Supporting information

Development of pH-Responsive Fluorescent False Neurotransmitters

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Part I: Structure and Synthesis of Coumarin Probes

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Part VIII: ¹H and ¹³C NMR of Coumarin Probes
Part I: Structure and Synthesis of Coumarin Probes

General

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich or Strem and used without further purification. When necessary, solvents were dried by passing them through a column of alumina under argon. Flash chromatography was performed on SILICYCLE silica gel (230–400 mesh). Nuclear Magnetic Resonance spectra were recorded at 300 K (unless otherwise noted) on Bruker 300 or 400 Fourier transform NMR spectrometers. Proton chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to residual protium in the NMR solvent (CDCl₃, δ 7.26; CD₃OD, δ 3.30; DMSO, δ 2.49). Data for ¹H NMR are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, bs = broad singlet), and coupling constant in Hertz (Hz). Carbon chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to the carbon resonance of the NMR solvent (CDCl₃, δ 77.0; CD₃OD, δ 49.0; DMSO, δ 39.5). Mass spectra were recorded on a JEOL LCmate (ionization mode: APCI⁺) or on a JMSHX110 HF mass spectrometer (ionization mode: FAB⁺). Preparative HPLC was performed with a Waters 600 Controller on a Vydac C18 Protein & Peptide column (#218TP1022); fractions were detected with a Waters 2487 Dual λ Absorbance Detector and collected with a Waters 2767 Sample Manager. Data was analyzed using OpenLynx software. Isocratic elution or linear gradients of solvents A and B were used (A = HPLC grade acetonitrile or methanol containing 10% (v/v) de-ionized water (Millipore Simplicity 185, 18.2 MΩ); B = de-ionized water containing 0.1% (v/v) trifluoroacetic acid (ReagentPlus grade, 99%). Analytical HPLC was performed on a Vydac C18 Protein & Peptide column (#218TP54).
Figure S1. Structure of coumarin probes as trifluoroacetic acid salts
Compound series of Mini101–106 were synthesized via a von Pechmann type condensation of resorcinols with β-ketoester 1 or 2 (prepared by following the published methods)\(^1,2\) in methanesulfonic acid\(^3,4\) for 2–3 h at RT followed by purification by RP-HPLC to obtain the probes as TFA salts. Synthesis of Mini102 is described below as a representative example.

To a mixture of 4-chlororesorcinol (0.17 g, 1.2 mmol) and 1 (0.20 g, 0.8 mmol) was added methanesulfonic acid (1.3 mL, 20 mmol) at 0 °C. The clear brown solution gradually became dark orange within 3 h at which point the reaction mixture was diluted with cold ethyl ether (−20 °C, 10 mL), and centrifuged (3000 rpm) at 4 °C for 20 min. After removing the ether solvent by aspiration, the residual orange solid was dried under high vacuum, dissolved in H\(_2\)O (3 mL), and purified by RP-HPLC using an appropriate linear gradient of acetonitrile containing 10% de-ionized water (A) and 0.1% (v/v) TFA/H\(_2\)O.

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\(^2\) Moreau, R. J.; Sorensen, E. J. \textit{Tetrahedron} 2007, 63, 6446-6453.


(B) (3–50% A over 20 min followed by a steep gradient to 100% A and equilibrium back to 3% A). The fractions containing the product (retention time ~12.7 min) were collected, concentrated, and lyophilized to give Mini102 as a white solid (37%). Alternatively, for larger scale synthesis of Mini102, the residue resulting from precipitation by ethyl ether was recrystallized from 0.1 M aq. HCl (twice) to give Mini102•HCl as a pale pink solid.

\(^1\)H NMR (DMSO-\(d_6\), 300 MHz): \(\delta\) 11.55 (1H, bs), 7.86 (1H, s), 7.86 (3H, bs), 6.95 (1H, s), 6.25 (1H, s), 3.11 (2H, \(J = 6.2\) Hz), 3.03 (2H, \(J = 6.0\) Hz). \(^{13}\)C NMR (DMSO-\(d_6\), 75 MHz): \(\delta\) 160.5, 157.4, 154.3, 151.9, 126.4, 118.0, 113.4, 112.5, 104.5, 38.4, 29.7.

LRMS (APCI\(^+\)): Calc’d for C\(_{11}\)H\(_{10}\)ClNO\(_3\) 239.0 \(m/z\), measured 240.2 (\(M\)H\(^+\)).

**Mini101**

\(^1\)H NMR (DMSO-\(d_6\), 300 MHz): \(\delta\) 10.65 (1H, s), 7.84 (3H, bs), 7.65 (1H, d, \(J = 8.8\) Hz), 6.83 (1H, dd, \(J = 8.7, 2.4\) Hz), 6.75 (1H, d, \(J = 2.3\) Hz), 6.18 (1H, s), 3.19 – 3.09 (2H, m), 3.02 (2H, \(J = 6.8\) Hz). \(^{13}\)C NMR (DMSO-\(d_6\), 75 MHz): \(\delta\) 162.2, 161.0, 156.1, 152.7, 127.0, 113.9, 112.1, 111.7, 103.4, 38.3, 29.8. LRMS (APCI\(^+\)): Calc’d for C\(_{11}\)H\(_{11}\)NO\(_3\) 205.1 \(m/z\), measured 206.1 (\(M\)H\(^+\)).

**Mini103**

\(^1\)H NMR (DMSO-\(d_6\), 300 MHz): \(\delta\) 11.26 (1H, bs), 7.93 (3H, s), 7.68 (1H, d, \(J = 11.8\) Hz), 6.95 (1H, d, \(J = 7.5\) Hz), 6.26 (1H, s), 3.12 (2H, \(J = 6.6\) Hz), 3.01 (2H, \(J = 6.6\) Hz). \(^{13}\)C NMR (DMSO-\(d_6\), 75 MHz): \(\delta\) 159.9, 151.4, 150.8, 149.2 (d, \(J = 14.4\) Hz), 148.2 (d, \(J = 238.1\) Hz), 112.6, 111.2 (d, \(J = 21.4\) Hz), 110.2 (d, \(J = 7.3\) Hz), 104.9, 37.5, 29.0. LRMS (APCI\(^+\)): Calc’d for C\(_{15}\)H\(_{19}\)NO\(_3\) 223.1 \(m/z\), measured 224.3 (\(M\)H\(^+\)).

**Mini104**

\(^1\)H NMR (DMSO-\(d_6\), 300 MHz): \(\delta\) 10.73 (1H, s), 7.88 (3H, bs), 7.40 (1H, s), 6.79 (1H, s), 6.15 (1H, s), 3.22 – 3.12 (2H, m), 3.07 (2H, \(J = 6.9\) Hz), 1.39 (9H, s). \(^{13}\)C NMR (DMSO-\(d_6\), 75 MHz): \(\delta\) 160.2, 160.0, 153.2, 152.0, 133.3, 121.9, 110.7, 109.9, 103.1, 37.3, 34.4, 29.2, 28.6. LRMS (APCI\(^+\)): Calc’d for C\(_{13}\)H\(_{19}\)NO\(_3\) 261.1 \(m/z\), measured 262.1 (\(M\)H\(^+\)).
Mini105

$^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 10.67 (1H, s), 7.83 (3H, bs), 7.40 (1H, s), 6.74 (1H, s), 6.15 (1H, s), 3.22 – 3.03 (4H, m), 1.96 (2H, s), 1.43 (6H, s), 0.72 (9H, s). $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 160.5, 160.1, 153.2, 152.0, 132.5, 122.8, 110.6, 110.0, 102.9, 51.2, 38.5, 37.3, 32.0, 31.2, 30.8, 28.6. LRMS (APCI$^+$): Calc’d for C$_{19}$H$_{27}$NO$_3$ 317.2 m/z, measured 318.0 (M$^+$).

Mini106

$^1$H NMR (CD$_3$OD, 300 MHz): $\delta$ 7.49 (2H, d, $J$ = 11.2 Hz), 7.37 – 7.34 (2H, m), 7.23 – 7.12 (3H, m), 6.26 (1H, s), 4.20 (2H, s), 3.27 (2H, t, $J$ = 7.8 Hz), 3.10 (2H, t, $J$ = 7.5 Hz). $^{13}$C NMR (CD$_3$OD, 75 MHz): $\delta$ 162.7, 153.3, 150.6, 150.2 (d, $J$ = 256.7 Hz), 148.7 (d, $J$ = 3.6 Hz), 141.0, 129.7, 129.2, 127.1, 120.2, 113.4, 111.7 (d, $J$ = 7.7 Hz), 109.2 (d, $J$ = 22.4 Hz), 39.1, 30.5, 29.6. LRMS (APCI$^+$): Calc’d for C$_{18}$H$_{16}$FNO$_3$ 313.1 m/z, measured 314.3 (M$^+$).

Scheme S2. Synthesis of Mini107, 108, and 109. (d) formaldehyde (~37 wt. % in H$_2$O), NaBH(OAc)$_3$, CH$_2$Cl$_2$, RT, 6 h, 40%; (e) formaldehyde (~37 wt. % in H$_2$O), NaBH(OAc)$_3$, CH$_2$Cl$_2$, RT, 12 h, 76%; (f) formaldehyde (~37 wt. % in H$_2$O), NaBH(OAc)$_3$, CH$_2$Cl$_2$, RT, 15 h, 51%.

Compound series of Mini107–109 were synthesized via Eschweiler-Clarke dimethylation
of Mini101–103 in dichloromethane with formaldehyde and NaBH(OAc)$_3$. The reaction mixture was stirred for the indicated time (Scheme S2) at room temperature. Purification by HPLC provided the probes as TFA salts. Synthesis of Mini107 is described below as a representative example.

To Mini101 (40 mg, 0.13 mmol) in CH$_2$Cl$_2$ (5 mL, 0.026 M) were added formaldehyde (~37 wt. % in H$_2$O, 0.20 g, 2.5 mmol) and NaBH(OAc)$_3$ (1.3 g, 6.1 mmol). The reaction solution was stirred for 6 h at RT, and the crude mixture was extracted into H$_2$O (2 x 2 mL) and purified by RP-HPLC using an appropriate linear gradient of acetonitrile containing 10% de-ionized water (A) and 0.1% (v/v) TFA/H$_2$O (B) (3–25% A over 30 min followed by a steep gradient to 100% A and equilibrium back to3% A). The fractions containing the product (retention time ~18.1 min) were collected, concentrated, and lyophilized to give Mini107 as a white solid (40%). $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 10.76 (1H, bs), 9.85 (1H, bs), 7.71 (1H, d, $J = 8.8$ Hz), 6.84 (1H, dd, $J = 8.7$, 2.4 Hz), 6.75 (1H, d, $J = 2.3$ Hz), 6.21 (1H, s), 3.40 (2H, t, $J = 7.5$ Hz), 3.15 (2H, t, $J = 7.5$ Hz), 2.87 (6H, s). $^{13}$C NMR (DMSO-$d_6$, 100 MHz): $\delta$ 161.5, 160.1, 155.2, 151.9, 126.4, 113.1, 110.7, 110.6, 102.6, 54.6, 42.4, 25.8. LRMS (APCI$^+$): Calc’d for C$_{13}$H$_{15}$NO$_3$ 233.1 m/z, measured 234.2 (M$^+$)

Mini108

$^1$H NMR (DMSO-$d_6$, 400 MHz): $\delta$ 11.54 (1H, bs), 9.58 (1H, bs), 7.92 (1H, s), 6.94 (1H, s), 6.28 (1H, s), 3.47 – 3.33 (2H, m), 3.16 (2H, t, $J = 8.0$ Hz), 2.88 (6H, s). $^{13}$C NMR (DMSO-$d_6$, 100 MHz): $\delta$ 159.6, 156.6, 153.3, 151.1, 125.8, 117.3, 111.7, 111.6, 103.6, 54.4, 42.4, 25.6. LRMS (APCI$^+$): Calc’d for C$_{13}$H$_{14}$ClNO$_3$ 267.1 m/z, measured 268.2 (M$^+$)

Mini109

$^1$H NMR (DMSO-$d_6$, 400 MHz): $\delta$ 11.23 (1H, bs), 9.51 (1H, bs), 7.75 (1H, d, $J = 11.8$ Hz), 6.96 (1H, d, $J = 7.5$ Hz), 6.30 (1H, s), 3.46 – 3.34 (2H, m), 3.13 (2H, t, $J = 8.0$ Hz), 2.88 (6H, s). $^{13}$C NMR (CD$_3$OD, 75 MHz): $\delta$162.7, 152.6, 152.5, 151.3 (d, $J = 15.1$ Hz),
150.4 (d, $J = 239.5$ Hz) 113.0, 111.9 (d, $J = 21.7$ Hz), 111.8, 106.3 (d, $J = 1.8$ Hz), 56.6, 43.7, 27.5. LRMS (APCI$^+$): Calc’d for C$_{13}$H$_{14}$FNO$_3$ 251.1 $m/z$, measured 252.2 ($M^+$).

To 2,4-dihydroxybenzaldehyde (2.0 g, 14 mmol), NaI (1.0 g, 7.2 mmol), and NaHCO$_3$ (1.5g, 17 mmol) in acetonitrile (30 mL, 0.50 M) was added benzyl bromide (1.7 mL, 14 mmol). The reaction mixture was stirred at 80 °C for 18 h under argon, cooled to RT, diluted with EtOAc (100 mL), washed with H$_2$O and brine, dried over MgSO$_4$, and filtered. The filtrate was concentrated and purified by flash chromatography (silica, EtOAc:hexanes = 1:8) to provided compound 3 as a white solid (49%). $^1$H NMR (CDCl$_3$, 300 MHz): $\delta$ 11.48 (1H, s), 9.72 (1H, s), 7.51 – 7.32 (6H, m), 6.62 (1H, dd, $J = 8.7, 2.3$ Hz), 6.54 (1H, d, $J = 2.3$ Hz), 5.11 (2H, s). $^{13}$C
NMR (CDCl$_3$, 100 MHz): δ 194.5, 166.0, 164.6, 135.8, 135.4, 128.9, 128.5, 127.6, 115.5, 109.0, 101.8, 70.5. LRMS (APCI$^+$): Calc'd for C$_{14}$H$_{12}$O$_3$ 228.1 m/z, measured 229.2 (MH$^+$).

To Boc-GABA-OH (0.85 g, 4.2 mmol) in dichloromethane (35 mL) were added N,N'-diisopropylcarbodiimide (DIC, 0.70 mL, 4.6 mmol), 4-(dimethylamino)pyridine (DMAP, 0.11 g, 0.90 mmol), and compound 3 (0.80 g, 3.5 mmol) successively. The reaction mixture was stirred at RT for 9 h, after which time the reaction mixture was washed with H$_2$O and brine, dried over MgSO$_4$, filtered, and concentrated. The crude product was purified by flash chromatography (silica, EtOAc:hexanes = 1:5 → 1:3) to give compound 4 as a white solid (90%). $^1$H NMR (CDCl$_3$, 400 MHz): δ 9.91 (1H, s), 7.79 (1H, d, $J = 8.7$ Hz), 7.54 – 7.30 (5H, m), 6.96 (1H, dd, $J = 8.6$, 2.4 Hz), 6.79 (1H, d, $J = 2.3$ Hz), 5.13 (2H, s), 4.79 (1H, s), 3.36 – 3.15 (2H, m), 2.71 (2H, t, $J = 7.2$ Hz), 2.02 – 1.89 (2H, m), 1.45 (9H, s). $^{13}$C NMR (CDCl$_3$, 100 MHz): δ 187.8, 171.5, 164.4, 156.2, 153.1, 135.6, 133.8, 122.0, 113.0, 109.9, 79.4, 70.7, 39.7, 31.2, 28.5, 25.1.

To a solution of compound 4 (0.90 g, 2.2 mmol) in dry benzene (2.0 mL) was added 3Å molecular sieves (powder, 2.0 g). To this solution, 2,8,9-triisopropyl-2,5,8,9-tetraaza-1-phospha-bicyclo[3.3.3]undecane (0.29 g, 0.96 mmol) in dry benzene (2.0 mL) was added via syringe at 40 °C. After being stirred at 50 °C under argon for 2.5 h, the reaction mixture was cooled to RT and loaded directly onto a silica gel column. Purification by flash column chromatography (silica, EtOAc:hexanes = 1:3 → 1:2) yielded compound 5 as a white solid (41%). $^1$H NMR (CDCl$_3$, 400 MHz): δ 7.45 – 7.27 (7H, m), 6.87 (1H, dd, $J = 8.6$, 2.4 Hz), 6.81 (1H, d, $J = 2.4$ Hz), 5.06 (3H, s), 3.41 – 3.37 (2H, m), 2.70 (2H, t, $J = 5.2$ Hz), 1.39 (9H, s). $^{13}$C NMR (CDCl$_3$, 100 MHz): δ 162.5, 161.5, 156.4, 155.3, 140.7, 136.3, 129.1, 128.9, 128.7, 127.9, 123.5, 113.6, 113.5, 101.9, 79.5, 70.8, 39.6, 31.9, 28.8. LRMS (APCI$^+$): Calc’d for C$_{23}$H$_{25}$NO$_3$ 395.2 m/z, measured 396.3 (MH$^+$).
Compound 5 (0.36 g, 0.88 mmol) was treated with trifluoroacetic acid (5 mL), triisopropylsilane (0.15 mL), and H₂O (0.15 mL) at RT for 2 h, after which time the solvent was removed under reduced pressure and Et₂O was added to precipitate the Boc-deprotected compound as a white solid. After filtration, the residue was dried under high vacuum, dissolved in EtOH/MeOH (10 mL/10 mL), and treated with Pd/C (25 mg). After being stirred vigorously under H₂ (50 psi) at RT for 2 h using Parr hydrogenation apparatus, the reaction solution was filtered through celite, concentrated, and purified by PR-HPLC using an appropriate linear gradient of acetonitrile containing 10% de-ionized water (A) and 0.1% (v/v) TFA/H₂O (B) (3–25% A over 30 min followed by a steep gradient to 100% A and equilibrium back to 3% A). The fractions containing the product (retention time ~16.9 min) were collected, concentrated, and lyophilized to give Mini201 as a white solid (73% for two steps). ¹H NMR (DMSO-d₆, 400 MHz): δ 7.89 (3H, s), 7.79 (1H, s), 7.47 (1H, d, J = 8.5 Hz), 6.80 (1H, dd, J = 8.5, 2.3 Hz), 6.73 (1H, d, J = 2.2 Hz), 3.10 – 3.05 (2H, m), 2.72 (2H, t, J = 7.0 Hz). ¹³C NMR (DMSO-d₆, 75 MHz): δ 161.3, 160.9, 154.8, 141.9, 129.2, 119.2, 113.2, 111.6, 101.9, 37.3, 28.6. LRMS (APCI⁺): Calc’d for C₁₁H₁₁NO₃ 205.6 m/z, measured 206.1 (MH⁺).

To a solution of 2,4-dihydroxybenzaldehyde (3.0 g, 22 mmol) in Et₂O (100 mL, 0.22 M) was added dropwise sulfurylchloride (2.1 mL, 26 mmol) at 0 °C under argon. After being stirred at RT for 30 min, the reaction solution was poured into ice-chilled brine, washed with H₂O and brine, dried over MgSO₄, filtered, and concentrated. Purification by flash chromatography (silica, Et₂O:hexanes = 1:2) provided compound 6 as an ivory solid (47%). ¹H NMR (DMSO-d₆, 300 MHz): δ 11.38 (1H, s), 10.87 (1H, s), 9.97 (1H, s), 7.59 (1H, s), 6.58 (1H, s). LRMS (APCI⁺): Calc’d for C₇H₅ClO₃ 172.0 m/z, measured 173.1 (MH⁺).

To a suspension of compound 6 (0.65 g, 3.7 mmol), NaI (0.28 g, 1.9 mmol), and NaHCO₃ (0.37 g, 4.4 mmol) in MeCN (40 mL, 0.1 M) was added benzyl bromide (0.45 mL, 3.7 mmol) at RT under argon. The
reaction solution was stirred at 80 °C under argon for 16 h, cooled to RT, diluted with EtOAc, washed with H₂O and brine, dried over MgSO₄, filtered, and concentrated. Purification by flash chromatography (silica, EtOAc:hexanes = 1:10) provided compound 7 as a white solid (58%). ¹H NMR (DMSO-d₆, 300 MHz): δ 11.13 (1H, s), 10.02 (1H, s), 7.70 (1H, s), 7.49 – 7.34 (5H, m), 6.77 (1H, s), 5.27 (2H, s). LRMS (APCI⁺): Calc’d for C₁₄H₁₁ClO₃ 262.0 m/z, measured 263.1 (M⁺).

To a solution of compound 7 (2.4 g, 9.1 mmol), 4-(dimethylamino)pyridine (DMAP, 0.29 g, 2.6 mmol), and Boc-GABA-OH (2.2 g, 11 mmol) in dichloromethane (100 mL) was added N,N'-diisopropylcarbodiimide (DIC, 1.8 mL, 12 mmol) at RT under argon. The reaction mixture was stirred at RT for 1.5 h, washed with H₂O and brine, dried over MgSO₄, filtered, and concentrated. Purification by flash chromatography (silica, EtOAc:hexanes = 1:3) provided compound 8 as a white solid (81%). ¹H NMR (DMSO-d₆, 300 MHz): δ 9.90 (1H, s), 7.96 (1H, s), 7.50 – 7.37 (5H, m), 7.32 (1H, s), 6.95 (1H, t, J = 5.4 Hz), 5.30 (2H, s), 3.07 – 3.00 (2H, m), 2.68 (2H, t, J = 7.3 Hz), 1.81 – 1.72 (2H, m). LRMS (APCI⁺): Calc’d for C₂₃H₂₆ClNO₆ 447.1 m/z, measured 448.3 (M⁺).

To a suspension of compound 8 (1.6 g, 3.6 mmol) and 3 Å molecular sieves (powder, 3.0 g) in dry benzene (15 mL) was added 2,8,9-triisopropyl-2,5,8,9-tetraaza-1-phosphabicyclo[3.3.3]undecane (0.43 g, 1.4 mmol) in dry benzene (15 mL) via syringe at 40 °C under argon. After being stirred at 50 °C for 17 h, the reaction mixture was diluted with CH₂Cl₂ (100 mL), filtered through celite, and concentrated to give a yellow solid. Purification by flash chromatography (silica, EtOAc:hexanes = 1:4 → 1:3) provided compound 9 as an ivory solid (44%). ¹H NMR (CDCl₃, 300 MHz): δ 7.46 – 7.28 (7H, m), 6.84 (1H, s), 5.17 (2H, s), 4.91 (1H, s), 3.42 – 3.36 (2H, m), 2.70 (2H, t, J = 5.7 Hz), 1.39 (9H, s). ¹³C NMR (CDCl₃, 75 MHz): δ 156.0, 153.1, 139.2, 135.3, 128.8, 128.4, 127.7, 127.2, 127.1, 124.4, 119.6, 113.4, 101.6, 79.3, 71.1, 39.1, 31.6, 28.4. LRMS (APCI⁺): Calc’d for C₂₃H₂₆ClNO₅ 429.1 m/z, measured 430.8 (M⁺).
To compound 9 (0.28 g, 0.65 mmol) were added trifluoroacetic acid (3.0 mL), H₂O (0.15 mL), and triisopropylsilane (0.15 mL). The reaction solution was refluxed at 85 °C under argon for 16 h. After cooling to RT, Et₂O (40 mL) was added to the reaction solution to precipitate the crude product as a white solid. The emulsion was centrifuged (4 °C, 3000 rpm, 5 min.), and ether was decanted. The crude solid was dried under high vacuum before being dissolved in H₂O/MeOH (4 mL/8 mL) and purified by RP-HPLC using an appropriate linear gradient of methanol (A) and 0.1% (v/v) TFA/H₂O (B) (3–100% A over 25 min followed by an equilibrium back to 3% A). The fractions containing the product (retention time ~16.2 min) were collected, concentrated, and lyophilized to give *Mini202* as a white solid (66% for two steps). ¹H NMR (DMSO-d₆, 300 MHz): δ 11.39 (1H, s), 7.76 – 7.72 (5H, m), 6.92 (1H, s), 3.06 (2H, t, J = 7.2 Hz), 2.72 (2H, t, J = 6.9 Hz). ¹³C NMR (CD₃OD, 75 MHz): δ 163.4, 157.8, 154.9, 142.8, 129.6, 122.0, 119.4, 114.0, 104.3, 39.4, 30.2. LRMS (APCI⁺): Calc’d for C₁₁H₁₀ClNO₃ 239.0 [M+H]⁺, measured 240.3 (M+H)⁺.

*Scheme S4.* Synthesis of *Mini301, 302, and 401.* (q) Methanesulfonic acid (MSA), RT, 1.5 h, 56%; (r) MSA, RT, 2.5 h, 20%; (s) MSA, RT, 4 h, 5%.
To a mixture of 2,4-dihydroxybenzaldehyde 6 (50 mg, 0.36 mmol) and 1 (59 mg, 0.24 mmol) was added methanesulfonic acid (1.0 mL) at 0 °C. The clear brown solution gradually became dark orange within 1.5 h after which time the reaction mixture was diluted with cold (−20 °C) ethyl ether (10 mL), and the crude mixture was extracted into H₂O (3 mL) and purified by RP-HPLC using an appropriate linear gradient of acetonitrile containing 10% de-ionized water (A) and 0.1% (v/v) TFA/H₂O (B) (3–50% A over 20 min followed by a steep gradient to 100% A and equilibrium back to 3% A). The fractions containing the product were collected, concentrated, and lyophilized to give **Mini301** as a white solid (56%). ¹H NMR (DMSO-d₆, 300 MHz): δ 11.37 (1H, s), 8.68 (1H, s), 7.85 (1H, d, J = 8.6 Hz), 7.75 (3H, bs), 6.89 (1H, dd, J = 8.6, 2.2 Hz), 6.79 (1H, d, J = 2.1 Hz), 3.38 (2H, t, J = 6.8 Hz), 3.14 – 3.10 (2H, m). ¹³C NMR (DMSO-d₆, 75 MHz): δ 194.3, 164.8, 159.3, 157.5, 148.7, 133.1, 118.2, 114.6, 110.9, 102.0, 34.3. LRMS (APCI⁺): Calc’d for C₁₂H₁₁NO₄ 233.1 m/z, measured 234.1 (MH⁺).

To a mixture of 5-chloro-2,4-dihydroxybenzaldehyde (40 mg, 0.18 mmol) and 1 (29 mg, 0.12 mmol) was added methanesulfonic acid (1.0 mL) at 0 °C. The clear brown solution gradually became dark orange within 2.5 h after which time the reaction mixture was diluted with cold (−20 °C) ethyl ether (10 mL), and the crude mixture was extracted into H₂O (3 mL) and purified by RP-HPLC using an appropriate linear gradient of acetonitrile containing 10% de-ionized water (A) and 0.1% (v/v) TFA/H₂O (B) (3–50% A over 20 min followed by a steep gradient to 100% A and equilibrium back to 3% A). The fractions containing the product were collected, concentrated, and lyophilized to give **Mini302** as a yellow solid (20%). ¹H NMR (DMSO-d₆, 400 MHz): δ 8.65 (1H, s), 8.11 (1H, s), 7.75 (3H, bs), 6.95 (1H, s), 3.38 (2H, t, J = 6.7 Hz), 3.14 – 3.07 (2H, m). ¹³C NMR (CD₃OD, 75 MHz): δ 195.6, 161.3, 160.9, 157.5, 149.3, 132.6, 120.7, 120.5, 113.1, 104.0, 40.4, 35.9. LRMS (APCI⁺): Calc’d for C₁₂H₁₀ClNO₄ 267.0 m/z, measured 268.2 (MH⁺).
To a mixture of 2,7-dihydroxynaphthalene (0.20 g, 1.3 mmol) and I (0.20 mg, 0.83 mmol) was added methanesulfonic acid (1.3 mL) at 0 °C. The clear brown solution gradually became dark orange within 4 h after which time the reaction mixture was diluted with cold (−20 °C) ethyl ether (10 mL), centrifuged (3000 rpm) at 4 °C for 20 min. After removing the ether by aspiration, the residual orange solid was dried under high vacuum, dissolved in H$_2$O (3 mL), and purified by RP-HPLC using an appropriate linear gradient of acetonitrile containing 10% de-ionized water (A) and 0.1% (v/v) TFA/H$_2$O (B) (3–50% A over 20 min followed by a steep gradient to 100% A and equilibrium back to 3% A). The fractions containing the product were collected, concentrated, and lyophilized to give **Mini401** as a white solid (5%). $^1$H NMR (DMSO-$d_6$, 400 MHz): δ 10.21 (1H, s), 8.08 (1H, d, $J = 8.9$ Hz), 7.94 (1H, d, $J = 8.8$ Hz), 7.84 (3H, bs), 7.72 (1H, s), 7.33 (1H, d, $J = 8.8$ Hz), 7.18 (1H, dd, $J = 8.8$, 2.0 Hz), 6.44 (1H, s), 3.54 (2H, t, $J = 6.8$ Hz), 3.32 – 3.29 (2H, m). $^{13}$C NMR (DMSO-$d_6$, 75 MHz): δ 159.2, 157.6, 155.1, 153.0, 153.9, 131.9, 133.0, 131.5, 130.8, 125.3, 117.0, 115.2, 114.0, 111.9, 107.9, 37.2, 33.9. LRMS (APCI$^+$): Calc’d for C$_{15}$H$_{13}$NO$_3$ 255.1 m/z, measured 256.2 ($MH^+$).

**Part II: HPLC Analysis of Coumarin Probes**

As an estimate of the compounds’ purity, all the synthetic probes were analyzed by analytical reverse phase HPLC (column is from GRACE VyDAC protein&peptide C18, cat.# 218TP54) using an appropriate linear gradient of acetonitrile containing 10% de-ionized water (A) and 0.1% (v/v) TFA/H$_2$O (B) (for Mini101–104, 107–109, and 201–302: 3–30% A over 40 min followed by equilibrium back to 3% A, for Mini105, 106, and 401: 3–50% A over 40 min followed by equilibrium back to 3% A).
Figure S2. Analytical RP-HPLC chromatograms of the coumarin probes. Detection at $\lambda_{\text{abs}} = 254$ nm.
Part III: Photophysical Characterization and Measurement of log $D$ values

General

Ultraviolet absorption spectra were measured on a Molecular Devices SPECTRAmax Plus 384 UV-Visible spectrophotometer operated through a Dell Pentium PC by SOFTmax software. Fluorescence measurements (emission/excitation) were carried out on a Jobin Yvon Fluorolog fluorescence spectrofluorometer.

Absorption

UV absorption spectra were taken by adding probe (2 $\mu$L of 10 mM stock solution in DMSO) to 998 $\mu$L of PBS buffer at different pH values (final probe concn = 20 $\mu$M) in quartz cuvette.

Emission/Excitation

Excitation/Emission spectra were taken by adding probe (20 $\mu$L of 0.1 mM stock solution in distilled water) to 980 $\mu$L of PBS buffer of different pH values (final probe concn = 2 $\mu$M) in quartz cuvette.

$pK_a$

The $pK_a$ values of probes were determined from the absorption spectra. The absorbance ratio at two $\lambda_{abs, \text{max}}$ was plotted versus pH of the PBS solution; the data were fit to a sigmoid curve using KaleidaGraph (Synergy Software, Reading, PA) nonlinear regression analysis program to determine the $pK_a$ value.

$log D$
The log $D$ values were determined by a traditional shake flask method. Each measurement was performed in duplicate as follows. First, 20 µM probe solution in 1 mL PBS (pH 7.4) was prepared to which 1 mL of $n$-octanol was added and mixed thoroughly. The mixture was kept in dark for 3 days for complete equilibrium, and the concentrations of probe in each layer were determined based on the UV absorbance. Log $D$ values were determined based on the following equation; \( \log D = \log [\text{probe}]_{\text{oct}} - \log [\text{probe}]_{\text{PBS}} \), where \([\text{probe}]_{\text{oct}}\) and \([\text{probe}]_{\text{PBS}}\) are the concentrations of the probe in $n$-octanol and PBS, respectively.

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<th>compound</th>
<th>$\lambda_{\text{abs, max}}$</th>
<th>$\lambda_{\text{em, max}}$</th>
<th>$\lambda_{\text{ex, max}}$</th>
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<td>—</td>
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*Table S1.* Summary of photophysical properties, log $D$, and pKa values.
Part IV: Protocols for Fluorescence Microscopy Assay in HEK and VMAT2-HEK cells and Assay Results Summary

HEK GNTI (nonglycosylating) cell line stably expressing VMAT2 (VMAT2-HEK) and HEK GNTI cell line stably transfected with TetR (HEK) to serve as a control were kindly provided by the laboratory of Robert Edwards at UCSF. Cells were grown in DMEM + Glutamax (Invitrogen #10569) with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 100 U/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen). For fluorescence microscopy experiments, cells were plated on poly-D-lysine (Sigma Aldrich, concn = 0.1 mg/mL) coated six-well dishes at a density of 1.0 x 10^5 cells per well and grown at 37 °C in 5% CO₂. After 5 days, the cells looked fibroblastic and had reached ~70% confluence. The medium was removed by aspiration, and the cells were washed with PBS (2 mL per well). To investigate the inhibitory effect of tetrabenazine (TBZ) and reserpine, cells were incubated in 1 mL of experimental media (DMEM minus phenol red (Invitrogen) with 4 mM L-glutamine (Invitrogen) and 1% charcoal/dextran-treated FBS (Atlanta Biologicals)) containing inhibitor (1 µM or 0 µM as a control, prepared from 10 mM stock solution in DMSO) at 37 °C in 5% CO₂ for 1 h. Then, the probe uptake was initiated by adding 0.1 mL of experimental media containing probe (220 µM, prepared from 10 mM stock solution in DMSO, final concn = 20 µM in the uptake assay). After incubating at 37 °C for 30 min, the media was removed by aspiration, and the cells were washed with PBS (2 mL per well) and treated with probe-free experimental media. Fluorescence images were taken by using Leica FW 4000 equipped with Chroma custom filter cube (ex = 350 ± 25 nm, em = 460 ± 25 nm) and Hamamatsu digital camera C4742-95. The fluorescent images and bright field images were acquired for 2000 ms and 37 ms, respectively. All images were adjusted using the same contrast and brightness level using ImageJ (National Institute of Health).

For the chloroquine-induced Mini202 redistribution experiment, after probe loading and cell washing with PBS, 1 mL of experimental media containing 300 µM chloroquine was added to the cells at RT for 3 min, after which time fluorescent images were taken by the same procedure described above.
The observations summarized in Figure S3 indicated that probes Mini101–103, Mini106, Mini201, and Mini202 are VMAT2 substrates. These probes are too polar to be accumulated in HEK cells lacking VMAT2. In contrast, in cells where VMAT2 can pump the probes into acidic organelles, we hypothesize that this transporter secondarily facilitates the net influx across the plasma membrane of even hydrophilic probes via passive diffusion. Compounds that are relatively lipophilic show non-selective uptake (i.e., Mini104, 105, and 401). More polar compounds that are not VMAT2 substrates show no uptake in either cell lines (Mini107–109, Mini301 and 302).

Figure S3. Summary of results from screening of Mini probes using VMAT2-HEK and HEK cells. Six probes exhibited VMAT2-dependent uptake affording fluorescent puncta, i.e., they were taken up by VMAT2-HEK cells but not control HEK cells and the uptake in VMAT2-HEK cells was abolished by VMAT inhibitors tetrabenazine (TBZ) and reserpine (red signs). Three probes were taken up as fluorescent puncta by both of VMAT2-HEK cells and HEK cells, regardless of the presence/absence of tetrabenazine or reserpine (yellow signs). Five probes showed no uptake by either VMAT-HEK or HEK cells (black signs).
Part V: Protocols for Fluorescence Microscopy in PC-12 cells

PC-12 cells were purchased and maintained according to the protocols provided by American Type Culture Collection (ATCC, CRL-1721).² PC-12 cells were grown in RPMI-1640 (Invitrogen, #11875) with 10% horse serum (Invitrogen, #16050-114), 5% fetal bovine serum (FBS) (Atlanta Biologicals), 100 U/ml penicillin/streptomycin (Invitrogen). For fluorescence microscopy experiments, cells were plated on poly-D-lysine (Sigma Aldrich, concn = 0.1 mg/mL) coated six-well dishes at a density of 5.0 x 10⁵ cells per well and grown at 37 °C in 5% CO₂. After 6 days when the cells reached ~70% confluence, the medium was removed by aspiration, and the cells were washed with PBS (2 mL per well). To investigate the inhibitory effect of reserpine, cells were incubated in 1 mL of experimental media (RPMI-1640 minus phenol red supplemented with 2 mM L-glutamine (Invitrogen #11835), 0.5% charcoal/dextran-treated FBS (Atlanta Biologicals), 1 % charcoal/dextran-treated horse serum (Invitrogen), 100 U/ml penicillin/streptomycin (Invitrogen)) containing reserpine (final concn = 1 µM prepared from 10 mM stock solution in DMSO) at 37 °C in 5% CO₂ for 1 h. Then, the probe uptake was initiated by adding 0.1 mL of experimental media containing Mini202 (220 µM, prepared from 10 mM stock solution in DMSO, final concn = 20 µM in the uptake assay) to the cells. After incubating the cells at 37 °C for 1 h, the medium was removed by aspiration, and the cells were washed with PBS (2 mL per well) and treated with dye-free experimental media. Fluorescence images were acquired by using Leica FW 4000 equipped with Chroma custom filter cube (ex = 350 ± 25 nm, em = 460 ± 25 nm) and Hamamatsu digital camera C4742-95. The fluorescent images and bright field images were acquired for 2000 ms and 37 ms, respectively. All images were adjusted using the same contrast and brightness level using ImageJ (National Institute of Health).

² Among the two variations of PC-12 provided by ATCC, the experiments were carried out using the one displaying loosely adherent (with no PDL coating) and multicellular-aggregating phenotype (CRL-1721).
Part VI: Protocols for Two-photon Fluorescence Microscopy and pH Measurement of Secretory Vesicles in PC-12 Cells

In Situ Calibration Curve

PC-12 cells were grown on 35×10 mm tissue culture dishes (Becton Dickinson Labware) under the cell maintenance conditions as described in Part V. To calibrate Mini202 fluorescence intensity ratio by dual excitation (692 nm and 760 nm) for a range of pH values in situ, vesicles of PC-12 cells were loaded with Mini202 by incubating the cells with 20 µM Mini202 in 1 mL experiment medium for 1 h at 37 °C. After washing the cells with dye-free PBS (1 mL per well), the extracellular media was replaced with 1mL of calibration buffer of known pH in the presence of monensin, the Na⁺/H⁺ ionophore, and nigericin, the K⁺/H⁺ ionophore, which act to equilibrate the pH of cytosol and vesicles with that of extracellular media. This pH calibration buffer contains 125 mM KCl, 20 mM NaCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 5 µM nigericin, 5 µM monensin, and 25 mM buffer (acetate for pH 4.27, 4.69, 5.39; MES for pH 5.74, 6.15; HEPES for pH 6.41, 6.95, 7.52). The cells were treated with the calibration buffer for 8–10 min at RT, and fluorescent images were acquired by a two-photon fluorescence microscope (Prairie Ultima multiphoton microscope operated with Prairie View 3.0.0.3 software for scan control and image collection (Prairie Technologies, Middleton, WI) with Mai Tai HP Ti:sapphire laser (Spectra-Physics, Newport Instruments, Irvine, CA) (excitation 692 or 760 nm, emission 430–510 nm) and water-immersion, IR-corrected objective from Olympus designated LUMPlanFl/IR 60x/0.90 NA on an Olympus BX61W1 microscope). For each 35 mm plate, two pairs of fluorescent images by 692 nm and 760 nm excitation were collected at a given pH value, and this process was repeated at least 2–3 times using a new plate of cells each time. The ratio of fluorescence intensity by excitation at 760 nm and 692 nm (I₇₆₀/I₆₉₂) was plotted versus pH of the calibration solution; the data were fit to a sigmoid curve using KaleidaGraph (Synergy Software, Reading, PA) nonlinear regression analysis program to construct a calibration curve (Figure 6A in the manuscript, ratio(760/692) = 0.09+1.43/(1+(pH/a)ᵇ); a = 5.91; b = −16.10; R² = 0.98). The calibration curve was constructed three times on three different days to determine the in
in situ pKₐ value of Mini202 in the LDCVs of PC-12 cells (pKₐ = 5.93 ± 0.04, n = 3).

Ratiometric pH measurements of LDCVs in PC-12 cells in situ

After PC-12 cells were incubated with 20 µM Mini202 for 1 h at 37 °C in 5% CO₂, fluorescent images containing ~10 cells were acquired by dual excitation (692 nm and 760 nm) using the Prairie multiphoton microscope, as described above. Fluorescence signals from LDCVs were manually selected using Volocity version 4.4 software (Perkin Elmer, Waltham, MA) with an appropriate object-selection parameter settings (e.g., the object size and fluorescence intensity). At this step, the total area and the punctate pattern selected as LDCVs from the two images (by 692 nm and 760 nm) were assured to be similar each other. Mean fluorescence intensity of the LDCVs from 7–10 cells were determined, which in turn was used to get fluorescence intensity ratio \( I_{760}/I_{692} \). This process was repeated three times in duplicate, using new plate of cells for each measurement.

In order to measure the pH change induced by methamphetamine, the PC-12 cells preloaded with Mini202 were washed with PBS, treated with 1mL of experimental media containing 100 µM methamphetamine (prepared from 50 mM stock solution in DMSO) for 5 min at RT, and imaged by the Prairie multiphoton microscope. Methamphetamine

![Figure S4](image-url). Two-photon fluorescence image of PC-12 cells treated with 100 µM methamphetamine for 5 min at RT with two-photon excitation (a) at 692 nm and (b) at 760 nm. (c) Pseudocolor image of \( I_{760}/I_{692} \) and corresponding pH values. The vesicular pH in PC-12 cells increased from 5.9 to 6.4 by the effect of methamphetamine.
treatment resulted in diffusion of Mini202 from the vesicles to cytoplasm to some extent, however, only fluorescence signals from LDCVs were selected as objects and taken account into the pH calculation (Figure S4).

**Part VII: Preparation and Uptake Test of the Hydrochloride Salt of Mini202**

In order to avoid possible toxic effects of trifluoroacetic acid present with Mini202 as a TFA salt, we prepared Mini202 as a hydrochloride salt from the corresponding TFA salt as described below. Mini202•TFA (20 mg) was dissolved in mixture of 0.8 M HCl aqueous solution (5 mL) and methanol (0.2 mL). The cloudy solution became clear after being heated at 80 °C for 5 min. The solution was cooled down to RT and lyophilized to afford Mini202•HCl as a white solid (12 mg). The purity of the compound was checked by 1H nmr spectroscopy, analytical reverse-phase HPLC, and mass spectrometry. 19F nmr was obtained to confirm the absence of TFA in the Mini202•HCl salt.

The activity of Mini202•HCl salt toward VMAT2 was tested in VMAT2-HEK and HEK cells by the same method described in Part IV. Both Mini202•TFA and Mini202•HCl salts showed the same pattern of punctate uptake in a VMAT2-dependent manner. This uptake was abolished in the presence of VMAT2 inhibitors (1 μM TBZ or 1 μM reserpine), which suggests the salt conversion from TFA to HCl does not affect Mini202’s activity as a VMAT2 substrate in VMAT2-HEK cells (Figure S5).
Figure S5. Uptake study of Mini202•TFA (a–f) and Mini202•HCl (g–l) in VMAT2-HEK cells and HEK cells. 20 µM Mini202•TFA or Mini202•HCl was incubated in VMAT2-HEK cells for 30 min to afford fluorescent puncta (a, g), and preincubation of the VMAT2-HEK cells with VMAT2 inhibitor TBZ (b, h) or reserpine (c, i) abolished the uptake of Mini202•TFA and Mini202•HCl. In HEK cells, both salt forms of Mini202 showed no uptake in the absence (d, j) or presence of TBZ (e, k) and reserpine (f, l). $\lambda_{ex} = 350 \pm 25$ nm, $\lambda_{em} = 460 \pm 25$ nm.
Part VIII: $^1$H and $^{13}$C NMR of Coumarin Probes

![Coumarin Probes NMR Spectra](image)
Mini301
$^1$H nmr

Mini301
$^{13}$C nmr