Supporting Information

Divergent Synthesis of Multifunctional Molecular Probes Enabled Elucidation of the Enzyme Specificity of Dipeptidic $\gamma$-Secretase Inhibitors on Presenilin-Type Aspartic Proteases

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Experimental Procedures for Chemical Synthesis
General remarks. All reactions sensitive to air and/or moisture were carried out under an atmosphere of argon in oven-dried glassware with anhydrous solvents unless otherwise noted. Anhydrous tetrahydrofuran, dichloromethane and \(N,N'\)-dimethylformamide (DMF) were purchased from Wako Pure Chemicals Co. Inc. and used without further drying. Methanol, pyridine, triethylamine and diisopropylethyl amine were distilled from CaH\(_2\) under an atmosphere of argon prior to use. All other reagents purchased were of the highest commercial quality and used as received unless otherwise stated. Analytical thin layer chromatography was carried out using E. Merck silica gel 60 F\(_{254}\) plates (0.25 mm thickness). Flash chromatography was carried out using Fuji Silysia silica gel BW300 (200–400 mesh). Reverse-phase C18 chromatography was performed using Wakogel 100C18 (particle size: 63–212 \(\mu\)m). Melting points were taken on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared spectra were recorded on a JASCO FT/IR–420 spectrometer. \(^1\)H NMR spectra were recorded on a Varian INOVA500 or INOVA600 spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane and coupling constants \((J)\) are reported in hertz (Hz). Undeuterated solvent signals were used as references (7.24 ppm for \(CHCl_3\); 3.31 ppm for \(CHD_2OD\); 7.58 ppm for \(C_5H/D_4N\)). The following abbreviations are used to designate the multiplicities: \(s\) = singlet, \(d\) = doublet, \(t\) = triplet, \(m\) = multiplet, \(br\) = broad. Low- and high-resolution mass spectra were recorded on a JEOL SX–102A mass spectrometer under fast atom bombardment (FAB) conditions using \(m\)-nitrobenzyl alcohol (NBA) as a matrix.
**General procedure for the synthesis of biotinylated azides 6a-c:** The synthesis of 6a is representative. To a solution of alcohol 4 (341.9 mg, 1.2792 mmol) in THF (12 mL) were added pyridine (0.100 mL, 1.2792 mmol) and p-nitrophenylchloroformate (284.0 mg, 1.2068 mmol). After being stirred at room temperature overnight, the reaction mixture was diluted with EtOAc, washed with H2O and brine, dried over Na2SO4, filtered, and concentrated under reduced pressure. The residue was passed through a pad of silica gel column eluting with 25% EtOAc/benzene to give mixed carbonate 5 (431.3 mg, 78%). This unstable material was immediately used in the next reaction.

To a solution of the above mixed carbonate 5 (181.3 mg, 0.4186 mmol) in DMF (5 mL) were added Et3N (0.600 mL, 4.223 mmol) and biotinylated amine (derived from the corresponding Boc derivative (181.1 mg, 0.4223 mmol) by deprotection of the Boc group with TFA). After being stirred at room temperature overnight, the reaction mixture was concentrated under reduced pressure. Purification by flash chromatography (silica gel, MeOH/AcOH/CHCl3 = 5/2/95) gave biotinylated azide 6a (205.3 mg, 79%).

**6a:** colorless powder; mp 141—143 °C; 1H NMR (500 MHz, C5D5N) δ 8.32 (dd, J = 5.5, 5.5 Hz, 1H), 8.18 (dd, J = 5.5, 5.5 Hz, 1H), 7.86 (dd, J = 8.5, 3.0 Hz, 4H), 7.57 (d, J = 8.0 Hz, 2H), 7.52 (s, 1H), 7.48 (d, J = 8.0 Hz, 2H), 7.48 (s, 1H), 5.42 (s, 2H), 4.51 (dd, J = 7.5, 5.0 Hz, 1H), 4.49 (s, 2H), 4.34 (dd, J = 5.5, 5.0 Hz, 1H), 3.42 (dd, J = 13.0, 7.0 Hz, 2H), 3.37 (ddd, J = 13.0, 7.0, 7.0 Hz, 2H), 3.20—3.16 (m, 1H), 2.91 (dd, J = 12.0, 5.0 Hz, 1H), 2.84 (d, J = 13.0 Hz, 1H), 2.32 (dd, J = 7.5, 7.0 Hz, 2H), 1.93—1.73 (m, 4H), 1.66—1.53 (m, 6H), 1.45—1.39 (m, 2H); 13C NMR (125 MHz, C5D5N) δ 195.4, 173.3, 172.6, 164.2, 157.0, 150.1, 143.0, 140.7, 137.6, 137.1, 130.6, 130.4, 128.3, 127.5, 123.7, 65.3, 62.3, 60.4, 56.2, 54.1, 41.3, 41.0, 39.3, 36.1, 30.0, 29.8, 28.9, 28.8,
26.0, 24.5, 21.3; HRMS (FAB) calcd for C$_{31}$H$_{40}$O$_5$N$_7$S$_1$ [(M+H)$^+$] 622.2733, found 622.2812.

6b: colorless powder; mp 163—165 °C; $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.80 (dd, $J$ = 11.5, 8.0, Hz, 4H), 7.54 (d, $J$ = 8.5 Hz, 4H), 5.21 (s, 2H), 4.53 (s, 2H), 4.48 (dd, $J$ = 8.0, 5.0 Hz, 1H), 4.29 (dd, $J$ = 8.0, 4.5 Hz, 1H), 3.63—3.54 (m, 8H), 3.37—3.16 (m, 5H), 2.91 (dd, $J$ = 12.5, 5.0 Hz, 1H), 2.695 (d, $J$ = 12.5 Hz, 1H), 2.21 (dd, $J$ = 7.5, 7.5 Hz, 2H), 1.76—1.55 (m, 4H), 1.45—1.39 (m, 2H); $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 198.4, 197.3, 197.2, 196.4, 195.3, 176.9, 174.3, 166.9, 159.5, 144.5, 143.1, 139.3, 138.9, 132.3, 132.0, 130.0, 129.2, 72.1, 71.7, 71.4, 67.4, 64.1, 62.4, 57.8, 55.8, 42.6, 41.8, 41.1, 37.5, 30.6, 30.3, 27.6; HRMS (FAB) calcd for C$_{32}$H$_{42}$O$_7$N$_7$S$_1$ [(M+H)$^+$] 668.2866, found 668.2868.

6c: colorless powder; mp 107—109 °C; $^1$H NMR (500 MHz, C$_5$D$_5$N) $\delta$ 8.37 (brs, 1H), 8.25 (brs, 1H), 7.88 (d, $J$ = 8.0 Hz, 4H), 7.60 (d, $J$ = 12.0 Hz, 2H), 7.55 (s, 1H), 7.50 (d, $J$ = 7.5 Hz, 2H), 7.41 (s, 1H), 5.47 (s, 2H), 4.54 (s, 1H), 4.52 (s, 2H), 4.37 (s, 1H), 3.49—3.42 (m, 4H), 3.23—3.19 (m, 1H), 3.21 (m, 1H), 2.93 (dd, $J$ = 12.0, 5.0 Hz, 1H), 2.87 (d, $J$ = 12.5 Hz, 1H), 2.38 (dd, $J$ = 7.0, 7.0 Hz, 2H), 1.68—1.58 (m, 6H), 1.38—1.29 (m, 4H), 1.21 (s, 4H), 1.17 (s, 4H); $^{13}$C NMR (125 MHz, C$_5$D$_5$N) $\delta$ 200.2, 195.4, 172.6, 164.1, 157.0, 143.0, 140.7, 137.6, 137.1, 135.8, 135.0, 130.6, 130.4, 128.3, 127.5, 123.8, 123.0, 65.4, 62.3, 60.4, 56.3, 54.1, 41.4, 41.0, 39.6, 36.2, 30.4, 30.2, 29.6, 29.46, 29.45, 29.0, 28.9, 27.2, 27.1, 26.1; HRMS (FAB) calcd for C$_{36}$H$_{50}$O$_5$N$_7$S$_1$ [(M+H)$^+$] 692.3594, found 692.3593.

**Alcohol 9a:** To a solution of alcohol 7 (110.3 mg, 0.4795 mmol) in DMF (5 mL) were added K$_2$CO$_3$ (119.2 mg, 0.8631 mmol) and N-($t$-butoxycarbonyl)-8-bromooctylamine (272.3 mg, 0.8631 mmol). After being stirred at 60 °C for 105 min, the reaction mixture
was cooled to room temperature and diluted with diethyl ether. The organic layer was washed with saturated aqueous NH₄Cl, saturated aqueous NaHCO₃ and brine, dried over Na₂SO₄, filtered and concentrated under reduced pressure. The residue was passed through a pad of silica gel column eluting with 10% EtOAc/hexane to give 8a contaminated with excess N-(t-butoxycarbonyl)-8-bromo-octylamine, which was used in the next reaction without further purification.

To a solution of the above 8a in MeOH (5 mL) cooled at 0 °C was added NaBH₄ (18.6 mg, 0.4795 mmol). After being stirred at 0 °C for 80 min, the reaction was quenched with saturated aqueous NH₄Cl. The resulting mixture was extracted with diethyl ether and the organic layer was washed with saturated aqueous NH₄Cl, saturated aqueous NaHCO₃ and brine, dried over Na₂SO₄, filtered and concentrated under reduced pressure. Purification of the residue by flash chromatography (silica gel, 10 to 40% EtOAc/hexane) gave alcohol 9a (202.9 mg, 92% for the two steps) as a colorless oil. 9a: ¹H NMR (500 MHz, CDCl₃) δ 7.31 (d, J = 8.0 Hz, 1H), 6.76 (d, J = 8.0 Hz, 1H), 6.59 (s, 1H), 4.67 (d, J = 6.5 Hz, 2H), 4.50 (brs, 1H), 3.97 (dd, J = 6.5, 6.5 Hz 2H), 3.12 —3.06 (m, 2H), 2.37—2.34 (m, 1H), 1.78 (dd, J = 13.5, 7.5, Hz, 1H), 1.77 (dd, J = 13.5, 7.5 Hz, 1H), 1.47—1.40 (m, 4H), 1.42 (s, 9H), 1.36—1.28 (m, 6H); HRMS (FAB) calcd for C₂₂H₃₃O₄N₃F₃ [(M+H)+] 460.2423, found 460.2427.

Alcohol 9b: Prepared according to the procedure described for 9a. 96% yield for the two steps. 9b: ¹H NMR (500 MHz, CDCl₃) δ 7.27 (d, J = 7.5 Hz, 1H), 6.78 (d, J = 7.5 Hz, 1H), 6.66 (s, 1H), 4.98 (br, 1H), 4.35 (s, 2H), 4.16 (m, 2H), 3.86 (m, 2H), 3.68 (m, 2H), 3.62 (m, 2H), 3.53 (m, 2H), 3.30 (m, 1H), 1.41 (s, 9H); HRMS (FAB) calcd for C₂₀H₂₉F₃N₆O₅ [(M+H)+] 489.2073, found 489.2076.
Azide 10a: To a solution of 9a (74.1 mg, 0.1612 mmol) in CH$_2$Cl$_2$ (5 mL) cooled at 0 °C were added Et$_3$N (0.700 mL, 0.4836 mmol) and MsCl (0.025 mL, 0.3224 mmol). After being stirred at 0 °C for 1 h, the reaction mixture was quenched with saturated aqueous NH$_4$Cl. The organic layer was diluted with diethyl ether, washed with H$_2$O, saturated aqueous NaHCO$_3$ and brine, dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure. The residue was passed through a pad of silica gel (20% EtOAc/hexane) to give crude mesylate, which was used in the next reaction without further purification.

To a solution of the above mesylate in DMF (4 mL) were added NaN$_3$ (30.0 mg, 0.3224 mmol) and n-Bu$_4$NI (5.6 mg, 0.016 mmol). After being stirred at 60 °C for 70 min, the reaction mixture was cooled to room temperature and diluted with diethyl ether. The organic layer was washed with H$_2$O and brine, dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure. Purification of the residue by flash chromatography (silica gel, 5 to 10% EtOAc/hexane) gave azide 10a (57.7 mg, 78%) as a colorless oil. 10a: $^1$H NMR (500 MHz, CDCl$_3$) δ 7.26 (d, $J = 7.5$ Hz, 1H), 6.76 (d, $J = 7.5$ Hz, 1H), 6.60 (s, 1H), 4.47 (brs, 1H), 4.33 (s, 2H), 3.95 (dd, $J = 6.5$, 6.5 Hz, 2H), 3.12—3.08 (m, 2H), 1.79 (dddd, $J = 14.0$, 7.0, 7.0, 7.0 Hz, 2H), 1.49—1.40 (m, 13H), 1.38—1.28 (m, 6H); HRMS (FAB) calcd for C$_{22}$H$_{32}$O$_3$N$_6$F$_3$ [(M+H)$^+$] 485.2488, found 485.2488.

Azide 10b: Prepared according to the procedure described for 10a. 56% yield. 10b: $^1$H NMR (500 MHz, CDCl$_3$) δ 7.26 (d, $J = 7.5$ Hz, 1H), 6.78 (d, $J = 8.0$ Hz, 1H), 6.66 (s, 1H), 4.96 (s, 1H), 4.35 (s, 2H), 4.16 (dd, $J = 5.0$, 5.0 Hz, 2H), 3.86 (dd, $J = 5.0$, 5.0 Hz, 2H), 3.69—3.67 (m, 2H), 3.62—3.61 (m, 2H), 3.53 (dd, $J = 5.5$, 5.5 Hz, 2H), 3.30 (d, $J$
= 5.0 Hz, 2H), 1.41 (s, 9H); HRMS (FAB) calcd for C<sub>20</sub>H<sub>28</sub>O<sub>5</sub>N<sub>6</sub>F<sub>3</sub> [(M+H)<sup>+</sup>] 489.2073, found 489.2076.

**Biotinylated azide 11a**: To a solution of azide 10a (596.9 mg, 1.232 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (6 mL) cooled at 0 °C was added TFA (6 mL). After being stirred at room temperature for 1 h, the reaction mixture was concentrated under reduced pressure to give the corresponding TFA salt, which was used in the next reaction without purification.

To a solution of the above TFA salt in DMF (12 mL) cooled at 0 °C were added (+)-biotin pentafluorophenyl ester (515.0 mg, 1.257 mmol) and Et<sub>3</sub>N (1.70 mL, 12.3 mmol). After being stirred at room temperature overnight, the reaction mixture was concentrated under reduced pressure. Purification of the residue by flash chromatography (silica gel, 5 to 8% MeOH/CHCl<sub>3</sub>) gave biotinylated azide 11a (556.7 mg, 74% for the two steps) as a colorless powder. **11a**: mp 150—152 °C; <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ 7.29 (d, J = 8.0 Hz, 1H), 6.79 (d, J = 8.0 Hz, 1H), 6.66 (s, 1H), 4.40 (dd, J = 8.0, 5.0 Hz, 1H), 4.30 (s, 2H), 4.21 (dd, J = 8.0, 4.5 Hz, 1H), 3.96 (dd, J = 6.5, 6.5 Hz, 2H), 3.14—3.07 (m, 3H), 2.84 (dd, J = 12.5, 5.0 Hz, 1H), 2.62 (d, J = 12.5 Hz, 1H), 2.11 (dd, J = 7.0, 7.0 Hz, 2H), 1.76 (dd, J = 13.0, 6.0, 6.0 Hz, 2H), 1.68—1.30 (m, 16H); HRMS (FAB) calcd for C<sub>27</sub>H<sub>38</sub>O<sub>3</sub>N<sub>8</sub>F<sub>3</sub>S<sub>1</sub> [(M+H)<sup>+</sup>] 611.2739, found 611.2745.

**Biotinylated azide 11b**: Prepared according to the procedure described for 11a. 88% yield. **11b**: mp 115—117 °C; <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ 7.32 (d, J = 7.5 Hz, 1H), 6.84 (d, J = 8.0 Hz, 1H), 6.73 (s, 1H), 4.40 (dd, J = 7.5, 5.0 Hz, 1H), 4.35 (s, 2H), 4.21 (dd, J = 8.0, 4.5 Hz 1H), 4.13 (dd, J = 4.5, 4.5 Hz, 2H), 3.82 (dd, J = 4.5, 4.5 Hz, 2H), 3.66—3.64 (m, 2H), 3.58—3.56 (m, 2H), 3.47 (d, J = 5.5, 5.5 Hz, 2H), 3.28 (dd, J = 5.5,
5.5 Hz, 2H), 3.11 (ddd, J = 8.5, 5.0, 5.0 Hz 1H), 2.84 (dd, J = 12.5, 5.0 Hz, 1H), 2.62 (d, J = 12.5 Hz, 1H), 2.12 (dd, J = 7.5, 7.5 Hz, 2H), 1.66—1.47 (m, 4H), 1.36 (ddd, J = 15.5, 8.0, 8.0 Hz 2H); HRMS (FAB) calcd for C_{25}H_{34}O_{5}N_{8}F_{3}S_{1} [(M+H)^{+}] 615.2325, found 615.2326.

**Carbamate 13:** To a solution of azide 12 (2.90 g, 7.30 mmol) in THF/H_{2}O (1/1, v/v, 100 mL) was added Ph_{3}P (2.30 g, 8.77 mmol). After being stirred at room temperature overnight, K_{2}CO_{3} (1.51 g, 10.9 mmol) and Boc_{2}O (3.35 mL, 14.6 mmol) were added, and the resultant mixture was further stirred at room temperature for 1.5 h. The reaction mixture was diluted with EtOAc, washed with H_{2}O and brine, dried over Na_{2}SO_{4}, filtered and concentrated under reduced pressure. Purification of the residue by flash chromatography (silica gel, 20 to 30% EtOAc/hexane) to give carbamate 13 (3.62 g, ~quant.) as a colorless viscous oil. 13: \(^{1}\)H NMR (500 MHz, CDCl_{3}) δ 7.47—7.39 (m, 3H), 7.31—7.28 (m, 4H), 7.17—7.10 (m, 2H), 6.91 (d, J = 9.0 Hz, 2H), 6.61 (d, J = 9.0 Hz, 2H), 6.49 (d, J = 8.5 Hz, 1H), 5.61 (d, J = 15.5 Hz, 1H), 5.36 (d, J = 8.5 Hz, 1H), 4.68 (d, J = 15.5 Hz, 1H), 3.63 (s, 3H), 1.42 (s, 9H); HRMS (FAB) calcd for C_{28}H_{30}O_{4}N_{3} [(M+H)^{+}] 472.2236, found 472.2237.

**Lactam 14:** To a solution of carbamate 13 (3.62 g, ~7.30 mmol) in CH_{3}CN/H_{2}O (3/1, v/v, 80 mmol) cooled at –15 °C was added CAN (20.0 g, 36.5 mmol) in H_{2}O (45 mL) dropwise. After being stirred at –15 °C for 3 h and then at 0 °C for 1.5 h, the reaction mixture was diluted with EtOAc, washed with H_{2}O and brine, dried over Na_{2}SO_{4}, filtered and concentrated under reduced pressure. Purification of the residue by flash chromatography (silica gel, 30 to 50% EtOAc/hexane) gave crystals that was washed with diisopropyl ether to give lactam 14 (1.95 g, 76% for the three steps) as a colorless powder. 14: mp 221—224 °C; \(^{1}\)H NMR (500 MHz, CDCl_{3}) δ 9.19 (br, 1H), 7.62—7.51
(m, 4H), 7.44—7.41 (m, 2H), 7.34—7.30 (m, 2H), 7.22 (m, 1H), 6.37 (d, $J = 8.0$ Hz, 1H), 5.33 (d, $J = 8.0$ Hz, 1H), 1.44 (s, 9H); HRMS (FAB) calcd for C$_{20}$H$_{22}$O$_3$N$_3$ [(M+H)$^+$] 352.1661, found 352.1668.

**Alkyne 15:** To a solution of lactam 14 (0.70 g, 1.99 mmol) in DMF (10 mL) were added Cs$_2$CO$_3$ (0.97 g, 2.98 mmol) and propargyl bromide (0.225 mL, 2.99 mmol). After being stirred at rt overnight, the reaction mixture was diluted with EtOAc. The organic layer was washed with H$_2$O and brine, dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure. Purification of the residue by flash chromatography (silica gel, 30% EtOAc/hexane) gave crystals that was washed with diisopropyl ether to give alkyne 15 (0.54 g, 70%) as a colorless powder. 15: mp 201—203 °C; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.65 (d, $J = 8.0$ Hz, 1H), 7.64—7.57 (m, 3H), 7.43 (m, 1H), 7.37—7.31 (m, 3H), 7.24 (m, 1H), 6.43 (d, $J = 9.0$ Hz, 1H), 5.33 (d, $J = 8.5$ Hz, 1H), 4.69 (d, $J = 17.5$ Hz, 1H), 4.57 (d, $J = 17.5$ Hz, 1H), 2.24 (s, 1H), 1.43 (s, 9H); HRMS (FAB) calcd for C$_{23}$H$_{24}$O$_3$N$_3$ [(M+H)$^+$] 390.1818, found 390.1819.

**Carbamate 16:** To a solution of alkyne 15 (0.49 g, 1.26 mmol) in CH$_2$Cl$_2$ (12 mL) cooled at 0 °C was added TFA (6 mL). After being stirred at room temperature for 1 h, the reaction mixture was concentrated under reduced pressure. The residue was partitioned between CH$_2$Cl$_2$ and 1 M aqueous NaOH, and the aqueous layer was extracted with CH$_2$Cl$_2$. The combined organic layer was dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure. The crude amine thus obtained was used in the next reaction without further purification.

To a solution of the above amine in THF (15 mL) cooled at 0 °C were added Boc-L-alanine (357.6 mg, 1.89 mmol), HOBt (255.4 mg, 1.68 mmol), EDC (362.3 mg, 1.89 mmol) and Et$_3$N (0.53 mL, 3.80 mmol). After being stirred at room temperature
overnight, the reaction mixture was diluted with EtOAc, washed with H₂O and brine, dried over Na₂SO₄, filtered and concentrated under reduced pressure. Purification of the residue by flash chromatography (silica gel, 50% EtOAc/hexane) gave carbamate 16 (0.56 g, 97%) as a colorless oil, which crystallized upon standing. 16: ¹H NMR (500 MHz, CDCl₃) δ 7.71 (m, 1H), 7.65 (d, J = 8.5 Hz, 1H), 7.61—7.55 (m, 3H), 7.44 (m, 1H), 7.38—7.33 (m, 3H), 7.28—7.24 (m, 2H), 5.53 (apparent t, J = 8.0 Hz, 1H), 5.06 (m, 1H), 4.70—4.55 (m, 3H), 4.37 (br, 1H), 2.25 (m, 1H), 1.45 (s, 9H); HRMS (FAB) calcd for C₂₆H₂₉O₄N₄ [(M+H)+] 461.2189, found 461.2193.

Alkyne 17: To a solution of carbamate 16 (0.42 g, 0.916 mmol) in CH₂Cl₂ (9 mL) cooled at 0 °C was added TFA (9 mL). After being stirred at room temperature for 1 h, the reaction mixture was concentrated under reduced pressure. The residue was partitioned between CH₂Cl₂ and 1 M aqueous NaOH. The aqueous layer was extracted with CH₂Cl₂. The combined organic layer was dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude amine thus obtained was used in the next reaction without further purification.

To a solution of the above amine in THF (15 mL) cooled at 0 °C were added 3,5-difluorophenylacetic acid (236.5 mg, 1.37 mmol), HOBT (185.7 mg, 1.21 mmol), EDC (263.4 mg, 1.37 mmol) and Et₃N (0.38 mL, 2.73 mmol). After being stirred at room temperature for 4 h, the reaction mixture was diluted with EtOAc, washed with H₂O and brine, dried over Na₂SO₄, filtered and concentrated under reduced pressure. Purification of the residue by flash chromatography (silica gel, 70% EtOAc/hexane) followed by recrystallization from EtOAc/hexane gave alkyne 17 (0.41 g, 87%) as a colorless powder. 17: mp 198—200 °C; ¹H NMR (500 MHz, CDCl₃) δ 7.65—7.53 (m, 5H), 7.44 (m, 1H), 7.37—7.24 (m, 4H), 6.80 (m, 2H), 6.67 (m, 1H), 6.45, 6.42 (d, J =
6.5 Hz, 0.5H x 2), 5.48 (m, 1H), 4.67—4.55 (m, 3H), 3.55—3.48 (m, 2H), 2.24 (s, 1H), 1.45, 1.44 (d, J = 7.0 Hz, 1.5H x 2); HRMS (FAB) calcd for C_{29}H_{25}O_{3}N_{4}F_{2} [(M+H)^+] 515.1894, found 515.1899.

**Alkyne 19:** To a solution of alcohol 18 (0.29 g, 0.612 mmol) in DMF (8 mL) were added K_{2}CO_{3} (422.6 mg, 3.06 mmol) and propargyl bromide (0.230 mL, 3.05 mmol). After being stirred at 60 °C for 2 h, the reaction mixture was cooled to room temperature and diluted with EtOAc. The organic layer was washed with H_{2}O and brine, dried over Na_{2}SO_{4}, filtered and concentrated under reduced pressure. Purification of the residue by flash chromatography (silica gel, 70% EtOAc/hexane) gave alkyne 19 (0.32 g, quant.), which was recrystallized from EtOAc/hexane to give a colorless powder. 19: mp 135—137 °C; \(^1\)H NMR (500 MHz, CDCl\textsubscript{3}) \(\delta\) 7.53—7.47 (m, 3H), 7.39—7.36 (m, 2H), 7.24—7.18 (m, 2H), 6.98 (d, J = 7.5 Hz, 1H), 6.92 (dd, J = 6.5, 2.5 Hz, 1H), 6.80—6.79 (m, 2H), 6.69 (m, 1H), 6.25 (d, J = 7.0 Hz, 0.5H), 6.20 (d, J = 6.5 Hz, 0.5H), 5.22 (apparent d, J = 5.5 Hz, 1H), 4.75 (s, 2H), 4.67 (m, 1H), 3.51 (d, J = 6.0 Hz, 2H), 3.33, 3.32 (s, 1.5H x 2), 2.57 (m, 1H), 1.50, 1.40 (d, J = 6.5 Hz, 1.5H x 2); HRMS (FAB) calcd for C_{29}H_{26}O_{4}N_{3}F_{2} [(M+H)^+] 518.1891, found 518.1893.

**General procedure for azide/alkyne cycloaddition (aqueous conditions):** The synthesis of 21a is representative. To a solution of alkyne 17 (20.0 mg, 0.0389 mmol) and azide 6a (24.2 mg, 0.0389 mmol) in MeOH/H\(_2\)O (2:1, v/v, 0.75 mL) were added sodium ascorbate (0.156 mL of 0.1 M solution in H\(_2\)O, 0.0156 mmol), copper(II) sulfate (0.078 mL of 0.1 M solution in H\(_2\)O, 0.0078 mmol) and tris(triazolyl)amine ligand 20 (8.2 mg, 0.0155 mmol). After being stirred at room temperature for 12 days, the reaction mixture was diluted with cold H\(_2\)O. The precipitate formed was collected and washed with diethyl ether and cold H\(_2\)O. Further purification was performed by
reverse-phase C18 chromatography eluting with 50-80% MeOH/H₂O. The desired fractions were collected and concentrated under reduced pressure. The resultant powder was finally washed with diethyl ether to give photoaffinity probe 21a (39.5 mg, 89%) as a colorless powder.

**General procedure for azide/alkyne cycloaddition (anhydrous conditions):** The synthesis of 21a is representative. To a solution of alkyne 17 (20.0 mg, 0.0389 mmol) and azide 6a (24.2 mg, 0.0389 mmol) in MeOH (1.5 mL) were added i-Pr₂NEt (0.068 mL, 0.390 mmol) and CuI (2.2 mg, 0.0116 mmol). After being stirred at room temperature for 20 h, the reaction mixture was concentrated under reduced pressure. The residue was purified by reverse-phase C18 chromatography eluting with MeOH/H₂O. The desired fractions were collected and concentrated under reduced pressure. The resultant powder was finally washed with diethyl ether to give photoaffinity probe 21a (40.4 mg, 92%) as a colorless powder.

21a: mp 131—133 °C; ¹H NMR (500 MHz, CD₃OD) δ 7.74—7.69 (m, 2H), 7.61—7.58 (m, 3H), 7.48—7.40 (m, 4H), 7.34—7.09 (m, 7H), 6.99 (dd, J = 8.0, 3.5 Hz, 2H), 6.86—6.82 (m, 2H), 6.71—6.68 (m, 1H), 5.47—5.39 (m, 3H), 5.28 (d, J = 10.5 Hz, 1H), 5.09 (s, 2H), 4.98 (d, J = 15.0 Hz, 1H), 4.45 (dd, J = 11.5, 6.5 Hz, 1H), 4.35 (dd, J = 8.0, 5.0 Hz, 1H), 4.16 (dd, J = 7.0, 5.0 Hz, 1H), 3.51—3.48 (m, 2H), 3.07—3.00 (m, 6H), 2.79 (dd, J = 13.0, 5.0 Hz, 1H), 2.57 (d, J = 13.0 Hz, 1H), 2.06 (dd, J = 8.0, 8.0 Hz, 2H), 1.60—1.23 (m, 14H); HRMS (FAB) calcd for C₆₀H₆₄O₈N₁₁F₂S₁ [(M+H)⁺] 1136.4627, found 1136.4630.

21b: mp 108—110 °C; ¹H NMR (500 MHz, CD₃OD) δ 7.76—7.71 (m, 2H), 7.64—7.61 (m, 3H), 7.50—7.44 (m, 4H), 7.36—7.14 (m, 7H), 7.02 (dd, J = 8.0, 3.0 Hz, 2H), 6.86 (dd, J = 6.0, 5.5 Hz, 2H), 6.73—6.69 (m, 1H), 5.49 (s, 2H), 5.47 (s, 2H), 5.44 (d, J
= 16.0 Hz, 1H), 5.31 (d, J = 11.0 Hz, 1H), 5.12 (s, 2H), 5.01 (d, J = 16.0 Hz, 1H), 4.49 – 4.45 (m, 1H), 4.37 (dd, J = 8.0, 5.0 Hz, 1H), 4.18 (dd, J = 7.5, 4.0 Hz, 1H), 3.53 – 3.37 (m, 1H), 3.09 – 3.06 (m, 2H), 2.80 (dd, J = 13.0, 5.0 Hz, 1H), 2.59 (d, J = 12.5 Hz, 1H), 2.10 (dd, J = 7.5, 7.0 Hz, 2H), 1.64 – 1.46 (m, 4H), 1.39 – 1.29 (m, 6H); HRMS (FAB) calcd for C_{61}H_{65}O_{10}N_{11}F_{2}S_{1} [(M+H)^+] 1182.4728, found 1182.4686.

21c: mp 143 – 145 °C; \(^1\)H NMR (500 MHz, CD_{3}OD) δ 7.71 – 7.69 (m, 2H), 7.58 – 7.54 (m, 3H), 7.43 – 7.38 (m, 4H), 7.29 – 7.23 (m, 3H), 7.19 – 7.14 (m, 3H), 7.08 (d, J = 7.5 Hz, 1H), 6.97 (d, J = 8.0 Hz, 2H), 6.82 (dd, J = 8.0, 8.0 Hz, 2H), 6.69 – 6.65 (m, 1H), 5.44 (s, 2H), 5.39 (d, J = 15.0 Hz, 1H), 5.28 (d, J = 11.0 Hz, 1H), 5.07 (s, 2H), 4.96 (dd, J = 15.5, 3.5 Hz, 1H), 4.49 – 4.43 (m, 1H), 4.35 (dd, J = 8.0, 5.0 Hz, 1H), 4.16 (dd, J = 7.5, 4.0 Hz, 1H), 3.48 (dd, J = 8.5, 2.5 Hz, 2H), 3.08 – 2.98 (m, 5H), 2.78 (dd, J = 13.0, 5.0 Hz, 1H), 2.57 (d, J = 12.5 Hz, 1H), 2.08 – 2.05 (m, 2H), 1.62 – 1.17 (m, 25H); HRMS (FAB) calcd for C_{65}H_{73}O_{8}N_{11}F_{2}S_{1} [(M+H)^+] 1206.4510, found 1206.4513.

21d: mp 114 – 115 °C; \(^1\)H NMR (500 MHz, CD_{3}OD) δ 7.75 (dd, J = 8.0, 7.5 Hz, 1H), 7.60 (dd, J = 7.5, 7.0 Hz, 1H), 7.54 (d, J = 3.5 Hz, 1H), 7.41 (brrs, 1H), 7.29 – 7.23 (m, 5H), 7.15 (d, J = 7.5 Hz, 1H), 6.88 – 6.83 (m, 3H), 6.72 (dd, J = 9.0, 7.5 Hz, 1H), 6.60 – 6.58 (m, 2H), 5.40 – 5.27 (m, 4H), 5.99 (d, J = 15.5 Hz, 1H), 4.48 (ddddd, J = 15.5, 7.5, 7.5, 7.5 Hz, 1H), 4.37 (dd, J = 7.0, 5.5 Hz, 1H), 4.19 (dd, J = 7.0, 4.5 Hz, 1H), 3.84 – 3.81 (m, 2H), 3.54 – 3.49 (m, 2H), 3.08 – 3.06 (m, 3H), 2.81 (dd, J = 13.0, 5.0 Hz, 1H), 2.60 (d, J = 13.0 Hz, 1H), 2.09 (dd, J = 7.5, 7.0 Hz, 2H), 1.61 – 1.38 (m, 21H); HRMS (FAB) calcd for C_{56}H_{62}O_{6}N_{12}F_{5}S_{1} [(M+H)^+] 1125.4556, found 1125.4557.

21e: mp 130 – 132 °C; \(^1\)H NMR (500 MHz, CD_{3}OD) δ 7.75 – 7.71 (m, 2H), 7.61 (dd, J = 8.0, 7.5 Hz, 1H), 7.41 – 7.39 (m, 1H), 7.28 – 7.21 (m, 5H), 7.13 – 7.10 (m, 1H),
6.97 (dd, J = 7.5, 4.5 Hz, 1H), 6.87 (d, J = 7.0 Hz, 2H), 6.73 (ddd, J = 9.5, 9.5, 2.5 Hz, 1H), 6.67 (d, J = 8.0 Hz, 1H), 6.57 (d, J = 8.0 Hz, 1H), 5.42—5.29 (m, 4H), 4.97 (dd, J = 15.0, 6.5 Hz, 1H), 4.54—4.48 (m, 1H), 4.37 (dd, J = 8.0, 5.0 Hz, 1H), 4.17 (dd, J = 6.0, 4.5 Hz, 1H), 4.02—3.96 (m, 1H), 3.90—3.86 (m, 1H), 3.67 (dd, J = 4.0, 4.0 Hz, 1H), 3.62—3.50 (m, 7H), 3.44—3.42 (m, 2H), 3.06 (dddd, J = 9.5, 5.5, 5.5, 5.5 Hz, 1H), 2.80 (dd, J = 12.5, 5.5 Hz, 1H), 2.58 (d, J = 12.5 Hz, 1H), 2.05 (dd, J = 7.5, 7.5 Hz, 2H), 1.61—1.43 (m, 5H) 1.37 (dd, J = 7.0, 4.0 Hz, 3H), 1.28 (dddd, J = 15.0, 7.0, 7.0, 7.0 Hz, 2H), HRMS (FAB) calcld for C_{54}H_{57}O_{8}N_{12}F_{5}S_{1} [(M+H)^+] 1129.4141, found 1129.4148.

22a: mp 130—132 °C; ^1H NMR (500 MHz, CD_{3}OD) δ 8.09 (s, 1H), 7.71—7.62 (m, 4H), 7.51—7.10 (m, 9H), 7.04—6.99 (m, 2H), 6.86 (dd, J = 7.5, 6.0 Hz, 2H), 6.73—6.67 (m, 1H), 5.65 (s, 2H), 5.22 (s, 2H), 5.11—5.07 (m, 3H), 4.51—4.42 (m, 2H), 4.36 (ddd, J = 13.0, 8.0, 8.0 Hz, 1H), 4.17 (ddd, J = 13.0, 8.0, 8.0 Hz, 1H), 3.56—3.49 (m, 2H), 3.08—3.01 (m, 6H), 2.83—3.77 (m, 1H), 2.58 (dd, J = 12.5, 7.5 Hz, 1H), 2.08 (ddd, J = 15.0, 7.0, 7.0 Hz, 2H), 1.64—1.25 (m, 14H); HRMS (FAB) calcld for C_{60}H_{64}O_{9}N_{10}F_{2}S_{1} [(M+H)^+] 1139.4625, found 1139.4635.

22b: mp 121—123 °C; ^1H NMR (500 MHz, CD_{3}OD) δ 8.09 (s, 1H), 7.67—7.62 (m, 4H), 7.49—7.10 (m, 9H), 7.04—6.99 (m, 2H), 6.86 (dd, J = 6.0, 6.0 Hz, 2H), 6.73—6.67 (m, 1H), 5.65 (s, 2H), 5.22 (s, 2H), 5.09 (s, 1H), 5.08 (s, 2H)4.50—4.44 (m, 1H), 4.35 (dd, J = 8.0, 5.0 Hz, 1H), 4.15 (dd, J = 7.5, 4.5 Hz, 1H), 3.53—3.41 (m, 10H), 3.25—3.18 (m, 13H), 3.05 (ddd, J = 9.5, 4.5, 4.5 Hz, 1H), 2.78 (dd, J = 12.5, 5.0 Hz, 1H), 2.56 (d, J = 12.5, Hz, 1H), 2.08 (dd, J = 7.5, 7.5 Hz, 2H), 1.61—1.43 (m, 4H), 1.35—1.28 (m, 5H); HRMS (FAB) calcld for C_{61}H_{67}O_{11}N_{10}F_{2}S_{1} [(M+H)^+] 1185.4679, found 1185.4685.
**22c**: mp 127—129 °C; $^1$H NMR (500 MHz, CD$_3$OD) δ 8.09 (s, 1H), 7.66—7.62 (m, 4H), 7.49—7.10 (m, 9H), 7.04 (dd, $J$ = 2.5, 1.5 Hz, 1H), 7.00 (dd, $J$ = 8.5, 2.5 Hz, 1H), 6.85 (dd, $J$ = 7.5, 7.5 Hz, 2H), 6.73—6.67 (m, 1H), 5.65 (s, 2H), 5.22 (s, 2H), 5.10—5.07 (m, 3H), 4.51—4.42 (m, 1H), 4.36 (dd, $J$ = 7.5, 5.0 Hz, 1H), 4.20—4.16 (m, 1H), 3.53—3.51 (m, 2H), 3.18 (s, 3H), 3.07—3.00 (m, 6H), 2.82—2.78 (m, 1H), 2.61—2.57 (m, 1H), 2.07 (dd, $J$ = 7.5, 7.0 Hz, 2H), 1.63—1.20 (m, 30H); HRMS (FAB) calcd for C$_{65}$H$_{74}$O$_9$N$_{10}$F$_2$S$_1$ [(M+H)$^+$] 1209.5407, found 1209.5409.

**22d**: mp 160—162 °C; $^1$H NMR (500 MHz, CD$_3$OD) δ 7.90 (s, 1H), 7.49—7.46 (m, 2H), 7.35—7.13 (m, 4H), 7.04 (s, 1H), 6.99 (dd, $J$ = 9.0, 2.5 Hz, 1H), 6.88 (dd, $J$ = 7.0, 6.5, Hz, 2H), 6.81 (d, $J$ = 7.5, Hz, 1H), 6.75—6.68 (m, 1H), 6.65 (s, 1H), 5.53 (s, 2H), 5.18 (s, 2H), 5.12 (d, $J$ = 3.0, Hz, 1H), 4.54—4.46 (m, 1H), 4.36 (dd, $J$ = 7.0, 5.5 Hz, 1H), 4.17 (dd, $J$ = 8.0, 5.0 Hz, 1H), 3.88 (dd, $J$ = 6.5, 5.5 Hz, 2H), 3.58—3.51 (m, 2H), 3.23 (s, 3H), 3.09—3.01 (m, 3H), 2.79 (dd, $J$ = 12.5, 5.0 Hz, 1H), 2.59 (d, $J$ = 12.5, Hz, 1H), 2.04 — 1.99 (m, 2H), 1.64 — 1.22 (m, 21H); HRMS (FAB) calcd for C$_{56}$H$_{63}$O$_7$N$_{11}$F$_5$S$_1$ [(M+H)$^+$] 1128.4553, found 1128.4562.

**22e**: mp 115—117 °C; $^1$H NMR (500 MHz, CD$_3$OD) δ 8.14 (s, 1H), 7.51—7.47 (m, 2H), 7.35—7.13 (m, 4H), 7.05—7.01 (m, 2H), 6.87 (ddd, $J$ = 7.0, 7.0, 7.0 Hz, 3H), 6.76—6.70 (m, 2H), 5.57 (s, 2H), 5.19 (s, 2H), 5.12 (s, 1H), 4.50 (dddd, $J$ = 14.0, 7.0, 7.0, 7.0 Hz, 1H), 4.35 (dd, $J$ = 7.5, 5.5 Hz, 1H), 4.17—4.08 (m, 3H), 3.76 (dd, $J$ = 4.0, 3.5 Hz, 2H), 3.60—3.52 (m, 6H), 3.37 (dd, $J$ = 6.0, 5.5 Hz, 2H), 3.27—3.19 (m, 5H), 3.07—3.02 (m, 1H), 2.80—2.76 (m, 1H), 2.58 (d, $J$ = 12.5 Hz, 1H), 2.01—1.96 (m, 2H) 1.57—1.24 (m, 9H); HRMS (FAB) calcd for C$_{54}$H$_{58}$O$_9$N$_{11}$F$_5$S$_1$ [(M+H)$^+$] 1132.4138, found 1132.4142.
**SPP reporter assay:** Cell-based SPP activity assay was performed as described previously (Nyborg et al., 2004). Briefly, expression vectors for ATF6-based SPP substrate and *Renilla* luciferase (phRL-TK, Promega) were cotransfected with ATF6 driven luciferase reporter (Yoshida et al., JBC 1998) into HEK293 cells, and incubated with each compound at indicated concentrations for 12 hours. Then cells were lysed and analyzed the luciferase activity. Relative levels of the luciferase activity compared with mock treatment were shown.