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In collaboration with :

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**Master of Science Thesis**

# *A grafted versus supramolecular moiety of Porphyrin , Laccase and β-cyclodextrin assembly for $O_2$ reduction*

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**ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ**

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**ΜΑΣΣΑΛΙΑ, ΓΑΛΛΙΑ**


**Μεταπτυχιακό Δίπλωμα Ειδίκευσης**

# *Ένα εμβολιασμένο έναντι ενός υπερμοριακού συνόλου πορφυρίνης, λακκάσης και συναρμολόγηση αυτού με β-κυκλοδεξτρίνη με σκοπό την αναγωγή οξυγόνου*

**Νικηφόρου Αγάπη**

Υπό την επίβλεψη των : Αθανάσιος Γ. Κουτσολέλος, Pierre Rousselot Pailley, Thierry Tron

Ηράκλειο , Οκτώβριος 2016

Dedicated to my beloved ones

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# ABSTRACT

In recent years, scientific community's attention has been drawn to the development and design of new synthetic systems which catalyze reactions using solar energy as a sole source of energy. These novel technologies are catalytically active for reactions like hydrogen oxidation, dioxygen reduction or water splitting to molecular $O_2$ and $H_2$. Various studies have demonstrated that the combination of photosensitizers and enzyme can support reactions like $O_2$ reduction or $H_2$ oxidation.

Photosynthesis is a complex procedure taking place in plants where the sunlight is the source of energy triggering the decomposition of water into molecular $O_2$ and $H_2$. The equation is shown below

$$2H_2O \rightarrow 2H_2 + O_2$$

By mimicking the natural photosynthesis, solar energy can be used to drive catalysis and convert light to stored chemical energy. Our target is to develop robust, stable systems powered by sunlight associating an electron donor to a unit capable to react with dioxygen. Photosensitizers are excellent candidates as electron shuttle since they are capable of harvesting solar energy. On the other hand, metalloenzymes like laccase act as "oxygen catalytic units".

In the present study, we report two hybrid photocatalytic systems designed to function in visible-light driven dioxygen reduction. The first system – the so called grafted system- contains a photosensitizer covalently bound on the surface of an enzyme (laccase). In the second system β-cyclodextrin is grafted on laccase and a suitable photosensitizer was used to interact deeply into the cavity of cyclodextrin, affording that way a supramolecular system.

Porphyrins (Zn-P and $COOCH_3$_NH_Pyrene in particular) were selected as photosensitizers since they demonstrate high stability, high absorption in the visible spectrum and display long life time in their excited states. Laccases, on the other side, are robust oxidoreductases which catalyze the oxidation of various organic and inorganic substrates with the concomitant reduction of dioxygen into water as by-product (abundant, not dangerous, non toxic, inexpensive).

At first, activity tests were performed to determine the percentage of activity laccase carries after the coupling we made in both systems. It was revealed that in covalent attachment of Zn-porphyrin_aldehyde on enzyme's surface, 80% of the initial activity of the enzyme was kept whereas in covalent attachment of cyclodextrin_aldehyde we have only 40%. Fluorescence titrations showed a 1 : 1 $COOCH_3$_NH_Pyrene – β-cyclodextrin inclusion complex. Last but not least, some dioxygen consumption experiments were conducted in order to investigate these two systems for their ability to function in visible light driven dioxygen reduction. Grafted system showed the best performance whereas the effectiveness of supramolecular system was almost absent, possibly due to the powerless and weak intermolecular forces developed among the components.

**Keywords**: porphyrin, laccase, multi-copper oxidases, dioxygen reduction, supramolecular chemistry, photoreduction.

# ΠΕΡΙΛΗΨΗ

Τα τελευταία χρόνια, το ενδιαφέρον της επιστημονικής κοινότητας έχει στραφεί στην ανάπτυξη και το σχεδιασμό νέων συνθετικών συστημάτων που καταλύουν αντιδράσεις χρησιμοποιώντας την ηλιακή ενέργεια ως μόνη πηγή ενέργειας. Αυτές οι καινούριες τεχνολογίες είναι καταλυτικά ενεργές σε αντιδράσεις όπως οξείδωση υδρογόνου, αναγωγή οξυγόνου ή διάσπαση νερού σε μοριακό $O_2$ και $H_2$. Διάφορες μελέτες έχουν δείξει ότι ο συνδυασμός φωτοευαισθητοποιητών και ενζύμου μπορεί να υποστηρίξει αντιδράσεις όπως αναγωγή $O_2$ ή οξείδωση $H_2$.

Η φωτοσύνθεση είναι μια πολύπλοκη διαδικασία που λαμβάνει χώρα στα φυτά όπου το ηλιακό φως είναι η πηγή της ενέργειας που πυροδοτεί την αποσύνθεση του νερού σε μοριακό οξυγόνο και υδρογόνο. Η αντίδραση απεικονίζεται παρακάτω

$$2H_2O \rightarrow 2H_2 + O_2$$

Μιμούμενοι τη λειτουργία της φωτοσύνθεσης, η χρήση της ηλιακής ενέργειας μπορεί να εφαρμοστεί στην κατάλυση και σε φωτοβολταϊκές κυψελίδες. Στόχος μας είναι να αναπτύξουμε σταθερά συστήματα τροφοδοτούμενα από το φως του ήλιου που συσχετίζουν ένα δότη ηλεκτρονίων με μια μονάδα ικανή να αντιδράσει με το οξυγόνο. Οι φωτοευαισθητοποιητές είναι εξαιρετικοί υποψήφιοι ως φορείς ηλεκτρονίων επειδή είναι ικανοί στη συγκομιδή ηλιακής ενέργειας. Από την άλλη πλευρά, μεταλλοένζυμα όπως η λακκάση λειτουργούν ως «καταλυτικές μονάδες οξυγόνου".

Στην παρούσα μελέτη, αναφέρουμε δύο υβριδικά φωτοκαταλυτικά συστήματα σχεδιασμένα για να λειτουργούν στην φωτοεπαγόμενη αναγωγή μοριακού οξυγόνου. Το πρώτο σύστημα –το αποκαλούμενο εμβολιασμένο σύστημα- περιέχει ένα φωτοευαισθητοποιητή ομοιοπολικά συνδεδεμένο στην επιφάνεια ενός ενζύμου (λακκάση). Στο δεύτερο σύστημα η β-κυκλοδεξτρίνη είναι εμβολιασμένη επί της λακκάσης και ένας κατάλληλος φωτοευαισθητοποιητής χρησιμοποιήθηκε για να εισαχθεί μέσα στην κοιλότητα της κυκλοδεξτρίνης, αποδίδοντας με αυτόν τον τρόπο ένα υπερμοριακό σύστημα.

Οι πορφυρίνες (Zn-P και $COOCH_3$_NH_Pyrene ειδικότερα) επελέγησαν ως φωτοευαισθητοποιητές δεδομένου ότι επιδεικνύουν υψηλή σταθερότητα, υψηλή απορρόφηση στο ορατό φάσμα και εμφανίζουν μεγάλη διάρκεια ζωής σε διεγερμένες καταστάσεις τους. Οι λακκάσες, από την άλλη πλευρά, είναι ανθεκτικές οξειδοαναγωγάσες που καταλύουν την οξείδωση διαφόρων οργανικών και ανόργανων υποστρωμάτων με την ταυτόχρονη αναγωγή του οξυγόνου σε νερό ως παραπροϊόν (άφθονο, ασφαλές, φθηνό).

Αρχικά, δοκιμές ενεργότητας διεξήχθησαν για να προσδιοριστεί το ποσοστό της δραστικότητας της λακκάσης μετά τη σύζευξη που κάναμε και στα δύο συστήματα. Απεδείχθη ότι στην ομοιοπολική δέσμευση μεταλλωμένης πορφυρίνης-αλδεΰδης πάνω στην επιφάνεια του ενζύμου, 80% της αρχικής ενεργότητας του ενζύμου διατηρήθηκε ενώ στην ομοιπολική δέσμευση της κυκλοδεξτρίνης_αλδεύδης έχουμε μόνο 40%. Τιτλοδοτήσεις φθορισμού έδειξαν το σχηματισμό συμπλόκου εγκλεισμού μεταξύ $COOCH_3$_NH_Pyrene – β-κυκλοδεξτρίνης σε αναλογία 1:1. Τελευταίο αλλά όχι λιγότερο σημαντικό, πειράματα κατανάλωσης οξυγόνου διεξήχθησαν για να ερευνήσουμε την ικανότητά των δυο αυτών συστημάτων στη φωτοεπαγόμενη αναγωγή μοριακού οξυγόνου. Το εμβολιασμένο σύστημα παρουσίασε την καλύτερη απόδοση, ενώ η αποτελεσματικότητα του υπερμοριακού συστήματος ήταν αμελητέα, πιθανότατα λόγω των ανίσχυρων και αδύναμων διαμοριακών δυνάμεων που αναπτύσσονται μεταξύ των μορίων που το αποτελούν.

**Λέξεις-κλειδιά**: πορφυρίνη, λακκάση, πολύ-πυρηνικές οξειδάσες χαλκού, αναγωγή μοριακού οξυγόνου, υπερμοριακή χημεία, φωτοαναγωγή.

# Table of Contents

# ABBREVIATIONS

| | |
|---|---|
| **B&R** | Britton & Robinson buffer |
| **Cdx** | cyclodextrin |
| **Cu** | copper |
| **d** | doublet |
| **ε** | molar absorptivity |
| **$E^0$** | redox potential |
| **eq** | equivalents |
| **EDTA** | ethylenediaminotetraacetic acid |
| **$^1$H NMR** | proton nuclear magnetic resonance |
| **fluo** | fluorescence |
| **J** | coupling constant |
| **kDa** | kiloDalton |
| **m** | multiplet |
| **MALDI** | matrix assisted laser spray desorption ionization |
| **Mw** | molecular weight |
| **MS** | mass spectrometry |
| **Lac3** | isoform of laccase |
| **$NaN_3$** | sodium azide |
| **NHE** | normal hydrogen electrode |
| **s** | singlet |
| **SGZ** | syringaldazine |
| **t** | triplet |
| **T1** | Type 1 (copper site) |
| **T2/T3** | Type 2/Type 3 (copper site) |
| **TOF** | time-of-flight |
| **Unik161** | mutated laccase |
| **UV-Vis** | ultraviolet/visible |
| **ZnP** | zinc porphyrin |

# Chapter 1 – Introduction

# I. LACCASE

## *1.1 Overview*

Laccase (p- diphenol: dioxygen oxidoreductase) has been the subject of study since the end of 19$^{th}$ century. It is a glycoprotein ubiquitous in nature. It was demonstrated for the first time in the exudates of Rhus vernicifera the Japanese lacquer tree, by Yoshida in 1883.[1] Few years later, in 1896, both Bertrand and Laborde demonstrated that the laccase was a fungal enzyme.[2] Also, laccases have been identified in insects and bacteria.[3,4]

Laccase belongs to a group of polyphenol oxidases containing copper atoms in their catalytic centre and usually called multicopper oxidases (MCO). Other members of this group are the mammalian plasma protein ceruloplasmin and ascorbate oxidases of plants. This blue copper enzyme catalyzes the oxidation of a wide variety of organic and inorganic substrates including mono-, di-, and polyphenols, amino phenols, methoxy phenols, aromatic amines and ascorbate with the concomitant reduction of molecular oxygen into water (**see Fig 1**).

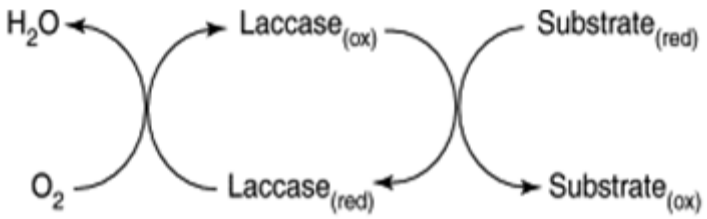



**Fig 1: Schematic representation of laccase-catalyzed oxidation redox cycles for substrates oxidation.[5]**

Substrate oxidation performed by laccase is a one-electron reaction generating a free radical. The overall outcome of the catalytic cycle is depicted in **eq 1** and constitutes the reduction of one molecule of oxygen to two molecules of

water without hydrogen peroxide production. Simultaneously, oxidation of four substrate molecules into the production of four radicals takes place.

$$4H^+ + 4\ substrate + O_2 \rightarrow 2H_2O + 4\ substrate^+ \quad \textbf{eq 1}$$

These reactive intermediates are unstable and they can undergo further oxidation, dimerization or oligomerization.

### *1.2 Distribution in nature*

As mentioned above, laccase has been isolated from plants, insects, bacteria and fungi. For instance, laccases have been found in trees, potatoes, apples, asparagus and other vegetables. Various insects such as *Oryctes* and *Sarcophaga* as well as *ascomycetes*, *deuteromycetes* and *basidiomycetes* fungi contain this particular enzyme. Among the latter ones, white rot fungi are the most important laccase producers. Well known producers are the wood rot fungi, for example *Trametes versicolor, Trametes villosa.*[6]

The function of laccases in insects is during sclerotization processing for epidermal cuticle synthesis. Laccases in bacteria play a role in copper homeostasis, morphogenesis processes, pigment biosynthesis and in the protection afforded by the spore coat against UV light and hydrogen peroxide. In fungi, laccases carry out a lot of functions like sporulation, pigment production, fruit body formation, plant pathogenesis, lignin and cellulose degradation.[7]

### *1.3 Structure and active sites*

To the best of our knowledge, multicopper oxidases have multidomain structures with cupredoxin-like domains as

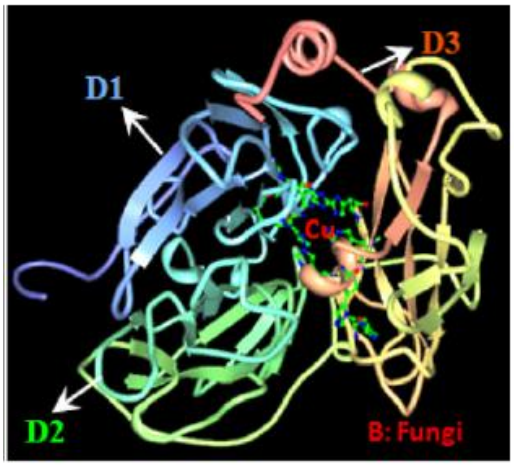



**Fig 2: 3-D structure of fungi laccase (Tramates versicolor)[9]**

structural units and they are classified into three categories based on their domain organization and functions: two domain (2dMCO), three domain (3dMCO) and six-domain (6dMCO) structures. These cupredoxin domains are mainly formed by β-barrels (Greek key motif), consisting β-sheets and β-strands, arranged in sandwich conformation. Laccase contains four copper ions per enzyme and has a three domain structure.[6,8] The three domain structure of fungi laccase is depicted in **Fig 2** and it is colored from blue (domain 1 D1) to green (D2) to red (orange) (D3).

The molecular weight of laccases extracted from fungi ranges from 60 to 100kDa with isolectric points (pI) from 3 to 7. Laccase is a glycosylated protein generally having fewer saccharide compounds (10-25%) in fungi and bacteria than in plants (20-25%). Mannose is the most predominant carbohydrate amongst the others attached to laccase.[7]

In terms of structure, these four copper ions can be classified into three types based on their unique spectroscopic features (electronic absorption and EPR). Type I or blue copper (T1), which is located in domain 3, Type II or normal copper (T2) and Type III (T3), which is basically a pair of type III coppers or binuclear copper site. T2 and T3 sites are organized into a trinuclear copper cluster (TNC) located between domain 1 and 3. The distance between the T2 and T3 sites of the enzyme is 4 Å and the T1 copper ion is located 13 Å away from them (**see Fig 3**).[10] All copper ions are involved in the transfer of electrons from the substrates to oxygen, which is the final electron acceptor. T1 site is responsible for the substrate oxidation and the TNC is the place where the reduction of oxygen occurs.

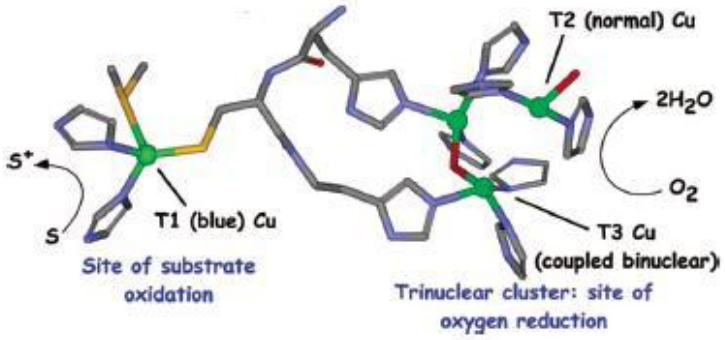



**Fig 3: Active sites of multicopper oxidases. Crystal structure of ascorbate oxidase. Cu sites are shown in green spheres.[10]**

### *1.4 The T1 blue active site*

The T1 copper is characterized by a strong and intense absorption at the wavelength of 600nm (ε ~ 5600$M^{-1}cm^{-1}$) in the electronic absorption spectrum and by a weak parallel superfine splitting in the EPR spectra. The Type 1 Cu site is present at +2 oxidized state in the resting enzyme. It shows coordination with two nitrogens of imidazoles from two histidines and one sulfur from a cysteine, forming a trigonal structure (**see Fig 4**). The strong π covalent bond formed between T1 copper and sulfur with an intense $S_{Cys} \rightarrow Cu^{II}$ charge transfer band at 600 nm is accounted for the blue color of the enzyme.

In many multi-copper oxidases, a fourth ligand, methionine, is coordinated axially resulting in a four coordinate trigonally elongated tetrahedral geometry, whereas in others there is an uncoordinated ligand, leucine or phenylalanine. In our case, methionine residue is absent.[8-12]

### *1.5 T2/T3 active sites*

T2 site is coordinated to two nitrogens from imidazoles from two histidines and one water ligand. It is electron paramagnetic resonance (EPR) active and exhibits only weak absorption in visible region.

The T3 copper ions form a binuclear site where each copper ion is fourfold coordinated to three histidine ligands and one hydroxyl group functioning as bridge between the two copper centers (**see Fig 4**). Because of a strong anti-ferromagnetic coupling, it is EPR silent. Regarding the UV region of the spectrum, there is a shoulder at 330nm (ε ~ 5000$M^{-1}cm^{-1}$).

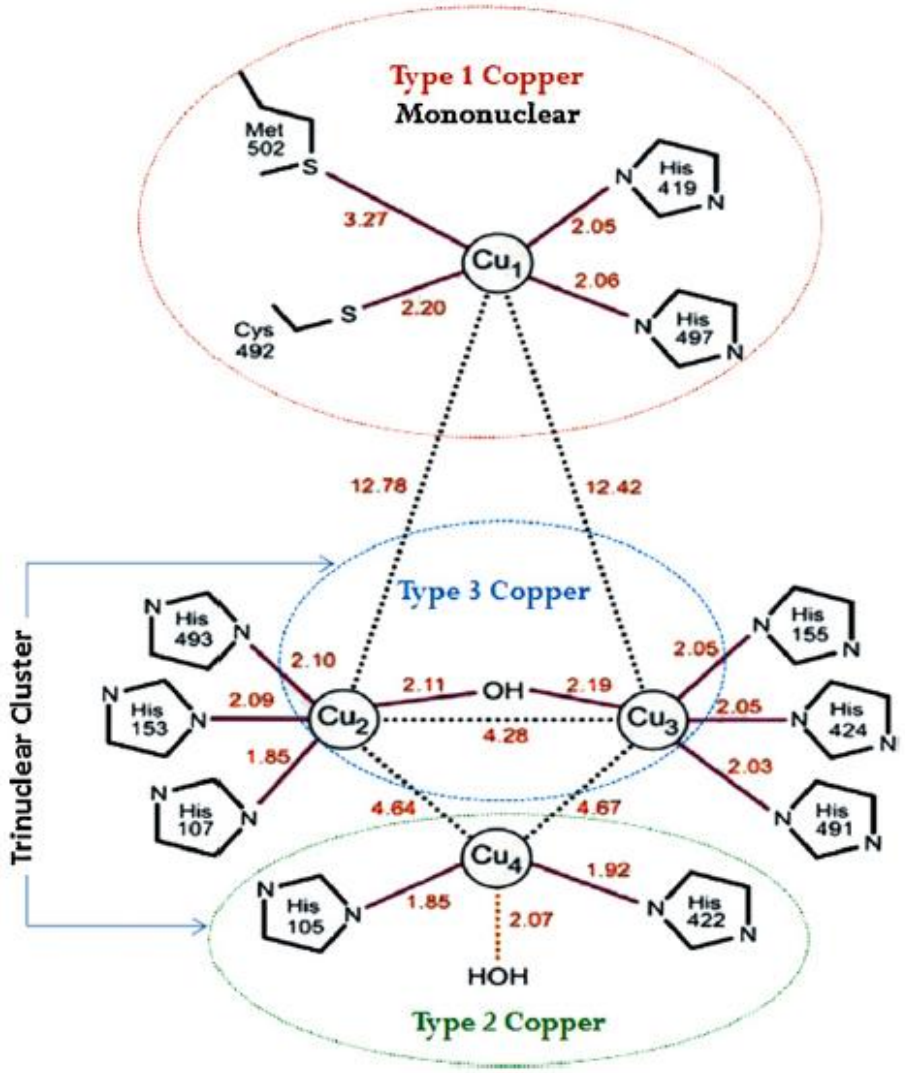



**Fig 4: Schematic representation of copper active sites including interatomic distances.**[9]

Laccases display a peak at the wavelength of 280nm (ε ~ 90000 $M^{-1}cm^{-1}$) as a result of the absorbance of aromatic amino acids.[10]

### *1.6 Laccase's redox potential*

Generally speaking, the redox potentials, $E^0$, of blue copper sites in laccases range from ~400 to ~800 mV versus NHE. Among other factors, the ligand environment of the T1 copper site influence the redox potential of laccase to a more significant extent. To be more specific, the presence of axial methionine as the

fourth ligand at the coordination sphere of T1 Cu decreases the redox potential of the enzyme. It has also been reported that substitution of methionine by phenylalanine or leucine contributes to an enhancement of potential. Solvation, electrostatic interactions, source of laccase (plants, fungi, bacteria), hydrophobicity of nearby residues play a role in the reduction potentials exhibited across T1 Cu sites. Data about redox potentials of copper sites in TNC are rare but both T2 Cu and T3 Cu display redox potentials comparable to that of T1 Cu site.[8,12]

### *1.7 Mechanism of action of laccases*

The reaction mechanism of laccases involves three main electron transfer (ET) steps. Firstly, the substrate is oxidized by the T1 Cu site, meaning that laccase withdraws the electron from the substrates and converts them in free radicals. The extracted electron is stored at T1 Cu site, serving as electron uptake site from diverse substrates.

Oxidation of four substrate molecules is required to produce complete reduction of molecular oxygen to water so after receiving four electrons in total, the electron transfer takes place.

Next step is the internal electron transfer from T1 Cu site to the trinuclear cluster formed by the copper atoms T2 and T3. The distance between the substrate oxidation site and the trinuclear cluster (TNC) is approximately 13Å and the two sites are connected through a histidine– cysteine– histidine tripeptide (H– C– H) involved in the coordination of the metal ions. Then, the dioxygen reduction by the T2/T3 cluster occurs (**see Fig 5**).[7-10,13] For accuracy's sake, dioxygen reacts with the fully reduced TNC to generate $Cu^{II}$– $O_2$– $Cu^{II}$ intermediate preceding the cleavage of O–O bond which triggers movements of protonated oxygen atoms away from the cluster.[14]

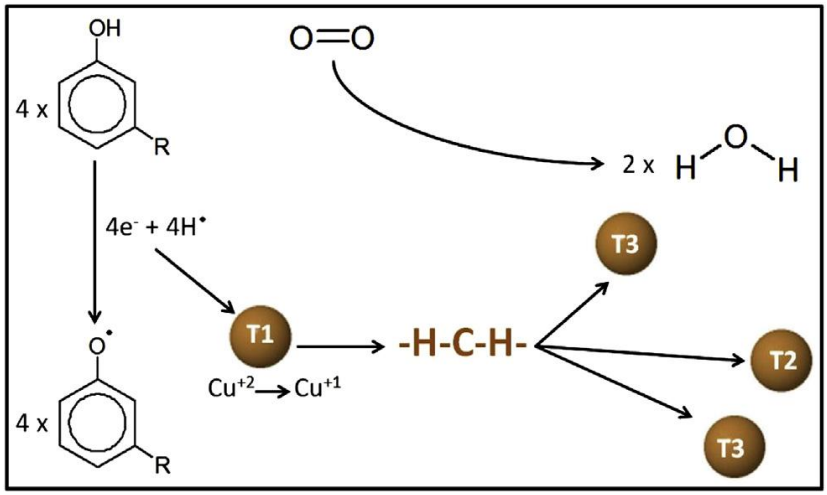



**Fig 5: Schematic representation of catalytic action of laccase. Copper atoms are depicted in brown**

Based on studies and experimental results, the joining pocket for oxygen is quite selective. Oxidizing agents other than $O_2$ are not able to penetrate and fit in the pocket. On the contrary, the binding pocket for the oxidation of the substrates is characterized by low specificity and selectivity.[7,8]

## *1.8 Factors affecting laccase's activity and stability*

I. Temperature

Depending on the strain, the optimal temperature of laccase differs. Our laccase, from the fungus *Trametes* sp. C30, has been found to be stable at 30–40 $^{o}$C for at least 24 hours, but rapidly loses activity at temperatures above 50 $^{o}$C.[7]

II. Inhibitors

Several examples of laccase's inhibitors have been reported in literature. Some of them interrupt the internal electron transfer by binding to the copper site 2 and 3. Small anions like fluoride, chloride, hydroxide anions and azide as well, act like that. On the other hand, metal ions (e.g. $Ba^{+2}$, $Co^{+2}$, $Zn^{+2}$), glutathione, thiourea, fatty acids and cationic quaternary

ammonium detergents chelate the $Cu^{II}$ atoms or cause conformational change in the glycoprotein. EDTA has no significant effect on laccase activity.[7,9]

Sodium azide was used as inhibitor in our study.

III. pH factor

The pH dependence curve is bell shaped. The optimum pH depends on the substrate. In the fungal laccase case, the stability is higher in acidic pH, because $OH^-$ ions bind to T2/T3 coppers, resulting in the inhibition of the laccase activity.[7]

## *1.9 Applications of laccase*

Various technological applications of laccases have been reported since now. They mainly include delignification of lignocellulosic compounds, paper pulping and biobleaching, degradation of explosives and pesticides, prevention of wine decoloration, transformation of colorants in the textile industry, removal of xenobiotics and pollutants found in industrial waste and water. They can function also as biosensors.[8]

# II. PORPHYRINS

## *2.1 Overview*

Porphyrins are the most important class of biological systems in nature and one of the most widely-studied macrocyclic systems. They are composed of four pyrrole rings which are mutually connected via methane bridges (=CH-) creating a tetrapyrrole ring. The structure of the basic macrocycle called porphine according to the nomenclature by Fischer, was developed in the tenth of 20 and 30.[15] Later, in 1943, Corwin has adopted the name "porphyrin" for the system of porphine which prevails today. Below is the numbering of the atoms of the porphyrin and the distinction between the pyrrole rings, according to Corwin (**see Fig 6**). [16]

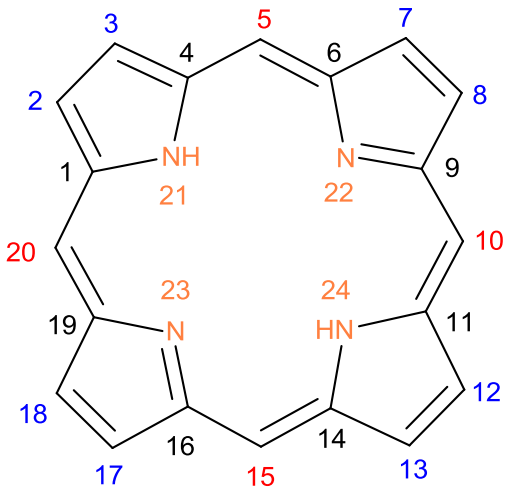



**Fig 6: Skeleton and numbering of carbons of the porphyrin core according to Corwin**

Porphyrins are considered as derivatives of porphine by replacing the exo-hydrogens at positions 2,3,7,8,12,13,17,18, or methine hydrogens at positions 5,10,15,20. The first are known as beta-substituted porphyrins while the latters as alpha- or

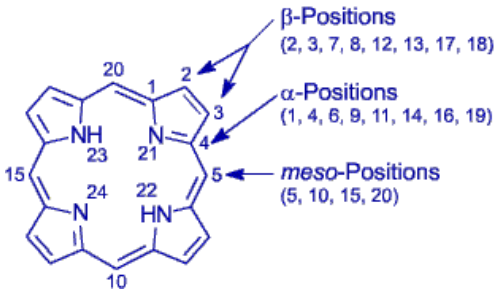



**Fig 7: Molecular structure of porphyrin**

meso substituted porphyrins (**see Fig 7**).

Furthermore, porphyrins are aromatic compounds that obey the Hückel rule (4n+2). They have 18 π electrons which are delocalized over the entire circumference of the porphyrin ring. Generally, porphyrins participate in electrophilic and radical reactions due to the aromatic nature of the porphyrin ring. Meso- positions have higher electron density, so they are more reactive. However if meso- positions are occupied, β-positions can participate in electrophilic reactions.[17]

Porphyrins are unsaturated tetradented macrocyclic ligands which can bind divalent metal ions that behave as Lewis acids. The introduction of metal ions is an easy process including the departure of the two acidic protons which are coordinated to two out of four nitrogen atoms of the porphyrin core (**see Fig 8**).


**Fig 8: Reaction of porphyrin's metallation**

## *2.2 Porphyrins and Spectroscopy*

Due to their high conjugation, porphyrins and their derivatives absorb in the visible region of light which gives their color. The characteristic purple color they have is responsible for their name. Due to the intense color and the fact that they are present in every kind of living cells on our planet, they are named "The Color of Life."

By $^{1}$H-NMR spectrometry, because of the anisotropic effect of the porphyrin ring, the protected N-H protons appear at high fields (-2, -4ppm), in the NMR

spectrum of a porphyrin. The rest of the protons appear at low fields (8, 10ppm), owing to the deprotection which exists as a result of the aromatic ring current phenomenon.[18]

The UV-visible spectra of these molecules consist of two distinct regions: in the near ultraviolet and in the visible region. Porphyrins exhibit a strong absorption band in 400nm called *Soret Band*,[19] as a result of delocalization because of the porphyrin ring current. In the visible region, between 450-800nm, four weak bands are found, the so-called *Q Bands*.

### *2.3 Porphyrins: essential molecules*

Porphyrins possess some remarkable features which render them a unique and important kind of molecules to living organisms. The most notable of them are the following:

- The stability of the ring that can adopt different geometric conformations (planar or twisted structure).[20] Carbon atoms as well as nitrogen atoms participating in the porphyrin skeleton have $sp^2$ hybridization. As a result, all the bond lengths range from 134-145 pm and angles from $107^o$-$126^o$. As tetra-dented chelating substituents, tetra-pyrrole rings are able to be assemble in order to stabilize "unstable" metal ions or metal ions with unusual oxidation states.
- Macrocyclic ligands show selectivity with respect to the size of the bound metal ion. This also applies to the porphyrin rings and gives them a significant stability, due to the conjugation of double bonds. Structural studies and computational models showed that ions with spherical radius of 60-70 pm are located in the central cavity of the tetrapyrrole ring. In case of a bigger radius, like in lanthanides (85-106pm), ions are located out of the plane defined by the four nitrogens of the porphyrin ring[21-24] (**see Fig 9**).

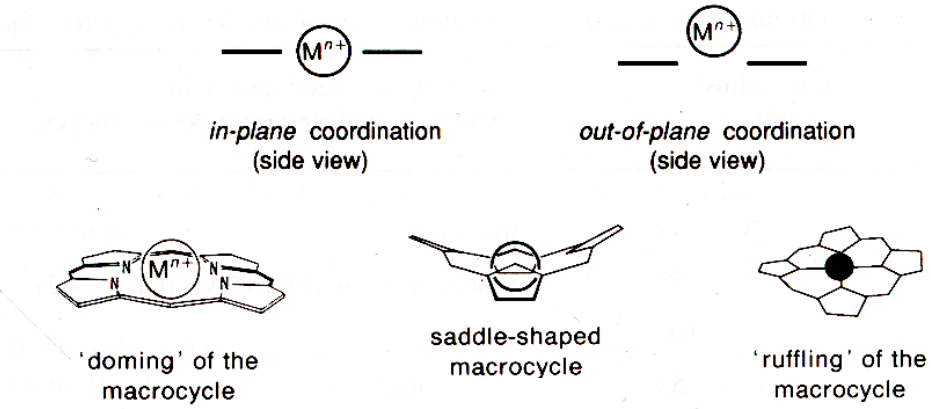



**Fig 9: Typical geometric configurations of tetrapyrrolic complexes**

These molecules prefer an almost planar configuration when attached (linked) to a metal ion. This feature, provided that the coordination number is 6 in octahedral geometry, leaves two empty coordination sites X, Y in axial positions allowing in that way *trans* substitution to occur in various reactions (**see Fig 10**).

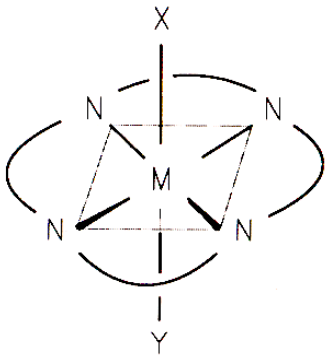



**Fig 10: Axial positions X, Y at six coordinated complexes**

Porphyrins are capable of "taking" and "giving" electrons, so the processes of the first oxidation and the first reduction are taking place with great ease. The formed anions or cations, respectively, are quite stable.

Therefore, the absorption of light and the easy redox processes make these tetrapyrrole molecules the most important energy convertors in biological systems.

## *2.4 Porphyrins in nature*

Porphyrin rings are often found in nature. They are the most favorite macrocycle ring of nature because they are important in fundamental biological processes that sustain life on the planet, such as photosynthesis and the transportation and storage of oxygen in living organisms.

In the human body there are three forms of porphyrin: protoporphyrin (PROTO), uroporphyrin (URO) and coproporphyrin (COPRO). Protoporphyrin has two isomers, I and IX. The protoporphyrin IX corresponds to the heme molecule b, which can be found in catalases, cytochrome b, globin and peroxidase. Heme c is distributed in cytochrome c and oxidoreductases (**see Fig 11**).

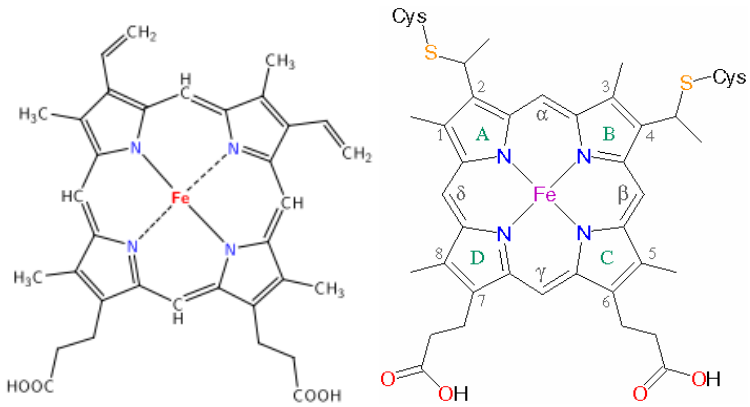
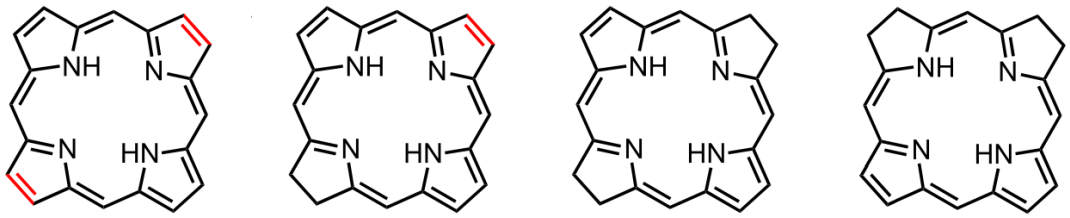



**Fig 11: Chemical structures of hemes b and c**

Hemes molecules assembled with iron are the active sites of proteins hemoglobin and myoglobin, which are responsible for the transportation and elimination of oxygen in organisms.[25]

In cytochromes enzymes, monoatomic oxygen binds to the iron of heme, displaying various roles like hormones synthesis.

Derivatives of porphyrins in which one of the groups of pyrrole has been reduced (pyrroline) are called *chlorins*. Magnesium-containing chlorins are called chlorophylls. They ensure photosynthesis in plants by triggering photochemical electron transfer events. The photosynthesis takes place in the chloroplast, where the principal receptor is chlorophyll A. Related compounds, with two pyrroles and two pyrrolines (where one double bond has been reduced to a single bond) in the macrocycle are called bacteriochlorins and isobacteriochlorins (**see Fig 12**).




**(a)** **(b)** **(c)** **(d)**

**Fig 12: Chemical structures of (a) porphyrin (b) chlorin (c) bacteriochlorin (d) isobacteriochlorin**

## *2.5 Applications*

Porphyrins are applied to numerous fields and some of the most notable applications are in medicine, molecular sensors, molecular electronics, supramolecular chemistry, molecular recognition, information storage, organic reactions catalysis, biochemistry.

In medicine for instance, many porphyrin derivatives are used against photodynamic therapy (PDT), because these derivatives absorb light strongly, which is further converted to energy and heat. By this therapy, the disease of age related

macular degeneration can be treated. The porphyrin derivate used is the verteporfin (**see Fig 13**).

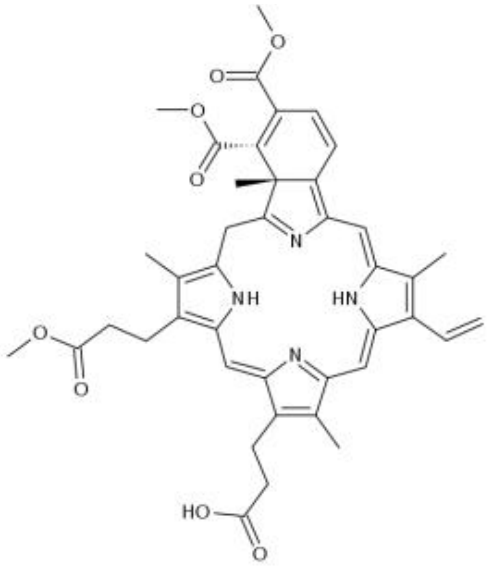



**Fig 13: Chemical structure of verteporfin**

In supramolecular chemistry, synthesis and study of complex macrocyclic organic and organometallic molecules takes place. Porphyrins and their derivatives function as precursors or catalysts for macrocyclic reactions.[26]

In molecular electronics, the derivatives of porphyrins are used in the manufacturing of solar cells that can be applied to photovoltaic systems. In the field of photovoltaics, metalloporphyrins are used in order to absorb the solar radiation and convert it into solar energy.

In the field of synthesis of new materials, porphyroids play an important role.[27] During last decades, various porous materials have been developed. These materials are basically polymers, containing porphyrin derivatives as monomers. The affording polymer has a variety of different structures and properties due to the complex structure which characterizes porphyrin derivatives. Such materials are useful in photoelectronics, in separation procedure of gas mixtures, heterogeneous catalysis, photocatalysis.

# III. CYCLODEXTRINS

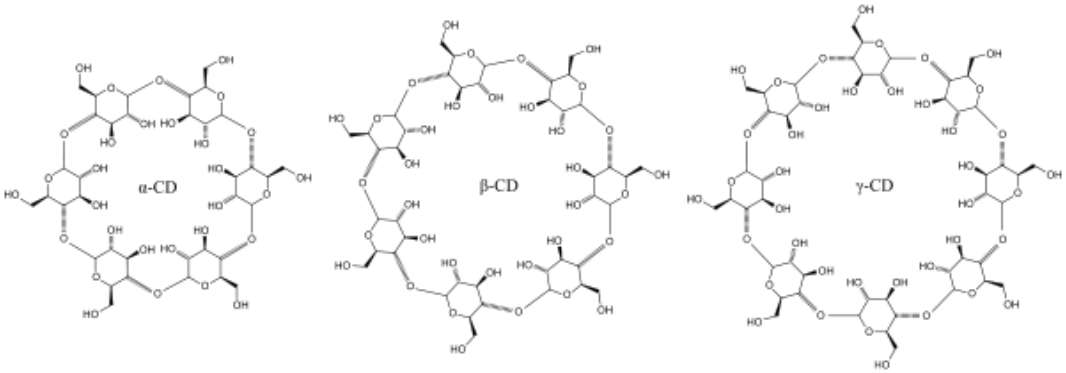
## *3.1 Historical background*

Cyclodextrins, also known as Schardinger dextrins, cycloamyloses, and cycloglucoamyloses, comprise a family of cyclic oligosaccharides obtained from starch by enzymatic degradation. They were discovered as unwanted crystals in 1891 by the French chemist and pharmacist Antoine Villiers during experiments on the degradation and reduction of carbohydrates under the action of ferments, [28] but the first detailed description of the preparation and isolation was made in 1903 by the Austrian chemist Schardinger.[29] He is also considered as the ''Founding Father'' of cyclodextrin chemistry as he shed light on the fundamental properties of cyclodextrins as well as he introduced the basis of their chemistry.

## *3.2 Synthesis of Cyclodextrins*

As mentioned above, cyclodextrins can be obtained by the enzymatic degradation of the most essential polysaccharides, starch.[30] It is a linear polysaccharide consisting of α(1➔4) linked glycose units arranged into a left-threaded screw with six glycose units per turn. The starch is treated by cyclodextrin glycosyl transferase enzyme (CGTase), a type of amylase, which is produced from microorganisms such as *Bacillus macerans*, the *Klebsiella oxytaca* and *Bacillus circulans*. CGTase hydrolyzes off the starch helix and link afterwards the two ends of this fragment to give a cyclic molecule. Given that this kind of enzyme is not very specific as to the site of hydrolysis, the resulting cyclodextrins contain 6-12 glycose units per ring. A five-membered cyclodextrin has not been observed due to steric hindrance and ring strain.[31] The main fractions are α-, β-, γ-, and δ- cyclodextrins, which are comprised of six, seven, eight, and nine glucose units. Indicatively, the chemical structures of α-, β- and γ- cyclodextrins are depicted in **Figure 14**. These three major types of cyclodextrins are frequently referred as parent or first generation cyclodextrins and their isolation is accomplished by selective precipitation with appropriate organic

compounds.[32-34] Cyclodextrins with 10-12 glucose units were also identified by chromatographic methods.[35]




(i) (ii) (iii)

**Figure 14: Chemical structure of (i) α-CD (ii) β-CD and (iii) γ-CD**

### *3.3 Structure*

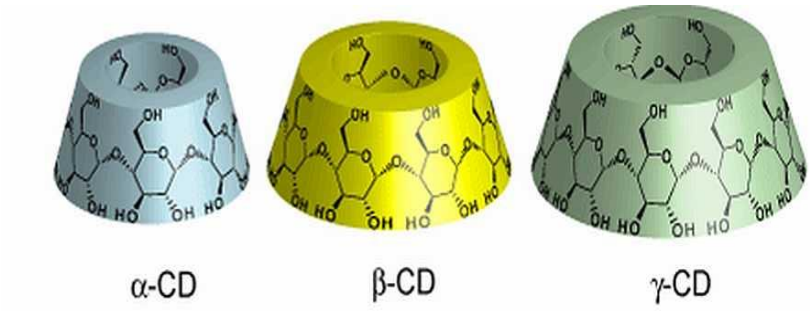



**Figure 15: Schematic presentation of the main three CDs**

As their appearance suggests (**see Fig 15**), the glucose units are linked by α-1,4 bonds in the cyclodextrin molecules. A cyclodextrin molecule resembles a hollow truncated cone and does not have a cylindrical shape due to the lack of free rotation of the bonds between glucopyranose units. Each glucopyranose unit has three hydroxyl groups. Two of them are secondary and are located at carbon 2 ($C_2$) and 3 ($C_3$) and one is primary and located at carbon 6 ($C_6$).The secondary hydroxyl groups ($C_2$ and $C_3$) are located on the wider

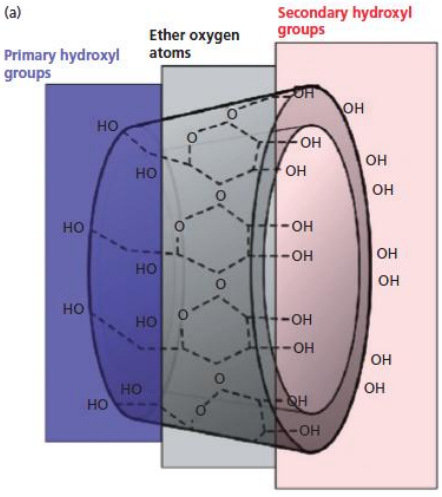



**Fig 16: Division of oxygen atoms in β-cyclodextrin structure[36]**

edge of the ring and the primary hydroxyl groups ($C_6$) on the other edge (narrower). Additionally, the apolar $C_3$ and $C_5$ hydrogens and ether-like oxygens are at the inside of the torus-like molecules. The conformation of the truncated cone of β-cyclodextrin is shown in **Fig 16.**[36] As a result, cyclodextrin is a molecule with apolar cavity, providing a hydrophobic matrix, and hydrophilic outside which can dissolve in water. This ''micro heterogeneous environment'' is a characteristic feature of cyclodextrins.[37]

Obviously, the number of glycose units is determinant concerning the cavity's dimension and size. As the cyclodextrin molecule is getting bigger, its molecular dimensions are increasing. The height of the torus remains constant at 0.79nm. The cavity diameter is 0.57nm for α-cyclodextrin, 0.78nm for β-cyclodextrin and 0.95nm for γ-cyclodextrin. Furthermore, there are significant differences with respect to the outer diameter (**see Fig 17**).

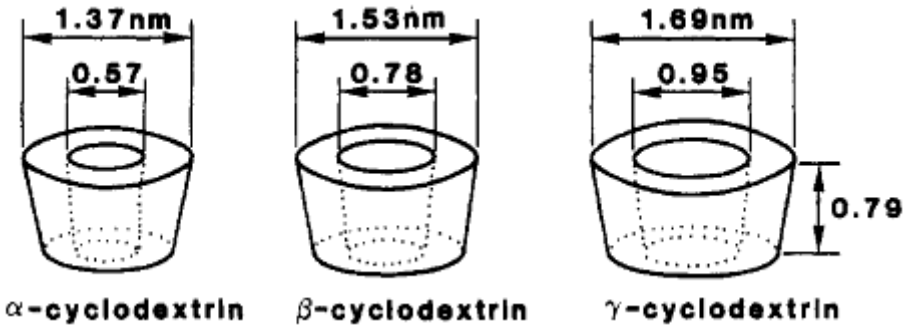



**Fig 17: Geometric dimensions of cyclodextrins**

It is worth noting also the fact that a ring of hydrogen bonds is formed intramolecularly between the 2-hydroxyl and the 3-hydroxyl groups of adjacent glucose units (**see Fig 18**). The remarkable rigid structure of cyclodextrins is attributed to this

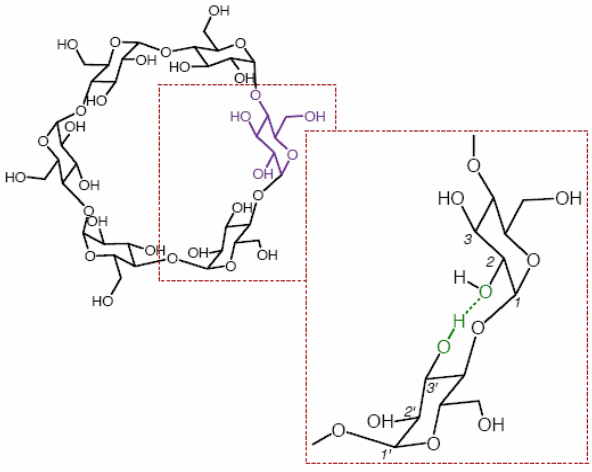


**Fig 18: Hydrogen bonds developed between the hydroxyl group of C-2 of a glucopyranose unit and the hydroxyl group of C-3 of the adjacent glucopyranose group in the molecule of beta-cyclodextrin**

hydrogen bonding ring.

## *3.4 Properties*

Cyclodextrins have some unique physical and chemical properties as a consequence of the above structural features. To begin with, cyclodextrins are poorly to moderately soluble in water, methanol, and ethanol, and readily soluble in strongly polar aprotic solvents like dimethyl sulfoxide (DMSO), dimethylformamide (DMF), N,N-dimethylacetamide, and pyridine as well as mixtures of these.[38]

What's more, the solubility of the naturally occurring cyclodextrins (α-, β-, γ-) can be improved by the production of some derivatives of them. Cyclodextrins are derivatized in order to vary their solubility behavior, to modify their complexation properties, and to introduce groups with certain specific functions, like catalytic function. Esterification, etherification or amination of the primary and secondary hydroxyl groups enhance the solubility of parent cyclodexrins.[28]

Among the cyclodextrins, β-cyclodextrin is the most accessible, lowest priced and generally the most useful. It is the most common in pharmaceutical formulations and thus, probably the best studied cyclodextrin in humans.

## *3.5 Host- guest chemistry in cyclodextrins*

A supramolecular structure results from defined noncovalent interactions between several individual molecules. Host -guest complexes are important examples of this type of structure. [39] We can meet the formation of those structures in cyclodextrins as the most notable property of them is their ability to form inclusion compounds with a variety of molecules. The only condition they have to satisfy is the following : they must fit entirely or at least partially into the cyclodextrin cavity. Aldehydes, ketones, alcohols, organic acids, fatty acids, aromatics, gases, and polar compounds such as halogens, oxyacids and amines are examples of compounds that can enter the cavity.[40]

Complex formation has a beneficial effect on the characteristic properties of the included substance. That means that the guest takes advantage of the inclusion and improves its physicochemical properties, which are not achievable otherwise. Such properties are the stabilization of labile guests against the degradative effects of oxidation, visible or UV light and heat, control of volatility, physical isolation of incompatible compounds, chromatographic separations and controlled release of drugs and flavours.[40]

The binding of guest molecules within the host cyclodextrin is a dynamic equilibrium process which can be illustrated by **eq 2**, where CD is cyclodextrin, G is the guest molecule, and CD-G is the inclusion complex:

$$CD + G \Leftrightarrow CD\text{-}G \quad \textbf{eq 2}$$

The stability of the inclusion complex can be described in terms of a formation constant $K_f$ or a dissociation constant $K_d$ as defined in **eqs 3 and 4:**

$$K_f = [CD\text{-}G]/( [CD]*[G] ) \quad \textbf{eq 3}$$

$$K_d = 1/K_f = ( [CD]*[G] ) / [CD\text{-}G] \quad \textbf{eq 4}$$

For determining the binding strength, several factors should be taken into consideration. The polarity of the guest molecule and its geometric capability, the medium and the temperature play a key role in the stability of the inclusion complex. Only molecules less polar than water can penetrate the cavity. The stability of a complex is proportional to the hydrophobic character of the guest molecule. Highly hydrophillic molecules complex very weakly or not at all. In addition, if the guest is too small, it will very easily pass in and out the cavity with little or no bonding at all. In the case of quite larger molecules than the cavity, complex formation may be plausible but only a part of the guest will manage to enter the cavity.

Complexes can be formed either in solution or in the crystalline state and water is typically the solvent of choice. The more soluble the cyclodextrin is in the solvent, the more molecules become available for complexation. The substrate must be able to displace the solvent from the cyclodextrin cavity if the solvent forms a complex

with the cyclodextrin. Water, for example is very easily displaced. It is true that some guests are not very well solubilized in water, making complexation either very slow or impossible. The use of an organic solvent to dissolve the guest is highly advisable.

With respect to temperature, it is generally acceptable that heating can increase the solubility of the complex but simultaneously destabilizes the complex. These effects often need to be balanced.

## *3.6 Molar Ratios in complexation Process*

Usually only one guest molecule interacts with one cyclodextrin molecule to give an inclusion compound. But it is not always the case. When the substrate has high molecular weight, more than one host molecule may bind to the substrate (2:1). On the other hand, in the case of low molecular weight molecules, more than one guest molecule may fit into the cavity (1:2). Formation of inclusion complex in a ratio of 2:2 has been noticed, too (**see Fig 19**).[41]

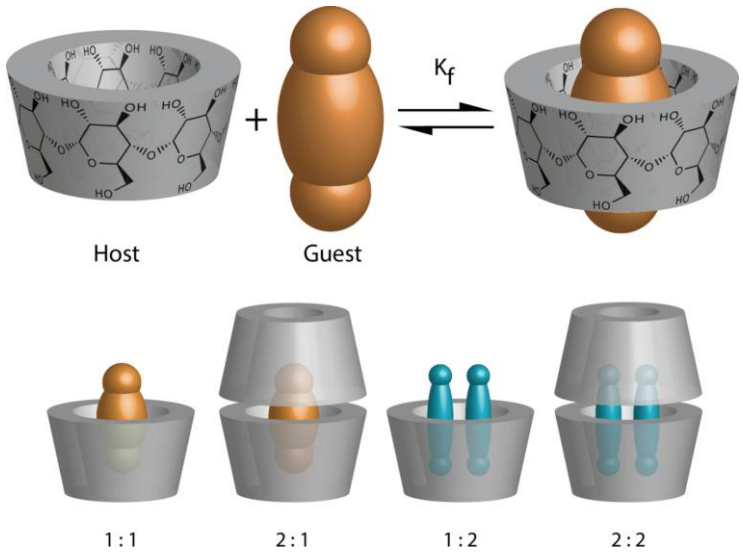



**Fig 19: Schematic illustrations of the association of free cyclodextrin ("host") and substrate ("guest") to form substrate–CD complexes**

## *3.7 Driving Forces involved in the complex formation*

During the formation of an inclusion complex, no covalent bonds are formed or broken.[42] In spite of that fact, a net energetic force should exist in order to pull the substrates being entrapped inside the cavity. The entrapment of a part or the bioactive substance in its wholeness in the hydrophobic cavity of cyclodextrin is associated with a series of phenomena-interactions. Studies carried out in this field have led to the conclusion that the formation of inclusion complexes is attributed to

- ✓ van der Waals' interaction
- ✓ Hydrophobic interactions
- ✓ Hydrogen bonds
- ✓ Release of water molecules rich in enthalpy

**van der Waals' forces**

The van der Waals' forces have no directional characteristic and appear when two atoms have a distance of around 3 or 4 Å between them. They are caused mainly by correlations in the fluctuating polarizations of nearby particles. Although they are weaker than the electrostatic interactions and hydrogen bonds, they are not less important for the formation of inclusion complexes. This type of forces becomes significantly important when large number of atoms of a particle approaches atoms of another molecule. This can occur only if the shape of the involved molecules is suitable for interaction. In other words, the effectiveness of the van der Waals bonds depends on the complementarity of molecules in space and how well they fit each other. Thus, although there is no specificity in a van der Waals type bond, specificity resulting from the opportunity to create many van der Waals' interaction simultaneously inside the cavity of the cyclodextrin.[43,44]

**Hydrophobic Interactions**

With the term ‘’hydrophobic interactions’’, we describe the tendency of hydrophobic molecules or lipophilic parts of molecules to avoid water because they cannot be adapted to the characteristic structure of water with hydrogen bonds. As a consequence, large lipophilic molecules are coupled each other in order to avoid water, forming in that way structures where their polar hydrophilic parts interact with water molecules. This interaction leads to a positive enthalpy and entropy change and this change is considered as an indication of existence of hydrophobic interactions. Firstly, the fact that during the formation of inclusion complexes in cyclodextrins’ cavity the change of the two thermodynamic parameters is negative, led to the wrong conclusion that the contribution of hydrophobic interactions during complexation is very small, almost negligible. Afterwards, it has been reported that hydrophobic interactions play an essential role in complexation process and that the positive growth of entropy and enthalpy outweighed by the negative changes due to the release of molecules rich in enthalpy and van der Waals interactions. However, the unique "source" of increase of entropy is the hydrophobic interaction.[43,44]

**Hydrogen bonds**

The definition of hydrogen bond according to IUPAC is : ‘’ The hydrogen bond is an attractive interaction between a hydrogen atom from a molecule or a molecular fragment X–H in which X is more electronegative than H, and an atom or a group of atoms in the same or a different molecule, in which there is evidence of bond formation’’. Indeed, a hydrogen bond is the electrostatic attraction between polar groups that occurs when a hydrogen (H) atom bound to a highly electronegative atom such as nitrogen (N), oxygen (O) or fluorine (F) experiences attraction to some other nearby highly electronegative atom. This type of bond is stronger than van der Waals’ forces but weaker compared to covalent bonds. The hydrogen bond typically has between 5 and 30 kJ/mole in thermodynamic terms. In case of cyclodextrins, it plays an important role in the

complexation since hydrogen bonding is found between the polar functional groups of guest molecules and the hydroxyl groups of the host.[44]

**Exclusion of cavity-bound high-energy water**

The main driving force of complex formation is considered to be the release of enthalpy rich water molecules from the cavity. The water molecules located inside the cavity cannot satisfy their hydrogen bonding potentials and therefore are of higher enthalpy. The energy of the system is lowered when these enthalpy-rich water molecules are replaced by suitable guest molecules which are less polar than water.[41,44] In more details, when the water molecules are expelled from the cavity and enter the larger pool of water solvent, they are rearranged and then form hydrogen bonds each other so the number of those bonds increase. Meanwhile, as the upcoming entrapped molecule approaches the cyclodextrin cavity, a reduction of the repulsive interactions between the hydrophobic guest and the aqueous environment is taking place. Lastly, an increase in the hydrophobic interactions as the guest inserts itself into the apolar cyclodextrin cavity is being noticed.

## *3.8 Applications*

The practical application of cyclodextrins has gained in importance over the past few years. The unique property of complex formation which results in beneficial changes in the characteristic properties of the included substance renders the cyclodextrin suitable for applications in cosmetics and toiletry, food, agricultural and chemical industry.

In terms of cosmetics and toiletry, cyclodextrins have been widely used for stabilization of fragrances by a further complexation of the inclusion compound formed between the cavity of the host and the substrate- fragrance, with calcium phosphate. Thus, the fragrance is being stabilized for manufacturing bathing

preparations.[45] Furthermore, several cosmetic products composed by cyclodextrins have been used for the reduction of body odours.[46] Other applications include odor control of washed items by cyclodextrin- based laundry detergent,[47]use in skin creams, paper towels, tooth paste, diapers.[30] In the field of food industry, β-cyclodextrin is a perfect tool for removing cholesterol from milk and dairy products. What's more, it has the ability to reduce bitterness and stabilize flavors when they are subjected to long term storage.[48] As a potential sweetener, cyclodextrins are accounted for the enhancement of beer's and whiskey's flavor.[49]

Cyclodextrins complexes are formed also with several agricultural chemicals causing a delay in the rate of germination of seed.[30] In addition, they can be found in the stationary phases of some analytical methods (GC, HPLC) for separating enantiomers as well as chiral shift agents in NMR.[30] Toxic substances that may be found in industrial waste can be removed by complexation with cyclodextrins. Moreover, the application of cyclodextrins in environmental science ranges from the solubility improvement of organic pollutants to removal of heavy metals from water, soil or even atmosphere.[50]

## *3.9 Click chemistry concept: fundamental and mechanistic aspects*

Sharpless in 2001 was the first who introduced the term ''click chemistry'', one of the most versatile and modular approaches to couple two reactive partners in a facile, quick, selective, reliable and high yield reaction under mild conditions. [51] Click chemistry reactions include cycloadditions, nucleophilic ring openings, carbonyl chemistry of non-aldol type and carbon–carbon additions.[52] Click chemistry finds many applications in a great variety of fields, such as the chemistry of nanomaterials, chemical biology, drug delivery, and medicinal chemistry.[21-26] Copper catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) is the best-known reaction from this set and is often referred as "click chemistry". Click reaction originates from the 1,3-dipolar cycloaddition of azides with dipolarophiles, that was reported by Huisgen in 1963.[54] Sharpless and Meldal independently proved that the 1,3-dipolar cycloaddition of azides with terminal alkynes could be further enhanced when catalyzed by copper(I) complexes. [52, 55]

<u>$Cu^{I}$ -CATALYZED HUISGEN 1,3-DIPOLAR CYCLOADDITION OF AZIDES AND TERMINAL ALKYNES</u>

The traditional 1,3-dipolar Hüisgen cycloaddition takes place at high temperatures but it has been demonstrated that milder conditions can be used (0-160$^0$C) producing minimal byproducts. What's more, CuAAC reaction can be performed in a variety of solvents such as water, ethanol or *tert*-butyl alcohol using a wide range of pH values (5-12). [17]

In the presence of a copper complex, the cycloaddition between an azide and a terminal alkyne group takes place resulting to a 1,2,3-triazole cycle (**see Fig 20**).[56]

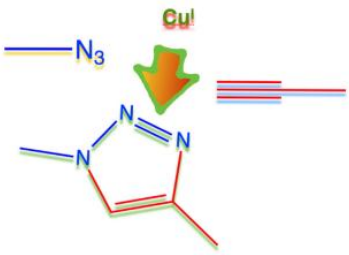



**Fig 20: Representation of the copper catalyzed azide-alkyne 1,3- dipolar cycloaddition (CuAAC)**

This particular reaction is regiospecific since it exclusively forms 1,4-substituted products.

As far as the mechanism of CuAAC is concerned, Fokin and his co-workers have proposed a detailed one.[57] According to their work, the first step of the reaction mechanism is the coordination of alkynyl to one copper, forming copper acetylide species. Afterwards, the azide is activated by coordination to a second copper atom. In the next step, the first C-N bond is formed. Cyclisation takes place and $Cu^{I}$ is excluded – through reductive elimination- for the formation of the triazole ring (**see Fig 21**). After the release of the $Cu^{I}$ catalyst from the 1,2,3-triazole product, a second catalytic cycle with different substrate can be done.

The cycloaddition product is chemically inert or stable towards redox reactions, has strong dipole moment, hydrogen bond accepting ability and aromatic character.[58]

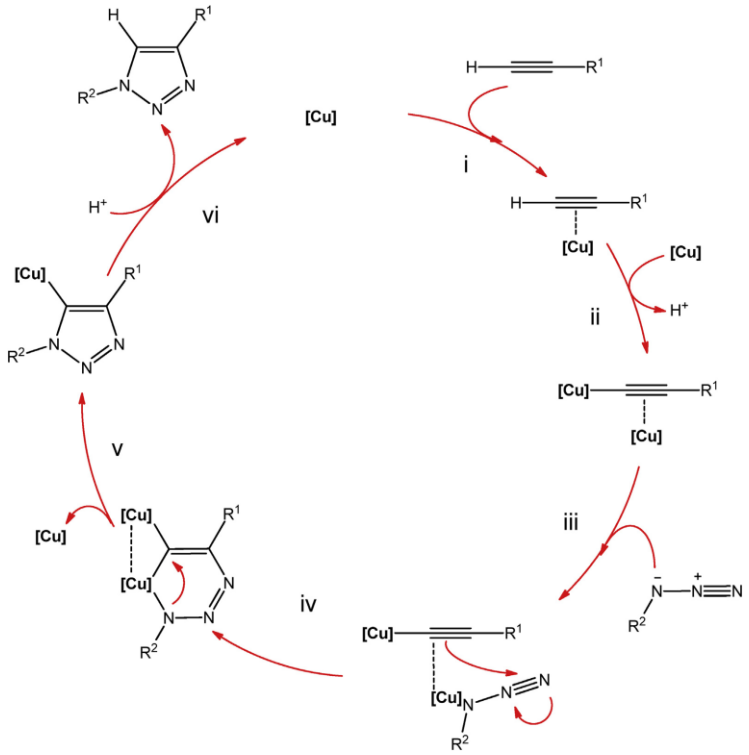



**Fig 21: Schematic representation of mechanism of the CuAAC reaction**

The re-generation of the $Cu^{I}$ catalyst is essential to take place. One commonly used way is to reduce $Cu^{II}$ salts, like $CuSO_4 \cdot 5H_2O$, in situ to form $Cu^{I}$ salts. Sodium ascorbate is typically utilized as the reducing agent in a large excess among several others.[59] It is a cheap method, it can be performed in water, and it does not require deoxygenated atmosphere.[59] A second way to create the catalyst is to directly add $Cu^{I}$ salts. CuBr, CuI are some of the characteristic compounds which have been used so far.[52] No reducing agent is required, but it has to be done in a deoxygenated environment and in an organic or a mixed solvent.

## Cyclodextrins and copper-catalyzed azide–alkyne cycloaddition (CuAAC)

As mentioned above, cyclodextrins constitute important building blocks in organic chemistry and by exploiting their unique properties, scientists have introduced them in innumerable areas. On the other hand, click reaction denotes a set of reactions widely used, able to link moieties together in a very efficient and reliable way. It was inevitable, then, to be involved in the cyclodextrins's chemistry.

Apparently, when marrying cyclodextrins with click chemistry, a functionalisation needs to take place. A chemical modification in the cyclostructure such as the substitution of hydroxyl groups, is necessary in order to proceed with the click. One of the most useful functionalization is the introduction of an azide group. In that way, the obtained azido-CDs are good candidates for the coming click chemistry and for attaching CDs together or to more complex structures such as polymers.

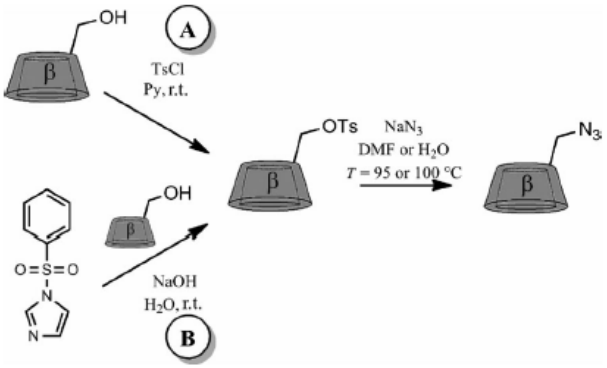



**Fig 22: ways for the synthesis of mono-$N_3$-cdx**

One, two or more azido group can be introduced. Regarding the monosubstitution reaction, numerous publications have described in details the synthetic method which was followed. Generally speaking, two ways are predominant (**see Fig 22**). According to the first one, cdxs are first tosylated classically with tosyl chloride (TsCl) and then azidated by using sodium azide ($NaN_3$) to give the corresponding mono (6-azido-6-deoxy)-β-CD ($N_3$-β-CD).[60] Based on the second one, the preactivation of TsCl with imidazole is done.[61] This strategy ensures that only a small amount of the undesired 6-*O*-ditosyl-β-CD is formed compared to other procedures. Despite the fact that no chromatography steps are involved, the yield is lower than classical tosylation yields (20%).

# Aim of the project

The demands in energy are increasing rapidly due to the growing global population. Therefore, one of the most important challenges scientific community facing in the 21th century is the supply of secure, clean, sustainable energy.

Sunlight is the most abundant and one of the cleanest sources of energy. Solar energy is providing more energy in 1 hour to the earth than all of the energy consumed by humans in an entire year.[62] By mimicking the natural process of photosynthesis, solar energy can be used to drive catalysis and convert light to stored chemical energy.

Our goal is to design and develop such stable systems in which light absorption triggers electron transfer events that, at the same time, lead to the activation of a catalytic center. There are several publications which have associated photosensitizers with transition metal complexes for photocatalysis purposes.[63] Moreover, one direction of research focuses on design of photo-driven systems using photosensitizer and enzyme for catalyzing chemical transformations using water or dioxygen as oxygen atom source.[64]

All the above systems have a common feature: they demand the presence of a sacrificial electron acceptor and inert conditions. Even the production of reactive oxygen species should be taken into account. So, the development of a system where the atmosphere's dioxygen could function as the final electron acceptor, in such catalytic systems, is highly desirable.

Scientists have been interested in designing properly a hybrid catalyst associating a photosensitizer with a unit capable of react with dioxygen. Metallo-enzymes such as laccase are excellent examples of ''oxygen catalytic units''. In this field, Gray et al. pioneered the coupling of photoactive units to metalloenzymes to access the buried active sites leading to great advances on the understanding of electron transfer

processes in biological systems.[65] According to an interesting scientific work performed in the laboratory, a photoinjection of four electrons into a multicopper oxidase (laccase) using a Ru–based photosensitizer was documented for the first time by Simaan and co-workers.[66]

Substitution of the Ru-based photosensitizer by a Zn- porphyrin has led to a zinc porphyrin- laccase system, catalytically active for dioxygen reduction into water.[67] Until now, 3 approaches have been developed. The first one is the bimolecular system, where the multicopper oxidase laccase and the porphyrin were mixed in solution. Within this system, for the first time electronic communication between a metallosensitizer (Zn-porphyrin) and laccase was observed upon light irradiation (**see Fig 23**). [67]

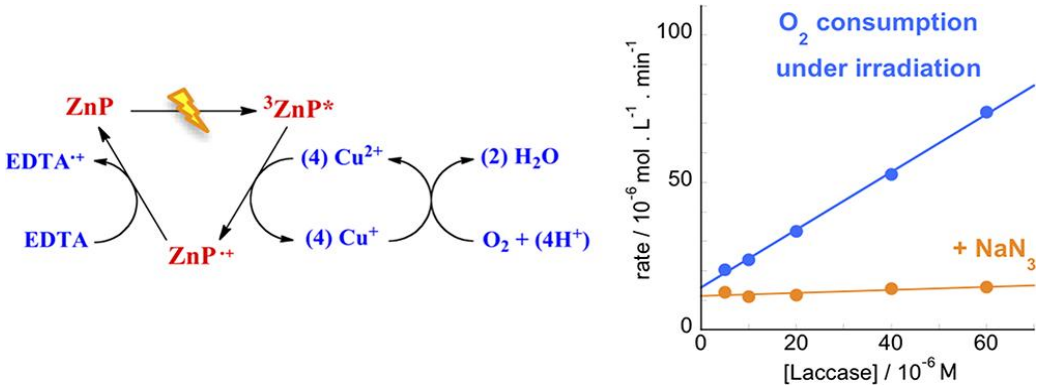



**Fig 23: Schematic representation of charge transfer upon irradiation and dioxygen consumption rate in presence and absence of $NaN_3$**

The second approach is the grafted one, where the porphyrin is covalently linked on the surface of laccase which is appropriately modified (**see Fig 24**). A suitable strategy was settled for the successful reaction between the enzyme and the photosensitizer, obtaining 90% yield. Through this hybrid system, it was proven that laccase is active, maintaining 80% of the initial activity of the unmodified enzyme.

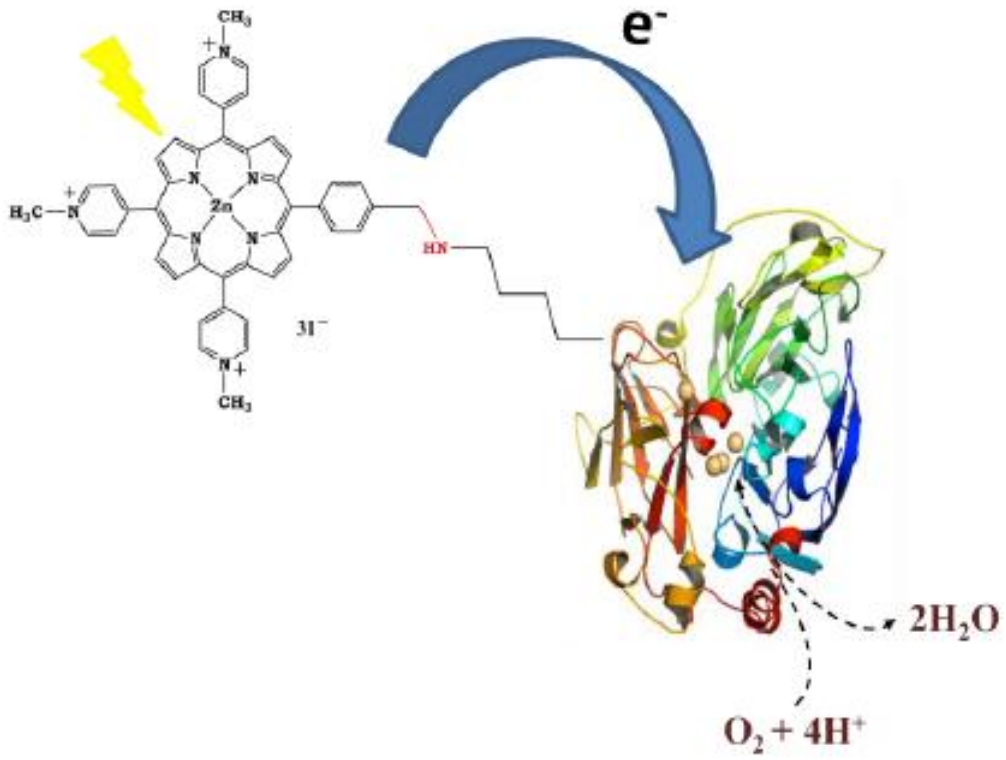



**Fig 24: Dioxygen reduction representation. Zn-porphyrin and laccase covalently linked.**

Last but not least, is the co-adsorption of porphyrin and enzyme to the surface of titanium oxide nanoparticles ($TiO_2$). In that case, visible light excites the photosensitizer which injects electrons into the $TiO_2$ conduction band and the electrons are then transferred into the enzyme (**see Fig 25**). As far as the oxidized dye is concerned, it is recovered by the sacrificial electron donor. The enzyme remains 97% active.

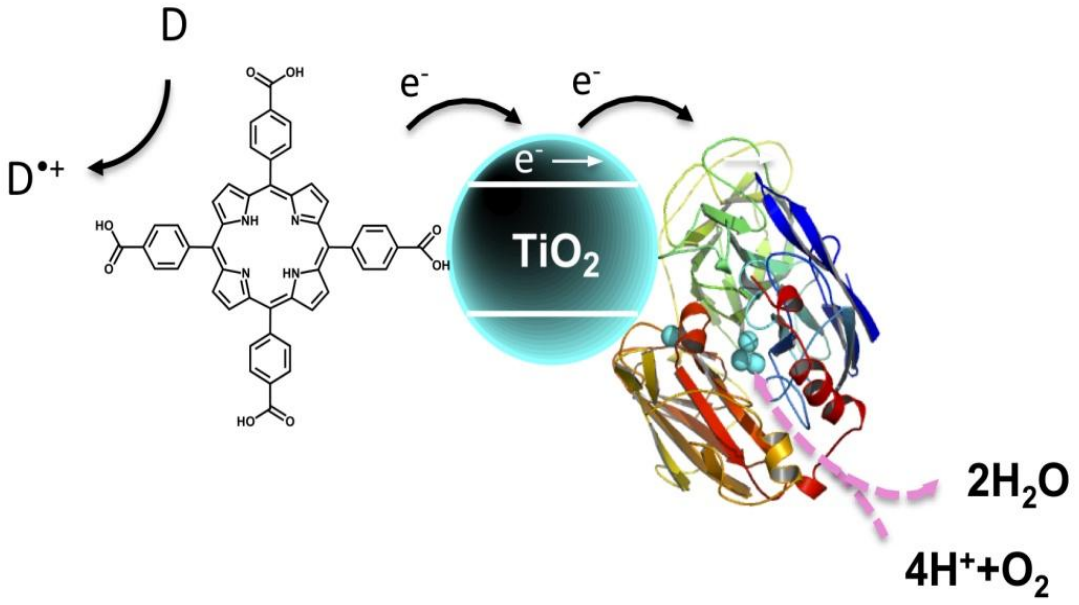



**Figure 25: Dioxygen reduction representation of the third approach**

So, the next system we wanted to explore its potential regarding the dioxygen reduction is a supramolecular system constituted by a photosensitizer, laccase and beta-cyclodextrin.

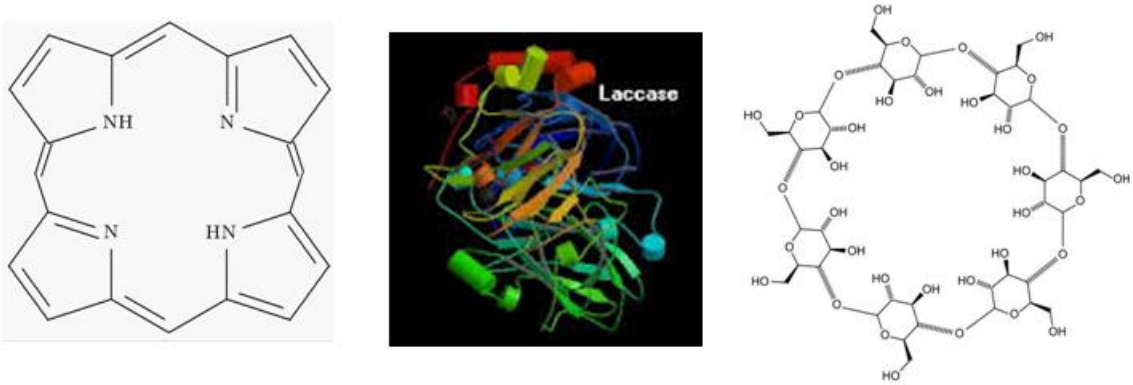
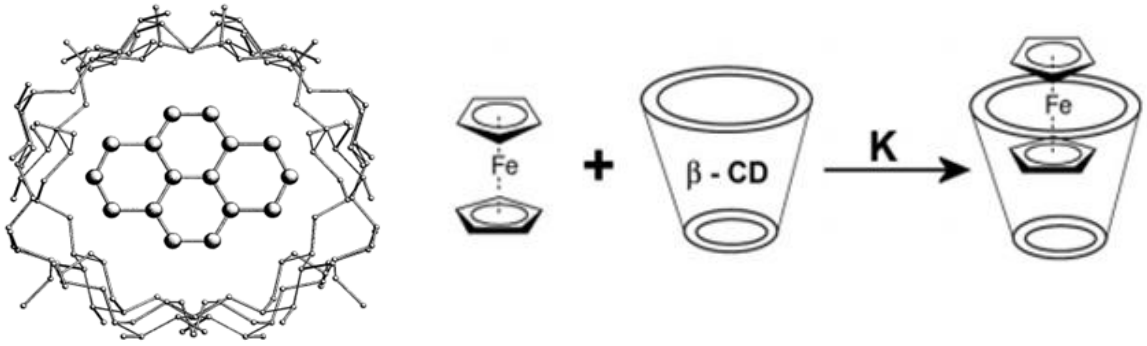



**Fig 26: Represantation of (a) main porphyrin core (b) 3-D laccase structure and (c) β-cyclodextrin structure**

Innumerable publications have confirmed that pyrene and ferrocene are characteristic molecules than can enter the cavity of cyclodextrin forming inclusion complexes (**see Fig 27**).[68-71] Hence, our synthetic approach concerning the photosensitizer relies on the incorporation of these two groups at the porphyrin core.




**(a)** **(b)**

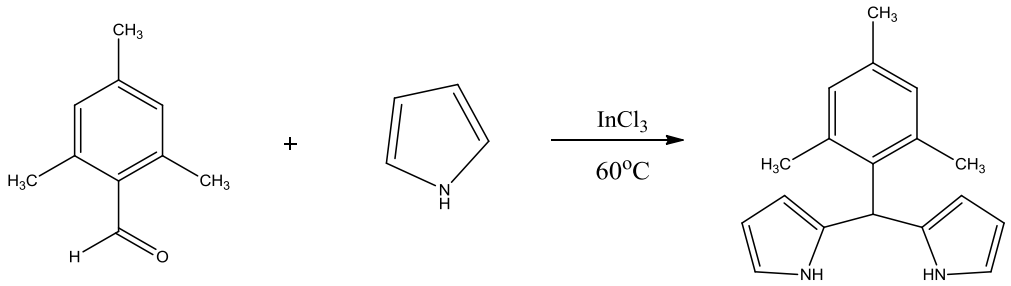
**Fig 27: (a) View of the pyrene molecular orientation with respect to that of the β-cyclodextrin molecule and (b) Schematic Interpretation of the host-guest interaction between ferrocene and β-cdx**

# Chapter 2 - Experimental Section

# Synthesis of Photosensitizers

In this chapter, the stepwise synthesis of the appropriate photosensitizers is going to be presented.

**Synthesis of 5-mesityl dipyrromethane (1)**




**(1)**

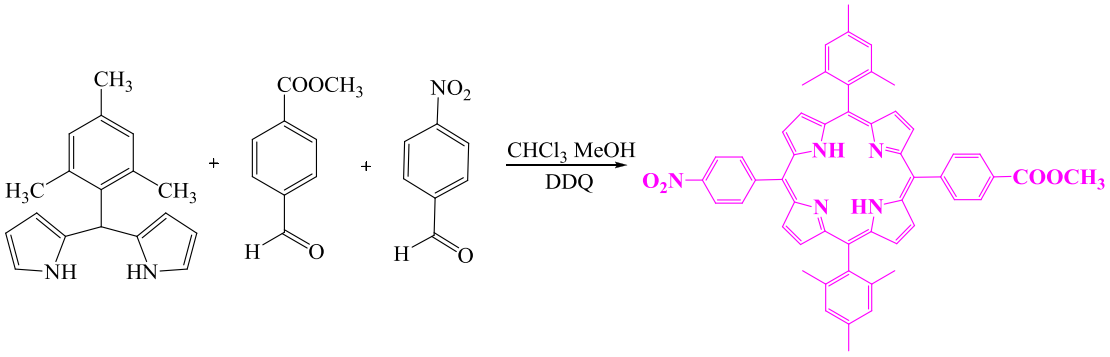
A 250ml two necked round bottom flask equipped with a magnetic stirrer , was placed over a hot plate in an oil bath. It was degassed with a stream of argon for 10 minutes at room temperature. Then, pyrrole (175ml, 2522.4mmol) and mesitylaldehyde (3.7ml, 25mmol) were added. Meanwhile, the flask was covered from light with aluminum foil in order to avoid a plausible oxidation of pyrrole. After 15minutes, $InCl_3$ (1.66gr, 7.55mmol) was added. The reaction mixture was stirred for 5h at $60^0$C. It colored yellow at that time. After this time, the heat source was removed and NaOH (9gr, 0.15mol) was added. The mixture was stirred for 1 hour. The addition of NaOH was made to neutralize the $InCl_3$ (Lewis acid) and so the mixture became neutral. The color of the mixture changed to dark green due to the production pyrrole's polymers. The green precipitate formed was filtered through a Buchner funnel and washed with pyrrole several times. Last step was the vacuum distillation of pyrrole filtrate using liquid nitrogen for the proper refrigeration of the experimental device. The distillation was done at $60^0$C.

A solution of ethyl acetate / hexane 1: 4 (400ml) was prepared. Originally 100ml of the solution were transferred in the flask and dissolved to ultrasound. The process was repeated two more times. Filtration to the solution through a chromatographic column regularly checking by TLC on silica plates was followed. After filtration, the yellow solution was collected and distillation under vacuum ($30^0$C, 400pressure) was carried out. 60ml of ethanol / water solution in a ratio of 4: 1 were added and heated for 2 minutes at 100°C in a water bath. The mixture was left to rest for 1h at room temperature. Lastly, another filtration took place performing several washes with ethanol / water solution (4: 1). The resulting solid was collected and dried under vacuum (2,2gr, 32.04% yield).

MS (MALDI-TOF): m/z calcd. for $C_{18}H_{20}N_2$ $[M+ H]^+$ 264.36, found 264.30

$^1$H NMR (300 MHz, $CDCl_3$): 2.34 (9H, s), 5.0 (2H, s), 5.34 (1H, s) , 5.72 (2H, d) , 5.99 (2H, m), 6.69 (2H, d), 6.79 (2H, s)

**Synthesis of 5(4-methoxycarbonylphenyl)- 15(4-nitrophenyl)- 10,20-bis(2,4,6- trimethylphenyl) porphyrin (2)**




**(1)** **(2)**

In a 1000ml round bottom flask dipyrromethane (1gr, 4mmol) and nitro-benzaldehyde (0.3gr, 2mmol) were dissolved in MeOH (400mL). Subsequently, a solution of HCl (36 %, 20mL) in 200ml $H_2O$ was added, and the reaction mixture was

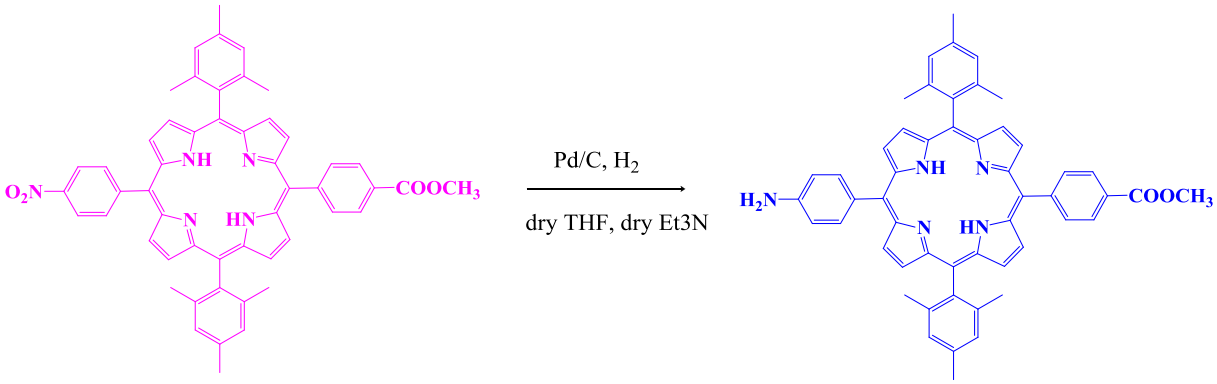
protected from light by aluminum foil and stirred at room temperature for 2 hours. The yellow precipitate formed was filtered through a Buchner funnel and washed with Ethanol (400ml). Methyl-4-formyl- benzoate (0.4gr, 2mmol) was added and after the dissolution of the mixture, a solution of HCl (36%, 20ml) in 200ml $H_2O$ was added and the reaction mixture was stirred at room temperature for 3 hours. The yellow precipitate formed was filtered through a Buchner funnel again and washed with dichloromethane (550ml). Tetrachloro-1,4-benzoquinone (1.5gr, 6mmol) was added and the solution was stirred overnight at room temperature. The reaction mixture was concentrated to half of its volume and filtered through a silica gel column eluted with $CH_2Cl_2$ and evaporated. The residue was purified by column chromatography (silica gel eluted with $CH_2Cl_2$/Hexane 6/4) affording porphyrin **(2)** as a purple solid (360mgr, 11.2% yield).

MS (MALDI-TOF): calcd. for $C_{52}H_{43}N_5O_4$ 801.3315 [M]+, found 801.3304

$^1$H NMR (500 MHz, $CDCl_3$): δ = 8.75 (m, 4 H), 8.72 (d, J = 4.5 Hz, 2 H), 8.70 (d, J = 5 Hz, 2H), 8.63 (d, J = 8.5 Hz, 2 H), 8.43 (d, J = 8.5 Hz, 2 H), 8.41 (d, J= 8.5 Hz, 2 H), 8.31 (d, J = 8 Hz, 2 H), 7.29 (s, 4 H), 4.11 (s, 3 H),2.63 (s, 6 H), 1.84 (s, 12 H), –2.64 (s, 2 H) ppm

$^{13}$C NMR (125 MHz, $CDCl_3$): δ = 167.5, 149.2 147.9, 146.8, 139.4, 138.2, 135.2, 134.6, 130.9, 130.7, 129.8, 128.1, 128.0, 122.0, 119.3, 118.8, 116.4, 52.6, 21.8, 21.6 ppm

**Synthesis of 5(4-methoxycarbonylphenyl)- 15(4-aminophenyl)- 10,20-bis(2,4,6- trimethylphenyl) porphyrin (3)**




(2) (3)

To a solution of 5-(4-nitrophenyl)-10,20-dimesityl-15-(4-methoxy-carbonyl-phenyl) porphyrin (360 mg, 0.449mmol) in anhydrous THF (72mL) and anhydrous $Et_3N$ (270 µL), Pd/C 10% (79,2 mg) was added. The reaction mixture was stirred overnight, under a $H_2$ atmosphere, at room temperature.

The solution was passed through Celite and concentrated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel ($CH_2Cl_2$/Hexane 6/4) to obtain the desired product as a purple solid (190 mg, 54.7% yield).

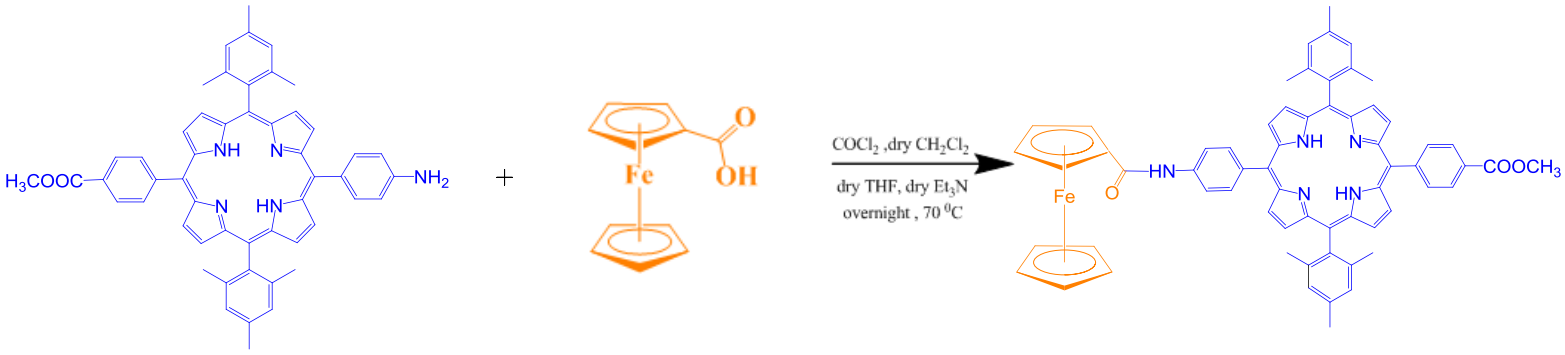
MS (MALDI-TOF): calcd. for $C_{52}H_{45}N_5O_2$ 771.3573 [M]+, found 771.3606

$^1$H NMR (500 MHz, $CDCl_3$): δ =8.91 (d, J = 4.5 Hz, 2 H), 8.72 (m, 6 H), 8.44 (d, J = 8 Hz, 2 H),8.32 (d, J = 8.5 Hz, 2 H), 8.01 (d, J = 8 Hz, 2 H), 7.29 (s, 4 H), 7.06 (d, J = 8 Hz, 2 H), 4.12 (s, 3 H), 2.64 (s, 6 H), 1.85 (s, 12 H),–2.58 (s, 2 H) ppm

$^{13}$C NMR (125 MHz, $CDCl_3$): δ = 167.5, 147.2, 146.2, 139.5, 138.6, 137.9, 135.7, 134.7, 132.3, 131.8, 130.9, 130.4, 130.1, 129.6, 128.0, 127.9, 120.6, 118.5, 117.5, 113.6, 52.5, 21.8 21.6 ppm

UV/Vis ($CH_2Cl_2$): *λmax*/nm (ε/$M^{-1}$*$cm^{-1}$): 421 (381.3), 516(18.0), 553 (9.4), 593 (5.5), 649 (5.4) nm

**Synthesis of 5(4-methoxy carbonyl phenyl)- 15(4- ferrocene-2-carbonyloxy)phenyl)- 10,20-bis(2,4,6-trimethyl phenyl) porphyrin (4)**




**(3)** **(4)**

To a solution of ferrocene carboxylic acid (119.35 mg, 0.52mmol) in dry $CH_2Cl_2$ (10.4 mL), oxalyl chloride (72.6 µl, 0.8mmol) was added and the solution was stirred under nitrogen at $40^0$C for 1h. Then the solvent and excess oxalyl chloride were removed and the mixture was dried under vacuum for 1 h, to yield the ferrocene carboxylic acid chloride, which was dissolved in dry THF (41.50mL). Porphyrin **(3)** (80 mg, 0.10mmol) and triethylamine (270µl, 1.8mmol) were added and the solution was stirred overnight at $70^0$C. After removal of the solvent the residue was purified by silica column chromatography with $CH_2Cl_2$/ Hexane 8/2 elution. The desired product **(4)** was obtained with $CH_2Cl_2$ as a purple solid (65.5 mg, 64.1% yield).

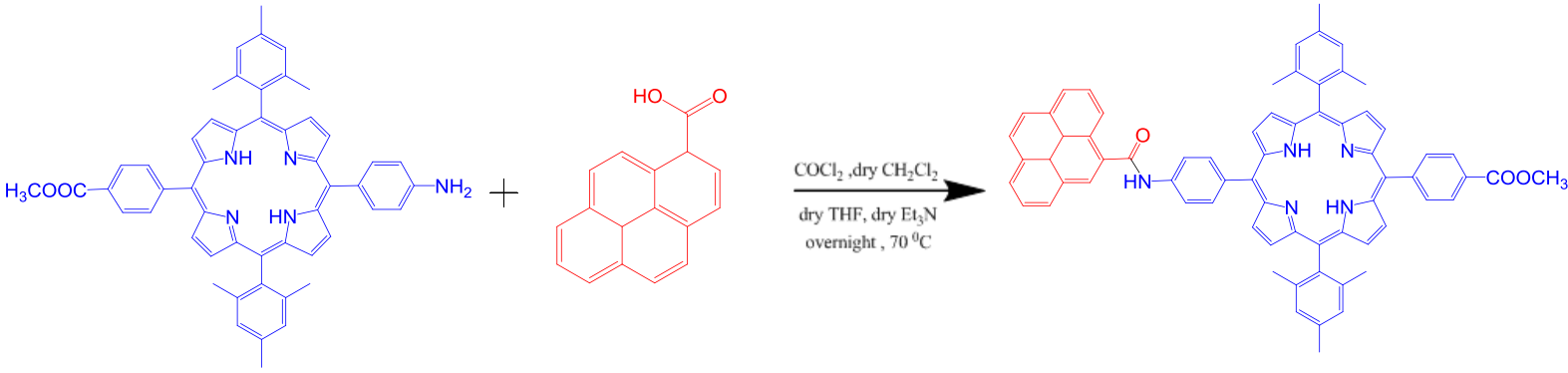
MS (MALDI-TOF): m/z calcd. for $C_{63}H_{53}FeN_5O_3$ $[M+H]^+$ 983.34, found 983.95

$^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 8.88 (d, $J$ = 4.77 Hz, 2 H), 8.73 (m, 6 H), 8.43 (d, $J$ = 8.16 Hz, 2 H), 8.31 (d, $J$ = 8.16 Hz, 2 H), 8.22 (d, $J$ = 8.34 Hz, 2 H), 7.99 (d, $J$ = 8.46 Hz, 2 H), 7.74 (s, 1H), 7.29 (s, 4 H), 4.93 (t, $J$ = 1.80 Hz, 2 H), 4.53 (t, $J$ = 1.80 Hz, 2 H), 4.39 (s, 5 H), 4.11 (s, 3 H), 2.63 (s, 6 H), 1.84 (s, 12 H), –2.62 (s, 2 H) ppm

$^{13}$C NMR (300 MHz, $CDCl_3$): $\delta$ 169.20, 167.51, 147.06, 139.49, 138.46, 138.02, 137.92, 137.70, 135.31, 134.67, 131.05, 129.61, 128.03, 127.92, 119.38, 118.70, 118.22, 117.86, 76.41, 71.25, 70.17, 68.60, 52.54, 21.76, 21.59

UV/Vis ($CH_2Cl_2$): $\lambda max$/nm ($\varepsilon/M^{-1}*cm^{-1}$): 420 (442500), 515.5 (17850), 551 (8269), 591.5 (5262), 647.5 (4529.7)

**Synthesis of 5(4-methoxy carbonyl phenyl)- 15(4-pyrene- 2-carbonyl oxy)phenyl)- 10,20-bis(2,4,6-trimethyl phenyl) porphyrin (5)**




**(3)** **(5)**

To a solution of pyrene carboxylic acid (79.85 mg, 0.32mmol) in dry $CH_2Cl_2$ (5.0mL) oxalyl chloride (44.9µl, 0.52mmol) was added and the solution was stirred under nitrogen at 40$^0$C for 1h. Then the solvent and excess oxalyl chloride were removed and the mixture was dried under vacuum for 1 h, to yield the pyrene carboxylic acid chloride, which was dissolved in THF (10mL). Porphyrin **(3)** (50 mg, 0.065mmol) and triethylamine (65µL, 2.2mmol) were added and the solution was stirred overnight at 70$^0$C. After removal of the solvent, the residue was obtained by

silica column chromatography using $CH_2Cl_2$ as eluent. 41.7 mg (64.2% yield) of the product **(5)** were obtained.

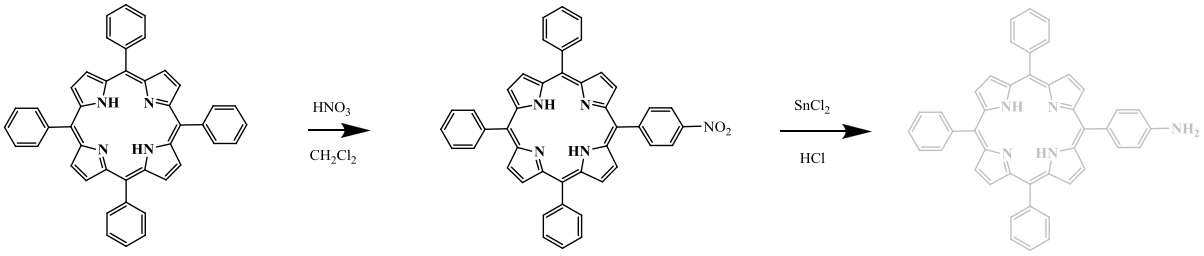
MS (MALDI-TOF): m/z calcd. for $C_{69}H_{55}N_5O_3$ $[M+H]^+$ 1001.43, found 1001.70

$^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 8.91 (d, *J* = 4.47 Hz, 2 H), 8.85 (d, *J* = 9.33 Hz, 1 H), 8.74 (t, *J* = 3.78 Hz, 6 H), 8.26 (m, 17 H), 7.31 (s, 4 H), 4.11 (s, 3 H), 2.6 (s, 6H), 1.8 (s, 12 H), –2.5 (s, 2 H ) ppm

$^{13}$C NMR (300 MHz, $CDCl_3$): $\delta$ = 168.5, 167.5, 147.0, 139.5, 138.4, 138.3, 138.1, 137.9, 135.3, 134.6, 133.1, 131.3, 130.9, 130.8, 130.6, 130.4, 129.6, 129.3, 129.2, 129.0, 128.0, 127.9, 127.2, 126.6, 126.2, 126.1, 125.0, 124.8, 124.6, 124.4, 119.2, 118.7, 118.3, 117.9, 52.5, 21.8, 21.6 ppm

UV/Vis ($CH_2Cl_2$): $\lambda max$/nm (ε/$M^{-1}$*$cm^{-1}$): 243.50 (82080) 278.5 (54500), 346.5 (52300), 420 (528000), 515 (24170), 550.5 (11260), 591.5 (7110), 648 (6610)

**Synthesis of 5-(4-aminophenyl)-10,15,20-triphenyl porphyrin (6)**




**(6)**

Tetraphenyl porphyrin (2gr, 3.25mmol) was dissolved in dichloromethane (300mL). Nitric acid 65% (4.2mL, 61.75mmol) was added with a dropping funnel at 0 °C over a 2 h period. The reaction was monitored by TLC and when it was converted to the desired product the solution was washed 3 × 150mL with saturated solution of

$NaHCO_3$ and then 3 × 150mL with $H_2O$ and dried using sodium sulfate. The solvent was removed under reduced pressure and the crude product was dissolved in HCl (65mL), tin chloride (2gr, 10.5mmol) was added and the solution was refluxed overnight. The mixture was neutralized by addition of aqueous ammonia, and washed with ethyl acetate (5 × 150mL) dried and solvent was removed. The product (6) was isolated by silica column chromatography $CH_2Cl_2$ to obtain the desired product as a purple solid (1.3 g, 63%).

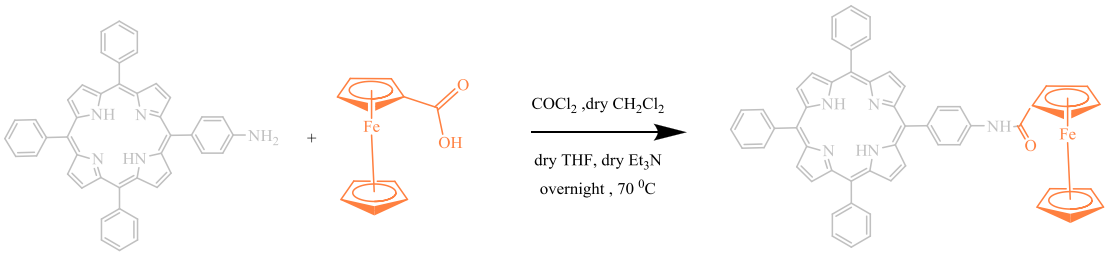
MS (MALDI-TOF): m/z calcd. for calcd for $C_{44}H_{32}N_5$ [M + H]+ 630.26, found 630.26

$^1$H NMR (500 MHz, CDCl3): δ 8.96 (d, *J* = 4.5 Hz, 2 H), 8.86 (s, 6 H), 8.24 (m, 6 H), 8.00 (d, *J* = 8.5 Hz, 2 H), 7.77 (m, 9H), 7.05 (d, *J* = 8.5 Hz, 2 H), 3.99 (s, 2H), −2.71 (s, 2 H) ppm

$^{13}$C NMR (500 MHz, CDCl3): δ 146.2, 142.4, 135.8, 134.7, 132.5, 131.2, 127.8, 126.8, 121.0, 120.1, 119.9, 113.6 ppm

UV/vis (toluene) : *λmax*/nm (ε/$M^{-1}$*$cm^{-1}$) : 422 (258.2), 516 (12.5), 552 (6.8), 594 (3.6), 650 (2.8)

**Synthesis of 5-(4-aminophenyl)-10,15 bisphenyl- 20-ferrocene phenyl porphyrin (7)**




(6) **(7)**

To a solution of ferrocene carboxylic acid (73.04 mg, 0.32mmol) in dry $CH_2Cl_2$ (5.0mL), oxalyl chloride (44 µl, 0.8mmol) was added and the solution was stirred under nitogen at $40^0$C for 1h. Then the solvent and excess oxalyl chloride were removed and the mixture was dried under vacum for 1 h, to yield the ferrocene carboxylic acid chloride, which was dissolved in dry THF (10mL). Porphyrin (6) (40 mg, 0.063mmol) and triethylamine (63.5 µl, 1.8mmol) were added and the solution was stirred overnight at $70^0$C. After removal of the solvent the residue was purified by silica column chromatography with $CH_2Cl_2$/ Hexane 7/3 elution. 33.3 mg (59% yield) of the desired product **(7)** were obtained.

MS (MALDI-TOF): m/z calcd. for $C_{58}H_{49}FeN_5O$ $[M+ H]^+$ 841.25, found 841.52

$^1$H NMR (500 MHz, $CDCl_3$) : $\delta$ = 8.93 (d, *J* = 4.6 Hz, 2 H), 8.86 (m, 6 H), 8.22 (m, 8 H), 8.00 (d, *J* = 8.45 Hz, 2 H), 7.76 (m, 10 H), 4.93 (t, *J* = 3.75 Hz, 2 H), 4.52 (t, *J* = 3.75 Hz, 2 H), 4.4 (s, 5 H), –2.75 (s, 2 H) ppm

$^{13}$C NMR (500 MHz, $CDCl_3$): $\delta$ 169.14, 142.3, 138.0, 137.99, 135.4, 134.7, 131.2, 127.8, 126.8, 120.3, 120.2, 119.7, 118.1, 76.43, 71.25, 70.17, 68.55 ppm

UV/Vis ($CH_2Cl_2$): $\lambda max$/nm ($\varepsilon$/$M^{-1}$*$cm^{-1}$): 252 (61580), 418 (1234700), 515 (45459.2), 551 (22398), 590 (13316), 646 (10969.4)

# Materials and Methods

***Laccase production***

The laccase used in this study is $LAC_3$ from fungus *Trametes* sp. strain C30 produced by heterologous expression of the laccase in the filamentous fungus *Aspergillus niger* in large – scale production (300mg/L). [72]

***Dioxygen consumption***

Dioxygen consumption was measured by polarography using a model 781 oxygen meter (Strathkelvin Instruments) with a micro Clark electrode fitted to a temperature controlled glass chamber (1.5ml). Irradiation of the sample was performed through the glass chamber using a Dolan-Jenner MI-150 illuminator (Edmund) equipped with a 150W EKE Quartz Halogen lamp with optic fibers (0.8 cm) adapted to the respiratory chamber. A power density of about 230 $mW/cm^2$ was measured with a power meter Vector H410 connected to a Scientech head after a 2 cm thick water lens corresponding to the water cooled glass respiratory chamber.

***Laccase – Unik161***

The enzyme variant was prepared at Biosciences. It was provided ready and pure at the initiation of this project.

***Activity test of grafted laccase***

Laccase activity was assayed at room temperature using siringaldazine (SGZ, namely 4-Hydroxy-3,5-dimethoxybenzaldehyde azine) as substrate. Oxidation of SGZ was detected by following the absorbance at 525 nm ($\varepsilon$=65.000 $cm^{-1}M^{-1}$) during 2 minutes using VARIAN, Cary 50 spectrophotometer using disposable plastic cuvettes of 1.00 cm path-length. The reaction mixture (1mL) contained 10 µL of appropriately diluted enzyme sample, 960 µL of acetate buffer (200mM, pH 5.5) and 30 µL of 1mg/mL SGZ in MeOH at 30°C. The reaction started after the addition of SGZ solution and immediate mixing by inversion. One unit (U) of laccase was defined as one micromole of substrate oxidized per minute in these described conditions.

***NMR spectra***

$^{1}$H NMR spectra were recorded on a Bruker AMX-500 MHz or AMX-300 MHz and $^{13}$C NMR spectra on Bruker AMX-500 MHz spectrometer.

$^{1}$H NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform-d (δ 7.26, singlet). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet) or m (multiplet). Coupling constants are reported as a J value in Hertz (Hz). The number of protons (n) for a given resonance is indicated as nH, and is based on spectral integration values. $^{13}$C NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform-d (δ 77.0, triplet).

***Mass spectra***

High-resolution mass spectra were recorded on a Bruker UltrafleXtreme MALDI-TOF/TOF spectrometer.

***Photophysical measurements***

UV-Vis experiments were performed on a VARIAN Cary 50 spectrophotometer. Fluorescence measurements were recorded at $25^{0}$C on a Horiba Jobin- Yvon Fluoromax spectrometer. The excitation wavelength was 350nm (absorption peak of pyrene) with a slit width of 10nm for both excitation and emission. The wavelength range was 370-500nm. The excitation spectrum was monitored at the two porphyrin emission peaks at 398nm and 444nm. However, the only data used for $K_d$ extraction were the ones that correspond to 398nm as a real emission peak. Titrations experiments were performed using an appropriate diluted solution of grafted enzyme (lac3 and Unik161) or free cyclodextrin in 20mM Acetate buffer (pH 5.5). As stock solution in all cases was used 1ml of 5μM or 1μM photosensitizer (**$COOCH_3$_NH_Pyrene**). For all the titrations we have that *5μl=0.0881eq*. These experiments were repeated three times for each condition.

For the analysis of fluorescence results, the following thinking was followed:

*The equilibrium considered is shown below*

| | X-CD | + PYR | = | XCD-PYR |
|---|---|---|---|---|
| t0 | $c_1$ | $c_0$ | | |
| teq | $c_1$-$\alpha c_0$ | $c_0(1-\alpha)$ | | $\alpha c_0$ |

*The relation between dissociation constant $K_d$ and factor α is:*

$$\alpha = \frac{(C_1 + C_0 + K_d) - \sqrt{(C_1 + C_0 + K_d)^2 - 4C_0C_1}}{2C_0}$$

*We have now to find a way to get α from experimental data.*

*Initial fluorescence intensity is proportional to the concentration of pyrene:*

$I_0 = f_1 * C_1$

*Maximum intensity (all pyrene is complexed, therefore it is proportional to XCD-PYR maximum concentration)*

$I_{max} = f_2 * C_0$

*At any other moment:* $I = f_1*(c_1-\alpha c_0) + f_2 * \alpha c_0$

*We have therefore:* $$\alpha = \frac{I - I_0}{I_{max} - I_0}$$

*We therefore have to convert the data and plot $I-I_0$ versus $C_1$ and fit with the following equation where $\Delta I_{max}$ and $K_d$ are adjusted during the fitting process.*

*So, the fluorescence changes were analyzed by curve fitting (GraphPad Prism 4.0) according to eq 5*

$$\alpha = \Delta I_{max} \frac{(C_1 + C_0 + K_d) - \sqrt{(C_1 + C_0 + K_d)^2 - 4C_0C_1}}{2C_0} \qquad \textbf{eq 5}$$

*where $C_0$ is the concentration of pyrene, $C_1$ is the concentration of cyclodextrin, $K_d$ is the dissociation constant and $\Delta I_{max}$ the maximum fluorescence quenching at high cofactor concentration (infinite limit). $K_d$ was obtained from curve-fitting analysis.*

*The model just described does not contain any approximation (General Model).*

*In Prism model for one binding site the equation used is* $Y=B_{max}*X/(K_d + X)$

*In this case, we have an approximation which is that* $[CD]=[CD]_0$ *, which is not true though.*

*In Prism model for two binding sites, we have*

$Site_2=B_{maxLo}*X/(K_{dLo}+X)$

$Y=Site_1 + Site_2$

*Same approximation as above but difficult to express the equation without approximations.*

## Grafting approach

The chemical modifications of proteins are almost an ancient art. A wide variety of procedures for protein modification have been developed and used all these years.

Given the range of chemical modification methods available, it is now possible to decide which residue to target and which modification to attach in order to confer the desired property/ function.

Chemical modification of proteins is based on two things: firstly, on the electrophilic reagents which target the nucleophilic functional groups present in amino side chains and secondly, on the differences in the reactivities of the individual amino acid side chains. These include the imidazole moiety of histidine, the indole of tryptophan, the p-hydroxyphenyl of tyrosine, the thioether of methionine, the thiol group of cysteine, the disulfide bond of cystine, the carboxyl groups of glutamic and aspartic acids, of carboxyl-terminal amino acids and amino groups of both lysine and amino-terminal amino acids.

Some representative protein bioconjugation methods for the modification of cysteine and lysine residues are shown in **Fig 28**.[73]

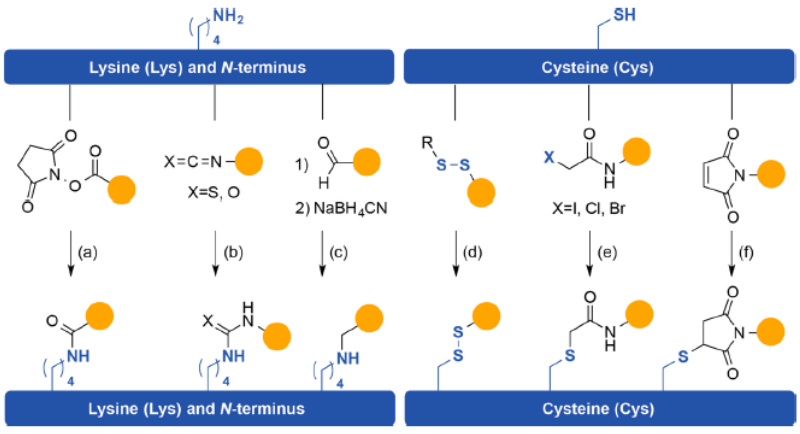



**Fig 28: Classical Methods for the Modification of Cys and Lys: (a) Amide Formation, (b) Urea and Thiourea Formation, (c) Reductive Amination, (d) Cys-Specific Disulfide Exchange, (e) Alkylation, and (f) Conjugate Addition to a Representative Maleimide Michael Acceptor.**[74]

In our case, the modification on laccase's surface was accomplished through lysine residue. The laccase available in our lab (LAC3 from *Trametes* sp.) only contains three free amine groups included the N – terminus and two lysine residues which are located away from T1 Cu site. Construction of a site directed mutant with a unique lysine near the T1 Cu site (K161), removing the two others (K40 and K71) was achieved at Biosciences and is named UniK. Thus, the oriented functionalization on surface of laccase can be done using this particular mutant.

A question arising at this point is how it is possible to maintain the activity of the enzyme after performing a chemical reaction on a biological macromolecule. Obviously, some ideal requirements should be fulfilled. To begin with, the reaction should be performed in aqueous media. Enzymes, in general, can tolerate a small quantity of alcohol-based solvent or DMSO, but the inclusion of these solvents can cause several structural changes. In addition, it would be advisable if pH is between 6 and 8 and the temperature should not exceed $40^0$C due to the fact that enzymes will not survive at a higher value. Last but not least, low concentration of the reactants is of utmost importance because these molecules can be deleterious to the enzyme at high concentrations.[75]

So, a suitable method which fulfills the above conditions needs to be chosen. Since our laccase is glycosylated, a lot of functionalized groups which can react with amine groups, can react with hydroxyl groups on sugars as well. Consequently, our grafting approach relies on the reductive amination of an aldehyde. Meaning that our goal is to perform a reaction between an aldehyde bearing molecule and an amine coming from a lysine residue pointing on the surface of laccase. That was exactly the reason why compound **8** (**see Fig 29**) was synthesized, as an ''aldehyde containing''

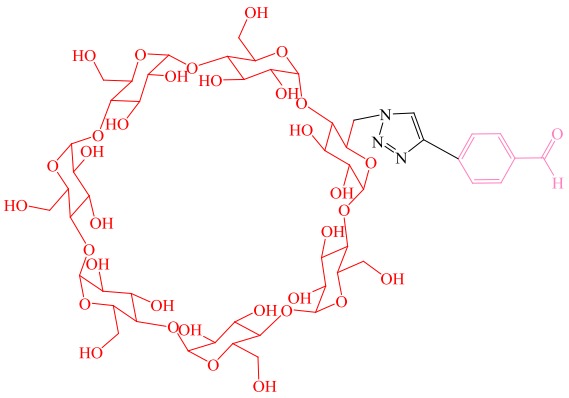


**Fig 29: structure of compound 8**

molecule. The synthetic pathway is given in details in the section **'' Synthesis of templates with grafting purposes ''**.

Apart from that, another compound was used to graft it on the surface of laccase. *5-(4-formyl-phenyl)-10,15,20- tris-methyl-(4-pyridyl) porphyrin zinc iodine* (**see Fig 30**) was provided ready and pure at the beginning of this project.

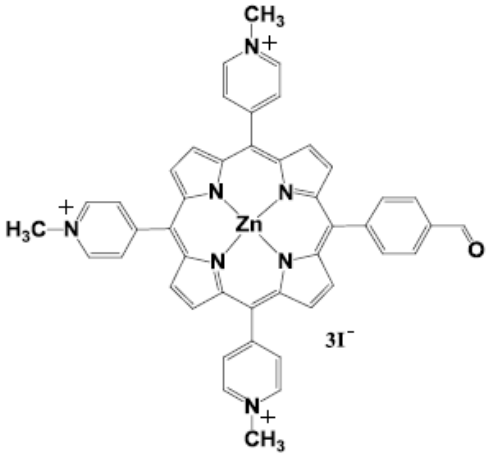



**Fig 30: Structure of aldehyde-bearing porphyrin 9**

## Reductive Amination

Reductive amination involves the condensation of lysine amino groups or the N-terminus with aliphatic or aromatic aldehydes.[75,76] Imine or Schiff base formation occurs rapidly and reversibly with both lysine and N terminus. In a second step, the imine is irreversibly reduced into the secondary amine using water-compatible hydrides such as $NaBH_4$. High concentrations of this highly reactive reducing agent may be harmful for the enzymes. Numerous transition metal catalysed reactions performed in aqueous media and minimal quantities of non toxic reagents have been published.[76] $NaBH_4$ could be replaced by the less harmful sodium formate. This reducing agent fails to react alone on an imine, thus the system requires the addition of a transition metal catalyst such as an iridium complex. The

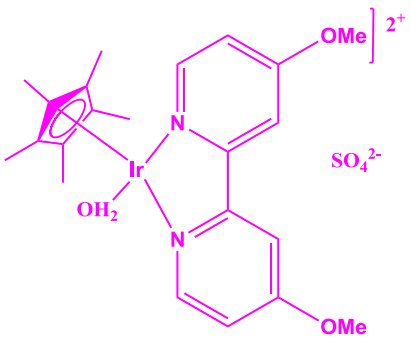


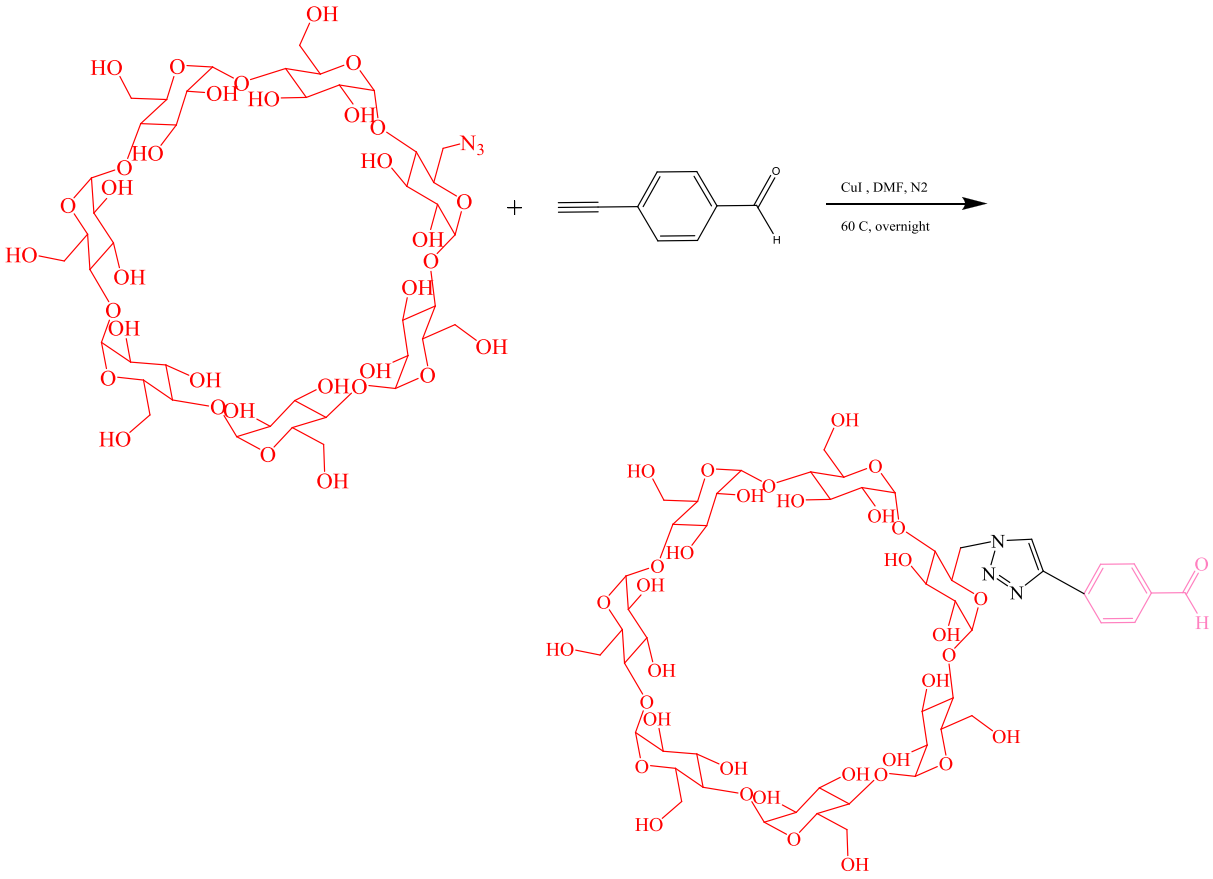

**Fig 31: structure of Ir-catalyst used in this study**

method is based on a classical method for reduction of an imine through a hydride transfer type mechanism.[77]

McFarland and Francis have shown that $Ir^{III}$ complexes can be used as an alternative reagent to reduce imines in the reductive amination of lysines and we selected this catalyst for our derivatization reactions. The catalyst was provided ready and pure at the initiation of the present work (**see Fig 31**). [78]

# Synthesis of templates for grafting purposes

**Synthesis of mono-6-deoxy-6- triazole-benzaldehyde- beta-cyclodextrin (8)**




**(8)**

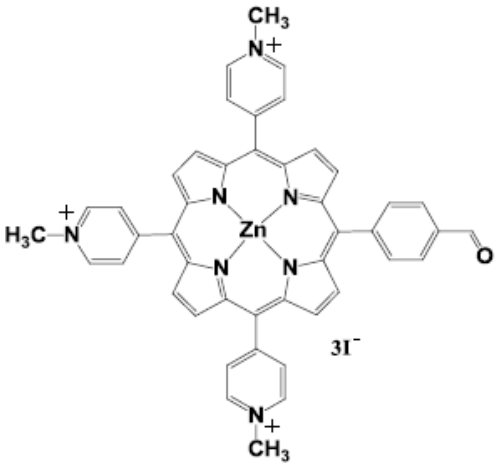
In a 50ml three necked flask, azido-cyclodextrin (0.2gr, 0.1724mmol), 4-ethynyl benzaldehyde (0.0224gr, 0.1724mmol) and dry DMF (15ml) were added. The mixture was degassed with a stream of argon for 30 minutes at room temperature. Then, the addition of CuI (0.0656gr, 0.3448mmol) was performed under $N_2$. The reaction mixture was stirred overnight at 60°C. The TLC detection indicated the disappearance of starting materials. After reaction, the DMF solvent was evaporated by rotary evaporator affording 78mgr (35.1% yield) of the click product.

The purity of the desirable crude product was checked by Mass Spectroscopy.

MS (MALDI-TOF): m/z calcd. for $C_{51}H_{75}N_3O35$ $[M+H]^+$ 1290.14, $[M+Na]^{+\,1312.40}$ found 1312.56

The compound was observed as Na adduct.

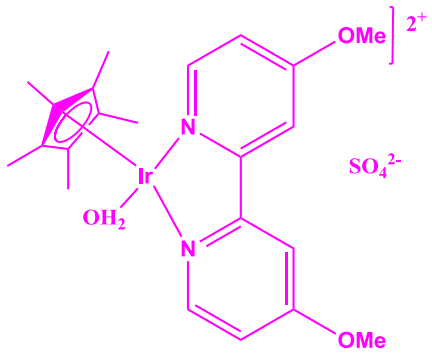
**Synthesis of 5-(4-formyl-phenyl)-10,15,20- tris-methyl-(4-pyridyl) porphyrin zinc iodine (9)**




**(9)**

*Firstly carbonyl functionalized water soluble porphyrin was obtained which then was reduced to hydroxyl functionalized porphyrin and finally reoxidized to the formyl one. The last step was the metallation and methylation of the porphyrin*[79,80].

**Synthesis of Cp*Ir(4,4'-dimethoxy-2,2'- bipyridine)$H_2O.SO_4$**




*An adapted procedure of Dadci et al* [81] *was used. The desirable structure was obtained.*

*The complex was prepared and purified to use it at the beginning of these experiments.*

## Grafting conditions of aldehyde-bearing molecule on the enzyme's surface

In a 10ml round bottom flask and in 2.5 ml aqueous phosphate buffer 50mM (total volume) set at pH=7.4, 50µM of enzyme (lac3/UniK161), 10eq of aldehyde molecule, 500µM of Ir-catalyst solution and 25mM HCOONa were mixed. The reaction mixture was left under stirring for 48h at 30$^{o}$C.

After this time, the reaction mixture was passed through PD10 (G-25) size exclusion column, the buffer was exchanged from 50 mM $Na_2HPO_4$ pH 7.4 to 0,1M Britton Robinson buffer (B&R) pH 4 containing 200mM NaCl using Sartorius Stedin Biotech Vivaspin 500, with a cut – off 30.000 MWCO and by centrifugation. The concentrations of the grafted products were estimated by UV–Visible Perkin–Elmer Lambda 650 spectrophotometer and 1.0 cm path–length quartz micro cuvettes (100µl).

# Chapter 3 – Results and Discussion

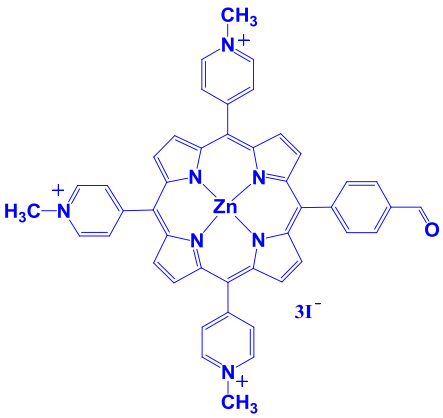
## *A little introduction…*

In this chapter of the present work, we will try to make a ''*compare and contrast*'' between a known grafted system (covalent attachment of a Zn- porphyrin on enzyme's surface) and a supramolecular moiety consisted of a photosensitizer (porphyrin), enzyme laccase and β-cyclodextrin assembly.
Both systems aim at the reduction of dioxygen. Based on some dioxygen consumption measurements, we intend to reach to a conclusion concerning the effectiveness of each of the systems on the transformation (reduction) of dioxygen to water. Additionally, we will try to figure out if the rate of dioxygen consumption depends on the enzyme used, mutant laccase (UNIK) or not.

After the successful synthesis of the templates needed for the grafting on the surface of the enzyme (**compounds 8 and 9**), our attempts normally should be focused on the investigation and development of reaction conditions which would afford the best yield in the minimum derivatization time maintaining the activity of the enzyme. Questions like which is the optimum ratio between enzyme and templates, the effect of the temperature on the reaction yield and rate, the duration of the experiment as well as the finding of the best pH value have to be answered. That is not the case though, because a previous work in the laboratory examined all these factors providing a protocol which has to be followed in any grafting procedure in the future.

## *Grafted system with Zn-porph_aldehyde*

The process of grafting an aldehyde-bearing molecule and more particularly, a porphyrin_ aldehyde on the surface of laccase, has been achieved previously in the laboratory with great success. The porphyrin used in that case was the *5-(4-formyl-phenyl)-10,15,20-tris-methyl-(4-*




**(9)**

*pyridyl) porphyrin zinc iodine* **(9)**.

It was found that the concentration of the porphyrin-aldehyde has to be set 10 times higher than that of the enzyme. That's the optimum ratio between the two components, in which the least unreacted enzyme was obtained.

The best concentrations of reactants were obtained using 50 μM of mutant laccase (UNIK), 10 equivalents of porphyrin, 5 equivalents of Ir catalyst and 25mM of hydride source (formate). What's more, after extensive studies and experiments, the temperature and pH values, under which the best reaction yield was obtained, were determined (30$^{o}$C, pH 7.4). The aqueous media was phosphate buffer and the grafting is done after 48h with yield of 90%. Below, you can see schematically the general conditions of targeted functionalization (**see Fig 32**).

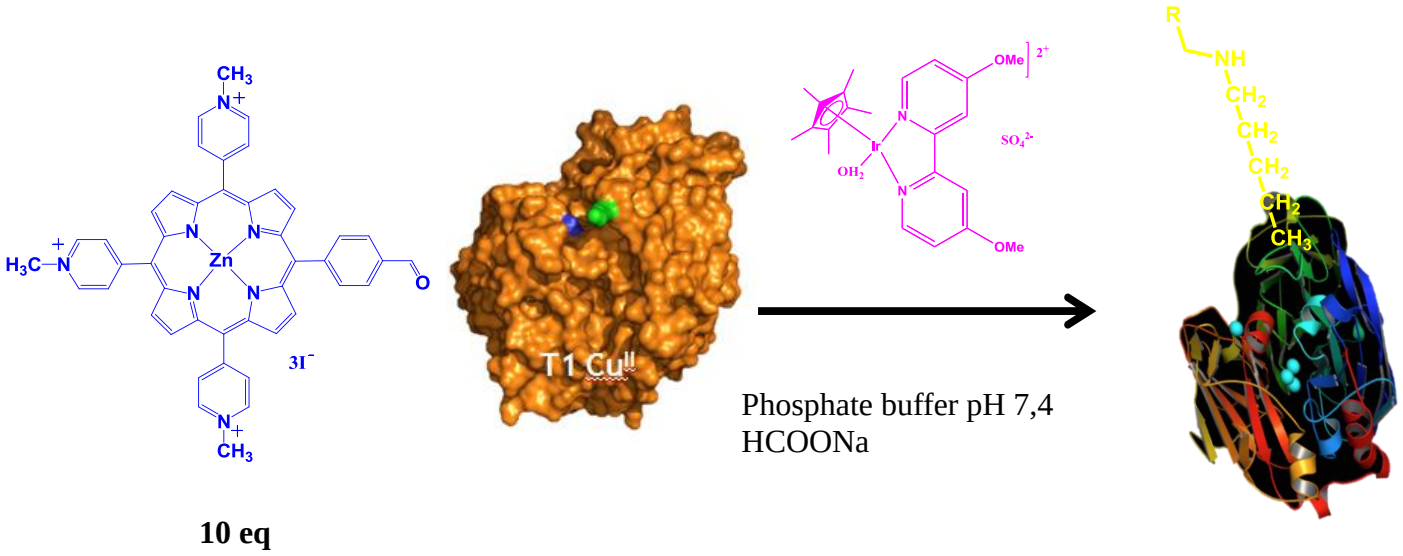



**Fig 32: General conditions of targeted functionalization**

Subsequently, the concentration of the porphyrin grafted on mutated laccase needs to be measured. Therefore, it was essential to remove the unreacted (free) porphyrin. The separation of the enzyme (grafted or not) from the unreacted Zn-porphyrin was achieved using size exclusion chromatography column. Atomic absorption and ICP – MS measurements proved the modification of UNIK as the ratio between Zn and Cu metal ions ([Zn]/[Cu]) ranges from 0.18 to 0.25. Namely, the hybrid system contains approximately 1 Zn ion –due to the porphyrin- and 4 copper ions- owing to the enzyme- per molecule - system. Various experiments were ran

concerning the activity of the enzyme. It was documented that it remained active at a percentage of roughly 80% at the end of the grafting reaction.

Taking into account all the above results about the experimental conditions required and within the context of the idea of this project, we grafted once more this particular Zn porphyrin-aldehyde (**9**) on the surface of both laccase and the single – lysine containing mutated laccase, unik161. The concept for running two grafting procedures simultaneously was to see if there is a noticeable difference in the dioxygen consumption rate between the two enzymes, since the impairment we caused in case of unik161 is close enough to the T1 copper center. So as the porphyrin is attached on the residue 161 and not far away from the active site, a different effect on dioxygen consumption is possible. The results coming from consumption experiments will be presented in the chapter ''**Dioxygen Consumption Measurements**''. At this point, I would like to mention that bearing in mind the sensitivity of Zn- porphyrin aldehyde to light, the grafting was performed under light protection. Moreover activity test was not performed for the grafted system as the maintenance of laccase's activity has already been reported.

## *Grafted System with cdx_aldehyde*

The same strategy was followed for the grafting of cyclodextrin_aldehyde molecule (**8**) on the surface of both laccase and its mutant, unik161. In other words, the reaction conditions used were similar in every detail with the ones above for the simpler grafted system. Contrariwise, no light protection was needed for this particular grafting.

By the end of the reaction, the concentration of the grafted products was estimated by UV-Vis Spectroscopy, meaning, by monitoring the absorbance at 608nm ($\varepsilon$ = 5600 $M^{-1}*cm^{-1}$). Knowing the concentration of these stock solutions and making the appropriate dilutions, we were able to proceed with enzyme's activity estimation.

### *Activity tests of grafted lac3/unik – cdx*

Right away after the end of the reaction, the activity of grafted products in the supramolecular approach was estimated by using syringaldazine as substrate, where the hydroxyl groups (-OH) get oxidized into carbonyl groups (**see Fig 33**).

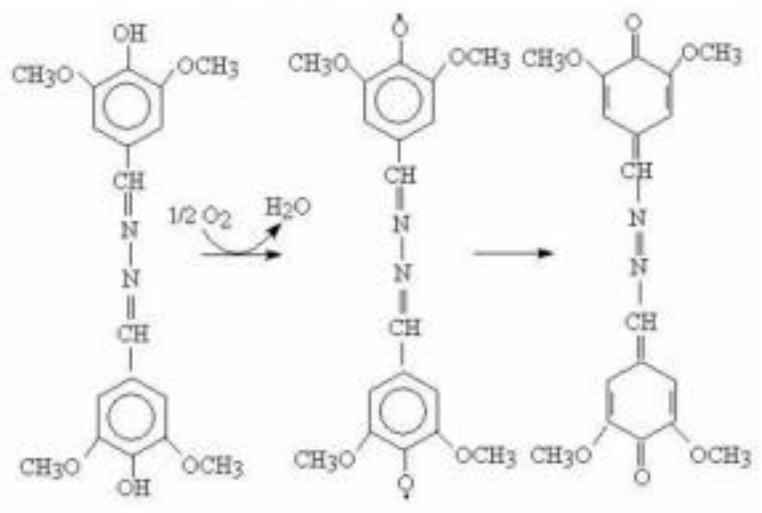



**Fig 33: Catalyzed oxidation of syringaldazine by laccase to the corresponding quinone**

Specific activity values for the enzymes (grafted or not) are shown in **Tables 1,2**.

| | Specific Activity Values (U/mgr) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Enzyme* | First Grafting | | | Second Grafting | | | Third Grafting | | |
| ***Non Grafted Lac3*** | **43,27** | **41,95** | **42,7** | **50,36** | **44,24** | **46,37** | **61,56** | **62,71** | **59,40** |
| ***Grafted Lac3*** | **19,21** | **15,48** | **15,73** | **27,87** | **28,76** | **27,72** | **21,37** | **22,30** | **25,02** |

**Table 1: Calculated activity values for grafted lac3_cdx or non- grafted lac3 in 3 independent grafting processes**

| | Specific Activity Values (U/mgr) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Enzyme* | First Grafting | | | Second Grafting | | | Third Grafting | | |
| ***Non Grafted Unik161*** | 41,61 | 43,34 | 47,68 | 65,04 | 67,45 | 67,63 | 35,96 | 33,79 | 32,98 |
| ***Grafted Unik161*** | 14,72 | 14,923 | 13,741 | 29,77 | 32,05 | 32,15 | 20,75 | 21,20 | 23,23 |

**Table 2: Calculated activity values for grafted unik-cdx or non- grafted unik in 3 independent grafting processes**

From the above results, it is totally clear a distinguished difference between the activity of grafted enzyme (lac3 or unik) and the non grafted version of it. Meaning that the induced impairment affects drastically the activity level of laccase.

The activity loss percentage was estimated. The results are depicted in **Table 3**.

| | % activity loss | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ***Enzyme*** | First Grafting | | | Second Grafting | | | Third Grafting | | |
| ***Lac3*** | 55.6 | 63.1 | 63.1 | 44.6 | 35 | 40.2 | 65.3 | 64.4 | 57.8 |
| ***Unik161*** | 64.6 | 65.5 | 71.2 | 54.2 | 52.5 | 52.5 | 42.3 | 37.3 | 29.5 |

**Table 3: % activity loss in case of lac3 and unik161 in 3 independent grafting processes**

Having caused modification to the enzyme near the active site, a higher loss in activity was expected in unik compared to lac3. A change -whatever that is- close enough to the site where the visible light driven dioxygen reduction initiates, affects greatly the enzyme's activity. The estimated loss in **third** grafting was not in conjunction with what we were waiting for and drew our attention. In more details,

lac3 lost approximately *62%* of its initial activity whereas unik around *37%*, a pretty debatable outcome. On the other hand the first two reactions at least complied with the general perspective.

With respect to various derivatization reactions performed in the laboratory, the activity of grafted laccase is not significantly decreasing. In our case though, whatever the number of grafting we studied, that loss cannot be easily neglected, it is unusually too high. That is the reason why we decided to run a last grafting, the so- called blank grafting. In the blank grafting no template was used. In a 10ml round bottom flask, only laccase, Ir catalyst and phosphate buffer were added. The concept is that through this type of grafting (absence of template), we can check if the grafting conditions themselves are deleterious to laccase leading to an uncommon plunge of activity or the template we chose to graft (cyclodextrin-aldehyde) causes a severe damage to laccase resulted in a great inactivation of enzyme. Meanwhile, just for comparison reasons, we ran also a new grafting of cdx_aldehyde on the surface of both laccase and its mutant, unik161.

The specific activity values we received were the following:

- Grafted lac3_cdx → **48 U/mgr**
- Grafted Unik161_cdx → **33 U/mgr**
- Blank laccase → **37 U/mgr**

To begin with, the higher activity of laccase_cdx compared to unik_cdx, is something which is expected, reasonable and rational (arguments set above). The fact that the blank grafting gave us an activity of 37 U/mgr, lower than 48 U/mgr which is attributed to the grafted lac3, verifies the assumption we made above about the role grafting conditions play in the determination of the enzyme activity. That means that the chosen, for grafting, template of cyclodextrin-aldehyde does not cause an impairment or damage to the enzyme. On the contrary, activity appears reinforced in presence of this particular template. Consequently, it is a well chosen molecule for grafting purposes without being detrimental to laccase.

To conclude with, average activity loss of grafted products, taking into account only the first two grafting reactions, is reported in **Table 4**.

| % average activity loss | | | |
|---|---|---|---|
| *Grafted enzyme* | First Grafting | Second Grafting | Final average grade |
| *Lac3* | 60.6 | 40 | 50.3 |
| *Unik161* | 67.1 | 53.1 | 60.1 |

**Table 4: % average activity loss of grafted lac3 and unik161**

Obviously, laccase after this particular grafting keeps *half of the initial activity* whereas mutated laccase, unik, keeps *40%* of it. With that remaining enzyme activity, we proceeded to fluorescence titrations and dioxygen consumption measurements.

## *Fluorescence Titrations*

Among various optical spectroscopy techniques, fluorescence spectroscopy is a powerful tool to elucidate the cdx complexation phenomena. A chromophore present inside the cdx molecule can exhibit some variations in its optical spectra upon experiencing a more hydrophobic environment due to complexation. Taking advantage of this, several fluorescence measurements were carried out. These experiments were performed in order to determine if there is any difference in fluorescence between pyrene_porphyrin alone and in the complex with β-cyclodextrin.

Having established some laccase activity, our next move was to get insight into the formation of inclusion complex between the pyrene_porphyrin (5) and the hydrophobic cavity of cyclodextrin, a molecule grafted on the surface of enzyme. Trials to extract dissociation constant values ($K_d$) have also been made. But at first,

we had to check the interaction –if there is one- between our chromophore with free non grafted β-cyclodextrin.

As reported in the **''Introduction''** part, the binding of guest molecules within the host cyclodextrin is a dynamic equilibrium process which can be illustrated by **eq 2**, where CD is cyclodextrin, G is the guest molecule, and CD-G is the inclusion complex:

$$CD + G \Leftrightarrow CD\text{-}G \quad \textbf{eq 2}$$

The stability of the inclusion complex can be described in terms of a formation constant $K_f$ or dissociation constant $K_d$ as defined in **eqs 3 and 4:**

$$K_f = [CD\text{-}G]/([CD]^*[G]) \quad \textbf{eq 3}$$

$$K_d = 1/K_f = ([CD]^*[G]) / [CD\text{-}G] \quad \textbf{eq 4}$$

When the value of $K_f$ is high, the inclusion complex is more stable and less separation can occur. The value of the $K_f$ varies with the size of the CDs opening and the guest molecule and how well the guest molecule fits in the CDs cavity. Depending on the size, the guest molecule will enter the cavity at the primary hydroxyl group or at the secondary hydroxyl group.

## *Pyrene*

Pyrene is a common and useful fluorescent probe because of its vibronic fine structure and sensitivity to microenvironmental changes. Hypothetical speaking, the same features find application to our photosensitizer (**see Fig 34**).

## *Porphyrin + cdx alone*

The first control experiment conducted was the titration of a stock solution of **$COOCH_3$_NH_Pyrene 5µM** by β-cdx solution.
In the following graph, the fluorescence intensity of porphyrin was plotted against the equivalents of

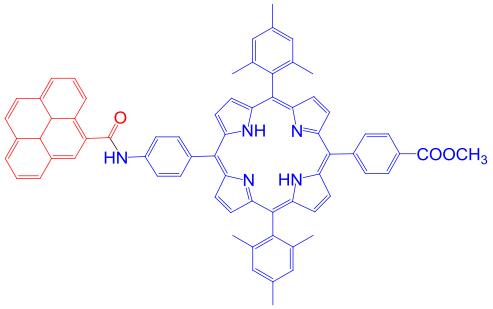



**Fig 34: Structure of photosensitizer used in fluorescence measurements**

host added, monitoring at the porphyrin's emission peak, 398nm (**see Fig 35**).

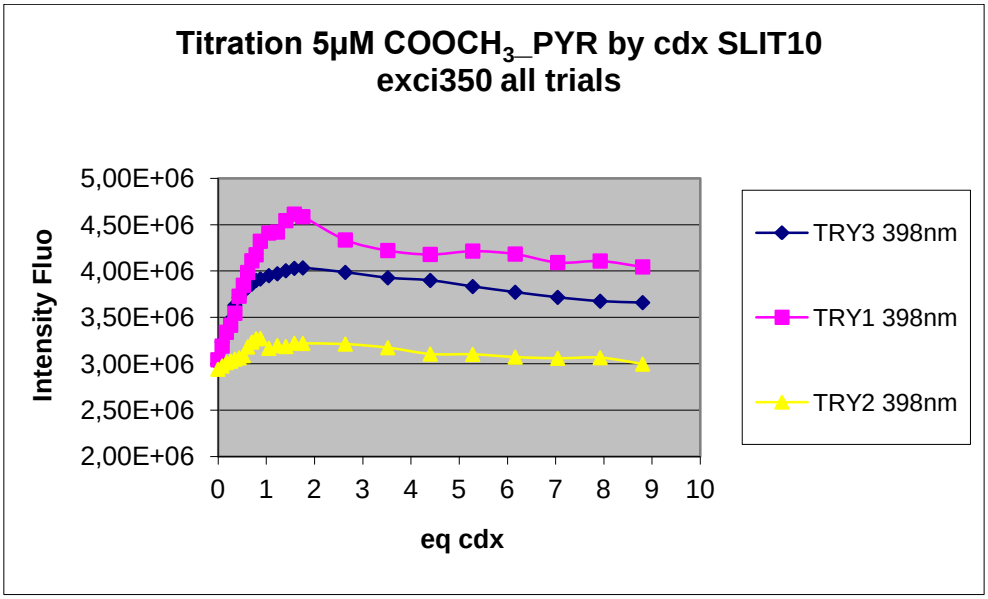



**Fig 35: Plot of the Fluorescence Intensity of photosensitizer (guest) in presence of increasing amount β-cyclodextrin (host)**

It is clear from the above figure that a significant increase in fluorescence signal of fluorophore is taking place and afterwards (around 1 equivalent of cdx added), the intensity reaches a plateau.

Judging from the molecular sizes of pyrene and β-cdx (internal diameter 7.0 Å), a part of pyrene is embedded in the β-cdx cavity, not pyrene in its wholeness, or even along with the porphyrin core.

Normally, as the pyrene part of our photosensitizer is penetrating deeply into the β-cdx cavity –given that the concentration of the host is getting higher- a loss in fluorescence intensity of pyrene is expected. That is because a partially trapped pyrene molecule is kind of hidden and cavity protected, resulting in a reduction of its initial fluorescence signal, compared to a totally free molecule in solution. This is not the case though. Surprisingly, a marked enhancement in fluorescence intensity was observed. Searching through the bibliography in our trial to give answer to this question, we found out that an increase in a concentration of host (β-cyclodextrin)

changes its microenvironment for guest (pyrene), meaning that the interaction of pyrene with apolar cavity of β-cyclodextrin increases, resulting in enhancement of fluorescence.

More specifically, as pyrene is particularly sensitive to media polarity changes, a minor variation in its environment affects greatly its behavior. The remarkable fluorescence enhancement of $COOCH_3$_NH_Pyrene upon addition of β-cdx implies that the environment around the guest alters and becomes considerably hydrophobic by increasing amount of the host. Indeed, cyclodextrins provide a less polar and more rigid protective environment enhancing the fluorescence of the guest molecule by shielding the excited species from the quenching and the pyrene fluorophore from non radiative interactions with water molecules, while the latter at the same time are expelled from the cdx cavity. Consequently, the observed emission enhancement of the hydrophobic pyrene originates in the protection from the quenching of the pyrene emission by water molecule owing to apolar cyclodextrin cavity.

## *$K_d$ extraction*

Fluorescence quenching upon addition of β-cyclodextrin was recorded and the data were analyzed using **eq 5** to determine the dissociation constant $K_d$ and to reach a conclusion regarding the molar ratio in complexation process.

According to ***General Model***, which does not contain any approximation, a strong association ($K_d$ < 1μM) was revealed between the guest and the host, with a maximum reached when 1 equivalent of β-cdx was added (**see Fig 36**). It seemed that a 1 : 1 $COOCH_3$_NH_Pyrene – cyclodextrin inclusion complex was formed.

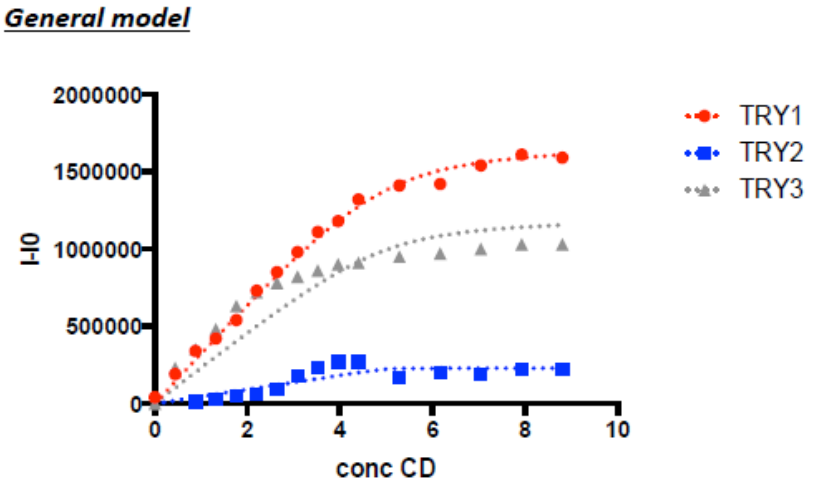



**Fig 36: Diagram derived from General model indicating changes in the fluorescence intensity upon addition of β-cyclodextrin**

On the contrary, if ***Prism's Model*** was taken into consideration, where we have that [CD]= $[CD]_0$, we got $K_d$ = 5.9, 5.6 and 1.9 µM respectively (**see Fig 37**).

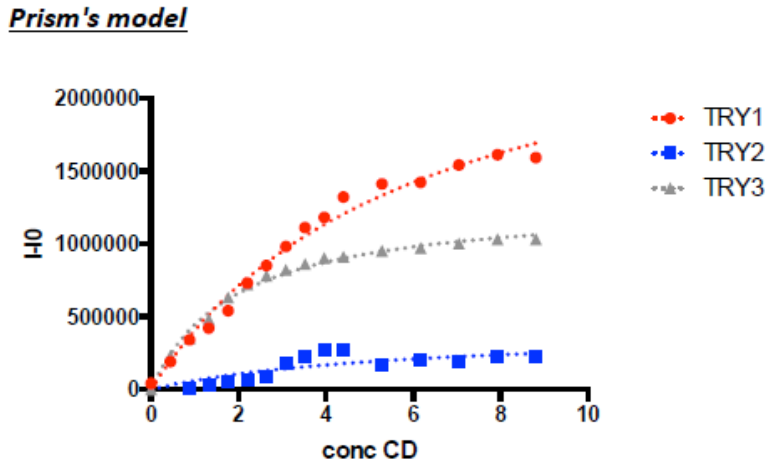
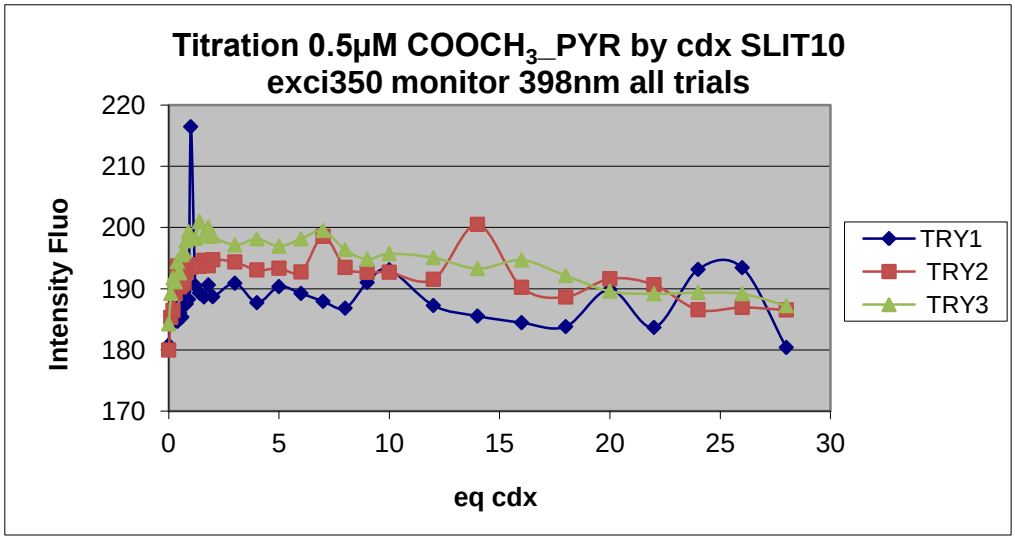



**Fig 37: Diagram derived from Prism's model indicating changes in the fluorescence intensity upon addition of β-cyclodextrin**

Despite the fact that the effect of cyclodextrin was similar in both models, the obtained $K_d$ values were different but of the same order of magnitude . Our differential fluorescence results suggested that probably we were not in good conditions to ''properly'' use the Prism's model and made the approximation that

the free cdx concentration was equal to the initial cdx concentration ([CD] = $[CD]_0$). Therefore, it would be advisable to reiterate the experiment using less initial concentration of the fluorophore (for instance 0.5µM or even 0.1µM porphyrin) to be capable of making approximations. These titrations have been completed, but the calculation of $K_d$ not yet. The graphs are presented below (**see Fig 38**).



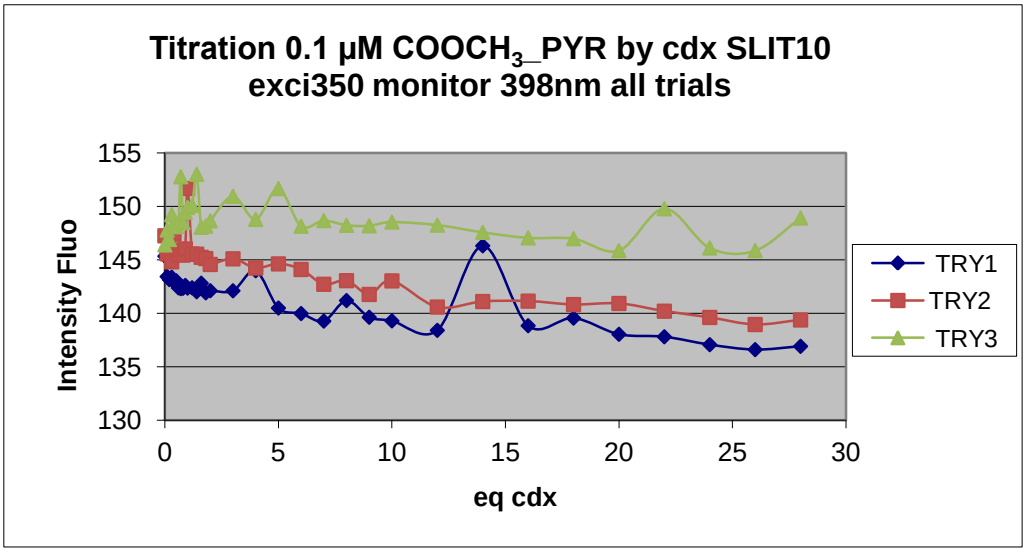




**Fig 38: Plot of the Fluorescence Intensity of 0.5µM and 0.1µM photosensitizer (guest) against increasing amount of β-cyclodextrin (host)**

Both figures are ''messy'' with quite noisy signal due to the low initial concentration of the fluorophore. In spite of this, the required approximations can be done so a more rational calculated $K_d$ is expected.

At this point, I would like to mention that the addition of host came to an end when the cuvette used was full and could not accommodate higher amount of solution.

### *Porphyrin + grafted Unik/lac3_cdx*

The behavior of β-cdx bounded on the surface of the enzyme -and not free in solution as above- had to be checked. In case of mutated enzyme, there is only one cyclodextrin molecule per unik, to interact with pyrene as there is one and only binding site (one lysine residue) available for reaction with the template (8) we synthesized. On the other hand, in lac3 we have 2 lysines so we expect to have 2 cdxs molecules grafted on the enzyme. The relevant titrations are presented below.

To begin with, 5μM was the starting concentration of pyrene_porphyrin (5).

In **Fig 39** the fluorescence intensity of porphyrin was plotted against the equivalents of grafted enzyme added, monitoring at 398nm.

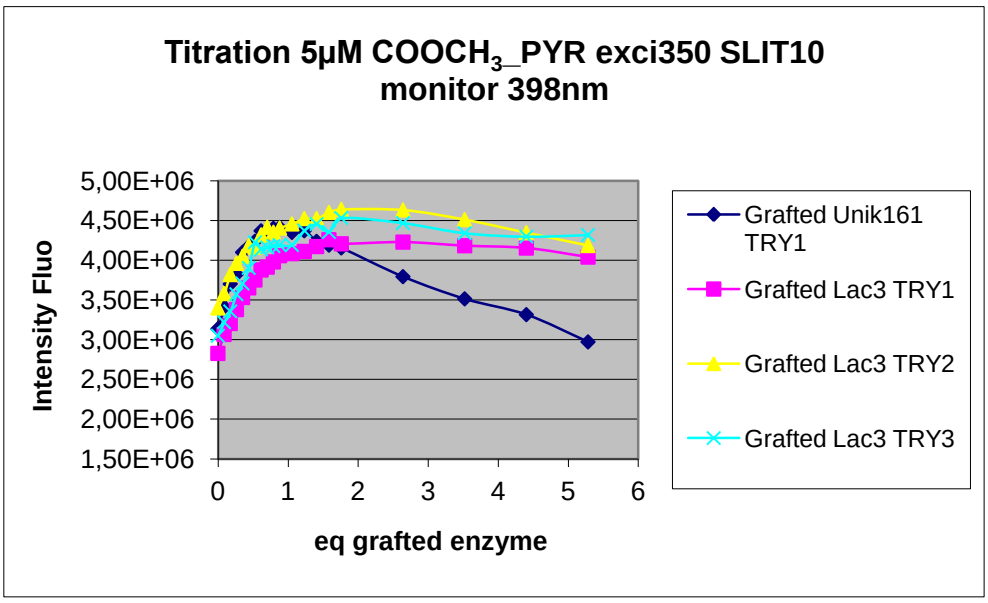



**Fig 39: Plot of the Fluorescence Intensity of 5μM photosensitizer (guest) against increasing amount of grafted unik/lac3_cdx (host)**

Unfortunately, due to quantity limit, only one titration was accomplished relating to grafted unik161. As far as the dissociation constant is concerned, data could not be reproduced by the models used, since the decrease of intensity began before 1 equivalent of unik was added. So, no $K_d$ extraction occurred for unik. Additionally, the ***Prism model for two binding sites*** for grafted lac3_cdx did not work. The fit of data was vague and ambiguous and also too many parameters needed to be taken. All these facts led us to believe that the current concentration of pyrene-porphyrin was not the appropriate one for $K_d$ extraction.

1μM of $COOCH_3$_NH_Pyrene seemed to be a right choice to serve our purpose. Once again, in **Fig 40** fluorescence signal was plotted against the addition of both laccase and its mutant, unik161.

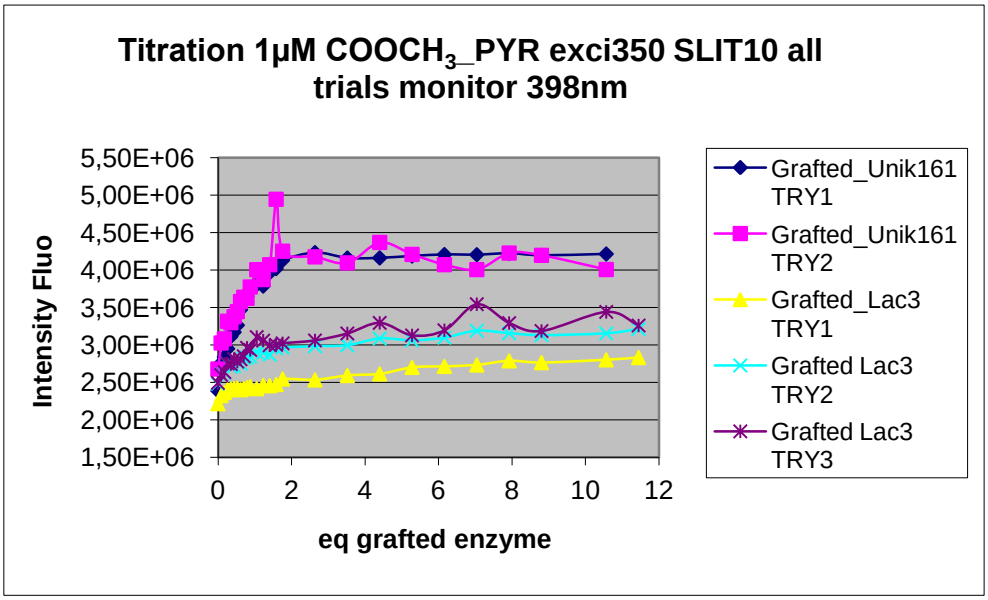



**Fig 40: Plot of the Fluorescence Intensity of 1μM photosensitizer (guest) against increasing amount of grafted unik/lac3_cdx (host)**

First of all, the two trials for unik were identical based on the graph. On the contrary, the reproducibility in lac3 was not particularly satisfactory, something that may affect the fitting of the data for $K_d$ calculations.

To get a better view upon the molar ratio in complexation procedure, the same graph as above is presented in **Fig 41** – until approximately 6eq.

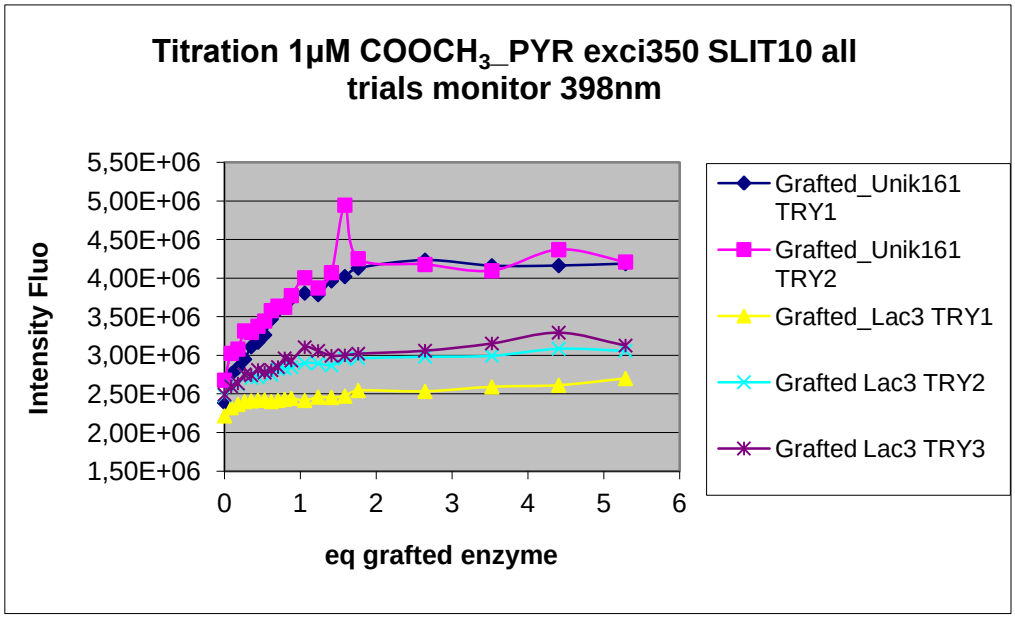


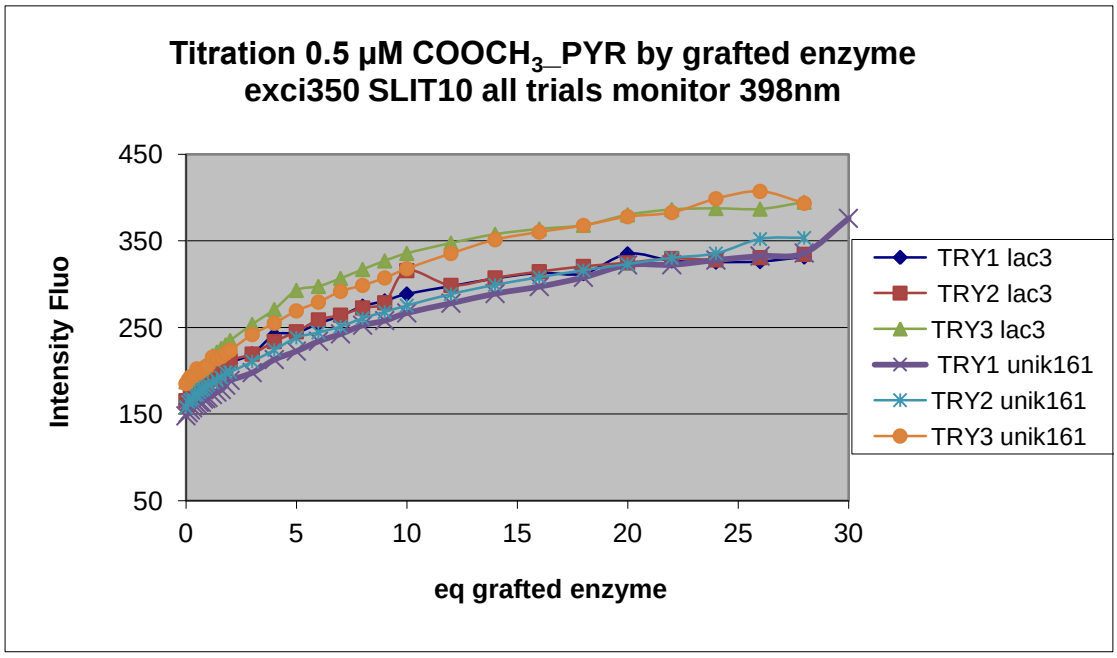

**Fig 41: Plot of the Fluorescence Intensity of 1µM photosensitizer (guest) against the addition of up to 6 equivalents grafted unik/lac3_cdx (host)**

The slopes correspond to the mutant, unik, appear to be much more sharper than the ones for lac3, an observation beyond doubt. Such a behavior has not been noticed in titration of 5µM porphyrin (Fig 38). The discrimination among the slopes back then was not even visible. However, at 1µM starting concentration, it seems that the gradual increase in concentration of the host induces a more rapid and faster enhancement in fluorescence signal in case of grafted unik. Probably this sharpness is directly linked to the stronger association unik_cdx has with pyrene for some reason. An argument suggested for such a behavior is that perhaps this is linked to the fact that by taking advantage of the unique lysine residue unik carries, (the only one binding site it bears actually), we assembled one cyclodextrin molecule on its surface, too close to the active center. Hence, the grafted system (composed by enzyme and cyclodextrin bounded on it) becomes more sensitive to the inclusion process. Meaning that whatever the change in cyclodextrin environment, it affects the other component of the system, laccase. It is known that a complexation to

occur, release of enthalpy rich water molecules is required and at a further step, upcoming entrapped molecules approach the cdx cavity. So, placing a cyclodextrin near enough to T1 Cu site and while pyrene penetrates the cavity, it is likely that the resulting supramolecular system is more influenced in contrast to lac3 supramolecular system where cdx is located in great distance from the active center.

We ran also a last set of titrations with lower concentration of porphyrin (0.5 and 0.1 μM). We repeated these titrations with concentration of pyrene_porphyrin lower than 1μM in order to be capable to make approximations.

The results are shown in **Fig 42**.



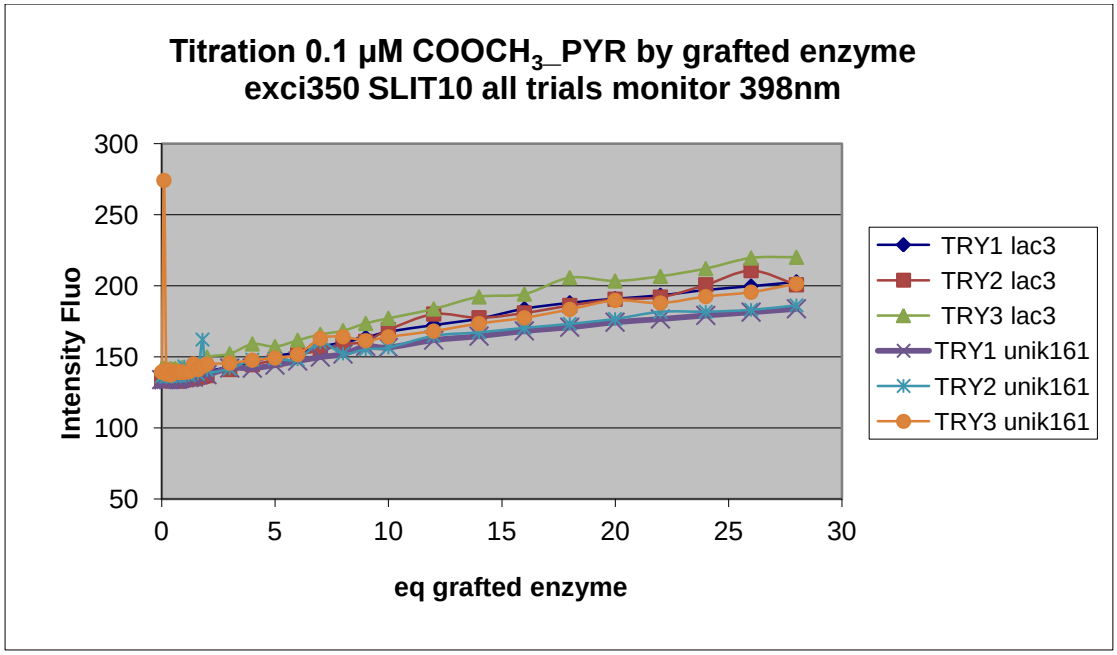



**Fig 42: Plot of the Fluorescence Intensity of 0.5µM and 0.1µM photosensitizer (guest) against the addition of grafted unik/lac3_cdx (host)**

Clearly, the differentiation in slopes between lac3 and unik does not exist. With the exception of *try 3 for both lac3 and unik in 0.5µM porphyrin*, where the two lines are out, in comparison with the rest of the trials, it seems that, laccase and its mutant behave the same way during complexation process, something which is opposed to the behavior unik showed at 1µM porphyrin.

## *$K_d$ extraction*

Grafted unik -cdx

Calculations have been made for approaching the strength of association between the host and the guest. Taking into account the results received from the titration of **1µM** $COOCH_3$_NH_Pyrene with grafted unik and trying to fit the data, ***general model*** proposed an association with exceptional strength, as the resulting value of $K_d$ implies ($K_d$ << 1µM). The maximum reached when 1 equivalent of grafted unik is added, proposing a ***1 : 1*** inclusion complex formation.

Grafted lac3 -cdx

Similarly, attempts for $K_d$ extraction has been done. **Prism Model for two binding sites** proposed 2 $K_d$ values, $K_{dHi}$=0.2μM and $K_{dLo}$=5μM. The maximum reached when 1 equivalent of grafted lac3_cdx was added, suggesting a 1 : 1 inclusion complex formation. The discrepancy in $K_d$ values between the 2 binding sites of laccase can be attributed to the fact that probably one site is more accessible to the cdx_aldehyde than the other. Thus, cdx_aldehyde reacts more easily with an amine coming from a lysine resulting in a stronger association between cdx cavity and pyrene_porphyrin so a low $K_d$ is expected in that case.

Results of $K_d$ extraction are expected when our potential guest was fixed at 0.5μM and 0.1μM. Calculations are in progress. It is believed that by setting pyrene at a concentration lower than 1μM, the approximations needed can be done, so we will get a better insight into the extent of the association growing between pyrene and cdx cavity. It seems, with the current results, that when cdx is attached on surface of unik, closer to the enzyme's active site, pyrene_porph is more strongly entrapped inside the cavity compared to lac3, where 2 molecules of the host are in great distance from T1 Cu site. So, probably the higher association of pyrene with cdx cavity in case of unik, lead us to expect a better catalytic performance on dioxygen reduction.

## *What about the ferrocene bearing porphyrins ?*

Among several porphyrins we synthesized, two of them bear a ferrocene group (**see Fig 43, 44**). Since these molecules had no fluorescence properties, the only way to investigate a possible interaction with cyclodextrin cavity was by electrochemistry. Water was chosen to be the medium in which this study took place owing to the solubility of the host in it.

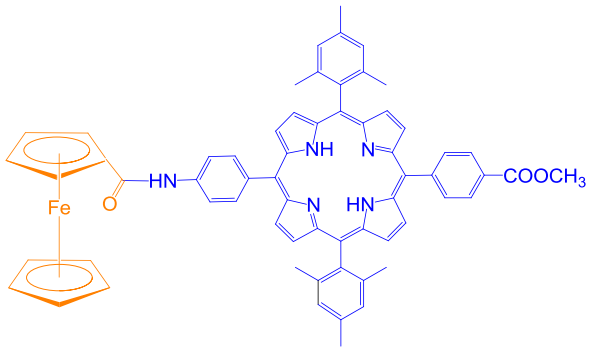



**Fig 43: Chemical structure of porphyrin (4)**

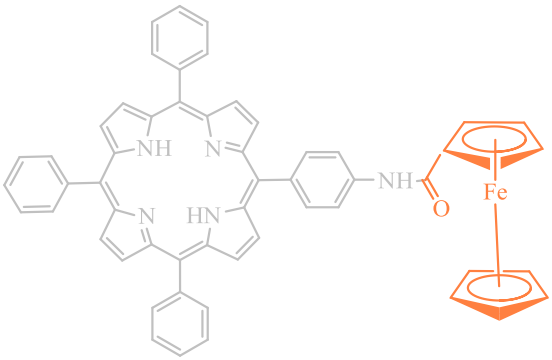



**Fig 44: Chemical structure of porphyrin (7)**

Sad to say that these particular porphyrins were hardly solubilized in water as they ''hate'' it. Another issue we had to face was the sticking of the photosensitizers onto the electrode surface. Due to the above reasons, we decided to deal only with the pyrene bearing porphyrin and letting behind the ferrocene ones.

## *Dioxygen Consumption Measurements*

The principle of the systems studied, grafted and supramolecular, is that visible light excites the photosensitizer affording the excited state of the dye. Electrons are then transferred into the active site of enzyme at which four electrons as well as four protons are used to reduce a molecule of dioxygen with the concomitant formation of two water molecules. The oxidized dye is recovered by the sacrificial electron donor, EDTA.

In our measurements, the photodriven dioxygen consumption rates were monitored as a function of time. The experiments were conducted in the presence or absence of sodium azide ($NaN_3$), a strong inhibitor of laccases. For each sample, the experiment was carried out three times. In the absence of light or in the absence of EDTA, no significant dioxygen consumption was detected under the same experimental conditions. In this chapter, the results of visible light –driven dioxygen consumption measurements are going to be presented analytically for each system that we study. Furthermore, deductions about the effectiveness of each system on reduction of dioxygen will be made.

### *Supramolecular System and $O_2$ Consumption*

To begin with, we firstly checked the effectiveness of the supramolecular system on dioxygen consumption. Based on some previous experimental results running in the lab which confirmed the optimal ratio between a photosensitizer and enzyme, we studied our system in a 1 : 1 Grafted enzyme_cdx / $COOCH_3$_NH_Pyr ratio (20μM each). Higher concentration than this could not be used due to solubility limit of our porphyrin in B&R.

- *20μM grafted lac3/unik_cdx + 20μM $COOCH_3$_NH_Pyrene + 100 eq 4mM EDTA*

Our best shots for this system are shown in **Fig 45**. One of them describes the rate of oxygen consumption in case of grafted unik_cdx and the other one corresponds to grafted lac3. Just for comparison reasons we plotted all these data in the same figure. % dissolved dioxygen was plotted against duration of the measurement (min). The appropriate amount of enzyme, porphyrin and EDTA were added inside the chamber glass since the beginning. Then, the irradiation started. No inhibitor was used in this series of experiments.

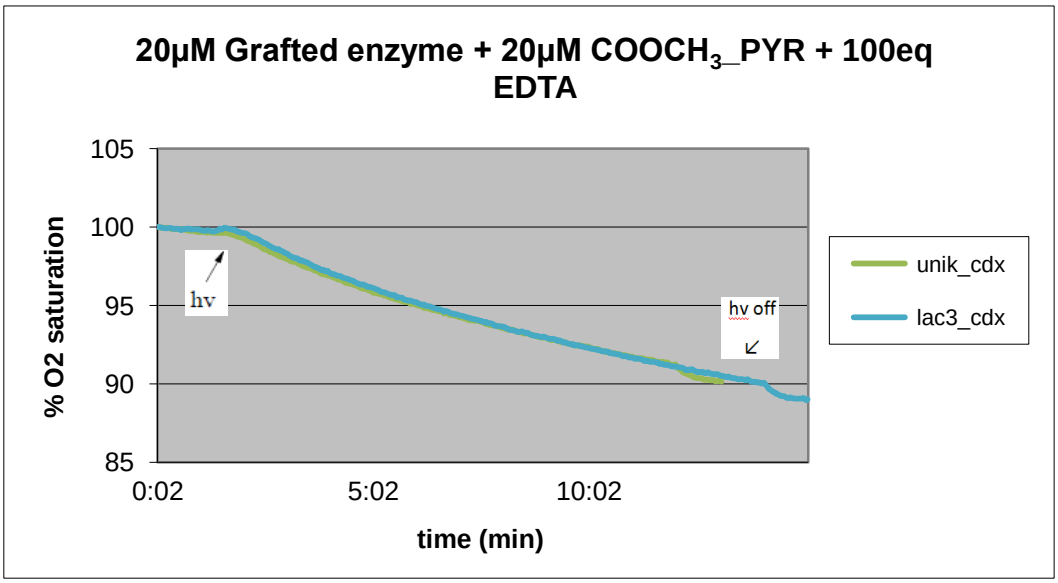



**Fig 45: Dioxygen consumption rate in systems of unik_cdx + porphyrin (5) + EDTA and lac3_cdx + porphyrin (5) + EDTA**

Upon irradiation of a solution containing 20μM grafted enzyme_cdx (lac3 or unik), 20μM $COOCH_3$_NH_Pyrene and 100 equivalents EDTA in a 1.5ml of B&R buffer pH 4, the dioxygen concentration decreases in a very small rate. To be more specific, the rate of consumption was found to be **-3 μmol $O_2$/L/min** for **lac3 system** and **-2.8 μmol $O_2$/L/min** for **unik**. In other words, that means that our chosen supramolecular system does not consume dioxygen, the effectiveness of it, is almost absent. The position also of cdx on the surface of enzyme (in a short distance or far away from T1 Cu center) seems not to be crucial to the consumption rate, at least for this

particular system as the found rates are almost identical for both systems. Surprisingly, a curve is formed –not so visible to see due to the insignificant rate- especially in the very beginning of irradiation, implying an oxidation process, not linked to the phenomenon studied. It was the first time that such a curve was observed during this kind of experiments. In order to shed light on this issue and figure out what is happening, we cut the measurement into pieces, trying to evaluate the contribution of each component to that strange curve and to the resulting consumption rate.

- *20µM grafted lac3/unik + 20µM $COOCH_3$_NH_Pyrene first*
  *+100 eq 4mM EDTA later*
  *+100µl 1M $NaN_3$* at the end

In **Fig 46** the behavior of unik and lac3 is presented.

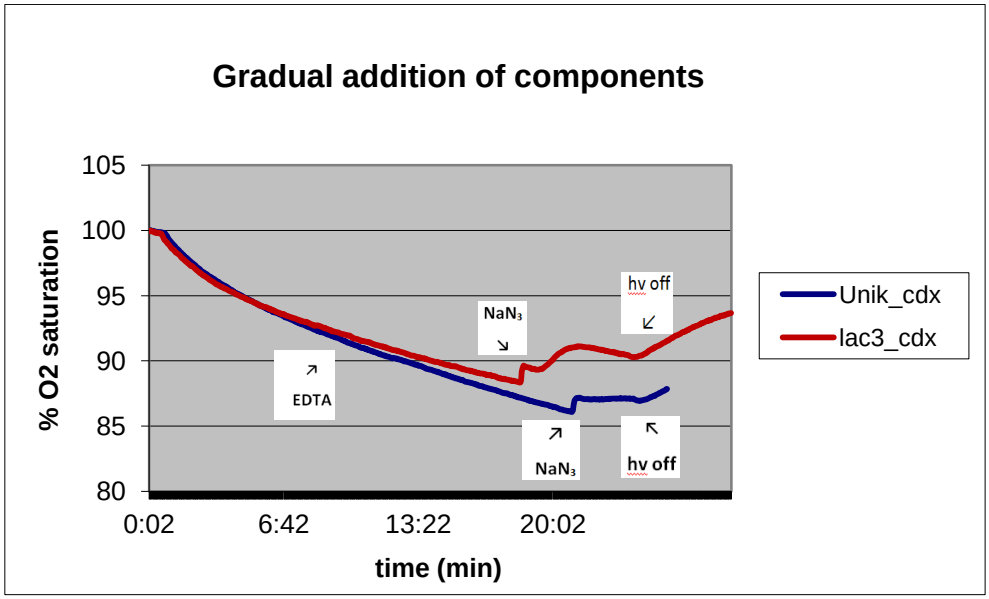



**Fig 46: Dioxygen consumption rate with gradual addition of EDTA and $NaN_3$ into the solution of grafted enzyme and pyrene_porphyrin in B&R buffer pH 4**

The data for both grafted lac3_cdx and unik_cdx are plotted together. Upon irradiation of a solution containing 1 : 1 ratio grafted enzyme/ photosensitizer, dioxygen concentration decreases faster in the very beginning with a rate of **-4.4**

**µmol $O_2$/L/min** for **unik_cdx** and **-4.3 µmol $O_2$/L/min** for **lac3_cdx** and then slows down reaching **-2 µmol $O_2$/L/min** for both systems, resulting this unusual curve. It seems that whatever the process occurs when we open the lights, it is going faster at the start. That curve gives the impression that something is oxidisable, even if EDTA is absent from the solution. Some kind of molecules is formed as a consequence of the interaction of $COOCH_3$_NH_Pyrene with laccase. This unexpected behavior is not completely understood yet though.

Subsequently, EDTA was added, affording a straight line with **-1.5 µmol $O_2$/L/min** for **unik_cdx** and **-1.4 µmol $O_2$/L/min** for **lac3_cdx**. Normally, an increase in dioxygen consumption was expected by adding EDTA in solution, as EDTA contributes to the regeneration of the excited state of our photosensitizer, $COOCH_3$_NH_Pyrene. Thus, after the regeneration, porphyrin is ready to be excited once again upon irradiation, losing a single electron per time and injecting it to the enzyme and whenever four electrons are transferred, a full catalytic cycle is accomplished. In fact, this addition did not have the expected effects, indicating that probably everything did not go through the enzyme but the chosen photosensitizer itself had an internal activity.
Last to add, was the inhibitor, $NaN_3$. Unfortunately, as the above graph indicates, air was inserted into the chamber glass of Clark electrode by mistake, perturbing the system. In spite of this, the dioxygen consumption rate caused by the presence of inhibitor was around **-0.5 µmol $O_2$/L/min** for **unik_cdx** and **-0.6 µmol $O_2$/L/min** for **lac3_cdx**. In other words, an inhibition of enzyme was noted. A last thing to check was to investigate if $COOCH_3$_NH_Pyrene alone has an internal activity, causing the formation of this uncommon curve. That's why we performed the following experiment.

- *20µM $COOCH_3$_NH_Pyrene first*

  *+100 eq 4mM EDTA later*

The relevant figure is the following (**see Fig 47**).

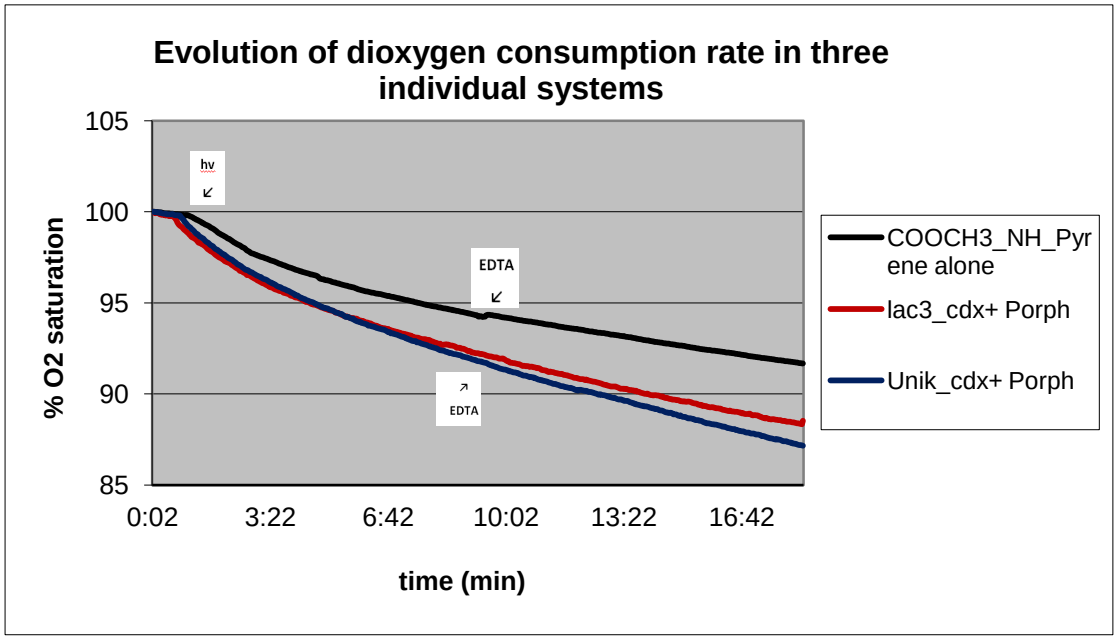



**Fig 47: Dioxygen consumption rate in systems of a) pyrene_porphyrin alone b) lac3_cdx + porphyrin c) unik_cdx + porphyrin**

It is evident that by irradiating a solution of pyrene_porphyrin alone, this curve is obtained again. Such a behavior denotes an oxidation procedure of an internal electron donor. Our porphyrin is oxidisable so it is subjected to this process, affording molecules that probably interact with the enzyme. That interaction may be responsible for the sharper slope we got in *lac3/unik_cdx + porphyrin* system, compared to the one which corresponds to porphyrin. In terms of rate values, porphyrin alone -in the very beginning of irradiation- consumes dioxygen with a rate of **-4 µmol $O_2$/L/min** which turns to **-1.6 µmol $O_2$/L/min** at the end. If we attempt to make a comparison between the consumption rates caused by porphyrin only on the one hand and grafted lac3 / unik_cdx on the other (we are exclusively referring to the rates coming from the curve part- before adding any other chemical), we end up with the **Table 5**:

| | Consumption Rates (µmol $O_2$/L/min) | |
|---|---|---|
| **system** | In the very beginning | At the end |
| ***Porphyrin_alone*** | **-4** | **-1.6** |
| ***Lac3_cdx*** | **-4.3** | **-2** |
| ***Unik_cdx*** | **-4.4** | **-2** |

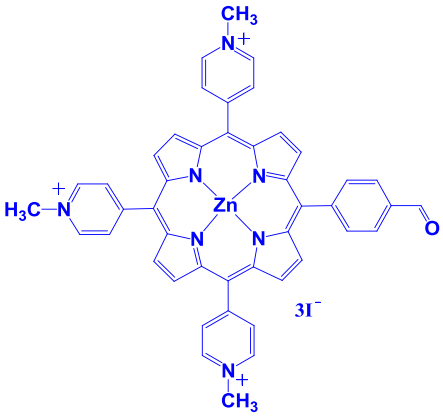
**Table 5: Summary table of consumption rates data for each system studied**

The contribution of $COOCH_3$_NH_Pyrene to dioxygen consumption is considered to be important based on the table. At least in the beginning of the light driven consumption measurements, the main reason consumption of dioxygen is noticed, is attributed to the presence of porphyrin in the solution and the interaction developing between laccase and the excited species of porphyrin. In **grafted lac3_cdx** system a rate of about **-0.3 µmol $O_2$/L/min** is due to the enzyme itself and in **grafted unik_cdx** system we have **-0.4 µmol $O_2$/L/min**.

All these data verify two things: first, the chosen photosensitizer has a noteworthy internal activity so it contributes greatly to the phenomenon studied and secondly, the specific grafting of a cyclodextrin molecule on enzyme's surface does not afford an efficient consumption system and also no discrepancy in consumption rates between laccase and unik is revealed as we expected based on fluorescence results.

It seems that the interaction between cyclodextrin and pyrene_porphyrin is too dynamic, meaning that the rate of the forward reaction (formation of the inclusion complex) is equal to the rate of the reverse reaction (dissociation of inclusion complex). Thus, pyrene_porphyrin is constantly getting in and out the host cavity so the placement of the host near enough to the active center has no impact on dioxygen conversion rate.

**Grafted system and $O_2$ consumption**



**(9)**

In this section, the effectiveness of the grafted system (porphyrin_aldehyde **9** grafted on laccase's surface) will be investigated. The experimental conditions used for all our trials were *20μM lac3/unik161 _aldehyde + 100 eq 4mM EDTA + 50μl (or even 100μl) 1M $NaN_3$.* An interesting graph is shown below, where there is a distinct and clear differentiation in the dioxygen consumption rate induced by lac3 and the mutated enzyme, unik161 (**see Fig 48**).

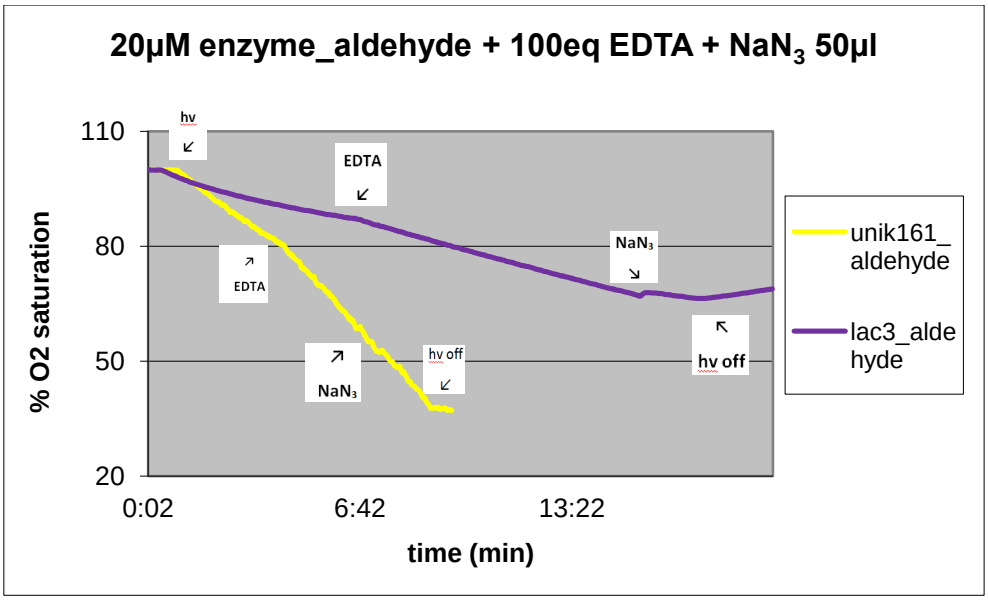



**Fig 48: Dioxygen consumption rate in systems of a) grafted unik_aldehyde and b) grafted lac3_aldehyde**

A lot of useful deductions can be made from the above figure. First of all, it is pretty obvious that when porphyrin_aldehyde is attached near enough to the T1 Cu center, the system consumes dioxygen in a really fast rate compared to lac3, where aldehyde is located far away from the active site. So it seems that the closer to the active site modification is done, the faster decrease in $O_2$ concentration is noticed. Something which is expected since upon light irradiation to this system,

photosensitizer (porph_aldehyde) is excited and the electrons are transferred to T1 Cu site. T1 Cu center is the main entrance for electrons in enzymes. Once reduced, it transfers electrons to the trinuclear cluster. So if the electrons are easily and fast transferred to T1 Cu site, the more efficient will be the system in dioxygen conversion. Thus, having attached the porphyrin too close to T1 Cu active site through covalent bond, the electrons are injected immediately to that site and then the internal electron transfer to the trinuclear cluster is taking place, resulting in a fast $O_2$ reduction. In case of laccase, where the photosensitizer is covalently placed a long way from T1 Cu site, electrons have to ''travel'' some way to get to that site. That's why the whole process in case of lac3 lacks in effectiveness.

Back to the graph, the slope in its wholeness is much sharper in case of mutated enzyme as shown. In presence of **unik_aldehyde** alone in the chamber glass the rate reaches **-16 μmol $O_2$/L/min** whereas in **lac3_aldehyde** alone **-6 μmol $O_2$/L/min**. With the addition of sacrificial electron donor, EDTA, both systems' rates enhance. In more details, in **unik_aldehyde** we have a rate of **-33 μmol $O_2$/L/min** (double value) and in **lac3_aldehyde -8 μmol $O_2$/L/min** respectively. As explained above, it is rational to have an increase in dioxygen consumption rate with the addition of EDTA into the solution. The magnitude of the increase though is quite different among the systems. Those values certify that each time porphyrin_aldehyde obtains its initial state, the electron transfer and consequently the reduction of dioxygen into water is a more rapid and extremely faster process in unik than in lac3. The location of photosensitizer in the system plays a crucial role to this differentiation. Probably another factor is the presence of covalent bond as the intermediate link between enzyme and porphyrin. Meaning that such a strong chemical bond, holds together and joints the two components in a direct manner and allows the electrons flow through it easily and quickly from porph_aldehyde to enzyme's active site.

With respect to $NaN_3$, as Fig 48 indicates, the activity of lac3_aldehyde system was inhibited by adding it afterwards. We obtained a straight line with a considerable reduction in system's activity (**-2.8 μmol $O_2$/L/min**), implying that everything is going through the enzyme. On the other side, surprisingly, despite the large excess of

inhibitor added, no inhibition was observed in system of unik. Even when the adding amount doubled (100µl instead of 50µl), the system did not demonstrate any inhibition. Another fact that we should bear in mind is that it has been proved that most of the times it's better to incubate first $NaN_3$ with the enzyme and then irradiate in order to see an effect on the rate. Various tests have been running in the laboratory confirming this statement. For some reason, when an inhibitor – whichever that is- is added afterwards, no inhibition is noticed.

Hence, we performed one last measurement. In this experiment, sodium azide (100µl) was added in a solution containing 20µM unik_aldehyde since the beginning.

- 20µM grafted unik_aldehyde
  +100µl $NaN_3$
  +100eq 4mM EDTA (last to add)

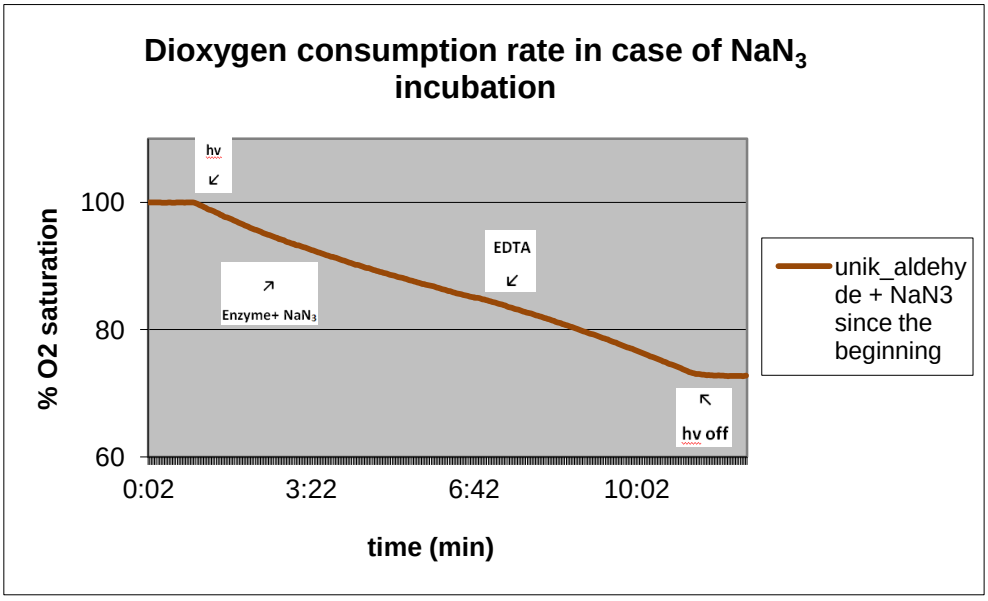



**Fig 49: Dioxygen consumption rate in the grafted system of unik_aldehyde + $NaN_3$ (incubation). EDTA was added later.**

Having investigated the effectiveness of unik_aldehyde alone (*-16 µmol $O_2$/L/min*) or even in presence of EDTA (*-33 µmol $O_2$/L/min*) on dioxygen consumption, a clear and apparent reduction in activity was expected in presence of the inhibitor. Indeed, $O_2$

concentration decreased with a rate of about -11 µmol $O_2$/L/min, a value which was sufficiently lower than the ones obtained in absence of any inhibitor. After EDTA addition, no big changes in rate has been noticed (-12 µmol $O_2$/L/min), which was absolutely normal as the enzyme has been inactivated so even if porphyrin has been excited and has injected the electrons to T1 Cu site, $O_2$ reduction by the trinuclear cluster could not take place.

To summarize, it turned out that supramolecular system is not an efficient system in visible-light driven dioxygen reduction. The grafting of cyclodextrin molecule on enzyme's surface did not have any significant contribution to visible-light-driven conversion. On the contrary, when porphyrin_aldehyde is covalently attached to laccase, provides a quite useful system for dioxygen consumption. A plausible explanation lies in the type and strength of interaction developing between photosensitizer- enzyme and secondarily in the percentage of activity grafted products carry. In case of enzyme_aldehyde hybrid system, laccase is active and keeps 80% of the initial activity of the unmodified enzyme whereas in supramolecular system, the coupling of cyclodextrin with laccase causes a drastic reduction in activity. Unik_cdx system is only 40% active and lac3_cdx 50%. With such low values the ability of the chosen photosensitizer to function in visible-light driven dioxygen reduction is probably limited.

Concerning the kind of grafting we made, in grafted system the photosensitizer is directly linked to the enzyme through a strong chemical bond, covalent bond. Therefore, the interaction between laccase and porphyrin is direct and quite powerful. In the case of supramolecular system, there is no such a bond between laccase and porphyrin. Cyclodextrin is grafted on laccase, not the photosensitizer ($COOCH_3$_NH_Pyrene). The latter is added to the system after the grafting is done and diffuses deeply into the cavity. Hence, a chemical system made up of a discrete number of assembled subunits is formed. The kind of interactions which are developed between laccase and porphyrin are weak and reversible non covalent interactions. So, as the enzyme_cdx system is weaker in terms of laccase- porphyrin association, we can attribute the ''failure'' of supramolecular system in dioxygen

reduction to these strengthless intermolecular forces. Taking advantage of the existence of covalent bond on the other system and the direct interaction between the components, the electrons meet a path and are injected immediately and easily to enzyme's active site affording a system with great efficacy.

The dynamic equilibrium at which the supramolecular system reaches is another factor. Pyrene as substrate is going in and out the cavity with the same rate providing a very dynamic interaction between cdx-porphyrin. Hence, wherever the template is located (far away or in a great proximity to the T1 Cu site) does not play a role in the determination of dioxygen consumption rate.

# Chapter 4- Conclusions

In conclusion, two systems have been evaluated for their ability to consume dioxygen upon light irradiation. The hybrid system of enzyme_aldehyde (grafted), where porphyrin (9) was covalently attached on laccase's surface, showed efficient visible-light-driven conversion with an exceptional consumption rate particularly when the porphyrin was attached on the surface of a single- lysine containing mutated laccase (unik161). In supramolecular case, where compound (8) was grafted on laccase- enzyme and photosensitizer was added later to the system, dioxygen conversion into water was not effectual. The intermolecular bonds (van der Waals, hydrophobic *etc*) which held together the photosensitizer with the enzyme_cdx were weak affecting the efficiency of the system. Another key factor was the dynamic interaction between the host and the guest, allowing pyrene get in and out the cavity so the proximity of the template to T1 Cu site had no impact on dioxygen conversion.

Furthermore, activity tests have been running to estimate how much active enzyme remained after the coupling of compound (8) with it. Results showed that laccase kept half of its initial activity and mutated laccase, unik was 40% active. In order to have a view upon the formation of inclusion complex between pyrene_porphyrin (5) and the hydrophobic cavity of cyclodextrin which was grafted on laccase, several fluorescence measurements have been made. A remarkable fluorescence enhancement of $COOCH_3$_NH_Pyrene upon addition of β-cdx was observed and a 1 : 1 $COOCH_3$_NH_Pyrene – cyclodextrin inclusion complex was formed. When $COOCH_3$_NH_Pyrene titrated by grafted lac3/unik_cdx an inclusion complex with a ratio 1 : 1 was formed in both cases. The $K_d$ value for the association unik_cdx/ pyrene_porph has an exceptional strength, as the resulting value of $K_d$ implies ($K_d$ << 1μM) whereas in lac3_cdx / pyrene_porph (2 binding sites) we have $K_{dHi}$=0.2μM and $K_{dLo}$=5μM. In addition, $K_d$ calculations when our potential guest is fixed at 0.5μM and 0.1μM are in progress.

# APPENDIX

## *Preparation of aqueous buffer solutions*

- Deionized water used was ultrapure and prepared by Millipore Milli-Q system.

- To adjust the pH of the buffer solutions was used Mettler Toledo meter, since it has been calibrated with buffers pH 4 and pH 7 derived from the company.

- The preparation of Britton - Robinson (B&R) buffer solutions were made as follows:

  0.1 M aqueous solution of acetic acid
  0.1 M aqueous solution of phosphoric acid
  0.1 M aqueous solution of boric acid

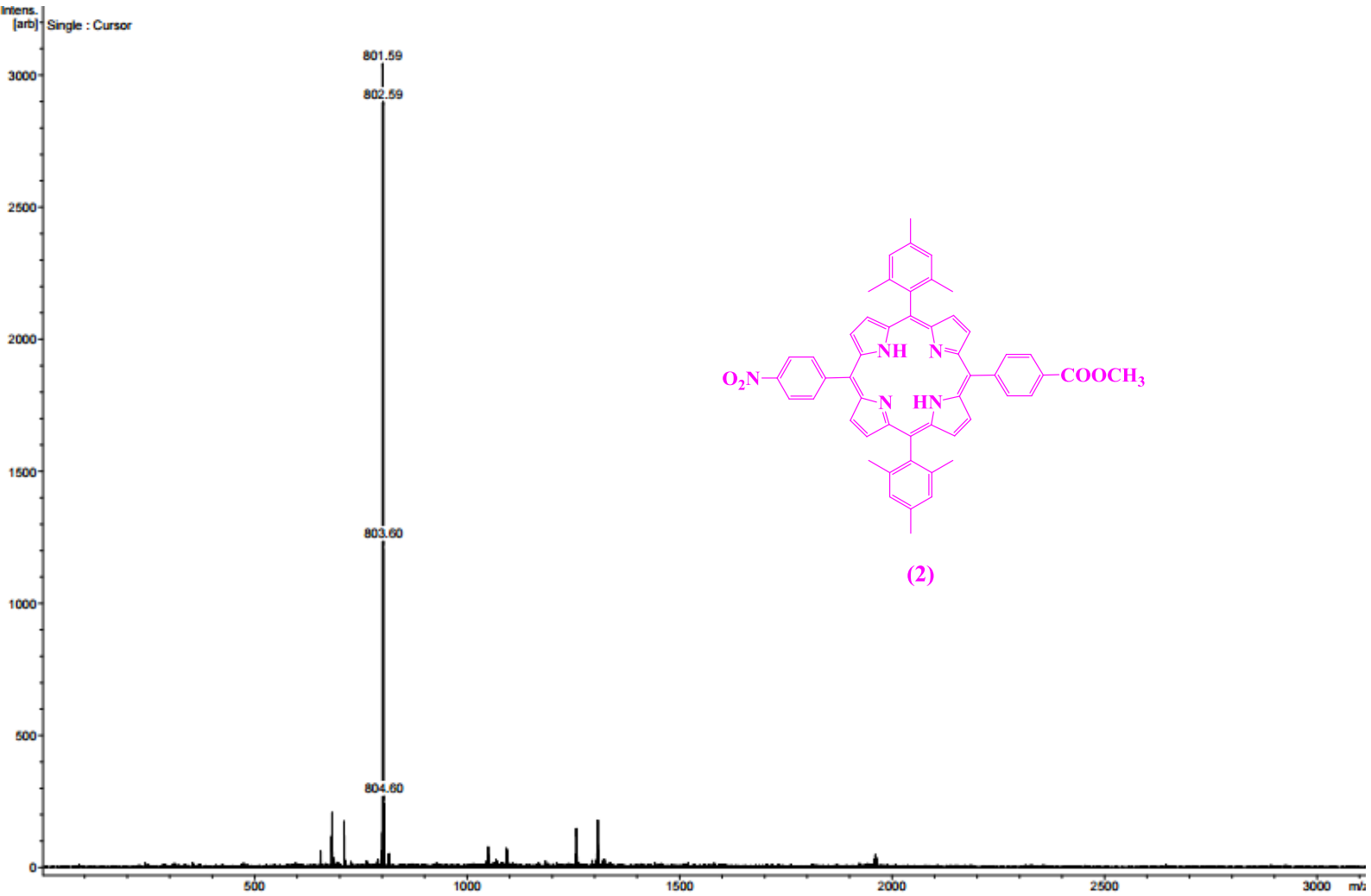
  Then they were mixed in a 1:1:1 ratio and the pH adjusted to the desired value by adding aqueous solution NaOH 1M.

- The preparation of aqueous sodium acetate solution (acetate buffer) at desired pH value was done by mixing aqueous solution of 0.2 M $CH_3COONa$ (NaOAc) and 0.2 M $CH_3COOH$ (HOAc) in a ratio according to the following table:

| **pH** | **ml 0.2M NaOAc** | **ml 0.2M HOAc** |
|---|---|---|
| 3.6 | 10 | 90 |
| 3.8 | 12 | 88 |
| 4 | 18 | 82 |
| 4.2 | 26.5 | 73.5 |
| 4.4 | 37 | 63 |

| | | |
|---|---|---|
| 4.6 | 49 | 51 |
| 4.8 | 59 | 41 |
| 5 | 70 | 30 |
| 5.2 | 79 | 21 |
| 5.4 | 86 | 14 |
| 5.6 | 91 | 9 |

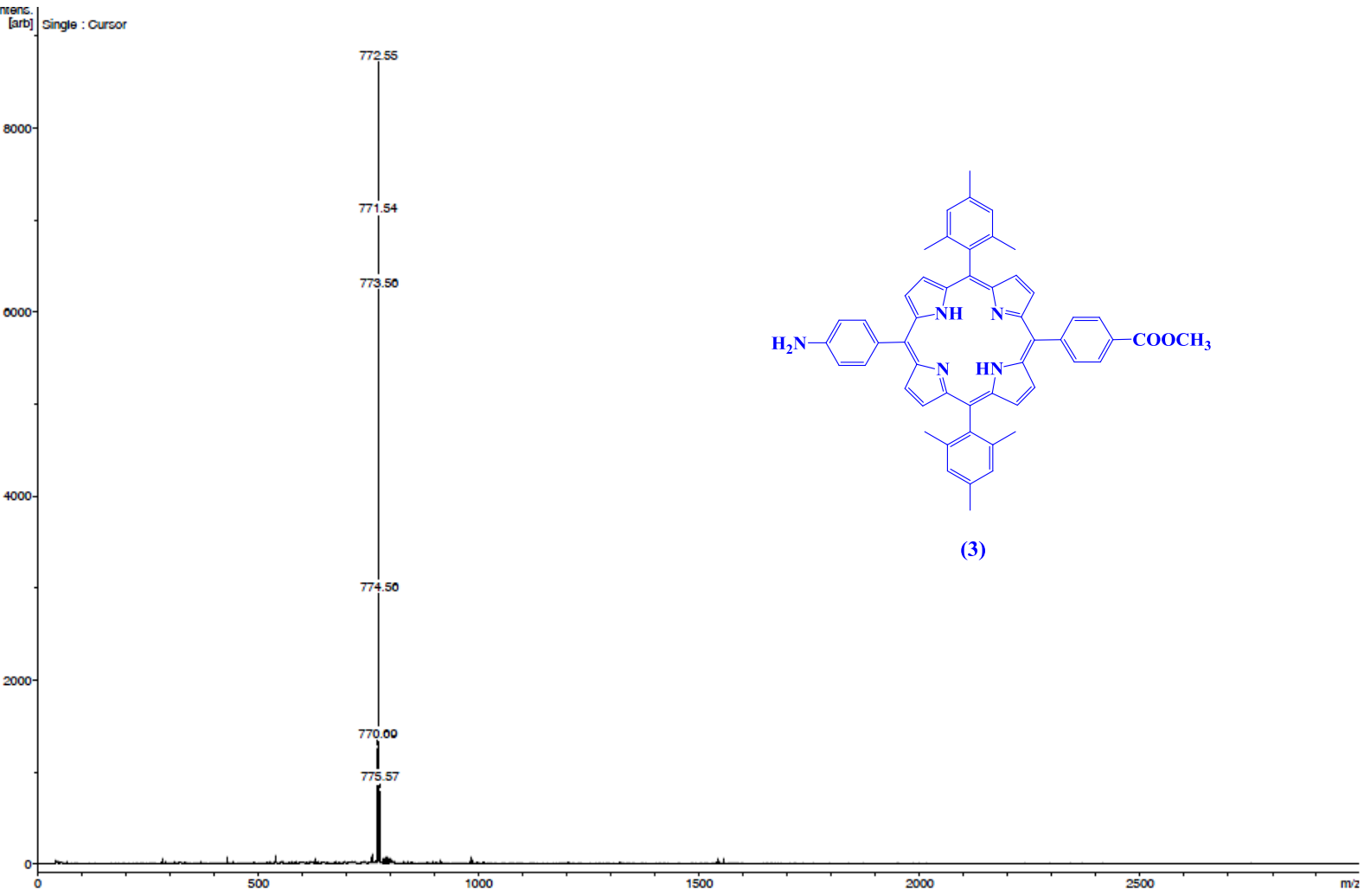
## HRMS (MALDI-TOF) Images of synthesized compounds







(2)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770.09
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2000
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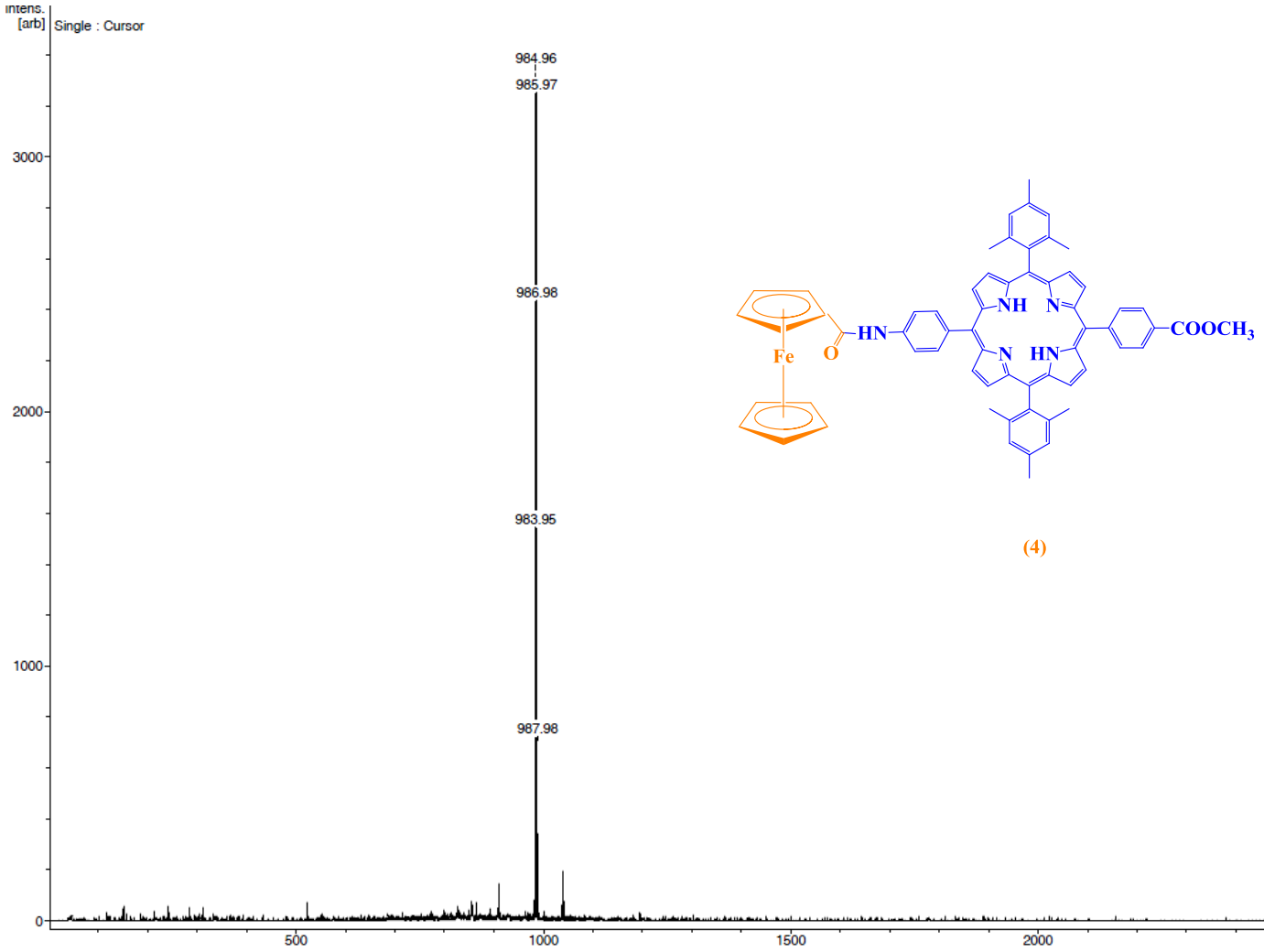
500
1000
1500
2000
2500
m/z


H2N
NH
N
N
HN
COOCH3

**(3)**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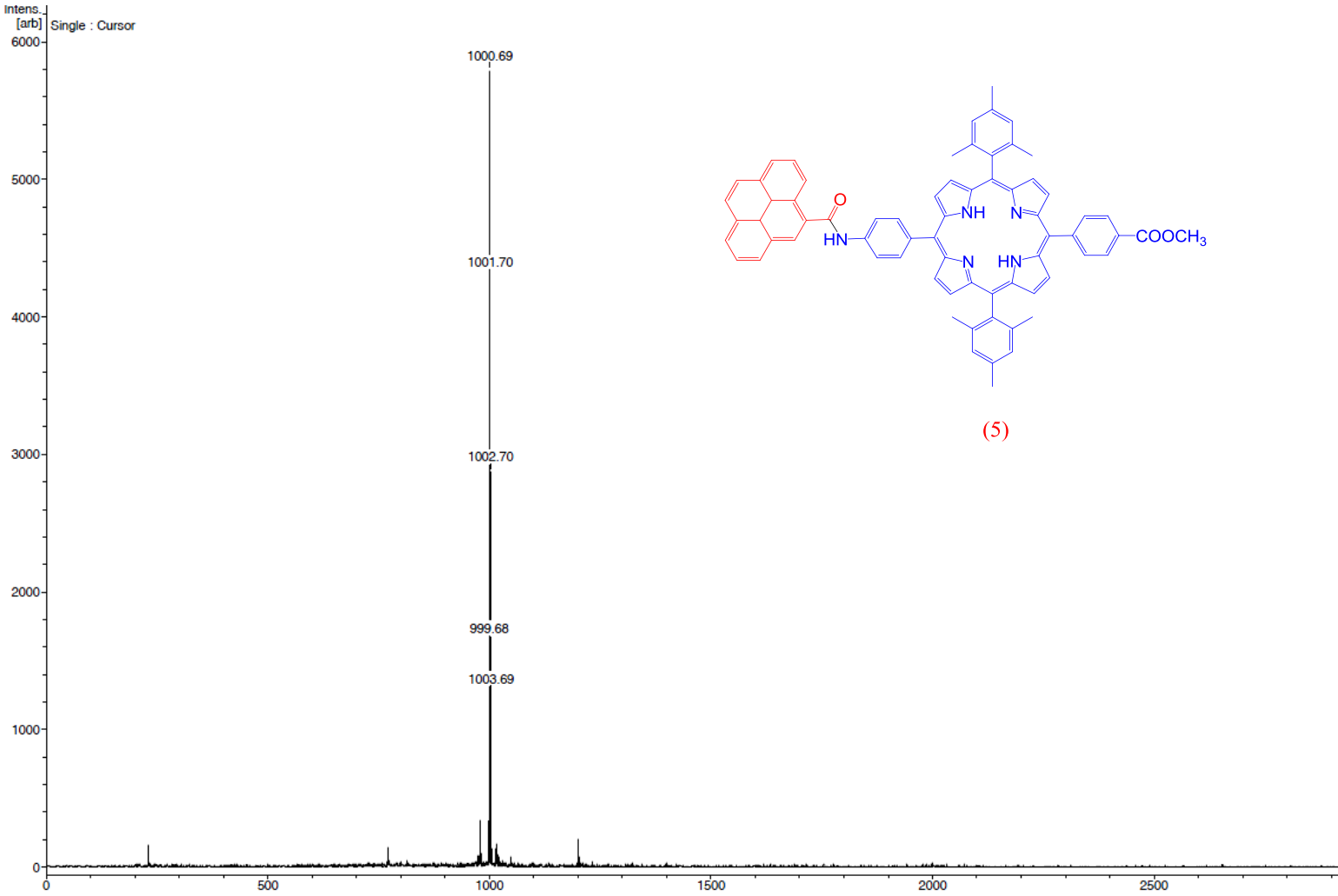
(4)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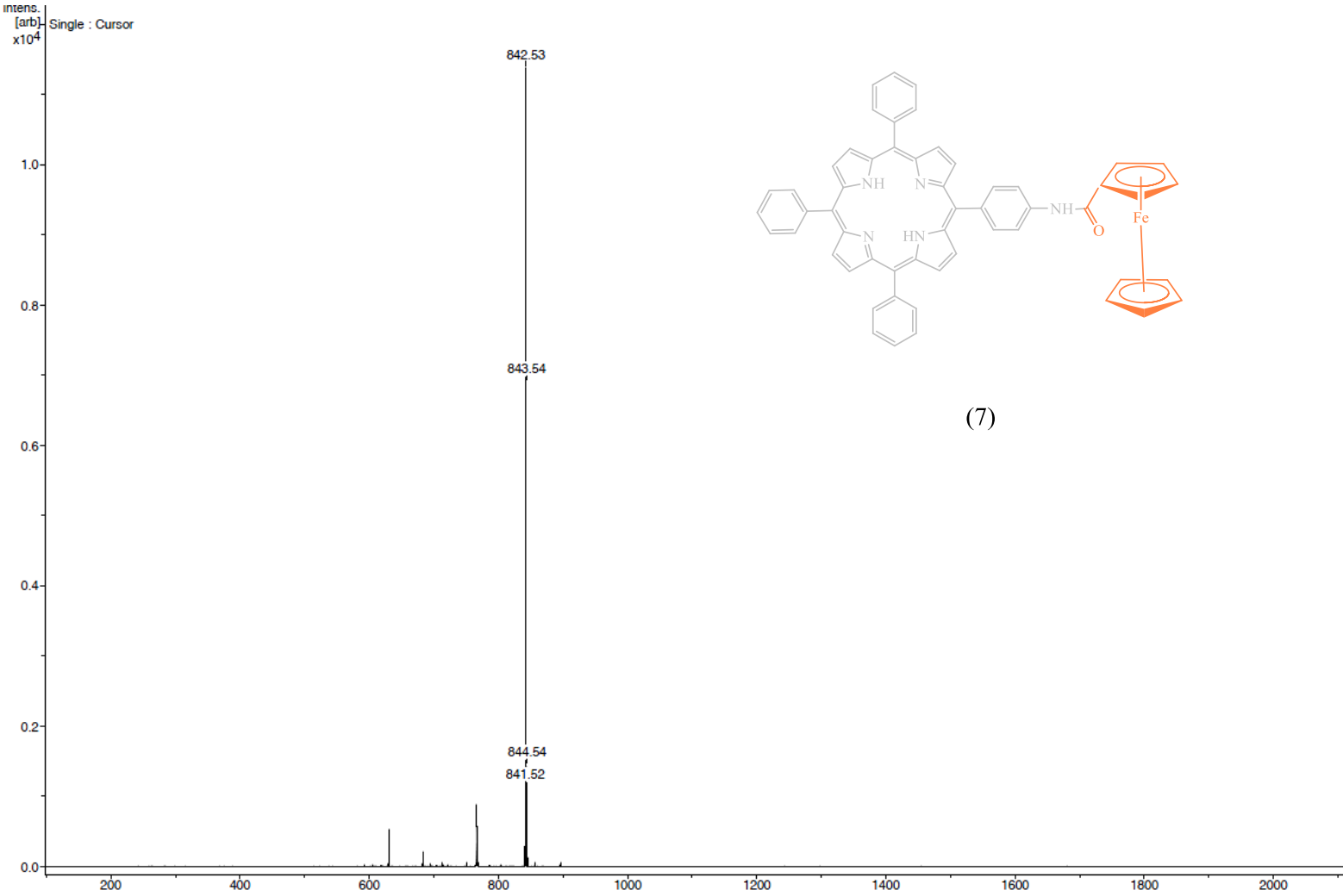
COOCH3

(5)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1600
1800
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NH
N
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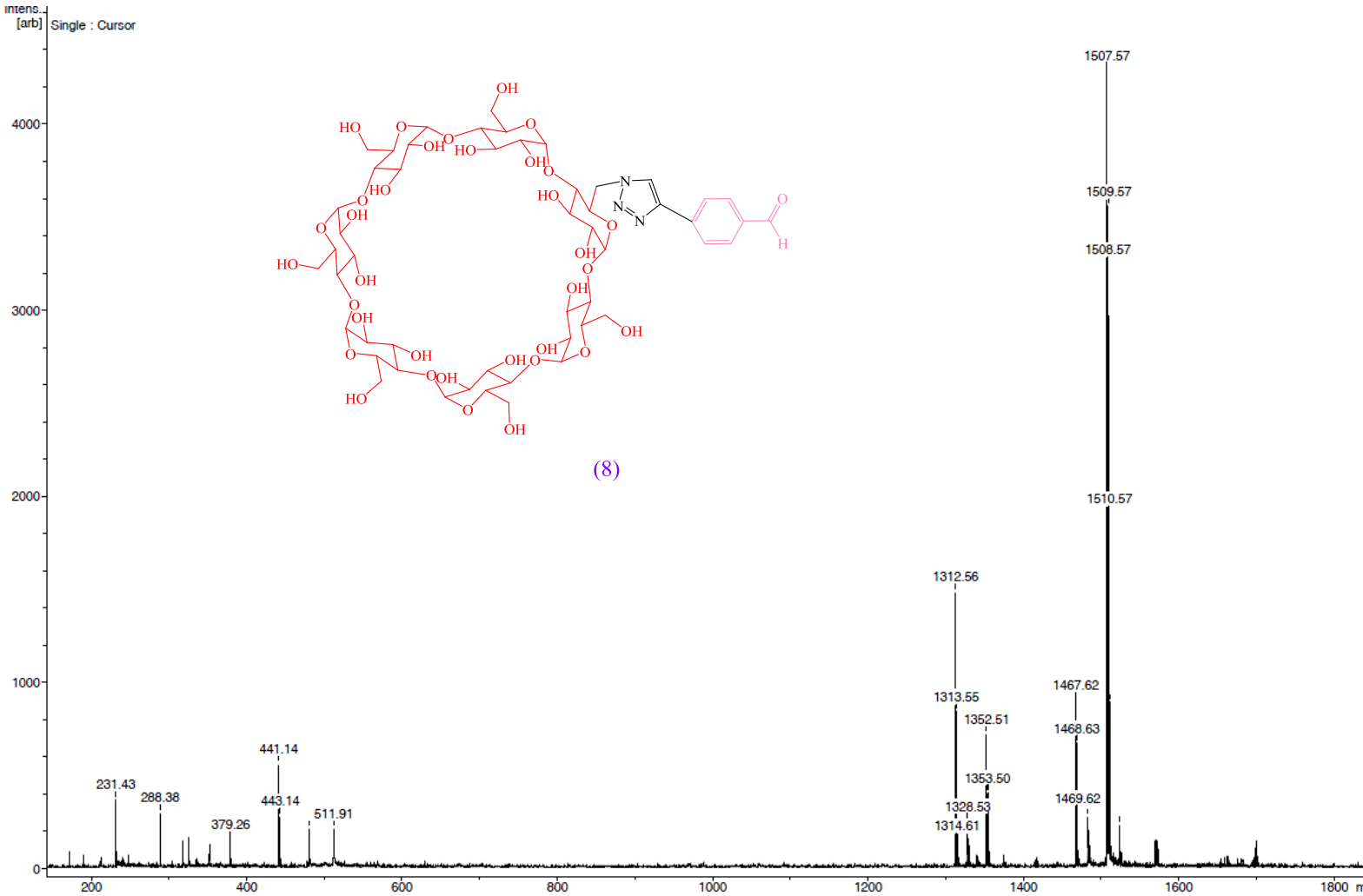
NH
O
Fe

(7)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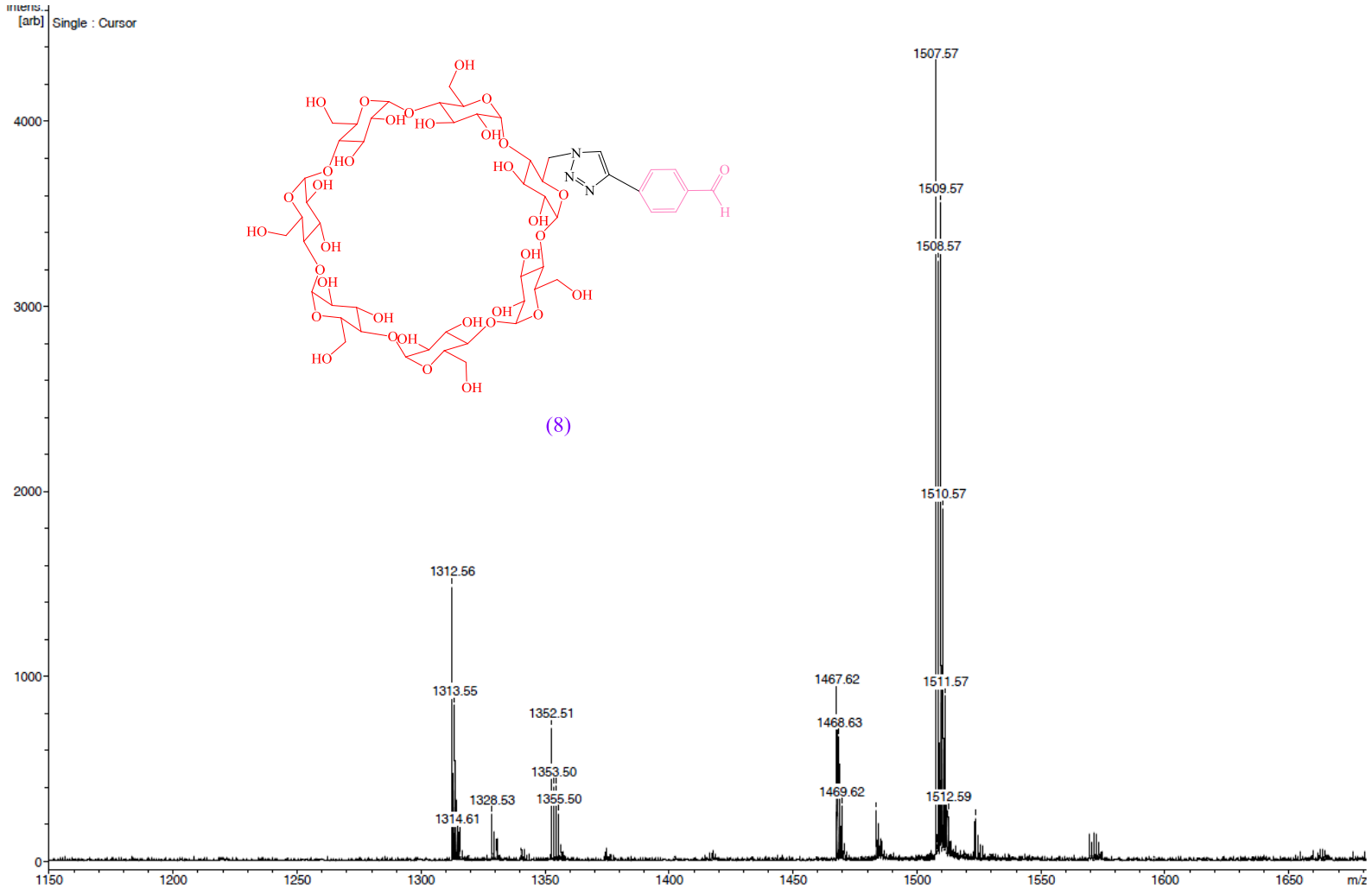
1509.57
1508.57
1510.57
1312.56
1313.55
1352.51
1353.50
1328.53
1314.61
1467.62
1468.63
1469.62
441.14
443.14
231.43
288.38
379.26
511.91

(8)

**Zoom**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1512.59
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1313.55
1314.61
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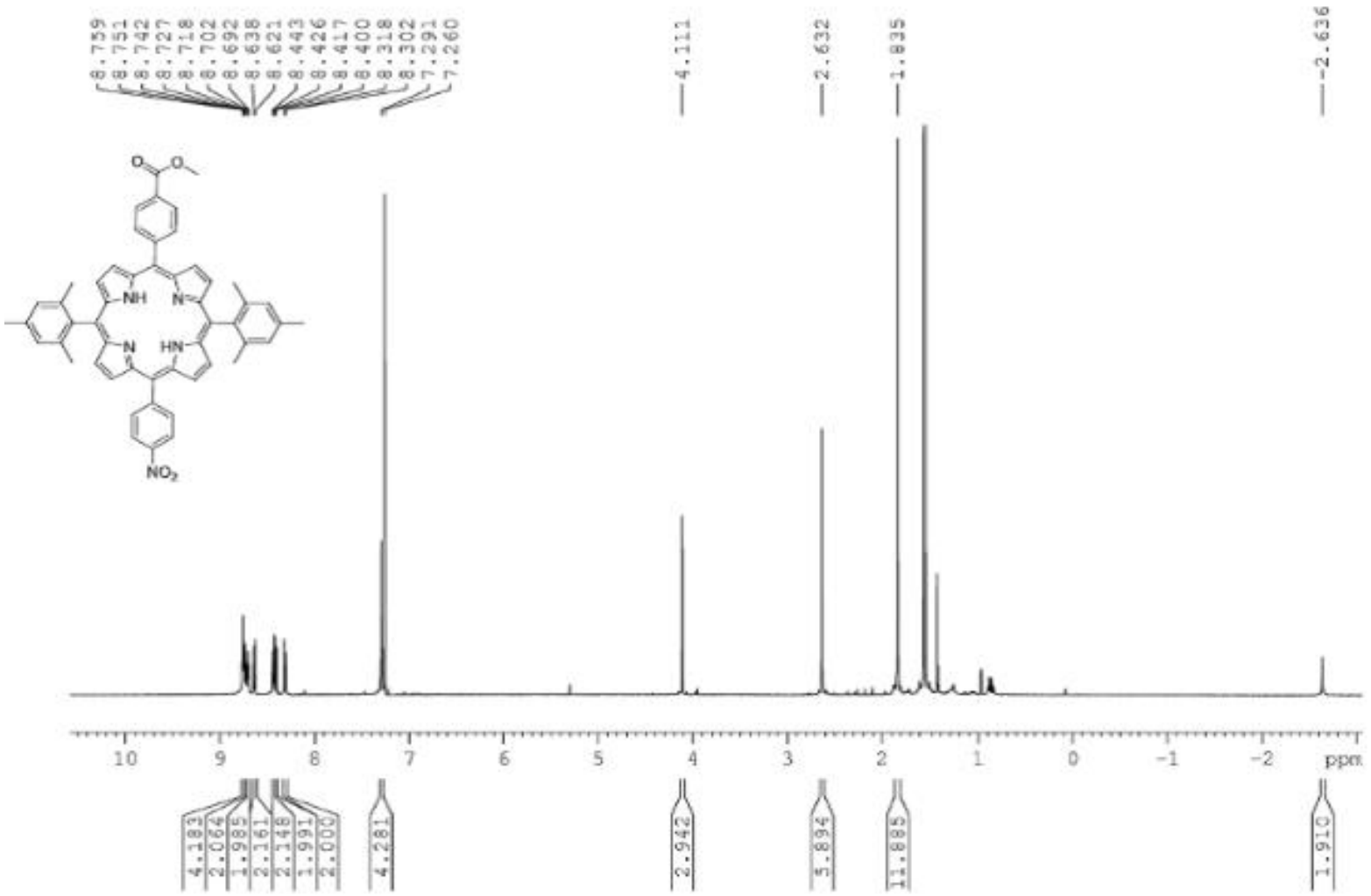
1467.62
1468.63
1469.62

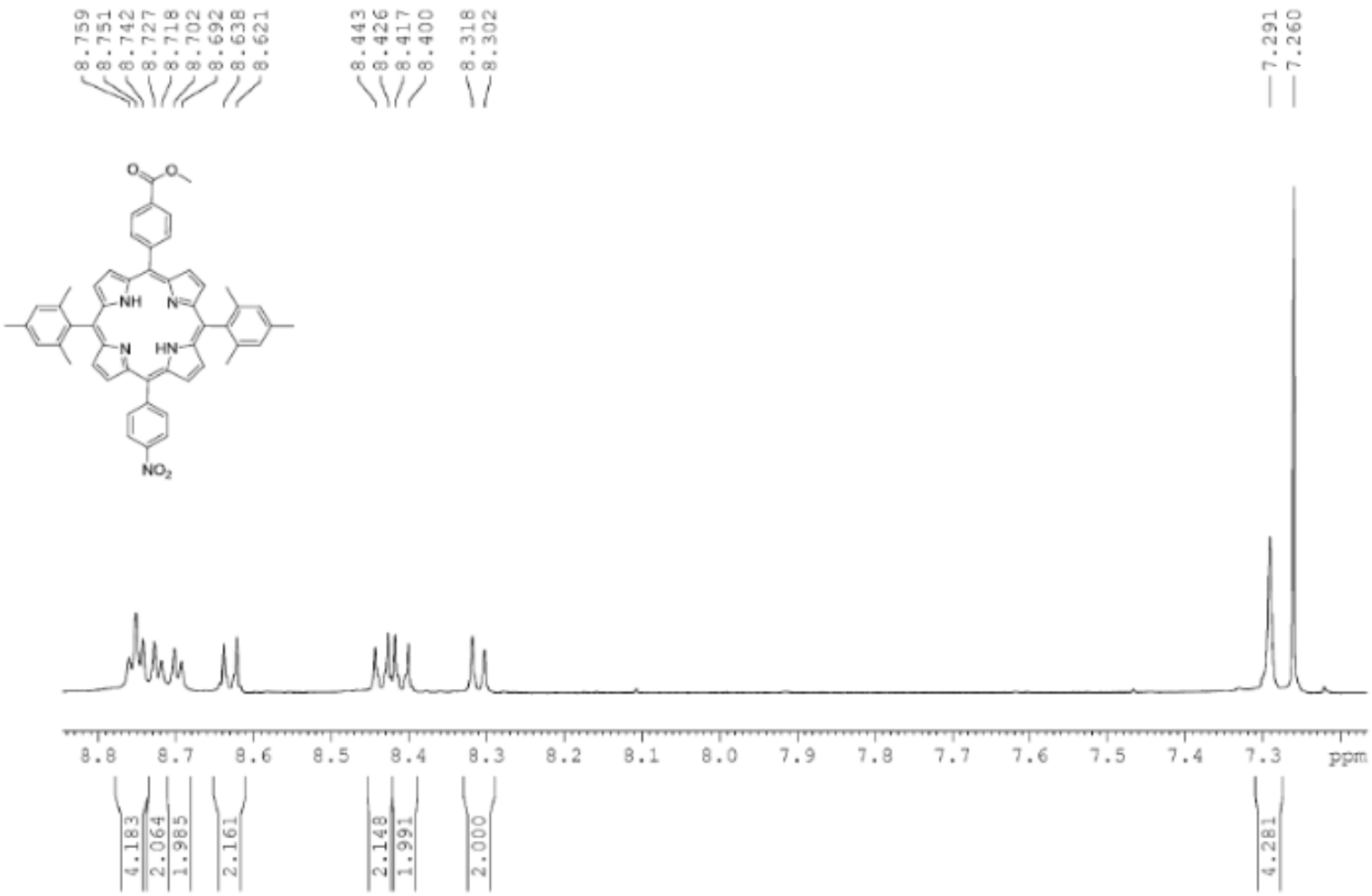
(8)

## NMR spectra

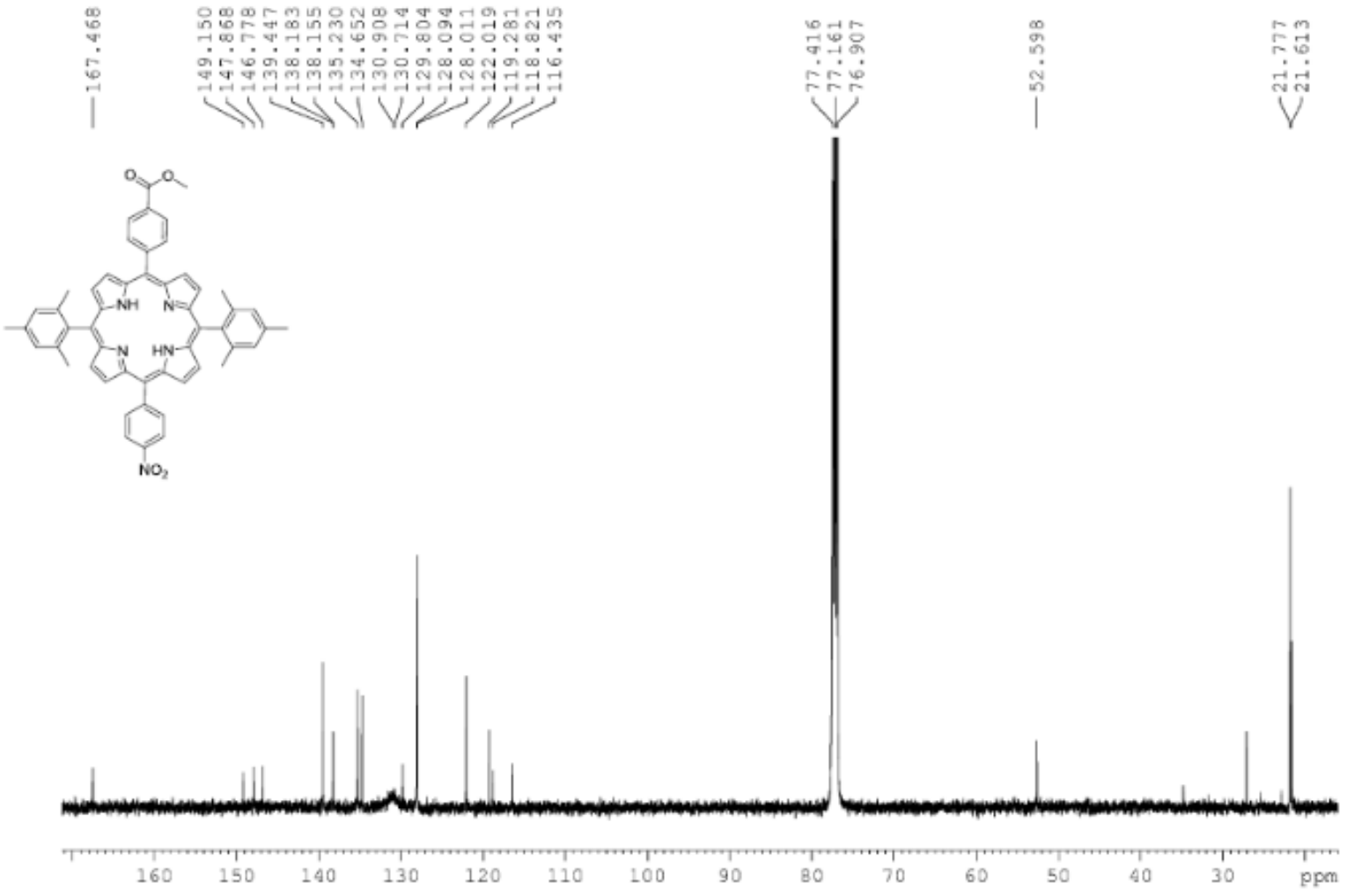
*$^1$H NMR spectrum of compound 2 in $CDCl_3$*



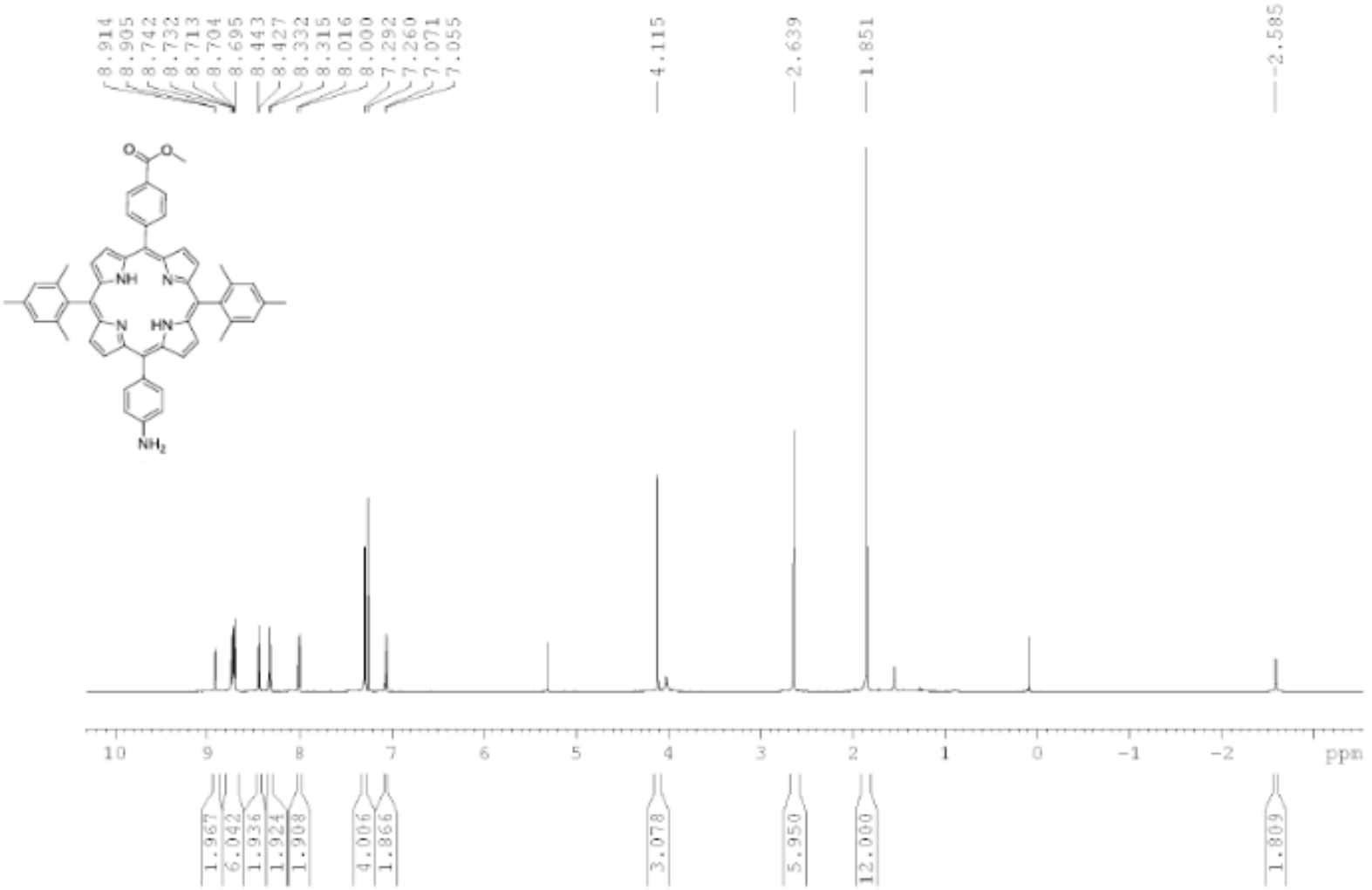
*H NMR spectrum (**zoom**) in the aromatic region of the spectrum of compound 2 in $CDCl_3$*



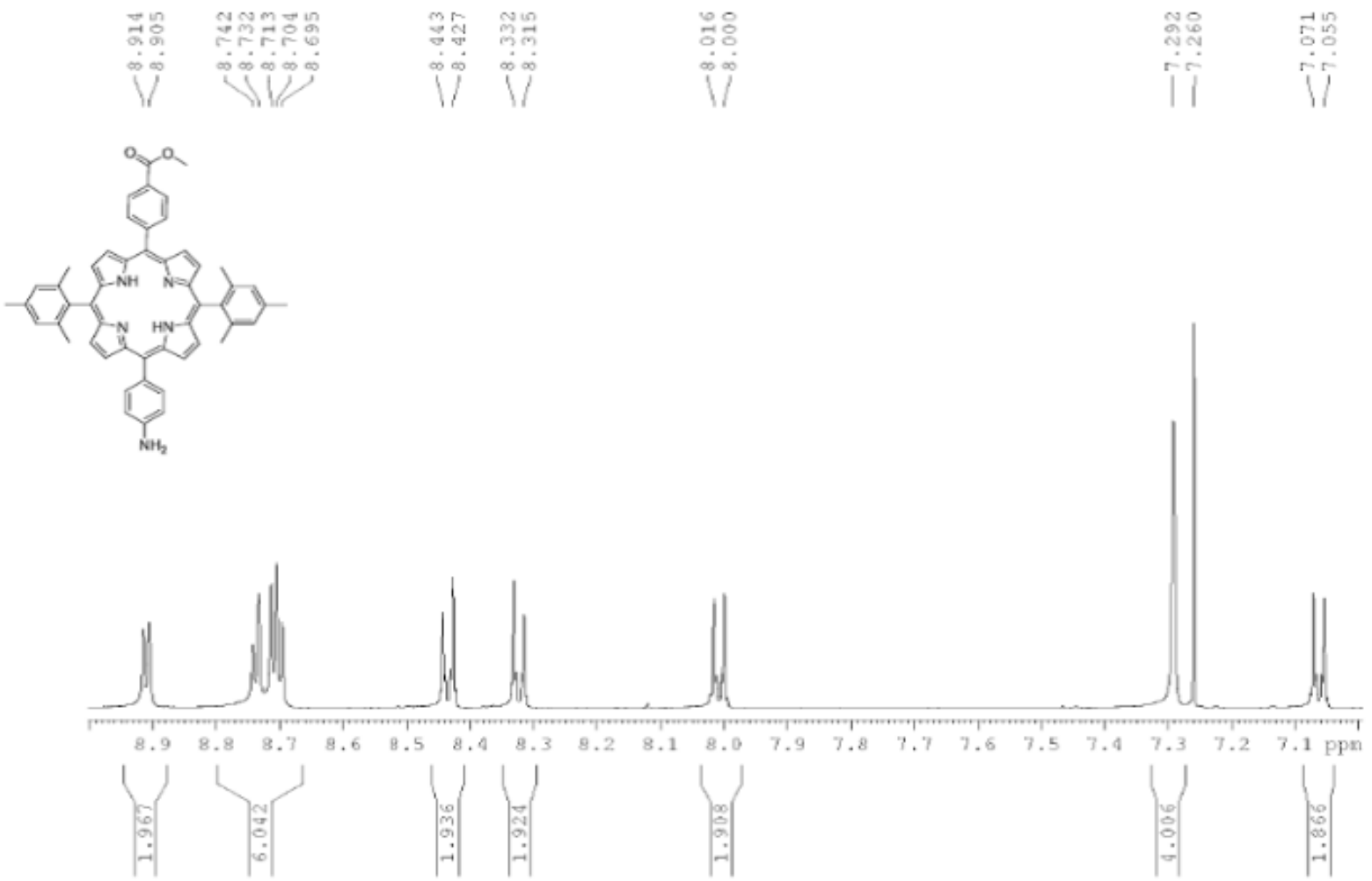
*$^{13}C$ NMR spectrum of compound 2 in $CDCl_3$*



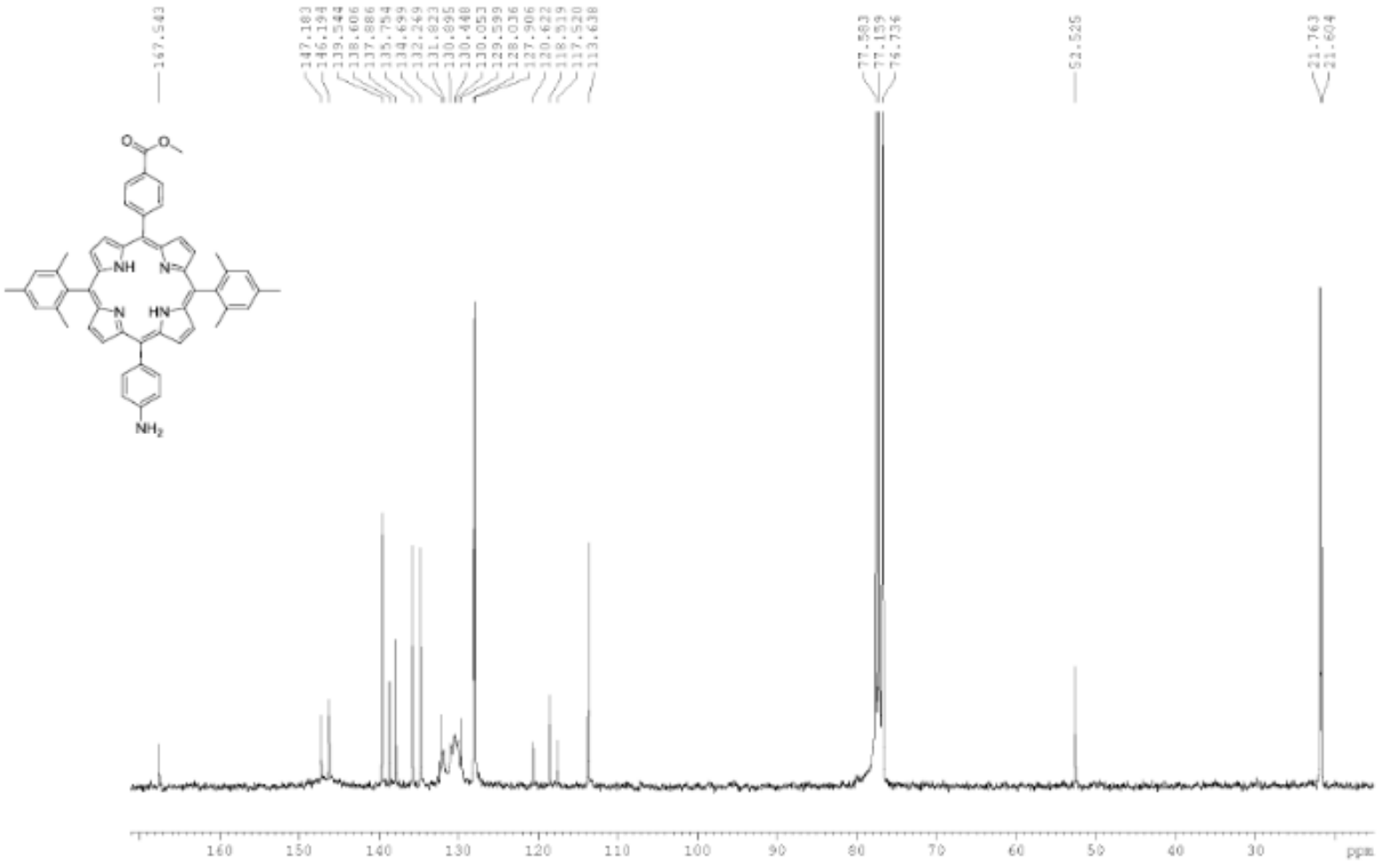
*$^{1}H$ NMR spectrum of compound 3 in $CDCl_3$*



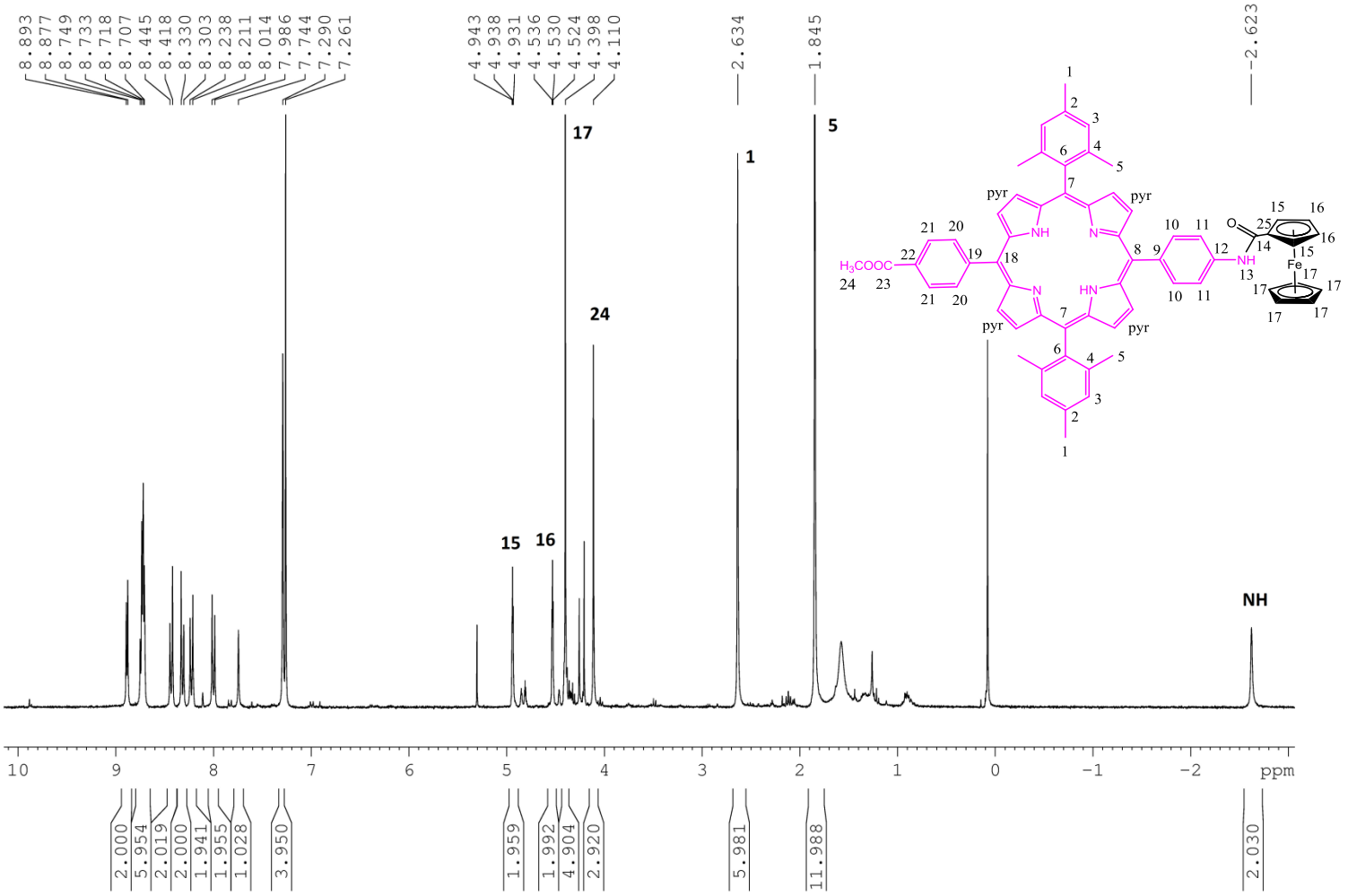
*$^{1}H$ NMR spectrum (**zoom**) in the aromatic region of the spectrum of compound 3 in $CDCl_3$*



*$^{13}C$ NMR spectrum of compound 3 in $CDCl_3$*



*$^{1}H$ NMR spectrum of compound 4 in $CDCl_3$*



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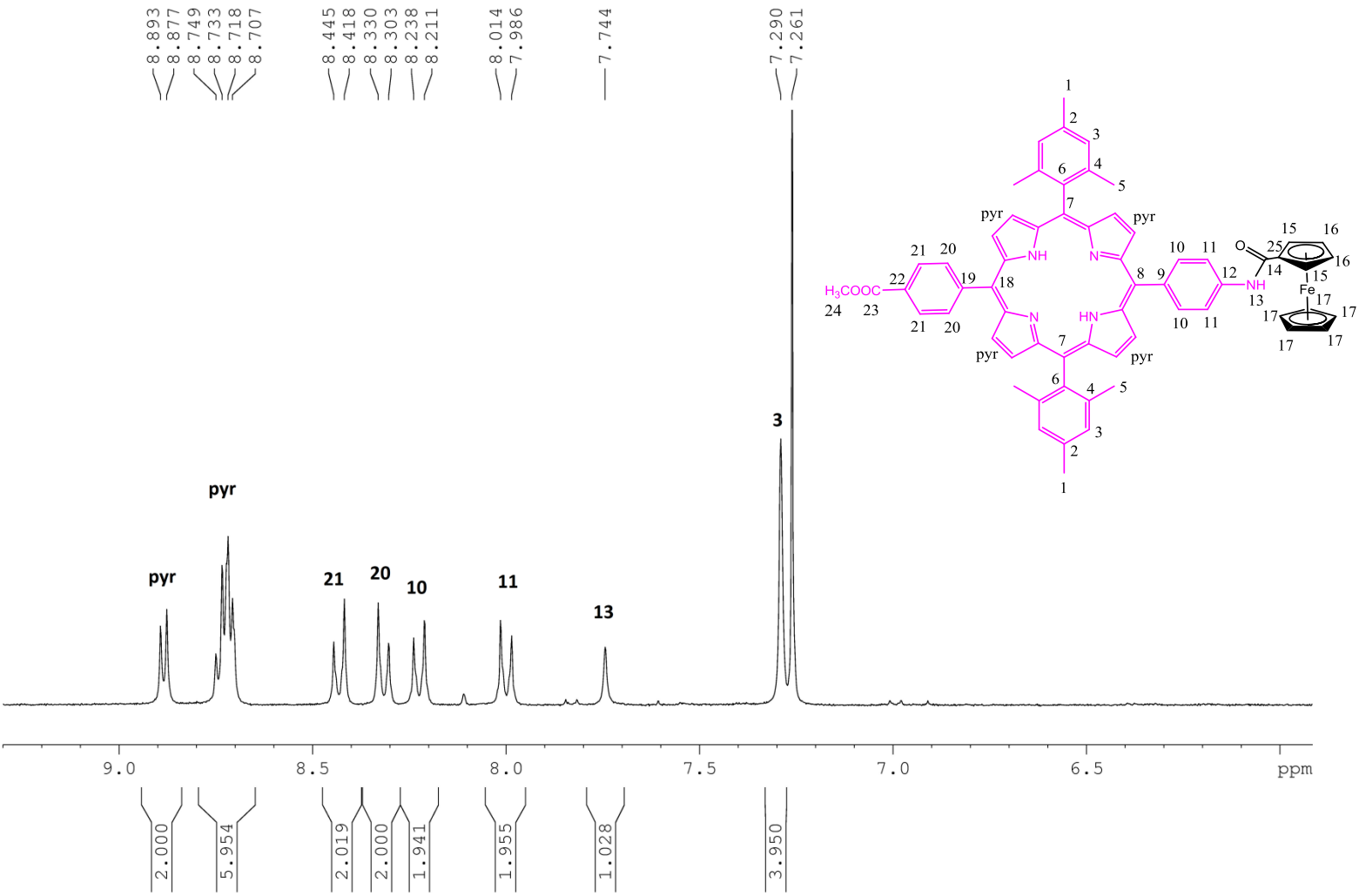

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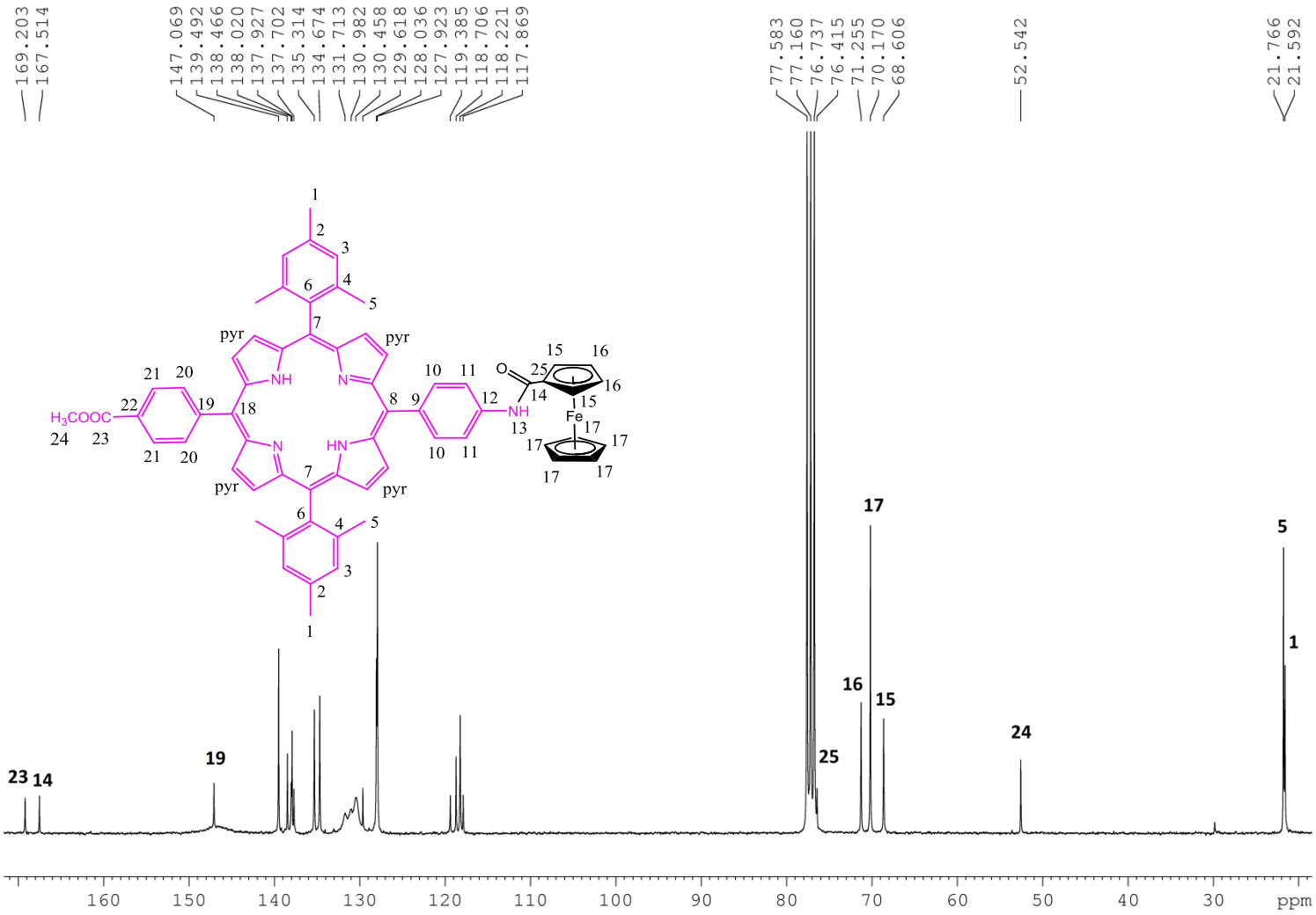
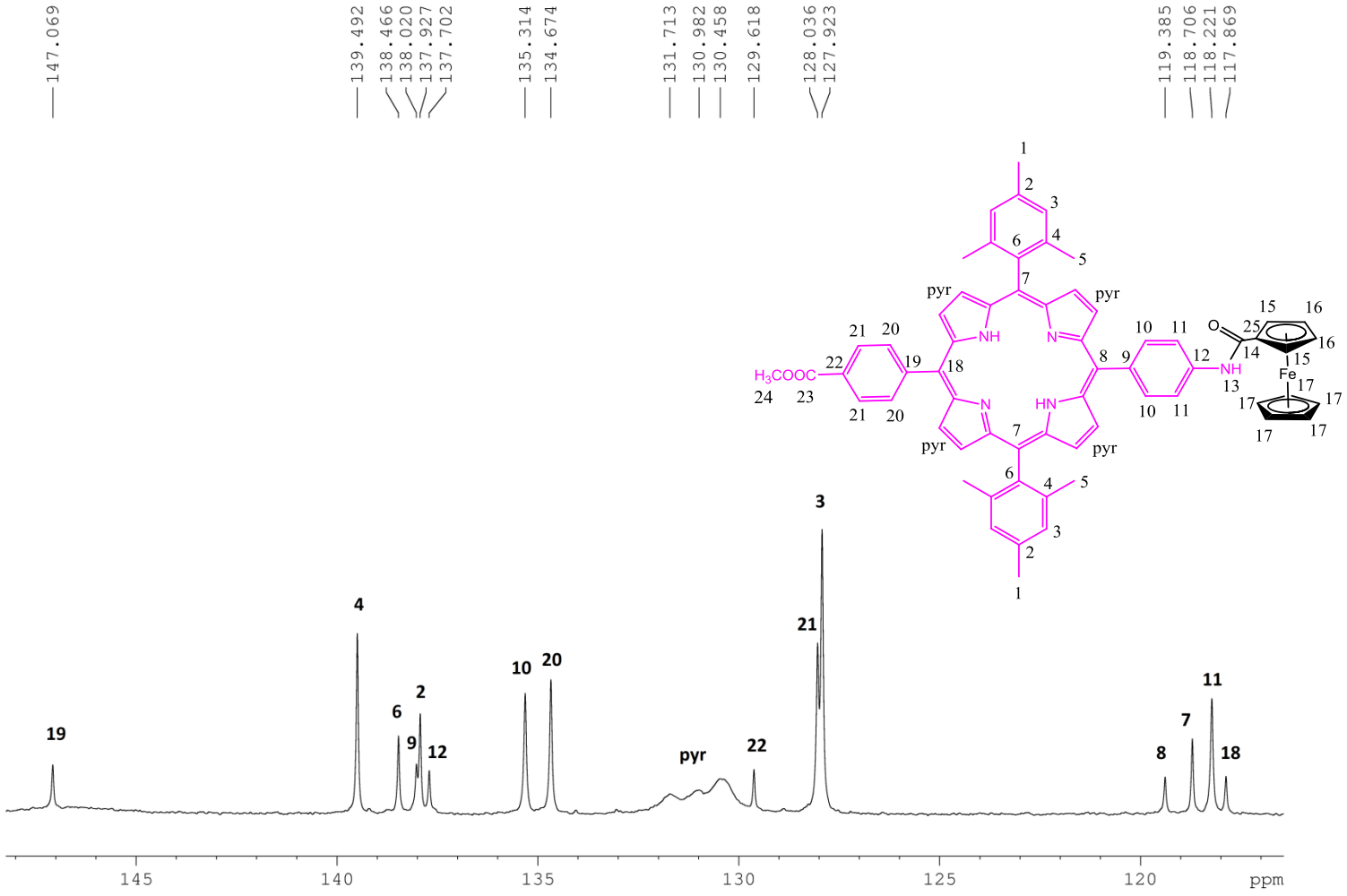
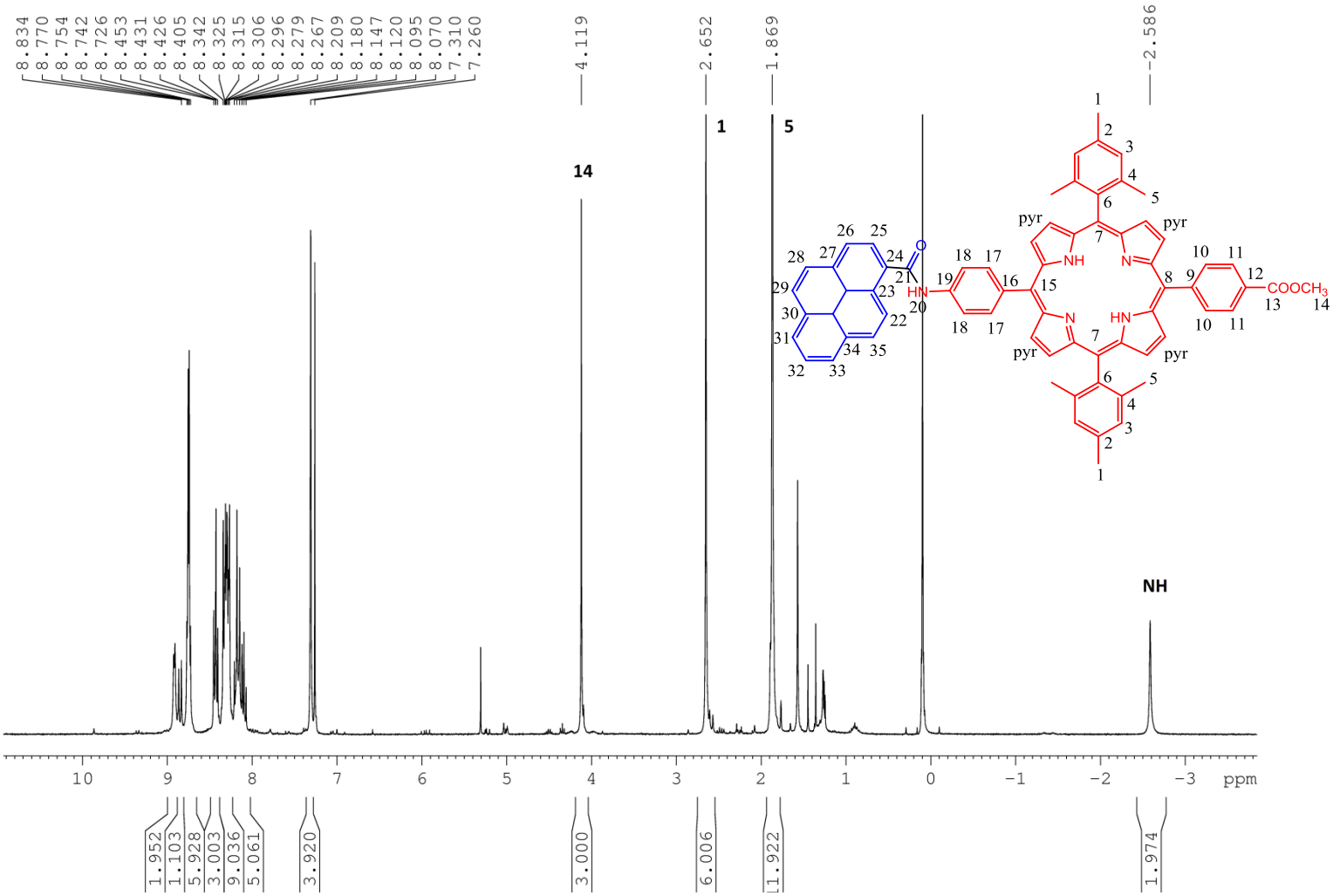


*$^{13}C$ NMR spectrum (**zoom**) of compound 4 in $CDCl_3$*



*$^{1}H$ NMR spectrum of compound 5 in $CDCl_3$*



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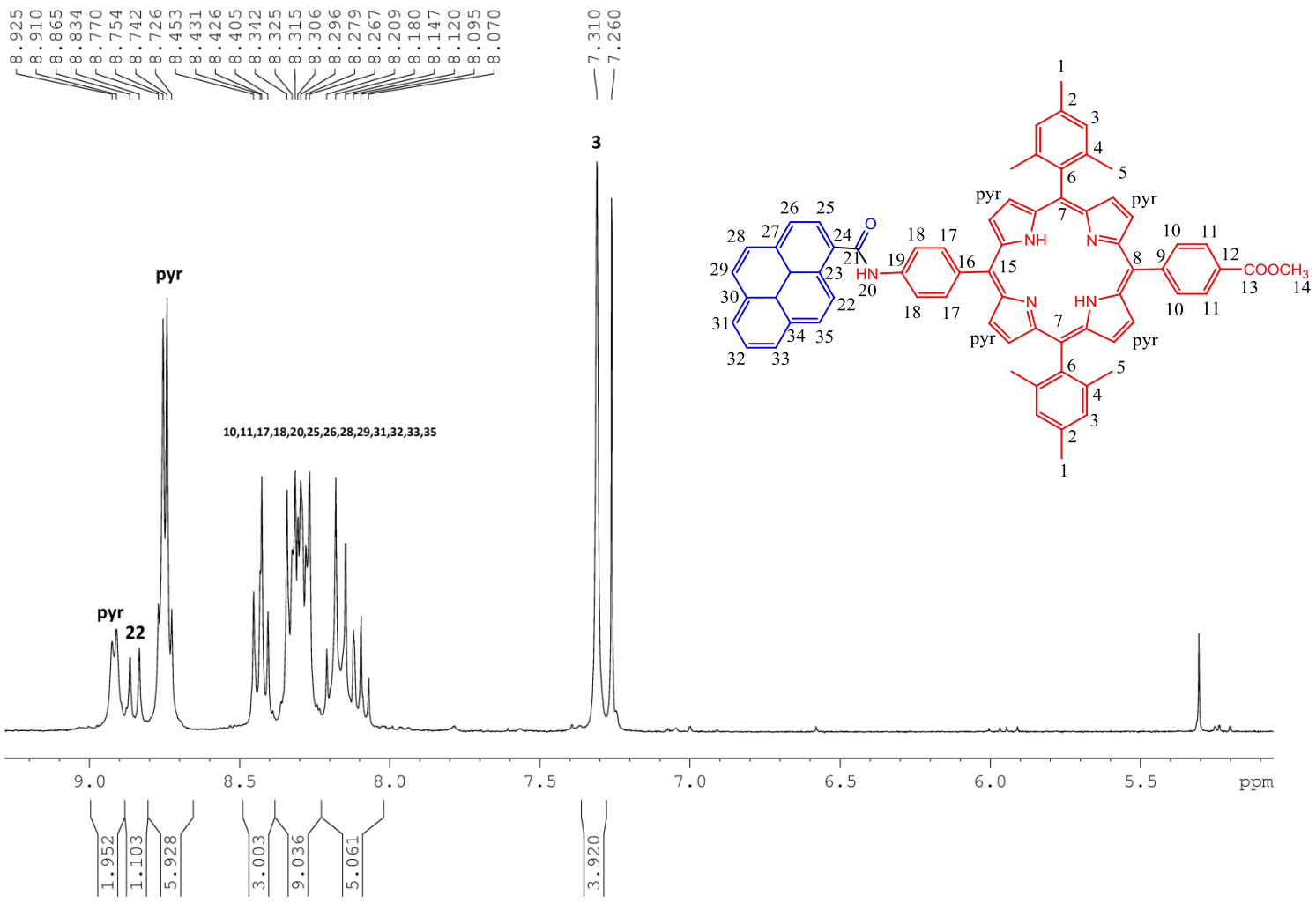

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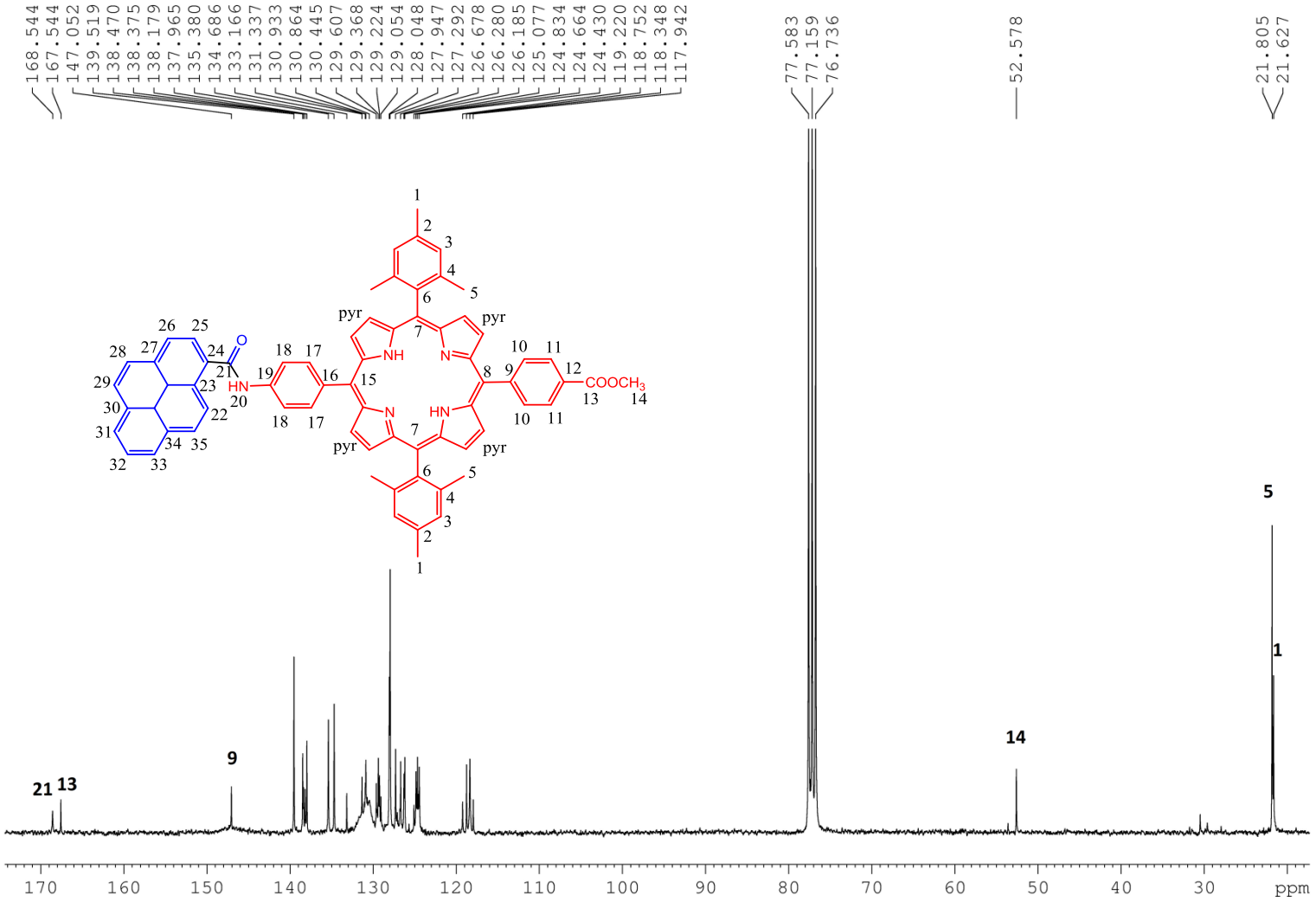

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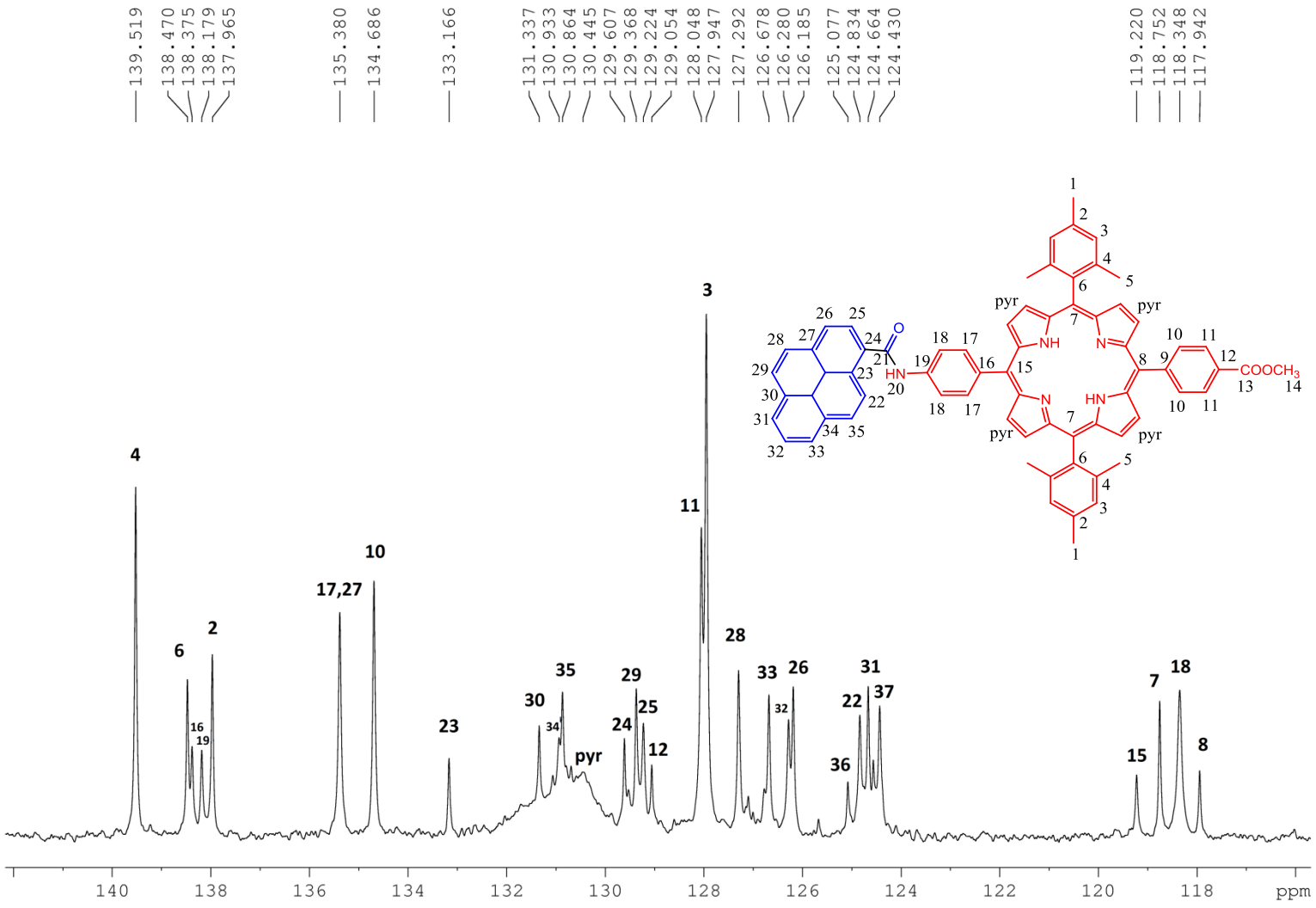

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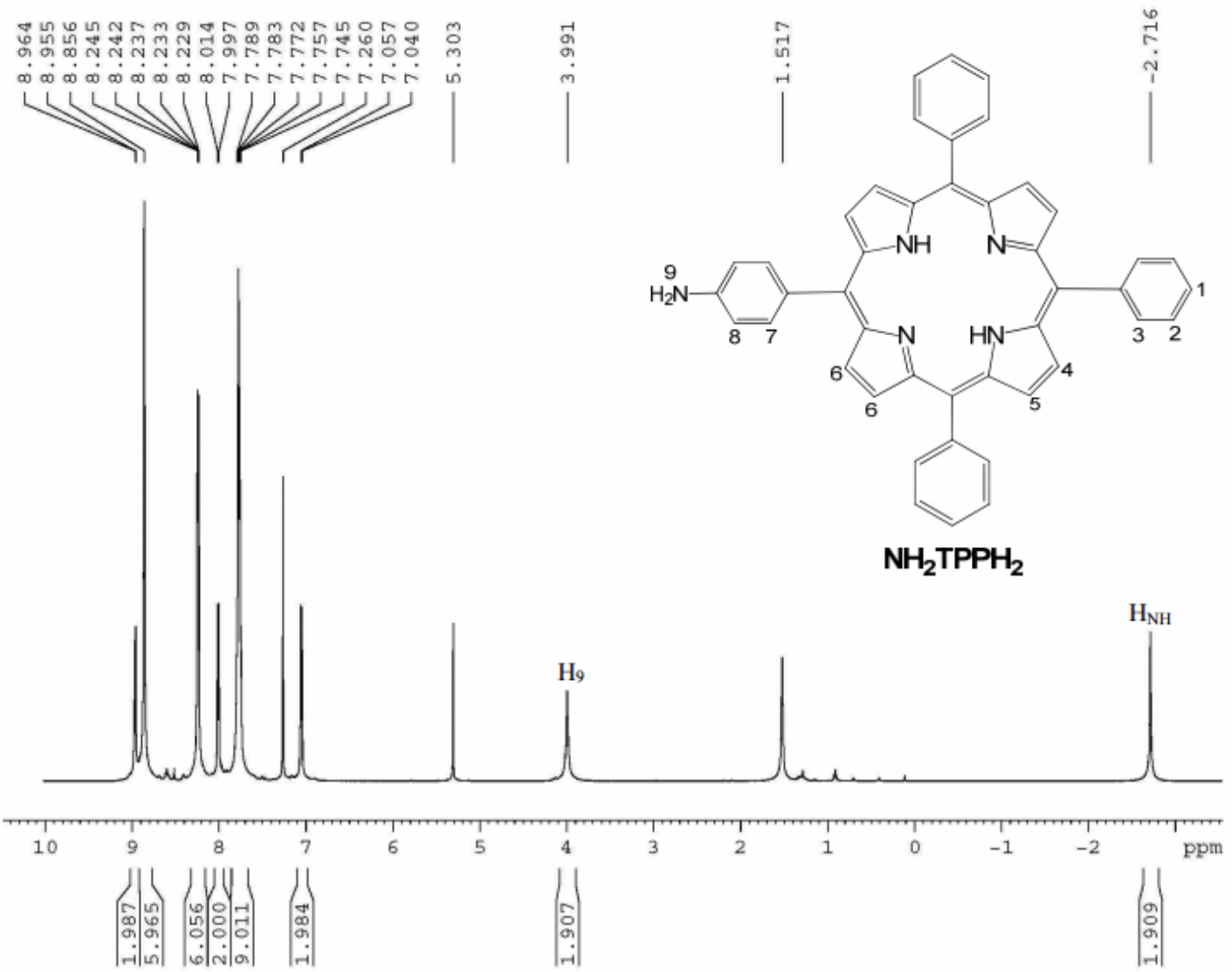

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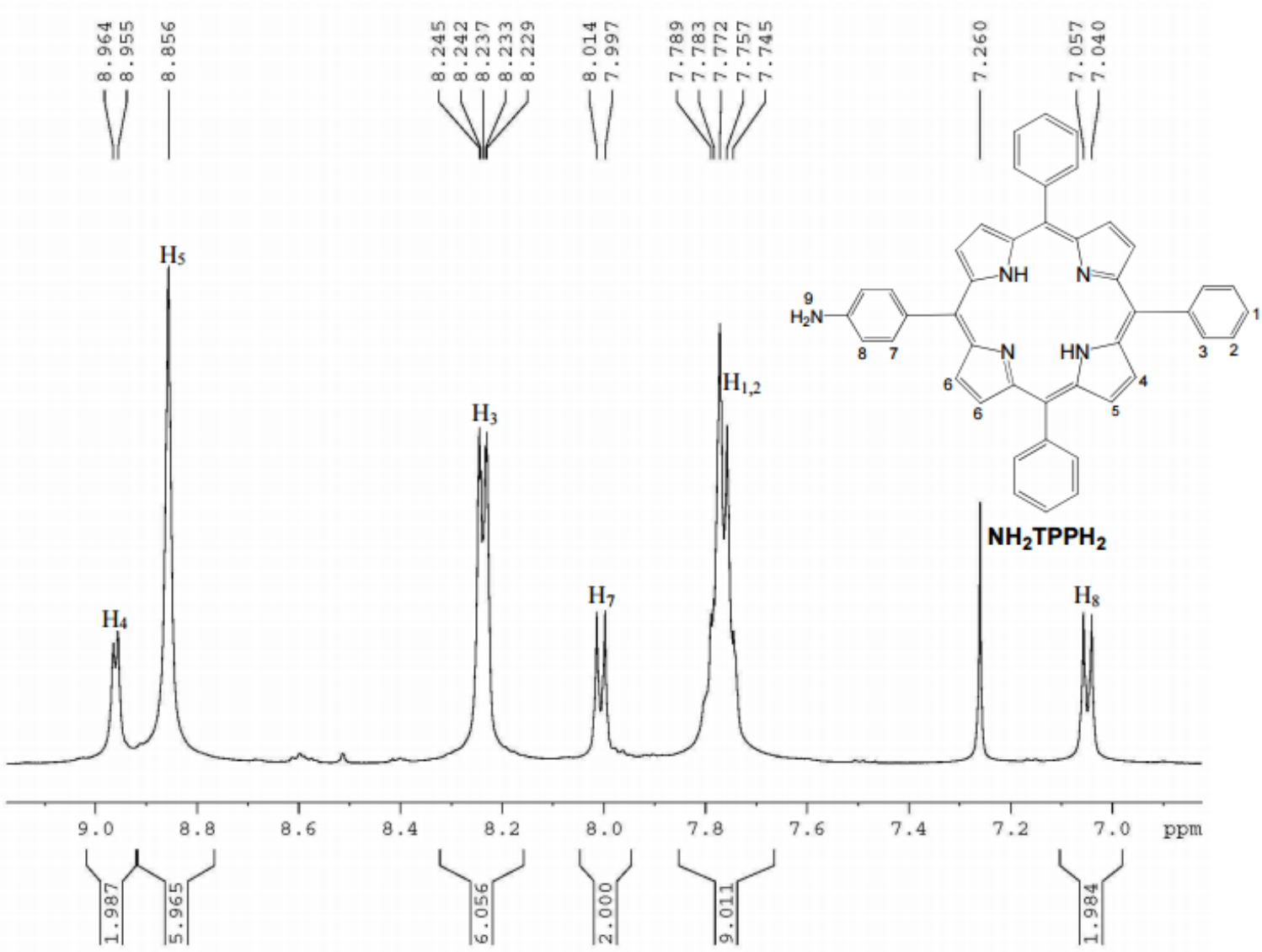

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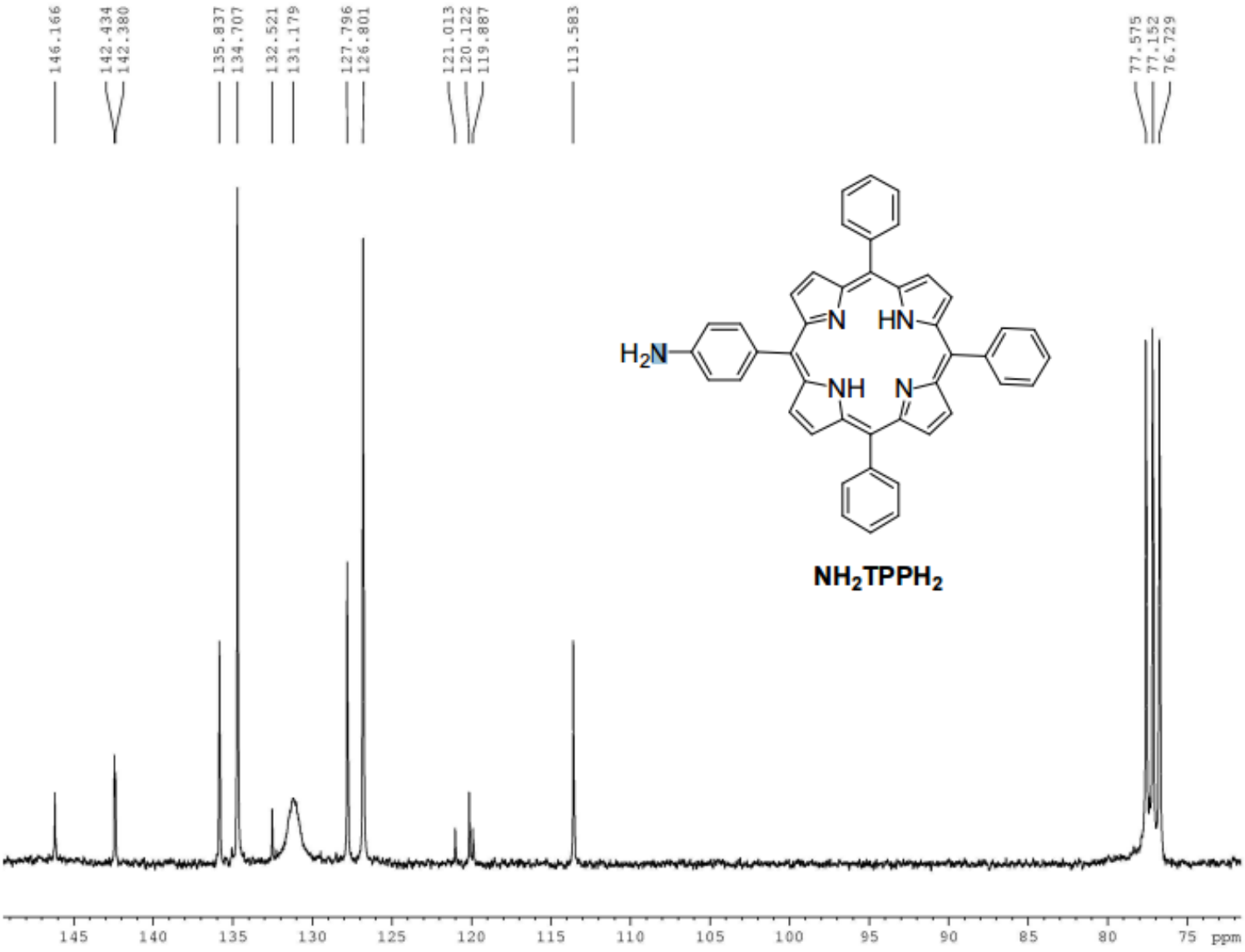
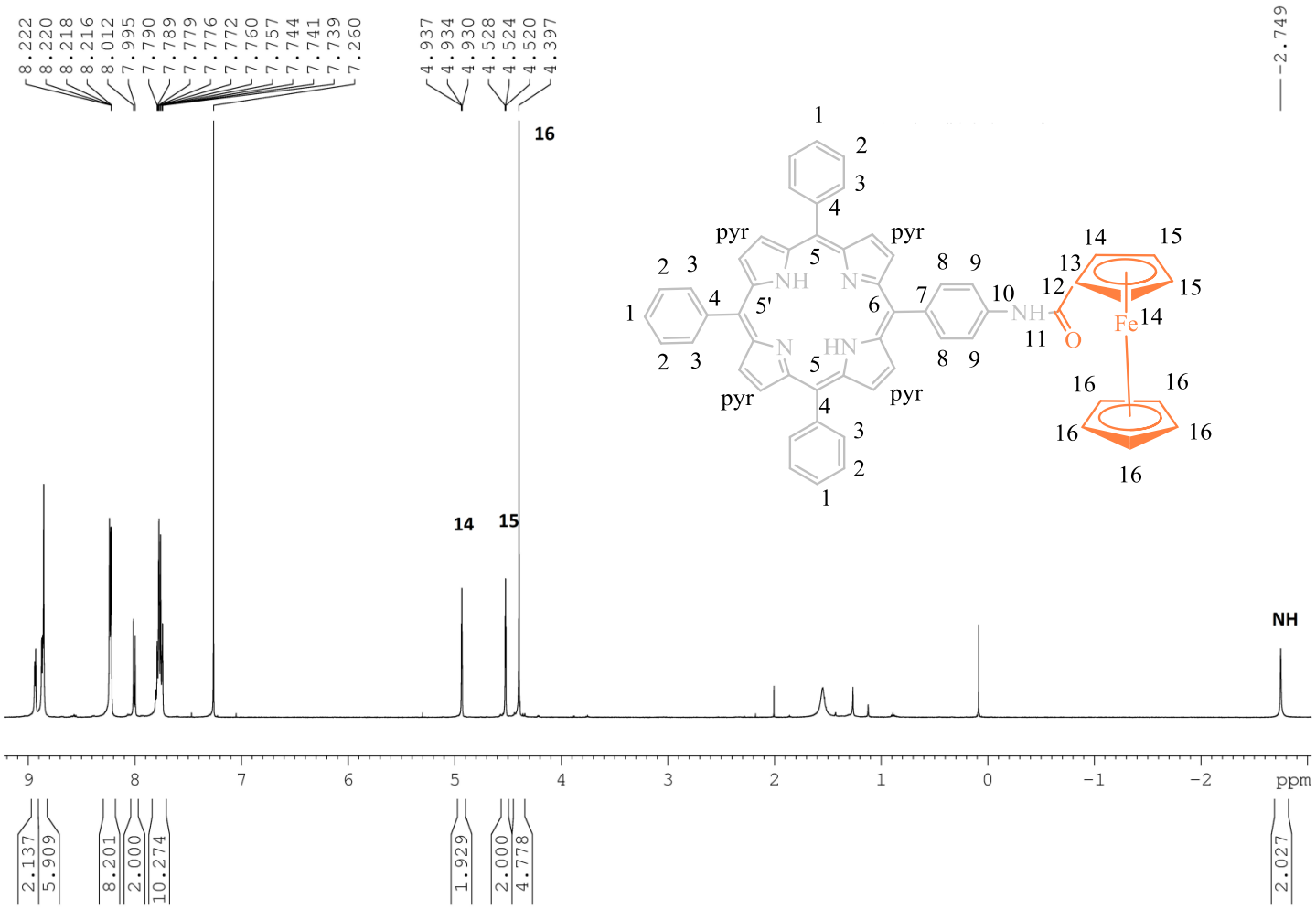
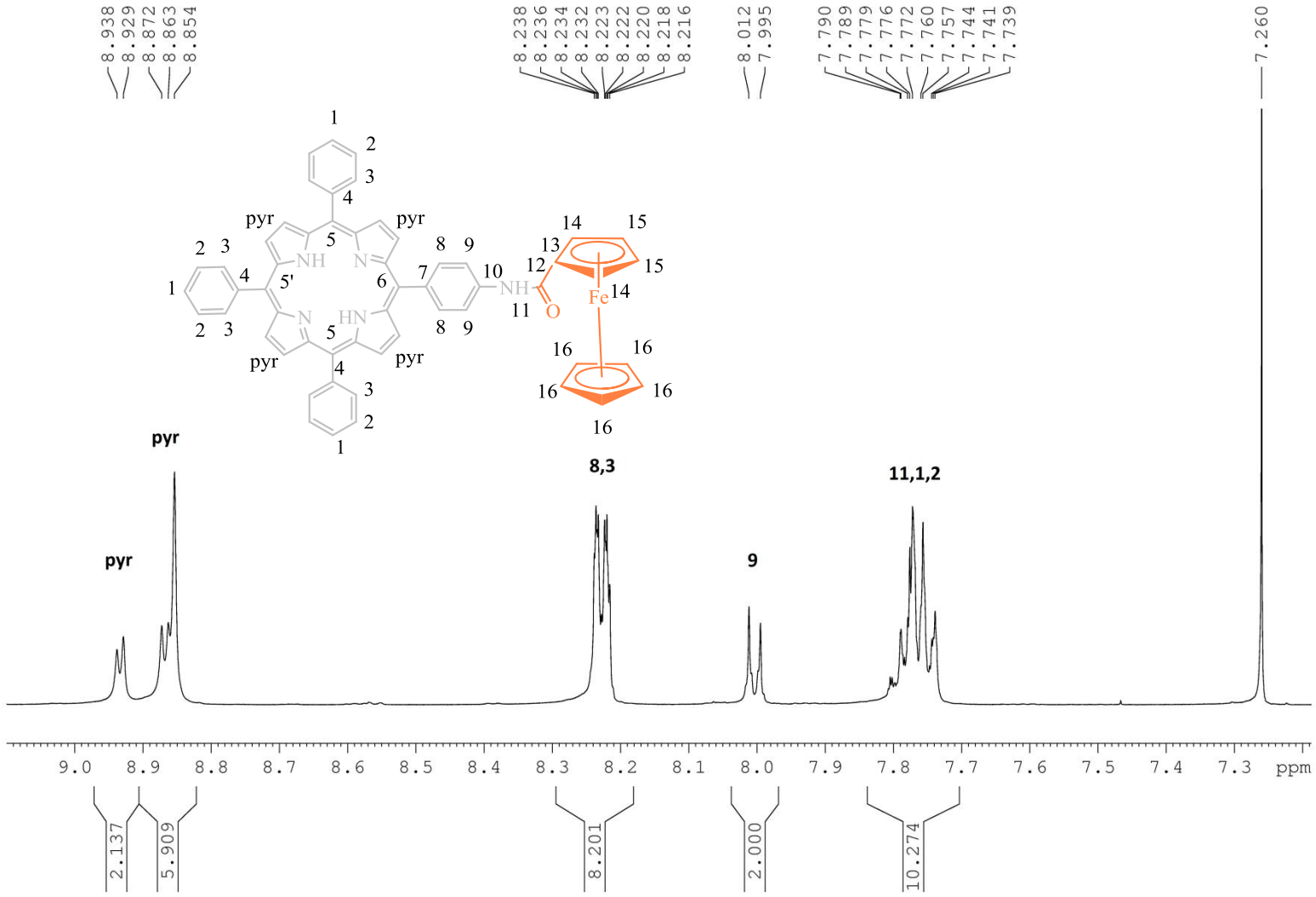


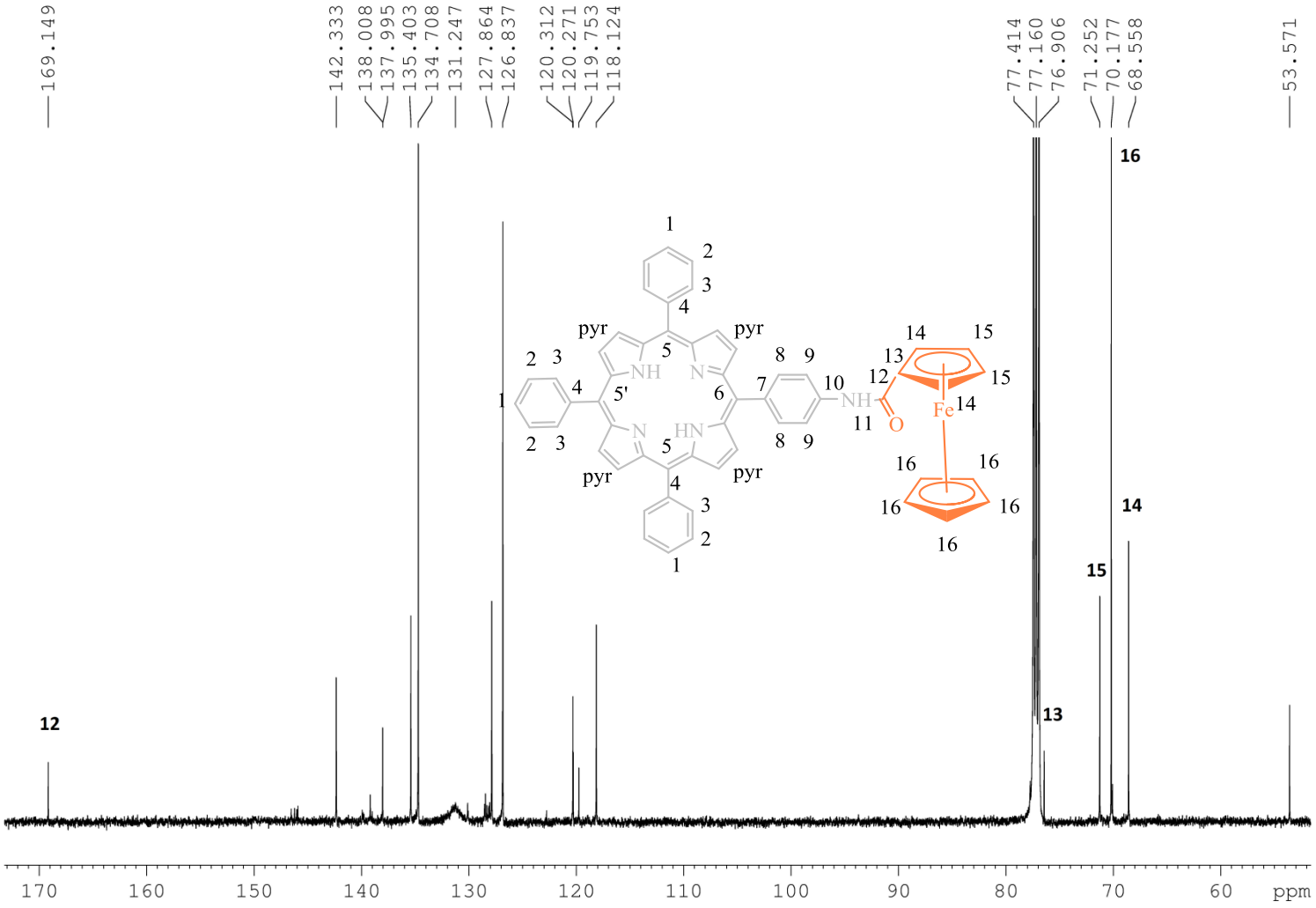
*$^{1}H$ NMR spectrum of compound 7 in $CDCl_3$*



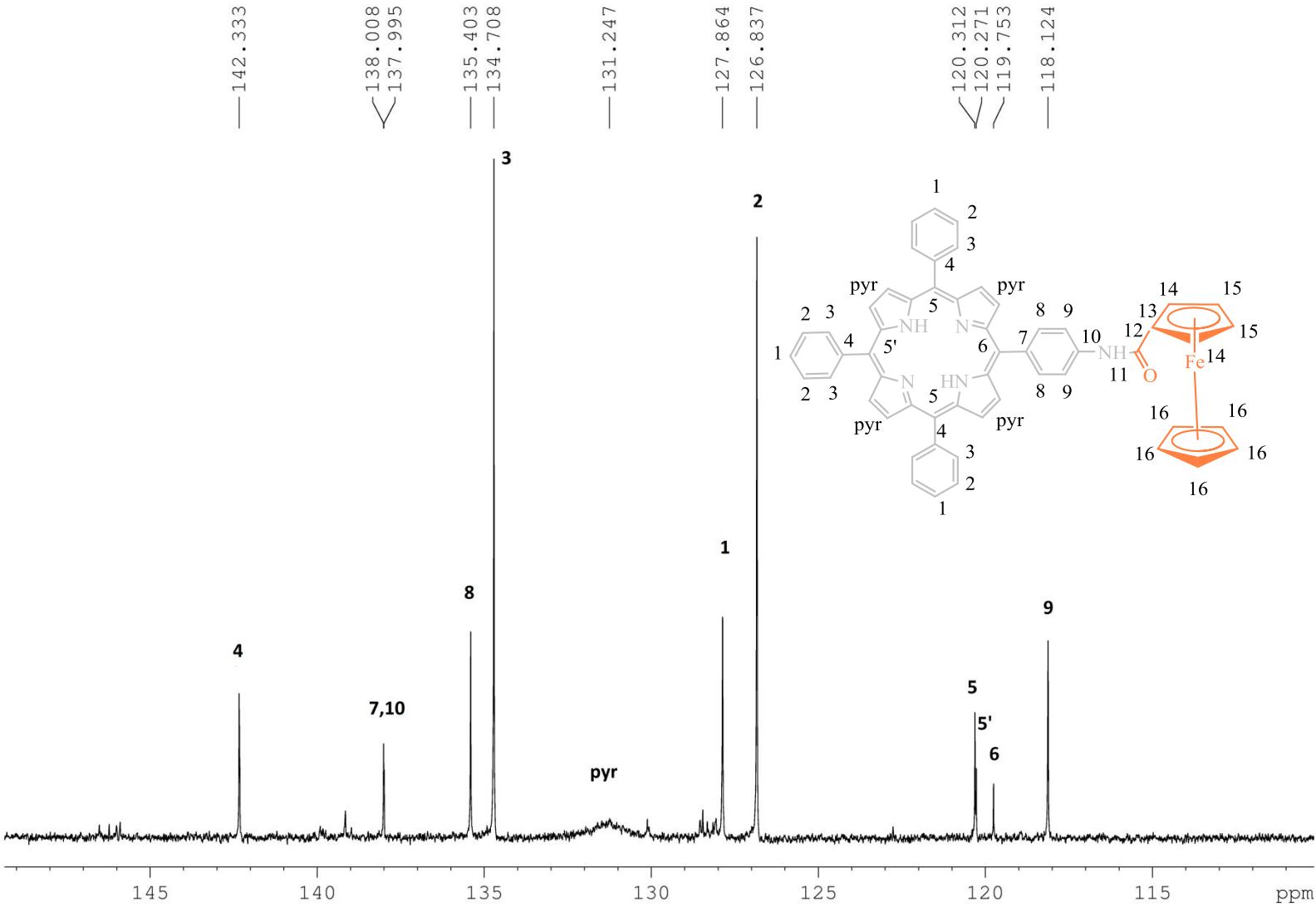
*$^{1}H$ NMR spectrum (**zoom**) in the aromatic region of the spectrum of compound 7 in $CDCl_3$*



*$^{13}C$ NMR spectrum of compound 7 in $CDCl_3$*



*$^{13}C$ NMR spectrum (**zoom**) of compound 7 in $CDCl_3$*



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