

Northumbria Research Link

Citation: Cellier, Marie, Gignoux, Amandine, James, Arthur, Orenga, Sylvain, Perry, John, Robinson, Shaun, Stanforth, Stephen and Turnbull, Graeme (2015) 2-(Nitroaryl)benzothiazole and benzoxazole derivatives as fluorogenic substrates for the detection of nitroreductase activity in clinically important microorganisms. *Bioorganic & Medicinal Chemistry Letters*, 25 (24). pp. 5694-5698. ISSN 0960-894X

Published by: Elsevier

URL: <http://dx.doi.org/10.1016/j.bmcl.2015.10.099>
<<http://dx.doi.org/10.1016/j.bmcl.2015.10.099>>

This version was downloaded from Northumbria Research Link:
<http://nrl.northumbria.ac.uk/id/eprint/24362/>

Northumbria University has developed Northumbria Research Link (NRL) to enable users to access the University's research output. Copyright © and moral rights for items on NRL are retained by the individual author(s) and/or other copyright owners. Single copies of full items can be reproduced, displayed or performed, and given to third parties in any format or medium for personal research or study, educational, or not-for-profit purposes without prior permission or charge, provided the authors, title and full bibliographic details are given, as well as a hyperlink and/or URL to the original metadata page. The content must not be changed in any way. Full items must not be sold commercially in any format or medium without formal permission of the copyright holder. The full policy is available online: <http://nrl.northumbria.ac.uk/policies.html>

This document may differ from the final, published version of the research and has been made available online in accordance with publisher policies. To read and/or cite from the published version of the research, please visit the publisher's website (a subscription may be required.)

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

Marie Cellier, Amandine Gignoux, the late Arthur L. James, Sylvain Orenge, John D. Perry, Shaun N. Robinson, Stephen P. Stanforth* and Graeme Turnbull

Supplementary information

Synthetic work

¹H-NMR spectra (400 MHz) and ¹³C-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 F₂₅₄. 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 C₁₄H₉N₂O₅⁺, M+H: 285.0506; IR ν_{\max} (cm⁻¹): 3000 (OH), 1688 (C=O), 1530, 1417, 1294, 1276; ¹H-NMR (400 MHz, d₆-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*); ¹³C-NMR (101 MHz, d₆-DMSO) δ_{C} 167.2 (C=O), 161.1 (C), 150.6 (C), 149.2 (C), 145.0 (C), 134.0 (CH), 133.9 (CH), 132.0 (CH), 129.4 (C), 127.0 (CH), 125.1 (CH), 120.7 (CH), 120.0 (C), 112.9 (CH).

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 C₁₄H₉N₂O₅⁺, M+H: 285.0506; IR ν_{\max} (cm⁻¹): 3108 (OH), 1672 (C=O), 1534, 1554, 1268, 1172; ¹H-NMR (400 MHz, d₆-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); ¹³C-NMR (101 MHz, d₆-DMSO) δ_{C} 167.3 (C=O), 160.3 (C), 153.5 (C), 149.1 (C), 141.6 (C), 133.9 (2 x 2CH), 132.0 (CH), 128.8 (C), 128.3 (CH), 125.2 (CH), 122.1 (CH), 120.1 (C), 111.9 (CH).

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 °C after purification by column chromatography over silica gel (eluent: ethyl acetate); HRMS found M+H:

303.0407. Calcd. for C₁₄H₈FN₂O₅⁺, M+H: 303.0412; IR ν_{\max} (cm⁻¹): 3000 (OH), 1679 (C=O), 1541, 1496, 1268, 1216; ¹H-NMR (400 MHz, d₆-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); ¹³C-NMR (101 MHz, d₆-DMSO) δ_{C} 167.1 (C=O), 165.1 (d, *J* = 254.0 Hz, CF), 160.3 (C), 150.6 (C), 145.6 (C), 144.8 (C), 129.7 (C), 128.5 (d, *J* = 9.6 Hz, CH), 127.1 (CH), 123.2 (d, *J* = 10.5 Hz, C), 120.9 (CH), 120.7 (CH), 119.4 (d, *J* = 26.8 Hz, CH), 112.9 (CH).

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 C₁₄H₈FN₂O₅⁺, M+H: 303.0412; IR ν_{\max} (cm⁻¹): 1683 (C=O), 1537, 1292; ¹H-NMR (400 MHz, d₆-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); ¹³C-NMR (101 MHz, d₆-DMSO) δ_{C} 167.2 (C=O), 163.8 (d, *J* = 254.0 Hz, CF), 159.2 (C), 153.5 (C), 145.5 (d, *J* = 2.9 Hz, C), 141.4 (C), 128.9 (C), 128.5 (CH), 128.4 (CH), 123.1 (d, *J* = 10.5 Hz, C) 122.2 (CH), 120.7 (d, *J* = 23.0 Hz, CH), 119.3 (d, *J* = 26.8 Hz, CH), 112.0 (CH).

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, D₂O) 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 NaHCO₃ (2.5 equiv) were added to THF and H₂O (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 NaHCO₃ (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 C₁₈H₁₆N₃O₄S⁺, M+H: 370.0856; IR ν_{max} (cm⁻¹): 3000 (OH), 1728 (C=O), 1594, 1504, 1309; ¹H-NMR (400 MHz, d₆-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-CH), 3.54 (1H, m, N-CH), 3.43 (1H, m, N-CH), 2.26 (1H, m, CH), 2.11 (1H, m, CH), 1.97 (2H, m, 2 x CH); ¹³C-NMR (101 MHz, d₆-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 (CH), 127.1 (CH), 126.2 (CH), 123.6 (CH), 122.8 (CH), 114.6 (CH), 113.1 (CH), 60.9 (N-CH), 49.0 (N-CH₂), 30.7 (CH₂), 23.9 (CH₂).

*(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 NaHCO₃ (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 C₁₈H₁₆N₃O₄S⁺, M+H: 370.0856; IR ν_{max} (cm⁻¹): 3338 (OH), 1730 (C=O), 1596, 1500, 1309; ¹H-NMR (400 MHz, d₆-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-CH), 3.60 (2H, m, 2 x N-CH), 2.30 (1H, m, CH), 2.15 (1H, m, CH), 2.00 (2H, m, 2 x CH); ¹³C-NMR (101 MHz, d₆-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 (CH), 127.1 (CH), 126.2 (CH), 123.6 (CH), 122.8 (CH), 114.6 (CH), 113.1 (CH), 60.9 (N-CH), 49.0 (N-CH₂), 30.7 (CH₂), 23.8 (CH₂).

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 NaHCO₃ (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 C₁₈H₁₈N₃O₄S⁺, M+H: 372.1013; IR ν_{\max} (cm⁻¹): 2919 (OH), 1718 (C=O), 1597, 1493, 1302; ¹H-NMR (400 MHz, d₆-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-CH₂), 3.09 (3H, s, N-CH₃), 2.29 (2H, t, *J* = 7.3 Hz, CH₂-CO₂H), 1.77 (2H, quintet, *J* = 7.3 Hz, CH₂); ¹³C-NMR (101 MHz, d₆-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 (CH), 127.0 (CH), 126.1 (CH), 123.6 (CH), 122.8 (CH), 113.8 (CH), 112.2 (CH), 51.5 (N-CH₂), 38.9 (N-CH₃), 31.0 (CH₂), 22.0 (CH₂).

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

Compound **4c** (1 equiv), an appropriate amine (1.1 equiv) and NaHCO₃ (2.5 equiv) were added to EtOH and H₂O (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 H₂O and then brine, dried (MgSO₄) 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 NaHCO₃ (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 C₁₈H₁₆N₃O₅⁺, M+H: 354.1084; IR ν_{\max} (cm⁻¹): 2858 (OH), 1728 (C=O), 1586, 1287; ¹H-NMR (400 MHz, d₆-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-CH), 3.59 (1H, m, N-CH), 3.49 (1H, m, N-CH), 2.31 (1H, m, CH), 2.17 (1H, m, CH), 2.02 (2H, m, 2 x CH); ¹³C-NMR (101 MHz, d₆-DMSO) δ_{C} 173.7 (C=O), 161.2 (C), 151.0 (C), 150.8 (C), 141.5 (C), 136.0 (C), 128.3 (CH), 126.3 (CH), 125.6 (C), 125.4 (CH), 120.6 (CH), 115.0 (CH), 113.8 (CH), 111.6 (CH), 60.9 (N-CH), 49.1 (N-CH₂), 30.7 (CH₂), 23.8 (CH₂).

(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 NaHCO₃ (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; 1H -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-CH), 3.60 (1H, m, N-CH), 3.50 (1H, m, N-CH), 2.32 (1H, m, CH), 2.18 (1H, m, CH), 2.02 (2H, m, 2 x CH); ^{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 (CH), 126.3 (CH), 125.6 (C), 125.4 (CH), 120.6 (CH), 115.0 (CH), 113.8 (CH), 111.6 (CH), 60.9 (N-CH), 49.1 (N-CH₂), 30.7 (CH₂), 23.8 (CH₂).

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 NaHCO₃ (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; 1H -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, NCH₂), 3.10 (3H, s, CH₃), 2.30 (2H, t, $J = 7.3$ Hz, CH₂-CO₂H), 1.78 (2H, quintet, $J = 7.3$ Hz, CH₂); ^{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 (CH), 126.2 (CH), 126.0 (CH), 125.3 (CH), 120.6 (CH), 114.2 (CH), 112.9 (CH), 111.6 (CH), 51.5 (N-CH), 39.0 (N-CH₂), 31.0 (CH₂), 22.1 (CH₂).

Microbiological work

Agar plate preparation

Each substrate (10 mg) was dissolved in a minimal volume of 1-methyl-2-pyrrolidone (200-400 μ L) and added to molten Columbia agar (100 mL) (Oxoid, Basingstoke) at 50 °C 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 10⁸ colony forming units (cfu) per mL using a densitometer. Each agar plate was then inoculated with 1 μ L 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 μ L 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 μ L, 100 μ L, 50 μ L, 25 μ L and 12.5 μ L. 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.