CHALMERS
UNIVERSITY OF TECHNOLOGY

Design and Synthesis of DNA-binding Cyanine Dyes

Chemical Modification of BOXTO

Master's thesis within the Materials Chemistry master's programme

VICTOR BJÄRKNEMYR

CHALMERS UNIVERSITY OF TECHNOLOGY
Department of Chemistry and Chemical Engineering
Gothenburg, Sweden, 2017

Master's Thesis 2017

Design and Synthesis of DNA-binding Cyanine Dyes

Chemical Modification of BOXTO

Victor Bjärknemyr

CHALMERS
UNIVERSITY OF TECHNOLOGY

Supervisors
Filip Nylander
Karin Sahlin

Examiner
Prof. Gunnar Westman

CHALMERS UNIVERSITY OF TECHNOLOGY
Department of Chemistry and Chemical Engineering
Gothenburg, Sweden, 2017

Design and Synthesis of DNA-binding Cyanine Dyes
Chemical Modification of BOXTO

Victor Bjärknemyr

Department of Chemistry and Chemical Engineering
Chalmers University of Technology
SE-412 96 Göteborg, Sweden
Phone: +46 (0)31-772 10 00

Cover: Picture of the synthesized chemical analog to BOXTO produced within this project.

Printed at the department of Chemistry and Chemical Engineering.
Chalmers University of Technology, Gothenburg, Sweden 2017

Design and Synthesis of DNA-binding Cyanine Dyes

Chemical Modification of BOXTO

Victor Bjärknemyr

Department of Chemistry and Chemical Engineering
Chalmers University of Technology

Abstract.

Fluorescent DNA-binding cyanine dyes are of great use as DNA-probes in quantitative PCR, making it possible to observe the increase of DNA in real-time instead of after the PCR process is completed. However, the dyes commercially available at present are still far from perfect and there is a lot of room for improvement, both in terms of properties and cost of the dyes. A dye which in the past have shown promising DNA-binding and fluorescent is a cyanine dye called BOXTO. In this project a chemical analog to the already existing BOXTO has been synthesized via synthesis of a benzothiazolium salt and a quinolinium salt followed by a condensation of these into the final dye. The syntheses have been designed with a potential scale up in mind and all structures were confirmed with NMR spectroscopy measurements.

Keywords: Cyanine, qPCR, organic synthesis, fluorescent, DNA-binding, NMR spectroscopy.

Acknowledgements.

First and foremost I would like to express my deepest gratitude to my examiner and main supervisor Gunnar Westman who has been extremely helpful and supportive during the duration of the project. A huge thanks also to my supervisors Filip Nylander and Karin Sahlin for bearing with me frequently asking them to help me with various tasks. Last, but not least, I would like to thank the rest of the students in “The Westman Group” for excellent company and many laughs during our meetings and coffee breaks.

Victor Bjärknemyr

Contents

1 Introduction.

DNA-binding molecules have always been of great interest due to their possible use in a variety of different research fields, including cell biology, biomedicine, pharmaceutics or environmental science [1]. Cyanine dyes is a family of molecules that have proven to be especially useful in quantitative polymerase chain reaction (qPCR) and in vivo cancer imaging, where they can seek out and attach to tumor cells with high precision [1,2]. Their usefulness is thanks to their ability to bind to double stranded DNA, either via intercalation between the base pairs in the helix or via groove binding, where they bind to either the minor or major groove of the helix. When they bind to DNA their fluorescence increases significantly compared to when they are free in solution, where they are basically non-fluorescent [3]. The increase in fluorescence is caused by the conformational change that the dye undergoes when it binds to DNA. The exact nature of this conformational change is not known but it is believed to be related to a restriction in rotation about the methine bridge between the two moieties of the compound [4, 5].

In an article by H. J. Karlsson et al. a series of novel asymmetrical cyanine dyes have been synthesized that, instead of intercalating in DNA, binds to the minor grooves whilst maintaining the high fluorescence and absorption at long wavelengths typical for intercalating cyanine dyes [4]. This means that background scattering is less of a problem since this usually occur near the UV-region, where traditional groove-binding dyes usually absorbs. It is also believed that, unlike intercalating dyes, these groove-binding dyes doesn't lengthen the DNA-helix significantly, which might be of importance since lengthening of the DNA-helix by intercalation affects properties such as stiffness and hydrophobicity. [4, 6]

Of the different cyanine dyes synthesized by Karlsson et al. the one which showed the most promising properties was the benzoxazole-based dye named BOXTO. It showed a high degree of minor groove binding as well as high fluorescence quantum yield.

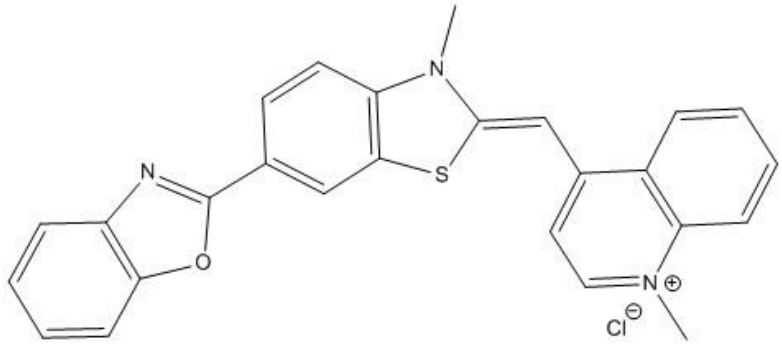


Figure 1: The cyanine named BOXTO synthesized by Karlsson et al.

It is still, however, not entirely known what it is that affects these properties of the dyes and whether potential modifications to them would improve or worsen their properties are highly speculative.

1.1 Aim of study.

The goal of this project is to synthesize chemical analogs to the cyanine dye BOXTO and compare their properties to current dyes. By doing this, the hope is to extend the knowledge of what parts in these dyes have a positive and/or negative impact on the dyes' properties. It is also desired to attempt optimization of the route of synthesis as well as making it viable for large scale synthesis by simplifying the reactions and purification steps as much as possible.

2 Theory.

2.1 Cyanine dyes.

Cyanine dye is the non-systematic name of a family of fluorescent dyes with excellent photophysical properties such as high fluorescence quantum yield, emission at long wave lengths and high photostability. In general, they are composed of two moieties, often containing nitrogen and/or oxygen, connected by a polymethine bridge which forms a conjugation between them. Depending on their structure, cyanine dyes can be divided into three different types: streptocyanines (or open chain cyanines), hemicyanines and closed chain cyanines. [7]

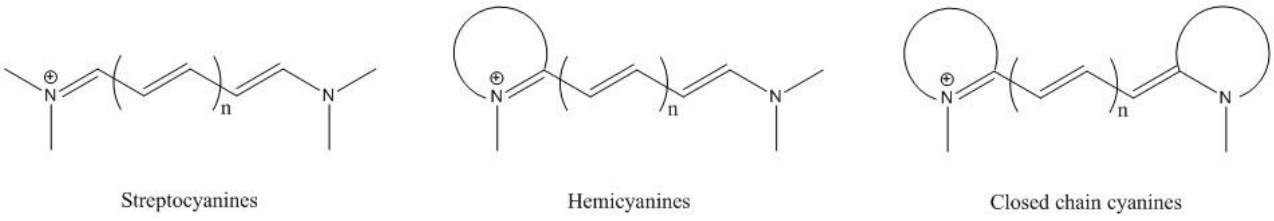


Figure 2: Three different types of cyanine dyes.

There are several areas of application for these types of dyes. Historically, they've been used mostly within photography but they have recently started to be used as fluorescent probes in areas using biomolecular labeling, such as PCR, in vivo imaging, genetic analysis and so forth. [7]

2.2 Fluorescence

Fluorescence is a type of photoluminescence which occurs when an orbital electron of a molecule is excited, by absorbing some sort of energy, and then relaxes to its ground state emitting energy in the form of photons. These emitted photons usually have a shorter wavelength than the absorbed light, meaning they have lower energy. Two important terms when talking about fluorescence are the fluorescence lifetime and the quantum yield of the fluorophore. The fluorescence lifetime is the average time the fluorophore spends in its excited state, usually around 10 nanoseconds, and the quantum yield is the number of emitted photons relative to the number of absorbed photons, meaning it can have a maximum value of 1 and a minimum value of 0. [8]

2.3 Real-Time Polymerase Chain Reaction.

Real-time PCR, also called quantitative PCR or qPCR, is a method commonly used in the field of biochemistry to reproduce strands of DNA or RNA in larger quantities. The method is very useful due to the fact that real-time PCR relatively fast since the method doesn't require any extra efforts when the amplification is completed. In addition to this, the method also provides a high sensitivity, due to detection with chemical probes instead of size analysis with gels, good reproducibility and high dynamic quantification. [5, 9]

There are a few different methods of qPCR that can be used, but the cheapest and simplest one is based on mixing the PCR-mixture with a DNA-binding fluorescent dye [5, 9]. The dye binds to double stranded DNA in one of two different ways: either via intercalation into the DNA or via covalent bonding to the minor groove of the DNA [4, 5]. When these dyes bind to DNA they undergo a conformational change, resulting in a large increase in fluorescence of a specific wavelength. This means that with each cycle of replication, the level of fluorescence will increase exponentially until it eventually reaches a plateau where the net synthesis is approximately zero. This eventual decrease in net synthesis happens due to the increasing chance that amplicons re-associate as the concentration of product increases [5]. The fluorescence is usually plotted on a logarithmic scale against the number of PCR-cycles and to remove background noise a fluorescence threshold is set in order to only measure DNA-based fluorescence. The number of cycles it takes for the level of fluorescence to reach the threshold is usually called the "quantification point" (C_q) [5, 10].

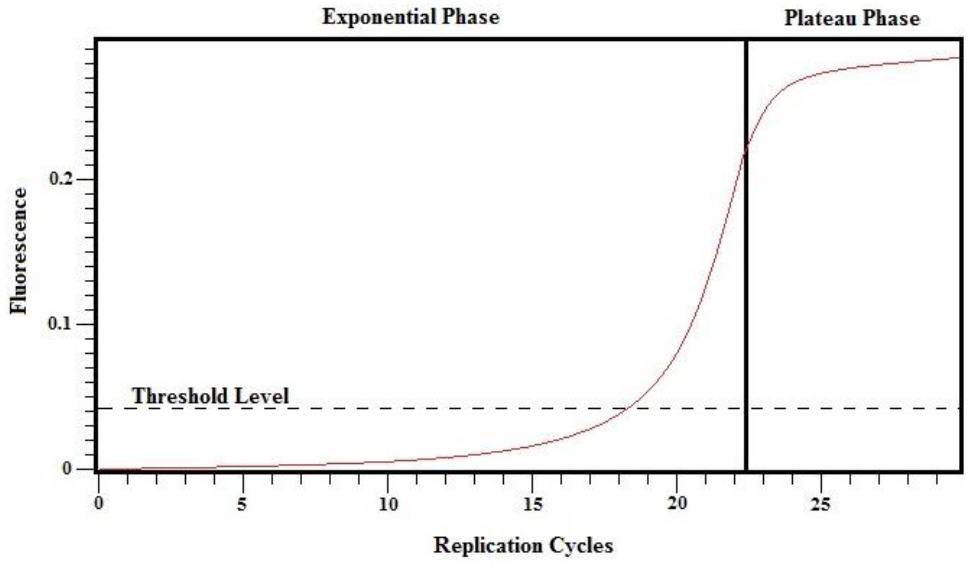


Figure 3: qPCR-plot of fluorescence against the number of replication cycles.

2.4 Modes of DNA-binding.

There are two major ways for molecules to bind to DNA which are relevant for this project: Groove binding, where molecules bind to either the minor or major grooves of the double stranded DNA helix, and intercalation, where molecules are sandwiched in between the base pairs of the DNA helix. [6, 11]

2.4.1 Groove binding

In general, a DNA-binding molecule binds to either the minor grooves or to the major grooves of the double stranded DNA-helix (more commonly the minor groove). Which one it binds to is largely size dependent as smaller DNA-binding molecules tend to bind either in or via the minor groove while larger molecules such as proteins or oligonucleotides tend to target the major groove. The minor groove is also better suited for smaller DNA-binding molecules since many of them are cationic and fairly flat, which fits well with the minor grooves' size, hydrophobicity and electrostatic potential. [6, 11]

2.4.2 Intercalation

In intercalation, a molecule is sandwiched in between two adjacent base pairs in the DNA-helix. This interaction is stabilized by stacking π-π interactions as well as dipole-dipole interactions between the bases and the electron deficient planar aromatic ring system which is characteristic for these intercalating molecules [6, 11]. As to be expected, this mode of binding affects the properties of DNA as the intercalation lengthens the DNA, leading to stiffening of the helix as well as a decrease in twisting between the base pair layers, which in turn leads to changes of the DNA's hydrodynamic properties which might be undesirable for certain studies [4, 6, 11]. These changes to the DNA are usually fully reversible though, as long as the structure of the helix is not permanently damaged when the intercalator is removed.

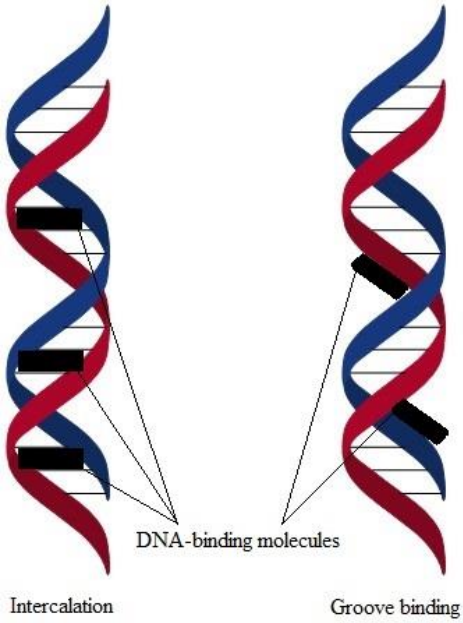


Figure 4: Illustration of two modes of DNA-binding: intercalation and groove binding.

3 Experimental.

3.1 Chemicals and Instruments.

For NMR-measurements, a Varian 400 MHz NMR system (Varian, US) was used. All water used was de-ionized. The rotary evaporator used was of the model Büchi rotavapor RE-111.
The following chemicals were used as reagents or solvents in the syntheses without further purification or drying. The chemicals used as reagents were: 4-aminobenzoic acid (99%, Sigma-Aldrich), 2-aminophenol (99%, Sigma-Aldrich), Ammonium hydroxide (~25%, Honeywell Fluka), Sodium perborate monohydrate (powder, 20-100 mesh,Sigma-Aldrich), Potassium bromide (99%, Sigma-Aldrich), Potassium o-ethyl dithiocarbonate (produced in-house), Hydrochloric acid (1M, diluted in-house), Chloroform (99.0-99.4%, Sigma-Aldrich), Iodomethane (≥99%, Sigma-Aldrich), Potassium carbonate (min. 99%, Scharlau), Triethylamine (≥99.5%, Sigma-Aldrich), Allyl bromide (99%, Sigma-Aldrich), 1,4-Dimethylquinolinium tosylate (produced in-house), Methyl Tosylate (98%, Sigma-Aldrich), 4-Methylquinoline (≥99%, Sigma-Aldrich), 1,3-Dibromopropane (99%, Sigma-Aldrich).

The chemicals used as solvents were: Polyphosphoric acid (Sigma-Aldrich), Ethanol (99.7%, Sigma-Aldrich), Acetic acid (≥99.8%, Sigma-Aldrich), Dimethyl formamide (>99.8%, Honeywell Fluka), Dichloromethane (99.98%, Thermo Fisher Scientific), Acetonitrile (99.99%, Thermo Fisher Scientific), Acetone (99.98%, Thermo Fisher Scientific), Diethyl ether (99.98%, Thermo Fisher Scientific).

Solvents used for NMR were: Chloroform-d (99.8 Atom% D, Sigma-Aldrich), Dimethyl sulfoxide-d_6 (99.5 Atom% D, Sigma-Aldrich), Methanol-d_4 (99.8 Atom% D, Sigma-Aldrich).

3.2 Laboratory methods.

Several common laboratory methods were used during this project. The most important of these were: rotary evaporation, recrystallization and nuclear magnetic resonance, which will be described briefly below.

3.2.1 Rotary evaporation.

Rotary evaporation is a very common method used in chemistry to remove solvents from samples. The flask with the sample is attached to a rotary evaporator with a cooler, a vessel for collecting the solvent, a rotor, a heated water bath and a vacuum pump attached to it. When the vacuum pump is activated it lowers the pressure in the closed system, reducing the boiling point of the solvents in the sample which makes it possible to evaporate at a lower temperature than normally would be required. The sample is then lowered into the water bath where it is heated during rotation until the solvent starts to evaporate. The vapor is led away from the sample flask and condensed by the cooler, whereafter the condensate is collected by the collection vessel.

3.2.2 Recrystallization.

Recrystallization is a separation technique which is very commonly used to remove impurities and defects from chemical products. The idea is that both the product and the impurities are dissolved in the smallest volume possible of a single or mixture of hot solvent(s). The mixture is then allowed to cool slowly which gradually lowers the solubility of compounds in the solution, causing them to slowly crystallize and precipitate. Ideally, the solid crystals are then pure product and all impurities are left in solution which means the solid crystals can be collected by filtration and the filtrate can be discarded. If the product still contains impurities, the recrystallization can be repeated until an acceptable purity is achieved.

3.2.3 Nuclear magnetic resonance spectroscopy (NMR-spectroscopy).

Nuclear magnetic resonance, or NMR, is one of, if not the most important spectroscopic analysis methods for organic chemists. It gives information about the number of atoms that is being studied (most commonly hydrogen or carbon) as well as information about the near chemical environment of each distinct type of nuclei which is important in either determining the structure of an unknown substance or to confirm the structure of a product. The principle is that different atomic nuclei have different spin states which are not equivalent in energy when a magnetic field is applied since the magnetic moment will be aligned either with or against the applied field. The applied magnetic field also causes the nucleus to resonate at a frequency which is characteristic for each isotope. This is important since the chemical environment of the nucleus affects the frequency at which this resonance happens, meaning that it's possible to tell similar nuclei apart. The frequencies at which these resonances are happening are measured as chemical shifts in parts per million (ppm) in relation to a reference sample of tetramethylsilane (TMS) and is directly proportional to the strength of the applied magnetic field and how far they are shifted from the reference (See equation 1). [12]

$$\delta(ppm) = \frac{Chemical\ shift\ in\ Hz}{Spectrometer\ frequency\ in\ MHz} \quad \text{(Equation 1)}$$

3.3 Synthesis.

The synthesis of the cyanine dye can be divided into different parts: the route of synthesis of the benzothiazolium salt, the synthesis of the quinolinium salt and the condensation of the two salts into the final cyanine dye. All reactions were performed under air at atmospheric pressure.

3.3.1 Synthesis of benzothiazolium salt 7.

The benzothiazolium salt 6-(Benzoxazol-2-yl)-3-methyl-2-thiomethyl-benzothiazolium tosylate **(7)** was synthesized in five consecutive steps.

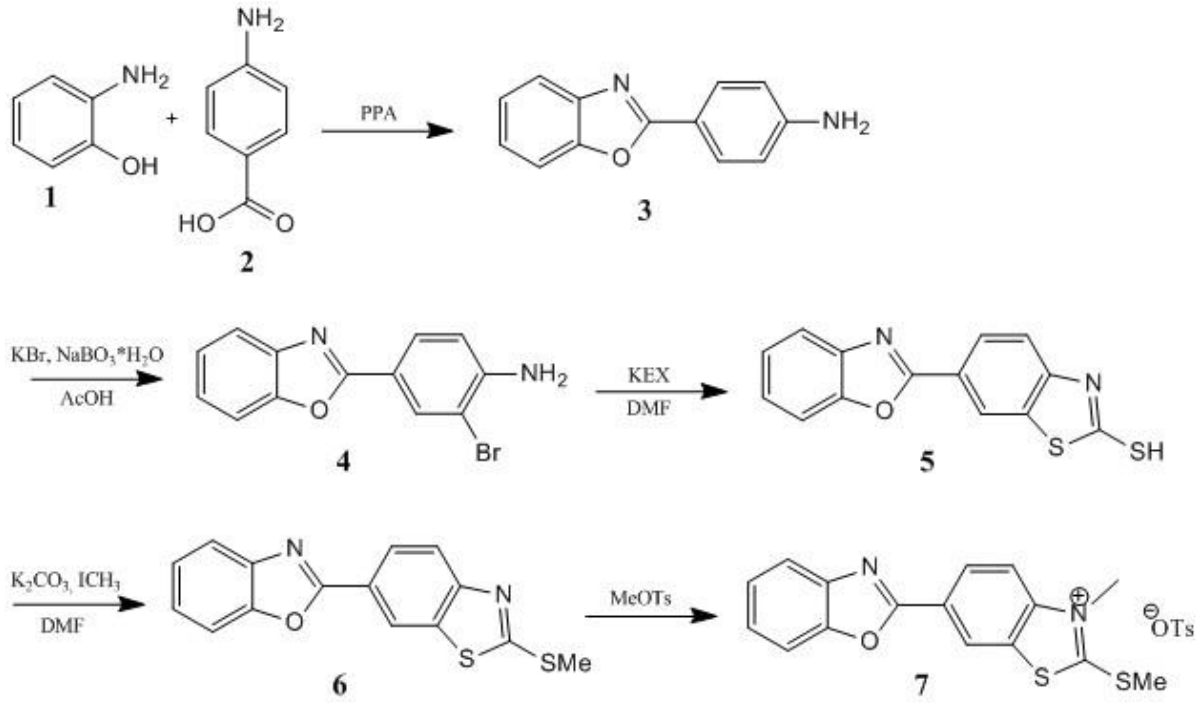


Figure 5: Route of synthesis for the benzothiazolium salt.

3.3.1.1 2-(4-aminophenyl)benzoxazole 3.

2-aminophenol **(1)** (5.1970g, 47,62mmol) and 4-aminobenzoic acid **(2)** (6.5323g, 47.63mmol) were added in portions to 50ml polyphosphoric acid during heating and stirring. The mixture was stirred at 205°C for 4 hours. After cooling slightly the solution was poured into ice water while being stirred vigorously and the pH was adjusted to ~8 using 25% ammonium hydroxide. The pink precipitate formed was collected by filtration and washed with water. The filter cake was dissolved in ethanol and evaporated in vacuo to remove water and residual solvents. (7.877g, 78.8% yield). ^{1}H NMR ($CDCl_3$): δ (ppm) 4.05 (2H, s, Ar-NH_2), 6.77 (2H, d, J=8.8, Ar-H), 7.30 (2H, m, Ar-H), 7.52 (1H, d, J=7.6, Ar-H), 7.70 (1H, d, J=7.6, Ar-H), 8.06 (2H, d, J=8.8, Ar-H). [13, 14]

3.3.1.2 2-(4-amino-3-bromophenyl)benzoxazole 4.

Sodium perborate monohydrate (2.6158g, 26,21mmol) was added to a solution of potassium bromide (3.4010g, 28,58mmol) and 2-(4-aminophenyl)benzoxazole **(3)** (5.0052g, 23.81mmol) in 5ml acetic acid. The solution was left to stir at room temperature for 21 hours and was then quenched by the addition of ice water. The mixture was filtered and washed with water to afford the product as a brown powder (6.7059g, 97.4% yield). ^{1}H NMR ($CDCl_3$): δ (ppm) 4.50 (2H, s, Ar-NH_2), 6.84 (1H, d, J=8.4, Ar-H), 7.31 (2H, m, Ar-H), 7.53 (1H, d, J=7.2, Ar-H), 7.71 (1H, d, J=7.2, Ar-H), 7.99 (1H, dd, J_1=8.4, J_2=1.94), 8.34 (1H, ds, J=1.94, Ar-H). [4]

3.3.1.3 6-(Benzoxazol-2-yl)-2-mercaptobenzothiazole 5.

Potassium o-ethyl dithiocarbonate (2.4462g, 15.26mmol) was added to a solution of 2-(4-amino-3-bromophenyl)benzoxazole **(4)** (2.0058g, 6.94mmol) in 15ml anhydrous DMF. The solution was heated to 140°C and left to stir for 4 hours. After cooling, the mixture was diluted by addition of 15ml water and 1M HCl was added dropwise to initiate precipitation. The precipitate formed was collected via filtration and washed with chloroform to afford a bright orange-brown powder which was left to air dry (1.790g, 90.7% yield). ^{1}H NMR (DMSO-d_6): δ (ppm) 7.40 (2H, m, Ar-H), 7.46 (1H, d, J=8.6, Ar-H), 7.77 (2H, m, Ar-H), 8.19 (1H, dd, J_1=8.6, J_2=1.7, Ar-H), 8.54 (1H, sd, J=1.7, Ar-H). [4]

3.3.1.4 6-(Benzoxazol-2-yl)-2-thiomethylbenzothiazole 6.

Iodomethane (3.0203g, 21.52mmol) was added to a solution of 6-(Benzoxazol-2-yl)-2-mercapto-benzothiazole **(5)** (5.10g, 17.94mmol) and potassium carbonate (3.718g, 26.9mmol) in ~50ml anhydrous DMF. The solution was left to stir at room temperature for 2 hours whereafter 50ml of water was added. The precipitate formed was filtrated and washed with water before being recrystallized in ethanol. The mixture was then filtered again, washed with ethanol and left to air dry in order to afford the product as a yellow solid. (3.4293g, 64.1% yield). ^{1}H NMR ($CDCl_3$): δ (ppm) 2.83 (3H, s, S-CH_3), 7.36 (2H, m, Ar-H), 7.59 (1H, m, Ar-H), 7.78 (1H, m, Ar-H), 7.97 (1H, d, J=8.6, Ar-H), 8.31 (1H, dd, J_1=8.6, J_2=1.7, Ar-H), 8.68 (1H, d, J=1.7, Ar-H). [4]

3.3.1.5 6-(Benzoxazol-2-yl)-3-methyl-2-thiomethylbenzothiazolium tosylate 7.

Methyl tosylate (1.16g, 6.23mmol) was added dropwise to 6-(benzoxazole-2-yl)-2-thiomethylbenzothiazole **(6)** (0.6185g, 2.07mmol) dissolved in 1.5ml acetonitrile while stirring. The solution was then heated to 100°C and left to reflux for 17 hours. This resulted in thick, brown-orange paste which was recrystallized in acetonitrile, filtered and left to air dry to afford the pure product as a light brown powder (0.3218g, 32.0% yield). ^{1}H NMR (DMSO-d_6): δ (ppm) 2.26 (3H, s, Ar-CH_3), 3.16 (3H, s, S-CH_3), 4.13 (3H, s, N-CH_3), 7.08 (2H, d, J=7.8, Ar-H), 7.47 (4H, m, Ar-H), 7.85 (2H, t, J_1=7.8, J_2=8.1, Ar-H), 8.38 (1H, d, J=8.7, Ar-H), 8.57 (1H, dd, J_1=8.9, J_2=1.7, Ar-H), 9.25 (1H, s, Ar-H). [4]

3.3.2 Synthesis of 1-allyl-4-methylquinolinium bromide 10.

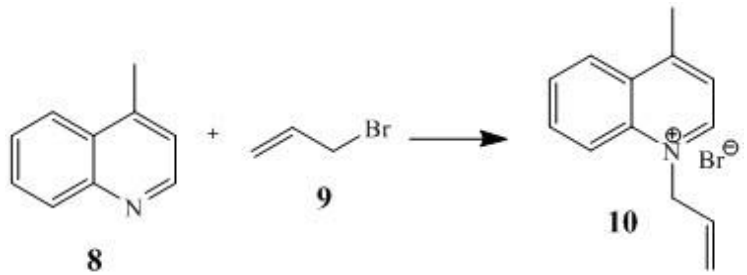


Figure 6: Synthesis of quinolinium salt from lepidine and allyl bromide.

4-methylquinoline **(8)** (1.083g, 7.01mmol) and allyl bromide **(9)** (1.000g, 8.27mmol) were mixed and refluxed at 80°C while stirring for 4 hours. The dark blue product was dissolved in acetonitrile

and filtered. The filtrate was evaporated in vacuo and the quinolinium salt **(10)** was afforded as dark blue crystals (1.7317g, 93.5% yield). ^{1}H NMR ($CDCl_3$): δ (ppm) 3.00 (3H, s, Ar-CH_3), 5.36-5.41 (2H, m, N-CH_2), 6.04 (2H, d, J=5.6, C=CH_2), 6.14-6.21 (1H, m, –CH=), 7.95 (1H, t, J=7.8, Ar-H), 8.02 (1H, d, J=6, Ar-H), 8.15 (1H, t, J=8, Ar-H), 8.34 (1H, d, J=8.5, Ar-H), 8.42 (1H, d, J=9, Ar-H), 10.27 (1H, d, J=6, Ar-H).

3.3.3 Condensation of cyanine dye 4-[6-(Benzoxazol-2-yl-(3-methyl-)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-allyl-quinolinium bromide 11.

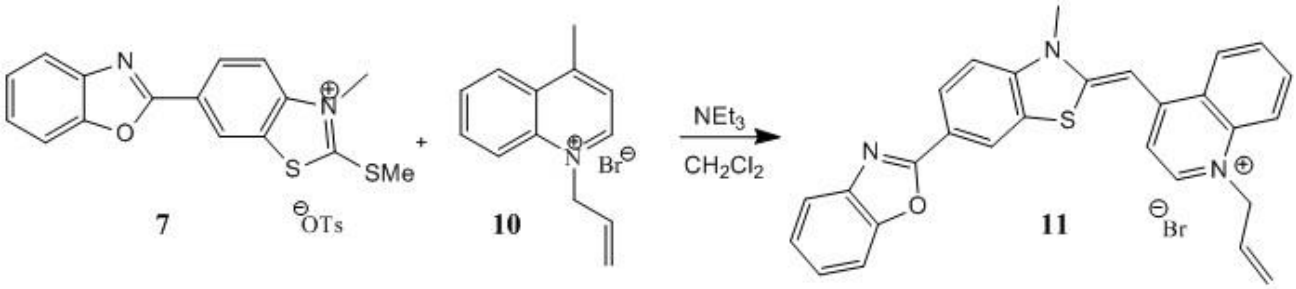


Figure 7: Condensation of benzothiazolium salt and quinolinium salt into cyanine dye.

Triethylamine (0.11ml, 0.8mmol) was added dropwise to a solution of the benzothiazolium salt **(7)** (0.0979g, 0.2mmol) and Allylquinolinium bromide **(10)** (0.0541g, 0.2mmol) in 5ml DCM after which the solution changed color from dark brown to deeply red. The solution was left to stir at room temperature for 20 hours. The solvent was evaporated in vacuo and the remaining product was recrystallized, first in a water/ethanol mixture (1:1 ratio) and then in acetonitrile and left in the fridge overnight to precipitate. The product was afforded as red crystals via filtration and washing with acetonitrile and was left to air dry (0.0484g, 45.3% yield). ^{1}H NMR (CD_3OD): δ (ppm) 3.95 (3H, s, N-CH_3), 5.21 (2H, d, J=5.26, N-CH_2), 5.25 (1H, dd, J_1=17.2, J_2=1.1, =CH), 5.42 (1H, dd, J_1=10.6, J_2=1.1, =CH), 6.17 (1H, m, –CH=), 6.74 (1H, s, –CH=), 7.24 (2H, m, Ar-H), 7.37 (1H, d, J=7.1, Ar-H), 7.47 (1H, m, Ar-H), 7.52 (1H, m, Ar-H), 7.69 (2H, m, Ar-H), 7.87 (1H, m, Ar-H), 7.93 (1H, dd, J_1=8.9, J_2=1.2, Ar-H), 8.25 (1H, dd, J_1=8.7, J_2=1.7), 8.46 (1H, d, 7.1, Ar-H), 8.51 (2H, m, Ar-H). [4]

4 Results.

4.1 Results.

In order to synthesize a modified version of the cyanine dye BOXTO, previously created by Karlsson et al., a series of organic syntheses were carried out [4]. Initially, another target molecule than the one finally achieved was designed and a plan of synthesis based on previously reported work was prepared. During the course of the project this initial plan was revised a series of times based on less than desirable results in some steps in the route of synthesis until the target was finally set on the cyanine dye **(11)** reported previously.

4.1.1 Initial goal.

Initially, the plan was to modify BOXTO with allyl, which theoretically then readily could be converted into a s-propyl ethanethioate group, and bromopropyl side chains (see fig. 8) as other reported cyanine dyes with these groups have proven to have good fluorescence quantum yield when bound to DNA in relation to when free in solution [15, 16, 17].

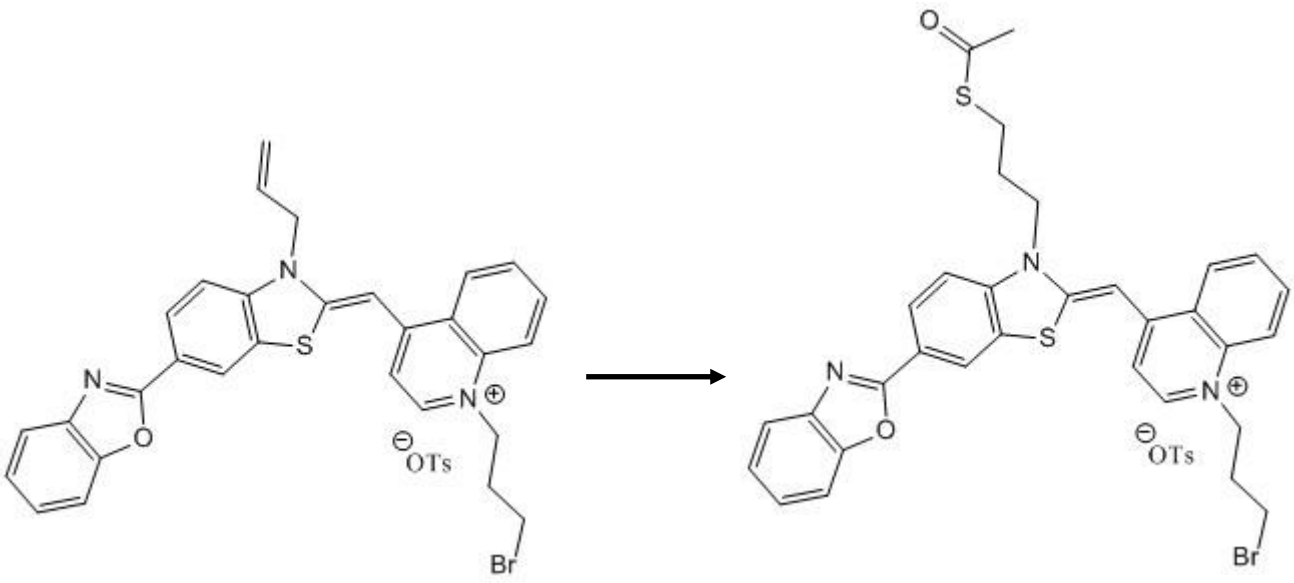


Figure 8: To the left is BOXTO modified with an allyl and a bromopropyl group. To the right is the same molecule with the allyl group converted into an s-propyl ethanethioate group.

This was supposed to be accomplished via a condensation reaction between a 1-(3-bromopropyl) lepidinium salt and the benzothiazolium salt **(7)** but with an allyl group instead of a methyl group on the benzothiazole nitrogen and with bromide as counter ion instead of tosylate. Despite several attempts however, neither the synthesis of the lepidinium salt nor the allylation of the benzothiazole were successful. Following this setback, the initial plan was abandoned and it was decided that it should be attempted to synthesize **(7)** and **(10)** instead and then try to combine these compounds into the cyanine dye **(11)**.

4.1.2 Synthesis of BOXTO analog.

4.1.2.1 Benzothiazolium salt (11)

The route of synthesis of the benzothiazolium salt **(11)** started with the condensation reaction of **(1)** and **(2)** in PPA into the aniline **(3)**. The reaction conditions were based on previously reported syntheses and resulted, after being quenched in ice water, basified with ammonium hydroxide, filtered and washed, in a yield of approximately 78.8% after purification with ethanol [13, 14]. The structure of the compound was verified with ^{1}H NMR in deuterated chloroform (see fig. 9, 10) and the data was consistent with previously reported compounds [4].

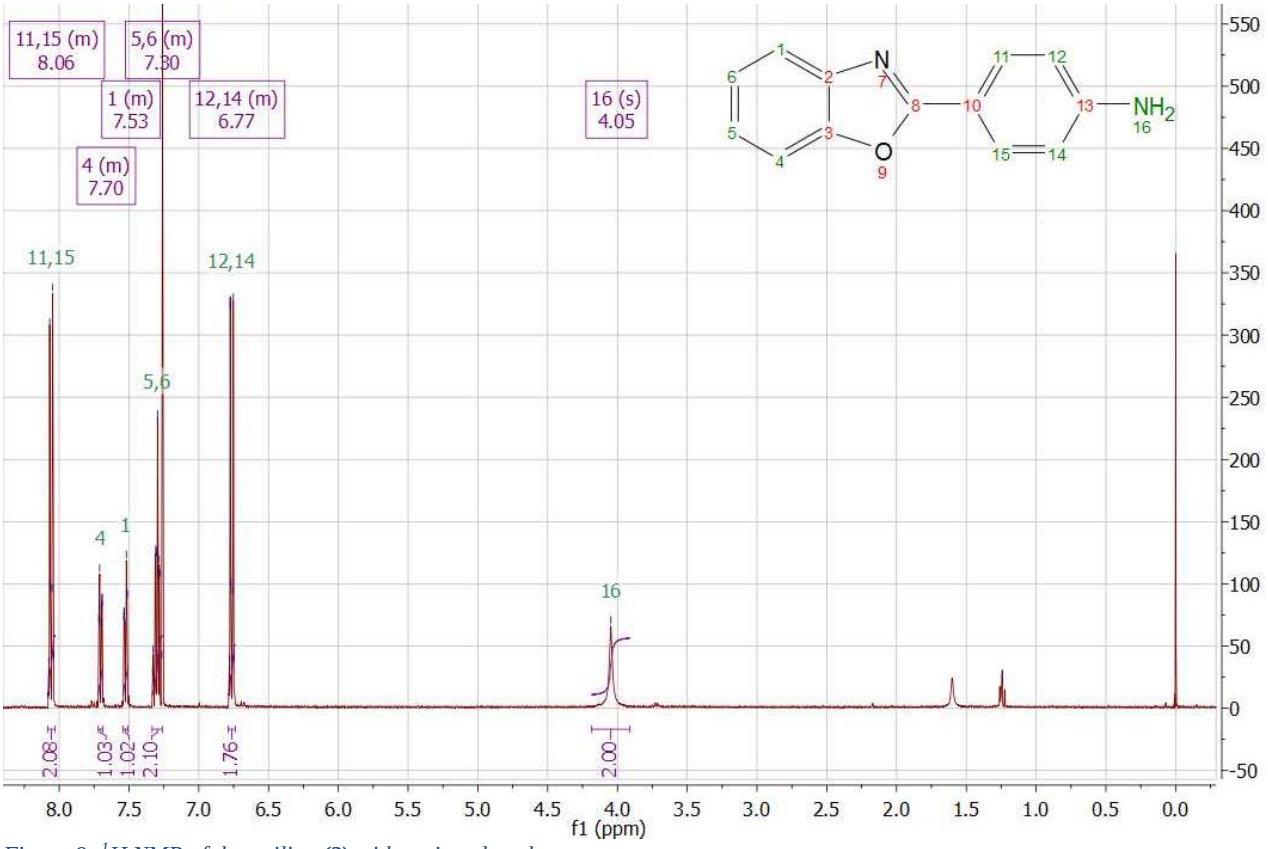


*Figure 9: ^{1}H NMR of the aniline **(3)** with assigned peaks.*

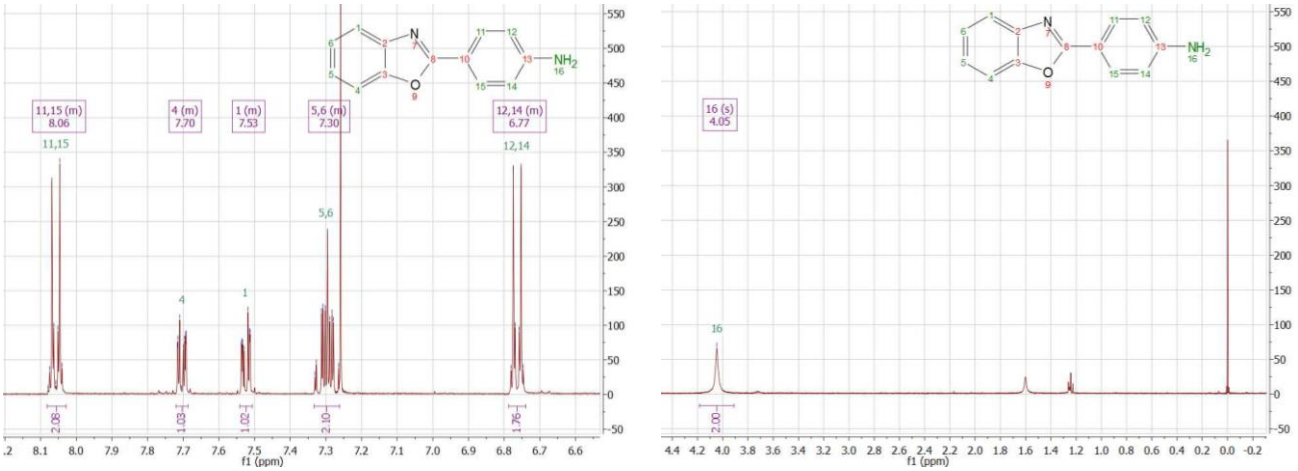


Figure 10: Magnification of the aromatic (left) and non-aromatic (right) parts of fig. 9.

The aniline was then brominated with potassium bromide and sodium perborate monohydrate in acetic acid, as previously described, before being quenched with ice water, filtered and washed to afford the bromoaniline **(4)** with a yield of approximately 97.4%**.** The structure was verified with ^{1}H NMR in deuterated chloroform (see fig. 11, 12) and the data received was consistent with previously prepared compounds. [4]

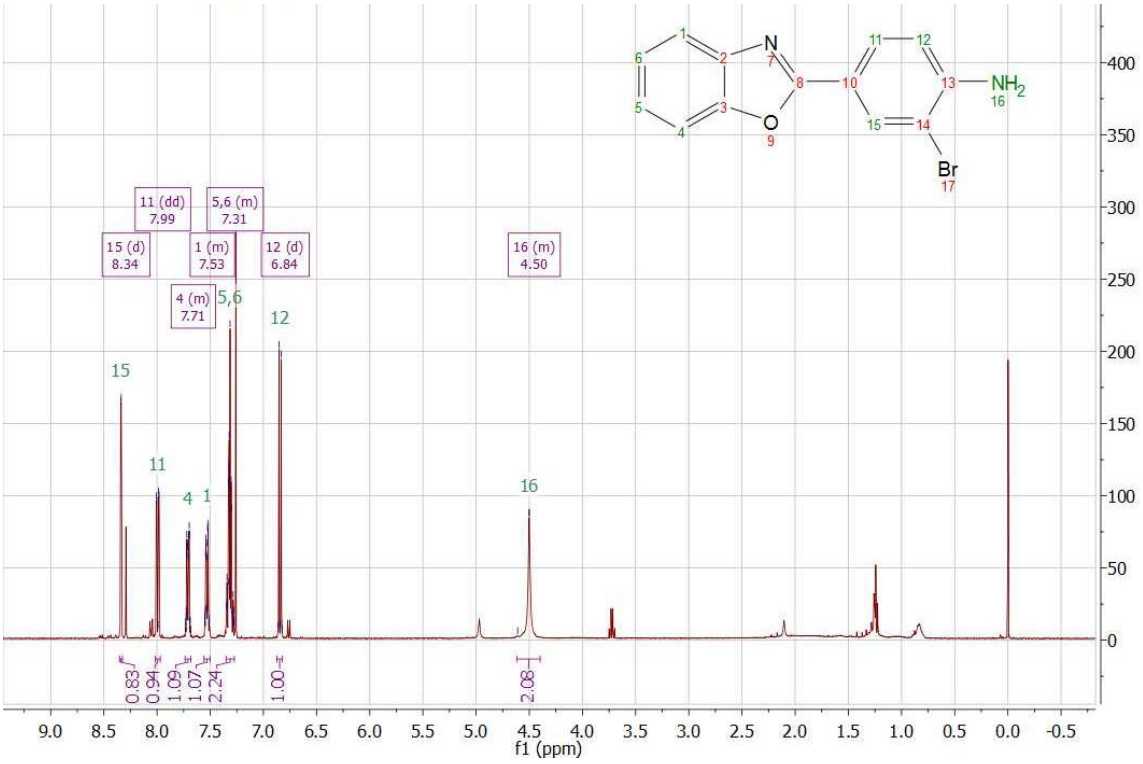


*Figure 11: ^{1}H NMR of the bromoaniline **(4)** with assigned peaks.*

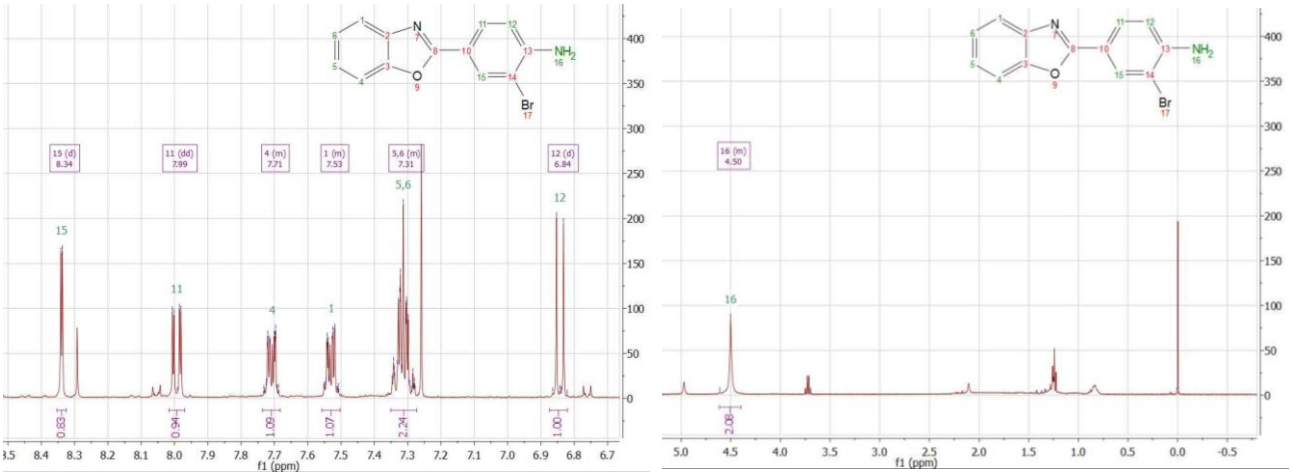


Figure 12: Magnification of the aromatic (left) and non-aromatic (right) parts of fig. 11.

In order to ring-close the bromoaniline **(4)** into the mercaptobenzothiazole **(5)**, it was reacted with potassium o-ethyl dithiocarbonate in DMF, in accordance with previously described methods, before being diluted with water, acidified with HCl, filtered and washed to afford the product in

approximately 90.7% yield. The structure was verified with ^{1}H NMR in deuterated DMSO (see fig. 13, 14) and the data received was consistent with previously prepared compounds. [4]

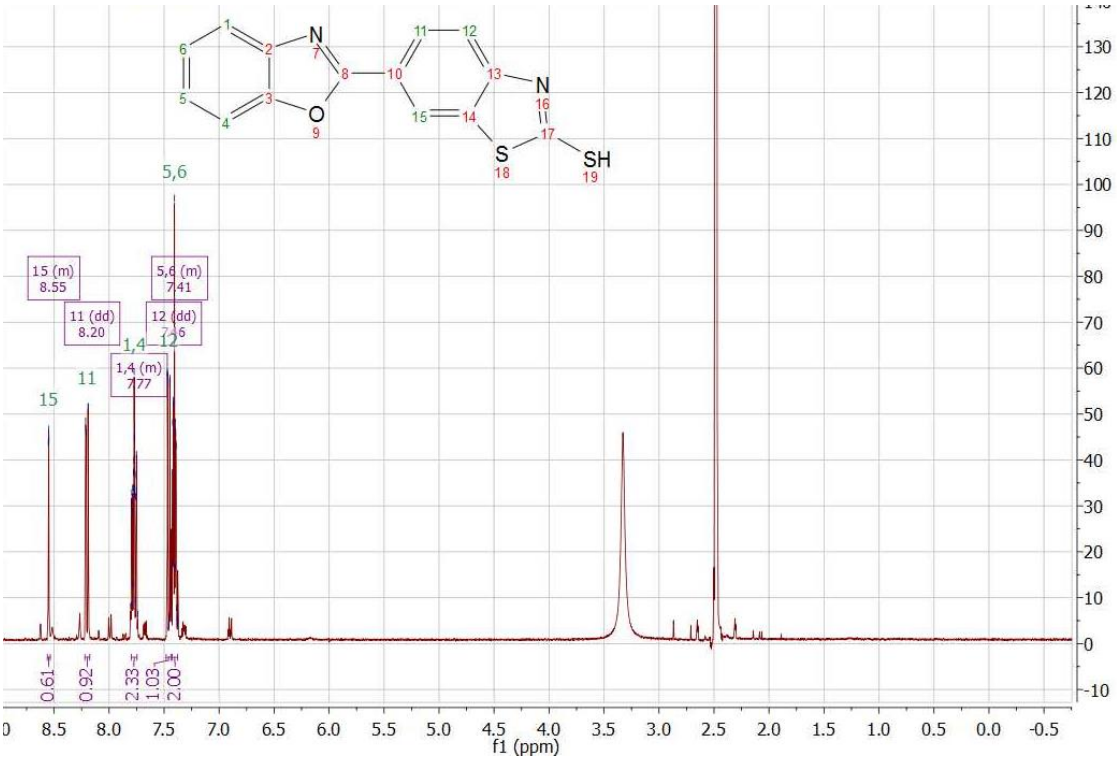


*Figure 13: ^{1}H NMR spectrum of the ring-closed mercaptobenzothiazole **(5)** in DMSO-d_6 with assigned peaks.*

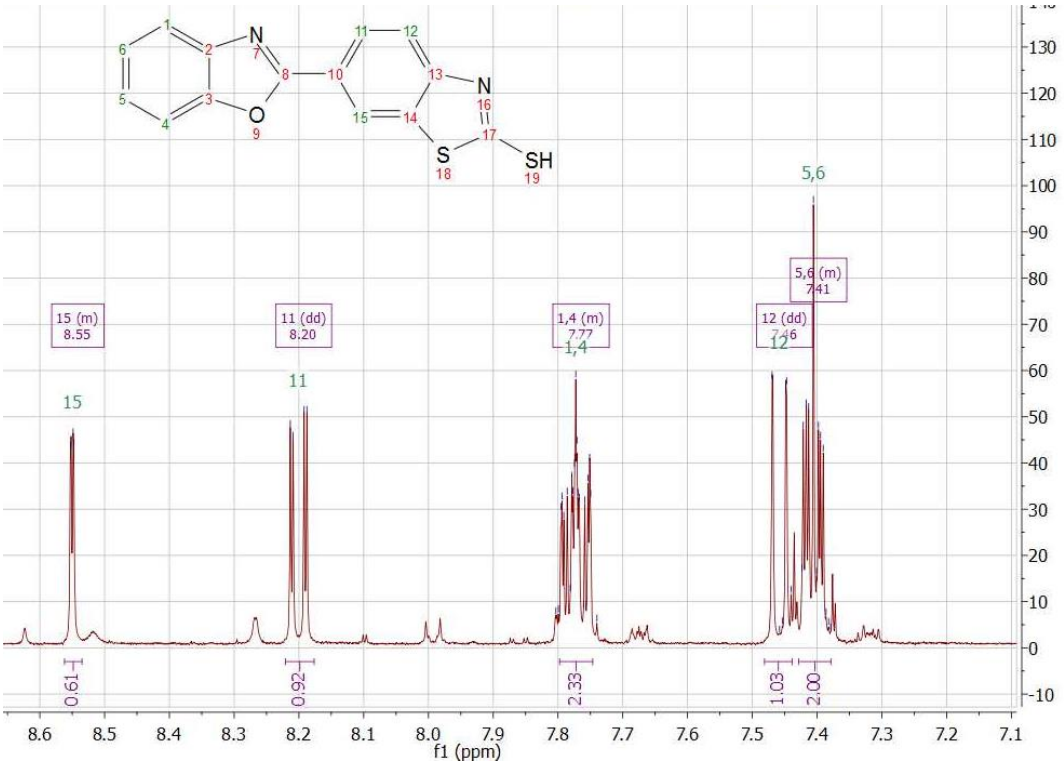


Figure 14: Magnification of the aromatic region from the NMR spectrum in fig. 13.

The mercaptobenzothiazole **(5)** was converted into the thiomethyl benzothiazole **(6)** via methylation with iodomethane and potassium carbonate in DMF in accordance with previously described methods. The mixture was diluted with water, filtered and washed before being recrystallized in ethanol due to impurities. The resulting mixture was then filtered and washed again to afford a pure product in approximately 64.1% yield. The structure of the product was confirmed with ^{1}H NMR in deuterated chloroform (see fig. 15, 16) and the data received from the spectra was consistent with previously determined compounds. [4]

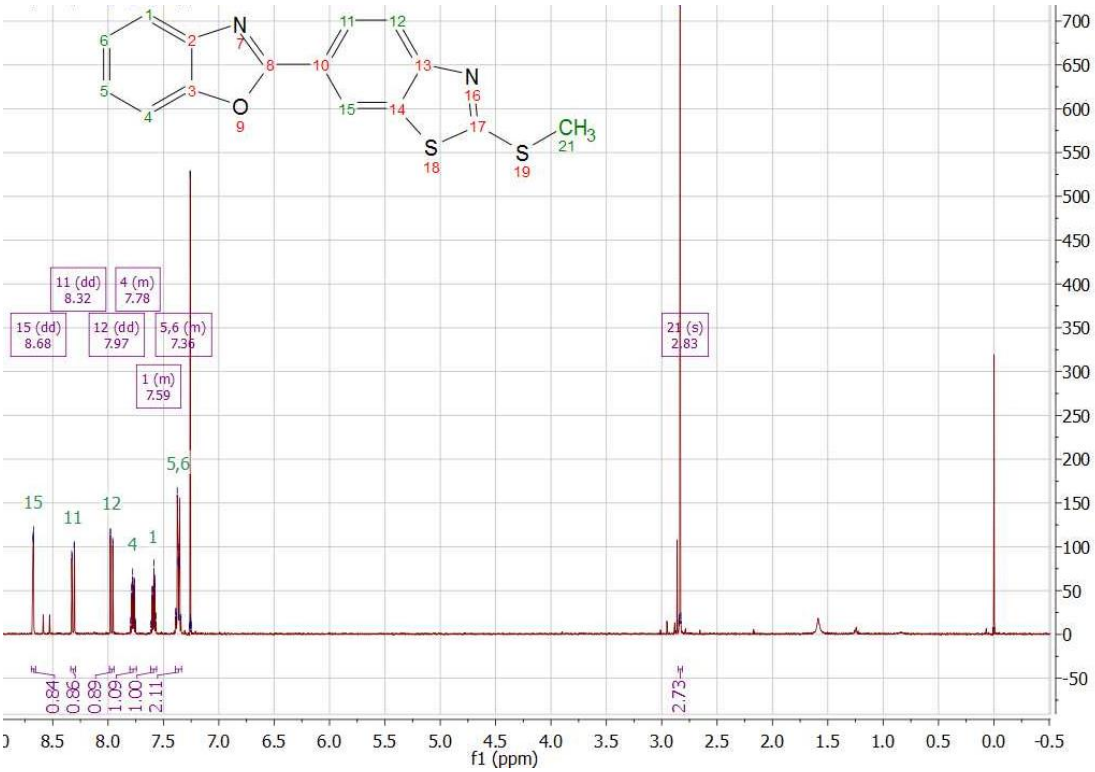


*Figure 15: ^{1}H NMR spectrum of the methylated thiomethyl benzothiazole **(6)** in $CDCl_3$ with assigned peaks.*

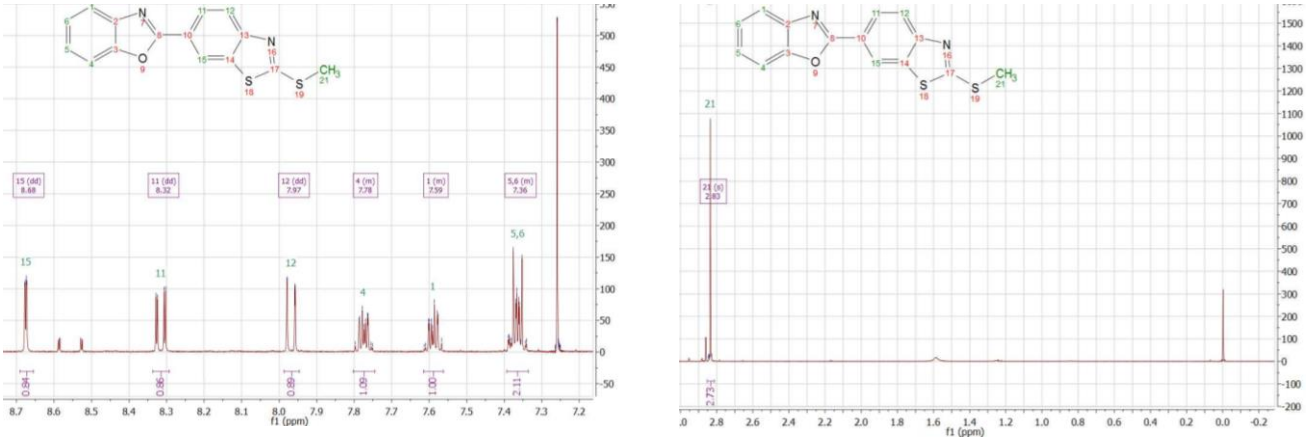


Figure 16: Magnification of the aromatic (left) and non-aromatic (right) region of the spectrum in fig. 15.

To obtain the final benzothiazolium salt **(7)**, the thiomethyl benzothiazole **(6)** was methylated with methyl tosylate in a small volume of acetonitrile. In order to remove impurities, the product was recrystallized in acetonitrile, the precipitate was collected via filtration and dried to afford a pure

product (apart from some residual water which was removed via further drying). The structure of the salt was confirmed with ^{1}H NMR in deuterated DMSO and the data received matched previously reported data of the same compound. [4]

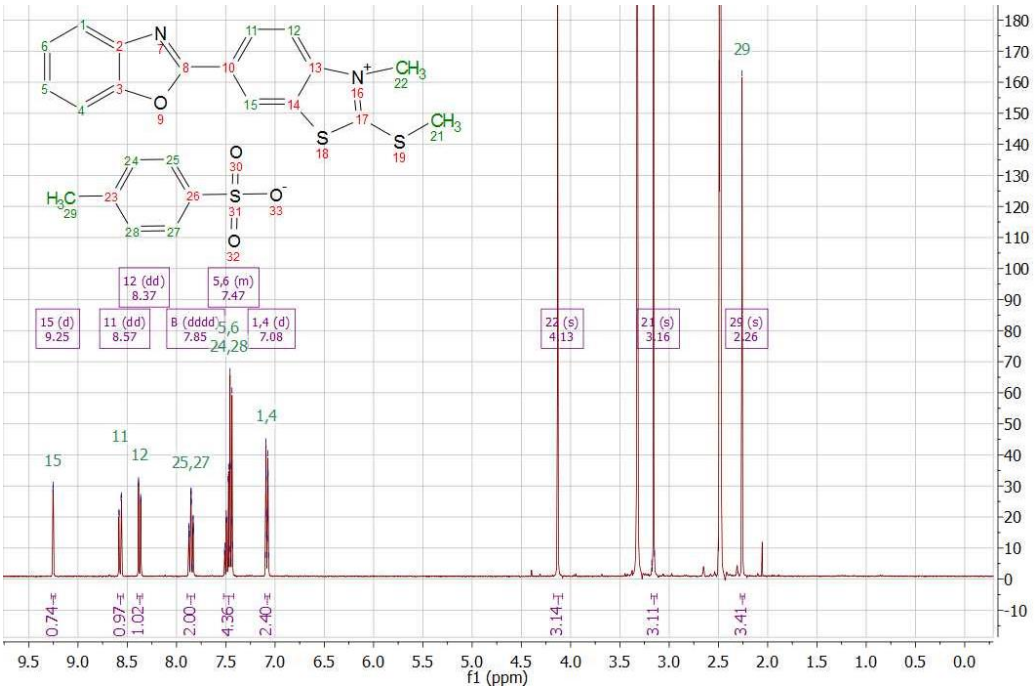


*Figure 17: ^{1}H NMR spectrum of the benzothiazolium salt **(7)** in DMSO-d_6 with assigned peaks.*

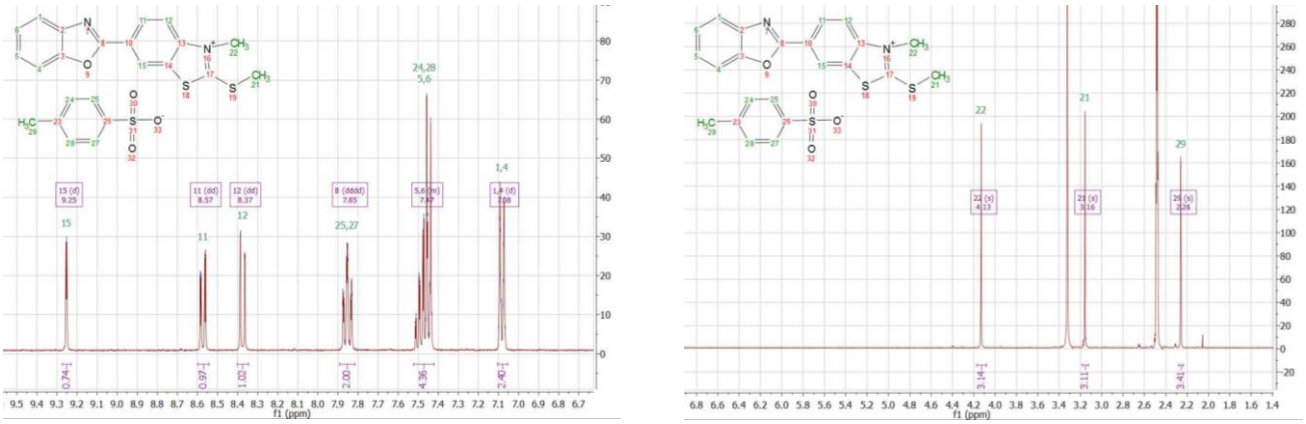


Figure 18: Magnification of the aromatic (left) and non-aromatic (right) region of the spectrum in fig. 17.

4.1.2.2 Synthesis of quinolinium salt.

The synthesis of the quinolinium salt **(10)** was carried out in a single step by simply refluxing allyl bromide with 4-methylquinoline. The salt was obtained as a dark blue solid with a yield of approximately 93.5%. The structure was then verified with ^{1}H NMR and ^{1}H COSY in deuterated chloroform as no data from previously reported compounds were found to compare with. All hydrogens could, however, be found in the spectra and assigned to their corresponding peaks (see fig. 18). The product also contained very small traces of starting material but was deemed pure enough to continue with as the impact this could have in the condensation of the cyanine dye would be negligible.

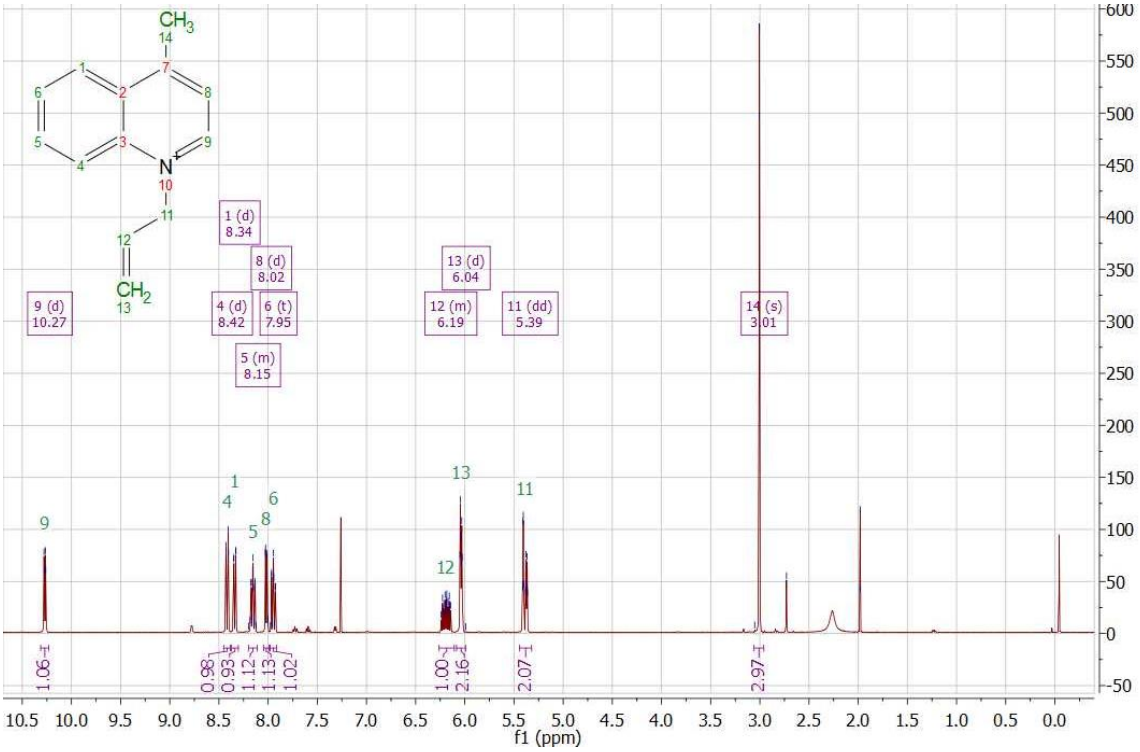


Figure 19: [1]H NMR spectra of allylated quinolinium salt (10) in $CDCl_3$ with assigned peaks. For COSY spectrum, see appendix A2.

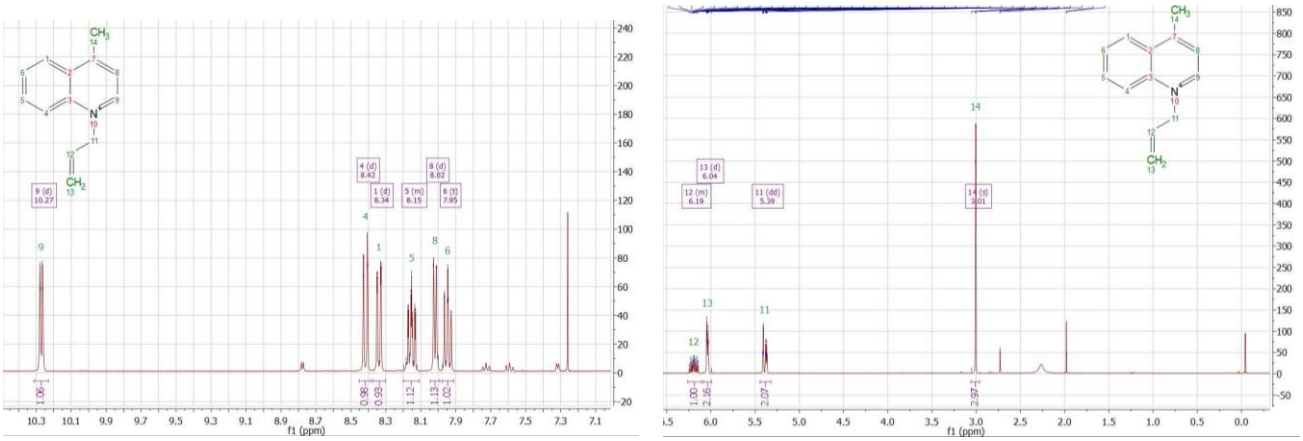


Figure 20: Magnification of the aromatic (left) and non-aromatic (right) region of the spectrum in fig. 19.

4.1.2.3 Cyanine dye condensation (11).

The BOXTO-analog was synthesized by dissolving the benzothiazolium salt **(7)** and the quinolinium salt **(10)** in dichloromethane and adding triethylamine, similarly to previously described methods [4]. After the solvent was evaporated the product it was observed that the sample contained impurities. The product was therefore dissolved in an ethanol/water mixture to attempt recrystallization. This resulted in no precipitation and the solvent was removed in vacuo in order to attempt recrystallization in acetonitrile instead. After cooling in the fridge overnight a precipitate had formed which was collected via filtration, giving the product as red crystals in approximately 45.3% yield. A ^{1}H NMR as well as a ^{1}H COSY was taken on the sample in deuterated methanol to confirm the

structure of the product (see fig. 20). The ^{1}H NMR spectrum showed signs of the sample containing a finished cyanine dye, but also residual solvents such as ethanol and acetonitrile as well as a lot of what was believed to be water and hydrogen. No relevant information could be retrieved from the ^{1}H COSY, leading to an inability to accurately assign the peaks of the ^{1}H NMR to their corresponding hydrogen.

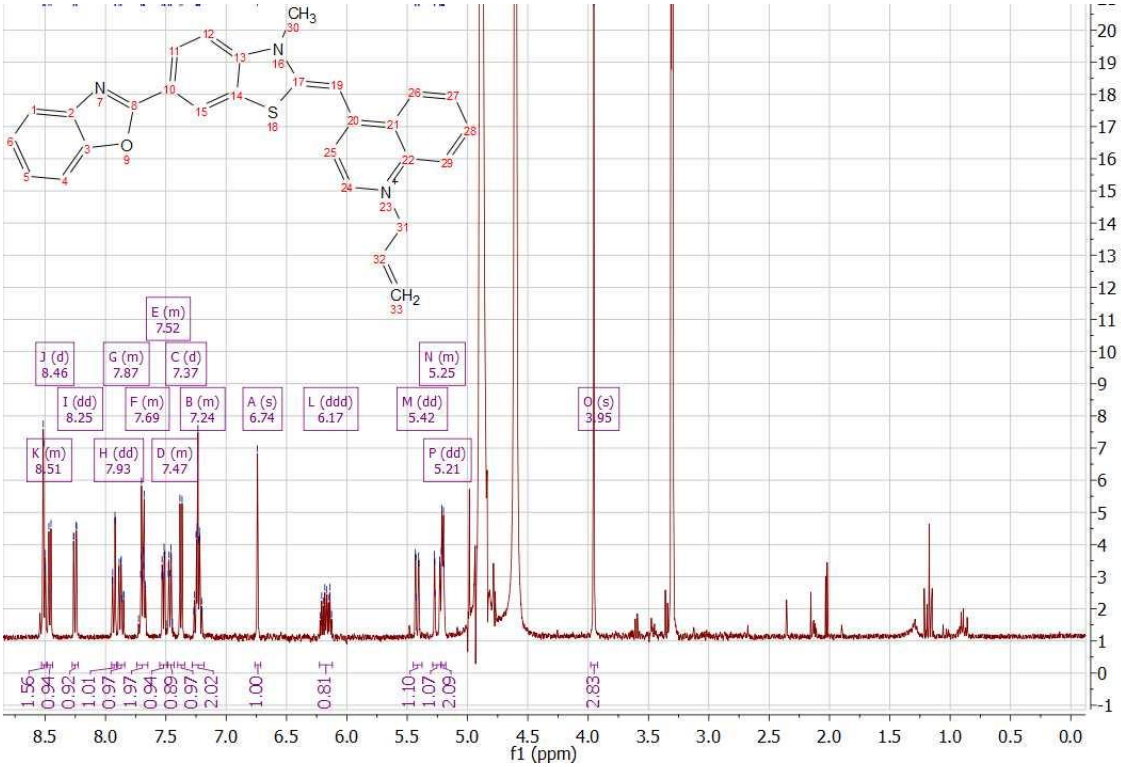


*Figure 21: ^{1}H NMR spectrum of the cyanine dye **(11)** in CD_3OD. For ^{1}H COSY of the same compound see appendix A2.*

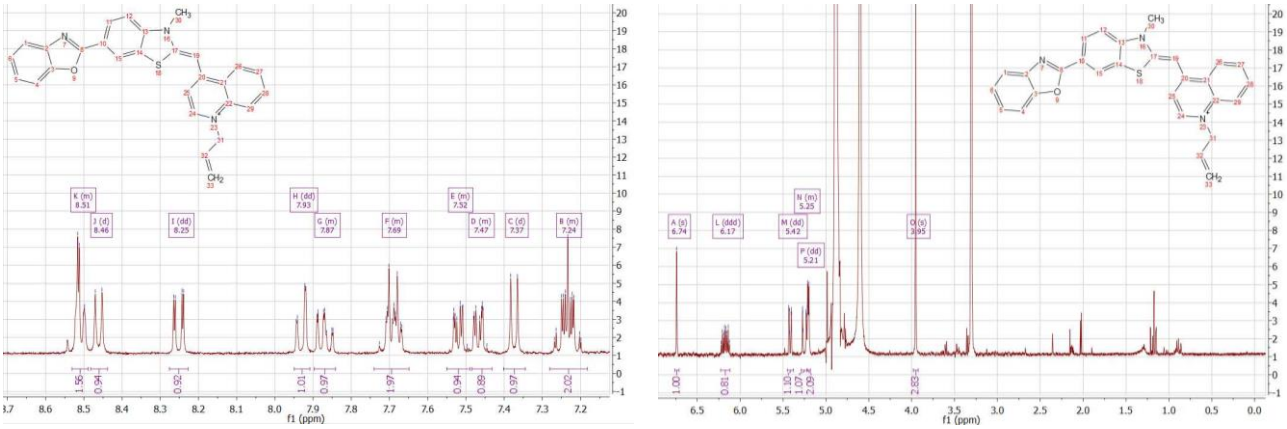


Figure 22: Magnification of the aromatic (left) and non-aromatic (right) region of the spectrum in fig. 21.

5 Discussion.

5.1 Cyanine dye structure.

Based on the information from the ^{1}H NMR on the cyanine dye (fig. 21 and 22) as well as the color of the crystals and the instant color change upon addition of triethylamine in the synthesis, it is pretty safe to say that the synthesis of the BOXTO-analog was successful. Even though the lack of information retrieved from the ^{1}H COSY spectrum (due to the large amount of water and hydrogen in the sample making it impossible to detect the other interactions) makes it hard to assign the aromatic peaks in the ^{1}H NMR of fig. 21 and 22 to specific hydrogens, the other peaks can be assigned with fairly high accuracy. The singlet corresponding for 3 hydrogens at 3.95 ppm is most likely from the methyl group (numbered 30) on the nitrogen of the benzothiazole group, due to it being the only methyl group on the molecule, and the multiplet at 6.17 ppm corresponds to the single hydrogen on the alkene group (numbered 32) since it's the only alkene hydrogen neighboring to more than one other hydrogen. However, the easiest peak to assign, and probably the one which is the most indicative of a successful reaction, is the singlet at 6.74 ppm corresponding to one hydrogen. This has to be the hydrogen on the carbon in the methine bridge (numbered 19) as this has not appeared in any of the spectra for the respective starting materials (fig. 17-20), in contrast to the ones previously mentioned which both appears in the spectrum for the quinolinium salt (albeit at different chemical shifts), meaning that a reaction creating a new compound has taken place.
This together with the fact that the amount of hydrogen signals found in the spectrum (13 in the aromatic region, 6 in the alkene or near alkene region and 3 in the alkane region as a single singlet) provides enough proof to assume that the desired cyanine product has been formed.

5.2 Purity and yield.

After the solvent had been evaporated in the final synthesis, a ^{1}H NMR was taken which showed the presence of impurities in the sample. These included mostly water and hydrogen but also starting material and solvent. A decision was therefore made to try to recrystallize the product in a water/ethanol mixture which, in retrospective, probably was a bad call. Not only did the recrystallization not work due to no precipitation of the product, but it also led to a very tedious evaporation process trying to evaporate the water from the collected filtrate where some of the sample was lost due to extensive foam formation and boiling, contributing to a lower yield. After the sample was recovered and had been successfully recrystallized in acetonitrile, the impurity from starting material was almost entirely gone but the water/hydrogen content was even higher than before (most likely due to the ethanol/water-mixture treatment). This made it difficult to read the NMR measurements as the baseline in the ^{1}H NMR became very bumpy and the ^{1}H COSY was only able to detect the water/hydrogen interaction with the methanol solvent and the methyl group.

As of now, no real weight can be put on the calculated yield of the dye due to the high water content. The sample would have to be further purified before a significant yield could be calculated. This yield would probably also be smaller than the actual yield due to all the minor losses of sample during the extensive purification process. At a larger scale these losses would be negligible but with

such a small sample scale described here, they have a real impact on the yield. If the yields of the other syntheses are compared with those published by Karlsson et al., most of them are nearly equal which is to expect since most of them used are based on said article [4]. The only large differences are in the synthesis of the benzoxazole aniline **(3)** and the thiomethyl benzothiazole **(6)**. In the case of the thiomethyl though, it could be the case of a one-time occurrence since another synthesis of the same compound on a smaller scale gave a much higher yield (around 80%), closer to the one reported in the article [4]. The aniline in this project was synthesized in a single step in contrast to the two step synthesis in the article previously mentioned and had an increase in yield of nearly 30 percentage points. With the purification steps being nearly identical, the only real tradeoffs of the single step method is the solvent (polyphosphoric acid), which is much more hazardous than NMP and ethanol, and the higher temperature, which might pose a problem if the experiment is scaled up.

5.3 Scale up.

Cyanine dyes used for DNA-probing are in general very expensive, thanks a lot to them being tricky to produce on a large scale. If a route of synthesis for a good dye with properties as good as or better than the ones commercially available to date was able to be scaled up to an industrial level in a viable fashion, the prices would most likely drop significantly. Looking at the route of synthesis described in this project, there are no real signs to indicate that a scale up of the synthesis steps wouldn't work as there aren't really any synthesis or purification steps limited by sample size, which could be the case if i.e. chromatography had been used. In fact, some of the yields might even improve as the experiments are scaled up as the impact of all minor losses which are bound to happen during all synthesis steps will decrease as the scale increases. The recrystallizations are more time consuming than size limited and could probably be scaled up without problem. The only things that might be of an issue are the high temperatures of some of the reactions as this could lead to a very high energy consumption on a large scale. Most of these reaction times are relatively short though (a few hours) and give high yields making the high temperatures less impactful.
If only a successful method for purifying the finished cyanine dye in an efficient manner (perhaps recrystallization in acetonitrile as well as some kind of drying process) it would certainly seem plausible that the entire route of synthesis could be scaled up.

5.4 Strategy of synthesis.

As is often the case in organic synthesis, everything seldom works out exactly as planned on the first attempt. Initially, the synthesis of the benzoxazole aniline **(3)** was supposed to be a two-step process with a nitrophenyl benzoxazole, synthesized from 2-aminophenyl and 4-nitrobenzoylchloride, followed by a reduction into the aniline (see fig. 23).

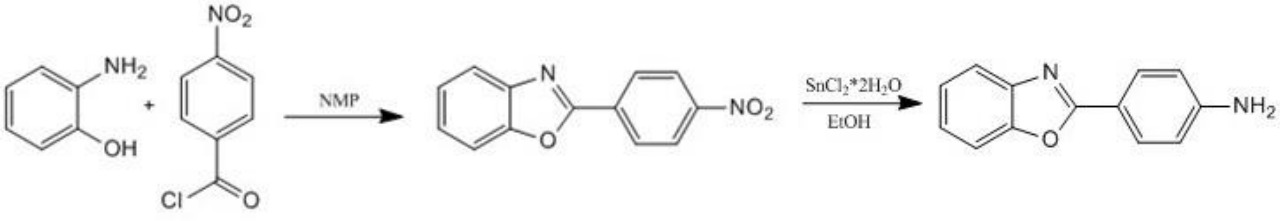


*Figure 23: Two-step synthesis of **(3)** via nitrophenyl benzoxazole intermediate.*

However, both the synthesis of the nitrophenyl as well as the reduction worked poorly, giving products with low purity and low yield. It was therefore attempted to create the aniline in a single step instead, according to the method previously described by Shi et al. [13, 14]. This resulted both in a reduced number of synthesis steps as well as a significantly higher yield and purity. Another synthesis that didn't work as planned was the attempted allylation of the methylthio benzothiazole **(6)** (see fig. 24). At first, allyl bromide was used but this didn't work very well, resulting in the wrong product. It was instead decided to try with allyl tosylate, since previous work had showed that methylation of the same molecule with methyl tosylate worked. The synthesis of the allyl tosylate was, however, not very successful and the following allylation didn't work. This led to the idea of allylating **(6)** being scrapped in favor of methylating it, which had already been proven to work [4].

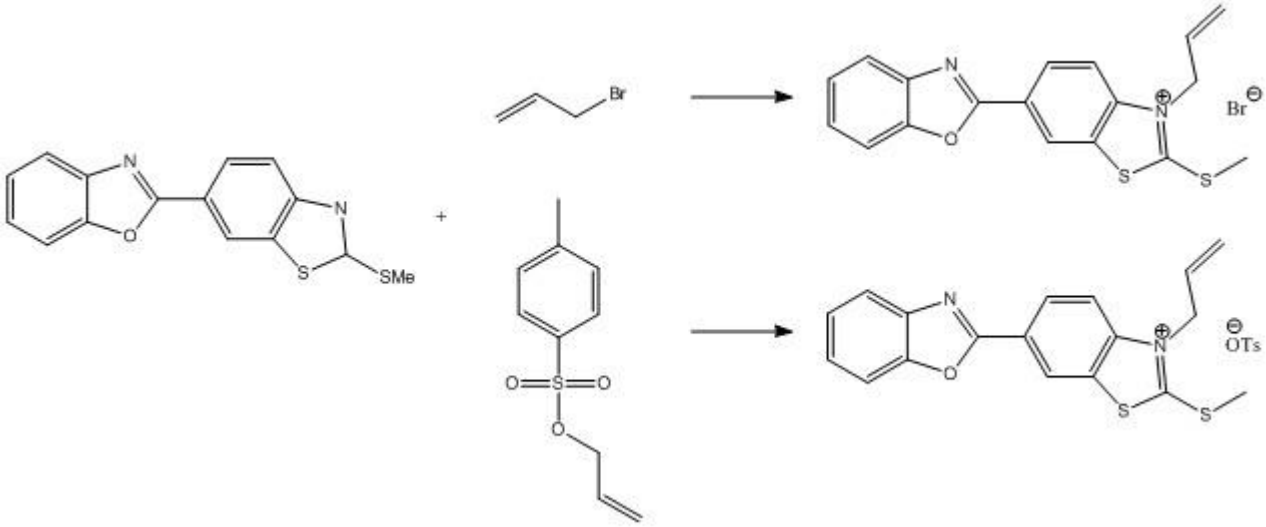


*Figure 24: Allylation of **(6)** with allyl bromide and allyl tosylate respectively.*

5.5 Future studies.

First and foremost, a way to purify/dry the synthesized BOXTO-analog needs to be found. It would then be desirable to measure the DNA-binding and fluorescent properties of the dye and compare the results to those of the original BOXTO cyanine dye. There is still room for optimization of the syntheses as most of them have only been run a few times, meaning that the yields probably can be improved. It would also be interesting to have another go at the analogs described in section 4.1.1 and perhaps take another approach to their syntheses as these analogs theoretically could have good properties. It would also be preferable to continue the research around cyanine dyes in general as there is a lot of room for improvement, both in the properties of the dyes but also in the manufacturing process which could (hopefully) be made a lot cheaper.

6 Conclusion.

Based on the information given from NMR spectroscopy measurements and visual observations as well as comparison with previously published data, the conclusion that all the reported syntheses have been successful and that a chemical analog to the cyanine dye BOXTO has been synthesized can be drawn. However, further purification of this product is still required before measurements on DNA-binding and fluorescent properties, as the water/hydrogen content of the sample still is very high along with some other minor impurities.

If an efficient way of purifying the cyanine dye **(11)** can be found, there should be no reasons as to why this route of synthesis couldn't be scaled up to a larger scale. Apart from the final synthesis, it gave products of acceptable purity without containing any size limiting purification steps. The syntheses also gave yields which were near equivalent to those of syntheses for similar compounds and, in some cases, even better. The yield of most syntheses are also expected to increase as the scale increases.

In the near future, the goal is to perform measurements on the properties of the dye (after purifying it) to see how the modification of BOXTO has affected them. Further research surrounding the area of cyanine dyes in general would also be very interesting since there still is a lot of room for improvement, both in their properties and their cost.

References.

1. Socher, E., Jarikote, D. V., Knoll, A., Röglin, L., Burmeister, J., & Seitz, O. (2008). FIT probes: peptide nucleic acid probes with a fluorescent base surrogate enable real-time DNA quantification and single nucleotide polymorphism discovery. Analytical biochemistry, 375(2), 318-330.

2. Yang, X., Shi, C., Tong, R., Qian, W., Zhau, H. E., Wang, R., ... & Henary, M. (2010). Near IR heptamethine cyanine dye–mediated cancer imaging. *Clinical Cancer Research*, *16*(10), 2833-2844.

3. Fürstenberg, A., Deligeorgiev, T. G., Gadjev, N. I., Vasilev, A. A., & Vauthey, E. (2007). Structure–fluorescence contrast relationship in cyanine DNA intercalators: toward rational dye design. *Chemistry–A European Journal*, *13*(30), 8600-8609.

4. Karlsson, H. J., Bergqvist, M. H., Lincoln, P., & Westman, G. (2004). Syntheses and DNA-binding studies of a series of unsymmetrical cyanine dyes: structural influence on the degree of minor groove binding to natural DNA. *Bioorganic & medicinal chemistry*, *12*(9), 2369-2384.

5. Logan, J., Edwards, K. J., & Saunders, N. A. (2009). *Real-time PCR: current technology and applications*. Horizon Scientific Press.

6. Richards, A. D., & Rodger, A. (2007). Synthetic metallomolecules as agents for the control of DNA structure. *Chemical Society Reviews*, *36*(3), 471-483.

7. Demchenko, A. P. (Ed.). (2010). *Advanced fluorescence reporters in chemistry and biology I: Fundamentals and molecular design* (Vol. 8). Springer Science & Business Media. doi: 10.1007/978-3-642-04702-2.

8. Lakowicz, J. R. (1999), *Principles of Fluorescence Spectroscopy, Second Edition*, Kluwer Academic/Plenum Publishers, ISBN: 978-1-4757-3063-0

9. Klein, D. (2002). Quantification using real-time PCR technology: applications and limitations. *Trends in molecular medicine*, *8*(6), 257-260.

10. Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., ... & Vandesompele, J. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry*, *55*(4), 611-622.

11. Kim, S. K., & Nordén, B. (1993). Methyl green: a DNA major-groove binding drug. *FEBS letters*, *315*(1), 61-64.

12. Pavia, D. L., Lampman, G. M., Kriz, G.S. & Vyvyan, J.R. (2015). *Introduction to Spectroscopy, Fifth Edition*. ISBN: 9781285460123.

13. Shi, D. F., Bradshaw, T. D., Wrigley, S., McCall, C. J., Lelieveld, P., Fichtner, I., & Stevens, M. F. (1996). Antitumor benzothiazoles. 3. 1 Synthesis of 2-(4-aminophenyl) benzothiazoles and evaluation of their activities against breast cancer cell lines in vitro and in vivo. *Journal of medicinal chemistry*, *39*(17), 3375-3384.

14. Malcolm, F. G., Shi, D. F., Castro, A. (1996). Antitumour benzothiazoles. Part 2. Formation of 2, 2′-diaminobiphenyls from the decomposition of 2-(4-azidophenyl) benzazoles in trifluoromethanesulfonic acid. *Journal of the Chemical Society, Perkin Transactions 1*, (1), 83-93.

15. Deligeorgiev, T., Gadjev, N., Vasilev, A., Drexhage, K. H., & Yarmoluk, S. M. (2006). Synthesis of novel monomeric cyanine dyes containing mercapto and thioacetyl substituents for nucleic acid detection. *Dyes and pigments*, *70*(3), 185-191.

16. Fürstenberg, A., & Vauthey, E. (2007). Ultrafast excited-state dynamics of oxazole yellow DNA intercalators. *The Journal of Physical Chemistry B*, *111*(43), 12610-12620.

17. Camerano, J. A., Casado, M. A., Ciriano, M. A., Tejel, C., & Oro, L. A. (2005). Peripheral SH-functionalisation of carbosilane dendrimers including the synthesis of the model compound dimethylbis (propanethiol) silane and their interaction with rhodium complexes. *Dalton Transactions*, (18), 3092-3100.

A. Appendix

Appendix A1: Experimental data from syntheses in 4.1.1.

3-allyl-[6-(benzoxazol-2-yl)-2-methylthio]benzothiazolium bromide:

Allyl bromide (0.091ml, 0.1272g, 1.05mmol) was added dropwise to 6-(Benzoxazol-2-yl)-2-thiomethylbenzothiazole (0.1051g, 0.352mmol). The mixture was heated to 100°C and left to stir for 4 hours. After cooling, acetone was added and the mixture was filtered and washed with acetone. The filtrate was evaporated in vacuo and the crude product was collected as an oily film in the round bottom flask (0.0676g, 45.4% yield). ^{1}H NMR (DMSO-d_6):

4-((3-allyl-6-(benzooxazol-2-yl)benzothiazol-2(3H)-ylidene)methyl)-1-methylquinolinium tosylate:

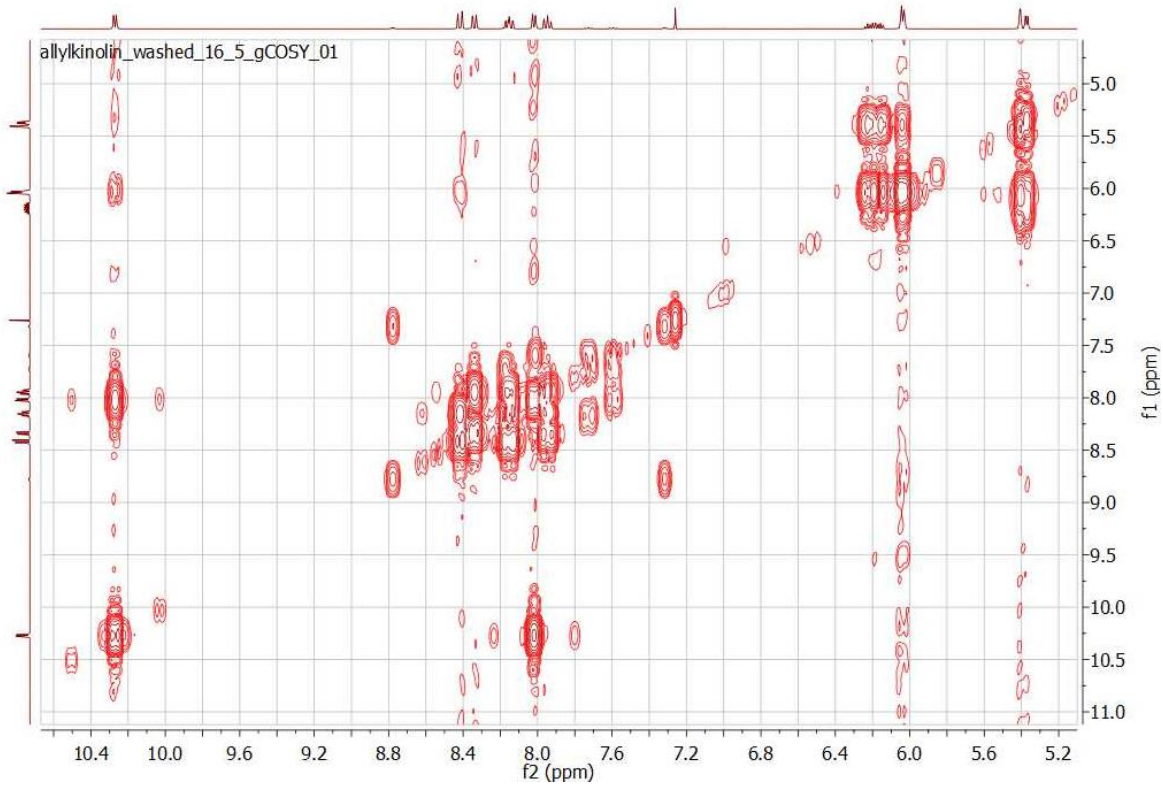
Triethylamine (0.08ml, 0.574mmol) was added dropwise to a solution of 3-allyl-[6-(benzoxazol-2-yl)-2-methylthio]benzothiazolium bromide (0.0676g, 0.16mmol) and 1,4-dimethylquinolinium tosylate (0.0521g, 0.158mmol) in 1.3ml DCM. The color of the solution instantly changed from brown to dark red upon adding triethylamine and the solution was left to stir at room temperature for 20 hours. The solvent was evaporated in vacuo, after which a mixture of ethanol and water (1:1) was added to the remaining solid. The mixture was filtered and a dark red powder was collected.

1-(3-bromopropyl)lepidinium bromide:

4-methylquinoline (1.0668g, 7.45mmol) and 1,3-dibromopropane (4.4878g, 22.2mmol) was dissolved in 25ml acetonitrile. The solution was heated to 120°C and was left to stir for 21 hours. The resulting black mass was dissolved in a small amount of ethanol whereafter diethyl ether was added (roughly 5 times the volume of ethanol). After mixing the phases were allowed to separate and the ether phase was removed. The remaining solution was evaporated in vacuo, resulting in a black solid.

Appendix A2: Additional ^{1}H COSY spectra.

^{1}H COSY spectra of allylated quinolinium salt (10):



^{1}H COSY spectra of the cyanine dye (11):