substrates up to heptapeptides, narrowing near the active site comprising the classical catalytic triad, the Cys 25, the His 159 and Asn 175 (Figure 3. 19, red square). The hydrophobic pocket (Figure 3. 19, green square) can accommodate large hydrophobic side chains such as phenylalanine. It is composed of hydrophobic amino acids.¹⁷⁶

5.4.2 Papain inhibitors and substrates

The model inhibitor **3.58** on which our work is based was reported by Hanzlik in 1984 (Scheme 3. 67).¹⁷⁷⁻¹⁷⁸ It comprises *N*-Acetyl phenylalanine bound onto which an ethyl acrylate as Michael acceptor which reacts with the Cys 25 in the enzymatic pocket in 1,4-fashion. He also reported that papain has specificity for substrates with a large hydrophobic side chain, such as a benzyl group due to the hydrophobic pocket. Suspecting that the recognition would be coming

from the *N*-acetyl phenylalanine, our design envisioned to replace the Michael acceptor by an allylic acetate, similarly to the **Joelinib**. The compound **3.59** was named **Celinib**.

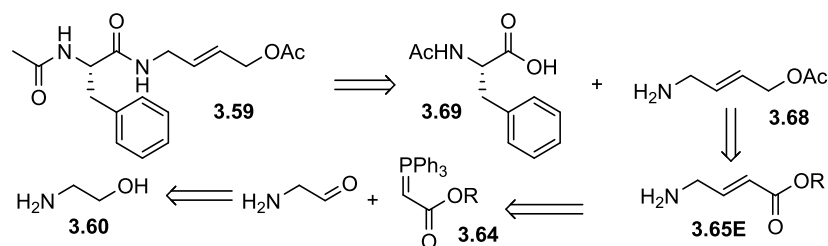


Scheme 3. 67 A: Hanzlik's inhibitor and mode of action. B: **Celinib** design and mode of action

5.4.3 Synthesis of Celinib and the model inhibitor

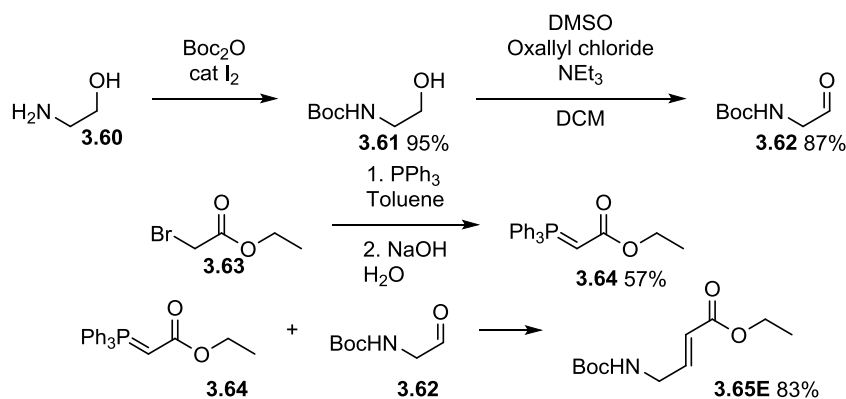
5.4.3.1 Celinib Synthesis

On contrary to phenol, amides are typically not very nucleophile due to the delocalization.¹⁷⁹ The disconnection had to be done one bond further away to involves an amide bond synthesis (Scheme 3. 68). The allylic amino acetate could come from an (E)-selective Wittig reaction.



Scheme 3. 68 Retrosynthetic analysis of **Celinib**

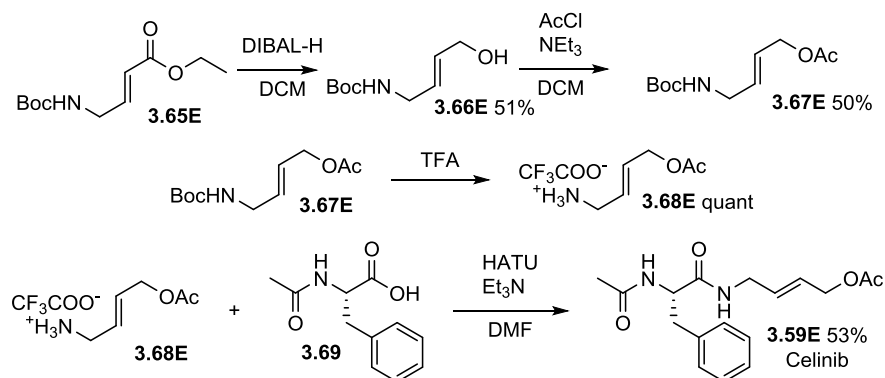
The ethanolamine **3.60** was protected by a Boc using iodine catalysis prior to the Swern oxidation to give the intermediate **3.62** (Scheme 3. 69).¹⁸⁰ Then, ethyl bromoacetate **3.63** was converted into the corresponding Wittig salt by treatment with triphenylphosphine, followed by a basic workup to give the ylide **3.64**.¹⁸¹ The coupling between **3.62** and **3.64** gave the Wittig adduct (E) as seen by the coupling constant analysis in ¹H-NMR.¹⁸² This ethyl acrylate **3.65E** was a key intermediate and was used for the synthesis of the Hanzlik inhibitor **3.58**.



Scheme 3. 69 Synthesis of the **3.65E** intermediate

For the **Celinib**, the ester **3.65E** was reduced with DIBAL-H¹⁸³ and the alcohol **3.66EE** was acetylated with AcCl and Et₃N (Scheme 3. 70). Treatment by TFA was used for the deprotection of the Boc and a brown sticky oil **3.68E** was obtained. The coupling between

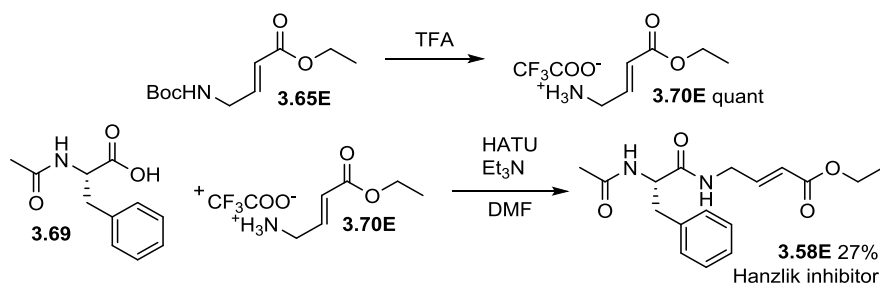
3.68E and **3.69** was easily achieved in DMF using HATU as a coupling agent. HATU was used as it was readily available and does not epimerize stereocentres.



Scheme 3.70 Synthesis of (*E*)-**Celinib** via HATU coupling

5.4.3.2 Reference inhibitor synthesis

Hanzlik's reference inhibitor **3.58E** was synthesized from the key intermediate **3.65** (Scheme 3.71). It was simply deprotected using TFA and coupled to *N*-acetyl phenylalanine using HATU.



Scheme 3.71 Synthesis of Hanzlik inhibitor **3.58E** using the ethyl acrylate key intermediate **3.65E**

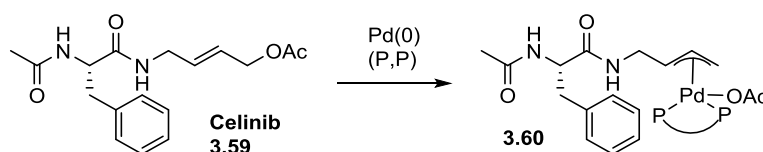
5.4.4 Papain inhibition tests

The global concept of the project was to inhibit the papain with a targeted covalent inhibitor after a preliminary activation of the pro-electrophile allylic acetate by a metal catalyst to get the π -allyl acetate complex, which will react with the cysteine of the enzymatic pocket

and create a covalent bond. The released palladium will thus be able to do another cycle.¹⁷⁹

5.4.4.1 Synthesis and isolation of the active species

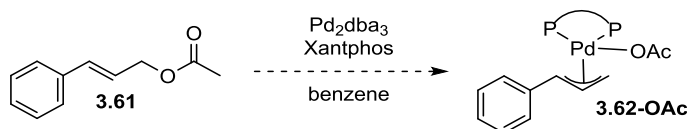
Since the activated electrophile is also the key intermediate of the Tsuji-Trost reaction, we decided to start by synthesizing the π -allyl activated species of **Celinib** (Scheme 3. 72).



Scheme 3. 72 Active electrophilic π -allyl palladium derived from **Celinib**

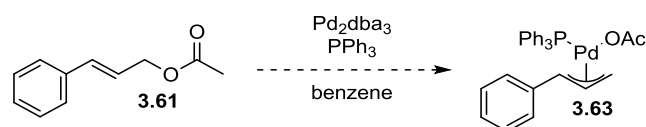
We started by developing a procedure on a model substrate prior to a transposition on **Celinib**. Cinnamyl acetate **3.61** was chosen.

We made the first try by treating the **3.61** by $\text{Pd}(\text{dba})_2$ and Xantphos, (Scheme 3. 73). However, we could neither precipitate it selectively nor purify **3.62-OAc** by flash chromatography, as it is possible with **PdA01** or its analogues. NMR analyses were not fully conclusive as all the key signals were masked by the dba peaks.



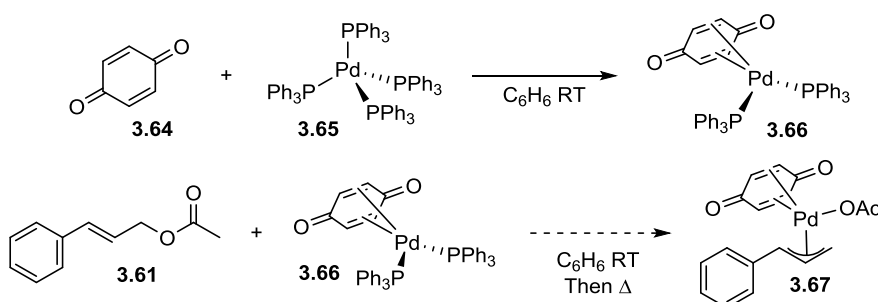
Scheme 3. 73 π -allyl synthesis on a model substrate with a Pd (0) source and Xantphos

No improvement was observed while triphenyl phosphine was used (Scheme 3. 74).



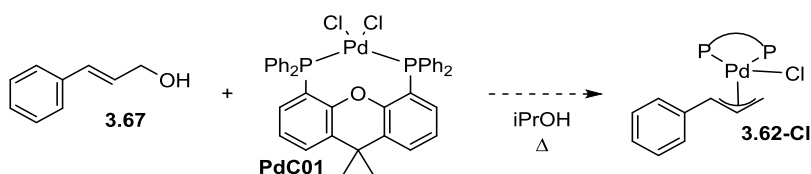
Scheme 3. 74 π -allyl synthesis on a model substrate with a Pd (0) source and triphenylphosphine

To simplify the NMR spectrum, we decided to avoid using dba containing palladium sources. We tried to react $\text{Pd}(\text{PPh}_3)_4$ **3.65** or $(\text{PPh}_3)_2\text{Pd}(\text{benzoquinone})$ **3.66** with **3.61**, but only traces of π -allyl palladium species **3.67** were observed by ^1H -NMR (Scheme 3. 75).



Scheme 3. 75 π -allyl synthesis **3.67** on a model substrate with a new Pd (0) source

Our last attempt consisted of using isopropanol to reduce **PdC01** and leaving acetone as by-product (Scheme 3. 76).¹⁸⁴ We followed the literature procedure with **3.67** and **PdC01**, but we did not observe any product by NMR.



Scheme 3. 76 π -allyl **3.62-OAc** synthesis on a model substrate with a Pd (II) source in-situ reduction by $i\text{PrOH}$

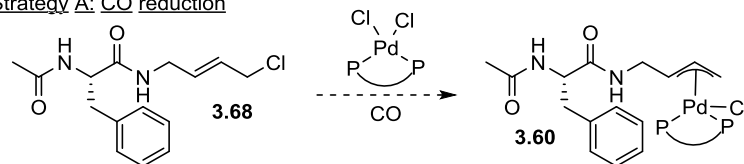
By lack of time, we did not try additional routes. There are some future possibilities.

- We think that the π -allyl palladium acetates are less stable compared to their chloro surrogates, making them impossible

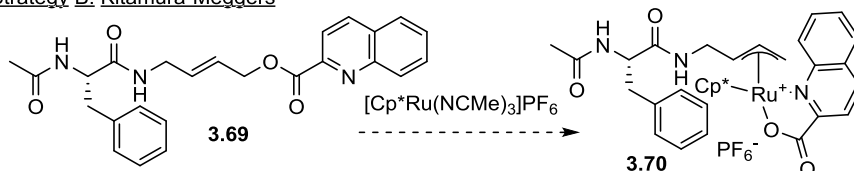
to separate from the dba ligand by flash column chromatography or even by precipitation. The direct first try would be to start from the allyl chloride analogue.

- Since $[\text{AllylPdCl}]_2$ seems to be synthesized from Na_2PdCl_4 and allyl chloride in presence of CO as reducing agent, we hypothesized that we could simply employ the allylic chloride **3.68** and the complex $(\text{L,L})\text{PdCl}_2$ and carry out the reduction with *in-situ* generated CO (Scheme 3. 77A). The advantage would be to synthesize a wide range of substrate of and an easy purification as the only by-products would be CO_2 and NaCl.
- Similar to the synthesis of the 3rd generation of Meggers complexes, our last idea would involve starting from the allylic ester of the Kitamura-Meggers ligand **3.69** and treatment with $\text{Cp}^*\text{Ru}(\text{MeCN})_3]^+(\text{PF}_6)^-$ (Scheme 3. 77B).⁴⁰ The active species **3.70** would be a Ru (IV) complex, similarly to Kitamura-Meggers 3rd generation catalyst.

Strategy A: CO reduction



Strategy B: Kitamura-Meggers



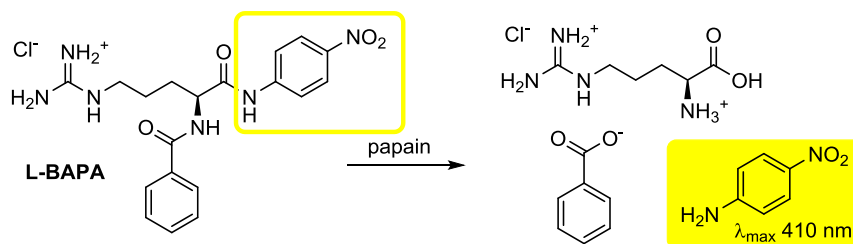
Scheme 3. 77 Two additional strategies: A) CO reduction, B) Meggers-Kitamura

5.4.4.2 Enzymatic tests

Without the key π -allyl, and by lack of time, we decided to move to the enzymatic tests, adding one more challenge to the test as we have not studied the formation and the stability in solution of π -allyl palladium acetates.

a) Chromogenic test

We based our test on a chromogenic reaction in order to determine the activity of the papain. Therefore, L-BAPA (benzoyl-L-arginine-p-nitroaniline) is a commercially available substrate of papain which releases 4-nitroanilin ($\lambda_{\text{max}} = 410 \text{ nm}$) upon enzymatic hydrolysis, and it can be followed by colorimetry (Scheme 3. 78).



Scheme 3. 78 Chromogenic reaction to follow the papain activity

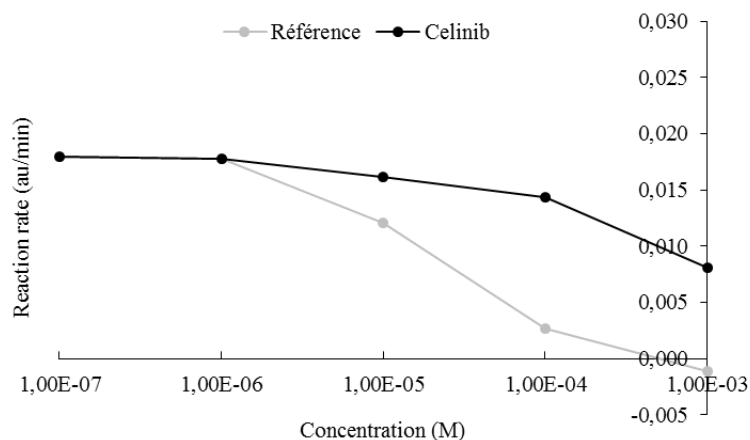
The only kinetic data which will be analysed in this study is the initial reaction rate. The reaction is launched and the absorbance is recorded for 15 min and the $v_{\max}$ is extracted by calculating the slope.

b) Measure of the IC_{50} of the inhibitors

At first, we wanted to determine the IC_{50} of the **Celinib** and the Hanzlik's inhibitor. The protocol was developed by Bouazza es Saadi.

- Pre-incubation of papain (50 mM in acetate buffer at pH 4.5) with the inhibitor (concentration ranging between 0.1 μ M and 1 mM).
- Addition of the L-BAPA at 10^{-3} M.
- Addition of neutral buffer to activate the papain (acidic buffer prevents self-digestion).
- Measure of the absorbance at 410 nm for 15 min and extraction of the slope.

In our case, the **Celinib** had an IC_{50} in the millimolar range whereas the reference inhibitor had an IC_{50} around 20 μ M. That means that at 100 μ M, we have a total inhibition coming from the reference inhibitor and virtually no effect coming from the **Celinib**.



Graph 3.31 IC_{50} determination for **Celinib** and the 3.58

c) Catalytic tests

A set of four catalysts were chosen: **PdA01**, **PdB03**, **PdB07** and the Meggers ruthenium complex (Figure 3. 20). They were chosen as the Xantphos was deemed to be the best ligand for catalytic activity and as they have three different modes of activation. The ruthenium catalyst was chosen for its smaller size.

The protocol was adapted to give time to the active form of **Celinib** to form in solution and to react with the papain.

- Pre-incubation of papain (50 mM in acetate buffer at pH 4.5) with the inhibitor (concentration ranging between 0.1 μ M and 1 mM) and the catalyst at 1.8 mM for 15 min.
- Addition of the L-BAPA.
- Addition of neutral buffer to activate the papain.
- Measure of the absorbance for 15 min and extraction of the slope.

A control experiment was performed with the catalyst alone to see if the catalysts were inducing an inhibition.

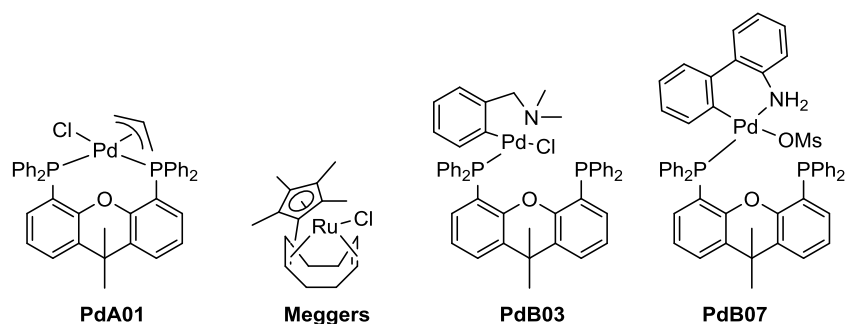
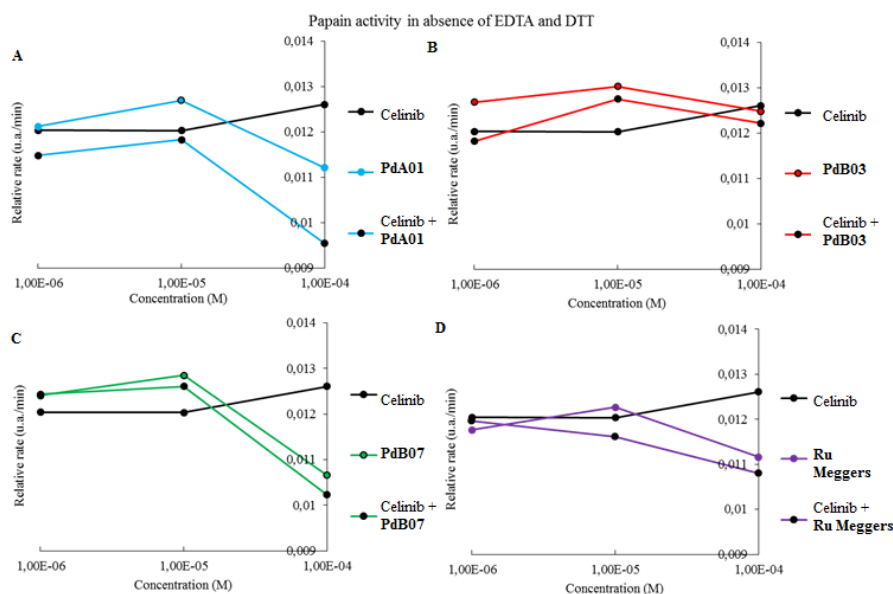


Figure 3. 20 Selected catalysts for the papain inhibition assay

While analysing the results, we noticed that in each case, the catalyst had an inhibitory effect and the combination with the **Celinib** did not make any significant difference. Two explanations were proposed.

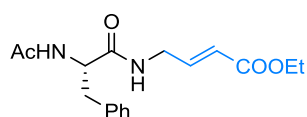
- We thought that the π -allyl did not have enough time to form or to react with papain. To verify this hypothesis, we decided to analyse the effect of the pre-incubation time of the catalyst and **Celinib**. However, even though we changed the time from 0 seconds to 30 min, strictly no effect was seen on the two catalysts we tested (**PdB07** and Meggers).
- We thought that the EDTA, present in the buffer, inactivates the catalyst. Also, the role of the thiol DTT is ambiguous: although it protects the enzyme for redox changes in its cysteines, it can also attack π -allyl palladium complexes, including the activated form of **Celinib**. Therefore, the optimization was carried out again without EDTA and DTT. Although a slightly lower activity of the papain was observed, no difference was observed.



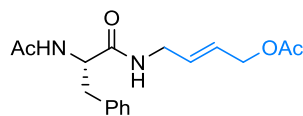
Graph 3. 32 Impact of the different catalysts in combination with the **Celinib** on the activity of the papain

5.4.5 Discussion

Due to the question whether the catalyst was able to meet the **Joelinib** or not, we decided to change the model and focus on a commercial and isolable cysteine protease, which is papain. It is a widely available cysteine protease, frequently used as a model cysteine protease for which many inhibitors are known in the literature. We wanted to carry out an *in vitro* test on the purified enzyme to avoid problems of cell-permeability. In the literature, we found an inhibitor featuring a Michael acceptor which could be re-designed with an allylic acetate as a π -allyl palladium precursor and synthesize it. It was called **Celinib**.



Hanzlik inhibitor **3.58**
Covalent inhibitor: Michael acceptor



Celinib **3.59**
Pro-electrophilic allylic acetate
activation by Pd(0)

The questions of the meeting of the catalyst and the inhibitor, the formation of the π -allyl and its reaction with the enzyme shall be discussed with this simplified system.

To avoid the tedious question of whether the catalyst and the inhibitor meet prior to any inhibition, we decide to pre-synthesize the π -allyl palladium intermediate. However, we were not able to isolate a stable π -allyl palladium complex on a model substrate and we did not try on **Celinib**. As discussed previously, the choice of the Xantphos and the acetate were not optimal and caused problems in the isolation of the π -allyl palladium complex on the model substrate.

As we could not obtain π -allyl palladium complexes, we moved on catalytic tests without any knowledge about the formation of π -allyl palladium complexes. Therefore, we still have no idea whether they form or not in our experimental conditions. Therefore, a first step would be to synthesize the π -allyl palladium complexes *ex situ* in solution and react them directly with the papain.

The enzymatic tests are not completely reliable as time was spent to develop a methodology with transition metals and EDTA in the buffer, which is obviously incompatible. The test was carried out again without EDTA and DTT, and we did not meet any success. The major problem hampering the reading of the high concentration enzyme of 50 mM. A new optimization should be carried out with a lower concentration to avoid stoichiometry problems between the inhibitor and the enzyme.

Finally, improvements should be made about the design. To avoid the trap of **Joelinib**, which was too close to a highly potent and selective inhibitor of EGF-R, we chose a covalent inhibitor as

template. From the literature, we think that the N-acetyl phenylalanine has no binding or recognition role to play, but to sit in the broad hydrophobic pocket. It means that the activated form of **Celinib** cannot be called a true TCI, but a covalent inhibitor. To make a true TCI, we should use a hydrophobic drug-like fragment instead of N-Acetyl phenylalanine. Some new design as those presented on Figure 3. 21 could become candidates (Figure 3. 21).

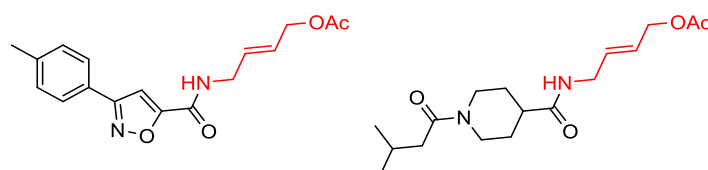


Figure 3. 21 New proposed inhibitors containing a drug-like recognition fragment (black) and a pro-warhead (red)

6 Conclusions

The second part of this thesis followed the same workflow as the first one, but instead of hydride transfers, focused on the implementation of discrete palladium complexes in the context of Alloc deprotection through Tsuji-Trost reaction. At the time we started to work on the topic, there had been seminal reports of Meggers, using ruthenium complexes, of Bradley, with supported nanoparticles, and Mascareñas with a few ruthenium complexes. Little was known about palladium (II) complexes, as they need to undergo a pre-activation to reach the oxidation stage (0) to become active. In our eyes, it could be seen as a challenge and an opportunity to selectively activate the catalyst in the context of vectorization.

In the first section of this work, we summarized our progress in different projects regarding the choice of an ideal fluorogenic for *in vitro* and *in cellulo* assays. As we needed to improve existing fluorogenic models, we developed several new probes based on a coumarin, a rhodamine or a xanthone derivative. Moreover, we were able to rationalize the criteria to discern good and bad fluorophores. The criteria are (i) an excitation within the visible range, (ii) a Stokes shift of min 20 nm, (iii) no degradation, (iv) no fluorescence quenching, (v) an on-off ration above 20-50, (vi) solubility in buffered medium with 1-2% DMSO and (vii) a one-step deprotection. For biology, a good model is expected to be cell permeable and lipophilic. We had two fluorophores matching most of the criteria, namely the **Rho(Boc-Alloc)** and the **Maudamine** (Figure 4. 1).

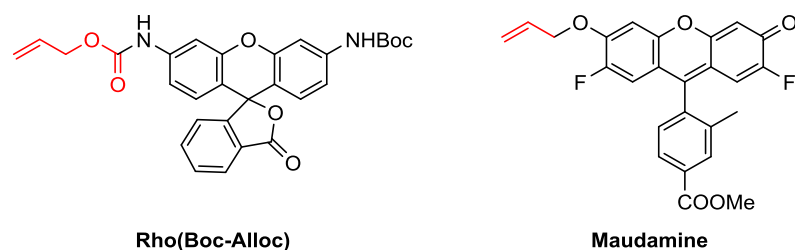
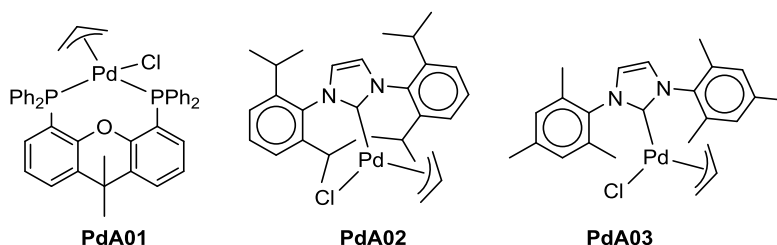
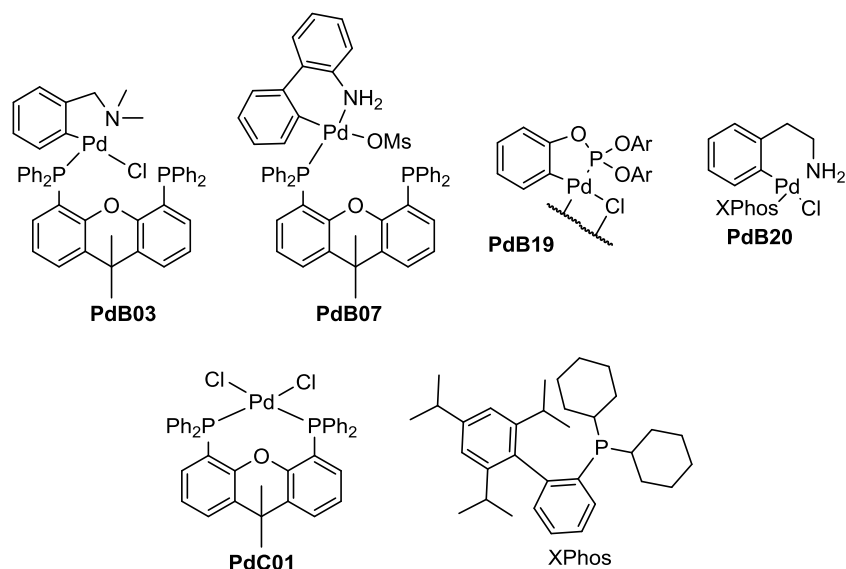


Figure 4. 1 In house developed fluorogenic models

With proper fluorogenic probes in hands, we were able to screen a library of 70 catalysts, mostly cantered on palladium, with diversity regarding the activity ligands (phosphines, NHC) and the activation mechanisms (Scheme 3. 79). At first, we found the structure of AllylPdCl(Xantphos), **PdA01**, as a good hit, we carried out an optimization regarding the catalyst amount and the nucleophile (glutathione). Valuable information could be taken, such as the existence of side reactions involving the catalyst and a 0.5 order respectively to the glutathione. A first screening enlightened the impact of the bite angle while biphosphines and showed that nitrogenated ligands were inefficient at catalysing the reaction. A broader screening showed the omnipresence of the Xantphos ligand and revealed different modes of activation. Among them, the π -allylpalladium complexes showed superiority, followed by various palladacycles and palladium dichlorides.



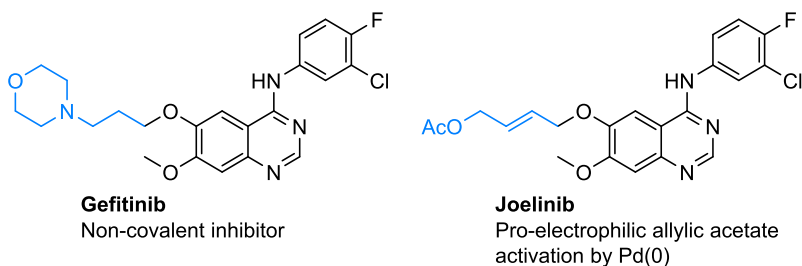


Scheme 3. 79 Hit catalysts found in the fluorogenic screening and validated in *cellulo*

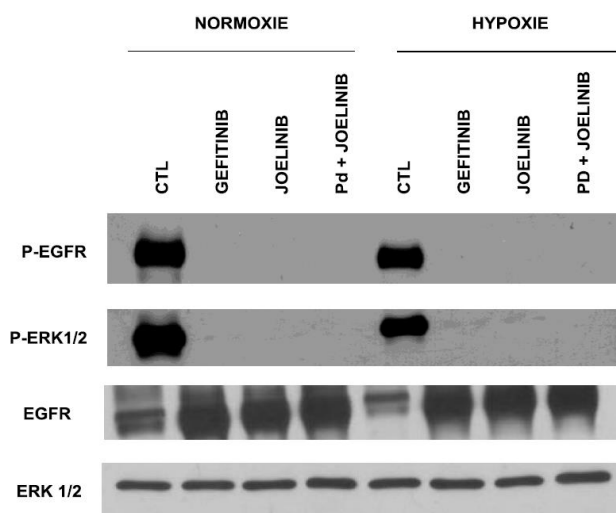
With a good picture *in vitro*, the best hits were tested *in cellulo*. Remarkably, some catalyst, like the Buchwald palladacycle or the XantphosPdCl₂, **PdC01**, performed differently *in cellulo* and *in vitro*. It is highly assumed that **PdC01** is bio-activated in the cells through an unknown mechanism. The diversity in the modes of activation is promising to control the activation to make sure that it activates at the desired time and place. To conclude the development phase, a fluorophore with selectivity for the endoplasmic reticulum was used for validation.

With tools developed, we wanted to move back towards our main goal, which is the use of the catalyst in a therapeutic context. Two approaches were selected. The first was focused on the use of a toxigenic and targeted prodrug, with attempts to vectorise the catalyst. The second aimed to develop a drug able to bind covalently to its probe thanks to a Tsuji Trost reaction.

and the binding mechanism was unknown (Scheme 3. 82). Due to the deeply buried and intracellular pocket, we don't know if the palladium penetrates in sufficient concentrations, meet the **Joelinib**, and if the **Joelinib**- π -allyl is small enough to fit in the pocket.



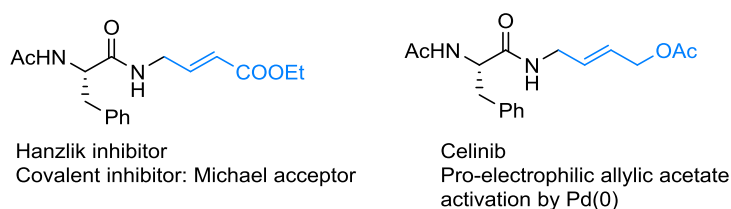
Scheme 3. 81 Design of **Joelinib** a targeted covalent inhibitor based on **Gefitinib**



Scheme 3. 82 Results obtained with **Joelinib** by Western blot: it inhibits the phosphorylation of EGF-R without the help of **PdA01**

We then moved to another inhibitor based on papain, which is a commercially available cysteine protease with a wide pocket capable of accommodating many different substrates with low specificity. Our design aimed to replace the Michael acceptor of a known inhibitor by an allylic acetate as a π -allyl precursor (Scheme 3. 83). Although we did not get convincing results due to several unseen flaws when the

experiments were done, we think that we used the wrong type of catalysts, as we used catalysts with a wide bite angle, thus making them reactive against any nucleophile present in larger quantities than the active site cysteine. New inhibitors with different palladium parts are being synthesized now.



Scheme 3. 83 Design of **Celinib**, a targeted covalent inhibitor of papain which needs to be activated by **PdA01**

If we come back to the initial goals of this project, we have been able to successfully find some new palladium catalysts through a screening process. We have been able to study their mechanism through an optimization. Their design was rationalized through two different screenings and for the first-time shed light on the rational design of this type of catalyst. *In cellulo* assays verified that the catalysts found were working in cells and, with optimization of the reaction conditions, could work in a biocompatible fashion.

Regarding the application from a therapeutic perspective, we have good signs that it will work if more work is put on this topic. We were able to design a first-generation of prodrug, which could be uncaged by our catalyst. However, solubility issues hampered the final stage of the release. Our first attempt of vectorization was unsuccessful due to a risky design and the incompatibility of the Pd and the Cu in the click reaction. Therefore, new designs were proposed to continue the work.

Regarding the use of π -allyl palladium complexes as electrophilic warheads, the results were more mitigated. Although the design and synthesis of inhibitors were not really an issue, making the system work was much harder to achieve. Indeed, we were unable to design an assay where enough parameters were controlled to truly determine whether the inhibitor was acting as designed. This work needs more in-depth work to synthesize π -allyl complexes, study their stability in function of the ligand, and see if those complexes can form covalent bonds with cysteines of small peptides before moving to applications on real biomolecules. The catalysts needed for this section have different requirements than for the previous one, unlike we thought at first.

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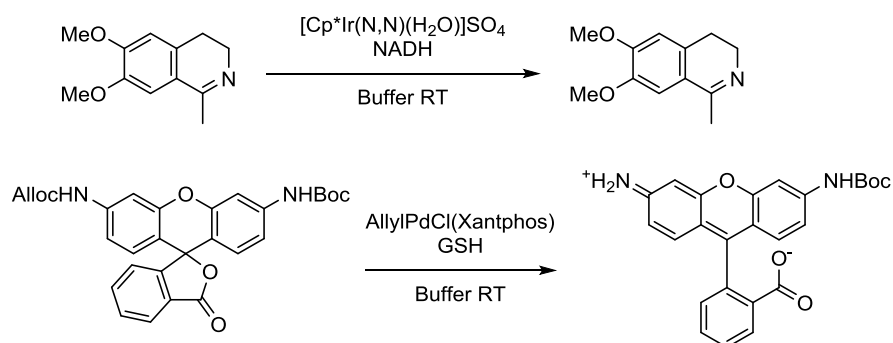
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Conclusions

Throughout this work, we have been involved in the development of new catalysts, mostly based on palladium, as well as therapeutical applications of the Tsuji-Trost deprotection of allyl carbamates in biocompatible conditions. We have been involved in many different steps from the design of new reactions to the proof of concepts and eventually evolving our systems to render them therapeutically relevant.

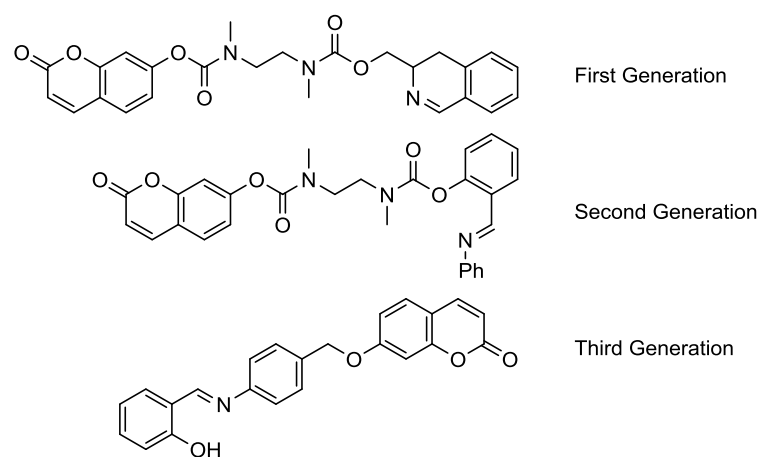
At first, we have set out to develop one new reaction of imine reduction, borrowing the concept of hydride transfer from NADH to transition metal complexes developed by Sadler and the classical works on transfer hydride reduction of carbonyls and imines (Scheme 4. 1). We also successfully implemented a series of new palladium catalysts to the allyl carbamate deprotection in the concept of Tsuji-Trost reaction, filling the gap of what we thought to be the most logical family of catalyst for *in cellulo* applications. Working with close-to-bioorthogonal reactions was facilitated with the experience our team had in developing copper catalysis over the last two decades. However, he had to innovate regarding the screening methods to increase the throughput of analysis, but more about how to observe all the conditions to make the reaction biocompatible. The use of fluorogenic reactions turned out to be capital, as it enabled us to follow up to 72 reactions at the time and follow the kinetics of all them with accuracy. It enabled us to gain more knowledge about the

mechanism with rudimentary tools, but with a robust mathematical treatment. We can regard this first goal as achieved.



Scheme 4.1 Model reactions studied in this work.

Then, the second phase consisted of the implementation of the catalytic systems in the cellular environment. Regarding the transfer hydride part, we were unable to reach this stage due to the failure to design fluorogenic probes (Scheme 4. 2). Our design contained some flaws due to the hydrolytic behaviour of imines and the few examples from the literature used more simple probes, with less good desirability on the perspective of the fluorescence properties.



Scheme 4.2 Fluorogenic reducible prodrugs.

Regarding the palladium part, we were able to successfully transfer the Alloc decaging to cells and validate the system in three

phases (Figure 4. 2). At first, the activity of the catalyst was measured by assessing the fluorescence of each test. Then, a Presto Blue test aims to demonstrate the viability of the cells after the test. Lastly, fluorescence imaging demonstrated that the fluorescence evolution was intracellular.

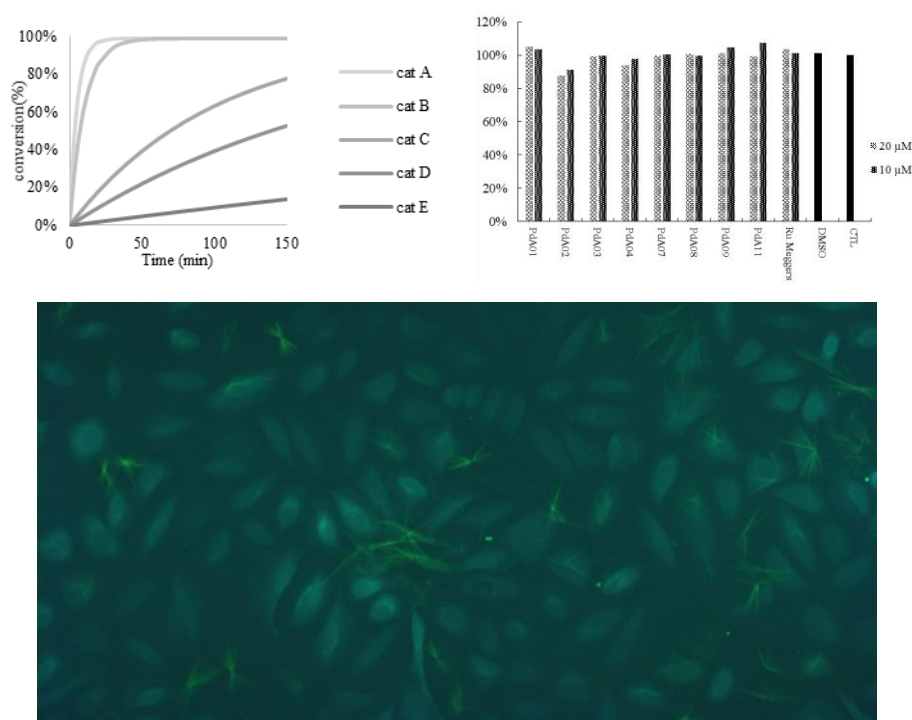
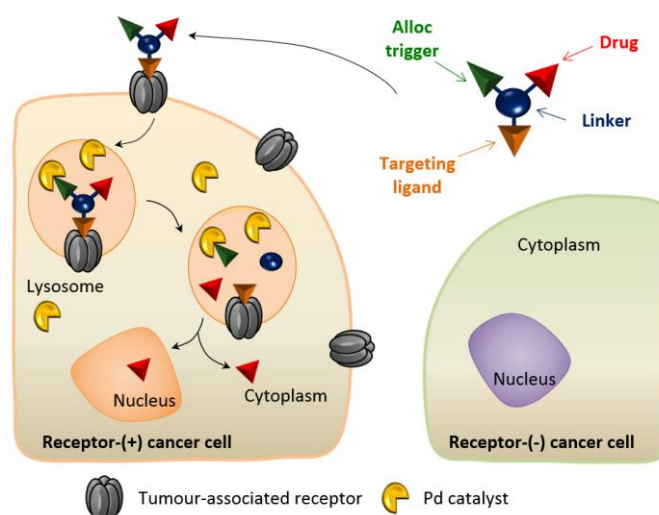


Figure 4. 2 To left: fluorogenic screening. Top right: Viability assay. Bottom. Fluorescence microscopy images

Later, two therapeutical applications were developed. In the first, we aimed to implement selectivity to the prodrug side by tethering a folic acid to the whole structure and used a potent cytotoxic, **MMAE**, as a payload (Scheme 4. 3). The prodrug was found to be significantly less toxic than the drug itself, allowing to work within a wide range of concentration. However, two problems were encountered. At first, the toxicity of the catalyst, found to be negligible over 4 h, started to be more intense while working in 72-

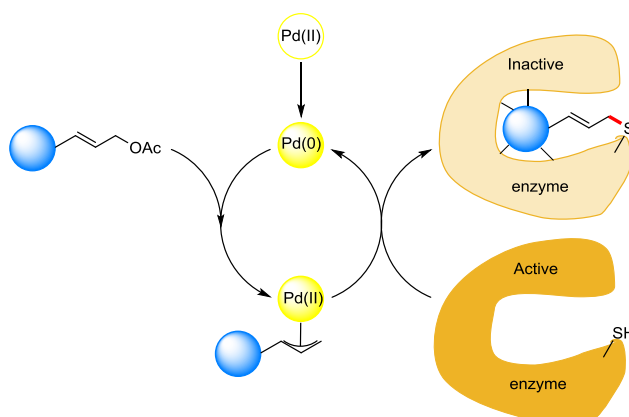
hours reactions. The second problem was related to the design of the probe itself, whose penultimate intermediate was not eliminating as expected, possibly due to solubility issues.



Scheme 4. 3 Molecular programmed system activated by bioorthogonal palladium catalysis

The second application suffered from being compared to the previous one. We were trying to use catalytic amounts of palladium to catalyse the formation of a covalent bond between the cysteine of a protein target and an inhibitor (Scheme 4. 4). Two systems were designed and the first one, targeting EGF-R, was tested *in cellulose*, and the second one, targeting papain, was tested *in vitro* on the isolated enzyme. The tests were carried out in a short period, and we did not have time to try enough and to study in detail the formation of π -allyl palladium complexes. A systematic stepwise approach should be more strictly followed, starting from the isolation of the reactive intermediate and their assay on the isolated target, followed by a study of the π -allyl palladium complexes formation *in-situ*, followed by *in vitro* tests if the previous ones are successful. These projects

enlightens that a more precise understanding of the behaviour of the complexes in a buffer or cellular medium is crucial to understand what they are capable of.



Scheme 4. 4 Catalyzed enzyme inhibition via Tsuji-Trost reaction.

To conclude, research on bioorganometallic catalysis should be supported. As every type of novel ideas, it deserves to have groups involved in the topic, launching projects, coming with innovative ideas and therapeutic applications to evaluate their feasibility without bias. In the same idea, transition metal complexes as anticancer (such as *cis*-platinum), PROTACs (proteolysis targeting chimaera) and ADC (antibody-drug conjugates) were considered simply as a curiosity invented by academic researchers. Nowadays, there are 2 ADC which are now approved drugs. PROTACs have brought solid proof of concept and are about to become marketed drugs in the next decade. We may not expect before 2050 the first patient treated by bioorthogonal chemistry.

Perspectives

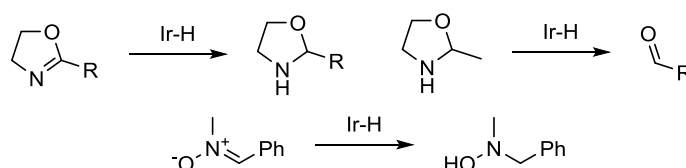
As the work was divided into two parts, the perspectives will be divided into two parts as well.

1 First part

Regarding the imine reduction itself, we have been to get a good picture of the mechanism, and what has not been done by us has been done by others, such as studies about the hydride formation (by Do and Fukuzumi). What is still missing in our work is a more systematic screening of the conditions. Now, this work has been made possible by the implementation of the plate reader, especially given that the model imine 1.3 has been found to be fluorescent. Using such a method would enable us to get, as we did for the second part, a better understanding of the whole reaction, with more conditions, and especially test the system at lower concentrations, which is crucial for biological applications.

In overcoming the problem of the prodrugs, we need to re-think about the trigger part from zero (Scheme 4. 5). We need to search for reducible and non-hydrolytic C=N bonds where the N is not nucleophilic and where the reduced form would be more nucleophilic. We did not spend a substantial amount of time on other substrates. Although we tried, the water solubility was always an issue as we did not want to develop a reaction working with organic co-solvents although targeting applications *in cellulo* where few co-solvents (DMSO or DMF) can be tolerated (max 1%). Therefore, besides

aldehydes and imines which are reported now, we could see if more exotic functions such as oxazolines and oxazolidines (the former being a masked carboxylic acid and the latter being a masked aldehyde), but also nitrones and in a more general way, any type of insaturations of the type C=X where X is a heteroatom.



Scheme 4. 5 Potential new triggers for a reduction-sensitive prodrug

Then, by working on the catalyst, we could develop the enantioselective version (Figure 4. 3). Although chiral bipyridines exist, they are not performant, and therefore phosphines could be better candidates as there is a huge variety regarding the stereo electronic parameters. We still doubt that there would be a better catalyst in terms of yield as the reaction is now thought to be limited by an equilibrium as all the steps are potentially reversible. However, enantioselectivity has no purpose in prodrug decaging but would be interesting if we work on a prodrug which needs an enantioselective reduction performed *in cellulo* to confer its toxicity.

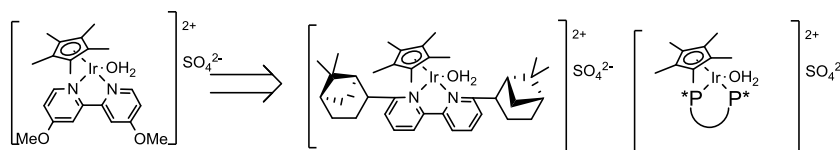


Figure 4. 3 Proposition of chiral ligands for asymmetric reductions

Regarding the prodrug, there is still a lot of room for improvement. For the moment, we still have not found a design for imine prodrug which meets the appropriate requirements, such as being stable, reactive by reduction and resistant to hydrolysis. There is

an opportunity by increasing the complexity of the trigger part to find a cyclic imine which would react after its reduction only (Figure 4. 4). Whereas we faced problems with cyclization trigger, there is room for improvement if we can prevent intramolecular attack by increasing the steric bulk near the imine and by using electron withdrawing groups to diminish the electronic density of the imine and make it non-reactive. However, increasing the complexity of the trigger increases the complexity of the synthesis, as well as the molecular weight, which could hamper cell penetration.

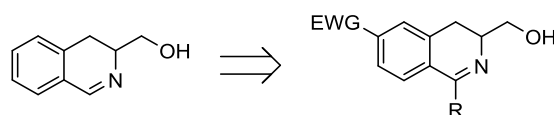


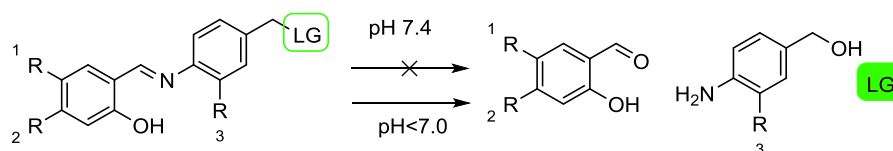
Figure 4. 4 Proposition of new cyclic imine trigger with improved properties

Work can be also done on the choice of the cytotoxic group of the prodrug only if the problem of the trigger is solved. As we learned from the work of Papot, cytotoxins such as monomethyl auristatin E, cyclopamine or MS-275 could be candidates, as well as Doxorubicin.

Regarding the catalyst, if the previous projects are working as expected, we would need to spend some effort on the catalyst to find one which meets all the criteria simultaneously: non-toxicity, cell penetration and activity. Therefore, studies like those performed for metallodrugs, but adapted to catalysts, should be performed.

However, at the short term, the chances to design and synthesize such a prodrug with a C=N bond as the trigger does not have many chances on the paper. Regarding the catalyst, any catalyst showing cell-penetration and stability may start to get unacceptably toxic due to ROS generation. Therefore, the only perspective that we see is to turn our disadvantages into advantages and search for a

trigger which hydrolyses at a narrow range of pH. Since imine hydrolysis is promoted by acids, we would be able to gain selectivity for tumoral cells, which classically trigger acidosis in the surrounding tissues provided the hydrolysis threshold is located below physiologic pH near 7.0 or even a bit below (Scheme 4. 6). On contrary to prodrugs which respond to an enzymatic trigger such as hydrolases, the release phenomenon may still happen at a basal level at physiological pH. Nevertheless, the absence of transition metal in such a process would make the system easier to study by HPLC and fluorescence spectroscopy. Although the design of the third generation of prodrug was flawed as the prodrug was already fluorescent, the easy synthesis would enable us to rapidly make modifications and thus increase the chances to find a suitable candidate due to the commercial availability of the building blocks.



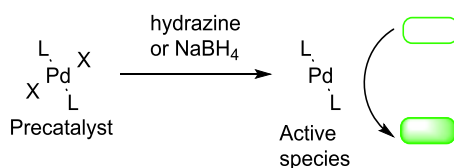
Scheme 4. 6 Hydrolytic imine-based prodrugs

2 Second part

In this second part, many goals have been achieved, demonstrated by the implementation of catalysts working in the cellular medium for Alloc deprotection, but also by all the backstage rigorous methodology of screening and the mechanistic investigation. Although the toughest part has been achieved, there are still some improvements which can be done to fully understand the behaviour of the catalysts, which is a preliminary to any clinical applications.

At first, their behaviour towards biomolecules in buffered medium must be understood better to get more insights about how the *in vitro* and the *in cellulo* activity differs in some cases. Then, ICM-MS studies would help us to understand better the internalization and the cellular distribution over the different cell compartments. Finally, there is still a bit of work related to the optimization of the *in vitro* test, as there is too much fluorescence evolving out the cells, making direct fluorescence imaging nearly impossible without multiple items of washing. With the most efficient concentrations and times of incubation found for *in cellulo* assays, it would then greatly improve the microscopy images, especially if confocal microscopy can be used in that case.

Throughout this study, we have used a relatively fast and easy to operate method based on brute force screening. However, as the activity is the product of activation x activity x stability x non-toxicity, we may have missed *in vitro* some hit structures which would give an activity if they are activated. We privileged in this project biocompatible conditions to seek for hits which activate in those conditions. The next idea would be to use a strong reducing agent, such as hydrazine or NaBH₄, to bypass the activation step and for the active species in quantitative yield and study its intrinsic activity and thus determine the contribution of the activity coming from the ligand (Scheme 4. 7). Then, taking the best ligand for activity, the next step would aim to study the mode of activation by trying many different activation ligands, such as π -allyl, dichlorides, or palladacycles, to see which one activates the best *in vitro* in a controlled way and in cells in their complex environment.



Scheme 4. 7 New screening principle based on the total reduction of the palladium by hydrazine or NaBH_4

Remarkably, during our studies, we were forced to notice that some of our complexes were far more cytotoxic than others. Among them, we noticed that **PdA01** and **PdB07** were really toxic but also our best candidates in terms of activity (Figure 4. 5). Since we know that those complexes are reduced (since we observed the catalytic activity which is only possible by Pd (0)), the toxicity may come either from the Xantphos Pd (0) or the residue of the activation ligand.

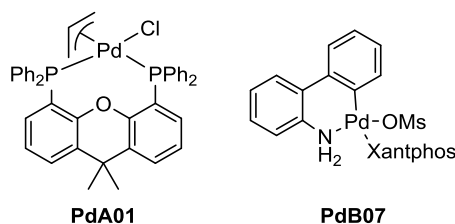
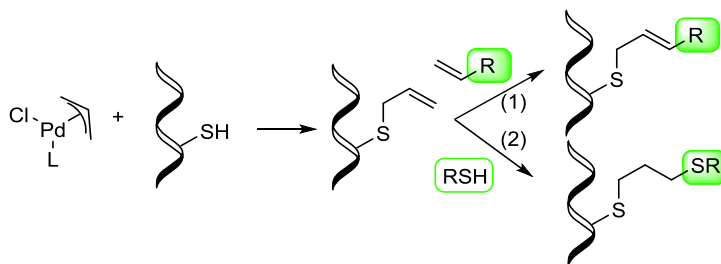


Figure 4. 5 Complexes **PdA01** and **PdB07**

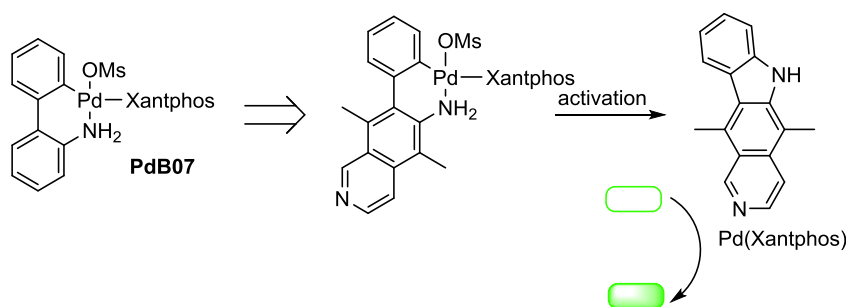
In the case of **PdA01**, the π -allyl is electrophile and should be easily captured by S-containing nucleophiles such as GSH or even cysteine of proteins. In case we can reduce the reactivity of the π -allyl by playing on the bite angle, we may consider developing complexes which can deliver their π -allyl to a specific target provided it has enough residence time in it thanks to a recognition moiety (Scheme 4. 8). It would act as an irreversible inhibitor by allylating a crucial residue in or near the active site. This can be also used as an anchor for click-thiol ene (2) reaction of metathesis (1) for post-modification of the target protein. In a more general way, it would be interesting to

see if we can direct a π -allyl palladium complex towards a specific cell compartment, such as mitochondrion, nucleus or endoplasmic reticulum and see if we can deliver the toxicity with a site selectivity for precise biological effects.



Scheme 4. 8 Palladium-catalysed protein allylation and post-functionalization

About **PdA07**, we think that the toxicity comes from the released ligand, the carbazole, which is a known DNA binder by intercalation in the minor groove. It could, however, be turned to our advantage as there are several approved drugs such as Ellipticin which contain a carbazole in their scaffold. It is, therefore, possible to envision a synergistic effect between this Pd-derivatized Ellipticin prodrug and a second Alloc-based prodrug (Scheme 4. 9).

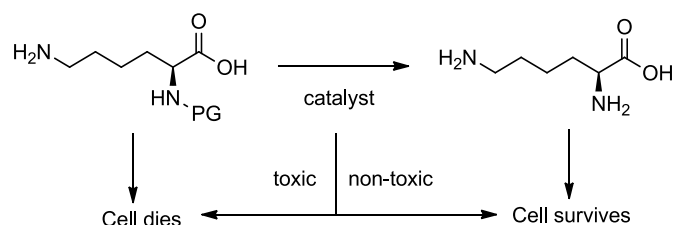


Scheme 4. 9 Bifunctional Ellipticin prodrug

As demonstrated by Hartinger, there is a possibility that some palladium complexes may bind to a specific target without bearing a bioactive ligand (Plecstatin is a selective metallodrug inhibiting plectin). It would be interesting to study *via* a pulldown experiment

coupled to *shotgun proteomics* the potential binding targets and determine the mode of action and see if there is any selectivity for this type of metallodrugs. Unlike the so-called small molecules, whose behaviour can be predicted thanks to a lot of tools, we do not have any predictive to know their activity, their mode of action or their properties in biological medium.

In a more global context, we also need to improve our biological tests in terms of precision, high throughput and reliability. One idea would involve using auxotroph cells in a nutriment or an amino acid, such as lysine, which would be provided in a protected form to the cell (Scheme 4. 10). If the catalyst is efficient, then the cell could survive. If the catalyst is toxic or inactive, the cell could die. By a simple one experiment readout with a simple test such as Presto Blue[®], it would be possible to get a binary response whether it works or not, increasing the throughput and leaving only the positive cases for detailed studies.

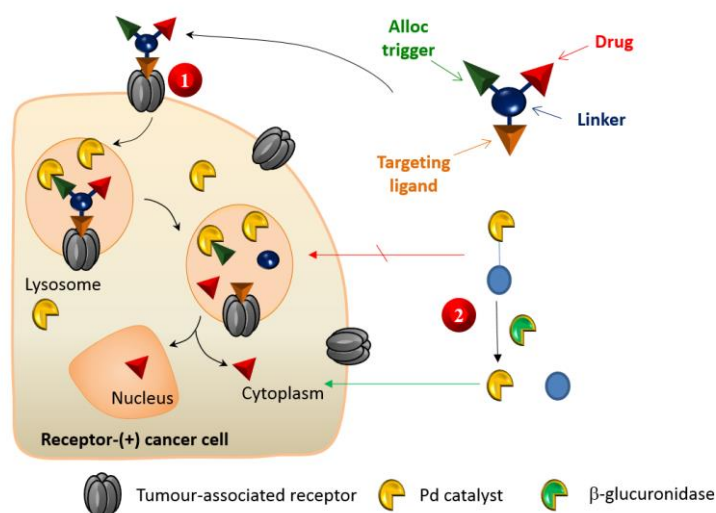


Scheme 4. 10 High throughput screening based on auxotroph cells.

One application among the two featuring the second part of this work seems to be the closest to achieve our final goal exposed in the objective section. The main question to be addressed is how to bring the prodrug and the catalyst to the cancer cells, leaving the healthy cells untouched. Weissleder opted for the classical micelle

strategy through EPR effect. Two strategies are available as a conclusion of this work.

The first one will rely on a double selectivity for the folate receptor thanks to the prodrug and the β -glucuronidase for the catalyst (Scheme 4. 11). The first step is to design an improved version of the prodrug by fixing the design flaws of the first generation, by shortening the PEG from 10 units and by circumventing the aniline intermediate which could be prone to precipitation. By this way, we would obtain the exact same intermediate as Papot, and his properties are fully known by the team.

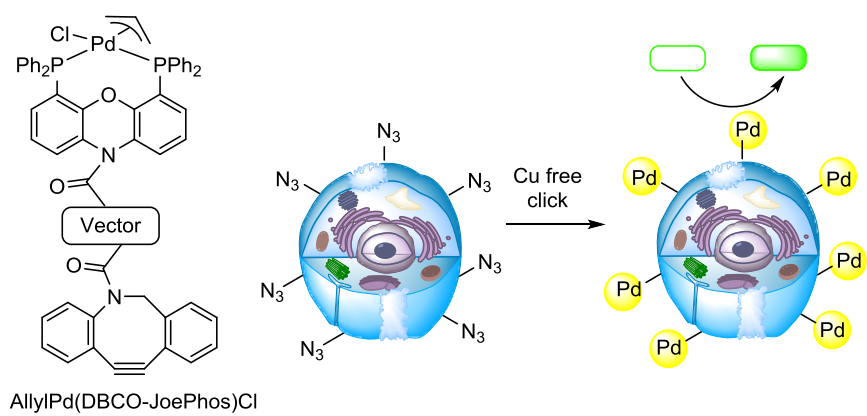


Scheme 4. 11 Double targeting. 1: Folic acid-receptor mediated internalization.
2: β -glucuronidase activation of the catalyst, which is then able to cross the membrane

Then, the ligand of the catalyst would need to be modified to permit its tethering to a β -glucuronide vector, like the early generation prodrug reported by Papot without folic acid. *N*-Xantphos would need to be elongated by a linker featuring an amine, given that the phenoxazine is very unreactive.

Such a method would enable us to get a double selectivity: the prodrug would be unable to cross the membranes due to its large molar mass (around 1600) and would need to bind to the folate receptor before internalization *via* endocytosis. The catalyst would be very polar due to the glucuronic acid and would be unable to be internalized. The extracellular β -glucuronidase exclusively in the tumoral microenvironment would be able to cut off the sugar part, making the catalyst able to cross the membranes of tumour cells.

The second perspective involves glycoengineered cells exposing azide functions on their outer membrane glycoproteins' sugars. The idea would be to use a clickable catalyst thanks to a cyclooctyne side chain. In that case, the reaction will be extracellular, and the cells should keep their activity even after being washed (Scheme 4. 12). The prodrug, if successful in the previous step, would feature a very hydrophilic chain instead of the PEG to prevent from its internalization. This means that if the catalyst is injected first, it would either bind to the glyco-engineered cancer cells or be eliminated, and after a subsequent prodrug injection, the extracellular uncaging would only take place near the tumour.



Scheme 4.12 Glycoengineered cell tagging via copper free click chemistry for prodrug release

Experimental part

Experimental part

1 General considerations

Commercial reagents were purchased from Acros, Sigma-Aldrich, TCI and Strem and used without purification unless stated otherwise. Unless specifically mentioned, the reactions were performed under an argon atmosphere using flame-dried glassware and distilled solvent. Toluene was distilled from sodium under an argon atmosphere. THF was distilled on sodium/benzophenone under an argon atmosphere. Dichloromethane and acetonitrile were distilled on CaH_2 under an argon atmosphere. Solvents used for work up were of technical grade.

Column Chromatography was carried out on silica gel (ROCC 60, 40-63 Dm). Thin Layer Chromatography analyses were performed on commercial aluminium plates bearing a 0.25 mm layer of Merck Silica gel 60_F 254. Components were visualized by illumination with a short wavelength UV light and/or staining in a solution of potassium permanganate or vanillin stain followed by heating.

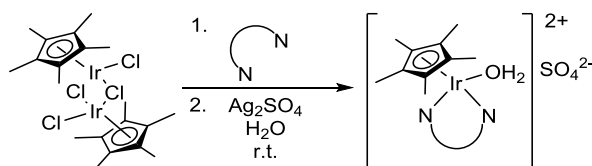
All ^1H and ^{13}C -NMR spectra were recorded on Bruker Advance 300 or 500 spectrometer. ^1H -NMR and ^{13}C -NMR chemical shifts were reported on the scale in ppm relative to TMS (0 ppm), CDCl_3 (7.26 ppm for ^1H , 77.16 ppm for ^{13}C) or CD_2Cl_2 (5.32 ppm). Spectral features were designed as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and bs = broad singlet.

2 First part

2.1 Imine reduction

2.1.1 Synthesis of catalysts

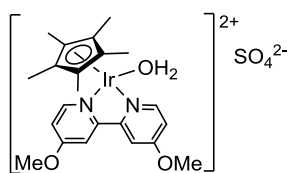
2.1.1.1 General procedure



The catalysts were synthesised according to a literature procedure.⁹⁵⁻⁹⁶ In a typical procedure for **Ir1**, (Cp*IrCl₂)₂ (20 mg, 0.25 μmol), was dissolved in 2.5 mL of MeOH in a GC vial under stirring under air. Then, the N,N ligand (0.5 mmol, 2 equiv.) was added. The heterogeneous mixture was stirred for 15 min, after which the yellow-orange solution turned bright yellow. The mixture was evaporated to dryness to give a bright yellow solid. Then, 3.3 mL of distilled water was added, followed by the addition of Ag₂SO₄ (15.8 mg, 50 μmol, 2 equiv.). The mixture turned rapidly cloudy as a white solid appears. The mixture was stirred under air in the dark overnight, after which the solution was filtered on celite to remove AgCl, and the solution was evaporated to dryness to yield the desired complex as a bright yellow powder. The spectra were compared to those reported in the literature.

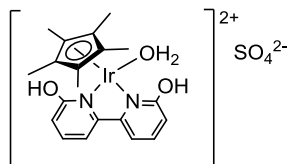
2.1.1.2 Characterizations

a) [Cp*Ir(4,4'-diMeObpy)(H₂O)]SO₄ (**Ir1**)



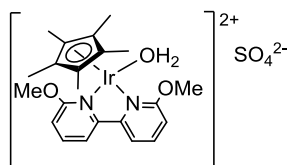
Bright yellow powder (30.8 mg, 94%). ¹H NMR (300 MHz, D₂O) δ 8.72 (d, *J* = 6.6 Hz, 2H), 7.87 (d, *J* = 2.7 Hz, 2H), 7.30 (dd, *J* = 6.6, 2.7 Hz, 2H), 4.04 (s, 6H), 1.59 (s, 15H). ¹³C NMR (75 MHz, D₂O) δ 169.3, 157.6, 149.8, 117.1, 111.6, 88.1, 59.2, 8.7

b) [Cp*Ir(2,2'-diOHbpy)(H₂O)]SO₄ (Ir2)



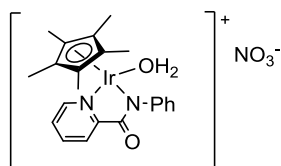
Bright yellow powder (90%). ¹H NMR (300 MHz, Deuterium Oxide) δ 7.97 – 7.77 (m, 2H), 7.64 (d, *J* = 7.6 Hz, 2H), 7.01 (d, *J* = 8.4 Hz, 2H), 1.43 (s, 15H).

c) [Cp*Ir(2,2'-diMeObpy)(H₂O)]SO₄ (Ir3)



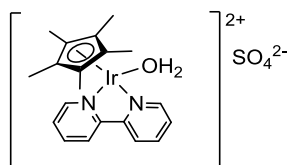
Bright yellow powder (90%). ¹H NMR (300 MHz, Deuterium Oxide) δ 8.29 – 8.14 (m, 2H), 8.00 (d, *J* = 7.7 Hz, 2H), 7.39 (d, *J* = 8.6 Hz, 2H), 4.20 (s, 6H), 1.50 (s, 15H).

d) [Cp*Ir(picolinamine)(H₂O)]NO₃ (Ir4)



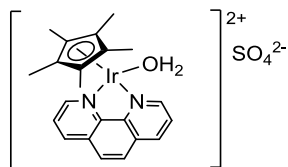
Bright yellow powder (95%). ¹H NMR (300 MHz, Deuterium Oxide) δ 8.90 (d, *J* = 5.0 Hz, 1H), 8.17 (td, *J* = 7.8, 1.4 Hz, 1H), 8.00 (d, *J* = 7.1 Hz, 1H), 7.81 – 7.65 (m, 1H), 7.47 (t, *J* = 7.8 Hz, 2H), 7.34 – 7.20 (m, 3H), 4.15 (dq, *J* = 14.3, 7.1 Hz, 1H), 1.35 (s, 15H).

e) [Cp*Ir(bpy)(H₂O)]SO₄



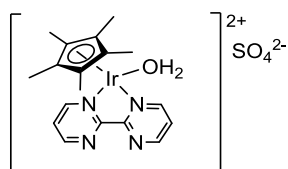
Bright yellow powder (28.4 mg, 97%), ¹H NMR (300 MHz, D₂O) δ 9.09 (dd, *J* = 5.4, 1.3 Hz, 2H), 8.56 – 8.47 (m, 2H), 8.30 (td, *J* = 8.0, 1.5 Hz, 2H), 7.91 – 7.81 (m, 2H), 1.64 (s, 15H). ¹³C NMR (75 MHz, D₂O) δ 156.2, 151.7, 141.8, 129.4, 124.5, 89.5, 7.8.

f) [Cp*Ir(phen)(H₂O)]SO₄



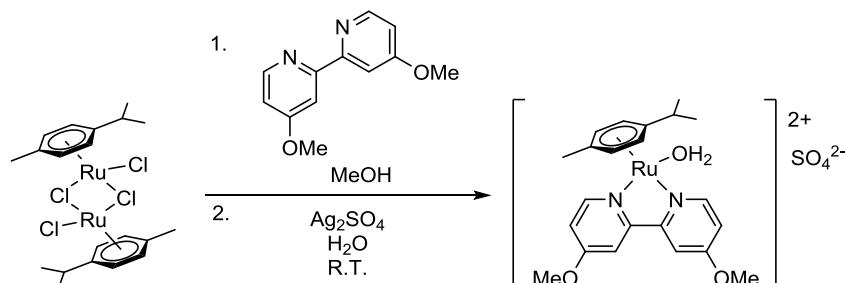
Bright yellow powder (28.5 mg, 93%), ¹H NMR (300 MHz, D₂O) δ 9.38 (dd, *J* = 5.3, 1.2 Hz, 2H), 8.78 (dd, *J* = 8.3, 1.2 Hz, 2H), 8.11 (s, 2H), 8.07 (dd, *J* = 8.3, 5.3 Hz, 2H), 1.61 (s, 15H). ¹³C NMR (75 MHz, D₂O) δ 152.0, 147.3, 140.8, 131.4, 128.3, 127.5, 89.4, 8.0.

g) [Cp*Ir(bpm)(H₂O)]SO₄



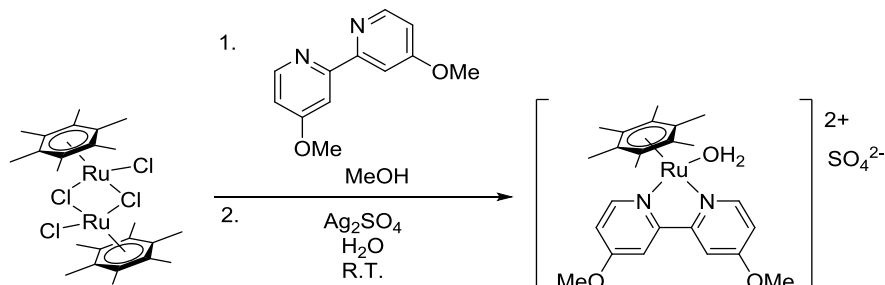
Orange powder (24.4 mg, 83%), ¹H NMR (300 MHz, D₂O) δ 9.37 (dd, *J* = 5.8, 2.0 Hz, 2H), 9.27 (dd, *J* = 5.0, 2.0 Hz, 2H), 8.03 (dd, *J* = 5.7, 4.9 Hz, 2H), 1.63 (s, 15H). ¹³C NMR (75 MHz, D₂O) δ 161.5, 159.4, 159.2, 125.8, 89.8, 7.9.

h) [p-cym Ru(4,4'-diMeObpy)(H₂O)]SO₄ (Ru2)



The complex was synthesised according to a literature procedure.⁹⁵⁻⁹⁶ (56.1 mg, 99%). ¹H NMR (300 MHz, D₂O) δ 9.32 (d, *J* = 6.7 Hz, 2H), 7.83 (d, *J* = 2.7 Hz, 2H), 7.33 (dd, *J* = 6.6, 2.7 Hz, 2H), 6.17 (d, *J* = 6.4 Hz, 2H), 5.93 (d, *J* = 6.4 Hz, 2H), 4.03 (s, 6H), 2.60 – 2.31 (m, 1H), 2.17 (s, 3H), 0.91 (d, *J* = 6.9 Hz, 6H). ¹³C NMR (75 MHz, D₂O) δ 169.1, 156.7, 156.2, 114.1, 110.8, 104.6, 99.1, 87.0, 82.6, 56.9, 30.5, 21.2, 17.8.

i) [(hmb)Ru(4,4'-diMeObpy)(H₂O)]SO₄ (Ru3)



The complex was synthesised according to a literature procedure.⁹⁵⁻⁹⁶ (50.6 mg, 85%). ¹H NMR (300 MHz, D₂O) δ 8.87 (d, J = 6.6 Hz, 2H), 7.81 (d, J = 2.7 Hz, 2H), 7.37 (dd, J = 6.6, 2.7 Hz, 2H), 4.03 (s, 6H), 2.07 (s, 18H). ¹³C NMR (75 MHz, D₂O) δ 168.3, 155.8, 154.0, 114.0, 110.1, 95.2, 56.7, 14.9.

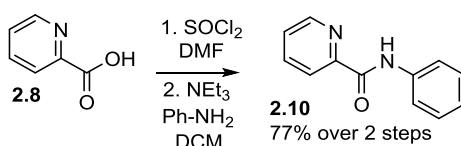
2.1.1.3 Catalyst Ru1

The complex **Ru1** was synthesized according to a literature procedure. A 25 mL round bottom flask is filled with ((*p*-cym)RuCl₂)₂ (51.4 mg, 83.6 μmol), 12 mL of distilled DCM, (S,S)-TsDPEN (61.8 mg, 167 μmol) and fine powder KOH (68 mg, 1.18 mmol). The orange solution is stirred for 20 min under Ar. Then, 1.2 mL of distilled water is added and the solution is stirred vigorously for 5 more min. It turns from dark orange to dark purple in 1-2 min. Water (20 mL) and DCM (20 mL) are added to the mixture and the water phase is extracted by 2x20 mL DCM. The organic phases are combined, dried by Na₂SO₄, and concentrated under reduced pressure to yield a dark purple solid (61 mg, 61%). The catalyst needs to be conserved in the fridge for 1.5 week, after which it turns dark brown).

¹H-NMR (CDCl₃): 6.61(m,1H), 6.49-7.00 (m,12H), 6.31 (d, 2H, J=9.2Hz), 5.67-5.84 (m, 4H), 3.66 (d, 1H, J=1.8Hz) , 3.54 (dd, 1H, J=10.8 and 10.8Hz), 3.26 (m, 1H) , 3.07(m, 1H), 2.19 (s, 3H), 2.28 (3H), 1.32 and 1.34 (d,6 H, J=5.2Hz)

2.1.2 Synthesis of ligands

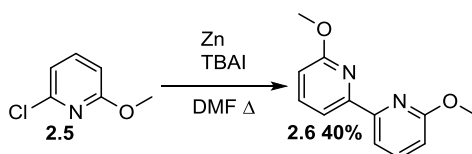
2.1.2.1 Do's ligand (2.10)



Do's ligand (**2.10**) was synthesized according to a literature procedure.⁶⁷ Yield = 77% (white powder)

¹H NMR (300 MHz, Chloroform-*d*) δ 10.03 (s, *J* = 44.8 Hz, 1H), 8.61 (d, *J* = 4.7 Hz, 1H), 8.30 (d, *J* = 7.8 Hz, 1H), 7.90 (td, *J* = 7.7, 1.7 Hz, 1H), 7.79 (dd, *J* = 8.6, 1.0 Hz, 2H), 7.48 (ddd, *J* = 7.6, 4.8, 1.2 Hz, 1H), 7.39 (dd, *J* = 8.0 Hz, 2H), 7.15 (dd, *J* = 7.7 Hz, 1H).

2.1.2.2 Yamaguchi's ligand (**2.7**)



The Yamaguchi ligand *bis* methoxy (**2.6**) was synthesized according to a literature procedure.¹⁸⁵⁻¹⁸⁶ Yield = 43% (white powder)

¹H NMR (300 MHz, Chloroform-*d*) δ 8.02 (d, *J* = 7.3 Hz, 2H), 7.78 – 7.58 (t, 6.9 Hz, 2H), 6.75 (d, *J* = 8.1 Hz, 2H), 4.04 (s, 6H).

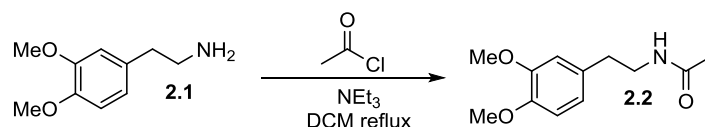


The Yamaguchi ligand (**2.7**) was synthesized according to a literature procedure.¹⁸⁵⁻¹⁸⁶ Yield = 54% (grey powder)

The compound was too insoluble in DMSO for a ¹H-NMR.

2.1.3 Synthesis of substrates

2.1.3.1 *N*-(3,4-dimethoxyphenethyl)acetamide (**2.2**)

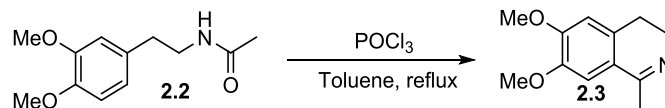


N-(3,4-dimethoxyphenethyl)acetamide (**2.2**) was prepared according to a literature procedure.¹⁸⁷ Pale-yellow solid (*m* = 12.4 g, 97%) and was used without any further purification in the next step.

¹H NMR (300 MHz, CDCl₃) δ 6.81 (d, *J* = 8.2 Hz, 1H), 6.73 (m, 2H), 5.49 (s, 1H), 3.86 (d, *J* = 2.1 Hz, 6H), 3.49 (dd, *J* = 13.0, 6.9 Hz, 2H),

2.76 (t, $J = 7.0$ Hz, 2H), 1.94 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 170.0, 149.0, 147.7, 131.3, 120.6, 111.9, 111.3, 55.9, 40.8, 35.2, 23.4

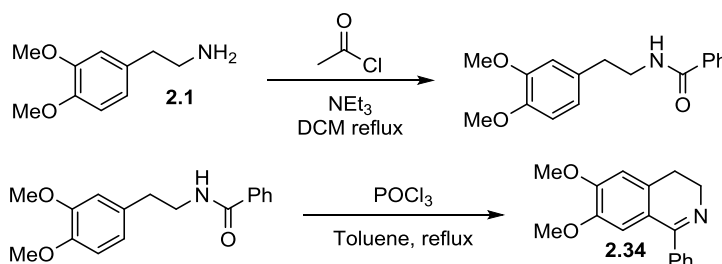
2.1.3.2 6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline (2.3)



6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline (**2.3**) was prepared according to a literature procedure.¹⁸⁸ Long transparent needles (m = 1.115 g, 81%).

^1H NMR (300 MHz, CDCl_3) δ 6.99 (s, 1H), 6.68 (s, 1H), 3.91 (d, $J = 3.0$ Hz, 6H), 3.63 (td, $J = 7.6, 1.3$ Hz, 2H), 2.64 (m, 2H), 2.38 (t, $J = 1.3$ Hz, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 151.7, 147.7, 131.4, 121.8, 110.3, 110.3, 109.3, 56.2, 46.0, 25.7, 22.8.

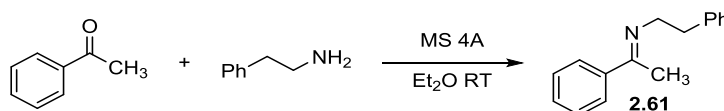
2.1.3.3 6,7-dimethoxy-1-phenyl-3,4-dihydroisoquinoline (2.34)



The same procedure was employed as the one to synthesize the 6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline, starting from 5.3 mmol of the 2-(3,4-dimethoxyphenyl)ethan-1-amine. Beige powder (1.07g, 76%)

^1H NMR (300 MHz, CDCl_3) δ 7.67 – 7.54 (m, 2H), 7.48 – 7.39 (m, 3H), 6.79 (d, $J = 3.5$ Hz, 2H), 3.95 (s, 3H), 3.87 – 3.76 (m, 2H), 3.72 (s, 3H), 2.83 – 2.65 (m, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ 166.7, 150.9, 147.1, 139.1, 132.6, 129.3, 128.8, 128.2, 121.6, 111.5, 110.2, 56.1, 56.0, 47.7, 26.0.

2.1.3.4 (E)-N-benzyl-1-phenylethan-1-imine (2.61)

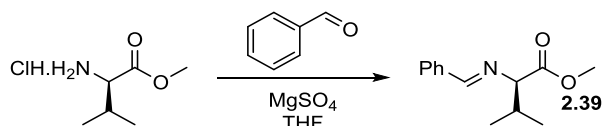


The imine (**2.61**) was synthesized according to a literature procedure.¹⁸⁹ Yellow solid (760 mg, 33%).

^1H NMR (300 MHz, CDCl_3) δ 7.80 – 7.70 (m, 2H), 7.37 (dd, $J = 3.8, 2.7$ Hz, 4H), 7.29 (d, $J = 3.2$ Hz, 3H), 7.26 – 7.16 (m, 1H), 3.74 (t, $J =$

7.5 Hz, 2H), 3.08 (t, $J = 8.0, 7.1$ Hz, 2H), 2.12 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 165.9, 141.4, 140.7, 129.5, 129.1, 128.7, 128.6, 128.4, 128.3, 126.7, 126.1, 54.2, 37.5, 15.6.

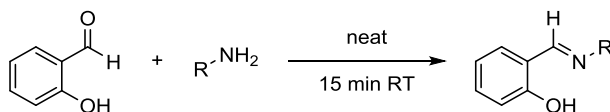
2.1.3.5 Methyl (R,E)-2-(benzylideneamino)-3-methyl-butanoate (2.39)



The imine (**2.39**) was prepared according to a literature procedure.¹⁹⁰ Transparent thick oil (267 mg, 49%).

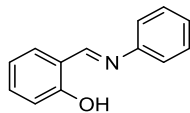
^1H NMR (300 MHz, CDCl_3) δ 8.25 (s, 1H), 7.93 – 7.72 (m, 2H), 7.50 – 7.37 (m, $J = 5.0, 2.3$ Hz, 3H), 3.75 (s, 3H), 3.67 (d, $J = 7.3$ Hz, 1H), 2.49 – 2.29 (m, 1H), 0.95 (dd, $J = 8.9, 6.8$ Hz, 6H). ^{13}C NMR (75 MHz, CDCl_3) δ 172.6, 163.4, 135.8, 131.2, 128.7, 128.7, 80.6, 52.1, 31.8, 19.6, 18.8.

2.1.3.6 Salicylidene series



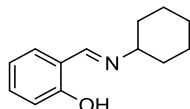
The salicylideneaniline substrates were synthesized following literature methods.¹¹³ A glass vial was filled under air with the amine (10 mmol). Then, the salicylaldehyde (10 mmol) was added by syringe during which the reaction mixture turned bright yellow. The reaction mixture was stirred with a spatula. The reaction was complete upon total solidification of the reaction mixture (in case of solid product) or when a bright yellow emulsion was obtained (in case of liquid products).

a) (E)-2-((phenylimino)methyl)phenol (2.37)



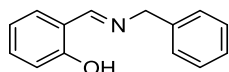
Yellow crystalline solid (99%), ^1H NMR (300 MHz, CDCl_3) δ 13.26 (s, 1H), 8.58 (s, 1H), 7.46 – 7.32 (m, 4H), 7.32 – 7.21 (m, 3H), 7.02 (dd, $J = 8.8, 1.1$ Hz, 1H), 6.92 (td, $J = 7.4, 1.1$ Hz, 1H). ^{13}C NMR (75 MHz, CDCl_3) δ 162.8, 161.2, 148.6, 133.2, 132.4, 129.5, 127.0, 121.3, 119.3, 119.2, 117.4.

b) (E)-2-((cyclohexylimino)methyl)phenol (2.58)



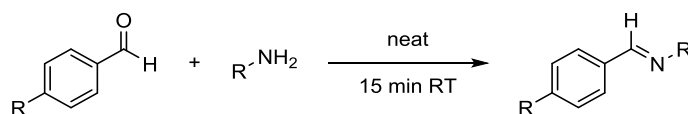
Yellow oil (93%), ^1H NMR (300 MHz, CDCl_3) δ 13.86 (s, 1H), 8.38 (s, 1H), 7.38 – 7.22 (m, 2H), 6.97 (d, J = 8.2 Hz, 1H), 6.88 (td, J = 7.5, 1.1 Hz, 1H), 3.42 – 3.12 (m, J = 9.9, 3.6 Hz, 1H), 1.94 – 1.75 (m, 4H), 1.73 – 1.21 (m, 6H). ^{13}C NMR (75 MHz, CDCl_3) δ 162.3, 161.6, 132.1, 131.2, 119.1, 118.4, 117.2, 67.6, 34.4, 25.7, 24.5.

c) (E)-2-((benzylimino)methyl)phenol (2.57)



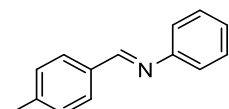
Yellow oil (90%), ^1H NMR (300 MHz, CDCl_3) δ 13.41 (s, 1H), 8.43 (s, 1H), 7.40 – 7.23 (m, 7H), 6.97 (d, J = 8.2 Hz, 1H), 6.88 (td, J = 7.5, 1.1 Hz, 1H), 4.80 (s, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ 165.7, 161.2, 138.3, 132.5, 131.5, 128.8, 128.7, 127.9, 127.5, 127.2, 126.9, 119.0, 118.8, 117.2, 63.3, 46.7.

2.1.3.7 N-benzylideneamines



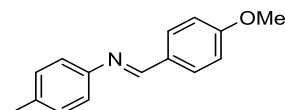
The *N*-benzylideneamines substrates were synthesized following an adapted literature method.¹¹³

a) N-phenyl-1-(p-tolyl)methanimine (2.47)



Pale-yellow powder (92%), ^1H NMR (300 MHz, CDCl_3) δ 8.42 (s, 1H), 7.79 (d, J = 8.1 Hz, 2H), 7.39 (dd, J = 8.5, 6.9 Hz, 2H), 7.32 – 7.17 (m, 5H), 2.42 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 160.5, 152.4, 142.0, 133.8, 129.7, 129.3, 128.9, 125.9, 121.0, 21.8.

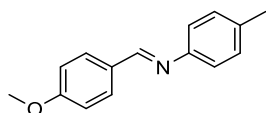
b) N-(4-methoxyphenyl)-1-(p-tolyl)methanimine (2.48)



Pale-yellow powder (87%), ^1H NMR (300 MHz, CDCl_3) δ 8.48 (s, 1H), 7.97 – 7.82 (m, 2H), 7.54 – 7.39 (m, 3H), 7.24 (d, J = 8.9 Hz,

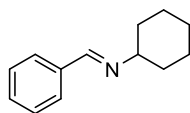
2H), 6.93 (d, $J = 8.9$ Hz, 2H), 3.83 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 158.6, 158.4, 145.0, 136.6, 131.2, 128.9, 128.7, 122.3, 114.5, 55.6.

c) 1-(4-methoxyphenyl)-N-(p-tolyl)methanimine (2.46)



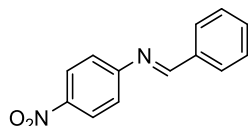
Pale-yellow powder (85%), ^1H NMR (300 MHz, CDCl_3) δ 8.38 (s, 1H), 7.83 (d, $J = 8.8$ Hz, 2H), 7.18 (d, $J = 8.2$ Hz, 2H), 7.11 (d, $J = 8.4$ Hz, 2H), 6.97 (d, $J = 8.8$ Hz, 2H), 3.86 (s, 3H), 2.36 (s, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 162.2, 159.1, 149.9, 135.5, 130.5, 129.8, 129.5, 120.9, 114.3, 55.5, 21.1.

d) N-cyclohexyl-1-phenylmethanimine (2.45)



Transparent oil (99%); ^1H NMR (300 MHz, CDCl_3) δ 8.32 (s, 1H), 7.79 – 7.67 (m, 2H), 7.44 – 7.35 (m, 3H), 3.20 (tt, $J = 10.4, 4.1$ Hz, 1H), 1.94 – 1.50 (m, 7H), 1.48 – 1.16 (m, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 158.7, 136.7, 130.4, 128.6, 128.2, 70.2, 34.5, 25.8, 25.0.

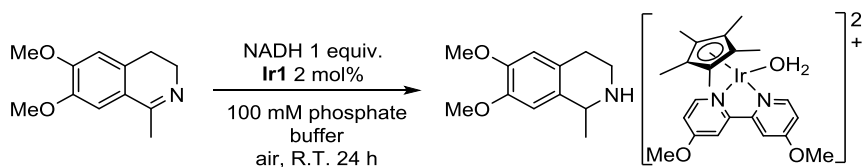
e) N-(4-nitrophenyl)-1-phenylmethanimine (2.51)



Yellow powder (92%), ^1H NMR (300 MHz, CDCl_3) δ 8.56 (s, 1H), 8.34 (d, $J = 8.8$ Hz, 2H), 8.09 (d, $J = 8.8$ Hz, 2H), 7.44 (t, $J = 7.6$ Hz, 2H), 7.37 – 7.23 (m, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 157.5, 151.1, 149.4, 141.7, 129.6, 129.5, 127.2, 124.2, 121.1.

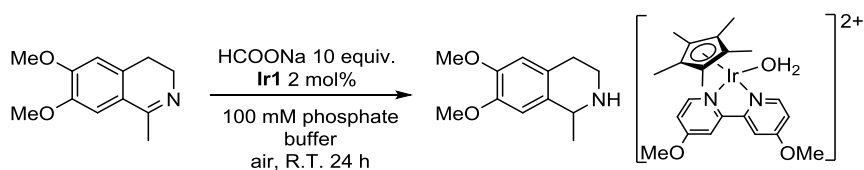
2.1.4 Catalytic tests

2.1.4.1 Reduction of 2.3 with NADH



In a typical procedure, a 3.5 mL vial equipped with a stirring bar was filled under air with the imine **2.3** (10.4 mg, 50 μ mol), pH 7 phosphate buffer 100 mM (3.3 mL, 15 mM in substrate) and upon stirring, NADH was added (50 μ mol, 1 equiv., 35.8 mg), followed by the **Ir1** (0.7 mg, 2 mol %). The mixture turned instantaneously bright yellow and the cap was closed without any Ar flush. The mixture was stirred for 24 h, during which TLC follow-up was carried out using DCM/MeOH 95:5. After 24 h, the reaction was poured in 3 mL of 2M NaOH, during which the reaction medium turned pale-yellow. The aqueous phase was extracted by 3x15 mL DCM and the organic phase was dried with Na₂SO₄ and was concentrated under reduced pressure to dryness. A known amount of dimethyl terephthalate was added as internal standard. The pale-yellow residue was dissolved in 1 mL of CDCl₃ and was analysed by ¹H NMR. The NMR yield was calculated by comparing the aromatic peak of the dimethyl terephthalate (δ = 8.09 ppm) with the aromatic peaks of the starting material (δ = 6.98 and 6.68 ppm) and the aromatic peaks of the product (δ = 6.62 and 6.56 ppm).

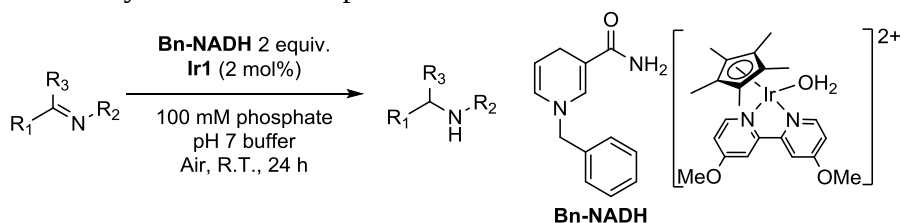
2.1.4.2 Reduction of **2.3** with HCOONa



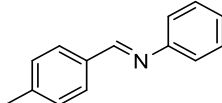
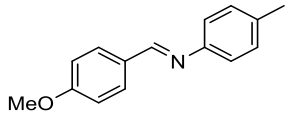
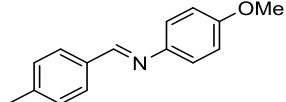
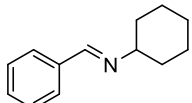
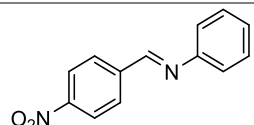
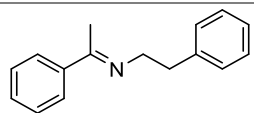
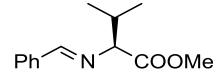
A 3.5 mL vial equipped with a stirring bar was filled under air with the imine **2.3** (20.7 mg, 100 μ mol), pH 7 phosphate buffer 100 mM (6.7 mL, 15 mM in substrate) and upon stirring, HCOONa was added (1 mmol, 10 equiv., 69.5 mg), followed by the **Ir1** (1.3 mg, 2 mol %). The mixture turned instantaneously bright yellow and the cap was closed without any Ar flush. The mixture was stirred for 24 h, during which TLC follow-up was carried out using DCM/MeOH 95:5. After 24 h, the reaction was poured in 3 mL of 2M NaOH, during which the reaction medium turned pale-yellow. The aqueous phase was extracted by 3x15 mL DCM and the organic phase was dried with Na₂SO₄ and was concentrated under reduced pressure to dryness. A known amount of dimethyl terephthalate was added as internal standard. The pale-yellow residue was dissolved in 1 mL of CDCl₃ and was analysed by ¹H NMR. The product was identified by comparison from a sample made by other methods and literature data.

2.1.4.3 Reduction with Bn-NADH

In a typical procedure, a 50 mL round bottom flask was filled with the imine (0.2 mmol) under air. The solvent (pH 7 phosphate buffer 100 mM, 13.3 mL) was added, immediately followed by the addition of **Bn-NADH** (2 equiv., 86.6 mg, 0.4 mmol) and **Ir1** (2.7 mg, 2 mol %). The heterogeneous mixture was stirred under air for 24 h during which TLC follow up was carried out with the appropriate solvent system. After 24 h, 3 mL of 2 M NaOH was added and the mixture was stirred for 5 min. Then, the aqueous medium was extracted by 3x15 mL of DCM, dried with Na₂SO₄ and evaporated to dryness. The yellowish residue was purified by flash column using the appropriate eluent to yield the desired product.



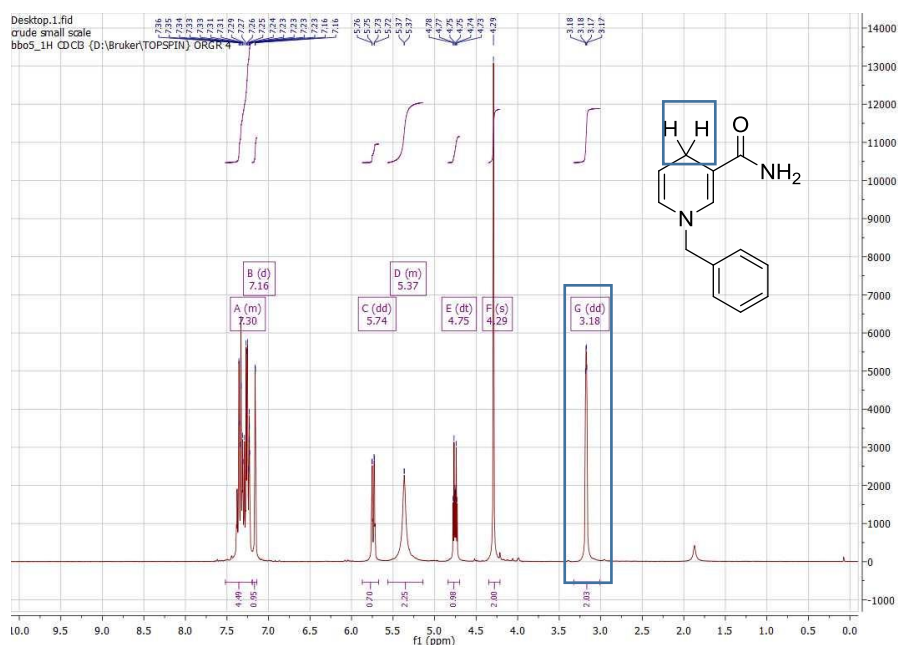
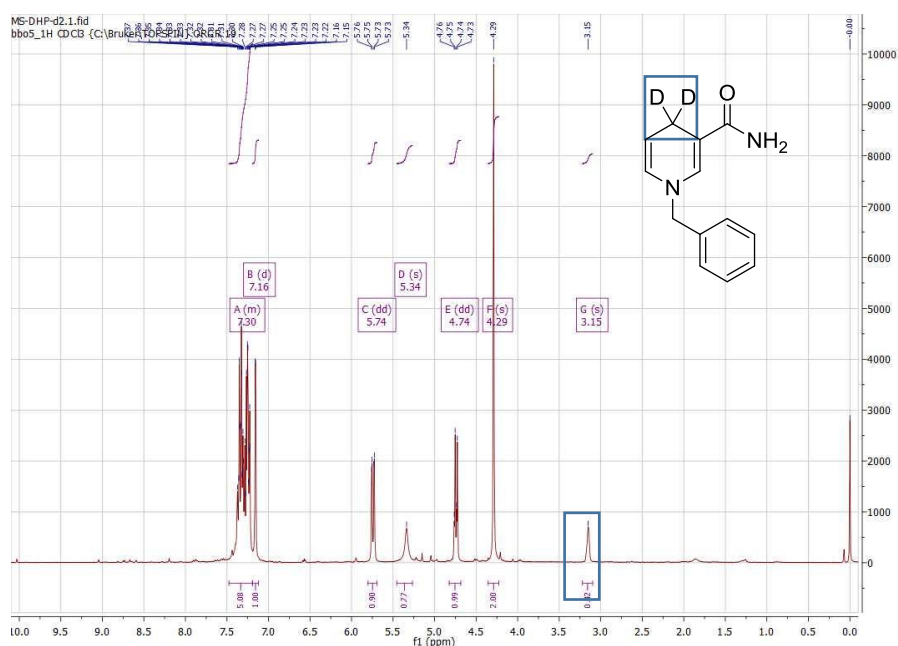
Entry	Substrate	Molecule n°	Yield (%)
1		2.3	83 ^a 99
2		2.41	87
3		2.34	99
4		2.37	72
5		2.58	99
6		2.57	99
7		2.38	97

8		2.47	78
9		2.46	82
10		2.48	81
11		2.45	99
12		2.51	91
13		2.61	60
14		2.39	88

Substrate 15 mM in 100 mM phosphate buffer under air, 2 equiv. of **Bn-NADH**, isolated yields after flash column chromatography, ^a 1 equiv. **Bn-NADH**

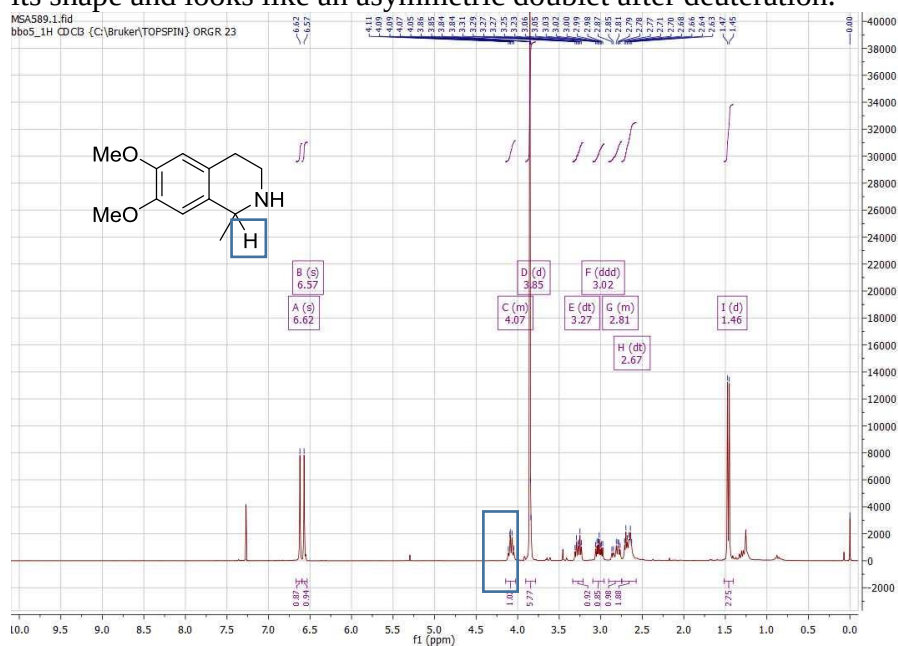
2.1.4.4 Deuteration experiment with **Bn-NADH-*d*₂**

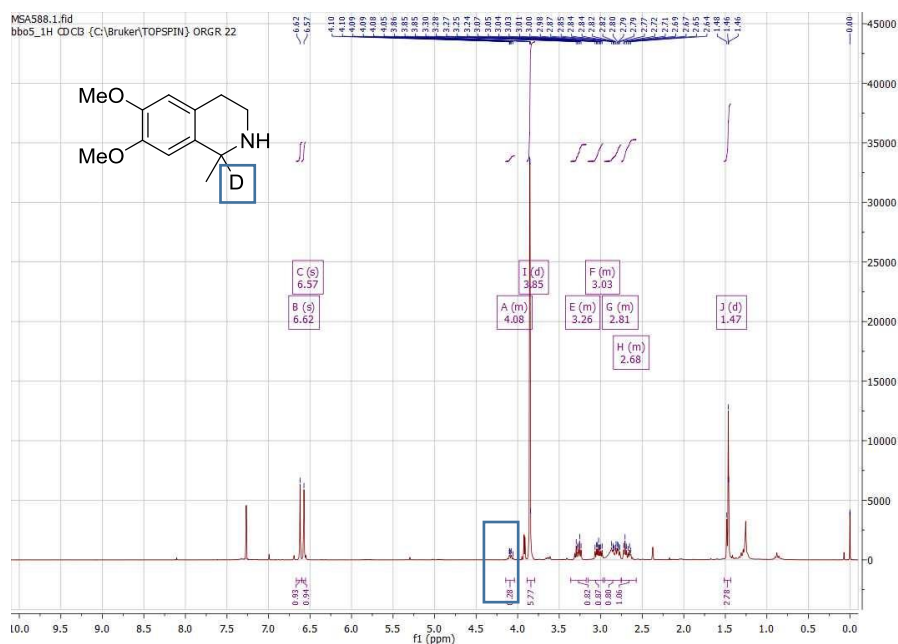
The **Bn-NADH-*d*₂** was synthesized according to a literature procedure. A total of 3 cycles oxidation-reduction were performed. A ¹H-NMR was recorded in CDCl₃. The peak at 3.15 ppm corresponds to the 4 position of the dihydropyridine ring (integral = 2). After deuteration, the integral is equal to 0.42, meaning that the deuteration is 79%.



For the reduction, the standard procedure described was used. A 25 mL round bottom flask was filled with the imine **2.3** (0.1 mmol) under air. The solvent (pH 7 phosphate buffer 100 mM, 6.7 mL) was added, immediately followed by the addition of **Bn-NADH-d₂** (2 equiv., 43.3 mg, 0.2 mmol) and the **Ir1** (1.3 mg, 2 mol %). The heterogeneous

mixture was stirred under air for 24 h during which TLC follow up was carried out with the appropriate solvent system. After 24 h, 3 mL of 2 M NaOH was added and the mixture was stirred for 5 min. Then, the aqueous medium was extracted by 3x15 mL of DCM, dried with Na₂SO₄ and evaporated to dryness. NMR was taken in CDCl₃. The peak at 4.07 ppm corresponds to the proton/deuterium at the benzylic position in α position relative to the methyl (blue square on the spectrum). The signal of the methyl at 1.47 ppm is also affected in its shape and looks like an asymmetric doublet after deuteration.





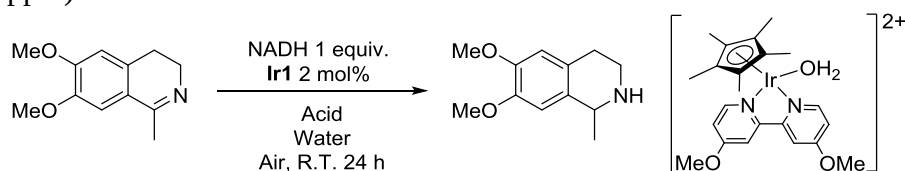
2.1.4.5 Impact of the acid

a) Impact of the pKa

At first, the reduction of **2.3** was carried out using the optimised conditions (1 equiv. of NADH, 2 mol % of **Ir1**) and various acids ranging from pKa = -0.3 to pKa = 9.95 using 1, 2 or 3 equivalents. A control experiment was carried out without acid and gave 10% of yield.

To study the impact of the acid, in a typical procedure, the imine **2.3** (50 μ mol, 10.4 mg) was dissolved in 3.3 mL of distilled water. The acid was added, and the reaction mixture was stirred for 1 min at R.T. under air, after which the NADH (35.8 mg, 1 equiv., 50 μ mol) was added in one portion. After dissolution, **Ir1** (0.7 mg, 2 mol %) was added and the mixture was stirred for 24 h under air, during which TLC follow-up was carried out using DCM/MeOH 95:5. After 24 h, the reaction was poured in 3 mL of 2 M NaOH, during which the reaction medium turned pale-yellow. The aqueous phase was extracted by 3x15 mL DCM and the organic phase was dried with Na₂SO₄ and was concentrated under reduced pressure to dryness. A known amount of dimethyl terephthalate was added as internal standard. The pale-yellow residue was dissolved in 1 mL of CDCl₃ and was analysed by ¹H NMR. The NMR yield was calculated by

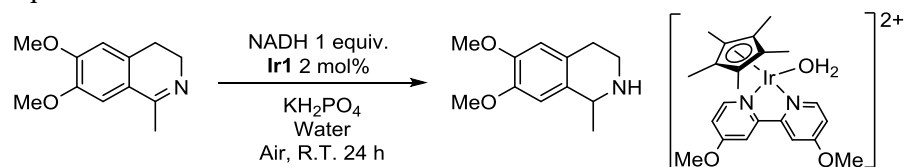
comparing the aromatic peak of the dimethyl terephthalate ($\delta = 8.09$ ppm) with the aromatic peaks of the starting material ($\delta = 6.98$ and 6.68 ppm) and the aromatic peaks of the product ($\delta = 6.62$ and 6.56 ppm).



	0 equiv.	1 equiv.	2 equiv.	3 equiv.
TFA	10%	43%	8%	0%
NaHSO₄	10%	31%	14%	0%
Citric acid	10%	37%	13%	9%
PhCOOH	10%	26%	40%	32%
AcOH	10%	30%	49%	43%
KH₂PO₄	10%	35%	54%	50%
PhOH	10%	12%	13%	15%

NMR yields versus dimethyl terephthalate as an internal standard.

The optimal acid was found to be KH₂PO₄. A set of experiments were carried out using increasing amounts of KH₂PO₄ ranging from 0 to 3 equivalents. The standard procedure (See 2.1.4.1 for more details) was used for the reaction setup and the yield calculation. An optimal of 2.5 equiv. was found.



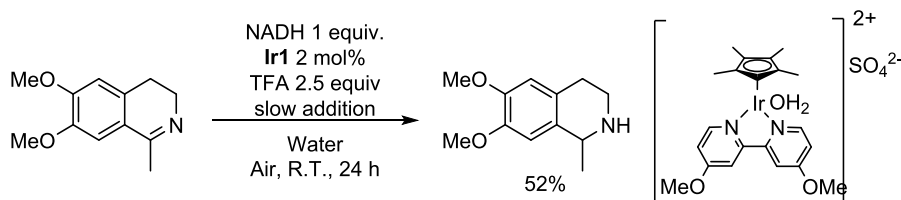
Entry	Equiv. of KH ₂ PO ₄	NMR Yield
1	0	10%
2	0.25	18%
3	0.50	21%
4	0.75	29%
5	0.9	30%
6	1.0	35%
7	1.1	43%
8	1.3	44%
9	1.5	52%
10	1.6	51%

11	1.7	56%
12	1.8	55%
13	1.9	56%
14	2.0	54%
15	2.5	60%
16	3.0	50%

NMR yields versus dimethyl terephthalate as an internal standard.

b) Slow addition of a strong acid

The hypothesis was made that the first equivalent was immediately consumed for the activation of the imine. In order to verify this hypothesis, an experiment was carried out where the acid was added by slow addition over 5 h using a syringe plunger after the addition of the NADH and **Ir1**.

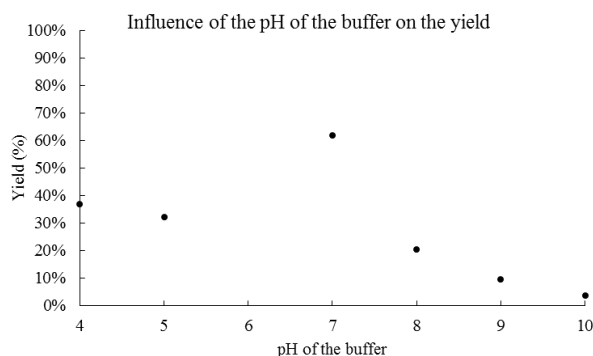


A 3.5 mL vial equipped with a stirring bar was filled under air with the imine **2.3** (10.4 mg, 50 μ mol), water (2.3 mL, 15 mM in substrate) and upon stirring, NADH was added (50 μ mol, 1 equiv. 35.8 mg), followed by the **Ir1** (0.7 mg, 2 mol %). The mixture turned instantaneously bright yellow. Then, a syringe was filled with trifluoroacetic acid (9.7 μ L, 2.5 equiv.) diluted in 1 mL of water. The latter solution was added dropwise over 5 h using a syringe plunger. The bright yellow solution was stirred for 19 more hours at R.T. under air, during which TLC follow-up was carried out using DCM/MeOH 95:5. After 24 h, the reaction was poured in 3 mL of 2M NaOH, during which the reaction medium turned pale-yellow. The aqueous phase was extracted by 3x15 mL DCM and the organic phase was dried with Na₂SO₄ and was concentrated under reduced pressure to dryness. A known amount of dimethyl terephthalate was added as internal standard. The pale-yellow residue was dissolved in 1 mL of CDCl₃ and was analysed by ¹H NMR. The NMR yield was calculated by comparing the aromatic peak of the dimethyl terephthalate (δ = 8.09 ppm) with the aromatic peaks of the starting material (δ = 6.98 and 6.68 ppm) and the aromatic peaks of the product (δ = 6.62 and 6.56 ppm). The calculated yield was 52%.

c) Use of buffers from pH 4 to 10

The buffers were prepared using the Henderson Haselbach equation, $pH = pKa - \log\left(\frac{C_A}{C_B}\right)$ and the pH was adjusted using NaOH 1M or HCl 1M. For pH 4-5, the couple was AcOH/AcONa, for pH 7-8 it was KH_2PO_4/Na_2HPO_4 and for 9-10, it was NH_4Cl/NH_3 . To study the impact of the pH of the buffer, in a typical procedure, the imine (50 μ mol, 10.4 mg) was dissolved in 3.3 mL of the appropriate buffer. Then, NADH (35.8 mg, 1 equiv., 50 μ mol) was added in one portion. After dissolution, **Ir1** (0.7 mg, 2 mol %) was added and the mixture was stirred for 24 h under air, during which TLC follow-up was carried out using DCM/MeOH 95:5. After 24 h, the reaction was poured in 3 mL of 2 M NaOH, during which the reaction medium turned pale-yellow. The aqueous phase was extracted by 3x15 mL DCM and the organic phase was dried with Na_2SO_4 and was concentrated under reduced pressure to dryness. A known amount of dimethylterephthalate was added as internal standard. The pale-yellow residue was dissolved in 1 mL of $CDCl_3$ and was analysed by ^1H NMR. The NMR yield was calculated by comparing the aromatic peak of the dimethylterephthalate ($\delta = 8.09$ ppm) with the aromatic peaks of the starting material ($\delta = 6.98$ and 6.68 ppm) and the aromatic peaks of the product ($\delta = 6.62$ and 6.56 ppm).

pH	Couple	yield	Ca (M)	Cb (M)	n eq Ac
4	AcOH/AcONa	37%	0.085	0.015	5.7
5	AcOH/AcONa	32%	0.036	0.064	2.4
7	KH_2PO_4/Na_2HPO_4	62%	0.062	0.038	4.1
8	KH_2PO_4/Na_2HPO_4	20%	0.014	0.086	0.9
9	NH_4Cl/NH_3	9%	0.064	0.036	4.3
10	NH_4Cl/NH_3	4%	0.015	0.085	1.0



2.1.4.6 Reductive alkylation

a) Optimisation of the reductive alkylation of the amine 2.4 (2.61-Ph)

For the design of the experiments, we chose to work on 3 key parameters: the quantity of **Bn-NADH**, the equivalents of dihydro cinnamaldehyde, the equivalents of AcOH, and the use of a buffered or unbuffered medium. Those latter parameters were set to arbitrary values of 2 discrete levels: 2 or 3 equiv. of **Bn-NADH**, 1 or 3 equiv. of aldehyde, 2 or 4 equiv. of AcOH and unbuffered or buffered phosphate pH 7 medium. The screening was designed to highlight the possible interactions between the different factors.



	Equiv. Bn-NADH	Equiv. Ald	Equiv. AcOH	medium	NMR yield
1	2	1	2	pH 7	74%
2	3	1	2	pH 7	63%
3	2	3	2	pH 7	79%
4	2	1	4	pH 7	38%
5	2	1	2	H ₂ O	67%
6	3	3	2	pH 7	81%
7	3	1	4	pH 7	51%
8	3	1	2	H ₂ O	72%
9	2	3	4	pH 7	75%
10	2	3	2	H ₂ O	93%
11	2	1	4	H ₂ O	31%
12	2	3	4	H ₂ O	52%
13	3	1	4	H ₂ O	30%
14	3	3	2	H ₂ O	89%
15	3	3	4	pH 7	74%
16	3	3	4	H ₂ O	87%

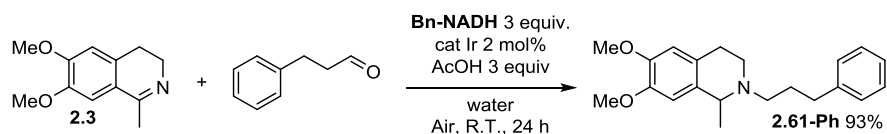
NMR yields versus dimethyl terephthalate as an internal standard.

Thus, the optimal conditions found were:

A 10 mL round bottom flask was filled with **2.3** (28.3 mg, 0.135 mmol) freshly synthesized, water (9.0 mL). Then, hydrocinnamaldehyde (54 μ L, 0.406 mmol, 3 equiv.) was added, immediately followed by AcOH (24 μ L, 3 equiv., 0.406 mmol), **Bn-NADH** (59 mg, 0.270 mmol, 2 equiv.) and **Ir1** (1.8 mg, 2 mol %). The mixture was vigorously stirred for 24 h and was followed by TLC. NaOH 2 M (3 mL) were added and the medium was extracted with 3x20 mL DCM, after which it was dried with Na₂SO₄ and evaporated in vacuo. Purification by flash column using DCM/EA 7:3 100 mL, DCM/EA 5:5 100 mL, DCM/MeOH 95:5 200 mL.

Yield: 93%, bright yellow oil, ¹H NMR (300 MHz, CDCl₃) δ 7.37 – 7.12 (m, 5H), 6.56 (s, 1H), 6.53 (s, 1H), 3.84 (s, 7H), 3.18 – 2.97 (m, 1H), 2.82 (q, *J* = 6.5 Hz, 2H), 2.71 – 2.56 (m, 5H), 1.97 – 1.78 (m, 2H), 1.32 (d, *J* = 6.7 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 147.5, 147.4, 142.3, 131.7, 128.5, 128.4, 125.9, 125.8, 111.4, 110.4, 56.1, 56.1, 56.0, 53.1, 43.7, 33.8, 29.8, 29.2, 26.4, 19.4.

b) Optimisation of the reductive alkylation of the imine 2.3 (2.61-Ph)



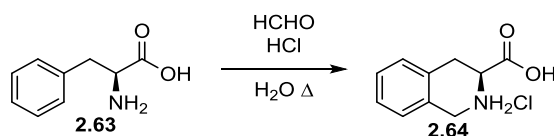
A 10 mL round bottom flask was filled with **2.3** (41.9 mg, 0.200 mmol) and water (13.3 mL) and the mixture was stirred until the solution became clear. Then, hydrocinnamaldehyde (80 μ L, 0.6 mmol, 3 equiv.) and AcOH (35 μ L, 3 equiv., 0.6 mmol), were added. The mixture was stirred for 1 min, after which **Bn-NADH** (130 mg, 0.6 mmol, 3 equiv.) and **Ir1** (2.7 mg, 2 mol %) were added. The mixture was vigorously stirred for 24 h and was followed by TLC (DCM/MeOH 97:3). For the workup, NaOH 2 M (3 mL) was added and the medium was extracted with 3x20 mL DCM. The combined organic phases were dried with Na₂SO₄ and the solvent was removed under reduced pressure. To the crude product, dimethyl terephthalate was added as internal standard and NMR was carried out in CDCl₃. NMR yield: 52%. The yield was calculated by integrating the aromatic peaks of the dimethyl terephthalate (δ = 8.09 ppm) with the aromatic peaks of the dihydroisoquinoline (δ = 6.98 and 6.68 ppm), the aromatic peaks of the tetrahydroisoquinoline (δ = 6.62 and 6.56 ppm) and the aromatic peaks of the reductive alkylation product (δ =

6.55 and 6.53 ppm). The spectrum was found to be in agreement with the one obtained in **a**).

2.2 Reducible prodrugs

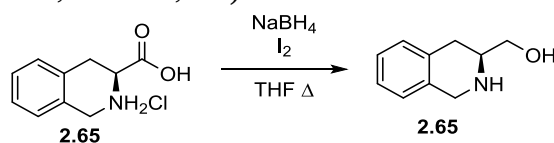
2.2.1 Synthesis of the first-generation prodrug

2.2.1.1 Trigger synthesis



The Pictet-Spengler reaction was carried out according to a literature procedure. The compound **2.64** was obtained as a white powder, yield = 78%.

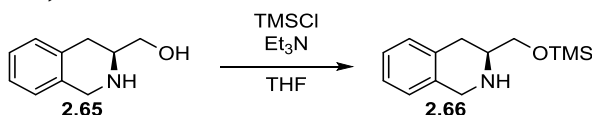
^1H NMR (300 MHz, D_2O) δ 7.31 – 7.09 (m, 4H), 4.38 (t, J = 11.5 Hz, 2H), 4.26 (dd, J = 11.2, 5.6 Hz, 1H), 3.35 (dd, J = 17.4, 5.4 Hz, 1H), 3.13 (dd, J = 17.3, 11.1 Hz, 1H).



The reduction was carried out using an adapted literature procedure.¹⁹¹ A 250 mL 3-neck round-bottom flask (equipped by an addition funnel and a refrigerant) was filled under Ar with THF (140 mL, 0.27 M), and NaBH_4 (5.25 g, 137 mmol, 2.5 equiv.). Then, the amino acid **2.64** (11.7 g, 55 mmol) was added by portions. The mixture was cooled at 0 °C. Then, the iodine solution in THF (14 g of I_2 , 55 mmol, 1 equiv., 60 mL THF) was added dropwise through the addition funnel. The solution was allowed to return to room temperature and then was refluxed overnight.

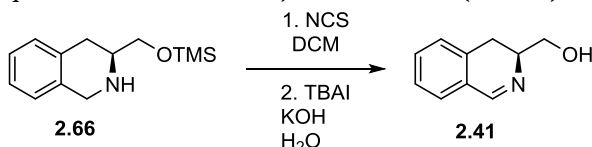
The reaction mixture was concentrated under reduced pressure on the next morning, and subsequently re-dissolved in 20% KOH and the mixture was stirred for 4 h at room temperature. The mixture was extracted 3 times by DCM, and the organic phase was dried by MgSO_4 and the volume was reduced. The solution was filtered on silica gel. The first elution by EtOAc led to the removal of an impurity. The second elution was performed with DCM/MeOH 95:5 and the collected fraction was the pure product **2.65** in the form of a white powder (3.6 g, 40%).

^1H NMR (300 MHz, CDCl_3) δ 7.23 – 6.97 (m, 4H), 4.08 (s, 2H), 3.83 (dd, J = 10.9, 3.8 Hz, 1H), 3.56 (dd, J = 10.9, 8.1 Hz, 1H), 3.21 – 3.06 (m, J = 12.2, 8.1, 4.0 Hz, 1H), 2.84 – 2.63 (m, 2H), 2.62 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 135.2, 133.9, 129.5, 126.5, 126.2, 126.1, 65.6, 55.3, 47.7, 30.8.



In a flame dried round bottom flask under argon, the amino alcohol **2.65** (1.76 g, 12.6 mmol) was dissolved in distilled THF (22 mL, 0.5 M). The mixture was cooled down to 0 °C and triethylamine (1.8 mL, 12.8 mmol, 1.2 equiv.) was added. Then, TMSCl (1.5 mL, 12.8 mmol, 1.2 equiv.) was added dropwise. The mixture was stirred under Ar and was allowed to reach room temperature for 2 h 30. The mixture became white and cloudy when the $\text{NEt}_3\cdot\text{HCl}$ precipitated. TLC following was carried out with DCM/MeOH 95:5. Upon completion (usually 3 h), the reaction mixture was filtered on celite to give a clear solution. The filtrate was concentrated under reduced pressure and dried under vacuum to yield the desired product **2.66** as a pale orange oil (yield: 2.16 g, 86%).

^1H NMR (300 MHz, CDCl_3) δ 7.23 – 6.96 (m, 4H), 4.09 (t, J = 9.3 Hz, 2H), 3.76 (dd, J = 10.0, 3.8 Hz, 1H), 3.58 (dd, J = 9.9, 7.3 Hz, 1H), 3.06 (dq, J = 7.4, 4.4 Hz, 1H), 2.80 – 2.54 (m, 2H), 0.17 (s, 9H).



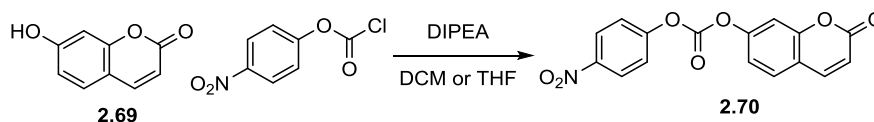
The imino alcohol **2.41** was prepared following a literature procedure.¹¹⁰ The trimethylsilyl derivative **2.66** (3.2 g, 18 mmol) was dissolved in distilled DCM (22 mL, 0.85 M), and to the DCM solution was added NCS (2.6 g, 26.6 mmol, 1.5 equiv.). The solution turned from light orange to dark orange. After 1 h, Bu_4NI (503 mg, 1.3 mmol) and 20% aqueous KOH (21 mL) were added to the mixture. The reaction mixture was stirred for 1 h, and the organic layer was washed with water and was dried (Na_2SO_4) and evaporated under vacuum. TLC was carried out using DCM/MeOH 95:5, a revelation in UV light 254 nm. The crude product was dissolved in 50 mL THF and 7 mL HCl 1 M was subsequently added. The mixture was stirred for 2 h. The mixture was basified by NaHCO_3 sat and was extracted by DCM, dried with MgSO_4 and evaporated under reduced pressure. The

crude mixture (which was the desired product) was purified by a flash column using DCM/MeOH 95:5 to afford the desired product **2.41** as a yellow oil. It was found to precipitate as an orange solid (38%, 660 mg).

^1H NMR (300 MHz, CDCl_3) δ 8.40 (d, J = 2.8 Hz, 1H), 7.50 – 7.08 (m, 4H), 4.02 (dd, J = 10.7, 4.5 Hz, 1H), 3.78 (dd, J = 10.6, 6.8 Hz, 1H), 3.66 (ddd, J = 8.7, 6.4, 3.6 Hz, 1H), 3.28 (s, 1H), 2.81 – 2.65 (m, 2H).

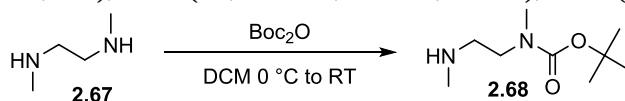
^{13}C NMR (75 MHz, CDCl_3) δ 160.9, 136.2, 131.6, 128.5, 127.9, 127.6, 127.3, 66.0, 58.9, 27.3

2.2.1.2 Probe and linker part



The compound **2.70** was synthesized the procedure described by Robillard.⁹ In a vacuum dried round bottom flask under argon, umbelliferon **2.69** (3.3 g, 20 mmol) was dissolved in distilled THF (100 mL, 0.2 M). Then, PNP-chloroformate (5.0 g, 24 mmol, 1.2 equiv.) was added. Then, DIPEA (5.1 mL, 30 mmol, 1.5 equiv.) was added dropwise. The mixture was subsequently stirred under Ar at room temperature and followed by TLC (petroleum ether/EtOAc 7:3). The cloudy white mixture became limpid upon addition of DIPEA. After 5min stirring at room temperature, a white solid precipitated. The reaction mixture was filtered and washed by THF. The white solid was re-dissolved in DCM and was washed by water twice. The water phases were extracted by DCM 3 times. The organic phases were combined, dried over MgSO_4 and evaporated under reduced pressure and dried under vacuum. (**2.70** White powder, 3.6 g, 55%)

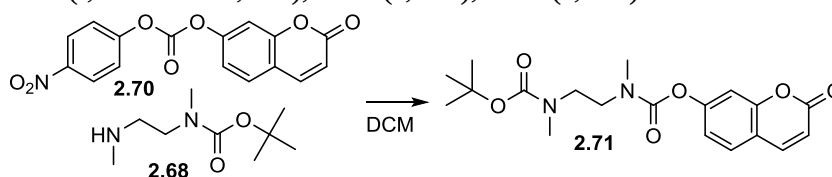
^1H NMR (300 MHz, CDCl_3) δ 8.49 – 8.24 (m, 2H), 7.75 (d, J = 9.6 Hz, 1H), 7.59 (d, J = 8.5 Hz, 1H), 7.56 – 7.49 (m, 2H), 7.35 (d, J = 2.3 Hz, 1H), 7.27 (dd, J = 8.9, 1.9 Hz, 1+1H), 6.47 (d, J = 9.6 Hz, 1H).



The mono Boc-protected derivative **2.68** was synthesized according to a literature method.¹⁰⁴ The commercially available *N*, *N'*-dimethylethylenediamine **2.67** (5.5 mL, 50 mmol) was dissolved in DCM (53 mL) and the solution was cooled by an ice-water bath. Boc_2O (3.9 mL, 16.8 mmol) in 27 mL of DCM was added dropwise at 0 °C with a

syringe plunger. When the addition ended, the reaction was allowed to warm to room temperature and was stirred overnight. The solvent was then removed under reduced pressure and the crude mixture was dissolved in EtOAc and was washed with brine. The organic solution was dried over MgSO₄ and the solvent was removed under reduced pressure to yield the mono-Boc- *N, N'*-dimethyl-ethylenediamine **2.68** compound as a pale-yellowish oil (2.4 g, 75%).

¹H NMR (300 MHz, CDCl₃) δ 3.33 (t, *J* = 5.9 Hz, 2H), 2.87 (s, 3H), 2.72 (t, *J* = 6.5 Hz, 2H), 2.45 (s, 3H), 1.45 (s, 9H).

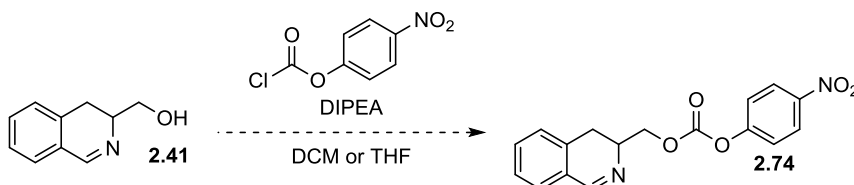


The compound **2.71** was synthesized following a literature procedure.¹¹¹ The compound **2.70** (1.55 g, 4.1 mmol) was dissolved in DCM (10 mL, 0.4 M) and **2.68** (776 mg, 4.1 mmol, 1 equiv.) was added. The reaction was stirred at room temperature for 2 h and was monitored by TLC (petroleum ether/EtOAc 3:1). After completion, the mixture was washed several times by NaHCO₃ sat until there was no more yellow colour in the water phase. The organic phase was dried by MgSO₄ and was evaporated in vacuo. The crude **2.71** was purified by flash column chromatography (EtOAc 30% in petroleum ether). The first fraction contained the by-product **2.72** where the umbelliferon plays the role of the leaving group. The second fraction was the pure product **2.71** (transparent sticky resin, 51%, 783 mg).

¹H NMR (300 MHz, CDCl₃) δ 7.71 (d, *J* = 9.6 Hz, 1H), 7.49 (d, *J* = 8.4 Hz, 1H), 7.23 – 7.03 (m, 1+1H), 6.40 (d, *J* = 9.5 Hz, 1H), 3.55 (bs, 4H), 3.30 – 2.86 (m, 6H), 1.48 (d, *J* = 5.8 Hz, 9H).

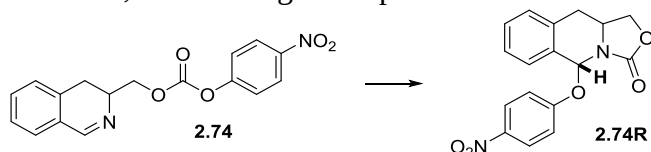
¹³C NMR (75 MHz, CDCl₃) δ 160.55, 154.64, 153.63, 143.00, 128.36, 118.53, 116.12, 115.69, 110.25, 80.04, 46.89, 35.40, 28.42.

2.2.1.3 First strategy: activation of the trigger



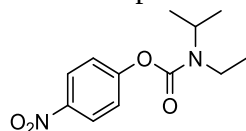
The method used to synthesize **2.74** was adapted from the literature.⁹ A GC vial was filled with the imino-alcohol **2.41** (100mg, 0.62mmol) and distilled THF (3.1 mL). Then, PNP-chloroformate (151.6 mg,

0.74 mmol, 1.2 equiv.) was added under argon. Eventually, pyridine (81 μ L, 0.74 mmol, 1.2 equiv.) was added dropwise at 0 °C. The mixture was stirred at room temperature for 3 h. TLC follow up was carried out using EtOAc/petroleum ether 1:9. Then, the organic phase was evaporated to half washed 3 times by water and was concentrated under reduced pressure. The crude mixture constituents were separated by flash column chromatography using EA/EP 1:9. However, the rearrangement product **2.74-R** was isolated (100 mg).



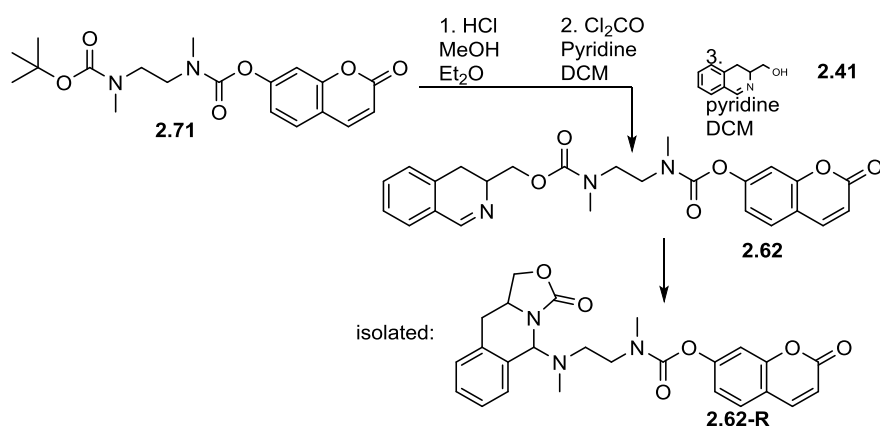
Spectrum of **2.74-R**: ^1H NMR (300 MHz, CDCl_3) δ 8.37 (d, J = 9.18 Hz, 1H), 8.19 (d, J = 9.05 Hz, 2H), 7.53 (d, J = 9.18 Hz, 1H), 7.32 (d, J = 9.18 Hz, 1H), 6.93 (d, J = 9.05 Hz, 2H), **6.08 (s, 1H)**.

The other product was also isolated and identified:



^1H NMR (300 MHz, CDCl_3) δ 8.11 (d, J = 7.5 Hz, 2H), 7.27 (d, J = 7.5 Hz, 2H), 3.78 (dq, J = 16.2, 6.1 Hz, 2H), 3.21 (q, J = 6.3 Hz, 1H), 1.35 (d, J = 6.0 Hz, 6H), 1.26 (t, J = 6.3 Hz, 3H).

2.2.1.4 Second strategy: activation of the linker by HCl

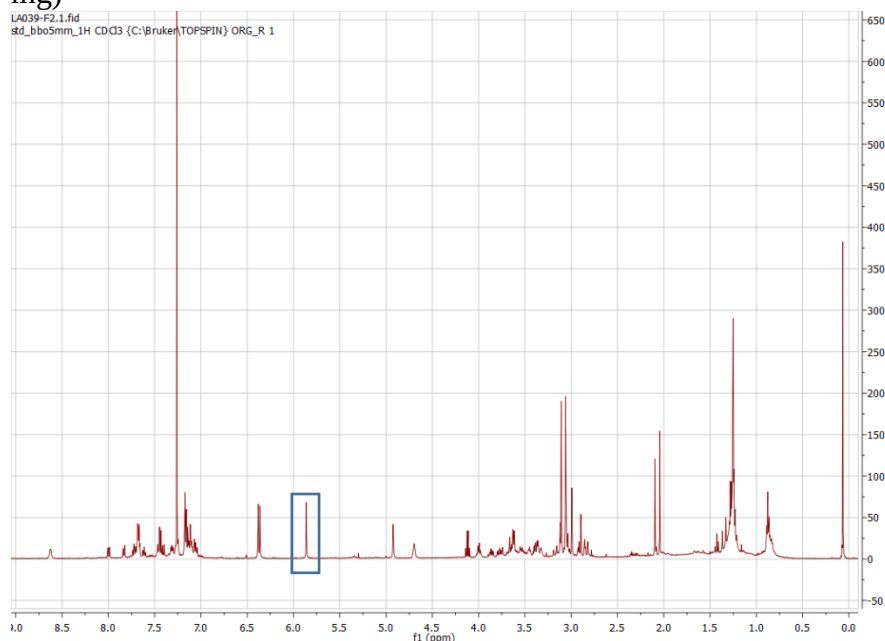


The first and second steps are described in paragraph 2.2.2

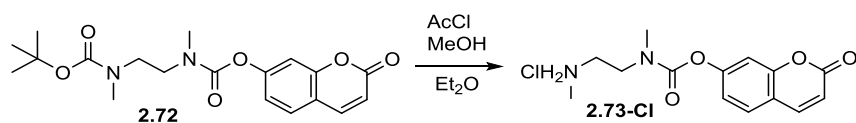
The last step:

A 10 mL round-bottom flask was filled at 0 °C with the carbamoyl chloride **2.75** obtained from the second step, pyridine and the imino

alcohol **2.41**. The yellow solution was stirred at 0 °C for 15 min, then was allowed to warm to room temperature and a slight precipitate appeared after 3 h. On the next day, the reaction medium was diluted in EtOAc and washed several times with HCl 1M, was dried by Na_2SO_4 and the solvent was evaporated in vacuum. The crude ^1H -NMR shows the absence of the peak at 8.3 ppm and shows a new peak at 6 ppm. The crude oil was purified by flash column chromatography using a gradient of petroleum ether/EtOAc 95:5 to 70:30 and the pure rearrangement product **2.62-R** was isolated as a yellow thick oil. (170 mg)



2.2.2 Synthesis of the second-generation prodrug

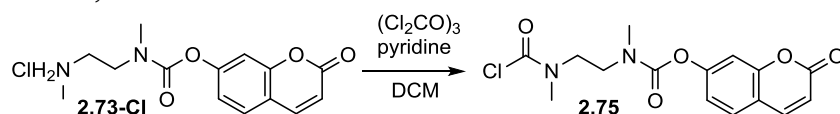


A known literature method was used for the deprotection.¹¹² The Boc-protected derivative **2.72** (1.09 g, 2.9 mmol, 1 equiv.) was dissolved in anhydrous MeOH (4.8 mL, 40 equiv.) and Et_2O (10 mL) under Ar. The reaction was cooled down to 0 °C by an ice bath. After 5 min stirring, acetyl chloride (4.1 mL, 58 mmol, 20 equiv.) was added dropwise and the reaction was stirred for 5 min at 0 °C and the batch was later removed. A white precipitate appeared. After 2 h stirring at room temperature, the reaction medium was filtered and the solid was

collected and washed by Et₂O to give **2.73-Cl** in the aspect of a fluffy white powder (900 mg, quant).

¹H-RMN (300 MHz, D₂O) δ 7.92 (d, J = 9.6 Hz, 1H), 7.60 (dd, J = 8.5, 1.5 Hz, 1H), 7.23 – 6.98 (m, 2H), 6.37 (d, J = 9.5 Hz, 1H), 3.69 (t, J = 46.1, 5.8 Hz, 2H), 3.24 (t, J = 7.8, 5.6 Hz, 2H), 2.99 (d, J = 40.7 Hz, 3H), 2.64 (t, J = 13.7 Hz, 3H).

HRMS (m/z): Calculated mass for C₁₅H₁₆O₅N₂³⁵Cl₁ [M+H]⁺: 277.1182, obtained 277.1182.

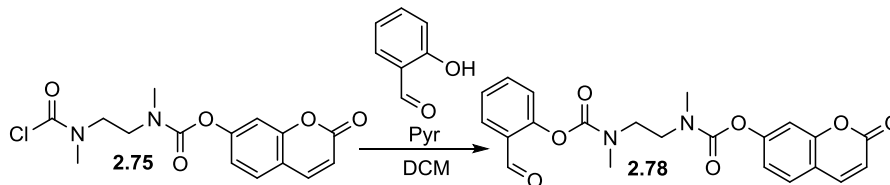


The crude protonated amine **2.73-Cl** (white powder, 85 mg, 0.274 mmol) was introduced in a 50 mL round-bottom flask under Ar and suspended in dry DCM (3 mL, 0.1 M). Then, triphosgene (83 mg, 0.274 mmol, 1 equiv) was carefully introduced in one portion. The heterogeneous mixture was subsequently cooled down to 0 °C by an ice bath and pyridine (65 µL, 2.5 equiv.) The mixture was stirred for 15 min at 0 °C and then allowed to warm up to room temp and stirred overnight under Ar, during which the mixture turned heterogeneous yellowish with some solid.

On the next morning, the mixture was filtered through a sintered funnel and a white powder corresponding to the non-consumed salt was removed. The yellow filtrate was evaporated to dryness to remove traces of phosgene and was re-dissolved in DCM, was washed by acidic water once to remove the remaining pyridine. The organic phase was dried by Na₂SO₄ and was evaporated under reduced pressure. The yellow oil was analysed by ¹H NMR and HRMS and indicated it was the carbamoyl chloride **2.75** as a yellow foam (Yield: 95%)

¹H-RMN (300 MHz, CDCl₃) δ 7.63 (d, J = 9.6 Hz, 1H), 7.41 (d, J = 8.4 Hz, 1H), 7.11 – 6.95 (m, J = 10.4, 6.5, 2.0 Hz, 2H), 6.32 (d, J = 9.5 Hz, 1H), 3.81 – 3.42 (m, 4H), 3.20 – 2.93 (m, 6H).

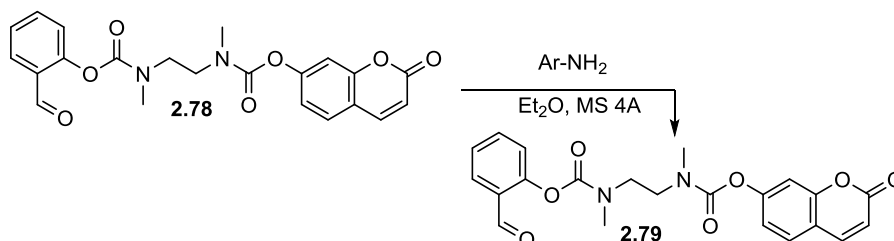
HRMS (m/z): Calculated mass for C₁₅H₁₆O₅N₂³⁵Cl₁ [M+H]⁺: 339.0742, obtained 339.07423.



A round-bottom flask under Ar was filled with the crude carbamoyl chloride **2.75** (200 mg, 0.59 mmol, 1 equiv). Then, salicylaldehyde (84 μ L, 0.77 mmol, 1.3 equiv.) was added dropwise, followed by pyridine (0.5 mL) and the reaction was stirred overnight at room temperature. TLC was carried out using petroleum ether/EtOAc 7:3. On the next morning, the reaction medium was diluted with 20 mL EtOAc and was washed with HCl 1M and H₂O, before being dried with Na₂SO₄. After evaporating the solvents, the crude product was purified by flash column chromatography using petroleum ether/EtOAc 7:3 to 5:5 to give the pure product **2.78** in the form of a transparent oil (201 mg, 81%).

¹H-RMN (300 MHz, CDCl₃) δ 10.07 (d, J = 10.2 Hz, 1H), 7.88 – 7.26 (m, J = 26.8, 18.4, 11.3, 4.7 Hz, 6H), 7.17 – 6.93 (m, 3H), 6.32 (d, J = 9.6 Hz, 1H), 3.79 – 3.48 (m, 4H), 3.26 – 2.95 (m, 6H).

HRMS (m/z): Calculated mass C₂₂H₂₁O₇N₂ [M+H]⁺: 424.1270, obtained 425.1343.



A round-bottom flask was filled under Ar with the aldehyde **2.78** (217 mg, 0.51 mmol), 8 pebbles of 4A molecular sieve and distilled aniline (3 mL). The yellow reaction medium was stirred overnight at room temperature. TLC was carried out using petroleum ether/EtOAc 7:3. Then, after 18 h of stirring, the reaction medium was filtered on a celite pad and the pad was rinsed two times with diethyl ether. Then, the remaining aniline was removed by horizontal distillation using a Kugel Rohr apparatus (50 °C). Finally, the obtained oil was dissolved in a minimum of MeCN and was washed by pentane twice to remove the grease. After evaporation, the pure product **2.79** was obtained as a pale-yellow sticky oil. (202.8 mg, 80 %).

¹H-RMN (300 MHz, CDCl₃) δ 8.58 (s, 1H), 7.74 – 7.28 (m, 5H), 7.25 – 6.78 (m, 10H), 6.60 (d, J = 8.3 Hz, 2H), 6.41 – 6.23 (m, 1H), 3.66 (td, J = 22.1, 18.6 Hz, 6H), 3.31 – 2.99 (m, 8H).

¹³C-RMN (75 MHz, CDCl₃) δ 160.51 (s), 151.40 – 150.65 (m), 148.83 (d, J = 84.0 Hz), 143.43 (d, J = 68.0 Hz), 135.37 (s), 132.52 – 131.32 (m), 125.76 (d, J = 12.0 Hz), 123.01 (d, J = 22.0 Hz), 120.73 (dd, J =

29.3, 18.6 Hz), 118.85 – 118.20 (m), 115.24 (s), 46.87 (s), 35.39 (d, J = 36.2 Hz), 20.75 (d, J = 41.6 Hz).

HRMS (m/z): Calculated mass for $C_{28}H_{26}O_6N_3$ $[M+H]^+$: 499.1740, obtained 500.1816.

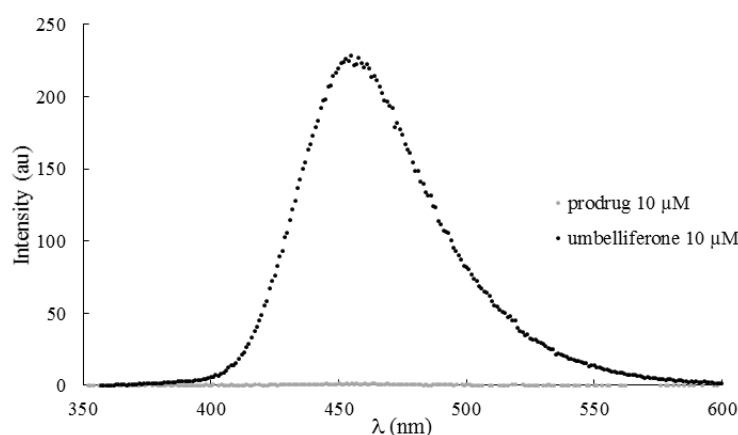
2.2.3 Prodrug testing

2.2.3.1 Excitation-emission of the prodrug

A 10 μ M solution of the prodrug **2.79** (2% DMSO) in water was prepared and was analysed in the spectrometer with the following parameters.

Parameter	Value
Excitation slits	2.5 mm
Emission slits	2.5 mm
Voltage	Medium
Scan mode	Emission
Excitation	325 nm
Delay between 2 scans	0.5 min
Number of scans	10
Temperature	25 °C

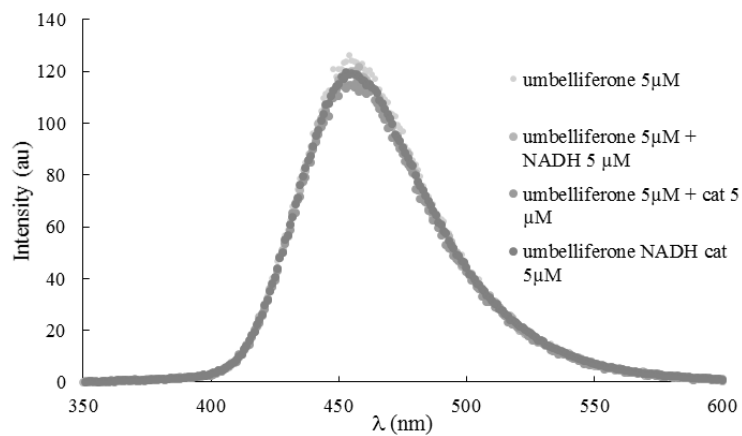
The ten excitation and emission spectra were averaged, and a correction was applied to set $I(600\text{ nm}) = 0$. This correction was very minor if the spectrum was properly zeroed prior to the acquisition. The following spectrum was obtained.



2.2.3.2 Fluorescence quenching test

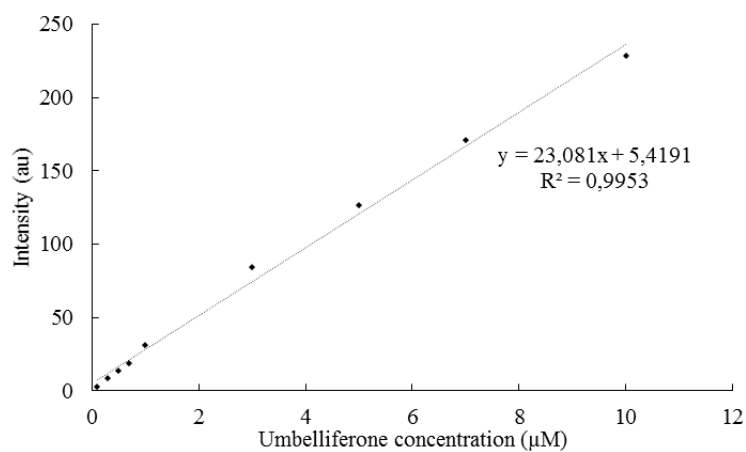
Parameter	Value
Excitation slits	2.5 mm
Emission slits	2.5 mm
	400

Voltage	Medium
Scan mode	Emission
Excitation	325 nm
Delay between 2 scans	0.5 min
Number of scans	5
Temperature	25 °C



2.2.3.3 Calibration curve

Parameter	Value
Excitation slits	2.5 mm
Emission slits	2.5 mm
Voltage	Medium
Scan mode	Emission
Excitation	325 nm
Delay between 2 scans	0.5 min
Number of scans	5
Temperature	25 °C

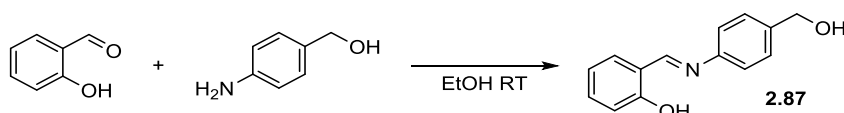


2.2.3.4 Kinetics

Parameter	Value
Excitation slits	2.5 mm
Emission slits	2.5 mm
Voltage	Medium
Scan mode	Emission
Excitation	325 nm
Delay between 2 scans	0.5 min
Number of scans	5
Temperature	25 °C

2.2.4 Synthesis of third generation prodrug

2.2.4.1 Synthesis of the trigger and linker adduct (2.87)

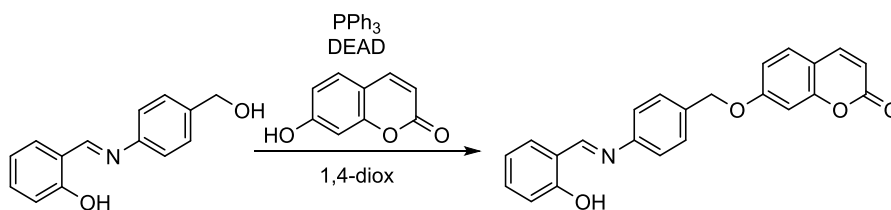


(E)-2-(((4-(hydroxymethyl) phenyl) imino) methyl) phenol **2.87** was synthesized according to a literature procedure.¹¹⁹ Yellow crystals. Yield: 200 mg, 88%

¹H NMR (300 MHz, CDCl₃) δ 13.24 (s, 1H), 8.63 (s, 1H), 7.47 – 7.35 (m, 4H), 7.33 – 7.24 (m, 2H), 7.03 (dt, *J* = 7.6, 1.1 Hz, 1H), 6.95 (td, *J* = 7.5, 1.1 Hz, 1H), 4.73 (d, *J* = 4.5 Hz, 2H), 1.74 (s, 1H).

¹³C NMR (75 MHz, CDCl₃) δ 162.73, 161.26, 148.05, 139.75, 133.33, 132.42, 128.23, 121.47, 119.33, 119.24, 117.41, 77.16, 65.05.

2.2.4.2 Mitsunobu coupling of the umbelliferon (2.84)



The compound **2.84** was synthesized using an adapted literature protocol.¹²⁰ A 25 mL 3-necks round-bottom flask was flame dried and purged by Ar. Then, it was filled with **2.87** (113 mg, 0.5 mmol), umbelliferon (164 mg, 1 mmol, 2 equiv.), PPh₃ (221 mg, 0.84 mmol, 1.7 equiv.) and 1,4-dioxane (11 mL, 0.044 M). Then, DEAD (120 μL, 0.75 mmol, 1.5 equiv) was added at room temperature and the bright orange mixture was heated at 110 °C for 2 h. Afterwards, it was cooled down to room temperature and the solvent was evaporated. It was re-dissolved in EtOAc and washed with brine 2 times, dried by

Na₂SO₄ and the solvent was removed under reduced pressure. The compound was purified by flash column chromatography using petroleum ether/EtOAc 8:2 to 6:4. The yellow solid containing DEAD.H₂ was then powdered, suspended in Et₂O and sonicated for 15 min, then stirred for a weekend. The solid **2.84** was collected by filtration on a filter paper and washed by Et₂O. Yellow powder 70%
¹H NMR (300 MHz, CDCl₃) δ 13.14 (s, 1H), 8.62 (s, 1H), 7.63 (d, *J* = 9.4 Hz, 1H), 7.49 (d, *J* = 8.3 Hz, 2H), 7.45 – 7.35 (m, 3H), 7.35 – 7.28 (m, 2H), 7.07 – 7.00 (m, 1H), 6.99 – 6.90 (m, 2H), 6.88 (d, *J* = 2.4 Hz, 1H), 6.25 (d, *J* = 9.5 Hz, 1H), 5.14 (s, 2H).
¹³C NMR (75 MHz, CDCl₃) δ 163.18, 161.83, 161.25, 155.92, 148.72, 143.49, 134.50, 133.48, 132.51, 128.97, 128.83, 121.66, 119.28, 119.24, 117.40, 113.43, 113.37, 112.94, 102.04, 70.18.

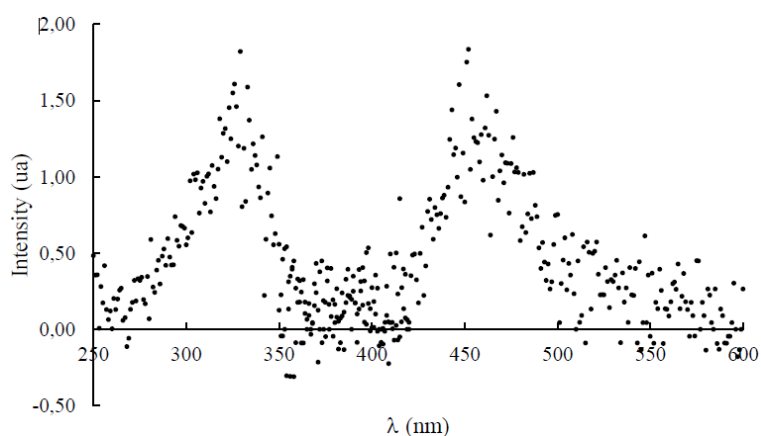
2.2.5 Prodrug testing

2.2.5.1 Photophysical parameters of the prodrug 2.84

A sample of 3.7 mg of the prodrug **2.84** was dissolved in 2 mL DMSO and was further brought to 100 mL with 50 mM pH 7 phosphate buffer. The solution was milky yellow and was sonicated to dissolve as much as possible. Then, 1 mL was sampled and transferred to a second 100 mL flask and diluted with 50 mM pH 7 phosphate buffer containing 2% DMSO and was sonicated for 15 min to give a clear solution at 1 μM. A spectrum was recorded with the following parameters:

Parameter	Value
Excitation slits	2.5 mm
Emission slits	2.5 mm
Voltage	Medium
Scan mode	Emission (with excitation at 325 nm) Excitation (with emission at 354 nm)
Number of scans	1
Temperature	25 °C

The following spectrum was obtained.

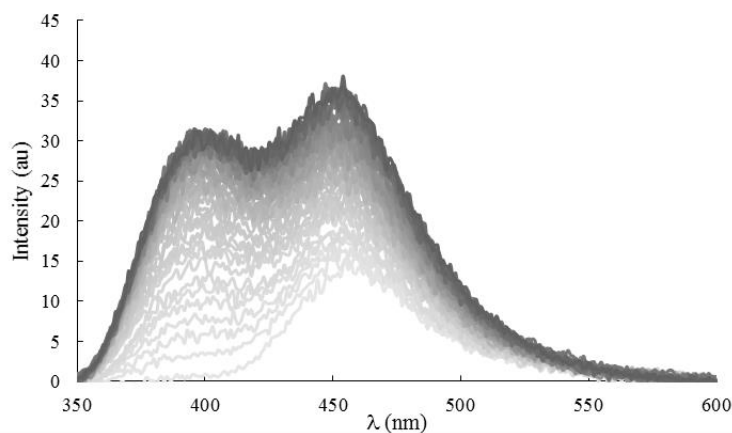


2.2.5.2 Hydrolysis assay

A sample of 3.7 mg of prodrug **2.84** was dissolved in 25 mL of DMSO to constitute the solution A at 400 μ M (100% DMSO). Then, 0.3 mL of the solution A were taken to a 10 mL flask, to which 50 mM phosphate buffer at pH 7 was added to give the solution B at 12 mM. For the experiment, a quartz cell was filled with 1 mL of solution B and 2 mL of pH 7 phosphate buffer 50 mM and the spectrometer was set up with the following parameters:

Parameter	Value
Excitation slits	2.5 mm
Emission slits	2.5 mm
Voltage	Medium
Scan mode	Emission
Excitation	325 nm
Delay between 2 scans	15 min
Number of scans	55
Temperature	25 °C

The following spectra were obtained



2.2.5.3 Quenching test

The following stock solutions

- A sample of 4.1 mg of umbelliferon was dissolved in 25 mL of pH 7 phosphate buffer with 2% DMSO (0.5 mL) to give a stock solution at 1 mM. It was further diluted to 30 μ M by diluting the stock solution.
- An NADH solution was prepared by dissolving 2.1 mg NADH in 10 mL of 50 mM phosphate buffer pH 7 with 2% DMSO. The concentration is 300 μ M.
- A solution of the catalyst was prepared by dissolving 0.5 mg of **Ir1** in 10 mL of 50 mM phosphate buffer pH 7 with 2% DMSO. The concentration is 30 μ M

From the following samples, three samples were prepared:

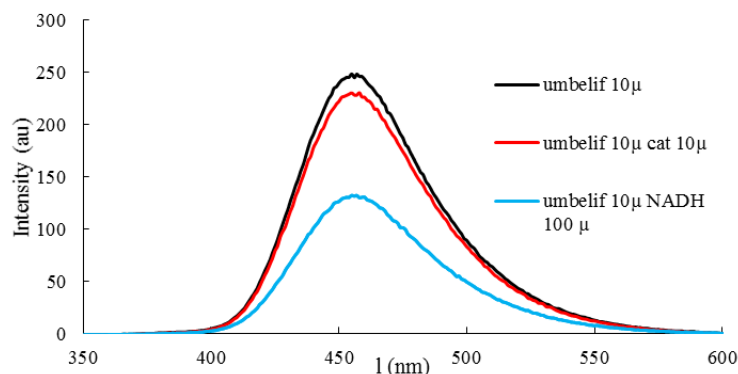
- Umbelliferon 10 μ M: 1 mL of umbelliferon 30 μ M solution + 2 mL 50 mM phosphate buffer pH 7 with 2% DMSO
- Umbelliferon 10 μ M + cat 10 μ M: 1 mL of umbelliferon 30 μ M solution+ 1 mL of **Ir1** 30 μ M solution + 1 mL 50 mM phosphate buffer pH 7 with 2% DMSO
- Umbelliferon 10 μ M + **Ir1** 10 μ M + NADH 10 μ M: 1 mL of umbelliferon 30 μ M solution+ 1 mL of **Ir1** 30 μ M solution + 1 mL NADH 300 μ M solution

The three samples were analysed using the following parameters:

Parameter	Value
Excitation slits	2.5 mm
Emission slits	2.5 mm
Voltage	Medium
Scan mode	Emission
Excitation	325 nm
Delay between 2 scans	0.5 min

Number of scans	5
Temperature	25 °C

The following curves were obtained



2.2.5.4 Calibration curve

At first, we envisioned using a standard calibration, using only the umbelliferon, but the quenching observed above made us re-consider our choice and opt for a calibration taking account of umbelliferon, the prodrug and NADH.

The following solutions, whose preparation are described below, were used:

- 3.7 mg of the prodrug **2.84** in 10 mL DMSO (1 mM)
- 21.3 mg NADH in 10 mL 50 mM phosphate buffer pH 7 with 2% DMSO (3 mM)
- 4.1 mg umbelliferon in 25 mL DMSO (1 mM)

The calibration samples were prepared according to the following protocol: (2 % DMSO, 0.47 mL of DMSO added to each sample to reach 2%)

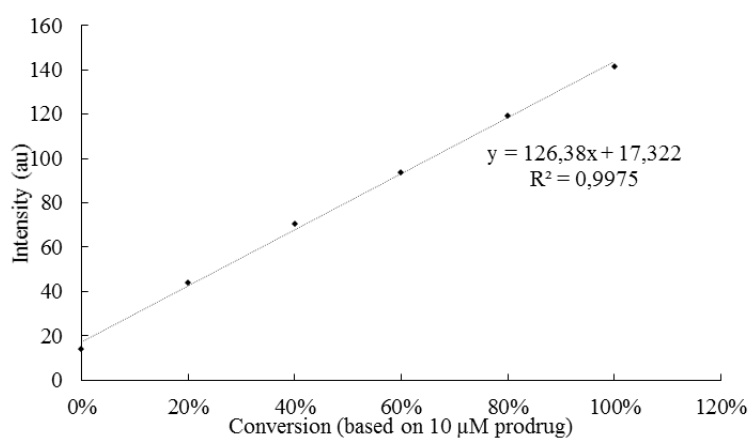
Conversion	C 2.84 (μ M)	C umbelliferon (μ M)	C NADH (μ M)	V stock 2.84 (mL)	V stock umbelliferon (mL)	V stock NADH (mL)
0%	10	0	100	0,50	0,00	1,67
20%	8	2	98	0,40	0,10	1,63
40%	6	4	96	0,30	0,20	1,60
60%	4	6	94	0,20	0,30	1,57
80%	2	8	92	0,10	0,40	1,53
100%	0	10	90	0,00	0,50	1,50

For each sample, a spectrum was recorded with the following parameters

Parameter	Value
Excitation slits	2.5 mm

Emission slits	2.5 mm
Voltage	Medium
Scan mode	Emission
Excitation	325 nm
Delay between 2 scans	0.5 min
Number of scans	5
Temperature	25 °C

The value at 454 nm of the 5 spectra was averaged after correction of the zero points and was plotted against the concentration of umbelliferon, which was a measurement of the conversion according to the table above.



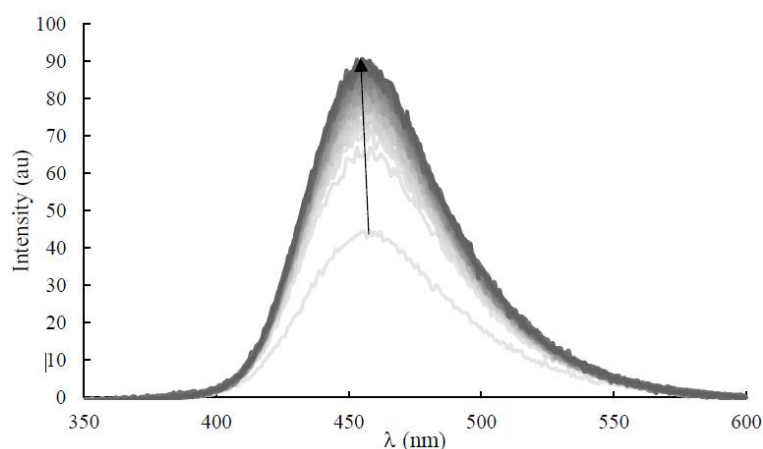
2.2.5.5 Fluorimetric assay

- 3.7 mg of the prodrug 1.93 in 10 mL DMSO (1 mM)
- 21.3 mg NADH in 10 mL 50 mM phosphate buffer pH 7 with 2% DMSO (3 mM)
- 0.5 mg **Ir1** in 25 mL 50 mM phosphate buffer pH 7 with 2% DMSO (3 mM)

1 mL of each solution was mixed in a quartz cell. The following parameters were applied:

Parameter	Value
Excitation slits	2.5 mm
Emission slits	2.5 mm
Voltage	Medium
Scan mode	Emission
Excitation	325 nm
Delay between 2 scans	15 min
Number of scans	50
Temperature	25 °C

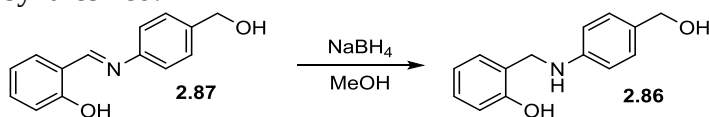
The following spectrum was obtained.



2.2.5.6 HPLC assay

a) Compound 2.86 preparation

All the following compounds were either commercially available, synthesis intermediates or final product, except **2.86** which was synthesized.



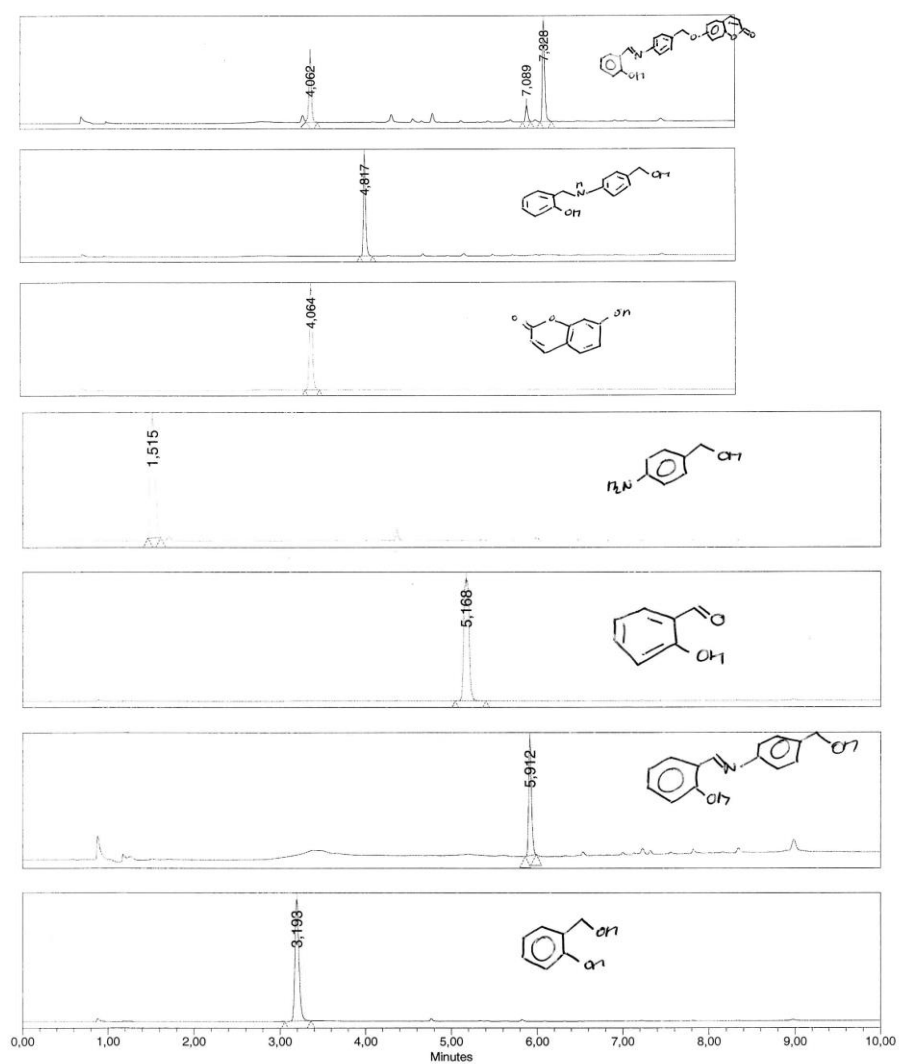
A 5 mL round-bottom flask was filled with the N-Salicylidene **2.87** (23.1 mg, 0.1 mmol), 2 mL MeOH (0.05 M) and NaBH₄ (19.6 mg, 5 equiv.). The solution decolourized and it was stirred for 40 min. Then, the solution was diluted in DCM and washed by water, dried by Na₂SO₄ to give the pure product **2.86** (19.4 mg, 83%).

¹H NMR (300 MHz, CDCl₃) δ 7.22 (d, *J* = 9.2 Hz, 2 H), 7.16 (m, 2 H), 6.89 (m, 2 H), 6.83 (d, *J* = 9.1 Hz, 2 H), 4.60 (s, 2H), 4.42 (s, 2H)

¹³C-RMN (75 MHz, CDCl₃) δ 156.75, 146.97, 133.33, 129.39, 128.87, 128.76, 122.95, 120.26, 116.76, 116.01, 65.26, 48.76.

b) References

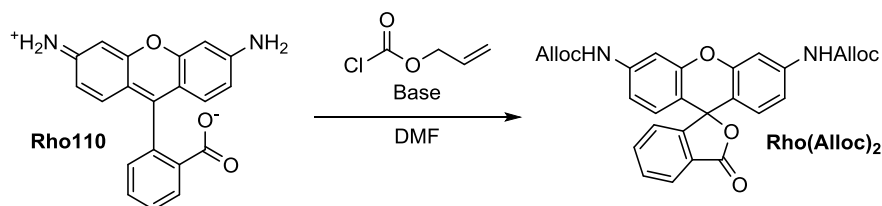
The compounds were analysed by reverse phase HPLC with a C18 Sunfire column XBridge REF21 with a gradient of H₂O/MeCN 90:10 to 10:90 in 10 min.



3 Second part

3.1 Synthesis of the fluorogenic probes

3.1.1 Rho(Alloc)₂



The **Rho(Alloc)₂** was synthesized according to an adapted literature procedure.¹⁹² White solid (20 mg, 19%).

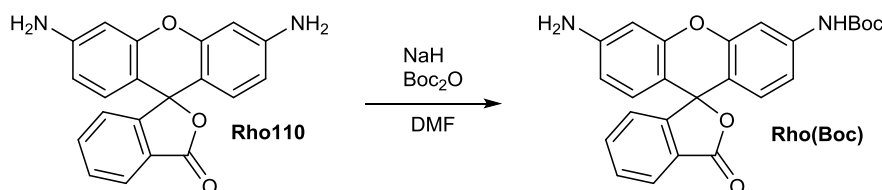
¹H NMR (300 MHz, DMSO-*d*₆) δ = 10.08 (s, 1H), 8.02 (d, *J* = 7.08 Hz, 1H), 7.77 (td, *J* = 7.09, 1.26 Hz, 1H), 7.71 (td, *J* = 7.09, 1.1 Hz, 2H), 7.56 (d, *J* = 2.04 Hz, 2H), 7.26 (d, *J* = 7.47 Hz, 1 H), 7.14 (dd, *J* = 8.7, 2.1 Hz, 2H), 6.69 (d, *J* = 8.67 Hz, 2H), 5.97 (m, 2H), 5.36 (ddt, *J* = 17.19, 1.65, 1.68 Hz, 2H), 5.23 (ddt, *J* = 10.41, 1.53, 1.53 Hz, 2H), 4.62 (dt, *J* = 5.43, 1.4 Hz, 4H).

¹³C NMR (75 MHz, DMSO-*d*₆) δ = 168.70, 153.11, 152.53, 150.96, 141.47, 135.73, 133.06, 130.23, 128.55, 125.75, 124.79, 123.81, 117.84, 114.50, 112.47, 105.13, 81.98, 64.97

MS ESI (+) *m/z* calcd for C₂₈H₂₂N₂O₇ [M + Na]⁺ 521.47; found 521.12

3.1.2 Rho(Boc+Alloc)

3.1.2.1 Rhodamine 110 mono Boc, Rho(Boc)



Rho(Boc) was synthesized according to a literature procedure.¹²⁶ Orange powder (126.8 mg, 74%).

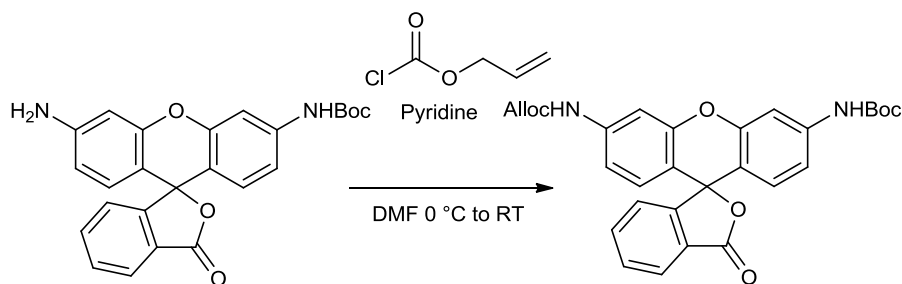
¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 9.65 (bs, 1H), 7.97 (d, *J* = 8.1 Hz, 1H), 7.78 (td, *J* = 7.4, 1.5 Hz, 1H), 7.70 (td, *J* = 7.5, 0.7 Hz, 1H), 7.52 (d, *J* = 1.8 Hz, 1H), 7.24 (d, *J* = 7.9 Hz, 1H), 7.09 (dd, *J* = 8.8, 2.5 Hz, 1H), 6.59 (d, *J* = 8.8 Hz, 1H), 6.43 (d, *J* = 1.9 Hz, 1H),

6.36 (d, $J = 8.5$ Hz, 1H), 6.32 (dd, $J = 8.5, 2.4$ Hz, 1H), 5.66 (bs, 2H), 1.48 (s, 9H).

^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ (ppm): 168.84, 152.64, 152.52, 151.98, 151.36, 151.30, 141.61, 135.46, 129.93, 128.49, 128.22, 126.34, 124.54, 124.00, 113.93, 112.55, 111.21, 105.26, 104.93, 99.08, 83.55, 79.64, 28.03 (3C).

HRMS (ESI): m/z 431.1593 (MH^+ [$\text{C}_{25}\text{H}_{23}\text{N}_2\text{O}_5$] = 431.1607).

3.1.2.2 Rhodamine 110 Boc-Alloc, Rho(Boc-Alloc)



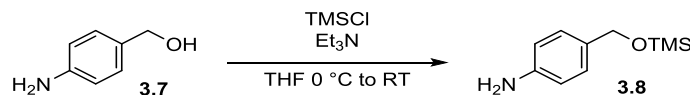
Rho(Boc-Alloc) was synthesized according to a literature procedure.¹⁹² A small RB flask was filled by home-synthesized **Rho(Boc)** (27 mg, 63 μmol) under Ar. Then, anhydrous DMF (0.4 mL, 0.2 M) and pyridine (50 μL) was added to give a bright yellow solution. Then, the reaction mixture was cooled at 0 $^{\circ}\text{C}$ with an ice bath, after which allyl Chloroformate (50 μM) was added by syringe at 0 $^{\circ}\text{C}$, during which the solution became bright red. The ice bath was removed 15 min after and the reaction was stirred overnight at room temperature. On the next morning, diethyl ether was added, and the organic phase was washed by HCl once, by NaHCO_3 once, then, by NaCl once and by LiCl 10% once. The organic phase was dried by Na_2SO_4 and the solvent was evaporated to give the **Rho(Boc-Alloc)** in the form of an orange-pink powder (yield: 88%, 28.7 mg)

^1H NMR (300 MHz, CDCl_3) δ 8.05 – 7.95 (m, 1H), 7.68 – 7.54 (m, 2H), 7.45 (d, $J = 2.2$ Hz, 2H), 7.24 (s, 1H), 7.16 – 7.07 (m, 1H), 6.98 (dd, $J = 8.6, 2.2$ Hz, 1H), 6.95 – 6.87 (m, 2H), 6.66 (dd, $J = 8.6, 4.8$ Hz, 2H), 6.09 – 5.82 (m, 1H), 5.34 (dd, $J = 17.2, 1.5$ Hz, 1H), 5.24 (dd, $J = 10.4, 1.4$ Hz, 1H), 4.65 (dt, $J = 5.8, 1.4$ Hz, 2H), 1.50 (s, 9H).

^{13}C NMR (75 MHz, CDCl_3) δ 169.78, 153.39, 153.07, 152.52, 151.99, 151.95, 140.73, 140.16, 135.25, 132.28, 129.87, 128.77, 128.64, 126.49, 125.15, 124.05, 118.60, 114.35, 113.66, 113.09, 106.47, 106.25, 82.92, 81.25, 77.16, 66.21, 60.56, 28.41.

3.1.3 Umbelliferon probe: 1st generation

3.1.3.1 4-aminobenzyl alcohol TMS protection (3.8)

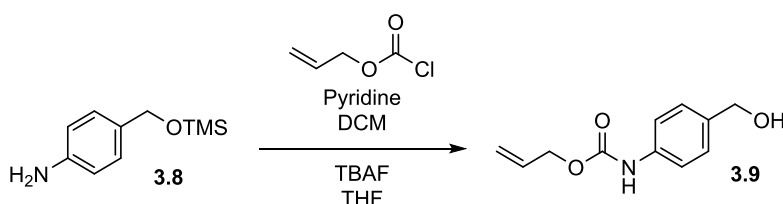


4-(((trimethylsilyl)oxy)methyl)aniline **3.8** was synthesized according to a routine procedure. A 25 mL round bottom flask was filled with 4-aminobenzyl alcohol **2.7** (250 mg, 2 mmol) under Ar atmosphere. Then, dry THF (4 mL, 0.5 M), followed by Et₃N (570 μ L, 4 mmol, 2 equivalents), was added. Then, the reaction mixture was cooled at 0 °C with an ice bath, after which TMSCl (0.3 mL, 23. mmol, 1.2 equiv.) was added dropwise at 0 °C. The reaction mixture became dark orange and an abundant white precipitate formed (Et₃N.HCl). The ice bath was removed, and the reaction was stirred at room temperature overnight. On the next morning, the reaction mixture was filtered on celite, and the pad was washed by Et₂O. The volatiles was removed under reduced pressure to give the crude **3.8** in the form of orange oil (350 mg, 74%).

¹H NMR (300 MHz, CDCl₃) δ 7.12 (d, *J* = 8.6 Hz, 2H), 6.65 (d, *J* = 8.4 Hz, 2H), 3.63 (s, 2H), 0.14 (s, 9H). ¹³C NMR (75 MHz, CDCl₃) δ 145.71, 131.12, 128.43, 115.14, 64.82, -0.15.

¹³C NMR (75 MHz, CDCl₃) δ 145.71, 131.12, 128.96, 128.43, 115.14, 64.82, -0.15.

3.1.3.2 Alloc protection of 4-aminobenzyl trimethylsilyl ether (3.9)



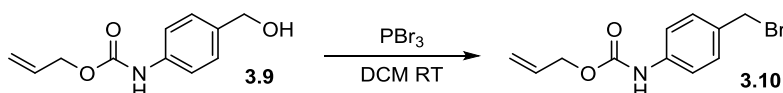
Allyl (4-(hydroxymethyl)phenyl)carbamate **3.9** was synthesized according to a routine procedure. A 25 mL round-bottom flask charged with the crude **3.8** (350 mg, 1.4 mmol) was purged by Ar 3 times before adding dry DCM (5 mL, 0.3 M) and pyridine (240 μ L, 3 mmol, 2 equiv.). Then, the reaction mixture was cooled at 0 °C with an ice bath, after which allyl Chloroform (180 μ L, 1.6 mmol, 1.1 equiv.) was added dropwise at 0 °C. After 15 min stirring, the ice bath was removed, and the reaction was stirred overnight at room

temperature. On the next morning, TBAF (1 M in THF, 1.2 equiv., 1.8 mL, 1.8 mL) was added at room temperature and the reaction was stirred for 1 more hour at room temperature. The organic phase was then diluted by EtOAc and the organic phase was washed by HCl 1 M 2 times, by NaHCO₃ sat and then dried by Na₂SO₄. Then the volume was reduced to a minimum and the product was filtered on a silica gel pad with 10% EtOAc in DCM to give the pure **3.9** (57 %, off-white solid).

¹H NMR (300 MHz, CDCl₃) δ 7.38 (d, *J* = 8.5 Hz, 2H), 7.31 (d, *J* = 8.9 Hz, 2H), 6.65 (s, 1H), 5.97 (ddt, *J* = 17.2, 10.4, 5.7 Hz, 1H), 5.37 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.27 (dq, *J* = 10.4, 1.3 Hz, 1H), 4.70 – 4.63 (m, 2+2H), 1.60 (t, *J* = 5.8 Hz, 1H).

¹³C NMR (75 MHz, CDCl₃) δ 153.43, 137.36, 136.29, 132.55, 128.07, 118.98, 118.39, 66.00, 64.93, 59.08, 24.24, 19.87, 13.81.

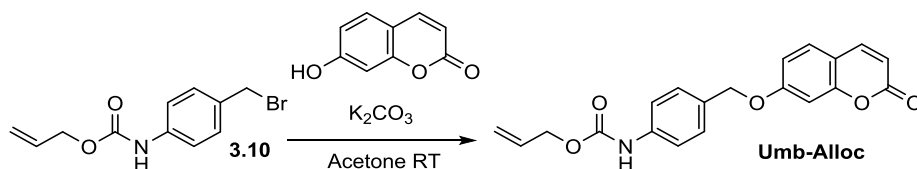
3.1.3.3 Bromination of the benzylic alcohol (**3.10**)



Allyl (4-(bromomethyl)phenyl)carbamate **3.10** was synthesized according to a routine procedure. A 25 mL round bottom flask was filled by **3.9** (103 mg, 0.5 mmol) under Ar atmosphere. Then, dry DCM (5 mL, 0.1 M), followed by PBr₃ (52 μL, 0.55 mmol, 1.1 equivalents), was added. The reaction was stirred at room temperature for one hour. Afterwards, 2 mL of H₂O was added, and the reaction was stirred for 1 more minute. Then, the phases were separated, and the organic phase was diluted in Et₂O and washed by brine 3 times, after which it was dried by Na₂SO₄ and the organic phase was concentrated under reduced pressure to give the product as an off-white powder (quantitative yield, 315 mg). The crude **3.10** was used as such in the next step without further purification.

¹H NMR (300 MHz, CDCl₃) δ 7.42 – 7.30 (m, 4H), 6.67 (s, 1H), 5.97 (ddt, *J* = 17.2, 10.4, 5.7 Hz, 1H), 5.37 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.32 – 5.23 (m, 1H), 4.67 (dt, *J* = 5.8, 1.4 Hz, 2H), 4.48 (s, 2H).

3.1.3.4 Coupling with umbelliferon (Umb-Alloc)



The compound **Umb-Alloc** was synthesized according to a routine procedure. The crude bromide **3.10** (134.3 mg, 0.49 mmol) was dissolved in acetone (5 mL, 0.1 M) in an open flask. Then, umbelliferon (90 mg, 0.54 mmol, 1.1 equiv.) and K₂CO₃ were added and the bright yellow heterogeneous mixture was stirred overnight at room temperature. On the next morning, EtOAc was added and the organic phase was washed by saturated NaHCO₃ until the organic phase became colourless. Afterwards, the organic phase was dried by Na₂SO₄ and the solvent was removed under reduced pressure. The prodrug **Umb-Alloc** was obtained as a white powder (168.5 mg, 97%). TLC: 35% EtOAc/petroleum ether.

¹H NMR (300 MHz, CDCl₃) δ 7.63 (d, *J* = 9.5, 1H), 7.43 (d, *J* = 8.7 Hz, 2H), 7.36 (d, *J* = 8.5 Hz, 3H), 6.94 – 6.85 (m, 2H), 6.84 (bs, 1H), 6.25 (d, *J* = 9.5 Hz, 1H), 5.96 (ddt, *J* = 17.2, 10.4, 5.7 Hz, 1H), 5.36 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.26 (dq, *J* = 10.4, 1.3 Hz, 1H), 5.06 (s, 2H), 4.67 (dt, *J* = 5.7, 1.4 Hz, 2H).

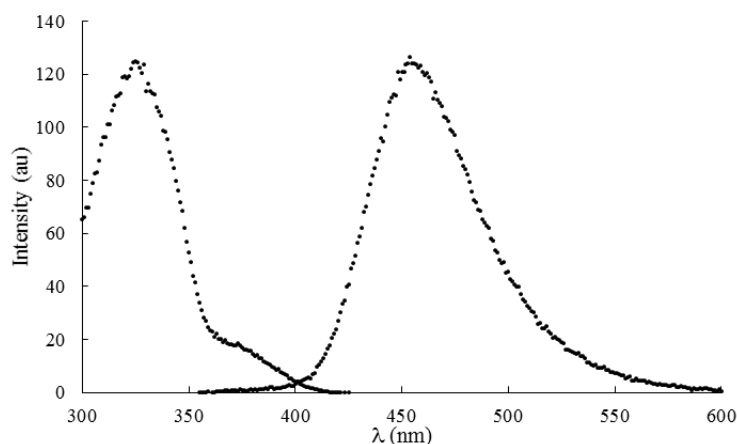
¹³C NMR (75 MHz, CDCl₃) δ 161.94, 161.36, 155.90, 153.27, 143.54, 138.17, 132.44, 130.76, 128.91, 128.73, 118.95, 118.46, 113.38, 113.31, 112.83, 102.01, 70.28, 66.06.

3.1.3.5 Photophysical parameters of umbelliferone

A 10 μ M solution of umbelliferon (2% DMSO) in water was prepared and was analysed in the spectrometer with the following parameters.

Parameter	Value
Excitation slits	2.5 mm
Emission slits	2.5 mm
Voltage	Medium
Scan mode	Emission (with excitation at 325 nm) Excitation (with emission at 354 nm)
Number of scans	10
Delay between 2 scans	0.5 min
Temperature	25 °C

The ten excitation and emission spectra were averaged, and a correction was applied to set I(600 nm) = 0. The following spectrum was obtained.



3.1.3.6 Photophysical parameters of the prodrug

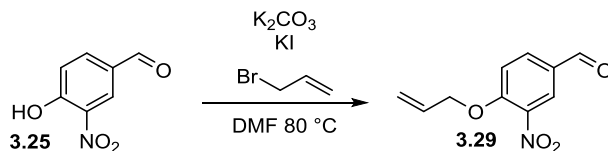
A 10 μM solution of umbelliferon (2% DMSO) in water was prepared and was analysed in the spectrometer with the following parameters.

Parameter	Value
Excitation slits	2.5 mm
Emission slits	2.5 mm
Voltage	Medium
Scan mode	Emission (with excitation at 325 nm) Excitation (with emission at 354 nm)
Number of scans	10
Delay between 2 scans	0.5 min
Temperature	25 $^{\circ}\text{C}$

The ten excitation and emission spectra were averaged, and a correction was applied to set $I(600\text{ nm}) = 0$. This correction was very minor if the spectrum was properly zeroed prior to the acquisition. The following spectrum was obtained.

3.1.4 Umbelliferon probe: 2nd generation

3.1.4.1 4-hydroxy-3-nitrobenzaldehyde allylation (3.29)



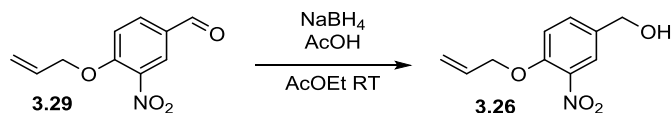
4-(allyloxy)-3-nitrobenzaldehyde **3.29** was synthesized according to a routine procedure. 4-hydroxy-3-nitrobenzaldehyde **3.25** was dissolved in DMF (2.5 mL, 0.6 M) in a 25 mL round-bottom flask. Then, K_2CO_3 (826 mg, 2.5 equiv., 6 mmol) and KI (80mg, 20 mol %, 0.48 μmol) were added, followed by allyl bromide (1 mL, 12 mmol, 5 equiv.).

The reaction was stirred at 100 °C overnight. On the next morning, the reaction medium was diluted with EtOAc and the organic phase was washed by brine 3 times, then by LiCl 10% once, after which the organic phase was dried with Na₂SO₄ and the solvent was removed under reduced pressure to afford the desired product **3.29** as a pale-yellow solid (480 mg, 97%).

¹H NMR (300 MHz, CDCl₃) δ 9.94 (d, *J* = 0.5 Hz, 1H), 8.35 (d, *J* = 2.1 Hz, 1H), 8.06 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.21 (d, *J* = 8.7 Hz, 1H), 6.04 (ddt, *J* = 17.3, 10.6, 5.0 Hz, 1H), 5.52 (dq, *J* = 10.6, 1.4 Hz, 1H), 5.39 (dq, *J* = 10.6, 1.4 Hz, 1H), 4.80 (dt, *J* = 5.0, 1.6 Hz, 2H).

¹³C NMR (75 MHz, CDCl₃) δ 188.87, 156.27, 134.59, 130.83, 129.22, 127.67, 119.37, 115.06, 70.62.

3.1.4.2 4-(allyloxy)-3-nitrobenzaldehyde reduction (3.26)

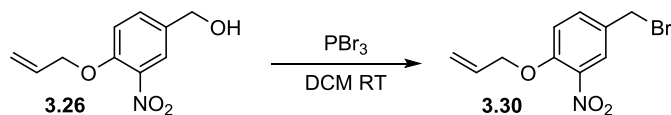


(4-(allyloxy)-3-nitrophenyl)methanol **3.26** was synthesized according to a standard procedure. To an Erlenmeyer containing 7 mL of EtOAc (0.18 M) was added NaBH₄ (156 mg, 3 equiv., 4.1 mmol). Then, acetic acid (0.7 mL, 9 equiv., 12.4 mmol) was added dropwise and effervescence was observed. When the effervescence stopped, the aldehyde **3.29** in solution in 3 mL EtOAc was added dropwise. Then, the reaction was stirred for 2 h at room temperature. Then, the reaction medium was transferred to a separatory funnel and the organic phase was washed by water twice, after which it was dried by Na₂SO₄. After evaporation of the solvent, the pure desired product **3.26** was afforded in the form of a white powder (97%, 279 mg).

¹H NMR (300 MHz, CDCl₃) δ 7.91 (dd, *J* = 2.3, 0.6 Hz, 1H), 7.56 (ddd, *J* = 8.6, 2.3, 0.6 Hz, 1H), 7.07 (d, *J* = 8.7 Hz, 1H), 6.15 – 5.93 (m, 1H), 5.49 (dq, *J* = 17.3, 1.7 Hz, 1H), 5.34 (dq, *J* = 10.6, 1.4 Hz, 1H), 4.89 (td, *J* = 6.2, 3.6 Hz, 1H), 4.70 (dt, *J* = 5.0, 1.6 Hz, 2H), 2.71 – 2.58 (m, 2H), 2.44 (d, *J* = 3.7 Hz, 1H), 2.11 (t, *J* = 2.6 Hz, 1H).

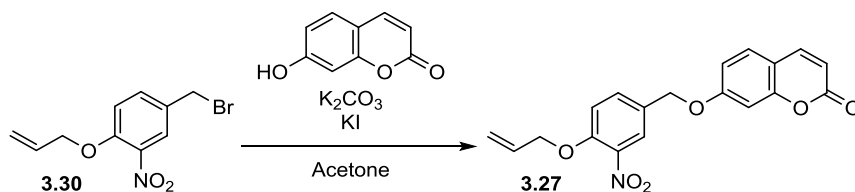
¹³C NMR (75 MHz, CDCl₃) δ 151.19, 139.57, 133.61, 132.66, 131.71, 124.04, 118.37, 115.03, 77.16, 70.13, 63.46.

3.1.4.3 (4-(allyloxy)-3-nitrophenyl)methanol bromination (3.30)



1-(allyloxy)-4-(bromomethyl)-2-nitrobenzene **3.30** was synthesized according to a routine procedure. A 25 mL round bottom flask was filled by **3.26** (279 mg, 1.33 mmol) under Ar atmosphere. Then, dry DCM (10 mL, 0.1 M), followed by PBr₃ (140 μ L, 1.47 mmol, 1.1 equivalents), was added. The reaction was stirred at room temperature for one hour. Afterwards, 2 mL of H₂O was added, and the reaction was stirred for 1 more minute. Then, the phases were separated, and the organic phase was diluted in Et₂O and washed by brine 3 times, after which it was dried by Na₂SO₄ and the organic phase was concentrated under reduced pressure to give the product as an off-white powder (quantitative yield). The crude **3.30** was used as such in the next step without further purification and characterization.

3.1.4.4 Umbelliferon second generation of the prodrug (**3.27**)

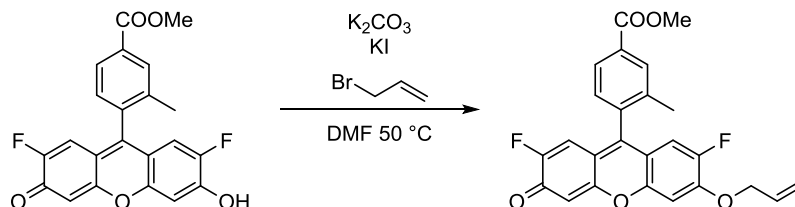


7-((4-(allyloxy)-3-nitrobenzyl)oxy)-2H-chromen-2-one **3.27** was synthesized according to a routine procedure. Compound **3.30** (1.33 mmol) was dissolved in acetone at room temperature in a 50 mL round-bottom flask. Then, K₂CO₃ (460 mg, 3.33 mmol, 2.5 equiv.) and KI (44 mg, 0.27 mmol, 20 mol%) were added, followed by the addition of umbelliferon (238 mg, 1.47 mmol, 1.1 equiv.). The mixture became bright yellow and was stirred overnight at room temperature. On the next day, the solvent was removed under reduced pressure and water was added and the mixture was vigorously stirred, and the pure product **3.27** was filtered on a paper and washed with distilled water. White powder (83%, 395 mg).

¹H NMR (300 MHz, DMSO-*d*₆) δ 8.03 (d, *J* = 2.2 Hz, 1H), 7.98 (d, *J* = 9.6, 1H), 7.75 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.64 (d, *J* = 8.6 Hz, 1H), 7.39 (d, *J* = 8.7 Hz, 1H), 7.10 (d, *J* = 2.4 Hz, 1H), 7.03 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.30 (d, *J* = 9.5 Hz, 1H), 6.03 (ddt, *J* = 17.3, 10.2, 5.0 Hz, 1H), 5.43 (dq, *J* = 17.3, 1.8 Hz, 1H), 5.29 (dq, *J* = 10.6, 1.5 Hz, 1H), 5.21 (s, 2H), 4.77 (dt, *J* = 5.0, 1.6 Hz, 2H).

¹³C NMR (75 MHz, DMSO) δ 161.13, 160.23, 155.30, 150.61, 144.26, 139.28, 134.09, 132.46, 129.56, 128.83, 124.65, 117.95, 115.59, 112.96, 112.74, 112.66, 101.66, 69.50, 68.36.

3.1.5 Maudamine



Maudamine was synthesized according to a routine procedure. Pennsylvania Green methyl ester (100 mg, 0.25 mmol) was dissolved in 1 mL DMF in a 5 mL round-bottom flask. Then, K_2CO_3 (87 mg, 0.63 mmol, 2.5 equiv.) and KI (8 mg, 20 mol %, 50 μ mol) were added, followed by allyl bromide (110 μ L, 1.26 mmol, 5 equiv.). Then, the reaction was stirred at 50 °C under Ar overnight. On the next morning, the DMF was evaporated and the bright red residue was re-dissolved in a minimum of DCM and washed by H_2O once and dried with Na_2SO_4 . Finally, the solvent was evaporated under reduced pressure and the bright orange solid was purified by flash column chromatography on silica gel using DCM/MeOH 95:5 as eluent. The product was obtained as an orange powder (95.6 mg, 86%)

^1H NMR (300 MHz, $CDCl_3$) δ 8.12 (s, 0H), 8.07 (d, J = 7.9 Hz, 0H), 7.26 (t, J = 3.9 Hz, 2H), 7.11 (d, J = 6.9 Hz, 1H), 6.69 (d, J = 10.9 Hz, 1H), 6.60 (d, J = 6.9 Hz, 1H), 6.54 (d, J = 10.6 Hz, 1H), 6.19 – 5.98 (m, 1H), 5.51 (dq, J = 17.3, 1.5 Hz, 1H), 5.42 (dq, J = 10.5, 1.1 Hz, 1H), 4.76 (dt, J = 5.4, 1.5 Hz, 2H), 3.99 (s, 3H), 2.13 (s, 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 176.37, 176.10, 166.43, 158.59, 157.35, 155.03, 152.26, 152.09, 151.51, 150.00, 148.21, 147.95, 136.75, 136.61, 132.11, 131.96, 131.17, 129.29, 127.75, 119.74, 118.10, 112.93, 112.65, 109.68, 109.38, 106.97, 102.51, 70.64, 52.65, 19.70.

^{19}F NMR (282 MHz, $CDCl_3$) δ -122.31.

3.2 Development of new biocompatible palladium complexes

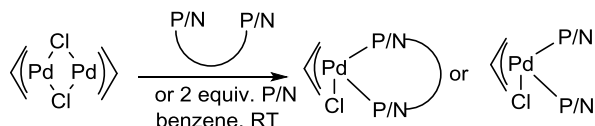
3.2.1 Synthesis of palladium complexes

3.2.1.1 Origin of the catalysts

In this project, several catalysts were commercially available, some others were synthesized by Dr Fatakanwa and some were synthesized more recently for the presented work. In this section, we will only describe the catalysts which we (re-)synthesized ourselves. For the others, see the thesis of Dr Fatakanwa.¹⁹³

3.2.1.2 Family A: π -Allyl palladium chlorides

a) General procedure



All the allylpalladium chloride (N, N) or (P, P) were prepared following an adapted literature procedure.¹⁹⁴ A glass vial with a magnetic bar was filled with η^3 -allylpalladium chloride dimer (20 mg, 0.055 mmol), followed by 3 mL benzene. After complete dissolution, the biphosphine (1 equiv.) or the monophosphine (2 equiv.) was added in one portion. The mixture was stirred at RT for typically 1 hour. In the case of biphosphines, a yellow to orange solid precipitates and was collected by filtration on paper, followed by washing with Et₂O. In the case of monophosphines, no precipitate was observed, and the solution was reduced under reduced pressure and precipitation was made adding *n*-pentane. The solid was filtered on a filter paper and dried under vacuum.

b) Characterizations

PdA01 (Xantphos)

¹H NMR (300 MHz, CD₂Cl₂) δ 7.63 (dd, *J* = 7.8, 1.4 Hz, 2H), 7.37 – 7.20 (m, 20H), 7.15 (t, *J* = 7.8 Hz, 2H), 6.56 (t, *J* = 7.6 Hz, 2H), 5.66 (dd, *J* = 19.5, 9.5 Hz, 1H), 3.70 – 3.53 (m, 4H), 1.65 (s, 6H). ³¹P NMR (121 MHz, DMSO-*d*₆) δ 3.37.

PdA02 (N-Xantphos)

¹H NMR (300 MHz, DMSO-*d*₆) δ 9.26 (s, 1H), 7.56 – 7.37 (m, 12H), 7.27 – 7.15 (m, 8H), 7.02 (d, *J* = 2.9 Hz, 4H), 6.09 (t, *J* = 10.1 Hz, 1H), 5.97 (ddd, *J* = 8.1, 6.2, 3.0 Hz, 2H), 3.59 (d, *J* = 10.0 Hz, 2H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 133.23, 133.16, 131.23, 129.47, 128.91, 125.88, 125.34, 117.53. ³¹P NMR (121 MHz, DMSO-*d*₆) δ 1.99.

PdA14 (JoePhos)

¹H NMR (300 MHz, CDCl₃) δ 7.34 – 7.27 (m, 20H), 7.06 (dd, *J* = 8.1, 1.3 Hz, 2H), 7.01 – 6.93 (m, 2H), 6.19 (td, *J* = 7.9, 7.3, 2.4 Hz, 2H), 5.78 (q, *J* = 10.1 Hz, 1H), 4.46 (d, *J* = 2.5 Hz, 2H), 3.64 (d, *J* = 6.6 Hz, 2H), 2.55 (d, *J* = 11.9 Hz, 2H), 2.36 – 2.24 (m, 1H). ¹³C NMR (75 MHz, CDCl₃) δ 148.03, 135.54, 133.27, 132.25, 128.86, 128.45, 126.70, 124.82, 117.94, 115.42, 109.03, 78.97, 77.36, 74.39, 34.37.

PdA07 (DPEPhos)

^1H NMR (300 MHz, DMSO- d_6) δ 7.58 – 7.39 (m, 16H), 7.33 – 7.25 (m, 8H), 7.13 – 6.99 (m, 4H), 6.57 (ddd, J = 9.4, 6.2, 1.6 Hz, 2H), 6.07 (p, J = 10.3 Hz, 1H), 3.81 (d, J = 62.0 Hz, 4H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 158.34, 134.51, 133.80, 131.68, 129.68, 129.61, 129.54, 128.91, 125.83, 123.62, 121.46, 79.28. ^{31}P NMR (121 MHz, DMSO- d_6) δ 12.85.

PdA22 (dppbz)

^1H NMR (300 MHz, DMSO- d_6) δ 7.84 (ddt, J = 13.1, 6.1, 2.6 Hz, 4H), 7.62 – 7.43 (m, 20H), 5.93 (p, J = 10.6 Hz, 1H), 4.17 (dt, J = 10.3, 4.6 Hz, 4H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 134.98, 133.52 (t, J = 6.8 Hz), 132.29, 131.73 – 130.67 (m), 130.08 (t, J = 5.5 Hz), 128.91. ^{31}P NMR (121 MHz, DMSO- d_6) δ 50.79.

PdA23 (dppe)

^1H NMR (300 MHz, DMSO- d_6) δ 8.02 – 7.41 (m, 20H), 5.95 (t, J = 10.6 Hz, 0H), 4.66 – 4.00 (m, 1H), 2.71 (t, J = 20.0 Hz, 2H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 134.02, 133.34, 132.65, 132.45, 131.08, 129.99, 129.44, 128.91. The aliphatic carbons were missing and were probably hidden by DMSO- d_6 . ^{31}P NMR (121 MHz, DMSO- d_6) δ 30.74.

PdA04 (PPh₃)

^1H NMR (300 MHz, DMSO- d_6) δ 7.49 (td, J = 6.6, 3.6 Hz, 15H), 5.79 (p, J = 10.0 Hz, 1H), 4.54 (s, 1H), 3.68 (s, 1H), 3.06 (s, 2H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 134.26 (d, J = 12.7 Hz), 132.81 (d, J = 40.7 Hz), 131.19, 129.27 (d, J = 10.0 Hz), 119.44. ^{31}P NMR (121 MHz, DMSO- d_6) δ 22.75.

PdA25 (Fur₃P)

^1H NMR (300 MHz, DMSO- d_6) δ 8.04 (s, 3H), 7.18 (t, J = 2.9 Hz, 3H), 6.63 (dt, J = 3.5, 1.8 Hz, 3H), 5.87 (p, J = 10.0 Hz, 1H), 3.79 (s, 4H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 150.11 (d, J = 5.0 Hz), 144.00 (d, J = 64.0 Hz), 124.57 (d, J = 23.7 Hz), 119.68, 112.05 (d, J = 8.3 Hz). ^{31}P NMR (121 MHz, DMSO- d_6) δ -33.95.

PdA05 ((4-OMe-C₆H₄)₃P)

^1H NMR (300 MHz, DMSO- d_6) δ 7.48 – 7.37 (m, 6H), 7.08 – 6.96 (m, 6H), 5.74 (p, J = 10.0 Hz, 1H), 4.51 (s, 1H), 2.99 (s, 2H). One proton as singlet masked by the water signal ^{13}C NMR (75 MHz, DMSO- d_6) δ 161.41, 135.71 (d, J = 14.1 Hz), 128.91, 124.46 (d, J = 45.7 Hz), 119.10 (d, J = 5.2 Hz), 114.79 (d, J = 11.1 Hz), 55.86. ^{31}P NMR (121 MHz, DMSO- d_6) δ 18.98.

PdA08 (dppf)

^1H NMR (300 MHz, CDCl_3) δ 7.87 (td, J = 8.6, 4.3 Hz, 9H), 7.74 – 7.43 (m, 20H), 4.54 (s, 3H), 4.24 (s, 2H). ^{13}C NMR (75 MHz, CD_2Cl_2) δ 134.66, 131.35, 128.23, 128.40, 75.87, 73.58. ^{31}P NMR (121 MHz, CDCl_3) δ 39.45, 28.61.

PdA09 (CocoPhos- CF_3)

^1H NMR (300 MHz, CD_2Cl_2) δ 7.93 (t, J = 9.5 Hz, 8H), 7.75 (d, J = 8.1 Hz, 8H), 6.03 – 5.60 (m, 1H), 4.60 (s, 4H), 4.50 (t, J = 2.0 Hz, 4H), 4.15 (dt, J = 10.0, 4.7 Hz, 4H). ^{31}P NMR (121 MHz, CD_2Cl_2) δ 91.18.

PdA15 (C042)

^1H NMR (300 MHz, CDCl_3) δ 7.98 – 7.75 (m, 1H), 7.54 (d, J = 6.3 Hz, 8H), 7.40 – 7.18 (m, 12H), 5.70 – 5.48 (m, 1H), 4.00 (s, 3H), 3.84 (s, 3H), 2.63 (t, J = 7.4 Hz, 2H), 1.88 (s, 2H), 1.44 – 1.21 (m, 2H), 1.12 – 0.96 (m, 1H), 0.80 (t, J = 7.3 Hz, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ 133.46, 133.38, 133.30, 130.36, 128.60, 128.53, 128.46, 119.48, 77.16, 71.10, 70.88, 70.66, 62.83, 62.69, 54.57, 54.31, 54.04, 27.81, 20.39, 14.09. ^{31}P NMR (121 MHz, CDCl_3) δ 3.73.

PdA18 (bpy)

^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 8.97 (s, 2H), 8.70 (d, J = 8.4 Hz, 2H), 8.35 (s, 2H), 7.78 (s, 2H), 6.08 (t, J = 9.8 Hz, 1H), 4.40 (s, 2H), 3.59 (s, 2H). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ 154.92, 141.71, 128.49, 124.33, 120.87, 64.30, 63.46.

PdA11 (MeObpy)

^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 8.73 (d, J = 6.3 Hz, 2H), 8.27 (d, J = 2.7 Hz, 2H), 7.29 (dd, J = 6.3, 2.6 Hz, 2H), 5.99 (p, J = 9.9 Hz, 1H), 4.33 (s, 2H), 4.04 (s, 6H), 3.44 (d, J = 12.5 Hz, 2H). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ 168.76, 156.65, 156.08, 139.36, 119.87, 113.54, 111.02, 62.43, 57.68.

PdA12 (phen)

^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 9.33 (dd, J = 5.0, 1.5 Hz, 2H), 8.95 (dd, J = 8.2, 0.8 Hz, 2H), 8.26 (s, 2H), 8.11 (dd, J = 8.3, 4.9 Hz, 2H), 6.16 (p, J = 9.8 Hz, 1H), 4.59 (s, 2H), 3.69 (s, 2H). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ 155.28, 145.55, 140.59, 130.52, 128.15, 127.11, 120.83, 65.80, 63.47.

PdA24 (bpa)

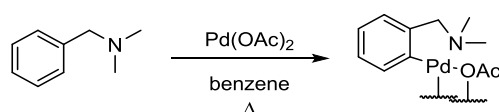
^1H NMR (300 MHz, CDCl_3) δ 12.37 (s, 1H), 8.38 (d, J = 8.6 Hz, 2H), 8.18 (ddd, J = 5.9, 1.9, 0.7 Hz, 2H), 7.75 (ddd, J = 8.8, 7.1, 1.8 Hz, 2H), 6.86 (ddd, J = 7.2, 5.8, 1.3 Hz, 2H), 5.99 (s, 1H), 3.89 (d, J = 7.1 Hz, 2H), 3.37 (d, J = 12.3 Hz, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ 151.47, 139.73, 118.20, 117.45, 77.23, 62.26.

PdA19 (bpa-CN)

^1H NMR (300 MHz, $\text{DMSO-}d_6$) δ 8.30 (s, 1H), 7.79 (dd, $J = 7.6, 1.3$ Hz, 2H), 7.69 (t, $J = 6.9$ Hz, 1H), 7.54 (dd, $J = 7.7, 1.4$ Hz, 2H), 7.43 (d, $J = 7.9$ Hz, 2H), 7.40 – 7.34 (m, 2H), 7.30 (d, $J = 8.4$ Hz, 1H), 6.99 (s, 1H), 5.83 (p, $J = 9.9$ Hz, 1H), 5.57 (s, 1H), 4.12 (d, $J = 45.1$ Hz, 2H), 3.44 (s, 2H). ^{13}C NMR (75 MHz, $\text{DMSO-}d_6$) δ 133.77, 119.54, 68.21. Not all the peaks were visible due to low concentration in the tube

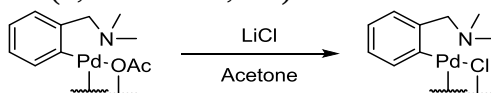
3.2.1.3 Family B: palladacycles

a) PdB01



It was synthesized according to a literature procedure.¹⁹³ A round-bottom flask was filled with N, N-dimethyl-1-phenylmethanamine (28.9 mg, 0.214 mmol, 1.2 equiv.) followed by benzene (3 mL, 0.06 M). Then, palladium (II) acetate (40 mg, 0.178 mmol, 1 equiv) was added. The bright orange mixture was heated in a closed vessel at 50 °C overnight during which it turned brown in one hour and then very dark yellow on the next morning. The mixture was cooled down to room temperature and filtered on a celite pad with DCM for washing. The solution was concentrated to a minimum volume and a yellow solid was obtained after the addition of hexanes. The bright yellow solid was obtained by filtration of paper. A second cycle could be performed. It was obtained as a bright yellow powder. Yield: 50%, 28 mg

^1H NMR (300 MHz, CDCl_3) δ 6.99 (ddd, $J = 16.8, 7.3, 1.4$ Hz, 2H), 6.93 – 6.82 (m, 2H), 3.57 (d, $J = 13.7$ Hz, 1H), 3.08 (d, $J = 13.8$ Hz, 1H), 2.80 (s, 2H), 2.05 (d, $J = 4.8$ Hz, 6H).

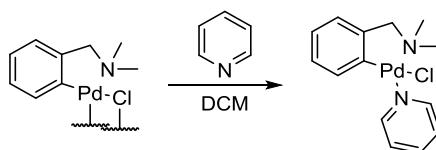


The chloro dimer intermediate was synthesized according to a routine procedure.¹⁹³ A round-bottom flask was filled with the palladacycle-acetate (28 mg, 0.047 mmol) followed by 3 mL of a saturated solution of LiCl in acetone (30 mg in 3 mL, 15 equiv.). The heterogeneous yellow mixture was stirred overnight protected from light in an aluminium foil. On the next day, the mixture seemed to be a darker yellow. The solvent was removed by rotavapor and DCM was added to fully re-dissolve the palladacycle. The inorganic salts were removed

by celite filtration. The bright yellow solution was concentrated to a minimum volume and a yellow solid was obtained after the addition of hexanes. The bright yellow solid was obtained by filtration of paper. A second cycle could be performed. It was obtained as a bright yellow powder. Yield: 90%, 23.3 mg.

^1H NMR (300 MHz, CDCl_3) δ 7.23 – 7.10 (m, 1H), 6.96 (dd, J = 6.9, 1.3 Hz, 1H), 6.87 (t, J = 6.4 Hz, 3H), 3.93 (s, 3H), 2.85 (d, J = 8.3 Hz, 10H).

^{13}C NMR (75 MHz, CDCl_3) δ = 147.03, 133.46, 125.22, 124.80, 121.64, 73.38, 52.95

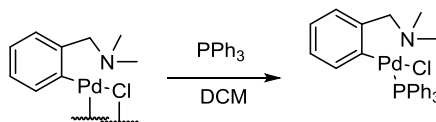


PdB01 was synthesized according to a routine procedure.¹⁹³ The chloro palladacycle (25.8 mg, 0.047 mmol) was dissolved in 1 mL DCM (0.02 M) in a round bottom flask. Then, pyridine (30 μL , 8 equiv., 0.880 mmol) was added by syringe. The yellow solution decolorizes within 2 seconds and turned very pale yellow. After 15 min of stirring, the volume of solvent was reduced to 25% and diethyl ether was added, during which an abundant white precipitate appears. The precipitate was obtained by filtration of paper. A second cycle could be performed. It was obtained as a fluffy white powder. The complex was purified by flash chromatography with 5-20% EtOAc in DCM. Yield: 22.8 mg, 69%. A suitable crystal for X-Ray Diffraction was obtained by slow diffusion of diethyl ether in a DCM solution.

^1H NMR (300 MHz, CDCl_3) δ = 8.87 (d, J = 5.01 Hz, 2H), 7.81 (t, J = 7.59 Hz, 1H), 7.37 (t, J = 7.02 Hz, 2H), 6.98 (d, J = 4.26 Hz, 2H), 6.79-6.74 (m, 1H), 5.99 (d, J = 7.62 Hz, 1H), 3.97 (s, 2H), 2.93 (s, 6H).

^{13}C NMR (75 MHz, CDCl_3) δ = 153.74, 149.06, 147.68, 137.89, 132.31, 125.46, 125.29, 124.65, 121.74, 74.17, 52.87.

b) **PdB02**

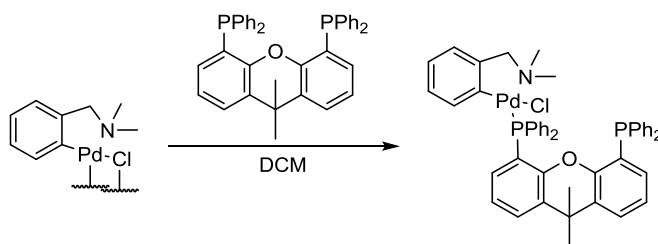


The same procedure as the one for **PdB01** was used.¹⁹³

^1H NMR (300 MHz, CDCl_3) δ 7.81 – 7.61 (m, 6H), 7.51 – 7.30 (m, 9H), 7.00 (dd, J = 7.4, 1.5 Hz, 1H), 6.82 (td, J = 7.2, 1.5 Hz, 1H), 6.47 – 6.24 (m, 2H), 4.07 (s, 2H), 2.85 (s, 6H). ^{13}C NMR (75 MHz, CDCl_3)

δ 150.76, 148.48, 137.98, 137.83, 135.39, 135.24, 131.73, 131.07, 130.51, 128.09, 127.95, 124.94, 124.87, 123.81, 122.35, 73.24, 50.58.
 ^{31}P NMR (121 MHz, CDCl_3) δ 42.97.

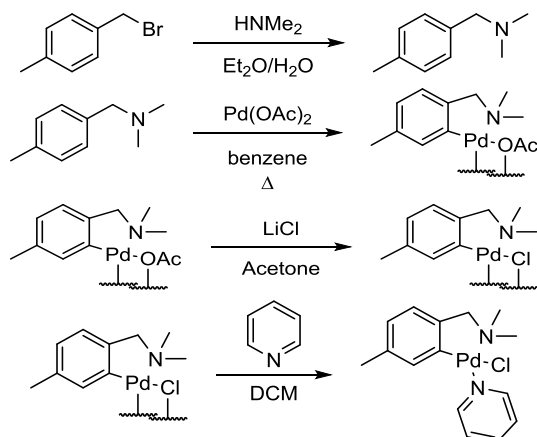
c) PdB03



The same procedure as the one for **PdB01** was used.¹⁹³

^1H NMR (300 MHz, CDCl_3) δ 7.50 – 7.32 (m, 9H), 7.22 (t, J = 7.3 Hz, 4H), 7.10 (t, J = 7.5 Hz, 8H), 6.96 (t, J = 7.7 Hz, 2H), 6.88 (d, J = 7.3 Hz, 1H), 6.83 – 6.68 (m, 3H), 6.31 – 6.23 (m, 2H), 3.84 (s, 2H), 2.63 (t, J = 1.4 Hz, 6H), 1.56 (s, 6H). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ 134.46, 130.85, 129.86, 128.66, 123.78, 49.96, 12.66. Not all the peaks were visible due to solubility issues in $\text{DMSO}-d_6$. ^{31}P NMR (121 MHz, CDCl_3) δ 22.08, 3.56 (broad).

d) PdB04

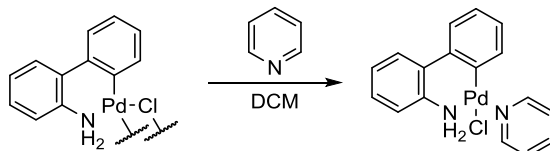


The same procedure as the one for **PdB01** was used.¹⁹³

^1H NMR (300 MHz, $\text{Chloroform}-d$) δ 8.88 (dt, J = 5.0, 1.6 Hz, 2H), 7.82 (tt, J = 7.7, 1.7 Hz, 1H), 7.46 – 7.33 (m, 2H), 6.87 (d, J = 7.5 Hz, 1H), 6.80 (d, J = 7.5 Hz, 1H), 5.79 (s, 1H), 3.94 (s, 2H), 2.91 (s, 6H), 2.07 (s, 3H).

^{13}C NMR (75 MHz, CDCl_3) δ 153.72, 148.91, 144.59, 137.88, 134.86, 132.97, 125.34, 125.25, 121.45, 77.16, 73.90, 52.79, 21.49.

e) **PdB06**

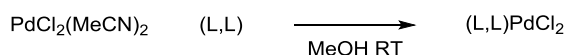


The same procedure as the one for **PdB01** was used.¹⁹³

¹H NMR (300 MHz, CDCl₃) δ 9.81 (s, 2H), 8.91 – 8.73 (m, 4H), 7.98 – 7.81 (m, 2H), 7.56 – 7.40 (m, 4H), 7.00 (d, *J* = 8.2 Hz, 2H), 6.52 (dd, *J* = 8.2, 2.4 Hz, 2H), 5.73 (d, *J* = 2.4 Hz, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 153.04, 138.44, 138.44, 126.33, 125.72, 118.80, 111.10, 109.98.

3.2.1.4 Family C: Palladium bis halides and various

a) **General procedure:**



PdCl₂(MeCN)₂ (20 mg) and the biphosphine (1 equiv.) or the monophosphine (2 equiv.) are suspended in dichloromethane (2 mL). After 30 min stirring, the mixture was poured in pentane and a yellow solid was collected by filtration as a yellow powder (yield: quantitative).

PdC20 was obtained by de-symmetrisation of the chloro-dimer with pyridine in DCM with the same method as **PdB01**.

b) **Characterizations**

PdC01 (Xantphos)

¹H NMR (400 MHz, CDCl₃) δ 7.66 (dt, *J* = 7.7, 1.0 Hz, 2H), 7.47 (ddd, *J* = 9.1, 7.7, 1.3 Hz, 2H), 7.37 (d, *J* = 10.0 Hz, 8H), 7.31 – 7.24 (m, 2H), 7.23 – 7.15 (m, 4H), 7.05 (td, *J* = 8.0, 7.6, 2.4 Hz, 8H), 1.86 (s, 6H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 130.84, 128.76. Only those two peaks were visible as it was not soluble enough in DMSO-*d*₆. ³¹P NMR (121 MHz, DMSO-*d*₆) δ 22.29.

PdC02 (rac-Binap)

¹H NMR (300 MHz, DMSO-*d*₆) δ 7.70 (dt, *J* = 10.9, 7.2 Hz, 8H), 7.64 – 7.41 (m, 12H), 7.16 (dt, *J* = 14.2, 8.7 Hz, 4H), 6.93 (t, *J* = 7.5 Hz, 2H), 6.75 (d, *J* = 7.8 Hz, 4H), 6.62 (d, *J* = 8.6 Hz, 2H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 135.25, 135.12, 134.16, 131.46, 131.09, 130.29, 129.73, 128.57, 128.44, 128.35, 128.19, 127.99, 127.55, 127.37, 122.80, 122.06. ³¹P NMR (121 MHz, DMSO-*d*₆) δ 28.36.

PdC03 (dppm)

^1H NMR (300 MHz, $\text{DMSO-}d_6$) δ 8.10 – 7.91 (m, 8H), 7.66 – 7.38 (m, 12H), 4.95 (t, J = 11.5 Hz, 2H). ^{13}C NMR (75 MHz, $\text{DMSO-}d_6$) δ 133.44 (t, J = 6.1 Hz), 132.90, 129.64 (t, J = 6.0 Hz), 127.68 (t, J = 26.8 Hz). ^{31}P NMR (121 MHz, $\text{DMSO-}d_6$) δ -52.28.

PdC04 (dppp)

^1H NMR (300 MHz, $\text{DMSO-}d_6$) δ 7.92 – 7.67 (m, 8H), 7.63 – 7.09 (m, 12H), 2.85 – 2.58 (m, 2H), 2.38 – 2.11 (m, 2H). No decent ^{13}C NMR could be recorded due to low solubility. ^{31}P NMR (121 MHz, $\text{DMSO-}d_6$) δ 13.06,

PdC05 (dppb)

^1H NMR (300 MHz, $\text{DMSO-}d_6$) δ 7.82 (ddt, J = 9.6, 6.4, 1.7 Hz, 8H), 7.62 – 7.44 (m, 13H), 2.80 – 2.63 (m, 4H), 1.47 (d, J = 23.0 Hz, 5H). No decent ^{13}C NMR could be recorded due to low solubility. ^{31}P NMR (121 MHz, $\text{DMSO-}d_6$) δ 32.29.

PdC06 (dppbz)

^1H NMR (300 MHz, $\text{DMSO-}d_6$) δ 7.88 – 7.67 (m, 10H), 7.66 – 7.46 (m, 10H). ^{13}C NMR (75 MHz, $\text{DMSO-}d_6$) δ 134.79, 134.31, 132.82, 129.73. ^{31}P NMR (121 MHz, $\text{DMSO-}d_6$) δ 64.79.

PdC07 (Fur₃P)

^1H NMR (300 MHz, $\text{DMSO-}d_6$) δ 7.92 (d, J = 2.0 Hz, 1H), 6.93 (t, J = 2.9 Hz, 1H), 6.57 (dt, J = 3.5, 1.6 Hz, 1H). ^{13}C NMR (75 MHz, $\text{DMSO-}d_6$) δ 150.54, 125.49 (d, J = 20.4 Hz), 112.32 (d, J = 8.8 Hz). ^{31}P NMR (121 MHz, $\text{DMSO-}d_6$) δ -21.43.

PdC08 ((C₆F₅)₃P)

No protons. The peaks were not visible on the ^{13}C spectrum. ^{31}P NMR (121 MHz, $\text{DMSO-}d_6$) δ -74.92 (p, J = 35.3 Hz). ^{19}F NMR (282 MHz, $\text{DMSO-}d_6$) δ 122.21.

PdC09 ((4-MeO-C₆H₄)₃P)

^1H NMR (300 MHz, $\text{DMSO-}d_6$) δ 7.47 (ddd, J = 9.0, 5.9, 3.7 Hz, 12H), 7.01 (d, J = 8.4 Hz, 12H), 3.77 (s, 18H). ^{13}C NMR (75 MHz, $\text{DMSO-}d_6$) δ 161.62, 131.83, 114.46, 55.89. ^{31}P NMR (121 MHz, $\text{DMSO-}d_6$) δ 20.60.

PdC12 (phen)

^1H NMR (300 MHz, $\text{DMSO-}d_6$) δ 9.32 (dd, J = 5.3, 1.3 Hz, 2H), 8.95 (dd, J = 8.2, 1.4 Hz, 2H), 8.26 (s, 2H), 8.11 (dd, J = 8.2, 5.3 Hz, 2H). No decent ^{13}C -NMR was recorded due to low solubility in $\text{DMSO-}d_6$

PdC13 (bpy)

^1H NMR (300 MHz, $\text{DMSO-}d_6$) δ 9.04 (d, J = 5.6 Hz, 2H), 8.50 (d, J = 8.1 Hz, 2H), 8.27 (t, J = 7.8 Hz, 2H), 7.81 – 7.57 (t, J = 7.8 Hz, 2H).

^{13}C NMR (75 MHz, DMSO- d_6) δ 156.94, 150.21, 141.76, 127.83, 124.43.

PdC14 (dmmda)

^1H NMR (300 MHz, DMSO- d_6) δ 4.93 (s, 1H), 2.63 (s, 6H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 97.38, 51.20.

PdC15 (dmeda)

^1H NMR (300 MHz, DMSO- d_6) δ 2.69 (s, 4H), 2.61 (s, 6H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 62.34, 51.07.

3.2.2 *In vitro* assay of palladium complexes: general protocol for a catalyst screening using the Varian Carry Eclipse Spectrometer

3.2.2.1 Preparation of the solutions

If we consider the model deprotection of the umbelliferon prodrug by (Allyl)PdCl(Xantphos) in presence of glutathione, we need to prepare 4 solutions for the experiment: one solution of prodrug, one of umbelliferon, one of glutathione and one of the catalyst. The prodrug concentration was 10 μM as we know that umbelliferon was detected with a linear response with this concentration. As the volume of the quartz cell was 3 mL, it was easier to prepare stock solutions with a concentration of 3 times what was used in the reaction, so that we simply need to mix 1 mL of each solution to launch the experiment.

The conditions for the reaction are described as following:

	C stock solution (μM)	C reaction (μM)	Equivalents
Prodrug	30 μM	10 μM	1 eq.
Glutathione (GSH)	150 μM	50 μM	5 eq.
Palladium ((Allyl)PdCl(Xantphos)) M_w = 761.56 g/mol	6 μM	2 μM	20 mol %

The mass needed for this protocol are described in the table below:

	C stock solution (μM)	m (mg)	Procedure	DMSO
Prodrug	30	5.3	m in 10 mL DMSO bring to 100 mL (water) 20 mL of diluted in 100 mL	2 %
Glutathione	150	4.6	m in 100 mL (water)	0%

Palladium ((Allyl)PdCl(Xantphos)) Mw = 761.56 g/mol	6	2.3	m in 5 mL DMSO bring to 100 mL (water) 20 mL of dil. in 100 mL	1%

The solution of the prodrug was stable if stored at 4°C but the other solutions need to be used rapidly after preparation.

For the calibration, one stock solution of umbelliferon. Since the calibration was conducted with concentrations ranging from 0 to 12 µM, we prepared a stock solution at 100 µM which was diluted 10 times. The stock solution was prepared by dissolving 16.2 mg of umbelliferone in 10 mL DMSO and makeup to 100 mL with water (10 % DMSO). A 10-fold dilution was then performed to obtain a *stock* solution (10 µM, 1% DMSO).

3.2.2.2 Catalyst assay

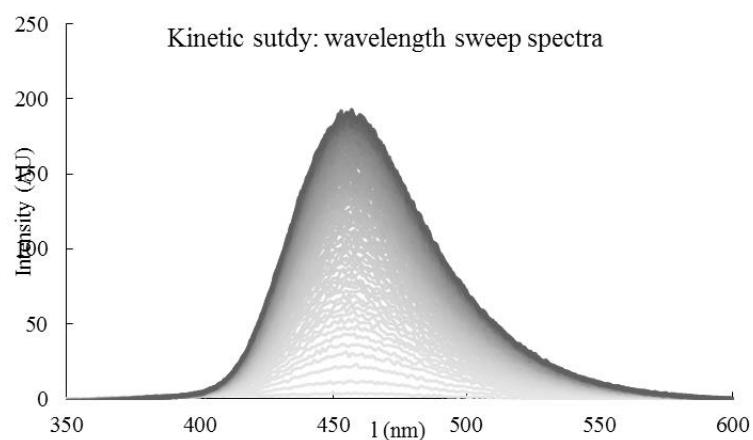
Once the three solutions were prepared of catalyst, prodrug and GSH were prepared, 1 mL of each was introduced in the fluorescence cell with a small stirring bar. The temperature was set to 25 °C and the stirring to medium. Spectra were recorded from 350 to 600 nm with an excitation at 325 nm every 5 min for 18 h. The slits were set to 2.5 mm.

3.2.2.3 Experimental result and data analysis

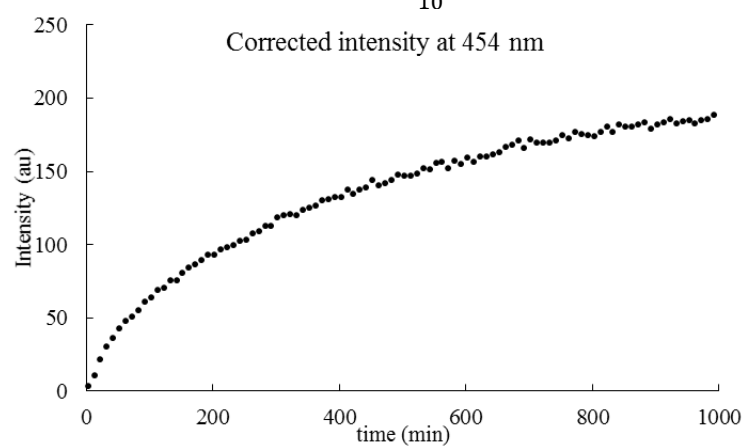
The kinetic study provides a larger matrix with the wavelengths in the first column and the time in the

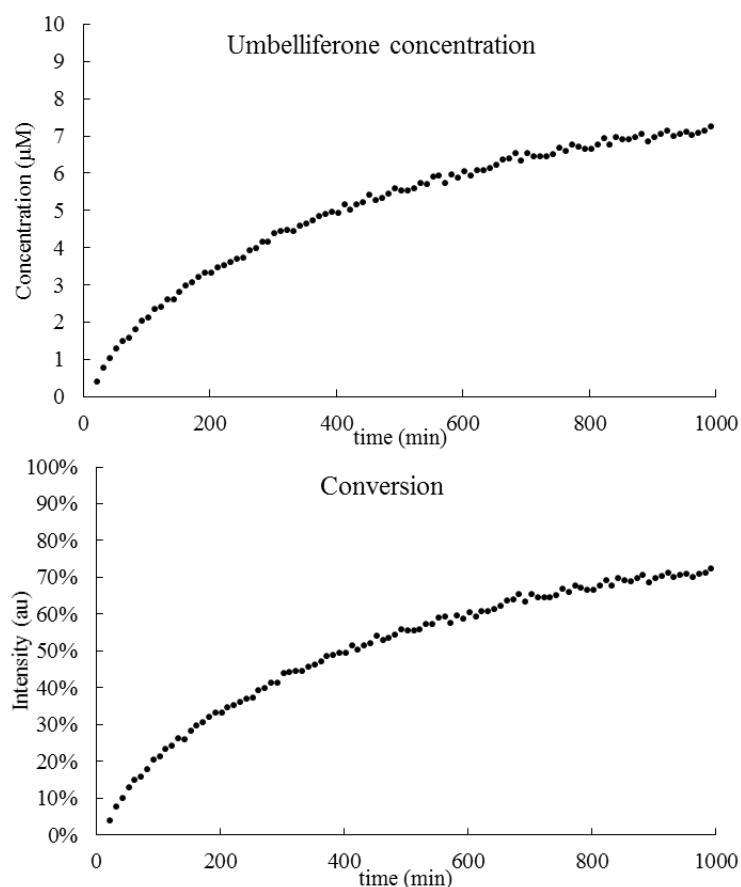
	1	2	...	n-1	n
350					
351					
352					
...					
...					
599					
600					

Firstly, the spectrum had to be recalibrated $I(\lambda)^{corrected} = I(\lambda) - I(600\text{ nm})$. This calibration was usually minor. Secondly, an automatic colour scale should be applied on those data in order to easily identify the intensity of the signal. This was a visual way to judge whether the experiment worked properly and quickly identify λ_{max} and compare this value to the expected one.



Then, the row $I(454 \text{ nm}) = f(t)$ has to be extracted and plotted in function of the time. The function $I_{\lambda_{max}} = f(t)$ was obtained. The concentration of umbelliferon can be found by using the calibration curve previously obtained. Then, knowing that C_0 was $10 \mu\text{M}$, the conversion was given by $conv(t) = \frac{c(t)}{10}$





3.2.3 *In vitro* assay of palladium complexes: general protocol for a catalyst screening using the multi-well plate reader

3.2.3.1 Description of the apparatus

The apparatus consists of a SpectraMax multi-well plate fluorimeter. It comprises a solution dispenser, but it was not used as it was not functional. It was equipped with excitation and emission filters which can be chosen prior to the experiment (for the rhodamine model, filters at 490 nm and 520 nm were available and chosen. A shaking run was performed prior to any experiment for 10 seconds with 240 rpm and 7 mm of diameter. Temperature control during the kinetics and shaking between two measurements were not available.

3.2.3.2 Preparation of the solutions

a) Plate plan

A typical plate plan consists of six rows for the catalytic tests and 2 rows for the calibration. The advantage if this typical plan was that the calibration was read at every measurement to take account of any fluctuation of the detector or the light source and that every catalyst was studied in triplicates, for which an average can be calculated.

	1	2	3	4	5	6	7	8	9	10	11	12
	Catalyst screening											
A	cat1	cat2	cat3	cat4	cat5	cat6	cat7	cat8	cat9	cat10	cat11	cat12
B												
C												
D	cat13	cat14	cat15	cat16	cat17	cat18	cat19	cat20	cat21	cat22	cat23	cat24
E												
F												
	Calibration (μM)											
G	0	0,4	0,8	1,2	1,6	2	2,4	2,8	3,2	3,6	4	4,4
H	0	0,4	0,8	1,2	1,6	2	2,4	2,8	3,2	3,6	4	4,4

b) Probe

The concentration of probe was set to 4 μM for several reasons. At first, the calibration curve was found to be linear with $R^2 > 0.99$ throughout all the range, a sign of the absence of aggregation and self-quenching phenomenon. Then, the ratio signal/noise was excellent as there was no background fluorescence and the **Rho(Boc-Alloc)** was 300 times less fluorescent than **Rho(Boc)**. Eventually, the probe was found to be fully soluble in those conditions, with 2% of DMSO during the kinetic study.

To prepare the stock solution, 3.98 mg of **Rho(Boc-Alloc)** was dissolved in 10 mL DMSO to reach a concentration of 0.8 mM. This solution was found to be stable and could be stored for weeks at 4 °C. Then the daughter solution was prepared by diluting 1 mL of mother solution to 50 mL with pH 7 10 mM phosphate buffer. The concentration was 16 μM with 2% DMSO.

A solution of **Rho(Boc)** was also prepared for the calibration. So, 1.72 mg was dissolved in 5 mL DMSO to make a 0.8 mM mother solution.

Then, 1 mL was diluted in diluting 1 mL of mother solution to 50 mL with pH 7 10 mM phosphate buffer. The concentration was 16 μ M with 2% DMSO.

c) Glutathione

The glutathione solution was prepared by dissolving 6.15 mg of GSH in 50 mL of 10 mM pH 7 phosphate buffer to reach 0.4 mM with 2% DMSO.

d) Catalysts

A typical procedure for **PdA01**. The solution was prepared by dissolving 0.91 mg of **PdA01** in 2 mL of DMSO prior to bringing the volume to 100 mL with 10 mM phosphate buffer to reach 12 μ M with 2% DMSO.

e) Calibration

As mentioned before, the strength of our screening method was to use a calibration curve in the two last horizontal rows of the plate were used for calibration and were measured every 5 minutes to get the regression parameters as a function of time. For this, as our kinetic tests use 4 μ M of **Rho(Boc-Alloc)**, we made 11 calibration standards ranging from 0 to 4.4 μ M, each standard was done in duplicate.

3.2.3.3 Plate preparation

The optimization leads us to find the optimal conditions as follows: **Rho(Boc-Alloc)** 4 μ M, 15 mol % of catalyst and 25 equivalents of glutathione.

a) Spectrometer setup

In this section, the general setup for any plate reading spectrometer will be detailed. Once the plate was ready, it was advisable to shake it either manually, but most spectrometer could include a shaking run at the beginning of a program. It was also recommended to apply temperature control throughout all the reaction. We kept the temperature at 25 °C during all the catalytic tests.

We opted for a kinetic assay rather than a single point reading for the catalyst screening. For clarity purposes in the paper, we presented only the values after 1 hour and after 13 h. The spectrometer was set to take a measurement every 5 minutes.

For our fluorescent probe **Rho(Boc)**, the available excitation and emission filters of 485 nm and 520 nm respectively were available and selected.

b) Catalyst assay

Each catalyst was tested in triplicate. Each well was successively filled with: 90 μL of 10 mM pH 7 phosphate buffer with 2% DMSO, 50 μL of GSH solution (400 μM), 10 μL of **PdA01** solution (12 μM). All the wells should be filled identically with the catalyst and GSH. The reaction itself was launched by adding 50 μL of prodrug solution as quickly as possible using a 12-channel micropipette. At this moment, the spectrometer program was launched.

c) Calibration

Each calibration sample was made in duplicate in the last two rows of the plate.

C cal (μM)	V Rho(Boc) (μL)	V buffer (μL)
0,0	0,0	200,0
0,4	5,0	195,0
0,8	10,0	190,0
1,2	15,0	185,0
1,6	20,0	180,0
2,0	25,0	175,0
2,4	30,0	170,0
2,8	35,0	165,0
3,2	40,0	160,0
3,6	45,0	155,0
4,0	50,0	150,0
4,4	55,0	145,0

3.2.3.4 Experimental results

a) Calibration

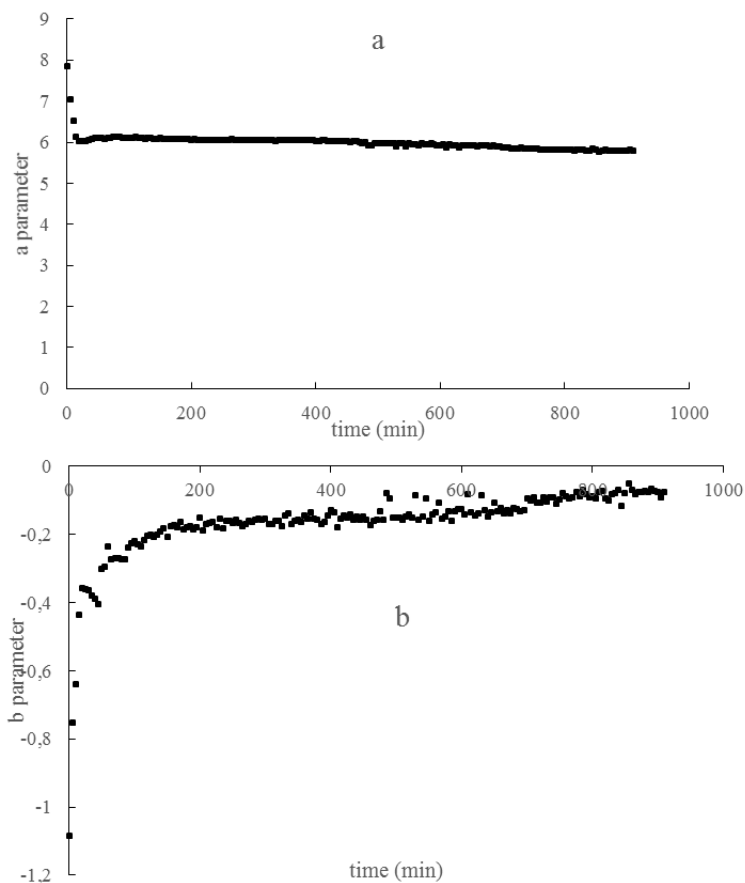
For each temporal set of data, the parameters a and b of the equation $I = a.C + b$ could be extracted and plotted in function of the time to obtain $a(t)$ and $b(t)$. As shown below, a tends to decrease quickly and then remain almost constant. It therefore highlights the importance of using this type of calibration to get accurate conversion values.

Based on this, taken on all the points, we could calculate average a and b parameters with uncertainties based on Student distribution.

$$a = 5.99 \mu\text{M}^{-1} \pm 0.014$$

$$b = -0.164 \pm 0.08$$

$$R^2 = 0.99845 \pm 7E - 0.5$$

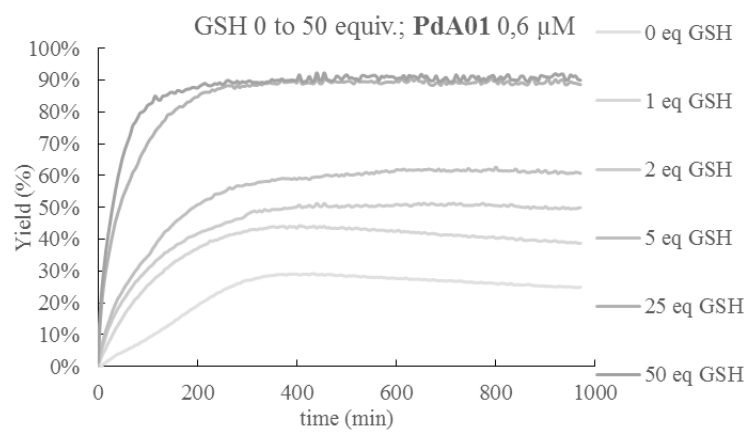
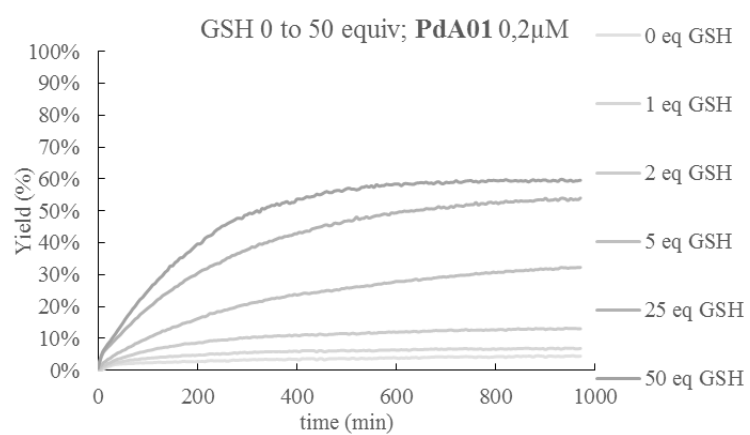
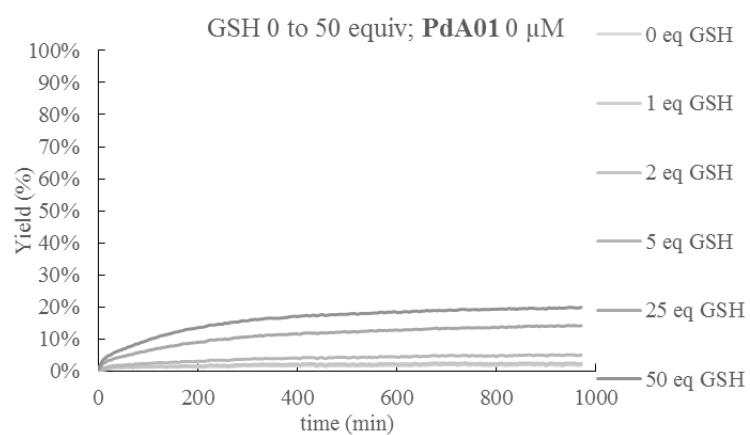


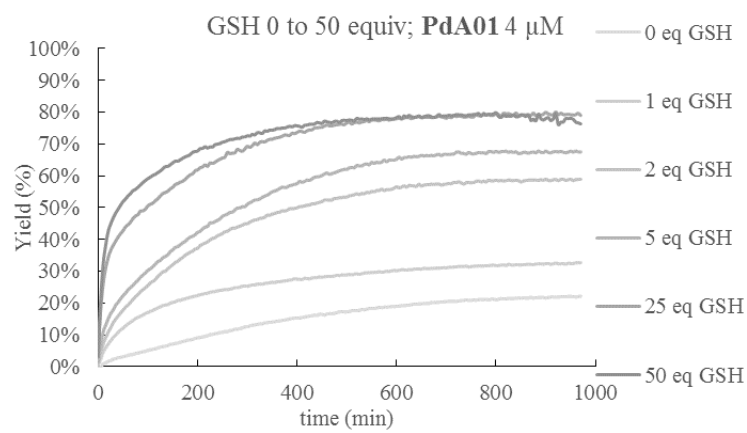
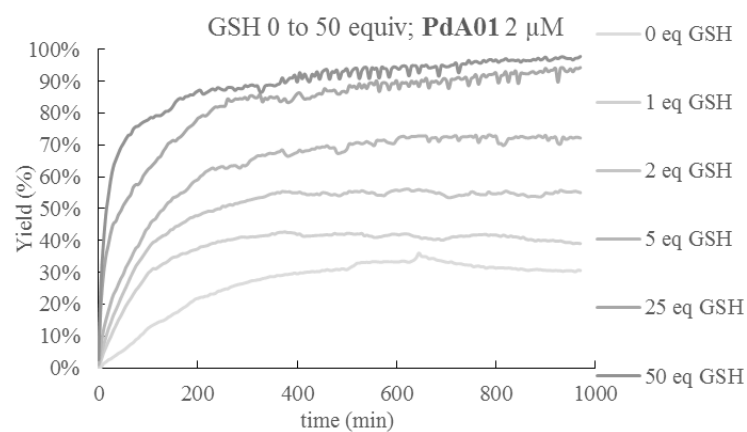
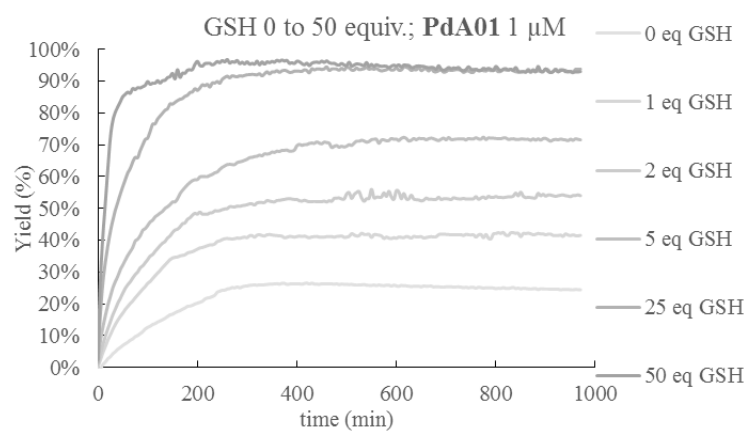
b) Data analysis

For each catalyst, we obtained 3 curves $I_i(t)$ for $i = 1$ to 3. Then, an average was performed $I(t) = \sum_{i=1}^3 \left(\frac{I_i}{3}\right)$. Then using the calibration curve, $C(t) = \frac{I(t)-b(t)}{a(t)}$, from which $conv(t) = \frac{C(t)}{C^0}$

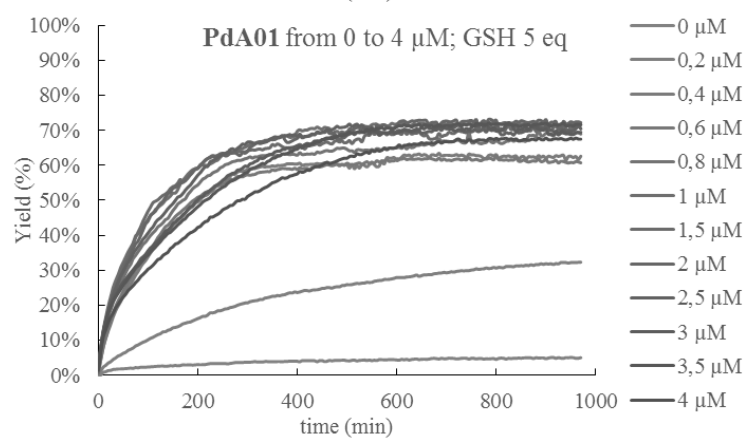
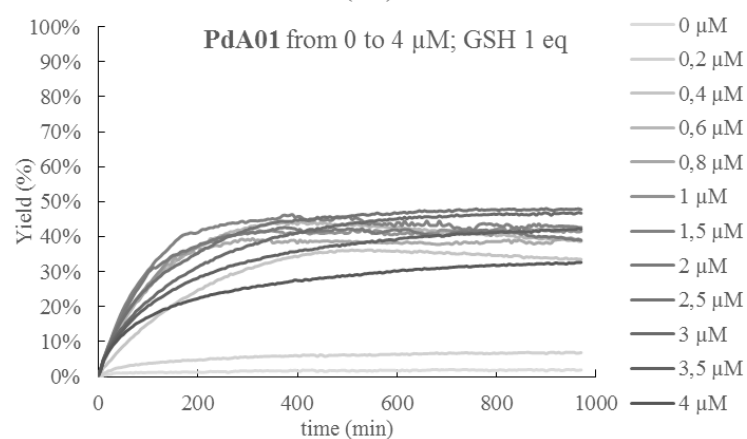
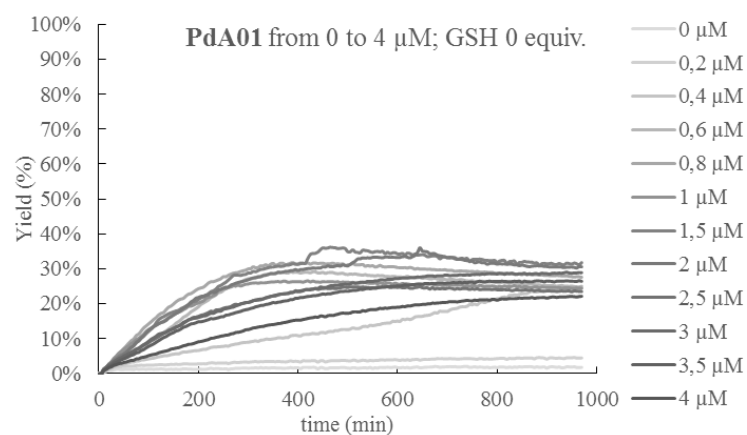
c) Kinetic curves – optimization

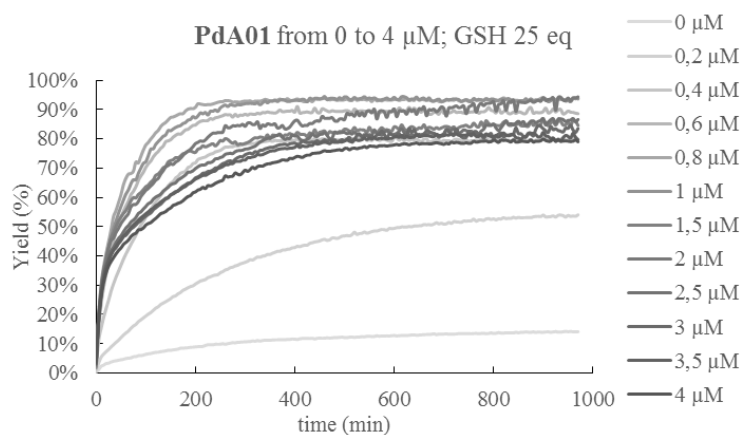
Selected curves for a constant amount of **PdA01** and variable GSH amount:





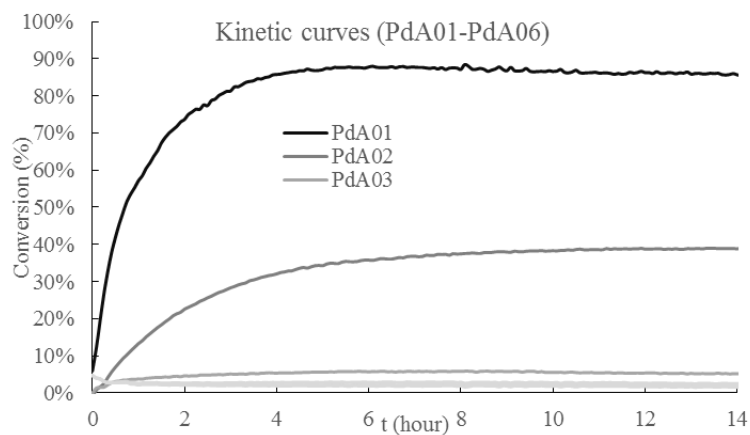
Selected curves for a constant amount of GSH and variable **PdA01** amount:

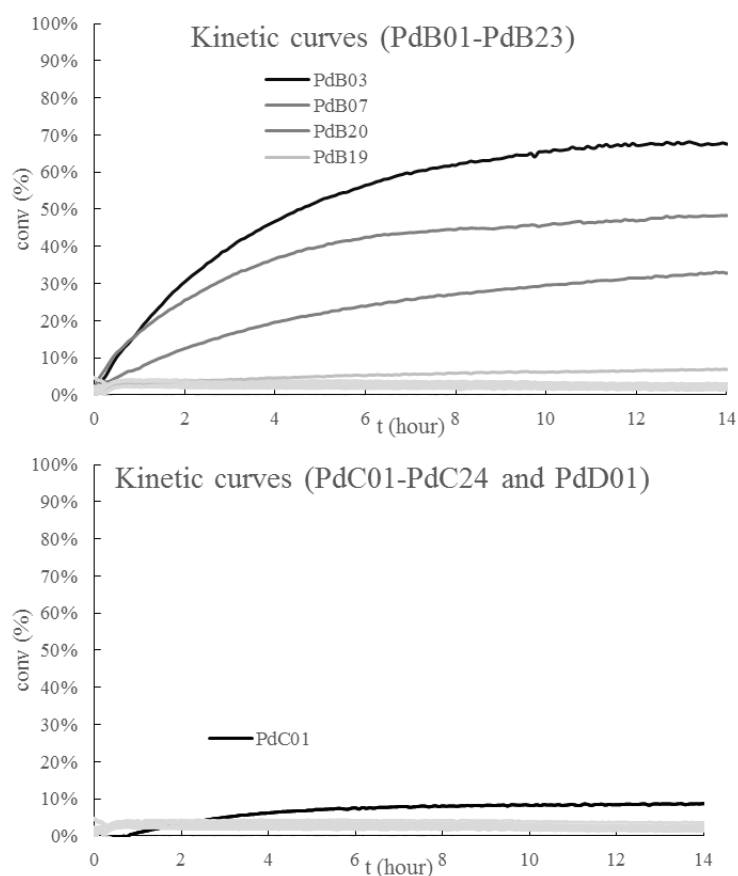




d) Kinetic curves - screening

For the sake of clarity, only the curves of the catalysts giving a conversion were annotated, the other ones giving no conversion were kept light grey. Each family of catalyst was represented on a separated graph. Each curve was the average of 3 simultaneously measured replicates for which an average was calculated.





e) Extraction of catalytic parameters from the curves

The conversion is the easiest parameter to get from the data as it does not require the whole curve. It is extracted from the final point by using the calibration curve. In case there are side reactions, it could be better to take the $I_{\max}$ instead of the I_{final} , by using the =Max(Range) function on Excel. However, we did not do it, and all our calculations were made on the t_{final} .

$$I_{\text{final}} = a \cdot C_{\text{final}} + b$$

$$C_{\text{final}} = \frac{I_{\text{final}} - b}{a}$$

$$\text{conv} = \frac{C_{\text{final}}}{C_{\text{prodrug}}^0}$$

The TON can be easily calculated by the following formula:

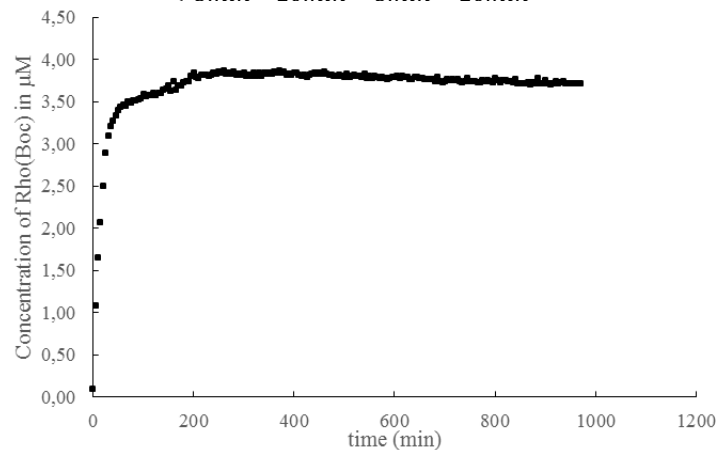
$$TON = \frac{C_{final}}{C_{cat}} = \frac{conv}{cat\ loading}$$

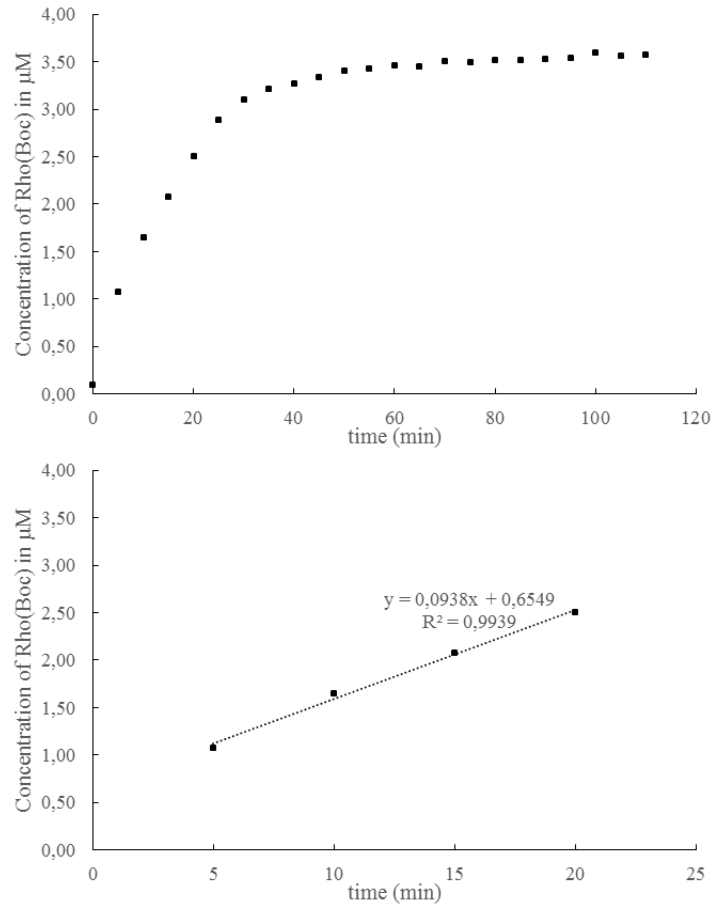
The reaction rates must be extracted from the curves by taking the temporal derivative:

$$v = \frac{dC(t)}{dt}$$

In our case, we assumed that the concentration or the conversion plot was linear for 15 min, between $t = 5$ and $t = 20$ min as the first point contains the most uncertainty due to the device itself, and was the maximal speed, as the slope of the curve decreases over the time. Therefore, the following formula was used to determine the v_{max} (where the first TRUE means that the regression curve is not forced to go by (0:0) and the second TRUE allows the function to display other parameters such as the standard deviation on a and b, but also R^2 and F, if needed):

$$= LINEST(I_{5min}:I_{20min}; t_{5min}:t_{20min}; TRUE; TRUE)$$





If an analytical function could be found for $I = f(t)$, we would then rewrite the general equation into:

$$v_{max} = \lim_{t \rightarrow 0} \frac{dC(t)}{dt} = \lim_{t \rightarrow 0} \frac{d\left(\frac{I(t) - b}{a}\right)}{dt}$$

Then, to extract the TOF, it is easy once the v_{max} is obtained, as it is directly derived from this value and does not require any other manipulation on the curved. It is given by

$$TOF = \frac{v_{max}}{C_{cat}^0}$$

3.2.4 *In cellulo* assay of palladium complexes

3.2.4.1 General Protocols:

a) Cell culture

SiHa cells (human cervix squamous carcinoma) were routinely cultured in powder Dulbecco's Modified Eagle Medium (DMEM) supplemented with D(+) glucose (10 mM), HEPES (25 mM), PIPES (25 mM), L-glutamine (2 mM), foetal serum bovine (FBS, 10%) and antibiotics (penicillin, 10 units/mL and streptomycin, 10 µg/mL). Cell cultures were performed in a 5% CO₂ atmosphere incubator at 37 °C. Media, sera and antibiotics were purchased from Lonza, Thermo Fisher Scientific and Sigma-Aldrich.

The day before the assays, the medium was removed, and cells were washed with Hank's Balanced Salt Solution (HBSS, 5 mL). To detach the cells from the flask, trypsin/EDTA 0.05% (3 mL) was added and then incubated for 3 minutes at 37 °C. Cells were counted using the automated counting system Cellometer Auto T4 (Nexcelom Bioscience) wherein viable cells were enumerated by Trypan Blue exclusion. Cells were then diluted with normal culture medium to the appropriate concentration.

b) Fluorimetric assay

SiHa cells were seeded in black polystyrene 96-well plates (Greiner Bio-one) at a density of 10,000 cells per well.

- Caged drug **Rho(Alloc)₂** was solubilized in DMSO to a concentration of 25 mM. This solution was diluted in cell culture medium to reach the concentration of **50 µM**.
- Catalysts were solubilized in DMSO to a concentration of 10 mM. These solutions were diluted in cell culture medium to reach the desired concentration, typically **10 µM**. The final concentration of DMSO was about 1/500 vol.

Caged drug diluted the medium was added (100 µL/well), followed by 1 h or an overnight (18 h) incubation at 37 °C. Thereafter, the culture medium was removed, and cells were washed 3 times with phosphate-buffered saline (PBS, 200 µL/well). Catalyst solutions were then added (100 µL/well). The fluorescence intensity was read with the multi-mode platform SpectraMax i3 (Molecular Devices). For the **Rho110** model; the excitation and emission wavelengths used were 505 ± 15 nm and 540 ± 15 nm respectively.

c) Cell viability assay

After every experiment, cell viability was performed using Presto Blue® reagent (Invitrogen by Thermo Fisher Scientific). 10% Presto Blue® was added to each well for 1 h incubation at 37 °C and absorbance were measured using Perkin Elmer Victor X4 Multilabel plate reader. Each experiment was performed in triplicates for each condition.

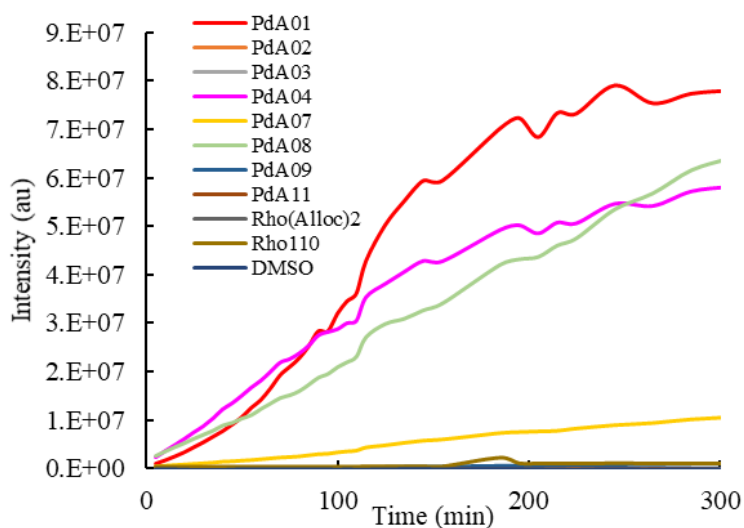
3.2.4.2 Cellular assays

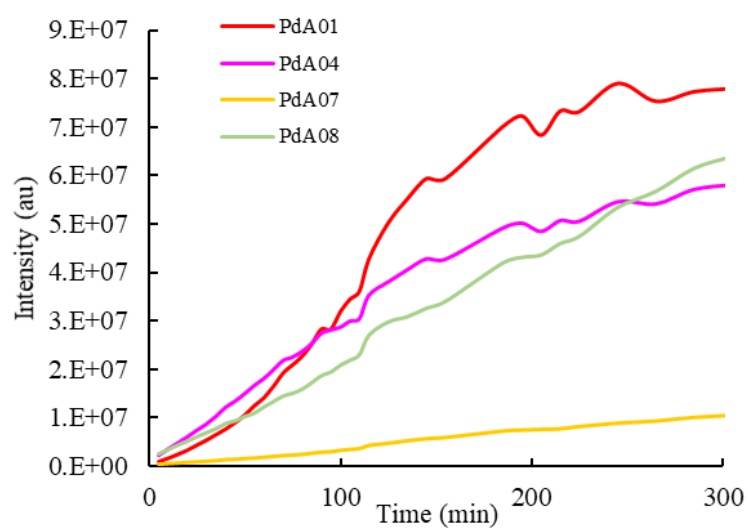
a) *In cellulo* catalyst screening

The standard protocol was used for the cell culture, the fluorimetric assay and the Presto Blue assay. Three screenings were performed:

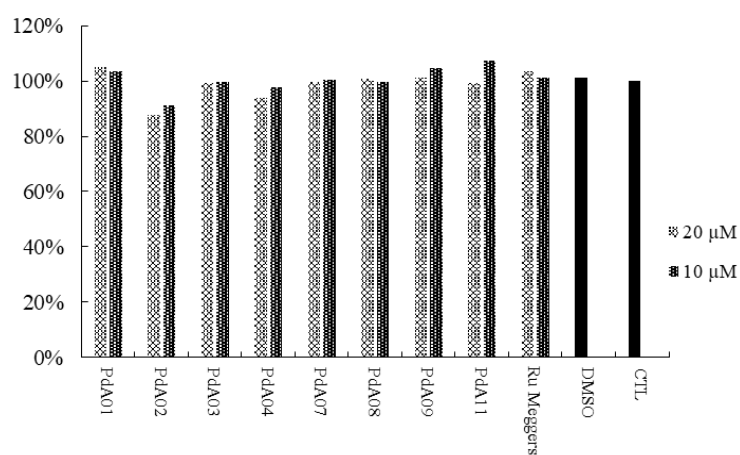
- First screening: First attempt with 8 catalysts at 20 μM of catalyst concentration. The stock solutions of the catalysts were prepared in DMSO at 10 mM. The list consisted of active and inactive catalysts from the *in vitro* screening on the rhodamine model.

Then, the catalysts were diluted as follows: 4 μL of the stock solution was diluted in 4 mL of culture medium to reach 20 μM . Then, the kinetics and the Presto Blue were performed on two separate but identical plates.

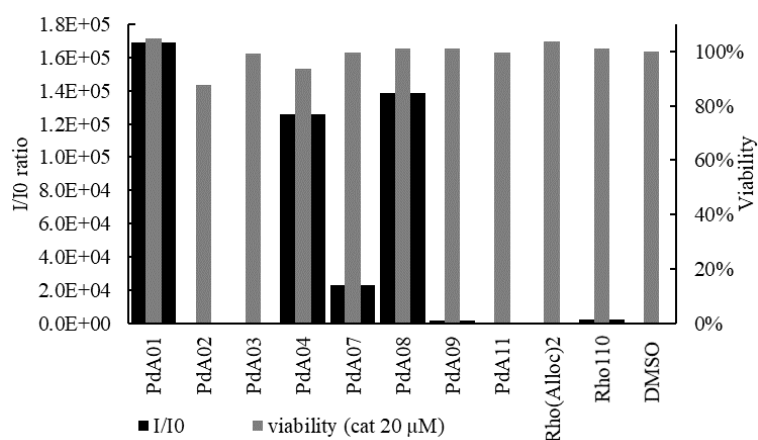




Presto Blue data of the catalysts alone



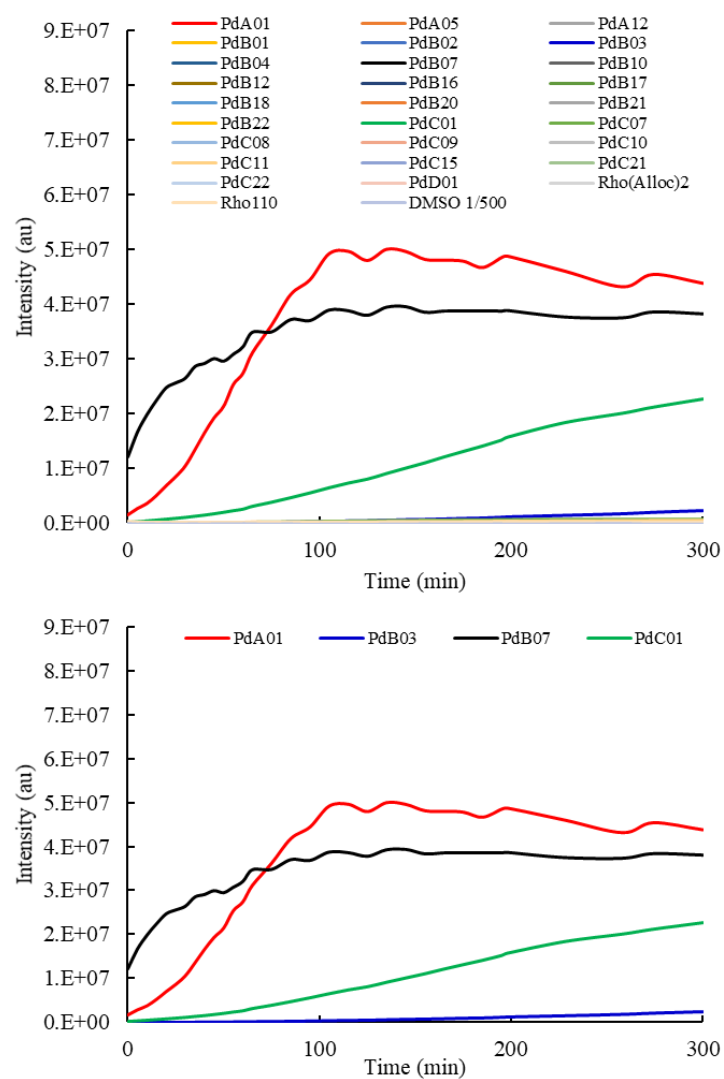
By combining I/I_0 and the toxicity of the catalysts alone at 20 μM , the following graph was obtained:



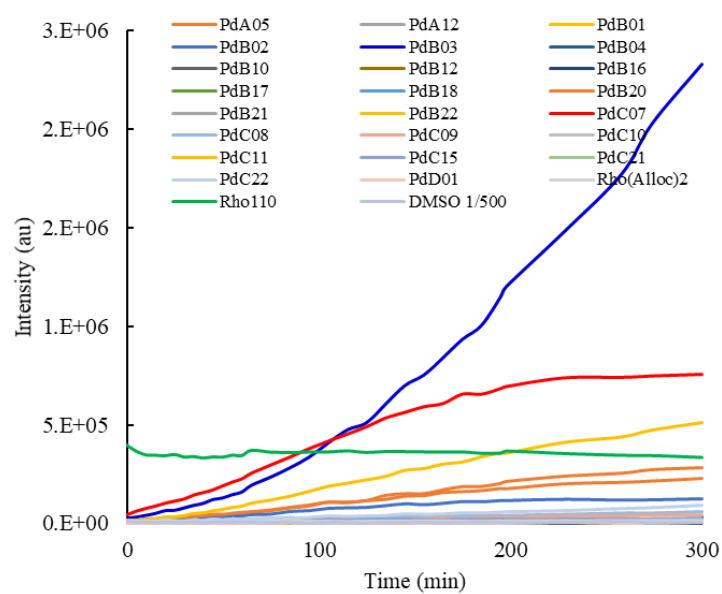
- Second Screening:** The conditions from above were adjusted to reduce the toxicity. The catalyst concentration was lowered to 10 µM. So, a set of 26 catalysts were selected, screened for their intrinsic toxicity against SiHa cells at 10 µM, and those which were not excluded were tested on the **Rho(Alloc)₂** model. A Presto Blue assay was performed on a second plate which is a replicate of the experiment, but which was kept in the incubator to avoid any contamination and allow to reaction to occur in conditions which maintain the highest cell viability.

The catalysts were diluted as follows: 2 µL of the stock solution was diluted in 2 mL of culture medium to give 10 µM solutions.

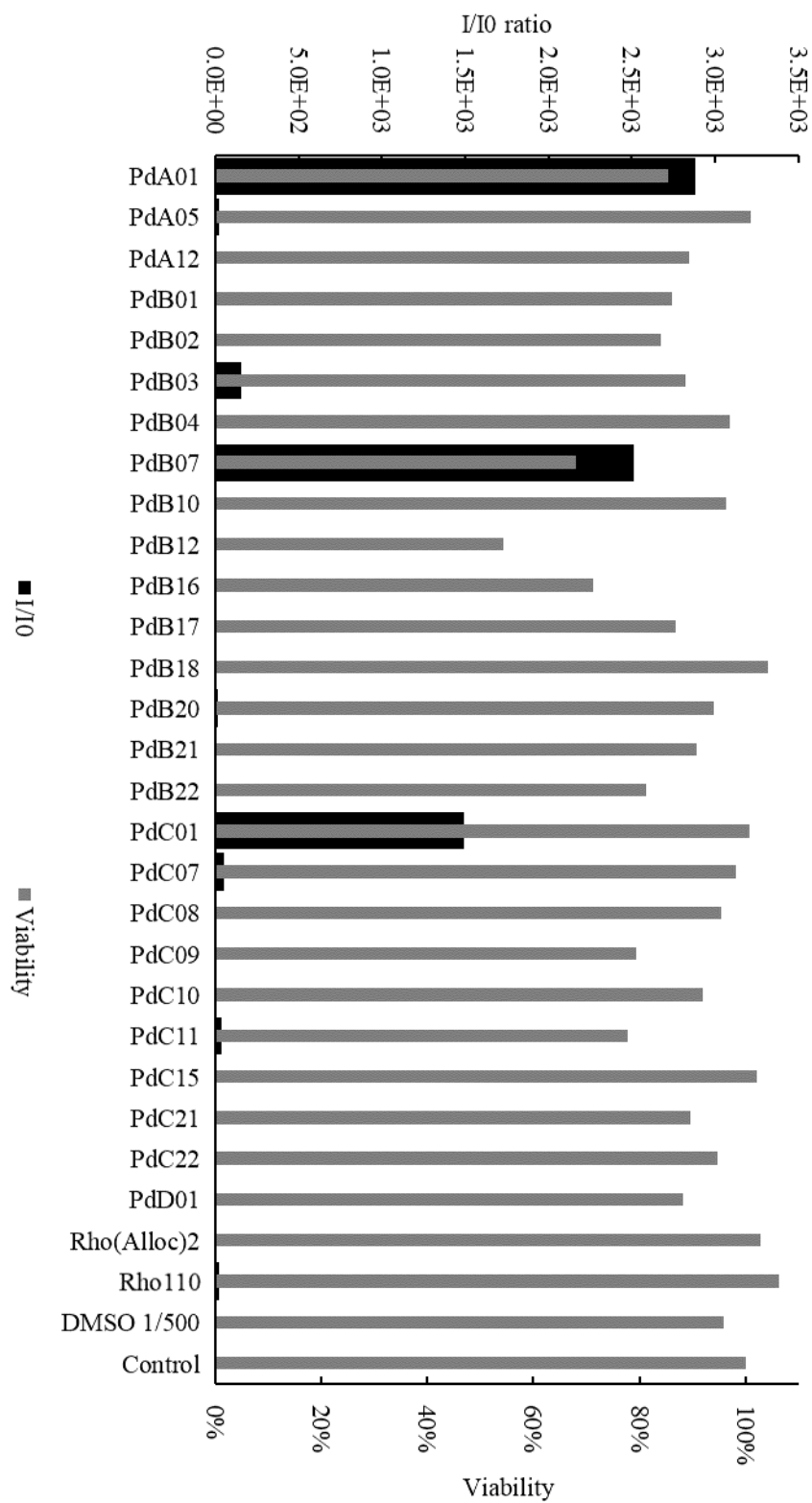
One plate was used for the kinetics and the other was used for the Presto Blue assay. Another plate was also incubated with just the catalyst to perform a Presto Blue after 5 h of incubation.



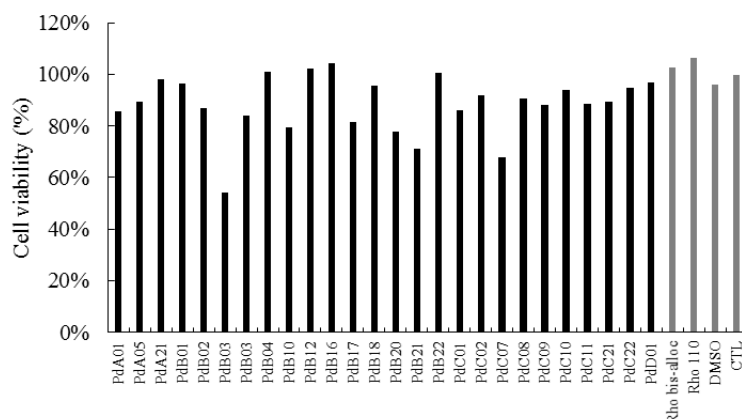
And when the first three hits are removed:



By combining the Presto Blue on the kinetics and the I/I_0 ratio, we obtained the following graph:



Presto Blue



b) *In cellulo* catalyst optimization

The hit catalysts (**PdA01**, **PdA04**, **PdA08**, **PdB07**, **PdC01**) were taken in an optimization run using the optimized procedure corresponding to the one used for the screening, but the stock solution of catalyst was used to prepare standards at various concentrations (10 – 5 – 1 – 0.5 μM). A Presto Blue after the test aimed to measure the cell viability on a replicate of the plate used for the assay but kept in the incubator for 5 h of measurement.

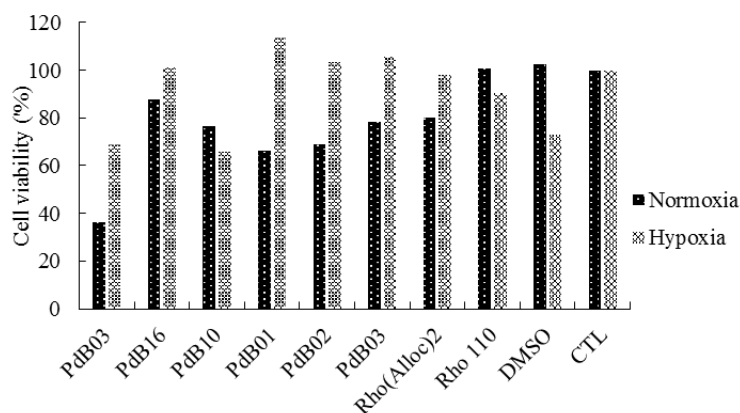
The DMSO control was prepared by diluting 4 μL of DMSO in 2 mL of total volume (1/500 volume)

c) *In cellulo* catalyst activity in normoxia vs hypoxia – Rhodamine model

This experiment was set exactly like the previous one in three replicates, but with the difference that once **Rho(Alloc)₂** was added:

- One plate was incubated overnight at 37 °C in a conventional incubator: one for the fluorimetric assay the Presto Blue assay.
- Two plates were in incubated in a Ruskin INVIVO₂ 400 hypoxia chamber (1% O₂, 5% CO₂): one for the t₁ of the fluorimetric assay and one for the t₂ and the Presto Blue assay.

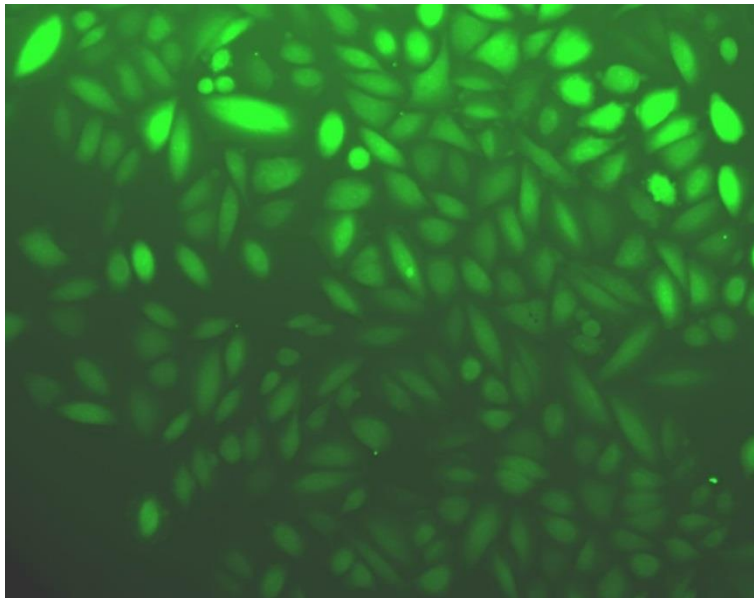
The difference is that there is no kinetic measurement, but two punctual measurements at t₁ = 60 min and t₂ = 300 min. The experiment in hypoxia can be used for the two plate readings and the Presto Blue as the residence time out of the incubator was short enough. However, for the hypoxia experiment, after the t₁ is measured, the plate had to be discarded, and the second plate was used for the t₂ measurement and the Presto Blue assay.



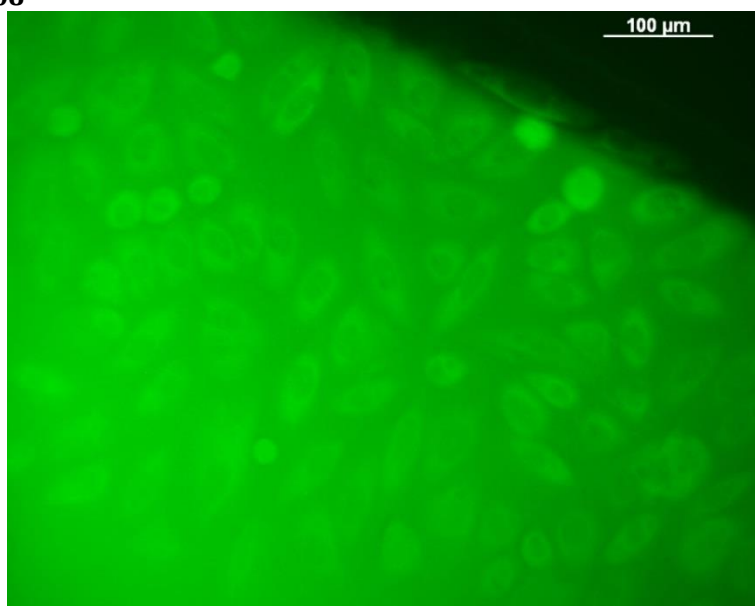
d) Fluorescence images of SiHa cells with the Rhodamine model

SiHa cells were seeded in black polystyrene 96-well plates (Greiner Bio-one) at a density of 10,000 cells per well. **Rho(Alloc)₂** was solubilized in DMSO to a concentration of 25 mM. This solution was diluted in cell culture medium to reach the concentration of 50 μ M. Catalysts were solubilized in DMSO to a concentration of 5 mM. These solutions were diluted in cell culture medium to reach the concentration of 10 μ M. The final concentration of DMSO was about 1/500 v/v. **Rho(Alloc)₂** diluted the medium was added (100 μ L/well) and incubated overnight at 37 °C. Thereafter, the culture medium was removed, and cells were washed 3 times with PBS (200 μ L/well). Catalyst solutions were then added (100 μ L/well). After 2h15 incubation, cells were washed again with PBS (200 μ L/well) and images were taken with fluorescence microscopy Axiovert S100 (Zeiss).

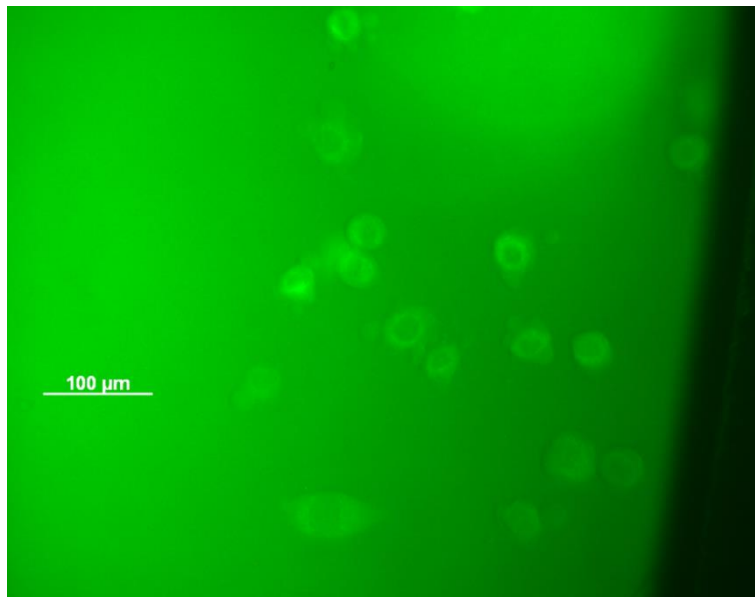
PdA01



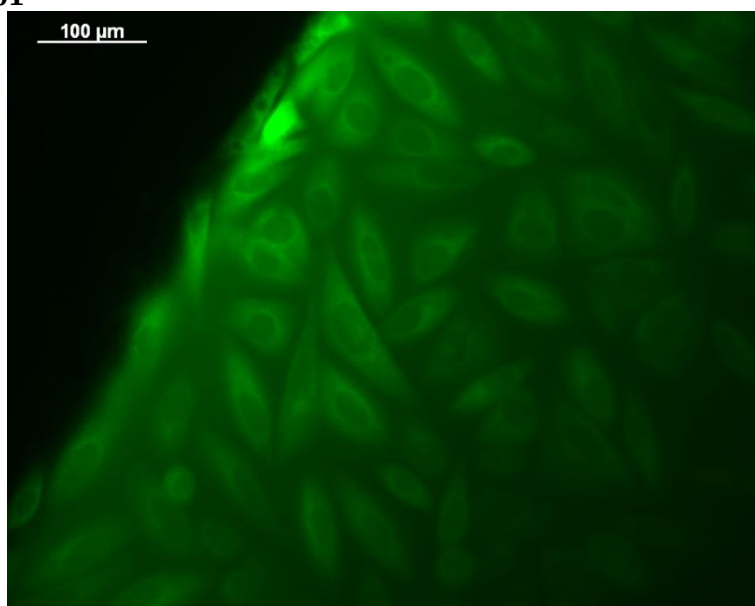
PdA08



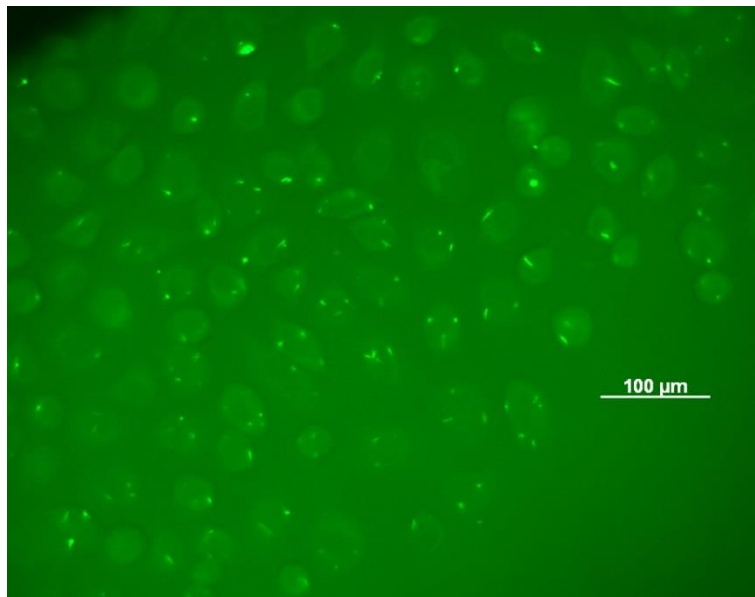
PdA04



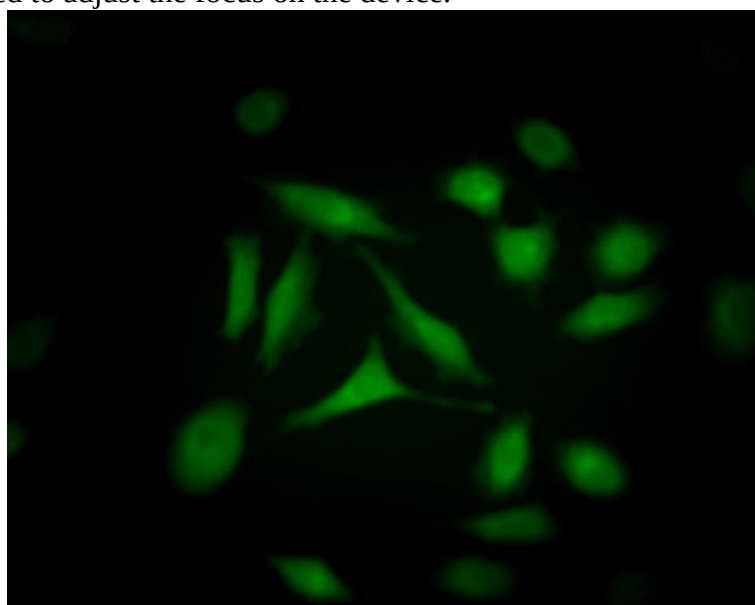
PdC01

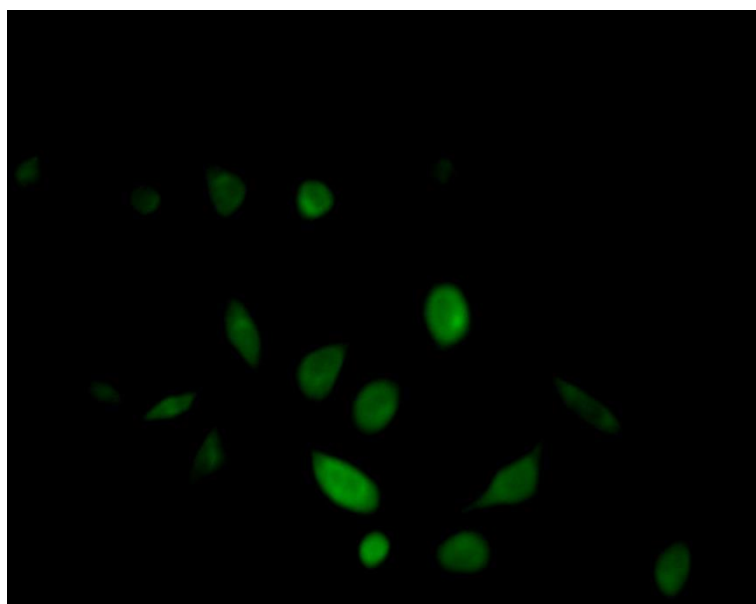


PdB03



Due to the excessive leaching of fluorescence outside the cells, a new attempt was performed with 50 μM of **Rho(Alloc)₂** incubated for only 2 h. The catalyst **PdA01** was kept at 10 μM . The second difference is that the first images were recorded after 30 min, which is the time needed to adjust the focus on the device.

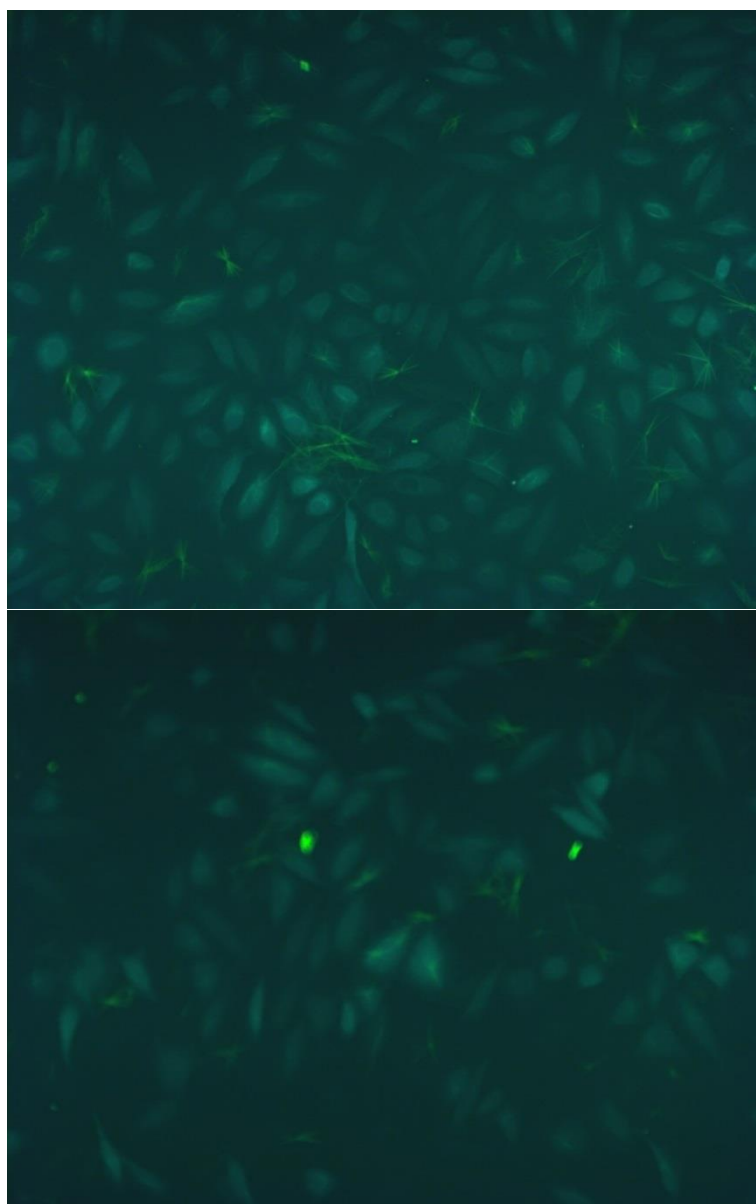




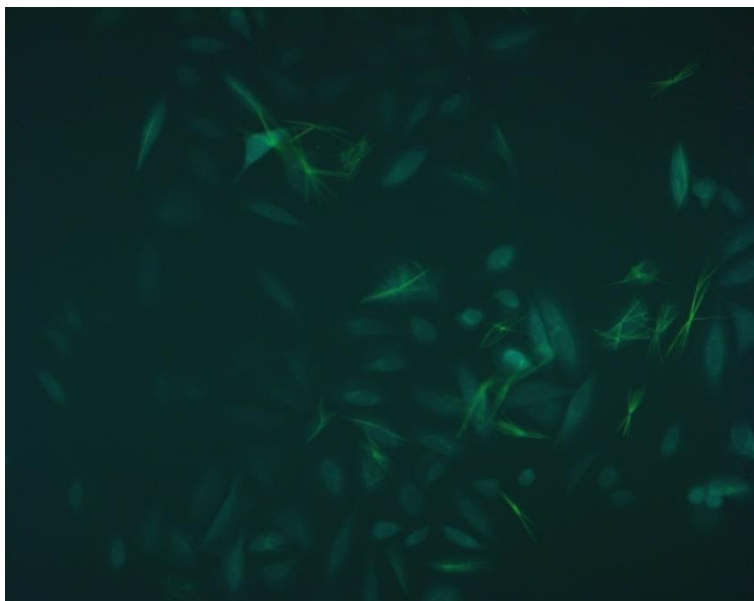
e) **Fluorescence images of SiHa cells with the Maudamine model**

SiHa cells were seeded in black polystyrene 96-well plates (Greiner Bio-one) at a density of 10,000 cells per well. **Maudamine** was solubilized in DMSO to a concentration of 25 mM. This solution was diluted in cell culture medium to reach the concentration of 20 μ M. Catalyst **PdA01** was solubilized in DMSO to a concentration of 5 mM. These solutions were diluted in cell culture medium to reach the concentration of 10 μ M. The final concentration of DMSO was about 1/500 v/v. **Maudamine** diluted the medium was added (100 μ L/well) and incubated overnight at 37 °C. Thereafter, the culture medium was removed, and cells were washed 3 times with PBS (200 μ L/well). Catalyst solutions were then added (100 μ L/well). After 2h15 incubation, cells were washed again with PBS (200 μ L/well) and images were taken with fluorescence microscopy Axiovert S100 (Zeiss).

Incubation with **Maudamine** 20 μ M 6 h, washing, then 10 μ M **PdA01**.



Incubation with **Maudamine** 20 μ M 6 h, washing, then 10 μ M **PdA01**.

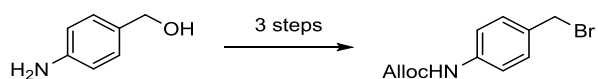


3.3 Folic-acid targeting molecular programmed systems

3.3.1 First-generation of prodrug

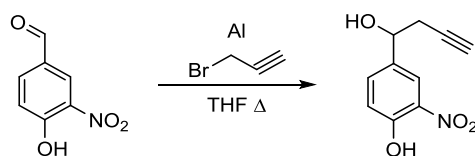
3.3.1.1 Synthesis

a) Bromo intermediate (3.10)



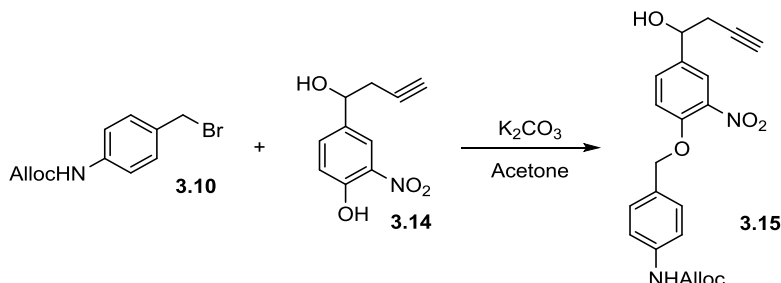
Allyl (4-(bromomethyl)phenyl)carbamate **3.10** was synthesized in 3 steps according to routine procedures described in paragraph 3.1.3.

b) Synthesis of the central unit (3.14)



4-(1-hydroxybut-3-yn-1-yl)-2-nitrophenol **3.14** was synthesized according to a literature procedure.¹⁵⁴

c) Coupling of the central unit and the trigger (3.15)

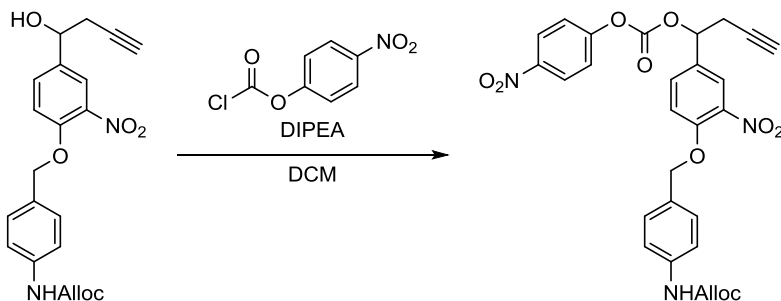


Allyl 4-((4-(1-hydroxybut-3-yn-1-yl)-2-nitrophenoxy)methyl)phenyl carbamate **3.15** was synthesized according to a routine procedure. A 25 mL round-bottom flask was filled with **3.14** (107.4 mg, 0.52 mmol) in 5 mL of acetone (0.1 M) to give a bright yellow solution. Then, K_2CO_3 (180 mg, 1.3 mmol, 2.5 equiv.) was added and the solution became dark yellow. Then, the bromide **3.10** (140 mg, 0.52 mmol, 1 equiv.) was added and the reaction was stirred vigorously overnight. On the next morning, EtOAc was added and the organic phase was washed with Na_2CO_3 3 times, and the organic phase was dried by Na_2SO_4 . The solvent was removed under reduced pressure to give **3.15** a very pale-yellow powder, no purification was needed. (205.3 mg, 99%).

^1H NMR (300 MHz, $CDCl_3$) δ 7.93 – 7.87 (m, 1H), 7.58 – 7.50 (m, 1H), 7.39 (s, 4H), 7.09 (d, J = 8.7 Hz, 1H), 6.69 (s, 1H), 6.08 – 5.83 (m, 1H), 5.44 – 5.30 (dq, J = 10.4, 1.2 Hz, 2H), 5.27 (dq, J = 10.4, 1.2 Hz, 2H), 5.18 (s, 2H), 4.87 (td, J = 6.2, 3.6 Hz, 1H), 4.67 (dt, J = 5.7, 1.4 Hz, 2H), 2.69 – 2.58 (m, 2H), 2.47 (d, J = 3.7 Hz, 1H), 2.10 (t, J = 2.6 Hz, 1H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 151.53, 140.07, 137.98, 135.36, 132.44, 131.48, 130.59, 128.22, 123.37, 118.98, 118.53, 115.38, 79.81, 71.96, 71.12, 70.98, 66.10, 29.56.

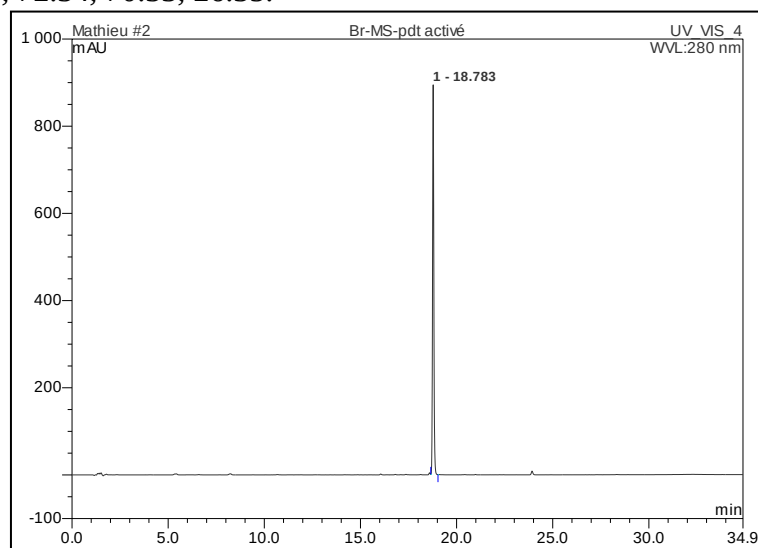
d) Activation of the trigger (3.16)



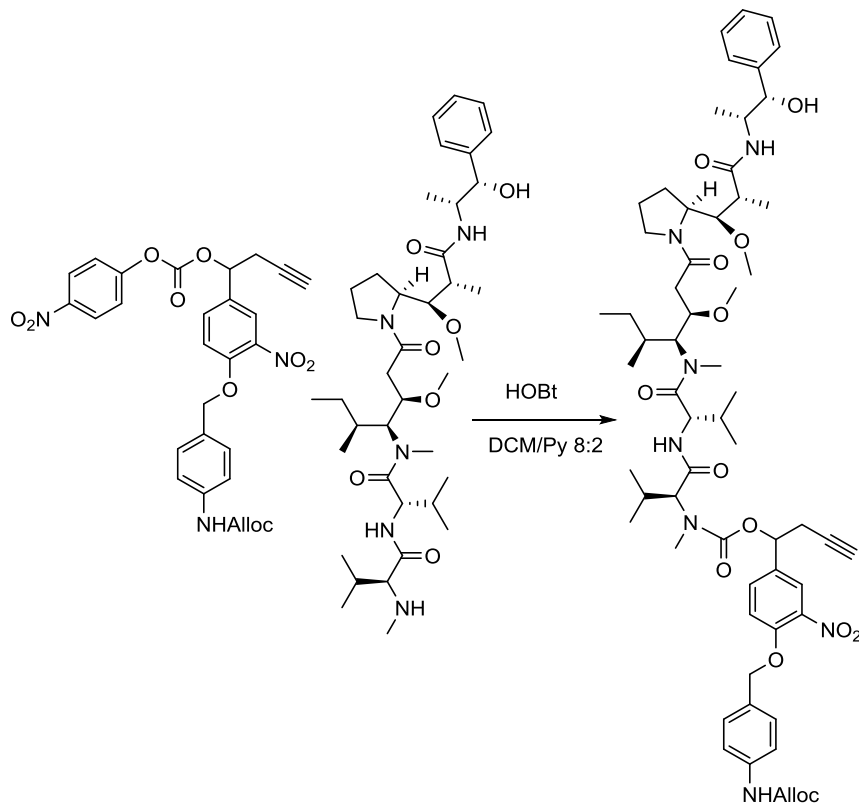
1-(4-((4-(((allyloxy)carbonyl)amino)benzyl)oxy)-3-nitrophenyl)but-3-yn-1-yl 4-nitrobenzoate **3.16** was synthesized according to a routine procedure. The compound **3.15** (110 mg, 0.28 mmol) was dissolved in DCM (2.8 mL, 0.1 M) under Ar in a 10 mL round-bottom flask. Then, PNP-chloroformate (69.2 mg, 0.33 mmol, 1.2 equivalent) was added, immediately followed by Et₃N (73 μ L, 0.42 mmol, 1.5 equiv.). The reaction was followed by TLC (petroleum ether/EtOAc 70:30) and stirred overnight at room temperature. On the next day, the reaction mixture was diluted with EtOAc and washed twice with 1 M HCl and dried with Na₂SO₄. The solvent was then evaporated under reduced pressure. The crude **3.16** was purified on silica gel by flash column chromatography using 30% EtOAc in petroleum ether. The product **3.16** was obtained as a pale grey foamy solid (145 mg, 93%).

¹H NMR (300 MHz, CDCl₃) δ 8.28 (d, *J* = 9.2 Hz, 2H), 7.99 (d, *J* = 2.3 Hz, 1H), 7.59 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.49 – 7.33 (m, 6H), 7.15 (d, *J* = 8.7 Hz, 1H), 6.69 (s, 1H), 5.98 (ddt, *J* = 17.2, 10.4, 5.7 Hz, 1H), 5.80 (t, *J* = 6.6 Hz, 1H), 5.38 (dd, *J* = 17.2, 1.5 Hz, 1H), 5.30 (d, *J* = 1.3 Hz, 0H), 5.22 (s, 2H), 4.68 (dt, *J* = 5.7, 1.4 Hz, 2H), 3.08 – 2.78 (m, 2H), 2.18 (s, 1H), 2.09 (t, *J* = 2.6 Hz, 1H).

¹³C NMR (75 MHz, CDCl₃) δ 155.35, 152.50, 151.65, 145.66, 139.96, 132.59, 131.49, 129.92, 125.47, 124.30, 121.82, 118.81, 115.77, 115.14, 72.34, 70.33, 26.35.



e) **Coupling of Monomethyl Auristatin E (3.17)**



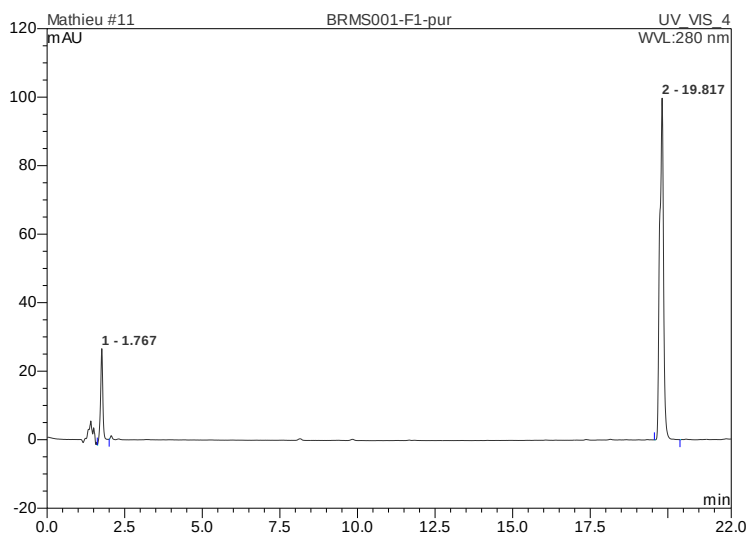
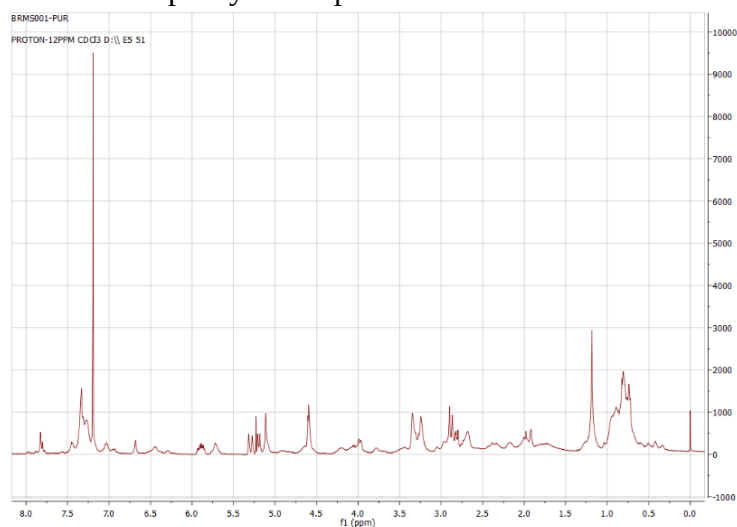
The loaded prodrug **3.17** was synthesized according to a literature procedure.¹⁵⁰

NB: extreme care must be taken while handling the **MMAE** as it has picomolar toxicity. So, 3 pairs of gloves must be worn, the ventilation must be switched off and the surroundings of the balance must be covered with paper. The wastes must be carefully collected, put into a closed bag and disposed of properly.

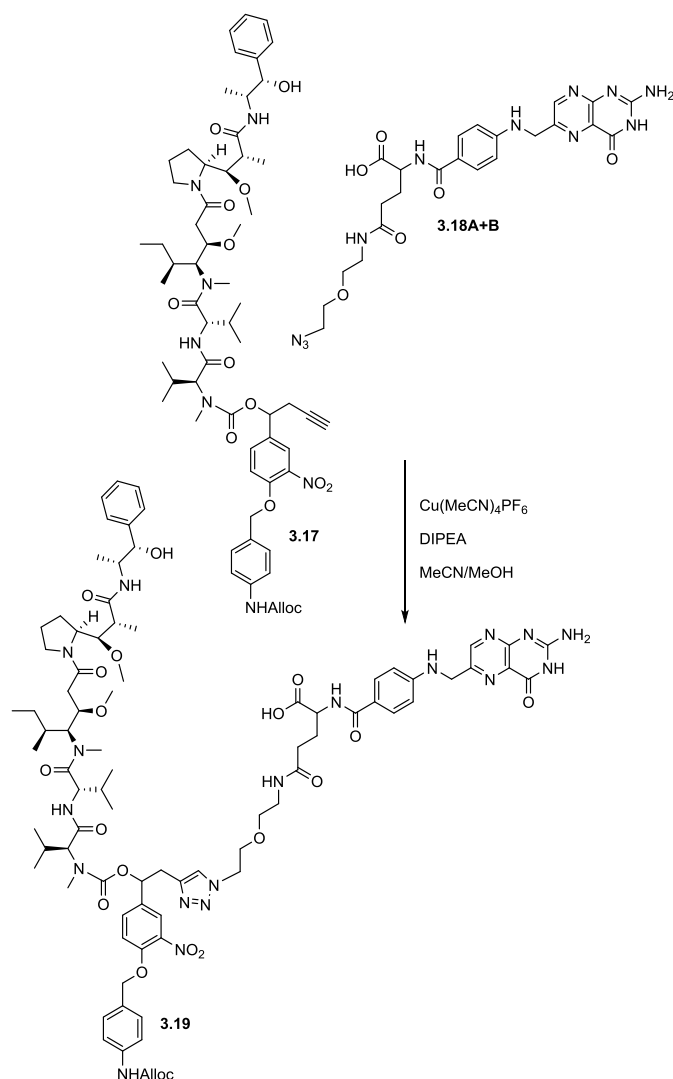
A 5 mL round-bottom flask was filled with the carbonate **3.16** (20 mg, 36 μ mol), **MMAE** (25.6 mg, 1 equiv. 36 μ mol) and HOBt (4.9 mg, 36 μ mol, 1 equiv.). Then, DMF (0.8 mL) was added by syringe and vacuum was applied to degas the solution. Then, it was filled with Ar. Afterwards, pyridine (0.2 mL) and DIPEA (7 μ L, 1.2 equiv. 43 μ mol) were added and the mixture was stirred for 72 h at room temperature, during which HPLC (C18, 10% MeCN to 0% MeCN in 30 min) was performed to follow the reaction. Upon completion, the solvent was removed under high vacuum to get a thick brown paste. It was then

purified on a 4 g 15 μ m flash column using DCM/MeOH. The product **3.17** was obtained as a transparent thick oil (28.6 mg, 70%)

For analysis, a ^1H -NMR was taken but the peaks could not be assigned due to the resolution of the spectrometer. Allylic peaks were seen between 5 and 6 ppm. ^{13}C -NMR could not be recorded due to the small quantity of product and the high molar mass. The HPLC traces were indicative of the purity of the product.



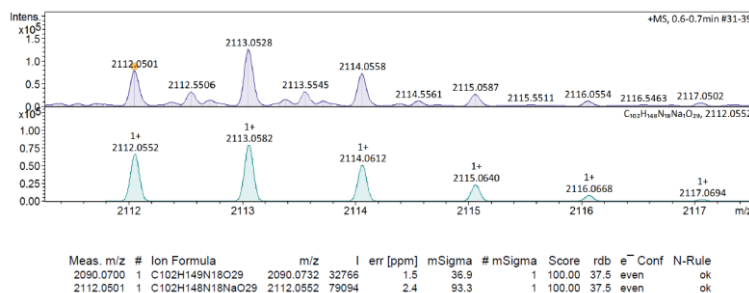
f) **Coupling with the folic acid –PEG-10 by click chemistry
(3.19)**

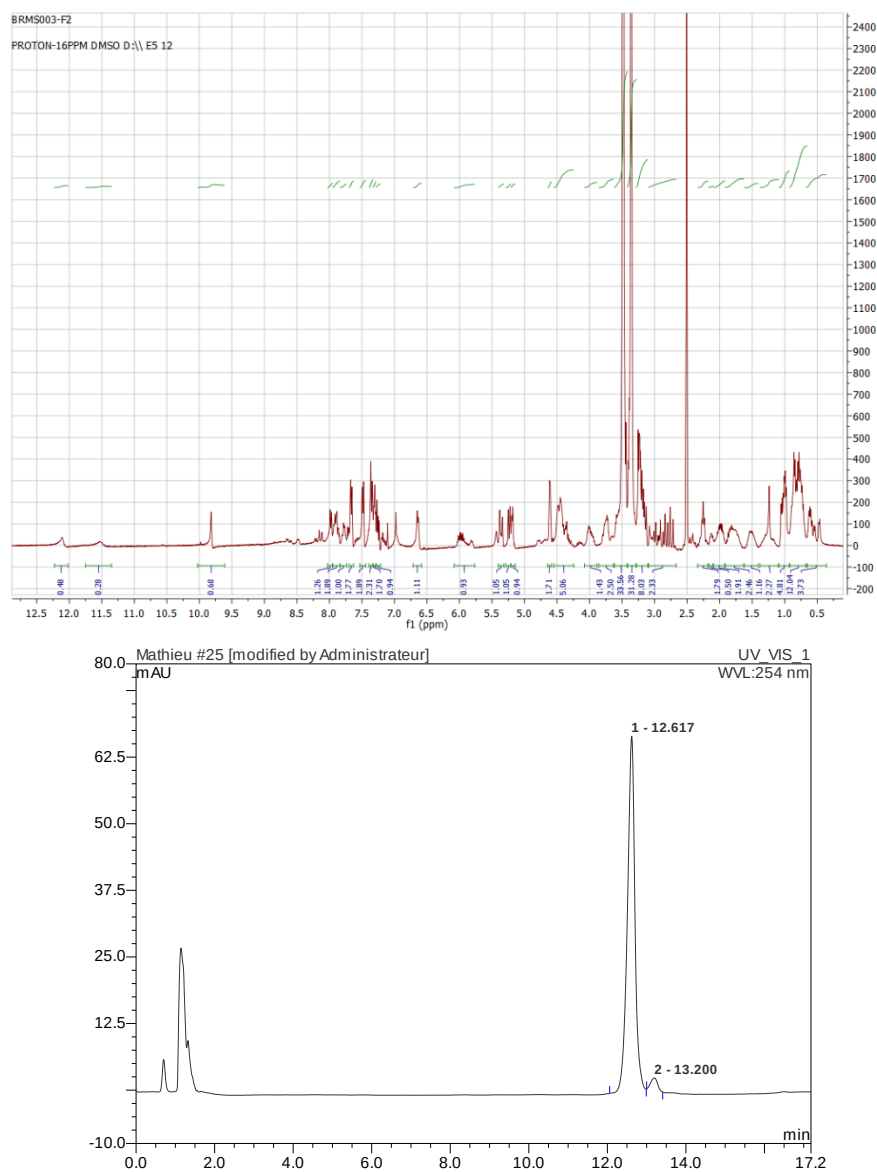


The prodrug **3.19** was synthesized employing a literature procedure.¹⁵⁰ The folic acid-PEG-10-N₃ **3.18** (14.3 mg, 0.95 equiv., 15.74 μmol) was dissolved in 0.15 mL dry MeOH under Ar (heating at 60 °C and sonication was necessary to ensure a good dissolution), after which dry DCM was added to reach 1.5 mL (0.01 M, max 10 % MeOH in DCM). Then, still under Ar at room temperature, the solution was added to another flask containing the **MMAE** derivative **3.17** (18.9 mg, 16.6 μmol, 1 equiv.). Then, Cu(MeCN)₄PF₆ (6.8 mg, 18.2 μmol, 1.1 equiv.) was added and the solution turned dark brown. The

mixture was stirred for 2-3 days and was followed by HPLC. If the reaction slows down, one more equivalent of $\text{Cu}(\text{MeCN})_4\text{PF}_6$ can be added. Upon total consumption of the azide **3.18**, DCM was added, as well as Quadrapure IDA resin and the solution was stirred until it became yellow. Then, the resin was filtered off, and the organic phase was dried with Na_2SO_4 and was concentrated under vacuum. The crude yellow residue was purified by flash column chromatography using a 4 g column containing 15 μM particles with a gradient from DCM/MeOH 100:0 to 0:100. The compound **3.19** was obtained as a dark yellow plastic-like solid (20 mg, 60%).

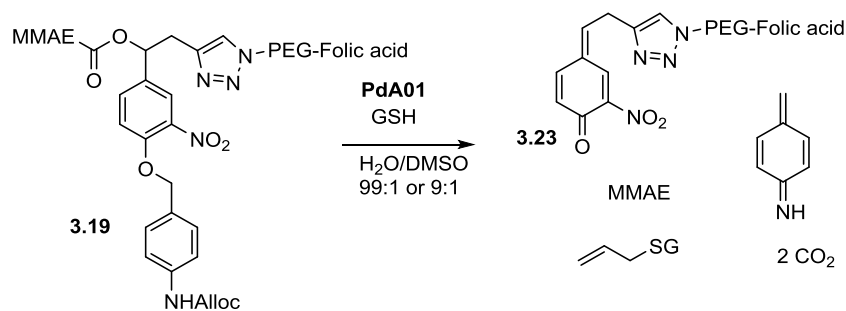
For analysis, a ^1H -NMR was taken but the peaks could not be assigned due to the resolution of the spectrometer. Allylic peaks were seen between 5 and 6 ppm. ^{13}C -NMR could not be recorded due to the small quantity of product and the high molar mass. The mass spectrum coupled with the HPLC traces were indicative of the purity of the product.





3.3.1.2 HPLC assay

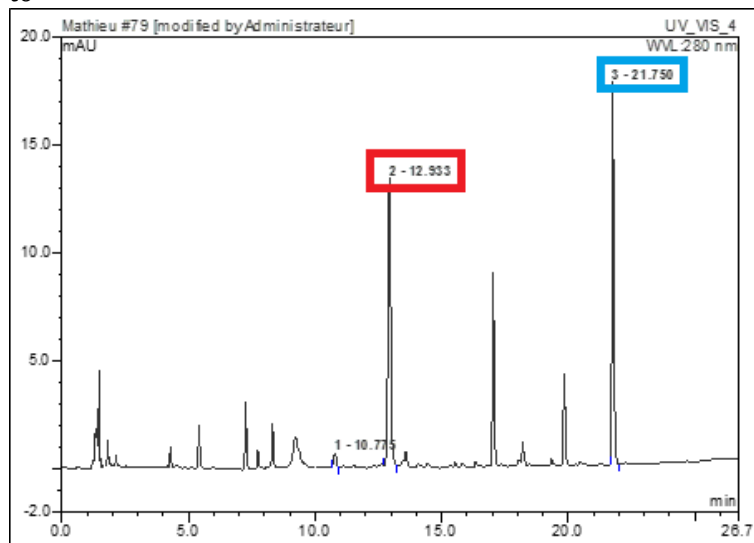
The decaging reaction of the prodrug was set up using the following concentrations, the total containing 1% DMSO as it is the maximum tolerated.



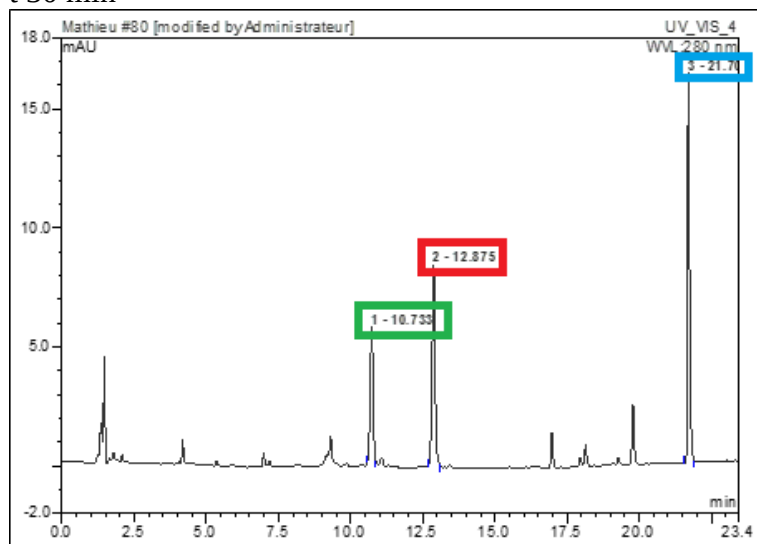
Compound	Stock solution (μM)	Concentration in reaction	Volume for the reaction (mL)	HPLC retention time (min)
Prodrug 3.19	30	10	10	12.9 (red)
Catalyst	30	10	10	21.7 (blue)
GSH	300	100	10	

A first trial was performed using the standard HPLC without filtration. **MMAE** is known to be invisible in HPLC with simple UV detectors. Moreover, a new peak was observed at 10.8 min (green) and it increased.

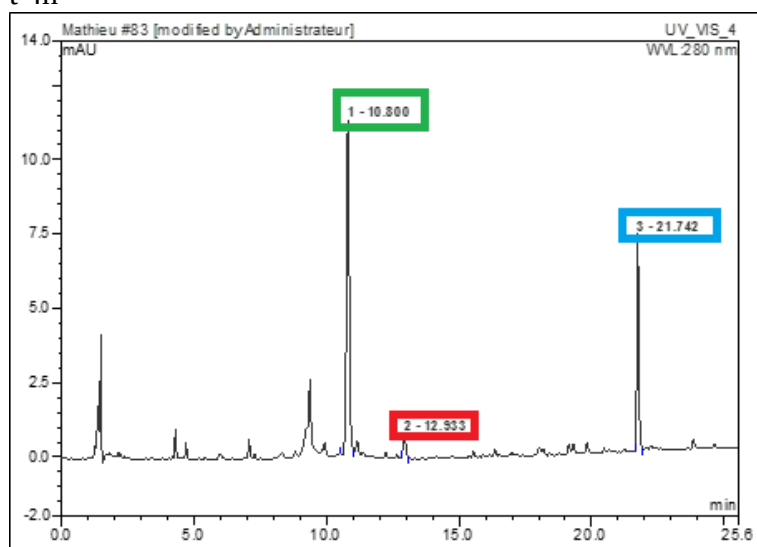
t₀



t 30 min



t 4h



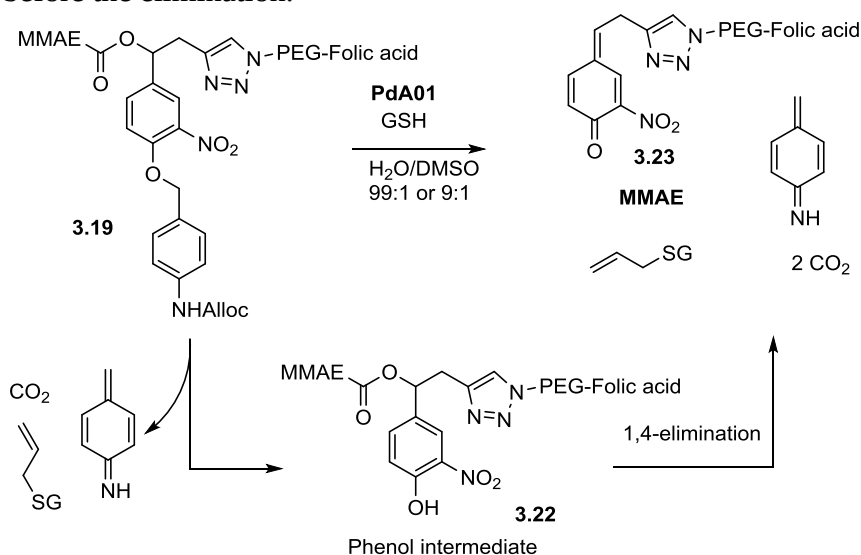
3.3.1.3 HPLC-MS assay

Solution preparation

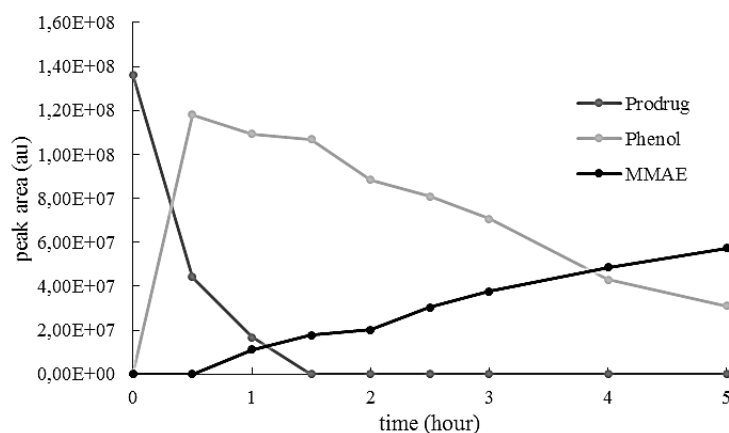
Compound	Stock solution (μM)	Concentration in reaction	Volume for the reaction (mL)	HPLC retention time (min)
Prodrug 3.19	30	10	10	12.9 (red)
Catalyst	30	10	10	21.7 (blue)
GSH	300	100	10	

The three solutions were mixed and were reacted in a round-bottom flask. 1.5 mL were sampled and taken into a glass vial. It is important to mention that the samples were not filtered, otherwise no signal accounting for the prodrug was observed in HPLC-MS. Then, for the specified times mentioned above, 10 μ L were sampled and introduced in HPLC-MS. A diver-valve was used to send to the waste the polar compounds containing GSH (2 minutes of elution) without overloading the mass spectrometer. Then, the rest was analysed at first in TIC (total ion count) to see the position of the peaks and take a mass spectrum to confirm the identity of the species.

The identity of the catalyst peak and prodrug peak was confirmed. The newly appearing peak was found to be the phenolic intermediate before the elimination.



The integrals of the peaks corresponding to the prodrug **3.19**, the catalyst, the phenol intermediate **3.22** and the MAME were calculated and plotted in function of the time.



3.3.1.4 Biological assay

a) Cell lines

Human KB tumour cells (nasopharynx, ATCC XXX number) were purchased from LGC Standards and A549 (lung carcinoma, ATCC XXX number) from Sigma-Aldrich.

b) Defrosting and cell culture

Thawing of human tumour cells KB (FR+, nasopharynx) and A549 (FR-, lung carcinoma) was performed in RPMI 1640 Glutamax medium (Gibco) containing 10% FCS and 100 U / mL penicillin/streptomycin (Lonza). After centrifugation for 7 min at 1000 rpm, the cells were seeded in the same medium in a petri dish and placed in a humid atmosphere incubator and at 37 °C and 5% CO₂. When they reached 80% confluency, the cells were enzymatically stripped with 0.25% trypsin containing 0.9 mM EDTA (Gibco). After centrifugation for 7 min at 1000 rpm and automated counting (Countess II from Life Technologies), the cells were seeded at the desired concentration of RPMI 1640 and complete Glutamax and were again placed in a humid atmosphere at 37 °C and 5% CO₂.

c) Measuring cell viability

In order to determine the effectiveness of the different catalysts to activate the **MMAE**-derived vector, the viability of the cells was measured using the Cell Proliferation Kit Ki II, XTT from Roche. It is a test based on the reduction of tetrazolium salts (XTT) in an orange compound, formazan. The optical density of this formazan can then be determined spectrophotometrically at 490 nm. The reduction of XTT in formazan being ensured by enzymes, the dehydrogenases,

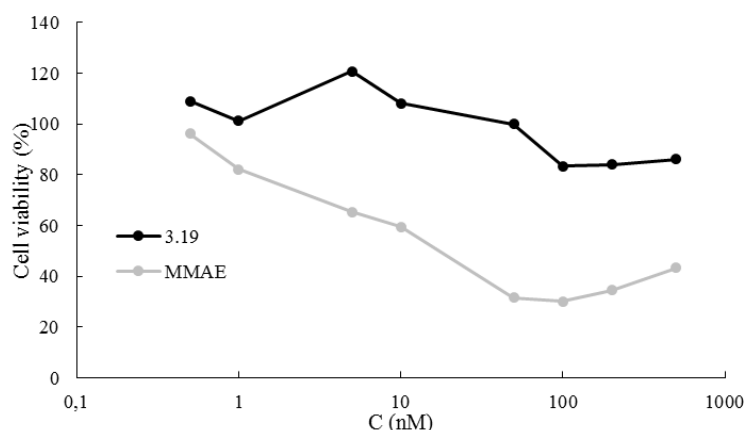
contained in the mitochondria of metabolically active cells, the amount of formazan produced is then proportional to the number of living cells.

To carry out this test, 3000 cells per well were seeded in 96-well plates in 100 μ L of RPMI 1640 + complete Glutamax and incubated for 24 h in a humid atmosphere and at 37 °C and 5% CO₂. After treatment of the cells with the various compounds, 25 μ L of the XTT solution was added to each well. The cells were then incubated for 4 h in a humid atmosphere at 37 °C and 5% CO₂ before the absorbance at 490 nm (formazan) was spectrophotometrically measured on a Multiskan Go reader from Thermo Scientific. Each experiment was performed in triplicate and reproduced 3 times. For each experiment, the average value of the absorbance of each triplicate was calculated. The average value obtained for the wells containing only the medium (blank means without cells) is deducted from all the other averages. The results were expressed as percent viability and normalized to untreated cells which represent 100% viability.

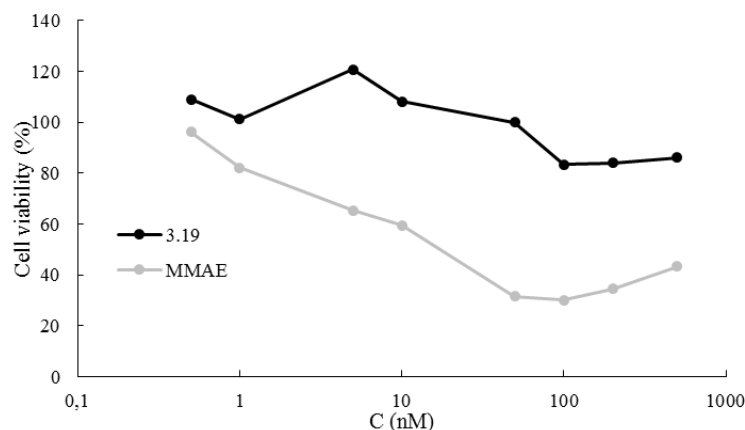
d) Experiments

A first control experiment aimed to measure the intrinsic IC₅₀ of the prodrug **3.19** and of **MMAE** in the conditions. Therefore, solutions of **MMAE** and of the prodrug **3.19** ranging from 500 pM to 500 μ M by diluting a stock solution in some fresh culture medium. The cells were incubated for 72 h, after which the viability was measured with an MTT assay. This experiment allowed us to establish that there are 2 orders of magnitude between the toxicity of the prodrug and **MMAE** and the **MMAE** itself, meaning that it is a good on-off toxicogenic probe.

KB Cells



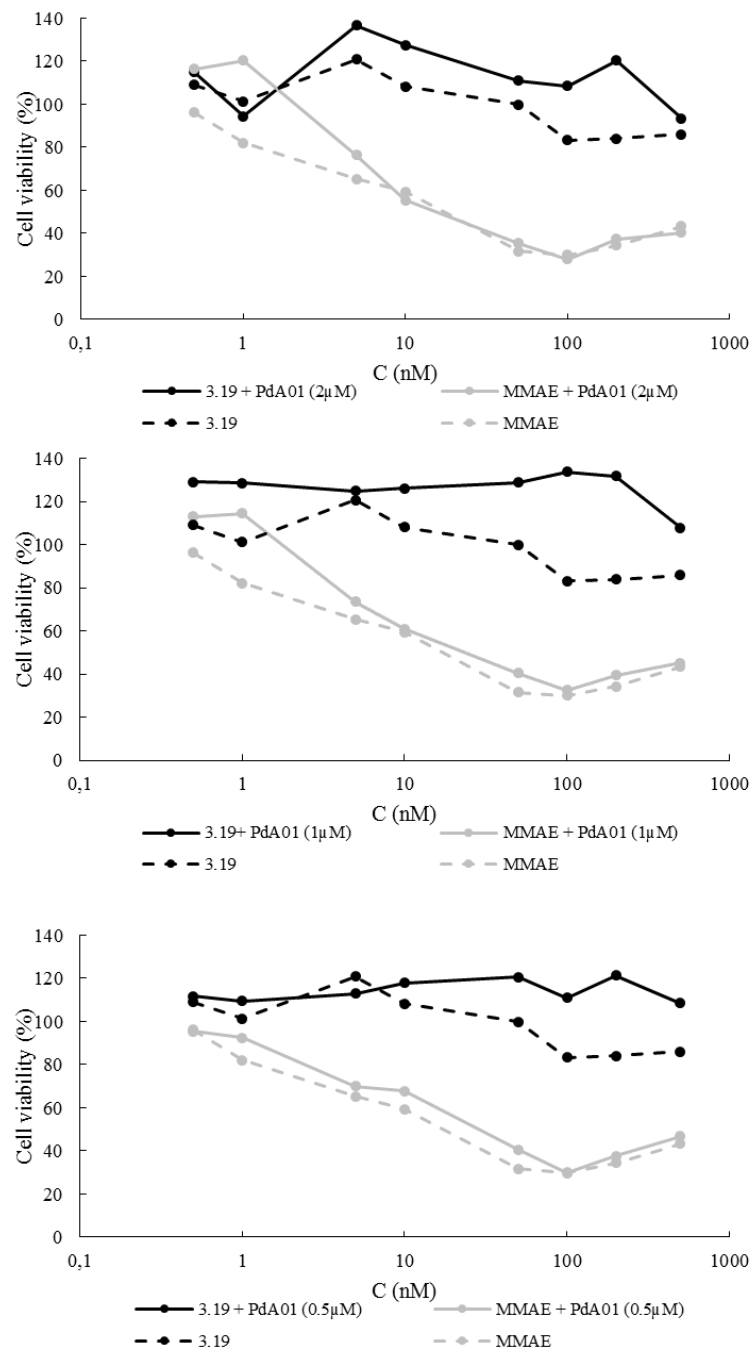
A549 cells



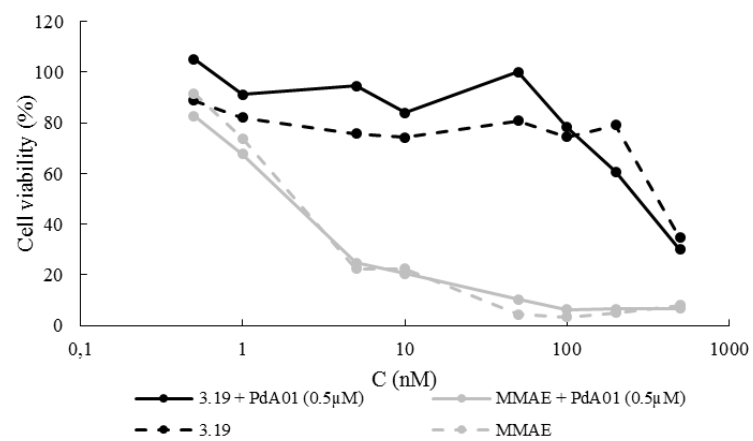
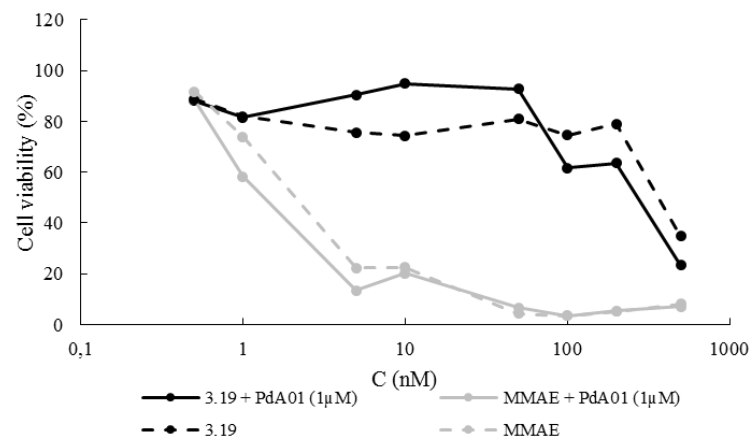
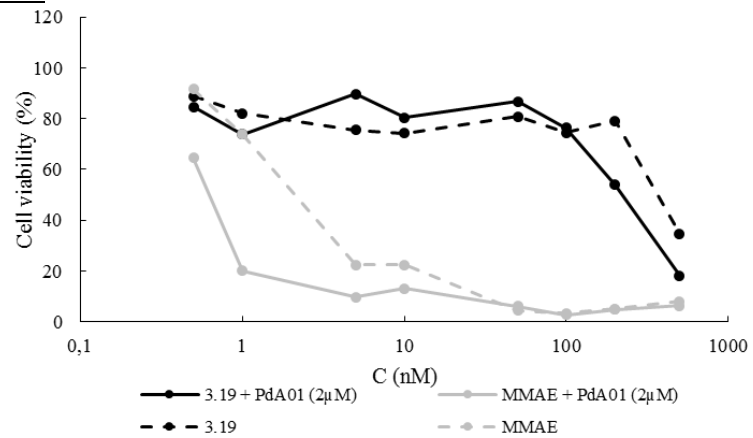
On the A549 cells, **MMAE** showed lower toxicity, with an IC_{50} around 20 nM, whereas the **MMAE** prodrug did not show significant toxicity as no trend was observed in the curve representing the prodrug **3.19** and we did not reach the IC_{50} value. We cannot exceed those concentrations as the prodrug **3.19** would start to enter *via* passive diffusion and the selectivity would be lost.

For the uncaging assay, we performed each experiment with 3 concentrations of catalyst **PdA01** (2 μ M, 1 μ M and 0.5 μ M), and perform a control experiment with **MMAE** and the **PdA01** at the same concentration as we know that both do induce toxicity, especially the catalyst while being incubated for 72 h. For the assay, the cells were incubated with **3.19** for 1 hour at the appropriate concentration, then washed 3 times by PBS, and were incubated with **PdA01** at the selected concentration for 72 h. In parallel, the cells were incubated for 72 h with **MMAE** and **PdA01** at the appropriate concentrations. Then, an MTT assay determined the cell viability in each well. The survival rate was determined by comparison with a control experiment with untreated cells.

A549 cells



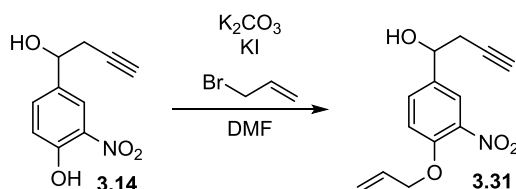
KB cells



3.3.2 The second generation of prodrug

3.3.2.1 Synthesis

a) Allylation of the phenol of the central unit (3.31)

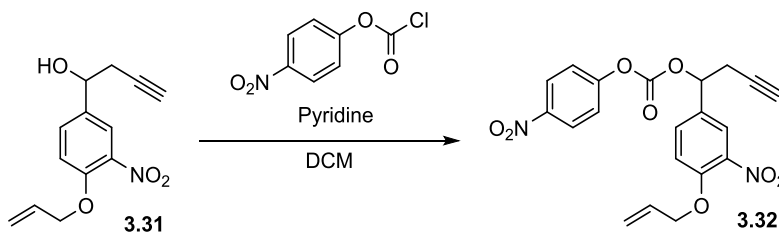


1-(4-(allyloxy)-3-nitrophenyl)but-3-yn-1-ol **3.31** was synthesized according to a routine procedure. Compound **3.14** (390 mg, 1.9 mmol) was dissolved in DMF (2 mL) and K_2CO_3 (650 mg, 2.5 equiv., 4.71 mmol) and KI (62 mg, 20 mol %, 376 μ mol) were added. After 1 min stirring at room temperature, allyl bromide (180 μ L, 2 mmol, 1.1 equiv.) was added by syringe and the mixture was stirred overnight at 60 °C. Then, on the next morning, the reaction was diluted with EtOAc and washed by brine 3 times and by LiCl 10% once to remove the DMF. After drying with Na_2SO_4 , the crude solid was purified by flash chromatography on silica gel using 10 to 30 % of EtOAc in petroleum ether to give the pure product **3.31** as a white powder (387 mg, 83%)

^1H NMR (300 MHz, $CDCl_3$) δ 7.91 (dd, J = 2.3, 0.6 Hz, 1H), 7.56 (ddd, J = 8.6, 2.3, 0.6 Hz, 1H), 7.07 (d, J = 8.7 Hz, 1H), 6.19 – 5.85 (m, 1H), 5.49 (dq, J = 17.3, 1.7 Hz, 1H), 5.34 (dq, J = 10.6, 1.4 Hz, 1H), 4.89 (td, J = 6.2, 3.6 Hz, 1H), 4.70 (dt, J = 5.0, 1.6 Hz, 2H), 2.73 – 2.52 (m, 2H), 2.44 (d, J = 3.7 Hz, 1H), 2.11 (t, J = 2.6 Hz, 1H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 151.58, 139.81, 135.30, 135.17, 131.75, 131.49, 123.36, 118.57, 115.01, 79.81, 71.92, 70.95, 70.26, 29.53, 29.48.

b) Activation of the central unit with 4-nitrophenyl chloroformate (3.32)



1-(4-(allyloxy)-3-nitrophenyl)but-3-yn-1-yl (4-nitrophenyl) carbonate **3.32** was synthesized according to a routine procedure. Compound **3.31** (320mg, 1.3 mmol) was introduced in a 50 mL round-bottom flask with 10 mL of distilled DCM. Then, pyridine (230 μ L, 2.61 mmol, 2 equiv.) and PNP-Chloroform (290 mg, 1.1 equiv., 1.44 mmol) were added and the reaction was stirred at room temperature overnight under Ar. On the next morning, the reaction was diluted with EtOAc and washed by HCl (1 M) once and by brine once, after which the organic phase was dried by Na₂SO₄ and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on a silica gel pad using a gradient of 10% to 40% EtOAc in petroleum ether. The product **3.32** was obtained as a pale-yellow powder (486 mg, 90%)

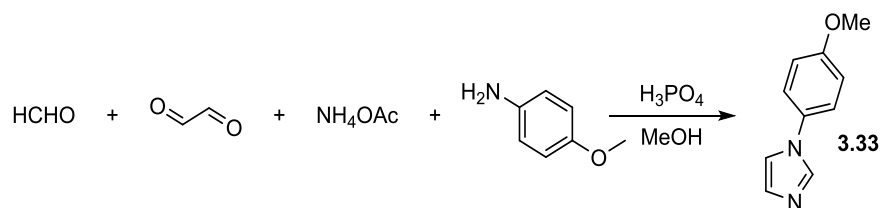
¹H NMR (300 MHz, CDCl₃) δ 8.47 – 8.16 (m, 2H), 7.98 (dd, J = 2.3, 1.3 Hz, 1H), 7.62 (ddd, J = 8.7, 2.3, 0.9 Hz, 1H), 7.42 – 7.34 (m, 2H), 7.12 (d, J = 8.7 Hz, 1H), 6.22 – 5.93 (m, 1H), 5.81 (t, J = 6.6 Hz, 1H), 5.58 – 5.45 (m, 1H), 5.36 (ddq, J = 10.6, 2.1, 1.3 Hz, 1H), 4.72 (dq, J = 4.7, 1.5 Hz, 2H), 3.06 – 2.74 (m, 2H), 2.09 (t, J = 2.6 Hz, 1H).

¹³C NMR (75 MHz, CDCl₃) δ 155.35, 152.50, 151.65, 145.66, 139.96, 132.59, 131.49, 129.92, 125.47, 124.30, 121.82, 118.81, 115.14, 72.34, 70.33, 26.35.

3.3.3 Synthesis of vectorizable complexes

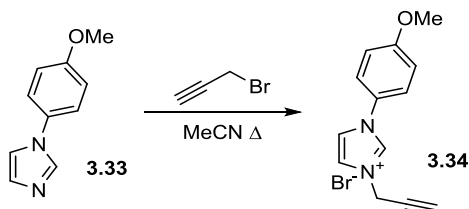
3.3.3.1 NHC

a) N-aryl imidazole mounting (3.33)



1-(4-OMe-C₆H₄)-1H-imidazole **3.33** was synthesized according to a literature procedure.¹⁵⁷ Beige powder (1.05 g, 12%). ¹H NMR (300 MHz, CDCl₃) δ 7.75 (t, J = 1.1 Hz, 1H), 7.29 (d, J = 9.0 Hz, 2H), 7.19 (dt, J = 6.3, 1.3 Hz, 2H), 7.05 – 6.88 (m, 2H), 3.84 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 159.01, 135.96, 130.82, 130.18, 123.31, 118.85, 114.98, 55.70.

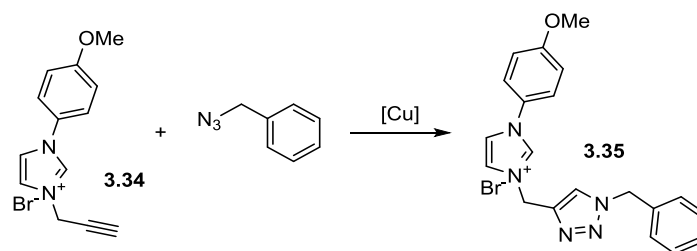
b) Imidazolium synthesis (3.34)



1-(4-OMe-C₆H₄)-3-propargyl-1H-imidazol-3-ium **3.34** bromide was synthesized according to a literature procedure.¹⁵⁷ Beige flakes (646 mg, 80%)

¹H NMR (300 MHz, DMSO-*d*₆) δ 9.87 (s, 1H), 8.29 (t, *J* = 1.9 Hz, 1H), 8.07 (t, *J* = 1.8 Hz, 1H), 7.73 (d, *J* = 9.1 Hz, 2H), 7.19 (d, *J* = 9.1 Hz, 2H), 5.29 (d, *J* = 2.6 Hz, 2H), 3.90 (d, *J* = 2.6 Hz, 1H), 3.84 (s, 3H). ¹³C NMR (75 MHz, DMSO) δ 160.07, 135.31, 127.71, 123.74, 122.81, 121.97, 115.08, 79.19, 75.87, 55.76.

c) Click on the ligand without metal (3.35)



• Procedure A¹⁵⁷

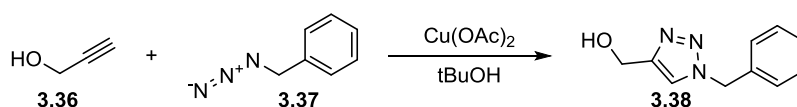
A 25 mL round-bottom flask was filled with acetonitrile (5 mL, 0.1 M), CuSO₄·5H₂O (125 mg, 0.5 mmol, 1 equiv.) and sodium ascorbate (150 mg, 0.75 mmol, 1.5 equiv) and the heterogeneous mixture was stirred at 60 °C for 20 min, during which the mixture turned blue to green-yellow. Then, **3.34** (0.5 mmol, 1 equiv.) was added and the mixture was stirred for one more hour at 60 °C. Then, benzyl azide (0.5 mmol, 64 μ l, 1 equiv.) was added by microsyringe and the reaction was then stirred at 60 °C for 2 days. During the first day, the mixture remained yellow and became dark on the second day with a sticky goo on the walls of the flask. Workup aimed the dissolve the solid and partition the organic phase between DCM and water, but the sticky goo was insoluble in either solvent. The organic phase was washed 3 times with water and was dried with Na₂SO₄ and was finally concentrated under reduced pressure. Very little material was isolated,

and ^1H -NMR did not give any clue about the identity of the compound as it was messy.

- Procedure B

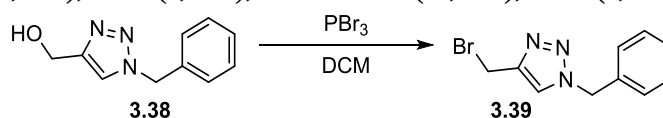
A 25 mL round-bottom flask was filled with 6 of $i\text{PrOH}$ and 3 mL H_2O and the solvent was degassed 3 times by freeze-pump-thaw cycles. Another round-bottom flask was filled with the **3.34** (117 mg, 0.4 mmol) and the degassed solvent was added under Ar, immediately followed by Bn-N_3 (51 μL , 0.4 mmol, 1 equiv.). The mixture was stirred for 5 min, after which $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (6 mg, 5 mol %) and sodium ascorbate (16 mg, 20 mol %) were added. The reaction was then stirred overnight with an aluminium foil to protect from light. On the next morning, the reaction was diluted with water and extracted 3 times with DCM. The combined DCM phases were washed with 10% NH_3 once and with EDTA once, after being dried with Na_2SO_4 . The solvent was evaporated, but all the product was gone in the water phase on contrary to the previous reaction.

d) An alternative synthesis of the clicked ligand (3.38)



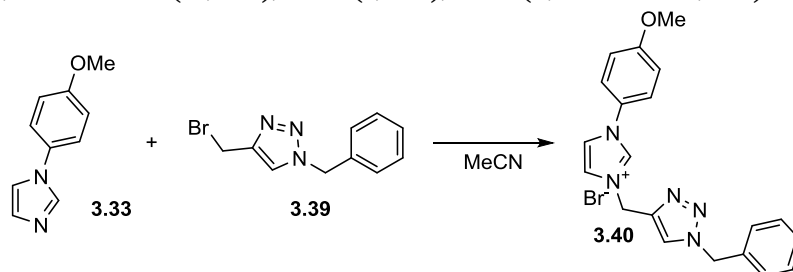
Compound **3.38** was synthesized according to a literature procedure.¹⁵⁸ ($R_f = 0.45$ in DCM/MeOH 95:5, 83%, 235 mg)

^1H NMR (300 MHz, CDCl_3) δ 7.48 (s, 1H), 7.33 – 7.21 (m, 3H), 7.23 – 7.13 (m, 2H), 5.39 (s, 2H), 5.07 – 4.92 (m, 1H), 4.65 (s, 2H).



Compound **3.39** was synthesized using the standard bromination method described previously. White solid (95%)

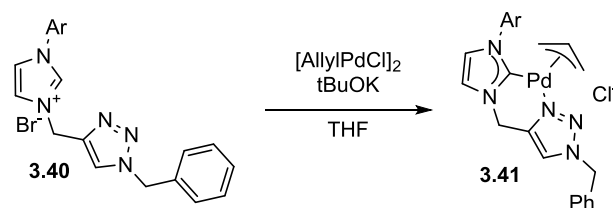
^1H NMR (300 MHz, CDCl_3) δ 7.49 (s, 1H), 7.39 (dd, $J = 5.1, 1.9$ Hz, 3H), 7.33 – 7.23 (m, 2H), 5.52 (s, 2H), 4.55 (d, $J = 0.5$ Hz, 2H).



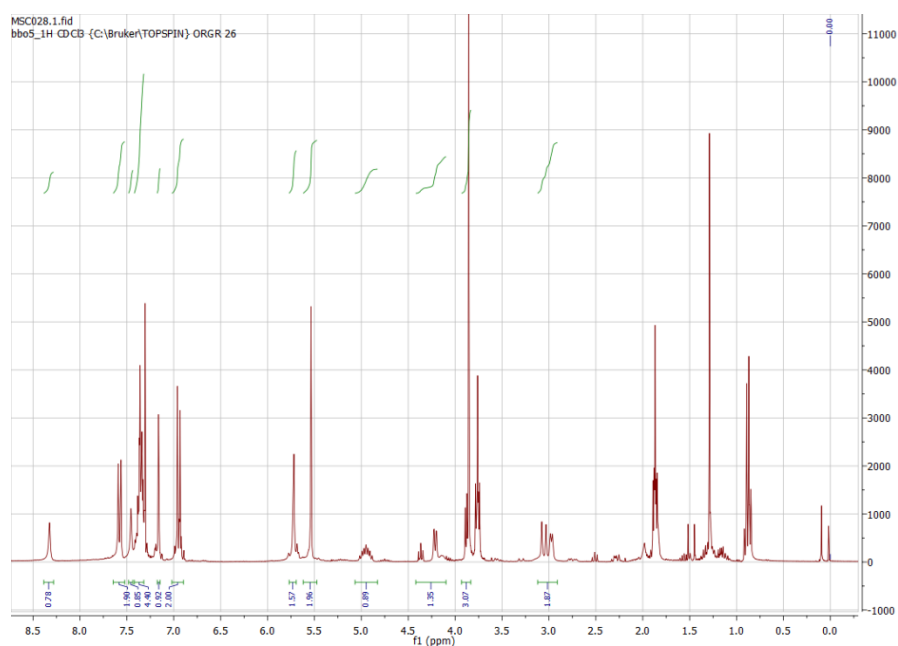
The ligand **3.40** was synthesized according to an adapted literature procedure.¹⁵⁷ A 10 mL round-bottom flask was filled with the bromide **3.39** (317 mg, 1.26 mmol, 1 equiv) and the imidazole **3.33** (219 mg, 1.26 mmol, 1 equiv) in 4 mL of distilled MeCN under Ar. The mixture was refluxed for 36 h during which an orange-brown solution was obtained. After reduction of the solvent volume, attempts to precipitate the product with Et₂O or hexane were unsuccessful. So, the product **3.40** was purified by flash chromatography on silica gel pad using DCM/MeOH 95:5 and it was obtained as a brown form (455 mg, 85%).

¹H NMR (300 MHz, DMSO-*d*₆) δ 9.87 (t, *J* = 1.7 Hz, 1H), 8.38 (s, 1H), 8.24 (t, *J* = 1.9 Hz, 1H), 7.99 (t, *J* = 1.8 Hz, 1H), 7.80 – 7.56 (m, 2H), 7.45 – 7.29 (m, 5H), 7.17 (d, *J* = 9.1 Hz, 2H), 5.64 (s, 2H), 5.60 (s, 2H), 3.82 (s, 3H).

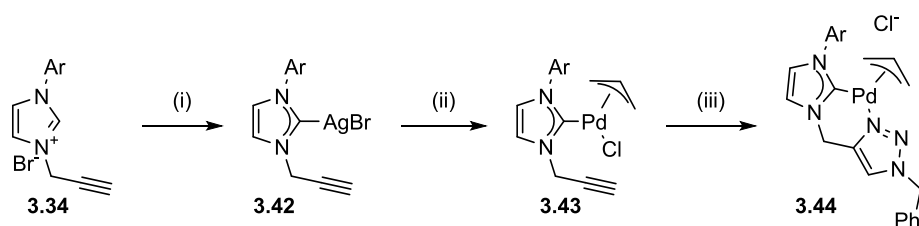
¹³C NMR (75 MHz, DMSO-*d*₆) δ 160.64, 141.17, 136.28, 135.96, 129.40, 128.87, 128.70, 128.34, 125.43, 124.20, 123.75, 122.40, 115.72, 56.36, 53.60, 44.73.



The synthesis of **3.41** was carried out using a modified literature method.¹⁵⁷ A flame dried Schlenk tube was charged under Ar with the imidazolium salt **3.40** (42.6 mg, 0.1 mmol), tBuOK (13.3 mg, 1.1 equiv, 0.11 mmol) and [(Allyl)PdCl]₂ (17.8 mg, 0.05 mmol, 0.5 equiv.), followed by distilled THF (1 mL). The yellow mixture was stirred at room temperature for 4 h under Ar. Then, the yellow mixture was filtered on a Millipore filter and the yellow solution was concentrated under reduced pressure. Then, it was re-dissolved in a minimum of DCM and Et₂O was added and a white solid appeared. It was collected on a filter paper, but it quickly decomposed to a sticky brown solid. ¹H-NMR indicated a degradation.

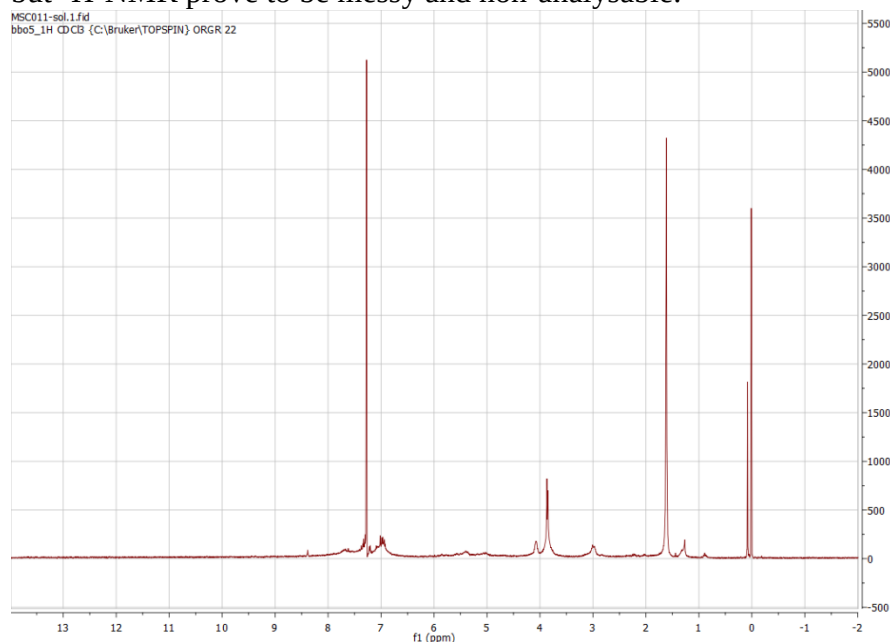


e) **One pot synthesis of the NHC palladium allyl and click (3.44)**

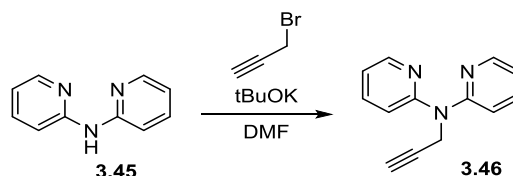


The synthesis of **3.44** was attempted without isolating the intermediates, as the NHC-Ag **3.42** and NHC-Pd **3.43** were unstable¹⁵⁷. A Schlenck tube was filled with DCM (2.5 mL, 0.2 M) and was degassed by three freeze-pump-thaw cycles. Then, when still frozen and under an Ar flow, the imidazolium salt **3.39** (Ar = Mes, 154 mg, 0.5 mmol), Ag₂O (59 mg, 0.25 mmol, 0.5 equiv.) and powdered MS 4Å. The tube was then flushed with Ar with 3 pump-Ar cycles at -196 °C and then was allowed to warm to room temperature, after which the mixture (containing **3.42**) was stirred overnight at room temperature under Ar. On the next morning, the reaction medium was collected in a syringe and was transferred to another flame-dried Schlenck tube containing [(Allyl)PdCl]₂ (94.3 mg, 0.25 mmol, 0.5 equiv.) under Ar after filtration on a Millipore filter. The

brown solution became bright yellow (containing **3.43**) upon transfer to the Pd-containing Schlenk tube. After 1 hour stirring at room temperature under Ar, the reaction medium was collected in a syringe and transferred to another Schlenk tube containing CuI (96.2 mg, 0.5 mmol, 1 equiv.) under Ar after filtration on a Millipore filter. The mixture became dark orange and did not change its colour when DIPEA (88 μ L, 0.5 mmol, 1 equiv.) was added. Then, 2,6-diisopropyl azide (100 μ L, 0.5 mmol, 1 equiv.) was added by syringe and the colour changed to dark brown and the mixture was stirred for 24 h at room temperature under Ar. For the workup, the organic phase was washed by H₂O once, then by NH₃ 10% after getting dried with Na₂SO₄ and concentration under reduced pressure. The compound was isolated by precipitation with Et₂O from a DCM solution (60 mg) but ¹H-NMR prove to be messy and non-analysable.



3.3.3.2 Bipyridylamine propargyl (**3.46**)

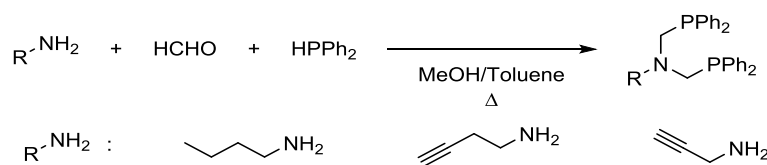


The ligand **3.46** was synthesized according to an adapted literature procedure.¹⁵⁹ Beige solid (88 mg, 42%). The compound **3.46** was unstable and decomposed if not properly stored under Ar at – 18 °C.

^1H NMR (300 MHz, CDCl_3) δ 8.40 (ddd, $J = 4.9, 2.0, 0.9$ Hz, 2H), 7.57 (ddd, $J = 8.4, 7.2, 2.0$ Hz, 2H), 7.23 (dt, $J = 8.4, 0.9$ Hz, 2H), 6.92 (ddd, $J = 7.3, 4.9, 1.0$ Hz, 2H), 5.14 – 4.89 (m, 2H), 2.15 (t, $J = 2.4$ Hz, 1H).

^{13}C NMR (75 MHz, CDCl_3) δ 156.43, 148.55, 137.53, 117.75, 114.63, 70.55, 37.75.

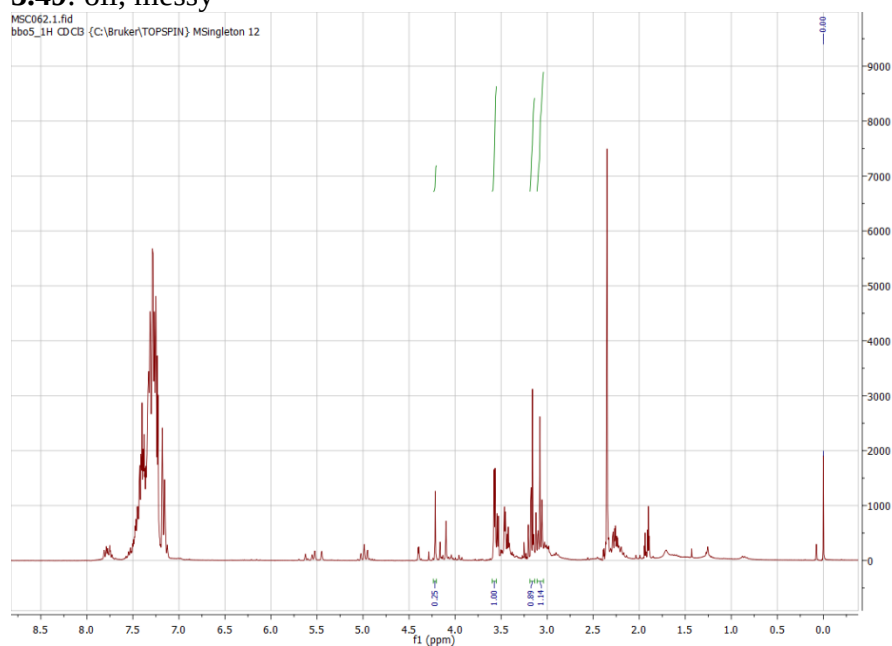
3.3.3.3 Dppm butylamine (3.51)

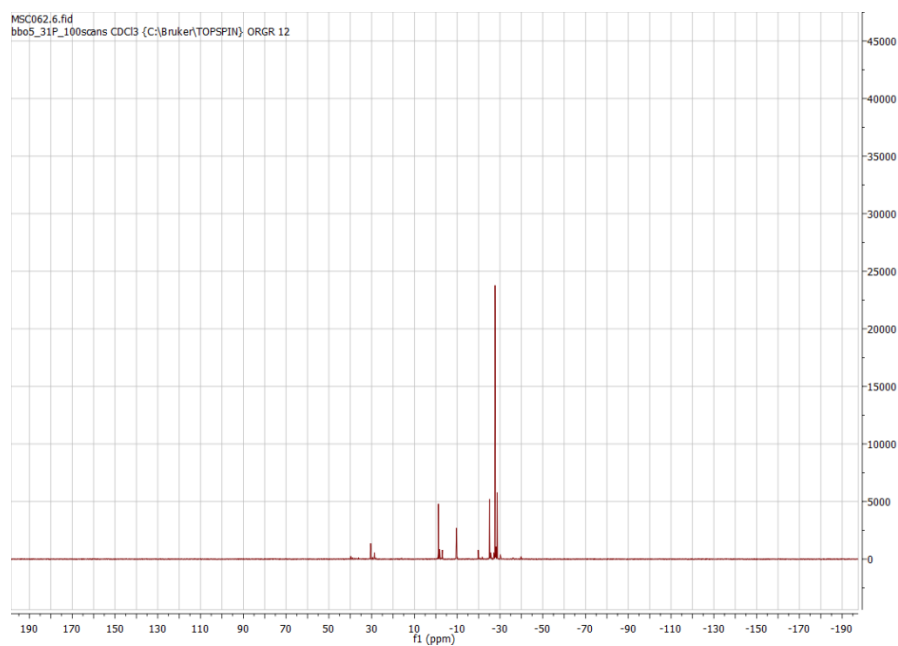


Compounds were synthesized according to a literature procedure.¹⁶⁰

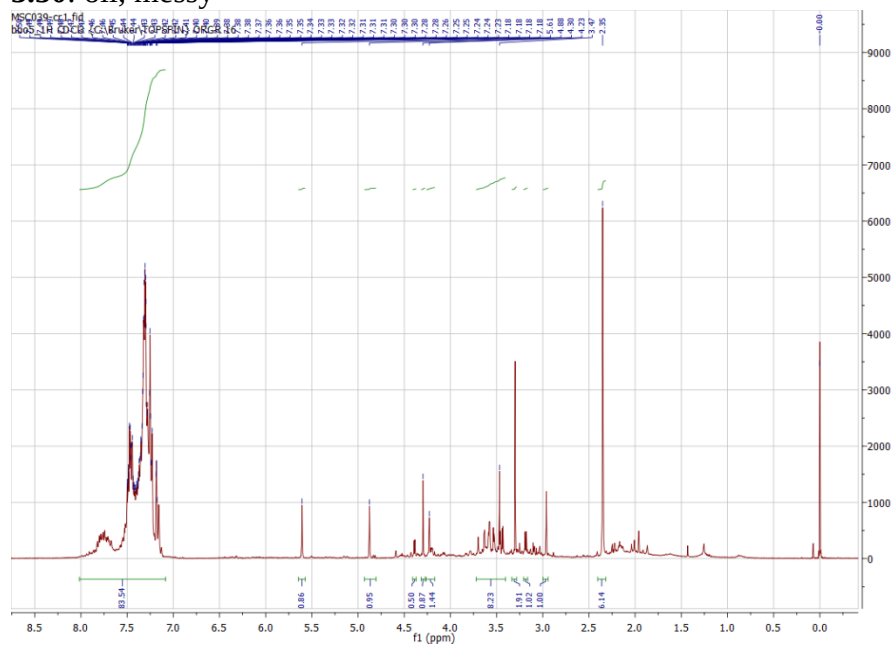
3.48: quantitative yield, thick colourless oil. ^1H NMR (300 MHz, CDCl_3) δ 7.53 – 7.23 (m, 20H), 3.66 – 3.38 (m, 4H), 2.84 (t, $J = 7.3$ Hz, 2H), 1.53 – 1.24 (m, 2H), 1.24 – 1.08 (m, 2H), 0.81 (t, $J = 7.3$ Hz, 3H). ^{31}P NMR (121 MHz, CDCl_3) δ -27.90.

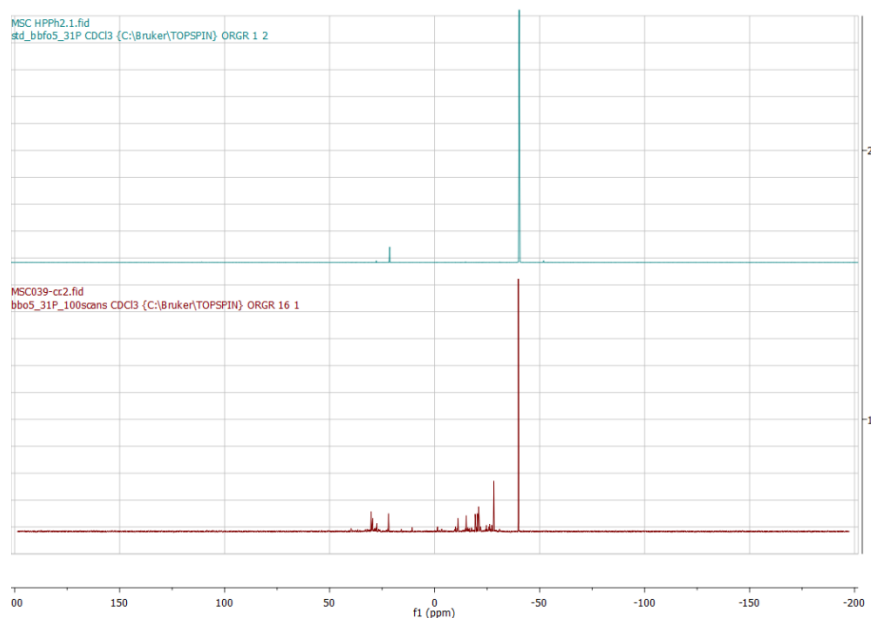
3.49: oil, messy



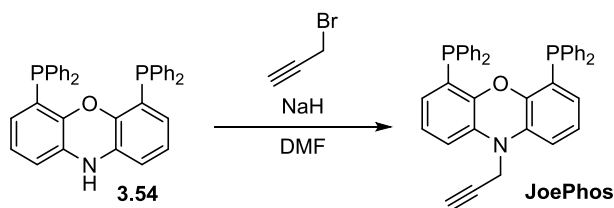


3.50: oil, messy





3.3.3.4 JoePhos



JoePhos was synthesized according to a routine procedure. A 10 mL round-bottom flask was filled with *N*-Xantphos **3.54** (103 mg, 0.186 mmol) and dry DMF (2.6 mL, 0.08 M). Then, the reaction was cooled down to 0 °C with an ice bath, and then NaH (80% in mineral oil, 10 mg, 0.3 mmol, 1.5 equiv.) was added and the reaction medium turned dark yellow, then green and eventually dark blue. The reaction was then stirred at 0 °C for 1 hour. Propargyl bromide (80% in toluene, 35 μ L, 0.37 mmol, 2 equiv.) was added and the reaction was stirred for 3 more h at room temperature. Then, EtOAc was added and the organic phase was washed with brine 3 times and by LiCl 10% once, after which it was dried with Na₂SO₄ and concentrated under reduced pressure. The crude dark brown solid was dissolved in a minimum of petroleum ether/EtOAc 8:2 and then deposited on a silica gel pad. It was eluted with 100 mL of petroleum ether/EtOAc 8:2 and the pure product **JoePhos** was obtained as a white powder after evaporation of the solvent. Yield = 80 mg, 72%

^1H NMR (300 MHz, CDCl_3) δ 7.25 (qq, J = 4.8, 2.6, 2.1 Hz, 20H), 6.81 (t, J = 7.7 Hz, 2H), 6.73 (dd, J = 7.9, 1.6 Hz, 2H), 6.17 (dd, J = 7.6, 1.8 Hz, 2H), 4.30 (d, J = 2.5 Hz, 2H), 2.29 (s, 1H).

^{13}C NMR (75 MHz, CDCl_3) δ 148.20, 148.06, 147.92, 137.04, 136.96, 136.87, 134.18, 134.04, 133.91, 133.29, 128.45, 128.39, 128.35, 128.30, 126.20, 125.49, 125.36, 125.23, 124.09, 112.69, 77.98, 73.03, 34.78.

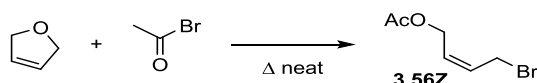
^{31}P NMR (121 MHz, CDCl_3) δ -18.44.

3.4 Targeted covalent inhibitors

3.4.1 Synthesis of target covalent inhibitors

3.4.1.1 Joelinib

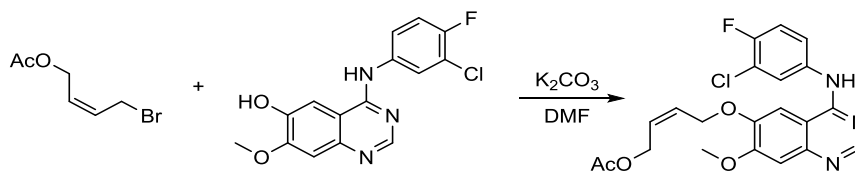
a) Synthesis of the allylic side chain (3.56Z)



(Z)-4-bromobut-2-en-1-yl acetate **3.56Z** was synthesized according to a literature procedure.¹⁹⁵ Transparent oil (689 mg, 30%).

^1H NMR (300 MHz, CDCl_3) δ 6.98-5.89 (m, 1H), 5.69 (dtt, J = 10.77, 6.92, 0.88, 1H), 4.70 (dd, J = 6.88, 1.42 Hz, 2H), 4 (d, J = 8.18 Hz, 2H), 2.08 (s, 3H).

b) Coupling to the targeting unit



Joelinib was prepared following literature.¹⁹⁶ Compound **3.57** (142.3 mg, 0.45 mmol) and **3.56Z** (85.2 mg, 0.446 mmol) were dissolved in DMF (1 mL) at room temperature. The reaction mixture was warmed up to 70 °C and stirred for 4h. The solvent was removed under high vacuum. DCM (5 mL) was added for filtration on silica. After purifying with silica flash column DCM/ EtOAc 6:4 to 5:5 and drying under high vacuum, the **Joelinib** was obtained as a solid (93 mg, 48%).

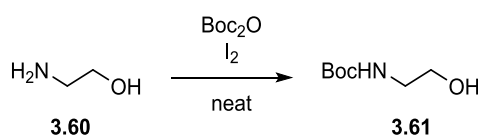
^1H NMR (300 MHz, CDCl_3) δ 8.59 (s, 1H), 8.49 (s, 1H), 7.77 (dd, J = 6.6, 2.6 Hz, 1H), 7.69 – 7.58 (m, 1H), 7.38 (s, 1H), 7.26 (d, J = 1.6 Hz, 1H), 7.17 (t, J = 8.8 Hz, 1H), 5.81 – 5.69 (m, 1H), 5.69 – 5.56 (m,

1H), 5.21 – 5.10 (m, 2H), 4.73 (dd, $J = 6.9, 1.2$ Hz, 2H), 4.04 (s, 3H), 1.96 (s, 3H).

^{13}C NMR (75 MHz, CDCl_3) δ 153.68, 131.22, 126.51, 125.76, 123.59, 116.38, 108.05, 101.21, 64.84, 60.97, 56.36, 21.10.

3.4.1.2 Celinib

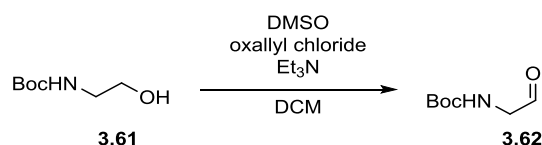
a) Ethanolamine N protection (3.61)



Tert-butyl (2-hydroxyethyl)carbamate **3.61** was synthesized according to a literature procedure.¹⁹⁷ Colourless thick oil (3.36 g, 95%). The crude was used as such in the next step without further purification.

^1H NMR (300 MHz, CDCl_3) δ 4.96 (s, 1H), 3.70 (q, $J = 5.2$ Hz, 2H), 3.28 (q, $J = 5.4$ Hz, 2H), 2.47 (s, 1H), 1.44 (s, 9H).

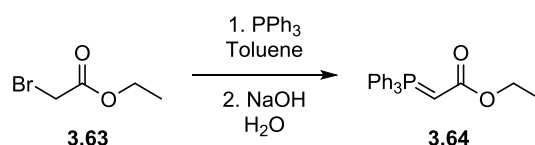
b) Swern oxidation (3.62)



Tert-butyl (2-oxoethyl)carbamate **3.61** was synthesized according to a literature procedure.¹⁸⁰ Yellow oil (4.85 g, 87%).

^1H NMR (300 MHz, CDCl_3) δ 9.65 (s, 1H), 4.08 (d, $J = 5.05$ Hz, 2H), 1.45 (s, 9H).

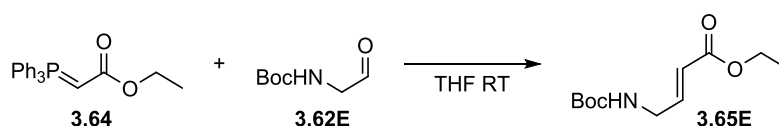
c) Wittig salt preparation (3.64)



Ethyl 2-(triphenyl-λ5-phosphaneylidene)acetate **3.64** was synthesized according to a literature procedure.¹⁹⁸ White solid (16 g, 57%).

^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 7.79 (m, 15H), 4.04 (q, $J = 7.07$ Hz, 2H), 0.97 (t, $J = 7.16$ Hz, 3H).

d) Wittig coupling (3.65E)

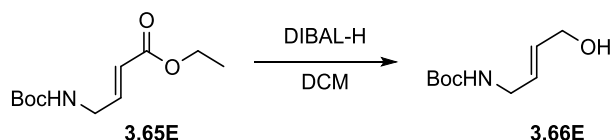


Ethyl (E)-4-((tert-butoxycarbonyl)amino)but-2-enoate **3.65E** was synthesized according to a literature procedure.¹⁷⁷ Compound **3.62E** (2.5 g, 16 mmol) was dissolved in THF (32 mL) in a 100 mL round-bottom flask under Ar and **3.64** (5.85g, 16.8 mmol) was added. The reaction mixture was stirred overnight at room temperature. On the next morning, the solvent was removed under high vacuum and diethyl ether was added and the reaction mixture was cooled down in the freezer at -20 °C, during which a white solid (phosphine oxide) appeared. After filtering off the precipitate on paper, the solvent was evaporated under high vacuum and the crude yellow oil was purified by flash column chromatography using EtOAc/DCM 6:3. Then, **3.65E** was obtained as a smelly yellow oil (3.1 g, 83%).

¹H NMR (300 MHz, CDCl₃) δ 6.9 (dt, J = 15.82, 4.67 Hz, 1H), 5.93 (dt, J = 15.71, 1.83 Hz, 1H), 4.2 (q, J = 7.11 Hz, 2H), 3.92 (s, 2H), 1.45 (s, 9H), 1.28 (t, J = 7.38 Hz, 3H).

¹³C NMR (75 MHz, CDCl₃) δ 166.23, 155.70, 144.88, 121.45, 80.02, 77.16, 60.59, 41.46, 28.48, 27.55, 14.36.

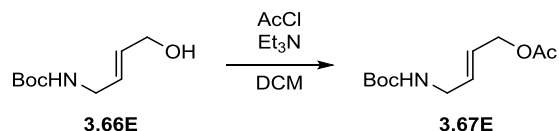
e) DIBAL-H reduction (3.66E)



Tert-butyl (E)-(4-hydroxybut-2-en-1-yl)carbamate **3.66E** was synthesized according to a literature procedure.¹⁸³ Compound **3.65E** (2.5 g, 11mmol) was dissolved in DCM (27 mL) at 0 °C in a 100 mL round-bottom flask. DIBAL in 1 M hexane (23.4 mL, 23 mmol) was added dropwise at 0 °C and the reaction mixture was stirred overnight at room temperature. The reaction mixture was slowly added to a cold HCl solution (2 M), after which the phases were separated, and the organic phase was washed with 3 x HCl (2M) and 1 x saturated NaCl. DCM was added to aqueous phase for extraction, organic phases were brought together and dried with Na₂SO₄. After purifying with silica flash column EtOAc/DCM 5% -> 10% -> 15% -> 20% -> 50%, DCM/MeOH 10%, **3.66E** was obtained as a yellow oil (1.05 g, 51%).

¹H NMR (300 MHz, CDCl₃) δ 5.75 (ddt, J = 15.60, 8.18, 5.07 Hz, 2H), 4.63 (s, OH), 4.14 (s, 2H), 3.75 (t, J = 5.05, 2H), 1.44 (s, 9H).

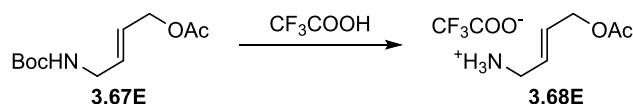
f) Acetylation (3.67E)



(E)-4-((tert-butoxycarbonyl)amino)but-2-en-1-yl acetate **3.67E** was synthesized according to a routine procedure. **3.66E** (1.05 g, 5.6 mmol) was dissolved in DCM (19 mL) in a 100 mL round-bottom flask and triethylamine (2 mL, 14 mmol) was added. The flask was then cooled to 0 °C with an ice bath. Acetyl chloride (0.48 mL, 6.7 mmol) was then added dropwise at 0 °C and the reaction mixture was stirred at room temperature for 30 min. After washing with hydrochloric acid, saturated sodium bicarbonate and saturated sodium chloride, drying by Na₂SO₄ and removing of the solvent under high vacuum and purifying with silica flash column EtOAc/petroleum ether 15:85, **3.67E** was obtained (0.64 g, 50%) as a solid.

¹H NMR (300 MHz, CDCl₃) δ 5.74 (ddt, J = 15.45, 9.19, 4.72 Hz, 2H), 4.54 (d, J = 5.12, 2H), 3.76 (t, J = 4.58, 2H), 2.06 (s, 3H), 1.44 (s, 9H).

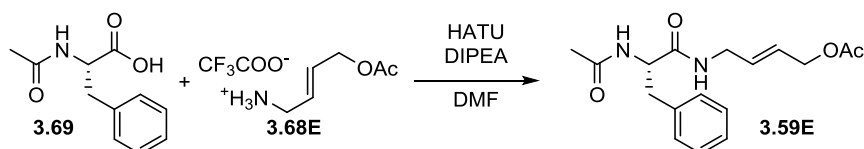
g) Boc deprotection in TFA (3.68E)



(E)-4-acetoxybut-2-en-1-aminium 2,2,2-trifluoroacetate **3.68E** was synthesized according to a routine procedure. Compound **3.67E** (0.64 g, 2.8 mmol) was dissolved in DCM (8 mL) and TFA (2.9 mL, 14 mmol) was added dropwise at 0 °C. The reaction mixture was stirred for 2 h and the solvent was removed under high vacuum to give (E)-4-acetoxybut-2-en-1-aminium 2,2,2-trifluoroacetate as a brown oil (1g, 147%). The crude **3.68E** was used as such in the next step without further purification.

¹H NMR (300 MHz, D₂O) δ 5.80 (ddt, J = 24.73, 15.75, 5.21 Hz, 2H), 4.89 (dd, J = 5.43, 1.05, 2H), 3.49 (d, J = 6.22, 2H), 1.97 (s, 3H).

h) Coupling to N-Acetyl-L-Phenylalanine: Celinib (3.59)



Celinib 3.59E was synthesized by a home-developed procedure. Ac-Phe **3.69** (85 mg, 0.4 mmol) and HATU (156 mg, 0.4 mmol, 1 equiv.) were dissolved in DMF at 0°C under Ar. After 10 min of stirring, the amine **3.68E** (100 mg, 0.4 mmol, 1 equiv.) in solution in DMF was added dropwise, immediately followed by the addition of DIPEA (0.36 mL, 2 mmol, 5 equiv.). The reaction was allowed to stir overnight during which it was allowed to warm to room temperature; the reaction mixture turned orange. On the next morning, the reaction mixture was poured in brine. The water phase was extracted 3 times with EtOAc, followed by 3 items of washing with brine and one washing with LiCl 10% to remove the remaining DMF. The combined organic phases were dried over Na₂SO₄ and evaporated under reduced pressure to give a yellow oil. The crude compound was purified by flash column chromatography on silica gel with a gradient starting at 3% MeOH in DCM to 10% MeOH in DCM. The **Celinib 3.59E** was obtained as a white powder (69 mg, 53%).

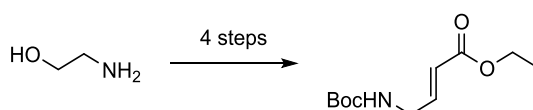
¹H NMR (300 MHz, CDCl₃) δ 7.27-7.23 (m, 5H), 5.54 (ddt, J = 15.44, 13.82, 5.58, 2H), 4.61 (q, J = 8.28, 1H), 4.47 (d, J = 5.45, 2H), 3.77 (tt, J = 20.1, 5.48, 2H), 3.05 (ddd, J = 20.1, 13.96, 6.26, 2H), 2.06 (s, 3H), 1.98 (s, 3H);

¹³C (75 MHz, CDCl₃) δ 170.02, 170.01, 136.57, 129.81, 129.26, 128.70, 127.05, 126.20, 64.03, 54.79, 40.52, 38.59, 23.21, 20.93;

FTMS (*m/z*) (ES⁺) calculated for C₁₇H₂₃O₄N₂ *m/z* = 319.16523. Found *m/z* = 319.16515.

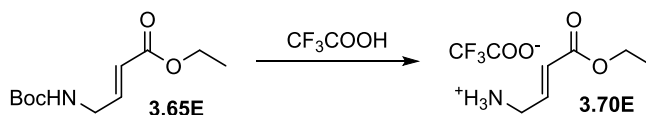
3.4.1.3 Reference inhibitor

a) Boc-ester synthesis (3.65E)



Compound **3.65EE** was synthesized according to an already described methodology reported in paragraph 3.4.1.2d).

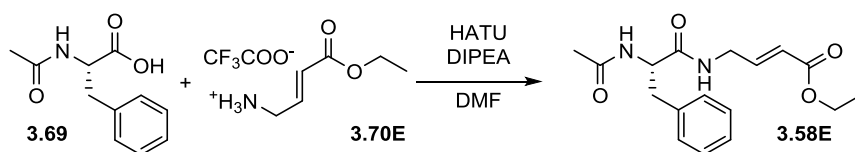
b) Boc deprotection in TFA (3.70E)



The (E)-4-ethoxy-4-oxobut-2-en-1-aminium trifluoroacetate **3.70E** was synthesized by a home-developed procedure. The ester **3.65E** (156 mg, 0.68 mmol) was dissolved in DCM (2 mL, 0.34 M) at 0 °C.

Then, trifluoroacetic acid was added dropwise at 0 °C and the reaction was stirred at 0 °C overnight while the reaction was allowed to go back to room temperature. The colour changed from colourless to brown. On the next morning, the solvent was evaporated under reduced pressure. Toluene (5 mL) was optionally added and then evaporated to remove TFA traces. The product **3.70E** was obtained after drying under vacuum as a thick brown oil (quantitative yield).

c) Coupling to N-Acetyl-L-Phenylalanine: Reference inhibitor (3.58E)



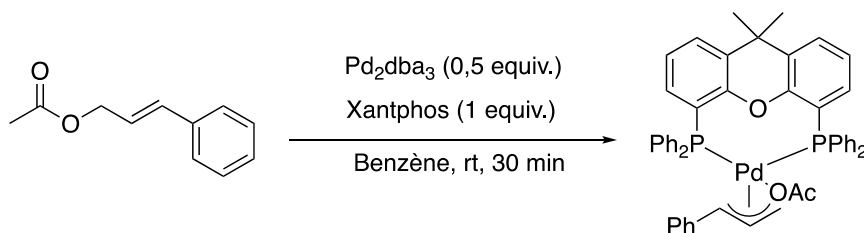
For the procedure to synthesize the compound **3.58E**, see 3.4.1.2h)
Yield: 27% (due to a loss at the workup). Aspect: white powder

^1H NMR (300 MHz, CDCl_3) δ 7.32-7.23 (m, 5H), 6.73 (dt, $J = 15, 9.5$, 5.40 Hz, 1H), 6.12 (s, NH), 5.91 (s, NH), 5.70 (dt, $J = 15.68$, 1.70, 1H), 4.64 (q, $J = 8.36$, 1H), 4.18 (q, $J = 7.54$, 2H), 3.93 (t, $J = 5.14$, 2H), 3.07 (dd, $J = 19.77$, 6.67, 2H), 2.00 (s, 3H), 1.29 (t, $J = 7.24$, 3H)
 ^{13}C NMR (75 MHz, CDCl_3) δ 171.75, 171.07, 166.27, 143.55, 136.54, 129.29, 128.70, 127.12, 121.77, 60.64, 54.99, 40.09, 38.43, 22.99, 14.27.

FTMS (m/z) (ES^+) calculated for $\text{C}_{17}\text{H}_{22}\text{O}_4\text{N}_2^{23}\text{Na}$ $m/z = 341,14718$.
Found $m/z = 341,14707$.

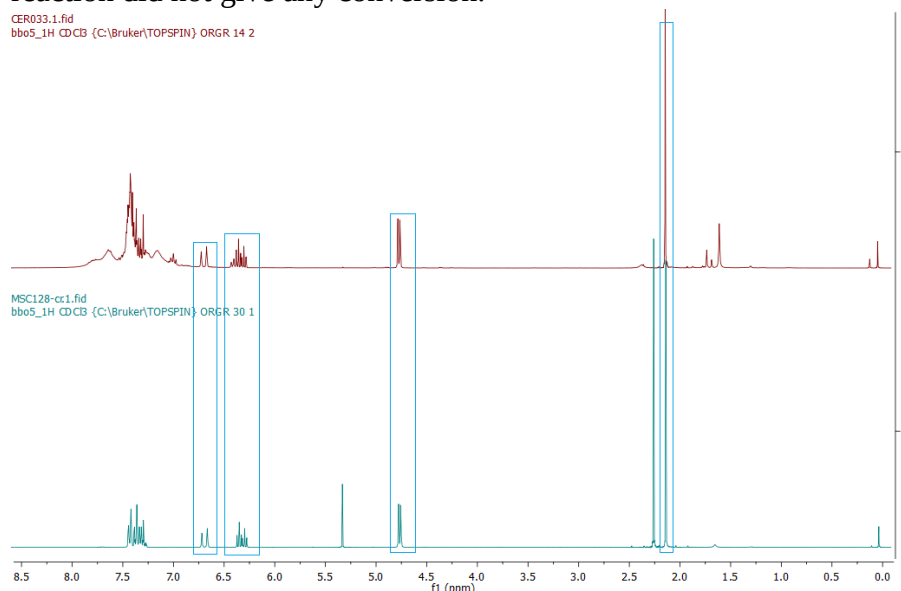
3.4.1.4 Synthesis of π -allyl palladium complexes

a) Pd_2dba_3 and Xantphos (3.62-OAc)

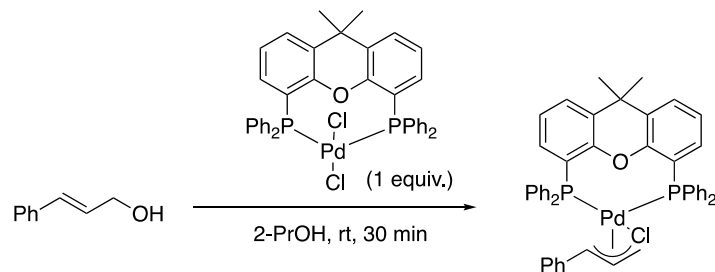


Cinnamyl acetate **3.61** (0.01 mL, 86.26 μmol) was dissolved in benzene (1 mL) and Pd_2dba_3 (0.039 g, 43.13 μmol) and Xantphos (0.05 mg, 86.26 μmol) were added. The reaction mixture was stirred for 30 min at room temperature. The precipitate was filtered on celite and the solvent was removed under high vacuum. The spectrum in

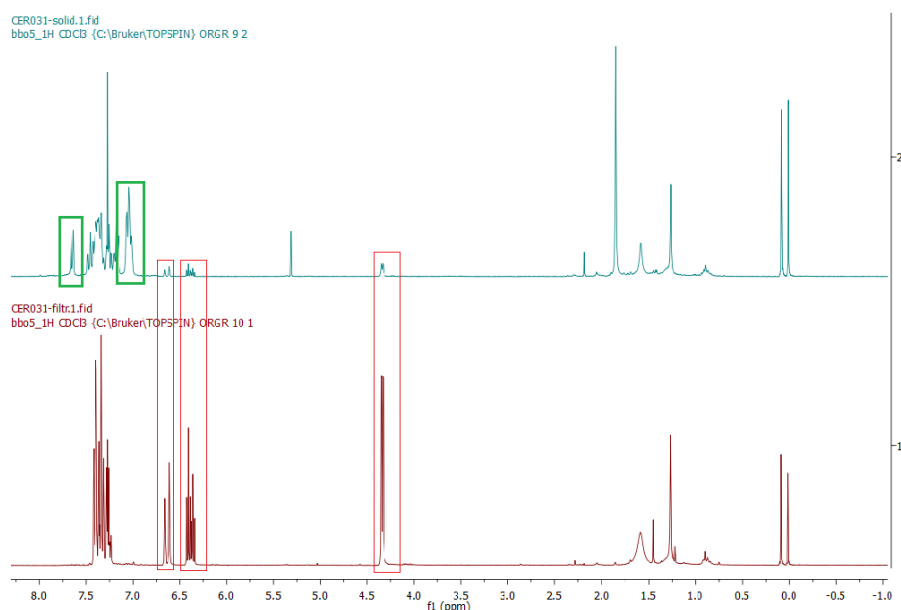
blue shows cinnamyl acetate, and all the non-aromatic peaks are found in the crude spectrum in red of the reaction, which is a sign that the reaction did not give any conversion.



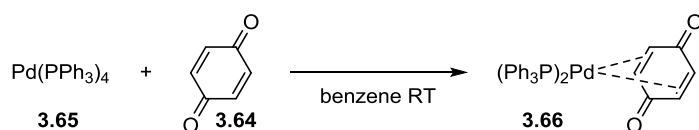
b) Pd(Xantphos)Cl₂ (PdC01) and iPrOH (3.62-Cl)



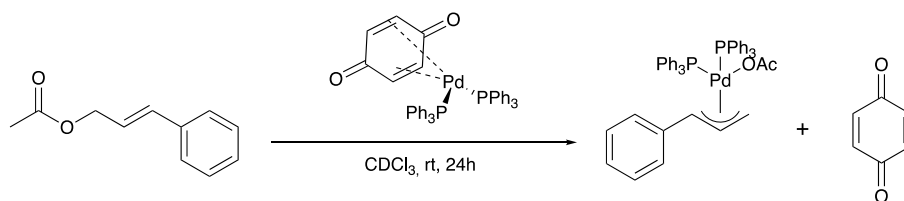
The π -allyl complex **3.62-Cl** was prepared following the literature.¹⁸⁴ (*E*)-3-phenylprop-2-en-1-ol **3.67** (0.009 g, 66.23 μ mol) and Pd(Xantphos)Cl₂ (0.01, 13.23 μ mol) were dissolved in isopropanol and the reaction mixture was stirred for 30 min. Hexane was added and a yellow precipitate was filtered. Both the filtrate and the solids were analysed by ¹H-NMR. The filtrate (#1) contains the pure cinnamyl alcohol, and the solid (#2) is composed of (Xantphos)PdCl₂ (**PdC01**), presumably non-reacted, and a bit of cinnamyl alcohol **3.67**, represented in red for the non-aromatic part. The reaction did not form any π -allyl palladium **3.62-Cl**.



3.4.1.5 Quinone-based catalyst (3.67)

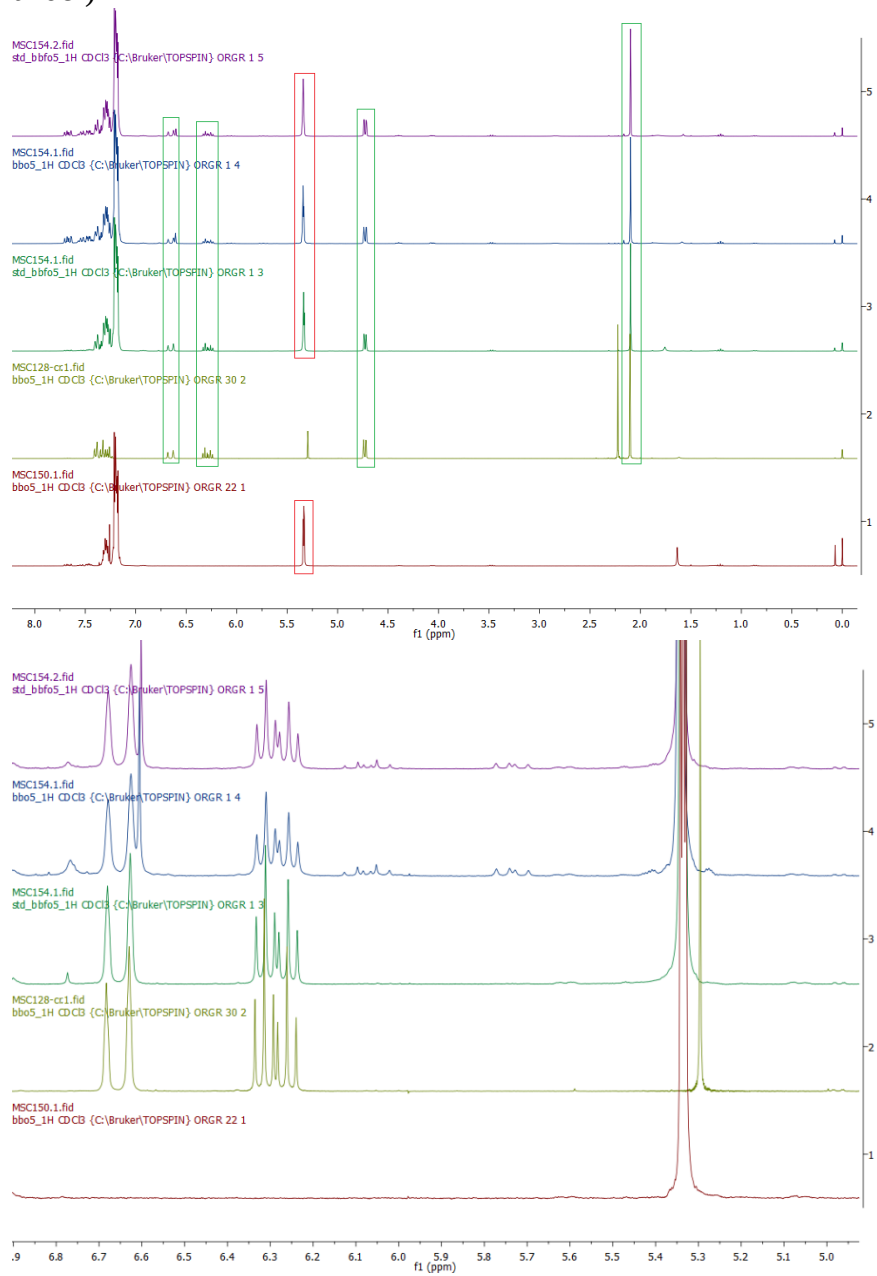


The complex **3.66** was synthesized according to a literature procedure.¹⁹⁹ Bright red powder (45%). ^1H NMR (300 MHz, CDCl_3) δ 7.40 – 7.05 (m, 30H), 5.44 – 5.28 (m, 4H). ^{31}P NMR (121 MHz, CDCl_3) δ 31.11.



The complex **3.66** (0.05 g, 67.65 μmol , spectrum #1) was dissolved in CDCl_3 (0.8 mL) and cinnamyl acetate **3.61** (0.01 mL, 67.65 μmol , spectrum #2) was added. A spectrum was recorded shortly after (#3), then after 24 h (#4). Only the **3.61** and **3.66** were visible on ^1H NMR and just some traces of π -allyl. The reaction mixture was heated up, but no change was observed (#5). The peaks of the quinone ligand of the palladium are reported in red and the peaks of the non-aromatic part of the cinnamyl did not change even after heating. However, while making a zoom between 5 and 7 ppm, some small signals at 5.7

and 6.1 ppm could be attributed to traces of π -allyl palladium. (See under)



3.4.2 Testing of target covalent inhibitors

3.4.2.1 Joelinib

a) Western Blot

i Cell lysis to extract protein

Protein could be extracted from different kinds of samples, such as tissue or cells. Below was the protocol to extract proteins from adherent cells.

Adherent cells:

Cells were washed in the culture flask by adding cold phosphate buffered saline (PBS) and rocking gently (Keep tissue culture dish on ice throughout). PBS was discarded. Then, 90 µL of ice-cold cell Ripa lysis buffer, 10 µL phosphatase inhibitor cocktail and 1 µL of protease inhibitor cocktail were added. After 20 minutes, a cell scraper was used to dislodge the cells. The mixture into microcentrifuge tubes was pipetted. After incubating for 30 minutes on ice, the lysate was clarified by spinning for 10 minutes at 13,000 RPM, at 4°C. The concentration of protein was measured using a spectrophotometer (Pierce™ 660nm Protein Assay).

Sample preparation:

Using $concentration = \frac{masse}{volume}$, the volume of protein extract was determined to ensure 300 µg in each well. Then, 25 µL Leammli (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH approx. 6.8.) 6x to the sample was added and make the volume in each lane equalized using cell lysis buffer. Heat the samples with dry plate for 7 minutes at 97 °C.

Gel preparation:

*, APS: Ammonium Persulfate; TEMED: Tetramethyl ethylenediamine; SDS: Sodium dodecyl sulphate

Gel preparation		8%	10%
Separating gel	dd H ₂ O ₂	5.1 mL	4.37 mL
	Glycerol	0.28 mL	0.28 mL
	Tris.Cl/SDS* (pH. 8.8)	2.815 mL	2.815 mL
	30% Acr/0.8% Bis	3 mL	3.75 mL
	20% APS*	55 µL	55 µL
	TEMED*	5 µL	5 µL
10% Stacking gel	dd H ₂ O ₂	2.95 mL	

Tris.Cl/SDS (pH. 6.8)	1.175 mL
30% Acr/0.8% Bis	0.515 mL
20% APS	47 μ L
TEMED	5 μ L

After preparing the 10% separating gel solution, the rack for gel solidification was assembled. Separating gel solution was added carefully until the level was equal to the green bar holding the glass plates. H₂O was added to the top. After waiting for 15–30 minutes until the gel turned solid, the separating gel was overlaid with the stacking gel after removing the water. The comb was inserted, ensuring that there were no air bubbles. After waiting 5 minutes the gel was turning into solid.

Electrophoresis:

The running buffer into the electrophorator was poured. The gel was placed inside the electrophorator and connected to a power supply. Make sure the gel was completely covered, and the comb was carefully removed. The marker (10 μ L) was loaded followed by the samples (10 μ L) into each well. The gel was run with low voltage (70 V) for stacking gel; a higher voltage (140 V) was used for separating gel. The gel was run for approximately an hour, or until the dye front run off the bottom of the gel.

Electrotransfer:

4 filter sheets were cut to fit the measurement of the gel, and one polyvinylidene fluoride (PDVF) membrane with the same dimensions. The sponge and filter paper were wet in transfer buffer and wet the PDVF membrane in methanol. Glass plates were separated, and the gel was retrieved. A transfer sandwich was created as follows: Sponge, 2 Filter Papers, Gel, PVDF, 2 Filter Papers. It was ensured that there were no air bubbles between the gel and PVDF membrane and squeeze out extra liquid. The sandwich was relocated to the transfer apparatus, which should be placed near an ice pack to maintain 4 °C. Transfer buffer was added to the apparatus and ensure that the sandwich was covered with the buffer. Electrodes were placed on top of the sandwich, ensuring that the PVDF membrane was between the gel and a positive electrode. The transfer was done for 100 minutes (360 mA).

Blocking and antibody incubation:

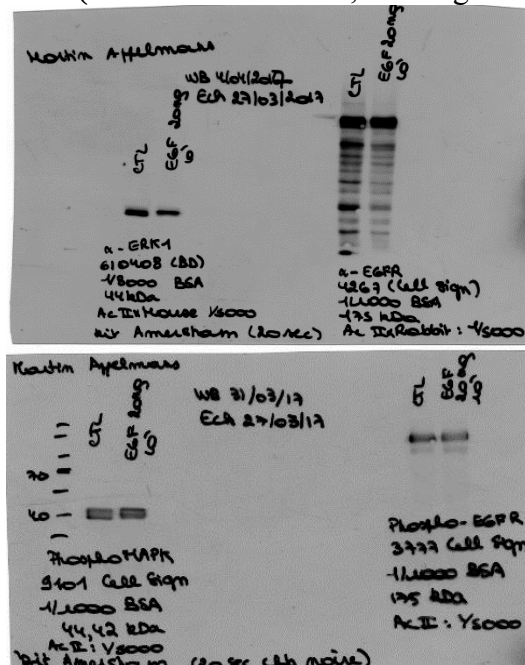
The membrane was blocked in 5% bovine serum albumin (BSA) for 1 hour. The primary antibody was added in 5% bovine serum albumin

(BSA) and incubated overnight at 4 °C on a shaker. The membrane was washed with TBST (Tris-Buffered Saline Tween-20) for 10 minutes, 3 times. The secondary antibody was added in TBST and incubated for 1 hour. The membrane was washed with TBST for 5 minutes, 3 times. ECL mix (following the proportion of solution A and B provided by the manufacturer) was prepared. The membrane was incubated for 1–2 min. The result was visualized in the dark room.

ii **Western Blot Analysis: Optimization of the method**

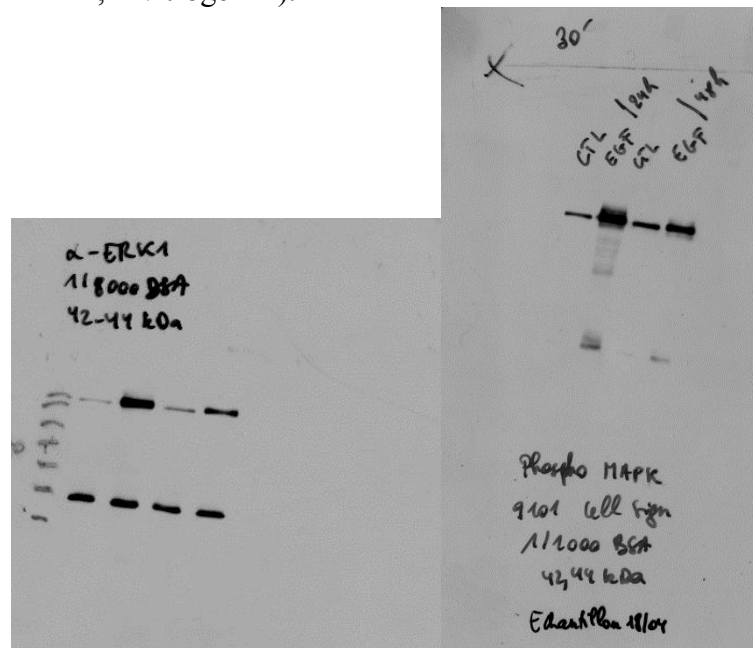
Procedure A:

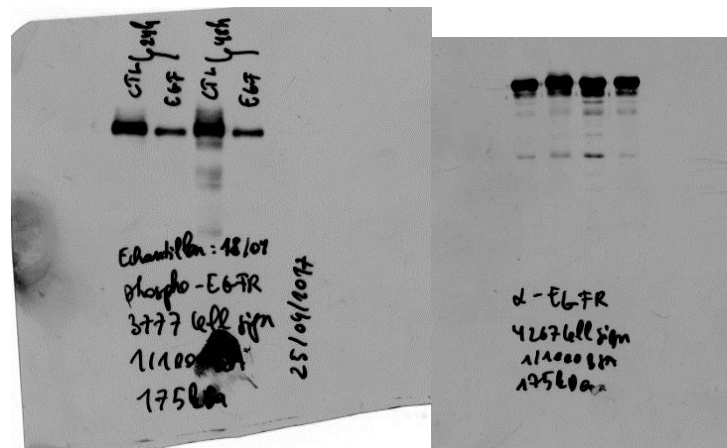
To examine EGF-stimulated phosphorylation of proteins, confluent tumour cells were cultured in medium supplemented with 10% FBS for 24 h. The cells were exposed to 20 ng/ml EGF for 10 min at 37 °C. The cells were then rinsed with ice-cold PBS and lysed in Ripa buffer. The cell lysates were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA). After transfer, the blots were incubated with blocking solution and probed with various antibodies followed by washing. Proteins were visualized with HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (CL-XPosure™ Film, Invitrogen™).



Procedure B:

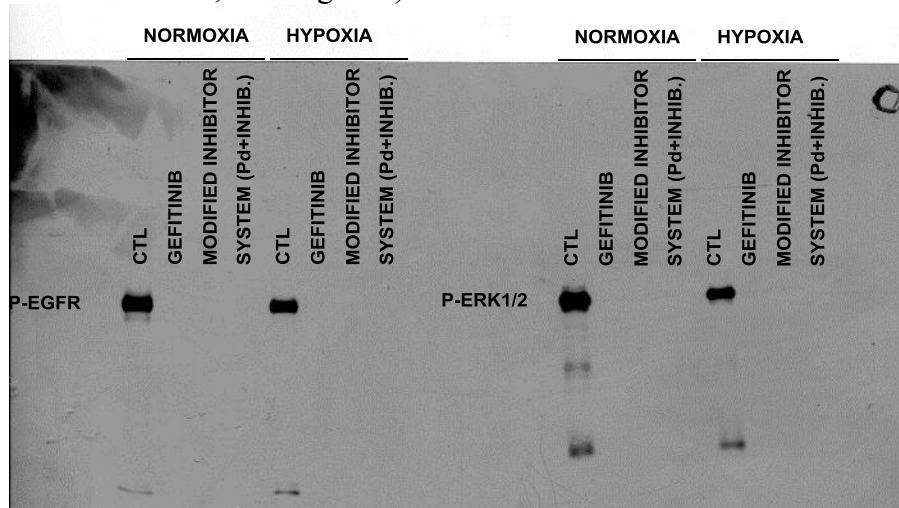
To examine EGF-stimulated phosphorylation of proteins, confluent tumour cells were cultured in serum-free medium for 24 and 48 h. The cells were exposed to 20 ng/ml EGF for 10 min at 37 °C. The cells were then rinsed with ice-cold PBS and lysed in Ripa buffer. The cell lysates were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA). After transfer, the blots were incubated with blocking solution and probed with various antibodies followed by washing. Proteins were visualized with HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (CL-XPosure™ Film, Invitrogen™).





iii Western Blot Analysis

To examine EGF-stimulated phosphorylation of proteins, confluent tumour cells were cultured in serum-free medium for 24 h in normoxic and hypoxic conditions. The cells were pre-treated with **Gefitinib**, **PdA01** and **(Z)-Joelinib** in serum-free medium solutions at concentrations ranging from 1 μ M up to 10 μ M for 1 h before exposure to 20 ng/ml EGF for 10 min at 37 °C. The cells were then rinsed with ice-cold PBS and lysed in RIPA buffer. The cell lysates were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA). After transfer, the blots were incubated with blocking solution and probed with various antibodies followed by washing. Proteins were visualized with HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (CL-XPosure™ Film, Invitrogen™).



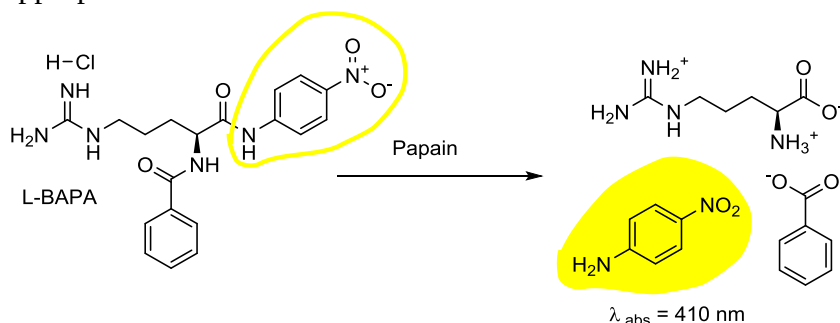
b) Cell Viability Assay

Presto Blue® Cell Viability Reagent (Invitrogen™) was used to evaluate cytotoxicity A431 cell lines. 200 μL aliquots of exponentially growing cell suspension ($6\text{--}8 \times 10^3$ cells) were seeded into 96-well plates, and 24 h later, various concentrations of **Gefitinib**, **PdA01** and **Joelinib** in serum-free medium solutions at concentrations ranging from 1 μM up to 10 μM were added. After incubation for 24 h at 37 °C, 100 μL of Presto Blue® reagent was added and fluorescence measured with a Multilabel counter (VICTOR™ X Multilabel Plate Reader). Each experiment was performed in three replicate wells for each drug concentration.

3.4.2.2 Celinib

a) Description of the papain assay

In this project, the papain activity was assayed using a chromogenic substrate, which was suitable for papain, trypsin and many other proteases. The 4-nitroaniline which was released has an absorbance maximum at 410 nm (in the purple range) and can thus be detected at that wavelength in a 96-well plate with a plate reader with an appropriate filter.



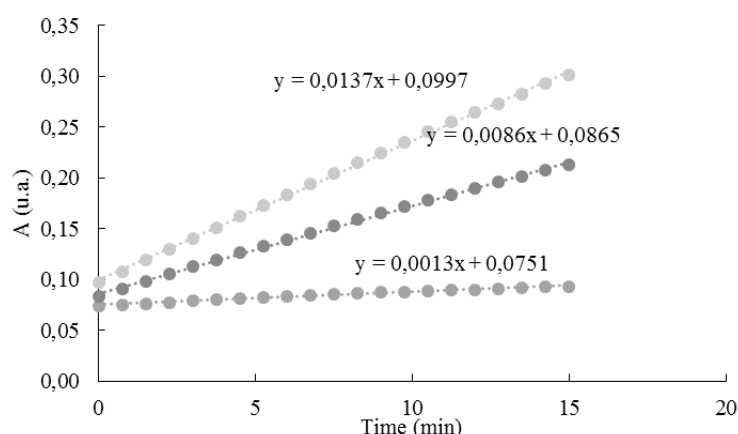
b) Development of the method

The method was first developed in absence of an inhibitor to test the activity of the papain and our ability to detect it. The following stock solutions were prepared:

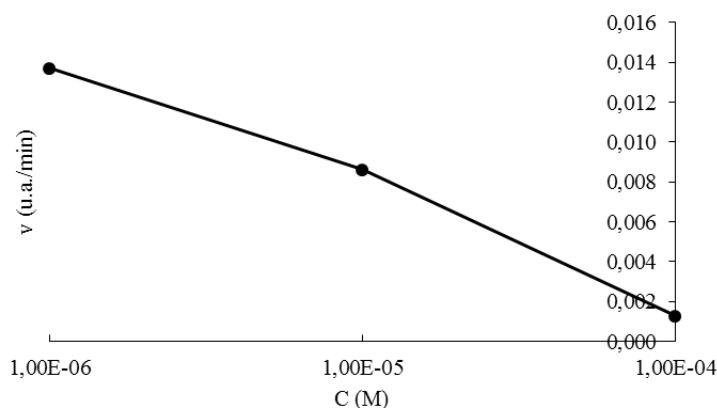
- Papain: 62.4 mg in 10 mL of pH 4.5 acetate buffer ($C = 0.05 \text{ M}$). At this pH, the papain is inactive, and it prevents autodigestion. Later, it was found that it represents roughly 10^{-4} M , which is enormous for a protein assay.
- L-BAPA was solubilized in water (4.3 mg , 10^{-3} M).

- Activity buffer (10^{-4} M sodium phosphate, $2 \cdot 10^{-3}$ M EDTA, $2 \cdot 10^{-3}$ M DTT)

In a papain activity assay, papain (65 μ L of stock solution) was introduced in a well of a 96-well plate. Then, 85 μ L of activity buffer was added, followed by the substrate (50 μ L of stock solution). The absorbance was followed at 410 nm for 15 min, during which it was found to be linear.



The slope corresponded to the activity of the papain in absorbance unit per minute.



Later, as it was found that EDTA and DTT would interfere with the reaction. They were simply omitted, and the rest was prepared in a similar way.

c) Evaluation of Celinib and the reference inhibitor

In a typical assay, a stock solution of **Celinib** and the reference inhibitor were prepared in DMSO. A well of a 96-well plate was filled with the inhibitor (20 μ L of stock solution), activity buffer (65 μ L)

and papain (65 μL). The solution was incubated at 37 °C for 10 min, after which the substrate (50 μL) was added and the absorbance was followed at 410 nm for 15 min, during which it was found to be linear. The slopes were extracted from each curve with the LINEST function and were plotted in function of the concentration in logarithmic scale. The IC_{50} was determined by calculating where the curve intersects the 50% threshold.

d) Evaluation of the catalysts

In a typical assay, the following catalysts were prepared in stock solutions following the plan:

Compound	m (mg)	V (μL)	C (M)
Celinib (20 μL)	2.2	691	10^{-2}
Reference inhibitor	1.9	597	10^{-2}
Celinib (10 μL)	2.7	424	$2 \cdot 10^{-2}$
PdA01	1.3	854	$2 \cdot 10^{-3}$
Ru Meggers	2.1	276	$20 \cdot 10^{-2}$
PdB07	2.2	1160	$2 \cdot 10^{-3}$
PdB03	2.3	1346	$2 \cdot 10^{-3}$

In a typical assay, a stock solution of **Celinib** and the reference inhibitor were prepared in DMSO. A well of a 96-well plate was filled with the inhibitor (20 μL of stock solution) the catalyst (20 μL), activity buffer (65 μL) and papain (65 μL). The solution was incubated at 37 °C for 30 sec to 2 h, after which the substrate (50 μL) was added and the absorbance was followed at 410 nm for 15 min, during which it was found to be linear.

The slopes were extracted from each curve with the LINEST function and were plotted in function of the concentration in logarithmic scale. The IC_{50} was determined by calculating where the curve intersects the 50% threshold.

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