# *Novel activity-based probes for N-acylethanolamine acid amidase*

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## Supporting Information

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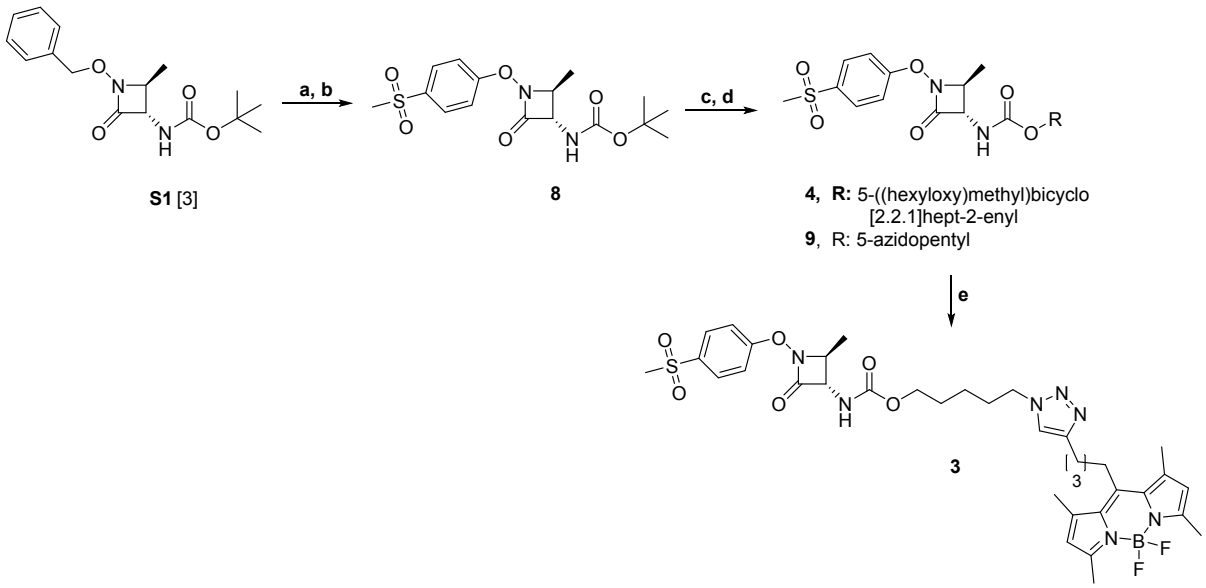
## Experimental section - Chemistry

### Chemicals, materials and methods.

All the commercial available reagents and solvents were used as purchased from vendors without further purification. Dry solvents (THF, $Et_2O$, $CH_2Cl_2$, DMF, DMSO, MeOH) were purchased from Sigma-Aldrich. Automated column chromatography purifications were performed using a Teledyne ISCO apparatus (CombiFlash® Rf) with pre-packed silica gel columns of different sizes (from 4 g up to 120 g). Mixtures of increasing polarity of cyclohexane (Cy) and ethyl acetate (EtOAc) or cyclohexane and methyl *tert*-butyl ether (MTBE) were used as eluents. NMR experiments were run on a Bruker Avance III 400 system (400.13 MHz for $^1H$, and 100.62 MHz for $^{13}C$), equipped with a BBI probe and Z-gradients. Spectra were acquired at 300 K, using deuterated dimethylsulfoxide (DMSO-$d_6$) or chloroform ($CDCl_3$) as solvents. UPLC/MS analyses were run on a Waters ACQUITY UPLC/MS system consisting of a SQD (single quadrupole detector) mass spectrometer equipped with an electrospray ionization interface and a photodiode array detector. The PDA range was 210-400 nm. Analyses were performed on an ACQUITY UPLC BEH $C_{18}$ column (100 x 2.1mmID, particle size 1.7 mm) with a VanGuard BEH $C_{18}$ precolumn (5 x 2.1 mmID, particle size 1.7 mm). Mobile phase was 10 mM $NH_4OAc$ in $H_2O$ at pH 5 adjusted with $CH_3COOH$ (A) and 10 mM $NH_4OAc$ in $CH_3CN$-$H_2O$ (95:5) at pH 5.0. Electrospray ionization in positive and negative mode was applied. Purifications by preparative HPLC/MS were run on a Waters Autopurification system consisting of a 3100 single quadrupole mass spectrometer equipped with an Electrospray Ionization interface and a 2998 Photodiode Array Detector. HPLC system included a 2747 sample manager, 2545 binary gradient module, system fluidic organizer and 515 HPLC pump. PDA range was 210-400 nm. Purifications were performed on a XBridge™ Prep $C_{18}$ OBD column (100 x 19 mmID, particle size 5 mm) with a XBridge™Prep $C_{18}$ (10 x 19 mmID, particle size 5 mm) guard cartridge. Mobile phase was 10 mM $NH_4OAc$ in MeCN-$H_2O$ (95:5) at pH 5. Electrospray ionization in positive and negative mode was used. Optical rotations were measured on a Propol automatic polarimeter using a sodium lamp (589 nm) as the light source, using $CHCl_3$ as a solvent and a 1 dm cell.

### Synthesis of Activity Based Probes (3-4) and inhibitors of NAAA (1-2 and 5-7).

Compounds **1** (CC-ABP)[1], **2** (ARN726)[2], **5**[3], **6**[3] and **7**[4] were synthesized according to described procedures and their spectroscopic data are in agreement with those previously reported.




**Scheme S1.** Reagents and conditions: a) Cyclohexene, 10 % palladium on activated carbon (Pd/C), EtOH, rt, 2h; b) 4-methylsulphonylphenyl-$B(OH)_2$, CuCl, pyridine, dry DCM, rt, 3h, 68%; c) *p*-toluensulfonic acid, TFA, rt, 15 min; d) 5-azido pentyl chloroformate (**S2**) or 5-((hexyloxy)methyl)biciclo [2.2.1]hept-2-enyl chloroformate (**S7**), DIPEA, dry DCM, rt, 15h, 57% for **4**, 60% for **9**; e) BODIPY alkyne, $CuSO_4.5H_2O$, sodium ascorbate, DMF, room temperature, 15h, 38%.

**BODIPY-ABP. 5-(4-(4-(5,5-difluoro-1,3,7,9-tetramethyl-5*H*-4l4,5l4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)butyl)-1*H*-1,2,3-triazol-1-yl)pentyl ((2*S*,3*S*)-2-methyl-1-(4-(methylsulfonyl)phenoxy)-4-oxoazetidin-3-yl)carbamate (3)**

Compound **S1** was synthesized according to a previously reported procedure.[3]

*Step 1. tert-butyl ((2S,3S)-2-methyl-1-(4-(methylsulfonyl)phenoxy)-4-oxoazetidin-3-yl)carbamate (**8**)*

A solution of benzyl derivative **S1** (0.837 g, 2.85 mmol) in absolute EtOH (40.0 mL) was submitted to ten cycles of vacuum and argon. Subsequently cyclohexadiene (2.69 mL, 28.5 mmol) and palladium on activated charcoal (0.837 g) were added to the solution and three more cycles of vacuum and argon were performed. The reaction was left to stir under argon atmosphere for 2 hours at room temperature. The solution was then further diluted with EtOAc, filtered over celite and evaporated to dryness. The resulting white powder was added to a round bottom flask, previously charged with freshly activated 4 Å molecular sieves (0.3 g), copper chloride (0.383 g, 2.85 mmol) and (4-methylsulfonylphenyl)boronic acid (1.14 g, 5.7 mmol) in dry $CH_2Cl_2$ (10.0 mL). The suspension was stirred 20 min at room temperature. Subsequently, pyridine (0.252 mL, 3.13 mmol) was added dropwise and immediately the solution turned into a brown coloration. The mixture was left to stir at open air 3 hours at room

temperature. The brown solution was diluted with $CH_2Cl_2$, filtered over a pad of silica and washed with 1N HCl aqueous solution; the organic phase was washed with brine, dried over $Na_2SO_4$ filtered and evaporated to dryness. The crude compound was absorbed over silica and purified by flash chromatographic column using a mixture of cyclohexane and EtOAc (from 100:0 to 70:30). The desired compound was obtained as a white solid (0.718 g, 68%). MS (ESI) m/z: 371.2 $[M-H]^+$, 388.3 $[M-NH_4]^+$. $^1$H NMR (DMSO-$d_6$, 400 MHz): $\delta$ 7.93 (d, $J$ = 8.9 Hz, 2H), 7.81 (d, $J$ = 8.2 Hz, 1H), 7.51 (d, $J$ = 8.9 Hz, 2H), 4.36 (d, $J$ = 8.2 Hz, 1H), 4.10 (dd, $J$ = 6.1, 2.0 Hz, 1H), 3.21 (s, 3H), 1.42 (s, 9H), 1.38 (d, $J$ = 6.1 Hz, 3H) ppm). $^{13}$C NMR (DMSO-$d_6$, 100 MHz): $\delta$ 163.8, 158.3, 154.1, 153.0, 129.9, 114.5, 61.6, 60.1, 42.5, 28.3, 15.8 ppm.

*General procedure (G1) for chloroformate preparation.*

To a stirred solution of triphosgene (0.4 equiv) in toluene (5 mL/mmol), pyridine (1.25 equiv) was added. After stirring for 1 h at 0 °C under argon, the alcohol was added dropwise. The mixture was stirred for 17 h at room temperature before being filtrated. The precipitate was rinsed twice with toluene and concentration of the filtrate under vacuum afforded crude chloroformate, which was directly used in the next step.

*Step 2. 5-azidopentyl N-[(2S,3S)-2-methyl-1-(4-methylsulfonylphenoxy)-4-oxo-azetidin-3-yl]carbamate (**9**)*

5-Azido 1-pentanol was synthesized according to described procedure[5] and its spectroscopic data are in agreement with those previously reported.

*a) Pentyl azido chloroformate (**S2**).*

**S2** was obtained following the general procedure G1, employing pentyl azido alcohol (1.0 g, 7.74 mmol), triphosgene (0.918 g, 3.09 mmol), pyridine (0.778 mL, 9.67 mmol). The compound was used immediately after preparation. $^1$H-NMR ($CDCl_3$, 400 MHz,): $\delta$ 4.33 (t, $J$ = 7.6 Hz, 2H), 3,31 (t, $J$ = 7.6 Hz, 2H), 1.79 (q, $J$ = 7.6 Hz, 2H), 1.63 (q, $J$ = 7.6 Hz, 2H), 1.50 (q, $J$ = 7.6 Hz, 2H). $^{13}$C-NMR ($CDCl_3$, 100 MHz): $\delta$ 152.1, 71.9, 51.3, 28.5, 28.1, 23.0 ppm.

*b) Tosylate salt preparation and coupling with pentyl azido chloroformate.*

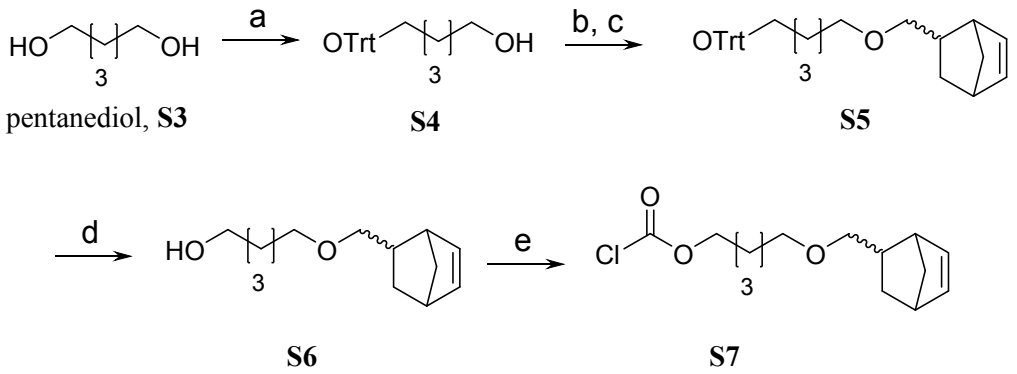
Compound **8** (0.200 g, 0.54 mmol) was mixed, in a heart shaped flask purged with nitrogen, with *p*-toluensulfonic acid (0.098 g, 0.57 mmol) and cooled to 0° C. Subsequently trifluoroacetic acid (1.89 mL) was added dropwise over 5 minutes and the reaction was stirred at 0 °C for 15 minutes. The solution was rotary evaporated maintaining the bath below 30 °C and the obtained oil was left under high vacuum for 1 hour. $Et_2O$ (10.0 mL) was added to the obtained crude and a white precipitate was observed. After washing with $Et_2O$ (5 x 10 mL) the

desired tosylate salt was recovered after filtration as white solid and re-suspended in dry $CH_2Cl_2$ (5.0 mL). Subsequently DIPEA (0.111 mL, 0.64 mmol) was added, followed by the pentyl azido chloroformate **S2** (0.309 g, 1.62 mmol). The reaction was left to stir under nitrogen atmosphere at room temperature for 15 h. The solvent was evaporated and the crude product was absorbed over silica gel and purified by standard column chromatography, eluting with pentane/EtOAc (from 100:0 to 50:50) to afford the titled compound **9** (0.137 g, 60%), as colorless oil. MS (ESI) m/z: 426.2 $[M-H]^+$, 443.3 $[M-NH_4]^+$, 424.2 $[M-H]^-$. $[\alpha]_{D20}$ ($c$ = 20, $CHCl_3$): -0.058. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 7.93 (d, $J$ = 8.6 Hz, 2H), 7.34 (d, $J$ = 8.6 Hz, 2H), 5.39 (s, 1H), 4.17-4.39 (m, 1H), 4.12 (t, $J$ = 7.2 Hz, 2H), 3.29 (t, $J$ = 6.6 Hz, 2H), 3.03 (s, 3H), 1.78 – 1.57 (m, 4H), 1.52 (d, $J$ = 5.9 Hz, 3H), 1.53 – 1.34 (m, 2H). $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ 162.6, 155.7, 135.9, 130.2, 114.5, 65.7, 62.8, 61.1, 51.4, 44.9, 28.6. 28.5, 23.2, 16.4 ppm.

*Step 3. Synthesis of BODIPY-ABP **3**.*

To a solution of **9** (0.012 g, 0.028 mmol) and BODIPY alkyne [5] (0.009 g, 0.028 mmol) in DMF (1.0 mL), sodium ascorbate (0.008 g, 0.042 mmol, 1M sol.) and $CuSO_4 \cdot 5H_2O$ (0.0014 g, 0.0056 mmol, 200 mM sol.) were subsequently added. The solution was stirred at room temperature for 15 h. The crude was evaporated and purified by HPLC (Solvent A: $H_2O$/MeCN 95:5 + 0.1% TFA. Solvent B MeCN. Flow rate 5 mL/min. Gradient 30% B till 90% B) affording the title compound as clean product (0.008 g, 38%). MS (ESI) m/z: 754.0 $[M-H]^+$. $[\alpha]_{D20}$ ($c$ = 0.2, $CHCl_3$): -8.0. $^1$H NMR (DMSO-$d_6$, 400 MHz): $\delta$ 8.06 (d, $J$ = 8.1 Hz, 1H), 7.94 (d, $J$ = 8.8 Hz, 2H), 7.86 (s, 1H), 7.51 (d, $J$ = 8.8 Hz, 2H), 6.23 (s, 2H), 4.40 (dd, $J$ = 8.1, 2.2 Hz, 1H), 4.30 (t, $J$ = 7.0 Hz, 2H), 4.14 (d, $J$ = 5.1 Hz, 1H), 3.99 (t, $J$ = 6.4 Hz, 2H), 3.17 (s, 3H), 3.04 – 2.86 (m, 2H), 2.75 – 2.63 (m, 2H), 2.40 (s, 6H), 2.38 (s, 6H), 1.90 – 1.73 (m, 4H), 1.67 – 1.54 (m, 4H), 1.39 (d, $J$ = 5.1 Hz, 3H), 1.33-1.21 (m, 2H). $^{13}$C NMR (DMSO-$d_6$, 100 MHz): $\delta$ 164.3, 162.1, 155.7, 153.1, 146.7, 146.4, 140.8, 135.9, 131.6, 129.8, 129.7, 121.3, 113.7, 64.1, 61.35, 60.01, 49.0, 48.6, 43.0, 30.7, 29.5, 27.9, 27.3, 24.5, 22.3, 15.8, 15.5, 14.1 ppm.

***5-(bicyclo[2.2.1]hept-5-en-2-ylmethoxy)pentyl ((2S,3S)-2-methyl-1-(4-(methylsulfonyl)phenoxy)-4-oxoazetidin-3-yl)carbamate (4)***




pentanediol, **S3** **S4** **S5**

**S6** **S7**

**Scheme S2.** Reagents and conditions: a) Trityl Chloride, DMAP, $Et_3N$, dry DCM, room temperature, 5 h; b) Mesyl chloride, $Et_3N$, dry DCM, room temperature, 20 h; c) Norbornene methanol, NaH, dry THF, 0 °C to room temperature, 15 h; d) *p*-toluensulphonic acid, DCM/MeOH 1:1, room temperature, 3 h; e) triphosgene, pyridine, dry toluene, 1 h at 0 °C then room temperature for 15 h.

*Step 1. Synthesis of 5-(2-Bicyclo[2.2.1]hept-5-enylmethoxy)pentyl carbonochloridate (**S7**)*

Intermediate **S4** was synthesized following previous reported procedure and its spectroscopic data are comparable to the literature.[6]

*a) (1S,4S)-5-(((5-(Trityloxy)pentyl)oxy)methyl)Bicyclo[2.2.1]hept-2-ene (**S5**)*

To a cooled (0 °C) solution of monotritylated pentane-1,5-diol **S4** (0.817 g, 2.36 mmol) in dry pyridine (10.6 mL), methanesulfonyl chloride (0.365 mL, 4.72 mmol) was added dropwise over 10 min. The reaction was stirred under nitrogen atmosphere for 3 h. The mixture was poured into 20 mL of iced water; the solution was then acidified with 2 N HCl until reaching pH 4. The aqueous phase was extracted with EtOAc (20.0 mL) and the collected organic layers were dried over $Na_2SO_4$, filtered and evaporated to dryness.

To a stirred solution of NaH (0.190 g, 4.75 mmol) in THF, at 0 °C, under argon atmosphere, was added 5-norbornene-2-methanol (570 µL, 4.71 mmol) and the reaction mixture was stirred for 30 min. the mesylate crude of the monotritylated pentane-1,5-diol was then carefully added, and the resulting mixture was stirred overnight at room temperature. A $NH_4Cl$ saturated solution (10 mL) and EtOAc (10 mL) were then added and the aqueous phase was extracted with EtOAc (2 x 10 mL). The combined organic phases were washed with brine (30 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The crude was purified using standard column chromatography (pentane/EtOAc, 90:10) to afford the desired compound as a colorless oil (0.613 g, 57%). MS (ESI) m/z: 453.3 $[M-H]^+$. $^1$H-NMR ($CDCl_3$, 400 MHz): $\delta$ 7.49-7.39 (m, 12H), 7.35-7.11 (m, 18H), 6.17-6.01 (m, 2H), 5.92 (dd, $J$ = 5.8, 2.9 Hz, 1H), 3.54-3.23 (m, 5H), 3.17-2.94 (m, 6H), 2.90-2.88 (m, 1H), 2.81-2.72 (m, 2H), 2.40-2.23 (m, 1H), 1.81 (ddd, $J$ = 11.5, 9.2, 3.8 Hz, 1H), 1.72-1.38 (m, 15H), 1.34-1.19 (m, 12H), 1.09 (dt, $J$ = 11.6, 3.9 Hz, 2H), 0.92-0.81 (m, 4H), 0.48 (ddd, $J$ = 11.6, 4.5, 2.6 Hz, 1H) ppm. $^{13}$C-NMR ($CDCl_3$,

100MHz): $\delta$ 144.6, 137.2, 136.8, 136.7, 132.6, 128.8, 127.8, 126.9, 86.4, 75.7, 74.7, 71.1, 71.0, 63.7, 49.5, 45.1, 45.1, 44.1, 43.08, 42.3, 41.7, 39.0, 38.9, 30.0, 29.9, 29.7, 29.3, 23.1 ppm.

*b) 5-((1S,4S)-Bicyclo[2.2.1]hep-5-e-2-ylmethoxy)pentan-1-ol (**S6**)*

To a dry solution of the tritylated compound **S5** (0.613 g, 1-35 mmol) in DCM/MeOH (1:1, 0.1 M) was added *p*-TsOH (0.080 g, 0.42 mmol) and the resulting mixture was stirred at room temperature, under argon, for 2 h. It was then quenched with $Et_3N$ and evaporated under vacuum. The crude was then purified using standard column chromatography (pentane/EtOAc, from 90:10 to 80:20) to yield the desired alcohol as a colorless oil (0.196 g, 69%). MS (ESI) m/z: 209.6 $[M-H]^-$. $^1$H-NMR ($CDCl_3$, 400 MHz): $\delta$ 6.14-5.97 (m, 2H), 5.90 (dd, $J$ = 5.8, 2.9 Hz, 1H), 3.63 (t, $J$ = 6.5 Hz, 4H), 3.50-3.20 (m, 5H), 3.11 (dd, $J$ = 9.3, 6.59 Hz, 1H), 2.99 (t, $J$ = 9.1 Hz, 1H), 2.90-2.83 (m, 1H), 2.81-2.65 (m, 2H), 2.36-2.22 (m, 1H), 1.79 (ddd, $J$ = 11.6, 9.2, 3.9 Hz, 3H), 1.66-1.46 (m, 7H), 1.43-1.38 (m, 4H), 1.31-1.15 (m, 3H), 1.07 (dt, $J$ = 11.6, 3.9 Hz, 1H), 0.46 (ddd, $J$ = 11.6, 4.5, 2.6 Hz, 1H) ppm. $^{13}$C-NMR ($CDCl_3$, 100MHz): $\delta$ 137.2, 136.7, 132.5, 75.7, 74.7, 71.1, 71.0, 62.9, 49.5, 45.1, 44.1, 43.8, 41.6, 38.9, 38.8, 32.6, 29.8, 29.5, 29.4, 29.3, 22.5 ppm.

*c) Synthesis of 5-(2-Bicyclo[2.2.1]hept-5-enylmethoxy)pentyl carbonochloridate (**S7**)*

**S7** was obtained following the general procedure G1, employing 5-((1*S*,4*S*)-Bicyclo[2.2.1]hep-5-e-2-ylmethoxy)pentan-1-ol **S6** (0.150 g, 0.71 mmol), triphosgene (0.083 g, 0.28 mmol), pyridine (0.0718 mL, 0.89 mmol). The compound was used immediately after preparation. $^1$H-NMR ($CDCl_3$, 400 MHz): $\delta$ 6.14-5.94 (m, 2H), 5.93-5.76 (m, 1H), 4.38-4.14 (m, 3H), 3.50-3.19 (m, 4H), 3.08 (t, $J$ = 7.8 Hz, 1H), 2.95 (td, $J$ = 9.2, 2.1 Hz, 1H), 2.84 (s, 1H), 2.74-2.68 (m, 3H), 2.39-2.09 (m, 1H), 1.85-1.30 (m, 12H), 1.28-1.10 (m, 3H), 1.12-0.90 (m, 1H), 0.43 (dt, $J$ = 11.7, 2.7 Hz, 1H). $^{13}$C-NMR ($CDCl_3$, 100 MHz): $\delta$ 150.6, 145.7, 141.1, 137.1, 136.6, 132.4, 127.2, 75.6, 74.6, 72.3, 70.4, 45.0, 44.0, 43.7, 42.2, 41.5, 38.8, 38.7, 29.7, 29.1, 28.1, 22.4 ppm.

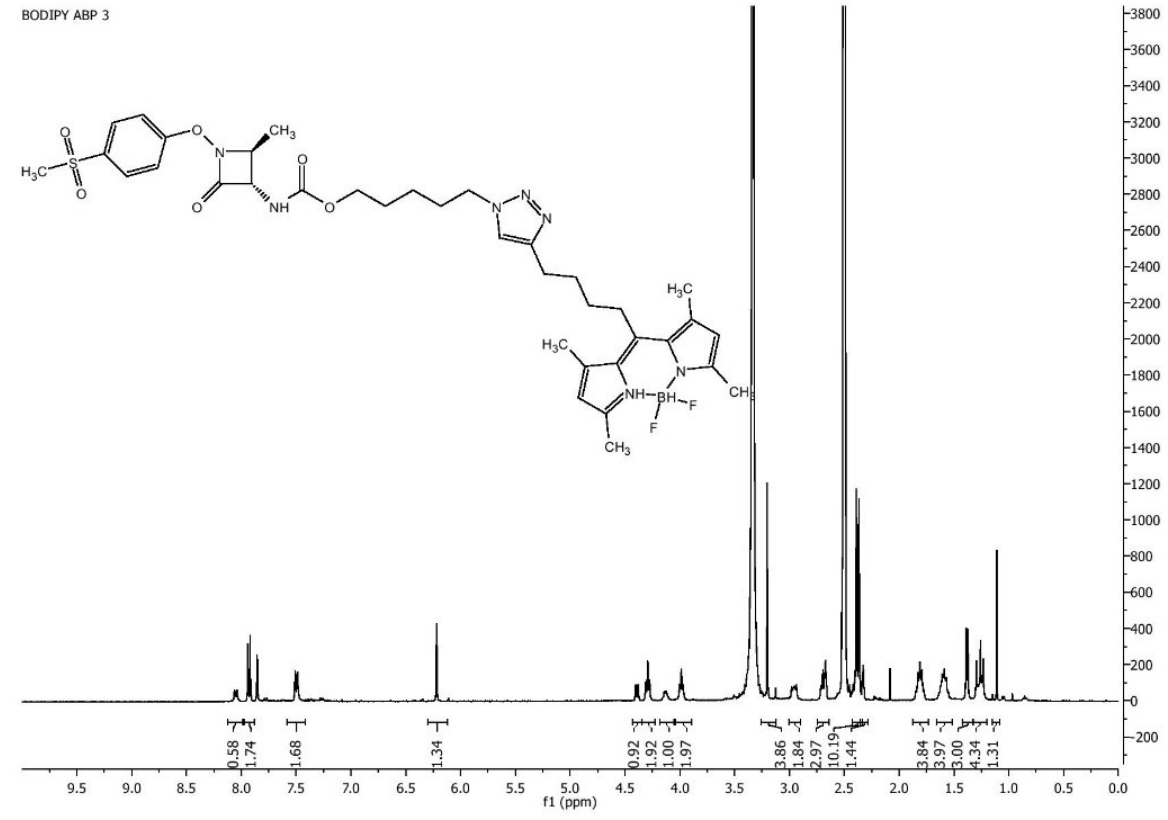
*Step 2. 5-(Norbornan-2-ylmethoxy)pentyl N-[(2S,3S)-2-methyl-1-(4-methylsulfonylphenoxy)-4-oxo-azetidin-3-yl]carbamate (**4**)*

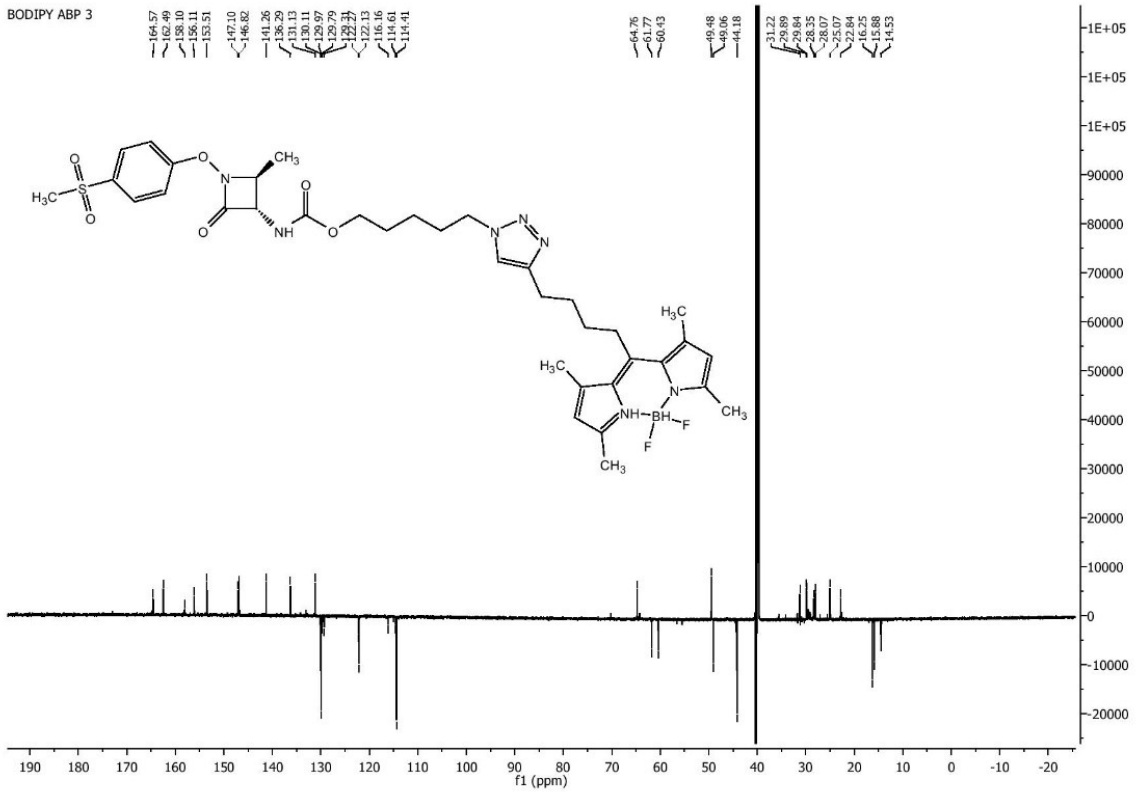
Under a nitrogen atmosphere, a solution of the intermediate β-lactam tosylate salt (Scheme S1, Step 2 (b) for the synthesis of **3**) (0.070 g, 0.16 mmol) in dry $CH_2Cl_2$ (3.0 mL) was treated with DIPEA (0.042 mL, 0.24 mmol) followed by the addition of **S7** (0.124 g, 0.48 mmol). The

reaction was stirred under nitrogen atmosphere at room temperature for 15 h. The solvent was evaporated and the crude product was absorbed over silica gel and purified by standard column chromatography, eluting with pentane/EtOAc (from 100:0 to 50:50) to afford the titled compound (0.047g, 60%), as a colorless oil. MS (ESI) m/z: 507.2 $[M-H]^+$, 524.3 $[M-NH_4]^+$, 505.2 $[M-H]^-$. $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ 8.05 (d, $J$ = 8.2 Hz, 1H), 8.01-7.89 (m, 2H), 7.51 (d, $J$ = 8.8 Hz, 2H), 6.17-6.09 (m, 1H), 6.06 (dd, $J$ = 5.6, 3.1 Hz, 1H), 5.91 (dd, $J$ = 5.8, 3.1 Hz, 1H), 4.40 (dd, $J$ = 8.2, 2.4 Hz, 1H), 4.14 (d, $J$ = 4.4 Hz, 1H), 4.02 (t, $J$ = 6.3 Hz, 2H), 3.45-3.29 (m, 4H), 3.06 (dd, $J$ = 9.2, 6.7 Hz, 1H), 2.94 (t, $J$ = 9.0 Hz, 1H), 2.83 (s, 1H), 2.76 (s, 1H), 2.71-2.64 (m, 1H), 2.28 (s, 1H), 1.92-1.71 (m, 1H), 1.67-1.48 (m, 3H), 1.39 (d, $J$ = 6.1 Hz, 4H), 1.31 (d, $J$ = 8.0 Hz, 1H), 1.21 (d, $J$ = 8.2 Hz, 2H), 0.55-0.33 (m, 1H) ppm. $^{13}C$-NMR ($CDCl_3$, 100 MHz): $\delta$ 162.5, 160.3, 155.7, 154.1, 137.4, 136.8, 136.7, 132.6, 130.1, 114.5, 100.15, 75.8, 74.2, 70.9, 70.8, 66.1, 61.2, 49.6, 45.9, 44,9, 44.1, 42.3, 39.1. 38.9, 29.9, 29.5, 29.3, 28.9, 22.8, 16.6 ppm.

**$^1H$- and $^{13}C$- NMR spectra**

**ABP 3**



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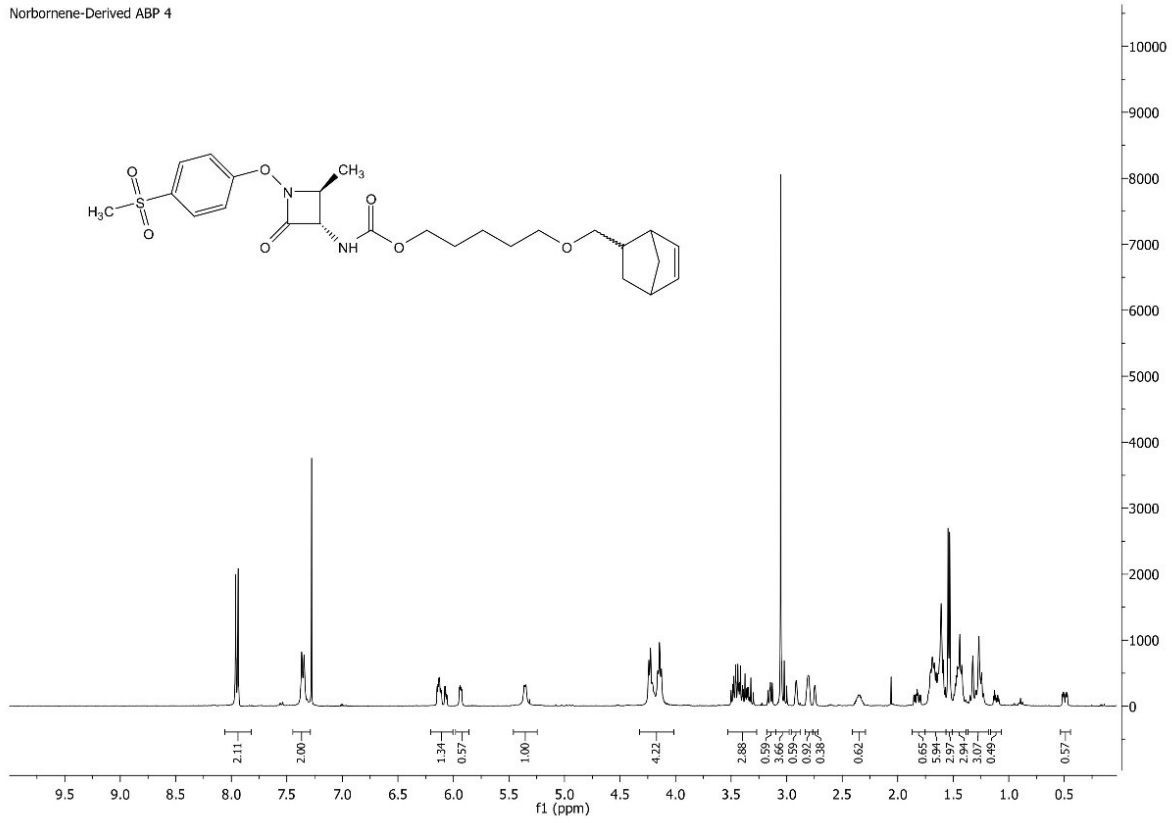

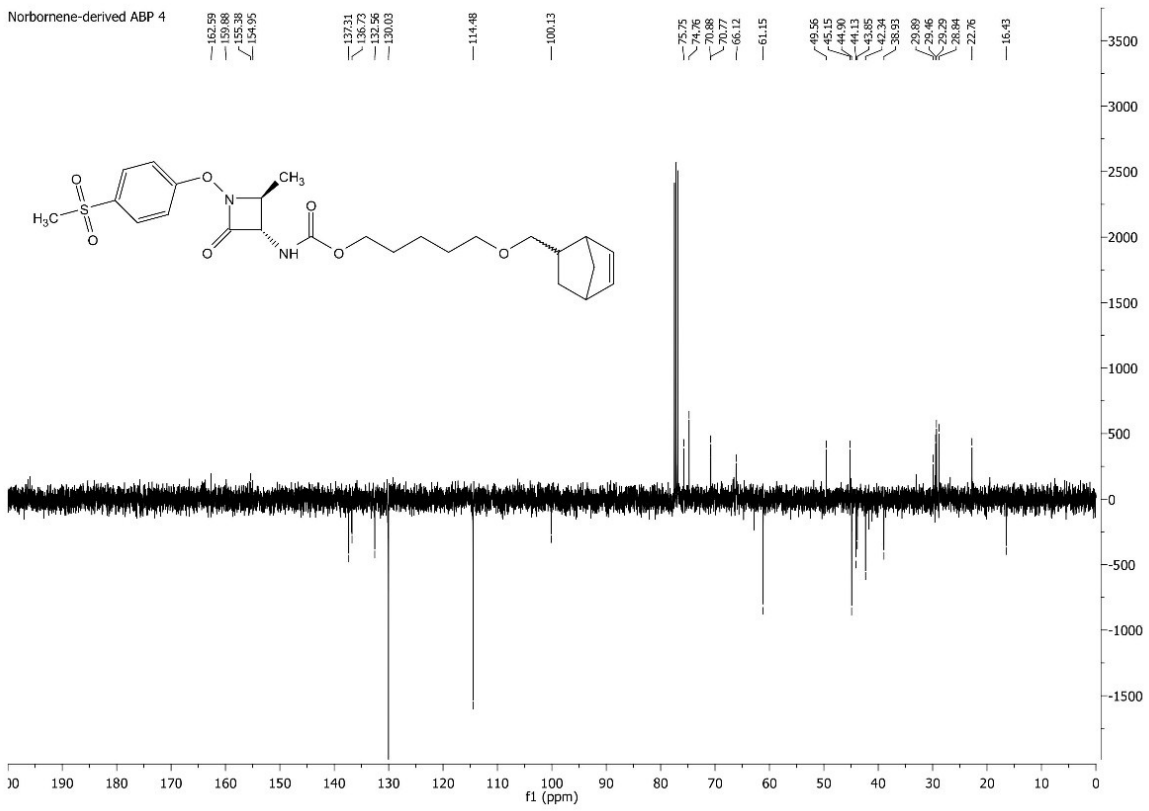


## Experimental section - Biochemistry

### Material and methods

**Cell extracts preparation.** For lysosome extract preparation, cell pellets from around 2.5x$10^8$ cells were resuspended in 9 volumes of 0.32 M Sucrose in Phosphate Buffer Saline (PBS) pH 7.4 and sonicated 5 x 10 sec. After centrifuging 20 min at 800xg, the supernatant was centrifuged again at 12,000xg 30 min at 4 °C. The obtained pellet was resuspended in around 2 volumes of PBS and stored at -80 °C for 1 h. After thawing, samples were frozen at -80 °C for another hour and thawed again. Finally, the suspension was centrifuged for 1 h at 100,000x*g* at 4 °C and the soluble fraction was taken. Next, the PBS buffer was exchanged with NAAA activation buffer [200 mM, NaCl, 100 mM phosphate, 100 mM sodium citrate, 3 mM 1,4-Dithiothreitol (DTT), 0.025% Triton X-100, pH4.5] using desalting columns (Zeba spin desalting columns, Thermo Fisher Scientific). Samples were centrifuged to eliminate precipitated proteins and the soluble fraction was quantified using Bradford assay kit (Thermo Fisher Scientific).

Total lysates were obtained by solubilizing cell pellets in NAAA activation buffer. After 10 min of incubation in ice, samples were centrifuged 10 min at 1000x*g* and the supernatants were stored.

**Experimental set up for probes 3 and 4.** For the titration curve of **3**, 15 μg of lysosome protein extract (1 mg/ ml) was incubated with **3** at 5, 2.5, 1, 0.5, 0.25, and 0 μM (5% DMSO final concentration) for 30 min at 37 °C. The analysis of the linearity of the signal was performed by plotting the intensity value of the obtained bands (ImageJ Version 1.46r, National Institutes of Health, Bethesda, MD, USA) against the probe concentrations.

For the *in vitro* experiment using probe **4,** 15 μg of lysosomal extract (1mg/ml) was incubated with 0, 1, 5 or 10 μM of probe **4**, 30 min at 37 °C. Next, BODIPY GREEN-tetrazine was added at 1, 5, or 10 μM, 30 min at 37 °C. All samples were analyzed by in gel florescent scanning (see below).

**Competitive ABPP assay using probe 3.** 15 μg of lysosomal extract (1 mg/ ml) was pre-incubated with the selected inhibitors (10 μM) for 1 h at 37 °C. Next, probe **3** was added at 1 μM final concentration for 30 min at 37 °C. For the dose/ response experiment, 15 μg of lysosomal extract was pre-incubated with decreasing concentrations of compound **7** for 1 h at 37 °C. Next, probe **3** was added at 1 μM final concentration for 30 min at 37 °C. The bands' intensity was calculated using image analysis software (ImageJ Version 1.46r, National Institutes of Health, Bethesda, MD, USA). Intensity of sample, which was not incubated with the inhibitor, was set as 100% of NAAA activity and other values were calculated consequently. Concentration-response curve was obtained by plotting the relative intensity values against the logarithm of the inhibitor concentration. GraphPad Prism software was used for curve fitting and parameter calculations.

**In-cell ABPP studies with probe 4.** $3x10^5$ HEK293 or NAAA-HEK293 cells were plated in 10 $cm^2$ wells with 2.4x2.4 cm polylysinated coverslips inserted in, and cultivated for an O/N in DMEM medium supplemented with 10% FBS and 2mM Glutamine. The day after, medium was aspired and fresh DMEM medium containing 1% FBS and 1 μM **4** was added for 1 h at 37 °C. After incubation time cells were either lysed for in-gel analysis and western blot or fixed for subsequent imaging studies (see below). For the in-gel analysis, cells were lysed in NAAA activation buffer as described above and 10 μM of BODIPY GREEN-tetrazine was added to 10 μg of total lysate. Samples were analyzed by both in-gel florescence scanning and western blot. For western blot, proteins were transferred onto a nitrocellulose membrane, which was next probed with an anti-NAAA specific antibody (MAB4494, R&D Systems).

**In-gel fluorescence scanning.** Samples were added with Laemmli Sample Buffer (1 x final concentration), boiled 5 min at 95 °C and loaded in a 4-12% Bis-tris gel (Thermo Fisher Scientific) to perform SDS-PAGE. An infra-red protein standard ladder (product # 26635,

Thermo Fisher Scientific) was used for protein mass check. Gel was fixed in 8% acetic acid and fluorescence was acquired using FUJIFILM FLA-9000 image scanner, excitation wavelengths were 488 nm for probes and 680 nm for protein ladder. If subsequent western blot analysis was required, the fixing step with acetic acid was skipped and, immediately after fluorescence acquisition, proteins were transferred onto a nitrocellulose membrane.

**Cell imaging studies.** For the microscopy study, cells which were treated with probe **4** as described above, were fixed with 4% paraformaldehyde in PBS pH 7.4 20 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS pH 7.4 20 min at room temperature, blocked with 5% donkey serum in 0.1% Tween-20/ PBS, 30 min at RT, and incubated with anti-NAAA antibody (MAB4494, R&D Systems) for an O/N at 4 °C. After washing 3 times with PBS, cells were incubated with the secondary reagent donkey anti-mouse Ig, Alexa Fluor 647, 1 h at room temperature. Cells were washed three times with PBS and then incubated with 5 μM of BODIPY GREEN-tetrazine dissolved in PBS, 30 min at RT. Finally, cells were washed six times with PBS and coverslips were mounted with DAPI (Thermo Fisher Scientific). Images were acquired using Nikon A1 confocal microscopy (Nikon) at the following wavelengths: 405 nm for DAPI, 490 for probe, and 640 for anti-NAAA.

## Supplementary figures

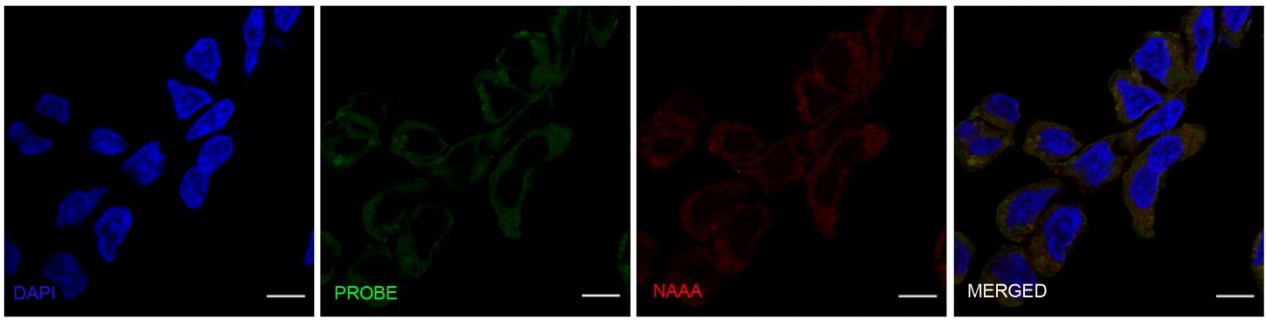


**Fig. S1** Fluorescence microscopy analysis of wild type HEK293, which were treated with probe **4** (1 μM). Cells were fixed and incubated with an anti-NAAA antibody and an Alexa Fluor 647 secondary reagent (in red) followed by 5 μM of tetrazine-BODIPY (in green). Nuclei were marked with DAPI (blue). Scale bar = 10 μm.

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