Summary of Ph.D. Thesis

Design, synthesis and application of conjugatable fluorescent dyes

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Szeged
2026

1. Introduction and aims

The molecular-level tracking and interpretation of processes occurring in biological systems is of paramount importance for medical biology and medicinal chemistry. These fields increasingly rely on highly sensitive, non-radioactive imaging techniques that can be monitored using optical tools. Although radiolabeling offers certain advantages, its use is increasingly limited due to safety, regulatory, and waste management concerns, bringing alternative labeling strategies, such as fluorescent tagging, to the forefront. Efficient application requires the selection and preparation of dyes possessing appropriate photophysical (absorption/emission properties, quantum yield), chemical (stability, functionalizability, solubility), and biological (compatibility, targetability) characteristics. The aim of our work was to synthesize conjugatable BODIPY, rhodamine, and rosamine derivatives, as well as *aza*-BODIPYs emitting in the red/near-infrared range, which could be used both for selective labeling of biomolecules and in photodynamic therapy as theranostic agents. To this end, we designed dye scaffolds bearing functional groups suitable for conjugation with various modified biomolecules. Derivatives containing terminal alkyne or phenolic hydroxyl groups were prepared either via scaffold synthesis strategies or by post-functionalization. To optimize reaction conditions, we minimized the use of solvents and halogenated compounds and, whenever possible, facilitated the reactions with microwave irradiation. During our work, we employed, among others, transition-metal-catalyzed Sonogashira reactions, Vilsmeier-Haack formylation, and Pinnick oxidation. Estradiol was modified at its 3-hydroxyl group and equipped with linkers of varying lengths, with the aim of preserving its original biological activity. Conjugates were constructed using CuAAC reactions or ether synthesis. In collaboration with partners, we planned to characterize the spectroscopic properties of the new fluorophores and their conjugates, perform high-resolution microscopy studies, measure the affinity of biologically active molecules for the estrogen receptor and confirm the appropriate binding conformation using computational chemistry methods, and investigate the biological effects of the fluorophores in photodynamic therapy.

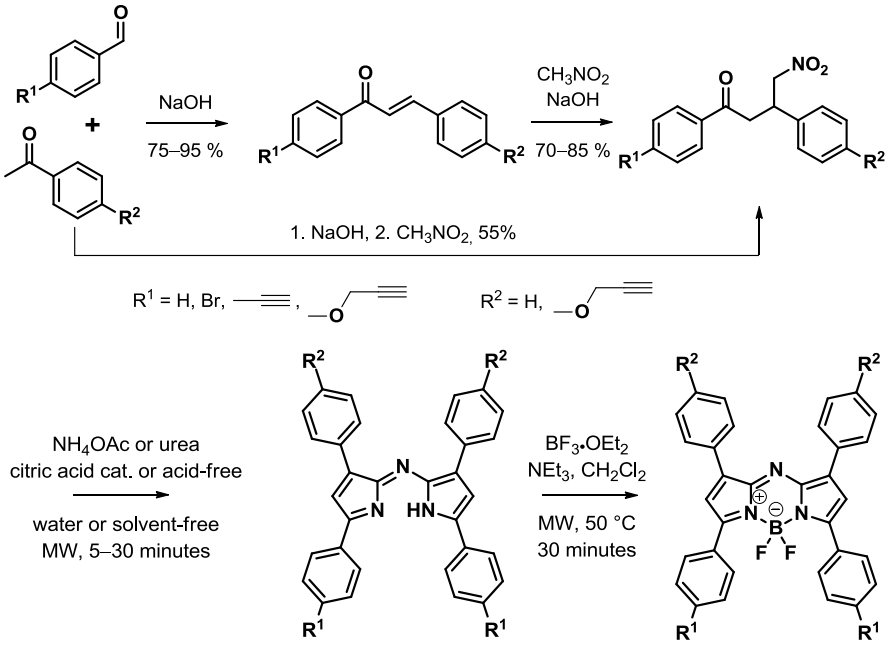
2. Experimental methods

The organic syntheses were carried out on a millimolar scale, and the progress of the reactions was monitored by thin-layer chromatography. Palladium-catalyzed and CuAAC reactions were performed under conventional heating conditions or in a microwave reactor. Reaction mixtures were purified and products were isolated using column chromatography. The

structures of the compounds were confirmed by one- and two-dimensional NMR spectroscopy, as well as by MS or HR-MS measurements.

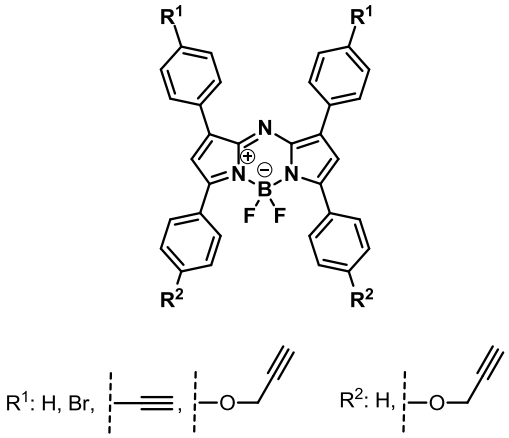
3. Novel scientific results

3.1. We have successfully improved the production efficiency of thetetraphenyl *aza*-BODIPY derivative (Scheme 1). A novel sequential one-pot procedure was developed for the sodium hydroxide-catalyzed condensation of benzaldehyde and acetophenone, followed by a Michael addition using an excess of nitromethane. Using the conventional two-step procedure with intermediate purification as a reference, it was observed that the overall yield decreased by approximately 20%; however, in accordance with the principles of green chemistry, the one-pot procedure proved to be more efficient. The feasibility of the subsequent dimerization step using either ammonium acetate or urea under various conditions was investigated. It was demonstrated that the solvent can be omitted or replaced with water instead of the commonly used ethanol or butanol, and that the reaction time can be shortened from 1–2 days to minutes by applying microwave irradiation. The best yield (51%) was obtained in aqueous solvent with citric acid catalysis and the addition of excess urea. Furthermore, it was demonstrated that the final complexation step with boron trifluoride diethyl etherate can be carried out in dichloromethane under microwave conditions at 50 °C for 30 minutes, affording a comparable yield (90%) to the conventional heating procedure in toluene under reflux for several hours. (**3rd publication**)



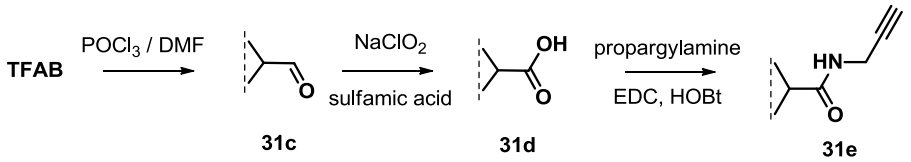
Scheme 1

3.2. We developed a framework synthesis strategy for the preparation of conjugatable *aza*-BODIPY derivatives. Functional groups suitable for subsequent conjugation were introduced already in the first reaction step. To incorporate a terminal alkyne function, 4-bromobenzaldehyde was reacted with trimethylsilylacetylene under Sonogashira coupling conditions. Propargyl ethers were obtained by reacting 4-hydroxybenzaldehyde or 4-hydroxyacetophenone with propargyl bromide. We verified that, using these building blocks, the procedure described in Section 3.1 can be performed with comparable efficiency, yielding *aza*-BODIPY-alkynes (Scheme 2). We observed that the trimethylsilyl group cleaves off by the end of the reaction sequence, eliminating the need for its removal with fluoride ions. (**Publication 1**)



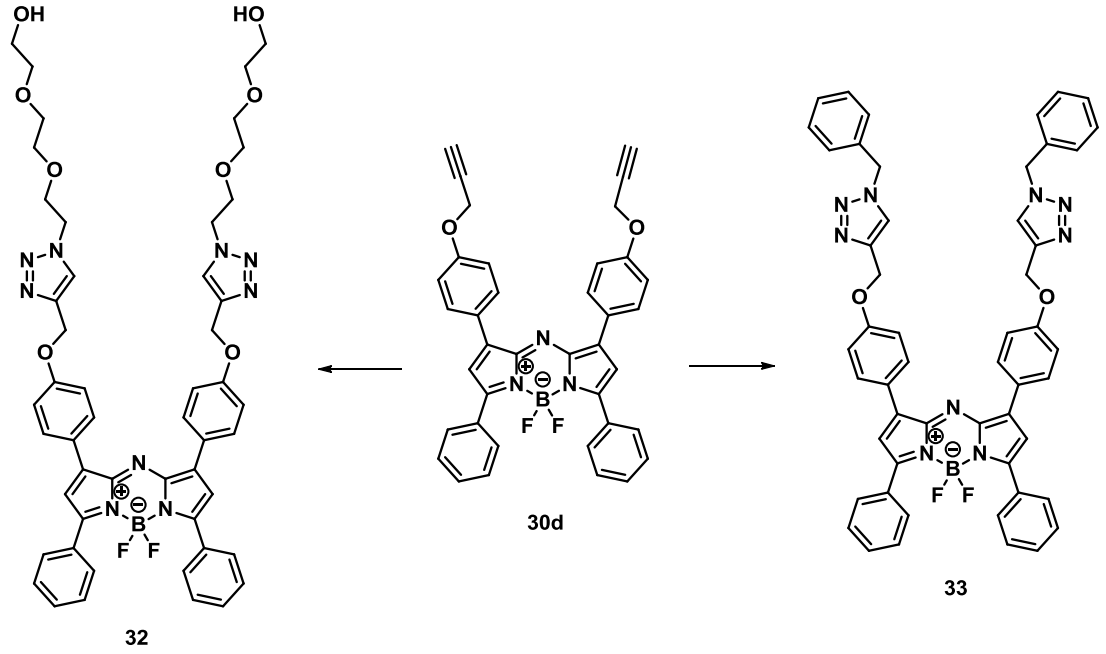
Scheme 2

3.3. We investigated the possibilities of post-functionalizing the *aza*-BODIPY core and confirmed that selective formylation of one of the pyrrole rings is achievable (Scheme 3). For this purpose, the framework was modified via a Vilsmeier-Haack (VH) reaction: after refluxing with $POCl_3$ in a DMF/1,2-dichloroethane solution, the reaction mixture was further stirred in aqueous medium at room temperature. We observed that the formyl group was introduced exclusively on one of the rings. The crude product was subjected to Pinnick oxidation with $NaOCl_2$, which efficiently produced the corresponding carboxylic acid derivative in 84% yield. Incorporation of the terminal alkyne function using propargyl amine and the coupling agents EDC/HOBt was successfully achieved. (**Publication 3**)



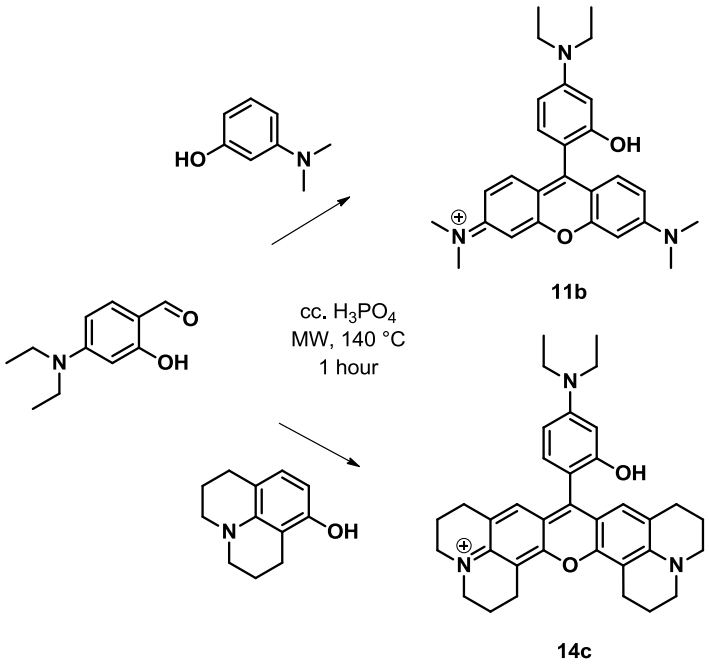
Scheme 3

3.4. We successfully carried out the CuAAC coupling of the TFAB *bis*-propargyl derivative (**30d**) using benzyl azide or the triethylene glycol azide derivative as reaction partners. We demonstrated that, in the presence of a Cu(I) catalyst (0.05 equiv.), a PPh_3 ligand (0.2 equiv.) and DIPEA as base, the triazole conjugates can be obtained in good yields (62% and 66%) by refluxing in toluene for 3 hours. (**Publication 1**)



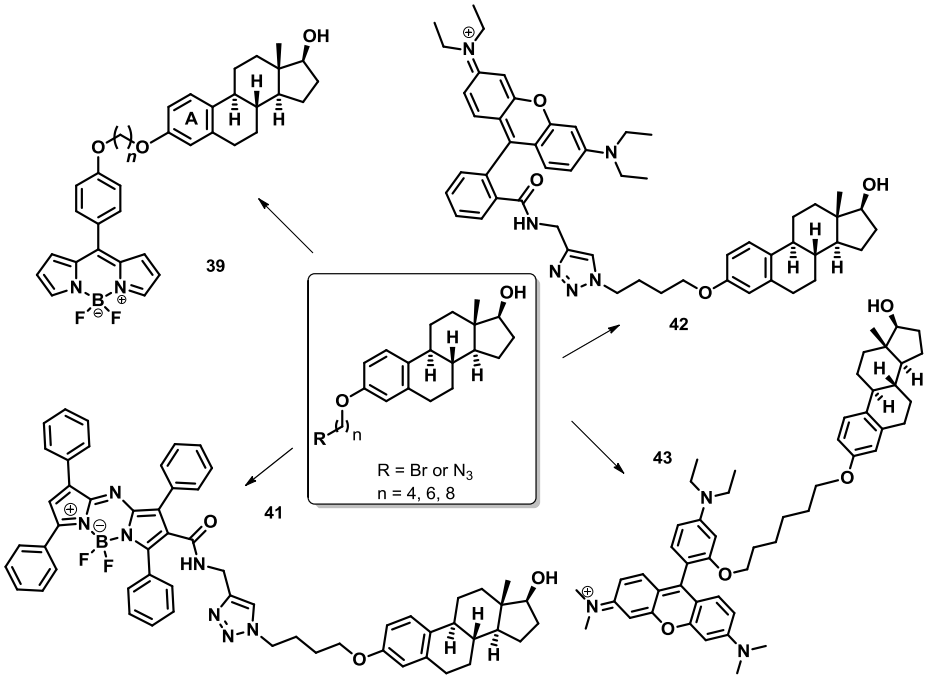
Scheme 4

3.5. We investigated the preparation of rosamine-type dyes containing a phenolic hydroxyl group and found that the best results were achieved under concentrated phosphoric acid, using microwave irradiation at 140 °C for 1 hour (Scheme 5). With this procedure, rosamine derivatives suitable for conjugation were synthesized (**11b, 14c**) in acceptable yields (~30%). We demonstrated that the starting salicylaldehyde undergoes a condensation reaction with m-dimethylaminophenol or 8-hydroxyjulolidine, leading to the formation of the xanthene scaffold.



Scheme 5

3.6. We successfully carried out the synthesis of various BODIPY-, *aza*-BODIPY-, rhodamine, and rosamine-E2 conjugates (Scheme 6). Spacer elements of different alkyl chain lengths were introduced in all cases at the phenolic hydroxyl group of the steroid. BODIPY and rosamine derivatives containing phenolic hydroxyl groups were coupled to the steroid via ether linkage, whereas *N*-propargyl *aza*-BODIPY and rhodamine derivatives were attached using CuAAC reaction. The conjugation reactions provided the desired fluorescently labeled E2 derivatives in good yields. (**Publications 2-3**)



Scheme 6

4. Results of collaborative biological and optical studies

4.1. Several of the synthesized compounds exhibited significant activity in photodynamic tumor therapy on the A431 human epidermoid carcinoma cell line. The TFAB propargyl and benzyl derivatives (**30d, 33**) showed light-induced cytotoxicity comparable to TFAB, with IC_{50} values in the micromolar range. Among them, compound **32** stood out, displaying an IC_{50} of 3.66 nM, which, to the best of our knowledge, surpasses previously reported results in the literature. We observed that the compounds exhibited negligible dark toxicity, and the most promising derivative remained non-toxic even at concentrations ten thousand times higher.

4.2. The dye-E2 conjugates (**39a-c, 41**) were subjected to in vitro estrogen activity assays in collaborative studies. We confirmed that the BODIPY conjugates retained their estrogenic activity despite fluorescent labeling, exhibiting IC_{50} values in the submicromolar range. Using radioligand binding assays, we demonstrated for the first time in the literature that the BODIPY-E2 ether conjugate containing a four-carbon linker (**39a**) can displace tritiated E2 from the human ERα estrogen binding site in a dose-dependent manner. These results provide evidence that E2 conjugates lacking a free phenolic hydroxyl group can bind the receptor's estrogen binding site with high affinity, even though the literature indicates that this hydroxyl group is essential for optimal binding. Preliminary biological tests suggest that the rosamine-E2 conjugate (**43**) exhibits comparable estrogenic activity; however, studies to clarify this are

still ongoing. The newly synthesized E2-BODIPY conjugates (**39a-c**) may serve as starting points for developing suitable probes for investigating estrogen uptake, transport, biotransformation, and protein-binding properties. Monitoring these processes is not only of critical importance for understanding estrogenic effects but also for other biological contexts, including neuroprotective mechanisms. Moreover, the high detectability of fluorescent estrogens may enable the identification of novel and significant biological effects. The green- and red-emitting BODIPY-E2 conjugates produced in this study complement each other chemically, as probes functioning in different wavelength ranges may be suitable for distinct biomedical applications. Fluorescent conjugates emitting at longer wavelengths - owing to their deeper tissue penetration - could provide a basis for live-cell imaging techniques, enabling the acquisition of high spatial and temporal resolution images.

4.3. The rosamine derivative containing a free phenolic hydroxyl group exhibited strong pH-dependent fluorescence. In acidic medium, its fluorescence intensity was exceptionally high, then gradually decreased with increasing pH and reached a minimum around pH 5–6. Based on the data, we determined the acid dissociation constant of the compound using sigmoid fitting, which resulted in pKa = 3.75.

5. Scientific publications directly related to the Ph.D. Thesis (MTMT identifier:)

1. **Hlogyik T**, Laczkó-Rigó R, Bakos É, Poór M, Kele Z, Özvegy-Laczka Cs, Mernyák E. Org. Biomol. Chem., **2023**, 21, 6018–6027. **IF = 2.9**

2. Peřina M, Börzsei R, Ágoston H, **Hlogyik T**, Poór M, Rigó R, Özvegy-Laczka Cs, Batta G, Hetényi Cs, Vojáčková V, Jorda R, Mernyák E. Eur. J. Pharm. Sci. **2024**, 199, 106813. **IF = 4.7**

3. **Hlogyik T**, Bózsity N, Börzsei R, Kovács B, Labos P, Hetényi Cs, Kiricsi M, Huliák I, Kele Z, Poór M, Erostyák J, Hunyadi A, Zupkó I, Mernyák E. Int. J. Mol. Sci. **2025**, 26(15), 7075. **IF = 4.9**

Total IF = 12.5

6. Other scientific publications

1. Náfrádi M, **Hlogyik T**, Farkas L, Alapi T. J. Environ. Chem. Eng**. 2021**, 9, 106684. **IF = 6.8**

2. Farkas J, Náfrádi M, **Hlogyik T**, Pravda BC, Schrantz K, Hernádi K, Alapi T. Environ. Sci.: Water Res. Technol. **2018**, 4, 1345–1360.

IF = 3.3

Total IF = 10.1

7. Lectures and posters related to the Ph.D. Thesis

Lectures

1. **Hlogyik Tamás**, Mernyák Erzsébet – Biomolekulák zöld- vagy vörös-emittáló fluorofórral való jelölése – XLVIII. Kémiai előadói napok, Szeged, 2025.10.27-29.
2. **Hlogyik Tamás**, Mernyák Erzsébet – *Aza*-BODIPY típusú fényérzékenyítő anyagok szintézise – XLVI. Kémiai előadói napok, Szeged, 2023.10.17-19.
3. **Hlogyik Tamás**, Mernyák Erzsébet – Vörös emittáló fluorofórok szintézise és fotodinámiás alkalmazhatóságuk – Ifjú Szerves Kémikusok Előadóülése, 2023.05.09.
4. **Hlogyik Tamás**, Mernyák Erzsébet – BODIPY-típusú fluorofórok etinil-származékainak szintézise és konjugálása – Ifjú Szerves Kémikusok Előadóülése, 2022.05.18.

Posters

1. **Tamás Hlogyik**, Máté Vágvölgyi, Labos Péter, Noémi Bózsity, István Zupkó, Attila Hunyadi, Erzsébet Mernyák – Novel Fluorescent-labeled estradiol derivatives. 30th International Symposium on Analytical and Environmental Problems, Szeged, 2024.10.7-8.
2. **Hlogyik Tamás**, Mernyák Erzsébet – Fluoreszcensen jelölt 17β-ösztradiol származékok előállítása – Vegyészkonferencia, Eger, 2024.06.10-12.
3. **Hlogyik Tamás**, Mernyák Erzsébet – Biomolekulák konjugálására alkalmas *aza*-BODIPY típusú fluorofórok szintézise – Vegyészkonferencia, Eger, 2022.06.15-17.
4. **Tamás Hlogyik**, Erzsébet Mernyák – Synthesis of red-emitting fluorophores - 28th International Symposium on Analytical and Environmental Problems, Szeged, 2022.1114-15.