

Halogenated 2,1,3-benzoxadiazoles as Potential Fluorescent Warheads for Covalent Protease Inhibitors [†]

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Abstract: Recently there has been a growing interest in covalent protease inhibitors in both industry and academia, caused by their longer residence times, their higher potency and their high ligand efficiency. Covalently reactive moieties which interact with activated amino acid residues such as serine or cysteine in enzymes like proteases or esterases mostly act through nucleophilic addition, substitution or ring opening. In contrast, nucleophilic aromatic substitution (S_NAr) is rarely employed. In our previous work, we prepared and investigated electrophilic “warheads”, which contain aromatic, heteroaromatic or quinoid fragments. Some of them show potent inhibition constants for cathepsin L, cathepsin B, rhodesain or dengue-protease, and depending on the exact nature of the electrophile they exhibit reversible covalent or irreversible inhibition modes. In the present work, we demonstrate the synthesis of fluorescent “warhead” candidates based on 2,1,3-benzoxadiazoles and the investigation of their physicochemical and photophysical properties. These molecules shall serve as probes for the detailed analysis of association/dissociation mechanism and of the kinetic parameters of the bond forming event.

Keywords: 2,1,3-benzoxadiazoles; covalent protease inhibitors; fluorescent properties

1. Introduction

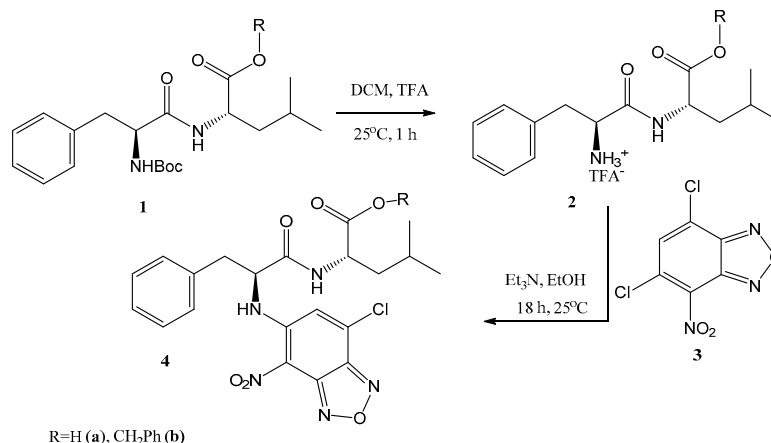
Alkyl halides, Michael acceptors, alkyl boronates, nitriles, sulfonyl fluorides, epoxides or aziridines have been used as reactive groups for the design of covalent inhibitors of proteases or esterases [1,2]. In contrast, only a small number of compounds are known to react with proteins in an S_NAr -reaction. Nevertheless, the general feasibility of S_NAr -based covalent protein ligands has recently been demonstrated [3]. For this reason, we have synthesized a series of different aromatic, heteroaromatic or quinoid electrophilic compounds, which exhibited a strong inhibitory activity on some proteases [4].

While the latter electrophilic moieties did not show a significant fluorescence, we became interested in the design and synthesis of fluorescent S_NAr reactive groups to investigate the association and the bond-forming event with the target enzyme in detail. It is known that derivatives of 2,1,3-benzoxadiazoles can be used as fluorescent labels for various biomolecules or -objects. For example, in our previous work, we showed the possibility of introducing fragments of 2,1,3-benzoxadiazoles into nucleosides and oligonucleotides [5,6]. They are also used for the synthesis of fluorescent amino acids, glucosides or protein binders [7–9].

Here, we report the synthesis of new fluorescent protease inhibitor candidates based on dihalogenated 2,1,3-benzoxadiazoles and the investigation of their photophysical properties.

2. Results and Discussion

The synthesis of electrophilic protease inhibitors was carried out according to Scheme 1.



Scheme 1. Synthesis of compounds **4**.

Compounds **1** and **2** were synthesized according to a procedure by Lawesson et al. [10], compound **3** was obtained by a method developed by Boulton and Katritzky [11]. The structures of Compounds **4a,b** were also confirmed by physicochemical analysis methods. To assess the possibility of using substances **4** as fluorescent labels which shall allow the detailed analysis of the various stages of covalent protein inhibition, we studied the luminescent properties of these compounds. Fluorescence spectra were recorded in MeOH and MeCN, shown in Figures 1 and 2. Maxima of emission are in the region of 475–485 nm. Compounds **4a,b** have similar curve profiles in different solvents, but there is a slight shift in maximum emissions towards the long-wave region in MeCN.

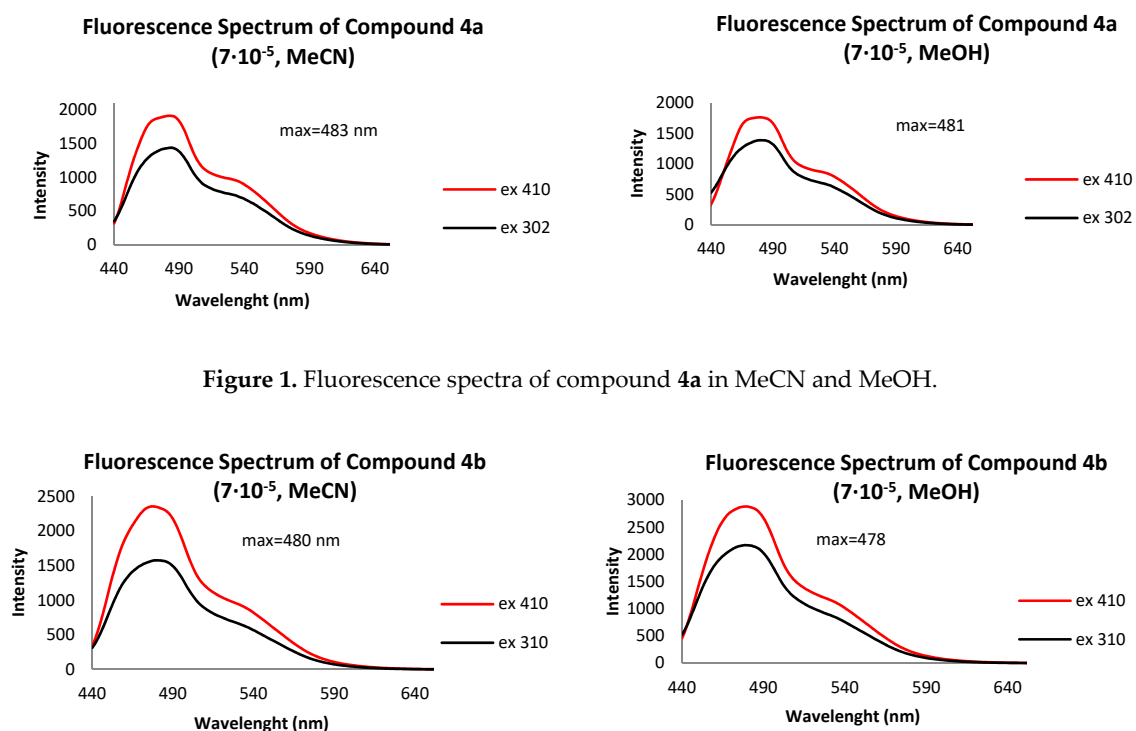
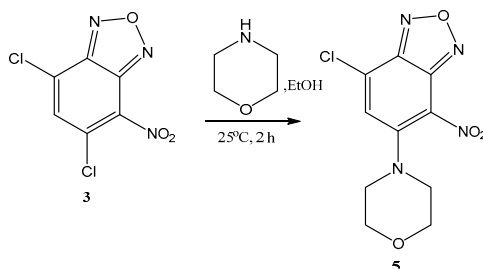


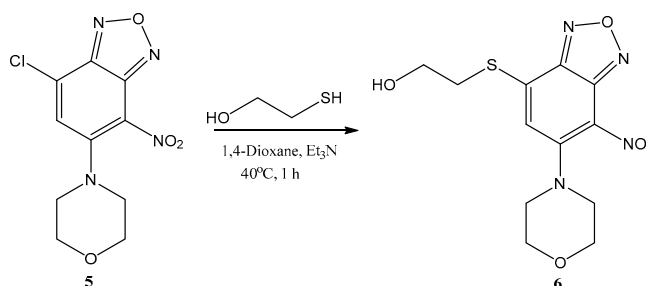
Figure 2. Fluorescence spectra of compound **4b** in MeCN and MeOH.

The location of the maximum emissions in a relatively narrow wavelength region is possibly caused by the presence of a hydrogen bond between the N-terminal amino group of the dipeptide and the nitro group located on the benzofurazan. The existence of this bond is also confirmed by the value of the chemical shift of the NH proton which is found strongly downfield shifted at 10.6 ppm. To exclude the influence of the hydrogen bond on the fluorescent properties of derivatives of 2,1,3-benzoxadiazoles, we synthesized a model compound from a secondary amine and studied its photophysical properties as well. 7-Chloro-5-morpholino-4-nitrobenzo[c][1,2,5]-oxadiazole (**5**) was synthesized according to Scheme 2.



Scheme 2. Synthesis of compound **5**.

The emission maximum of compound **5** is at 546 nm. Furthermore, we studied the S_NAr reaction of this compound with mercaptoethanol simulating the active cysteine residues in certain proteases and also studied the impact on the fluorescent properties (Scheme 3).



Scheme 3. Synthesis of compound **6**.

The nucleophilic displacement of the second chlorine by thiolate doesn't change the emission wavelength but increases the fluorescence intensity (the determination of the quantum yield is currently underway in our laboratory). The fluorescence spectra of compounds **5** and **6** are depicted in Figure 3.

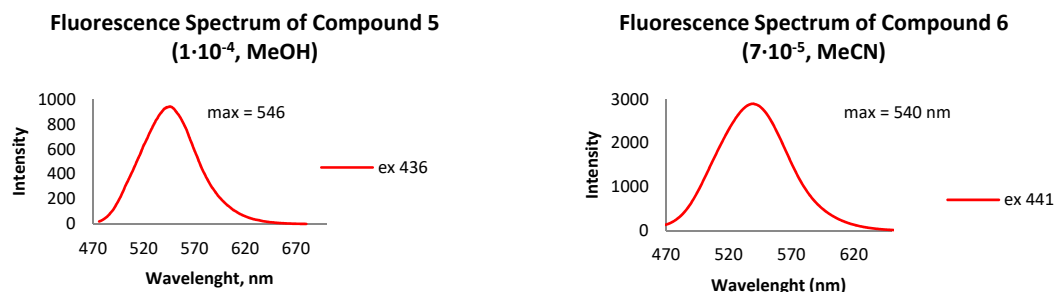


Figure 3. Fluorescence spectra of compounds **5,6**.

Quantum yields were calculated relative to quinine sulfate for compounds **4a,b** and to fluorescein for compounds **5** and **6**. Values of quantum yields for all compounds are lower than 1%. Such small values are possibly caused by the presence of a nitro group—adjacent to an amino function.

3. Methods

All reagents and solvents were obtained from commercial suppliers (Sigma Aldrich, Alfa Aesar, TCI chemicals, ABCR, Acros Organics and Fischer Scientific) and used without further purification. Analytical thin-layer chromatography (TLC) was performed on Merck silica gel plates (60 F₂₅₄) with defined solvent mixtures and visualized under ultraviolet (UV) light irradiation and/or TLC staining reagents. The melting points were determined in an open capillary tube. Nuclear magnetic resonance (NMR) experiments were performed on a 300 MHz (300 MHz ¹H and 75.4 MHz ¹³C) or a 600 MHz (600 MHz ¹H and 153.8 MHz ¹³C) spectrometer from Bruker using deuterated solvents ((residual) solvent signals: CDCl₃: δH = 7.26 ppm, δC = 77.16 ppm; (CD₃)₂SO: δH = 2.50 ppm, δC = 39.52 ppm) as an internal reference and reported in parts per million (ppm, δ) relative to tetramethylsilane (TMS, δ = 0.00 ppm). The absorption spectra were recorded on Thermo Scientific Evolution 201 using a 1 cm path length quartz cuvette. Samples were analyzed in MeCN or MeOH, concentration 7 × 10^{−5} mol L^{−1} for compounds **4a,b**; in MeCN, concentration 1 × 10^{−4} mol L^{−1} for compound **5**, and in MeCN, concentration 7 × 10^{−5} mol L^{−1} for compound **6**. All fluorescence spectra were recorded on a JASCO FP-8300 spectrofluorometer equipped with a xenon arc lamp using a 1 cm path length quartz cuvette. We used excitation (Ex) and emission (Em) bandwidths of 10 nm for compounds **4a,b** and Ex, Em bandwidths of 20 nm for compounds **5** and **6**.

(7-Chloro-4-nitrobenzo[c][1,2,5]oxadiazol-5-yl)-L-phenylalanyl-L-leucine: Crude L-Phenylalanyl-L-leucine trifluoroacetic acid salt (51.43 mg, 0.185 mmol, 1 eq.), 5,7-dichloro-4-nitrobenzo[c][1,2,5]oxadiazole (65 mg, 0.28 mmol, 1.5 eq.) were dissolved in ethanol (5 mL) and triethylamine (50 µL) was added. After stirring for 18 h at room temperature, the solvent was removed under reduced pressure. Column chromatography (SiO₂, DCM:MeOH; 20:1) gave the pure product (29.1 mg, 61 µmol, 33%).

¹H NMR, COSY (600 MHz, DMSO-d₆): δ/ppm = 10.83 (s, 1H, NH^{Phe}), 8.77 (s, 1H, NH^{Leu}), 7.23–7.17 (m, 5H, H^{Ar}, 1H, CH⁵), 5.19 (m, 1H, α-CH^{Phe}), 4.36 (m, 1H, α-CH^{Leu}), 3.38 (dd, 13.7, 8.8 Hz, 1H, β-CH₂^{Phe}), 3.06 (dd, J = 13.7, 8.8 Hz, 1H, β-CH₂^{Phe}), 1.64–1.23 (m, 3H, β-CH₂, γ-CH^{Leu}), 0.94–0.86 (m, 6H, CH(CH₃)₂).

¹³C NMR, HSQC, HMBC (150 MHz, DMSO-d₆): δ/ppm = 174.1 (COOH), 169.1 (C=O^{Phe}), 150.1 (C-4a), 145.8 (C-7a), 145.6 (C-7), 136.0 (CH^{Ar}), 130.4 (C-6), 129.0 (CH^{Ar}), 127.7 (CH^{Ar}), 124.8 (C-5), 112.6 (C-4), 58.46 (α-CH^{Phe}), 51.0 (α-CH^{Leu}), 24.7 (γ-CH^{Leu}), 23.41, 21.50 (δ-CH₃^{Leu}).

R_f = 0.14 (DCM:MeOH, 10:1).

mp: 213–215 °C.

ESI-MS: m/z = 476.2 (100%, [M + H]⁺).

UV spectrum (MeCN, 1 × 10^{−5} mol L^{−1}), λ_{max}, nm: 303 nm, 412 nm.

Fluorescence spectrum, λ_{max}, nm: 481 (MeOH), 483 (MeCN).

Benzyl (7-chloro-4-nitrobenzo[c][1,2,5]oxadiazol-5-yl)-L-phenylalanyl-L-leucinate: Crude benzyl L-phenylalanyl-L-leucinate trifluoroacetic acid salt (103 mg, 0.21 mmol, 1 eq.), 5,7-dichloro-4-nitrobenzo[c][1,2,5]oxadiazole (75 mg, 0.32 mmol, 1.5 eq.) were dissolved in ethanol (6 mL) and triethylamine (100 µL) was added. After stirring for 18 h at room temperature, the solvent was removed under reduced pressure. Column chromatography (SiO₂, Cyclohexane: Ethyl acetate, 2:1) gave the pure product (109 mg, 0.20 mmol, 90%).

¹H NMR, COSY (300 MHz, CDCl₃): δ/ppm = 10.57 (d, J = 6.7 Hz, 1H, NH^{Phe}), 7.38–7.30 (m, 10 H, H^{Ar}), 6.98 (s, 1H, CH⁵), 6.23 (d, J = 8.4 Hz, 1H, NH^{Leu}), 5.15 (m, 2H, OCH₂^{Bz}), 4.75 (m, 1H, α-CH^{Leu}), 4.54 (m, 1H, α-CH^{Phe}), 3.39 (dd, J = 14.1, 4.7 Hz, 1H, β-CH₂^{Phe}), 3.29 (dd, J = 14.1, 4.7 Hz, 1H, β-CH₂^{Phe}), 1.70–1.50 (m, 3H, β-CH₂, γ-CH^{Leu}), 0.93 (m, 6H, CH(CH₃)₂).

¹³C NMR, HSQC, HMBC (75 MHz, CDCl₃): δ/ppm = 172.0 (C=O^{Leu}), 168.7 (C=O^{Phe}), 149.21 (C-4a), 145.4 (C-7a), 144.8 (C-6), 135.0 (OCC^{Leu}), 132.2 (C-7), 129.4, 128.6 (CH^{Ar}), 121.9 (C-5), 113.3 (C-4), 67.5 (CH₂^{Bz}), 60.5 (α-CH^{Phe}), 51.3 (α-CH^{Leu}), 41.0 (β-CH₂^{Leu}), 39.5 (β-CH₂^{Phe}), 24.9 (γ-CH^{Leu}), 27.3, 21.63 (δ-CH₃^{Leu}).

Rf = 0.35 (EtOAc: Cyclohexane, 1:2).

mp: 169–170 °C.

ESI-MS: m/z = 566.2 (62%, [M + H]⁺), 588.2 (26%, [M + Na]⁺).

UV-spectrum (MeCN, 1×10^{-5} mol L⁻¹), λ_{\max} , nm: 302 nm, 411 nm.

Fluorescence spectrum, λ_{\max} , nm: 478 (MeOH), 480 (MeCN).

7-Chloro-5-morpholino-4-nitrobenzo[c][1,2,5]oxadiazole: 5,7-Dichloro-4-nitrobenzo[c][1,2,5]oxadiazole (100 mg, 0.43 mmol, 1 eq.) was dissolved in ethanol (5 mL) and morpholine (56 μ L, 0.65 mmol, 1.5 eq.) was added. After stirring for 2 h at room temperature, the solvent was removed under reduced pressure. Crystallization from ethanol afforded the title compound (85 mg, 0.30 mmol, 70%).

¹H NMR, COSY (300 MHz, DMSO-d₆): δ /ppm = 8.20 (s, 1H, CH-5), 3.79 (m, 4H, CH₂O), 3.58 (m, 4H, CH₂N).

¹³C NMR, HSQC, HMBC (75 MHz, CDCl₃): δ /ppm = 151.2 (C-4a), 146.6 (C-6), 146.3 (C-7a), 129.3 (C-5), 126.7 (C-7), 114.6 (C-4), 66.63 (CH₂O), 52.8 (CH₂N).

Rf = 0.48 (DCM:MeOH, 10:1).

mp: 184–186 °C.

UV-spectrum (MeCN, 1×10^{-5} mol L⁻¹), λ_{\max} , nm: 312 nm, 440 nm.

Fluorescence spectrum, λ_{\max} , nm: 546 (MeOH).

2-((6-Morpholino-7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)thio)ethan-1-ol:

7-Chloro-5-morpholino-4-nitrobenzo[c][1,2,5]oxadiazole (22 mg, 78 μ mol, 1 eq.), mercaptoethanol (8.3 μ L, 0.12 mmol, 1.5 eq.) were dissolved in 1,4-dioxane (3 mL) and 1 drop of trimethylamine was added. After stirring for 1 h at 40 °C, the solvent was removed by co-evaporation with cyclohexane (5 mL). Crystallization from propanol-2 afforded the title compound (20 mg, 61 μ mol, 79%).

¹H NMR, COSY (300 MHz, DMSO-d₆): δ /ppm = 7.34 (s, 1H, CH-5), 5.24 (t, J = 5.6 Hz, 1H, OH), 3.80 (m, 6H, CH₂O, CH₂OH), 3.60 (m, 4H, CH₂N), 3.46 (m, 2H, CH₂S).

¹³C NMR, HSQC, HMBC (75 MHz, CDCl₃): δ /ppm = 151.7 (C-7a), 146.3 (C-6), 146.2 (C-4a), 136.3 (C-4), 120.5 (C-5), 114.7 (C-7), 66.44 (CH₂O), 59.68 (CH₂OH), 52.87 (CH₂N), 34.36 (CH₂S).

Rf = 0.29 (Toluene:EtOAc, 4:1).

mp: 177–179 °C.

UV spectrum (MeCN, 1×10^{-5} mol L⁻¹), λ_{\max} , nm: 372 nm, 441 nm.

Fluorescence spectrum, λ_{\max} , nm: 540 (MeCN).

4. Conclusions

Compounds **4a,b** were synthesized as potential protease inhibitors allowing a multiparameter optical read-out, and their photophysical properties were investigated. The emission maxima of these compounds are located at relatively short wavelengths due to the presence of an intramolecular hydrogen bond. In contrast, derivatives devoid of NH-protons have longer emission wavelengths and a second S_NAr reaction with a thiolate leads to an increase in the fluorescence intensity while keeping the emission wavelengths essentially unchanged.

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