

Supplemental material

Development of Potent Reversible Selective Inhibitors of Butyrylcholinesterase as Fluorescent Probes

Stane Pajk,^a Damijan Knez,^a Urban Košak,^a Maja Zorović,^b Xavier Brazzolotto,^c
Nicolas Coquelle,^d Florian Nachon,^c Jacques-Philippe Colletier,^e Marko Živin,^b
Jure Stojan,^f Stanislav Gobec^{a*}

^a*Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia;*

^b*Institute of Pathological Physiology, Faculty of Medicine, University of Ljubljana, Vrazov
trg 2, 1000 Ljubljana, Slovenia;* ^c*Institut de Recherche Biomédicale des Armées, Département
de Toxicologie et Risques Chimiques, 91223, Brétigny sur Orge, France;* ^d*Institut Laue
Langvein, 71 Avenue des Martyrs, 38000 Grenoble, France;* ^e*Université Grenoble Alpes,
CNRS CEA, IBS, F-38044 Grenoble, France;* ^f*Institute of Biochemistry, Faculty of Medicine,
University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia*

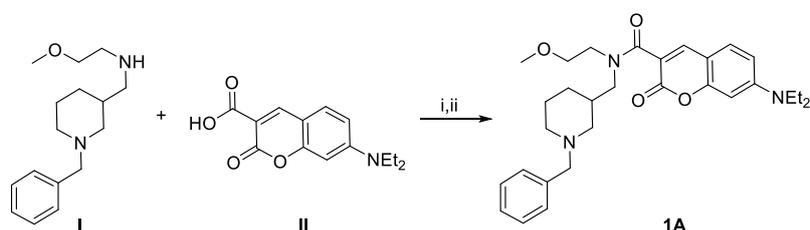
*correspondence: stanislav.gobec.@ffa.uni-lj.si

1. Synthesis

1.1 General

Chemicals from Sigma-Aldrich, TCI, and Acros were used without further purification. All reactions were performed under argon atmosphere, unless otherwise stated. Analytical TLC was performed on Merck silica gel (60 F₂₅₄) plates (0.25 mm) and visualized with ultraviolet light (254 or 366 nm). ¹H and ¹³C NMR spectra were recorded on a Bruker AVANCE III 400 MHz NMR spectrometer in CDCl₃ and DMSO-*d*₆ solution, with TMS or residual non-deuterated solvent as the internal standards. Mass spectra were recorded using a VG-Analytical Q-TOF Premier mass spectrometer.

1.2 Synthesis of compound **1A**



Scheme S1. Reagents and conditions: (i) oxalyl chloride, DMF, DCM, 0 °C to reflux; (ii) Et₃N, 0 °C to rt, 15 h.

Compounds **I**[1] and **II**[2] were synthesized according to the published procedures.

***N*-((1-benzylpiperidin-3-yl)methyl)-7-(diethylamino)-*N*-(2-methoxyethyl)-2-oxo-2H-chromene-3-carboxamide (**1A**)**. 7-(Diethylamino)-2-oxo-2H-chromene-3-carboxylic acid (**I**) (112 mg, 0.43 mmol, 1.1 equiv) was suspended in CH₂Cl₂ (5 mL) and cooled to 0 °C. The solution was stirred and dimethylformamide (10 μL) was added, followed by dropwise addition of oxalyl chloride (48 μL, 0.56 mmol, 1.3 equiv). The reaction mixture was stirred at 0 °C for 5 minutes, then at reflux conditions for 30 minutes. Solvent was removed under reduced pressure and the residue was dissolved in CH₂Cl₂ (5 mL) and cooled to 0 °C. This solution was added dropwise to the solution of *N*-((1-benzylpiperidin-3-yl)methyl)-2-methoxyethan-1-amine (**II**) (113 mg, 0.39 mmol, 1.0 equiv) and Et₃N (119 μL, 0.86 mmol, 2.0 equiv) in CH₂Cl₂ (5 mL) cooled to 0 °C. The reaction mixture was allowed to reach room temperature and stirred for 15 h. The mixture was transferred to separating funnel, washed with water (20 mL), saturated aqueous NaHCO₃ (20 mL), brine (10 mL) and dried over anhydrous Na₂SO₄. Solvent was evaporated to produce the crude product, which was purified by flash column chromatography using DCM/MeOH (from 100:1 to 30:1, v/v) as the eluent to produce 78 mg of compound **1A** as a yellow solid (39% yield).

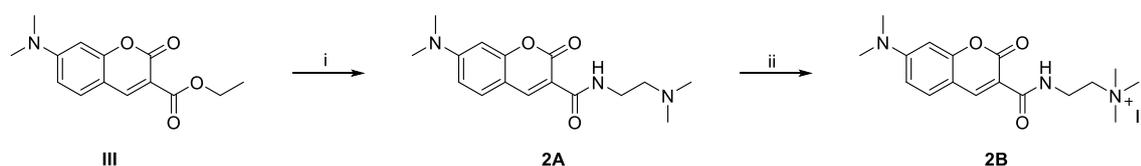
¹H NMR (400 MHz, CDCl₃) δ 8.47 (s, 0.03H), 7.54 (s, 0.47H), 7.51 (s, 0.50H), 7.37 – 7.09 (m, 6H), 6.51 (dd, *J* = 8.9, 2.3 Hz, 1H), 6.38 (dd, *J* = 5.3, 2.3 Hz, 1H), 3.68 – 3.47 (m, 3H),

3.47 – 3.24 (m, 9H), 3.23 – 3.06 (m, 3H), 2.95 – 2.50 (m, 2H), 2.26 – 1.87 (m, 2H), 1.84 – 1.34 (m, 4H), 1.22 – 0.92 (m, 7H).

^{13}C NMR (101 MHz, CDCl_3) δ 167.13, 166.79, 159.17, 159.00, 156.94, 156.87, 151.33, 151.27, 143.40, 143.22, 137.50, 136.45, 129.72, 129.52, 129.14, 128.24, 128.11, 127.36, 127.02, 117.49, 109.15, 107.59, 107.48, 96.95, 70.46, 70.19, 63.20, 62.94, 58.90, 58.80, 57.27, 57.08, 53.61, 53.53, 53.48, 48.41, 48.03, 45.74, 45.40, 44.83, 35.09, 34.18, 28.14, 27.83, 24.44, 24.21, 12.37, 8.81.

HRMS (ESI): m/z calcd for $\text{C}_{30}\text{H}_{40}\text{N}_3\text{O}_4$ $[\text{M}+\text{H}]^+$ 506.3019; found 506.3011.

1.3 Synthesis of compounds **2A** and **2B**



Scheme S2. Reagents and conditions: (i) N^1,N^1 -dimethylethane-1,2-diamine, EtOH, reflux, 15 h; (ii) MeI, DCM, rt, 24 h.

Compound **III**[3] was synthesized according to the published procedure.

7-(Dimethylamino)-*N*-(2-(dimethylamino)ethyl)-2-oxo-2*H*-chromene-3-carboxamide

(2A). Ethyl 7-(dimethylamino)-2-oxo-2*H*-chromene-3-carboxylate (300 mg, 1.15 mmol, 1.0 equiv) and N^1,N^1 -dimethylethane-1,2-diamine (286 μL , 2.3 mmol, 2.0 equiv) were suspended in dry ethanol (30 mL) and refluxed for 15 h. Solvent was removed under reduced pressure and the residue was dissolved in DCM (50 mL). The solution was washed with water (5 \times 50 mL), brine (50 mL) and dried over anhydrous Na_2SO_4 . The solvent was evaporated and the product was purified by flash column chromatography using DCM/MeOH (from 20:1 to 10/1, v/v) as the eluent to produce 225 mg of compound **2B** as a yellow solid (64% yield).

^1H NMR (400 MHz, MeOD) δ 7.54 (d, $J = 9.0$ Hz, 1H), 6.81 (dd, $J = 9.0, 2.4$ Hz, 1H), 6.55 (d, $J = 2.3$ Hz, 1H), 3.56 (t, $J = 6.6$ Hz, 2H), 2.61 (t, $J = 6.6$ Hz, 2H).

^{13}C NMR (101 MHz, TFA- d) δ 167.36, 163.93, 156.82, 152.12, 148.66, 135.67, 122.26, 120.32, 120.18, 112.06, 61.12, 49.32, 45.83, 38.21.

HRMS (ESI): m/z calcd for $\text{C}_{16}\text{H}_{22}\text{N}_3\text{O}_3$ $[\text{M}+\text{H}]^+$ 304.1656; found 304.1647.

2-(7-(Dimethylamino)-2-oxo-2H-chromene-3-carboxamido)-N,N,N-trimethylethan-1-aminium iodide (2B)

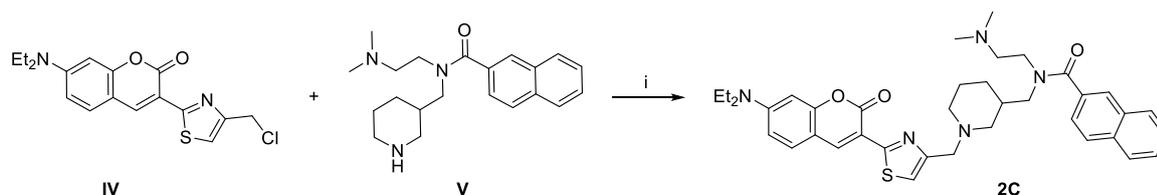
7-(Dimethylamino)-*N*-(2-(dimethylamino)ethyl)-2-oxo-2H-chromene-3-carboxamide (**2A**) (100 mg, 0.33 mmol, 1.0 equiv) was dissolved in DCM (10 mL) followed by addition of MeI (27 μ L, 0.43 mmol, 1.3 equiv). Product started to precipitate while reaction mixture was stirred at room temperature for 24 h. Product was filtered off and dried to give 95 mg of compound **2C** as a yellow solid (65% yield).

^1H NMR (400 MHz, MeOD) δ 7.60 (d, J = 9.0 Hz, 1H), 6.87 (dd, J = 9.0, 2.5 Hz, 1H), 6.61 (d, J = 2.3 Hz, 1H), 3.92 – 3.87 (m, 2H), 3.58 (t, J = 6.6 Hz, 2H).

^{13}C NMR (101 MHz, TFA-d) δ 166.03, 163.79, 156.68, 152.31, 148.63, 135.55, 122.33, 120.58, 120.45, 112.37, 66.25, 55.68, 49.09, 36.49.

HRMS (ESI): m/z calcd for $\text{C}_{17}\text{H}_{24}\text{N}_3\text{O}_3$ $[\text{M}]^+$ 318.1812; found 318.1805.

1.4 Synthesis of probe **2C**



Scheme S3. Reagents and conditions: (i) K_2CO_3 , ACN, 60 $^\circ\text{C}$, 48 h.

Compounds **IV**[4] and **V**[5] were synthesized according to the published procedures.

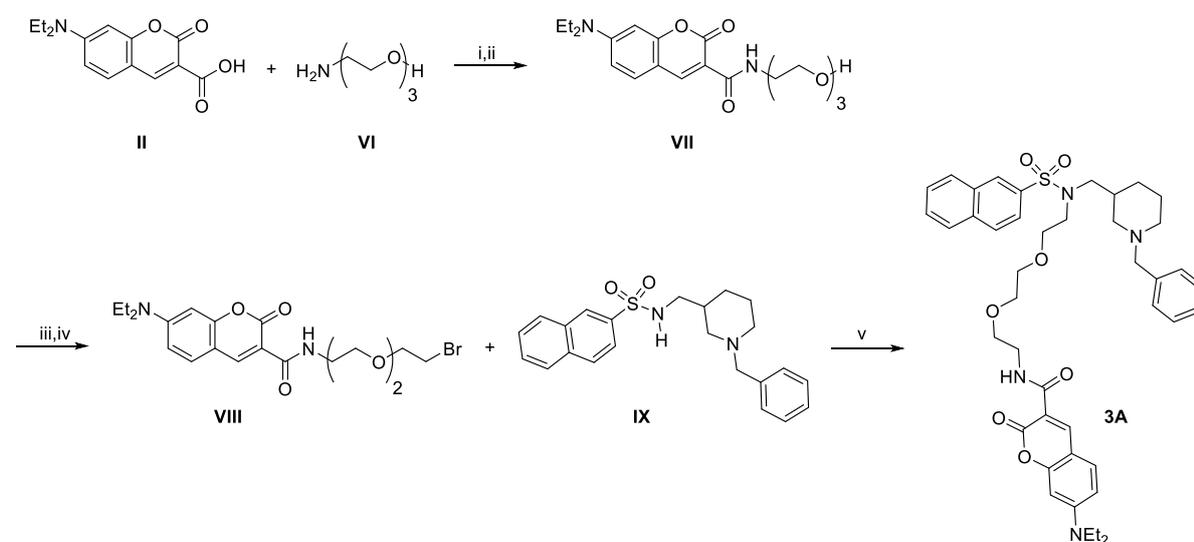
N-((1-((2-(7-(Diethylamino)-2-oxo-2H-chromen-3-yl)thiazol-4-yl)methyl)piperidin-3-yl)methyl)-*N*-(2-(dimethylamino)ethyl)-2-naphthamide (**2C**). K_2CO_3 (47 mg, 0.34 mmol, 2.0 equiv), 3-(4-(chloromethyl)thiazol-2-yl)-7-(diethylamino)-2H-chromen-2-one (**IV**) (62 mg, 0.18 mmol, 1 equiv), and *N*-(2-(dimethylamino)ethyl)-*N*-(piperidin-3-ylmethyl)-2-naphthamide (**V**) (60 mg, 0.18 mmol, 1 equiv) were suspended in acetonitrile (5 mL) and the reaction mixture was stirred at 60 $^\circ\text{C}$ for 48 h. The solvent was removed under reduced pressure and the crude product was purified by flash column chromatography using DCM/MeOH/ $\text{NH}_4(\text{aq})$ (100:5:1, v/v) as the eluent to produce 38 mg of probe **2C** as an orange solid (32% yield).

^1H NMR (400 MHz, CDCl_3) δ 8.61 (s, 1H), 7.88 – 7.57 (m, 4H), 7.53 – 6.94 (m, 5H), 6.65 – 6.25 (m, 2H), 3.82 – 3.12 (m, 9H), 2.97 (dd, J = 47.7, 8.5 Hz, 1H), 2.85 – 2.67 (m, 1H), 2.56 (bs, 1H), 2.37 – 2.17 (m, 4H), 2.14 – 2.04 (bs, 1H), 1.96 – 1.80 (m, 5H), 1.76 – 1.31 (m, 4H), 1.27 – 1.01 (m, 7H).

^{13}C NMR (101 MHz, CDCl_3) δ 172.42, 161.07, 160.56, 156.36, 153.15, 151.45, 140.46, 140.18, 134.32, 133.41, 133.35, 132.72, 132.66, 130.44, 129.45, 129.19, 128.31, 127.87, 127.11, 126.88, 126.65, 126.04, 124.54, 124.07, 118.15, 117.67, 112.89, 109.63, 108.58, 96.86, 63.58, 58.91, 57.72, 57.47, 56.79, 54.81, 53.54, 50.65, 48.16, 47.37, 45.78, 45.60, 45.02, 43.04, 35.30, 28.48, 25.00, 12.54.

HRMS (ESI): m/z calcd for $\text{C}_{38}\text{H}_{45}\text{N}_5\text{O}_3\text{S}$ $[\text{M}+\text{H}]^+$ 652.3321; found 652.3320.

1.5 Synthesis of probe **3A**



Scheme S4. Reagents and conditions: (i) oxalyl chloride, DMF, DCM, 0 °C to reflux; (ii) Et_3N , 0 °C to rt, 15 h; (iii) mesyl chloride, DCM, 0 °C to rt, 1 h; (iv) LiBr , acetone, 60 °C, 15 h; (v) Cs_2CO_3 , ACN, 60 °C, 24 h.

Compounds **VI**[6] and **IX**[1] were synthesized according to the published procedures.

7-(Diethylamino)-N-(2-(2-(2-hydroxyethoxy)ethoxy)ethyl)-2-oxo-2H-chromene-3-carboxamide (**VII**).

7-(Diethylamino)-2-oxo-2H-chromene-3-carboxylic acid (**II**) (515 mg, 1.97 mmol, 1.0 equiv) was suspended in CH_2Cl_2 (25 mL) and cooled to 0 °C. The solution was stirred and dimethylformamide (50 μL) was added, followed by dropwise addition of oxalyl chloride (334 μL , 3.94 mmol, 2.0 equiv). The reaction mixture was stirred at 0 °C for 5 minutes, then at reflux conditions for 30 minutes. Solvent was removed under reduced pressure and the residue was dissolved in CH_2Cl_2 (10 mL) and cooled to 0 °C. This solution was added dropwise to the solution of 2-(2-(2-aminoethoxy)ethoxy)ethan-1-ol (**VI**) (440 mg, 2.95 mmol, 1.5 equiv) and Et_3N (327 μL , 2.24 mmol, 1.2 equiv) in CH_2Cl_2 (25 mL) cooled to 0 °C. The reaction mixture was allowed to reach room temperature and stirred for 2 h. The mixture was

transferred to separating funnel, washed with 1 M HCl (25 mL), brine (15 mL) and dried over anhydrous Na₂SO₄. Solvent was evaporated to produce the crude product, which was purified by flash column chromatography using DCM/MeOH (from 50:1 to 10:1, v/v) as the eluent to produce 422 mg of compound **VII** as a yellow oil (54% yield).

¹H NMR (400 MHz, CDCl₃) δ 9.15 – 9.05 (m, 1H), 8.70 (s, 1H), 7.43 (d, *J* = 9.0 Hz, 1H), 6.65 (dd, *J* = 9.0, 2.4 Hz, 1H), 6.50 (d, *J* = 2.4 Hz, 1H), 3.80 – 3.75 (m, 2H), 3.75 – 3.60 (m, 10H), 3.45 (q, *J* = 7.1 Hz, 4H), 1.24 (t, *J* = 7.1 Hz, 6H).

HRMS (ESI): *m/z* calcd for C₂₀H₂₉N₂O₆ [M+H]⁺ 393.2026; found 393.2020.

***N*-(2-(2-(2-Bromoethoxy)ethoxy)ethyl)-7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxamide (VIII).**

7-(Diethylamino)-*N*-(2-(2-(2-hydroxyethoxy)ethoxy)ethyl)-2-oxo-2*H*-chromene-3-carboxamide (**IX**) (403 mg, 1.03 mmol, 1 equiv) was dissolved CH₂Cl₂ (15 mL) and cooled to 0 °C. The solution was stirred and Et₃N (185 μL, 1.34 mmol, 1.3 equiv) was added, followed by dropwise addition of mesyl chloride (88 μL, 1.13 mmol, 1.1 equiv). The reaction mixture was allowed to reach room temperature and stirred for 1 h. The mixture was transferred to separating funnel, washed with 1 M HCl (25 mL), brine (20 mL) and dried over anhydrous Na₂SO₄. Solvent was evaporated and the residue was dissolved in acetone (20 mL). LiBr (1.77 g, 20.6 mmol, 20 equiv) was added portionwise to the stirred solution. The reaction mixture was heated to 60 °C and stirred for 15 h. Solvent was evaporated, and the residues suspended in EtOAc (30 mL). The mixture was transferred to separating funnel, washed with water (30 mL), brine (20 mL) and dried over anhydrous Na₂SO₄. Solvent was evaporated to produce the crude product, which was purified by flash column chromatography using EtOAc/Hex (from 1:2 to 3:1, v/v) as the eluent to produce 194 mg of compound **VIII** as a yellow oil (41% yield).

¹H NMR (400 MHz, CDCl₃) δ 9.03 (t, *J* = 4.7 Hz, 1H), 8.69 (s, 1H), 7.42 (d, *J* = 9.0 Hz, 1H), 6.64 (dd, *J* = 9.0, 2.5 Hz, 1H), 6.49 (d, *J* = 2.3 Hz, 1H), 3.85 (t, *J* = 6.3 Hz, 1H), 3.75 – 3.62 (m, 4H), 3.50 (t, *J* = 6.3 Hz, 1H), 3.45 (q, *J* = 7.1 Hz, 2H), 1.24 (t, *J* = 7.1 Hz, 3H).

HRMS (ESI): *m/z* calcd for C₂₀H₂₈N₂O₅Br [M+H]⁺ 455.1182; found 455.1185.

***N*-(2-(2-(2-(*N*-((1-Benzylpiperidin-3-yl)methyl)naphthalene-2-sulfonamido)ethoxy)ethoxy)ethyl)-7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxamide (3A).**

N-((1-Benzylpiperidin-3-yl)methyl)naphthalene-2-sulfonamide (**IX**) (48 mg, 0.105 mmol, 1 equiv), Cs₂CO₃ (68 mg, 0.21 mmol, 2 equiv) and *N*-(2-(2-(2-bromoethoxy)ethoxy)ethyl)-7-(diethylamino)-2-oxo-2*H*-chromene-3-carboxamide (**VIII**) (42 mg, 0.105 mmol, 1 equiv) were dissolved in ACN (3 mL) and stirred at 60 °C for 24 h. The solvent was evaporated and

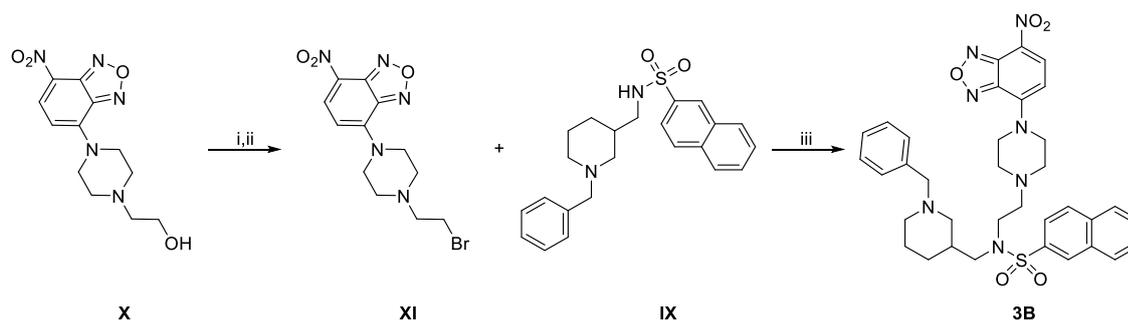
the product was purified by flash column chromatography using DCM/MeOH (from 60:1 to 30:1, v/v) as the eluent to produce 55 mg of probe **3A** as a yellow oil (68% yield).

^1H NMR (400 MHz, CDCl_3) δ 8.97 (t, $J = 5.2$ Hz, 1H), 8.66 (d, $J = 0.4$ Hz 1H), 8.41 – 8.35 (m, 1H), 8.01 – 7.85 (m, 3H), 7.77 (ddd, $J = 8.7, 3.7, 1.9$ Hz, 1H), 7.65 – 7.54 (m, 2H), 7.39 (d, $J = 9.0$ Hz, 1H), 7.31 – 7.18 (m, 5H), 6.61 (dd, $J = 9.0, 2.3$ Hz, 1H), 6.45 (d, $J = 2.3$ Hz, 1H), 3.64 – 3.51 (m, 6H), 3.50 – 3.46 (m, 4H), 3.42 (q, $J = 7.1$ Hz, 4H), 3.36 (td, $J = 6.3, 2.2$ Hz, 2H), 3.19 – 3.08 (m, 2H), 2.91 – 2.78 (m, 2H), 2.75 – 2.51 (m, 2H), 2.16 – 1.91 (m, 1H), 1.88 – 1.37 (m, 5H), 1.21 (t, $J = 7.1$ Hz, 6H), 1.10 – 0.92 (m, 1H).

^{13}C NMR (101 MHz, CDCl_3) δ 163.32, 162.60, 157.70, 152.56, 148.09, 138.12, 137.89, 136.80, 136.70, 134.78, 134.73, 132.23, 132.19, 131.19, 129.51, 129.29, 129.19, 128.76, 128.67, 128.48, 128.42, 128.33, 128.23, 127.96, 127.91, 127.57, 127.51, 127.19, 127.07, 122.72, 122.40, 110.30, 109.95, 108.38, 96.58, 70.44, 70.31, 69.86, 69.80, 63.39, 57.76, 57.26, 53.93, 53.22, 48.21, 47.24, 45.12, 39.44, 34.77, 28.22, 24.59, 24.16, 12.49.

HRMS (ESI): m/z calcd for $\text{C}_{43}\text{H}_{53}\text{N}_4\text{O}_7\text{S}$ $[\text{M}+\text{H}]^+$ 769.3635; found 769.3630.

1.6 Synthesis of probe **3B**



Scheme S5. Reagents and conditions: (i) mesyl chloride, DCM, 0 °C to rt, 30 min; (ii) LiBr, acetone, 50 °C, 1 h; (iii) Cs_2CO_3 , ACN, 60 °C, 24 h.

Compound **X**[7] was synthesized according to the published procedure.

4-(4-(2-Bromoethyl)piperazin-1-yl)-7-nitrobenzo[c][1,2,5]oxadiazole (**XI**).

2-(4-(7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)piperazin-1-yl)ethan-1-ol (**X**) (560 mg, 1.91 mmol, 1.0 equiv) was dissolved CH_2Cl_2 (5 mL) and cooled to 0 °C. The solution was stirred and Et_3N (528 μL , 3.82 mmol, 2.0 equiv) was added, followed by dropwise addition of mesyl chloride (155 μL , 2.0 mmol, 1.05 equiv). The reaction mixture was allowed to reach room temperature and stirred for 30 min. DCM (25 mL) was added to the reaction mixture, and the solution was transferred to separating funnel, washed with water (20 mL), saturated aqueous

NaHCO₃ solution (20 mL), brine (15 mL) and dried over anhydrous Na₂SO₄. Solvent was evaporated and the residue was dissolved in acetone (10 mL). The solution was stirred, and LiBr (1.66 g, 19.1 mmol, 10 equiv) was added. The reaction mixture was heated to 50 °C and stirred for 1 h. Solvent was evaporated and the residue was suspended in EtOAc (20 mL). The mixture was transferred to separating funnel, washed with water (20 mL), brine (20 mL) and dried over anhydrous Na₂SO₄. Solvent was evaporated to produce 467 mg of crude product (69%), which was used immediately in the next reaction step without further purification.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.47 (d, *J* = 9.2 Hz, 1H), 6.69 (d, *J* = 9.2 Hz, 1H), 4.13 (apps, 4H), 3.63 (t, *J* = 6.9 Hz, 2H), 2.81 (apps, 2H), 2.74 (apps, 4H).

***N*-((1-Benzylpiperidin-3-yl)methyl)-*N*-(2-(4-(7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)piperazin-1-yl)ethyl)naphthalene-2-sulfonamide (3B).**

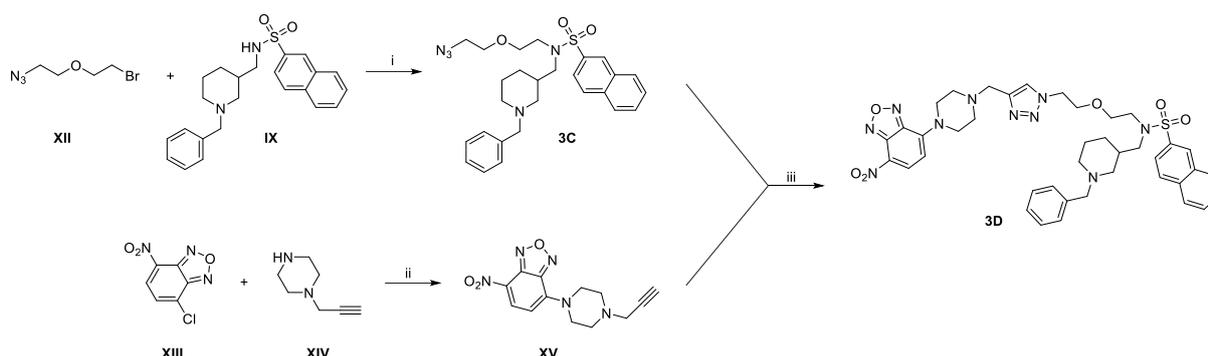
N-((1-Benzylpiperidin-3-yl)methyl)naphthalene-2-sulfonamide (**IX**) (180 mg, 0.45 mmol, 1.0 equiv), Cs₂CO₃ (222 mg, 0.68 mmol, 1.5 equiv) and 4-(4-(2-Bromoethyl)piperazin-1-yl)-7-nitrobenzo[*c*][1,2,5]oxadiazole (**XI**) (162 mg, 0.45 mmol, 1.0 equiv) were dissolved in ACN (5 mL) and stirred at 60 °C for 24 h. The solvent was evaporated under reduced pressure and the residue suspended in EtOAc (30 mL). The mixture was transferred to separating funnel, washed with water (20 mL), saturated aqueous NaHCO₃ solution (20 mL), brine (20 mL) and dried over anhydrous Na₂SO₄. The solvent was evaporated and the product was purified by flash column chromatography using DCM/MeOH/NH₄(aq) (from 100:2:1 to 100:10:1, v/v) as the eluent to produce 47 mg of probe **3B** as a yellow solid (15% yield).

¹H NMR (400 MHz, CDCl₃) δ 8.38 – 8.36 (m, 1H), 8.34 (d, *J* = 9.1 Hz, 1H), 8.00 – 7.87 (m, 3H), 7.76 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.67 – 7.58 (m, 2H), 7.30 – 7.13 (m, 5H), 6.22 (d, *J* = 9.1 Hz, 1H), 4.06 – 3.92 (m, 4H), 3.44 (d, *J* = 2.8 Hz, 2H), 3.37 – 3.19 (m, 2H), 3.11 (d, *J* = 7.6 Hz, 2H), 2.90 – 2.42 (m, 8H), 2.05 – 1.85 (m, 2H), 1.80 – 1.61 (m, 3H), 1.60 – 1.44 (m, 1H), 1.08 – 0.89 (m, 1H).

¹³C NMR (101 MHz, CDCl₃) δ 145.13, 144.75, 138.27, 136.32, 135.25, 134.71, 132.16, 129.45, 129.17, 129.14, 128.87, 128.46, 128.19, 127.95, 127.71, 127.01, 123.21, 122.50, 102.62, 63.43, 57.74, 56.81, 54.09, 53.26, 52.76, 49.31, 46.26, 35.13, 28.43, 24.72.

HRMS (ESI): *m/z* calcd for C₃₅H₄₀N₇O₅S [M+H]⁺ 670.2812; found 670.2821.

1.7 Synthesis of compounds **3C** and **3D**



Scheme S6. Reagents and conditions: (i) Cs_2CO_3 , ACN, 60 °C, 3 d; (ii) K_2CO_3 , THF, 0 °C to rt, 24 h; (iii) CuBr, *N*-methylmorpholine, EtOAc, 40 °C, 24 h.

Compounds **XII**[8] and **XIV**[9] were synthesized according to the published procedures.

***N*-((1-benzylpiperidin-3-yl)methyl)naphthalene-2-sulfonamide (**3C**).**

N-((1-benzylpiperidin-3-yl)methyl)naphthalene-2-sulfonamide (**IX**) (100 mg, 0.25 mmol, 1.0 equiv), 1-azido-2-(2-bromoethoxy)ethane (**XII**) (74 mg, 0.38 mmol, 1.5 equiv) and Cs_2CO_3 (165.5 mg, 0.51 mmol, 2.0 equiv) were suspended in ACN (5 mL), and stirred at 60 °C for 3 days. Solvents were removed under reduced pressure and the crude product was purified by flash column chromatography using DCM/MeOH (from 9:1 to 9:2, v/v) as the eluent to produce 71 mg of azide **3C** as a yellow viscous oil (56% yield).

^1H NMR (400 MHz, CDCl_3) δ 8.41 – 8.35 (m, 1H), 8.01 – 7.86 (m, 3H), 7.76 (dd, $J = 8.6, 1.9$ Hz, 1H), 7.68 – 7.58 (m, 2H), 7.43 – 7.18 (m, 5H), 3.58 (t, $J = 6.2$ Hz, 2H), 3.51 – 3.46 (m, 4H), 3.37 – 3.20 (m, 4H), 3.13 (d, $J = 7.5$ Hz, 2H), 2.77 (dd, $J = 32.4, 9.2$ Hz, 2H), 2.05 – 1.90 (d, 2H), 1.85 – 1.40 (m, 4H), 1.08 – 0.92 (m, 1H).

HRMS (ESI): m/z calcd for $\text{C}_{27}\text{H}_{34}\text{N}_5\text{O}_3\text{S}$ $[\text{M}+\text{H}]^+$ 508.2377; found 508.2384.

4-Nitro-7-(4-(prop-2-yn-1-yl)piperazin-1-yl)benzo[*c*][1,2,5]oxadiazole (XV**).**

Solution of NBDCl (**XIII**) (50 mg, 0.25 mmol, 1.0 equiv) in THF (5 mL) was added dropwise to the mixture of 1-(prop-2-yn-1-yl)piperazine (**XIV**) (48.5 mg, 0.30 mmol, 1.2 equiv) and K_2CO_3 (172 mg, 1.25 mmol, 1.5 equiv) in THF (5 mL). During the addition, the reaction mixture was vigorously stirred and cooled on an ice bath. Following the addition, the temperature of the reaction mixture was allowed to reach room temperature and stirring was continued for 24 h. The solvent was evaporated under reduced pressure and the residue suspended in DCM (30 mL). The mixture was transferred to separating funnel, washed with

water (20 mL), saturated aqueous NaHCO₃ solution (20 mL), brine (20 mL) and dried over anhydrous Na₂SO₄. The solvent was evaporated under reduced pressure to produce 51 mg of the desired product **XV** as a red powder (71% yield).

¹H NMR (400 MHz, DMSO-d₆) δ 8.48 (d, *J* = 9.2 Hz, 1H), 6.69 (d, *J* = 9.2 Hz, 1H), 4.19 – 4.12 (m, 4H), 3.40 (d, *J* = 2.4 Hz, 2H), 3.21 (t, *J* = 2.4 Hz, 1H), 2.72 – 2.68 (m, 4H).

MS (ESI): *m/z* calcd for C₁₃H₁₄N₅O₃ [M+H]⁺ 288.11; found 288.21.

***N*-((1-Benzylpiperidin-3-yl)methyl)-*N*-(2-(2-(4-((4-(7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)piperazin-1-yl)methyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)ethyl)naphthalene-2-sulfonamide (3D)**

4-Nitro-7-(4-(prop-2-yn-1-yl)piperazin-1-yl)benzo[*c*][1,2,5]oxadiazole (**XV**) (8.0 mg, 0.025 mmol, 1.0 equiv) and azide **3C** (12.7 mg, 0.025 mmol, 1.0 equiv) were dissolved in EtOAc (20 mL). *N*-Methylmorpholine (5.5 μl, 0.05 mmol, 2.0 equiv) and catalytic amount of CuBr (~ 1 mg) were added to the stirred mixture at room temperature. The reaction mixture was then stirred at 40 °C for 1 day. The solvents were removed under reduced pressure and the crude product was purified by flash column chromatography using DCM/MeOH (from 40:1 to 10:1, v/v) as the eluent to produce 17 mg of compound **3D** as an orange solid (86% yield).

¹H NMR (400 MHz, CDCl₃) δ 8.35 (d, *J* = 9.0 Hz, 1H), 8.33 (d, *J* = 1.5 Hz, 1H), 8.00 – 7.86 (m, 3H), 7.79 (s, 1H), 7.72 – 7.58 (m, 3H), 7.36 – 7.20 (m, 5H), 6.23 (d, *J* = 9.0 Hz, 1H), 4.49 (t, *J* = 5.0 Hz, 2H), 4.18 – 4.00 (m, 4H), 3.79 (s, 2H), 3.79 (t, *J* = 5.0 Hz, 2H), 3.86 – 3.44 (m, 4H), 3.42 – 3.18 (m, 2H), 3.14 – 2.86 (m, 4H), 2.81 – 2.72 (m, 4H), 2.15 – 1.15 (m, 4H), 1.40 – 1.15 (m, 2H), 1.05 – 0.70 (m, 1H).

HRMS (ESI): *m/z* calcd for C₄₀H₄₇N₁₀O₆S [M+H]⁺ 795.3395; found 795.3398.

2. Crystal structure refinement statistics

Table S1. Data collection and refinement statistics calculated using Phenix (Adams *et al* Acta Cryst. D 2010;66:213-221). $R\text{-work} = \frac{\sum |F_o - |F_c||}{\sum |F_o|}$, F_o and F_c are observed and calculated structure factors, R-free set uses about 5% randomly chosen reflections. Statistics for the highest-resolution shell are shown in parentheses.

	hBChE 2C	hBChE 3A	hBChE 3B
Data collection			
X-ray source	ESRF ID23-1	ESRF ID23-2	ESRF ID23-1
Wavelength	0.9724	0.8726	0.9724
Resolution range	47.89 - 2.5 (2.589 - 2.5)	44.93 - 2.75 (2.848 - 2.750)	55.15 - 2.474 (2.562 - 2.474)
Space group	I 4 2 2	P 21 21 21	I 4 2 2
Unit cell	154.93 154.93 128.03 90 90 90	76.29 80.2 231.35 90 90 90	154.22 154.22 127.85 90 90 90
Total reflections	176432 (18342)	195803 (20184)	200463 (22509)
Unique reflections	27001 (2689)	37475 (3702)	26287 (2723)
Multiplicity	6.5 (6.8)	5.2 (5.5)	7.6 (8.3)
Completeness (%)	98.95 (99.67)	99.10 (99.87)	94.63 (99.96)
Mean I/sigma(I)	14.58 (2.55)	12.71 (2.27)	19.30 (2.24)
Wilson B-factor	56.69	58.52	60.11
R-merge	0.07755 (0.6376)	0.09612 (0.6969)	0.07009 (0.9361)
R-meas	0.08423 (0.6882)	0.107 (0.7712)	0.07524 (0.9993)
R-pim	0.03107 (0.2458)	0.04593 (0.3244)	0.02689 (0.3458)
CC1/2	0.998 (0.823)	0.997 (0.746)	0.999 (0.758)
CC*	1 (0.95)	0.999 (0.924)	1 (0.929)
Refinement statistics			
Reflections used in refinement	26943 (2689)	37465 (3702)	26279 (2725)
Reflections used for R-free	1078 (107)	1122 (116)	1296 (129)
R-work	0.1922 (0.2379)	0.1811 (0.2546)	0.1823 (0.2711)
R-free	0.2572 (0.3616)	0.2375 (0.3398)	0.2275 (0.3606)
CC(work)	0.960 (0.880)	0.941 (0.832)	0.958 (0.841)
CC(free)	0.916 (0.724)	0.907 (0.696)	0.945 (0.715)
Number of non-hydrogen atoms	4588	9029	4521
macromolecules	8385	4216	4222
ligands	469	214	194
solvent	175	91	172
Protein residues	537	1053	537
RMS(bonds)	0.009	0.006	0.009
RMS(angles)	1.05	1.85	1.04
Ramachandran favored (%)	95.04	95.33	94.85
Ramachandran allowed (%)	4.96	4.39	4.77
Ramachandran outliers (%)	0.00	0.29	0.38
Rotamer outliers (%)	0.00	1.11	0.00
Clashscore	9.23	4.99	9.32
Average B-factor	62.11	53.55	66.64
macromolecules	51.13	65.01	60.62
ligands	99.87	100.27	96.15
solvent	45.13	63.40	60.39

3. Rat Brain Slices Experiments

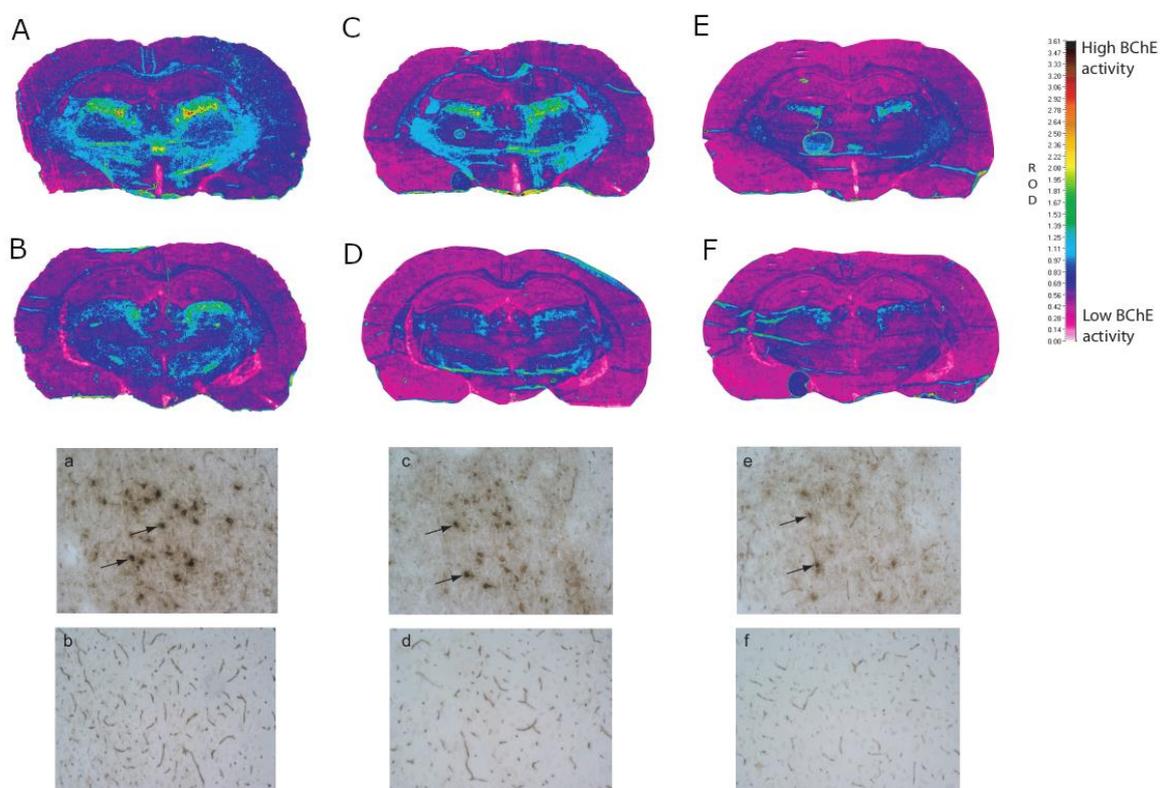


Figure S1. BChE activity histochemical staining of coronal cryosections (10 μm) of a rat brain from two regions at the level of the thalamus. All sections were processed with 0.1 mM BW-284C51 in Koelle solution to completely block the AChE activity (A, B). For the inhibition experiment, the sections were incubated with compounds **2C** (1 mM, C, D) and **3B** (1 mM, E, F), respectively. The relative optical density (ROD) pseudocolor scale for the staining intensity of BChE activity is shown on the right. The photomicrographs show BChE-rich neurons in the laterodorsal thalamic nucleus (arrows) and the capillaries in the cerebral cortex in the absence (a, b) and the presence of compounds **2C** (c, d) and **3B** (e, f). Magnification of the photomicrographs is 20 \times .

4. Titration of hBChE with probe **2C**

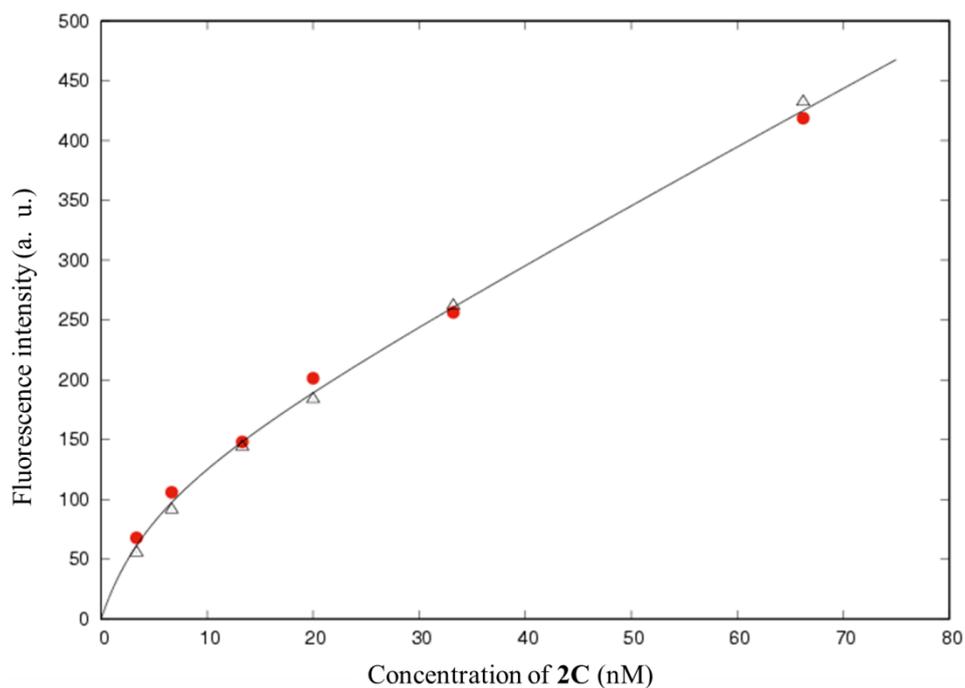


Figure S2. The fluorescence titration data, in duplicate, were simultaneously fitted to the sum of hyperbolic and linear equation. The determined binding constant for probe **2C** was 5.5 ± 1.6 nM, remarkably similar to the inhibition constant for the binding to the free enzyme (3.3 nM, see Table 1 in the main text).

5. References

1. Košak U, Brus B, Knez D, et al. Development of an *in-vivo* active reversible butyrylcholinesterase inhibitor. *Sci Rep.* 2016;6:39495.
2. Yao H, Wei G, Liu Y, et al. Synthesis, Biological Evaluation of Fluorescent 23-Hydroxybetulinic Acid Probes, and Their Cellular Localization Studies. *ACS Med Chem Lett.* 2018;9(10):1030-1034.
3. Hori Y, Norinobu T, Sato M, et al. Development of Fluorogenic Probes for Quick No-Wash Live-Cell Imaging of Intracellular Proteins. *J Am Chem Soc.* 2013;135(33):12360-12365.
4. Nishizono N, Oda K, Kato Y, et al. Synthesis of Fluorescent Derivatization Reagents: Reaction of Isatin with 3-Aryl-7-diethylaminocoumarins and Their Fluorescent Properties. *Heterocycles.* 2004;63.
5. Knez D, Coquelle N, Pišlar A, et al. Multi-target-directed ligands for treating Alzheimer's disease: Butyrylcholinesterase inhibitors displaying antioxidant and neuroprotective activities. *Eur J Med Chem.* 2018;156:598-617.
6. Puchkov PA, Kartashova IA, Shmendel EV, et al. Spacer structure and hydrophobicity influences transfection activity of novel polycationic gemini amphiphiles. *Bioorg Med Chem Lett.* 2017;27(15):3284-3288.
7. Schininà B, Martorana A, Colabufo NA, et al. 4-Nitro-2,1,3-benzoxadiazole derivatives as potential fluorescent sigma receptor probes. *RSC Adv.* 2015;5(58):47108-47116.
8. Yu X, Eymur S, Singh V, et al. Flavin as a photo-active acceptor for efficient energy and charge transfer in a model donor-acceptor system. *Phys Chem Chem Phys.* 2012;14(19):6749-6754.
9. Zheng S, Lingyue G, Ong MJH, et al. Divergent synthesis of 5',7'-difluorinated dihydroxanthene-hemicyanine fused near-infrared fluorophores. *Org Biomol Chem.* 2019;17(17):4291-4300.