Metode sinteze i oksidacije piridilporfirina

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Methods of pyridylporphyrin oxidation and synthesis

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Pegi Pavletić

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Special thanks to my family who supported me throughout my studies,

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1. Assistant professor dr. Ivana Ratkaj– head of the committee
2. Assistant professor dr. Rozi Andretić- Waldowski- member
3. Associate professor dr. Nela Malatesti– member, supervisor

Summary

Porphyrins are organic compounds that can be used as photosensitizers in photodynamic therapy (PDT). PDT involves photosensitizer (PS) administration into the body, where they penetrate tumour tissues and, once illuminated, cause tumour degradation.

Most common way of porphyrin synthesis in our laboratory includes modified Adler-Longo method, since it is relatively easy to conduct, it is fast and gives us products with impurities separable with a column chromatography. Recently we have found that zwitterionic *N*-oxidised pyridylporphyrins as PSs in PDT exhibit low "dark" toxicity, as opposed to their cationic analogues, *N*-methylated pyridylporphyrins, thus we decided to focus our research on this perspective group of porphyrin PSs.

One of the requirements for porphyrins to be used in PDT efficiently, is their high uptake into targeted cells. Therefore, *N*-oxidised pyridylporphyrins we are particularly interested in have lipophilic component, and their synthesis in this work includes adding long alkyl chains derived from dodecanoyl and hexadecanoyl chlorides.

In this work different methods of pyridylporphyrin *N*-oxidation were analysed to investigate which method is the best in terms of yields and ease of purification for preparation of new potential photosensitisers for the use in PDT. Four methods were assessed for *N*-oxidations of porphyrins using: *meta*-chloroperoxybenzoic acid (*m*-CPBA), dimethyldioxirane (DMD); hydrogen peroxide, sodium hydroxide and *meta*-chloroperoxybenzoic acid; or acetic acid. Only methods using *m*-CPBA and DMD showed promising results, providing high yields of oxidised pyridylporphyrins (65 % for *m*-CPBA and 70 % for DMD) in the reactions that were easy to optimize and conduct. Method including H_2O_2, NaOH and *m*-CPBA gave a yield of (oxydo)pyridylporphyrin of 3,92 % and the reaction using CH_3COOH did not occur.

UV/ Vis spectroscopic analysis showed no difference between the spectra of symmetric porphyrin oxidised by the different methods.

We are concluding from this work that both methods 1 (*m*-CPBA) and 2 (DMD) provide good yields. Although method 2 is more complicated to perform due to constant pH adjustments, the product contains less impurities, needs less additional purification and the yield is up to 5 % higher in comparison to the method 1.

Key words: porphyrin, *m*-CPBA, dimethyldioxirane, PDT, synthesis

Sažetak

Porfirini su organski spojevi koji se mogu koristiti kao fotoosjetljivi spojevi (PS) u fotodinamičkoj terapiji (PDT). PDT uključuje administraciju PS-a u tijelo, gdje se isti lokalizira u tumorskom tkivu te, nakon osvjetljavanja svjetlošću određene valne duljine, uzrokuje odumiranje tumorskog tkiva.

Najčešća metoda sinteze porfirina u našem laboratoriju je modificirana Adler- Longo reakcija koja se relativno jednostavno izvodi, brza je i daje nečistoće koje se mogu pročistiti stupčanom kromatografijom. Zwitterionski oksidirani porfirini imaju malu toksičnost u "mraku", što je bitno za fotodinamičku terapiju (PDT), u usporedbi sa svojim kationskim analozina, *N*-metiliranim piridilporfirinima. Stoga, smo naše istraživanje usmjerili na proučavanje upravo ove grupe porfirinskih PS-ova.

Jedan od zahtjeva za porfirine koji se efikasno primjenjuju u PDT, je njihov visoki unos u ciljane stanice. Stoga, *N*-oksidirani piridilporfirini koji su nam od interesa imaju lipofilnu komponentu, a njihova sinteza u okviru ovog istraživanja uključuje adiciju dugih alkilnih lanaca koji potječu od dodekanoil klorida ili heksadekanoil klorida.

U ovom radu, različite metode *N*-oksidacije piridilporfirina su analizirane kako bi se istražilo koja metoda rezultira najvećim iskorištenjem oksidiranih porfirina s minimalnom prisutnošću nečistoća za potencijalnu primjenu u PDT-u. Četiri analizirane metode uključuju *N*-oksidaciju pomoću: *meta*-kloroperbenzojeve kiseline (*m*- CPBA); dimetildioksirana (DMD); hodikovog peroksida, natrijeva hidroksida i *meta*- kloroperbenzojeve kiseline; ili pomoću octene kiseline. Samo su se metode *N*-oksidacije pomoću m-CPBA i DMD-a pokazale optimalnima, jer su rezultirale visokim iskorištenjem piridilporfirina (65 % za *m*-CPBA i 70 % za DMD) u reakcijama koje su se lagano mogle optimizirati i izvesti. Metoda koja uključuje upotrebu H_2O_2, NaOH i *m*-CPBA, dala je iskorištenje od 3,92% a reakcija s CH_3COOH nije dala produkte.

UV/Vis spektroskopska analiza nije pokazala razliku između spektara simetričnog porfirina oksidiranima upotrebom različitih metoda.

Zaključak istraživanja jest kako metoda 1 (m-CPBA) i 2 (DMD) daju zadovoljavajuća iskorištenja. Iako je oksidacija metodom 2 zahtjevnija zbog potrebe za stalnim podešavanjem pH vrijednosti, daje produkt s manje onečišćenja, te je iskorištenje veće za do 5%.

Ključne riječi: porfirin, *m*-CPBA, dimetildioksiran, PDT, sinteza

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1. Porphyrins in photodynamic therapy

1.1. Historical overview of porphyrin discovery and research

Porphyrins, as naturally occurring compounds, were mentioned for the first time in 460-370 BC, by the famous philosopher and scientist Hippocrates, who had described a case of a young woman, who had symptoms resembling menstrual-related acute porphyria.(1) Following the modern scientific interest in the field, porphyrins are mentioned for the first time during the 19th century, in a medical context, by a researcher named Laennec who had diagnosed a patient with anaemia caused by lead poisoning. In 1841, Scherer isolated the first iron-free hemine compound, and after the discovery of haemoglobin in 1864, porphyrins have been isolated and researched.

First visible spectrum of porphyrins was taken in 1867, alongside red fluorescence, by Thudichum who had called porphyrin: "cruentine" due to the blood-red fluorescence colour of the compound. Fluorescence of the compound was not found interesting at that time, and it will take more than a decade until it was assessed again by Hoppe-Seyler (1879) who had studied chlorophylls at that time and noticed the resemblance between the stated compounds. He had also, in 1871, prepared porphyrins from blood samples and noticed their tetrapyrrolic nature. Hoppe-Seyler named isolated compounds "Hämatoporphyrin", meaning "blood-purple", and used different prefixes on the "porphyrin" name base, which has become an official nomenclature of the researched compounds.

Soret discovered in 1883 that hemins absorb light at the wavelength of 400 nm, which is characteristic trait for all porphyrins. At this time, from 1890s to 1930s, lots of research of porphyrins were connected to physiological effects, and porphyria was first recognised and categorised as a disease by Günther. The structure of porphyrins was at this time still unknown, although protoporphyrin had been successfully synthesized and

coproporphyrin, *meso*-porphyrin and haemoglobin were known to exist and were isolated.

In 1912, W. Küster derived a haemin formula in which he had proposed a structure of hemin, constituted of four pyrrole rings linked with 4 methine bridges on the third position in the pyrrole ring. In the centre of the molecule, connected to the inward-facing nitrogen atoms of pyrroles is a proposed FeCl group.(2) In 1929, Fischer and Zeile had successfully synthesized hemine that confirmed Küster`s proposed porphyrin structure and connections to the structures of heme and chlorophyll were made. Fischer prepared uroporphyrin ester from urine of his patient named Petry, who suffered from porphyria. He received Nobel prize in 1930 for his work on porphyrins.(3) He also found that uroporphyrin has lower photosensitivity in comparison to the hematoporphyrin. Körbler had noticed, in 1931, that there is a significant uptake of porphyrin in the tumour tissue, which marked the era of research of porphyrins as a possible therapy for tumours.

Looking back at the historical timeline of porphyrin research, it is evident that the long initial research period of porphyrin-based compounds was a result of not only the lack in technology, but mostly due to use of different terminology for the compounds.(3) In this regard, Hoppe-Seyler marked a beginning of porphyrin era, after which most significant scientists for the era were Günter, whom after congenital porphyria was named Günter`s disease due to his classification of porphyrias, and Fischer. Fischer contributed greatly to the field by introducing the first porphyrin nomenclature (trivial names and isomer nomenclature) (4) and establishing a research facility for this type of compounds. After him, porphyrin research had developed fast in structure research and nomenclature optimization.

Porphyrin is built of four pyrrolic rings linked by four methine bridges, and it is substituted on one of the twelve possible positions on the inner ring. Eight of those positions are referred to as the *β*- positions and only four are located on the methine bridges, called *meso* positions. Accordingly, *β*-

substituted porphyrins are named *β*-porphyrins and *meso*-substituted porphyrins are called *meso*-porphyrins.

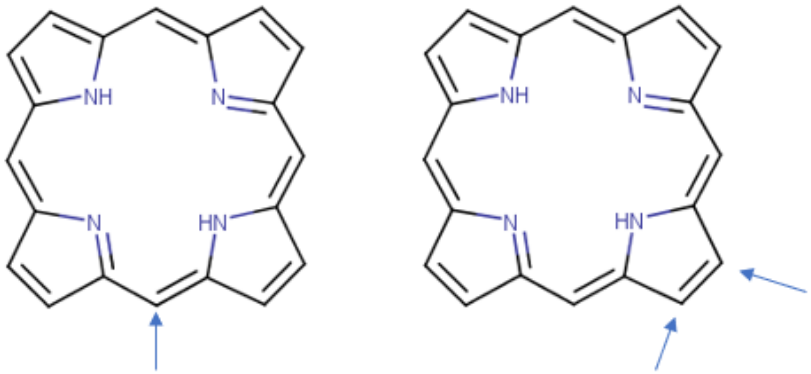


Figure 1: meso-substitution position on porphyrin (left) and β-substitution positions on porphyrin (right)

If the porphyrin is not substituted, its structure is called porphine. Naturally occurring porphyrins are mostly *β*-substituted, but *meso*-porphyrins are easier to produce and are so far used more as potential drugs in PDT.

1.2. Porphyrin synthesis

There are several ways to synthesize porphyrins, and throughout the years, scientists have tried to find new ways of synthesis, that would result in higher yields and the ones that require less purification to isolate clean products. Depending on the structure of porphyrin product we are trying to synthesize, there are different methods we can use to produce them. Two types of occurring porphyrins are *meso*-substituted and *β*-substituted, latter of which are more similar to the natural porphyrin, such as protoporphyrin IX. The focus of the research in this work are *meso*-substituted porphyrins with applications in photodynamic therapy (PDT).

a) Rothemund`s method

In 1936, scientist Paul Rothemund concluded that the best method for porphyrin synthesis is a reaction of a pre-synthesized pyrrole and a substance with aldehyde function (formaldehyde, acetaldehyde etc.) at different temperatures (mostly 90- 95 °C). This refers only to the *meso*-

substitution of aldehyde groups to the α,β,γ and δ positions in a porphyrin ring.(5) The reaction occurs in a presence of nitrogen and in an absence of oxidant. Rothemund method for porphyrin synthesis results in creation of parallel products to the desired porphyrin, mostly chlorins, which results in lower porphyrin yields.(5),(6) Chlorins can be oxidized to related porphyrins afterwards. Yield of porphyrin in this reaction is up to 10%.

b) Adler-Longo method

After Rothemund had developed a method for symmetric porphyrin synthesis, in 1960s, Adler and Longo used the condensation reaction of pyrrole and benzaldehyde in acid (mainly acetic acid or acetic acid with metal salts). The reaction was conducted under reflux.(6) Reaction has two steps.

First step is a self-assembly of the four pyrrole and four aldehyde molecules into porphyrinogen. In this, concentration-dependent process, eight C-C bonds are formed.

Second step includes oxidation of porphyrinogen to porphyrin in the presence of an oxidant. Light and oxygen must be present in the reaction system.(7)

Nowadays, this method is improved by the use of propionic acid as a solvent, and this reaction requires oxygen for the formation of porphyrins, as it oxidises the porphyrinogen intermediate.(8) Yields of porphyrins using this method are up to 20%, thus higher in comparison to the Rothemund`s method.(7)

c) Lindsey's method

About 20 years after the Adler-Longo method, Lyndsey has created a method for porphyrin synthesis under milder conditions than Rothemund and Adler-Longo. It is a reaction in the presence of oxygen-donor and includes pyrrole condensation in dichloromethane (DCM) or chloroform, catalysed by a Lewis acid. Reaction undergoes reflux conditions for one hour

after the addition of an oxidant. This allows for the formation of a tetrapyrrolmethane. Yields in this reaction can be up to 40 % or 50%.(8) Products in the reaction are a mix of different asymmetric porphyrins that are *meso*-substituted.

1.3. *N*-oxidation of pyridylporphyrins

Previous research has found that *N*-oxides of pyridylporphyrins, exhibit lower "dark" toxicity in comparison to the *N*-methylated pyridylporphyrins.(9) However, their synthesis proved to be difficult, especially purification of the products, thus we wanted to study current and investigate new methods for the preparation of this group of compounds.

N-oxidation of pyridylporphyrins includes preparation of (oxido)pyridylporphyrins using different oxidants. Oxidation results in the formation of different products, thus there is a need for the isolation of the wanted products and its purification. Success in the oxidation reactions toward preparation of (oxido)pyridylporphyrins is followed on Thin Layer Chromatography (TLC), and the isolation from the reaction mixture and product purification is carried out by column chromatography, using the appropriate solvent ratios.

In the aforementioned oxidation reactions, the addition of an excess oxidant can lead to the degradation of the pyridylporphyrin, thus we have to take this into account when considering existing and new methods, and their optimisation. Furthermore, (oxido)pyridylporphyrins are more soluble than their parent porphyrins in polar solvents.(10)

In this work, *N*-oxidations were carried out using following reagents:

- *meta*-chloroperoxybenzoic acid (*m*-CPBA)
- Oxone
- Hydrogen peroxide
- *m*-CPBA and hydrogen peroxide

a) *N*-oxidation using *meta*-chloroperoxybenzoic acid

m-CPBA is an abbreviation and a trivial name for the compound 3-chloroperbenzoic acid, whose molecular mass is 156,57 g/mol. It belongs to the class of organic compounds named halobenzoic acids, due to the position of the chloride on the aromatic ring (*Figure 2*). It is a white solid soluble in aqueous solutions and in some organic solvents, such as DCM.

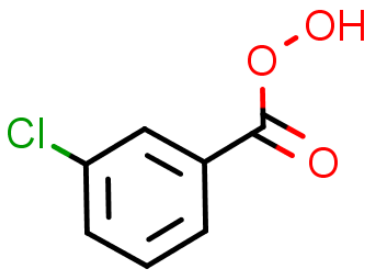


Figure 2: Structure of m-CPBA

In 1999. Posakony *et al*. used *m*-CPBA for *N*-oxidation of porphyrins containing one to four 4-pyridyl groups. Mixed aldehyde condensation was used to synthesize *meso*-substituted porphyrin, and then *N*-oxidation was performed using *m*-CPBA to produce five (oxido)pyridylporphyrin compounds and seven porphyrin-*N*-oxides. *m*-CPBA was used in a solution of CH_2Cl_2 and CH_3OH at room temperature, its aliquot being 1-2 equivalents of porphyrin. This resulted in formation of (oxido)pyridylporphyrin, whilst the addition of several aliquots resulted in the formation of porphyrin-*N*-oxides. Using *m*-CPBA as an oxidant included the use of triethylamine to quench the reaction to prevent over-oxidation. After the reaction completion, some by-products appeared, such as *meta*-chlorobenzoic acid which had to be removed from the reaction mixture with the extraction using NaOH solution. Triethylamine was absorbed on the silica gel of the column, straight after the *N*-oxidation, to remove it from the mixture and to stop its further oxidation. Addition of *m*-CPBA after the completion of the reaction led to the degradation of porphyrin.(10)

In the same paper, the authors state that using Lindsey method of porphyrin synthesis had given impure products, resulting in the formation of unknown compounds, resulting in lower porphyrin yields.

Oxidopyridyl group is, in comparison with phenyl group, less susceptible to oxidative degradation, which means that (oxido)pyridylporphyrins are stable enough to be isolated. *m*-CPBA was shown to provide stable products and this method is reproducible on large number of compounds. Expected values of ^{1}H NMR of (oxido)pyridylporphyrins created using *m*-CPBA include a complex *β*-hydrogens region, caused by the proximity of different *meso*-substituents, which appears as broad area of one or more peaks in the spectrum. The hydrogens from within the porphyrin ring appear as singlets at δ ~-2,85. (10)

b) *N*-oxidation using dimethyldioxirane

Dimethyldioxirane (DMD) was first synthesized in 1985. by Robert W. Murray and Ramasubbu Jeyaraman who defined dioxiranes as the smallest cyclic peroxide systems.(11) Caroate-acetone system was used to produce DMD *in situ* with the yield up to 90% at room temperature. The caroate addressed here is peroxymonosulfate, potassium caroate or trivially-Oxone. DMD is yellow in a solution and they have determined that the maximum absorption peak of DMD is at λ= 335 nm. Proton NMR analysis showed single peak at δ = 1,65, and ^{13}C NMR analysis shows peaks at 22,72 (quartet) and 214,4 ppm (singlet). They have also noticed stereospecificity in reactions they have conducted.(11)

In 1988. Curci and Edwards have created Methyl(trifluoromethyl)dioxirane, a form of dioxirane compound up to 1000 times more reactive than DMD, by the reaction of caroate and acetone. Curci also discovered that fluorinated ketones have an impact on the stability and reactivity of the synthesized dioxirane.(12) It is also recognised that the stability of the DMD is not high, therefore more of the reactants are used to produce it; however, the cost of these compounds is low.(12) After this, in 1992. Murray

developed a new method for DMD synthesis from cyclic compounds, such as cyclohexane.

DMD is often used in the reactions of epoxidation (13), but it is also an effective oxidising agent for the oxidation of porphyrins.(14) DMD exhibits stereoselectivity and chemoselectivity and is desirable oxidant for the creation of oxidised porphyrin compounds. The preparation of DMD *in situ* is achieved in the reaction of Oxone with ketone under hydrolytic conditions.(15) DMD undergoes the conversion to a solvent during the reaction of its preparation.(16) Preparing DMD prior to its use in reactions allows for its use in non-aqueous reactions. However, for the purpose of this research, we opted for *in situ* generation of DMD in a non-miscible one pot (CH_2Cl_2 and H_2O) reaction system, after which the purification of the product was conducted. In such preparation procedure, the pH needs to be between 7,0 and 8,0 to assure the formation of (oxido)pyridylporphyrin, and buffers such as sodium bicarbonate or phosphate buffer are mostly used in a solution with excess ketone. Reaction lasts anywhere between 0,5 and 72 hours, and many variables effect the success of the reaction. Besides this method of synthesis, it is possible to derive DMD in a system using benzene.(17) Isolation method for the DMD is used only if the substrate or the product are unstable in biphasic conditions, which was not the case in this research. The excess of Oxone and the excess of ketone do not affect the rate of conversion to DMD, neither increase the product yield. The mechanism of dioxirane formation involves the nucleophilic attack of Oxone at the carbonyl carbon, resulting in the loss of potassium hydrogen sulphate.(17)

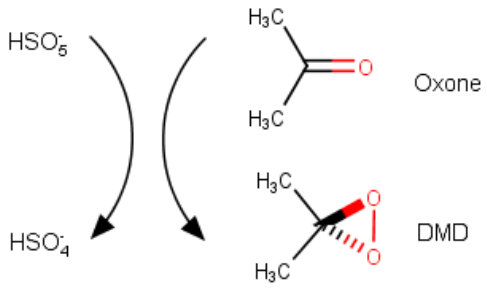


Figure 3: Conversion of Oxone to DMD

Paredes *et al*. have compared diastereoselectivity between products obtained with the use of DMD and *m*-CPBA in 1997 and have concluded that the difference is not very high between the two.(18) However, DMD is more sensitive on the steric interactions and its regiospecificity is determined by the electronic effects in the molecule subjected to oxidation, whilst the latter does not influence the oxidation using *m*-CPBA.

Conditions that effect production of DMD are: pH, time of oxidant addition, ketone type and lipophilicity and steric interactions. (17) Temperature is not considered to have an impact on the production of oxidised compounds nowadays, although some previous research stated that it has an impact.(17)

In literature, there are divided opinions on the temperature impact on the success of the reaction. Some of the new sources (15) have provided a detailed research into this topic, where it was concluded that the reactions at room temperature give the same yields of products as do the reactions at 0 °C. However, in the older research and sometimes, in the new processes for porphyrin oxidations, it is advised to conduct the reactions at 0 °C throughout the time of the oxidant addition to the mixture.(17) In our research, no metalloporphyrins were used, so it was concluded that the temperature of the reaction will be kept at room temperature, since only metalloporphyrins undergo thermal decomposition in solutions in the presence of dioxiranes.(19)

1.4. Photochemical properties of porphyrins

Photodynamic therapy (PDT) is a non-invasive form of photosensitized reactions that have a positive effect on human health. Ancient Egyptians, Chinese and Indians were known to use plants orally for the treatment of vitiligo and psoriasis in direct sunlight. Scientist Niels Finsen used light therapy (red part of the spectrum) to limit spreading of smallpox pustules in patients, during the end of 18th century. For his research, he was awarded the Nobel prize in 1903.(20) Many consider Oscar Raab to be the first to study PDT, having discovered that acridine dyes are the cause of death of mono cellular organisms, such as *Paramecium*. Scientists R. L. Lipson and S. Schwartz who worked in 1960 at the Mayo Clinic are the first who have discovered that preparations containing hematoporphyrin's cause fluorescence in tumour tissue of the treated patients.(21)

Modern understanding of PDT involves three components: photosensitizing agent, light of the specific wavelength and molecular oxygen. The photochemistry behind the PDT can be explained with the use of Jablonski diagram (*Figure 4*), which serves as a visual representation of the PDT principle.

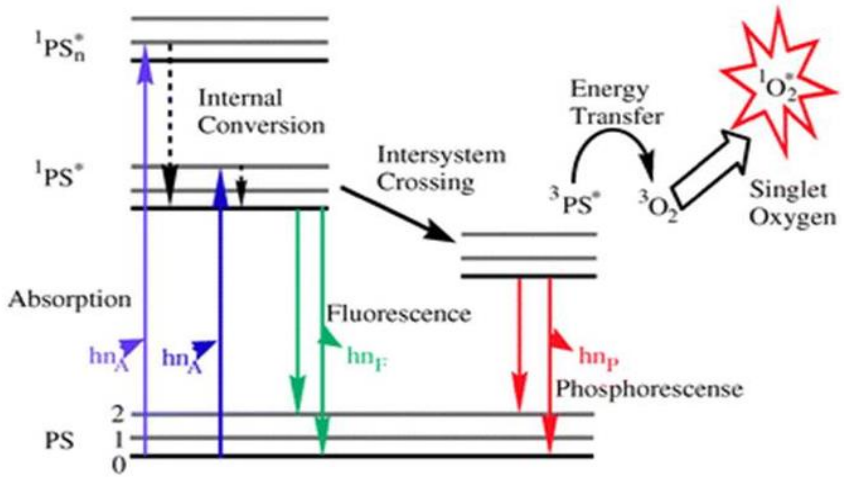


Figure 4: Scheme of modified Jablonski diagram(22)

Chromophore (an atom or a molecular group that gives colour to the compound), is illuminated with the light of a specific wavelength that excites

chromophore to one of the excited singlet states. The excited chromophore may relax to the singlet ground state by the emission of photon in a process called fluorescence, or it can convert to the excited triplet state in a process called intersystem crossing. Relaxation from the triplet state back to the ground state is named phosphorescence, and it is caused by the emission of photon from the excited triplet state of the chromophore. The loss of energy, except from phosphorescence, may happen if the excited chromophore transfers its energy to another molecule. In PDT of porphyrins, that molecule is triplet ground-state oxygen ($^3O_2$) that converts to the singlet oxygen ($^1O_2$).(23) Ian J. Macdonald and Thomas J. Dougherty state that: "The energy required for the triplet to singlet transition in oxygen is 22 kcal/mol, which corresponds to a wavelength of 1274 nm (infrared light). Thus relatively low energy is needed to produce singlet oxygen. Photochemical reactions of this type are known as Type-II photoreactions and are characterized by dependence on the oxygen concentration (O_2)".

Type I photoreactions involve a reaction between a triplet-state photosensitizer with superoxide radicals to produce superoxide anions. Type I and type II photoreactions are depicted in the *Figure 5*. They can then react with other molecules to create hydroxide radicals.(23) Type I sensitizers are subject to photoinduced electron transfer, upon which $O^{2\bullet-}$ and $HO^{-\bullet}$ are being produced. In comparison to that, type II reactions are characterized as energy-transfer process to oxygen, causing the formation of $^1O_2$. Both types are oxygen-dependent reactions.(24)

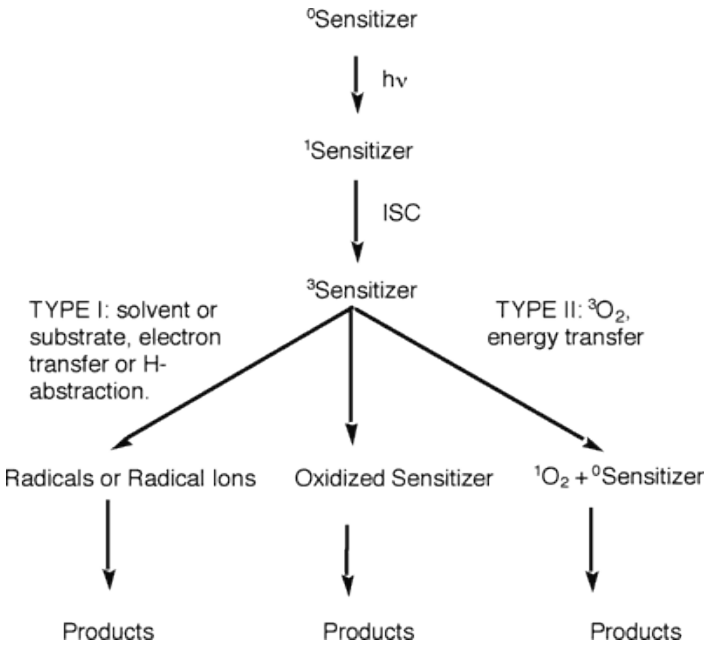


Figure 5: Type I and type II photoreactions (25)

Singlet oxygen (product of the type II reaction) that forms in PDT is formed by the inversion of the electron's spin on the outer electron of the molecule. In the magnetic field, there are three different configurations of electrons possible, which are indistinguishable when magnetic field is not present. Those are:

- Electronic configuration with both spins oriented downwards
- Electronic configuration with both spins oriented upwards
- Electronic configuration with one spin oriented upwards and the other downwards

Due to these three possibilities, oxygen is a triplet in its ground state. Oxygen is very reactive due to two electrons pairing into π^*_{2p} antibonding orbitals in the same spin, which is not in accordance to the Hund`s rule and the Pauli`s exclusion principle, and it is destabilising the molecule. Lifetime of this oxygen is quite long, up to 10-100 μs in organic solvents.(23)

2. Photosensitizers

Photosensitizers (PS) used in PDT should have as much as possible absorption in the red field of electromagnetic spectrum and have the possibility of producing singlet oxygen. Red light allows for deeper penetration into the tissue thus having better healing effect in tumour application. Most used PDT photosensitizers are mostly planar tetrapyrroles: porphyrin, bacteriochlorin and chlorin. Porphyrins mostly absorb visible light at approximately 400-450 nm, visible on the spectroscopy chromatogram, and known as Soret band. This wavelength, however, since it is in the blue area of visible light on the electromagnetic spectrum, is not so relevant for PDT applications, as this type of light does not penetrate the tissue as deep as the red light, thus not achieving the desirable effect. Aside from Soret band, there are four more bands characteristic for porphyrins, ranging from 600-800 nm on chromatogram, known as Q-bands. They are in the red part of the spectra and are more relevant for most of the PDT applications. These compounds successfully create singlet oxygen in line with the singlet oxygen quantum yield, marked with the symbol φ. This yield determines the number of oxygen molecules formed per photon upon the absorption of energy.(23)

The properties of PSs differ significantly between solvents and biological systems, making them harder to study. Ideal PS exhibits no effect until the application of light, i.e. they should not express the so called "dark" toxicity. PSs do not bind to the receptors within the body, but there is a link between lipophilicity and drug uptake into serum by binding to low density lipoprotein (LDL) serum constituents. LDL is increased in cancer tissue due to growth of cancer`s vasculature. From the circulation, PSs move into cells. After the irradiation of the tumour tissue, immunological response occurs in the accumulation of platelets, macrophages, and the release of prostaglandins and cytokines, which cause the damage to the tumour vasculature. Apoptosis is triggered in tumour cells, but it is not achieved fully only by PDT. Besides apoptosis, necrosis may occur in tumour tissue. Duration and

intensity of irradiation as well as the amount of PS used have impact on the effectiveness of tumour PDT.(26)

Apart from LDL, some researchers believe that low pH value and the presence of macrophages can also contribute to the PS localisation in tumour tissue.(27) With the increase in porphyrin hydrosolubility, their accumulation in tumour tissue grows. On the other side, if lipophilic carriers are used for PSs transport at the temperature of 37 °C, binding to LDL is the primary path of their tissue distribution. Tumour cells possess a high number of LDL receptors on their surface, making them even more susceptible to the binding of PS. By endocytosis, PSs pass through the membrane and bind to the mitochondrial membrane, the Golgi apparatus and the endoplasmic reticulum. It is debatable which transporters to the tumour tissue are better, for there are examples of good transporters that do not bind to LDL (*meso*-tetraphenylporphin tetrasulfonate is one example) and of some poor transporters, such as hematoporphyrin. Additional factor to take into account is the availability of oxygen in the tumour tissues, which can drop significantly *in vivo* if PS are applied in full dose and irradiated. Photobleaching helps by limiting the initial response of the applied PS, which causes PS to activate through a certain period, thus helping in maintaining sufficient oxygen levels in tumour tissue. Oxygen levels can be controlled by modifying the duration and repetition of light during therapy, for example 15-20 seconds with pauses between the application. (27)

PS must exhibit properties such as: having an absorption peak in the red area of electromagnetic spectra (>650 nm), having high quantum yield for the formation of triplet state and singlet oxygen production, low toxicity in the dark, selectivity for tumour tissues, fast reactivity, fast and easy elimination from the body, easy and affordable synthesis, optimal pharmacokinetics, low to no side-effects for the patient, long-term beneficiary effect for the patient, or easy administration.

- First generation PSs

First generation photosensitizers include only porphyrin compounds, amongst which Photofrin IX and Hematoporphyrin. These compounds were created for the purpose of suboptimal tissue penetration and prolonged skin photosensitization, mostly for the detection of cancer due to their fluorescent nature.(28) These compounds were not selective enough, so their ingestion caused displacement in different tissues and unwanted photoactivation wherever PS was absorbed. The problem which occurred with Photofrin was that it had to be administered in large dose to be effective in tumour therapy, and it had an exceptionally long half-life (up to 452 hours), which caused undesirable photosensitization in patients amongst other side-effects. Photofrin is still used today as a golden standard, despite its many flaws, such as low absorption in the red spectrum and shallow tissue penetration (1,5 mm).(28) Mechanism of PDT action of these PSs includes the formation of ROS (mostly singlet oxygen) that damage tumour cells and tumour tissue vasculature.

- Second generation PSs

Second generation PSs were created to try and resolve the deficiencies of the first generation of PSs. Bacteriochlorins and chlorins are such compound types. PS Tookad is a bacteriochlorin and it has better properties than Photofrin. It penetrates the tissue moderately (up to 4,0 mm), clears from the body quicker and has a much shorter half-life (up to 20 minutes).(28) These compounds target tumour vasculature causing hypoxia, necrosis and cell death. Foscan, a chlorin compound, has similar properties to Tookad, as well as Protoporphyrin IX. Foscan, in comparison to Tookad, had longer half-life and could cause moderate skin problems weeks after its administration. Second generation PSs could not completely resolve the problems of the first generation, but only limit certain parameters, such as tissue penetration, clearance, higher generation of ROS, length of half-life, dark-toxicity, higher yields etc. Second generation PSs include both porphyrin and non- porphyrin compounds.

- Third generation PSs

Specificity of a third generation PSs is targeted delivery of the second generation of PSs to the tumour tissues in carrier molecules such as monoclonal antibodies, polymer bodies and liposomes. The goal of the third generation is to make PSs more hydrophilic at physiological pH for a more efficient delivery.

Due to occurrence of specific antigens on the surface of tumour cells, antibodies can detect them and bind to their receptors on the surface. In this way, healthy tissues are protected from the targeted action of PS. Polyethyleneglycol (PEG) nanocarriers are used widely, and PEG liposomes are being developed. Photofrin is an example of a third generation PS that was used incorporated into polyacrylamide nanoparticles and applied in the therapy of a rat brain tumour. Encapsulating photofrin caused better efficiency in tumour therapy which significantly reduced the time of action of the drug. Besides PEG, phthalocyanines and amine-functionalized polyacrylamide are used as carriers.(28) Third generation PSs include both porphyrin and non-porphyrin compounds, and they are still being developed today. Although they seem very promising in therapy, there is a possibility that closing the PS in a capsule may lead to the lower level of produced ROS.(29)

3. Research objective

The purpose of this research is to find, implement and optimize the reaction of *N*-oxidation of pyridylporphyrins.

Four methods will be tested and compared in terms of product yields and ease of purification. These methods will use following oxidants:

- *meta*-chloroperoxybenzoic acid
- dimethyldioxirane
- *meta*-chloroperoxybenzoic acid (elution with NaOH)
- H_2O_2 and acetic acid

All aforementioned methods will be tested on symmetric 5,10,15,20-tetrakis(3-pyridyl)porphyrin to evaluate the success of the reaction (percentage of yield), whereas only the most successful method, or the one most similar to *m*-CPBA set as the standard, will be further tested on porphyrins substituted with dodecanoyl chloride and palmitoyl chloride.

Asymmetric pyridylporphyrins with long alkyl chains are more effective in photodynamic therapy, as they can penetrate membranes more successfully than symmetric hydrophilic porphyrin, thus it is our aim to optimise their synthesis and preparation.

4. Materials and methods

4.1. General information

Reagents and chemical substances used in this work are commercially available, and originate from manufacturers: Sigma-Aldrich, VWR Chemicals, Acros Chemicals and Carlo Erba Reagents. For Thin Layer Chromatography (TLC), aluminium plates with thin layer of silica gel were used (Macherey-Nagel, 0.20 mm silica gel 60 Å with fluorescent indicator UV254), alongside UV lamp at wavelengths of 254 and 365 nm. Column chromatography was performed in columns of different diameters filled with silica gel from Macherey-Nagel (Silica 60 Å, 0.04-0.06). Elution was carried out with dichloromethane and methanol solution in different ratios.

Ultra-violet and visible (UV/Vis) spectrometry was performed on the Agilent Cary 60 UV-Vis Spectrophotometer in the visible spectre, between 350 nm and 750 nm. The dilutions of the compounds were prepared in the following manner, from the 10 μM solution of porphyrin:

a) First vial: 10 μM solution of porphyrin
b) Second vial: 8 μM solution of porphyrin
c) Third vial: 6 μM solution of porphyrin
d) Fourth vial: 4 μM solution of porphyrin
e) Fifth vial: 2 μM solution of porphyrin
f) Sixth vial: 1 μM solution of porphyrin
g) Seventh vial: BLANK, CH_2Cl_2

Quartz cuvette was used for the analysis of the porphyrin samples.

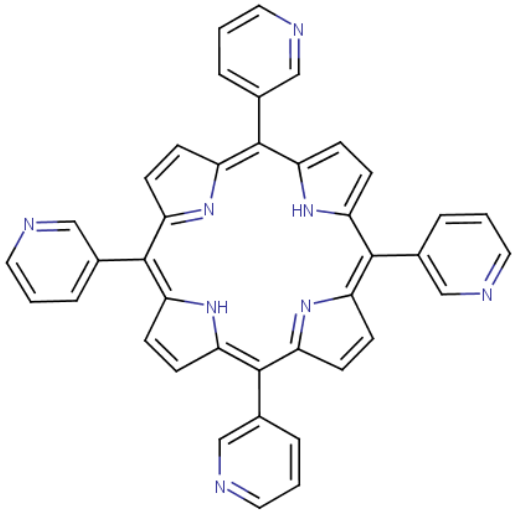
Agilent Cary Eclipse Fluorescence Spectrophotometer was used for measuring the fluorescence of the synthesized porphyrins, at the maximum wavelength of the Soret band of the mentioned porphyrin. Range in which the fluorescence emission was recorded was between 500 nm and 800 nm, for the concentration of 1μM porphyrin.

Nuclear magnetic resonance (NMR) analysis was conducted at the Chemistry Department of the University of Zagreb, Faculty of science; in the Laboratory for NMR spectroscopy, with Bruker Avance III HD 400 MHz. Solvents used for the preparation of samples were deuterated chloroform and deuterated methanol. Analysis of the NMR spectres was performed using the program MestReNova 14.0.1.

Computational chemistry involved the use of ChemAxon`s Marvin Sketch programme. 3D projections of the compounds were done in Avogadro.

4.2. Porphyrin synthesis

a) 5,10,15,20-tetrakis(3-pyridyl)porphyrin



Molecular weight: 618,704 g/ mol

Molecular formula: $C_{40}H_{26}N_8$

Polar surface area: 108,92

5,10,15,20-tetrakis(3-pyridyl)porphyrin (porphyrin **1**) is prepared by dissolving 4-acetaminobenzaldehyde (0,90 g; 5,52 mmol; 1 eq.) and 3-pyridinecarboxaldehyde (1,78 g; 0,02 mol; 3 eq.) in propionic acid (70 ml). The reaction is conducted at two temperatures, whilst the reaction mixture is being gradually heated:

- When the temperature reaches 20°C - 30°C, distilled pyrrole (1,54 ml; 0.02 mol; 4 eq.) is added to the mixture slowly and in small amounts within 10 minutes

- When the temperature reaches 90°C, it is maintained for 45 minutes approximately, after which the reaction is stopped.

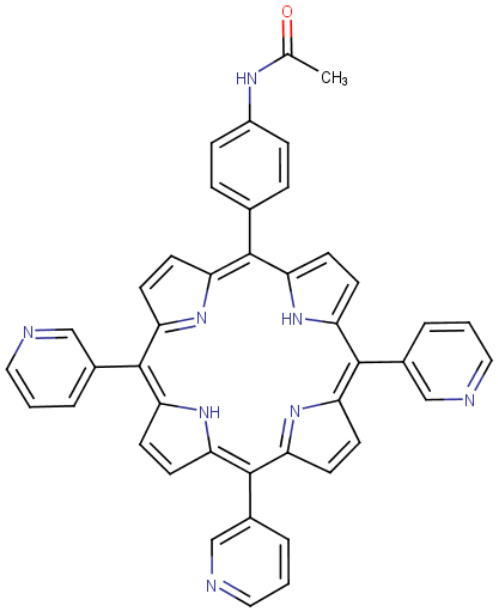
Light and oxygen are necessary during this process to allow the oxidation of the porphyrinogen to porphyrin.

After the confirmation of product formation on TLC, solvent is removed *in vacuo*. Two column chromatography procedures are conducted subsequently for product purification. On the column, porphyrin **1** is separated as dark purple, crystalline solid (0,182 g; 5,12%).

UV/ Vis spectroscopy analysis showed the presence of Soret band at 418 nm, with Q bands at next λ_{max}/ nm: 514, 549, 589, 645. Extinction coefficient was calculated: ε= 0,31. Fluorescence measured at the excitation wavelength of the Soret band gave two peaks at λ_{max}/nm= 650, 715.

^{1}H NMR was measured in solvent CD_3Cl and next peaks were observed: δ/ppm 9,49 (d; *J* = 2,2 Hz; 3H; Py-2-H); 9,10 (dd; *J* = 4.9; 1.7 Hz; 3H; Py-4-H); 8,89 (s; 8H; β-H); 8,56 (d, *J* = 7.7 Hz; 3H; Py-6-H); 7,81 (dd, *J* = 7,8; 4,9 Hz; 3H; Py-5-H); -2,81 (s, 2H, pyrrole NH).

b) 5-(4-acetamydophenyl)-10,15,20-tris(3-pyridyl)porphyrin



Molecular weight: 674,768 g/ mol

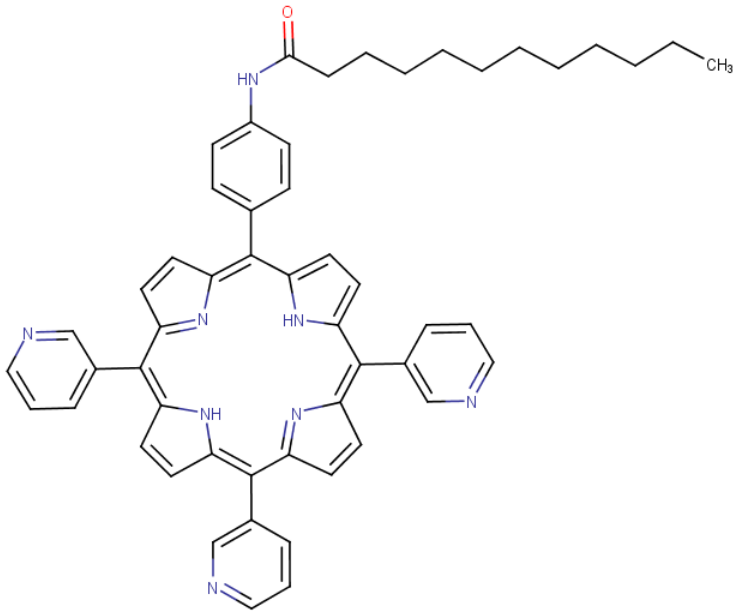
Molecular formula: $C_{43}H_{30}N_{8O}$

Polar surface area: 125,13

5-(4-acetamydophenyl)-10,15,20-tris(3-pyridyl)porphyrin (porphyrin **2**) is separated from the same column as porphyrin **1**, as a second fraction. This fraction is also isolated as a dark purple crystalline matter (0,131 g; 3,74%).

UV/ Vis spectroscopy analysis shows the presence of Soret band at 418 nm, with Q bands at next λ_{max}/ nm: 515, 550, 590, 647. Extinction coefficient was calculated: ε= -0,29. Fluorescence measured at the excitation wavelength of the Soret band gave two peaks at λ_{max}/nm= 651, 717.

c) 5-(4-dodecanamidophenyl)-10,15,20-tris(3-pyridyl)porphyrin



Molecular weight: 815,038 g/ mol

Molecular formula: $C_{53}H_{50}N_{8O}$

Polar surface area: 125,13

5-(4-dodecanamidophenyl)-10,15,20-tris(3-pyridyl)porphyrin (porphyrin **3**) is synthesized by adding dodecanoyl chloride (0,083 g; 0,377 mmol; 5 eq.) to porphyrin **2** (0,051 g; 0,078 mmol; 1 eq.) in dichloromethane at 0 °C through 45 minutes in a single round flask. The reaction completion was followed by TLC. Solvent for TLC was CH_2Cl_2: CH_3OH= 9:1. The oxidised

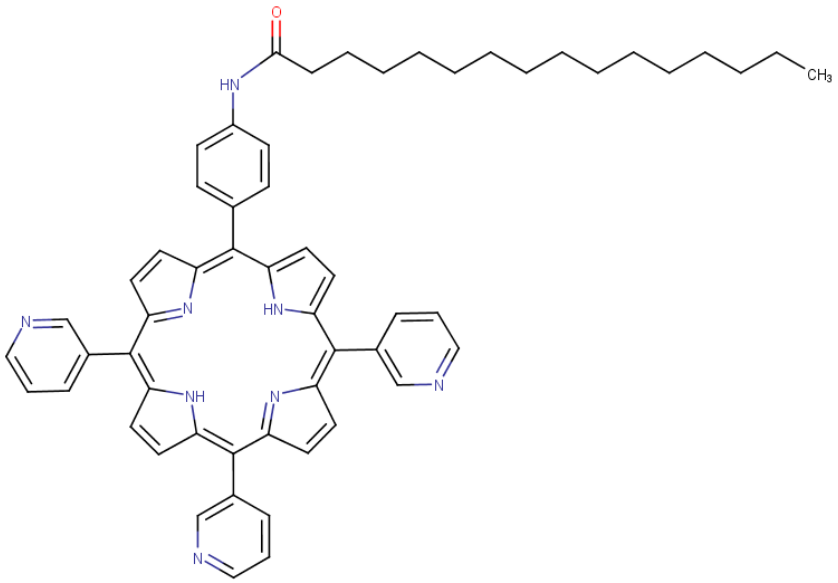
compound is purified by extraction with water (3 times) and Na_2SO_4, filtered and the solvent is removed *in vacuo*. Column chromatography is used for purification of the synthesized porphyrin **3**, obtained from the column as dark purple fraction (0,073 g; 87,95%).

UV/ Vis spectroscopy analysis shows the presence of Soret band at 419 nm, with Q bands at next λ_{max}/ nm: 515, 551, 591, 646. Extinction coefficient was calculated: ε= 0,38. Fluorescence measured at the excitation wavelength of the Soret band gave two peaks at λ_{max}/nm= 649, 715.

^1H NMR was measured in solvent CD_3Cl and next peaks were observed:

δ/ppm 9,49 (t; *J*= 2,5 Hz; 3H; Py-2-H); 9,09 (d; *J*= 3,7 Hz; 3H; Py-4-H); 8,99- 8,80 (m; 8H; *β*-H); 8,55 (d, *J*= 5,76 Hz, 3H; Py-6-H); 8,20 (d; *J*= 6,6 Hz; 2H; Ar-2,6-H); 7,96 (bd, 2H; Ar-3,5-H); 7,79 (t; *J*=5,13 Hz; 3H; Py-5-H); 7,74 (s; 1H; amide N-H); 2,57 (t; *J* = 5,64 Hz; 2H; C^2H_2); 1,91 (t; *J*= 3,02 Hz; 2H; R-C^3H_2); 1,49 – 1,29 (m; 18H; R-H: C^4H_2, C^5H_2, C^6H_2, C^7H_2, C^8H_2, C^9H_2, $C^{10}H_2$, $C^{11}H_2$); 0,92 (t; *J*= 5,19 Hz; 3H; R-C-H_3); -2,78 (s; 2H; pyrrole N-H)

d) 5-(4-hexadecanamidophenyl)-10,15,20-tris(3-pyridyl)porphyrin



Molecular weight: 871,146 g/ mol

Molecular formula: $C_{57}H_{58}N_{8O}$

Polar surface area: 125,13

5-(4-hexadecanamidophenyl)-10,15,20-tris(3-pyridyl)porphyrin (porphyrin **4**) is synthesized in a reaction of hexadecanoyl chloride (palmitoyl chloride) (0,107 g; 0,388 mmol; 5 eq.) and porphyrin **2** (0,052 g; 0,078 mmol; 1 eq.) in dry CH_2Cl_2 at 0 °C through 45 minutes in a single round flask. The reaction completion was followed by TLC. Solvent for TLC was CH_2Cl_2: CH_3OH= 9:1. The oxidised compound was extracted with water (3 times) and dried over Na_2SO_4. Drying agent was filtered and the solvent was removed *in vacuo*. Column chromatography was used for purification of the synthesized porphyrin **4**, which was obtained from the column as dark purple fraction (0,047 g; 90,38%).

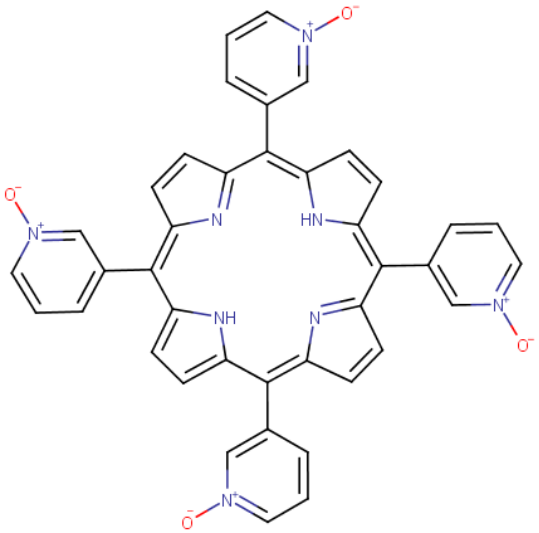
UV/ Vis spectroscopy analysis showed the presence of Soret band at 419 nm, with Q bands at next λ_{max}/ nm: 517, 554, 593, 648. Extinction coefficient was calculated: ε= 0,40. Fluorescence measured at the

excitation wavelength of the Soret band gave two peaks at λ_{max}/nm= 658, 722.

^{1}H NMR was measured in solvent CD_3Cl and next peaks were observed:

δ/ppm 9,49 (s; 3H; Py-2-H); 9,09 (d; *J*=3,75 Hz; 3H; Py-4-H); 8,98- 8,83 (m; 8H; β-H); 8,55 (d, *J*= 5,67 Hz, 3H; Py-6-H); 8,20 (d; *J*= 6,6 Hz; 2H; Ar-2,6-H); 7,964 (bd, 2H; Ar-3,5-H); 7,79 (t; *J*= 2,90 Hz; 3H; Py-5-H); 7,70 (s; 1H; amide N-H); 2,57 (t; *J* = 5,64 Hz; 2H; C^2H_2); 1,91 (t; *J*= 5,78 Hz; 2H; R-C^3H_2); 1,42 – 1,28 (m; 24H; R-H: C^4H_2, C^5H_2, C^6H_2, C^7H_2, C^8H_2, C^9H_2, $C^{10}H_2$, $C^{11}H_2$, $C^{12}H_2$, $C^{13}H_2$, $C^{14}H_2$, $C^{15}H_2$); 0,90 (t; *J*= 5,19 Hz; 3H; R-C-H_3); -2,78 (s; 2H; pyrrole N-H).

e) 5,10,15,20-tetrakis(1-oxydopyrid-3-yl)porphyrin



Molecular weight: 682,702 g/ mol

Molecular formula: $C_{40}H_{26}N_4[N^+]_4[O^-]_4$

Polar surface area: 165,12

Method 1:

Porphyrin **1** is oxidised into 5,10,15,20-tetrakis(1-oxydopyrid-3-yl)porphyrin (porphyrin **5**) using *m*-CPBA in a round flask, on room temperature, under nitrogen atmosphere. Porphyrin **1** (0,080 g; 0,119

mmol; 1 eq.) was dissolved in dry CH_2Cl_2 and *m*-CPBA (0,615 g; 3,567 mmol; 30 eq.) was added slowly over 45 minutes. The oxidation reaction was followed on TLC. Upon completed oxidation, reaction was quenched by triethylamine (1,5 ml). Solvent was removed *in vacuo*, and the reaction mixture was purified on the column using CH_2Cl_2 and CH_3OH in ratio 20:1, with the addition of small amount of triethylamine. Porphyrin **5** was obtained as purple compound (0,052 g; 65,0 %).

Method 2:

We undertook a few steps in the oxidation reaction of porphyrin using DMD. In a round flask, under room temperature, porphyrin **1** (0,020 g; 0,032 mmol; 1 eq.) was dissolved in solvent CH_2Cl_2: CH_3OH= 1:1 (10 ml) and stirred on a magnetic stirrer. Oxone (0,180 g; 0,650 mmol; 20 eq.) was dissolved separately in 0,01 M NaOH (1 ml). Acetone (3 ml) was added in the flask in excess and the pH in the flask was set to approximately 7,0 using 0,01M NaOH (the flask was set under nitrogen conditions). Oxone was injected slowly into the flask over the course of 30 minutes and the pH value was reset periodically.

After the addition of Oxone, the oxidation reaction was followed on TLC. To purify the product from the watery layer, filtration is conducted, followed by the extraction with water (3x) and drying the organic extract over Na_2SO_4. The dried solvent was removed *in vacuo*. To purify the product additionally, column chromatography was performed using the solvent mixture CH_2Cl_2:CH_3OH = 20:1 for elution. Porphyrin **5** (0,014 g; 70,0%) was obtained as a purple solid.

Method 3:

H_2O_2 was used in the reaction of oxidation of porphyrin **1** as an additional oxidant. In a round flask, under nitrogen conditions, porphyrin **1** (0,051 g;

0,082 mmol; 1 eq.) is dissolved in the solvent (dry CH_2Cl_2), and *m*-CPBA (0,423 g; 2,450 mmol; 30 eq.) was added to the reaction mixture within 45 minutes. 2 ml of H_2O_2 were sufficient for full oxidation of 5,10,15,20-tetrakis(3-pyridyl)porphyrin in 45 min. TLC was used to confirm that the reaction was completed. To remove the excess of H_2O_2, extraction was performed using 0,01 M NaOH (1x), H_2O (2x) and the product was dried with Na_2SO_4. The excess of solvent was removed *in vacuo*. Column chromatography was performed twice with the ratio of solvents CH_2Cl_2: CH_3OH= 15:1 to purify the porphyrin **5** (0,002 g; 3,92%) additionally.

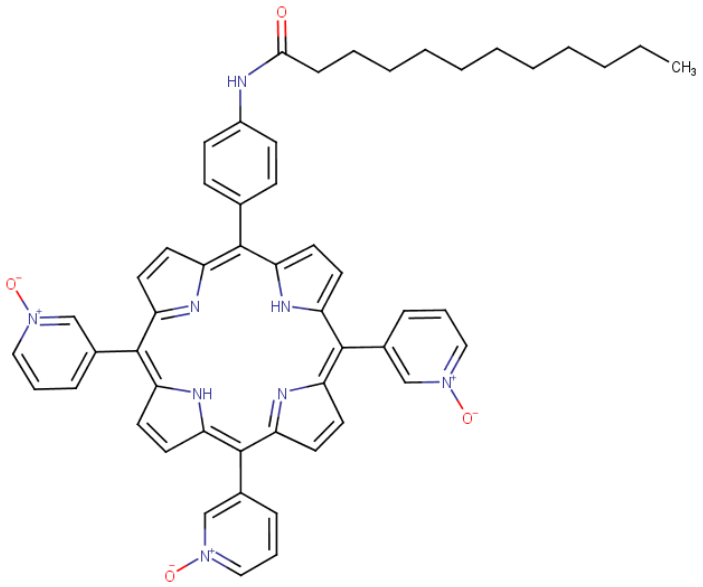
Method 4:

The reaction was performed in a round flask, wherein porphyrin **1** (0,012 g; 0,019 mmol; 1 eq.) was dissolved in CH_3COOH (10 ml). To this mixture, H_2O_2 (0,013 g; 0,384 mmol; 20 eq.) was added in small amounts through 45 minutes. TLC was used to check the progress of the reaction. No oxidation occurred in the flask and no product was isolated.

UV/ Vis spectroscopy analysis showed the presence of Soret band at 418 nm, with Q bands at next λ_{max}/ nm: 513, 547, 589, 647. Extinction coefficient was calculated: ε= 0,32. Fluorescence measured at the excitation wavelength of the Soret band gave two peaks at λ_{max}/nm= 648, 712.

^{1}H NMR was measured in solvent CD_3Cl and next peaks were observed: δ/ppm 9,02 (s; 4H; Py-2-H); 8,92 (bs; 8H; β-H); 8,70 (d; *J*= 4,02 Hz; 4H; Py-4-H); 8,20 (d; 4H; Py-6-H); 7,84 (t; *J*= 4,86 Hz; 4H; Py-5-H)

f) 5-(4-dodecanamidophenyl)-10,15,20-tris(1-oxopyrid-3-yl)porphyrin



Molecular weight: 863,035 g/ mol

Molecular formula: $C_{53}H_{50}N_5[N^+]_3O[O^-]_3$

Polar surface area: 167,28

Method 1:

Porphyrin **3** was oxidised into 5-(4-dodecanamidophenyl)-10,15,20-tris(1-oxopyrid-3-yl)porphyrin (porphyrin **6**) using *m*-CPBA in a round flask, at room temperature, under nitrogen atmosphere. Porphyrin **3** (0,034 g; 0,042 mmol; 1 eq.) was dissolved in dry CH_2Cl_2 and *m*-CPBA (0,144 g; 0,836 mmol; 20 eq.) was added slowly over 45 minutes. The success of the oxidation was tracked on TLC. Upon completion, the reaction was quenched using triethylamine (1 ml). Solvent was removed *in vacuo*, and the compound was then isolated by purification through the column using CH_2Cl_2 and CH_3OH in ratio 20:1, with the addition of small amount of triethylamine. Porphyrin **6** was obtained as purple solid (0,024 g; 70,59 %).

Method 2:

We undertook following steps in porphyrin *N*-oxidation using DMD: in a single round flask, under room temperature, porphyrin **3** (0,011 g; 0,014 mmol; 1 eq.) was dissolved in solvent CH_2Cl_2: CH_3OH= 1:1 (10 ml) and stirred on a magnetic stirrer; after which Oxone (0,129 g; 0,421 mmol; 25 eq.) was dissolved separately in 0,01 M NaOH (1 ml). Acetone (3 ml) is added in the flask in excess and the pH in the flask was set to approximately 7,0 using 0,01M NaOH, and the flask was set under nitrogen conditions. Oxone was injected slowly into the flask over the course of 30 minutes and the pH value is reset periodically.

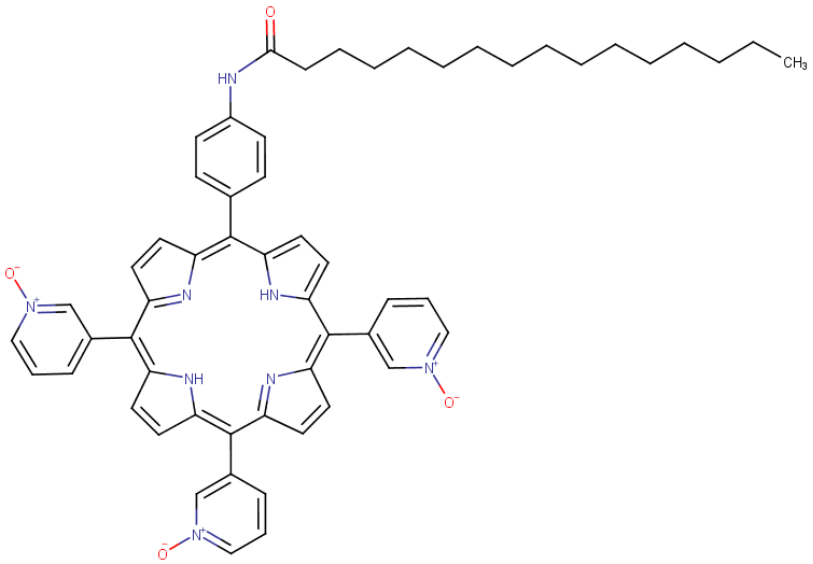
After the addition of Oxone, the success of the oxidation was followed on TLC. To extract the product from the organic layer, and remove the water and hydrophilic impurities, filtration was conducted, followed by the extraction with water (3x) and Na_2SO_4 (1x). The rest of the organic solvent after the removal of water solvent was removed *in vacuo*. To purify the product additionally, column chromatography was performed using the solvent CH_2Cl_2:CH_3OH = 20:1. Porphyrin **6** (0,008 g; 72,72%) was obtained as a purple solid.

UV/ Vis spectroscopy analysis showed the presence of Soret band at 420 nm, with Q bands at next λ_{max}/ nm: 514, 550, 589, 646. Extinction coefficient was calculated: ε= 0,25. Fluorescence measured at the excitation wavelength of the Soret band gave two peaks at λ_{max}/nm= 649, 715.

^{1}H NMR was measured in solvent CD_3Cl and next peaks were observed:

δ/ppm 9,09 (s; 3H; Py-2-H); 9,03 – 8,87 (m; 8H; β-H); 8,72 (d; *J*=4,77 Hz; 3H; Py-4-H); 8,18 (d; *J*= 5,97 Hz; 3H; Py-6-H); 8,12 (bd; 2H; Ar-2,6H); 7,96 (bd; 2H; Ar-3,5-H); 7,74 (t; *J*= 4,86 Hz; 3H; Py-5-H); 7,67 (s; 1H; amide N-H); 2,59 (t; *J*= 5,69 Hz;2H; C^2H_2); 1,53 (t; *J*= 5,69 Hz; 2H; C^3H_2); 1,48- 1,29 (m; 16H; R-H: C^4H_2, C^5H_2, C^6H_2, C^7H_2, C^8H_2, C^9H_2, $C^{10}H_2$, $C^{11}H_2$); 0,91 (t; *J*= 5,18 Hz; 3H; R-C-H_3); -2,94 (s; 2H; pyrrole N-H)

g) 5-(4-hexadecanamidophenyl)-10,15,20-tris(1-oxopyrid-3-yl)porphyrin



Molecular weight: 919,143 g/ mol

Molecular formula: $C_{57}H_{58}N_5[N^+]_3O[O^-]_3$

Polar surface area: 167,28

Method 1:

Porphyrin **4** was oxidised into 5-(4-hexadecanamidophenyl)-10,15,20-tris(1-oxopyrid-3-yl)porphyrin (porphyrin **7**) using *m*-CPBA in a round flask, at room temperature, under nitrogen atmosphere. Porphyrin **4** (0,057 g; 0,065 mmol; 1 eq.) was dissolved in dry CH_2Cl_2 and *m*-CPBA (0,225 g; 1,300 mmol; 20 eq.) was added slowly over 45 minutes. The success of the oxidation was followed on TLC. Reaction was stopped using triethylamine (1 ml). Solvent was removed *in vacuo*, and the compound was isolated by column chromatography using CH_2Cl_2 and CH_3OH in ratio 20:1, with the addition of small amount of triethylamine. Porphyrin **7** was obtained as purple solid (0,041 g; 71,93 %).

Method 2:

The steps we undertook in porphyrin *N*-oxidation using DMD are: in a round flask, under room temperature, porphyrin **4** (0,017 g; 0,019 mmol; 1 eq.) was dissolved in solvent CH_2Cl_2: CH_3OH= 1:1 (10 ml) and stirred on a magnetic stirrer and Oxone (0,180 g; 0,584 mmol; 25 eq.) was dissolved separately in 0,01 M NaOH (1 ml). Acetone (3 ml) was added in the flask in excess and the pH in the flask is set to approximately 7,0 using 0,01M NaOH. Flask was set under nitrogen conditions and Oxone was injected slowly into the flask over the course of 30 minutes and the pH value was adjusted periodically.

After the addition of Oxone, the success of the oxidation was followed on TLC. To extract the product from the organic layer, and remove the water and hydrophilic impurities, filtration was conducted, followed by the extraction with water (3x) and Na_2SO_4 (1x). The rest of the organic solvent after the removal of water solvent was removed in vacuo. To purify the product additionally, column chromatography was performed using the solvent mixture CH_2Cl_2: CH_3OH= 20:1. Porphyrin ***7*** (0,013 g; 76,47 %) was obtained as a purple solid.

UV/ Vis spectroscopy analysis showed the presence of Soret band at 421 nm, with Q bands at next λ_{max}/ nm: 514, 552, 591, 650. Extinction coefficient was calculated: ε= 0,19. Fluorescence measured at the excitation wavelength of the Soret band gave two peaks at λ_{max}/nm= 648, 716.

^{1}H NMR was measured in solvent CD_3Cl and next peaks were observed:

δ/ppm 9,09 (s; 3H; Py-2-H); 9,04- 8,87 (m; 8H; β-H); 8,72 (d; *J*= 5,04 Hz; 3H; Py-4-H); 8,18 (d, *J*= 6,60 Hz, 3H; Py-6-H); 8,12 (bd; *J*= 6,6 Hz; 2H; Ar-2,6-H); 8,01 (bd; 3H; Ar-3,5-H); 7,75 (d; *J*= 2,67 Hz; 3H; Py-6-H); 7,73 (s; 1H; amide N-H); 2,59 (t; *J*= 5,69 Hz; 2H; C^2H_2); 1,53 (t; *J*= 6,32 Hz; 2H; C^3H_2); 1,46- 1,26 (m; 24H; R-H: C^4H_2, C^5H_2, C^6H_2, C^7H_2, C^8H_2,

$C^{9}H_2$, $C^{10}H_2$, $C^{11}H_2$, $C^{12}H_2$, $C^{13}H_2$, $C^{14}H_2$, $C^{15}H_2$); 0,90 (t; *J*= 5,18 Hz; 3H; R-C-H_3); -2,94 (s; 2H; pyrrole N-H)

5. Results and discussion

5.1. Porphyrin synthesis

Modified Adler-Longo method was chosen for the condition reaction to prepare starting material porphyrins, since it is easy to conduct, it is relatively fast and gives us products whose impurities is possible to separate on a column. Adler-Longo method gives better yields of porphyrins in comparison to the Rothemund method.(30) Propionic acid under the reflux conditions and the oxygen from the air creates the initial mixture of porphyrins from pyrrole, 4- acetamidobenzaldehyde and 3-pyridylbenzaldehyde; afterwards separated by the column chromatography.(31) Acetamidobenzaldehyde gives 4-acetamidophenyl group and 3-pyridylbenzaldehyde gives three pyridyl groups on the *meso* positions on the porphyrin ring.

Two porphyrins were isolated from the reaction mixture:

- Symmetric porphyrin/ porphyrin **1**: 5,10,15,20-tetrakis(3-pyridyl)porphyrin
- Asymmetric porphyrin/ porphyrin **2**: 5-(4-acetamydophenyl)-10,15,20-tris(3-pyridyl)porphyrin

The yield of porphyrin **1** (*Figure 6*) was calculated to be 5,12 % and the yield of porphyrin **2** (Figure 7) is 3,74 %.

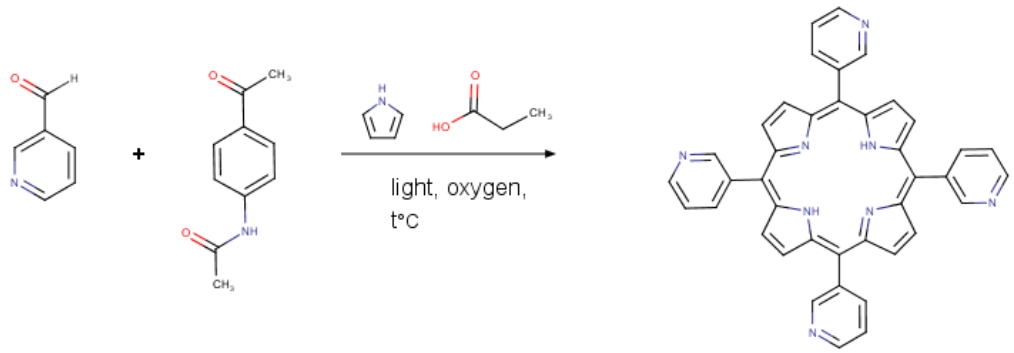


*Figure 6: Synthesis of porphyrin **1***

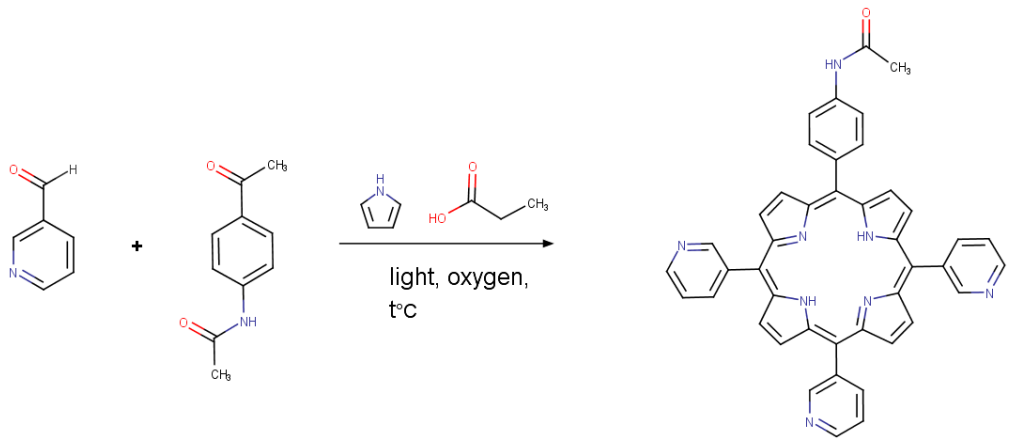


*Figure 7: Synthesis of porphyrin **2***

Hydrolysis of the amide group on the porphyrin **2** is performed in order to add longer alkyl chains onto the phenyl *meso* substituent of the porphyrin. Hydrolysis is conducted using 18% hydrochloric acid under reflux and the product with free amino group is then used for the addition of chains deriving from dodecanoyl chloride or hexadecanoyl chloride/ palmitoyl chloride.

The reaction of dodecanoyl chloride and palmitoyl chloride with amino group on the porphyrin leads to formation of lipophilic porphyrins, which should penetrate biological cells better then porphyrin **1** in PDT applications. Metabolism of lipids in tumour cells is also affected by the use of conjugated fatty acids onto the porphyrin structures. Yields in addition of the chains are high: for the reaction with dodecanoyl chloride it amounts to 87,95% (*Figure 8*) and with palmitoyl chloride amounts to 90,38 % (*Figure 9*). The correct structure of each new porphyrin was confirmed using ^{1}H NMR spectroscopy. Obtained data for porphyrins **1** and **2** is in accordance with published results.(9)

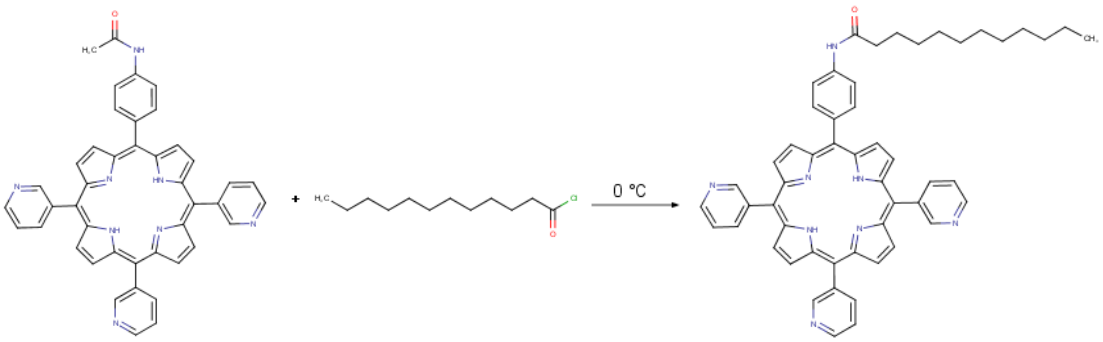


*Figure 8: Synthesis of porphyrin **3***

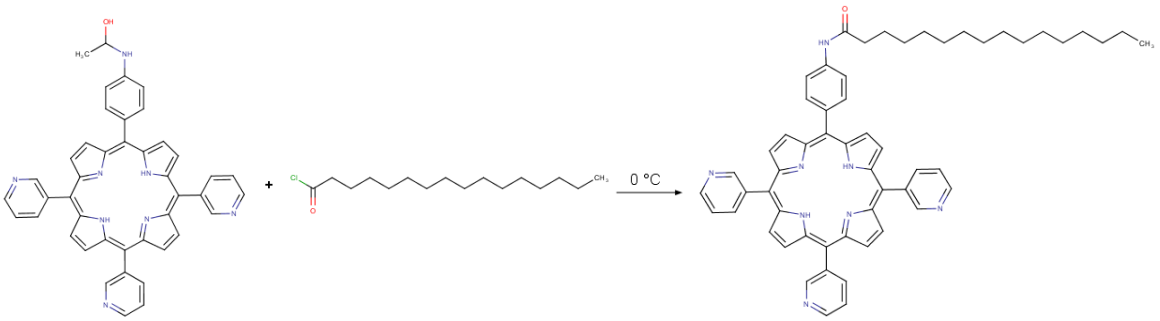


*Figure 9: Synthesis of porphyrin **4***

Both synthesized porphyrins (porphyrin **3** and **4**) have higher polarity than the compounds they originate from, which is visible from the polar surface calculated in Chemaxon Marvin Sketch programme.

5.2. Methods of *N*-oxidation of pyridylporphyrins and optimization

Four methods were assessed for *N*-oxidation of the synthesized pyridylporphyrins, based on the literature research.

a) Oxidation of pyridylporphyrins using meta-chloroperoxybenzoic acid

First method includes *m*-CPBA use as an oxidant of asymmetric porphyrins (porphyrins **3** and **4**). This method has been previously often used in the laboratories and the parameters for a successful reaction were known. *m*-CPBA acts as an oxidant in the reactions of *N*-oxidation with pyridyl

compounds, and the surplus of *m*-CPBA per reaction varies between 60% and 85% per reaction. Regarding porphyrin compounds, oxidation is successful if several equivalents of *m*-CPBA are added, resulting in relatively good yields of porphyrin-*N*-oxides.(10) Syntheses of porphyrin **5** (*Figure 10*), porphyrin **6** (*Figure 11*) and porphyrin **7** (*Figure 12*) using *m*-CPBA are depicted below.

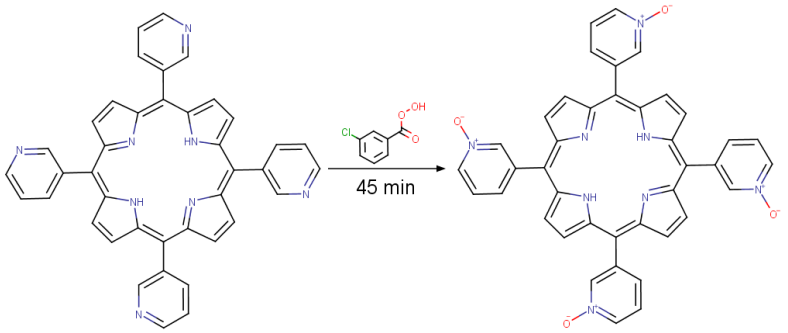


*Figure 10: Synthesis of porphyrin **5** using method 1*

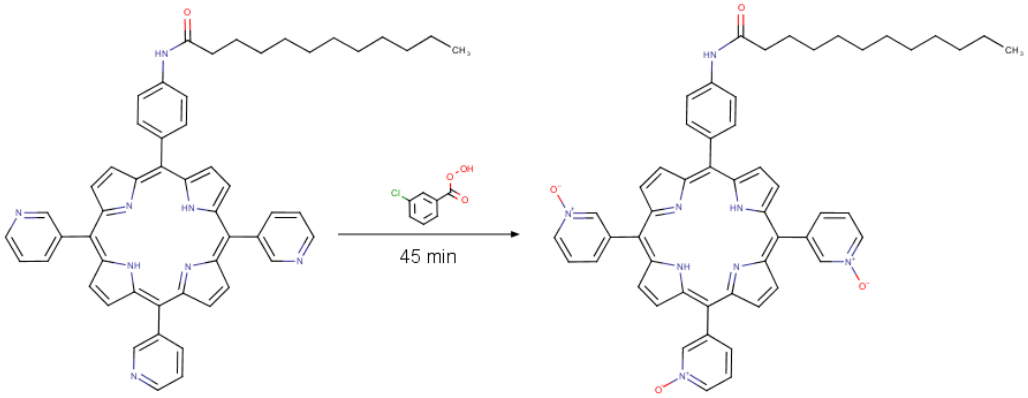


*Figure 11: Synthesis of porphyrin **6** using method 1*

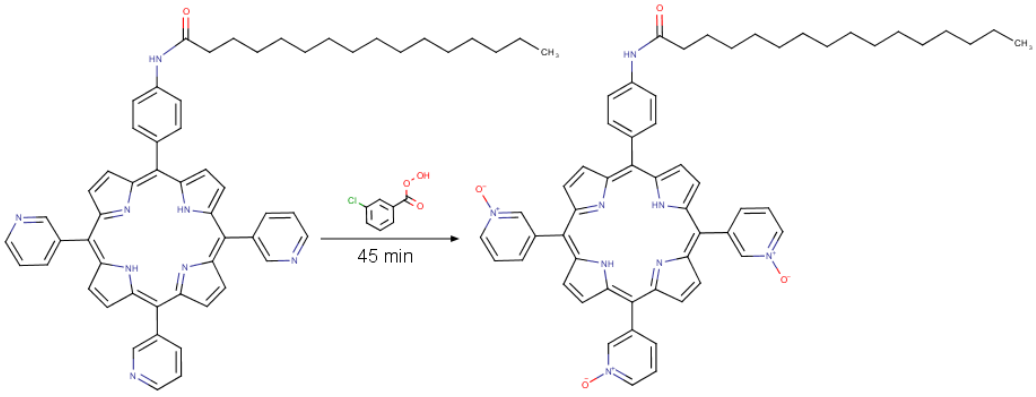


*Figure 12: Synthesis of porphyrin **7** using method 1*

Following this oxidation, immediate purification on the column was performed to remove the excess of oxidant, making the oxidation process last up to 13 hours or more, to properly purify the porphyrin. Similar to reported autoxidation of trimethylamine (32), triethylamine is autoxidised in aqueous solutions, and this is why dry DCM is used. In the presence of a strong oxidant such as *m*-CPBA, triethylamine is converted to triethylamine oxide, which is very hard to remove and presents an obstacle for successful use of our asymmetric porphyrins. This was a motivation to try other methods of oxidation that could give pure products with less or no impurities. Impurities were visible on the NMR spectra of porphyrins **3** and **4**.

b) Oxidation of pyridylporphyrins using dimethyldioxirane

Second assessed method included oxidation using DMD, for which we took several parameters into account during the *in situ* preparation of DMD from Oxone. It took some time before the optimal method was created that could work both on the porphyrin **1** and porphyrins **3** and **4**. Primarily, decision on which temperature will be used for the addition of the oxidant was discussed. It was decided room temperature will be assessed first and in the repeated oxidation procedure, 0 °C will be applied. The reaction was optimized in 4 consecutive attempts, and it was found that the temperature had no impact on the yield of the oxidised products (porphyrins **6** and **7**).

Secondly, pH had to be set between 7,0 and 8,0, and 0,01M NaOH was used, which was easy to prepare and to add into the reaction mixture. The only problem encountered was the lack of pH meter to calculate the exact pH of the coloured mixture. pH strips were used, but the colour of porphyrin was, at times, too intensive to read out the most probable pH value with certainty. Non-miscible layers had different pH values, which could cause confusion if the pH strip was not in contact with the bottom, non-polar layer where the porphyrin and DMD were dissolved. Upper, polar layer had

slightly higher pH value, even if the reaction was stirred, and it could cause false reading of the pH strip to presume the pH value is around 7,5 when in fact, it is lower and insufficient for Oxone conversion. We found that the pH value is the most important parameter to be set for *in situ* generation of DMD and adding more Oxone and/or ketone had no influence on the increase in DMD formation. Acetone had to be added to assure the DMD generation, and a surplus of acetone was not a big problem for the reaction system. However, acetone changed the pH value of the reaction, so adding acetone had to be followed by the adjustment of pH. Syntheses of porphyrin **5** (*Figure 13*), porphyrin **6** (*Figure 14*) and porphyrin **7** (*Figure 15*) using DMD formed *in situ* are depicted below.

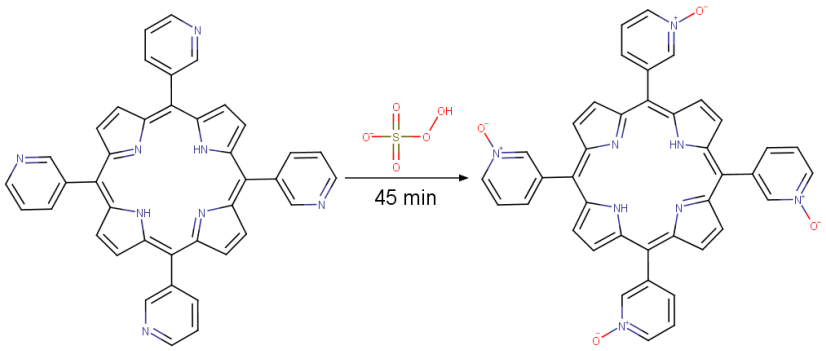


*Figure 13: Synthesis of porphyrin **5** using method 2*

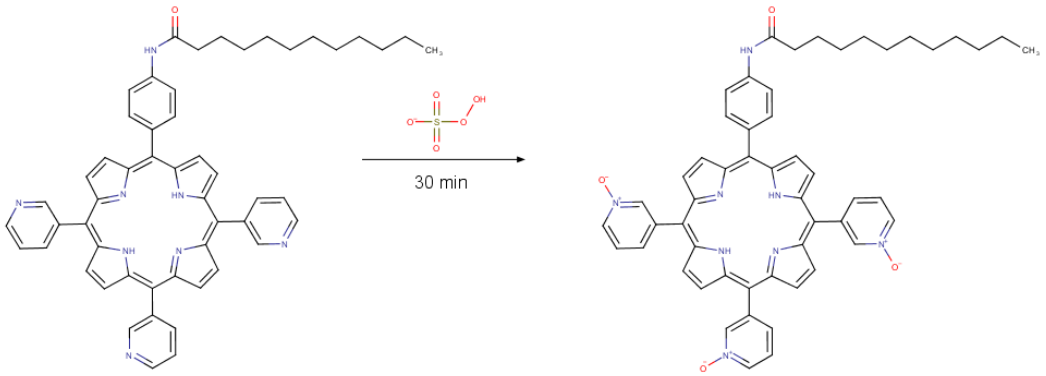


*Figure 14: Synthesis of porphyrin **6** using method 2*

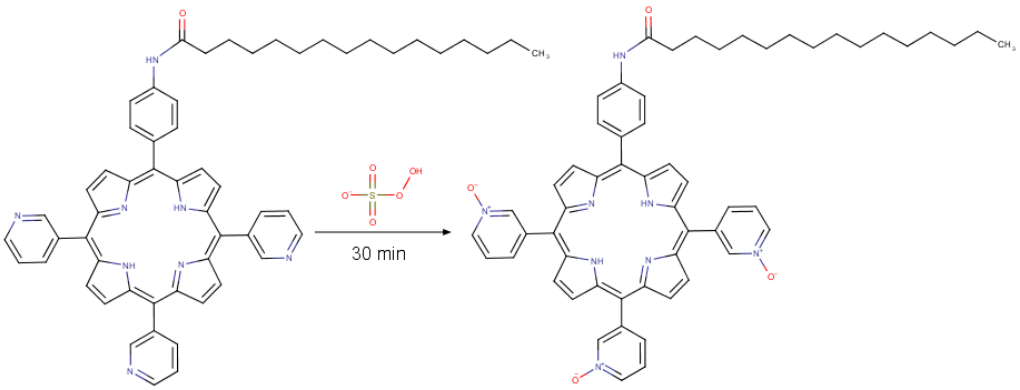


*Figure 15: Synthesis of porphyrin **7** using method 2*

Oxone is a white hygroscopic granular crystalline powder with the pH value of around 1,5 and it is soluble in polar solvents, such as water. Adding Oxone to the reaction mixture was performed by dissolving it beforehand. The most efficient way of dissolving Oxone was its dilution in 1 ml of 0,01M NaOH and its injection using syringe to the mixture in small portions within 45 minutes, alongside pH adjustments. Oxone is added in surplus and some of it remains unused as a consequence of variating pH value and its reactivity under those conditions. Unused Oxone forms a white layer between polar and non-polar phase and is removed after the reaction.

c) Oxidation of pyridylporphyrins using hydrogen peroxide and meta-chloroperoxybenzoic acid

Third method we assessed also involved the use of *m*-CPBA, because of the simplicity of its use, and hydrogen peroxide was used as an additional oxidant. We expected that H_2O_2 would not significantly change the method, but we wanted to try and resolve the problem of the addition of triethylamine, by applying the method of purification using NaOH to stop the oxidation.(10) Product formation was confirmed successfully on TLC using *m*-CPBA synthesized porphyrin **5**. This reaction was performed twice:

- First time, extraction was performed three times with 0,01M NaOH and once with dry Na_2SO_4. During this process, in the first extraction, yellow-coloured impurities were washed out, but in the next

extractions, porphyrin was eluted in the polar phase and this led to the loss of product. It was decided to limit the number of extractions with NaOH.

- The products in the second reaction were only once washed with the 0,01 M NaOH and impurities were washed without porphyrins in the polar phase. Two additional extractions were performed with water and eventually excess water was removed with Na_2SO_4.

Although this method seemed promising, it was found that after the product is stored at room temperature overnight (both before and after column chromatography), large quantity of porphyrin was bleached and the loss of product is considerable. Therefore, yield of porphyrin **5** (*Figure 16*) for this method was lowest of all successful reactions of synthesis (3,92%). Regarding the latter, we decided not to pursue this method as a potential method for oxidation of porphyrins **3** and **4**.

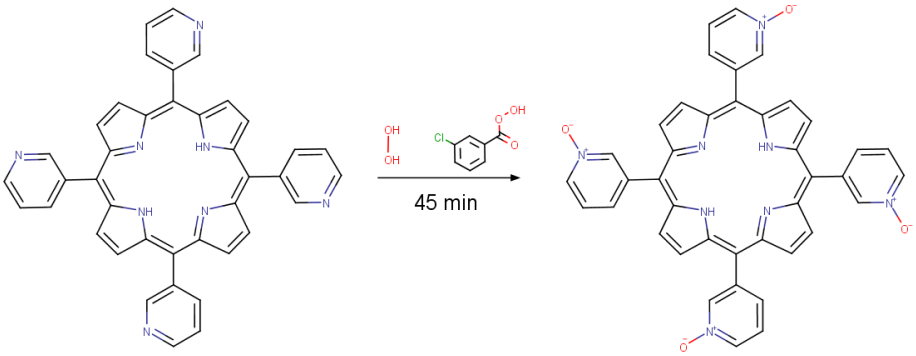


*Figure 16: Synthesis of porphyrin **5** using method 3*

d) Oxidation of pyridylporphyrins using acetic acid and hydrogen peroxide

We performed this reaction under acidic conditions, which was different compared to the other oxidation methods conducted in neutral or mildly alkaline conditions.(33) No oxidation occurred in the flask no matter which condition was changed: the addition of oxidant, the concentration and the amount of acetic acid, change of the pH value, temperature, presence of light. After removing the solvent *in vacuo*, the presence of brown crystals was noticed, but after dissolving the compound in CH_2Cl_2 and purification

on the column, product was stretched, and TLC could not confirm any presence of porphyrin. This method was abandoned as potential method for oxidation (*Figure 17*).

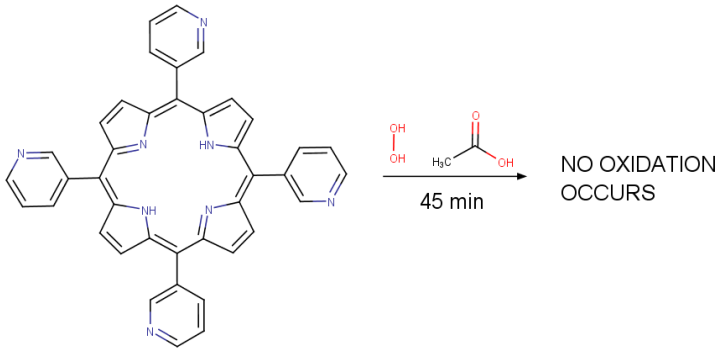


Figure 17: Synthesis of porphyrin **5** *using method 4*

5.3. UV/Vis spectroscopy

Spectroscopic analysis was done to evaluate the absorbance of the porphyrin compounds in the visible spectrum of electromagnetic radiation. Soret band of the porphyrin **1** has a maximum at 418 nm, which is consistent with the known value of Soret band at 420 nm. Four Q-bands are spread between 450 and 670 nm, with maximum absorbances at 514 nm, 549 nm, 589 nm and 645 nm (*Figure 18*).

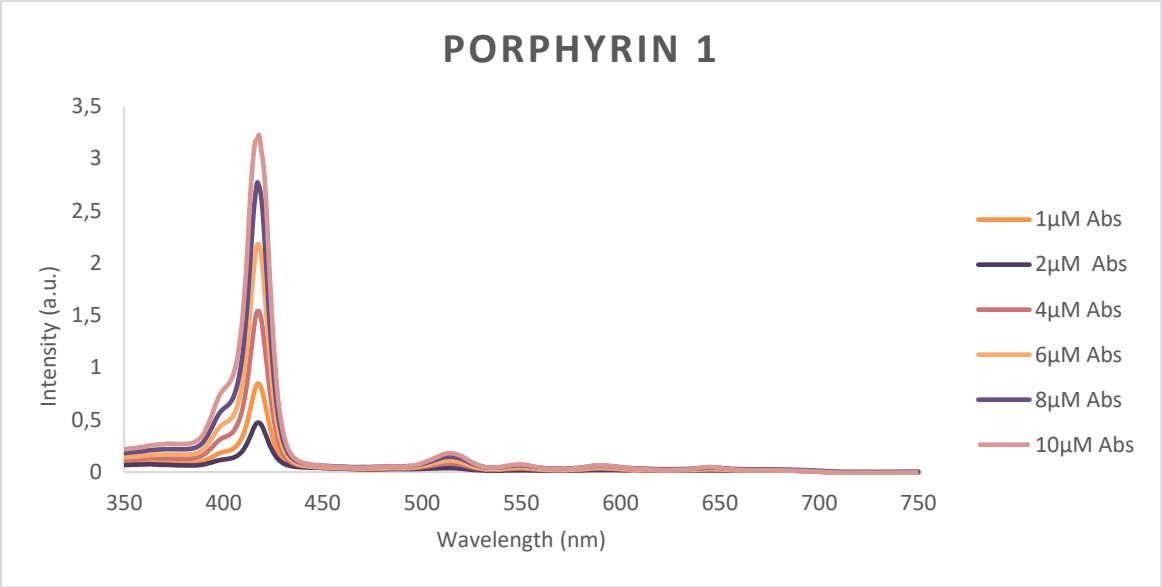


*Figure 18: UV/ Vis spectra of porphyrin **1** in different concentration taken between wavelengths 350 nm and 750 nm*

Fluorescence was measured on a sample with the porphyrin concentration of 1μM, with the excitation wavelength of 418 nm. Two emission peaks are present, one at 650 nm and the other one, less intensive, at 715 nm (*Figure 19*).

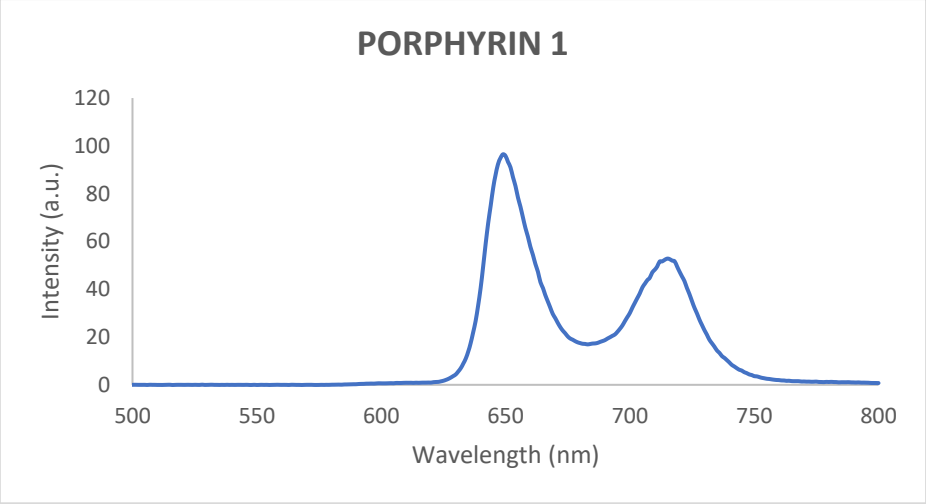


*Figure 19: Fluorescence spectrum of porphyrin **1** at the excitation wavelength of 418 nm, with the emission wavelength between 500 nm and 800 nm*

Oxidised symmetric porphyrin (porphyrin **5**) has lower absorbance than non-oxidised. Its four Q- bands are at 513 nm, 547 nm, 589 nm and 647 nm. Soret band is present at 418 nm (*Figure 20*).

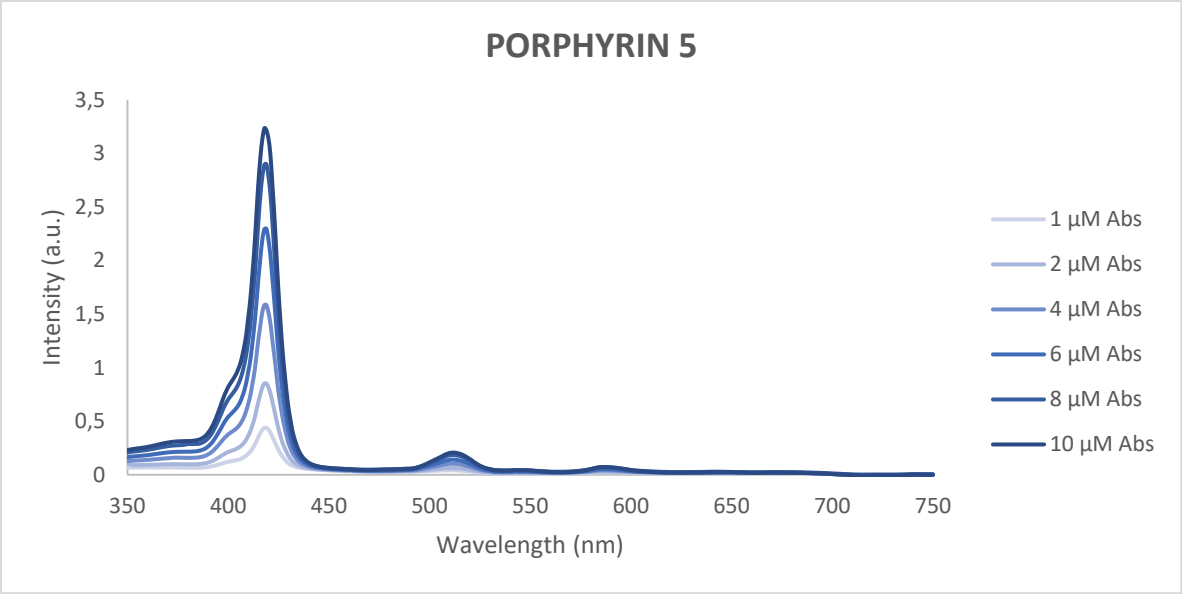


*Figure 20: UV/ Vis spectra of porphyrin **5** in different concentration taken between wavelengths 350 nm and 750 nm*

Fluorescence spectrum of 1µM porphyrin **5** does not show a significant drop after oxidation. Maximum of porphyrin **1** is at 650 nm and has the intensity of 95,79 a.u.. However, once oxidised into porphyrin **5**, it changes its maximum to the peak at approximately 713 nm, reaching the maximal intensity of 38,50 a.u. (*Figure 21*).

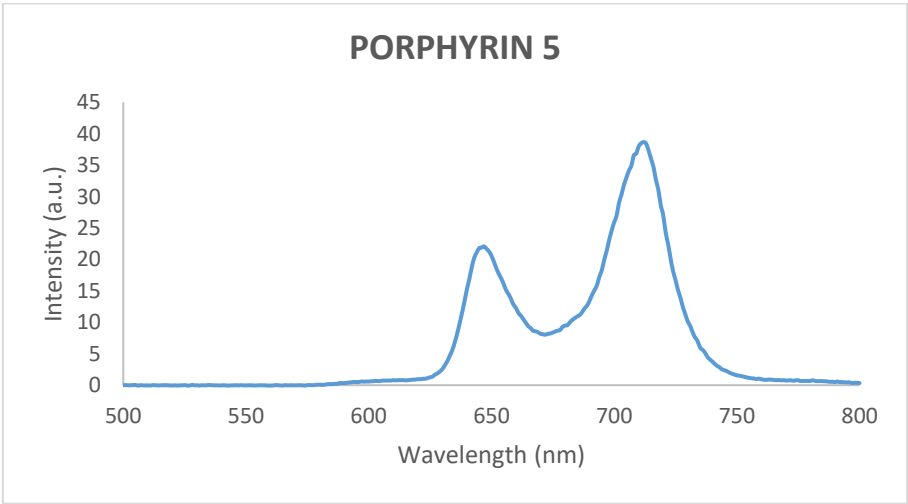


*Figure 21: Fluorescence spectrum of porphyrin **5** at the excitacion wavelength of 418 nm, with the emission wavelength between 500 nm and 800 nm*

Soret band for porphyrin **3** is at 419 nm, with Q-bands at 515 nm, 551 nm, 591 nm and 646 nm (*Figure 22*), which is similar to the values of

porphyrin **1**. Soret band for porphyrin **4** was detected at 419 nm, and Q bands were detected at 517 nm, 554 nm, 593 nm and 648 nm (*Figure 23*).

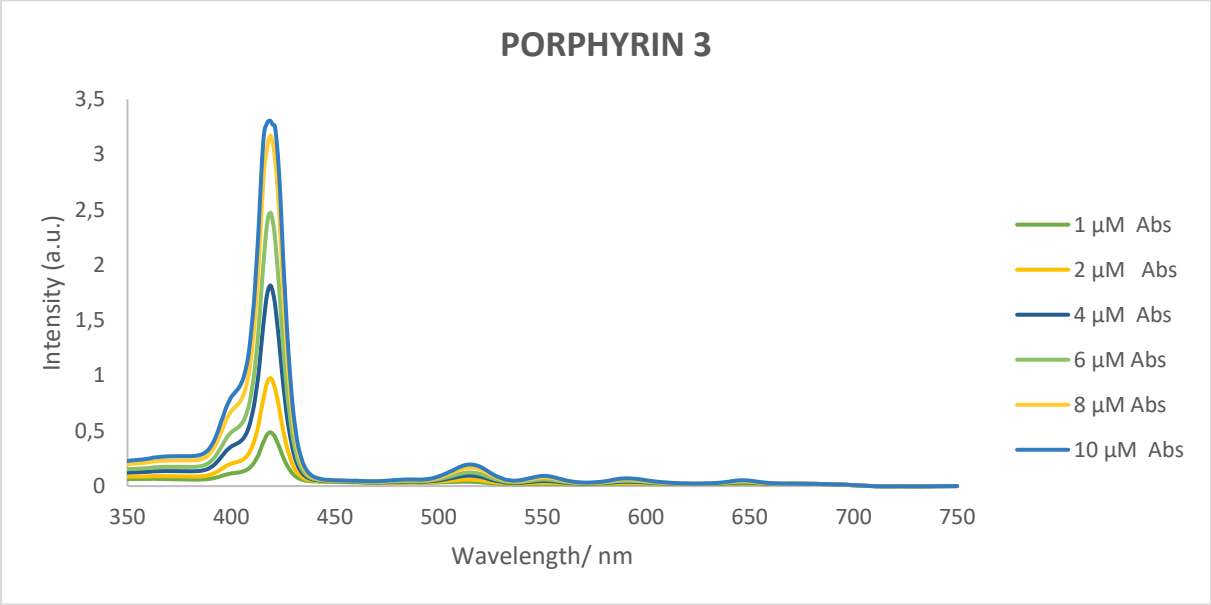


*Figure 22: UV/ Vis spectra of porphyrin **3** in different concentration taken between wavelengths 350 nm and 750 nm*

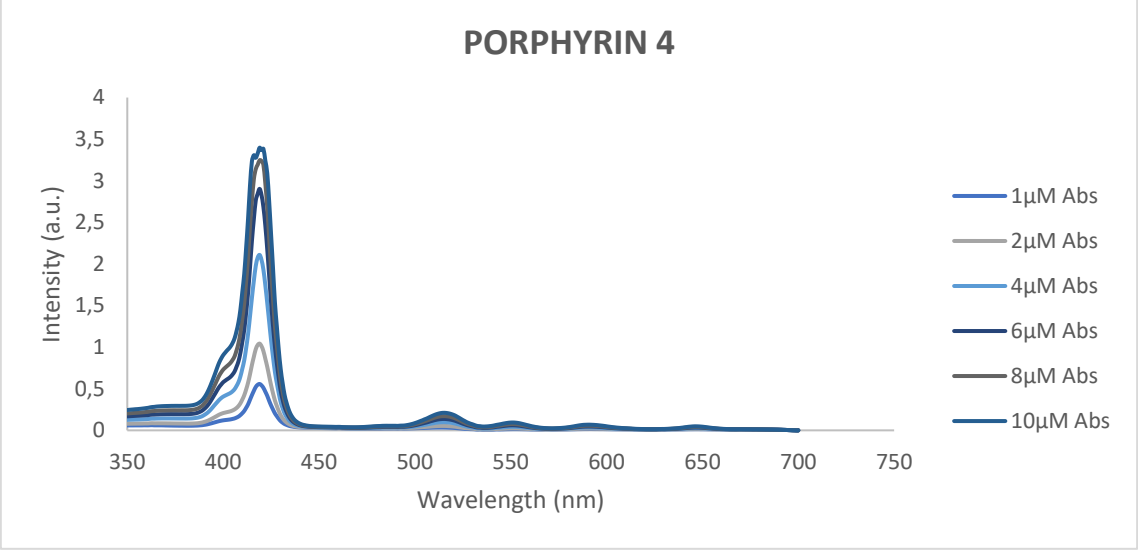


Figure 23: UV/ Vis spectra of porphyrin 4 in different concentration taken between wavelengths 350 nm and 750 nm

Fluorescence of the porphyrin **3** was spread between 600 and 800 nm, like in symmetric porphyrin. Maximum peak was at 649 nm and the lower peak was at 715 nm (*Figure 24*).

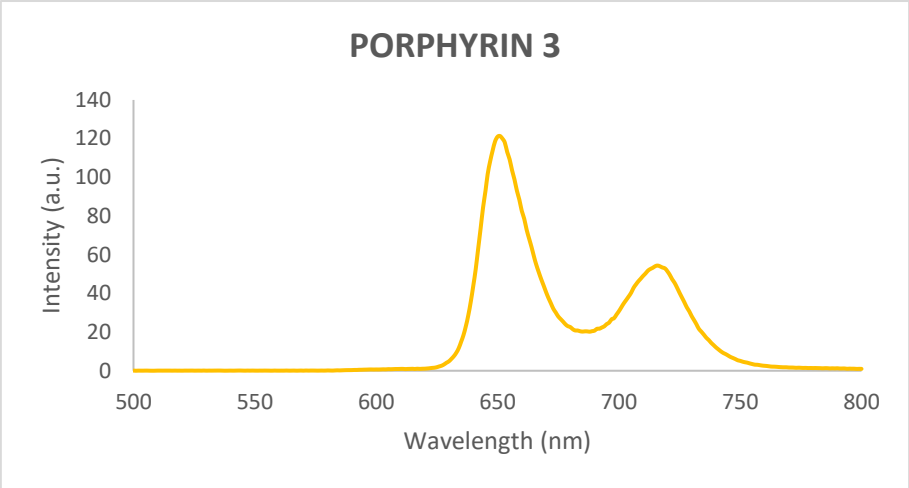


*Figure 24: Fluorescence spectrum of porphyrin **3** at the excitation wavelength of 419 nm, with the emission wavelength between 500 nm and 800 nm*

Excitation wavelength was 419 nm, corresponding to the wavelength of the Soret band for porphyrin **3**. Porphyrin **4** had the same excitation wavelength and gave similar values of both peaks (658 nm and 722 nm), only slightly shifted towards the red values of the electromagnetic spectrum (*Figure 25*).

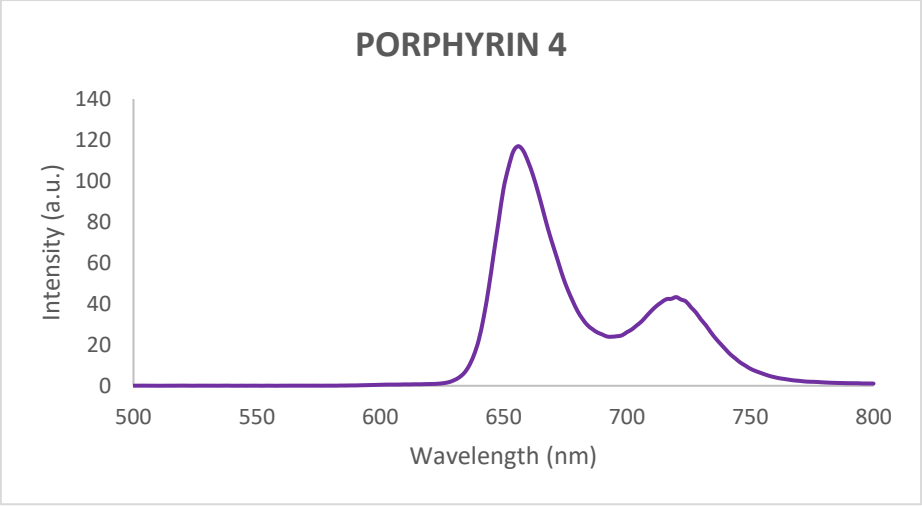


*Figure 25: Fluorescence spectrum of porphyrin **4** at the excitation wavelength of 419 nm, with the emission wavelength between 500 nm and 800 nm*

Generally, oxidised porphyrins **6** and **7** show very similar values when measured with spectrometer, both when oxidised with *m*-CPBA and DMD. To avoid replication of data, only one set of data was used, for the new method subjected to testing (oxidation using DMD).

Porphyrin **6**`s Soret band has a maximum peak at 420 nm, with Q bands at 514 nm, 550 nm, 589 nm and 646nm (*Figure 26*).

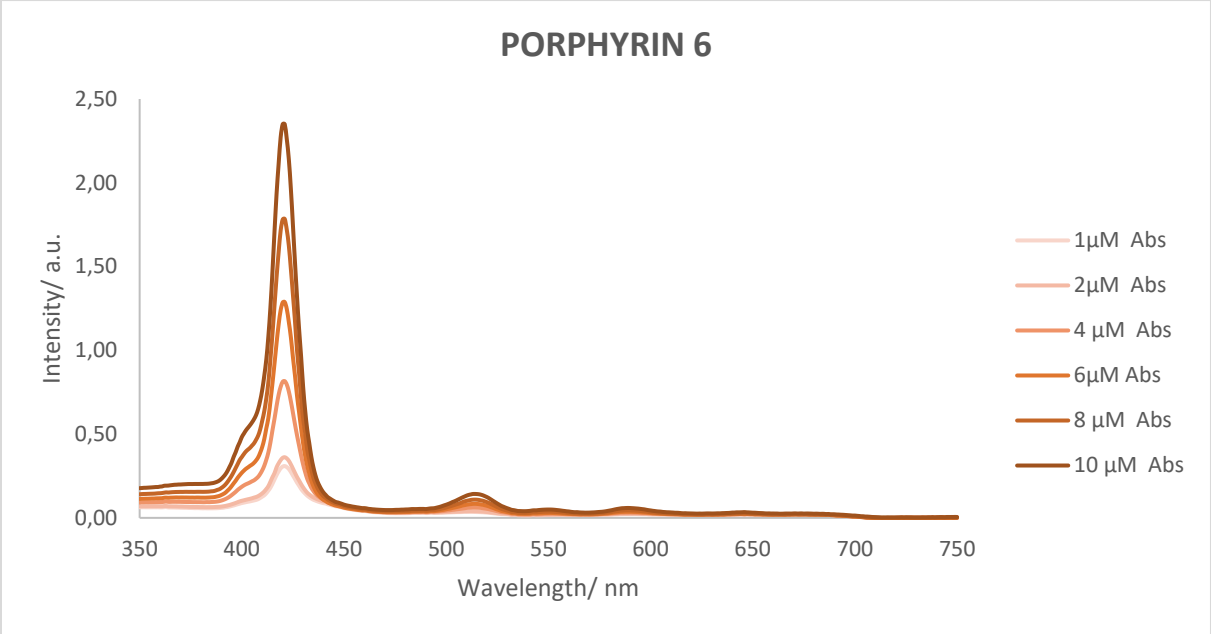


*Figure 26: UV/ Vis spectra of porphyrin **6** in different concentration taken between wavelengths 350 nm and 750 nm*

Total intensity of porphyrin **6** is lower than the intensity of porphyrin **3**. Porphyrin **7** has a Soret band at 421 nm, with Q bands at 514 nm, 552 nm, 591 nm and 650 nm (*Figure 27*).

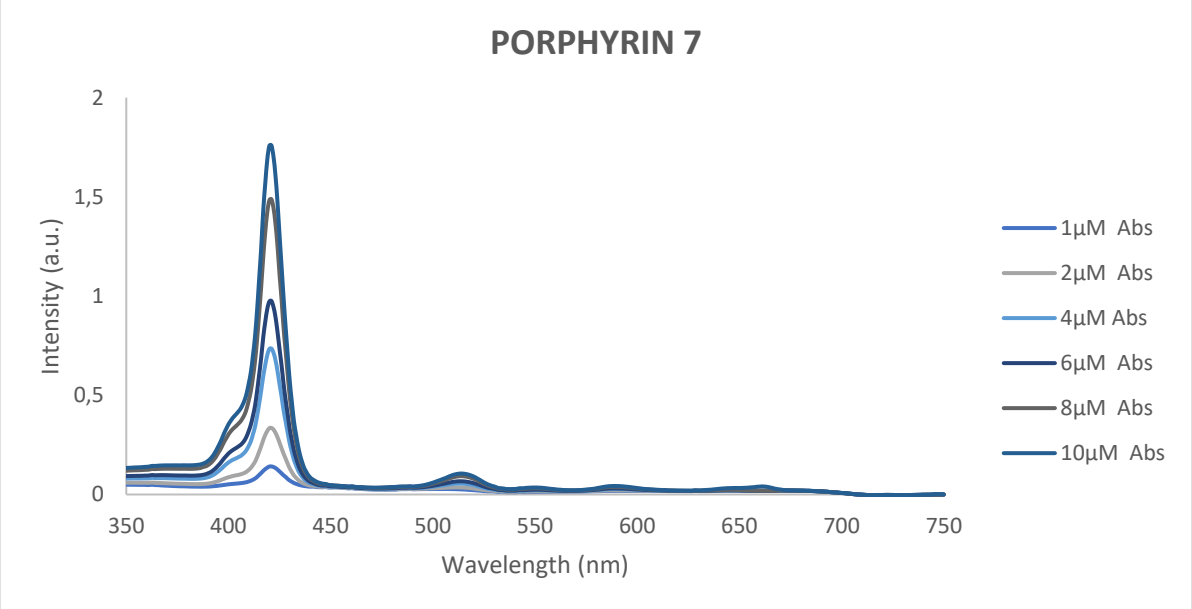


*Figure 27: UV/ Vis spectra of porphyrin **7** in different concentration taken between wavelengths 350 nm and 750 nm*

On the *Figure 28* , it is visible that the intensity of the porphyrin **6** is higher in comparison to porphyrin **7**, which means that it absorbs more light in comparison to the porphyrin **7**. Porphyrin **5** has quite higher value of intensity, reaching total of 3,21 a.u., whereas the value drops proportionally to the length of the substituted chain.

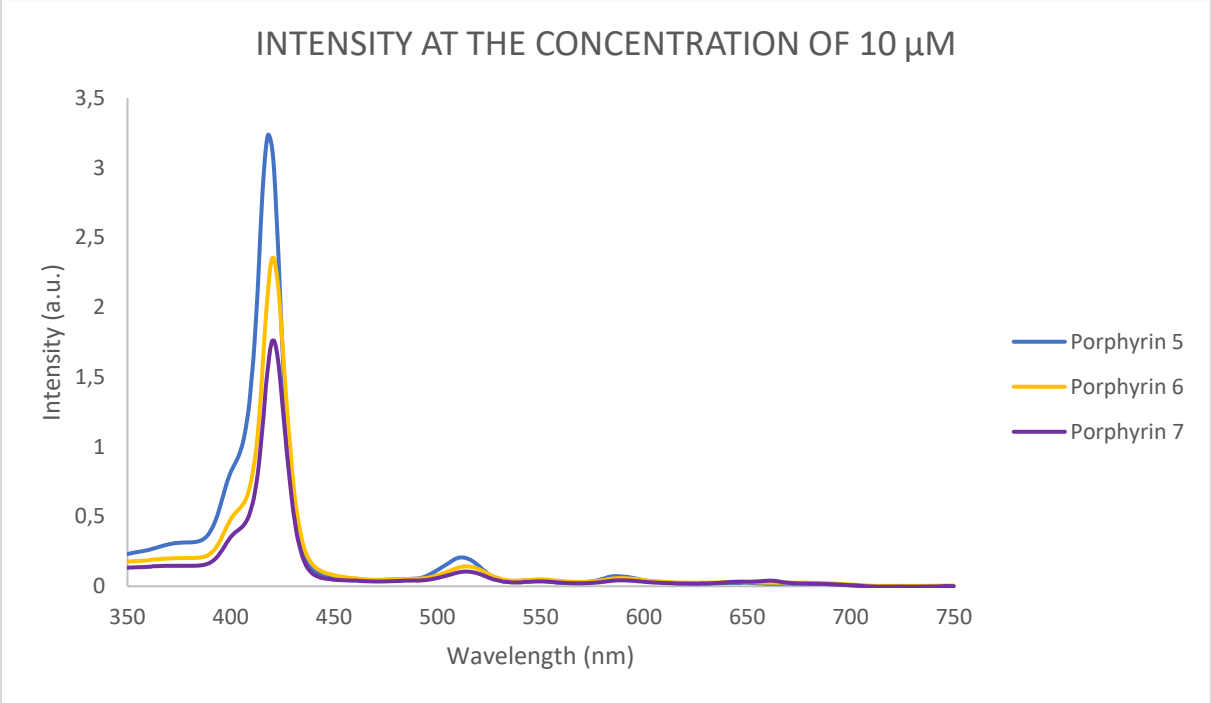


*Figure 28: Intensity (a.u.) of 10 M solutions of porphyrins **5,6** and **7** at wavelength between 350 nm and 750 nm*

At the excitation wavelength of 420 nm, porphyrin **6** did not reach threshold of 50 a.u. Peaks were noticed below threshold at 649 nm and 715 nm (*Figure 29*).

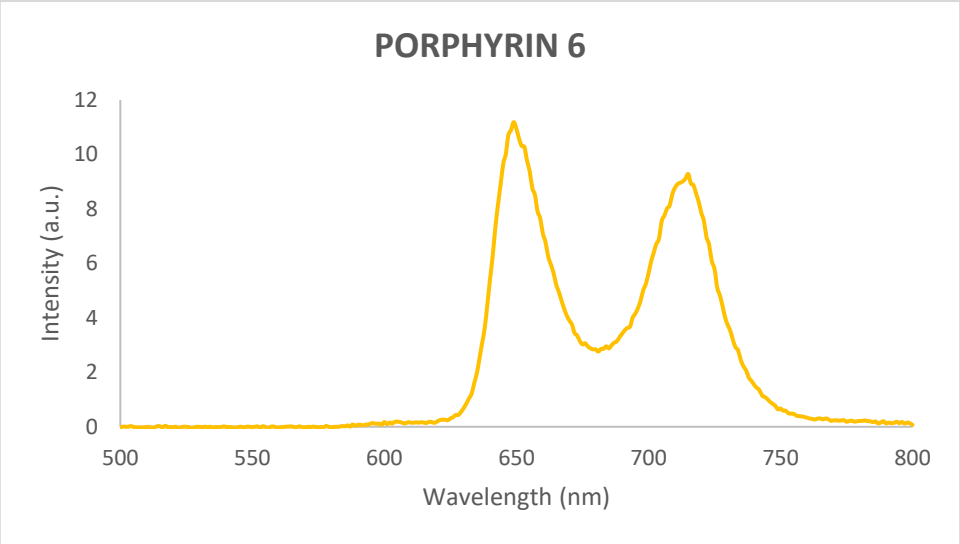


*Figure 29: Fluorescence spectrum of porphyrin **6** at the excitation wavelength of 420 nm, with the emission wavelength between 500 nm and 800 nm*

Fluorescence of the porphyrin **7** was also beneath the threshold of 50 a.u., and it was measured for the concentration of 1μM at the 421 nm excitation wavelength. Measured maximums of the two peaks were at 648 nm and 716 nm (*Figure 30*).

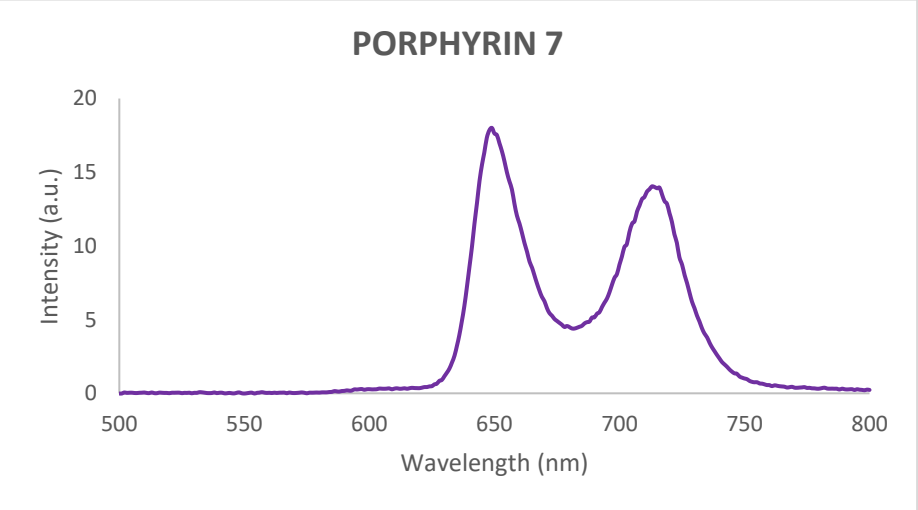


*Figure 30: Fluorescence spectrum of porphyrin **7** at the excitacion wavelength of 421 nm, with the emission wavelength between 500 nm and 800 nm*

Lower fluorescence of the porphyrins is noticed after the addition of the chains to the structure of porphyrin **2**, and the subsequent oxidation of the compounds to porphyrins **6** and **7**.

If the values of fluorescence intensity for porphyrins **5**, **6** and **7** are compared, same trend is noticeable as with the intensity of measured absorbance: the values of fluorescence drop proportionally to the length of the substituted chain on the porphyrin structure. Oxidised products have lower intensities. The difference between the heights of two peaks for the same porphyrin in oxidised and non-oxidised states vary: the difference is inverted in favour of the peak between 700 nm and 750 nm for symmetric porphyrin, whereas porphyrin **6** and **7** exhibit smaller growth of the same peak but it does not pass the value of the peak between 630 nm and 670 nm (*Figure 31*).

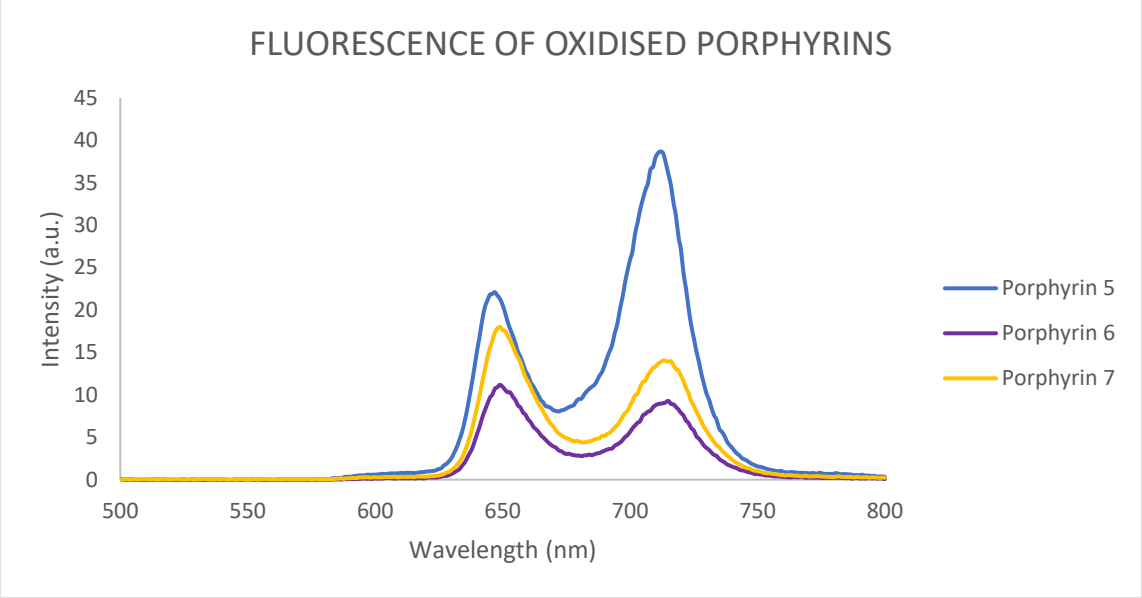


*Figure 31: Fluorescence spectra of oxidised porphyrins (porphyrins **5,6** and **7**) at wavelength between 500 nm and 800 nm*

No difference was evident between spectroscopy results obtained from compounds oxidised with *m*-CPBA and those oxidised with DMD. All graphs gave one Soret band and four Q-bands at approximately the same wavelength and of approximately the same intensity, making it impossible to distinguish the difference between methods and measurements.

ε were higher for porphyrins **3** and **4** than for porphyrins **6** and **7**, while the value of ε remained similar for both porphyrins **1** and **5**. This indicates that the amount of light absorbed by a porphyrin is higher in non-oxidised

asymmetric porphyrins than in oxidised compounds. This is in correlation with the UV/Vis spectra which shows that the oxidised compounds absorb less visible light.

5.4. Nuclear Magnetic Resonance

NMR spectra were collected for porphyrins **3**,**4**,**5**,**6** and **7**.

Two spectra were obtained for porphyrin **5**, one representing method 2 (*Figure 32*), and the other one representing method 3 (*Figure 33*).

In the *Figure 32*, where DMD was used as an oxidant, it is visible that impurities are present in the synthesized porphyrin **5**, and the spectrum peaks are visible at higher values than the ones in *Figure 33*. Impurities have lower values quantitatively than hydrogen atoms present in the molecule of porphyrin. In the *Figure 33*, peak of triethylamine oxide hydrogens from CH3 group is present at approximately 3,35 ppm, and hydrogens from CH2 groups are located between 3,35 and 3,90 ppm. Mentioned hydrogens are present as an impurity from triethylamine that could not be efficiently separated after the reaction of the *N*-oxidation using *m*-CPBA was performed. Such peak is not visible when DMD is used as an oxidising agent, which can, after the purification of porphyrin **5**, give a clean product.

In the *Figures 34* and *35* aromatic hydrogens appear as broad doublets. Shift is noticed towards lower values, and remarkable similarities are noticed between both spectra (porphyrin **6** and **7**) in the position of the peaks. *β*-hydrogens are shifted towards higher values and peaks representing impurities are in the following range: 7,37; 4,38; 2,98-2,91 (d). Water is present from 1,95-1,80 and $CDCl_3$ is at 7,28 ppm. The peak of methine protons might appear at 2,98 and 2,94 ppm on ^1H NMR spectrum, the peak of methine protons might appear, but it is unclear why those peaks would not appear also on the spectra with oxidation method 1.(34)

Comparing the NMR spectra of porphyrin **3** and porphyrin **6**, due to proximity of hydrogen atoms both to oxygen molecules and nitrogen in the pyridyl rings it is visible that the values (ppm) of hydrogen atoms are slightly shifted towards the higher values. Oxidation of porphyrins **3** (*Figure 36*) and **4** (*Figure 37*) with DMD resulting in formation of porphyrins **6** and **7** gives products with less impurities, which is visible in the spectra of the synthesized porphyrins (*Figure 34* and *35*). *β*-hydrogens are shifted right, towards lower shift values of the spectrum, as oxygen added to the molecule does not impact them as much as pyridyl hydrogens. Part of the spectrum in which aliphatic hydrogens are detected does not change significantly. Only the hydrogen on the nitrogen from the amide bond shifts towards higher values.

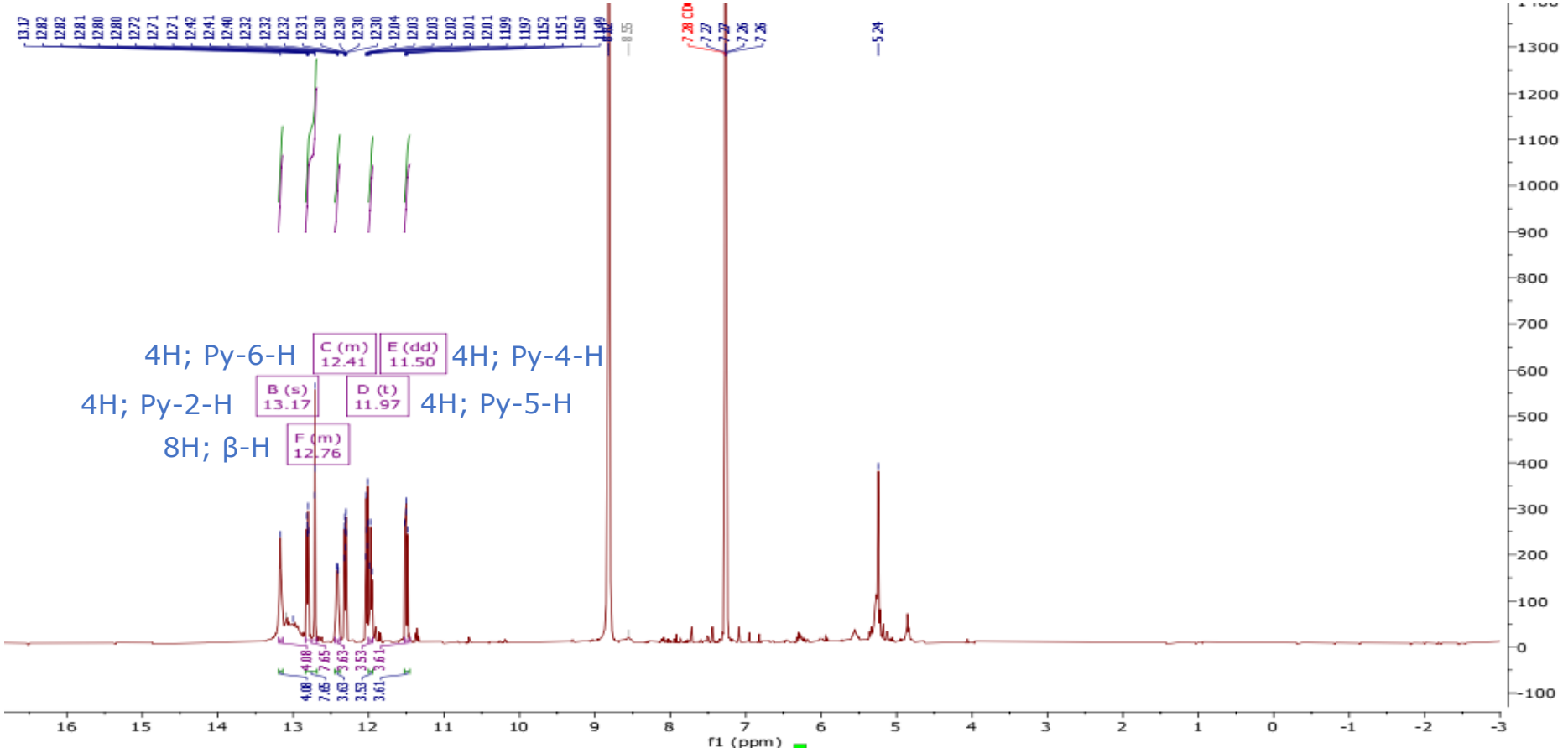


*Figure 32: NMR spectrum of porphyrin **5** oxidised using method 2 (DMD)*

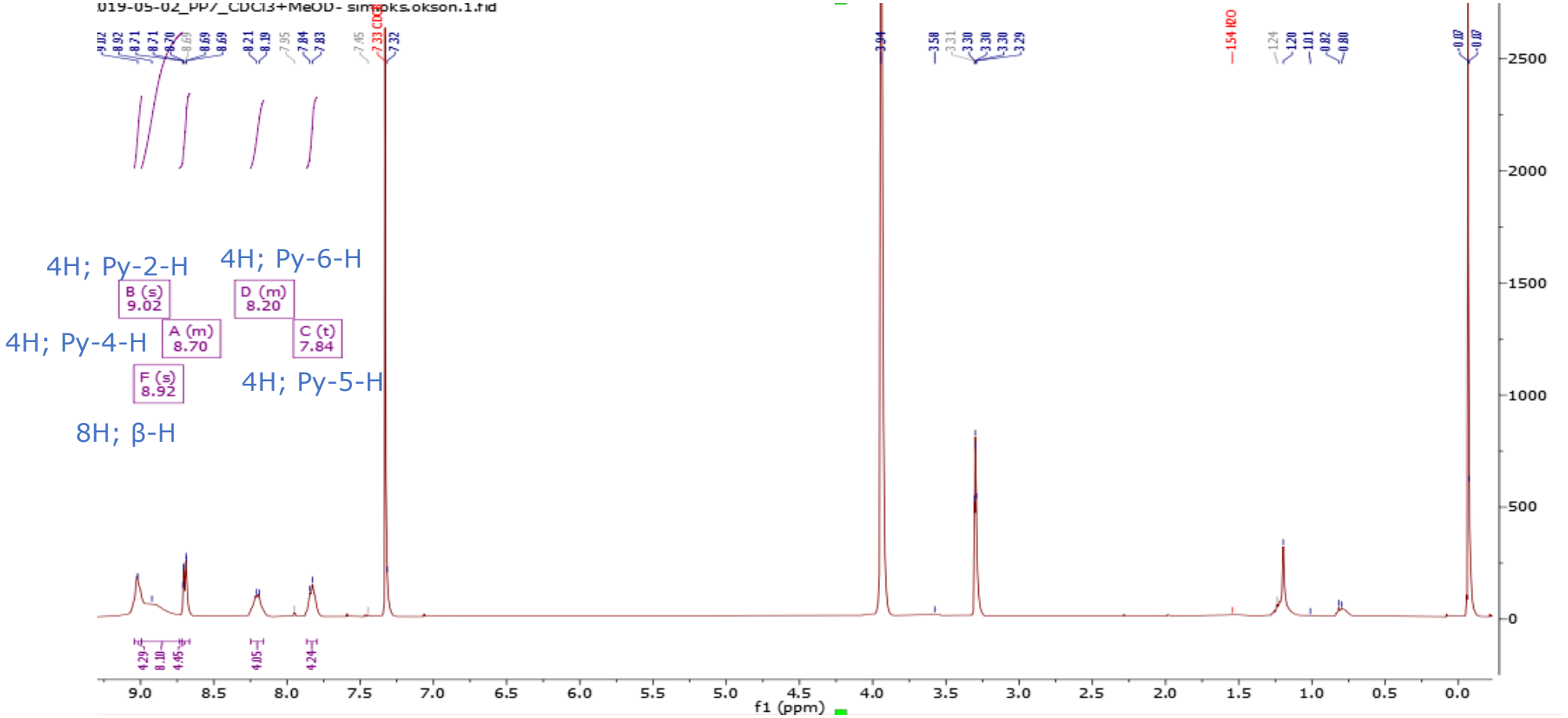


*Figure 33: NMR spectrum of porphyrin **5** oxidised using method 3 (m-CPBA and H_2O_2)*

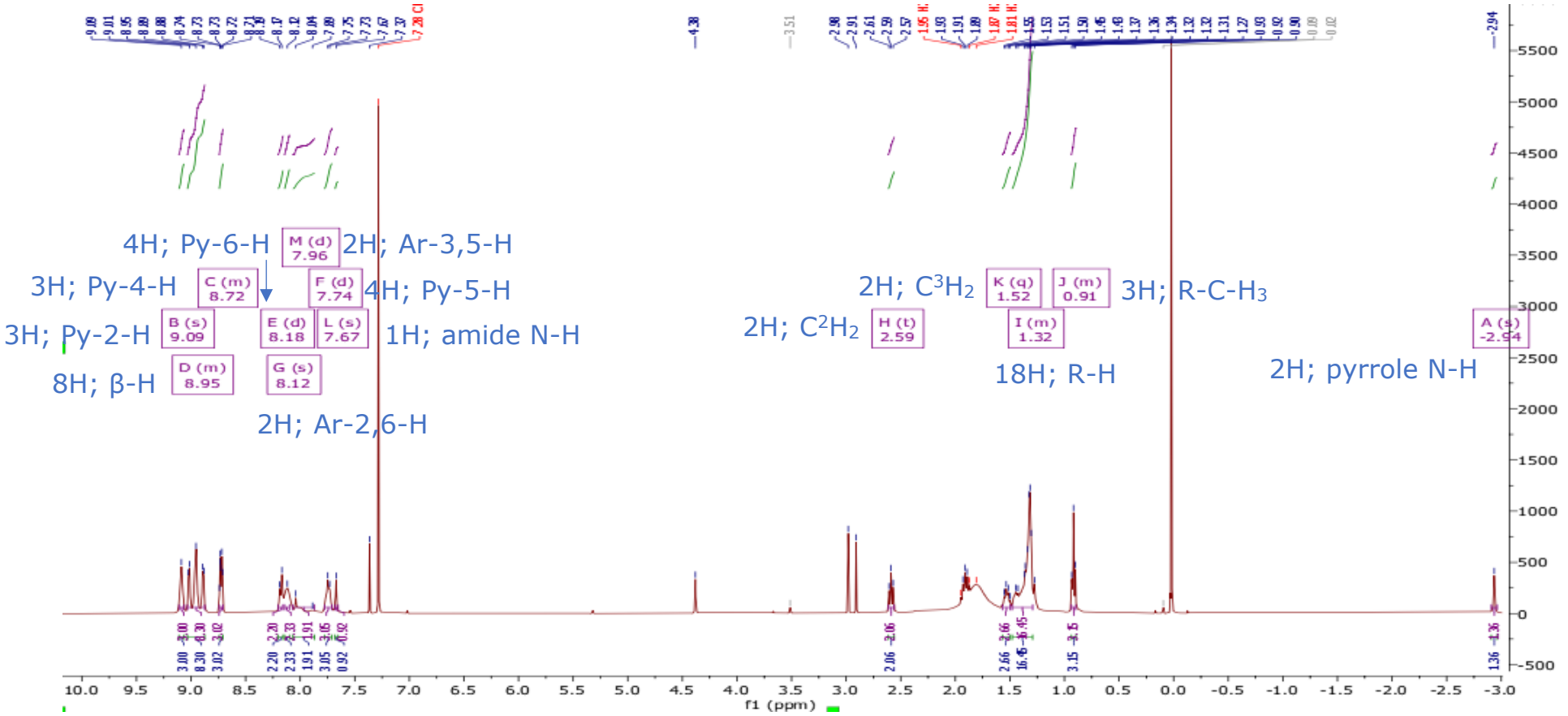


*Figure 34: NMR spectrum of porphyrin **6** oxidised using method 2 (DMD)*

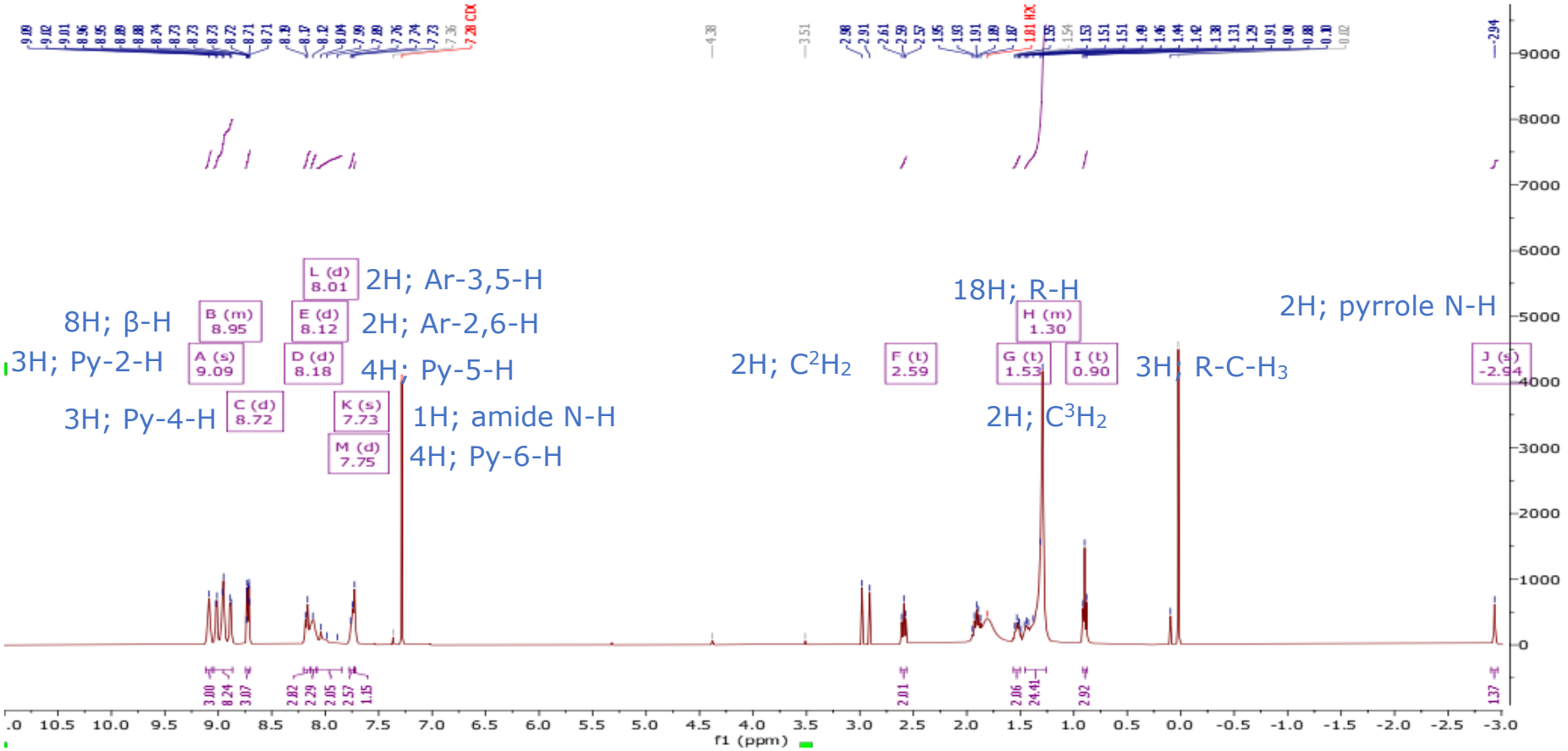


*Figure 35: NMR spectrum of porphyrin **7** oxidised using method 2 (DMD)*

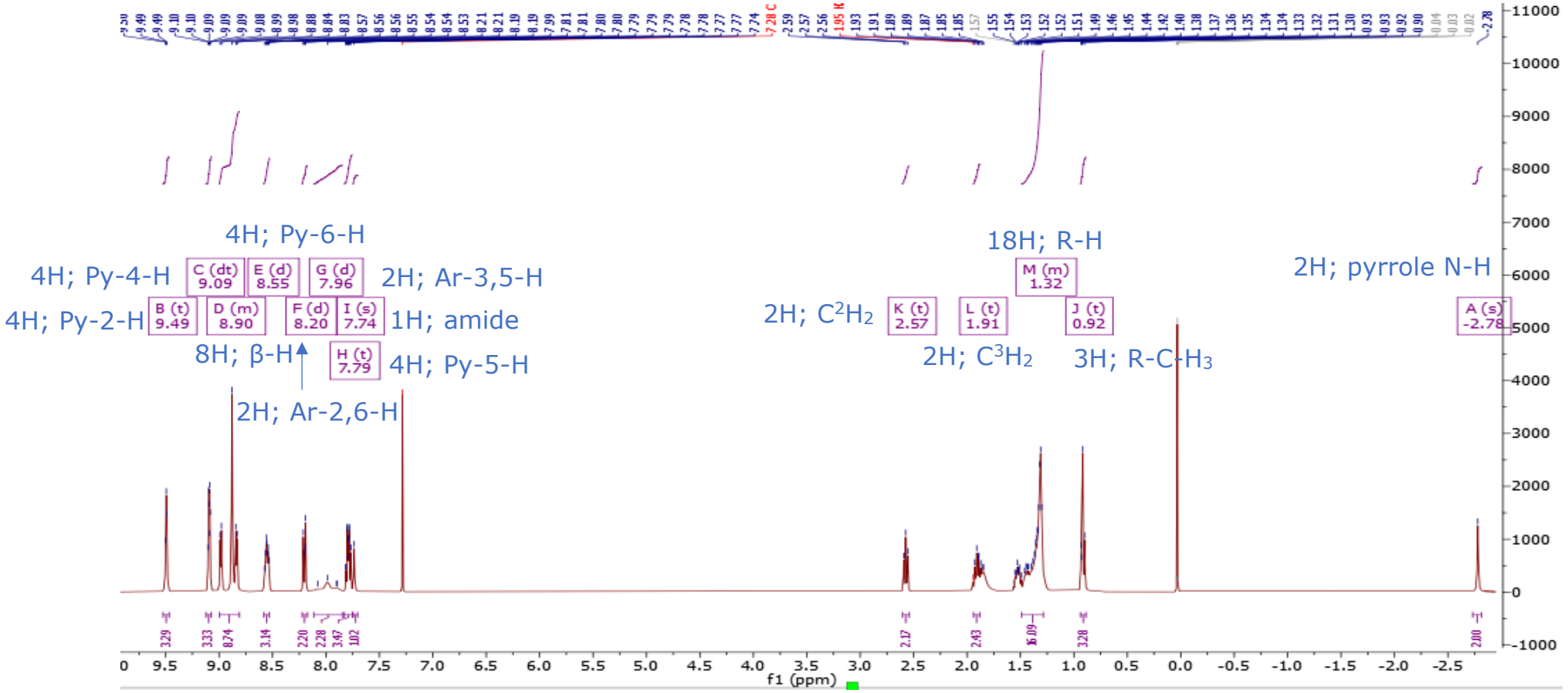


*Figure 36: NMR spectrum of porphyrin **3***

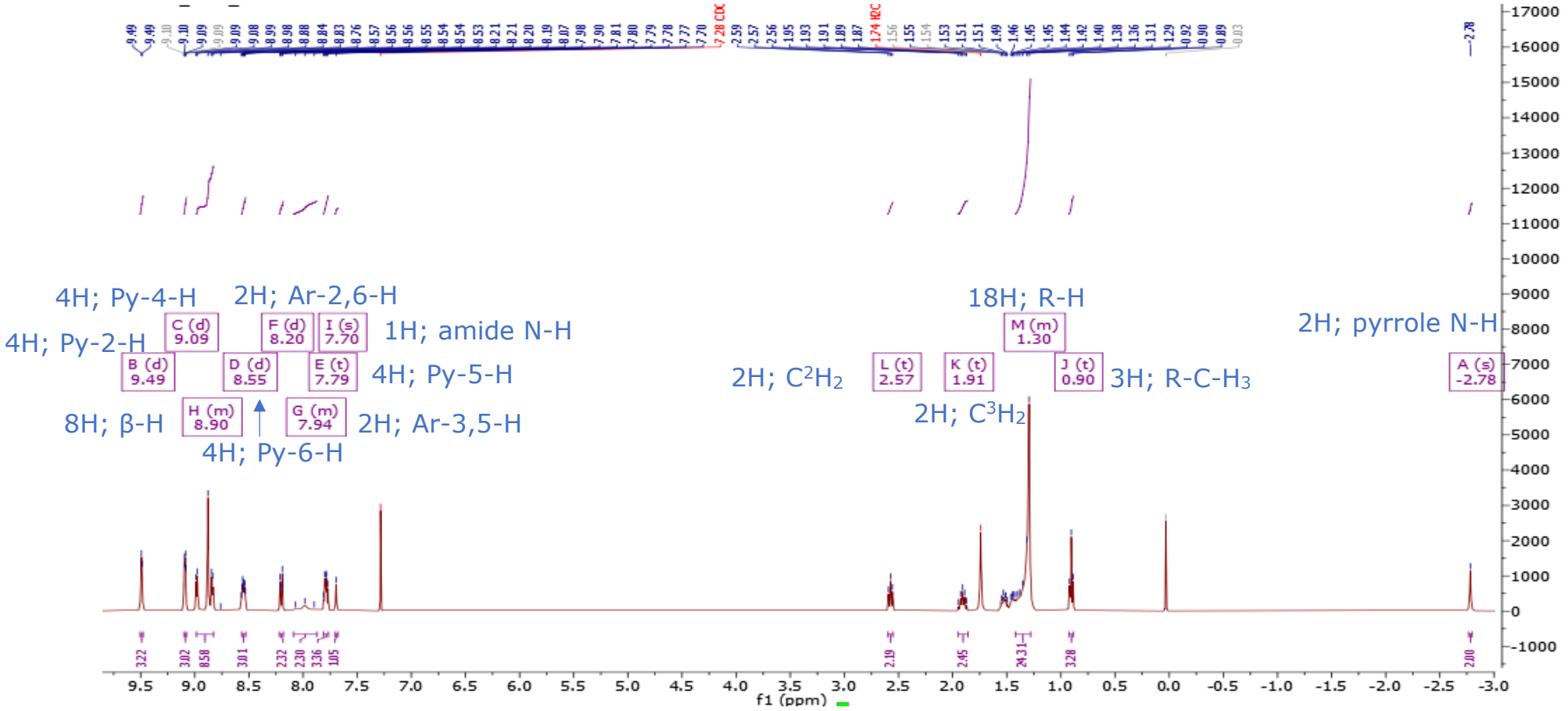


*Figure 37: NMR spectrum of porphyrin **4***

6. Conclusion

The aim of this work was to develop an alternative method for *N*-oxidation of pyridylporphyrins, and to assess different oxidation methods currently used.

Four different methods were assessed on the symmetric pyridylporphyrin:

- Method 1: Oxidation using *m*-CPBA (current standard)
- Method 2: Oxidation using DMD
- Method 3: Oxidation using *m*-CPBA and NaOH (H_2O_2 was used as an oxidant)
- Method 4: Oxidation using CH_3COOH

Out of all methods of oxidation, method using DMD stood out as the reaction was relatively quick to perform and gave satisfying porphyrin yields (70,00%). Yield of porphyrin **1** oxidised using *m*-CPBA was 65,00%. After optimization of the reaction, it was concluded that the most important factor for it was the pH value. Neither the fact that the reaction is performed in a non-miscible reaction system, or that the acetone is added, did not cause unsuccessful reaction. DMD was prepared successfully *in situ* from Oxone. Reaction of oxidation using method 1 was faster in comparison to the method 2, however, it required more steps and was more sensitive than the method 1 to the conditions of the reaction. Purification of the product using method 2 is significantly shorter (up to several hours shorter), due to the fact triethylamine is not used in the reaction. Triethylamine oxidises when added to reaction mixture with *m*-CPBA and porphyrin, to triethylamine oxide, which sticks to the column and cannot be subsequently removed from the product, as seen on the NMR spectrum (*Figure 33*). Method 2 involves different methods of purification (filtration, extraction, second filtration, column chromatography), more than method 1 (extraction, filtration, column chromatography).

We measured yields of porphyrin **6** and **7**, and for porphyrin **6**, yield using method 1 was 70,59% and using method 2 was 72,72%, while for porphyrin **7**, yield using method 1 was 71,93% and using method 2 was 76,47%.

Oxidation process in which *m*-CPBA and NaOH were used gave a poor yield of oxidised symmetric porphyrin (3,92%), and no product was formed with the use of CH_3COOH. These reactions were not pursued for optimization as a new potential method of oxidation.

Analysis of UV/Vis spectres of porphyrins oxidised by *m*-CPBA and by DMD has shown no difference between the spectra; therefore UV/Vis analysis was not a criterion in deciding which method is more convenient for porphyrin oxidation.

NMR analysis was performed on porphyrins **3**,**4**,**5**,**6**, and **7**, leading to the conclusion that the method 2 contributes less to the creation of total amount of impurities, and those impurities can subsequently be removed. Method 1 gave expected results, giving less different impurities, but exhibited impurities originating from triethylamine oxide, which cannot be removed from the final product effectively. Part of the NMR spectrum in which aromatic hydrogens can be found changes significantly by the oxidation of porphyrins, shifting the spectrum peaks towards higher values. Aliphatic hydrogens do not change significantly.

Considering all the outcomes, we conclude that the method 2 is better in the terms of producing compounds with less impurities and in much shorter period than method 1. However, economically, more resources are used, which can be a negative side of this method.

More research should be dedicated to the optimization of a method 2, making it more practical and reducing the need for repeated purification of a product. Future steps would be to investigate and optimize methods 3 and 4 to give the wanted product and to be able to fully assess the methods of porphyrin oxidation.

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Common European Framework of Reference for Languages

ADDITIONAL INFORMATION

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- Stipend of the Ministry of war veterans in academic years 2017/2018 and 2018/2019
- Rector`s award for student activism