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Identification of fluorogenic and quenched benzoxadiazole reactive chromophores

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ABSTRACT

The Sharpless–Meldal reaction was employed to generate triazole-substituted, alkynyl, azido and triazolyl-benzoxadiazole as well as nitro-benzoxadiazole fluorophores. Linkage of the triazole to the benzoxadiazole ring at C4 gave chromophores which were fluorogenic, while attachment through N1 resulted in quenching. The 4-azido-7-nitrobenzoxadiazole underwent a 470–fold decrease in quantum yield upon conversion to the triazole. While, 5-ethynyl-benzoxadiazole exhibited a 48–fold enhancement of quantum yield upon formation of triazole. The modulating effects of solvent polarity, conjugation, and attachment point of the fluorochrome to the triazole were examined.

1. Introduction

The development of bioconjugate strategies for the ligation of fluorescent labels to biomolecules is a growing area of research. Successful strategies for use in biological assays require high specificity and sensitivity to enable the detection of single species in complex mixtures. Applications for studying proteins, nucleic acids, carbohydrates, and lipids are known [1–6]. Dyes with improved sensitivity, selectivity, or unique spectral properties will expand the toolkit of bioconjugate chemists. Advances in these areas enable researchers to address novel questions in molecular biology, a field where heterogeneous environments impose immense challenges to characterisation.

Renewed focus has been devoted to bioorthogonal chemical reactions which can retain their specificity in aqueous environments and in the presence of common biomolecular functional groups [7,8]. The condensation of ketones and aldehydes with amine nucleophiles, such as aminooxy or hydrazide compounds, is a classic example of a bioorthogonal reaction [7,9]. However, this methodology is often limited to cell surface applications due to the presence of endogenous carbonyls within the cytoplasm [7]. The Staudinger ligation is a popular bioorthogonal reaction employing the selective conjugation of an azide with a phosphine [3]. It has been successfully used for numerous labelling experiments and is amenable to use on live cells. The main limitation of the Staudinger ligation is that it suffers from relatively slow reaction kinetics [7,10]. The Sharpless–Meldal reaction, which couples azides and alkynes through a Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition, has rapidly gained in popularity [11,12]. This reaction has been dubbed a click reaction as it requires only mild aqueous reaction conditions [13], allowing its use in biochemical studies with an array of tags and fluorophores [14–17]. Although the click reaction has shown remarkable versatility in biological studies, chromophores with improved sensitivity are still needed.

The formation of a triazole product from the Sharpless–Meldal reaction has been shown to alter the spectral properties from the initial azide or alkyne [18–21]. Examples are known of both azido and alkynyl substituted fluorophore cores that exhibit altered photophysical properties following triazole formation [18–22]. In 2004, Zhou and Fahrni showed an example of a 7-alkynyl coumarin that underwent an 18-fold increase in quantum yield and bathochromic shift of emission by 27 nm [19]. Later, Sivakumar and colleagues synthesized a series of click-ligation products from a library of terminal alkynes and 3-azido coumarin profluorophores. These authors found emission intensities significantly increased upon triazole formation, in the best cases the quantum yield

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increased from 0.0 to 0.3 [18]. Iterations of this methodology have been applied to several other fluorophore classes, including napthalimides[22] and anthracenes [20]. We class these examples as development of *reactive chromophores*, structures which exhibit significant changes in photophysical properties upon chemical reaction. Reactive chromophores offer the potential to greatly simplify biochemical assays and improve sensitivity [23,24]. Reactive chromophores have been used in biological samples with high background and complexity for the specific labelling of proteins, lipids, nucleic acids and virus particles [20,22–24]. The increased excitation or emission maxima or altered quantum yield of reactive chromophores have clear advantages; however, it remains difficult to predict desired spectral changes in specific chromophores. We have previously addressed this problem by generating a series of structurally similar fluorophores which incorporated a conjugated alkyne on a coumarin backbone [21]. Upon conversion of these fluorophores to the corresponding triazole, we found that subtle differences in substitution were able to modulate the effect arising from triazole formation. Comparison of the photophysical properties of these compounds after triazole formation identified increases in quantum yields of up to 9-fold, and bathochromic shifts of as much as 23 nm. Although these dyes are useful for fluorogenic labelling strategies, their relatively low brightness and short excitation wavelength inspired us to examine a new series of alkynyl- and azido-benzoxadiazole fluorophores which we expected to have longer wavelength emission.

Nitrobenzoxadiazole (NBD) fluorophores are an important class of small molecule labels used in chemical biology and bioanalytical studies [25–27]. NBDs are environmentally sensitive dyes which exhibit dramatic changes in excitation and emission spectra due to the polarity and hydration of their environment [28]. Their small size, strong fluorescence, and sensitivity to environmental changes make them ideal for biological applications. We chose to examine the photophysical properties of a series of alkynyl-, azido-, and triazolyl-benzoxadiazole and nitro-benzoxadiazole fluorophores. Nitro substitution at the 4-position and alkyne or azide substitution at the 5- and 7- positions were selected as they have previously been shown to strongly influence fluorescent properties [29,30]. We examined the modulating effects of solvent polarity, conjugation and attachment point of the fluorochrome to the triazole. Characterization of these compounds revealed that ligation to model alkynes or azides by Sharpless–Meldal reaction imparts dramatic photophysical changes to these dyes. We were able to identify reactive chromophores which act as fluorogenic, quenched, and invariant fluorescent labels. Fluorogenic dyes with increases of intensity of almost 50-fold, and quenched dyes which show an almost 500-fold loss of intensity were identified. This is the largest quantified increase in quantum yield for a reactive alkynyl dye, and the largest quenching effect known upon triazole formation. These reaction-sensitive dyes should be of interest for use in bioconjugate chemistry.

2. Experimental

2.1. General

Reagents were of reagent grade purity obtained from Sigma–Aldrich (Oakville, Ont) and were used without additional purification. ^{1}H and ^{13}C NMR were performed on Varian 300, 400, or 500 MHz instruments at room temperature as noted. Deuterated solvents were obtained from Cambridge Isotope Laboratories (Andover, MA). CD_3OD, $(CD_3)_2SO$, and $CDCl_3$ solvent peaks (3.31, 2.50, and 7.26 ppm for ^{1}H; 49.0, 39.5, and 77.2 ppm for ^{13}C, respectively) were used as internal chemical shift references. Some spectra contain small amounts of contaminating solvents [36]. Mass spectrometry was performed using an MS-50G positive electron impact instrument from Kratos Analytical (Manchester, UK) and a Mariner Biospectrometry positive ion electrospray instrument from Applied Biosystems (Foster City, CA).

2.2. Spectroscopy

The absorbance spectra of all compounds were collected at room temperature using a Hewlett–Packard (Palo Alto, CA) model 8453 diode array UV–visible spectrophotometer or Varian (Walnut Creek, CA) Cary 50 spectrophotometer. Absorbance measurements were taken using Eppendorf (Hamburg, Germany) UVette cuvettes (220–1600 nm) or NSG Precision Cells (Farmingdale, NY) ES quartz cuvettes (190–2000 nm). Fluorescence spectra for all compounds were collected at room temperature with a Photon Technology International model MP1 steady-state fluorimeter. Fluorescence measurements were taken using NSG Precision Cells (Farmingdale, NY) ES quartz cuvettes (190–2000 nm).

2.3. Synthesis

2.3.1. 5-(Azidomethyl)benzo[c][1,2,5]oxadiazole (**2**)

5-(Bromomethyl)benzo[c][1,2,5]oxadiazole (200 mg, 0.93 mmol, 1 equiv) was dissolved in DMSO (15 mL), followed by the addition of sodium azide (0.57 g, 8.8 mmol, 1.5 equiv). The reaction mixture was heated to 40 °C and then quenched after 45 min using distilled water. Extraction was performed with diethyl ether, followed by water and brine washes. The organic layer was dried over $MgSO_4$ and concentrated in vacuo giving a yellow oil (133 mg, 82%).; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.90 (d, 1H, ^{3}J = 10.4 Hz), 7.80 (s, 1H), 7.36 (d, 1H, ^{3}J = 10.4 Hz), 4.51 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 149.0, 148.7, 139.5, 131.7, 117.3, 114.2, 54.2. IR (neat film): ν = 3064, 2107, 1635 cm^{-1}; EI-HRMS calculated for $C_7H_5N_5O$: 175.0494; observed: 175.0495. R_f = 0.50 (1:3 EtOAc/hexanes).

2.3.2. 5-Azidobenzo[c][1,2,5]oxadiazole (**4**)

5-bromobenzo[c][1,2,5]oxadiazole (200 mg, 1 mmol, 1 equiv) was dissolved in dimethylformamide (20 mL). Sodium azide (195 mg, 3 mmol, 3 equiv) was then added and the reaction mixture was heated at 85 °C for 24 h. The reaction was quenched with distilled water and extracted with ethyl ether. The organic layer was then dried over $MgSO_4$, concentrated in vacuo giving a yellow solid which was purified by gradient flash column chromatography (EtOAc/hexanes). The product was obtained in 99% yield as a brown-yellow powder (160 mg, 99%). 98.9–100.0 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (dd, 1H, ^{4}J = 0.96 Hz, ^{3}J = 9.6 Hz), 7.39 (dd, 1H, ^{4}J = 0.96 Hz, ^{4}J = 1.96 Hz), 7.07 (dd, 1H, ^{4}J = 1.96 Hz, ^{3}J = 9.6 Hz); APT ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.5, 147.9, 144.0, 127.8, 118.6, 101.9; IR (microscope): ν = 3119, 3058, 3050, 2177, 2136, 1626, 1536 cm^{-1}; EI-HRMS calculated for $C_{12}H_{14}O$: 161.0338; observed: 161.0337. R_f = 0.71 (1:3 EtOAc/hexanes).

2.3.3. 4-Azido-7-nitrobenzo[c][1,2,5]oxadiazole (**6**)

4-Chloro-7-nitrobenzo[c][1,2,5]oxadiazole (250 mg, 1.25 mmol, 1 equiv) was dissolved in 10 mL of 1:1 acetone/methanol solution. To this was added, slowly, sodium azide (90 mg, 1.38 mmol, 1.1 equiv) dissolved in 1:2:1 acetone/methanol/water solution. The reaction was allowed to proceed at room temperature for 1 h until the starting material was not visible by TLC. Solvent was removed under vacuum and the resulting residue was recrystallized with 100% ethanol and allowed to cool at −20 °C giving brown-yellow crystals (252 mg, 98%). m.p. 95.3–97.0 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.53 (dd, 1H, ^{4}J = 0.64 Hz, ^{3}J = 8.0 Hz), 7.08 (dd, 1H, ^{4}J = 0.64 Hz, ^{3}J = 8.0 Hz); APT ^{13}C NMR (100 MHz, $CDCl_3$): δ 145.9, 143.5, 138.1, 132.5, 132.1, 114.9; IR (cast film): ν = 3092, 2139, 2118,

1536, 1338 1321 cm^{-1}; EI-HRMS calculated for $C_6H_2N_6O_3$: 206.0188; observed: 206.0187. $R_f = 0.33$ (1:3 EtOAc/hexanes).

2.3.4. N-(3-Bromopropyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**7**)

4-Chloro-7-nitrobenzo[c][1,2,5]oxadiazole (200 mg, 1 mmol, 1 equiv) and bromopropyl amine HBr (658 mg, 3 mmol, 3 equiv) were dissolved in methanol (8 mL) and heated to 40 °C. DIPEA (0.2 mL, 1.14 mmol, 1.1 equiv) was then added, turning the reaction mixture a dark red colour. Upon the consumption of starting materials as detected using TLC (approximately 6 h), the solvent was removed under vacuum. The resulting red oily residue was purified by column chromatography (EtOAc/hexanes), obtained as an orange-brown powder (180 mg, 60%). m.p. 72.1–73.8 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 8.46 (d, 1H, $^3J = 8.8$ Hz), 6.33 (d, 1H, $^3J = 9.2$ Hz), 3.71 (broad s, 2H), 3.59 (t, 2H, $^3J = 6.8$ Hz), 3.31 (q, 2H, $^4J = 1.64$ Hz), 2.30 (q, 2H, $^3J = 6.8$ Hz); ^{13}C NMR (100 MHz, CD_3OD): δ 146.6, 145.9, 145.5, 138.3, 123.5, 99.8, 43.2, 32.4, 31.1; IR (microscope): ν = 3382, 3096, 2967, 2922, 1621, 1586, 1502, 1357 and 1334, cm^{-1}; ES-HRMS calculated for $C_9H_9N_4O_3Na$: 322.9750; observed: 322.9755. $R_f = 0.67$ (3:2 EtOAc/hexanes);

2.3.5. N-(3-Azidopropyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**8**)

N-(3-Bromopropyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (76 mg, 0.254 mmol, 1 equiv) and sodium azide (50 mg, 0.762 mmol, 3 equiv) were dissolved in dimethylformamide and heated at 80 °C for 3 h. The reaction was quenched with distilled water and extracted with ethyl ether. After water and brine washes, the organic layer was dried over $MgSO_4$ and concentrated in vacuo. The sample was purified by column chromatography (EtOAc/hexanes). The product was obtained as an orange powder (50 mg, 76%). m.p. 68.7–70.4 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.49 (d, 1H, $^3J = 8.4$ Hz), 6.60 (broad s, 1H), 6.24 (d, 1H, $^3J = 8.8$ Hz), 3.68 (q, 2H, $^3J = 6.8$ Hz), 3.62 (t, 2H, $^3J = 6.8$ Hz), 2.11 (q, 2H, $^3J = 6.8$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 144.5, 144.1, 144.0, 136.7, 124.4, 99.0, 49.4, 41.9, 28.0; IR (microscope): ν = 3325, 3101, 2973, 2933, 2856, 2137, 2108, 1620, 1586, 1362 cm^{-1}; ES-HRMS calculated for $C_9H_{10}N_7O_3$: 263.0840; observed: 264.0843. $R_f = 0.35$ (1:1 EtOAc/hexanes).

2.3.6. 7-Nitro-N-(prop-2-ynyl)benzo[c][1,2,5]oxadiazol-4-amine (**9**)

4-Chloro-7-nitrobenzo[c][1,2,5]oxadiazole (500 mg, 2.5 mmol, 1 equiv) was dissolved in acetonitrile (20 mL). Propargylamine (3-amino-1-propyne; 0.32 mL, 5 mmol, 2 equiv) was then added and the ensuing dark green coloured mixture was stirred at room temperature for 1.5 h. The solvent was removed under vacuum and the crude product was purified by column chromatography (EtOAc/hexanes). Product was obtained as an orange-brown powder (600 mg, 33%). m.p. 134.0–137.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.56 (d, 1H, $^3J = 8.4$ Hz), 6.4 (broad s, 1H), 6.38 (br s, 1H, $^3J = 8.4$ Hz), 4.35–4.33 (dd, 2H, $^4J = 2.4$ Hz, $^3J = 5.6$ Hz), 2.54 (t, 1H, $^4J = 2.4$ Hz,); ^{13}C NMR (100 MHz, $CDCl_3$): δ 144.6, 144.0, 142.5, 136.1, 100.2, 74.4, 33.7. IR (microscope): ν = 3392, 3366, 3310 and 3291, 3077, 3038, 2124, 1621, 1585 cm^{-1}; ES-HRMS calculated for $C_9H_6N_4O_3$: 241.0332; observed: 241.0334. $R_f = 0.10$ (1:3 EtOAc/hexanes).

2.3.7. 5-((Trimethylsilyl)ethynyl)benzo[c][1,2,5]oxadiazole (**10**)

5-Bromobenzo[c][1,2,5]oxadiazole (150 mg, 0.75 mmol, 1 equiv) was dissolved in acetonitrile (12 mL). $PdCl_2(PPh_3)_2$ (52 mg, 0.075 mmol, 0.1 equiv), DIPEA (0.50 mL, 2.8 mmol, 3.77 equiv), CuI (15 mg, 0.075 mmol, 0.1 equiv) and trimethylsilyl acetylene (0.52 mL, 3.75 mmol, 5 equiv) were then added and the reaction mixture was degassed using three, freeze–thaw cycles and then heated at 80 °C for 24 h. The reaction mixture was diluted with EtOAc, washed with NH_4Cl and dried over $MgSO_4$. The crude product was concentrated in vacuo, and purified by gradient flash column chromatography (EtOAc/hexanes) (150 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.93 (s, 1H), 7.77 (d, 1H, $^3J = 10.5$ Hz), 7.38 (d, 1H, $^3J = 10.5$ Hz), 0.27 (s, 9H); EI-HRMS calculated for $C_{11}H_{12}N_2OSi$: 216.0718; observed: 216.0717. $R_f = 0.68$ (1:9 EtOAc/hexanes).

2.3.8. 5-Ethynylbenzo[c][1,2,5]oxadiazole (**11**)

5-((Trimethylsilyl)ethynyl)benzo[c][1,2,5]oxadiazole (73 mg, 0.34 mmol, 1 equiv) was dissolved in methanol (7 mL) and tetrabutylammonium fluoride (0.29 mL, 1 mmol, 3 equiv) was then added; the reaction mixture was heated at 60 °C for 20 min, until starting material was no longer visible as judged by TLC. The reaction was quenched with distilled water, and concentrated in vacuo. The crude product was extracted with dichloromethane, dried over $MgSO_4$, concentrated in vacuo and purified by column chromatography (EtOAc/hexanes) giving a brown-yellow powder (35 mg, 72%). m.p. 66.0–66.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (s, 1H), 7.84 (d, 1H, $^3J = 10.4$ Hz), 7.43 (d, 1H, $^3J = 10.4$ Hz), 3.37 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.0, 148.4, 134.4, 125.8, 120.7, 116.9, 82.1, 82.0; IR (neat film): ν = 3290, 3270, 3109, 3054, 2110 cm^{-1}; EI-HRMS calculated for $C_8H_4N_2O$: 144.0324; observed: 144.0320. $R_f = 0.29$ (1:9 EtOAc/hexanes).

2.3.9. 5-((4-Hexyl-1H-1,2,3-triazol-1-yl)methyl)benzo[c][1,2,5]oxadiazole (**12**)

5-(Azidomethyl)benzo[c][1,2,5]oxadiazole (40 mg, 0.228 mmol, 1 equiv) was dissolved in 1:1 water/methanol (5 mL) and *n*-octyne (0.17 mL, 1.14 mmol, 5 equiv) was added. Copper sulphate (7 mg, 0.046 mmol, 0.2 equiv) and ascorbic acid (12 mg, 0.068 mmol, 0.3 equiv) were then added. The white precipitate which formed was filtered after stirring for 40 min at room temperature and then purified by column chromatography (EtOAc/hexanes). The product was obtained as a white powder (41 mg, 63%). m.p. 92.0–92.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87 (d, 1H, $^3J = 10.0$ Hz), 7.67 (s, 1H), 7.34 (s, 1H),7.33 (d, 1H, $^4J = 1.24$ Hz, $^3J = 9.2$ Hz), 5.62 (s, 2H), 2.74 (t, 2H, $^3J = 8.4$ Hz), 1.66 (m, 3H), 1.33 (m, 6H), 0.88 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 150.0, 149.2, 148.9, 139.3, 131.3, 121.1, 118.1, 115.1, 53.7, 31.8, 29.5, 29.1, 26.0, 14.3; IR (microscope): ν = 3109, 3055, 2965, 2956, 2854, 1556 cm^{-1}; ES-HRMS calculated for $C_{15}H_{20}N_5O$: 286.1662; observed: 286.1666. $R_f = 0.61$ (3:2 EtOAc/hexanes).

2.3.10. 5-(4-Hexyl-1H-1,2,3-triazol-1-yl)benzo[c][1,2,5]-oxadiazole (**13**)

5-Azidobenzo[c][1,2,5]oxadiazole (30 mg, 0.19 mmol, 1 equiv) was dissolved in 1:1 water/methanol (5 mL) and *n*-octyne (0.14 mL, 0.93 mmol, 5 equiv) was added. Copper sulphate (6 mg, 0.037 mmol, 0.2 equiv) and ascorbic acid (10 mg, 0.056 mmol, 0.3 equiv) were then added. The reaction was stirred at room temperature for 6 h and the reaction was then quenched with distilled water. The crude product was extracted with chloroform followed by water and brine washes. The organic layer was then dried over $MgSO_4$ and concentrated in vacuo. The sample was purified by column chromatography (EtOAc/hexanes). The product was obtained as white crystals (25 mg, 50%). m.p. 113.2–115.4 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.19 (dd, 1H, $^4J = 3.8$ Hz, $^4J = 1.84$ Hz), 8.06 (m, 2H), 7.91 (s, 1H), 2.85 (t, 2H, $^3J = 8.0$ Hz), 1.77 (m, 2H), 1.31–1.47 (m, 6H), 0.91 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ150.5, 149.0, 148.4, 139.1, 126.8, 119.1, 118.9, 104.5, 31.8, 29.4, 29.1, 25.9, 22.8, 14.3; IR (microscope): ν = 3147, 3114, 3095, 3059, 2956, 2929, 2857, 1637, 1540 cm^{-1}; ES-HRMS calculated for $C_{14}H_{18}N_5O$: 272.1506; observed: 272.1513. $R_f = 0.51$ (1:3 EtOAc/hexanes).

*2.3.11. 4-(4-Hexyl-1H-1,2,3-triazol-1-yl)-7-nitrobenzo[c][1,2,5] oxadiazole (**14**)*

A solution of *n*-octyne (0.143 mL, 0.97 mmol, 5 equiv), copper sulphate (6 mg, 0.039 mmol, 0.2 equiv), and ascorbic acid (10 mg, 0.06 mmol, 0.3 equiv) in 1:1 water/methanol (5 mL) was stirred at room temperature for 5 min 4-azido-7-nitrobenzo[c][1,2,5]oxadiazole (40 mg, 0.194 mmol, 1 equiv) was then added slowly and a green precipitate formed after 45 min. The reaction mixture was stirred for 1.5 h after which time, the solvent was removed in vacuo, and the crude product was purified by column chromatography (EtOAc/hexanes), giving a yellow powder. The purified product was obtained as a green-yellow powder (41 mg, 68%). m.p. 108.3–109.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.75 (s, 1H), 8.72 (d, 1H, $^3J = 8.0$ Hz), 8.52 (d, 1H, $^3J = 8.0$ Hz), 2.89 (t, 2H, $^3J = 8.4$ Hz), 1.80 (q, 2H, $^3J = 8$ Hz), 1.40 (m, 6H), 0.93 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 151.0, 144.1, 143.9, 135.0, 131.9, 130.8, 122.8, 117.7, 31.8, 29.3, 29.1, 25.8, 22.8, 14.3; IR (microscope): ν = 3159, 3114, 3090, 2955, 2930, 2855, 1597, 1559, 1542, 1337 cm^{-1}; ES-HRMS calculated for $C_{14}H_{17}N_6O_3$: 317.1357; observed: 317.1350.$R_f = 0.43$ (3:2 EtOAc/hexanes).

*2.3.12. N-(3-(4-hexyl-1H-1,2,3-triazol-1-yl)propyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**15**)*

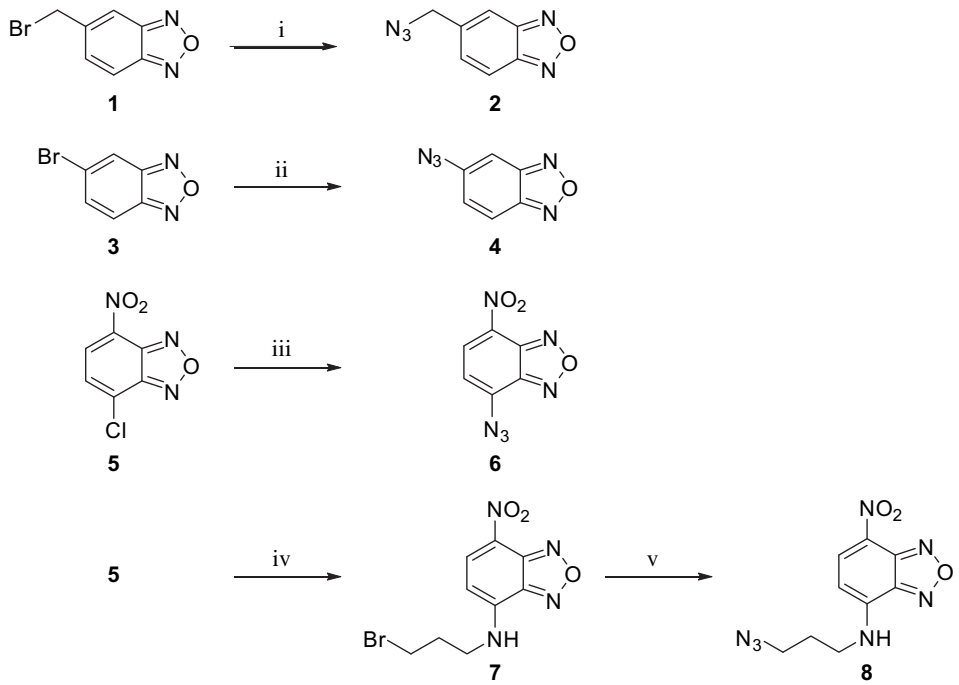
N-(3-Azidopropyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (20 mg, 0.076 mmol, 1 equiv), *n*-octyne (0.06 mL, 0.38 mmol, 5 equiv), copper sulphate (3 mg, 0.015 mmol, 0.2 equiv) and ascorbic acid (4 mg, 0.023 mmol, 0.3 equiv) were dissolved in 1:1 water/methanol solution (5 mL). The reaction mixture was stirred at room temperature for 6 h and the solvent then removed under vacuum, and the crude product was extracted with chloroform, then washed with water and brine. The organic layer was then dried over $MgSO_4$ and concentrated in vacuo. The sample was purified by column chromatography (EtOAc/hexanes). The product was obtained in 32% yield as an orange powder. m.p. 125.5–126.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.47 (d, 1H, $^3J = 10.0$ Hz), 7.39 (s, 1H), 6.94 (broad s, 1H), 6.19 (d, 1H, $^3J = 8.8$ Hz), 4.57 (t, 2H, $^3J = 7.6$ Hz), 3.66 (m, 2H), 2.73 (t, 2H, $^3J = 7.6$ Hz), 2.45 (q, 2H, $^3J = 6.4$ Hz), 2.04 (broad s, 1H), 1.67 (q, 2H, $^3J = 6.4$ Hz), 1.30 (m, 6H), 0.90 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.3, 144.5, 144.1, 144.0, 136.5, 124.5, 121.4, 99.2, 47.6, 41.3, 31.8, 29.6, 29.2, 28.9, 25.8, 22.8, 14.3; IR (microscope): ν = 3357, 3213, 3142, 3064, 2956, 2930, 2859, 1619, 1590, 1529, 1307 cm^{-1}; ES-HRMS calculated for $C_{17}H_{24}N_7O_3$: 374.1935; observed: 374.1933. $R_f = 0.56$ (3:2 EtOAc/hexanes).

*2.3.13. N-((1-Benzyl-1H-1,2,3-triazol-4-yl)methyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine (**16**)*

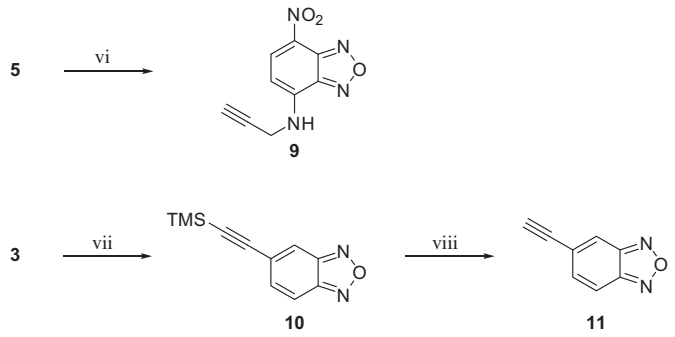
7-Nitro-*N*-(prop-2-ynyl)benzo[c][1,2,5]oxadiazol-4-amine (20 mg, 0.092 mmol, 1 equiv), benzyl azide (0.05 mL, 0.46 mmol, 5 equiv), copper sulphate (3 mg, 0.02 mmol, 0.2 equiv) and ascorbic acid (5 mg, 0.028 mmol, 0.3 equiv) were dissolved in a 1:1 water/methanol solution (5 mL). The reaction mixture was stirred at room temperature for 6 h. The solvent was then removed under vacuum, and the crude product was extracted with chloroform, and washed with water and brine. The organic layer was then dried over $MgSO_4$ and concentrated in vacuo. The sample was purified by column chromatography (EtOAc/hexanes). The product was obtained in as a yellow powder (15 mg, 46%). m.p. 198.8–199.7 °C; ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 8.54 (d, 1H, $^3J = 8.8$ Hz), 8.08 (s, 1H), 7.35 (m, 5H), 6.63(d, 1H, $^3J = 8.8$ Hz), 5.62 (s, 2H), 4.94 (s, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 144.4, 144.1, 137.6, 135.9, 128.7, 128.1, 127.9, 123.6, 121.3, 99.9, 52.8, 40.1; IR (microscope): ν = 3265, 3204, 3142, 3062, 2961, 2899, 1620, 1588, 1527, 1296 and 1274; ES-HRMS calculated for $C_{16}H_{14}N_7O_3$: 352.1153; observed: 352.1152. $R_f = 0.26$ (3:2 EtOAc/hexanes).

*2.3.14. 5-(1-Benzyl-1H-1,2,3-triazol-4-yl)benzo[c][1,2,5]-oxadiazole (**17**)*

5-Ethynylbenzo[c][1,2,5]oxadiazole (26 mg, 0.18 mmol, 1 equiv) was dissolved in 1:1 water/methanol and benzyl azide (0.095 mL, 0.90 mmol, 5 equiv) was added. Copper sulphate (6 mg, 0.036 mmol, 0.2 equiv) and ascorbic acid (10 mg, 0.025 mmol,



Scheme 1. Synthetic scheme for the generation of azido benzoxadiazoles. i. NaN_3, DMSO, 45 °C, 0.75 h, 82%; ii. NaN_3, DMF, 85 °C, 24 h, 99%; iii. NaN_3, acetone/MeOH/water, rt, 1 h, 98%; iv. Bromoproylamine·HBr, MeOH, DIPEA, 40 °C, 6 h, 60%; v. NaN_3, DMF, 80 °C, 3 h, 76%.



Scheme 2. Synthetic scheme for the generation of akynyl benzoxadiazoles. vi. Propargyl amine, CH_3CN, rt, 1.5 h, 33%; vii. TMS acetylene, CuI, $PdCl_2(PPH_3)_2$, DIPEA, CH_3CN, 80 °C, 24 h, 92%; viii. TBAF, MeOH, 60 °C, 0.5 h, 72%.

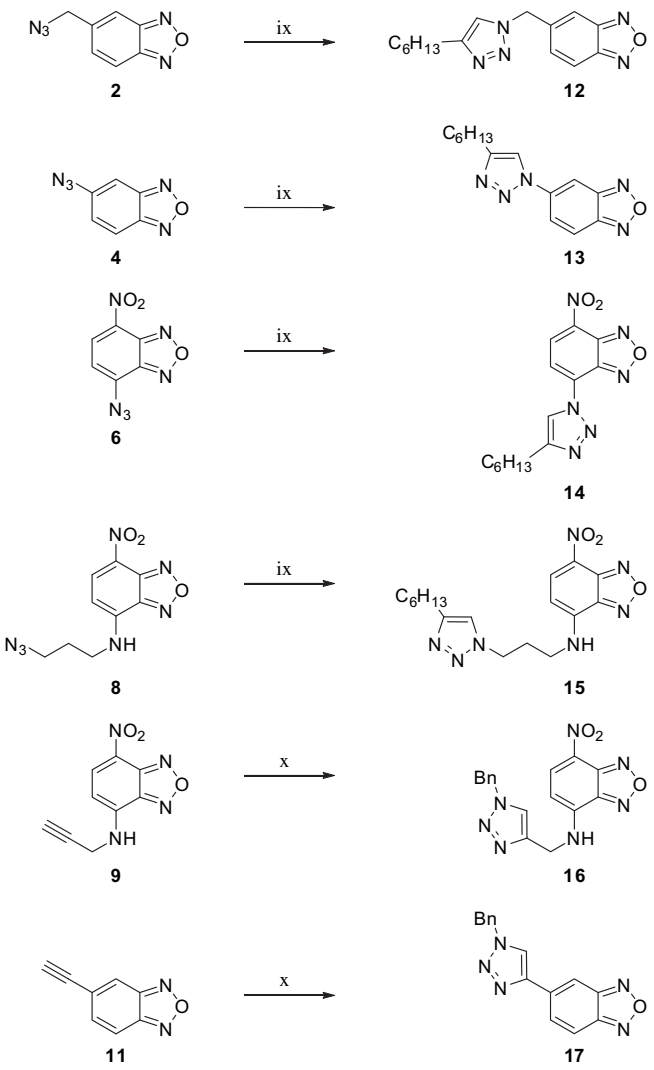
0.3 equiv) were then added to the solution. The reaction mixture was stirred at room temperature for 1.5 h, becoming white in colour. The solvent was removed in vacuo and the crude product was dissolved in chloroform, washed with water, dried over $MgSO_4$ and concentrated in vacuo. The sample was purified by column chromatography (EtOAc/hexanes). Product was obtained as a white powder in (30 mg, 60%). m.p. 149.5–148.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.19 (s, 1H), 8.02 (dd, 1H, $^4J = 1.2$ Hz, $^3J = 9.6$ Hz), 8.01 (dd, 1H, $^4J = 1.2$ Hz, $^3J = 9.6$ Hz), 7.88 (s, 1H), 7.40–7.48 (m, 3H), 7.34–7.40 (m, 2H), 5.64 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.6, 149.0, 146.2, 133.8, 131.2, 129.6, 129.4, 128.5, 121.4, 117.3, 111.3, 54.8; IR (microscope): ν = 3138, 3122, 3071, 3040, 2924, 2853, 1630, 1564 cm^{-1}; ES-HRMS calculated for $C_{15}H_{12}N_5O$: 278.1036; observed: 278.1032. $R_f = 0.18$ (1:3 EtOAc/hexanes).

3. Results and discussion

3.1. Fluorophore Synthesis

The most direct route to substituted benzoxadiazoles was identified as nucleophilic substitution of the appropriate halo starting materials (Scheme 1). The 5-azidomethyl-benzoxadiazole **2** was synthesized from commercially available 5-bromomethyl-benzoxadiazole **1** using sodium azide in DMSO at 45 °C in good yield. Direct substitution of the aromatic ring was accomplished by nucleophilic aromatic substitution (S_NAr) of a commercially available 5-bromo-benzoxadiazole **3**, to provide the 5-azido-benzoxadiazole **4** in quantitative yield. We employed the 4-chloro-7-nitro-benzoxadiazole (NBD-Cl) **5** as a common substrate for S_NAr reactions. The para-nitro substitution of the ring in addition to the oxadiazole ring allowed for excellent yields of the 4-azido-7-nitro-benzoxadiazole **6** upon reaction with sodium azide at room temperature. A chromophore with an unconjugated azido-group was generated by first introducing an alkylbromide via S_NAr of NBD-Cl **5** with bromopropylamine, providing the *N*-(3-bromopropyl)-7-nitro-benzoxadiazole-4-amine, **7**. The bromide was then converted by nucleophilic displacement with sodium azide to provide *N*-(3-azidopropyl)-7-nitro-benzoxadiazole-4-amine, **8**, in good yield. We generated a propargyl-amine derivative by an analogous route, by reaction of NBD-Cl **5** with propargyl-amine which provided *N*-(propynyl)-7-nitro-benzoxadiazole-4-amine, **9**, in moderate yield (Scheme 2). Yields were low due to the polar amine being partly retained on silica during flash chromatography. To install an alkyne group directly onto the benzoxadiazole, we investigated application of Pd-catalyzed Sonogashira cross coupling [21]. The 5-trimethylsilylethynyl-benzoxadiazole, **10**, was generated from bromide **3** by reaction with trimethylsilyl acetylene under standard conditions in excellent yield. Compound **10** was then deprotected using tetrabutylammonium fluoride (TBAF) to afford the 5-ethynyl-benzoxadiazole, **11**, in good yield. Direct alkynylation was attempted on NBD-Cl, **5**, using a variety of conditions, however the extreme reactivity of this substrate towards S_NAr substitution prevented isolation of the desired products.

With azide and alkyne benzoxadiazole derivatives (**2**, **4**, **6**, **8**, **9**, **11**) in hand, we examined the properties of the dyes and their click reaction products. Azide and alkyne dyes were reacted with either *n*-octyne or benzylazide, respectively (Scheme 3). A variety of conditions are known for the Sharpless–Meldal reaction; however, care must be taken when choosing appropriate reaction conditions to avoid undesired 5,5-bistriazole side products when using alkynyl fluorophores [31–33]. Variables can include the choice of copper source, reducing agent, ligand/base, and solvent conditions. Copper sulphate with ascorbic acid in a 1:1 methanol/water mixture gave consistent yields in our hands [21]. The alkynes (**9**, **11**) were reacted with benzyl azide, a substrate that lacks conjugation to the alkyne in order to mimic the triazole product of a bioconjugate addition. The azide benzoxadiazoles (**2, 4, 6**, **8**) were subjected to click reaction conditions with *n*-octyne, an alkynyl mimic of a lipid acyl chain. Yields and reaction times varied between 32–68% and



Scheme 3. Synthetic scheme for the generation of triazole click products. ix. *n*-octyne, $CuSO_4$, ascorbic acid, MeOH/H_2O, rt, 0.75–6 h, 32–68%; x. Benzyl azide, $CuSO_4$, ascorbic acid, MeOH/H_2O, rt, 1.5–6 h, 46–60%.

Table 1
Spectral properties of benzoxadiazoles in ethanol.

Compound	Absorbance (nm)[a]	Emission (nm)	ε	Φ[b]	$\Delta\Phi$[c]	Brightness	Δ Brightness[d]
2	**278**, 290	nd	7100	nd	na	na	na
12	**278**, 289	nd	8400	nd	na	na	na
4	**286**, 298, 329	481	30 400	0.003	na	91.2	na
13	**301**	406	7800	0.003	1.0	0.2	0.02
6	330, **454**	530	14 100	0.47	na	6630	na
14	267, **371**	539	26 000	<0.001	<0.002	<52	<0.008
8	331, **461**	524	22 100	0.51	na	11 300	na
15	329, **461**	524	38 600	0.52	1.0	20 100	1.83
9	324, **453**	517	34 300	0.57	na	19 600	na
16	326, **453**	522	16 300	0.58	1.0	9450	0.48
11	**300**	428	45 600	0.001	na	31.9	na
17	293, **305**, 325	421	18 300	0.048	48	878	28

[a] Absorbance maxima are listed with the most intense peak shown in bold face.
[b] Quantum yield standards were quinine sulphate in 0.5 M sulphuric acid, fluorescein in 0.1 M sodium hydroxide or rhodamine 6G in ethanol.
[c] $\Delta\Phi$ calculated as $\Phi_{\text{click product}}/\Phi_{\text{azide or alkyne}}$.
[d] Δ Brightness calculated as $\text{brightness}_{\text{click product}}/\text{brightness}_{\text{azide or alkyne}}$.

0.75–6 h for the fluorophore substrates. Compounds **4**, **8** and **9** gave the lowest yields and had the longest reaction times. Some of the more polar triazole derivatives, such as **15**, were obtained in low yield due to loss in chromatography.

3.2. Fluorophore characterization

To quantitate the utility of these dyes as reactive chromophores, changes in photophysical properties were determined after triazole formation. We isolated and characterized the alkynyl (**9**, **11**), azido (**2**, **4**, **6**, **8**), and triazolyl compounds (**12**–**17**) in both polar and non-polar solvents. Absorbance, fluorescence emission, molar absorption coefficient, quantum yield, and brightness were determined (Table 1). Spectral characterization was performed in ethanol and *n*-hexane, using quinine sulphate in 0.5 M sulphuric acid, fluorescein in 0.1 M sodium hydroxide or rhodamine 6G in ethanol as fluorescence standards.

In general, the NBD fluorophores showed greater absorbance, emission, quantum yield and brightness, and had greater solubility in ethanol (Tables 1 and 2). Substitution of the nitro group on the benzoxadiazole ring system resulted in longer wavelength excitation/emission and increased the emission intensity and quantum yield. These observations correspond well with previous reports linking enhanced fluorescence to an electron rich and polarized benzoxadiazole ring, presumably via an intramolecular charge transfer fluorescent excited state [30,34]. Strong electron donating or withdrawing groups, such as the nitro group in compounds **6**, **8**, and **9**, increase the dipole moment contributing to greater charge transfer character. The observed enhancement in the more polar ethanol may be attributed to relaxation of this charge transfer excited state by the solvent dipole [26]. The NBD core can still have a remarkably strong absorbance even in the absence of the nitro group, as seen in compound **4**, however the low quantum yield of this dye reduces its overall brightness.

Our results show several interesting trends upon fluorophore conversion from alkyne or azide to triazole when measured in ethanol. Azide **6** has been recently examined for its use as a photoactivatable probe, and was observed to undergo photochemical conversion to a more fluorescent amine upon UV irradiation [35]. In our experiments we examined the properties of this dye before and after conversion to a triazole derivative. Conjugated azido-benzoxadiazoles (**4**, **6**) exhibited a significant quenching effect upon triazole formation (**13**, **14**) (Fig. 1). The quantum yield of compound **6** decreases from 0.47 to <0.001 upon forming the click reaction product **14** (470-fold). To the best of our knowledge, this is the first example of such a significant quenching effect upon triazole formation. This trend was also observed in *n*-hexane, but to a lesser extent (30-fold). In contrast, the conjugated 5-ethynyl-benzoxadiazole **11** shows a 48-fold increase in quantum yield upon formation of triazole **17** (Fig. 2). These results demonstrate the extreme sensitivity of the benzoxadiazole fluorophore, as triazole products **13** and **17**, differ only in the triazole attachment point to the fluorochrome. From these observations we conclude that linkage of the triazole through *N*1 results in quenching and attachment through *C*4 results in fluorogenicity. This trend differs from that previously seen with 1,8-napthalimides, where linkage of

Table 2
Spectral properties of benzoxadiazoles in *n*-hexane.

Compound	Absorbance (nm)[a]	Emission (nm)[b]	ε[c]	Φ	$\Delta\Phi$[d]	Brightness	Δ Brightness
2	**278**, 289	nd	24 300	nd	na	na	na
12	**278**, 289	nd	11 200	nd	na	na	na
4	**275**, 285, 297, 325	424	9000	0.01	na	304	na
13	290, **300**, 318	370	18 900	0.002	0.2	15.6	0.05
6	260, **375**	522	30 500	0.03	na	915	na
14	260, **380**	501	21 900	<0.001	<0.03	<21.9	0.02
8	305, **420**	523	12 200	<0.001	na	<12.2	na
15	nd	nd	nd	nd	na	na	na
9	296, **410**	520	17 100	0.28	na	4790	na
16	347, 467, **504**	525	28 300	0.02	0.07	566	0.12
11	**283**, 298	406	4800	0.008	na	38	na
17	293, **305**, 324	375	9000	0.008	1.0	72	1.9

[a] Absorbance maxima are listed with the most intense peak shown in boldface.
[b] Quantum yield standards were quinine sulphate in 0.5 M sulphuric acid, fluorescein in 0.1 M sodium hydroxide or rhodamine 6G in ethanol.
[c] $\Delta\Phi$ calculated as $\Phi_{\text{click product}}/\Phi_{\text{azide or alkyne}}$.
[d] Δ Brightness calculated as $\text{brightness}_{\text{click product}}/\text{brightness}_{\text{azide or alkyne}}$.

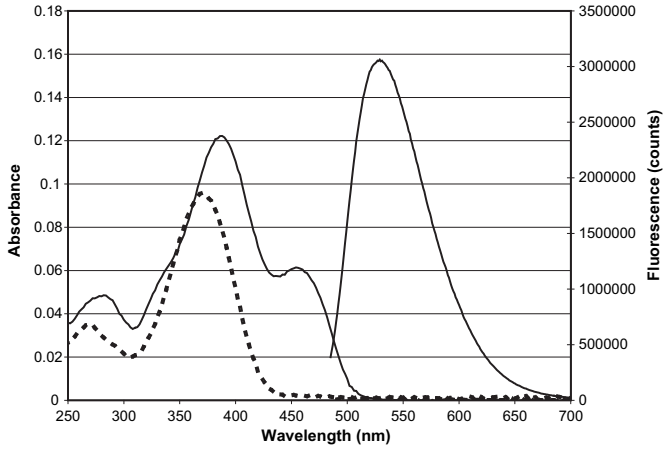


Fig. 1. Equimolar Absorbance and fluorescence emission of azide 6(–), and triazole structure **14**(…). Spectra were obtained at equimolar concentrations in EtOH, excitation 470 nm.

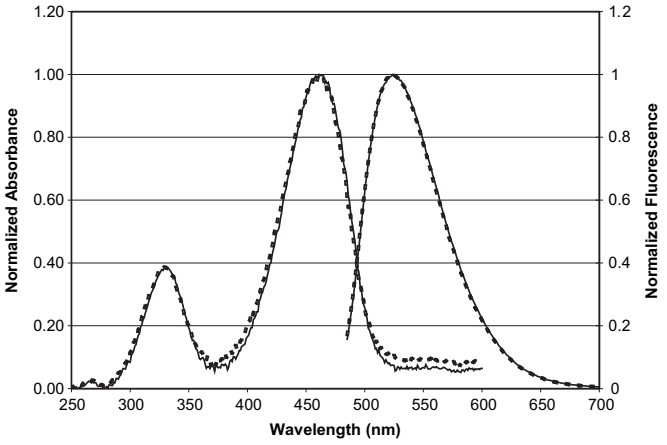


Fig. 3. Normalized Absorbance and fluorescence emission of azide 8(–), and triazole structure **15**(…). Spectra were obtained at equimolar concentrations in EtOH, excitation 470 nm.

the triazole through the *N*1 and *C*4 both elicit an increase in fluorescence [22]. However, it should be noted that a larger quantum yield (0.36) was observed for the *C*4 linked 1,8-napthalimide than the *N*1 linked (0.29) [22]. Fluorophores which feature alkyne and azide moieties that are not conjugated to the benzoxadiazole core (**2**, **8**, **9**) exhibited only minor changes in molar absorption coefficient (ε) upon triazole formation, resulting in small changes to their brightness, while their quantum yields remained fairly constant (Fig. 3). Although azide **2** and triazole **12** have very low fluorescence in both *n*-hexane and ethanol, a phospholipid analogue labelled with azide **2** exhibited stronger fluorescence in other solvent environments [16]. The absorption coefficient and brightness decreased approximately 52% upon conversion of azide **8** to triazole **15**. However, the opposite effect was observed for alkyne **9**, nearly doubling upon conversion to triazole **16**.

Our results add important new examples of reactive chromophores based upon triazole formation as a result of the Sharpless–Meldal reaction. We have identified a series of reactive chromophores based on the NBD core, exhibiting a range of activities including dramatic quenching and fluorogenic effects. Notably, the quenching observed upon conversion of compound **6** to **14** exceeds the fluorogenic increase observed in previous reports [18,19,22]. As well, the 48-fold increase in quantum yield and 28-fold increase in brightness upon conversion of alkyne **11** to triazole **17** significantly exceeds the results observed in our previous studies with a series of alkynyl coumarin derivatives and other alkynyl fluorophores in previous reports [19,21].

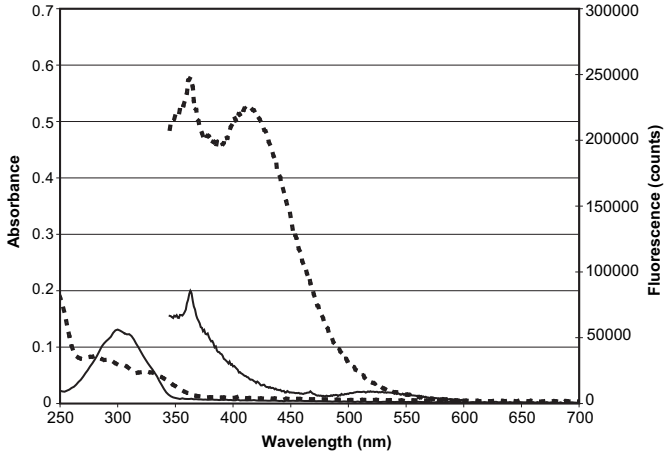


Fig. 2. Equimolar Absorbance and fluorescence emission of alkyne 11(–), and triazole structure **17**(…). Spectra were obtained at equimolar concentrations in EtOH, excitation 330 nm.

4. Conclusions

The reactive chromophores studied here will be useful for a variety of labelling strategies in bioconjugate chemistry. The benzoxadiazole derivatives reported were generated in good yield using nucleophilic substitution of commercially available starting materials. The 5-ethynyl-benzoxadiazole, **11**, shows a dramatic fluorogenic increase in quantum yield (48-fold) and brightness upon conversion to the triazole, **17**. While the NBD-azide, **6**, undergoes a dramatic quenching (470-fold) after conversion to triazole **14**. The alkynyl- and azido-NBD amines (**8** and **9**) and their triazole products (**15** and **16**) exhibit high quantum yields and brightness, and should be suitable for use with 488 nm laser line.

Acknowledgements

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