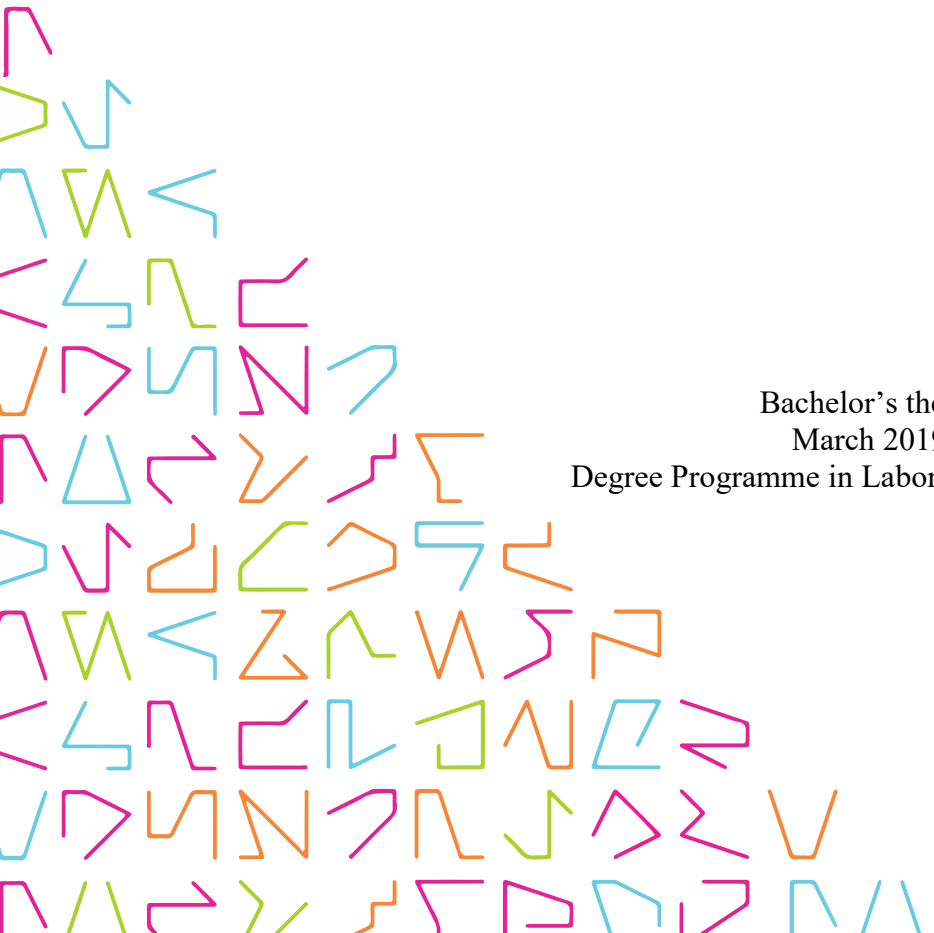
TAMPERE UNIVERSITY
OF APPLIED SCIENCES

SYNTHESIS AND TESTING OF A PHOTOANTIMICROBIAL DYE

Noora Lamminen



Bachelor's thesis
March 2019
Degree Programme in Laboratory Engineering

ABSTRACT

Tampereen ammattikorkeakoulu
Tampere University of Applied Sciences
Degree Programme in Laboratory Engineering

LAMMINEN, NOORA:
Synthesis and Testing of a Photoantimicrobial Dye

Bachelor's thesis 37 pages, appendices 2 pages
March 2019

This thesis was done at the Laboratory of Chemistry and Biotechnology in the Tampere University of Technology in 2018. The objective of this thesis was to synthesise as much as possible of a phthalocyanine based antimicrobial dye. The purpose was through a multistep synthetic chain to synthesise a phthalocyanine based antimicrobial dye and to study the structure and purity of the synthesised products. Several methods were used to achieve this, including mass, UV-VIS, and NMR spectroscopy and thin layer chromatography.

The development of new antimicrobial materials is vital. Many of the conventional antimicrobial materials such as antibiotics are losing their effectiveness to the growth of antimicrobial resistance.

In this thesis a phthalocyanine-based light-activated antimicrobial dye was synthetized with a five-step synthetic chain. The potential of phthalocyanines as possible new antibacterial materials has been studied because of the good quantum yields of the singlet oxygen they produce. Phthalocyanines are also currently used in photodynamic cancer therapy.

Combined 29 reactions were examined in this thesis. The completion of the reactions and the purity of the products were studied with many different techniques in different steps of the synthetic chain. All abovementioned testing methods were used.

A combined amount of 11,5 grams of products was synthesized. 1,13 grams of this consisted of the final product, the dye. A number of difficulties were encountered in the third step of the synthetic chain when the catalyst used in the reaction ran out and a new one had to be purchased. With a new batch of catalyst, the yield of the reaction dropped by over 50% and the purification of the product became more difficult. Different parameters of the reaction such as the starting materials and temperature were experimented with, but the yield did not reach acceptable levels. In the end a different method that had been in use for this reaction was taken into use and it provided promising results. Further experimentation is needed to study this step of the reaction.

The synthesised dye has previously been proven to be effective against microbes and it is water soluble. In the future different practical uses and the safety of using the dye need to be investigated.

Key words: antimicrobial, phthalocyanine, singlet oxygen, photosensitizer

TIIVISTELMÄ

Tampereen ammattikorkeakoulu
Tampere University of Applied Sciences
Laboratoriotekniikan koulutus

LAMMINEN, NOORA:
Valoaktiivisen antimikrobisen väriaineen synteesi ja testaus

Opinnäytetyö 37 sivua, joista liitteitä 2 sivua
Maaliskuu 2019.

Opinnäytetyö tehtiin Tampereen teknillisen yliopiston kemian ja biotekniikan laboratoriossa loppuvuodesta 2018. Opinnäytetyön tavoitteena oli valmistaa mahdollisimman paljon ftalosyaniini pohjaista antimikrobista väriainetta. Tarkoituksena oli useiden välireaktioiden kautta valmistaa ftalosyaniini pohjaista antimikrobiallista väriainetta ja tutkia valmistettujen aineiden rakennetta ja puhtautta. Käytössä olleita menetelmiä olivat massa-, UV-VIS- ja NMR-spektroskopia, sekä ohutlevy kromatografia.

Uusien antimikrobisten aineiden kehitys on hyvin tärkeää. Useat perinteiset antimikrobiset aineet kuten antibiootit menettävät tehokkuuttaan antibioottiresistenssin kasvaessa.

Työssä valmistettiin ftalosyaniini pohjaista valoaktiivista antimikrobista väriainetta erilaisten orgaanisten synteesireaktioiden kautta. Ftalosyaniineja tutkitaan mahdollisina uusina antimikrobisina aineina niiden hyvän singlettihapen tuoton takia. Niitä käytetään nykyään myös esimerkiksi syövän valoaktiivisessa hoidossa.

Reaktioketju koostui viidestä reaktiosta. Reaktioita suoritettiin yhteensä 29 kappaletta. Reaktioiden onnistumista ja lopputuotteiden puhtautta sekä rakennetta tutkittiin edellä mainituilla menetelmillä reaktioketjun eri vaiheissa.

Työssä valmistettiin yhteensä 11,5 grammaa tuotteita joista 1,13 grammaa oli lopputuotetta. Reaktioketjun kolmannessa vaiheessa synteesissä esiintyi ongelmia käytettävän reagenssin vaihtuessa. Reaktiosta saatu lopputuotteen määrä väheni yli 50% ja tuotteen puhdistaminen vaikeutui. Menetelmää testattiin muuttamalla eri parametreja kuten reaktion lähtöaineita ja lämpötilaa, mutta saantoa ei saatu riittävän korkealle tasolle. Lopulta siirryttiin käyttämään toista aiemmin käytössä ollutta menetelmää, jolla saatiin lupaavia tuloksia. Kolmatta reaktiota tulisi jatkossa suorittaa ja tutkia lisää.

Työssä valmistetun väriaineen on aikaisemmin todettu olevan tehokkaasti antimikrobinen ja vesiliukoinen. Tulevaisuudessa on tärkeää tutkia väriaineelle sopivia mahdollisia käyttökohteita ja käyttöturvallisuutta.

Asiasanat: antimikrobinen, ftalosyaniini, singlettihappi, valoherkiste

CONTENTS

ABBREVIATIONS AND TERMS

ABR	Antibacterial resistance
AMR	Antimicrobial resistance
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
HAI	Healthcare-associated infection
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance spectroscopy
PACT	Photodynamic antimicrobial chemotherapy
Pc	Phthalocyanine
PDT	Photodynamic cancer therapy
ROS	Reactive oxygen species
TLC	Thin layer chromatography
TMS	Tetramethyl silane
ZnPc	{4,4′,4′′,4′′′-(29H,31H-phthalocyanine-1,8,15,22-tetrayl-K4N29,N30,N31,N32)tetrakis[1-methylpyridiniumato(2-)]}zinc(4+)tetraiodide

1 INTRODUCTION

This thesis was done in the Tampere University of Technology (now Tampere University) in the chemistry and new materials teams Light activated antimicrobial materials -project. The project is led by Dr. Alexander Efimov.

The objective of this thesis was to synthesise as much as possible of a phthalocyanine based antimicrobial dye. The purpose was thought a multistep synthetic chain to synthesise a phthalocyanine based antimicrobial dye and to study the structure and purity of the synthesised products. Methods for this include mass-, UV-VIS- and NMR-spectroscopy and thin layer chromatography. The novel dye had first been synthesised by the group in 2017 and published in their research and in the doctoral thesis of Dr. Lijo George (2018). It was important to synthetize more of the dye for further research.

The wide use of antibiotics, antimicrobial sprays and disinfectants make developing resistance easier for microbes (WHO 2014). This is a growing threat around the world and innovative products are needed to help fight it. This thesis describes the method for producing such a product.

All the reactions in this thesis are referred to with the initials NL and a consecutive number corresponding to each reaction. In total there are 29 reactions that are referred to in this thesis.

2 THEORETICAL BACKROUND

2.1 Conventional antimicrobial agents

Antibiotics are widely used in treating bacterial infections. The first antibiotics were found in nature. Penicillin for example is produced by fungi to fight other microbes for space and nutrients. Antibiotics work in different ways. Some kill the bacteria and others prevent them from reproducing by disrupting the cells normal metabolism. For example, penicillin weakens the cell membrane, so it ruptures killing the bacteria. Antibiotics are not universally effective. They need the bacteria to have specific receptors to bind to in order to work and bacteria are always evolving leading to resistance. (Lumio Antibiootit 2018)

Antimicrobial resistance (AMR) is the phenomenon that happens when a given microbe develops resistance to a drug it was previously susceptible to. AMR is a growing global threat. For example, reports of resistance in *Staphylococcus aureus* were made only three years after penicillin was first sold globally and only two years after methicillin was developed. (Maisch 2015, 1519). When microbes develop AMR infections and diseases, they cause become harder to treat, spread easier and cost more both economically and socially. (WHO 2014)

Some microbes have developed AMR to many different antibiotics making them very difficult to treat. For example, MRSA is methicillin resistant *Staphylococcus aureus*. In addition to methicillin it is also resistant to penicillin and cephalosporins. MRSA is common healthcare-associated infection (HAI). MRSA can survive on dry surfaces for weeks though it is more often transmitted through nursing staff from an affected patient to a new patient. (Lumio MRSA 2018)

Microbes are not only susceptible to synthetic organic drugs. Humans have been using copper as an antimicrobial material for centuries. Copper works as an antimicrobial material through contact killing. Contact killing is not completely understood yet as a process but the belief is that copper ions and reactive oxygen species (ROS) created by the copper are involved in the process. The rate of killing for certain microbes has been observed as

being at least 7 to 8 logs per hour. This means that the copper reduced the number of microbes in a logarithmic scale. 7 logs per hour means the number of microbes was 10 000 000 times smaller after an hour contact with the copper. In 2008 some copper alloys like brass and bronze were accepted by U.S. Environmental Protection Agency as the first solid antimicrobial material. (Grass etc 2011, 1541)

Copper has been in experimental use in hospitals as a surface material with positive results. Compared to control materials copper containing surfaces like door handles and taps produced clear diminishing off bacteria. (Grass etc 2011, 1544-1546)

2.2 Singlet oxygen and photodynamic inactivation of microbes

Singlet oxygen is a reactive oxygen species (ROS). Another example of ROS is hydrogen peroxide which is known to be an antimicrobial substance. The antimicrobial effect of ROS is based on killing cells by oxidation. The singlet oxygen is formed when a photosensitiser, a substance that induces a chemical charge in another nearby substance, transfers energy produced by light irritation into O_2 molecules in the air surrounding the photosensitiser. (Ishii 2012, 1556; Tirkkonen 2017, 3)

Singlet oxygen has been studied as a candidate for photodynamic antimicrobial chemotherapy (PACT) and photodynamic cancer therapy (PDT) because of its ability to kill all types of microbes effectively. Both PACT and PDT are treatment methods in which a photosensitiser, light and oxygen are used. The advantage of PACT and PDT is that the treatment can be localised to only involve the affected areas. The drug can travel in the bloodstream but will only affect the places that are irritated with light. A fibre optic laser is usually used as a light source. (Ishii 2012, 1556; Wainwright 1998, 13)

It is very likely that microbes will not be able to develop resistance against singlet oxygen for multiple reasons. The effect of the singlet oxygen is so fast that the bacteria have no time to produce protective proteins. Unlike antibiotics singlet oxygen does not only affect a cell in specific spots or only if the cell has certain receptors. Singlet oxygen has also been proven to be effective against MRSA and other HAIs. (Eichner 2012, 135; Maisch 2015, 1518)

2.3 Phthalocyanines

Phthalocyanines (Pc) are aromatic compounds that can be synthetically made from phtalonitriles as in figure 1. They are often made with a metal complex. These metals can be Zn, Si, Al, Ga, etc. Pc have shown good potential as photosensitizers and they are known for being efficient producers of ROS. The before mentioned diamagnetic metals enhance the singlet oxygen yield. (Masilela etc 2013, 500; Ormond & Freeman 2013, 829)

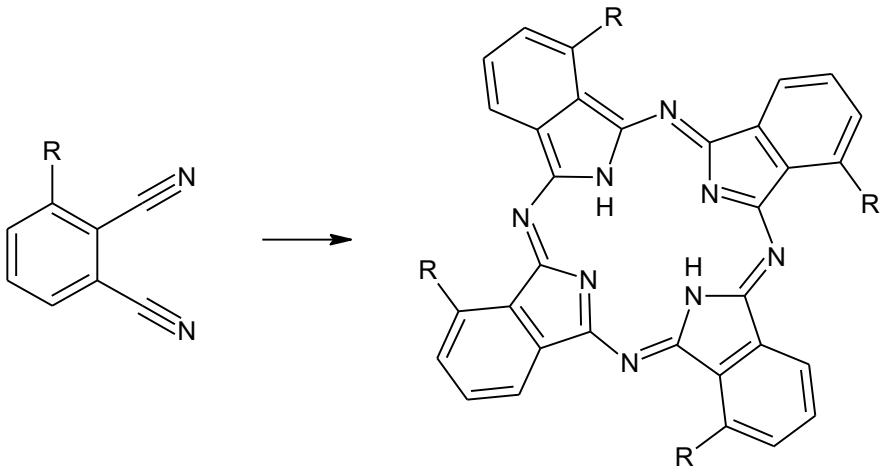


FIGURE 1. Formation of phthalocyanine (George 2018, 32, modified)

A Pc containing aluminium has been in use in Russia for the treatment of several types of cancer under the name Photosens. Photosens causes skin phototoxicity as a side effect for several weeks. A silicon Pc is in trials for the treatment of multiple diseases such as skin cancer and Bowen's disease. It has completed Phase I medical trials. (Ormond & Freeman 2013, 829)

2.4 Analytical methods

Different kinds of tests can be performed on synthesis products to verify the completion of the reaction and to determine the purity of the product. In chemical synthesis the amount of starting material used can be called the scale of the reaction. The yield of a reaction is the number of moles of the product obtained from the reaction divided by the theoretical maximum possible amount from the reaction. It gives out a percentage that can be used to describe how efficient the reaction is.

2.4.1 Thin layer chromatography

Thin layer chromatography (TLC) is one of the simplest ways to do chromatography. The basic idea in chromatography is to separate a sample containing different components by transporting it thought a stationary phase with a mobile phase called the eluent. In TLC the stationary phase is a plate that has been coated with a thin layer of adsorbent material. (Jaarinen & Niiranen 2008, 150-151.)

The sample is dissolved in the eluent or another liquid it dissolves well in. A place for each studied sample is marked in the TLC plate with a pencil on the same level. It is important to leave enough space between the samples, so the plate stays clear and is easy to read (FIGURE 2).

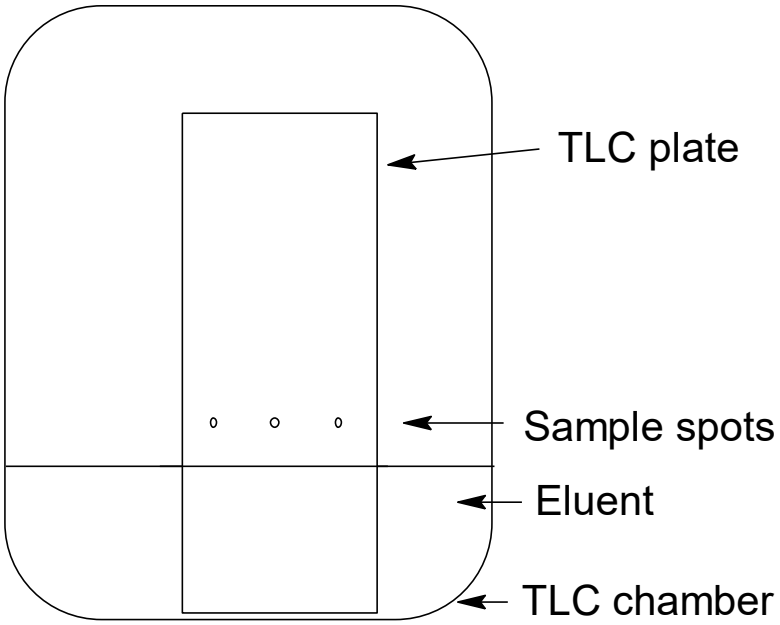


FIGURE 2. Thin layer chromatography

A small amount of the eluent is poured to the bottom of a TLC chamber. It is important that the samples are higher on the plate then the surface of the eluent reaches (FIGURE 2). The chamber is closed. The eluent begins to rise on the plate because of the capillary forces. The eluent is allowed to rise about 10 – 15 cm on the plate. The plate is then removed from the chamber and allowed to dry. (Jaarinen & Niiranen 2008, 150-151.)

If the sample spots are coloured and visible the results can be seen instantly. Sometimes the visualising of the spots needs staining. If the compounds are fluorescent the spots can be seen under a UV lamp. Sometimes the plate is dyed after the elution to visualize the spots. (Jaarinen & Niiranen 2008, 150-151.)

2.4.2 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) as all spectroscopy is the study of how matter interacts with electromagnetic radiation. Some atoms, like hydrogen (proton), carbon and fluorine, have nuclei that have a small own magnetic moment μ. This is caused by the nuclei having a non-zero spin. (Zumdahl 2005, 691)

The NMR spectrometer has a superconducting magnet which creates a strong magnetic field B. When the dipole μ of the nuclei is placed in the strong magnetic field B the nucleus can only exist in two states. The low energy state is aligned with B and the high energy state against the field B. The nucleus can shift from the low energy state to the high energy state by absorbing electromagnetic radiation of the right wavelength. (Zumdahl 2005, 691; Keeler 2010, 483)

Different chemical environments affect the local magnetic field around the nuclei. This affects the absorbance of the electromagnetic radiation which is observed. This is how NMR can be used to tell which kinds of atoms and bonds are present in the studied sample. (Zumdahl 2005, 691-693)

NMR is a non-invasive method that requires a very small sample. It can be used in determining the structure of the sample. If the structure is known, it can also be used to look for impurities in the sample.

2.4.3 Mass spectroscopy

Mass spectroscopy (MS) is the measurement of the molecular mass of the sample. It is based on ionizing the sample and passing it through a mass analyzer. The weight of the ions can be determined from the way they interact with the magnetic field, electronic field or a combination of both in the mass analyzer. (Jaarinen & Niiranen 2008, 122; Zumdahl 2005, 51)

The identification of products with mass spectroscopy is done by comparing the result with results for known compounds. Modern mass spectrometers can also predict the spectra a specific molecule could produce. This prediction can be compared to the results of a measured sample. (Jaarinen & Niiranen 2008, 122)

2.4.4 Absorption coefficient

Absorption of light is a phenomenon which occurs when energy from light (photon) excites an atom or a molecule into a higher energy state. This can only happen when the energy of the photon is the same as the energy that is required for the atom or molecule to transition to a higher state. (Jaarinen & Niiranen 2008, 48.)

In Lambert-Beer law

$$A(\lambda) = \varepsilon(\lambda) \cdot C \cdot b \tag{1}$$

absorbance is A, the molar absorptivity or molar absorption coefficient is ε, the concentration of the studied sample is C and the length that the light travels in the sample is b. The length b is often 1 cm in spectroscopy. (Thermo Fisher 2013)

From formula 1 we can calculate the molar absorption coefficient in which

$$\varepsilon = \frac{A}{C} \tag{2}$$

we can leave out b if the length of the cuvette used is 1 cm. The absorption coefficient is used to determine how much light the substance that is being studied absorbs per mole in a solution.

The molar absorption coefficient can be used for many applications. For example, in protein research if the specific molar absorption coefficient of the studied compound is known, estimating the concentration of a sample or determining what fractions have the wanted product. It can also be used to compare the measured results of the absorption coefficient to a known absorption coefficient. (Thermo Fisher 2013)

3 METHODS

3.1 The synthetic route

The process of synthesising the antimicrobial dye is a multistep process (FIGURE 3) that is made up of five reactions. The chain begins from commercial 3-nitrophthalonitrile.

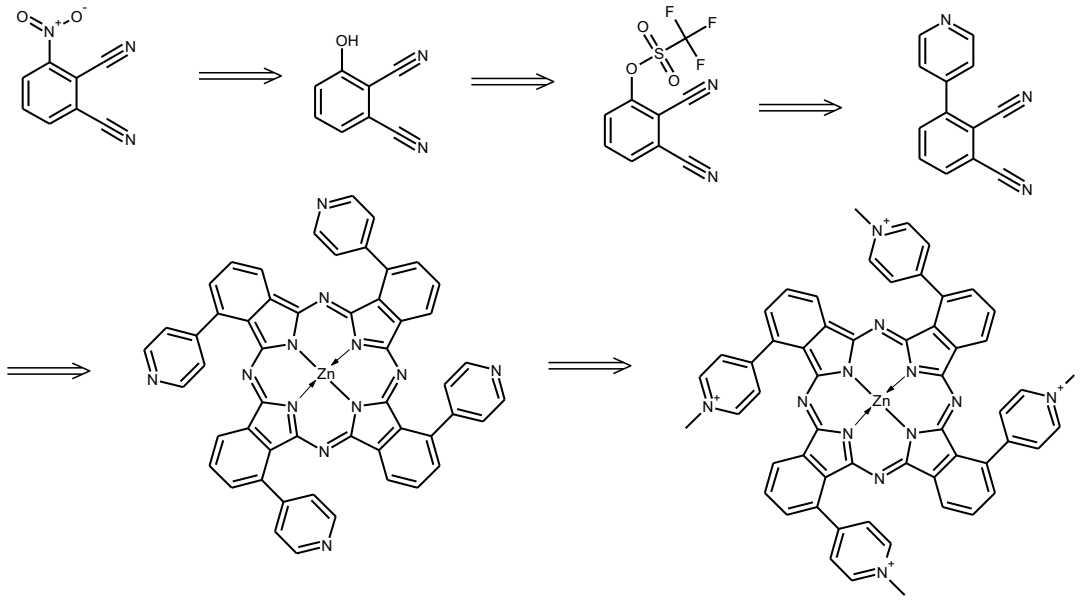


FIGURE 3. The synthetic chain

3.2 The synthesis of 3-hydroxyphthalonitrile

The first step of the chain is the reaction that converts 3-nitrophtalonitrile to 3-hydroxyphthalonitrile. The reaction scheme for this reaction is presented in figure 4.

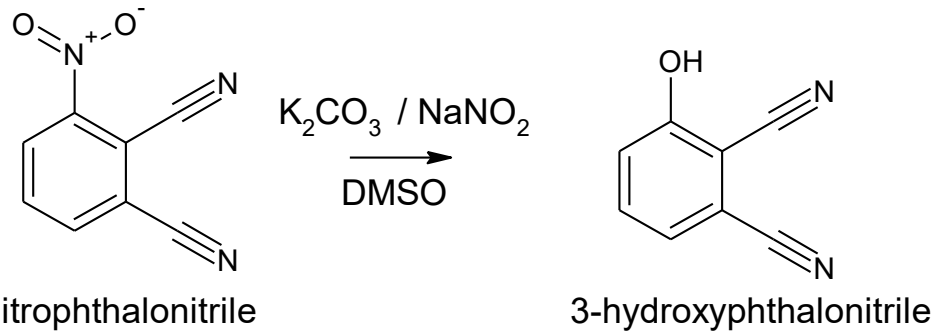


FIGURE 4.

500 mg of 3-nitrophtalonitrile, 440 mg of potassium carbonate, and 220 mg of sodium nitrate were weight and dissolved in 8 ml of dimethyl sulfoxide (DMSO) in a round bottom flask. The reaction mixture was heated and stirred under argon flow for 1 hour.

After the mixture was cooled to room temperature it was placed in an ice bath and approximately 3 ml of 2 M hydrochloric acid was added. The pH of the solution was approximately 4. Sodium chloride was added to enhance the precipitation of the product. The cold mixture was centrifuged, and the settled material was washed 4 times with cold water. The product was dried in a vacuum desiccator.

The dry product was transferred to a round bottom flask and recrystallized with acetonitrile and water. The solid was dried. A TLC was done to make sure the reaction has happened correctly and to see there were no impurities.

3.3 The synthesis of 2,3-dicyanophenyl trifluoromethanesulfonate

The second step of the reaction chain is the transformation of 3-hydroxyphthalonitrile to 2,3-dicyanophenyl trifluoromethanesulfonate. This reaction is presented in figure 5.

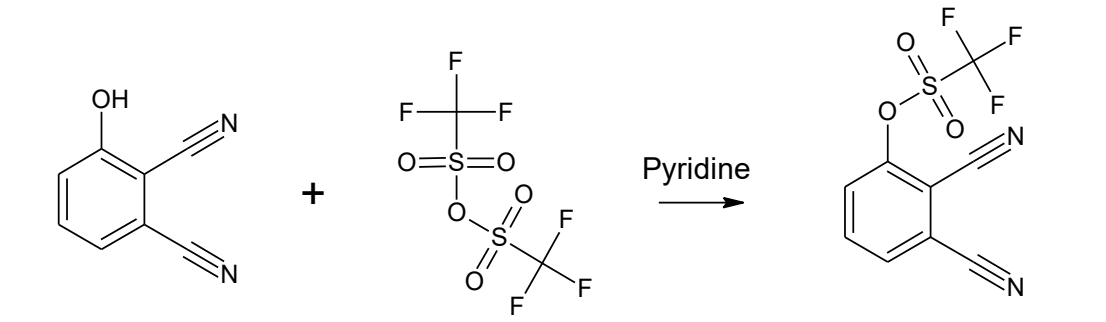


FIGURE 5.

200 mg of 3-hydroxyphthalonitrile was weighted and dissolved in 2,5 ml of pyridine in a flame dried vial. The mixture was stirred and cooled in an ice bath under argon flow for half an hour. 250 µl of trifluoromethanesulfonic anhydride was added to the reaction mixture dropwise over 15 minutes period of stirring. The argon flow was removed, and the cap was covered with parafilm to preserve the argon atmosphere. The reaction mixture was stirred at room temperature overnight.

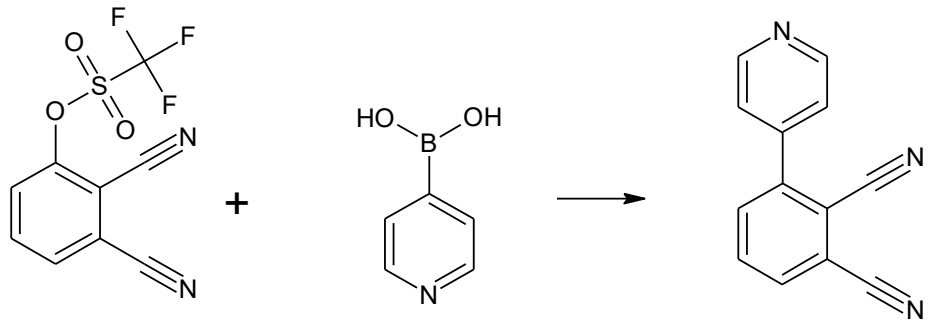
A TLC was done to confirm the reaction completion. The mixture was transferred into a separation funnel and distributed between equal amounts of chloroform and water. The product went into the chloroform. The chloroform fraction was dried by filtration though sodium sulfate and transferred into a round bottom flask. Chloroform was removed with rotavapor. The product was then run through a silica 60 column with chloroform as an eluent. The fractions were checked with TLC and the ones containing the product were collected and concentrated with rotavapor. The purity of the product was checked with TLC and NMR.

3.4 The synthesis of 3-(pyridine-4-yl)phthalonitrile

The third step of the chain is the reaction to transform the 2,3-dicyanophenyl trifluoromethanesulfonate into 3-(pyridine-4-yl) phthalonitrile. This reaction was done with two different methods.

3.4.1 The first method for 3-(pyridine-4-yl)phthalonitrile

This method was used because it gave a good yield and the reagents for this reaction were less expensive than in the other method. The reaction scheme is presented in figure 6.



2,3-dicyanophenyl trifluoromethanesulfonate 4-pyridylboronic acid 3-(pyridin-4-yl)phthalonitrile

FIGURE 6.

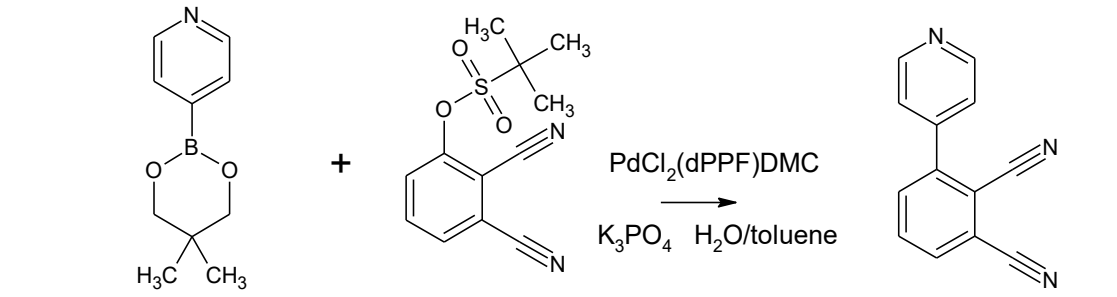
200 mg of 2,3-dicyanophenyl trifluoromethanesulfonate, 107 mg of 4-pyridylboronic acid, 150 mf of potassium carbonate and 85 mg of tetrakis(triphenylphosphine)palladium were weight and dissolved in 10 ml of a 4:1 mixture of water and dimethyl ether (DME). The vial was closed with a cap and a septum. The air was removed and replaced with argon from a balloon 5 times. The mixture was stirred and heated in a heating block at 80 - 100 °C depending of the reaction for 1 hour.

The reaction was verified with a TLC. The mixture was then transferred into a separation funnel and distributed between chloroform and water. The chloroform fraction was collected, and the chloroform was removed with rotavapor. The dry mixture was then recrystallized with chloroform and hexane. A TLC was done. The recrystallization was repeated if some impurities were present. The final product was checked with TLC and NMR.

Slightly different methods with catalysts from different manufacturers and different heating temperatures were used in reactions NL-15, NL-16, NL-17, NL-25 and NL-26. These are all presented in table 7 (appendix 1).

3.4.2 The second method for 3-(pyridine-4-yl)phthalonitrile

This method had been in use in the production of 3-(pyridine-4-yl)phthalonitrile before the less expensive method was taken into use. It however was more reliable, provided stable yields and it was finally taken into use. The reaction scheme is presented in figure 7.



4-(5,5-dimethyl-1,3,2-dioxaborinen-2-yl) pyridine

2,3-dicyanophenyl trifluoromethane sulfonate

3-(pyridin-4-yl)phthalonitrile

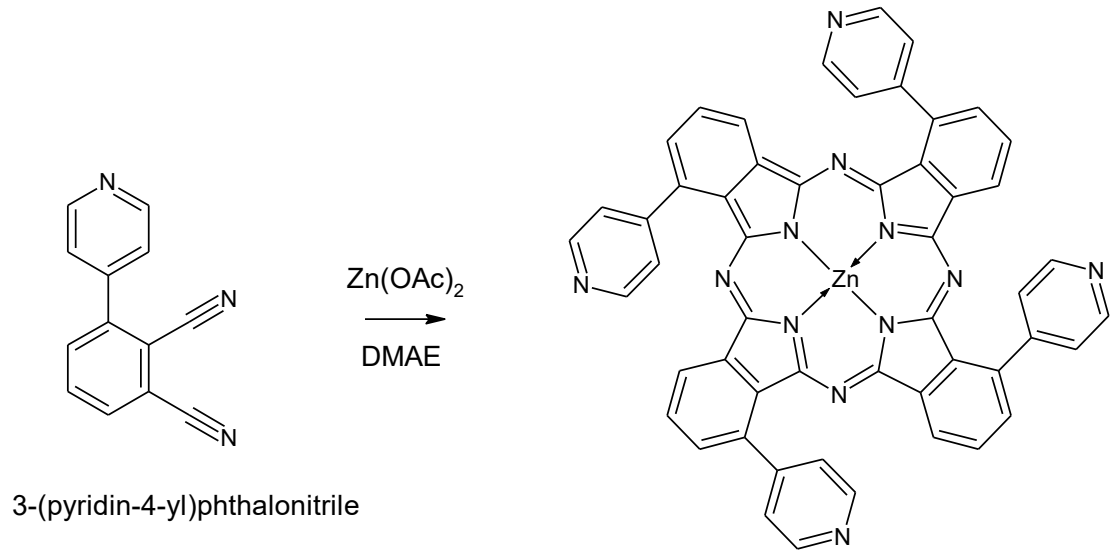
FIGURE 7.

1,15 g of tripotassium phosphate was dissolved in 31 ml of water. The 500 mg of 2,3-dicyanophenyl trifluoromethanesulfonate, 415 mg of 4-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl) pyridine and 37 mg of [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II), complex with dichloromethane were weighted into a round bottomed flask (RB) and 31 ml toluene was added. The water solution of tripotassium phosphate was added to the RB. The flask was equipped with a condenser and sealed with a septum. Air was removed from the reaction vessel by vacuum pumping and replaced with argon from a balloon four times. Some reactions were done under argon flow and some under an argon balloon. The reaction was heated in a glycerol bath at 95 - 100 °C for two hours.

The reaction mixture was diluted with chloroform and then washed with water in a separation funnel. The organic layer was collected to a beaker. The aqueous layer was neutralized with 2 M hydrochloric acid and extracted with chloroform. The organic layers were combined and washed with brine solution and evaporated to dryness. The solid material was recrystallized with chloroform and hexane. The final product was checked with TLC and NMR.

3.5 The synthesis of tetrapyridyl phthalocyanine zinc

The forth step of the reaction is the transformation of 3-(pyridine-4-yl)phtalonitrile to tetrapyridyl phthalocyanine zinc. The reaction formula for this reaction is presented in figure 8.



3-(pyridin-4-yl)phthalonitrile

tetrapyridyl phthalocyanine zinc

FIGURE 8.

250 mg of 3-(pyridine-4-yl)phthalonitrile and 268 mg zinc acetate were weighted and dissolved in 2.6 ml dimethyl amino ethanol (DMAE). The mixture was heated and stirred in a heating block at 140 °C for 15 hours. 20 ml of 9:1 methanol:water was added to the cooled reaction mixture and stirred for 30 minutes. The precipitate was separated by centrifugation, and the liquid was discarded. Water was added to the precipitate and stirred for 30 minutes. The mixture was centrifuged, and the liquid was discarded again. The solid was washed with methanol 3 times. The final product was allowed to dry completely. In reactions NL-12 and NL-27 the product was boiled in methanol before drying.

3.6 The synthesis of {4,4′,4′′,4′′′-(29H,31H-phthalocyanine-1,8,15,22-tetrayl-κ4N29,N30,N31,N32)tetrakis[1-methylpyridiniumato(2-)]}zinc(4+)tetraiodide

The methylation of tetrapyridyl phthalocyanine zinc is the last step in the synthetic chain. The reaction scheme is presented in figure 9.

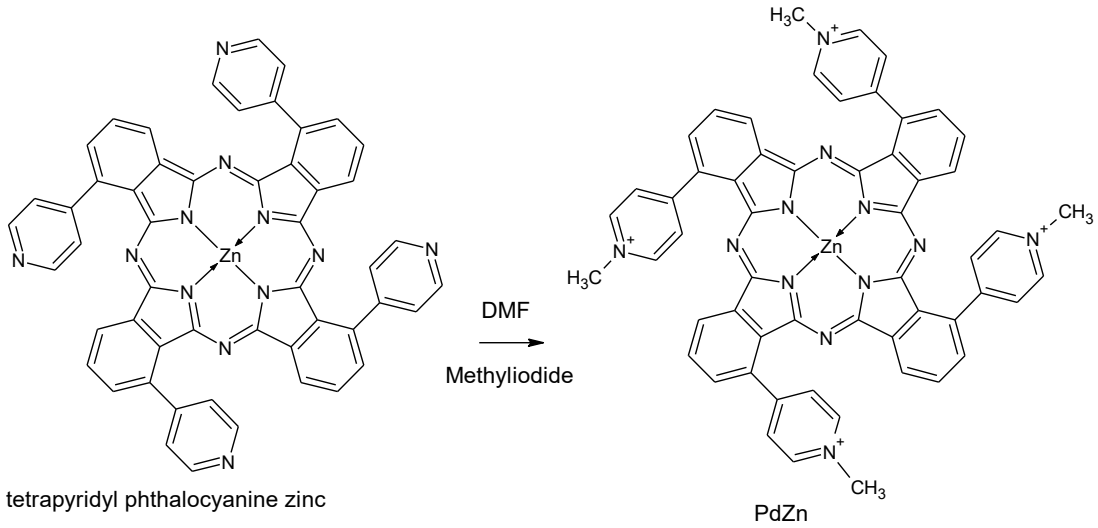


FIGURE 9.

310 mg of tetrapyridyl phthalocyanine zinc was weighted and 14 ml of methyl iodide and 40 ml of dimethylformamide (DMF) are added to a round bottomed flask. The RB was with a condenser and heated at 45 °C for 18 hours at stirring.

A small sample of the product was taken and precipitated with diethyl ether and dissolved in water. It was checked with the MS to confirm the reaction and to check if there are any unwanted isomers.

Diethyl ether was added with a dropper to the cooled mixture placed in ice. The mixture is centrifuged, and the liquid part is poured out. The solid matter is washed with diethyl ether three times. The diethyl ether is centrifuged out. The solid is left to dry. The dry product is recrystallized with acetone and water on a watch glass. This was usually done twice. The absorption coefficient of the product was measured.

3.7 The applications of the analytical methods.

Silica 60 plates with 18:1 chloroform:ethanol eluent were used for all the TLC tests. A UV-lamp was used for visualising the results. Deuterated chloroform was used in H^1 NMR. Approximately 5 mg of the sample was weighted and then dissolved into 750 µl of chloroform-d before the measurement. The mass analysis was done from a small precipitated sample of the reaction mixture. The precipitate was dissolved in ultra-high purity water before the measurement.

For the measurement of the absorption coefficient approximately 1 mg of the studied substance was weighted. It was dissolved in 5 ml of dimethylformamide (DMF). For the analysis of the absorption coefficient the concentration needed to be 0,005 mM. The absorption coefficient was measured against the absorption of pure DMF in quartz cuvettes. The absorption of the sample was measured from 250 nm to 750 nm. The highest absorption, around 700 nm, was taken into the calculation of the absorption coefficient. Molar absorption of phthalocyanines is over 100 000. It had been decided that the absorption coefficient needed to be over 100 000 for the product to be considered as sufficiently pure.

4 RESULTS

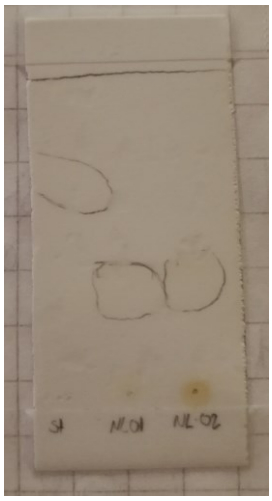
4.1 The results of the synthesis of 3-hydroxyphthalonitrile

The synthesis of 3-hydroxyphthalonitrile was done two times. The results of the synthesis are presented in table 1. The first synthesis was done in a smaller scale of 500 mg and the second in ten times the scale with 5 g of the starting material. In total 2,83 g of 3-hydroxyphthalonitrile was produced with the average yield of 67%.

TABLE 1. The synthesis of 3-hydroxyphthalonitrile

Name	Starting material	End product	Yield
NL-1	509,6 mg	314,3 mg	74%
NL-2	4,9997 g	2,517 g	60%

The products of this synthesis were tested with TLC. The tests for both NL-1 and NL-2 were done on the same TLC plate (PICTURE 1). The spots that were visible under the UV light were marked on the plate with a pencil.



PICTURE 1. TLC plate of the testing of NL-01 and NL-02

In the case of the synthesis of 3-hydroxyphthalonitrile the samples on the plate (PICTURE 1) were the starting material, NL-1 and NL-2. This TLC shows that NL-1 and NL-2 were the same product and that it was not the same substance as the starting material because they are on different levels.

4.2 The results of the synthesis of 2,3-dicyanophenyl trifluoromethanesulfonate

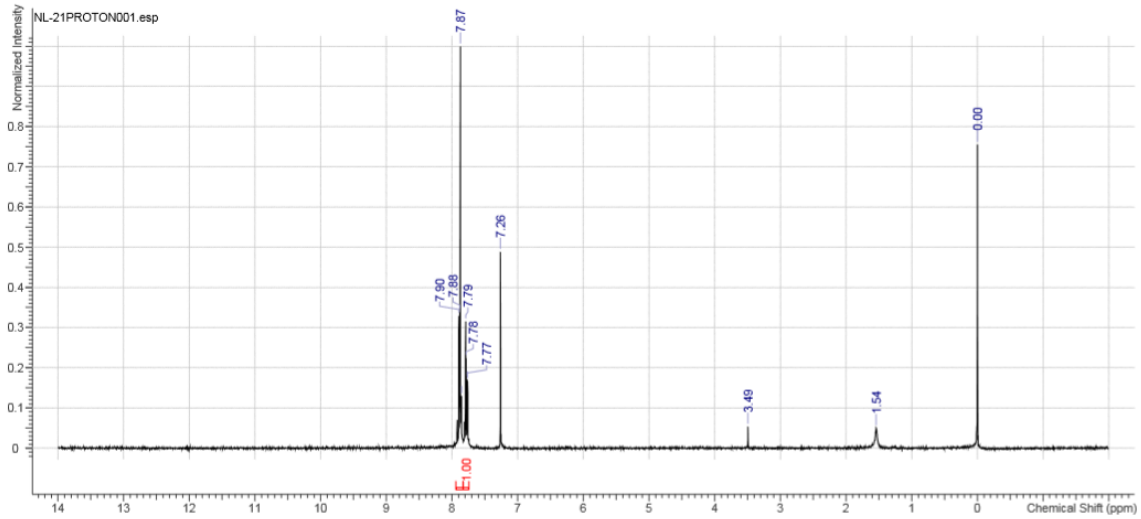
The synthesis of 2,3-dicyanophenyl trifluoromethanesulfonate was done 9 times. The results of the synthesis are presented in table 2. The synthesis was done in 200 mg and in 500 mg scales. In total 5,82 g of 2,3-dicyanophenyl trifluoromethanesulfonate was produced with the average yield of 83%.

TABLE 2. The synthesis of 2,3-dicyanophenyl trifluoromethanesulfonate

Name	Starting material	End product	Yield
NL-3	199,9 mg	301,1 mg	79%
NL-18	200,4 mg	341,3 mg	89%
NL-19	501,6 mg	785,3 mg	82%
NL-20	200 mg	305,7 mg	80%
NL-21	503,8 mg	776,4 mg	80%
NL-22	502,7 mg	748,6 mg	78%
NL-23	504,1 mg	813,5 mg	84%
NL-28	558,3 mg	926,8 mg	86%
NL-29	501,9 mg	825,9 mg	85%

The products of this synthesis were preliminarily tested with TLC in many stages of the purification. When the TLC showed only a spot for the 2,3-dicyanophenyl trifluoromethanesulfonate the product was also tested with H^1 NMR. The NMR used was a Varian Mercury 300 MHz spectrometer.

Picture 2 shows the H^1 NMR spectrum of 2,3-dicyanophenyl trifluoromethanesulfonate. The visible peaks are around 8 ppms because the compound is aromatic. The strong peak at 0 ppms is tetramethyl silane (TMS) that is used as an internal standard in the deuterated chloroform and the peak around 7.2 is the chloroform-d. The small peaks at 3,5 ppms and 1,55 as most likely small amounts of methanol and water.



PICTURE 2. The NMR spectra for pure 2,3-dicyanophenyl trifluoromethanesulfonate

All synthetized 2,3-dicyanophenyl trifluoromethanesulfonate was tested with the NMR and were not considered sufficiently pure until the NMR spectra was similar to picture 2.

4.3 The results of the synthesis of 3-(pyridine-4-yl)phthalonitrile

The synthesis of 3-(pyridine-4-yl)phthalonitrile was done using two different methods. The first method used 4-pyridylboronic acid and the second used 4-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl) pyridine.

4.3.1 The results for the first method for 3-(pyridine-4-yl)phthalonitrile

The synthesis of 3-(pyridine-4-yl)phthalonitrile was done twice with the old batch of catalyst from one manufacturer, and then five times using new batches of catalyst from three different manufacturers. The attempts with the new chemicals provided significantly

lower or no yields at all. The scale of the synthesis was kept small to avoid losing significant amounts of the starting material 2,3-dicyanophenyl trifluoromethanesulfonate. The results of the synthesis are presented in table 3.

TABLE 3. The synthesis of 3-(pyridine-4-yl)phthalonitrile, first method

Name	Starting material	End product	Yield
NL-4	199,9 mg	83 mg	56%
NL-14	200,2 mg	92,8 mg	62%
NL-15	100,4 mg	24,8 mg	33%
NL-16	100,5 mg	23,7 mg	32%
NL-17	99,9 mg	-	-
NL-25	99,6 mg	-	> 30%
NL-26	100,5 mg	-	> 30%

The products from NL-15, NL-16, NL-17, NL-25 and NL-26 could not be purified sufficiently with the purification steps presented earlier. NL-17 was completely discarded as it produced no 3-(pyridine-4-yl)phthalonitrile.

4.3.2 The results for the second method for 3-(pyridine-4-yl)phthalonitrile

The synthesis of 3-(pyridine-4-yl)phthalonitrile was realized only once with this method. The resulting yield of the synthesis presented in table 4 was the highest for the synthesis of 3-(pyridine-4-yl)phthalonitrile.

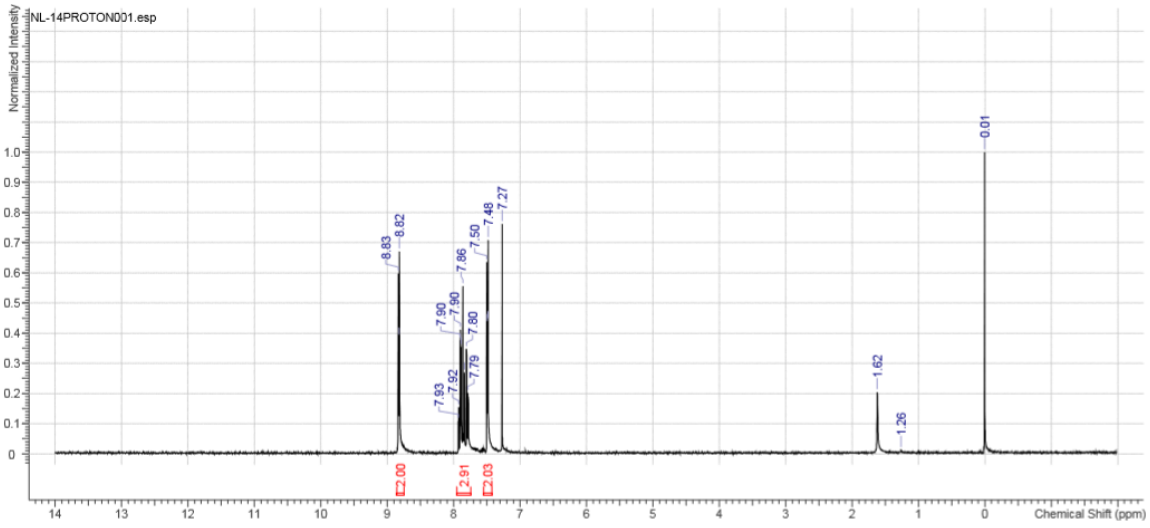
TABLE 4. The synthesis of 3-(pyridine-4-yl)phthalonitrile, second method

Name	Starting material	End product	Yield
NL-30	500,4 mg	296,8 mg	80%

This synthesis was done in a scale of 500 mg. The 4-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl) pyridine used in this synthesis had been synthetized in the laboratory.

4.3.3 The testing for the synthesis of 3-(pyridine-4-yl)phthalonitrile

Though the two methods for synthesising and purifying 3-(pyridine-4-yl)phthalonitrile were different the testing was done in the same manner for both methods. The preliminary test were done with the TLC and the final products checked with H^1 NMR. In picture 3 a sufficiently pure sample of 3-(pyridine-4-yl)phthalonitrile is presented. The peaks around 8 ppms are the aromatic compound. The peak at 0 ppms is TMS, the peak around 7,2 is the chloroform and the peaks at 1, 6 and 1,2 are most likely residual solvents.



PICTURE 3. NMR spectra for pure 3-(pyridine-4-yl)phthalonitrile

In total 470 mg of 3-(pyridine-4-yl)phthalonitrile was produced with these two methods with the average yield of 53%. The average yield for the first method was 46% and the yield for the second method was 80%. All 3-(pyridine-4-yl)phthalonitrile was tested with NMR and not accepted as sufficiently pure until the spectra resembled that shown in picture 3.

4.4 The results of the synthesis of tetrapyridyl phthalocyanine zinc

The synthesis of tetrapyridyl phthalocyanine zinc was done six times. The results of the synthesis is presented in table 5. The scale of the synthesis was between 250 – 300 mg. The weight yields of these reactions were systematically higher then theoretical maximum. This means that the product contained residual water, zinc acetate, and other impurities and side-products. In total 1,52 g of tetrapyridyl phthalocyanine zinc was prepared with the average "yield" of 107%.

TABLE 5. The synthesis of tetrapyridyl phthalocyanine zinc

Name	Starting material	End product	Yield
NL-5	249,9 mg	310 mg	115%
NL-8	250 mg	302,9 mg	112%
NL-10	249,8 mg	298,1 mg	111%
NL-12	283,9 mg	320,2 mg	105%
NL-27	292 mg	289,9 mg	92%

The purity of products of this synthesis were not tested. The compounds were used in the next step of the synthetic chain without further purification. It is clear there was more substance than there should have been in reactions NL-5, NL-8, NL-10 and NL-12.

4.5 The results of the synthesis of {4,4′,4′′,4′′′-(29H,31H-phthalocyanine-1,8,15,22-tetrayl-K4N29,N30,N31,N32)tetrakis[1-methylpyridiniumato(2-)]}zinc(4+)tetraiodide

The synthesis of {4,4′,4′′,4′′′-(29H,31H-phthalocyanine-1,8,15,22-tetrayl-K4N29,N30,N31,N32)tetrakis[1-methylpyridiniumato(2-)]}zinc(4+)tetraiodide (ZnPc) was done 5 times. The results of the synthesis are presented in table 6. The scale of the synthesis depended mostly on the available amount of the starting material tetrapyridyl phthalocyanine zinc. The combined amount of ZnPc synthesised was 1,13 g. The average yield for this synthesis was 48%.

TABLE 6. The synthesis of ZnPc

Name	Starting material	End product	Yield
NL-6	310 mg	175,6 mg	35%
NL-7	266,3 mg	224 mg	51%
NL-9	302,9 mg	196,7 mg	40%
NL-11	292,4 mg	227,7 mg	51%
NL-13	311,1 mg	310,8 mg	61%

This synthesis was tested with the MS before purification to see that the product was the correct one. An example of this is presented in picture 4 (appendix 2) in which the first row is a prediction of the product by the MS based on the compound's chemical formula. The second row is the measured spectra for NL-07 that has been processed by the MS according to the internal standard. The last row is of the raw data. All the spectrums have the peaks beginning at approximately 236,06 so the reaction has happened. Because the product is not purified before this test there are plenty of other peaks from the reaction mixture. The MS used was a Waters LCT Premium XE ESI-TOF benchtop mass spectrometer.

After purification the product was tested for the absorption coefficient and was only accepted as pure when its absorption coefficient was calculated to be over 100 000. The absorption coefficients were measured with Shimadzu UV-3600 UV-VIS-NIR spectrophotometer. For example, the highest absorption for NL-11 after the second recrystallization was 0,5300. The concentration of the sample was 0,005 mM which is 0,000005 M. We can place these values into formula 2

$$\varepsilon = \frac{0{,}5300}{0{,}000005\ \mathrm{M}}$$
$$\varepsilon = 106\,000\ \mathrm{M}^{-1} \qquad (3)$$

and get the absorption coefficient of NL-11 106 000. The product is therefore sufficiently pure. All ZnPc produced was tested for its absorption coefficient and the purification was not complete before the result was >100 000. In other words, the highest absorption of the sample needed to be over 0,5.

5 DISCUSSION

The objective and purpose of this thesis were accomplished. A total of 11,5 grams of various products were obtained. The target product was prepared in the amount 1,13 grams.

The first step of the chain was accomplished with the average yield of 67%. The products were tested only preliminarily with TLC. This means that the possibility of these product not being completely pure is quite high. In future it might be useful to test these products more as it is likely that having a pure starting material for the next step of the synthetic chain would result in the highest possible yield.

The second step of the chain was done with the average yield of 83%. The results of the reaction did not have a lot of variation and the yield was consistently on the same level. Several tests were used to determine the product was as pure as possible.

The third step of the chain had some problems. This was because the catalyst for the reaction, tetrakis(triphenylphosphine)palladium, ran out. New batch of the same catalyst from the same manufacturer was ordered but the reaction did not work with this new catalyst. The same problem occurred with different manufacturers catalysts. The most likely explanation for this is that as the original catalyst that had been in use for several years had most likely degraded or changed over time. The second method was used only once in this thesis, but it provided promising results. In the future the second method needs to be tested more and possibly taken into use for the synthesis of 3-(pyridine-4-yl) phthalonitrile.

A very similar synthetic chain has been presented in an earlier study (George et al 2017, 335-336). In this study the second method of synthesizing 3-(pyridine-4-yl) phthalonitrile was used. The amount of the palladium catalyst however was twice (5 mole percent) what was used in this thesis (2,5 mole percent). The yield for this reaction was 60 % in the study and 80 % in this thesis (George et al 2017, 336). The reaction seems to produce improved results with a smaller amount of catalyst. This assumption needs to be confirmed in the future with further research.

The fourth step of the reaction consistently had yields that were impossible. The yield of a reaction cannot be over a 100%. This means there was an impurity left in the product. This impurity was possibly zinc acetate that was used in synthesis. Probably, zinc ions coordinated between nitrogen atoms at meso- and pyridyl-positions of phthalocyanines, which made these complexes very stable and hard to destroy. The added step of boiling the product in methanol lowered the yield to acceptable levels. In the fore mentioned study, the yield was >90% which corresponds with the results in this thesis (George etc 2017, 336). It is not known if the product became completely pure because it was not tested. All the available tetrapyridyl phthalocyanine zinc was turned into the final product when it was sufficiently dry. The final product was thoroughly tested.

The final step of the chain had the average yield of 48%. The yields of the reactions got almost consistently higher. This may have been because the starting material for these reactions was likely purer as their yields came down to a reasonable level. The average yield is very similar as in the previously published study where the yield was 45% (George et al 2017, 336). These products were preliminarily tested with the MS to see that the reaction had happened. The MS is more accurate than the TLC for preliminary tests. The purity of these products was tested with the absorption coefficient. This is a good test for the product because the product absorbs light well.

The antimicrobial effect was strong. A filter paper with the dye load of 80 mg/m^2 demonstrated a 3 log inactivation against *Escherichia coli* and *Acinetobacter baylyi* in 1 hour illumination (George et al 2017, 341). This is not as high as coppers 7 to 8 log reduction (Grass et al 2011, 1541). But with higher dye loads the efficacy was so high that it was impossible to calculate a reduction because there were not bacteria left (George et al 2017, 341). With the amount of dye produced in this thesis it would be possible to make over 14 m^2 of self-disinfecting light-activated filter paper. This could be used to cover the surfaces of a small hospital room. However, at this point in time the dye needs to be used for further research.

In the future the dye can be applied to different materials. For example, phthalocyanines have been embedded in polymer membranes with promising results in singlet oxygen production. In a polymer membrane the phthalocyanine could be used in water purification. The singlet oxygen based effect against microbes has been proven effective against HAIs so it is very likely this dye will also be effective against them. This would make the dye excellent for use in hospital settings as a surface material. The user safety of the dye also needs to be studied to make sure it does not cause irritation like Photosens. (Eichner et al 2013, 135; Ormond & Freeman 2013, 829; Mafukidze et al 2016, 212)

In conclusion this thesis shows that the dye can be manufactured in a scale that is sufficient for further studies. In the future the steps with the lowest yields might require further optimisation to produce the best possible results. Methods for large scale production can be based on the methods presented in this thesis.

The many good qualities of this type of material like the effectiveness against microbes, the unlikeliness of resistance developing and the fact the effect can be controlled simply by controlling the light make this an important field of study in the future (Eichner et al 2013, 135; Maisch 2015, 1518). Like with PACT and PDT only the illuminated dye produces singlet oxygen.

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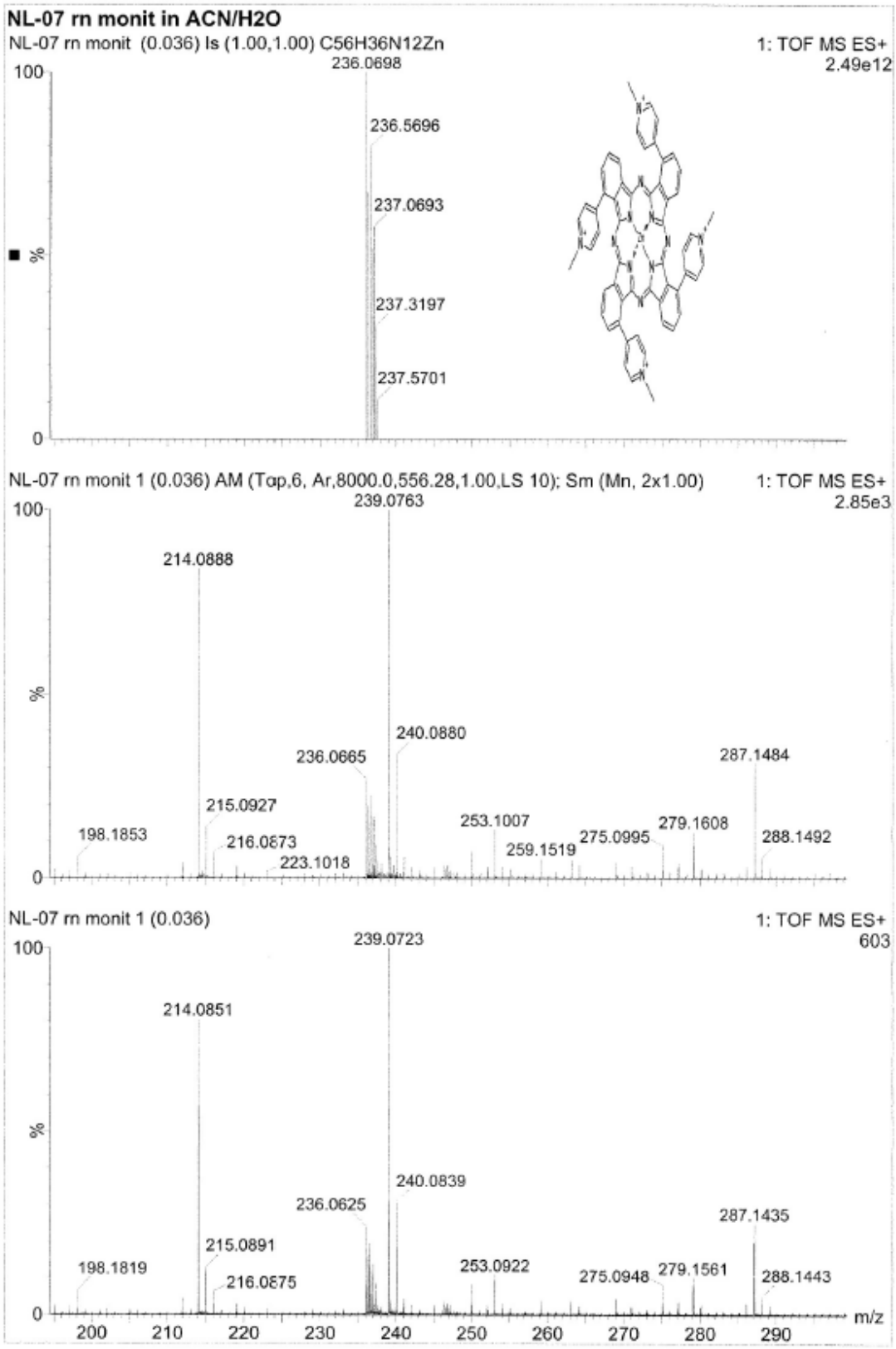
APPENDICES

Appendix 1. Experiments with the synthesis of 3-(pyridine-4-yl)phthalonitrile

TABLE 7. Experiments with the synthesis method of 3-(pyridine-4-yl)phthalonitrile

NL-15	New catalyst Same amount of catalyst as in the original method Temperature of the reaction 100 ºC Reaction time 1 hour
NL-16	New catalyst that was filtered and left at room temperature over-night to "age" Same amount of catalyst as in the original method Temperature of the reaction 90 ºC Reaction time 1 hour
NL-17	Palladium (II) chloride and triphenyl phosphine oxide as catalyst instead of tetrakis(triphenylphosphine)palladium 2 mole percentage of catalyst Temperature of the reaction 90 ºC Reaction time 1 hour
NL-25	Old catalyst 10 times less catalyst as in the original method Temperature of the reaction 90 ºC Reaction time 1 hour
NL-26	New catalyst 10 times less catalyst as in the original method Temperature of the reaction 100 ºC Reaction time 1 hour

Appendix 2. Example of the results of the mass spectrometer analysis



PICTURE 4. Example of the results of the mass spectrometer analysis of NL-07