Halogenated 2,1,3-benzoxadiazoles as potential fluorescent warheads for covalent protease inhibitors

Anastasiia Kuznetsova[1], Philipp Klein[2], Till Opatz[2]*

1. National Research Tomsk Polytechnic University, 30 Lenin Ave., Tomsk, 634050, Russia
2. Johannes Gutenberg-University Mainz Institute of Organic Chemistry, Duesbergweg 10-14, Mainz, D-55128, Germany

e-mail:opatz@uni-mainz.de

Abstract

Recently there is a growing interest in covalent protease inhibitors in 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, heteroaromaric 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 and of the kinetic parameters of the bond forming event.

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

Introduction

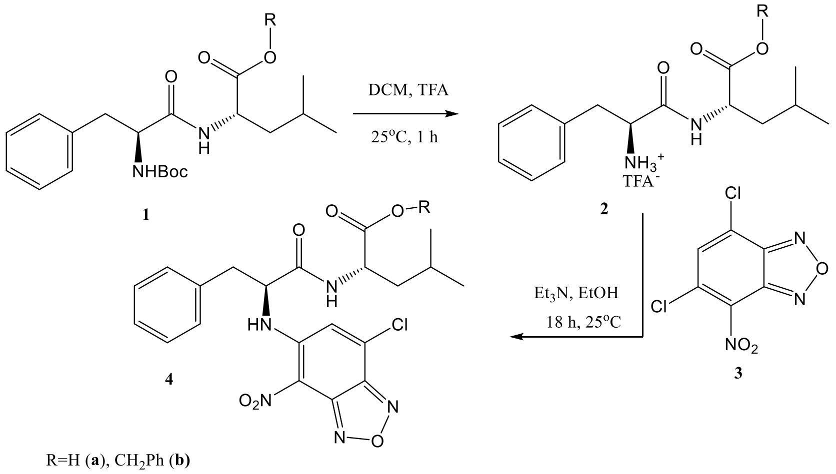
Alkyl halides, Michael acceptors, alkyl boronates, nitriles, sulfonyl fluorides, epoxides or aziridines can be used as reactive groups for the design of covalent inhibitors of proteases or esterases [1-2]. In contrast, only a small number of compounds are known to react with proteins by mechanism of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

While the latter electrophilic moieties did not show 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, our previous work showed the possibility of introducing fragments of 2,1,3-benzoxadeazoles into nucleosides and oligonucleotides [5-6]. They are also used for the synthesis of fluorescent amino acids, glucosides or protein binders [7-9].

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

Results and discussion

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



Scheme 1

Compounds **1** and **2** were synthesized according to a procedure by Lawesson et al. [10], compound **3** was obtained by a method developed by Bolton and Katritzky [11]. Structures of compounds **4** (**a,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 inihibition, we have studied the luminescent properties of these compounds. Fluorescence spectra were recorded in MeOH and MeCN and they are showed in **Fig. 1-2**. Maxima of emission are in region 475-485 nm. The 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.

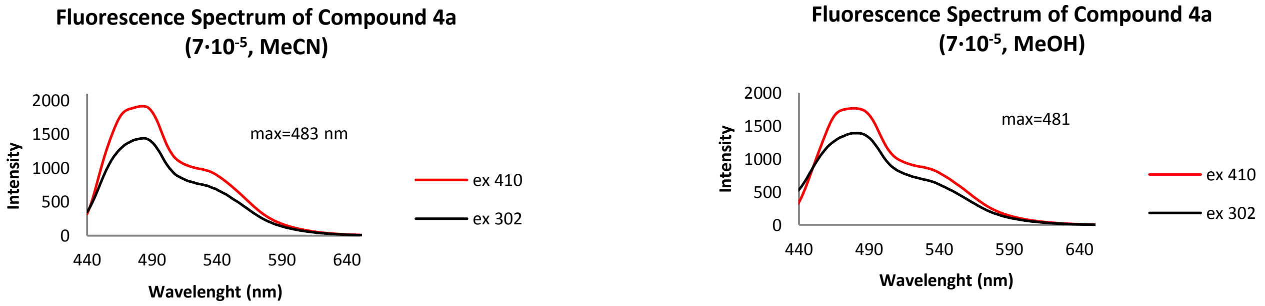


Fig.1 Fluorescence spectra of compound **4a** in MeCN and MeOH

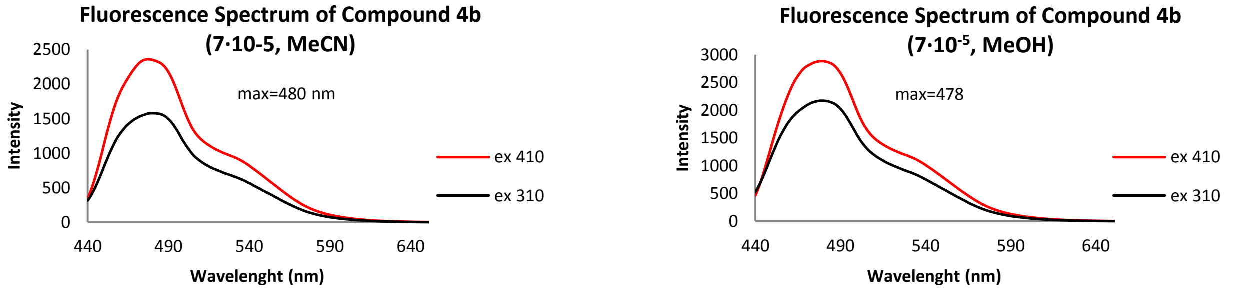
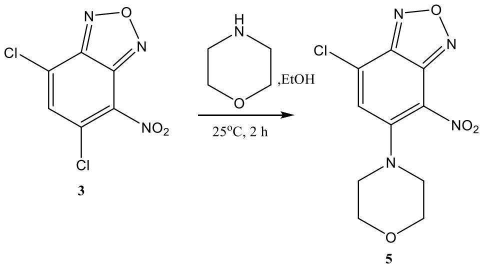


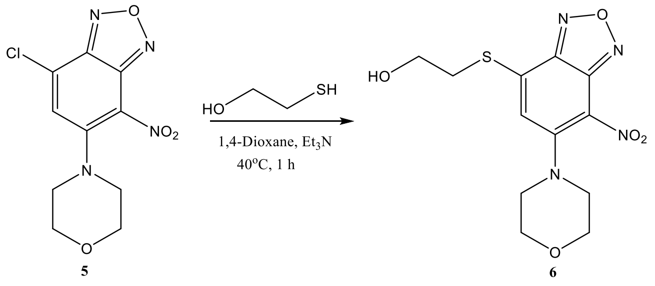
Fig.2 Fluorescence spectra of compound **4b** in MeCN and MeOH

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 chemical shift of the NH proton which is found at a strongly downfield shifted position at 10.6 ppm. For an exclusion of an 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 out according to Scheme 2.



Scheme 2

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

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 substance **5** and **6** are depicted in **Fig. 3.**

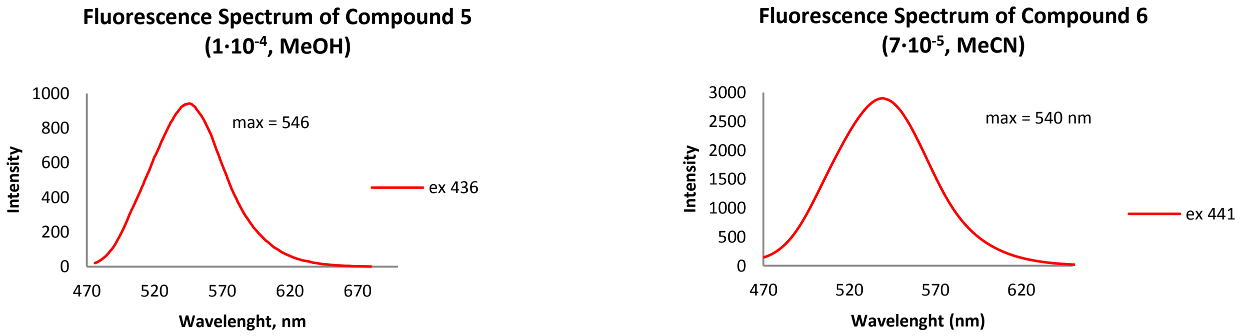


Fig.3 Fluorescence spectra of compounds **5,6**

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 F_{254}) with defined solvent mixtures and visualized under UV light irradiation and/or TLC staining reagents. Melting points were determined in open capillary tube. NMR experiments were performed on a 300 MHz (300 MHz ^{1}H and 75.4 MHz ^{13}C) or a 600 MHz (600 MHz ^{1}H and 153.8 MHz ^{13}C) spectrometer from Bruker using deuterated solvents ((residual) solvent signals: $CDCl_3$: δH = 7.26 ppm, δC = 77.16 ppm; $(CD_3)_2SO$: δH = 2.50 ppm, δC = 39.52 ppm;) as internal reference and reported in parts per million (ppm, δ) relative to tetramethylsilane (TMS, δ = 0.00 ppm).[1] Absorption spectra were recorded on Thermo Scintific Evolution 201 using a 1 cm path length quartz cuvette. Samples were analyzed in MeCN, concentration $1 \cdot 10^{-5}$. All fluorescence spectra were recorded on a JASCO

FP-8300 spectrofluorimeter equipped with a xenon arc lamp using a 1 cm path length quartz cuvette.

(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 50µL triethylamine was added. After stirring for 18 h at room temperature, the solvent was removed under reduced pressure. Column chromatography (SiO_2, DCM:MeOH; 20:1) gave the pure product (29.1 mg, 61 µmol, 33 %).

^{1}H NMR, COSY (600 MHz, DMSOd$_6$): δ/ppm = 10.83 (s,1H, NH^{Phe}), 8.77 (s,1H, NH^{Leu}), 7.23 – 7.17 (m, 5H, H^{Ar}, 1H, CH^5), 5.19 (m, 1H, α-CH^{Phe}), 4.36 (m, 1H, α- CH^{Leu}), 3.38 (dd, 13.7, 8.8 Hz, 1H, β-CH_2^{Phe}), 3.06 (dd, *J* = 13.7, 8.8 Hz, 1H, β-CH_2^{Phe}), 1.64-1.23 (m, 3H, β-CH_2, γ-CH^{Leu}), 0.94 – 0.86 (m, 6H, CH(CH_3)$_2$).

^{13}C NMR, HSQC, HMBC (150 MHz, DMSOd$_6$): δ/ppm = 174.1 (*C*OOH), 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-*C*H^{Phe}), 51.0 (α-*C*H^{Leu}), 24.7 (γ-*C*H^{Leu}), 23.41, 21.50 (δ-*C*H$_3^{Leu}$).

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

mp: 213-215 ^{o}C.

ESI-MS: m/z = 476.2 (100%, $[M+H]^+$).

UV spectrum (MeCN, 1·10^{-5}), 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)-D-phenylalanyl-D-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 (SiO_2, Cyclohexane: Ethyl acetate, 2:1) gave the pure product (109 mg, 0.20 mmol, 90 %).

^{1}H NMR, COSY (300 MHz, CDCl$_3$): δ/ppm = 10.57 (d, *J*=6.7Hz,1H, NH^{Phe}), 7.38 – 7.30 (m, 10 H, H^{Ar}), 6.98 (s, 1H, CH^5), 6.23 (d, *J*=8.4 Hz, 1H, NH^{Leu}), 5.15 (m, 2H, OCH_2^{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-CH_2^{Phe}), 3.29 (dd, *J* = 14.1, 4.7 Hz, 1H, β-CH_2^{Phe}), 1.70-1.50 (m, 3H, β-CH_2, γ-CH^{Leu}), 0.93 (m, 6H, CH(CH_3)$_2$).

^{13}C NMR, HSQC, HMBC (75 MHz, CDCl$_3$): 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 (OC*C*^{Leu}), 132.2 (*C*-7), 129.4, 128.6 (*C*H^{Ar}), 121.9 (*C*-5), 113.3 (*C*-4) 67.5 (*C*H$_2^{Bz}$), 60.5 (α-*C*H^{Phe}), 51.3 (α-*C*H^{Leu}), 41.0 (β-*C*H$_2^{Leu}$), 39.5 (β-*C*H$_2^{Phe}$), 24.9 (γ-*C*H^{Leu}), 27.3, 21.63 (δ-*C*H$_3^{Leu}$).

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, $1\cdot10^{-5}$), 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 µL, 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 %).

^{1}H NMR, COSY (300 MHz, DMSOd_6): δ/ppm = 8.20 (s, 1H, C*H*-5), 3.79 (m, 4H, CH_2O), 3.58 (m, 4H, CH_2N).

^{13}C NMR, HSQC, HMBC (75 MHz, $CDCl_3$): 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 (CH_2O), 52.8 (CH_2N).

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

mp: 184-186°C.

UV-spectrum (MeCN, $1\cdot10^{-5}$), 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 µmol, 1 eq.), mercaptoethanol (8.3 µL, 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 ^{o}C, the solvent was removed by co-evaporation with cyclohexane (5 ml). Crystallization from propanol-2 afforded the title compound (20 mg, 61 µmol, 79 %).

^{1}H NMR, COSY (300 MHz, DMSOd_6): δ/ppm = 7.34 (s, 1H, C*H*-5), 5.24 (t, *J*=5.6 Hz, 1H, O*H*), 3.80 (m, 6H, CH_2O, CH_2OH), 3.60 (m, 4H, CH_2N), 3.46 (m, 2H, CH_2S).

^{13}C NMR, HSQC, HMBC (75 MHz, $CDCl_3$): 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 (CH_2O), 59.68 (CH_2OH), 52.87 (CH_2N), 34.36 (CH_2S).

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

mp: 177-179°C.

UV spectrum (MeCN, $1\cdot10^{-5}$), λ_{max}, nm: 372 nm, 441 nm.

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

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.

Acknowledgements

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