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Radicaux stables de type trityle en tant qu'agents de contraste pour l'imagerie moléculaire par RPE : synthèse de dérivés fonctionnalisés, élaboration de conjugués peptidiques, et caractérisations physico-chimiques

Thèse présentée en vue de l'obtention

du grade de Docteur en Sciences

par

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Abstract

Biomedical electron paramagnetic resonance (EPR) spectroscopy and imaging (EPRI) are techniques allowing *in vivo* assessment of certain physiological parameters such as oxgen concentration, pH, tissues redox status, etc. By contrast with magnetic resonance imaging (MRI), widely used in clinics, which uses the nuclear spin of endogen water's protons, *in vivo* EPR spectroscopy and EPRI need the use of a spin label (a free radical).

This multidisciplinary work focuses on the development of new spin labels, of type triarylmethyl (TAM) radical, for biomedical EPR applications.

We developed a water soluble phosphonated TAM radical used to measure pH *in vivo* in a physiological range while fluorinated TAMs were synthetized to asset oxygen concentration with high sensitivity using biocompatible perfluorocarbon formuations.

The static and dynamic stereochemistry of TAM alcohols, alkanes and radicals were also studied. Theses molecules adopt a propeller shape conformation allowing to atropoisomeric conformations to occur that feature enantiomeric relationship. Due to high steric hindrance, the enantiomerization between the two enantiomers is restricted, a phenomenon which is responsible for the chiral properties of TAM compounds.

Finally, we synthesized tumor targeted spin labels by binding the TAM radical to targeting moieties such as a small peptide containing the Arg-Gly-Asp (RGD) sequence or a home made peptidomimetic of this sequence.

Résumé

La spectroscopie et l'imagerie par résonance paramagnétique électronique (RPE) biomédicales sont des techniques permettant la mesure *in vivo* et de façon non invasive de certains paramètres physiologiques tels que l'oxygénation, le pH ou le status rédox. Par opposition à l'imagerie de résonance magnétique (IRM) nucléaire, largement utilisée en clinique, et qui met à profit les propriétés magnétiques de l'eau endogène au patient, la RPE nécessite l'utilisation d'un traceur (un radical stable).

Ce travail de thèse a été consacré au développement de nouveaux traceurs de type triarylméthyle (TAM) pour la RPE biomédicale.

Nous avons développé un radical TAM phosphoré soluble en milieu physiologique permettant la mesure du pH alors que la synthèse de TAMs fluorés a permis la mesure de l'oxygénation tissulaire avec une haute sensibilité en utilisant des formulations de perfluorocarbones.

La stérochimie statique et dynamique des composés TAM a également été étudiée. Ces molécules adoptent une conformation en hélice. En raison de l'encombrement stérique, l'isomérisation entre les deux conformations énantiomériques (hélice droite et hélice gauche) est limitée. Ce phénomène est responsable de la chiralité de ces molécules.

Enfin, des traceurs TAMs liés de façon covalente à un peptide de séquence Arg-Gly-Asp (RGD) ou peptidomimétique de cette séquence ont été synthétisés de façon à diriger ces traceurs vers des cellules cancéreuses.

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Abréviations et acronymes

Français

AM	Acétoxyméthyle
NADPH	Nicotinamide adénine dinucléotide phosphate
OEM	Onde électromagnétique
QM	Quinone méthide
RPE	Résonance paramagnétique électronique
RMN	Résonance magnétique nucléaire
SN	Substitution nucléophile
SQM	Semi-quinone-méthide

Anglais

ACN	Acetonitrile
a_X	Hyperfine splitting constant
BOC	Butoxycarbonyl
BOLD	Blood oxygen level dependent
BOP	(benzotriazol-1-yloxy)tris(diethylamino)phosphonium hexafluorophosphate
CD	Circular dichroism
CMC	Carboxy methyl cellulose
COSY	Correlation spectroscopy
CSA	Chiral solvating agent
CSP	Chiral solvating phase
CW-EPR	Continuous wave EPR
DCE	Dichloroethane
DCM	Dichloromethane
DFT	Density fonctional theory
DIEA or DIEPA	Diisopropylethylamine
DMP	Dess-Martin periodinane
EDTA	Ethylenediaminetetraacetic acid
EPRI	EPR imaging
EXSY	Exchange spectroscopy
FMT	FLuoresence mediated tomography
FT-EPR	Fourier transform EPR
HetCor	Heteronuclear correlation
HFB	Hexafluorobenzene
Hfs	Hyperfine splitting
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HMDS	Hexamethyldisilazane also used for Hexamethyldisiloxane
HMPA	Hexamethylphorphoramide
HMQC	Heteronuclear multiple-quantum correlation
HPLC	High performance liquid chromatography
IR	Infra-red
MS	Mass spectrometry

MR	Magnetic resonance
MRI	Magnetic resonance imaging
NIRS	Near infra-red spectroscopy
NLO	Non linear optical response
NMR	Nuclear magnetic resonance
nOe	Nuclear Overhauser effect
NOESY	Nuclear Overhauser effect spectroscopy
NR	Nitroxide
OMRI	Overhauser magnetic resonace imaging
PDA	Photodiode aray
PDMS	Polydimethylsiloxane
PET	Positron emission tomography
PFC	Perfluorocarbon
PFOB	Perfluorooctylbromide
pO2	Oxygen partial pressure
PTM-TE	Perchlorotriphenylmethyl triester
PVP	Polyvinyl pyrolidinone
RP	Reverse phase
RT	Retention time also used for room temperature
SM	Starting material
SPECT	Single-photon emission computed tomography
TAM	Triarylmethyl
TLC	Thin layer chromatography
TFA	Trifluoroacetic
THF	Tetrahydrofurane
TMEDA	Tetramethylethylenediamine
TMS	Trimethylsilyl
TS	Transition state
USPIO	Ultra small particules of iron oxyde

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1. Introduction

Le travail de recherche entrepris lors de cette thèse de doctorat a été consacré à la conception, la synthèse et l'évaluation de nouveaux radicaux stables de type tétrathiatriarylméthyle **1** (Figure 1) utilisés comme sondes paramagnétiques pour des applications en résonance paramagnétique électronique (RPE) biomédicale.

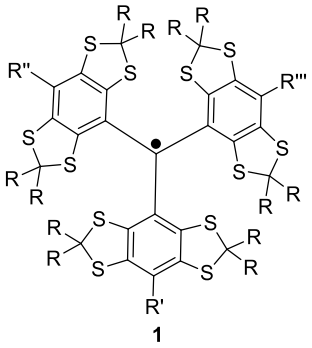


Figure 1: Radicaux tétrathiatriarylmétyles utilisés en RPE biomédicale.

La richesse et la complexité de ce thème de recherche résident dans une interaction étroite entre disciplines très variées comme la pharmacie, la biologie, la physique ou la chimie. C'est davantage avec un éclairage de chimiste que cette thématique sera présentée. Ainsi, l'introduction de ce manuscrit est consacrée à la synthèse et à la réactivité des radicaux tétrathiatriarylméthyles. Nous verrons avec quelques exemples comment ces dernières années, quelques modifications structurales ont permis d'étendre le champ d'applications de ces molécules. Cependant, afin de rendre compréhensible la suite du manuscrit pour le lecteur non initié à la RPE, la première partie de ce chapitre s'attachera à présenter très brièvement les principes de base de la RPE ainsi que deux applications dans le domaine biomédical en lien direct avec cette thèse, à savoir, l'oxymétrie et la pH-métrie par RPE.

1.1 La résonance paramagnétique électronique biomédicale[1]

La première expérience de résonance paramagnétique électronique fut réalisée par le physicien Russe Yevgeny Konstantinovich Zavoisky en 1944. Cette technique est basée sur l'absorption d'une onde électromagnétique (OEM) de fréquence appropriée par un échantillon paramagnétique placé dans un champ magnétique. La RPE est donc utilisée pour l'étude de systèmes possédant un ou plusieurs électrons célibataires.

Le principe est le suivant: l'électron possède un spin (un moment angulaire), il se comporte comme s'il tournait sur lui-même; à ce moment angulaire est associé un moment magnétique. L'électron peut être imaginé comme une petite toupie aimantée tournant sur elle-même. L'électron étant une particule quantique, son comportement est régi par les lois de la mécanique quantique et ces lois nous apprennent que pour l'électron, deux états de spin désignés par les lettres grecques α et β existent. Ces deux états diffèrent dans l'orientation des moments angulaires dans l'espace qui sont de sens opposés, mais pas dans leur grandeur (Figure 2).

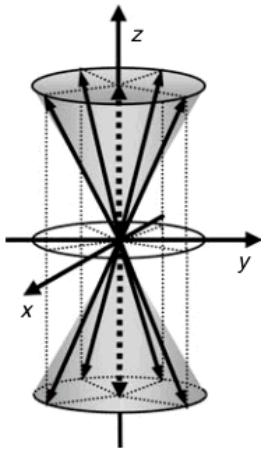


Figure 2: Représentation des moments angulaires de spin α et β de l'électron (*reproduit de ref[1]).*

En absence de champ magnétique extérieur, ces deux états possèdent la même énergie (ils sont dégénérés). Par contre, en présence d'un champ magnétique extérieur B_0, il y a une levée de dégénérescence, appelée effet Zeeman (Figure 3), et les deux états se séparent d'une énergie $\Delta E=g|\mu_B|B_0$ où g est le facteur de Landé (souvent simplement appelé facteur

g). Ce facteur est caractéristique de l'espèce étudiée et analogue au déplacement chimique en RMN, μ_B est le magnéton de Bohr et B_0 le champ magnétique extérieur. (Remarque: le champ magnétique en RPE est souvent exprimé en gauss (CGS) et correspond à 10^{-4} tesla (SI)).

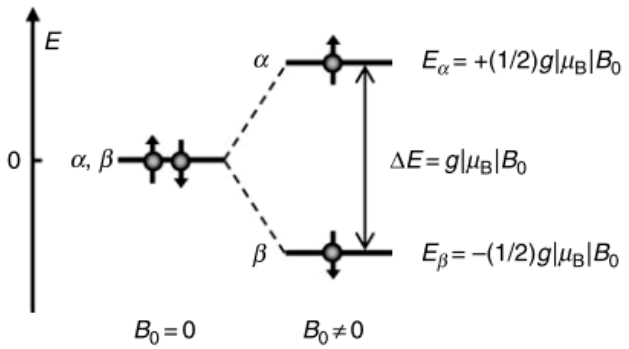


Figure 3: Effet Zeeman sur le spin électronique *(reproduit de ref[1]).*

Dans un échantillon macroscopique, les spins électroniques α et β se répartissent suivant une distribution de Maxwell-Boltzmann, à température ambiante, il en résulte un excès de spins dans l'état β, de plus faible énergie (1/1000 à température ambiante et pour un champ magnétique de 3000 G). Une onde électromagnétique d'énergie égale à la différence entre ces deux niveaux $h\nu = \Delta E = g|\mu_B|B_0$ sera capable de provoquer des transitions de type β → α s'accompagnant de l'absorption d'un photon hν et des transitions de type α → β s'accompagnant de l'émission d'un photon hν (émission spontanée). Les spins β étant majoritaires, il en résulte une absorption nette de l'onde électromagnétique (nombre de transitions β → α > α → β). En pratique, la condition de résonance $h\nu = g|\mu_B|B_0$ est déterminée par irradiation constante de l'échantillon alors que le champ magnétique est balayé: c'est l'expérience RPE à onde continue (CW-EPR). La détection de l'absorption est rendue plus sensible par l'utilisation d'un dispositif de modulation de champ et fournit le spectre d'absorption sous la forme de sa dérivée première (Figure 4).

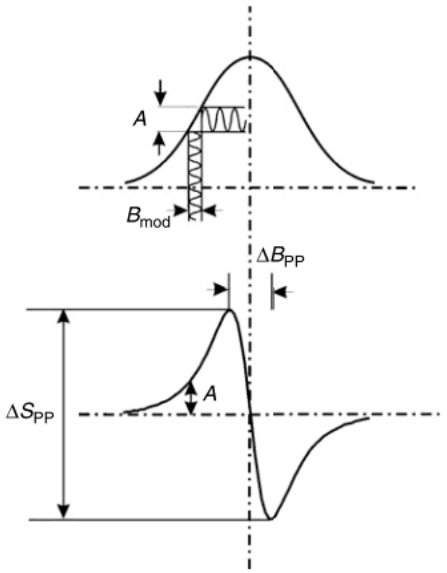


Figure 4: Spectre d'absorption et sa dérive première *(reproduit de ref[2]).*

Plus récemment, des spectromètres RPE à transformée de Fourier (FT-EPR) ont été développés et sont disponibles commercialement; cependant, leur utilisation se limite à l'étude d'espèces paramagnétiques caractérisées par des temps de relaxation longs.

L'irradiation à la fréquence de résonance du système de spins va avoir comme conséquences d'une part de tendre à égaliser les populations de spins α et β et d'autre part de provoquer une précession en phase des moments magnétiques. Une fois excité, le système tend à revenir à son état d'équilibre thermodynamique. On distingue la relaxation T_1 (ou spin-réseau), correspondant à la restauration de l'excès de population des spins β, alors que la relaxation T_2 (ou spin-spin) correspond à un déphasage progressif des moments magnétiques.

L'intensité du signal augmente avec la différence de population des spins α et β, par conséquent, plus le champ magnétique extérieur sera important, plus le signal sera intense et plus la fréquence de l'OEM pour satisfaire à la condition $h\nu = g|\mu_B|B_0$ sera élevée. Le spectromètre le plus classique utilise un champ magnétique de 3400 Gauss, correspondant à une fréquence de l'onde électromagnétique de ≈ 9.5 GHz (gamme des μ-ondes) appelée bande-X (Table 1). A cette fréquence, l'absorption non résonante par des solvants à constante diélectrique élevée comme l'eau pose problème pour des applications *in-vivo.* Alors qu'en bande-X, la pénétration de l'OEM dans un système aqueux est de l'ordre du

millimètre, elle atteint le centimètre en bande-L (≈ 1 GHz). Des spectromètres bande-L ou de fréquences encore plus faibles sont utilisés pour des études *in-vivo*. Malheureusement, le prix à payer est une diminution de la sensibilité. A l'inverse, des spectromètres de hautes fréquences existent pour des études *in-vitro*.

Fréquence (GHz)	**Champ magnétique (G)**	**Désignation**
1	360	Bande-L
9.5	3400	Bande-X
36	13000	Bande-Q
95	34000	Bande-W

Table 1: Fréquences et champs magnétiques de spectromètres RPE couramment utilisés.

Cette propriété de spin n'est pas l'apanage de l'électron, certains noyaux possèdent également un moment magnétique de spin non nul. Cette propriété est à la base de la résonance magnétique nucléaire (RMN). Le moment angulaire nucléaire peut prendre 2*I* + 1 orientations, ou *I* est le nombre quantique de spin nucléaire et est une caractéristique du noyau étudié (table 2).

Isotope	*I*
^{1}H, ^{19}F, ^{31}P, ^{15}N, ^{13}C	1/2
^{2}H, ^{14}N	1
^{12}C, ^{32}S	0

Table 2: Nombres quantiques de spin de quelques noyaux.

Lorsque l'électron célibataire est en interaction avec un noyau possédant un spin nucléaire non nul, une multiplicité apparaît sur le spectre RPE, faisant apparaître 2*I* + 1 raies. Ce sera le cas d'un radical conjugué à un ^{14}N, le spectre se présentera sous la forme de 3

raies séparées d'une distance a_N appelée constante de couplage hyperfine. En plus de la position du signal traduite par la valeur du facteur g, la ou les constantes de couplage sont également caractéristiques de l'espèce étudiée (Figure 5).

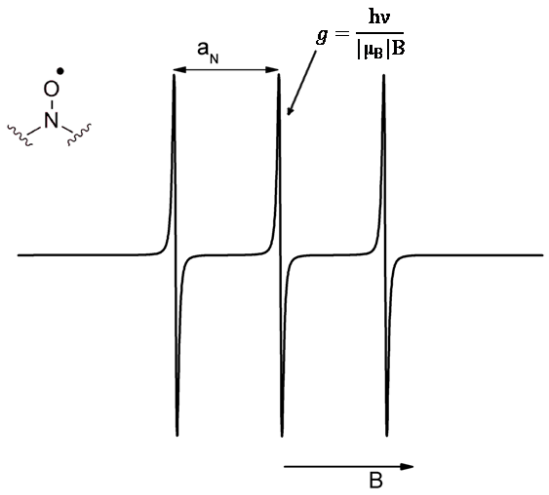


Figure 5: Spectre simulé d'un radical nitroxide en interaction avec un ^{14}N.

Si l'électron est en interaction avec n_a noyaux équivalents A et n_b noyaux équivalents B, le nombre de raies obtenues sera: $(2n_aI_A + 1)*(2n_bI_B + 1)$. De manière générale, le nombre de raies N sera:

$$N = \prod(2n_i I_i + 1) \qquad [1]$$

1.1.1 Oxymétrie par RPE[3, 4]

L'oxygène est caractérisé par un état fondamental paramagnétique possédant deux électrons célibataires dans deux orbitales moléculaires antiliantes (Figure 6). Alors qu'il est possible d'enregistrer le spectre RPE de l'oxygène en phase gazeuse et pour une faible pression partielle en oxygène (ex. 1 mmHg),[5] aucun spectre de l'oxygène n'a été rapporté en solution en raison de temps de relaxation extrêmement courts. En effet on peut démontrer que la largeur de raie RPE est inversement proportionnelle au T_2. Pour l'oxygène, les raies sont si larges qu'elles sont confondues avec la ligne de base.

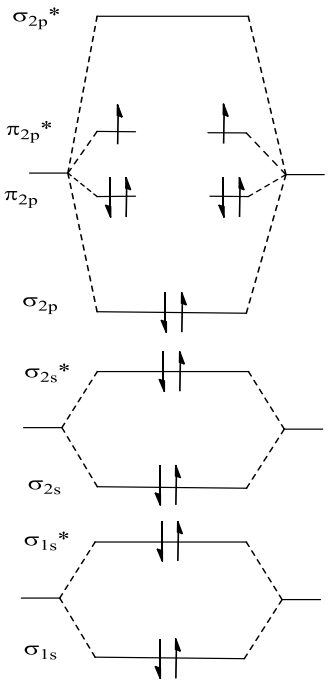


Figure 6: Configuration électronique de l'oxygène à l'état fondamental.

La mesure de l'oxygène par RPE se base sur une méthode indirecte: l'oxygène agit comme agent de relaxation sur une autre espèce paramagnétique qui servira de sonde. L'interaction entre l'oxygène triplet et une autre espèce paramagnétique utilisée comme sonde va induire une diminution des temps de relaxation T_1 (relaxation spin-réseau) et T_2 (relaxation spin-spin) de cette sonde. L'importance de cet effet pourra être reliée à la quantité d'oxygène en interaction avec la sonde. La méthode la plus aisée pour évaluer l'effet de l'oxygène est de mesurer son incidence sur le T_2, ceci par mesure de la largeur de raie RPE à mi-hauteur pour le spectre en absorption et la distance pic à pic B_{pp} pour le spectre en dérivée première. **L'oxygène aura donc pour effet un élargissement du signal RPE d'une autre espèce paramagnétique introduite comme sonde**. Après calibration *in vitro*, la mesure de la largeur de raie de la sonde introduite *in vivo* permet d'évaluer l'oxygénation (Figure 7). Deux types de sonde sont couramment utilisés pour cette

application, les sondes particulaires ((na)phtalocyanines de lithium, charbons, noirs de carbone) et les sondes solubles (radicaux nitroxides et triarylméthyles (TAM)).[4]

Pour les sondes solubles, il existe une relation linéaire entre la largeur de raie et la concentration en oxygène dissous. G.E. Pake et T.R. Tuttle[6] ont défini la relation entre l'élargissement de la largeur de raie en fonction de la vitesse de collision radical-radical en solution comme étant :

$$LW = kp\omega + N. \quad [2]$$

Où LW est la largeur de raie RPE ("linewidth"), k est une constante de proportionnalité, p est la probabilité que l'échange se fasse à chaque collision ($0 \leq p \leq 1$) et ω est la fréquence de collision. N représente d'autres contributions à la largeur de raie RPE indépendantes de ω. La variation de largeur de raie en fonction de la variation de vitesse de collision est donc :

$$\Delta LW = kp\Delta\omega. \quad [3]$$

La vitesse de collision est quant à elle gouvernée par l'équation de Smoluchowski :

$$\omega = 4\pi R\left(D_{O_2} + D_{sonde}\right)[O_2]. \quad [4]$$

Où R est la distance d'interaction oxygène-sonde, D_{O_2} et D_{Sonde} sont les constantes de diffusion de l'oxygène et de la sonde paramagnétique, $[O_2]$ est la concentration en oxygène dissous. Une variation de concentration en oxygène engendrera une variation de la vitesse de collision selon :

$$\Delta\omega = 4\pi R\left(D_{O_2} + D_{sonde}\right)\Delta[O_2]. \quad [5]$$

En combinant [3] et [5] on obtient :

$$\Delta LW = 4\pi Rkp\left(D_{O_2} + D_{sonde}\right)\Delta[O_2]. \quad [6]$$

On a donc une variation linéaire de la largeur de raie en fonction de la concentration en oxygène (Figure 7).

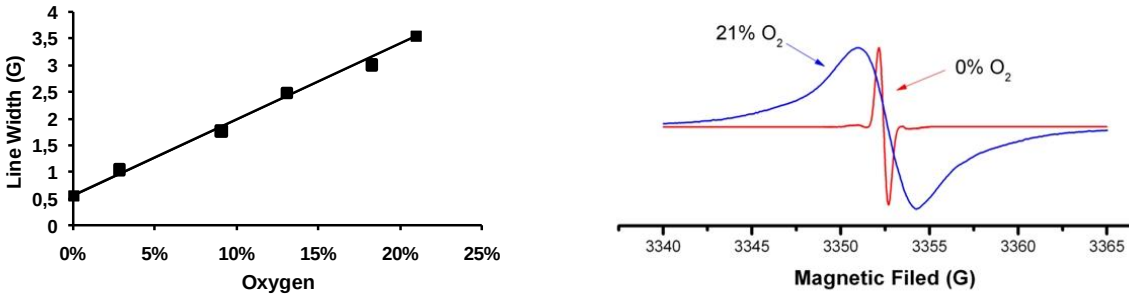


Figure 7 : Effet de l'oxygène sur la largeur de raie.

Pour les sondes particulaires, la relation entre largeur de raie et la quantité d'oxygène est plus complexe et dépend du matériau utilisé. Mis à part pour certains dérivés de (na)phtalocyanine de lithium, cette relation n'est pas linéaire et une courbe de calibration est utilisée.

Les sondes particulaires peuvent être d'origine synthétique, semi-synthétique ou naturelle. De manière générale, elles possèdent comme avantage de présenter une grande stabilité *in vivo*, une grande sensibilité à l'oxygène et une grande densité de spin permettant une bonne sensibilité de détection. Leur spectre RPE se présente souvent sous la forme d'un singulet ; parmi ces sondes on retrouve les cristaux de phtalocyanine ou naphtalocyanine de lithium d'origine synthétique. (Figure 8) Dans cette famille, les dérivés "oxo" substitués sont les plus stables, il a ainsi été montré que le dérivé LiNc-OBu présente une stabilité *in vivo* de plus de 6 mois. [7]

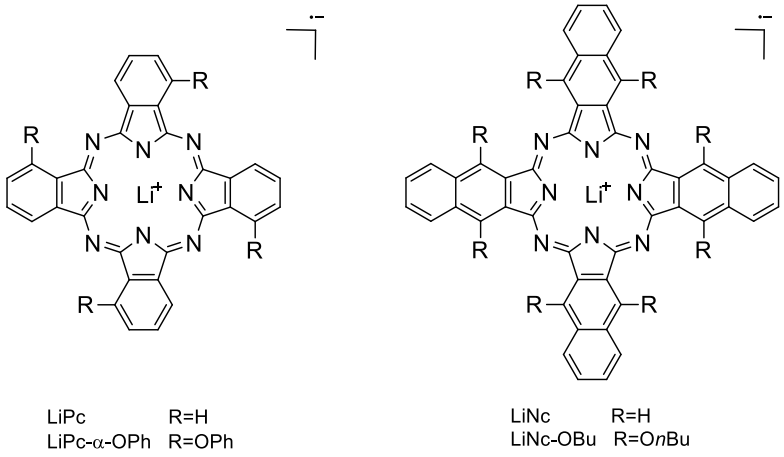


Figure 8 : Phatlocyanines et naphtalocyanines d'origine synthétique.

Une voie d'accès à ces dérivés est la cyclotétramérisation d'un dérivé *ortho*-dicyano suivie d'une oxydation mono-électronique. Le schéma 1 présente en exemple, la synthèse en deux étapes de LiPc-α-OPh par cyclotétramérisation du 3-phenoxyphtalonitrile suivie d'une oxydation électrochimique.[8]

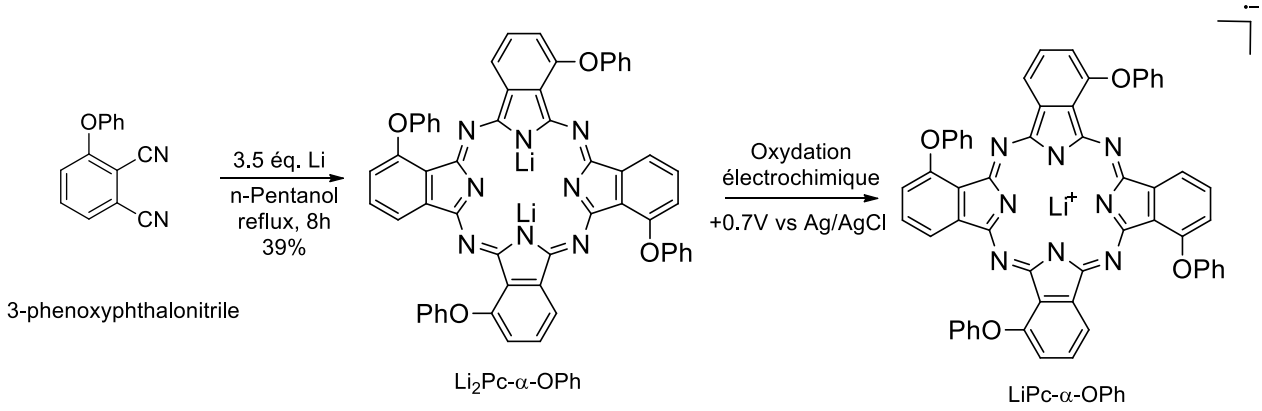


Schéma 1: Synthèse du LiPc-α-OP.

Une autre classe de sondes particulaires utilisées en oxymétrie par RPE est constituée par des dérivés du charbon ; ils peuvent être d'origine naturelle, issus de la lente minéralisation de végétaux au court du temps et extraits des couches géologiques profondes (les funisites[9] et gloxy[10]) ou être d'origine semi-synthétique, obtenus par la carbonisation de matériaux (charbons de bois[11], charbons de polysaccarides[12], etc.).

Certaines encres qui sont des suspensions de noir de carbone présentent également des spectres RPE sensibles à l'oxygène. Les premiers essais d'oxymétrie RPE *in vivo* chez l'homme ont été réalisés avec des encres de Chine.[13] Elles présentent l'avantage d'être déjà autorisées pour un usage clinique en Europe et aux USA puisqu'elles sont utilisées comme agents de marquage chirurgical ou pour des tatouages. Ces premières encres ne possèdent cependant pas des propriété optimales pour l'oxymétrie RPE (faible densité de spin, spectre multi-composants) ; des nouvelles encres plus performantes composées de suspension de noir de carbone dispersé dans une matrice de polymère biocompatible ont été developpées (ex: Printex U dispersé dans une matrice (CMC: carboxy methyl celulose ou PVP: polyvinyl pyrrolidinone).[14]

La sensibilité à l'oxygène varie fortement d'un type de sonde à l'autre (Figure 9), de plus les charbons et noirs de carbone sont plus sensibles aux faibles pO_2 alors que les dérivés de (na)phtalocyanine présentent une relation plus linéaire.

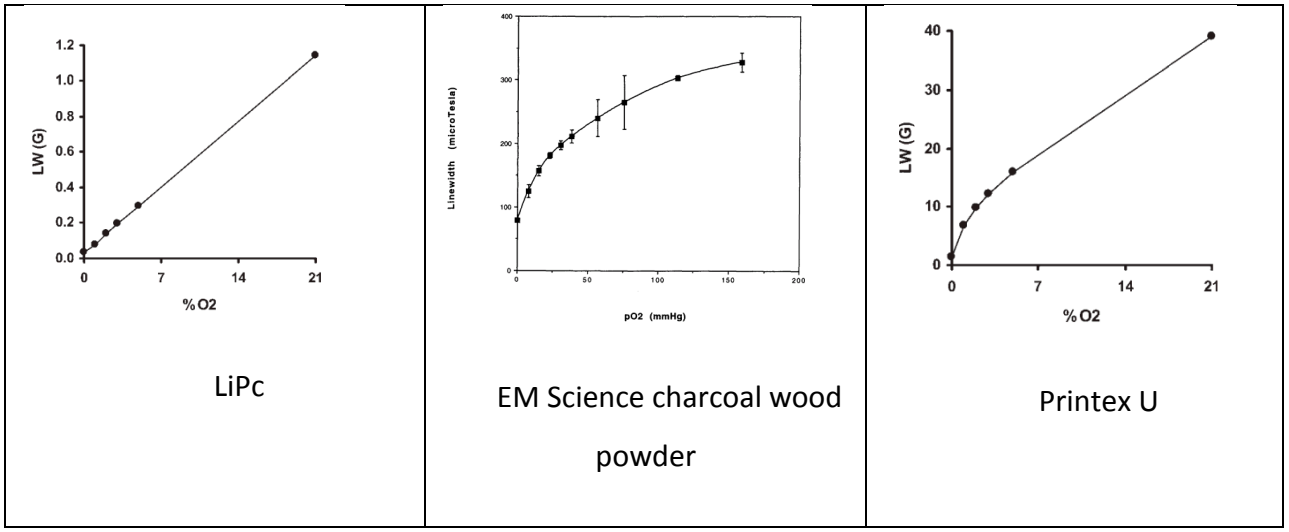


Figure 9: Calibration de trois sondes particulaires; Phalocyanine de lithium (gauche, reproduit de ref [3]), charbon (centre, reproduit de ref[11]), noir de carbone (droite, reproduit de ref[3]).

Les sondes solubles utilisées en oxymétrie RPE sont de type nitroxide ou triarylméthyle (TAM). Ces sondes ont une stabilité et une sensibilité à l'oxygène plus faible que les sondes particulaires ; elles peuvent cependant être éliminées de façon naturelle. Les nitroxides (Figure 10) sont les premiers à avoir été utilisés pour l'oxymétrie RPE ; il en existe une large variété structurale permettant de moduler leurs propriétés. Par exemple, un nitroxide chargé ne pourra pas traverser la membrane cellulaire et sera utilisé pour mesurer l'oxygénation extracellulaire alors qu'un nitroxide neutre se distribuera dans les deux compartiments (extra- et intracellulaire). Ils peuvent également être ciblés vers un organe ou un tissu particulier.[15, 16]

Le nitroxide provient généralement de l'oxydation d'une amine ou hydroxylamine secondaire[17] et ne possède pas de proton en position alpha afin d'éviter les réactions de dismutation.

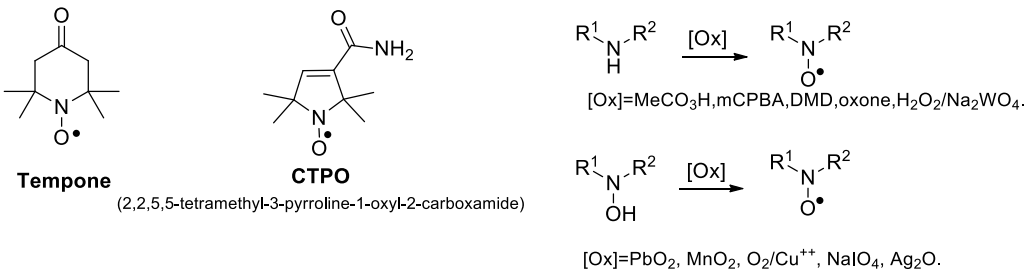


Figure 10 : Examples de radicaux nitroxides.

Contrairement aux sondes particulaires, le spectre des nitroxides présente généralement trois raies de même intensité en raison d'un couplage hyperfin avec un atome ^{14}N. Typiquement, la largeur de raie est de l'ordre de 1 G et sa variation entre une solution désoxygénée et une solution contenant 210 µM d'O_2 (une solution physiologique en équilibre à l'air) est de l'ordre de 0.1 G, ce qui est relativement modeste (10% de variation). Cette variation de 0.1 G est limitée par la solubilité de l'oxygène en milieu physiologique et est similaire pour toutes les sondes solubles (équation 6). Une autre difficulté rencontrée avec les nitroxides est leur rapide réduction en milieu biologique (généralement en quelques minutes) pour conduire à une hydroxylamine non paramagnétique. Cette caractéristique peut cependant être utilisée pour évaluer le statut rédox d'un tissu en observant la cinétique de disparition du signal RPE.[18] Le rapport signal sur bruit et la sensibilité à l'oxygène peuvent être augmentés en utilisant des nitroxides perdeutérés caractérisés par des raies plus fines. La substitution de l'atome ^{14}N (S=1) par un^{15}N (S=1/2) permet également de gagner en rapport signal sur bruit en diminuant de trois à deux le nombre de raies (Figure 11). La largeur de raie de ces nitroxides perdeutérés peut dans certains cas être de l'ordre de 0.2 G en absence d'oxygène et de ≈ 0.3 G en équilibre à l'air, la variation est alors de 50%.

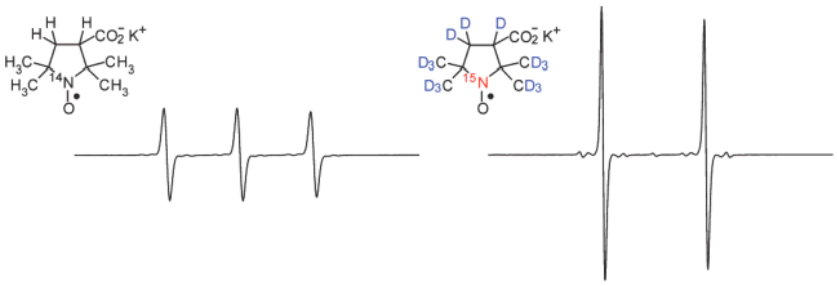


Figure 11: Effet de la substitution isotopique sur le spectre RPE (reproduit de ref[19]).

Le schéma 2 montre un exemple de synthèse d'un nitroxide perdeutéré possédant un ^{15}N.[19] L'azote marqué de la fonction nitroxide et les atomes de deutérium sont introduits à la première étape par condensation de l'acétone-d_6 avec de l'ammoniaque marqué ^{15}N. Une autre étape-clé est la contraction de cycle utilisant un réarrangement de Favorskii pour mener à la pyrrolidine **7**.

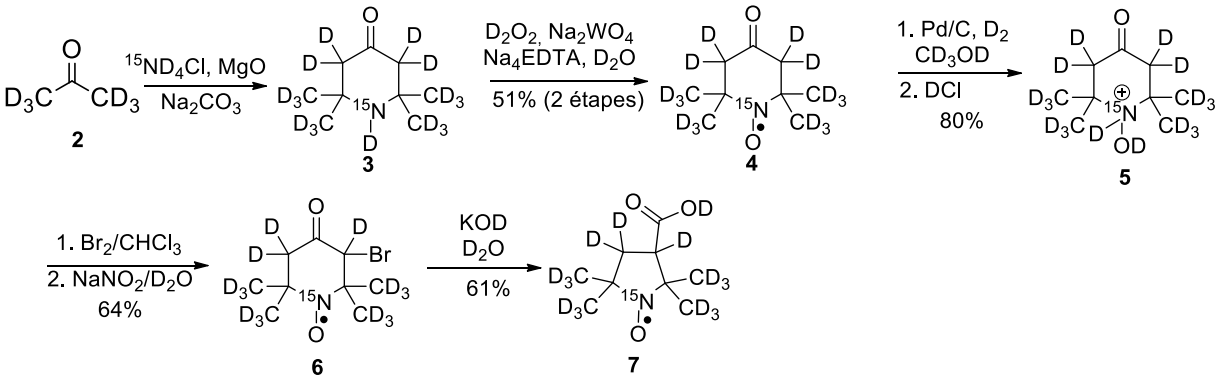


Schéma 2 : Example de synthèse d'un radical nitroxide perdeutéré et possédant un ^{15}N.

Les radicaux triarylméthyles (TAM) de type tétrathiatriarylméthyle constituent le deuxième type de sondes solubles utilisées en RPE et sont d'utilisation beaucoup plus récente que les nitroxides. Ceux-ci ont été développés dans les années 1990 par Nycomed Innovation (aujourd'hui General Electric Healthcare).[20-27] Parmi les dérivés synthétisés, **Ox063**, **CT-03** et son équivalent deutéré **CT-03-d_{36}** sont les plus couramment rencontrés dans la littérature (Figure 12) et leurs synthèses seront présentées au paragraphe 1.2.1.

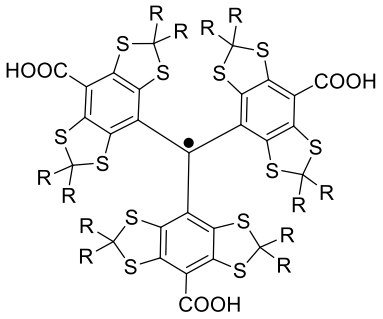


Figure 12 : Radicaux TAMs synthétisés par Nycomed.

Par comparaison avec les nitroxides, ces radicaux sont plus stables et le paragraphe 1.2.2 sera consacré à la stabilité et au métabolisme *in vitro* de ces dérivés. De plus, leurs spectres

se présentent sous la forme d'un singulet dont la largeur de raie est inférieure à 100 mG, synonyme de haute sensibilité de détection (au µM en bande-X) et plus haute sensibilité à l'oxygène (100% de variation) (Figure 13).

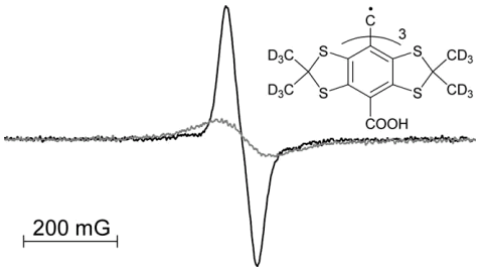


Figure 13: Spectre RPE (bande-L) de CT-03-d_{36} en solution aqueuse (0.1 mM dans PB) à l'équilibre à l'air (gris) et en solution dégazée (noir) (reproduit de ref[28]).

En plus de l'interaction sonde-oxygène, l'interaction sonde-sonde peut également induire une augmentation de la largeur de raie. Les radicaux tétrathiatriarylméthyles sont moins sensibles à ce phénomène que les radicaux nitroxides. Par exemple, l'élargissement de raie de **CT-03-d_{36}** en fonction de sa concentration est de 33 mG/mM[29] contre 167 mG/mM pour **mHCTPO** (Figure 14).[30] Ces radicaux sont également considérés comme non toxiques. La dose létale évaluée pour **Ox063** chez la souris étant de 8 mmol/Kg (≈ 11 g/Kg).[31]

mHCTPO	**CT-03 d36**
ΔLW_L = 167 mG/mM **mCTPO**[a]	ΔLW_L = 33 mG/mM **CT-03-d_{36}**[b]

Figure 14 : Les radicaux TAMs sont moins sensible à l'auto élargissement de raie que les nitroxides.

[a] Composante Lorentzienne de la largeur de raie. Mesurée à 27°C en solution aqueuse. Raie RPE analysée suivant une fonction de Voigt, convolution entre une fonction lorentzienne et Gausienne. Lorsque la largeur de raie est uniquement fonction de la relaxation T_2, alors la raie d'absorption est dite homogène et est décrite selon une fonction Lorentzienne. Si la raie est une superposition de signaux (multiples conformations, couplages non résolus, etc.) alors la raie est dite inhomogène et d'autres fonctions sont utilisées : Gausienne, Voigt, Tsallian, etc.).

[b] Composante Lorentienne de la largeur de raie. Mesurée à 37°C en solution isotonique.

En raison de leurs caractéristiques particulières (raie unique et fine, solubilité, faible toxicité, stabilité et sensibilité à l'oxygène), ces radicaux sont idéaux pour des applications d'imagerie. En effet, moyennant l'utilisation de gradients de champ magnétique, il est possible de réaliser une image *in vivo* de la distribution d'une sonde paramagnétique[c]. La largeur de raie (T_2) dans chaque pixel de l'image permet de dresser une carte de l'oxygénation tissulaire. Ceci est illustré ci-dessous avec l'image d'une patte de souris portant une tumeur réalisée avec **Ox063**.[32]

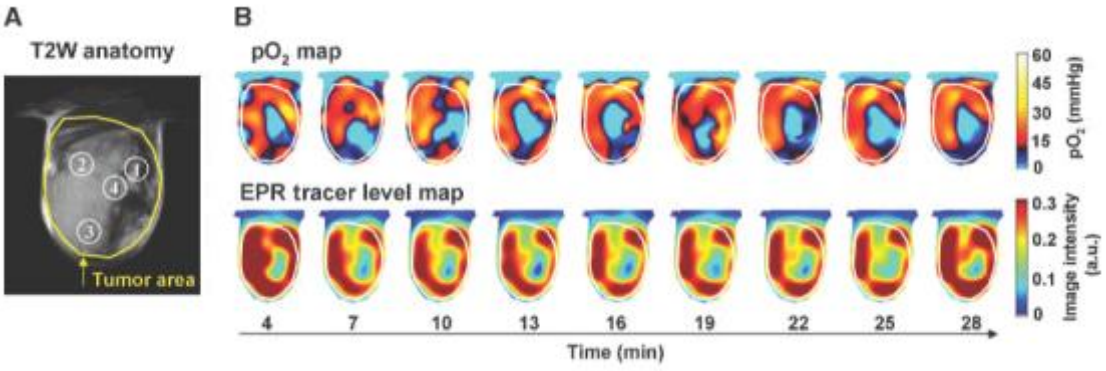


Figure 15: (A) image IRM. (B) image par résonance paramagnétique électronique (reproduit de ref[32]).

Des radicaux trityles perchlorés tel que le perchlorotriphénylmétyle triester (PTM-TE) (Schéma 3) ont également été utilisés pour l'oxymétrie RPE mais de façon beaucoup plus limitée. Le PTM-TE est synthétisé en utilisant une réaction de Friedel-Crafts pour la formation du cœur trityle. Le radical est quant à lui généré par oxydation de l'anion formé par la déprotonation du proton benzylique (Schéma 3). Ce radical PTM-TE n'est pas soluble dans l'eau et doit donc être utilisé en formulation: injection dans un perfluorocarbone[33], dans une micelle d'hexaméthyldisiloxane (HMDS) ou incorporé dans un polymère de polydiméthylsiloxane (PDMS).[34] La formulation de sondes est une technique couramment utilisée pour modifier certaines propriétés telles que stabilité, toxicité, sensibilité à l'oxygène, distribution ou biocompatibilité.[35]

[c] Contrairement à l'IRM par RMN, il n'y a pas d'espèce paramagnétique endogène en quantité et temps de vie suffisant (à l'exception de la mélanine) pouvant être analysée directement *in vivo* par RPE ; une sonde exogène paramagnétique doit donc être introduite pour pouvoir réaliser une image.

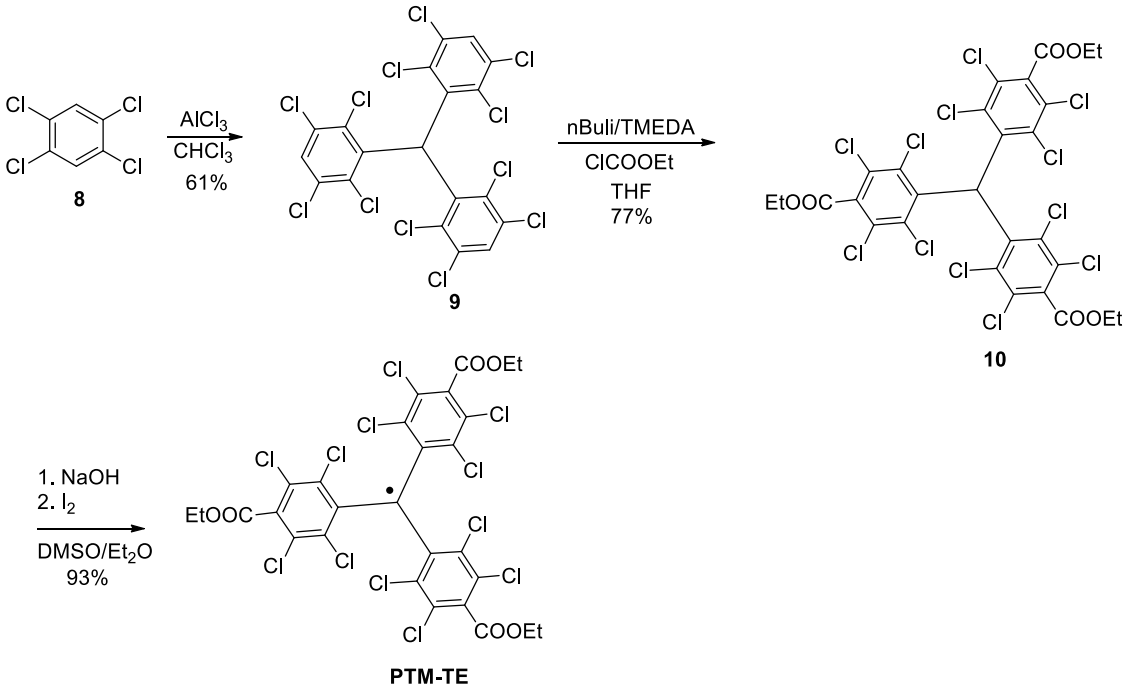


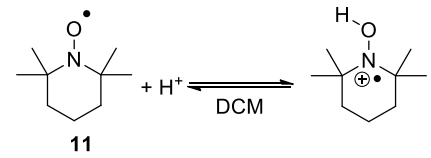
Schéma 3 : Synthèse du PTM-TE.

Pour le choix de la sonde, plusieurs paramètres sont à prendre en compte :

- La sonde doit être **non toxique.**
- Une haute **sensibilité à l'oxygène** est idéale pour mesurer de faibles variations d'oxygénation avec grande précision. Par contre, des variations plus importantes entraînent une forte diminution du signal (intensité du signal inversement proportionnelle au carré de la largeur de raie) et une sensibilité moindre est alors préférable.
- Un **signal fin et unique** ainsi qu'une **haute densité de spins** permettent d'obtenir un bon rapport signal/bruit.
- La **stabilité** et la **distribution** doivent être adaptées à l'application visée. Les sondes particulaires sont idéales pour des mesures locales sur de longues périodes de temps alors que les sondes solubles, en raison de leur diffusion tissulaire, sont mieux adaptées pour des applications d'imagerie.

1.1.2 pH-métrie par RPE[36-40]

La technique de mesure du pH par RPE est basée sur l'utilisation d'une sonde paramagnétique dont les propriétés spectrales sont sensibles au pH. La première observation de l'effet du pH sur un spectre RPE a été reportée en 1969 pour le nitroxide **11.** La protonation du fragment nitroxide lui-même en milieu fortement acide provoque une modification de la constante de couplage a_N et du facteur g (Schéma 4). De plus, un couplage supplémentaire apparait en raison de la présence supplémentaire d'un atome ^1H (S=1/2) à proximité du radical. [41]



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$a_N = 15.87 \pm 0.10$ G
$g = 2.0059 \pm 0.002$

$a_N = 21.80 \pm 0.20$ G
$a_H = 3.30 \pm 0.10$ G
$g = 2.0042 \pm 0.002$

Schéma 4 : Protonation du fragment nitroxide.

Les sondes couramment utilisées *in vivo* pour la mesure du pH par RPE sont des dérivés nitroxides de type imidazoline ou imidazolidine (Figure 16). L'effet de l'ionisation sur le spectre RPE est illustré de manière qualitative à la figure 16. L'électron célibataire d'un radical nitroxide est localisé dans une orbitale moléculaire π résultant du recouvrement de deux orbitales atomiques 2p de l'oxygène et de l'azote ($\rho^{\pi}_O \approx 0.6$; $\rho^{\pi}_N \approx 0.4$). La protonation en N-3 stabilise la forme de résonance non ionique du radical nitroxide, il en résulte une modification de la densité électronique sur l'oxygène et l'azote et par conséquent, une modification de la constante de couplage a_N et du facteur g.

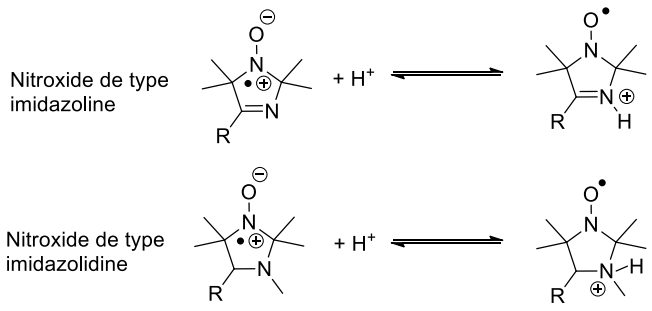
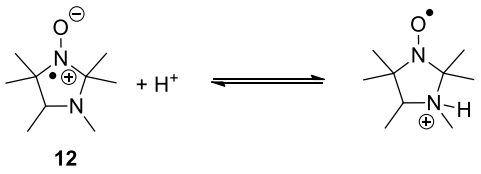


Figure 16 : Nitroxides de type imidazole et imidazolidine.

Pour des pH proches du pKa de la sonde, les deux espèces ioniques sont présentes en solution. Si l'échange de proton entre ces deux espèces est lent par rapport à l'échelle de temps RPE, alors le spectre se présentera sous la forme d'une superposition des deux espèces. Au contraire, si l'échange est rapide, seule une forme moyenne sera présente sur le spectre. Il a été démontré par V. V. Khramtsov que pour le cas de nitroxides où les raies sont séparées de ≈ 1 G, la condition pour avoir un équilibre d'échange lent est: $3 \leq pKa \leq 11$ (pour une vitesse d'échange de protons limitée par la diffusion). Etant donné que le pKa désiré pour une application biologique est souvent dans la zone du pH physiologique, le spectre obtenu est souvent une superposition des deux espèces ioniques. Ceci est illustré à la figure 17. Le spectre du nitroxide **12** a été enregistré pour un pH proche du pKa (pKa = 4.6) pour différentes fréquences de spectromètre. A très haute fréquence (140 GHz, Bande-D), on observe une résolution parfaite des deux espèces ioniques en raison de valeurs de couplage a_N et de facteur g différents entre les deux espèces ioniques. La position de la raie centrale des deux espèces étant séparée de la valeur Δg, la raie à bas champ de $\Delta g - \Delta a_N$ et la raie à haut champ de $\Delta g + \Delta a_N$. Par contre, en utilisant des spectromètres opérant à une plus basse fréquence, la résolution est progressivement perdue. Cela s'explique par la dépendance du Δg à la fréquence du spectromètre : le Δg sera d'autant plus grand que la fréquence sera élevée. Au contraire, le Δa_N est un facteur indépendant de la fréquence du spectromètre.



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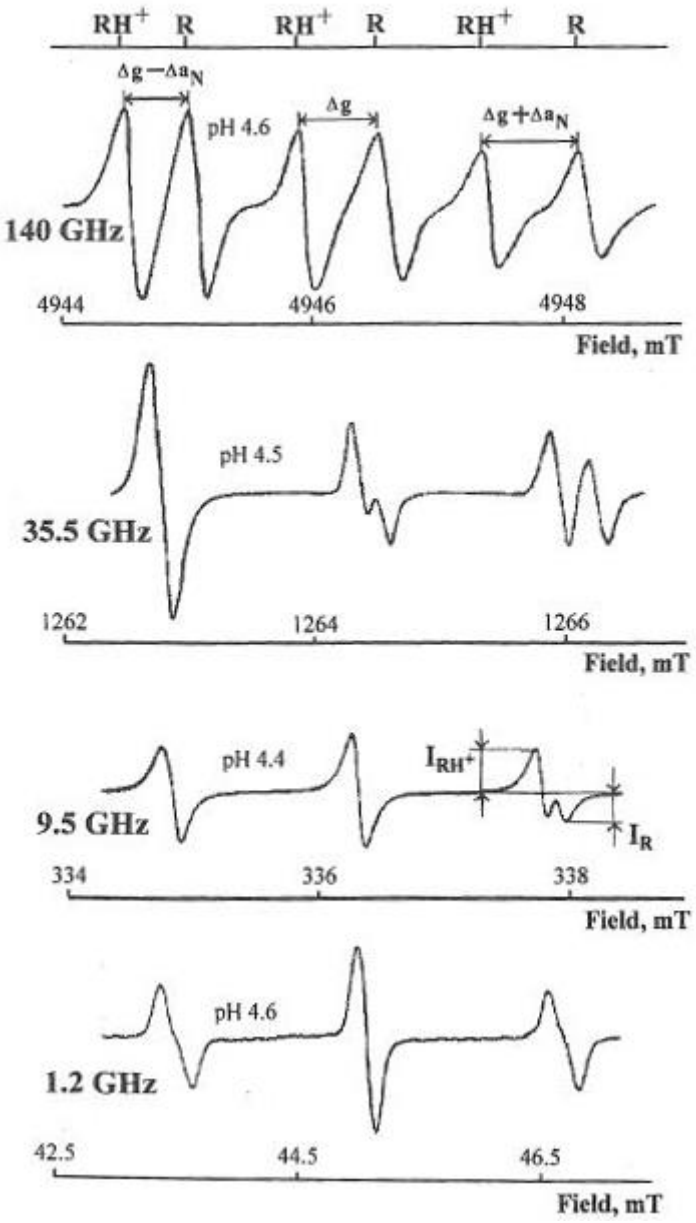


Figure 17: Effet de la protonation sur le spectre *(reproduit de ref[37]).*

Il faut encore mentionner que le régime d'échange peut être modifié par certains facteurs (p.ex. la température, le solvant). Le nitroxide **12** dans un tampon dont le pKa est proche du sien (acétate de potassium) passera d'un équilibre d'échange lent à rapide en

augmentant la concentration en tampon (participation à l'échange par le tampon) (Figure 18).

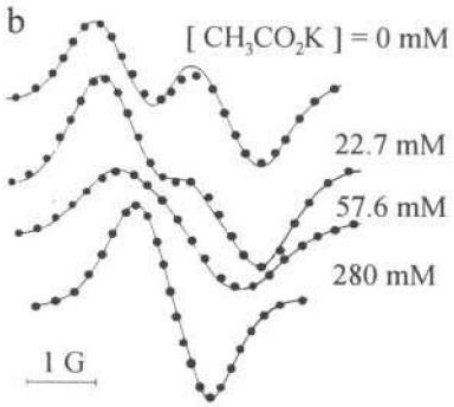
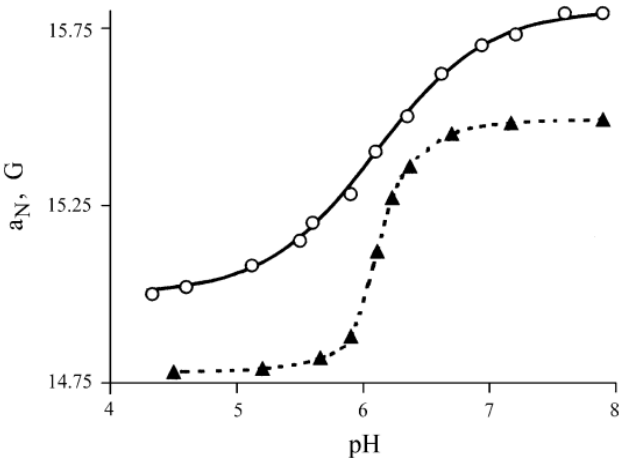
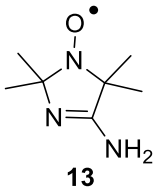


Figure 18: Raie haut-champ du nitroxide 12 à différentes concentrations en tampon *(reproduit de ref[38]).*

La détermination du pH peut se réaliser de différentes façons. En principe, il est possible de modéliser la forme du signal RPE pour deux espèces en équilibre dynamique ($R \leftrightarrows RH^+$), la comparaison du spectre expérimental avec un spectre simulé par ordinateur permettant de déterminer le rapport RH^+/R et donc le pH. En pratique, cette méthode souffre de grosses limitations *in vivo*. Par exemple, il est difficile de tenir compte d'une modification de la forme du signal en raison de difficultés techniques comme une inhomogénéité de champ ou encore un mouvement de l'animal. En pratique, on préfère réaliser une calibration d'un paramètre répondant au pH. En cas de résolution même partielle des espèces ioniques, on peut calibrer le rapport d'intensités des signaux des espèces R et RH^+ (I_R/I_{RH+}) en fonction du pH (voir figure 17). A bas champ (*in vivo*), on utilise plutôt la calibration de la constante de couplage apparente entre les raies non résolues en fonction du pH. Cette calibration est représentée pour le radical nitroxide **13** à la figure 19.[36] Il est important de noter que la courbe obtenue est fonction des paramètres d'acquisition et doit donc être réalisée dans les mêmes conditions que pour la mesure du pH dans l'application visée.





13

Figure 19: Courbes de calibrage de la constante de couplage aN en fonction du pH réalisé en bande-X (o) et bande-L (▲) pour le radical 13 (pKa = 6.1) *(reproduit de ref[36]).*

Idéalement, pour pouvoir être utilisée *in-vivo,* une sonde paramagnétique pour la mesure du pH devrait satisfaire à un certain nombre de conditions, parmi lesquelles:

1. Une **bonne sensibilité spectrale** à l'équilibre acide-base pour pouvoir mesurer de faibles variations de pH avec précision.
2. Un **spectre simple**, idéalement deux raies fines pour avoir un rapport signal sur bruit le plus grand possible.
3. Un **pKa dans la zone à étudier** ; l'effet du pH est limité autour du pKa, où les deux espèces ioniques coexistent.
4. Une **absence de toxicité**.
5. Une bonne **stabilité en milieu biologique**.
6. Une **bonne biodistribution** dans la zone à étudier.

1.2 Radicaux tétrathiatriarylméthyles: origine, synthèse, stabilité et applications

Les radicaux trityles possèdent une très longue histoire en chimie organique qui débuta en 1900 lorsque Moses Gomberg fit la découverte du premier radical, le radical triphénylméthyle **16**.[42, 43] En essayant de synthétiser l'hexaphényléthane, Moses Gomberg

remarqua que quand le chlorotriphénylméthane était traité par certains métaux, de préférence le zinc, un hydrocarbure particulièrement réactif vis-à-vis de l'oxygène ou des halogènes était formé, le trityle **16** (Schéma 5).

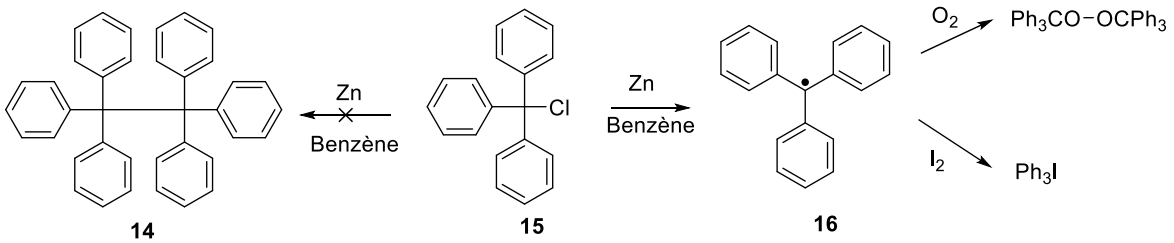


Schéma 5 : Formation du radical trityle au départ du chlorure trityle.

Ce radical **16** ne peut cependant pas être isolé tel quel puisqu'il est présent très largement sous forme dimérique en solution, alors qu'il l'est complètement à l'état solide. La structure de ce dimère a d'ailleurs été sujette à controverse durant de nombreuses années. Des trois hypothèses **14**, **17**, et **18**, (Schéma 6) la mauvaise structure **14** (hexaphényléthane) a été retenue pendant plus d'un demi-siècle avant que l'hypothèse de Jacobson **17** ne soit définitivement prouvée en 1968. Ce dimère est caractérisé par une structure asymétrique tête-queue et une énergie de liaison intra-dimère faible de ≈11 kcal.mol^{-1}.[44]

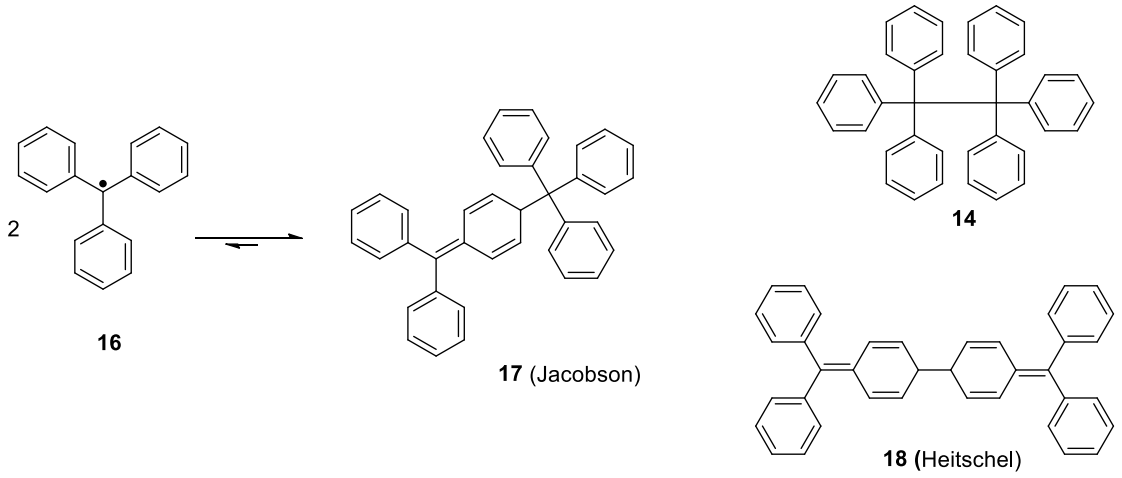
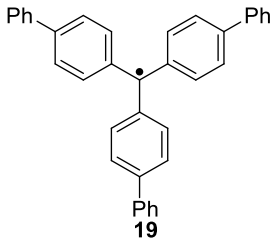


Schéma 6 : Les différentes hypothèses structurales du dimère.

L'existence du radical triphénylméthyle fut également sujette à discussion et fut définitivement prouvée en 1910 par W. Schlenk qui réalisa la synthèse du trityle **19** (Figure 20) présent presque exclusivement sous forme dissociée en solution en raison de son

encombrement stérique.[45] La découverte de Gomberg fut le point de départ du développement de la chimie radicalaire et a permis de confirmer une hypothèse émise au 19ème siècle selon laquelle le carbone pouvait exister sous la forme trivalente mais qui avait fini par être abandonnée faute de pouvoir le prouver. Malgré l'importance considérable de sa découverte et de multiples nominations pour le prix Nobel, il ne le reçut jamais ![46]

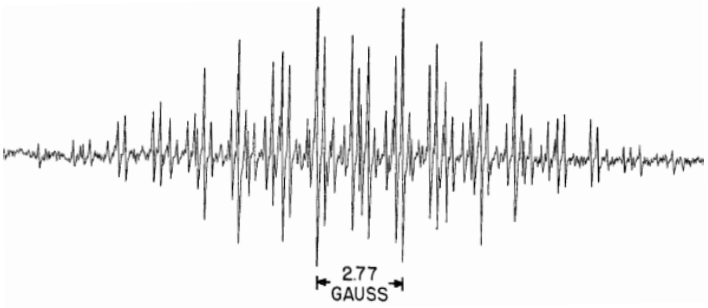


19

Figure 20 : Structure du trityle synthétisé par Schlenk.

Le radical trityle **16** n'est cependant pas un radical stable ; une définition pragmatique a été donnée par K.U. Ingold. Selon cette définition, un radical pour être qualifié de stable doit pouvoir être isolé et stocké tel quel. *A contrario* un radical persistant doit avoir un temps de vie suffisamment long pour pouvoir être observable.[47] Le trityle **16** doit donc être qualifié de persistant plutôt que stable, malheureusement cette définition n'est pas largement suivie dans la littérature et les termes stable et persistant se confondent.

Le trityle de Gomberg n'est cependant pas adapté pour l'oxymétrie par RPE en raison de sa réactivité et de la complexité de son spectre RPE. En effet, la présence d'atomes d'hydrogène (^{1}H S=1/2) a pour effet de produire un éclatement du signal en 196 raies (Figure 21).[48]



Constantes de couplage hyperfines

a_{Ho}=2.53 G
a_{Hm}=1.11 G
a_{Hp}=2.77 G

6H*ortho*, 6H*meta*, 3H*para*.
N=7x7x4=196 raies.

Figure 21 : Spectre RPE (Bande-X) du radical trityle **16** dans le toluène à -20°C (reproduit de ref[48]).

Les sondes **Ox063**, **CT-03** et **CT-03-d$_{36}$** présentées au schéma 7 sont issues d'une large gamme de radicaux triarylméthyles développés par Nycomed Innovation. Par rapport au trityle de Gomberg, la substitution de tous les atomes d'hydrogène par des atomes à spin nul (^{32}S, ^{12}C) permet de supprimer tous les couplages ainsi que d'augmenter la stabilité par effet stérique et électronique. L'introduction de trois fonctions acide carboxylique permet une hydrosolubilisation à pH physiologique.

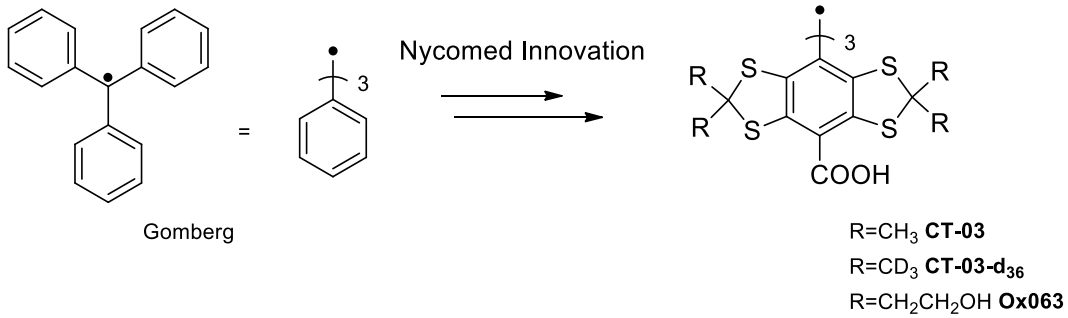


Schéma 7: Modification de Nycomed.

Initialement, ces radicaux ont été développés pour être utilisés comme agents de polarisation pour l'imagerie IRM. En effet, le moment magnétique de l'électron étant 658 fois supérieur à celui du proton, il en résulte que pour le même champ magnétique extérieur B_0, la différence de population entre spins α et spin β (la polarisation) est plus importante pour l'électron que pour n'importe quel noyau. La sensibilité étant proportionnelle à cette polarisation, la RPE est intrinsèquement une technique plus sensible que la RMN.

Si à un échantillon à analyser par RMN on y ajoute un radical et qu'on l'irradie à sa fréquence de résonance, on peut réaliser un transfert de polarisation de l'électron vers le noyau et ainsi augmenter le signal RMN.[49] Cette technique est applicable en IRM et en spectroscopie. La polarisation nucléaire dynamique qui est une utilisation majeure de ces radicaux TAMs ne sera pas développée ici.[50, 51] Signalons quand même que l'importance de ce transfert de polarisation est fonction de la largeur de raie du radical et donc de la concentration en oxygène situé à proximité du radical. Ceci est la base de l'oxymétrie par polarisation nucléaire dynamique.[31]

Nous allons maintenant nous attarder sur la synthèse de ces molécules. La stabilité, le métabolisme oxydatif et réductif seront ensuite abordés. Finalement, nous présenterons

quelques exemples de modifications chimiques qui ont permis de modifier certaines propriétés et d'étendre le champ d'application de ces molécules.

1.2.1 Synthèse de CT-03, CT-03-d_{36} et Ox063

Les synthèses de ces trois dérivés ont été décrites en premier lieu dans la littérature des brevets par Nycomed,[20-27] et plus tard dans la littérature scientifique.[52-56] La retrosynthèse imaginée par Nycomed (Schéma 8) fait intervenir l'alcool TAM **21** comme précurseur du radical. Cet alcool provient de l'addition de trois entités bisthioacétal **22** sur un dérivé carbonylé.

Les synthèses initiales et les modifications proposées par la suite seront présentées de manière intégrée. Seules les stratégies de synthèse seront présentées, les modifications mineures seront volontairement laissées de côté.

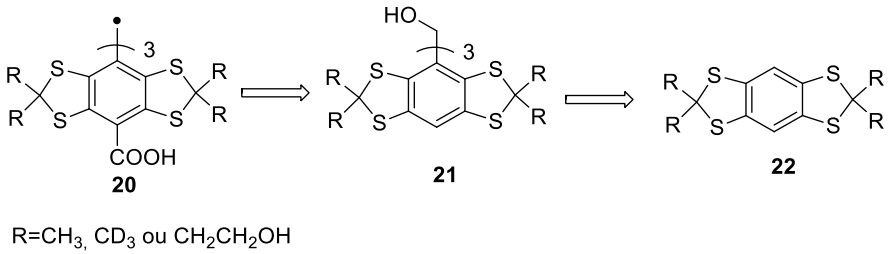


Schéma 8 : Rétrosynthèse de Nycomed.

Synthèse de **CT-03** et **CT-03-d_{36}**

L'approche de Nycomed fait intervenir le bisthiocarbonate **24** synthétisé par une séquence en deux étapes proposée par K. Bechgaard (Schéma 9).[57] La synthèse démarre par une substitution nucléophile aromatique (S_{Nar}) sur le 1,2,4,5-tétrachlorobenzène par l'isopropylthiolate. Le tétra(isopropylthio)benzène est ensuite désalkylé par réduction au sodium métallique à haute température (100-110°C) dans la pyridine. Le tétrathiolate de sodium ainsi formé est piégé par le phosgène, réactif hautement toxique, pour former le bisthiocarbonate **24**. Par la suite, une hydrolyse basique suivie d'un *work-up* acide permet

d'isoler le tétrathiolbenzène **25**, réactif hautement sensible à l'oxydation, particulièrement en solution.[58] Celui-ci est directement mis en réaction avec de l'acétone en milieu acide pour conduire à l'intermédiaire-clé bisthioacétale **26**.

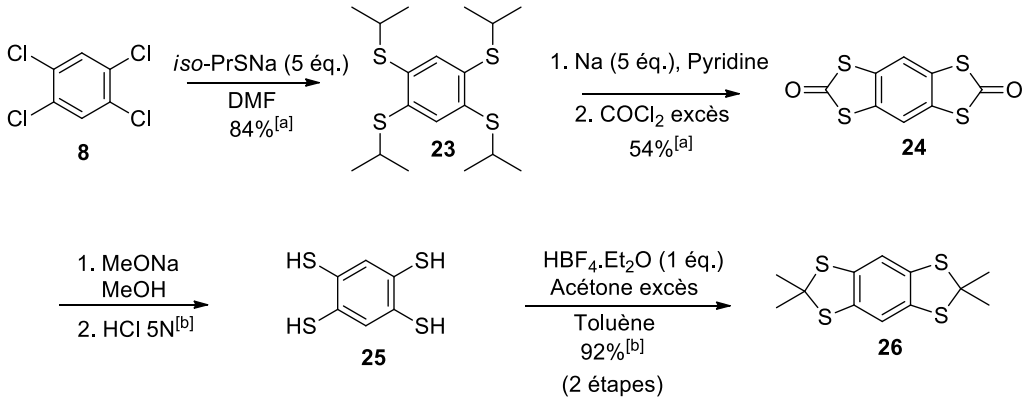


Schéma 9: Synthèse du bisthioacétale 26 [a] selon ref[57], [b] selon ref[20].

La synthèse du tétrathiol **25** directement par réaction du tétrachlorobenzène **8** avec l'isopropylthiolate dans l'HMPA suivie d'une désalkylation *one pot* au sodium a été reportée par Tiecco.[59] Des problèmes de reproductibilité ont cependant été rapportés.[57, 58]

D'autres stratégies de synthèse ont été développées pour la synthèse du tétrathiol **25**. Par exemple, le téta(méthylthio)benzène est connu pour être une excellente forme protégée de **25** pour son stockage (Schéma 10). Il peut être synthétisé au départ du téta(isopropylthio)benzène ou directement par S_{Nar} entre le méthylthiolate et le 1,2,4,5-tétrafluorobenzène. L'utilisation de l'équivalent fluoré, plus réactif, mais également plus onéreux, se justifie par le fait que les conditions plus dures nécessaires à la S_{Nar} sur le dérivé chloré sont suffisantes pour provoquer une déméthylation d'une partie des sulfures aromatiques formés, ceci par S_{N2} entre le méthylthiolate et le sulfure.[58]

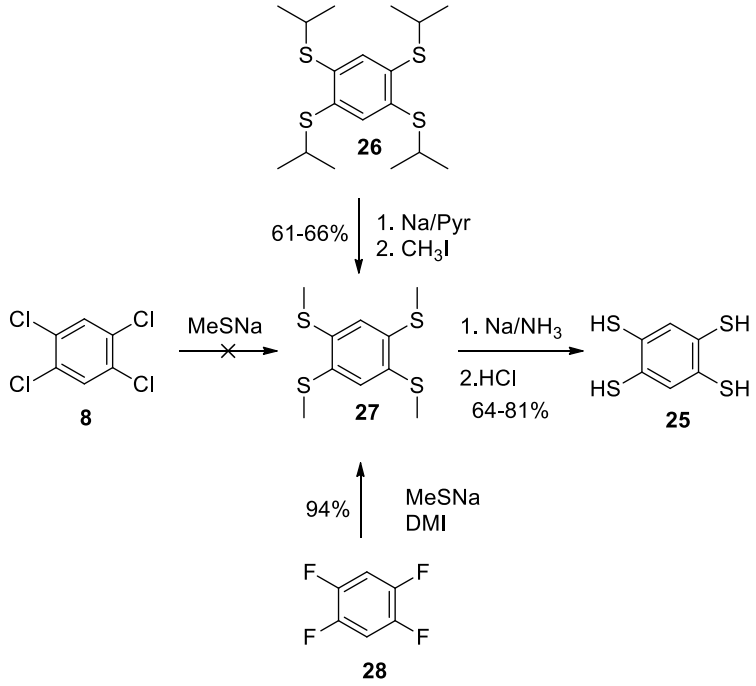


Schéma 10 : Synthèse de 25.

Plus tard, une amélioration de la synthèse de l'intermédiaire bisthioacétale **26** a été proposée par V. H. Rawal.[52] Celui-ci propose de réaliser la substitution nucléophile aromatique sur le tétrachlorobenzène avec le *tert*-butylthiolate (Schéma 11) au lieu de l'isopropyl thiolate. Cette petite modification permet de déprotéger les thiols en milieu acide et de réaliser la condensation directement avec l'acétone en une seule étape. Cette avancée permet non seulement de réduire de trois étapes la synthèse de **26** mais évite également d'utiliser du phosgène, du sodium métallique à haute température ou encore d'isoler le tétrathiol **25**, réactif sensible.

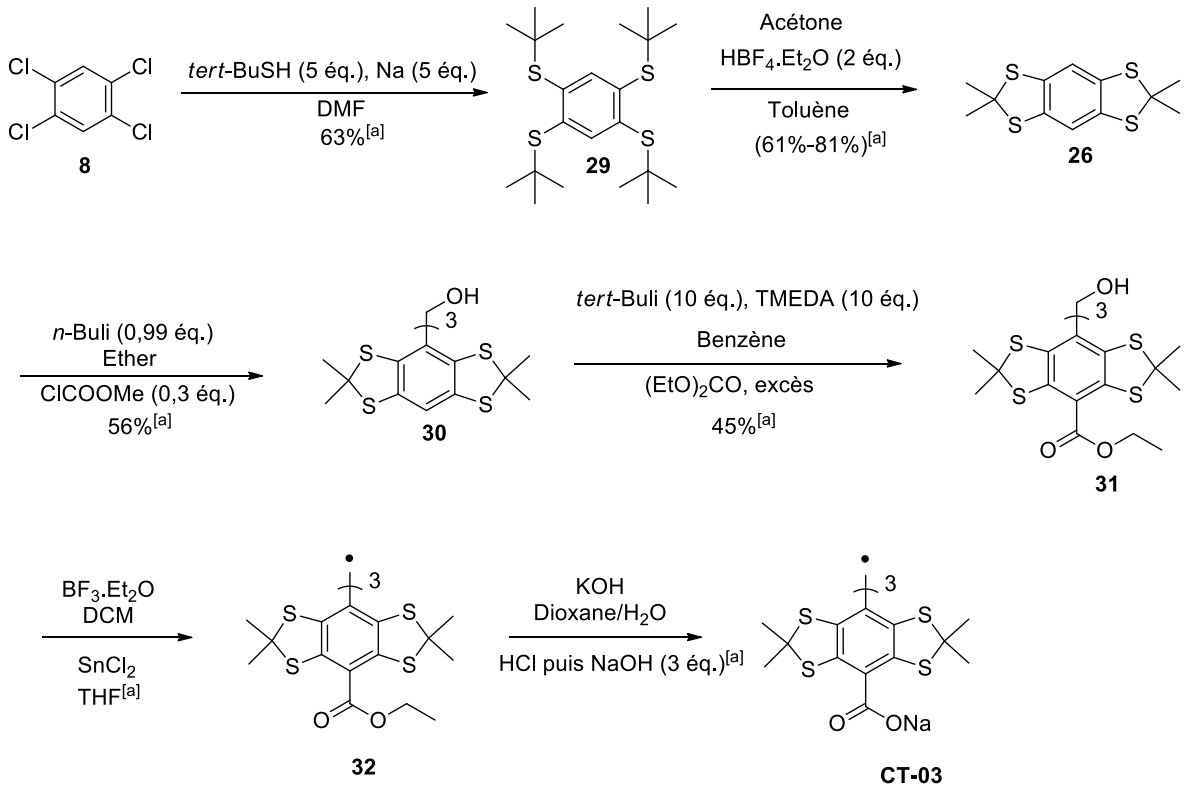


Schéma 11: Synthèse de **CT-03** par Reddy *et al* [a] selon ref[52].

Ensuite, une lithiation de **26** par le *n*-BuLi suivie de trois additions successives du lithien formé sur le chloroformate de méthyle permet de former l'alcool TAM **30**. Dans l'étape suivante, les esters précurseurs des acides carboxyliques sont introduits pas une nouvelle lithiation en utilisant un excès de *tert*-BuLi en présence de tétraméthyléthylènediamine (TMEDA) ; le lithien formé est ensuite additionné sur le carbonate diéthylique, utilisé en excès pour éviter les additions multiples. Le radical est formé dans l'étape suivante par traitement de l'alcool TAM **31** par le $BF_3.Et_2O$, et le cation ainsi généré est réduit par le chlorure d'etain^{II}. Finalement les esters sont hydrolysés pour conduire à **CT-03** (Schéma 11).

Une deuxième modification pour la synthèse de **CT-03** à grande échelle implique l'intermédiaire de la benzophénone **33**.[54] En effet l'étape de formation du cœur triarylméthyle par addition consécutive de trois lithiens **34** sur le chloroformate de méthyle est critique (Schéma 12), la troisième addition étant particulièrement lente. En effet, alors que thermodynamiquement la cétone **33** devrait être plus réactive, l'encombrement stérique rend difficile l'attaque nucléophile du lithien **34** sur le carbonyle et la cétone est souvent obtenue en produit secondaire, limitant le rendement.

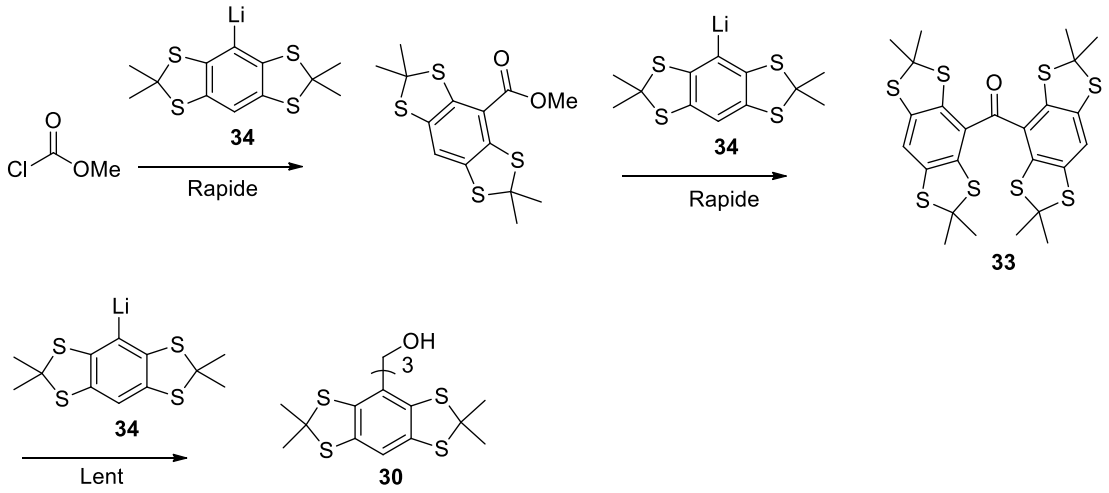


Schéma 12: Formation du trityle par additions successives de trois aryle lithien sur le chloroformate.

Cette modification propose de synthétiser la cétone de manière sélective dans un premier temps en augmentant la quantité de chloroformate et d'ensuite de favoriser la formation du trityle en ajoutant un excès de lithien **34** sur cette cétone (Schéma 13). Enfin les mêmes auteurs proposent de remplacer les esters éthyliques par des esters *tert*-butyliques afin de bloquer le carbonyle de l'ester vis-à-vis de l'addition nucléophile d'un lithien restant en solution. De plus, la déprotection des esters *tert*-butyliques par de l'acide trifluoroacétique (TFA) mène à la formation concomitante du radical. Cette méthode n'est d'ailleurs pas spécifique aux esters *tert*-butyliques et a été reportée pour d'autres substituants.[54, 60-62]

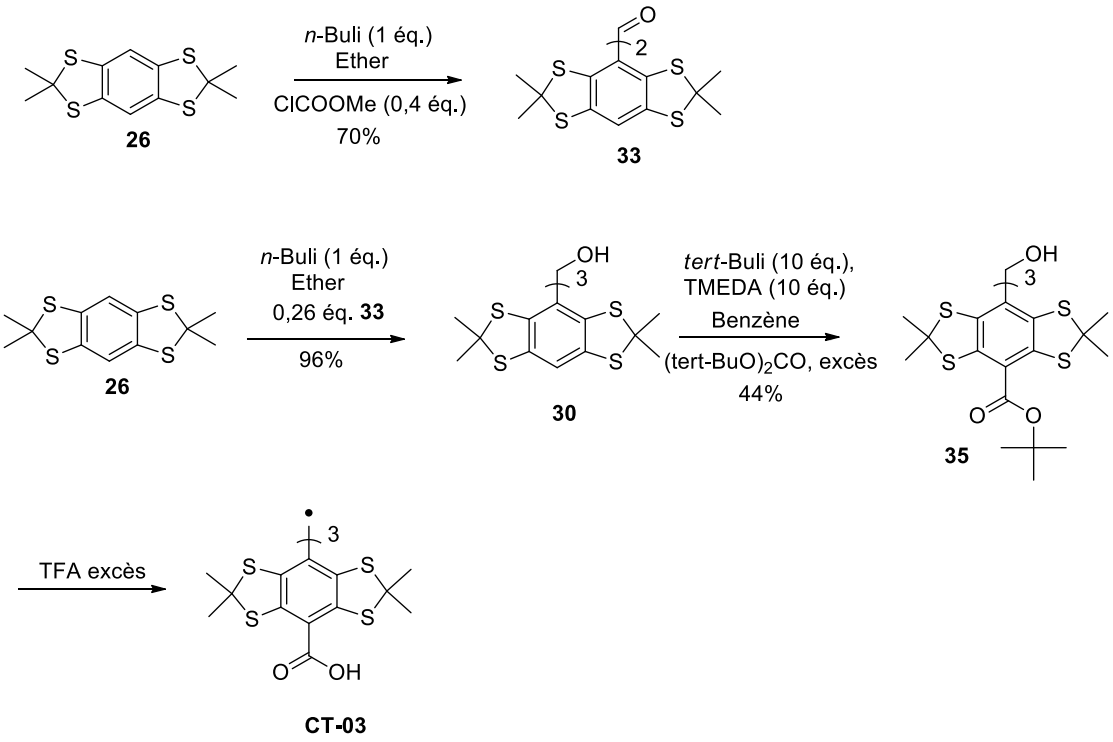


Schéma 13 : Alternative propose par I. Dhimitruka et al.

La substitution isotopique des 36 protons des fonctions thioacétal par des atomes de deutérium permet d'affiner encore le signal ; la largeur de raie mesurée dans les mêmes conditions (Bande-X, 1 mM dans le DMSO dégazé, mêmes paramètres d'acquisition) est de 95 mG pour **CT-03** et 65 mG pour **CT-03-d$_{36}$**.[56] Sa synthèse ne demande le remplacement que d'un seul réactif, à savoir l'utilisation d'acétone-d$_6$ lors de la formation du bisthioacétal (Schéma 14).[21, 56]

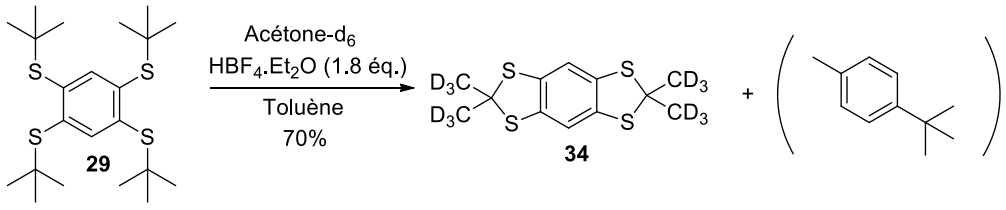


Schéma 14 : Formation du dérivé deutéré **34** par l'utilisation d'acétone-d$_6$.

Il est intéressant de constater que maximum 5% des deutériums sont échangés par des protons lors de cette réaction.[56] Cet échange provient de l'équilibre céto-énol de l'acétone en milieu acide, trois sources de protons peuvant être identifiées: l'acide fluoroborique, le

cation *tert*-butyle en se décomposant en isobutène ou en réagissant avec le solvant pour donner le *para*-*tert*-butyltoluène, un sous-produit de la réaction.

Synthèse d'Ox063

Ox063 est le composé le plus hydrophile des trois sondes présentées, ceci en raison de la présence de douze fonctions alcool supplémentaires par rapport à **CT-03(-d_{36})**. Sa synthèse est légèrement plus longue et n'est décrite que dans la littérature des brevets[25, 27] (Schéma 15). Elle se différencie à partir du tétrathiol **25** qui est condensé avec le diéthylacétone dicarboxylate. Les fonctions esters de **37** sont ensuite réduites en alcools (**38**) qui sont protégés sous forme d'éthers *tert*-butyliques (**39**). Une lithiation par le *n*-BuLi et addition sur de l'iode moléculaire permet de former l'intermédiaire **40**. Ensuite, un échange halogène métal suivi de l'addition du lithien formé sur le carbonate d'éthyle mène à la formation de l'alcool TAM **41**. Les fonctions acides carboxyliques sont introduites à ce stade par une nouvelle lithiation et addition sur du dioxyde de carbone (42). Enfin, le radical est formé par traitement de **42** à l'acide triflique et chlorure d'étain^{II}. Sous ces conditions, les alcools sont également déprotégés pour conduire à **OX063**.

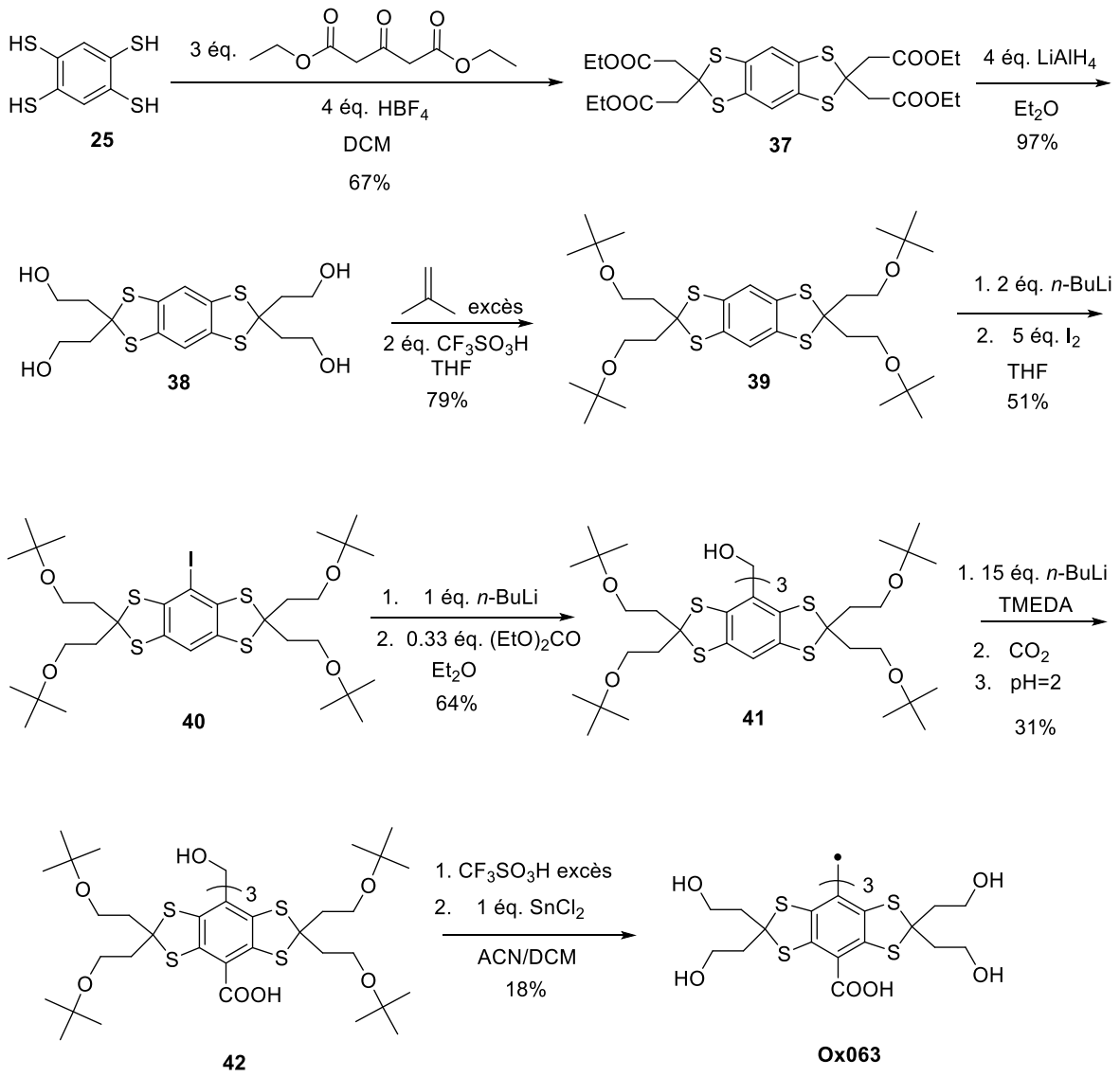


Schéma 15 : Synthèse du radical Ox063 par Nycomed.

1.2.2 Stabilité et métabolisme in vitro

Comme nous l'avons déjà mentionné, ces radicaux tétrathiatriarylméthyles présentent une stabilité remarquable. Par exemple, le temps de demi-vie de **CT-03-d_{36}** dans du sang humain à 37°C est de plus de 24h.[29] Des études menées sur **Ox063** et **CT03** ont montré qu'ils sont stables vis-à-vis de nombreux oxydants, réducteurs et espèces réactives et l'oxygène présents dans les systèmes biologiques tels que le radical hydroxyle, le peroxyde d'hydrogène, le NADPH, l'acide ascorbique, le glutathion, etc. Par contre une instabilité vis-à-vis de l'ion superoxyde ($O_2^{\bullet -}$) et dans une moindre mesure les radicaux peroxyles ($ROO^{\bullet}$) a

été observée[63, 64]. L'utilisation d'**Ox063** pour la détection d'ions superoxydes a d'ailleurs été proposée.[63, 65] Très récemment, les recherches menées sur le métabolisme *in vitro* de **CT-03** et **Ox063** ont permis de comprendre l'origine de cette instabilité et de mettre en évidence les voies de dégradation de ces molécules en présence de microsomes de foie et de diverses peroxydases/hémoproteines.[66-68]

1.2.2.1 Décarboxylation oxydative en présence d'ions superoxydes et de radicaux peroxyles[66]

En présence d'ions superoxydes et de radicaux peroxyles, **CT-03** et **Ox063** se dégradent en composés de type quinone-méthide (QM) non paramagnétiques (Schéma 16). Le mécanisme de dégradation implique l'addition du radical superoxyde ou peroxyle en position *para* de l'aromatique suivie d'une décarboxylation oxydative conduisant à la formation de la quinone-méthide (QM). Le suivi UV-Vis de cette réaction s'accompagne de la disparition du pic caractéristique du radical (469 nm pour **CT-03** et 466 nm pour **Ox063** dans le PB pH = 7.4) accompagnée de l'apparition du pic caractéristique de la quinone-méthide (542 nm pour **CT-03** et 546 nm pour **Ox063** dans le PB pH = 7.4).

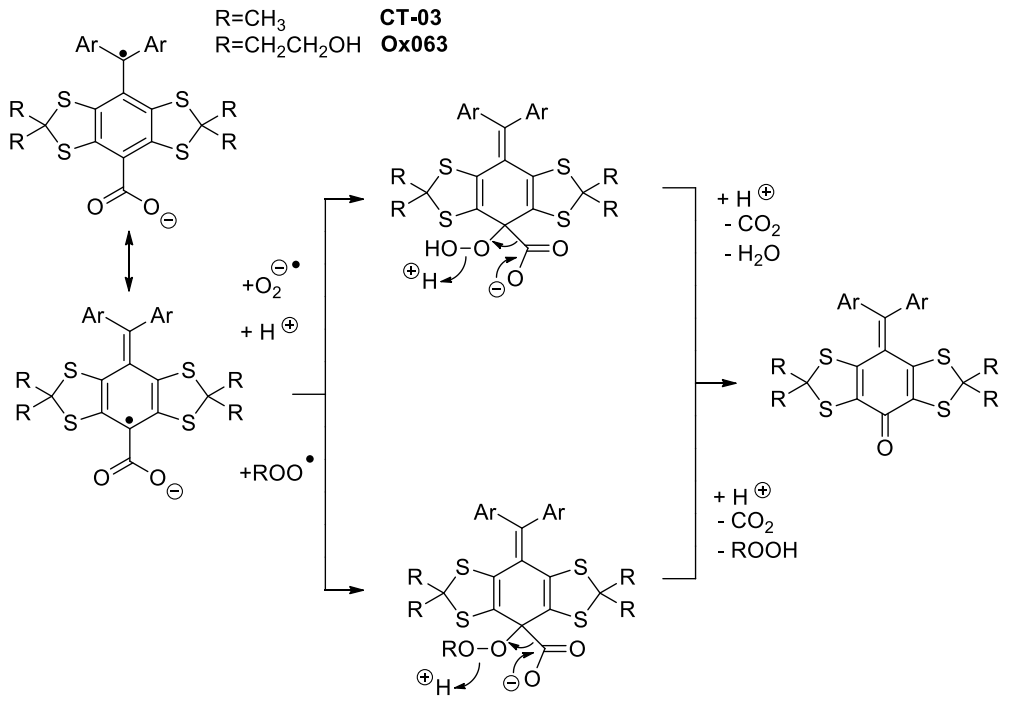


Schéma 16 : Décarboxylation oxydative.

Il convient de noter qu'une *S*-oxydation plus conventionnelle a quand même été observée avec des oxydants puissants tels que $KHSO_5$ et $NaIO_4$.[66]

1.2.2.2 Métabolisme microsomal[67]

Lorsqu'ils sont incubés en présence de microsomes de foie (de rat, porc, humain) et de NADPH, **CT-03** et **Ox063** montrent une nouvelle fois une réactivité similaire. Deux voies de transformation se dégagent suivant la présence ou l'absence d'oxygène dans le milieu. En condition aérobie, on observe la formation de QM (**45**, **46**) alors qu'en condition anaérobie, c'est le triarylméthane (**43**, **44**), produit de réduction, qui est formé (Schéma 17).

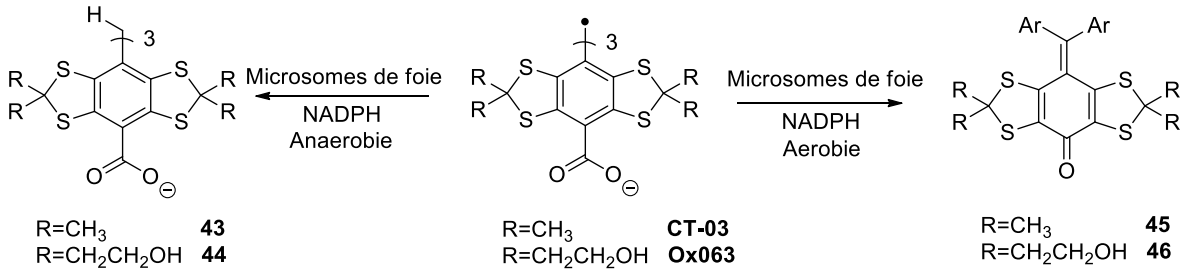


Schéma 17 : Réduction en triarylmethyle et oxydation en quinone méthide.

Il a été démontré que le métabolisme réductif est inhibé par la présence d'oxygène et implique les cytochromes P_{450} ainsi que leurs réductases, le NADPH jouant quant à lui le rôle de donneur d'électrons. En condition aérobie l'oxydation en QM est principalement due à l'ion superoxyde (à hauteur de ≈80%) produit par les cytochromes P_{450} et leurs réductases, le NADPH étant toujours le donneur d'électrons. L'activité résiduelle indépendante de l'ion superoxyde n'a pas pu être déterminée mais pourrait être catalysée également par les cytochromes P_{450}. Il a également été démontré que la QM **45** en présence de microsomes de foie et de NADPH pouvait être réduite en condition anaérobie pour donner la semi-quinone-méthide (SQM) **46** paramagnétique. Ce composé est capable de reformer la QM **45** en condition aérobie par réduction de l'oxygène et pourrait ainsi être à l'origine de stress oxydant, de par la formation d'ions superoxydes (Schéma 18).

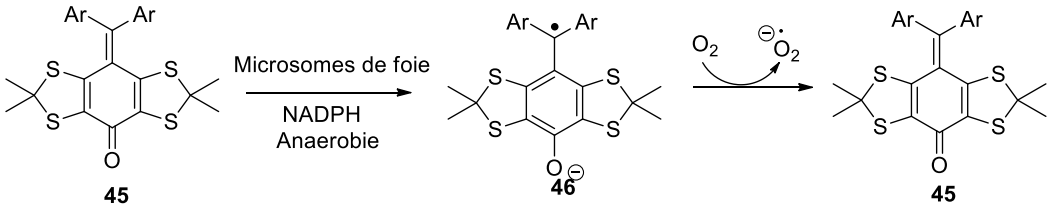


Schéma 18 : La SQM peut être à l'origine de stress oxydant.

1.2.2.3 Métabolisme peroxydasique[68]

En présence de diverses peroxydases et hémoprotéines[d] activées par un substrat hydroperoxyde [e], **CT-03** et **Ox063** subissent une oxydation mono-électronique pour donner le cation triarylméthyle possédant une bande d'absorption caractéristique à 744 nm (PB pH = 7.4). Ce cation évolue ensuite pour donner la QM. Des expériences de marquage à l'oxygène 18 ($^{18}O_2$) ont permis de déterminer que l'oxygène incorporé provenait à la fois de l'eau et de l'hydroperoxyde (Schéma 19). Le mécanisme proposé pour rendre compte de cette observation est le suivant: après formation du cation, le peracide peut s'additionner en position *para* de l'aromatique et conduite à une décarboxylation oxydative pour donner la QM (Voie A). Si c'est l'eau qui s'additionne la décarboxylation de l'intermédiaire conduit à l'anion trityle qui subirait une oxydation à deux électrons pour donner la QM (Voie B).

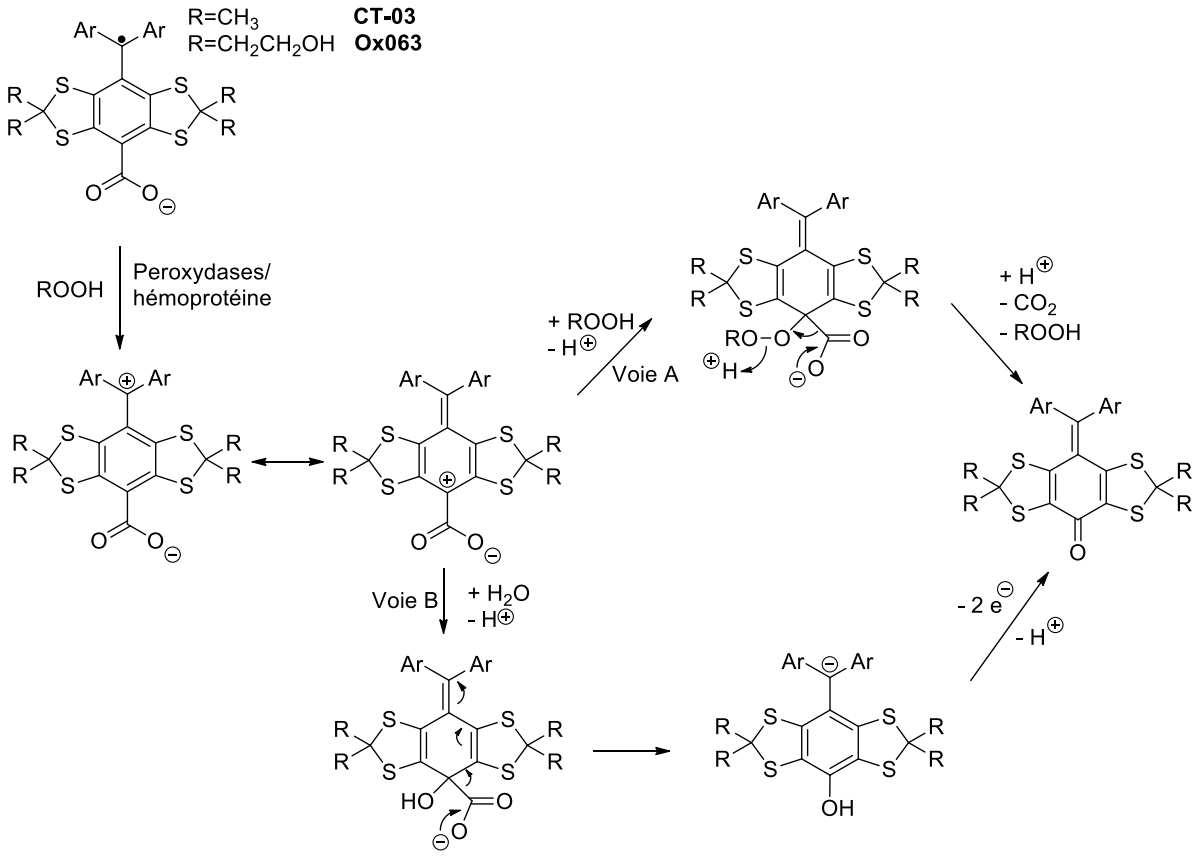


Schéma 19

[d] Horseradish peroxydase (HRP), lactoperoxydase (LPO), prostaglandine H synthase (PGHS, methémoglobine (metHb), metmyoglobine (metMb), catalase, hémine.

[e] H_2O_2, tert-BuOOH, hydroperoxyde de cumyle.

La nature de l'accepteur d'électrons n'a pu être déterminée mais il pourrait s'agir de la peroxydase/hémoprotéine ou encore du cation trityle. En effet, il a été observé que le cation trityle formé en solution aqueuse n'était pas stable et conduisait à la formation d'environ 75% de radical et 25% de QM (Schéma 20). L'anion pourrait être oxydé en QM par deux équivalents de cation TAM^+ donnant un rapport théorique de 66% de radical et 33% de QM. Notons que le cation formé par oxydation du radical par le complexe d'iridium (IV) K_2IrCl_6 peut être transformé en méthide-quinone par l'utilisation d'hydroperoxyde et NaOH ou quantitativement réduit en radical par l'acide ascorbique (Schéma 20).

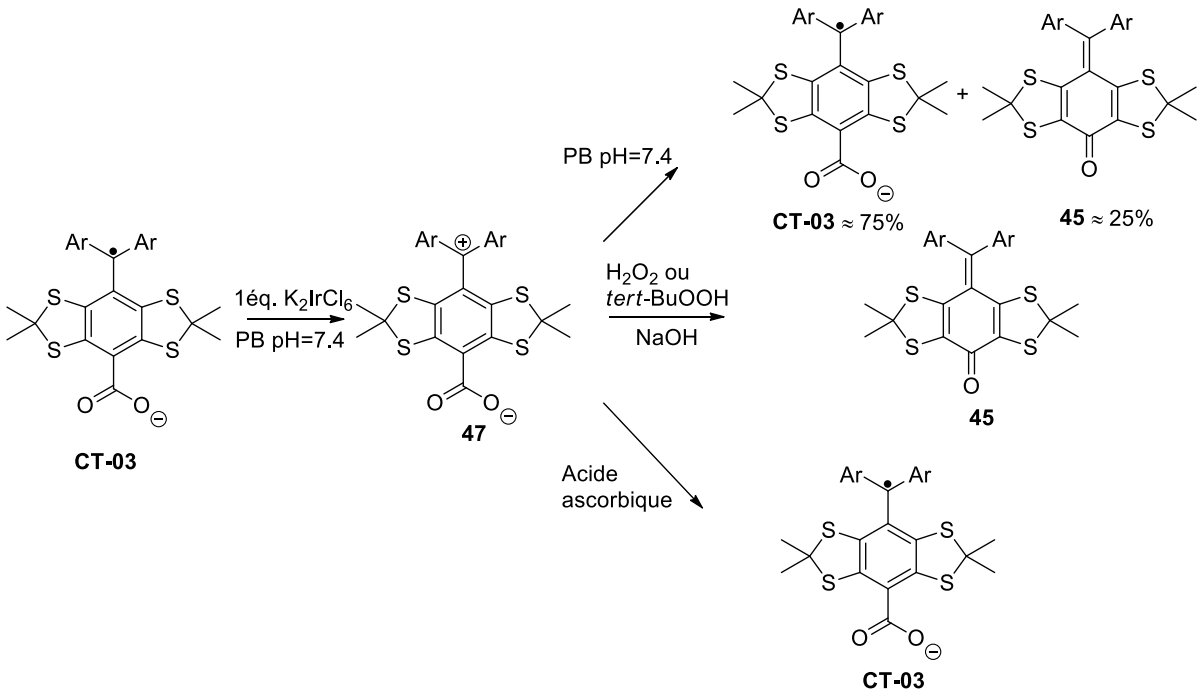


Schéma 20

Le système peroxydase/hémoprotéine en présence d'un substrat hydroperoxyde d'alkyle peut également former des radicaux peroxydes et pourrait donc aussi être à l'origine de la formation de la QM sans passer par le cation.

1.2.3 Modifications chimiques

Nous allons maintenant présenter quelques modifications chimiques de ces sondes qui ont permis de modifier certaines propriétés et/ou d'étendre le champ d'application de ces molécules.

1.2.3.1 Mesure du pH[60, 62]

Une première modification est la synthèse de radicaux tétrathiatriarylméthyles substitués par des fonctions amine permettant la mesure simultanée de la [O_2] et du pH. Ce travail fait suite à l'observation que **CT-03** et **Ox063** présentaient une sensibilité au pH. En effet la valeur du facteur g, et donc la position du signal est fonction de l'état d'ionisation des fonctions acide carboxylique (Figure 22). Les spectres RPE des composés **48** et **49** issus d'une substitution non complète du cœur TAM présentent une multiplicité en raison de couplages hyperfins avec un (**48**) ou deux (**49**) atomes d'hydrogène. En plus du facteur g, la valeur de la constante de couplage est elle aussi fonction du pH (Figure 23). Malheureusement, de nombreux inconvénients limitent l'utilisation de ces radiaux comme sonde à pH. Premièrement, la zone de sensibilité est centrée sur le pKa de l'acide carboxylique (pKa ≈ 2.5) limitant les applications dans les zones où le pH est fortement acide, l'estomac par exemple. Ensuite **CT-03**, **48** et **49** précipitent sous forme protonée en milieu aqueux. Enfin, les variations de g et a_H sont relativement modestes (Figure 22 et 23) et diminuent quand le nombre de fonctions acide carboxylique diminue.

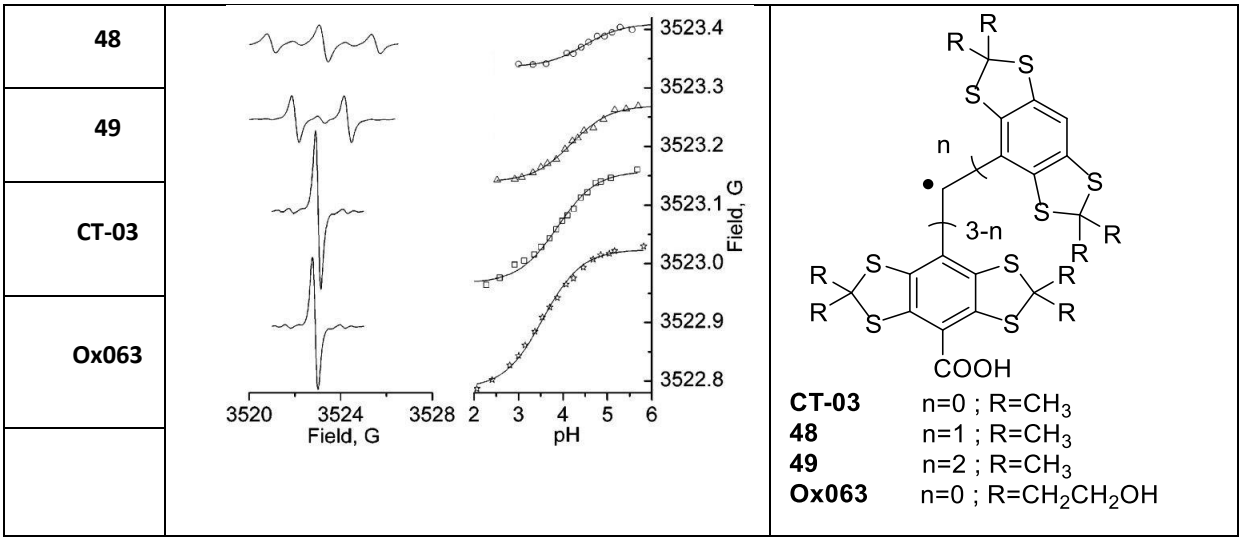


Figure 22: Position du signal RPE en bande-X en fonction du pH pour **48**, **49**, **CT-03** et **Ox063** en solution aqueuse en présence de 25% d'EtOH afin d'éviter la précipitation sous la forme protonée. (reproduit de ref[60]).

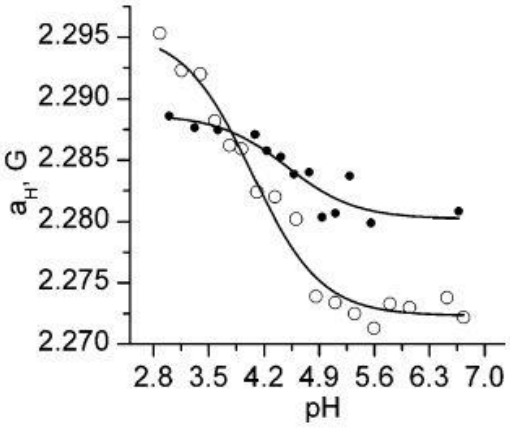
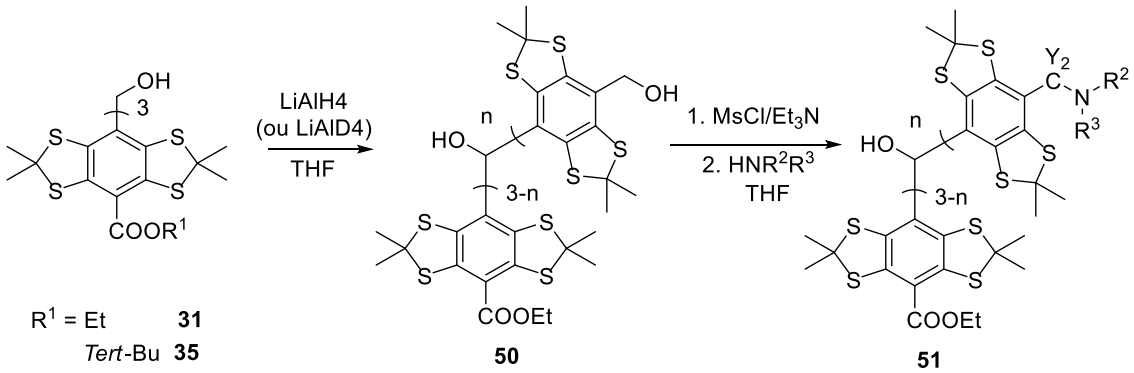


Figure 23: Constante de couplage en fonction du pH pour 48 (•) et 49 (o) en bande-X (reproduit de ref[60]).

Rappelons que la variation de g en fonction du pH est un paramètre dépendant de la fréquence du spectromètre et il devient pratiquement insensible en bande-L. De plus, l'utilisation de la variation de la position du signal nécessite la présence d'un autre radical utilisé comme référence dont le spectre n'est pas sensible au pH. Toutes ces raisons rendent l'utilisation *in vivo* de ces molécules très difficile.

Afin d'augmenter la solubilité et de disposer d'un pKa plus proche du pH physiologique, des radicaux tétrathiatriarylméthyles substitués par des fonctions amines ont été synthétisés (Schéma 21).



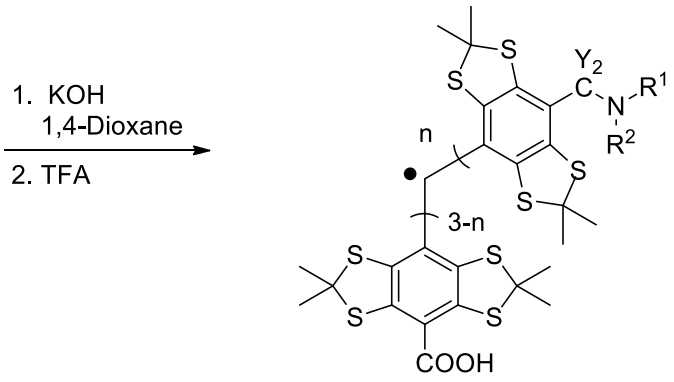
R^1 = Et **31**
Tert-Bu **35**

50

51

Composé	n	Y
50a	3	H
50b	2	H
50c	1	H
50d	1	D

Composé	n	R^2	R^3	Y
51a	3	*Tert*-Bu	H	H
51b	2	*Tert*-Bu	H	H
51c	1	*Tert*-Bu	H	H
51d	1	Et	Et	H
51e	1	Et	Et	D



52

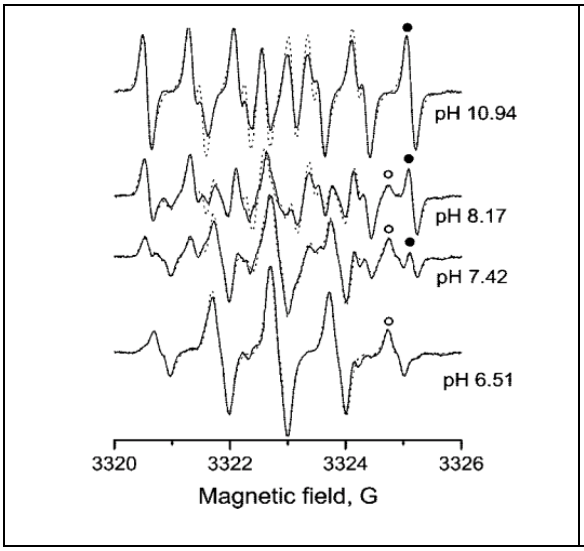
Composé	n	R^2	R^3	Y
52a	3	H	H	H
52b	2	*Tert*-Bu	H	H
52c	1	*Tert*-Bu	H	H
52d	1	Et	Et	H
52e	1	Et	Et	D

Schéma 21: Synthèse des dérives aminés.

La synthèse commence par la réduction des esters. L'utilisation d'un excès d'hydrure d'aluminium permet une triple réduction des esters *tert*-butyliques **35** alors que le traitement de **31** (ester éthylique) par un équivalent de $LiAlH_4$ permet d'isoler le produit de mono-réduction **50c** et de di-réduction **50b**. Le produit de substitution isotopique des protons benzyliques **50d** est quant à lui obtenu par l'utilisation de $LiAlD_4$. Dans l'étape suivante, après activation, le(s) alcool(s) est (sont) substitué(s) par la *tert*-butylamine (**51a-c**) ou la diéthylamine (**51d-e**). Enfin, le(s) ester(s) est (sont) hydrolysé(s) par le KOH (sauf pour **52a**) et un traitement au TFA permet de générer le radical ainsi que de déprotéger *one pot* les amines *tert*-butyles de **51a**. De manière surprenante, le TFA s'est avéré incapable de déprotéger les amines *tert*-butyliques de **51b** et **51c**.

Les spectres de **52a-e** enregistrés en bande-X se révèlent relativement compliqués en raison de nombreux couplages avec des noyaux ^1H, ^2H et ^{14}N, limitant le rapport signal/bruit. Les composés **52c-e** sont de ce fait plus intéressants, car ils ne possèdent qu'une seule fonction conduisant à des couplages. De plus, la présence de deux fonctions acide carboxylique permet de garder la molécule à l'état ionisé favorisant la solubilité en milieu aqueux. Seul le composé **52d** a été évalué comme sonde pour la mesure simultanée du pH et de la concentration en oxygène. Les spectres RPE de **52d** enregistrés en bande-X sous

azote sont représentés à la figure 24. Conformément à la condition: 3 ≤ pKa ≤ 11 (voir paragraphe 1.1.2), l'équilibre entre les formes ioniques est lent et les deux espèces ($RNEt_2$/$RNHEt_2^+$) sont visibles pour un pH proche du pKa (pKa = 8). La différence de spectre entre les deux espèces est la conséquence de la valeur de g et de constantes de couplages différentes entre les deux formes ioniques. De plus, la protonation de l'amine induit un couplage supplémentaire avec le proton issu de la réaction acide-base.



Constantes de couplages hyperfines

a. Forme $RNEt_2$
- a_N = 0.78 mG
- a_{H1} (CH_2) = 95 mG
- a_{H2} (CH_2) = 2.05 G

b. Forme $RNHEt_2^+$
- a_N = 1.01 mG
- a_{H1} (CH_2) = 97 mG
- a_{H2} (CH_2) = 1.05 G
- a_{H3} ($NHEt_2$) = 0.15 G

Figure 24: Spectres bande-X de **52d** mesurés à différents pH à 37°C sous atmosphère d'azote (reproduit de ref[62]).

Etant donné la complexité des spectres obtenus, la mesure simultanée du pH et de la concentration en oxygène se réalise par comparaison entre le spectre expérimental et un spectre simulé permettant de déterminer les proportions des espèces ioniques et donc le pH (Figure 25, gauche)). La largeur de raie de la composante Lorentzienne du pic donne quant à elle, accès à la concentration en oxygène en interaction avec la sonde (Figure 25, droite).

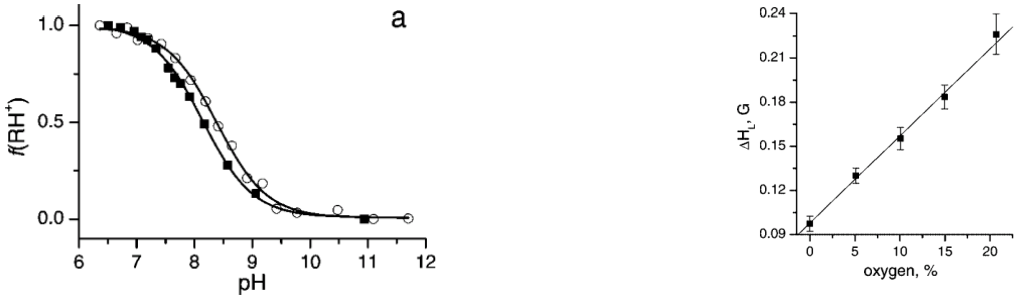


Figure 25: A gauche, fraction molaire de RH^+ en fonction du pH à 23°C (o) et 37°C (▪). En trait continu, la régression non linéaire selon l'équation $f(RH^+) = 1/(1+10^{pH-pKa})$ ou pKa =

8.4 à 23°C et 8.0 à 37°C. A droite, la calibration de la largeur de la composante Lorentzienne du pic en fonction du $\%O_2$. Les deux espèces ioniques à différents pH ont été utilisées pour la calibration ce qui montre le caractère indépendant de la mesure du pH et de la $[O_2]$ (reproduit selon ref[62]).

1.2.3.2 Augmentation de la sensibilité à l'oxygène[61]

La deuxième modification que nous allons présenter avait pour but une augmentation de la sensibilité à l'oxygène. La variation de largeur de raie est limitée par l'équation de Smolushowski. Cependant, le radical **53** substitué par deux fonctions acide carboxylique et une fonction aldéhyde présente un spectre sous la forme d'un doublet qui n'est pas complètement résolu. Cette structure résulte d'un couplage entre le radical et le proton de l'aldéhyde avec une constante de couplage hyperfine de 245 mG et une largeur de raie en condition anoxique de 105 mG. Cette signature particulière permet d'utiliser le rapport d'intensité (I_{in}/I_{out}) (Figure 26) pour la mesure de la concentration en oxygène. Ce rapport est un paramètre plus sensible que la largeur de raie. En effet, l'intensité du signal est inversement proportionnelle au carré de la largeur de raie. En théorie, l'intensité du signal peut être utilisée pour mesurer la concentration en oxygène. Cependant, l'intensité du signal est également fonction de la concentration en sonde. En pratique, on préfère utiliser la largeur de raie à mi-hauteur (spectre d'absorption) ou la largeur de raie pic à pic (spectre en dérivée première) qui sont indépendants de la concentration (tout comme I_{In}/I_{out}).

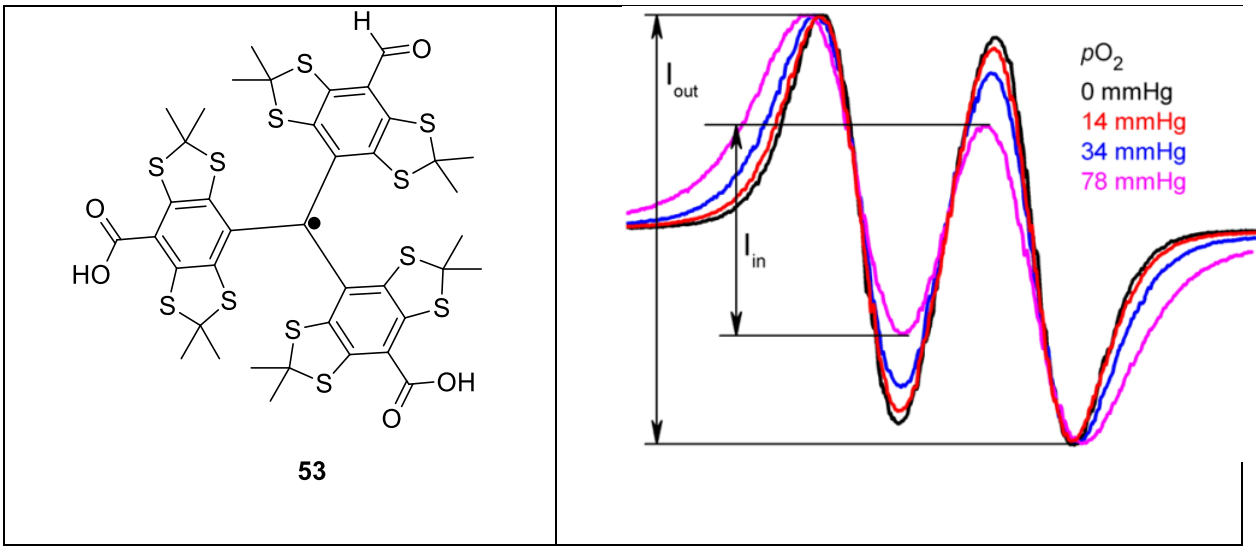


Figure 26: A gauche, structure de **53**. A droite, spectres bande-X montrant la variation du rapport I_{in}/I_{out} en fonction de la pO_2 (reproduit de ref[61]).

De plus, ce rapport a l'avantage de garder une bonne sensibilité à très basse concentration en oxygène. En effet, la largeur de raie des radicaux **CT-03-(d36)** et **Ox063** présente un seuil en dessous duquel la sensibilité est limitée. Ceci s'explique par le fait qu'une partie de la largeur de raie, la composante gaussienne, n'est pas fonction du T_2 (et donc de la concentration en oxygène). Cette composante devient prépondérante à faible concentration en oxygène. La figure 27 montre cette limite pour **Ox063** qui, dans les conditions de mesure, est presque insensible à la variation de concentration en oxygène en dessous d'une pression partielle inférieure à 20 mm Hg (26 µM O_2) alors que le rapport de I_{in}/I_{out} de **53** garde une bonne sensibilité. Cette dernière molécule est donc particulièrement adaptée pour la mesure de faibles concentrations en oxygène.

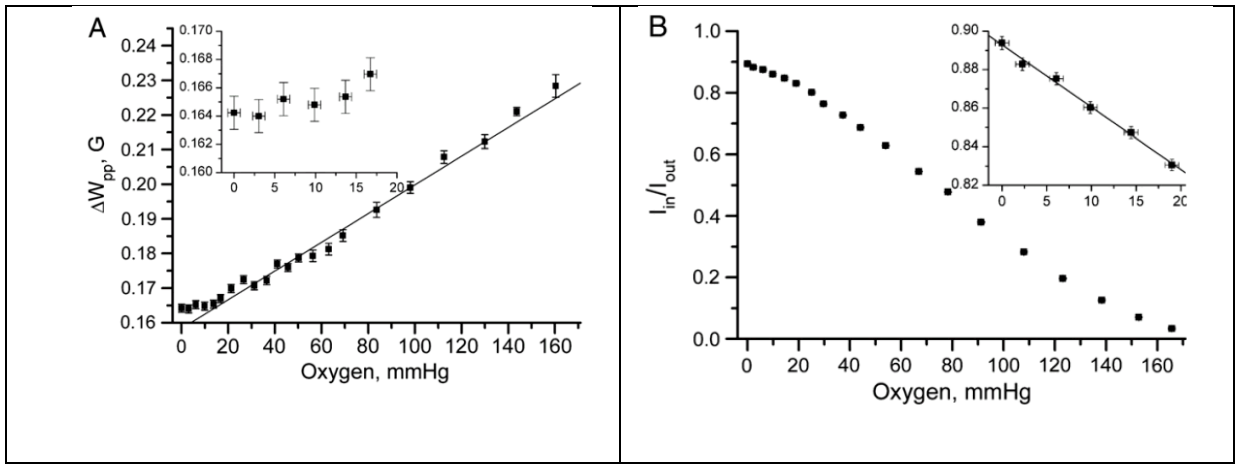


Figure 27: A gauche, dépendance de la largeur de raie pic à pic en fonction de la pO_2 pour **Ox063**. A droite, dépendance du rapport Iin/Iout en fonction de la pO_2 pour **53** (reproduit de ref[61]).

D'un point de vue synthétique, le radical **54** est accessible en trois étapes au départ du composé **50c** déjà présenté. Une oxydation de l'alcool primaire par le réactif de Dess-Martin permet d'isoler l'aldéhyde **53**. Par la suite, les esters sont hydrolysés et le radical est généré par traitement au TFA pour fournir **54** avec un rendement global de 55% sur les trois étapes (Schéma 22).

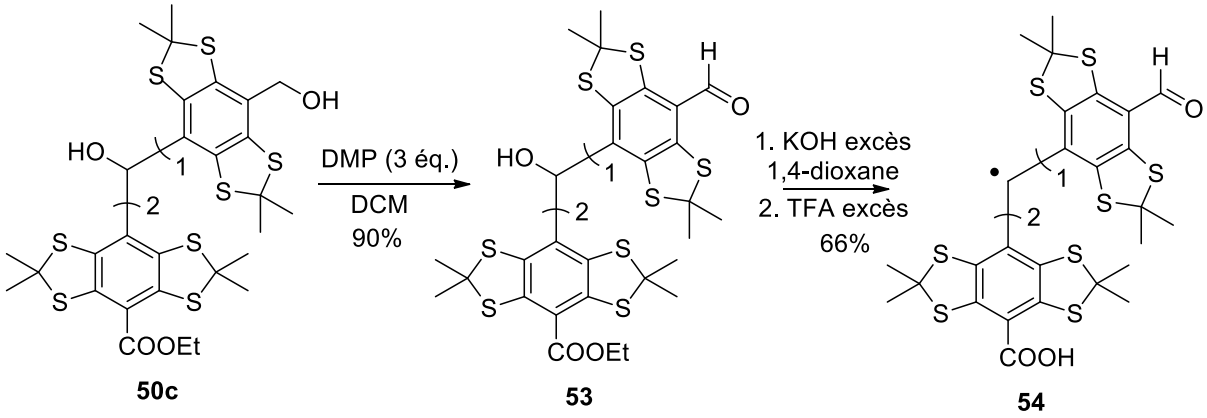


Schéma 22 : Synthèse du dérivé alhéhyde 54.

1.2.3.3 Mesure intra-cellulaire de la concentration en oxygène[69, 70]

De par leur nature chargée, les radicaux tétrathiatriarylméthyles ne peuvent traverser la membrane cellulaire.[70, 71] De ce fait, les mesures de la concentration en oxygène sont limitées au milieu extra-cellulaire. Afin de rendre possible la mesure intra-cellulaire de

la [O_2], des radicaux TAMs, plus lipophiles, substitués par des fonctions esters ont été synthétisés (Schéma 23). Le radical **55** substitué par trois esters *tert*-butyles est synthétisé par traitement de l'alcool TAM correspondant **35** par le $BF_3.OEt_2$ et chlorure d'étain. L'ester méthylique est formé quant à lui par estérification de **CT-03** par l'iodure de méthyle en présence de KOH.

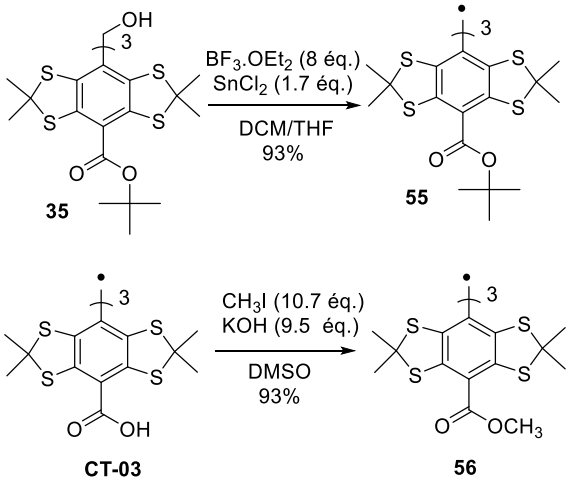


Schéma 23 : Dérivés esters de CT-03, plus lipophiles.

Une stratégie couramment utilisée pour masquer une fonction acide carboxylique d'une molécule pour la faire traverser la membrane cellulaire est d'utiliser son ester acétoxyméthyle (AM) qui est lui plus lipophile. Une fois internalisé, une hydrolyse enzymatique par des estérases permet de libérer l'acide carboxylique. Cette stratégie a déjà été utilisée pour internaliser des radicaux nitroxides.[72] L'estérification de **CT-03** par le bromure de méthyle acétate en présence de K_2CO_3 permet d'isoler les composés **57** et **58** possédant, une et deux fonctions AM, respectivement (Schéma 24).

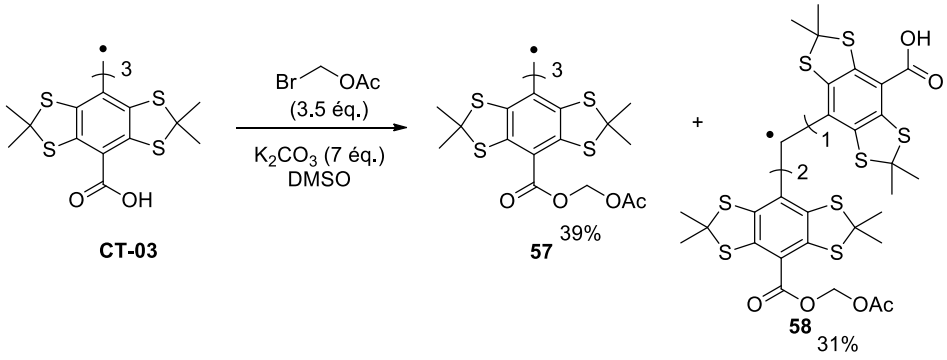


Schéma 24 : Esters acétoxyméthyles (AM).

Dans le DMSO, tous ces radicaux montrent un spectre sous la forme d'un fin singulet. Un couplage faiblement résolu avec les protons des groupements méthyles (a_H = 106 mG) est cependant visible dans le solvant dégazé pour **56**. Les largeurs de raies en condition anoxique s'élèvent à 102 mG, 171 mG, 203 mG et 393 mG pour **55** (BT-03), **57** (AMT-03), **58** (AMT-02) et **56** (MT-03). L'origine de ces variation est très probablement due à un couplage non résolu ou résolu (**56**) entre le radical et les protons alkoxys des esters plutôt qu'à une variation de temps de relaxations.

L'évaluation *in vitro* de ces sondes sur des cellules endothéliales aortiques bovines a montré que toutes sont capables de traverser la membrane cellulaire. Cependant, des largeurs de raie très importantes, de l'ordre de plusieurs gauss ont été obtenues pour **55**, **56** et **57**, probablement en raison d'une précipitation. En effet, ce phénomène d'augmentation très importante de la largeur de raie a déjà été observé pour **CT-03** lors de sa précipitation en milieu acide. Par contre, **58** présente un meilleur potentiel, en effet ce radical TAM possède un caractère amphiphile composé d'une partie aromatique lipophile et d'une fonction acide carboxylique hydrophile. Ces caractéristiques permettent une meilleure solubilité sans bloquer le passage à travers la membrane cellulaire. De plus, et contrairement aux autres TAMs testés, l'hydrolyse de **58** en **CT-03** est observée (Figure 28). Cependant, l'excrétion de **CT-03** hors de la cellule est aussi observée, mais peut être limitée par l'ajout d'un inhibiteur de transporteur membranaire d'anions (probenecid) ce qui tend à prouver l'implication de ces transporteurs dans l'excrétion de **CT-03**.

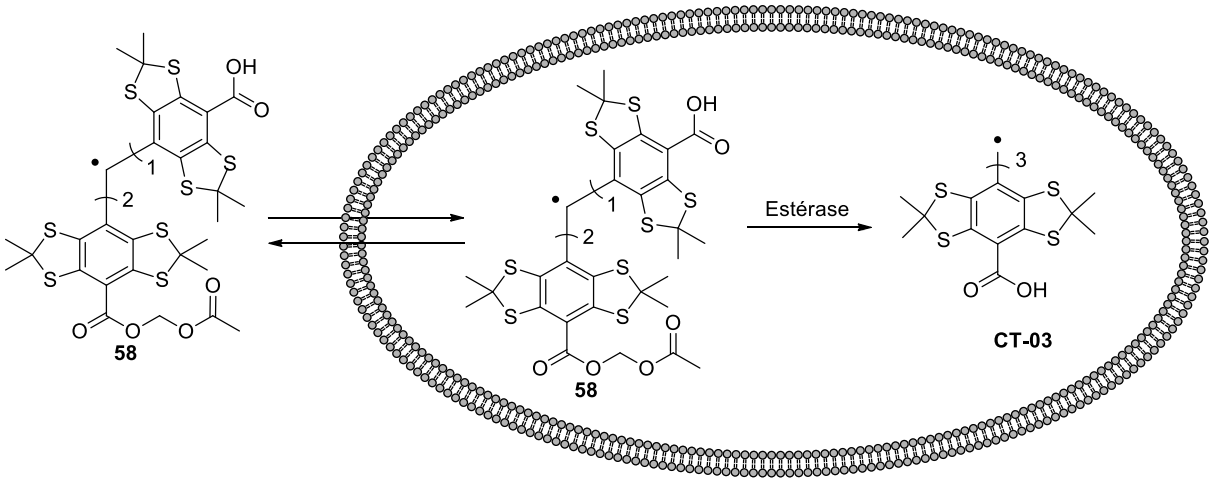


Figure 28 : Le dérivé 58 est capable de rentrer dans une cellule, l'hydrolyse de l'ester par des estérases du cytosol permet de former **CT-03**.

1.2.3.4 Autres modifications et ipso-substitution aromatique

D'autres modifications ont encore été réalisées, par exemple, des dendrimères covalents de TAMs (**DTR1**, **DTR2**, **TG, TdG, dTdG, dTdG PEG**) présentant une plus grande stabilité ont été synthétisés (Figure 29).[64 73]

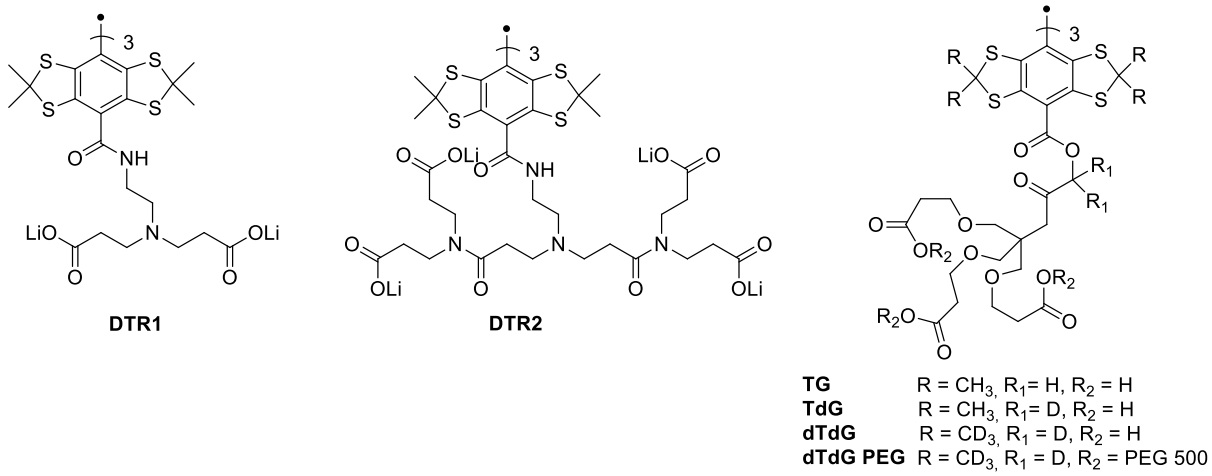


Figure 29: Dérivés dendrimères.

Les biradicaux TAM-nitroxide **TN1**, **TN2**, **TNN14**, et **TNN15** ont été développés pour la mesure du statut rédox par RPE.[74, 75] (Figure 30).

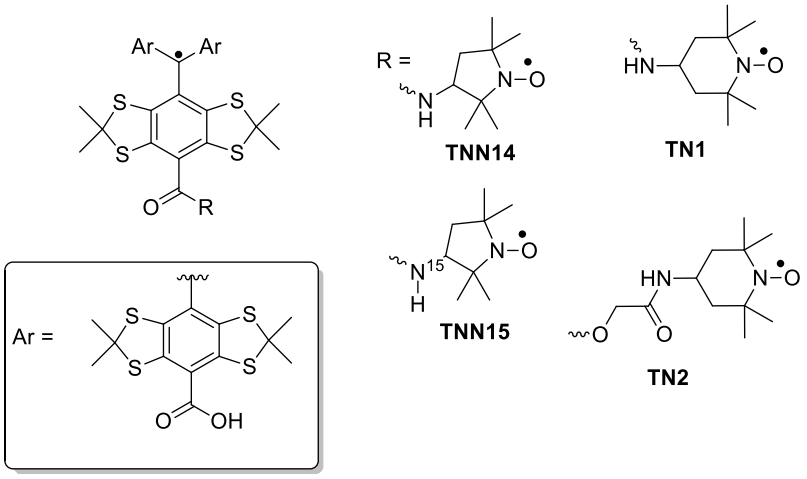


Figure 30: Biradicaux TAM-nitroxide.

Les biradicaux TAM-TAM **TSST** ou TAM-nitroxide **TSSN** liés par un pont disulfure sont utilisés pour la détection de fonction thiol[76] (Figure 31). Ou encore pour la mesure de la distance inter-spins (**59**)[77] (Figure 32). En particulier, **CT-02-TP** est utilisé comme spin label de protéine permettant d'obtenir des informations structurelles (Figure 32).[78]

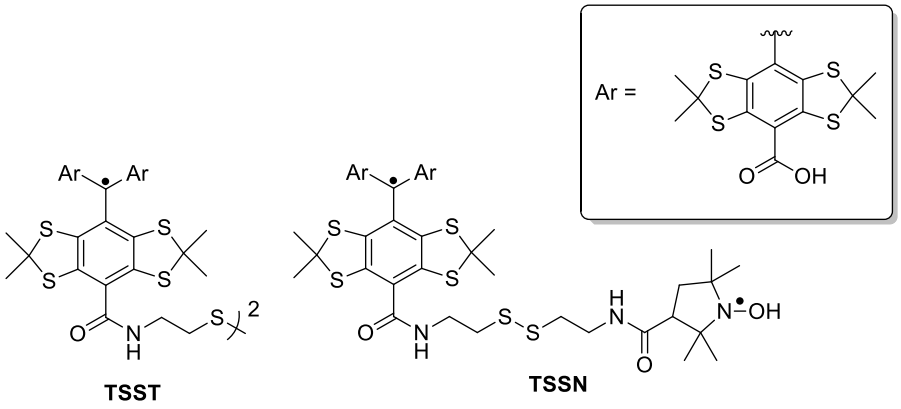


Figure 31: Radicaux TAMs pour la detection de thiols.

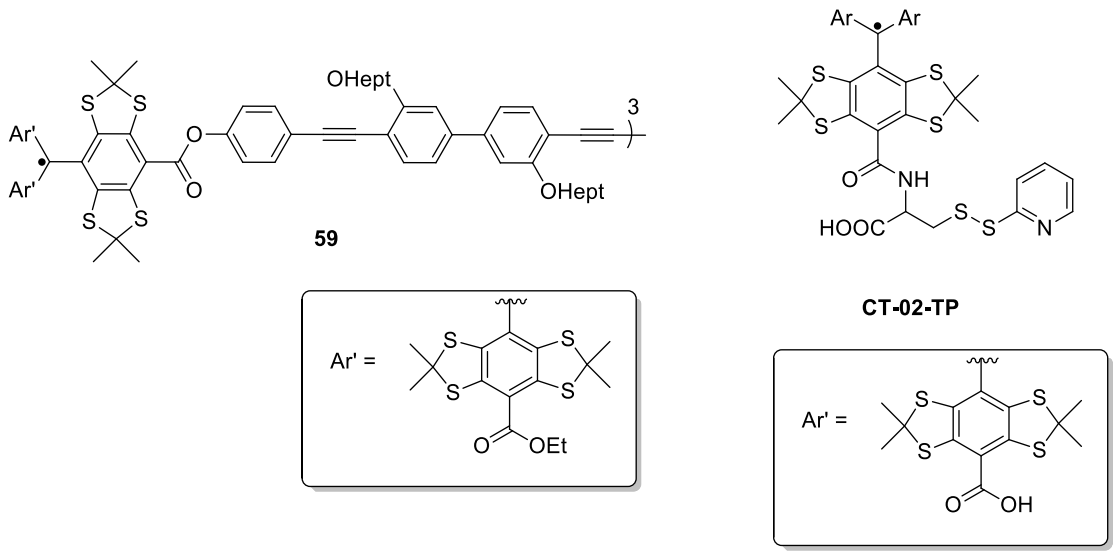


Figure 32: Radicaux TAMs pour la mesure de distance.

Les biradicaux TAM-TAM **60**, **61**, **62** ont été évalués comme agents de polarisation en DNP (Figure 33).[79] Ce domaine de recherche reçoit un développement soutenu par les communautés académique et industrielle ; de nouveaux radicaux triarylmétyles inspirés de ceux développés initialement par Nycomed ont encore été brevetés en 2012.[80]

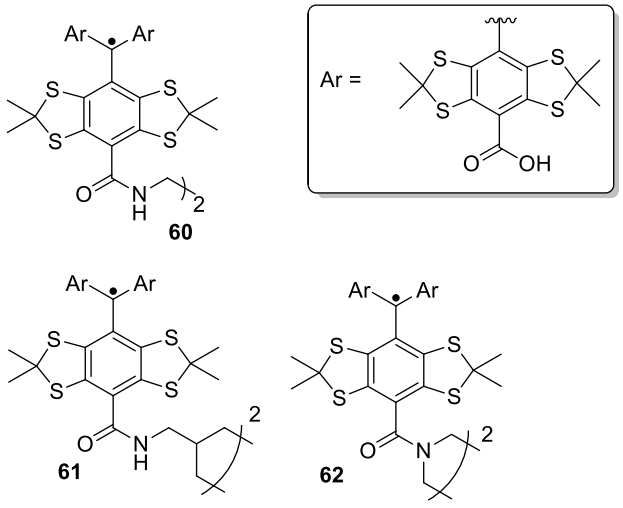


Figure 33: Radicaux TAMs pour la polarisation nucléaire dynamique.

1.3 Références

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2 Objectifs et stratégies

2.1 Contexte général

Le travail entrepris dans cette thèse de doctorat s'inscrit dans le contexte général du développement d'outils permettant la caractérisation du micro environnement tumoral en utilisant la résonance paramagnétique électronique (RPE). Ce travail est le fruit d'une collaboration fructueuse entre deux laboratoires ayant chacun acquis une expérience particulière dans leur domaine de recherche, à savoir le laboratoire de résonance magnétique médicale (REMA) et le laboratoire de chimie organique et médicinale (CHOM).

Nous avons présenté dans l'introduction les deux types de sondes utilisées en RPE biomédicale, premièrement sondes particulaires (charbon, noir de carbone, phtalocyanines de lithium) et les sondes solubles (radicaux nitroxides et triarylmethyles (TAM)). Alors que les sondes particulaires sont mieux adaptées pour des mesures localisées sur des longues périodes, les sondes solubles, en raison de leur diffusion tissulaire, sont mieux adaptées pour des applications d'imagerie. Dans cette dernière catégorie, les radicaux TAMs (ex. **CT-03** ou **Ox063**) (Figure 1) possèdent l'avantage par rapport aux radicaux nitroxides d'une plus grande stabilité, et un signal unique et fin, permettant une meilleure sensibilité de détection. Dans ce travail de thèse, nous proposons de développer de nouveaux radicaux TAMs aux propriétés améliorées, offrant la mesure de l'oxygénation et du pH tissulaire par spectroscopie et imagerie de résonance paramagnétique électronique. Nous proposons également de conférer une sélectivité tissulaire à ces radicaux. Le radical **CT-03** a été sélectionné pour cette modification en raison d'une synthèse plus courte que **Ox063**, bien que déjà relativement compliquée.

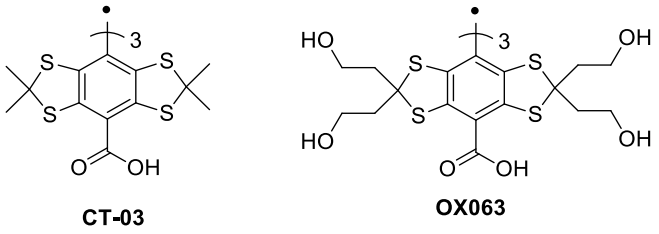


Figure 1: Structure de **CT-03** et **Ox063.**

2.2 Nouveaux radicaux TAMs sensibles au pH

La mesure du pH par RPE se base sur la modification induite par le pH sur le spectre d'un composé paramagnétique.[1,2] Ce sont principalement des radicaux nitroxides qui ont été développés et utilisés pour cette application. Récemment, l'utilisation des radicaux TAM pour la mesure du pH a également été proposée.[3,4] Cependant, le manque de solubilité sous forme acide, la faible sensibilité au pH ainsi que le pKa très faible (pKa **Ox063** = 2.6) des dérivés para-carboxyl limite grandement l'utilisation de ces dérivés. Des composés para-amino ont permis la mesure du pH dans sa gamme physiologique et amélioré la solubilité dans cette gamme. Cependant, la complexité des spectres obtenus rendent l'application pratique difficile. Afin de rendre la mesure du pH dans sa gamme physiologique plus efficace et aisée, il est nécessaire de disposer d'une sonde présentant un pKa ≈ 7, une bonne solubilité et un spectre simple. A nos yeux, la substitution des acides carboxyliques de **CT-03** (Figure 2) par des acides phosphoniques devrait représenter une amélioration majeure. En effet, la présence de trois atomes de phosphore (S_n = ½) devrait éclater le signal de **CT-03** en un quadruplet, structure simple, permettant la mesure du pH de par son effet sur la constante de couplage de ce quadruplet. De plus, les valeur de pKa d'un acide phosphonique aromatique (pKa_1 ≈ 2 , pKa_2 ≈ 7) devraient permettre de mesurer le pH autour de ses deux valeurs. L'hydrophilicité de la fonction acide phosphonique devrait permettre une bonne solubilité dans l'eau.

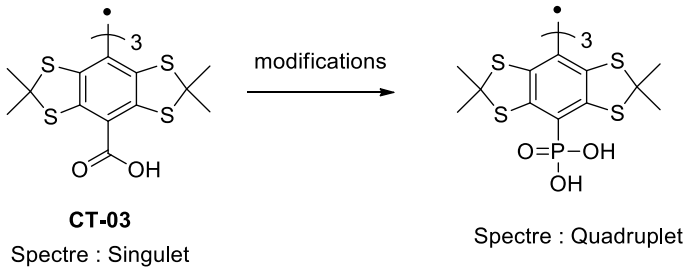
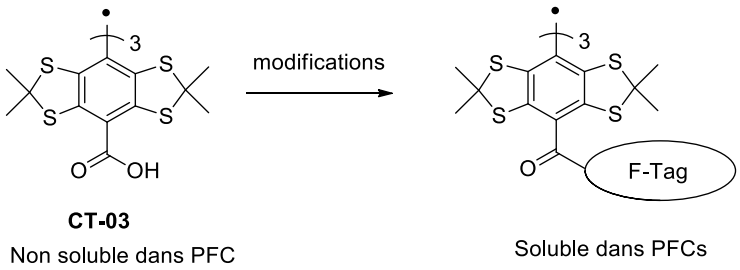


Figure 2 : Modification de **CT-03** pour la mesure du pH.

2.3 Nouveaux radicaux TAMs fluorés pour la mesure de l'oxygénation utilisant des formulations de perfluorocarbones

La mesure de la concentration en oxygène par RPE se base sur la modification de la largueur de raie d'une sonde paramagnétique en fonction de la concentration en oxygène. La sensibilité à la concentration en oxygène des sondes solubles est limitée par la solubilité de l'oxygène dans le milieu (équation de Smolushowski).[5] Celle-ci se limite à une variation d'environ 100 mG entre une solution aqueuse dégazée et une solution en équilibre avec l'air ambiant (21% d'oxygène). Le laboratoire REMA développe des formulations de perfluorocarbones (PFC) biocompatibles permettant d'augmenter la sensibilité à l'oxygène d'une sonde soluble. En effet, les solvants fluorés sont connus pour dissoudre de très grandes quantités d'oxygène. Alors qu'une solution aqueuse dissout 3,1 vol% (25°C) d'oxygène moléculaire, un solvant fluoré tel que le bromure de perfluorooctyle, d'usage clinique, est capable de dissoudre environ 50 vol% d'oxygène (25°C). En encapsulant une sonde à oxygène dans une micelle nanométrique de perfluorocarbone, on augmente la sensibilité à l'oxygène. En effet, l'oxygène étant plus soluble dans le solvant fluoré que dans le milieu biologique extérieur, une variation de la concentration d'oxygène dans celui-ci engendrera une plus grande variation de concentration en oxygène au sein de la micelle. Par conséquent, la variation de largeur de raie sera également plus importante pour la sonde à l'intérieur de la micelle que si elle se situait dans le milieu extérieur. Ceci permettra la mesure de plus faibles variations de la concentration en oxygène. Cette application nécessite donc une sonde présentant une grande affinité pour les solvants fluorés. Les radicaux TAMs synthétisés jusqu'à présent possédant une très faible solubilité dans des solvants fluorés, il est donc nécessaire d'en synthétiser de nouveaux, présentant une meilleure fluorophilicité.

L'introduction de groupements perfluorés permet de rendre une molécule plus affine pour une phase fluorée. L'introduction de chaînes perfluorées sur le radical **CT-03** (Figure 3) au niveau des fonctions carbonyles devrait augmenter la solubilité dans un solvant fluoré et permettre son incorporation dans une micelle de PFC.



CT-03
Non soluble dans PFC

Soluble dans PFCs

Figure 3 : Composés TAMs fluorés.

2.4 Etude de la chiralité des dérivés TAMs

Un autre objectif de cette thèse est de conférer une certaine sélectivité tissulaire à un radical TAM en liant le radical à un ligand spécifique pour un récepteur membranaire. Ces ligands étant souvent des molécules présentant un ou plusieurs centres chiraux, cela nous amène à considérer dans un premier temps la chiralité de conformation du motif triarylmethyle. En effet, en raison de leur grand encombrement stérique, les composés trityles ou TAMs adoptent une conformation en hélice. Ce type de conformation engendre un type de chiralité moins souvent rencontré en chimie organique que le centre stéréogénique. En effet, la conformation hélice peut prendre deux conformations images miroirs : hélice droite et hélice gauche (Figure 4). L'introduction d'un ligand présentant un centre chiral pourrait conduire à la formation de deux diastéréoisomères aux propriétés physique, chimique et biologique différentes. L'équilibre entre ces deux conformations est en général rapide pour le tritytle, ce qui rend ce motif non chiral. Cependant, la très grande gêne stérique des radicaux TAMs utilisés en RPE pourrait limiter l'équilibre entre ces deux conformations et rendre ces molécules chirales. Il conviendra d'étudier cet équilibre et les facteurs structuraux l'influençant afin d'anticiper la formation de stéréoisomères lors de la

liaison du TAM à un ligand chiral. L'étude expérimentale sera complétée par une étude en chimie théorique en collaboration avec le Prof. R. Robiette (IMCN/CHOM)

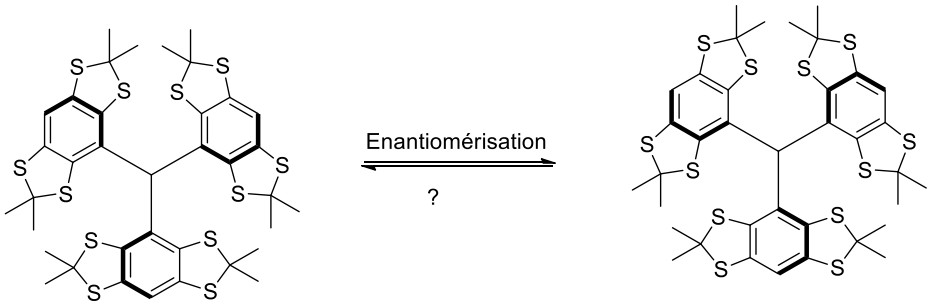


Figure 4 : Les deux conformations énantiomériques des radicaux TAMs.

2.5 Synthèse de radicaux TAMs vectorisés

Alors que certains radicaux nitroxide vectorisés ont été décrits,[6,7] jusqu'à présent seuls des radicaux TAMs présentant une distribution non spécifique ont été synthétisés. Nous pensons conférer une sélectivité tissulaire au radical **CT-03** en le liant d'une manière covalente à un ligand qui sera reconnu spécifiquement par un récepteur membranaire. En particulier, c'est l'angiogenèse tumorale qui est visée. Le laboratoire CHOM a développé une série de peptidomimétiques présentant une grande affinité pour l'intégrine $\alpha_v\beta_3$.[7] Cette glycoprotéine est impliquée dans le phénomène d'adhésion entre cellules endothéliales et entre la matrice extracellulaire et les cellules endothéliales. Ce récepteur est également exprimé à la surface de certaines cellules cancéreuses invasives. Cette intégrine reconnaît spécifiquement la séquence tri-peptidique RGD de protéines présentes dans la matrice extra-cellulaire telles que la vitronectine et la fibronectine. Des composés peptidiques cycliques présentant cette séquence RGD, tel que le Cilengitide (Figure 4) ont été synthétisés comme antagonistes de l'intégrine $\alpha_v\beta_3$. La structure cristalline du Cilengitide complexé à la partie extra-cellulaire de l'intégrine $\alpha_v\beta_3$[8] a permis le développement d'une série de peptidomimétiques mimant la séquence RGD et présentant une grande affinité et sélectivité pour cette intégrine. Le peptidomimétique **3a** est le pharmacophore présentant la meilleure affinité pour l'intégrine $\alpha_v\beta_3$. Une IC_{50} de 0,1 nM a été mesurée vis-à-vis d'intégrines humaines isolées lors d'un test de binding. Ce composé peut être muni d'un bras d'ancrage

de type oligo éthylène glycol permettant son accrochage à une matrice ou autre molécule sans perdre d'activité (IC_{50} de 0,3 nM pour **3b** et 0,7 nM pour **3c**).

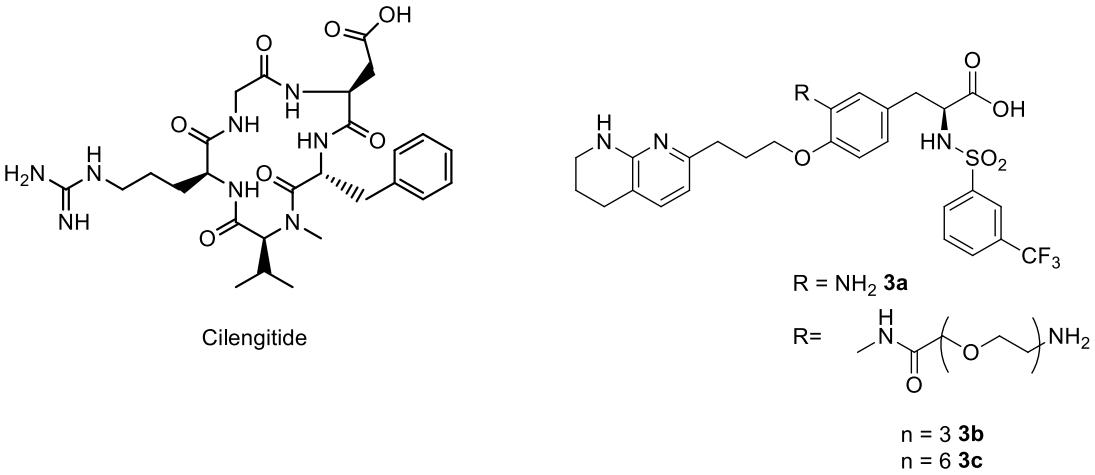


Figure 5 : Structure du Cilengitide et peptidomimetiques RGD développés au laboratoire.

La liaison du radical **CT-03** à un peptide possédant la séquence RGD ou au peptidomimétique **3** par l'intermédiaire d'un bras espaceur devrait nous fournir un radical TAM vectorisé vers l'intégrine $\alpha_v\beta_3$ qui sera utilisé en imagerie RPE (Figure 6).

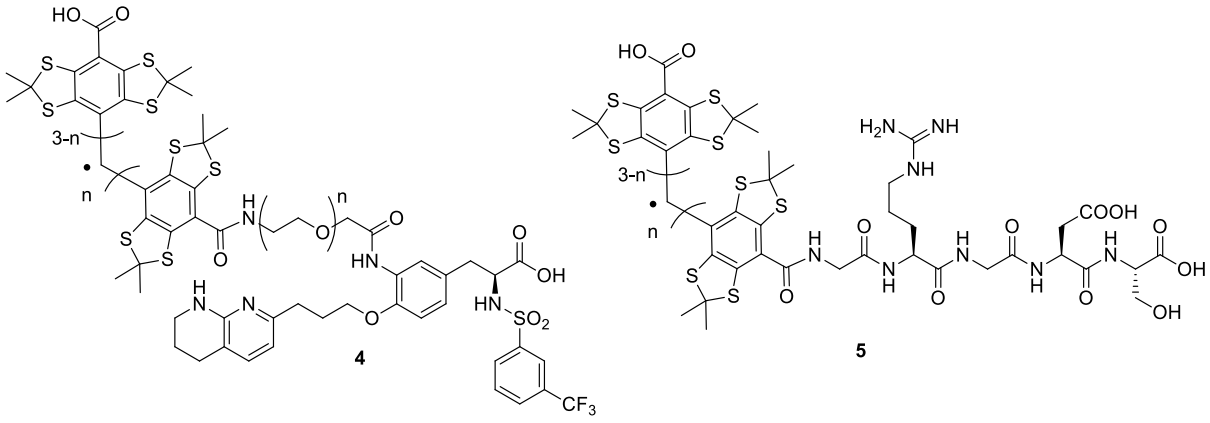


Figure 6 : Structure des TAMs vectorisés.

2.6 Plan du manuscrit

La partie résultats est constituée des articles publiés ou soumis pour publication. Les parties expérimentales seront introduites à la fin de chaque article. Les parties expérimentales volumineuses de certains articles seront allégées des informations non essentielles. (Spectre HRMS, RMN, coordonées cartésiennes des molécules, etc.). Les versions complètes sont disponiblles gratuitement en ligne.

Le **chapitre 3** est consacré aux nouveaux radicaux **TAMs sensibles au pH**.

Article 1 :B. Driesschaert, V. Marchand, P. Levêque, B. Gallez, J. Marchand-Brynaert, Chem. Commun. **2012**, *48*, 4049-4051

Le **chapitre 4** est consacré au radicaux **TAMs fluorés** pour la mesure de l'oxygénation utilisant des formulations de perfluorocarbones.

Article 2 B. Driesschaert, N. Charlier, B. Gallez, J. Marchand-Brynaert, Bioorg. Med. Chem. Lett. **2008**, *18*, 4291-4293.

Article 3 N. Charlier, B. Driesschaert, N. Wauthoz, N. Beghein, V. Préat, K. Amighi, J. Marchand-Brynaert, B. Gallez, J Magn. Reson. **2009***, 197*, 176-180.

Le **chapitre 5** est consacré à l'étude de la **chiralité des dérivés TAMs**.

Article 4 B. Driesschaert, R. Robiette, F. Lucaccioni, G. Gallez, J. Marchand-Brynaert, Chem. Commun. **2011**, *47*, 4793-4795.

Article 5 B. Driesschaert, R. Robiette, C. S. Le Duff, L. Collard, K. Robeyns, B. Gallez, J. Marchand-Brynaert, Eur. J. Org. Chem. **2012**, *33*, 6516.

Le **chapitre 6** est consacré à la synthèse de radicaux **TAMs vectorisés**.

Article 6 B. Driesschaert, P. Levêque, B. Gallez, J. Marchand-Brynaert, Tet. Lett. accepté

Article 7 B. Driesschaert, P. Levêque, B. Gallez, J. Marchand-Brynaert à soumettre dans Chemistry an Asian Journal

Article 8 V. Rerat, S. Laurent, C. Burtéa, B. Driesschaert, V. Pourcelle, L. Vander Elst, R. N. Muller, J. Marchand-Brynaert, Bioorg. Med. Chem. Lett. **2010**, *20*, 1861-1865.

La **chapitre 7** cloture la manuscrit par les **conclusions et perpectives** de ce travail.

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3. Radicaux TAMs sensibles au pH

Cette partie du manuscrit décrit la synthèse d'un radical phosphoré et sa caractérisation physico-chimique pour une utilisation comme sonde à pH par spectroscopie RPE.

Ce travail a été publié dans le journal " Chemical Communications" . Son importance a été soulignée par les referees et il a fait l'objet de la couverture du journal.

Ma contribution personnelle à ce travail a été le design d'une nouvelle molécule pour la mesure du pH par RPE, l'imagination de sa voie de rétrosynthèse et finalement sa synthèse totale. L'étape clé est l'introduction des fonctions phosphonates par addition du lithien TAM sur un chlorophosphate. Ce travail a été inspiré par l'expertise acquise par le laboratoire CHOM dans la synthèse et la purification de dérivés phosphorés.

La courbe d'étalonnage en RPE bande-X a été construite avec l'aide de V. Marchand (UCL/REMA) qui possède une expertise dans ce domaine. V. Marchand a également évalué l'influence de la force ionique sur la constante de couplage. Les mesures préliminaires ont été réalisées par le Dr. P. Levêque (UCL/REMA).

Je remercie le Prof. V. Khramtsov pour ses précieux conseils donnés lors du congrès Spin 2011 (Marseille, France) concernant la construction de la courbe d'étalonnage. La synthèse de radicaux TAMs phosphorés a été reprise par le groupe du Prof. Khramtsov, et deux articles directement inspirés de notre travail pionnier ont été publiés par la suite.

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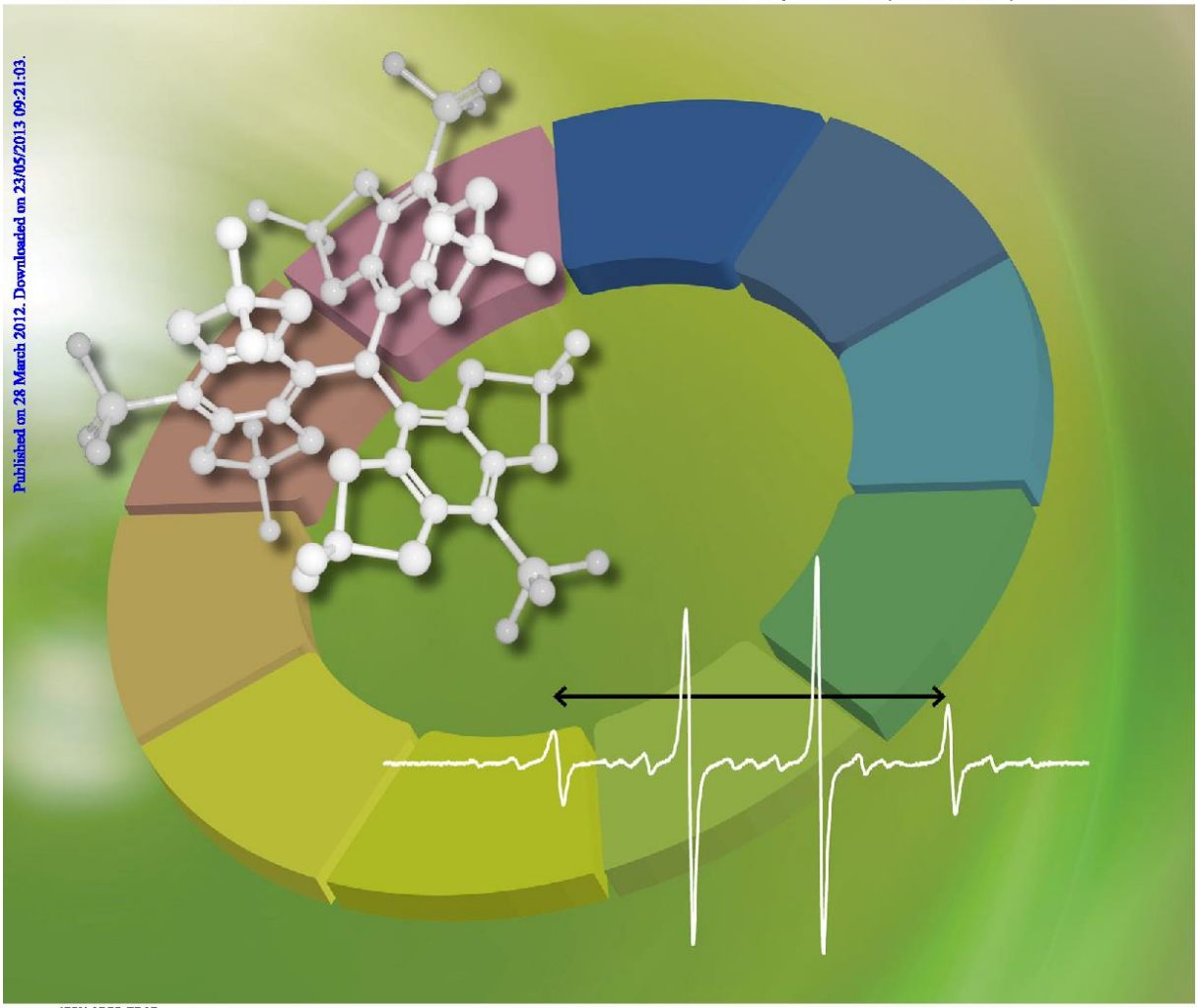

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COMMUNICATION

J. Marchand-Brynaert *et al.*

A phosphonated triarylmethyl radical as a probe for measurement of pH by EPR

Phosphonated Triarylmethyl Radical as Probe for Measurement of pH by EPR

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Abstract: A new water soluble phosphonated tetrathiatriarylmethyl radical has been synthesized and its application for pH measurement in a physiological range by EPR is reported.

Local pH plays a key role in the physiology of living organisms. Many cell activities, cellular organelles and enzymes are closely dependent of the pH. For these reasons, living beings have developed mechanisms to regulate the local proton concentration. Deviance from a given pH is also known to be tightly linked to numerous diseases such as tumor, chronic heart failure, inflammation, etc.. Therefore, non-invasive measurement of pH is of great physiological and pathophysiological interest. Most of the non-invasive methods for pH assessment rely on endogenous and/or exogenous ionizable probes displaying different spectral properties in their basic (R^-) and acidic (RH) forms[1]. Absorption and fluorescence spectroscopy using pH sensitive dyes are suitable for cellular and sub-cellular levels *in vitro* while magnetic resonance (MR) techniques are particularly convenient for non-invasive measurement of pH *in vivo*. The modification of ^{31}P NMR chemical shift of endogenous inorganic phosphate (Pi) with pH has proven to be the most appropriate method for measuring the intracellular pH but also suffers from limitations, including a low sensitivity, the fact that the chemical shift changes with ionic strength and the variation of Pi concentration with the metabolism and ischemia. EPR has an advantage over NMR because it has higher intrinsic sensitivity for the same probe

concentration. The pH measurement by EPR is based on the modification of g-factor and nitrogen hyperfine splitting (hfs) constant a_N of particular ionizable nitroxides with proton concentration. The value of pH can be extracted from the ratio $[R^-]/[RH]$ determined by spectral simulation or by calibration of certain spectral parameters (peaks intensity ratio $[R^-]/[RH]$ or nitrogen hfs constant a_N)[2]. The range of measurable pHs is limited to three units centered on the pKa of the probe which became a critical characteristic for this application. Imidazoline and imidazolidine type nitroxides have been widely used to accurately determine pH values *in vivo*.[3] However, the use of nitroxides is limited *in vivo* by their fast bioreduction into EPR silent hydroxylamines. Tetrathiatriarylmethyl radicals such as water soluble **Ox063** (Fig. 1) are newly developed carbon-centered radicals possessing remarkable properties. Indeed, by comparison with nitroxides, these radicals have a higher biostability and display a lower concentration-dependent broadening of their EPR lines. Furthermore, they exhibit longer relaxation times, which results in narrower EPR lines and thus a higher analytical resolution (as the EPR signal intensity is inversely proportional to the square of the linewidth)[4]. These compounds initially developed as hyperpolarizing agents for Overhauser-enhanced magnetic resonance imaging (OMRI)[5] have found applications for EPR measurements of intracellular[6] and extracellular[7] oxygenation, superoxide anion[8], redox status[9], thiol[10] and pH[11, 12]. The concept of using tetrathiatriarylmethyl radicals for measuring pH by EPR has been introduced by Khramtsov *et al*.[11] The ionization of the carboxylic functions of **Ox063** and **cTAM$_n$** (Fig. 1) results in a modification of the g-factor and the proton hfs constant a_H. However, biological applications are limited by the low value of pKa (pKa_{Ox063}=2.6)[11], the relatively small sensitivity to pH variations and the low aqueous solubility of $cTAM_n$ derivatives in their acidic forms (RH). The synthesis of amino derivatives ($aTAM_n$) improved water solubility and allowed pH measurement in a physiological range but hyperfine splittings with ^1H, ^2H and ^{14}N render the spectra very complex, limiting practical applications[12].

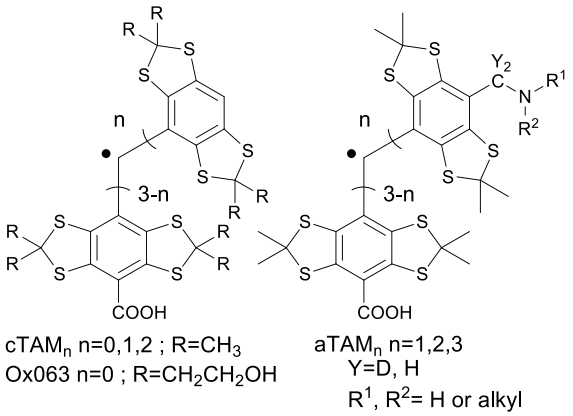
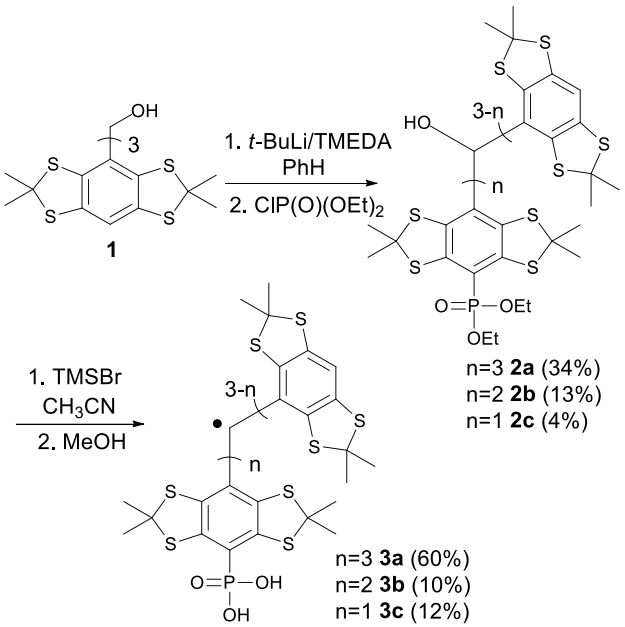


Fig. 1 Structures of TAM radicals used as pH probes.

We hypothesized that a tetrathiatriarylmethyl radical substituted by three phosphonic acids should be the ideal candidate as probe for measuring the pH by EPR in a physiological range (pH≈7). The presence of three phosphorus atoms with a nuclear spin of ½ should split the EPR signal into a quartet, allowing the assessment of the pH by measuring the modification of the phosphorus hfs constant a_P.

Herein, we report a straightforward synthesis of a new water soluble phosphorus-containing TAM derivative and its application as a "molecular pH-meter". The synthesis was achieved in a two-step sequence starting from **1** as depicted in Scheme 1. Trityl alcohol **1** has been synthesized according to a procedure described by Dhimitruka *et al*.[13] The deprotonation of the three aromatic protons with *t*-BuLi and TMEDA followed by addition of the resulting anion to a solution of diethyl chlorophosphate led to the formation of the desired phosphonic ester **2a**, mixed with smaller quantities of **2b** and **2c** resulting from an incomplete substitution of the trityl core. The mono, di- and tri-substituted trityl alcohols **2** were separated by semi-preparative HPLC. In the second step, the ethyl ester functions of **2** were cleaved by treatment with TMSBr. It is noteworthy that under these conditions, the trityl alcohol was converted into a radical. This observation is consistent with previous results regarding the quantitative conversion of tetrathiatriarylmethyl alcohol into radical under acidic conditions (e.g. TFA).[12-14]



Scheme 1 Synthesis of phosphonated TAM radicals.

The EPR spectra of newly synthesized TAMs derivatives were recorded at X-band in 10 mM PBS for **3a** and **3b** and in methanol for the less hydrophilic structure **3c**. (Fig. 2). According to its structural properties, radical **3a** exhibits a simple quartet spectral feature resulting from the coupling between the unpaired electron with three ^{31}P nuclei. At pH=7.5 the apparent a_P measured is 3.5 G. Residual ^{1}H atoms of **3b** and **3c** render their spectra much more complicated due to additional coupling and reduce their water solubility. The triplet of doublet of **3b** arises from the coupling of the unpaired electron with two ^{31}P and one ^{1}H nucleus while a doublet of triplet is observed for **3c** arising from the coupling of the unpaired electron with one ^{31}P and two ^{1}H nuclei. The observed a_H=2.3 G is similar to that report for $cTAM_n$ (n=1,2).[11] The ideal probe for measuring pH should exhibit high water solubility and a simple EPR spectrum. Complete substitution of the trityl core of **3a** results in the simplest EPR pattern and a very good water solubility. Thus, only compound **3a** has been selected for further evaluation as probe for pH measurement.

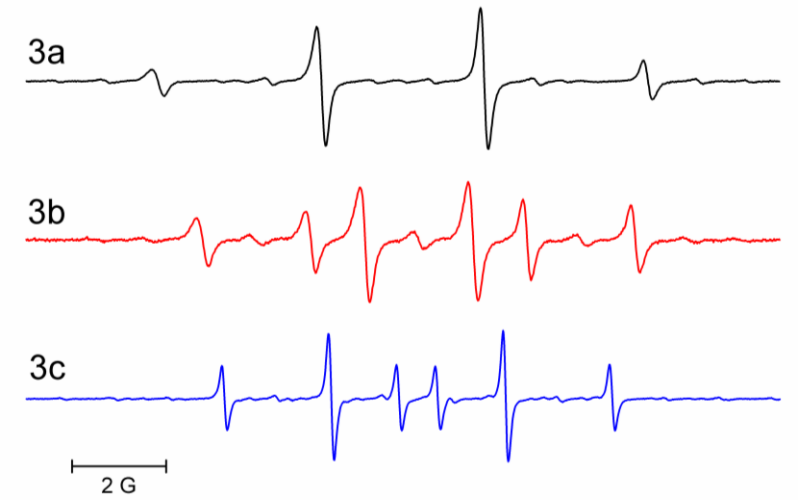


Fig. 2 X-band spectra of 100 µM phosphonated TAM radicals in 10 mM PBS (**3a**, **3b**) and degassed MeOH (**3c**).

In aerated PBS (10 mM, pH=7.5), radical **3a** exhibits a narrow linewidth (< 170 mG) and shows excellent stability as no degradation was observed after 48h at 25°C. Since the phosphonic acid possesses two pKa values pH measurements in two ranges of pH should be possible.

In order to evaluate the spectral sensitivity to pH, a titration curve has been performed in PBS 10 mM at X-band (Fig. 3). The apparent a_P, measured as the distance between the two high-field components of the quartet, is affected by the pH around the two pKa values. The first deprotonation corresponding to pka_1 results in a decrease of the apparent a_P of 133 mG (Δa_{P1}=133 mG). The variation observed for pKa_2 is higher and reaches Δa_{P2}=258 mG. The pKa values determined by fitting the experimental titration curve give pKa_1=1.3 and pKa_2=7.1.

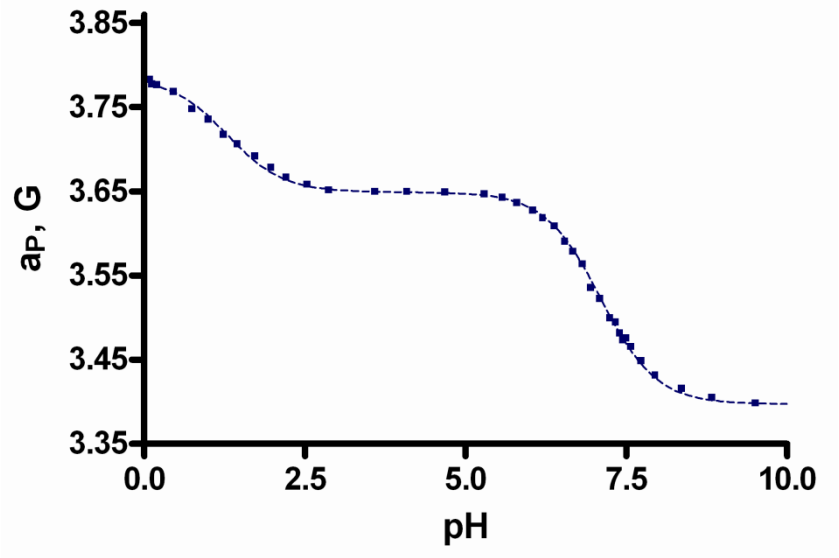


Fig. 3 The pH dependence of the hyperfine splitting constant of **3a** at 25°C measured as the distance between the two high-field components of the quartet referred to as a_P. Spectra were recorded at X-band for 100 µM of **3a**.

The second pKa is particularly interesting as many biological applications require measuring pH in a physiological range while the low value of the first pKa is useful for preventing self-aggregation over a wide range of pH by keeping the molecule in an ionic form. Indeed, no precipitation was observed during the titration while protonation of the carboxylic functions of cTAM for pH<4 induces precipitation in aqueous media limiting its applications as a pH probe[13]. Enhanced sensitivity to pH is obtained by measuring the distance between the low- and high-field components of the quartet referred to as $3a_P$ (Fig. 4). In that case $\Delta 3a_{P1}$=400 mG and $\Delta 3a_{P2}$=774 mG, with no modification of the ESR-determined pKa values. The modification of the hyperfine splitting constant with pH is also affected by spectrometer frequency and acquisition settings which should be optimized for a particular application. Detection at modulation amplitude higher than the linewidth results in a line broadening and an increase of the signal to noise ratio (SNR). By increasing the modulation amplitude from 0.02G to 0.6G, the signal intensity is increased by one order of magnitude and results in a slight smoothing of the calibration curve (Fig. 4).

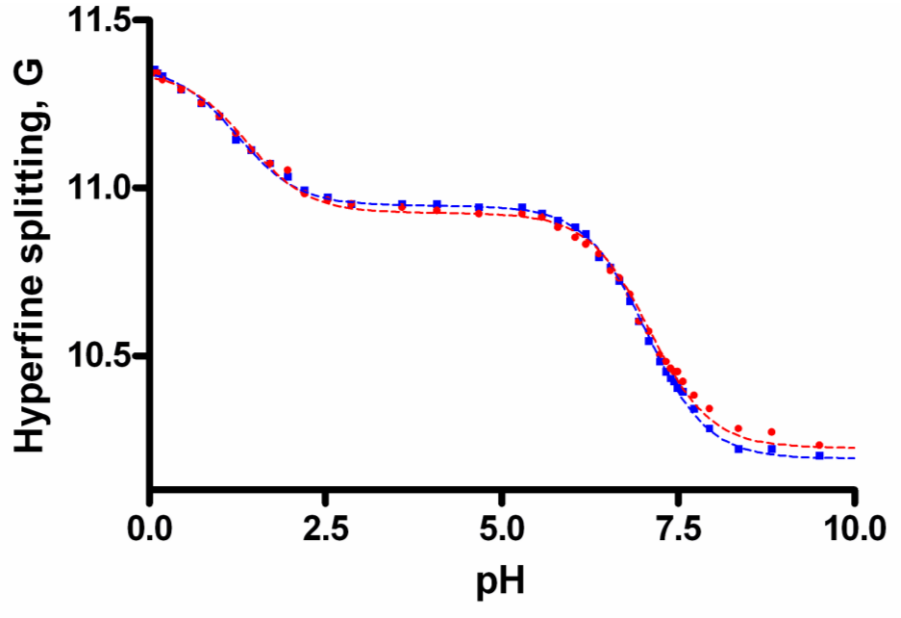


Fig. 4 The pH dependence of the hyperfine splitting of **3a** at 25°C measured as the distance between the low- and high-field components of the quartet, referred to as $3a_P$. Spectra were recorded at X-band for 100 µM of **3a** at 0.02 G (▪) and 0.60 G (•) modulation amplitude.

Trityl radical **3a** possesses three phosphonic acids which can be ionized independently which results in four ionic species present together for pH close to the pKa (See ESI†). These ionic species are visible for the second pKa at a low concentration of phosphate buffer (Fig. 5). The splitting of the two low-field spectral components results of the superposition of the four ionic forms with different a_P and g. This observation is due to a slow proton exchange rate between these species on the EPR time scale. On the contrary, an average spectrum is observed for the first pKa which is the consequence of a fast proton exchange rate.[2] By increasing the phosphate buffer concentration, a coalescence of the EPR signal of the ionic forms corresponding to the second pKa is observed as a consequence of an increase of the proton frequency exchange by using a buffer with a pKa close to the probe's pKa.

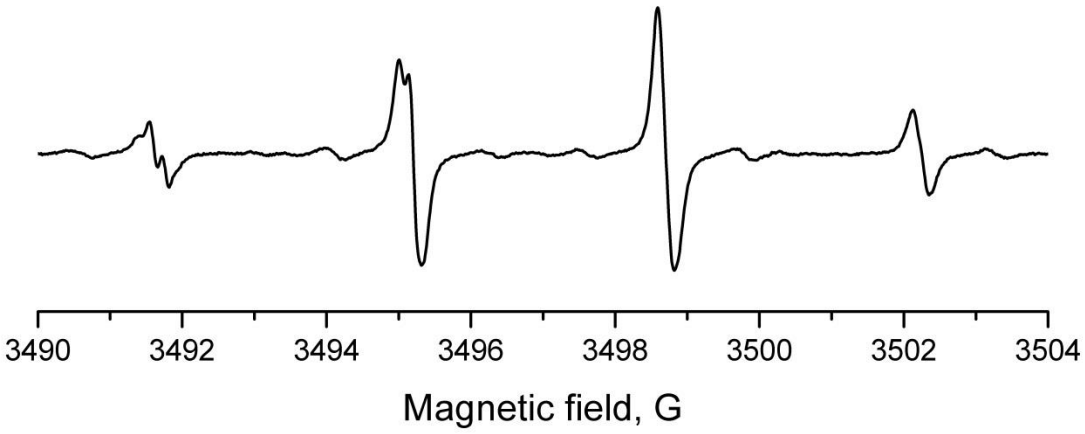


Fig. 5 X-band spectra of 100 µM phosphonated TAM radical **3a** in 2 mM phosphate buffer pH=7.5 under normal air condition.

In principle, ratiometric approach can be used for pH measurement, however, the presence of four ionic species makes the calibration of the hyperfine splitting method more convenient. As it is the case for ^{31}P NMR chemical shift of inorganic phosphate, the hyperfine splitting of **3a** was found to be dependent of the ionic strength (see ESI†). Ideally, the calibration should be performed in solution that mimics the condition of pH measurement (ionic strength, temperature, etc.). The intracellular/extracellular localization as well as the complete evaluation of the ability to measure pH and oxygen *in vivo* is under study and will be reported elsewhere.

In conclusion, we have synthesized a very promising EPR probe allowing pH measurement around its two pKa values. Phosphonated trityl radical **3a** combines for the first time a very simple spectrum, an excellent water solubility and high stability, with one pKa in the physiological range. Because of these unique properties, stable radical **3a** has to be considered for non-invasive pH measurement by EPR.

This work is supported by grants from the Université catholique de Louvain (UCL), the Belgian National Fund for Scientific Research (F.R.S.-FNRS), the Televie, the Fonds Joseph Maisin, the Saint-Luc Foundation, the Actions de Recherches Concertées-Communauté Française de Belgique-ARC 09/14-020, and the Pôle d'attraction Interuniversitaire PAI VI (P6/38).

Notes and references

† Electronic Supplementary Information (ESI) available: Experimental procedures for the synthesis, HPLC chromatograms, NMR, EPR and MS spectra, details of the titration, g-factors determination and the influence of ionic strength.See DOI: 10.1039/b000000x/

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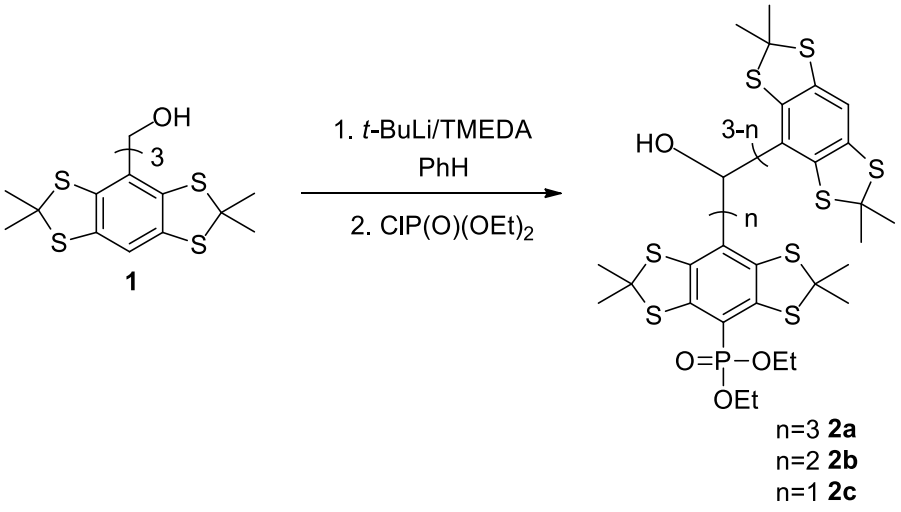
Experimental Part

General information

All reactions were carried out in flame-dried glassware under argon atmosphere. Anhydrous grade solvents were used for reactions and HPLC grade for purifications. All commercially available reagents were used as received without further purification. NMR spectra were recorded on a BRUKER Avance II (^{1}H: 300 MHz, ^{13}C: 75 MHz, ^{31}P 121 MHz) and a BRUKER Avance DPX (^{1}H: 500 MHz, ^{13}C: 125 MHz). Chemical shifts (δ) are reported in parts per million (ppm), referenced to NMR solvents, chloroform-d (δ ^{1}H=7.27 ppm, ^{13}C=77.2 ppm) and to H_3PO_4 85% (δ ^{31}P=0 ppm). The following abbreviations are used: s=singlet, d=doublet, t=triplet, m=multiplet. Coupling constants (J) are reported in Hertz (Hz). Infrared (IR) spectra are recorded using a Shimadzu spectrometer FTIR-8400S; products were analyzed as thin films deposited on a Se−Zn crystal by evaporation from the solvent. High resolution mass spectrometry (HRMS) analyses were performed at the University College London. Analytical HPLC was carried out on a Waters Alliance 2690 separations module equipped with a Waters 2998 PDA detector. Semi-preparative HPLC was carried out on a Waters 600 Pump equipped with a Waters 486 UV detector, a Waters fraction collector III and a Hitachi L-7200 HPLC Autosampler. EPR spectra were recorded on an X-Band BRUKER EMX spectrometer equipped with a ER4119HS resonator. pH was measured with a inoLab pH 730 pH-meter.

Chemistry

Synthesis of trityl alcohols 2a, 2b and 2c



Trityl alcohol **1** (containing 1.3 eq. of benzene) (400 mg, 0.405 mmol, 1eq.) was dissolved in dry benzene (5 mL), TMEDA (611 µL, 4.052 mmol, 10 eq.) was added. The solution was cooled at 0°C and t-BuLi 1.7 M in pentane (2.38 mL, 4.052 mmol, 10 eq.) was added dropwise under stirring. The mixture was stirred at 0°C for 2h then added to a solution of diethyl chlorophosphate (2.93 mL, 20.263 mmol, 50 eq.) in dry benzene (5 mL) at 0°C. The

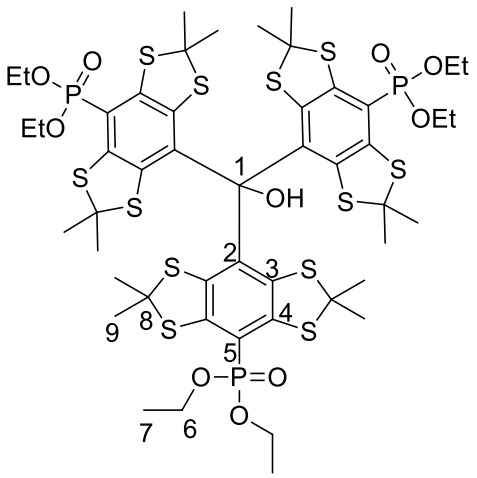
mixture was stirred 30 min at 0°C and 1h30 at room temperature. The reaction was quenched with $KH_2PO_{4sat.}$ (10 mL) and the layers were separated, the organic layer was washed with $NH_4Cl_{sat.}$ (1 x 10 mL), Na_2CO_3 (1 x 10 mL), H_2O (1 x 10 mL). The organic layer was dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The dark-orange oil was heated at 85°C under vacuum (5 x 10^{-2} mbar) to remove volatile compounds then semi-preparative HPLC afforded **2a** (178 mg, 34%), **2b** (61 mg, 13%) and **2c** (17 mg, 4%).

The conditions of purification were as follows:

Column: XBridge Prep C18 5µm 10 x 100 mm at ambient temperature equipped with a guard column XBridge Prep C18 5µm 10 x 20 mm, helium sparge 30 mL/min, UV detection at 364 nm. Gradient elution:

Time	Flow	H_2O	ACN	Curve (Waters)
0.00 min	5 mL/min	40%	60%	
1.00 min	5 mL/min	40%	60%	6 (linear)
13.00 min	5 mL/min	0%	100%	6
16.00 min	5 mL/min	0%	100%	6
16.10 min	5 mL/min	40%	60%	6

Tris(8-diethoxyphosphoryl-2,2,6,6-tetramethylbenzo[1,2-d;4,5-d']bis[1,3]dithiol-4-yl)methanol (2a)



Aspect: Orange solid.
Rf (SiO_2): 0.13 DCM/EtOAc (80:20) UV (254 nm).
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.33 (t, *J*=6.9 Hz, 18H, H-7), 1.59 (s, 9H, H-9), 1.67 (s, 9H, H-9'), 1.73 (s, 9H, H-9''), 1.76 (s, 9H, H-9'''), 4.05-4.24 (m, 12H, H-6), 6.51 (s, OH).
^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 16.4 (m, C-7), 27.1 (C-9), 29.0 (C-9'), 32.2 (C-9''), 35.1 (C-9'''), 61.1 (C-8), 61.3 (C-8'), 62.9 (m, C-6), 84.4 (C-1), 118.8 (d, *J*=184.6 Hz, C-5), 134.4 (d, *J*=2.3 Hz, C-2), 140.0 (d, *J*=14.6 Hz, C-3), 140.6 (d, *J*=14.2 Hz, C-3'), 143.1 (d, *J*=9.0 Hz, C-4), 144.6 (d, *J*=8.7 Hz, C-4').
^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm): 16.3.
ATR-IR (cm^{-1}): 2978, 2908, 1452, 1364, 1294, 1252, 1211, 1018.
HRMS (TOF MS ES+) *m/z*: $[M+H]^+$ calcd for $C_{49}H_{68}O_{10}P_3S_{12}$: 1293.0674, found: 1293.0542.
RP-HPLC: RT=1.1 min, column: XBridge C18 2.5 µm 4.6 x 50mm equipped with a guard column XBridge C18 2.5 µm 4.6 x 20mm. Column temperature: 40°C, UV detection at 254 nm.

Time	Flow	H_2O	ACN	Curve (Waters)
	1.5 mL/min	20%	80%	
4.00 min	1.5 mL/min	0%	100%	6 (linear)
6.00 min	1.5 mL/min	0%	100%	6
6.10 min	1.5 mL/min	20%	80%	6

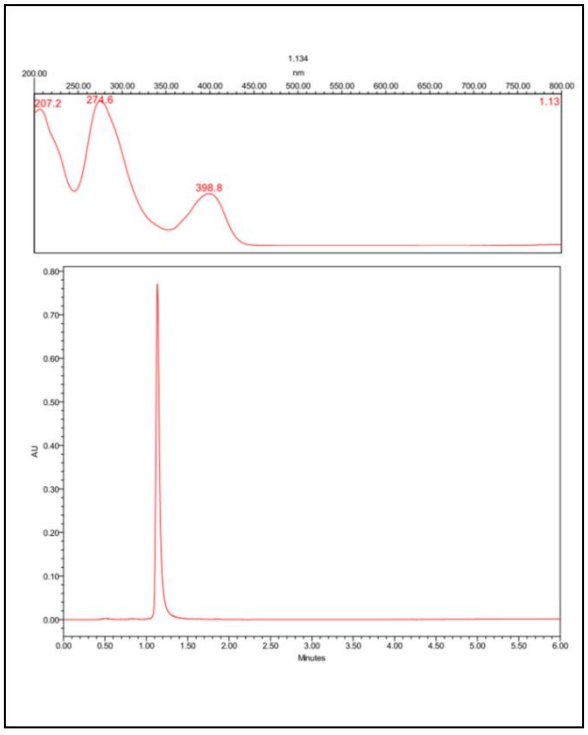
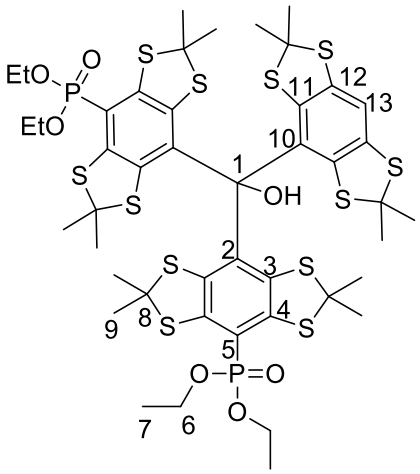


Figure 1: HPLC/UV of **2a**

Bis(8-diethoxyphosphoryl)tris(2,2,6,6-tetramethylbenzo[1,2-d;4,5-d']bis[1,3]dithiol-4-yl)methanol (2b)



Aspect: Orange solid.
Rf (SiO_2): 0.33 DCM/EtOAc (80:20) UV (254 nm).
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.35 (t, *J*=7.1 Hz, 12H, H-7), 1.60 (s, 3H, H-9), 1.62 (s, 3H, H-9'), 1.67 (s, 3H, H-9''), 1.68 (s, 3H, H-9'''), 1.68 (s, 3H, H-9$^{4'}$), 1.72 (s, 3H, H-9$^{5'}$), 1.73 (s, 3H, H-9$^{6'}$), 1.76 (s, 3H, H-9$^{7'}$), 1.78 (s, 3H, H-9$^{8'}$), 1.79 (s, 3H, H-9$^{9'}$), 1.81 (s, 3H, H-9$^{10'}$), 1.81 (s, 3H, H-9$^{11'}$), 4.08-4.24 (m, 8H, H-6), 6.43 (s, OH), 7.20 (s, 1H, H-13).
^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 16.5 (m, C-7), 27.1 (C-9), 27.4 (C-9'), 27.5 (C-9''), 28.5 (C-9$^{3'}$), 29.1 (C-9$^{4'}$), 29.8 (C-9$^{5'}$), 31.9 (C-9$^{6'}$), 32.1 (C-9$^{7'}$), 32.7 (C-9$^{8'}$), 34.9 (C-9$^{9'}$), 35.2 (C-9$^{10'}$+C-9$^{11'}$), 60.9 (C-8), 61.1 (C-8'+C-8''), 61.3 (C-8$^{3'}$), 62.9 (m, C-6), 63.7 (C-8$^{4'}$), 64.7 (C-8$^{5'}$), 84.2 (C-1), 118.6 (C-13), 118.6 (d, *J*=184.5 Hz, C-5), 118.7 (d, *J*=184.2 Hz, C-5'), 131.6 (C-ArH), 134.6 (d, *J*=2.5 Hz, C-2), 134.8 (d, *J*=2.5 Hz, C-2'), 137.6 (C-ArH), 138.1 (C-ArH), 138.5 (C-ArH), 138.9 (C-ArH), 140.2 (d, *J*=14.5 Hz, C-3), 140.3 (d, *J*=14.1 Hz, C-3'+C-3''), 141.4 (d, *J*=14.2 Hz, C-3'''), 143.0 (d, *J*=9.1 Hz, C-4), 143.1 (d, *J*=9.1 Hz, C-4'), 144.2 (d, *J*=8.7 Hz, C-4''), 144.6 (d, *J*=8.8 Hz, C-4''').
^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm): 16.5, 16.6.
ATR-IR (cm^{-1}): 2976, 2921, 1452, 1365, 1296, 1249, 1211, 1018.
HRMS (TOF MS ES+) *m/z*: $[M+H]^+$ calcd for $C_{45}H_{59}O_7P_2S_{12}$: 1157.0385, found: 1157.0323.
RP-HPLC: RT=2.5 min, column: XBridge C18 2.5 µm 4.6 x 50mm equipped with a guard column XBridge C18 2.5 µm 4.6 x 20mm. Column temperature: 40°C, UV detection at 254 nm.

Time	Flow	H_2O	ACN	Curve (Waters)
	1.5 mL/min	20%	80%	
4.00 min	1.5 mL/min	0%	100%	6 (linear)
6.00 min	1.5 mL/min	0%	100%	6
6.10 min	1.5 mL/min	20%	80%	6

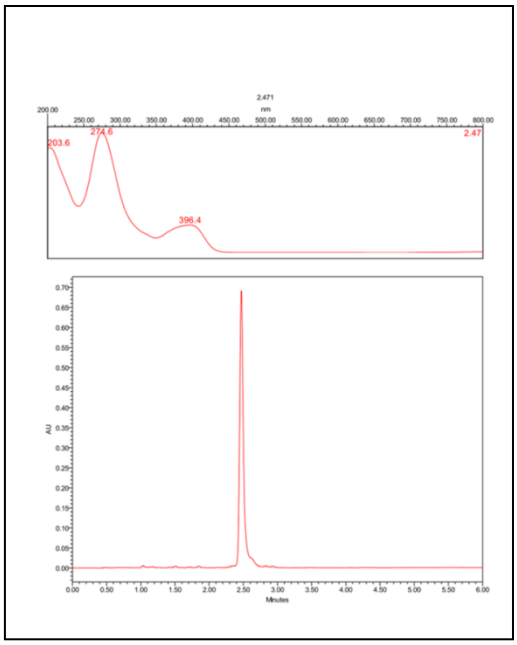
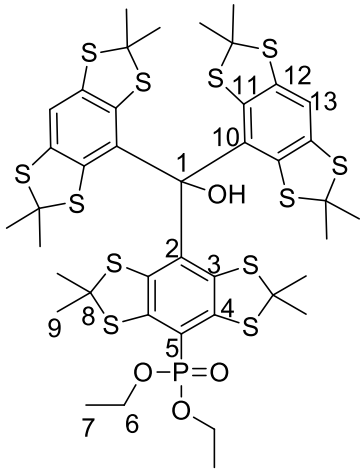


Figure 2: HPLC/UV of **2b**

8-diethoxyphosphoryl-tris(2,2,6,6-tetramethylbenzo[1,2-d;4,5-d']bis[1,3]dithiol-4-yl)methanol (2c)



Aspect: Orange solid.
Rf (SiO_2): 0.55 DCM/EtOAc (90:10) UV (254 nm).
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.35 (t, *J*=7.1 Hz, 6H, H-7), 1.61 (H-9), 1.67 (H-9'), 1.69 (H-9''), 1.69 (H-9'''), 1.72 (H-$9^{4'}$), 1.73 (H-$9^{5'}$), 1.77 (H-$9^{6'}$), 1.78 (H-$9^{7'}$), 1.79 (H-$9^{8'}$), 1.80 (H-$9^{9'}$), 1.83 (H-$9^{10'}$), 1.84 (H-$9^{11'}$), 4.05-4.25 (m, 4H, H-6), 6.33 (s, OH), 7.19 (s, 1H, H-13), 7.19 (s, 1H, H-13').
^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 16.5 (m, H-7), 27.3 (C-9), 27.5 (C-9'), 27.9 (C-9''), 28.6 (C-9'''), 29.3 (C-$9^{4'}$), 29.8 (C-$9^{5'}$), 31.8 (C-$9^{6'}$), 32.5 (C-$9^{7'}$), 32.6 (C-$9^{8'}$), 34.9 (C-$9^{9'}$), 34.9 (C-$9^{10'}$), 35.2 (C-$9^{11'}$), 61.0 (C-8), 61.0 (C-8'), 62.9 (m, C-6), 63.6 (C-8''), 63.6 (C-8'''), 64.2 (C-$8^{4'}$), 64.6 (C-$8^{5'}$), 84.0 (C-1), 118.4 (d, *J*=184.1 Hz, C-5), 118.5 (C-13), 118.6 (C-13'), 131.7 (C-ArH), 131.9 (C-ArH), 134.9 (d, *J*=2.4 Hz, C-2), 137.5 (C-ArH), 137.5 (C-ArH), 138.1 (C-ArH), 138.2 (C-

ArH), 138.4 (C-ArH), 138.5 (C-ArH), 138.6 (C-ArH), 139.7 (C-ArH), 140.4 (d, *J*=14.3 Hz, C-3), 141.1 (d, *J*=14.2 Hz, C-3'), 143.0 (d, *J*=9.1 Hz, C-4), 144.2 (d, *J*=8.9 Hz, C-4').
^{31}P NMR (121 MHz, $CDCl_3$) δ (ppm): 16.8.
ATR-IR (cm^{-1}): 2959, 2918, 1452, 1365, 1250, 1149, 1020.
HRMS (TOF MS ES+) *m/z*: $[M+H]^+$ calcd for $C_{41}H_{50}O_4P_1S_{12}$: 1021.0095, found: 1021.0020.
RP-HPLC: RT=3.8 min, column: XBridge C18 2.5 µm 4.6 x 50mm equipped with a guard column XBridge C18 2.5 µm 4.6 x 20mm. Column temperature: 40°C, UV detection at 254 nm.

Time	Flow	H_2O	ACN	Curve (Waters)
	1.5 mL/min	20%	80%	
4.00 min	1.5 mL/min	0%	100%	6 (linear)
6.00 min	1.5 mL/min	0%	100%	6
6.10 min	1.5 mL/min	20%	80%	6

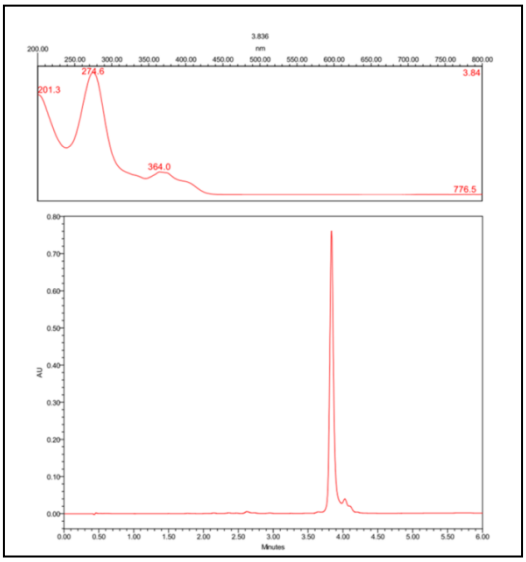
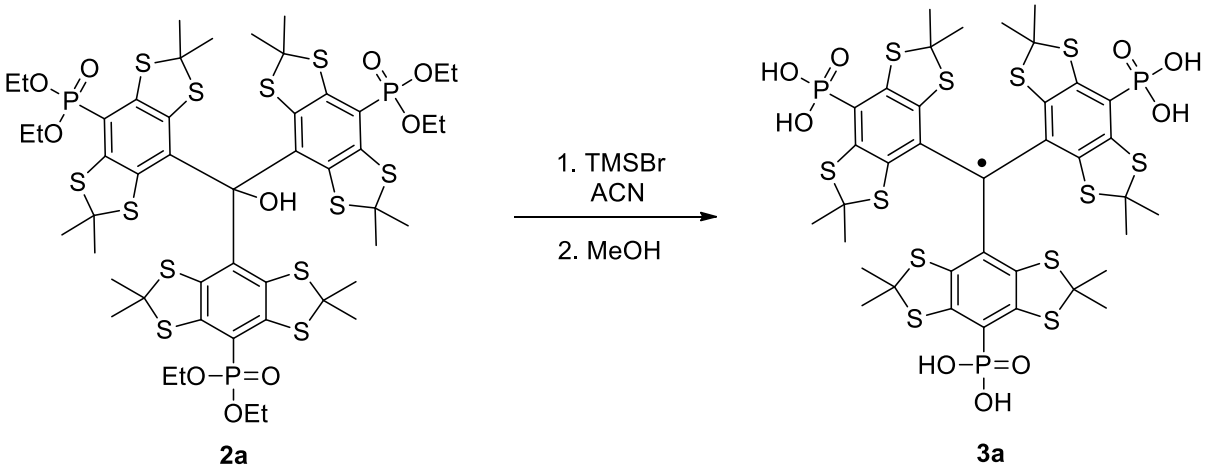


Figure 3: HPLC/UV of **2c**

Synthesis of Tris(8-phosphono-2,2,6,6-tetramethylbenzo[1,2-d;4,5-d']bis[1,3]dithiol-4-yl)methyl (3a)



2a **3a**

To a solution of trityl alcohol **2a** (110 mg, 0.085 mmol, 1 eq.) in CH_3CN (12 mL) was added TMSBr (169 µL, 1.275 mmol, 15 eq.) The mixture was refluxed for 22h, the orange solution gradually turned green. The solvent was removed under reduced pressure and the green residue was then dissolved and stirred at room temperature for 2 hours in MeOH (15 mL). After evaporation of MeOH, the green residue was purified by semi-preparative HPLC to afford the title compound (59 mg, 60%) as an ammonium salt.

The conditions of purification were as follows:

Column: XBridge Prep C18 5 µM 10 x 100 mm at ambient temperature equipped with a guard column XBridge Prep C18 5 µM 10 x 20 mm, helium sparge 30 mL/min, UV detection at 254 nm. For high purity, the product was collected from the half-height of the upward slope to the half-height of the downward slope. Gradient elution:

Time	Flow	H_2O	ACN	NH_4OAc 500 mM	Curve (Waters)
0.00 min	5 mL/min	85%	5%	10%	
6.00 min	5 mL/min	75%	15%	10%	6 (linear)
8.00 min	5 mL/min	75%	15%	10%	6
9.00 min	5 mL/min	10%	90%	0%	6
11.00 min	5 mL/min	10%	90%	0%	6
11.10 min	5 mL/min	85%	5%	10%	6

N.B: small amounts of deprotected phosphonic acids with residual trityl alcohol might be present in the crude. In that case, trityl alcohol can be converted into a radical by dissolving the crude mixture in TFA (15 mL) for 1h before purification.

Aspect: Green solid.
HRMS (TOF MS ES+) *m/z*: $[M+H]^+$ calcd for $C_{37}H_{43}O_9P_3S_{12}$: 1107.8769, found: 1107.9026.
RP-HPLC: RT=1.6 min, column: XBridge C18 2.5 µm 4.6 x 50mm. Column temperature: 40°C, UV detection at 254 nm.

Time	Flow	H_2O	ACN	NH_4OAc 50 mM	Curve (Waters)
	1.5 mL/min	85%	5%	10%	
3.00 min	1.5 mL/min	75%	15%	10%	6 (linear)
4.00 min	1.5 mL/min	0%	90%	10%	6
6.00 min	1.5 mL/min	0%	90%	10%	6
6.10 min	1.5 mL/min	85%	5%	10%	6

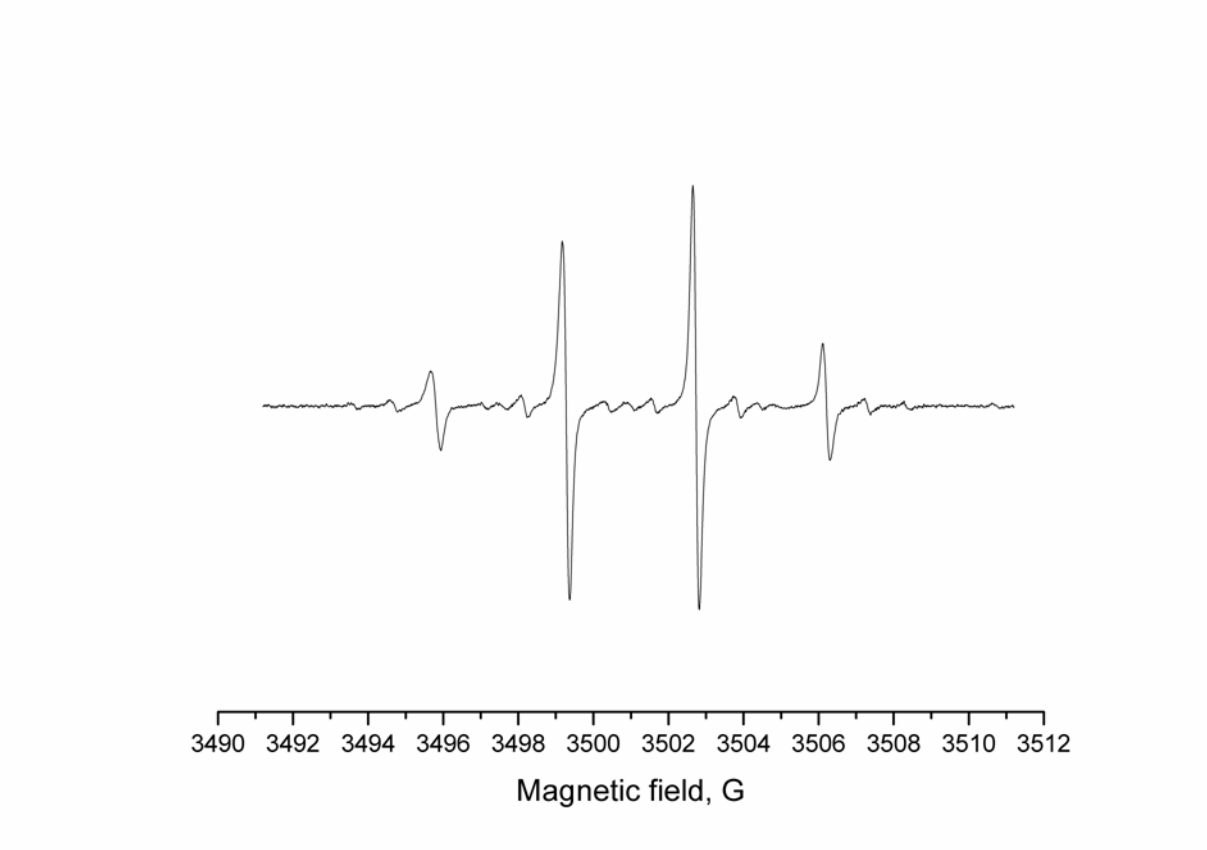


Figure 4: X-Band EPR spectrum of **3a** 100 µM in PBS 10 mM, pH=7.51

Acquisition settings were: power, 0.5 mW; sweep width, 20 G; modulation frequency, 10 kHz; modulation amplitude, 0.02 G; time constant, 20.48 ms; conversion time, 20.48 ms; sweep time, 41.94 sec; number of points, 2048; number of scans, 3; room temperature; normal air.

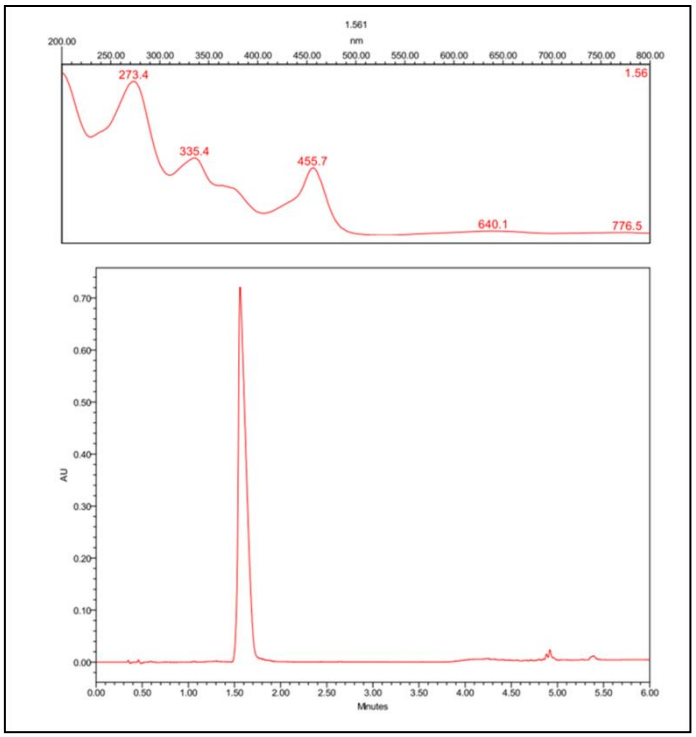
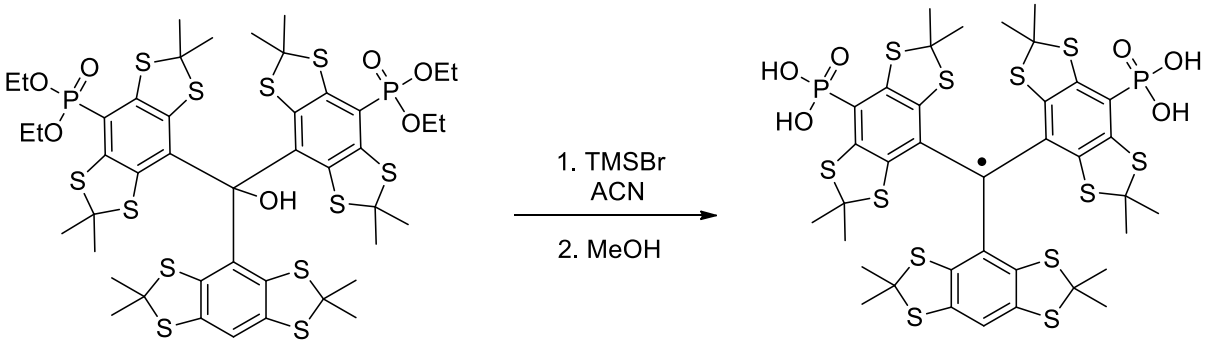


Figure 5: HPLC/UV of **3a**

Synthesis of Bis(8-phosphono)tris(2,2,6,6-tetramethylbenzo[1,2-d;4,5-d']bis[1,3]dithiol-4-yl)methyl (3b)



2b **3b**

To a solution of trityl alcohol **2b** (35 mg, 0.030 mmol, 1 eq.) in CH_3CN (3 mL) was added TMSBr (60 µL, 0.453 mmol, 15 eq.) The mixture was refluxed for 22h, the orange solution gradually turned green. The solvent was removed under reduced pressure and the green residue was then dissolved and stirred at room temperature for 2 hours in MeOH (5 mL). After evaporation of MeOH, the green residue was purified by semi-preparative HPLC to afford the title compound (3.4 mg, 11%) as an ammonium salt.

The conditions of purification were as follows:

Column: XBridge Prep C18 5 µM 10 x 100 mm at ambient temperature equipped with a guard column XBridge Prep C18 5 µM 10 x 20 mm, helium sparge 30 mL/min, UV detection at 273 nm. For high purity, the product was collected from the half-height of the upward slope to the half-height of the downward slope. Isocratic elution: 33%ACN/57%H_2O/10%NH_4OAc 500 mM, flow: 5 mL/min.

N.B: small amounts of deprotected phosphonic acids with residual trityl alcohol might be present in the crude. In that case, trityl alcohol can be converted into a radical by dissolving the crude mixture in TFA (5 mL) for 1h before purification.

Aspect: Green solid.
HRMS (TOF MS ES+) *m/z*: $[M+H]^+$ calcd for $C_{37}H_{42}O_6P_3S_{12}$: 1027.9105, found: 1027.9109.
RP-HPLC: RT=5.1 min, column: XBridge C18 2.5 µm 4.6 x 50mm equipped with a guard column XBridge C18 2.5 µm 4.6 x 20mm. Column temperature: 40°C, UV detection at 254 nm.

Time	Flow	H_2O	ACN	NH_4OAc 50 mM	Curve (Waters)
	1.5 mL/min	85%	5%	10%	
10.00 min	1.5 mL/min	0%	90%	10%	6 (linear)
12.00 min	1.5 mL/min	0%	90%	10%	6
12.10 min	1.5 mL/min	85%	5%	10%	6

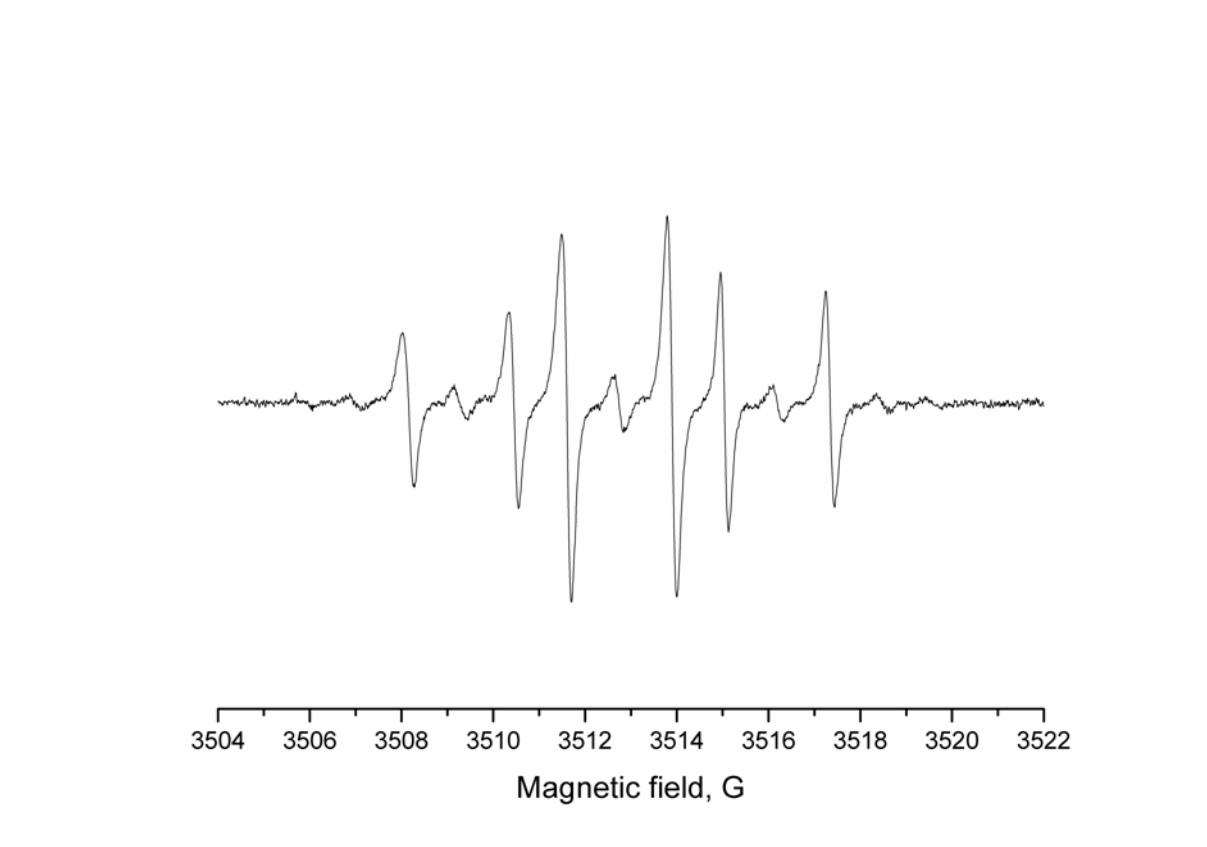


Figure 6: X-Band EPR spectrum of **3b** 100 µM in PBS 10 mM, pH=7.46

Acquisition settings were: power, 0.5 mW; sweep width, 20 G; modulation frequency, 10 kHz; modulation amplitude, 0.02 G; time constant, 20.48 ms; conversion time, 20.48 ms; sweep time, 41.94 sec; number of points, 2048; number of scans, 10; room temperature; normal air.

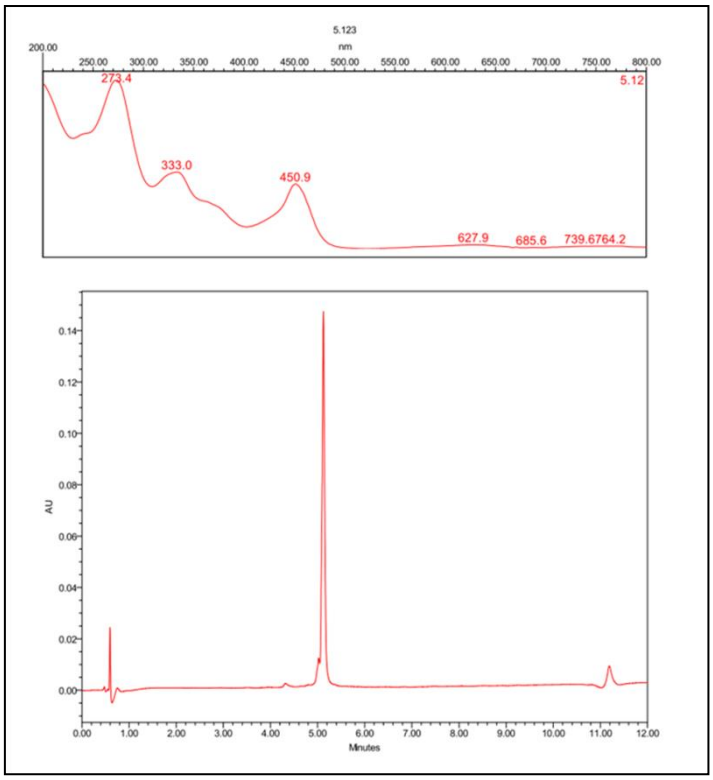
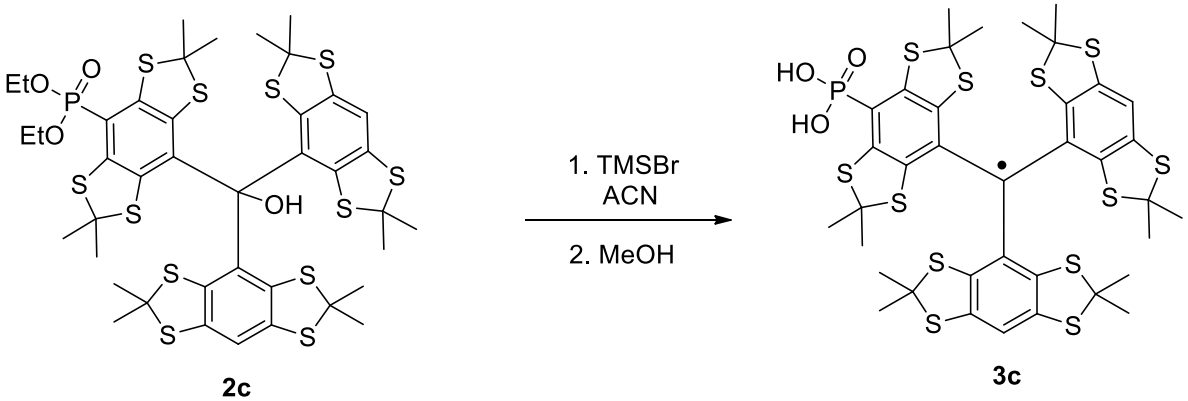


Figure 7: HPLC/UV of **3b**

Synthesis of 8-phosphono-tris(2,2,6,6-tetramethylbenzo[1,2-d;4,5-d']bis[1,3]dithiol-4-yl)methyl (3c)



2c **3c**

To a solution of trityl alcohol **2c** (9 mg, 0.009 mmol, 1 eq.) in CH_3CN (1 mL) was added TMSBr (17.5 µL, 0.132 mmol, 15 eq.) The mixture was refluxed for 22h, the orange solution gradually turned green. The solvent was removed under reduced pressure and the green residue was then dissolved and stirred at room temperature for 2 hours in MeOH (5 mL).

After evaporation of MeOH, the green residue was purified by semi-preparative HPLC to afford the title compound (1.1 mg, 12%) as an ammonium salt.

The conditions of purification were as follows:

Column: XBridge Prep C18 5 µM 10 x 100 mm at ambient temperature equipped with a guard column XBridge Prep C18 5 µM 10 x 20 mm, helium sparge 30 mL/min, UV detection at 273 nm. For high purity, the product was collected from the half-height of the upward slope to the half-height of the downward slope. Isocratic elution: 65%ACN/25%H_2O/10%NH_4OAc 500 mM, flow: 5 mL/min.

N.B: small amounts of deprotected phosphonic acids with residual trityl alcohol might be present in the crude. In that case, trityl alcohol can be converted into a radical by dissolving the crude mixture in TFA (5 mL) for 1h before purification.

Aspect: Green solid.
HRMS (TOF MS ES+) *m/z*: $[M+H]^+$ calcd for $C_{37}H_{41}O_3PS_{12}$: 947.9442, found: 947.9473.
RP-HPLC: RT=9.1 min, column: XBridge C18 2.5 µm 4.6 x 50mm equipped with a guard column XBridge C18 2.5 µm 4.6 x 20mm. Column temperature: 40°C, UV detection at 254 nm.

Time	Flow	H_2O	ACN	NH_4OAc 50 mM	Curve (Waters)
	1.5 mL/min	85%	5%	10%	
10.00 min	1.5 mL/min	0%	90%	10%	6 (linear)
12.00 min	1.5 mL/min	0%	90%	10%	6
12.10 min	1.5 mL/min	85%	5%	10%	6

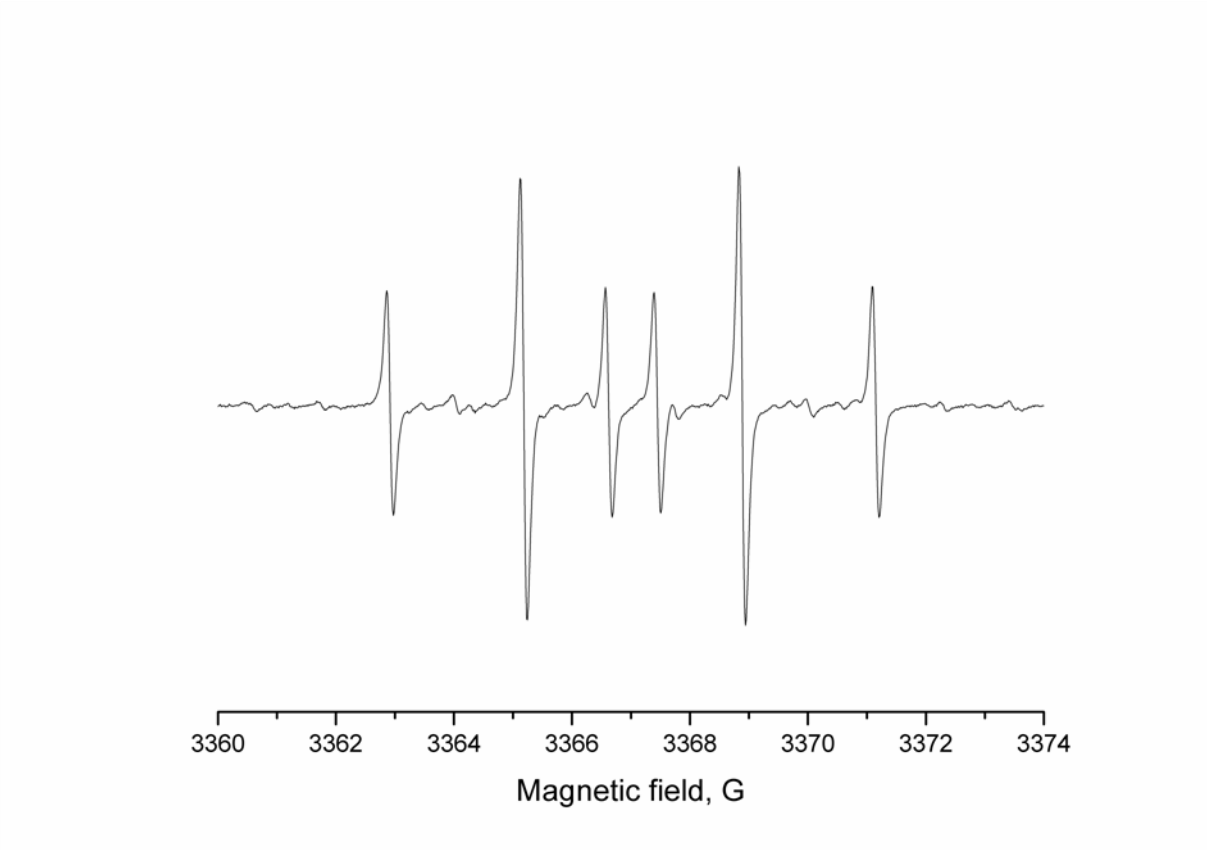


Figure 8: X-Band EPR spectrum of **3c** 100 µM in degassed MeOH.

Acquisition settings were: power, 0.3 mW; sweep width, 26 G; modulation frequency, 10 kHz; modulation amplitude, 0.03 G; time constant, 20.48 ms; conversion time, 40.96 ms; sweep time, 83.89 sec; number of points, 2048; number of scans, 3; room temperature.

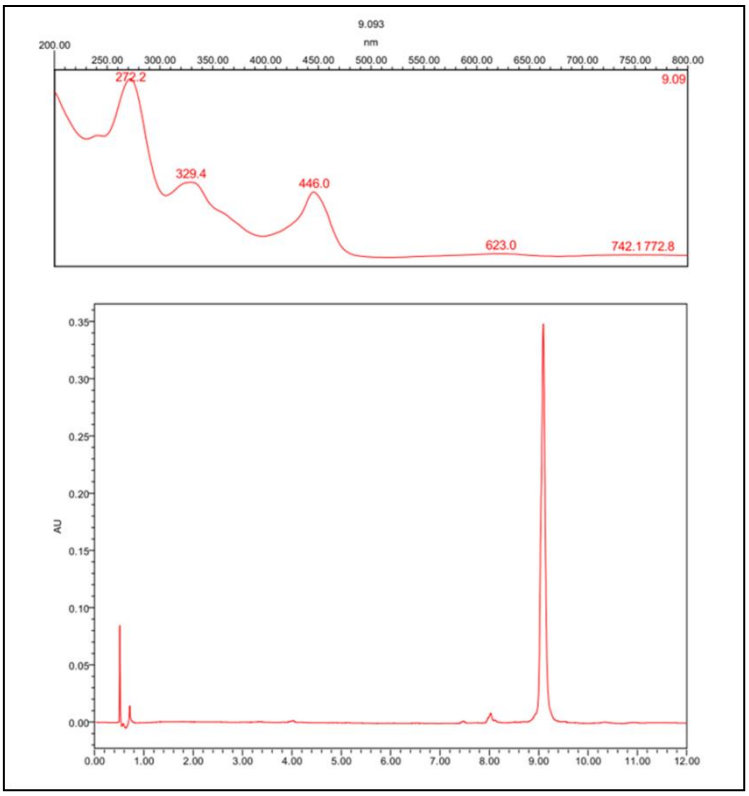


Figure 9: HPLC/UV of **3c**

Evaluation of 3a as EPR pH probe

Saturation

Saturation plot of **3a** 100 µM in PBS (10 mM PB, 138 mM NaCl, 2.7 mM KCl) pH=7.46

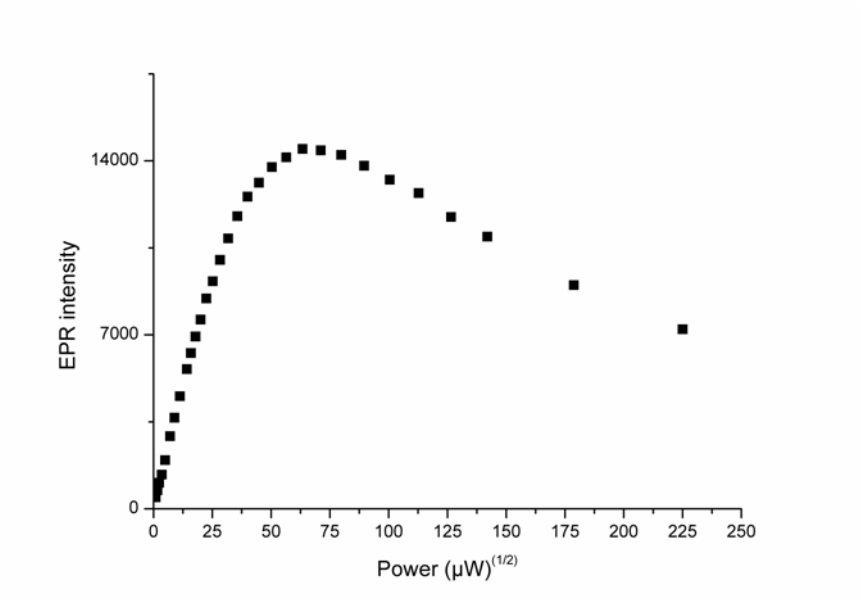


Figure 10: Saturation of **3a** under normal air condition

Acquisition settings were: power, from 1 µW to 50.6 mW; sweep width, 20 G; modulation frequency, 10 kHz; modulation amplitude, 0.02 G; time constant, 20.48 ms; conversion time, 20.48 ms; sweep time, 41.94 sec; number of points, 2048; number of scan, 1; room temperature; normal air.

Titration of 3a in PBS 10 mM

The X-band pH dependence of the apparent hyperfine splitting a_P and $3a_P$ were determined by conventional titration procedure of compound **3a** 100 µM in aerated PBS (10 mM PB, 138 mM NaCl, 2.7 mM KCl) at room temperature using NaOH and HCl.

Apparent hyperfine splitting constants (a_P) were measured as the distance between the two high-field components after integration of the signal as depicted in figure S26.

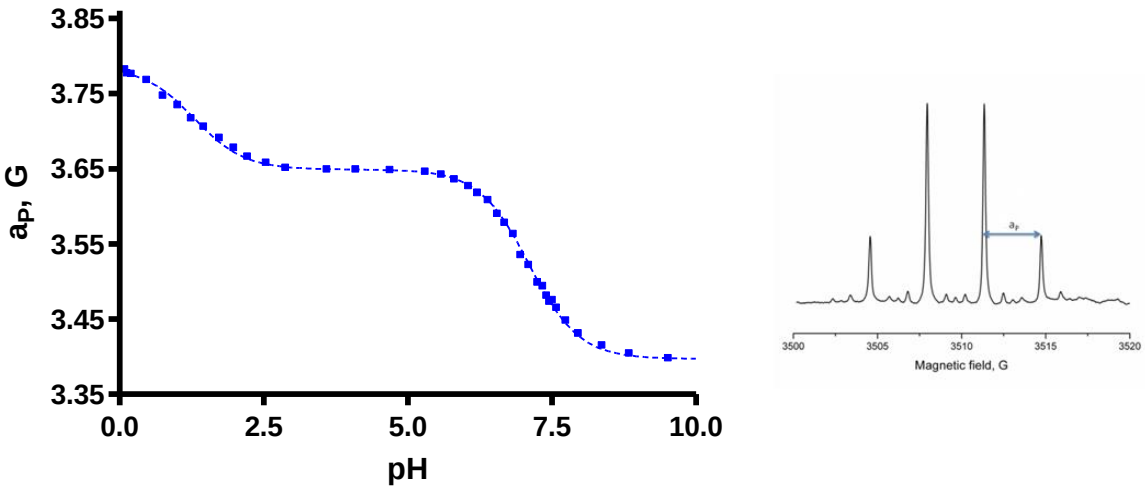


Figure 11: The X-band pH dependence of the apparent a_P

Acquisition settings were: power, 0.5 mW; sweep width, 20 G; modulation frequency, 10 kHz; modulation amplitude, 0.02 G; time constant, 20.48 ms; conversion time, 20.48 ms; sweep time, 41.94 sec; number of points, 2048; number of scans, 2.

Experimental data were fitted (dotted line) according equation (1). This equation is the standard titration dependence for a probe with two pKa values in the case of fast frequency proton exchanges in the EPR time scale. However, this equation is used as a good approximation for the case of moderate and slow frequency exchanges when the exchangeable lines are partially overlapped. The parameters of fitting were a_{P1} =3.785 G, a_{P2} =3.649 G, a_{P3} =3.397 G, pKa_1 = 1.293 and pKa_2 = 7.104.

$$a_P(pH) = \frac{a_{P_1} + a_{p_2} \times 10^{(-pKa_1+pH)} + a_{P_3} \times 10^{(-pKa_1-pKa_2+2\times pH)}}{1 + 10^{(-pKa_1+pH)} + 10^{(-pKa_1-pKa_2+2\times pH)}} \quad (1)$$

Apparent hyperfine splittings ($3a_P$) were measured as the distance between the low- and high-field components after integration of the signal as depicted in figure S27. Two modulation amplitudes were used, 0.02 G and 0.6 G.

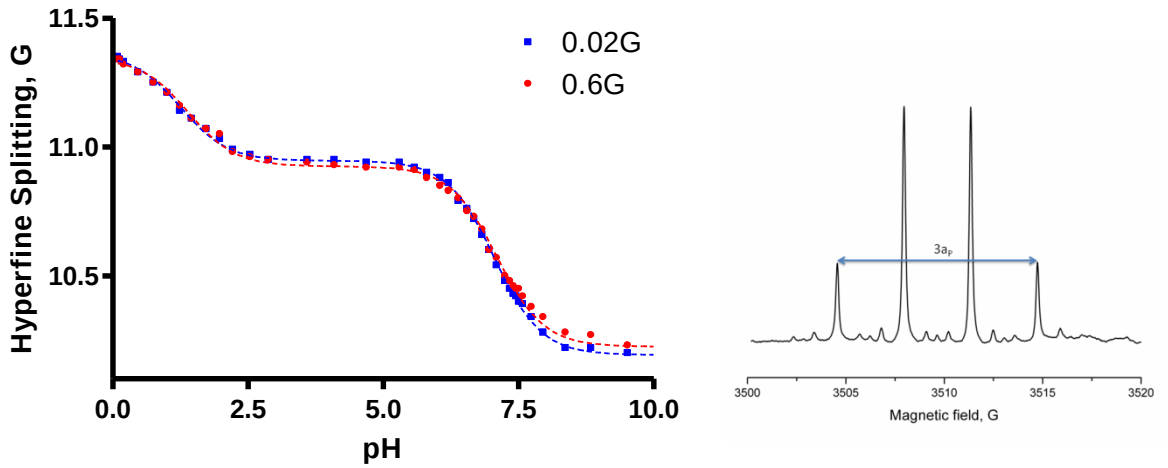
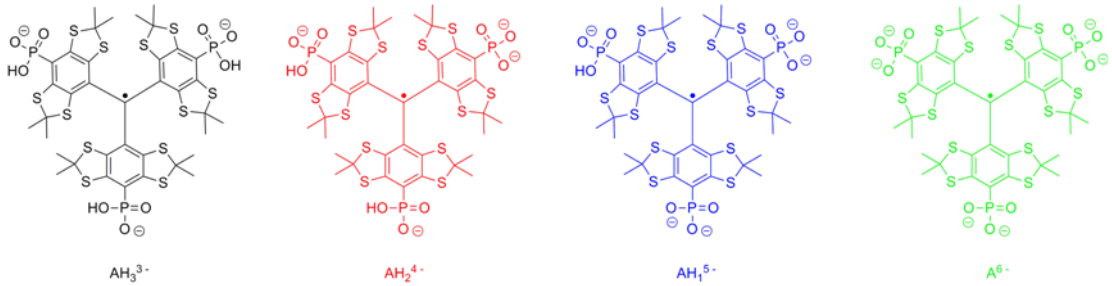


Figure 12: The X-band pH dependence of the apparent $3a_P$ at 0.02 G and 0.6 G modulation amplitude

Acquisition settings were: power, 0.5 mW; sweep width, 20 G; modulation frequency, 10 kHz; modulation amplitude, 0.02 G or 0.6 G; time constant, 20.48 ms; conversion time, 20.48 ms; sweep time, 41.94 sec; number of points, 2048; number of scans, 2.

X-band spectra of 3a 100 µM in PB 2 mM

In phosphate buffer 2 mM for pH close to the second pKa, the four ionic species (AH_3^{3-}, AH_2^{4-}, AH^{5-}, A^{6-}) are partially resolved on the spectrum under normal air conditions as a consequence of a slow proton frequency exchanges on the EPR time scale.



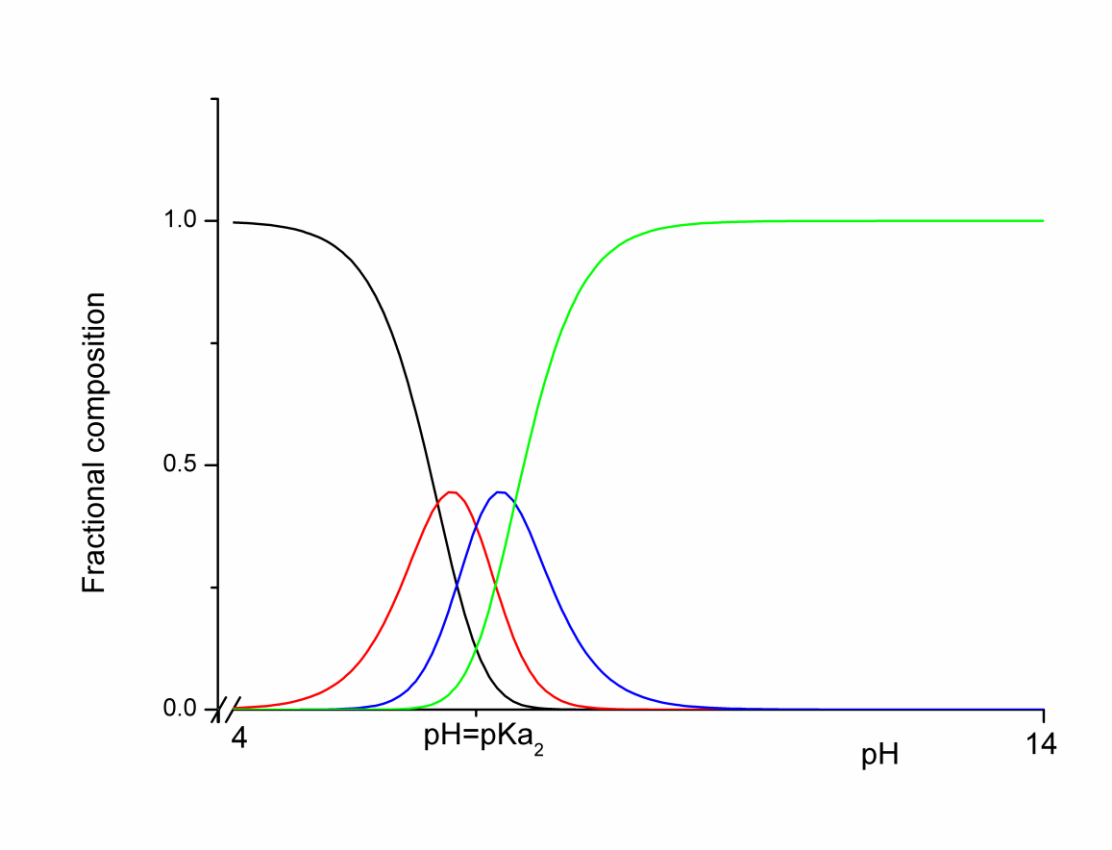


Figure 13: Theoretical fractional composition of AH_3^{3-}, AH_2^{4-}, AH^{5-}, A^{6-} ionic species with respect to the pH

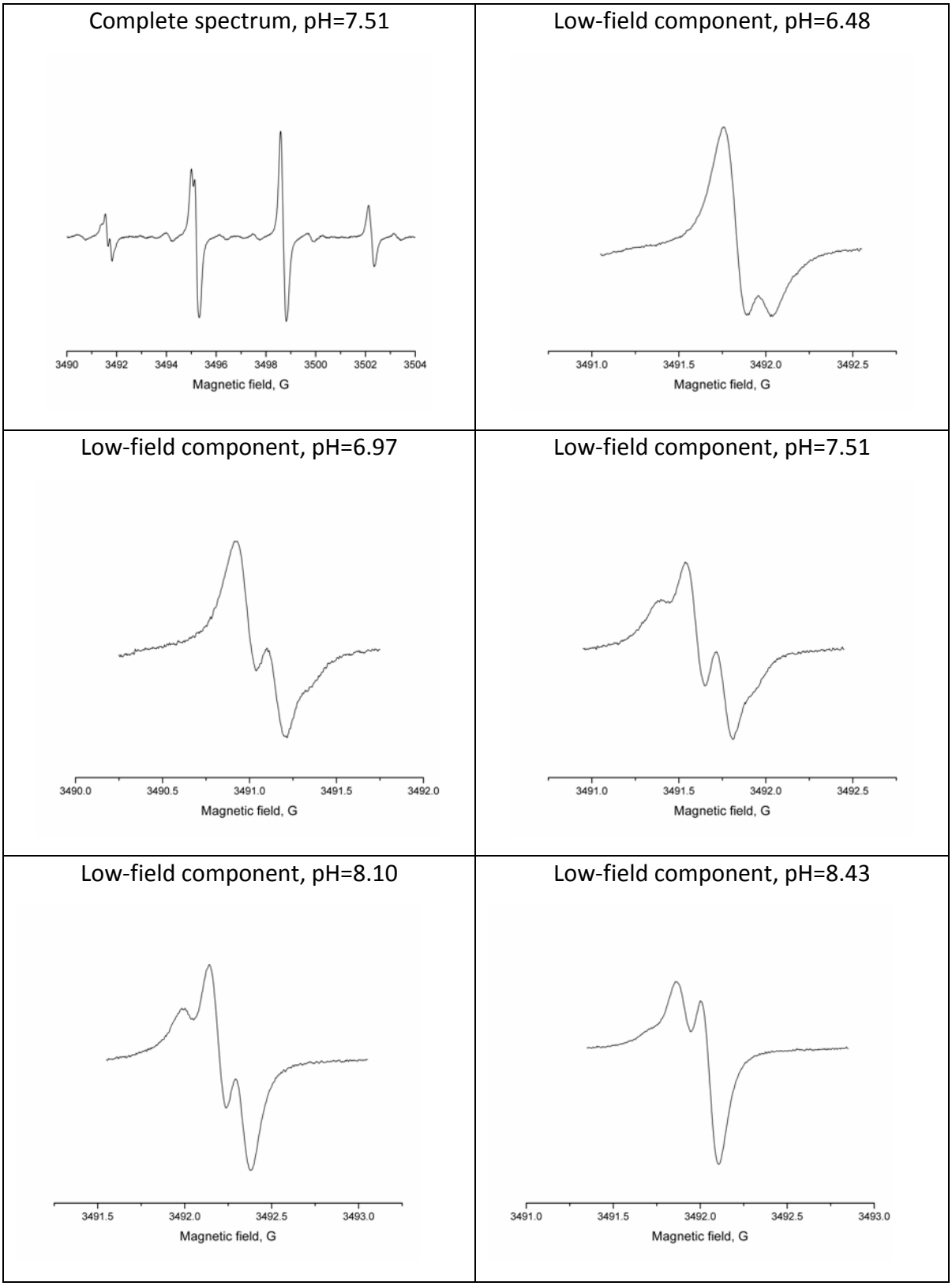


Figure 14: X-band EPR spectrum of **3a** in PB 2 mM at pH 7.51 and the low-field component of the quartet at various pH values.

Acquisition settings were (a) for the complete spectrum: power, 0.5 mW; sweep width, 20 G; modulation frequency, 10 kHz; modulation amplitude, 0.02 G; time constant, 20.48 ms; conversion time, 20.48 ms; sweep time, 41.94 sec; number of points, 2048; number of scans, 5; room temperature; normal air. (b) For the low-field component: power, 0.5 mW; sweep width, 1.5 G; modulation frequency, 10 kHz; modulation amplitude, 0.02 G; time constant, 20.48 ms; conversion time, 20.48 ms; sweep time, 10.49 sec; number of points, 512; number of scans, 16; room temperature; normal air.

g-factor measurements

The g-factors of **3a** at pH=0.11, 4.98 and 10.50 were measured relatively to TEMPOL (g= 2.00590)[f] used as internal standard. The g-factors were measured in PB 2 mM at X-band.

pH	Ionic form	g (±0.0001)
10.50	A^{6-}	2.00273
4.98	AH_3^{3-}	2.00302
0.11	AH_6	2.00286

Table 1

Acquisition settings were : power, 2.5 mW; sweep width, 80 G; modulation frequency, 10 kHz; modulation amplitude, 0.03 G; time constant, 20.48 ms; conversion time, 20.48 ms; sweep time, 83.89 sec; number of points, 4096; number of scans, 3; room temperature; normal air.

Influence of ionic strength

The X-band EPR spectra of **3a** 100 µM were recorded in PB 10 mM with 0 mM, 70 mM and 138 mM of NaCl at room temperature under normal air condition. The apparent phosphorus hyperfine splitting constant (a_P) was found to be dependent of the ionic

[f] *Phys. Rev. B*, **2005**, 72, 085307

strength (Table 2). This is the consequence of a modification of the pKa associated with the phophonic acids when ionic strength varies. This results in a modification of the fractional composition of ionic species for a given pH and thus a modification of the apparent hyperfine splitting constant. The effect is similar to that observed for the ^{31}PNMR chemical shift dependence of inorganic phosphate with ionic strength.

Solvent	a_P (G)	$3a_P$ (G)
PB 10 mM, pH=7.51, NaCl 0 mM	3.508	10.50
PB 10 mM, pH=7.51, NaCl 70 mM	3.481	10.43
PB 10 mM, pH=7.51, NaCl 138 mM	3.462	10.36

Table 2

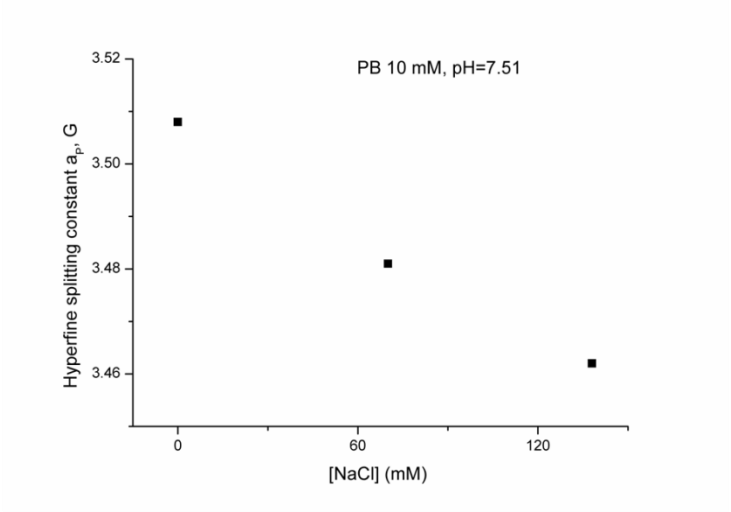


Figure 15: Influence of ionic strength

Acquisition settings were: power, 0.5 mW; sweep width, 20 G; modulation frequency, 10 kHz; modulation amplitude, 0.02 G; time constant, 20.48 ms; conversion time, 20.48 ms; sweep time, 41.94 sec; number of points, 2048; number of scans, 3.

4. Radicaux TAMs fluorés pour la mesure de l'oxygénation utilisant des formulations de perfluorocarbones

Cette partie du manuscrit décrit la synthèse de radicaux TAMs fluorés utilisés sous forme de formulations de perfluorocarbones (PFC) pour la mesure de la concentration en oxygène avec une grande sensibilité.

Ce travail a été publié dans les journaux" Bioorganic and Medicinal Chemistry Letters" (partie synthèse) et "Journal of Magnetic Resonance" (partie formulation et évaluation *in vivo*).

Cette recherche fait suite à une demande du laboratoire REMA qui souhaitait disposer de radicaux TAMs possédant une grande affinité pour les solvants fluorés afin d'être utilisés sous forme d'émulsions de PFC. Ma contribution personnelle a été l'imagination de la voie de synthèse et la synthèse des deux radicaux TAMs fluorés. La caractérisation RPE, la formulation et l'utilisation *in vivo* ont été réalisées par le Dr. N. Charlier (UCL/REMA) *et al*.

La méthode de synthèse de ces premiers radicaux TAMs fluorés décrits à ce jour repose sur une étape d'aminolyse d'un ester éthylique TAM par une amine perfluorée. Cette réaction permet d'éviter l'hydrolyse de l'ester et le couplage de l'acide obtenu avec une amine en utilisant un agent de couplage comme cela se fait généralement en chimie médicinale. Cette méthode permet la protection *in situ* de l'alcool trityle (fonction labile) par sa déprotonation.

Synthesis of Two Persistent Fluorinated Tetrathiatriarylmethyl (TAM) Radicals for Biomedical EPR Applications

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Abstract: Tetrathiatriarylmethyl radicals are attractive spin probes extensively used in biomedical magnetic resonance applications. We report a straightforward synthesis of two original tetrathiatriarylmethyl radicals incorporating respectively 15 and 45 fluorine atoms, and thus possessing a high affinity to fluorous media. **F15T-03** and **F45T-03** exhibit a single sharp EPR spectrum and their EPR line broadening is highly sensitive to molecular oxygen. These spin probes are specially designed for assessment of tumor oxygenation using perfluorocarbon formulations.

Molecular oxygen plays one of the most important roles in the metabolism of living organisms. Abnormal tissue oxygenation is closely linked to number of diseases (e.g., cancer, stroke, ischemic diseases).[1] Therefore, it is of particular importance for pO_2 *In vitro* and *in vivo* assessment to rely upon accurate methods. The techniques for measuring oxygen partial pressure in biological media include both non magnetic and magnetic resonance (MR) based methods. Typically polarographic oxygen electrodes, fluorescence quenching, phosphorescence quenching, near infra-red spectroscopy (NIRS) or the use of bioreductive markers such as 2-nitroimidazole derivatives belong to the former series of methods, while ^{19}F-NMR spectroscopy/imaging, blood oxygen level dependent (BOLD) imaging or electron paramagnetic resonance (EPR) and Dynamic nuclear polarisation (DNP) using oxygen sensitive spin probes belong to the latter.[2] EPR methods have the advantage of the non

invasiveness combined with a high sensitivity and specificity. They are mostly based on the broadening of the signal caused by Heisenberg exchange between molecular oxygen and the spin probe to determine pO_2. Two different types of spin probes are used in EPR oximetry, either particulate materials like lithium phthalocyanine, chars, coals, carbon black, or soluble molecules, namely nitroxides and the triarylmethyl (trityl) radicals. By the late 90s, Nycomed Innovation AB featured original Gomberg's trityl radical in order to avoid hydrogen hyperfine coupling and enhance its stability and water solubility.[3] A new family of trityl spin probes was synthesized, also known as tetrathiatriarylmethyl (TAM), bearing four sulfur atoms on the phenyl ring (Figure 1). The most representative members are water soluble **CT-03**, **deutered CT-03** and **OX063** which exhibit a very narrow EPR linewidth, non toxic properties and are less sensitive to bioreduction than nitroxides.[4,5] Due to their unique properties, TAM type radicals have found numbers of MR applications as oxygen/pH sensitive spin probes or as contrast agents in electron paramagnetic resonance imaging (EPRI) and Overhauser magnetic resonance imaging (OMRI).[5,6] Moreover, TAM radicals have also been used for measuring superoxide radical by EPR spectroscopy or by spectrophotometry.[7] Recently, creative efforts have be done for the synthesis of these complex molecules.[8,9] **CT-03** can now be synthesized in large-scale in an efficient way.[10]

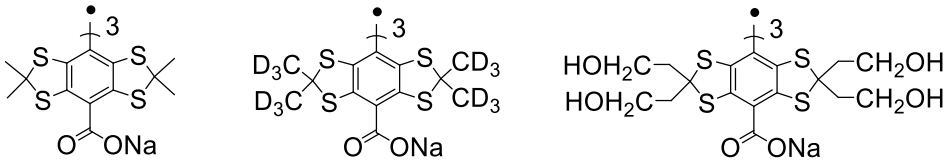


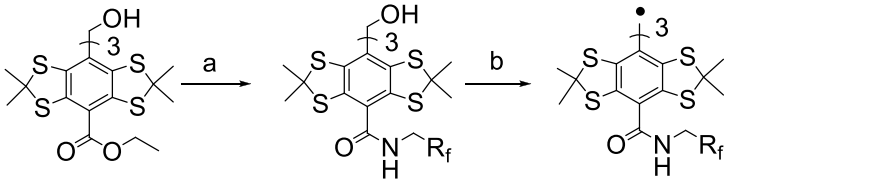
Figure 1. Representative Nycomed's trityl radicals.

Among useful solvents, perfluorinated ones are well-known for their excellent capacity to dissolve a high quantity of non polar gases like O_2. Many of them can at physiological temperature dissolve up to 40 – 50 % *v/v* oxygen at 1 atm.[11] For instance, several PFCs have been used *in vivo* for their excellent oxygen solubility, such as hexafluorobenzene (HFB) that is utilized in animal models, and perfluorooctylbromide

(PFOB) that is in clinical use. Recently, the group of Kuppusamy took advantage of that special property in the synthesis of a triethoxycarbonyl perchlorotriarylmethyl radical (PTM-TE) and the use of PTM-TE/HFB formulation for high-resolution oxygen mapping in tumor using EPR spectroscopy.[12,13] This group reported a high sensitivity of the line broadening with molecular oxygen in HFB. The sensitivity in HFB is at least 10 times as high as in DMSO, mainly due to the high solubility of oxygen in this solvent.

In the course of our research on the development of new tools for the assessment of tumor oxygenation by EPR spectroscopy using biocompatible perfluorocarbon (PFC) emulsions, we sought to enhance the affinity of the oxygen spin probe for a PFC formulation by attaching a perfluorinated tag on the trityl radical. Indeed, the radical derived from trityl ethyl ester **1** is not soluble in PFCs (solubility inferior to 0.1 mM in PFOB), so introducing a fluorous label on a molecule is the usual strategy to enhance its fluorophilicity.[14] Hereby we report a straightforward access to original fluorinated trityl oxygen probes.

Two new highly fluorinated TAM radicals, **F15T-03** (20 % fluorine by weight) and **F45T-03** (40 % fluorine by weight) were efficiently synthesized in a two-step sequence from precursor **1** as depicted in Scheme 1. Trityl ester **1** was obtained according to the procedure described in the literature.[8] The aminolyse of ethyl ester **1** by perfluoroamine ($H_2NCH_2R_f$) in presence of trimethylaluminum, resulted in the formation of fluorinated trityl alcohols **2** (R_f= C_2F_5) and **3** (R_f=C_7F_{15}) in 95% and 66% yields respectively, after purification by preparative thin layer chromatography (TLC).[15] It is noteworthy to mention the low nucleophilicity of these two commercially available fluoroamines. Nevertheless, our conditions allowed to reach good to excellent isolated yields. In this first step, the labile trityl alcohol was protected *in situ* by the formation of an aluminum alcoholate. Then, treatment of the trityl alcohols **2-3** with $BF_3.Et_2O$ gave the corresponding carbocations which are subsequently reduced by $SnCl_2$ to afford the persistent captodative radicals **F15T-03** and **F45T-03**.[16]



1

2 ($R_f = C_2F_5$)
3 ($R_f = C_7F_{15}$)

F15T-03 ($R_f = C_2F_5$)
F45T-03 ($R_f = C_7F_{15}$)

Scheme 1. Reagents and conditions: (a) $AlMe_3$, $H_2NCH_2R_f$, DCE, reflux, overnight (95%, $R_f = C_2F_5$; 66%, $R_f = C_7F_{15}$). (b) 1–$BF_3.Et_2O$, DCM, 1 h, 2–$SnCl_2$/THF, 30 min.

The EPR properties of the two new TAM-type radicals were comparable to those of other tetrathiatriarylmethyl radicals described previously, such as radical of compound **1**.[8] A single sharp peak was observed for **F15T-03** and **F45T-03**. The sensitivity of the EPR linewidth to oxygen was measured by carrying out the calibration of line broadening ΔB_{pp} (G) versus pO_2 (mmHg). Calibration curves were built in HFB and PFOB, for **F15T-03** and **F45T-03** respectively, according to their respective high solubility in these solvents. **F15T-03** in HFB showed a single sharp peak with a linewidth of 0.550 G under anaerobic conditions and 3.55 G in ambient room air (21 % oxygen) (Figure 2). The slope of the calibration curve (18.7 mG/mmHg) is consistent with the results already published on the PTM-TE in HFB (Figure 3).[12] **F45T-03** in PFOB showed a single sharp peak with a linewidth of 0.545 G under anaerobic conditions and 3.34 G in ambient room air with a slope of the calibration curve of 17.5 mG/mmHg. The sensitivity of compound **1** radical was not measurable using the same conditions due to its poor solubility in PFCs. For comparison, the sensitivity of **CT-03** in water was 0.64 mG/mmHg. Thus, our novel probes dissolved in PFCs are about 30-fold more sensitive. As mentioned previously the better sensitivity of line broadening to pO_2 in perfluorous liquids is the consequence of the better solubility of oxygen in such media. While water dissolves 3.1 vol%, (25°C) of oxygen, HFB is able to dissolve 46.8-48.8 vol% (25°C) and PFOB 50.0-52.7 vol% (25°C) of molecular oxygen.[17]

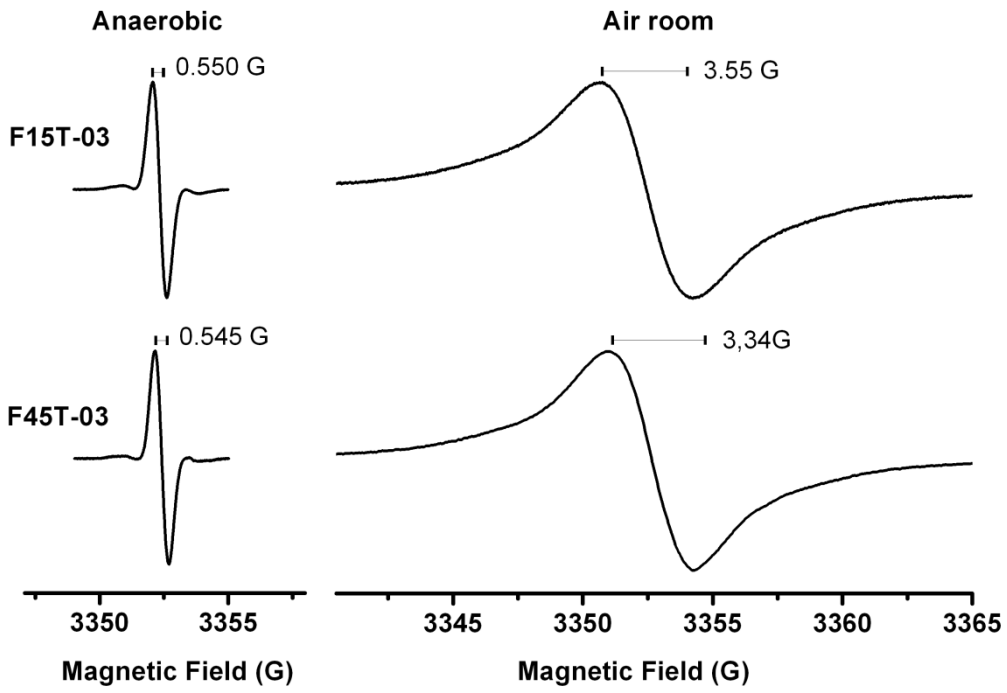


Figure 2. X-band EPR spectra, linewidths (ΔB_{pp}) of **F15T-03** in HFB, **F45T-03** in PFOB under anaerobic and air room conditions. The EPR acquisition settings were (a) F15T-03 in HFB; 1 mM filtered on 0.2 µm (Pall Ghp Acrodisc); anaerobic conditions; sweep width: 10 G; power: 200 µW; temperature: 310 K; frequency modulation: 10 kHz; modulation amplitude: 0.15 G; time constant: 10.24 ms; conversion time: 10.24 ms. (b) F45T-03 in PFOB; 0.25 mM filtered on 0.2 µm (Pall Ghp Acrodisc); anaerobic conditions; sweep width: 6 G; power: 50 µW; temperature: 310 K; frequency modulation: 100 kHz; modulation amplitude: 0.1 G; time constant: 20.48 ms; conversion time: 20.48 ms.

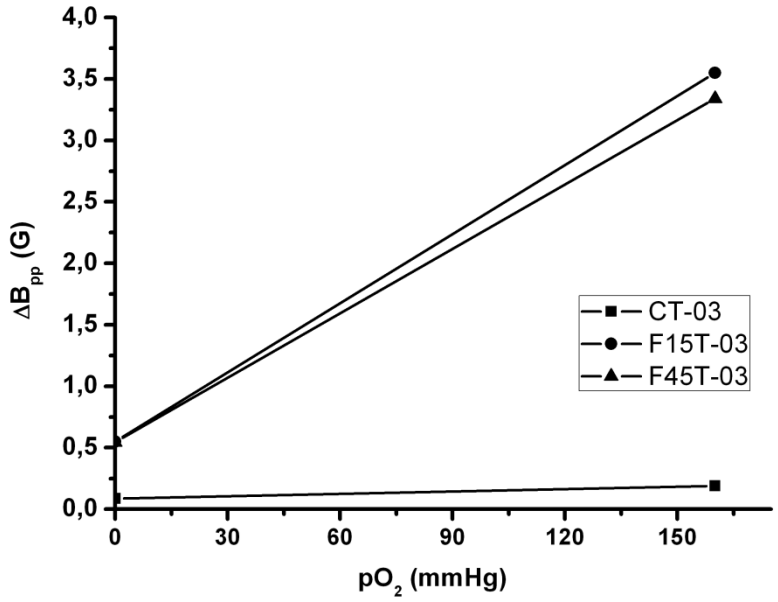


Figure 3. Calibration curves of linewidth ΔB_{pp} (G) as a function versus pO_2 (mmHg) for (■) **CT-03** (in water), (●) **F15T-03** (in HFB) and (▲) **F45T-03** (in PFOB).

Further developpement of the present study is to use these fluorinated tetrathiatriarylmethyl radicals as components of nanocapsules containing PFCs. These systems should present high sensitivity to oxygen and should be biocompatible and injectable to living systems.

In conclusion, we have disclosed an efficient synthesis of two new TAM based radicals possessing a high affinity to fluorous media. These radicals are specially designed for assessment of tumor oxygenation using PFC formulations. Moreover, the sensitivity of line broadening is higher in PFC liquids than in water in agreement with the higher solubility of oxygen in these solvents.

Acknowledgment

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research associate of the Belgian FRS-F.N.R.S (Fond National de la Recherche Scientifique, Belgium).

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15. (a) Preparation of **2**: To a stirred solution of 2,2,3,3,3-pentafluoropropan-1-amine (68 mg, 0.454 mmol, 10 eq.) in dichloroethane (DCE) (1 mL) was slowly added at room temperature a 2 M solution of trimethylaluminium in toluene (0.227 mL, 0.454 mmol, 10 eq.). The mixture was stirred at room temperature for 15 min and trityl ester **1** (50 mg, 0.045 mmol, 1 eq.) in DCE (1 mL) was added. The mixture was stirred at room temperature for 1 h and refluxed overnight. After cooling at to room temperature, the resulting orange mixture was diluted with dichloromethane (DCM) (5 mL) and carefully treated with saturated aqueous KH_2PO_4 (5 mL), and the organic layer was separated. The aqueous layer was extracted with DCM (3×5 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The orange residue was purified by preparative TLC (plate 20 × 20 cm, silicagel 60 F_{254}, 1 mm, Merck) using DCM to provide 60.8 mg (95%) of compound **2** as an orange solid. ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm) : 1.69 (s, 18H), 1.81 (s, 18H), 4.18 (m, 6H), 6.52 (s, 1H), 6.67 (t, ^{3}J = 6.1 Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm) : 27.5, 29.1, 32.2, 34.7, 39.2 (t, ^{2}J = 24 Hz), 62.6, 63.2, 84.1, 113.0 (tq, ^{1}J = 253.3 Hz, ^{2}J = 37.3 Hz), 118.8 (qt, ^{1}J = 284.3 Hz, ^{2}J = 34.7 Hz), 125.6, 133.0, 137.6, 138.0, 140.3, 141.0, 167.1. ^{19}F-NMR

(282 MHz, $CDCl_3$) δ (ppm) : -121.61 (s, 6F), -84.63 (s, 9F). ATR-IR (cm^{-1}) : 3350, 2960, 2923, 2864, 1670, 1527, 1195, 1151. HRMS (TOF MS ES+) *m/z* $[M+Na]^+$ calcd for $NaC_{49}H_{46}F_{15}N_3O_4S_{12}$: 1431.9795, found 1431.9755.

Preparation of **3**: The previous procedure was applied to the synhesis of **3** using a solution of 1H,1H-perfluorooctylamine (181 mg, 0.454 mmol, 10 eq.) treated with a 2 M solution of trimethylaluminium in toluene (0.227 mL, 0.454 mmol, 10 eq.) and then with trityl ester **1** (50 mg, 0.045 mmol, 1 eq.). The crude product was purified by preparative TLC (plate 20 × 20 cm, silicagel 60 F_{254}, 1 mm, Merck) using DCM/petroleum ether (2:1) to provide 65 mg (66%) of compound **3** as an orange solid. ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm) : 1.69 (s, 18H), 1.82 (s, 18H), 4.23 (m, 6H), 6.53 (s, 1H), 6.68 (t, ^3J = 6.3 Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm) : 27.6, 29.1, 32.2, 34.7, 39.6 (t, ^2J = 23.3 Hz), 62.6, 63.3, 84.2, 100-125 (21C), 125.7, 133.1, 137.6, 138.0, 140.3, 141.0, 167.2. ^{19}F-NMR (282 MHz, $CDCl_3$) δ (ppm) : -126.59 (s, 6F), -123.70 (s, 6F), -123.18 (s, 6F), -122.48 (s, 6F), -122.21 (s, 6F), -117.75 (s, 6F), -81.23 (s, 9F). ATR-IR (cm^{-1}) : 3342, 2962, 2920, 2851, 1670, 1527, 1225, 1201, 1147. HRMS (ESI+) *m/z* $[M+1]^+$ calcd for $C_{64}H_{47}F_{45}N_3O_4S_{12}$: 2159.9491, found 2159.9451, (b) For an example of using trimethylaluminium for conversion of esters to amides, see: Basha, A.; Lipton, M.; Weinreb, S. M. *Tetrahedron Lett.* **1977**, *18*, 4171.

16. Preparation of **F15T-03**: To a stirred solution of **2** (120 mg, 0.085 mmol, 1 eq.) in DCM (20 mL) was added dropwise at room temperature $BF_3.Et_2O$ (0.085 mL, 0.680 mmol, 8 eq.). After stirring for 1 h, the resulting dark solution was treated with a solution of $SnCl_2$ (27 mg, 0.145 mmol, 1.7 eq.) in THF (4 mL). The mixture was stirred at room temperature for 30 min. Saturated aqueous KH_2PO_4 (10 mL) was added and the organic layer was separated. The aqueous layer was extracted with DCM (3×10 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to provide 118 mg of the crude trityl radical **F15T-03** as a dark green solid. ATR-IR (cm^{-1}) : 3335, 2960, 2918, 2849, 1670, 1524, 1196, 1151. HRMS (TOF MS ES+) *m/z* $[M]^{\cdot+}$ calcd for $C_{49}H_{45}F_{15}N_3O_3S_{12}^{\bullet}$: 1391.9870, found 1391.9854. Preparation of **F45T-03**: To a stirred solution of **3** (43 mg, 0.020 mmol, 1 eq.) in DCM (8 mL) was added dropwise at room temperature $BF_3.Et_2O$ (0.020 mL, 0.159 mmol, 8 eq.). After stirring for 1 h, the resulting dark solution was treated with a solution of $SnCl_2$ (6 mg, 0.034 mmol, 1.7 eq.) in THF (1 mL). The mixture was stirred at room temperature for 30 min and

worked up as previously (F15T-03) to provide 43 mg of the crude trityl radical **F45T-03** as a dark green solid. ATR-IR (cm^{-1}) : 3335, 2959, 2922, 2851, 1670, 1522, 1236, 1205, 1148. Cyclic voltammetry was carried out using about 0.5 mM of **F15T-03** and **F45T-03** in degassed DCM containing 0.1 M of tetrabutylammonium tetrafluoroborate. The redox potentials were measured with a glassy carbon working electrode, a platinum-foil counter electrode, and Ag/AgCl in EtOH LiCl saturated as the reference electrode. Scan rate was 50 mV/s. Similar cyclic voltammograms to the ester derivatives[9] were obtained with a $E(ox)_{1/2}$= + 0.9 V and $E(red)_{1/2}$ = − 0.4 V.

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Nano-emulsions of fluorinated trityl radicals as sensors for EPR oximetry

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Abstract : This article reports the development and evaluation of two nano-emulsions (F45T-03/HFB and F15T-03/PFOB) containing fluorinated trityl radicals dissolved in perfluorocarbons. Preparation with a high-pressure homogenizer conferred sub-micronic size to both nano-emulsions. In vitro and in vivo EPR spectroscopy showed that the nano-emulsions had much greater oxygen sensitivity than the hydrophilic trityl, CT-03. In vivo experiments in rodents confirmed the ability of the nano-emulsions to follow the changes in oxygen concentration after induced ischemia. Histological evaluation of the tissue injected with the nano-emulsions revealed some acute toxicity for the F45T-03/HFB nano-emulsion but none for the F15T-03/PFOB nano-emulsion. These new formulations should be considered for further EPR oximetry experiments in pathophysiological situations where subtle changes in tissue oxygenation are expected.

1. Introduction

The role played by oxygen in physiopathological processes and therapeutics is of crucial importance. The development of non-invasive tools for monitoring tissue oxygenation may have profound diagnostic and therapeutic implications in oncology and in cardiovascular diseases. Existing methods for estimating pO_2 in tissues include magnetic resonance (MR)-based and non-MR-based techniques. Non-MR-based oximetry methods

include polarographic oxygen electrodes, fluorescence quenching, phosphorescence quenching, near infra-red spectroscopy (NIRS), and the use of bioreductive markers selectively trapped in hypoxic regions. MR-based techniques include ^{19}F-NMR spectroscopy/imaging, blood oxygen level-dependent (BOLD) NMR imaging, electron paramagnetic resonance (EPR) spectroscopy/imaging, and dynamic nuclear polarization (DNP) which involves both NMR and EPR (for a review, see [1], [2] and [3]).

In EPR oximetry, the oxygen content in tissues is measured by the effect of oxygen on the EPR spectrum of a paramagnetic oxygen sensor. These oxygen sensors can be classified into two groups: Particulate paramagnetic materials and soluble paramagnetic materials. The particulate probes include lithium phthalocyanine [4] and naphthalocyanine crystals [5], natural charcoals [6] and [7], analytical charcoals [8] and [9], India inks [10] and [11], and carbon blacks [12]. These materials possess high sensitivity to changes in oxygen concentration (large variation in EPR line width [LW] as a function of the pO_2), in vivo inertness, and a high spin density providing a high signal-to-noise ratio (SNR). However, for imaging applications, it may be interesting to use soluble probes that can diffuse inside tissues and provide information about the spatial distribution of oxygen within a tissue. Among soluble materials, two types of structure are particularly interesting: The nitroxides and the triarylmethyl (trityl or TAM) radicals. Various chemical properties of the nitroxides can be manipulated to modulate intracellular accumulation or tissue selectivity [13] and [14]. Nitroxides are metabolically converted to the EPR-silent hydroxylamines. Trityls are characterized by a narrower LW and a higher stability compared to nitroxides [15]. Trityls have been used as oxygen/pH probes [16], [17], [18], [19] and [20], and as contrast agents in EPR [21] and DNP [22] and [23] imaging. Soluble paramagnetic probes are less sensitive to oxygen changes compared to particulate probes. This limited sensitivity to oxygen can be problematic for evaluating subtle changes in tissue oxygenation. To overcome this limitation, Liu and colleagues proposed encapsulating nitroxides in lipophilic environments [24]. Consistent with the Smoluchowski equation, as oxygen is more soluble in lipophilic environments than in water, an increase in sensitivity can be achieved using these systems. For this proof-of-concept, Liu et al. used nitroxides dissolved in common organic solvents and encapsulated in denaturized albumin microspheres with a size of 2.5 µm. More recently, Kuppusamy's group reported the synthesis [25] and use of a trityl radical

(perchlorotriphenylmethyl-triester radical or PTM-TE in HFB). The authors also used bovine serum albumin (BSA) in order to encapsulate a solution of PTM-TE dissolved in HMDS [26]. HFB belongs to the class of perfluorocarbons (PFC) that have very high oxygen solubility. PFCs are able to dissolve up to about 50% their volume of oxygen [27].

It could be desirable to extend this concept to other PFCs which are biocompatible and already used clinically as blood substitutes (e.g., perfluorooctyl bromide (PFOB)). Moreover, it could be interesting to use a biocompatible nano-capsule as a vehicle, because BSA can generate immunogenic responses [28]. Furthermore, a nano-size formulation is suitable for parenteral administration and decreases uptake by the reticuloendothelial system after intravenous administration. To achieve these goals, we sought to enhance the affinity of the oxygen spin probe for a PFC formulation by attaching a perfluorinated tag on trityl radicals [29]. Introducing a fluorine label on a molecule enhances its fluorophilicity [30]. Two new trityl radicals (Fig. 1), called F15T-03 and F45T-03 (which contain three fluorine chains with 5 and 15 fluorine atoms, respectively), were recently synthesized by our group [29]. These radicals exhibit a single, sharp EPR spectrum. Calibration was performed in PFC solvents according to the respective solubility of the compounds. The oxygen sensitivities of F15T-03 in HFB and F45T-03 in PFOB were 1.87 µT/mm Hg and 1.75 µT/mm Hg, respectively [29]. This sensitivity was markedly increased compared to the hydrophilic trityl, CT-03 (0.064 µT/mm Hg). The aim of the present work was to develop and optimize the preparation of nano-emulsions containing these fluorinated trityls included in PFC solvents. These new oxygen sensor systems were characterized for their size, stability, and oxygen sensitivity in vitro and in vivo; and histological assessment of tissue reaction after administration was also conducted.

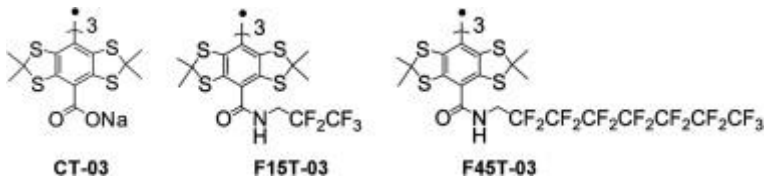

Fig. 1. Chemical structures of hydrophilic trityl CT-03, F15T-03 and FT45-03 fluorinated trityl.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Fluka unless specified and were all ⩾99% grade products. F15T-03 and F45T-03 were synthesized as described previously [29]. The hydrophilic trityl, CT-03, was kindly provided by HJ Halpern (University of Chicago).

2.2. Nano-emulsion preparation

F15T-03 and F45T-03 have different solubilities in PFC. F15T-03 was soluble in HFB and not in PFOB, while F45T-03 was soluble in both solvents. The highest concentration of the compounds was chosen according to their solubility in PFC and the need to avoid spin–spin exchange: F15T-03 1 mM in HFB and F45T-03 0.25 mM in PFOB. Solubilization was performed overnight. The solutions were filtered on a 0.2 μm GHP filter (Pall Acrodisc) before further manipulation. The nano-emulsions were prepared using 3-sn-phosphatidylcholine from dried egg yolk (purity ⩾ 50%). 3-sn-phosphatidylcholine (3.120 g) was dispersed for 1 h in 100 ml PBS buffer. Eighteen (F15T-03/HFB nano-emulsion) or 16 (F45T-03/PFOB nano-emulsion) ml of the phospholipid dispersion were collected and further homogenized for 10 min with an Ultraturax (13,500 rpm). Two or 4 ml of the PFC solution (F15T-03 in HFB and FT45-03 in PFOB, respectively) containing the fluorinated trityl were added drop by drop to the phospholipid dispersion. The emulsion was stirred for 30 min with the Ultraturax before being placed in an EmulsiFlex C-5 high-pressure homogenizer (Avestin) working at 20,000 psi for 12–20 min (20–35 passes) (adapted from [34]). Final concentrations were 0.1 μmol F15T-03/0.1 ml HFB/25 mg lecithin and 0.05 μmol F45T-03/0.2 ml PFOB/28 mg lecithin for 1 ml of nano-emulsion.

2.3. Size measurement

Particle size was assessed using a Zetasizer Nano ZS (Malvern). The nano-emulsions were diluted (10 times) in PBS buffer to obtain 1 ml for the measurement. Values are expressed as a mean of three measurements ± standard deviation (SD).

2.4. In vitro EPR oximetry

As the oxygen solubility is temperature dependent, all in vitro and in vivo experiment were made at 37 °C. Calibration measurements (EPR LW as a function of the pO_2) were performed at 9.3 GHz with a Bruker EMX EPR spectrometer equipped with a variable temperature controller BVT-3000. The sample (nano-emulsion containing F15T-03 or FT45-03, or aqueous solution of the hydrophilic CT-03) was placed in a gas-permeable Teflon tube (0.625 mm inner diameter; 0.05 mm wall). This tube was folded twice at both ends and placed in a quartz tube open at both ends. Measurements were taken at 310 K and at 21% and 0% oxygen. The oxygen content in the gas was analyzed using a Servomex oxygen analyzer OA540. Typical spectrometer parameters were modulation amplitude less than one third of the peak-to-peak LW, and incident microwave power of 100 µW. The frequency modulation was set at 10 kHz for CT-03, and 100 kHz for the F15T-03 and FT45-03 nano-emulsions. A kinetics experiment was also performed to check that the phospholipid shell allowed equilibrium with the oxygen environment [31]. Air was flushed at 400 l/h into the EPR spectrometer cavity and a first spectrum was acquired. The flushing gas was then changed to N_2 and an EPR spectrum was recorded every minute. After equilibrium, the gas in the cavity was changed back to 21% oxygen.

2.5. In vivo EPR oximetry

Animal experiments were carried out in compliance with national care regulations and the local ethics committee (protocol 2004/UCL/MD/026). NMRI male mice (Janvier) and Wistar rats (Animalerie Facultaire, Université catholique de Louvain) were used for these experiments. The animals were anesthetized with isoflurane before administration of the nano-emulsions into the gastrocnemius muscle (150 µl in mice and 500 µl in rats). In vivo EPR spectra were recorded using an EPR spectrometer (Magnettech, Berlin, Germany) with a low-frequency microwave bridge operating at 1.2 GHz and an extended loop resonator. Local hypoxia was induced by restriction of the blood supply in the muscle; the base of the thigh was reversibly tied with an elastic band to restrict blood flow through the femoral arteries. Measurements (n = 5 per group) were carried out during normoxia (3 min after nano-emulsion injection) and 5 min after inducing ischemia. Infra-red lights have been used in order to maintain the temperature of the animals. Parameters for experiments with CT-03

were: center field: 53 mT; modulation amplitude: 0.00315 mT; modulation frequency: 10 kHz; power: 600 μW. For F15T-03 and F45T-03 nano-emulsions, the acquisition parameters were: center field: 53 mT; sweep width: 1.0 mT; modulation amplitude: 0.021 mT; modulation frequency: 100 kHz; power: 23.8 mW.

2.6. Histological evaluation

The effect of the nano-emulsions on the muscle tissue was assessed by histological examination. Thirty microliters of F15T-03/HFB or F45T-03/PFOB were administered intramuscularly. Four or 24 h later, mice were sacrificed and the muscle into which the nano-emulsion had been administered was carefully removed. After fixing the samples (n = 2) in 10% formalin for 48 h, they were embedded in paraffin blocks and prepared into histological slices (5 μm thickness). Slices were stained using a classical hematoxylin–eosin procedure. Picture captions were taken using an optical microscope (Nikon Alphashot-2 Y52) (magnification 100×) and a digital photo camera, Nikon CoolPix 4500.

3. Results and discussion

3.1. Size measurement and stability of the nano-emulsions

The homogenizing procedure was critical in order to obtain a stable nano-emulsion. Without high-pressure homogenization, it was impossible to obtain a stable emulsion. After the nano-emulsion had been processed, particle size measurements were performed. The F15T-03/HFB nano-emulsion was characterized by an average diameter of 112 ± 1 nm and a polydispersity index (PDI) of 0.223 on the day of preparation. For the F45T-03/PFOB nano-emulsion, the average diameter was 182 ± 3 nm and the PDI 0.158. This distribution size would allow intravascular administration of these nano-emulsions. The size distribution remained stable for at least one month for the F15T-03/HFB nano-emulsion and two weeks for the F45T-03/PFOB nano-emulsion (data not shown).

3.2. In vitro EPR oximetry

When changing the oxygen environment, the nano-emulsions containing F15T-03/HFB and F45T-03/PFOB had larger variations in LW compared to the hydrophilic CT-03 trityl. The ΔLWs when changing from air to nitrogen were 0.2336 mT (0.0554 mT and 0.289 mT at 0% and 21% oxygen) for the F15T-03/HFB nano-emulsion, 0.0962 mT

(0.0548 mT and 0.151 mT at 0% and 21% oxygen) for the F45T-03/PFOB nano-emulsion and 0.010 mT (0.009 mT and 0.019 mT at 0% and 21% oxygen) for the CT-03 aqueous solution. From these data, it is possible to calculate the % increase in LW compared to the values measured at 0% oxygen. The increase in LW were 521%, 275% and 211% for the nano-emulsion F15T-03/HFB the F45T-03/PFOB nano-emulsion and the CT-03 solution, respectively. These data demonstrate that both nano-emulsions have increased sensitivity to oxygen variation.

Interestingly, the LW equilibrium values in air were comparable for F15T-03 when dissolved in HFB or encapsulated in the nano-emulsions. However, the LW values obtained for F45T-03 were larger when this compound was dissolved in PFOB [29] than when it was encapsulated in PFOB (0.334 mT in PFOB versus 0.151 mT in the nano-emulsion). Importantly, the calibration (LW as a function of pO_2) remained stable over time (more than one week, data not shown). The kinetics experiment clearly showed that the phospholipid shell around the dispersed PFC nano-droplets was totally permeable to gaseous oxygen exchange. As shown in Fig. 2, the equilibrium of the nano-emulsion of F15T-03 with the gas environment was achieved within less than 5 min when changing from air to nitrogen and vice-versa.

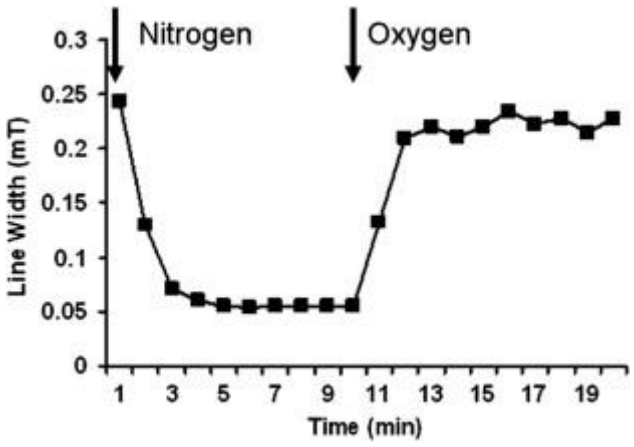

Fig. 2. Kinetic of fast oxygen exchanges in nano-emulsions of F15T-03. Nitrogen was flushed into the chamber for 10 min starting from time 0 (first arrow); at 11 min oxygen was flushed (second arrow).

3.3. In vivo EPR oximetry

After intramuscular administration of hydrosoluble CT-03 trityl or F15T-03 or F45T-03 nano-emulsions, the animals were placed in our L-band EPR spectrometer. At this frequency, EPR can be carried out with a depth of penetration of about 1 cm, which is optimal for studies in rodents. The LWs (means ± SD) observed in normoxic muscle were 0.0134 ± 0.0011 mT for the CT-03 solution, 0.092 ± 0.0044 mT and 0.079 ± 0.0025 mT for F15T-03 and F45T-03 nano-emulsions, respectively. These values correspond to pO_2 values (means ± SD) of 70.3 ± 18.2 mmHg for the CT-03 solution, 25.0 ± 3.0 mm Hg and 40.8 ± 4.1 mm Hg for F15T-03 and F45T-03 nano-emulsions, respectively. These values are consistent with previously published data recorded in muscles of rodents anesthetized with isoflurane [32] and [33]. The differences between the two nano-emulsions are not significant in term of pO2. The apparent discrepancy could come from the inflammatory reaction that has been observed with the use of the F15T-03/HFB nano-emulsion. The important standard deviation using CT-03 can be attributed to the limited resolution accuracy when measuring very small LW.

The variation in LW after interruption of blood flow was significantly lower with the hydrophilic CT-03 than with the nano-emulsions. The hydrophilic CT-03 solution had a ΔLW of 0.0038 ± 0.0023 mT, compared to 0.0208 ± 0.0096 mT and 0.0194 ± 0.0071 mT for F15T-03 and F45T-03, respectively (Fig. 3). This corresponds to a fivefold increase in sensitivity to oxygen variations in vivo. This increase in oxygen sensitivity is appreciable when monitoring subtle modulations of oxygen in vivo. However, a disadvantage of using nano-emulsions is the decrease in signal intensity obtained in vivo compared to the hydrophilic compound, CT-03. This is the result of three different phenomena: (1) an intrinsically larger LW even at low pO_2 (the signal intensity is inversely proportional to the square of the EPR LW); (2) the need to avoid a spin–spin exchange in the starting PFC solution (concentration < 5 mM); (3) the dilution factor when preparing an emulsion of PFC in water solutions. This difference in signal intensity is illustrated in Fig. 4.

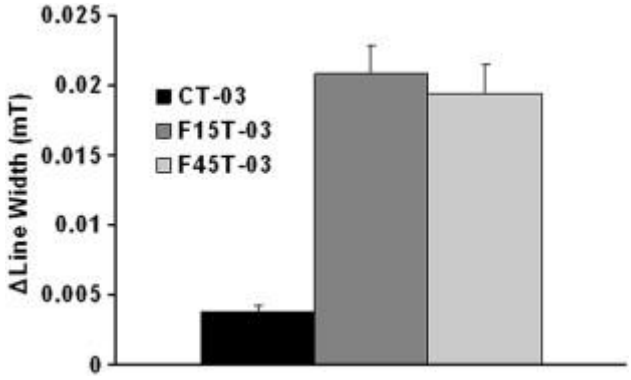

Fig. 3. In vivo ΔLW of measurement at normoxia and after leg ligation in mice injected with the trityl CT-03 (black columns), or the F15T-03/HFB nano-emulsion (dark gray columns), and rats injected with the F45T-03/PFOB nano-emulsion (light gray columns).

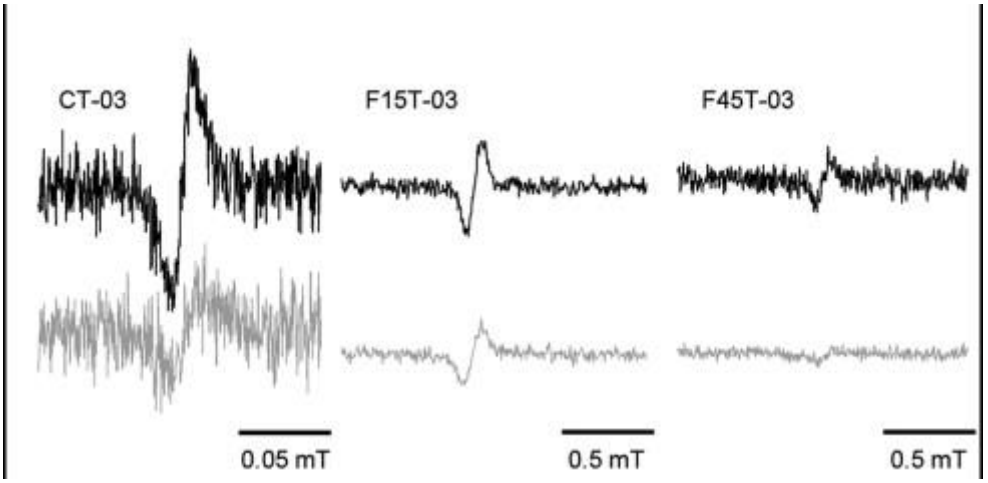

Fig. 4. In vivo spectrum of measurement at normoxia (gray) and after leg ligation (black). Left: CT-03; center: F15T-03/HFB; right: F45T-03/PFOB.

3.4. Reactivity in tissues and histology

After intramuscular administration, the F15T-03/HFB emulsions rapidly produced edema in the muscle, but there was no visible reaction with the F45T-03/PFOB nano-emulsions. To better characterize this phenomenon, a preliminary histological examination was carried out. No inflammation was detected either at 4 or at 24 h after injection (Fig. 5, left panel) in mice which had received the F45T-03/PFOB nano-emulsion. This is in accordance with the considerable amount of work investigating the use of this PFC as a blood substitute [34]. The situation was different in the group that received F15T-03/HFB. As shown in Fig. 5 (right panel), an inflammatory process was observed after administration of

this compound, characterized by considerable edema, eosinophilia, abnormalities in the muscle cell structure, and lack of cell nucleus. This situation persisted 24 h after administration of the nano-emulsion.

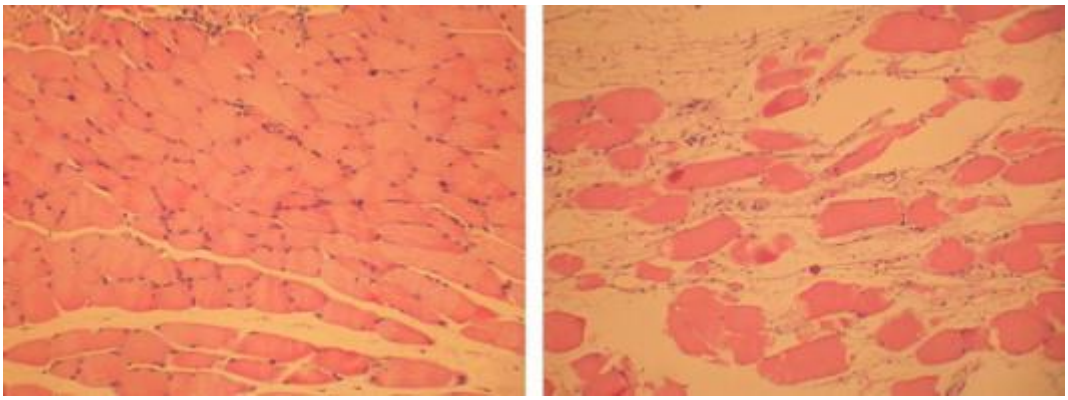

Fig. 5. Histological sections of muscles after administration of F45T-03/PFOB at 4 h (left) and F15T-03/HFB at 4 h (right).

4. Conclusions

We have developed new systems that incorporate fluorine trityls in nano-emulsions containing PFC. These compounds were evaluated for their performance as EPR oxygen sensors. Stable formulations were achievable using high-pressure homogenization, and nano-emulsions were usable for at least two weeks after preparation. A large increase in sensitivity to oxygen was observed both in vitro and in vivo when using these nano-emulsions compared to hydrophilic trityls. When comparing the two nano-emulsions, the F15T-03/HFB nano-emulsion had the highest sensitivity in vitro to oxygen variation. Moreover, its higher solubility (up to 1 mM in HFB) allowed preparation of a nano-emulsion detectable in vivo with a high signal-to-noise ratio. However, in vivo this compound induced an inflammatory reaction and edema. The F45T-03/PFOB nano-emulsion, while being apparently less efficient, is still five times more sensitive to oxygen than hydrophilic trityls. This formulation did not induce any inflammatory response in preliminary tests, and should be considered further as a system to monitor subtle changes in tissue oxygenation in vivo.

Acknowledgments

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5. Chiralité des dérivés TAMs

Cette partie du manuscrit décrit les propriétés de chiralité des radicaux TAMs.

Ce travail a fait l'objet de deux publications, la première dans le journal " Chemical Communications" et la deuxième dans le journal " European Journal of Organic Chemistry" . L'importance du travail a été soulignée par les referees et a fait l'objet de la couverture du journal EJOC.

Ma contribution a été la vision d'une possible chiralité de ces molécules (stériquement très enconbrées) associée à leurs conformations, la synthèse de différentes famille de dérivés, la résolution des énantiomères et la cinétique de racémisation des deux premiers composés étudiés. J'ai assisté L. Collard (UCL/CHOM) spécialiste en HPLC pour la résolution et la cinétique des deux derniers composés étudiés. J'ai également assisté le Dr. Cécile Le Duff (UCL/ASM) expert en RMN pour les expériences de spectroscopie d'échange (EXSY). Le Prof. R. Robiette (UCL/CHOM) spécialiste en chimie organique et en chimie théorique a réalisé l'étude théorique suite à ma demande de collaboration.

La découverte de la chiralité de conformation des molécules TAM trouve son origine dans la rencontre entre le monde de la RPE biomédicale qui avait déjà largement étudié les radicaux TAMs et le monde de la chimie organique plus souvent confronté à d'autres types de chiralité que le centre stéréogénique. Ce travail largement multidisciplinaire et très enrichissant est également le fruit d'une belle collaboration entre la chimie organique (B.D.), théorique (R.R.), analytique (HPLC, L.C. et B.D.) et spectroscopique (RMN, C.L et B.D.). La chimie organique a permis de fournir des composés TAMs de type radical, alcool ou alcane. La résolution par HPLC chirale et le suivi cinétique de racémisation par HPLC a permis de déterminer la stabilité conformationelle de ces molécules. La spectroscopie d'échange a permis de déterminer le mécanisme d'isomérisation des composés alcanes. Après vérification de la bonne adéquation entre les valeurs expérimentales et théoriques, l'étude théorique menée par le Prof. R. Robiette a permis d'étendre l'analyse aux molécules et isomères qui ne sont pas facilement accessibles expérimentalement ou ne peuvent pas être

analysés par RMN (radicaux). Tout ce travail a permis de connaitre la stabilité conformationelle, les facteurs structuraux influençant cette stabilité et le mécanisme d'isomérisation de molécules qui n'étaient pas connues comme chirales auparavant par leurs utilisateurs issus du milieu biomédical.

Chiral Properties of Tetrathiatriarylmethyl Spin Probes

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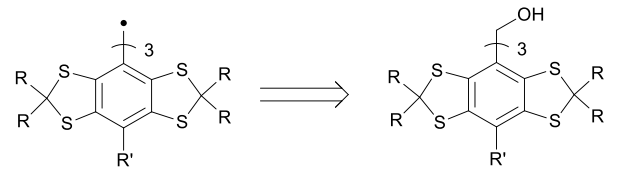
Abstract : We report that tetrathiatriarylmethyl (trityl) EPR probes are chiral molecules at room temperature, the two stereoisomers that differ in their helicity being configurationally stable enough to be separated and stored independently.

Stable tetrathiatriarylmethyl radicals **1a** (Fig. 1) belong to a family of trityl radicals extensively used for electron paramagnetic resonance imaging (EPRI),[1] oximetry,[2] dynamic nuclear polarization (DNP)[3] and the detection of superoxide radical anion.[4] These water soluble paramagnetic species have unique properties, namely an exceptional biostability, a narrow EPR linewidth (the anoxic linewidth is <100 mG), and display a low concentration-dependent broadening of the EPR line.[5]



1a R = CH_3, CD_3 or CH_2OH, R' = COONa
1b R = CH_3, R' = $COOC_2H_5$
1c R = CH_3, R' = $COOCH_3$
1d R = CH_3, R' = H

2b R = CH_3, R' = $COOC_2H_5$
2d R = CH_3, R' = H

Fig. 1 Representative tetrathiatriarylmethyl radicals of biomedical use (**1a**) and precursors.

Accordingly, this class of carbon-centered radicals has been selected by several laboratories for chemical modifications with the aim of diversifying their applications and improving their performances. In this context, a series of new tetrathiatriarylmethyl radicals have recently been synthesized for various applications: dendritic trityl radicals

that possess a higher stability,[6] ester-derivatized forms used for intracellular oxygen measurement,[7] amino-derivatized forms allowing dual pH/oxygen assessment,[8] fluorinated variants used to quantify tissue oxygenation with high sensitivity in perfluorocarbon formulations,[9] aldehyde derivative with enhanced sensitivity to oxygen[10] and trityl-nitroxide biradicals dedicated to the simultaneous measurement of redox status and oxygenation.[11]

Surprisingly, during these numerous developments of trityl radicals for biomedical use, the fundamental question of potential chirality associated with their helicoidal conformation has never been addressed. It has however been shown that tetrathiatriarylmethyl radicals adopt a propeller shape in which all rings have the same direction of twist.[12] This geometrical arrangement allows two conformations, namely the right-handed (*P*) and left-handed (*M*) helices which feature an enantiomeric relationship (Fig. 2). If conformational equilibration between these two helices is rapid, then the molecules will not display chiral properties. However, since the large steric bulk of the three aryl moieties, one can expect the rotation around the three carbon-carbon bonds connected to the central carbon to be restricted, thus limiting the stereoisomerisation of the two helices. If this is the case and interconversion between the two enantiomeric helices is slow, then the two enantiomeric helices could potentially be isolated separately. It is therefore crucial to study the dynamic of this conformational equilibrium since chirality could have important consequence on applications, indeed different enantiomers can display different biological activities. In addition, the connection of chiral substituants to the trityl core would result in the formation of diastereoisomeric mixtures[11] with possibly different chemical, biological and physical properties (*e.g* different EPR spectra). Finally, chirality associated with an open-shell system would confer to tetrathiatriarylmethyl radicals interesting chirooptical properties as nonlinear optical (NLO) responses.[13]

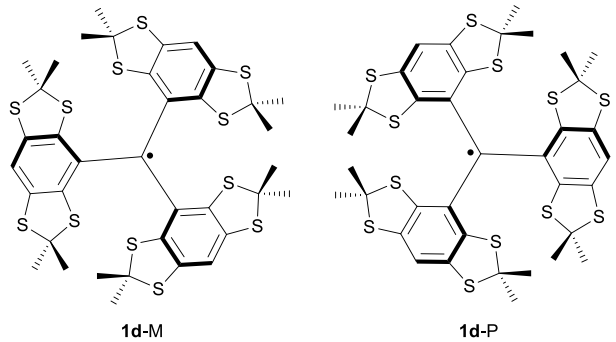


Fig. 2 The two conformational enantiomers of tetrathiatriarylmethyl radical **1d**.

As trityl alcohol is also a propeller shape molecule,[14] tetrathiatriarylmethyl alcohols, the direct precursors of the radicals (see Fig. 1), might similarly be chiral if the rotations around the three carbon-carbon bonds connected to the central carbon are restricted. Indeed, when we added Pirkle's alcohol, a known chiral solvating agent (CSA), to a solution of **2d**[15] in $CDCl_3$, we observed the splitting of ^1H and ^{13}C NMR signals on spectra measured at room temperature (see ESI†) indicating the formation of two diastereoisomeric solvatation complexes. This observation shows that the helicity interconversion does not occur rapidly on the NMR time scale at 25°C. Further experimental evidence of the chirality of tetrathiatriarylmethyl alcohols was obtained for alcohol **2b**[15] by analytical HPLC carried out on a chiral stationary phase (CSP HPLC) at room temperature. Indeed, the resolution of the peaks corresponding to the two enantiomers of **2b**‡ shows that there is no rapid isomerisation process on the HPLC time scale either (Fig. 3, a). Under the same conditions, resolution of the tetrathiatriarylmethyl radical **1b** was also well visible (Fig. 3, b). As expected for enantiomers the CD spectra recorded by stopped-flow spectrometry at the maximum of CD intensity for the two peaks of **1b** are mirror images (see ESI†). This is the first direct experimental proof of the chirality of a tetrathiatriarylmethyl radical at room temperature.

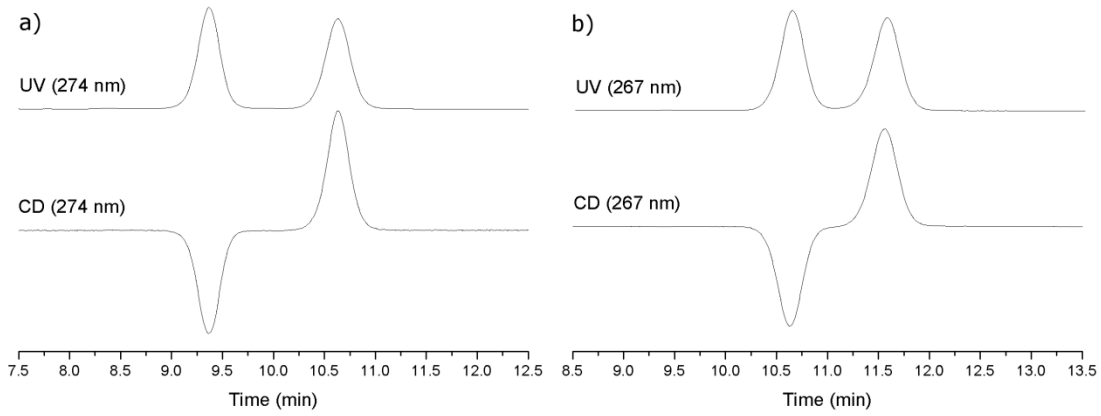


Fig. 3 CSP HPLC chromatograms with UV and CD detection of trityl alcohol **2b** at 274 nm (a) and radical **1b** at 267 nm (b).

In order to study the kinetic of isomerization, we first investigated the conformational pathway using computational (DFT) methods.[§] There are four potential pathways for interconversion: the n-ring flip mechanisms (n = 0, 1, 2, 3) in which zero, one, two or three aromatic ring(s) rotate(s) in a conrotatory fashion across the plane perpendicular to the one formed by the three aromatic carbons linked to the central carbon while the other ring(s) rotate(s) in the opposite direction through the reference plane (Fig. 4, for complete pathways see ESI[†]).[16] We have investigated all these four possible mechanisms for radical **1d** (model compound). Transition state structures could be found for the two- and the three-ring flip mechanisms, **TS-two** and **TS-three**, respectively. For the other mechanisms, our calculations revealed that bringing two of the rings in the same plane would imply such a spatial proximity between thio substitutents of these aryl groups (<0.5 Å between two S atoms) that these structures are simply not realistic. Calculated transition states energy shows that the lower lying pathway for conformational equilibrium is the one involving a two-ring flip (Table 1). It is interesting to note that our calculation suggest that solvation should not have an important effect on the activation free energy.

Table 1 Computed energy barrier ($\Delta E^{‡}$) and activation free energy ($\Delta G^{‡}$) in the gas phase and in solution for radicals **1d** and, in brackets, **1c** (energies are given in kcal mol^{-1} relative to reactant).

	$\Delta E^{‡}$	$\Delta G^{‡}$	$\Delta G^{‡}$ (EtOAc)
TS-zero	-[a]	-[a]	-[a]
TS-one	-[a]	-[a]	-[a]
TS-two	25.6(27.8)	28.3	29.5
TS-three	74.8	77.9	79.6

[a] All our attempts to obtain such a transition state structure showed the unfeasibility of having two (or three) of the aromatic rings on the same plane without breaking a bond. This enantiomerization pathway is thus not accessible.

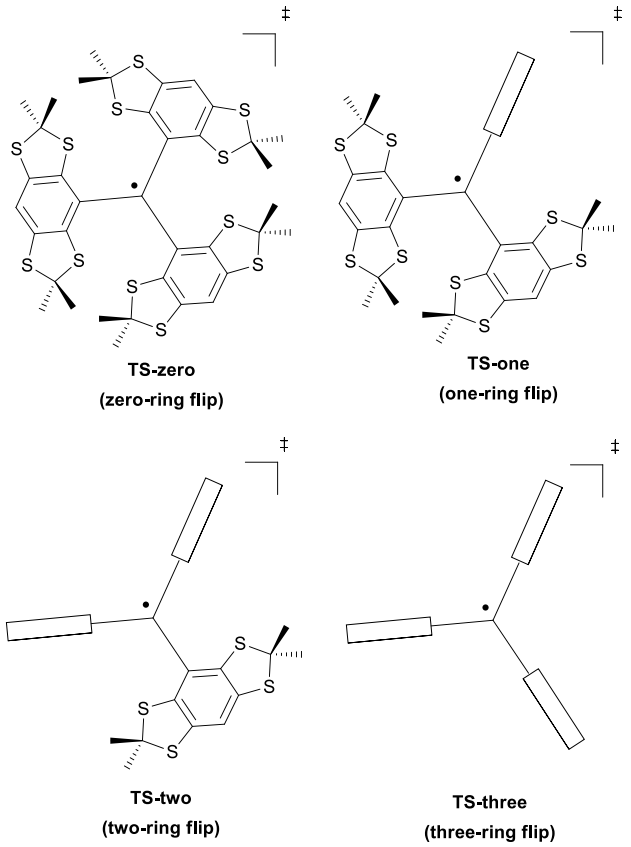
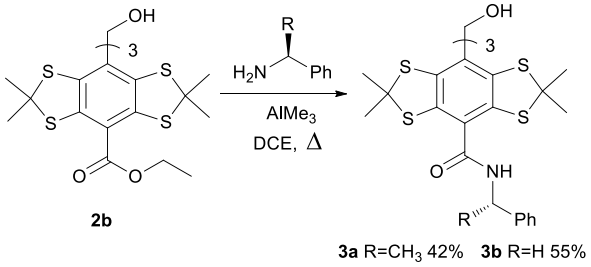


Fig. 4 The four potential transition states for isomerization of radical **1d**.

According to the calculated activation free energy in solution (29.5 kcal mol^{-1}), the two enantiomers of **1d** should be configurationally stable enough to be isolated and stored separately. We thus undertook the resolution of a tetrathiatriarylmethyl radical on a preparative scale.

We first envisioned to synthesize diastereoisomers by derivatization of the trityl core with an enantiomerically pure compound. Our strategy consisted in derivatizing corresponding trityl alcohol instead of the radical because of its higher chemical stability; the radical could be easily generated from alcohol[17] after separation of diastereoisomers. Accordingly, trityl alcohol **3a** has been synthesized by aminolysis of **2b** with (S)-α-methylbenzylamine as chiral derivatizing agent (Scheme 1). ^{1}H and ^{13}C NMR spectra of **3a** clearly show the presence of two diastereoisomers by constrast with the benzylamine analogue **3b** that does not possess additional chiral center (see ESI†). Unfortunately, all our attempts to separate diastereoisomers **3a** by crystallization or chromatography failed. It is noteworthy that no induction of asymmetric carbons on the helicity has been observed as the ratio of diastereoisomers of **3a** was 50:50 (determined by NMR)



Scheme 1

We then investigated the possibility of direct resolution of enantiomers using semi-preparative CSP HPLC. Again, the resolution of trityl alcohol **2b** instead of the radical **1b** has been considered since the better resolution obtained for alcohol **2b** by analytical CSP HPLC (see Fig. 3). Transposition of the analytical CSP HPLC method to a semi-preparative scale allowed us to obtain the two enantiomers of **2b** in milligram amounts with an enantiomeric excess of 100% (see ESI†). Further scaling-up is limited due to the low solubility of **2b** in the eluant. The two optically pure enantiomers of **2b** were then

separately converted into the corresponding enantiomers of radical **1b** by treatment with neat trifluoroacetic acid, a known procedure for quantitative conversion of tetrathiatriarylmethyl alcohols into radicals.[17] Analytical CSP HPLC of the two enantiomers confirmed their enantiomeric purity (see ESI†).

Having these two enantiomers **1b** in hand, we investigated their conformational stability in solution by following their kinetic of racemization (see ESI†). In the event, we found that, at room temperature, **1b** isomerizes slowly in EtOAc ($t_{1/2\ enantio} \approx 1$ month), confirming thus that the two enantiomers of **1b** can be stored separately for months in the freezer. The free activation energy calculated from the obtained kinetic constant, using Eyring equation, is 25.98±0.02 kcal mol^{-1} at 25°C. This experimental value is in good agreement with our calculations and thus supports the two-ring flip as the enantiomerization mechanism. A complete study of enantiomerization mechanism and determination of activation parameters for other substituted tetrathiatriarylmethyl radicals, tetrathiatriarylmethanols and tetrathiatriarylmethanes are currently under study and will be reported elsewhere.

In conclusion, we have shown that tetrathiatriarylmethyl radicals, very attractive spin probes, are chiral molecules at room temperature. The two enantiomers that differ in their helicity are configurationally stable enough to be separated and stored independently for months in the freezer; slow racemization occurs however in solution at room temperature. These observations are of primary importance and should be taken into account in further development and derivatization of these species for biomedical use.

This work was supported by the Université catholique de Louvain (UCL) and the Fonds de la Recherche Scientifique-FNRS (F.R.S.-FNRS). R.R. and J.M.-B. are Chercheurs qualifiés F.R.S.-FNRS. We are grateful to L. Collard for technical assistance in HPLC and Dr. P. Levêque for recording the EPR spectra of radical **1b.**

Notes and references

† Electronic Supplementary Information (ESI) available: details of experimental procedures, characterizations and computational details. See DOI: 10.1039/b000000x/

‡ (right- and left-handed helices showing opposite helipticity in the circular dichroism (CD) chromatogram and identical UV spectra (see ESI†)).

§ Geometry optimization was carried out using the UB3LYP hybrid density functional as implemented in Jaguar 7.5 with the standard split valence polarized 6-31G* basis. Single point energy calculations were carried out at the UB3LYP-D level of theory (including an approximation correction for dispersion) with the larger 6-311+G** basis, using ORCA package. Solvation effects were estimated at the UB3LYP-D/6-31G* level using the conductor-like screening model (COSMO) as implemented in ORCA, using the parameters appropriate for ethyl acetate. See Electronic Supplementary Information for full computational details and related references.

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Experimental Part

Chemistry

All reactions were carried out in flame-dried glassware under argon atmosphere and were monitored by thin layer chromatography (TLC) using aluminum sheets coated with silica gel 60 F254 (Merck). Anhydrous grade solvents were used for reactions and HPLC grade for purifications. All commercially available reagents were used as received without further purification. Preparative TLCs were done on plate 20 x 20 cm, silicagel 60 F254, 1 mm, (Merck). NMR spectra were recorded on a BRUKER Avance II (^{1}H: 300 MHz, ^{13}C: 75 MHz, ^{19}F 282 MHz) and a BRUKER Avance DPX (^{1}H: 500 MHz, ^{13}C: 125 MHz). Chemical shifts (δ) are reported in parts per million (ppm), referenced to NMR solvents: chloroform-d (δ ^{1}H=7.27 ppm, ^{13}C=77.2 ppm). The following abbreviations are used: s=singlet, d=doublet, t=triplet, m=multiplet. Coupling constants (J) are reported in Hertz (Hz). Infrared (IR) spectra are recorded using a Shimadzu spectrometer FTIR-8400S; products were analyzed as thin films deposited on a Se−Zn crystal by evaporation from the solvent. High resolution mass spectrometry (HRMS) analyses were performed at the University College London. Analytical HPLC was carried out on a Waters Alliance 2690 separations module equipped with a Waters 2998 PDA detector and a Gilson HPLC system (two Pumps 306, manometric module 805, dynamic mixer 811D, system interface 506C) equipped with a Jasco CD-2095 Plus chiral detector. Semi-preparative HPLC was carried out on a Waters 600 Pump equipped with a Waters 996 PDA detector. EPR spectrum was recorded on an X-Band BRUKER EMX spectrometer operating at ~9.46 GHz equipped with a ER4119HS resonator.

Computational methods

The bulk of the computations has been carried out using the Jaguar 7.5 pseudospectral program package.[g] All species have been fully optimized and the Cartesian coordinates are supplied in Section 8.

Geometry optimization was carried out using the well established UB3LYP hybrid density functional[h] as implemented in Jaguar with the standard split valence polarized 6-31G* basis.[i] Single point energy calculations were carried out at the UB3LYP-D level of theory (including an approximation correction for dispersion[j]) with the larger 6-311+G** basis, using ORCA package.[k]

In order to validate our chosen methodology, additional single energies have been evaluated for radical **1d** at several level of theory: UB3LYP/6-311+G**, UB3LYP-D/6-31G*, UB3LYP-D/6-31G**, RO-B3LYP-D/6-31G**, UBP86/6-31G** and LMP2/6-31G*. These results are reported in Section 8.1.

Vibrational frequencies were computed for all stationary points for radical **1d** at the UB3LYP/6-31G* level of theory and are used to include zero-point energy, thermal and entropic corrections to compute ideal gas free energies. The correct nature of stationary points was confirmed by checking the curvature and its eigenvectors: Ground state structures were found to be minima with zero imaginary frequency, while transition state structures were found to be first-order saddle points with a single imaginary frequency.

Solvation effects were estimated by performing additional single point energy calculations using the ORCA program package, at the UB3LYP-D/6-31G* level. These calculations were performed using the conductor-like screening model (COSMO) as implemented in ORCA,[l] using the parameters appropriate for ethyl acetate, the solvent used for racemization kinetics.

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[g] Jaguar, version 7.5, Schrödinger, LLC, New York, NY, 2008.

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For the large reaction systems there are usually several local minima or saddle points corresponding to each intermediate or transition state. This is due to the possibility of multiple conformations. In particular, the non-planarity of the two five-member rings on each phenyl groups allows two different conformations for each ring. Test calculations showed that energy differences between the various accessible conformers[m] and energy barriers connecting them are much lower than barriers to enantiomerization. We have made a systematic attempt to locate all possible local saddle points, with the data presented referring to the lowest energy form.

NMR spectroscopy analysis of compound 2d with a chiral solvating agent

To a solution of 50 mg of trityl alcohol **2d** (containing 1.3 eq. of C_6H_6) in 0.6 mL of $CDCl_3$ was added 1,3,6,8 or 10 equivalents of (+)-2,2,2-trifluoro-1-(9-anthryl)ethanol (the Pirkle's alcohol)[n] as chiral solvating agent (CSA)[o] and ^{1}H,^{13}C and ^{19}F NMR spectra were recorded at 500 MHz, 125 MHz and 282 MHz, repectively.

[m] In most cases, the more stable conformer is the one in which the five-member rings on each phenyl group are twisted in opposite directions so that the whole substituted phenyl group is in a chair-like conformation.

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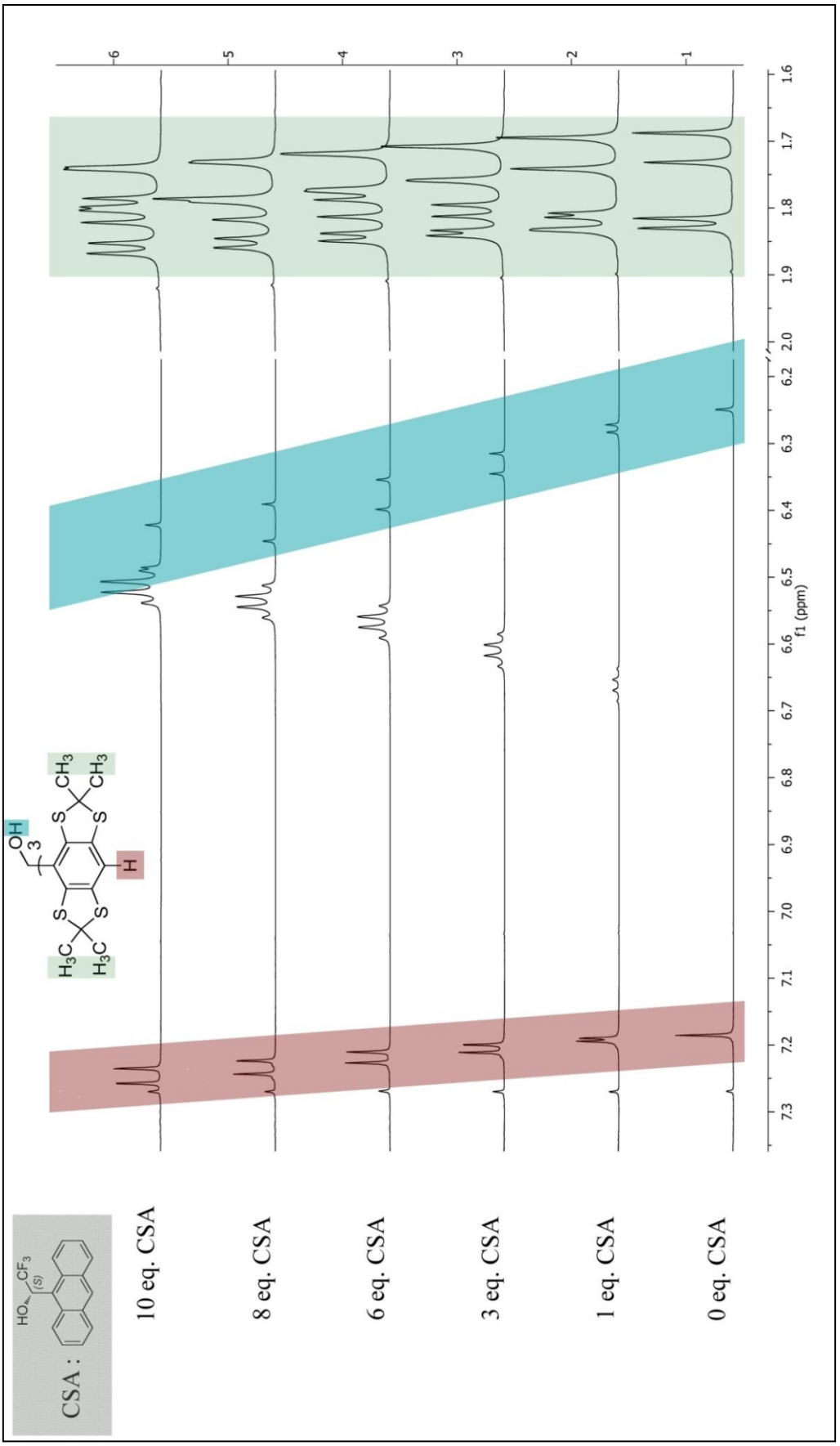
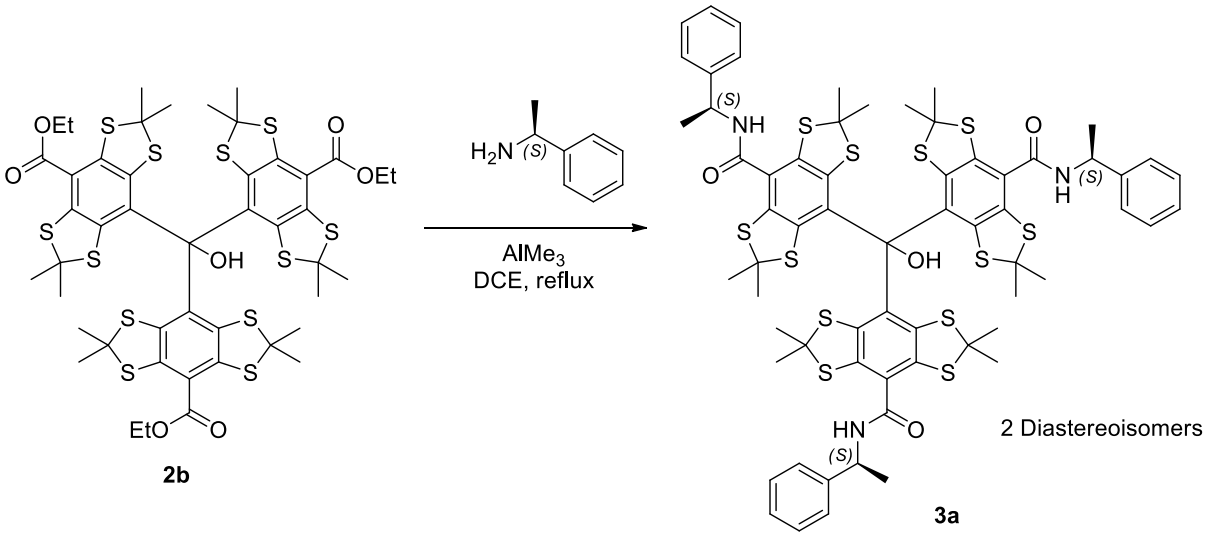


Figure 1

Syntheses and characterizations of compounds 3a and 3b

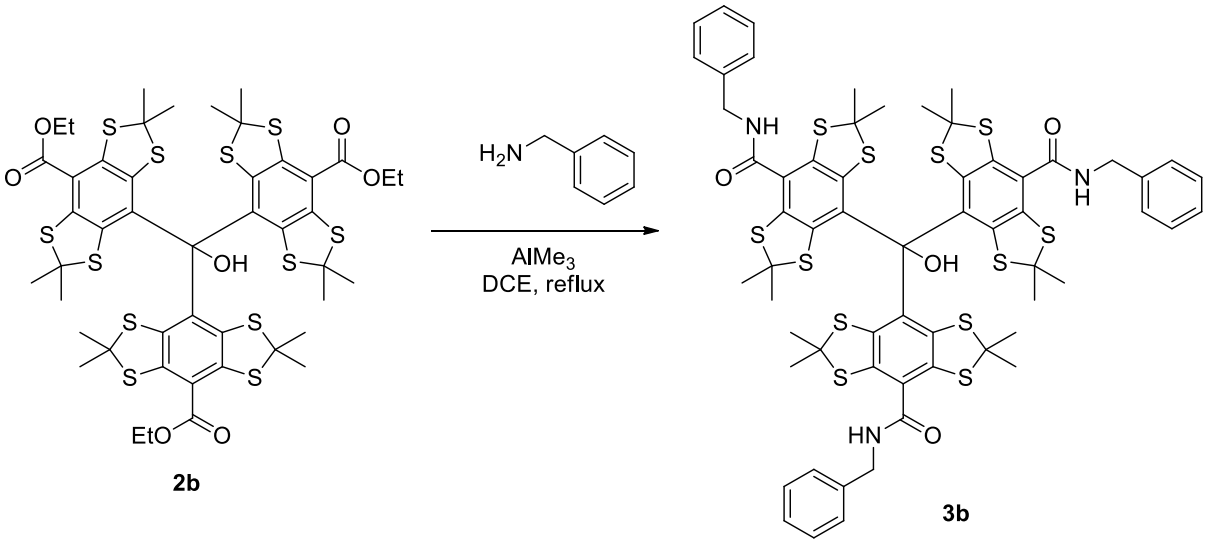
Synthesis of tris(8-(S)-α-methylbenzylaminocarbonyl-2,2,6,6-tetramethylbenzo[1,2-d;4,5-d']bis[1,3]dithiol-4-yl)methanol **3a**



To a solution of (S)-(−)-α-methylbenzylamine (117 µL, 0.908 mmol, 20 eq.) in dichloroethane (DCE) (1 mL) was added $AlMe_3$ 2M in toluene (0.454 mL, 0.908 mmol, 20 eq). The solution was stirred for 10 min and trityl alcohol **2b** (50 mg, 0.045 mmol, 1 eq.) in DCE (1 mL) was then added. The orange solution was stirred 1 h at room temperature and refluxed for 19 h. After cooling the solution to room temperature KH_2PO_4 sat. (2 mL) was carefully added. The mixture was diluted with dichloromethane (DCM) (3 mL) and the organic layer was separated. The aqueous layer was extracted with DCM (3x3 mL). The combined organic layers were dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude mixture was purified by preparative TLC with EtOAc 5% in DCM to provide 25 mg (42%) of the title compound **3a** as an orange solid. R_f : 0.35 DCM/EtOAc (95:5) UV (254,366 nm). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.63-1.80 (several signals, 45H), 5.25-5.36 (m, 3H), 6.47-6.57 (m, 4H, OH+NH), 7.28-7.47 (m, 15H). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 21.9+22.0, 27.8+28.1, 29.2+29.2, 32.1, 34.5+34.6, 50.1+50.2, 62.4+62.5, 63.0+63.1, 84.2+84.2, 126.6+126.7, 126.9+127.1, 127.7+127.7, 128.9+128.9, 132.7, 137.3+137.4, 137.5+137.7, 139.8+139.9, 140.8+140.8, 142.7+142.8, 166.1. HRMS (TOF MS ES+) *m/z* :

$[M+H]^+$ calcd for $C_{64}H_{68}N_3O_4S_{12}$: 1326.1858, found: 1326.1954. ATR-IR (ν_{MAX}/cm^{-1}): 3331, 2960, 2924, 1655, 1522, 1495, 1452, 1230.

Synthesis of tris(8-benzylaminocarbonyl-2,2,6,6-tetramethylbenzo[1,2-d;4,5-d']bis[1,3]dithiol-4-yl)methanol **3b**



To a solution of benzylamine (49 µL, 0.454 mmol, 10 eq.) in DCE (1 mL) was added $AlMe_3$ 2M in toluene (0.227 mL, 0.454 mmol, 10 eq). The solution was stirred for 10 min and trityl alcohol **2b** (50 mg, 0.045 mmol, 1 eq.) in DCE (1 mL) was then added. The orange solution was stirred for 1 h at room temperature and refluxed for 16h30. After cooling the solution to room temperature, KH_2PO_4 sat. (2 mL) was added carefully. The mixture was diluted with DCM (3 mL) and the organic layer was separated. The aqueous layer was extracted with DCM (3x3 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The crude mixture was purified by preparative TLC with MeOH 4% in DCM to provide 32 mg (55%) of the title compound **3b** as an orange solid. R_f : 0.10 DCM/MeOH (99 :1); 0.34 DCM/MeOH (98:2); 0.51 DCM/MeOH(96:4) UV (254,366 nm). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.66 (s, 9H), 1.68 (s, 9H), 1.78 (s, 18H), 4.67 (d, *J*=5.7 Hz, 6H), 6.55 (s, 1H, OH), 6.60 (t, *J*=5.6 Hz, 3H, NH), 7.30-7.50 (m, 15H). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) : 27.9, 29.1, 32.1, 34.6, 44.4, 62.5, 63.0, 84.1, 126.8, 127.8, 128.2, 128.9, 132.8, 137.3, 137.6, 137.8, 139.9, 140.8, 167.0. HRMS (TOF MS ES+) *m/z* : $[M-OH]^+$ calcd for $C_{61}H_{60}N_3O_3S_{12}$: 1266.1278, found: 1265.7814, $[M+H]^+$: calcd for $C_{61}H_{62}N_3O_4S_{12}$: 1284.1389, found: 1283.7803, $[M+Na]^+$: calcd for $C_{61}H_{61}N_3NaO_4S_{12}$: 1306.1208, found: 1305.7568. ATR-IR (ν_{MAX}/cm^{-1}): 3342, 2959, 2920, 1651, 1526, 1497, 1452, 1229, 910.

Resolution of trityl alcohol 2b by CSP HPLC

The two enantiomers of **2b** were separated on a semipreparative CHIRALPAK® IB column 10x250 mm 5µm equipped with a precolumn CHIRALPAK® IB 10x20 mm 5µm. The conditions of resolution were as follows: flow rate: 4.7 mL/min; eluent: *i*-hex 93%/EtOAc 4%/MeOH 3% (premixed); UV detection: 274 nm; injection volume: 50 µL; column temperature: ambient; sample: 5.5 mg/mL of EtOAc. Fraction 1 corresponding to the first peak, was collected in a flask cooled at -78°C from the beginning of the upward slope to the half-height of the downward slope as depicted in Figure 2. Fraction 2 corresponding to the second peak, was collected in a flask cooled at -78°C starting from the half-height of the upward slope to the end of the downward slope (Figure S9).

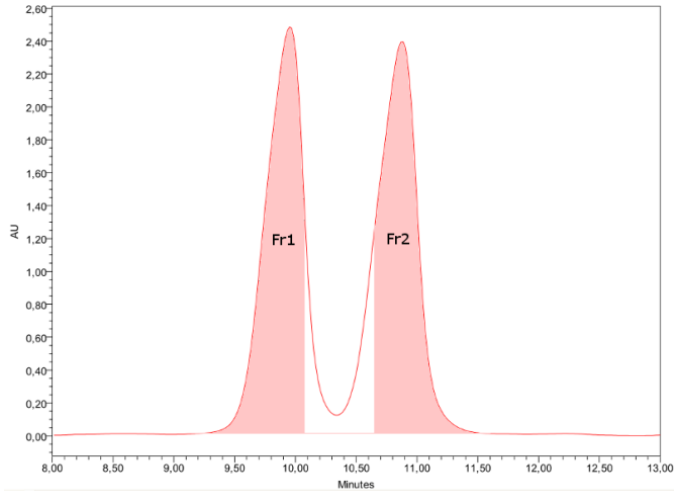


Figure 2

In the optimized final procedure, 50 µL of the racemate were injected every 8 min without stopping the run (Figure S10). About 1.5 mg of each enantiomers was recovered.

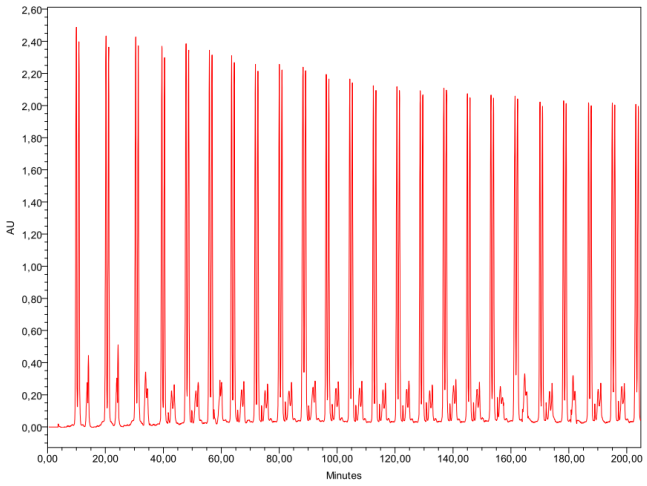
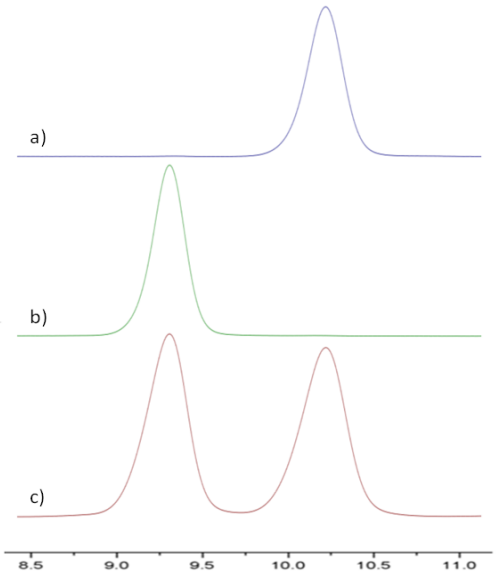


Figure 3

The enantiomeric purity of the two enantiomers **2b** was controlled by analytical CSP HPLC (Figure S11) on a CHIRALPAK® IB column 4.6x250 mm 5µm equipped with a precolumn CHIRALPAK® IB 4.6x20 mm 5µm. The conditions were as follows; flow rate: 1 mL/min; eluent: *i*-hex 93%/EtOAc 4%/MeOH 3% (premixed); UV detection: 274 nm; injection volume: 20 µL; column temperature: 25°C; sample: Fr1 and Fr2 directly from the semipreparative CSP HPLC. The enantiomeric excess was 100% for both fraction 1 and fraction 2.



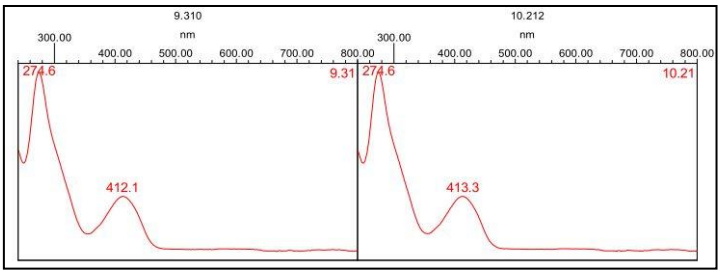
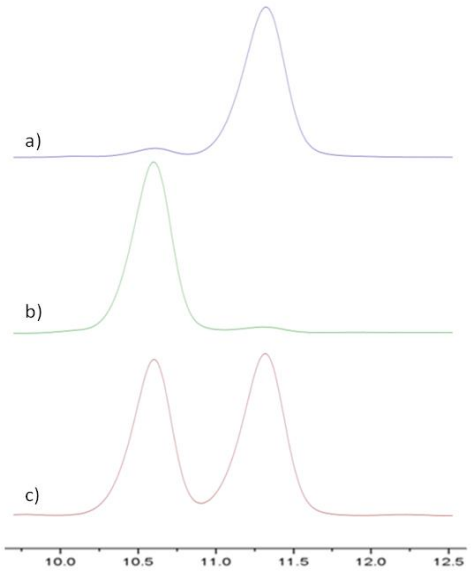


Figure 4. Stacked analytical chromatograms and UV spectra of the two fractions collected from the semipreparative CSP HPLC and racemate. a) Fraction 2; ee=100%, Rt=10.21 min b) Fraction 1; ee=100%, Rt=9.31 min c) racemate.

Conversion of optically pure trityl alcohols 2b into radicals 1b

Approximately 0.25 mg of one pure enantiomer of **2b** (Fr1 or Fr2) was dissolved in 1 mL of trifluoroacetic acid (TFA) at 0°C. After 1h30 at 0°C, TFA was removed under reduced pressure and the resulting trityl radical was dissolved in 1.5 mL of EtOAc and the optical purity was controlled by analytical CSP HPLC on a CHIRALPAK® IB column 4.6x250 mm 5µm equipped with a precolumn CHIRALPAK® IB 4.6x20 mm 5µm. The conditions were as follows: flow rate: 1 mL/min; eluent: *i*-hex 93%/EtOAc 4%/MeOH 3% (premixed); UV detection: 485 nm; injection volume: 10 µL; column temperature: 25°C. The enantiomeric excess of the two enantiomers of trityl radical **1b** were 94% and 91% for Fraction 1 and fraction 2, respectively.



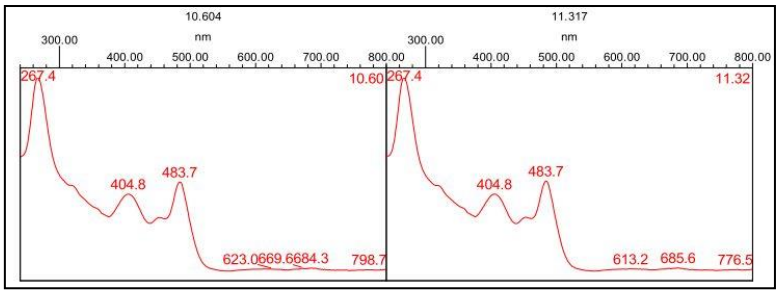


Figure 5: Stacked analytical chromatograms and UV spectra of the highly optically enriched enantiomers of trityl radical **1b** and racemate. a) Fraction 2 of trityl alcohol treated with TFA; ee=91%, Rt=11.32 min, b) Fraction 1 of trityl alcohol treated with TFA; ee=94%, Rt=10.60 min, c) trityl alcohol racemate treated with TFA.

Kinetics of racemization for trityl radical **1b**

The irreversible racemization processes follow a first-order kinetic[p] and thus, the racemization of enantiomers **1b** (Figure S13) has been treated according the following relation:

$$ee = ee_0 e^{-k_{rac}t}$$

Where, ee is the enantiomeric excess

ee_0 is the initial enantiomeric excess

k_{rac} is the rate constant of the racemization

t is the time

k_{rac} was determined by a nonlinear modeling using SAS JMP® 7.0 software.

The relation between the rate constant of racemization (k_{rac}) and enantiomerization (k_{enant}) is:

$$k_{rac} = 2\, k_{enant}$$

The half-lifes of racemization and enantiomerization are given by:

[p] C. Wolf, in *Dynamic Stereochemistry of Chiral Compounds*, RSC Publishing, Cambridge, UK, 2008, ch. 3, pp. 29-36.

$$t_{1/2rac} = \frac{ln\,2}{k_{rac}} = \frac{ln\,2}{2k_{enant}} \text{ and } t_{1/2enant} = \frac{ln\,2}{k_{enant}}$$

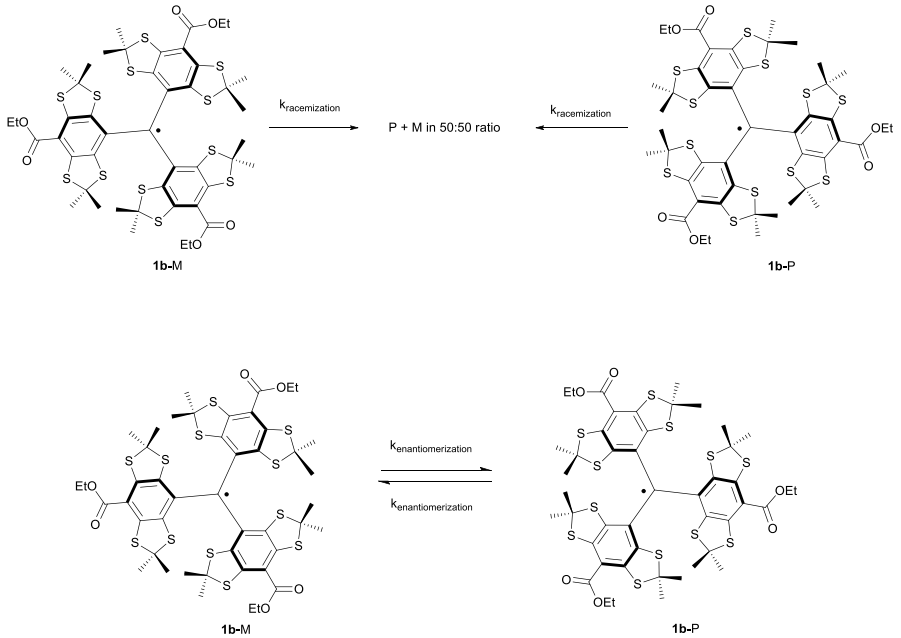


Figure 6

The racemization of trityl radical **1b** in EtOAc at 25°C, 35°C and 40°C was followed by analytical CSP HPLC on a CHIRALPAK® IB column 4.6x250 mm 5µm equipped with a precolumn CHIRALPAK® IB 4.6x20 mm 5µm.

Approximately 0.25 mg of one pure enantiomer of **2b** (Fr1 or Fr2) was dissolved in 1 mL of TFA at 0°C. After 1h30 at 0°C, TFA was removed under reduced pressure and the resulting trityl radical was dissolved in an HPLC vial (VWR™ 1.5 mL, 32 x 11.6 mm) with 1.5 mL of EtOAc at 25°C, 35°C or 40°C. The vial was then put inside the HPLC oven where the sample temperature was controlled to 25°C, 35°C or 40°C. Chromatograms were recorded periodically over the time, the enantiomeric excess was obtained by integration of the two enantiomers at 485 nm. The first chromatogram was considered to be t=0. When necessary, the vial was topped-up with solvent at 25°C, 35°C or 40°C. The conditions were as follows:

flow rate: 1 mL/min; eluent: *i*-hex 93%/EtOAc 4%/MeOH 3% (premixed); UV detection: 485 nm; injection volume: 10 µL; column temperature: 25°C.

Fr1 40°C		**Fr2 40°C**		**Fr2 35°C**		**Fr2 25°C**	
time (min)	ee (%)	time (min)	ee (%)	time (min)	ee (%)	time (min)	ee (%)
0	93.82	0	91.38	0	93.92	0	91.14
169	90.48	169	88.56	104	92.46	108	90.98
338	87.54	338	86.04	238	90.34	428	89.14
537	84.5	537	82.98	566	87.16	868	86.92
796	80.16	796	79.66	760	85.36	1521	84.48
1116	76.68	1115	75.5	918	84.04	2353	81.52
1675	70.04	2433	61.94	1373	79.74	3097	79.14
2440	61.7	3198	54.76	2158	73.72	3846	77.26
3205	53.76	3963	48.38	2782	69.26	4616	75.9
3970	46.9	4728	42.6	3553	64.38	5369	74.02
4736	41.42	5494	37.82	4325	59.24	6123	71.88
5501	36.24	6269	33.62	5096	55.24	6503	71.52
6277	32.12	7035	29.76	5868	51.28	7271	69.56
7042	28.3	7800	26.72	6640	47.96	8025	68.28
7808	24.8	8267	23.8	7412	44.62	8779	66.76
8275	23	9891	19.16	8183	41.46	9532	65.26
9898	18.14	11300	14.52	8859	39.16	9711	65.16
11307	13.32	18389	4.68	9630	36.6	10464	63.38
18396	4.22	/	/	9829	36	11218	62.5
/	/	/	/	12809	26.88	11971	60.4
/	/	/	/	16774	18.82	12646	58.68
/	/	/	/	19953	12.38	13400	57.3
/	/	/	/	22887	8.76	14152	55.94
/	/	/	/	26835	5.76	14906	54.24

/	/	/	/	/	/	15660	53.12
/	/	/	/	/	/	16413	51.26
/	/	/	/	/	/	16594	50.78

Table 1

Fr1 40°C

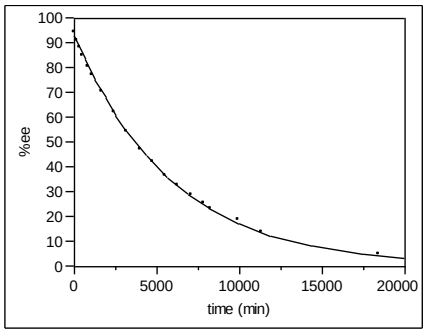


Figure 7

Parameter	Estimate	ApproxStdErr	Lower CL	Upper CL
ee0	92.849821397	0.20392574	92.4195222	93.280662
krac	0.0001696465	8.7453e-7	0.0001678	0.0001715

Fr2 40°C

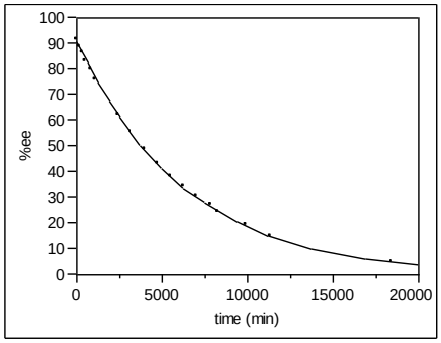


Figure 8

Parameter	Estimate	ApproxStdErr	Lower CL	Upper CL
ee0	90.806728653	0.16624645	90.4545031	91.1592601
krac	0.0001592682	6.78006e-7	0.00015784	0.00016071

Fr2 35°C

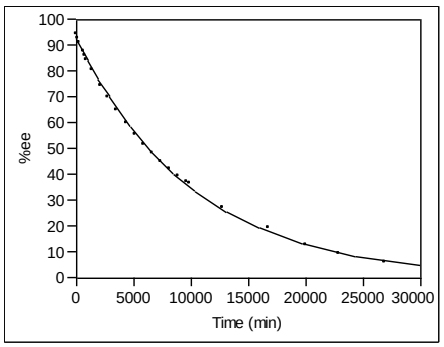


Figure 9

Parameter	Estimate	ApproxStdErr	Lower CL	Upper CL
ee0	92.271367902	0.31723797	91.6137091	92.930463
krac	0.0000985793	8.6452e-7	9.67939e-5	0.00010039

Fr2 25°C

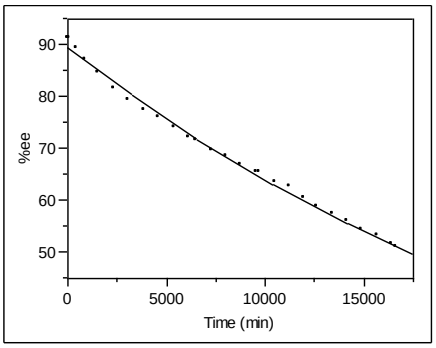


Figure 10

Parameter	Estimate	ApproxStdErr	Lower CL	Upper CL
ee0	89.503665787	0.32548101	88.8331384	90.1754959
krac	0.0000336519	4.5625e-7	3.27113e-5	3.45946e-5

Summary of k_{rac} found at 25°C, 35°C and 40°C

	T (°C)	T(K)	k (min^{-1})	k_{min} (min^{-1})	k_{max} (min^{-1})	k (sec^{-1})	k_{min} (sec^{-1})	k_{max} (sec^{-1})
Fr1	40	313.15	0.00016965	0.0001678	0.0001715	2.8274E-06	2.7967E-06	2.8583E-06
Fr2	40	313.15	0.00015927	0.00015784	0.00016071	2.6545E-06	2.6307E-06	2.6785E-06
Fr2	35	308.15	9.8579E-05	9.6794E-05	0.00010039	1.643E-06	1.6132E-06	1.6732E-06
Fr2	25	298.15	3.3652E-05	3.2711E-05	3.4595E-05	5.6087E-07	5.4519E-07	5.7658E-07

Table 2

Determination of $t_{1/2}$ enantiomerization and $t_{1/2}$ racemization

Racemization								
	T(°C)	T(K)	k (min^{-1})	k_{min} (min^{-1})	k_{max} (min^{-1})	$t_{1/2}$ (min)	$t_{1/2max}$ (min)	$t_{1/2min}$ (min)
Fr1	40	313.15	0.00016965	0.0001678	0.0001715	4086	4131	4042
Fr2	40	313.15	0.00015927	0.00015784	0.00016071	4352	4391	4313
Fr2	35	308.15	9.8579E-05	9.6794E-05	0.00010039	7031	7161	6905
Fr2	25	298.15	3.3652E-05	3.2711E-05	3.4595E-05	20598	21190	20036

Enantiomerization								
	T(°C)	T(K)	k (min^{-1})	k_{min} (min^{-1})	k_{max} (min^{-1})	$t_{1/2}$ (min)	$t_{1/2max}$ (min)	$t_{1/2min}$ (min)
Fr1	40	313.15	8.4823E-05	0.0000839	0.00008575	8172	8262	8083
Fr2	40	313.15	7.9634E-05	0.00007892	8.0355E-05	8704	8783	8626
Fr2	35	308.15	4.929E-05	4.8397E-05	5.0195E-05	14063	14322	13809
Fr2	25	298.15	1.6826E-05	1.6356E-05	1.7297E-05	41195	42380	40073

Table 3

Determination of $\Delta G^{\ddagger}$ according to the Eyring equation

$$k = \frac{k_B T}{h} e^{\frac{-\Delta G^{\ddagger}}{RT}}$$

Where, k is the rate constant

k_B is the Boltzman's constant

T is the temperature

h is the Planck's constant

R is the ideal gas constant

	T (°C)	T (K)	$\Delta G^{\ddagger}$ (kJ mol^{-1})*	$\Delta G^{\ddagger}_{max}$ (kJ mol^{-1})*	$\Delta G^{\ddagger}_{min}$ (kJ mol^{-1})*	$\Delta G^{\ddagger}$ (kcal mol^{-1})	$\Delta G^{\ddagger}_{max}$ (kcal mol^{-1})	$\Delta G^{\ddagger}_{min}$ (kcal mol^{-1})
Fr1	40	313.15	110.0844	110.112885	110.05611	**26.31**	**26.32**	**26.30**
Fr2	40	313.15	110.24875	110.272194	110.22528	**26.35**	**26.36**	**26.34**
Fr2	35	308.15	109.67623	109.72306	109.6296	**26.21**	**26.22**	**26.20**
Fr2	25	298.15	108.69947	108.76975	108.63099	**25.98**	**26.00**	**25.96**

*Calculated by http://www-jmg.ch.cam.ac.uk/tools/magnus/eyring.html

Table 4

Complete enantiomerization pathways for radical 1d

The four potential pathways for enantiomerization of **1d** (the Kurland's flip mechanisms) are depicted in figure 11.

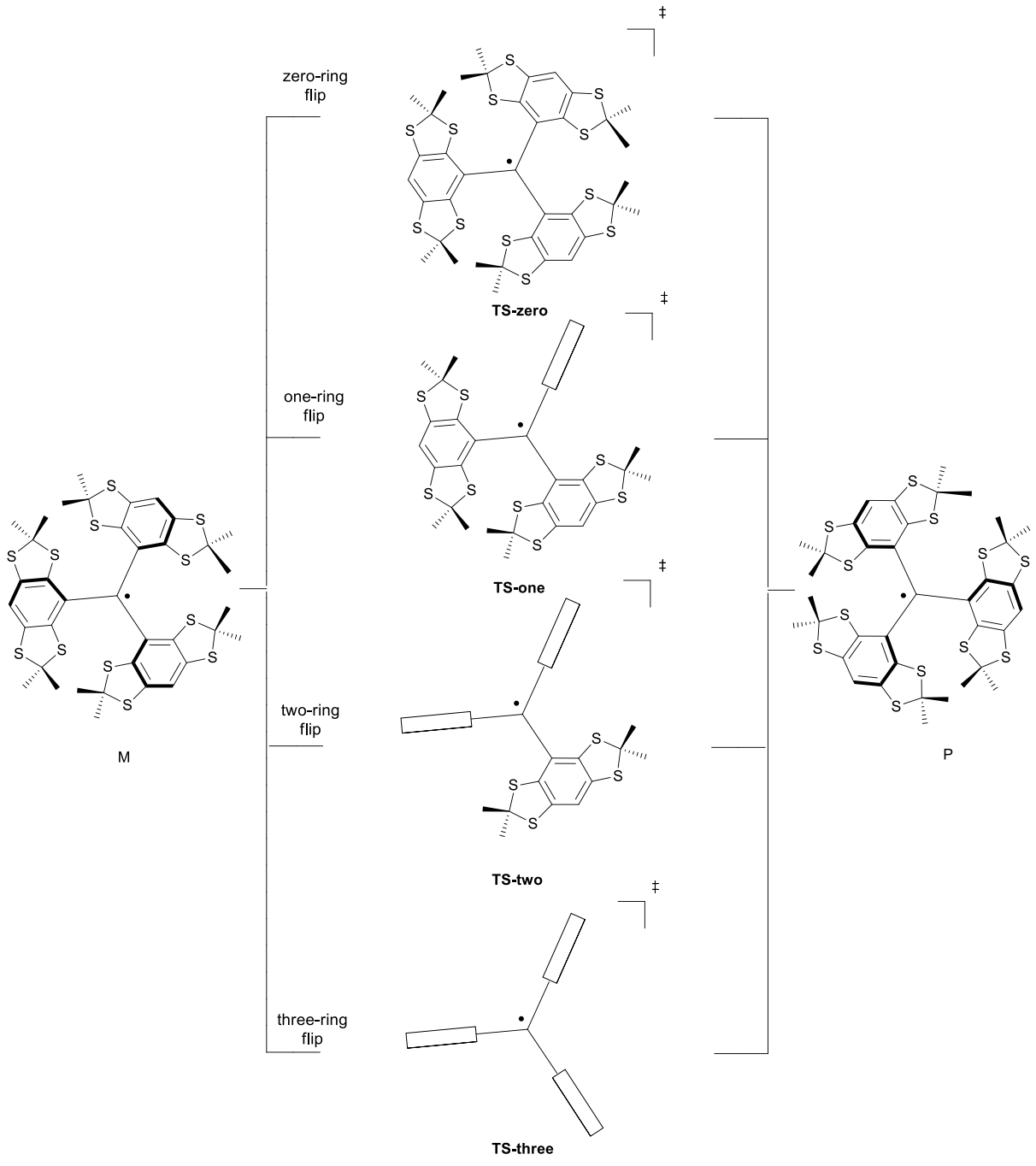


Figure 11: The four potential pathways for enantiomerization on trityl radical **1d**.

Computed optimized geometries and energies

Single point calculations

Energy barriers ($\Delta E^{\ddagger}$) for isomerization of radical **1d** computed at different levels of theory (energies in kcal mol^{-1})

Mechanism	TS-two	TS-three
UB3LYP/6-31G*	28.9	72.0
UB3LYP/6-311+G**	27.2	72.3
UB3LYP-D/6-31G*	25.7	74.2
UB3LYP-D/6-31G**	25.6	74.4
UB3LYP-D/6-311+G**	25.6	74.8
RO-B3LYP-D/6-31G**	26.0	74.5
UBP86/6-31G**	25.5	76.6
LMP2/6-31G*	23.1	59.7

Table 5

Obtained UB3LYP and UB3LYP-D energies indicate that dispersion effects have a rather limited influence on the energy barrier to isomerization.

Obtained energies varying the basis set reveal no significant basis set effects.

The low variation in energy barriers observed with the various methods supports the appropriateness of the chosen methodology to describe the studied system.

Obtained RO-B3LYP-D energies and $\langle S^2 \rangle$ values (always within 3% of the expected s(s+1) values) indicate that spin contamination is negligible in our calculations.

Radical 1d

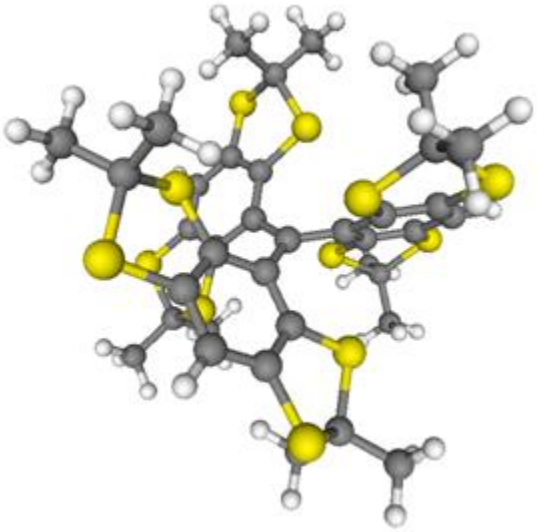


Figure 12: Optimized structure of **1d** (UB3LYP/6-31G*)

E(UB3LYP-D/6-31G*) = -6210.110014

E(UB3LYP-D/6-31G*(AcOEt)) = -6210.128250

E(UB3LYP-D/6-31G**) = -6210.163852

E(UB3LYP-D/6-311+G**) = -6210.163824

E(RO-B3LYP-D/6-31G**) = -6210.161620

E(UB3LYP/6-31G*) = -6211.704131

E(UB3LYP/6-311+G*) = -6212.3669944

E(UBP86/6-31G**)=-6211.864957

E(LMP2/6-31G*)= -6200.030085

TS-two for enantiomerization of radical 1d

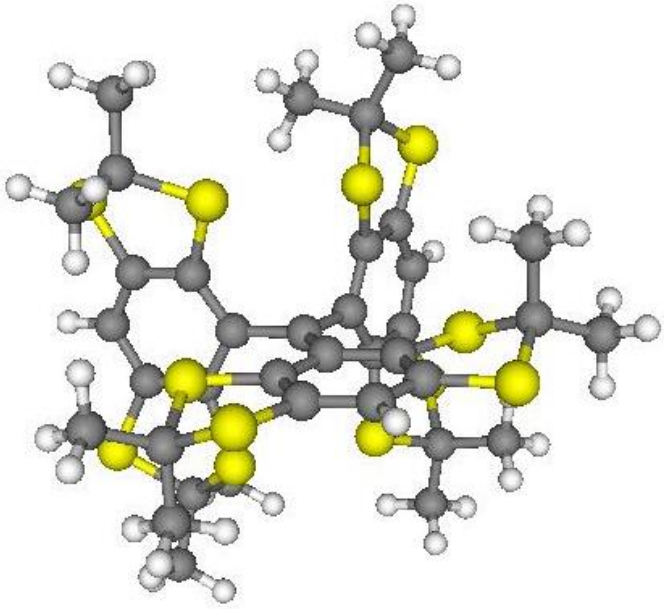


Figure 13: Optimized two-ring flip TS for radical **1d** (UB3LYP/6-31G*)

E(UB3LYP-D/6-31G*) = -6210.069052

E(UB3LYP-D/6-31G*(AcOEt)) = -6210.085014

E(UB3LYP-D/6-31G**) = -6210.122962

E(UB3LYP-D/6-311+G**) = -6210.122962

E(RO-B3LYP-D/6-31G**) = 26.032059

E(UB3LYP/6-31G*) = -6211.658027

E(UB3LYP/6-311+G*) = -6212.323687

E(UBP86/6-31G**)= -6211.824249

E(LMP2/6-31G*)= -6199.993303

TS-three for enantiomerization of radical 1d

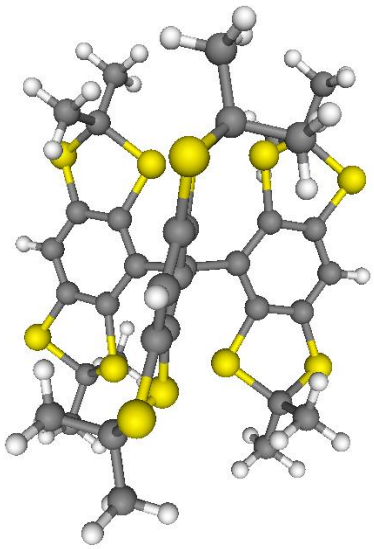


Figure 13: Optimized three-ring flip TS for radical **1d** (UB3LYP/6-31G*)

E(UB3LYP-D/6-31G*) = -6209.991736

E(UB3LYP-D/6-31G*(AcOEt)) = -6210.006901

E(UB3LYP-D/6-31G**) = -6210.045749

E(UB3LYP-D/6-311+G**) = -6210.044629

E(RO-B3LYP-D/6-31G**) = -6210.042829

E(UB3LYP/6-31G*) = -6211.589339

E(UB3LYP/6-311+G*) = -6212.251843

E(UBP86/6-31G**)= -6211.742808

E(LMP2/6-31G*)= -6199.934930

Radical 1c

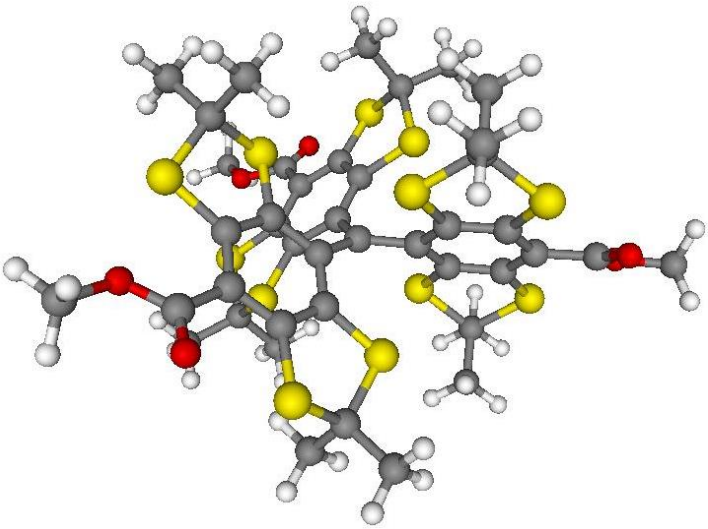


Figure 14: Optimized structure of radical **1c** (UB3LYP/6-31G*)

E(UB3LYP-D/6-31G**) = -6893.470095

E(UB3LYP-D/6-311+G**) = -6893.470095

E(UB3LYP/6-31G*) = -6895.321938

TS-two for enantiomerization of radical 1c

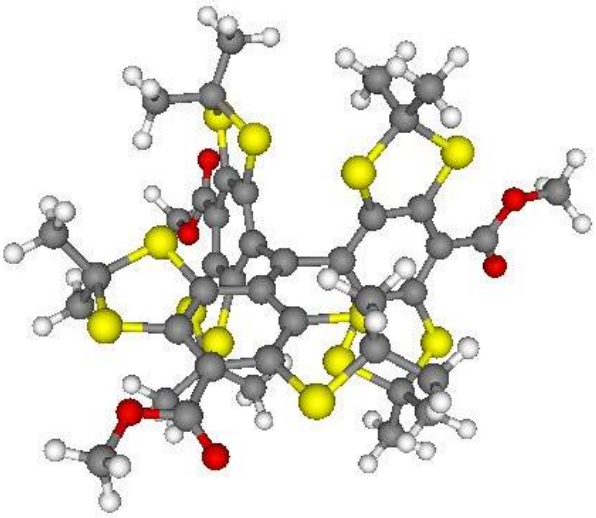


Figure 15: Optimized two-ring flip TS for radical **1c** (UB3LYP/6-31G*)

E(UB3LYP-D/6-31G*) = -6893.364650

E(UB3LYP-D/6-311+G**) = -6893.425740

E(UB3LYP/6-31G*) = -6895.273954

Circular dichroism spectra of radical 1b

The CD spectra of the two enantiomers **1b** were recorded by stopped-flow spectrometry on a CHIRALPAK® IB column 4.6x250 mm 5µm equipped with a precolumn CHIRALPAK® IB 4.6x20 mm 5µm. The conditions were as follows: flow rate: 1 mL/min; eluent: *i*-hex 93%/EtOAc 4%/MeOH 3% (premixed); UV and CD detection: 267 nm. The flow was stopped at the maximum of the CD signal for each enantiomer and the circular dichroism spectra were recorded from 220 to 440 nm with a Jasco CD-2095 Plus chiral HPLC detector. As expected for two enantiomers, the two spectra are mirror images.

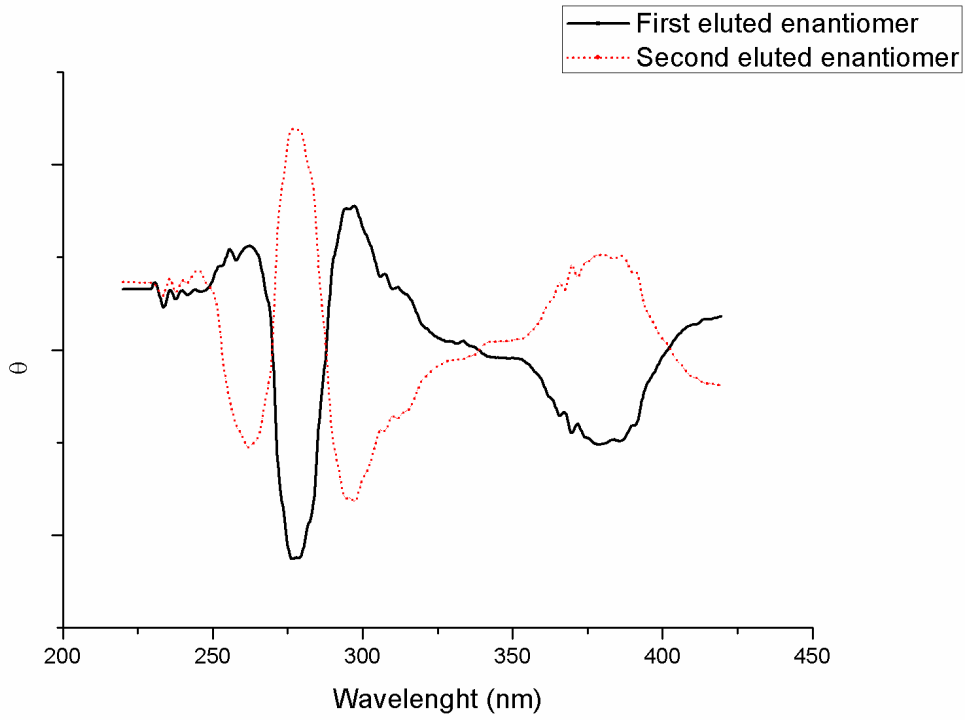


Figure 16: CD spectra of enantiomers **1b**.

Conversion of alcohol 2b to radical 1b with neat TFA

Tetrathiatriarylmethyl radical **1b** has been synthesized from the corresponding alcohol **2b** by treatment with neat trifluoroacetic acid (TFA). This unclassical but straightforward method is widely used for quantitative conversion of a tetrathiatriarylmethyl alcohol into radical.[q] However, the mechanistic aspects of this transformation remain unknown.

5.5 mg of **2b** was dissolved in 2 mL of neat TFA and stirred for 2h. TFA was then removed under reduced pressure and the dark green residue dissolved in 1 mL of EtOAc, the EPR spectrum was then recorded and the quantitative conversion was check by RP HPLC.

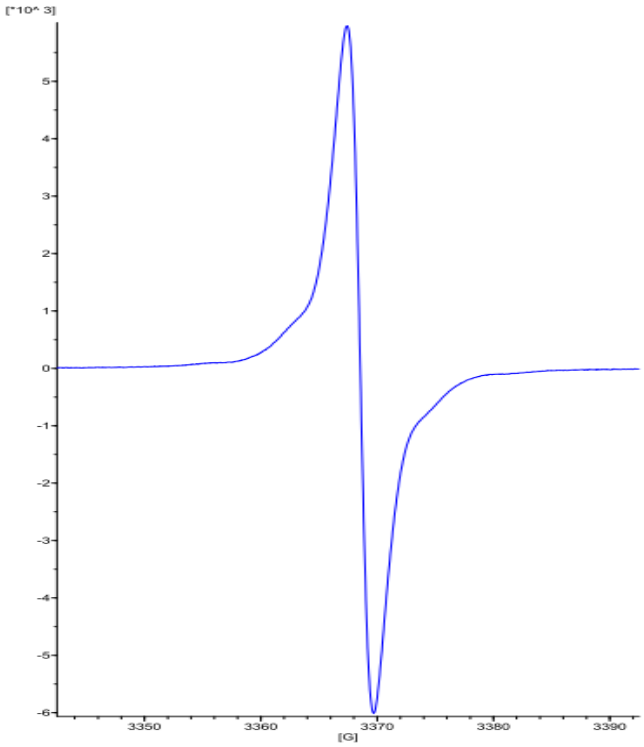


Figure 17: X-Band EPR spectrum of radical **1b** in EtOAc. The acquisition settings were; sweep width: 50 G, center field: 3367.5 G, resolution: 1024 pts, power: 1.269 mW, modulation

[q] (a) I. Dhimitruka, M. Velayutham, A. A. Bobko, V. V. Khramtsov, F. A. Villamena, C. M. Hadad and J. L. Zweier, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 6801-6805. (b) A. A. Bobko, I. Dhimitruka, J. L. Zweier and V. V. Khramtsov, *J. Am. Chem. Soc.*, 2007, **129**, 7240-7241.(c) I. Dhimitruka, A. A. Bobko, C. M. Hadad, J. L. Zweier and V. V. Khramtsov, *J. Am. Chem. Soc.*, 2008, **130**, 10780-10787. (d) Y. Liu, F. A. Villamena, J. Sun, Y. Xu, I. Dhimitruka and J. L. Zweier, *J. Org. Chem.*, 2008, **73**, 1490-1497. (e) A. A. Bobko, I. Dhimitruka, T. D. Eubank, C. B. Marsh, J. L. Zweier and V. V. Khramtsov, *Free Radical Biol. Med.*, 2009, **47**, 654-658.

frequency: 10 kHz, modulation amplitude: 0.25 G, conversion time: 20.48 ms, time constant: 20.48ms.

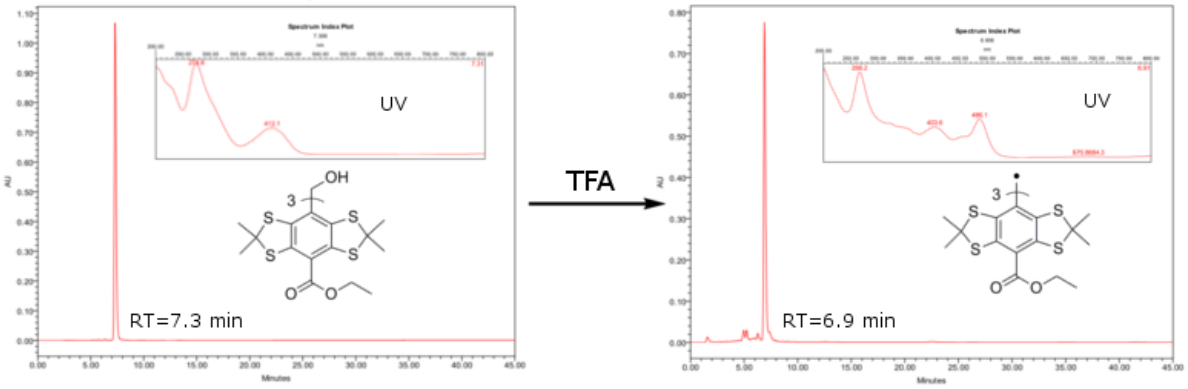


Figure 18: RP HPLC Chromatograms of **2b** and **1b**. The conditions were as follows: Column Symmetry C18 (4.6 x 250 mm) 5 µm, ACN100%, 1mL/min, 25°C, 254 nm.

[33]

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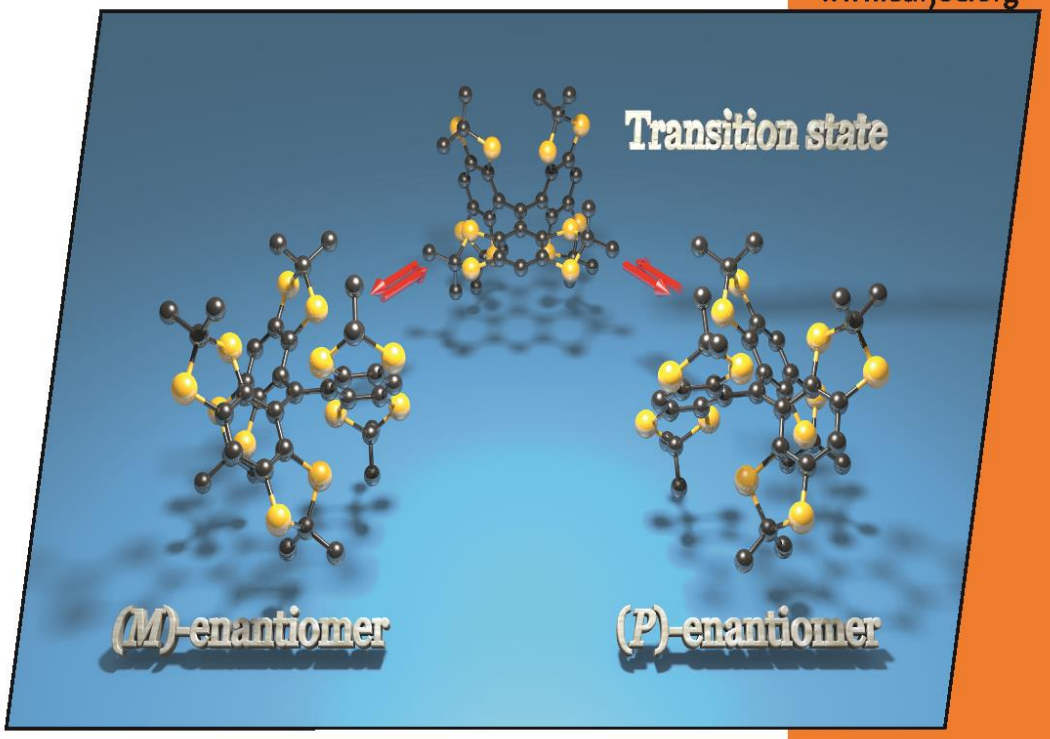

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Configurationally Stable Tetrathiatriarylmethyl Molecular Propellers

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Abstract: Tetrathiatriarylmethyl alcohols and alkanes are direct precursors and metabolites, respectively, of tetrathiatriarylmethyl radicals which are very attractive paramagnetic spin probes used for many magnetic resonance applications. Due to steric hindrance, these propeller-shaped molecules display slow interconversion between their two enantiomeric conformations (right-handed and left-handed propeller conformations). Syntheses and HPLC resolutions carried out on a chiral stationary phase have been achieved. The influence of hybridization of the central carbon as well as the *para*-substitution on the conformational stability were experimentally and theoretically studied. Depending on their particular structure, the free activation energy for the isomerization process determined by kinetic of racemization varies from 24.4 to 26.4 kcal.mol^{-1} and corresponds to racemization half-lifes of 12 h and 343 h, respectively. Computational and NOESY/EXSY spectroscopy studies are consistent with a two-ring flip mechanism for the isomerization process of tetrathiatriarylmethyl alcohols, radicals and alkanes. Introduction

Stable tetrathiatriarylmethyl radicals are members of a family of trityl radicals which have found many magnetic resonance (MR) applications during the last decade. Their

structures were inspired by the Gomberg's trityl radical in which each phenyl group has been ortho- and meta- substituted by sulphur atoms and *para*-substituted by a carboxylic acid (Figure 1). These modifications were made in order to avoid hydrogen hyperfine splittings, enhance stability by electronic and steric effects and finally for making these molecules water soluble. These radicals, initially developed by Nycomed Innovation to be used as hyperpolarizing agents for Overhauser magnetic resonance imaging (OMRI),[1] possess exceptional properties. They exhibit a single sharp EPR line of less than 10 µT in deoxygenated water which is typically one order of magnitude less than nitroxide radicals which represent the second class of soluble spin probes used for biomedical EPR. As a result, the sensitivity of detection is very high since the signal intensity is inversely proportional to the square of the linewidth. In addition, depending on their particular structure, the half-life in human blood of tetrathiatriarylmethyl radicals at 37°C varies from few hours to more than 24 h. Finally, they display a low concentration-dependent broadening of their EPR line.[2, 3] Due to these unprecedented properties, original Nycomed's and newly synthesized tetrathiatriarylmethyl radicals have been used as spin probes for electron paramagnetic resonance imaging (EPRI)[4], for the measurement of oxygen[5], pH[6, 7], thiol[8], superoxide anion[9], redox status[10] and for dynamic nuclear polarization (DNP).[11]

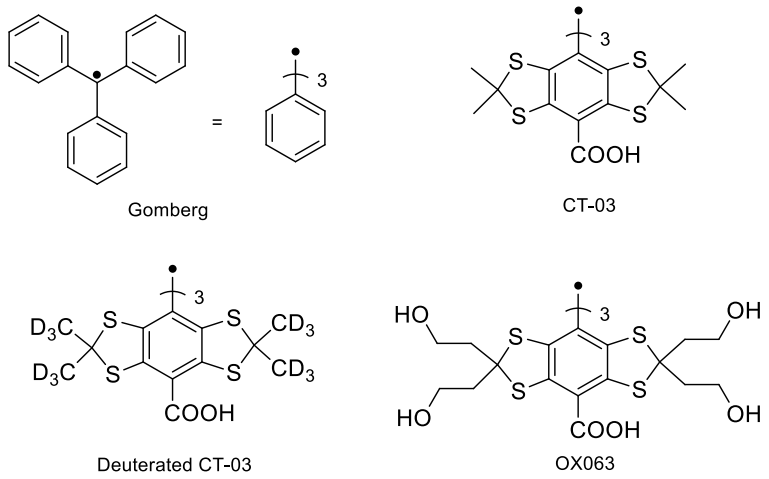


Figure 1: Gromberg's trityl and representative Nycomed's tetrathiatriarylmethyl radicals of biomedical uses.

The maximum of resonance would be in favour of a planar conformation. However, due to steric hindrance, trityl radicals adopt a propeller like conformation in which all rings have the same direction of twist. This conformation was suggested by G.R. Lewis *et al.* in 1944 based on the UV spectrum[12] and confirmed later by other techniques (X-Ray[13], EPR[14], etc.). Tetrathiatriarylmethyl radicals are sterically crowded with two five-membered rings on each phenyl rings. This results in a high twist angle, lowering the conjugation of the central carbon bearing consequently most of the spin density of the unpaired electron.[3] The X-Ray structure of **1b**[15] which is the methyl ester derivative of **CT-03** (Figure 2) shows that the rings are twisted by 45.6°, relative to the plane of the central carbon and its three bonds. This arrangement allows two conformations to occur, namely the right-handed (*P*) and left-handed (*M*) propeller conformations (Figure 2).

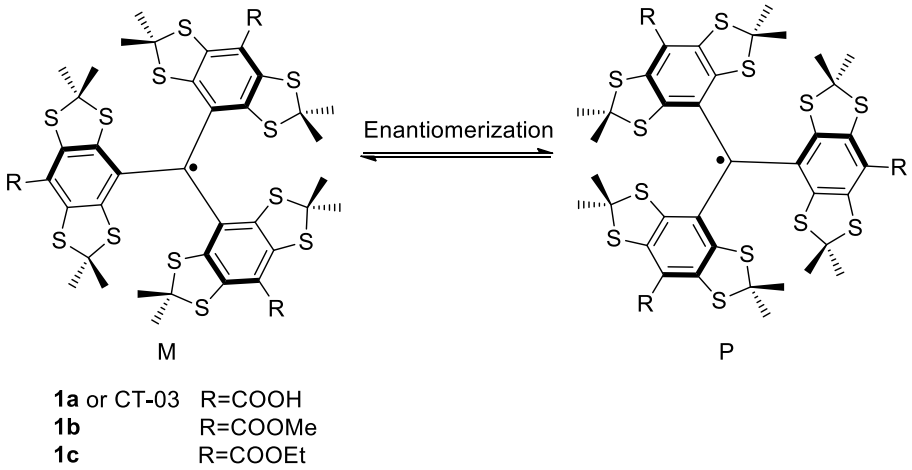
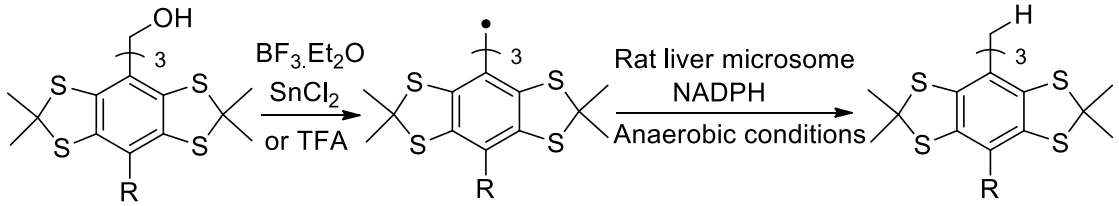


Figure 2: The two enantiomeric conformations of tetrathiatriaylmethyl radicals.

These two conformations feature enantiomeric relationship. The study of static and dynamic stereochemistry of three-bladed propellers has been launched by Mislow and co-workers more than 30 years ago.[16] In most cases three-bladed propellers have a low isomerization barrier which prevents the separation of stereoisomers. However, a few examples of configurationally stable three-bladed propellers bearing bulky aromatic moieties have been reported in the literature and stereoisomers could be isolated in optically pure form in some cases.[17, 18] Recently, we reported that tetrathiatriarylmethyl radicals and their direct diamagnetic precursor tetrathiatriarylmethanols[19] (Figure 3), are chiral molecules at room temperature. The propeller shape conformation associated with a

slow interconversion was responsible for this chirality. Semi-preparative HPLC carried out on a chiral stationary phase (CSP) allowed isolation of the two enantiomers of radical **1c** and alcohol **2c**. The half-live of racemization determined for radical **1c** in EtOAc at room temperature was *ca* 15 days. This result could have important consequences because enantiomers can exhibit different biological properties; furthermore diastereoisomers are formed if a chiral group are connected to the trityl core[10, 20] with possibly different physical, biological and chemical properties.

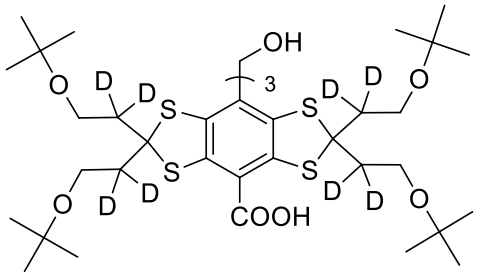
Very recently V.M. Tormyshev *et al.* used exchange spectroscopy (NOESY/EXSY) to study the enantiomerization of tetrathiatriarylmethanols **2d** and **2e** (Figure 3), the half life of racemization reaching 8.4 h at room temperature for **2d** in [d_6]-DMSO while a high influence of the steric hindrance of the side-ring was observed for **2e**.[21] This compound shows a higher conformational stability, the racemization half-live reaching 1.36 years in the same conditions. However, no information has been reported concerning the influence of *para*-substitution and the enantiomerization of tetrathiatriarylmethanes, which are metabolites of radicals produced under reducing conditions.[22]



2a R=COOH
2b R=COOMe
2c R=COOEt
2d R=H

1a R=COOH
1b R=COOMe
1c R=COOEt
1d R=H

3a R=COOH



2e

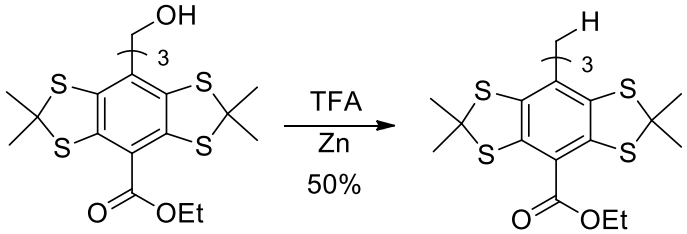
Figure 3: Structures of few tetrathiatriarylmethyl alcohols, radicals and alkane.

Herein, we report studies on the dynamic stereochemistry of propeller shaped tetrathiatriarylmethyl radicals, alcohols and methanes. DFT and experimental approaches are used in order to gain insight into the nature of the mechanism of stereoisomerization. The influence of *para*-substitution and hybridization of the central carbon are also discussed.

Results and Discussion

Synthesis

The synthesis of compounds **1** and **2** has already been reported.[15, 21, 23, 24] Compound **3c** has been synthesized by reduction of the corresponding alcohol **2c** using trifluoroacetic acid (TFA) and metallic zinc (Figure 4) and was isolated in 50% yield after purification by HPLC. It is known that tetrathiatriarylmethanols are quantitatively converted into radicals by TFA,[24] the radical being subsequently reduced by Zn; the resulting anion is protonated by TFA leading to the tetrathiatriarylmethane **3c**.



2c **3c**

Figure 4: Synthesis of **3c**.

Propeller shape conformation

^{1}H NMR spectra of diamagnetic tetrathiatriarylmethyl alcohols **2** or methanes **3** show, except for accidental isochrony, four resonances associated to the methyl groups of the thioacetonide moiety. The non equivalence of the four methyl groups is consistent with a propeller shape conformation featuring a C_3 symmetry. In this conformation, the three aryl groups are equivalent but the four methyl groups of each aryl group are magnetically different as they are localized in different spatial environments. The same observation can be made on the ^{13}C NMR spectra where four resonances are observed for the methyl groups, six for the phenyl rings and two for the quaternary carbon of the thioacetonide.

Crystallization from THF afforded crystals of alcohol **2d** suitable for X-Ray diffraction. The structure reveals a propeller-like conformation and two molecules of THF co-crystallized (Figure 5). As expected the right- and the left-handed propellers are present in the crystal. The three aromatic moieties are twisted in the same direction; however, the structure lacks an exact 3-fold symmetry. The three dihedral angles θ_1-θ_3, defined as the angle between the two nearest C_{ipso}-C_{ortho} bonds of two adjacent rings, are different (Table 1). A difference in the bond length between the central carbon and the three aromatic rings is also observed. In addition, the two five-membered rings connected to the phenyl group are twisted in the opposite direction for two of the three phenyl rings, while they are twisted in the same direction for the last one.

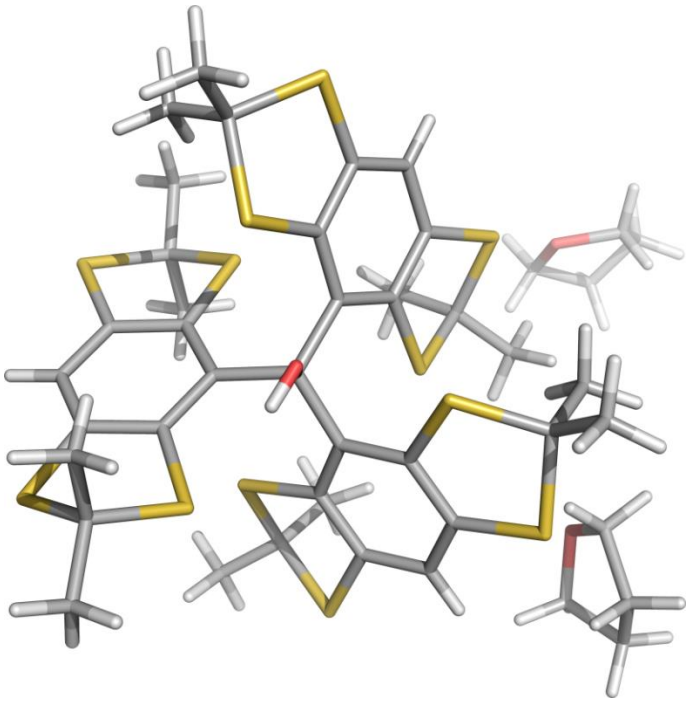


Figure 5: Pymol view of X-Ray structure of **2d**.

The X-Ray data are available in the literature for the paramagnetic derivatives **1b**[15] and **1e.**[25] While the methyl ester derivative **1b** exhibits a strict C_3 rotation axis, the nitro derivatives **1e** partly diverge from a C_3 symmetry as the phenyl rings are twisted with three different angles (Table 1). The three bonds connecting the phenyl rings to the central carbon also have a slightly different length. However all the dihedral angles θ_1-θ_3 have the same sign which reveals a propeller conformation.

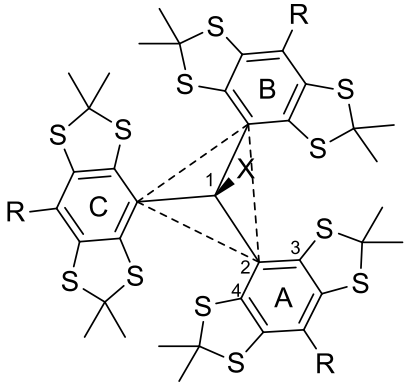


Table 1: Crystallographic data

Cpd	R, X	θ_1 (C_{A3}-C_{A2}-C_{B2}-C_{B4}) θ_2(C_{B3}-C_{B2}-C_{C2}-C_{C4}) θ_3(C_{C3}-C_{C2}-C_{A2}-C_{A4})	d(C_1-C_{2A}) d(C_1-C_{2B}) d(C_1-C_{2C})	Ref.
2d	R=H	-70.7(4)°	1.550(6) Å	
	X=OH	-69.9(4)°	1.546(6) Å	
		-75.3(4)°	1.561(5) Å	
1b	R=COOMe	-74.0(3)°	1.449(3) Å	[15]
	X=single electron	-74.0(3)°	1.449(3) Å	
		-74.0(3)°	1.449(3) Å	
1e	R=NO_2	-72.3(4)°	1.450(7) Å	[25]
	X=single electron	-86.6(4)°	1.468(6) Å	
		-71.9(4)°	1.461(6) Å	

Resolution by CSP HPLC

The resolution of two peaks for tetrathiatriarylmethyl radical **1c** and alcohol **2c** on HPLC carried on a cellulose carbamate chiral stationary phase (CHIRALPAK IB) was the first experimental proof of the chirality of tetrathiatriarylmethyl compounds (Figure 6).[26] The resolution for tetrathiatriarylmethane **3c** is also possible at room temperature (Figure 6), indicating that there is no rapid isomerization on the HPLC timescale for the reduced derivative either. Resolutions of ethyl ester derivatives (R = COOEt) **1c**, **2c** and **3c** were achieved using a cellulose carbamate chiral stationary phase (CHIRALPAK IB) but this column failed to separate the enantiomers of the unsubstituted derivatives **2d** (R = H). The

resolution of compound **2d** was in fact achieved using an amylose carbamate chiral stationary phase (CHIRALPAK IA) (Figure 6).

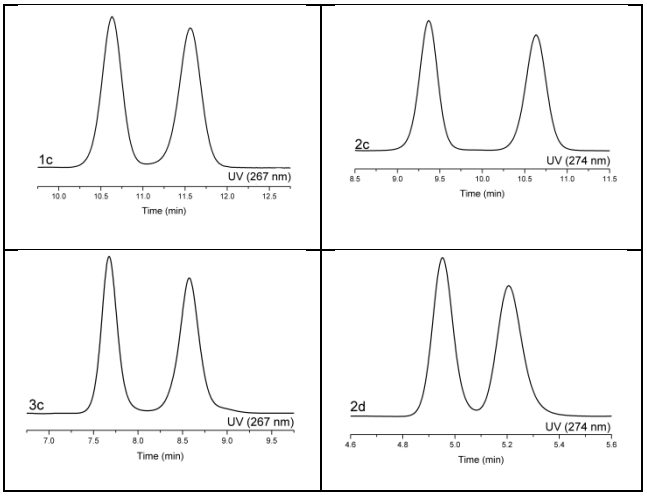


Figure 6: CSP-HPLC chromatograms with UV detection. Resolution was carried out on a CHIRALPAK IB column for compounds **1c**, **2c**, **3**c and on a CHIRALPAK IA column for **2d**.

In order to further evaluate the influence of hybridization of the central carbon and substitutions on the conformational stability, semi-preparative resolution of tetrathiatriarylmethanols **2c** and **2d** and tetrathiatriarylmethane **3c** was undertaken. A few milligrams of each enantiomer of alcohol **2c** was obtained with an enantiomeric ratio (e.r.) of 100:0 using a semi-preparative HPLC (CHIRALPAK IB 10 x 250 mm, 5 µm) accordingly to a procedure previously reported.[26] The semi-preparative HPLC resolution of **3c** affords the two optically enriched enantiomers in milligrams scale. The first eluted enantiomer was isolated with an e.r. of 88.4:11.6 while the second eluted enantiomer was isolated with an e.r. of 94.0:6.0 using a semi-preparative column (CHIRALPAK IB 10 x 250 mm, 5 µm). The amount of each enantiomer that can be isolated is limited by the low solubility of **2c** and **3c** in the eluent. The resolution of unsubstituted alcohol **2d** was carried on an analytical column (CHIRALPAK IA 4.6 x 250 mm, 5 µm). Half a milligram of each of the two enantiomers was isolated with an enantiomeric ratio of 85.6:14.4 for both enantiomers.

Determination of configurational stability

A free activation energy of $\Delta G^{\ddagger}_{(25°C)}$=26.4 kcal.mol^{-1} for the enantiomerization process of radical **1c** was determined by following kinetics of racemization after isolation of enantiomers.[26] This value is sufficiently high to store independently the two enantiomers for months in the freezer. The kinetics of resolution have been extended to alcohols **2c** and **2d** and to alkane **3c** in order to determine the influence of the nature of central carbon and the influence of aromatic substitution. The racemization was followed by HPLC at 25°C in EtOAc for each compound (Figure 7). The racemization rates were determined by fitting the experimental decay of enantiomeric excess (% ee) according to equation 1.

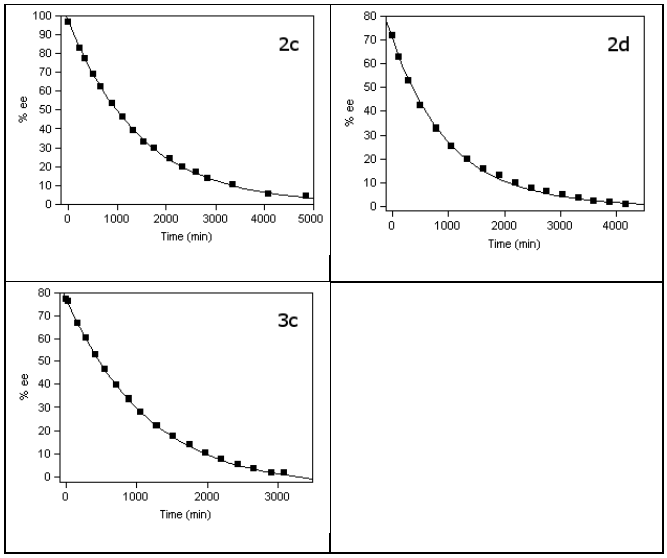


Figure 7: CSP-HPLC kinetics of racemization for compounds **2c**, **2d**, **3c**.

$$\%ee = \%ee_0 \; e^{k_{rac}t} \qquad (1)$$

Table 2: Kinetic parameters and enantiomerization free activation energy for compounds **1c**, **2c**, **2d**, **3c** at 25°C.

Cpd	$k_{rac.}$ (h^{-1})	$t_{(1/2)\ rac.}$ (h^{-1})	$k_{enant.}$ (h^{-1})	$\Delta G^{\ddagger}_{enant.}$ (kcal.mol^{-1})	**Method**
1c[a] (in EtOAc)	0.0020	343.3	0.0010	26.4	HPLC
2c (in EtOAc)	0.0410	16.9	0.0205	24.6	HPLC
2d[b] (in [d_6]DMSO)	0.0823	8.4	0.0411	24.2	NOESY/EXSY
2d (in EtOAc)	0.0572	12.1	0.0286	24.4	HPLC
3c (in EtOAc)	0.0513	13.5	0.0257	24.5	HPLC

[a]Ref[26]. [b] Ref[21].

The racemization and enantiomerization processes are closely linked. The racemization corresponds to a macroscopic and non-reversible process leading to an enantiomeric excess of 0 % which is the thermodynamic equilibrium. The enantiomerization is related to a microscopic and reversible process which is completed when all of the molecules have been converted into the other enantiomer. The relationship between the rate constants is $k_{rac.} = 2\ k_{enant}$. The experimental rates of racemization are very close for all diamagnetic compounds **2c**, **2d**, and **3c** indicating that there is no strong influence of the *para*-substitution (H or COOEt) or of the nature of the group connected to the central carbon (H or OH) on the isomerization process. The half-life of racemization in EtOAc varies from 12.1 h for **2d** to 16.9 h for **2c**, the rate of **2d** in EtOAc found by CSP-HPLC being in good agreement with the rate found by V.M. Tormyshev *et al.* in dimethylsulfoxide using exchange spectroscopy[21]. However, the rate of isomerization is one order of magnitude lower for the paramagnetic derivative **1c**.

Determination of the isomerization mechanism by exchange spectroscopy

The isomerization for three-bladed propellers can be described by the so-called Kurland's flip mechanism.[16] There are four pathways leading to helicity reversal, namely, the zero-, one-, two- or three-ring flips. These mechanisms involve a conrotatory rotation of zero, one, two or three aromatic rings across a plane perpendicular to the one formed by the three C_{ipso} (the reference plane) while the other rings rotate in the opposite direction, through the reference plane (Figure 8). The two-ring flip is considered as the lower energy mechanism for the helicity reversal, however, exceptions exist.[27]

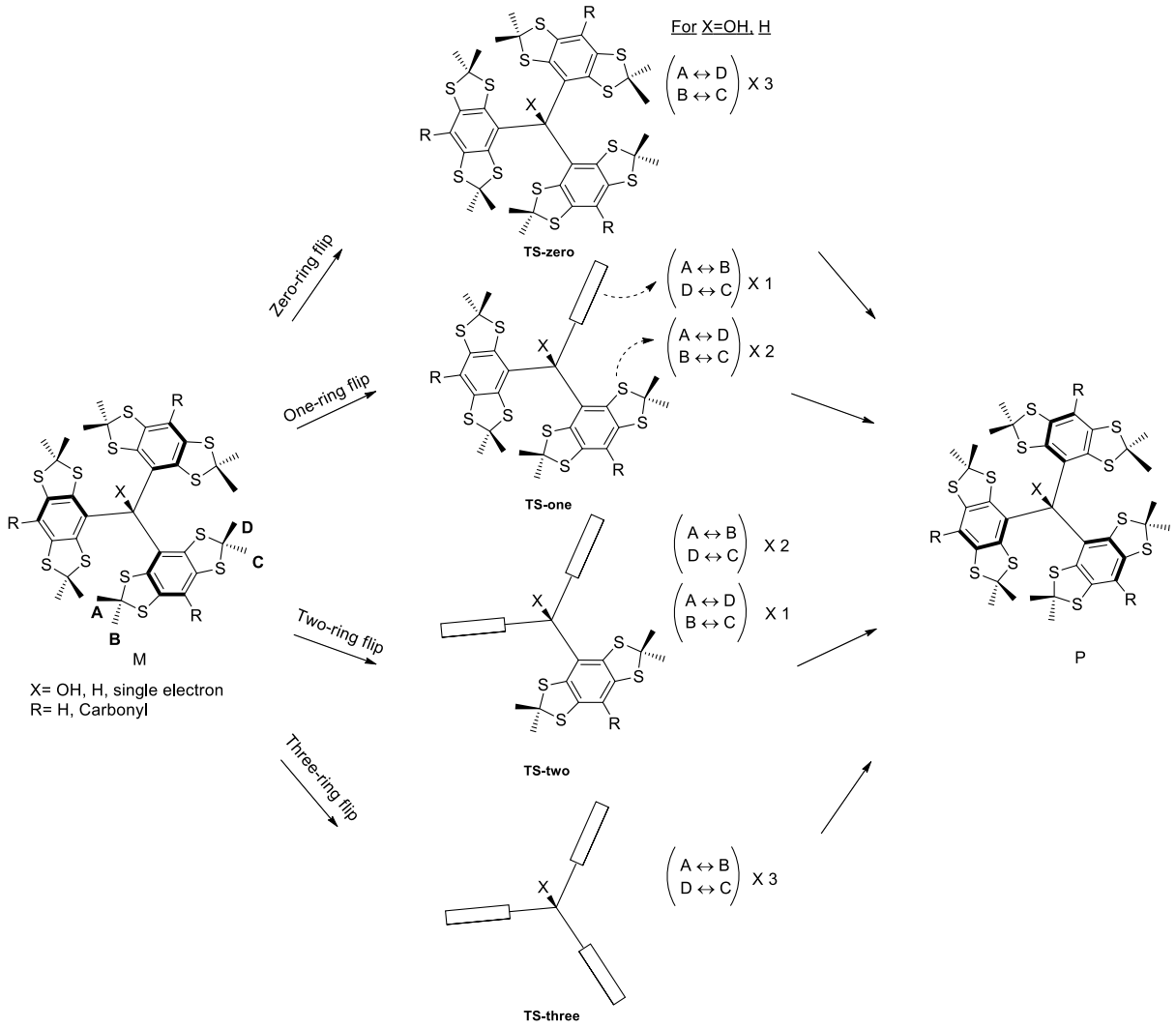


Figure 8: the n-ring flip mechanisms and exchanged positions.

In the case of a tetrahedral configuration of the central carbon for diamagnetic compounds **2** (X=OH) and **3** (X=H), the four methyl groups (A, B, C, D, figure 8) are localized in different spatial environments, resulting in four distinct peaks in the ^{1}H and ^{13}C NMR spectra. Degeneracy arises for the radical derivatives **1**, the planar geometry of the central carbon making the methyl groups A equivalent to C and methyl groups B equivalent to D. Analysis of isomerization mechanisms for tetrathiatriarylmethyl derivatives **2** and **3** shows an exchange of the position of methyl groups A and B and an exchange of position of methyl groups D and C when the rings rotate through the plane perpendicular to the reference plane while the rotation in the opposite direction involves an exchange of position of methyl groups A and D and methyl groups B and C. Tormyshev *et al.* used exchange spectroscopy to determine the ratio of flipping rings passing through the plane perpendicular to the reference plane to rings flipping through the reference plane[21]. A ratio of 2:1 was determined for tetrathiatriarylmethanol **2d** which corresponds to a two-ring flip mechanism.
A similar strategy to determine the mechanism of isomerization of tetrathiatriarylmethane **3c** was used. The ^{1}H NMR spectrum of **3c** at 500 MHz shows four resonances for the methyl groups in deuterated chloroform but only three in [d_6]-DMSO because of accidental isochrony of two methyl groups. However, by increasing the temperature, a small change in peak frequency is observed leading to complete resolution of the methyl groups. Correlations on 2D ^{1}H-^{13}C HMBC spectrum reveals that methyl groups 1 and 2 (Figure 9) are attached to the same carbon; in the same fashion methyl groups 3 and 4 are attached to the same carbon.

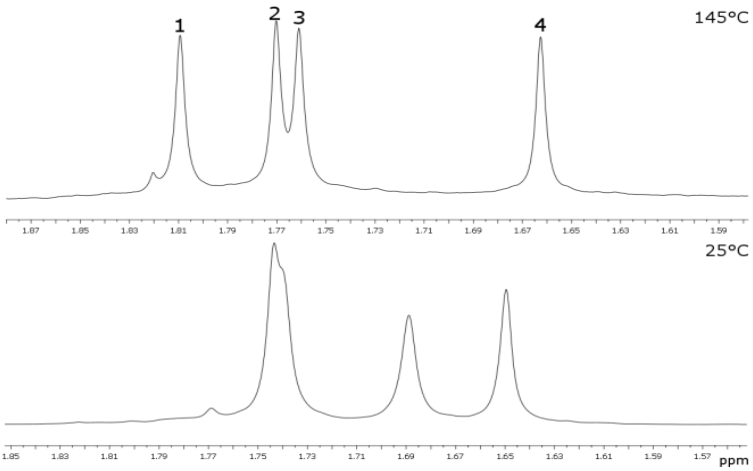


Figure 9: Zoom on the methyl groups region of **3c** in [d_6]-DMSO at 25°C and 145°C (500 MHz).

A high resolution 2D ^{1}H-^{1}H NOESY/EXSY spectrum was recorded at 145°C in [d_6]-DMSO on a spectrometer operating at 500 MHz for ^{1}H, with mixing time of 600 ms. The acquisition region was centered on the methyl groups with a spectral width of 0.5 ppm in both F1 and F2 dimensions. On such spectrum, cross-peaks that have the same sign as the diagonal are chemical exchange peaks (EXSY) while cross-peaks that have a sign opposite to the diagonal are nOe peaks (NOESY). At 145°C, 8 correlations with the same sign as the diagonal are observed corresponding to chemical exchange (Figure 10). The 2D NOESY/EXSY spectrum being symmetrical across the diagonal, the same information is found twice on such spectrum, so it is only essential to describe 4 of the 8 correlations. First of all, an EXSY cross-peak is observed between geminal methyl groups 4 and 3 with an intensity of 2 while an EXSY cross-peak is observed between methyl groups 4 and 1 with an intensity of 1. Furthermore, two EXSY cross-peaks are obtained for methyl group 3, one with methyl group 4 (intensity of 2) and one with methyl group 2 (intensity of 1). For each methyl group, there is exchange between its geminal position with intensity of 2, which corresponds to a rotation across the plane perpendicular to the reference plane and exchange with a position on the opposite side of the aryl moiety with intensity of 1 which corresponds to the rotation through the reference plane. The only mechanism that fits these observations is the two-ring flip mechanism (see Figure 8).

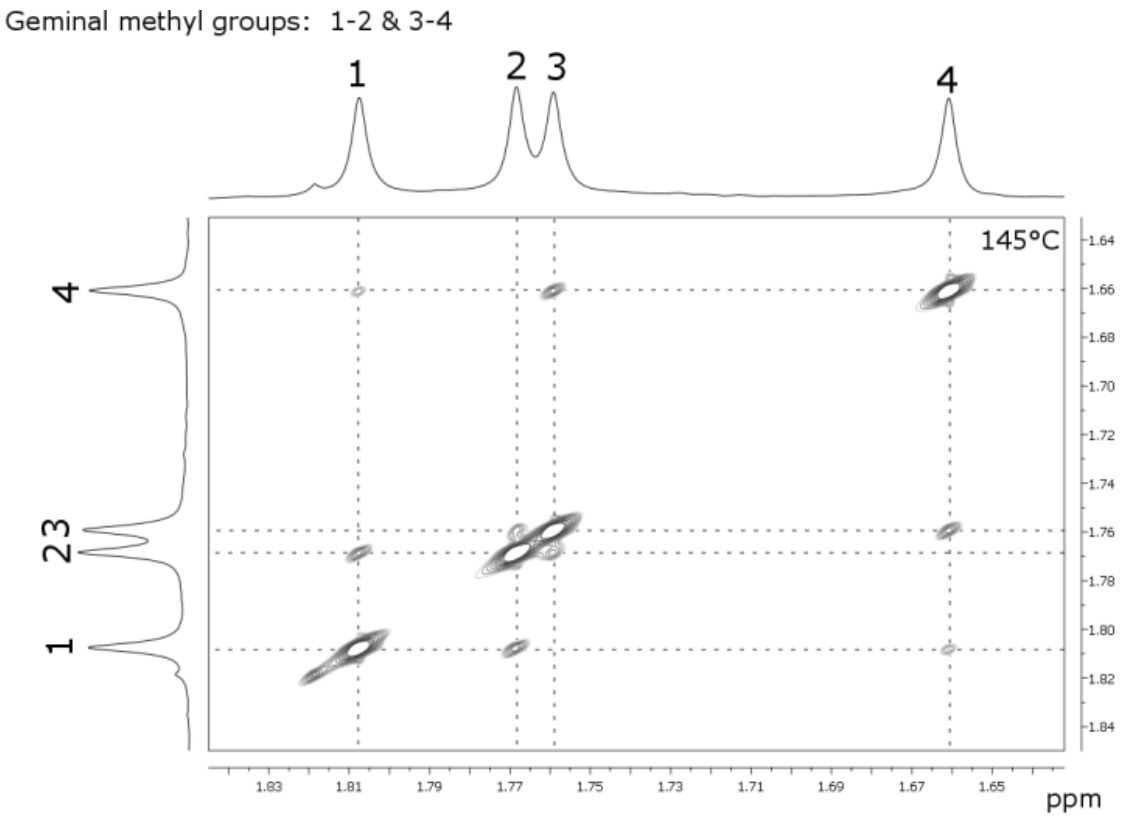


Figure 10: 2D ^{1}H-^{1}H NOESY/EXSY spectrum of **3c** at 145°C (500 MHz).

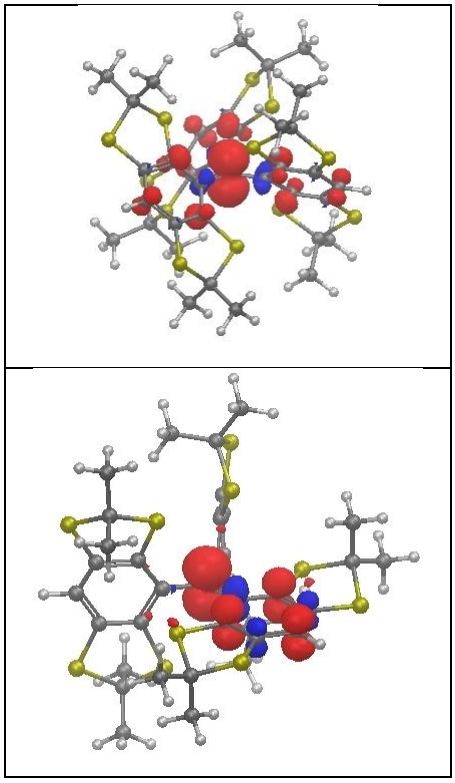
Computational chemistry

We first investigated the mechanism of isomerization by exploring the four potential pathways for interconversion for radical (**1d)** and alcohol (**2d)** derivatives using DFT methods. Transition state structures could be found for the two- and three-ring flip mechanisms. For the other mechanisms (zero- and one-ring flip), our calculations revealed that bringing two of the aromatic rings in the same plane would lead to such a spatial proximity between thio substituents of these aryl groups (<0.5 Å between two S atoms) that these structures are simply not realistic. Calculated transition states energy confirms that the lower lying pathway for helicity reversal is, in both cases, the one involving a two-ring flip mechanism (Table 3). This can be accounted for by the difference in steric strain between the two transition state structures. Indeed, the three-ring flip TS involves a close proximity of *ortho* thio substituents of the three aromatic ring which highly destabilizes this structure. In the case of radical **1d**, there is also a difference in spin delocalization between the two- and three-ring flip transition state structures; the two-ring flip TS allows

delocalization of spin density into one aromatic ring whereas in the three-ring flip TS the three aromatic rings are perpendicular to the reference plane preventing any conjugation (Figure 11). Stabilization by delocalization into one aromatic ring can be estimated by computing the energy barrier of rotation around the CH_2-Ar single bond of the model paramagnetic compounds 2,2,6,6-tetramethylbenzo[1,2-d:4,5-d']bis([1,3]dithiol-4-yl)methyl radical. Obtained energy barrier is 13.3 kcal.mol^{-1} at the B3LYP/6-31G* level.

Table 3: Computed energy barrier ($\Delta E^{\ddagger}$) to isomerization for **1d** and **2d** (energies are in kcal.mol^{-1} relative to reactants).

Mechanism	**1d**	**2d**
Two-ring flip	28.9	30.3
Three-ring flip	73.6	82.4



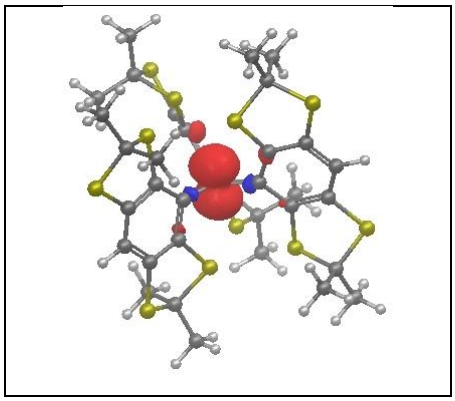


Figure 11: Spin density surface of ground state (top) and TS for two-ring (middle) and three-ring (bottom) flip mechanisms for **1d**.

Computed energy barrier to isomerization is lower for radical **1d** than for its alcohol analogue **2d** (computed energy barrier in solution is 27.6 and 30.2 kcal.mol^{-1}, respectively; Table 4).[28] Calculation of activation free energy shows however that isomerization of radical **1d** is entropically disfavoured: inclusion of zero-point energy, thermal and entropic contributions increases the barrier by 2.7 kcal.mol^{-1}. For alcohol **2d** these contributions are much lower (0.3 kcal.mol^{-1}). It results that, although energy barrier is slightly lower for radical **1d**, in terms of free energy, the barrier to isomerization is similar for both non-substituted derivatives (**1d** and **2d**).

Table 4: Computed energy barrier ($\Delta E^{\ddagger}$) and activation free energy ($\Delta G^{\ddagger}$) to isomerization (energies are in kcal.mol^{-1} relative to reactants).

Cpd	R, X	$\Delta E^{\ddagger}$	$\Delta E^{\ddagger}_{(AcOEt)}$	$\Delta G^{\ddagger}_{(EtOAc)}$
1d	R=H X=single electron	28.9	27.6	30.3
1b	R=CO_2Me X=single electron	30.1	29.4	
2d	R=H X=OH	30.3	30.2	30.5
2b	R=CO_2Me X=OH	30.6	30.5	

In order to investigate the influence of *para*-substitution on the aromatic moieties, we computed the energy barrier to isomerization for **1b**, **2b**. Substitution was found to have no significant effect on the rate of interconversion of alcohol derivatives. This is in good agreement with our experimental results which showed that free activation energy for **2d** and **2c** are 24.4 and 24.6 kcal/mol, respectively. Energy barrier to helicity reversal for paramagnetic derivatives is, on the contrary, sensitive to *para*-substitution: ester substituted radical derivative **1b** is computed to be less reactive (energy barrier increased by 1.8 kcal.mol^{-1}) than its non-*para*-substituted analogues **1d**. Whereas no difference in reactivity between radical and alcohol is predicted for non-substituted derivatives, the rate of isomerization for ester substituted radial (**1b**) should thus be lower than its alcohol analogues (**2b**). Indeed, experiments showed that the rate of isomerization is one order of magnitude lower for the ester substituted paramagnetic derivative **1c**, as compared to alcohol derivative **2c**.

Conclusions

Tetrathiatriarylmethyl alcohols, radicals and alkanes adopt a propeller like conformation. Slow interconversion of right-handed (*P*) and left-handed (*M*) propellers is responsible for the chirality of these molecules at room temperature. Analytical and semi-preparative CSP-HPLC allows resolution of enantiomers for alcohol, radical and alkane derivatives. The enantiomerization free activation energies determined by kinetics of racemization using CSP-HPLC varies from 24.4 kcal.mol^{-1} for alcohol **2d** to 26.4 kcal.mol^{-1} for radical **1c**. Computational chemistry results are in good agreement with experimental results and show an influence of the *para*-substitution for the paramagnetic derivatives while no significant effect was found for the alcohol derivatives. Computational and NOESY/EXSY spectroscopy studies are consistent with a two-ring flip mechanism for the isomerization process of tetrathiatriarylmethyl alcohols, radicals and alkanes.

Experimental Section

Generals: all commercially available reagents were used as received without further purification. NMR spectra were recorded on a BRUKER Avance DPX (^{1}H: 500 MHz, ^{13}C: 125 MHz). Chemical shifts (δ) are reported in parts per million (ppm), referenced to NMR solvents, [d]-chloroform (δ ^{1}H=7.27 ppm, ^{13}C=77.2 ppm), [d_6]-dimethylsulfoxide (δ ^{1}H=2.50 ppm, ^{13}C=39.52 ppm). The following abbreviations are used: s=singlet, d=doublet, t=triplet, m=multiplet. Coupling constants (J) are reported in Hertz (Hz). High resolution mass spectrometry (HRMS) analyses were performed at the University College London. Analytical HPLC was carried out on a Waters Alliance 2690 separations module equipped with a Waters 2998 PDA detector. Semi-preparative reverse phase HPLC of **3c** was carried out on a Waters 600 Pump equipped with a Waters 486 UV detector, a Waters fraction collector III and a Hitachi L-7200 HPLC autosampler. Semi-preparative CSP HPLC was carried out on a Waters 600 Pump equipped with a Waters 996 PDA, a Waters 717 autosampler and a Waters fraction collector III.

Computational methods: computations have been carried out using the Jaguar 7.5 pseudospectral program package.[29] All species have been fully optimized and the Cartesian coordinates are supplied in the Supporting Information.

Geometry optimization was carried out using the well established UB3LYP hybrid density functional[30, 31] as implemented in Jaguar with the standard split valence polarized 6-31G* basis.[32] This methodology has already been shown to describe properly these systems.[26]
Vibrational frequencies were computed for all stationary points for non substituted derivatives (R = H) **1d** and **2d** at the UB3LYP/6-31G* level of theory and are used to include zero-point energy, thermal and entropic corrections to compute ideal gas free energies. The correct nature of stationary points was confirmed by checking the curvature and its eigenvectors. Ground state structures were found to be minima with zero imaginary frequency, while transition state structures were found to be first-order saddle points with a single imaginary frequency.
Solvation effects were estimated by performing additional single point energy calculations (UB3LYP/6-31G*) using the Poisson-Boltzmann polarizable continuum method and the parameters appropriate for ethyl acetate (probe radius = 2.69, dielectric constant = 6.02), the solvent used for racemization kinetics.
For the large reaction systems there are usually several local minima or saddle points corresponding to each intermediate or transition state. This is due to the possibility of multiple conformations. In particular, the non-planarity of the two five-membered rings on each phenyl groups allows two different conformations for each ring. Test calculations showed that energy differences between the various accessible conformers[33] and energy barriers connecting them are much lower than barriers to enantiomerization. We have made a systematic attempt to locate all possible local saddle points, with the data presented referring to the lowest energy form.

X-Ray structure determination: crystals suitable for X-ray analysis were obtaind by slow evaporation from THF. The colourless plate like crystal (0.24 x 0.410 x 0.04 mm) was flash cooled in a 120K nitrogen flow prior to the diffraction experiment. A total of 91 images ($\Delta\phi$ 2°) were collected on a Mar345 image plate using Mo Kα radiation (Rigaku UltraX 18 rotating anode).
The reflections of the images were indexed and integrated using the CrysalisPro package (Agilent Technologies).[34] Data were scaled and corrected for absorption using the integrated scale3 abspack procedure.

The structure was solved by direct methods (SHELXS) and refined by full-matrix least squares on $|F^2|$ (SHELXL)[35]. Non-hydrogen atoms were anisotropically refined and the hydrogen atoms were placed on calculated positions with temperature factors fixed at 1.2 times U_{eq} of the parent atoms and 1.5 times U_{eq} for methyl groups.

The structure was twinned around the reciprocal c axis and was integrated as such. The ratio of both twin domains refined to 48.5%.

CCDC-883661 contains 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.

Crystal data for 2d C_{37} H_{40} O S_{12}, 2(C_4 H_8 O), M_r = 1029.62 g mol^{-1}, monoclinic, space group $P2_1/c$, a = 14.0782(11), b = 12.6254(8), c = 27.718(4) Å, β = 98.575(11)°, V = 4871.6(8) $Å^3$, Z = 4, ρ = 1.404 g cm^{-3}, μ(Mo-Kα) = 0.578 mm^{-1}, T = 120 K, reflections: 11216 collected, 11216 unique, R_{int} = unmerged, $R_1(|F|>2sigma(F))$ = 0.0736, $wR_2(|F|>2sigma(F))$ = 0.1683.

Synthesis of Tris(8-ethoxycarbonyl-2,2,6,6-tetramethylbenzo[1,2-d;4,5-d']bis[1,3]dithiol-4-yl)methane: to the tetrathiatriarylmethanol **2c** (25 mg, 0.023 mmol) was added TFA (5 mL) and metallic zinc foil (120 mg) and the solution was stirred for 48 h. TFA was then removed under reduced pressure and the crude was purified by semi-preparative HPLC to give 12.5 mg (50%) of the title compound as a orange solid. The conditions of purification were as follows: Column XBridge Prep C18 (10 x 100 mm 5 µm) equipped with a guard column XBridge Prep C18 (10 x 20 mm 5 µm), helium sparge 30 mL/min, UV detection at 267 nm, flow rate: 5 mL/min, eluent: ACN 86%/H_2O 14%. ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) : 1.47 (t, J=7.1 Hz, 9H, OCH_2CH_3), 1.67 (s, 9H, CH_3), 1.73 (s, 9H, CH_3), 1.77 (s, 9H, CH_3), 1.79 (s, 9H, CH_3), 4.35-4.50 (m, 6H, OCH_2CH_3), 5.39 (s, 1H, CH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) : 14.5 (OCH_2CH_3), 29.0 (CH_3), 29.3 (CH_3), 33.0 (CH_3), 34.2 (CH_3), 62.2 (CH), 62.3 (SCS), 62.5 (OCH_2CH_3), 62.9 (SCS), 120.4 (CAr), 130.0 (CAr), 139.3 (CAr), 140.1 (CAr), 140.6 (CAr), 141.8 (CAr), 166.5 ($CO_2CH_2CH_3$). HRMS (TOF MS ES+) m/z: $[M+H]^+$ calcd for $C_{46}H_{53}O_6S_{12}$: 1085.0491, found: 1085.0607. RP HPLC: Rt= 6.14 min (254nm, 1mL/min, ACN 100%, Symmetry C18 (4.6 x 250 mm), 25°C).

Exchange spectroscopy: the 2D ^{1}H-^{1}H NOESY/EXSY was recorded at 145°C using the Bruker noesygpph pulse sequence, mixing time d8=0.6 s, spectral width SW=0.5 ppm, centre of spectrum O1P=1.71 ppm , TD=1024 points in the direct-detect ^{1}H dimension, TD=256 increments in the indirect ^{1}H dimension, number of scans ns=32, number of dummy scans ds=16. A Sine Bell window function shifted by 90**°** was used to process both dimensions of the spectrum. Zero filling was used to lead a final resolution of 0.12Hz/point in the direct ^{1}H dimension and 0.24Hz/point in the indirect ^{1}H dimension.

Resolution of enantiomers: the two enantiomers of **2c** have been separated using a procedure previously reported[26]. Enantiomers of **3c** have been separated on a semipreparative CHIRALPAK IB column 10 x 250 mm 5 µm equipped with a guard column CHIRALPAK IB 10 x 20 mm 5 µm. The conditions of resolution were as follows: flow rate: 4.7 mL/min; eluent: *i*-hex 88%/EtOAc 10%/EtOH 2%; UV detection: 268 nm; column temperature: ambient. Enantiomers of **2d** have been separated on an analytical CHIRALPAK IA column 4.6 x 250 mm 5 µm equipped with a guard column CHIRALPAK IA 4.6 x 20 mm 5 µm. The conditions of resolution were as follows: flow rate: 1 mL/min; eluent: *i*-hex 88%/EtOAc 10%/EtOH 2%; UV detection: 275 nm; column temperature: ambient. Enantiomers of **2c**, **2d**, **3c** were collected using a fraction collector in flask cooled at -78°C.

HPLC Kinetics: racemization of **2c**, **2d** and **3c** were followed by CSP HPLC according to this general procedure: one enantiomer was dissolved in an HPLC vial (1.5 mL) with EtOAc at 25°C. The vial was then placed in the HPLC oven where the temperature was controlled to 25°C. This time was considered to be t=0. Chromatograms were recorded periodically over the time and the enantiomeric excess calculated by integration of the two enantiomers at 274 nm for **2c**, 274 nm for **2d** and 267 nm for **3c**. Kinetics was stopped when %ee=0. The conditions of resolution were as follows: (**2c**) Column: CHIRALPAK IB (4.6 x 250 mm 5 µm) equipped with a guard column CHIRALPAK IB (4.6 x 20 mm 5 µm), flow rate: 1 mL/min; eluent: *i*-hex 93%/EtOAc 4%/MeOH 3%. (**2d**) Column: CHIRALPAK IA (4.6 x 250 mm 5 µm) equipped with a guard column CHIRALPAK IA (4.6 x 20 mm 5 µm), flow rate: 0.5 mL/min; eluent: *i*-hex 90%/EtOAc 7%/EtOH 3%. (**3c**) Column: CHIRALPAK IB (4.6 x 250 mm 5 µm) equipped with a guard column CHIRALPAK IB (4.6 x 20 mm 5 µm), flow rate: 1 mL/min; eluent: *i*-hex 88%/EtOAc 10%/EtOH 2%.

Supporting Information (see footnote on the first page of this article): ^{1}H NMR, ^{13}C NMR, HRMS spectra and HPLC chromatogram of **3c**; optimized Cartesian coordinates and corresponding energies for all species discussed in the text.

Acknowledgments

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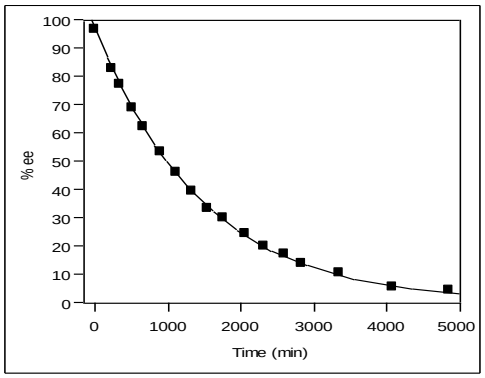
Kinetic of racemization

2d		2c		3c	
Time (min)	**%ee**	**Time (min)**	**%ee**	**Time (min)**	**%ee**
0	71.12	0	95.98	0	76.84
109	62.32	239	82.5	29	75.94
271	52.06	347	76.94	177	66.22
494	41.88	515	68.56	286	59.64
776	32.26	667	61.86	420	52.68
1058	24.9	895	53	559	46.2
1341	19.54	1107	45.7	727	39.36
1623	15.5	1336	39.16	895	33.3
1906	12.64	1548	32.76	1063	27.8
2188	9.46	1760	29.34	1292	21.72
2470	7.28	2048	24.02	1520	17
2752	5.52	2320	19.24	1749	13.58
3035	4.26	2593	16.5	1977	9.78
3317	2.94	2824	13.54	2206	7.04
3600	1.62	3336	10.16	2434	4.68
3882	1.4	4085	5.26	2662	2.84

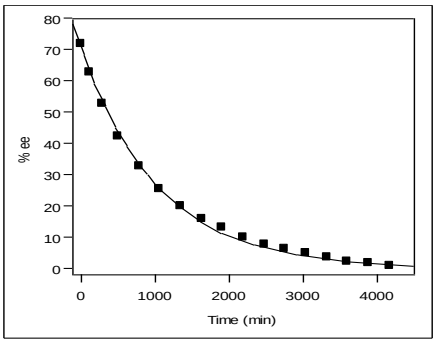
4165	0.14	4855	3.76	2915	1.14
				3100	1.28

2c



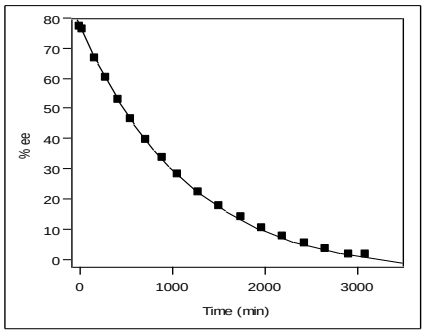
Parameter	Estimate	ApproxStdErr	Lower CL	Upper CL
ee0	96.950396695	0.33645567	96.235748	97.6671021
krac	0.0006832698	4.26623e-6	0.00067426	0.00069237

2d



Parameter	Estimate	ApproxStdErr	Lower CL	Upper CL
ee0	69.232784171	0.653654	67.8370062	70.6371916
krac	0.0009529357	1.69669e-5	0.00091701	0.00099042

3c



Parameter	Estimate	ApproxStdErr	Lower CL	Upper CL
ee0	83.034706364	0.36276223	82.2692596	83.8137108
krac	0.0008550389	1.07273e-5	0.00083238	0.0008779
theta3	-5.434827008	0.38641822	-6.2709275	-4.6294052

Note: the equation $\%ee = \%ee_0\ e^{k_{rac}t} + theta3$ has been used for **3c** to take into account a systematic error induce by an impurity in the peak.

6. Radicaux TAMs vectorisés

Cette partie du manuscrit décrit la synthèse de radicaux TAMs liés de façon covalente à une entité vectrice. Deux ligands ont été couplés, le pentapetpide Gly-Arg-Gly-Asp-Ser (GRGDS) se liant à 8 des 24 types d'intégrines (récepteur d'adhésion) et un peptidomimetique RGD sélectif du sous-type d'intégrines $\alpha_v\beta_3$.

Ce travail fera l'objet de deux publications. La première consacrée à la synthèse d'un radical TAM couplé au peptide GRGDS accepté dans le journal Tetraheron Letter" et la deuxième consacrée à la synthèse d'un radical TAM couplé à un peptidomimétique RGD soumise dans " Chemistry an Asian Journal" .

Ma contribution à ce travail est le design, l'imagination d'une voie de synthèse et la réalisation de la synthèse des deux radicaux TAMs vectorisés. Le Dr. P. Levêque (UCL/REMA) a réalisé les tests d'adhésion cellulaire *in vitro*.

La synthèse des premiers radicaux TAMs vectorisés décrits à ce jour se base sur deux voies de synthèse fondamentalement différentes. Premièrement, le radical TAM **CT-03** a été couplé trois fois au peptide GRGDS. Cette méthode de synthèse repose sur l'activation des fonctions acides de **CT-03** sous forme d'esters de type pentafluorophénol et réaction avec le pentapetide GRGDS totalement déprotégé ! Cette voie de synthèse est compatible avec le centre radicalaire puisqu'elle se réalise directement sur le radical **CT-03**. De plus, les esters de type pentafluorophénol sont connus pour être stables en milieu aqueux. Cette voie de synthèse rapide et efficace pourra être utilisée pour coupler directement le radical **CT-03** à toute autre molécule, possédant une amine nucléophile, en solvant organique ou aqueux.

La deuxième voie de synthèse, plus longue, utilisée pour coupler le petpidomimétique RGD se base sur une étape de désymétrisation du motif TAM permettant de n'ajouter qu'un seul ligand. De plus, une bonne partie de la synthèse se réalise sur l'alcool TAM permettant une bonne caractérisation par RMN des intermédiaires. Les conjugés TAM-

Vecteur sont des molécules de très haute complexité obtenues de manière pure, ce qui n'est pas classique pour des molécules de cette taille.

Une troisième publication parue dans le journal " Bioorganic and Medicinal Chemitry letters" est consacrée à la fonctionnalisation de particules de fer avec le même peptidomimétique que celui couplé aux radicaux TAMs. Ma contribution à cet article se limite au travail sur la synthèse du peptidomimétique couplé à un bras oligoéthylène glycol qui a pu être améliorée. Ainsi seule la partie expérimentale de l'article concernant la synthèse du peptidomimetique est incorporée dans cette thèse.

RGD-conjugated triarylmethyl radical as probe for electron paramagnetic imaging

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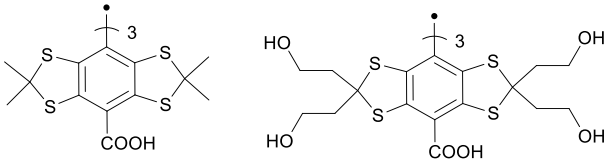
Abstract. A stable RGD-conjugated triarylmethyl (TAM) radical **3** has been synthesized in a straightforward three-step sequence. **CT-03** (Finland trityl radical) which is a very popular contrast agent for electron paramagnetic resonance imaging has been covalently bound to the NH_2-ending of a pentapeptide commonly used to target integrins and confer tissue selectivity. Moreover, this new TAM radical is stable and sensitive to molecular oxygen.

Low frequency electron paramagnetic resonance (EPR) imaging is a technique that allows *in vivo* mapping of the distribution of a paramagnetic species, and other physiological parameters such as oxygen concentration, pH and redox status[1,2]. The physical basis of EPR is similar to the well known nuclear magnetic resonance (NMR). However, the magnetic moment of the electron is 658 times greater than that of the proton, which makes EPR intrinsically more sensitive than NMR for the same probe concentration. By contrast with magnetic resonance imaging (MRI) by NMR, there is no naturally occurring paramagnetic species in sufficient concentration and long-enough half-life to be imaged *in vivo* by EPR imaging (EPRI), with the exception of melanin.[3,4] Thus an exogenous paramagnetic spin probe is needed. Among the water soluble radicals used in EPRI, the triarylmethyl (TAM) radicals bearing four sulphur atoms on each phenyl ring such as **CT-03** and **Ox063** (Fig. 1) are the most attractive. [5]



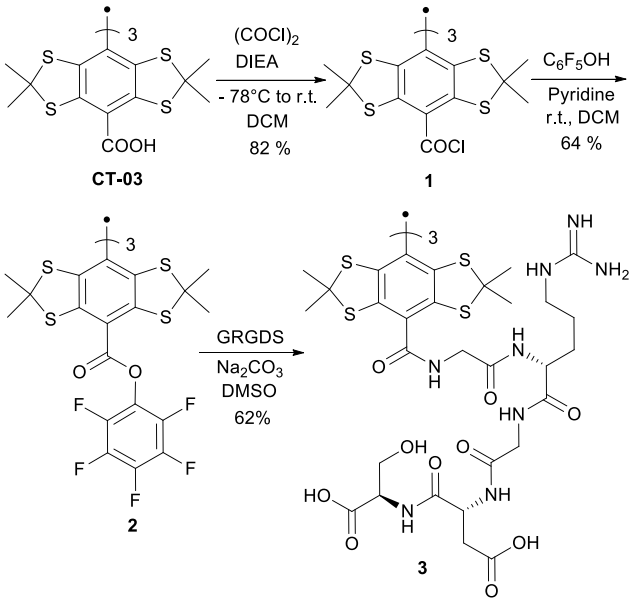
CT-03 OX063

Fig. 1 Chemical structure of **CT-03** and **Ox063**.

These compounds initially developed to be used as hyperpolarizing agents for Overhauser-enhanced magnetic resonance imaging (OMRI)[6] exhibit a good stability, a single narrow EPR line of less than 100 mG under anoxic conditions (0% oxygen), a low concentration-dependent line-broadening and non toxic properties.[5] Indeed, a narrow EPR line confers high sensitivity of detection, particularly useful *in vivo,* since the intensity of the signal is inversely proportional to the square of the linewidth. Yet, the synthesis of such complex molecules remains challenging and new structures are regularly reported. Original Nycomed's TAMs and subsequent chemically modified TAMs have been used for EPR measurements of intracellular[7] and extracellular[8] O_2 concentrations, pH[9,10], thiol[11], superoxide anion[12], redox status[13], spin-spin interdistance[14], for EPRI[15] and employed as hyperpolarizing agents[6]. However, to date, only triarylmethyl radicals with unspecific distribution have been reported.

Hereby, we describe a straightforward synthesis of the pentafluorophenol ester derivative **2** of **CT-03**. This activated form of **CT-03** can be used to bind the trityl core to a targeting moiety designed to achieve tissue selectivity. Compound **2** has been used to conjugate the TAM radical to the pentapeptide GRGDS (Gly-Arg-Gly-Asp-Ser) including the RGD sequence, known to bind some of the trans-membrane proteins called integrins which are commonly used as targets for molecular imaging.[16]

The synthesis has been achieved as depicted in scheme 1. **CT-03** is synthesized according to a procedure described in the literature.[17] Activation of the carboxylic acid functions of **CT-03** by oxalyl chloride and diisopropylethylamine (DIEA) leads to the formation of **1** with 83 % yield after filtration on a small silica gel path. Then, esterification of **1** with pentafluorophenol and pyridine furnishes **2** which is stable enough to be purified by column chromatography. This compound was recovered in pure form with 64 % yield.



Scheme 1 Synthesis of **3**.

Compound **2** is an activated form of **CT-03** and can be used to conjugate the TAM radical to any carrier device. In order to prove the concept, **2** was mixed in DMSO with a small excess of unprotected pentapeptide GRGDSin the presence of sodium carbonate. The triaryalmethyl radical **3** coupled to three GRGDS peptides, most probably through the reactive terminal NH_2 function, was formed with more than 90% conversion. Purification on semi-preparative HPLC carried out on reverse-phase gives **3** in pure form (Fig. 2a) with 62 % yield. While only one peak is visible on the chromatogram obtained under gradient elution (Fig. 2a), two peaks with the same areas are obtained under isocratic elution (Fig. 2b). These two peaks correspond to diasteroisomers associated with the helicoidal chirality of the trityl core.[18,19]

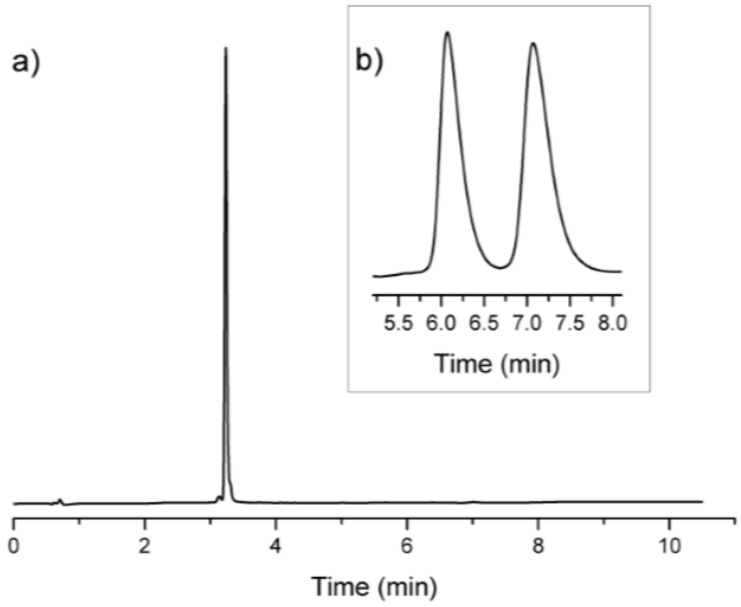


Fig. 2 HPLC chromatograms of **3** under gradient (a) and isocratic (b) elution.

Indeed, the trityl core displays a propeller conformation where all rings have the same direction of twist. Both right-handed (*P*) and left-handed (*M*) propeller conformations exist and display enantiomeric relationship. We have previously shown that for the tetrathiatriarylmethyl core, the isomerization between the two enantiomeric conformations is slow, which confers chiral properties to these molecules.[18,19] As GRGDS is an enantiomerically pure molecule, two diastereoisomers are obtained for the conjugated compound **3.**

The EPR spectrum of **3** has been recorded at X-band in phosphate buffer saline (PBS) (Fig. 3). The spectrum shows a single EPR line with a peak-to-peak linewidth ΔB_{pp} = 705 mG under air room condition (21 % O_2). The wider linewidth obtained for TAM **3** than for **CT-03** can be explained by unresolved hyperfine splitting with nitrogen atoms of the amide functions. This observation has been already reported for dendritic TAM radicals.[20] Bimolecular collision between a TAM radical and molecular oxygen induces a broadening of the EPR line of the TAM spin probe. This phenomenon can be used to assess oxygen concentration by EPR. According to the Smoluchowski equation there is a linear relationship between the linewidth and O_2 concentration for a soluble spin probe. With 0% of oxygen,

the linewidth of **3** reaches ΔB_{pp} = 630 mG, indicating that compound **3** keeps its sensitivity to oxygen and can be used as an EPR oxymetric probe.

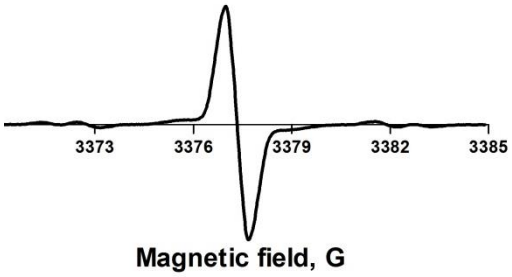


Fig. 3 EPR spectrum of **3** recorded in PBS (pH = 7.4) at X-band.

Recent studies about *in vitro* metabolism of **CT-03** and **Ox063** have shown that they could be reduced under anaerobic conditions in the presence of liver microsomes to the corresponding triarylmethane through formation of the anion.[21] Furthermore, a TAM cation is obtained through oxidation of radicals by peroxydases and related hemoproteins.[22] The cation can subsequently react with nucleophiles such as water, peroxides, phosphines, thiols, amines, etc. The redox potential of compound **3** has been measured in PBS (pH = 7.4) by cyclic voltammetry (CV) (Fig. 4) and compared with **CT-03**.

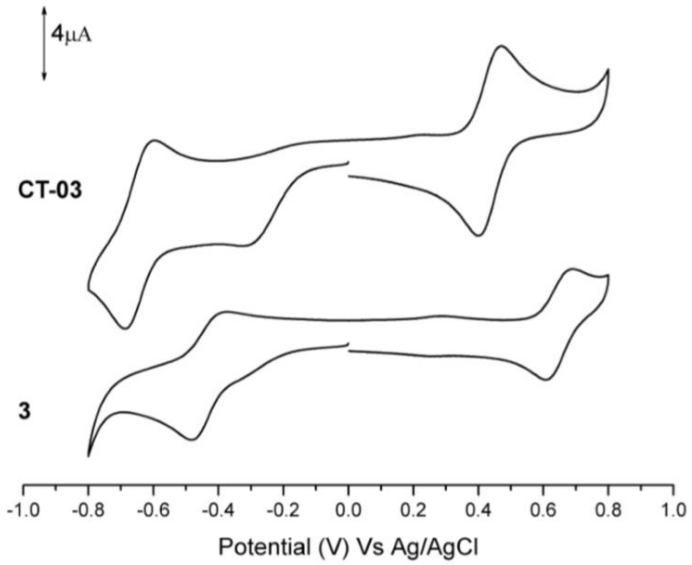


Fig. 4 Cyclic voltamogram of **CT-03** and **3**.

The results show that compound **3** can be reduced more easily than **CT-03** but is harder to oxidise than **CT-03** (Table 1). This can be explained by the higher electron-attracting power of amide functions of **3** by comparison with the carboxylate functions of **CT-03** in aqueous medium. Thus, radical **3** appears be more stable than **CT-03** under oxidation conditions.

Table 1 (Redox potentials ($E_{1/2}$) relative to Ag/AgCl (KCl 3M) determined by CV

Compound	**Solvent**	**$E_{1/2}$ ox**[a]	**$E_{1/2}$ red**[a]
CT-03	PBS	+0.434	-0.642
3	PBS	+0.648	-0.429

[a] calculated according to $E_{1/2}$ = (Epa + Epc)/2

In conclusion, we have synthesized for the first time a tetrathiatriarylmethyl radical linked to three carrier molecules. Compound **3** has been synthesized by mixing pentafluorophenol ester **2** with an unprotected pentapeptide sequence, followed by purification by semi-preparative HPLC. The RGD-conjugated **3** keeps the sensitivity to oxygen. Its oxidation potential is higher than that of **CT-03** while its reduction potential is lower. Thus radical **3** could be considered as a potential spin label possessing tissue selectivity for EPR O_2 measurements and for EPRI. Moreover, the activated ester **2** is a valuable key-intermeiate which can be used to bind other carrier molecules to the TAM radical.

Acknowledgements: We gratefully acknowledge funding from the Université catholique de Louvain, the Fond de laRecherche Scientifique (F.R.S-F.N.R.S). JMB is a senior research associate for the F.R.S-F.N.R.S. Prof. Z. Mekhalif (F.U.N.D.P-Namur, Belgium) is gratefully acknowledged for the recording of cyclic voltammograms.

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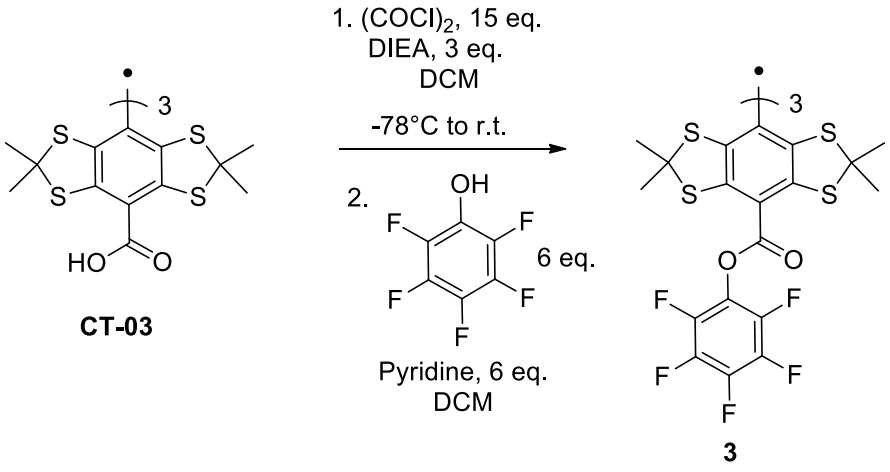
Experimental part

General information

All reactions were carried out in flame-dried glassware under argon atmosphere. Anhydrous grade solvents were used for reactions and HPLC grade for purifications. All commercially available reagents were used as received without further purification. Infrared (IR) spectra are recorded using a Shimadzu spectrometer FTIR-8400S; products were analyzed as thin films deposited on a Se−Zn crystal by evaporation from the solvent. High resolution mass spectrometry (HRMS) analyses were performed at the University College London. Analytical HPLC was carried out on a Waters Alliance 2690 separation module equipped with a Waters 2998 PDA detector. Semi-preparative HPLC was carried out on a Waters 600 Pump equipped with a Waters 486 UV detector, a Waters fraction collector III and a Hitachi L-7200 HPLC Autosampler. EPR spectra were recorded on an X-Band BRUKER EMX spectrometer equipped with a ER4119HS resonator.

Syntheses

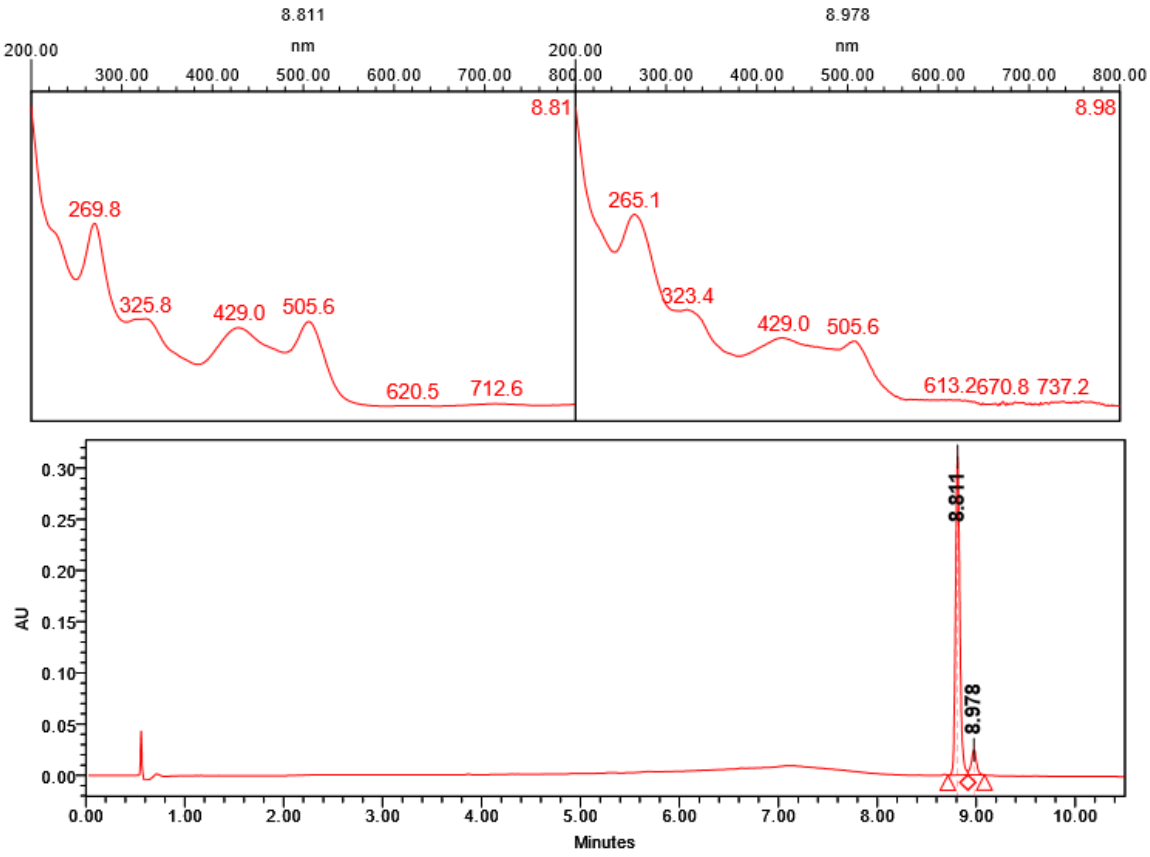
Synthesis of 2



To a stirred suspention of **CT-03** (100 mg, 0.100 mmol, 1 equiv.) in degased DCM (6 mL) was added DIEA (52 µL, 0.300 mmol, 3 equiv.). The solution was cooled to -78°C and oxalyl chloride (128 µL, 1.500 mmol, 15 equiv.) was added. The mixture was stirred at -78°C for 10 min, then the cooling bath was removed and the solution were stirred for 4 hours at room temperature. Afterward, the solvent and the excess of oxalyl chloride were removed under reduced pressure and the dark-red solid was purified on a short silica gel path (5 × 2 cm) using DCM as eluent to provide 85 mg (82%) of acyl chloride which was checked by mass spectrometry (ESI, positive mode) and then directely dissolved in degased DCM (2 mL). This red solution of trityl acyl chloride (85 mg, 0.081 mmol, 1 equiv.) was added at room temperature to a solution of pentafluorophenol (89 mg, 0.484 mmol, 6 equiv.) in degassed DCM (4 mL). After the addition of pyridine (39 µL, 0.484 mmol , 6 equiv.), the red solution was stirred one hour at room temperature. The solvent was removed under reduced pressure and the dark-red residue was purified by flash chromatography using PE/DCM (from 2:1 to 1:1, v/v) to give 77.5 mg (64%) to the title compound as an orange solid.

R_f value : 0.13 DCM/PE (1:2) UV(254 nm) or visible, 0.55 (DCM/PE) (1:1) UV (254 nm) or visible
HRMS (TOF MS ES+) *m/z* :$[M+1]^+$ calcd for $C_{58}H_{37}F_{15}O_6S_{12}$: 1497.8999, found 1497.9170
ATR-IR (cm^{-1}): 1736, 1520, 1229, 1200, 1094, 1028, 997.
RP-HPLC : RT = 8.8 min, column : XBridge C18 2.5µm 4.6 x 50mm equipped with a precolumn XBridge C18 2. 5µm 4.6 x 20mm. Column temperature : 40°C

Time	Flow	ACN	H_2O	H_2O (1%TFA)
	1.5 mL/min	80%	10%	10%
1.00 min	1.5 mL/min	80%	10%	10%
6.00 min	1.5 mL/min	0%	90%	10%
7.00 min	1.5 mL/min	0%	100%	0%
10.50 min	1.5 mL/min	0%	100%	0%
10.60 min	1.5 mL/min	80%	10%	10%

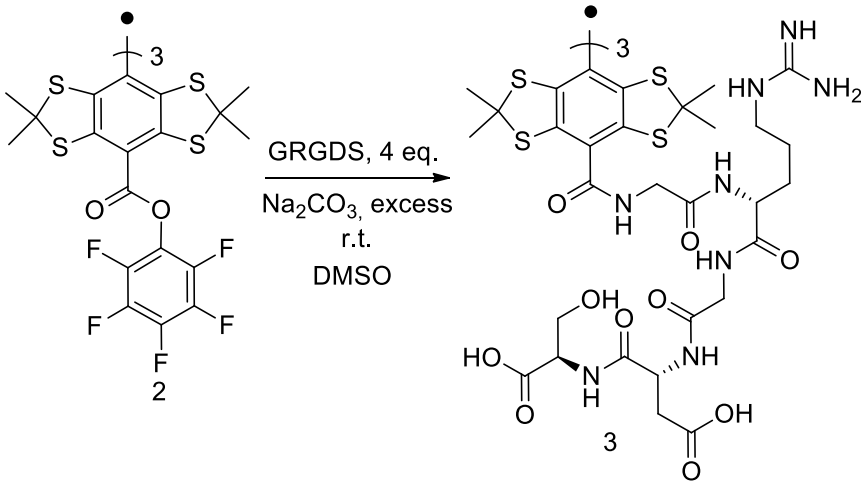


Peak Results

	Name	RT	Area	Height	Amount	Units	% Area	Int Type
1		8.811	930731	311211			91.38	bv
2		8.978	87815	24339			8.62	vb

Figure S1 : HPLC chromatogram of **2**

Synthesis of 3



To a stirred solution of **2** (26 mg, 0.017 mmol, 1 equiv.) in degased DMSO (10 mL) was added GRGDS (50 mg, 0.07 mmol, 4 equiv.) purchased from Polypeptide and Na_2CO_3 (18 mg). Thered solution becomes rapidly dark green. The mixture was stirred overnight and water was added (500 mL) and stirred for 2 h. The solution was lyophilized and the dark green residue purified by semi-preparative HPLC to give 29.5 mg (62%) of **3** in pure form.

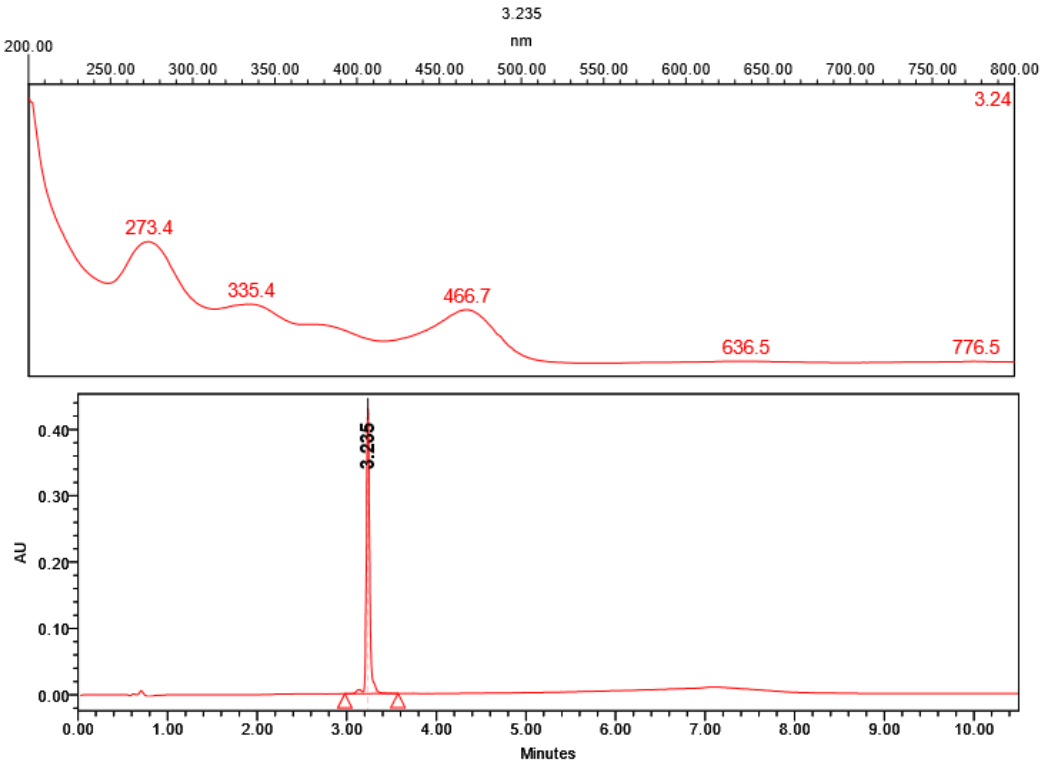
The HPLC purification conditions were as follows : Column XBridge 5 µm 10 x 100 mm equipped with a precolumn XBridge 5 µm 10 x 20 mm, ambient temperature, helium sparge 30 mL/min, detection 254 nm, run of 15 min, gradient elution :

Time	flow	H_2O(0.1% TFA) 90%/ACN(0.1% TFA)10%	ACN(0.1% TFA)
0 min	5 mL/min	100	0
10 min	5 mL/min	30	70
10.1 min	5 mL/min	100	0

HRMS (TOF MS ES+) *m/z* :$[M+3]^{3+}$ calcd for $C_{91}H_{126}N_{24}O_{30}S_{12}^{\bullet}$: 806.19067, found 806.85650; $[M+2]^{2+}$ calcd for $C_{91}H_{125}N_{24}O_{30}S_{12}^{\bullet}$: 1208.7821, found 1209.28195
RP-HPLC : RT = 3.2 min, column : XBridge C18 2.5 µm 4.6 x 50mm equipped with a precolumn XBridge C18 2.5µm 4.6 x20mm. Column temperature : 40°C, UV detection at 254 nm

Time	Flow	H_2O	ACN	H_2O (1%TFA)	Curve (Waters)
	1.5 mL/min	80%	10%	10%	

1.00 min	1.5 mL/min	80%	10%	10%	6
6.00 min	1.5 mL/min	0%	90%	10%	6
7.00 min	1.5 mL/min	0%	100%	0%	6
10.50 min	1.5 mL/min	0%	100%	0%	6
10.60 min	1.5 mL/min	80%	10%	10%	6



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area	Int Type
1		3.235	1101445	429941			100.00	bb

Figure S3: HPLC chromatogram of **3**

Cyclic voltammetry

Oxydation and reduction potentials of **CT-03** and **3** were measured by cyclic voltammetry using a potentiostat and computer-controlled electroanalytical system (CH instruments, Potentiostat Model 440). The solution of TAM radical partially degased using Argon flux was placed in a 2 mL cell equipped with Ag/AgCl electrode (KCl 3M+AgCl saturated solution) as the reference electrode, platinum foil as the counter electrode and gold electrode as working electrode.

CVs recorded at 20, 50, 100 mV/s for CT-03

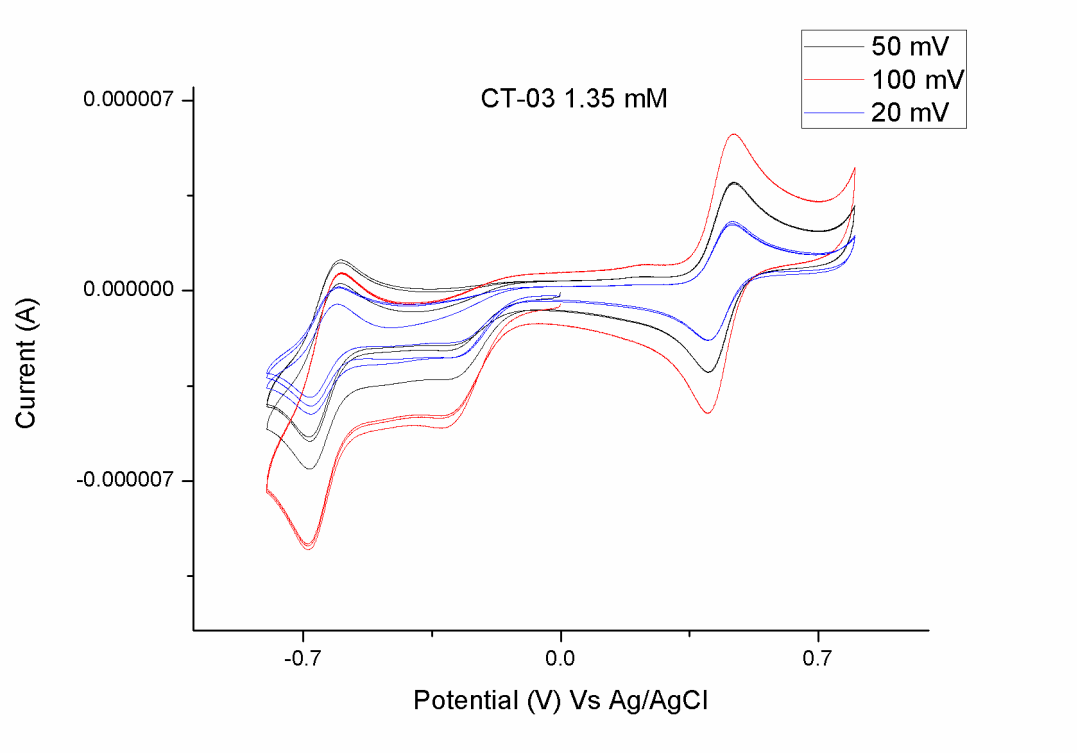


Figure S5: Cyclic voltammogram of radical **CT-03** (1.35 mM in PBS (0.01M, pH=7.4)), scan rate=20, 50, 100 mV/s

CVs recorded at 20, 50, 100 mV/s for 3

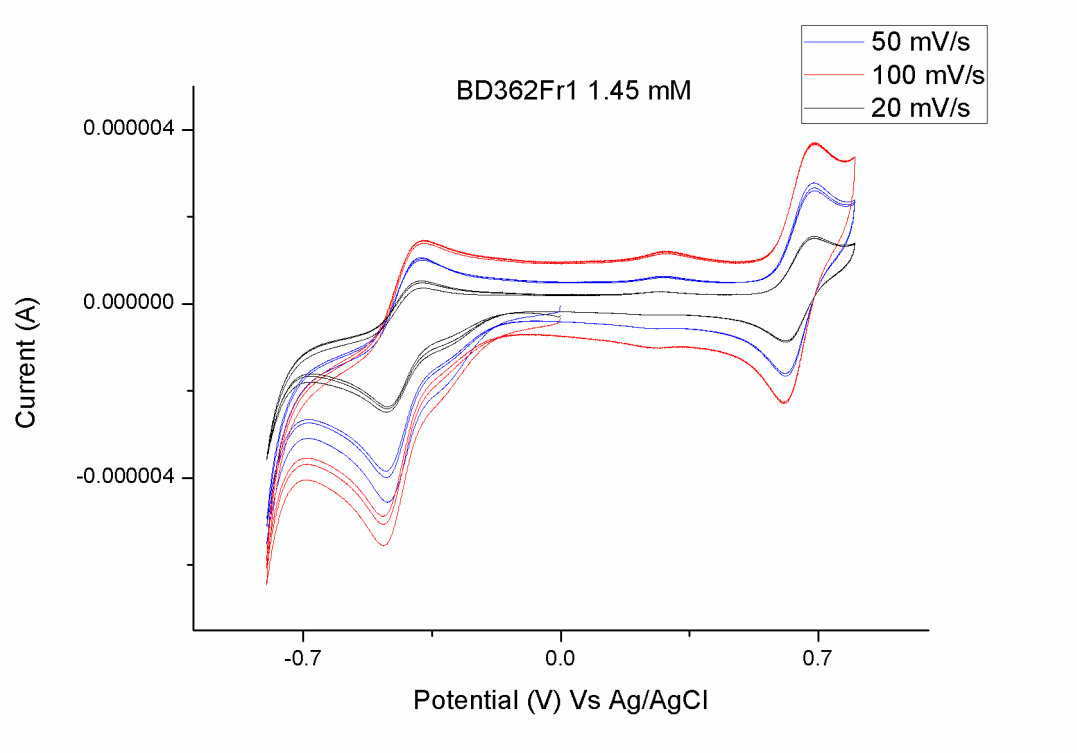


Figure S6: Cyclic voltammogram of radical **3** (1.45 mM in PBS (0.01M, pH=7.4)), scan rate=20, 50, 100 mV/s

X-band EPR spectra

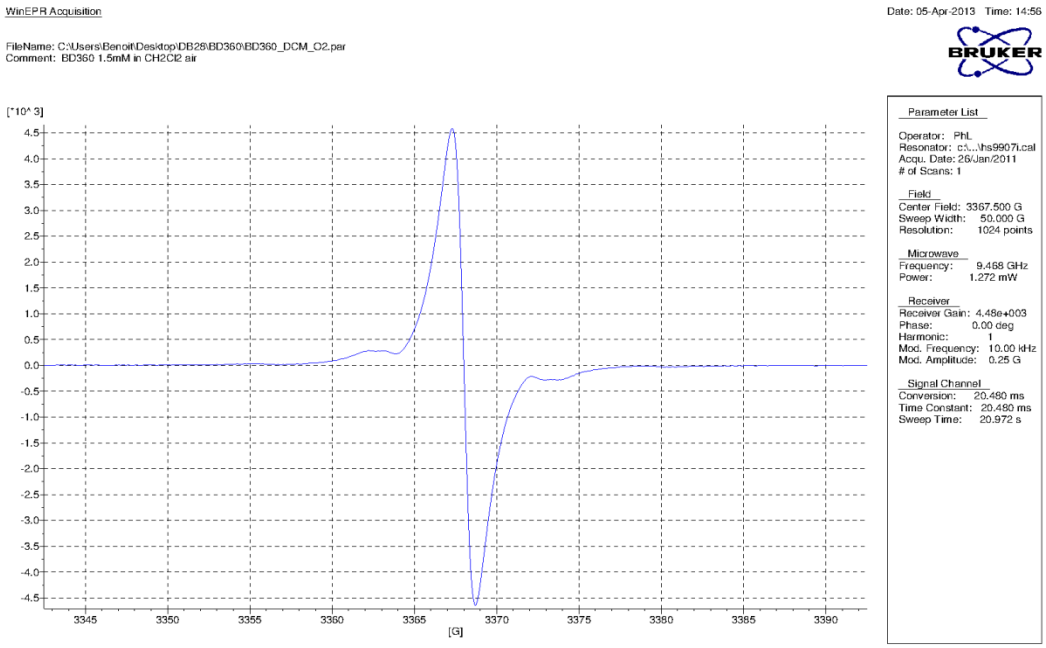


Figure S7: X-band EPR spectrum of **2** in DCM under air room condition

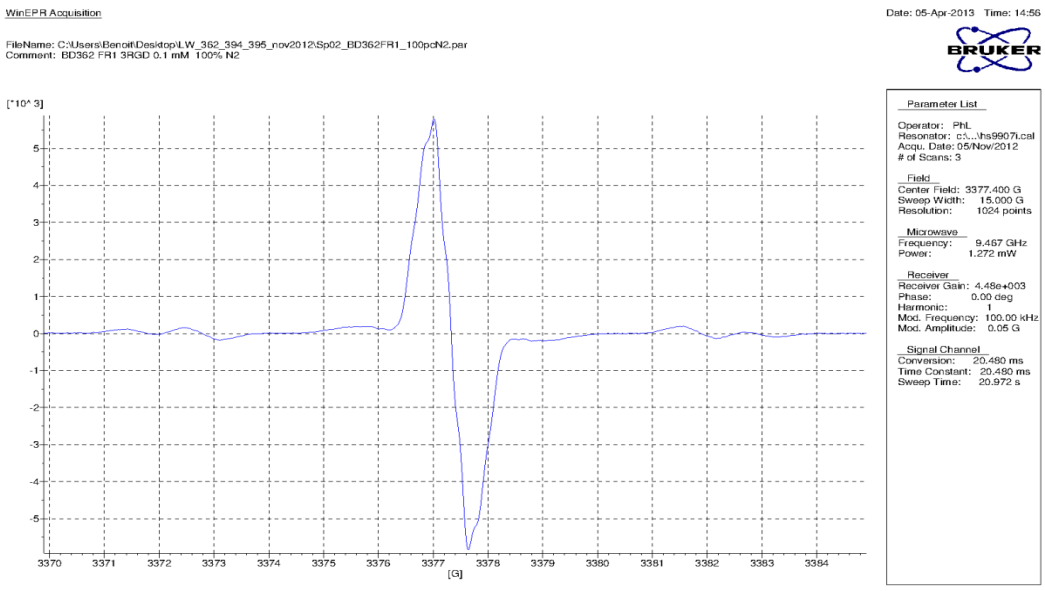


Figure S8: X-band EPR spectrum of **3** in PBS under N_2 atmosphere

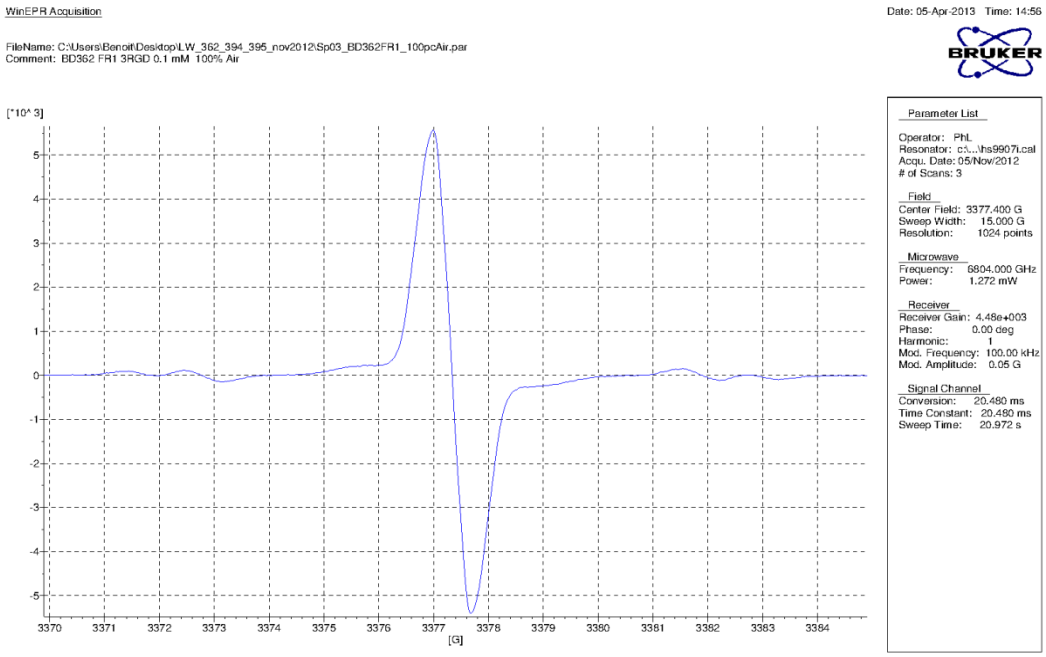


Figure S9: X-band EPR spectrum of **3** in PBS under air room condition

Stable triarylmethyl radicals conjugated to RGD peptidomimetic: synthesis and EPR properties

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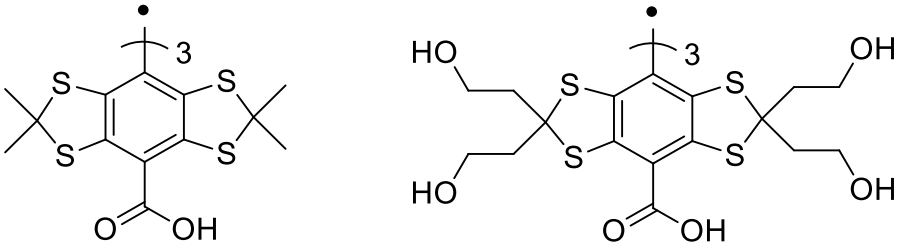
Abstract : Triarylmethyl (TAM) radicals are favourite spin labels for electron paramagnetic resonance imaging. In this work, we report a straightforward synthesis of new TAM radicals bound to a RGD-peptidomimetic possessing a high affinity to integrin $\alpha_v\beta_3$. These new radicals are stable in solution and exhibit a single EPR line.

Introduction

Electron paramagnetic resonance imaging (EPRI) is an emerging technique allowing to map the distribution of paramagnetic species.[1] Regarding the well-known nuclear magnetic resonance imaging (MRI) extensively used in clinical environment, the EPRI technique possesses the advantages of a higher intrinsic sensitivity and the absence of background. Indeed, as there is no endogenous paramagnetic species in sufficient concentration and long enough half-life, with the exception of melanin,[2] an exogenous paramagnetic spin label has to be injected for EPRI application.[3] Two classes of water-soluble paramagnetic probes are currently used, namely nitroxide (NR) radicals and triarylmethyl (TAM) radicals. The later radicals such as **CT-03** and the more hydrophilic compound **Ox063** (Figure 1) are superior spin probes to nitroxide radicals because of their higher stability in biological media and the lower intrinsic linewidth of their single EPR line

resulting in a higher signal-to-noise ratio (signal intensity is inversely proportional to the square of the linewidth).[4]



CT-03 **OX063**

Figure 1: Chemical structure of **CT-03** and **Ox063**

To the best of our knowledge, TAM radicals conjugated to a targeting moiety are not yet described in the literature, although few examples of such conjugates exist in the case of nitroxide radicals. For instance, NRs have been coupled to arabinogalactan[5] or fatty acid moieties[6] to target the liver cells.

The highly challenging chemistry of TAM radicals could explain this lack of synthetic investment. Hereby, we report the first synthesis of TAM radicals coupled to a peptidomimetic molecule targeting the $\alpha_v\beta_3$ integrin.

Integrins are transmembrane receptors playing crucial roles in cell-to-cell and cell-to-matrix adhesion phenomena.[7] Specifically, the $\alpha_v\beta_3$ integrin is over-expressed on the spreading endothelial cells of novel capillaries in the angiogenic process linked to tumour growth and development.[8] In addition, it is also expressed on certain invasive tumour cells.[9] Therefore the $\alpha_v\beta_3$ integrin has been considered as an appealing target for anticancer therapy (drugs) and cancer diagnosis (probes).[10] The $\alpha_v\beta_3$ integrin interacts with several extracellular matrix proteins through the recognition of the RGD (Arg-Gly-Asp) tripeptide sequence.[11] RGD containing cyclic peptides, such as Cilengitide[12] (in clinical trials for treatment of glioblastoma) and peptidomimetics of this sequence have been intensively developed as potent antagonists of $\alpha_v\beta_3$ integrin for therapeutic purpose mainly.[13] However, some molecules bearing a spacer-arm for grafting on devices or matrices are also used in various biotechnological applications.[14]

Guided by the X-ray structure[15] of the extracellular part of $\alpha_v\beta_3$ integrin in complex with Cilengitide, our group has previously designed RGD peptidomimetics based on the (L)-Tyrosine scaffold. The parent compound **1**, showed an excellent ability to bind isolated human $\alpha_v\beta_3$ integrin, with an IC_{50} value of 0.1 nM. The derived compounds **2** equipped with oligoethylene glycol spacer-arms keeped the antagonist activity (IC_{50} = 0.3-0.7 nM).[16] Moreover, the anchorage of **2** on the surface of culture substrates allowed the strong adhesion of human endothelial cells and their retention under shear stress.[17]

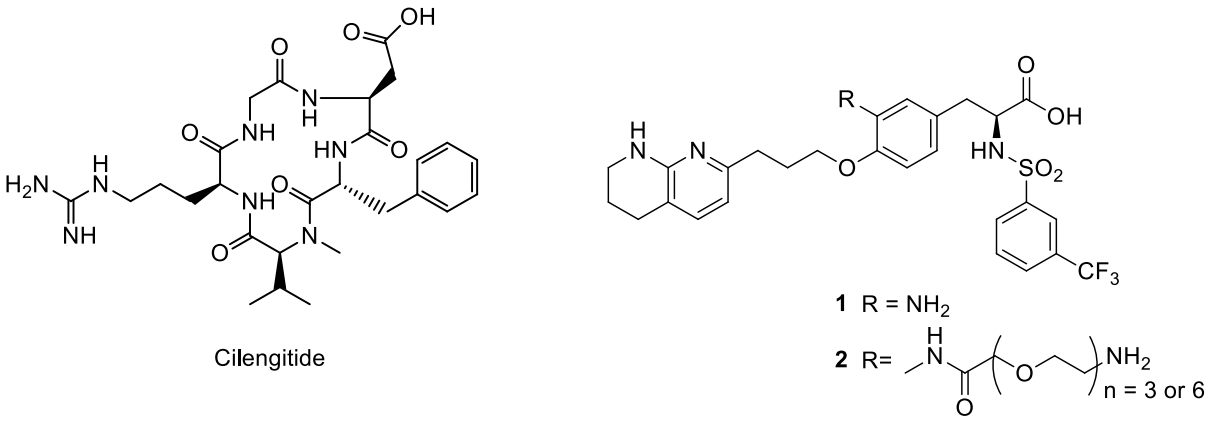
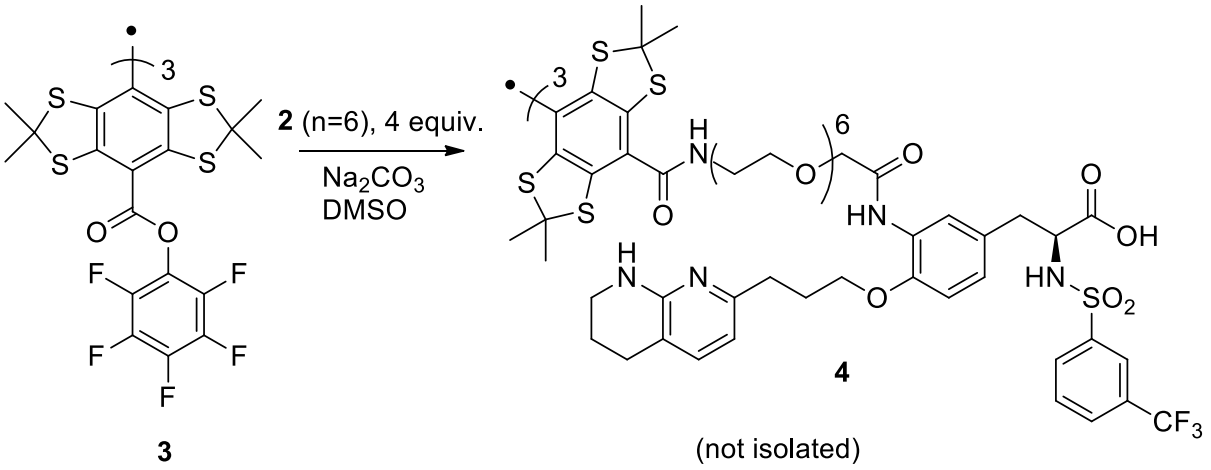


Figure 2: Structure of Cilengitide and peptidomimetic.

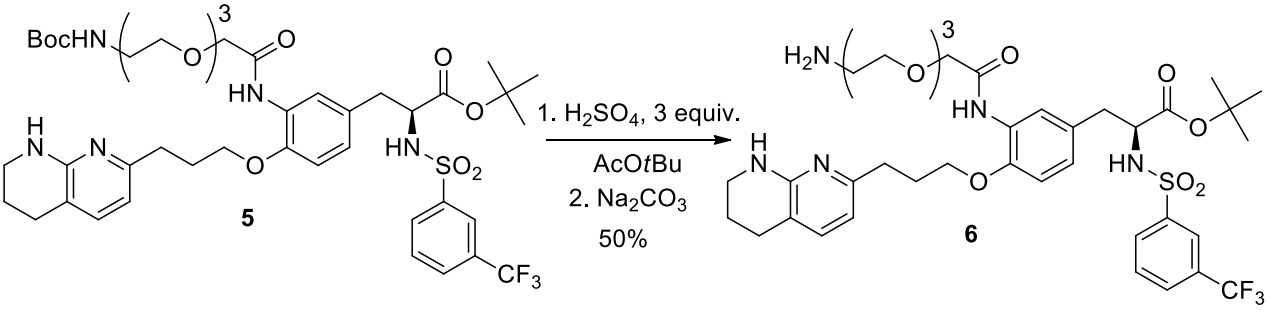
Results and discussion

The graftable RGD-peptidomimetic **2** (n=6) has been synthesized as described before.[18] Since all the polar functions are unprotected, we envisaged as first strategy, the reaction of **2** with an activated ester of TAM radical, namely the pentafluorophenol triester derivative **3** (see supporting information for synthesis). **3** was reacted with four equivalents of peptidomimetic **2** (bis TFA salt) and Na_2CO_3 in degased DMSO (Scheme 1). A change of color from red to green appeared during the reaction as a result of the coupling of **2** through the terminal primary amine of its OEG spacer. The product **4** featuring three peptidomimetic moieties was detected in the crude mixture by mass spectrometry (calculated for $[M+2]^+/2$: 1821.4931, found: 1821.2750). However, we failed to purify this compound by flash chromatography on silica gel, crystallization or semi-preparative HPLC on reverse phase. This can probably be explained by the high molecular weight of the molecule and the presence of several acidic and basic functions giving multiple ionic species for all the values of pH we screened for HPLC purification.



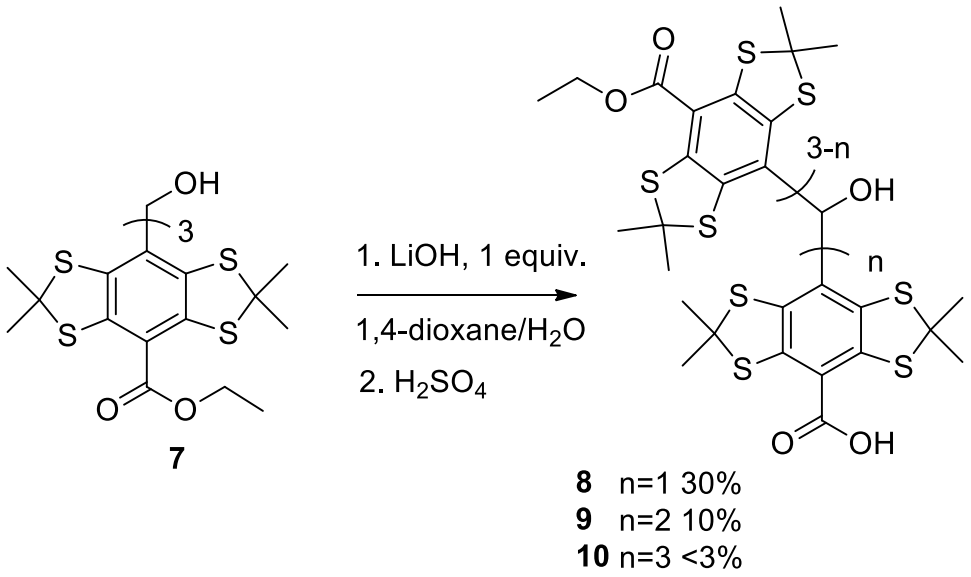
Scheme 1: First strategy envisaged for the synthesis of a TAM radical bound to the peptidomimetic **2**.

In order to overcome the purification difficulties, an alternative synthetic strategy was imagined with a double objective. Firstly, in order to limit the number of ionic species, and to allow purification on silica gel, we decided to attach only one peptidomimetic moiety on the TAM core and to use an acid-protected form of the peptidomimetic **2** (n=3). Secondly, in order to keep the possibility of analysing the molecule by nuclear magnetic resonance (NMR) spectroscopy, we chose to use the TAM alcohol instead of the radical for the coupling reaction. Starting from the bis-protected intermediate **5**,[16] the selective Boc deprotection was performed by treatment with three equivalents of sulphuric acid in *tert*-butyl acetate (Scheme 2).[19] Under these particular conditions, amine deprotection is complete because the resulting amine is stabilised by protonation, while the deprotection of the carboxylic acid is reversible. Indeed, the *tert*-butyl cation, produced from the reaction between *tert*-butyl acetate and sulphuric acid, can re-esterify the carboxylic acid into *tert*-butyl ester. After a basic work-up, the selectively Boc-deprotected compound **6** was isolated in 50% yield by flash chromatography.



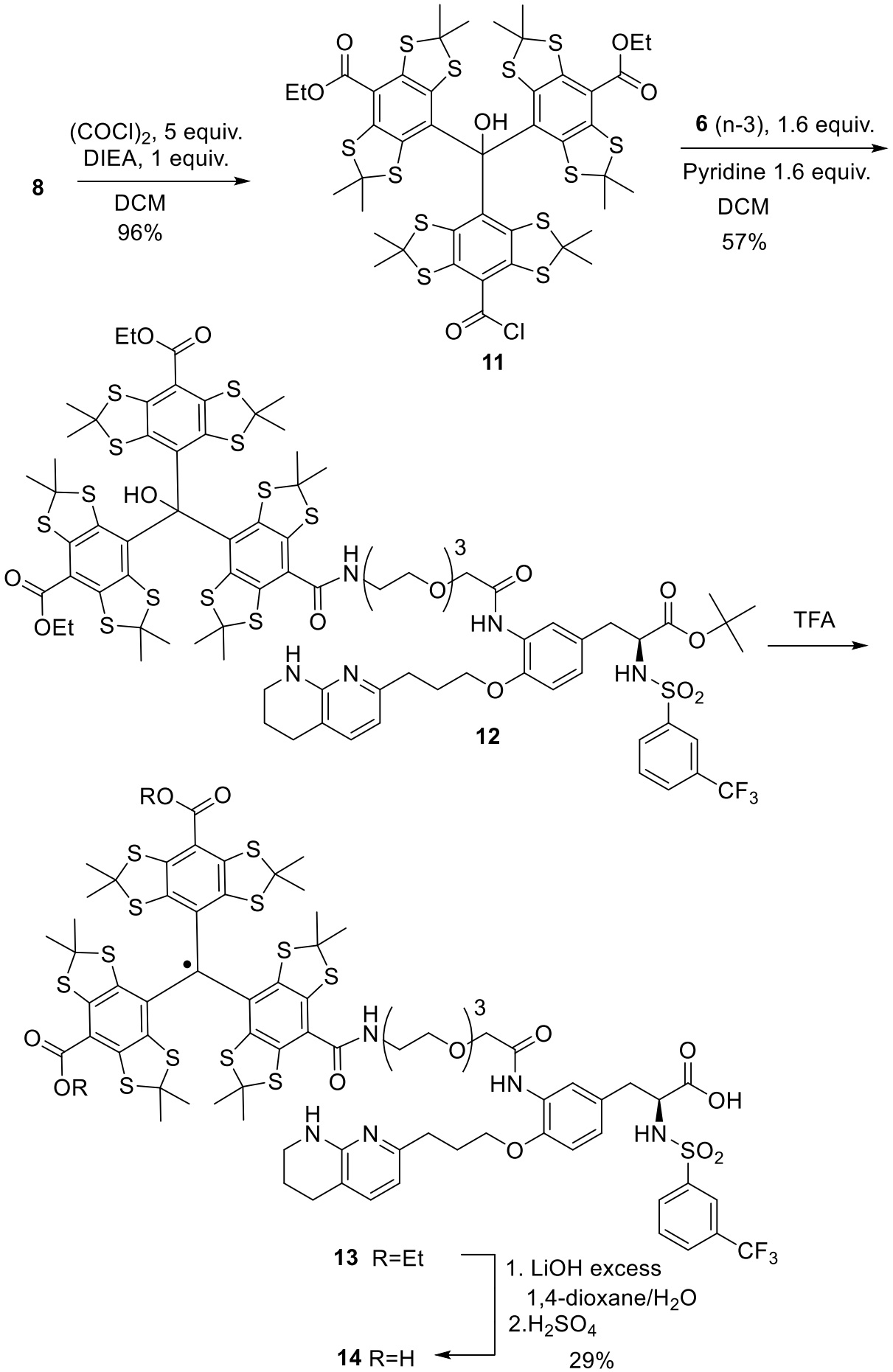
Scheme 2: Selective *N*-Boc deprotection of the *bis*-protected pepidomimetic **5**.

The tri-ethyl ester of TAM alcohol **7** has been synthesized using a procedure described in the literature[20] with slight modifications and identification of side products never reported before (see supporting Information). Treatment of **7** with only one equivalent of aqueous LiOH in refluxing 1,4-dioxane led to the partial, statistical hydrolysis of **7** (Scheme 3). Flash chromatography allowed the recovery of about 40% of the starting material **7**, 30% of the desired mono-hydrolysed compound **8** and 10% of the di-hydrolysed compound **9**. Due to the high polarity of the tri-hydrolyzed compound **10**, it was not recovered from the silica gel column.



Scheme 3: Partial hydrolysis of TAM alcohol **7**.

Activation of the mono-hydrolysed compound was achieved by treating **8** with an excess of oxalyl chloride and one equivalent of diisopropylethylamine (DIEA) in dichloromethane (Scheme 4). The acyl chloride **11** was recovered in 96% yield by simple filtration on a small silica gel path. It is worth noting that no reaction was observed between the trityl alcohol and oxalyl chloride, probably due to the steric protection of the hydroxyl function.


8
(COCl)2, 5 equiv.
DIEA, 1 equiv.
DCM
96%
11
6 (n-3), 1.6 equiv.
Pyridine 1.6 equiv.
DCM
57%
12
TFA
13 R=Et
14 R=H
1. LiOH excess
1,4-dioxane/H2O
2.H2SO4
29%

Scheme 4: Activation of TAM alcohol **8**, coupling with peptidomimetic **6** and deprotection of the *tert*-butyl ester with concomitant formation of the TAM radical followed by hydrolysis of the ethyl esters.

In the next step, the selectively deprotected peptidomimetic **6** was reacted with the activated TAM compound **11** in the presence of pyridine in dichloromethane. The resulting coupling product **12** can be purified by flash chromatography and isolated in 57% yield (Scheme 4). The assignment of the NMR signals of this complex molecule was realized using 2D sequences such as COSY, HMBC, HMQC (see supporting information). Then, treatment of **12** by neat trifluoroacetic acid (TFA) led, simultaneously to the deprotection of the *tert*-butyl ester and the conversion of the alcohol into the TAM radical (Scheme 4). The formation of tetrathiatriarylmethyl radical by treatment of tetrathiatriarylmethyl alcohol with neat TFA has been previously reported.[21] Purification by semi-preparative HPLC carried out on reverse phase allowed the isolation of the pure trityl spin probe **13** bound to the RGD-peptidomimetic moiety in 39% yield. In order to enhance the hydrophilicity of this new spin label, the two remaining ethyl esters were hydrolysed by an excess of LiOH. The bis-acid **14** was purified by semi-preparative HPLC carried out on reverse phase to give the final compound in 29% yield (Scheme 4). The relative low yields of the two last steps can be explained by the loss of material during the HPLC purification (fraction collected at the maximum of the peak).

The two novel radicals **13** and **14** were isolated in high purity as shown by their chromatograms obtained under gradient elution (Figure 3). In addition, the radicals are stable in a methanol solution at room temperature for at least 48h (no trace of degradation products from the HPLC analysis). The pure radicals can be store for months in the freezer.

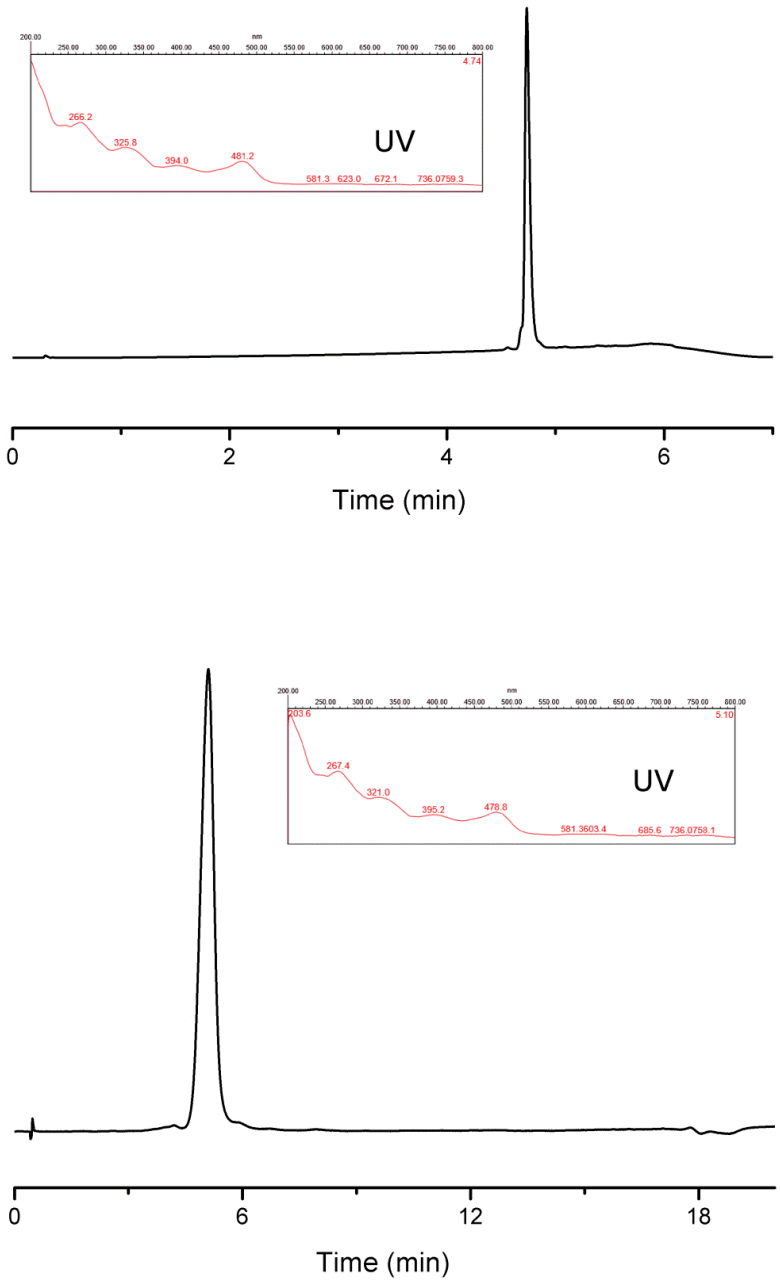


Figure 3: Chomatograms and UV spectra of TAM radicals **13** (up) and **14** (down) obtained under gradient elution.

The EPR spectra of compounds **13** and **14** have been recorded in X-band (Figure 4). Under normal air room conditions (21% oxygen), the spectra showed a single EPR line for both radicals with a peak-to-peak linewidth (ΔB_{pp}) of 0.0930 µT and 0.0654 µT for compounds **13** and **14**, respectively. These observed linewidths are larger than for traditional

derivatives (**CT-03** and **Ox063**). This fact can be explained by hyperfine splitting between the radical and the nitrogen atom of the amide function of **14**. For **13**, the broadening can arise from hyperfine splitting between the radical and the methylene protons of the ethyl esters as well as the nitrogen of the amide function. The spectra recorded under nitrogen (0% oxygen) are given as supplementary data.

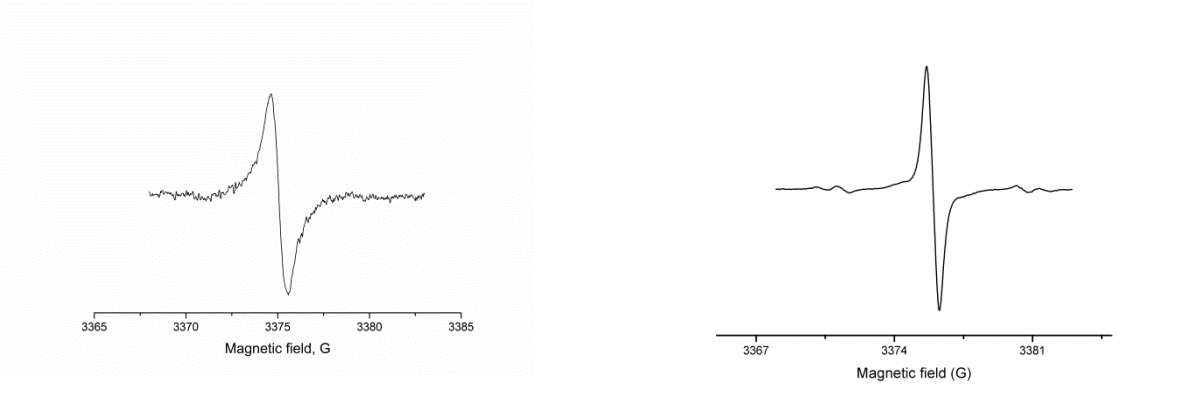


Figure 4: EPR spectra of **13** and **14** (500 µM) recorded at X-band in ACN/PBS 1:1 for **13** (left) and MeOH/PBS (1:1) for **14** (right)under normal air condition.

Conclusion

We have synthesized two original TAM radicals conjugated to a RGD-peptidomimetic molecule whose antagonist activity against $\alpha_v\beta_3$ integrin has been demonstrated in previous publications. The present work represent the first example of EPR spin labels linked to a specific addressing motif of receptor, although probes conjugated to ligands of $\alpha_v\beta_3$ integrin have already been described in the context of other imaging techniques such as MRI, SPECT (single photon emission tomography), PET (positron emission tomography) and FMT (fluorescence mediated tomography).[22] Generally, RGD-peptides and cyclopeptides are used; RGD-peptidomimetics have been considered in few cases, as exemplified by the coupling of **2** (n=6) to ultra small particules of iron oxide (USPIO).[18]

The stability of radicals **13** and **14**, and their EPR characeristics are adequate for the further development of imaging applications. The success of our synthetic strategy is built on several chemical tricks which can be useful in general for the difficult TAM radical chemistry. By keeping the alcohol function on the trityl core for most of the reactions, we allow the

accurate characterization of intermediates by NMR spectroscopy. Moreover activation of the carboxyl function with oxalyl chloride is an efficient method to produce the acyl chloride in high yield with no alteration of the TAM hydroxyl function. By contrast, all attempts to perform the coupling reaction with *in situ* activation reagents (BOP, carbodiimides) failed (data not shown) although they are reported to be efficient on the TAM radical.[23] Finally, the partial hydrolysis of TAM **7** allows isolation of the mono-acid **8** in 52% yield (based on the recovery of starting material (SM)) and the bis-acid **9** in 17% yield (based on the recovery of SM). While **8** was used here to bind only one carrier molecule, compound **9** could be similarly used to link the trityl core to two dedicated entities.

Acknowledgement

This work was supported by the Université catholique de Louvain (UCL) and the fond de la recherche scientifique (F.R.S-FNRS, Belgium). Jacqueline Marchant-Brynaert is a senior research associate of the F.R.S-FNRS. The authors gratefully acknowledge Laurent Collard for his help in HPLC purifications as well as Dr. Cecile Le Duff and Martin Poncelet for helpful discussions.

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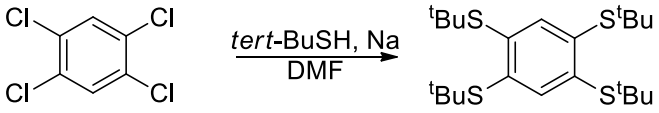
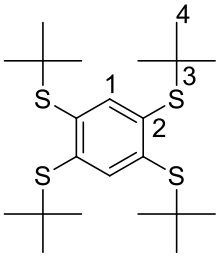
Experimental part

Synthesis of S1



S1

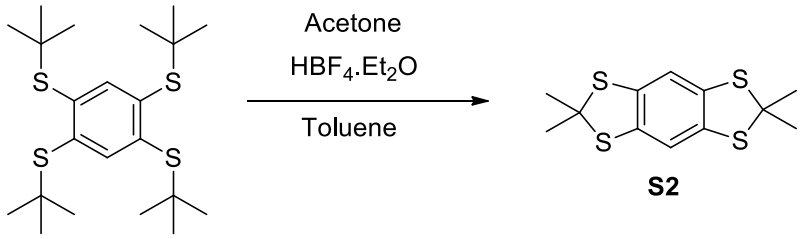
To metallic sodium (22.3 g, 0.97 mol, 6 equiv.) in dry DMF (500 mL) at 0°C was added 2-methyl-2-propanethiol (109 mL, 0.97 mol, 6 equiv.), The mixture was stirred at 0°C for 4h and overnight at r.t. Then, 1,2,4,5-tetrachlorobenzene (35.0 g, 162 mmol, 1 equiv.) was added and the resulting mixture was refluxed for 24h. The mixture was allowed to reach r.t. and water (450 mL) was added to precipitate the product. The mixture was filtered and the solid washed with water. Then, the white solid was dissolved in DCM and dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure to give 41.5 g (59%) of the title compound S1.



S1

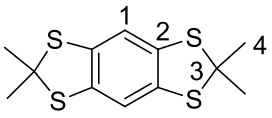
Appearance : White solid.
R*f* value : 0.50 hexane/DCM (1 :1).
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) : 1.37 (s, 36H, H-4), 7.95 (s, 2H, H-1).
^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) : 31.5 (C-4), 48.3 (C-3), 139.5 (C-2), 145.0 (C-1).
RP HPLC : Rt= 15.0 min (254nm, 1mL/min, ACN 100%, Symmetry C18 (4.6 x 250 mm), 25°C).

Synthesis of S2



S1 **S2**

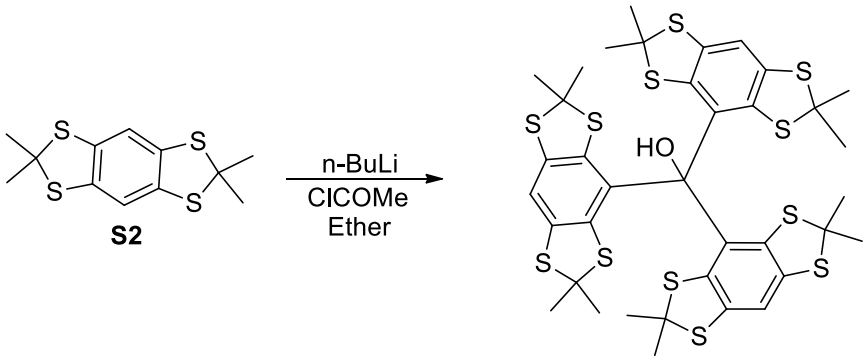
To a suspension of S1, (25 g, 0.058 mol, 1 equiv.) in toluene (230 mL) was added acetone (23 mL) and fluoroboric acid diethyl ether complex (15.93 ml, 0.116 mol, 2 equiv.). The solution was stirred at r.t. for 5h and then refluxed overnight. The mixture was allowed to reach r.t. and was quenched with $NaHCO_3$ sat. (250 mL). The aqueous layer was extracted with diethyl ether (3 x 200 mL). The combined organics layer were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The by-product, *p-tert*-butyltoluene was totally evaporated by heating the crude yellow solid under vacuum (10^{-3} mbar). The resulting solid was washed successively with *n*-hexane and methanol until a clean white product was obtained (12.82 g, 77%) The combined filtrates were concentrated under reduced pressure and 2.22 g (13%) of pure title compound was recovered by flash chromatography using DCM/hexane (1:2).



S2

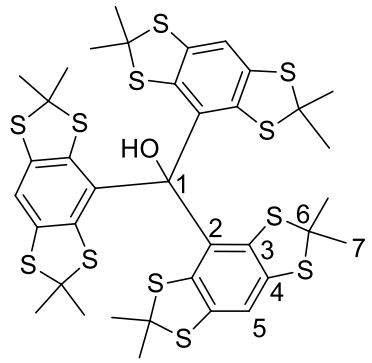
Appearance : White solid
R*f* value : 0.58 hexane/DCM (1 :1)
0.47 hexane/DCM (2 :1)
^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) : 1.89 (s, 12H, H-4), 7.03 (s, 2H, H-1).
^{1}H NMR (300 MHz, C_6D_6) δ (ppm) : 1.59 (s, 12H, H-4), 6.67 (s, 2H, H-1).
^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) : 31.5 (C-4), 65.9 (C-3), 117.0 (C-1), 135.9 (C-2).
^{13}C NMR (75 MHz, C_6D_6) δ (ppm) : 31.2 (C-4), 65.7 (C-3), 117.2 (C-1), 136.3 (C-2).
RP HPLC : Rt = 4.9 min (254nm, 1mL/min, ACN 100%, Symmetry C18 (4.6 x 250 mm), 25°C).

Synthesis of S3



S2 **S3**

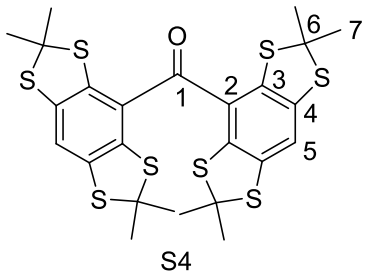
To a refluxed suspension of S2 (16 g, 0.0558 mol, 3 equiv.) in diethyl ether (400 mL) was added *n*-Buli in pentane (23.5 mL, 0.0058 mol, 3 equiv.) (n.b: BuLi was titrated prior to use and was 2.371 M). The mixture was refluxed for 2h, the heating was stopped and chloroformate (1.44 mL, 0.018 mol, 1 equiv.) in diethyl ether (15 mL) was added over 20h45 using a syringe pump. The solution was stirred at r.t. for 36h and was then quenched with KH_2PO_4 sat.(400 mL). The mixture was stirred until the solid disappeared. The organic layer was separated and the aqueous layer was extracted with diethyl ether (3 x 400 mL). The combined organic layers were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude product (17.7 g) was washed with a refluxed solution of THF/CH_3CN (95:5) during 30 min. The heterogeneous solution was allowed to reach r.t. and was keep without stirring overnight. The solid was filtered, washed with *n*-hexane (15 mL), then dissolved in dry benzene. Evaporation of the solvent under reduced pressure gave 8.016 g of S3 containing 1.6 equiv. of benzene (43%). The filtrate was concentrated under reduced pressure and 3.286 g of the starting material was recovered by column chromatography using DCM/*n*-hex (1:2) to (1:1) as eluent. The ketone S4 was also recovered from the column chromatography.



S3

Appearance : Pale yellow solid.
R*f* value : 0.33 hexane/DCM (1 :1.)
^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) : 1.70 (s, 9H, H-7), 1.75 (s, 9H, H-7), 1.83 (s, 9H, H-7), 1.85 (s, 9H, H-7), 6.26 (s, 1H, OH), 7.20 (s, 3H, H-5).
^{1}H NMR (300 MHz, C_6D_6) δ (ppm) : 1.42 (s, 9H, H-7), 1.49 (s, 9H, H-7), 1.70 (s, 9H, H-7), 1.73 (s, 9H, H-7), 6.66 (s, 1H, OH), 6.98 (s, 3H, H-5).
^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) : 27.7 (C-7), 29.3 (C-7) 32.4 (C-7) 35.0 (C-7), 63.5 (C-6), 64.2 (C-6), 83.8 (C-1), 118.3 (C-5), 132.0, 137.4, 138.0, 138.5, 139.4 (C-2, C-3, C-3, C-4, C-4).
^{13}C NMR (75 MHz, C_6D_6) δ (ppm) : 27.5 (C-7), 29.1 (C-7) 32.1 (C-7) 34.9 (C-7), 63.5 (C-6), 64.1 (C-6), 84.1 (C-1), 118.6 (C-5), 132.6, 137.7, 138.7, 139.2, 139.4 (C-2, C-3, C-3, C-4, C-4).
RP HPLC : Rt= 13.0 min (254nm, 1mL/min, ACN 100%, Symmetry C18 (4.6 x 250 mm), 25°C).

By product S4:

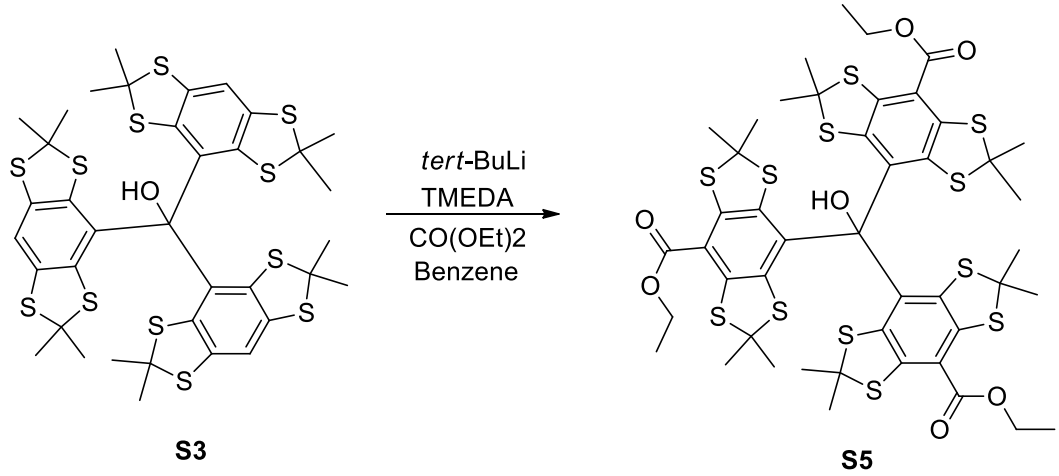


S4

Appearance : Orange solid
R*f* value : 0.28 hexane/DCM (1 :1)
^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) : 1.82 (s, 24H, H-7), 7.17 (s, 2H, H-5).
^{1}H NMR (300 MHz, C_6D_6) δ (ppm) : 1.56 (s, 24H, H-7), 6.68 (s, 2H, H-5).
^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) : 31.3 (C-7), 65.5 (C-6), 119.5 (C-5), 127.4, 137.7, 137.8 (C-2, C-3, C-4), 193.7 (C-1).
^{13}C NMR (75 MHz, C_6D_6) δ (ppm) : 30.9 (C-7), 65.3 (C-6), 119.6 (C-5), in C_6D_6 signal, 138.1, 138.2 (C-2, C-3, C-4), 193.3 (C-1).

RP HPLC : Rt = 6.9 min (254nm, 1mL/min, ACN 100%, Symmetry C18 (4.6 x 250 mm), 25°C).

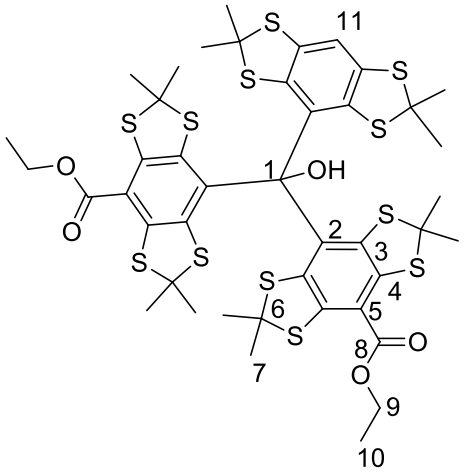
Synthesis of S5



S3 **S5**

To a stirred solution of S3 containing 1.6 of benzene (3g, 0.0031 mol, 1equiv.) in benzene (40 mL), was added TMEDA (4.7mL, 0.031 mol, 10 equiv.). The solution was cooled at as 0°C and *t*-BuLi in pentane (titrated to 2.1 M) solution (14.43 mL, 0.031 mol, 10 equiv.) was added. The solution was allowed to reach r.t. and was stirred for 2h30. This solution was cooled at 0°C and added dropwise to a solution of diethyl carbonate (19 mL) in benzene (35 mL) at 0°C. The mixture was stirred at r.t. overnight. KH_2PO_4 sat. (80 mL) was added and the layers separated. The organic layer was extracted with DCM (3 x 80 mL). The combined organic layers were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude solid was heated at 55°C under 10^{-3} mbar. The residual solid was recrystallized in ACN (75 mL) to recover 1.122g of S5. The filtrate was concentrated under reduced pressure and the residue purified by column chromatography using DCM/n-hex (3 : 1) to DCM 100%. Compound S5 recovered by chromatography was recrystallized in ACN (20 mL) to give 671 mg of pure S5. Total yield: 52%. By-product S6, S7 and S8 was also recovered from chromatography.

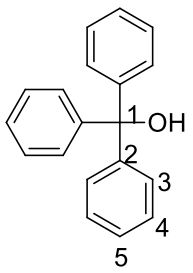
By product S6 :



S6

R*f* value : 0.6 DCM
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) : 1.46 (t, *J*=7.06, 3H, H-10), 1.46 (t, *J*=7.05, 3H, H-10), 1.64 (s, 3H, H-7), 1.67 (s, 3H, H-7), 1.67 (s, 3H, H-7), 1.68 (s, 6H, H-7), 1.74 (s, 3H, H-7), 1.75 (s, 3H, H-7), 1.78 (s, 6H, H-7), 1.79 (s, 6H, H-7), 1.82 (s, 3H, H-7), 4.45 (m, 4H, H-9), 6.72 (s, 1H, OH), 7.20 (s, 1H, H-11).

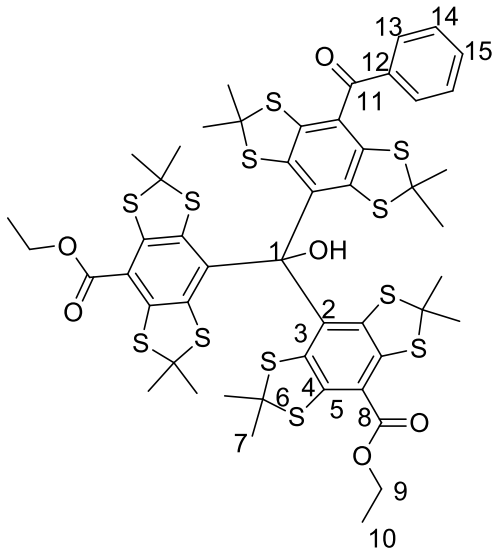
By product S7



S7

^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 2.95 (s, 1H, OH), 7.37 (m, 15H, H-ph),

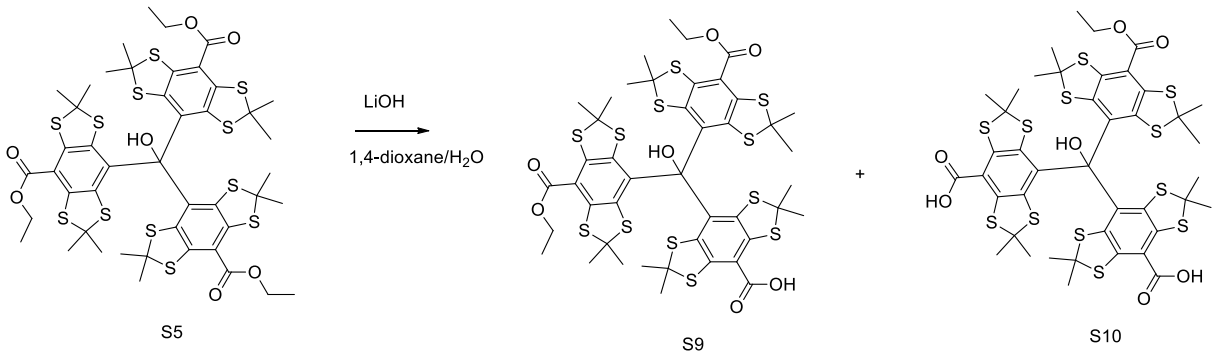
By product S8



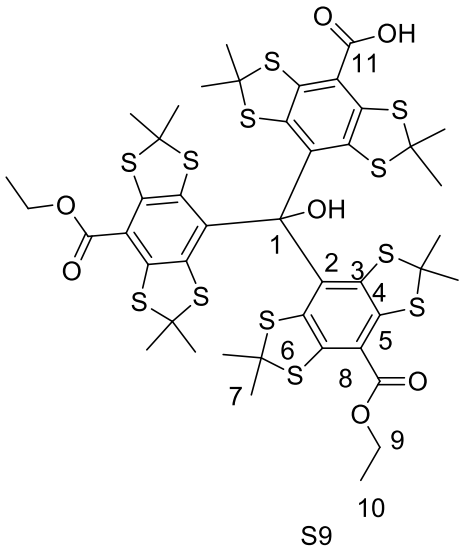
S8

Appearance : Orange solid.
R*f* value : 0.45 DCM
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.48 (t, J=7.0 Hz, 3H, H-10), 1.48 (t, J=7.0 Hz, 3H, H-10), 1.55 (s, 6H, H-7), 1.62 (s, 6H, H-7), 1.67 (s, 3H, H-7), 1.68 (s, 3H, H-7), 1.74 (s, 3H, H-7), 1.71 (s, 3H, H-7), 1.76 (s, 3H, H-7), 1.80 (s, 3H, H-7), 1.81 (s, 3H, H-7), 1.84 (s, 3H, H-7), 1.84 (s, 3H, H-7), 4.45 (m, 4H, H-9), 6.80 (s, 1H, OH), 7.43 (t, J=7.6 Hz, 2H, H-14), 7.62 (m, 1H, H-15), 7.99 (d, J=7.1 Hz, 2H, H-13).
HRMS (TOF MS ES$^-$) : m/z calcd for [$C_{50}H_{51}O_6S_{12}$] 1131.0334, found: 1131.0396.

Synthesis of S9

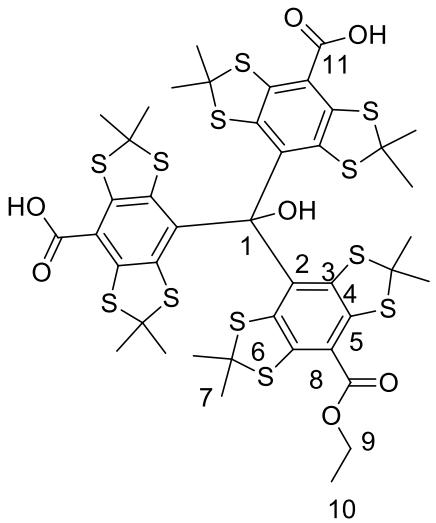


To a stirred solution of S5 (490 mg, 0.444 mmol, 1 equiv.) in dioxane (15 mL) was added LiOH (10.6 mg, 0.444 mmol, 1 equiv.) in water (5 mL).The mixture was refluxed for 20 h. The solvent was removed under reduced pressure and EtOAc (20 mL) and H_2SO_4 1M (5 mL) were added. The layers were separated and the aqueous layer extracted with EtOAc (3 x 10 mL). The combined organic layers were dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue purified by column chromatography using DCM 100% to DCM /MeOH (7:3) to recover 196 mg (40% of the starting material), 147 mg (30%) of the desired S9 compound and finally 10% of the dihydrolyzed compound S10.



S9

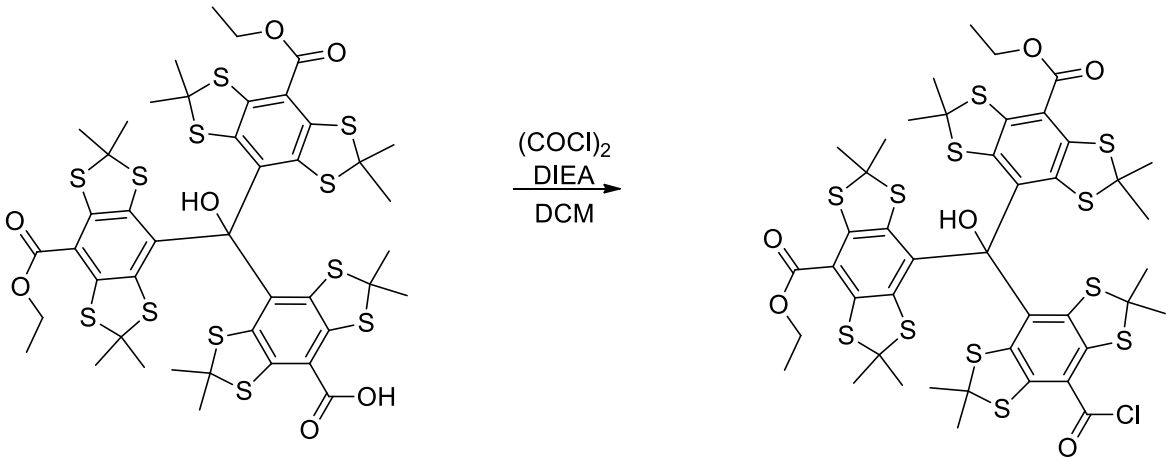
Appearance: Orange solid.
R*f* value: 0.5 DCM/MeOH (9:1).
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.45 (t, *J*=7.10, 6H, H-10), 1.65-1.77 (several singlets, 36H, H-7), 4.44 (m, 4H, H-9), 6.79 (s, 1H, OH).
^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 14.4 (C-10), 28.4 (C-7), 28.8 (C-7), 28.9 (C-7), 29.1 (C-7), 29.1 (C-7), 29.8 (C-7), 31.4 (C-7), 32.2 (C-7), 32.3 (C-7), 33.6 (C-7), 34.0 (C-7), 34.3 (C-7), 60.9 (C-6), 60.9 (C-6), 61.0 (C-6), 61.0 (C-6), 61.0 (C-6), 61.4 (C-6), 62.5 (C-9), 62.5 (C-9), 84.4 (C-1), 121.4 (3C, C-Ar), 133.8 (C-Ar), 134.1 (C-Ar), 134.7 (C-Ar), 138.9 (C-Ar), 139.5 (C-Ar), 139.6 (C-Ar), 140.3 (C-Ar), 140.4 (C-Ar), 140.6 (C-Ar), 141.6 (C-Ar), 141.7 (C-Ar), 141.9 (C-Ar), 142.0 (C-Ar), 142.4 (C-Ar), 143.0 (C-Ar), 166.3 (C-8), 171.2 (C-11).
HRMS TOF ES^{-} : m/z calcd for [$C_{44}H_{47}0_7S_{12}$] 1070.9970, found: 1070.9915.



S10

Appearance : Orange solid.
R*f* value: 0.3 DCM/MeOH (7:3).
^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) : 1.34 (t, *J*=7.08, 3H, H-10), 1.54 (s, 3H, H-7), 1.54 (s, 3H, H-7), 1.57 (s, 3H, H-7), 1.58 (s, 6H, H-7), 1.62 (s, 3H, H-7), 1.63 (s, 3H, H-7), 1.64 (s, 9H, H-7), 1.69 (s, 3H, H-7), 1.70 (s, 3H, H-7), 4.33 (m, 2H, H-9), 6.76 (s, 1H, OH).
^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 14.0 (C-10), 27.4 (C-7), 27.5 (C-7), 27.9 (C-7), 28.0 (C-7), 28.7 (C-7), 29.7 (C-7), 30.5 (C-7), 31.8 (C-7), 32.1 (C-7), 34.2 (C-7), 34.2 (C-7), 24.4 (C-7), 59.2 (C-6), 59.5 (C-6), 59.7 (C-6), 59.9 (C-6), 60.4 (C-6), 60.7 (C-6), 62.1 (C-9), 83.5 (C-1), 120.6 (3C, C-Ar), 130.0-145.0 (15C, C-Ar), 165.3 (C-8), 169.6 (C-11).
HRMS (TOF MS ES$^-$) m/z: calcd for [C42H4307S12] 1042.9657, found: 1042.9702.

Synthesis of S11



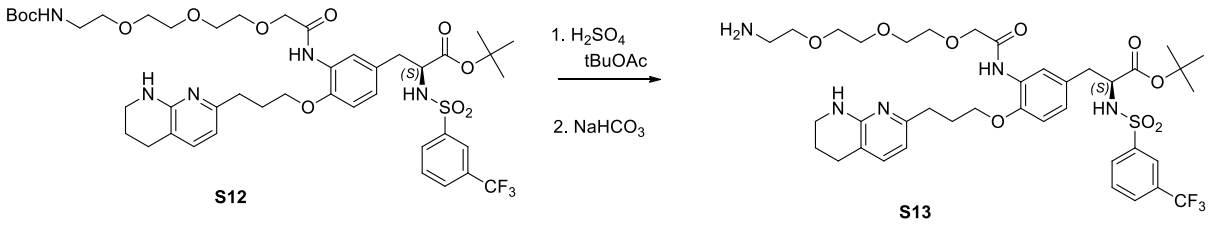
S9 **S11**

To a stirred solution of S9 (147 mg, 0.137 mmol, 1 equiv.) in DCM (10 mL) was added DIEA (13 µL, 0.137 mmol, 1 equiv.). The solution was cooled to -78 °C and oxalyl chloride (59 µL, 0.685 mmol, 5 equiv.) was added. The red solution was stirred at -78°C for 15 min and

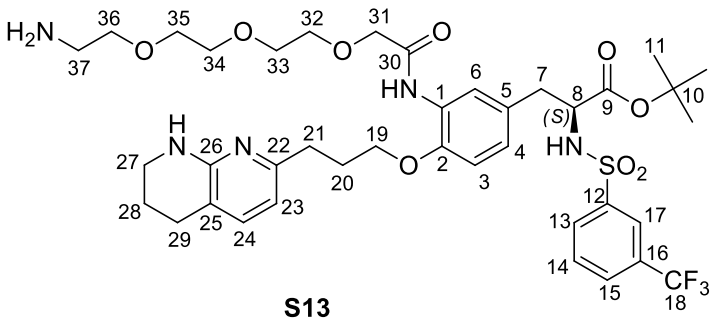
overnight at r.t. The solvent was removed under reduced pressure and the residue was purified by elution on a small path of silicagel (5 cm) using DCM. After evaporation of DCM 143 mg (96%) of S11 were recovered.

Apearance: Red solid.
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) : 1.47 (t, *J*=8.10, 3H, H-10), 1.47 (t, *J*=8.10, 3H, H-10), 1.66 (s, 3H, H-7), 1.67 (s, 6H, H-7), 1.68 (s, 3H, H-7), 1.70 (s, 3H, H-7), 1.72 (s, 3H, H-7), 1.75 (s, 3H, H-7), 1.77 (s, 9H, H-7), 1.79 (s, 3H, H-7), 1.80 (s, 3H, H-7), 1.80 (s, 3H, H-7), 4.44 (m, 4H, H-9), 6.78 (s, 1H, OH).
^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 14.4 (C-10), 28.2 (C-7), 28.3 (C-7), 28.5 (C-7), 29.1 (C-7), 29.6 (C-7), 30.5 (C-7), 30.8 (C-7), 32.3 (C-7), 32.8 (C-7), 33.3 (C-7), 34.0 (C-7), 34.3 (C-7), 61.0 (C-6), 61.1 (C-6), 61.2 (C-6), 61.8 (C-6), 62.6 (C-9), 63.3 (C-6), 63.6 (C-6), 84.3 (C-1), 121.5 (3C, C-Ar), 128.0 (C-Ar), 133.7 (C-Ar), 134.1 (C-Ar), 137.5 (C-Ar), 138.4 (C-Ar), 138.5 (C-Ar), 139.5 (C-Ar), 140.2 (C-Ar), 140.4 (C-Ar), 141.1 (C-Ar), 141.4 (C-Ar), 141.8 (C-Ar), 142.0 (C-Ar), 142.3 (C-Ar), 166.3 (C-8), 167.5 (C-11).

Synthesis of S13



To a pale yellow solution of S12 (400 mg, 0.433 mmol, 1 equiv.) in tBuOAc (30 mL) at 0°C was added dropwise a solution of H_2SO_4 (70 µL, 1.299 mmol, 3 equiv.) in tBuOAc (4 mL) over 10 min at 0°C. A white precipitate was formed during addition of sulfuric acid. The heterogeneous solution was stirred at 0°C for 10 min and 1 h at room temperature. The reaction was quenched with $NaHCO_{3\ sat.}$ and the mixture was vigorously stirred until total dissolution of the white-yellow solid. EtOAc (15 mL) was added and the layers separated. The aqueous layer was extract with EtOAc (3 x 20 mL). The combined organic layers were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The residue was purified by flash chromatography on deactived silica gel using DCM 90%/MeOH 10% to remove starting material and impurities, then the title compound was eluted with DCM 89%/MeOH 10%/Et_3N 1% to get 169 mg (47%) of S13 as a pale yellow oil.



S13

Appearance: Pale-yellow oil

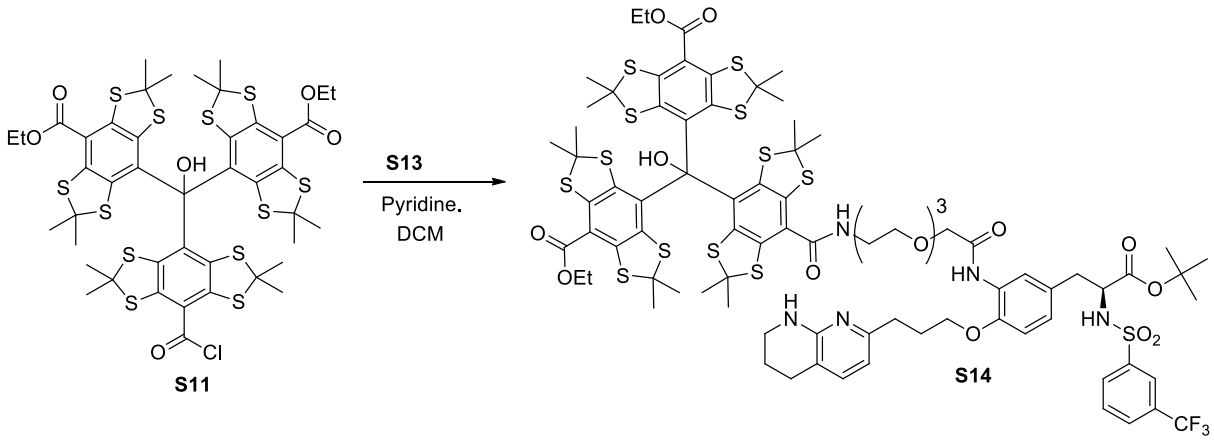
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.27 (s, 9H, H-11), 1.90 (m, 2H, H-28), 2.19 (m, 2H, H-20), 2.69 (t, *J*=6.3 Hz, 2H, H-29), 2.74 (t, *J*=7.5 Hz, 2H, H-21), 2.80 (m, 2H, H-37), 2.92 (dd, *J*= 6.6, 14.0 Hz, 1H, H-7), 2.99 (dd, *J*= 5.4, 14.0 Hz, 1H, H-7), 3.30 (bs, 3H, NH), 3.40 (m, 2H, H-27), 3.44 (t, *J*=5.2 Hz, 2H, H-36), 3.50-3.90 (m, 8H, H-32 to H-35), 4.00 (t, *J*= 6.4 Hz, 2H, H-19), 4.09 (dd, *J*=5.6, 6.4 Hz, 1H, H-8), 4.14 (bs, 2H, H-31), 5.17 (bs, 1H, NH), 6.35 (d, *J*=7.3 Hz, 1H, H-23), 6.71 (d, *J*=8.4 Hz, 1H, H-3), 6.81 (dd, *J*=2.1, 8.3 Hz, 1H, H-4), 7.06 (d, *J*=7.2 Hz, 1H, H-24), 7.54 (t, *J*=7.8 Hz, 1H, H-14), 7.72 (d, *J*=7.7 Hz, 1H, H-15), 7.94 (d, *J*=7.9 Hz, 1H, H-13), 8.02 (s, 1H, H-17), 8.10 (d, *J*=2.0 Hz, 1H, H-6), 9.00 (s, 1H, NH).

^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 21.5 (C-28), 26.5 (C-29), 27.8 (C-11), 29.2 (C-20), 33.8 (C-21), 38.8 (C-7), 41.7 (C-27+C-37), 57.5 (C-8), 68.0 (C-19), 70.3-71.3 (C-31 to C-35), 73.3 (C-36), 82.9 (C-10), 111.1 (C-3), 111.4 (C-23), 113.9 (C-25), 121.1 (C-6), 123.4 (q, *J*=272.9 Hz, C-18), 124.2 (q, *J*=3.9 Hz, C-17), 125.3 (C-4), 127.0 (C-1), 127.9 (C-5), 129.0 (q, *J*= 3.4 Hz, C-15), 129.7 (C-14), 130.6 (C-13), 131.4 (q, *J*=33.4 Hz, C-16), 136.9 (C-24), 141.9 (C-12), 147.0 (C-2), 156.0 (C-26), 156.6 (C-22), 167.8 (C-30), 170.0 (C-9).

^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm): -63.30 (s, 3F, F-18).

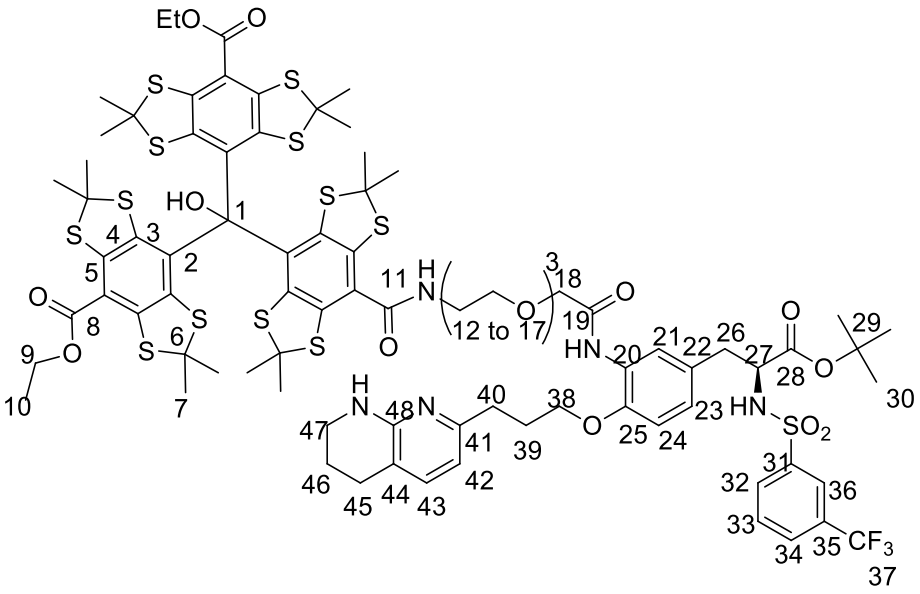
HRMS (TOF MS ES+) *m/z* : calcd for $C_{39}H_{53}F_3N_5O_9S$: 824.3516, found 824.3500.

Synthesis of S14



To a stirred solution of S13 (170 mg, 0.206 mmol, 1.6 equiv.) in DCM (6 mL) was added pyridine (16 µL, 0.206 mmol, 1.6 equiv.) and S11 (143 mg, 0.131 mmol, 1 equiv. in DCM (1 mL). The solution was stirred at r.t. for 24h then NH_4Cl sat.(3 mL) was added and the layers

were separated. $NaHCO_3$ sat. (3 mL) was added to the organic phase and the layers were separated. The aqueous phase was extracted with EtOAc (2x5mL). The combined organic layers were dried over $MgSO_4$ and the solvent removed under reduced pressure. The residue was purified by column chromatography using Acetone/EtOAc (from 1 :4 to 1 :1) as eluent to give 140 mg of S14 (57%).

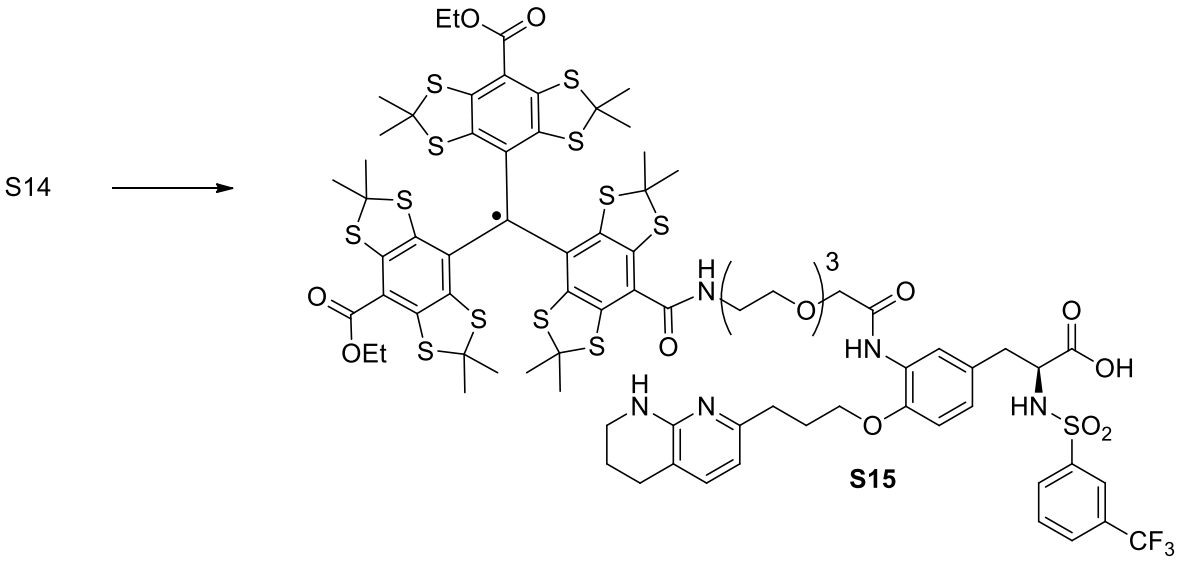


S14

Appearence : Orange solid
R*f* value : 0.25 EtOAc/Acetone (5 :1)
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.24 (s, 9H, H-30), 1.44 (t, *J*=7.0Hz, 6H, H-10), 1.61-1.76 (several singlets, 36H, H-7), 1.87 (m, 2H, H-46), 2.17 (m, 2H, H-39), 2.66 (t, *J*=6.1Hz, 2H, H-45), 2.73 (t, J=7.3Hz, 2H, H-40), 2.86 (dd, *J*=7.0, 13.9Hz, 1H, H-27), 2.98 (dd, *J*= 5.2, 13.9Hz, 1H, H-27), 3.37 (m, 2H, H-47), 3.54-3.77 (m, 12H, H-17 to H-12), 3.98 (t, *J*=6.1Hz, 2H, H-38), 4.05 (m, 1H, H-27), 4.11 (s, 2H, H-18), 4.42 (m, 4H, H-9), 5.44 (s, 1H, NH), 6.31 (d, *J*=7.2Hz, 1H, H-42), 6.68 (d, *J*=8.3Hz, 1H, H-24), 6.73 (s, 1H, OH), 6.8 (dd, *J*=1.7, 8.3Hz, 1H, H-23), 7.05 (d, *J*=7.2Hz, 1H, H-43), 7.53 (t, *J*=7.9Hz, 1H, H-33), 7.71 (d, J=7.7Hz, 1H, H-34), 7.91 (d, J=8.0Hz, H-32), 7.99 (s, 1H, H-36), 8.12 (s, 1H, H-21), 8.94 (s, 1H, NH).
^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm):14.4 (C-10), 21.0 (C-46), 26.3 (C-45), 27.7 (C-7), 27.8 (C-30), 29.3 (C-7), 29.4 (C-7), 29.7 (C-7), 30.5 (C-7), 31.8 (C-7), 32.7 (C-7), 32.7 (C-40), 33.0 (C-7), 33.8 (C-7), 34.4 (C-7), 34.9 (C-7), 38.9 (C-26), 39.8 (C-12), 41.5 (C-47), 57.3 (C-27), 60.9 (C-6), 61.1 (C-6), 61.3 (C-6), 61.9 (C-6), 62.0 (C-6), 62.6 (C-9), 67.6 (C-38), 69.5-71.5 (6 peaks: C18 to C13), 83.1 (C-29), 84.3 (C-1), 111.1 (C-24), 111.1 (C-42), 120.8 (C-21), 121.4 (3 C-Ar), 123.3 (q, J=273.1 Hz, C-37), 124.3 (q, J=3.2 Hz, C-36), 125.3 (C-23), 127.0 (C-20), 127.7 (C-22), 129.2 (q, J=2.8 Hz, C-34), 129.8 (C-33), 130.7 (C-32), 131.5 (q, J=33.6 Hz, C-35), 131.8 (C-Ar), 134.5 (C-Ar), 136.7 (C-Ar), 137.2 (C-Ar), 137.8 (C-43), 138.1 (C-Ar), 139.3 (C-Ar), 140.4-142.2 (9 peaks:

8 C-Ar + C-31), 146.7 (C-44), 155.1 (C-41+C-48), 166.3 (C-8), 166.3 (C-8), 167.1 (C-11), 167.6 (C-19), 169.9 (C-28).
HRMS (TOF MS ES$^+$) m/z: calcd for [$C_{83}H_{99}N_5O_{15}F_3S_{13}$] 1878.3459, found: 1878.3258

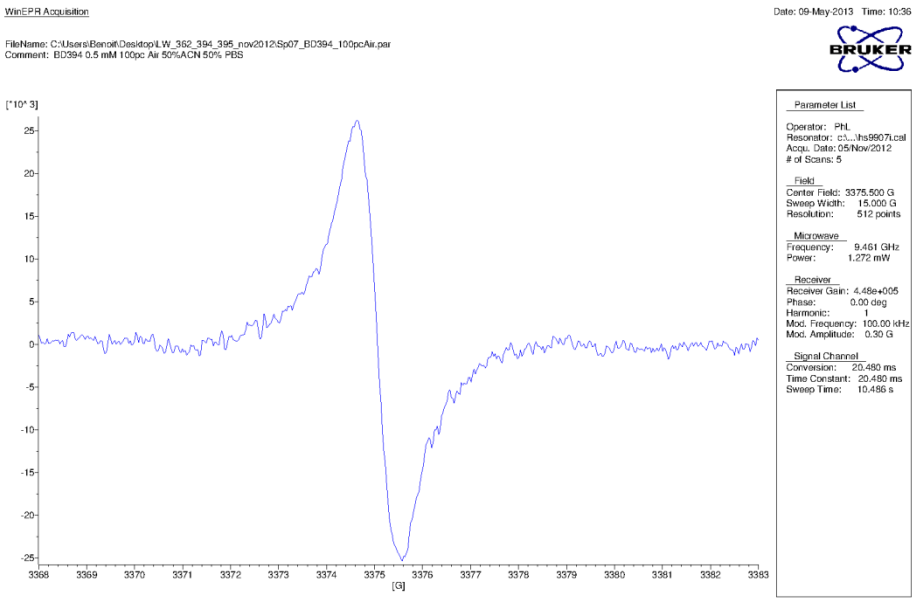
Synthesis of S15



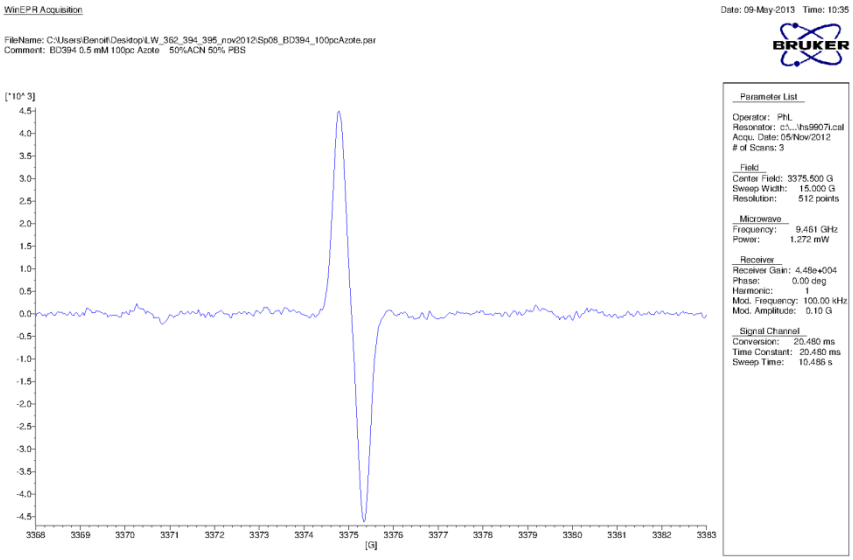
70 mg of S14 was dissolved in TFA (10 mL). The solution was stirred for 2h and then the TFA was removed under reduced pressure. The crude product was purified by semi-preparative HPLC to get 27 mg of pure S15. The conditions of purification were as folows; column XBride 10 x 100 mm 5 µm equipped with a guard column 10 x 20 mm 5 µm, UV detection at 485 nm, flow rate 5 mL/min. Isocratique elution with 70% ACN / 20 % H_2O / 10% H_2O containing 1% of TFA. (Fraction corresponding to the maximum of peak was collected).

HRMS (TOF MS ES$^+$) m/z: calcd for [$C_{79}H_{90}N_5O_{14}F_3S_{13}$] 1805.28001, found: 1805.27957

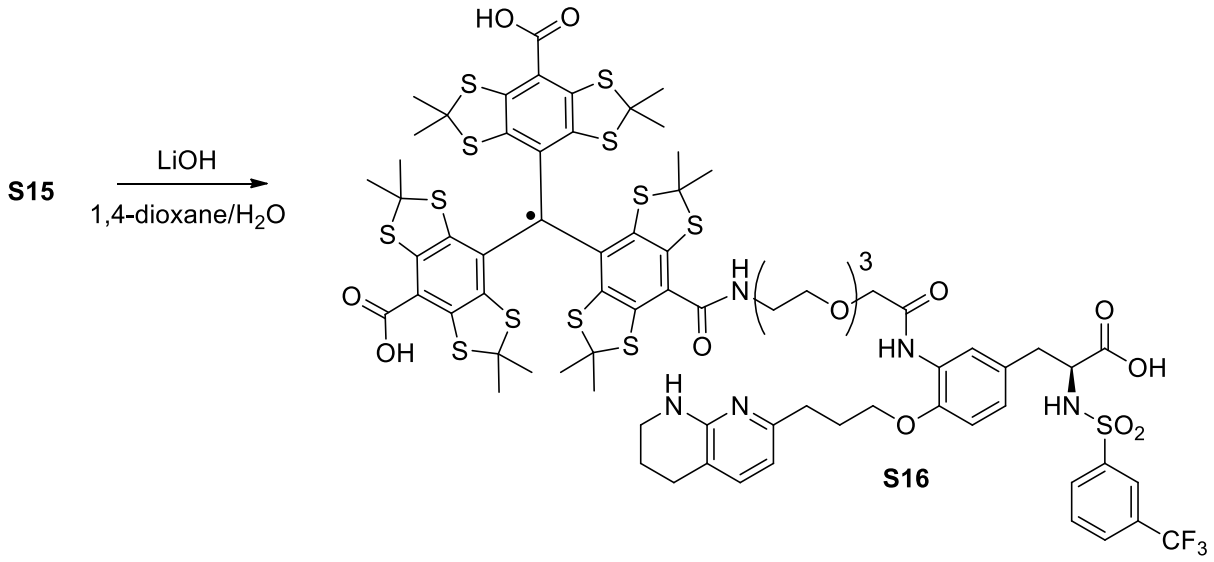
EPR X-Band 500 µM in ACN/PBS 1:1 under air room condition:



EPR X-Band 500 µM in ACN/PBS 1:1 under nitrogen condition:



Synthesis of S17



15 mg of S15 were dissolved in dioxane (2 mL). LiOH 1M (1 mL) was added and the solution was stirred overnight. HCl 1M (2 mL) and EtOAc (3 mL) were added and the layers were separated. The aquous layer was extracted with EtOAc (3 x 6 mL). The combined organic layers were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude product was purified by semi-preparative HPLC to give 9.3 mg (29%) of S16.

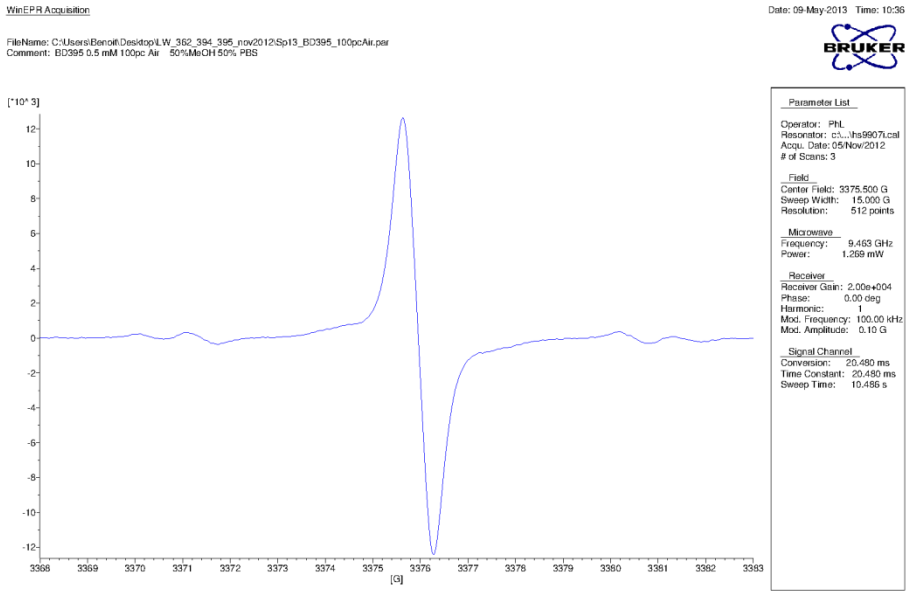
The conditions of purification were as follows: UV detection 486 nm, column XBridge 10 x 100 mm 5 µM equipped with a guard column 10 x 20 mm 5 µM. Gradient elution:

Time (min)	Flow (mL/min)	MeOH (%)	H_2O (%)	H2O 1%TFA (%)
/	5	68	22	10
10	5	68	22	10
11	5	90	0	10
14	5	90	0	10
14.1	5	68	22	10

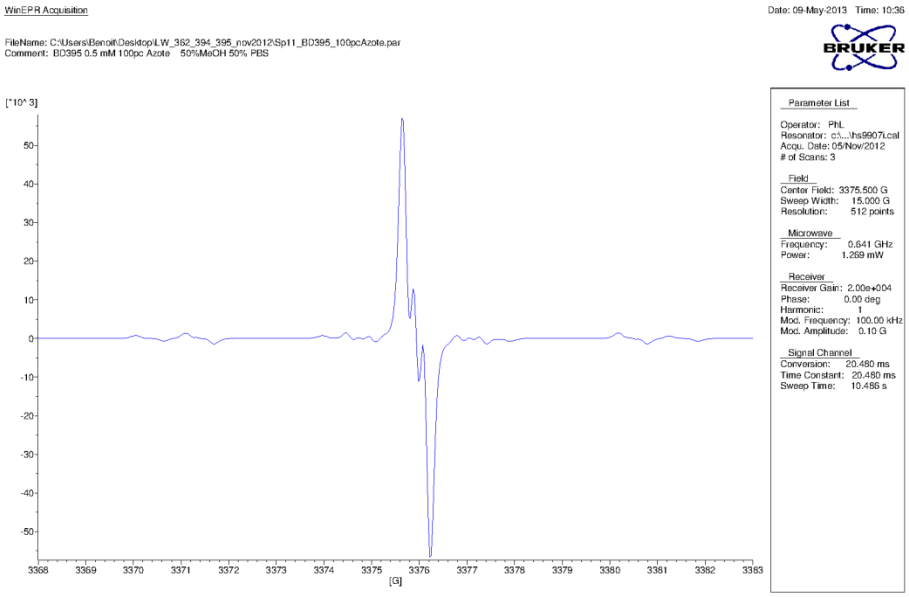
(Fraction corresponding to the maximum of the peak was collected)

HRMS (TOF MS ES$^+$) m/z: calcd for $[C_{75}H_{82}N_5O_{14}F_3S_{13}]$ 1749.21741, found: 1749.21683

EPR X-Band 500 µM in MeOH/PBS under air room condition



EPR X-Band 500 µM in MeOH/PBS under nitrogen condition



Partie expérimentale de l'article :

Ultrasmall particle of iron oxide-RGD peptidomimetic conjugate: synthesis and characterization

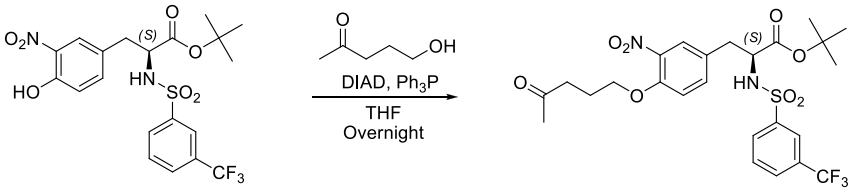
V. Rerat, S. Laurent, C. Burtéa, B. Driesschaert, V. Pourcelle, L. Vander Elst, R. N. Muller, J. Marchand-Brynaert, Bioorg. Med. Chem. Lett. **2010**, 20, 1861-1865.

General methods

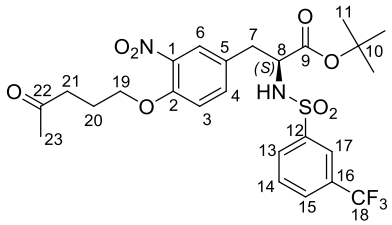
All reactions were carried out in flame-dried glassware under argon atmosphere and were monitored by thin layer chromatography (TLC) using aluminum sheets silica gel 60 F_{254} (Merck). Anhydrous grade solvents were used for reactions and HPLC grade for purifications. All commercially available reagents were used as received without further purification. Column chromatographies were done using Merck Silica Gel 60 (230-400 mesh). NMR spectra were recorded on a BRUKER AC-300 Avance II (^{1}H: 300 MHz, ^{13}C: 75 MHz, ^{19}F: 282 MHz) and a BRUKER AM-500 (^{1}H: 500 MHz, ^{13}C: 125 MHz). Total assignment of NMR signals were done through 2D analysis (COSY, HMQC, HMBC, HETCOR). Chemical shifts (δ) were reported in parts per million (ppm), referenced to NMR solvents: chloroform-d (δ ^{1}H=7.27 ppm, ^{13}C=77.2 ppm), methanol d-4 (δ ^{1}H=3.31 ppm, ^{13}C=49.05 ppm) or $CFCl_3$ (δ ^{19}F=0.00 ppm). The following abbreviations were used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, dd=doublet of doublets, ddd=doublet of doublet of doublets, bs=broad singlet etc. Coupling constants (*J*) were reported in hertz. The specific rotation values at 589 nm $[\alpha]_D$ were measured with a Perkin Elmer 241 MC Polarimeter and are given in deg $dm^{-1}g^{-1}cm^3$, concentrations are given in g/100mL.

The synthesis of starting material **1** tert-butyl (2S)-3-(4-hydroxy-3-nitrophenyl)-2-{[3-(trifluoromethyl)benzene]sulfonamido}propanoate was described by Biltress *et al*. (see ref 19b in text).

Synthesis of tert-butyl (2S)-3-{3-nitro-4-[(4-oxopentyl)oxy]phenyl}-2-{[3-(trifluoromethyl)benzene]sulfonamido}propanoate 2.



To a stirred solution of (*t*-butyl-(S)-3-(4'-hydroxy-3'-nitro-phenyl)-2-(3'-trifluoromethyl-1-benzenesulfonylamino)-propionate (4.770 g, 9.726 mmol, 1 eq.) in THF (140 mL) was added, triphenylphosphine (3.061 g, 11.671 mmol, 1.2 eq.) and 3-acetyl-1-propanol (986 µL, 9.726 mmol, 1 eq.). Diisopropyl azodicarboxylate (DIAD) (2.298 mL, 11.671 mmol, 1.2 eq.) was then added dropwise and the mixture was stirred at room temperature overnight. The solvent and volatile compounds were removed under reduced pressure. The residue was purified by flash chromatography using Et_2O/n-hexane (85:15) to afford 2.867 g (51%) of the title compound as a pale yellow foam or oil. R*f* value : 0.23 Et_2O/n-Hex (85:15) UV (254 nm).



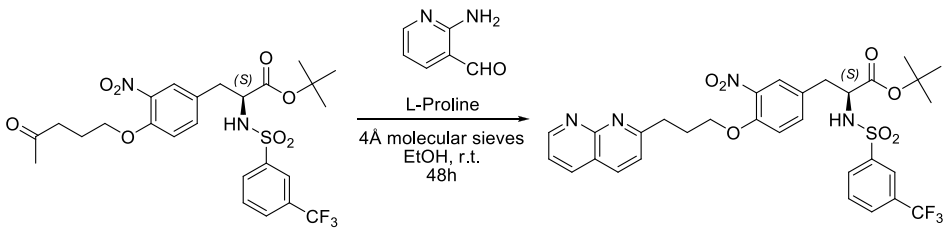
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) : 1.25 (s, 9H, H-11), 2.07 (m, 2H, H-20), 2.18 (s, 3H, H-23), 2.72 (t, *J*=6.9 Hz, 2H, H-21), 2.96 (dd, *J*=6.7, 14.1 Hz, 1H, H-7), 3.06 (dd, *J*=5.4, 14.1 Hz, 1H, H-7'), 4.06 (ddd, *J*=5.5, 6.7, 9.0 Hz, 1H, H-8), 4.09 (t, *J*=5.9 Hz, 2H, H-19), 5.59 (d, *J*=9.0 Hz, 1H, NH-SO_2), 6.95 (d, *J*=8.6 Hz, 1H, H-3), 7.36 (dd, *J*=2.2, 8.6 Hz, 1H, H-4), 7.58 (d, *J*=2.2 Hz, 1H, H-6), 7.61 (t, *J*=7.9 Hz, 1H, H-14), 7.79 (d, *J*=7.8 Hz, 1H, H-15), 7.96 (d, *J*= 7.9 Hz, 1H, H-13), 8.02 (s, 1H, H-17).

^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) : 23.0 (C-20), 27.8 (C-11), 30.2 (C-23), 38.1 (C-7), 39.4 (C-21), 57.1 (C-8), 68.4 (C-19), 83.8 (C-10), 114.7 (C-3), 123.2 (q, *J*=272.9 Hz, C-18), 124.2 (q, *J*=3.8 Hz, C-17), 126.6 (C-6), 127.7 (C-5), 129.5 (q, *J*=3.3 Hz, C-15), 130.1 (C-14), 130.6 (C-13),

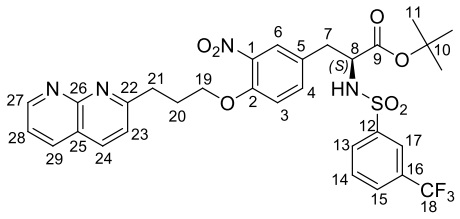
131.7 (q, *J*= 33.5 Hz, C-16), 135.7 (C-4), 139.2 (C-1), 141.1 (C-12), 151.6 (C-2), 169.3 (C-9), 208.4 (C-22).

^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) : -63.31 (s, 3F, F-18).

Synthesis of tert-butyl (2S)-3-{4-[3-(1,8-naphthyridin-2-yl)propoxy]-3-nitrophenyl}-2-{[3-(trifluoromethyl)benzene]sulfonamido}propanoate **3**.



To a stirred solution of tert-butyl (2S)-3-{3-nitro-4-[(4-oxopentyl)oxy]phenyl}-2-{[3-(trifluoromethyl)benzene]sulfonamido}propanoate (2.867 g, 4.990 mmol, 1 eq.) containing 4 Å molecular sieves in EtOH (50 mL) was added, 2-aminopyridine-3-carbaldehyde (670 mg, 5.489 mmol, 1.1 eq.) and L-proline (287 mg, 2.495 mmol, 0.5 eq.). The reaction mixture was refluxed for 48h, filtered and the solvent was removed under reduced pressure. 1.805 g (55%) of the pure Friedländer condensation product was recovered by flash chromatography using AcOEt/Me_2CO (94:6). R*f* value: 0.28 AcOEt/Acetone (94:6) UV (254 nm). Appearance : White solid.



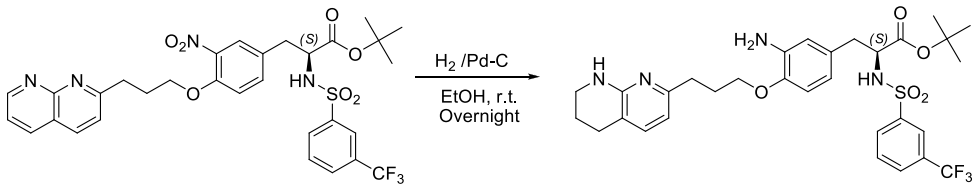
^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) : 1.26 (s, 9H, H-11), 2.53 (m, 2H, H-20), 2.98 (dd, *J*=6.2, 14.1 Hz, 1H, H-7), 3.06 (dd, *J*=5.5, 14.1 Hz, 1H, H-7'), 3.22 (t, *J*=7.3 Hz, 2H, H-21), 4.07 (ddd, *J*= 5.4, 6.3, 8.8 Hz, 1H, H-8), 4.22 (t, *J*=6.0 Hz, 2H, H-19), 5.32 (d, *J*=8.9 Hz, 1H, HN-SO_2), 6.99 (d, *J*=8.6 Hz, 1H, H-3), 7.34 (dd, *J*=2.2, 8.6 Hz, 1H, H-4), 7.47 (t, *J*=4.0 Hz, 1H, H-28), 7.48 (d, *J*=8.2 Hz, 1H, H-23), 7.59 (d, *J*=2.3 Hz, 1H, H-6), 7.62 (t, *J*=7.9 Hz, 1H, H-14), 7.79 (d, *J*=7.8 Hz, 1H, H-

15), 7.98 (d, *J*=7.9 Hz, 1H, H-13), 8.05 (s, 1H, H-17), 8.12 (d, *J*=8.3 Hz, 1H, H-24), 8.19 (dd, *J*=1.9, 8.0 Hz, 1H, H-29), 9.10 (dd, *J*=1.9, 4.3 Hz, 1H, H-27).

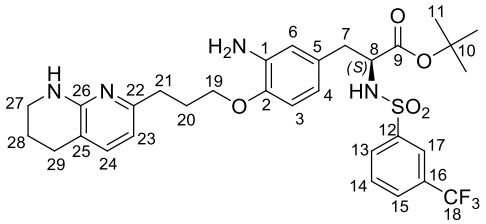
^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) : 27.8 (C-20), 27.9 (C-11), 34.9 (C-21), 38.3 (C-7), 57.0 (C-8), 68.9 (C-19), 84.0 (C-10), 114.8 (C-3), 121.4 (C-25), 121.8 (C-28), 123.3 (C-23), 124.4 (q, *J*=3.9 Hz, C-17), 126.6 (C-6), 127.4 (C-5), 129.6 (q, *J*=3.3 Hz, C-15), 130.1 (C-14), 130.6 (C-13), 131.9 (q, *J*=33.8 Hz, C-16), 135.7 (C-4), 137.1 (C-29), 137.4 (C-24), 139.4 (C-1), 141.1 (C-12), 151.9 (C-2), 153.5 (C-27), 156.0 (C-26), 165.5 (C-22), 169.3 (C-9), C-18 not detected.

^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) : -63.39 (s, 3F, F-18).

<u>Synthesis of tert-butyl (2S)-3-{3-amino-4-[3-(5,6,7,8-tetrahydro-1,8-naphthyridin-2-yl)propoxy]phenyl}-2-{[3-(trifluoromethyl)benzene]sulfonamido}propanoate **4a**.</u>



To a stirred solution of tert-butyl (2S)-3-{4-[3-(1,8-naphthyridin-2-yl)propoxy]-3-nitrophenyl}-2-{[3-(trifluoromethyl)benzene]sulfonamido}propanoate (1.805 g, 2.732 mmol) in ethanol (50 mL) was added palladium 10% on activated carbon (1 g). The heterogeneous mixture was hydrogenated at 1 bar overnight. Afterward, the reaction mixture was filtrated through a celite path and the solvent was removed under reduced pressure to provide 1.643 g (95%) of pure reduced compound as a pale yellow foam or oil. R*f* value: 0.30 AcOEt 100% UV (254, 366 nm). $[\alpha]_d$=-11.9 (c=2.39, $CHCl_3$).

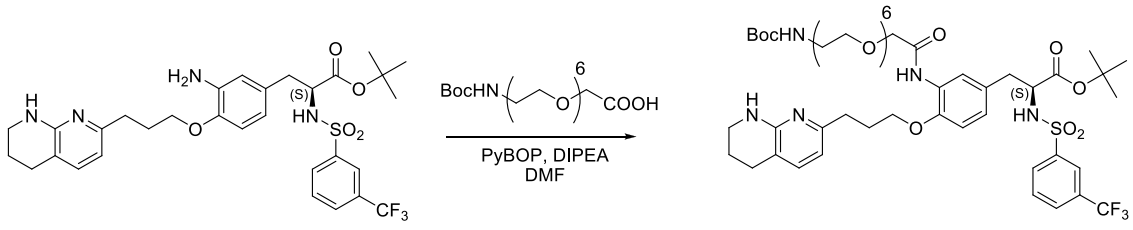


^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) : 1.24 (s, 9H, H-11), 1.91 (m, 2H, H-28), 2.18 (m, 2H, H-20), 2.70 (t, *J*=6.3 Hz, 2H, H-29), 2.74 (t, *J*=7.5 Hz, 2H, H-21), 2.89 (m, 2H, H-7), 3.41 (m, 2H, H-27), 3.75 (s, 2H, NH), 3.97 (t, *J*=6.3 Hz, 2H, H-19), 4.07 (dd, *J*=5.9, 5.9 Hz, 1H, H-8), 4.93 (s, 1H, NH), 5.25 (s, 1H, NH), 6.39 (m, 2H, H-4, H-23), 6.44 (d, *J*=1.9 Hz, 1H, H-6), 6.59 (d, *J*=8.1 Hz, 1H, H-3), 7.08 (d, *J*=7.3 Hz, 1H, H-24), 7.57 (t, *J*=7.9 Hz, 1H, H-14), 7.76 (d, *J*=7.6 Hz, 1H, H-15), 7.92 (d, *J*=7.9 Hz, 1H, H-13), 8.06 (s, 1H, H-17).

^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) : 21.6 (C-28), 26.5 (C-29), 27.8 (C-11), 29.4 (C-20), 34.2 (C-21), 38.9 (C-7), 41.7 (C-27), 57.4 (C-8), 67.8 (C-19), 82.8 (C-10), 111.4 (C-3 or C-23), 111.5 (C-3 or C-23), 113.6 (C-25), 116.0 (C-6), 119.3 (C-4), 123.3 (q, *J*=272.9 Hz, C-18), 124.3 (q, *J*=3.6 Hz, C-17), 127.5 (C-5), 129.2 (q, *J*=3.0 Hz, C-15), 129.8 (C-14), 130.6 (C-13), 131.5 (q, *J*=33.3 Hz, C-16), 136.5 (C-1), 136.9 (C-24), 141.6 (C-12), 146.0, (C-2), 155.9 (C-26), 157.3 (C-22), 170.1 (C-9).

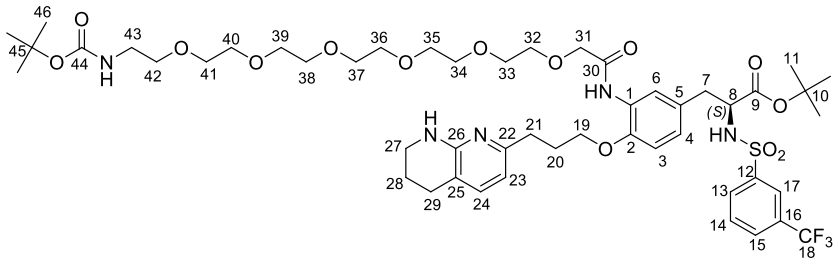
^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) : -63.22 (s, 3F, F-18).

Synthesis of tert-butyl (2S)-3-[3-(20-{[(tert-butoxy)carbonyl]amino}-3,6,9,12,15,18-hexaoxaicosanamido)-4-[3-(5,6,7,8-tetrahydro-1,8-naphthyridin-2-yl)propoxy]phenyl]-2-{[3-(trifluoromethyl)benzene]sulfonamido}propanoate **5a**.



To a stirred solution of 20-{[(tert-butoxy)carbonyl]amino}-3,6,9,12,15,18-hexaoxaicosanoic acid (60 mg, 0.136 mmol, 1.2 eq.) in DMF (1 mL) was added DIEPA (24 µL, 0.136 mmol, 1.2 eq.) and PyBOP (71 mg, 0.136 mmol, 1.2 eq.). A solution of tert-butyl (2S)-3-{3-amino-4-[3-(5,6,7,8-tetrahydro-1,8-naphthyridin-2-yl)propoxy]phenyl}-2-{[3-(trifluoromethyl)benzene]sulfonamido}propanoate (72 mg, 0.114 mmol, 1 eq.) in DMF (1 mL) was subsequently added dropwise to the mixture. The pale yellow solution was stirred at room temperature for 15 h. Afterward, 6 mL of AcOEt and 6 mL of aqueous LiCl. 5% was added and the layers were separated. The organic layer was washed with $LiCl_{aq.}$ 5% (3 X 6 mL) and saturated $NaHCO_{3aq.}$ (1 X 6 mL). The combined organic layers were dried over $MgSO_4$

and the solvent removed under reduced pressure. The crude yellow oil was purified by flash chromatography using Acetone/AcOEt (from 3:7 to 6:4) to give 40 mg (33%) of the coupling product as a pale yellow oil. R*f* value : 0.48 Acetone/AcOEt (1:1) UV (254, 366 nm). $[\alpha]_d$=+2.5 (c=2.60, $CHCl_3$).

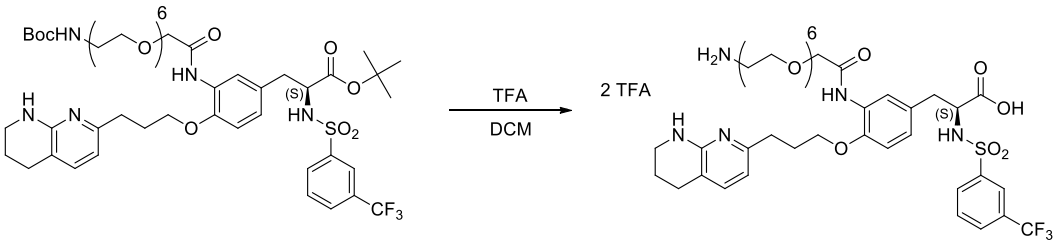


^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) : 1.26 (s, 9H, H-11), 1.43 (s, 9H, H-46), 1.90 (m, 2H, H-28), 2.19 (m, 2H, H-20), 2.69 (t, *J*=6.2Hz, 2H, H-29), 2.75 (t, *J*=7.4Hz, 2H, H-21), 2.88 (dd, *J*=7.0, 13.9 Hz, 1H, H-7), 3.00 (dd, *J*=5.3, 14.0 Hz, 1H, H-7'), 3.30 (m, 2H, H-43), 3.40 (m, 2H, H-27), 3.52 (t, *J*=4.9 Hz, 2H, H-42), 3.55-3.80 (m, 20H, H-32 to H-41), 3.99 (t, *J*=6.4 Hz, 2H, H-19), 4.07 (dd, *J*=6.1, 6.1 Hz, 1H, H-8), 4.13 (s, 2H, H-31), 5.12 (s, 1H, NH), 5.24 (s, 1H, NH), 5.45 (s, 1H, NH), 6.35 (d, *J*=7.3 Hz, 1H, H-23), 6.70 (d, *J*=8.4 Hz, 1H, H-3), 6.81 (dd, *J*=2.0, 8.3 Hz, 1H, H-4), 7.07 (d, *J*=7.3 Hz, 1H, H-24), 7.54 (t, *J*=7.9 Hz, 1H, H-14), 7.73 (d, *J*=7.8 Hz, 1H, H-15), 7.92 (d, *J*=7.9 Hz, 1H, H-13), 8.01 (s, 1H, H-17), 8.13 (d, *J*=1.9 Hz, 1H, H-6), 8.97 (s, 1H, NH).

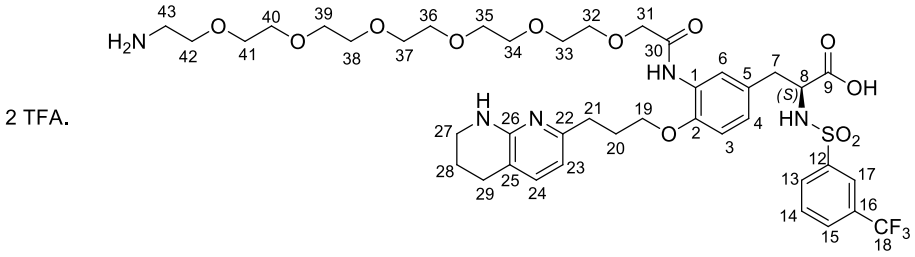
^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) : 21.4 (C-28), 26.4 (C-29), 27.8 (C-11), 28.6 (C-46), 29.1 (C-20), 33.6 (C-21), 38.9 (C-7), 40.5 (C-43), 41.7 (C-27), 57.4 (C-8), 67.8 (C-19), 70.3-71.3 (C-31 to C-42), 79.3 (C-45), 83.1 (C-10), 111.1 (C-3), 111.4 (C-23), 114.1 (C-25), 120.7 (C-6), 123.3 (q, *J*=272.8 Hz, C-18), 124.3 (q, *J*=3.8 Hz, C-17), 125.2 (C-4), 127.1 (C-1), 127.6 (C-5), 129.2 (q, *J*=3.4 Hz, C-15), 129.8 (C-14), 130.7 (C-13), 131.5 (q, *J*=33.4 Hz, C-16), 137.1 (C-24), 141.4 (C-12), 146.8 (C-2), 155.8 (C-26), 156.2 (C-22+C-44), 167.6 (C-30), 169.9 (C-9).

^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) : -63.26 (s, 3F, F-18).

<u>Synthesis of bis(2,2,2-trifluoroacetate); 2-{3-[2-(20-azaniumyl-3,6,9,12,15,18-hexaoxaicosanamido)-4-[(2S)-2-carboxy-2-{[3(trifluoromethyl)benzene]sulfonamido}ethyl]phenoxy]propyl}-5,6,7,8-tetrahydro-1,8-naphthyridin 1-ium **6b**.</u>



To a stirred solution of tert-butyl (2S)-3-[3-(20-{[(tert-butoxy)carbonyl]amino}-3,6,9,12,15,18-hexaoxaicosanamido)-4-[3-(5,6,7,8-tetrahydro-1,8-naphthyridin-2-yl)propoxy]phenyl]-2-{[3-(trifluoromethyl)benzene]sulfonamido}propanoate (39 mg, 0.037 mmol) in DCM (1 mL) was added trifluoroacetic acid (1 mL). The solution was stirred at room temperature for 4h, then, the solvent and excess of TFA was removed under reduced pressure to give 41 mg (100%) of the title compound as a pale yellow oil.



^{1}H NMR (300 MHz, CD_3OD) δ (ppm) : 1.92 (m, 2H, H-28), 2.23 (m, 2H, H-20), 2.60-2.81 (m, 3H, H-7+H-29), 2.94 (t, *J*=7.1 Hz, 2H, H-21), 3.04 (dd, *J*=4.4, 13.8 Hz, 1H, H-7'), 3.13 (m, 2H, H-43), 3.46 (m, 2H, H-27), 3.55-3.85 (m, 22H, H-32 to H-42), 4.02-4.14 (m, 3H, H-8+H-19), 4.20 (s, 2H, H-31), 6.60 (d, *J*=7.3 Hz, 1H, H-23), 6.80 (d, *J*=8.3 Hz, 1H, H-3), 6.88 (d, *J*=8.5 Hz, 1H, H-4), 7.54 (d, *J*=7.3 Hz, 1H, H-24), 7.60 (t, *J*=7.9 Hz, 1H, H-14), 7.80 (d, *J*=7.7 Hz, 1H, H-15), 7.87 (d, *J*=8.0 Hz, 1H, H-13), 7.93 (bs, 2H, H-6+H-17).

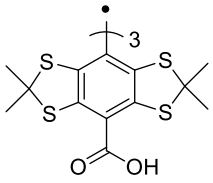
^{13}C NMR (75 MHz, CD_3OD) δ (ppm) : 20.5 (C-28), 26.4 (C-29), 29.3 (C-20), 30.5 (C-21), 39.3 (C-7), 40.7 (C-43), 42.1 (C-27), 59.3 (C-8), 67.9, 68.2 (C-19 and C-42), 70.9 to 72.0 (C-31 to C-41), 111.8 (C-23), 112.5 (C-3), 120.8 (C-25), 122.8 (C-6), 124.7 (q, *J*=3.7 Hz, C-17), 127.1 (C-4), 127.4 (C-1), 129.8 (q, *J*=3.3 Hz, C-15), 130.4 (C-5), 131.1 (C-14), 131.5 (C-13), 132.0 (q, *J*=33.0 Hz, C-16), 142.7 (C-24), 143.9 (C-12), 148.4 (C-2), 148.9 (C-22), 153.0 (C-26), 170.2 (C-30), 174.1 (C-9), C-18 not detected.

^{19}F NMR (282 MHz, CD_3OD) δ (ppm) : -75.30 (s, 6F, CF_3COO^-), -62.49 (s, 3F, F-18).

7. Conclusions et perspectives

7.1 Conclusions

L'objectif de cette thèse était la modification chimique d'un radical stable utilisé en RPE biomédicale de la famille des tetrathiatriarylmethyles : le radical **CT-03** (Figure 1), le but visé étant d'améliorer les performances et d'en étendre le champ d'applications.



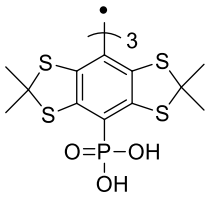
CT-03

Figure 1 : Structure de CT-03

7.1.1 Nouveau radical TAM sensible au pH

La première modification avait pour but de produire un radical permettant la mesure du pH. L'utilisation de radicaux TAMs pour mesurer le pH par RPE a été récemment démontrée par le Professeur V. Khramtsov (Ohio State University, US. of A.) avec les radicaux TAMs para-carboxyle et para-amino[1,2]. Cependant, la faible solubilité de la forme acide, la faible sensibilité et une valeur de pKa bas limite les applications alors que la complexité des spectres obtenus pour les radicaux TAMs para-amino rend l'utilisation pratique complexe. Nous avons synthétisé un radical TAM **1** substitué par trois fonctions acide phosphonique (Figure 2). Ce composé représente une avancée majeure pour la mesure du pH par RPE. Le spectre RPE se présente sous la forme d'un quadruplet, structure nettement plus simple que celle des dérivés TAMs para-amino, permettant une bonne sensibilité de détection. La variation de la constante de couplage hyperfine en fonction de l'état d'ionisation de la fonction acide phosphonique permet de mesurer le pH autour des deux valeurs de pKa (pKa_1 = 1,3 et pKa_2 = 7,1, déterminée expérimentalement). Alors que la deuxième valeur de pKa

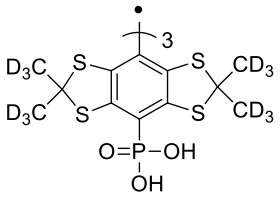
est particulièrement intéressante pour la mesure du pH dans la gamme physiologique, la première valeur permet de garder la molécule sous forme ionisée pour des pH supérieurs à 2,5 et garantit d'une bonne solubilité en milieu aqueux. Cette molécule a représenté la première sonde de type TAM pour la mesure du pH par RPE et est actuellement en cours d'évaluation pour la mesure du pH *in vivo* par V. Marchand (UCL/REMA).



1

Figure 2 : Structure de **1**

Ce travail pionnier a constitué la principale source d'inspiration de V. Khramtsov qui, en réalisant la synthèse de l'analogue deutérié,[1] a permis de mesurer de manière simultanée le pH et la concentration en oxygène. En effet, la structure quadruplet de **1** pour un pH d'environ 7 n'est pas un quadruplet pur mais le résultat d'une superposition de quatre quadruplets non résolus (Figure 3). La substitution isotopique des protons par le deutérium permet d'affiner le signal (comme c'est le cas pour **CT-03** et **CT-03d36**). Cette modification rend possible la résolution des quadruplets et par conséquent la mesure de la $[O_2]$ par son effet sur la largeur de raie.



1 d36

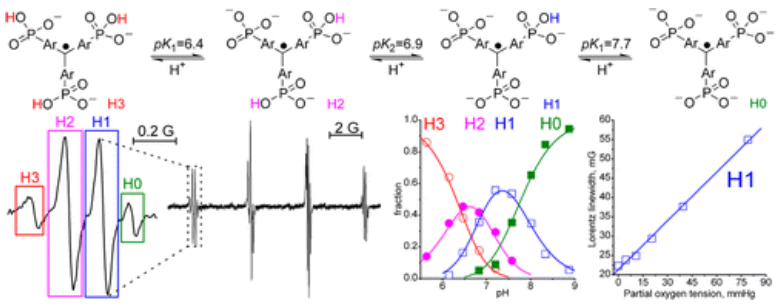
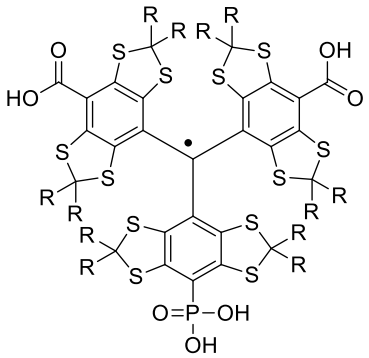


Figure 3 : Structure de 1-d36, forme ioniques et sensibilité à l'oxygène (reproduit de ref[3])

Très récemment, V. Khramtsov a proposé la synthèse d'un radical TAM **2** substitué par une seule fonction acide phosphonique[4] et deux fonctions acide carboxylique (Figure 4). Cette amélioration transforme le quadruplet de **1** en un doublet augmentant de ce fait la sensibilité de détection, et diminuant à deux le nombre d'espèces ioniques pour un pH autour de 7, et facilitant la mesure de la $[O_2]$.



2

Figure 4 : Structure de 2

7.1.2 Nouveaux radicaux TAMs pour la mesure de l'oxygénation utilisant des formulations de perfluorocarbones

Le développement de formulation perfluorocarbones (PFC) incorporant une sonde à oxygène par le Dr Nicolas Charlier (UCL/REMA) nécessitait une sonde à haute affinité pour une phase fluorée. Nous avons réalisé la synthèse de deux radicaux TAM possédant une chaîne perfluorée. Ces deux radicaux **F15T-03** et **F45T-03** (Figure 5) contenant respectivement 20% et 40% en poids de fluor ont une bonne affinité pour les solvants fluorés (bromure de perfluorooctyle et hexafluorobenzene). Alors que la sensibilité à l'oxygène de **CT-03** est de 0,64 mG/mmHg, celle des deux TAMs fluorés est de 18,7 mG/mmHg pour FT15-03 dans HFB et 17,5 mG/mmHg pour F45T-03 dans le bromure de perfluorooctyle. Cette augmentation de sensibilité s'explique par une plus grande solubilité de l'oxygène pour une phase fluorée.

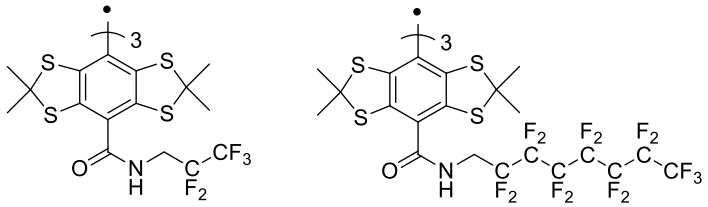


Figure 5: Structure des deux radicaux TAM fluorés synthétisés

Par la suite, ces radicaux ont été utilisés sous forme de formulations biocompatibles pour la mesure de l'oxygénation tissulaire *in vivo* avec une grande sensibilité par le Dr Nicolas Charlier. Malheureusement, le faible rapport sur bruit des spectres obtenus en raison d'une relative faible incorporation de trityles dans la micelle de PFC limite les applications en imagerie RPE. Une piste d'optimisation du concepte serait d'incorporer des trityles dans une micelle formée au départ d'un copolymère à blocs, composé d'un co-polymère hydrophyle et d'un autre co-polymère perfluoré.

7.1.3 Etude de la chiralité des dérivés TAMs

Bien que des radicaux TAMs aient été largement utilisés, la question relative à la chiralité inhérente à la conformation hélicoïdale de ces molécules n'avait pas encore été investiguée. Nous avons démontré que l'encombrement stérique limite fortement l'isomérisation entre les deux conformations énantiomériques de ces composés dans leur différentes formes (alcool, alcane et radical) rendant ces molécules chirales. Les deux enantiomères sont suffisamment stables pour être résolus par HPLC chirale. En vue d'étudier l'énergie d'énantiomérisation, les composés **3** (radical), **4**, **5** (alcools) et **6** (alcane) (Figure 6) ont été séparés par HPLC chirale semi-préparative. Des valeurs expérimentales de $\Delta G^{\ddagger}$ associés au processus d'énantiomérisation allant de 24,4 à 26,4 kcal.mol^{-1} ont été déterminées par des suivis cinétiques de racémisation. Ces valeurs représentent un temps de ½ vie de racémisation allant de 12,1 à 343,3 h. Ce résultat est à prendre en considération pour des transformations futures puisque la liaison entre composés TAMs et un groupement chiral conduira à la formation de diastéréoisomères pouvant comporter des propriétés physiques, chimiques et biologiques différentes.

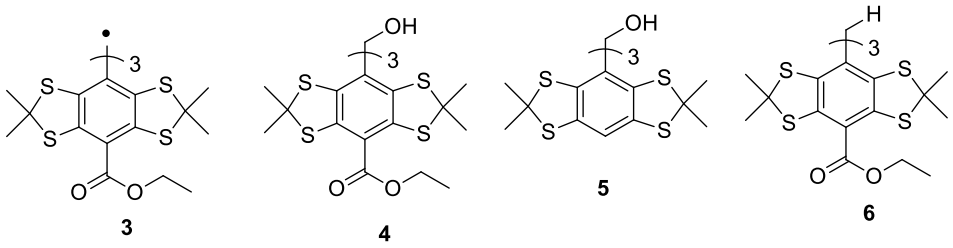


Figure 6: Structure des composés TAM ayant été isolés optiquement purs pour la déterminaition du $\Delta G^{\ddagger}$ associés au phénomène d'énantiomerisation

Le mécanisme d'énantiomérisation a été déterminé par calculs théoriques en collaboration avec le Prof. R. Robiette et par spectroscopie d'échange 2D ^{1}H-^{1}H NOESY/EXSY en collaboration avec le Dr. C. S. Le Duff. Conformément à ce qu'il est généralement observé pour les composés présentant une structure en hélice à trois palmes, c'est le mécanisme "two ring flip" qui est le mécanisme effectif des dérivés tetrathiatriarylmethyle (Figure 7).

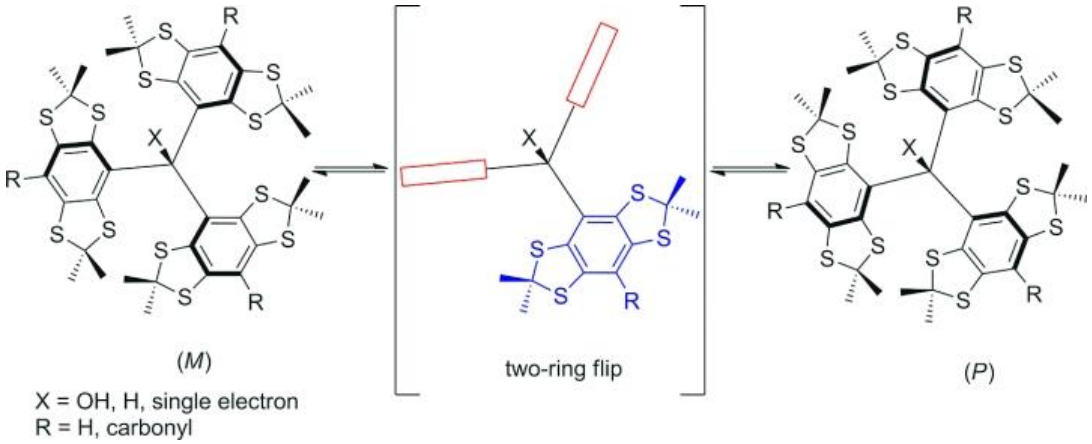


Figure 7 : Mécanisme d'énantiomerisation de type "two ring flip" opérant pour les dérivés TAMs

7.1.4 Synthèse de radicaux TAMs vectorisés

Le dernier objectif de cette thèse visait à conférer une sélectivité tissulaire au radical **CT-03**. Dans un premier temps, celui-ci a été couplé au peptide commercial GRGDS (Figure 8), comportant la séquence RGD, séquence universelle de reconnaissance des intégrines. Ce couplage a été réalisé par l'utilisation de l'ester pentafluorophenol activé de **CT-03**. Cet

intermédiaire a permis de réaliser les couplages en utilisant directement le pentapeptide totalement déprotégé. Cet ester activé peut être utilisé pour réaliser le couplage avec toute autre molécule présentant une fonction nucléophile. Conformément à notre étude sur la chiralité des TAMs, nous avons montré que le produit de couplage **8** se présente sous forme de deux diastéréoisomères. Le spectre obtenu garde des propriétés idéales pour l'imagerie par résonance paramagnétique électronique.

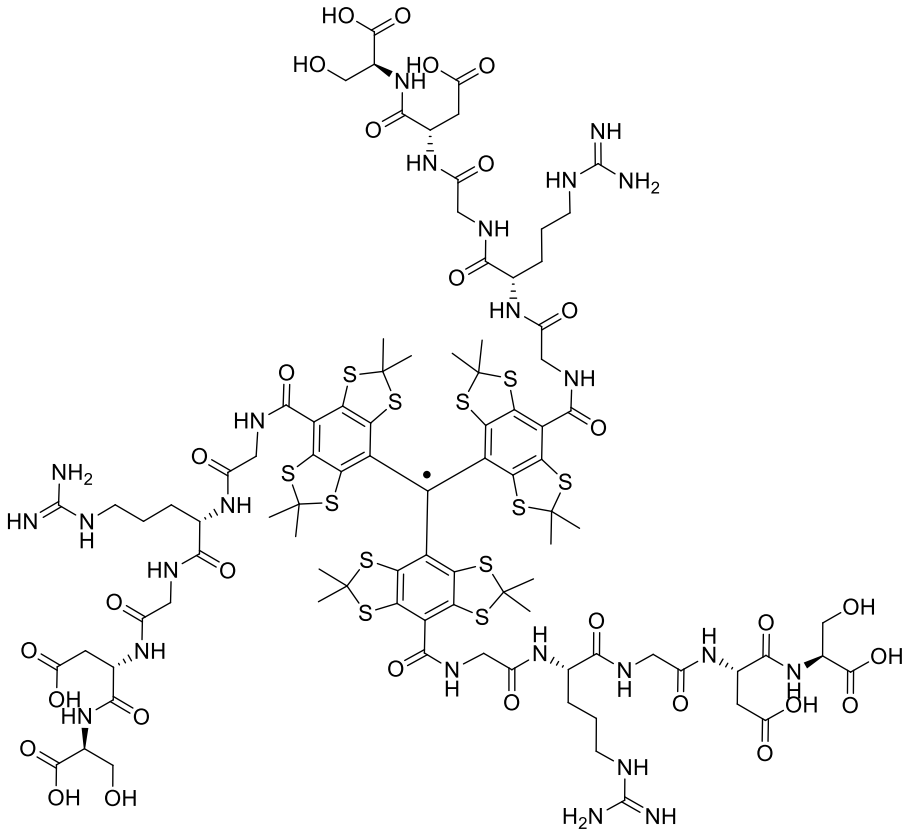


Figure 8 : Structure de **8**

Une deuxième voie de synthèse a permis de coupler le radical **CT-03** avec un peptidomimétique RGD, antagoniste sélectif de l'intégrine $\alpha_v\beta_3$ (Figure 9). Cette molécule a été synthétisée suivant une voie totalement différente, l'objectif étant de préserver la fonction alcool du trityle le plus longtemps possible afin de garder l'outil d'analyse RMN permettant une bonne caractérisation des intermédiaires. Notre schéma de synthèse comporte une étape de désymétrisation statistique qui offre la possibilité de fixer un seul motif d'adressage. Malheureusement, durant la rédaction de ce manuscrit, cette mono-hydrolyse a été décrite par un autre groupe.[5] Cette séquence a permis de fournir le premier

radical TAM portant un ligand spécifique d'un récepteur membranaire. Ce radical présente également des propriétés spectrales adéquates pour l'EPRI.

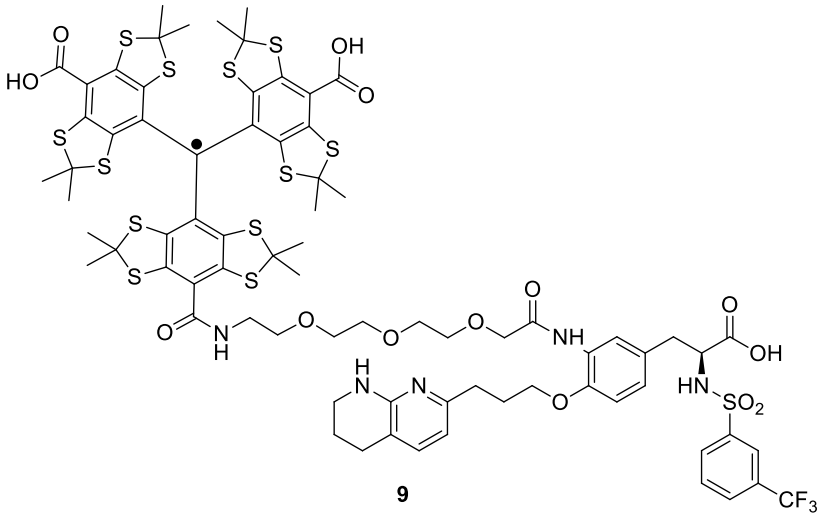


Figure 9 : Structure de **9**

Ces deux premiers radicaux TAMs vectorisés de structures hautement complexes ont été obtenus avec une grande pureté par purification par HPLC semi-préparative. La capacité de ses molécules à se lier spécifiquement à des cellules exprimant l'intégrine $\alpha_v\beta_3$ est étudiée par le Dr. Philippe Levêque (UCL/REMA) mais n'a pas pu être démontrée à ce jour.

7.2 Perspectives

7.2.1 Vectorisation

Devant l'incapacité actuelle de mettre en évidence un phénomène d'adhésion pour nos radicaux TAM **8** et **9** face à des cellules exprimant l'intégrine $\alpha_v\beta_3$, ces deux structures pourraient être revues afin d'éloigner davantage le pharmacophore du radical trityle, ceci en ajoutant un "linker" entre le GRGDS et le centre paramagnétique de **8**. La longueur du bras oligoéthylène glycol de **9** pourrait être augmentée (n>3) (Figure 10).

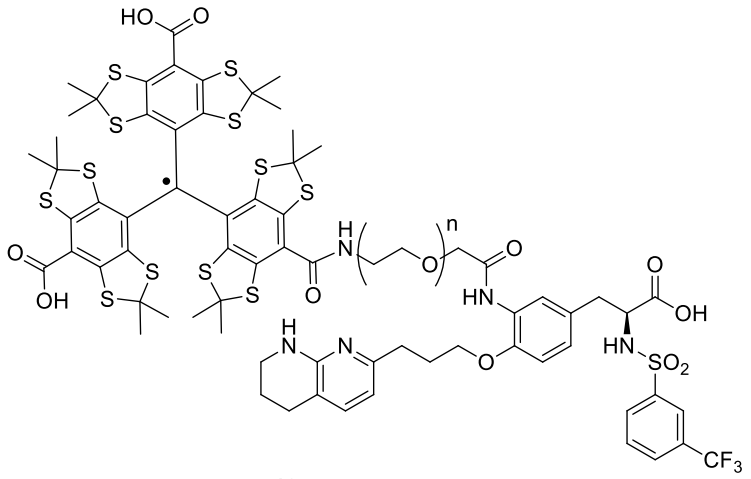


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Figure 10: L'introduction d'un "linker" de plus grande longueur pourrait permettre une meilleure interaction entre le peptidomimétique RGD et l'intégrine.

Une autre stratégie pour vectoriser un radical TAM vers l'intégrine $\alpha_v\beta_3$ serait de l'incorporer dans une nanoparticule possédant à sa surface un peptidomimétique RGD, un peptide ou même un anticorps vectorisé vers l'intégrine $\alpha_v\beta_3$. Cette stratégie a été utilisée au laboratoire pour délivrer spécifiquement des substances actives.[6] Dans un contexte plus général, d'autres ligands pourraient être utilisés comme l'acide folique, des porphyrines, des arabinogalactans, etc.

7.2.2 Sonde à pH

L'inconvénient majeur de l'utilisation de TAMs substitués par des fonctions acide phosphonique pour la mesure du pH est la grande capacité de ces fonctions à complexer les métaux. Ceci peut résulter en une modification de la constante de couplage sans variation du pH. Un effet important de la force ionique a été mis en évidence (voir partie expérimentale de l'article). Une amélioration serait de s'inspirer de façon large des sondes de type aminophosphonique utilisées pour la mesure du pH par RMN du ^{31}P (Figure 11)[7] ou c'est l'ionisation de l'amine qui provoque une modification du déplacement chimique du ^{31}P. Les deux radicaux **11** et **12** devraient présenter un spectre RPE sous la forme d'un doublet. L'ionisation de la ou des fonctions amine d'un pKa ~ 7 (simulation Marvin Sketch) devrait modifier la constante de couplage a_P et permettre la mesure du pH.

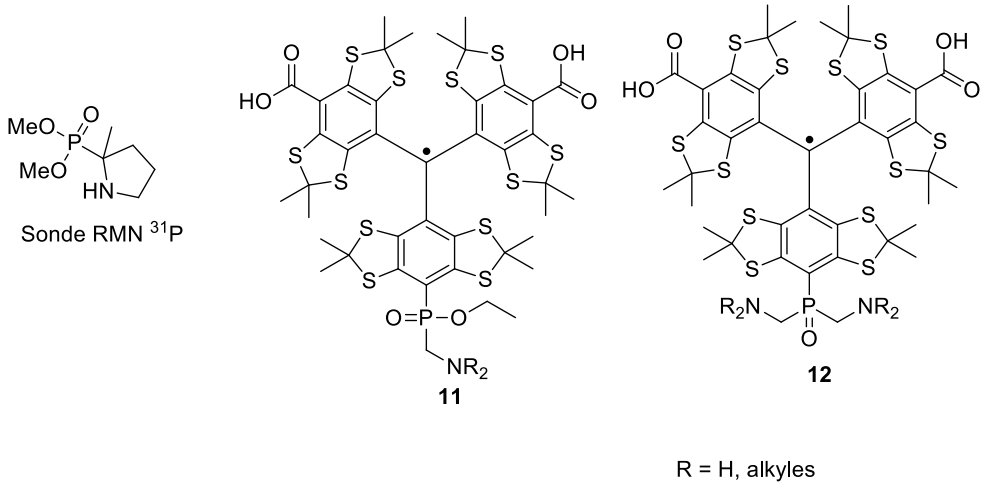

Figure 11: Structure de deux sondes à pH potentielles

7.2.3 Mesure de la concentration en oxygène

Une largeur de raie fine est synonyme d'une plus grande sensibilité de détection et une plus grande sensibilité à la [O_2] (% de variation plus important). Cette largeur de raie est fonction de la structure du radical, Il serait intéressant d'évaluer d'autres structures proches en veillant à ne pas introduire d'atome à spin nucléaire non nul à proximité du centre radicalaire. Quelques structures possibles en fonction de synthèses réalistes qui pourraient être envisagées au laboratoire sont représentées à la Figure 12. Les composés ne présentant pas de symétrie C_2, dont l'axe est aligné avec la liaison C-$C_{radical}$, sur le groupement aromatique (**14** et **15**) sont à proscrire en raison de la possibilité de former de nombreux stéréoisomères.

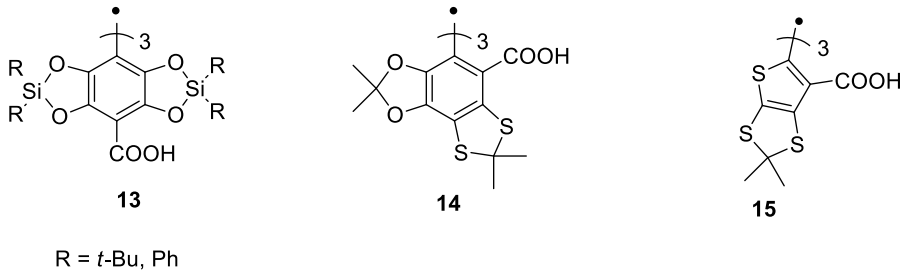

Figure 12: Structures proposées de quelques radicaux TAM potentiels pour l'oxymétrie RPE (spectroscopie et imagerie)

Le laboratoire du Prof. Boucher (Paris Descartes, France) a mis en évidence que c'est la fonction acide carboxylique qui est à l'origine de l'instabilité des radicaux TAM **CT-03** et **OX063**,[8] la suppression de ces fonctions devrait engendrer une augmentation de stabilité. Les composés **16** et **17** de la figure 13 sont deux exemples de molécules qui pourraient être synthétisées au laboratoire. La mono-fonctionalysation pourrait se faire par une réaction d'ipso-substitution de la fonction acide carboxylique, réaction découverte dans le même laboratoire.

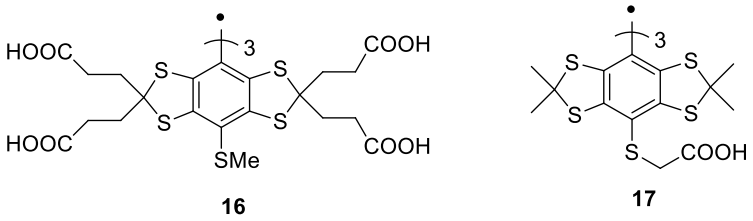

Figure 13: Structures proposées de quelques radicaux TAM potentiels pour l'oxymétrie RPE (spectroscopie et imagerie)

7.3 References

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