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# Novel Phenoxazine Dyes: Laccase-Mediated Synthesis, Physico-Chemical Properties and Applications

February 2010

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## Acknowledgements

This work would not have been possible without the support of many people. First of all I would like to thank my supervisor, Prof. J. Marchand-Brynaert, who welcomed me again, after 3 years of an insightful journey in the pharmaceutical industry, and gave me the opportunity to perform a research work in the extremely interesting and challenging domain of biocatalysis. Without her precious confidence, constant support and helpful guidelines, this work would have never seen neither the beginning nor the end.

I have a special thought for the Dr. Sophie Vanhulle and for her family. Sophie Vanhulle, unfortunately deceased in September 2009, had a vision which resulted in the SOPHIED project that she initiated and in which she kindly welcomed me. Without this research project, this thesis would not have been possible.

I also thank my committee members, Prof. Gohy, Prof. Iacazio, Prof. Devillers, Prof. Courtoy, Prof. Elias and the Prof. Robiette for the helpful suggestions. During this project I had the chance to work with a number of competent, kind and very helpful peoples that I would like to specially thanks: Ludovic Billottet, Ludovic D'Auria, Estelle Enaud and the members of the Microbiology Unit, Van Tran-Trieu for training me in the science of HPLC, Bernard Tinant for his insightful help in X-ray structures analysis, Sabrina Devouge and Chantal Delaet for who I'm deeply indebted for their constant help in administrative tasks. All the NMR team, i.e. Cecile Leduff, Alain Jancquart, but also the 'mass team', i.e. Raoul Rozenberg and Alexandre Spote, for their help and training in the LC-MS/MS$^2$ analysis.

I would like to thanks also the members of the group ORGJ: “the old ones” yet younger than myself for their helpful discussions, their energy and joy and the all fun activities we shared outside the lab. This goes especially for Frédéric, Benoit, Vincent, Martin and Xavier. I also thank the younger ones for cheering the lab atmosphere, sometime at their expense.

I thank my apprentice, Murielle and a former co-worker Olivier who did learn me how to teach to “younger one”.

A special thought goes to my family, especially my parents for giving me the love of science and their constant support, but also to my friends for their support and their affection.

# RESUME

Les Laccases font partie de la famille des oxydases à cuivre, enzymes principalement impliquées dans de nombreuses voies de biosynthèse chez les champignons. Parmi les isozymes répertoriés, une laccase a permis de réaliser avec succès la formation in vitro d'un pigment d'origine naturelle dénommé « acide cinnabarinique ». Ce composé comprend un motif hétérocyclique identique à celui du groupement chromophore de l'Actinomycin D qui s'avère être un puissant agent antitumoral d'origine fongique. Le motif hétérocyclique commun dénommé 2-amino-*3H*-phenoxazin-3-one, possède un potentiel avéré pour une application telle que le marquage par fluorescence dans le domaine de la biologie cellulaire et/ou moléculaire. Une excellente solubilité en milieux aqueux représente une caractéristique incontournable pour de telles applications biologiques. Dés lors, de nouvelles voies de synthèses présentant simultanément originalité et efficacité doivent être continuellement élaborées afin de produire de nouveaux marqueurs fluorescents hydrophiles inspirés de pigments naturels.

Dans le cadre d'un projet de recherche européen nommé SOPHIED, nous avons élaboré la synthèse biomimétique de nouveaux colorants de type aminophenoxazinone possédant une solubilité en milieux aqueux modulable grâce aux groupements fonctionnels présents sur ceux-ci. Notre voie de synthèse implique une étape clé de cascade oxydante pour un total de six électrons initiée par un bio-catalyseur, une laccase. Nos blocs de constructions, des molécules de type aminophénols présentant des groupements fonctionnels dérivés d'acides sulfoniques et phosphoniques, ont été synthétisés avec un contrôle total de la régio-sélectivité. Ces substrats hydrophiles ont été transformés par une laccase en nouveaux colorants fluorescents avec une complète sélectivité et d'excellents rendements.

Une investigation des potentialités et limitations de notre synthèse bio-catalytique a permis de suggérer une proposition mécanistique pour la cascade oxydante, principalement une variante de celle communément acceptée pour la production de pigments naturels. Néanmoins, bien que cette proposition ait le mérite d'expliquer certains de nos résultats plutôt surprenant, elle reste une suggestion et en aucun cas une quelconque affirmation. Les propriétés de fluorescence de ces composés hétérocycliques ont été évaluées et testés comme marqueurs fluorescents de structures cellulaires vivantes. Grâce à cette application, un de ces hétérocycles s'est révélé prometteur en tant que traceur pour endocytose avec une localisation intracellulaire finale dans les lysosomes. Finalement, la découverte accidentelle d'une cyclisation intramoléculaire due à un effet activant des groupes fonctionnels acides, type sulfonique et phosphorique, dans nos substrats aromatiques à mené à une brève investigation d'une voie alternative de synthèse de benzoxazoles car ce motif structural est largement représenté dans diverses molécules bioactives d'origine naturelle ou synthétique.

# ABSTRACT

Laccases are members of the multicopper oxidase family which are involved in numerous biosynthetic pathways in fungi. Among these, one laccase has been shown to produce in vitro a natural pigment, namely the cinnabarinic acid, which is related to the actinomycin chromophore. The core structure of this chromophore, i.e 2-amino-3H-phenoxazin-3-one, was found to possess a marked potential for the fluorescent labelling in the field of molecular biology. As water-solubility is one of the critical features for biological application, efficient and original synthetic routes are needed to provide new hydrophilic fluorophores inspired from natural pigments.

**In the frame of the European research project SOPHIED, we have investigated the biomimetic synthesis of novel aminophenoxazinone dyes featuring tuneable water-solubility. Our synthetic route involves a key step of biocatalysed oxidative cascade with a total of $6e^-$ oxidation mediated by a laccase. Sulfonylated and phosphorylated aminophenol building-blocks were synthesised with a total regioselectivity control and further processed as water-soluble substrates by our biocatalyst to afford novel fluorophores.**

The investigation of the scope and limitations of our biocatalytic process led to a new mechanistic proposal for the key step of oxidative cyclisation initiated by the laccase. Fluorescent properties of these heterocyclic scaffolds were evaluated and further assayed in a live-cell imaging application. This results in the discovery of a promising fluorophore, presumably a bulk endocytic tracer with targeting by default to lysosomes. Lastly the serendipitous discovery of an intramolecular cyclisation due to a neighbouring substituent effect of the acidic group ($SO_3H$ and $PO(OH)_2$) in our aminophenol derivatives led to the development of an alternative synthetic route towards novel benzoxazole derivatives of potential interest in medicinal chemistry.

## Abbreviations list

2-HMA : 2-hydroxymetanilic acid

3ASA : 3-aminosalicylic acid

3HAA : 3-hydroxyanthranilic acid

3HOA : 3-hydroxyorthanilic acid

aa : amino acid

ABTS : 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid

Acac : Acetyl acetonate

AcOEt : Ethyl acetate

Actinocin : derivatives with the same chromophore as actinomycin D

AIBN : Azo-bis(1-amidinopropane)

APCI : Atmospheric pressure chemical ionisation

APX : 2-aminophenoxazine

Aq : Aqueous

ATR : Attenuated total reflection

Bn : Benzyl

BTIB : bis(trifluoroacetoxy)iodobenzene

BuLi : Butyllithium

CA : cinnabarinic acid

CAN : Ceric ammonium nitrate

CHO : Chinese Hamster Ovary

CI : Chemical ionisation

CV : Cyclic voltametry

CVs : Cyclic voltamograms

DBU : 1,8-Diazabicyclo[5.4.0]undec-7-ene

DCE : Ethylene chloride

DCM : Methylene chloride

DDQ : 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone

DEPTQ : Distorsionless Enhancement by Polarization Transfer Including the Detection of Quaternary Nuclei

DIEA : Diisopropylethylamine

DME : 1,2-dimethoxy ethane

DMEM/F12 : Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12

DMF : Dimethylformamide

DMG : Directing metallation group

DMSO : Dimethylsulfoxide

DMVP : Dimethylvinylphosphonate

DPPA : diphenyl phosphoryl azide

DPQ : Diphenoquinone

EI : Electronic Impact

Eq : Equivalents

ESIPT : Excited state electron proton transfer

ET : Electron Transfer

EtOH : Ethanol

FT-IR : Fourier transformed infrared

GFP : Green Fluorescent Protein

GSA : guaiacol sulfonic acid

Hbpx : 2-(2’-Hydroxyl-phenyl)benzoxazole

HMBC : Heteronuclear Multiple Bond Correlation

HMQC : Heteronuclear Multiple Quantum Coherence

HPLC : High performance liquid chromatography

HRMS : High resolution mass spectrometry

i-PrOH : 2-Propanol or isopropanol

$K_m$ : Affinity constant (mol/L) in the Michaelis-Menten equation

LAO : Laccase Orange Dye

Litmus : mixture of dyes isolated from lichens

LLCT : Ligand to ligand charge transfer

LMS : Laccase mediated system

M : Molar (mol/L)

M W : Molecular Weight

MB : Methylene Blue

MCO : Multicopper oxidase

MNK : Miyazaki-Newman-Kwart

MS : Mass spectrometry

MSA : Methanesulfonic acid

NIR : Near-Infrared

NMP : N-Methylpyrrolidone; solvent

NMR : Nuclear magnetic resonance

NRU : Neutral Red Uptake

Orcein : mixture of dyes isolated from natural source

PBS : Phosphate buffered saline

PDA : Photodiode array

PM3 : Parameterized Model number 3

PMF : Potential Mean Force

PMP : para-methoxyphenyl

PPO : Polyphenol oxidase

RP18 : Reverse Phase C18

STTAC : Sulfur trimethylamine complex

$TBABF_4$ : Tetrabutylammonium tetrafluoroborate

TEA : Triethylamine

TFA : Trifluoroacetic acid

TfOH : Trifluoromethanesulfonic acid

THF : tetrahydrofuran

TLC : Thin layer chromatography

TMEDA : N,N,N',N'-Tetramethylethylenediamine

TMS : Trimethylsilyl

TMSOTf / DMS : Trimethysilyl trifluoromethane sulfonate / Dimethyl sulfide

TSTU : (O-(N-Succinimidyl)-1,1,3,3-tetramethyl uranium tetrafluoroborate

TvL : *Trametes versicolor*

XYD : 2,5-dimethylaniline

$\varepsilon$ : Molar extinction coefficient

$\lambda$ : wavelength (nm)

$\Phi_F$ : relative quantum yield of fluorescence

ClogP (Calculated logP) : ClogP is the calculated log octanol/water partition coefficient, which is a measure for the lipophilicity of a compound. The logP is an important physicochemical parameter for oral absorption, since it relates to solubility and influences the ability of a compound to permeate through cell membranes including those of the intestinal epithelial cells. Too hydrophilic compounds (negative logP) are not able to pass through membranes, as they hardly enter the hydrophobic interior (mimicked by the octanol phase in the octanol/water system) of the lipophilic bilayer.

## Brief description of the Laccases used

In the following manuscript, we will report experimental results which were obtained with three different laccases. Each one of them was chosen for specific reasons which are hinted below. Furthermore their known biochemical properties, commercial or academic sources, purity are briefly summarised.

Laccase [EC 1.10.3.2]

**Laccase TvL1 or Laccase Bioscreen**,

Commercial source:

Bioscreen is a commercial laccase from *Trametes versicolor*, purchased from BIOSCREEN, Germany, and delivered under the commercial trademark Oxizym LA – batch N° LA 2000/001

Purity: unknown as the specific activity of the enzyme preparation

Optimal pH : 3 Optimal Temperature : 55°C

Km (for ABTS oxidation) : 30.8

Activity (unit/liter or $U.L^{-1}$) : one unit is defined as the amount of enzyme that oxidises 1µmol of ABTS (4mM) per minute in tartaric buffer (100mM) at pH 4.5 at 25°C.(Enaud *et al*, *Dyes and Pigments*, **2010**, *85*, 99-108).

Remark: The Bioscreen laccase has been shown to possess similar biochemical properties as the major isozyme TVB (ref 119b in chapter 1) suggesting that the commercial enzyme Bioscreen could be an inducible form of laccase as TVB (ref 176 in chapter 5)

**Laccase TvL2**

Commercial source:

Laccase Tvl2 was purchased from SIGMA-ALDRICH, Belgium under the trademark of 38429-Laccase from Trametes versicolor.

Cas : 80498-15-3

Specification:

Biochemika powder, light brown, ≥ 0.5 units/mg (stored temp. 2-8°C)

Activity: 1 unit is defined as the amount of enzyme which converts 1µmol catechol per minute at pH 6.0 and 25°C. (SIGMA-ALDRICH.com)

**Laccase PS (*Pleurotus sajor caju*)**

Academic source:

**Laccase PS** was produced and used by Professor A. Rescigno from the Università di Cagliari, Dipartimento di Scienze e tecnologie Biomediche, Cittadella Universitaria, 090402 Monserrato (CA), Italy.

The laccase was partially purified following published procedures.(see chapter 4, ref 28,29, 40, 41)

Activity: 1 unit was defined as the amount of enzyme which converts 1 µmol of syringaldazine (50µM) in a buffered solution (50 mM $Na_2PO_4$) at pH 6.5 at 25°C.

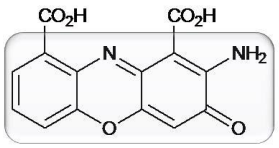
Optimal pH : 6.5 Optimal Temperature : unknown

Bibliographical reference (genetic): Hoegger et al, *FEBS journal* **2006**, *273*, 2308-2326.

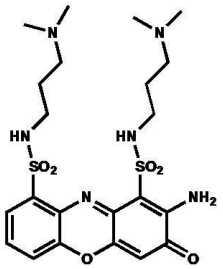
## Introductory note

Fungal laccases are extracellular oxidases mainly involved in several detoxification processes and the production of secondary metabolites having interesting physico-chemical properties. Originating from the tryptophan metabolism, the natural pigment named cinnabarinic acid (**1**) formally comes from the oxidative dimerisation of a toxic metabolite which is mediated by a laccase (Figure 1). The planar heterocyclic framework, namely the 2-amino-*3H*-phenoxazin-3-one (**APX**, in box), have attracted a special attention as a lead for the development of novel synthetic dyes and fluorophores. A major drawback still lies in the poor solubility of natural pigments derived from the 2-amino-3H-phenoxazin-3-one scaffold as much as the poor water-solubility of synthetic fluorescent dyes related to that scaffold. As such, the development of novel water-soluble fluorophores, derived from the natural framework **APX**, holds a great potential as promising alternative for creating a new class of aminophenoxazinone dyes. Through this manuscript, we will describe how to insert hydrophilic functional groups in the **APX** framework in order to modulate the final water-solubility (said in the manuscript as tuneable water-solubility). From the created library of dyes, one of them (**in bold**) gave interesting results as a fluorescent marker in biological media. Let us first introduce the reader with the field of fluorescent dyes derived from natural and synthetic frameworks.

Figure 1. 2-amino-*3H*-phenoxazin-3-one scaffold




Cinnabarinic acid



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# CHAPTER 1. INTRODUCTION

## 1.1 How light and colour shape the world

"You can observe a lot by watching". The quote was cleverly used by Martin Chalfie at the beginning of his Nobel lecture after Osamu Shimomura, Roger Y. Tsien and himself were awarded with the Nobel prize in chemistry of 2008. Their involvement in the discovery and development of the green fluorescent protein (GFP) indeed paved the way to improvement of the non invasive imaging of cells physiology.[1]

Truth it is, the natural world conceals complex and fascinating life forms among which coral reefs provide the most spectacular natural colours. Colours result from the reflection of different wavelengths of visible light, and they cannot be detected in total darkness.[2] Coloured life-forms contain small organic molecules responsible for their appearance, the so-called natural pigments. More precisely, in biology, a pigment is defined as any material producing colours in plant or animal cells, and is indeed the result of selective absorption. The chemist will mostly define those pigments as compounds with absorptions in the UV-A and UV-B regions, and most importantly in the visible region. In addition, the chemist will use, for an identical compound, the term of pigment when the molecule is not dissolved into a vehicle (i.e. a solvent) or dye when dissolution is achieved.[3] These coloured molecules can be classified according to the chemical nature of the chromophore group they contain.[3]

[1] Shimomura, O.; Chalfie, M.; Tsien, R. Y., *Angew. Chem. Int. Ed*. **2009**, 31, 5555.

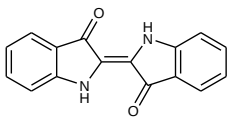
[2] Zollinger H., *Color Chemistry: Syntheses, Properties and Applications of Organic dyes and pigments*, 2nd edition, edn. VCH publishers, New York, **1991.**

[3] Riegel E.R., *Riegel's handbook of industrial chemistry*, 9th edition, edn. New York (N.Y.), **1992.**

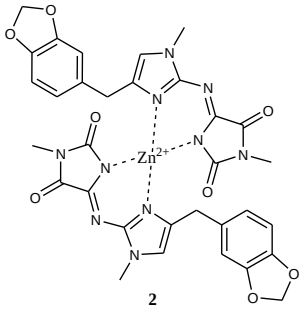
### *1.1.1. The chromophore*

A chromophore is a molecular framework where the energy difference between the frontier orbitals (HOMO and LUMO) falls within the range of the visible spectrum. Visible light that hits the chromophore can thus be absorbed by exciting one electron from its ground state into an excited state. A brief review of the basic principles of light absorption and emission can be found in the supporting material of the manuscript. Chromophores almost always arise in one of two possible forms: conjugated π systems and metal complexes (Schem 1).[2] In the former, the energy levels (that the electrons jump between) are extended π orbitals created by a series of alternating single and double bonds, often in aromatic systems (e. g. indigo **1**). The metal complex chromophores often arise from the splitting of d-orbitals by binding of a transition metal to ligands (e.g. clathridine **2**). Two molecules, indigo and chlorophyll, are the most emblematic ones for the colours blue and green respectively and are still the subject of intense research.[4] As expected, chemists have tried for a long time now to reproduce synthetically those natural pigments.

Scheme 1. Indigo (**1**) and Clathridine Zn-complex (**2**)



**1**



**2**

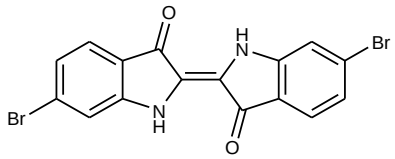
[4] a) Sexias de Malo, J. S.; Burrows, H. D. ; Serpa, C.; Arnaut, L.G., *Angew. Chem. Int. Ed*. **2007**, 46, 2094-2096. b) Ciba Foundation Symposium 61, *Chlorophyll Organization and Energy Transfer in Photosynthesis*., Book Series: Novartis Foundation Symposia, **2008**.

### *1.1.2 The colour industry*

#### *1.1.2.1 Historical background*

Until recently, the study of natural pigments was empirical and often the "chemistry" of the pigments was poorly described.[13] Still, natural pigments were at the origin of the very first colour industry. The use of fine purple dye for instance has a long history. The ancient "Tyrian Purple", described by Reaumur in 1771 was made from the collected fluid of dying molluscs, especially the *Murex trunculus*, *Mitra* and *Purpura* species. The Phenicians were the first to create an industry for its extraction, and the Babylonians to use the dye. The Tyrian Purple dye was used as a dye for centuries until it was identified as 6,6'-dibromoindigo **3** and synthesised (Figure 2).[14] Noteworthy, the precursor of **3** exists in the molluscs as a colourless precursor which, when exposed to the sunlight (UV light), is transformed into the purple species by a sulfatase enzyme. In other word, this is already a natural example of a so-called "biocatalytic transformation".[15]

Figure 2. Tyrian Purple Dye (**3**)

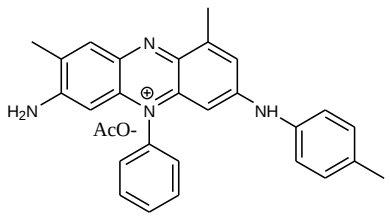



**3**

[13] de Reaumur, R. A., *Mem. Acad. R. Sci*. **1771**, 118.
[14] a) Friedlandler, P.; *Monatsh. Chem.* **1907**, 28, 991. b) Friedlandler, P., *Ber. Dtsch. Chem. Ges*. **1909**, 42, 765.
[15] Fouquet, F.; Bielig, H. J.; *Angew. Chem. Int. Ed*. **1971**, 10, 816-817.

In 1856, a young chemist named William Henry Perkin (1838-1907) tried to synthesise the quinine with the aim to provide a synthetic drug to the English troops located in India. Using *N*-allyltoluidine as starting material, through oxidative coupling reaction initiated by potassium dichromate, Perkin failed to produce the desired quinine compound. Instead, a new synthetic compound red-brown coloured was isolated.[16] The young chemist had just obtained the first synthetic dye **4** which featured good properties for the dyeing of textiles. Following his discovery, he created the first textile industry in the 19$^{th}$ century and became immensely wealthy (Figure 3).

Figure 3. Revised structure of Mauveine **4** [17]




**4**

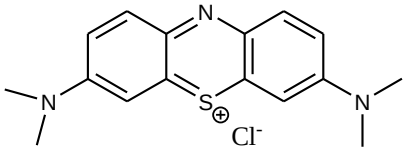
The German chemists were the first ones to follow Perkin's footsteps. Among the research workers who have contributed directly or indirectly to the unique development of the tar-dyestuff industry, the place of honour goes to the Professor at Munich University, Adolf von Baeyer, for his researches on the composition of indigo as well as on the triphenyl methane dyestuffs.

[16] Sousa, M. M.; Melo, M. J.; Parola,A. J.; Morris, P. J. T.; Rzepa; H. S.; de Melo, J. S. S., *Chem Eur J*. **2008**, 14, 8507-8513.
[17] Meth-Cohn, O.; Smith, M., *J. Chem. Soc., Perkin Transactions 1* **1994**, 5.

Indigo, the gorgeous pigment of the indigo plant, has been considered the most important of all organic pigments on account of its beauty and colour fastness. To reproduce the pigment by synthetic methods and make it more easily obtainable was therefore an exceptionally inviting task for chemical research. The Nobel prize winner of chemistry in 1905, succeeded in producing indigo synthetically in three principal ways, namely from *ortho*-nitrophenylacetic acid, from *ortho*-nitrocinnamic acid and from *ortho*-nitrobenzaldehyde and acetone.[18] This paved the way for the production of indigo from raw material obtainable without much difficulty from coal tar.

Historically, a last major masterpiece in dyestuff chemistry was brought by Paul Ehrlich in the late 19$^{th}$ century. Again the Nobel prize winner of physiology and Medicine in 1908 took the dyestuff one step further by demonstrating the use of several molecules in medicinal chemistry. Among other things, he was the first to report the antimalarial action of methylene blue (**MB**) **5** (Figure 4). [19]

Figure 4. Methylene Blue **5**




**5**

[18] *Nobel Lectures, Chemistry **1901-1921***, Elsevier Publishing Company, Amsterdam, **1966**.

[19] Atamna, H.; Krugliak, M.; Shalmiev, G.; Deharo, E.; Pescarmona, G.; Ginsburg, H., *Biochem. Pharmacol*. **1996**, *51*, 693-700.

#### *1.1.2.2 Challenge of the dyestuff industry today*

The mention of the colour industry usually leads to the deceptive view of a chemical industry producing textile dyes and dyes for the food industry. Likely, everyone knows that coloured molecules are actually present in any synthetic material today. Yet the dyestuff industry also produces numerous dyes for the field of molecular biology, histochemistry and medicinal chemistry. As for the food industry, those last three fields point out more acutely the problem of dye purity.[20]

The problem of dye purity and toxicity has partially been tackled by the European Commission which has set up a new law enforcement (Directive 2002/61/CE 19 July 2002). This mostly focuses on dyes which produce upon cleavage one or more aromatic amines beyond a concentration of 30 ppm.[21] To date, only 5% of the whole azoïc dyes are concerned by the E.U. directive and have been removed of the E.U. market. Obviously the synthetic dyes purity and toxicity problem apply to the whole pool of dyes and beside the azoïc ones, all of them are submitted to in-vitro toxicological assays to determine their hazard potential.[22]

[20] a) Merck & Co. Merck Index, Encyclopedia, Inc. edn. **2001.** b) Lyon, H., *Biotech. Histochem*. **2002**, *77*, 57-80.

[21] EPA Office of Compliance, Notebook Project: Profile of the Textile Industry. **1997**.

[22] a) Gottlieb A.; Shaw C.; Smith A.; Wheathley A.; Forsythe S., *J. Biotechnol.* **2003**, 101, 49-56. b) Riu, I.; Schönsee, I.; Barceló, D.; Ràfols, C., *Trends in Anal. Chem*. **1997**, 16, 405-419.

### *1.1.2.3 Dyes classification*

Whatever their applications are, the dyestuff are commonly classified either depending of the chromophores they contain or depending on the material they are applied to. Whereas several recent dyes mostly used in the biological field (e. g. BODIPY dyes)[23] are only found using the first (structural) classification, most of the dyes falls in one of the groups defined by the second classification (dyeing application).[24] Noteworthy, in 1974 the Biological Stain Commission concluded that the dyes used in cytology and biomedicine were not fine chemicals, but usually mixtures of variable and often undefined composition.[25] More than 10 years ago the US department of Health and Human Services, Food and Drug Administration (FDA) has published rules to classify/reclassify analyte specific reagents and immunohistochemical reagents.[26]

Structurally the most representative groups are the azoïc, stilbene, nitro and nitroso dyes but also heterocyclic cores such as anthraquinone, acridine, indigoïd, thiazole, thiazine, oxazine, quinoline, dipyromethene, triarylmethane and phtalocyanine are found.[23,24]

[23] a) Loudet, A.; Burgess, K., *Chem. Rev*. **2007**, 107, 4891–4932. b) Gonçalves, M. S. T., *Chem. Rev*. **2009**, *109*, 190–212.

[24] a) Abrahart, E. N., Dyes and their intermediates, 2nd ed. Edward Arnold, London, **1977**, 236-237,. b) Venkataraman K., The chemistry of synthetic dyes I. **1952**.

[25] Mowry, R. W.; Kasten, F., *Stain Technol*. **1975**, 50, 64-81.

[26] a) Federal Register, **1996**, Proposed rules to classify/reclassify analyte specific reagents. 61, N°. 051. b) Federal Register, **1996**, Proposed rules to classify / Reclassify immunohistchemical reagents and kits. 61, N° 116.

Beside the azoïc ones, the major class of dyes which will be of interest in the following part of this manuscript is the class of so called "acid dyes".[24] *Acid dyes* are water-soluble anionic dyes that are applied to fibres such as silk, wool, nylon and modified acrylic fibres using neutral to acid dyebaths. Attachment to the fibre is attributed, at least partly, to salt formation between anionic groups in the dyes and cationic groups in the fibre. *Acid dyes* are not substantive to cellulosic fibres. Most synthetic food colours fall in this category. The same applies for most of the water-soluble dyes used in the field of molecular biology.[23b] Now that we have shed some light upon the colour industry, let us say a few words about its future and current axes of research.

#### *1.1.2.4 Field of research today*

Among the current fields of research about dyes, a marked trend appears focusing on a more exhaustive isolation and study of natural pigments.[27,28] This relies upon the logical postulate that nature is more efficient than any chemical process and that among the myriads of coloured molecules produced, a major part should be more easily applied as they are likely to be less toxic than the synthetic products.

[27] Duran, N.; Teixeira, M. F.S.; De Conti, R.; Esposito E., *Crit. Rev. Food Sc. Nutri.* **2002**, *42*, 53-66.
[28] Baker, R. A.; Tatum, J. H., *J. Ferment. Bioeng*. **1998**, *85*, 359-361.

As stated in the beginning, the marine life forms provide a tremendous field of investigations. In the terrestrial life forms, the fungi offer a similar advisableness due to the diversity of species and their marked potential to grow in bioreactors.[29] For all bioactive compounds obtained from fungi, terrestrial or marine organisms, the knowledge of the biosynthesis of the molecules of interest likely ensures an efficient way to scale up their production at a scale useful for the human society.[30] The advantage of fungi is the simultaneous ability of species to detoxify organic compounds through oxidative pathways which ultimately either afford polymers or pigments.[31] The European project SOPHIED[32] which partially funded the experimental work of research described later in this manuscript is an excellent illustration of such current trend.

## 1.2 Nature as a source of inspiration

Education always influences our vision. As a member of a community of chemists involved in the field of organic chemistry at the interface of synthesis, medicinal chemistry and biological chemistry, our tendency is to look at organic compounds with biosynthetic aspects.

[29] Hajjaj, H.; Blanc, P. J.; Groussac, E.; Goma, G.; Uribelarrea, J. L.; Loubiere P., *Biotechnol. Bioeng*. **1999**, *64*, 497-50.
[30] Wesenberg, D.; Kyriakides, I.; Agathos S., *Biotechnol. Adv*. **2003**, *22*, 161–187.
[31] http://www.sophied.net/
[32] a) Jose, J.; Burgess, K., *J. Org. Chem.* **2006**, *71*, 7835-7839. b) Simmonds, A. C.; Miller, J. N.; Moody, C. J.; Swann, E.; Briggs, M. S. J.; Bruce, I., Patent WO 9729154, 1997. c) Wardman, P., *Free Radical Biol. Med*. **2007**, *43*, 995-1022. d ) Umezawa, K.; Nakamura, Y.; Citterio, D.; Suzuki, K., *Chem Eur J.* **2009**, *15*, 1096-1106. e) Peneva, K.; Nolde, F.; Rocha, S.; Hotta, J.-I.; Braeckmans, K.; Hofkens, J.; Uji-i, H.; Herrmann, A.; Müllen, K., *Angew. Chem*. **2008**, *120*, 3420-3423.

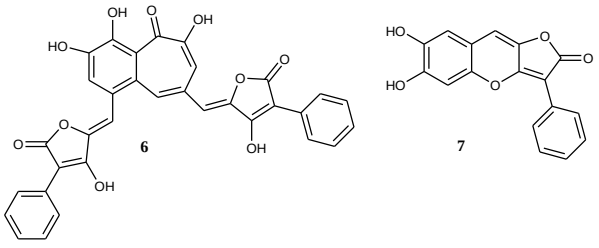
Reviewing here the multitude of chromophores used in the field of biology and imaging of cells would be deceptive as their number increases steadily each day.[32]

Similarly, an exhaustive review of the natural pigments is not achievable here as every year several reviews focus on revised information gained upon the biosynthesis of already known compounds and more recently discovered ones are regularly published.[33] Instead, we would like to expose here several natural structures which are of interest to synthetic chemists and molecular and cellular biologists. Furthermore, several of these will be related to synthetic dyes which are currently the most promising ones due to constant research efforts and their fields of applications.[32]

### *1.2.1. Pigments of fungi*

Pulvinic acids and related butenolide compounds were isolated from the red-caps of *Tricholoma aurantium*. Those are related to the unique red pigment aurantricholone **6**, rather unstable, which is the first naphthalenoid pulvinic acid found so far (Scheme 2).[33] Like other metabolites of this species, **6** occurs as a metal chelate; in this case the predominant counter ion is calcium.

Scheme 2. Auranthricolone (**6**) and Auranthricolide A (**7**)




[33] a) Gill, M., *Nat. Prod. Rep.* **1999**, 16, 301-317. b) Gill, M., *Nat. Prod. Rep.* **2003**, 20, 615-639. c) Gill, M., *Aust. J. Chem.* **2001**, 54, 721–734. d) Bandaranayake, W. M., *Nat. Prod. Rep.* **2006**, 23, 223-255.

Likely more interesting are few cometabolites of **6** found in *Tricholoma aurantium* such as auranthricholide A (**7**) which is intensely fluorescent. Being biogenetically related to compound **6,** the unambiguous structure of **7** has been confirmed by total synthesis.[34]

Through the biotechnology techniques, anthraquinones have been isolated from a number of fungi that are *Trichoderma*, *Drechslera*, *Aspergillus*, and *Curvularia* strains.[35] Biosynthetically related to the polyketides pigments, the anthraquinones, napthoquinones, and naphtopyrans represent the largest classes of natural pigments already used.

Numerous total syntheses have been developed to confirm or revise the proposed structures of compounds isolated either from terrestrial or marine sources.[36] Among those pigments isolated from the *Dermocibe* species, hypericin **8**, a purple quinone compound, has been defined as a photosensitising pigment of great interest (scheme 3).[37] Because hypericin accumulates preferentially in cancer tissues, it is used as an indicator of cancerous cells after injection in the body. Beside several bioactivities, hypericin is now under study as an agent for photodynamic therapy, where a drug is absorbed by an organism to be later activated with spectrum-specific light from dedicated lamps or laser sources. Therefore, synthetic routes toward hypericin and derivatives drive a lot of researches.

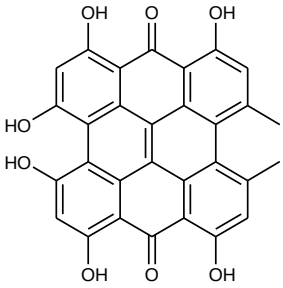
[34] Klostermeyer, D.; Knops, L.; Sindlinger, T.; Polborn, K.; Steglich, W., *Eur. J. Org. Chem*. **2000**, 603 – 609.

[35] Gill, M., *Nat. Prod. Rep.* **1996**, 16, 513-528.

[36] a) Duran, N.; Song P. S., *Photochem. Photobiol*. **1986**, 43, 677-680. b) Falk, H., *Angew. Chem. Int. Ed.* **1999**, 38, 3116-3136.

[37] a) Motoyoshiya, J.; Masue, Y.; Nishi, Y.; Aoyama, H., *Nat. Prod. Commun*. **2007**, 2, 67-70. b) Aigner, S.; Falk, H., *Monatsh. Chem*. **2008**, 139, 991-993.

Scheme 3. Hypericin (**8**)



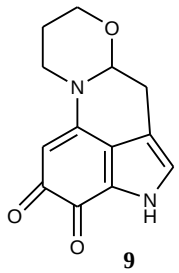

**8**

Among the indolic pigments, the red compound haematopodin **9**, synthesised from 6,7-bis(benzyloxy)indole, is the first pyrroloquinone alkaloid found in a fungus (scheme 4).[33,35] Haemotopodin, from *Mycena haematopus* likely arises in the decomposition of the native pigment of the sap. Compounds of this type, which also occur in marine sponges, can be synthesised by intramolecular cyclisation of 6,7-dihydroxy-tryptamine - a potential model for their biosynthesis.[38] Haematopodin occurs only in traces (if at all) in the fresh fruiting bodies of the fungus, and seems to arise from another pigment not identified. If a crude extract is left for some weeks in methanol in a sealed flask exposed to light, it is converted quantitatively into **9**. This led to the assumption that similar groups of tetrahydropyrrolo[4,3,2-de]quinolinequinones resulted also from intramolecular oxidative cyclisation.[39]

[38]. Baumann, C.; Bröckelmann, M.; Fugmann, B.; Steffan, B.; Steglich, W.; Sheldrick, S., *Angew. Chem. Int. ed*. **1993**, 32, 1087-1089. b) Hopmann, C.; Steglich, W., *Liebigs Annalen* 1**996**, 7, 1117-1120.

[39] Peters, S.; Spiteller, P., *Eur.J. Org. Chem*. **2007**, 10, 1571 – 1576.

Scheme 4. Haematopodin (**9**)



**9**

The siderophores are some of the best-known families of strong small chelates. A broad range of molecules, including macrolides and many pigments, with a variety of donor groups capable of metal binding, have been isolated from marine and terrestrial fungi.[40] The donor groups vary from low molecular weight bidentate and multidentate chelates to proteins containing catechol side groups.[41] Pyrrole groups and related nitrogen heterocycles are frequently found in many pigments. They are excellent sources of inspiration for novel synthetic dyes, as shown for the BODIPY ones.

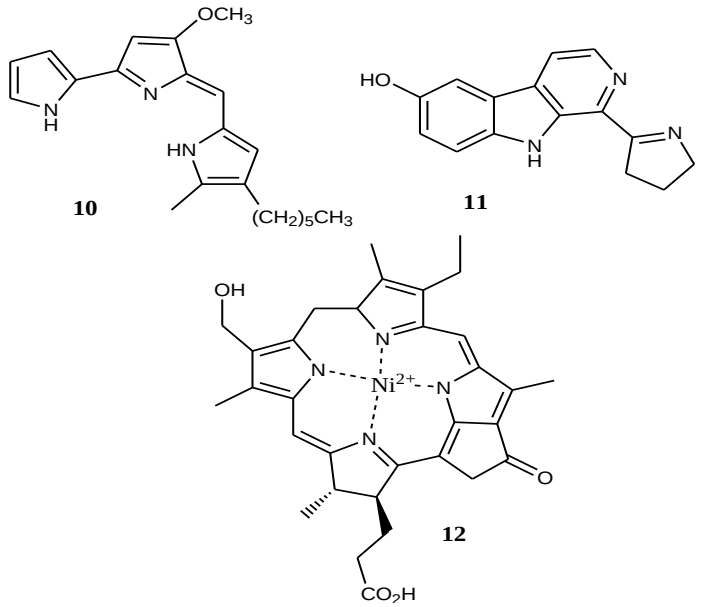
For example, the deeply-red coloured pigment prodiogiosin **10**, has a similar Fe(II) group chelator as the pyrimine one of Eudestomin **11** isolated from a *Pseudomonas* species (scheme 5).[41] Whereas those are isolated without the metal, the blue-green pigment tunichlorin **12** are one of the few compounds to be isolated under the metal-containing complex which is quite exceptional.[43]

[40] Michael, J. P.; Pattenden, G., *Angew. Chem. Int. Ed.* **1993**, 32, 1 (& references cited herein).

[41] Hawkins, J., *Pure Appl. Chem*. **1988,** 60, 1227. (& reference cited herein).

[42] Rescigno, A.; Sanjust, E., *O-aminophenol-type tryptophan metabolites: 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and their role in living organisms Studies in Natural Products Chemistry. In Bioactive Natural Products*, Atta-ur-Rahman, Eds.' Elsevier, **2002,** Volume 26, Part 7, 965-1028.

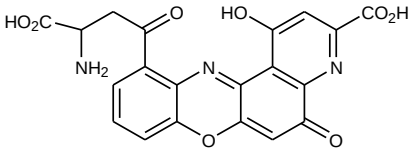
Scheme 5. Selected heterocyclic scaffolds of several siderophores



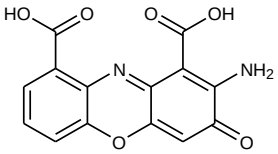

Lastly, let us say a few words about the omonochrome pigments which can be subdivided into two groups from which the yellow, orange, bright red, and brownish-violet, alkali-labile ommatins (e. g. xanthommatin **13**) are the most represented in the animals that also synthesise melanins (scheme 6).[42] The omnochromes are typically insoluble in water and neutral solvents.

They arise biogenetically from the metabolism of tryptophan and are associated with proteins. Related to those heterocycles is the oxazine pigment cinnabarinic acid **14** (scheme 6).[44] Noteworthy, laboratory cultures of the fungus *Plectosphaerella cucumerina* obtained from marine sediments collected in Barkley Sound, British Columbia, yielded recently the novel alkaloid plectosphaeroic acid A (**15**).[43] The molecule has a marked bioactivity as novel indoleamine 2,3-dioxygenase inhibitor.

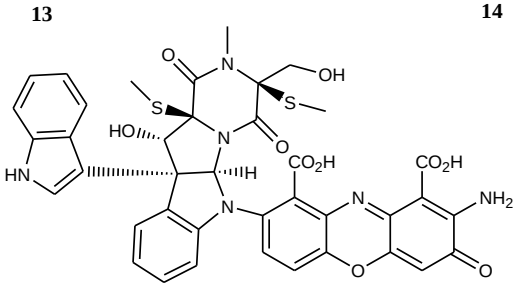
Scheme 6. Omnochromes pigments



**13**



**14**



**15**

### *1.2.2 Synthetic dyes at the heart of researches today*

The use of organic molecules in nonfluorescent labelling, in the ultraviolet and visible regions, is important in several applications. However, in recent years, detection based on fluorescence techniques has received special attention and notable progresses have been made in both fluorescence instrumentation and synthesis of new fluorophores.[7,9,10,11,23,32] In addition to the intrinsic physico-chemical interest of new fluorophores, development of fluorophores with absorption and emission at long wavelengths is of extreme importance for biological purposes.[44]

[43] Carr, G.; Tay, W.; Bottriell, H.; Andersen, S. K.; Mauk, A. G.; Andersen, R. J., *Org. Lett*. **2009**, 11, 2996–2999.

[44] a) Boonacker, E.; Van Noorden, C. J. F., *J. Histochem. Cytochem*. **2001**, 49, 1473–1486. b) Yee, D. J.; Balsanek, V.; Bauman, D. R.; Penning, T.; Sames, D., *PNAS* **2006**, 36, 13304–13309.

[45] Barondeau, D. P.; Putnam, C. P.; Kassmann, C. J.; Tainer, J. A.; Getzoff, E. A., *PNAS* **2003**, 10, 12111–12116.

In the previous sections, we have exposed natural pigments which have either inspired directly some of the current synthetic dyes being now developed or have a promising interest due to their likely biosynthetic origin. Indeed, whether or not those last pigments are developed in their synthetic versions, they all involve, like the GFP chromophore, oxidative intramolecular cyclisations.[45] As we will see later on, this should likely be an alternative for the synthesis of novel synthetic dyes, independently of the photo-physical properties wanted today. On the other hand, most of the dyes still failed to offer a tuneable water-solubility which is somehow the Holy Grail for several classes of fluorophores.[46]

#### *1.2.2.1 Xanthenes and derivatives*

Fluorogenic peptidic substrates have emerged as efficient tools for mapping enzymatic activities.[48] Among the commonly used fluorophores are the derivative of C-terminally capped coumarin derivatives. Still the replacement of coumarin dyes remains a challenge.[48] A recent publication reports the excellent synthesis and application of a new derivative of "rhodamine 110" and "2-Me Tokyo green" dye.[49] The new dye, named "Singapore green" allows the easy grafting to a surface of choice with the concomitant linkage of a peptidic moiety. "Singapore green" synthesis is disclosed in the Scheme 7 hereafter. Whereas its synthesis is not so much trivial, the resulting dye possess a quantum yield value of 0.5, with excitation and emission maxima at 494 and 521 nm respectively in water. Those are compatible with the 488 nm conventional argon-ion laser source.

[46] Jose, J.; Burgess, K., *Tetrahedron* **2006**, 62, 11021.

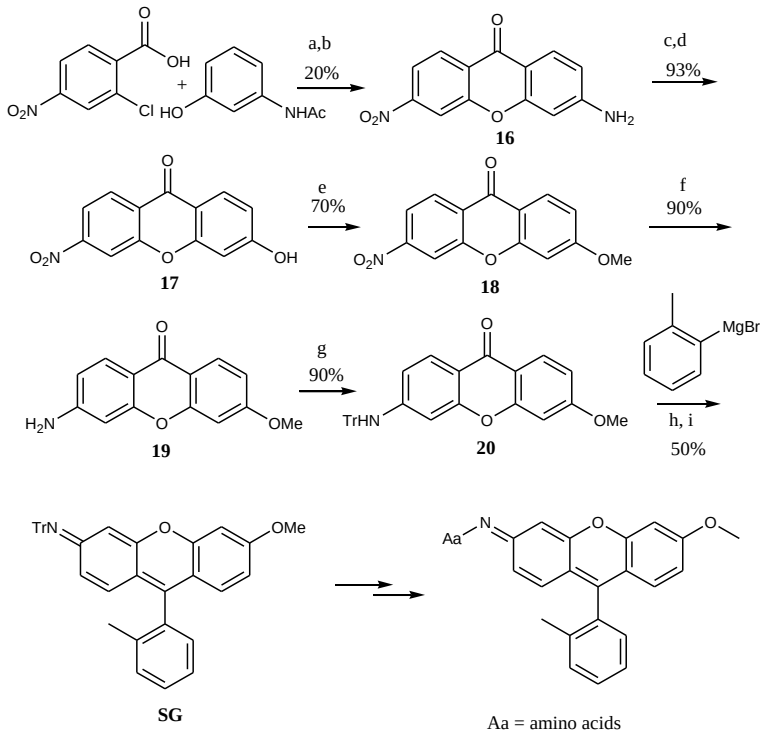
[47] Harris, J. J.; Backes, B. J.; Leonetti, F.; Craik, C. S.; Ellman, J. A., *PNAS U.S.A* **2000**, 97, 7754-7759.

[48] Diamond, S., *Curr. Opin. Chem. Biol.* **2007**, 11, 46-51. (& reference herein)

[49] Li, J.; Yao, S. Q., *Org. Lett.* **2009**, 11, 405-408.

Yet for the application in aqueous solution, the water-solubility will remain the critical drawback. [50]

Scheme 7. Synthesis of "Singapore Green dye"




Reagents. a) $K_2CO_3$, Cu, DMF, 130°C; b) $H_2SO_4$ 80°C; c) $NaNO_2$, 50% $H_2SO_4$, 0°C; d) $H_2O$; e) $K_2CO_3$, DMF, rt, $(MeO)_2SO_2$; f) $H_2$, 10% Pd/C, EtOAc, rt; g) $C(Ph)_3Cl$, $NEt_3$, DCM; h) RMgBr, THF, 50°C, i) DCM, TFA, $H_2O$, rt.

[50] Valdes-Aguilera, O.; Neckers, D. C.; *Acc. Chem. Res.* **1989**, 22, 171-177.

#### *1.2.2.2 Borondipyromethene derivatives*

The development of multicolour fluorescent dyes as standards is expected to significantly contribute to the progress of fundamental and practical technologies. Additionally, NIR fluorescent dyes have been often spotlighted as new revolutionary tools for noninvasive and simple in vivo optical imaging.[51] In this field of research, tailor-made and constricted borondipyromethene derivatives ( Schemes 8 and 9) hold a special place. Inspired by natural pigments derived from pyrrole building blocks, they offer several of the desired properties of the "Holy Grail" of the fluorophores, namely:

- High fluorescence quantum yield (near 1.0)
- Sharp fluorescence spectra (full width at half max height around 500 nm)
- Large molar extinction coefficients (over $10^5$ $M^{-1}cm^{-1}$)
- Ease of tuning the fluorescence emission over a wide spectral range (Vis/NIR).

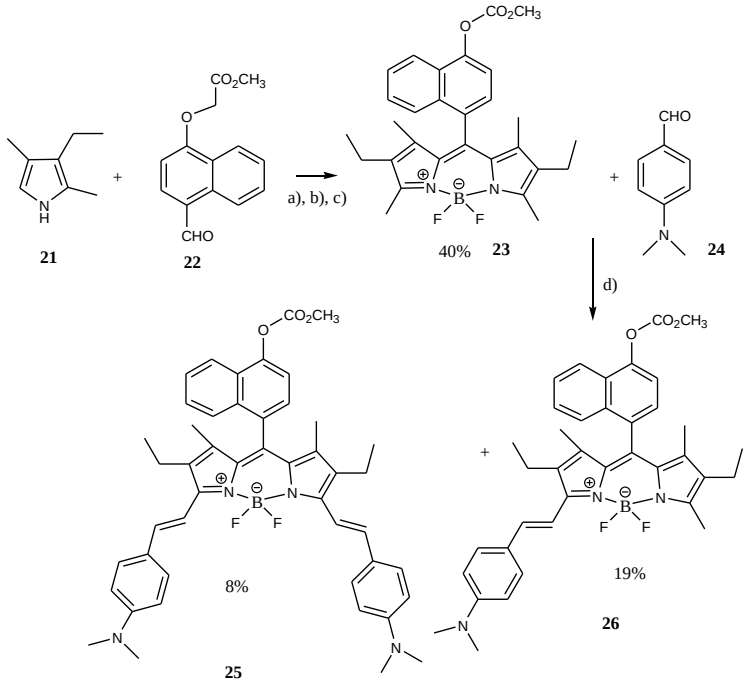
Yet, as stated in the latest published results, the synthetic routes remain disappointing in terms of yields.[51] Whereas the excellent photo-physical properties may level-off such drawbacks, there are still limitations regarding the stability and the water solubility. The use of borondipyrromethene compounds in bioimaging applications requires their grafting to biopolymers.[51, 52]

[51] Weissleder, R.; Tung, C. H.; Mahmood, U.; Bogdanov, A., *Nat. Biotechol.* **1999**, 17, 375-378.
[52] Zheng, Q.; Xu, G.; Prasad, P. N., *Chem Eur J.* **2008**, 14, 5812-5819.

The introduction of a naphthalenyl group on the *meso* position increased considerably the fluorescence quantum yield, whereas the condensation reaction with a benzaldehyde derivative allowed the fine tuning of the absorption and emission wavelengths to the near-infrared visible range (scheme 8).

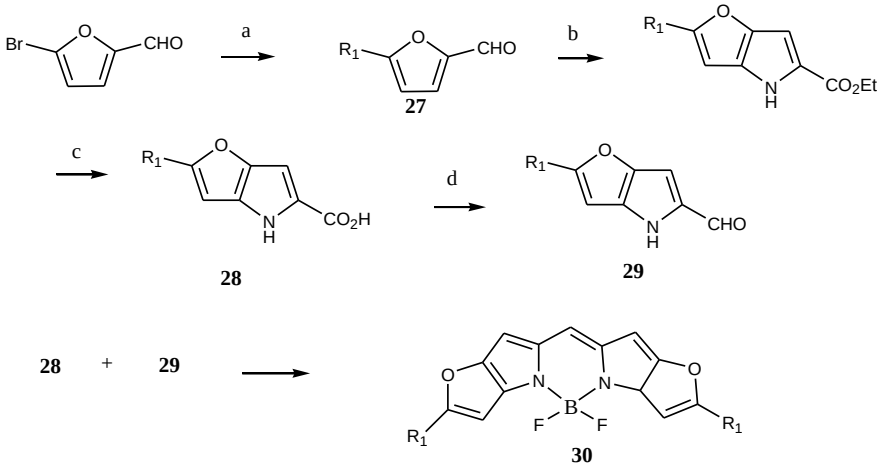
The scheme 9 disclosed the nice synthetic route towards new heteroaryl-fused BODIPY named "Keio Fluors" with anchorage sites for electron-donating and/or withdrawing groups.[32d]

Scheme 8. Synthesis of constricted Borondipyromethene




Reagents; a) TFA, DCM, 12h, rt; b) DDQ; c) $NEt_3$, $BF_3.Et_2O$; d) piperidine, AcOH, toluene, molecular sieves, reflux, 24h

Scheme 9. Keio Fluor BODIPY dyes




Reagents; a) R-$B(OH)_2$, $PdL_2$, $Na_2CO_3$ aq, toluene/EtOH, 80°C; b) $N_3CH_2CO_2Et$, EtONa, EtOH, 0°C, toluene reflux; c) NaOH, EtOH/$H_2O$, reflux; d) TFA, 50°C, $CH(OEt)_3$, 50°C; e) TFA, $POCl_3$, 50°C; $BF_3$ complex, TEA, DCE, 80°C.

Briefly, aryl substitutions were performed in the first step by Suzuki-Miyaura coupling to give intermediates **27**. The furopyrrole 5-carboxylic **28** motif was synthesised by Hemetsberger-Knittel reaction[53] and α-formylation of furopyrrole was performed in the presence of alkylorthoformate and trifluoroacetic acid to give **29**. The title dyes **30** were obtained through α-formylated furopyrroles and α-carboxylated furopyrroles condensation in the presence of trifluoroacetic acid and trichlorophosphine oxide. Using such synthetic route, the authors were able to prepare a set of BODIPY dyes by varying the substituents and hence they covered the whole range of the UV-Visible spectrum.

[53] Gribble, G. W., *J. Chem. Soc., Perkin Trans. 1* **2000**, 1, 1045-1075.

[54] Huisgen, R., *Angew. Chem. Int. Ed.* **1986**, 25, 297-311.

The photo-physical properties are again among the best reported so far. Unfortunately, the breakthrough is levelled off by the almost total absence of water-solubility.

#### *1.2.2.3 Nile Blue derivatives*

In this presentation of the most interesting heterocyclic scaffolds devoted to fluorescence imaging applications, we have begun with the xanthene scaffolds discovered by A. Von Baeyer in 1871 which do not have natural counterparts.[54] As stated before, the BODIPY dyes are directly inspired from natural pigments based on the pyrrole building block. To finish the brief survey, let us say few words about a third class of heterocyclic frameworks which are unavoidable in colouring chemistry. Directly based on the omnochrome natural pigments, the class of benzo[a]phenoxazine dyes is the third winner in the history of the synthetic heterocyclic dyes featuring excellent photo-physical properties.[54, 55]

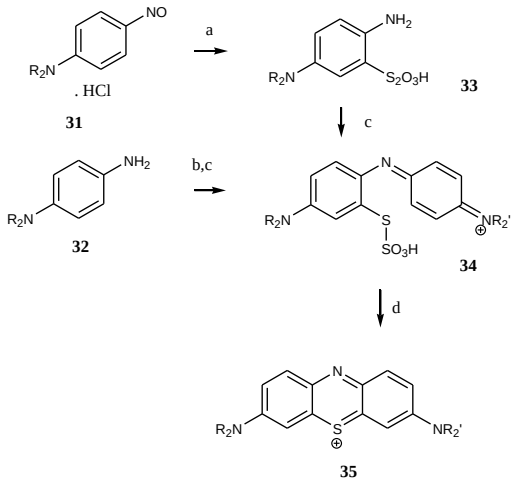
Likely inspired from the work of Ehrlich on Methylene Blue scaffolds, i. e. phenothiazonium salts, those compounds are rather poorly water-soluble, similarly to their natural counterparts.[12,32,46] Typically, benzophenoxazine are water-soluble in the range of $10^{-5}$ M to sub-micromolar concentrations. We will see later on that intense researches focused upon the increase of water-solubility. In this context, the two following examples found in the recent literature markedly show how the synthetic routes have remained unmodified since the 19$^{th}$ century. In addition, the dyes exposed in both cases are reported as water-soluble which is, after a careful inspection of the experimental data, strictly true in the nanomolar to micromolar range.

[55] a) Meldola, R., *J. Chem. Soc.* **1879**, 2065-2066. b) Wainwright, M., *Photosensitisers in Biomedicine*, Ed John Wiley & Sons, Ltd, **2009**, Chapt 4.

***1.2.2.3.1 Phenothiazinium salts***

The traditional approach to phenothiazinium synthesis employs a *p*-phenylenediamine derivative as the starting material (Scheme 10). This is dissolved in acidic media and oxidised with thiosulfate and an aniline derivative to give the corresponding thiosulfonic acid derivative of Binschedler's Green (the indaminethiosulfonic acid). Yet, the thiosulfate derivative of the *p*-phenylenediamine may also be obtained directly via treatment of the corresponding 4-nitrosoaniline hydrochloride in acetic acid with thiosulfate.[56] Similarly, the common synthesis of phenoxazinium salts, useful as dyes or potential therapeutic agents, involves the so-called nitrosative condensation (Scheme 11). As expected, those synthetic processes typically give low yields of heterocyclic frameworks and involve tedious purifications.

Scheme 10. Traditional syntheses of phenothiazinium dyes

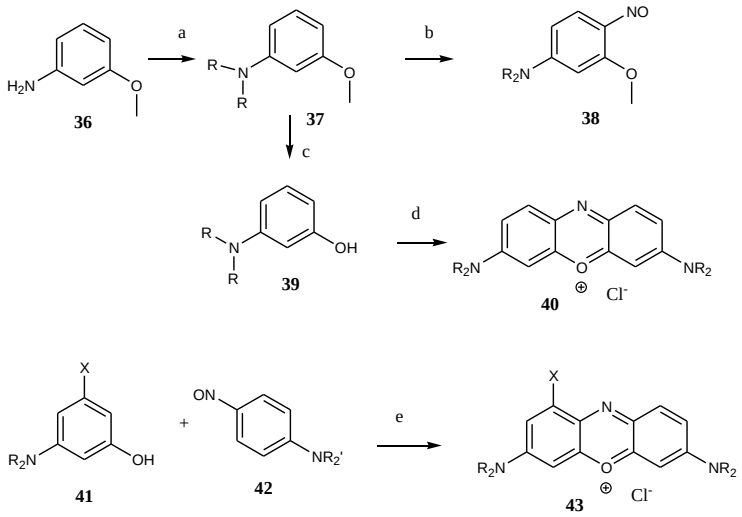



Reagents: (a) $Na_2S_2O_3$, AcOH; (b) $Na_2S_2O_3$, $Na_2Cr_2O_7$, $H^+$; (c) $C_6H_5NR_2$', $Na_2Cr_2O_7$, $H^+$; (d) $CuSO_4$

[56] Wainwright, M.; Giddens, R. M., *Dyes and Pigments* **2003**, 57, 245–257. And reference herein.

Yet, such syntheses are still to date envisaged for producing compounds devoted to medical purposes. The cationic character of the molecules ensures the so-called water-solubility in the range of $10^{-6}$ M.[57]

Scheme 11. Water-Soluble phenoxazin-5-ium derivatives




Reagents: a) $Br(CH_2CH_2)_nBr$, $(i\text{-}Pr)_2NEt$ toluene, reflux. b) i. HCl, $NaNO_2$; ii. $K_2CO_3$. c) i. HI, HCl, reflux, ii. $Na_2CO_3$. d) HCl, 90% *i*-PrOH, reflux, 4h. e) i. HX, EtOH, air, ii. Ion exchange resin

[57] Ge, J. F.; Arai, C.; Kaiser, M.; Witlin, S.; Brun, R.; Ihara, M., *J. Med. Chem*. **2008**, 51, 3654-3658. (and references herein).

#### *1.2.2.3.2 Nile blue derivatives*

Among the long-wavelength emitting dyes, oxazine derivatives such as benzophenoxazines and benzo[a]phenoxazines are now experiencing a remarkable growth in research interest.[58]

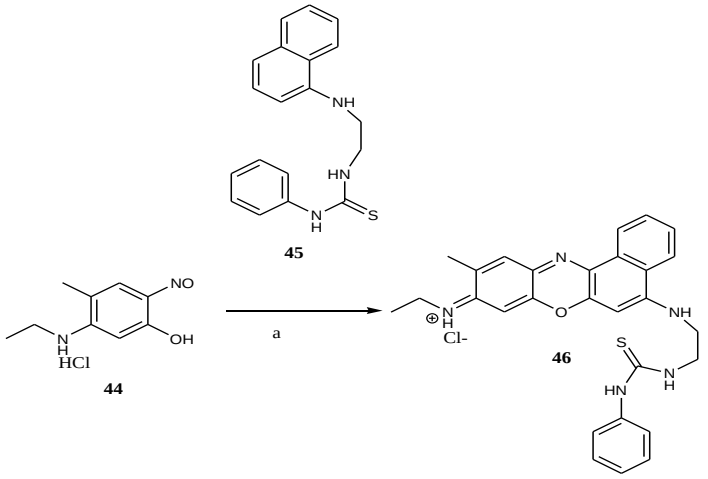
These cationic fluorophores have been used as biomarkers in a wide scope of applications, i. e. nucleic acid detection, labelling of proteins, cellular membranes etc...[59] Directly inspired from the phenoxazinium dyes by keeping the core structure of Nile Red or Nile Blue, the challenge tackled in the recent years was the introduction of functional groups in order to allow an easy grafting, or derivatisation, and an increase of water-solubility.

An elegant example published recently by Kim and his co-workers reports the high yielding synthesis of a nanomolar Hg(II) probe **46** based on the Nile Blue scaffold (Scheme 12).[60] This new fluorogenic dye, water-soluble at $10^{-6}$ M as the hydro-chloride salt, absorbs and emits light at 630 and 652 nm respectively with a reported quantum yield value of 0.2.

Now the presence of $Hg^{2+}$ ions induces a desulfurisation-based cyclisation (Scheme 13) which simultaneously gives a red-shift displacement and an increase of quantum yield to 0.35. The dye is selective for mercury detection which is the direct consequence of the oxidative cyclisation.

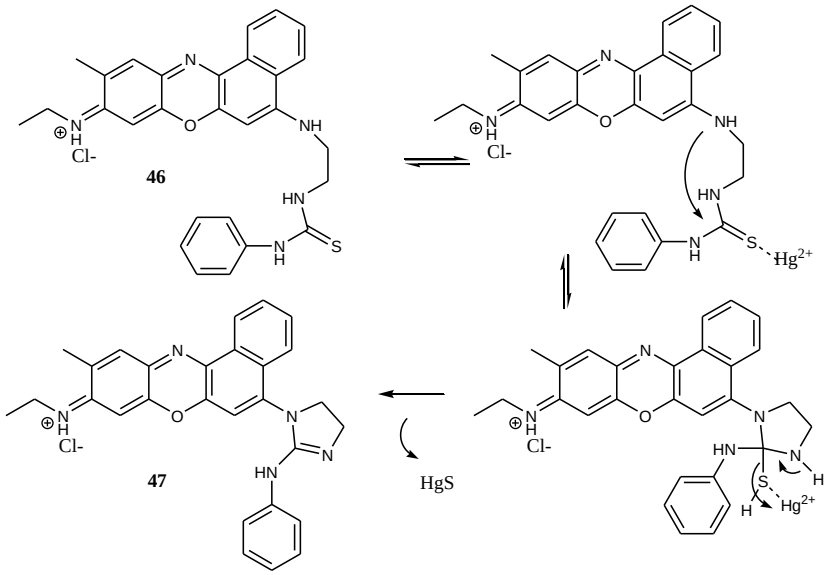
[58] a) Frade, V. H. J.; Goncalves, S. T.; Moura, J., *Tetrahedron Lett.* **2005**, 46, 4949-4952 and *Tetrahedron Lett*. **2006**, 47, 8567-8570. Mukherjee, H.; Raghuraman, H.; Chattopadhyay, A., *Biochim. Biophys. Acta* **2007**, 1769, 59-66.
[59] Ho, N. H.; Weissleider, R.; Tung, C. H., *Tetrahedron* **2006**, 62, 578-585.
[60] Lee, M. H.; Lee, S. W.; Kim, S. H.; Kang, C.; Kim, S. J., *Org. Lett*. **2009**, 11, 2101-2104.

Scheme 12. Synthetic route to dye **46**




Reagents : a) Ethanol, H+, reflux, (90% reported yield).

Scheme 13. Intramolecular cyclisation induced by $Hg^{2+}$



## 1.3 Pitfalls and prospects

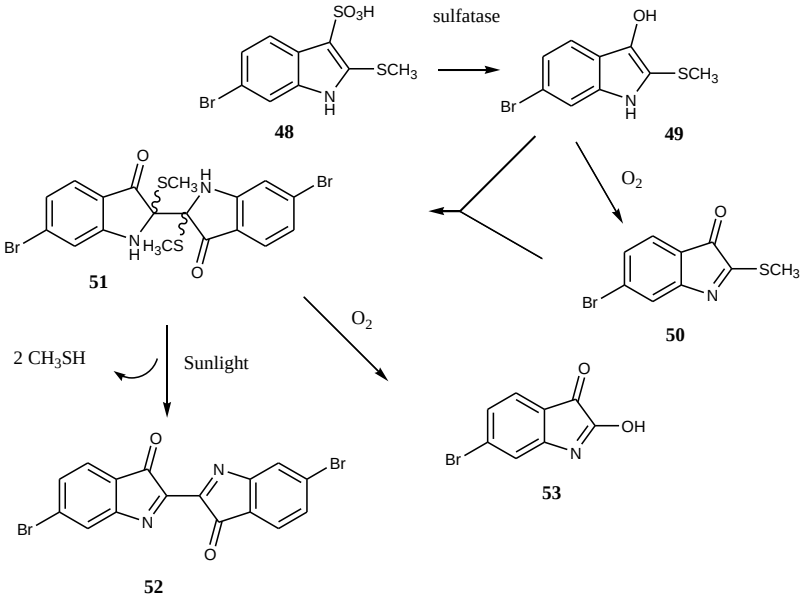
This survey of the scientific literature pointed out the remaining pitfalls in the development of efficient fluorophores or simple coloured molecules. Despite the facts that almost all the tactics have been employed for their synthesis and that their photophysical properties are almost totally mastered, the poor solubility of dyes in aqueous solution remains an unresolved problem. Looking at the natural molecules, one can quickly perceive that the same question arises for those so-called "natural pigments". Considering the biosynthetic pathways, the nature has used many stratagems to circumvent the problem. For example, the pigments are grafted on biomaterials such as carbohydrates or nucleic acids.[61a] The chromophore may be sealed in a hydrophobic "box" as in the GFP.[61b] Otherwise, formation of sulfate or phosphonate esters is a method of choice allowing the purposely release at will of the coloured molecules.[61c,d]

Another tactic of choice is nicely illustrated by the biosynthesis of the Tyrian Purple precursors from water-soluble sulfonated building blocks.[61a] Here, nature create hydrophilic substrate by inserting an ionised sulfonic acid group which ensure total solubility in water. Next, specific enzymes play a critical role by ensuring a complex transformation which is the substitution of the free sulfonate group by a hydroxyl one (Scheme 14). This is the closest method, while use in a reverse path, to the ones mainly used for synthetic routes towards water-soluble dyes.

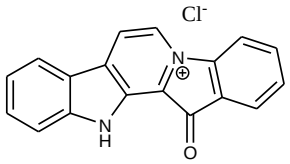
[61] a) Musso, H., *Tetrahedron*, **1979**, Volume 35, 2843-2853. b) Lopez, X.; Marques, M.; Castro, A.; Rubio, A., *J. Am. Chem. Soc*. **2005**, 127, 12329–12337. c) Smith, I. R.; Sutherland, M. D.; *Aust. J. Chem*. **1971** 24, 1487. d) Christophersen, C.; Anthoni, U., *Sulf. Rep*. **1986,** *4*, 365 (& reference herein).

Regarding the presence of quaternised nitrogen atoms as water-soluble motifs, the biosynthetic pathways yielding pigments with such features are widely found in the marine life forms as illustrated by the yellowish-brown pigment fascaplysin **53** (Scheme 14').[33d]

Scheme 14. Postulated biosynthetic pathway towards Tyrian Purple




Scheme 14'. Fascaplysin **53**




**53**

### *1.3.1 Tactics to increase the water-solubility: look at the tail*

We have briefly exposed the common strategies chosen by nature to create or increase the required water-solubility. As far as synthetic compounds are concerned, the chemist must face a choice which can be summarised as following: either the grafting of a hydrophilic tail of various lengths and natures, or the direct binding of the ionisable group on the core structure. Let us discuss first the "tail" strategy which opens a way to the dissolution into aqueous medium without a marked change of the other physico-chemical properties of the compound.

#### *1.3.1.1 Grafting of carbohydrate tails*

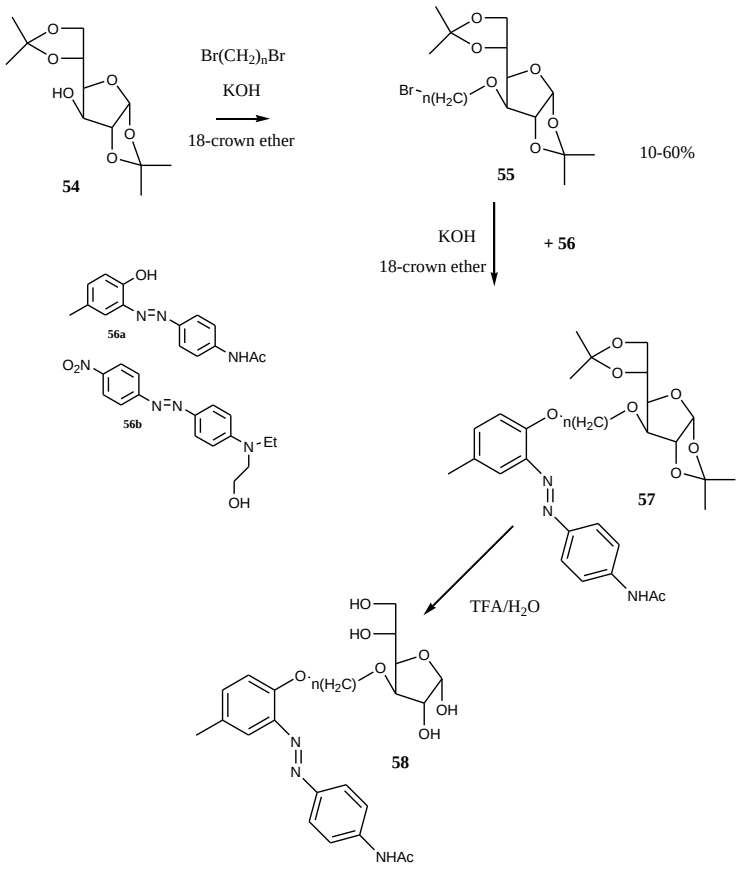
Just as the nature uses the "conjugation tactic" to biopolymers, the easiest way to "get into water" is nicely illustrated by Bianchini and his co-workers.[62] They obviously tackled the current problem of toxicity regarding azoïc dyes. Noteworthy their "naturalisation" process only focused on the improvement of the dyeing process by reducing the common additives. The Scheme 15 details the reagents and model dyes used to create the so-called naturalised dye. Typically, the synthesis involves nucleophilic substitution reactions on the protected sugar in presence of crown-ether as phase-transfer agent. Still, the yields are variable and by-products are usually formed. The tinctorial tests were successful for the modified dye **58** with a carbohydrate tail.

[62] Bianchini, R.; Catelani, G.; Cecconi, R.; D'Andrea, F.; Guazzeli, L.; Isaad, J.; Rolla, M., *Eur. J. Org. Chem* **2008**, 444-454.

### *1.3.1.2 Grafting of unnatural peptides*

Here, we describe an efficient way to impart water-solubility to the class of BODIPY dyes without impairing their excellent photo-physical properties.[63]

Scheme 15. Naturalised azodyes




[63] Niu, S. L.; Ullrich, G.; Ziessel, R.; Kiss, A.; Renard, P. Y.; Romieu, A., *Org. Lett.* **2009**, 11, 2049-2052.

Romieu and his group are dedicated to create new fluorogenic probes. Following several publications of Burgess's group, they proposed three ways to generate water-soluble BODIPY dyes among which the first two synthetic routes are directly inspired from strategies used by Burgess. The first tactic is the use a Heck-type coupling reaction based upon the prior functionalisation of the pyrrole building blocks.

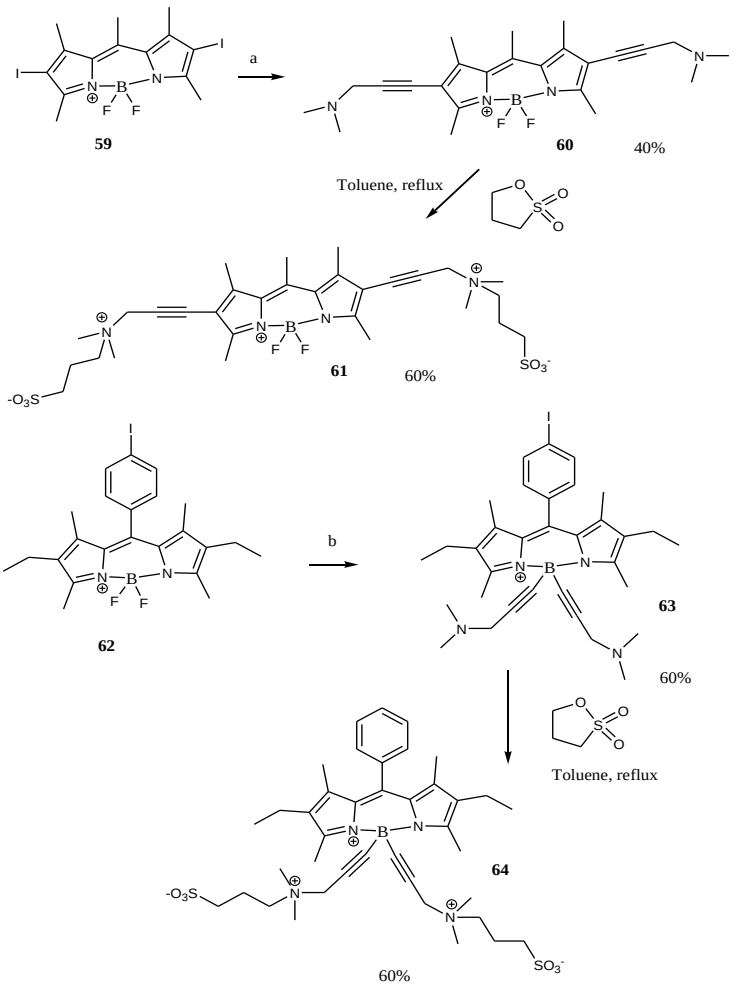
In the Scheme 16, the electrophilic aromatic iodination gave the intermediate **59** which is coupled afterward with 1-dimethylamino-2-propyne. Noteworthy the same reagent is used in the second tactic for the fluorine atoms displacement leading to alkylated boron atom.[64] Next, the amino groups are alkylated with a common reagent, namely the 1,3-propane sultone. In this way, Romieu and co-workers synthesised new water-soluble dyes with the simultaneous presence of ionised sulfonated groups and quaternised nitrogen atoms.

The third tactic is to insert a carboxylic group upon the aromatic nucleus usually found at the *meso* position of the BODIPY dyes. The activation of this acid and further coupling with oligopeptidic derivatives of the well known α-sulfo-β-alanine unnatural amino acid impart the water-solubility.[65]

[64] a) Thivierge, C.; Bandichhor, R.; Burgess, K., *Org. Lett*. **2007**, 9, 2135-2138. b) Li, L.; Nguyen, B.; Burgess, K., *Bioorg. Med. Chem. Lett*. **2008**, 18, 3112-3116. c) Li, L.; Han, J.; Nguyen, B.; Burgess, K., *J. Org. Chem*. **2008**, 73, 1963-1970.

[65] Wagner, D.; Gertner, D.; Zilkha, A., *Tetrahedron Letters* **1968**, 9, 4479-4480.

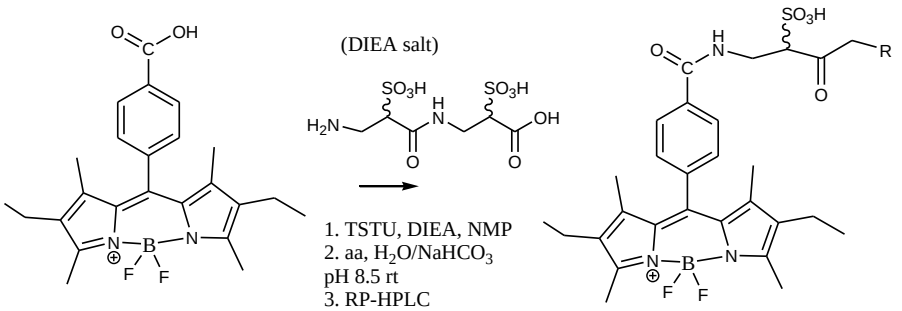
Scheme 16. Two “tail” tactics used for BODIPY




Reagents: a) 1-dimethylamino-2-propyne, [$Pd(PPh_3)_2Cl_2$] 10%, CuI 10%, benzene, TEA, 60°C. b) 1-dimethylamino-2-propyne, EtMgBr, THF, 60°C .

The synthetic route is illustrated in the Scheme 17. Yet, the photo-physical properties of the new BODIPY derivatives in simulated physiological conditions were not as good as desired. Derivative **61** (Scheme 16) does not show any alteration while the derivative **64** (Scheme 16) forms heavy aggregates which result in a fluorescence quenching. Lastly, the derivative **66** (Scheme 17) also exhibits the phenomenon of aggregation which somehow decreases the efficiency of the method.

Scheme 17. Grafting of unnatural amino acids

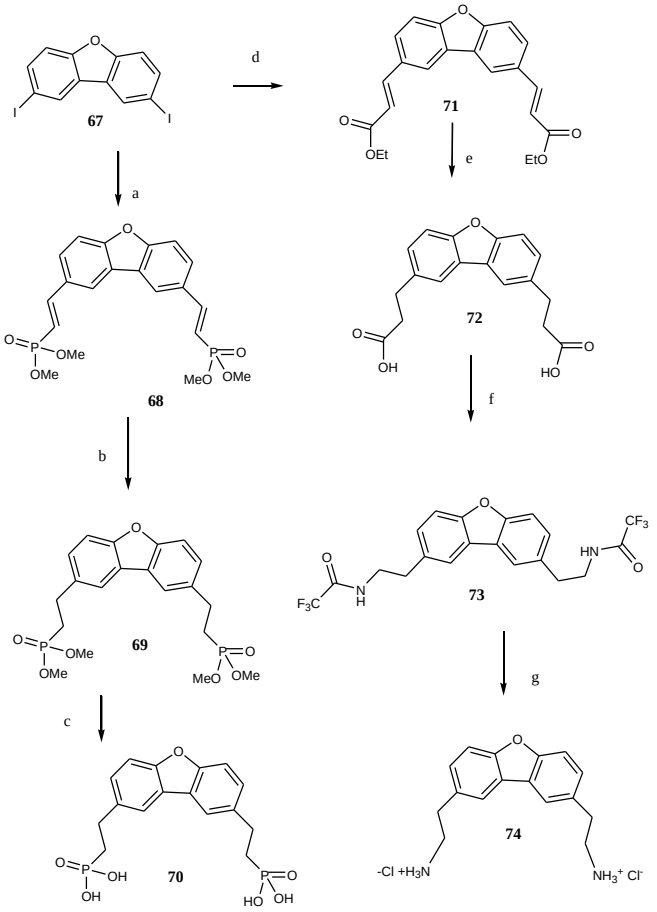



### *1.3.1.3 Others "tail" methods*

Lastly, let us close the section with synthetic routes involving the insertion upon aromatic nucleus of hydrophilic tails with either ammonium or phosphonates groups. Yet, such procedure has only been used for a model compound, i.e. benzofuran, in order to study exhaustively the water-solubility imparted by several ionisable groups.[66] Again, a Heck-type coupling strategy with dimethyl vinylphosphonate or ethyl acrylate was applied.

The crude intermediates are directly hydrogenated upon Pd/C. The deprotection of the phosphonate ester requires strong acidic conditions to give the free phosphonate **70** in moderate yields (Scheme 18).

Scheme 18. Tails bearing phosphonate and ammonium groups




Reagents: a) i. DMVP, $Pd(OAc)_2$, TEA, ii. NaOH, EtOH; b) $H_2$, Pd/C; c) TMSOTf-DMS, *m*-cresol, EDT, TFA; d) Ethyl acrylate, $Pd(OAc)_2$, TEA; e) i. NaOH, ii. $H_2$ , Pd/C; f) DPPA, TEA, TFA; g) $NaBH_4$

[66] LaBrenz, S. R.; Bekele, H.; Kelly, J. W., *Tetrahedron* **1998**, 54, 8671-8678.

On the other hand, the carboxylic groups are submitted to Curtius rearrangement giving the isocyanate intermediates which are trapped with trifluoroacetic acid and reduced with sodium borohydride to give the compound 7**4** in excellent yield. Those methodologies are indeed suitable for synthetic compounds and easily implemented. Yet, such synthetic routes are not widely reported compared to the direct modification of the core structure of the dyes.

### *1.3.2 Direct insertion of hydrophilic groups*

We have seen how the "tail tactic" has been used in organic synthesis. The second strategy mainly focuses upon the bioisosters of the carboxyl function, i.e. the sulfonic and the phosphonic acids. Here, we will not mention the nitration and reduction processes leading to cationic amino groups. Indeed, most of the dyes are aromatic compounds and the corresponding arylamines are of less interest knowing they are prone to toxicity and side effects.

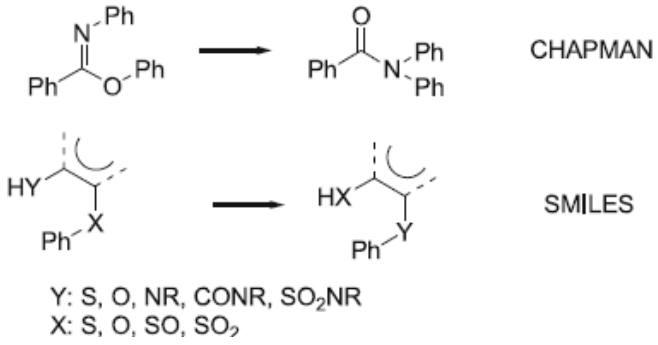
#### *1.3.2.1 Sulfonation reactions*

The oldest synthetic process for the sulfonation of organic molecules is the well-known electrophilic substitution in the aromatic series. Excellent reviews exist and we will not expose here the common reaction mechanism.[67-68] Yet, let us remind the reader that this reaction is the only one being reversible among the electrophilic substitution. Another fact is the constant production of regioisomeric mixtures in which the *para* derivatives are always favoured. The possibilities to circumvent this problem rely upon the (anchimeric) assistance of a functional group adjacent to the desired position for sulfonation, or to create the regioselectivity by a prior functionalisation of the aromatic nucleus.[68] The first strategy encompass rearrangement reactions such as the Miyazaki-Newman-Kwart (MNK) rearrangement.[69]

#### *1.3.2.1.1 Rearrangement reactions*

Historically, the possibility of obtaining intramolecular rearrangements came from the observations of Chapman and Smile (Scheme 19).

Scheme 19. Chapman and Smile rearrangements




In his early work, Chapman was able to demonstrate that the rearrangement of O,N-*bis*-aryl imidates into *N,N'-bis*-aryl amides undertakes an intramolecular pathway, i.e. via a spirocyclic intermediate. Later Smiles found another reaction causing the 1,3 shift of a substituent in the aromatic ring, involving a similar intermediate. Over the years, the MNK reaction has emerged as a reliable method, especially for the conversion of aromatic alcohols into aromatic thiols (and oxidised derivatives).

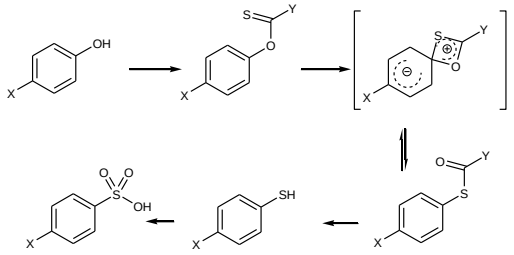
[67] LeRoi, N., *Sulfonation, in G. A. Olah, Friedel-Crafts and related Reactions*, Interscience Publishers, New York London Sidney, **1964,** Vol III, Part 2, 1373-1374.

[68] a) Pearson, D. E.; Buehler, C. A., *Synthesis* **1971**, 455-47. b) Bennetau, B.; Dunogues, J., *Synlett* **1992**, 171-176.

[69] Zonta, C.; De Luchi, O.; Volpicelli, R.; Cotarca, L., *Top Curr Chem* **2007**, 275, 131–161.

The mechanism of the MNK rearrangement consists in an *ipso* nucleophilic displacement of the oxygen substituent on the aromatic ring by the sulfur atom of the thiono-ester through a four-membered ring transition state. The greater strength of the bond between the carbon and sulfur compared to the one with oxygen is the driving force of the reaction towards a single product where sulfur has completely substituted the oxygen. Yet, the strategy is less efficient when *ortho* substituents are present in the aromatic nucleus. In addition, the reaction needs thermal activation to take place, usually above 200°C.

Scheme 20. Miyazaki-Newman-Kwart rearrangement[69]

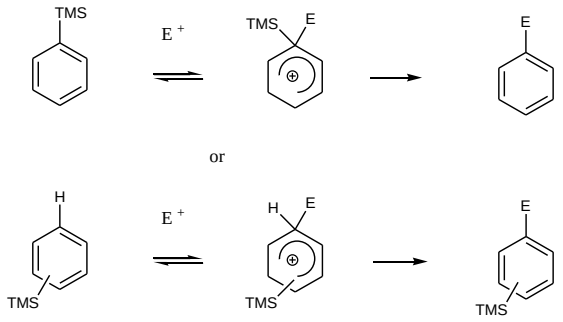



***1.3.2.1.2 Electrophilic aromatic substitution of silyl derivatives***

Electrophilic substitution can be achieved by taking advantage of the so-called "ipso" effect of the trimethylsilyl group (TMS). Aryl silicon bonds are cleaved by electrophilic reagents, probably via a similar mechanism as the one of normal aromatic electrophilic substitution. The consequence is the unavoidable competition between silyl and hydrogen atoms toward substitution. Yet, for most of the electrophiles, the "ipso" substitution occurs faster than the replacement of a hydrogen atom. This results from the cationic intermediate stabilisation by the electronic effect (β effect) of the TMS group (Scheme 21).[70]

[70] a) Eaborn, C., *J. Chem. Soc. Com*. **1972**, 1255. b) Bassindale, A. R.; Taylor, P. G., *In the Chemistry of Organosilicon Compounds, The Activating and Directing Effect of Silicon*, J Wiley & Sons, Chichester, **1989**, Part 2, Chap 4.

Scheme 21. Ipso substitution




Exploiting the "ipso" effect requires the preparation of a suitable organosilicon precursor. In laboratory, the most common route involves the reaction with TMS-Cl of Grignard reagents (or other metallated reagents) obtained from the corresponding aryl halides.[71] The general pathway is shown below (Scheme 22). To avoid the stoichiometric use of metals, alternative procedures have been developed such as the reductive silylation/oxidative rearomatisation (silicon-Birch reduction) (Schema 23).[72]

This reaction has been extensively studied and optimised by the group of Bennetau.[72] Application to the synthesis of Ketoprofene (anti-inflammatory drug) is shown in the Scheme 23. Lastly, the preparation of arylsilanes by proton abstraction from activated arenes has been discovered by Gilman and Wittig.[73] Such strategy has been widely used in the preparation of polysubstituted aromatic compounds, mostly by Snieckus and his group (Scheme 24).[74]

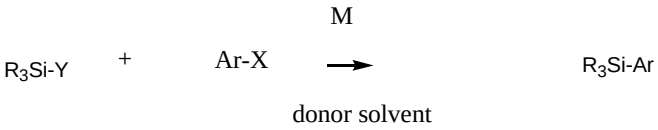
[71] Birkofer, L.; Stuhl, O., *In The Chemistry of Organosilicon Compounds, General Synthetic Pathways to Organosilicon Compounds*, J Wiley & Sons, Chichester, **1989,** Part 2, Chap 1.

[72] Benneteau, B.; Krempp, M.; Dunogues, J., *Tetrahedron* **1990**, 8131.

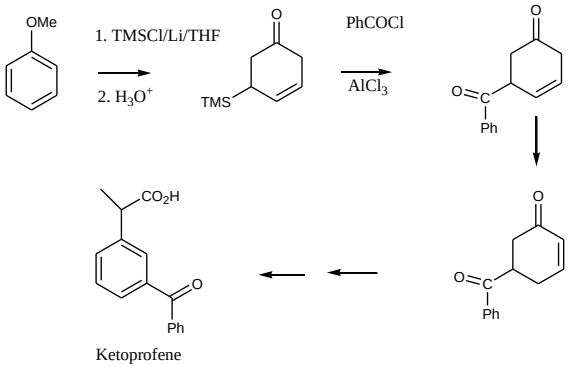
[73] Gilman, H.; Bebb, R., *J. Am. Chem. Soc*. **1939**, 61, 109.

[74] Snieckus, V., *Chem. Rev*. **1990**, 90, 879.

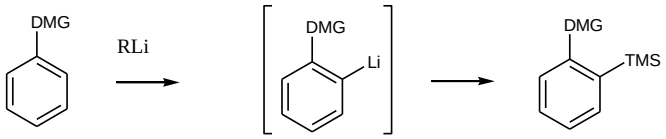
Scheme 22. Traditional synthesis of organosilicon




Scheme 23. Silicon-Birch reduction in fine chemical synthesis



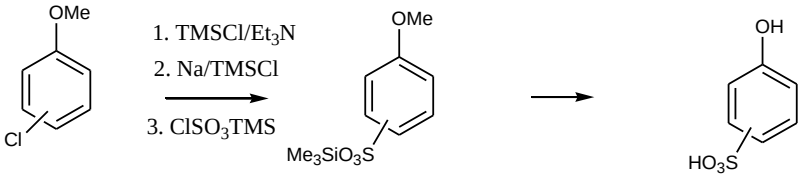

Scheme 24. Directed metallation reaction and silylation




DMG = NHCOR, OR, $CO_2H$, ...

The use of the "*ipso*" effect is illustrated in the Scheme 25 where the regioselective sulfonation has been achieved for the first time.[75]

Scheme 25. Regioselective preparation of sulfonated phenols



#### *1.3.2.1.3 Insertion of oxidised sulfur into carbon-metal bond*

As stated in the previous section, the "ipso" effect provides an excellent strategy to control the selectivity in the sulfonation process. Yet, if one could achieve the desired regiochemistry without the prior organosilicon synthesis, the overall yield will be increased. The principle of the directed metallation reaction (see Scheme 24) has been used now to perform the insertion of sulfur electrophiles. Following the seminal work of Gilbert, numerous groups have dedicated researches to the use of aryllithium species for the insertion of oxidised sulfur electrophiles.[76] The nature of the organometallic species plays a minor role and usually the same yields are obtained either with Grignard reagents or alkyllithium reagents.[77] The nature of the electrophilic species has a more prominent role. Sulfuryl chlorides have been used with variable success for the production of sulfonyl chlorides.[78] Gaseous sulfur dioxide usually gives the best results; yet the process remains poorly practical and rather expensive.[79]

[75] Babin, P.; Bennetau, B.; Bourgeois, P.; Rajarison, F.; Dunogues, J., *Bull. Soc. Chim. Fr*. **1992**, 129, 25.

[76] Gilbert, E. E., *Chem. Rev*. **1962**, 62, 549.

[77] a) Scott, R.; Gayle, J.; Heller, M.; Lutz, R., *J. Org. Chem*. **1955**, 20, 1165–1168. b) Truce, W. E.; Murphy, A. M., *Chem. Rev*. **1951**, 48, 69.

[78] Pandya, R.; Murashima, T.; Tedeschi, L.; Barrett, A., *J. Org. Chem*. **2003**, 68, 8274-8276.

[79] Jin, Q.; Nie, H.; McCleland, B. W.; Widdowson, K. L.; Palovich, M. R.; Elliott, J. D.; Goodman, R. M.; Burman, M.; Sarau, H. M.; Ward, K. W.; Nord, M.; Orr, B. M.; Gorycki, P. D.; Busch-Petersen, J., *Bioorg. Med. Chem. Lett*. **2004**, 14, 4375-4378.

The use of sulfur trioxide (in the form of various solid complexes) has been reported in aliphatic chemistry: addition to alkenes giving sultones[80-82], and aminosulfonation of alkenes in the presence of acetonitrile[83-84], for instance. The quenching with $SO_3$ of organometallic species in the aromatic series remains to be exploited.

#### *1.3.2.2 Phosphorylation reactions*

Phosphorus compounds are important species in agricultural and medicinal chemistry.[85-86] Their stability and activity have been attributed to the relatively inert nature of the C-P bond and to the structural similarity of phosphonic and phosphinic acids to the biologically important phosphate esters. Moreover phosphonate salts are highly water-soluble.

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[80] Bakker, B. H.; Cerfontain, H., *Eur. J. Org. Chem*. **1999**, 91-96.

[81] Suter, C. M.; Evans, B. P.; Kiefer, J. M., *J. Am. Chem. Soc*., **1938**, 60, 538-540.

[82] Thaler, W. A.; duBreuil, C., *J. Polym. Sci. Polym Chem. Ed*., **1984**, 22, 3905-3919.

[83] Cordero, F. M.; Cacciarini, M.; Machetti, F.; De Sarlo, F., *Eur J Org Chem* **2002**, 1407-1411.

[84] a) Enders, D.; Harnying, W.; Vignola, N.; *Synlett* **2002**, 1727-1729. b) Enders, D.; Berner, O. M.; Vignola, N.; Harnying, W., *Synthesis* **2002**, 1945-1952. c) Enders, D.; Harnying, W.; Vignola, N., *Eur. J. Org. Chem*. **2003**, 3939-3947.

[84] d) Enders, D.; Harnying, W., *Arkivoc* **2004**, 2, 181-188. e) Enders, D.; Harnying, W.; Raabe, G., *Synthesis* **2004**, 590-594. f) Enders, D.; Vignola, N.; Berner, O. M.; Harnying, W., *Tetrahedron* **2004**. g) Enders, D.; Harnying, W., *Synthesis* **2004**. h) Enders, D.; Hoffman, K. *Eur. J. Org. Chem*. **2009,** 1665–1668. (& reference herein).

[85] Savignac, P.; Iorga, B., *Modern Phosphonate Chemistry*, CRC Press, Boca Raton, **2003**.

[86] Nowack, B., *Water Research* **2003**, 37, 2533–2546.

Prior to 1950 no important new methods for preparing this class of compounds have been disclosed.[87] The principal traditional methods given for preparing phosphonic acids were the Friedel-Crafts and the Michaelis-Arbuzov reactions, first described in 1879 and 1808, respectively. In 1950 Mikhailov and Ihcherova reported that a separable mixture of arylphosphonic and diarylphosphinic acids can be obtained through the reaction between an aryllithium reagent and phosphoropiperidin dichloride ($C_5H_{10}NPOCl_2$).[88] Noteworthy, the efficient syntheses of aromatic phosphonic acids are still to date based either upon the Arbuzov reaction or the reaction between aryllithium and dialkylchlorophosphate species. [89]

The transformations were improved regarding the practical point of view, but novel strategies for the formation of C-P bonds did not appear in the last decades. For the alkylphosphate derivatives, C-P bond forming reactions generally fall into several traditional categories listed below (list 1).[90,91] The development of methods dedicated to the regioselective synthesis of aryl-phosphonates remains a present-day research subject.

[87] a) Freedman, L. D.; Doak G. O., *Chem. Rev*., **1957**, 57, 479–523. b) Bhattacharya, A. K.; Thyagarajan, G., *Chem. Rev*. **1981**, 81, 415–430.

[88] Michaelov, B. M.; Kucherova, N. F.; *Doklady Akad. Nauk* S.S.S.R7.8, 709 (**1951**); *Chem. Abstracts* **1962**, 46, 2004.

[89] Bonnaventure, I. ; Charette, A. B., *J. Org. Chem*. **2008**, 73, 6330-6340.

[90] a) Engel, R., *Chem. Rev*. **1977**, 77, 349. b) Worms, K. H. and Schmidt-Dunker, M., *Organic Phosphorus Compounds*; G. M. Kosolapoff and L. Mair, Eds.; John Wiley & Sons: New York, **1976**; 1-486. c) Crofts, P. C., *Organophosphorus Compounds;* G. M. Kosolapoff and L. Mair, Eds.; John Wiley & Sons: New York, **1973**; 1-209.

[91] Fields, S. C., *Tetrahedron* **1999**, 55, 12237-12273.

List 1: Traditional C-P forming bond reaction in aliphatic series:

Michaelis-Arbusov reaction of trialkylphosphites with alkylhalides:

$(RO)_3P + RX \rightarrow R\text{-}P(O)(OR)_2$

Michaelis-Becker reaction of alkali salts of dialkylphosphonates with alkylhalides:

$NaP(O)(OR)_2 + RX \rightarrow R\text{-}P(O)(OR)_2$

Michael addition of either trialkylphosphites or dialkyl phosphonates to unsaturated C=C bonds:

$(RO)_3P$ or $H\text{-}P(O)(OR)_2$ + $R'\text{-}CH{=}CH_2$ -> $R'\text{-}CH_2CH_2\text{-}P(O)(OR)_2$

Addition of trialkylphosphites or dialkyl phosphonates to open aziridines and epoxides:

$(RO)_3P$ or $NaP(O)(OR)_2$ + 3-ring heterocycle -> $X\text{-}CH_2CH_2\text{-}P(O)(OR)_2$

X = heteroatom

### *1.3.3 Recent examples of water-soluble dyes*

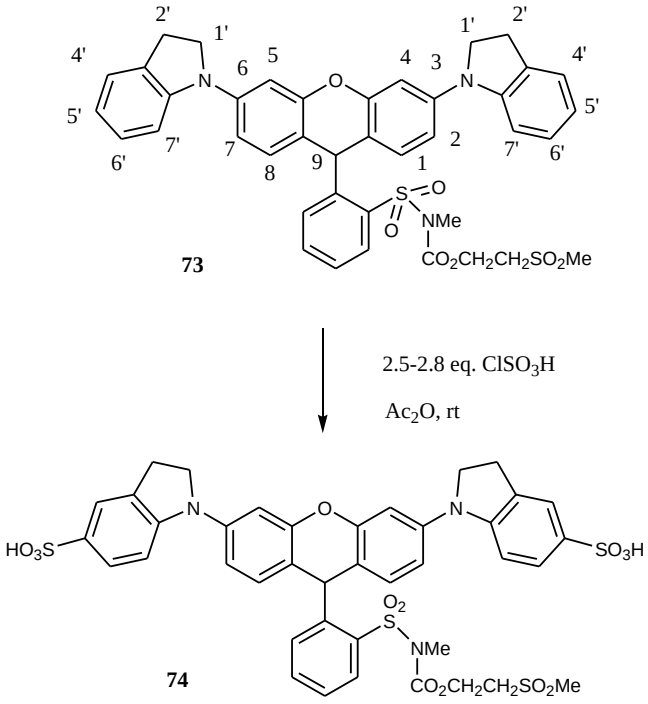
We have already illustrated the insertion of hydrophilic groups upon dyes of interest with the "tail approach", the most widely used. Examples of direct sulfonation or phosphorylation on expensive heterocyclic structures are less common. Burgess and his group have already published the sulfonation of borondipyrromethene scaffolds using chlorosulfonic acid which allows either the monosulfonation or the disulfonation depending on the experimental conditions.[64c]

Few recent papers are focused upon the direct sulfonation or phosphorylation of the xanthenes dyes, a method which is not suitable in the case of benzophenoxazinone dyes.[93] In general, the regiochemistry of sulfonation is subject to kinetic or thermodynamic control, depending on the reaction conditions. However, in polycyclic aromatic substrates the regiochemistry of sulfonation is often unclear, and mixtures of regioisomers are sometimes obtained. Thus, it is important to understand the responsible factors for controlling the regioselectivity. An interesting case is provided by the study of xanthene-derived base-bleachable dyes such as **73**.[94] The sulfonation was carried out with chlorosulfonic acid in acetic acid at room temperature. Since the sulfonation is carried out below 30 °C, kinetic factors are likely responsible for the observed selectivity. After theoretical investigation and experimental studies with indoline used as model (Scheme 27), it appeared that steric issues must be the reason for such a high selectivity. Indeed, electronic densities calculated through modelisation predicted the competitive sulfonation with the xanthene backbone. This is one of the very few examples of selective sulfonation upon such heterocyclic framework.

[93] Ho, N. H.; Weissleder, R.; Tung, C.-H., *Tetrahedron* **2006**, 62, 578-585.
[94] Carlier, P. R.; Lockshin, M. P.; Filosa, M. P., *J. Org. Chem*. **1994**, 59, 3232-3236.

Considering the precursor **73** (Scheme 26). There are four pairs of activated positions: 2/7 and 4/5 on the xanthene and the 5'/5'' and 7'/7'' on the indolines. Amine substituents are known to be *meta*-directing under strong acidic conditions, due to their protonation. Thus, substitutions at the 6'/6" and 4'/4" positions on the indolines remain a possibility depending of the pH of the solution. It is important to note that the high yield obtained from the sulfonation reaction of **73** (97% isolated yield) indicates that the regioselectivity in favour of the position 5'/5" (product **74**) is quite high.

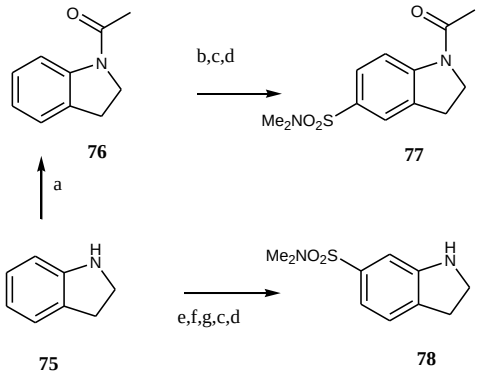
Scheme 26. Regioselective sulfonation of xanthene derivative




**73**

**74**

Scheme 27. Sulfonation of indoline as a model reaction

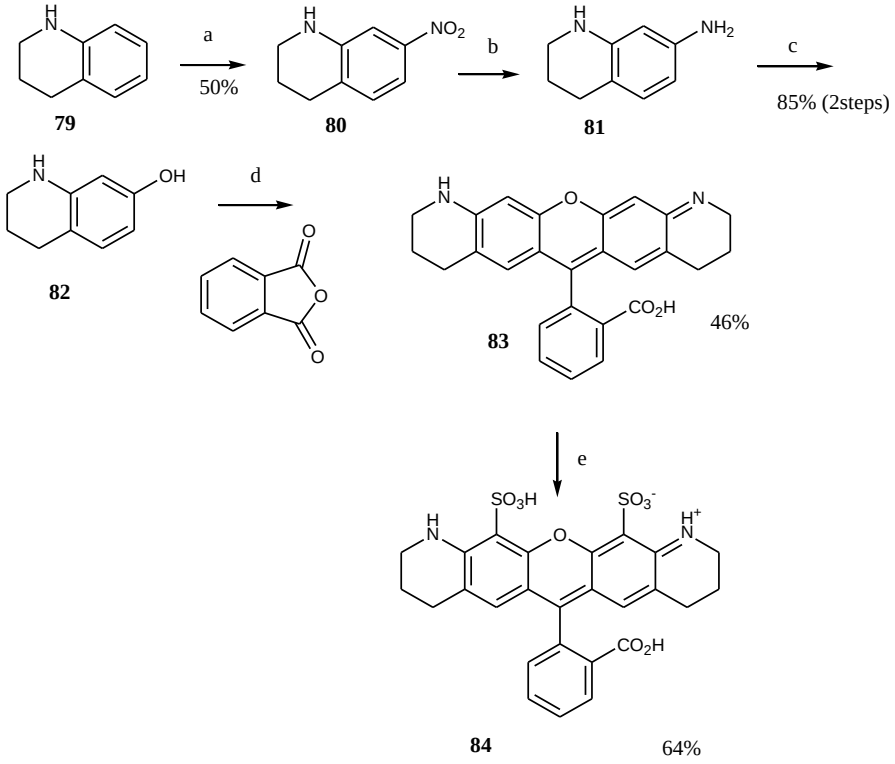



Reagents: (a) $HCO_2H$; (b) $ClSO_3H$; (c) $Me_2NH(aq)/CH_2Cl_2$; (d) $HCl(g)$; (e) $H_2SO_4$, 27-33% $SO_3$; (f) $Ac_2O$, AcOH; (g) $POCl_3$.

Another example recently published describes the efficient synthesis of water-soluble labels based on a sulfonated rhodamine dye. The rhodamine 110, i.e. a 3,6-diaminoxanthene derivative with unsubstituted amino groups, smoothly reacts with 30% $SO_3$ in $H_2SO_4$ giving the corresponding 4,5-disulfonated product.[95] Due to the very high cost of the xanthene dyes ( > 1000 € for 50 mg) , Boyarskiy and co-workers devised the production of new water-soluble derivatives as illustrated in the Scheme 28. With the key intermediate **84**, they could easily create molecular diversity for subsequent labelling experiment using the free carboxylic function.

[95] Boyarskiy, V.; Belov, V.; Medda, R..; Hein, B.; Bossi, M.; Hell, S., *Chem. Eur. J*. **2008**, 14, 1784-1792.

Scheme 28. Synthesis of water-soluble rigidified xanthene dye intermediate




Reagents: a) 100% $HNO_3$, cc $H_2SO_4$, 7°C; b) $N_2H_4.H_2O$, Raney Ni, MeOH, reflux; c) 85% $H_3PO_4$, 150°C, 24h; d) 170°C, 3h, then 85% $H_3PO_4$, 170°C, 3h; e) 30% $SO_3$ in $H_2SO_4$, 0°C, 12h.

## 1.4 Oxidative cyclisation in natural pigments

We have exposed in the previous sections how the natural pigments were used as templates to create new synthetic dyes. We also briefly highlighted the similarities and differences between the tactics chosen by the nature and the organic chemist respectively to achieve a suitable solubility in aqueous solutions. In the beginning, we mentioned the GFP protein as an extraordinary tool used today in cells mapping-physiology. The formation of the chromophore actually involves a biosynthetic pathway using an oxidative cyclisation.

Now, natural products biosyntheses always begin with ubiquitous biomolecules. The interconnectivity of natural products implies that a synthetic strategy based on a biomimetic total synthesis represents a general solution to the preparation of a wide scope of natural products. As we highlighted before, by delving more deeply into the biosyntheses of natural pigments, oxidative coupling reactions can usually be observed. Another remarkable feature is the common interconnections between the enzymes involved in such oxidative steps which allow exchanging one for another in some cases.[96]

Hereafter, several biomimetic syntheses of natural pigments will be exposed and compared to their respective syntheses via purely chemical routes. This will point out two features: the potential of biocatalytic synthesis for novel heterocyclic scaffolds and the potential of suitable oxidases known for their wide scope in terms of processed substrates.

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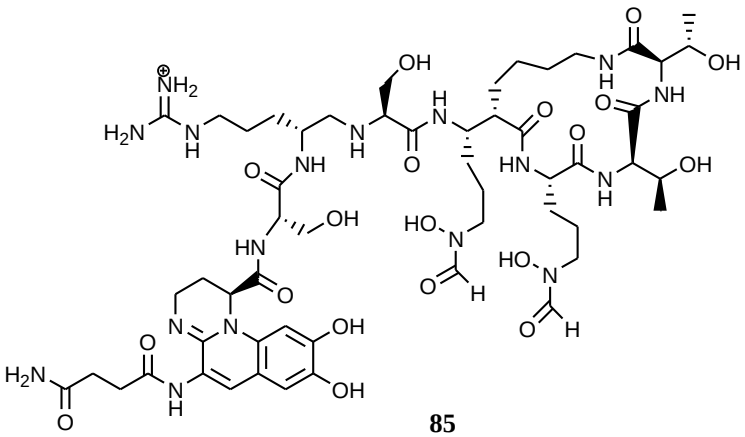
[96] a) Skyler, D.; Heathcock, C. H., *J. Nat. Prod*. **2002**, 65, 1573-1581. b) Gademann, K., *ChemBioChem* **2005**, 6, 913-919. c) Gademann, K.; Bethuel, Y., *Angew. Chem. Int. Ed*. **2004**, 43, 3327-3329. d) Gademann, K.; Bethuel, Y., *Org. Lett.* **2004**, 6, 4707-4710. e) Drechsel, H.; Jung, G., *J. Pept. Sci*. **1998**, 4, 147-181. f) Dorrestein, P.; Begley, T. P., *Bioorg Chem*. **2005**, 33, 136-148.

Let us begin with the biomimetic syntheses of a natural siderophore which is usually found in bacterial life forms. Whereas the compound is not produced by fungi source, it still possesses a chromophore which is related to an ubiquitous building block, i. e. the dihydroxybenzene ring, found in all life forms (catechol motif).

### *1.4.1 Synthesis of the pyoverdin chromophore*

The pyoverdins are siderophores secreted by fluorescent *Pseudomonas sp.* under conditions of iron starvation.[97] Structurally they comprise a species-specific peptide of 6-19 residues containing D-amino acids, hydroxamate functions and a characteristic 2,3-dihydro-1H-pyrimido[1,2-a]quinoline chromophore as exemplified in the Scheme 29. Incorporation studies have demonstrated that the pyoverdine chromophore is derived from tyrosine metabolism and L-2,4-diaminobutyric acid. [98]

Scheme 29. Pyoverdine derivative **85**



**85**

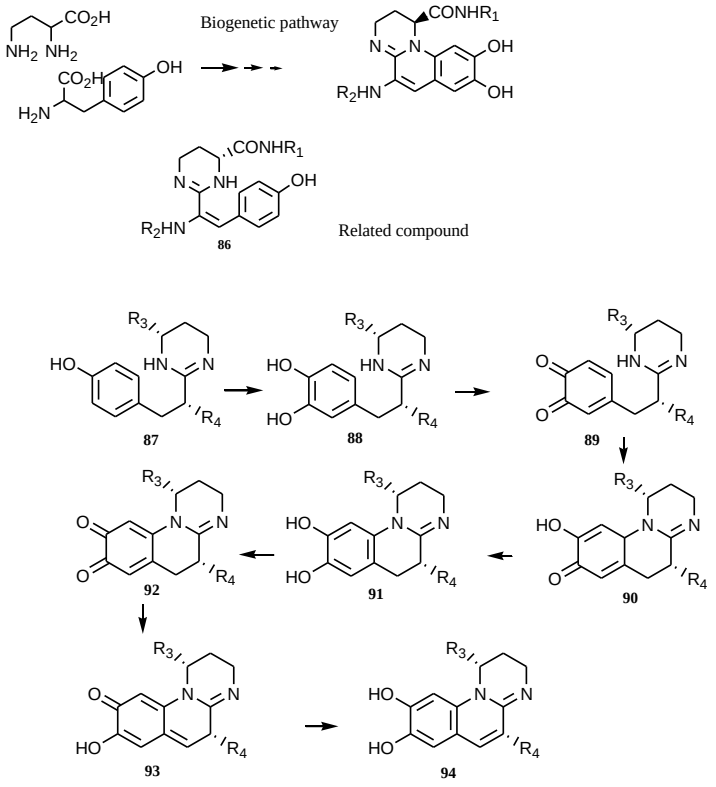
[97] Budzikiewicz, H., *FEMS Microbiol. Rev*. **1993**, 104, 209-228.

[98] Thompson, B. N.; Gould, S. J., *Tetrahedron* **1994**, 50, 9865-9872.

### *1.4.1.1 Biomimetic synthesis using oxidase*

It has been demonstrated that compound **86**, among several other compounds, is a precursor of pyoverdine. Looking at the building blocks, Dorrestein and co-workers, among which T. Begley is well known for his work upon the phenoxazinone synthase, have postulated a mechanistic route in 2003 which is illustrated in the scheme 30.[99] To reinforce their elegant proposal, they performed the last stage of the synthesis using polyphenol oxidase (PPO) with success, albeit in low yield (6%) (see Scheme 30').

Scheme 30. Mechanistic proposal



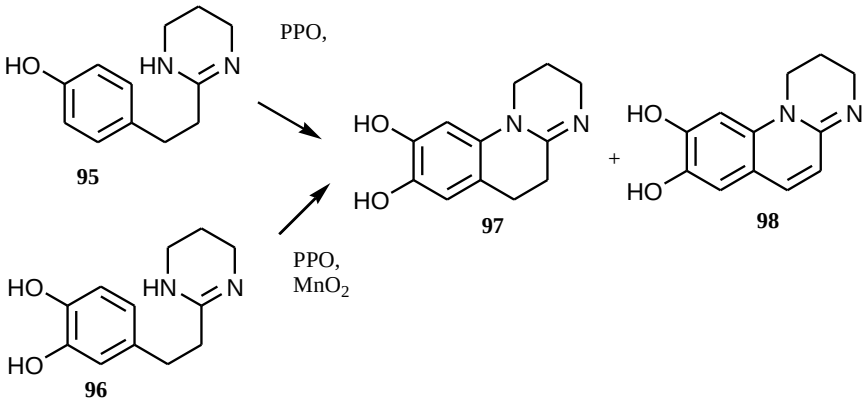

[99] Dorrestein, P.; Poole, K.; Begley, T. J., *Org. Lett*. **2003**, 5, 2215-2217.

Briefly, the enzymatic hydroxylation of **87** would give the catechol intermediate **88** which is submitted to $2e^-$ oxidation and conjugated addition to give the intermediate **90**. Tautomerisation reestablishes the catechol motif which is again oxidised to give the *o*-quinone species **92**.

A sequence of two successive tautomerisation reactions yields the final chromophore **94** as a highly blue fluorescent product. This cascade would be analogous to the one found in the biosynthesis of the actinomycin chromophore. Likely a polyphenol oxidase is also involved here as in the formation of melanin pigments and the pyridoacridine derivatives such as styelsamine. [100]

As validition of their proposal, Dorrestein and co-workers were able to cyclise a simpler analogue using respectively PPO, $MnO_2$ and cell free extract of bacterial cells as oxidising agents (Scheme 30').

Scheme 30'. Oxidative cyclisation



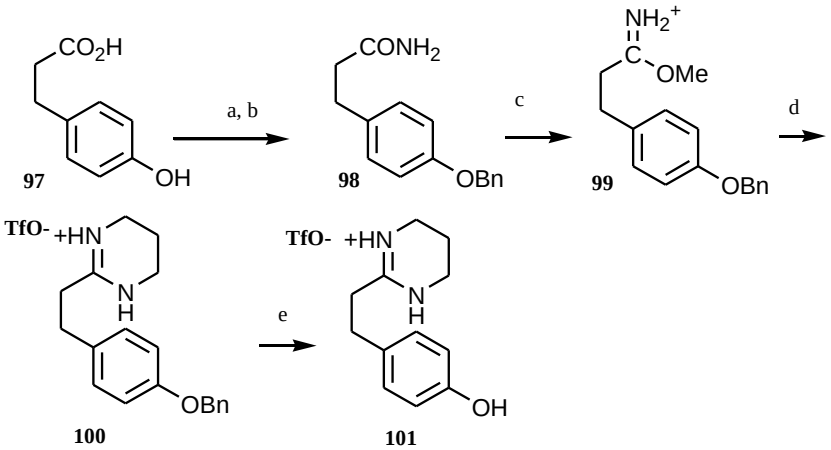

[100] Fatibello,-Filho, O.; Viera, I. C., *Analyst* **1997**, 122, 345-350.

### *1.4.1.2 Biomimetic synthesis by oxidative cascade*

This year, Cole and co-workers published experimental results obtained in the biomimetic synthesis of the pyoverdine chromophore using I(III) as reagent. The research was based on their experience acquired in the methodology of phenolic oxidation mediated by hypervalent iodine.[101]

The required model substrate was efficiently synthesised as shown in the Scheme 31.

Scheme 31. Chemical route towards pyoverdine analogues

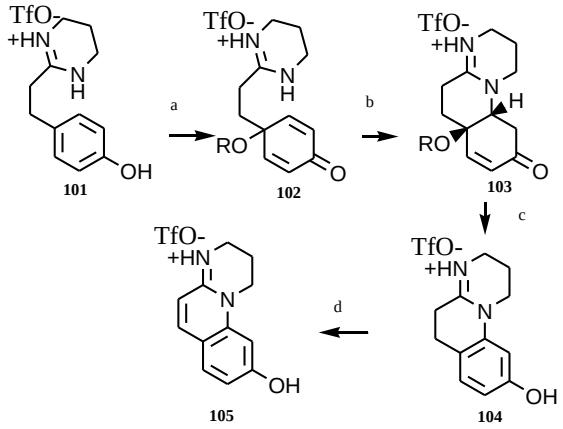



Reagents: a) BnBr, $K_2CO_3$, acetone, reflux; NaOH, reflux; b) $(COCl)_2$, DMF cat, then $NH_3$ aq; c) MeOTf, DCM, reflux; d) $H_2N(CH_2)_3NH_2$, EtOH reflux; e) $H_2$, Pd/C, MeOH

[101] Coles, S. J. *et al*, *Org. Lett.*, **2009**, 11, 1519-1522.

Based on their experience in the oxidative synthesis of cyclic amidines they were confident to readily cyclise their precursor **101**. Yet the initial trials were unsuccessful and they selected an oxidation procedure in the presence of a nucleophilic alcoholic solvent. Treatment of **101** with hypervalent iodine gave an unstable dienone compound **102** which cyclised during chromatographic separation upon basic alumina. The isolated product **103** contains an ether group resulting from the addition of the solvent. Subsequent treatment of **103** with trifluoromethanesulfonic acid afforded in moderate yield the intermediate **104** which was finally submitted to dehydrogenation. The chromophore **105** was obtained by this sequence, in about 10% of overall yield. So an organic chemical reagent is not more efficient than PPO in such case (Scheme 32).

Scheme 32. Chemical synthesis




Reagent: a) BTIB, 20 °C, ROH; b) Basic alumina; c) TfOH, reflux; d) DDQ, dioxane, reflux.

### *1.4.2 Synthesis of the pyridoacridine chromophore*

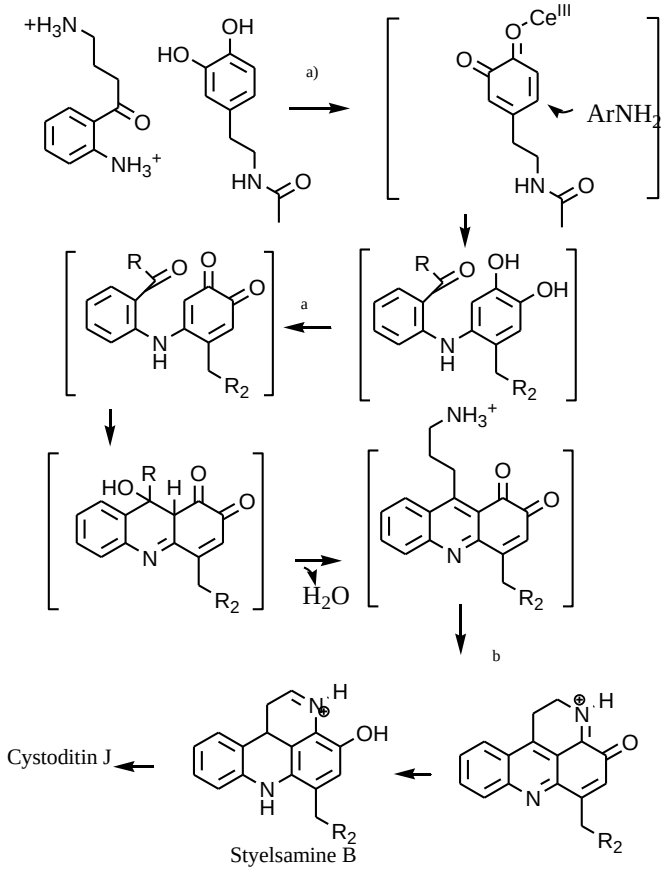
In 2001, C. Heatcock who has a long-standing experience in the biogenesis of marine natural products related to the pyridoacridine family, proposed an elegant biomimetic synthesis of styelsamine B and cystodytin J.[102] The rapid and low cost total synthesis takes advantage of the well known metabolism of kynurenine derivatives (see Scheme 33). With a view to clarify the biosynthetic pathway, the authors realised an oxidative merging transformation of kynurenine with benzoquinone. The biomimetic synthesis used a silver salt (and cerium chloride as Lewis acid) as oxidising reagent and up to date, no biocatalysed transformation has been published for comparison. Yet, as we will see later, copper oxidase would likely be an alternative of choice. The conversion of styelsamine B to cystodytin J was again performed with $Ag_2O$ as the oxidising reagent to give a good yield of the final product. This work paves the route towards a general synthesis of pyridoacridine derivatives.

### *1.4.3 Synthesis of the phenoxazinone chromophore and derivatives*

The first phenoxazinone-type colouring matters discovered in nature were the ommochromes, a class of photosensitive pigments occurring in arthropods, cephalopods and other invertebrates. The exact structure of the closely related ommins,[103] which constitute the red–violet fraction of cephalopod eye pigments, is still unknown today. A certain number of natural and synthetic antiproliferative compounds also contain the phenoxazinone ring system. Indeed, it has been shown that the antineoplastic activity is correlated with the ability of the planar heterocyclic core to intercalate into double-stranded DNA.

[102] Skyler, D.; Heatcock, C. H., *Org. Lett.*, **2001**, 3, 4323-4324.

Scheme 33. Biomimetic synthesis of pyridoacridine derivative




Reagents: a) MeOH:AcOH (2:1), 15% mol $CeCl_3$, $Ag_2O$ (2 equ), 40 °C, $N_2$ atmosphere, b) HCl 6N, 90 °C, 30 min.

[103] a) Schaefer, W., *Prog. Org. Chem*. **1964**, 6, 135. b) Ionescu, M.; Mantsch, H., *Adv. Heterocycl. Chem*. **1967**, 8, 83. c) Prota, G., *Marine Natural Products: Chemical Biological Perspectives*, ed. P. J. Scheuer, Academic Press, New York, **1980**, vol. 3, 141.

In the actinomycins,[104] an important family of antibiotics produced by actinomycetes, the phenoxazinone skeleton is linked to two pentapeptide chains.As such, novel derivatives of the actinomycin planar chromophore have raised a marked interest which results in numerous scientific publications and patents reporting "actinocin" compounds with various bioactivities.[105]

Dyestuffs such as *orcein and litmus* (names for dyes extracted from several species of lichen, representative structures are given in Scheme 34) also feature a phenoxazinone core whose electronic distribution is strongly influenced by the nature of the substituents.[106] The phenoxazine structure can suffer an enzymatic single-electron reduction. The resulting free radical intermediate is able to transfer one electron to $O_2$ for producing superoxide anion.[107]

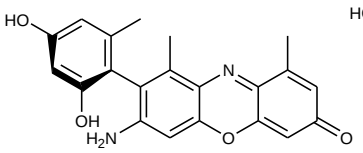
[104] a) Chabner, B. A.; Allegra, C. J.; Curt G. A.; Calabresi, P., *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 9th edn., eds. J. G. Hardman, L. E. Limbird, P. B. Molinoff, R. W. Ruddon and A. G. Gilman, McGraw-Hill, NewYork, **1996**, 1233. b) Graf, E.; Schneider, K.; Nicholson, G.; Strobele, M.; Jones, A. L.; Goodfellow, M.; Beil, W.; Sussmuth, R. D.; Fiedler, H.-P., *J Antibiot.* **2007**, 60, (4), 277-284.

[105] a) Bolognese, A.; Correale, G.; Manfra, M.; Lavecchia, A.; Mazzoni, O.; Novellino, E.; Barone, V.; Pani, A.; Tramontano, E.; La Colla, P.; Murgioni, C.; Serra, I.; Setzu, G.; Loddo, R., *J. Med Chem*. **2002**, 45, 5205. b) Bolognese, A.; Correale, G.; Manfra, M.; Lavecchia, A.; Mazzoni, O.; Novellino, E.; Barone, V.; La Colla P.; Loddo, R., *J. Med Chem*. **2002**, 45, 5217. c) Yavorskaya, N. P.; Golubeva, I. S.; Kubasova, I. Yu.; Glibin E. N., *Pharmaceutical Chemistry Journal* **1996**, 30, 22-26. d) Avorskaya, N.P.; Golubeva, I.S.; Kubasova, I.Y.; Ovchinnikov, A.V.; Plekhanova, N.G.; Glibin, E.N., *Pharmaceutical Chemistry Journal* **2001**, 35, 305-307. e) Golubeva I.S.; Kubasova I.Y.; Yavorskaya N.P.; Glibin E.N.; Popova E.B., *Pharmaceutical Chemistry Journal* **2000**, 34, 339-341. f) Rinehart, K. L.; McMillan, M. W.; Witty, T. R.; Tipton, C. D.; Shield, L. S.; Li, L. H.; Reusser, F., *Bioorg. Chem.* **1977**, 6, (3), 353-369.

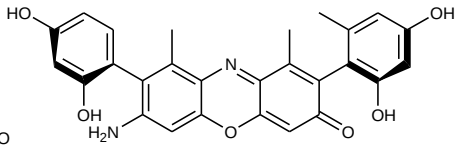
[106] a) Nakazawa, H.; Chou, F.; Andrews, P. A.; Bachur, N. R., *J. Org Chem.* **1981**, 46, 1493-1496. b) Sehgal, R.; Dieter, R. K.; Sengupta, S. S., *J. Het. Chem*. **1984**, 21, 1661. c) Sehgal, R.; Dieter, R. K.; Sengupta, S. S., *J. Het. Chem.* **1986**, 23, 329. d) Bolognese, A.; Liberatore, R., *J. Het. Chem.* **1988**, 25, 1243. e) Bolognese, A.; Liberatore, R., *J. Het. Chem*. **1988**, 25, 1251.

The chemical syntheses of actinocin derivatives always involve the functionalisation of *o*-nitro phenols, the subsequent catalytic hydrogenation into anilines and the oxidative dimerisation using a strong oxidising reagent (Scheme 35).[103-107] Now methods based on the post-modification of the phenoxazinone scaffold or on the oxidative dimerisation of a pre-functionalised nitro building block have their limitations.

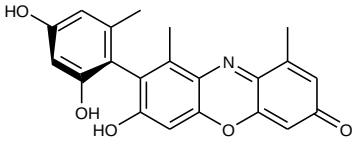
Scheme 34. Orceins and litmus dyes



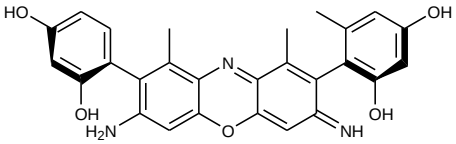

α-amino orcein



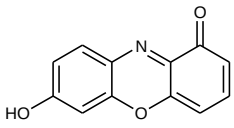

β-amino orcein




α-hydroxy orcein



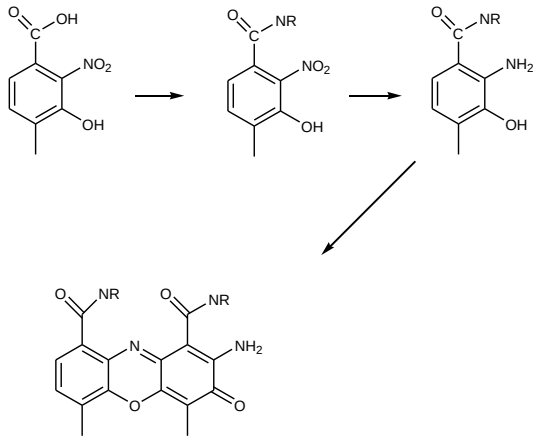

β-amino orceinimine




7-hydroxyphenoxazone chromophore of litmus components

[107] Bachur, N. R.; Gordon, S. L.; Gee, M., *Cancer Res*. **1978**, 38, 1745.

Scheme 35. Actinocin synthetic routes




Organic synthesis is challenging and tandem transformations in which several steps occur in a single vessel, always fascinate the chemist. As such, the phenoxazinone synthase, a copper-oxidase, which catalyses the $6e^-$ oxidation of aminophenol to generate the chromophore of actinomycin has been used as a model in bioinorganic chemistry for a long time.[108]

The potential use of a copper oxidase as the biocatalyst to reproduce the tandem reactions involved in the actinomycin biosynthesis appears as a promising tool also in organic synthesis. [109]

[108] a) Mukherjee, C.; Weyhermuller, T.; Bothe, E.; Chaudhuri, P., *Comptes Rendus Chimie Catalyse biomimitique d'oxydation* **2007**, 10, 313-325.b) Horvath, T.; Kaizer, J.; Speier, G., *J. Mol. Catal. A: Chemical* **2004**, 215, 9-15. c) Szigyarto,

I. C.; Simandi, T. M.; Simandi, L. I.; Korecz, L.; Nagy, N., *J. Mol. Catal. A: Chemical The 9th International Symposium Activation of Dioxygen and Homogeneous Catalytic Oxidation* **2006**, 251, 270-276. d) Simandi, L. I.; Simandi, T. M.; May, Z.; Besenyei, G., *Coordination Chemistry Reviews The 35th International Conference on Coordination Chemistry* **2003**, 245, 85-93.

The detailed mechanism of action of the phenoxazinone synthase has been elucidated for a long time and will be exposed later in the manuscript.[110] This enzyme continues to raise a great interest as its X-ray structure has been resolved in 2006 by Smith and co-workers.[111]

Whereas the phenoxazinone synthase is recognised as a member of the copper oxidase family, like laccases and tyrosinases, an uncertainty remains regarding the exact nature of the catalytic sites. Thanks to the work of Smith *et al*, it has been shown that phenoxazinone synthase is almost identical to laccase when comparing their catalytic sites which are formed of the copper site T1, and the trinuclear copper sites T2/T3. A fifth copper atom has been found in the phenoxazinone synthase which only serves as stabilising agent for the protein supramolecular structure. In the next section, we will detail the specific features of the copper oxidases and specifically the laccase ones.

[109] a) Christen, S.; Southwell-Keely, P. T., *Biochemistry* **1992**, 31, 8090-8097. b) Eggert, C.; Temp, U.; Dean, J. F. D.; Eriksson, K.-E. L., *FEBS Letters* **1995**, 376, 202-206. c) Osiadacz, J.; Al-Adhami, A. J. H.; Bajraszewska, D.; Fischer, P.; Peczynska-Czoch, W., *J. Biotechnol*. **1999,** 72, 141-149. d) Salzman, L.; Weissbach, H.; Katz, E., *Archives of Biochemistry and Biophysics* **1969**, 130, 536-546. e) Tomoda, A.; Hamashima, H.; Arisawa, M.; Kikuchi, T.; Tezuka, Y.; Koshimura, S., *Biochimica et Biophysica Acta (BBA) - General Subjects* **1992**, 1117, 306-314.

[110] a) Barry, C., E.; Nayar, P. G.; Begley, T. P., *Biochemistry* **1989**, 28, 6323-6333. b) Sivak, A.; Miloni, M. L.; Nobili, F.; Katz, E., *Biochimica et Biophysica Acta* **1962**, 57, 283-289.
[111] Smith, A. W.; Camara-Artigas, A.; Wang, M.; Allen, J. P.; Francisco, W. A., *Biochemistry* **2006**, 45, 4378-4387.

## 1.5 Laccase as a biocatalyst of choice in synthesis

Oxidases catalyse reactions involving direct activation of oxygen (from air), and within this group, the multi-copper oxidases catalyse the four-electron reduction of oxygen into water with the concomitant four successive one-electron oxidation of substrates.[112]

The terminology used for naming these enzymes in the literature, and particularly early references to phenol-oxidising enzymes, can be somewhat confusing due to a lack of differentiation between laccases, phenol oxidases (PO) and polyphenol oxidases (PPO). In addition, the range of substrates of the various enzymes overlap broadly, and the enzymes isolated from different sources are often not characterised to the point where the phenol type oxidising activity is well defined.

### *1.5.1 Multicopper Oxidase and Oxygenase*

Copper, an essential trace element in living systems, is a key cofactor of enzymes and implied in diverse arrays of biological oxidation-reduction reactions. These involve either outer-sphere electron transfer, as in the blue copper protein, or inner-sphere electron transfer in the binding, activation and reduction of dioxygen.[112] Now copper sites have been traditionally divided in three classes based on their spectroscopic features. These reflect the geometric and electronic structures of the active sites: type 1 or blue copper (T1), type 2 or normal copper (T2) and type 3 (T3) or coupled binuclear copper centers.[113]

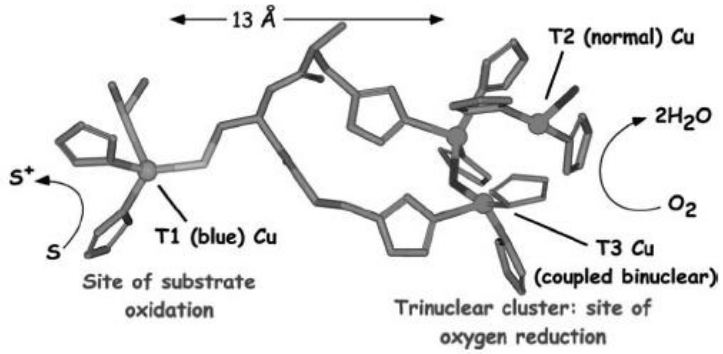
---

[112] Quintanar, L.; Stoj, C.; Taylor, A. B.; Hart, P.; Kosman, D.; Solomon, E. I., *Acc. Chem. Res*. **2007**.445-452.

[113] a) Solomon, E. I.; Baldwin, M. J.; Lowery, M. D., *Chem. Rev*. **1992**, 92, (4), 521-542. b) Holm, R.; Kennepohl, P.; Solomon, E., *Chem Rev*. **1996**, 96, 2239.

In multicopper oxidases the copper site T2 exhibits an electronic structure similar to simple planar Cu(II) complexes whereas the type 3 is a binuclear Cu(II)-Cu(II) cluster which is diamagnetic due to the antiferromagnetic coupling through a hydroxide bridge. Those copper sites T2 and T3 are the sites of molecular dioxygen reduction (Figure 4).

Figure 4. Active sites of Multicopper oxidases [112]




The main electron transfer (ET) steps in the reaction mechanism of multicopper oxidase are: (i) reduction of the T1 copper site by the substrate, (ii) channelling the electron through peptidic backbone to the trinuclear site T2/T3, (iii) reduction of the molecular dioxygen. The role of the T1 copper site is mainly to shuttle electrons from a substrate being present in the binding cavity to the core of the enzyme.[114] The coordination sphere of the T1Cu always contains two histidines and one cysteine. The cysteine is involved in a highly covalent Cu-S bond with an intense ligand-metal charge transfer band at ~600 nm giving the characteristic blue colour of multicopper oxidase. The covalency of the T1Cu-cysteine bond is thought to facilitate the electron transfer from metal to peptidic backbone.[115]

[114] Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E., *Chem. Rev.* **1996**, 96, 2563-2605.

Lastly, the T1Cu site can come with or without a fourth ligand, in axial position, which is thought to tune the oxidation potential of the oxidase. Methionine for example will give a long Cu-$S_{Meth}$ bond which ultimately leads to a decrease of oxidation potential of about few hundred millivolts. If the axial ligand is a non coordinating amino acid, the oxidation potential raises to the usual value of about 790 mV.[116]

### *1.5.2 Reactivity of T1Cu site of Multicopper Oxidase*

The reactivity of the T1 site can be probed using electrochemical methods.[117] The electron transfer (ET) can be described according to the classical theory for the rate of intermolecular ET ($k_{ET}$) developed by Marcus.[118] The equation contains a term which describes the equilibrium formation of the enzyme-substrate complex ($K_A$), a steric term (S), the reorganisation energy associated with the valence change at the enzyme and substrate sites (λ), and finally the free energy difference ( ΔG°) which is the driving force and an electronic term which quantifies the efficiency of the ET pathway ($H_{DA}$).[118]

$$k_{ET} = K_A S \sqrt{4\pi^3/(h^2 \lambda k_B T)} (H_{DA})^2 \, e^{[-(\Delta G^\circ + \lambda)^2/(4\lambda k_B T)]} \quad (1)$$

[115] Palmer, A. E.; Randall, D. W.; Xu, F.; Solomon, E. I., *J. Am. Chem. Soc.* **1999**, 121, 7138-7149.

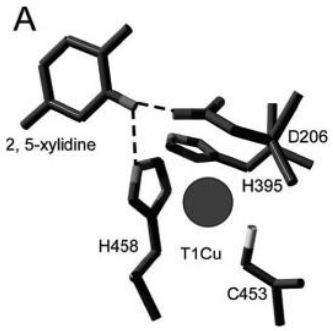
[116] Xu, F.; Palmer, A. E.; Yaver, D. S.; Berka, R. M.; Gambetta, G. A.; Brown, S. H.; Solomon, E. I., *J. Biol. Chem*. **1999**, 274, 12372-12375.

[117] a) Johnson, D. L.; Thompson, J. L.; Brinkmann, S. M.; Schuller, K. A.; Martin, L. L., *Biochemistry* **2003**, 42, 10229-10237. b) Leiger, C.; Bertrand, P., *Chem. Rev*. **2008**, 108, 2379-2438.

Among the contributions which will influence the rate of ET, the efficient binding ($K_A$) of an organic substrate is an important feature. The binding of a substrate in a laccase can be inferred by comparison with the structures of several fungal laccases which were crystallised with or without a ligand at the vicinity of the active site.[119] In most of the laccase structures, one histidine residue is solvent-exposed at the bottom of the cavity.

There, a possible H-bond contact can be expected with amino or alcohol group of the ligand.[120] This has experimentally been observed in the X-ray structure of *Trametes versicolor* enzyme with 2,5-xylidine. The structure shows that the substrate amine group is H-bonded to the protein via the residues His395, Asp206 and His458.[119] This is illustrated in the figure 4. One can expect similar interactions in the case of phenolic substrates.

Figure 5. Accepted Ligand binding to the T1Cu site[119b]




[118] a) Rees, N. V.; Clegg, A. D.; Klymenko, O. V.; Coles, B. A.; Compton, R. G., *J. Phys. Chem. B* **2004**, 108, 13047-13051. b) Stoj, C. S.; Augustine, A. J.; Zeigler, L.; Solomon, E. I.; Kosman, D. J., *Biochemistry* **2006**, 45, 12741-12749.

[119] a) Matera, I.; Gullotto, A.; Tilli, S.; Ferraroni, M.; Scozzafava, A.; Briganti, F., *Inorg. Chim. Acta* **2008**, 361, 4129-4137. b) Bertrand, T.; Jolivat, C.; Briozzo, P.; Caminade, E.; Joly, N.; Madzak, C.; Mougin, C., *Biochemistry* **2002**, 41, 7325-7333. c) Piontek, K.; Antorini, M.; Choinowski, T., *J. Biol. Chem*. **2002**, 277, 37663-37669.

The structure of the substrate-enzyme complex is consistent with a relatively rapid ET, given the efficient orientation parameters, i.e. well solvent-exposed T1Cu site, favourable substrate orientation, and direct ET pathway into T1Cu site. The substrate positioning of 2,5-xylidine has been used for modelisation and theoretical investigations of electron transfer and oxidation of other organic molecules.[120] As phenoxazinone synthase shares an almost identical arrangement of the T1Cu site, it is expected that similar interactions are involved for the efficient oxidative dimerisation of actinomycin building blocks. A major difference here compared to the tyrosinase family is the absence of direct interaction between the phenolic substrate and the copper atom. This is important as tyrosinases have found a wide scope of applications in organic synthesis due to such direct interaction which is beneficial for selectivity in several oxidative cyclisations.[121]

### *1.5.3 Laccase, blue copper oxidase as biocatalyst*

Laccase is a member of the oxidoreductase family whose applications as biocatalyst in organic synthesis has been neglected in the past. It likely results from the paucity of commercial source. Today, as the enzyme is more easily available to scientific community, its potential has been highlighted through a steadily increasing number of scientific publications.[122] The wide scope of substrate tolerance is certainly the critical feature to account for such scientific interest.

[120] Reynisson, J.; Steenken, S., *Org. Biomol. Chem.* **2004**, 2, 578 - 584.
[121] a) Burton, S. G., *Curr. Org. Chem.* **2003**, 7, 1317-1331. b) Duran, N.; Rosa, M. A.; D'Annibale, A.; Gianfreda, L., *Enz. Microb. Technol.* **2002**, 31, 907-931.
[122] a) Yaropolov, A. I.; Skorobogat'ko, O. V.; Vartanov, S. S.; Varfolomeyev, S. D., *App. Biochem. Biotechnol*. **1994**, 49, 257-280. b) Xu, F.; Li, K.; Elder, T. J., *Prog. Biotechnol*. **2002**, 21, 89-104. c) Baldrian, P., *FEMS Microbiol. Rev*. **2006**, 30, 215-242. d) Morozova, O.; Shumakovich, G.; Shleev, S.; Yaropolov, Y., *App. Biochem. Microbiol*. **2007**, 43, 523-535.

Laccase (EC 1.10.3.2) belongs to the so-called blue-copper family of enzymes; these are extracellular glycoproteins which are ubiquitous in nature. They were first reported in higher plants and virtually in all fungi which were studied for them.[123] The first description of a laccase goes back to the end of the 19th century when Yoshida and his group identified the protein as a component of the resin duct of the lacquer tree *Rhus vernicifera*.[124]

Yet, it has been used from centuries and traces of it were found in the polychromy of the Chinese Terracotta Army in Lintong of the first Chinese emperor Quin Shihuandgdi.[125]

In light of the exhaustive reviews written regularly each year for a decade now, one can see that laccase has found a wide range of application fields ranging from oxidising catalyst for waste-water treatment-biosensor production-electrochemical cells components, to oxidising reagent in organic chemistry-catalyst for biomaterial productions and catalyst for polymerisation processes. These last fields of application have stirred more deeply our interest and some examples will be briefly exposed in the next sections.

#### *1.5.3.1 Laccase as catalyst in polymerisation reactions*

Traditionally laccase has been involved in the polymerisation /depolymerisation of natural phenolic type materials such as lignin and lignin building blocks. Catalytic oxidative polymerisation of phenols has significant advantages such as the use of halogen-free monomers, moderate reaction temperatures, and the production of water as by-product.

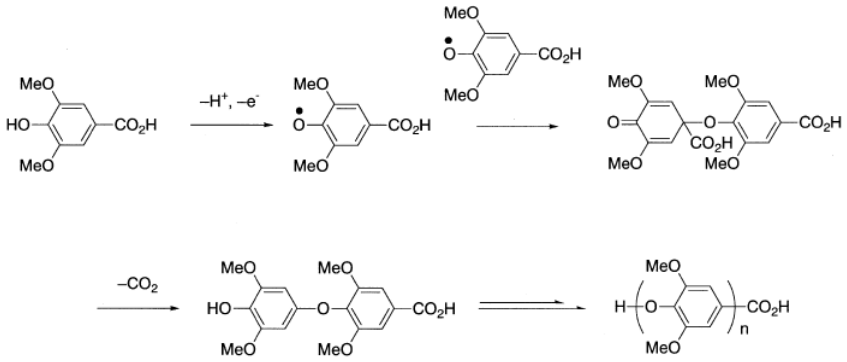
[123] Thurston, C. F., *Microbiol.* **1994**, 140, 19-26.

[124]Yoshida, H., *J. Chem. Soc.* ,**1883**, 43, 472-486.

The catalytic oxidative polymerisation, therefore, is to be regarded as a clean and low-loading process for synthesis of phenolic polymers.[126] The laccase-catalyzed polymerization of 2,6-$Me_2$Phenol in a mixture of acetone and a buffer (pH 5) produced P-2,6-$Me_2$P with a Mn of 2.700, and DPQ (diphenoquinone) with 57% and 40% yields, showing that the C–C coupling significantly occurs under acidic conditions. The oxidative polymerisation of syringic acid by laccase catalysis with dioxygen produced the polymer with a Mn higher than 10,000.

The postulated reaction mechanism was explained as follows (Scheme 36); the phenoxyl radical of syringic acid is formed by hydrogen abstraction and couples with another radical to give the quinoide-type intermediate. Carbon dioxide is liberated from the intermediate to afford a dimer, followed by further coupling to lead to polymer formation.

Scheme 36. Oxidative polymerisation of a phenolic substrate [126]



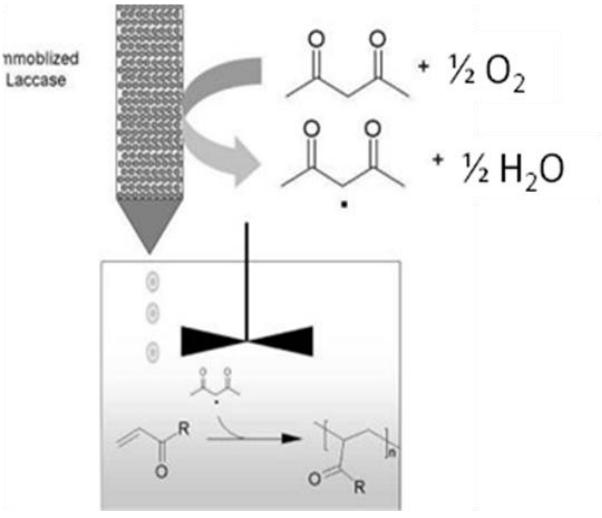

[125] Langhals, H.; Bathelt, D., *Angew. Chem. Int. Ed* **2003**, 42, 5676.

[126] Kobayashi, S.; Higashimura, H., *Prog. Polym. Sci*. **2003**, 28, 1015–1048.

Regarding another polymerisation process of industrial interest, it is to be mentioned that laccase induces radical polymerisation of acrylamide with or without mediator.[127] Poly(acrylates) are produced every year on a multi-thousand ton scale preferably via radical chain polymerisation. Polymerisation initiators are chosen from organic peroxides or azo compounds, like benzoylperoxide or AIBN. Such compounds are however somewhat questionable from environmental and work-safety point-of-views. Taking the seminal work of Kobayashi *et al* one step further, Holmann and Thum have developed a catalytic process based upon laccase in order to evaluate the scope of this reaction for industrial production of poly(acrylates).[128]

As a model reaction they chose the laccase-initiated polymerisation of acrylamide in the so-called laccase mediator system (LMS, Scheme 37) using 2,4-pentanedione (acetylacetone, Acac) as mediator. The feasibility of this reaction has been clearly demonstrated.[129]

Scheme 37. Proof of concept: laccase-mediated poly(acrylates) synthesis[129]



The polymerisation proceeded smoothly to full conversion of the substrate within less than 2 h. The polymer average molecular weight varied from 250,000 g/mol at the beginning of the reaction and to 150,000 g/mol after full conversion; thus, on average, 2100-mers were formed. Still, at present stage the LMS not (yet) fulfils the requirements of economic feasibility at industrial scale. A critical drawback remains to date which is the laccase catalytic activity with Acac needing significant improvement.

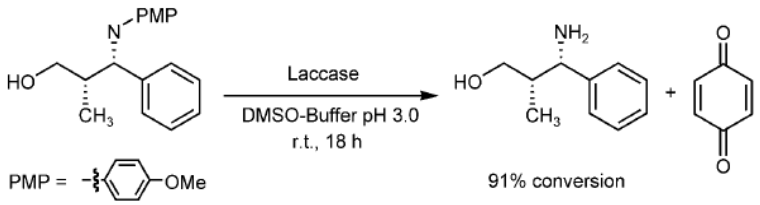
Lastly, a merging research of graft-polymerisation and biomaterial synthesis using laccase has been published recently. This is a pioneering work devoted to laccase-mediated surface modification of (bio)materials. Briefly, enzymatic covalent binding is investigated, after grafting methacrylate monomers with different amino groups (primary, tertiary, and quaternary) onto a polypropylene surface using plasma pretreatment.[130] The coupling of GSA (guaiacol sulfonic acid) in the presence of a laccase is proven by a 10-fold increase in sulfur content compared to the control.

[127] Ikeda, R.; Tanaka, H.; Uyama, H.; Kobayashi, S., *Macromol. Rap. Commun.* **1998**, 19, 423–425.
[128] Tsujimoto, T.; Uyama, H.; Kobayashi, S., *Macromol. Biosci.* **2001**, 1, 228–232.
[129] Hollmann, F.; Gumulya, Y.; Tolle, C.; Liese, A.; Thum, O., *Macromolecules* **2008**, 41, 8520-8524.
[130] Schroeder, M.; Fatarella, E.; Kova, J.; Guebitz, G. M.; Kokol, V., *Biomacromolecules*, **2008**, 9, 2735–2741.

### *1.5.3.2 Laccase as oxidising catalyst in organic synthesis*

A first example of the potential of laccase as a catalyst appeared in the protecting group chemistry. The use of laccase as a selective oxidative deprotection reagent for peptide synthesis has been reported. Semenov et al.[131] have shown that laccase can be used to remove phenylhydrazide protecting groups of carboxyl groups. The deblocking method was performed under mild conditions in aqueous medium and at pH 7.0, in the presence of oxygen. This deprotection method proceeds without alteration of the amino acid side chains. Another example concerns the deprotection of the para-methoxyphenyl (PMP) group usually performed by oxidation with cerium ammonium nitrate (CAN), a highly toxic reagent. Here again, laccase makes easily the job (Scheme 38).[132]

Scheme 38. Laccase for deprotection reaction




Laccase has been shown to catalyse the oxidative cross-coupling reaction between different molecules.[132]

[131] Semenov, A. N.; Lomonsova, I. V.; Berezin, V. I.; Titov, M. I., *Biotechnol. Bioeng*. **1993**, 42, 1137 – 1141.

[132] Witakrayan, S.; Ragauskas, A. J., *Adv. Synth. Catal*. **2009**, 351, 1187 – 1209.

Moreover, the dimerisations of penicillin X, totarol, flavonolignan silybin, and salicylic esters by laccase have already been reported.[133] Laccase has also been shown to easily perform the bioconversion of indole compounds into trimeric products. The synthetic route provides in good yield the 2,2-bis(3'-indolyl)indoxyl product which has been isolated from natural source, i.e. bacterial life forms.[134] This supports the postulated involvement of oxidases in the biosynthesis of such natural products.

Another elegant application of the biocatalyst has been recently exposed with the oxidative coupling reaction yielding the critical dimer intermediate **108** precursor of anhydrovinblastine **109**.[135] The efficient enzyme-catalysed coupling of the indolic alkaloids, catharanthine **105** and vindoline **107**, was carried out by exploiting an oxidoreductase (laccase) and atmospheric oxygen. Following $NaBH_4$ reduction of the eniminium cationic intermediate **108**, the title product **109**, was isolated in good yields (Scheme 39). The biocatalysed process provides an alternative to the traditional chemical oxidation which can be achieved with peracids (the so-called Potier–Polonovski route) or $Fe^{3+}$ salts.

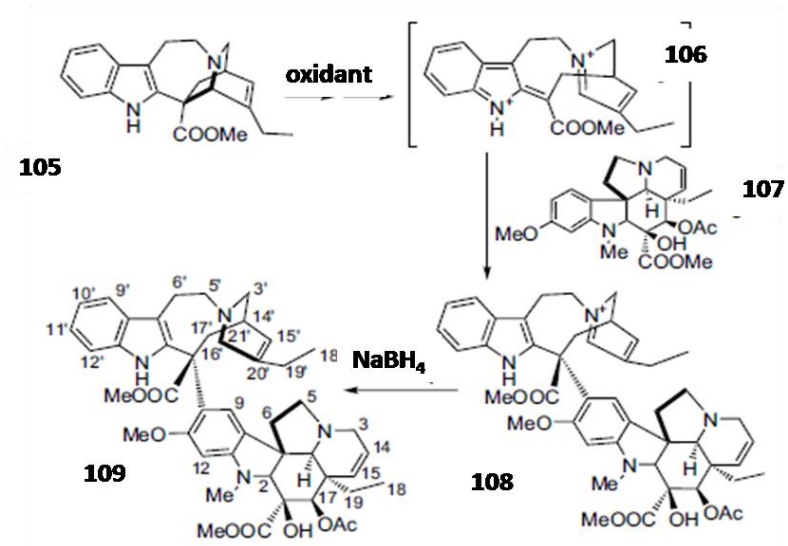
[133] Chung, J. E.; Kurisawa, M.; Uyama, H.; Kobayashi, S., *Biotechnol. Lett*. **2003**, 25, 1993 – 1997. b) Agematu, H.; Tsuchida, T.; Kominato, K.; Shibamoto, N.; Yoshioka, T.; Nishida, H.; Okamoto, R.; Shin, T.; Murao, S., *J. Antibiot.* **1993**, 46, 141 – 148. c) Ncanana, S.; Baratto, L.; Roncaglia, L.; Riva, S.; Burton, S. G., *Adv. Synth. Catal.* **2007**, 349, 1507 – 1513.

[134] Ganachaud, C.; Garfagnoli, V.; Tron, T.; Iacazio, G., *Tetrahedron Letters*, **2008**, 2476-2478.

[135] Sagui, F.; Chirivı`, C.; Fontana, G.; Nicotra, S.; Passarella, S.; Riva, S.; Danieli, S., *Tetrahedron*, **2009**, 65312–317.

Another interesting use of laccase in organic synthesis is the in situ oxidation of phenolic substrates to the corresponding quinones. The quinonic derivative is then quenched with other reagents to provide the desired final product. Two groups have demonstrated the wide potential of the strategy. Beifuss *et al* have developed an efficient approach to 3,4-dihydro-7,8-dihydroxy-2H-dibenzofuran-1-ones through the laccase-initiated domino reaction of 1,3-diketones with catechols using air as the oxidant (Scheme 40).[136]

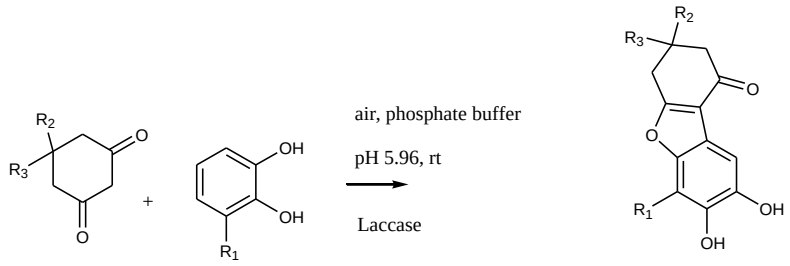
Scheme 39. Alternative to the Potier- Polonovski route [135]




[136] Leutbecher,H.; Hajdok, S.; Braunberger, C.; Neumann, M.; Mika, S.; Conrad, J.; Beifuss, U., *Green Chem*., **2009**, 11, 676.

Suteera and Ragauskas have disclosed the tandem synthesis of naphthoquinones, involving the reaction of laccase-generated quinones and acyclic dienes via the Diels–Alder (DA) reaction (Scheme 41). The reaction was carried out under mild conditions in aqueous medium and yielded naphthoquinones up to 80%.[137]Such green chemistry is directly inspired from the seminal work of Waldmann. Indeed, the combination of enzymatic with nonenzymatic transformations for tandem reactions was first reported by Waldmann and co-workers in 1998.[138]

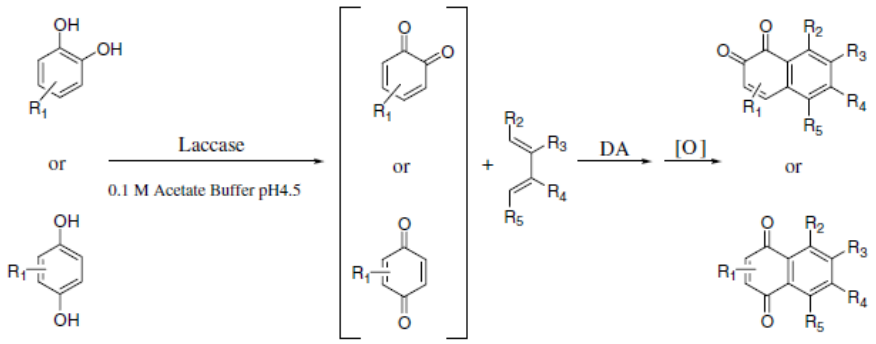
Scheme 40. Tandem reaction initiated by laccase [136]




R1: H, Me, OMe
R2: H, Me
R3: H, Me, Ph

[137] Witayakran, S.; Zettilib, A.; Ragauskas, A. J., *Tetrahedron Letters*, **2007**, 48 2983–2987.
[138] Müller, G. H.; Lang, A.; Seithel, D. R.; Waldmann, H., *Chem. Eur. J.* **1998**, 4, 2513–2522.

Scheme 41. Tandem reaction with D.A. transformation [137]




With these selected examples, we have highlighted the attractive potential of laccase as a biocatalyst. Its ability to produce reactive quinonic species which can be used subsequently in tandem reactions or dimerisation transformations provides a powerful synthetic route towards natural products and analogues. As laccase shares with phenoxazinone synthase the same specific organisation of the catalytic copper sites, these oxidases are readily exchangeable in synthetic strategies devoted to fine chemical production.

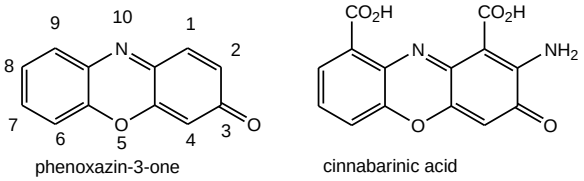
# CHAPTER 2. OBJECTIVES AND STRATEGY

## 2.1 Context of the research

Linked to the European project SOPHIED (NMP2-CT2004-505899), our field of research focuses on the development of alternative synthetic routes towards novel dyes featuring tuneable water-solubility.[31] By mimicking the nature which produces numerous heterocyclic pigments through oxidation reactions, our synthetic route should involve an oxidative-cascade initiated by an enzyme such as copper oxidase.[96, 99, 100] In collaboration with the SOPHIED partners, the choice of a laccase as biocatalyst has been settled.

Regarding the natural pigments whose production involves a copper oxidase, the cinnabarinic acid pigment appeared as a valuable lead compound for the development of our bio-catalysed synthetic process (Figure 6).[109] The core structure is a phenoxazin-3-one chromophore which is ubiquitous in the large class of the synthetic oxazone dyes. [56-60, 139]

Figure 6. Phenoxazin-3-one chromophore and cinnabarinic acid

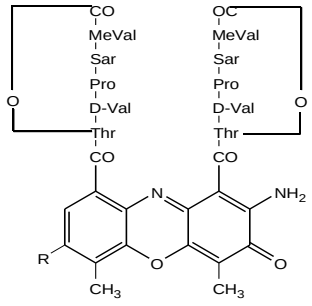



In the literature, the in-vitro productions of the 2-aminophenoxazin-3-one, the cinnabarinic acid, and the related chromophore of the natural actinomycin-D using a laccase were previously published.[109]

Yet, there is no report of an exhaustive investigation of a laccase-mediated synthesis of 2-aminophenoxazin-3-ones bearing side chains which do not consist of a carboxylic amide group. Moreover, the anchoring positions of those functional groups remain restricted to the carbon atoms C1 and C9.[105-109]

Finally, the actinomycin-D chromophore is known to exhibit slight fluorescence properties whereas its derivative, the 7-amino-actinomycin-D, possesses a stronger fluorescence ( $\Phi_F = 0.016$, $\lambda_{em}$ = 505 nm, pH 7 ) which has been used in the development of probes in the field of histochemistry and molecular biology.[140] Such a photophysical property linked to the tuneable water-solubility could open the way to a new class of fluorogenic probes (reactive or not) related to the phenoxazin-3-one chromophores.

Figure 7. Fluorogenic chromophores of actinomycin derivatives




R = H ; Actinomycin D
R = $NH_2$ ; 7-amino-actinomycin D

[139] a) Otsuki, S.; Taguchi, T., *J. Photochem. Photobiol. A: Chemistry* **1997,** 104 189- 195.

[140] a) Gill, J. E.; Jotz, M. M.; Young, S. G.; Modest, E. J.; Sengupta, S. K., *J. Histochem. Cytochem.* **1975,** *23*, 793-799. b) Philpott, N.; Turner, A.; Scopes, J.; Westby, M.; Marsh, J.; Gordon-Smith, E.; Dalgleish, A.; Gibson, F., *Blood* **1996**, 87, 2244-2251. c) Yoo, H.; Rill, R., *J. Biochem. Mol. Biol*. **2003**, 36, 305-311.

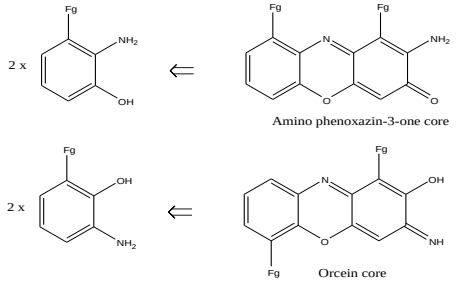
## 2.2 Objectives

As disclosed in the introduction, a large number of natural pigments arise from oxidative pathways in which enzymes such as copper oxidases play a prominent role. More precisely, in the more specific class of the pigments named omnochromes, the building blocks for the oxidative coupling reactions are aminophenol derivatives.

### *2.2.1 Laccase-mediated synthesis*

Our objective is to develop an efficient biocatalytic system based on the oxidative-cascade initiated by a laccase using original sulfonated (or phosphorylated) substrates. Functionalisation of the adjacent position of the aniline or phenol groups is therefore a prerequisite feature for our building blocks. In the first case, the starting material is a simple aminophenol derivative (or at least one of those functional groups must be present). In the second case, the successive insertion of the side chain and the precursor of the missing atom, i.e. N or O, has to be realised in high yield and a minimum number of synthetic steps.

Scheme 41. Novel dyes featuring tuneable water-solubility




Fg = Fuctional group

Fg = $SO_3H$, $PO(OH)_2$, $SO_2NH_2$
Fg = $SO_2NH$-cyclohexyl, $SO_2NH$-$(CH_2)_2$-$NH_2$, $SO_2NH$-$(CH_2)_2CH_2OH$
Fg = $SO_2NH$-$(CH_2)_3NMe_2$
Fg = $SO_2NH$-$(CH_2)CO_2Me$

An innovative synthetic route towards a 2-aminophenoxazin-3-one derivatives displaying water-solubility implies that our oxidative cascade initiated by the enzyme must be the last synthetic step. This rules out the production of unsubstituted 2-aminophenoxazin-3-one by laccase oxidation, and subsequently its derivatisation by an electrophilic aromatic substitution to insert other functional groups ($SO_3H$, $NO_2$, halogen). [105-106]

Similarly, using the free carboxylic acid group of cinnabarinic acid to link a hydrophilic moiety such as α-sulfo-β-alanine is feasible.[63] Yet, the laccase production of cinnabarinic acid does not exceed a yield of 70% and the expensive starting material is not easily produced.[109, 141] Hence the synthesis of several sulfonated or phosphorylated building blocks which should be coupled afterward by a copper oxidase, here a laccase, remains the more challenging synthetic route.

### *2.2.2 Tuneable water-solubility*

The common way to increase the water solubility of pigments takes advantage of the direct incorporation of ionisable hydrophilic groups i.e. carboxylic, phosphonic, sulfonic, and ammonium functions.[62-66] The alternative would be to link the hydrophobic chromophores to hydrophilic polymers such as carbohydrates, oligonucleotides and polyethylene glycols.[63] Both strategies have been widely used with the classes of benzo[a]phenoxazine and xanthene dyes for example. [93-95] From the literature, it is obvious that cinnabarinic acid which contains two free carboxylic groups in positions C1 and C9 is still a pigment.[109] Even worse, the molecule is sparingly soluble in aqueous solutions and organic solvents.

[141] Nguyen, T.-H.; Chau, N. T. T.; Castanet, A.-S.; Nguyen, K. P. P.; Mortier, J., *J. Org. Chem.* **2007**, 72, 3419-3429.

Since numerous water-soluble fluorogenic probes contain ionisable acidic groups we have chosen to concentrate on the incorporation of sulfonic and phosphonic acids into the core structure of the chromophore.[23, 46] Sulfonic and phosphonic acids being bioisosters of the carboxylic group, we hope that the enzyme will efficiently initiate the oxidative cascade with our substrates.[86]

Considering that the laccases were reported to oxidise sulfonated dyes and to produce colourful molecules from sulfonated aniline, we intend to investigate whether or not the laccase could accommodate sulfonated side chains of different natures. Indeed, we plan to tune the water-solubility of our novel dyes using side chains such as primary sulfonamide, alkyl-sulfonamide and aryl-sulfonamide. The alkyl-sulfonamides will be chosen so that the end of the side chain displays functional groups such as an amine or hydroxyl group, a carboxylic ester and an *N*-dialkyl moiety.

### *2.2.3 Mechanistic insight of the catalysed dimerisation*

The mechanism of 3-hydroxyanthranilic acid dimerisation through laccase catalysis has been postulated in the literature.[109] Such mechanism, which somehow differs from authors regarding the first site of electron abstraction is mainly inspired from the one described for the actinomycin-D chromophore synthesis by the phenoxazinone synthase.[110] In this mechanistic investigation, the authors described an oxidative cascade realised in three successive $2e^-$ oxidation processes. In addition, the first coupling step is proposed to occur at the entry of the catalytic site of the phenoxazinone synthase. [110,142]

[142] a) Le Roes-Hill, M.; Goodwin, C.; Burton, S., *Trends Biotechnol*. **2009**, 27, 248-258. b) Suzuki, H.; Furusho, Y.; Higashi, T.; Ohnishi,Y.; Horinouchi, S., *J. Biol. Chem*. **2006**, 281, 824–833.

In the case of laccases, a common mechanism of four successive $1e^-$ oxidation reactions linked to the reduction of molecular dioxygen into water is generally accepted.[112-115] Such mechanism is mainly based upon data obtained with phenol and aniline derivatives.[143] For 3-hydroxyanthranilic acid, the postulated mechanism involves two phenoxyl radicals produced by the laccase which could react together to give a quinonoid species and a reduced aminophenol.[109] Those phenoxyl radicals may diffuse from the catalytic site towards the bulk of the solution over a long distance.[109, 144] This mechanism was postulated bearing in mind a role of 3-hydroxyanthranilic acid as a mediator in the radical depolymerisation of the lignin by a laccase from white-rot fungi. Recently, such depolymerisation has been observed in mutants that do not produce any 3-hydroxyanthranilic acid.[145] So several questions remain regarding the laccase production of cinnabarinic acid and related heterocyclic scaffolds:

- Does the oxidation involve 1 or $2e^-$ abstraction when pro-quinonoid compound are specifically involved.

- Does the first 1,4 addition occur in the catalytic site of the laccase or further in the bulk of the solution.

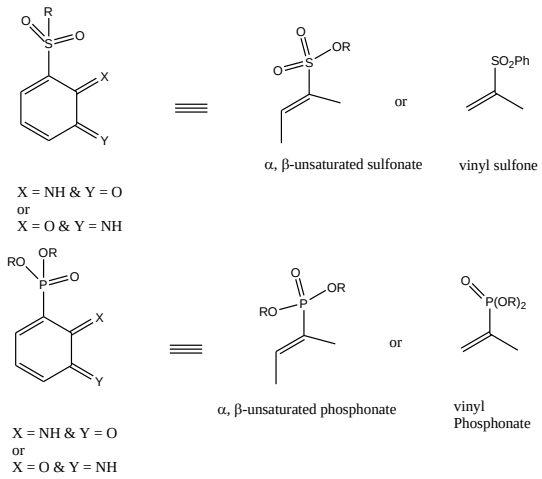
- Does the first 1,4 addition occur regioselectively at the carbon adjacent to the sulfonated (phosphorylated) group (Figure 8).

[143] Xu, F., *Biochemistry* **1996**, 35, 7608-7614.

[144] Tatiana, L. I. S.; Simándi, M.; Gyr, M.; Rockenbauer,A.; Gömöry, A., *Dalton Trans.* **2004**, 1056-1060.

[145] Li, K.; Horanyi, P. S.; Collins, R.; Phillips, R. S.; Eriksson, K., *Enz. Microb. Technol*. **2001**, 28, 301-307.

Figure 8. Likely structural equivalences for the electrophile partner




### *2.2.4 Fluorescence labelling in molecular biology*

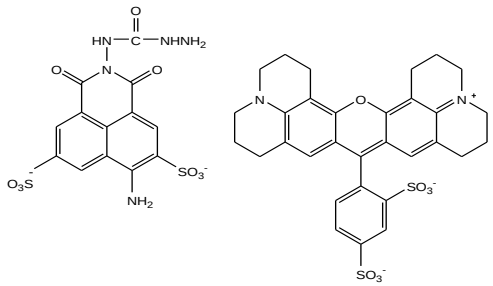
Fluorescent organic dyes are widely used as non radioactive and non invasive labels in biological analysis.[23, 46] Owing to the tuneable water-solubility of our potential dyes, a broad range of applications may be targeted. More specifically, the novel dyes could be tested in a live-cell imaging application in molecular biology. Indeed, the fluorescence labelling of living cells is of critical importance to study the cellular metabolism. Several sulfonated dyes like Lucifer yellow or the red sulforhodamine (Figure 9) are routinely used as fluid phase endocytosis tracers.[146] We can directly compare our dyes with one of those commercial dyes used as a reference. Moreover, such application is well suited to quickly assess few photochemical properties and the possible cellular toxicity of our molecules.

[146] a) Gitan, R.; Eide, D., *Biochem. J*. **2000**, 346, 329-336.

b) Vertut-Doi, A.; Ohnishi, S.-I.; Bolard, J., *Ant. Chemotherap* **1994** 2373-2379.

Depending on the results obtained, we will keep in mind that an aromatic substitution of our chromophores at the position C7, similarly to the 7-amino-actinomycin-D produced from actinomycin-D, is likely to improve their fluorescence properties if required. [106, 140]

Figure 9. Lucifer yellow (left) and sulforhodamine (right) dyes




## 2.3 Strategy

### *2.3.1 Selective synthesis of the building blocks*

Considering aromatic building blocks, several synthetic procedures were first rejected:

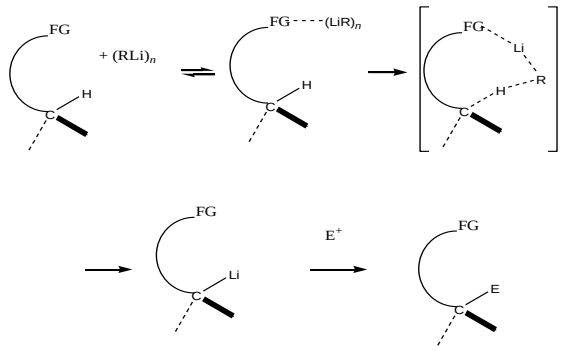
- The electrophilic aromatic substitution was discarded due to the lack of control of the regiochemistry with aminophenols.[67, 94]

- The Miyazaki-Newman-Kwart rearrangement which converts a phenol into a thiol is a viable alternative but it requires a sacrificial phenol on the right position. [69]

- An unusual electrophilic substitution in the aromatic series via organosilicon intermediates, based upon the ipso silyl effect, could offer the desired selectivity. Yet, it would require the prior insertion of a silyl group.[67, 68]

The most straightforward and convenient procedure we retained involves the proton abstraction from activated arenes.[68] The organometallic intermediates are generated by hydrogen-metal exchange and the regiochemistry is provided by various metallation directing groups, usually heteroatoms.[74] Depending on the nature of the heteroelements present on the activated arenes, an optional selectivity may be achieved.[147] The marked advantage of the reaction is the ability to readily insert an oxidised sulfur or phosphorus group into the carbon-metal bond with high yield.[148]

Scheme 43. Deprotonation of an organic substrate with organolithium reagent. FG = coordinating functional group, $E^+$ = electrophile.



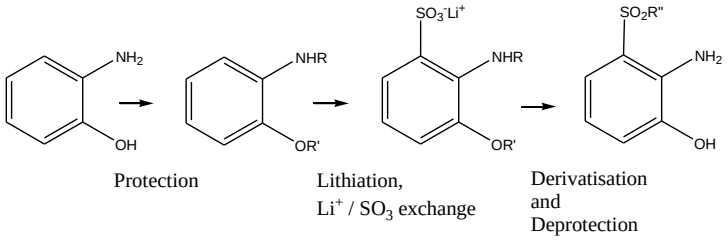

[147] a) Schlosser, M., *Organometallics in synthesis. A manual*, 2nd ed., Chichester, **2002**. b) Maggi, R.; Schlosser, M., *J. Org. Chem*. **1996**, 61, 5430-5434.

[148] Smith, K.; Hou, D., *J. Org. Chem*. **1996**, 61, 1530-1532.

*2.3.1.1 3-hydroxyorthanilic derivatives*

The synthetic route proposed includes first one or two high yielding steps of protection. Afterward, the selective metallation and subsequent metal exchange with the commercial and safe sulfur trioxide trimethylamine (STTAC) complex gives the sulfonated intermediate. The conversion of the free sulfonic acid into sulfonamide is followed by two steps of deprotection to give the desired building blocks.

Scheme 44. Synthetic scheme



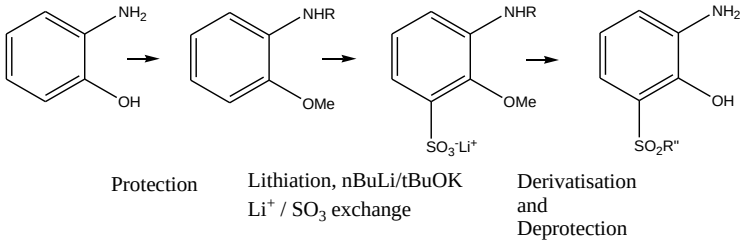

The same strategy can be applied for the preparation of the corresponding phosphorylated derivatives.

*2.3.1.2 2-hydroxymetanilic derivatives*

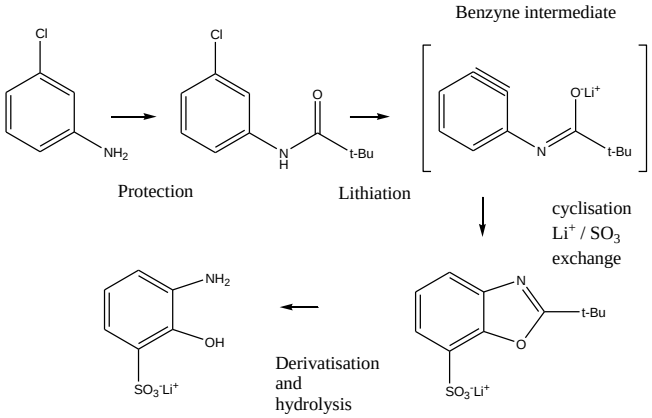
For the synthesis of those substituted aminophenols, two synthetic routes were considered (respectively Scheme 45 and 46). The first synthetic scheme is identical to the one proposed for the 3-hydroxyorthanilic derivatives but with an inversion of the regioselectivity in the directed metallation reaction. This could be achieved by using the superbasic mixture of Schlosser.

Briefly, the superbase is produced by mixing equivalent quantities of potassium *tert*-butanolate and *n*-butyllithium. This mixed base is highly sterically hindered and if the protecting group of the phenol is small, like a *O*-methyl ether, selectivity may be obtained in favour of the adjacent position to the protected phenol.

Scheme 45. First synthetic scheme [147]




Scheme 46. Selected benzyne pathway




While reported in the literature, the complete inversion of selectivity may quite be difficult to achieve. In addition, such regioselectivity has been observed with a good electrophile, i.e. methyl iodide, but there is no information on the behaviour of more exotic reagents such as oxidised sulfur or phosphorus groups.[147]

Therefore, an alternative synthetic route was envisaged from a different starting material (Scheme 46). The key step remains the directed metallation reaction but using the more challenging benzyne pathway.[149] A benzyne intermediate is highly reactive and the intramolecular cyclisation followed by metal/$SO_3$ exchange can be realised with caution to decrease likely competitive side reactions. The process has been successfully used with gaseous sulfur dioxide as the electrophile.[79]

Still, no report is available on attempts to produce such sulfonated benzoxazole intermediate using another oxidised sulfur group. The resulting sulfonated benzoxazole can be converted into the desired sulfonamides and benzoxazole ring hydrolysis gives the desired sulfonated building blocks.

We have now settled the choices of our synthetic routes towards the required substituted aminophenols which will be used as building blocks for the coupling reactions. Let us say a few words regarding our key step of biocatalysed cascade reaction. The principle is easily perceived but the method to study the reactions is still to debate.

### *2.3.2 HPLC analytical method*

The oxidative cascade will be developed in aqueous solution, preferably a buffered medium to maintain the stability of our laccase. The biocatalyst will be of commercial source to ensure the reproducibility and the generality of the bioprocess. An efficient and informative analytical method is required to follow the progress of the reaction. The most obvious method is the UV-Vis spectroscopy.

[149] Yoshida, Y.; Hamada, Y.; Umezu, K.; Tabuchi, F., *Org. Process Res. Dev.* **2004,** 8, 958-961.

The knowledge of the specific absorption pattern for the 2-aminophenoxazin-3-one core structure readily offers a way to detect the formation of our dyes. Yet, we expect to form in the non optimised conditions, several oxidised products which will likely be also colourful. Hence, we need a method making the unambiguous distinction between our desired dyes and the side oxidised products.

We have chosen to develop a suitable HPLC analytical method which will ensure the separation and clear distinction between starting materials, desired dyes and side products. The method will be developed bearing in mind the requirement to perform separations at the semi-preparative scale, after the preliminary trials made at very small scales. The HPLC analysis of sulfonated dyes and aromatics is an arduous task. Several reviews point out the need to conceive specific methodologies for different classes of ionised organic sulfonic acid.[150]

Briefly, we will make use of a reverse-phase HPLC column dedicated to separations in the presence of high proportions of water and strong acids. As an additive, we have selected the commercial methanesulfonic acid to ensure a sufficient equilibrium of protonation of our molecules. Indeed, under the protonated form, the retention and separation are largely improved.

[150] a) Wangkarn, S.; Soisungnoen, P.; Rayanakorn, M.; Grudpan, K., *Talanta* **2005**, 67, (4), 686-695. b) Chen, M.; Moir, D.; Benoit, F. M.; Kubwabo, C., *J. Chrom. A* **1998**, 825, (1), 37-44. c) Christen, S.; Stocker, R., *Anal. Biochemistry* **1992**, 200, (2), 273-279. d) Comas, E.; Gimeno, R. A.; Ferre, J.; Marce, R. M.; Borrull, F.; Rius, F. X., *J. Chrom. A* **2003**, 988, (2), 277-284. e) Reemtsma, T., *J. Chrom. A* **1996**, 733, (1-2), 473-489.

### *2.3.3 Evaluation of the dyes in fluorescence labelling*

In collaboration with the group of Professor P.J. Courtoy, we will evaluate our compounds as surrogate fluorophores of commercial dyes. The selected application will be the fluorescence labelling of the fluid phase endocytosis pathway i.e. the way how cellular membranes create invaginations and internalise extracellular fluids and molecules.

The sub-cellular location of phenoxazines will be likely linked to their solubility properties (hydrophobic-hydrophilic balance). The ability of our water-soluble dyes to efficiently label sub-cellular compartments will be directly evaluated. Prior the assay with living cells, the relative quantum yields of our novel dyes will be determined using the dye rhodamine 6g as a reference ($\Phi_F = 0.95$).

Now that the objectives of this work have been settled and the selected strategies to produce and evaluate our target chromophores have been described, we will report the experimental results obtained in this thesis work. The manuscript organisation is presented below.

## 2.4 Content of the manuscript

The previous section 1 and this one have presented respectively the literature on the subject, and our working plan. The next sections corresponding to the results and discussions have been divided into five chapters, each focusing on a particular topic exposed in the objectives. Noteworthy, four chapters concern works either already published (chapters 3 and 4), or close to be submitted for publication (chapters 6 and 7) in peer reviewed journals. One chapter, (chapter 5) discloses experimental results which are briefly reported and linked to a proposal of mechanism having the merit to plausibly explain part of several puzzling results.

- The chapter 3 focuses on the proof of concept of our biocatalysed synthetic route. The development of the regioselective synthesis of our model compound, 3-hydroxyorthanilic acid, is reported. (F. Bruyneel *et al*, *Eur. J. Org. Chem*. **2008**, 1, 72-78)

- The chapter 4 exposes the scope and limitations of our catalytic system. The syntheses of the remaining building blocks, the structural elucidations of the formed dyes, and several physico-chemical properties of the substrates and products are reported. (F. Bruyneel *et al*, *Chem. Eur. J.* **2009**, 15, 8283-8295)

- The chapter 5 unveils the several experimental results obtained in cross-coupling experiments. Interestingly, those results plausibly give a better insight to the mechanism involved in our catalytic system. Chemical oxidations are also compared to the laccase-mediated air oxidation.

- The chapter 6 brings to light the first synthesis of a bis-phosphorylated dye. This original compound, along with the sulfonylated dyes were tested in a live-cell imaging application. (F. Bruyneel *et al*, *ChemBioChem*, to be submitted)

- The chapter 7 uncovers results focused on the synthesis of new water-soluble 2-substituted-benzoxazoles. We use an acid catalysed condensation reaction which was serendipitously discovered in the chapter C. (F. Bruyneel *et al*, *Synlett*, to be submitted)

The manuscript will be ended by a chapter 8 of conclusions and outlooks which will summarise the pertinent results obtained and the more attractive perspectives. In the supporting material, a section I will briefly detail the basic principles of light absorption and emission phenomena, few experimental results are disclosed in a section J which were not reported in the publications. Residual sections are the supporting material related to full papers published or soon to be published.

# CHAPTER 3. PROOF OF CONCEPT

## 3.1 Foreword

The results presented hereafter were obtained in the frame of the project SOPHIED (NMP2-CT2004-505899) funded by the Sixth Framework Program of the European Commission (www.sophied.net/). SOPHIED is a joint research and innovation initiative, led by the Université catholique de Louvain, and has as an objective the development of novel and sustainable bio procedures for the European colour industry.

The project which partially funded our research work focuses on the development of a new bioprocess based on oxidase enzymes i.e. laccases. As part of the multidisciplinary group of research, our task consisted mainly in developing the proof of concept for the synthesis of novel water-soluble dyes displaying good colouring properties and a decreased toxicity. The key step has to use a laccase for performing the biomimetic synthesis of phenoxazine frameworks in aqueous solution. As a member of this scientific project we were encouraged to take an active part into the intellectual protection of the discoveries (Patent, PCT/EP2009/058640) and the dissemination of our scientific results in the international scientific community.

Hence, the full paper disclosed hereafter was published in the European Journal of Organic Chemistry in January 2008. The publication directly followed the scientific communication we presented at the 8$^{th}$ International Symposium on Biocatalysis and Biotransformations which was held from the 8 to 13 July 2007 in Oviedo (Spain).

## 3.2 Main topics addressed in the publication

The first SOPHIED milestone consisted in the development of a selective synthesis of water-soluble building-blocks for the subsequent reaction of oxidative cyclising dimerisation. The regioselectivity was achieved through the use of the Directed Metallation Reaction which has proven to be a selective and high yielding process when the starting material contains an aromatic nucleus. The insertion of an oxidised sulfur group into the carbon-metal bond was performed with good yields, as usually reported in the aniline series.

Inspired by the synthetic researches devoted to monobactam antibiotics, which are *N*-sulfonated azetidinone compounds, we developed a methodology focused on the isolation of a free organic sulfonic acid. The process, easily scalable, involves an ion-pair extraction using a lipophilic salt (*n*-$Bu_4HSO_4$). Adsorption of the lipophilic salt upon an ion exchange resin bearing cationic groups allows first the recovery of the amino counter ion (*n*-$Bu_4Cl$). The subsequent elution in acidic water affords the free organic acid as a pure compound.

While performing the cleavage of a methyl ether (phenol protecting group), the serendipitous discovery of an intramolecular cyclisation leading to 2-alkyl-4-sulfonic acid-benzoxazole occurred. The specific position of the acid ($SO_3H$) on the aromatic nucleus and its strong properties as Brönsted acid probably catalysed the dehydrating cyclisation involving the vicinal amide chain. This property is exploited in the section G of the manuscript to synthesise novel water-soluble 2-aryl-4 or 7-sulfonated benzoxazoles.

Finally, similarly to the biosynthesis of Actinomycin-D antibiotic, we set up the first laccase-mediated synthesis of a water soluble aminophenoxazinone dye.

The pure compound could be isolated after careful optimisation of the biocatalysed reaction, using a HPLC method specially designed for our molecules.

## 3.3 Full Paper

***Journal***

*Eur. J. Org. Chem*. **2008**, 1, 72-78.

***Title***

Regioselective synthesis of 3-hydroxyorthanilic acid and its biotransformation with laccase into a novel phenoxazinone dye.

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***Abstract***

A natural phenoxazinone derivative, namely cinnabarinic acid **2** (CA, R=$CO_2H$), could be obtained in vitro with purified laccase from the fungi Pycnoporus cinnabarinus by oxidative dimerization of 3-hydroxyanthranilic acid **1** (3-HAA, R=$CO_2H$). With the aim of entering into a new class of water-soluble chromophores and potential bioactive molecules, the regioselective sulfonation of 2-hydroxyaniline was investigated. Straightforward insertion of sulfonate group into carbon-metal bond provided an efficient and selective process for the synthesis of 3-hydroxyorthanilic acid **3** (3-HOA, R = $SO_3H$). This sulfonated compound was then submitted to laccase oxidation in order to mimic the cinnabarinic acid synthesis. A practical HPLC method was developed to study the oxidized products and the major product of biotransformation, namely 2-amino-3H-phenoxazin-3-one-1, 9-disulfonic acid **4** (LAO, R = $SO_3H$), was isolated and identified as the sulfonic analogue of cinnabarinic acid.

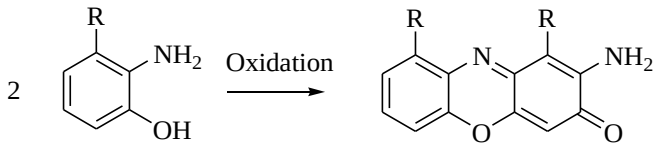
***Keywords***

Laccase / Phenoxazinone / Lithiation / Biotransformation / Aromatic sulfonation reaction.

## *1. Introduction*

Cinnabarinic acid **2** is a red phenoxazinone compound responsible of the Pycnoporus species colour.[1-3] White rot fungi produce cinnabarinic acid from 3-hydroxyanthranilic acid **1** (3-HAA) through oxidative dimerization catalysed by laccase (Scheme 1). Furthermore, phenoxazinones have been identified in various biological systems including pigments and antibiotics. For instance, actinomycin is an antibiotic which is formed by phenoxazinone synthase, an enzyme of Streptomyces species.[1] Other phenoxazines of industrial interest comprise the Meldola Blue and Nile Red dyes.[4]

Scheme 1. Biomimetic synthesis of phenoxazinone dyes




**1**: R = $CO_2H$ (3-HAA)

**2**: R = $CO_2H$ (CA)

**3**: R = $SO_3H$ (3-HOA)

**4**: R = $SO_3H$ (LAO)

The search of water-soluble long-wavelength fluorochromophores for the detection of biological and organic molecules has recently drawn high interest. Indeed, fluorescence-based techniques are sensitive, simple and selective. In particular, phenoxazinone chromophores represent potentially good leads for the development of fluorescent probes for the detection of hydroxyl radicals.[5] These radicals are largely responsible for DNA damage by endogenous effect but also by several indirect effects.[6]

However, the poor solubility of the currently available phenoxazinone derivatives represents a drawback for biological applications. One way to render these molecules soluble in aqueous media is to add a highly polar substituent such as a sulfonic group.

To our knowledge, selective sulfonation on a specific position of phenoxazinones has never been reported before. Considering the direct sulfonation of the phenoxazinone core, the extent and selectivity of the reaction may be hard to control. Similar reactions for the synthesis of sophisticated analogs of 3-H-phenoxazin-3-one by halogenation failed.[7-9]

Furthermore, the usual chemical synthesis of phenoxazine derivatives involves the condensation of nitroso compounds at elevated temperature.[10-12] Such processes are highly toxic and energy consuming. Moreover, the cyclization of sulfonated nitrosoanilines as building blocks is not very effective.[12] On the other hand, it is possible to regioselectively introduce a sulfonic group on a phenoxazine precursor[13] which could further undergo an oxidative coupling in presence of the appropriate catalyst.[2, 3]

Nowadays, the use of enzymes integrated with traditional synthetic methods is studied in order to develop more environmental friendly processes.[14, 15] The aim of this study was to investigate the biomimetic synthesis of a sulfonated phenoxazinone derivative using laccase. A regioselective sulfonation method was developed to synthesize 3-hydroxyorthanilic acid (3-HOA), the sulfonic analogue of 3-hydroxyanthranilic acid. The laccase catalysed oxidation of this compound was further studied.

## *2. Results and Discussion*

### *2.1 Synthesis of 3-hydroxyorthanilic acid (3-HOA)*

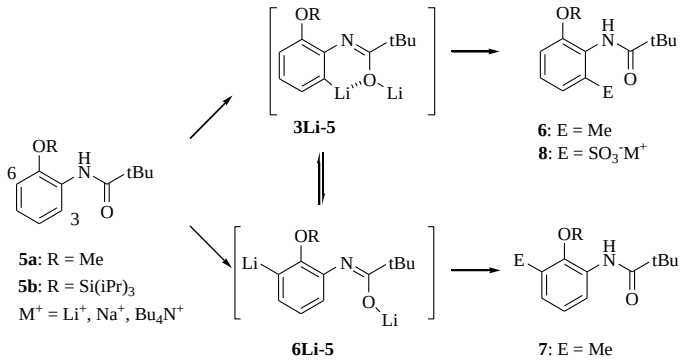
The sulfonation reaction is a rather old chemical process. Sulfonic acids clearly impact the industrial chemistry in various subfields of organic synthesis. They are used as protecting groups, as ligand modifiers[16, 17] and as bioisosteres in medicinal chemistry.[18-20]

Direct sulfonation of aromatic compounds is still the most common approach used when the aryl core is slightly complex. The regiochemistry is then controlled by the ring substituents. Another efficient method for polysubstituted aromatic compounds proceeds through the production of a sulfonated intermediate from a commercial sulfonic acid. This intermediate is then broken down in a selective way. Using such reactions, 3-HOA (**3**) has been previously reported as a by-product from the reduction of an azoïc dye.[21]

However the sulfonation regiochemistry can be also controlled by either modulating the sulfur trioxide reactivity by mean of dioxane,[22] or by using a silane substituent already present on the ring to direct the insertion reaction.[23] The ipso substitution of arylsilanes by various electrophiles has recently led to numerous interesting transformations. But, this method requires at first the introduction of a silane group on the right position by an ortho-directed metallation reaction. Therefore, the direct insertion of oxidized sulfur in the carbon-metal bond, as reported before on unsusbstituted *N*-protected aniline[13], would be the most straightforward strategy for producing 3-HOA.

Thus, our selected synthetic path starts from commercial *o*-aminophenol which already displays the required substituents for a dimerization process catalysed by laccase (Scheme 1). Minimal number of steps, inexpensive protecting groups and purification methods are required for further scaling up. Pivaloyl amide was chosen to protect the amine and a few classical protecting groups (alkyl and silyl ethers) were tested for masking the phenol. Through this preliminary screening, both combinations of methyl ether-pivalamide[24, 25] (**5a**) and triisopropylsilyl ether-pivalamide (**5b**) were selected for the development of a direct sulfonation process (Scheme 2).

Scheme 2. Direct sulfonation: quenching of the di-anion intermediate



Metallation of **5** followed by quenching of the di-anion intermediate with methyl iodide was first studied in order to optimize the experimental conditions and to control the regioselectivity (Table 1). Deprotonation of **5a** at -78 °C in THF with the couple *n*-BuLi-TMEDA or with the more basic

*t*-BuLi failed; the starting material was recovered (entries 1-2). Similar result was obtained with **5b** (entry 3). When the solution of 5a and *t*-BuLi was warmed up to –20 °C before addition of MeI at the same temperature, methylation products 6a and **7a** (scheme 2) were formed in 7:2 ratio with a conversion rate of 90% (entry 4). At –10 °C, the regioselectivity in favour of the desired regioisomer **6a** was enhanced to 8:1 (entry 6). In the case of the more hindered precursor **5b**, the yield was slightly lower (entry 5) at –20 °C and the reaction could not be performed at –10°C, due to the instability of the silyl ether. Raising the temperature of the di-anion solution seems to allow the equilibration of **3Li-5** and **6Li-5** intermediates favouring the di-anion stabilized by intramolecular complexation.

Since *n*-BuLi is more prone to be handled at room temperature than *t*-BuLi,[26] the next reactions were performed with the less basic *n*-BuLi. Thus deprotonation of **5a** at 0 °C to 25 °C and quenching at 25 °C furnished 80% of **6a**, without detectable regioisomer **7a** (entry 7). Similarly, the yield of **6b** (entry 8) was 70%. The following conditions were retained thus for deprotonation of **5**: treatment at low temperature (≤ 0 °C) with *n*-BuLi (2.4 equiv.) in THF, then reaction at 25 °C for 3h.

Table 1. Experimental conditions of ortho-metallation and quenching[a]

| | | | | E = Me[e] | | | | E = $SO_3^-M^+$ [f] | |
|---|---|---|---|---|---|---|---|---|---|
| Entry (series) | Base (2.4eq) | T0, T1[c] | T2[d] | **5** | **6** | **7** | **Others**[g] | **5** | **8** |
| 1 **(a)** | *n*-BuLi/ TMEDA | - 78, -78 | - 78 | 100% | - | - | - | | |
| 2 **(a)** | *t*-BuLi | - 78, -78 | - 78 | 100% | - | - | - | | |
| 3 **(b)** | *t*-BuLi | - 78, -78 | - 78 | 80% | - | - | 20% | | |
| 4 **(a)** | *t*-BuLi | -78, -20 | -20 | 10% | 70% | 20% | - | | |
| 5 **(b)** | *t*-Buli | - 78, - 20 | - 20 | 20% | 50% [b] | - | 30% | | |
| 6 **(a)** | *t*-BuLi | -78, -10 | -10 | 10% | 80% [b] | 10% | - | | |
| 7 **(a)** | *n*-BuLi | 0, 25 | 25 | 20% | 80% [b] | - | - | | |
| 8 **(b)** | *n*-BuLi | 0, 25 | - 20 | 20 % | 70% [b] | - | 10% | | |
| 9 **(a)** | *n*-BuLi | 0, 25 | -78 | | | | - | 85% | 15% [b] |
| 10 **(a)** | *n*-BuLi | 0, 25 | - 20 | | | | - | 30% | 70% [b] |
| 11 **(a)** | *n*-BuLi | 0, 25 | 25 | | | | - | 40% | 60% [b] |
| 12 **(b)** | *n*-BuLi | 0, 25 | -20 | | | | 10% | 40% | 50% [b] |

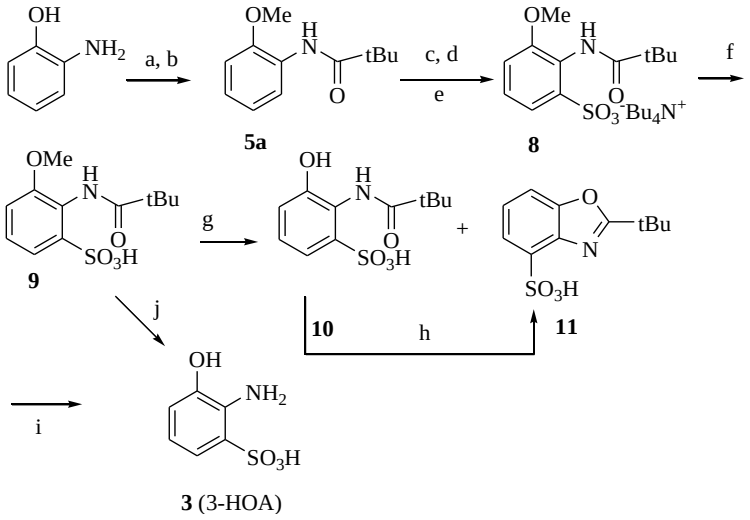
[a] NMR yields in $CDCl_3$: ratios of conversion were determined by comparison of residual proton signals of H-3, (8.42 ppm and 8.44 ppm respectively for **5a** and **5b**) with H-4 (6.83 ppm) and $CH_3$ (2.20 ppm) for **6a** ; with H-3 ( 8.22 ppm) and $CH_3$ (2.30 ppm) for **7** ; with H-4 (6.81 ppm) and $CH_3$ (2.17 ppm) for **6b**. [b] Products isolated and structures confirmed by MS, $^1H$ NMR and $^{13}C$ NMR. [c] The base was allowed to react with **5a-b** at temperature T0 for 15 min, then for 3 h at temperature T1 (°C). [d] The di-anion was allowed to react with the electrophile for 2 h at temperature T2 (°C). [e] Quenching was done by addition of 1 eq of MeI to the di-anion solution. [f] Quenching was made by addition of the di-anion solution to a suspension of sulfur electrophile ($SO_3.C_3H_9N$), the reaction time of 2 h was extended by 15 h at 25 °C.[g] Products arising from partial deprotection of the silyl ether. TMEDA = tetramethylethylenediamine

Insertion of oxidized sulfur in a carbon-metal bond is usually achieved with gazeous sulfur dioxide or trioxide. While such reagents are troublesome to use, solid sulfur trioxide complexes[27] are much easier to handle and give good yields in general. Synthesis of the sulfonated intermediate **8** ($M^+ = Li^+$) was performed by addition of the di-anion solution to $SO_3$.trimethylamine complex as the electrophile, at low temperature (Table 1). Quenching of **3Li-5a** at –78 °C was unsuccessful (entry 9). Quenching between –20 °C and +25 °C furnished 60-70% of sulfonate **8a** (entries 10-11) and only 50% of sulfonate **8b** (entry 12), without detectable contamination by the 6-regioisomers.

Hence, precursor **5a** was selected for large-scale synthesis (Scheme 3). The di-anion was produced under the optimized conditions, then this solution was added to $SO_3.Me_3N$ at –10 °C and the reaction mixture was kept at low temperature for two hours before running overnight at room temperature. Isolation of the sulfonic acid was achieved by forming its tetrabutylammonium salt **8a** ($M^+ = Bu_4N^+$), a method inspired from the production of monobactam antibiotics.[28] The following steps involved the so-called "cosmetic reactions". (Scheme 3)

The methyl ether cleavage of 8a by $BBr_3$[29] or $BCl_3$ in methylene chloride gave the expected product in high yield. However, subsequent hydrolysis of the pivalamide, either in strong acidic or basic media, was sluggish and the isolation of the final product rather difficult by chromatography on reversed phase, due to the presence of non identified by-products. This prompted us to investigate the use of a less expensive process to isolate the free sulfonic acid **9** before performing the deprotections. An anion exchange resin bearing quaternary ammonium groups was chosen in order to quench the aromatic sulfonate. Deprotonated **9** could be trapped on the column cationic residues and purified by removing all other organic compounds. Free sulfonic acid **9** was then eluted in quantitative yield.

Scheme 3. Optimised synthesis of 3-hydroxyorthanilic acid




*Reagents and conditions :*

a) 1.1 eq *t*-BuCOCl, 3 eq $NaHCO_3$, $H_2O$/EtOAc, 25 °C 4 h ; b) 1.1 eq MeI, 1.1 eq $K_2CO_3$, DMF, 0 °C, 1 h then 25 °C, 8 h ; c) 2.4 eq *n*-BuLi, THF, 0 °C, 10 min then 25 °C, 3 h ; d) 1.5 eq $C_3H_9N.SO_3$, – 10 °C to 0 °C, 2 h then 25 °C, 15 h ; e) 0.9 eq $HSO_4.N(Bu)_4$ ; f) anion exchange resin IRA-900, MeOH/$H_2O$ then HCl/$H_2O$ ; g) 3 eq $BCl_3$, $CH_2Cl_2$, -20 °C, 1 h then 25 °C, 8 h ; h) 0.5 eq TFA, MeOH, 80 °C, 3 h ; i) HCl 6 N, 80 °C, 20 h ; j) HBr 48% in water, 120 °C, 17 h.

A sulfonic acid is poorly soluble in organic solvents; nevertheless the methyl ether cleavage of **9** with $BCl_3$ could be worked up in methylene chloride. Purification by filtration on RP18 silica gel yielded the desired compound. Interestingly, it was present as a 7:3 mixture of non cyclised (**10**) and cyclised product (**11**). The sulfonic acid in *ortho* position seems to activate the amide function versus an intramolecular nucleophilic attack from the phenol and the loss of a water molecule.

Total cyclisation into 2-tert-butyl-benzoxazole-4-sulfonic acid **11** was done by gentle heating in the presence of a catalytic amount of trifluoroacetic acid. Compared with the challenging synthesis of benzoxazole-4-carboxylic derivatives'[19, 30] this sulfonic analogue offers a promising entry into a new class of valuable intermediates for fine chemicals.

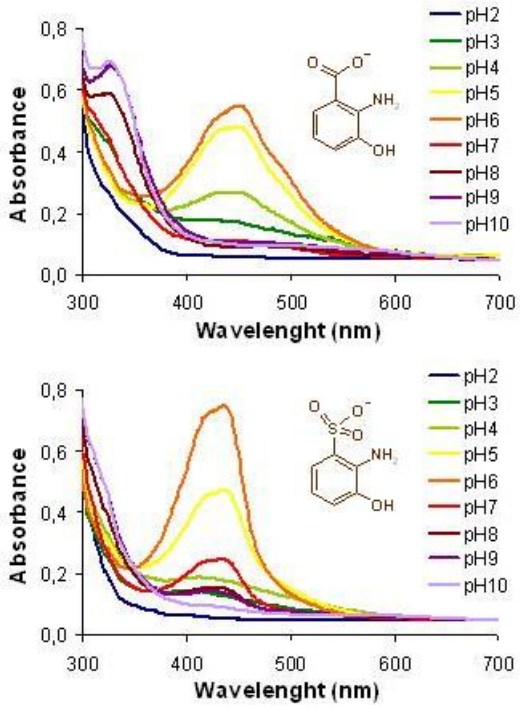
The last step of our synthesis consisted in a clean acidic hydrolysis of the imidate bond of **11** with 6N HCl. Alternatively, simultaneous deprotection of both functions could be achieved from precursor **9** by heating in aqueous HBr[31] solution. The final target 3-HOA was obtained with an overall yield of 50%. The purity of compound **3** was about 95% as determined by HPLC.

The structures of the sulfonated products **8-11** and **3** were unambiguously determined by NMR spectroscopy. The question whether the sulfonated group was inserted into the position *ortho* to the amino or to the hydroxyl group was best answered by HMQC and HMBC spectra performed on **3** (3-HOA). The signals of aromatic protons $H_4$, $H_5$ and $H_6$ were assigned to their relative carbons by HMQC. A $^3J$ correlation between the carbon at position $C_2$ (substituted with $NH_2$) and both the proton signals appearing as doublets in positions $C_4$ and $C_6$ was observed.

### *2.2 Biotransformation of 3-HAA and 3-HOA using fungal laccase – HPLC analysis*

3-HAA and 3-HOA (1 mM) were subjected to laccase (100 U L-1) oxidation in phosphate 0.1 M solution, during 24 h at 25 °C, at pH ranging from 2 to 10 (Scheme 1). The biotransformations were followed by UV spectroscopy (Figure 1). With both substrates, colour apparition was rapidly observed at pH 3 to 5.

Figure 1. UV-visible spectra of 3-HAA and 3-HOA oxidation media.




Reaction media contained 1 mM substrate, 100 U $L^{-1}$ laccase and 100 mM phosphate at pH ranging from 2 to 10. After 24 h incubation at 25°C, the spectra of 10 fold diluted solutions were recorded.

Indeed, the commercial laccase Oxizym LA displays its maximum of activity towards a reference compound, namely 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS)), at pH 3 (data not shown). 3-HAA containing media became progressively orange/brown between pH 3 and 7 while 3-HOA containing media became bright yellow in the same conditions.

After 24 h incubation maximal colouration was obtained at pH 6-7. It can be noticed that at lower pH, the colour of the media did not evolve significantly after 15 h. It seems that the replacement of the carboxylic function by the sulfonic one did not drastically affect the substrate reactivity.

3-HAA containing media showed a light coloration upon storage at pH 8 to 10, even in the absence of laccase. This phenomenon is probably due to substrate auto-oxidation.[32] Such reaction was less visible in the case of 3-HOA. The UV-visible spectrum shape of 3-HAA oxidation medium around pH 6-7, strongly resembled this of pure cinnabarinic acid (CA)[2], while spectra recorded at lower pH differed significantly. Similarly, with 3-HOA, UV-visible spectra shape of the media seemed to indicate that several by-products are present at lower pH. This suggests that more neutral conditions favour the apparition of the highly coloured phenoxazinone product; this has been checked more accurately through HPLC analyses.

The HPLC method used for 3-HAA oxidation products analysis was inspired from Iwahashi et al.[32] The biotransformation media showed the apparition of cinnabarinic acid (CA), and several by-products which amounts increased when reactions were performed at low pH. The identification of CA as oxidation product through different processes was already studied.[33- 35]

We confirmed that the main product of 3-HAA oxidation by laccase is CA by comparison of the UV-visible scan with data reported by Eggert et al[2] and by mass spectrometry analysis (APCI, positive mode) which showed the expected peak [M+H] at 301 m/z in accordance with the previous results of the literature.[2, 3, 32]

Analysis of 3-HOA biotransformation media required to set up an appropriate method. Separation of organic sulfonic acid by HPLC is not a trivial issue.[36, 37] Our optimal analytical conditions allowed the elution of the substrate after a few minutes while the oxidized products were detected during the 40 min run (Table 2).

All the oxidized products were detected at 220/254 nm and the orange-red coloured compounds were also monitored at 440/460 nm (Table 2, entries 1-5). HPLC analyses showed the apparition of at least three orange-coloured compounds in the crude media at retention times (RT) of 2.1, 23 and 26 min. As observed in the case of 3-HAA, the number and amount of by-products were higher at lower pH. At pH 6 no residual substrate could be detected and the compound eluting at 23.3 min was produced with a conversion ratio of about 85%.

Preparative HPLC was envisaged in order to purify this main oxidation product. The presence of phosphate salts from the buffer caused problems when concentration of the crude reaction mixtures was necessary. The laccase oxidation reaction of 3-HOA was therefore assayed in absence of buffer, the pH being adjusted to pH = 4, 5 and 6 by addition of NaOH/HCl. The colouration of the unbuffered media followed almost the same kinetics than the buffered ones and no significant pH modification was observed during the biotransformations.

Table 2. HPLC studies of biotransformation reactions[a]

| Entry | pH | buffer | Retention time (RT min)[b] and ratio of peak area (%)[c] | | | |
|---|---|---|---|---|---|---|
| | | | 3-HOA RT = 1.9 | By-products 2< RT < 24 | LAO RT = 23-24 | By-products RT > 25 |
| 1[d] | 3 | Yes | 2% | 25% | - | 50% |
| 2[d] | 4 | Yes | 2% | 30% | 7% | 40% |
| 3[d] | 5 | Yes | - | 15% | 75% | 10% |
| 4[d] | 6 | Yes | - | 6% | 85% | 10% |
| 5[d] | 7 | Yes | - | 25% | 40% | 20% |
| 6[e] | 4 | No | 10% | 55% | 10% | 20% |
| 7[e] | 5 | No | - | 15% | 48% | 20% |
| 8[e] | 6 | No | - | 2% | 70% | 25% |
| 9[f] | 6 | No | - | 13% | 65% | 20% |
| 10[g] | 6 | No | - | 3% | 85% | 9% |
| 11[h] | 6 | no | - | - | 90% | 7% |

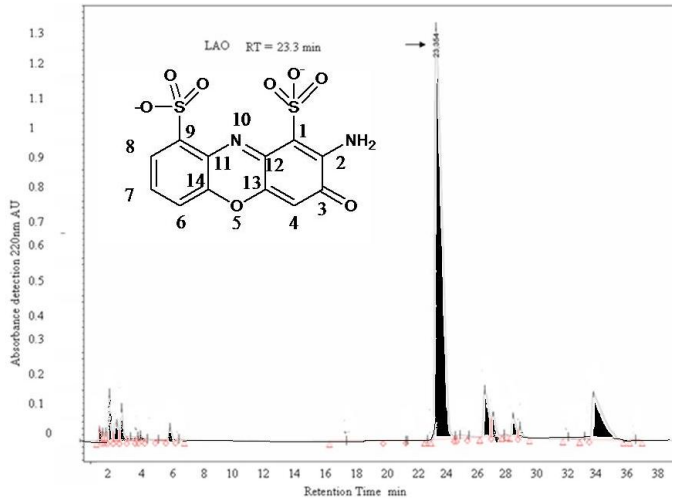
[a] The elution system for the analysis of 3-HOA and its oxidation products consisted of: A (H2O/ MSA 100 / 0.1 v/v), B (H2O/CH3CN/MSA 75/25/0.01 v/v/v), C (H2O). After a 5 min isocratic step at 100 % A, a linear gradient to 100 % C was applied during 5 min followed by a second gradient to 100 % B in 15 min. A 10 min isocratic step at 100 % B was applied to ensure the elution of all oxidation products, on-line UV-scans were performed, samples were taken after 24 h. [b] Retention times obtained as triplicates. [c] % area of peaks detected at 235 nm. [d] Sample diluted at 0.5 mM with MSA/H2O. [e] Sample at 5 mM, directly taken from crude reaction mixture. [f] Sample of 5 mM concentration from crude medium of large scale biotransformation. [g] Sample at 5 mM of fraction collected after chromatography on RP18. [h] Sample at 5 mM of fraction collected after preparative HPLC.

HPLC analyses confirmed that the same products were obtained in almost similar amounts (Table 2, entries 6-8). The UV-visible spectrum of the major red compound at RT = 23.3 min was similar to the one of a 2-aminophenoxazinone.[2, 3] Mass spectrum indicated the presence of a dimeric structure.

### *2.3. Identification of the main product obtained through 3-HOA oxidation by laccase*

A large-scale biotransformation was conducted in a higher concentration of substrate (5mM) and enzyme (200 U $L^{-1}$) at pH 6 in phosphate-free medium. The reaction was stopped after 24 h by decreasing the pH with MSA in order to inactivate the laccase. The ratio of substrate conversion was determined by analytical HPLC of the crude mixture (Figure 2).

Figure 2. HPLC chromatogram




Bioconversion reaction with commercial laccase Bioscreen (Trametes versicolor) at 25 °C with 5 mM of substrate 3-HOA. pH of 6 (adjusted with HCl/NaOH), sample taken after 24 h of reaction. LAO, major red compound of interest. Elution conditions from Table 2. (area under the peaks are filled in black for clarity)

Detection at 220/254 nm clearly showed the complete consumption of 3-HOA. The major red compound, named LAO (which stands for laccase acid orange), was well obtained and the ratio of by-products was rather low (Table 2, entry 9). Freeze drying gave a residual red powder which HPLC analysis assessed a purity of about 65% in LAO. A portion of this residue was then submitted to a chromatography on reversed phase silica RP-18 with water as solvent.

The major red fraction was collected again and analysed by HPLC. LAO was isolated with a purity of about 80% (table 2, entry 10). However, another unidentified oxidized product of red colouration was still present (RT = 26 min). Finally preparative HPLC followed by filtration over a short pad of reversed phase for removing the residual MSA allowed the recovery of LAO with a purity of about 90 % (Table 2, entry 11).

ESI mass spectrum of purified LAO showed a peak at 371 m/z with 100% intensity corresponding to the structure **4** (Scheme 1) minus one atom of hydrogen. Adducts of sodium and potassium could also be detected in lower proportions. $^{1}H$ NMR in DMSO showed the two hydrogen atoms of the amino group as non equivalents at 9.9-9.4 ppm. $^{1}H$ NMR in $D_2O$ gave the proton signals corresponding to the expected core for the sulfonic analogue of cinnabarinic acid. $^{13}C$ NMR showed clearly the twelve carbon atoms of phenoxazinone **4** (Table 3).

2D correlation experiments (HMQC and HMBC) provided unambiguous structural assignment of the novel dye LAO. The $^{3}J$ coupling observed by HMBC established a correlation between the proton $H_7$ and both carbons $C_9$ and $C_{14}$. Similar correlations could be detected for the protons $H_6$ and $H_8$ and the carbons $C_8/C_{11}$ and $C_6/C_{11}$ respectively. Finally, a correlation between the proton $H_4$ and both carbons $C_{12}$ and $C_2$ allowed the complete characterization of LAO.

Table 3. NMR data of 2-amino-3H-phenoxazin-3-one-1,9-disulfonic acid 4 (LAO)

| H No. | δ ppm[a] | δ ppm[b] | C No. | δ ppm[c] |
|---|---|---|---|---|
| - | - | - | 1 | 123.2 |
| 2 | - | 9.87 (bs), 9.45 (bs) | 2 | 139 |
| - | - | - | 3 | 176.3 |
| 4 | 6.56 (s) | 6.65 (s) | 4 | 105 |
| - | - | - | 13 | 150.1 |
| - | - | - | 14 | 141.6 |
| 6 | 7.76 (d), J = 8 Hz | 7.74 (d), J = 7.5 Hz | 6 | 117 |
| 7 | 7.43 (t), J = 8 Hz | 7.5 (dd), J = 7.5 Hz ; 6.9 Hz | 7 | 127 |
| 8 | 7.57 (d), J = 8 Hz | 7.58 (d), J = 6.9 Hz | 8 | 123.8 |
| - | - | - | 9 | 136.8 |
| - | - | - | 11 | 118.3 |
| - | - | - | 12 | 153.7 |

[a] 500 MHz, $D_2O$, [b] 300 MHz, DMSO/$CD_3OD$ (95/5 v/v) , [c] 75 MHz DMSO/$CD_3OD$ (95/5 v/v), D1 = 5s ; for atoms numbering, see Figure 2.

## 3. Conclusion

We have disclosed the first efficient synthesis of 3-hydroxyorthanilic acid (3-HOA), in five steps from 2-aminophenol, with an overall yield of 50%. This compound was previously mentioned only as a by-product, and was not fully characterized.

Our process, making use of the selective ortho-metallation reaction for installing the sulfonate group, was feasible on multi-gram scale. Moreover, the study of O/N deprotection conditions of intermediate **9** led to the discovery of a novel benzoxazole derivative **11**, which will be further exploited for the preparation of tetra-substituted benzenesulfonic acids. Considering 3-HOA as a good potential substrate for laccase, similarly to 3-HAA, we were able to prepare a new red dye of phenoxazinone type (LAO) resulting from an oxidative dimerisation of the precursor.

This biotransformation mimics the well-known synthesis of cinnabarinic acid (CA). Our study has established the best experimental conditions (pH, concentrations,...) for producing high yield of LAO, with a minimum amount of side-products, as well as analytical and preparative conditions for HPLC analysis and purification of sulfonated products. The mechanism of laccase oxidation is still under investigation. For instance, the increased reactivity of aminophenol derivatives under acidic pH, suggests that their protonation state could modify the relative oxidation potentials of aromatic amine and hydroxyl functions.[38] The primary oxidation product would be a protonated arylimino-radical rather than the protonated aryloxy-radical usually obtained in neutral solutions.

The new phenoxazinone derivative (LAO) offers interesting colour properties: it has high molar extinction coefficient at 450 nm and is totally water soluble as required for biological detection systems.

## *4. Experimental Section*

### *4.1 General methods*

$^{1}H$ and $^{13}C$ NMR spectra were recorded with a Bruker Avance 500 or a Bruker Avance 300 spectrometer. Spectra were obtained in DMSO-$d_6$, $CDCl_3$, $D_2O$ or $CD_3OD$. Chemical shifts are reported in ppm relative to TMS.[40] $^{13}C$ NMR were obtained with broadband proton decoupling (D1 = 5 sec). Low resolution mass spectra were acquired using a Thermo Finnigan LCQ spectrometer, either in positive mode APCI, negative mode ESI or EI methods (70 eV). High Resolution Mass Spectrometry (HRMS) analysis using ESI method was performed at the University of Mons Hainaut (Belgium). Melting points were recorded with an Electrothermal apparatus calibrated with benzoic acid. Infrared spectra were recorded with a Shimadzu FT-IR 84005 spectrometer using KBr plates. The HPLC system consisted of Waters pumps, Waters 996 photodiode array detector (for analytic separations) or Waters 486 absorbance detector (for semi-preparative HPLC) (Waters, Milford, Massachusets, USA). Analytical separations were performed with Waters XterraMSC18 column (4.6 × 100 mm, 5 µm) (Waters, Milford, Massachusets, USA) equipped with a conventional pre-column. Detection was performed at 220/254 nm with control at 440/460 nm and on-line UV-Visible absorbance scans were performed. Flow rate was of 1 mL $min^{-1}$. Semi-preparative separation was performed with a Waters Xterra-PrepMSC18 OBD column (30 × 100 mm, 5 µm) (Waters, Milford, Massachusets, USA) equipped with a conventional pre-column. Detection was performed at 300 nm. All the solvents were of HPLC grade. Flow rate was of 15 mL $min^{-1}$ for semi-preparative separation.

### *4.2. Organic synthesis*

For standard working practice, see recent publication.[40] Reactions with organolithium reagents were carried out under argon atmosphere in flame-dried glassware. Anhydrous tetrahydrofuran and methylene chloride were purchased from Fluka and chemicals reagents and reactants were from Aldrich and Acros companies. Thin layer chromatography was carried out on aluminium-backed silica gel plate F254 (Merck) and visualized by UV irradiation or by iodine vapor. Column chromatography was carried out with either flash silica gel (60-200 µm) or silica gel 60-RP18 (40-60 µm) as the stationary phase and eluted using the flash chromatography technique.

### *4.3. Biotransformation*

Laccase from Trametes versicolor was purchased as a brownish powder form Bioscreen e.K. (Uebach-Palenberg, Germany) under the commercial trademark Oxizym LA (batch n° LA 2000/001). Laccase activity was determined by oxidation of ABTS [2',2-azino-bis-(3-ethylbenzo thiazoline-6-sulfonic acid)] (4 mM) (Sigma-Aldrich) into a stable cationic radical ABTS●+ (absorption coefficient, $\varepsilon_M$ = 34 220 $M^{-1}$ $cm^{-1}$) in tartaric buffer (100 mM) at pH 4.5, at 25 °C. One unit was defined as the amount of enzyme that oxidizes 1 µmol of ABTS per minute. Analytical biotransformations were performed at 25 °C in solutions containing 1 mM 3-HAA or 3-HOA, 0.1 M phosphate buffer and 100 U $L^{-1}$ Laccase, unless otherwise mentioned. All reactions were monitored through spectrophotometry between 300 and 800 nm using a Beckman DU-800 spectrophotometer (Analis, Namur, Belgium) connected to a high performance temperature controller.

### *4.4. N-(2-Hydroxy-phenyl)-2, 2-dimethyl-propionamide*

To a solution of 2-aminophenol (36.7 mmol; 4 g) in ethyl acetate/water (250/300 mL) were added $NaHCO_3$ (110.1 mmol; 9.2 g) and pivaloyl chloride (40.37 mmol; 4.9 mL). The mixture was stirred 4 h at room temperature. The layers were separated and the organic layer was washed with aqueous HCl (1 N). The organic layer was dried over $Na_2SO_4$ and evaporated in vacuo. The residue was purified by column chromatography on flash silica gel (cyclohexane/ethyl acetate 7:3) to give the title compound as a white solid.[41] Yield = 91% (6.5 g). MW: $C_{11}H_{15}NO_2$, 193.11 g/mol. EI-MS: (m/z) (%) = 193.3 (55) $[M]^+$, 136 (7), 109 (15), 85 (45), 57.1 (40). $^1$H NMR 500 MHz (DMSO) δ = 9.79 (s, 1 H, OH), 8.54 (s, 1 H, NH), 7.76 (dd, $J_{H,H}$ = 7.8 Hz & 1.9 Hz, 1 H, Ar-H), 6.93 (td, $J_{H,H}$ = 7.8 Hz & 1.9 Hz, 1 H, Ar-H), 6.86 (dd, $J_{H,H}$ = 6.8 Hz & 1.9 Hz, 1 H, Ar-H), 6.77 (td, $J_{H,H}$ = 7.8 Hz & 1.9 Hz, 1 H, Ar-H), 1.23 (s, 9 H, 3 × $CH_3$) ppm.$^{13}$C NMR 75 MHz (DMSO) δ= 176.1 (CO amide), 147.4 (C1), 126.2 (C2), 124.29 (C5), 121.5 (C4), 118.8 (C3), 115.2 (C6), 38.7 ($C(CH_3)_3$), 27 (3 × $CH_3$) ppm.

### *4.5. N-(2-Methoxy-phenyl)-2, 2-dimethyl-propionamide (5a)*

To a solution of N-(2-hydroxy-phenyl)-2, 2-dimethyl-propionamide (3.1 mmol; 600 mg) in DMF (2 mL) were added $K_2CO_3$ (3.4 mmol; 472 mg) and methyl iodide (3.4 mmol; 0.21 mL). The solution was stirred 24 h at 20 °C. The reaction mixture was diluted with water (15 mL) and extracted with ethyl acetate (3 × 15 mL). The organic layer was dried over $MgSO_4$ and evaporated in vacuum. The residue was purified by column chromatography on flash silica gel (cyclohexane/ethyl acetate 9:1, 5:5 and 0:10) to give the title compound.[42] Yield = 94%. MW: $C_{12}H_{17}NO_2$, 207.27 g/mol. EI-MS: (m/z) (%) = 207.1 (100) $[M]^+$, 176 (10%), 164 (7%), 150 (15%), 123 (45%), 107.9 (40%), 57.1 (48%). $^1$H NMR 300 MHz ($CDCl_3$) δ = 8.42 (d, $J_{H-H}$ = 8 Hz, 1 H, Ar-H), 8.13 (s, NH), 7.03 (t, $J_{H-H}$ = 7.8 Hz, 1 H, Ar-H), 6.95 (t, $J_{H-H}$ = 6.8 Hz, 1 H, Ar-H), 6.87 (d, $J_{H-H}$ = 7,8Hz, 1 H,

Ar-H), 3.89 (s, 3 H, $OCH_3$), 1.31 (s, 9 H, 3 × $CH_3$) ppm. $^{13}C$ NMR 75 MHz ($CDCl_3$) δ = 176.4 (CO amide), 147.9 (C1), 127.8 (C2), 123.3 (C5), 121.07 (C4), 119.4 (C3), 109.7 (C6); 55.7 (O-$CH_3$), 39.9 ($C(CH_3)_3$), 27.5 (3 × $CH_3$) ppm.

### *4.6. 2-(2,2-Dimethyl-propionylamino)-3-methoxy-benzenesulfonic acid (9)*

To a solution of **5a** (19.3 mmol; 4 g) in anhydrous THF (40 mL), under argon atmosphere, at -10°C, was added dropwise *n*-Butyllithium 2.5M in cyclohexane (40.5 mmol; 16.2 mL). The solution was kept 15 min at low temperature (between -10 °C and 0 °C), then warmed to 25 °C and kept 3 h at room temperature. The di-anion solution was added dropwise at – 10 °C to a stirred suspension of cristalline sulfur trioxide trimethylamine complex (powder dried prior use under vacuum for 1 h) (28.95 mmol; 4.028 g) in anhydrous THF (10 mL). The reaction mixture was reacted 2 h at low temperature (between -10 °C and 0 °C) (white-orange suspension) then allowed to warm up to room temperature for 20 h (orange-brown solution). Then, 10 % (w/w) aqueous NaOH (150 mL) was added and the mixture was extracted with diethyl ether (3 × 100 mL) to remove residual starting material. To the aqueous solution, tetrabutyl ammonium hydrogenosulfate was added (17.37 mmol; 5.89 g) and the mixture was extracted with ethyl acetate (3 × 100 mL). The combined organic phases were dried over $MgSO_4$ and evaporated (black oil). This residue was purified on anion exchange resin IRA-900 (chloride form) with MeOH/$H_2O$ (1/1 v/v) as neutral eluant and MeOH/HCl 1 N (1/1 v/v) as acidic eluant. IRA mass needed was 100g. First the resin was laid in 200 mL $H_2O$ and allowed to swell for 24 h, then the resin was put in a column (diameter of 2 cm) and washed with 150 mL of HCl 1 N, followed by 150 mL of MeOH/HCl 1 N (1/1 v/v). The crude salt **8**, dissolved in minimum of MeOH, was added dropwise onto the resin. The resin was allowed to stand 30 min unmoved after complete percolation of the mixture into the resin. Afterward the resin was

washed by 500 mL of neutral eluant to recover the tetrabutylammonium chloride salt. The resin was then washed by 500 mL of acidic eluant and the solution was evaporated under vacuo to yield the title compound as a brown gummy solid. Yield: 70% (3.9 g). MW: $C_{12}H_{17}NO_5S$, 287 g/mol. HRMS-ESI negative mode: m/z $[M-H]^-$ Calcd for $C_{12}H_{16}NO_5S$: 286.0749; found: 286.0758. ESI-MS: (m/z) (%) = $[M-H]^-$ 286.1 (100), 270.9 (25), 214 (70), 206 (40). $^1H$ NMR 300 MHz ($CD_3OD$) $\delta$ = 7.48 (dd, $J_{H-H}$ = 7.8 Hz & 1.5 Hz,1 H, Ar-H), 7.30 (t, $J_{H-H}$ = 8.4 Hz, 1 H, Ar-H), 7.17 (dd, $J_{H-H}$ = 7.8 Hz & 1.5 Hz, 1 H, Ar-H), 3.83 (s, 3 H, $OCH_3$); 1.33 (s, 9 H, 3 × $CH_3$) ppm. $^{13}C$ NMR 125 MHz ($CD_3OD$) $\delta$ = 179.5 (CO amide), 156.4 (C3), 141.8 (C1), 127.9 (C5), 124.1 (C2), 119.7 (C6), 115.5 (C4), 56.5 ($OCH_3$), 39.9 ($C(CH_3)_3$), 27.6 (3 × $CH_3$) ppm.

### *4.7. 2-(2,2-Dimethyl-propionylamino)-3-hydroxy-benzenesulfonic acid (10)*

To a solution of **9** (13.5 mmol; 1.02 g) in $CH_2Cl_2$ (15 mL) kept at 0 °C, was added dropwise $BCl_3$ 1 M (10.78 mmol, 10.8 mL). The mixture was left 1 h at 0 °C then overnight at 25 °C. The reaction was cooled to 0°C and $H_2O$ (20 mL) was added dropwise to quench the residual $BCl_3$. After evaporation, the residue was dissolved in MeOH (6 mL) and added to RP18 (0.1 g) and concentrated under vacuum. The residue was filtered on 0.5 µm filter by MeOH/ $H_2O$ (1/1 v/v) (60 mL) and the solution was evaporated to yield the title compound as a brown solid. Yield = 91% (0.87 g).

NOTE: The compound is partially under the form of benzoxazole **11** in a ratio of 70/30 in favour of product **10**. MW: $C_{11}H_{15}NO_5S$, Mol. Wt.: 273.3055 g/mol. $^1H$ NMR 300 MHz ($CD_3OD$) $\delta$ = 7.52 (d, $J_{H-H}$ = 6.8 Hz, 1 H, Ar-H), 7.16 (t, $J_{H-H}$ = 7.8 Hz, 1 H, Ar-H), 7.05 (d, $J_{H-H}$ = 6.8 Hz, 1 H, Ar-H), 3.68 (s, 0.5 H), 1.36 (s, 9 H, 3 × $CH_3$) ppm.

### *4.8. 2-tert-Butyl-benzoxazole-4-sulfonic acid (11)*

To a solution of a mixture **10 + 11** as dry powder (3.6 mmol; 1 g) in MeOH (10 mL) was added TFA (1.8 mmol, 0.2 mL). The mixture was stirred 3 h at 80 °C. The reaction was then cooled to 25 °C and evaporated under vacuo to yield the title compound as a pale yellow solid; Yield = 99% (0.92 g). MW: $C_{11}H_{13}NO_4S$, 255 g/mol. HRMS-ESI negative mode: m/z calcld: 278.0463 for $C_{11}H_{12}NO_4NaS$, found: 278.0466. ESI-MS: (m/z) (%) = 254.2 [M-H] (100), 190.2 (15). $^1$H NMR 300 MHz ($CD_3OD$) δ = 7.86 (d, $J_{H-H}$ = 7.5 Hz ;1 H, Ar-H), 7.77 (d, $J_{H-H}$ = 7.5 Hz, 1 H, Ar-H), 7.48 (t, $J_{H-H}$ = 7.8 Hz, 1 H, Ar-H), 1.56 (s, 9 H, 3 × $CH_3$) ppm. $^{13}$C NMR 125 MHz ($CD_3OD/CDCl_3$) δ = 176.8 (C2), 151.1 (C1),134.3 (C4), 125.3 (C6), 124.7 (C3), 113.3 (C5,C7), 35.8 ($C(CH_3)_3$), 20.5 (3 × $CH_3$) ppm. M. p. = 293-295 °C. IR (KBr): υ = 3448, 2977, 2360, 1566, 1473, 1427, 1253, 1191, 1141, 1045, 891, 802, 682 $cm^{-1}$

### *4.9. 2-Amino-3-hydroxy-benzenesulfonic acid (3) (3-HOA). Method i*

To a solution of 6 N HCl (21 mL) was added **11** (3.1 mmol; 0.8 g). The mixture was heated at 80 °C for 24 h. Then the solution was cooled down and evaporated. The residue was dissolved in MeOH (20 mL), activated charcoal was added (50 mg). The mixture was stirred at 40 °C for 30 min. The solution was then filtered over a short pad of celite and the pad was washed with hot methanol (30 mL). The filtered solution was evaporated and dried under vacuo to yield the title compound as a light brown solid. Yield: 90% (0.53 g). Purity of the compound can be controlled by HPLC (RT: 1.8 min). MW: $C_6H_7NO_4S$: 189,1 g/mol. HRMS-ESI negative mode: m/z calcld 188.0018 for $C_6H_6NO_4S$; found: 188.0018. ESI-MS: (m/z) (%) = 188.07 [M-H] (100), 376.8 [2M-2 H] (75), 187.1 (6), 108 (5), 79.8 (15). $^1$H NMR 300 MHz ($CD_3OD$) δ = 7.41 (dd $J_{H-H}$ = 7.8 Hz & 1.35 Hz,1 H, Ar-H), 7.34 (t, $J_{H-H}$ = 8.1 Hz, 1H, Ar-H), 7.07 (dd, $J_{H-H}$ = 7.8 Hz & 1.35 Hz, 1 H, Ar-H) ppm. $^{13}$C NMR 125 MHz ($CD_3OD$) δ =

152.6 (C3), 140.6 (C1), 130.6 (C5), 119.4 (C6), 118.8 (C4), 115.8 (C2) ppm. M.p.= 210-215 °C (slow decomposition). IR (KBr): υ = 3487, 3436, 3220, 3066, 2931, 2692, 1635, 1490, 1469, 1365, 1307, 1191, 1064, 1037, 902, 798, 675 $cm^{-1}$.

### *4.10. 2-Amino-3-hydroxy-benzenesulfonic acid (3) (3-HOA). Method j.*

To a solution of 48 % HBr (6 mL) was added **9** (1.04 mmol, 0.3 g). The mixture was heated at 120 °C for 13 h. Afterward the reaction was cooled down. Part of the product was recovered as a solid from precipitation *in situ*. The filtered solution was evaporated and the residue was dissolved in MeOH (20 mL). Activated charcoal was added (50 mg) and the mixture was agitated at 40 °C for 30 min. The solution was then filtered over a short pad of celite and celite was washed with hot methanol (30 mL). The filtered solution was evaporated and dried under vacuum to yield the title compound as a brown solid. Yield: 95% (0.17 g including 20 % as hydrobromide salt (0.05 g) from precipitation *in situ*.

### *4.11. 2-Amino-3H-phenoxazi-3-one-1,9-disulfonic acid (4) (LAO)*

A solution of laccase (200 U $L^{-1}$) and 3-HOA (5.29 mmol; 1.02 g) in a final volume of 100 mL of water adjusted at pH 6 with HCl and NaOH was prepared. The stirred mixture was kept 24 h at 25 °C. The reaction was stopped by decreasing the pH with MSA (pH~2). The crude mixture was freeze dried to give a red powder (1 g). Part of this powder (400 mg) was purified by chromatography on reversed phase (water as eluant) to yield an almost pure fraction of LAO (270 mg). Part of this fraction (70 mg) was submitted to preparative HPLC to yield pure title compound as a deep red solid (30 mg). Yield of about 70% determined by HPLC. MW: $C_{12}H_8N_2O_8S_2$, 372,3305 g/mol. HRMS-ESI negative mode: m/z calcld 370.9644 for $C_{12}H_7N_2O_8S_2$; found: 370.9641. ESI-MS: (m/z) (%) = 371.16 [M- H) (100); 393.1 [M –H + Na] (40); 291.2 (20). $^{1}H$ NMR and $^{13}C$ NMR : see Table 3.

M.p. > 300 °C. IR (KBr): υ = 3433, 2364, 1635, 1593, 1473, 1415, 1218, 1053. UV: $\lambda_{max}$ at 435 and 339 nm ( 0.1 M phosphate buffer, pH 7). 435 nm ($\varepsilon$ = 13981 L $mol^{-1}$ $cm^{-1}$). UV: $\lambda_{max}$ at 460 and 330 nm ( 0.1 M phosphate buffer, pH 2). 460 nm ($\varepsilon$ = 5426 L $mol^{-1}$ $cm^{-1}$).

### *4.12. Supporting information*

$^{1}H$ and $^{13}C$ NMR spectra of compounds **9** (Figure S2, Figure S3); **11** (Figure S4, Figure S5); **3** (Figure S6, Figure S7); **4** (Figure S8, Figure S9) are provided as additional material. UV-Vis spectra of compound **4** at pH 7 & pH 2 (Figures S10) are also furnished (see footnote on the first page of this article).


### *5. Acknowledgements*

This research was supported by the European Commission, Sixth Framework Program (NMP2-CT2004-505899, SOPHIED).Trieu-Van Tran, Muriel Isaac and Dominique Ingels are kindly acknowledged for their technical assistance. J. Marchand-Brynaert is a senior research associate of FRS - FNRS (Belgium).

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## 3.4 Evaluation of the acute toxicity of LAO

In the frame of the E.U. project SOPHIED, the proof of concept of the bioprocess towards new synthetic dyes implied the simultaneous evaluation of their toxicity. A preliminary testing was undertaken using standard procedures which are currently applied both by the EU and by the FDA agency.

### *3.4.1 General dyes toxicity evaluation*

REACH is a new European Community Regulation on chemicals and their safe use (EC 1907/2006). It deals with the Registration, Evaluation, Authorisation and Restriction of Chemical substances. The new law entered into force on 1 June 2007. The aim of REACH is to improve the protection of human health and the environment through the better and earlier identification of the intrinsic properties of chemical substances. A survey of oral acute toxicity of 4,461 dyes, evaluated by the lethal dose for 50% of treated rats, has revealed that azo and cationic dyes are the most toxic.

As the ingestion of contaminated water would lead to a direct contact with the human intestine, toxicity assays are usually carried out on human Caco-2 cells, which are considered as a validated in vitro model for the human intestinal epithelium. It represents a useful and well-accepted tool for studying toxicity of pollutants. [151]

[151] Velarde, G.; Ait-Aissa, S.; Gillet, C.; Rogerieux, F.; Lambre, C.; Vindimian, E.; Porcher, J.M. *Toxicol in Vitro* **1999**, 13:719–722.

### *3.4.2 In vitro cytotoxicity: the Caco-2 cells viability assay*

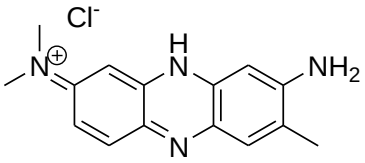
The cytotoxicity has been defined as the adverse effects resulting from interference with structures and/or processes essential for cell survival, proliferation, and/or function. These effects may involve the integrity of membranes and the cytoskeleton, cellular metabolism, the synthesis and degradation or release of cellular constituents or products, ion regulation, and cell division. The cytotoxicity is determined using a protocol based upon the Neutral Red Uptake (N.R.U.) principle in conjunction with Caco-2 cells. Caco-2 cells monolayers spontaneously differentiate to express morphological and functional characteristics of mature small-intestinal enterocytes. The Caco-2 cells grown on permeable filter supports form tight junctions, and the permeability across Caco-2 cells monolayers is used to predict human permeability of drug.

Neutral red (see Figure 10) is weakly cationic water-soluble dye that stains living cells [152, 153]. It readily diffuses through the plasma membrane and concentrates in lysosomes where it electrostatically binds to the anionic lysosomal matrix. Toxicants can alter the cell surface or the lysosomal membrane to cause lysosomal fragility and other adverse changes that gradually become irreversible. Thus, cell death and/or inhibition of cell growth decreases the amount of neutral red retained by the culture. The NRU assay, which was standardized for a 96-well plate format was described as a rapid, reliable, inexpensive, and reproducible in vitro test method for screening potentially toxic agents

[152] Borenfreund, E. Puerner, J. A. *Toxicol Lett* **1985**, 24, 119-124.

[153] Fernando, J.; Morgan, W. S.; Hausser, J. W., *J. Org. Chem*. **1967**, 32, 1120–1123.

Figure 10. Neutral Red dye




### *3.4.3 LAO toxicity results*

A brief description of the general protocol is outlined below. A detailed description of the test and its implementation can be found in the scientific literature published by the members of the SOPHIED project involved in the field of toxicology. [154]. Here, the test was realised with two different cells lines, i.e. the Caco-2 cells and the Rainbow Trout Gonad (RTG) cells which are common for the detection of pollutants.

The cells were seeded in their respective standard cell culture medium at an appropriate density, and allowed to attach and start proliferation in 96-well microplates in such a way that 20% confluence was reached in 24 hours. Subsequently, the cells were exposed to a series of concentrations of the tested compounds, and two blanks, for at least 48 hours until 90% confluence was attained in the blanks. The highest concentration tested being 0.5 g/L (1mM). Thereafter, the exposure medium containing the tested compounds was decanted and the cells in the microplate wells were gently washed with phosphate buffered saline solution. The cells were exposed to a solution of neutral red in cell culture medium for 3 hours.

[154] Vanhulle, S.; Trovaslet, M.; Enaud, E.; Lucas, M.; Sonveaux, M.; Decock, C.; Onderwater, R.; Schneider, Y. J.; Corbisier, A. M., *World J Microbiol Biotechnol* **2008** , 24, 337–344.

Finally, the neutral red solution was decanted, the wells were again gently washed and then a solution of 1% acetic acid in 50% ethanol was added to the wells to dissolve the neutral red taken up by the viable cells. The absorbance of each well at 540 nm was measured with an appropriate microplate reader. The amount of neutral red present in each well is an indication of the number of viable cells and when compared to the values of the blanks, can be used to calculate a percentage of cell death; an $IC_{50}$ value, i.e. the concentration of the tested compounds at which 50% of the cells are killed, is calculated.

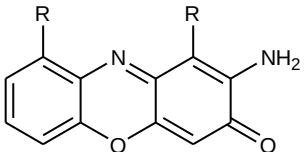
Table 1 Significant toxicity results

| Dye [a] | NRU Caco-2 cells | | NRU RTG-2 cells | |
|---|---|---|---|---|
| | $IC_{50}$ ± S.D. | Mean $IC_{50}$extrapol | $IC_{50}$ ± S.D. | Mean $IC_{50}$ extrapol |
| LAO | 0.55± 0.01 | 0.55 | >0.78 | / |
| CA | 0.48± 0.02 | 0.48 | 0.55 ± 0.09 | 0.55 |
| Congo red (91%) | 0.28 ± 0.02 | 0.28 | 0.123 ± 0.0004 | / |

[a] Congo red is a commercial dye used as a reference in the test. Congo red itself is moderately toxic [$LD_{50}$ intra-venously is 190 mg/kg in rats].[155]

CA = cinnabarinic acid; R = $CO_2H$

LAO = Amino-3H-phenoxazin-3-one-1,9-disulfonic acid; R = $SO_3H$

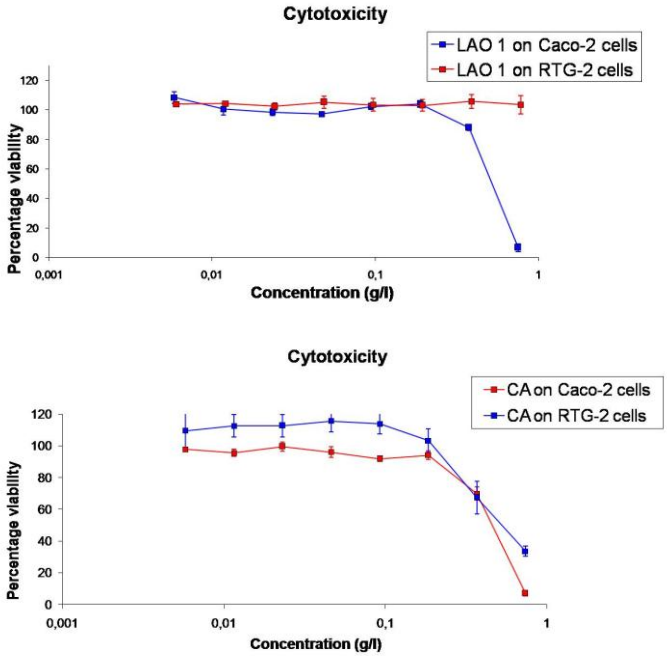



[155] Frid, P.; Animisov, S. V.; Popovic, N., *Brain Res. Rev.* **2007**, 53, 135-160.

When expressing $IC_{50}$ values in g/L, in the laboratory (and in this particular in vitro method) a value of less than 0.01 g/L is attained by highly toxic compounds, a value of less than 0.1 g/L by significantly toxic ones and values above 0.1 g/L by compounds with no significant in vivo toxicity. The compounds shown in the table have $IC_{50}$ values which are significantly higher than 0.1 g/L and thus can be considered as non-toxic.

The decrease of toxicity of LAO compared to the one observed for the natural orange pigment CA probably comes from the increase of water-solubility. Such results are encouraging for further potential application in molecular biology or in the field of medicinal chemistry provided that a marked activity or photochemical property could be determined for the new water-soluble aminophenoxazinone dye.

Figure 11. Results of cytotoxicity for aminophenoxazinone dyes



# CHAPTER 4. SCOPE AND LIMITATIONS

## 4.1 Foreword

In the former section, we have demonstrated how the laccase as a biocatalyst could be valuable in the synthesis of new water-soluble dyes. The replacement of the carboxylic function by a sulfonic one, based on the principle of bio-isosterism, gave an excellent result.[156] This was not totally unexpected in light of the growing number of publications related to the oxidative degradation of synthetic acid dyes by laccases. The wide-scope of substrate tolerance which is characteristic of the laccase family is an obvious advantage of such copper oxidases in the field of organic synthesis.

Obviously the new water-soluble dye 1,9-disulfonylated-2-amino-3-oxo-phenoxazinone is strongly similar to the natural orange pigment cinnabarinic acid. The question whether a laccase could accommodate in its binding cavity few aminophenol compounds, either bearing larger side chains or having the sulfonyl group anchored in the adjacent position of the aromatic OH, remains open. To date, there is no investigation dedicated to the production of water-soluble orcein-like dyes using a laccase. Noteworthy, few patents have disclosed the dyeing processes with mixtures of dyes obtained from laccase-mediated oxidation of aminosalicylic derivatives as starting materials.[157]

[156] a) Patani, G. A.; Lavoie, E. J., Chem. Rev. **1996,** 96, 3147-3176. b) Sheridan, R. P., *J. Chem. Inf. Comput. Sci.* **2002,** 42, 103-108.

[157] Barfoed, M; Kirk, O., WO/1997/023684, **1997.**

These publications can be formally considered as an illustration of oxidised compounds produced by a copper oxidase using as substrate 3-aminosalicylic acid (3ASA), i.e. the regiosomer of 3HAA. Still, the exact structures of the resulting coloured compounds are not known. Whereas the preliminary results with 3ASA showed a marked increase of the formation of side products, we have still synthesised a pool of sulfonated analogues of 3ASA to investigate their interaction with our biocatalyst.

## 4.2 Main topic addressed in the publication

Hereafter, we have synthesised a small library of 3-hydroxyorthanilic derivatives taking advantage of the key-intermediate 2-*tert*-butyl-benzoxazole-4-sulfonic acid as starting material. Its quantitative conversion into the corresponding sulfonyl chloride and subsequent substitution by ammonia, alkyl and aryl-amines rapidly created the desired molecular diversity. Different functional groups were also chosen bearing in mind the potential linkage of the resulting dyes, after laccase initiated dimerisation, on various vehicles (fibres, synthetic or natural polymers, ...).

Regarding the orcein-like pigments, the synthesis of the required building blocks was first investigated using the *N*-*tert*-butyl-anisidine as starting material. Yet, the desired optional selectivity, i.e. the ability to selectively insert the oxidised sulfur group in the adjacent position of the methoxy group, could not be achieved.[158]

Whereas the superbasic mixture, namely the Schlosser base, led to the selective insertion of a methyl group, the insertion of the oxidised sulfur group never took place.[159 ] Instead, we used the "benzyne pathway" as an alternative synthetic route.[160 ] This reaction was successfully applied to produce the required 2-*tert*-butyl-benzoxazole-7-sulfonyl chloride, in moderate yield. The choice of sulfuryl chloride as the electrophilic reagent shortened the overall synthesis. Yet, such advantage was levelled-off by an increased proportion of side products. [ 161]

Using the laccase in the optimal conditions previously disclosed, we could produce a first family of novel 1,9-disubstituted aminophenoxazinone dyes featuring tuneable water-solubility. Two X-ray structures were obtained which unambiguously confirmed the general structure of those dyes (head to head or head to tail dimers).

[158] a) Nguyen,T-H.; Thanh Chau, N T.; Castanet, A.-S.; Phung Nguyen, K. P.; Mortier, J., *J. Org. Chem.* **2007**, 72, 3419-3429. b) Gorecka-Kobylinska, J.; Schlosser, M., *J. Org. Chem.* **2009**, 74, 222–229.

[159] Seyferth, D., *Organometallics* **2009,** 28, 2–33.

[160] a) Bunnett, J. F.; Hrutfiord, B. F., *J. Am. Chem. Soc.* **1961**, 83, 1691–1697. b) Clarck, M. D.; Caroon, J. M., *J. Org. Chem.* **1982**, 47, 2804-2806. c) Yoshida, Y.; Hamada, Y.; Umezu, K.; Tabuchi, F., *Org. Process Res. Dev.* **2004**, 8, 958-961.

[161] a) Jones, R. G.; Gilman H., *Chem. Rev.* **1954**, 54, 835–890. b) Scott, R. B.; Gayle, J. B.; Heller, M. S.; Lutz, R. E., *J. Org. Chem.* **1955**, 20, 1165–1168. c) Pandya, R.; Murashima, T.; Tedeschi, L; Barrett, Anthony G. M., *J. Org. Chem.* **2003**, 68, 8274-8276.

## 4.3 Full Paper

The paper was published in *Chemistry–A European Journal* **2009**, *15*, 8283-8295.

***Title***

Laccase-Mediated Synthesis of Novel Substituted Phenoxazine Chromophores Featuring Tuneable Water-Solubility.

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***Abstract***

Laccases are members of the blue copper oxidases family found in nature. They commonly oxidize a wide range of phenol and aniline derivatives which, in turn, are involved in oxidative coupling reactions. Yet, laccases remain scarcely described as biocatalysts in organic synthesis. This paper describes the chemical preparation of original sulfonated aminophenol substrates and their enzyme-mediated dimerization into phenoxazine chromophores featuring tunable water-solubility as a function of the sulfonyl substituent nature. Scope and limitations of the biocatalyzed synthetic process are outlined. Kinetic data were collected to evaluate the influence of physicochemical parameters. The structure of the novel phenoxazine dyes ("head to head" or "head to tail" dimer) was assessed by NMR analysis. Two crystalline compounds were analyzed by X-ray diffraction. Such laccase-mediated synthesis (green chemistry process) was proven to be more efficient than the chemical oxidation of *o*-aminophenols with silver oxide.

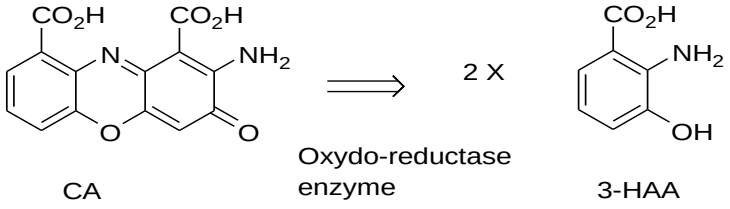
***Keywords***

Heterocyclic compd. / oxidation / condensation react. / chromophores / enzymes.

*1. Introduction*

As part of the mammalian metabolism, multicopper oxidases are involved in the oxidation of 3-hydroxyanthranilic acid (3-HAA) into cinnabarinic acid pigment (CA) to protect cells from oxidative damages (Figure 1).[1,2] Such phenoxazinone and related heterocycles have also been produced in vitro by means of laccases.[3,4] The potential applications of those natural compounds span from the therapeutic field[5] to the one of molecular probe,[6,7] but their poor water-solubility remains a major drawback.

Figure 1. Phenoxazinone pigment




Nowadays, the great potential interest of laccases as green catalysts is well recognized,[8-9] and their use for the assembly of complex molecules is growing steadily.[10-12] Upon laccase-mediated oxidation, *o*-aminophenols are converted into the corresponding *o*-quinone imines which are extremely reactive species. In-situ nucleophiles rapidly add to those oxidized intermediates leading to products of oxidative coupling reactions.

Often, *o*-quinone imines spontaneously react with their reduced forms to produce phenoxazine derivatives (Figure 1). This oxidative condensation formally corresponds to a total of $6e^-$ oxidation.[13] While the first $2e^-$ oxidation is an enzymatic process, chemical models of phenoxazine synthase also catalyse such reactions.[14,15]

In the literature, there is no systematic investigation of the effect of side chains at adjacent positions of the aniline and/or phenol functions on the laccase-mediated dimerization process. Yet, the production of diversely substituted phenoxazine dyes is highly wished for the development of new applications. Up to now, the diversity has been obtained through the chemical synthesis of such poly-heterocycles starting from nitrosophenols or anilines.[16,17]

In the present work, we detail the regioselective synthesis of new *o*-aminophenols bearing side chains anchored on a sulfonic function, and their laccase-mediated oxidative coupling under mild conditions. Scope and limitations of the enzyme-initiated oxidative cascade are presented along with optimal conditions for the synthesis of original heterocyclic frameworks in good yields. Those types of phenoxazine chromophores feature tuneable water-solubility thanks to their sulfonyl substituents.

## *2. Results*

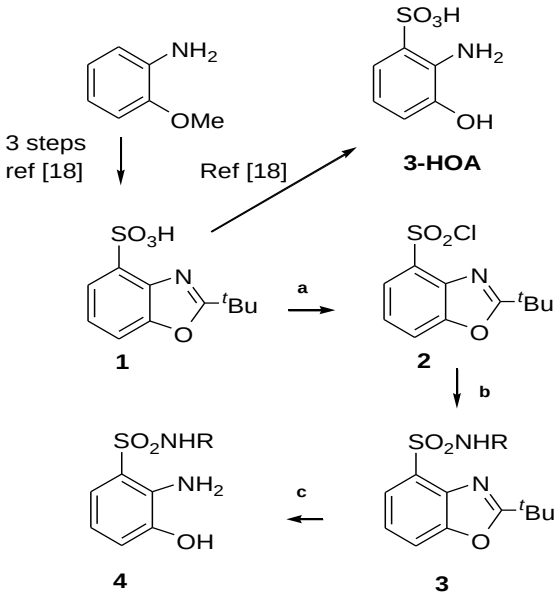
### *2.1 Preparation of substituted benzenesulfonic acids*

To draw trends in the type of substrates susceptible to be efficiently processed by a laccase, new compounds derived from 3-hydroxyorthanilic acid (**3-HOA**) were synthesized. The preparation of **3-HOA** and the key intermediate **1** were previously published.[18] Compounds **4a** to **4g** (Table 1) were obtained using benzoxazole **1** as the starting material (Scheme 1).

The sulfonic acid was converted into the corresponding sulfonyl chloride **2** in high yield which was directly submitted to substitution by ammonia, alkyl or aryl amines in a biphasic medium. The reactions proceeded well and the crude products **3** could be directly engaged in the hydrolysis step of the oxazole function. A limitation of our syntheses occurred during the acidic hydrolysis of **3** in refluxing 6N HCl.

Indeed the primary and the aryl sulfonamides, **4a** and **4d** respectively, were obtained with moderate to poor yields while the other alkyl sulfonamides (**4b-g**) were recovered in about 85% yields. This probably comes from a competitive hydrolysis of the sulfonamides which seem too labile for such experimental conditions. Cleavage of aryl sulfonamide was reported under harsh conditions and long reaction times. Yet such cleavages were often not observed with ortho substituents.[19,20] Except compound **4d**, all the crude aminophenol derivatives obtained were pure enough to be directly used in the biotransformations. Nevertheless, all the compounds were purified by flash chromatography to rule out any external artefacts in the reactions performed with laccases.

Scheme 1. Synthesis of substrates **4a-g** from 2-tert-butylbenzoxazole-4-sulfonic acid.[18]




Reagents: a) 5eq $PCl_5$, 5eq $OPCl_3$, $CH_2Cl_2$, 4h at 40°C, 95% ; b) $RNH_2$ 10%, $CH_2Cl_2$, overnight at 25°C, 95% ; c) HCl 6N, overnight at 100°C, 50% for **4a**, 30% for **4d**, 85% for **4b**, **c**, **e**, **f**, **g** (see Table 1).

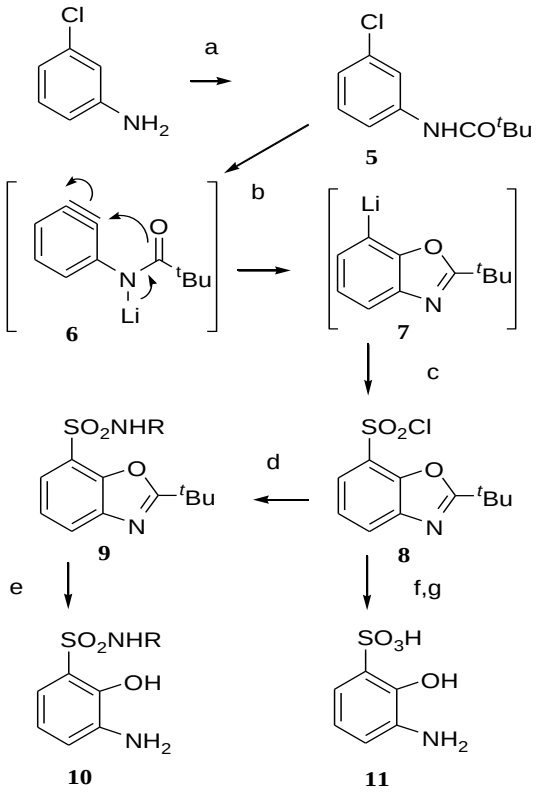
While 3-hydroxyanthranilic acid (3-HAA) is commonly reported as a natural substrate for multicopper oxidases, similar data for its regioisomer, 3-aminosalicylic acid, are not available. The actinocin derivatives biosynthesized with enzymes only possess a hydrogen atom or a methyl group adjacent to the phenol OH.[3,4] To further assess the potential of our methodology, some regioisomers were required. 2-Hydroxymetanilic acid (**11**) has been previously reported as a by-product of azoïc dyes reduction.[21]

We first tried to synthesize a sulfonated intermediate from *N*-pivaloyl-anisidine as the starting material. Indeed, directed *ortho*-metallation reaction was successfully used for the regioselective sulfonation of aniline and derivatives.[18,22] Optional selectivity in site of metallation has been reported before for *N*-(*tert*-butoxycarbonyl)-anisidine.[23] In our hand, the use of Schlosser superbasic mixture gave no satisfactory results (data not shown). Some selectivity in favour of the adjacent site to methoxy group could be detected by quenching with methyl iodide.

But no sulfonation was obtained with sulfur trioxide trimethylamine complex (STTAC) used as the electrophile. So, an alternative synthesis of **11** was used which involves a benzyne intermediate (Scheme 2).[24,25]

The acylation of 3-chloroaniline under solvent-free conditions gave pure intermediate **5** in excellent yield after recrystallization. Next, **5** was submitted to deprotonation at the most activated C2 position with an organolithium reagent at -78°C and the dianion obtained was slowly warmed up to -45°C over 2 hours. Upon warming, expulsion of the chlorine yields the benzyne intermediate **6** and an intramolecular quenching by the imidate led to the lithiated benzoxazole **7**. Control experiments by quenching with methyl iodide gave excellent yields.

Scheme 2. Synthesis of substrates 10b-c and 11 from 3-chloro-aniline.




Reagents: a) 0.5eq ZnO, 1eq *t*BuCOCl, 4h neat , 85% ; b) 2.1eq *n*BuLi -78°C to -45°C over 2h, 90% by quenching with MeI ; c) 5eq $SO_2Cl_2$ at -78°C to 25°C overnight 50%, d) $RNH_2$ 10%, $CH_2Cl_2$ overnight at 25°C, 85% ; e) HCl 6N, 100°C overnight 85% ; f) MeOH, 2h at 80°C, quantitative ; g) HCl 6N at 100°C overnight, 95%

When the sulfur complex (STTAC) was used as the electrophile, the only product isolated was 2-*tert*-butylbenzoxazole. It seems that either the electrophile is not reactive enough or that the *tert*-butyl moiety induces steric hindrance which in turn prevents the sulfonation. To shorten the synthesis, we chose to quench directly the lithiated benzoxazole **7** with sulfuryl chloride. Intermediate **8** was obtained with a moderate yield of 50% after chromatographic purification.

From compound **8**, the desired sulfonamides **10b** and **10c** were obtained in high yields as described above. Intermediate 2-*tert*-butyl-7-sulfonic benzoxazole could be quantitatively formed by heating **8** at reflux in methanol. The methylsulfonate, formed in situ, is deprotected by HCl via a SN2 substitution by chloride anion. Then acidic hydrolysis gave quantitatively the desired compound **11**.

Lastly, the directed *ortho*-sulfonation methodology was applied to *N*-4-pivaloyl anisidine with success. Good selectivity was observed in the lithiation/sulfonation process leading to compound **12** (Table 1) after usual deprotections (see supporting information).

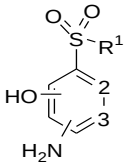
### *2.2 Biotransformation*

The biotransformation of the library of aminophenol substrates (Table 1) was investigated using the commercial laccase as received (benzenediol:oxygen oxidoreductase, EC 1.10.3.2). To make practical our laccase-mediated catalytic system, some adjustments were needed in regard of the previously reported method.[18] A phosphate buffered medium was initially discarded since phosphate salts would impair the isolation of the desired water-soluble heterocycles.

Table 1. Substrates structures

| Compounds | $R^1$ | aminophenols | | ClogP[a] |
|---|---|---|---|---|
| | | $NH_2$ | OH | |
| **3HOA** | OH | C2 | C3 | 0.02 |
| **4a** | $NH_2$ | C2 | C3 | -0.55 |
| **4b** | NH-cyclohexyl | C2 | C3 | 1.47 |
| **4c** | $NH(CH_2)_3N(Me)_2$ | C2 | C3 | -0.25 |
| **4d** | NH-phenyl | C2 | C3 | 1.33 |
| **4e** | $NH(CH_2)_2NH_2$ | C2 | C3 | -1.13 |
| **4f** | $NHCH_2CO_2Me$ | C2 | C3 | -0.71 |
| **4g** | $NH(CH_2)_2CH_2OH$ | C2 | C3 | -0.96 |
| **10b** | NH-cyclohexyl | C3 | C2 | 1.47 |
| **10c** | $NH(CH_2)_3N(Me)_2$ | C3 | C2 | -0.25 |
| **11** | OH | C3 | C2 | -1.44 |
| **12** | OH | C2 | C5 | 0.67 |

[a] CLogP was calculated for the non ionic species with the Plugin LogP calculator of chemaxon Ltd (chemaxon.com). Plugin uses a set of experimental data from the publication of Viswanadhan, V. N. & al (doi:10.1021/ci00063a006).




However, working in pure water led to slight variations of pH during the reactions. Furthermore, the previously isolated dye LAO, for Laccase Acid Orange dye[18] (Table 2 and Scheme 3), when placed in water evolved in time between two protonated forms (NMR study not shown). Thus, a reaction medium made of ammonium acetate buffer was used here to ensure a fixed pH in the optimal conditions of the biotransformation without impairing the enzyme stability.

The presence of ammonium was thought to be the best choice in light of the strong ionic pair formed with the sulfonate moieties.

Indeed, the yield of conversion of **3-HOA** into **LAO** dye (considered as the standard reaction) was improved up to 95% in those conditions. The course of reaction was followed by UV-vis spectroscopy at 440 nm ($\lambda_{max}$ of **LAO**). Purity of the freeze-dried crude product was checked by HPLC and the NMR spectroscopic data confirmed that the product was stable in this buffered solution over days.

Next, the library of synthesized compounds (Table 1) was used as substrates for biotransformations with commercial laccase into heterocycles **13a-g**, **14b**, and **15** (see Scheme 3 and Scheme 4). The crude reaction mixtures and the course of compounds oxidation in presence of laccase were monitored (see supporting information) by analytical HPLC with UV-Vis detection at 220, 280 and 440 nm. Except for compounds **10c**, **11** and **12**, all the molecules were readily converted over a pH range of 2 units and the yields of isolated dyes were moderate to good (Table 2). Depending on the hydrophobic nature of the substrate, the enzyme ratio had to be adjusted to ensure a conversion within one day.

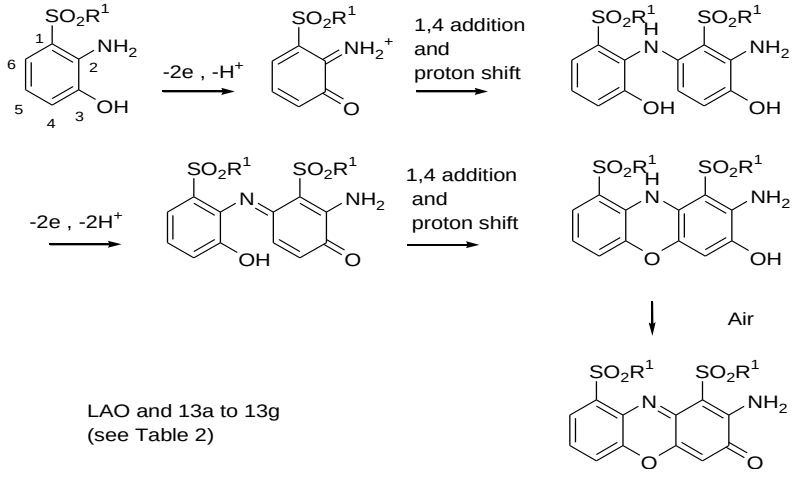
Compounds **4b**, **4d** and **10b** corresponding to the most hydrophobic ones (see ClogP in Table 1) tend to precipitate during the oxidation reactions, which in turn may prevent the enzyme to work properly. For those molecules, higher amounts of laccase were used to increase the rate of oxidation and biotransformation. Otherwise, all the water-soluble molecules could be converted even when the catalyst loading was reduced. For all those substrates, the corresponding heterocycles biosynthesized were partially soluble above a concentration of 1 mM and suspensions were usually obtained at the end of the reactions.

Table 2. HPLC results and yields of phenoxazinones in the optimized conditions

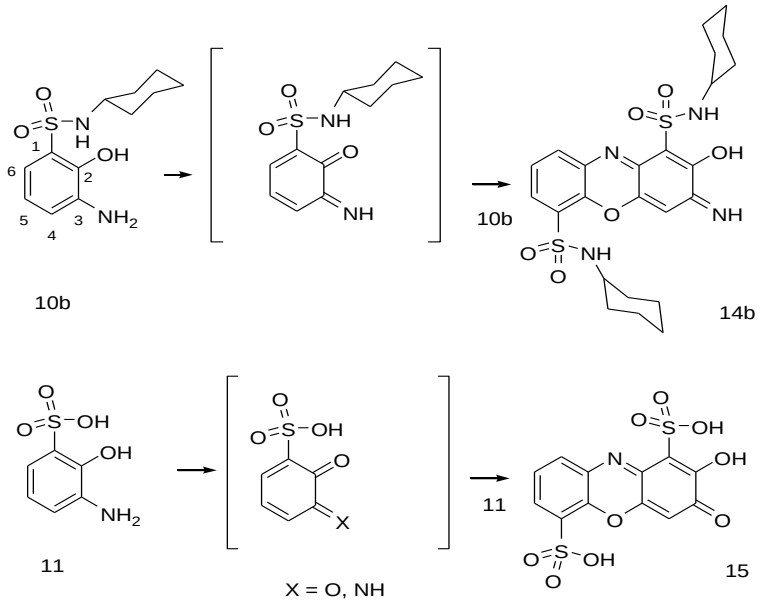
| Cpd[a] | HPLC[b] | pH[c] | Laccase activity[d] | Yields (%) Conver.[e] | Isolated[f] | Product |
|---|---|---|---|---|---|---|
| 3HAA | 1 | 4–4.5 | 20–100 | 70 | - | CA |
| 3HOA | 2 | 5–6 | 20–100 | 95 | 93 † | LAO |
| 4a | 2 | 4–6 | 100 | 80 | 60 £ | 13a |
| 4b | 3 | 4–6 | 400 | 80 | 86 ¥ | 13b |
| 4c | 2 | 4–6 | 20–100 | 90 | 90 † | 13c |
| 4d | 3 | 4–6 | 400 | 70 | 40 £ | 13d |
| 4e | 2 | 4–6 | 20–100 | 90 | 87 † | 13e |
| 4f | 2 | 4–6 | 100 | 70 | 58 £ | 13f |
| 4g | 2 | 4–6 | 20–100 | 90 | 90 † | 13g |
| 10b | 3 | 4–6 | 400 | 80 | 73 ¥ | 14b |
| 10c | 2 | 4–6 | 200–800 | 0 | - | - |
| 11 | 2 | 4 | 800 | 20 | 4 £ | 15 |
| 12 | 2 | 4–6 | 20–100 | 0 | - | - |

[a] 3HAA from commercial source, 3HOA previously synthesized[18] others from this work, see Table 1 [b] 1: Published method.[32]; 2 & 3 see supporting information for description of the HPLC methods, [c] optimum range of pH. [d] Laccase activity was measured following a known method, see experimental section. [e] Yields of conversion are qualitative and were evaluated by HPLC from the ratio of integration area detected at a wavelength of 280 nm. [f] Yields of isolated products are calculated from recovered mass, purities (>95%) were determined by HPLC and NMR after either freeze drying (†) or extraction (¥) or chromatography (£), see experimental.

Scheme 3. 6e oxidative cascade for the synthesis of phenoxazines LAO and 13a-g




Scheme 4. Laccase-mediated synthesis of phenoxazines 14b and 15



The case of **11** appeared to be less favourable. While the molecule was oxidized at a low rate at pH 4, it was the only pH value allowing the formation of the desired product. Increasing the catalyst loading did not improve the transformation (data not shown). The maximum conversion yield of 20% was obtained with a laccase activity of 800 U $L^{-1}$. This would suggest that the situation of the electron withdrawing group ($SO_2R^1$) adjacent to the phenol is not well tolerated by the biocatalyst.

Heterocycle **15** was isolated in small quantity after tedious purification using semi-preparative HPLC. The structural analysis of **15**, HRMS in particular, indicated the hydrolysis of the imine function. Whether this hydrolysis occurs during the oxidative cascade or the purification step is not known. The oxidation of **12** occurred in the medium but no coupling products could be detected. This would suggest that the *p*-iminoquinone species is too stable or that the aniline is not nucleophilic enough to realize the 1, 4-Michael addition required for coupling.

Finally, compound **10c** in the presence of laccase did not give any cyclized product. Varying the experimental conditions, we could not detect any coupling reaction of this compound. The behavior of this regioisomer is consistent with the observed poor reactivity of **11**. But surprisingly, both regioisomers **10b** and **4b** reacted well.

### *2.3 Structural analysis of heterocycles*

Using the optimal conditions, the syntheses of the tricyclic heterocycles were realized at the scale of 0.1 to 0.2 g. The crude products were usually pure enough to obtain clear NMR analyses. $^1$H NMR of the products **LAO**, **13b**, **14b**, and **15** are presented in Table 3 and the detailed $^{13}$C NMR assignments of the tricyclic framework of **LAO**, **13a-g**, **14b** and **15** can be found in the experimental section.

$^1$H NMR spectra of **LAO** and **15** (recorded in deuterated water) showed significant differences attributed to different frameworks. In the case of **15**, all the proton signals show a shift toward higher ppm values in comparison to those recorded for **LAO**. Proton signal of H9 in **15** is less well resolved comparatively to the signal of the corresponding proton H6 in **LAO**. A possible reason could be the protonation of N10, leading to H10 interaction with the nearby H9 in the tautomeric keto form.

$^1$H NMR spectra of **13b** and **14b** were recorded in deuterated acetone. Again, the specific pattern of a phenoxazine ring i.e proton signals of H8/7 and H6/9 as doublets, H7/8 as a doublet of doublet and H4 as singlet in **13b** and **14b** respectively, was identified.

Table 3. $^1$H NMR assignments of heterocycles LAO, 13b, 14b and 15

| | **LAO**[a] [c] | **15**[a] [d] | **13b**[b] [c] | **14b**[b] [d] |
|---|---|---|---|---|
| Proton | | | | |
| $NH_2$/OH | (labile) | (labile) | 7.57 & 8.30 | (labile) |
| =NH | _ | _ | _ | 6.66 (s) |
| $H_4$ | 6.56 (s) | 6.76 (s) | 6.54 (s) | 6.70 (s) |
| $H_6$ | 7.57 (d) | _ | 7.78 (d) | _ |
| $H_7$ | 7.43 (t) | 7.85 (d) | 7.70 (dd) | 8.05 (d) |
| $H_8$ | 7.77 (d) | 7.47 (t) | 7.95 (d) | 7.69 (dd) |
| $H_9$ | _ | 7.75 ( br d) | _ | 8.05 (d) |
| $SO_2NH$ | _ | _ | 6.65 & 6.52 (d) | 7.28 & 6.51 (d) |
| Alkyl (CH) | _ | _ | 3.08 & 3.19 (m) | 3.36 & 3.15 (m) |
| Alkyl ($CH_2$) | _ | _ | 1.1-1.6 (m) | 1.2-1.8 (m) |

[a] [$D_2$]$H_2O$; [b] [$D_6$]Acetone ; [c] framework “head to head” for LAO and **13b**; [d] framework “head to tail” for **15** and **14b**. JH-H coupling are detailed in the experimental section. Atom numbering is given in Schemes 3 and 4.

Downshield was observed for all the proton signals of these heterocycles comparatively to their respective parent compounds **LAO** and **15**. The $SO_2$-NH proton signals of the alkylsulfonamide groups in **13b** and **14b** are quite different: two doublets at 6.6 and 6.5 ppm, and 7.3 and 6.5 ppm respectively. Two proton signals of non equivalent hydrogen atoms of primary amino group at C2 are well visible in **13b**, while only one proton signal at 6.66 ppm (imine group at C3) is observed for **14b**.

Enzyme-processing of 1-sulfonyl-2-amino-3-phenol derivatives led to the phenoxazines with "head to head" framework, similarly to the biosynthesis of cinnabarinic acid from 3-hydroxy-anthranilic acid, while 1-sulfonyl-3-amino-2-phenol regioisomers gave "head to tail" dimers. This structure is unusual as laccase-oxidation product but typical of orcein pigments. [26]

Some heterocycles were further purified by chromatography and submitted to recrystallization. Two crystalline structures were analyzed by X-ray diffraction, respectively **13f** and **13g** (Figures 2 and 3). There is no previous crystallographic data on such phenoxazinones. The folding of the molecules is somehow similar to the one observed for natural actinomycin compounds and the one of actinocin derivatives obtained from computational studies.[27] A detailed discussion of the X-ray data is provided as supporting information.

Lastly, the phenoxazine derivatives were characterized by UV-Visible spectroscopy (see experimental section and supporting information). The parent compound LAO and the sulfonamide chromophores **13-14** showed similar spectra in water (pH 7.4) or polar organic solvents with absorption bands at 225, around 420 and 440 nm.

Figure 2. Molecular structure of one independent molecule of compound 13f ($R^1$ = $NHCH_2CO_2Me$) obtained from recrystallization in a mixture of $CH_2Cl_2$/MeOH (8/2) by slow evaporation. Ellipsoid drawn at 50% probability with ORTEP.

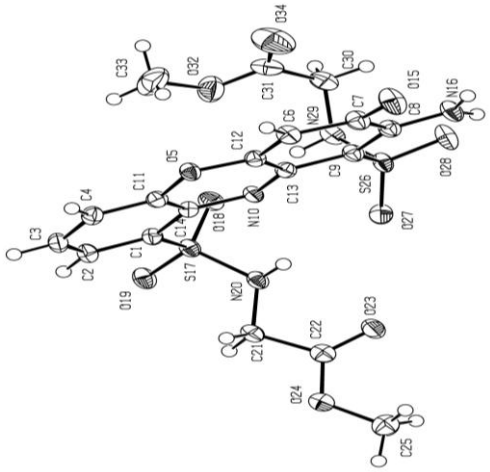
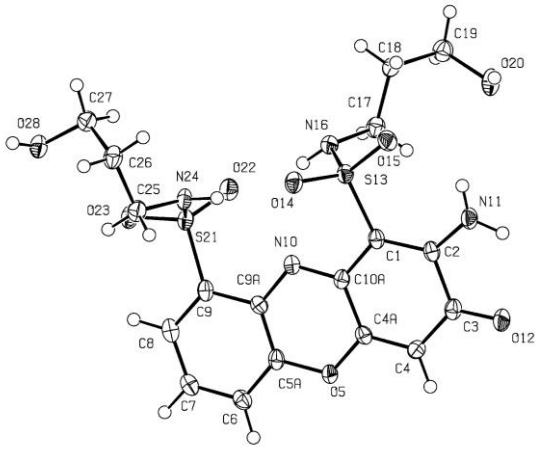



Figure 3. Molecular structure of compound 13g ($R^1$ = $NH(CH_2)_3CH_2OH$) obtained from recrystallization in a mixture of $CDCl_3$/$CD_3OD$ (8/2) by slow evaporation. Ellipsoid drawn at 50% probability with ORTEP.



The water solubility of these novel dyes depends on the sulfonyl substituent nature. Solutions up to $10^{-2}$ M concentration could be reached in water (pH 7.4) with LAO ($R^1$ = OH), **13c** ($R^1$ = $NH(CH_2)_3NMe_2$) and **13e** ($R^1$ = $NH(CH_2)_2NH_2$), in methanol with **13a** ($R^1$ = $NH_2$) and **13c** ($R^1$ = $NH(CH_2)_3NMe_2$), and in acetone with **13b** and **14b** ($R^1$ = NH-cyclohexyl). All compounds gave millimolar solutions in methanol (Table 4).

Table 4. Solubility of phenoxazinones

| Cpd | ClogP[a] | Range of solubility [b] | | |
|---|---|---|---|---|
| | | Buffer pH=7.4 | MeOH | Acetone |
| **LAO** | -0.39 | +++ | ++ | - |
| **13a** | -1.54 | ++ | +++ | + |
| **13b** | 2.51 | - | ++ | +++ |
| **13c** | -0.93 | +++ | +++ | ++ |
| **13d** | 2.23 | - | ++ | ++ |
| **13e** | -2.68 | +++ | ++ | ++ |
| **13f** | -1.84 | + | ++ | ++ |
| **13g** | -2.35 | + | ++ | ++ |
| **14b** | 3.16 | - | ++ | +++ |

[a] CLogP was calculated for the non ionic species with the Plugin LogP calculator of chemaxon Ltd.[b] (+++) means between $5.10^{-3}$ M and $1.10^{-2}$ M, (++) means above $1.10^{-3}$ M , (+) means between $2.10^{-4}$ M and $5.10^{-4}$ M , (-) means less than $1.10^{-4}$ M.

For a definition of CLogP, see page 12 of the manuscript

### *2.4 Kinetic data*

Kinetic data were collected using a non commercial laccase from *Pleurotus sajor caju*. The enzyme was fully purified following a previously published method[28,29] and its activity was determined at an optimal pH of 6 to 6.5. The selected laccase has been shown to be significantly similar to the commercial one from *Trametes*[30] while some differences remain in term of substrate affinity. Few representative substrates were chosen and submitted to the laccase mediated oxidation (Table 5, UV-vis spectra available as supporting information).

Table 5. Kinetic data

| Substrate | $K_m^{app}$ (mM) | $V_{max}$ (Δ Abs/min) |
|---|---|---|
| **3-HAA** | [a] | [a] |
| **3-HOA** | 0.075 ± 0.008 | 0.08 ± 0.002 |
| **4a** | 0.066 ± 0.001 | 0.033 ± 0.001 |
| **4c** | 0.069 ± 0.01 | 0.036 ± 0.002 |
| **4e** | 0.087 ± 0.01 | 0.028 ± 0.001 |
| **10b** | 0.042 ± 0.003 | 0.056 ± 0.001 |
| **11** | 1.89 ± 0.31 | 0.053 ± 0.005 |

[a] Km for natural substrate 3-HAA could not be obtained in the same experimental conditions due to poor water solubility of the conpound. In presence of 5% of DMSO, an apparent Km of 0.573 ± 0.096 mM and a Vmax of 0.290 ± 0.021 were measured. At such concentration of DMSO, laccase lost 34% of its activity which does not allow a direct comparison with the substrates in this work.

The pronounced importance of the position of the sulfonyl side chain was confirmed: an almost three fold increase of the apparent Km is recorded when changing the position of the sulfonate from **3-HOA** to **11**. The affinity for the substrate slightly decreased when passing from the derivatives **4a** to **4c**, **3-HOA** and **4e**.

This trend doesn't correlate with the scale of hydrophobicity and could suggest an interaction more or less favourable between the side chains and residues in the binding cavity of the enzyme. More surprising, the apparent Km of **4b** could not be determined due to its lower water-solubility while the apparent Km of the regioisomer **10b** could be determined. The affinity seemed even better for this substrate than for the previous ones. This would support to some extend the discrimination of substrates by preferential positioning into the active site.

### *2.5 Chemical oxidation*

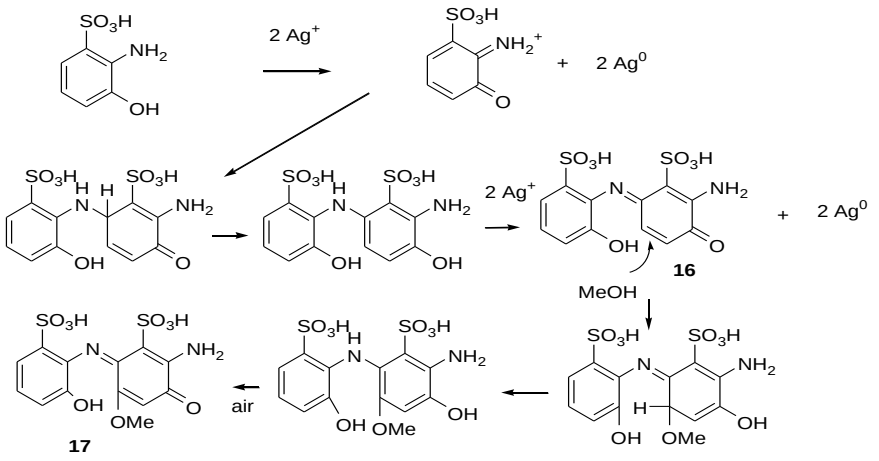
To further assess the scope of our methodology, **3-HOA** oxidation was performed with common chemical oxidants. The oxidative dimerization of **3-HAA** has been reported with moderate yields through potassium ferricyanide oxidation,[31,32] benzoquinone[33] and acidic ferric chloride in the presence of alkylsulphinate.[34] When ferricyanide and ferric chloride are used in aqueous solution, side products from extensive polymerization and competitive addition of alkylsulphinates were respectively observed.

Benzoquinone-mediated oxidation was performed in alcoholic solvent which is not possible for the biocatalytic system. Nevertheless, we tried to oxidize **3-HOA** in presence of ferricyanide in aqueous or alcoholic solvent. No aminophenoxazinone could be detected in both cases (see supporting information).

A biomimetic synthesis of natural products has been recently published with excellent yields using silver oxide as the reagent for oxidation of a catechol moiety.[35] In our case, the silver oxide (5eq) mediated oxidation of **3-HOA** yielded a side-product along with the desired compound **LAO** (well separated in the HPLC analyses).

The maximum ratio of side-product was observed when the reaction was performed in dry alcohol (see experimental annexe I.5). The crude mixture was partially purified by semi-preparative HPLC and a small quantity of product **17** was collected and identified by NMR analysis (Scheme 5).

Scheme 5. Oxidation of 3-HOA with $Ag_2O$ in methanol. The phenoxazine derivative LAO is produced alongside with a major product 17 which formation is shown here




This molecule would result from an imino-quinone intermediate **16** suffering 1,4 addition of methanol upon the carbon C5. Thus, after the first 2e oxidation followed by 1,4 addition of the aniline and a proton shift, a second 2e oxidation occurred and competition between addition of solvent and intramolecular phenol could be observed in the presence of pure methanol.

Our enzyme-catalyzed system supersedes such oxidative coupling reaction using a chemical oxidant since no side-product is formed with laccase.

## 3. Discussion

To mimic efficiently the synthesis of natural phenoxazine derivatives in aqueous solutions, the scope and limitations of the laccase-catalyzed system must be defined. Non natural aminophenols are expected to react differently depending on their physicochemical properties and structures. While it is commonly accepted that the binding cavity of laccase is large enough to adapt to a wide variety of substrates,[36,37] we found that some discriminations are still present among closely related structures. As mentioned in the literature, ortho-derivatives are better substrates for laccase than the ones having subtituents in para position such as **12**.[37]

From the experimental data recorded (Tables 2 and 5), major trends can be drawn. First, the sulfonated compounds **3-HOA** and **4a**, **c**, **e-g** are still better converted into the corresponding phenoxazines (**LAO**, **13a**, **c**, **e-g**) than **3-HAA** (carboxylated reference) in the same experimental conditions. Thus, water-solubility of the substrate is a favourable factor. Secondly, the position and the nature of the side group either adjacent to the amino function (**3-HOA**, **4a-g**) or to the phenol (**10b**, **10c**, and **11**) seem to have a prominent importance in this process. Substrates bearing the sulfonyl substituent in *ortho*-position versus $NH_2$ are always readily converted, while the regioisomers bearing the sulfonyl substituent in *ortho*-position versus OH are either unreactive (**10c**), very poorly reactive (**11**) or quite reactive (**10b**). Lastly, the hydrophobic nature of the substrates does not impair too much the reaction as long as the ratio of catalyst is sufficient.

Scheme 3 details the formal 6e oxidative cascade involved in the synthesis of heterocycles **LAO**, **13a** to **13g**. The *o*-iminoquinone species formed in situ rapidly reacts by Michael addition of the aniline substrate on its most electrophilic carbon C6, followed by a proton shift yielding the first coupling intermediate.

The second 2e oxidation is expected to be also mediated by laccase such as in the synthesis of actinomycin by the phenoxazinone synthase.[38] Intramolecular Michael addition of the phenol to the C5 atom leads to the fully reduced aminophenoxazinone which is spontaneously air-oxidized to produce the desired heterocycle. Similarly, laccase mediated 2e oxidation of **10b** yields the iminoquinone species (Scheme 4).

The aniline group of the substrate adds to the most electrophilic carbon C6 which is still the adjacent one to the sulfonyl side chain. The subsequent proton shift aromatizes the first oxidized intermediate as above. 2e Oxidation followed by intramolecular Michael addition of the phenol yields again a tricyclic framework which spontaneously oxidizes into the imine product **14b**. The phenoxazine obtained is thus cyclized in a "head to tail" manner which is confirmed by the $^{1}H$ NMR data (Table 3).

Why such process is not observed for the related substrate **10c** remains puzzling. The oxidative transformation of substrate **11** into the desired heterocycle was highly difficult (Scheme 4). Apparent Km of **11** suggests that free sulfonic acid (comparatively to the sulfonamide) in this precise regiochemistry, strongly handicapes the laccase mediated 2e oxidation. Furthermore, the imine group of the oxidized species has an increased susceptibility toward hydrolysis. The resulting *o*-quinone should be more reactive toward any nucleophile present in situ. This could explain the high amount of side products formed during the laccase-mediated oxidation of **11**.

Those side products were difficult to separate from the desired product and semi-preparative HPLC was required in order to isolate a small quantity of the phenoxazine **15**. HRMS analysis confirmed the molecular formula of $C_{12}H_7NO_9S_2$ which corresponds to the hydrolyzed product of the expected aminophenoxazinone ($C_{12}H_8N_2O_8S_2$).

The phenoxazines produced, except for the most hydrophilic **LAO**, **13c**, **13e** and **15**, are partially water-soluble at a concentration of 1 mM and thus precipitated during the bioprocess, but without perturbing the enzyme work. All the derivatives are oxidized over a range of 2 units of pH which correlates well with the previously reported bell-shaped laccase activity-pH relationship.[37] As the pH increases, phenols become more prone to oxidation, but the concentration of hydroxide ions and subsequent inhibition of laccase also increase.

## 4. Conclusion

An efficient biocatalytic system working under mild conditions is proposed for the synthesis of substituted phenoxazine derivatives bearing sulfonyl groups able to tune the solubility properties. Good substrates are ortho-aminophenols and not para-aminophenols, substituted with a sulfonyl moiety (acid or amides) next to the $NH_2$ group preferably to the OH group. The formation of “head to head“ dimers can be predicted in the first case (**LAO**, **13**) and “head to tail“ dimers in the latter one (**14, 15**).

The enzyme-catalyzed oxidative cascade, as a green chemistry process, has been shown to replace the industrial nitrosophenol mediated syntheses of aminophenoxazine dyes, and to be more selective than the recently proposed $Ag_2O$-mediated oxidation of *o*-substituted phenols. Investigation of the scope and limitations of our process led to the biocatalyzed synthesis of new water-soluble chromophores. Moreover, the presently synthesized heterocycles **13c**, **13e-g** contains chemical reactive functions which could be used in further derivatisations such as conjugation to polypeptides, carbohydrates, nucleic acids or linking to other labels, polymers or devices.[7b]

## 5. Experimental Section

### *5.1 General method*

$^1H$ and $^{13}C$ NMR spectra were recorded with a Bruker Avance 500 or a Bruker Avance 300 spectrometer. Spectra were obtained in [$D_6$]DMSO, [$D_6$]acetone, [D]$CHCl_3$, [$D_2$]$H_2O$ or [$D_4$]$CH_3OH$. Chemical shifts are reported relative to TMS.[39] $^{13}C$ NMR were obtained with broadband proton decoupling (D1 = 5 sec) or DEPTQ. Low resolution mass spectra were acquired using a Thermo Finnigan LCQ spectrometer, either in positive mode APCI, negative mode ESI or EI methods (70 eV). High Resolution Mass Spectrometry (HRMS) analyses using ESI or FAB methods were performed at the University of Mons Hainaut (Belgium) or at the University of Oxford (UK). Melting points were recorded with a Büchi Melting point B-540 apparatus calibrated. Infrared spectra were recorded with a Shimadzu FT-IR 84005 spectrometer using KBr plates. The HPLC system consisted of Waters Alliance 2699 separation module, Waters 2998 photodiode array detector (for analytical separations) or a Waters 600 pump, a waters 717

autosampler and Waters 486 absorbance detector (for semi-preparative HPLC) (Waters, Milford, Massachusets, USA).

Analytical separations were performed with Waters XterraMSC18 column (4.6 × 100 mm, 5 µm) (Waters, Milford, Massachusets, USA) equipped with a conventional pre-column. Detection was performed at 220/254/280 nm with control at 440/460 nm and on-line UV-Visible absorbance scans were performed. Semi-preparative separation was performed with a Waters Xterra-PrepMSC18 OBD column (30 × 100 mm, 5 µm) (Waters, Milford, Massachusets, USA) equipped with a conventional pre-column. Detection was performed at 300 nm. All the solvents were of HPLC grade. Flow rate was of 1 $mL.min^{-1}$ for analytical separation and 15 $mL.min^{-1}$ for semi-preparative separations.

### *5.2. Biotransformation*

Laccase from *Trametes versicolor* was purchased as a brownish powder form Bioscreen e.K. (Uebach-Palenberg, Germany) under the commercial trademark Oxizym LA (batch n° LA 2000/001). Laccase activity was determined by oxidation of ABTS [2',2-azino-bis-(3-ethylbenzo thiazoline-6-sulfonic acid)] (4 mM) (Sigma-Aldrich) into a stable cation radical ABTS●+ (absorption coefficient, $\varepsilon M = 34\ 220\ M^{-1}\ cm^{-1}$) in tartaric buffer (100 mM) at pH 4.5, at 25 °C. One unit was defined as the amount of enzyme that oxidizes 1 µmol of ABTS per minute. Analytical biotransformations were performed at 25 °C in solutions containing 1 mM of substrate, 0.1 M phosphate buffer or 0.1 M acetate buffer and 100 $U\ L^{-1}$ Laccase, unless otherwise mentioned. All reactions were monitored through spectrophotometry between 300 and 800 nm using a Beckman DU-800 spectrophotometer (Analis, Namur, Belgium) connected to a high performance temperature controller.

### *5.3. Preparation of laccase from Pleurotus sajor-caju:*

Laccase was partially purified from a culture medium of the fungus Pleurotus sajor-caju, a basidiomycetes belonging to the class of white-rot fungi capable of degrading lignin when grown on lignocellulosics.[40] The culture medium containing the extracellular laccase was submitted to two consecutive chromatographic steps with Macro-Prep DEAE and Sephacryl HR-200 (Pharmacia, Sweden). Laccase activity was measured spectrophotometrically by monitoring the absorbance increase at 525 nm when 50 μM syringaldazine was present as the substrate in a final volume of 1 mL of a buffered (50 mM potassium phosphate, pH 6.5) solution. One laccase unit is defined as the amount of the enzyme capable of forming 1 μmol of product per minute.[41]

### *5.4 Spectrophotometric measurements*

Scans and kinetic measurements were carried out with an Ultrospec 2110 pro UV/Vis spectrophotometer (Amersham Biosciences, Milan, Italy). For each compound to be tested, 1 mM stock solution in 10% EtOH was prepared. The reaction mixtures contained 100 μM of each compound in the presence of 50 mM potassium phosphate buffer (pH 6.5). The reaction started by adding laccase (28 U/mL), in 1 mL as the final volume. UV/visible absorption spectra were recorded immediately after mixing (t=0) and at prearranged times, and the visible maximum absorption was determined for each compound. In order to verify possible phenomena of autoxidation, each measurement was also followed in the absence of laccase for the same period of time.

### *5.5 Determination of kinetic parameters Km and Vmax*

Compounds **3-HOA, 11, 4a, 4c, 4e, 4f** were dissolved in 10% EtOH at a concentration of 20 mM. Kinetic assays were carried out by measuring the product formation at the selected wavelength for each compound.

Selected wavelengths for the product formation were determined after inspection of each absorption spectrum (see above). The Km value for **4b** could not be determined due to insolubility of this substrate at concentrations above 2 mM. Such a concentration was not enough to allow the reaction to reach the $V_{max}$.

### *5.6 Statistical analysis and software:*

All experiments were run at least in triplicate. Origin 7.0 (Origin Corporation, Northampton, MA, USA) was used for statistical analysis. Lineweaver-Burk data were analyzed with GraFit 4.0.21, Erithacus Software Ltd, UK.

### *5.7 X-ray crystallography*

The X-ray intensity data were collected at 120K for both structures with a MAR345 image plate using MoK□ (□= 0.71069Å) radiation. The crystal was chosen, mounted in inert oil and transferred to the cold gas stream for flash cooling. The crystal data and the data collection parameters are summarized in Table 7 which can be found in the supporting information. The unit cell parameters were refined using all the collected spots after the integration process. The data were not corrected for absorption, but the data collection mode partially takes the absorption phenomena into account. Both structures were solved by Patterson method and refined by full-matrix least squares on F2 using SHELXL97. All the non-hydrogen atoms were refined anisotropically. For **13f**, there are two independent molecules in the asymmetric part of the unit cell. For this structure, the H atoms of the N-H groups were located by Fourier difference and all the other hydrogen atoms were calculated with AFIX. The H atoms were included in the refinement with a common isotropic temperature factor (B = 0.028 $Å^2$). For **13g**, one molecule of methanol is co-crystallized. All the H atoms were located by Fourier difference and included in the refinement with a common isotropic temperature factor (B = 0.029 $Å^2$).

The details of the refinement and the final R indices are presented in table 7. CCDC 719578 (**13f**) and CCDC 719579 (**13g**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif." Molecular structures are drawn with Ortep software.

### *5.8 Organic synthesis:*

Reactions with organolithium reagents were carried out under argon atmosphere in flame-dried glassware. Anhydrous tetrahydrofuran and methylene chloride were purchased from Fluka and chemicals reagents and reactants were from Aldrich and Acros companies. Thin layer chromatography was carried out on aluminium-backed silica gel plate F254 (Merck) and visualized by UV irradiation or by iodine vapor. Column chromatography was carried out with either flash silica gel (60-200 µm) or silica gel 60-RP18 (40-60 µm) as the stationary phase and eluted using the flash chromatography technique.

### *5.9. 2-tert-butylbenzoxazole-4-sulfonyl chloride (2)*

To a solution of 1[18] (3.92 mmol; 1 g) in anhydrous $CH_2Cl_2$ (20 mL) was added phosphorus pentachloride (11.76 mmol; 2.4 g) and phosphorus oxychloride (11.76 mmol; 1.1 mL) and the resulting mixture was heated at reflux for 4 hours. The reaction was then cooled to room temperature and the solution was poured over ice. The ice was washed with diethyl ether and the organic phases were collected and dried over anhydrous sodium sulphate. Evaporation of the solvent gave a residue which was purified by column chromatography on flash silica gel (cyclohexane/ethyl acetate 9:1) to give the title compound as a gummy solid. Yield = 80%. (0.799 g). MW: $C_{11}H_{12}ClNO_3S$, 273.7 $g.mol^{-1}$ ; Rf=0.3 (cyclohexane/AcOEt); $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ = 7.97 (dd, $J_{H-H}$ = 8 & 0.9 Hz, 1 H, $H_5$), 7.88 (dd, $J_{H-H}$ = 8 & 0.9 Hz, 1 H, $H_7$), 7.50 (t, JH-H = 8 Hz, 1 H, $H_6$),

1.60 (s, 9 H, 3 x $CH_3$) ppm. $^{13}C$ NMR (75 MHz, $CDCl_3$) δ = 177.3 ($C_2$), 152.5 ($C_8$), 139.1 ($C_4$), 133.9 ($C_9$), 124.2 ($C_6$), 123.9 ($C_5$), 117.9 ($C_7$), 35.1 ($C_{10}$), 28.8 ($C_{11}$) ppm. IR (KBr) ν~ = 720-680 (1,2,3-Ar), 1180 & 1203 ($SO_2Cl$), 1357-1427 ($SO_2Cl$), 1542 (benzoxazole) $cm^{-1}$; APCI-MS: (m/z) (%) : 273.96 (100) [M+H], 275.92 (40), 270.12 (40), 201.95 (25), 174.05 (40), 118.01 (7), 89.98 (10); HRMS-ESI: clcld for $C_{11}H_{12}NO_3NaSCl$ = 296.0124, found 296.0121

### *5.10. General procedure for the synthesis of compounds 3a-g:*

A solution of **2** (1 mmol) in anhydrous $CH_2Cl_2$ (5 mL) was added dropwise at 0 °C to an aqueous solution of amine (10% w/w) in excess. The reaction is allowed to warm to room temperature overnight. The organic phase was collected and washed with HCl 1N (3 x 20 mL). The organic phases were pooled together and dried over anhydrous sodium sulphate to give the title compound.

### *5.11 2-tert-butylbenzoxazole-4-sulfonic acid amide (3a).*

White solid. Yield = 80%. MW: $C_{11}H_{14}N_2O_3S$, 254.3 $g.mol^{-1}$; m.p. 133.6°C; $^1H$ NMR (300 MHz, DMSO) δ = 7.97 (d, $J_{H-H}$ = 8.1 Hz,1 H, $H_5$), 7.75 (d, $J_{H-H}$ = 7.7 Hz, 1 H, $H_7$), 7.50 (dd, $J_{H-H}$ = 7.7 & 8.1 Hz, 1 H, $H_6$), 7.40 (bs, 2 H, $SO_2NH_2$), 1.50 (s, 9 H, 3 x $CH_3$) ppm. $^{13}C$ NMR (75 MHz DMSO) δ = 175.2 ($C_2$), 151.8 ($C_8$), 137.7 ($C_4$), 134.9 ($C_9$), 125.2 ($C_6$), 122.8 ($C_5$), 115.4 ($C_7$), 34.9 ($C_{10}$), 29.0 ($C_{11}$) ppm. IR (KBr) ν~ = 740-790 ($SO_2NH$), 1120-1160 (1,2,3-Ar), 1203-1253 ($SO_2NH$), 1346, 1420, 1542 (benzoxazole), 3448 (NH) $cm^{-1}$. ESI-MS: (m/z) (%) = 254.63 (80) [M+•], 276.61 (100), 308.65 (45), HRMS-ESI: clcld for $C_{11}H_{14}N_2O_3NaS$ = 277.0623, found 277.0621

***5.12 2-tert-butylbenzoxazole-4-sulfonic acid cyclohexyl amide (3b).***

White solid. Yield = 80%. MW: $C_{17}H_{24}N_2O_3S$, 336.4 g.mol$^{-1}$.; m.p. = 130°C; $^1$H NMR (300 MHz DMSO) δ = 7.95 (d, $J_{H-H}$ = 8.1 Hz,1 H, $H_5$), 7.74 (d, $J_{H-H}$ = 7.8 Hz, 1 H, $H_7$), 7.52 (d, $J_{H-H}$ = 7.6 Hz, 2 H, $SO_2NH$), 7.50 (dd, $J_{H-H}$ = 7.6 & 7.7 Hz, 1 H, $H_6$), 3.60 (bs, 1 H, $H_{12}$), 1.60 (m, 4 H, $H_{13}$ & $H_{17}$), 1.50 (s, 9 H, $H_{11}$), 1.15 (m, 6 H, $H_{14-16}$ ) ppm. $^{13}$C NMR (75 MHz DMSO) δ = 174.8 ($C_2$), 151.5 ($C_8$), 137.5 ($C_4$), 133.7 ($C_9$), 125.0 ($C_6$), 123.3 ($C_5$), 115.2 ($C_7$), 52.5 ($C_{12}$), 34.4 ($C_{10}$), 34.2 ($C_{13}$ & $C_{17}$), 28.5 ($C_{11}$), 25.2 ($C_{14-16}$) ppm. IR (KBr) ν~ = 675-929 ($SO_2NH$, 1,2,3-Ar), 1145, 1203-1292 ($SO_2NH$), 1562-1604 (benzoxazole), 2920 (alcane), 3305 (NH) cm$^{-1}$. ESI-MS: (m/z) (%) = 337.01 (70) [M+H], 255.14 (100), 256.09 (35), 270.02 (15). HRMS-ESI: clcld for $C_{17}H_{24}N_2O_3NaS$ = 359.1412, found 359.1405

***5.13 2-tert-butylbenzoxazole-4-sulfonic acid (3-dimethylamino-propyl) amide (3c).***

White gummy solid. Yield = 80%. MW: $C_{16}H_{25}N_3O_3S$, 339.4 g.mol$^{-1}$. $^1$H NMR (300 MHz DMSO) δ = 7.99 (d, $J_{H-H}$ = 8.1 Hz,1 H, $H_5$), 7.80 (m, 1 H, $SO_2NH$), 7.75 (d, $J_{H-H}$ = 7.8 Hz, 1 H, $H_7$), 7.52 (dd, $J_{H-H}$ = 7.8 & 8.1 Hz, 1 H, $H_6$), 3.12 (m, 2 H, $H_{12}$), 2.99 (m, 2 H, $H_{14}$), 2.66 (s, 6 H, $H_{15}$), 1.83 (m, 2 H, $H_{13}$), 1.5 (s, 9 H, $H_{11}$) ppm. $^{13}$C NMR (75 MHz DMSO) δ = 175.0 ($C_2$), 151.5 ($C_8$), 137.7 ($C_4$), 131.9 ($C_9$), 125.0 ($C_6$), 123.7 ($C_5$), 115.5 ($C_7$), 54.7 ($C_{12}$), 42.5 ($C_{15}$), 39.1 ($C_{14}$), 34.5 ($C_{10}$), 28.6 ($C_{11}$), 25.1 ($C_{13}$) ppm, IR (KBr) ν~ = 670-760 ($SO_2NH$), 1099-1180-1253 ($SO_2NH$ & $NMe_2$), 1542-1604 (benzoxazole), 2920, 3440 cm$^{-1}$. ESI-MS: (m/z) (%) = 340.36 (70) [M+H], 295.24 (10), 266.96 (10). HRMS-ESI: clcld for $C_{16}H_{26}N_3O_3S$ = 340.1695, found 340.1695

***5.14 2-tert-butylbenzoxazole-4-sulfonic acid phenyl amide (3d).***

Brown solid. Yield = 80%. MW: $C_{17}H_{18}N_2O_3S$, 330.4 $g.mol^{-1}$; m.p. = 189°C; $^1H$ NMR (300 MHz DMSO) δ = 10.40 (s, 1 H, $SO_2NH$), 7.95 (dd, $J_{H-H}$ = 8.1 & 0.9 Hz, 1 H, $H_5$), 7.80 (dd, $J_{H-H}$ = 7.9 & 0.6 Hz, 1 H, $H_7$), 7.50 (dd, $J_{H-H}$ = 8.1 & 7.9 Hz, 1 H, $H_6$), 7.14 (m, 4 H, $H_{13,14,16,17}$), 6.93 (m, 1 H, $H_{15}$), 1.44 (s, 9 H, $H_{11}$) ppm. $^{13}C$ NMR (75 MHz DMSO) δ = 175.2 ($C_2$), 151.3 ($C_8$), 137.9 ($C_4$), 130.3 ($C_9$), 129.2 ($C_{13,17}$), 125.0 ($C_6$), 124.1 ($C_5$), 120.0 ($C_{14-16}$), 116.0 ($C_7$), 34.5 ($C_{10}$), 28.5 ($C_{11}$) ppm. IR (KBr) ν~ = 650-756 (Ar & $SO_2NH$), 1203-1485 ($SO_2NH$ & Ar), 1594 (benzoxazole), 3450 (NH) $cm^{-1}$. ESI-MS: (m/z) (%) = 331.09 (100) [M+H], 393.14 (10), 496.18 (25), HRMS-ESI: clcld for $C_{17}H_{18}N_2O_3NaS$ = 353.0936, found 353.0943

***4.15 2-tert-butylbenzoxazole-4-sulfonic acid (2-amino-ethyl) amide (3e).***

White solid. Yield = 75%. MW: $C_{13}H_{19}N_3O_3S$, 297.1 $g.mol^{-1}$.; m.p. = 90°C; $^1H$ NMR (300 MHz $CDCl_3$) δ = 7.84 (d, $J_{H-H}$ = 7.7 Hz,1 H, $H_5$), 7.70 (d, $J_{H-H}$ = 8.2 Hz, 1 H, $H_7$), 7.41 (dd, $J_{H-H}$ = 7.7 & 8.2 Hz, 1 H, $H_6$), 3.01 (dd, $J_{H-H}$ = 5.0 Hz & 6.5 Hz, 2 H, $H_{12}$), 2.80 (dd, $J_{H-H}$ = 5 Hz & 6.4 Hz, 2 H, $H_{13}$), 1.50 (s, 9 H, $H_{11}$) ppm. $^{13}C$ NMR (75 MHz DMSO) δ = 176.1 ($C_2$), 151.8 ($C_8$), 137.8 ($C_4$), 130.7 ($C_9$), 124.6 ($C_6$), 124.0 ($C_5$), 115.2 ($C_7$), 46.4 ($C_{12}$), 41.7 ($C_{13}$), 34.9 ($C_{10}$), 28.8 ($C_{11}$) ppm, IR (KBr) ν~ = 736-756 ($SO_2NH$), 933, 1087-1203 ($SO_2NH$), 1427 (alcane), 1558 (benzoxazole), 3280 (NH) $cm^{-1}$. ESI-MS: (m/z) (%) = 298.1 (100) [M+H], 320 (7), 281.24 (5), 594.64 (5). HRMS-ESI: clcld for $C_{13}H_{19}N_3O_3NaS$ = 320.1045, found 320.1053

***5.16 (2-tert-butylbenzoxazole-4-sulfonylamino)-acetic acid ethyl ester (3f).***

White solid. Yield = 70%. MW: $C_{15}H_{20}N_2O_5S$, 340.4 g.mol$^{-1}$.; m.p. = 92°C; $^1$H NMR (300 MHz $CDCl_3$) δ = 7.84 (d, $J_{H-H}$ = 7.8 Hz,1 H, $H_5$), 7.70 (d, $J_{H-H}$ = 8.4 Hz, 1 H, $H_7$), 7.40 (dd, $J_{H-H}$ = 7.8 & 8.4 Hz, 1 H, $H_6$), 5.90 (m, 1 H, $SO_2NH$), 4.10 (dd, $J_{H-H}$ = 7.1 & 14.3 Hz, 2 H, $H_{14}$), 4.01 (d, $J_{H-H}$ = 5.4 Hz, 2 H, $H_{12}$), 1.51 (s, 9 H, $H_{11}$), 1.20 (t, $J_{H-H}$ = 7.1 Hz, 3 H, $H_{15}$) ppm. $^{13}$C NMR (75 MHz $CDCl_3$) δ = 175.7 ($C_2$), 168.6 ($C_{13}$), 151.4 ($C_8$), 137.8 ($C_4$), 130.1 ($C_9$), 124.1 ($C_6$), 123.4 ($C_5$), 115.0 ($C_7$), 61.6 ($C_{14}$), 44.8 ($C_{12}$), 34.5 ($C_{10}$), 28.4 ($C_{11}$), 14.0 ($C_{15}$) ppm, IR (KBr) v~ = 694-101 ($SO_2NH$ & 1,2,3-Ar), 1122-1253 & 1334 ($SO_2NH$ & alcane), 1423-1550 (benzoxazole), 1740 (CO), 2977 (Hydrogen bonding), 3271(NH) cm$^{-1}$. ESI-MS: (m/z) (%) = 298.1 (100) [M+H], 341.32 (60), 363.11 (100) [M + $Na^+$], 702.61 (100), HRMS-ESI: clcld for $C_{15}H_{20}N_2O_5NaS$ = 363.0991, found 363.0995

***5.17 2-tert-butylbenzoxazole-4-sulfonic acid (3-hydroxy-propyl) amide (3g).***

White solid. Yield = 70%. MW: $C_{14}H_{20}N_2O_4S$, 312.2 g.mol$^{-1}$.; m.p. = 140°C; $^1$H NMR (300 MHz $CDCl_3$) δ = 7.90 (d, $J_{H-H}$ = 7.8 Hz, 1 H, $H_5$), 7.72 (d, $J_{H-H}$ = 7.8 Hz, 1 H, $H_7$), 7.43 (t, $J_{H-H}$ = 7.8 Hz, 1 H, $H_6$), 5.32 (bs, 1 H, $SO_2NH$), 3.76 (t, $J_{H-H}$ = 5.7 Hz, 2 H, $H_{12}$), 3.10 (t, $J_{H-H}$ = 5.7 Hz, 2 H, $H_{14}$), 1.75 (m, 2 H, $H_{13}$), 1.53 (s, 9 H, $H_{11}$) ppm. $^{13}$C NMR (75 MHz $CDCl_3$) δ = 176.2 ($C_2$), 151.7 ($C_8$), 137.7 ($C_4$), 130.5 ($C_9$), 124.7 ($C_6$), 124.2 ($C_5$), 115.3 ($C_7$), 60.4 ($C_{14}$), 41.1 ($C_{12}$), 32.3 ($C_{13}$), 30.1 ($C_{10}$), 28.4 ($C_{11}$) ppm, IR (KBr) v~= 694-101 ($SO_2NH$ & 1,2,3-Ar), 1122-1253 & 1334 ($SO_2NH$ & alcane), 1423-1550 (benzoxazole), 2977 (alcane), 3290-3460 (NH & OH) cm$^{-1}$. ESI-MS: (m/z) (%) = 313.1 (100) [M+H], 335.1 (60) [M + $Na^+$], HRMS-ESI: clcld for $C_{14}H_{21}N_2O_4S$= 313.1222, found 313.1218

### *5.18 General procedure for the synthesis of compounds 4a-g.*

To a solution of 6 N HCl (100 mL) was added (1 mmol) of **3a-g**. The mixture was heated at 100 °C overnight. Then the solution was cooled down, filtered, and evaporated. The residue was purified by column chromatography on flash silica gel (diethyl ether/ ethyl acetate 7:3) to give the title compound. Purity of the compound was check by HPLC.

### *5.19 2-Amino-3-hydroxy-benzenesulfonamide (4a).*

Yellow powder.Yield: 30%. MW: $C_6H_8N_2O_3S$, 188.2 g.mol$^{-1}$; m.p. = 192.1°C; $^1$H NMR (300 MHz $CD_3OD$) δ = 7.20 (dd $J_{H-H}$ = 8.1 Hz & 1.2 Hz, 1 H, $H_6$), 6.83 (dd, $J_{H-H}$ = 7.8 & 1.5 Hz, 1H, $H_4$), 6.56 (dd, $J_{H-H}$ = 8.1 & 7.7 Hz, 1 H, $H_5$) ppm. $^1$H NMR 300 MHz (DMSO) δ = 7.20 (bs, 1 H), 7.10 (dd $J_{H-H}$ = 8.1 Hz & 1.3 Hz, 1 H, $H_6$), 6.82 (dd, $J_{H-H}$ = 7.7 & 1.2 Hz, 1H, $H_4$), 6.50 (dd, $J_{H-H}$ = 8.1 & 7.6 Hz, 1 H, $H_5$), 5.31 (d, $J_{H-H}$ = 4.9 Hz, 2 H, $SO_2NH_2$) ppm. $^{13}$C NMR (75 MHz DMSO) δ = 145.2 ($C_3$), 135.1 ($C_1$), 125.1 ($C_2$), 118.6 ($C_5$), 116.7 ($C_6$), 115.1 ($C_4$) ppm. IR (KBr) v~= 667-720 ($SO_2NH$), 1130-1180 & 1298-1323 ($SO_2NH$ & 1,2,3-Ar), 1500, 1620 ($ArNH_2$), 3230-3430 ($NH_2$) cm$^{-1}$. UV/Vis ($H_2O/CH_3SO_3H$): $\lambda_{max}$ = 310 nm, ESI-MS: (m/z) (%) = 187.1 [M-H] (10), 188.08 [M•$^+$] (100), 79.8 (45). HRMS-ESI: clcld for $C_6H_9N_2O_3S$ = 189.0334, found 189.0331

### *5.20 2-Amino-N-cyclohexyl-3-hydroxy-benzenesulfonamide (4b).*

Rose-colored foam. Yield: 95%. MW: $C_{12}H_{18}N_2O_3S$, 270.3 g.mol$^{-1}$; m.p. = 165.6°C; $^1$H NMR 300 MHz ($CD_3OD$) δ = 7.50 (dd $J_{H-H}$ = 8.1 & 1.1 Hz, 1 H, $H_6$), 7.20 (d, $J_{H-H}$ = 7.7 & 1.1 Hz, 1H, $H_4$), 6.91 (dd, $J_{H-H}$ = 7.9 & 7.7 Hz, 1 H, $H_5$), 5.21 (bs, 4 H, ), 3.84 (q, $J_{H-H}$ = 7 Hz, 1 H, $H_7$), 2.01 (m, 4 H, $H_8$, & $H_{12}$), 1.51 (m, 6 H, $H_{9-11}$) ppm. $^{13}$C NMR 125 MHz ($CD_3OD$) δ = 146.6 ($C_3$), 135.6 ($C_1$), 122.7 ($C_2$), 119.8 ($C_5$), 117 ($C_6$), 115.8 ($C_4$), 52.6 ($C_7$), 33.8 ($C_8$ & $C_{12}$), 25.4 ($C_9$ & $C_{11}$), 25.1 ($C_{10}$) ppm. IR (KBr) v~= 650-780 ($SO_2NH$), 1080-1240

& 1296-1326 ($SO_2NH$), 1440-1480 (alcane), 1616 ($ArNH_2$), 2800-3040 (alcane), 3200-3400 (NH) $cm^{-1}$. UV/Vis ($H_2O/CH_3SO_3H$): λmax = 319 nm, ESI-MS: (m/z) (%) = 270.90 [M+H] (100), 204.11 (15), 189.01 (20). HRMS-ESI: clcld for $C_{12}H_{18}N_2O_3NaS$ = 293.0936, found 293.0931

### *5.21 2-Amino-N-(3-dimethylamino-propyl)-3-hydroxy-benzenesulfonamide (4c).*

Rose-colored foam. Yield: 95%. MW: $C_{11}H_{19}N_3O_3S$, 273.3 $g.mol^{-1}$.; m.p. = 105°C; $^1H$ NMR (300 MHz $CD_3OD$) δ = 7.02 (dd $J_{H-H}$ = 7.9 & 1.4 Hz,1 H, $H_6$), 7.33 (t, $J_{H-H}$ = 7.9 Hz, 1H, $H_5$), 7.21 (dd, $J_{H-H}$ = 7.9 & 1.4 Hz, 1 H, $H_4$), 3.20 (td, $J_{H-H}$ = 1.6 & 3.3 Hz, 2 H, $H_7$), 3.06 (t, $J_{H-H}$ = 6.4 Hz, 2 H, $H_9$), 2.90 (s, 6 H, $H_{10}$), 1.91 (m, 2 H, $H_8$) ppm. $^{13}C$ NMR (125 MHz DMSO) δ = 146.7 ($C_3$), 133.9 ($C_1$), 121.1 ($C_2$), 119.2 ($C_5$), 117.3 ($C_6$), 116.2 ($C_4$), 54.9 ($C_7$), 42.7 ($C_{9-10}$), 23.9 ($C_8$) ppm. IR (KBr) v~= 650-790 ($SO_2NH$), 1072-1326 ($SO_2NH$ & $NMe_2$), 1420-1480 (alcane), 1620 ($ArNH_2$), 2711-3300 & 3400 ( NH, hydrogen bonding) $cm^{-1}$. UV/Vis ($H_2O/CH_3SO_3H$): λmax = 317 nm, ESI-MS: (m/z) (%) = 274.13 [M+H] (100), 275.14 (20), 340.16 (7). HRMS-ESI: clcld for $C_{11}H_{20}N_3O_3S$ = 274.1225, found 274.1229

### *5.22 2-Amino-N-phenyl-3-hydroxy-benzenesulfonamide (4d).*

Red gummy solid. Yield: 30% MW: $C_{12}H_{12}N_2O_3S$, 264.3 $g.mol^{-1}$; 1H NMR (500 MHz DMSO) δ = 7.20 (m, 2 H, $H_8$ & $H_{12}$), 7.04 (m, 3 H, $H_9$ & $H_{11}$ & $H_6$), 6.96 (m, 1 H, $H_{10}$), 6.80 (dd, $J_{H-H}$ = 7.6 & 1.1 Hz, 1 H, $H_4$), 6.43 (dd, $J_{H-H}$ = 7.6 & 7.9 Hz, $H_5$), 5.41 (bs, 2 H, $NH_2$) ppm. $^{13}C$ NMR (125 MHz DMSO) δ = 145.7 ($C_3$), 138.7 ($C_1$), 136.7 ($C_2$), 129.8 ($C_8$ & $C_{12}$), 124.1 ($C_7$), 120.5 ($C_5$), 119.8 ($C_9$ &$C_{11}$), 119.7 ($C_{10}$), 117.8 ($C_6$), 115.3 ($C_4$) ppm. IR (KBr) v~= 670-729 ($SO_2NH$), 921 (Ar), 1080 (Ar), 1137-1184 (Ar), 1276-1315 ($SO_2NH$), 1496, 1620 ($ArNH_2$), 3200, 3300 (NH) $cm^{-1}$. UV/Vis ($H_2O/CH_3SO_3H$): $\lambda_{max}$ = 320 nm, ESI-MS: (m/z) (%) = 265.02 [M+H] (100), 266.06 (10),

197.22 (10), HRMS-ESI: clcld for $C_{12}H_{13}N_2O_3S$ = 265.0647, found 265.0640

***5.23 2-Amino-N-(2-amino-ethyl)-3-hydroxy-benzenesulfonamide (4e).***

Rose-colored foam. Yield: 95%. MW: $C_8H_{13}N_3O_3S$, 231.3 $g.mol^{-1}$; m.p. = 176.1°C; $_1$H NMR (300 MHz $CD_3OD$) δ = 7.42 (dd $J_{H-H}$ = 7.8 & 1.2 Hz, 1 H, $H_6$), 7.30 (dd, $J_{H-H}$ = 7.8 & 8 Hz, 1H, $H_5$), 7.22 (dd, $J_{H-H}$ = 8.0 Hz, 1.2 H, $H_4$), 3.20 (m, 2 H, $H_7$), 3.10 (m, 2 H, $H_8$) ppm. $^{13}$C NMR (125 MHz DMSO) δ = 146 ($C_3$), 135.1 ($C_1$), 120.5 ($C_3$), 119.6 ($C_5$), 117.7 ($C_6$), 116.1 ($C_4$), 49 ($C_7$), 38.8 ($C_8$) ppm. IR (KBr) v~= 657 ($SO_2NH$), 786 (1,2,3-Ar), 1130-1326 ($SO_2NH$), 1469-1488 (alcane), 1620 ($ArNH_2$), 3000-3200 ($NH_2$, $SO_2NH$) $cm^{-1}$. UV/Vis ($H_2O/CH_3SO_3H$): $\lambda_{max}$ = 320 nm, ESI-MS: (m/z) (%) = 232.13 [M+H] (100), HRMS-ESI: clcld for $C_8H_{14}N_3O_3S$ = 232.0756, found 232.0765

***5.24 (2-Amino-3-hydroxy-benzenesulfonylamino)-acetic acid methyl ester (4f).***

Rose-colored foam. Yield: 95%. MW: $C_9H_{12}N_2O_5S$, 260.2 $g.mol^{-1}$; m.p. = 195.8°C; $^1$H NMR (300 MHz DMSO) δ = 7.01 (dd $J_{H-H}$ = 8.1 & 1.2 Hz,1 H, $H_6$), 6.93 (dd, $J_{H-H}$ = 7.7 & 1.2 Hz, 1 H, $H_4$), 6.58 (dd, $J_{H-H}$ = 7.7 & 8.1 Hz, 1H, $H_5$), 3.63 (bs, 2 H, $H_7$), 3.52 (s, 3 H, $H_9$) ppm. $^{13}$C NMR (125 MHz DMSO) δ = 169.9 ($C_8$), 146.0 ($C_3$), 134.7 ($C_1$), 121.5 ($C_2$), 119.4 ($C_5$), 117.7 ($C_6$), 116.2 ($C_4$), 52.2 ($C_9$), 43.9 ($C_7$) ppm. ESI-MS: (m/z) (%) = 261.11 [M+H] (75), 283.12 (35), 327.14 (100), 349.09 (35). IR (KBr) v~ = 1114-1342 ($SO_2NH$), 1420-1480 (alcane), 1620 ($ArNH_2$), 1716 (CO), 2900-2990, 3100 & 3400 (NH) $cm^{-1}$. UV/Vis ($H_2O/CH_3SO_3H$): $\lambda_{max}$ = 320 nm, HRMS-ESI: clcld for $C_9H_{13}N_2O_5S$ = 261.0545, found 261.0551

### *5.25 2-Amino-N-(3-hydroxy-propyl)-3-hydroxy-benzenesulfonamide (4g).*

Rose-colored foam. Yield: 95%. MW: $C_9H_{14}N_2O_4S$, 246.3 $g.mol^{-1}$.; m.p. = 170°C; $^1H$ NMR (300 MHz DMSO) δ = 7.03 (dd $J_{H-H}$ = 8.1 & 1.2 Hz, 1 H, $H_6$), 6.93 (dd, $J_{H-H}$ = 7.7 & 1.2 Hz, 1 H, $H_4$), 6.60 (dd, $J_{H-H}$ = 8.1 Hz, 1 H, $H_5$), 3.34 (t, $J_{H-H}$ = 6.2 Hz, 2 H, $H_7$), 2.70 (t, $J_{H-H}$ = 7.2 Hz, 2 H, $H_9$), 1.50 (m, 2 H, $H_8$) ppm. $^{13}C$ NMR (125 MHz DMSO) δ = 146.5 ($C_3$), 134.4 ($C_1$), 122.4 ($C_2$), 120.0 ($C_5$), 118.0 ($C_6$), 117.0 ($C_4$), 59.0 ($C_9$), 40.1 ($C_7$ in the solvent peak), 32 ($C_8$) ppm. IR (KBr) v~=1070-1300 ($SO_2NH$), 1420-1480 (alcane), 1620 ($ArNH_2$), 2700 (alcane), 3100 ($NH_2$, Hydrogen bonding) $cm^{-1}$. UV/Vis ($H_2O/CH_3SO_3H$): $\lambda_{max}$ = 315 nm, ESI-MS: (m/z) (%) = 247.38 [M+H] (100), 313.33 (70); HRMS-ESI: clcld for $C_9H_{13}N_2O_4S$= 245.0596, found 245.0605

### *5.26 N-(3-Chloro-phenyl)-2, 2-dimethyl-propionamide (5) [42].*

To a mixture of ZnO (dry powder, 50 mmol; 4.06 g) and pivaloyl chloride (100 mmol; 12.3 mL), 3-chloro-aniline (100 mmol; 10.5 mL) was added. The reaction mixture was stirred with a mechanical stirrer for 2 h at room temperature. The solid mass (ZnO) was then eluted with $CH_2Cl_2$ (1 L) and the $CH_2Cl_2$ extract was concentrated under vacuum to a volume of 500 mL. The resulting organic phase was washed with an aqueous solution of sodium bicarbonate (2 x 500 mL) and dried over anhydrous sodium sulfate. Evaporation of solvent furnished almost pure title compound which was recrystallized in cold diethyl ether to furnish pure product as a white solid. Yield = 80% (16.4 g). MW: $C_{11}H_{14}ClNO$, 211.7 $g.mol^{-1}$. $^1H$ NMR (300 MHz $CDCl_3$) δ = 7.70 (t, $J_{H,H}$ = 2 Hz , 1 H, ArH), 7.42 (bs, 1 H, NH), 7.36 (ddd, $J_{H,H}$ = 8.1 & 1.9 & 0.9 Hz, 1 H, Ar-H), 7.20 (t, $J_{H,H}$ = 8 Hz, 1 H, Ar-H), 7.10 (ddd, $J_{H,H}$ = 7.8 & 1.9 Hz & 0.9 Hz, 1 H, Ar-H), 1.31 (s, 9 H, 3 x CH3) ppm.$^{13}C$ NMR (75 MHz DMSO) δ= 176.8 (CO amide), 139.1, 134.5, 129.8, 124.2, 120.2, 118, 39.7 ($C(CH_3)_3$), 27.54 (3 x CH3) ppm.

### *5.27 2-tert-butylbenzoxazole-7-sulfonyl chloride (8).* [25]

To a solution of **6** (4.7 mmol; 1 g) in anhydrous THF (20 mL), under argon atmosphere, at -78°C, was added dropwise *n*-Butylithium 2.5M in cyclohexane (11.3 mmol; 4.5 mL). The solution was kept 15 min at-78 °C then warmed to c.a -20 °C over 45 min and allowed to react at -20 °C for 2 h. The lithiated benzoxazole solution was cooled again to -78 °C and neat sulfuryl chloride (23.5 mmol; 2.5 mL) was added dropwise at – 78 °C to the stirred solution. The reaction mixture was reacted 2 h at -78 °C and then allowed to warm up to room temperature overnight. The reaction is stopped by diluting the mixture into 40 mL of $CH_2Cl_2$ and pouring the resulting solution over ice. The organic phase was collected and dried over anhydrous sodium sulphate. Evaporation of the solvent gave a residue which was purified by column chromatography on flash silica gel (cyclohexane/ethyl acetate 9:1) to give the title compound as an yellow oil. Yield = 50%. ( 0.15 g). MW: $C_{11}H_{12}ClNO_3S$, 273.7 g.mol$^{-1}$. Rf = 0.5; $^1$H NMR (300 MHz $CDCl_3$) δ = 8.10 (dd, $J_{H-H}$ = 7.9 & 1 Hz,1 H, $H_6$), 7.90 (dd, $J_{H-H}$ = 8 & 1.0 Hz, 1 H, $H_4$), 7.51 (dd, $J_{H-H}$ = 7.9 & 8 Hz, 1 H, $H_5$), 1.61 (s, 9 H, $H_{11}$) ppm. $^{13}$C NMR (75 MHz $CDCl_3$) δ = 174 ($C_2$), 147.4 ($C_8$), 142.5 ($C_4$), 124.8 ($C_6$), 124.7 ($C_5$), 118.1 ($C_7$), 115 ($C_9$), 34.9 ($C_{10}$), 28.7 ($C_{11}$) ppm. IR (KBr) ν~ = 720-680 (1,2,3-Ar), 1180 & 1203 ($SO_2Cl$), 1357-1427 ($SO_2Cl$), 1542-1660 (benzoxazole) cm$^{-1}$.MS-ESI (%): 274.08 (100) [M+H], 218.19 (25).

### *5.28 General procedure for the synthesis of compounds 9b, c:*

A solution of **8** (1 mmol) in anhydrous CH2Cl2 (5 mL) was added dropwise at 0 °C to an aqueous solution of amine (10% w/w) in excess. The reaction is allowed to warm to room temperature overnight. The organic phase was collected and washed with HCl 1N (3 x 20 mL). The organic phases were pooled together and dried over anhydrous sodium sulphate to give the title compound.

***5.29 2-tert-butylbenzoxazole-7-sulfonic acid cyclohexyl amide (9b).***

White solid. Yield = 80%. MW: $C_{17}H_{24}N_2O_3S$, 336.4 $g.mol^{-1}$.; m.p. = 105°C; $^1H$ NMR (300 MHz $CDCl_3$) δ = 7.90 (dd, $J_{H-H}$ = 7.9 & 1 Hz, 1 H, $H_5$), 7.80 (dd, $J_{H-H}$ = 7.8 & 1 Hz, 1 H, $H_4$), 7.41 (dd, $J_{H-H}$ = 7.9 & 7.8 Hz, 1 H, $H_6$), 4.90 (d, 1 H, $SO_2NH$), 3.30 (m, 1 H, $H_{12}$), 1.70-1.11 (m, 10 H, $H_{13-17}$), 1.50 (s, $H_{11}$) ppm. $^{13}C$ NMR (75 MHz $CDCl_3$) δ = 174.5 ($C_2$), 145.8 ($C_8$), 142.7 ($C_4$), 125 ($C_9$), 124.3 ($C_6$), 124.2 ($C_5$), 124 ($C_7$), 52.7 ($C_{12}$), 34.4 ($C_{10}$), 33.9 ($C_{13}$ & $C_{17}$), 28.5 ($C_{11}$), 25.2 ($C_{14}$ & $C_{16}$), 24.4 ($C_{15}$) ppm, IR (KBr) v~= 650-1000 ($SO_2NH$ and 1,2,3-Ar), 1090-1300 ($SO_2NH$), 1420 (cyclohexyl), 1560-1600 (benzoxazole), 2850-2970 (cyclohexyl), 3200 (NH) $cm^{-1}$. ESI-MS: (m/z) (%) = 337.01 (30) [M+H], HRMS-ESI: clcld for $C_{17}H_{25}N_2O_3S$= 337.1586, found 337.1583

***5.30 2-tert-butylbenzoxazole-7-sulfonic acid (3-dimethylamino-propyl) amide (9c).***

White gummy solid. Yield = 80%. MW: $C_{16}H_{25}N_3O_3S$, 339.4 $g.mol^{-1}$; $^1H$ NMR (300 MHz $CDCl_3$) δ = 9.80 (bs, 1 H), 7.81 (dd, $J_{H-H}$ = 7.9 & 0.9 Hz, 1 H, $H_5$), 7.76 (dd, $J_{H-H}$ = 7.8 & 0.9 Hz , 1 H, $H_7$), 7.40 (dd, $J_{H-H}$ = 7.9 & 7.8 Hz, 1 H, $H_6$), 3.20 (m, 2 H, $H_{12}$), 3.00 (m, 2 H, $H_{14}$), 2.61 (s, 6 H, $H_{15}$), 1.83 (m, 2 H, $H_{13}$), 1.50 (s, $H_{11}$) ppm. $^{13}C$ NMR (75 MHz $CDCl_3$) δ = 176.3 ($C_2$), 145.9 ($C_8$), 142.7 ($C_4$), 124.2 ($C_6$), 124 ($C_5$), 123.9 ($C_7$), 55.1 ($C_{12}$), 42.4 ($C_{15}$), 40.1 ($C_{14}$), 34.4 ($C_{10}$), 28.5 ($C_{11}$), 25.1 ($C_{13}$) ppm, IR (KBr) v~ = 1090-1300 ($SO_2NH$), 1400-1480 (alkane), 1560-1600 (benzoxazole), 2930-1000 (alkane), 3200 (NH) $cm^{-1}$. ESI-MS: (m/z) (%) = 340.22 (100) [M+H], HRMS-ESI: clcld for $C_{16}H_{26}N_2O_3S$= 340.1695, found 340.1709

***5.31 General procedure for the synthesis of compounds 10b, c:***

To a solution of 6 N HCl (100 mL) was added (1 mmol) of **9a, b**. The mixture was heated at 100 °C overnight. Then the solution was cooled down, filtered, and evaporated. The residue was purified by

column chromatography on flash silica gel (diethyl ether/ ethyl acetate 7:3) to give the title compound. Purity of compounds was check by HPLC.

***5.32 2-Hydroxy-N-cyclohexyl-3-amino-benzenesulfonamide (10b).***

White foam. Yield: 95%. MW: $C_{12}H_{18}N_2O_3S$, 270.3 g.mol$^{-1}$; m.p. 220-225°C; $^1$H NMR (300 MHz $CD_3OD$) δ = 7.80 (dd $J_{H-H}$ = 8.1 & 1.5 Hz, 1 H, $H_6$), 7.61 (dd, $J_{H-H}$ = 8.1 & 1.5 Hz, 1H, $H_4$), 7.12 (t, $J_{H-H}$ = 8.1 Hz, 1 H, $H_5$), 3.12 (m, 1 H, $H_7$), 1.60- 1.11 (m, 10 H, $H_{8-12}$ ) ppm. $^{13}$C NMR (125 MHz $CD_3OD$) δ = 149.4 ($C_2$), 129.9 ($C_5$), 129.5 ($C_6$), 128.5 ($C_1$), 122 ($C_3$), 121.6 ($C_4$), 54 ($C_7$), 34.6 ($C_8$ & $C_{12}$), 26.1 ($C_9$ & $C_{11}$), 25.8 ($C_{10}$) ppm. (KBr) v~ = 650-720 ($SO_2N$), 750-900 (1,2,3 Ar), 1087-1315 ($SO_2N$ and cyclohexyl), 1420-1480 (1,2,3-Ar), 1620 ($ArNH_2$), 2800-2920 (cyclohexyl) 3100 (NH) cm$^{-1}$. UV/Vis ($H_2O/CH_3SO_3H$): λmax = 310 nm, ESI-MS: (m/z) (%) = 269.10 [M-H] (100), 270.09 (10), 303.11 (7), 170.98 (10), 107.01 (5). HRMS-ESI: clcld for $C_{12}H_{19}N_2O_3S$= 271.1116, found 271.1108

***5.332-Hydroxy-N-(3-dimethylamino-propyl)-3-amino-benzenesulfonamide (10c).***

Brown oil. Yield: 95%. MW: $C_{11}H_{19}N_3O_3S$, 273.3 g.mol$^{-1}$. $^1$H NMR (300 MHz $CD_3OD$) δ = 7.81 (dd $J_{H-H}$ = 8.1 & 1.4 Hz, 1 H, $H_6$), 7.66 (dd, $J_{H-H}$ = 7.8 & 1.4 Hz, 1H, $H_4$), 7.21 (dd, $J_{H-H}$ = 8 & 7.8 Hz, 1 H, $H_5$), 3.20 (m, 2 H, $H_7$), 3.10 (m, 2 H, $H_9$), 2.91 (s, 6 H, $H_{10}$), 1.90 (m, 2 H, $H_8$ ) ppm. $^{13}$C NMR (125 MHz $CD_3OD$) δ = 149.3 ($C_3$), 129.8 ($C_5$ & $C_1$), 126.7 ($C_3$), 122.2 ($C_6$), 121.6 ($C_4$), 56.1 ($C_7$), 43.2 ($C_{10}$), 40.6 ($C_9$), 25.6 ($C_8$) ppm, IR (KBr) v~= 650-730 & 1080-1307

($SO_2NH$), 1400-1480 (1,2,3-Ar), 1627 ($ArNH_2$), 3100-3500 (NH, and H bonding) cm$^{-1}$. UV/Vis ($H_2O/CH_3SO_3H$): λmax = 320 nm. ESI-MS: (m/z) (%) = 274.18 [M+H] (100), 275.14 (20). HRMS-ESI: clcld for $C_{11}H_{20}N_3O_3S$= 274.1225, found 274.1232

***5.34 2-tert-butylbenzoxazole-7-sulfonic acid.***

A stirred solution of **8** (3.65 mmol; 1 g) in methanol (20 mL) heated at reflux was allowed to react over 4 h. The reaction was stopped and the solvent was evaporated. The residue was dried under vacuo to afford the title compound as white oil. Yield = 95%. ( 0.93 g). MW: $C_{11}H_{13}NO_4S$, 255.29 g.mol$^{-1}$. $^1$H NMR (300 MHz $CDCl_3/CD_3OD$) $\delta$ = 7.90 (d, $J_{H-H}$ = 7.9 Hz, 1 H, $H_6$), 7.84 (d, $J_{H-H}$ = 7.6 Hz, 1 H, $H_4$), 7.42 (dd, $J_{H-H}$ = 7.9 & 7.6 Hz, 1 H, $H_5$), 6.10 (1 H, $SO_3H$), 1.53 (s, 9 H, $H_{11}$) ppm. $^{13}$C NMR (75 MHz $CDCl_3/CD_3OD$) $\delta$ = 173.6 ($C_2$), 146.3 ($C_8$), 141.9 ($C_7$), 132.1 ($C_9$), 123.6 ($C_6$), 123.1 ($C_5$), 120.5 ($C_4$), 35.2 ($C_{10}$), 28.2 ($C_{11}$) ppm. IR (KBr) v~ 690 (1,2,3 Ar), 1045 & 1161-1226 ($SO_3H$), 1523-1639 (benzoxazole), 3200 cm$^{-1}$. ESI-MS: (m/z) (%) = 254.14 (100) [M-H], 272.18 (20) 238.13 (30). HRMS-ESI Calcd for $C_{11}H_{14}NO_4S$= 256.0644; found: 256.0651

***5.35 2-Hydroxy-3-amino-benzenesulfonic acid (11).***

To a solution of 6 N HCl (21 mL) was added 2-*tert*-butylbenzoxazole-4-sulfonic acid (3.1 mmol; 0.8 g). The mixture was heated at 80°C for 24 h. Then the solution was cooled down, filtered, and evaporated. The residue was dissolved in methanol and evaporated again before drying the solid under vacuo to yield the title compound as a light grey powder. Yield: 90% (0.53 g). MW: $C_6H_7NO_4S$: 189.1 g.mol$^{-1}$; m.p. 290-300°C $^1$H NMR (300 MHz DMSO) $\delta$ = 11.36 (bs, 1 H, OH), 7.51 (dd $J_{H-H}$ = 7.8 Hz & 1.4 Hz, 1 H, $H_6$), 7.31 (dd, $J_{H-H}$ = 7.8 & 1.3 Hz, 1H, $H_4$), 6.93 (t, $J_{H-H}$ = 7.8 Hz, 1 H, $H_5$) ppm. $^{13}$C NMR (125 MHz DMSO) $\delta$ = 147.4 ($C_2$), 132.3 ($C_1$), 127.3 ($C_5$), 125.6 ($C_6$), 119.7 ($C_4$ & $C_3$) ( ppm. IR (KBr) v~ = 670-870 (1,2,3-Ar), 1018-1230 ($SO_3H$), 1630 (aniline), 3100-3400 (hydrogen bonding) cm$^{-1}$. UV/Vis ($H_2O/CH_3SO_3H$): $\lambda$max = 290 nm, ESI-MS: (m/z) (%) = 188.08 [M-H] (100), 376.6 [2M-2 H] (25), 254.1 (35), 108 (15), 79.8 (25). HRMS-ESI clcld for $C_6H_8NO_4S$= 190.0174 found: 190.0177. For synthesis of compound **12**, see supporting information.

***5.36 2-Amino-3oxo-3H-phenoxazine 1,9-disulfonic acid (LAO for Laccase Acid Orange dye)[18].***

A solution of **3-HOA** (1.06 mmol; 0.2 g) in 25 mL of MeOH was diluted to a final volume of 500 mL with water and 250 mL of 0.2 mM of ammonium acetate buffer at pH 6. The reaction was started by adding 5 mL of a solution of laccase (104 U $L^{-1}$) and the stirred mixture was kept 24 h at 25 °C under air. The reaction was stopped by adding an equivalent volume of methanol and concentrated under vacuum to a volume of 300 mL. The crude mixture was freeze dried to give a red powder (0.2 g) with an isolated yield of 93 %. The isolated product is obtained as a salt of ammonium sulfonate (MW: $C_{12}H_8N_2O_8S_2.[NH_4^+]_2$) , 406.4 $g.mol^{-1}$) with residual traces of acetic acid. **LAO**: MW: $C_{12}H_8N_2O_8S_2$, 372.3 $g.mol^{-1}$; m.p > 250°C (slow degradation). $^1$H NMR (300 MHz DMSO) δ = 8.60 (bs, 1 H, $NH_2$), 7.90 (d $J_{H-H}$ = 7.8 Hz,1 H, $H_8$), 7.61 (d, $J_{H-H}$ = 7.8 Hz, 1H, $H_6$), 7.58 (dd, $J_{H-H}$ = 7.8 Hz, 1 H, $H_7$) ppm, 7.10 (1 H, $NH_2$) 6.20-7.30 (bs 8 H, 2 x $NH_4^+$), 6.41 (s, 1 H, $H_4$), 1.41 (s, < 1 H, $CH_3CO_2^-$). $^{13}$C NMR (125 MHz DMSO) δ = 179.2 ($C_3$), 148.9 ($C_{11}$), 145.2 ($C_{14}$), 145.0 ($C_2$), 143.0 ($C_9$), 141.8 ($C_{12}$), 130.3 ($C_{13}$), 128.3 ($C_7$), 125.6 ($C_8$), 118.3 ($C_6$), 111.6 ($C_1$), 102.9 ($C_4$), 28.7 ($CH_3CO_2^-$) ppm. IR (KBr) v~ = 1051 & 1207 ($SO_3^-$), 1388-1400 & 1595 (iminoquinone), 2330-2360, 3100-300 & 3440 ($NH_4^+$ salt & $NH_2$) $cm^{-1}$. UV/Vis ($H_2O$, pH = 7.4): $\lambda_{max}$ (ε) = 440 nm (9840 $mol^{-1}dm^3cm^{-1}$ ), ESI-MS: (m/z) (%) = 371.16 [M- H] (100); 393.1 [M – 2 H + Na] (40); 291.2 (20). HRMS-ESI calcld for $C_{12}H_7N_2O_8S_2$ = 370.9644; found: 370.9641.

***5.37 General procedure for the synthesis of 13a, c, e.***

A solution of **4a, c, e** (1.06 mmol) in 25 mL of MeOH was diluted to a final volume of 500 mL with 220 mL of water and 250 mL of 0.2 mM of ammonium acetate buffer at pH 4. The reaction was started by adding 5 mL of a solution of laccase ($10^4$ U $L^{-1}$) and the stirred mixture was kept 24 h at 25 °C under air. The reaction was stopped by adding an equivalent volume of methanol and concentrated

under vacuum to a volume of 300 mL. The crude mixture was freeze dried to give the title compound.

### *5.38 2-Amino-3-oxo-3H-phenoxazine-1,9-disulfonic acid diamide (13a).*

From **4a** (0.563 mmol). The crude product (0.2 g), an orange powder, is obtained as a salt of acetate (MW: $C_{12}H_{10}N_4O_6S_2.[(CH_3CO_2H)_3]$, 550.3 $g.mol^{-1}$) with residual trace of acetic acid. The product was purified by column chromatography on flash silica gel ($CH_2Cl_2$/ MeOH 95:5) to give the title compound (0.08 g) with an isolated yield of 60%. MW: $C_{12}H_{10}N_4O_6S_2$, 370.3 $g.mol^{-1}$; m.p = >280°C (decomposition). $^1H$ NMR 300 MHz (DMSO) $\delta$ = 8.10 (bs, 2 H, $NH_2$), 7.91 (dd $J_{H-H}$ = 7.7 & 1.2 Hz,1 H, $H_8$), 7.82 (dd, $J_{H-H}$ = 8.3 & 1.2 Hz, 1H, $H_6$), 7.65 (dd, $J_{H-H}$ = 8.3 & 7.7 Hz, 1 H, $H_7$) ppm, 7.20 (bs, 4 H, $SO_2NH_2$), 6.62 (s, 1 H, $H_4$) ppm. $^{13}C$ NMR (125 MHz DMSO) $\delta$ = 178.7 ($C_3$), 150.4 ($C_{14}$), 146.3 ($C_2$), 145.1 ($C_{11}$), 143.1 ($C_{12}$), 139.0 ($C_9$), 129.6 ($C_7$), 129.3 ($C_{13}$), 124.0 ($C_8$), 121.1 ($C_6$), 107.6 ($C_1$), 105.5 ($C_4$), 29.8 & 27.2 ($CH_3CO_2^-$) ppm. IR (KBr) v~ = 1143 & 1191-1315 ($SO_2NH$), 1540-1595 (iminiquinone), 3200-3303 & 3402-3420 ($NH_2$) $cm^{-1}$. UV/Vis ($H_2O$, pH 7.4): $\lambda$max ($\varepsilon$) = 440 nm (4014 $mol^{-1}dm^3cm^{-1}$) nm, ESI-MS: (m/z) (%) = 365.1 (100), 370.89 [M + H] (25), 393.16 [M + Na] (100). HRMS-ESI calcld for $C_{12}H_{10}N_4O_6NaS_2$ = 392.9939; found: 392.9947.

### *5.39 2-Amino-3-oxo-3H-phenoxazine-1,9-disulfonic acid bis-[(3-dimethylamino-propyl)-amide] (13c).*

From **4c** (0.42 mmol). The product (0.13 g), an orange powder, is obtained as a salt of acetate (MW: $C_{22}H_{32}N_6O_6S_2.[(CH_3CO_2H)_2]$, 660.7 $g.mol^{-1}$) with residual trace of acetic acid with an isolated yield of 90%. MW: $C_{22}H_{32}N_6O_6S_2$, 540.6 $g.mol^{-1}$; m.p = 205-220°C. $^1H$ NMR (300 MHz Acetone-$d_6$ / $D_2O$) $\delta$ = 7.91 (dd $J_{H-H}$ = 7.5 & 1.2 Hz,1 H, $H_8$), 7.81 (dd, $J_{H-H}$ = 8.3 & 1.2 Hz, 1 H, $H_6$), 7.71 (dd, $J_{H-H}$ = 7.5 & 8.3 Hz, 1 H, $H_7$), 6.60 (s, 1 H, $H_4$), 2.75-3.15 (m, 8 H, $CH_2$), 2.80-

2.60 (s,12 H, $N[CH_3]_2$), 1.9 (m, 4 H, $CH_2$), 1.9 (s, 6 H, $CH_3CO_2H$) ppm. $^{13}C$ NMR (125 MHz Acetone-$d_6$ / $D_2O$) δ = 177.9 ($C_3$), 150.5 ($C_{14}$), 148.8 ($C_2$), 145.2, 143.2 ($C_{12}$), 134.5 ($C_1$,$C_9$), 129.6 ($C_7$), 129.5 ($C_{13}$), 126.3 ($C_8$), 121.7 ($C_6$), 105.5 ($C_4$), 55.5 & 55.4 ($SO_2NH$-$CH_2$), 43.0 ($NMe_2$), 40.6 ($CH_2NMe_2$), 24.8 & 24.6 ($CH_2$), 21.0 ($CH_3CO_2H$) ppm. IR (KBr) v~ = 1139 & 1155 & 1313 ($SO_2NH$ & $NMe_2$), 1400-1473 & 1600 (iminoquinone), 2705 (alcane), 3288 & 3411 ($NH_2$) $cm^{-1}$. UV/Vis ($H_2O$, pH 7.4): λmax (ε) = 440 nm (7219 $mol^{-1}dm^3cm^{-1}$), ESI-MS: (m/z) (%) = 541.07 [M + H] (100). HRMS-ESI calcld for $C_{22}H_{33}N_6O_6S_2$ = 541.1903; found: 541.1881.

### *5.40 2-Amino-3-oxo-3H-phenoxazine-1,9-disulfonic acid bis-[(2-amino-ethyl)-amide] (13e).*

From **4e** (0.54 mmol). The product (0.15 g), a brown powder, is obtained as a salt of acetate (MW: $C_{16}H_{20}N_6O_6S_2.[(CH_3CO_2H)_2]$, 576.6 $g.mol^{-1}$) and residual trace of acetic acid with an isolated yield of 87%. MW: $C_{16}H_{20}N_6O_6S_2$, 456.1 $g.mol^{-1}$; m.p. > 300 °C. $^1H$ NMR (300 MHz Acetone-$d_6$ / $D_2O$) δ = 7.93 (d $J_{H-H}$ = 7.6 Hz,1 H, $H_8$), 7.81 (d, $J_{H-H}$ = 8.2 Hz, 1H, $H_6$), 7.70 (dd, $J_{H-H}$ = 7.6 & 8.2 Hz, 1 H, $H_7$), 6.61 (s, 1 H, $H_4$), 3.60 (m, 1 H, $SO_2NH$), 3.10-3.25 (m , 8 H, $CH_2$), 1.84 (s, 6 H, 2 x $CH_3CO_2H$) ppm. $^{13}C$ NMR (125 MHz Acetone-$d_6$ / $D_2O$) δ = 177.7 ($C_3$), 150.1 ($C_{14}$), 148.7 ($C_2$), 144.7 ($C_{11}$), 142.7 ($C_{12}$), 133.2 ($C_1$, $C_9$), 129.4 ($C_7$), 129.0 ($C_{13}$), 126.2 ($C_8$), 121.8 ($C_6$), 105.2 ($C_4$), 40.2 ($CH_2NH$), 39.2 & 39.0 ($CH_2NH_2$), 22.4 ($CH_3CO_2^-$) ppm. IR (KBr) v~ = 1130-1190 ($SO_2NH$), 1313 (alcane), 1404 & 1577-1602 (iminoquinone), 2360, 3047-3413 ($NH_2$) $cm^{-1}$. UV/Vis ($H_2O$, pH 7.4): $\lambda_{max}$ (ε) = 440 nm (6022 $mol^{-1}dm^3cm^{-1}$), ESI-MS: (m/z) (%) = 457.1 [M + H] (100). HRMS-ESI calcld for $C_{16}H_{21}N_6O_6S_2$ = 457.0964; found: 457.0957

### *5.41 General procedure for the synthesis of 13b, d, f, g and 14b.*

A solution of **4b, d, f, g** (1.06 mmol ) in 25 mL of MeOH was diluted to a final volume of 500 mL with water and 250 mL of 0.2 mM of ammonium acetate buffer at pH 6. The reaction was started by adding 5 mL of a solution of laccase ($10^4$ U $L^{-1}$) for **4f, g** and 20 mL of a solution of laccase ($10^4$ U $L^{-1}$) for **4b** or **4d** and the stirred mixture was kept 24 h at 25 °C under air. The reaction was stopped by adding a equivalent volume of methanol and concentrated under vacuum to a volume of 300 mL. The crude mixture was extracted with ethyl acetate (3 x 300 mL) and the organic phases were pooled together and dried over $MgSO_4$. The organic solvent was removed under vacuo to give the title compound.

### *5.42 2-Amino-3-oxo-3H-phenoxazine-1,9-disulfonic acid bis-cyclohexylamide (13b)*

From **4b** (0.443 mmol). The product (0.1 g), a yellow powder, is obtained with an isolated yield of 86%. MW: $C_{24}H_{30}N_4O_6S_2$, 534.6 $g.mol^{-1}$; m.p. = 135°C. $^1$H NMR (300 MHz Acetone-$d_6$) δ = 8.31 (bs, 1 H, $NH_2$), 7.95 (d, $J_{H-H}$ = 7.3 Hz, 1 H, $H_8$), 7.80 (d, $J_{H-H}$ = 7.2 Hz, 1H, $H_6$), 7.70 (dd, $J_{H-H}$ = 7.2 & 7.3 Hz, 1 H, $H_7$), 7.55 (bs, 1 H, $NH_2$), 6.65 & 6.51 (d, $J_{H-H}$ = 6.1, 2 H, $SO_2NH$), 6.53 (s, 1 H, $H_4$), 3.20 (m, 2 H, 2 x CH-N), 1.00-1.81 (m , 20 H, $CH_2$) ppm. $^{13}$C NMR (125 MHz Acetone-$d_6$) δ = 177.6 ($C_3$), 149.7 ($C_{14}$), 147.3 ($C_2$), 144.5 ($C_{11}$), 142.7 ($C_{12}$), 137.6 ($C_1$, $C_9$), 128.9 ($C_7$), 125.0 ($C_8$), 120.3 ($C_6$), 104.9 ($C_4$), 53.0 ($SO_2NH$-CH), 33.6 & 33.0 ($CH_2$-CH), 25.0 & 24.9 & 24.2 ($CH_2$) ppm. IR (KBr) ν~ = 669, 993, 1078-1325 ($SO_2NH$), 1577-1596 (iminoquinone), 2854-2931 (alcane), 3272-3444 ($NH_2$) $cm^{-1}$. UV/Vis ($H_2O$, pH 7.4): $\lambda_{max}$ (ε) = 440 nm (10821 $mol^{-1}dm^3cm^{-1}$), ESI-MS: (m/z) (%) = 557.19 [M + Na] (100), 535 [M + H] (10). HRMS-ESI calcld for $C_{24}H_{31}N_4O_6S_2$ = 535.1685; found: 535.1684

***5.43 2-Amino-3-oxo-3H-phenoxazine-1,9-disulfonic acid bis-phenylamide (13d).***

From **4d** (0.3 mmol). The product (0.05 g), was purified by column chromatography on flash silica gel ($CH_2Cl_2$/ MeOH 95:5) to give the title compound as an orange gummy solid with a isolated yield of 40%. MW: $C_{24}H_{18}N_4O_6S_2$, 522.5 $g.mol^{-1}$.; $^1H$ NMR (300 MHz DMSO) $\delta$ = 8.75 (bs, 1 H, $SO_2NH$), 8.64 (bs, 1 H, $SO_2NH$), 8.50 (bs, 1 H, $NH_2$), 8.31 (bs, 1 H, $NH_2$), 7.70 (m, 2 H, $H_6$ & $H_8$), 7.25-7.10 (m, 11 H, Ar-H & $H_7$), 6.71 (s, 1 H, $H_4$) ppm. $^{13}C$ NMR (125 MHz DMSO) $\delta$ = 177.9 ($C_3$), 150.2 ($C_{14}$), 149.7 ($C_2$), 145.9 ($C_{11}$), 139.6 ($C_{12}$), 132.7 ($C_1$, $C_9$), 130.8 ($C_{13}$), 130.3 (Ar), 130.2 (Ar), 129.2 ($C_7$), 126.6 (Ar), 126.3 ($C_8$) 122.7 (Ar), 122.4 (Ar), 121.9 ($C_6$), 106.6 ($C_4$) ppm. IR (KBr) v~ = 669- 800 (Ar) , 1078-1325 ($SO_2NH$), 1560-1600 (iminoquinone), , 3272 (NH) $cm^{-1}$. UV/Vis ($H_2O$, pH 7.4): $\lambda$max ($\varepsilon$) = 440 nm (482 $mol^{-1}dm^3cm^{-1}$), APCI-MS: (m/z) (%) = 556.9 (100), 523.07 [M + H] (35). HRMS-ESI calcld for $C_{24}H_{18}N_4O_6NaS_2$ = 545.0565; found: 545.0552

***5.44 8-Amino-[9-(methoxycarbonylmethyl-sulfamoyl)-7-oxo-7H-phenoxazine-1-sulfonylamino]-acetic acid methyl ester] (13f).***

From **4f** (0.538 mmol). The product (0.08 g), an orange powder, is obtained with an isolated yield of 58%. For recrystallization, the product is further purified on flash silica gel ($CH_2Cl_2$/MeOH 95/5) to yield the title compound as a orange foam (0.075 g). MW: $C_{18}H_{18}N_4O_{10}S_2$, 514 $g.mol^{-1}$; m.p. =160-170°C. $^1H$ NMR (300 MHz Acetone-$d_6$ / $D_2O$) $\delta$ = 7.90 (dd $J_{H-H}$ = 7.6 & 1.3Hz, 1 H, $H_8$), 7.81 (dd, $J_{H-H}$ = 8.4 Hz & 1.1Hz, 1 H, $H_6$), 7.71 (dd, $J_{H-H}$ = 8.4 & 7.6 Hz, 1 H, $H_7$), 6.61 (s, 1 H, $H_4$), 3.90 (s, 2 H, $CH_2$), 3.84 (s, 2 H, $CH_2$), 3.50 (s, 3 H, $CH_3$), 3.41 (s, 3 H, $CH_3$) ppm. $^{13}C$ NMR (125 MHz Acetone-$d_6$ / $D_2O$) $\delta$ = 177.5 ($C_3$), 168.8 ($CO_2Me$), 149.7 ($C_{14}$), 147.4 ($C_2$), 144.5 ($C_{11}$), 142.5 ($C_{12}$), 135.7 ($C_1$, $C_9$), 129.4 ($C_{13}$), 128.8 ($C_7$), 125.1 ($C_8$), 120.7 ($C_6$), 104.9 ($C_4$), 51.5 ($OCH_3$), 51.4 ($OCH_3$), 44 ($CH_2$) ppm. IR (KBr) v~= 1101-1236 ($SO_2NH$), 1316-1352 ($SO_2NH$), 1577-1596

(iminoquinone), 1718 & 1743 (CO), 2362, 3523-3438 (NH & $NH_2$) $cm^{-1}$. UV/Vis ($H_2O$, pH 7.4): $\lambda_{max}$ ($\varepsilon$) = 440 nm (714 $mol^{-1}dm^3cm^{-1}$), ESI-MS: (m/z) (%) = 537.04 [M + Na] (70), 514.9 [M + H] (35). HRMS-ESI calcld for $C_{18}H_{19}N_4O_{10}S_2$ = 515.0543; found: 515.0543

### *5.45 2-Amino-3-oxo-3H-phenoxazine-1,9-disulfonic acid bis-[(3-hydroxy-propyl)-amide (13g)*

From **4g** (1.3 mmol).The product (0.29 g), an orange powder, is obtained with an isolated yield of 90%. For recrystallization, part of the product (0.1 g) is purified on flash silica gel ($CH_2Cl_2$/MeOH 95/5) to yield the title compound as a orange foam (0.09 g). The title compound (0.02 g) is dissolved in a mixture of $CDCl_3$/MeOD (8/2) and recrystallized by slow evaporation. MW: $C_{18}H_{22}N_4O_8S_2$, 486.1 $g.mol^{-1}$.; mp.= 175-185°C. $^1$H NMR (300 MHz $CDCl_3$ / MeOD) $\delta$ = 7.93 (dd $J_{H-H}$ = 7.3 & 1.4 Hz, 1 H, $H_8$), 7.61 (d, $J_{H-H}$ = 8.4 Hz, 1 H, $H_6$), 7.54 (dd, $J_{H-H}$ = 7.3 & 8.4 Hz, 1 H, $H_7$), 6.52 (s, 1 H, $H_4$), 3.60 (dd, $J_{H-H}$ = 11.8 & 5.8 Hz, 4 H, $CH_2$-N), 2.90 (m, 4 H, $CH_2$-O), 1.66 (m , 4 H, $CH_2$) ppm. $^{13}$C NMR (125 MHz $CDCl_3$ / MeOD) $\delta$ = 177.3 ($C_3$), 149.2 ($C_{14}$), 147.4 ($C_2$), 144.1 ($C_{11}$), 142.3 ($C_{12}$), 135.4 ($C_1$,$C_9$), 129 ($C_7$), 128.8 ($C_{13}$), 126.2 ($C_8$), 120.6 ($C_6$), 105.5 ($C_4$), 58.7 (CH-N), 39.8 ($CH_2$-O), 31.7 ($CH_2$), 31.4 ($CH_2$) ppm. IR (KBr) v~ = 1070, 1139-1313 ($SO_2NH$), 1419, 1571-1595 (iminoquinone), 2854-2925 (alcane), 3265-3413 (NH, OH) $cm^{-1}$. UV/Vis ($H_2O$, pH 7.4): $\lambda_{max}$ ($\varepsilon$) = 440 nm (12634 $mol^{-1}dm^3cm^{-1}$), ESI-MS: (m/z) (%) = 509.014 [M + Na] (100), 487.1 [M + H] (35). HRMS-ESI calcld for $C_{18}H_{23}N_4O_8S_2$ = 487.0957; found: 487.0959.

### *5.46 2-Hydroxy-3-imino-3H-phenoxazine-1,6-disulfonic acid bis-cyclohexylamide (14b)*

From **10b** (0.462 mmol). The product (0.09 g), a red powder, is obtained with a isolated yield of 73%. MW: $C_{24}H_{30}N_4O_6S_2$, 534.6 $g.mol^{-1}$; m.p. = 140-150°C. $^1$H NMR (300 MHz Acetone-$d_6$) $\delta$ = 8.05 (m, 2 H, $H_6$ & $H_8$), 7.70 (t, $J_{H-H}$ = 7.8 Hz, 1 H, $H_7$), 7.30 & 6.50 (d, 2

H, $SO_2NH$), 6.65 (bs, NH), 6.70 (s, 1 H, $H_4$), 3.30 & 3.12 (m, 2 H, 2 x CH), 1.00-1.85 (m , 20 H, $CH_2$) ppm. $^{13}C$ NMR (125 MHz Acetone-$d_6$) δ = 175.6 ($C_3$), 147.3 ($C_{14}$), 146.9 ($C_{11}$), 146.3 ($C_{12}$), 137.8 ($C_{13}$), 134.7 ($C_6$), 132.7 ($C_8$), 130.1 ($C_2$), 129.1 ($C_7$), 125.7 ($C_9$), 116 ($C_1$), 100.3 ($C_4$), 52.8(CH-N), 52.6 (CH-N), 33.2 ($CH_2$), 25 ($CH_2$), 24.5 ($CH_2$), 24.3 ($CH_2$) ppm. IR (KBr) v~ =1080, 1143-1161 ($SO_2NH$), 1326, 1458 & 1544 & 1600-1623 (imine), 2853 (alcane), 2931 (alcane), 3263-3461 (NH & OH) $cm^{-1}$. UV/Vis ($H_2O$, pH 7.4): $\lambda_{max}$ (ε) = 440 nm (3954 $mol^{-1}dm^3cm^{-1}$), ESI-MS: (m/z) (%) = 557.19 [M + Na] (100), 535 [M + H] (10). HRMS-ESI calcld for $C_{24}H_{30}N_4O_6NaS_2$ = 557.1504; found: 557.1503.

### *5.47 2-Hydroxy-3-oxo-3H-phenoxazine-1,6-disulfonic acid (15).*

A solution of **11** (1.06 mmol; 0.2 g) in 25 mL of MeOH was diluted to a final volume of 500 mL with water and 250 mL of 0.2 mM of ammonium acetate buffer at pH 4. The reaction was started by adding 40 mL of a solution of laccase ($10^4$ U $L^{-1}$) and the stirred mixture was kept 24 h at 25 °C under air. The reaction was stopped by adding a equivalent volume of methanol and concentrated under vacuum to a volume of 300 mL. The crude mixture was freeze dried to give a red powder which was purified by semi-preparative HPLC and the resulting freeze dried solid was filtered over a short pad of RP18 to yield small quantity of the pure isolated product (0.008 g). Isolated yield of 4%. MW: $C_{12}H_7NO_9S_2$, 373.3305 $g.mol^{-1}$. $^1H$ NMR (300 MHz $D_2O$) δ = 7.85 (d $J_{H-H}$ = 7.8 Hz, 1 H, $H_7$), 7.75 (d-m, $J_{H-H}$ = 7.8 Hz, 1H, $H_9$), 7.46 (t, $J_{H-H}$ = 7.8 Hz, 1 H, $H_8$), 6.76 (s, 1 H, $H_4$) ppm. UV/Vis ($H_2O$, pH = 7.4): $\lambda_{max}$ = 239, 440 and 460 nm, ESI-MS: (m/z) (%) = 372.02 [M - H] (100); 394.00 (40); 291.13 (20). HRMS-ESI calcld for $C_{12}H_6NO_9S_2$ = 371.9484; found: 371.9485

### *5.48. Supporting information*

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200900681.

## 6. Acknowledgements

This research was supported by the European Commission, Sixth Framework Program (NMP2-CT2004-505899, SOPHIED) and partially, by the Concerted Research Programme ARC 08/13-009 (UCL, Belgium). Estelle Enaud and Sophie Vanhulle are kindly acknowledged for constructive discussions and assistance with the commercial laccase used. J. Marchand-Brynaert is a senior research associate of FRS–FNRS (Belgium).

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## 4.4 X-ray structures discussion

### *4.4.1 Previous published structures*

#### *2-amino-3H-phenoxazin-3-one X-ray data*

Several X-ray structures were previously published regarding the heterocyclic framework of the 2-amino-3H-phenoxazin-3-one (APX) chromophore. Here, we have selected one published result which simultaneously gives informative features about this heterocycle and an interesting synthesis. In 2001, a group of scientists reported the formation of a single crystal of the title compound through the oxidation of 2-aminophenol in methanol solution under irradiation by sunlight.[ 162] Whereas the yield of APX product did not exceed 12%, the observation of phenoxazinone formation in organic solvent through photosynthesis is of interest. At least, this could be a way to evaluate whether or not our unreactive substrate **10c** is able to give a phenoxazinone dye. Such oxidation reaction will be tried and the results are disclosed in the next chapter E.

Regarding the X-ray structure obtained, the remarkable features are similar to those previously reported for the 2-amino-7-methoxy-3H-phenoxazin-3-one.[163]

[162] a) Buckley, R. G.; Charalambous, J.; Henrick, K., *Acta Crystallogr* **1982**, B38, 289-291. b) Graf, E.; Schneider, K.; Nicholson, G.; Ströbele, M.; Jones, A. L.; Goodfellow, M.; Beil, W.; Smuth, R.; Fiedler, H. P., *J Antibiot* **2007**, 60, 277-284.

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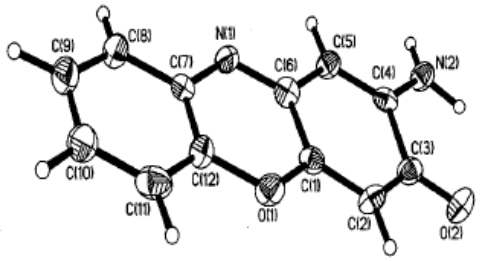
First, the APX (Figure 11) consists of three nearly coplanar rings, the dihedral angles between the heterocyclic and quinonic rings, and between the heterocyclic and benzene rings, being respectively of 1.8° and -5.6°. Interesting features are the bond distances in agreement with the Kekule formula showing a formal alternance of single and double bonds. The single bond distances between carbon atoms (~1.49 Å) are longer than those of the double bonds (~1.35 Å). The bond length between the carbon atom and the amino group (~1.33 Å) is shorter than a normal C-N bond length of about 1.47 Å. this shows a partial double bond character between the quinonic ring and the amino group.

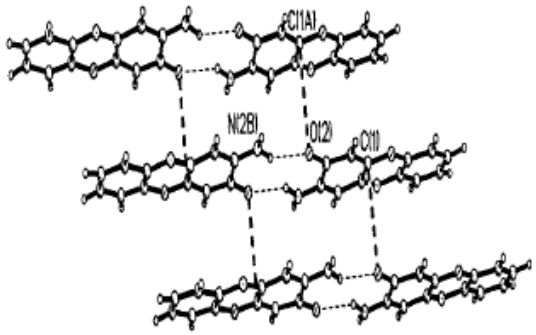
Figure 11. Ortep view of APX heterocycle




In the crystal, APX molecules are stacked upon each other, flipped horizontally by 180° and linked by intermolecular hydrogen bonds between the amino group of one molecule and the oxygen atom of the keto group of another one.

Lastly, the supramolecular arrangement observed shows Van der Waals contacts between chromophores with a distance of about 3.1 Å. We will see that similar features are observed with our molecules.

Figure 12. Supramolecular arrangement of APX




***Actinomycin D X-ray data***

Actinomycins are chromopeptides highly active as antibiotic and cytostatic agents, isolated from *Streptomyces* cultures. All possess the 2-amino-phenoxazin-3-one chromophore. Actinomycin D has been the most intensively investigated compound and recently untwined crystals were isolated by the group of professor Lackner[ 164]. This readily permits to compare the spatial structure and the folding of the side chains, the pentapeptide lactones, with the one observed in presence of DNA. Similarly to our X-ray structures, side chains pointed in opposite directions and were disposed upon one face of the heterocyclic core structure. Several X-ray structures of actinomycin D intercalated into a double-stranded of helical DNA showed how the side chains are folded and hydrogen bonded to the oligonucleotides.[165 ]

[164] a) Lackner, H., *Angew. Chem. Int. Ed.* **1975**, 14, 375-386. ) Schäfer, G. M. S. M.; Bahner, I.; Lackner, H., *Angew. Chem. Int. Ed.* **1998**, 37, 2381.

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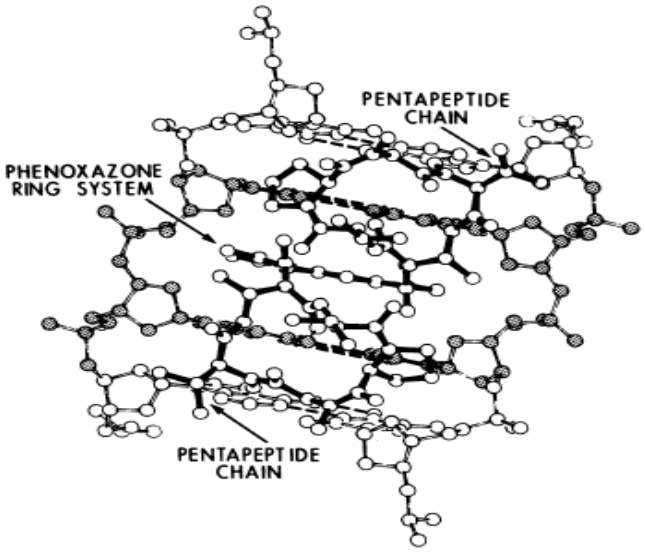
The interesting features of the structure will be briefly exposed as those are with our crystallised dyes. The unit cell of the crystal contains three independent molecules together with several solvent molecules. Two molecules form a dimer across a pseudo-twofold axis. The chromophores are stacked in parallel with a short distance of about 3.4 Å. Two chromophores are stacked with their quinone rings overlapping. This association is only visible in free actinomycin D, because in presence of DNA, a specific intercalation between GC bases takes place. [166]

Figure 13. Actinomycin intercalated into DNA



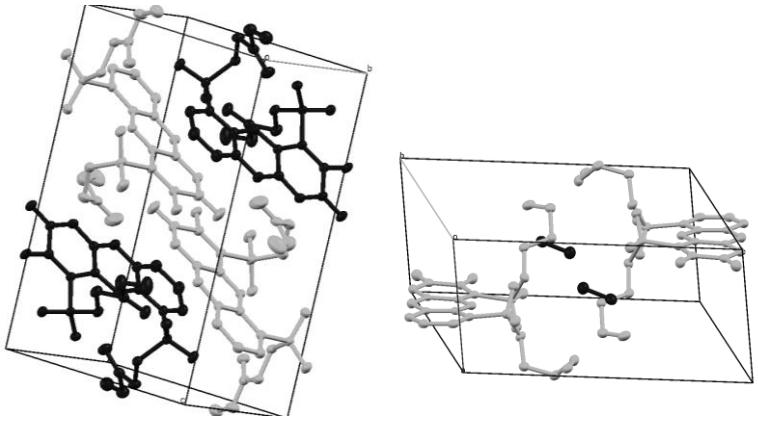

[166] a) Lybrand, T. P.; Brown, S. C.; Creighton, S.; Shafer, R. H.; Kollman, P. A., *J. Molecular Biol.* **1986,** 191, 495-507. b) Takusagawa, F.; Takusagawa, K. T.; Carlson, R. G.; Weaver, R. F., *Bioorg. Med. Chem*. **1997**, 5, 1197-1207. c) Maleev, V.; Semenov, M.; Kruglova, E.; Bolbukh, T.; Gasan, A.; Bereznyak, E.; Shestopalova, A., *J. Mol. Struct*. **2003**, 645, 145-158. d) Bendic, C.; Enache, M.; Volanschi, E., *J. of Mol. Graph. Model*. **2005**, 24, 10-16.

### *4.4.2 X-ray structures of phenoxazinone dyes*

The folding of our molecules is similar to the one observed for natural actinomycin compounds and the one of actinocin derivatives obtained from computational studies.[27] Compound **13f** and **13g** respectively crystallised from methylene chloride/methanol and deuterated chloroform/methanol as orange-red single crystals with the triclinic space group P-1. The unit cell of **13f** referred to subsequently as **13f*** contains 4 molecules (2 independent molecules of **13f** named α and β).

The disposition in space of the lateral chains is highly similar and pointing towards the outer space of the cell (Figure 14).

Figure 14. Packing of **13f** (left) and **13g** (right) in the cell, coloured following symmetry observed in the crystals (hydrogen atoms are omitted for clarity)



**13f** **13g**

On the other hand, the phenoxazine nuclei of the 2 molecules α located in the inner core of the cell, are stacked in parallel and totally flat whereas those of molecules β are slightly twisted along a line joining O105 and N110. Going from molecule β down to molecule β up, the disposition resembles somewhat to a helix shape in the crystalline lattice with a head to tail positioning between successive molecules. The plane of molecule β is deviated from the one of the molecule α by an angle of 10.4°.

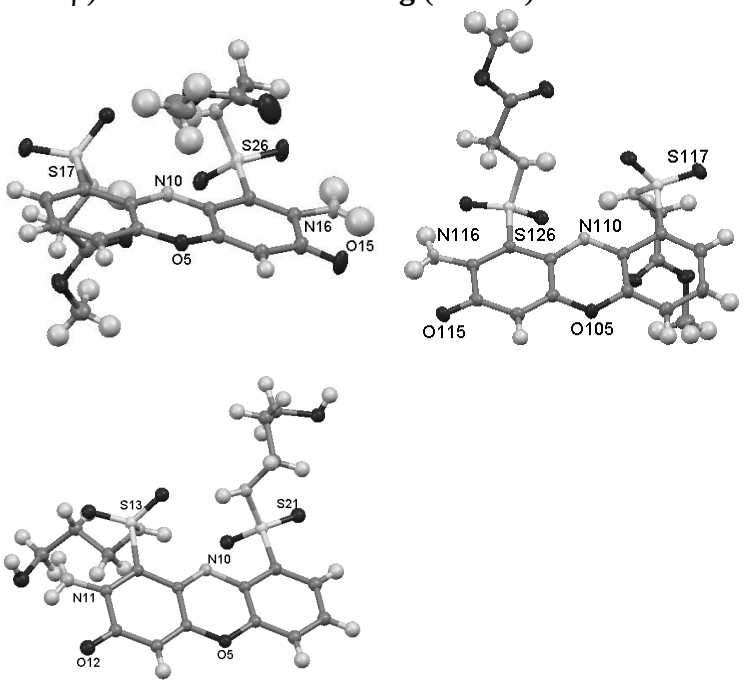
The torsions angles for the sulfonated side chains are significantly different between molecules α and β and probably result from the numerous intra and intermolecular hydrogen bonds observed. Finally, molecules β exhibit a deviation out of plane (0.103 Å) more pronounced than the slight one observed in molecules **13f** α (0.042 Å) and **13g** (0.048 Å) which are almost planar like the natural actinomycin molecules. The unit cell of **13g** referred to as **13g*** contains two molecules, co-crystallized with two molecules of methanol (Figure 14). Interestingly, both chromophores are disposed towards the exterior of the unit cell while lateral chains are pointing towards those of the other molecule. The chromophores are disposed in parallel to each other (angle between molecules of 3°) again in a head to tail manner. A plane of symmetry can be drawn by joining both molecules of methanol. Each of those solvent molecules is located between one of the aminopropanol side chains of both chromophores and hydrogen bonded to one of the side chains. A significant π-π stacking interaction is observed between molecules of adjacent unit cells with a distance ranging from 3.44 to 3.57 Å.

Selected features such as

- intra and intermolecular hydrogen bonds
- torsions angles
- deviation from the plane for the phenoxazine scaffold
- distance between aromatic ring ( π-π stacking)

are detailed in the annexe which collects published supporting informations and should be consulted for a more in depth description of the new compounds.

Figure 15. Perspective view of the independent molecules of **13f** (top, α and β) and of the molecule **13g** (bottom).



# CHAPTER 5 CROSS-COUPLING REACTIONS AND HETERODIMERIC AMINOPHENOXAZINONES DYES

## 5.1 Foreword

As mentioned in the chapter 2 dedicated to the objectives and strategies, this section exposes results which were not published or submitted yet for publication. Several of our experimental results obtained with cross-coupling reactions suggest a mechanism operating for the laccase-mediated synthesis of phenoxazinone and orcein dyes slightly different from those previously reported in the literature.

Based upon competitive experiments involving chosen pairs of substrates, our qualitative results suggest few variations in our case regarding the mechanism by which laccases mediate the oxidative cascade. Yet, there is a limitation of our experiments: having not mapped the entire sequence steps, this prevents us to make any affirmation regarding the whole sequence of oxidation-reduction.

Another limitation comes from the commercial preparations of laccase used for the experiments. We did not have the opportunity to check whether contamination of the laccase by other oxidases may be present. Furthermore, the process by which the laccase is produced being not known, we can't exclude the possibility to have different isozymes in the commercial reagents. Still, we have used few laccases from different sources and purities with our model compound suggesting in each case that the laccase is the main biocatalyst responsible for the oxidative dimerisation. The nature and properties of the laccases used are briefly given in the beginning of the manuscript (see pg 13).

Several aspects are addressed in the following section. The use of electrochemistry has been chosen to compare more closely the related substrates. We have used again HPLC as an analytical methodology to study the oxidative coupling reactions, particularly the cross-coupling reactions. Still, we did not collect exhaustive kinetics data regarding the oxidation and subsequent reactions of our substrates. It is important to stress that our proposal comes from chosen assumptions which are yet to be confirmed by further experimental studies. Those plausible assumptions are first disclosed hereafter while briefly mapping again the mechanistic proposal for the oxidative cascade involved in the synthesis of our water-soluble dyes.

Finally, as we assume a non radical pathway for the coupling reaction, the regioselectivity observed in the first conjugated addition is rationalised at best, thanks to the broad knowledge regarding the selectivity in the Michael addition reactions.[167] The selectivity observed in our biocatalysed reactions are in good agreement with the known behaviour of related electrophilic species. While we did not take into account the potential dismutation of two distinct radicals[42] produced by the laccase which would lead back to the reduced form (nucleophile) and the quinonic species (electrophile), this can't be exclude based upon our experimental results.

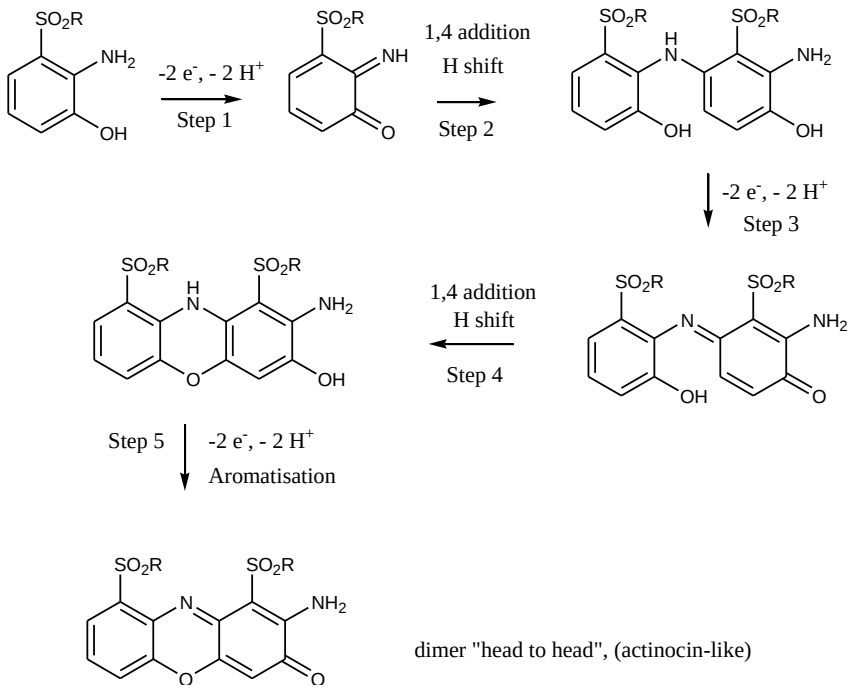
Again, we remind the reader here that this section should be considered as a tentative explanation for the puzzling experimental results observed. It has the merit to suggest plausible variants in the synthetic pathway which still need to be demonstrated. In no way this should be seen as an affirmative proposal.

[167] a) Enders, D.; Hoffman, K., *Eur. J. Org. Chem.* **2009**, 1665–1668 and references herein. b) Reddick, J. J.; Cheng, J.; Roush, W. R., *Org. Lett*. **2003**, *5*, 1967-1970.

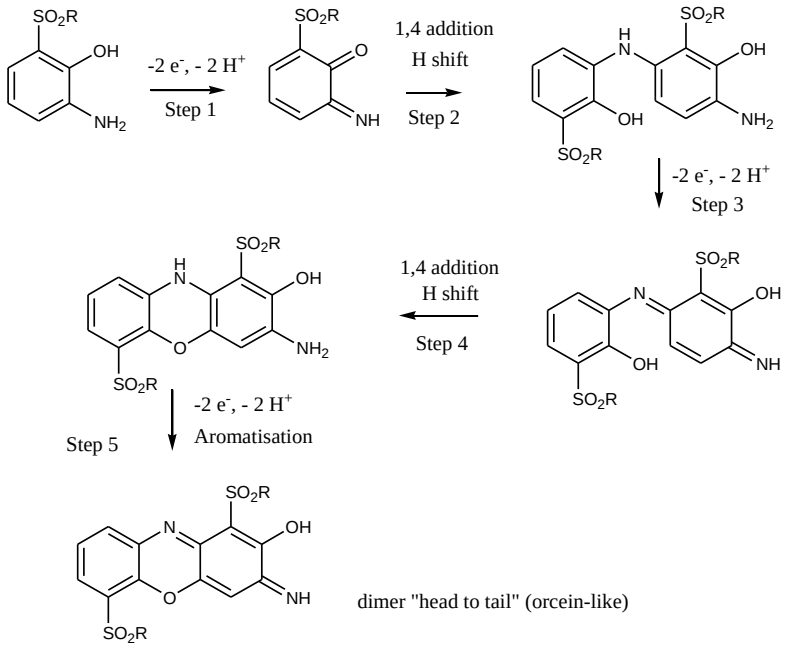
## 5.2 Assumptions made and proposal

In the chapter 4 dedicated to the scope and limitations of our biocatalytic system, puzzling discrepancies were observed for several regioisomeric substrates. Thus we try to investigate more in depth the behaviour of our substrates with several oxidants in order to get a better comprehension of the reaction (see Schemes 47 and 48).

Scheme 47. Proposal for the 6e$^-$ oxidation (actinocin)



Scheme 48. Proposal for the 6e$^-$ oxidation (orcein)




Step 1: First laccase-catalysed oxidation (1e$^-$ leading to distinct radicals in solution, or two successive 1e$^-$ oxidations leading to a quinonic species ?)

Step 2: Michael addition in solution ?

Step 3: Second oxidation (likely laccase-catalysed ?)

Step 4: Second Michael addition (in solution ?)

Step 5: Spontaneous aromatisation in solution (under air ?)

The likelihood of these proposed pathways is linked to several prior assumptions made which are briefly detailed here below.

**A/Step 1**- We assume that the main biocatalyst is a laccase and more probably that one isozyme is the major constituent of the enzymatic preparations we used. As similar results were obtained with the three enzymatic preparations, we suggest that similar interactions between the catalytic site of the enzymes and the chosen substrates exist. Yet we remind the reader that only one laccase was well characterised in term of purity whereas both TvL laccase preparations were not (see page 13-14). The possibility of a contamination of the preparation by other oxidases (e.g. peroxidase) can't be excluded. Control experiments were made to rule out an oxidation mediated by air but not by reactive species derived from molecular oxygen.

**B/Step 1**- The high similarity of the catalytic site T1 between laccase, more so for isozymes from a same fungal origin, compared to the one of phenoxazinone synthase (PHX) [110] led us to suggest a third assumption. We proposed in a similar fashion to the PHX two successive monoelectronic oxidations in the binding pocket giving a reactive quinonic species. Yet, the laccase could still oxidise two distinct molecules giving rise to two distinct radicals that could either combine themselves to create a new bond or react in a dismutation process leading to a reduced substrate and a quinonic species.[42] The latter proposition can't be distinguished here as it yields the same intermediate at the end of the step 1. Spin trapping experiment could help determine the true oxidation pathway. A successful application of such method has been published by Simandi et al in 2004.[169] This led to the observation of an intermediate being a radical in the oxidation of *o*-aminophenol with an iron catalyst.

[169] Simándi, T. M.; Simándi, L.; Gyor, M.; Rockenbauer, A.; Gömöry, A., *Dalton Trans.* **2004**, 1056-1060.

A previous mechanism reported for the laccase-mediated synthesis of cinnabarinic acid (**CA**) from 3-hydroxyanthranilic acid (**35**) suggests a $1e^-$ oxidation of the phenol group as first step, similarly to the route observed with peroxidases.[109] Such pathway, well suited for the MCOs, was suggested on the basis of a postulated involvement of **35** in the depolymerisation of lignin by white-rot fungi: **35**, a metabolite derived from tryptophan, would be a natural mediator, eliminated afterward by the production of CA.[109] Yet, mutants of the white-rot fungi provided an experimental proof that the depolymerisation of lignin could take place in absence of **35**.[170]

**C/Step 2**- We assume that the first new N-C bond forming reaction involves a nucleophilic addition of a reduced substrate to a reactive quinonic species. Whether the addition happen in the bulk of the solution, at the vicinity of the biocatalyst or even at the entry of the binding cavity can't be ascertain here. Yet, as we will see later, the cross-coupling products observed suggest that a major part of such addition happen in the bulk of the solution. Still, the possibility of a rapid oxidation -or oxidation and radical dismutation- yielding predominantly the quinonic species along with the total conversion of the reduced substrate may be ruled out here.[42,169] Such pathway does not account for the concomitant formation of the final dye in the early stage of the reaction. This has been confirmed by the HPLC analyse.

**D/Step 3**- We assume that the second oxidation is again performed by the laccase. Whether this yields directly a quinonic species or produce again distinct radicals that would react by dismutation can't be determined here. The intermediate being more electron rich, it could be more easily oxidise by the laccase.

[170] Li, K.; Horanyi, P. S.; Collins, R.; Phillips, R. S.; Eriksson, K.-E. L. *Enz. Microb. Technol.* **2001**, *28*, 301-307.

Yet, as seen for light oxidation of the simple *o*-aminophenol in absence of biocatalyst, the quinonic species may also come from air oxidation. Again, spin trapping experiment would be a method of choice to determine whether or not a radical intermediate could be stable enough and detected in the aqueous media. In addition, studies of the molecular oxygen consumption could provide interesting informations.

**E/Step4**- The second intramolecular nucleophilic addition is assumed to occur either in the bulk of the solution or in the binding cavity.

**F/Step5**- The last step of oxidation is assumed to be spontaneous in aerated solution. The driving force would be the rearomatisation leading to a stable dye as observed for non catalysed APX syntheses. We can't exclude the action of the laccase in this last step.

The air oxidation should produced transient Reactive Oxygen Species (ROS). The monitoring of oxygen consumption during the reaction could provide some information. In addition, several of the ROS likely produced could be trapped. If we assume first one electron reduction of the molecular oxygen, then superoxide dismutase (SOD) could be used to trap the superoxide anion formed. This would yields hydrogen peroxide which could also be converted in presence of catalase into water.[171]

[171] a) A. D. A. Mahelet A. Tadesse, C. Galli, P. Gentili, F. Sergi, *Org. Biomol. Chem.* **2008**, *60*, 868-878. b) Suresh, P. S.; Kumar, A.; Kumar, R.; Singh, V. P.; *J. Mol. Graph. Model*. **2008**, *26*, 845-849.

Clearly, the overall sequence is very complex and involves multiple steps for which a careful investigation should be conducted if one would like to detail a novel mechanism in an affirmative manner. Here, we are first interested by the investigation of the discrepancies observed between several substrates. Experimental studies of the reactivity of all of those substrates in similar conditions could give us some hints regarding the reason of such discrepancies. It is important to stress that most of our experimental results are obtained after few hours of reaction and that no kinetic data were collected in the early stage of the reaction.

Before trying to decipher part of the mechanism in play with our substrates, a first evaluation of their oxidation potential was performed. Cyclic voltametry, a powerful electrochemical method, readily gave pertinent evaluations of the anodic potential of our compounds.

Afterwards, we used a protocol established in the laboratory to evaluate the kinetics of 3-hydroxyorthanilic acid consumption. Our previous biocatalyst i.e the Bioscreen laccase (**TvL1**) was replaced here by a surrogate laccase of *Trametes versicolor*, commercially available from sigma Aldrich (**TvL2**). This underlines both the scope of our catalytic reaction and the production of the LAO dye in the earlier hours of the biotransformation. The subsequent conversions of several selected substrates were realised with **TvL2** and similar results were observed. The same discrepancies observed previously with **TvL1** were confirmed here. Then, to briefly map the problematic, we chose three qualitative approaches which are given hereafter.

A first approach of the problem is a manual docking of selected compounds into the laccase binding cavity. The approach could suggest, similarly to results published recently, the critical importance of an efficient location of the substrates at the vicinity of the copper site T1.[112, 119, 120,171]

The second approach is the experimental comparison between the biocatalysed oxidation of selected compounds with their chemical oxidation. Several chemical reagents were used to determine whether or not the oxidation can take place. The reactions were first studied by HPLC coupled to a PDA detector and afterwards analysed by HPLC-$MS^2$. Both techniques are complementary in our case and similar results were collected.

Lastly, the third approach involves cross-coupling reactions. Those were realised in order to consolidate the proposal for the oxidation of our compounds. In addition, this also helps to determine whether or not each aminophenol partner could act as a good nucleophile in the putative second step of the cascade (see schemes 47 and 48). Cross-coupled products were obtained which suggest that all aminophenols are able to add on an activated electrophile. Whereas the exact structures of the electrophilic species and intermediates are still unknown, the oxidised products detected through HPLC-PDA and HPLC-$MS^2$ analyses are always at least actinocin-like or orcein-like derivatives.

## 5.3 Reminder of the catalytic site of the laccase

Clearly, laccases which are members of the multicopper oxidase family (MCO) have an ability to oxidise a wide variety of molecules ranging from organic to inorganic compounds. Furthermore, several of those copper oxidases are involved in the synthesis of natural pigments having the phenoxazinone core structure. The laccases contain four copper atoms distributed in three copper sites (T1, T2 and T3) well characterised. The type 1 (T1) or "blue" Cu site holds a characteristic intense absorption around 600 nm due to the highly π covalent link between S(cys) and $Cu^{2+}$. The copper is strongly bound by two other ligands (His) to form a trigonal plane whereas a fourth ligand, involved in the tuning of the enzyme activity, varies greatly

among proteins. The site T1 is the primary acceptor of electron; it is slightly buried in the enzyme core near the binding cavity where small organic molecules are oxidised. Coppers sites T2 and T3 form a trinuclear cluster where channelled electrons from T1 are furnished to molecular oxygen which is reduced into water. Among the other copper oxidases involved in the chemistry of related chromophores both the phenoxazinone synthase[110, 111] and *o*-aminophenol oxidase GriF[172], respectively involved in the biosynthesis of actinomycin and grizoxazone, are the most interesting.

Based on the apparent spectroscopic properties of the phenoxazinone synthase, it was previously suggested that the arrangement of the Cu centres was different from other multicopper oxidases. The mechanistic investigation of the synthesis of the actinomycin chromophore, a phenoxazinone, led to the proposal of a cyclisation involving $6e^-$ for the total oxidation.

Oxidative coupling of two molecules of substituted *o*-aminophenol takes place via a series of three $2e^-$ oxidations, unique among the MCOs which usually catalyse $1e^-$ oxidation reactions. In 2006, the first X-ray structure of a phenoxazinone synthase was published.[111] The almost identical arrangements of the Cu centres to the one observed in laccases, except for a fifth Cu atom which only serves to further stabilise the enzyme tertiary structure, has been unambiguously demonstrated.

This opens new perspectives for developing a general method of laccase-mediated synthesis of diversely substituted phenoxazinones. Indeed, if a similar rapid two sequential $1e^-$ oxidations happen here, the quinonic species would be directly produced from aminophenol substrates.

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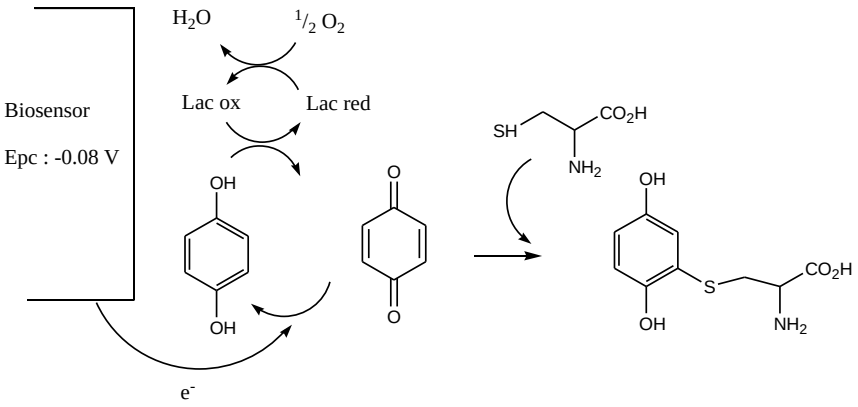
[172] Suzuki, H.; Furusho, Y.; Higashi, T.; Ohnishi, Y.; Horinouchi, S. *J. Biol. Chem.* **2006**, *281*, 824-833.

Moreover several biosensors based on laccase are currently developed. Among them, few have been respectively used to detect the presence of L-cysteine in pharmaceutical formulations or dopamine.[173] In the first case, the principle of the detection is the rapid oxidation of hydroquinone by laccase into the corresponding quinone. In the absence of chemical reaction, the quinone is reduced by electron transfer at the vicinity of the electrode. When cysteine is present, 1,4 addition of the sulfhydryl group to the quinone prevents the reduction step and thus decreases the current intensity (figure 16). A similar mechanism is involved for the detection of dopamine. Therefore, it is not deceptive to postulate a similar rapid oxidation into our quinonic species.

Figure 16. Detection of L-cysteine with laccase and electrode




[173] Santhiago, M.; Cruz Vieira, I. *Sensors and Actuators B* **2007**, *128*, 279-285.

## 5.4 Electrochemistry

### *5.4.1 Foreword*

Cyclic voltametry (CV) is a potentiodynamic electrochemical measurement. In a CV experiment the working electrode potential is increased linearly versus time. When CV reaches a selected step potential, the working electrode potential ramp is inverted. This ramping is termed the experiment's scan rate. The current at the working electrode (anode) is plotted versus the applied voltage to give the cyclic voltamogram trace.

The forward scan produces here a current peak for any analyte that can be oxidised through the range of scanned potentials. The anodic current will increase till the potential reaches the oxidation potential of the analyte, but then falls off as the concentration of the analyte is depleted at proximity of the electrode surface. If the redox couple is reversible, then the inversion of applied potential will reduce the oxidised product formed in the first oxidation reaction and hence produce a cathodic current. This reduction peak should have similar shape as the oxidation one. As a result, information about the redox potentials and electrochemical reaction rates of the compounds are obtained.

### *5.4.2 Method*

Briefly, solutions of the substrates (Figure 17) at a concentration of 15 milligrams per milliliter were made in dimethylformamide in the presence *n*-tetrabutyl ammonium tetrafluoroborate as the electrolyte salt, at a concentration of 0.1 M. The electrochemical system

consisted of a glassy carbon working electrode, a platinum foil counter electrode and a reference electrode (Ag/AgCl in EtOH saturated with LiCl) having a standard potential of 0.143 V.

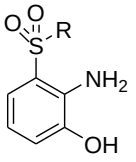
Cyclic voltamograms were recorded in triplicate at a scan rate of 150 mV per second with a potential of -0.5 V to 1.8 V. Accordingly to the literature, a positive shift toward higher potential was observed due to the organic nature of the solvent used in the electrochemical analysis.[174]

The anodic peaks of oxidation were measured in organic polar solvent and compared in the same condition to the one of 3-hydroxyanthranilic acid (3HAA / **35**) used as a reference.

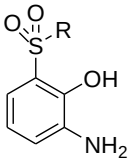
Those values are listed in Table 2 and correspond to the anodic peaks of oxidation recorded at the end of the principal oxidation waves. As expected, the observed voltamograms deviated strongly from the ones expected for reversible redox couples (see Figure 18). This suggests indeed the formation of short life-time quinonic species and their rapid subsequent chemical reactions likely to happen. We have different derivatives in hands to evaluate the effect of the organic medium chosen for the analysis. It is of common knowledge that the nature of the solvent affects both the rate of oxidation and the potential value, in fact mainly due to the rate of electrons and mass transfer.[170] Furthermore, several publications have already reported the electrochemical analysis of kynurenine derivatives in aqueous buffer.[175] We could easily compare our data with those published.

[174] Sripriya, R.; Chandrasekaran, M.; Noel, M. *Colloid Polym. Sci*. **2006**, *285*, 39-48.
[175] Giles, G. I.; Collins, C. A.; Stone, T. W.; Jacob, C. *Biochem. Biophys. Res. Commun.* **2003**, *300*, 719-724.

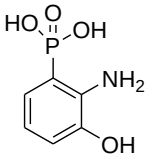
Figure 17. Aminophenols compounds studied by CV



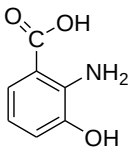

R = OH **1**
R = NH-cyclohexyl **2**
R = $NH(CH_2)_3NMe_2$ **3**
R = $NH_2$ **4**




R = OH **5**
R = NH-cyclohexyl **6**
R = $NH(CH_2)_3NMe_2$ **7**




**34**




**35**

R = NH-phenyl **30**
R = $NH(CH_2)_2NH_2$ **31**
R = $NHCH_2CO_2Me$ **32**
R = $NH(CH_2)_3OH$ **33**

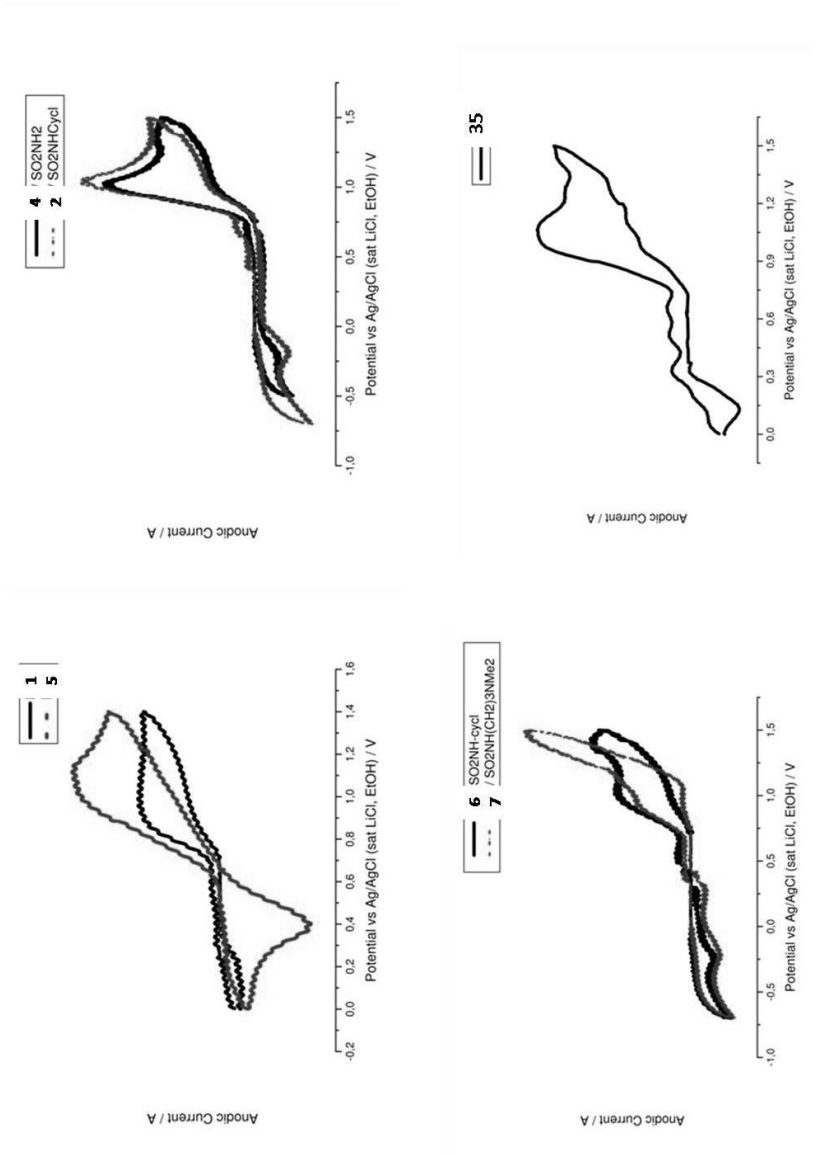
All the values measured for the anodic peaks of oxidation are rather similar (between 0.9 to 1.1 V). Several compounds are slightly more prone to oxidation but the majority of the aminophenol derivatives features the same $E_{pa}$ value as the reference 3HAA (**35**) in our system Regarding the tested molecules, significant reduction processes have not been observed consistently with the irreversible EC pathway (electron transfer and chemical reaction) characteristic of the transient iminoquinone species generated upon oxidation. The CVs results obtained show that all of our compounds are indeed prone to oxidation whereas no marked trends appear to correlate their susceptibility to oxidation and the yields collected in the laccase-mediated reactions.

Table 2. Properties of related aminophenols

| Cpd | $E_{pa}$/V [a] | Conversion(%) [b] | Isolated product (%)[c] |
|---|---|---|---|
| **1** | 1.00 | >95 | 90 |
| **2** | 1.04 | >80 | 86 |
| **3** | 1.03 | >90 | 90 |
| **4** | 1.03 | >80 | 60 |
| **5** | 0.96 | >20 | 4 |
| **6** | 1.02 | >80 | 73 |
| **7** | 0.98 | - | - |
| **30** | 1.03 | >70 | 40 |
| **31** | 1.10 | >90 | 87 |
| **32** | 1.05 | >70 | 58 |
| **33** | 0.98 | >90 | 90 |
| **34** | 0.98 | >95 | 90 |
| **35** | 1.03 | 70 | / |

. [a] ± 0.02 V , recorded in DMF, $TBABF_4$ as supporting electrolyte.
[b] Ratio of integration area at 280 nm. From reaction with **TvL1** (100 $U.L^{-1}$) followed by HPLC.
[c] Final dyes, phenoxazinones and related structures isolated

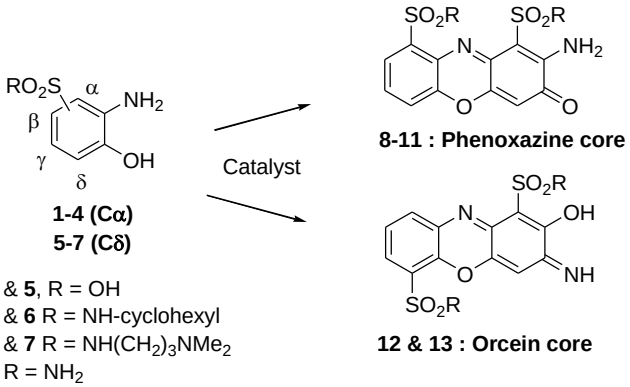
Figure 18. Selected Cyclic voltamograms among the studied substrates



## 5.5 3HOA consumption with Tvl2

The previous data were obtained using a commercial preparation of laccase (**TvL1**) from *Trametes versicolor* (TvL) for which unfortunately there is no exhaustive data collection regarding its kinetic characterisation.[176] Yet, the majority of laccases from TvL, independently of the organism, corresponds to few isozymes which are well thoroughly characterised and for which some X-ray structures have been published.[119] For practical reasons commercial laccase (**TvL2**) purchased from Sigma was assayed. First its catalytic properties were assessed regarding our previous methodology for the synthesis of 1,9 and 1,6-disubstituted heterocycles (see Scheme 49). The rate of consumption of our model compound **1** (R = OH) using both catalysts, in the same experimental conditions, was evaluated by analytical HPLC (see supporting material). The heterocyclic dye **8** (R = OH), previously isolated and fully characterised, was used as reference product. Several catalytic quantities of laccase, corresponding respectively to an activity of 100 $U.L^{-1}$, 50 $U.L^{-1}$ and 20 $U.L^{-1}$ were used.

Scheme 49. synthesis of model dyes using commercial laccase **TvL2**

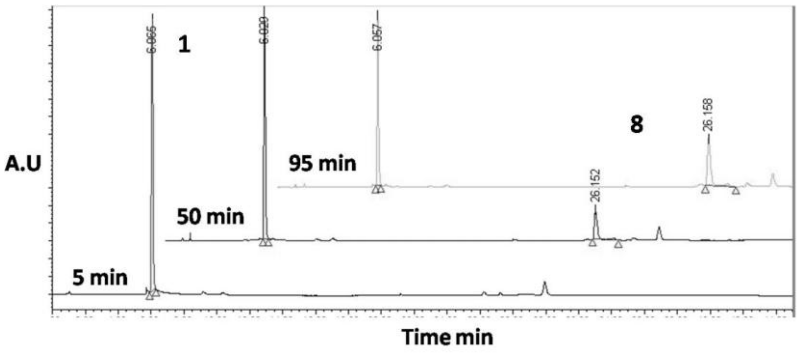



**1** & **5**, R = OH
**2** & **6** R = NH-cyclohexyl
**3** & **7** R = $NH(CH_2)_3NMe_2$
**4** R = $NH_2$

[176] Trovaslet, M.; Enaud, E. ; Bazes, A.; Jolivalt, C.; Van Hove, F.; Vanhulle, S. *Chem. Eng. Trans.* **2008**, *14*, 315-322.

The crude reaction mixtures were incubated at 25 °C and samples were taken every 45 min during the first 2 hours of reactions. The rates of the production of **8** were similar for both catalysts. Representative chromatograms illustrating the conversion of **1** into **8** at pH 6 in presence of 50 $U.L^{-1}$ are given in Figure 19.

Figure 19. Kinetics of consumption (HPLC-PDA) of **1** (1mM) by **TvL2** (50 $U.L^{-1}$) at pH 6 and the concomitant formation of phenoxazinone **8** in the first 2 hours of reaction (detection at 280 nm).




As stated in our second assumption, the first addition step could indeed be a nucleophilic conjugated addition (Michael addition) of the unreacted *o*-aminophenol (thus reduced) to its oxidised form (iminoquinone), rather than an electrophilic dimerisation of two iminoquinones as described for an *o*-aminophenol oxidase GriF, a tyrosinase homolog.[168] The latter mechanism, similar to the one observed with a phenoxazinone synthase model in organic solvent, implies a significant lag-phase before the apparition of the corresponding phenoxazinone.[108c,d]

Afterwards the efficiency of both TvL catalysts was compared for selected substrates using as references the corresponding isolated phenoxazinones previously characterised (chapter 4). The results of the HPLC-PDA analyses are summarised in Table 3, the results are showing similar trends with both catalysts. At the end of the reactions, the 1,9-disubstituted heterocycles are readily produced with similar conversion yields, whereas for the 1,6-disubstituted regioisomers the reactions are once again less selective for **5**, **6** or even inexistent for **7**. On the other hand, conversion of **5** into **12-red** or **12,** while still poisoned with side products, seemed to give higher yields. The nature and purity of the catalyst, unknown precisely here for both laccases, could explain part of those observations.

Table 3. Conversion of selected substrates with two different laccases

| cpd[a] | TvL activity | Yield[b] TvL1 | Yield[b] TvL2 | $K_m^{app}$ Psc[c] (mM) |
|---|---|---|---|---|
| 1 | 100 | . 90* | 90# | 0.075 ± 0.008 |
| 2 | 600 | 80* | 80# | N.D |
| 3 | 100 | 80* | 80# | 0.069 ± 0.014 |
| 4 | 100 | 70* | 80# | 0.066 ± 0.001 |
| 5 | 20-1000 | 5*,20# | 50# | 1.899 ± 0.313 |
| 6 | 600 | 80* | 60# | 0.042 ± 0.003 |
| 7 | 20-1000 | 0* | 0# | - |
| 3HAA/35 | 100 | 70# | 70# | - |

[a] See Scheme 1 for structure. [b] (*) mean isolated yield for a fully characterised product, (#) mean conversion yield. Those were evaluated by HPLC-PDA from the ratio of integration area detected at 280 nm. [c] Kinetic data were collected with a laccase from *Pleurotus sajor caju* (28 $U.mL^{-1}$) at pH 6 to 6.5. N.D means not soluble above 2mM, not enough for Km determination.

## 5.6 Manual docking

The ability of **7** to be oxidised, suggested by the electrochemical analysis, and the absence of product **15** in laccase-mediated reactions remains rather difficult to explain. To investigate a potential steric effect that would prevent the enzyme from processing **7**, we tried to perform a manual docking of selected substrates into the laccase active site. This would be a preliminary investigation. The oxidative cascade may be split into successive reactions. The regioselectivity is likely to outcome from the two first steps. These are the oxidation of one substrate molecule and then the conjugated addition of another native molecule leading to the formation of a stable dimeric intermediate (see Scheme 47).[110]

From the literature, the first $1e^-$ oxidation which probably occurs at the phenol group would be the rate limiting step.[177a] Regarding *o*-catechol and *o*-aminophenol, the subsequent $1e^-$ oxidation which converts the radical semiquinone into the reactive *o*-quinone and *o*-iminoquinone respectively should be even faster and energetically favoured.[177b] If the laccase could accommodate the substrate in the binding cavity, two sequential $1e^-$ oxidations may be available due to the fast channelling of electron through the peptidic backbone linking the Cu site T1 to the trinuclear Cu sites T2/T3. Those two electrons taken, the suggested iminoquinone formed could leave the cavity towards the bulk of solution to allow another substrate to be reduced. Two X-ray structures, 1GYC and 1KYA, of laccases from *Trametes versicolor* species can be found in the protein data bank with respectively a 2-propanol or a 2,5-dimethylaniline (XYD) molecule present in the active site.[119b,c]

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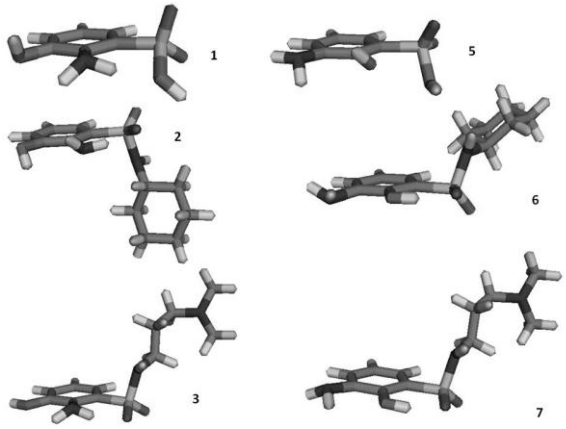
[177]a) Kurzeev, S. A.;. Vilesov, A. S.; Fedorova, T. V.; Stepanova, E. V.; Koroleva, V. C.; Bukh, O.; Bjerrum, M. J.; Kurnikov, I. V.; Ryabov, A. D. *Biochemistry* **2009**, 48, 4519-4527. b) Wright, J. S.; Johnson, E. R.; DiLabio, G. A. *J. Am. Chem. Soc*. **2001**, *123*, 1173-1183. b) Shadnia, H.; Wright, J. S. *Chem. Res. Toxicol.* **2008**, *21*, 1197-1204.

For the second one, the critical interactions of a small aromatic substrate with the amino acids of the binding cavity were deduced and the residues which determine the binding were thus identified. The catalytic sites being highly conserved among laccases from different species, such correlations are now commonly accepted as a general rule.[119b, 167] To investigate a manual docking of our substrates (see Figure 21 for representative views), three-dimensional structures were generated and their geometries fully optimised, first at a semi-empirical level using the PM3 method. Those optimised structures (see Figure 20) were in accordance with the minimised geometries usually observed for related compounds.[178] Manual docking (detailed in the experimental) was carried out using MOLDEN software.[179]

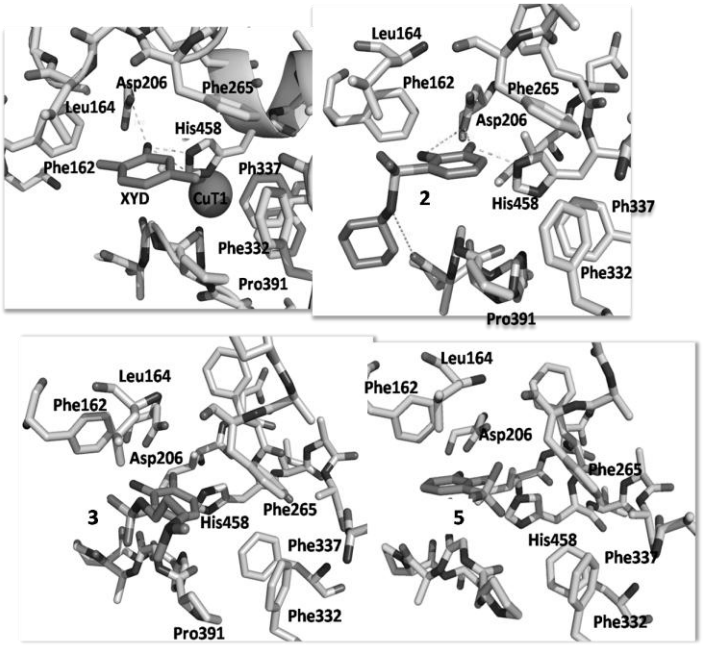
Figure 20. Optimised geometries of selected substrates




[178] a) Mora, M. A.; Mora-Ramírez, M. A.; Soto-Estrada, A. M.; Bertin, V. *Polymer* **2007**, *48*, 5431-5439. b) Hao, M.-H.; Haq, O.; Muegge, I. *J. Chem. Inf. Model.* **2007**, *47*, 2242-2252. c) Brameld, K. A.; Kuhn, B.; Reuter, D. C.; Stahl, M. *J. Chem. Inf. Model*. **2008**, *48*, 1-24.

[179] Schaftenaar, G.; Noordik, J.H. *J. Comput.-Aided Mol. Design* **2000**, *14*, 123-134.

Figure 21. Top, left: Representative picture of the ligand XYD in the binding cavity of 1KYA as observed in the X-ray structure. Top right: Manual docking of **2** into the binding cavity, RMS < 1Å. Dashed lines are the critical hydrogen-bond interactions. Bottom, left: Manual docking of **3** into the binding cavity. Bottom, right: Manual docking of **5** into the binding cavity. View elaborated with PyMol from crystallographic data.




The quality of this docking has been evaluated following a Potential-Mean-Force (PMF) scoring function. Such knowledge-based scoring function emerged as an alternative way of ranking protein-ligand complexes with known 3D structures according to their binding affinities.[180]

[180] a) Muegge, I.; Martin, Y. C.; Hajduk, P. J.; Fesik, S. W. *J. Med. Chem*. **1999**, *42*, 2498-2503. b) Muegge, I. *J. Med. Chem*. **2005**, *49*, 5895-5902.

Bearing in mind the critical interactions between the site of oxidation and the ligand (His458) of the T1 copper, an alignment of the aromatic core of our substrates to the one of the ligand XYD was performed. The phenol group was usually directed towards the His458 residue, otherwise the aniline function was located in such a manner. The aromatic core was allowed to move from less than 1 Å from its initial position when required. Conformational spaces of the side chains were not fully investigated; the so-called "tails" were likely to lie outside of the binding cavity.

Representative views of the docking of **2**, **3**, **5** compared to the one of XYD are shown in the Figure 21. One can distinguish the amino acids Pro391, Phe332 and Phe337 which form together the right wall of the cavity. The residues Phe265, Phe162 and Leu164 delimit the rest of the cavity, while the more embedded Asp206 and His458 are the critical amino acids interacting with the substrates. Ionised Asp206 may form hydrogen-bond interactions with the protons of the aniline or phenol group. This further stabilises the ligand. His458, directly linked to the T1 copper, also form hydrogen-bond interactions with the functional group from which electrons are taken. This may involves a Proton-Coupled Electron transfer (PCET).[181] The substrates **1-3**, **5** and **6** (see examples in Figure 21) could be docked easily without having to move the aromatic core beyond a distance of 0.5 Å. The compounds **1-3** were placed in a similar manner i.e the sulfonylated group pointing toward the entry of the cavity, the aniline directed to the residue Asp206 and the phenol facing the residue His458. For **5**, an interaction between the phenol and the residue His458 implied that the free sulfonic group may have to be much more embedded into the cavity.

[181] Reynisson, J.; Steenken, S. *Org. Biomol. Chem*. **2004**, *2*, 578-584.

Whereas the cyclohexyl group of **6** pointed away of the residue Leu164, for **3** the most effective disposition featured the dimethylaminopropyl side-chain pointing up toward this residue. The compound **6** could adopt a similar conformation to **3** resulting in the aniline facing both Asp206 and His458. Such arrangement could not be optimised. Finally, no docking of the compound **7** could be achieved within 1KYA binding cavity, whatever the dispositions assayed considering a RMS of less than 2 Å. Yet, a manual docking into the binding cavity of 1GYC could be achieved (data not shown). This may come from a single difference related to the peptidic backbone bearing Leu164. In the case of 1KYA, Leu164 is coming closer into the cavity whereas in 1GYC, the cavity appears slightly wider. Such difference has not been precisely explained.

At the end of this first approach, no affirmation or clear assumption could be made from it.

## 5.7 Chemical oxidation versus biocatalysed oxidation

The comparison between laccase-mediated and chemical oxidant-mediated reactions was undertaken here. The oxidised products were analysed by HPLC and part of them by LC-$MS^2$. Then, competitive reactions were performed by mixing equal quantities of **7**, suggested as non reactive by the enzymatic preparations used, with **1** or **2** and those pairs of substrates were submitted to oxidation (see scheme 50 for general description). The results being likely the most interesting are summarised in Table 4. When comparing air oxidation with a biocatalyst to a chemical oxidant, the selectivity of the laccase-mediated oxidations always seems to supersede those carried out with either silver oxide or $O_2$ and a Co(salen) catalyst.[182]

Table 4. Selected results of biocatalysed and chemical oxidations

| entry | cpd | Oxidant[a] | HPLC[b] RT (±0.1) min , (cpd) | Yield %[c] | LC-MS[d] m/z, parent ion (cpd) |
|---|---|---|---|---|---|
| 1 | 7 | $Ag_2O$ | < 1* | - | Polymers |
| 2 | 7 | Co(salen) | < 1* | - | Polymers |
| 3 | 7 | Air (light) | < 1* | - | 274 (**7**) ;541 (**15**) |
| 4 | 2 | TvL2 | 7.4 (**9**) | 80 | - |
| 5 | 7 + 2 | $Ag_2O$ | 2.7 (**22**) | 70 | 538 (**22**) |
| 5 | 7 + 2 | $Ag_2O$ | 7.4 (**9**) | 10 | 535 (**9**) |
| 6 | 7 + 2 | TvL2 | 2.7 (**22**) | 51 | 538 (**22**) |
| 6 | 7 + 2 | TvL2 | 7.4 (**9**) | 38 | 535 (**9**) |
| 7 | 6 | TvL2 | 5.4 (**unknow**) | 30 | 535 (**13**) |
| 7 | 6 | TvL2 | 6.1 (**13**) | 60 | |
| 8 | 6 | $Ag_2O$ | 4.5 ( **unknow**) | 50 | - |
| 8 | 6 | $Ag_2O$ | 6.1 (**13**) | 40 | |
| 9 | 6 | Co(salen) | 3.8 (**6**) | 80 | - |
| 9 | 6 | Co(salen) | 6.1 (**13**) | 10 | |
| 10 | 7 + 1 | $Ag_2O$ | 1.7* (**16**) | 40 | 538 (**16**) |
| 10 | 7 + 1 | $Ag_2O$ | 7.4* (**8**) | 30 | 535 (**9**) |
| 11 | 7 + 1 | TvL2 | 2.7* (**16**) | 40 | 538 (**16**) |
| 11 | 7 + 1 | TvL2 | 7.4* (**8**) | 20 | 535 (**8**) |

[a] TvL2 activity of 600 $U.L^{-1}$, 5eq $Ag_2O$ in MeOH, 0.03 eq of Co(salen) in MeOH. [b] (*) for method HPLC 3 (<1 means in the dead volume), otherwise method 2 was used, see supporting material. [c] Those are estimated from ratio of integration area at 280 nm detected by HPLC. [d] HPLC methods were transposed for LC-MS, see supporting information. Mass spectra were acquired in ESI positive mode $[M+H]^+$.

[182] Maruyama, T. M. K.: Mashino, T.: Nishinaga, A. *Chem. Lett.* **1996**, *25*, 819-820.

To oxidise those aminophenols, we chose a silver oxide ($Ag_2O$) and the well known oxidative catalyst "Co(salen)" working with $O_2$ or air (Figure 22). The first one, used in stoichiometric quantity, has been successfully applied for the biomimetic oxidative synthesis of a pyridoacridine compound.[102] The Co(salen) catalyst, in oxygenated solutions, is also known as a surrogate catalyst of an oxidase enzyme.[180] Finally, we left the compound **7** in solution, irradiated by sunlight, for a few days in presence of air to see whether or not a simple photo- and/or auto-oxidation process could generate the desired phenoxazinone dye **15**.[163]

Scheme 50. General cross coupling reactions

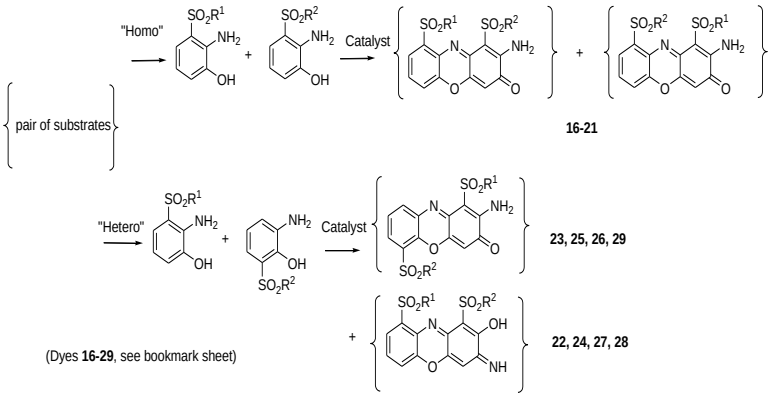
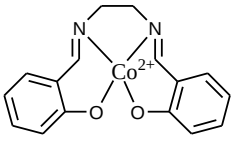



Figure 22. Co(salen) catalyst (see supporting material for preparation) [183]




Cosalen

[183]. Appleton, T. G.; *J. Chem. Educ*. **1977**, *54*, 443-444.

Herein, the Co(salen) mediated oxidations (rt, 8h) were performed under air atmosphere, and those reactions merely assessed the ability to produce even in low quantities the desired heterocycles. Except for compounds **7** (entry 2), all the substrates were partially oxidised leading to a yield of phenoxazinone of about 10% (identified by HPLC) (entry 6, for one example). Similarly, the use of an excess of silver oxide gave a complete consumption of all the substrates in 8 hours of reactions at rt (see entries 1, 5, 8). For **6** (entry 8), a lower yield of phenoxazinone **13** was observed along with unknown side products. Even **7** (entry 5) was significantly oxidised by such chemical reagent, but still no desired heterocycle **15** could be detected.

Photo/auto-oxidation of **7** (entry 3) in methanol under air for two days either at 1 mM or 20 mM concentrations seems mainly to result into the starting material unchanged (more than 90% from HPLC). But LC-MS of the reaction mixture, (at higher concentration i.e. 20 mM), allowed the detection of traces of the desired phenoxazinone **15**. This suggests that the cyclisation process is potentially available.

Now, competitive experiments with the pair of compounds **7** and **2** gave in all cases two cyclised products featuring the specific pattern of a phenoxazinone dye in UV-Visible spectroscopy ($\lambda_{max}$ absorption around 240 and 420 nm). The major compound (m/z = 537.17 detected here as $[M+H]^+$ with m/z = 538) is likely the 1,6-disubstituted heterodimeric phenoxazinone **22** arising from the first conjugated addition of **7** to the iminoquinone resulting from the oxidation of **2**. The structure was suggested by LC-MS. Relative proportions observed in the reaction mixtures suggest an efficient addition step of **7** to the oxidised reactive species.

Still, in both cases (chemical and enzymatic oxidation) a second compound was formed as a minor product namely the homodimers **9**. Same results can be observed by combining **7** and **1** (see entries 10 and 11) in both chemical and laccase-mediated oxidations. The major compound (m/z = 456.08 detected here as $[M+H]^+$ with m/z = 457) is likely the 1,6-disubstituted heterodimeric phenoxazinone **16**.

Those qualitative results suggest that the regioisomer **7,** which seems quite reluctant towards chemical or enzymatic oxidation, may behave as a nucleophilic reagent for quenching the iminoquinone formed in situ from the regioisomers **1** or **2**.Yet, why there are no phenoxazinone originating from an addition to an oxidised **7** remain unsolved.

## 5.8 Selected cross-coupling reactions with laccase

Four representative substrates were chosen, namely **2**, **4**, **6** and **7**, for competitive experiments in the presence of a commercial laccase. The pairs of substrates, in an equimolar ratio, were mixed with a catalytic quantity of oxidase in the same conditions (see Scheme 51).

From each pair of compounds, there are four heterocyclic frameworks potentially available (two homodimers and two heterodimers). Instead of an exhaustive investigation using all the potential permutations for the pair of substrates, we chose the regioisomers **2** and **6** as references for the preliminary comparison of the reactivity and selectivity, and for helping to detect specific fragmentation patterns in MS/MS analysis. Equimolar quantities of substrates (1 mM in a buffer at pH 6) were used in pair with laccase (100 $U.L^{-1}$). After few hours, the precipitated dyes were collected and dissolved in methanol. Both alcoholic and residual aqueous solutions were analysed through HPLC-PDA and HPLC-$MS^2$.

Using the pair of compounds **2** and **4** (Table 5, entry 1), both homodimeric dyes **9** and **11** (Scheme 51) appeared to be produced albeit in very minor proportions.

Scheme 51. Cross-coupling reactions between pairs of substrates are realised in the presence of laccase, in aqueous buffer at pH 6

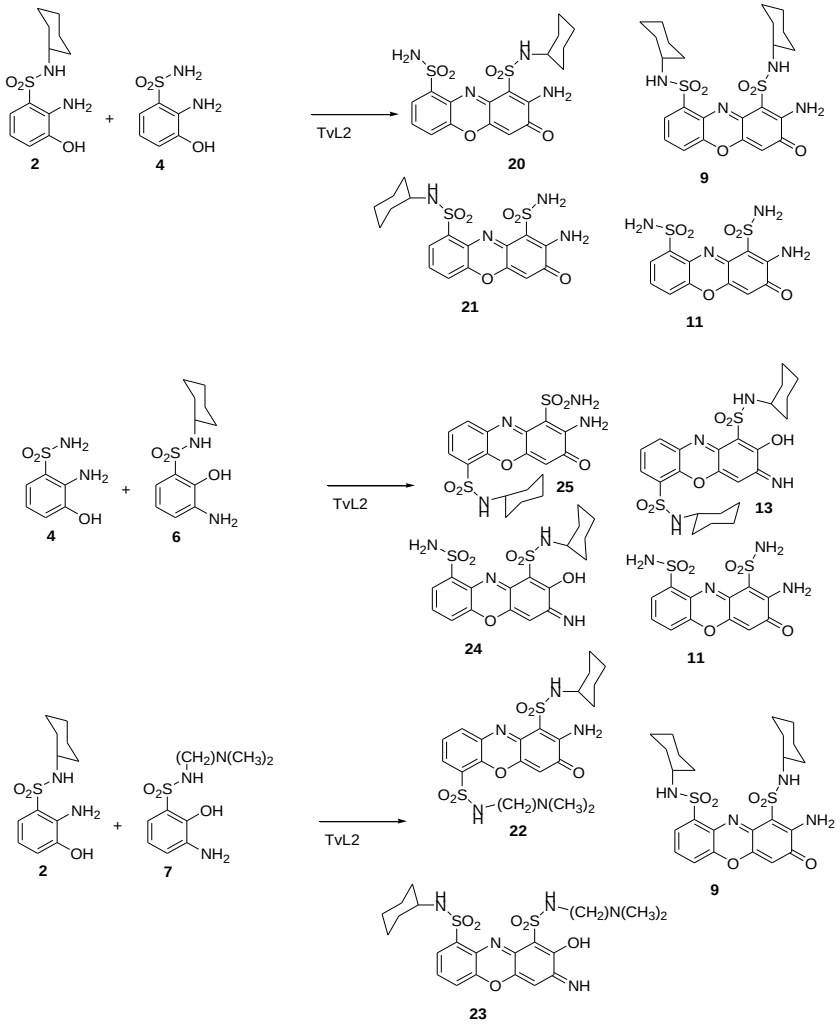


Table 5. Selected results regarding the heterodimeric dyes obtained from cross-coupled substrates [a]

| Entry | Cpd | Yields[b] | RT min ±(0.1) [c] | HPLC-MS$^2$ ( *first peak = [M+H]$^+$*) (**base peak = 100% intensity, bold**) |
|---|---|---|---|---|
| 1 | **2+4** | 30 (**20**) | 10.6 (**20**) | *453.2*, 370.79, 353.73, 305.75, **289.70**, 272.7, 208.70 |
| | | 40 (**21**) | 11.0 (**21**) | *453.2*, 370.79, 353.73, 305.75, **289.70**, 272.7, 208.70 |
| 2 | **6+4** | 60 (**24**) | 8.7 (**24**) | *453.20*, 387.90, **371.85**, 359.90, 289.70, 256.67 |
| | | | N.D (**25**) | *453.20*, 370.74, 353.76, 305.75, **289.70**, 272.73, 208.71 |
| 3 | **2-7** | 40 (**22**) | 6.8 (**22**) | ***538.11***, 455.97, 390.89, 374.86, 208.66 |
| | | | N.D (**23**) | ***538.11***, 456.08, 410.90, 373.61, 291.67, 164.70 |

[a] ESI detection in positive mode $[M+H]^+$, fragmentation yields consistently a loss of 82 m/z specific of a phenoxazinone dye with a cyclohexyl side chain.

[b] % of dimers detected by HPLC, (entry 1, homodimers **9** & **11** in about 10% respectively); (entry 2, homodimers **11** in about 15% and **13** in less than 5%); (entry 3, homodimer **9** in about 20%).

[C] Retention times for HPLC-PDA.

The major chromophores, identified as **20** and **21** through HPLC-MS$^2$ suggest a poor relative selectivity in the laccase-mediated reaction.

Now looking at the pair of compounds **6** and **4** (Table 5, entry 2), the only homodimeric dye detected is the heterocycle **11** while the dye **13** is almost absent of our reaction mixture. Moreover, only one heterodimeric phenoxazinone **24** could been identified by UV-Visible detection in HPLC with a retention time of 8.7 min, and the expected mass value of 453 m/z ($[M+H]^+$) by MS detection.

The $K_m$ of **6** appeared as the lowest among our substrates, and **6** seemed also to be the one undergoing the most rapid consumption in the first hours of the reaction. This could suggest a first addition step performed into the bulk of the solution where the concentration of the reduced form of **4** is expected to be higher and the quinonic species derived from oxidation of **6** could accumulate. If the addition occurs in the binding cavity, the ratio of dye **13** should be significantly higher and some proportion of a second heterodimer **25** should be observed. By LC-$MS^2$, the presence of this second compound is indeed detected albeit in traces.

Finally, we mixed **2** and **7** in presence of the biocatalyst (entry 3). Two compounds corresponding to a value of 538 m/z were identified in HPLC-$MS^2$ whereas only one major heterodimeric dye **22** appears when UV-Visible detection (PDA) is performed. Along with those heterocycles, the reference dye **9** (homodimer from **2**) was also produced, but not the dye **15** (homodimer from **7**). The major compound probably corresponds to the addition of the poor substrate **7** upon the quinonic form of **2**. Such heterocycle exhibits the ubiquitous pattern of fragmentation of a phenoxazinone bearing a cyclohexyl motif (loss of 82 m/z for cyclohexene).

Moreover, the characteristic fragment of 164.7 m/z, which seems specific of a side chain bearing the *N*-dimethyl group on position C1 of a phenoxazinone dye, is absent of the fragmentation pattern. But, the second product (**23**) detected in traces in LC-$MS^2$ could correspond to such fragmentation and likely arises from the addition of a reduced **2** upon the tenuous fraction of oxidised **7**.

## 5.9 Summary of the preliminary experimental results

In summary, the electrochemistry allowed us to evaluate the susceptibility of our substrates towards oxidation. Experimental results suggest that all compounds exhibit a similar potential of oxidation which don't explain the absence of laccase-mediated transformation of compound **7**.

A surrogate biocatalyst, a commercial enzymatic preparation of laccase from *Trametes versicolor* seemed to exhibit a similar behaviour towards chosen substrates as the commercial one previously used. The absence of a lag phase for the production of our model dye **8** seems consistent with the proposal made of a nucleophilic addition in the step 2 of our oxidative cascade provided that we rely upon the assumptions made in the section 5.1.

A manual docking was performed and seems to be coherent with the experimental results obtained. Yet, the inherent subjectivity due to the numerous choices and assumptions that must be made through the entire process does underline the fact that only suggestions can be retrieved from such method.

The oxidation of selected substrates with chemical reagents seems to reinforce the fact that similar behaviours are observed for the substrates when compared to their oxidation mediated by the laccase. The detection in traces of an aminophenoxazinone by the auto-oxidation of one substrate whereas it seemed to be non reactive towards a laccase may suggest a drawback related to the laccase-substrate interaction.

The experimental results obtained through the cross-coupling reactions seemed to suggest the reactivity of all of our substrates. Interestingly, these results also suggest the ability of all the aminophenol derivatives to act as nucleophile towards electrophilic species provided that we rely again to the assumptions of section 5.1. Based on those assumptions and the experimental results obtained

with a semi-purified laccase, one could propose a link between the $K_m$ of the substrates and the relative conversions observed for the cross-coupling reactions.

In conclusion, we have made a proposal regarding the oxidative cascade involved in the production of our actinocin-like and orcein-like dyes. This may account some of the experimental results observed only if we rely upon several assumptions. As the proposal is not affirmative in any ways but merely a plausible pathway, those assumptions and the overall proposal remain to be proved and further investigations should be made to either confirm or contradict those assumptions and the said proposal.

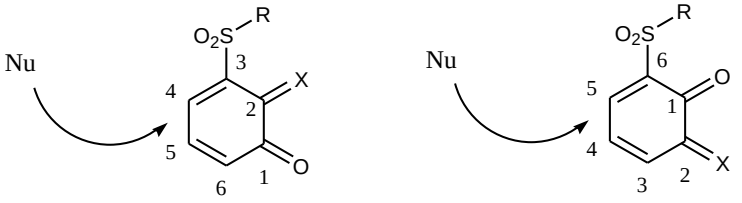
Yet, it seems that oxidation of **7** (electrochemical or chemical) may lead to an unstable species which does not lie enough to yield a phenoxazinone dye as a major product.

## 5.10 Observed regioselectivity

To close this section, as we made the assumption of a nucleophilic addition of a reduced aminophenol upon an electrophilic quinonic species, we will disclose here several trends which may account for the observed selectivity. Those trends are related to the 1,4-Michael addition process in organic solvent and may partially explain the regioselectivity. It has to be kept in mind that the reaction likely occurs in buffered aqueous media which is uncommon for most of the mechanistic investigations made regarding the 1,4-Michael addition.

Two regioisomeric iminoquinone intermediates could be produced by the oxidation of our aminophenol precursors (see Figure 23). These molecules likely behaved as good Michael acceptors and thus likely reacted readily with the aniline function of the substrates (Ar-$NH_2$ being more nucleophilic than Ar-OH). The experimentally observed chemoselectivity for the nucleophilic attack was in favour of the positions C4 and C5 respectively, for the intermediates I and II. This could suggest that the electron-withdrawing effect of the $SO_2R$ substituent is the determining factor. Otherwise, reactions at position C5 of isomer I and position C4 of isomer II could occur for steric reasons (less hindered positions) or electronic reasons depending on the protonation state of the iminoquinones (activation order: $H_2N^+$= > O= > HN=).

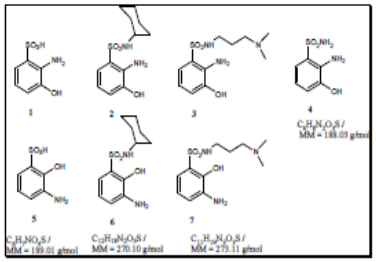
Figure 23. Iminoquinone intermediates of regioisomers I and II



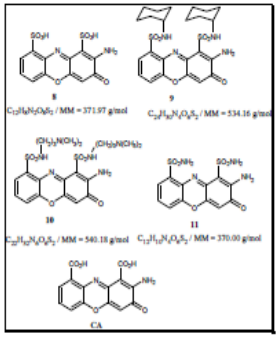

(I) X = NH or $NH_2^+$ (II)

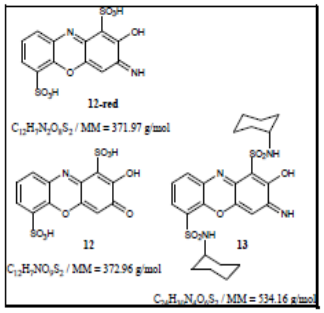
BOOKMARK RELATED TO CHAPTER 5: COMPENDIUM OF SUBTSRATES AND DYES MENTIONED IN THE CHAPTER 5:

***SUBSTRATES***

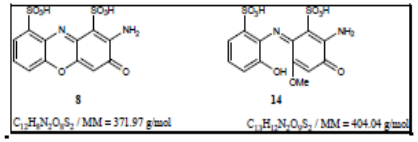


***ISOLATED PHENOXAZINONES "HEAD to HEAD" and "HEAD to TAIL" (SEE CHAPTERS 3 & 4)***

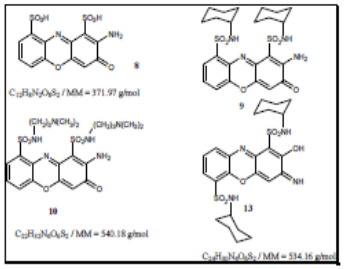




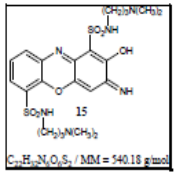

***PHENOXAZINONE & BY-PRODUCT ISOLATED FROM CHEMICAL OXIDATION WITH $Ag_2O$ ( from substrate 1)***



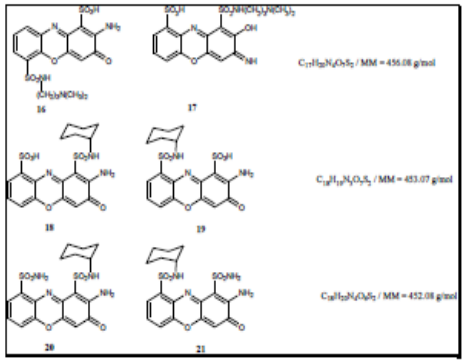

***PHENOXAZINONES IDENTIFIED BY HPLC-PDA & HPLC-MS IN CHEMICAL OXIDATION***

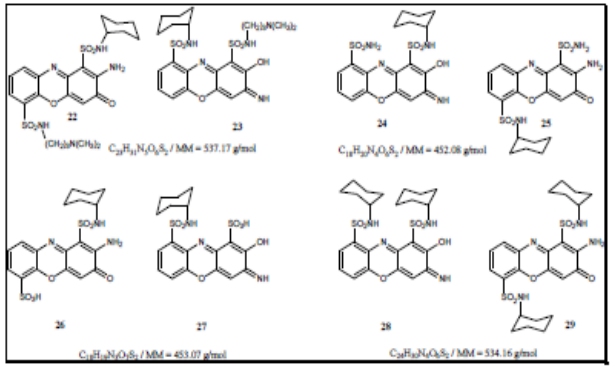



***PHENOXAZINONE IDENTIFIED BY HPLC-MS IN PHOTO-OXIDATION***

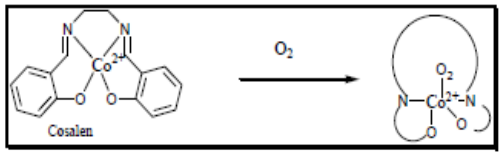


## *IDENTIFIED PHENOXAZINONES IN CROSS-COUPLING REACTIONS (METHOD OF IDENTIFICATION, HPLC-PDA & HPLC-MS²)*

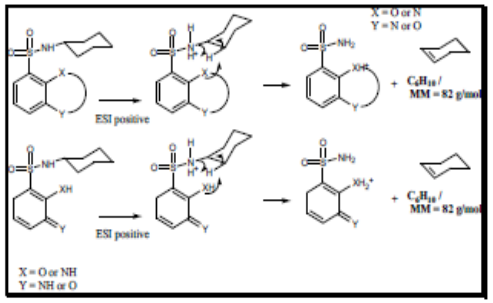




*Co(Salen) catalyst*




*Fragmentation Pattern specific to phenoxazinones bearing cyclohexyl*



# CHAPTER 6. LIVE CELL IMAGING

## 6.1 Foreword and topic addressed in the publication

In a precedent chapter, we disclosed the proof of concept of our strategy towards aminophenoxazinone dyes featuring tuneable-water solubility. Afterwards, we developed a small library of substrates and investigated the scope and limitations of our biocatalytic process. We were rewarded with the efficient syntheses of several novel dyes from which two compounds could be crystallised proving unambiguously the exact structures of the new dyes. Moreover, discrepancies observed for few substrates stimulated to the investigation of the mechanism of the laccase-mediated oxidative cyclisation.

Logically, we chose to investigate the biocatalysed synthesis of 1,9-diphosphorylated aminophenoxazinone dye which, to our knowledge, has never been reported in the literature. Hereafter, the efficient production of the first phosphorylated dye, soluble in aqueous medium at $10^{-2}$ M, is reported.

We have mentioned in the introduction the potential of actinocin derivatives, if water-soluble, for the fluorescent labelling in the field of cell biology. We had the opportunity to evaluate the suitability of our dyes (sulfonylated and phosphorylated) in the labelling of living cells, by mapping one of the most studied cellular processes, i.e fluid-phase endocytosis. Therefore, the relative quantum yields of our dyes were first determined. One sulfonated phenoxazinone (R = NH-$CH_2$-$CH_2$-$CH_2$-$NMe_2$) dyes appears particularly promising as a presumably bulk endocytic tracer with targeting to lysosomes by default. In addition, the novel phosphorylated dye proves to be an excellent chelating compound of at least two divalent cations.

## 6.2 Full Paper

***Journal***

The publication will be submitted to the journal ChemBioChem.

***Title***

Live-Cell Imaging with Water-Soluble Aminophenoxazinone Dyes Synthesized Through Laccase Biocatalysis.

***Authors***

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## Keyword

Biocatalysis • Dyes/Pigments • Fluorescent Probes • Endocytosis • Lysosomes.

***Abstract***

Aminophenoxazinone dyes featuring variable water solubilities were assayed for the first time in a live-cell imaging application. Among a library of ten sulfonylated chromophores, one compound gave excellent results as endocytic tracer. This compound led to a precise subcellular distribution. The compound was compared to four commercial vital tracers, including Lucifer Yellow. The first laccase-mediated regioselective synthesis of a diphosphorylated 2-aminophenoxazinone dye was also described. This compound, water-soluble at $10^{-2}$ M, displayed modest fluorescence properties and the ability to complex $Mg^{2+}$ and $Ca^{2+}$ cations, giving fluorescence quenching.

***Introduction***

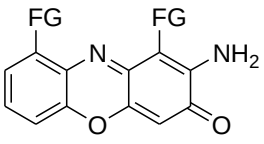
Fluorescent organic dyes are widely used as markers in the field of cellular and molecular biology and in biomedical research.[1,2] Such non radioactive probes allow both the labelling of biomolecules of interest and the imaging of cell physiology.[3,4] Indeed, sub-cellular distribution, co-localisation and specific interactions of biological molecules are of central importance. All these aspects are particularly relevant to the study of human disorders related to a defect in the endo-lysosomal system.[5-7] On a more applied perspective, live cell imaging using endocytic tracers may be proposed as potential applications whenever one desires to develop a new class of

fluorophores for cell biology. Whereas numerous available synthetic fluorescent dyes exhibit high quantum yields, narrow emission bands up to the near-infrared and good photostability, poor water-solubility is a severe drawback for most of them.[1] Improved water-solubility is obtained commonly through the insertion of ionisable hydrophilic groups into the fluorophore core structure, or by dye conjugation to biopolymers such as carbohydrates.[8,9] In the former strategy, functions such as sulfonic, and/or ammonium groups are the most popular ones and have been widely used in the case of benzo[a]phenoxazine and xanthene dyes leading to several families of water-soluble fluorophores endowed with high quantum yields.[10,11] The classical synthetic pathway towards benzo[a]phenoxazine dyes involves a nitrosative condensation which is poorly efficient with sulfonated building blocks.[12] This only improves solubility at the latest stage of the chemical synthesis and thus considerably limits the diversity of novel probes libraries.

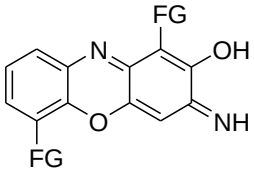
Aminophenoxazinone dyes and pigments are known for several decades, most of them being of natural sources.[13] Their potential use as fluorescent probes has been mainly prevented by a low water-solubility and the absence of a general methodology for the production of diversely substituted phenoxazinone scaffolds.[14] Recently, our group reported a novel synthetic approach, based on the use of a laccase as biocatalyst, that showed promising results for the "green" production of phenoxazinone dyes featuring tuneable water-solubility.[15] From a pool of diverse benzenesulfonyl derivatives as substrates, the last synthetic step corresponds to a reaction of cyclising oxidative dimerisation conveniently performed at room temperature, in aqueous solution, and with air as oxidant.

Herein, we report an extension of this methodology by the production of the first phosphorylated aminophenoxazinone dye. This water-soluble compound, along with the previously prepared series of sulfonylated phenoxazinone dyes (see Figure 1 for general core structures) were further studied for their optical properties and screened for their potential in fluorescence live cell imaging. The best candidate (the sulfonylated compound **8c**) was finally characterized as an endocytic tracer of the degradative pathway, by reference to known fluorescent tracers targeted to endosomes, lysosomes or the Golgi complex, using double-labelling experiments.

Figure 1. General structures (I) and (II) of studied fluorescent dyes. FG = functional group responsible of the solubility properties




I




II

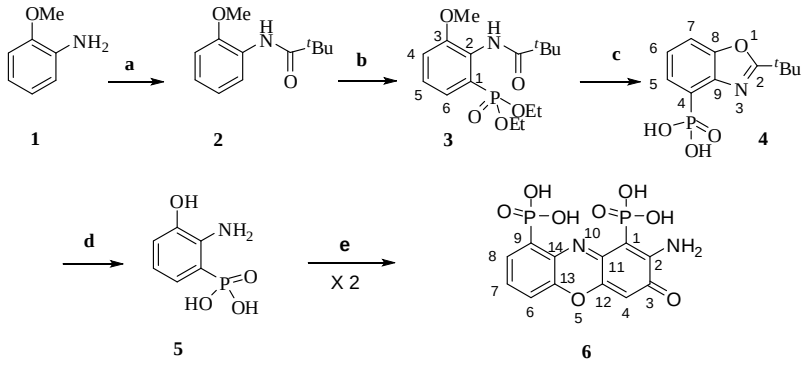
## *Results and discussion*

### *Chemistry*

From a synthetic point of view, the Michaelis-Arbuzov reaction was the traditional method when phosphorylated aromatic dyes had to be produced.[16] Yet this strategy implies the regioselective synthesis of aromatic halides as starting materials. The directed-metallation reaction has proven to be an alternative method of choice to insert selectively an oxidized phosphorous group in organometallic chemistry.[17] Based on a synthetic approach previously used by our group for selective aromatic sulfonation, we have taken advantage of the insertion of a lithiated intermediate into a P-Cl bond leading to the corresponding phosphorylated precursor in good yield and complete selectivity.

Next, a laccase was used as catalyst to promote the oxidative cyclisation of the substrate into the first diphosphorylated 2-aminophenoxazinone dye produced as a single regioisomer. The synthetic pathways for the efficient synthesis of our phosphorylated building block and its dimerisation are outlined in Scheme 1.

Scheme 1. Synthesis of aminophenoxazinone **6**. See experimental section for reagents and conditions.




Briefly, commercial *o*-anisidine (**1**) was quantitatively protected (step a) as *N*-pivaloyl derivative (**2**). The crude intermediate was submitted to regioselective lithiation due to the slight difference in directing ability in favour of the *N*-pivaloyl function over the *O*-methyl ether. Using 2.4 equivalents of *n*BuLi in the presence of TMEDA (tetramethylethylenediamine) as activating agent, the lithiated aromatic intermediate was produced in situ and then reacted with 2.4 equivalents of diethyl chlorophosphate (step b) to give the compound **3** with a good yield of 70% after chromatography. Next the methyl ether function was cleaved using an excess of boron reagent in dichloromethane (step c). After usual work-up, pure benzoxazole compound **4** was obtained in 78% yield after precipitation in diethyl ether. Interestingly, spontaneous intramolecular cyclisation was observed here, in the same way as previously described for the sulfonated analogue.[15a]

This original 2-*tert*-butyl-4-phosphonic acid benzoxazole was hydrolysed in a 12 N HCl solution at reflux (step d) to give the precursor **5** in a 70% yield, as a white powder, after concentration and precipitation.

To date, there is no example of a laccase-mediated oxidative coupling using organic phosphorylated compounds as substrates. Sulfonic and phosphonic groups being considered as bioisosters of the carboxylic function,[18] the previous conditions of our laccase-catalysed synthesis in water were applied to compound 5. In presence of a catalytic amount of laccase in an aqueous buffer at pH 6, substrate 5 was converted at room temperature (step e) into the corresponding 1,9-disubstituted phenoxazinone dye 6 as a >95% pure compound with an excellent yield of 90% after isolation. Briefly, the reaction was monitored by HPLC until complete conversion of 5; next the laccase was inactivated by adding 40% (v/v) of methanol and concentration with rotary evaporator before freeze-drying of the remaining solution. The residual red powder was fully characterised by HRMS (high resolution mass spectrometry), IR, NMR ($^{1}$H and $^{13}$C) and UV-visible spectroscopies. The novel *bis*-phosphorylated dye 6 is highly water-soluble: solutions of 6 up to a concentration of $10^{-2}$ M can be prepared in PBS or pure water. In methanol the highest concentration reached is about 5 x $10^{-4}$ M. Syntheses and structural elucidations of the related *bis*-sulfonylated dye 7 and derivatives 8a-g and 9 (see Table 1) were previously reported.[15b]

***Absorption and fluorescence properties***

The photophysical properties of the aminophenoxazinone dyes featuring tuneable water-solubility, depending on the nature of the FG substituents,[15b] were evaluated under simulated physiological conditions i.e in phosphate-buffered saline solution (0.15 M), pH 7.4.

The UV-visible spectra of compounds **6-9** showed absorptions between 420 and 450 nm with ε values (ε = Molar coefficient extinction) ranging from 480 to 12,600 $M^{-1}$ $cm^{-1}$ (Table 1 and Figure 1). Data collections were performed at concentrations ranging from 5 x $10^{-4}$ M to 1 x $10^{-6}$ M to ensure full solubility of all compounds. Rather low ε values were obtained for dyes **8a, 8d** and **9** whereas ε values for **8c**, **8e** and **8f** were slightly higher. The highest ε values were recorded for the dyes **6**, **7**, **8b** and **8g**. Still the absorbance for these newly synthesized compounds was 10- to 100-fold lower than for a classical benzo[a]phenoxazine dye (ε = 42.4 x $10^3$ $M^{-1}cm^{-1}$).[10a]

**Table 1.** UV-Vis absorption data of dyes **6-9** [a]

| Cpd | FG [b] | nnucleus[b] | ε x $10^3$ ($M^{-1}cm^{-1}$) | $\lambda_{max}$ (nm) [c] |
|---|---|---|---|---|
| **6** | $PO_3H$ | I | 8.80 | 241, <u>438</u> |
| **7** | $SO_3H$ | I | 9.80 | 240, 418, <u>441</u> |
| **8a** | $SO_2NH_2$ | I | 4.04 | 240, 436, <u>451</u> |
| **8b** | $SO_2NH$-cyclohexyl | I | 10.80 | 236, 424, <u>456</u> |
| **8c** | $SO_2NH(CH_2)_3N(Me)_2$ | I | 7.20 | 239, 422, <u>442</u> |
| **8d** | $SO_2NH$-phenyl | I | 0.48 | 240, <u>430</u> |
| **8e** | $SO_2NH(CH_2)_2NH_2$ | I | 6.02 | 240, 423, <u>442</u> |
| **8f** | $SO_2NHCH_2CO_2Me$ | I | 5.90 | 238, 422, <u>442</u> |
| **8g** | $SO_2NH(CH_2)_2CH_2OH$ | I | 12.60 | 240, 420, <u>440</u> |
| **9** | $SO_2NH$-cyclohexyl | II | 3.95 | 238, 420, <u>430</u> |

[a] Measured in PBS at pH value of 7.4; [b] See Figure 1; [c] The underlined value corresponds to the given ε (Molar coefficient extinction).

Next, the fluorescence properties of the functionalised aminophenoxazinone dyes **6-9** were evaluated at concentrations from 5 x $10^{-5}$ to 1 x $10^{-4}$ M using rhodamine 6g as reference (see Supporting Information; $\Phi_F$ (Quantum yield) = 0.95) results are summarised in Table 2.

**Table 2.** Fluorescence data of dyes **6-9** [a]

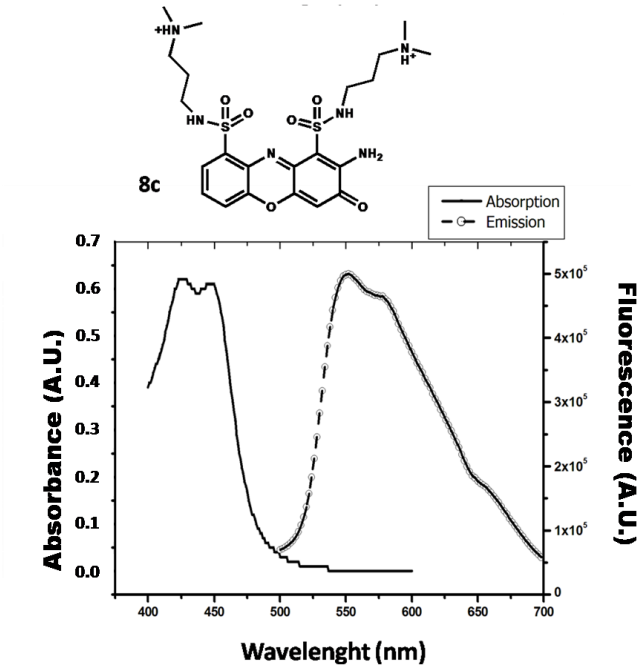
| Cpd | $\lambda_{exc}$ (nm) | $\lambda_{em}$ (nm) | $\Phi_F$ | Stokes shift |
|---|---|---|---|---|
| **6** | 488 | 500-650 | 0.003 | 110 nm |
| **7** | 488 | 500-600 | 0.001 | ~100 nm |
| **8a** | 488 | 520-680 | 0.009 | 122 nm |
| **8b** | 488 | 520-680 | 0.007 | 130 nm |
| **8c** | 488 | 500-700 | 0.017 | 116 nm |
| **8d** | 488 | 570-620 | 0.0004 | ~100 nm |
| **8e** | 488 | 500-700 | 0.007 | 146 nm |
| **8f** | 488 | 500-700 | 0.014 | 146 nm |
| **8g** | 488 | 500-700 | 0.005 | ~130 nm |
| **9** | 488 | 520-650 | 0.002 | ~100 nm |

[a] Measured in PBS (0.15 M) at pH 7.4

Dye concentrations were adjusted to yield an identical optical density of 0.06 in PBS. Firstly, emission spectra after excitation at $\lambda$ = 488 nm were recorded between 500 and 700 nm. All dyes exhibited some fluorescence, albeit with a rather low intensity. Clear emission maxima were observed around 560 nm while some dyes such as **7**, **8d**, **8g** and **9** emitted broadly from 500 to 650 nm without clear maxima. Similarly, relative quantum yields measured by reference to rhodamine 6g were in general rather low, with 10 to-100 fold difference in comparison to Lucifer Yellow (LY; $\Phi_F$ = 0.21, see Supporting Information).[19]

Two dyes, **8c** and **8f**, emerged as most promising based on this criterium, with quantum yields close to 0.02. Dyes **6**, **8a-c** and **8e** exhibited a large Stokes' shift, from 110 to almost 150 nm, depending on the functional groups inserted in the core structures (see Figure 2, example of **8c**). Compounds bearing sulfonylated side chains were always more efficient than the parent sulfonic acid **7**, whereas compound **6** bearing free phosphonic acids still gave a quite significant Stokes' shift. Hydrophobicity did not seem to play a major role either. Whereas both dyes **6** and **7** exhibited similar molar extinction coefficients, a puzzling difference in emission spectra was observed.

Figure 2. Absorption (solid line) and fluorescence spectra (dashed line) of **8c** in PBS at pH 7.4. **8c** at 5 x $10^{-5}$ M for absorption (left scale A.U. = arbitrary unit of absorbance) and emission (right scale, A.U.= arbitrary unit of fluorescence).




Although it is the first time that such heterocyclic chromophores are available for testing in biological media, it may seem brave to apply dyes **6-9** in fluorescence labelling experiments, given their rather limited quantum yield. Nevertheless, a first screen by live cell imaging was undertaken using CHO (Chinese Hamster Ovary) cells after 1 h of incubation at 37°C with the entire series of phenoxazinone dyes at 1 mg.mL$^{-1}$, except compound **8d**.

The results of this visual screening are summarised in Table 3. It immediately appeared that the significant hydrophobicity of compounds **8b** and **9** caused massive aggregation, preventing any application to biological systems. Despite the rather high final ethanol concentration in this screening, overall cell did not appear to be appreciably altered.

**Table 3.** Preliminary Live-cell imaging results, (**6**-**9**, ~ 5 X $10^{-3}$ M)[a]

| Cmpd | Fluorescence | Location | Aggregate | Cells state[b] |
|---|---|---|---|---|
| **6** | Null | - | - | - |
| **7** | Low | - | - | Good |
| **8a** | Low | Intracellular | - | Good |
| **8b** | High | Extracellular | Massive | Stressed |
| **8c** | Medium | Intracellular | - | Good |
| **8e** | Low | Intracellular | Small | Good |
| **8f** | Medium | Intracellular | - | Good |
| **8g** | Medium | Intracellular | - | Good |
| **9** | Null | Extracellular | Massive | Stressed |

[a] Excitation at 488 nm, Emission filter at 526-532 nm. Transmission at 5%; detection in the green channel; [b] In some cases, cells showed marked signs of stress. Due to the variable water solubility of dyes, final concentrations of ethanol were ranging from 0.5 to 5% for **8a**, **8b**, **8e**, **8f**, **8g**, **9**; below 0.5% for **6**, **7** and **8c**.

The candidate selected for further imaging was the water-soluble compound, **8c**, which also offers the highest quantum yield. Furthermore, its intracellular distribution in well-defined spots readily pointed to its potential as endocytic tracer. The puzzling result observed with compound **6** seemed a priori quite disappointing. Such absence of fluorescence while even compound **7** could be detected in the experiments led us to consider as an artefact the result recorded with **6**. Bearing in mind the well known ability of phosphonate groups to chelate divalent cations, we envisaged a quenching of fluorescence due to the presence of $Mg^{2+}$ and $Ca^{2+}$ ions in the CHO medium. Such loss of fluorescence due to the chelation of either $Mg^{2+}$ and/or $Ca^{2+}$ was indeed confirmed independently (see Supporting Information). Whereas **6** (4.6 mM) fluoresced in pure water and phosphate-buffered saline (PBS, 0.15 M NaCl, 0.8 mM $PO_4^{3-}$), quenching was complete in the presence of PBS doped with $Ca^{2+}/Mg^{2+}$ (3 mM) and similarly in aqueous solutions of the cations (3 mM).

***Subcellular localisation of compound 8c***

Several endocytic pathways, all based on membrane-bound vesicles pinching-off from the plasma membrane, allow bulk or selective internalisation of extracellular, membrane impermeant compounds.[5] Bulk (i.e. fluid-phase or non-specific adsorptive) endocytosis allows to simultaneously explore the various internalization portals and intracellular endocytic routes.[7] The endo-lysosomal system comprises early, sorting, late and recycling endosomes, secondary lysosomes and the trans-Golgi network. Whereas early endosomes are primarily involved in sorting internalized cargo and membrane, recycling endosomes ensure the homeostasis of the plasma membrane.[20] Lysosomes are usually the final destination where internalized macromolecules are sequestered and degraded. A large variety of fluorescent probes are available to analyze this complex system.[21]

Some of these, such as Lucifer Yellow (LY), are widely used as tracers for fluid-phase endocytosis.[22] Other fluorescent dyes show intrinsic properties that permit the selective labelling of well-defined intracellular compartments such as the recycling pathway (transferrin),[23] lysosomes,[24] or the Golgi complex, such as BODIPY-ceramide.[25]

To rule out diffusion as a mechanism contributing to intracellular labelling by compound **8c**, two classical endocytic controls were carried out: energy depletion and low-temperature block. As shown by Figure 3, cells incubated with 1.5 mM compound **8c** for 45 min in serum-free medium showed multiple intracellular dots, with preferential clustering around the nucleus. In contrast, neither ATP-depleted cells exposed to compound **8c** at 37 °C, nor untreated cells incubated with this compound at 4°C showed any fluorescence. The integrity of the plasma membrane was verified by simultaneous labelling with CellTracker Red, a fluorescent membrane-permeant probe which becomes membrane-impermeant after conjugation with glutathione (see Supporting Information S2 for chemical structure).

We next analysed the fate of internalized compound **8c**, by reference to vital tracers of recycling endosomes (Alexa568-transferrin), the Golgi complex (BODIPY-ceramide, a metabolic precursor of BODIPY-sphingomyelin) and LysoTracker Red (which, like chloroquine and acridine orange, is membrane-permeant in its non-protonated form and becomes sequestrated upon protonation in acidified closed compartments). As shown by Fig 4, compound **8c** fully segregated from recycling endosomes and the Golgi complex and was exclusively targeted to late endosomes/lysosomes.

Figure 3. Evidence for endocytic uptake of compound **8c**. Control (a, b, e, f) or ATP-depleted CHO cells (c, d) were incubated for 45 min with 1.5 mM compound **8c** at 37°C in control (a, b) or ATP-depleting medium (c, d); or at 4°C in the presence of 10 µM CellTracker, for global cell imaging (e, f). Figure shows dark-field fluorescent images (a, c, e), merged with transmission images (b, d, f). In the upper panel, control cells showed bright intracellular punctate labelling, mainly perinuclear (arrowheads). Boxed area at (a) is enlarged in the inset. As shown by the central and lower panels, ATP-depletion (c, d) or incubation at 4°C (e, f) abrogate green intracellular labelling. At f, integrity of the plasma membrane is demonstrated by cytosolic labelling with CellTracker Red. These experiments demonstrate that **8c** accumulates in cells by a temperature-and energy-dependent mechanism, consistent with endocytosis. (N, nucleus). Scale bar, 10 µm.

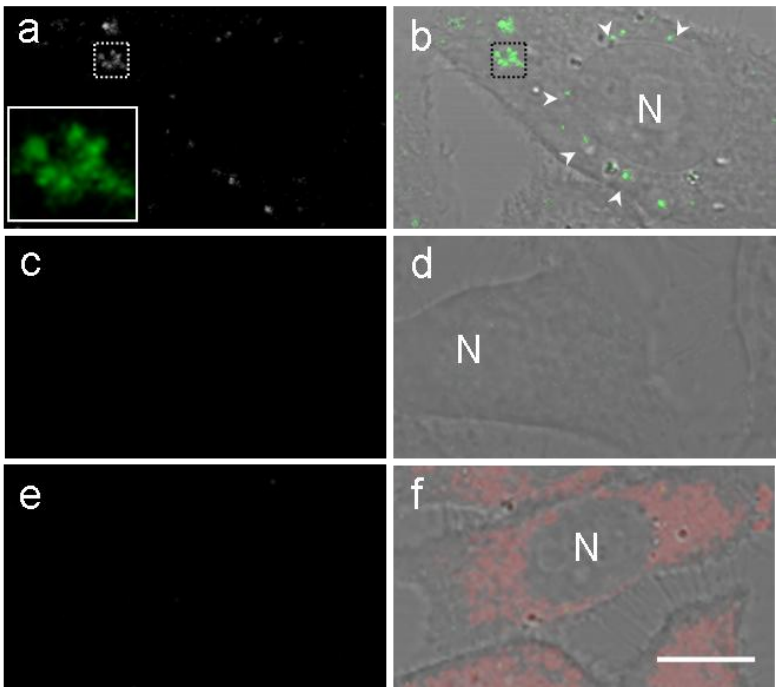
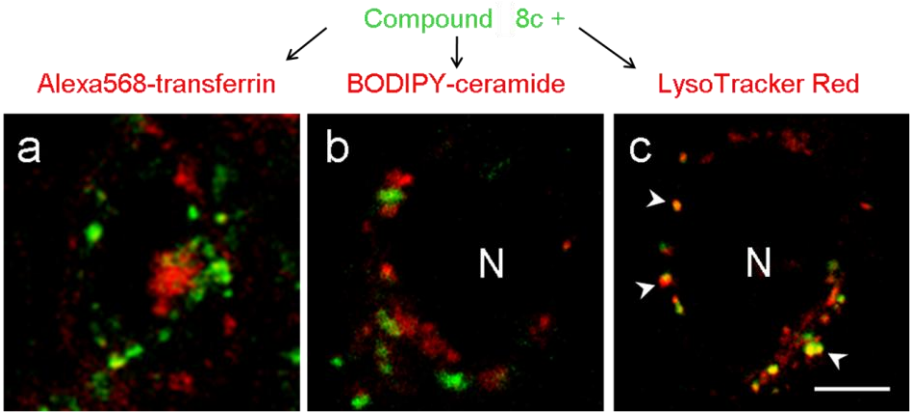
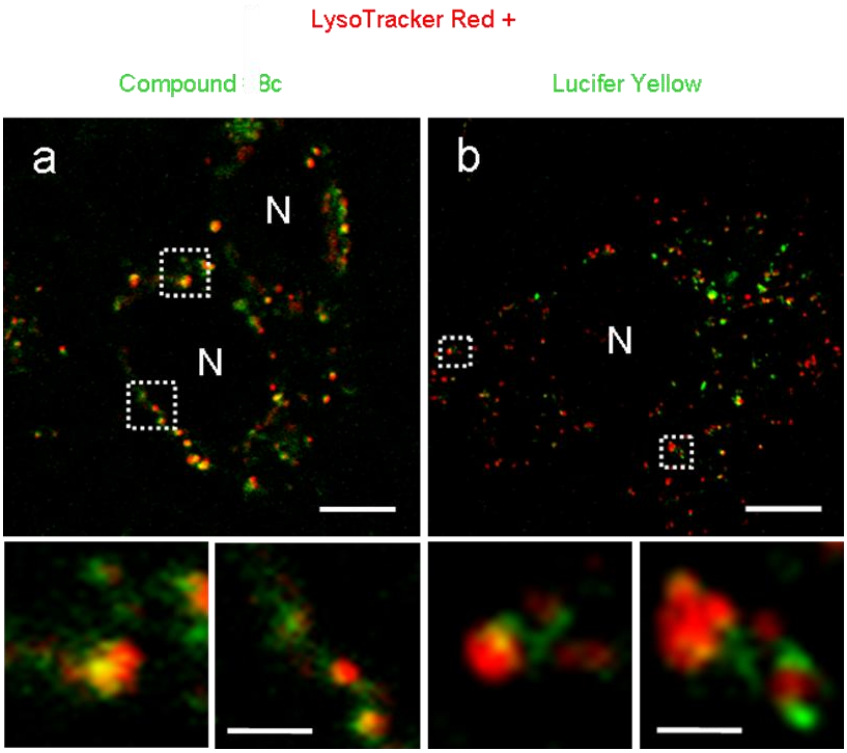


Figure 4. Internalized compound **8c** is targeted to late endosomes/lysosomes. CHO cells were incubated with **8c** at 37°C for 45 min together with (in red) 0.7 µM Alexa 568-transferrin (a, tracer of recycling endosomes), 5 µM BODIPY-ceramide (b, vital stain of the Golgi complex) or 50 nM LysoTracker (c, to label late endosomes/lysosomes by acidotropic sequestration). Notice that internalized **8c** segregates from recycling endosomes and the Golgi complex and is exclusively targeted to late compartments of the degradative pathway. (N, nucleus). Scale bar, 10 µm.




Finally, to test whether compound **8c** qualified as a bulk endocytic tracer, its endocytic trafficking was compared with that of Lucifer Yellow. Interestingly, although compound **8c** is a cationic species, due to protonation of its tertiary amino groups, whereas the bisulfonated compound Lucifer Yellow is an anionic species, both endocytic tracers labelled similar structures and apparently progressed towards lysosomes at a comparable kinetics (Figure 5).

Figure 5. Despite bearing opposite net charges, compound **8c** and Lucifer Yellow are targeted to late endosomes/lysosomes by default. CHO cells were incubated at 37°C for 45 min with 50 nM LysoTracker Red together with 1.5 mM compound **8c** (left) or 2.2 mM Lucifer Yellow (right), briefly washed and studied by live confocal microscopy. Optical sections at the level of the nucleus (N) are shown. Boxed areas at a,b, enlarged 10-fold below, suggest that pleiomorphic late endosomes, filled by endocytosis of the membrane-impermeant tracers, **8c** or Lucifer Yellow, dock with lysosomes concomitantly labelled by acidotropic sequestration of the membrane-permeant probe, LysoTracker Red is shown in Figure 4, fusion is expectedto follow shortly after. Scale bars; upper panel, 10 µm; lower panel 1 µm.



***Conclusion***

We here provide the first report on a series of water-soluble aminophenoxazinone dyes and of its evaluation for vital imaging in cell biology. These heterocyclic compounds were produced using an enzymatic synthetic strategy, as an alternative to the nitrosative or chemical oxidative coupling of phenolic precursors. Thus, the first 1,9-*bis*-phosphorylated aminophenoxazinone dye (**6**) has been synthesized with excellent yield by taking advantage of laccase-mediated cyclisation in water. The fluorescence quenching observed when this new dye **6** was mixed with $Mg^{2+}$ and/or $Ca^{2+}$ brought about a heavy handicap in the cell labelling application, but could lead to interesting developments as sensors for variations of divalent cation concentrations in the high range. Among the first generation of tested dyes, namely the sulfonylated compounds (**7**-**9**), one dye (**8c**) appears particularly promising as a presumably bulk endocytic tracer with targeting by default to lysosomes. Despite a 10-fold lower quantum yield as compared with Lucifer Yellow at neutral pH, compound **8c** generated intracellular signals of comparable intensity. However, it remains to be tested whether this useful "compensation" for an endocytic tracer could be ascribed to a difference the emission of **8c** in acidic pH or to the benefit of membrane adsorption due to charge complementarity with the cell surface glycocalyx. Indeed, these differences could be further used to analyse the endocytic apparatus. While the other sulfonylated water-soluble chromophores (**7**-**9**) suffer from much lower quantum yields, they may eventually reveal interesting properties yet to be explored. Finally, all these dyes feature at least one free reactive function for further conjugation to biomolecules and grafting to materials, which opens the route towards selective labelling experiments.[26]

***Experimental Section***

**General methods.** $^{1}H$, $^{13}C$ and $^{31}P$ NMR spectra were recorded with a Bruker Avance 500 or a Bruker Avance 300 spectrometer. Spectra were obtained in [$D_6$]DMSO, [D]chloroform, [$D_2$]water or [$D_4$]methanol. Chemical shifts are reported in ppm relative to TMS.[27] $^{13}C$ NMR were obtained with broadband proton decoupling (D1 = 5 sec) or DEPTQ. Low resolution mass spectra were acquired using a Thermo Finnigan LCQ spectrometer, either in positive mode ESI, or negative mode ESI. High Resolution Mass Spectrometry (HRMS) analysis using ESI or FAB methods were performed at the University of Mons Hainaut (Belgium) or at the University of Oxford (UK). Melting points were recorded with an Electrothermal apparatus calibrated with benzoic acid. Infrared spectra were recorded with a Shimadzu FT-IR 84005 spectrometer using KBr plates. The HPLC system consisted of Waters Alliance 2699 separation module, Waters 2998 photodiode array detector; analytical separations were performed with Waters XterraMSC18 column (4.6 × 100 mm, 5 µm) (Waters, Milford, Massachusets, USA) equipped with a conventional pre-column. Detection was performed at 220/254/280 nm with control at 440/460 nm and on-line UV-Visible absorbance scans were performed. Flow rate was 1 $mL.min^{-1}$. Initial conditions were ACN 100% for 5 min, then hyperbolic gradient toward 75/25 of solvents A and B, respectively water/$HCO_2H$ (100/0.1 v/v) for A and ACN/$HCO_2H$ (100/0.1 v/v) for B in 5 min, then hyperbolic gradient to 25/75 of A/B in 10 minutes. Then, run ends by returning to initial conditions in 5 minutes. Injection volume was of 10 µL.

**Materials.** Lucifer Yellow (LY) was purchased from Sigma. Other commercial fluorescent probes, namely Tfn-Alexa 568, ceramide-BODIPY-589, Cell-Tracker-Red and LysoTracker-Red were obtained from Invitrogen. Laccase from Trametes versicolor was purchased as a brownish powder form Bioscreen e.K. (Uebach-Palenberg, Germany) under the commercial trademark Oxizym LA (batch n° LA 2000/001).

**Fluorescence measurements.** UV-Vis spectra were recorded on a UV-Vis-NIR Varian-CARY spectrophotometer (λ given in nm). Emission fluorescence spectra were recorded on a Fluorolog®-3 spectrofluorometer (Jobin Yvon Horiba). The excitation spectra (emission at 500-700 nm, window of 526-534 nm used for $\Phi_F$) and emission spectra (excitation at 488 nm) of aminophenoxazinone dyes were determined in phosphate-buffered solution, pH 7.4 in a range of concentrations around $10^{-5}$ M (optical density of 0.06). The ε values were also obtained in phosphate-buffered solutions. The fluorescence intensities in pure water were measured from the corrected emission curve using the area function of the fluorometer. Relative fluorescence quantum yields were obtained by using Rhodamine 6g (ΦF = 0.95) as the reference.

**Quenching of fluorescence.** Fluorescence experiments of dyes **6**, **7**, **8a**, **8e**, **8g** in presence of $Mg^{2+}$ (3 mM) and/or $Ca^{2+}$ (3.6 mM) either in PBS or pure water were realised in triplicate using a 96-well plate. Fluorescence was recorded using a HP Fluorocount mounted with excitation filter at 488 nm and emission filter at 530 nm. Blanks were recorded with and without dyes in PBS or water. A quenching phenomenon was observed only for dye **6**.

**Cell culture and labelling.** CHO cells were propagated in DMEM/F-12 supplemented by 10% fetal calf serum and antibiotics as described elsewhere.[28] For experiments, cells were seeded at ~20,000/$cm^2$ on Lab-Tek chambers (Nunc, Roskilde, Denmark) and grown till ~ 90% confluency (2 days). For the primary screening, probes were dissolved in ethanol (20 $mg.mL^{-1}$), then diluted at 1 $mg.mL^{-1}$ in PBS. For the analysis of subcellular localisation of dye **8c**, this probe was dissolved from a stock solution at 200 $mg.mL^{-1}$ in ethanol into DMEM containing 1 $mg.mL^{-1}$ defatted BSA to prevent adsorption to the culture vessel, to a final concentration of 1 $mg.mL^{-1}$ (1.5 mM). For the comparison with the Lucifer Yellow, both compounds were presented in 0.5 % ethanol, final concentration.

**Live cell imaging by confocal microscopy.** Extensively washed cells were examined using a Zeiss LSM510 confocal microscope equipped with an incubation chamber set at 37°C (Zeiss; Incubator XL/LSM) and a 63x immersion oil, NA 1.4 objective. Images were recorded sequentially in the green then red channels. For commercial probes, laser was set at 2-5% (detector gain, 700; amplifier offset, -0.5). For aminophenoxazinone probes, laser was set at 4-8% (detector gain, 750; amplifier offset, -0.5).

***Organic Synthesis.***

***N*-(2-Methoxy-phenyl)-2,2-dimethyl-propionamide (2)**: To a solution of *o*-anisidine **1** (5.460 g; 5.00 mL; 44.33 mmol) in ethyl acetate/water (130/150 mL/mL) were added $NaHCO_3$ (3.0 eq; 11.173 g) and pivaloyl chloride (1.1 eq; 600 µL). The solution was stirred at room temperature for 6 h. The layers were separated and the organic layer was washed with aqueous 1 N HCl. The organic layer was dried over $MgSO_4$ and evaporated. Title compound was obtained with a quantitative yield (10.860 g). $^1$H NMR (300 MHz; [D]chloroforme): δ = 1.31 (s, 9H, *t*Bu), 3.87 (s, 3H, $OCH_3$), 6.85 (dd, 1H, $^3J$(H-H) = 7.9

Hz, $^{4}J$(H-H) = 1.8 Hz, $H_6$), 6.94 (td, 1H, $^{3}J$(H-H) = 7.6 Hz, $^{4}J$(H-H) = 1.8 Hz, $H_4$), 7.01 (td, 1H, $^{3}J$(H-H) = 7.9 Hz, $^{4}J$(H-H) = 1.8 Hz, $H_5$), 8.13 (s,1H, NH amide), 8.41 ppm (dd, 1H, $^{3}J$(H-H) = 7.6 Hz, $^{4}J$(H-H) = 1.8 Hz, $H_3$); $^{13}$C NMR (75 MHz; [D]chloroforme): δ = 27.6 ($C(\underline{CH_3})_3$), 40.0 ($\underline{C}(CH_3)_3$), 55.8 ($O\underline{CH_3}$), 109.8 (C6), 119.5 (C3), 121.1 (C4), 123.4 (C5), 127.9 (C2), 148.0 (C1), 176.5 ppm (CO amide).

**1-Diethoxyphosphoryl-2-(2,2-dimethyl-propionylamino)-3-methoxy-benzene (3):** The glassware was flame-dried under argon atmosphere. To a solution of **2** (4.000 g; 19.30 mmol) in anhydrous THF (40 mL) was added dropwise TMEDA (tetramethyl ethylenediamine) (2.4 eq; 6.95 mL) and a 2.5 M solution of *n*BuLi in hexane (2.4 eq; 18.5 mL) at -10 °C. The addition lasted 5 minutes. The mixture was stirred at -10 °C for 15 minutes, then at room temperature for 3 h. At -10 °C, diethylchlorophosphate (2.4 eq; 6.72 mL) was added dropwise to the solution. The reaction mixture was stirred at -10 °C for 2 h then warmed to room temperature during 17 h. An aqueous solution of 1 N HCl was added and the mixture was extracted with ethyl acetate. The organic phase was washed with water, dried over $MgSO_4$ and evaporated. The red oil (7.920 g) was purified by column chromatography on flash silica gel (eluent: cyclohexane/ethyl acetate 1/1, then ethyl acetate). Two fractions were obtained. The first one is an orange oil corresponding to the starting compound **2** (0.727 g; Recovery = 18.2%; Conversion = 81.8%). The second fraction is a yellow solid corresponding to the title compound **3** (4.566 g; Yield = 68.9%). $^{1}$H NMR (300 MHz; [$D_4$]methanol): δ = 1.30 (m, 6H, $PO(CH_2\underline{CH_3})_2$), 1.32 (s, 9H, *t*Bu), 3.83 (s, 3H, $O\underline{CH_3}$), 3.99-4.12 (m, 4H, $PO(\underline{CH_2}CH_3)_2$), 7.31-7.36 (m, 1H, $H_5$), 7.41-7.46 ppm (m, 2H, $H_4$ and $H_6$); $^{13}$C NMR (75 MHz; [D]chloroform): δ = 16.3 (d, $^{4}J$(C-P) = 6.8 Hz, $PO(CH_2\underline{CH_3})_2$), 27.6 ($C(\underline{CH_3})_3$), 39.8 ($\underline{C}(CH_3)_3$), 56.3 ($OCH_3$), 63.6 (d, $^{3}J$(C-P) = 5.3 Hz, $PO(\underline{CH_2}CH_3)_2$),

117.7 (d, $^{4}J$(C-P) = 2.6 Hz, C4), 124.2 (d, $^{1}J$(C-P) = 182. 0 Hz, C1), 124.4 (d, $^{2}J$(C-P) = 6.8 Hz, C6), 127.2 (d, $^{3}J$(C-P) = 16.8 Hz, C5), 129.4 (d, $^{2}J$(C-P) = 6.1 Hz, C2), 155.4 (d, $^{3}J$(C-P) = 16.2 Hz, C3), 176.6 ppm (CO amide); $^{31}$P NMR (121 MHz; [$D_4$]methanol): δ = 18.55 ppm (s, $PO(OEt)_2$); MS (ESI) m/z (%): 344.18 (100) $[M+H]^+$; HRMS (ESI): Calcd = 366.1446 for $C_{16}H_{26}NO_5NaP$, found = 366.1434.

**2-*tert*-Butylbenzoxazole-4-phosphoric acid (4):** The glassware was flame-dried under argon atmosphere. To a solution of compound **3** (2.132 g; 6.21 mmol) in anhydrous dichloromethane (70 mL) was added dropwise at 0 °C a 1 M solution of $BBr_3$ in dichloromethane (3.0 eq; ; 18.63 mL). The mixture was stirred at 0 °C for 1 h, then at room temperature for 18 h. The mixture was cooled to 0 °C and water was added dropwise to quench the residual $BBr_3$. The solution was evaporated and the residue was filtered on RP-18 silica gel with methanol/water 1/1, then methanol. After evaporation, both residues were dissolved several times in diethyl ether; each time, the formed white solid was filtered and dried. The title compound was isolated with an overall yield of 77.9% (1.234 g). M.p. = 204-207 °C; $^{1}$H NMR (300 MHz; [$D_4$]methanol): δ = 1.51 (s, 9H, *t*Bu), 7.41 (td, 1H, $^{3}J$(H-H) = 7.8 Hz, $^{4}J$(H-P) = 3.2 Hz, $H_6$), 7.74 (d, 1H, $^{3}J$(H-H) = 7.7 Hz, $H_7$), 7.79 ppm (dd, 1H, $^{3}J$(H-H) = 7.50 Hz, $^{4}J$(H-P) = 14.3 Hz, $H_5$); $^{13}$C NMR (75 MHz; [$D_4$]methanol): δ = 28.7 (*t*Bu), 35.4 ($\underline{C}$-$(CH_3)_3$), 115.0 (C7), 124.4 (d, $^{1}J$(C-P) = 185.8 Hz, C4), 125.2 (d, $^{3}J$(C-P) = 14.6 Hz, C6), 128.8 (d, $^{2}J$(C-P) = 7.8 Hz, C5), 143.2 (d, $^{2}J$(C-P) = 6.7 Hz, C9), 152.5 (d, $^{3}J$(C-P) = 15.2 Hz, C8), 175.7 ppm (C2); $^{31}$P NMR (121 MHz; [$D_4$]methanol): δ = 11.02 ppm (s, $PO(OH)_2$); MS (ESI) m/z (%): 256.18 (100) $[M+H]^+$; HRMS (ESI): Calcd = 256.0739 for $C_{11}H_{15}NO_4P$, found 256.0736.

**2-Amino-3-hydroxy-benzenephosphoric acid (5):** A solution of **4** (0.410 g; 1.61 mmol) in 12 N HCl (mL) was heated at 100 °C for 24 h. The reaction mixture was cooled down to room temperature and evaporated. The residue was added with water and the remaining solid was dissolved in methanol/diethyl ether. After evaporation under vacuum, the title compound was obtained as a white solid (0.210 g) with a yield of 69%. M.p. = 202-206 °C; $^{1}$H NMR (300 MHz; [$D_6$]DMSO): δ = 6.42 (td, 1H, $^{3}J$(H-H) = 7.6 Hz, $^{4}J$(H-P) = 4.08 Hz, $H_5$), 6.74 (dd, 1H, $^{3}J$(H-H) = 7.41 Hz, $^{4}J$(H-H) = 1.2 Hz, $H_4$), 6.88 ppm (ddd, 1H, $^{3}J$(H-H) = 7.6 Hz, $^{4}J$(H-H) = 1.2 Hz, $^{3}J$(H-P) = 12.1 Hz, $H_6$); $^{13}$C NMR (75 MHz; [$D_6$]DMSO): δ = 115.1 (d, $^{1}J$(C-P) = 177 Hz, C1), 116.1 (d, $^{3}J$(C-P) = 14.7 Hz, C5), 116.2 (C4), 122.7 (d, $^{2}J$(C-P) = 6.9 Hz, C6), 138 (d, $^{2}J$(C-P) = 8.5 Hz, C2), 144.6 ppm (d, $^{3}J$(C-P) = 16.5 Hz, C3); $^{31}$P NMR (121 MHz; [$D_6$]DMSO): δ = 15.87 ppm (s, $PO(OH)_2$); MS (ESI) m/z (%): 190.14 (100) $[M+H]^+$; HRMS (ESI): Calcd = 190.0269 for $C_6H_9NO_4P$, found 190.0273.

**2-Amino-3-oxo-3H-phenoxazine-1,9-diphosphoric acid (6)**: A solution of **5** (1.06 mmol; 0.2 g) in 25 mL of MeOH was diluted to a final volume of 500 mL with 220 mL of water and 250 mL of 0.2 mM ammonium acetate buffer at pH 6. The reaction was started by adding 5 mL of a solution of laccase ($10^4$ U $L^{-1}$) and the stirred mixture was kept 24 h at 25 °C under air. The reaction was stopped by adding 40% v/v of methanol and concentrated under vacuum to a volume of 300 mL. The crude mixture was freeze-dried to give a red powder (0.2 g) with a yield of 90%. The isolated product was obtained with traces of acetate which were removed after repeated freeze-drying. HPLC retention time = 7.78 min. M.p. >280 °C (degradation); $^{1}$H NMR (300 MHz; [$D_6$]DMSO/[$D_2$]water 1/1): δ = 6.37 (s, 1 H, $H_4$), 7.41 (ddd, 1 H, $^{3}J$(H-H) = 7.0, 8.0 Hz, $^{4}J$(H-P) = 3.6 Hz, $H_7$), 7.49 (d, 1H, $^{3}J$(H-H) = 7.9 Hz, $H_6$), 7.63 ppm (dd, 1 H, $^{3}J$(H-H) = 7.0 Hz, $^{3}J$(H-P) = 11.7 Hz, $H_8$); $^{13}$C NMR (125 MHz, [$D_6$]DMSO/[$D_2$]water 1/1) δ = 103.7

(C4), 103.9 (d, $^{1}J$(C-P) = 171.2 Hz, C1), 117.2 (C6), 128.0 (d, $^{2}J$(C-P) = 6.8 Hz, C8), 128.8 (d, $^{3}J$(C-P) = 15.0 Hz, C7), 133.2 (d, $^{2}J$(C-P) = 5.38 Hz, C14), 138.1 (d, $^{1}J$(C-P) = 170.2 Hz, C9), 141.5 (d, $^{3}J$(C-P) = 11.8 Hz, C13), 146.2 (d, $^{2}J$(C-P) = 5.6 Hz, C2), 148.0 (d, $^{2}J$(C-P) = 4.5 Hz, C11), 149.6 (d, $^{3}J$(C-P) = 12.8 Hz, C12), 181.3 ppm (d, $^{3}J$(C-P) = 15 Hz, C3); $^{31}$P NMR (121 MHz; [$D_6$]DMSO/[$D_2$]water): $\delta$ =9.87, 7.18 ppm (s, $PO(OH)_2$); IR (KBr) $\upsilon\sim$ = 960-1200, 1120-1220 ($RPO(OH)_2$), 1530-1700 (iminoquinone), 3030-3400 ($NH_2$) $cm^{-1}$; UV/Vis (phosphate buffer, pH 7.4): $\lambda_{max}$ ($\varepsilon$) = 241, 452 nm (8800); MS (ESI) m/z (%): = 370.96 (100) $[M-H]^-$, 392.97 (40) [M–2H+Na], 290.95 (55), 273.06 (30), 220.20 (60), 78.84 (30); HRMS (ESI, negative mode) calcd = 370.9834 for $C_{12}H_9N_2O_8P_2$; found: 370.9854.

***Acknowledgements***

This research was supported in part by the European Commission, Sixth Framework Program (NMP2-CT2004-505899, SOPHIED) and in part by the Concerted Research Programme ARC 08/13-009 (UCL, Belgium). J. Marchand-Brynaert is senior research associate of FRS (FNRS, Belgium). Investigations at the CELL Unit were supported by FNRS, Région wallonne, Région bruxelloise, Loterie nationale, Concerted Research Actions and Interuniversity Attraction Poles.

***Supporting information available***

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# CHAPTER 7. SYNTHESIS OF SULFONYLATED BENZOXAZOLES

## 7.1 Foreword

In the chapter C we have exposed the serendipitous discovery of an intramolecular cyclisation of 3-hydroxy-2-pivaloylamine-1-benzenesulfonic acid into 2-*tert*-butyl-4-sulfonic acid benzoxazole. Such reaction likely occurs during the acidic workup, i.e. the quenching of residual boron reagent after cleavage of the methyl ether. This hypothesis was reinforced by the variable proportions of cyclised and free phenol products observed depending on the type of boron reagent used. Indeed the use of boron tribromide results in 70% of benzoxazole whereas with the boron trichloride, the yield does not exceed 50%.

Noteworthy, the acidic work up includes heating the biphasic medium which likely activates the cyclisation. The combination of those parameters actually resembles the oldest common experimental conditions of benzoxazole synthesis.[184] Aminophenol derivatives are first acylated with an acyl chloride reagent and afterwards heated at elevated temperature in presence of a strong Brönsted acid. In a similar fashion, here the intramolecular cyclisation occurs through acid activation of the vicinal amide chain and subsequent intramolecular substitution by the nearby phenol group leading to the successive cyclisation and dehydration steps.

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Whereas it could be useful to acylate our model compounds, i.e. 3-HOA and 2-HMA, with a library of acyl reagents, one has to note that acylation of sulfonated aniline derivatives usually implies either hard thermal conditions, strong aqueous basic solutions or solid-phase chemistry.[185] Our compounds being quite sensitive toward air oxidation, particularly in presence of bases, this does not bode well for a general synthetic process. Therefore, we chose to develop an alternative synthetic route towards those original derivatives bearing specifically a sulfonic group on the carbon C4 or C7.

## 7.2 State of the art in the benzoxazole synthesis

Benzoxazoles form a class of heterocyclic frameworks found in numerous bioactive natural products. As such they have raised a marked interest for the development of general and easily implemented synthetic routes in pharmaceutical and agrochemical discovery programs.[186, 187] Presenting an exhaustive summary of the numerous synthetic routes towards benzoxazoles would be quite deceptive. Indeed, few general synthetic methods are constantly used with slight variations in the experimental conditions and afterwards regularly published months after months. Excellent reviews have been written and should be consulted for an exhaustive report. [188]

[185] So, Y. H.; Heeschen, J. P., *J. Org. Chem*., **1997**, 62, 3552–3561.

[186] a) Kloetzel, M.; King, W.; Wasserman, W.; Warren, C.; Larssen, P., *J. Org. Chem*. **1961**, 26, 607.

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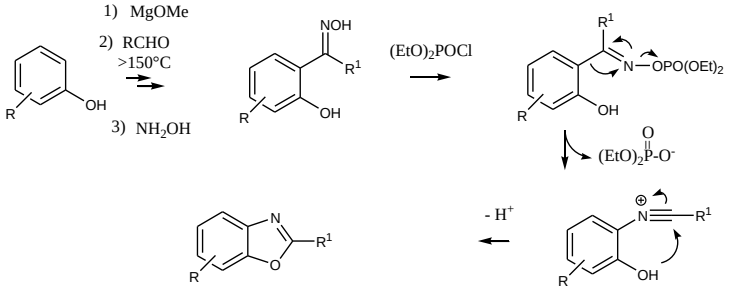
Hereafter, we will first disclose several uncommon syntheses which were published in the last years, and illustrate the constant desire to add synthetic processes to the existing arsenal of the chemist. Afterwards, few traditional synthetic routes will be exposed through recent examples which are of interest, either for the convenience of the procedure or for the elegant variant proposed. The direct C-H activation of 2H-benzoxazole through the use of transition metals will also be commented. Such organometallic approach bestows a marked advantage upon the conventional methods but suffers of time consuming optimisation of the catalytic systems.

### *7.2.1 Beckmann rearrangement of 2-hydroxyaryl ketoximes*

Along with the two synthetic routes which have been used the most used in the benzoxazole preparation, i.e. the dehydrative condensation between a carboxylic acid and aminophenol or the oxidative cyclisation of a phenolic Schiff base, a third route is related to the Beckmann rearrangement. Such methodology tried to eliminate the traditional drawbacks such as harsh conditions, excesses of reagents and lengthy procedures. The Beckmann rearrangement of *ortho*-phenolic ketoximes into benzoxazoles is usually achieved using Brönsted or Lewis acids with the simultaneous thermal activation through microwave irradiation.[189] The simple procedure exposed here, and reported as an excellent alternative, recommends to heat equimolar quantities of 2-hydroxyaryl compound and diethyl chlorophosphate in toluene.[190] The proposed mechanism involves the activation of the oxime by the phosphate reagent. The resulting nitrilium intermediate due to the Beckmann reaction is then intramolecularly quenched by the *ortho*-hydroxyl group, leading to the cyclised product (Scheme 52).

[189] Loupy, A.; Regnier, S., *Tetrahedron Lett.* **1999,** 40, 6221.
[190] Sardarian, A. R.; Shahsavari-Fard, Z., *Synlett*, **2008**, 9, 1391-1393 (and reference herein).

Scheme 52. Beckmann rearrangement of *ortho*-phenolic ketoxime




Yet, whereas the disclosed yields are rather excellent, the scope is limited to common aromatic or aliphatic derivatives due to the necessity to produce the suitable ketoximes as starting materials. [190]

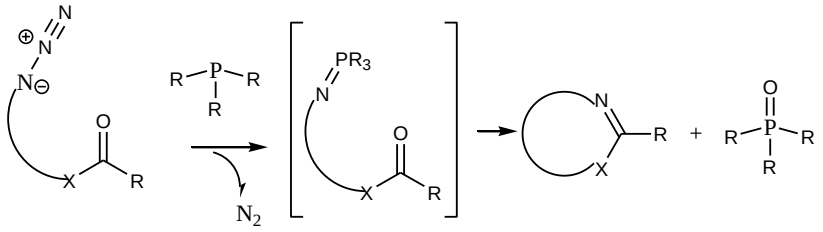
### *7.2.2 Catalytic aza-Wittig reaction*

Since the discovery in 1919 by Staudinger and Meyer of the iminophosphoranes, ($PhN=PPh_3$ for instance), which are the nitrogen analogues of the Wittig reagents, those reagents have been widely used in the synthesis of heterocyclic frameworks,[191] especially for the preparation of nitrogen containing heterocycles. Such intense development comes from the ease of conducting various reactions in neutral solvent, without catalyst, at mild temperature and with high yields. It was therefore logical to develop a methodology towards the synthesis of benzoxazole derivatives based upon those reagents. Yet, up to now the development of a general synthetic process had for major drawback the prerequisite synthesis of the iminophosphoranes using the Staudinger reaction between a phosphine and organic azides (see scheme 53).[192]

[191] Palacios, F.; Alonso, C.; Aparicio, D.; Rubiales,G.; de los Santos, J. M., *Tetrahedron* **2007**, 63, 523. b) Wamhoff, H.; Richardt, G.; Stølben, S., *Adv.Heterocycl. Chem*. **1995**, 64, 159–249.

[192] Fresneda, P. M.; Molina, P., *Synlett* **2004**, 1.

Scheme 53. Aza-Wittig intramolecular reaction



As stated previously, the catalytic version of the Staudinger reaction is mostly inexistent due to the strength of the phosphine oxide (P=O) bond which is great enough to drive the reaction to completion but requires on the other hand harsh reduction conditions for the production of useful P(III) reagents. The solution was to use a catalytic species which solely works at the oxidation state of P(V) as developed in the 1960s for the formation of carbodiimides through self-condensation of isocyanates.[193]

The proposed mechanism involves the in-situ formation of the iminophosphorane intermediate by a slow metathesis reaction between the phosphine oxide and an isocyanate reagent. The iminophosphorane then undergoes a rapid aza-Wittig reaction with a second isocyanate reagent which produces the carbodiimide and regenerates the phosphine oxide. Coming from those previously published results, Marsden and co-workers developed in 2008 the first aza-Wittig intramolecular cyclisation leading to five- and six-membered aromatic heterocycles containing a C=N bond.[194]

[193] a) Monagle, J. J.; Campbell, T. W.; McShane, H. F., *J. Am. Chem. Soc.* **1962,** 84, 4288. b) Monagle, J. J.; Mengenhauser, J. V., *J. Org. Chem*. **1966**, 31, 2321. c) Appleman, J. O.; DeCarlo, V. J., *J. Org. Chem*. **1967**, 32, 1505.

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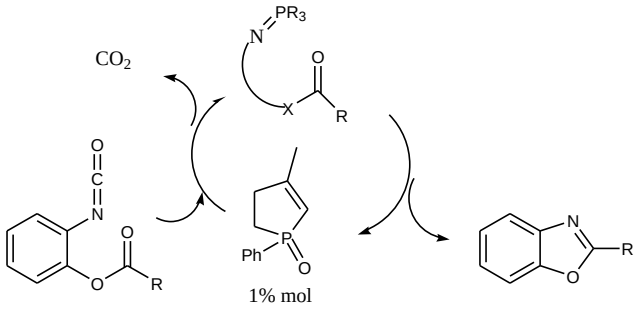
The heterocycle formation is illustrated in the Scheme 54, where the aromatic isocyanate bearing a pendant carbonyl group would also react with phosphine oxide to generate an iminophosphorane which could then be intercepted by intramolecular aza-Wittig reaction with the carbonyl.

Scheme 54. First aza-Wittig catalytic synthesis of benzoxazoles




The reaction functions well regardless of the nature of either the aryl substituents or the carbonyl substituents (primary and secondary alkyl, electron-poor and electron-rich aromatic, and heteroaromatic).

### *7.2.3 Oxidative cyclisation of phenolic Schiff bases*

#### *7.2.3.1 Direct synthesis in presence of activated charcoal*

Schiff bases resulting from the condensation of aminophenol compounds with aldehydes have been exhaustively used as intermediates for benzoxazoles syntheses.[193]

Numerous oxidants such as DDQ, $Mn(OAc)_3$, $PhI(OAc)_2$, $BaMnO_4$, $NiO_2$, and $Pb(OAc)_4$ were reported in the literature as efficient reagents. Yet all of those have to be used at least in stoichiometric quantities. A more effective process is still the subject of research. In this field, Hayashi *et al* reported in 2003 an excellent procedure which was ultimately used afterward for the convenient synthesis of original ligands in organometallic chemistry (see section 7.3). [196]

Following their work upon the Pd/C-catalysed oxidative aromatisation of 1,3,5-trisubstituted pyrazolines and Hantzsch 1,4-dihydropyridines, they sought to extent the process to the synthesis of 2-aryl benzoxazoles. Their first attempt to cyclise phenolic Schiff bases in the presence of 10 to 20 wt% Pd/C at 80 °C in acetic acid mainly resulted in the hydrolysis of the Schiff bases along with traces of benzoxazole derivatives. The ensuing screening of suitable solvents and catalysts led to the successful cyclisation in *m*-xylene at elevated temperature, yet in the absence of a metal catalyst. In the optimal conditions (see Scheme 55), both the aminophenol and the aldehyde are mixed in the presence of a suspension of activated charcoal in *m*-xylene, placed under oxygen atmosphere and heated for a few hours at 120 °C. The resulting 2-aryl benzoxazole is produced in high yield and easily isolated by recrystallisation after filtration of the charcoal reagent.

[193] Chen, Y.-X.; Qian, L.-F.; Zhang, W.; Han, B., *Angew. Chem. Int. Ed*. **2008**, 47, 9330 –9333.

[194] Kawashita, Y.; Nakamichi, N.; Kawabata, K.; Hayashi, M., *Org Lett*. **2003**, 5, 3713-3715.

### *7.2.3.2 Domino process through metals redox coupled reactions*

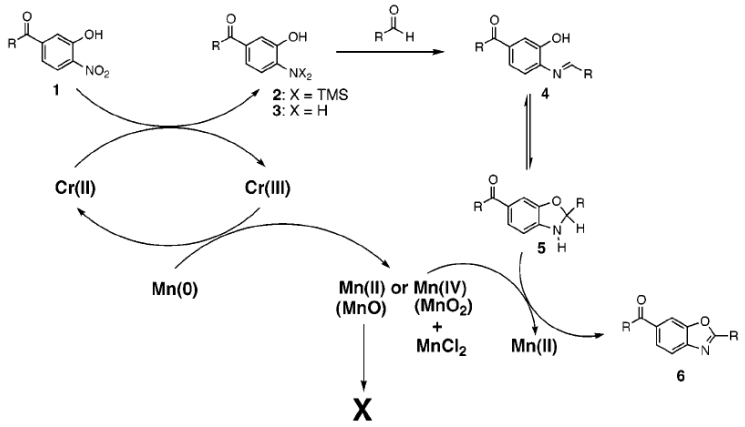
Still focused on Schiff bases as intermediates, an elegant one-pot synthesis of benzoxazoles was published in 2001 by the group of Miller.[197] The method is the direct outcome of a research program dedicated to the catalytic reduction of nitroarenes using a chromium/manganese redox couple, and is analogous to the one of Fürstner and coworkers for the Nozaki-Hiyama-Kishi coupling reaction.[198] The reduction of a nitroarene to aryl hexamethyldisilazane involves the oxidation (conversion) of Cr(II) to Cr(III). Subsequent reduction of the Cr(III) species occurs via oxidation of Mn(0). This needed to investigate the nature of the oxidised manganese species produced. Hence, the authors chose to add a functional group which could take advantage of the presumably oxidised species formed to create few additional bonds via a domino process (see Scheme 56).

The suspected oxidised species are either Mn(II) oxide (MnO), Mn(IV) oxide ( $MnO_2$) and possibly $MnCl_2$. A model compound, 2-nitro-phenol, mixed with the redox couple in the presence of hexamethyldisilazane would yield the expected *N*-silylated intermediate. Assuming the in situ conversion of this intermediate into the corresponding aniline, the presence of an aldehyde would yield an imine derivative which could then undergo equilibration with the aminal species. Such intermediate would quickly react with $MnO_2$ through a dehydrogenation reaction to give ultimately a benzoxazole. The method requires an aldehyde reagent but is quite tolerant regarding the nature of this derivative.

[197] Hari, A.; Karan, C.; Rodrigues, W. C.; Miller, B. L., *J. Org. Chem*. **2001**, 66, 991-996.

[198] Fürstner, A.; Shi, N. J., *Am. Chem. Soc*. **1996**, 118, 12349-12347.

Scheme 56. Domino process involving a Cr/Mn redox couple




On the other hand, the lack of strong electron-withdrawing groups upon the aromatic substrate markedly decreases the yield. It seems that whereas the nucleophilic character of the aniline (imine formation) is diminished, it would advantageously shift the equilibrium in favour of the aminal species (driving force of the reaction). Noteworthy, *o*-nitrophenol and aromatics bearing a strong electron withdrawing group in *ortho* position are poor substrates.

### *7.2.4 Transition metal catalysed domino annulation*

In a similar way, those general methods are based upon the acylation of *o*-haloanilines as starting materials.[199] Here, the oxygen atom of the heterocyclic ring of the benzoxazole directly comes from the acyl reagent.

Such synthetic route has attracted considerable attention due to the broad variety of starting materials available. The C-O bond originates in the intramolecular copper-catalysed cross coupling reaction under very mild conditions.[200]

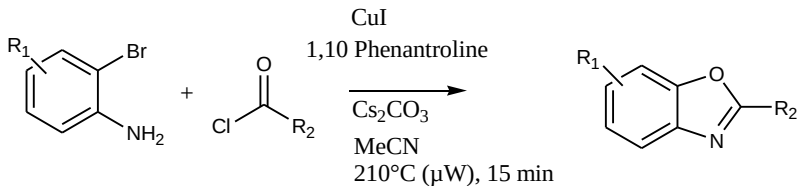
The approach described below has the potential advantage to use directly the acyl reagent in a one-pot reaction. This is rather unusual since acyl chlorides are much less represented in metal-catalysed transformations. Briefly, a *o*-bromoaniline derivative is reacted in DME at room temperature in the presence of an acyl chloride for 24 hours. Afterward, a catalytic amount of copper salt (5%), phenanthroline as the ligand (10%) and 2 equivalents of inorganic base such as $Cs_2CO_3$ were added.

After reaction for 24 hours again at 95 °C, the desired benzoxazole was isolated with residual part of anilide. The *o*-chloroaniline was, as expected, much less reactive. The screening of solvents identified acetonitrile as a good medium and the reaction was next developed as a one pot procedure with the use of microwave activation. Such modifications markedly increased the yields. Still the use of *ortho*-susbtituted acyl chloride reduces the efficiency of the reaction.

[199] a) Altenhoff, G.; Glorius, F., *Adv. Synth. Catal*. **2004**, 346, 1661 –1664. b) Evindar, G.; Batey, R. B., *J. Org. Chem*. **2006**, 71, 1802–1808. c) Bonnamour, J.; Bolm, C., *Org. Lett*., **2008**, 10, 2665-2667. d) Zhang, M., *Adv. Synth. Catal*. **2009**, ASAP.

[200] Viire, R. D.; Evindar, G.; Batey, R. A., *J. Org. Chem*. **2008**, 73, 3452-3459.

Scheme 57. Copper-catalysed one-pot synthesis of benzoxazoles



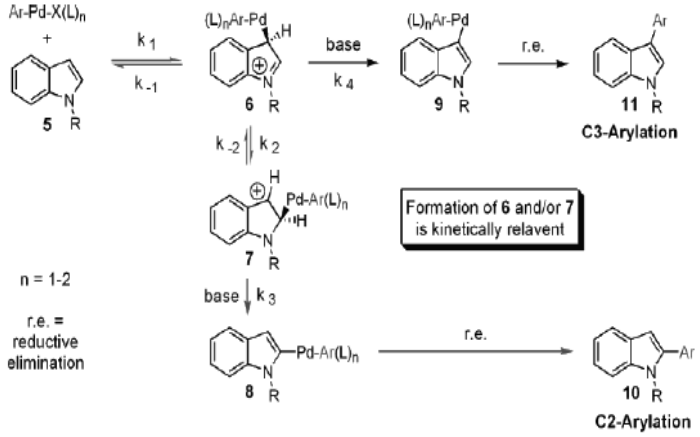

### *7.2.5 C-H activation through transition metal*

A short survey of the recent literature would not be complete if we did not expose the recent breakthrough observed with the synthesis of 2-aryl benzoxazole derivatives from simple 2H-benzoxazole compounds.

The direct arylation reaction mediated by transition metals has emerged as an appealing alternative method. Such reaction does not require pre-functionalisation as usually observed for the common cross-coupling reactions. Directly originating from the cross-coupling reaction of azole derivatives, the direct arylation of benzoxazoles mostly involves Pd species as the transition metal catalysts.[201, 202] Yet, numerous publications have widened the scope of catalyst with iron and nickel for example. Still, the use of palladium is the most common and it has considerably helped the organic chemist in the comprehension of the metal catalysed reaction.

Here we would like to say a few words about the reaction mechanism by detailing some of the results published by Zhuralev *et al* in 2007. The direct Pd-catalyzed arylation is currently believed to involve electrophilic palladation of the azole ring.[203] General pathway is illustrated below with the indole ring for which an uncommon electrophilic substitution was observed.

Scheme 58. Example of electrophilic palladation [204]




Zhuralev *et al* suggested, based upon Hammett correlation experiments and theoretical calculations, another mechanistic route which is detailed in the scheme 59. The studies were performed under standard conditions (2.5 mol % of $Pd(OAc)_2/PPh_3$, $Cs_2CO_3$, 100 °C, DMF) using 20 equiv of PhI. Earlier work has shown that imidazoles, thiazoles, and oxazoles are appreciably C-H acidic and can form ring-opened isomers upon deprotonation. Benzoxazole is particularly acidic (pKa < 16), and its conjugated base exists predominantly as 2-isocyanophenolate. Under the conditions of cross coupling, the intermediate is an open species. Equilibrium of deprotonation would give the *o*-isocyanophenolate[205] which is known to be excellent ligand for the Pd(II) species.[206]

[201] a) Lane, B. S.; Brown, M., A.; Sames, D., *J. Amer. Chem. Soc*., **2005**, 9. 127, 8053-8057. b) Daugulis, O.; Do, H.-Q.; Shabashov, D., *Acc. Chem. Res*., **2009**, 42, 1074–1086.

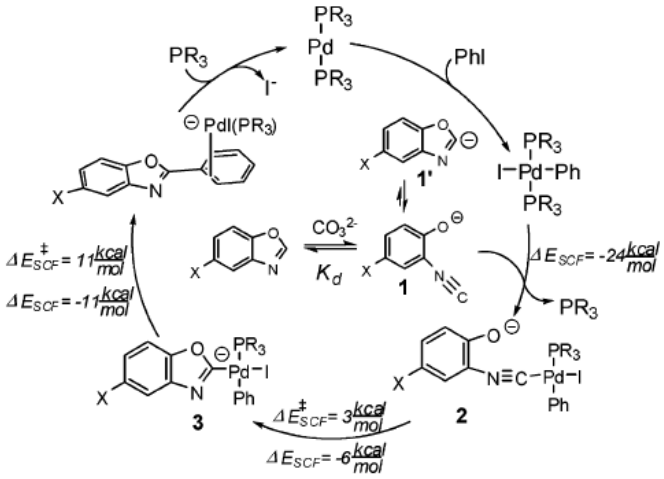
[202] Canivet, J.; Yamaguchi, J.; Ban, I.; Itami, K., *Org. Lett*., **2009**, 11, 1733–1736.

[203] Sanchez, R. S.; Zhurolev, F. A., *J. Am. Chem. Soc*. **2007**, 129, 5824-5829.

[204] Lane, B. S.; Brown, M. A.; Sames, D., *J. Am. Chem. Soc*. **2005**, 127, 8050–8057.

The intramolecular nucleophilic attack of the phenolate upon the activated carbon atom of the cyanide yields the palladate species **3**. The rate limiting step would be the reductive elimination leading to the benzoxazole derivative and the regeneration of the Pd(0) catalyst. Such result would suggest an efficient mechanism for which the C-H acidity would be the key feature. By tuning such C-H acidity, a mild arylation process could be developed which ultimately accommodates electron withdrawing groups on the substrate. Noteworthy, to date no aromatic substrate bearing a strong electron withdrawing group in *ortho* position has been reported for such direct arylation reaction. Moreover, derivatives such as nitroarenes already showed the limitation of the method as those are markedly less reactive.

Scheme 59. Pd-mediated direct arylation of benzoxazole [203]




[205] a) Pirrung, Ghorai, M. C. S.; *J. Am. Chem. Soc*., **2006**, 128, 11772–11773. b) Bayh, O.; Awad, H.; Mongin, F.; Hoarau, C.; Bischoff, L.; Trecourt, F.; Queguiner, G.; Marsais, F.; Blanco, F.; Abarca, B.; Ballesteros, R., *J. Org. Chem*. **2005**, 70, 5190-5196.

[206] a) Jutzi, P.; Gilge, U., *J. Organomet. Chem*. **1983**, 246, 159-162. b) Kernbach, U.; Lugger, T.; Hahn, F. E.; Fehlhammer, W. P., *J. Organomet. Chem*. **1997**, 541, 51-55.

We have herein described several general methods for the synthesis of the benzoxazole scaffold. A recurrent problem seems to be the difficulty to synthesise benzoxazoles bearing strong electron withdrawing groups in ortho position, i. e. in C4 or C7 positions. The oldest methodologies which usually imply harsh conditions remain useful when such limitation occurs.

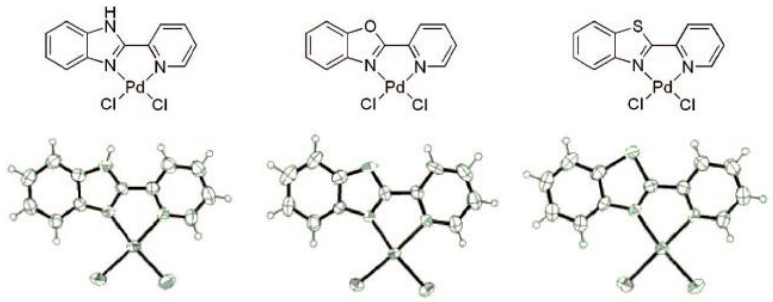
Still we would like to investigate the synthesis of our C4 or C7 sulfonated benzoxazoles using alternative methodologies. This will be the subject of the "soon-to-be submitted" publication which is detailed hereafter. Using a limited number of substrates, we have tried to determine whether or not novel pathways are operative in the case of *ortho*-sulfonated compounds. If so, the marked advantage is that such process will probably be also suitable to produce a new library of phosphorylated benzoxazoles at positions C4 or C7. Before we disclose the experimental results obtained, let us give several general examples of the potential applications of such heterocyclic scaffolds.

## 7.3 Applications of 2-aryl benzoxazoles in organic chemistry

Beside obvious application of benzoxazoles in medicinal chemistry [207], Hayashi and his group took their research one step further and used their methodology to produce a 2-(2-pyridyl)-benzoxazole. This compound along with the 2-(2-pyridyl)-benzazole and 2-(2-pyridyl)-benzothiazole were tested as suitable ligands for transition metal complexes. By mixing the ligands and $PdCl_2$, they could easily produce the corresponding palladacycles in high yield. The X-ray cristallographic data showed that all three complexes in the solid state exist as plain four-coordinated Pd complexes (scheme 60). The strength of the complexation was studied by $^1H$ NMR and this allowed determining the dissociation kinetics of the complexes. The rate of dissociation is similar for both benzoxazole and benzothiazole and markedly higher than the one observed for benzazole.

The palladacycles were tested as catalysts in a typical Mizoroki-Heck reaction between 4-bromotoluene and *tert*-butyl acrylate in DMF at 120 °C with $K_2CO_3$ as base. Excellent yields were obtained for the three different catalysts with a steadily increase going from the benzothiazole to benzoxazole and finally to the benzazole.[208]

Scheme 60. palladium catalysts used in Mizoroki-Heck reaction



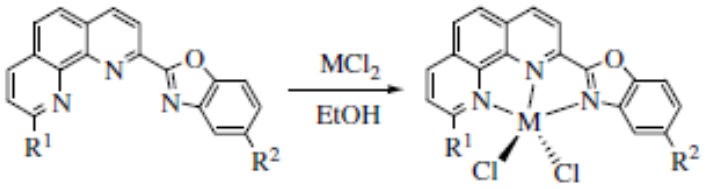
Similarly, series of 2-benzoxazole-1,10-phenanthrolines and 2-oxazoline-1,10-phenanthrolines were synthesised as novel ligands by Zhang and co-workers.[209] The metal complexes, nickel(II), cobalt(II) and iron(II), were characterised and subsequently used as catalysts for ethylene oligomerisation upon activation with appropriate aluminium cocatalysts. The variation of oxygen-containing group (benzoxazole or oxazoline) on the 2-position of the phenanthroline ring shows a great influence on the catalytic performance of those metal complexes (Scheme 61). This should be more thoroughly investigated in the following years.

[207] Carey, J. S.; Laffan, D.; Thomson, C.; Williams, M. T., *Org. Biomol. Chem*. **2006**, 4, 2337.

[208] Haneda, S.; Gan, Z.; Eda, K.; Hayashi, M., *Organometallics* **2007**, 26, 6551-6555.

[209] M. Zhang *et al., J. Organomet. Chem*., **2008**, 693 3867–3877.

Scheme 61. Benzoxazole catalysts for olefin oligomerisation



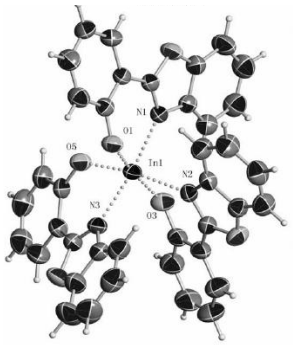

Another use of metal complexes of benzoxazoles is illustrated in photoluminescence applications.[210] Some luminescent organoindium complexes with benzimidazole derivatives have been discovered in recent years, implying that imidazole derivatives may be excellent organic chelating ligands for the preparation of new photoluminescent and/or electroluminescent complexes with indium element. [211] 2-(2'-Hydroxy-phenyl)benzoxazole or Hbpx is a similar N,O-chelating ligand and many complexes have been characterised and shown excellent photoluminescence and/or electroluminescence properties. The photoluminescent properties are ascribed to metal-perturbed π-π* ligand-to-ligand charge transfer (LLCT).[212] Hpbx emits at 494 nm in trichloromethane. For the neutral Hpbx, an extremely rapid excited state intramolecular proton transfer (ESIPT) via a cis enol structure with an intramolecular hydrogen bonding between the phenolic group and imidazole nitrogen atom is observed, manifested by the lack of the primary emission and the appearance of a strongly Stokes-shifted low-energy tautomeric fluorescence.

[210] Tong, Y. P.; Lin, Y. W., *Inorganica Chimica Acta* **2009**, 362, 2033–2038.

[211] (a) Tong, Y.-P.; Zheng, S.-L.; Chen, X.-M., *Inorg. Chem*. **2005**, 44, 4270. (b) Kim, W.S.; You, J.M.; Lee, B.-J.; Jang, Y.-K.; Kim, D.-E.; Kwon, Y.-S., *Thin Solid Films* **2007**, 515, 5070.

[212] Waluk, J., *Conformational Analysis of Molecules in Excited States*, Wiley-VCH New York, **2000**. and references cited therein.

Figure 24. Photoluminescent complex of Hbpx and In(III)




Why did we expose those results? Actually, several attempts to realise cross coupling reactions, e. g. the Mizoroki-Heck reaction, in water have been the focus of recent researches. Here we can link it back to our main focus in the thesis, the production of new water-soluble heterocycles. A potential application, if the corresponding 2-(2-pyridyl)-4-sulfonic acid-benzoxazole could be produced, would be the development of such catalysed cross coupling reactions in water.

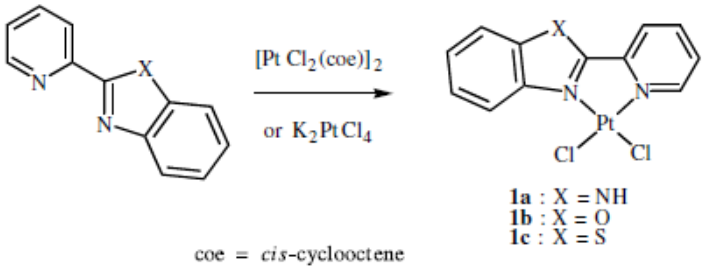
Finally, let us say a few words about the use of benzoxazoles as ligands in metal complexes devoted to medicinal chemistry. Cisplatin is one of the most studied bioactive complexes for cancer chemotherapy. The drug has to be administered intravenously. Dose-limiting side effects such as severe renal toxicity, neurotoxicity, and emesis arise due, in part, from the low solubility of cisplatin in water.[213]

Those facts along with the observation that cancer cells treated with cisplatin acquire resistance to the drug, have provided research impetus towards the development of a more effective platinum drug that can be administered orally and with reduced side effects. 2-Arylbenzimidazoles, -oxazoles, and -thiazoles constitute a remarkably important class of active compounds.[214] Westcott and co-workers focused on generating platinum complexes with biologically active carrier ligands.

Therefore this prompted them to make complexes containing arylbenzimidazoles, and related benzoxazole and benzothiazole groups.

The Scheme 62 below illustrates the synthesis of such complexes. Sadly, the authors could not obtain full X-ray data for all the complexes produced with 2-pyrid-2-yl-benzoxazole/benzothiazole/ benzimidazole and those were also poorly soluble either in aqueous or organic solvents. The metal complexes were characterised with $^{1}H$ NMR and FT-IR, On the other hand, the isomeric ligand, i.e. 2-pyrid-3-yl-benzoxazole/benzothiazole/benzimidazole yielded novel platinum complexes shown in the Figure 25. Further investigation has to be realised to determine whether or not those ligands are useful for chelation with the platinum ion.[215]

Scheme 62. Preparation of novel platinum (II) complexes

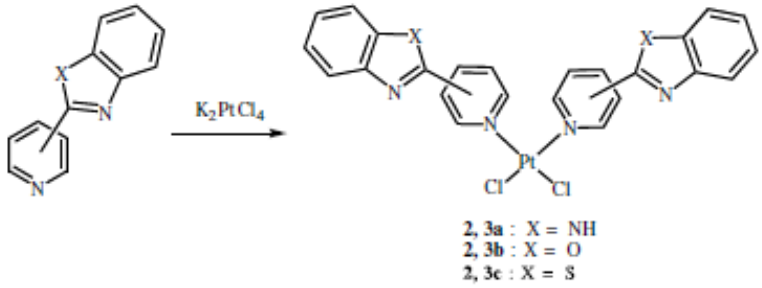



[213]. Jamieson, E.R.; Lippard, S.J., *Chem. Rev*. **1999**, 99, 2467.

[214] Westcott S.A. *et al*, *Can. J. Chem*. **1999**, 77, 1196.

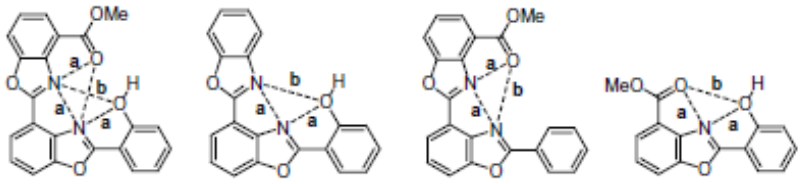
[215] He, X.-F.; Vogels, C. M.; Decken, A.; Westcott, S. A., *Polyhedron*, **2004**, 23 155–160.

Figure 25. Novel cis-dichlorobis(2-pyrid-3-ylbenzoxazole) platinum(II) complexes




Still focused on medicinal chemistry, the synthesis of sulfonated analogues of the critical UK-1 metabolite scaffold found in *Streptomyces* cultures are of interest (scheme 63). Such derivatives are expected to display chelating properties for several divalent cations ($Zn^{2+}$, $Mg^{2+}$, $Cu^{2+}$) and a marked ability to intercalate into double stranded DNA. The synthesis of sulfonated analogues of 2'-hydroxy-2-phenyl-benzoxazole would also open the way to novel water-soluble fluorescent dyes.

Scheme 63. UK-1 metabolite and simplified analogues




UK-1

It is notable that UK-1 and analogues all possess a triangular arrangement of Lewis-basic sites including heterocyclic nitrogen atoms and oxygen atoms, from ester or phenol groups. It has been suggested that these groups are involved in metal ion chelation, which is important for DNA binding and correlates with cytotoxicity.[216]

[216]a) Shibata, K.; Kashiwada, M.; Ueki, M.; Taniguchi, M. J., *Antibiot*. **1993**, 46, 1095–1100. b) Kumar, D.; Jacob, M. R.; Reynolds, M. B.; Kerwin, S. M., *Bioorg. Med. Chem*. **2002**, 10, 3997–4004. c) Oehlers, L.; Mazzitelli, C. L.; Brodbelt, J. S.; Rodriguez, M.; Kerwin, S., *J. Am. Soc. Mass Spectrom*. **2004**, 15, 1593–1603.

## 7.4 Full Paper
## Journal

The publication will be submitted in the journal Synlett.

***Title***

# Novel 4- and 7-sulfonated 2-substituted benzoxazoles.

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***Abstract***

The efficient synthesis of sulfonated benzoxazoles at positions C4 or C7 is reported. The condensation reactions involve original anilide acetal reagents which, upon acid catalysis, allow an easy cyclisation reaction with the sulfonylated *ortho*-aminophenol partner. This method circumvents the classical use of orthoesters which drawback is the limited access to aromatic reagents.

***Keyword***

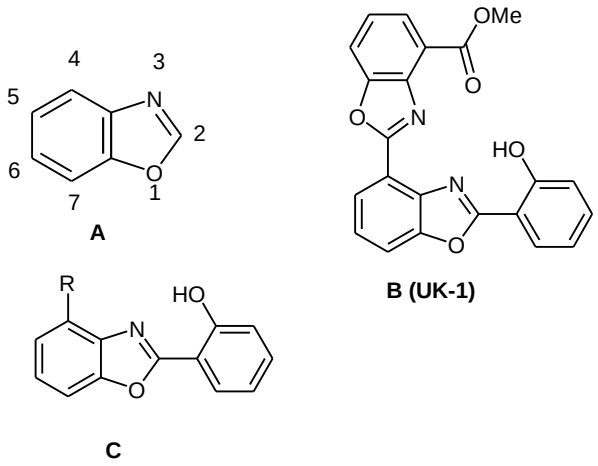
Acetals / Condensation / Cyclisation / Heterocycles / Substituent effects.

***Introduction***

The benzoxazole framework sporadically occurs in natural compounds exhibiting a broad spectrum of activities (Figure 1).[1] Synthetic efforts towards derivatives with an increased molecular diversity mainly focus on the 2-aryl substituted family.[2] The common syntheses of such oxazole heterocycles include the heat dehydration of *o*-acylaminophenols,[3] the Beckmann rearrangement of *o*-hydroxybenzophenone-oximes,[4] the oxidative ring closure of *o*-phenolic Schiff bases,[5] and the direct arylation reactions through transition-metal C2-H functionalisation.[6] Among those synthetic routes, little works have been dedicated to the selective insertion of hydrophilic groups on the benzoxazole core structure. Yet such derivatives could offer a high potential either to modulate pharmacological properties or to give access to water-soluble fluorophores.[1,7,8]

Sulfonated benzoxazoles derivatives on positions C5 and C6 are readily available through the aromatic electrophilic substitution process.[9] Up to now, synthetic routes toward sulfonated bezoxazoles on positions C4 and C7 have been scarcely reported. Those involve either a Newman-Kwart type rearrangement or a benzyne pathway.[10] Recently, the drawback of poor water-solubility of the natural metabolite UK-1 from *Streptomyces* (see Figure 1) was markedly pointed out.[7] This metabolite features two critical benzoxazole moieties which impart its cytotoxic activity. Given our recent researches involving the selective synthesis of phenoxazine scaffolds from sulfonated aminophenols, we report here a practical and efficient synthesis of functionalised benzoxazoles bearing free sulfonic acid, sulfonate ester or sulfonamide either on position C4 or C7.

Figure 1. The benzoxazole framework (**A**), its occurrence in the UK-1 metabolite (**B**) and simpler analogues (**C**).



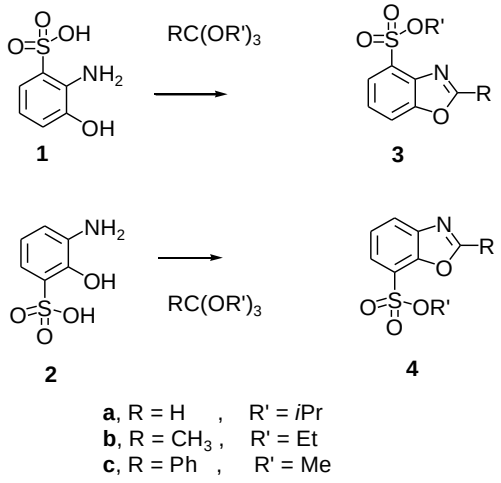

We have taken advantage of the sulfonic acid group present on the *ortho*-aminophenol precursors (**1** and **2**, see Scheme 1) to catalyse intramolecularly the oxazole ring formation by reaction with orthoesters.[11] We further developed anilide acetals as more versatile reagents for this Brönsted acid catalysed heterocyclisation. Lastly, we have illustrated our method with the synthesis of sulfonated analogues of the UK-1 metabolite benzoxazole core (see Figure 1, structure C with R = $SO_3H$, $SO_2NH_2$).

***Results and discussions***

The efficient and regioselective syntheses of our sulfonated building blocks, respectively 3-hydroxyorthanilic acid (**1**) and 2-hydroxymetanilic acid (**2**) were previously described (Scheme 1).[12] We first determined the optimal conditions for the acid catalysed

cyclisation of **1** and **2** with a series of commercial orthoesters. Whereas the common protocol for benzoxazole synthesis uses high temperature and strong acid, in the presence of a large excess of orthoester, in our case, the reaction proceeded well under very mild conditions. Typically, the compounds **1** or **2** were mixed with 3 equivalents of orthoester dissolved in a small volume of dioxane and allowed to react at 60 °C for a few hours. Then, the mixture was filtered and the residual solid washed with dichloromethane. The filtered solution was evaporated to give crude benzoxazoles in high purity (from NMR analysis), collected as the corresponding sulfonate ester.

Scheme 1. Synthesis of benzoxazoles sulfonated either in position C4 (**3a-c**) or C7 (**4a-c**) from commercial orthoesters. Reagents and conditions: see experimental section.




**a**, R = H , R' = *i*Pr
**b**, R = $CH_3$ , R' = Et
**c**, R = Ph , R' = Me

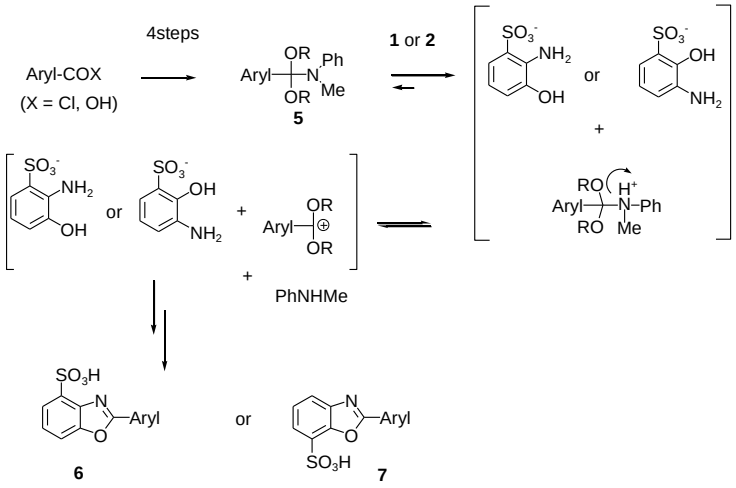
The compounds **3a-c** and **4a-c** were obtained with good to moderated yields, i.e. 80% for **3a-b** and **4a-b,** and 50% for **3c** and **4c.** The benzoxazoles **3c** and **4c** could be recrystallised in ethanol/dichloromethane. The simultaneous protection of the sulfonic

acid with orthoesters has been previously reported.[13] The lower yields obtained with trimethylbenzoate orthoester (a small amount of product was isolated as the free sulfonic acid) point out the limitation of the classical condensation reaction when aryl substituent is required at position C2. In addition, the relative difficulty to synthesise aromatic orthoesters bearing diverse functional groups does not bode well for a general synthetic route. For instance, aromatic orthoesters are not readily synthesised using the classical Pinner reaction.[14] An alternative route includes the formation of orthothioesters which are subsequently submitted to methanolysis in the presence of a silver salt and a base.[15]

An excellent (yet old) procedure was reported by MacClelland and his group. It involves the condensation of an acyl chloride with *N*-methylaniline followed by the preparation of benzanilide acetal by using standard amide acetal procedure.[16] The last step corresponds to the treatment of the previous crude acetal with alcohol and an excess of acid which ultimately gives the desired orthoester. Yet, the yields are largely dependent on the nature of the substituents present on the aromatic nucleus.

We reasoned that such a simple and fast synthetic route could be of interest for our acid catalysed cyclisation (illustrated in scheme 2). The desired benzanilide acetals **5** were synthesised in high yields and used directly in the condensation reaction with the sulfonated aminophenols **1** or **2**. The desired benzoxazoles **6** or **7** were isolated in good yields after purification by column chromatography. The results obtained with compounds **1** or **2** in the presence of several benzanilide acetals **5** are reported in Table 1. The synthesis of reagents **5** is given as supporting information. The access to one particular reagen (FG = *o*-OBn) is described in the Scheme 3. The Table 1 shows that three 4-sulfonic benzoxazoles (**6a-c**) and one 7-sulfonic benzoxazole (**7a**) were readily accessible, while three condensations failed (entries 3, 6 and 7) and the hydrolysed reagent (**5'**) was recovered.

Scheme 2. Synthesis of 2-arylbenzoxazole-sulfonic acids using anilide acetals as reagents (see Table 1)

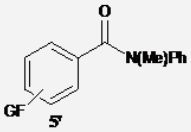



Increasing the amount of acetal **5** (3 to 10 equivalents), and heating at higher temperatures (60 to 80 °C) for a long time (17- 48 hours) did not help. The reaction most probably proceeds via an acid-base equilibrium as the first step, giving the intermediate aryl-(*bis*-ethoxy)carbenium ion. The formation of this reactive species (Scheme 2) should be favoured by electron-donating substituents on the aryl moety (i.e FG= *ortho*-OMe and *ortho*-OBn) and disfavoured by electron-withdrawing substituents (i.e FG = *ortho*-$NO_2$ and *meta*-OMe with a lesser importance). The difference of behaviour between the substrates **1** and **2** versus the *m*-OMe reagent **5** (entries 2 and 6) also suggests that the intramolecular acid catalysis for assisting the condensation/cyclisation is more efficient when the sulfonic acid group is next to the aniline function

Table 1. Condensation reactions with anilide acetals

| | Aminophenol | Benzanilide acetal [a] **5** FG = | Benzoxazoles [b] Yields isolated (**cpd**) | Comment [c, d c] |
|---|---|---|---|---|
| | **1** | ***o*-OMe** | **70 % (6a)** | |
| | **1** | ***m*-OMe** | **60% (6b)** | |
| | **1** | ***o*-$NO_2$** | - | **>80% benzanilide recovered** |
| | **1** | ***o*-OBn** | **60 % (6c)** | **containing about 20% of ethyl ester of 6c** |
| | **2** | ***o*-OMe** | **65% (7a)** | |
| | **2** | ***m*-OMe** | - | **>50% benzanilide recovered** |
| | **2** | ***o*-$NO_2$** | - | **> 80% benzanilide recovered** |

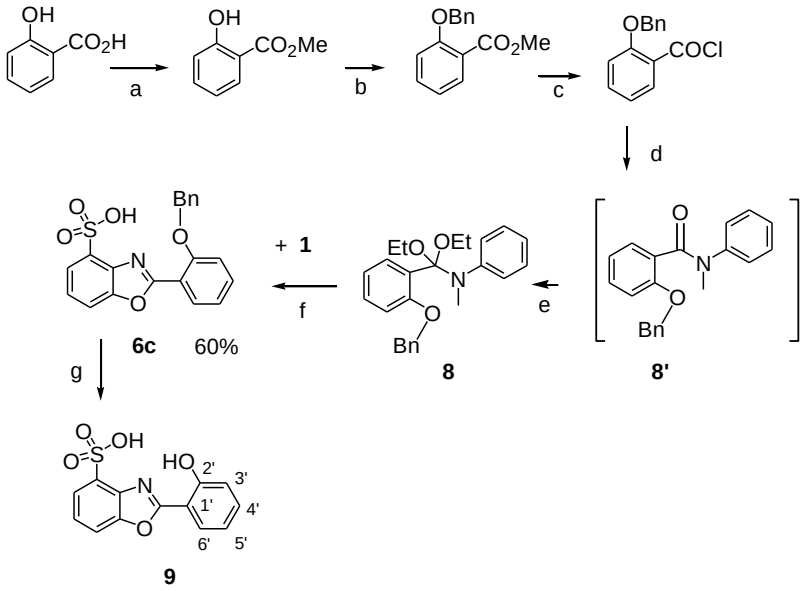
vere prepared from the corresponding benzoic acids, purity of the reagent was verified by $^{1}H$ & $^{13}C$




which a part of the bezoxazole was isolated as a sulfonate ester, all of the benzoxazoles gave free atives. [c] The nature of the side product was confirmed by NMR. [d] Benzanilide **5'** results from hydrolysis

The problem of chemoselective deprotection of a *O*–methyl ether function on benzoxazole derivatives has been previously reported in the literature.[1a] Accordingly, we synthesised the water-soluble sulfonated analogue of UK-1 scaffold using the benzyl ether as the protecting group.[7b] (scheme 3).

Scheme 3. Synthesis of 2-(2'-hydroxy-phenyl)-benzoxazole-4-sulfonic acid.



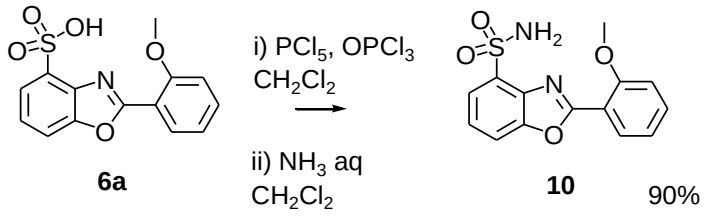

Reagents and conditions: a) THF, DBU, MeI;[18] b) BnBr, $K_2CO_3$, $CH_2Cl_2$/MeOH;[19] c) i. 1.4 N NaOH, dioxane, reflux; ii. Oxalyl chloride, $CH_2Cl_2$, DMF; d) *N*-Me aniline, $Et_3N$; e, f) see experimental conditions; g) Pd/C, $H_2$, ethanol, 6 hours. [19]

The required acetal **8** was synthesised from salicylic acid. First the acid protection was realised using DBU and methyl iodide in acetonitrile.[18] Next, benzylation of the phenol with benzyl bromide and $K_2CO_3$, followed by saponification gave the crude acid in excellent yield >90%.[19] This acid was converted into acyl chloride and substituted with *N*-methylaniline to give the required benzanilide **8'**. *O*-alkylation with ethyl triflate gave the imidatonium salt which was reacted with freshly made sodium ethoxide to produce the desired benzanilide acetal **8** in an overall yield of 80% for seven steps.

The condensation of **8** with the substrate **1**, as previously described, gave the desired heterocyclic scaffold **6c**. The benzyl ether of the benzoxazole **6c**, bearing the free sulfonic acid group, was quantitatively cleaved under usual hydrogenation conditions to afford the target compound.[19] This benzoxazole **9** is quite water-soluble (> $10^{-4}$ M) and features interesting fluorescence properties with blue emission from sunlight irradiation, like the well known 2'-hydroxy-2-phenylbenzoxazole dye, an intercalating agent into DNA duplex.[20]

The robustness of benzoxazole sulfonic acids **6** under standard experimental conditions has been further illustrated with the transformation of **6a** into primary sulfonamide **10** (Scheme 4). The free acid was converted into the corresponding sulfonyl chloride with $PCl_5/OPCl_3$ and then substituted by ammonia in a biphasic medium to give the derivative **10** with an overall yield of 90% (see scheme 4).

Scheme 4. Conversion into primary sulfonamide (reagents & conditions see supporting information).



Given the structural similarity of sulfonamide **10** to known carbonic anhydrase (CA) inhibitors, we tested our compound against the human CA-I enzyme; **10** was found inactive at 100 µM (see supporting material).

### *Conclusion*

In conclusion, we have disclosed two routes allowing the preparation of 4- or 7-sulfonated benzoxazoles under very mild conditions. The compounds present various substitutions at position 2 (H, alkyl, aryl) and an increased water-solubility. Further derivatisations have been illustrated with the synthesis of an analogue of UK-1 metabolite and a sulfonamide related to CA inhibitors. Works are in progress for similarly preparing phosphorylated benzoxazoles[21] and exploiting their metal chelation properties.

### *Acknowledgements*

This research was supported in part by the European Commission, Sixth Framework Program (NMP2-CT2004-505899, SOPHIED) and in part by the Concerted Research Programme ARC 08/13-009 (UCL, Belgium). J. Marchand-Brynaert is senior research associate of FRS (FNRS, Belgium).

***Experimental Section***

Solvent and reagents were used as purchased (Sigma or Acros). $^{1}H$ and $^{13}C$ NMR spectra were recorded with a Bruker Avance 500 or a Bruker Avance 300 spectrometer. Spectra were obtained in DMSO-$d_6$, $CDCl_3$, methanol-$d_4$. Chemical shifts are reported in ppm relative to TMS.[22]. Low resolution mass spectra were acquired using a Thermo Finnigan LCQ spectrometer, either in positive mode or negative mode ESI or EI methods (70 eV). High Resolution Mass Spectrometry (HRMS) analyses using ESI or CI methods were performed at the University of Oxford (UK). Melting points were recorded with a Büchi Melting point B-540 apparatus calibrated with benzoic acid. Infrared spectra were recorded with a Shimadzu FT-IR 84005 spectrometer (ATR-SeZn). TLC were carried out using silica gel 60 F254 plates (0.2 mm, Merck) and the spots were visualized using UV light (254 nm). Column chromatography was performed on Merck silica gel 60 (70–230 mesh).silica gel 60 F254 plates (0.2 mm, Merck) and the spots were visualized using UV light (254 nm).

***Synthesis of benzanilide acetals; General procedure (d-f)***

**Benzanilide acetals[15].**

Benzanilide (1 equiv.; 10 mmol) and ethyl trifluoromethanesulfonate (1.2 equiv.) were stirred in dry $CH_2Cl_2$ (6 mL) overnight. Dry ether (40 mL) was added at 0°C and the mixture was left to stand 1 hour until all the imidatonium salt had precipitated. This salt was separated from the solvent and dissolved into $CH_2Cl_2$ (6mL) which was added dropwise over a period of 30 min to a cooled (0°C) stirred solution made by addition of sodium (0.5 g) and ethanol (20 mL). The solvents were removed on a rotary evaporator and hexane was added (40 mL). This dissolved the crude anilide acetal and the excess of salt were filtered off. Evaporation gave the crude acetal.

***Cyclisation (orthoesters/ anilide acetals); General procedure***

Sulfonated aminophenols (1 equiv., 1 mmol) and orthoester (3 equiv.) were added to dioxane (3 mL) and the mixture was heated at 60°C overnight. The crude reaction mixture was filtered directly and the residual solid was triturated twice with dichloromethane and filtered again. The filtered solutions were pooled together and concentrated under vacuum. The residue was either recristallized in a mixture of dichloromethane/ethanol (8:2 v:v) or purified by column chromatography (silica gel, cyclohexane/ethyl acetate).

**2*H*-Benzoxazole-4-sulfonic acid isopropyl ester (3a).**

Gummy white solid. Yield 80% ; IR: 2850 , 1649, 1469, 1174, 975, 670 $cm^{-1}$; $^{1}H$ NMR (300 MHz, $CDCl_3$): δ 1.32 (d, 6H, J = 6.3 Hz, $CH(\underline{CH_3})_2$), 5.14 (td, 1H, J= 6.3 Hz, J= 12.5 Hz, $C\underline{H}(CH_3)_2$), 7.56 (t, 1H, J= 8.0 Hz, $H_6$), 7.90 (dd, 1H, J= 0.8 Hz, J= 8.3 Hz, $H_7$), 8.01 (dd, 1H, J= 0.7 Hz, J= 7.8 Hz, $H_5$), 8.32 (s, 1H, $H_2$).; $^{13}C$ NMR (75 MHz, $CDCl_3$): δ 22.9 ($C(\underline{CH_3})_2$), 78.8 ($\underline{C}(CH_3)_2$), 116.5 ($C_7$), 125.41 ($C_5$), 125.49 ($C_6$), 129.2 ($C_9$), 137.2 ($C_4$), 150.8 ($C_8$), 154.4 ($C_2$).; MS (EI, 70eV): *m/z* (%)= 242.04 (5) $[M]^{\bullet}$, 226.06 (10), 119.07 (15), 85.98 (65), 84 (100); HRMS-CI: *m/z* $[M + H]^{+}$ calcd for $C_{10}H_{12}NO_4S$: 242.04870; found: 242.04821.

**2-Methyl-benzoxazole-4-sulfonic acid ethyl ester (3b).**

Gummy white solid. Yield 80% ; IR: 2920, 1560, 1427, 1346, 1247, 1230, 1188, 912, 794 $cm^{-1}$; $^{1}H$ NMR (300 MHz, $CDCl_3$): δ 1.33 (t, 3H, J= 7.0 Hz, $CH_2\underline{CH_3}$), 2.74 (s, 3H, $CH_3$), 4.32 (q, 2H, J= 7.0 Hz, $C\underline{H_2}CH_3$), 7.43 (t, 1H, J= 7.9 Hz, $H_6$), 7.76 (d, 1H, J= 8.1 Hz, $H_7$), 7.88 (d, 1H, J = 7.78 Hz, $H_5$).; $^{13}C$ NMR (75 MHz, $CDCl_3$): δ 14.7 ($CH_2\underline{C}H_3$), 14.8 ($\underline{C}H_3$), 67.9 ($\underline{C}H_2CH_3$), 115.8 ($C_7$), 124.1 ($C_5$), 125.05 ($C_6$), 126.6 ($C_9$), 139.0 (C4), 151.7 ($C_8$), 166.6 ($C_2$).; MS (ESI): *m/z*

(%)= 263.94 (100) $[M + Na]^+$, 242.06 (30), 214.08 (30); HRMS-CI: *m/z* $[M + H]^+$ calcd for $C_{10}H_{12}NO_4S$: 242.04870; found: 242.04819.

**2-Phenyl-benzoxazole-4-sulfonic acid methyl ester (3c).**

Light brown needles. Yield 50%, Mp 150-155 °C; IR: 2918, 1548, 1423, 1348, 1199, 1166, 972, 794, 767, 690 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$): δ 4.05 (s, 3H, $CH_3$), 7.47-7.61 (m, 4H, $H_6$ & $H_{3',-5'}$), 7.89 (dd, 1H, J= 0.9 Hz, J= 8.2 Hz, $H_7$), 7.98 (dd, 1H, J= 0.9 Hz, J= 7.8 Hz, $H_5$), 8.36 (m, 2H, $H_{2'}$ & $H_{6'}$).; $^{13}C$ NMR (75 MHz, $CDCl_3$): δ 57.4 ($OCH_3$), 116.2 ($C_7$), 124.5 ($C_5$), 125.8 ($C_6$), 126.0 ($C_{4'}$), 126.4 ($C_{1'}$), 128.4 ($C_{2'}$ & $C_{6'}$), 129.0 ($C_{3'}$ & $C_{5'}$),132.6 ($C_9$), 139.8 ($C_4$), 151.6 ($C_8$), 165.3 ($C_2$).; MS (ESI): *m/z* (%)= 290.00 (100) $[M + H]^+$, 276.06 (10), 222.01 (10), 194.16 (5); HRMS-ESI: *m/z* $[M - H]^-$ calcd for $C_{14}H_{10}NO_4S$: 288.0331; found: 288.0321.

**2-Phenyl-benzoxazole-4-sulfonic acid.**

White solid. Yield 10%. $^1H$ NMR (300 MHz, DMSO-$d_6$): δ 7.37 (t, 1H, J= 7.8 Hz, $H_6$),7.64-7.70 (m, 3H, $H_{3',-5'}$), 7.76 (d, 1H, J= 7.8 Hz, $H_7$), 7.68 (d, 1H, J= 7.7 Hz, $H_7$), 7.76 (d, 1H, J = 7.8 Hz, $H_5$), 8.23 (m, 2H, $H_{2'}$ & $H_{6'}$).; $^{13}C$ NMR (75 MHz, DMSO-$d_6$): δ 112.3 ($C_7$), 123.6 ($C_5$), 125.2 ($C_6$), 127.4 ($C_{4'}$), 128.2 ($C_{2'}$ & $C_{6'}$), 130.2 ($C_{3'}$ & $C_{5'}$), 132.8 ($C_{1'}$), 138.7 ($C_9$), 140.1 ($C_4$), 151.5 ($C_8$), 162.6 ($C_2$).; MS (ESI): *m/z* (%)= 274.1 (100) $[M - H]^-$, 210.1 (30), 104.96 (11); HRMS-ESI: *m/z* $[M - H]^-$ calcd for $C_{13}H_8NO_4S$: 274.0174; found: 274.0166.

**2*H*-Benzoxazole-7-sulfonic acid isopropyl ester (4a).**

Gummy grey solid. Yield 80%.; IR: 2923, 1633, 1461, 1191, 1149, 1027, 675 $cm^{-1}$; $^1H$ NMR (300 MHz, DMSO-$d_6$): δ 1.19 (d, 6H, J = 6.2 Hz, $CH(\underline{CH_3})_2$), 4.83 (td, 1H, J= 6.2 Hz, J= 12.4 Hz, $C\underline{H}(CH_3)_2$), 7.66 (t, 1H, J= 7.9 Hz, $H_5$), 7.90 (d, 1H, J= 7.7 Hz, $H_4$), 8.24 (d, 1H, J= 7.9 Hz, $H_6$), 9.02 (s, 1H, $H_2$).; $^{13}C$ NMR (75 MHz, DMSO-$d_6$): δ 22.7 ($C(\underline{CH_3})_2$), 79.7 ($\underline{C}(CH_3)_2$), 121.1 ($C_9$), 125.7 ($C_4$), 126.16 ($C_6$),

127.1 ($C_5$), 142.0 ($C_7$), 145.3 ($C_8$), 156.0 ($C_2$).; MS (ESI): *m/z* (%)= 242.1 (25) $[M + H]^+$, 200.1 (25), 191.23 (40); HRMS-CI: *m/z* $[M + H]^+$ calcd for $C_{10}H_{12}NO_4S$: 242.04870; found: 242.04902.

**2-Methyl-benzoxazole-7-sulfonic acid ethyl ester** (**4b**)**.**

Gummy white solid. Yield 80%; IR: 2700, 1633, 1537, 1434, 1190, 1150, 1031, 736 $cm^{-1}$; $^1H$ NMR (300 MHz, DMSO-$d_6$): δ 1.22 (t, 3H, J= 7.0 Hz, $CH_2\underline{CH_3}$), 2.71 (s, 3H, $CH_3$), 4.21 (q, 2H, J= 7.0 Hz, $C\underline{H_2}CH_3$), 7.57 (t, 1H, J= 7.9 Hz, $H_5$), 7.80 (dd, 1H, J= 0.8 Hz & 7.8 Hz, $H_4$), 8.09 (dd, 1H, J = 0.9 & 8.0 Hz, $H_6$).; $^{13}C$ NMR (75 MHz, DMSO-$d_6$): δ 14.6 ($CH_2\underline{C}H_3$), 15.0 ($\underline{C}H_3$), 69.1 ($\underline{C}H_2CH_3$), 119.3 ($C_9$), 125.1 ($C_4$), 125.2 ($C_6$), 125.9 ($C_5$), 143.5 ($C_7$), 146.4 ($C_8$), 166.2 ($C_2$).; MS (ESI): *m/z* (%)= 264.07 (30) $[M + Na]^+$, 242.08 (100), 214.18 (35); HRMS-CI: *m/z* $[M + H]^+$ calcd for $C_{10}H_{12}NO_4S$: 242.04870; found: 242.04884.

**2-Phenyl-benzoxazole-7-sulfonic acid methyl ester** (**4c**)**.**

Light brown needles. Yield 50% Mp 140-145 °C; IR: 2923, 1565, 1427, 1369, 1220, 1207, 979, 769 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$): δ 3.93 (s, 3H, , $CH_3$), 7.49-7.60 (m, 4H, $H_4$ & $H_{3'\text{-}5'}$), 7.87 (dd, 1H, J= 0.8 Hz, J= 7.8 Hz, $H_4$), 8.05 (dd, 1H, J= 0.8 Hz, J= 8.0 Hz, $H_6$), 8.31 (dd, 2H, J= 1.6 Hz & 7.7 Hz, $H_{2'}$ & $H_{6'}$).; $^{13}C$ NMR (75 MHz, $CDCl_3$): δ 57.1 ($OCH_3$), 119.1 ($C_4$), 124.6 ($C_6$), 125.7 ($C_5$), 125.9 ($C_{4'}$), 128.1 ($C_{2'}$ & $C_{6'}$), 129.1 ($C_{3'}$ & $C_{5'}$), 132.4 ($C_{1'}$), 144.0 ($C_7$), 146.6 ($C_8$), 164.6 ($C_2$).; MS (ESI): *m/z* (%)= 290.20 (100) $[M + H]^+$,; HRMS-ESI: *m/z* $[M + H]^+$ calcd for $C_{14}H_{12}NO_4S$: 290.04870; found: 290.04873.

**2-(2'-Methoxy-phenyl)-benzoxazole-4-sulfonic acid (6a).**

White solid. Yield 70%. Mp slow decomp-225 °C; IR: 2923, 2352, 1647, 1548, 1419, 1240, 1105, 1049, 750 $cm^{-1}$; $^{1}H$ NMR (300 MHz, DMSO-$d_6$): δ 3.90 (s, 3H, $OCH_3$), 7.23 (ddd, 1H, J= 0.8 Hz, J=2.6 Hz, J= 8.3 Hz, $H_{3'}$), 7.37 (t, 1H, J= 7.9 Hz, $H_{5'}$), 7.56 (t, 1H, J= 8.0 Hz , $H_6$), 7.68 (dd, 1H, J= 1.0 Hz, J= 7.7 Hz, $H_7$), 7.71 (m, 1H, $H_{4'}$), 7.77 (dd, 1H, J= 1.0 Hz, J= 8.1 Hz, $H_{6'}$), 7.82 (dd, 1H, J= 1.0 Hz, J= 7.7 Hz, $H_5$).; $^{13}C$ NMR (75 MHz, DMSO-$d_6$): δ 56.3 ($OCH_3$), 112.2 ($C_{3'}$), 112.6 ($C_7$), 119.0 ($C_{5'}$), 120.6 ($C_{1'}$), 123.6 ($C_{4'}$), 125.2 ($C_{6'}$), 128.7 ($C_5$), 131.4 ($C_6$), 138.6 ($C_4$), 140.3 ($C_9$), 151.4 ($C_8$), 160.5 ($C_2$), 162.4 ($C_{2'}$).; MS (ESI): *m/z* (%)= 304.13 (100) $[M - H]^-$., 289.07 (25), 198.05 (10); HRMS-ESI: *m/z* $[M - H]^-$ calcd for $C_{14}H_{10}NO_5S$: 304.0280; found: 304.0267.

**2-(3'-Methoxy-phenyl)-benzoxazole-4-sulfonic acid (6b).**

Red solid. Yield 60%. Mp slow decomp-250 °C; IR: 2921, 1609, 1549, 1496, 1245, 1190, 1062, 744, 702 $cm^{-1}$; $^{1}H$ NMR (300 MHz, $CDCl_3$/methanol-$d_4$): δ 4.05 (s, 3H, $OCH_3$), 7.14 (t, 1H, J= 7.4 Hz, $H_{5'}$), 7.23 (d, 1H, J= 8.2 Hz, $H_7$), 7.43 (t, 1H, J= 8.0 Hz , $H_6$), 7.57 (m, 1H, $H_{4'}$), 7.74 (d, 1H, J= 8.1 Hz, $H_{6'}$), 7.84 (s, 1H, $H_{2'}$), 8.20 (d, 1H, J= 7.7 Hz, $H_5$).; $^{13}C$ NMR (75 MHz, $CDCl_3$/methanol-$d_4$): δ 56.0 ($OCH_3$), 112.3 ($C_7$), 112.4 ($C_{4'}$)113.9 ($C_{1'}$), 121.0 ($C_{5'}$), 122.3 ($C_{2'}$), 124.5 ($C_{6'}$), 130.6 ($C_6$), 133.4 ($C_5$), 136.9 ($C_8$), 149.4 ($C_{3'}$), 157.6 ($C_2$).; MS (ESI): *m/z* (%)= 304.13 (100) $[M - H]^-$, 289.12 (30), 225.21 (10); HRMS-ESI: *m/z* $[M - H]^-$ calcd for $C_{14}H_{10}NO_5S$: 304.0280; found: 304.0271.

**2-(2'-Benzyloxy-phenyl)-benzoxazole-4-sulfonic acid (6c).**

Yellow powder. Yield 60%. Mp 180-185 °C; IR: 2923, 1549, 1450, 1419, 1249, 1197, 1051, 752 $cm^{-1}$; $^{1}H$ NMR (300 MHz, Methanol-$d_4$/$CDCl_3$): δ 5.21 (s, 2H, $\underline{CH_2}Ph$), 6.64 (d, 1H, J= 8.4 Hz, $H_{3'}$), 6.74 (t, 1H, J= 7.4 Hz, $H_{5'}$) , 6.90-7.05 (m, 6H, Ar & $H_{4'}$), 7.29 (t,1H, J= 7.8 Hz, $H_6$), 7.39 (d, 1H, J=7.9 Hz, $H_7$), 7.55 (d, 1H, J= 7.6 Hz, $H_{6'}$), 7.75 (d, 1H, J= 7.1 Hz, $H_5$).; $^{13}C$ NMR (75 MHz, Methanol-$d_4$/$CDCl_3$): δ 72.2 ($\underline{C}H_2Ph$), 112.8 ($C_{3'}$), 115.6 ($C_7$), 115.7 ($C_{1'}$), 121.8 ($C_{5'}$), 123.2 ($C_{4'}$), 125.1 ($C_{6'}$), 127.7 (Ar), 127.8 (Ar), 128.1 (Ar), 130.5 ($C_5$), 133.1 ($C_6$), 135.1 ($C_4$), 136.3 ($C_9$), 150.0 ($C_8$), 155.7 ($C_2$), 162.6 ($C_{2'}$).; MS (ESI): *m/z* (%)= 380.07 (95) $[M - H]^-$, 289.36 (100); HRMS-ESI: *m/z* $[M - H]^-$ calcd for $C_{20}H_{14}NO_5S$: 380.0588; found: 380.0593.

**2-(2'-Benzyloxy-phenyl)-benzoxazole-4-sulfonic acid ethyl ester.**

Gummy grey solid. Yield 20%.; IR: .2920, 1608, 1541, 1450, 1350, 1242, 1207, 1168, 1002, 914, 750 $cm^{-1}$; $^{1}H$ NMR (300 MHz, $CDCl_3$): δ 5.21 (s, 2H, $\underline{CH_2}Ph$), 6.64 (d, 1H, J= 8.4 Hz, $H_{3'}$), 6.74 (t, 1H, J= 7.4 Hz, $H_{5'}$), 6.90-7.05 (m, 6H, Ar & $H_{4'}$), 7.29 (t, 1H, J= 7.8 Hz, $H_6$), 7.39 (d, 1H, J= 7.9 Hz, $H_7$), 7.55 (d, 1H, J= 7.6 Hz, H6'), 7.75 (d, 1H, J= 7.1 Hz, $H_5$).; $^{13}C$ NMR (75 MHz, $CDCl_3$): δ 15.0 ($OCH_2\underline{C}H_3$), 68.3 ($O\underline{C}H_2CH_3$), 70.7 ($\underline{C}H_2Ph$), 113.6 ($C_{3'}$), 115.7 ($C_7$), 121.1 ($C_{5'}$), 124.2 ($C_{1'}$), 125.2 ($C_5$), 126.9 (Ar), 127.2 ($C_6$), 127.8 (Ar), 128.6 (Ar), 128.1 (Ar), 132.2 ($C_{6'}$), 133.7 ($C_{4'}$), 136.4 ($C_4$), 139.5 ($C_9$), 151.4 ($C_8$), 157.9 ($C_2$), 164.3 ($C_{2'}$).; MS (ESI): *m/z* (%)= 409.96 (100) $[M + H]^+$, 320.22 (10), 91.06 (50).; HRMS-CI: *m/z* $[M + H]^+$ calcd for $C_{22}H_{19}NO_5S$: 410.10621; found: 410.10633.

**2-(2'-Methoxy-phenyl)-benzoxazole-7-sulfonic acid (7a).**

Red solid. Yield 65%, Mp .slow decomp-210°C; IR: 2923, 1602, 1552, 1480, 1419, 1220, 1199, 817, 794 .$cm^{-1}$; $^{1}H$ NMR (300 MHz, DMSO-$d_6$): δ 3.89 (s, 3H, $OCH_3$), 7.24 (dd, 1H, J= 2.5 Hz, J= 7.9 Hz, $H_{3'}$), 7.35 (t, 1H, J= 7.8 Hz, $H_{4'}$), 7.55-7.62 (m, 2H, $H_5$ & $H_{5'}$), 7.69 (m, 1H, $H_{6'}$), 7.78 (m, 2H, $H_6$ & $H_4$); $^{13}C$ NMR (75 MHz, DMSO-$d_6$): δ 56.3 ($OCH_3$), 112.9 ($C_{3'}$), 118.8 ($C_4$), 121.2 ($C_{5'}$), 124.4 ($C_{4'}$), 124.9 ($C_6$), 128.5 ($C_5$), 131.5 ($C_9$), 133.1 ($C_7$), 143.0 ($C_8$), 146.7 ($C_8$), 160.5 ($C_{2'}$), 163.1 ($C_2$).; MS (ESI): *m/z* (%)= 304.17 (100) $[M - H]^-$, 289.30 (70), 261.36 (30); HRMS-ESI: *m/z* $[M - H]^-$ calcd for $C_{14}H_{10}NO_5S$: 304.0280; found: 304.0269.

**2-(2'-hydroxy-phenyl)-benzoxazole-4-sulfonic acid (9).**

Brown solid. Yield 95% Mp 197-200 °C; IR: 3174, 2921, 1631, 1548, 1454, 1421, 1247, 1054, 746 $cm^{-1}$; $^{1}H$ NMR (300 MHz, Methanol-$d_4$): δ 7.02-7.07 (m, 2H, $H_{3'}$ & $H_{4'}$), 7.41-7.48 (m, 3H, $H_6$, $H_{5'}$, OH), 7.81 (d, 1H, J= 8.2 Hz, $H_7$), 7.87 (d, 1H, J= 7.7 Hz, $H_{6'}$), 8.06 (d, 1H, J= 7.9 Hz, $H_5$); $^{13}C$ NMR (75 MHz, Methanol-$d_4$): δ 112.8 ($C_{1'}$), 115.3 ($C_7$), 119.9 ($C_{3'}$), 125.8 ($C_{6'}$), 127.3 ($C_{4'}$), 130.0 ($C_{5'}$), 133.8 ($C_5$), 136.5 ($C_6$), 137.8 ($C_4$), 139.3 ($C_9$), 152.5 ($C_8$, $C_{2'}$), 166.0 ($C_2$).; MS (ESI): *m/z* (%)= 289.87 (100) $[M - H]^-$; HRMS-ESI: *m/z* $[M - H]^-$ calcd for $C_{13}H_8NO_5S$: 290.0234; found: 290.0123.

**2-(2'-Methoxy-phenyl)-benzoxazole-4-sulfonic acid amide (10).**

White powder. Yield 90% Mp .slow decomp-200 °C; IR: 2918, 1544, 1423, 1328, 1251, 1157, 1139, 754 $cm^{-1}$; $^{1}H$ NMR (300 M Hz, DMSO-$d_6$): δ 3.91 (s, 3H, $OCH_3$), 7.27 (ddd, 1H, J= 0.8 Hz, J= 2.6 Hz, J=8.3 Hz, $H_{3'}$), 7.55-7.60 (m, 4H, $SO_2NH_2$ & $H_7$ & $H_{4'}$), 7.79-7.85 (m, 2H, $H_{5'}$ & $H_6$), 7.91 (dd, 1H, J= 1.2 Hz, J= 6.6 Hz, $H_{6'}$), 8.07 (dd, 1H, J= 0.8 Hz, J= 8.2 Hz, $H_5$).; $^{13}C$ NMR (75 MHz, DMSO-$d_6$): δ 55.9 ($OCH_3$), 113.0 ($C_{3'}$), 115.2 ($C_7$), 119.2 ($C_{5'}$), 120.7 ($C_{1'}$), 122.9 ($C_5$), 125.5 ($C_6$), 127.4 ($C_{1'}$), 131.0 ($C_{4'}$), 134.7 ($C_4$), 138.0 ($C_9$), 151.3 ($C_8$),

160.1 ($C_2$), 163.8 ($C_{2'}$).; MS (ESI): *m/z* (%)= 304.90 (100) $[M + H]^+$, 242.09 (20); HRMS-CI: *m/z* $[M + H]^+$ calcd for $C_{14}H_{12}N_2O_4S$: 305.05960; found: 305.05976.

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# CHAPTER 8. CONCLUSION AND FORESIGHTS

## 8.1 Results review

This research work ends here after four years of intense and challenging scientific investigations in the field of organic synthesis and biocatalysis. As a milestone worth mentioning, the laboratory developed an expertise in the field of oxidative coupling reactions in aqueous solutions, based upon multicopper oxidase catalysis, and in the HPLC analytical study of these biotransformations. A second achievement is the proof of concept for the application of the novel water-soluble phenoxazinone dyes in the field of cell biology with a hit in the fluorescence labelling of cellular process like the endocytosis. Let us briefly summarise hereafter the main achievements.

### *8.1.1 Organic synthesis; selective insertion of hydrophilic groups*

In the first section of the experimental results detailed in the manuscript, we elaborated a high yielding synthetic route to produce aminophenols bearing a free sulfonic or phosphonic acid in the adjacent position of the aniline. As such, the linear sequence of seven steps involves a hydrogen/metal exchange and subsequent insertion of oxidised sulfur (or phosphorus) moiety in the carbon-metal bond with a total selectivity. An easy purification of the free aromatic sulfonate has been developed, based on ion-exchange chromatography, inspired by the traditional synthesis of monobactams derivatives. Noteworthy, the serendipitous discovery of an intramolecular cyclisation of the vicinal amide chain provided both a key intermediate for creating molecular diversity in the aminophenol family, and a new field of investigation in the benzoxazole synthesis.

In the second section of experimental results, we could devise a complementary synthetic route leading to another key intermediate, a 7-sulfonated benzoxazole, which increased further the molecular diversity and more importantly gave access to regioisomers of our first pool of sulfonated building blocks. This proved to be rewarding as this synthetic effort led to the discovery of puzzling results in presence of the biocatalyst.

Lastly, regarding the organic synthesis, we took the intramolecular cyclisation of our sulfonated aminophenol derivatives one step further by developing an alternative synthetic route towards water-soluble benzoxazoles. Beyond the validation of our synthesis of hydrophilic oxazole derivatives using commercial orthoesters, we could implement an acid catalysed condensation reaction with anilide acetal reagents bearing various substituents. This synthetic scheme gave access to various 2-aryl substituted benzoxazoles bearing free sulfonic acid or esters. The illustration of the potential for such methodology was readily given with the production of the first sulfonated analogue of UK-1 metabolite which displays interesting fluorescence properties.

### *8.1.2 Organic synthesis; laccase as a biocatalyst*

As we had no experience in biocatalysis, this research required considerable efforts to go deeply enough in the field of catalysis and copper oxidase. Starting from scratch, we developed several analytical methods using HPLC with UV-detection or $MS/MS^2$ detection to study the key step of the oxidative cascade. This investment in analytical chemistry has been quite demanding but showed clearly the importance of an efficient HPLC method for optimising our biocatalytic system.

From UV-detection based on the specific absorption pattern of the phenoxazinone chromophore, the unambiguous identification of the desired product considerably helped for determining optimal experimental conditions. Lastly, it should be mentioned that our analytical method could be advantageously transferred to semi-preparative scale which results in the rapid isolation and structural elucidation of few model dyes.

Regarding the use of the biocatalyst itself, we have shown how the copper oxidases, and laccase particularly, are indeed well suited for organic synthesis. Sulfonanylated and phosphorylated aminophenols, functionalised at the *ortho* position of the aniline, gave in excellent yields the corresponding sulfonanylated /phosphorylated analogues of the natural pigment cinnabarinic acid (CA). As for the regioisomers, functionalised at the *ortho* position of the phenol, discrepancies appeared that could be related to the fact that the "orcein like" chromophores are markedly more prone to hydrolysis and degradation in solution.

As stated before, using analytical methods based on HPLC-$MS^2$ and by realising cross-coupled reactions, we could propose that indeed the laccase processes the aminophenol substrates in a similar fashion as does the well known phenoxazinone synthase.

Lastly, in collaboration with the Prof. Rescigno, we could determine a relative affinity of laccase for several substrates based on kinetic data collected with a semi-purified fungal laccase. Whereas these results are probably not easily extended to other laccases, based on the use of three different enzymatic preparations of biocatalysts, they suggest a general trend in reactivity.

### *8.1.3 Physico-chemical evaluations of hydrophilic compounds*

By combining several experimental approaches, we could evaluate few physico-chemical properties of the sulfonated/phosphorylated substrates such as water-solubility (up to $10^{-2}$ M) and anodic oxidation potentials (between 0.9 and 1.1 V, compared to the natural mediator 3HAA (1.03 V)) for examples. Regarding our novel phenoxazine dyes, we have also evaluated their solubility and fluorescence properties. The relative quantum yields, while rather low in aqueous solution, were in the range of 0.001 to 0.02. Whereas these are markedly lower regarding commercial fluorophores, they still led to interesting applications. The phosphorylated analogue of cinnabarinic acid in particular bestows a marked chelating activity towards divalent cations which results in a quenching of its fluorescence.

### *8.1.4 Application in molecular biology and medicinal chemistry*

We here provide the first report of the synthesis of a series of water-soluble aminophenoxazinone dyes and of their evaluation for vital imaging in cell biology. Among the first generation of tested dyes, one dye appears particularly promising as a presumably bulk endocytic tracer with targeting by default to lysosomes. Despite a 10-fold lower quantum yield as compared with Lucifer Yellow, compound **8c** generated intracellular signals of comparable intensities.

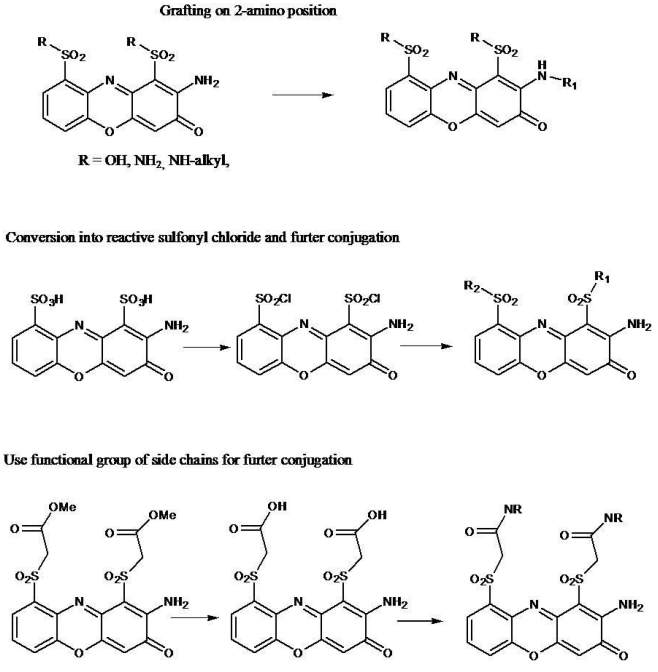
As mentioned previously, benzoxazoles are known for several bioactivities among which sulfonated oxazole derivatives bestows an inhibitory effect upon carbonic anhydrase enzymes. Therefore, we have tested the small library of sulfonated benzoxazoles for the inhibition of human CA-I. Unfortunately, these proved to be poor inhibitors for such isozyme.

## 8.2 Foresights

From the experimental results collected through these four years of scientific researches, there are few fields of investigations which could open new developments.

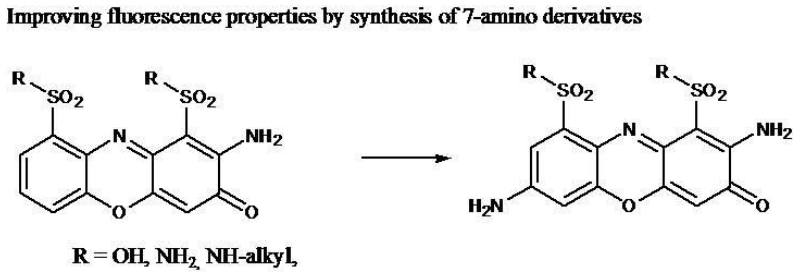
First, regarding the live cell imaging application, it remains to be tested whether this useful "compensation" (good labelling albeit having a lower relative quantum yield) observed with our best fluorophore could be ascribed to the benefit of membrane adsorption or to an effect of increased fluorescence in acidic media. All these dyes feature at least one free reactive function for further conjugation to biomolecules and grafting to materials, which opens the route towards selective labelling experiments (see scheme 64).

Scheme 64. Potential position for further conjugation of the dyes



If needed, using a similar method developed for the natural actinomycin D chromophore one could realise the insertion of amino group at the C7 position which has been shown to improve greatly the fluorescence properties of phenoxazinone compounds (see Scheme 65).[106, 140]

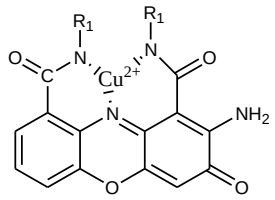
Scheme 65. 7-amino derivatives as lead for improved fluorophores



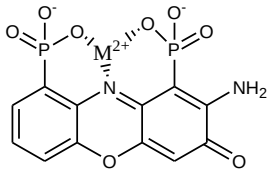

In addition, the quenching of fluorescence observed for the 2-amino-3-oxo-*3H*-phenoxazine-1,9-diphosphoric acid dye which happened in an aqueous media doped by $Mg^{2+}$ and/or $Ca^{2+}$ could inspire us for developing a probe of divalent cations. Regarding the plausible modes of metal chelation by the phosphorylated dye, few suggestions could be made. First, a recent publication linked the bioactivity of the actinomycin D to an efficient complexation of copper ion ($Cu^{2+}$). This could initiate oxidative stress at the proximity of the DNA.[217] Two copper binding sites were proposed based on ab-initio calculation and potentiometric experiments (See Scheme 66). Yet, this does not entail a same binding mode for $Mg^{2+}$ and $Ca^{2+}$ by the phosphorylated dye.

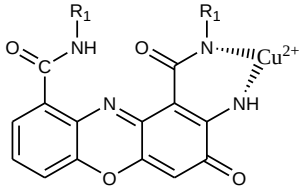
[217] Szcepanik, W.; Kaczmarek, P.; Jezowska-Bojczuk, M, *J. Inorg. Biochem.*. **2004**, *98*, 2141-2148.

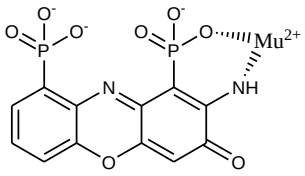
Scheme 66. Plausible copper binding sites of actinomycin D




actinomycin D complexation of copper



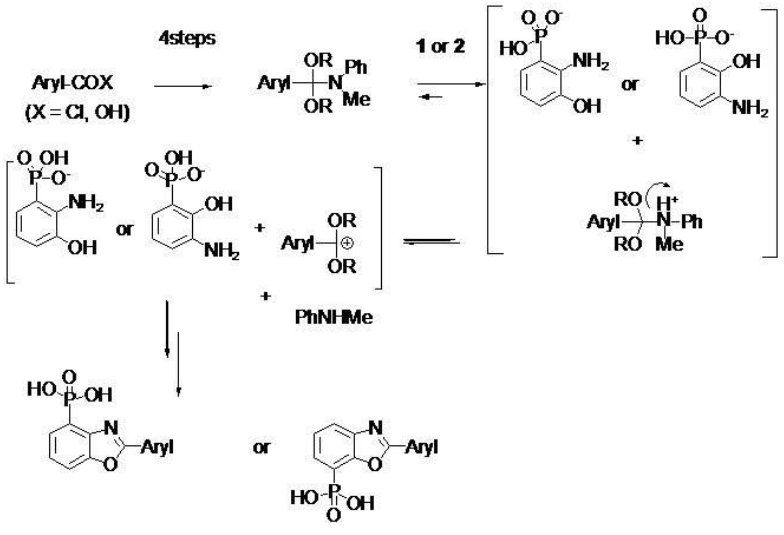



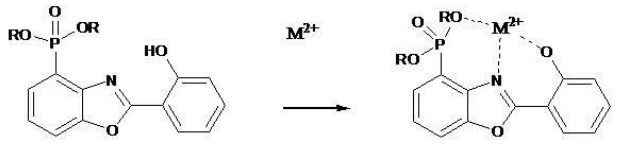

Plausible mode of complexation of divalent cations

Third, we have disclosed two routes allowing the preparation of 4- or 7-sulfonated benzoxazoles under very mild conditions. The compounds present various substitutions at position 2 (H, alkyl, aryl) and an increased water-solubility. Taking advantage of these synthetic routes it is possible to produce novel phosphorylated benzoxazoles and exploiting their metal chelation properties (see Scheme 67 and 68). This chelating ability may be of interests in bioactive compounds, like for the UK-1 cytotoxicity, or for producing ligand for metal catalysis in aqueous solutions (Mizoroki-Heck reaction). Here again, the bioactivity of the secondary metabolite UK-1, which has been shown to intercalate itself into DNA, is presumably linked to the specific chelation of copper ($Cu^{2+}$) or magnesium ($Mg^{2+}$). The copper chelate would be again a source of oxidative stress in the vicinity of the DNA. In the case of the $Mg^{2+}$ cation, it has been proposed that magnesium chelate further stabilise the intercalation of benzoxazole UK-1.

Scheme 67. Synthesis of novel phosphorylated benzoxazoles



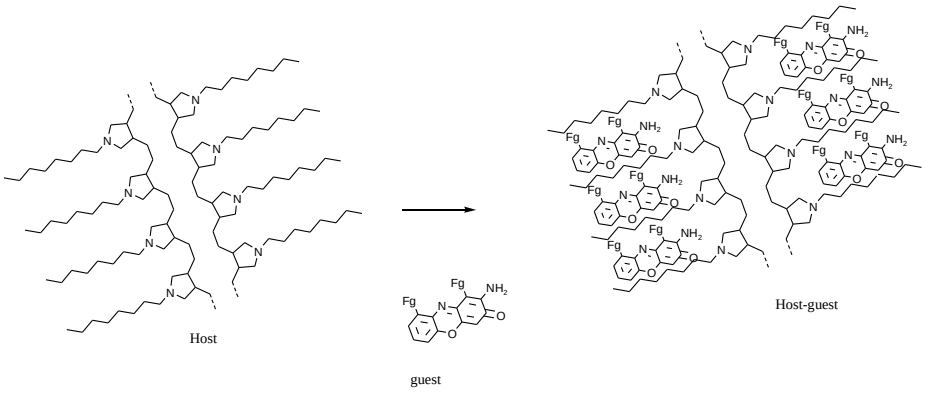

Scheme 68. Phosphorylated benzoxazoles as a lead for novel ligand




R = H, Alkyl

Fourth, several of our aminophenols derivatives and phenoxazinone dyes could be used in the frame of the Concerted Research Programme ARC 08/13-009 (UCL, Belgium). Based upon a « host-guest interactions » principle, by inserting selected organic molecules in an organic polymer as « smart host matrix » (see Scheme 69) we may develop "smart material" for specific detection ($O_2$, $Cu^{2+}$, $H_2O_2$, $Mg^{2+}$/$Ca^{2+}$ for examples) Taking advantage of the properties of a zwitterionic organic polymer matrix (Scheme 69) displaying supramolecular organisation (published results of F. Rullens *et al*)[218], the host matrix can be tuned in term of physical properties, solubility and functionality.

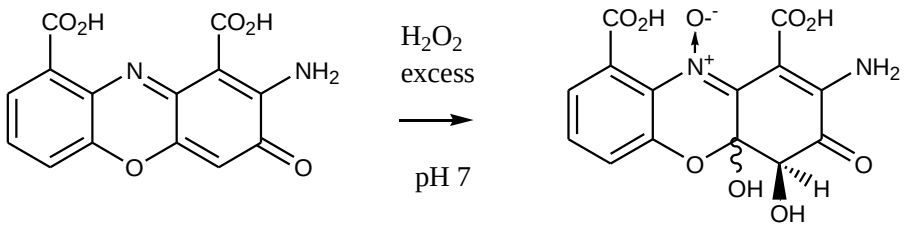
Scheme 69. Hypothetical lamellar arrangements of an amphiphilic organic polymers and the plausible insertion of a guest molecule




[218] Rullens, F.; Devillers, M.; Laschewsky, A, *Macromol. Chem. phys*, **2004**, *205*, 1155-1166.

Let us close the subject by adding few words regarding the aminophenoxazinone derivatives as potential probes for hydrogen peroxide. Manthey *et al* have shown that cinnabarinic acid, in presence of an excess of hydrogen peroxide and a buffered medium at pH 7, decompose itself to yield a mixture of two heterocyclic compounds highly fluorescent. The exact structures of those compounds were elucidated and show to be isomeric hemiketals Structures of those compounds are given in the scheme 70 below.

Scheme 70. Decomposition of cinnabarinic acid in presence of $H_2O_2$




moderatly fluorescent

Highly fluorescent

Cinnabarinic acid

A similar behaviour of our sulfonylated and phosphorylated dyes would open new perspectives either for developing a sensor of oxidative stress in cells or to produce doped polymers which would become highly fluorescent when submitted to an increase of the local concentration of hydrogen peroxide.

## 8.3 Publications and citation tracking

Hereafter, scientific publications are summarised and related either to previous research work or both the published results from the thesis work and the publications that will be shortly submitted.

In addition, the citations tracking related to our first publication in the field of biocatalysis is detailed. In 2009, three citations from others scientific groups were reported.

***Articles published during 2003-2009***

1. Jeanjot, P.; Bruyneel, F.; Arrault, A.; Gharbi, S.; Cavalier, J.-F. ; Abels, A.; Marchand, C.; Touillaux, R.; Rees, J.-F.; Marchand-Brynaert, J., N-(Alkyl)-2-amino-1,4-pyrazine Derivatives: Synthesis and Antioxidative Properties of 3- and 3,5-p-Hydroxyphenyl-Substituted Compounds. *Synthesis* **2003**, (04), 0513-0522.

2. Van Quaquebeke, E.; Simon, G.; Andre, A.; Dewelle, J.; Yazidi, M. E.; Bruyneel, F.; Tuti, J.; Nacoulma, O.; Guissou, P.; Decaestecker, C.; Braekman, J.-C.; Kiss, R.; Darro, F., Identification of a Novel Cardenolide (2‘‘3-Oxovoruscharin) from Calotropis procera and the Hemisynthesis of Novel Derivatives Displaying Potent in Vitro Antitumor Activities and High in Vivo Tolerance: Structure & Activity Relationship Analyses. *Journal of Medicinal Chemistry* **2005,** 48, (3), 849-856.

3. Bruyneel, F.; Enaud, E.; Billottet, L.; Vanhulle, S.; Marchand-Brynaert, J., Regioselective Synthesis of 3-Hydroxyorthanilic Acid and Its Biotransformation into a Novel Phenoxazinone Dye by Use of Laccase. *European Journal of Organic Chemistry* **2008**, (1), 72-79.

4. Bruyneel, F.; Payen, O.; Rescigno, A.; Tinant, B.; Marchand-Brynaert, J., Laccase-Mediated Synthesis of Novel Substituted Phenoxazine Chromophores Featuring Tuneable Water Solubility. *Chemistry - A European Journal* **2009,** 15, (33), 8283-8295.

5. Estelle Enaud, Marie Trovaslet, Frédéric Bruyneel, Ludovic Billottet, Rezzan Karaaslan, Mehmet E. Sener, Paul Coppens, Ana Casas, Ismene J. Jaeger, Christof Hafner, Rob C.A. Onderwater, Anne-Marie Corbisier, Jacqueline Marchand-Brynaert, Sophie Vanhulle, A Novel Azoanthraquinone Dye Made Through Innovative Enzymatic Process. *Dyes and Pigments* **2009**, submitted.

***Patent filled in 2009***

6. Phenoxazine dyes, F. Bruyneel, A. Jarosz, J. Polak, C.-M. Bols, S. Vanhulle, E. Enaud, R. Basosi, R. Pogni, J.I. Jager, C. Hercher, J. Marchand-Brynaert, (Patent, PCT/EP2009/058640).

***Publication to be submitted***

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***Supporting Material***

Supporting material for this manuscript can be obtained from the authors in the research group of Professor J. Marchand-Brynaert. It regroups mainly the already mentioned supporting material of published articles and those of the articles to be submitted. In addition, several experimental procedures of interest, for example analytical methods for HPLC analysis, may also be found.