2-(Nitroaryl)benzothiazole and benzoxazole derivatives as fluorogenic substrates for the detection of nitroreductase activity in microorganisms

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Supplementary information

Synthetic work

^{1}H-NMR spectra (400 MHz) and ^{13}C-NMR spectra (101 MHz) were recorded on a Jeol ECS400 instrument. High resolution mass spectrometry (HRMS) was performed by the EPSRC mass spectrometry service. Infrared spectra were obtained *via* a diamond anvil sample cell using a Perkin Elmer 1000 spectrometer. Melting points are reported uncorrected as determined on a Stuart SMP 1 melting point apparatus. Thin layer chromatography was performed on Merck plastic foil plates pre-coated with silica gel 60 F_{254}. Merck silica gel 60 was used for column chromatography. Compound **6a** was prepared following a literature procedure (ref. 13 in text). The synthesis of compounds **3c** and **4c** have previously been described by us (ref. 11 in text).

General method for the synthesis of substrates 7a-d.

A mixture of 3-amino-4-hydroxybenzoic acid or 4-amino-3-hydroxybenzoic acid (1.0 equiv) and an appropriate 2-nitrobenzaldehyde derivative (1.0 equiv) was heated in EtOH at reflux for 1 h. The reaction mixture was allowed to cool to room temperature and the resulting precipitate (compound **12**) was collected, washed with water, dried under vacuum overnight and used directly in the next step. The precipitate and DDQ (1.0 equiv) in anhydrous 1,4-dioxane was stirred at room temperature. The reaction mixture was then filtered and the filtrate was evaporated giving the substrate **7**.

*2-(2-Nitrophenyl)benzoxazole-6-carboxylic acid (**7a**).*

4-Amino-3-hydroxybenzoic acid (0.30 g, 1.96 mmol) and 2-nitrobenzaldehyde (0.30 g, 1.96 mmol) in ethanol (30 mL) at reflux for 16 hours gave compound **12a** which was reacted with DDQ (0.44 g, 1.96 mmol) in 1,4-dioxane (50 mL) for 16 h. Compound **7a** was obtained as a brown solid (0.53 g, 1.86 mmol, 95%), m.p. 252-254 °C; HRMS found M+H: 285.0510. Calcd. for $C_{14}H_9N_2O_5^+$, M+H: 285.0506; IR ν_{max} (cm^{-1}): 3000 (OH), 1688 (C=O), 1530, 1417, 1294, 1276; ^{1}H-NMR (400 MHz, d_6-DMSO) δ_H 8.32 (1H, d, *J* = 1.6 Hz, C7-*H*), 8.25 (1H, m, Ar-*H*), 8.18 (1H, m, Ar-*H*), 8.07 (1H, dd, *J* = 8.2 and 1.6 Hz, C5-*H*), 7.96 (3H, m, 3 x Ar-*H*); ^{13}C-NMR (101 MHz, d_6-DMSO) δ_C 167.2 (*C*=O), 161.1 (*C*), 150.6 (*C*), 149.2 (*C*), 145.0 (*C*), 134.0 (*C*H), 133.9 (*C*H), 132.0 (*C*H), 129.4 (*C*), 127.0 (*C*H), 125.1 (*C*H), 120.7 (*C*H), 120.0 (*C*), 112.9 (*C*H).

*2-(2-Nitrophenyl)benzoxazole-5- carboxylic acid (**7b**).*

3-Amino-4-hydroxybenzoic acid (1.00 g, 6.53 mmol) and 2-nitrobenzaldehyde (1.09 g, 7.18 mmol) in ethanol (50 mL) at reflux for 16 hours gave compound **12b** which was reacted with DDQ (0.95 g, 4.19 mmol) in 1,4-dioxane (50 mL) for 62 h. Compound **7b** was obtained as a brown solid (0.53 g, 1.86 mmol, 53%), m.p. 240-243 °C; HRMS found M+H: 285.0509. Calcd. for $C_{14}H_9N_2O_5^+$, M+H: 285.0506; IR ν_{max} (cm^{-1}) 3108 (OH), 1672 (C=O), 1534, 1554, 1268, 1172; ^{1}H-NMR (400 MHz, d_6-DMSO) δ_H 8.37 (1H, broad s, C4-*H*), 8.24 (1H, d, *J* = 7.3 Hz, Ar-*H*), 8.18 (1H, d, *J* = 7.3 Hz, Ar-*H*), 8.11 (1H, d, *J* = 8.7 Hz, Ar-*H*), 7.95 (3H, m, 3 x Ar-*H*); ^{13}C-NMR (101 MHz, d_6-DMSO) δ_C 167.3 (*C*=O), 160.3 (*C*), 153.5 (*C*), 149.1 (*C*), 141.6 (*C*), 133.9 (2 x 2*C*H), 132.0 (*C*H), 128.8 (*C*), 128.3 (*C*H), 125.2 (*C*H), 122.1 (*C*H), 120.1 (*C*), 111.9 (*C*H).

*2-(5-Fluoro-2-nitrophenyl)benzoxazole-6- carboxylic acid (**7c**).*

4-Amino-3-hydroxybenzoic acid (0.20 g, 1.31 mmol) and 5-fluoro-2-nitrobenzaldehyde (0.22 g, 1.31 mmol) in ethanol (30 mL) at reflux for 16 hours gave compound **12c** which was reacted with DDQ (0.30 g, 1.31 mmol) in 1,4-dioxane (50 mL) for 16 hours. Compound **7c** was obtained as a brown solid (0.04 g, 0.15 mmol, 11%), m.p. 236-238 ^{o}C after purification by column chromatography over silica gel (eluent: ethyl acetate); HRMS found M+H: 303.0407. Calcd. for $C_{14}H_8FN_2O_5^+$, M+H: 303.0412; IR ν_{max} (cm^{-1}): 3000 (OH), 1679 (C=O), 1541, 1496, 1268, 1216; ^{1}H-NMR (400 MHz, d_6-DMSO) δ_H 8.31 (2H, m, 2 x Ar-*H*), 8.11 (1H, dd, *J* = 8.2 Hz and 2.8 Hz, Ar-*H*), 8.07 (1H, dd, *J* = 8.2 Hz and 1.4 Hz, Ar-*H*), 7.98 (1H, d, *J* = 8.2 Hz, Ar-*H*), 7.83 (1H, m, Ar-*H*); ^{13}C-NMR (101 MHz, d_6-DMSO) δ_C 167.1 (*C*=O), 165.1 (d, *J* = 254.0 Hz, *C*F), 160.3 (*C*), 150.6 (*C*), 145.6 (*C*), 144.8 (*C*), 129.7 (*C*), 128.5 (d, *J* = 9.6 Hz, *C*H), 127.1 (*C*H), 123.2 (d, *J* = 10.5 Hz, *C*), 120.9 (*C*H), 120.7 (*C*H), 119.4 (d, *J* = 26.8 Hz, *C*H), 112.9 (*C*H).

*2-(5-Fluoro-2-nitrophenyl)benzoxazole-5- carboxylic acid (**7d**).*

Compound **7d** (95%) was prepared in a similar manner to compound **7c** and was obtained as a yellow solid m.p. 217-220 °C. HRMS found M+H: 303.0416. Calcd. for $C_{14}H_8FN_2O_5^+$, M+H: 303.0412; IR ν_{max} (cm^{-1}): 1683 (C=O), 1537, 1292; ^{1}H-NMR (400 MHz, d_6-DMSO) δ_H 8.32 (1H, d, *J* = 1.6 Hz, C4-*H*), 8.26 (1H, dd, *J* = 8.7 and 4.6 Hz, Ar-*H*), 8.07 (1H, dd, *J* = 8.7 and 1.6 Hz, C6-*H*), 8.04 (1H, dd, *J* = 8.7 and 2.9 Hz, Ar-*H*), 7.89 (1H, d, *J* = 8.7 Hz, C7-*H*), 7.76 (1H, qd, *J* = 7.8 and 2.9 Hz, Ar-*H*); ^{13}C-NMR (101 MHz, d_6-DMSO) δ_C 167.2 (*C*=O), 163.8 (d, *J* = 254.0 Hz, *C*F), 159.2 (*C*), 153.5 (*C*), 145.5 (d, *J* = 2.9 Hz, *C*), 141.4 (*C*), 128.9 (*C*), 128.5 (*C*H), 128.4 (*C*H), 123.1 (d, *J* = 10.5 Hz, *C*) 122.2 (*C*H), 120.7 (d, *J* = 23.0 Hz, *C*H), 119.3 (d, *J* = 26.8 Hz, *C*H), 112.0 (*C*H).

Potassium salt of carboxylic acid 7a.

Potassium 2-(2-nitrophenyl)benzoxazole-6-carboxylate

To compound **7a** (98 mg, 0.35 mmol) was added a solution of methanolic KOH solution (34 mM, 10.3 mL, 0.35 mmol) at room temperature with stirring. The solvent was evaporated to yield the potassium salt (106 mg, 94%) as a dark brown solid; δ_H (400 MHz, D_2O) 7.61-7.57 (2H, m, 2 x Ar-*H*), 7.55-7.48 (2H, m,2 x Ar-*H*), 7.37 (2H, m, 2 x Ar-*H*), 7.28 (1H, dd, *J* = 8.2 and 1.8 Hz, C7-*H*).

General procedure for the preparation of the benzothiazole derivatives 9a-9c.

Compound **3c** (1 equiv), an appropriate amine (1.1 equiv) and $NaHCO_3$ (2.5 equiv) were added to THF and H_2O (50 mL, 1:1) and the mixture was heated under reflux for 16 h. The reaction was allowed to cool and the THF was evaporated. The remaining solution was then acidified to pH 1-2 with 2M aqueous HCl. The resulting precipitate was collected giving the desired compound.

*(2R)-1-[3-(1,3- Benzothiazol-2-yl)-4-nitrophenyl]pyrrolidine-2-carboxylic acid (**9a**).*

Compound **9a** was prepared from compound **3c** (0.10 g, 0.37 mmol), L-proline (0.05 g, 0.40 mmol) and $NaHCO_3$ (0.08 g, 0.91 mmol). Compound **9a** was obtained as a yellow solid (0.13 g, 0.35 mmol, 95%), m.p. decomposes from 132 °C. HRMS found M+H: 370.0857. Calcd for $C_{18}H_{16}N_3O_4S^+$, M+H: 370.0856; IR ν_{max} (cm^{-1}): 3000 (OH), 1728 (C=O), 1594, 1504, 1309; ^{1}H-NMR (400 MHz, d_6-DMSO) δ_H 8.15 (1H, d, *J* = 7.3 Hz, Ar-*H*), 8.07 (1H, d, *J* = 9.2 Hz, Ar-*H*), 8.01 (1H, d, *J* = 7.8 Hz, Ar-*H*), 7.54 (1H, td, *J* = 6.9 and 1.4 Hz, Ar-*H*), 7.48 (1H, td, *J* = 6.9 and 1.4 Hz, Ar-*H*), 6.67 (2H, broad s, 2 x Ar-*H*), 4.49 (1H, dd, *J* = 8.7 and 2.3 Hz, N-C*H*), 3.54 (1H, m, N-C*H*), 3.43 (1H, m, N-C*H*), 2.26 (1H, m, C*H*), 2.11 (1H, m, C*H*), 1.97 (2H, m, 2 x C*H*); ^{13}C-NMR (101 MHz, d_6-DMSO) δ_C 173.9 (*C*=O), 165.2 (*C*), 153.3 (*C*), 150.5 (*C*), 136.2 (*C*), 136.0 (*C*), 131.5 (*C*), 128.3 (*C*H), 127.1 (*C*H), 126.2 (*C*H), 123.6 (*C*H), 122.8 (*C*H) , 114.6 (*C*H), 113.1 (*C*H), 60.9 (N-*C*H), 49.0 (N-*C*H_2), 30.7 (*C*H_2), 23.9 (*C*H_2).

*(2S)-1-[3-(1,3-Benzothiazol-2-yl)-4-nitrophenyl]pyrrolidine-2-carboxylic acid (**9b**).*

Compound **9b** was prepared from compound **3c** (0.10 g, 0.37 mmol), D-proline (0.05 g, 0.40 mmol) and $NaHCO_3$ (0.08 g, 0.91 mmol). Compound **9b** was obtained as a yellow solid (0.11 g, 0.30 mmol, 80%), m.p. decomposes from 132 °C. HRMS found M+H: 370.0850. Calcd. for $C_{18}H_{16}N_3O_4S^+$, M+H: 370.0856; IR ν_{max} (cm^{-1}): 3338 (OH), 1730 (C=O), 1596, 1500, 1309; ^{1}H-NMR (400 MHz, d_6-DMSO) δ_H 8.19 (1H, d, *J* = 7.3 Hz, Ar-*H*), 8.11 (1H, d, *J* = 9.6 Hz, Ar-*H*), 8.05 (1H, d, *J* = 7.8 Hz, Ar-*H*), 7.58 (1H, td, *J* = 7.8 Hz and 1.4 Hz, Ar-*H*), 7.52 (1H, td, *J* = 7.8 Hz and 1.4 Hz, Ar-*H*), 6.71 (2H, broad s, 2 x Ar-*H*), 4.53 (1H, dd, *J* = 8.7 and 2.3 Hz, N-C*H*), 3.60 (2H, m, 2 x N-C*H*), 2.30 (1H, m, C*H*), 2.15 (1H, m, C*H*), 2.00 (2H, m, 2 x C*H*); ^{13}C-NMR (101 MHz, d_6-DMSO) δ_C 173.9 (*C*=O), 165.2 (*C*), 153.3 (*C*), 150.5 (*C*), 136.2 (*C*), 136.0 (*C*), 131.5 (*C*), 128.3 (*C*H), 127.1 (*C*H), 126.2 (*C*H), 123.6 (*C*H), 122.8 (*C*H), 114.6 (*C*H), 113.1 (*C*H), 60.9 (N-*C*H), 49.0 (N-*C*H_2), 30.7 (*C*H_2), 23.8 (*C*H_2).

*4-{[3-(1,3- Benzothiazol-2-yl)-4-nitrophenyl](methyl)amino}butanoic acid (**9c**).*

Compound **9c** was prepared from compound **3c** (0.10 g, 0.37 mmol), *N*-methylaminobutyric acid hydrochloride (0.05 g, 0.40 mmol) and $NaHCO_3$ (0.08 g, 0.91 mmol). Compound **9c** was obtained as a yellow solid (0.11 g, 0.29 mmol, 79%), m.p. 163-166 °C. HRMS found M+H: 372.1014. Calcd for $C_{18}H_{18}N_3O_4S^+$, M+H: 372.1013; IR ν_{max} (cm^{-1}): 2919 (OH), 1718 (C=O), 1597, 1493, 1302; ^{1}H-NMR (400 MHz, d_6-DMSO) δ_H 8.19 (1H, d, *J* = 7.7 Hz, Ar-*H*), 8.09 (1H, d, *J* = 9.2 Hz, Ar-*H*), 8.06 (1H, d, *J* = 7.7 Hz, Ar-*H*), 7.58 (1H, td, *J* = 7.7 and 1.4 Hz, Ar-*H*), 7.52 (1H, td, *J* = 7.7 and 1.4 Hz, Ar-*H*), 6.97 (1H, dd, *J* = 9.2 and 2.8 Hz, Ar-*H*), 6.92 (1H, d, *J* = 2.8 Hz, C2′-*H*), 3.51 (2H, t, *J* = 7.3 Hz, N-CH_2), 3.09 (3H, s, N-CH_3), 2.29 (2H, t, *J* = 7.3 Hz, CH_2-CO_2H), 1.77 (2H, quintet, *J* = 7.3 Hz, CH_2); ^{13}C-NMR (101 MHz, d_6-DMSO) δ_C 174.7 (*C*=O), 165.6 (*C*), 153.3 (*C*), 152.5 (*C*), 136.1 (*C*), 135.4 (*C*), 131.8 (*C*), 128.4 (*C*H), 127.0 (*C*H), 126.1 (*C*H), 123.6 (*C*H), 122.8 (*C*H), 113.8 (*C*H), 112.2 (*C*H), 51.5 (N-*C*H_2), 38.9 (N-*C*H_3), 31.0 (*C*H_2), 22.0 (*C*H_2).

General procedure for the preparation of the benzoxazole derivatives 10a-c.

Compound **4c** (1 equiv), an appropriate amine (1.1 equiv) and $NaHCO_3$ (2.5 equiv) were added to EtOH and H_2O (50 mL, 1:1) and the mixture was heated under reflux for 16 h. The reaction was allowed to cool and the EtOH was evaporated. The remaining solution was then acidified to pH 1-2 with 2M aqueous HCl. EtOAc was then added and the organic layer was separated. The organic layer was washed sequentially with H_2O and then brine, dried ($MgSO_4$) and evaporated yielding the product.

*(2R)-1-[3-(1,3-Benzoxazol-2-yl)-4-nitrophenyl]pyrrolidine-2-carboxylic acid (**10a**).*

Compound **10a** was prepared from compound **4c** (0.50 g, 1.93 mmol), L-proline (0.25 g, 2.13 mmol) and $NaHCO_3$ (0.41 g, 4.83 mmol). Compound **10a** was obtained as a yellow solid (0.49 g, 1.38 mmol, 71%), 207-209 °C. HRMS found M+H: 354.1084. Calcd. for $C_{18}H_{16}N_3O_5^+$, M+H: 354.1084; IR ν_{max} (cm^{-1}): 2858 (OH), 1728 (C=O), 1586, 1287; ^{1}H-NMR (400 MHz, d_6-DMSO) δ_H 8.17 (1H, d, *J* = 9.2 Hz, Ar-*H*), 7.86 (1H, m, Ar-*H*), 7.78 (1H, m, Ar-*H*), 7.47 (2H, m, 2 x Ar-*H*), 6.93 (1H, broad s, Ar-*H*), 6.81 (1H, broad s, Ar-*H*), 4.55 (1H, dd, *J* = 8.2 Hz and 1.8 Hz, N-C*H*), 3.59 (1H, m, N-C*H*), 3.49 (1H, m, N-C*H*), 2.31 (1H, m, C*H*), 2.17 (1H, m, C*H*), 2.02 (2H, m, 2 x C*H*); ^{13}C-NMR (101 MHz, d_6-DMSO) δ_C 173.7 (*C*=O), 161.2 (*C*), 151.0 (*C*), 150.8 (*C*), 141.5 (*C*), 136.0 (*C*), 128.3 (*C*H), 126.3 (*C*H), 125.6 (*C*), 125.4 (*C*H), 120.6 (*C*H), 115.0 (*C*H), 113.8 (*C*H), 111.6 (*C*H), 60.9 (N-*C*H), 49.1 (N-*C*H_2), 30.7 (*C*H_2), 23.8 (*C*H_2).

*(2S)-1-[3-(1,3-Benzoxazol-2-yl)-4-nitrophenyl]pyrrolidine-2-carboxylic acid (**10b**).*

Compound **10b** was prepared from compound **4c** (0.50 g, 1.93 mmol), D-proline (0.25 g, 2.13 mmol) and $NaHCO_3$ (0.41 g, 4.83 mmol). Compound **10b** was obtained as a yellow solid (0.59 g, 1.68 mmol, 87%), m.p. 207-209 °C. HRMS found M+H: 354.1082. Calcd. for

$C_{18}H_{16}N_3O_5^+$, M+H: 354.1084; IR ν_{max} (cm^{-1}): 1727 (C=O), 1586, 1287; ^{1}H-NMR (400 MHz, d_6-DMSO) δ_H 8.18 (1H, d, *J* = 9.2 Hz, Ar-*H*), 7.87 (2H, m, 2 x Ar-*H*), 7.79 (1H, m, Ar-*H*), 7.48 (2H, m, 2 x Ar-*H*), 6.94 (1H, broad s, Ar-*H*), 6.80 (1H, broad s, Ar-*H*), 4.57 (1H, dd, *J* = 8.7 and 1.8 Hz, N-C*H*), 3.60 (1H, m, N-C*H*), 3.50 (1H, m, N-C*H*), 2.32 (1H, m, C*H*), 2.18 (1H, m, C*H*), 2.02 (2H, m, 2 x C*H*); ^{13}C-NMR (101 MHz, d_6-DMSO) δ_C 173.7 (*C*=O), 161.2 (*C*), 151.0 (*C*), 150.8 (*C*), 141.5 (*C*), 136.0 (*C*), 128.3 (*C*H), 126.3 (*C*H), 125.6 (*C*), 125.4 (*C*H), 120.6 (*C*H), 115.0 (*C*H), 113.8 (*C*H), 111.6 (*C*H), 60.9 (N-*C*H), 49.1 (N-*C*H_2), 30.7 (*C*H_2), 23.8 (*C*H_2).

*4-{[3-(1,3- Benzoxazol-2-yl)-4-nitrophenyl](methyl)amino}butanoic acid (**10c**).*

Compound **10c** was prepared from compound **4c** (0.50 g, 1.93 mmol), *N*-methylaminobutyric acid hydrochloride (0.33 g, 2.13 mmol) and $NaHCO_3$ (0.41 g, 4.83 mmol). Compound **10c** was obtained as a yellow solid (0.58 g, 1.64 mmol, 85%), m.p. 168-170 °C. HRMS found M+H: 356.1240. Calcd. for $C_{18}H_{18}N_3O_5^+$, M+H: 356.1241; IR ν_{max} (cm^{-1}): 2940 (OH), 1727 (C=O), 1587, 1308; ^{1}H-NMR (400 MHz, d_6-DMSO) δ_H 8.14 (1H, d, *J* = 9.2 Hz, Ar-H), 7.85 (1H, m, Ar-*H*), 7.78 (1H, m, Ar-*H*), 7.46 (2H, m, Ar-*H*), 7.12 (1H, d, *J* = 2.8 Hz, C2′-H), 7.02 (1H, dd, *J* = 9.6 and 2.8 Hz, Ar-*H*), 3.53 (2H, t, *J* = 7.3 Hz, NC*H*$_2$), 3.10 (3H, s, C*H*$_3$), 2.30 (2H, t, *J* = 7.3 Hz, C*H*$_2$-CO_2H), 1.78 (2H, quintet, *J* = 7.3 Hz, C*H*$_2$); ^{13}C-NMR (101 MHz, d_6-DMSO) δ_C 174.7 (*C*=O), 161.5 (*C*), 152.9 (*C*), 151.0 (*C*), 141.6 (*C*), 135.1 (*C*), 128.3 (*C*H), 126.2 (*C*H), 126.0 (*C*H), 125.3 (*C*H), 120.6 (*C*H), 114.2 (*C*H), 112.9 (*C*H), 111.6 (*C*H), 51.5 (N-*C*H), 39.0 (N-*C*H_2), 31.0 (*C*H_2), 22.1 (*C*H_2).

Microbiological work

Agar plate preparation

Each substrate (10 mg) was dissolved in a minimal volume of 1-methyl-2-pyrrolidone (200-400 µL) and added to molten Columbia agar (100 mL) (Oxoid, Basingstoke) at 50 ºC to a final concentration of 100 mg L^{-1}. Agar plates were then prepared and dried to remove excess moisture. Bacterial strains and yeasts obtained from various national culture collections (see Tables) were sub-cultured onto Columbia agar. Colonies of each strain were sampled using a loop and suspended in 0.85 % sterile saline to generate a suspension equivalent to 10^8 colony forming units (cfu) per mL using a densitometer. Each agar plate was then inoculated with 1 µL of this suspension using a multipoint inoculator that delivered suspensions of 20 strains per plate. Plates were incubated at 37 °C in air for 24 h. Control plates without the substrate were prepared and inoculated concomitantly.

Fluorescence response at varying concentrations

Compound **7a** (10 mg) was dissolved in 0.95 mL sterile deionised water and 50 µL of 1M sodium hydroxide solution was added to produce a solution of 10000 mg/L. The following volumes were added to brain heart infusion broth (Oxoid) to give a final volume of 20 mL: 200 µL, 100 µL, 50 µL, 25 µL and 12.5 µL. The final concentration range was 0.33 – 0.02

mmol/L. A Gram-negative isolate (*Enterobacter cloacae*) and a Gram-positive isolate (*Staphylococcus aureus*) were inoculated at a final inoculum of 10^5 CFU/mL and the broths were incubated overnight for 18 h at 37°C. Substrate-free and bacteria-free controls were included and tests were performed in duplicate. Fluorescence was read before and after incubation on a Synergy HT microplate reader using 365 nm excitation and 440 emission wavelengths.