Progress towards the synthesis and self-assembly of amphiphilic drug conjugates of Iodinin

Thesis for the Master's degree in Chemistry

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ABSTRACT

Despite recent improvement in therapy, acute myeloid leukaemia (AML) is still associated with high lethality. Iodinin (1, 6-dihydroxyphenazine 5, 10-dioxide) is a compound of interest in leukaemia research, as it is showing very promising anti-cancer properties with low toxicity.

However, since the drug is essentially water-insoluble, effective administration is problematic and requires other chemical modification or encapsulation in nanoparticles e.g. liposomes, micelles etc. To this end we seek to develop an amphiphilic drug conjugate by first alkylating the phenolic group on iodinin C-1 and lastly attaching a PEG unit on the iodinin C-6 phenolic group with the aim of altering the physiochemical properties i.e. electrostatic binding and hydrophobicity in iodinin, so that this drug conjugate can be further studied in a nanoparticle motif to be released slowly upon hydrolysis in biological systems.

Graphical abstract
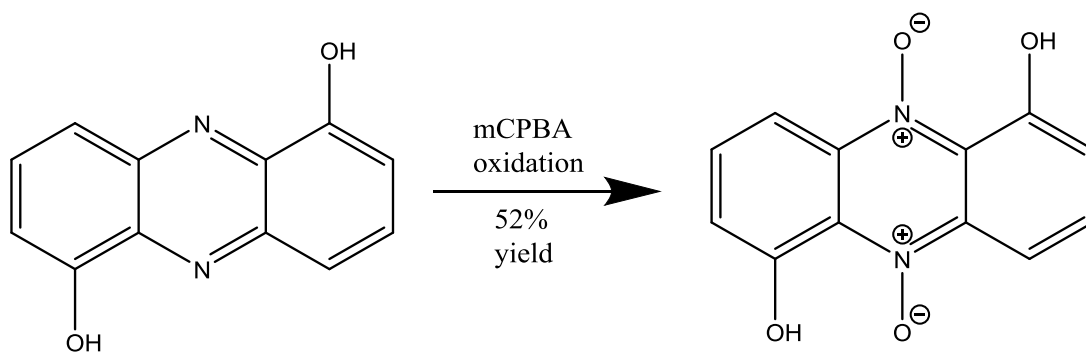
Iodinin

Abbreviations

5 | P a g e
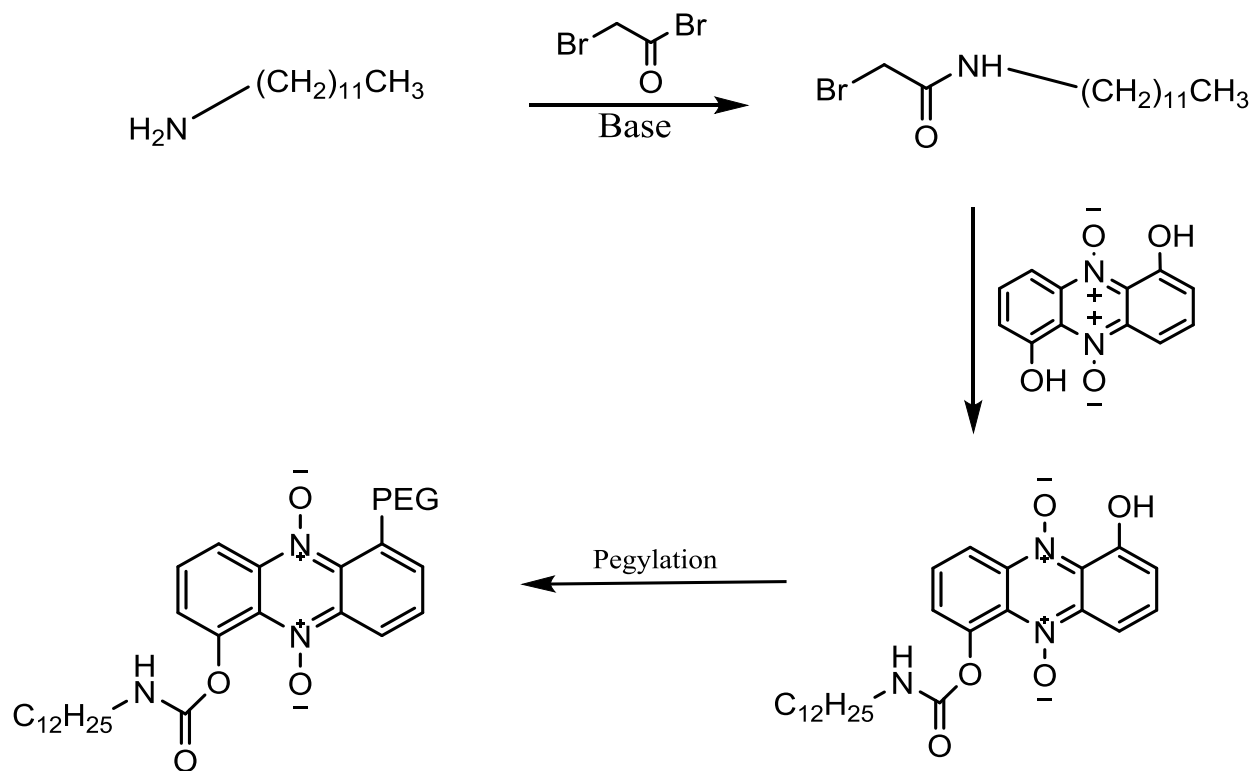
AML	Acute myeloid leukemia
AraC	Cytarabine
CoAS	Acetyl coenzyme A
CoASOC	Acetyl-CoA carboxylase
d	dublet
Da	Dalton
dd	double dublet
DCE	1,2-dichloromethane
DMF	Dimethylformamide
DMSO-*d6*	Deuterated dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
EC	Effective concentration
EI	Electron impact
eq.	Equivalent(s)
EtOAc	Ethylacetate
HMBC	Heteronuclear multiple bond-correlation
HSQC	Heteronuclear single quantum correlation
HMPT	Hexamethylphosphoric triamide
HR-MS	High resolution mass spectrometry
J	Coupling contant
m	Multiplet (NMR)
*m*CPBA	*meta*-chloroperbenzoic acid

MS	Mass spectrometry
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance spectroscopy
PEG	Poly (ethylene) glycol
PEO	Poly (ethylene) oxide
PLA	Poly (lactic acid)
PPO	Poly (propylene oxide)
PhMe	Toluene
ppm	Parts per million
r.t.	Room temperature
s	Singlet
t	Triplet
t-BuOK	Potassium *tert*-butoxide
TLC	Thin layer chromatography
UHP	Urea hydrogen peroxide

Contents

1 Background

Recently a potent anti-cancer candidate iodinin has been identified for effective treatment of acute myeloid leukaemia. Iodinin can be extracted from marine micro-organism but this method is expensive and yields limited quantities. This problem has been resolved by the SYNFAS Group (Tore Bonge-Hansen, department of chemistry), (Pål Rongved, department of pharmacy) who have prepared iodinin in larger quantities through new synthetic routes involving Buchwald-Hartwig condensation of 2-bromo-3-methoxyaniline to 1,6-dimethoxy-phenazine, which could be further de-methylated and oxidised to iodinin (scheme 1). This new preparation method enables synthesis of a variety of derivatives useful in the present project.
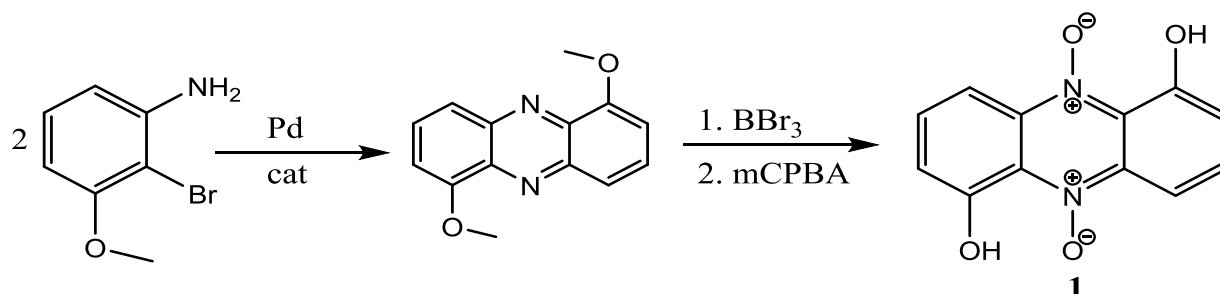
Scheme 1: Synthesis of iodinin

1.1 The aim of this project

The aim of the project is to synthesize amphiphilic iodinin conjugates by modifying one hydroxyl group with a C12 fatty acid amide and subsequently attaching a hydrophilic poly (ethylene oxide) chain to the other hydroxyl group of phenazine **1**.

1.2 Biological Background

A brief introduction of the biological background of the project will be presented, which will include a concise presentation on acute myeloid leukaemia, together with the current treatment methods. And lastly, the biological importance of phenazines, poly ethylene oxide, other related hydrophobic drugs and iodinin will be discussed.

1.2.1 Acute myeloid leukemia

Acute myeloid leukaemia (AML) is an aggressive neoplastic disorder of the hematopoietic stem cells characterized by an increase in the number of myeloid cells in the bone-marrow and an arrest in their maturation, resulting in hematopoietic insufficiency with or without leukocytosis.[1] The initial treatment of AML has remained largely unchanged in the past decades and resistance to cytotoxic agents emerged as one of the major causes of treatment failure.[2]

1.2.2 A brief history of AML therapy

The first formal attempt to treat leukemia was reported by Lissauer in 1865, who obtained complete response in a patient with chronic myeloid leukemia through the administration of arsenic trioxide.[2] However, in 1903, the same treatment seemed less effective in AML when compared with radiation therapy.[2] Consequently, AML was thought to be an incurable disease. Finally, in 1930, an 'American businessman' with AML was cured by Gloor with a combination of radiation therapy, arsenic, mesothorium and two blood cell transfusions: the patient achieved a complete response and died at the age of 102 after 52 years.[2] In the following years, a major breakthrough in AML therapy was made with the discovery of AraC, a deoxycytidine analogue, S-phase-specific anti-metabolite drug, that constituted the backbone of AML therapy as one of the most active agents. Finally, in 1969, daunorubicin was successfully tested in AML patients, initiating a series of studies that led to the most widely used AML scheme, the 3/7 DNR/AraC combination.[2]

1.2.3 Treatment of AML

Daunorubicin is an anthracycline antibiotic produced by *Streptomyces peucetius* and is known as one of the most effective chemotherapeutic agents widely used for the treatment of acute lymphoblastic or myeloblastic leukemias.[3] Similar to Doxorubicin, the mode of actions of Daunorubicin is mainly owing to its ability to intercalate with DNA, leading to inhibition of DNA replication and transcription or break down of double-strand DNA and inhibiting topoisomerase II.[3] Despite daunorubicin's extensive use for cancer treatment, its clinical effectiveness is limited, because of serious side effects such as cardiomyopathy, skin ulcerations, hematologic toxicity and infections, immunosuppressive and teratogenic

activities, congestive heart failure, as well as the appearance of multidrug resistance in tumor cells.[3]
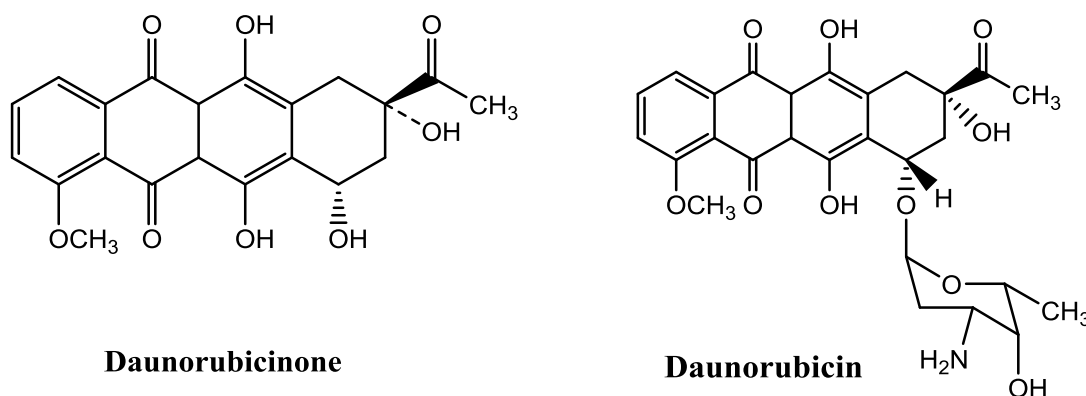
Figure 1: Structure of Daunorubicinone and Daunorubicin.[4, 5]

1.2.4 Phenazines

Phenazine (figure 2) and phenazine-like molecules have a wide spectrum of biological activity.[6] Several naturally occurring phenazine-containing antibiotics are known to react effectively with biopolymers. Some phenazine derivatives are capable of generating hydroxyl radicals under mild conditions, which enables them to be used as reagents for site-directed DNA scission.[6]
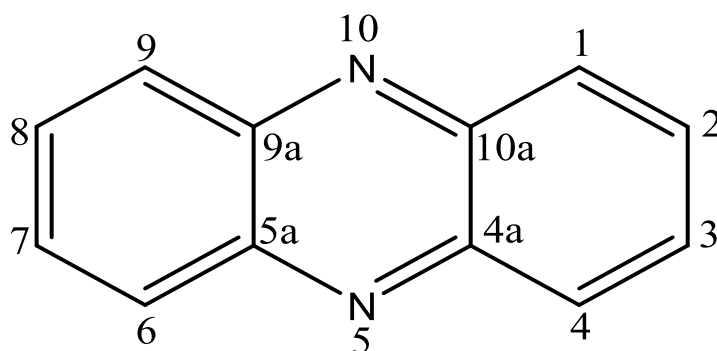
Figure 2: Phenazine with atomic numbering.[7]

The phenazine core and polar substituents such as amines at positions 2 and 8 are crucial for potent activity, possibly due to the possession of unique hydrogen bonding array.[8] The

reactivity of phenazine derivatives can be estimated by comparing the charge electron density (kinetic control) and nucleophilic localization energies (thermodynamic control).[6] Introduction of fragments containing substituted phenazines into biopolymers endows the hydroxyl radical generation with new, unique properties that allow one to use such conjugates for specific modification of biopolymers.[9] These considerations stimulate the search for new methods of polyfunctionalization of the phenazine ring, especially under conditions where biopolymers retain their biological activity.[6]

All of the ca. 100 natural phenazines are thought to originate from phenazine-1,6-dicarboxylic acid (PDC) or phenazine-1-carboxylic acid (PCA) Figure 3, 'core' phenazines that originate from two molecules of chorismic acid in a biosynthetic pathway.[10] Biomimetic synthesis of PDC by the intermolecular dimerization of a 2-aminocyclohexanone resembles the true biosynthetic substrate.[10]


PDC   **PCA**
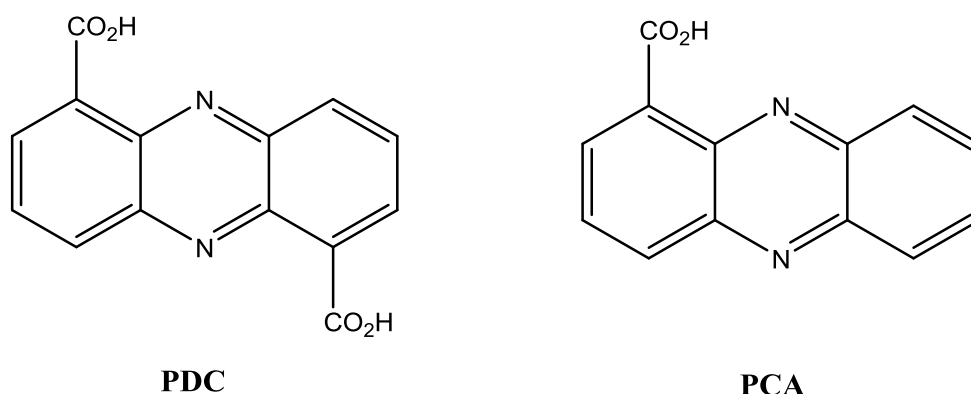
Figure 3: PDC and PCA.[10]

1.2.5 Introduction – Iodinin

Iodinin, 1,6-phenazinediol-5,10-dioxide (Figure 4) is a purple, red-glinting pigment, originally found to cover the colonies of isolated *Chromobacterium iodinum* on suitable solid media.[11] This pigment, the iodinin, was found to inhibit the growth of certain other bacteria.[12] It has been found to be one of the phenazine compounds and is known to be produced by microorganisms. Iodinin has been of great interest in the antibiotic field since it can be converted to a pharmaceutically valuable antibiotic, myxin, and a lot of effort has been put into developing a suitable process for its manufacture.[11]
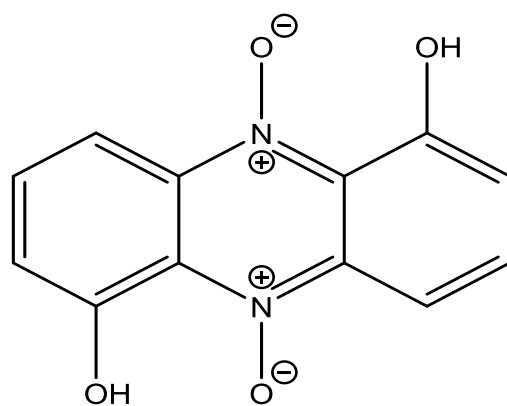
Figure 4: Iodinin

The most promising activity concerning iodinin is the antileukemia induction, especially sought in the acute myeloid leukemia and acute promyelocytic leukemia which are still associated with high lethality.[12] Iodinin showed selective toxicity to AML and acute promyelocytic (APL) leukaemia cells, with EC50 values for cell death up to 40 times lower for leukemia cells compared with normal cells.[13] Molecular modelling suggested that iodinin intercalate between the bases in the DNA, in a way similar to the anti-cancer daunorubicin (DNR) causing DNA strand to break.[13]
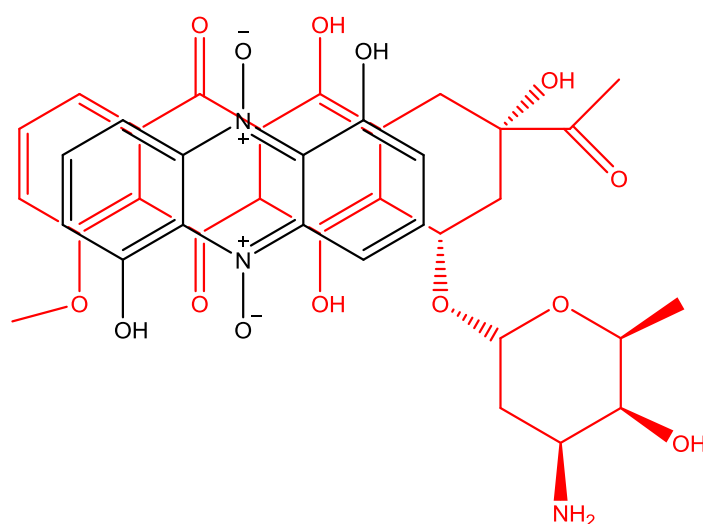
Figure 5: Structural similarities between anthracyclines (daunorubicin) and Phenazine-N,N`- dioxides (iodinin).

Before the development of any synthetic route to iodinin, the compound was isolated from bacteria growth.[14] The bacteria were grown on a solid agar medium, and after about 15 days had attained maximal pigmentation; the colouring matter was removed by washing with

water and extraction with chloroform, and, on concentration of the extract, separated as uniform crystals, deep purple in colour and with coppery lustre, in a yield of about 1 g. per sq. m. of medium. Analytical data indicate for the pigment an empirical formula $C_{12}H_8O_4N_2$.[14] The first synthetic route to iodinin was reported by Yoshioka and kidani in 1952 with several yields of 7.1%, 7.4%, and 26.3% which was the highest after various modifications.[15] Msc. Simen Snellingen at the department of chemistry UIO reported a synthetic route to iodinin in 2015, with a totally yield of 86%.[7]

Scheme 2: Oxidation of phenazine-1,6-diol occurs via mono-oxidised phenazine to give iodinin.[7]

1.2.6 Hydrophobic drugs

1.2.6.1 Introduction: Solubility enhancement of hydrophobic drugs

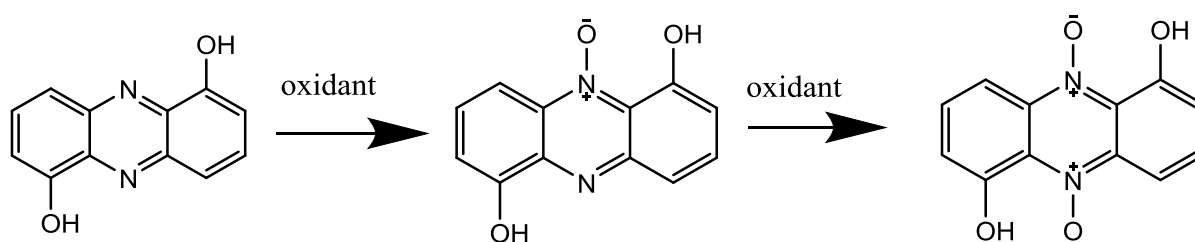Orally administered drugs completely absorb only when they show fair solubility in gastric medium and such drugs shows good bioavailability.[16] Iodinin is just one of the thousands of newly developed drug-candidates which face challenges with solubility. One of the biggest challenges facing pharmaceutical and biotechnology industries at present is the poor solubility of new and established chemical entities.[17] In recent times, block copolymers have emerged as a potential agent for targeted drug delivery and gene therapy. One such block copolymer proposed for controlled drug delivery is Pluronic, which has a triblock PEO−PPO−PEO structure.[18] At high temperatures, the central PPO block becomes hydrophobic, while the PEO blocks remain hydrophilic. Because of this amphiphilic nature, Pluronic molecules, above a critical temperature and concentration, self-aggregate in aqueous solutions to form spherical micelles with hydrophobic PPO cores surrounded by hydrophilic PEO coronas. As the copolymer concentration is increased, the micelles can arrange

themselves in cubic crystalline order.[18] Most recently, amphiphilic prodrugs have been designed by conjugating hydrophobic anticancer drugs to a short hydrophilic head, by which they could assemble to form Nano structures with controllable drug loading.[19] The present invention relates to improved pharmaceutical compositions of hydrophobic drugs which have enhanced solubility and to a method of preparing such composition.[17] For instance, Shen and co-workers reported the direct conjugation of camptothecin to the short oligomer ethylene glycol, in which the resultant amphiphilic prodrug could assemble into Nano capsules with a remarkably high drug loading capacity and improved cancer therapeutic efficacy.[19] It is estimated that up to 90% of new molecular entities and 40% of existing compounds can be categorised as Biopharmaceutics Classification System class II or IV, which means that they show poor and variable oral bioavailability in vivo.[17] Due to their low dissolution rate and poor availability, hydrophobic drugs are challenging to administer and formulate.[17] Thus, solubility is the intrinsic factor of a drug candidate which affects absorption of drug in the body fluids and its oral bioavailability.[20] As the absorption of a drug occurs at the pH at which it remains in the unionized state so the drug must be soluble at a particular body fluids pH from where drug can absorb and show its action. Some novel approach techniques to improve solubility are Nanosuspension, Super Critical Fluid, Cryogenic and Inclusion complex formation.[20]

1.3 Chemical Background

1.3.1 Introduction - Biosynthesis of phenazines

It has been shown that phenazine-1,6-dicarboxylic acid (PDC) and phenazine-1-carboxylic acid (PCA) are precursors for more complex phenazine metabolites.[21] Extensive studies support the hypothesis that these phenazine precursors are derived from the shikimic acid pathway, as outlined in Scheme 3, with chorismic acid (51) as the most probable branch point intermediate. Shikimic acid (50) is converted to chorismic acid (51) in known transformations that are part of the common aromatic amino acid biosynthetic pathway. The transformation from chorismic acid (51) to the phenazine precursors has been discussed and investigated through intensive biochemical studies; so far, no intermediates have been identified and little is known about the genetic origin and details of the phenazine biosynthesis.[21]
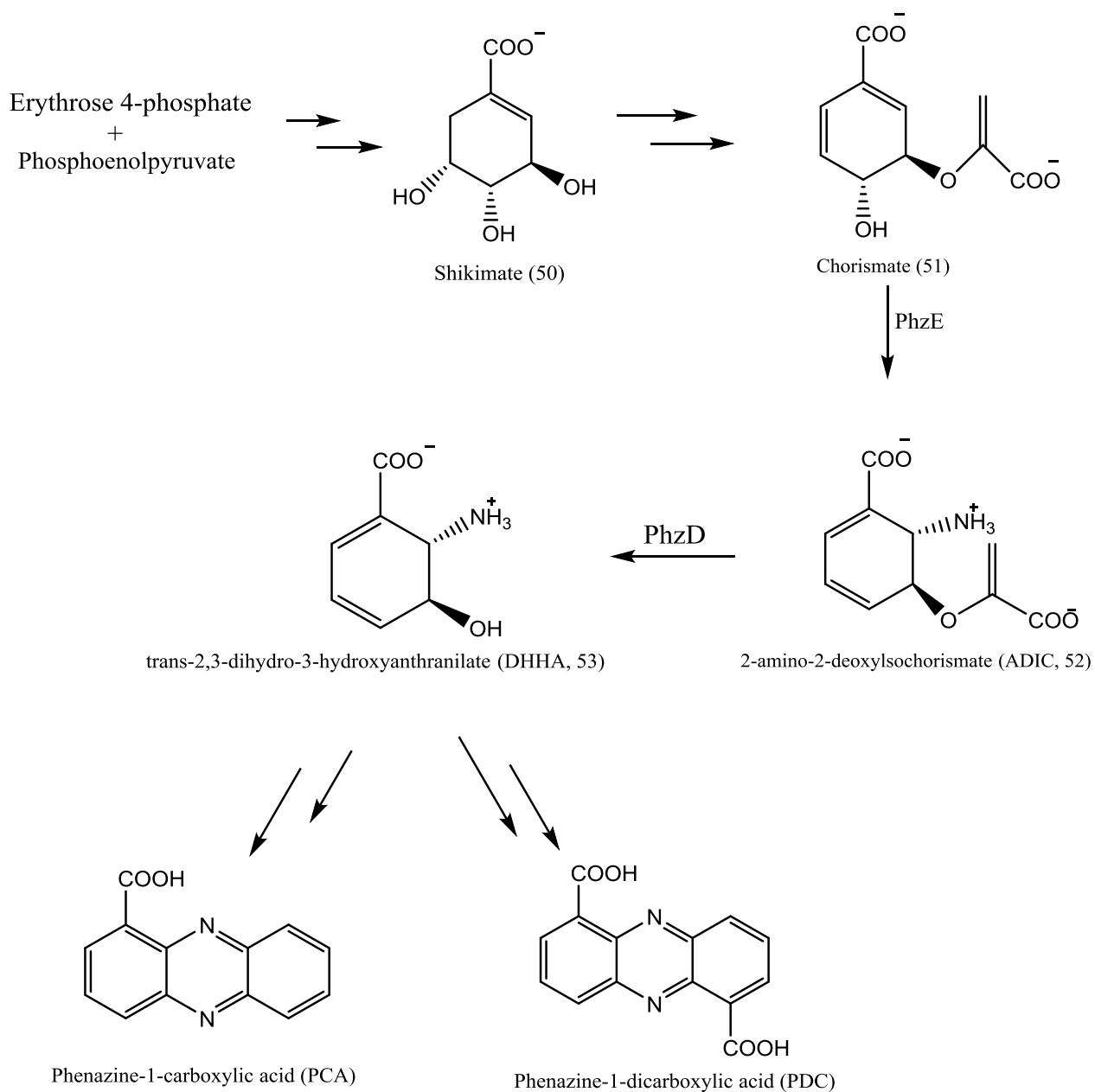
Scheme 3: Proposed General Biosynthetic Pathway of Common Phenazine Precursors, Phenazine-1-carboxylic Acid and Phenazine-1,6-dicarboxylic Acid.[21]

1.3.2 Chemistry of N-oxides

A significance insight was taken in the chemistry of heterocyclic N-oxides about 15 years ago. The discovery that the antibiotics iodinin and aspergillic acid were, respectively, a phenazine dioxide (1) and the cyclic hydroxamic acid (II) tautomer of a pyrazine oxide (III) attracted some attention.[22]

| (1) | (II) | (III) |
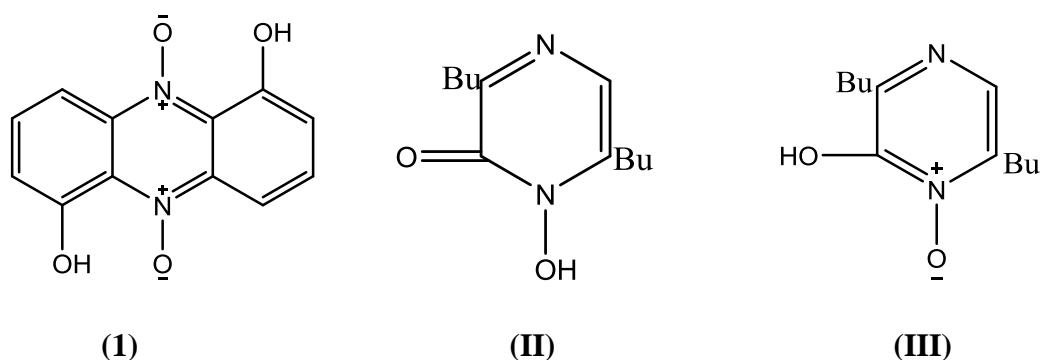
The fact that the N-oxide function is strongly polarisable in both directions is of considerable theoretical interests and in this respect; the $N^+\!\!-\!\!O^-$ group of an N-oxide is similar to a nitroso-group attached to a benzene ring. The ability of the N-oxide group both to accept or to donate electrons is clearly shown by a comparison of dipole moments of alkyl, phenyl, 4-pyridyl, and (4-pyridyl 1-oxide) compounds.[22] Hydrogen bonding enhances the positive nature in phenazine dioxide (1) nitrogen, thus increasing electron attraction, and also stabilizes the oxyanions formed during charge transfer. Compound (1) contains hydroxyl groups situated for hydrogen bonding via six-membered rings. The greater ease of reduction for compound (1) is expected to be due to the additional aromatic ring, the more acidic phenolic groups, and the greater degree of rigidity in the H-bonded system.[23]

1.3.3 Hydrogen bonding in heterocyclic N-oxides

Aromatic heterocyclic compounds, such as derivatives of pyridine (Py) and pyridine N-oxide (PyO), occupy in the field of H-bond research a very particular place.[24] A rich variety of H-bond related phenomena arises from the interplay between stronger and weaker bonds and their evolution upon media effects, clustering, bulk/surface effects, etc.[24] In the Cambridge Crystallographic Database (version 5.35, including November 2013 update) of organic structures containing N-oxides, 94 of the 112 structures was discovered with co-ordinates

deposited display hydrogen-bonding interactions, with the oxygen atom of the N-oxide moiety participating as a hydrogen-bond acceptor.[25] There is ample precedent of heterocyclic N-oxide CH...O close contacts in the solid state, Bodige *et al* noted the propensity of 2,2'-dithiobis(pyridine N-oxide) (DTPO) to self-associate via CH...O hydrogen bonding in its solid state. Both singly-bonded (IV) and doubly-bonded (V) CH...O modes of binding were observed in the DTPO-networked crystals.[26]

IV   **V**
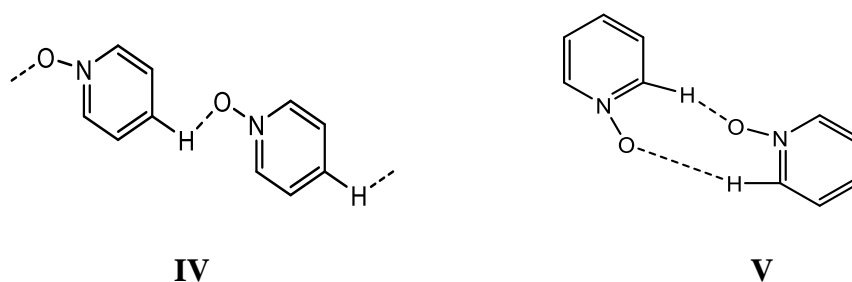
Figure 6: Singly-bonded and doubly-bonded CH...O modes of binding in the DTPO-networked crystals.[26]

1.3.4 Phenazine-Modifying Pathways

The modification of most phenazines precursor is specific to each phenazine-producing species. In general, hydroxyl groups can be introduced into the ring at any position, starting from phenazine- 1-carboxylic acid (PCA).[21] In Scheme 4 below the modification of the final product from phenazine-1,6-dicarboxylic acid (PDC) precursor after hydroxylative decarboxylation to give *iodinin* is of main interest in this project.
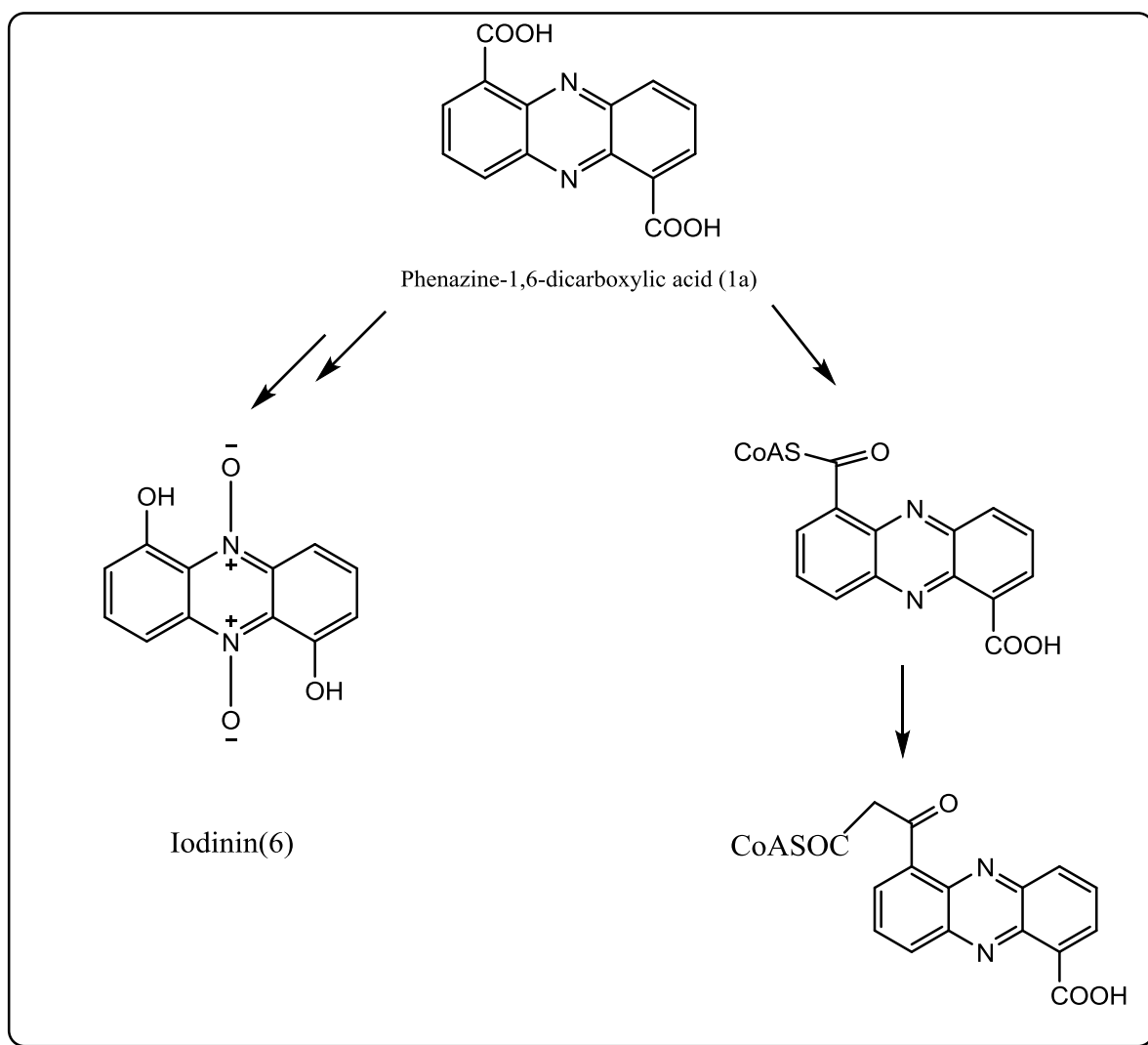
Scheme 4: Proposed Biosynthetic Routes to Selected Phenazine Natural Products.[21]

Methods of synthesizing the phenazine framework are presented in Scheme 5. One of the oldest methods is the reaction of anilines with nitroarenes under basic conditions (the Wohl–Aue reaction, (path a) [27]. The Holliman synthesis of phenazines (path b) is a base-induced

cyclization of *ortho*-nitrodiphenylamines [28]. In the Bamberger–Ham reaction (path c) nitrosobenzenes dimerize under acidic conditions to form phenazines. Other methods are the condensation of *ortho*-phenylenediamines with *ortho*-quinones (path d) , reaction of benzofuroxanes and phenols (the Beirut reaction, path e), and palladium-catalyzed cyclization of 2-amino-2′-bromophenylenediamines (path f).[29] Further elaboration on some selected routes regarding to this project is presented chapter 1.3.5.
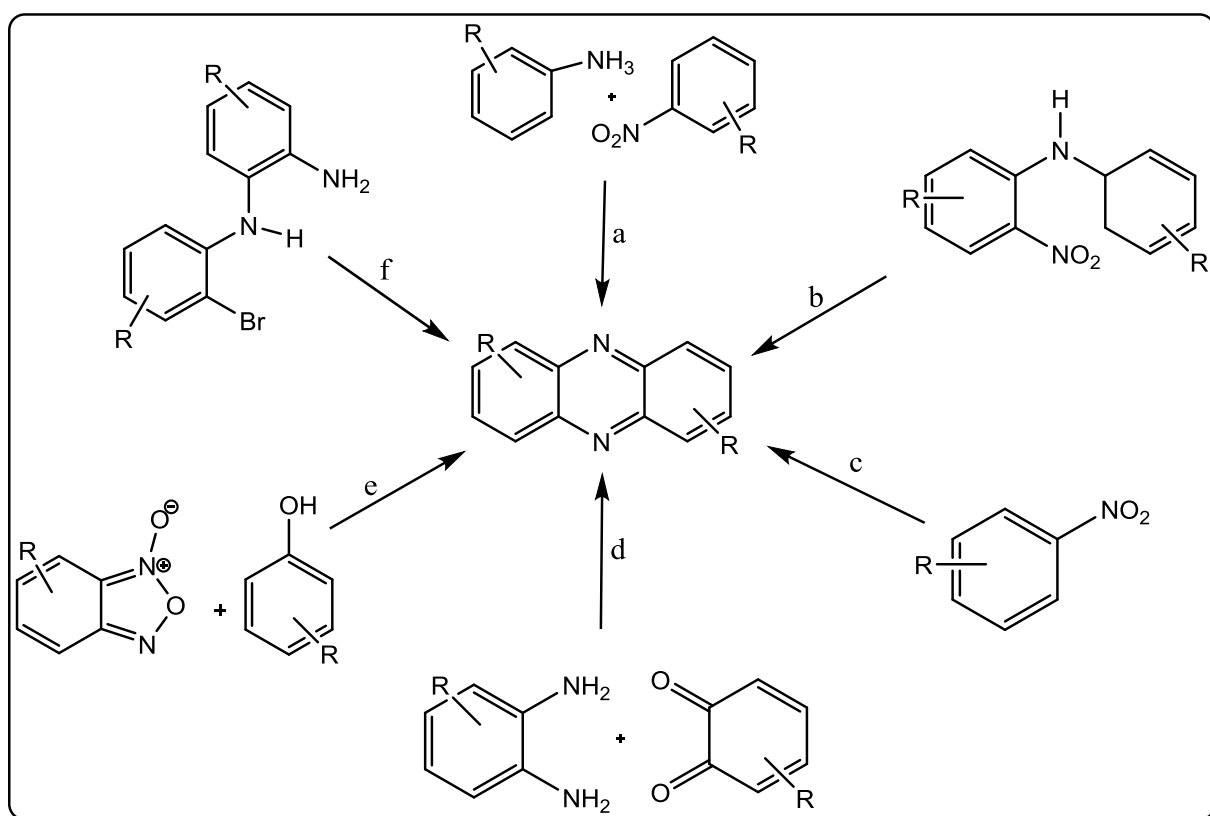
Scheme 5: Selected methods of synthesizing the phenazine framework. a) Wohl– Aue reaction, b) The Holliman synthesis of phenazines is a base-induced cyclization of orthonitrodiphenylamines, c) In the Bamberger–Ham reaction nitrosobenzenes dimerize under acidic conditions to form phenazines. d) The condensation of ortho-phenylenediamines with ortho-quinones, e) Reaction of benzofuroxanes and phenols 'the Beirut reaction', f) Palladium-catalyzed cyclization of 2-amino-2' – bromophenylenediamines. [29]

1.3.5 The Wohl-Aue reaction

The Wohl-Aue reaction is reaction between an aromatic nitro compound and aniline to form a phenazine in the presence of an alkali base. A typical example is the reaction of nitrobenzene and aniline (scheme 6).[30]
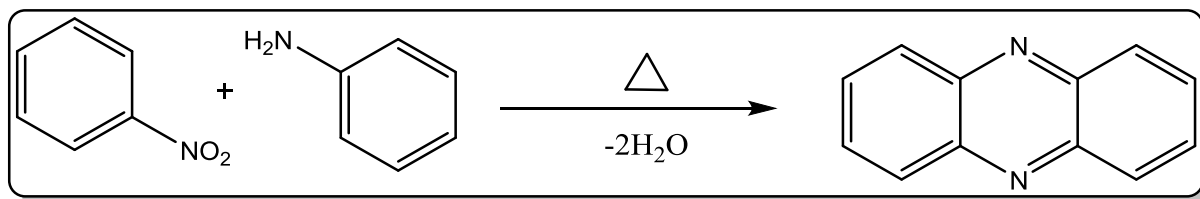
Scheme 6: The Wohl-Aue condensation reaction.[30]

The Wohl and Aue reaction also was adapted to the synthesis of 1,6-dimethoxyphenazine (v) and 1,6-dichlorophenazine (vi) in attempts to establish the structure of, and to conveniently synthesize, the antibiotic pigment iodinin (1,6- dihydroxyphenazine dioxide). In 1951, Irwin *et al* published a reaction where o-anisidine and o-nitroanisole were subjected to the mild conditions of the Wohl- Aue reaction and 1,6-dimethoxyphenazine was obtained in 12% yield.[31]

VI, R = OCH$_3$; VII, R = Cl
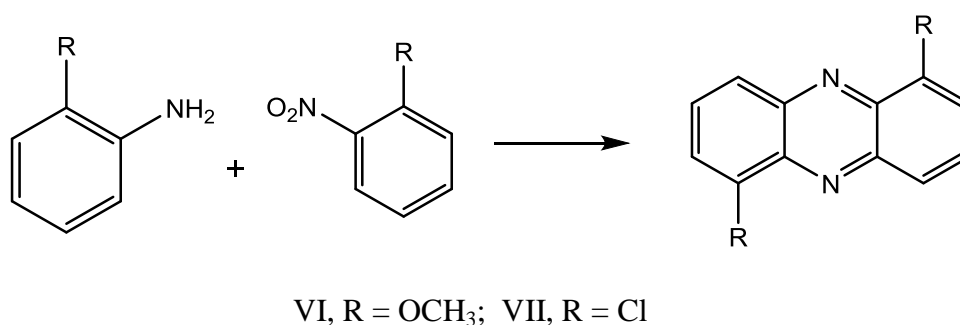
Scheme 7: Synthesis of 1,6-dimethoxyphenazine and 1,6-dichlorophenazine as reported by Irwin *et al*.[31]

1.3.6 Modified iodinin analogues – The path to prodrug

If iodinin is to enter in *vivo* studies, water soluble analogues must be developed.[7] A convenient synthesis of iodinin, myxin and their derivatives from commercially available building blocks, suitable for large scale production process has been developed.[32]
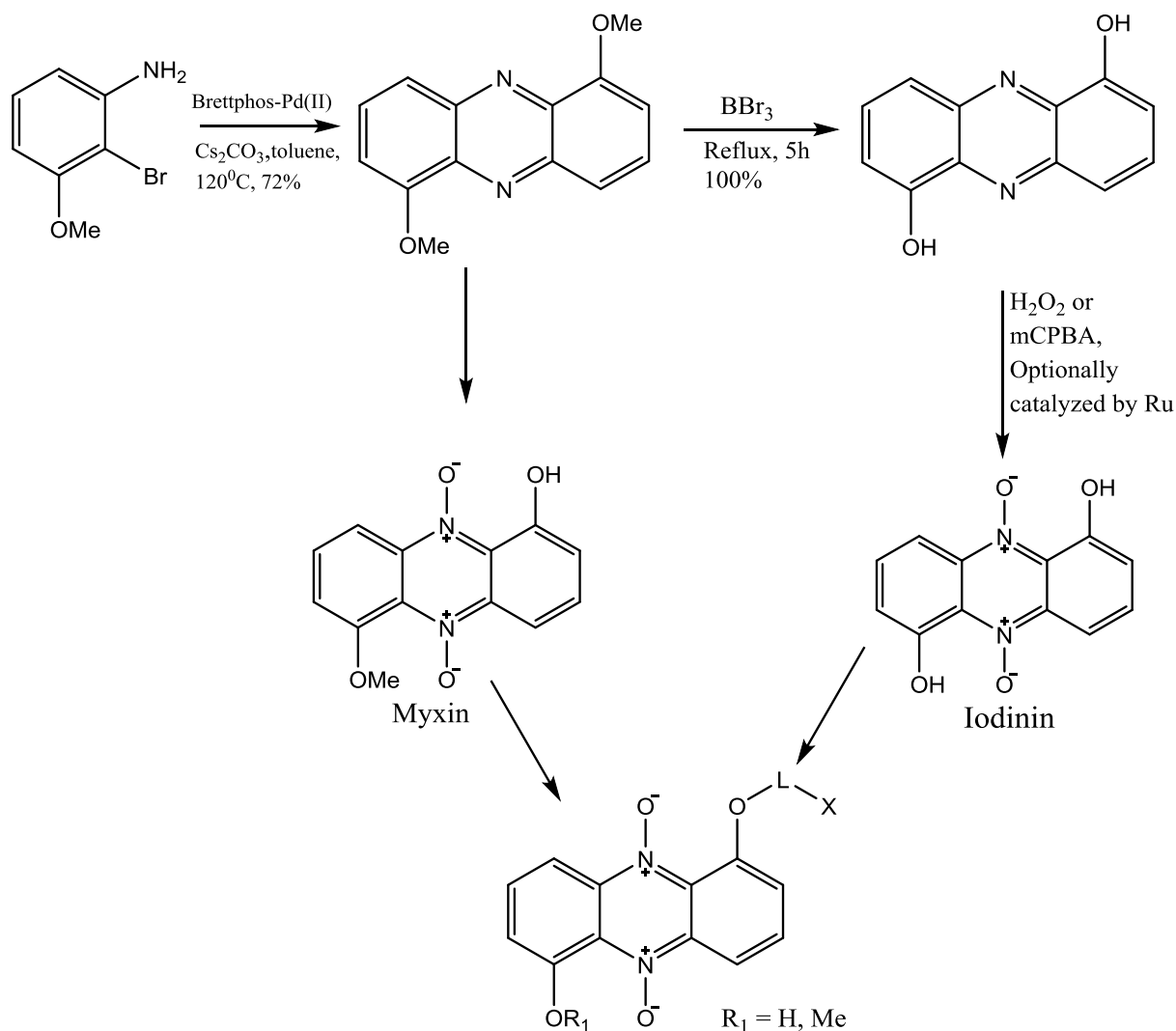
Scheme 8: A novel general process for iodinin, myxin and derivatives.[33]

Other preferred analogues are polymers that consist of iodinin e.g. with the polymeric form in scheme 8 which can be precipitated as nano colloids alone or together with functionalised polymers like poly-lactic-co-glycolic acid (PLGA) with iodinin to obtain surface

modifications.[32] Thus an effective way to increase the solubility of a drug is through synthesis of a prodrug.[16] Willy et al describe the synthesis of various iodinin derivatives through the Williamson Ether procedure (scheme 9) used base stable, acid labile protecting group to enable selective alkylation of the hydroxyl group of iodinin.

1.3.7 The Williamson Ether Synthesis

The Williamson Ether Synthesis is an old reaction, dating back to 1851, but hasn't been surpassed. It is an S_N2 reaction between a deprotonated alcohol ["alkoxide"] and an alkyl halide that forms ether. In Williamson Ether procedure we're forming and breaking a bond on carbon.[34]
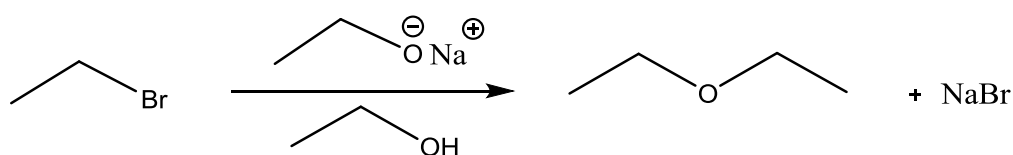
Scheme 9: General Williamson Ether Synthesis.[34]

1.3.8 Steglich esterification

The Steglich Esterification is a mild reaction, which allows the conversion of sterically demanding and acid labile substrates.**[35]** Vitor et al reported the synthesis of juglone ester from juglone and palmitic acid, consequently with other fatty acids.[36]

$R= n-(CH_2)_{10}CH_3; n-(CH_2)_{14}CH_3; n-(CH_2)_{16}CH_3$
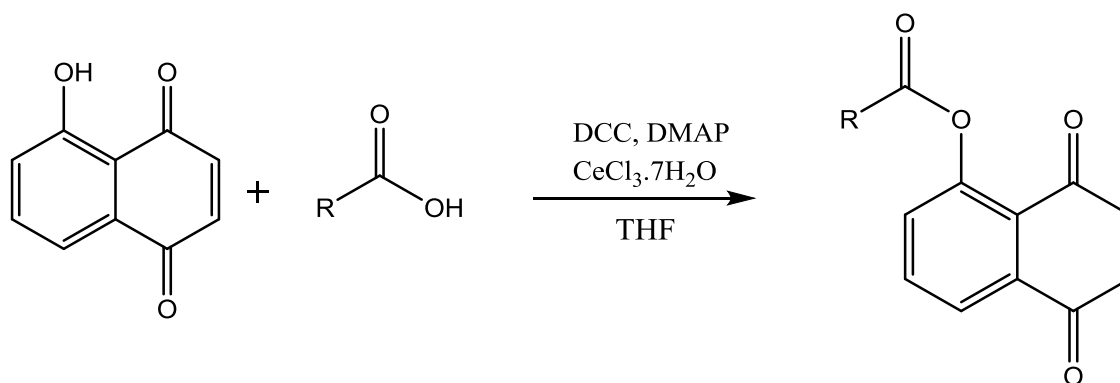
Scheme 10: Synthesis of new Juglone esters with fatty acids

1.3.9 Iodinin Analogues

The invention of novel processes for the synthesis of antimicrobial phenanzine N,N'-dioxides and to novel intermediates is useful in the synthesis iodinin.[37] 1, 6-Phenazinediol-5,10-dioxide (iodinin) is a well-known compound having broad spectrum antibacterial properties.[38] Willy Leimgruber *et al* reported various routes to iodinin derivatives by selective alkylation (scheme 10).[37]
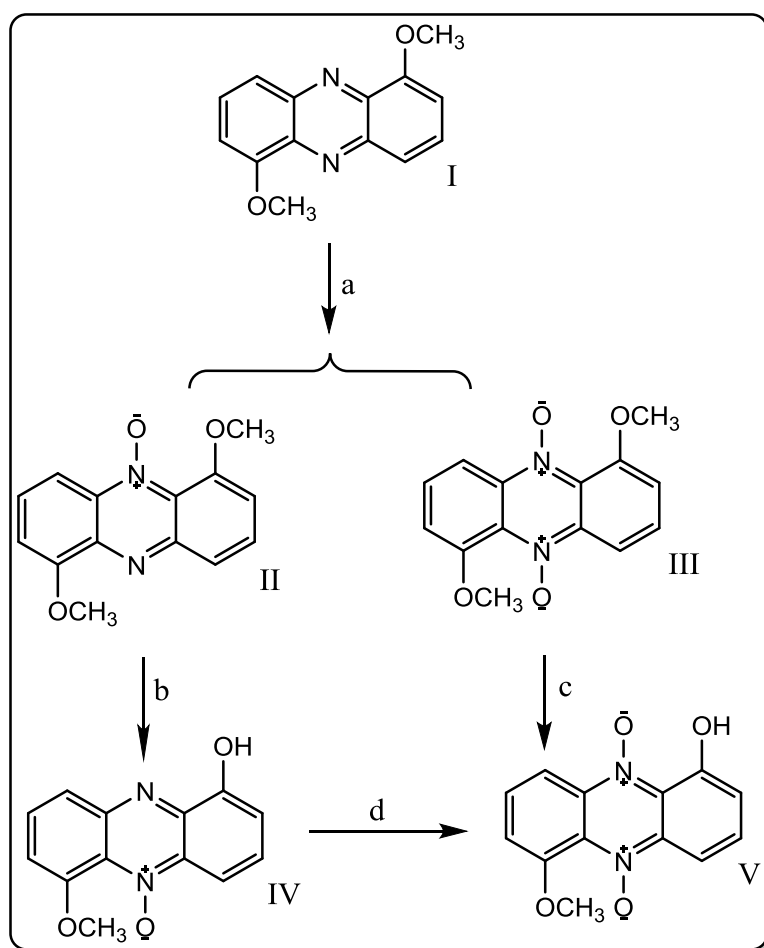
Scheme 11: Oxidation of compound I with per benzoic acid yields complex containing compounds II and III (path a). Further reaction of compound II and III in chloroform

with aluminium bromide and aluminium chloride yields compounds **IV** and **V** respectively (path b&c). Oxidation of compound **IV** in benzene with m-chloroperbenzoic acid yield pure product **V** (path d).[37]
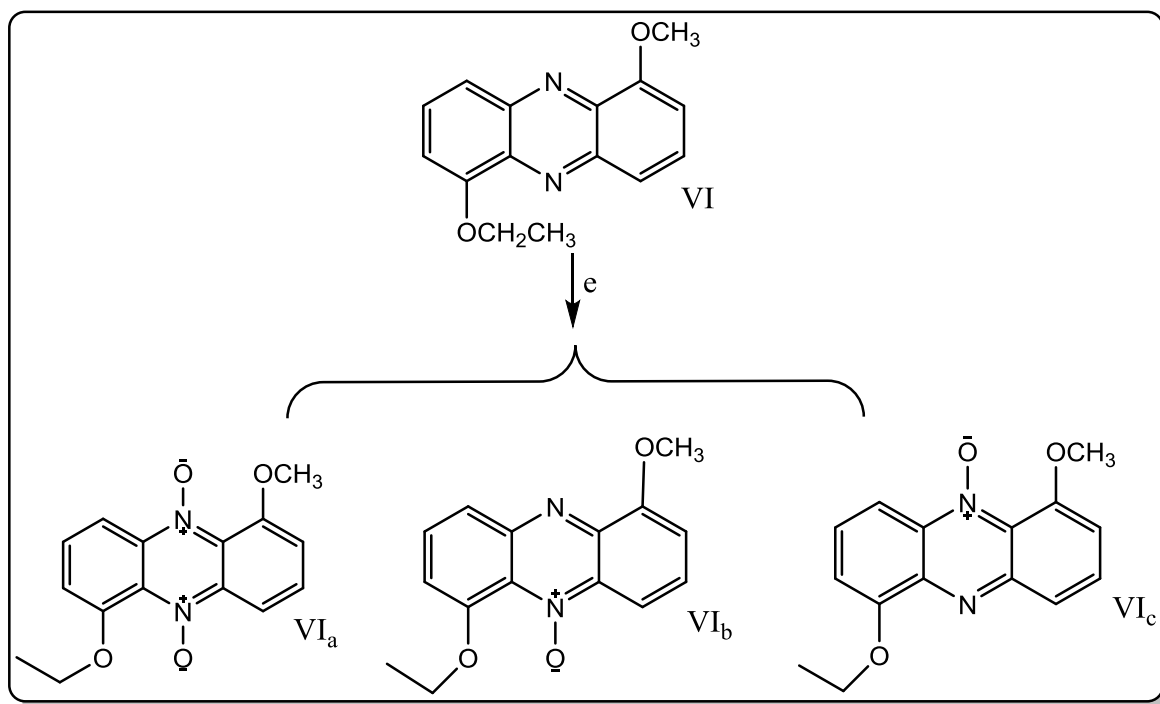
Scheme 12: Oxidation of compound VI in benzene with *m*-chloroperbenzoic acid yields complex containing compounds VI$_A$, VI$_B$ and VI$_C$ respectively (path e).[37]
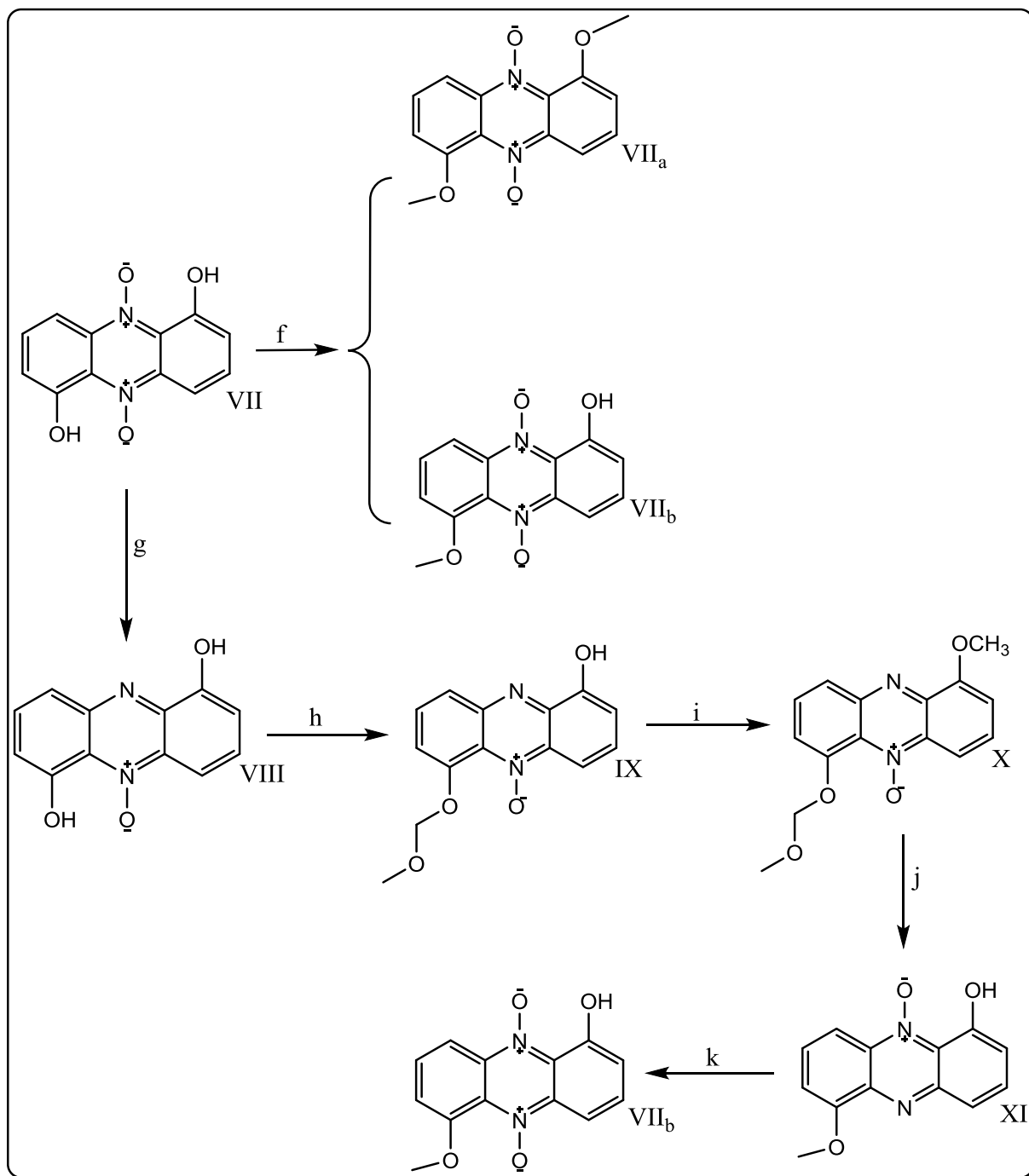
Scheme 13: Alkylation of compound **VII** yield complex which contains mainly compound **VII_B** and traces of compound **VII_A** (path f). Treatment of compound **VII** in HMPT with KOtBu yields compound **VIII** (path g). Further reaction of compound **VIII** in HMPT with KOtBu yields compound **IX** (path h). Treatment of compound **IX** in HMPT with KOtBu yields compound **X** (path i). Reaction of compound **X** in DMSO with hydrochloric acid yields compound **XI** (path j). Oxidation of compound **XI** in chloroform with *m*-chloroperbenzoicacid is identical to compound **VII_B** (path k).[37]

1.4   Polymer based drugs

Polymer-based drugs have emerged from the laboratory bench in the 1990s as a promising therapeutic strategy for the treatment of devastating human diseases.[39] They are becoming increasingly important in pharmaceutical applications especially in the field of drug delivery.[40] A number of such polymer therapeutics is presently on the market or undergoing clinical evaluations to treat cancer and other diseases. The polymers used in these formulations are usually considered biologically inert or often biodegradable e.g. PLA degrades into lactic acid which is harmless, excipients that protect biological agents from degradation, prolong exposure of biological agents to tissues, and enhance transport of biological agents into cells.[39] Polymer chain behaves either as the bioactive (a polymeric drug carrier/encapsulated) or, more commonly, as the inert carrier to which a therapeutic is covalently linked, as in the case of polymer-drug conjugates.[41]

1.4.1 Poly (ethylene) oxide

Poly (ethylene oxide) (PEO) very often somehow inaccurately referred to as poly (ethylene glycol) is the most widely used polymer in delivering anticancer drugs clinically.[42] It is non-toxic and non-biodegradeable.[43] Due to high aqueous solubility, a PEG polymer is considered as a versatile candidate for the prodrug conjugation. Ringdorf was the first to propose the rational model for pharmacologically active polymers in 1975.[42] Modifying a drug by attaching a functional PEG can enhance drug solubility, reduce protein aggregation, and decrease immunogenicity.[44] Also, "Pegylation" technology, linear or branched PEG derivatives are coupled to the surface of the protein to increase their stability towards proteases in vivo circulation time, has resulted in the development of clinically as well as commercially successful products such as pegylated asparaginase, PEGylated adenosine deaminase, PEGylated interferons, and pegylated granulocyte colony stimulating factor.[45] Linear PEGs are the simplest and most often used conjugate agents.[46]

Poly (ethylene glycol) (PEG)

Methoxy poly (ethylene oxide) (mPEO)

Figure 7: Structure of PEG and mPEO.[47]

PEG can be conjugated to different drugs by a variety of chemical reaction, including degradable bonds, such as, (a) hydrolysable esters and acid-sensitive acetals, imines (Schiff bases) e.t.c.[43]   Wenjun *et al* published in 2012 a configuration of pegylated zidovudine AZT,[47] an antiretroviral medication used to prevent and treat HIV/AIDS,[48] with the aim to improve the pharmacokinetics properties and to lower the dose related toxicity.[47]
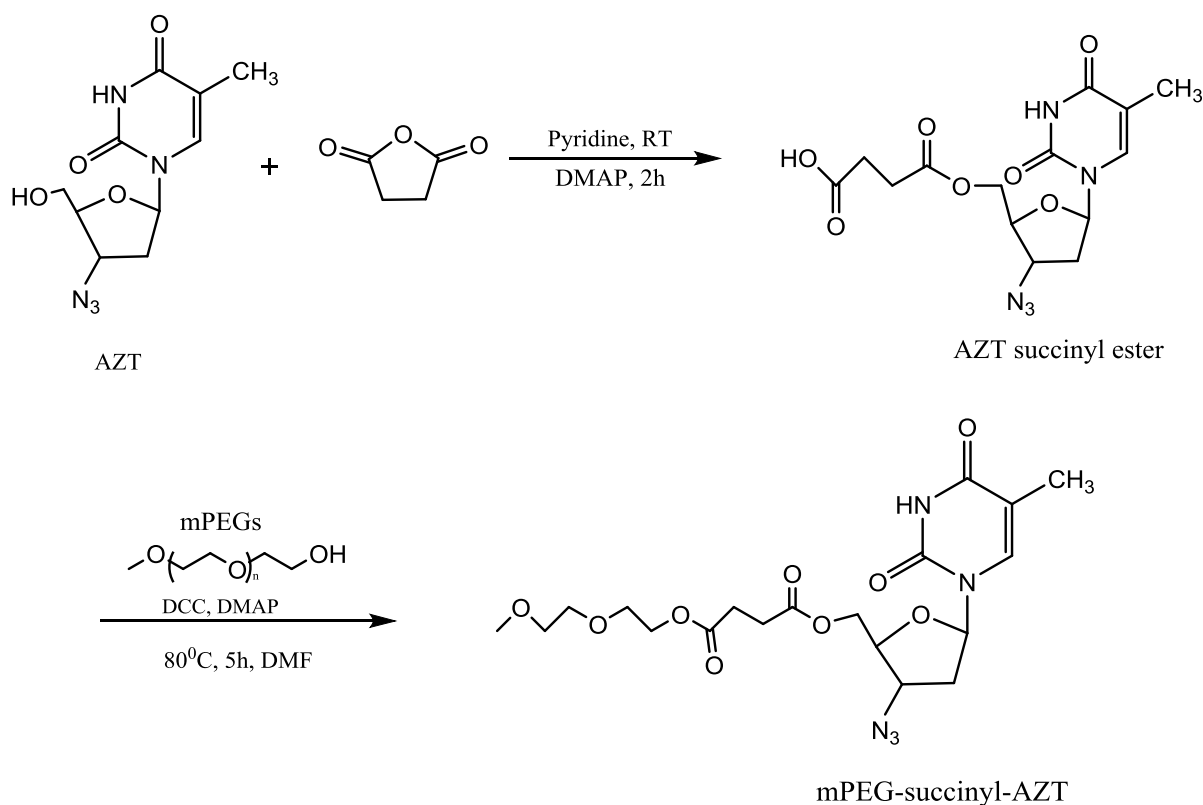
Scheme 14: The Synthesis route of mPEG-succinyl-AZT.[47]

2 Synthesis of iodinin derivatives

2.1 Renunciation – A note about conformity

 In an attempt to synthesize a phenazine **1** moiety with the facet of good water solubility, increased biodisponibility and hopefully increase the selectivity as a chemotherapeutic agent. We wanted to synthesize phenazine **1** prodrug from the outlook design in pathway **1** (figure 10) with the aim of meeting the later attributes mentioned of phenazine **1** in future research. The complete tasks and success in the synthesis of phenazine **3** and phenazine **4** was based on the experiences and findings of co-workers. Also, concise findings of co-workers were mentioned where necessary in this chapter.

2.2 Synthetic strategy

The first task was to synthesize phenazine **1** following the procedure from the project of M.Sc. Simen Snellingen at department of chemistry UIO, where a satisfactory yield of iodinin has been successfully performed from a readily available starting material phenazine **2**. Oxidation of phenazine **2** gave phenazine **1**, therefore the phenazine **1** synthesis reported in this project was well known from previous work. In order to achieve derivatives of phenazine **1** with a lipophilic head**,** two possible pathways were envisioned. Williamson ether synthesis discussed in section 1.3.7 was selected as the method to synthesize phenazine **3**, from phenazine **1** and compound **8**. Also, steglich esterification discussed in section 1.3.8 was selected as a method to synthesize phenazine **5**, from phenazine **1** and compound **9.**
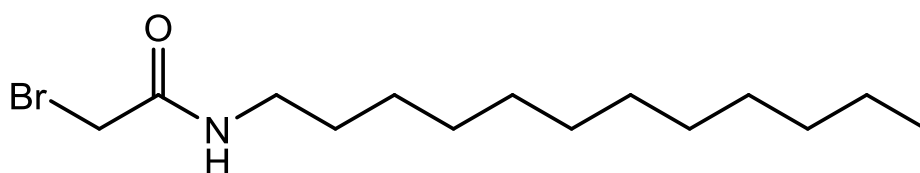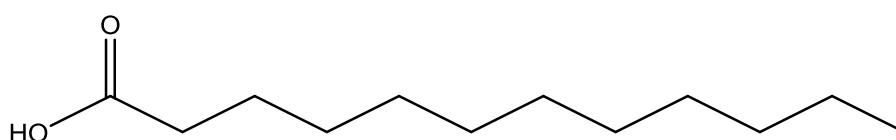
Figure 8: 2-Bromo-N-dodecylacetamide (8)

Figure 9: Dodecanoic acid (9)
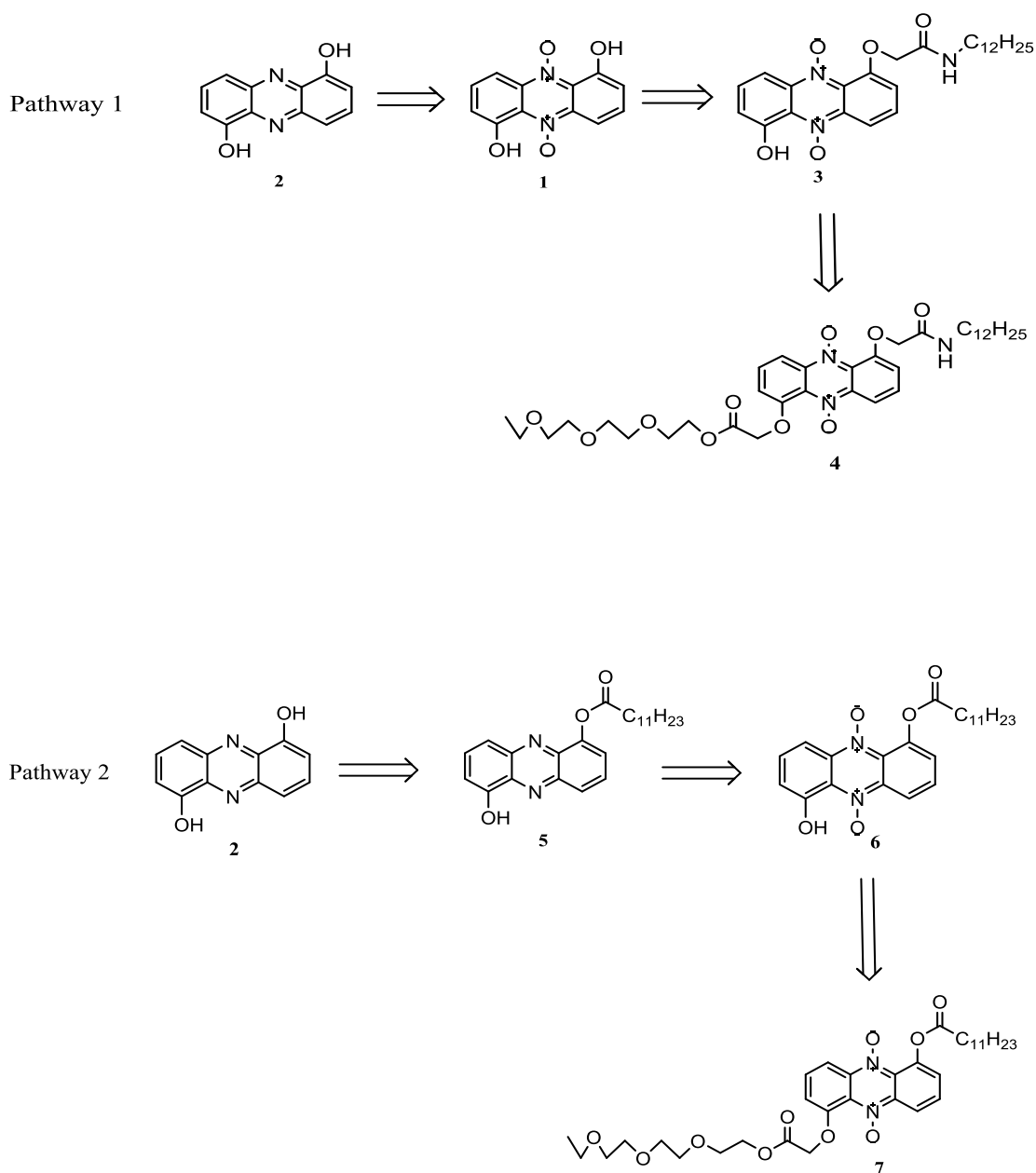
Pathway 1

Pathway 2

Figure 10: Retrosynthetic visualization of pathway 1 and 2

The N-oxidation of phenazine **2** was carried out by employing the procedure from Msc. Simen Snelling where mCPBA was used as the oxidizing agent and reported phenazine **1** with a total yield of 53% from the commercially available phenazine **2.[7]** After the implementation of the later procedure, then a total yield of 52% phenazine **1** was isolated subsequent to purification and characterization in this project. In the consideration to synthesize phenazine **3**, an alkylation reaction between phenazine **1** and compound **8** was carried out to give phenazine **3** by employing the procedure used in the synthesis myxin (an

analogue to envisioned phenazine **3)** (scheme 15) reported by Bendik Grøthe *et al* where potassium carbonate with 18-crown-6-ether was used as the alkylating agent.
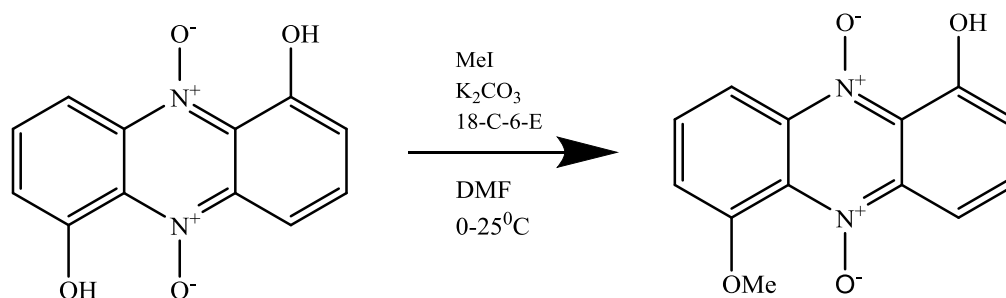
Scheme 15: The synthesis of Myxin.[49]

Moreover in an attempt to synthesize phenazine **5** by employing the Steglich esterification procedure discussed in chapter 1.3.8 from phenazine **2** and a commercially available compound **9**. The analysis of the first reaction was ambiguous i.e. phenazine **5** seems difficult to achieve from the envisioned pathway 2 and after several discussions with experienced co-workers we decided to abandon pathway 2, and focus was shifted to pathway 1. The result of pathway 1 will be presented here.

2.3   Synthesis of 1, 6-dihydroxyphenazine 5, 10-dioxide (1)

2.3.1   A known synthetic route to compound 1

It was noted that buying commercially available phenazine **2** was more cost and time effective than synthesizing it.[7] Therefore, phenazine **2** that was commercially available in high purity was oxidized. The commercially available phenazine **2** was used as the main source of phenazine **1** used in this project.
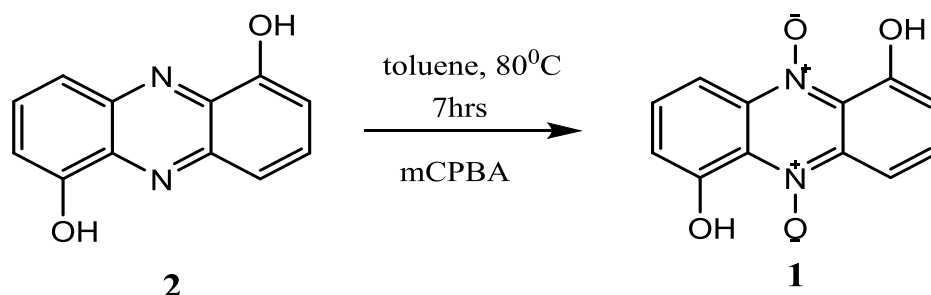
Consequent to the synthesis of N-dioxide of phenazine **2** which completes the synthesis of phenazine **1** by employing the procedure in Msc. Simen Snelling using mCPBA as oxidizing agent. Also the oxidation of phenazine **2** was reported to have occurred via mono-oxidized phenazine **8** (figure 11) intermediate to the final product phenazine **1**.[7]
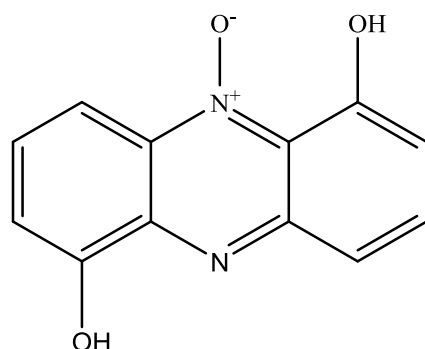
Figure 11: Mono-oxidized phenazine 8 intermediate

Basically, the oxidation of nitrogen's on phenazine **2** was successful, succeeding 52% yield isolated of phenazine **1** as reported in the experimental chapter pg.47 in this project. But, following the assumption that the hydrogen of the phenolic group on phenazine **2** stabilizes the N-oxide (figure 12), creating an intramolecular hydrogen bond in the phenazine N-oxide.
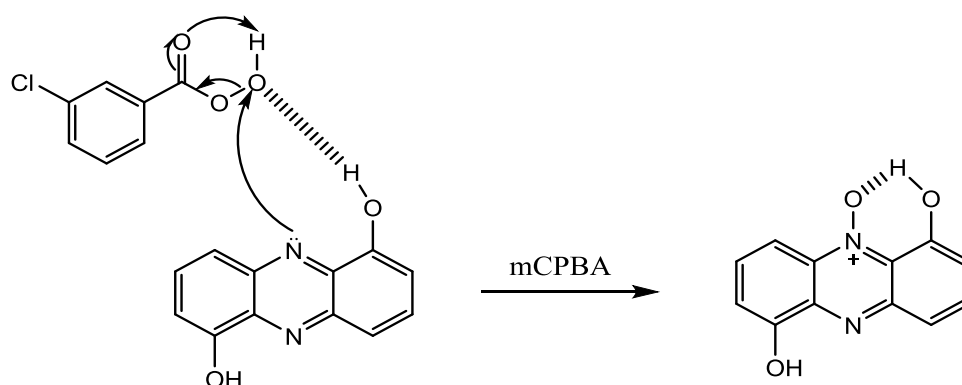
Figure 12: Intramolecular hydrogen bonding of phenolic group stabilizing N-oxide moiety.[7]

The intramolecular hydrogen bonding in phenazine **1** is presumably the cause for low solubility, given a less interaction between phenazine N-oxide and solvent. Msc. Simen Snelling concluded that selective oxidation of nitrogen in phenazine **1** to reach iodinin should be carried out prior to any further functionalization of the hydroxyl groups on phenazine **2** due to resilient of N-oxide.[7]

2.4 Synthesis of 2-bromo-N-dodecylacetamide (8)

2.4.1 A known synthetic route from literature

In the course of creating headway to the functionalization of the phenolic groups of phenazine **1**, we urged to selectively functionalize one of the phenolic groups on phenazine **1** with compound **8**. Compound **8** have a unique lipophilic property that was envisioned to enhance the dispersion of phenazine **1** in oil phase and also to facilitate further reaction on the other phenol end of phenazine **1**.

Following the procedure reported in Enzo Terreno *et al*.[50], an acetylation reaction (Scheme 17) between commercially available bromo acetyl bromide (a) and dodecylamine (b) (figure 13) gave compound **8**. After purification and characterization a total yield of 37% of compound **8** was isolated as reported in experimental chapter pg. 49 of this project.
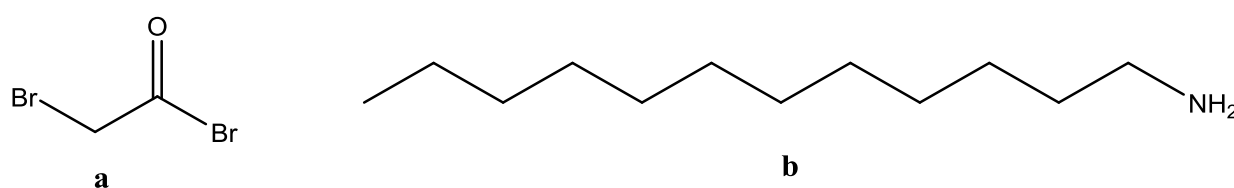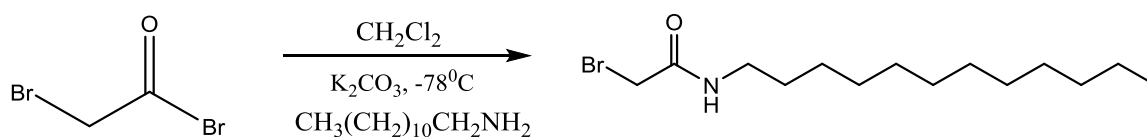

Figure 13: Commercially available Bromo acetyl bromide (a) and Dodecyl amine (b).

Scheme 17: Acylation of dodecyl amine to synthesize compound 8

2.5   Synthesis of 1-(2-(dodecyl amino)-2-oxoethoxy)-6-hydroxyphenazine 5, 10-dioxide (3)

One goal of the project was to synthesize a derivative of phenazine **1** with the aim to increase its solubility in polar solvent. Therefore, alkylation reaction was carried out between compound **8** and phenazine **1**. The selective alkylation of one phenolic group on phenazine **1** completes the synthesis of phenazine **3**.
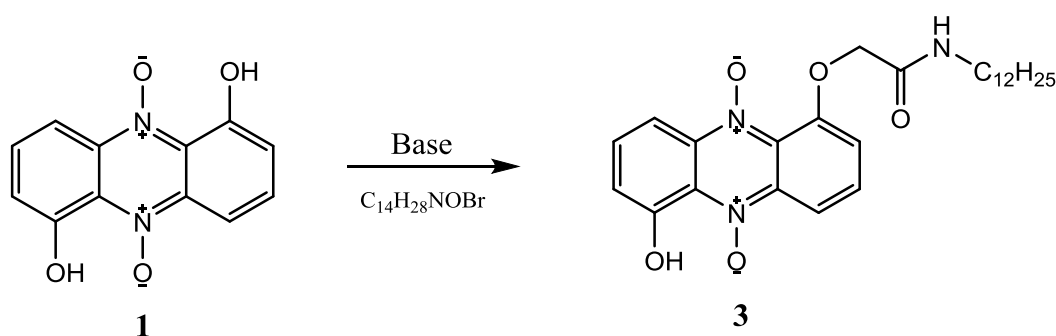
Scheme 18: Alkylation of phenazine 1

The conversion of the phenolic group in phenazine **1** into phenyl ethers was achieved by testing several bases in the alkylation process. Therefore, two bases were considered for initial testing on small scale proportions to see if compound **8** was selectively attached to one phenolic end on phenazine **1**.   Both cesium carbonate and potassium carbonate were employed.

Subsequently, the alkylation reaction where cesium carbonates was employed gave relatively low yield of phenazine **3**, whereas the alkylation reaction involving potassium carbonate with 18-crown-6-ether gave higher yield but still TLC analysis showed ample phenazine **1** present at the end of the reaction. Nevertheless, it was decided to go for potassium carbonate with 18-crown-6-ether as alkylating base when reaction was maximized. Unconverted phenazine **1** was collected, concentrated and used in supplementary reactions.

2.5.1 Alkylation of phenazine 1 using K_2CO_3 (18-Crown-6-ether)

The first alkylation reaction of phenazine **1** reported in this project with K_2CO_3 (18-crown-6-ether) in DMF gave up 24% yield of phenazine **3** (entry 1, Table 1). The other base tested was Cs_2CO_3, but this gave relatively poor yield of phenazine **3** (entry 2, Table 1). Therefore, it was proposed to have yield increase of phenazine **3** higher than that in (entry 1, Table 1). We then decided to conduct the reaction in a highly concentrated proportion under inert atmosphere as shown in (entry 3, Table 1) also; purification technique described in chapter 2.5.2 was thought to have enhanced the yield increase of phenazine **3** as seen in (entry 3, Table 1). At early hours of the reaction, TLC analysis showed several components present and at later stage of the reaction, four spots were noticed on the TLC plate, in which two of it overlapped and other two spots were confirmed to be the starting materials i.e. phenazine **1** and compound **8** prior purification.

Table 1: Alkylation of phenazine 1, Experiments where conditions were changed was solely noted.

Entry	Base	Phenazine1	Solvent	Notes	product
1	K_2CO_3(1.5eq)	100mg	DMF(5mL)	r.t 24hr	24% (3)[a], 52% (1)
2	Cs_2CO_3(1.5eq)	100mg	DMF(5mL)	r.t 24hr	60% (1)[b], traces of (3)
3	K_2CO_3(1.5eq)	250mg	DMF(5mL)	r.t 24hr	42% (3)[a], traces of (1)

a: Isolated yield.

b: Concluded from TLC analysis and NMR analysis of the crude product.

2.5.2 Purification and isolation of alkylated phenazine 1 products

The purification and isolation of phenazine **3** happened to be onerous and lingering, due to the hydrogen bonding present in the structure of phenazine **3** makes it tails on TLC and tailing also suspected to be acid-base interaction between compound **8** and silica surface. This causes co-elution during flash chromatography.

^{1}H-NMR spectra of the crude reaction mixture was difficult to interpret, due to the intricacy of interpreting the aromatic region. Although, it was clearly depicted in the –OH region the crude contains its N-mono-oxide phenazine **3** moiety.

On a long run, after two sets of flash chromatography purification, it was seen that separating phenazine **3** and its N-mono-oxide moiety cannot be achieve by flash chromatography, therefore, phenazine **3** was only isolated along with the N-mono-oxide moiety to be used in the next phase of the project. However, the NMR and mass data was fully reported in the experimental chapter pg. 51.

The compelling attribute of these compounds was the distinctive colors which makes isolation facile. On the other hand, phenazine **3** being a very polar compound, a major drawback is the enormous quantity of ethyl acetate used in purification, compared to the yield isolated of phenazine **3,** this is not feasible cost effective on industrial scale reactions.

2.5.3 Alternative purification methods for N-mono-oxide and N,'N-dioxide of phenazine 3 moieties

At the moment of this work, phenazine **3** stands to be a very precious material due to relatively arduous purification procedure considering the yield reported in this project.

Willy *et al* reported several alkylation and oxidation reaction of phenazine **1**(scheme 11-13)**.[37]** On several occasions in attempt to alkylate phenazine **1**, crude product analysis showed the presence of N-mono-oxide and N,'N-dioxide of the phenazine moiety.[37]

In an attempt to oxidized 1,6-dimethoxyphenazine (scheme 11) which is an analogue to phenazine **2** discussed in this project, the crude obtained contains a mixture of 1,6-dimethoxyphenazine-5-oxide and 1,6-dimethoxyphenazine-5,10-dioxide and it was separated on florisil chromatography. Florisil based chromatography is known for its efficient separation of phospholipids based compounds.[51] Also, preliminary experiments indicated that chromatography on Florisil gave good separations of naturally occurring lipids.[51]

Unfortunately, we do not have the time to carry out a purification technique of phenazine **3** N-mono-oxide and N,'N-dioxide isolated product on florisil chromatography. Since this is

not attempted by us, it is then a process worth exploring in future related studies. Therefore, isolated phenazine **3** complex was used in the next phase of the project discussed in chapter 3.

2.5.4 Conclusion of pathway 1

The selective alkylation of one of the hydroxyl group on phenazine **1** completed the synthesis of the target compound, phenazine **3**, in 42% yield. Further attempts to complete the last step of pathway 1 were fully discussed in chapter 3. The complete reaction sequence is summarized in Scheme 17.
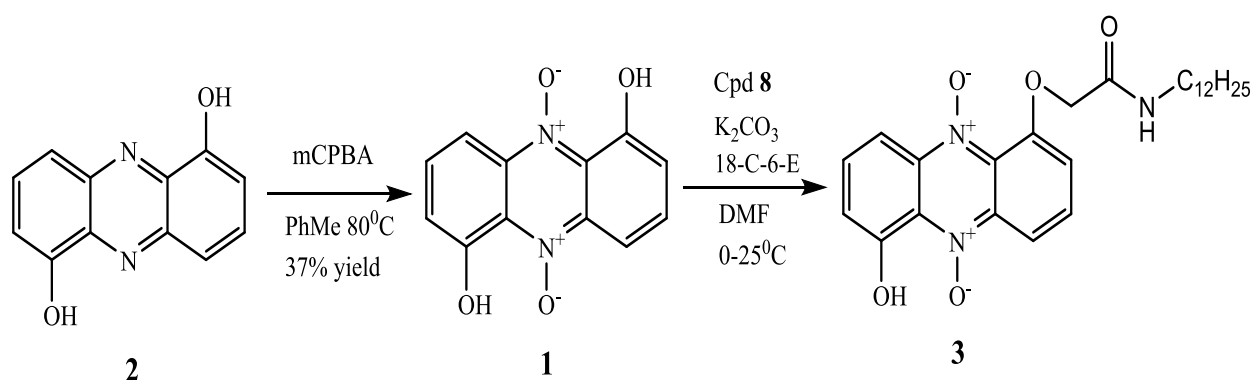
Scheme 19: A new mono-alkylated analogue (phenazine 3) of phenazine 1 with a C-12 fatty acid chain.

2.5.5 Discussion

In the course of this project, we observed that all the isolated phenazine **3** crude was a complex of N-mono-oxide and N,N'- dioxide moiety of phenazine **3** regardless the base used as alkylating agent. Basically, the separation of the N-mono-oxide and N,N'- dioxide moiety of phenazine **3** seems difficult based on the deductions from TLC analysis. Therefore the separation was not attempted nor reported in this project. Hence, phenazine **3** complex was used in the pegylation reactions discussed in chapter 3.

Moreover, apparent explanations as to why complex of N-mono-oxide and N,N'- dioxide phenazine **3** were formed and why the reaction was a success, will be briefly discussed here.

The alkylation of one hydroxyl group on phenazine **1** was ultimately a success (phenazine **3**) with a long fatty acid chain (compound **8**). Thus, we hypothesized that if the hydrogen of the phenolic groups is sort of stabilizing the transition of N-oxide in phenazine **1** as shown in figure 12. Hence, upon selective alkylation of one phenolic group on phenazine **1**, the presumably intramolecular hydrogen bond (figure 12) in the phenazine-N-oxide is then broken, thus the N-oxide is ephemeral, making alkylation occurred on both N-mono-oxide and N,N'- dioxide moiety of phenazine **1.** Thus, given rise to a complex of N-mono-oxide and N,N'- dioxide complex of phenazine **3** in the alkylation process.

3 Pegylation reactions – The path to a prodrug

3.1   Prodrugs

In spite of the promising biological effect of iodinin, it has a major disadvantage, it is practically water insoluble, which also hinder it for vivo test in mammals, also they have a non-selective bio distribution in vivo and lack of chemical functionality required to attach functional groups for regulating their bio distribution.[12] Pegylation technologies are widely used in drug modification, with an ever-increasing range in proteins, peptides, oligonucleotides and small organic molecules.[46] Veronese et al. synthesized a series of PEG-Doxorubicin conjugates with different molecular weight PEG through various peptidyl linkers where the study shows that all PEG conjugates were more than 10-fold less toxic than free Doxorubicin. [46] Since then over four pegylated small drugs have been taken into clinical trials.[47] To these points, synthesizing a pegylated iodinin moiety was therefore considered as the next goal of the project.

3.2 Attempted synthesis of 1-(2-(dodecylamino)-2-oxoethoxy)-6-((2-oxo-3,6,9,12-tetraoxatetradecyl)oxy)phenazine 5, 10-dioxide

2-(2-(2-ethoxyethoxy) ethoxy) ethyl 2-bromoacetate ($C_{10}H_{19}O_5Br$) with molecular weight 299Da was chosen to pegylate phenazine **3** with the aim of creating an amphiphilic compound (phenazine **4**) having a hydrophilic poly (ethylene oxide) block and a hydrophobic phenazine **1** block.
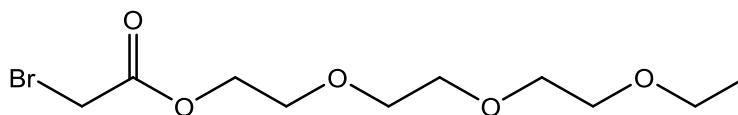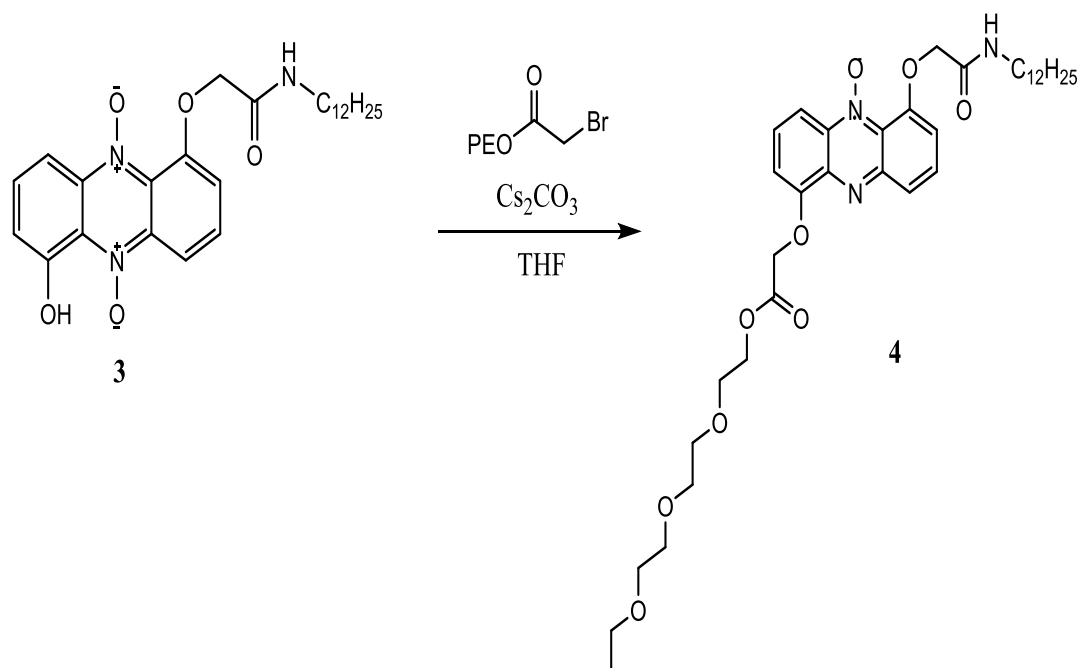

Figure 14: Structure of 2-(2-(2-ethoxyethoxy) ethoxy) ethyl 2-bromoacetate

This can be investigated in future studies, in the synthesis and self-assembly reactions of phenazine **4**, also biological response can be tested respectively. The visualize phenazine **4** synthesis in scheme 20.

Scheme 20: The visualize pegylation of phenazine 3.

An alkylation reaction was carried out with phenazine **3** (Scheme 20) that was slightly diluted in a relatively small amount of THF resulting in a highly concentrated solution, the choice of using THF was to circumvent creating micelles or rather to avoid polymerization. Also, two bases (cesium carbonate and potassium carbonate in 18-crown-6-ether) were tested to deprotonate phenazine **3** at the phenolic group on position 6. Regardless either of the bases, TLC analysis depicted large amount of starting material left after 2 hours at RT. The reactions was continuously monitored by TLC analysis, potassium carbonate based reaction shows faster conversion of the starting material compare to cesium carbonate based reaction. Both reactions were quenched after 24 hours at RT.

Reaction mixtures were diluted with dichloromethane and organic phase was extracted with brine separately. After purification and isolation, the TLC and ^{1}H-NMR analysis of potassium carbonate based product phenazine **4** showed that 18-crown-6-ether co-elutes with the product and further purification of the product was difficult, therefore it was abandoned with no other characterization done.

Upon purification and isolation of the cesium based product, the product formed yellow viscous oil that solidified below RT. Although, the phenazine **4** N-mono-oxide formed was not fully characterized. The structure was concluded to be phenazine **4** based on some few characterizations done as shown in the experimental page 54.

3.3 Attempted pegylation of phenazine 1 with a α-Methoxy-ω-bromo PEO

A reaction was carried out with phenazine **1** and a α-Methoxy-ω-bromo PEO with structural formula $CH_3O-(CH_2-O-CH_2)_n-Br$ (mw=750Da) at RT (scheme 19).
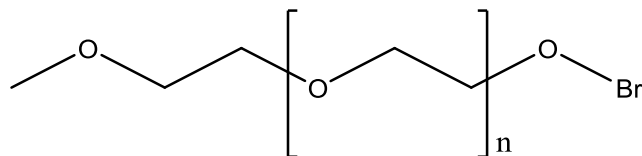

Figure 15: A α-Methoxy-ω-bromo PEO (n=14)

The mixture was stirred in THF so as to avoid polymerization. After 5 hours reaction time TLC analysis deduced substantial amount of unconverted phenazine **1.** It was decided to increase the temperature to 65^0C so as to boost the reactivity. After 24 hours reaction time, the mixture was diluted with CH_2Cl_2 hopefully to avoid polymerization and the organic phase was extracted with water. The crude was purified to remove unconverted phenazine **1.**
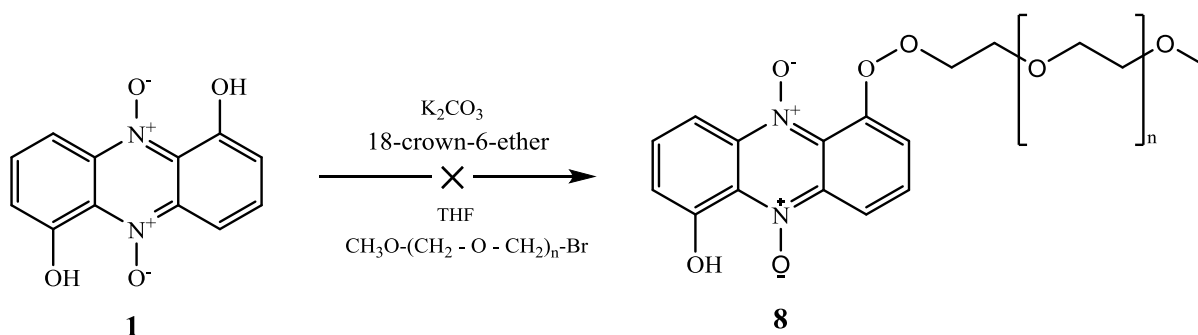
Scheme 21: Pegylation of iodinin with PEO-Br did not yield the desired product

TLC analysis of the isolated product showed an undistinguishable spot between PEO-Br and the envisage phenazine **8**, also the mass spectrum report showed no molecular ion in respect of phenazine **8** while ^{1}H-NMR analysis of the product was ambiguous, due to the fact that PEO-Br co-elutes critically with the suspected phenazine **8** formed so further purification of the product seemed difficult therefore it was not attempted, thus we decided to abandon this route.

3.4 Attempted pegylation of phenazine 3 with a α-Methoxy-ω-bromo PEO

Upon the successful synthesis of phenazine **4**, we decided to follow the pegylation route described in chapter 3.3, pg. 40, a reaction was set up in order to pegylate the phenolic group at position 6 of phenazine **3**, an α-Methoxy-ω-bromo PEO with molar mass 750Da dissolved in THF was charged into a flask containing phenazine **3** in THF in the presence of potassium carbonate and 18-crown-6-ether. (Scheme22). The reaction was carried out under inert atmosphere at 65^0C. After 2 hours reaction time TLC analysis showed no significant spot, and the reaction was left to stir for 24 hours under same condition. Crude was diluted with CH_2Cl_2 hopefully to avoid polymerization and organic phase was extracted with water.
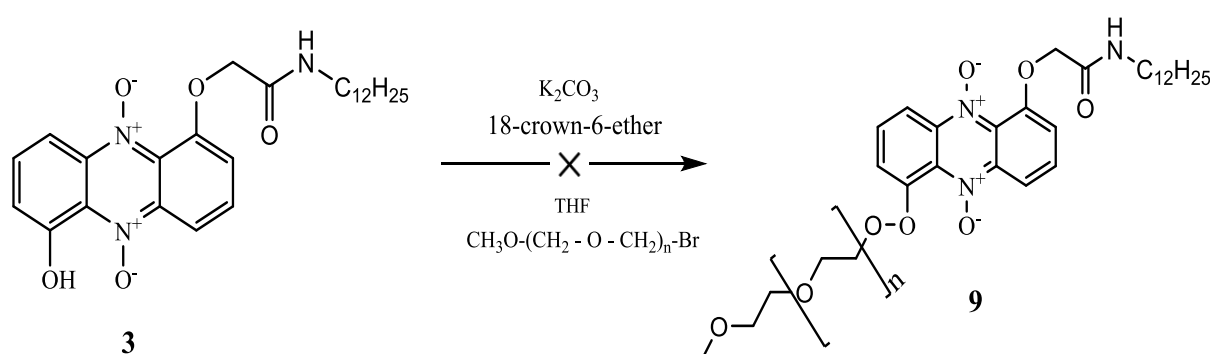
Scheme 22: Pegylation of phenazine 3 with PEO-Br did not yield the desired product

TLC analysis of the crude product showed two spots which correlates to phenazine 3 and α-Methoxy-ω-bromo PEO. Also, the crude NMR was ambiguous, thus this reaction was abandoned without any other purification or characterization.

3.5 Conclusion from the pegylation reactions

The pegylation of phenazine **3** with 2-(2-(2-ethoxyethoxy) ethoxy) ethyl 2-bromoacetate in figure 11 was successful, yielding phenazine **4** under the conditions described in chapter 3.2. Because 2-(2-(2-ethoxyethoxy) ethoxy) ethyl 2-bromoacetate is a short peg that contains highly acidic proton, which makes the alkylation of 2-(2-(2-ethoxyethoxy) ethoxy) ethyl 2-bromoacetate and the phenolic group on phenazine **3** rapid. Under milder conditions for this reaction an N-dioxide phenazine **4** moiety could be synthesized.

The pegylation of phenazine **1** with a α-Methoxy-ω-bromo PEO (mw=750Da) was unsuccessful due to low reactivity of the α-Methoxy-ω-bromo PEO, because the α-Methoxy-ω-bromo PEO has a very high molecular weight and the possibility of selectively alkylating it to phenazine **3** phenolic end could be difficult due to steric hinderance from the lipophilic end of phenazine **3** and also α-Methoxy-ω-bromo PEO contains no acidic proton, which will definitely makes it less reactive compare to short peg on phenazine **4**. On the other hand pegylation of phenazine **3** with α-Methoxy-ω-bromo PEO was inconclusive due to some traces of a new product formed suspected to be phenazine **9** deduced from the ^{1}H-NMR. Reaction was assumed to be unsuccessful because no characterization analysis was presented in this work.

4 Future aspects

4.1 Future synthetic work

There are various critically insight to the continuity of this project which lies in the development of a new synthetic route to improve the yields of phenazine **3** and phenazine **4** reported in this project. Also, other longer fatty acids than the one displayed in figure 11 or phospholipids could be used to functionalize phenazine **1**.

Furthermore, several purification techniques could be explored in order to separate phenazine **3** N-mono oxide and N-dioxide in its pure state before pegylation reactions.

Another feasible area is to focus on the pegylation reactions of phenazine **3** with a α-Methoxy-ω-bromo PEO. Reaction conditions and purification technique must be modified. This could create an amphiphilic moiety of phenazine **1** and spur the self-assembly characterization of the prodrug into micellar structure.

5   Conclusion

A new derivative of phenazine **1** with a long chain fatty acid (C=12) has been developed. The phenazine **3** was prepared by Williamson ether procedure from a pure synthesized phenazine **1** and a pure synthesized compound **8** that was prepared from the commercially available bromo acetyl bromide and dodecylamine (figure 13). The total yield of all phenazine **3** synthesized was 42%, being the first iodinin analogue with a long fatty acid chain selectively attached to one of two phenolic group on phenazine **1.**

Also, in an attempt to pegylate the phenolic group on position 6 of phenazine **3**, a successful synthesized phenazine **4** with total yield of 46% was achieved.

6 Experimental

General

Solvents used for reactions were delivered from Sigma-Aldrich unless stated otherwise. Already sealed Dry PhMe was bought from Sigma-Aldrich i.e. under inert atmosphere; Dry DMF and CH_2Cl_2 used were taken from the MB SPS-800 solvent purification system from Mbraun. All solvents used for extraction were technical grade. NMR-solvents were also delivered from Sigma-Aldrich and Cambridge isotope laboratories. . Chemicals used were 1,6 phenazinediol (2) bought from Accel pharmatech. Lauric acid was bought from Sigma-Aldrich (9), Bromo acetyl bromide and dodecylamine were delivered from Sigma-Aldrich (figure 13).

Thin layer chromatography was done on 60 F_{254} silica coated aluminiun plates from Merck. Flash chromatography was carried out on silica gel from Merck (silicagel 60, 40-0.60um, 460-520 m^2/g, pH 6.5-7.5) either manually or with the Isco Inc. Combiflash Companion with PeakTrak software (v.1.4.10). All eluents used were of good technical grade.

NMR spectra were recorded on Bruker Avance DPX300, AVIIHD400 and AVI600 instruments at 400 or 600 MHz for H-NMR and C-NMR. Chemical shift (∂) for the NMR solvent $CDCl_3$ at (7.24 ppm for H-NMR, and 77.0 ppm for C-NMR. All coupling constants are given in Hz in H-NMR. And all C-NMR spectra are decoupled. Peak assignment in H-NMR and 13C-NMR were based on the information from HMQC and HSQC experiments as needed. Peaks due to solvents such DCM, EtOAc and water are not included in the spectra interpretations.

MS spectra are recorded on a VG Prospec sector instrument from Fissions Instruments at 70eV (ESI) by Osamu Sekiguchi. HR-MS is recorded using perfluorokerosene as reference.
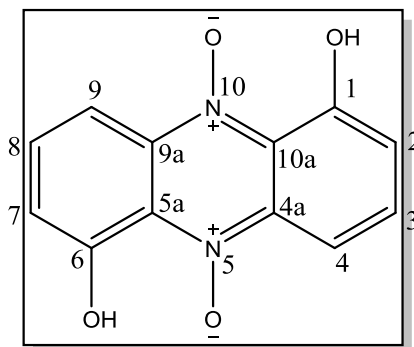
Melting points are recorded using a Buchi B-545 melting point apparatus, and are uncorrected.

And lastly, some of the compounds prepared in this project are already reported in the literature. References are given to published data. All data is according to literature unless stated otherwise.

Synthesized compounds

1, 6-dihydroxyphenazine 5, 10-dioxide (1)

A dry round bottomed flask containing anhydrous toluene (110ml, 1026.7 mmol) (with a reflux condenser) was loaded with phenazine **2** (1000mg, 4.72 mmol, 1eq.), at rt under argon atm. *mCPBA* (2g, 11.59 mmol, 2.5eq.) was added before the mixture was gradually warmed to 85^0C. *mCPBA* (800mg) was added in pulses every hour (repeated 4 times). After 5 hours at 85^0C, 1g of mCPBA was added and reaction mixture was stirred for additional 120minutes. The reaction mixture was cooled down (ice bath) and solvent was removed in *vacuo,* resulting in a dark and slurry crude material. Afterwards, the resulting crude was dispersed in 1:1 MeOH/EtO_2 and it was therefore filtered on a Buchner funnel in portions, each portion was approximately washed with 15ml $NaHCO_3$ (saturated aqueous solution) MeOH and EtO_2 respectively. These portions were collected and washed again with $NaHCO_3$, H_2O, MeOH and EtO_2. A homogenous dark-purple color was obtained when a clear transparent solvent runs through the filter paper. Product was dried on a *vacuo* affording (618mg, 52% yield) of iodinin as a deep-purple solid with a coppery luster.

Yield: 52%

R_f: 0.28 (50-50% Hex-CH_2Cl_2)

^{1}H-NMR: (400 MHz, $CDCl_3$) δ 14.09 (s, 2H, OH1/OH6), 8.03 (dd, 2H, *J*=8.0Hz, *J*=1.0Hz, H4/H9) 7.73 (dd, 2H, *J*=8.0Hz, *J*=7.0Hz, H3/H8) 7.19 (dd, 2H, *J*=8.0Hz, *J*=1.0Hz, H2/H7).

^{13}C-NMR: (600MHz, $CDCl_3$): δ 153.3 (C1/C6) 134.9 (C4a/C9a) 133.4 (C3/C8) 126.5 (C10a/C5a) 114.8 (C2/C7) 107.7 (C4/C9).[7]

MS (ESI) *m/z* (relative intensity): 267 [M+Na]$^+$ (43.7%), 265 (24), 251 (17), 102 (45)

HR-MS: 244.0467; Calculated value for $C_{12}H_8N_2O_4$: 244.0464 (-1.4ppm)

Melting point: 239-241^{0}C. Literature reference 238^{0}C[12]

Compound (1) is known in the literature[12]
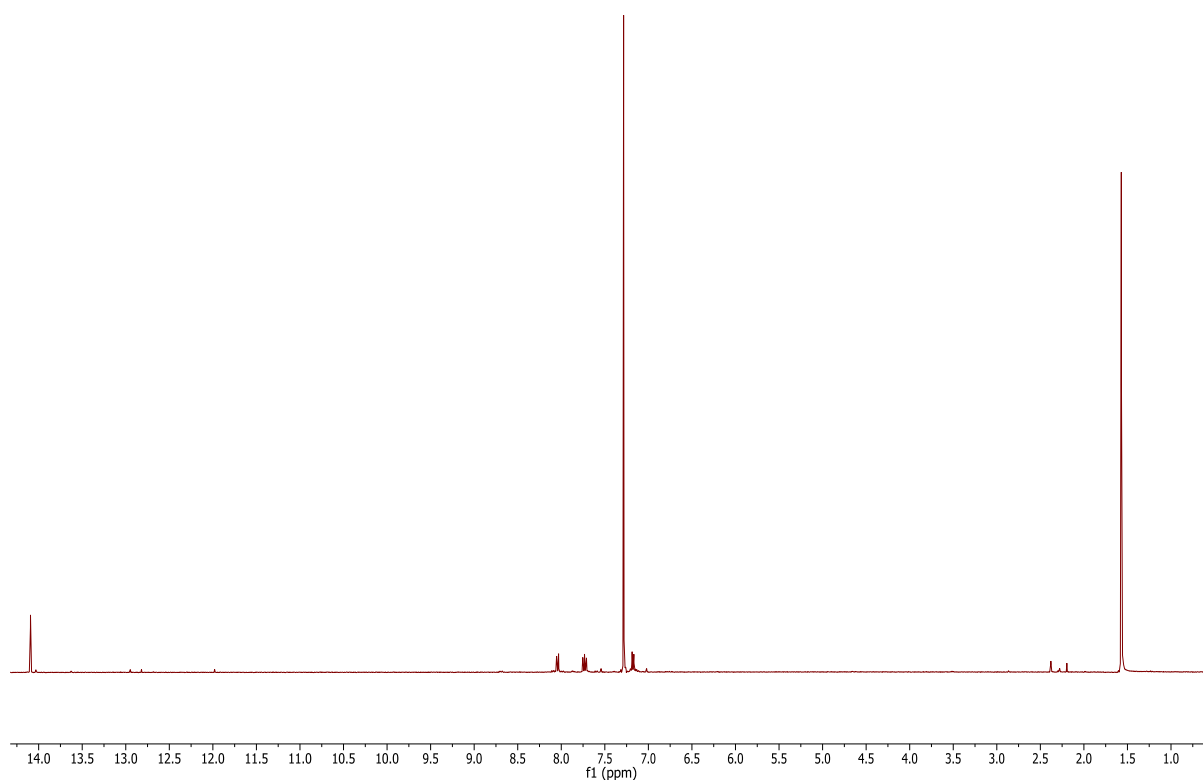
Spectra 1: The ^{1}H-NMR of phenazine 1 (400MHz, CDCl$_3$).

6 Experimental

2-bromo-N-dodecylacetamide (8)

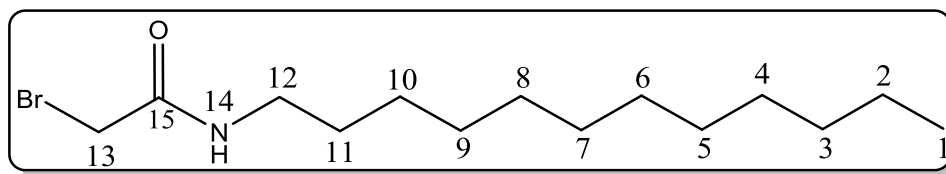A 100ml two-necked round bottomed flask was charged with bromoacetyl bromide (0.8g, 4.04mmol, 1.5eq), anhydrous CH_2Cl_2 (15mL) and K_2CO_3 (0.93g, 6.74mmol, 2.5eq) respectively. The mixture was stirred on ice bath at -78^0C for 5 minutes under argon atm. Dodecylamine which has been previously dissolved in 5mL anhydrous CH_2Cl_2 was added in drops to the reaction mixture for a period of 30 minutes. Stirring was the continued for a period of 120 minutes before quenching excess acid bromide with aqueous HCl (0.5M, 10mL). After stirring for additional 5 minutes, H_2O (5mL) and brine (5mL) were added, then the two transparent phases were transferred into a separatory funnel and the organic phase was drained off. The aqueous phase was extracted with CH_2Cl_2 (1×10mL), the joint organic phase was washed with $NaHCO_3$ (10%wt, 10mL) and brine (10mL), then brine (15mL). it was dried with $MgSO_4$ and concentrated on a *vacuo*. Crude afforded was 550mg.

Yield: 37%

R$_f$: 0.44 (100% DCM)

^{1}H-NMR (400MHz, $CDCl_3$) δ 6.69 (bs, 1H, H14) 3.95 (s, 2H, H13), 3.32 (q, 2H, $J = 6.8$ Hz, H12), 1.57 (quint, 2H, $J = 6.9$ Hz, H11), 1.34–1.28 (m, 18H, H10-H2), 0.9 (t, 3H, $J = 6.7$ Hz, H1).

^{13}C-NMR: (400MHz, $CDCl_3$) 165.38 [C15], 40.33 [CH_2, C13], 29.61 [CH_2, C12], 29.54 [$3 \times CH_2$, C11-C9], 29.48 [CH_2, C8], 29.34 [CH_2, C7], 29.32 [CH_2, C6], 29.23 [CH_2, C5], 29.21 [CH_2, C4], 26.79 [CH_2, C3], 22.67 [CH_2, C2], 14.09 [CH_3, C1].

MS (ESI) *m/z* (relative intensity): 328.12 [M+Na]$^+$ (100%), 267 (68), 206 (20), 146 (36)

HR-MS: 306.28; Calculated value for $C_{14}H_{28}NOBr$: 306.28 (0.1ppm)

Melting point: 49-51^{0}C (from CH_2Cl_2). Literature reference 48-50^{0}C.[50]

Compound is known in the literature.[50]
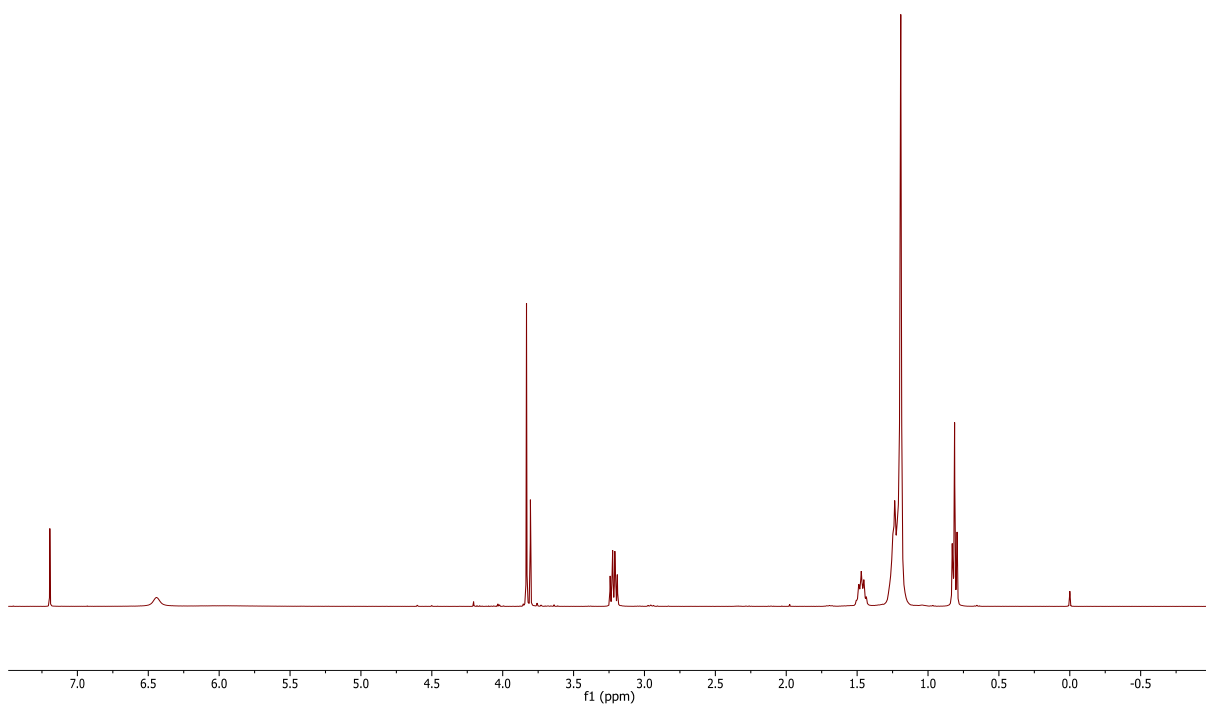
Spectra 2: The ^{1}H-NMR of compound 8 (400MHz, CDCl$_3$).
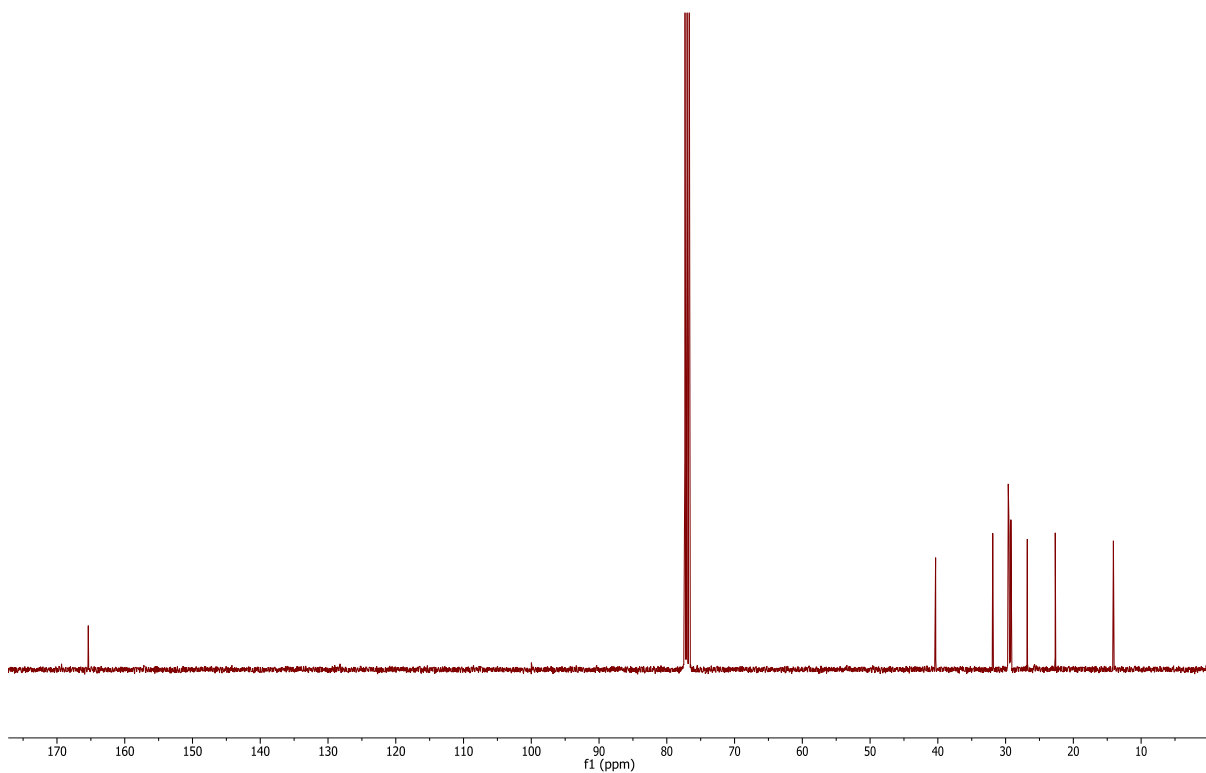
Spectra 3: The ^{13}C-NMR of compound 8 (400MHz, CDCl$_3$).
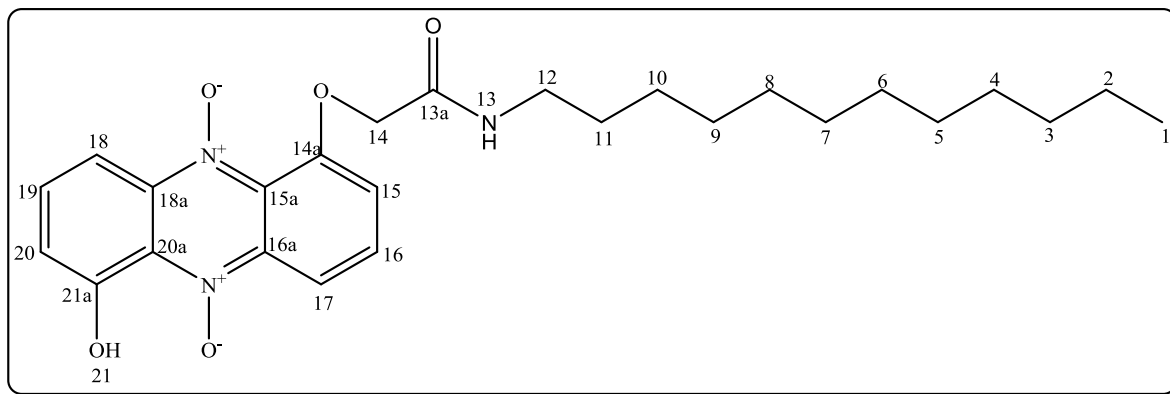
6 Experimental

1-(2-(dodecylamino)-2-oxoethoxy)-6-hydroxyphenazine 5, 10-dioxide (3)

A dry round bottomed flask was charged with phenazine **1** (250mg, 1.02mmol, 1eq), K_2CO_3 (212.3mg, 1.53mmol, 1.5eq) and 18-crown-6-ether (404.3mg, 1.53mmol, 1.5eq). The solids were dispersed in anhydrous DMF (6mL) under argon atm. and well shielded from light. After 30 minutes of stirring where the resulting mixture had turned color from dark violet to emerald green, a drop wise addition of 2-bromo-N-dodecylacetamide (471.2mg, 1.53mmol, 1.5eq) which has already been dissolved in 3ml anhydrous DMF, was carried out before the mixture was left to stir for another 24 hours at room temperature. EtOAc (50mL) was added to the reaction mixture and it was extracted with brine (5×10mL). The combined organic phase was dried with $MgSO_4$, filtered and concentrated on *vacuo*. The crude mixture was further purified by flash chromatography on silica gel (20-80% EtOAc/Hexane as eluent) to afford 54mg of compound (3) as a bright cherry-red solid.

Yield: 41.5%

R$_f$: 0.48 (100% EtOAc)

^{1}H-NMR: (400 MHz, CDCl$_3$) δ 14.45 (s, OH21), 8.03 (dd, 2H, *J*=8.0Hz, *J*=1.0Hz, H17/H18) 7.73 (dd, 2H, *J*=8.0Hz, *J*=7.0Hz, H16/H19) 7.19 (dd, 2H, *J*=8.0Hz, *J*=1.0Hz, H15/H20) 6.34 (bs, H13) 4.14 (s, 2H, H14) 3.46 (q, 2H, *J*= 8Hz, H12) 1.74-1.71 (m, 2H, H11) 1.37-1.25 (m, 18H, H10-H2) 0.92-0.88 (m, 3H, H1)

^{13}C-NMR: (400 MHz, CDCl$_3$) δ 177.2 [C13a], 171.8 [C14a], 167.3 [C21a], 166.1 [C16a], 153.8 [C18a], 152.2 C16], 150.8 [C19], 145.4 [C15a], 132.8 [C20a], 131.4 [C15], 130.7

[C20], 113.0 [C17], 111.6 [C18], 62.1 [C14], 29.6-29.2 [C12-C5], 26.9 [C4], 26.8 [C3], 22.6 [C2], 14.0 [C1].

MS (ESI) *m/z* (relative intensity): 492 [M+Na] $^+$ (19%), 476 (39), 413 (33), 266 (100)

HR-MS: 492.24; Calculated value for $C_{26}H_{35}N_3O_5$: 492.2 (0.2ppm)

Melting point: 99-101^{0}C
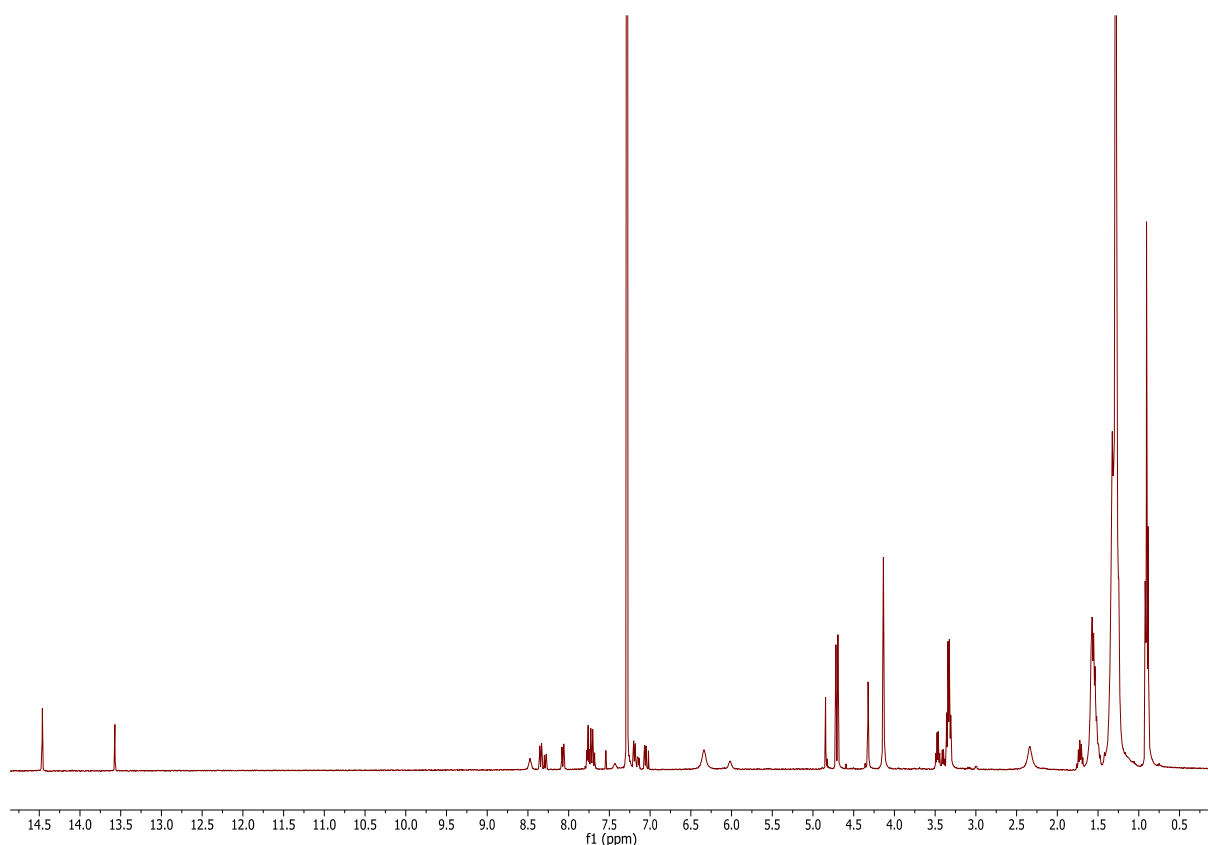
Spectra 3: The ^{1}H-NMR of phenazine 3 (400MHz, CDCl$_3$).
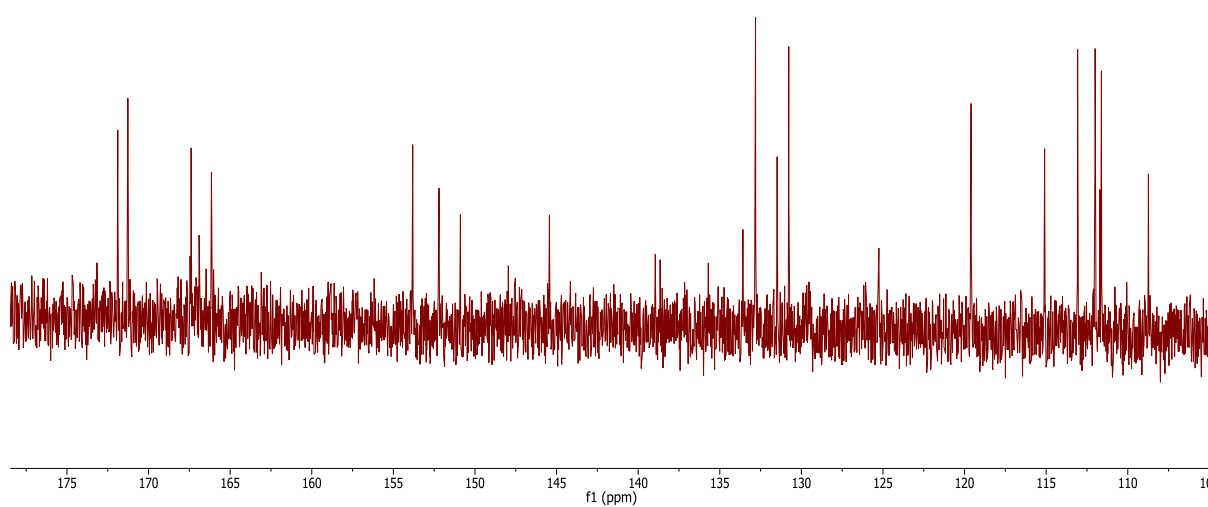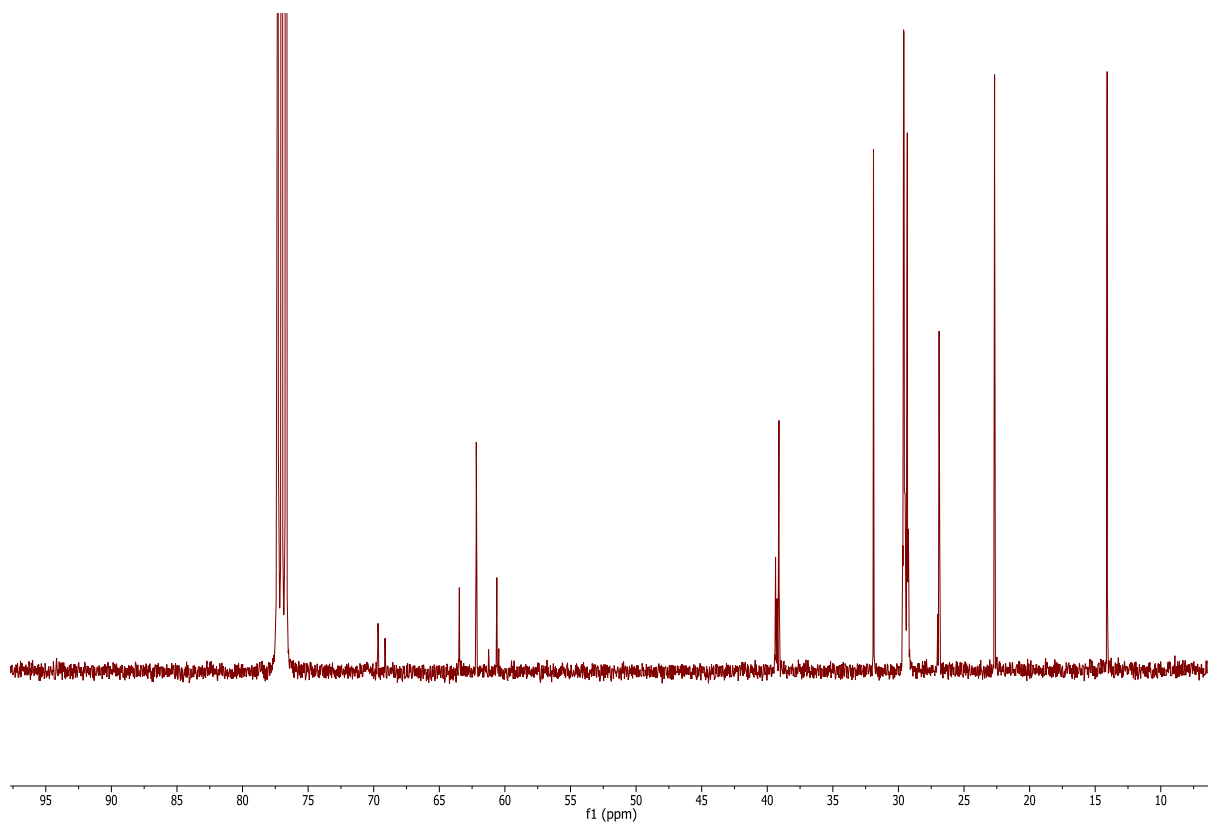
Spectra 4: The ^{13}C-NMR of phenazine 3 (400MHz, CDCl$_3$). The upper spectrum shows only the sp^3 carbons, while the lower spectrum shows the sp and sp^2 carbons.

1-(2-(dodecylamino)-2-oxoethoxy)-6-((2-oxo-3,6,9,12-tetraoxatetradecyl)oxy)phenazine 5, 10-dioxide (4)
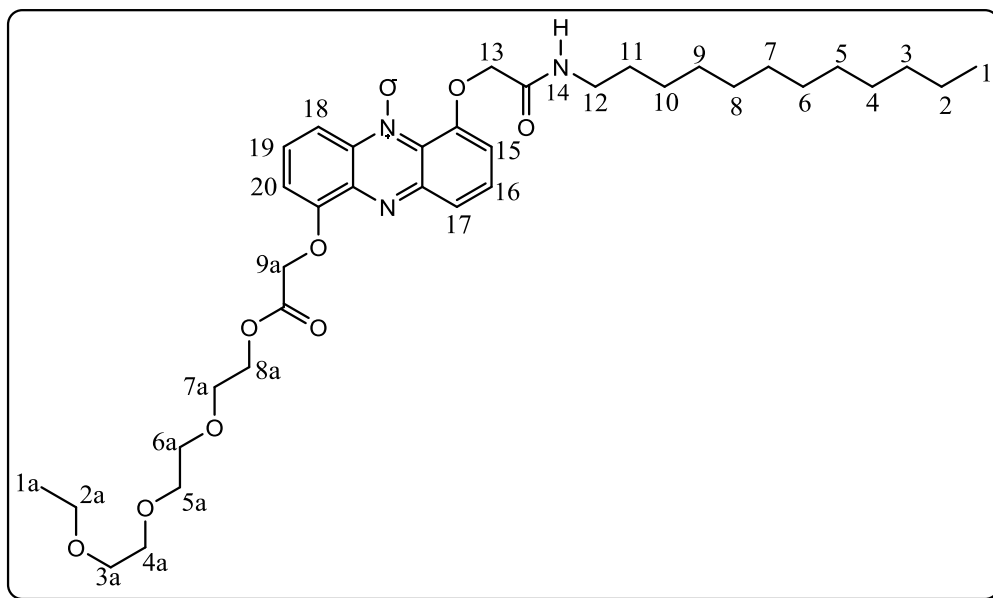
A dry round bottomed flask was charged with phenazine **3** (20mg, 0.02mmol, 1eq) and Cs_2CO_3 (9.7mg, 0.03mmol, 1.5eq). The solids were dispersed in anhydrous THF (1.5mL) under argon atm. and well shielded from light. After 10 minutes of stirring, a drop wise addition of 2-(2-(2-ethoxyethoxy)ethoxy)ethyl 2-bromoacetate (figure 14) (80µL, 0.08mmol, 2.0eq) was added to the reaction mixture while stirring, before the mixture was left to stir for another 24 hours at room temperature. DCM (5mL) was added to the reaction mixture and it was extracted with brine (4×5mL). The combined organic phase was dried with $MgSO_4$, filtered and concentrated on *vacuo.* The crude mixture was further purified by flash chromatography on silica gel (80-20% EtOAc/Hexane as eluent) to afford 13.5mg of phenazine (**4**) as a bright yellow viscous liquid.

Yield: 46%

R_f: 0.38 (100% EtOAc)

^{1}H-NMR: (600 MHz, CDCl$_3$) δ 8.35 (m, H18), 8.02 (m, H19) 7.95 (m, H16) 7.64 (m, 2H, H15/H20) 7.19 (m, H17) 4.35-4.33 (m, 2H, H8a) 4.30 (s, 2H, H9a)   4.21 (s, 2H, H13) 3.75-

3.73 (m, 2H, H7a) 3.69-3.66 (m, 6H, H6a/5a/4a) 3.62-3.60 (m, 2H, H3a) 3.57 (q, 2H, J=4MHz, H2a) 3.40 (q, 2H, J=6MHz H12) 1.59-1.53 (m, 2H, H11) 1.32-1.21 (m, 18H, H10-H2) 0.96-0.85 (m, 6H, H1a/H1)

MS (ESI) *m/z* (relative intensity): 694 [M+Na] $^{+}$ (100%), 548 (50), 477 (72), 413 (55)

HR-MS: 694.4; Calculated value for $C_{36}H_{53}N_3O_{10}$: 694.4 (0.1ppm)
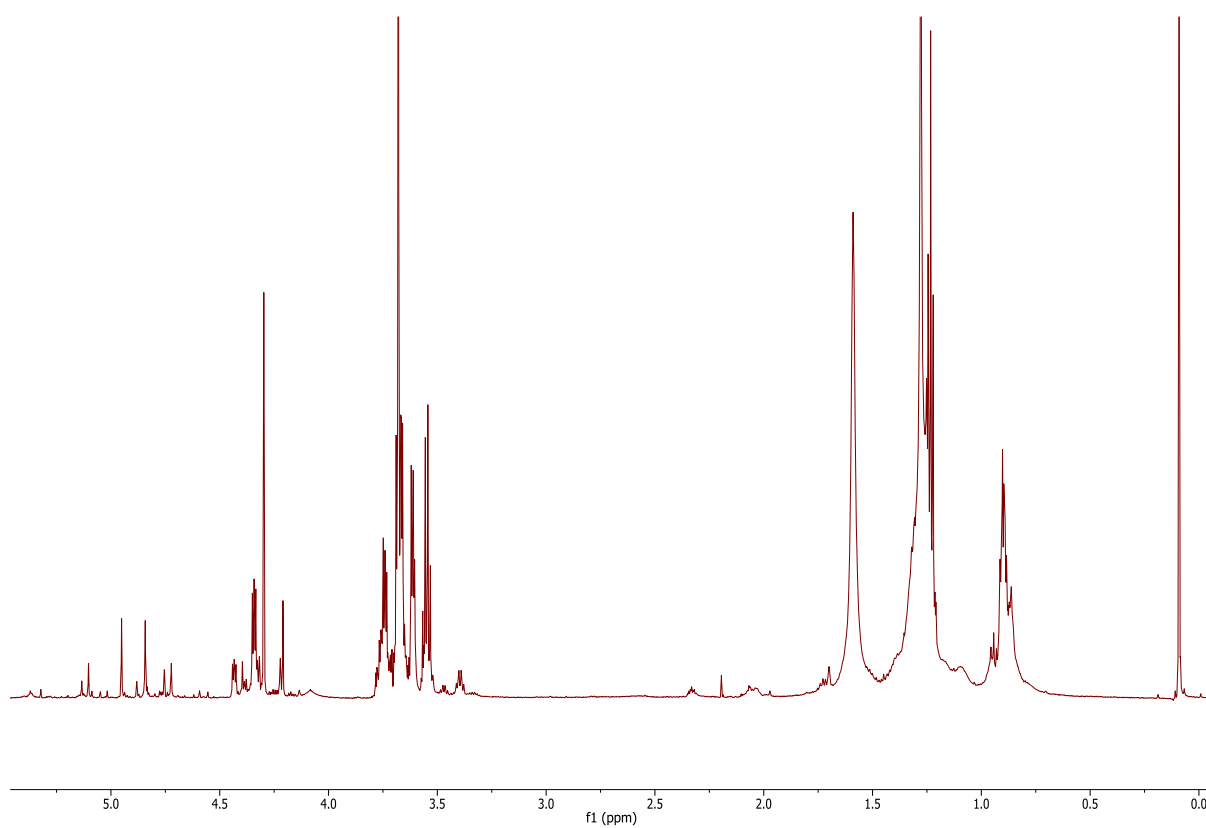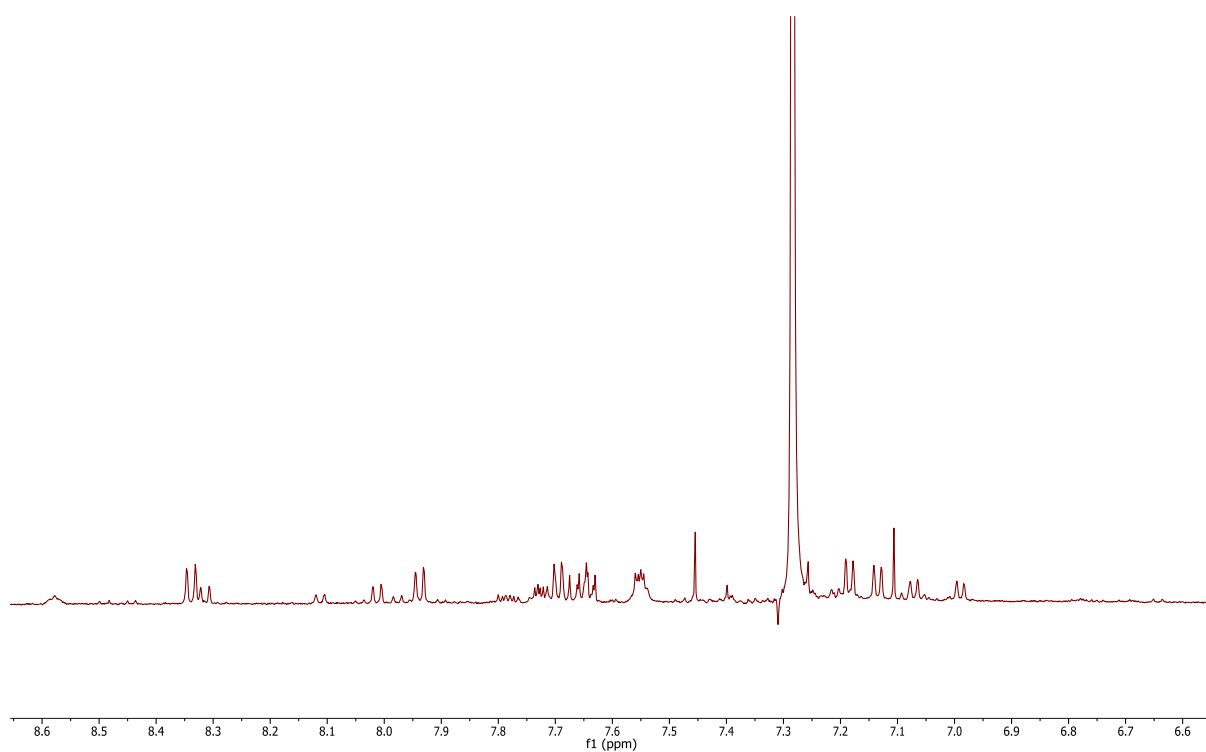
Spectra 5: The ^1H-NMR of phenazine 4 (600MHz, CDCl₃). The upper spectrum shows only the aromatic protons, while the lower spectrum shows the amide and PEO chain protons.

6 Experimental

Attempted synthesis

Phenazine 3 with a α-Methoxy-ω-bromo PEO
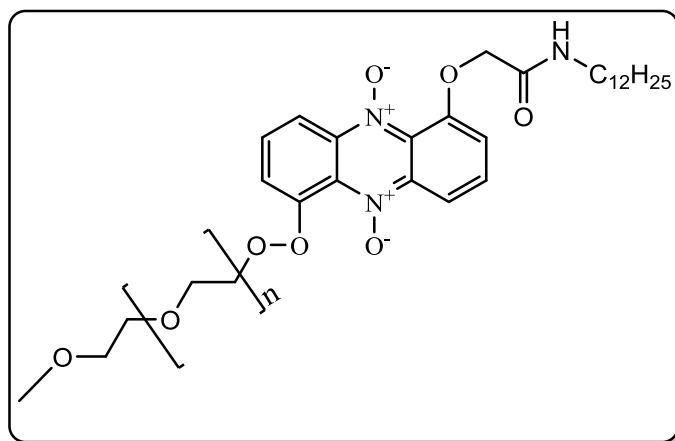
Phenazine **3** (2.1mg, 0.004mmol, 1 eq.) K_2CO_3 (0.9mg, 0.007, 1.5 eq.) and 18-crown-6-ether (1.7mg, 0.007mmol, 1.5 eq.) was added to 1mL dry THF and was stirred for 10 minutes at 65^0C under argon atmosphere, α-Methoxy-ω-bromo PEO (figure 15) (10.7mg, 0.009mmol, 2 eq.) previously dissolved in 1mL THF was added to the reaction mixture in drops. After 2 hours reaction time TLC analysis showed no significant spot, reaction was left to stir for 24 hours under same condition. Crude was diluted with CH_2Cl_2 hopefully to avoid polymerization and organic phase was extracted with water. ^{1}H-NMR of crude was ambiguous, therefore it was not analyzed and neither purification nor isolation was attempted afterwards.

6 Experimental

Attempted synthesis

Phenazine 1 with a α-Methoxy-ω-bromo PEO

Phenazine **1** (8.2mg, 0.034mmol, 1 eq.) K_2CO_3 (7mg, 0.051, 1.5 eq.) and 18-crown-6-ether (13.5mg, 0.051mmol, 1.5 eq.) was added to 1.5mL dry THF and was stirred for 10 minutes at 65^0C under argon atmosphere, α-Methoxy-ω-bromo PEO (figure 15) (81.5mg, 0.067mmol, 2 eq.) previously dissolved in 1.5mL THF was added to the reaction mixture in drops. After 2 hours reaction time TLC analysis showed no significant spot, reaction was left to stir for 24 hours under same condition. Crude was diluted with CH_2Cl_2 hopefully to avoid polymerization and organic phase was extracted with water. ^{1}H-NMR of crude was ambiguous, but from the –OH region we suspected phenazine **8** is present in the complex formed, due to the ^{1}H-NMR ambiguity it was therefore not analyzed further and neither purification nor isolation was attempted afterwards.

Spectra 6: The ^{1}H-NMR of phenazine 8 (400MHz, CDCl$_3$).

References

1. Bob Lowenberg, M.D., James R. Downing, M.D., and Alan Burnett, M.D, *Acute Myeloid Leukemia.* 1999.
2. Bertuzzi, C., et al., *Daunorubicin for the treatment of acute myeloid leukemia.* Expert opinion on orphan drugs, 2014. **2**(10): p. 1075-1087.
3. Chu, L.L., et al., *Synthetic analog of anticancer drug daunorubicin from daunorubicinone using one-pot enzymatic UDP-recycling glycosylation.* J. Mol. Catal. B: Enzym., 2016. **124**: p. 1-10.
4. LGC. *Daunorubicinone*. Available from: http://www.lgcstandards.com/GB/en/Daunorubicinone/p/EPD0120000.
5. katerina vavrova, J.R., Ing Galina Karabanovich, Katerna Hruskova. *Cardioprotective iron chelator*. Available from: http://portal.faf.cuni.cz/Groups/Cardioprotective-iron-chelators/Research/.
6. Garipova, I.Y. and V.N. Silnikov, *New synthetic approaches to multifunctional phenazinium salt derivatives.* Molecules, 2003. **8**(6): p. 505-519.
7. Snellingen, S., *Development of an improved Synthetic Route to Iodinin*, in *Chemistry department*. 2015, University of Oslo. p. 72.
8. Wuyi Wang, P.P., Nicolas Morin, Samir Mounir, Weizhong Cai and M. Arshad Siddiqui *Hepatitis C Viral IRES Inhibition by Phenazine and Phenazine-Like Molecules.* p. 1151-1154.
9. Lokhov, S.G., Podyminogin, M.A., Sergeev, D.S., Silnikov, V.N., Kutyavin, I.V., Shishkin, G.V., and V.P. Zarytova, *Synthesis and high stability of complementary complexes of N-(2-hydroxyethyl)phenazinium derivatives of oligonucleotides.* 1992. **3**.
10. Clark, T.W. and J. Sperry, *Biomimetic synthesis of phenazine-1,6-dicarboxylic acid (PDC).* Synlett, 2012. **23**(19): p. 2827-2829.
11. Tseng, M., et al., *Novel Acrocarpospora punica strain that produces the antitumor compound iodinin*. 2009, Food Industry Research and Development Institute, Taiwan . p. 9pp.
12. Rongved., F.P.-M.L.B.-P., *Iodinin (1,6-dihydroxyphenazine 5,10-dioxide) - origin, chemical/biological properties and synthetic studies. .*
13. Lene E. Myhren, G.N., Gro Gausdal, Håvard Sletta, Knut Teigen, kristin F. Degnes, *Iodinin (1,6-Dihydroxyphenazine 5, 10-Dioxide) from streptosporagium sp. Induces Apoptosis Selectively in Myeloid leukemia Cell Lines and Patient cells.* 2013: p. 35.
14. Clemo, G.R. and H. McIlwain, *Phenazine series. VII. The pigment of Chromobacterium iodinum; the phenazine di-N-oxides.* J. Chem. Soc., 1938: p. 479-83.
15. Yoshioka, I. and H. Otomasu, *Phenazines. IV. Synthesis of iodinin isomers. 1. Syntheses of 2,7- and 1,8-dihydroxyphenazine di-N-oxides.* Pharm. Bull., 1953. **1**: p. 66-9.
16. Anuj Kumar*, S.K.S., Kumud Padhee, Prithi Pal Singh Kochar, Ajit Satapathy andNaveen Pathak, *REVIEW ON SOLUBILITY ENHANCEMENT TECHNIQUES FOR HYDROPHOBIC DRUGS.* 2010.
17. Ikeda, M.S., Neeraj; Nolan, Frank, *Solubility Enhancement of hydrophobic drugs.* 2015.
18. Basak, R. and R. Bandyopadhyay, *Encapsulation of hydrophobic drugs in pluronic F127 micelles. Effects of drug hydrophobicity, solution temperature, and pH.* Langmuir, 2013. **29**(13): p. 4350-4356.
19. Shuo Fang, y.N., Wenjun Zhu, Yemin Zhang, Liangli Yu and Xinsong Li, *Liposomes Assembled from aDual Drug-tailed Phospholipid forCancer Therapy.* 2015: p. 1232 – 1238.
20. Pritika Sharma, A.K., Shilpi Bhargava, *A Review on: Solubility Enhancement by Implementing Solid Dispersion*

Technique for Poorly Water Soluble Drug. Research Journal of Pharmaceutical, Biological and Chemical

Sciences, 2012. **3**(1).

21. Jane Buus Laursen†, a.J.N., †, *Phenazine Natural Products: Biosynthesis, Synthetic Analogues, and*

Biological Activity. 2003.

22. A. R. KATRITZKY, M.A., D.Phil., B.Sc, *THE CHEMISTRY OF THE AROMATIC HETEROCYCLIC N-OXIDES.* p. 12.

23. MICHAEL D. RYAN*, R.G.S., AND PETER KOVACIC*", *Charge Transfer in the Mechanism of Drug Action Involving Quinoxaline Di-N-oxides.* Journal of Pharmaceutical Sciences 1984. **74**.

24. Balevicius, V., et al., *Hydrogen Bonding in Pyridine N-Oxide/Acid Systems: Proton Transfer and Fine Details Revealed by FTIR, NMR, and X-ray Diffraction.* J. Phys. Chem. A, 2012. **116**(34): p. 8753-8761.

25. Brock, E.A., et al., *The Synthesis and Crystal Structures of Two Hydrogen-Bonded N-Oxides.* J. Chem. Crystallogr., 2014. **44**(11-12): p. 548-554.

26. S.G. Bodige, M.A.Z., S.E. McKay, and S.C. Blackstock*, *HETEROCYCLIC N-OXIDE CH...O HYDROGEN BONDING (PART 3).*

CRYSTAL PACKING OF 2,2-BIPYRIDINE-1,1*-DIOXIDE AND*

PYRIDINE N-OXIDE. 1998.

27. Wohl A, A.W., *Chem Ber.* 1901. **34**.

28. Gaertner G, H.F., Gray A, *tetrahedron.* 1962. **18**: p. 1105.

29. Wojciechowski, Z.W.b.M.W.c.R.B.K., *Synthesis of pyrrolo[3,2-a]phenazines from 5-nitroindoles*

and anilines. 2013.

30. Encyclopedia. *The Wohl-Aue Reaction*. 1901 20 April, 2013; Available from: https://en.wikipedia.org/wiki/Wohl – Aue_reaction.

31. KLOETZE, I.J.P.A.M.C., *The Wohl-Aue Reaction. I. Structure of Benzo [a] phenazine Oxides and Syntheses*

of 1,6-Dimethoxyphenazine and 1 ,6-Dichlorophenazine. 1951. **73**.

32. Doeskeland, S.O., et al., *Preparation of phenazine derivatives and cyclodextrin nanoparticle delivery thereof for treatment of cancer and infection*. 2015, Universitetet i Oslo, Norway; Bergen Teknologioverfoering AS; Universite Claude Bernard Lyon 1; Golding, Louise . p. 51pp.

33. Doeskeland, S.O., et al., *Preparation of phenazine derivatives and cyclodextrin nanoparticle delivery thereof for treatment of cancer and infection*. 2015, Universitetet i Oslo, Norway; Bergen Teknologioverfoering AS; Universite Claude Bernard Lyon 1; Golding, Louise . p. 51pp.

34. Ashenhurst, J. *Master Organic Chemistry*. 2016; Available from: http://www.masterorganicchemistry.com/2014/10/24/the-williamson-ether-synthesis/.

35. *Steglich Esterification*. Available from: http://www.organic-chemistry.org/namedreactions/steglich-esterification.shtm.

36. Vitor Gilles , M.A.V., Valdemar Lacerda Jr.a , Eustáquio V. R. Castroa , Reginaldo B. Santosa , Ednilson Orestesb , José W. M. Carneirob , Sandro J. Greco, *A New, Simple and Efficient Method of Steglich Esterification of Juglone with Long-Chain Fatty Acids: Synthesis of a New Class of Non-Polymeric Wax Deposition Inhibitors for Crude Oil.* 2015. **26**.

37. Willy Leimgruber, M., and Manfred Weigele,, N.J. North Caldwell, assignors to Holfmann-La Roche, and N. Inc., NJ. , *Phenazine derivatives.* 1969.

38. Lechevalier, N.N.G.a.M.P., *1,6-Phenazinediol-5-oxide from Microorganisms.* 1965. **4**.

39. Kabanov, A.V., et al., *Polymer genomics: shifting the gene and drug delivery paradigms.* J. Controlled Release, 2005. **101**(1-3): p. 259-271.

40.   Jones, P.D. *Pharmaceutical Applications of Polymers for Drug Delivery*. 2004 24 march 2016; Available from: http://www.smithersrapra.com/publications/books/browse-by-category/review-reports/pharmaceutical-applications-polymers-drug-delivery.

41.   william B. Liechty, D.R.K., Brandon V. Slaughter, Nicholas A. Peppas, *polymers for drug delivery systems.* 2012.

42.   Shashwat S. Banerjeee, N.A., Rajesh Patil, Jayant khandare, *Poly(ethylene glycol)-Prodrug Conjugates: Concept, Design, and Applications.* journal of drug delivery, 2012. **2012**.

43.   Hiroshi saito and Allan S. Hoffman, H.I.O., *Delivery of Doxorubicin from Biodegradable PEG Hydrogels Having Schiff Base Linkages.* 2007. **22**.

44.   science, A.M., *Polymer for drug delivery.* p. 12.

45.   Haag, R. and F. Kratz, *Polymer therapeutics: concepts and applications.* Angew. Chem., Int. Ed., 2006. **45**(8): p. 1198-1215.

46.   Wenjun Li , P.Z., Erik De Clercq, Hongxiang Lou, Xinyong Liu, *Current drug research on PEGylation with small molecular agents.* 2013.

47.   Li, W., et al., *Synthesis, drug release and anti-HIV activity of a series of PEGylated zidovudine conjugates.* Int. J. Biol. Macromol., 2012. **50**(4): p. 974-980.

48.   *Zidovudine.*

49.   simen snelling, b.M.G., Elvar O, Anthony prandina, O Alexander H, Pål Rongved, tore bonge hansen, *The first efficient synthesis of 1,6-dihydroxy-N5,N10-dioxide (iodinin) and 1-hydroxy-6-methoxyphenazine-N5,N10-dioxide (Myxin).* 2015.

50.   Terreno, E., et al., *Determination of water permeability of paramagnetic liposomes of interest in MRI field.* J. Inorg. Biochem., 2008. **102**(5-6): p. 1112-1119.

51.   CARROL, K.K., *Separation of lipid classes by chromatography on florisil.* 1961. **2**: p. 7.