

Discovery of *in-vivo* chemical probes for treating Alzheimer's disease: Dual phosphodiesterase 5 (PDE5) and class I histone deacetylases-selective inhibitors.

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Protocol for preparative HPLC purification methods

The HPLC measurement was performed using Gilson 281 or SHIMADZU preparative HPLC, an autosampler and a UV detector. The fractions were detected by LC-MS. The MS detector was configured with an electrospray ionization source. The source temperature was maintained at 300-350 °C.

Method 1

Reversed phase HPLC was carried out on Luna (100 x 30 mm; 5 µm). Solvent A: water with 0.075% TFA; Solvent B: acetonitrile. Gradient: At room temperature, 28% of B to 58% of B within 10.5 minutes; then 58% B over 4 minutes. Flow rate: 25 mL/min.

Method 2

Reversed phase HPLC was carried out on Luna (100 x 30 mm; 5 µm). Solvent A: water with 0.075% TFA; Solvent B: acetonitrile. Gradient: At room temperature, 33% of B to 63% of B within 10.5 minutes; then 63% B over 4 minutes. Flow rate: 25 mL/min.

Method 3

Reversed phase HPLC was carried out on Luna (100 x 30 mm; 5 µm). Solvent A: water with 0.075% TFA; Solvent B: acetonitrile with 0.075% TFA. Gradient: At room temperature, 25% of B to 45% of B within 6 minutes; then 45% B over 4 minutes. Flow rate: 25 mL/min.

Method 4

Reversed phase HPLC was carried out on Luna (100 x 30 mm; 5 µm). Solvent A: water with 0.075% TFA; Solvent B: acetonitrile with 0.075% TFA. Gradient: At room temperature, 28% of B to 48% of B within 6 minutes; then 40% B over 4 minutes. Flow rate: 25 mL/min.

Method 5

Reversed phase HPLC was carried out on Luna (100 x 30 mm; 5 µm). Solvent A: water with 0.075% TFA; Solvent B: acetonitrile with 0.075% TFA. Gradient: At room temperature, 26% of B to 46% of B within 6 minutes; then 40% B over 4 minutes. Flow rate: 25 mL/min.

Method 6

Reverse phase HPLC was carried out on Waters Xbridge (150×25 mm; 5 μm). Solvent A: 10 mM NH₄HCO₃ in water; Solvent B: acetonitrile. Gradient: At room temperature, 40% of B to 70% of B within 12 minutes; then 70% of B over 2 minutes; then 70% of B to 95% of B within 0.2 minutes and maintained for 2 minutes; then decreased to 10% of B within 0.2 minutes and maintained 1.6 minutes. Flow rate: 20 mL/min.

Method 7

Reverse phase HPLC was carried out on Luna C18 (150×25 mm; 5 μm). Solvent A: water with 0.040% ammonia hydrate; Solvent B: acetonitrile with 0.040% ammonia hydrate. Gradient: At room temperature, 20% of B to 40% of B within 6 minutes at 20 mL/min; then 40% B at 25 mL/min over 2 minutes.

Method 8

Reverse phase HPLC was carried out on Luna C18 (100×30 mm; 5 μm). Solvent A: 10 mM NH₄HCO₃ in water; Solvent B: acetonitrile. Gradient: At room temperature, 45% of B to 65% of B within 12 minutes; then 65% of B over 2 minutes; then 65% of B to 95% of B within 0.2 minutes and maintained for 2 minutes; then decreased to 45% of B within 0.2 minutes and maintained 1.6 minutes. Flow rate: 20 mL/min.

Method 9

Reverse phase HPLC was carried out on Luna C18 (100×30 mm; 4 μm). Solvent A: water with 0.075% TFA; Solvent B: acetonitrile with 0.075% TFA. Gradient: At room temperature, 20% of B to 45% of B within 6 minutes; then 40% B over 3 minutes. Flow rate: 25 mL/min.

Method 10

Reverse phase HPLC was carried out on Waters Xbridge (150×25 mm; 5 μm). Solvent A: 6.7 mM NH₄HCO₃ in water; Solvent B: acetonitrile. Gradient: At room temperature, 35% of B to 60% of B within 8 minutes; then 60% of B over 2 minutes; then 60% of B to 100% of B within 0.2 minutes and maintained for 1.5 minutes; then decreased to 35% of B within 0.2 minutes and maintained 2 minutes. Flow rate: 25 mL/min.

Method 11

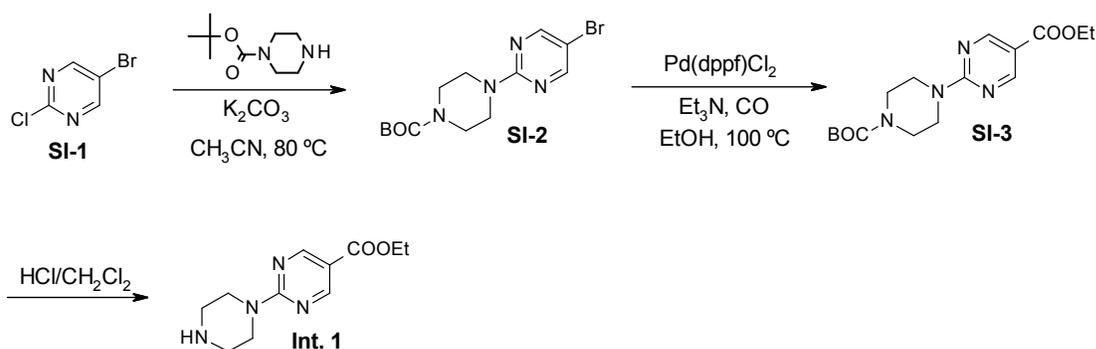
Reverse phase HPLC was carried out on Waters Xbridge (150×25 mm; 5 μm). Solvent A: 6.7 mM NH₄HCO₃ in water; Solvent B: acetonitrile. Gradient: At room temperature, 25% of B to 55% of B within 8 minutes; then 55% of B over 2 minutes; then 55% of B to 100% of B within 0.2 minutes and maintained for 1.5 minutes; then decreased to 25% of B within 0.2 minutes and maintained 2 minutes. Flow rate: 25 mL/min.

Method 12

Reversed phase HPLC was carried out on Luna (100 x 30 mm; 5 μm). Solvent A: water with 0.1% TFA; Solvent B: acetonitrile. Gradient: At room temperature, 35% of B to 65% of B within 12 minutes; then 65% B over 2 minutes. Flow rate: 20 mL/min.

Synthesis of intermediates 1 to 5

Synthesis of ethyl 2-piperazin-1-ylpyrimidine-5-carboxylate (**Int. 1**)



tert-butyl 4-(5-bromopyrimidin-2-yl)piperazine-1-carboxylate (**SI-2**)

To a solution of 5-bromo-2-chloropyrimidine (9.75 g, 50 mmol) in CH_3CN (100 mL) were added *tert*-butyl piperazine-1-carboxylate (9.25 g, 50 mmol) and K_2CO_3 (13.8 g, 100 mmol) and the reaction mixture was stirred at $80\text{ }^\circ C$ overnight. Then the reaction mixture was concentrated under vacuum and extracted with EtOAc. The organic phase was washed with water, dried over anhydrous Na_2SO_4 , filtered and concentrated to give compound **SI-2** (15 g 87%). ESI-MS m/z 343 $[M+H]^+$ calc. for $C_{13}H_{19}BrN_4O_2$

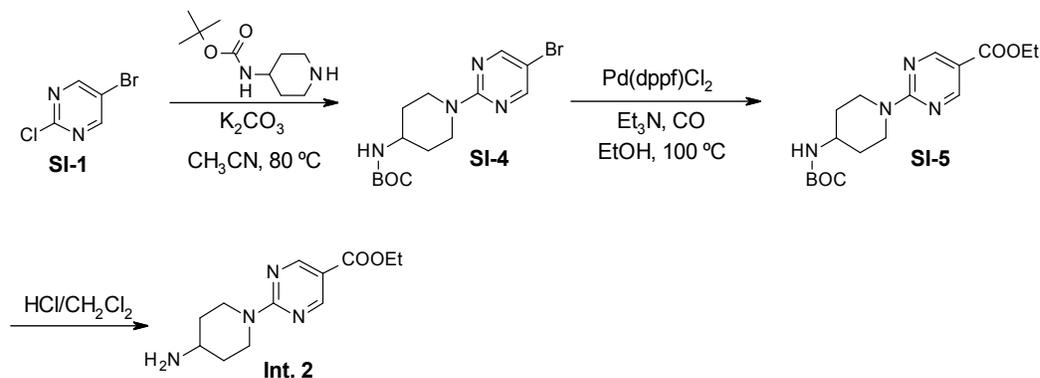
ethyl 2-(4-tert-butoxycarbonylpiperazin-1-yl)pyrimidine-5-carboxylate (SI-3)

To a solution of **SI-2** (6.00 g, 17.49 mmol) in EtOH (100 mL) were added Et₃N (1.77 g, 2.43 mL) and Pd(dppf)Cl₂ (5 g, 6.83 mmol) and the reaction mixture was stirred at 100 °C at 1.5 MPa under CO atmosphere overnight. The reaction mixture was filtrated and the filtrate was concentrated to give the desired product **SI-3** (5 g, 85%). ESI-MS *m/z* 337 [M+H]⁺ calc. for C₁₆H₂₄N₄O₄

ethyl 2-piperazin-1-ylpyrimidine-5-carboxylate (Int. 1)

A solution of **SI-3** (2.50 g, 7.44 mmol) in HCl/CH₂Cl₂ (4.0 M, 30 mL) was stirred at room temperature overnight. Then, the solution was concentrated to give the desired **Int. 1** (1.7 g, 97%). ¹H NMR (MeOD, 400 MHz): δ 8.88 (s, 2H), 4.37-4.33 (m, 2H), 4.17-4.13 (m, 4H), 3.32-3.28 (m, 4H), 1.37-1.35 (m, 3H). ESI-MS *m/z* 237 [M+H]⁺ calc. for C₁₁H₁₆N₄O₂.

Synthesis of ethyl 2-(4-amino-1-piperidyl)pyrimidine-5-carboxylate (Int. 2)



tert-butyl N-[1-(5-bromopyrimidin-2-yl)-4-piperidyl]carbamate (SI-4)

To a solution of 5-bromo-2-chloropyrimidine (9.75 g, 50 mmol) in CH₃CN (100 mL) were added *tert*-butyl piperidin-4-ylcarbamate (9.95 g, 50 mmol) and K₂CO₃ (13.8 g, 100 mmol) and the reaction mixture was stirred 80 °C overnight. Then, the reaction mixture was concentrated and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous Na₂SO₄, filtered and concentrated under vacuum to give the crude product which was purified through column chromatography to give **SI-4** (15 g, 84%). ESI-MS *m/z* 357 [M+H]⁺ calc. for C₁₄H₂₁BrN₄O₂

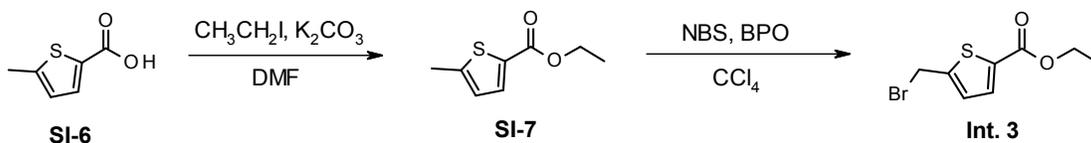
ethyl 2-[4-(tert-butoxycarbonylamino)-1-piperidyl]pyrimidine-5-carboxylate (SI-5)

To a solution of **SI-4** (5.00 g, 14.00 mmol) in EtOH (100 mL) were added Et₃N (1.77 g, 2.43 mL) and Pd(dppf)Cl₂ (512 mg, 0.7 mmol) and the reaction mixture was stirred at 100 °C at 1.5 MPa under CO atmosphere overnight. The reaction mixture was filtrated and the filtrate was concentrated to give the crude product which was purified through column chromatography to give desired **SI-5** (3 g, 61%). ESI-MS (M+1): 351.2 calc. for C₁₇H₂₆N₄O₄

ethyl 2-(4-amino-1-piperidyl)pyrimidine-5-carboxylate (Int. 2)

A solution of **SI-5** (3 g, 8.57 mmol) in HCl/CH₂Cl₂ (4.0 M, 30 mL) was stirred at room temperature overnight. Then, the solution was concentrated to give the desired **Int. 2** (2 g, 93%). ¹H NMR (CDCl₃, 400 MHz): δ 8.88 (s, 2H), 4.88 (m, 2H), 4.33-4.30 (m, 2H), 3.53-3.48 (m, 1H), 3.27-3.24 (m, 2H), 2.3-2.10 (m, 2H), 1.67-1.63 (m, 2H), 1.40-1.36 (t, 3H). ESI-MS *m/z* 251 [M+H]⁺ calc. for C₁₂H₁₈N₄O₂.

Synthesis of ethyl 5-(bromomethyl)thiophene-2-carboxylate (Int. 3)



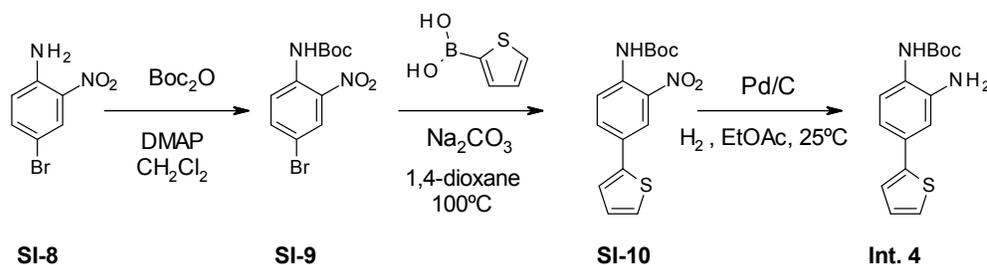
ethyl 5-methylthiophene-2-carboxylate (SI-7)

To a solution of commercially available 5-methylthiophene-2-carboxylic acid (9.2 g, 0.065 mol) in DMF (80 mL) was added K₂CO₃ (17.9 g, 0.13 mol) and then CH₃CH₂I (15.2 g, 0.98 mol) was added slowly. The reaction mixture was stirred at room temperature overnight. Then, the mixture was quenched with water and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous Na₂SO₄, filtered and concentrated to give compound **SI-7** (9.5 g, 86%) as a pale yellow oil. ¹H NMR (CDCl₃, 400 MHz): δ 7.62-7.61 (d, *J* = 4 Hz, 1H), 6.77-6.76 (d, *J* = 4 Hz, 1H), 4.35-4.23 (m, 2H), 2.52 (s, 3H), 1.38-1.35 (m, 3H). ESI-MS *m/z* 171.0 [M+H]⁺ calc. for C₈H₁₀O₂S

ethyl 5-(bromomethyl)thiophene-2-carboxylate (Int. 3)

To a solution of compound **SI-7** (1.9 g, 0.011 mol) in CCl_4 (30 mL) was added BPO (242 mg, 0.001 mol) and NBS (2.85 g, 0.016 mol) and the reaction mixture was stirred at reflux for 2 hours. Then, the mixture was extracted with CH_2Cl_2 and the organic layer was washed with aqueous NaHCO_3 and brine, dried over anhydrous Na_2SO_4 , filtered and concentrated to give the crude compound which was purified by silica gel chromatography to give pure compound **Int. 3** (1.3 g, 47%) as a yellow oil. ESI-MS m/z 249.1 $[\text{M}+\text{H}]^+$ calc. for $\text{C}_8\text{H}_9\text{BrO}_2\text{S}$.

Synthesis of tert-butyl *N*-[2-amino-4-(2-thienyl)phenyl]carbamate (**Int. 4**)



tert-butyl N-(4-bromo-2-nitro-phenyl)carbamate (SI-9)

To a mixture of 4-bromo-2-nitro-aniline (**SI-8**) (5 g, 23.1 mmol) and DMAP (5.64 g, 46.2 mmol) in CH_2Cl_2 (100 mL), was added Boc_2O (5.49 g, 25.4 mmol). The mixture was stirred at room temperature overnight. The mixture was extracted with CH_2Cl_2 (100 mL \times 3). The combined organic phase was washed with saturated brine (50 mL \times 2), dried with anhydrous Na_2SO_4 , filtered and concentrated in vacuum to give compound **SI-9** (2.02 g, 27%). ESI-MS m/z 317 $[\text{M}+\text{H}]^+$ calc. for $\text{C}_{11}\text{H}_{13}\text{BrN}_2\text{O}_4$.

tert-butyl N-[2-nitro-4-(2-thienyl)phenyl]carbamate (SI-10)

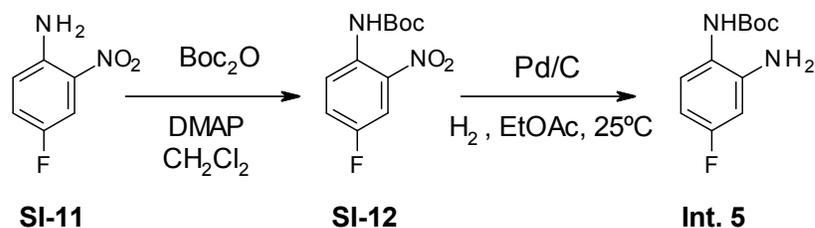
To a mixture of **SI-9** (2.02 g, 6.37 mmol) and 2-thienylboronic acid (937.28 mg, 7.32 mmol) in 1,4-dioxane (65 mL), was added Na_2CO_3 (1.58 g, 14.90 mmol) in one portion at room temperature under N_2 and then, heated to 100°C and stirred for 12 hours. The mixture was cooled to 25°C and concentrated in reduced pressure at 45°C . The residue was poured into ice-water and stirred for 20 minutes. The aqueous phase was extracted with EtOAc and the combined organic phase was washed with saturated brine, dried with anhydrous Na_2SO_4 ,

filtered and concentrated in vacuum. The residue was purified by silica gel chromatography to obtain compound **SI-10** (1.90 g, 93%) as yellow solid. ¹H NMR (CDCl₃, 400 MHz): δ 9.66 (s, 1H), 8.61-8.59 (d, *J* = 8Hz, 1H), 8.41 (d, *J* = 2Hz, 1H), 7.84-7.81 (m, 1H), 7.35-7.33 (m, 2H), 7.12-7.11 (m, 1H), 1.56 (s, 3H). ESI-MS *m/z* 321.1 [M+H]⁺ calc. for C₁₅H₁₆N₂O₄S.

tert-butyl *N*-[2-amino-4-(2-thienyl)phenyl]carbamate (**Int. 4**)

To a solution of **SI-10** (1.90 g, 5.93 mmol) in EtOAc (130 mL) was added Pd/C (10%, 0.65 g) under N₂. The suspension was degassed under vacuum and purged with H₂ several times. The mixture was stirred under H₂ (10 psi) at 25 °C for 16 hours. The reaction mixture was filtered and the filter was concentrated. The crude product was purified by silica gel chromatography to give pure compound **Int. 4** (1.60 g, 93%). ¹H NMR (CDCl₃, 400 MHz): δ 7.30 (d, *J* = 8.3 Hz, 1 H), 7.27-7.23 (m, 2H), 7.1-7.1 (m, 1H), 7.1 (dd, *J* = 3.3, 1.8 Hz, 1 H), 7.02 (d, *J* = 2.0 Hz, 1 H), 6.21 (br s, 1H), 3.80 (br s, 2H), 1.53 (s, 9H). ESI-MS *m/z* 291 [M+H]⁺ calc. for C₁₅H₁₈N₂O₂S.

Synthesis of *tert*-butyl *N*-[2-amino-4-(2-thienyl)phenyl]carbamate (**Int. 5**)



tert-butyl *N*-(4-fluoro-2-nitro-phenyl)carbamate (**SI-12**)

To a mixture of 4-fluoro-2-nitro-aniline (**SI-11**) (3.5 g, 22.4 mmol) and DMAP (5.47 g, 44.8 mmol) in CH₂Cl₂ (100 mL), was added Boc₂O (5.32 g, 24.4 mmol). The mixture was stirred at 25 °C overnight. Then, the mixture was extracted with CH₂Cl₂ and the combined organic phase was washed with saturated brine, with anhydrous Na₂SO₄, filtered and concentrated in vacuum to obtain compound **SI-12** (1.36 g, 24%). ESI-MS *m/z* 257 [M+H]⁺ calc. for C₁₁H₁₃FN₂O₄.

tert-butyl N-(2-amino-4-fluoro-phenyl)carbamate (Int. 5)

To a solution of **SI-12** (1.36 g, 5.31 mmol) in EtOAc (100 mL) was added Pd/C (10%, 0.3g) under N₂. The suspension was degassed under vacuum and purged with H₂ several times. The mixture was stirred under H₂ (10 psi) at 25 °C for 16 hours. The reaction mixture was filtered and the filter was concentrated. The crude product was purified by silica gel chromatography to give pure compound **Int. 5** (800 mg, 66.7%). ESI-MS *m/z* 227.1 [M+H]⁺ calc. for C₁₁H₁₅FN₂O₂.

High Resolution Mass Spectrometry of final compounds

High resolution mass spectrometry (HRMS) m/z determination was performed by a nanoLC Ultra HPLC system (Eksigent, Sciex, Ontario, Canada) coupled to a TripleTOF 5600+ system (Sciex, Ontario, Canada). Separation was carried out on an Acclaim PepMap™ 100 column (100 $\mu\text{m} \times 2 \text{ cm}$, 5 μm , 100Å, nanoViper, ThermoScientific) at 25°C. Solvent A: water with 0.1% formic acid; Solvent B: acetonitrile. Gradient: After 0.1 minutes at the initial condition of 90% A and 10% B solvent B was increased from 5 to 100% over 8 minutes, maintained at 100% for 2 minutes, then a linear gradient to initial conditions was applied for 0.5 minutes and maintained for 1.5 minutes to re-equilibrate the column (A: 95%, B: 5%), giving a cycle time of 12 minutes. Flow rate was 0.6 $\mu\text{L}/\text{min}$ during the complete run.

LC purities of final compounds

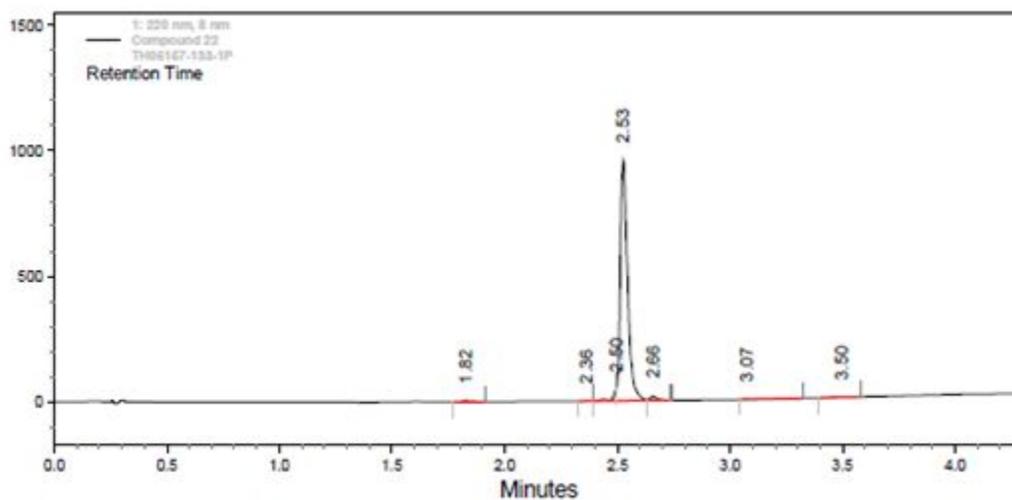
Method 1: HPLC-analysis was performed using a Shimadzu LC-20AB or LC-20AD with a Luna-C18(2), 5 μm , 2.0*50 mm column at 40 °C and UV detection at 215, 220 or 254 nm. Solvent A: water with 0.056% trifluoroacetic acid; Solvent B: acetonitrile with 0.056% trifluoroacetic acid. Gradient: After 0.1 minutes at the initial condition of 90% A and 10% B, solvent B was increased to 80% over 4 minutes, maintained at 80% for 0.9 minutes, then a linear gradient to initial conditions was applied for 0.02 minutes and maintained for 0.58 minutes to re-equilibrate the column, giving a cycle time of 5.50 minutes. Flow rate was 0.8 mL/min from 0.01 to 4.90 minutes, increased to 1.2 mL/min in 0.03 minutes and maintained until the end of the run.

Method 2: UHPLC analysis was performed using an Acquity UPLC™ system (Waters Corp., Milford, MA, USA) with thermostated autosampler. Separation was carried out on an Acquity UPLC™ BEH C18 column (50 mm \times 2.1 mm, 1.7 μm) at 40 °C and UV detection at 215, 220 or 254 nm. Solvent A: water with 0.1% formic acid; Solvent B: acetonitrile. Gradient: After 0.1 minutes at the initial condition of 90% A and 10% B solvent B was increased from 5 to 100% over 8 minutes, maintained at 100% for 2 minutes, then a linear gradient to initial conditions was applied for 0.5 minutes and maintained for 1.5 minutes to re-equilibrate the column (A: 95%, B: 5%), giving a cycle time of 12 minutes. Flow rate was 0.6 mL/min during the complete run.

Compound	Rt	Method	Purity (%)	HRMS
18a	2.53	1	95.66	673.2709
18b	2.44	1	95.04	687.2848
18c	2.40	1	95.08	616.2354
23a	2.24	2	100	673.2688
23b	2.19	1	97.42	687.2840
23c	2.14	1	98.19	616.2357
29a	3.20	1	96.35	537.2709
29b	3.41	1	95.08	543.1888
29c	3.01	1	98.57	515.2844
34c	2.42	1	99.09	515.2838
35a	3.16	1	95.15	543.2267
35b	3.03	1	95.92	521.2422
40a	2.95	1	97.25	543.2273
50a	2.82	1	100	685.2967
50b	2.84	1	95.28	697.3225
52a	2.82	1	95.99	619.2537
52b	5.05	2	95.38	555.2584
54	2.51	1	99.22	497.1834

LC traces for final compounds

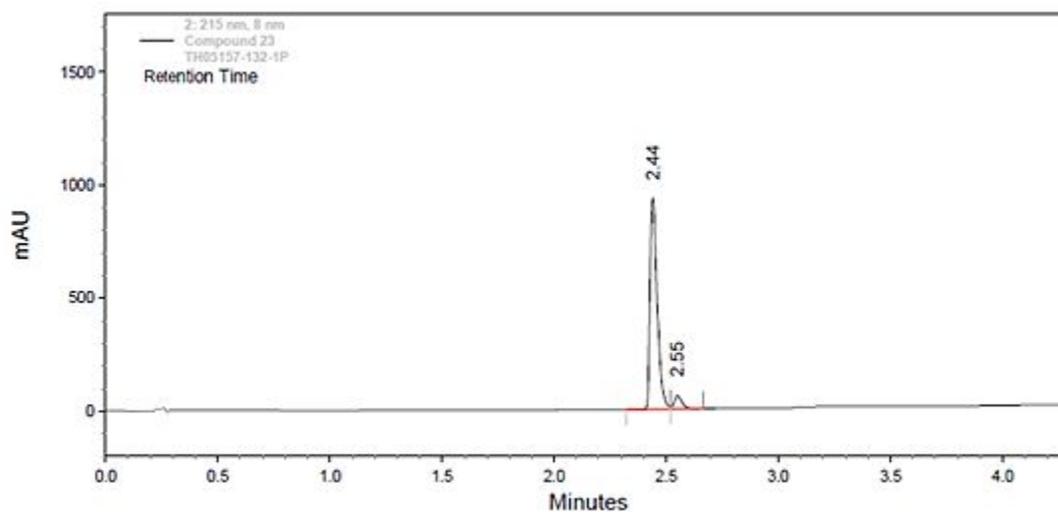
Compound 18a



λ : 220 nm, 8 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
1.82	2786	6317	0.28
2.36	748	1684	0.07
2.50	86751	51313	2.27
2.53	840506	2162376	95.66
2.66	11148	22919	1.01
3.07	1476	7783	0.34
3.50	1258	8194	0.36

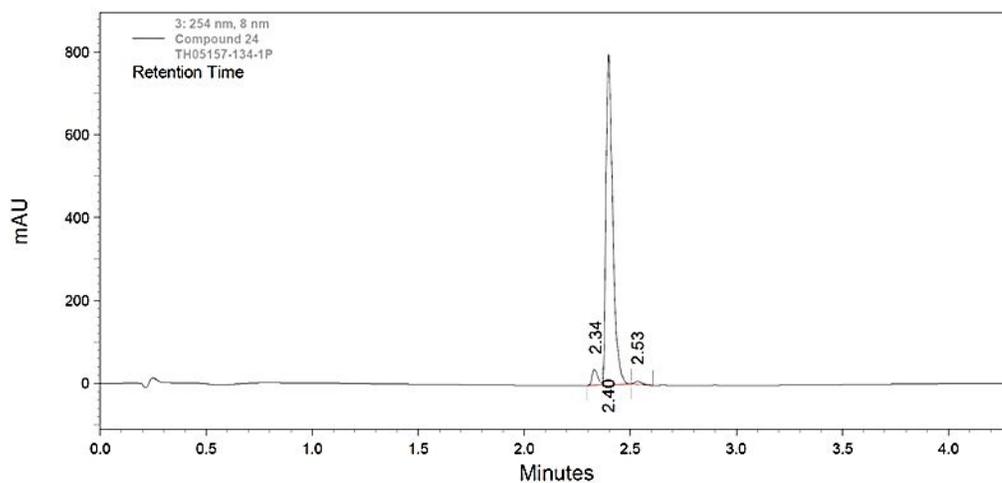
Compound 18b



λ : 215 nm, 8 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
2.44	903122	2278674	95.04
2.55	59073	118838	4.95

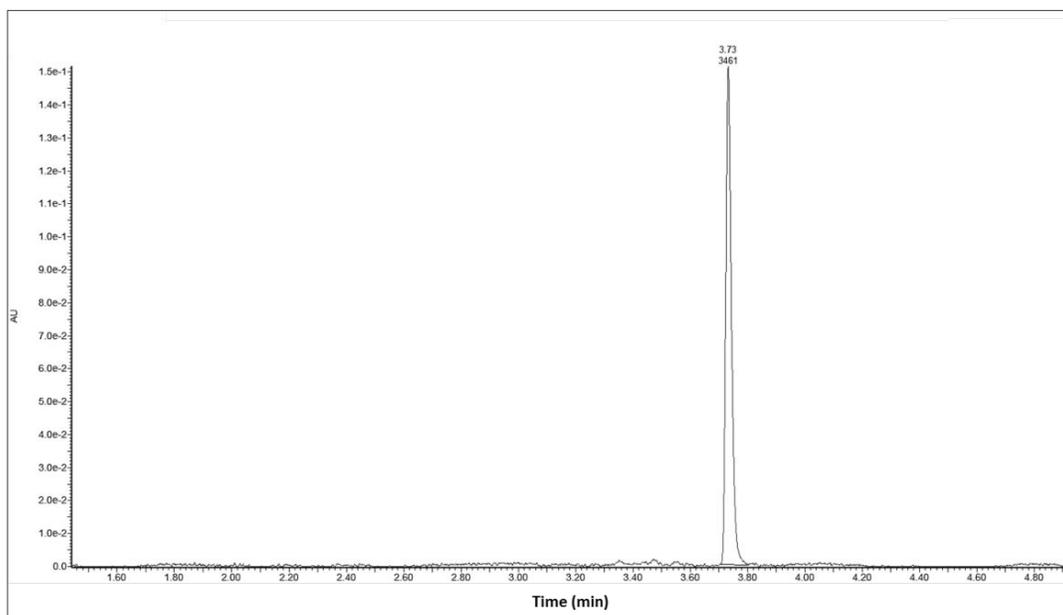
Compound 18c



λ : 254 nm, 8 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
2.34	36408	73064	3.99
2.40	786770	1740293	95.08
2.53	7331	16995	0.93

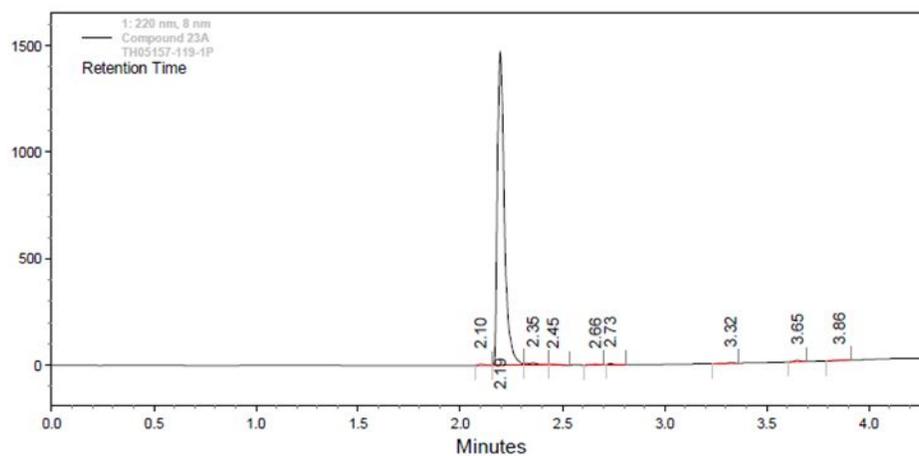
Compound 23a



λ : 220 nm, 1.2 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
3.73	151182	3460.95	100.00

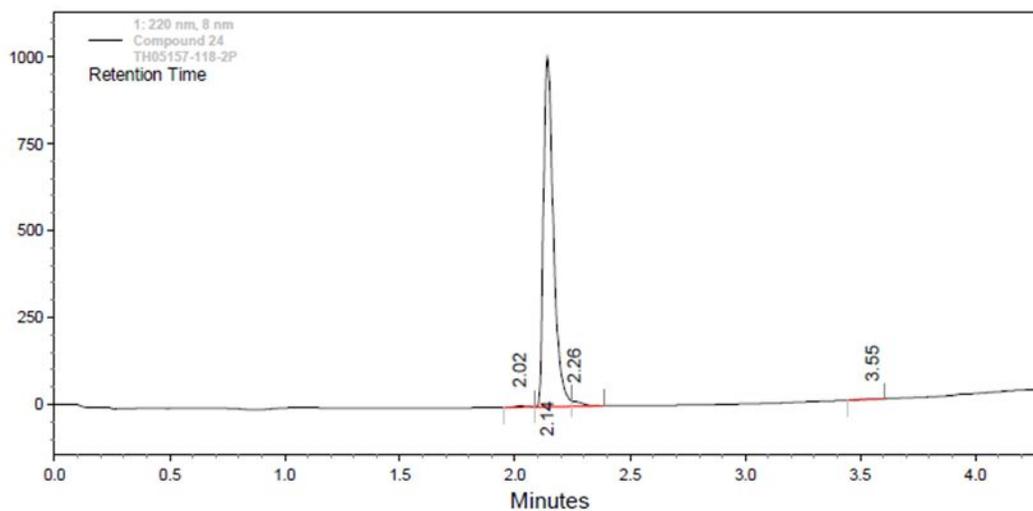
Compound 23b



λ : 220 nm, 8 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
2.10	6095	12897	0.36
2.19	1447070	3498163	97.42
2.35	9117	34288	0.95
2.45	2823	4800	0.13
2.66	3070	8032	0.22
2.73	5159	11444	0.32
3.32	2062	5144	0.14
3.65	5242	11397	0.32
3.86	1396	4651	0.13

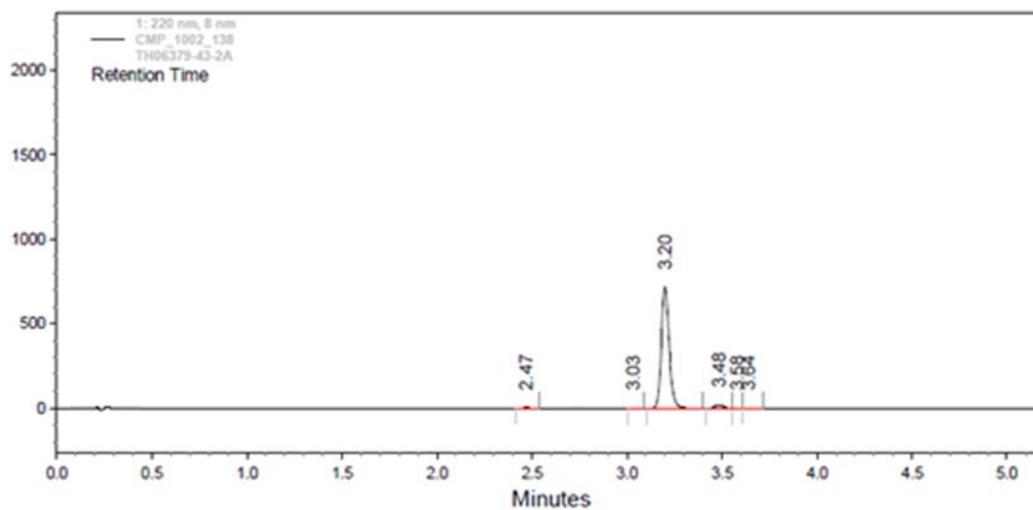
Compound 23c



λ : 220 nm, 8 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
2.02	3987	11332	0.37
2.14	927289	3028676	98.19
2.26	12953	40095	1.30
3.55	1178	4559	0.15

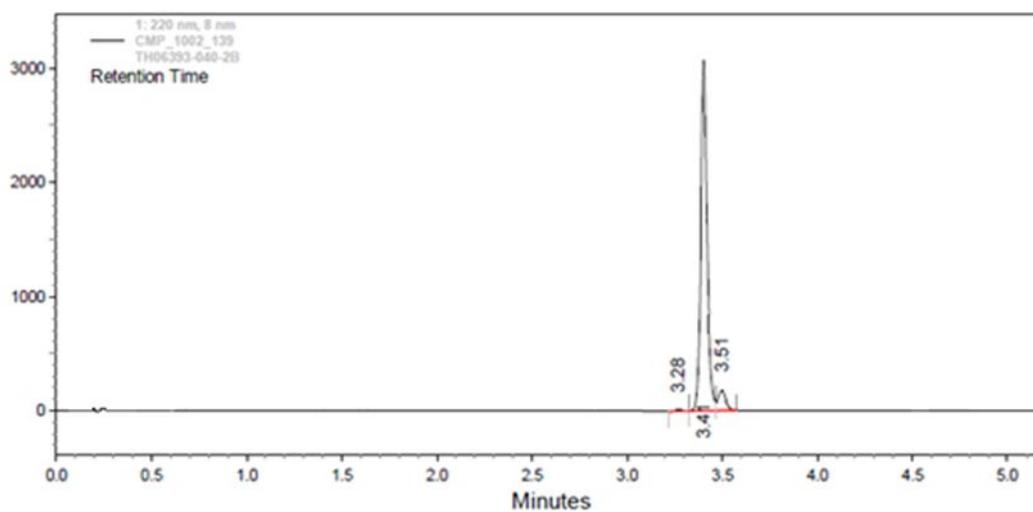
Compound 29a



λ : 220 nm, 8 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
2.47	3520	10695	0.50
3.03	1060	2418	0.11
3.20	720671	2051590	96.35
3.48	17193	52567	2.47
3.58	1051	3139	0.15
3.64	3256	8964	0.42

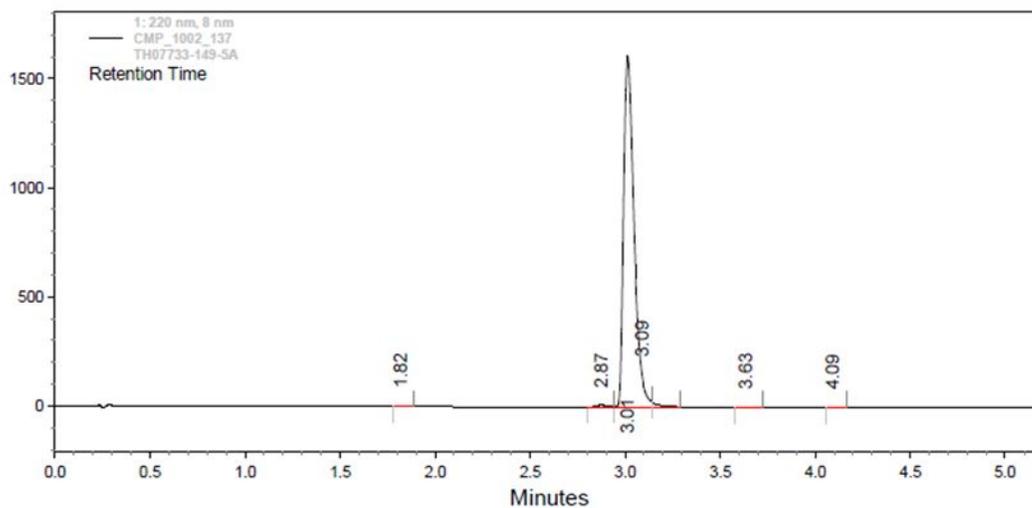
Compound 29b



λ : 220 nm, 8 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
3.28	9503	25056	0.32
3.41	3031252	7304141	95.08
3.51	176010	352161	4.58

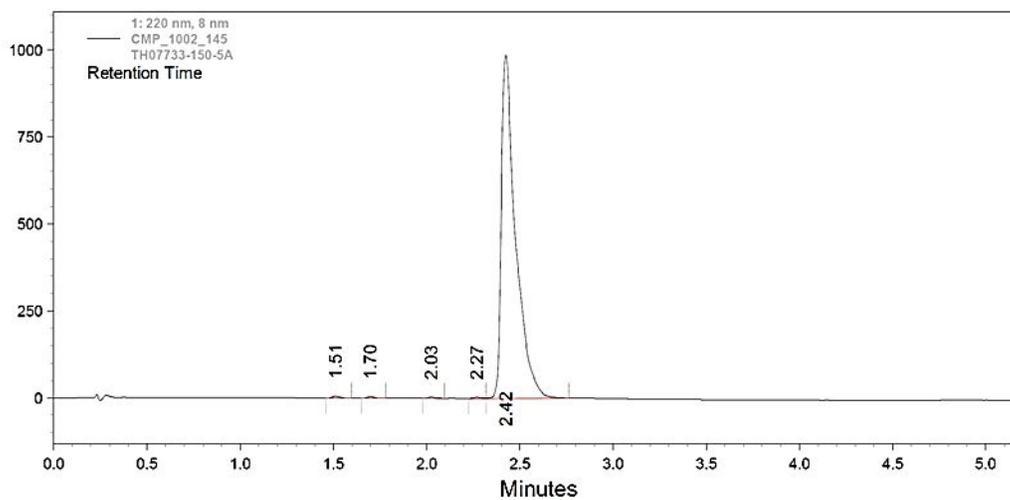
Compound 29c



λ : 220 nm, 8 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
1.82	1238	3513	0.06
2.87	7321	28739	0.46
3.01	1608796	6098006	98.57
3.09	132429	43058	0.70
3.63	2492	9999	0.16
4.09	1052	2963	0.05

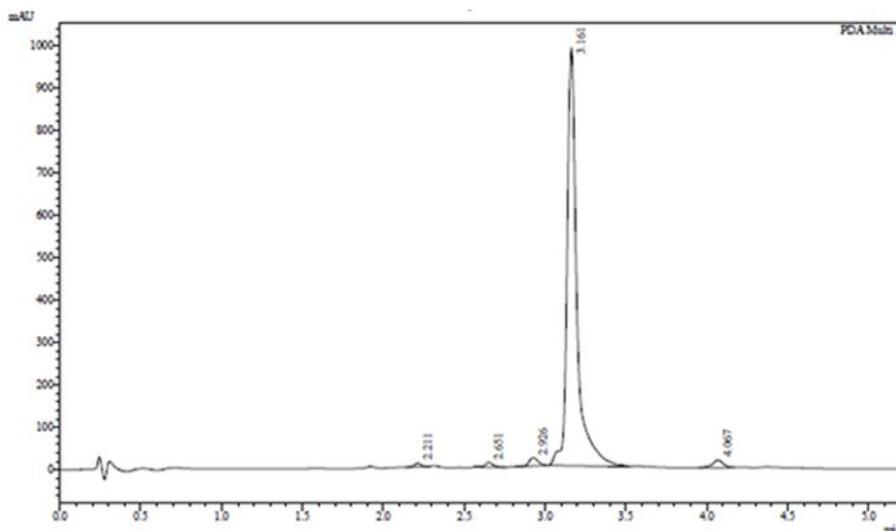
Compound 34c



λ : 220 nm, 8 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
1.51	5279	15595	0.29
1.70	4711	13328	0.25
2.03	3369	9362	0.18
2.27	3485	9862	0.19
2.42	986427	5261626	99.09

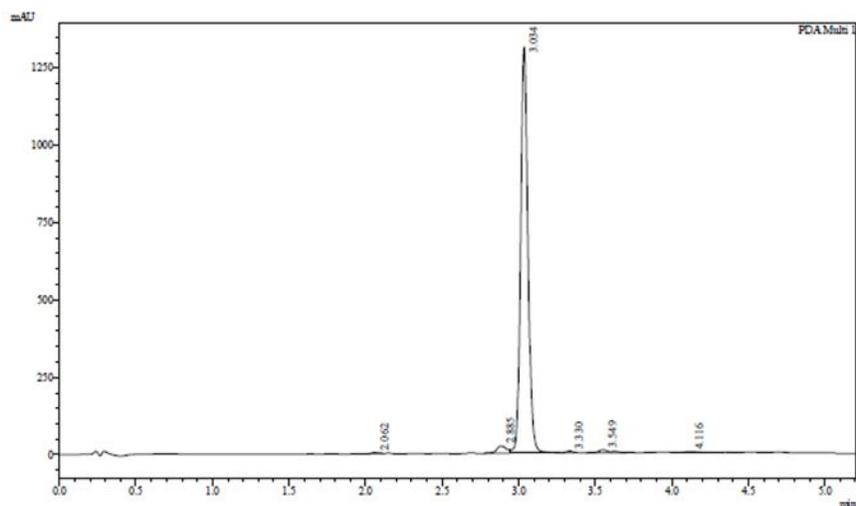
Compound 35a



λ : 254 nm, 4 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
2.211	8689	28463	0.549
2.651	10620	39503	0.800
2.926	18746	72058	1.642
3.161	937628	4139637	95.150
4.067	17525	81592	1.859

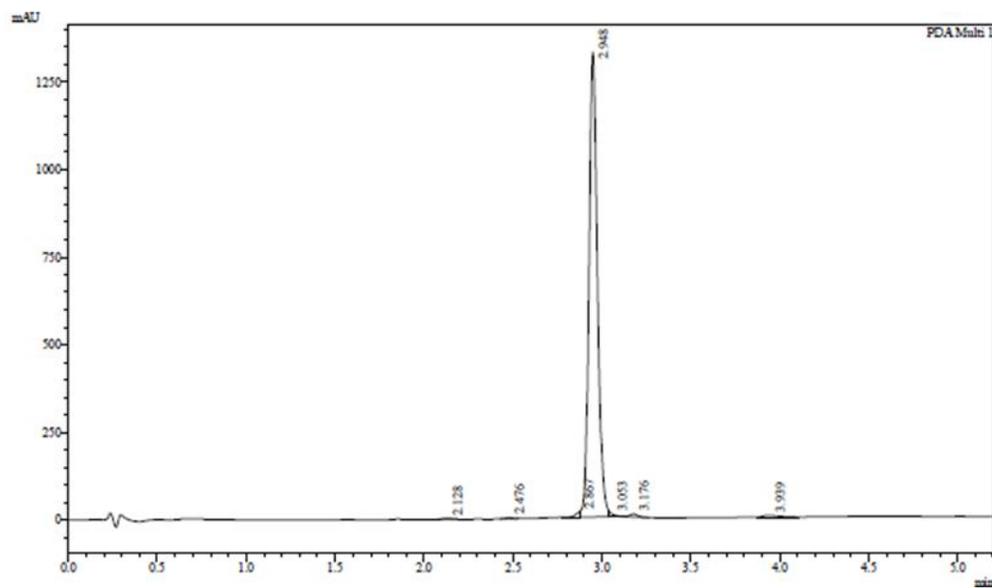
Compound 35b



λ : 220 nm, 4 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
2.062	2758	9254	0.202
2.885	23422	107147	2.343
3.034	1292909	4387160	95.922
3.330	4812	13040	0.285
3.549	9478	50117	1.096
4.116	1841	6964	0.152

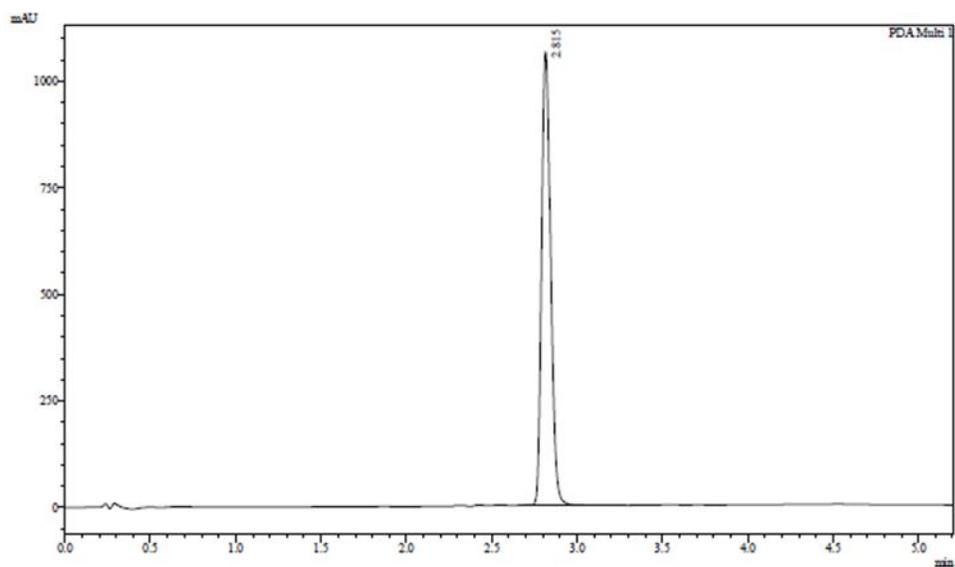
Compound 40a



λ : 220 nm, 4 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
2.128	2802	8696	0.192
2.476	2634	7324	0.162
2.867	10741	29620	0.653
2.948	1259232	4407749	97.245
3.053	11405	22193	0.490
3.176	7895	23978	0.525
3.939	5580	33258	0.734

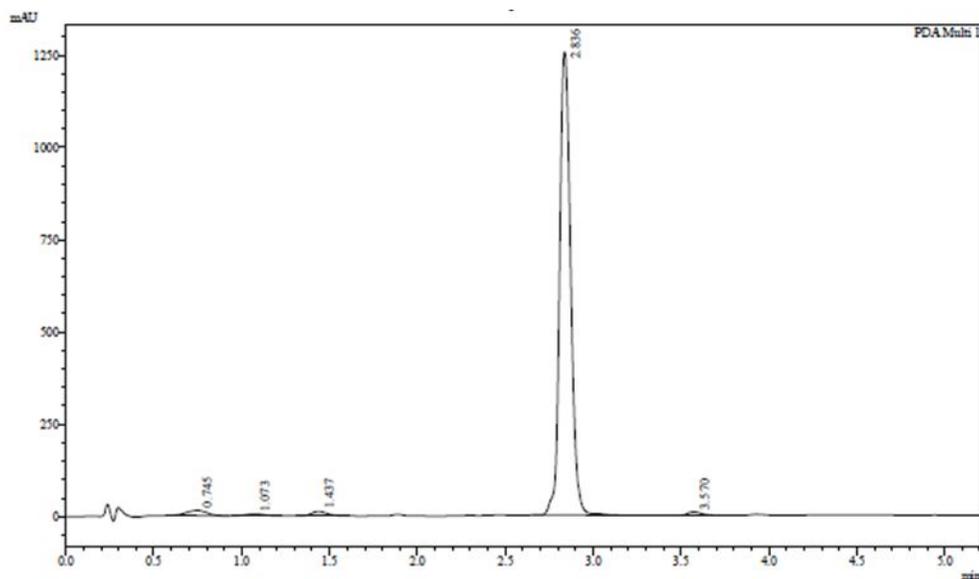
Compound 50a



λ : 220 nm, 4 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
2.81	1029276	3935599	100.00

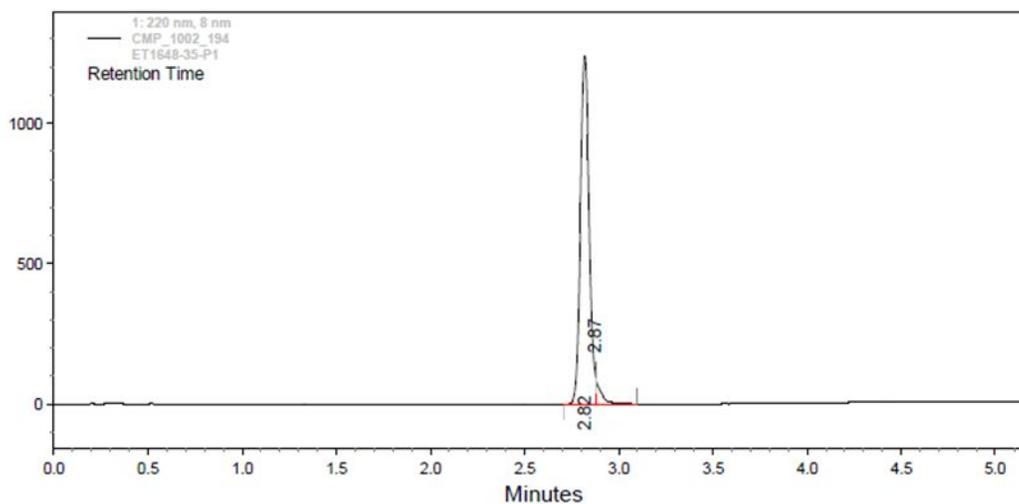
Compound 50b



λ : 220 nm, 4 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
0.745	14006	115329	2.021
1.073	5470	36042	0.632
1.437	11508	72328	1.268
2.836	1239091	5436582	95.283
3.570	8325	45410	0.796

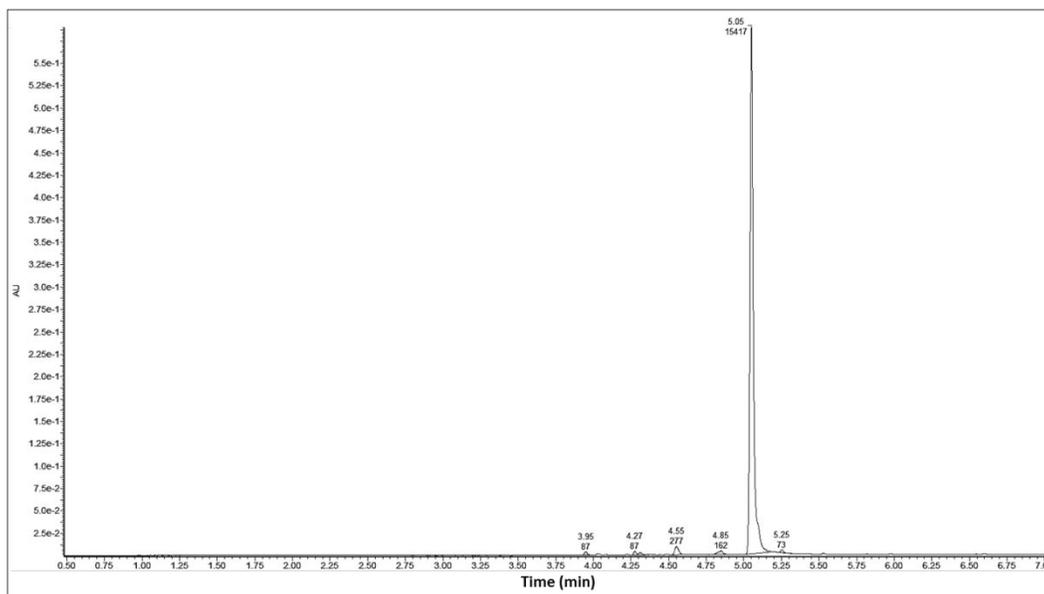
Compound 52a



λ : 220 nm, 8 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
2.82	1236493	4002073	95.99
2.87	105296	167061	4.01

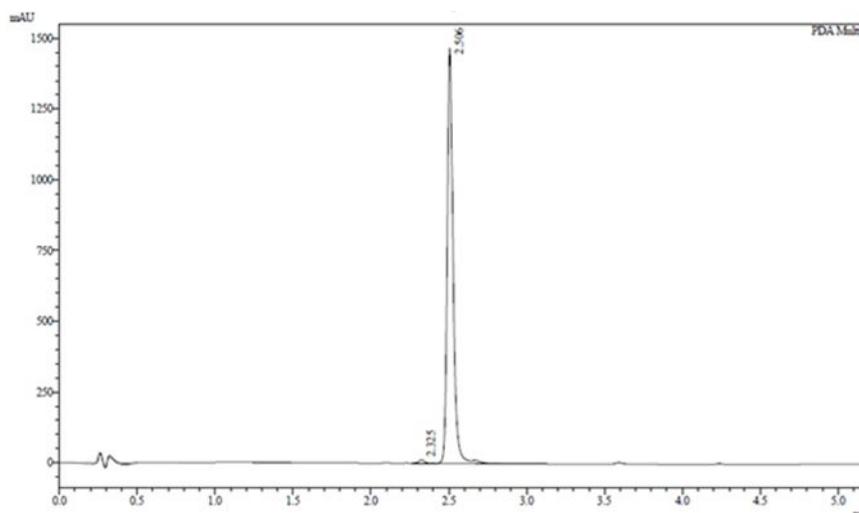
Compound 52b



λ : 254 nm, 1.2 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
3.95	3869	87.06	0.54
4.27	4066	87.18	0.54
4.31	2718	60.97	0.38
4.55	9326	276.94	1.71
4.85	3999	162.09	1.00
5.05	588937	15417.16	95.38
5.25	3382	73.00	0.45

Compound 54



λ : 220 nm, 4 nm

<i>Retention time</i>	<i>Height</i>	<i>Area</i>	<i>Area Percent</i>
2.325	11596	30116	0.781
2.506	1317163	3827437	99.219

Bioanalysis of compound 29a in plasma samples

Chromatographic separation was performed by gradient elution at 0.45 mL/min using a XSelect CSH C18 column (50 x 2.1 mm, 2.5 μ m particle size; Waters). The mobile phase consisted of A: water with 0.1% formic acid, B: methanol with 0.1% formic acid. The autosampler temperature was set at 10° C and column temperature at 40° C. For detection and quantification, the electrospray ionization operated in the positive mode was set up for multiple reaction monitoring (MRM). The collision gas used was ultra-pure argon at a flow rate of 0.15 mL min⁻¹.

Quantification was achieved by external calibration using matrix-matched standards. Concentrations were calculated using a weighted least-squares linear regression ($W = 1/x$). Calibration standards were prepared by adding the appropriate volume of diluted solutions of the compound (made in a mixture of methanol and water, 50:50, v:v) to aliquots of 10 μ L of blank plasma. The calibration standard and sample preparation is as follows: 200 μ L of 3% formic acid in methanol containing 50 nM tolbutamide, used as internal standard, were added to precipitate the proteins. The mixture was then agitated for 5 min and centrifuged at 13200 rpm for 10 min at 4° C. 180 μ L of the resulting supernatants were transferred to an Ostro plate (Waters, Manchester, UK), designed to remove phospholipids by solid phase extraction (SPE). The resulting eluents were evaporated at 37° C under a stream of nitrogen. Plasma sample residues were dissolved in 25 μ L of a mixture of methanol and water both with 0.1% formic acid (25:75, v:v). A 5 μ L aliquot of the resulting solution was injected into the LC-MS/MS system for analysis.

PK Parameters

Table 1. Compound **29a** plasmatic concentrations after intraperitoneal (i.p.) administration (20 mg/kg) (n= 5) (RSD: relative standard deviation)

Time (h)	Mean (nM)	SD (nM)	RSD (%)
0.25	934.4	133.6	14.3
0.75	476.5	159.4	33.4
2	240.5	96.9	40.3
5	120.1	43.7	36.3
8	27.6	11.1	40.0
24	22.7	8.9	39.3

Table 2. Compound **29a** pharmacokinetic parameters estimated by fitting the experimental data to a non-compartmental model (NCA) using Winnonlin software for pharmacokinetic analysis. The parameters are: Area Under the Curve computed to the last observation (AUClast); half-life of the product ($t_{1/2}$); clearance (Cl); volume of distribution (Vz). (n=5) (RSD: relative standard deviation)

Parameter	Mean	SD	%RSD
AUClast (nM·h)	1901.78	266.6	14.0
$t_{1/2}$ (h)	7.50	1.1	15.0
Cl/F (L/h) ^a	0.46	0.1	11.6
Vz/F (L) ^a	4.97	1.2	24.0
Plasma conc. at Tmax (nM)	1447.6	128.3	8.9
Brain conc. at Tmax (nmol/kg)	153.2	12.9	8.4
LogBB ^b	-0.98	0.01	1.15
Norm. Vz/F (L/kg)	199.0		
Norm. Cl/F (L/h·kg)	18.2		

^a For extravascular models (IP), the fraction of dose absorbed cannot be estimated and therefore, Volume and Clearance for these models are Volume/F and Clearance/F, where F is the fraction absorbed. Volume of distribution is the volume of distribution based on the terminal phase for these models (Vz).

^b Determined at Tmax.

Figure S1.

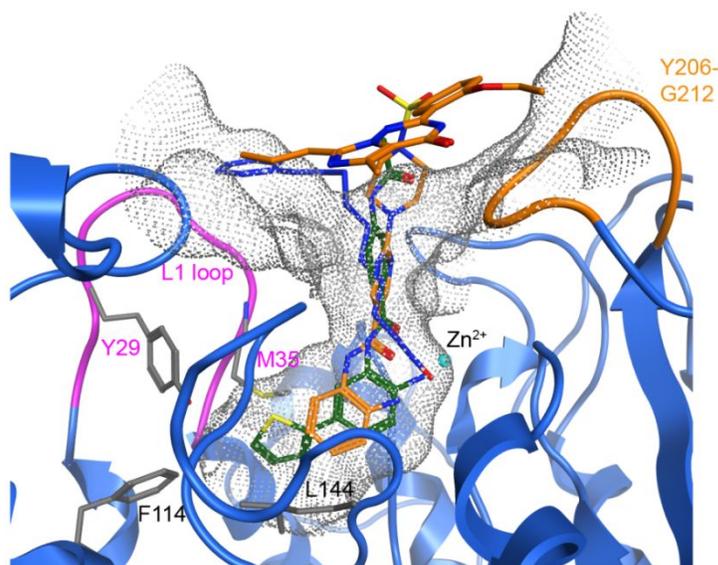


Figure S1. Comparison of the binding modes of compound **23a** (*orange*, predicted by docking), **4** (SAHA, *blue*, as superposed from PDB entry 4LXZ)[1] and 4-acetamido-N-[2-amino-5-(2-thienyl)phenyl]benzamide (*green*, as superimposed from PDB entry 4LY1)[1] into HDAC2. The capping group of **4** orients toward the L1 loop, while the predicted binding mode of **23a** orients the capping group towards the loop comprising residues Tyr206-Gly212. The benzamide inhibitor of PDB entry 4LY1 corresponds to the benzamide-based inhibitor with the largest “capping” group currently crystallized against any class I HDAC and serves as a reference to assess the prediction of the positioning of the capping group of **23a**.

- [1] B.E.L. Lauffer, R. Mintzer, R. Fong, S. Mukund, C. Tam, I. Zilberleyb, B. Flicke, A. Ritscher, G. Fedorowicz, R. Vallero, D.F. Ortwine, J. Gunzner, Z. Modrusan, L. Neumann, C.M. Koth, P.J. Lupardus, J.S. Kaminker, C.E. Heise, P. Steiner, Histone deacetylase (HDAC) inhibitor kinetic rate constants correlate with cellular histone acetylation but not transcription and cell viability., *J. Biol. Chem.* 288 (2013) 26926–43. doi:10.1074/jbc.M113.490706.

Table S1. Impact of incubation times, for reference compound **6**, on functional response (AcH3K9) at different concentrations.

Incubation Time	<i>50 nM</i>	<i>100 nM</i>	<i>500 nM</i>	<i>1 μM</i>
2 h	<i>0.93</i>	<i>1.17</i>	<i>1.05</i>	<i>0.8</i>
24 h	<i>1.464</i>	<i>2.9</i>	<i>3.14</i>	<i>5.1</i>

Effect of **6** on AcH3K9 levels (fold-change over basal) using SHSY-5Y cell line at different concentrations and incubation times.

Table S2. Dose-response study for compound **29a** *versus* its functional response on pCREB.

Concentration	100 nM	500 nM	1 μM
<i>4h</i>	<i>1.47</i>	<i>2.8</i>	<i>5.11</i>

Effect of **29a** on pCREB levels (fold-change over basal) using SHSY-5Y cell line (incubation time is 4 hours)