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Near-infrared benzodiazoles as small molecule environmentally-sensitive fluorophores

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Abstract The development of fluorophores emitting in the near-infrared spectral window has gained increased attention given their suitable features for biological imaging. In this work, we have optimised a general and straightforward synthetic approach to prepare a small library of near-infrared-emitting C-bridged nitrobenzodiazoles using commercial precursors. C-bridged benzodiazoles have low molecular weight and neutral character as important features that are not common in most near-infrared dyes. We have investigated their fluorescence response in the presence of a wide array of 60 different biomolecules and identified compound 3i as a potential chemosensor to discriminate between Fe2+ and Fe3+ ions in aqueous media.

Keywords fluorescence, probes, iron, screening, library

1 Introduction

The development of chemical structures showing enhanced fluorescence emission upon recognition of molecular analytes is an active area of research in analytical chemistry and molecular imaging [1–5]. In addition to their utility as highly sensitive analytical probes for environmental (e.g., metal ion detection) or biological measurements (e.g., quantification of biomarkers in clinical samples) [6–11], they have been increasingly used to derivatise bioactive molecules (e.g., peptides, proteins, antibodies) and to prepare complex molecular constructs [12–17]. The resulting architectures combine good selectivity for receptors and/or cells of interest as well as high sensitivity derived from environmentally-sensitive readouts, and have been reported as useful tools for in vivo imaging of specific biological processes or subpopulations of cells [18–24]. Among the different chemical structures employed for the development of fluorescent probes, those with emission wavelengths in the NIR (near-infrared) range (i.e., 650–900 nm) have received considerable attention [25–29]. NIR fluorophores benefit from the inherently low autofluorescence of biomolecules and tissues in a biological ‘silent’ window as well as the minimal toxicity derived from light irradiation at relatively long excitation wavelengths.

Our groups and many others have contributed to the synthesis and characterization of NIR fluorescent structures with very diverse chemical scaffolds (Fig. 1). Some of the most used include Si-rhodamines [30,31], phthalocyanines [32–34], squaraines [35,36], porphyrins [37], tricarbocyanines [38,39], and the recently reported flavylum polymethines with emission wavelengths over 1000 nm [40]. One common feature of all these NIR-emitting scaffolds is their relatively large hydrophobicity, which can result in limited water solubility (e.g., charged groups such as sulfonates are often needed to enhance solubility in aqueous media) as well as their large molecular size, which can hamper the retention of bioactivity when derivatising peptides and proteins. C-bridged nitrobenzodiazoles have been recently described as chemical structures with relatively small size ( < 300 Da) and neutral character [41]. These properties can facilitate their application for labeling metabolites (e.g., lipids, sugars) as well as their translation to NIR bioimaging. However, with only a couple of examples being reported to date, the systematic evaluation of the C-bridged nitrobenzodiazole scaffold for
the development of turn-on molecular probes has not been addressed.

Diversity-oriented fluorescent libraries have been described as a powerful approach for the discovery of new chemosensors [42]. Pioneered by Chang and coworkers, many turn-on fluorescent probes have been identified through the combinatorial derivatisation of fluorophore scaffolds and their systematic screening against biologically-relevant analytes [43–45]. One important advantage of this approach is its versatility, making it compatible with a broad range of chemical structures (including NIR fluorophores) and many different biological analytes. In the present work, we describe a synthetic methodology for the preparation of a small yet diverse collection of C-bridged nitrobenzodiazoles and their systematic spectral characterization against multiple biomolecules, including sugars, lipids, amino acids and metal ions, among others. Through an in vitro fluorescence screening, we identified the benzodiazole 3i as a fluorophore for recognition of Fe²⁺ ions. Our results demonstrate the potential utility of the C-bridged nitrobenzodiazole scaffold for the preparation of NIR-emitting activatable probes of smaller size than conventional NIR fluorophores.

2 Experimental

General materials. Commercially available reagents were used without further purification. Bioanalytes for the screening were obtained from Sigma-Aldrich, TCI and Junsei Chemical. Thin-layer chromatography was conducted on Merck silica gel 60 F254 sheets and visualized by UV (254 and 365 nm). Silica gel (particle size 35–70 µm) was used for column chromatography. ¹H NMR and ¹³C NMR spectra were recorded in a Bruker Avance 500 spectrometer (at 500 and 125 MHz, respectively). Data for ¹H NMR spectra are reported as chemical shift δ (ppm), multiplicity, coupling constant (Hz) and integration. Data for ¹³C NMR spectra reported as chemical shifts relative to the solvent peak. HPLC-MS analysis was performed on a Waters Alliance 2695 separation module connected to a Waters PDA2996 photodiode array detector and a ZQ Micromass mass spectrometer (ESI-MS) with a Phenomenex® column (C₁₈, 5 µm, 4.6 × 150 mm). High-resolution mass spectra (HRMS) were acquired using LTQ Orbitrap Velos with resolving power > 100000. High-throughput in vitro screening was performed on a FlexStation3 Multi-Mode Microplate Reader.

Synthesis of 3-fluoro-6-nitrobenzene-1,2-diamine (1). 3-Fluoro-6-nitrobenzoselenadiazole (2.9 mmol) were dissolved in HCl (concentrated, 30 mL) and HI (57%, 7.5 mL) was added dropwise. The reaction was stirred for 1 h at r.t. and then a saturated aqueous solution of Na₂SO₃ (100 mL) was added. Afterwards, an aqueous solution of 2 mol·L⁻¹ NaOH was added dropwise until the pH reached 8. The solution was filtered through Celite and extracted with EtOAc (4/100 mL). The organic phase was dried over anhydrous MgSO₄ and the solvent was removed under reduced pressure to render a dark red solid. Crude product were purified by column chromatography (hexane:EtOAc, 7:3) to give compound 1 as an orange solid (130 mg, 85% yield). ¹H NMR (500 MHz, DMSO-d₆, δ ppm) 7.40 (dd, J = 9.6, 5.8 Hz, 1H), 7.14 (s, 2H), 6.52 (t, J = 9.7 Hz, 1H), 5.15 (s, 2H). ¹³C NMR (125 MHz, DMSO-d₆, δ ppm) 152.6 (d, J_C-F = 242.0 Hz), 137.8 (d, J_C-F = 9.9 Hz), 128.6, 124.3 (d, J_C-F = 16.1 Hz), 114.2 (d, J_C-F = 10.1 Hz), 104.8 (d, J_C-F = 23.2 Hz). HRMS (m/z, ESI): calcd for C₆H₇FN₃O₂⁺ [M + H]⁺: 172.0444, found: 172.0442.

General procedure A for the synthesis of 2-fluorobenzazoles (2a–2c). To a solution of compound 1 (1 eq)
in EtOH (5 mL), Cu(OAc)$_2$ (0.05 eq) was added, followed by the corresponding ketone (10 eq). Reaction was heated at 80 °C overnight. Then, the reaction mixture was filtered through Celite and the solvent was removed under reduced pressure to give the crude products, which were purified by column chromatography. 2a. Red solid (10 mg, 80% yield); eluent: dichloromethane:hexane = 1:1. 2b. Red solid (3 mg, 6% yield); eluent: hexane:EtOAc = 8:2. 2c. Red solid (20 mg, 37% yield); eluent: dichloromethane: hexane = 7:3.

Scale-up synthesis of compound 2a. Following to the general procedure A and I (0.3 mmol, 51 mg, 1 eq), EtOH (5 mL), Cu(OAc)$_2$ (0.015 mmol, 3 mg, 0.05 eq), and acetone (30 mmol, 2.2 mL, 10 eq) were used. The yield of 2a was 70% (44 mg). $^1$H NMR (500 MHz, CD$_2$Cl$_2$, $\delta$ppm) 7.19 (dd, $J$ = 9.6, 4.6 Hz, 1H), 6.26 (dd, $J$ = 9.3, 8.4 Hz, 1H), 1.52 (s, 6H). $^{13}$C NMR (125 MHz, CD$_2$Cl$_2$, $\delta$ppm) 149.2 (d, $J_{C-F}$ = 243.8 Hz), 141.5 (d, $J_{C-F}$ = 11.2 Hz), 126.7 (d, $J_{C-F}$ = 17.1 Hz), 114.9 (d, $J_{C-F}$ = 8.5 Hz), 107.1 (d, $J_{C-F}$ = 22.2 Hz), 81.9, 30.2. HRMS (m/z, ESI) calculated for C$_9$H$_9$FN$_3$O$_2$ [M+H]$^+$: 210.0673, found: 210.0673.

3g. Yellow solid (3 mg, 34% yield); eluent: dichloromethane:MeOH = 95:5. 3h. Purple solid (1 mg, 15% yield); eluent: dichloromethane:MeOH = 95:5. 3i. Purple solid (1 mg, 24% yield); eluent: dichloromethane:MeOH = 95:5. 3j. Brown solid (1 mg, 7% yield); eluent: dichloromethane:MeOH = 95:5.

Scale-up synthesis of hit compound 3i. Following to the general procedure B and 2a (0.09 mmol, 19 mg, 1 eq), MeCN (1 mL), NaHCO$_3$ (0.23 mmol, 20 mg, 2.5 eq), H$_2$O (1 mL), morpholine (0.23 mmol, 18 µL, 2.5 eq) were used. The yield of 3i was 13% (3 mg). $^1$H NMR (500 MHz, CD$_2$Cl$_2$, $\delta$ppm) 8.22 (d, $J$ = 8.9 Hz, 1H), 7.48 (d, $J$ = 8.9 Hz, 1H), 4.07 – 3.97 (m, 4H), 3.78 – 3.74 (m, 4H), 1.50 (s, 6H). $^{13}$C NMR (125 MHz, CD$_2$Cl$_2$, $\delta$ppm) 154.7, 151.5, 149.6, 140.3, 115.3, 105.2, 100.3, 65.7, 48.4, 21.1. HRMS (m/z, ESI) calculated for C$_{13}$H$_{17}$N$_4$O$_3$ [M+H]$^+$: 277.1295, found: 277.1301.

3 Results and discussion

3.1 Chemical synthesis of a library of C-bridged nitrobenzodiazoles

Building on the recently reported SCOTfluors [41], which feature a benzodiazole core as a fluorogenic scaffold, we designed the synthesis of a small collection of C-bridged benzodiazoles using a two-step protocol with a readily accessible common precursor (compound 1, Fig. 2) and commercially available ketone and amine building blocks. We obtained 2-fluoro-4-nitro-o-phenylenediamine 1 in good yields and then utilized it in Cu-catalyzed condensations with ketones to render the intermediate C-bridged benzodiazoles (2, Fig. 2). Subsequently, we performed nucleophilic aromatic substitutions to conjugate an array of primary and secondary amines and render the final NIR-emitting products (3, Fig. 2). First, we employed acetone as the main carbonyl input to enable the formation of the smallest C-bridged benzodiazoles with methyl groups as substituents. In parallel, we also assessed Cu-catalyzed condensations with two aromatic ketones (i.e., acetophenone and 4-nitroacetophenone) to explore the scope of the reaction and analyze the effect of different substituents on the spectral properties of C-bridged benzodiazoles. While the condensation of the starting material 1 with acetone proceeded smoothly to afford compound 2a in 79% yield, acetophenones showed very poor reactivity, likely due to steric hindrance and electron-donating effects. As a result, compounds 2b and 2c were isolated only in poor yields (6% and 37%, respectively), even after prolonged heating for multiple days. Attempts at improving these yields by addition of fresh catalyst or additional equivalents of ketone did not improve the poor conversion rates.

Next, the conjugation reactions with a chemically diverse set of amine building blocks were run in water/acetonitrile in the presence of sodium bicarbonate as a base. These conditions ensured full solubility of the reagents and activation of the nucleophilic amine group. Initially, N,N'-diethylamine was employed to assess the reactivity of different benzodiazole intermediates (e.g., compounds 2a–2c). While 2a underwent a clean conversion into the corresponding 3a with yields over 80%, derivatives bearing aromatic substituents turned out to be problematic with yields for compounds 3b and 3c below 10%. We therefore choose to employ the compound 2a for further derivatisation and preparation of the C-bridged benzodiazole library. Simple primary amines (e.g., N-propylamine, aminoxylic acid) were found to react well, and both compounds 3d and 3e were isolated in moderate yields between 20% and 40%. On the other hand, secondary amines other than N,N'-diethylamine gave mixed results. Piperidine and N-methylallylamine derivatives 3f and 3g were also obtained in comparable yields, but the use of N-ethylnaphthoxalylamine rendered compound 3h in trace amounts. Finally, we also attempted the
formation of morpholine and benzylamine derivatives. Of note, during these reactions we observed the formation of double substitution products. For the morpholino derivative we could isolate both mono and disubstituted benzodiazoles (3i and 3j, respectively) whereas the benzylamine derivative was only isolated as the disubstituted analog (3k).

3.2 In vitro screening of C-bridged nitrobenzodiazoles with biological analytes

After the synthesis of a collection of C-bridged benzodiazoles, we analyzed their spectral properties. Most compounds exhibited absorbance maxima wavelengths around 570 nm and broad fluorescence emission in the 650–750 nm range. Comparative analysis within the library highlighted some differences between the differently substituted compounds 3a, 3b and 3c. Of note, compounds 3b and 3c, which incorporate an aromatic residue at the bridging position, displayed around 20 nm longer emission maxima wavelengths than compound 3a. On the other hand, the incorporation of different amines at the position 4 of the C-bridged benzodiazoles did not lead to major changes in their emission wavelengths (Table S1, cf. Electronic Supplementary Material, ESM).

Little is known about the potential of the C-bridged benzodiazole scaffold for the development of fluorescent biomolecules, so we decided to systematically investigate the fluorescence responses of all benzodiazoles against a broad range of biological analytes under physiological conditions.

![Fig. 2](image-url) Synthetic strategy for the preparation of NIR-emitting C-bridged benzodiazoles and chemical structures of the isolated compounds with their respective synthetic yields.
conditions. Diversity-oriented fluorescence libraries have been proven an effective approach for the identification of environmentally-sensitive fluorophores as well as analyte-responding probes [42]. First, we run a fluorescence response profiling analysis by monitoring the fluorescent intensity changes of 11 C-bridged benzodiazoles against 60 biometabolites including amino acids (asparagine, iso-leucine, tyrosine, lysine, tryptophan, alanine, arginine, asparagine, phenylalanine, methionine, proline, valine, glutamine, histidine, leucine, glutamate, serine, glycine and threonine), oxidant and reducing agents (cysteine, homocysteine, glutathione, glutathione disulfide (GSSG), \( \cdot \)OH, \( \cdot \)O2, NO, NaSH and \( \cdot \)O2), sugars (arabinose, glucose, fructose, galactose, sucrose, maltose, mannosse and glycogen), steroids (estradiol, chloric acid, dexamethasone, estrone, \( \beta \)-estradiol, 4-androsterone-3,17-dione), metal ions (Fe\(^{3+}\), Fe\(^{2+}\), Cu\(^{2+}\), Cu\(^{+}\), Ca\(^{2+}\), Mg\(^{2+}\), Zn\(^{2+}\), Hg\(^{2+}\), Sn\(^{2+}\) and Pd\(^{2+}\)), nucleic acids (DNA) and proteins (bovine serum albumin, human serum albumin, lysozyme and peroxidase). To maximise the reliability of our primary screening, we configured the assay conditions with four serial concentrations for each analyte, including steroids, 2–2000 \( \mu \)g \( \cdot \)mL\(^{-1}\) for proteins, 0.31–310 pg \( \cdot \)mL\(^{-1}\) for DNA and 1–1000 \( \mu \)mol \( \cdot \)L\(^{-1}\) for all other analytes.

A total of 2640 combinations (11 C-bridged benzodiazoles \( \times \) 60 analytes \( \times \) 4 concentrations) were examined. To simplify the visualization and analysis of the generated data, we calculated the fluorescence intensities for each compound as emission fold changes in the absence and in the presence of the different analytes and plotted them as a pseudo-colored heat map (Fig. 3). The overall average fold change observed for our library of compounds was determined as of 1.098, which indicates that the majority of benzodiazoles remained fluorescently silent and did not show bright emission and highlight their potential as fluorogenic probes. However, some of the screened compounds displayed moderate to good fluorescence increases upon incubation with specific analytes and we could identify several probe/analyte pairs with turn-on responses (for a detailed summary of the screening results, see Table S2 (cf. ESM)). Interestingly, compounds 3i and 3j, which both contain morpholine groups, were found to be a notable exception and displayed some remarkable reactivity against metal cations. Compound 3j showed bright fluorescence emission upon incubation with different metal ions (Fe\(^{2+}\), Cu\(^{2+}\), Cu\(^{+}\), Hg\(^{2+}\) and Pd\(^{2+}\)) but also displayed high reactivity with other analytes (GSSG, \( \cdot \)OH, NO, NaSH and Leu, among others). On the other hand, compound 3i showed a significant fluorescence emission increase upon 10 min incubation with Fe\(^{2+}\) ions in aqueous solution and good selectivity over other metal cations and biologically-relevant molecules (Fig. 3). In the view of these results, and due to the biological relevance of iron species and to the ability of this fluorophore to discriminate between very similar analytes (i.e., Fe\(^{2+}\) and Fe\(^{3+}\) ions), we scaled up the synthesis of compound 3i for further analytical characterization.

![Fig. 3](image-url) Representative heatmap with the fluorescence intensity of C-bridged benzodiazoles (2a-3k) at the highest concentration of analyte, including steroids (estradiol, chloric acid, dexamethasone, estrone, \( \beta \)-estradiol, 4-androsterone-3,17-dione) with 2% EtOH in phosphate buffered saline (PBS) pH 7.4; sugars (arabinose, glucose, fructose, galactose, sucrose, maltose, mannosse and glycogen); redox-related molecules (cysteine, homocysteine, glutathione, GSSG, H\(_2\)O\(_2\), OCl\(^{-}\), \( \cdot \)OH, \( \cdot \)O\(_2\), NO, NaSH and \( \cdot \)O\(_2\)); proteins (bovine serum albumin, human serum albumin, lysozyme and peroxidase); amino acids (asparagine, iso-leucine, tyrosine, lysine, tryptophan, alanine, arginine, asparagine, phenylalanine, methionine, proline, valine, glutamine, histidine, leucine, glutamate, serine, glycine and threonine); metal ions (Fe\(^{3+}\), Fe\(^{2+}\), Cu\(^{2+}\), Cu\(^{+}\), Ca\(^{2+}\), Mg\(^{2+}\), Zn\(^{2+}\), Hg\(^{2+}\), Sn\(^{2+}\) and Pd\(^{2+}\)) and DNA (all of those in PBS pH 7.4). The response of compound 3i to metal ions is highlighted in red.
3.3 Compound 3i shows a differential fluorescence response to \( \text{Fe}^{2+} \) and \( \text{Fe}^{3+} \) ions in aqueous media

Iron is an abundant and essential transition metal involved in several physiologic processes such as oxygen delivery, electron transport and enzymatic reactions [46]. Disruption of iron homeostasis leads to oxidative stress and cellular damage, which in turn play a significant role in the onset of cardiovascular diseases, neurodegenerative disorders and cancer [47]. Specifically, lysosomes require \( \text{Fe}^{2+} \) ions to catalyze Fenton-type reactions with hydrogen peroxide to generate reactive oxygen species and contain such species in high micromolar concentrations [48,49].

To assess the potential of compound 3i for the fluorescence detection of \( \text{Fe}^{2+} \) ions, we first recorded the fluorescence spectra of compound 3i in aqueous solutions containing different concentrations of the metal cation (Fig. 4). Because C-bridged benzodiazoles display low fluorescence quantum yields, concentrations of compound 3i in the high micromolar range (100–200 \( \mu \text{mol} \cdot \text{L}^{-1} \)) were needed to record proper emission spectra. As observed in the primary screening, a slight fluorescence increase was detected in the presence of \( \text{Fe}^{2+} \) ions (Fig. 4(a), quantum yield increased from 2.5% to 4.7%). Next, we analyzed whether compound 3i would respond differently to other iron species and observed a clear difference between the fluorescence and colorimetric response to \( \text{Fe}^{2+} \) and \( \text{Fe}^{3+} \) solutions at the same concentration, always using chloride as the counteranion. Compound 3i showed extinction coefficients around 7800 \( \text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1} \) at 560 nm (Fig. S2, cf. ESM) and a clear colorimetric shift from purple to blue from \( \text{Fe}^{2+} \) to \( \text{Fe}^{3+} \) together with a drastic reduction of its fluorescence emission (Fig. 4(b)). Notably, we replicated this response when we incubated compound 3i with solutions of \( \text{Fe}^{2+} \) that had been treated with ethylenediaminetetraacetic acid (EDTA), which suggests that the behavior of compound 3i might be related to the chelation of \( \text{Fe}^{2+} \) ions. We also performed a Job plot analysis to study the complex formed between compound 3i and \( \text{Fe}^{2+} \) and observed a maximal response for a 1:1 stoichiometry (Fig. S3, cf. ESM). Furthermore, to analyze whether compound 3i would show a differential aggregation profile in aqueous solutions that contained \( \text{Fe}^{2+} \) or \( \text{Fe}^{3+} \) ions, we performed dynamic light scattering experiments. In these assays, we observed the formation of large aggregates when compound 3i was incubated with aqueous \( \text{FeCl}_2 \) but no changes in aggregation for \( \text{FeCl}_3 \) solutions (Fig. 4(c)).

Altogether, our results indicate compound 3i as a fluorogenic molecule for the detection of \( \text{Fe}^{2+} \) in aqueous media with a clear differential response to other metal cations, particularly to \( \text{Fe}^{3+} \) ions. Future studies with compound 3i will involve its evaluation as a probe for imaging the trafficking of intracellular \( \text{Fe}^{2+} \) in live cells.

4 Conclusions

In summary, herein we report the synthesis of the first small collection of C-bridged benzodiazoles and their systematic characterization as potential fluorogenic molecules with NIR emission. Through a diversity-oriented fluorescence screening using 60 chemically-diverse biomolecules, we observed that C-bridged benzodiazoles are inherently silent molecules and with potential to turn on their fluorescence response upon incubation with different analytes. From our screening we observed that morpholino-containing benzodiazoles showed remarkable response against metal cations and we further studied the behavior of compound 3i for the detection of \( \text{Fe}^{2+} \) ions. Compound 3i showed increased fluorescence emission in the presence of equal amounts of \( \text{Fe}^{2+} \) ions, whereas the signals were drastically reduced in the presence of \( \text{Fe}^{3+} \).

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**Fig. 4** (a) Fluorescence spectra of compound 3i (200 \( \mu \text{mol} \cdot \text{L}^{-1} \)) after incubation with increasing concentrations of aqueous \( \text{FeCl}_2 \) (0, 0.125, 0.25, 0.5, 1 and 2 \( \mu \text{mol} \cdot \text{L}^{-1} \)), \( \lambda_{\text{exc}}: 570 \text{ nm} \); (b) fluorescence intensity of compound 3i (200 \( \mu \text{mol} \cdot \text{L}^{-1} \)) after incubation with different iron species (\( \text{FeCl}_2 \) and \( \text{FeCl}_3 \)); 1 \( \mu \text{mol} \cdot \text{L}^{-1} \); EDTA: 1 \( \mu \text{mol} \cdot \text{L}^{-1} \)), inset: pictograms of the different solutions under white light; (c) dynamic light scattering measurement of aggregates formed after 60-min incubation of compound 3i (200 \( \mu \text{mol} \cdot \text{L}^{-1} \)) with 2 \( \mu \text{mol} \cdot \text{L}^{-1} \) \( \text{FeCl}_2 \) or 2 \( \mu \text{mol} \cdot \text{L}^{-1} \) \( \text{FeCl}_3 \). Plots show the relative increase aggregate size when compared to an aqueous solution of compound 3i (200 \( \mu \text{mol} \cdot \text{L}^{-1} \)). Data presented as means ± SD (\( n = 3–6 \)).
ions. Mechanistic assays suggest that the response of compound 3i might be related to chelation — as seen in experiments with EDTA or aggregation-followed by light scattering experiments. The expansion of the C-bridged benzodiazole toolbox with additional commercially-available amine and/or ketone building blocks will accelerate the development of NIR chemosensors with multiple applications in analytical and biological chemistry.

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