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Hydrosolubilization of BODIPY for optical labelling of biomolecules

Hydrosolubilizace skeletu BODIPY pro optické značení biomolekul

Diploma thesis

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May, 2015

Prague

The experimental part of this work was done at Institute of Organic Chemistry and Biochemistry AS CR, v.v.i. in the laboratory of Synthetic Nanochemistry (Dr. Petr Cígler).

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně, a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, dne .....

Podpis .....

Acknowledgements

In the first place I would love to thank my family, especially my brothers and their wives. For all those countless cups of coffee, glasses of gin, bottles of beer or wine and above all, dinners.

It's a great pleasure to thank my supervisors and all my colleagues in lab(s) for support, ideas and cookies. Namely, I would love to praise Miloš for his advise about trashing (almost) everything on my desk including many presumptions.

I would like to thank my gifted roommate, who would persuade me that failed reactions are just inconvenience. Mostly because his upgrades of our room left me breathless for several days. Repeatedly. To all without their kind words I wouldn't have finished this intact, notably ŠsŠ, Honza, fellow organisers and participants of KSICHT and many other...

Special thanks belongs to University of California Irvine (Department of Chemistry) for their incredible accurate parody of chemist dreams **Chemist Know**.

"The Guide says there is an art to flying," said Ford, "or rather a knack. The knack lies in learning how to throw yourself at the ground and miss."
Douglas Adams

Abstract

This work aims at showing synthesis and potential use of water-soluble fluorescent probes based on BODIPY. The preparation of probes containing bioorthogonal mono- and heterobifunctional functional groups was demonstrated. Ground work was done at the optimisation of reliable, scalable and fast sulfonation of BODIPY in 2,6-positions. A protocol for handling sulfonated BODIPY has been established; especially for the exchange of counterions. In counterion selection, their relation to synthetic pathway and biocompatibility were taken into consideration.

The second part of the work shows series of water-soluble fluorescent probes, into which can be easily introduced bioactive or bioorthogonal functional groups. This can be used for click chemistry in connection with turn off/on probes or fluorescent sensing of molecules or ions. All this can be done in aqueous solution without organic solvents, which is relevant for biochemical, analytical and imaging applications.

Keywords BODIPY, bifunctional, water-soluble, fluorescent probe, solubilization, biocompatible probes, bioorthogonal reaction, BODIPY sulfonation

Abstrakt

Cílem této práce bylo ukázat syntézu a potenciální využití fluorescenčních sond rozpustných ve vodě založených na BODIPY. Konkrétně se jednalo o přípravu sond s mono- a heterobifunkčními funkčními skupinami. Bylo nezbytné optimalizovat podmínky pro spolehlivou, škálovatelnou a rychlou sulfonaci BODIPY v polohách 2,6. Dále byl vytvořen postup pro práci se sulfonovaným BODIPY, především výměnu protiiontů. Při výběru protiiontů byla uvažována jejich biokompatibilita, reakční cesta při syntéze a separace produktů.

Ve druhé části práce byla připravena série ve vodě rozpustných fluorescenčních sond, do kterých lze snadno zavést bioaktivní nebo bioortogonální funkční skupiny. Takto modifikované sondy lze využít v klik chemii ve spojení s off/on sondou nebo pro fluorescenční detekci molekul či iontů. Zásadní je, že navržené aplikace lze provádět ve vodných roztocích bez použití organických rozpouštědel, což je nezbytné pro biochemické, analytické a zobrazovací uplatnění.

Klíčová slova BODIPY, bifunkční, rozpustná ve vodě, fluorescenční sonda, solubilizace, biokompatibilní sonda, bioortogonální reakce, sulfonace BODIPY

List of Abbreviations

ACN Acetonitrile

BHT 2,6-Di-*tert*-butyl-4-methylphenol

BODIPY 4,4-Difluoro-4-bora-3a,4a-diaza-*s*-indacene

CV Column volume

DCC *N,N'*-Dicyclohexylcarbodiimide

DCM Dichloromethane

DCU *N,N'*-Dicyclohexylurea

DDQ 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone

DGA Diglycolic anhydride

DIPEA *N*-Ethyldiisopropylamine

DMF Dimethylformamide

DMSO Dimethyl sulfoxide

ESI Electrospray ionization

HEX Hexane

HMBC Heteronuclear Multiple Bond Correlation

HSQC Heteronuclear Single-Quantum Correlation

MeOH Methanol

NIR Near infrared

NMR Nuclear magnetic resonance

RP Reverse phase

RT Room temperature

TBTU *N,N,N',N'*-Tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate

TEA Triethylamine

TEAF Triethylammonium formate

TFA Trifluoroacetic acid

TLC Thin layer chromatography

TSTU *N,N,N',N'*-Tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate

Contents

1 | Introduction

The development of molecular imaging techniques in the recent years have brought enormous demand for fluorescent molecular probes which are highly sensitive, selective and bioorthogonal. Simultaneously, they must meet series of *usual* and new requirements such as [1]:

- Biocompatibility
- Water-solubility
- Chemoselectivity
- Brightness and high quantum yields
- Photochemical stability
- Reasonable reaction kinetics in physiological pH (if reacting covalently)
- Excitation and emission profiles in visible or near NIR region.

Fluorescent probes are molecules which exhibit characteristic fluorescent properties, such as fluorescence intensity, excitation and emission band wavelengths, lifetime or quantum yields etc. that can change as a result of an interaction with a target molecule. These interactions can be generally covalent or non-covalent. While we use a term probe, it is important to keep in mind the difference between a probe, indicator and label, as depicted in figure 1.1.

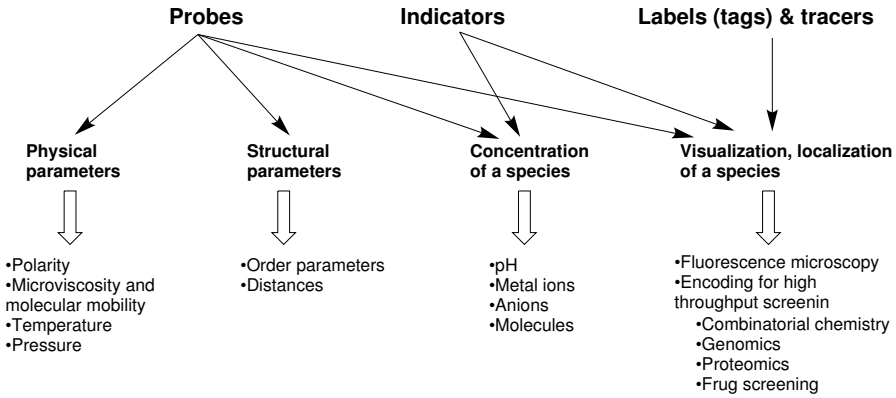

Figure 1.1: Difference between probes, labels and indicators. Redrawn from [2].

1.1 Fluorescent probes based on BODIPY

The class of fluorescent dyes called BODIPY, first discovered by Treibs and Kreuzer in 1968 [3], have experienced a tremendous boom in the last few years. There are several reasons for it: fast-developing methods for versatile substitution of BODIPY, excellent fluorescence properties and their tunability, high extinction coefficient, sharp excitation and emission bands, high quantum yield and photostability.

Moreover, recent studies (experimental [4] and theoretical [5, 6], section 1.1.1) showed trends in introducing the same (different) substituents in the same (different) positions which enables a quite precise prediction of spectral properties. When combined, for instance, with the detailed review on selective sensing of ions and molecules [7, 8], an excellent toolbox for preparing tailor-made fluorescent probes is available.

1.1.1 Applications of BODIPY

Fluorescent dyes based on BODIPY currently experience a great boom. In the last few years, the number of articles grew rapidly; therefore, only a few closely related topics will be covered in this introduction. The number of BODIPY applications, not only as a proof of principle, but also as readily applicable experiments, is growing fast.

Older reviews with broader scope will be often cited in this work, especially [9, 10, 11, 12], since the field of BODIPY has become more complex in the latest years. The most recent reviews cover only a small part of BODIPY field, rather than the diversity of BODIPY's physics, chemistry and biochemistry. For that reason, a short itemisation of current reviews is listed below.

Detailed information on specific areas can be found in the following: photodynamic therapy (PDT) in Ref. [13], photovoltaics in Ref. [14] and a comprehensive overview for design strategies

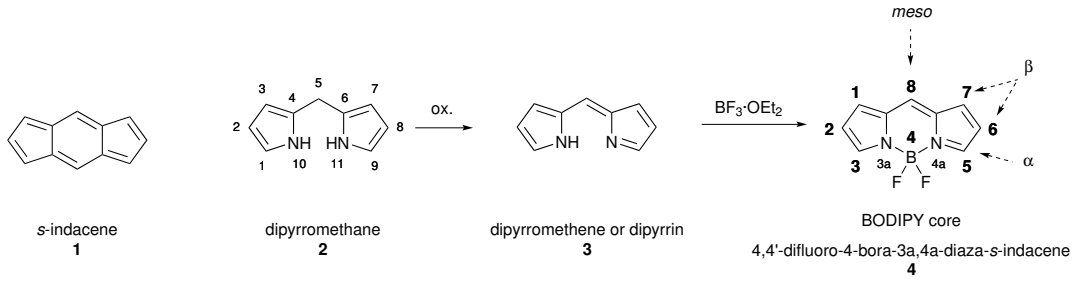

Figure 1.2: Structural motive of BODIPY (from left to right): terminological origin (**1**), synthetic precursor (**2**), its oxidised form (**3**) and BODIPY with IUPAC name and numbering (**4**).

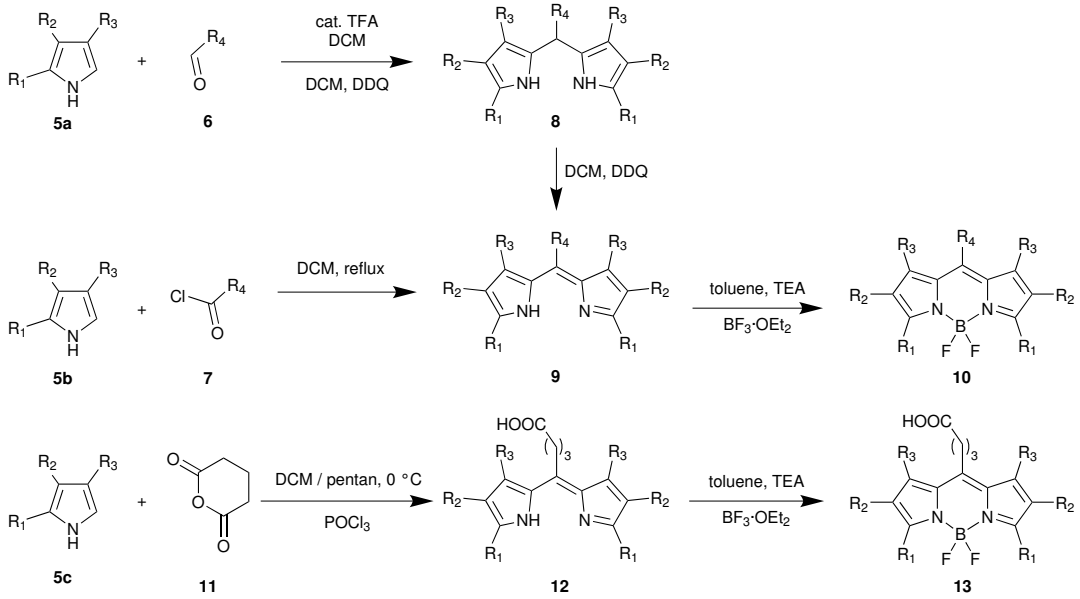

Figure 1.3: Simplified scheme of synthesis of a symmetrical BODIPY core.

for organic photovoltaic in small-molecule and bulk-heterojunction solar cell in Ref. [15]. Biologically oriented applications on pH sensing (intracellular) are review in Ref. [16, 8], strategies for shifting BODIPY fluorescence into far red and NIR in [4] (pH probes), for comprehensive review including calculations and prediction of properties see [6].

1.2 Preparation of BODIPY

The structural motive of boron-dipyrromethene, traditionally abbreviated as BODIPY **4** (Figure 1.2), is by IUPAC nomenclature derived from *s*-indacene (**1**). Synthetically, there is no connection to the structure; the preparation of BODIPY core is done in multi-step synthesis, starting with the condensation of (un)substituted pyrrole. This is usually done as a one-pot reaction without isolation of a less stable dipyrromethane (**2**) or oxidised dipyrromethene (dipyrrin) (**3**). The last step is the addition of a tertiary base (TEA, DIPEA) followed by boron trifluoride to form fluorescent **4** (BODIPY). An unsubstituted BODIPY **4** resisted synthetic efforts up to the recent time (2009), because it is missing stabilisation from substituents on the rest of the BODIPY scaffold (mainly a *meso*-substituent or methyl groups on pyrrole units) [17, 18, 19].

1.2.1 BODIPY core

From the synthetic point of view, the condensation of pyrrole yielding dipyrromethane leads to low yields (great number of byproducts) and it is highly dependent on a substituent in a *meso*-position. Lower yields can be generally observed, when unsubstituted pyrrole is used

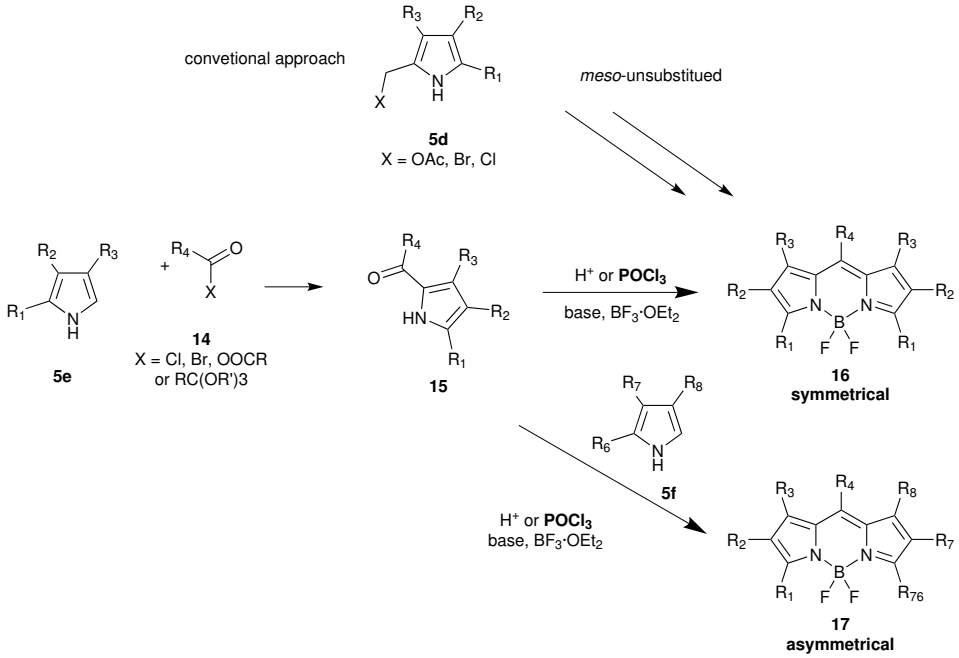

Figure 1.4: Scheme of synthesis of both symmetrical and asymmetrical BODIPY.

for condensation (*meso*-position contain still substituent). This is caused by a poor sterical hindrance, which leads to porphyrine-like adducts [20].

Therefore, if it's suitable for further synthetic modification, substituted pyrrole (mainly 2,4-dimethyl-pyrrole) is used. A detailed description of reactions is provided in [9, 12].

To prepare a symmetrically substituted core, condensation can be done by the reaction of pyrrole (**5b**) with acylchloride (**7**) or aldehyde (**6**) (Figure 1.3). In case of the reaction using aldehyde **6**, resulting dipyrromethane **8** must be oxidised to dipyrromethene **9**, which creates more byproducts. The advantages of these approaches comprise higher yields (usually above 50 %) and a feasibility to use *meso*-substituent as a starting point for further functionalisation [21]. If an aryl substituent in *meso*-position is not required, condensation can be done with anhydride **11** and pyrrole **5c**. Oxidised dipyrromethene (**9,12**) is then commonly complexed with boron trifluoride forming fluorescent BODIPY **10** or **13**.

Symmetrical BODIPY **16** (Figure 1.4) can be prepared by conventional self-condensation of pyrrole-2-carbaldehyde formed by oxidation **5d**, derivative **16** prepared this way will be *meso*-unsubstituted. By preparation of ketopyrrole **15** from correspondingly substituted pyrrole **5e** and ketone **14**, both symmetrical and asymmetrical BODIPY can be prepared. Dependent on the addition of pyrrole **5c** to ketopyrrole **15**, asymmetrical derivative **17** can be prepared. Reaction of **15** can be acid catalysed (commonly used) or catalysed with addition of phosphoryl chloride. The latter catalysis is reported to provide better yields. It was first reported in 2008 [22] and the mechanism was proposed in Ref. [12].

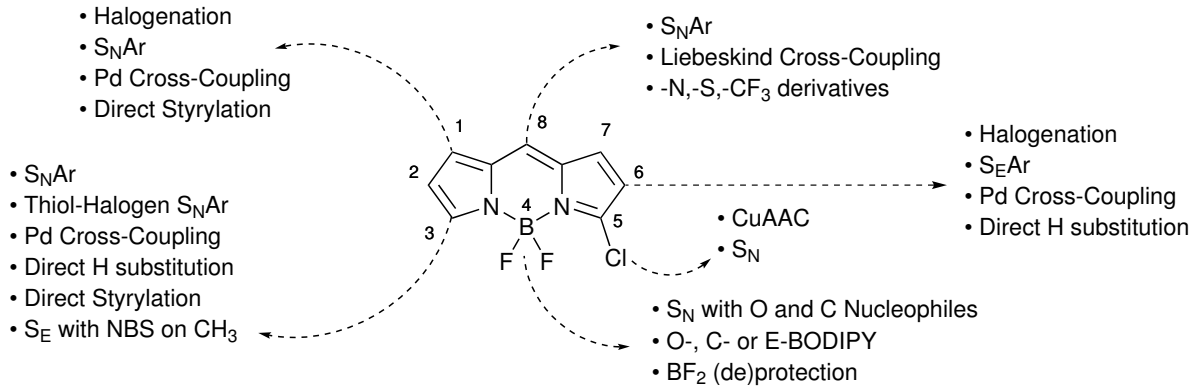

Figure 1.5: Synthetically available functionalizations of BODIPY. Redrawn from [15].

To prepare aryl-substituted BODIPY, we chose a path using acylchloride **7** because we could avoid the oxidation step, which produce a significant amount of by products and the reaction can be done in one pot (Figure 3.1, Route C).

1.2.2 Functionalization of core

Great variation of fluorescent properties can be achieved on BODIPY by condensation properly substituted pyrroles (see section 1.2). Overview of synthetically available derivatization of BODIPY is showed figure 1.5, especially the modification at 3,5- and 8-positions are well described in the literature. The modifications prior complexation, are often not highlighted , for example, bromination of positions 1,2,3,5,6,7 to introduce sterically crowded BODIPY [23] or chlorination of positions 3,5 in order to prepare library of 120 compounds (e.g. **18**) [24].

1.2.3 Positions 3,5

Positions 3,5 can undergo electrophilic substitution (selectively) with NBS introducing bromine to methyl group in positions 3,5 CH_3, resulting into preparation of derivatives **19** [25] or oxidative nucleophilic substitution to unsubstituted positions 3,5 [26]. Vicarious nucleophilic substitution (VNS) of hydrogen can be done, if the nucleophile carries a suitable leaving group (Br, Cl, SPh). This results into compounds such as **20** [27], to direct palladium-catalysed C–H (het)arylation forming **21** [28] or dimer **22** which have fluorescence shifted to red (absorption max. 621 nm, emission max. 707 nm) [29]. Thiol–halogen nucleophilic substitution can be also performed, forming **23** [30], or iridium-catalyzed borylation can be performed which is selective to positions 3,5 or 2,6 producing compounds **24a** and **24b**, resulting also into shift of fluorescence to red [31]. Last reported is a new procedure of radical C-H arylation, which easily introduces aromatic substituent(s) into positions 3,5 [32].

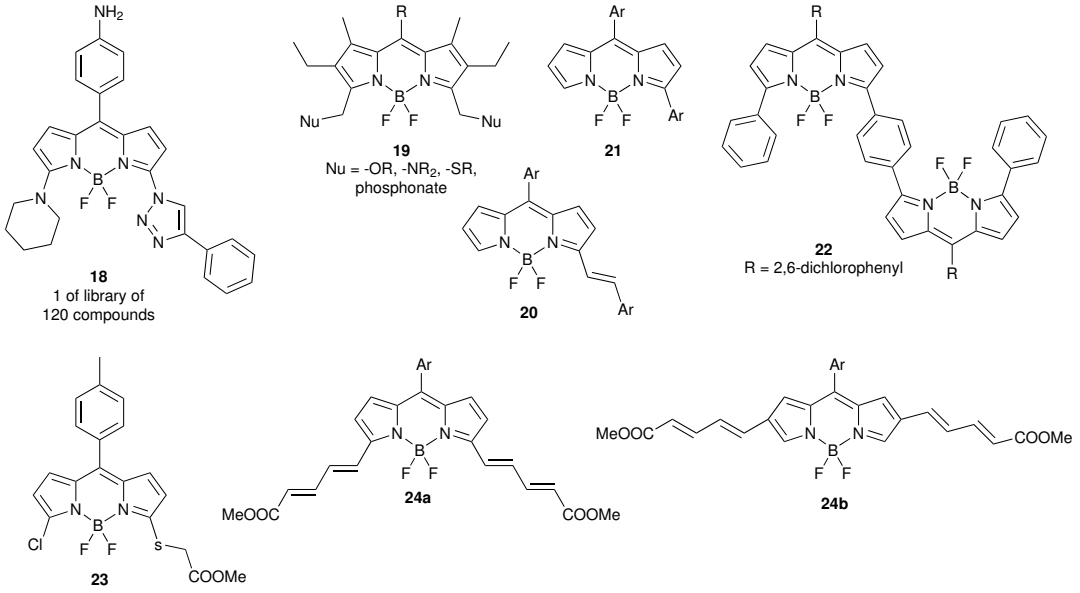

Figure 1.6: Modifications at 3,5-positions (with exception of compound **24b**).

1.2.4 Position 8 (*meso*)

Modifications at *meso*-position are interesting for several reasons: i) substitution on aryl substituent significantly change fluorescent properties in dependence on photoinduced electron transfer (e.g. off/on) [33] ii) it is synthetically accessible to functionalization iii) it is suitable for preparation of e.g. pH probes, metal-chelators, selective (chemo)sensors or biomolecule conjugating molecules [9]. Derivatives substituted in position 8 do not show distinctive changes of spectral characteristics (shift of fluorescence maxima), because of the orthogonal geometry of *meso* substituent and BODIPY core. However, the insertion of ortho-substituents on the *meso*-phenyl ring, bulkier aromatic groups or the introduction of 1,7-substituents, improve significantly fluorescence emission efficiency. This is caused by restricted intramolecular rotation and steric hindrance between the substituents [21, 34].

As mentioned in section 1.2.1, a variety of substituents can be introduced via acid-catalysed condensation of pyrrole and this method is widely used. Various studies of effects of electron-rich or electron-deficient groups have been done so far [33, 35, 36]. Except the commonly used substitutions, introducing hetero atom is of interest – for example "exchange" of C-*meso* for nitrogen creates subclass called aza-BODIPY **25** [37]. These dyes have similar structure to phthalocyanines and can be refered as "semi-phthalocyanines". Aza-BODIPYs have absorption and emission bands shifted to 650-850 nm range, high molar extinction coefficients and moderate fluorescence quantum yields (ϕ_{fl} = 0.23-0.36). In addition they have a low sensitivity to the solvent polarity. Thus, they present interesting alternative to BODIPY dyes, especially for use in NIR region [38]. Red-shift of absorption and emission maxima is result of stabilisation of the HOMO-LOMO energy gap by nitrogen [11].

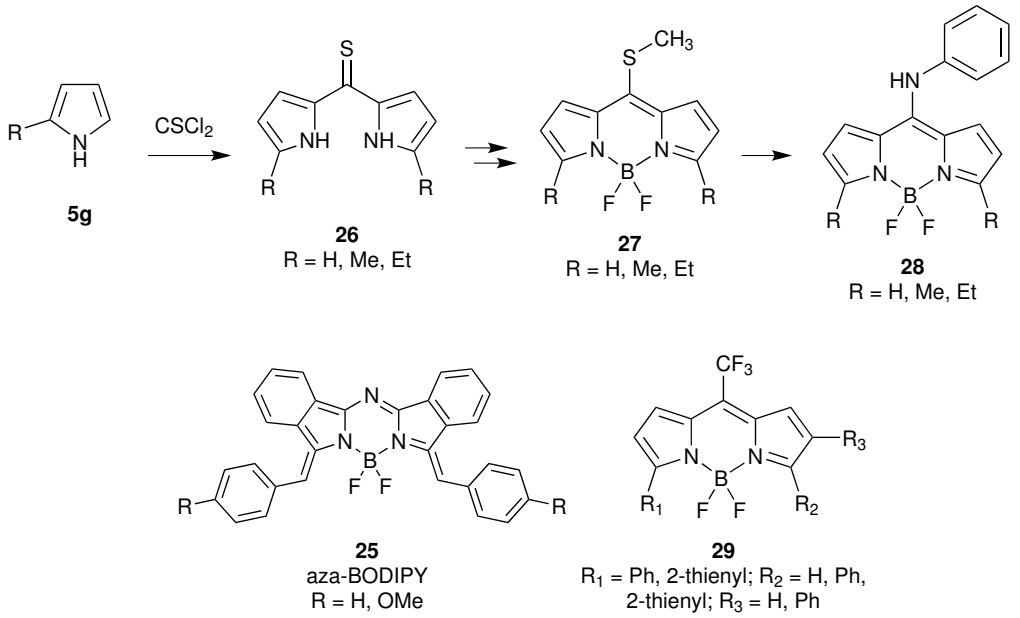

Figure 1.7: Modifications at the 8-position (*meso*).

Another variation is to introduce a heteroatom next to C-*meso* carbon, such as sulfur, which is done by condensation of substituted pyrrole **5d** with thiophosgene forming thioketone **26**, subsequent reaction enable to prepare S or N-*meso*-BODIPY [39]. Derivative **27** was employed in Liebeskind-Srögl cross-coupling and series of (aromatic) substituents were introduced to *meso*-position within few hours with high yields [40]. Using the same procedure and to avoid harsh conditions of oxidation by Lindsay process, a series of secondary and tertiary amines was prepared in *meso*-position (e.g. **28**) from **27** by S_NAr-like reaction. Fluorescence at blue edge of the visible spectrum was observed for derivatives like **28** [41]. An introduction of *meso*-CF_3 was also reported, both symmetrical and asymmetrical **30** [42].

1.2.5 Positions 1,7

Wider selection of functionalization reactions was missing for these positions for longer time. Finally, more options for modification were accessed by halogenation (compound **30** in figure 1.8), followed by nucleophilic aromatic substitution (S_NAr) or Pd-catalysed cross-coupling reaction [43] resulting in **31**. Another reported modification of positions 1,7 is by direct styrylation of methyl groups **51-53** in figure 1.11 [44].

1.2.6 Positions 2,6

Positions 2,6 bear the least positive charge on BODIPY and can undergo electrophilic attack (compound **32** in Figure 1.9); the extent of selectivity is not confirmed, though. In the majority

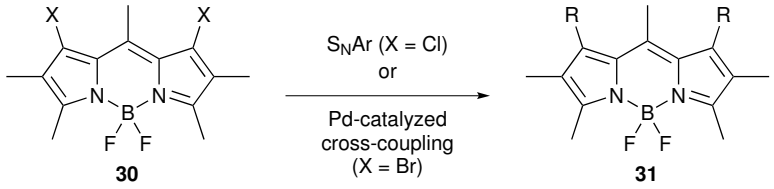

Figure 1.8: Modifications of positions 1,7.

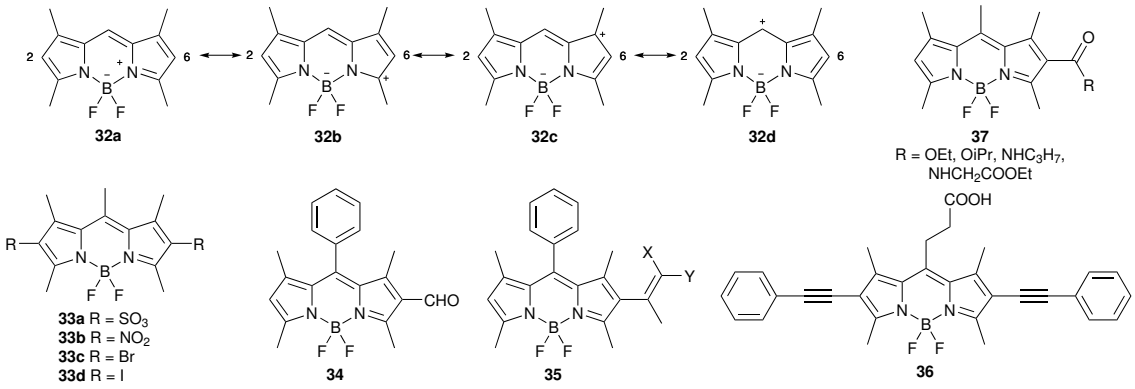

Figure 1.9: Mesomeric structures of BODIPY, positions 2,6 are more susceptible for electrophilic attack that other positions. Bottom: example of accessible derivatives for positions 2,6.

of the cases, other positions of pyrrole were blocked by methyl or ethyl substituents. The list of reported types of reactions is thus shorter: sulfonation (Section 1.3.4), nitration, bromination and iodation (compounds **33a**, **33b** [45], **33c** [45, 46], **33d** [47], respectively). Another reactions are formylation and Pd-catalysed cross-coupling accessing alkyne substituents like compound **36** [48, 49].

Introduction of sulfonate groups does not virtually influence spectral properties and quantum yields of prepared derivatives. On the other side, introduction of nitro or halo groups quenches drastically fluorescence and shifts absorption and emission maxima to red. In the case of halogen atoms, it can be attributed to the internal heavy atom effect. One of the applications of iodo-BODIPY is generation of singlet oxygen in photodynamic therapy [13, 50], the second one is the introduction of carbonyl derivatives from mono-iodo **33d** derivatives to prepare **37** via carbopalladation [51]. Bromo-BODIPY was used as reactant for Suzuki-Miyaura coupling [46]. Formylation of hydrogen at 2,6-positions was done via Vilsmeier-Haack reaction with mixture DMF and $POCl_3$ forming **34** and further functionalized via Knoevenagel condensation leading to **35** [52].

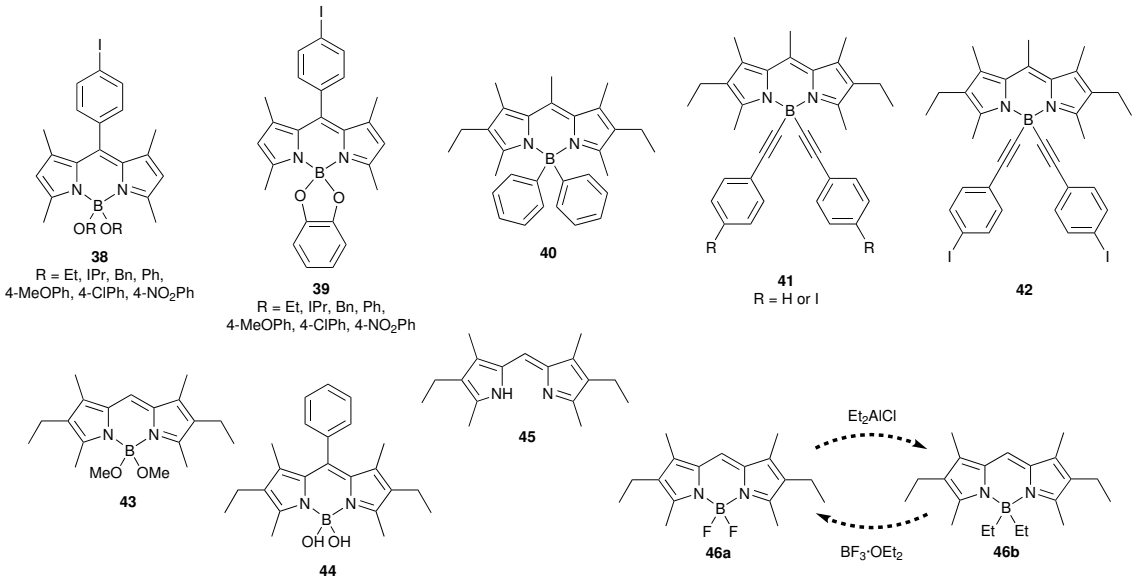

Figure 1.10: Synthetically available substitutions of fluorine atoms at position 4.

1.2.7 Position 4

At the 4-position, it is possible to exchange fluorine atoms by the means of nucleophilic substitution (S_N). In the case of substitution for alkoxide (or aryloxy), the so called O-BODIPY is prepared, in the case of aryl and ethynyl (ethynylaryl) substitution, C-BODIPY and E-BODIPY are prepared, respectively.

To prepare O-BODIPY, it is sufficient to treat BODIPY with aluminium chloride and corresponding alcohol producing compound **38-39** (Figure 1.10), which can improve inherent problems of BODIPY, especially small Stokes shift and fluorescence quenching [53, 38] and can be also used for creating energy-cassettes [54]. Similar results can be achieved using aryl-Grignard or organo-lithium reagents yielding compounds such as **40** [55]. To prepare E-BODIPY (e.g. **41-42**), there are several pathways, well described in e.g. [56, 57, 58].

The previous modifications are known for about one decade.

Optimisation of microwave assisted substitution for alkoxide was recently reported (e.g. compound **43**) and characterisation of dihydroxy BODIPY **44** by X-ray was reported, even it was suspected to be unstable. The implementation of BF_2-deprotection (compound **45)** and reversibility of the substitution seems even more interesting (**46a**↔**46b**). This can be used as a masking tool for BODIPY, since substitution of a fluorine atom for alkyl usually leads to stabilisation of BODIPY and more harsh reaction conditions can be employed [59, 60, 61].

1.3 Strategies leading to water-soluble probes

Many applications of fluorescent dyes, such as pH probes, biomolecule labelling, *in vivo* imaging, chemosensors, metal ion detection etc., rely on solubility in water, while maintaining fluorescence quantum yields at high as possible. BODIPY has several advantages in comparison to other fluorescent dyes, such as strong absorption in visible region on the edge of "the imaging window", narrow emission bands, high quantum yields and excellent physiological stability [62, 10]. The majority of prepared derivatives are well soluble in organic solvents (usually from DCM to MeOH), but insoluble in water.

High solubility of BODIPY in water can be achieved by introducing various hydrophilic groups, such as the sulfonate group. The introduction of sulfonate groups to positions 2,6, is discussed in section 1.3.4. There have been reported only two studies concerning solubility of BODIPY, so far. One is a short review of prepared BODIPY compounds [62], the other one discusses the effects of different hydrophilics groups, while the rest of BODIPY remains unchanged. The authors convey an opinion that strongly anionic dyes may enter cell uneasily and suggest using different groups, such as sulfobetain or trimethylalkylammonium [63].

1.3.1 Carboxylic groups

The carboxylic group was first introduced into 2,6-positions by Komatsu et. al **47** (Figure 1.11) and this derivative was introduced by means of S_{N2} reaction conversed to amide and ester. All three compounds are soluble in water. The cleavage of ester **48** to form carboxylate **47** resulted in 20 nm red shift, which is caused by weaker electron withdrawing ability of carboxylate and this is suitable for development of new ratiometric fluorescent probes [64]. Another application of BODIPY is off/on fluorescent probe **49**, which have emission shifted to NIR (emission maxima 670 nm). Fluorescence of the probe **49** is turned off when calcium ions are not present. When the probe is exposed to calcium ions in live cell, an increase of fluorescence was observed ($\phi_{fl,\text{off}} = 0.002 \rightarrow \phi_{fl,\text{on}} = 0.24$) [65].

1.3.2 Quaternary ammonium, phosphonate groups and oligo-ethylenglycol chains

The use of quaternary ammonium groups with BODIPY is scarce, they are often used with other functional groups such as sulfonate or oligo-ethylenglycol moieties, for instance compounds **50** (Figure 1.11) or **51** with excess of sulfonate groups. By coupling with styryl substituents in 3,5-positions, dyes such as **51** were prepared, which are water-soluble with emission shifted to red region [66, 67]. The situation is similar for phosphonate derivatives, a little work was done also with phosphonate groups, example of a such derivative is **52**. Solubility was also significantly

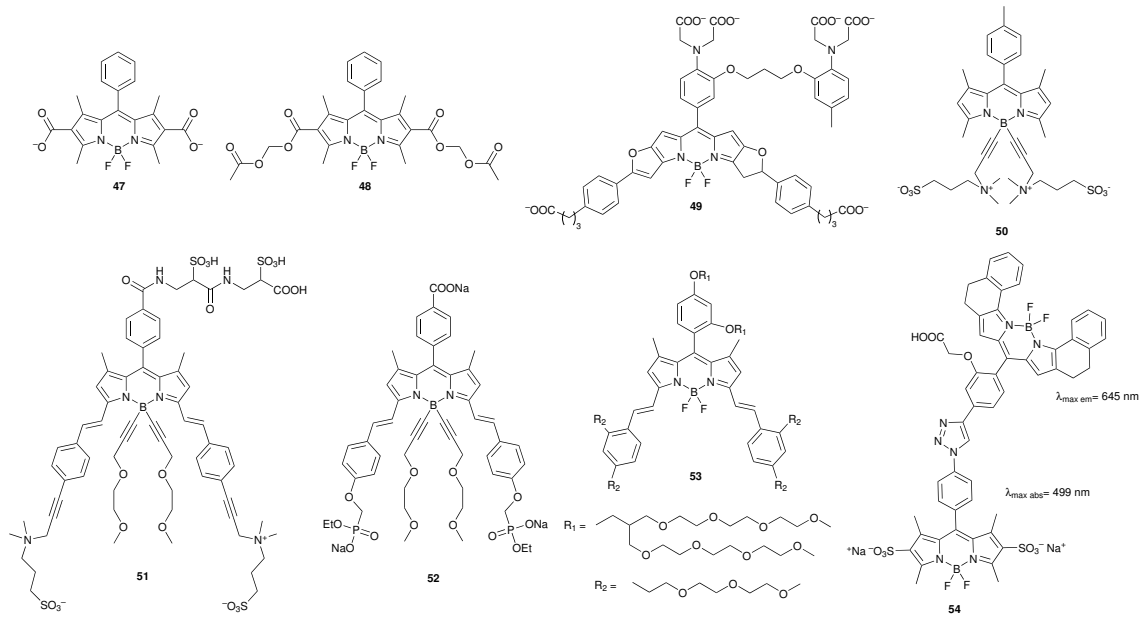

Figure 1.11: Different ways of preparing water soluble BODIPY.

improved by phosphonate groups and combined with oligo-ethyleneglycol groups [68]. The use of oligo-ethyleneglycol was reported, e.g. **53**. The other advantages can be ascribed to steric hindrance that reduces aggregation and to the neutral hydrophilic groups what facilitate a cell entry [69].

1.3.3 Sulfonate groups

BODIPY sulfonated in positions 2,6 has almost the same properties as non-sulfonated BODIPY, especially high fluorescent quantum yield, e.g **55** in Figure 1.12 has fluorescent quantum yield in water $\phi_{fl} = 0.85$. For the application in biomolecule imaging a suitable option is representated by **62** (Figure 1.13) and especially by the product of click reaction **63**, with relative high fluorescent quantum yield ($\phi_{fl} = 0.61$ in water) [70]. Different use of sulfonated BODIPY is demonstrated by Burgess group. BODIPY was used for through-bond energy transfer (TBET) cassettes. This represents an alternative to the fluorescence resonance energy transfer (FRET). TBET is not dependent on the overlap of fluorescence emission of the donor and absorption of the acceptor, but depends on transfer of energy over π-electron system of **54** (Figure 1.11) [54].

1.3.4 Overview of sulfonated BODIPY

The first reported sulfonation was done by the authors of BODIPY themselves, although they did not further worked with it **55** (Figure 1.12 on the following page) [3]. This free sulfonic acid degrades on air if prepared by sulfonation with chlorosulfuric acid. Stabilisation of BODIPY

was crucial for any further studies with sulfonated derivatives, which was done by neutralisation forming **56**, this was the first reported chemistry with sulfonated BODIPY [71]. For physical studies compounds **55-58** were utilised [45, 72]. Compound **56** became commercially available (Molecular Probes, Exciton, now Invitrogen) and it was used in many studies, without any further modification [73, 74, 75, 76, 77]. There exist also a patent using BODIPY as multiphoton device, namely derivative **59** [78].

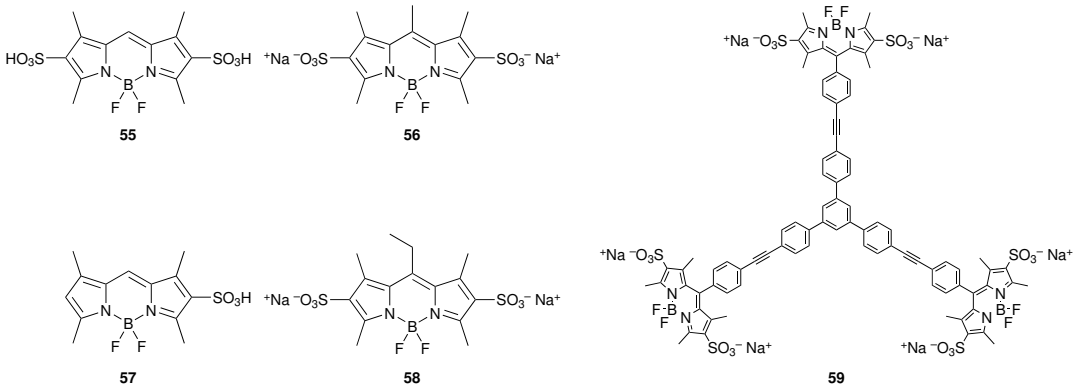

Figure 1.12: Reported sulfonated BODIPY before 2008.

Over 40 years to 2007 only five unique derivatives were reported . From this point of view, when Li *et. al.* published in 2008 series of sulfonated compounds (Compounds **60-67** in figure 1.13), this can be seen as breakthrough [70]. Although Li *et. al.* prepared three times more sulfonated derivatives* than was so far reported, group of Kevin Burgess also depicted the major issues in this area. Two reasons given in conclusion are rather self-explanatory and discouraging:
"i) inappropriate conditions give mixtures of products and
ii) sulfonic acid derivatives of BODIPYs can be hard to purify."

This closure can be easily explained, using experience based on the work done previously and in this thesis: the use of silica gel often generates new byproducts rather than purify the sulfonation mixture. Other techniques must be employed to obtain pure product (see section 3.2). Later, Burgess published several papers containing derivatives originated from the same precursor **64**, for instance **54, 68** in figure 1.14 [79, 54], or tried to use **60** in photodynamic therapy [50]. From 2008 there have been reported dozen of derivatives, namely for sensing Diels-Alder reaction in biological system. Anthracene was used as a off switch of BODIPY fluorescence (compounds **69-70**), the product Diels-Alder reaction **71** restored its fluorescence [80].

The synthesis of derivative **72** seems to be more appealing, although BODIPY was sulfonated in the last step of synthesis, radical scavenger (BHT) was added to stabilise the reaction mixture [81]. The last found reported sulfonation applies to **73**, with low yield using *standard* conditions

*If we consider mono- and disulfonated derivative as two.

[82]. No further optimisations of sulfonation using chlorosulfuric acid and its separations were found in literature.

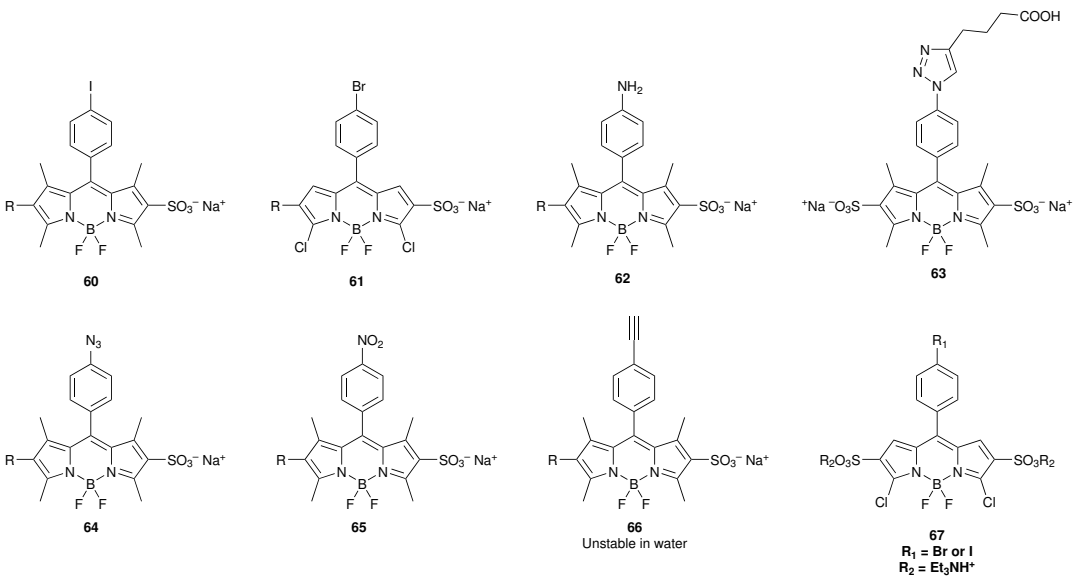

Figure 1.13: Reported sulfonated BODIPY in 2008, where R = H or $SO_3^-Na^+$ [70].

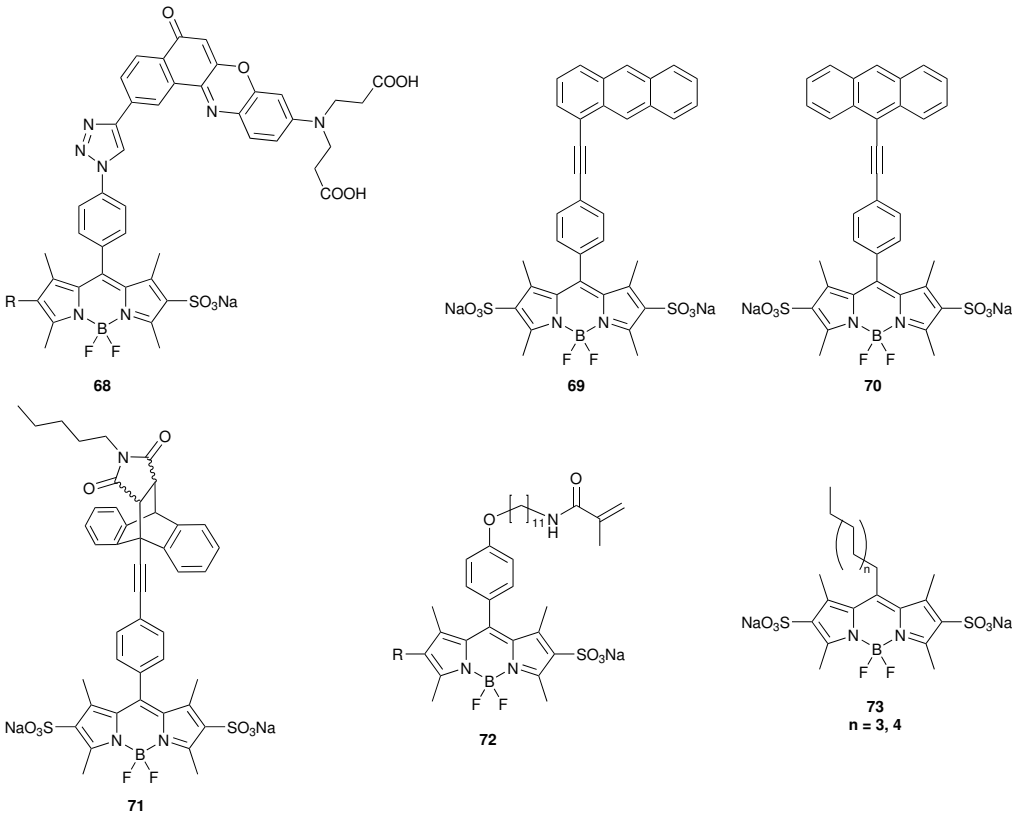

Figure 1.14: Reported sulfonated BODIPY after 2008, where R = H or $SO_3^-Na^+$ [70].

Different approach to sulfonation

Interestingly, there was reported a different approach to introduce sulfonate group to compound **74** (Figure 1.15) in 2014 [83]. The reaction with 93 % yield was performed with a high excess (20 eq.) of sulfur trioxide-pyridine complex. This could be, once again, a turning point for sulfonation, because the reaction undergoes easily. It is not known, whether monosulfonated BODIPY can be isolated this way and how will the yield vary with different derivatives of BODIPY. Experimental procedure gives the impression, that sulfonated BODIPY **75** can be manipulated outside inert conditions (performing preparative TLC) even in the form of acid (pyridine is not present in the spectra in supporting information). To our best knowledge, no similar reaction has been reported so far.

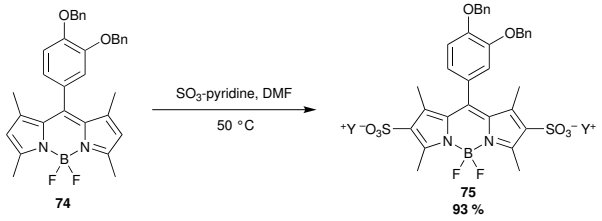

Figure 1.15: Different approach to sulfonation. It is not apparent if $Y^+ = H^+$ or Na^+.

2 | Aims of the study

In this work we aim at finding a simple, water-soluble fluorescent probes, which could be easily integrated in other molecules. A suitable option is BODIPY that meets all the previously mentioned requirements (Figure 2.1). First, we focused on finding a reliable methodology for sulfonation and is scale up. Afterwards, we aimed at creating a series of sulfonated derivatives and developing new chemistry of highly water-soluble fluorescent probes. These goals can be summarised as follows:

1. To establish scaled up protocol for sulfonation.
2. To investigate the effect of different counterions and develop the methodology for exchange of counterions.
3. To find conditions for alkylation of amines with BODIPY.
4. To prepare following small, water-soluble fluorescent probes:
   4.1. Monofunctional
   4.2. Heterobifunctional and bioorthogonal
   4.3. Off/on

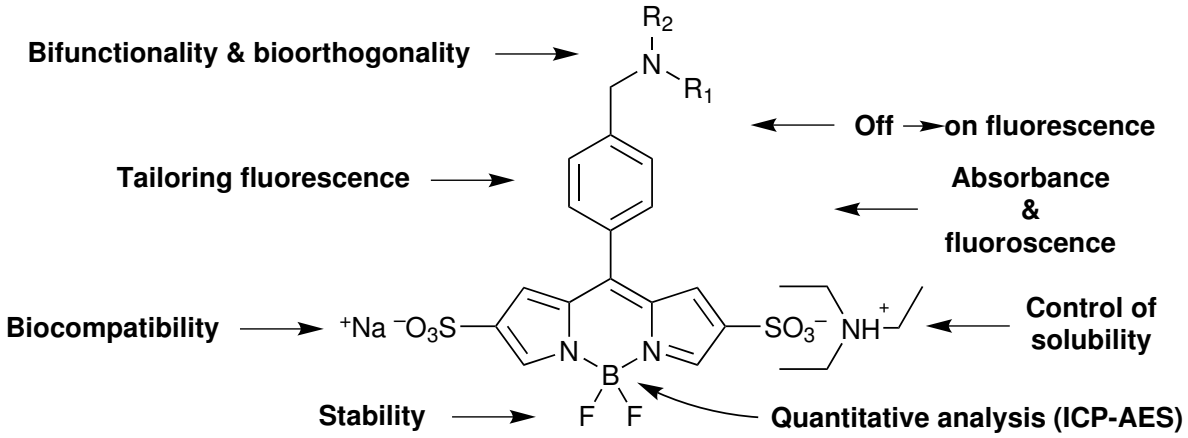

Figure 2.1: Possible modification of BODIPY and accessible analytical methods.

3 | Results & discussion

50 years after the discovery of BODIPY have been reported in the literature only about 30 sulfonated derivatives (see 1.3.4) Furthermore, the majority of them was prepared as a last step in a synthesis, not as a starting point of synthetic pathways. The sulfonated derivatives are reported only scarcely because their synthesis is extremely troublesome and tedious.

In the following text, we describe general and individual procedures for the preparation of water-soluble fluorescent probes based on sulfonated BODIPY.

First, the synthesis and efficient purification of BODIPY dye is described. Then the sulfonation procedure and main challenges in it are explained; the essential part of the procedure is swift and reproducible purification of product. Optimal conditions are no less important for alkylation of amines by BODIPY, these conditions made possible further modification of sulfonated BODIPY.

3.1 Synthesis of BODIPY dye

There are several approaches to prepare aryl-substituted BODIPY, which are described in well known reviews [9, 12, 11]. In our synthesis, we employed three approaches to obtain **B1** (Figure 3.1).

Route A is the most universal [53] and can be used to prepare differently substituted (aromatic) groups, only by preparing differently substituted ketopyrroles [43]. This option proves useful when planning applications of a probe (e.g. preparing *ortho*-metallated complexes (section 3.7 on page 26). Overall yields of **route B** are generally lower as it was demonstrated in [21]. Separation of intermediate product **B0** before complexation with boron trifluoride is counterproductive.

We tried to prepare **B1** from benzaldehyde derivative [84] (**Route B**) and then to employ a common procedure using DDQ and a catalytic amount of TFA. This lead to moderate yields and higher amount of byproducts. Therefore, several synthetic optimisations were needed, which resulted in change of starting reagents and avoided oxidation step (**Route C**). The outcome

was a simplified and more elegant reaction procedure, including extraction of toluene with alkaline aqueous solution to neutralise residues of boron trifluoride. These refinements drastically reduced the mass of byproducts, less than 80 wt% of byproducts compared to **Route B**. Further, the mixture was purified in a single flash chromatography run. We found and optimized crystallisation conditions and obtain a pure product in the form of small orange glittering crystals. These optimised conditions were used to scale up the synthesis to several grams of **B1** per week.

Different changes of colour were observed during reaction: *from transparent to yellowish (after mixing reactants), red to dark red (50 °C), light red (toluene, triethylamine), greenish-like fluorescence of dark purple solution (after adding 3 eq. of boron trifluoride), resulting in a brown tar.*

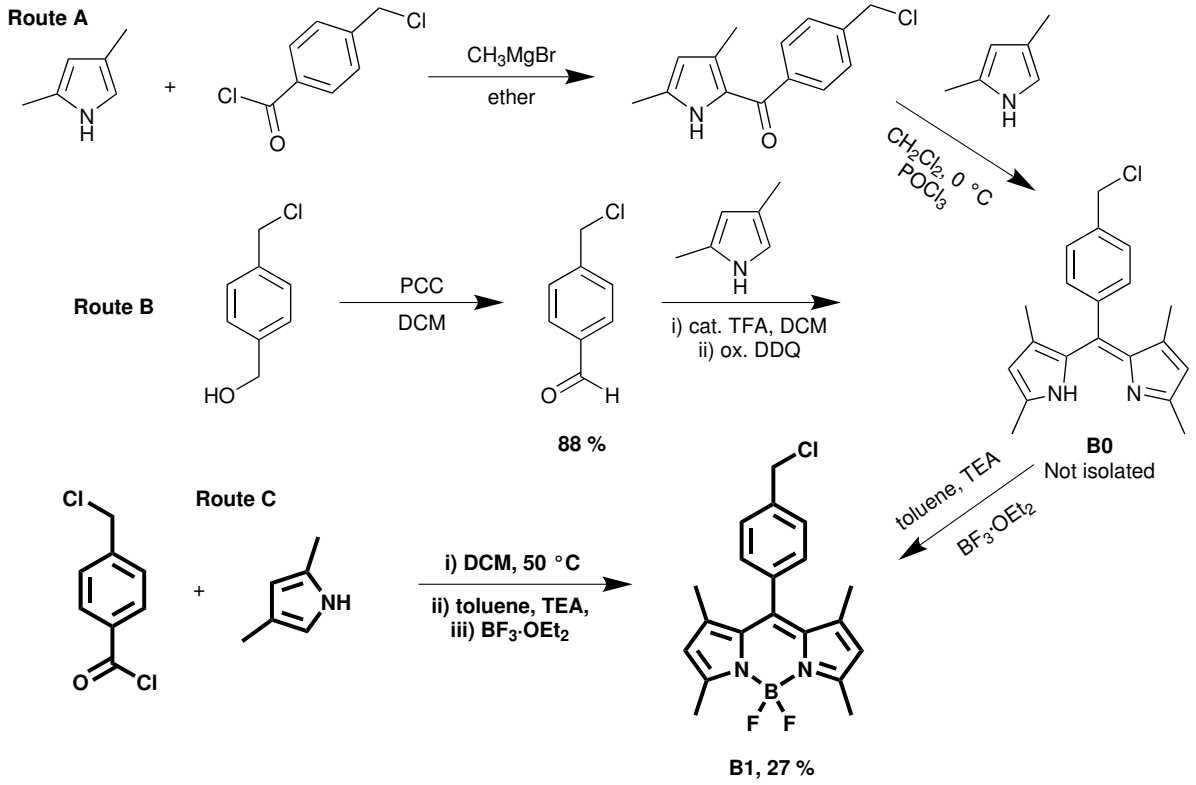

Figure 3.1: Different approaches to the synthesis of **B1**: Routes A, B and C. The latter was fully optimised.

3.2 Sulfonation

Sulfonation required a tedious optimisation to be reproducible and scalable, see section 1.3. We present here a procedure of sulfonation and its workup. Besides, we will mention less efficient or unsuccessful procedures so that they can be avoided. As a base for experimental procedures we used articles from Li *et. al.* (2008, group of Kevin Burgess) [70] and Sauer *et. al.* (2008) [81].

The reaction mixture (product) tends to decompose if it contains sulfonic acid or its derivatives, see section 1.3.4. The solubility of the product is highly affected by available counterions. For these reasons, we decided to prepare several sulfonate salts by neutralisation of reaction mixture with sodium carbonate, DIPEA, TEA, pyridine or Proton sponge (Figure 3.2).

The sulfonation of BODIPY was done using standard Schlenk procedures with dry solvents. Because the dyes have typically very high absorption coefficients, the reactions were performed in dark (the reaction flask was covered with aluminium foil). Products were stored as a solid, or if necessary in solution, then in a freezer. Although it's usually reported that sulfonation can proceed only with a freshly prepared solution of chlorosulfuric acid, we performed the reaction with 3 months old dichloromethane stock solution with uninfluenced yield. On the other hand, BHT was used in excess as radical scavenger; if it was not added, formation of black tar on tube wall was observed. Only vacuum grease was not used, since it is easily dissolved. PTFE head inserts or joint sleeves were used strictly — for the reactions as well as for storing dry solutions and solvents.

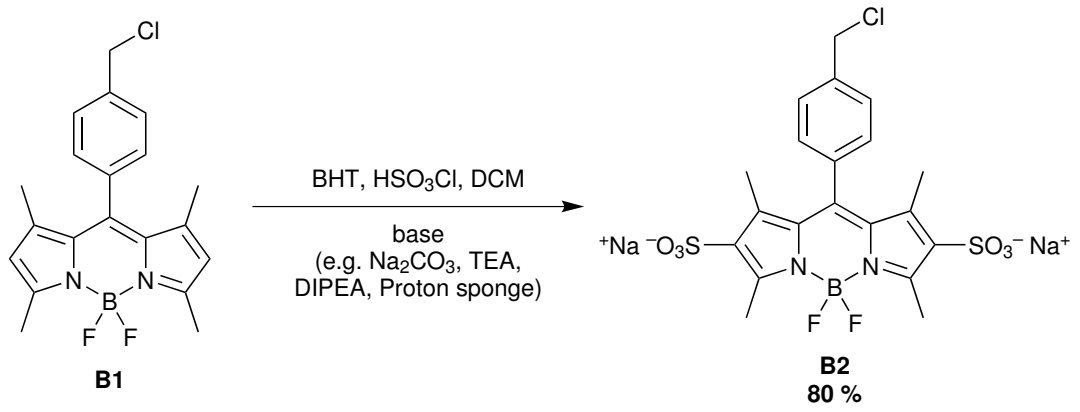

Figure 3.2: Preparing **B2** by optimised sulfonation procedure.

3.2.1 Procedure of sulfonation

The optimised reaction setup of the sulfonation (Figure 3.2) is quite simple — reactants in a reaction flask were dried in vacuum for 1 hour, the reaction flask was covered with an aluminium foil and reactants were dissolved in dichloromethane. Then the reaction flask was cooled down to –40 °C followed by the addition of diluted chlorosulfuric acid. Slowly, the cooling bath was heated up to 0 °C and the formation of precipitate was observed. Suspension was diluted with dichloromethane and transfered to S4 frit* by a double-tip needle. The suspension was dispersed and rinsed on a frit three times with dichloromethane. Then the filter cake was dissolved in a more polar solvent (ACN or DMF) and neutralised with the excess of sodium carbonate.

*Also referred to sintered glass filter

A more complex apparatus (addition funnels, glass bend adaptors) lead to leaks, as well as any glass joints with grease tend to *freeze*, especially while rotating the bottom of flask up, so the suspension would be transffered to frit. Reactants and apparatus must be thoroughly dried, especially when performing reaction in smaller amounts. Therefore, a simple Schlenk tube or just a tube with Suba-seal® septum with an argon needle provided the best results.

The reaction proceeds quantitatively around –30 °C. If cooled below this temperature, chlorosulfuric acid can be added in a swift manner. Then the temperature is slowly risen to 0 °C ca. in 30 minutes, until the formation of a precipitate is completed[†]. If not heated further, the reaction reaches equilibrium without any observable decomposition, only the particles of precipitate enlarge. The precipitate is usually too fine to be filtered out after less than 30 minutes. Optimal time for reaction was found about one hour.[‡] The smallest amount of solvent as possible was used achieve maximal conversion. On the other hand, for the most efficient filtration of reaction mixture was the volume of solvent in reaction flask to doubled or tripled prior filtration. This cause

i) smaller losses of the product,
ii) better extraction of BHT and mixture of acids,
iii) prevents the creation of a sinter on a frit.

The latter is important for rinsing precipitate on a frit, thus washing away byproducts and later reduce neutralisation heat. A supernatant should be yellowish-to-dark red and be disposed after slow neutralisation. The precipitate can be dissolved in a polar solvent and flushed down into a flask with the solution of sodium carbonate or dissolved in a polar solvent with a base. Neutralisation takes approximately 1 hour and a gas is evolved. At this point, the evaporating or lyophilising the mixture lead to a virtually pure product with some excess of base (purity more than 90 %, depending on filtration and rinsing step). Purification of this mixture can be tricky and lead to side products, as will be explained in the following section 3.2.2.

3.2.2 Separation of sulfonated BODIPY

Many separation approaches were tried: filtration, crystallisation or precipitations, ion exchange, chromatography on alumina, silica gel, and finally, reverse phase, which solved practically all separation problems.

Different approaches in separation

Simple filtration of a reaction mixture is sufficient, if some impurities are tolerable and the mixture will be used immediately in the next reaction step. Evidently, the mass of the product

[†]The aluminium foil should not be removed before filtration, even cooled solution with acid can degrade in light in 20 minutes. Once the mixture of acids in the reaction mixture is removed, the product is more stable.

[‡]If the temperature exceeds +10 °C, decomposition and lower yields were observed.

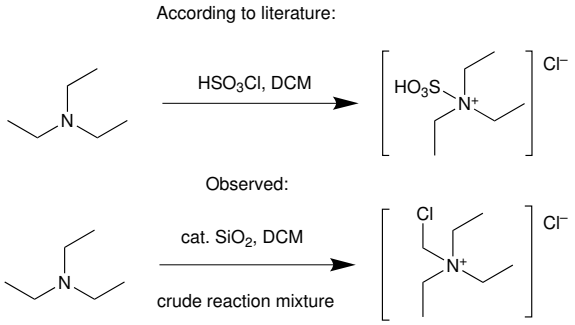

Figure 3.3: Byproducs of sulfonation and separation.

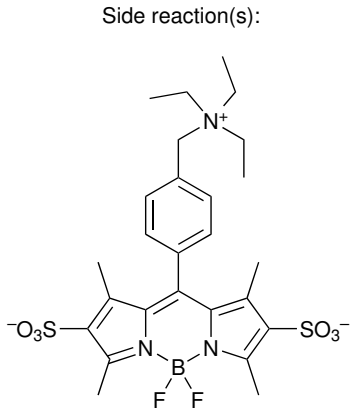

Figure 3.4: One of observed side reactions.

can be only guessed since some excess of a salt is present.

Suitable conditions for the precipitation were not found. Several attempts were done with a strongly acidic macroreticular cation exchange resin, giving poor purification results, low loading capacity and permanent staining of resin with dyes. It is also a time-consuming method, in comparison to flash chromatography. Although TLCs seemed promising, the use of basic or neutral alumina with and without modifier, resulted in tailing and significant losses of product.

Huge effort was invested into silica gel separations; especially, using triethylamine as a counterion which has optimal solubility for further organic synthesis. Due to low solubility, the liquid load was out of question. Solid load on silica gel (1:5 ratio of mass mixture:silica gel) was followed by isocratic and/or gradient flash chromatography in DCM/EA:MeOH and brought mediocre result. The compound was pure enough for the synthesis and NMR characterisation, but not for elemental analysis or even biochemical experiments.

The yield varied significantly (from 5 to 85 %) on the reaction setup and workup. Seek of responsible step for loss of product exposed several weaknesses of whole procedure. While searching for the step responsible for a product loss we revealed weaknesses of the whole procedure. Firstly, a significant loss of sulfonated compound was observed after loading the mixture into silica gel. This lead to a speculation that the sulfonated mixture degrades. Fumes were observed during workup after the addition of non-dried dichloromethane (for extraction). This would indicate, that the reaction mixture neutralized by the excess of triethylamine contained byproducts which can degrade the product.

The search through the literature revealed several articles addressing the unanticipated reaction of triethylamine with chlorosulfuric acid (Figure 3.3, top) [85]. Also the reaction of silica gel with chlorosulfuric acid [86] and catalysis done on sulfonated silica gel was described [87], which explained the decomposition of the reaction mixture. Alkylation of triethylamine by a fragment of dichloromethane was observed (Figure 3.3, bottom) [88, 89], as well as side reaction

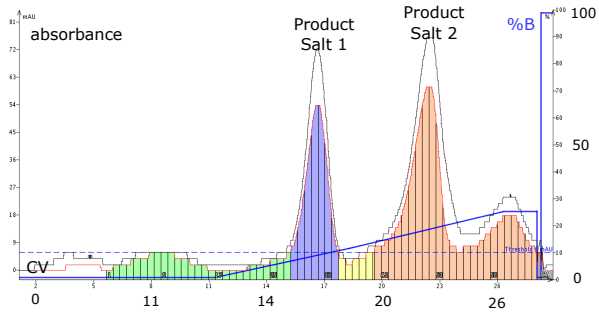

Figure 3.5: RP chromatography of single chemical species with different counterions.

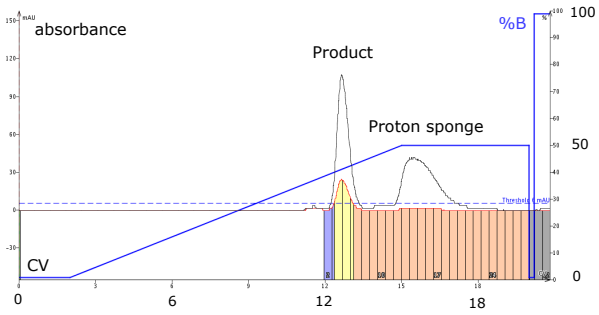

Figure 3.6: Chromatogram of RP chromatography used for ion exchange – desalination. Collected yellow fraction containing products equals ~30 ml.

triethylamine with BODIPY (Figure 3.4 on the preceding page).

This was, unfortunately, an excellent demonstration of a bad choice of triethylamine as a counterion in the first place. From this, we made remedy and finally stopped using silica gel and acquired reverse phase column for flash chromatography.

Purification and ion exchange protocol using RP

The use of a reverse phase (RP) solved problems, which ion exchanger failed surprisingly to solve, such as complete exchange of ions. Furthermore, RP combined functions, in principle inaccessible to an ion exchanger:

- Salt of choice e.g. Na $\rightleftharpoons$ TEA $\rightleftharpoons$ Protonsponge.
- Easy loading in water, even in larger volumes. In the case of bad solubility was sufficient to add few drops of methanol.
- Fast, reliable salt exchange & chemical purification of sulfonated product in one (max. two) flash chromatography runs.
- Sharp elution bands (less than 1CV = 45 ml) by application of MeOH gradient.
- Desalination.
- Cheap and scalable purification.
- Freeze-drying gives stable and easy-to-handle powder.

This brings a significant enhancement to the whole purification process and can be easily generalised. The optimised procedure is rather trivial: add into water 0.1 % modifier of choice (base or buffer: e.g. DIPEA, ammonium formate), precondition column with 95 to 100 % of water, load a sample dissolved in water. The crucial part is to wash the column with 3-10 CV of water (Figure 3.5). The progress of ion exchange can be sometimes observed visually on the column, even if not, apply gradient and collect product with a single counterion. Sometimes this can lead to worse separation resolution, if product is not pure enough, the removal of polar

solvent (methanol) and loading the sample again without isocratic part solved problem, as showed in figure 3.6 on the previous page. Freeze-drying yields the final product as stable, light powder. Volatile compounds (e.g. DIPEA, ammonium formate) were in this way also removed. For biochemical applications the same procedure can be applied using preparative HPLC.

3.3 Comparison of non-sulfonated and sulfonated BODIPY

In the course of work, crystal structures of non-sulfonated (**B1**, Figure 3.7) and salt of (1,8-bis(dimethylamino)naphthalene and propargylamine of sulfonated (**B2**, Figure 3.8) BODIPY were obtained. By comparing both structures, no significant changes were found (for data of X-ray analysis see table 5.1). Length of bonds are nearly the same, angles between planes of BODIPY core and aromatic substituent in *meso*-position differ slightly, changing from 84° (**B1**) to 88°(**B2**).

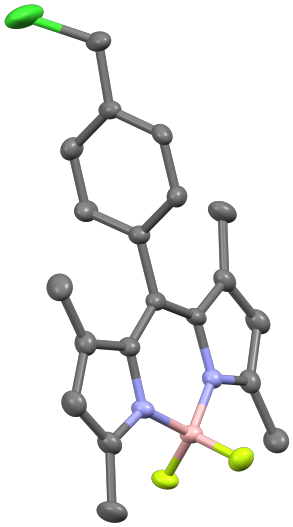

Figure 3.7: Non-sulfonated BODIPY **B1**

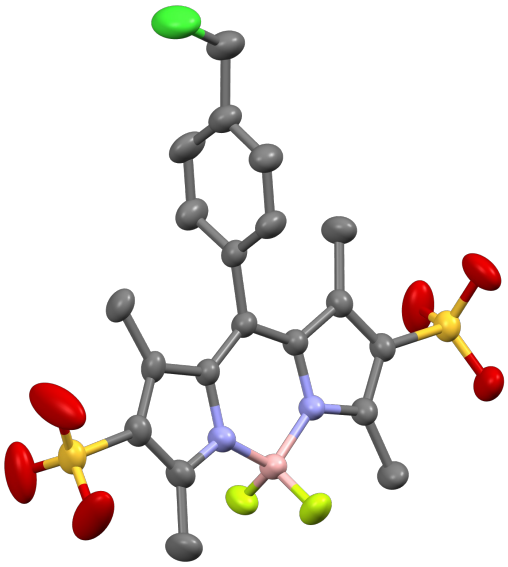

Figure 3.8: Sulfonated BODIPY **B2**

Sulfonation did not significantly affected fluorescent spectra, since there is a minimal overlap between delocalised electrons of BODIPY core and sulfonate group (see figures 3.9 and 3.10). Fluorescent spectra of **B1** and **B2** in methanol (Figure 3.9) and water (Figure 3.10) were measured, although solubility of **B1** in water is poor. Red shift of absorption, excitation and emission spectra from **B1** to **B2** is measurable, but small. Similar red shift was also reported in the literature [70]. The most significant change of em. maxima is in methanol (8 nm), while in water it is decreased (3 nm) (Table 3.1 on the facing page). Stokes shift of **B2** is slightly increased, about 1-2 nm.

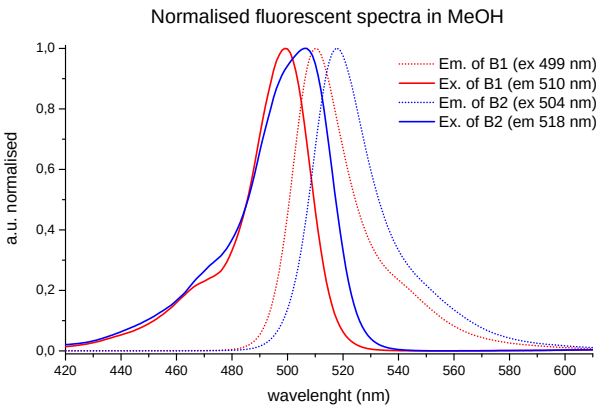

Figure 3.9: Fluorescence spectra of **B1** and **B2** in methanol.

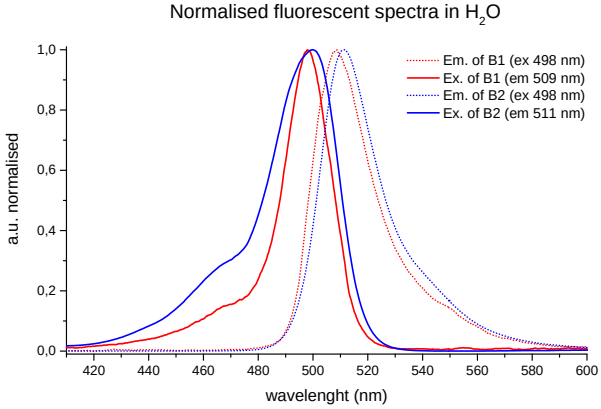

Figure 3.10: Fluorescence spectra of **B1** and **B2** in water.

compound	λ_{max} (abs) (nm)	λ_{max} (ex) (nm)	λ_{max} (em) (nm)	solvent
B1	499	499	510	MeOH
B2	504	506	518	MeOH
B1	499	498	509	H_2O
B2	498	500	511	H_2O

Table 3.1: Spectroscopic properties of **B1** and **B2** in different solvents.

3.4 Alkylation by sulfonated BODIPY

Alkylation of amine with chloromethyl group turned out to be more complex. As a first approach, a small amount of **B2-DIPEA** or **B2-TEA** was dissolved in ACN with amine (dimethylamine or propargylamine). The reaction was monitored on TLC, followed by chromatography on silica gel, but the product was not isolated. Because of low reactivity, chloride was substituted by iodide, prior to the addition of amine. Formation of precipitate was observed and low conversion of **B7** was confirmed in the mixture dry of ACN and MeOH. In other solvents was observed none reaction (ACN, DMF, MeOH) at 50°C.

More thorough approach was chosen using the well-defined **B2-Na** and HPLC confirm zero conversion in different solvents and temperatures even with more than 20 equivalents of amine. This fact revealed a fundamental flaw in the approach — the buffering capacity of counterion of sulfonate group. Simple addition of stronger base (1,8-bis(dimethylamino)naphthalene, commercially called Proton sponge) deprotonated the amine and reaction proceed as was originally expected. Reactions (Figure 3.11 on the next page) were carried out using crude mixture of sulfonated **B1** neutralised by 1,8-bis(dimethylamino)naphthalene, or by the addition of the excess of 1,8-bis(dimethylamino)naphthalene to purified **B2-Na**. We obtained reasonable yields which varied from 10 to 50 % (Sections 3.5, 3.6, 3.7).

Despite a high excess of amine, part of amine in reaction mixture is probably protonated

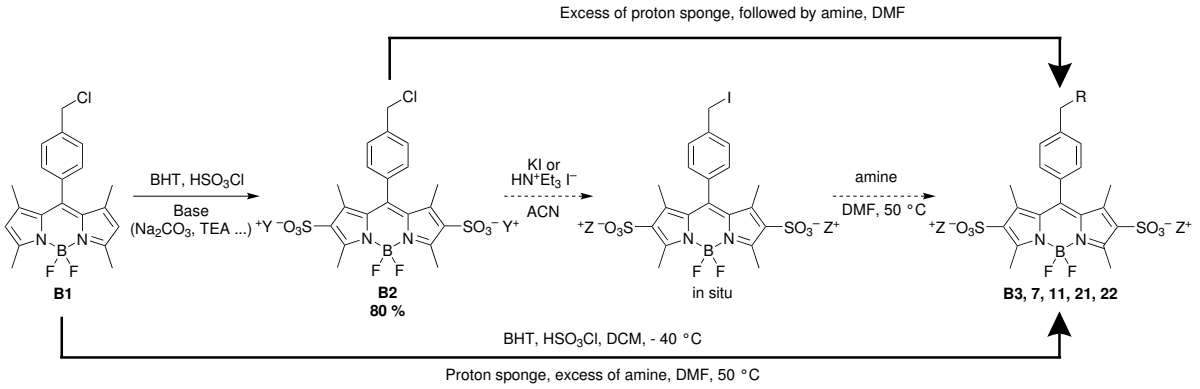

Figure 3.11: Different pathways to alkylate primary or secondary amines with sulfonated BODIPY, Y^+ = used protonated base after sulfonation, Z^+ = mixture of salts used in previous steps together with used amine.

and therefore it is unavailable for alkylation. This can turn to a disadvantage for more precious amines. Stronger base (1,8-bis(dimethylamino)naphthalene) was used as a compromise between basicity and solubility. This can be partially circumvented by ion exchange at RP chromatography, efficiently recovering unreacted amine.

3.5 Monofunctional probe

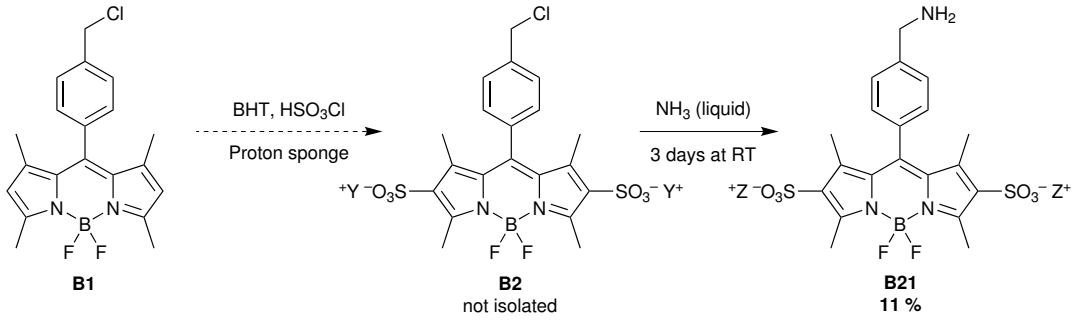

Figure 3.12: Preparation of **B21**, Y^+ = [Proton sponge-H]$^+$, Z^+ = NH_4^+.

We pursued several objectives while preparing compound **B21** (Figure 3.12). Firstly, we wanted to create a showcase of a simple, small, water-soluble monofunctional probe. Secondly, we aimed to prepare a fluorescent probe with short, electronically disconnected link, which can be easily introduced into biomolecules or polymers. From the experimental point of view, we wanted to find an optimal counterion and procedure, which would be suitable for spectroscopic measurements.

From the experimental point of view, we wanted to find an optimal salt and its purification suitable for spectroscopic measurements and to develop a purification protocol.

We started from reported procedures using coincident aromatic substituents with good yields and performed reaction in condensed ammonia [90, 91]. We avoid competitive hydrolysis by performing reaction at RT in pressure flask with dried ammonia.

Low yield of preparation of **B21** was caused by several factors, i) reaction scheme, which at that time consisted from two consecutive reactions without separation, ii) mishap by use of triethylamine as modifier during flash chromatography, which with ellegantly demonstrated the ability of to alkylate triethylamine by primary amines (**B2**), as mentioned before in section 3.2.

Repeated purifications using flash chromatography and HPLC, in order to establish optimal purification procedure, decreased the final yield. Sufficient amount of sample was obtained for characterisations and ongoing collaborative experiments.

3.6 Heterobifunctional probes

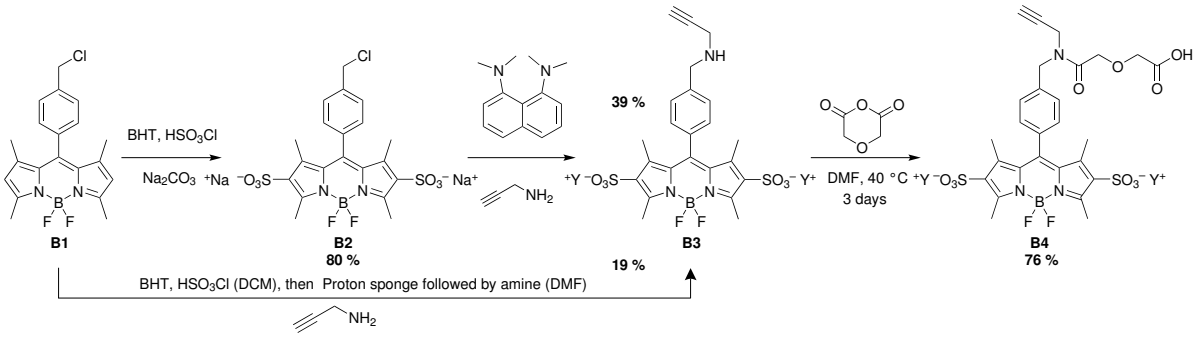

Figure 3.13: Synthesis of **B4** and **B5**, where Y = $[TEA\text{-}H]^+$.

The first heterobifunctional probe (Figure 3.13) was prepared in several steps. First, alkylation of propargylamine provided compound **B3** with reasonable yield by separation after sulfonation of **B1**. The second functional group was introduced to **B3** by a reaction with diglycolic anhydride (DGA). Due to poor solubility of **B3** in ACN or less polar solvents (toluene, THF), those solvents were not used, although good yields were reported [92, 93]. Slightly lower yield [94] (76 % in comparison to 87 %) of target compound **B4** was obtained. Purification of the product was done using gradient flash chromatography with 0.1% of ammonium formate in water as a mobile phase modifier, obtaining pure **B4** in form of ammonium salt without any further problems.

Evaporation residue was dissolved in water (5 ml) and loaded on a reverse phase column (C18, 40 g, H_2O:MeOH, gradient from 100 % to 55 % of H_2O) using 0.1% ammonium formate in water as a mobile phase modifier. Title product **B4** (35 mg, 0.050 mmol, 76 %) was preconcentrated on rotary evaporator, lyophilised and obtained as orange powder.

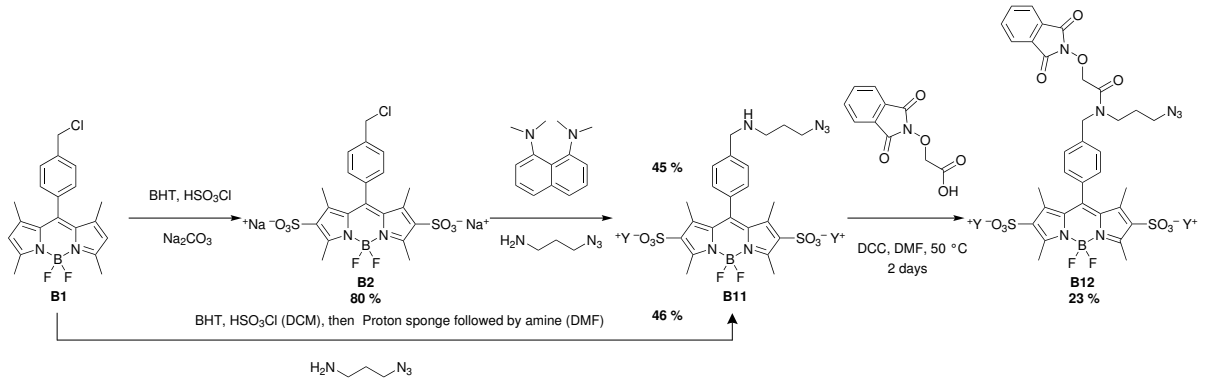

Figure 3.14: Synthesis of **B11** and **B12**, where Y = $[TEA\text{-}H]^+$.

Preparing heterobifunctional probe containing azide group and reactive aminooxy group (**B12)** turn out to be more complicated. Same yields of **B11** were obtained regardless to the alkylation procedure since alkylation procedure of **B2** was optimised. Unambiguous assignment of signals in proton and carbon spectra was done with due to HMBC and HSQC experiments.

Amide coupling of aminooxy acetic acid with secondary amine turn out to be tricky. Jakub Hývl, previous membrer of the group, done similar (unpublished) work on non-sulfonated BODIPY, which was excellent starting point. Use of standard coupling procedures with 1-hydroxybenzotriazole, TBTU or TSTU, no product was observed. Surprisingly, the most simple conditions were required for successful preparation of **B12** using DCC and 2-((1,3-dioxoisoindolin-2-yl)oxy)acetic acid. Keeping in mind high reactivity of aminooxy group towards aldehydes and ketones, triethylammonium formate (TEAF) buffer was used and removal of solvent was done carefully with rotary evaporator followed by freeze-drying. On the other hand, removing DCU using RP was simple. Reactions were so far done in small scale and small amount of **B12** was prepared (2 mg). Exchange of isoindoline-1,3-dione for proton in hydrazine was not performed, since such small amount of **B12** would easily react with impurities in solvents forming corresponding oxime.

3.7 Off/on probes

Synthetic pathway showed in 3.16 aims to prepare water-soluble probe for selective imaging of CO in living cells [1]. In the previous work, the reported synthesis was successfully reproduced, indicating the increase of fluorescence roughly by two orders of magnitude. In connection with minimal change of fluorescent properties (Section 3.3), this nudge us to prepare similar complex with sulfonated BODIPY. Unfortunately the reaction conditions could not be simply transferred. Different approach for ortho-palladation as well for separation was employed.

During alkylation of $HN(CH_3)_2$, byproduct **B22** was also prepared. This was probably caused by small excess of dimethylamine or better availability of **B7**, which underwent second alkyla-

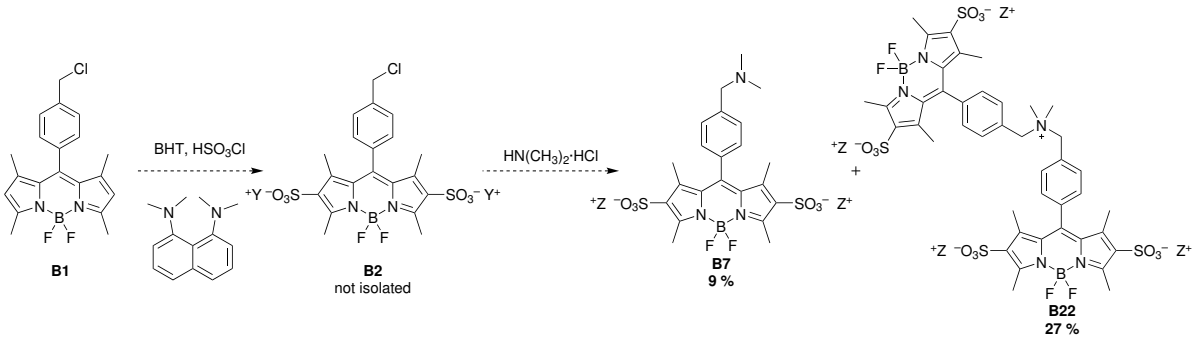

Figure 3.15: Synthesis of **B7**, where Y^+ = [Proton sponge-H]$^+$, Z^+ = [TEA-H]$^+$.

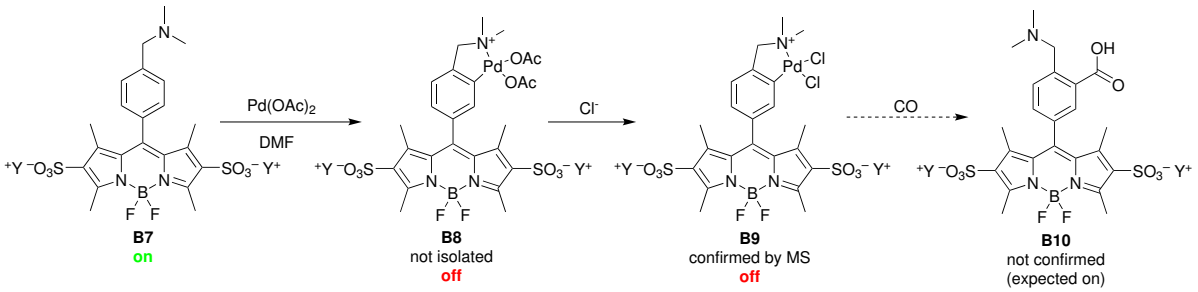

Figure 3.16: Attempts on synthesis of compounds **B7-10**, where Y = [TEA-H]$^+$.

tion (Figure 3.15). Lesson was taken from this reaction and for similar reactions, reactant **B2** was slowly added into excess of deprotonated amine.

The preparation of **B8** or **B9** was due to insolubility unfeasible in hexane and optimal condition for the reaction in polar solvents were needed to be found. From several selected papers, procedure from Cope *et. al.* [95] was relevant, since it employed the reaction in polar solvents. Reported Pt and Pd complexes were even separated on silica gel without decomposition. Series of reactions were done in order to find optimal conditions for ortho-palladation (Figure 3.16). Different solvents, temperatures (RT or 50 °C) and reactants were mixed in NMR tubes, monitoring conversion by changes in proton spectra in aromatic region. As apparent from table 3.2, feasible conditions employed DMF at 50 °C with $Pd(OAc)_2$ in less than 3 hours — in other reactions was observed no conversion after 3 days at 50 °C.

Reaction conversion was monitored by NMR spectroscopy and product was confirmed by MS spectroscopy. Unfortunately, impurities in reaction mixture prevented to assign signals unambiguously. Separation using silica gel as well as reverse phase were done with partial success. Amount and purity of isolated product were not sufficient for spectroscopic characterisation and preparation of intended compound **B10**.

Solvent \ Reagent	$PdCl_2$	Na_2PdCl_4	$Pd(OAc)_2$
H_2O	✗	✗	✗
MeOH	✗	✗	✗
ACN	✗	✗	✗
DMF	✗	✗	✓

Table 3.2: Search for feasible conditions for ortho-palladation at RT or 50 °C for 3 days.

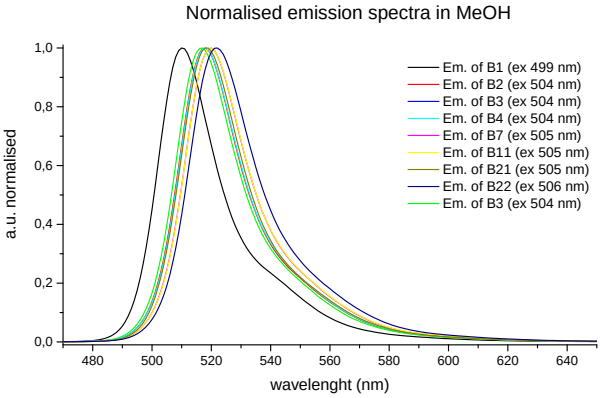

Figure 3.17: Normalised emission spectra of prepared compounds in MeOH.

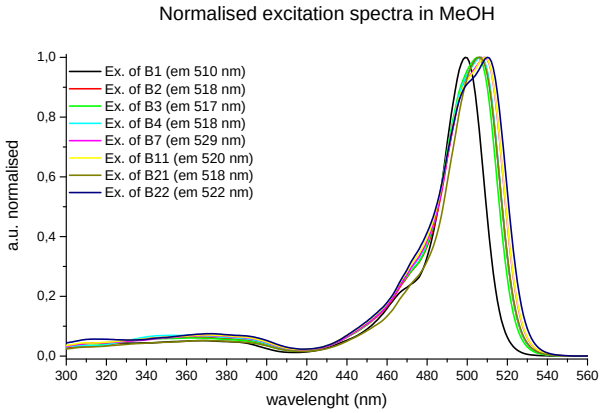

Figure 3.18: Normalised excitation spectra of prepared compounds in MeOH.

3.8 Fluorescent spectra of prepared compounds

Brief look at fluorescent spectra (Figures 3.17,3.18,3.19,3.20) of prepared derivatives of BODIPY are very similar in excitation or emission spectra. Measured maxima of prepared compounds in different solvents are listed table 3.3. There are two molecules, which elude from previous – non-sulfonated BODIPY **B1**[§] and dimer **B22**.

[§]Due to poor solubility in water, quality of emission and excitation spectrum is poor, absorption was not possible to measure.

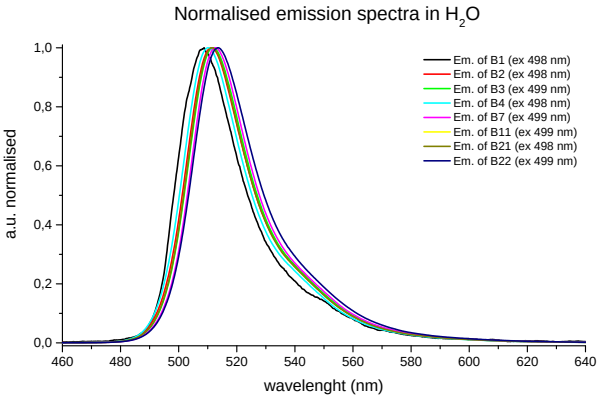

Figure 3.19: Normalised emission spectra of prepared compounds in water.

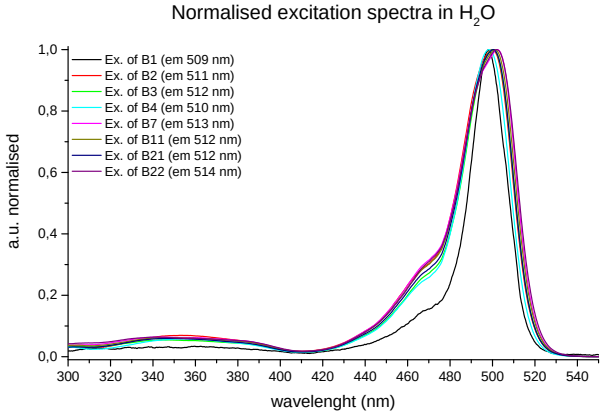

Figure 3.20: Normalised excitation spectra of prepared compounds in water.

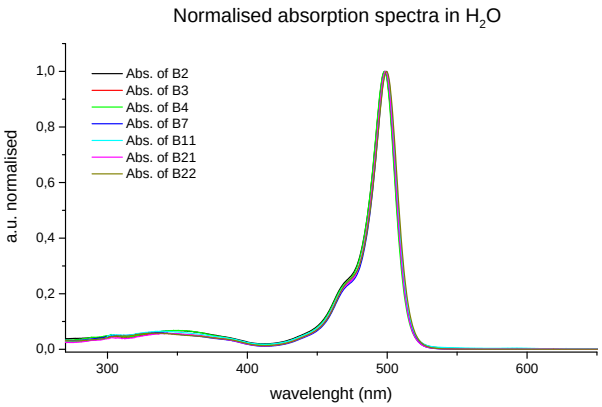

Figure 3.21: Normalised absorption spectra of prepared compounds in water.

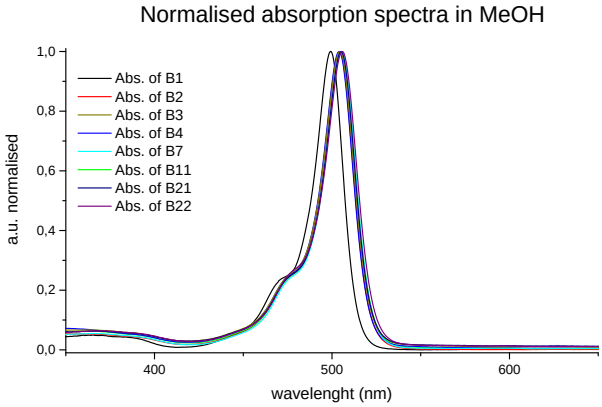

Figure 3.22: Normalised absorption spectra of prepared compounds in water.

In figure 3.17, emission spectra in MeOH of two compounds slightly differ from the rest, band of non-sulfonated **B1** is shifted to the blue region (left), and sulfonated dimer **B22** is red shifted (right). The same trend can be observed in excitation spectra in MeOH (Figure 3.17). Moreover, new shoulder is observed for **B22**. This could indicate slight interaction between two BODIPY cores, but calculation is needed to support such claim. Emission spectra in figure 3.17 are more similar to each other, although the same tendency can be observed for **B1** and **B22**. On the other hand, excitation spectra in figure 3.20 are resembling each other, only shoulder of **B1** have lower intesity, but shift is almost identical and no tendency can be attributed to substitutions. Normalised absorption spectra in methanol are same with exception of non-sulfonated **B1** (Figure 3.22) and absorption spectra in water are identical as apparent from figure 3.21).

This confirm expectations, that influence from substituting electronically disconnected part of molecule has virtually no effect on absorption and fluorescence spectra in water and slight effect on spectra measured in MeOH.

compound	λ_{max} (abs) (nm)		λ_{max} (ex) (nm)		λ_{max} (em) (nm)	
	MeOH	H_2O	MeOH	H_2O	MeOH	H_2O
B1	499	499	499	498	510	509
B2	504	498	506	500	518	511
B3	505	499	508	499	519	511
B4	505	498	506	498	518	510
B7	506	500	509	502	520	513
B11	505	499	509	501	520	512
B21	505	499	506	501	518	512
B22	506	500	510	502	522	513

Table 3.3: Spectroscopic properties of prepared compound in water and MeOH.

4 | Experimental part

4.1 Material and methods

4.1.1 General information

Chemicals and solvents used were supplied by Sigma-Aldrich, Penta, Merck, Lachema, Fluka, Acros Organics, Fisher Scientific, Linde or Chemotrade and used as received. Solvents (e.g. toluene, DCM, MeOH) were dried by standard procedures [96] or bought dry (DMF) and then used without further purification.

Reactions were performed under standard inert atmosphere techniques [97] with argon (99.996%). Reported compounds were identified by their NMR spectra (often in different solvents, as explained in section 4.1.4. For unambiguous determination, HR-MS was used. Using ESI ionisation sulfonated BODIPY derivatives exhibited m/z peaks $[M + H]^-$ and $[M]^{2-}$.

Unless stated otherwise, reaction temperatures were measured directly in aluminium block or in ethanol-dry ice cooling bath. TLCs were carried out on Merck TLC Silica gel 60 F_{254} (silica gel on aluminium foil) and spots were detected by UV-lamp ($\lambda = 254$ nm). Interpretation of TLC in case of sulfonated derivatives was done with caution, accompanied always by other analytical method. Retention factors R_f given in experimental part were estimated from freshly dissolved samples – purified dye loaded in low concentration. Reaction monitoring was usually done with two spots, one with low and the other with high concentration of mixture, in order to rule out phantom spots.

Yield were determined for lyophilised samples, purity was checked by NMR spectroscopy.

4.1.2 List of Instruments

- Centrifuge: Eppendorf centrifuge 5430
- Chromatography: Biotage Isolera Prime and Buchi Sepacore® X50
- Fluorescent spectrometer: JASCO FP-6600 Spectrofluorometer

- HPLC: 515 HPLC pump with 2996 PDA (Waters)
- LC-MS: Shimadzu LC-MS 2020
- Lyophilisation: Labconco CentriVap Cold Trap
- MS: Q-TOF Micro (Waters)
- NMR: Bruker Avance III™HD 400 MHz equipped with Prodigy cryo-probe
- Sonicator: Elmasonic P60 H
- Spectrophotometer: Specord 250 Plus

4.1.3 Purification and separation methods

Removal of volatile solvents (e.g. HEX, DCM, MeOH) was done using rotary evaporator, less volatile compounds (e.g. water, DMSO, formic acid) were removed using lyophilisation. Flash chromatography separation were done using Biotage Isolera™ Prime or Buchi Sepacore® X50 system in case of compound **B1**. For SiO_2 separations prefilled Biotage SNAP Cartridges (HP-SIL) or self-packed columns filled with Merck silica gel 60 (0.040 – 0.063 mm) were used. For purification on revere phase column Reveleris™C18 Flash Cartridge was used. Ion exchanger Amberlyst 15 from Fluka was used. HPLC separations were carried out using Phenomenex® synergi Polar-RP column and for preparative HPLC purification was used YMC-Pack ODS-AM (C18, Silica) column.

4.1.4 NMR spectroscopy

Spectra were measured using Bruker Avance III™ HD 400 MHz equipped with Prodigy cryo-probe in several deuterated solvents due to great difference in solubility for different salts, as follows: D_2O (99,8 % D, Armar Chemicals), methanol$-d_4$ (99,80 % D, VWR Chemicals), acetone$-d_6$ (99,8 % D, Acros Organics), DMSO$-d_6$ (99,9 % D, Acros Organics), DMF$-d_7$ (99,5 % D, Acros Organics), $CDCl_3$ (99,96 % D, Sigma-Aldrich).

Non-sulfonated compounds were measured in chloroform (e.g. **B1**). Into deuterated water was added 0,25 % of 1,4-dioxane for referencing. In longer period of time (one week), complete exchange of CH_3 for CD_3 in deuterated water (methanol) was observed, for this reason some spectra were also measured in dimethyl sulfoxide.

Spectra of ^{1}H, ^{13}C were referenced to signal from solvent or internal standard as stated in table 4.1. For full assignment of ^{1}H and partial assignment of ^{13}C were used HSQC and HMBC experiments. NMR spectra of ^{19}F and ^{11}B were not referenced by external standard and used for purity control only, although all measured spectra are in good agreement with literature [80, 59, 60].

All chemical shifts δ are given in ppm and coupling constants in hertz [Hz]. Multiplicity of signals was describes as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet),

	TMS	1,4-Dioxane	$CHCl_3$	DMSO	DMF	CD_3OD
δ^1H [ppm]	0.00	3.75	7.26	2.50	8.03	3.31
δ^{13}C [ppm]	0.00	67.19	77.16	39.52	34.89	49.00

Table 4.1: Chemical shifts of internal standards or solvents.

dd (double doublet), tt (triplet of triplets). Multiplicity of signals in ^{19}F was described as non-binomial quartet (1:1:1:1) as is expected from ^{11}B [98].

4.1.5 Single-crystal X-ray diffraction

Measurement of compound **B1** was done using 4-circle diffractometer Bruker NONIUS KAPPA CCD at 150 K using $Mo_{K\alpha}$ ($\lambda = 0{,}71073$ Å) as a source of rays. Crystal structure was measured and solved by RNDr. Ivana Císařová, CSc., verified by doc. RNDr. Jan Kotek, PhD. Data were analysed by HKL package, solution and refining were done by SHELXS97, visualisation was done in Crystal Maker.

The single crystal data **B2** were collected 180K on Xcalibur PX diffractometer with the graphite monochromatized $Cu_{K\alpha}$ radiation ($\lambda = 1.54180$ Å). CrysAlisProCCD [99] was used for data collection, cell refinement and data reduction. The structure was solved by direct methods (SIR92) [100] and refined by full-matrix least-squares based on F with CRYSTALS [101] by Blanka Klepetářová PhD. All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were found on difference Fourier map, but those attached to carbon atoms were recalculated into idealized positions and refined with riding constraints.

4.1.6 Fluorescent spectroscopy

Samples for spectral analysis were dissolved from lyophilised samples (if possible) immediately before measurement. HPLC grade solvents were used (MeOH, DCM) and MilliQ water. Absorption spectra were measured on Specord 250 Plus, fluorescence spectra using JASCO FP-6600 Spectrofluorometer in range 300 – 700 nm. Band width was 1 nm for excitation, 2 nm for emission with high sensitivity and data pitch 1 nm for spectra in methanol, 0.2 nm for spectra in water. Ex/em wavelength was set for maximal intensity and scanning speed was 200 nm/min. Data were processed and normalised in Origin 9.1.

4.2 Synthesis of BODIPY dye

4.2.1 Optimised synthesis of BODIPY B1

Reaction was done by modified and optimised procedure described in literature [81, 70]. Into dried three-neck flask equipped with stirrer, addition funnel and condenser were placed with 2,4-dimethyl-pyrrole (5.28 g, 55.5 mmol, 2.1 eq.) in DCM (200 ml) under argon atmosphere. Then 4-(chlormethyl)benzoyl chloride (5.00 g, 26.6 mmol) was dissolved in 10 ml of DCM and added to reaction mixture. The flask was covered with aluminium foil and reaction mixture was refluxed 12 hours in oil bath at 50 °C. Solvent was distilled of and obtained residue was dissolved in 250 ml of toluene. Triethylamine (14.76 ml, 106 mmol) was added, followed by dropwise addition of boron trifluoride etherate ($BF_3{\cdot}OEt_2$) (19.59 ml, 159 mmol, 6 eq.) with cooling reaction flask in water bath. The reaction mixture was stirred at 50 °C for 4 hours, washed with 200 ml of solution of sodium carbonate and 3×200 ml with water. Organic phases were dried over $MgSO_4$, filtered and adsorbed into approx. 10 g of silica gel followed by slow removal of the solvent. The resulting dark powder was dry-loaded and used as precolumn on flash chromatography with 25g prepacked column.

Gradient chromatography was done using mobile phase HEX:DCM, starting from 100 % to 20 % of HEX yielding dark orange crystalline product. The product was dissolved in DCM (approx. 20 ml) and recrystallised after addition of 250 ml HEX and 100 ml MeOH (for better result mixture was slightly concentrated on rotary evaporator). Title product 1,3,5,7-tetramethyl-8-(4'-(chloromethyl)phenyl)-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (**B1**) was colleted by filtration (3.22 g, 8.64 mmol, 33 %) as orange crystals.

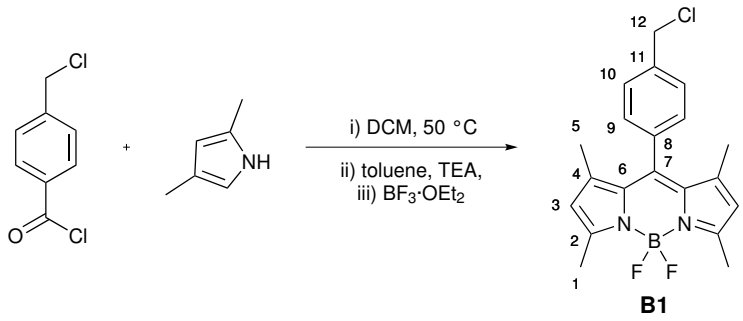

Figure 4.1: Compound **B1**.

Yield: 3.22 g orange crystals (8.64 mmol, 33 %).
TLC: DCM:HEX 1:2 $R_f = 0.22$; DCM:HEX 2:1 $R_f = 0.70$.
^{1}H-NMR (600 MHz, $CDCl_3$): $\delta = 7.52$ (d, 2 H, $^3J_{HH} = 7.6$, $\mathcal{H}^{10}$); 7.29 (d, 2 H, $^3J_{HH} = 7.7$, $\mathcal{H}^{9}$); 5.98 (s, 2 H, $\mathcal{H}^{3}$); 4.66 (s, 2 H, $\mathcal{H}^{12}$); 2.55 (s, 6 H, $\mathcal{H}^{1}$); 1.38 (s, 6 H, $\mathcal{H}^{5}$).
^{13}C-NMR (151 MHz, $CDCl_3$): $\delta = 155.8$ (s, 2 C, $\mathcal{C}^{2}$); 143.2 (s, 2 C, $\mathcal{C}^{4}$); 141.1 (s, 1 C, $\mathcal{C}^{7}$); 138.7 (s, 1 C, $\mathcal{C}^{11}$); 135.2 (s, 1 C, $\mathcal{C}^{8}$); 131.5 (s, 2 C, $\mathcal{C}^{6}$); 129.4 (s, 2 C, $\mathcal{C}^{10}$); 128.6 (s, 2 C, $\mathcal{C}^{9}$);

121.5 (s, 2 C, $\mathcal{C}^3$); 45.8 (s, 1 C, $\mathcal{C}^{12}$); 14.7 (s, 2 C, $\mathcal{C}^1$); 14.6 (s, 2 C, $\mathcal{C}^5$).
^{19}F-NMR (282 MHz, $CDCl_3$): $\delta = -146.3$ (non-binomial q., $J_{FB} = 33$).
^{11}B-NMR (128 MHz, D_2O): $\delta = 0.54$ (t, $J_{BF} = 33$).
MS (ESI): (+) *m/z* calc. for $C_{20}H_{20}BClF_2N_2$: 372.1, found: 373.2 $[M + H]^+$; 395.1 $[M + Na]^+$; 411.0 $[M + K]^+$; 767.2 $[2\,M + Na]^+$.
HR MS (ESI): (+) *m/z* calc. for $[C_{20}H_{21}BClF_2N_2]^+$: 373.14489, found: 373.14499; *m/z* calc. for $[C_{20}H_{20}BClF_2N_2Na]^+$: 395.12683, found: 395.12692.
UV-Vis: 499.0 nm (MeOH); 498.5 nm (H_2O); 502.5 nm (DCM).
Fluorescence: $\lambda_{ex,em}$ 499.0 nm, 510.0 nm (MeOH); $\lambda_{ex,em}$ 498.0 nm, 508.8 nm (H_2O); $\lambda_{ex,em}$ 505.4 nm, 515.4 nm (DCM).

4.3 Sulfonation of BODIPY dye

4.3.1 Synthesis of B2-Na from B1

Into Schlenk tube with stirrer was added **B1** (500 mg, 1.342 mmol) and 2,6-Di-*tert*-butyl-4-methylphenol (BHT) (296 mg, 1.342 mmol). Reactants were washed with dichloromethane (5 ml) into Schlenk tube. Apparatus was dried, reactants were dissolved under Ar atmosphere in dichloromethane (5 ml), flask was covered with aluminium foil and the mixture was cooled down to –40 °C. Chlorosulfuric acid (10 ml, 4.03 mmol, 0.4106M in DCM) was slowly transferred into Schlenk tube via double-tip needle. Cooling bath was left to warm up to –10 °C within 1 hour and formation of orange precipitate was complete.

Reaction mixture was cooled down, suspension was diluted with dichloromethane (40 ml) and reaction mixture was transferred via double tip needle to S4 frit under argon atmosphere with constant flow of dry DCM. Precipitate was resuspended and rinsed three times with DCM, then dried by argon flow. Filter cake was dissolved in dry, precooled acetonitrile and transferred into flask with sodium carbonate (1,42 g, 13.42 mmol) with crushed ice. The mixture was stirred for 3 h, then the solvents were removed.

The resulting mixture was dissolved in water, part of sodium carbonate was precipitated by addition of acetone and filtred out. Solvents were removed, mixture was dissolved in water (20 ml) and liquid-loaded on reverse phase column multiple times (C18, 40 g, H_2O:MeOH gradient from 100 % to 85 % of H_2O). Adequate fraction were preconcentrated on rotary evaporator and lyophilized, obtaining title product **(B2-Na)** (620 mg, 1.076 mmol, 80 %) as orange powder.

Yield: 620 mg bright orange powder (1.076 mmol, 80 %).
TLC: EA:MeOH 1:1 $R_f = 0.89$; EA:MeOH 2:1 $R_f = 0.64$.
^{1}H-NMR (600 MHz, D_2O): $\delta = 7.69 - 7.62$ (m, 2 H, $\mathcal{H}^{10}$); 7.32 – 7.25 (m, 2 H, $\mathcal{H}^9$); 4.76 (s, 2 H, $\mathcal{H}^{12}$); 2.76 (s, 6 H, $\mathcal{H}^1$); 1.63 (s, 6 H, $\mathcal{H}^5$).

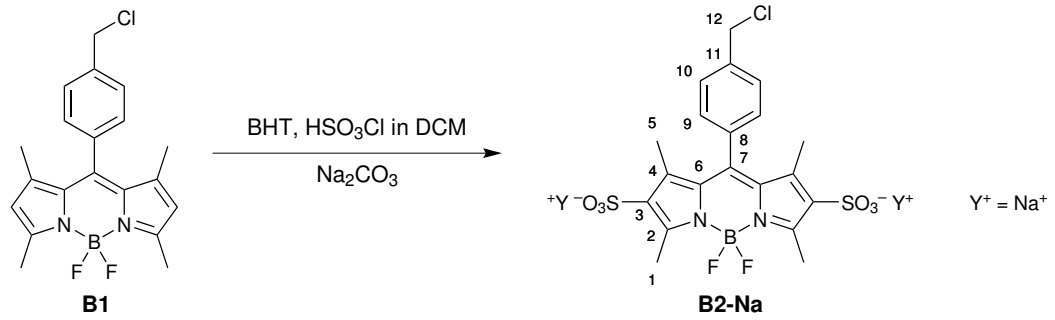

Figure 4.2: Compound **B2-Na**.

^{1}H-NMR (401 MHz, DMSO): δ =7.72 – 7.57 (m, 2 H, $\mathcal{H}^{10}$); 7.46 – 7.33 (m, 2 H, $\mathcal{H}^{9}$); 4.88 (s, 2 H, $\mathcal{H}^{12}$); 2.64 (s, 6 H, $\mathcal{H}^{1}$); 1.50 (s, 6 H, $\mathcal{H}^{5}$).
^{13}C-NMR (101 MHz, D_2O): δ = 155.92 (s, 2 C, $\mathcal{C}^{2}$); 146.89 (s, 1 C, $\mathcal{C}^{7}$); 144.43 (s, 2 C, $\mathcal{C}^{4}$); 140.47 (s, 1 C, $\mathcal{C}^{11}$); 134.00 (s, 1 C, $\mathcal{C}^{8}$); 133.23 (s, 2 C, $\mathcal{C}^{3}$); 131.15 (s, 2 C, $\mathcal{C}^{6}$); 130.57 (s, 2 C, $\mathcal{C}^{10}$); 128.69 (s, 2 C, $\mathcal{C}^{9}$); 46.22 (s, 1 C, $\mathcal{C}^{12}$); 14.19 (s, 2 C, $\mathcal{C}^{1}$); 13.39 (s, 2 C, $\mathcal{C}^{5}$).
^{13}C-NMR (101 MHz, DMSO): δ = 154.3 (s, 2 C, $\mathcal{C}^{2}$); 143.1 (s, 1 C, $\mathcal{C}^{7}$); 139.5 (s, 2 C, $\mathcal{C}^{4}$); 139.0 (s, 1 C, $\mathcal{C}^{11}$); 137.8 (s, 1 C, $\mathcal{C}^{8}$); 134.4 (s, 2 C, $\mathcal{C}^{3}$); 129.8 (s, 2 C, $\mathcal{C}^{10}$); 129.5 (s, $\mathcal{C}^{\mathrm{Ar}}$); 128.2 (s, 2 C, $\mathcal{C}^{9}$); 45.6 (s, 1 C, $\mathcal{C}^{12}$); 13.9 (s, 2 C, $\mathcal{C}^{1}$); 12.5 (s, 2 C, $\mathcal{C}^{5}$).
^{19}F-NMR (377 MHz, D_2O): δ = −141.2 (non-binomial q., J_{FB} = 30).
^{19}F-NMR (377 MHz, DMSO): δ = −142.1 (non-binomial q., J_{FB} = 32).
^{11}B-NMR (129 MHz, D_2O): δ = 0.68 (t, J_{BF} = 33).
^{11}B-NMR (129 MHz, DMSO): δ = 0.59 (t, J_{BF} = 33).
MS (ESI): (−) *m/z* calc. for $[(C_{20}H_{18}BClF_2N_2O_6S_2)^{2-}]$: 530.0, found: 531.0 $[M + H]^-$ 553.4 $[M + Na]^-$; 265.0 $[M]^{2-}$.
HR MS (ESI): (−) *m/z* calc. for $[(C_{20}H_{18}BClF_2N_2O_6S_2)^{2-}]$: 265.01834, found: 265.01816.
UV-Vis: 504.0 nm (MeOH); 498.0 nm (H_2O).
Fluorescence: $\lambda_{\mathrm{ex,em}}$ 506.0 nm, 518.0 nm (MeOH); $\lambda_{\mathrm{ex,em}}$ 499.8 nm, 511.2 nm (H_2O).

Synthesis of B2-DIPEA from B1

Into Schlenk tube with stirrer was added **B1** (509 mg, 1.36 mmol) and BHT (295 mg, 1.34 mmol). Apparatus with an addition funnel and reactants were dried in vacuum for 30 minutes, the argon atmosphere was established. Reactants were dissolved in small amount of DCM (10 ml), stiring was set. Flask was covered with aluminium foil and cooled down to –40 °C. Chlorosulfuric acid (29 ml, 2.93 mmol, 0.10M in DCM) was slowly added with the addition funnel into reaction mixture. Cooling bath was left to warm up to –10 °C within 1 hour, and held at this temperature until formation of orange precipitate was complete.

Reaction mixture was cooled down and transferred via glass bent adapter to S4 frit under argon atmosphere. Precipitate was rinsed three times with DCM. Filter cake was dissolved in

solution of dry DCM (50 ml) with DIPEA (0.5 ml, 2.73 mmol, 2 eq) and was quantitatively transferred with 50 ml of methanol into 250 ml flask. Solvents were removed on rotary evaporator and reaction mixture was purified multiple times on silica gel chromatography (SiO_2, 40 g, EA:MeOH, gradient from 95 % to 70 % of EA).

Volatile solvents were removed on rotary evaporator, obtaining title product **(B2-DIPEA)** (746 mg, 0.943 mmol, 69 %) as orange powder.

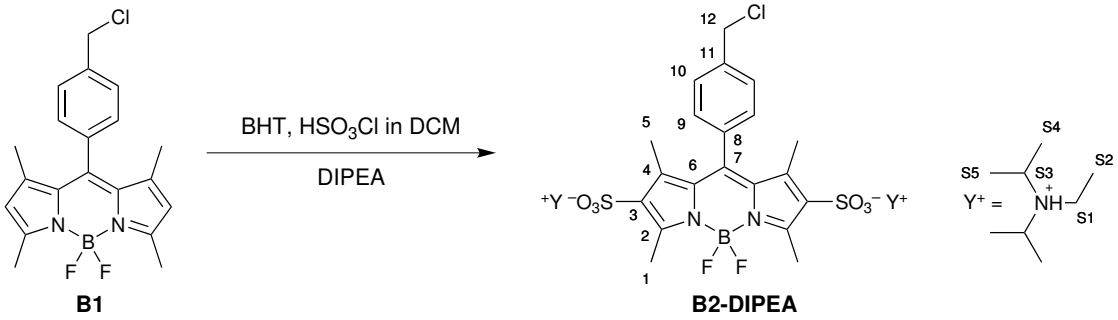

Figure 4.3: Compound **B2-DIPEA**.

Yield: 746 mg bright orange powder (0.943 mmol, 69 %).
^{1}H-NMR (600 MHz, MeOD): δ =7.72 – 7.57 (m, 2 H, $\mathcal{H}^{10}$); 7.46 – 7.32 (m, 2 H, $\mathcal{H}^{9}$); 4.77 (s, 2 H, $\mathcal{H}^{12}$); 3.82 – 3.57 (m, 4 H, $\mathcal{H}^{S3}$); 3.25 – 3.15 (m, 4 H, $\mathcal{H}^{S1}$); 2.79 (s, 6 H, $\mathcal{H}^{1}$); 1.68 (s, 6 H, $\mathcal{H}^{5}$); 1.38 – 1.28 (m, 30 H, $\mathcal{H}^{S2,4,5}$).
^{13}C-NMR (151 MHz, MeOD): δ = 156.75 (s, 2 C, $\mathcal{C}^{2}$); 146.14 (s, 1 C, $\mathcal{C}^{7}$); 143.24 (s, 2 C, $\mathcal{C}^{4}$); 141.48 (s, 1 C, $\mathcal{C}^{11}$); 136.16 (s, 1 C, $\mathcal{C}^{8}$); 135.82 (s, 2 C, $\mathcal{C}^{3}$); 131.63 (s, 2 C, $\mathcal{C}^{6}$); 131.01 (s, 2 C, $\mathcal{C}^{10}$); 129.64 (s, 2 C, $\mathcal{C}^{9}$); 55.85 (s, 4 C, $\mathcal{C}^{S3}$); 46.14 (s, 1 C, $\mathcal{C}^{12}$); 43.82 (s, 2 C, $\mathcal{C}^{S1}$); 55.85 (s, 4 C, $\mathcal{C}^{S3}$); 18.72, 17.28 (2·s, 8 C, $\mathcal{C}^{S4,5}$); 14.51 (s, 2 C, $\mathcal{C}^{1}$); 13.55 (s, 2 C, $\mathcal{C}^{5}$) 13.18 (s, 2 C, $\mathcal{C}^{S2}$).
^{19}F-NMR (282 MHz, D_2O): $\delta = -141.3$ (non-binomial q., $J_{FB} = 32$).
MS (ESI): (−) *m/z* calc. for $[(C_{20}H_{18}BClF_2N_2O_6S_2)^{2-}]$: 530.0, found: 531.1 $[M + H]^-$; 265.0 $[M]^{2-}$.

4.4 Synthesis of alkylated amines

4.4.1 General procedure for sulfonation followed by alkylation of amines

Into Schlenk tube with stirrer was transferred **B1** and BHT and the tube was sealed with septum. Apparatus with reactants was dried in high vacuum for 30 minutes and the argon atmosphere was established. Reactants were dissolved in small amount of DCM (5 – 10 ml), stirring was set. Flask was covered with aluminium foil and cooled down to –40 °C.

Diluted solution of chlorosulfuric acid (from 0.1M up to 0.5M in dry DCM) was slowly transferred via double-tip needle or syringe, in dependence on volume and safety of the work. Cooling bath was left to warm up to –10 °C within 40 minutes until formation of orange precipitate stopped.

Reaction mixture was diluted with DCM to double volume of reaction mixture and cooled down again. Suspension was transferred via double tip needle to S4 frit under argon atmosphere with constant flow of dry DCM from other double tip needle. Precipitate was resuspended and rinsed three times with DCM and dried with flow of argon. Filter cake was dissolved in dry, precooled acetonitrile and transferred into dry flask.

Into clear dark orange solution was added excess of 1,8-bis(dimethylamino)naphthalene, followed in 15 minutes by addition of amine. Reaction mixture was heated at 40 °C until no further conversion of reactant was observed (TLC or HPLC).

Solvent was removed, reaction mixture was dissolved in methanol followed by slow addition of water. Resulting precipitate was removed by centrifugation, solution was concentrated on rotary evaporator and liquid-loaded on reverse phase column (C18, 40 g, H_2O:MeOH, gradient from 100 % to 60 % of H_2O) with or without mobile phase modifier (TEA, ammonium formate). Purified product was preconcentrated on rotary evaporator and dried using lyophilisation.

4.4.2 Synthesis of B3-TEA from B1

Reaction was done in accordance with general procedure of in section 4.4.1.

Into Schlenk tube with stirrer was transferred **B1** (502 mg, 1.348 mmol) and BHT (296 mg, 1.343 mmol). Apparatus was dried, reactant were dissolved in argon atmosphere in DCM (5 ml), covered with aluminium foil and cooled down to –40 °C.

Diluted solution of chlorosulfuric acid (17.80 ml, 4.04 mmol, 0.227M in DCM) was added via syringe. Cooling bath was left to warm up to –10 °C within 30 minutes.

Suspension was transferred via double tip needle to S4 frit with constant flow of dry DCM. Part of a precipitate passed though frit, it was further treated by the same way as main fraction. Precipitate was resuspended and rinsed three times with DCM and dried in flow of argon. Precipitate was dissolved in 20 ml of acetonitrile with 1,8-bis(dimethylamino)naphthalene (1.443 g, 6.73 mmol) and transferred into 100ml flask. Filtration extension was replaced with septum and propargylamine (0.216 ml, 3.37 mmol) was added into transparent dark orange solution . Reaction mixture was heated at 50 °C for 2 days.

Solvent was removed, reaction mixture was dissolved in methanol and byproducts were crystallised by slow addition of water. Precipitate was removed, solution was concentrated on rotary evaporator and liquid-loaded on reverse phase column (C18, 40 g, H_2O:MeOH, gradient from

100 % to 60 % of H_2O) with 0.1 % TEA in both mobile solvents. Solution was preconcentrated on rotary evaporator and title product **(B3-TEA)** was obtained by lyophilisation (74 mg, 0.098 mmol, 7 %) as orange powder and characterised.

Product which passed frit was purified for further spectroscopic measurements two times by flash chromatography on reverse phase column (C18, 40 g, H_2O:MeOH, gradient from 100 % to 70 % of H_2O, 0.1 % ammonium formate in H_2O). Product **(B3-NH$_4$)** was obtained by lyophilisation (87.2 mg, 0.149 mmol, 11 %) as orange powder and characterised.

Synthesis of B3-TEA from B2-TEA

In small vial with prop-2-yn-1-amine (222.9 mg, 4.05 mmol) and 1,8-bis(dimethylamino)naphthalene (121 mg, 0.565 mmol, 3 eq.) were dissolved in DMF. In other vial was dissolved **B2** (100 mg, 0.188 mmol) and of 1,8-bis(dimethylamino)naphthalene (202 mg, 0.942 mmol, 5 eq.) in DMF. Solution of **B2** was slowly transferred into the first vial and heated at 50 °C for 12 hours.

Solvent was removed, resulting mixture was dissolved in methanol and byproducts were crystallised by slow addition of water. Precipitate was removed, solution was concentrated and liquid-loaded on reverse phase column (C18, 40 g, H_2O:MeOH, gradient from 100 % to 50 % of H_2O) with 0.1 % triethylamine in mobile solvent. Separation was done two times. Solvents were removed on rotary evaporator and dried by lyophilisation. Title product was obtained as triethylammonium salt **B3-TEA** (56 mg, 0.074 mmol, 39 %) in form of dark orange powder.

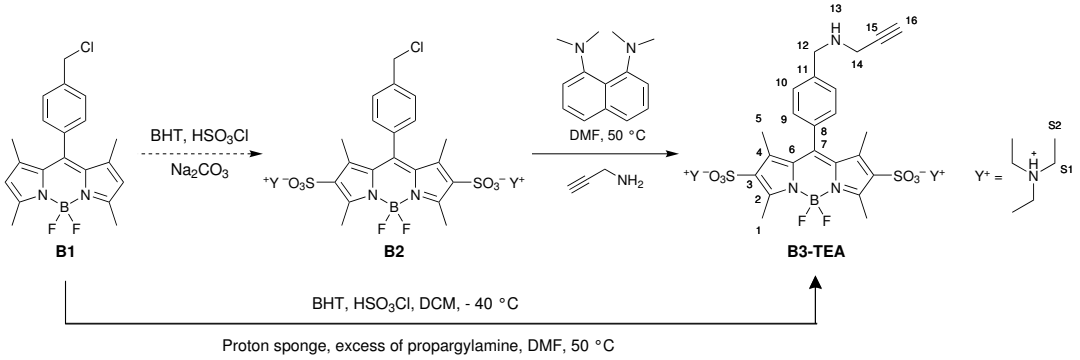

Figure 4.4: Compound **B3-TEA**.

Yield: 55.6 mg orange powder (0.074 mmol, 39 %).
TLC: EA:MeOH 1:1 $R_f = 0.85$; EA:MeOH 2:1 $R_f = 0.15$.
^{1}H-NMR (401 MHz, MeOD): δ =7.76 – 7.65 (m, 2 H, $\mathcal{H}^{10}$); 7.47 – 7.36 (m, 2 H, $\mathcal{H}^{9}$); 4.43 (s, 2 H, $\mathcal{H}^{12}$); 3.94 (d, 2 H, $^4J_{HH} = 2.6$, $\mathcal{H}^{14}$); 3.19 (q, 2 H, $^3J_{HH} = 7.3$, $\mathcal{H}^{S1}$); 3.09 (t, 1 H, $^4J_{HH} = 2.5$, $\mathcal{H}^{16}$); 2.76 (s, 6 H, $\mathcal{H}^{1}$); 1.61 (s, 6 H, $\mathcal{H}^{5}$) 1.27 (t, 3 H, $^3J_{HH} = 7.3$, $\mathcal{H}^{S2}$).
^{13}C-NMR (101 MHz, MeOD): δ = 156.54 (s, $\mathcal{C}^{Ar}$); 146.28 (s, $\mathcal{C}^{Ar}$); 144.37 (s, $\mathcal{C}^{Ar}$); 136.08 (s, $\mathcal{C}^{Ar}$); 134.29 (s, $\mathcal{C}^{Ar}$); 133.27 (s, $\mathcal{C}^{Ar}$); 132.46 (s, 2 C, $\mathcal{C}^{10}$); 131.28 (s, $\mathcal{C}^{Ar}$); 129.77 (s, 2 C,

$\mathcal{C}^{9}$); 79.54 (s, 1 C, $\mathcal{C}^{16}$); 73.89 (s, 1 C, $\mathcal{C}^{15}$); 50.46 (s, 1 C, $\mathcal{C}^{12}$); 47.63 (s, 6 C, $\mathcal{C}^{S1}$); 36.77 (s, 1 C, $\mathcal{C}^{14}$); 14.55 (s, 2 C, $\mathcal{C}^{1}$); 13.63 (s, 2 C, $\mathcal{C}^{5}$); 9.20 (s, 6 C, $\mathcal{C}^{S2}$).

^{19}F-NMR (377 MHz, MeOD): $\delta = -144.30$ (non-binomial q., $J_{\mathrm{FB}} = 32$).

^{11}B-NMR (129 MHz, MeOD): $\delta = 0.69$ (t, $J_{\mathrm{BF}} = 32$).

MS (ESI): (−) *m/z* $[(C_{23}H_{22}BF_2N_3O_6S_2)^{2-}]$: calc. for 549.1, found: 550.2 $[M + H]^-$; 274.6 $[M]^{2-}$.

HR MS (ESI): (−) *m/z* calc. for $[(C_{23}H_{23}BF_2N_3O_6S_2)^-]$: 550.10949 found: 550.10959; *m/z* calc. for $[(C_{23}H_{22}BF_2N_3O_6S_2)^{2-}]$: 274.55111 found: 274.55115.

UV-Vis: 504.5 nm (MeOH); 499.0 nm (H_2O).

Fluorescence: $\lambda_{\mathrm{ex,em}}$ 508.0 nm, 519.0 nm (MeOH); $\lambda_{\mathrm{ex,em}}$ 499.4 nm, 511.2 nm (H_2O).

4.4.3 Synthesis of B4-NH$_4$ from B1

Into vial equipped with stirrer was added **B3-TEA** (50 mg, 0.066 mmol) and excess of diglycolic anhydride (77 mg, 0.663 mmol, 10 eq.) and dissolved in 3 ml DMF. Argon atmosphere was established and reaction mixture was stirred for 3 days at 40 °C. Solvent was removed, evaporation residue was dissolved in water (5 ml) and loaded on reverse phase column (C18, 40 g, H_2O:MeOH, gradient from 100 % to 55 % of H_2O) using 0.1% ammonium formate in water as a mobile phase modifier. Title product **B4** (35 mg, 0.050 mmol, 76 %) was preconcentrated on rotary evaporator, lyophilised and obtained as orange powder.

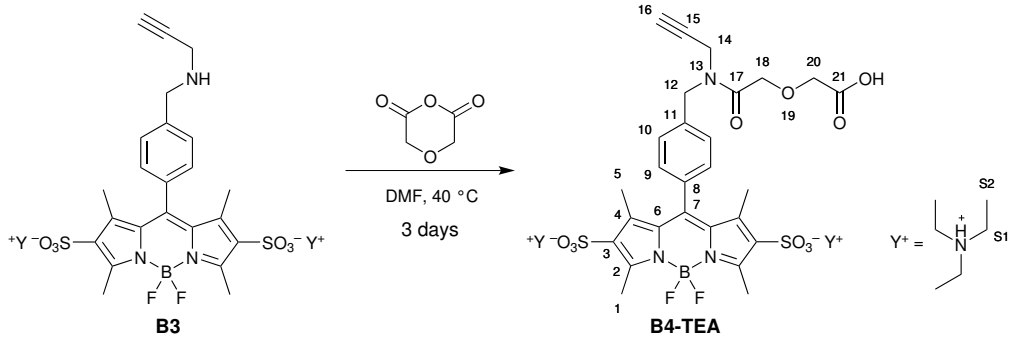

Figure 4.5: Compound **B4-TEA**.

Yield: 35.3 mg orange powder (0.050 mmol, 76 %).

TLC: EA:MeOH 1:1 $R_{\mathrm{f}} = 0.00$; EA:MeOH 1:2 $R_{\mathrm{f}} = 0.64$.

^{1}H-NMR (401 MHz, D_2O): δ =7.58 – 7.27 (2·m, 2·2 H, $\mathcal{H}^{9,10}$); 4.80, 4.75 (2·s, 2·2 H, $\mathcal{H}^{12}$); 4.57, 4.48 (2·s, 2·2 H, $\mathcal{H}^{18}$); 4.18, 4.14 (2·d, 2·2 H, $^4J_{\mathrm{HH}} = 2.5$, $\mathcal{H}^{14}$); 4.04, 4.01 (2·s, 2·2 H, $\mathcal{H}^{20}$); 3.19 (q, 36 H, $^3J_{\mathrm{HH}} = 7.3$, $\mathcal{H}^{S1}$); 2.69, 2.60 (2·t, 2·1 H, $^4J_{\mathrm{HH}} = 2.4$, $\mathcal{H}^{16}$); 2.75 (s, 12 H, $\mathcal{H}^{1}$); 1.64 (s, 12 H, $\mathcal{H}^{5}$); 1.26 (t, 24 H, $^3J_{\mathrm{HH}} = 7.3$, $\mathcal{H}^{S2}$).

^{13}C-NMR (101 MHz, D_2O): $\delta = 177.97, 177.81$ (2·s, 1 C $\mathcal{C}^{21}$); 172.38, 172.19 (2·s, 1 C $\mathcal{C}^{17}$); 155.79 (s, $\mathcal{C}^{\mathrm{Ar}}$); 144.49 (s, $\mathcal{C}^{\mathrm{Ar}}$); 138.42 (s, $\mathcal{C}^{\mathrm{Ar}}$); 137.77 (s, $\mathcal{C}^{\mathrm{Ar}}$); 133.69 (s, $\mathcal{C}^{\mathrm{Ar}}$); 133.06 (s, $\mathcal{C}^{\mathrm{Ar}}$); 131.22 (s, $\mathcal{C}^{\mathrm{Ar}}$); 129.94 (s, $\mathcal{C}^{\mathrm{Ar}}$); 129.39 (s, $\mathcal{C}^{\mathrm{Ar}}$); 128.84 (s, $\mathcal{C}^{\mathrm{Ar}}$); 128.60 (s, $\mathcal{C}^{\mathrm{Ar}}$); 79.06, 78.38

(2·s, 2·1 C, $\mathcal{C}^{16}$); 74.81, 73.57 (2·s, 2·1 C, $\mathcal{C}^{15}$); 70.43, 70.36 (2·s, 2·1 C, $\mathcal{C}^{20}$); 68.88, 68.78 (2·s, 2·1 C, $\mathcal{C}^{18}$); 50.88, 50.10 (2·s, 2·1 C, $\mathcal{C}^{12}$); 47.25 (s, 18 C, $\mathcal{C}^{S1}$); 36.25 (s, 1 C, $\mathcal{C}^{14}$); 14.17 (s, 2 C, $\mathcal{C}^{1}$); 13.44 (s, 2 C, $\mathcal{C}^{5}$); 8.84 (s, 12 C, $\mathcal{C}^{S2}$).
^{19}F-NMR (377 MHz, D_2O): $\delta = -141.21$ (non-binomial q., $J_{FB} = 29$).
^{11}B-NMR (129 MHz, D_2O): $\delta = 0.62$ (t, $J_{BF} = 30$).
MS (ESI): (−) *m/z* calc. for $[(C_{27}H_{26}BF_2N_3O_{10}S_2)^{2-}]$: 665.1, found: 666.2 $[M + H]^-$; 333.6 $[M]^{2-}$.
MS (ESI): (+) *m/z* calc. for $[(C_{27}H_{26}BF_2N_3O_{10}S_2)^{2-}]$: 665.1, found: 668.2 $[M + 3H]^+$.
HR MS (ESI): (−) *m/z* calc. for $[(C_{27}H_{27}BF_2N_3O_{10}S_2)^-]$: 666.12045 found: 666.12022; *m/z* calc. for $[(C_{27}H_{26}BF_2N_3O_{10}S_2)^{2-}]$: 332.55658 found: 332.55651.
UV-Vis: 504.0 nm (MeOH); 498.0 nm (H_2O).
Fluorescence: $\lambda_{ex,em}$ 506.0 nm, 518.0 nm (MeOH); $\lambda_{ex,em}$ 498.4 nm, 510.2 nm (H_2O).

4.4.4 Synthesis of B7-NH_4 from B1

Reaction was done in accordance with general procedure in section 4.4.1.

Into 100ml Schlenk tube with stirrer was transferred **B1** (500.24 mg, 1.342 mmol) and BHT (298 mg, 1.352 mmol). An apparatus was dried in vacuum, argon atmosphere was established and reactants were dissolved in DCM (5 ml), then the apparatus was covered with aluminium foil and cooled down to –40 °C.

Diluted solution of chlorosulfuric acid (17.74 ml, 4.03 mmol, 0.227M in DCM) was slowly added via syringe. Cooling bath was left to warm up to –10 °C within 1 hour.

Suspension was transferred via double tip needle to S4 frit with constant flow of dry DCM. Precipitate was resuspended and rinsed three times with DCM and dried by flow of argon. Precipitate was dissolved in 20 ml of acetonitrile with 1,8-bis(dimethylamino)naphthalene; (1438 mg, 6.73 mmol) and transferred into 100ml flask. Filtration extension was replaced with septum and into transparent dark orange solution was added dimethylamine hydrochloride (328 mg, 4.03 mmol). Reaction mixture was heated at 50 °C for 2 days.

Solvent was removed, resulting mixture was dissolved in methanol and byproducts were crystallised by slow addition of water. Precipitate was removed, solution was concentrated on rotary evaporator and liquid-loaded on reverse phase column (C18, 40 g, H_2O:MeOH, gradient from 100 % to 60 % of H_2O) with 0.1 % TEA in both mobile solvents. Main fraction was preconcentrated on rotary evaporator and dried by lyophilisation. It was characterised as compound **B22** (370 mg, 0.358 mmol, 27 %) in form of orange powder.

Title product **B7** was obtained as minor fraction in form of triethylamine salt (102 mg, 0.137 mmol, 10 %) as orange powder. Product was characterised and for spectroscopic measurement purified on reverse phase column (C18, 40 g, H_2O:MeOH, gradient from 100 % to 70 %

of H_2O with 0.1 % ammonium formate in H_2O). Solution was preconcentrated on rotary evaporator and title product **(B3-NH4)** was obtained by lyophilisation (69.3 mg, 0.120 mmol, 9 %) as orange powder.

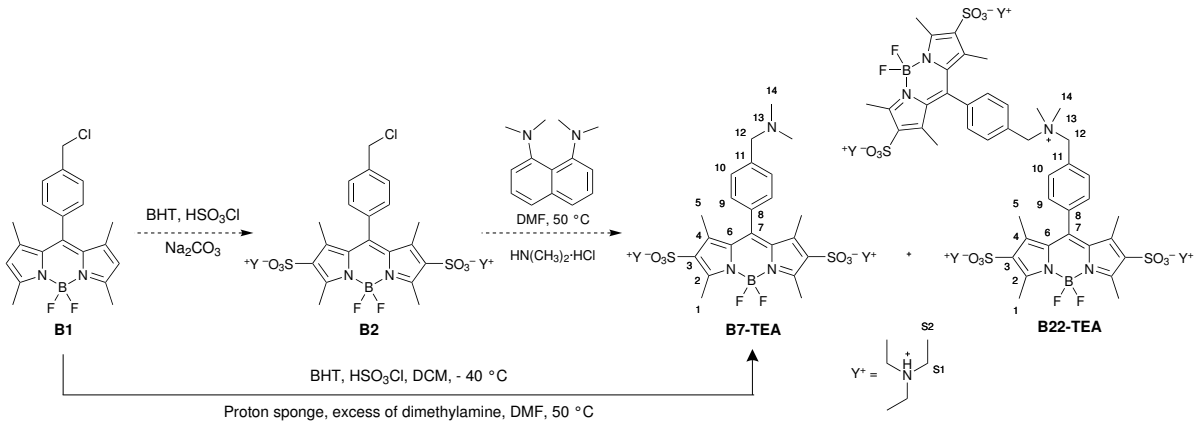

Figure 4.6: Compound **B7-TEA** and **B22-TEA**.

Yield: 69.3 mg dark orange powder (0.120 mmol, 9 %).
TLC: EA:MeOH 1:1 $R_f = 0.27$.
^{1}H-NMR (401 MHz, MeOD): δ = 7.77 – 7.69 (m, 2 H, $\mathcal{H}^{10}$); 7.53 – 7.45 (m, 2 H, $\mathcal{H}^{9}$); 4.44 (s, 2 H, $\mathcal{H}^{12}$); 3.21 (q, 12 H, $^3J_{HH}$ = 7.3, $\mathcal{H}^{S1}$); 2.90 (s, 6 H, $\mathcal{H}^{14}$); 2.82 (s, 6 H, $\mathcal{H}^{1}$); 1.65 (s, 6 H, $\mathcal{H}^{5}$) 1.31 (t, 18 H, $^3J_{HH}$ = 7.3, $\mathcal{H}^{S2}$).
^{13}C-NMR (101 MHz, MeOD): δ = 157.06 (s, $\mathcal{C}^{Ar}$); 145.32 (s, $\mathcal{C}^{Ar}$); 142.95 (s, $\mathcal{C}^{Ar}$); 137.80 (s, $\mathcal{C}^{Ar}$); 136.20 (s, $\mathcal{C}^{Ar}$); 133.39 (s, $\mathcal{C}^{Ar}$); 133.05 (s, 2 C, $\mathcal{C}^{10}$); 131.45 (s, $\mathcal{C}^{Ar}$); 130.42 (s, 2 C, $\mathcal{C}^{9}$); 61.60 (s, 1 C, $\mathcal{C}^{12}$); 47.86 (s, 6 C, $\mathcal{C}^{S1}$); 43.08 (s, 2 C, $\mathcal{C}^{14}$); 14.56 (s, 2 C, $\mathcal{C}^{1}$); 13.60 (s, 2 C, $\mathcal{C}^{5}$); 9.22 (s, 6 C, $\mathcal{C}^{S2}$).
^{19}F-NMR (377 MHz, MeOD): $\delta = -142.59$ (non-binomial q., $J_{FB} = 32$).
^{11}B-NMR (129 MHz, MeOH): δ = 2.03 (t, $J_{BF} = 32$).
MS (ESI): (−) *m/z* calc. for $[(C_{22}H_{24}BF_2N_3O_6S_2)^{2-}]$: 539.1, found: 541.2 $[M + 2H]^-$; 563.2 $[M + Na + H]^-$; 270.1 $[M]^{2-}$.
MS (ESI): (+) *m/z* calc. for $[(C_{22}H_{24}BF_2N_3O_6S_2)^{2-}]$: 539.1, found: 541.2 $[M + 3H]^+$.
HR MS (ESI): (−) *m/z* calc. for $[(C_{22}H_{25}BF_2N_3O_6S_2)^-]$: 540.12514, found: 540.12469; *m/z* calc. for $[(C_{22}H_{24}BF_2N_3O_6S_2)^{2-}]$: 269.55893, found: 269.55896.
UV-Vis: 505.5 nm (MeOH); 499.5 nm (H_2O).
Fluorescence: $\lambda_{ex,em}$ 509.0 nm, 520.0 nm (MeOH); $\lambda_{ex,em}$ 502.4 nm, 512.6 nm (H_2O).

Characterization of B22-TEA

Yield: 370 mg orange powder (0.358 mmol, 27 %).
TLC: EA:MeOH 1:2, $R_f = 0.38$.
^{1}H-NMR (401 MHz, MeOD): δ = 7.94 – 7.81 (m, 4 H, $\mathcal{H}^{10}$); 7.72 – 7.54 (m, 4 H, $\mathcal{H}^{9}$); 4.81 (s, 4 H, $\mathcal{H}^{12}$); 3.19 (q, 24 H, $^3J_{HH}$ = 7.3, $\mathcal{H}^{S1}$); 3.04 (s, 6 H, $\mathcal{H}^{14}$); 2.81 (s, 12 H, $\mathcal{H}^{1}$); 1.70 (s,

12 H, $\mathcal{H}^5$) 1.29 (t, 36 H, $^3J_{\mathrm{HH}} = 7.3$, $\mathcal{H}^{S2}$).
^{1}H-NMR (401 MHz, DMSO): δ = 7.96 – 7.70 (m, 4 H, $\mathcal{H}^{10}$); 7.65 – 7.34 (m, 4 H, $\mathcal{H}^9$); 4.77 (s, 4 H, $\mathcal{H}^{12}$); 3.09 (q, 24 H, $^3J_{\mathrm{HH}} = 7.3$, $\mathcal{H}^{S1}$); 2.89 (s, 6 H, $\mathcal{H}^{14}$); 2.66 (s, 12 H, $\mathcal{H}^1$); 1.56 (s, 12 H, $\mathcal{H}^5$) 1.16 (t, 36 H, $^3J_{\mathrm{HH}} = 7.3$, $\mathcal{H}^{S2}$).
^{13}C-NMR (101 MHz, MeOD): δ = 157.14 (s, $\mathcal{C}^{\mathrm{Ar}}$); 144.99 (s, $\mathcal{C}^{\mathrm{Ar}}$); 142.95 (s, $\mathcal{C}^{\mathrm{Ar}}$); 138.77 (s, 2 C, $\mathcal{C}^{11}$); 136.30 (s, 4 C, $\mathcal{C}^{10}$); 135.75 (s, $\mathcal{C}^{\mathrm{Ar}}$); 131.40 (s, 4 C, $\mathcal{C}^{10}$); 130.62 (s, $\mathcal{C}^{\mathrm{Ar}}$); 130.27 (s, 4 C, $\mathcal{C}^9$); 69.35 (s, 2 C, $\mathcal{C}^{12}$); 48.82 (s, 4 C, $\mathcal{C}^{14}$); 47.86 (s, 12 C, $\mathcal{C}^{S1}$); 14.56 (s, 4 C, $\mathcal{C}^1$); 13.69 (s, 4 C, $\mathcal{C}^5$); 9.23 (s, 12 C, $\mathcal{C}^{S2}$).
^{13}C-NMR (101 MHz, DMSO): δ = 154.48 (s, $\mathcal{C}^{\mathrm{Ar}}$); 142.38 (s, $\mathcal{C}^{\mathrm{Ar}}$); 139.61 (s, $\mathcal{C}^{\mathrm{Ar}}$); 137.89 (s, 2 C, $\mathcal{C}^{11}$); 136.67 (s, 4 C, $\mathcal{C}^{10}$); 134.20 (s, $\mathcal{C}^{\mathrm{Ar}}$); 129.35 (s, 4 C, $\mathcal{C}^{10}$); 128.80 (s, $\mathcal{C}^{\mathrm{Ar}}$); 128.74 (s, 4 C, $\mathcal{C}^9$); 67.26 (s, 2 C, $\mathcal{C}^{12}$); 48.47 (s, 4 C, $\mathcal{C}^{14}$); 45.74 (s, 12 C, $\mathcal{C}^{S1}$); 13.94 (s, 4 C, $\mathcal{C}^1$); 12.71 (s, 4 C, $\mathcal{C}^5$); 8.66 (s, 12 C, $\mathcal{C}^{S2}$).
^{19}F-NMR (377 MHz, DMSO): δ =−142.10 (non-binomial q., $J_{\mathrm{FB}} = 31$).
^{11}B-NMR (129 MHz, MeOH): δ = 0.62 (t, $J_{\mathrm{BF}} = 33$).
MS (ESI): (−) *m/z* calc. for $[(C_{42}H_{42}B_2F_4N_5O_{12}S_4)^{3-}]$: 1034.2, found 1080 $[M + 2\,Na]^-$; 1057 $[M + H + Na]^-$; 1037 $[M + 2\,H]^-$; 517.5 $[M + H]^{2-}$; 528.5 $[M + Na]^{2-}$; 344.6 $[M]^{3-}$.
UV-Vis: 506.0 nm (MeOH); 499.5 nm (H_2O).
Fluorescence: $\lambda_{\mathrm{ex,em}}$ 510.0 nm, 522.0 nm (MeOH); $\lambda_{\mathrm{ex,em}}$ 502 nm, 513.6 nm (H_2O).

4.4.5 Synthesis of B9-TEA from B7-NH$_4$

In vial with stirrer was transferred **B7-NH$_4$** (5.1 mg, 8.69 μmol) and $Pd(OAc)_2$ (11.7 mg, 0.052 mmol) and in 3 ml of dry DMF dissolved and heated at 50 °C for 3 hours. Solvent was removed and reaction mixture was dissolved in MeOH, black suspension filtered out and loaded on RP samplet (300 mg of C18 silica gel). Resulting fraction were analysed by NMR and HLPC-MS.

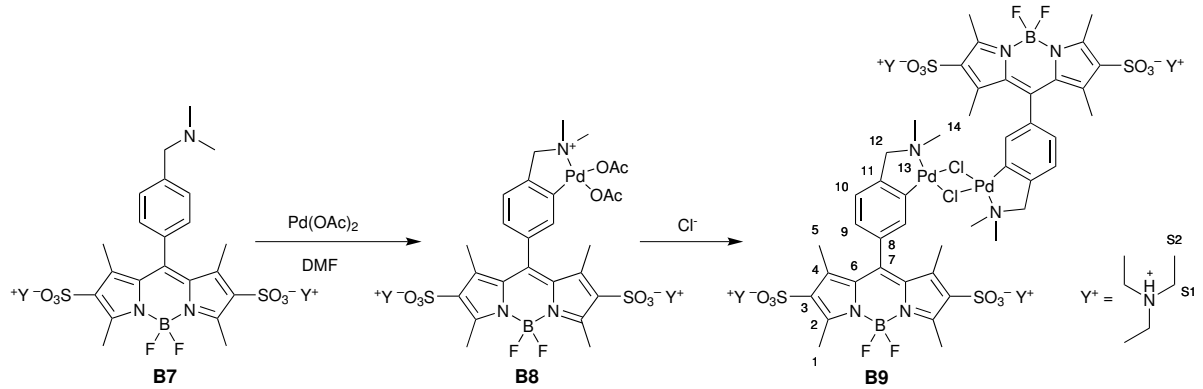

Figure 4.7: Compound **B9-TEA**.

MS (ESI): (−) *m/z* calc. for $[(C_{22}H_{23}BCl_2F_2N_3O_6PdS_2)^{2-}]$: 713.95, found: 714 $[M + H]^-$.

4.4.6 Synthesis of B11-TEA from B1

Reaction was done in accordance with general procedure in section 4.4.1.

Into 100ml Schlenk tube with stirrer was transferred **B1** (500.42 mg, 1.343 mmol) and BHT (296 mg, 1.343 mmol). Apparatus was dried, reactants were dissolved in argon atmosphere in DCM (5 ml), covered with aluminium foil and cooled down to –40 °C.

Diluted solution of chlorosulfuric acid (9.81 ml, 4.03 mmol, 0.4106M in DCM) was slowly added via syringe. Cooling bath was left to warm up to 0 °C within 30 minutes.

Suspension was diluted with DCM (20 ml) and transferred to S4 frit with constant flow of dry DCM. Precipitate was resuspended and rinsed three times with DCM and dried by flow of argon. Precipitate was dissolved in 20 ml solution of acetonitrile with 1,8-bis(dimethylamino)naphthalene (1.727 g, 8.06 mmol) and transferred into 100ml flask. Filtration extension was replaced by septum and into transparent dark orange solution was added 3-azidopropan-1-amine (336 mg, 3.36 mmol). Reaction mixture was heated at 50 °C for 2 days.

Solvent was removed, reaction mixture was dissolved in methanol and byproducts were crystallized by slow addition of water. Precipitate was removed, solution was concentrated. Reaction mixture was dissolved in water and liquid-loaded on reverse phase column (C18, 40 g, H_2O:MeOH, gradient from 100 % to 75 % of H_2O) with 0.1 % TEA in both mobile solvents. Main fraction was preconcentrated, dried by lyophilisation, and title compound **B11-TEA** (365 mg, 0.614 mmol, 46 %) was obtained in form of orange powder.

Synthesis of B11-TEA from B2-TEA

In small vial with 3-azidopropan-1-amine (56.6 mg, 0.565 mmol) and 1,8-bis(dimethylamino)naphthalene (121 mg, 0.565 mmol, 3 eq.) were dissolved in DMF. In other vial was dissolved **B2** (100 mg, 0.188 mmol) and of 1,8-bis(dimethylamino)naphthalene (203 mg, 0.942 mmol, 5 eq.) in DMF. Solution of **B2** was slowly transferred into the first vial and heated at 50 °C for 3 days.

Solvent was removed, resulting mixture was dissolved in methanol and byproducts were crystallized by slow addition of water. Precipitate was removed, solution was concentrated and liquid-loaded on reverse phase column (C18, 40 g, H_2O:MeOH, gradient from 100 % to 75 % of H_2O) with 0.1 % TEA in H_2O. Solvents were removed on rotary evaporator and dried by lyophilisation. Title product was obtained as triethylammonium salt **B11-TEA** (67 mg, 0.084 mmol, 45 %) in form of orange powder.

Yield: 67.01 mg orange powder (0.084 mmol, 45 %).
TLC: EA:MeOH 1:1 $R_f = 0.53$;EA:MeOH 2:1 $R_f = 0.15$.

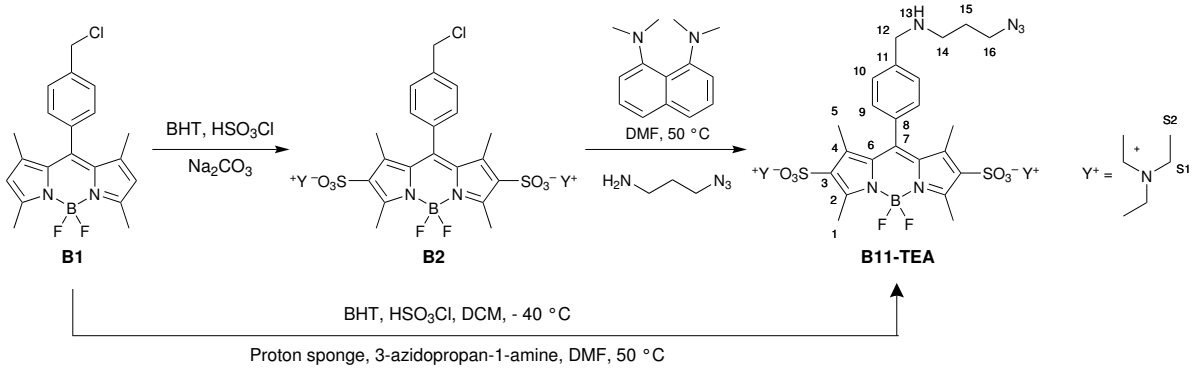

Figure 4.8: Compound **B11-TEA**.

^{1}H-NMR (401 MHz, D_2O): δ = 7.68 – 7.56 (m, 2 H, $\mathcal{H}^{10}$); 7.31 – 7.23 (m, 2 H, $\mathcal{H}^{9}$); 4.74 (s, 2 H, $\mathcal{H}^{12}$); 3.47 (t, 2 H, $^3J_{HH}$ = 6.3, $\mathcal{H}^{16}$); 3.07 (t, 2 H, $^3J_{HH}$ = 7.3, $\mathcal{H}^{14}$); 2.76 (s, 6 H, $\mathcal{H}^{1}$); 2.05 (tt, 2 H, $^3J_{HH}$ = 7.3 and 6.3, $\mathcal{H}^{15}$);1.62 (s, 6 H, $\mathcal{H}^{5}$).
^{13}C-NMR (101 MHz, D_2O): δ = 155.91 (s, $\mathcal{C}^{Ar}$); 146.85 (s, $\mathcal{C}^{Ar}$); 144.35 (s, $\mathcal{C}^{Ar}$); 140.51 (s, $\mathcal{C}^{Ar}$); 133.97 (s, $\mathcal{C}^{Ar}$); 133.29 (s, $\mathcal{C}^{Ar}$); 131.14 (s, $\mathcal{C}^{Ar}$); 130.60 (s, 2 C, $\mathcal{C}^{10}$); 128.63 (s, 2 C, $\mathcal{C}^{9}$); 46.18 (s, 1 C, $\mathcal{C}^{12}$); 37.89 (s, 1 C, $\mathcal{C}^{14}$); 26.70 (s, 1 C, $\mathcal{C}^{15}$); 48.84 (s, 1 C, $\mathcal{C}^{16}$); 14.22 (s, 2 C, $\mathcal{C}^{1}$); 13.42 (s, 2 C, $\mathcal{C}^{5}$).
^{19}F-NMR (377 MHz, D_2O): δ = −141.26 (non-binomial q., J_{FB} = 28).
^{11}B-NMR (129 MHz, D_2O): δ = 0.62 (t, J_{BF} = 29).
MS (ESI): (−) *m/z* calc. for $[(C_{23}H_{25}BF_2N_6O_6S_2)^{2-}]$: 594.1, found: 595.0 $[M + H]^-$; 297.2 $[M]^{2-}$.
HR MS (ESI): (−) *m/z* calc. for $[(C_{23}H_{26}BF_2N_6O_6S_2)^-]$: 595.14218, found: 595.14108; *m/z* calc. for $[(C_{23}H_{25}BF_2N_6O_6S_2)^{2-}]$: 297.06745, found: 297.06713.
UV-Vis: 505.0 nm (MeOH); 499.0 nm (H_2O).
Fluorescence: $\lambda_{ex,em}$ 509.0 nm, 520.0 nm (MeOH); $\lambda_{ex,em}$ 501.2 nm, 512.2 nm (H_2O).

4.4.7 Synthesis of B12-TEA from B11-TEA

In 20ml vial with septum was dissolved 2-((1,3-dioxoisoindolin-2-yl)oxy)acetic acid (5.3 mg, 0.024 mmol) in 2 ml of dry DMF under argon atmosphere followed by addition of DCC (4.9 mg, 0.024 mmol). Reaction mixture was heated at 40 °C for 30 minutes. Then **B11-TEA** (9.5 mg, 0.012 mmol) was added. Only minor conversion was observed, therefore DCC (9.82 mg, 0.048 mmol) and 2-((1,3-dioxoisoindolin-2-yl)oxy)acetic acid (10.5 mg, 0.048 mmol) were added into reaction mixture and stirred at 50 °C for 2 days.

Solvent was removed, resulting mixture was dissolved in water and liquid-loaded on reverse phase column (C18, 40 g, H_2O:MeOH, gradient from 100 % to 40 % of H_2O) with 0,2 % TEAF buffer in water. Major fraction was reactant **B11** and title product **B12-TEA** (2.3 mg, 2.76 μmol, 23 %) was obtained by lyophilisation as minor fraction.

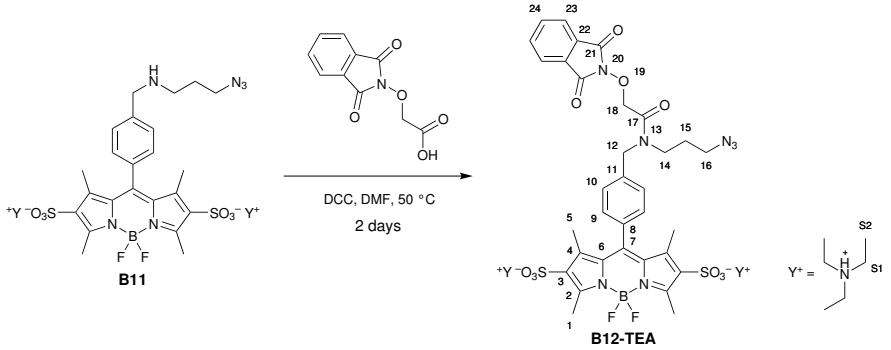

Figure 4.9: Compound **B12-TEA**.

Yield: 2.3 mg orange powder (2.76 m mol, 23 %).
TLC: EA:MeOH 2:1 $R_f = 0.76$.
^{1}H-NMR (401 MHz, D_2O): $\delta = 7.91 - 7.80$ (m, 4 H, $\mathcal{H}^{23,24}$); 7.61 – 7.57 (m, 2 H, $\mathcal{H}^{10}$); 7.35 – 7.31 (m, 2 H, $\mathcal{H}^{9}$); 5.02 (s, 2 H, $\mathcal{H}^{18}$); 4.79 (s, 2 H, $\mathcal{H}^{12}$); 3.54 – 3.46 (m, $\mathcal{H}^{16}$); 3.03 (t, 2 H, $^3J_{HH} = 7.0$, $\mathcal{H}^{14}$); 2.79 (s, 6 H, $\mathcal{H}^{1}$); 1.96 – 1.78 (m, 2 H, $\mathcal{H}^{15}$); 1.68 (s, 6 H, $\mathcal{H}^{5}$).
MS (ESI): (−) *m/z* calc. for $[(C_{33}H_{30}BF_2N_7O_{10}S_2)^{2-}]$: 797.2, found: 798.3 $[M + H]^-$; 398.6 $[M]^{2-}$.
MS (ESI): (+) *m/z* calc. for $[(C_{33}H_{30}BF_2N_7O_{10}S_2)^{2-}]$:, 797.2 found: 800.1 $[M + 3H]^+$; 822.1 $[M + 2H + Na]^+$.

4.4.8 Synthesis of B21-NH_4 from B1

Reaction was done in accordance with general procedure in section 4.4.1.

Into 100ml Schlenk tube with stirrer was transferred **B1** (150 mg, 0.403 mmol) and BHT (89 mg, 0.403 mmol). Apparatus was dried in vacuum, reactants were dissolved in argon atmosphere in DCM (5 ml), covered with aluminium foil and cooled down to –40 °C. Diluted solution of chlorosulfuric acid (3.5 ml, 1.208 mmol, 0.4106M in DCM) was slowly added via syringe. Cooling bath was left to warm up to –5 °C within 30 minutes.

Suspension was diluted with DCM (10 ml), transferred via double tip needle to S4 frit with constant flow of dry DCM. Precipitate was resuspended and rinsed three times with DCM and dried by flow of argon. Precipitate was dissolved in 10 ml of acetonitrile with 1,8-bis(dimethylamino)naphthalene (431 mg, 2.013 mmol) and transferred into 50ml pressure flask, solvent was removed.

Into reaction flask with cooling in dry-ice ethanol bath was condensed roughly 5 ml of liquid ammonia. Pressure flask was sealed off and cooling bath was removed. Reaction mixture was stirred for 3 days at RT, then liquid ammonia was removed.

Reaction mixture was dissolved in methanol and byproducts were crystallized by slow addition of water. Precipitate was removed, solution was concentrated. Reaction mixture was dissolved in water and liquid-loaded on reverse phase column (C18, 40 g, H_2O:MeOH, gradient from 100 % to 75 % of H_2O) with 0.1 % TEA in mobile solvents, then repeated with 0.1 % DIPEA in H_2O. Main fraction was preconcentrated on rotary evaporator and dried by lyophilisation. Title product was obtained as DIPEA salt of **B21** (53 mg, 0.074 mmol, 19 %) in form of orange powder. For spectroscopic characterisation was the title product **B21** purified on HPCL column (RP) with 0.1 % ammonium formate in H_2O and lyophilised, obtaining ammonium salt of **B21** (25 mg, 0.045 mmol, 11 %)

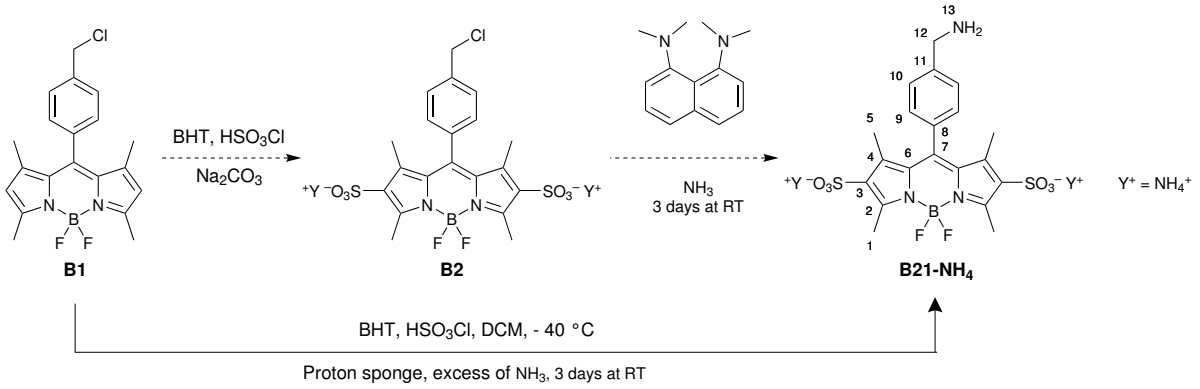

Figure 4.10: Compound **B21-DIPEA**.

Yield: 53 mg orange powder (0.074 mmol, 18 %). HPLC purification gave 25 mg of pure compound B-21:
Yield: 25 mg orange powder (0.045 mmol, 11 %).
TLC: EA:MeOH 1:1 $R_f = 0.30$; EA:MeOH 2:1 $R_f = 0.05$.
^{1}H-NMR (400 MHz, MeOD): $\delta = 7.70 - 7.65$ (m, 2 H, $\mathcal{H}^{10}$); 7.49 – 7.45 (m, 2 H, $\mathcal{H}^{9}$); 4.24 (s, 2 H, $\mathcal{H}^{12}$); 3.35 (s, 2 H, $\mathcal{H}^{14}$); 2.80 (s, 6 H, $\mathcal{H}^{1}$); 1.66 (s, 6 H, $\mathcal{H}^{5}$).
^{13}C-NMR (101 MHz, MeOD): $\delta = 156.92$ (s, $\mathcal{C}^{\mathrm{Ar}}$); 145.67 (s, $\mathcal{C}^{\mathrm{Ar}}$); 143.19 (s, $\mathcal{C}^{\mathrm{Ar}}$); 136.88 (s, 2 C, $\mathcal{C}^{11}$); 136.23 (s, $\mathcal{C}^{\mathrm{Ar}}$); 136.02 (s, $\mathcal{C}^{\mathrm{Ar}}$); 131.55 (s, $\mathcal{C}^{\mathrm{Ar}}$); 131.34 (s, 2 C, $\mathcal{C}^{10}$); 130.22 (s, 2 C, $\mathcal{C}^{9}$); 43.92 (s, 3 C, $\mathcal{C}^{12}$); 14.50 (s, 2 C, $\mathcal{C}^{1}$); 13.71 (s, 2 C, $\mathcal{C}^{5}$).
^{19}F-NMR (377 MHz, MeOD): $\delta = -144.50$ (non-binomial q., $J_{\mathrm{FB}} = 32$).
^{11}B-NMR (129 MHz, MeOD): $\delta = 0.70$ (t, $J_{\mathrm{BF}} = 32$).
MS (ESI): (+) *m/z* calc. for $[(C_{20}H_{20}BF_2N_3O_6S_2)^{2-}]$: 511.1, found: 512.1 $[M + H]^-$; 534.1 $[M + Na]^-$; 255.5 $[M]^{2-}$.
HR MS (ESI): (+) *m/z* calc. for $[(C_{20}H_{21}BF_2N_3O_6S_2)^-]$: 512.09384, found: 255.54350; *m/z* calc. for $[(C_{20}H_{20}BF_2N_3O_6S_2)^{2-}]$: 255.54328, found: 255.54350.
UV-Vis: 505.0 nm (MeOH); 498.5 nm (H_2O).
Fluorescence: $\lambda_{\mathrm{ex,em}}$ 506.0 nm, 518.0 nm (MeOH); $\lambda_{\mathrm{ex,em}}$ 500.6 nm, 511.6 nm (H_2O).

5 | Conclusions

In this thesis we:

i) established a protocol for reliable and scalable sulfonation of BODIPY,
ii) developed methodology to remove surplus salts and exchange of counterions,
iii) prepared a series of alkylated water-soluble species **B3**, **B7**, **B11**, **B21**,
iv) prepared series of water-soluble, biocompatibile fluorescent probes, which can be easily modified and used as bioorthogonal probes.

This was done with the respect to compatibility with organic synthesis, analytical purification and biological applications. Each branch of prepared compounds can be summed up in one of following categories:

i) water-soluble monofunctional fluorescent probe **B21**,
ii) precursors of heterobifunctional fluorescent probes **B4** and **B12**,
iii) water-soluble off/on probe **B9**, which has been confirmed by MS spectroscopy.

Supporting information

Compound	**B1**	**B2**
Formula	$C_{20}H_{20}BClF_2N_2$	$(C_{14}H_{18}N_2)(C_3H_5N)C_{20}H_{18}BClF_2N_2O_6S_2$
M_r	372.65	800.13
Shape, colour	prism, orange-reddish	prism, red
Crystal diameters (mm)	0.27 × 0.37 × 0.53	0.12 × 0.26 × 0.37
Crystal system	monoclinic	triclinic
Space group	$P2_1$	$P\bar{1}$
a (Å)	8.7446(6)	11.6302(4)
b (Å)	8.9986(5)	12.7156(4)
a (Å)	11.5351(7)	13.9621(6)
α (°)	90.00	76.631(3)
β (°)	94.195(2)	73.002(3)
γ (°)	90.00	82.527(3)
Cell volume (Å^3)	905.26(10)	1916.75(13)
Z	2	2
D_{calc} g· cm^{-3}	1.367	1.386
μ (mm^{-1})	0.236	2.427
T (K)	150	180
θ range (°)	1.77 – 27.49	4.397 – 75.944
Total refl.	4150	15241
Obsd. refl. ($I > 2\sigma(I)$)	3848	6080
GOF on F^2	1.027	1.051
R; wR ($I > 2\sigma(I)$)	0.0308; 0.0769	0.0659; 0.0749
R; wR	0.0349; 0.0794	0.0802; 0.0843

Table 5.1: Summary of X-ray diffraction data for **B1** and **B2**.

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