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Syntéza nových potenciálních léčiv atherosklerózy

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List of abbreviations

ALA	5-aminolevulinic acid
AlPcS	Aluminium phthalocyanin sulphonates
AMD	Age-related Macular Degeneration
ATP	Adenosine triphosphate
CVD	Cerebral vascular disease
DCM	Dichloromethane
DDQ	2,3-Dichloro-5,6-dicyanobenzoquinone
DMF	N,N-dimetheyformamide
DNA	Deoxyribonucleic acid
EDRF	Endothelial cell-derived relaxing factor
FDA	Food and Drug Administration
HDL	High density lipoprotein
HpD	Hematoporphyrin derivative
HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High Performance Liquid Chromatography
CHD	Coronary heart disease
INF-γ	Interferone-gamma
ISC	Intersystem crossing
LDL	Low density lipoprotein
MI	Myocardial infarction
NMR	Nuclear Magnetic Resonance
PCT	Photochemotherapy
PCTA	Percutaneous coronary transluminal angioplasty
PDT	Photodynamic Therapy
PS	Photosensitizer
PUVA	Psoralens and UV A
ROS	Reactive oxygen species
SMCs	Smooth Muscle Cells
TFAA	Trifluoroacetic acid
TIAs	Transient ischemic attacks
TLC	Thin Layer Chromatography
TPPS	Tetraphenyl porphine sulphonate

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Introduction - Goals of My Work

My work deals with porphyrins and there are a lot of aims in it: at the beginning of theoretical part I study porphyrins in general, their occurrence in nature, basic synthetically prepared skeletons and their nomenclature, properties and basic methods of preparation. In the next bigger unit I concentrate on usage of porphyrins in practice and I chose one specific field to describe it thoroughly, use of porphyrins as photosensitizers in photodynamic therapy (PDT). Here I encounter properties of the optimal photosensitizer, their division, history and principle of PDT. The crucial aim of my experimental part is preparation of potential drugs for curing of atherosclerosis with a new mechanism of action based on PDT, therefore as a nearly last and most important part of theoretical section, information about atherosclerosis and mechanism of action of motexafin lutetium is included. My work is closed with experimental section, describing performed reactions and specifications of my products.

Theoretical Part

The Porphyrins

The porphyrins are a class of naturally occurring macrocycles, usually tetrapyrroles, that are ubiquitous in our world. Porphyrins, in form of chlorophyll, are included in plants converting light energy into chemical energy while producing oxygen. This oxygen, evolved from photosynthesis, is transported, stored, and reduced by heme-containing proteins in many organisms, including mammals. Not surprisingly, therefore, these molecules are intensively studied and also a lot of their synthetic modifications were prepared[1].

We can divide them into two main groups: naturally occurring porphyrins and synthetically prepared porphyrins.

Naturally Occurring Porphyrins

Porphyrins occur in many forms in nature and they also bind different metal ions to the centre of their molecules, as we can see in examples shown below.

Chlorophyll

Chlorophyll is green pigment that gives most plants their colour and enables them to carry on the process of photosynthesis. Chemically, chlorophyll has several similar forms, the main two are named chlorophyll-*a* (bluish-black solid) and chlorophyll-*b* (dark green solid). Molecule of chlorophyll consists of chlorin-ring and lipophilic phytyl side chain. There is Mg^{2+} ion in the centre of chlorin-ring and one orthocondensed cyclopentanone ring in *m,n* chlorin position, which has its origin in propionic acid joined to C13. The chlorin-ring remains intact in treatment of chlorophyll with acids and bases, we can observe only remove of magnesium ion and further hydrolysis removing phytol. The absorption maxima of chlorophyll-*a* are 430 and 662nm, that of chlorophyll-*b* are at 453 and 642nm. Chlorophyll-*a* is pictured in figure *1a*.

Heme

Heme is a prosthetic group that consists of an iron atom contained in the centre of porphyrin ring. There are three biologically important kinds of heme: the most common type is heme *b (fig. 1b,c)*; the others are heme *a* and heme *c*. Heme *b* is the most abundant heme: both hemoglobin and myoglobin are examples of proteins that contain it. Heme *b* is not covalently bound to these proteins. Heme *a* differs from heme b in that a methyl side chain is oxidized into a formyl group, and one of the vinyl side chains has been replaced by an isoprenoid chain. Like heme *b*, heme *a* is not covalently bound to the apoprotein in which it is found (e.g. cytochrome *c* oxidase). Heme *c* differs from heme *b* in that the two vinyl side chains are covalently bound to the protein itself. Example of protein that contains heme *c* is cytochrome *c*. Heme is further included in these hemoproteins: catalase, peroxidase and tryptophan oxygenase.

Cytochromes

Cytochromes, big group of electron or proton-transferring proteins, include heme groups in their structure. Cytochromes can be classified according to their heme iron coordination, by heme type, and further by sequence similarity. Cytochromes *c* (cyt *c*), the most common, can be defined as electrontransfer proteins having one or several heme *c* groups, bound to the protein by one or, more commonly two, thioether bonds involving sulphydryl groups of cysteine residues *(fig. 1d)*. The fifth heme iron ligand is always provided by a histidine residue. Cyt *c* possesses a wide range of properties and functions in a large number of different redox processes[2].

Cyanocobalamin

Cyanocobalamin, more commonly known as vitamin B_{12}, is a very unusual biochemical that contains a corrin ring. In the centre of the corrin ring there is coordinated a cobalt ion that is also associated with a cyanide ion and a dimethylbenzimidazole nucleotide *(fig. 1e)*.

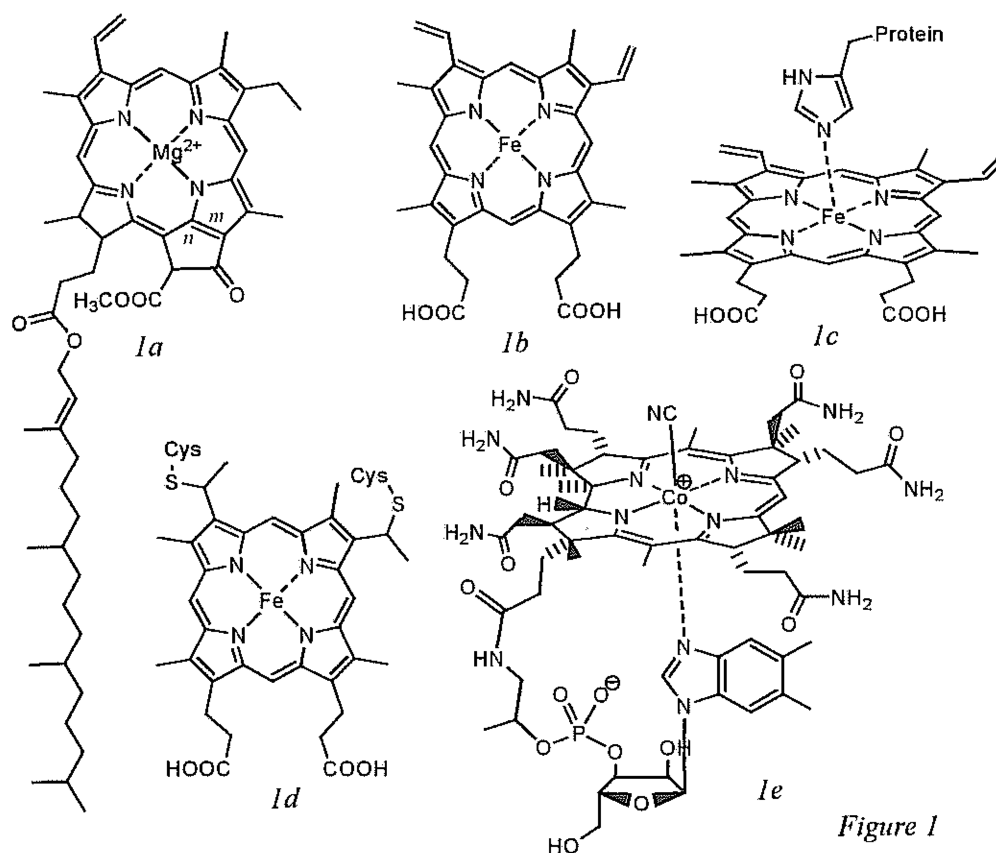


Figure 1

Synthetically Prepared Porphyrins

There is a wide variety of synthetically prepared porphyrins, that's why we need good nomenclature to describe them not interchangeably. Chemists have also developed a scheme, in which every porphyrin can be formally derived from annulenes and named it [18]annulene framework. Using this framework we can predict any porphyrin, although it wasn't synthesised yet *(fig. 2)*. From higher annulenes larger porphyrins called pentaphyrins and hexaphyrins can be derived[2,3].

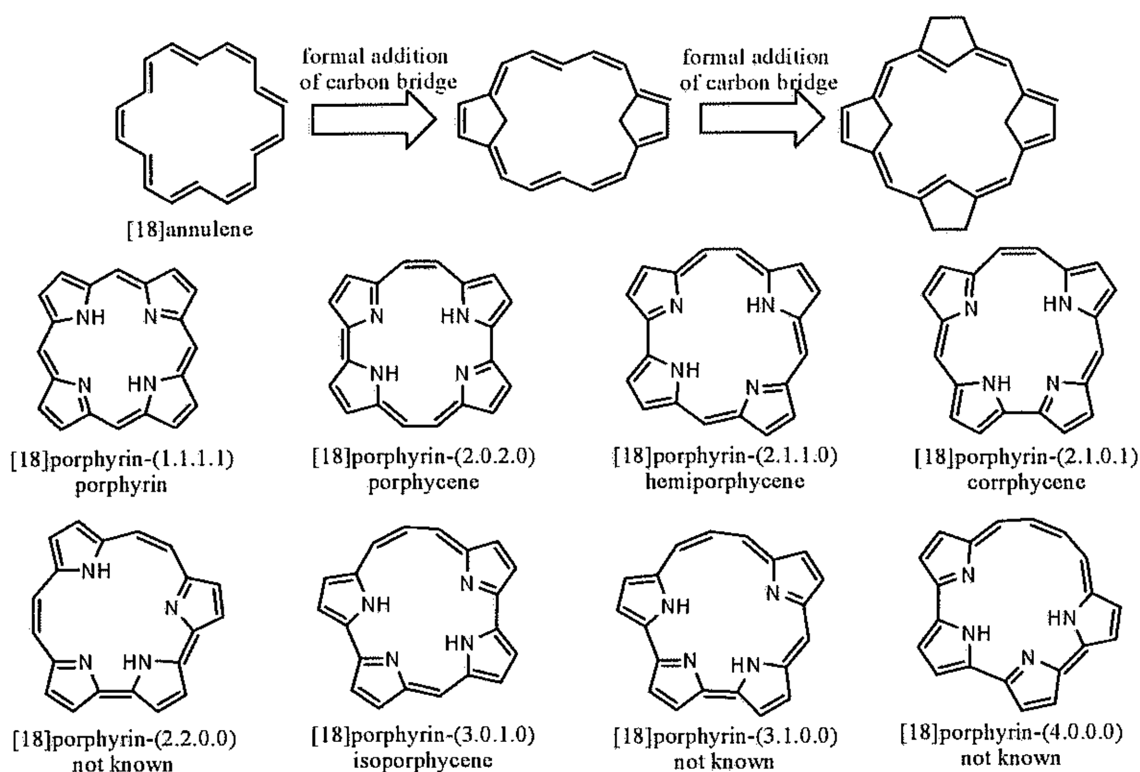


Figure 2

Nomenclature of Porphyrins

The term „tetrapyrroles" is used very often in connection with porphyrins, but the group of tetrapyrroles includes also linear bile pigment in addition to porphyrins. In 1930's „Fischer's nomenclature" evolved, which was full of trivial names. The basic ring structure was called porphine and it was numbered as shown in figure *3a*. This nomenclature wasn't appropriate any more, so since 1979 we have use IUPAC recommended nomenclature, which is partially based of Fischer's names. The basic skeleton of porphyrins is called porphyrin and it is numbered according to figure *3b*[4].

The 2,3,7,8,12,13,17 and 18 positions are commonly named as β-positions and those at 1,4,6,9,11,14,16 and 19 are α-positions (derived from trivial pyrrole numbering), while those at 5,10,15 and 20 are designated as meso-positions (*fig. 3c*). These generic terms are discouraged to avoid possible ambiguity with stereochemical designations (α,β).

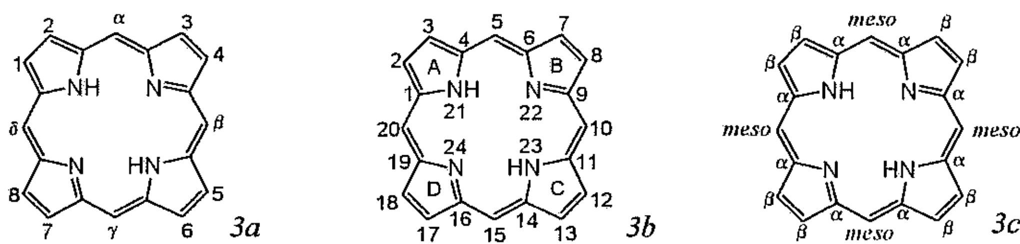


Figure 3

Other important structures are mentioned below *(fig. 4)*.

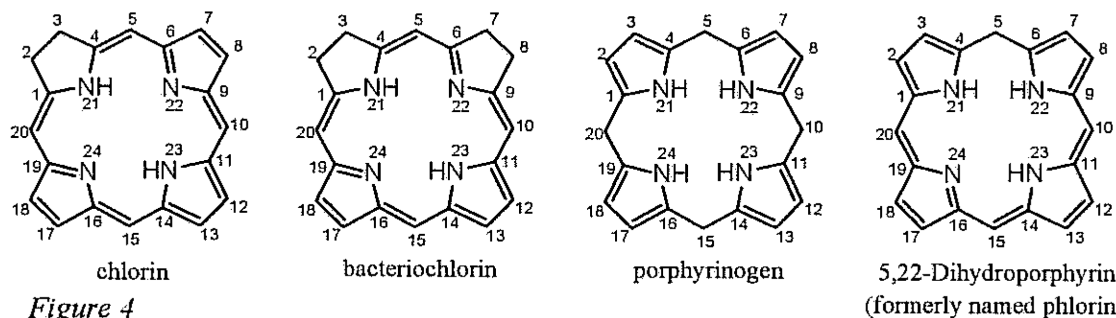
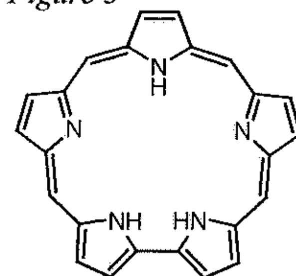


Figure 4

Owing to the complexity of IUPAC nomenclature it is still quite usual, that original discovers name their compounds with name derived from a word stem based on the colour or other feature of the macrocycle, followed by the suffix „phyrin“ or „rin“ taken from porphyrin. Woodward began this trend when he assigned the trivial name „sapphyrin“ to a pentapyrrolic macrocycle that he found crystallizes as a dark blue solid. Sessler followed this when he named the bright red, six-pyrrole-containing macrocycle „rubyrin“ and the large, „Texas-sized“ system „texaphyrin“. In effort to make systematic nomenclature Franck suggested this rule: for a given macrocycle, the number of pyrrole or pyrrole-like substituents is first determined and the system designated as being a porphyrin (4 subunits), pentaphyrin (5 subunits), hexaphyrin (6 subunits), ect. This compound name is then preceded by a number (in brackets) that indicates the shortest π-conjugation pathway available to the molecule, it is also followed by a sequence (in parentheses), which, starting with the largest bridging spacer and continuing around the ring, serves to define the number of carbon (or other) atoms between the heterocyclic subunits (see example for sapphyrin on *fig. 5*). This system together with rules for substitutive nomenclature is sufficient for systematic naming of compounds of this kind[1].

Figure 5



[22]pentaphyrin-(1.1.1.1.0)
sapphyrin

Chemical Properties of Porphyrins

General Porphyrin's Chemical Properties

The basic structure of porphyrin, with molecular formula $C_{20}H_{14}N_4$, consists of four pyrrole units linked by four methine bridges to make a ring. The porphyrin macrocycle is an aromatic system containing 22 π-electrons, but only 18 of them are involved in delocalization pathway (designated in red). It obeys Huckel's rule of aromaticity in that they possess 4n+2 π-electrons (n=4) which are delocalized over the macrocycle and this has also been proved by X-ray crystallography *(fig. 6)*[5].

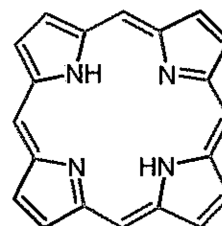


Figure 6

Isomerism in Porphyrins

There had been no isomer from porphyrin prepared until 1986, when Vogel and his co-workers synthesised [18]porphyrin-(2.0.2.0) called porphycene. No new examples of porphyrin isomers had appeared in literature till the end of 1993. But the year 1994 was very fruitful in the synthesis of them: four independent works reported together about 3 new isomeric compounds, namely: [18]porphyrin-(2.1.0.1), [18]porphyrin-(2.1.1.0) and [18]porphyrin-(3.0.1.0) with these trivial names: corrphycene, hemiporphycene and isoporphycene *(fig. 2)*.

A different type of porphyrin isomers has appeared recently, these isomers called "mutant" or "N-confused" porphyrins contain some pyrroles linked in an α-β' fashion, that means one or more nitrogen atom is located outside of the core of the macrocycle *(fig. 7)*[1].

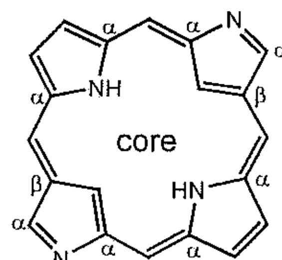


Figure 7

Conformations of Porphyrins

In spite of well proved porphyrin planarity, there occur numerous synthetic studies dealing with porphyrinoid molecules with non-planar features ranging from small localized distortions in mostly planar porphyrin macrocycles to grossly distorted macrocycles bearing little resemblance to planarity. At present, the most powerful analytical tool for evaluating the non-planarity of porphyrin 3-dimensional structure is X-Ray crystallography. Non-planarity of porphyrin is caused by many factors including: peripheral substitution, central metallation, substitution of the core nitro-

gens and axial ligation. The basic porphyrin conformations have following names: saddled, ruffled, domed and waved conformation and they are pictured in figure *8*[5].

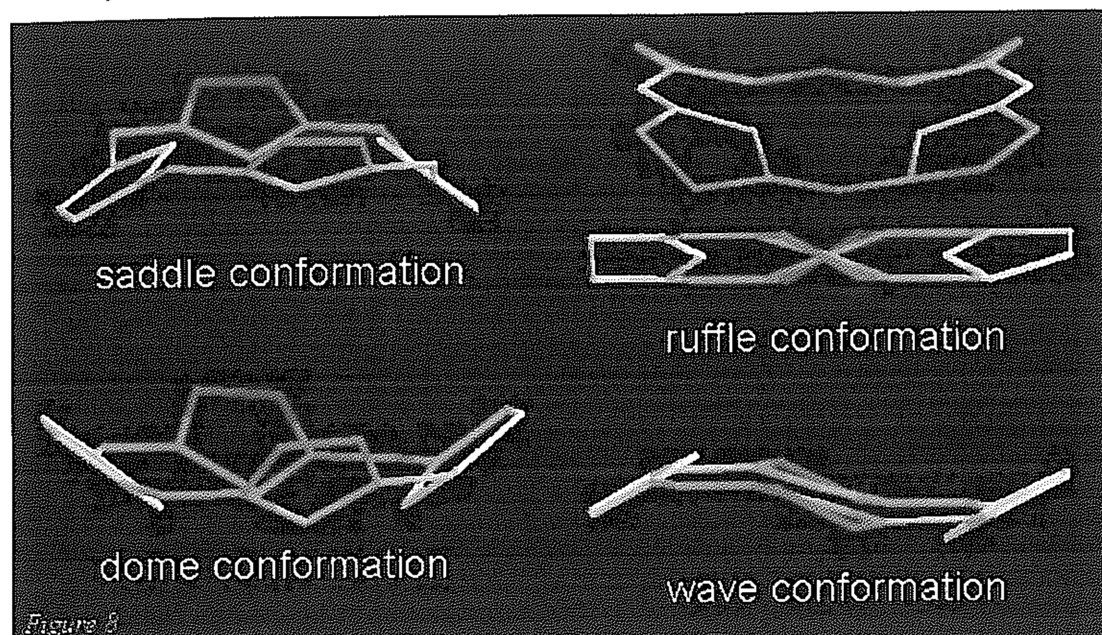


Figure 8

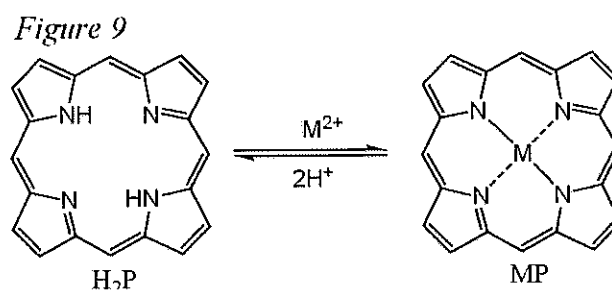
Stability and Acidobasic Properties of Porphyrins

The porphyrin ring is very stable to concentrated acids (e.g. sulphuric acid), and can exhibit amphoteric properties. Strong bases such as alkoxides can remove the protons (pK_a ~16) from the inner nitrogen atoms of a porphyrin to form a dianion. Conversely, the two pyrrolic nitrogen atoms bearing lone pairs of electrons (pK_b ~9) can be protonated easily with acids such as trifluoroacetic acid[5].

Complexation and Metalloporphyrins[6]

Any porphyrin derivative in which at least one of the central nitrogen atoms of a porphyrin (H_2P) forms a bond to a metal atom is called metalloporphyrin (MP). The simplest case is the monometallic metalloporphyrin, the synthesis of which is formulated in figure *9*. In most cases, the formation of such a metalloporphyrin involves the reaction of the porphyrin free acid (H_2P) with metal salt (MX_2), producing MP and corresponding acid molecules HX, which are liberated accordingly. This process is called metallation and its reverse is then a demetalation. Porphyrin molecule works as a ligand with four donor nitrogen atoms

Figure 9



arranged in square, the hole in the middle has diameter approximately 0,419nm. Two of N-atoms can make coordination-covalent bond and another two (NH-atoms) provide covalent bond.

By 1975, almost all metals and some semimetals had been combined with a porphyrin ligand, these elements are shown in the so-called „periodic table of metalloporphyrins“ *(fig. 10)*. The development of this periodic table took place in 5 stages:

Stage I: metalloporphyrins provided by nature: Mg (in chlorophyll), Fe (in heme), Ni and V (in mineral oils), Cu (Turacus indicus, bird from Central Africa), Mn (in blood), Zn (in yeast) and Co (vitamin B12).

Stage II: decade before the year 1913: artificial insertion of Fe, Co, Mn, Cu, Zn, Ni and Sn into porphyrins.

Stage III: culmination of Hans Fischer's work in 1940's: Na, K, Mg, Zn, Cd, Hg, Cu, Ag, Ni, Pd, Pt, Co, Fe, Mn, Al, Ga, In, Tl, V, Ge, Sn, Pb, As and Sb.

Stage IV: publication of Falk's monograph on porphyrins and metalloporphyrins in 1964 (discovery of axial ligands and possibility of addition, elimination and substitution of them), new metals: Li, Rb, Cs, Be, Ca, Sr, Ba, Si and Au.

Stage V: periodic table completed, new metals since 1964: Sc, Y, La, all the lanthanides, Ti, Zr, Hf, Th, Nb, Ta, Cr, Mo, W, Tc, Re, Ru, Os, Rh, Ir, P and Bi.

Stage I
Stage II
Stage III
Stage IV
Stage V
non-metals or other elements

Figure 10

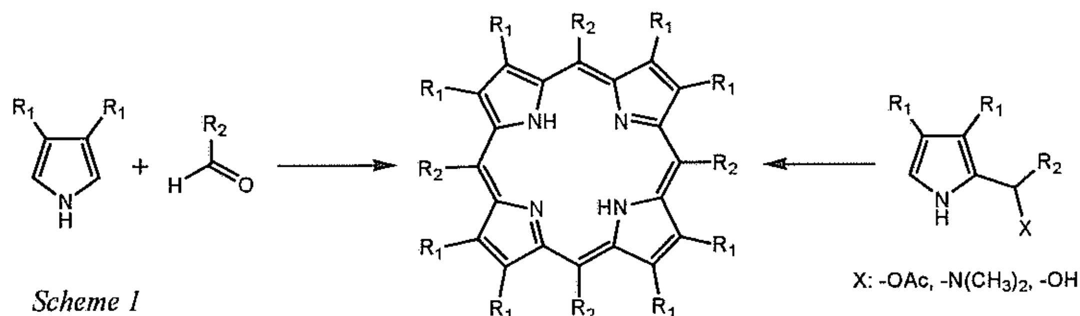
Syntheses of Porphyrins[6,7]

There are two general ways to obtain a desired porphyrin: by modification of a naturally occurring porphyrin (e.g. heme, chlorophyll) or by total synthesis. Although convenient, modification of naturally occurring porphyrins poses great limitations on the choice of peripheral substituents because certain substituents cannot be modified easily. In most cases, such limitations can be overcome by total synthesis, which involves the syntheses of the pyrrole subunits having the required substituents. The following are the methods commonly used in porphyrin total synthesis: tetrameriza-

tion of monopyrroles, condensation of dipyrrolic intermediates or cyclization of open-chain tetrapyrroles.

Tetramerization of Monopyrroles

This method is suitable for choosing if we want to obtain porphyrin containing only one type of *meso*-substituent, β-substituents on pyrrole rings or combination of these two possibilities. We use a pyrrole and an aldehyde providing the bridging methine carbons as a starting material. If we want to prepare the mixture of isomers we can start with two different aldehydes. Another approach of monopyrrole tetramerization involves the self-condensation of a 2-acetoxymethylpyrrole or 2-N,N-dimethylaminomethylpyrrole *(scheme 1)*.



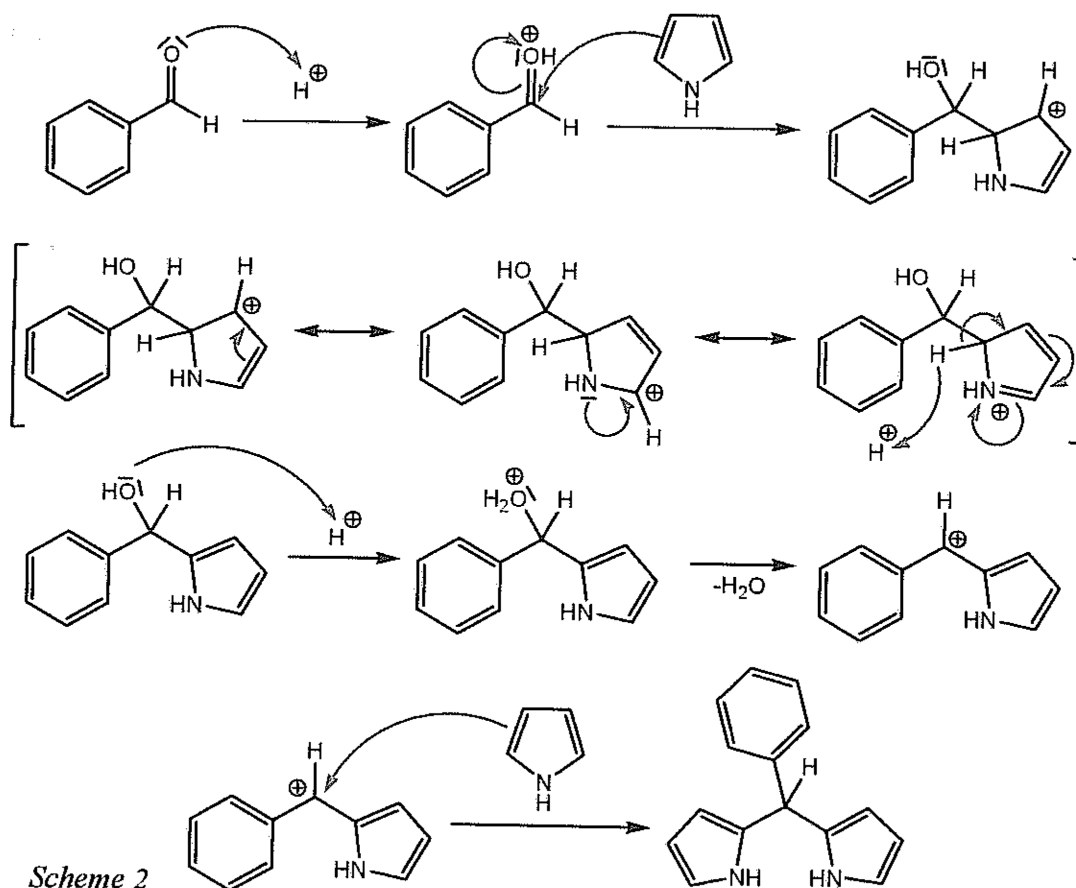
Scheme 1

There were a lot of suggested mechanisms for this reaction:

Badger, Jones and Laslett proposed production of substituted pyrrolylmethanols in first step, which loses water to give reactive double bond or oxidize to ketones. Carbonylic group of ketone provided condensation with another pyrrole, to make dipyrrylmethane. Then two molecules of dipyrrylmethane condense together in similar way to the occurrence of porphyrin.

Adler, Longo and Shergalis continued with study of aerobic condensation of pyrrole and benzaldehyde in acidic medium and they proved that the oxidizing equivalents necessary for the reaction were completely supplied by atmospheric oxygen.

Kim, Leonard and Longo suggested carbonium cation to be reactive species, which result from the reaction between protonated benzaldehyde and α-position of the pyrrole by loss of water. This carbonium ion then attacks the free α-position of another pyrrole *(Scheme 2, on the next page)*.



Scheme 2

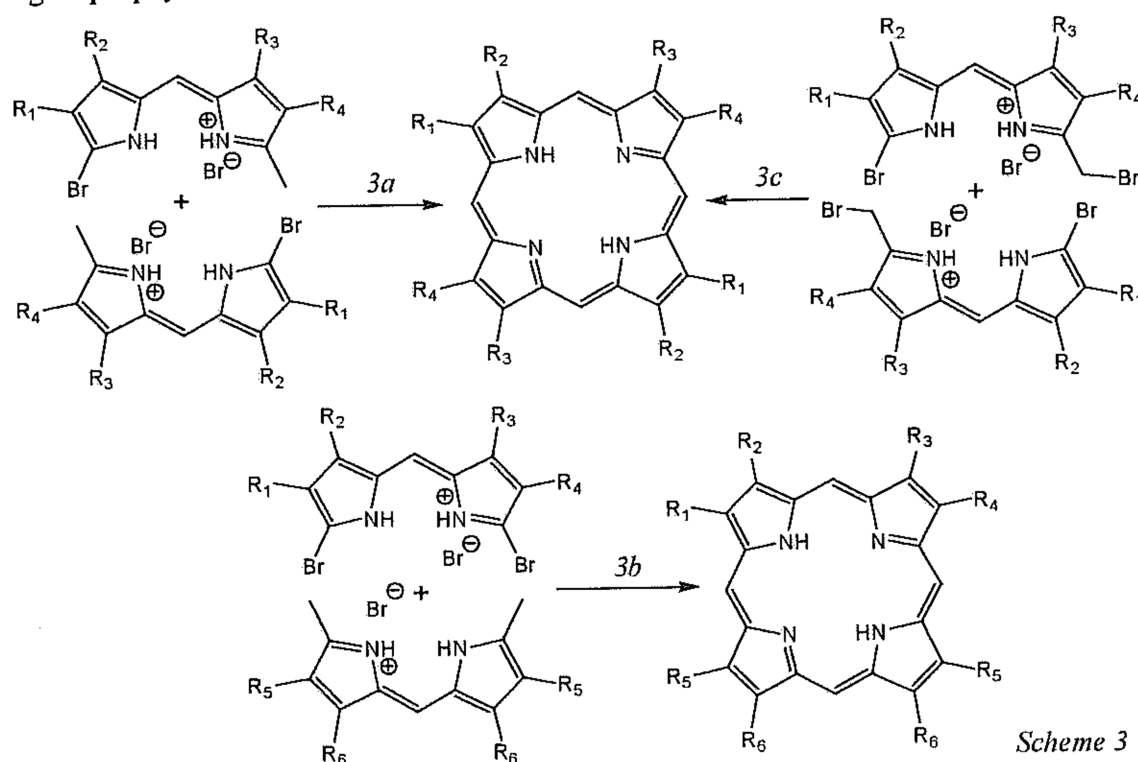
Chain building continues producing tetramers and longer oligomeric by-products, tetramers may be present at various stages of oxidation, can cyclize to form the porphyrinogen, chlorin, phlorin and other reduced cyclic tetrapyrroles. Adler et al. suggested that the ring closure resulted in formation of the phlorin which could be then rearranged to the chlorin or be oxidized to the porphyrin. This oxidation can be supported with some oxidizing agent (e.g. DDQ).

Condensation of Dipyrrolic Intermediates

This approach of porphyrin synthesis is convenient for the formation of porphyrins that are centrosymmetrically substituted as well as porphyrins that have symmetry in one or both halves of the molecule. Three types of dipyrrolic intermediates can be used: dipyrrins, dipyrromethanes and dipyrroketones.

Dipyrrins

Developed by Fischer, the self-condensation of 1-bromo-9-methyldipyrrins in an organic acid melt (e.g. succinic acid) at temperatures up to 200°C gives good yields of porphyrins *(Scheme 3a)*. By condensing a 1,9-dibromodipyrrin and a 1,9-dimethyldipyrrin, this method can also be used to synthesize porphyrins in which one or both halves of the molecule are symmetrical *(Scheme 3b)*. A variation of this method involves the reaction of 1-bromo-9-bromomethyldipyrrins in formic acid to give porphyrins in relatively high yields *(Scheme 3c)*.



Scheme 3

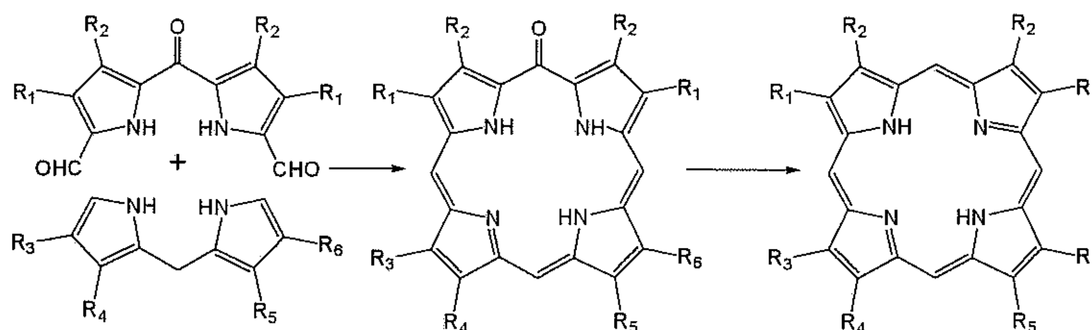
Dipyrromethanes

Although known in Fischer's time, the dipyrromethane route was not widely used because of the problem of pyrrole „redistribution" during the porphyrin formation leading to a mixture of products. However, this route became common after MacDonald, in 1960, had developed milder conditions for the reaction. The MacDonald synthesis involves the self condensation of 1-unsubstituted-9-formyldipyrromethanes or the condensation of a 1,9-diunsubstituted dipyrromethane and a 1,9-diformyldipyrromethane in the presence of an acid catalyst such as hydroiodic acid or p-toluenesulfonic acid. Reaction of two 1,9-diunsubstituted dipyrromethanes with aldehyde is also possible. These routes are widely used today also because the dipyr-

romethanes required for the MacDonald synthesis are often more easily prepared and purified than the corresponding dipyrrins.

Dipyrroketones

This synthetic route involving a dipyrroketone and a dipyrromethane is less convenient than the two discussed above because the initial product obtained is an oxophlorin, which needs to be converted into a porphyrin *(Scheme 4)*. The symmetry limitation and reaction condition follow those of the MacDonald synthesis with dipyrromethanes. It is also required that the dipyrroketone should contain the diformyl groups since 1,9-diunsubstituted dipyrroketones are not nucleophilic enough to react with 1,9-diformyldipyrromethanes.



Scheme 4

Physical Properties of Porphyrins

Absorption and Colour

Porphyrins are macrocycles with the system of highly conjugated π-electrons, which shows intense absorption (extinction coefficient > 200 000) in the UV-visible absorption spectrum at around 400 nm (the Soret band), followed by several weaker absorptions (Q Bands) at higher wavelengths (450 to 700nm)[3] (see *Spectrum 12*). The colour of porphyrins is determined by this absorption. The peripheral substituents on the porphyrin ring can vary the intensity and wavelength of these absorptions as well as protonation of two of the inner nitrogen atoms or insertion of a metal into the porphyrin cavity. So does the colour, it can range from purple over blue and green to black. Compounds I have synthesised in experimental part of my work are dark purple shiny crystals, purple in basic or neutral solution and green in acidic solution.

The disruption of π-electron conjugation (e.g. by hydrogenation of methine bridges) leads to roughly different absorption of these compounds connected with change of colour (e.g. porphyrinogens are colourless, because of this phenomenon).
The porphyrins dissolved in strong mineral acids or organic solvents and illuminated by UV light emit a typical strong red fluorescence in range from 620 to 750nm, which can also serve for their detection[8].

Usage of Porphyrins[9,10]

The group and chemical variety of porphyrins is very large as well as their application in many fields. Porphyrins are used in technology as supramolecular catalysts, transporters of anions and other bioinorganic substrates, liquid crystals, in microelectronics for organic light-emitting diodes (OLED), electrically conductive organic material, chemoresponsive materials and sensors and hybrid materials (porphyrins and fullerens) and dyes[11]. Further are synthesised porphyrins-containing compounds as models of haemoproteins: cytochrome P-450 model, hemoglobin and myoglobin models and biomimetic systems as photosynthesis models and for interactions between DNA and porphyrins (DNA intercalations, DNA cleavage). In medicine there are porphyrins used for diagnosis and as photosensitizers in therapy of tumours and other serious diseases.

Photosensitizers in PDT[12]

As I mentioned before photosensitizer is a kind of compound, which can, after its excitation, convert molecular oxygen to its reactive and cytotoxic form – singlet oxygen. The production of singlet oxygen depends on many factors, but mainly on properties of the photosensitizer. Looking for the optimal properties of photosensitizers is a very important way of how to decrease side-effects of PDT.

Properties of the Optimal Photosensitizer

Chemical Purity

PS should be chemically pure and of well known structure or composition. This was the problem of HpD. Hematoporphyrin derivative was a mixture of mono-, di- and oligomers of derivatised hematoporphyrin. Newly synthesized PSs are mostly well characterized compounds.

Photochemical Stability

Photobleaching is the common property of dyes and it means that dyes become more pale after long effect of the light and heat. Also PSs, which belong to this group of dyes, follow photobleaching, but we call it loss of intensity of absorption caused by light in this case. PSs undergo chemical changes (photoaddition, photocyclisation, irreversible photoreduction or most often oxidation) resulting in damage of chromophore.

We know two types of irreversible chemical changes: photomodification and so called „proper photobleaching". There is observable loss of absorption or fluorescence in specific wavelenght without any change of chromophore in the case of photomodification. In the case of „proper photobleaching" the chromophore is decomposed into small fragments, which don't have significant absorption in visible light spectrum. Thanks to this the PS loses its cytotoxic properties, but this on the other hand can help in excretion of PS from skin.

Minimal Toxicity in Darkness

Optimal PS should be cytotoxic only after irradiation by light of proper-defined wavelenght, which causes its activation. Any biological action without this activation we can consider as a side-effect.

Selective Accumulation

PS should be widely accumulated in its target (tumorous tissue, atherosclerotic plaque, etc.). Accumulation in another sites causes side-effects (e.g. deposition of PS in skin can make long and serious photosensitivity in patients). We can change the accumulation by changing of logP, or by using of another specific mechanism of absorption to the target tissue.

Rapid Excretion

PS remaining in blood circulation after accumulation in target tissue could cause systemic intoxication. Therefore its rapid excretion from the body is so important and desirable. This property also depends mainly on the hydrophilicity of PS.

Good Photochemical Properties

We can compare and quantify photochemical properties using Triplet state quantum yield (ΦT) and Triplet state lifetime (τT). Both of these quantities should be high for good production of singlet oxygen.

Good Spectral Properties

Vital tissues are not well permeable for electromagnetical radiation, that's why good PS should have large absorption with high extinction coefficient in longer wavelenghts (650–800nm).

Price, Availability and Modifiability

PS should not have to be very expensive, it must be easily available or not difficult to synthesize and suitable for simple modification of structure for improvement of pharmacokinetic parameters.

Division of Photosensitizers

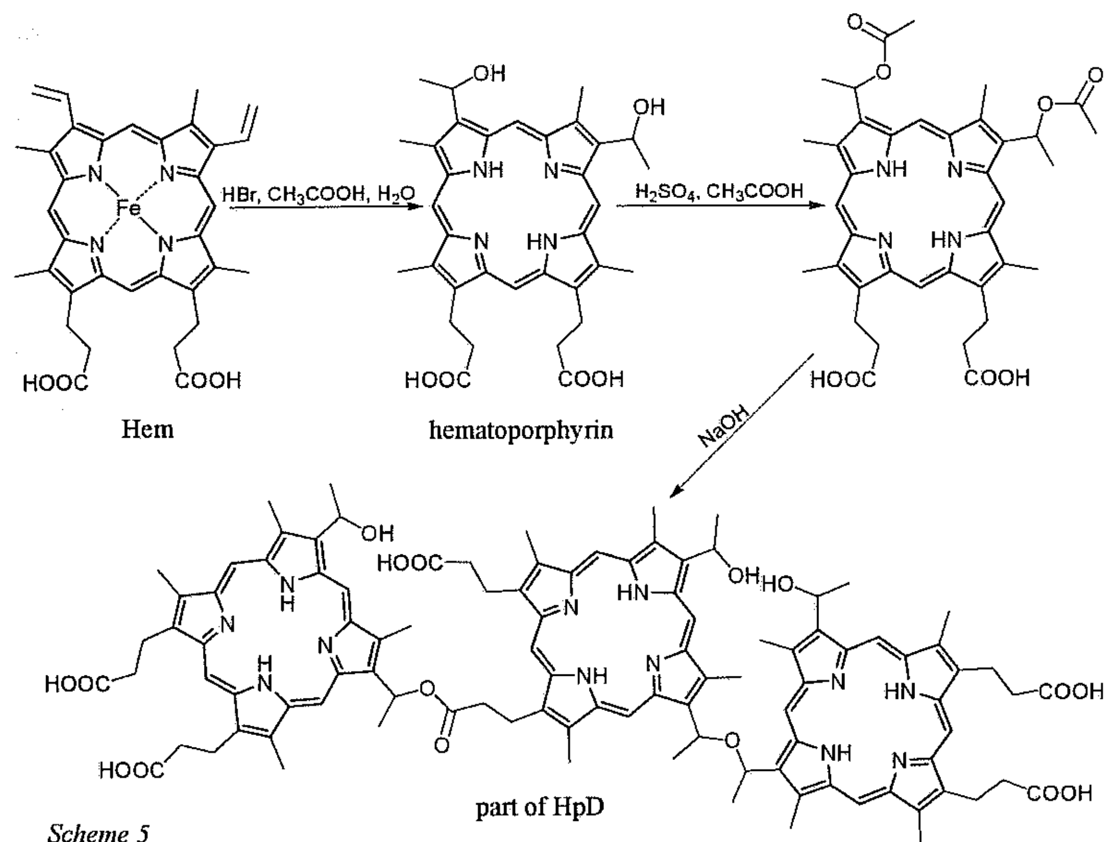
We divide photosensitizers into three generations at present time. First generation includes probably only HpD, which has some disadvantageous properties. Due to these properties, compounds of the second generation were prepared. In addition to porphyrins, compounds with another chemical structure belong to this group. Third generation consists of PSs of the second generation which are bounded to biomolecules which enable selective biodistribution in human body.

First Generation

HpD (=hematoporphyrin derivative, porfimer sodium, Photofrin®)
HpD is a mixture of products obtained from Hem by applying of procedures mentioned below. Hem is removed from hemoglobine, then in the second step the atom of iron is removed from the centre of Hem molecule using HBr in acetic acid. During this process addition of HBr to the peripheral double bonds takes place and in next step, in the aqueous medium the bromide is converted to hematoporphyrin. Hematoporphyrin has been used since the beginning as PS, but it was discovered later that not only this compound is responsible for the PDT effect. Besides of hematoporphyrin there were oligomeric fractions isolated from blood, which were mainly responsible for the effect. Schwartz[13] enriched isolated mixtures with dimers and oligomers

treating with H_2SO_4 in acetic acid followed by neutralisation with NaOH. This procedure led to complicated mixture of dimers and oligomers, in which the units were joined by ester of ether bonds. This mixture was partially purified by gel chromatography yielding a base of Photofrin® *(Scheme 5)*.

Photofrin® was developed by QLT PhotoTherapeutics (Vancouver, Canada) and established in therapy of cancer of urinary bladder in 1993 in Canada as a first compound in PDT. The patent was sold to AxcanPharma (Montreal, Canada) in 2000.



Scheme 5

Unfortunately, Photofrin® has several disadvantageous properties:

- Very low wavelength for its activation (λ~630nm) and also a very low extinction coefficient in this absorption maximum (1170 $M^{-1}cm^{-1}$). That's why we have to use high doses of Photofrin® as well as very good source of light.
- After purification there are still about 60 compounds in the mixture, so it isn't easy to reproduce its composition.
- Also the uptake to tumorous tissues isn't significantly selective, we can find it in liver, kidney, spleen and especially in skin in high concentration[14]. Accumulation of Photofrin® in skin causes its main side-effect, photosensitivity.

Photofrin® plays regardless the key role in treatment of precancerous lesions (Barrett's oesophagus, Cervical dysplasia) and also in treatment of cancer (cancer of cervix, oesophagus, urinary bladder, stomach and bronchus).

Photofrin® is further tested of using in treatment of another kinds of cancer (cancer of head and neck, intestine, skin, breast and in non-cancerous diseases like psoriasis and in prevention of arterial restenosis[15].

There are also derivatives of Photofrin® in use, e.g. Photohem® in Russia, which is used in curing of cancer of skin, breast, oesophagus, lungs, larynx and stomach.

Second Generation

PSs of this generation were prepared synthetically, therefore they are well characterised pure chemical entities. The wavelength of their absorption maxima is usually higher and they can be activated by the light deeper in the tissue. PSs of this group belong to tetrapyrroles, which include e.g. porphyrines, phthalocyanines, texaphyrins, porphycenes etc.

Verteporfin (BPD-MA, Visudyne™)

The basic structure of verteporfin *(fig. 11)* is close to porphyrins, the abbreviation BPD-MA came from benzoporphyrin-derivative monoacid ring A. Absorption maximum is moved to higher wavelength (λ~690nm), the light of this wavelength is approximately twice more tissue penetrating than the light at 630nm. The uptake of verteporfin to tumorous tissue is very quick, but so is the excretion from these tissues and also from whole body. That's why the possible skin photosensitive reaction is very short (max. a few days). As the biodistribution in the human body proceeds, the best concentration ratio between tumorous tissue and other tissues is achieved between 30 and 150 minute after parenteral application, then the concentration in the tumorous tissue falls down quickly and the selectivity to tumour isn't complete.

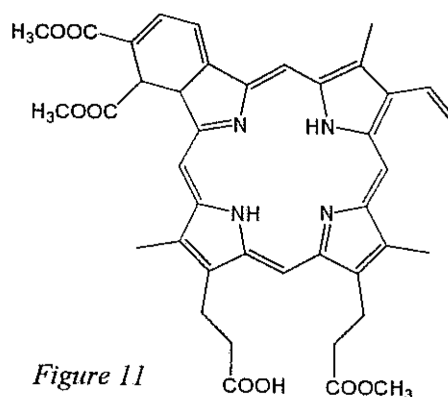


Figure 11

However the selectivity to tumours isn't ideal, this compound is in the beginning of clinical trial in cancer treatment. The biggest success was reached by QLT PhotoTherapeutics in ophthalmology. Verteporfin was introduced to the market in 1999,

since this time it was registered in 71 countries for curing of age-related macular degeneration (AMD). Its trademark is Visudyne™.

Temoporfin (mTHPC, Foscan®)

This compound *(fig. 12)*, which belongs to chlorins group, was prepared already in 1989 as one compound in a series of porphyrins and it appeared to be the most efficient. Temoporfin doesn't have such high absorption maximum wavelength (652 nm), but it has 10x higher absorption than verteporfin in absorption maximum (ε_{652} = 22400 $M^{-1}cm^{-1}$). That's why temoporfin is one of the most photocytotoxic compounds tested at present time and we need only small doses (cca 0.1 mg/kg) as well as only small doses of light. If we consider this advantage and quite high singlet oxygen production, we can say temoporfin is approximately 100x more efficient than verteporfin.

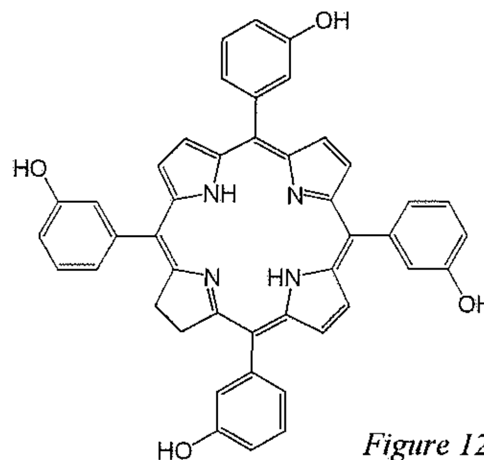


Figure 12

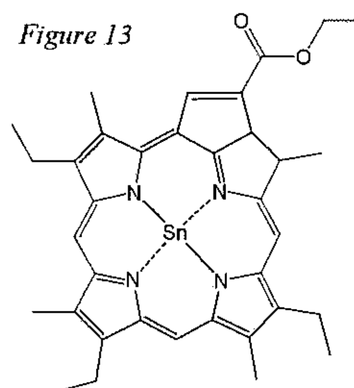
Althought temoporfin is weak in its pharmacokinetics, the uptake to the tumorous tissue is less selective (ratio between tumorous and healthy tissue is about 1.3–2.9)[16]. Also the time gap between application and irradiation (3-4 days) is too long. As a next disadvantage we can consider relatively long lasting skin photosensitivity in patients.

In spite of these unfavourable pharmacokinetic parameters Foscan® (Biolitec Pharma, Edinburgh, UK) was established in therapy of head and neck cancer, resistant to other kinds of therapy, in EU, Norway and Island in October 2001. Foscan® was refused by FDA in USA year before. Foscan® is tested in clinical trial for its use in treatment of Barrett's oesophagus dysplasia, cancer of stomach, oesophagus, skin, prostate gland and hyperplasia of prostate.

Rostaporfin (Tin ethyl etiopurpurin, SnET2, Photrex®)

Also this compound *(fig. 13)*, which belongs to the groups of metallochlorins, has large absorption in λ~660nm, however we need high doses of light for efficient therapy. Skin photosensitivity isn't much lesser than in the case of HpD (remains 10–14 days

Figure 13



after application).

Photrex® was tested by Miravant Medical Technologies (Santa Barbara, USA) as an agent for curing AMD. Phase III of clinical trial was completed successfully and FDA has accepted it. Photrex® is going to expand to Europe. Manufacturer company cooperate further with research groups at American College of Cardiology (New Orleans) dealing with photodynamic therapy in reduction of atherosclerotic plaque inflammation, induction plaque stabilization and promotion of plaque reduction[17]. SnET2 runs also phase II of clinical trial in testing for skin metastases of breast cancer and Kaposi's sarcoma in AIDS patients[18].

Talaporfin (mono-L-aspartyl chlorin e6, NPe6, Litx™)

Talaporfin *(fig. 14)* is a next member of synthetic chlorins group with large absorption in the area of higher wavelength (664nm, ε_{664} = 38000$M^{-1}cm^{-1}$). Similarly to verteporfin, the excretion is fast and the tissue is irradiated 4 hours after intravenous application. Using small doses of PS and light, the removal of tumorous tissue is only short-acting. Under higher doses the treatment can be complete, but the tumour selectivity is endangered. Skin photosensitivity is considered to be temporary[19].

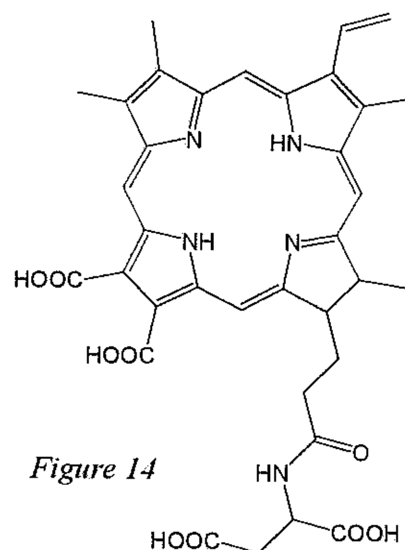


Figure 14

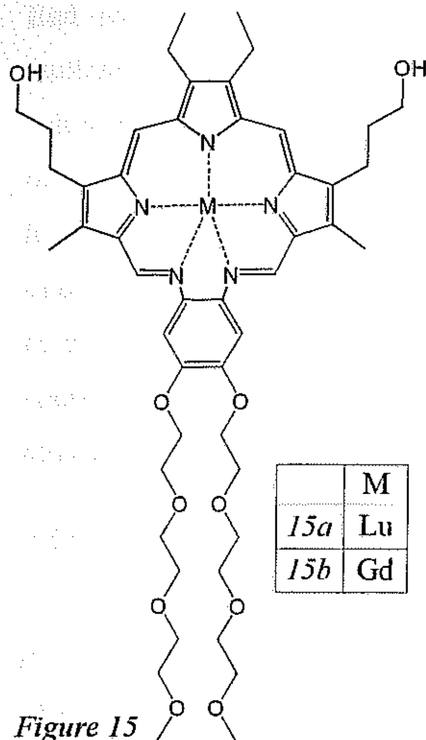
There is successfully finished phase III of clinical trial testing of talaporfin for early stadium of lung cancer and this compound is being prepared for commercial use in this indication in Japan. Light Sciences Corporation (Snoqualmie, USA) includes three subsidiary companies developing Litx™ for AMD and other eye diseases (phase I/II of clinical trial in progress), for primary liver cancer and colorectal liver metastases (phase II of clinical trial finished) and for atherosclerosis (research in the beginning)[20].

Motexafin Lutetium (Antrin®, injection previously was called Lu-Tex)

Motexafin lutetium *(fig. 15a)* having a little bit different chemical structure belongs to texaphyrins group and it has centrally chelated lutetium ion.

This PS is advantageous in its large absorption at longer wavelength (732nm), in good tumour tissue selectivity and also in good excretion from blood plasma. Due to fine pharmacokinetics we can apply the light irradiation 3 hours after application of PS, which is followed only with weak photosensitivity[21]. Very fast clearance of

motexafin lutetium enables repetitive PDT application, which shows better results than single application in animal models. Considering its high absorption wavelength it can be used for intensely pigmented tumours (malignant melanoma)[22].



	M
15a	Lu
15b	Gd

Figure 15

Motexafin lutetium, cumulating in vulnerable plaque, is tested by Pharmacyclics Inc. (Sunnyvale, USA) under trademark Antrin® for coronary artery disease and phase I of clinical trial was completed. The clinical trial suggests that the light-activated motexafin lutetium procedure can be incorporated into current medical practice; i.e., placement of the light delivery fibre can be accomplished with customary interventional techniques, such as PCTA. Current and future work on motexafin lutetium development is focused on the potential use of light-activated motexafin lutetium in the treatment of vulnerable plaque to reduce or eliminate atherosclerosis[23].

Motexafin Gadolinium (Xcytrin®, injection previously was called Gd-Tex)

Motexafin gadolinium *(fig. 15b)*, close relative of motexafin lutetium, is tested by Pharmacyclics Inc. (Sunnyvale, USA) under trademark Xcytrin® for oncological application. Clinical trial of Xcytrin® for brain metastases from lung cancer is in phase III and for glioblastoma (a type of primary brain cancer), paediatric brain tumours, lymphomas (including non-Hodgkin's lymphoma), chronic lymphocytic leukaemia (CLL), multiple myeloma, renal cell cancer, head and neck cancer, lung cancer and advanced stages of other solid tumours is in phase I or II[23].

Phthalocyanines

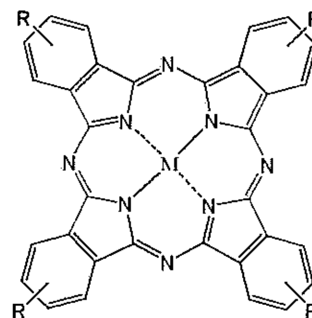


Figure 16

Phthalocyanines *(fig. 16)* are next group of PSs promising application in PDT. They have their maximal absorption shifted to the red part of light spectrum (670–700nm) and very large absorption in these wavelengths (ε up to $200000 M^{-1} cm^{-1}$). The central chelated ion or atom of metal or semimetal (e.g. Zn, Al, Si), prolonging the duration of triplet state of PS, is important for proper PDT effect. The disadvantage of

several phthalocyanines is their poor solubility in water and also in organic solvents. Bad solubility of phthalocyanines is solved by peripheral structural modification (sulfonation) or by encapsulation to liposomes. Sulfonated phthalocyanines are difficult to separate. In spite of this fact the mixture of products was tested for treatment of skin, breast, lung and digestive system cancer under name Photosense in Cancer Research Center AMS of Russia, Moscow. Badly soluble zinc complex of unsubstituted phthalocyanine (M = Zn, R = H) was tested, in form of liposomes in phase I/II of clinical trial (Ciba-Geigy, Basell, Switzerland and QLT PhotoTerapeutics, Vancouver, Canada), for therapy of squamous skin cells cancer[15].

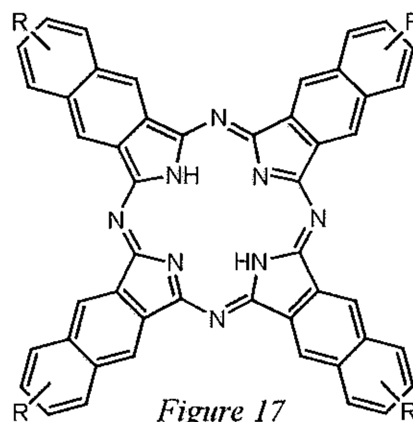


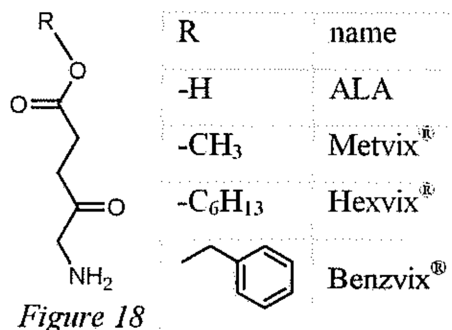
Figure 17

Naphthalocyanines

Naphthalocyanines can be formally created from phthalocyanines by addition of 4 next benzene rings. These compounds absorb the light of very long wavelength (up to 770nm) and it makes possible to use these compounds for therapy of highly pigmented tumours (e.g. melanoma).

5-aminolevulinic acid (ALA)

The structure of ALA differs from tetrapyrroles a lot, however, it is also used in PDT. ALA is endogenous intermediate participating on biosynthetic pathway of heme. This pathway is controlled by negative feedback: higher concentration of heme inhibits conversion of glycine and succinylcoenzyme A to ALA. Exogenous addition of ALA enables to avoid this control mechanism and leads to synthesis of protoporphyrin IX, which acts then as a potent PS. Ferrochelatase catalyses incorporation of iron atom to the centre of protoporphyrin IX and molecule of photodynamically inactive heme is created. Selectivity as well as efficiency of ALA therapy depends on many factors of tumorous tissue (e.g. increased permeability of abnormal keratin, increased level of porphobilinogen deaminase, decreased iron concentration and decreased ferrochelatase activity). These conditions stop conver-



R	name
-H	ALA
$-CH_3$	Metvix®
$-C_6H_{13}$	Hexvix®
[benzyl]	Benzvix®

Figure 18

sion to photodynamically inactive heme and lead to cumulation of protoporphyrin IX in tumorous tissue.

In 1997, DUSA Pharmaceuticals completed phase III study on Levulan® for actinic keratoses (skin precancerosis) and now there is Levulan® Kerastick®, topical solution and blue light illuminator, commercially available. Another product of DUSA Pharmaceuticals is BLU-U® 4170 which offers effective, non-invasive treatment for moderate inflammatory acne vulgaris using narrow band blue light (λ~417nm), which activates endogenous photosensitizers within the Propionibacterium acnes and kills this, for acne vulgaris primarily responsible, bacteria[24].

Because of ALA low lipophilicity, its esters showing much much better tissue permeability were synthesised. These esters were developed and are sold by PhotoCure ASA (Oslo, Norway) under names Metvix®, Hexvix® and Benzvix® *(fig. 18)*.

Metvix®, methyl aminolevulinate, was established for therapy of actinic keratoses in 2001, and later for therapy of basal cell carcinoma.

Hexvix®, hexyl aminolevulinate, is in use for detection of bladder cancer (induces tumour fluorescence, which makes it much easier for the urologist to find all relevant lesions in the bladder) since 2002. Usage of Hexvix® in therapy of bladder cancer as well as applications in gynaecology are further developed[25].

Benzvix®, benzyl aminolevulinate, is being developed as a product for photodiagnostics and photodynamic therapy for lesions in the gastrointestinal tract, and the first patients were enrolled in a pilot study in 2002.

Other Photosensitizers

Compounds mentioned above belong to main PSs, but there are also other PSs in use or in clinical trial. These natural or synthetic compounds are tested for PDT or modified in their properties to fulfil or approach properties of an ideal photosensitizer.

Methylene blue

This for humans non-toxic phenothiazine derivative (*fig. 19*) is used in the treatment of drug-induced methemoglobinemia, in microbiology as a bacteriological stain, in some diagnostic procedures as a detection dye. Methylene blue was also used with good results for herpes simplex inactivation and plasma decontamination[26]. Local photodynamic action of methyl-

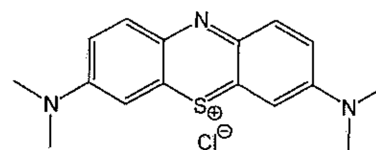


Figure 19

ene blue favourably modulates the postinterventional vascular wound healing response and helps prevent restenosis after balloon intervention[27]. Use of methylene blue is limited by enzymatic reduction to photodynamically inactive leucoform in cells.

Rhodamines

Rhodamine dyes are generally toxic. Because of their uptake by mitochondria they are used as fluorescent dyes with high fluorescence production. This behaviour isn't favourable for use in PDT, because it doesn't convert from S_1 to T_1-state, which plays the key-role in photodynamic action. This problem was partially solved by synthesis of brominated analogue *(fig. 20)*, which also shifted its absorption maximum closer to the red light area (λ_{max}=511nm, ε = 20000$M^{-1}cm^{-1}$). This analogue was applied in tests for autotransplant therapy of myeloid leukaemia[28].

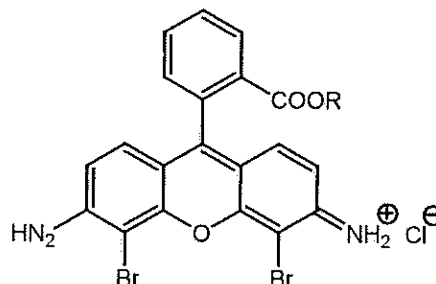


Figure 20

Porphycenes

Their absorption maximum fluctuates in range from 600 to 650nm. There were reached very good pharmacokinetic properties by peripheral methoxyethylation (increase of cellular uptake) and acetylation (increase of hydrophilicity), and 9-acetoxy-2,7,12,17-tetrakis-(beta-methoxyethyl)-porphycene (ATMPn, λmax = 630nm, ε_{630} = 52000 $M^{-1}cm^{-1}$) belongs to one of the best uptaken PS at all. ATMPn *(fig. 21)* is used in dermatology only for external application and enables therapy of basal cell carcinomas[29] and psoriasis.

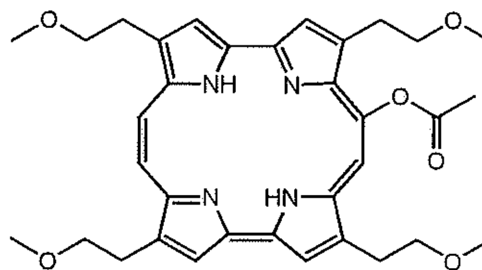


Figure 21

Hypericin

Hypericin *(fig. 22)* is a natural product found in Hypericum perforatum, known to cause phototoxicity appearing as cataract in calf after consumption of bigger amount of this medicinal herb. Hypericin

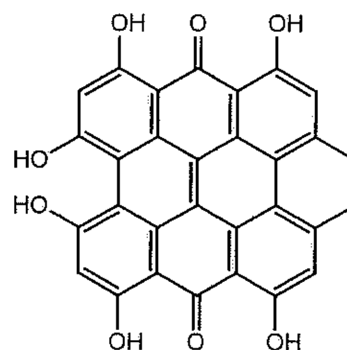


Figure 22

(λ_{max}=590nm, ε_{590} = 44000$M^{-1}cm^{-1}$) belongs to group of compound known as naphthodianthrones. In animal models, hypericin has shown to prevent replication of encapsulated viruses and anti-inflammatory activity as well. Hypericin is commonly used as an antidepressant and for novel ex vivo fluorescent cytological detection of bladder cancer[30].

Photoclor (HPPH)

Photoclor *(fig. 23)* belongs to chlorins group, chemically 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH). Analogues of pheophorbide-a are generally more active than similar compounds of chlorin-e6 group (e.g. talaporfin). Photoclor has λ_{max} = 665nm, ε_{665} = 50000$M^{-1}cm^{-1}$, it is highly selective for tumorous tissue and less skin phototoxic than Photofrin®[31]. Photoclor was tested for the treatment of malignant gliomas in rat models in 2001[32].

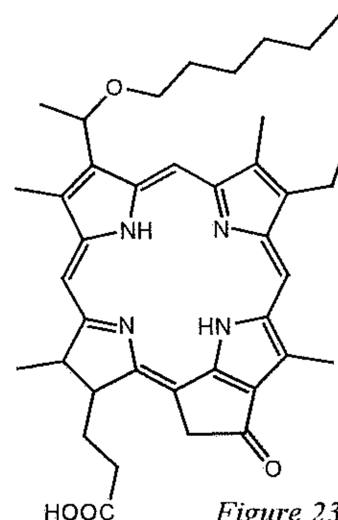


Figure 23

Sapphyrins

Sapphyrins *(fig. 24)* belong to expanded porphyrin group (as texaphyrins do). Their pentapyrrolic ring system with one direct bipyrrolic bond absorbs in range about 675nm. Some of them showed in tests with mice models high difference in concentration between tumorous and muscular tissue.

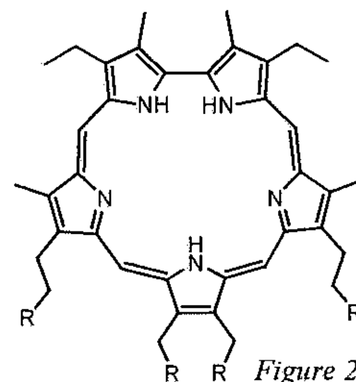


Figure 24

Psoralens

Psoralens *(fig. 25)* and their derivatives have been used for over 3000 years in the treatment of skin disorders and are still in use today. The cytotoxic action of these compounds stems from their ability to cross-link biomolecules, in particular DNA, following activation by ultraviolet light.

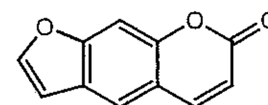


Figure 25

Basic information about these PS is summarized in the table on the next page. Particular values can differ according to the author[33].

Photosensitizer	Wavelengt (nm)	Extinction coefficient ($M^{-1}cm^{-1}$)	Application	Delivery vehicle	Typical dose (mg/kg)	Light dose (Jcm^{-2})	Time post-injection	Photosensitivity duration
HpD	630	3000	i.v. or topical	5% glucose	2-5	100-200	24-48h	2-3 months
Verteporfin	690	35000	i.v.	liposomes	0.1-2.0	100-200	30-150m	3-5 days
Temoporfin	652	30000	i.v.	PEG/EtOH/water	0.1-0.3	8-12	24-48h	< 6 weeks
Rostaporfin	660	28000	i.v.	lipid emulsion	1-2	100-200	24h	< 1 month
Talaporfin	664	40000	i.v.	water	0.5-3.5	25-100	4h	3-7 days
Texaphyrins	732	42000	i.v.	water	0.6-7.2	150	3-5h	minimal
Phthalocyanines	670-680	250000	i.v.	liposome or water	0.5-2	100	24-72h	8-10 days
Naphthalocyanines	750-780	>100000	i.v.	liposomes				
Protoporphyrin IX	635	<5000	topical, p.o. or i.v.	water	<60(p.o.) <30(i.v.)	100-200		1-2 days
Methylene blue	668	95000	ex vivo	water	1mM	50 klx		
Rhodamines	511	20000	ex vivo	water	25mM	1-10		
Porphycenes	630	52000	topical	liposomes	1-3			
Hypericin	590	44000	topical	liposomes				

Photodynamic Therapy

Photodynamic therapy is the combined application of specifically localized compound (photosensitizer) and light. Photosensitizer is a kind of compound, which can, after interaction with light, convert molecular oxygen (O_2) to its reactive form – singlet oxygen ($^1O_2$). Singlet oxygen is cytotoxic for cells, so it will kill them. Singlet oxygen, short-living product of the reaction between an excited photosensitizer molecule and oxygen, is cytotoxic for all kinds of cells of human or animal body, therefore application of photosensitizer must be well localized. In contrast to radiation therapy or chemotherapy, PDT has a low mutagenic potential and except for skin phototoxicity no serious side effects[34].

History of Using of the Light in Therapy

We can divide this field into three smaller groups: Phototherapy, Photochemotherapy and Photodynamic therapy.

Phototherapy [34]

Solar light has been used to treat a number of disorders such as vitiligo, psoriasis, rickets, skin cancer and even psychosis. Maybe heating have played psychological effect in these therapies, but the action of visible light and ultraviolet radiation on the skin was probably more important.

Phototherapy can be defined as the use of light alone for therapeutic purposes. Phototherapy has been applied by humans for 3000 years and was known by the Egyptians, the Indians and the Chinese. In Greece Herodotes called it „heliotherapy“ and recommended it for „restoration of health“ in the 2nd century BC. In the 18th century the effect of sunlight on rickets became known. In 1815 J. F. Carvin wrote that sunlight had a curing effect on „scrofula“ (=tuberculosis of nodules), rickets, rheumatism, scurvy, paralysis and muscle weakness. In 1903 the Dane Niels Finsen got the Nobel Prize for his work on the use of light from the carbon arc in the treatment of lupus vulgaris (=skin tuberculosis) and he is also acknowledged as the founder of modern phototherapy. He also described usage of red light as a prevention of suppuration of smallpox pustules. In the 1950s Richard Cremer in Essex, England intro-

duced phototherapy as a treatment of newborn baby's jaundice. This is most widely used form of phototherapy today.

Photochemotherapy

Photochemotherapy, using of photosensitizing drugs and the action of the light together, is also very old. Photosensitizing drugs have been known and applied in medicine for several thousand years. However, the scientific basis for such use was vague or non-existent before about 1900. One of India's sacred books, Atharavavéda (1400 BC) describes how seeds of the plant Psoralea corylifolia can be used for the treatment of vitiligo. Psoralens are the photoactive components of these seeds just as in the extracts of the plant, Ammi majus, which grows on the banks of the Nile, and was used by the Egyptians to treat vitiligo. For centuries phototherapy made no progress until 1974 when PUVA (= treatment with psoralens and UV A radiation) was reported to be an efficient treatment of psoriasis[35] and cutaneous T-cell lymphoma by extracorporeal photochemotherapy as well[36].

Photodynamic Therapy [34]

We can consider the PDT to be a subgroup of photochemotherapy. Besides photosensitizer and the action of light the presence of molecular oxygen is very important for the therapeutic effect.

In 1897 in Munich Oscar Raab, the medical student, was observing the toxic effects of the antimalaric drug chinin and some related drugs (acridine, phenylacridin adenosine) on paramecia. In one experiment the paramecia survived incubation with given acridine concentration for about 1.5 hours, while in another experiment they survived for about 15 hours under identical conditions. So he began to wonder whether light might play a role – and discovered the photodynamic action. It was characteristic in that time supervisors published the finding before the students. I happens also in this case - the term „photodynamic action“ („photodynamische Wirkung“) was introduced in 1904 by Professor Hermann von Tappeiner, Director of the Pharmacological Institute of the Ludwig-Maximilians University in Munich, Germany (and also Raab's supervisor). The word „dynamic“ should distinguish this action from the photographic process that had been discovered a few days earlier, but Tappeiner wasn't so satisfied with this word. This group then continued in work on photosensitization, they discovered that oxygen is required for the photodynamic effect and

summarized their work in a book (Die sensibilisierende Wirkung fluorescierender Substanzen untersuchungen über die photodynamische Erscheinung). During years 1903–1905 they also made attempts to apply PDT for the treatment of tumourous and other skin diseases. They tried a lot of dyes: eosin, fluorescein, sodium dichloroanthracene disulfonate and „Grubler's magdalene red". Application was mainly topical, but intratumoural injections were also attempted. In need for more correct and descriptive term they tried to replace PDT by „photochemotherapy", PCT. But it wasn't successful. Because of no follow up of this research and also because of discovery of ionizing radiation in cancer therapy, PDT was soon forgotten.

Hematoporphyrin and hematoporphyrin derivative (HpD) were the most important for the development of PDT. Hematoporphyrin was first produced, in impure form, by Scherer, who removed iron from dried blood in 1841 by treatment with sulphuric acid. Basic properties of this red substance, absorption spectrum and its fluorescence, was described by Thudichum in 1867. Hoppe-Seyler has carried out a lot of experiments with this substance (e.g. he has demonstrated how it sensitized paramecia, erythrocytes, mice, guinea pigs and humans to light) and also he gave it its name „hematoporphyrin" in the turn of 19th and 20th century. For instance, Hausmann in 1911 applied hematoporphyrin s.c. to mice and reported that light exposure of these mice gave skin reaction and symptoms (scratching). The German doctor, Friedrich Meyer Betz, injected 200mg hematoporphyrin into himself and became extremely photosensitive for more than 2 month.

Policard observed red fluorescence from endogenously produced porphyrins in tumours in 1924 and also an another experiment with application of ^{65}Zn hematoporphyrin to mice (mice with tumours tolerated larger doses of ^{65}Zn hematoporphyrin than mice without tumours) proved that HpD concentrated in primary and metastatic tumours as well as in lymph nodes. Animal tumours were successfully treated with light after injection of this dye. It was demonstrated that hematoporphyrin also had a tumour-localizing ability in human malignancies.

Much work on the tumour-localizing ability of porphyrins was published by Lipson and his group at the Mayo Clinic during the years 1960 – 1967. Their investigations were inspired by Dr. Samuel Schwartz, who defined the curing dose of hematoporphyrin for mice. (It was 50μg - neither low (10μg) nor high (250μg) doses didn't have stronger sensitizing effect.) Schwartz realized that commercial samples of hematoporphyrin were impure and tried to purify them. Then he found the pure hema-

toporphyrin as a pure tumour-localizer. Lipson and co-workers used hematoporphyrin derivatives (HpD, product of the treatment of dried blood with the mixture of acetic and sulphuric acid) for diagnostic and diagnostic purposes.

The debate about the composition of HpD lasted for several years. From the HPLC and fluorescence analyses was investigated composition of HpD and fluorescence quantum yields of constituents. HpD consists of monomers (hematoporphyrin stereoisomers, hematoporphyrin vinyl deuteroporphyrin isomers and protoporphyrin) which have high fluorescence quantum yield, but are poorly taken up by tumours and dimers, which have lower fluorescence quantum yield, but a higher tumour uptake. Generally non-fluorescent aggregates have the best tumour localizing properties but also have a low photosensitizing ability. The cellular uptake also increases with with increasing lipophilicity. Dougherty and his co-workers were further purifying HpD by removing monomers – resulting product, porfimer sodium, was called Photofrin®. The past decade is by many regarded as the „Golden Age of PDT" - on 19th April 1993 Photofrin® was approved for PDT of recurrent superficial papillary bladder cancer by the Canadian Health Protection Branch. It is considered as the official beginning of PDT. In next years it was established in treatment of lung cancer, esophageal and gastric cancer and it is probably still the most widely used sensitizer in the clinic. The pioneering work of Dougherty and his colleagues at the Roswell Park Memorial Cancer Institute in Buffalo, USA developed clinical acceptance of PDT.

In the „Second generation" [34,37] of photosensitizers began research for compound which have better pharmacokinetic a photodynamic properties than HPD has. One of the first tetraphenyl porphine sulphonate (TPPS) was synthesized, but it wasn't used because of suspicion of neurotoxicity. There was possibility of changing of properties of these compounds, because they were fully synthetic (e.g. by the addition of sulphonate groups to TPPS or aluminium phthalocyanin sulphonates (AlPcS) increased their water solubility). Also other parameters could be changed. Phthalocyanines are suitable for clinical use, mainly due to their strong absorption in red spectral band ($\lambda \approx 670$nm) where strong dye lasers are available and where tissue is more transparent than around 630nm.

Kennedy and Pottier successfully treated human skin tumours with topically ALA-based PDT (ALA = 5-aminolevulinic acid). Then ALA was approved by the FDA for topical PDT treatment of skin actinic keratosis, methyl aminolevulinate (Metvix®)

was approved in EU for advanced head and neck cancer. Many others (Verteporfin®, Hexviw®, ...)[34] were introduced for a lot of kinds of cancer.

Spears et al. were the first to introduce PDT into the field of cardiovascular diseases – inhibition of restenosis after angioplasty. Harriman, Sessler, and Mallouk were the first to suggest that expanded porphyrins could be useful as a PDT photosensitizers and lutetium(III)texaphyrin was produced by Pharmcyclics Inc. Preclinical studies have demonstrated that uptake and accumulation of the intravenously administered texaphyrin, motexafin lutetium, is selective for multiple sites of vascular plaque in animal models of atherosclerosis. Also, preclinical studies suggest that motexafin lutetium targets macrophages, which are metabolically active inflammatory cells known to accumulate in vulnerable plaque. Lastly, these studies suggest that motexafin lutetium is readily cleared from the rest of the body. A Phase I clinical study involving this substance is now on-going to prove its applicability in therapy of vulnerable plaque and atherosclerosis[23].

A number of other non-oncological diseases were treated including psoriasis, rheumatoid arthritis, menorrhagia and benign prostatic hyperplasia by PDT.

Principle of the PDT

In the idea of PDT combines the preferential accumulation of the photosensitiser in the target tissue with precise illumination by the light of suitable wavelength, which causes excitation of the photosensitizer to provide the selectivity of the treatment[38].The interaction of light with the photosensitizer molecule raises its energy state and in the presence of molecular oxygen leads to the formation of reactive oxygen species, primarily singlet oxygen ($^1O_2$), which can react with electron rich regions of many biomoleculs, giving rise to oxidized species, which loose their function. Since singlet oxygen has very short lifetime in cells, its intracellular targets are located close to molecules of photosensitiser[39].

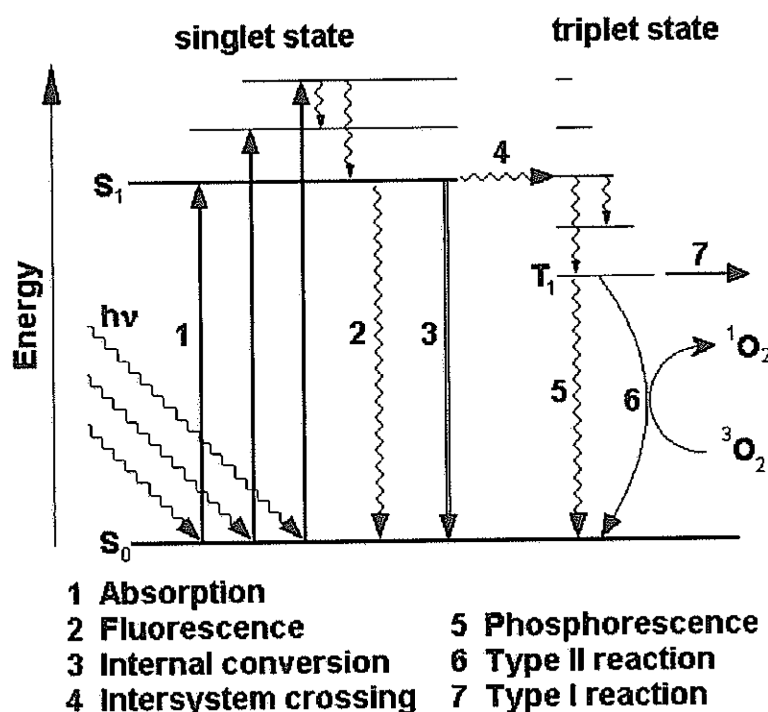
Physical Principle of the PDT[38,40,41]

Physical basement of the PDT is the excitation of the photosensitizer by the light of appropriate wavelength. The excitation of the photosensitizer as well as the effect of the treatment depends on its exposure to irradiation. Optimal tissue penetration by light apparently occurs between 650-800nm. The light with lower wavelength pene-

trates vital tissues only a few millimetres, because of its absorption caused by occurrence of natural endogenous chromophores in the tissue (e.g. hemoglobine). Also the light of higher wavelength isn't so efficient due to low energy of irradiation, which is not sufficient for excitation. Therefore one of the most important reasons for preparation of new photosensitizers is to enlarge absorption of the photosensitizer's molecule in the range of 650-800nm.

Photosensitizers have a stable electronic configuration which is in a singlet state (S_0) in their lowest or ground state energy level. Following absorption of a photon of light of specific wavelength a molecule is promoted to an excited state (S_1), which is also a singlet state and which is too short living (few nanoseconds) to make an interaction with surrounding molecules. The photosensitizer returns to the ground state by emitting a photon (fluorescence) or by internal conversion with energy loss as heat, which maybe also participates on the cellular response[42]. It is also possible that the molecule may convert to the triplet state (T_1) via intersystem crossing (ISC) which involves a change in the spin of an electron. The triplet state photosensitizer has lower energy than the singlet state, but has a longer lifetime (typically > 500 ns for photosensitizers) and this increases the probability of energy transfer to other molecules. Whole process is pictured in scheme *6*.

The tendency of a photosensitizer to reach the triplet state is measured by the triplet state quantum yield, which measures the probability of formation of the triplet state per photon absorbed (depending on the interaction of the singlet species with other substrates producing fluorescent quenching). The triplet state lifetime influences the amount of cytotoxic



Scheme 6

species produced by collision-induced energy transfer to molecular oxygen and other cellular components. A high intersystem crossing probability will produce an effective population of excited triplet state photosensitizer molecules whose energy can then be transferred by the two mechanisms described below. In addition, the photosensitizer is not destroyed but returns to its ground state without chemical alteration and is able to repeat the process of energy transfer to oxygen many times.

There are two mechanisms by which the triplet state photosensitizer can react with biomolecules; these are known as the Type I and Type II reactions.

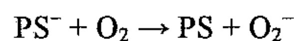
Type I reaction involves $H^{\bullet}$ transfer directly from the photosensitizer, producing ions, or $H^{\bullet}$ abstraction from a substrate molecule to form free radicals. These radicals then react rapidly, usually with oxygen, resulting in the production of highly reactive oxygen species (e.g. the superoxide and the peroxide anions). These radicals then attack cellular targets as described below.

Type II reactions produce the electronically excited and highly reactive state of oxygen known as singlet oxygen ($^1O_2$), which is considered to be the most important in the mechanism of action of PDT. Direct interaction of the excited triplet state photosensitizer with molecular oxygen (which, unusually, has a triplet ground state) results in the photosensitizer returning to its singlet ground state and the formation of singlet oxygen.

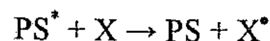
In PDT, it is difficult to distinguish between these two reaction mechanisms. There is probably a contribution from both Type I and II processes indicating the mechanism of damage is dependent on oxygen tension and photosensitizer concentration.

Type I reaction is preferred in conditions of low oxygen pressure. In environment without oxygen excited photosensitizer (PS^*) reacts directly with surrounding biological substrate (X) resulting in production of oxidised substrate (X^+) and reduced photosensitizer (PS^-). In hypoxic conditions PS^- can react with oxygen to transform it to the radical of superoxid anion ($O_2^{\bullet-}$), providing a highly reactive hydroxide radical ($OH^{\bullet}$), which strongly participates on oxidation of biomolecules. Other reactions mentioned in the scheme below are also possible:

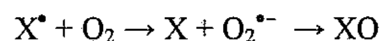
$$PS^* + X \rightarrow PS^- + X^+$$



$$PS^- + O_2 \rightarrow PS + O_2^-$$



$$PS^* + X \rightarrow PS + X^{\bullet}$$



$$X^{\bullet} + O_2 \rightarrow X + O_2^{\bullet-} \rightarrow XO$$

In the conditions with higher concentration of oxygen predominates Type II reaction with transformation of ground triple state of molecular oxygen ($^3O_2$) to the singlet oxygen ($^1O_2$). Both oxygen states are depicted by their molecular orbitals in Figure *26*. Singlet oxygen's lifetime differs according to the medium in which occurs: in water 2–4μs, in lipids 50–100μs, organic solvents 10–100μs and 0.6–0.04 μs in biological systems (values differs according to authors, because of difficult detectability of $^1O_2$). The most important is the idea, that $^1O_2$ can't penetrate biologic membrane, which explains together with selective accumulation of PS and localized irradiation the selectivity of effect of PDT.

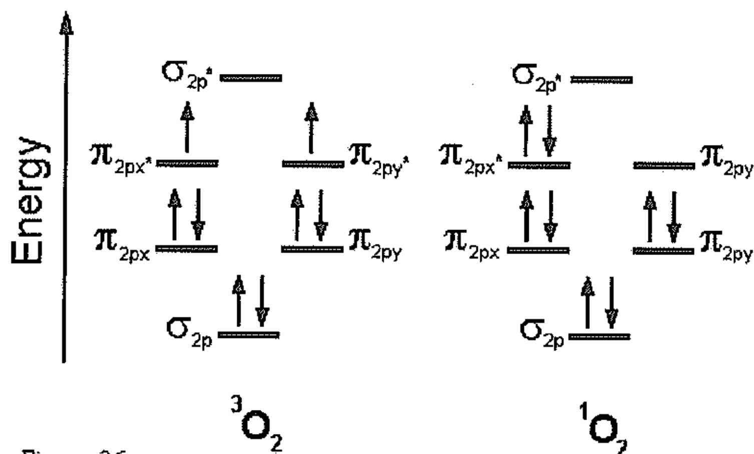


Figure 26

Photosensitizer in the triplet state can be also recycled and transferred to the ground state by phosphorescence.

Biological Principle of the PDT[38]

Cellular Photosensitisation

PDT produces cytotoxic effects through photodamage to subcellular organelles and biomolecules. These sites of photodamage may reflect the localisation of the photosensitizer in the cell. A variety of cellular components such as amino acids (particularly cysteine, histidine, tryptophan, tyrosine and methionine), nucleosides (mainly guanine) and unsaturated lipids can react with singlet oxygen. The diffusion distance of singlet oxygen is relatively short (about 0.1μm), therefore the photosensitizer must associate intimately with the substrate for efficient photosensitisation to occur. Although the Type II process is considered the more relevant reaction mechanism in PDT, cytotoxic species generated by the Type I reaction process can also act in a site-specific manner.

Many factors determine the cellular targets of photosensitizers. The incubation parameters and mode of delivery as well as the chemical nature of the photosensitizer can all influence subcellular localisation, creating a number of potential targets for photodamage. In cell culture studies with porphyrin based photosensitizers, short incubation times (up to 1 hour) prior to illumination leads primarily to membrane damage whereas extended incubation periods followed by light exposure results in damage to cellular organelles and macromolecules.

Hydrophobic (lipophilic) compounds preferentially bind membranes and will target structures such as the plasma membrane, mitochondria, lysosomes, endoplasmic reticulum and the nucleus. Oxidative degradation of membrane lipids can cause the loss of membrane integrity, resulting in impaired membrane transport mechanisms and increased permeability and rupturing of membranes. Cross-linking of membrane associated polypeptides may result in the inactivation of enzymes, receptors and ion channels.

Mitochondrial Localisation

The mitochondrion *(fig. 27)* has been shown to be a critical target in PDT. Lipophilic porphyrins have demonstrated intimate intracellular association with mitochondrial membranes, whilst cationic compounds such as rhodamines and cyanines may accumulate in these organelles due to mitochondrial membrane potential. Much work has focused on photosensitisation of mitochondria because these organelles perform vital functions in the cell. ATP is synthesised by oxidative phosphorylation in the mitochondria and it is required for energy requiring processes such as replication, protein synthesis, DNA synthesis and transport. Mitochondrial photosensitisation may cause the uncoupling of respiration and phosphorylation resulting in the impairment of ATP synthesis and subsequent loss of cellular function. At the molecular level several mitochondrial enzymes and carriers involved in ATP synthesis have displayed sensitivity to mitochondrial photosensitisation. Following PDT the loss of mitochondrial integrity has been observed to occur before the loss of plasma membrane integrity, underlining the importance of the mi-

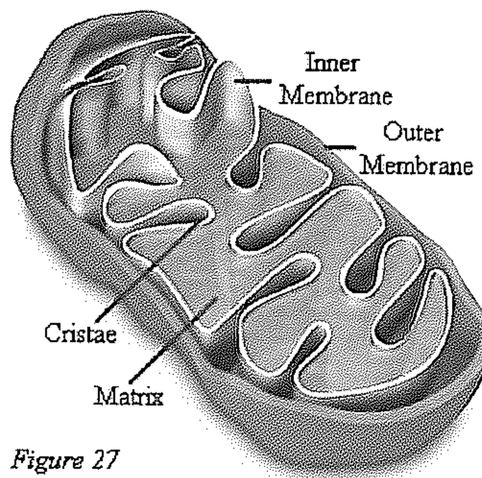


Figure 27

tochondria as targets for PDT. Mitochondrial damage can also induce nuclear chromatin condensation, and has been linked to the induction of apoptosis.

Lysosomal Localisation

Lysosomal localisation has been observed for a number of photosensitizers. Initially it was thought that cell death was due to the release of enzymes following lysosomal membrane photodamage, however cell survival has since been observed following photodamage to 80% of cellular lysosomes *(fig 28)*. Recent studies have demonstrated that photosensitizers are redistributed from the lysosomes to other cellular sites upon light exposure.

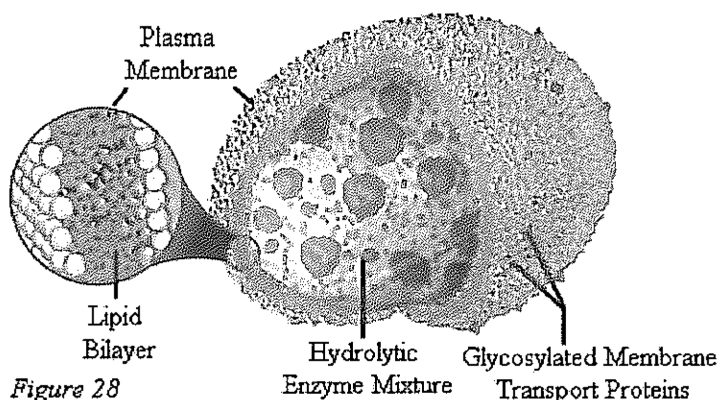


Figure 28

Nuclear Localisation

At the level of the nucleus, PDT has been shown to cause single/double stranded breaks and alkali-labile sites in DNA, as well as induction of sister chromatid exchanges and chromosomal aberrations. However, further studies have indicated that nuclear damage and/or repair is not generally a dominant factor in PDT mediated cytoxicity.

Atherosclerosis[43,44]

Definition

Atherosclerosis is a disease of the arteries in which fatty material and plaque (a combination of cholesterol, other fatty materials, calcium, and blood components) are deposited in the wall of an artery, resulting in narrowing and hardening of the arterial lumen and eventual impairment of blood flow. We can also define atherosclerosis as chronic inflammatory process, which is caused by various noxious substances affecting artery's wall and following lipid storage, proliferation of fibrous tissue and smooth muscle cell proliferation and migration. Atherosclerosis plays as a very important factor in several serious diseases including coronary heart disease (CHD),

myocardial infarction (MI), angina pectoris, cerebral vascular disease (CVD), thrombotic stroke, transient ischemic attacks (TIAs), insufficient blood supply to lower limbs and feet (claudication), organ damage, and vascular complications of diabetes. Because symptoms can be few or minor in the early stages, atherosclerosis is referred to as „the silent killer", because it can progress undetected for years, particularly in individuals who are at high risk for heart disease. Atherosclerosis and cardiovascular diseases remain a major health problem in industrialized countries.

Structure of the Intact Artery Wall

A large artery consists of three morphologically distinct layers. The intima, the innermost layer is bounded by a monolayer of endotelial cells on the luminal side a sheet of elastic fibres, the internal elastic lamina, on the peripheral side. The normal intima is very thin region and consists of extracellular connective tissue matrix, primarily proteoglycans and collagen. The media, the middle layer, consists of smooth muscle cells (SMCs). The adventitia, the outer layer, consists of connective tissues with interspersed fibroblasts and SMCs.

Risk Factors and Ethiology of Atherosclerosis

Four major mechanisms that are involved in the development of atherosclerosis are: oxidation of low density lipoprotein-cholesterol (LDL-C), homocysteine overload, abnormal platelet aggregation and inflammation. Another factors are very risky in formation of atherosclerosis: high intake of fatty and sweet food helps increase blood cholesterol and gives arise of obesity, cigarette smoking and exposure to tobacco smoke, diabetes mellitus, high blood pressure, stress, physical inactivity and genetic predisposition for vascular diseases.

According to most widely accepted hypothesis, there is a lesion at the beginning of the whole process caused by chemical (nicotine, free radicals, other harmful compounds from tobacco smoke, hypoxia, other chemicals, drugs, toxins and also by direct cytotoxic action of oxidized LDL), physical (high blood pressure, blood turbulation, mechanical damage) and biological (bacteriae (Chlamydia pneumoniae), viruses (cytomegalovirus infection)) stimuli.

The wall of artery is predisposed to these kinds of injury, because the cells of artery form only 30% of total artery wall mass, so the way for oxygen and nutrients leads through glycoproteins, colagen and elastin and is quite long, proteins of artery wall

are changing quickly, that's why there is bigger danger of proliferation. Another problem causes permanent contact with blood, which contains noxious particles mentioned above.

As a reply to the primary endotelial dysfunction there occurs higher endotelial permeability (for LDL, vasoactive mediators, monocytes, lymfocytes and other blood derivatives), loss of antiadhesive properties and higher production of adhesive molecules, decrease of activity of lipoprotein lipase, decrease of NO (vasodilatant) production, increased endotelin 1 production, increased production of cytokins activating cell proliferation, impaired release of SMC's migration and growth inhibitors. These facts lead to retention of LDL and other apolipoprotein B-containing lipoproteins in the vessel wall.

Oxidation of the LDL and Formation of Macrophages

The LDL undergoes oxidative modification as a result of interaction with reactive oxygen species (ROS) including products of 12/15 lipoxygenase such as HPETE and a lot of others coming from oxidative stress. Oxidation of LDL is inhibited by HDL, which contains the antioxidant protein serum paraoxonase (PON1). LDL is therefore often referred to as „bad cholesterol", while HDL is „good" one.

LDL oxidation involves modification of apolipoprotein B so that it is metabolized through macrophage scavenger, which receptors are recognizing modified LDL. Ox-LDL is also more susceptible to aggregation[45]. Ox-LDL is also cytotoxic to many cell types and chemotactic for monocyte macrophages. It means Ox-LDL stimulates the overlying endothelial cells to produce adhesion molecules (ICAM-1, P-selectin, E-selectin, PCAM-1 and VCAM-1), chemotactic proteins such as monocyte chemotactic protein-1 (MCP-1), and growth factors such as macrophage colony stimulating factor (M-CSF), resulting in the entry of monocytes to the vessel wall, where they undergo further differentiation (at least one day) to become multifunctional tissue macrophages. In addition, Ox-LDL can inactivate endothelial cell-derived relaxing factor (EDRF, NO) and cause apoptosis.

Foam-cell Formation

In next step of progress of atherosclerosis highly oxidized and aggregated LDL is recognized by macrophage scavenger receptors such as SR-A, CD 36 and CD 68. Macrophages intaking Ox-LDL give rise foam cells, which tend to die. The death of

foam cells leaves behind a growing mass of extracellular lipids and other cell debris. Higher plasma homocysteine levels support creation of LDL-homocysteine aggregates, which are well swallowed by macrophages.

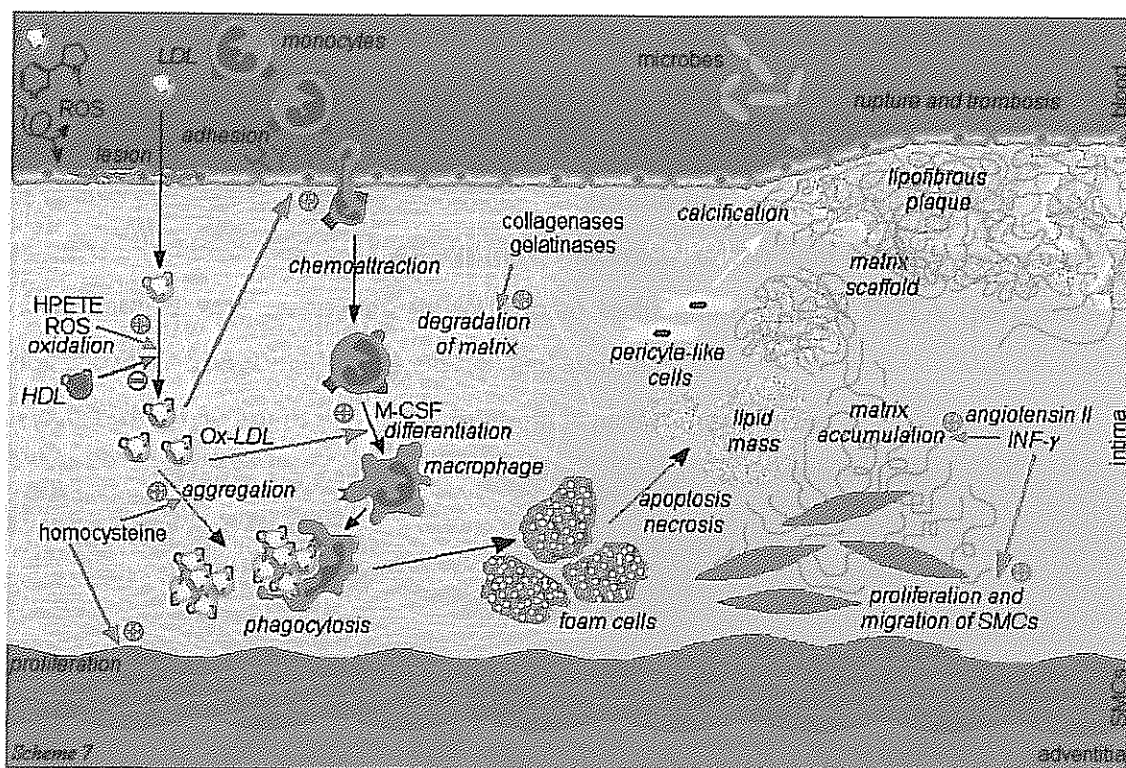
Formation of Fibrous Plaques

Death foam cells deposit in so called lipid stripes in intima. A number of risk factors, including elevated levels of homocysteine and angiotensin II (vasoconstrictor), stimulate the proliferation and migration of SMCs. Oestrogens exert beneficial effects on plasma lipoprotein levels and they also stimulate production of NO and prostacyclin (vasodilator, thrombocyte aggregation inhibitor) by endothelial cells (that's why women are affected more after menopause). The interaction of CD40 and CD40 ligand stimulates T lymphocytes (T cells) and macrophages to express cytokines such as INF-γ that can influence inflammation, SMC growth and matrix accumulation. The intimal SMCs secrete extracellular matrix and give rise lipofibrous plaques.

Complex Lesion and Thrombosis

Vulnerable plaques with thin fibrous caps result from degradation of matrix by various proteinases such as collagenases, gelatinases, strombolysin and cathepsins and by inhibition of matrix secretion. Among various factors that can destabilize plaques and promote thrombosis are infection, which may have systemic effects such as induction of acute phase proteins and local effects such as increased expression of tissue factor and decreased expression of plasminogen activator (fibrinolytic agent). The calcification of lesions appears to be an active, regulated process involving the secretion by pericyte-like cells in the intima of a scaffold for calcium phosphate deposition. The formation of a thrombus, consisting of adherent platelets and fibrin crosslinks, usually results from plaque rupture, exposing tissue factor in the necrotic core.

All these mechanisms are shortly summarized in Scheme *7*.



Mechanism of Action of Motexafin Lutetium

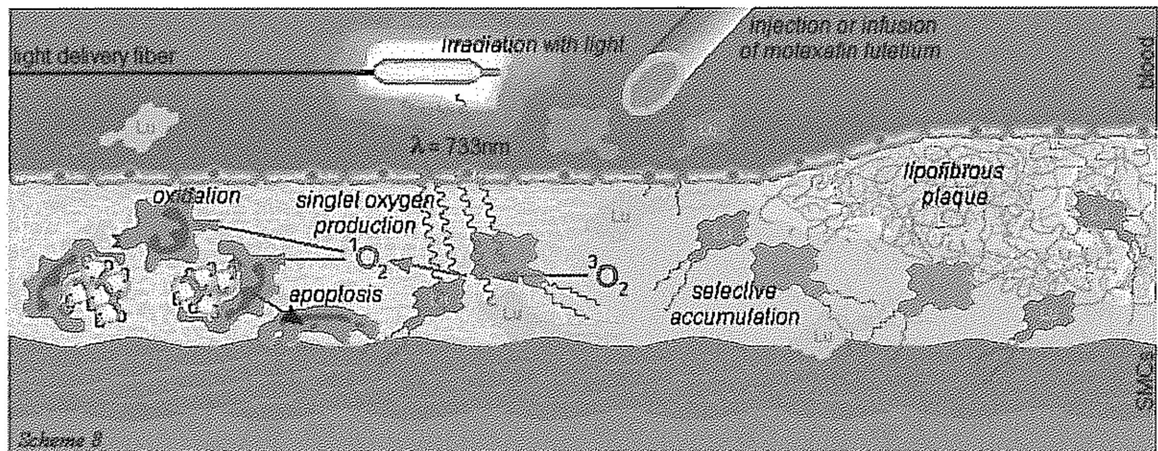
Motexafin Lutetium has good water solubility and apparent stability in solution. It has an absorption peak in aqueous solution centred at 733nm, which allows for deeper penetration of the activating light than many other photosensitizers being investigated[46] do. Preclinical studies have demonstrated that uptake and accumulation of the intravenously administered texaphyrin, motexafin lutetium, is selective for multiple sites of vascular plaque in animal models of atherosclerosis. Also, preclinical studies suggest that motexafin lutetium targets macrophages, which are metabolically active inflammatory cells known to accumulate in vulnerable plaque. Intracellular targets in macrophages are described in the chapter Biological principle of the PDT *(see p. 39)*. Lastly, these studies suggest that motexafin lutetium is readily cleared from the rest of the body.

Motexafin lutetium is activated by far-red light delivered by an optical fibre that is inserted into a blood vessel containing sites of vulnerable plaque. Activated motexafin lutetium causes the production of singlet oxygen ($^1O_2$) inside macrophages in vulnerable plaque. Because the intracellular production of singlet oxygen causes the macrophages in vulnerable plaque to undergo programmed cell death (apoptosis), treatment with activated motexafin lutetium may result in stabilization of vulnerable

plaque or decreased inflammation. In contrast, initial studies suggest that normal regions of the blood vessel are unaffected, so the treatment seems to have no serious adverse events.

It is also possible that the light-activated motexafin lutetium procedure can be incorporated into current medical practice; i.e., placement of the light delivery fibre can be accomplished with customary interventional techniques, such as PCTA (percutaneous coronary transluminal angioplasty) and stenting[23].

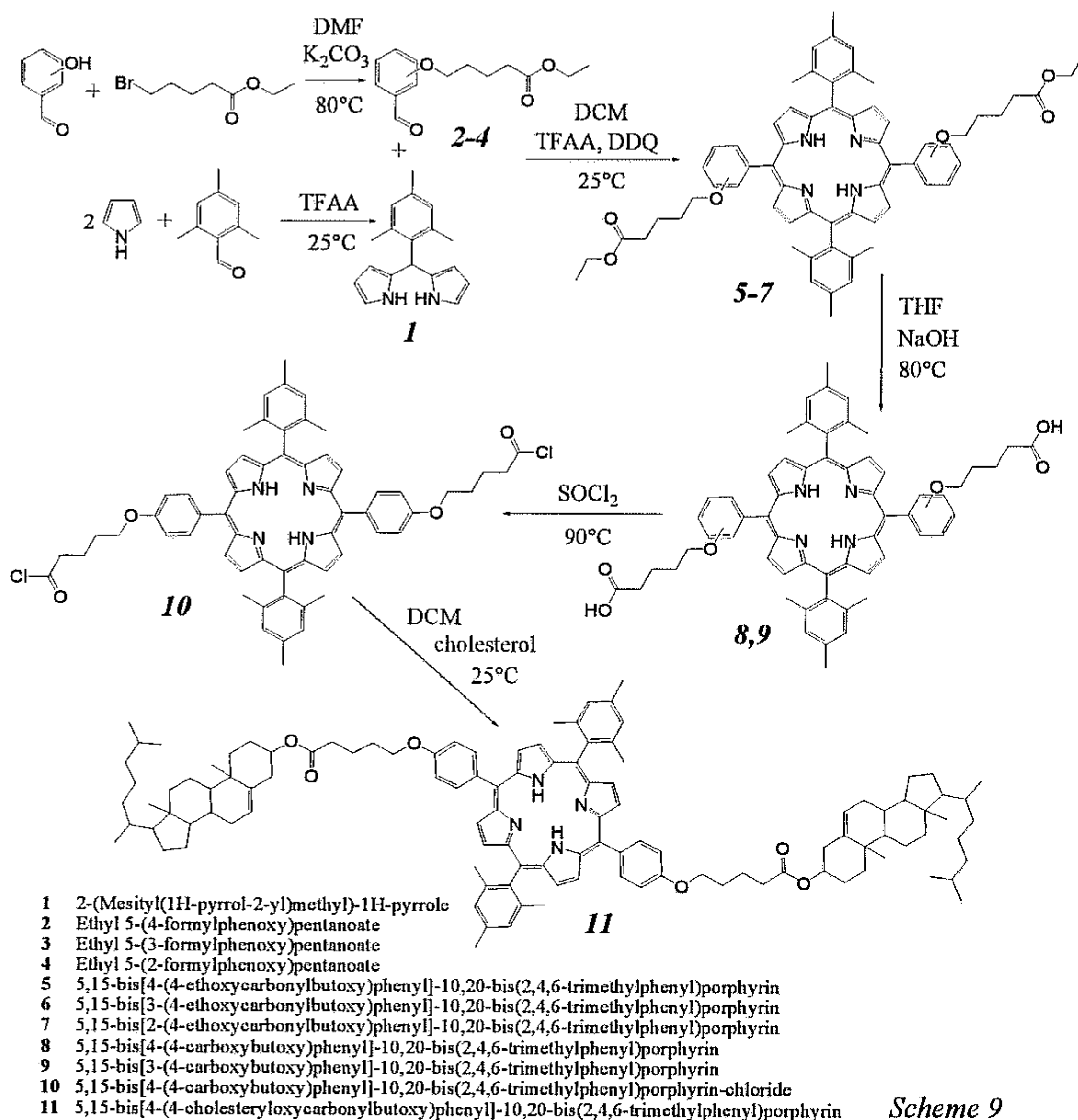
Mechanism of action of motexafin lutetium is pictured in Scheme *8*.



Scheme 8

Experimental Part

I should synthesise 4 precursors of porphyrins and 9 porphyrins in experimental part of my diploma project. There is an overview of reactions I have used in Scheme *9* and *10*.



Scheme 9

All organic solvents and chemicals used for the synthesis and work up were of analytical grade. TLC was performed on 25 TLC aluminium sheets (Silica gel 60 F254, Merck, Darmstadt, Germany). Merck Silica gel 60 (0.063–0.200 mm) and Aldrich Aluminium oxide, activated, basic brockmann I, standard grade, ca. 150 mesh was used for column chromatography. ^{1}H NMR spectra were recorded on Bruker AMX-

500. UV-VIS spectra were recorded on spectrophotometer MultiSpec-1501 Shimadzu.

Further there are included UV-VIS and ^{1}H NMR spectra of these compounds in a pictorial appendix below references *(see pages 63-77):*
^{1}H NMR: **1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 13**
UV-VIS: **5, 6, 7, 8, 9, 11, 13**

Method

2-(Mesityl(1H-pyrrol-2-yl)methyl)-1H-pyrrole (compound 1)

Solution of pyrrole (50ml; 720mmol) and 2,4,6-trimethylbenzaldehyde (2.65ml; 18mmol) was degassed by bubbling with argon for half an hour. Then TFAA (0.134ml; 1.8mmol) was added to start the reaction, the solution was stirred under argon at room temperature (25°C) for 1h and then the reaction was stopped by the addition of triethylamine.
The mixture was diluted with toluene (150ml) and the rest of triethylamine was removed by extraction with brine (5% (w/w) water solution of sodium chloride). Organic phase was dried with anhydrous sodium sulphate and solvents (pyrrole and toluene) was removed by vacuum distillation at 40°C. The residue (dark brown oil) was dissolved in minimal amount of DCM and filtered through a short pad of silica gel, using DCM as eluent. Yellow fraction was caught and then DCM was removed by vacuum distillation. Small yellow crystals were washed with hexane and cyclohexane and were collected by filtration and dried under vacuum. Yield 0.609g (12.8%) of yellow solid.
^{1}H NMR (500MHz, $CDCl_3$) δ 2.07 (6H, s, 6-H) 2.29 (3H, s, 8-H) 5.93 (1H, s, 5-H) 6.01 (2H, m, 4-H) 6.19 (2H, q, J_1=2.8, J_2=3.1, 3-H) 6.67 (2H, m, 2-H) 6.87 (2H, s, 7-H) 7.94 (2H, brs, 1-H). *(Spectrum 1)*

Ethyl 5-(4-formylphenoxy)pentanoate (compound 2)

The three-neck round-bottomed was dried under vacuum, then stored under argon till it got cold. Then 4-hydroxybenzaldehyde (3.66g; 30mmol) was put into the flask, dry DMF (14ml; 30mmol) and ethyl-5-bromopentanoate (5.7ml; 36mmol) were poured into the flask too. As a very last compound which started the reaction potassium car-

bonate (4.976g; 36mmol) was added. The reaction mixture was stirred for 1h at the temperature of 80°C. The presence of the product was proved by TLC using ethyl 5-(4-formylphenoxy)pentanoate as a standard and mixture of hexane and DCM (1:9) as an eluent.

The rest of potassium carbonate was removed from the reaction mixture by filtration and the solution was diluted with DCM and dried under the vacuum. The DCM served for azeotropic remove of the DMF and the whole procedure was twice repeated. After putting to the fridge very light brownish-white crystals appeared. Then the extraction between DCM (80ml) and water (2x40ml) was carried out. Organic phase was dried by distillation, brownish white crystals appeared again. Purity of the product was tested by TLC. Yield 7.302g (97.2%) was obtained.

^{1}H NMR (500MHz, $CDCl_3$) δ 1.25 (3H, t, J=7.1, 9-H) 1.82 (2H, m, 6-H) 1.84 (2H, m, 5-H) 2.38 (2H, t, J=7.0, 7-H) 4.05 (2H, t, J=5.9, 4-H) 4.13 (2H, q, J_1=7.1, J_2=7.1, 8-H) 6.97 (2H, d, J=8.7, 3-H) 7.82 (2H, d, J=8.8, 2-H) 9.87 (1H, s, 1-H). *(Spectrum 2)*

Ethyl 5-(3-formylphenoxy)pentanoate (compound 3)

The three-neck round-bottomed was dried under vacuum, then stored under argon till it got cold. Then 3-hydroxybenzaldehyde (2.0g; 16.38mmol) was put into the flask, dry DMF (7,5ml; 15mmol) and ethyl-5-bromopentanoate (3.11ml; 19.65mmol) were poured into the flask too. As a very last compound which started the reaction potassium carbonate (2.72g; 19.65mmol) was added. The reaction mixture was stirred for 1h at the temperature of 80°C.

Potassium carbonate was removed by filtration, maximum of DMF was evaporated by vacuum distillation and then extraction between DCM (80ml) and water (3x30ml) was carried out. DCM was removed by distillation and the liquid product was dried for NMR-analysis under the vacuum. NMR-analysis was carried out to show us, that the product wasn't pure, it was also certified by TLC. As a further purifying procedure column chromatography, using silica gel and mixture of DCM and hexane (2:8) as an eluent, was chosen. Fractions were collected according to testing by TLC and then evaporated. Yield 3.515g (85.7%) of yellow liquid was obtained.

^{1}H NMR (500MHz, $CDCl_3$) δ 1.25 (3H, t, J=7.1, k-H) 1.84 (4H, m, gh-H) 2.38 (2H, t, J=7.0, i-H) 4.03 (2H, t, J=5.8, f-H) 4.13 (2H, q, J_1=7.1, J_2=7.1, j-H) 7.16 (1H, m,

b-H) 7.37 (1H, d, J=1.9, c-H) 7.42 (1H, s, e-H) 7.44 (1H, d, J=2.7, d-H) 9.96 (1H, s, a-H). *(Spectrum 3)*

Ethyl 5-(2-formylphenoxy)pentanoate (compound 4)

The three-neck round-bottomed was dried under vacuum, then stored under argon till it got cold. Then 2-hydroxybenzaldehyde (2.0g; 16.38mmol) was added into the flask, dry DMF (7.5ml; 15mmol) and ethyl-5-bromopentanoate (3.11ml; 19.65mmol) were poured into the flask too. As a very last compound which started the reaction potassium carbonate (2.72g; 19.65mmol) was used. The reaction mixture was stirred overnight at the temperature of 80°C.

Then the maximum of solvent was removed by vacuum distillation, extraction between DCM (80ml) and water (4x30ml) was carried out. Organic phase was evaporated and TLC was carried out, one stain on TLC proved the purity of the compound as well as subsequent NMR-analysis. Yield 3.961g (96.6%) of yellow liquid was gained.

^{1}H NMR (500MHz, $CDCl_3$) δ 1.25 (3H, t, J=7.1, a-H) 1.89 (4H, m, ed-H) 2.39 (2H, t, J=7.1, c-H) 4.10 (2H, q, J_1=6.1, J_2=4.7, b-H) 4.13 (2H, t, J=7.1, f-H) 6.97 (1H, d, J=8.4, j-H) 7.01 (1H, t, J=7.5, i-H) 7.52 (1H, t, J=7.0, h-H) 7.83 (1H, d, J=7.7, g-H) 10.50 (1H, s, k-H). *(Spectrum 4)*

5,15-bis[4-(4-ethoxycarbonylbutoxy)phenyl]-10,20-bis(2,4,6-trimethyl-phenyl)porphyrin (compound 5)

Into a dry round-bottomed two-necked 500ml flask 1.00g (3.78mmol) of **1** and 0.946g (3.78mmol) of compound **2** was placed, then DCM (365ml) was poured into the flask. Mixture was stirred under argon for five minutes, then TFAA (0.506ml; 6.81mmol) was added to begin the cyclization and the mixture was stirred at the room temperature (24.5°C). After 1h DDQ (0.978g; 4.31mmol) was threw in and the reaction mixture was stirred overnight.

In the morning the reaction mixture was dark green and the solvent was removed by the rotary evaporator at elevated temperature. Remaining residue was diluted with minimum of DCM and the porphyrin was purified by column chromatography on basic aluminium oxide, using DCM as an eluent. The porphyrin had changed its colour due to acidobasic reaction to dark purple and purple fraction was collected and

subsequently DCM was removed. Purple porphyrin was recrystallized from n-hexane in yield 0.335g (19.0%). Purity was tested by TLC and then by NMR.

UV–VIS (CH_2Cl_2): λ_{max} (ε) 649 (4344), 593 (5199), 552 (7338), 517 (13426), 420 (361656). *(Spectrum 12)*

^{1}H NMR (500MHz, $CDCl_3$) δ -2.61 (2H, s, n-H) 1.32 (6H, t, *J*=7.1, a-H) 1.84 (12H, s, k-H) 2.02 (8H, m, de-H) 2.52 (4H, t, *J*=6.9, c-H) 2.63 (6H, s, m-H) 4.21 (4H, q, J_1=7.1, J_2=7.1, b-H) 4.27 (4H, t, *J*=5.7, f-H) 7.25 (4H, s, h-H) 7.28 (4H, s, l-H) 8.12 (4H, d, *J*=8.5, g-H) 8.68 (4H, d, *J*=4.7, i-H) 8.82 (4H, d, *J*=4.7, j-H). *(Spectrum 5)*

5,15-bis[3-(4-ethoxycarbonylbutoxy)phenyl]-10,20-bis(2,4,6-trimethyl-phenyl)porphyrin (compound 6)

Into a dry round-bottomed two-necked 500ml flask 0.6g (2.271mmol) of **1** and 0.5684g (2.271mmol) of compound **3** was placed, then DCM (220ml) was poured into the flask. Mixture was stirred under argon for five minutes, then TFAA (0.304ml; 4.088mmol) was added to begin the cyclization and the mixture was stirred at the room temperature (22.5°C). Reaction mixture became dark green. After one and half an hour DDQ (0.588g; 2.59mmol) was threw in and the reaction mixture was stirred overnight.

Then the solvent was removed by rotary evaporator at 60°C and the residue was chromatographed on a silica gel column using the mixture of DCM and hexane (7:3) with traces of ammonia (25% aqueous solution) as the eluent. Purple fractions were collected, evaporated and dried for NMR-analysis. Yield 0.2735g (24.4%) of dark purple shiny crystals was obtained.

UV–VIS (CH_2Cl_2): λ_{max} (ε) 646 (3132), 590 (4868), 549 (5413), 514 (15591), 419 (372085). *(Spectrum 13)*

^{1}H NMR (500MHz, $CDCl_3$) δ -2.64 (2H, s, p-H) 1.22 (6H, t, *J*=7.1, a-H) 1.84 (12H, s, m-H) 1.91 (8H, m, ed-H) 2.41 (4H, t, *J*=7.0, c-H) 2.63 (6H, s, o-H) 4.12 (4H, q, J_1=7.1, J_2=7.1, b-H) 4.17 (4H, t, *J*=5.8, f-H) 7.28 (4H, s, n-H) 7.31 (2H, d, *J*=1.7, i-H) 7.61 (2H, t, *J*=7.9, h-H) 7.77 (2H, s, j-H) 7.81 (2H, d, *J*=7.4, g-H) 8.68 (4H, d, *J*=4.7, l-H) 8.84 (4H, d, *J*=4.7, k-H). *(Spectrum 6)*

5,15-bis[2-(4-ethoxycarbonylbutoxy)phenyl]-10,20-bis(2,4,6-trimethyl-phenyl)porphyrin (compound 7)

Into a dry two-necked round-bottomed 500ml flask 0.6g (2.271mmol) of **1** and 0.5684g (2.271mmol) of compound **4** was placed, then DCM (220ml) was poured into the flask. Mixture was stirred under argon for five minutes, then TFAA (0.304ml; 4.088mmol) was added to start the cyclization of the porphyrin and the mixture was stirred at the room temperature (23°C). Reaction mixture became dark green. After 75 minutes DDQ (0.588g; 2.59mmol) was threw in and the reaction mixture was stirred overnight at room temperature.

Then the mixture was filtered through the short pad of aluminium oxide, and the filtrate was further purified by column chromatography on silica gel using the mixture of DCM and hexane (7:3) as the eluent. Both atropoisomers wasn't separated properly, therefore the column chromatography was repeated. But neither next separation was successful and both atropoizomers was obtained together as a purple solid in yield of 191.0mg (17.1%).

UV–VIS (CH_2Cl_2): λ_{max} (ε) 648 (2402), 591 (3389), 548 (3587), 515 (9642), 419 (222785). *(Spectrum 14)*

^{1}H NMR (500MHz, $CDCl_3$) δ -2.57 (2H, s, p-H) 0.86 (6H, m, a-H) 0.98 (4H, m, d-H) 1.10 (4H, m, e-H) 1.68 (4H, t, *J*=7.1, c-H) 1.84 (12H, s, m-H) 2.62 (6H, s, o-H) 3.71 (4H, m, b-H) 3.94 (4H, t, *J*=6.2, f-H) 7.27 (4H, s, n-H) 7.30 (2H, s, j-H) 7.32 (2H, s, i-H) 7.73 (2H, t, *J*=8.0, h-H) 8.00 (2H, d, *J*=5.5, g-H) 8.62 (4H, d, *J*=4.6, l-H) 8.73 (4H, d, *J*=4.6, k-H). *(Spectrum 7)*

5,15-bis[4-(4-carboxybutoxy)phenyl]-10,20-bis(2,4,6-trimethylphenyl)porphyrin (compound 8)

Into a two-necked round-bottomed 250ml flask 150mg (0.1519mmol) of **5** was placed and dissolved in THF (14.65ml; 0.191mol). Then 50ml of 80% ethanolic solution of sodium hydroxide (c=2mol/l; 0.1mol) was poured in. Then reaction mixture was stirred at the temperature of 80°C for 24h. The grade of hydrolysis was checked by TLC (mobile phase: DCM – methanol (8:2)). Then the reaction mixture was cooled down, diluted with water and acidified by aqueous solution of HCl (c=1mol/l) to get filterable precipitate. Raw product was obtained by filtration and was further purified by extraction between DCM and aqueous solution of HCl (c=1mol/l; 3x50ml). Organic phase was evaporated to get purple compound. Purity was tested

by TLC and product was further purified by recrystallisation from n-pentane. Yield 36.8mg (26.1%) of dark purple-red solid was obtained.

UV–VIS (CH_2Cl_2): λ_{max} (ε) 649 (1024), 594 (1148), 552 (1366), 516 (2142), 420 (368107). *(Spectrum 15)*

^{1}H NMR (500MHz, $CDCl_3$) δ -2.60 (2H, s, l-H) 1.83 (12H, s, i-H) 2.03 (4H, m, b-H) 2.04 (4H, s, c-H) 2.58 (4H, t, *J*=6.8, a-H) 2.62 (6H, s, k-H) 4.18 (4H, t, *J*=5.3, d-H) 7.14 (4H, d, *J*=8.4, f-H) 7.27 (4H, s, j-H) 8.00 (4H, d, *J*=8.4, e-H) 8.66 (4H, d, *J*=4.7, h-H) 8.76 (4H,d, *J*=4.6, g-H). *(Spectrum 8)*

5,15-bis[3-(4-carboxybutoxy)phenyl]-10,20-bis(2,4,6-trimethylphenyl)porphyrin (compound 9)

Into a round-bottomed 100ml flask 100mg (0.1013mmol) of **6** was placed and dissolved in THF (15ml). Then 405mg of sodium hydroxide (10.13mmol) was added and reaction mixture was stirred at room temperature (19°C) for 22h. The grade of hydrolysis was checked by TLC (mobile phase: DCM – methanol (8:2)), subsequently reaction mixture was stirred at temperature of 80°C for 6h more. Then the reaction mixture was cooled down, diluted with water and acidified by aqueous solution of HCl (c=1mol/l) to get filterable precipitate. Raw product was obtained by filtration and was further purified by extraction between DCM and aqueous solution of HCl (c=0.1mol/l; 3x50ml). Organic phase was evaporated to get purple compound. Purity was tested by TLC and product was further purified by column chromatography. Dark purple-red solid was obtained in yield 14.1mg (14.1%).

UV–VIS (CH_2Cl_2): λ_{max} (ε) 645 (3953), 590 (5798), 548 (5973), 515 (13967), 419 (271433). *(Spectrum 16)*

^{1}H NMR (500MHz, $CDCl_3$) δ -2.64 (2H, s, o-H) 1.84 (12H, s, k-H) 1.91 (8H, m, cb-H) 2.46 (4H, m, a-H) 2.62 (6H, s, m-H) 4.18 (4H, m, d-H) 7.28 (4H, s, l-H) 7.30 (2H, s, g-H) 7.60 (2H, m, f-H) 7.76 (2H, s, h-H) 7.80 (2H, d, *J*=7.0, e-H) 8.68 (4H, d, *J*=4.7, j-H) 8.84 (4H, q, J_1=2.4, J_2=2.0, i-H). *(Spectrum 9)*

5,15-bis[4-(4-carboxybutoxy)phenyl]-10,20-bis(2,4,6-trimethylphenyl)porphyrin-chloride (compound 10)

Into a two-necked round-bottomed under vacuum dried 100ml flask was put 150mg (0.1611mmol) of dry **8** and $SOCl_2$ (15ml) was added. Reaction mixture was stirred under argon at the temperature 90°C for 17 hours. During this time 10ml $SOCl_2$ was

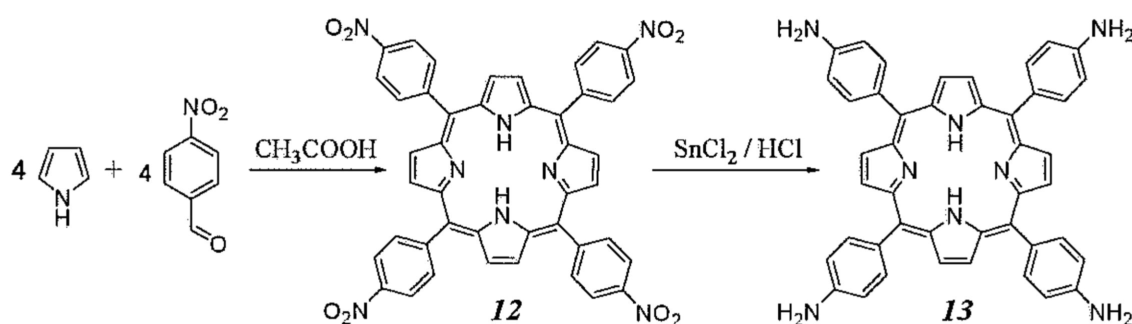
added. Reaction mixture became green-brown. After this the excess of $SOCl_2$ was evaporated under vacuum, product was dried and used directly for next reaction.

5,15-bis[4-(4-cholesteryloxycarbonylbutoxy)phenyl]-10,20-bis(2,4,6-trimethylphenyl)porphyrin (compound 11)

Whole amount of the intermediate from previous reaction was dissolved in dry DCM (45ml) and cholesterol (149.5mg; 0.384mmol) was added. Then dry triethylamine (0.0449ml; 0.3222mmol) was supplemented to catch forming HCl. Reaction mixture was stirred at room temperature (23°C) for 6 h. Occurrence of product was tested by TLC, using mixture of $Ce(SO_4)_2$, H_2SO_4, water and heat for detection. Reaction was stopped by evaporation of solvent and wash up (0.33M aqueous solution of HCl 2x40ml, water 2x80ml, solution of $NaHCO_3$ 1x40ml and water 2x80ml) was carried out. Subsequently the organic phase was dried with Na_2SO_4 and concentrated by evaporation. Then purifying by column chromatography was carried out, using DCM as an eluent. First fraction was taken, for NMR-analysis. NMR analysis didn't prove supposed structure. *(Spectrum 10)*

UV–VIS (CH_2Cl_2): λ_{max} (ε) 652 (3087), 594 (3504), 520 (7424), 423 (855055). *(Spectrum 17)*

General reaction scheme of another two reactions is shown in Scheme *10*:



12 5,10,15,20-tetrakis(4-nitrophenyl)porphyrin
13 5,10,15,20-tetrakis(4-aminophenyl)porphyrin

Scheme 10

5,10,15,20-tetrakis(4-nitrophenyl)porphyrin (compound 12)

A solution of 4-nitrobenzaldehyde (5g, 0.0331mol) in glacial acetic acid (100ml) was heated at a reflux open to the air. Pyrrole (2.3ml; 0.031mol) was then added dropwise to the boiling solution. The solution became dark and was heated under reflux for 20 minutes, chloroform (12ml) was then added after cooling down. Crystalline product

was isolated by filtration and washed with chloroform and dried and directly used as an intermediate for next reaction.

5,10,15,20-tetrakis(4-aminophenyl)porphyrin (compound 13)

The compound 12 (5.83g; 7.33mmol) was dissolved in concentrated HCl (300ml) at room temperature, followed by the addition of excess of tin(II)chloride dihydrate (23.918g; 106mmol). Resulting green mixture was quickly heated to 65-70°C and stirred for 25 minutes, after that the solution was neutralized with concentrated aqueous ammonia (261ml). Basic reaction of the mixture was tested and then ethylacetate was added (485ml) and stirred overnight. The aqueous layer was separated and then extracted with ethylacetate (3x100ml). Collected organic phases were dried by Na_2SO_4 and evaporated to dryness. Crystals were dissolved in minimal amount of chloroform, precipitated with hexane and subsequently filtered. Black crystalline product in yield 0.1418g (2.87%) was prepared for NMR-analysis to prove it structure.

UV–VIS (CH_2Cl_2): λ_{max} (ε) 564 (2625), 523 (2505), 428 (414207). *(Spectrum 18)*

^{1}H NMR (500MHz, $CDCl_3$) δ -2.72 (2H, s, 4-H) 7.07 (8H, d, *J*=8.2, 2-H) 8.00 (8H, d, *J*=8.1, 3-H) 8.89 (8H, s, 1-H) *(Spectrum 11)*

Testing for LDL and porphyrin interaction

I have used water soluble porphyrin (5,15-bis(N-methyl-4-pyridyl)-10,20-bis(4-(3-hydroxycarboxamidopropoxy)phenyl)porphyrin chloride) in concentration 1mg/10ml and low density lipoprotein from human plasma in scale of concentrations (1.8mg/10ml; 0.9mg/10ml; 0.45mg/10ml; 0.225mg/10ml; 0.1125mg/10ml and 0mg/10ml) for this experiment. I have put 1.5ml of each of these solutions together and after 5 minute incubation time I have controlled the interaction by measuring of UV-VIS spectra. I observed absorption shown in Spectrum *19*.

Discussion

My supervisor has tasked me with doing line of three positional isomers of substituted porphyrins with (ethoxycarbonylbutoxy)phenyl and mesityl functions in *meso*-position, compounds **2-4**. This part of my experiments was quite successful and I managed to prepare and get in pure form all of them except for purification of atropoizomers of compound 7.

Next task was to prepare corresponding free acids by hydrolysis of compounds **5** and **6** to increase polarity of these compounds. Hydrolysis took place in severe conditions, but fortunately it was successful too. Despite presence of two carboxylic functions in porphyrin's molecule, compounds **8** and **9** didn't reach water solubility, they were bit soluble in strongly basic aqueous solution of NaOH. These compounds also don't show good absorption in red part of visible spectrum.

In next step of my work I have tried to include structure of cholesterol into porphyrin's molecule to follow the idea with selective uptake to vulnerable plaque of atherosclerotic arteries. My effort to bind cholesterol by esteric bond to carboxylic function of compound **8** has failed, because of high molecular weight of intended compound (1668.4g/mol) as well as of difficult chromatographic separation procedure, which can be influenced by surface active properties of cholesterol.

In the end of my work I tried to test interaction of water soluble porphyrin with human LDL. I chose scale of LDL concentrations and let them interact with porphyrin. Interaction should appear as a change of UV-VIS spectrum, but I didn't observe any substantive difference.

Conclusion

I have prepared 12 compounds:

2-(Mesityl(1*H*-pyrrol-2-yl)methyl)-1*H*-pyrrole
Ethyl 5-(4-formylphenoxy)pentanoate
Ethyl 5-(3-formylphenoxy)pentanoate
Ethyl 5-(2-formylphenoxy)pentanoate
5,15-bis[4-(4-ethoxycarbonylbutoxy)phenyl]-10,20-bis(2,4,6-trimethylphenyl)porphyrin
5,15-bis[3-(4-ethoxycarbonylbutoxy)phenyl]-10,20-bis(2,4,6-trimethylphenyl)porphyrin
5,15-bis[2-(4-ethoxycarbonylbutoxy)phenyl]-10,20-bis(2,4,6-trimethylphenyl)porphyrin
5,15-bis[4-(4-carboxybutoxy)phenyl]-10,20-bis(2,4,6-trimethylphenyl)porphyrin
5,15-bis[3-(4-carboxybutoxy)phenyl]-10,20-bis(2,4,6-trimethylphenyl)porphyrin
5,15-bis[4-(4-carboxybutoxy)phenyl]-10,20-bis(2,4,6-trimethylphenyl)porphyrin-chloride
5,10,15,20-tetrakis(4-nitrophenyl)porphyrin
5,10,15,20-tetrakis(4-aminophenyl)porphyrin

Each of these eight porphyrins can serve as a model or starting point for further progress of the idea using porphyrins for PDT of atherosclerosis. Chiefly it is important to reach higher water solubility and absorption of light of longer wavelength.

Abstract

Náplní mé diplomové práce je studium vlastností, použití a syntéza porfyrinů.
V úvodu teoretické části jsem vzpomenul na přírodní původ rodiny porfyrinů a uvedl základní informace o čtyřech nejdůležitějších v přírodě se vyskytujících zástupcích: chlorofylu, hemu, cytochromech a vitaminu B_{12}. V kapitole věnované synteticky připraveným porfyrinům jsem se zabýval vývojem jejich názvosloví a jejich chemickými a fyzikálními vlastnostmi. Důkladně jsem informoval o amfoterních vlastnostech a planaritě související s konjugací 22 π–elektronů základního porfyrinového kruhu. Dále jsem naznačil, že již byly připraveny neplanární deriváty porfyrinů a stejně tak mnohé izomery. Neopomněl jsem ani informaci o důležité vlastnosti porfyrinů, kterou je jejich schopnost vystupovat jako ligand pro většinu kovů a některé nekovy periodického systému prvků. Následující část byla zasvěcena syntézám porfyrinů a zmíněny byly dva hlavní způsoby: syntéza z monomerů a syntéza z dimerů (dipyrriny, dipyrromethany, dipyrroketony). Z fyzikální vlastností jsem se blíže věnoval spektrálním vlastnostem porfyrinů, které se odrážejí v jejich barvě.
Kapitola o použití porfyrinů naznačila, že větší část mé práce bude věnována jejich použití jako fotosenzitizérů pro fotodynamickou terapii (PDT). Po historickém úvodu byly uvedeny vlastnosti ideálního fotosenzitizéru a rozdělení fotosenzitizérů doplněné o informace o průběhu klinických zkoušek a jejich aplikací. Následující kapitola poskytla informace o vzniku reaktivního singletového kyslíku a objasnila tak mechanismus účinku fotosenzitizérů, odhaleny byly i buněčné cíle $^1O_2$.
K vlastnímu tématu mé práce jsem se dostal až v kapitole Atheroskleróza, která byla věnována podrobnějšímu popisu rizikových faktorů a ethiologie tohoto velmi častého onemocnění. Celý proces tvorby aterogenního plátu, který ve finále působí ztrátu schopnosti cév zásobovat tkáně kyslíkem a vede k vážným stavům (infarkt myokardu, angina pectoris, cévní mozková příhoda, ...) jsem schematizoval v jednom obrázku. Následující obrázek shrnul mechanismus účinku motexafin lutecia, látky, která se jeví jako velmi slibný fotosenzitizér pro PDT atheroskerózy.
V experimentální části mé práce jsem měl za úkol nasyntetizovat řadu 3 polohových derivátů porfyrinu s esterickou funkcí na periferii. Tyto látky se též staly výchozím bodem pro syntézu porfyrinu nesoucího ve své struktuře 2 molekuly cholesterolu. V úmyslu bylo obdržet porfyrin, který by byl selektivněji akumulován v místě

účinku. Snahy o syntézu tohoto derivátu selhaly díky komplexnosti obdržené reakční směsi, kterou se nepodařilo rozdělit ani chromatografickou metodou. U všech obdržených produktů byla provedena NMR-analýza a u absorbujících porfyrinů změřena absorbce v UV-VIS spektru. Posledním provedeným experimentem bylo ověření interakce ve vodě rozpustného porfyrinu s humánním LDL, sledované změnou absorbce v UV-VIS oblasti. Série pokusů ukázala, že k interakci nedochází.

Na samém konci mé diplomové práce jsem uvedl seznam použité literatury a přiřadil spektra připravených látek jako obrazovou přílohu.

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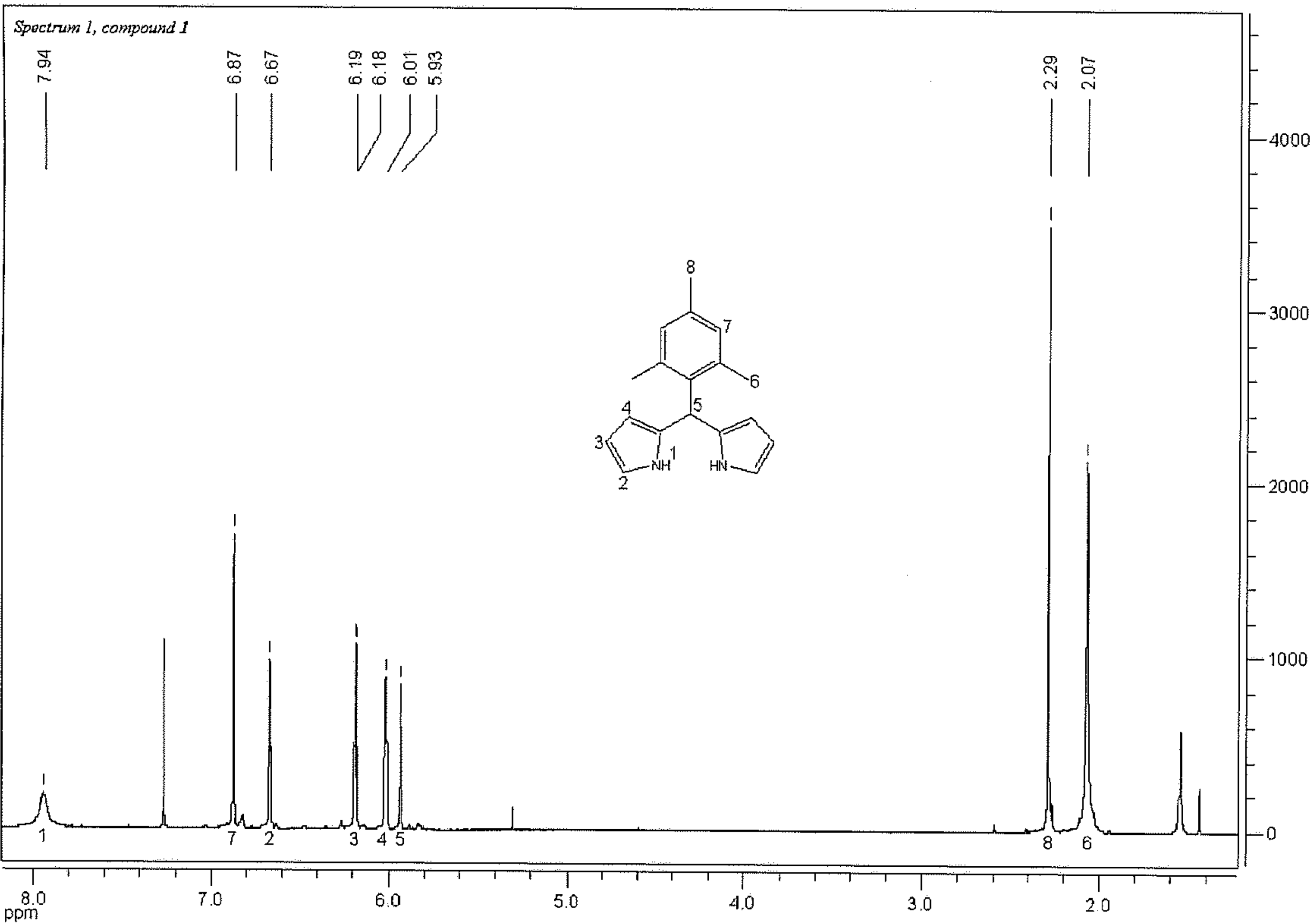

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6.87
6.67
6.19
6.18
6.01
5.93
2.29
2.07
8
7
6
5
4
3
2
1
NH
HN
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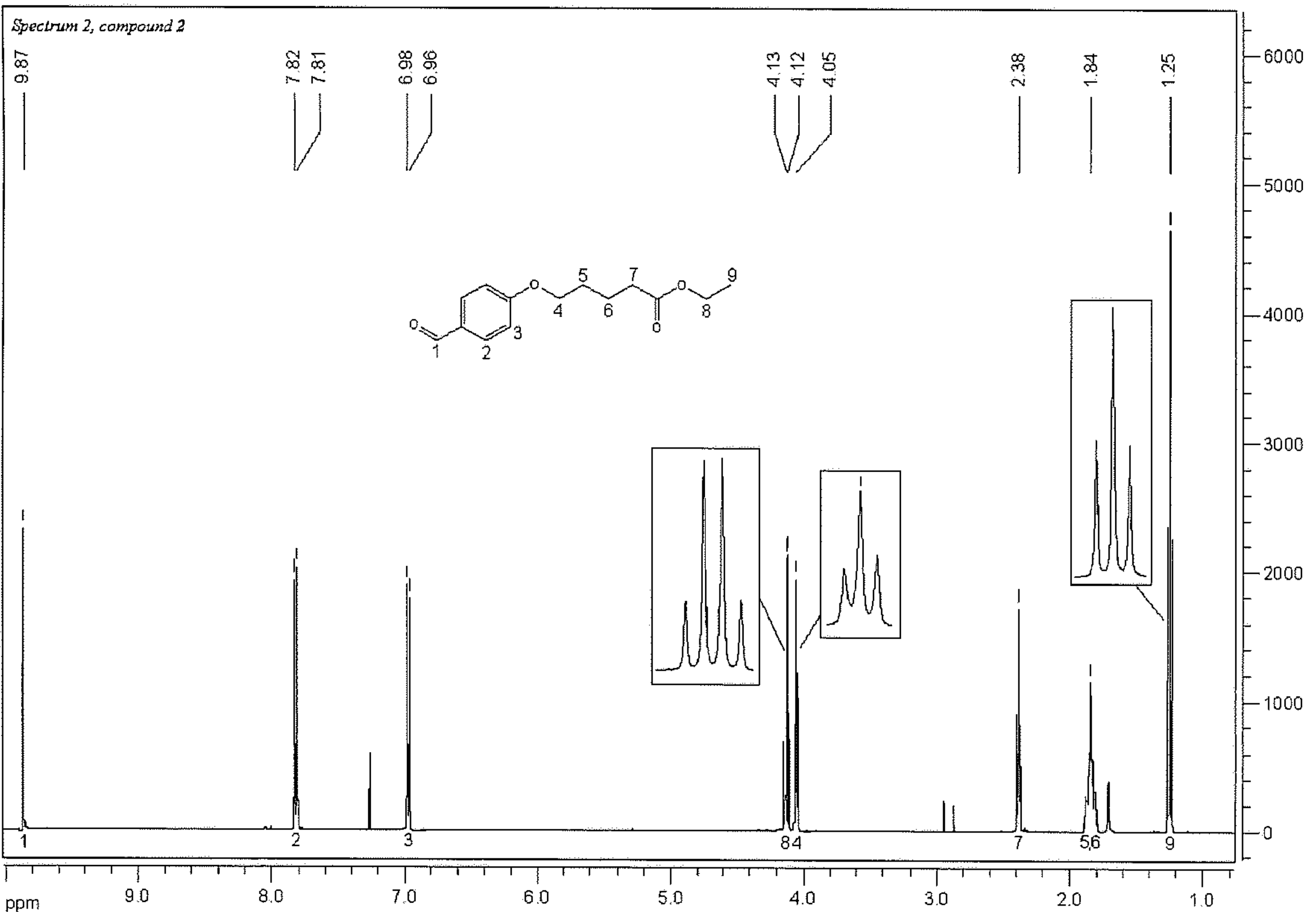
3000
2000
1000
0
8.0
ppm
7.0
6.0
5.0
4.0
3.0
2.0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7.81
6.98
6.96
4.13
4.12
4.05
2.38
1.84
1.25
6000
5000
4000
3000
2000
1000
0
1
2
3
84
7
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ppm
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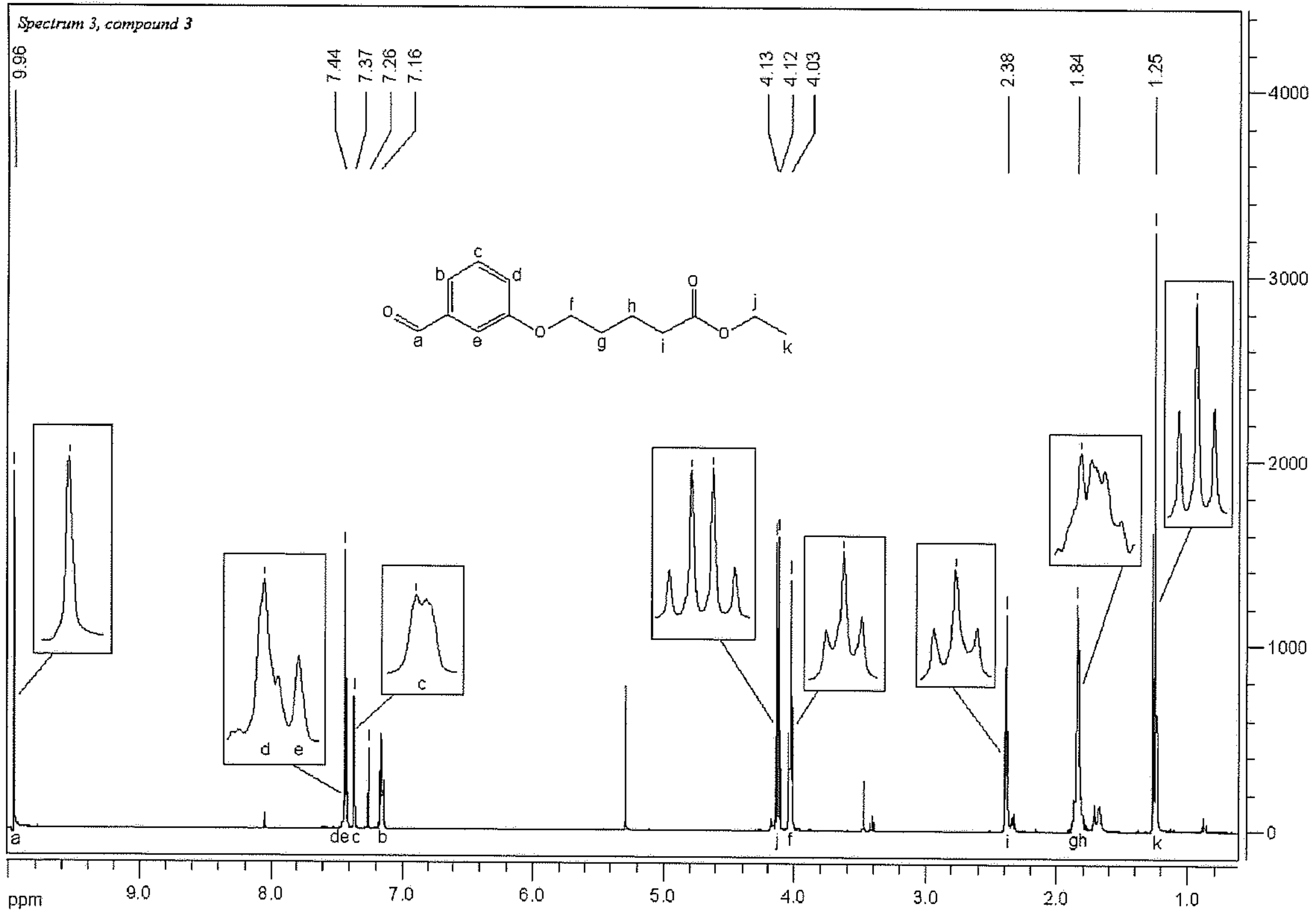
7.0
6.0
5.0
4.0
3.0
2.0
1.0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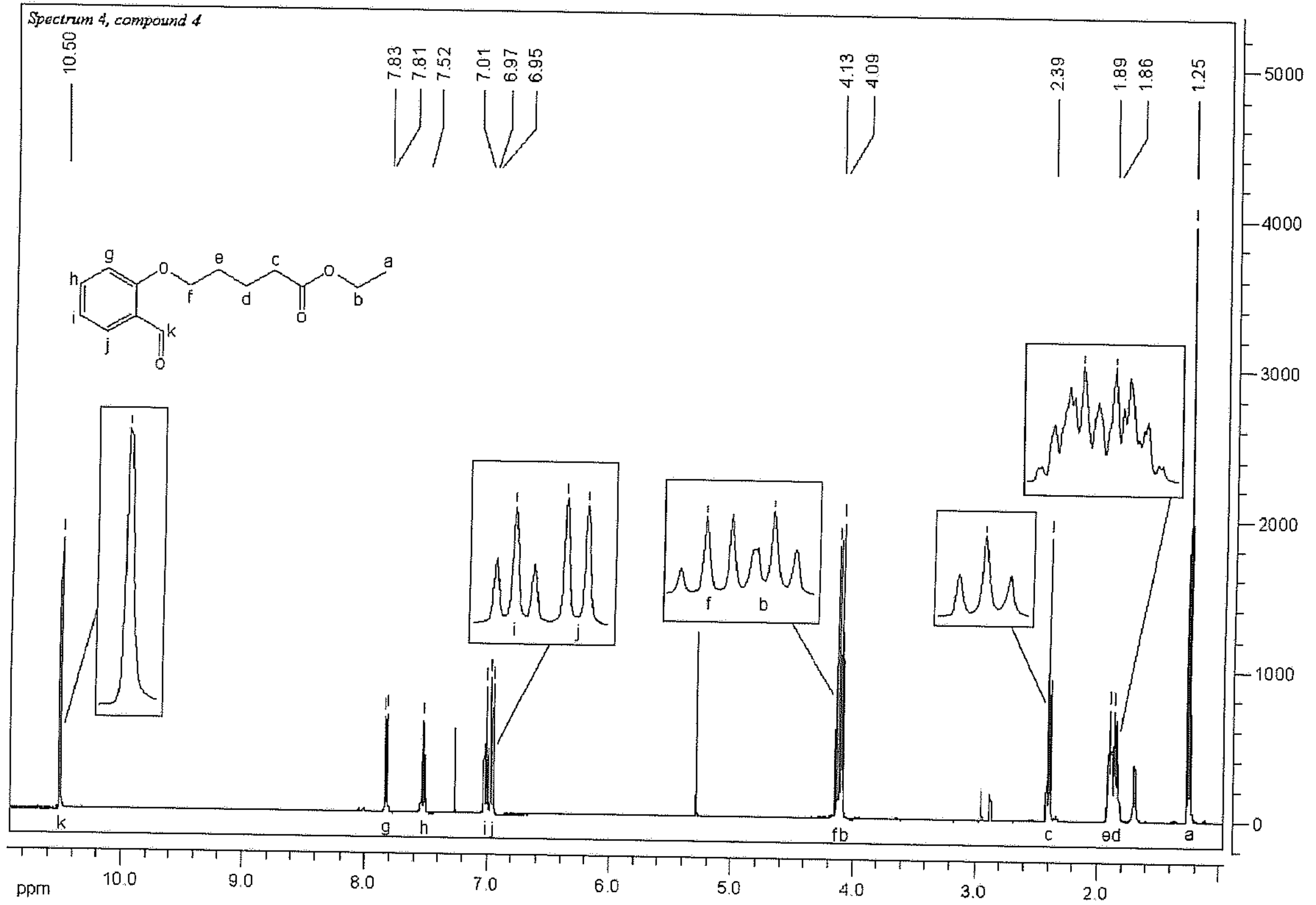
7.37
7.26
7.16
4.13
4.12
4.03
2.38
1.84
1.25
ppm
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
4000
3000
2000
1000
0
a
de c
b
j f
i
gh
k
d e
c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7.52
7.01
6.97
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4.09
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1.86
1.25
g
h
i
j
k
O
e
c
f
d
a
b
5000
4000
3000
2000
1000
0
k
g
h
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ppm
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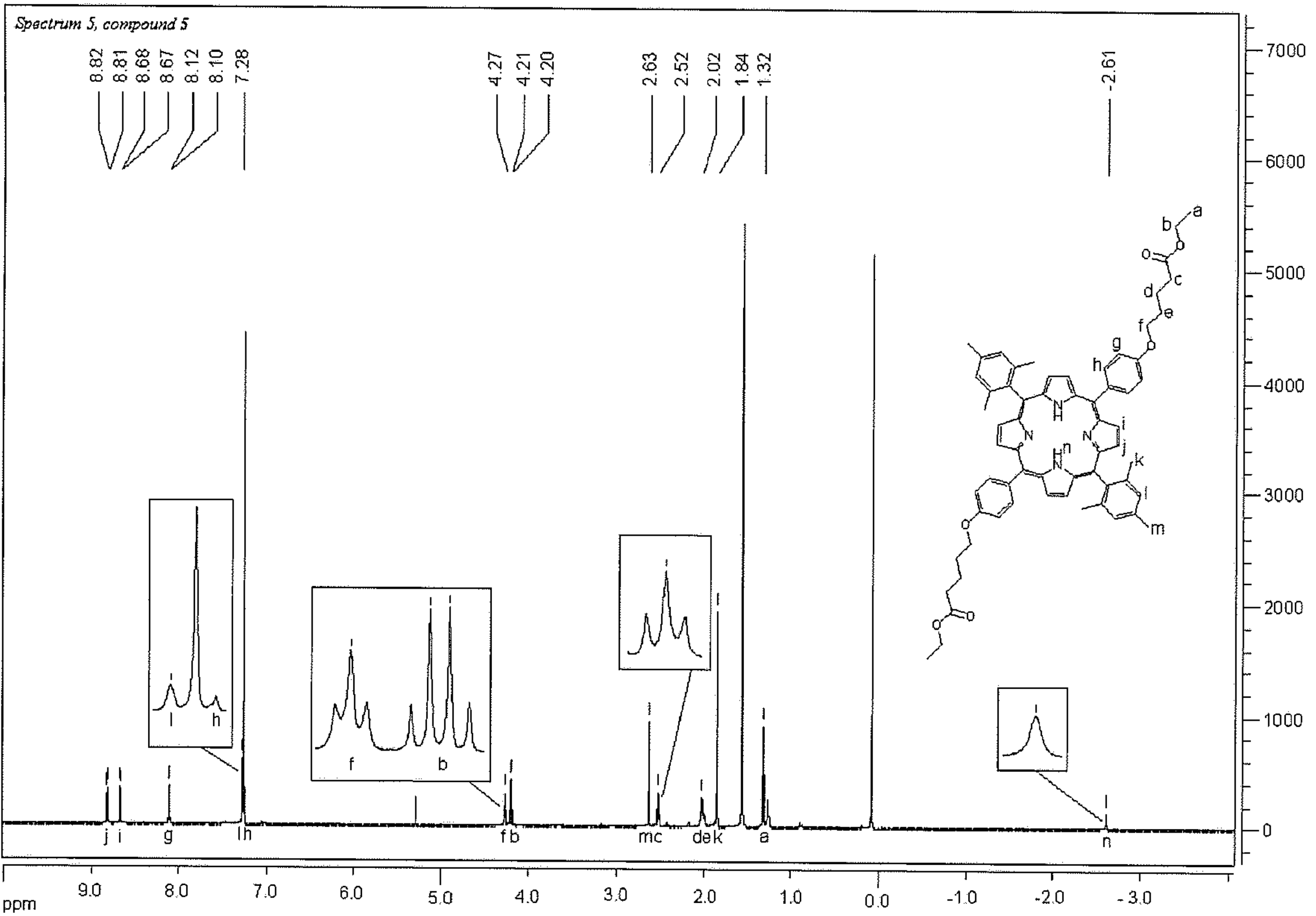
2.0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2.63
2.52
2.02
1.84
1.32
-2.61
j i
g
l h
f b
mc
dek
a
n
ppm
9.0
8.0
7.0
6.0
5.0
4.0
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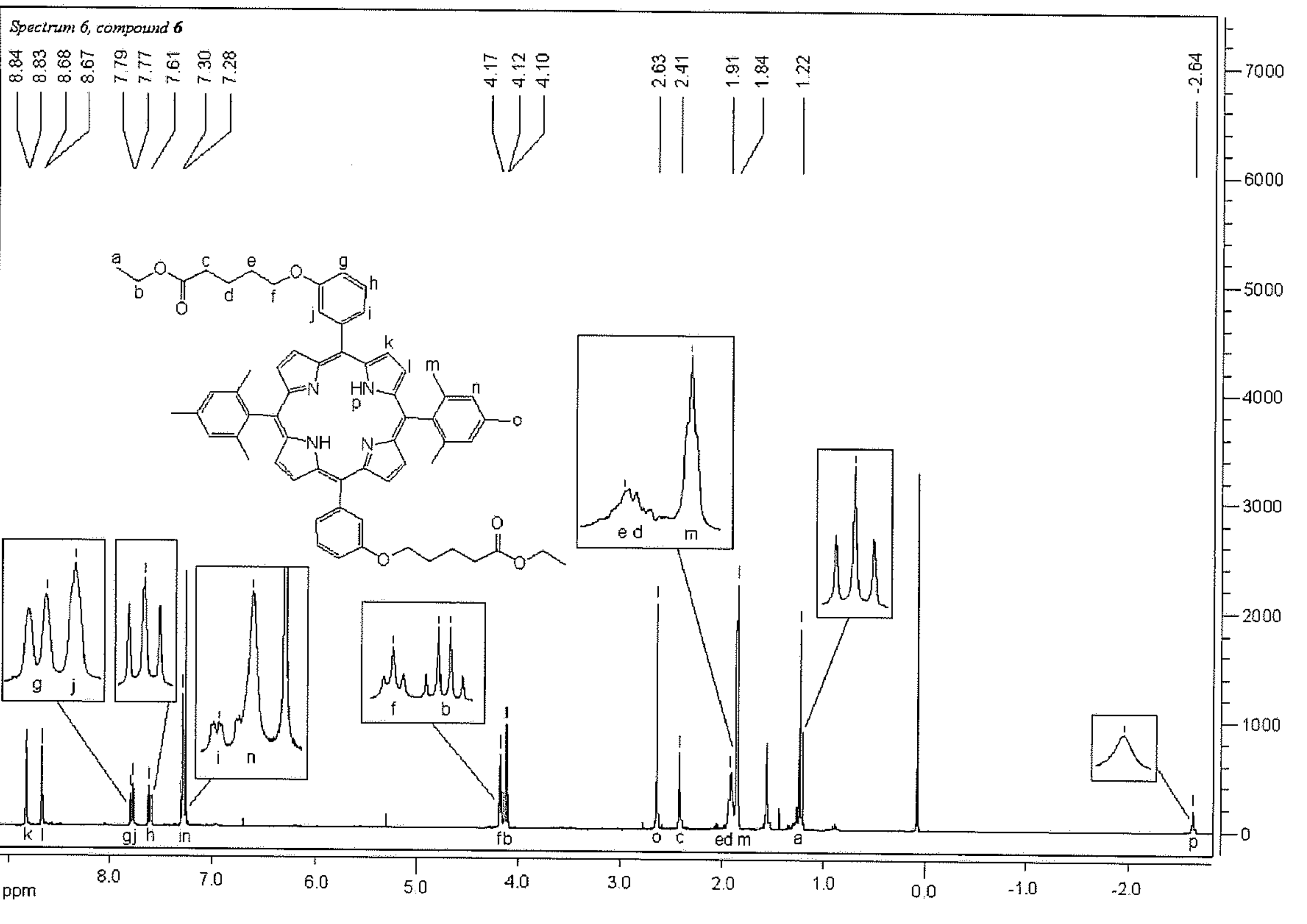
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8.83
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7.77
7.61
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7.28
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4.12
4.10
2.63
2.41
1.91
1.84
1.22
-2.64
7000
6000
5000
4000
3000
2000
1000
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a b c d e f g h i j k l m n o p
HN
NH
N
O
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g j
i n
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k l
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fb
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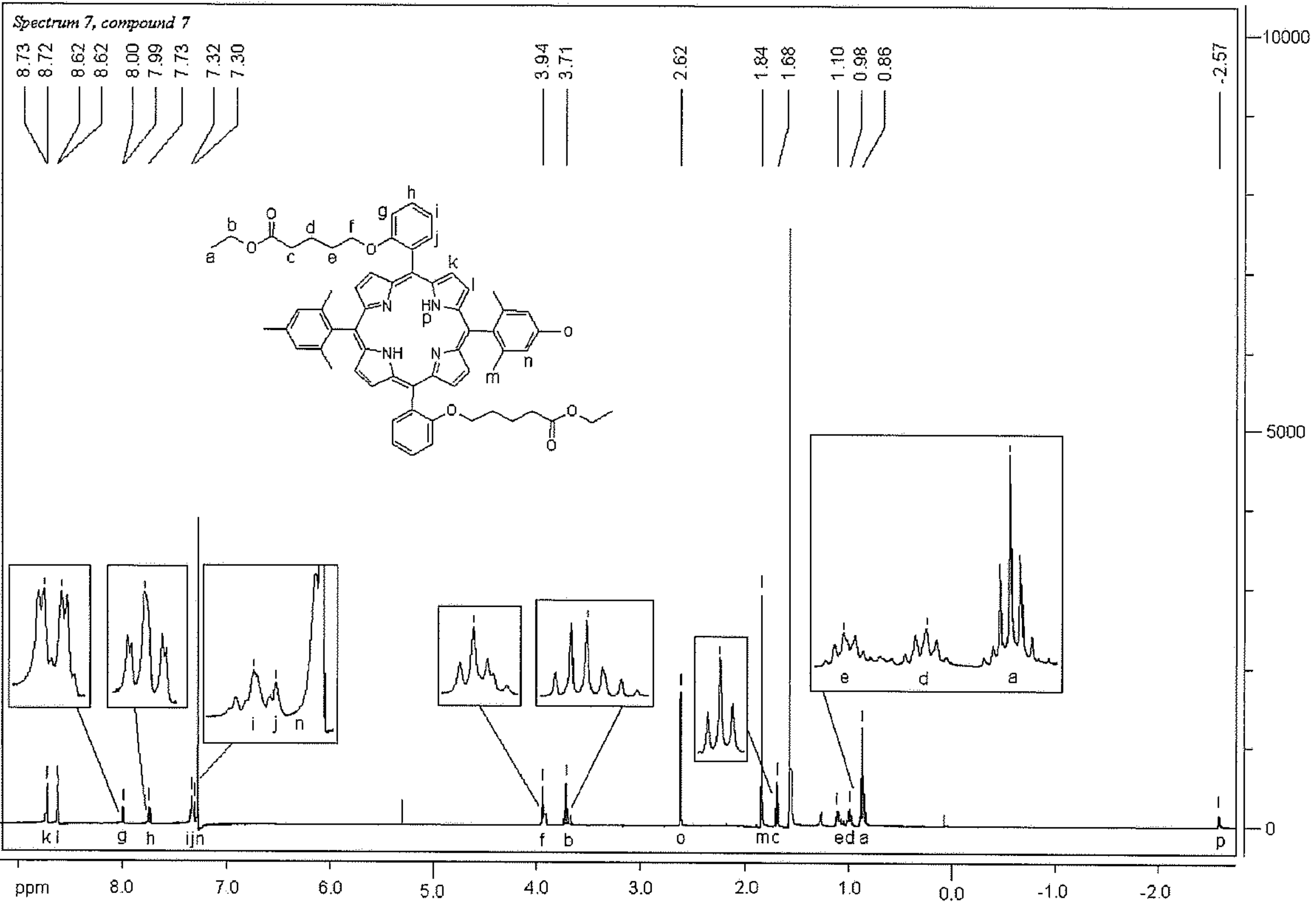
-2.0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3.71
2.62
1.84
1.68
1.10
0.98
0.86
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10000
5000
0
ppm
8.0
7.0
6.0
5.0
4.0
3.0
2.0
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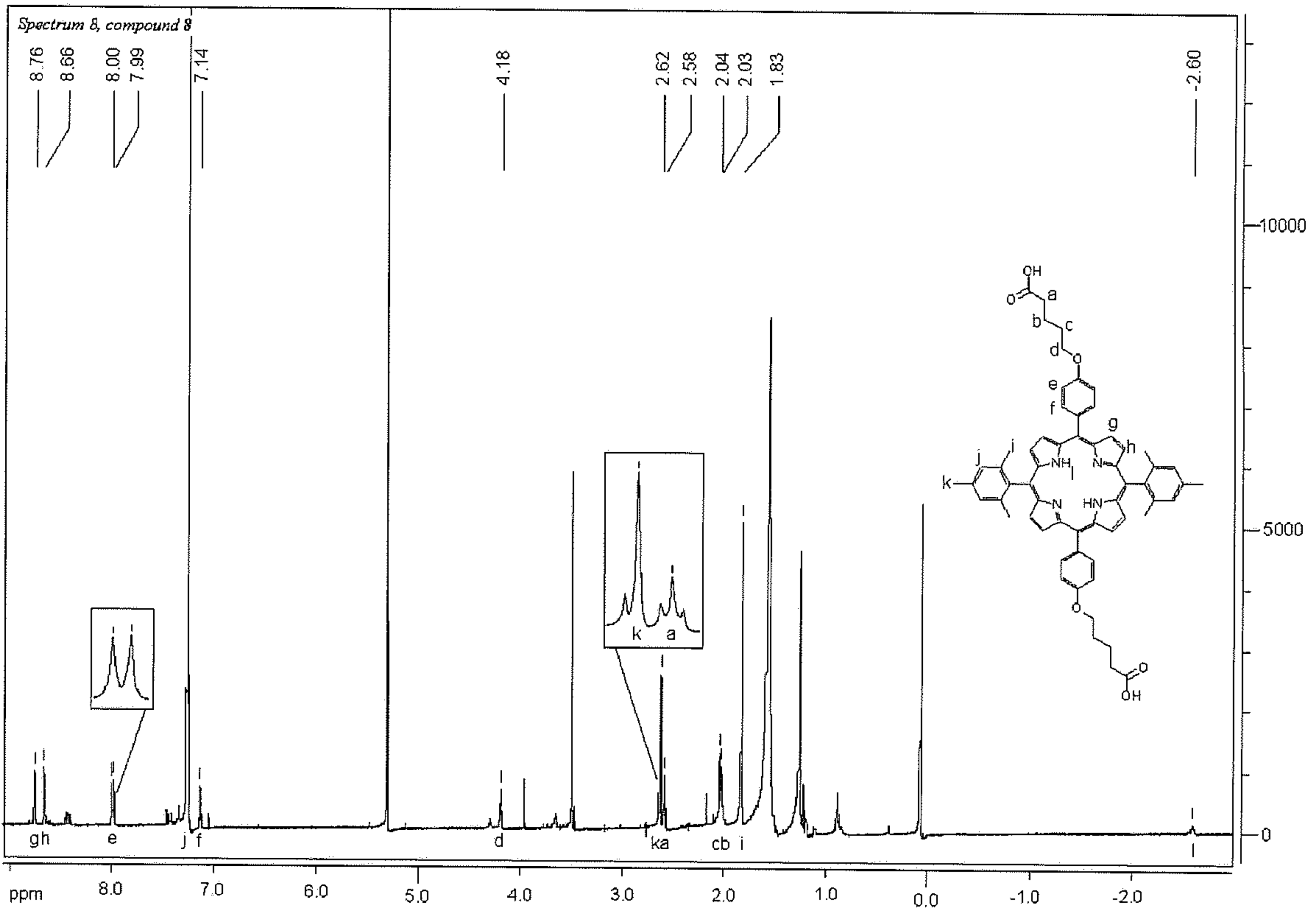
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2.58
2.04
2.03
1.83
-2.60
gh
e
j f
d
ka
cb i
k
a
ppm
8.0
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5.0
4.0
3.0
2.0
1.0
0.0
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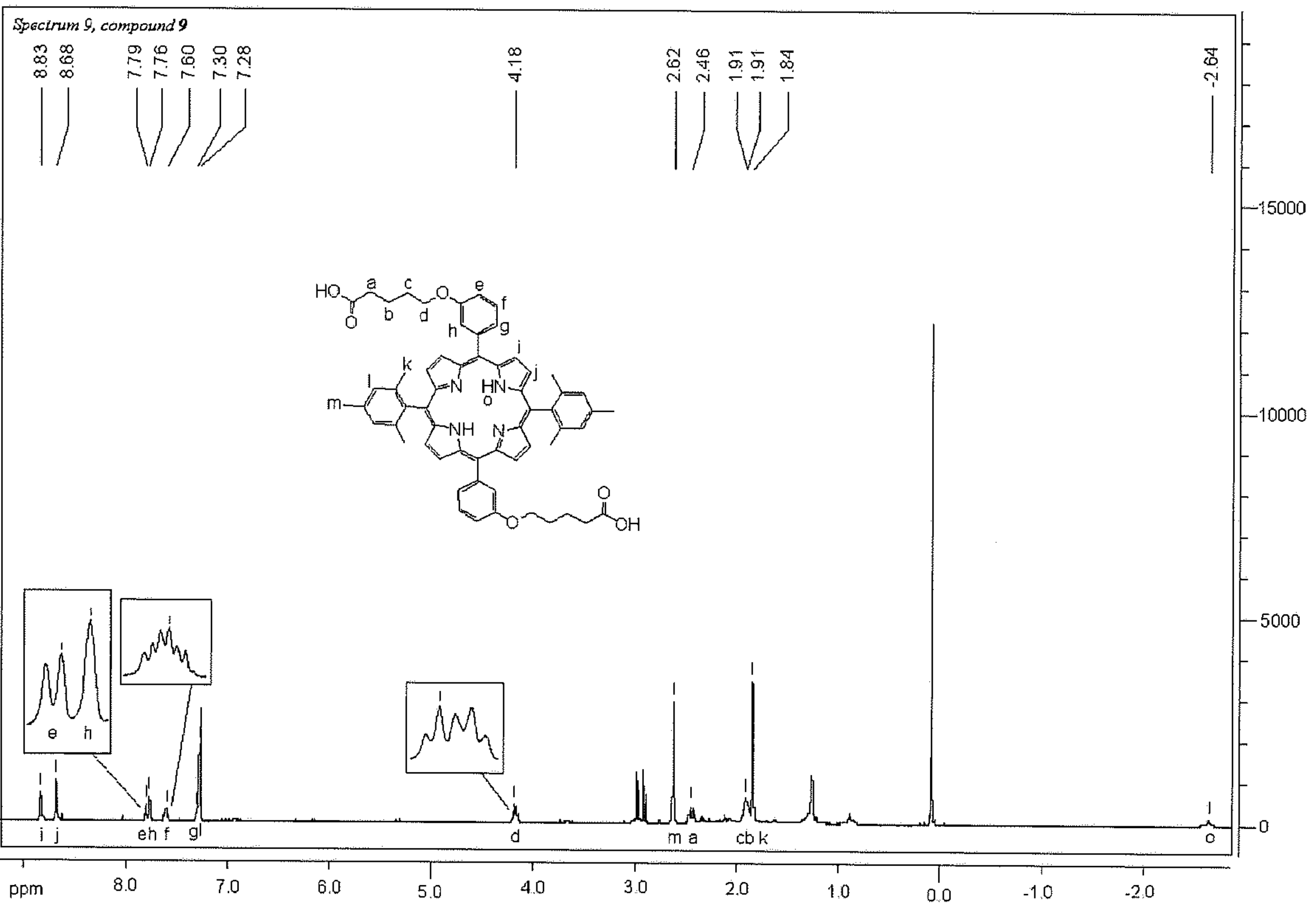
-2.0
10000
5000
0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8.68
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7.76
7.60
7.30
7.28
4.18
2.62
2.46
1.91
1.91
1.84
-2.64
15000
10000
5000
0
HO
a
b
c
d
O
e
f
g
h
i
j
k
l
m
N
HN
o
NH
N
OH
e h
i j
eh f
gl
d
m a
cb k
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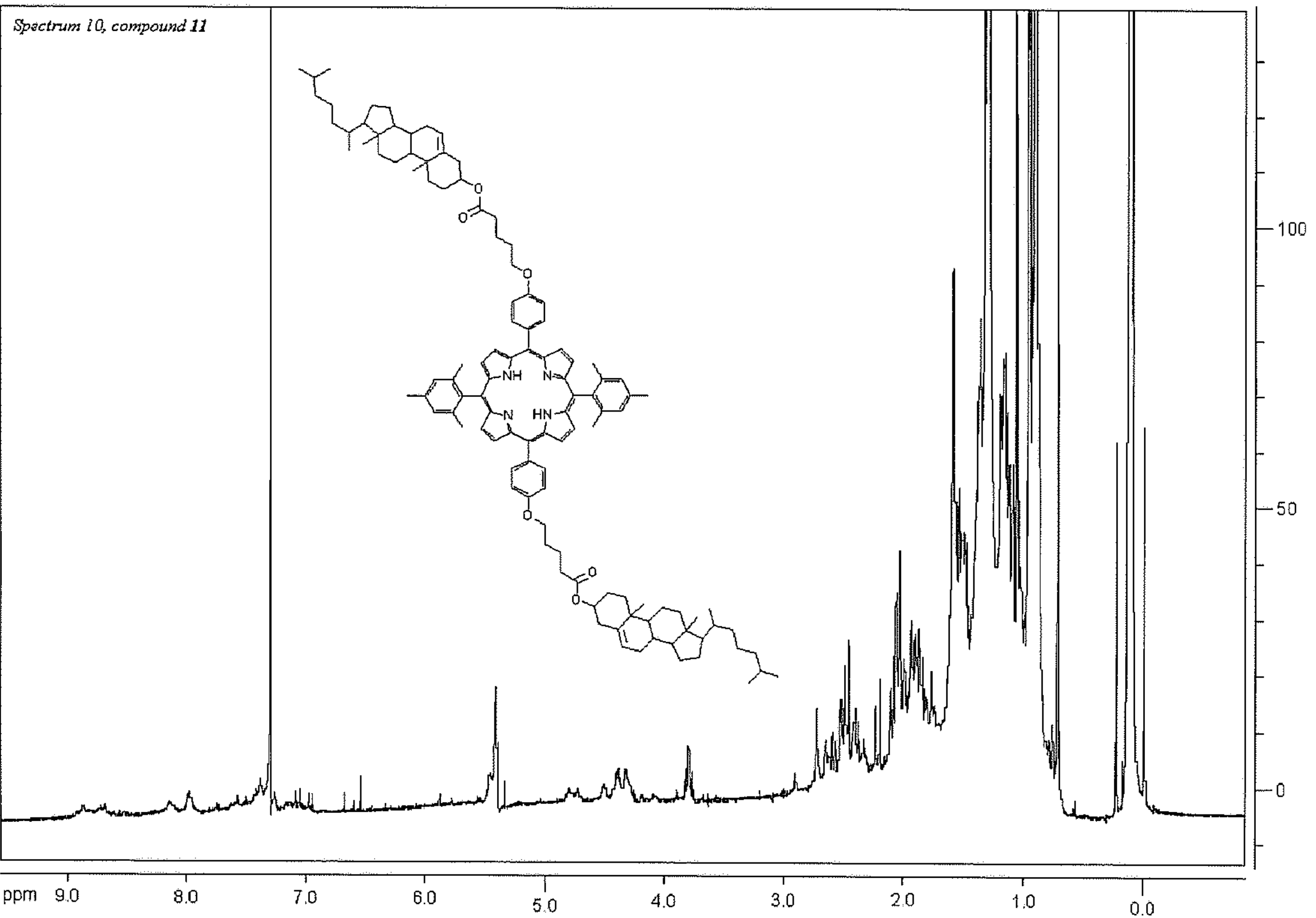
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N
HN
O
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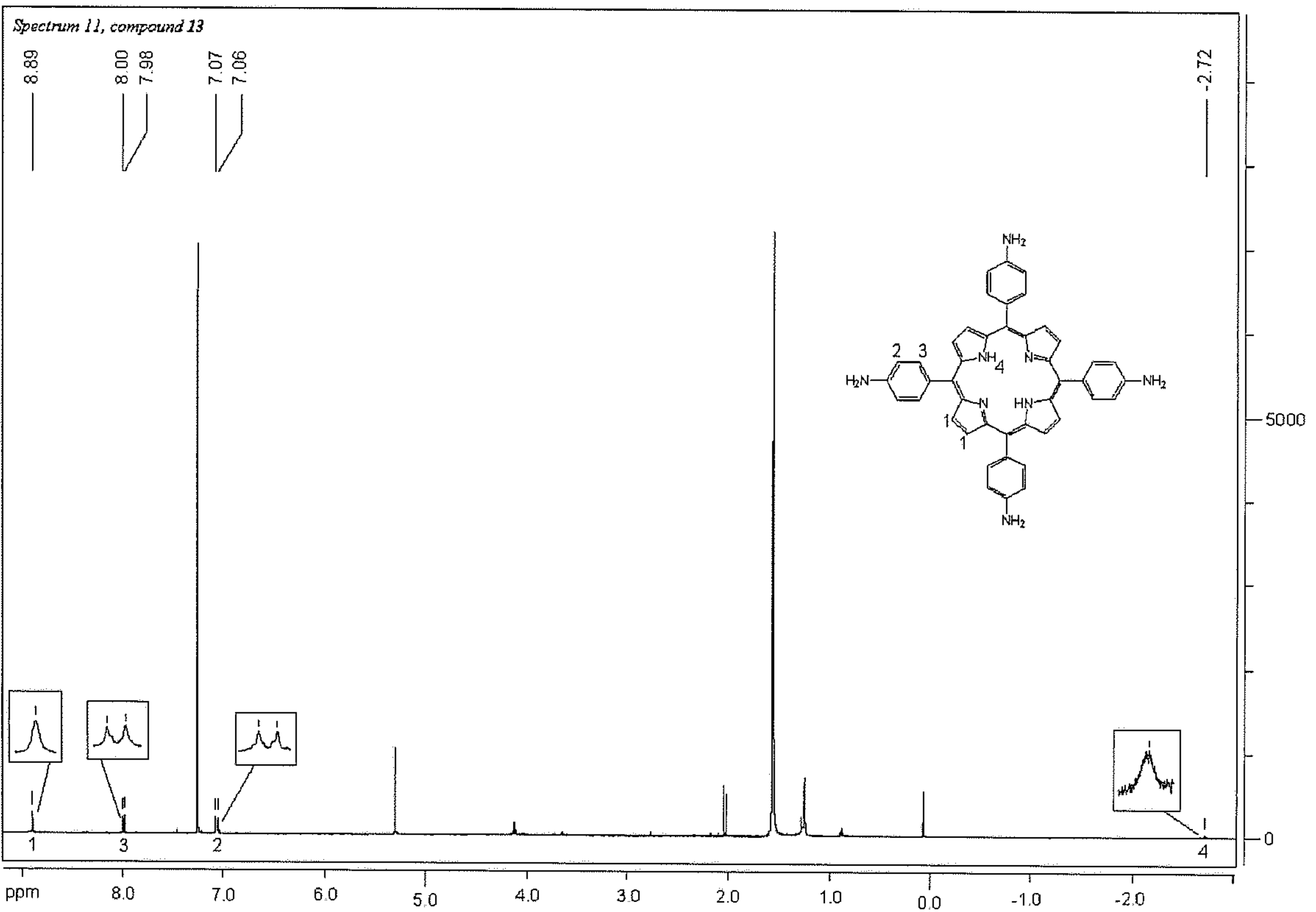
100
50
0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7.07
7.06
-2.72
NH_2
H_2N
NH
HN
N
1
2
3
4
5000
0
ppm
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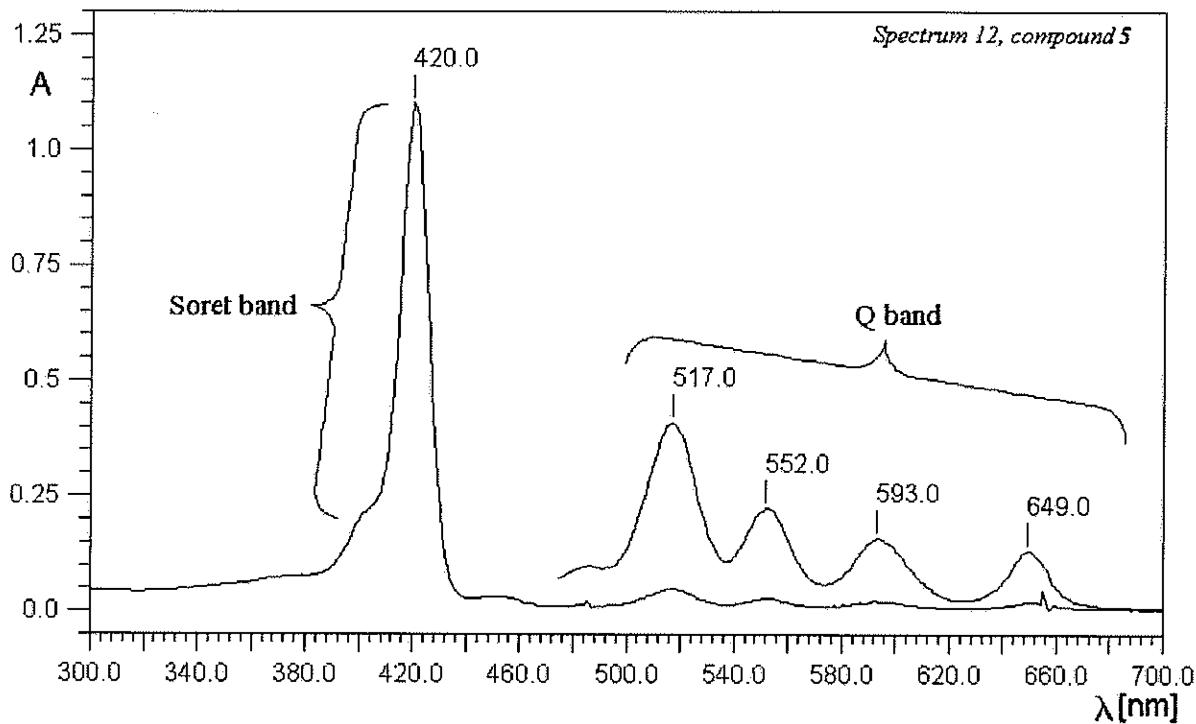
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0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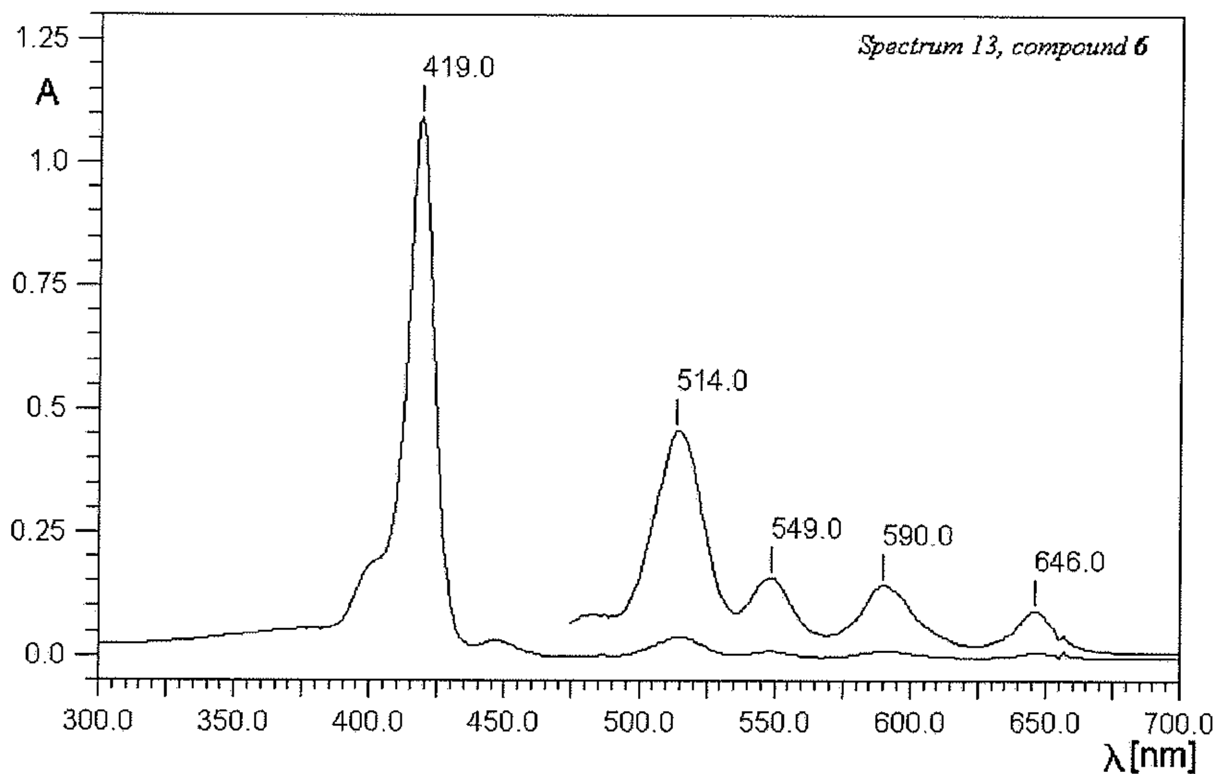
420.0
Soret band
Q band
517.0
552.0
593.0
649.0
1.25
1.0
0.75
0.5
0.25
0.0
300.0
340.0
380.0
420.0
460.0
500.0
540.0
580.0
620.0
660.0
700.0
λ[nm]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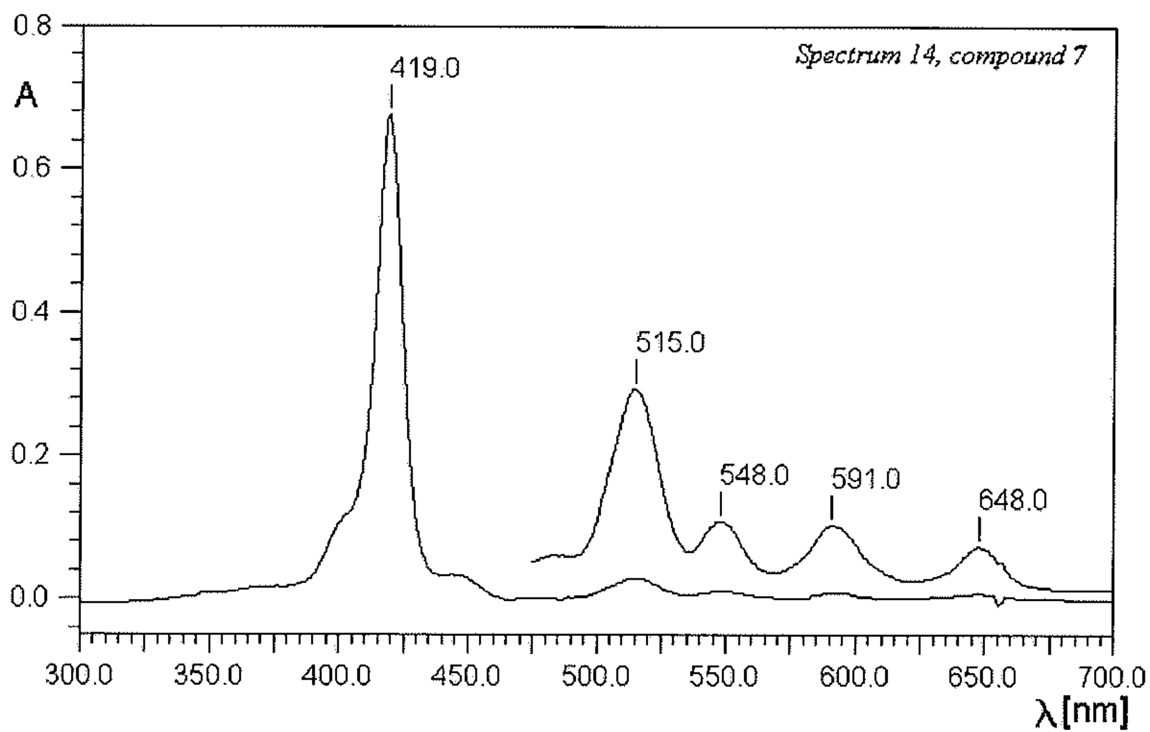
514.0
549.0
590.0
646.0
1.25
1.0
0.75
0.5
0.25
0.0
300.0
350.0
400.0
450.0
500.0
550.0
600.0
650.0
700.0
λ[nm]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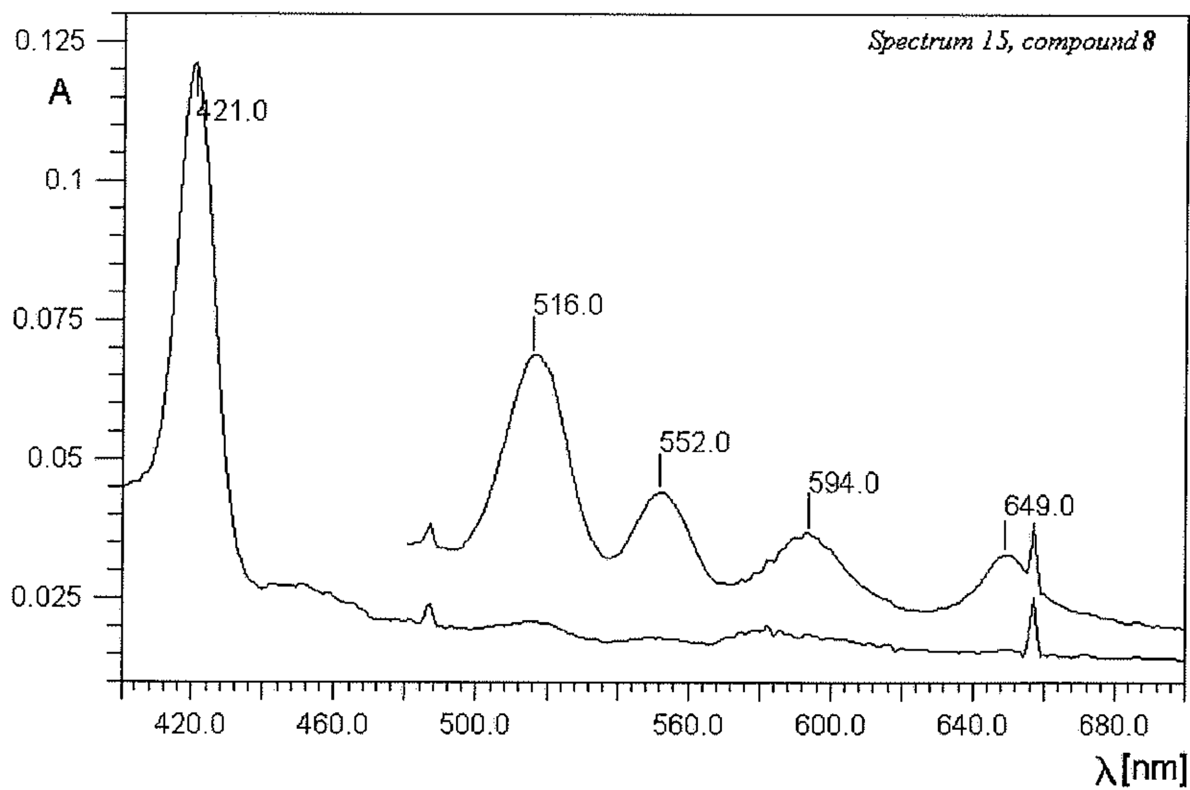
0.4
0.2
0.0
419.0
515.0
548.0
591.0
648.0
300.0
350.0
400.0
450.0
500.0
550.0
600.0
650.0
700.0
λ[nm]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0.1
0.075
0.05
0.025
421.0
516.0
552.0
594.0
649.0
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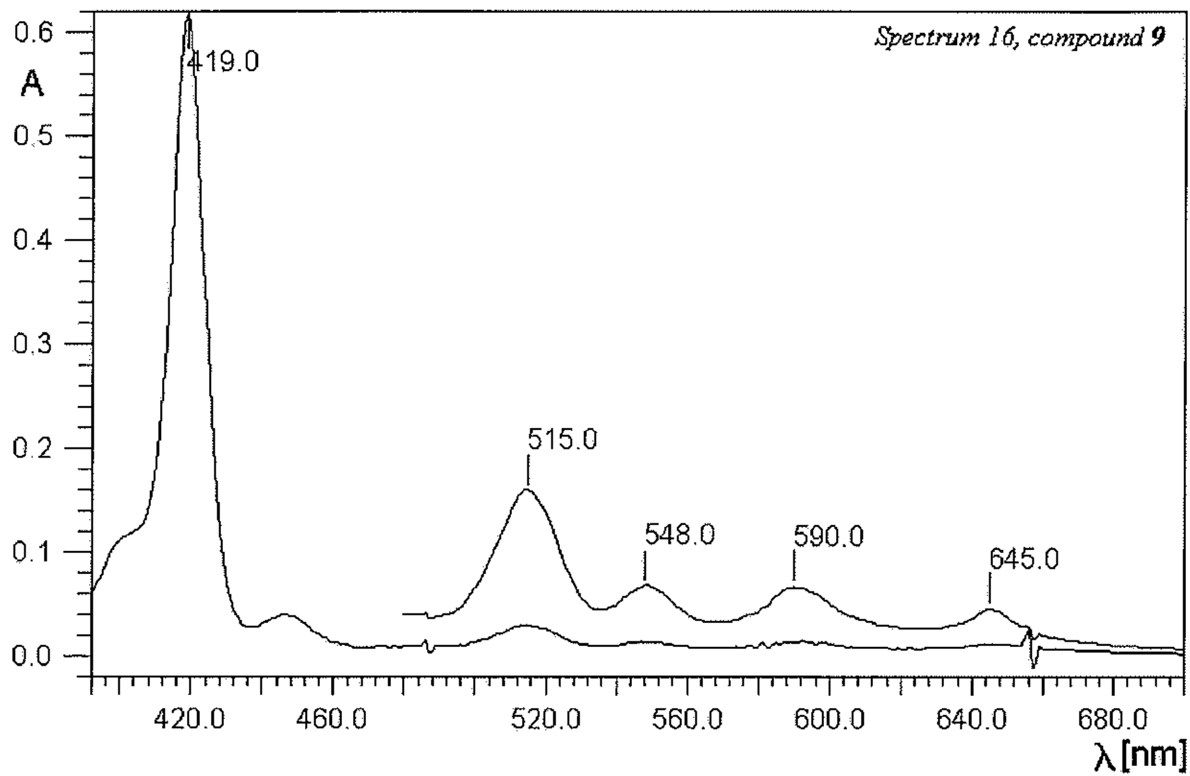
500.0
560.0
600.0
640.0
680.0
λ[nm]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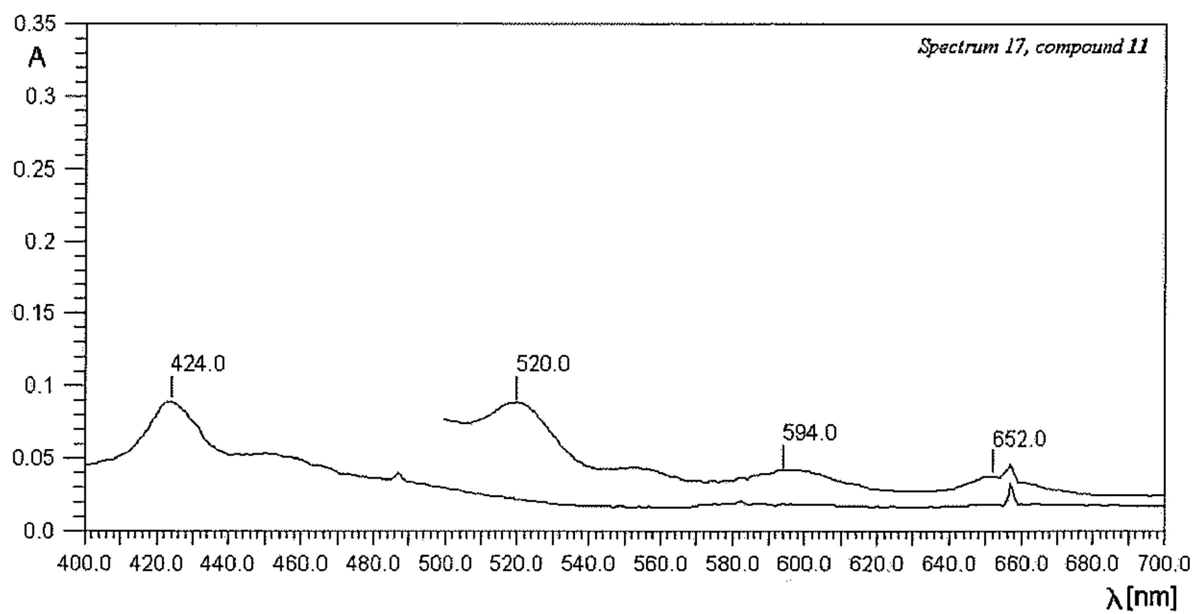
A
0.5
0.4
0.3
0.2
0.1
0.0
419.0
515.0
548.0
590.0
645.0
420.0
460.0
520.0
560.0
600.0
640.0
680.0
λ[nm]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A
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0.25
0.2
0.15
0.1
0.05
0.0
424.0
520.0
594.0
652.0
400.0
420.0
440.0
460.0
480.0
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540.0
560.0
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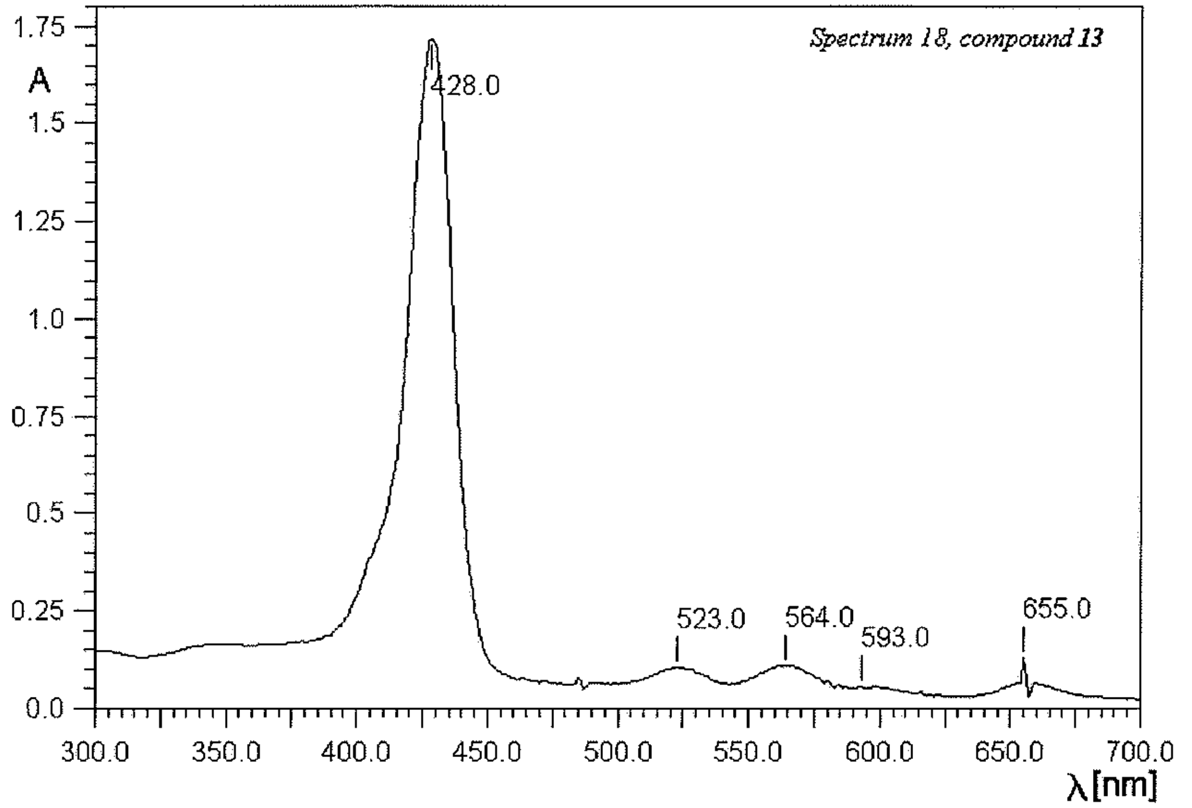
700.0
λ[nm]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1.5
1.25
1.0
0.75
0.5
0.25
0.0
428.0
523.0
564.0
593.0
655.0
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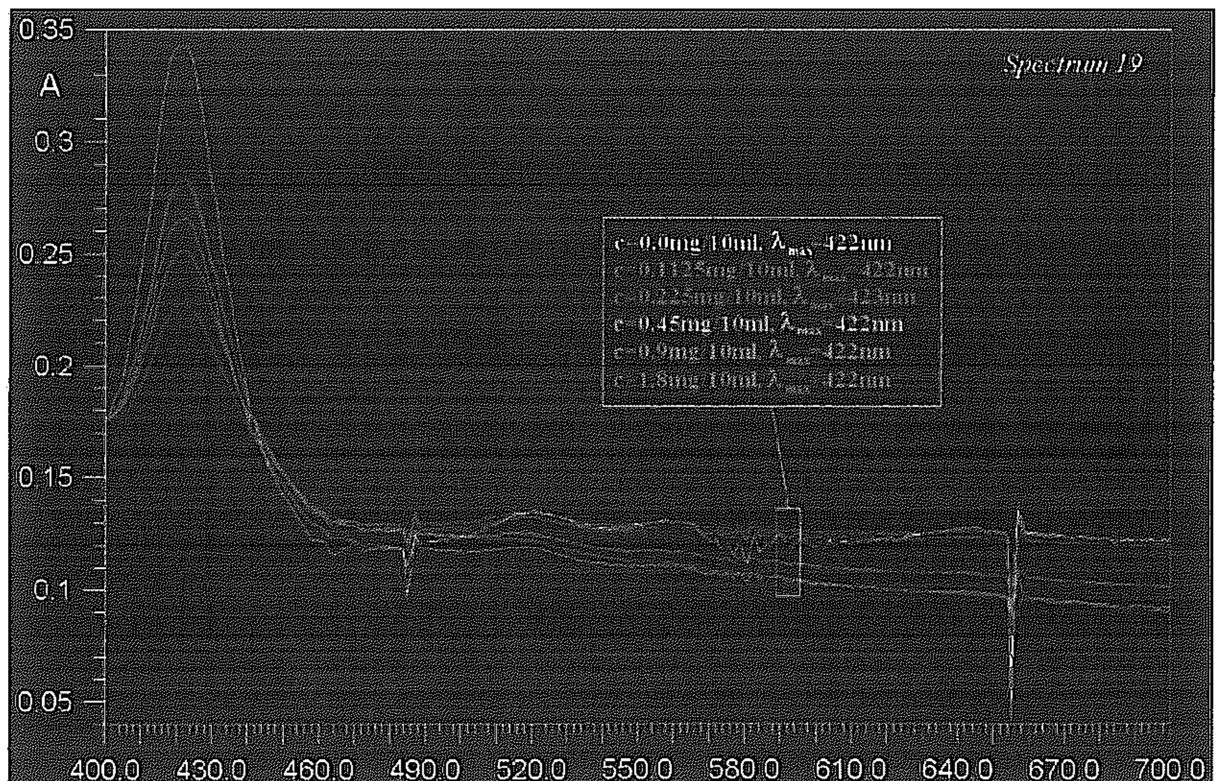
450.0
500.0
550.0
600.0
650.0
700.0
λ[nm]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0.25
0.2
0.15
0.1
0.05
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c=0.1125mg 10ml, λ_{max}=422nm
c=0.45mg 10ml, λ_{max}=422nm
c=0.9mg 10ml, λ_{max}=422nm
c=1.8mg 10ml, λ_{max}=422nm
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430.0
460.0
490.0
520.0
550.0
580.0
610.0
640.0
670.0
700.0