Membrane receptor probes: solid phase synthesis of biotin-Asp-PEG-arvanil derivatives.

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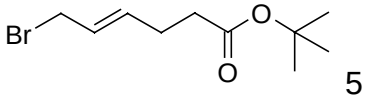
Supporting information

^{1}H and ^{13}C spectra were recorded on a Bruker AMX-300 spectrometer. Chemical shifts are reported as ppm to TMS as internal standard. Mass spectra were recorded on either a VG ZAB SE spectrometer (EI, FAB) or a Gilson- Finningan AQA LC-mass spectrometer using C-18 column (Hypersil BDS 100 x 4.6 mm, 5µm). Microanalysis was carried out by the Analytical Services Section, Department of Chemistry, University College London. Purification was by reverse-phase HPLC (Gilson) using preparative C-18 columns (Hypersil PEP 100 x 21 mm, 5µm). Compound **15** is commercially available but expensive; a convenient preparation is given below.

Preparation of the resin

6-Bromo-hex-4-enoic acid tert-butyl ester 5



5

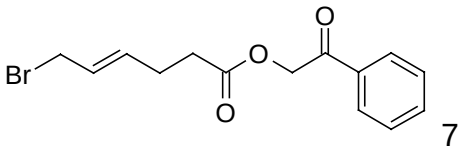
Acetic acid *tert*-butyl ester (5.00 g, 43 mmol) was dissolved in THF (100 mL) at –78°C. LDA (20.3 mL, 2M solution in THF) was added slowly keeping the temperature at –78°C and the reaction stirred for 1h. This reaction mixture was then added through cannula to a solution of dibromo-2-butane (18.3 g, 86 mmol) in THF (200 mL) at –78°C. The reaction mixture was then left to warm up to 20°C over 1.5 h. Cyclohexane (200 mL) was added and the mixture washed with 0.1 M hydrochloric acid (100 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure and the product was purified by column chromatography (SiO_2), eluting with cyclohexane / ethyl acetate (gradient 0 to 10% ethyl acetate) to give a yellow oil (9.18 g, 37 mmol, 86%). Previously prepared by Nakahara[1]

[1] Nakahara, Y.; Ando, S.; Itakura, M.; Kumabe, N.; Hojo, H.; Ito, Y.; Nakahara, Y. *Tetrahedron Letters* **2000**, *41*, 6489-6493

^{1}H NMR ($CDCl_3$); δ 1.33 (s, 9H), 1.98-2.42 (m, 4H), 3.81 (d, 2H, *J* = 5.6 Hz), 5.66 (m, 2H).

^{13}C NMR ($CDCl_3$); δ 27.84, 28.50, 34.99, 35.80, 80.84, 127.68, 127.68, 134.78, 172.36.

6-Bromo-hex-4-enoic acid 2-oxo-2-phenyl-ethyl ester 7



7

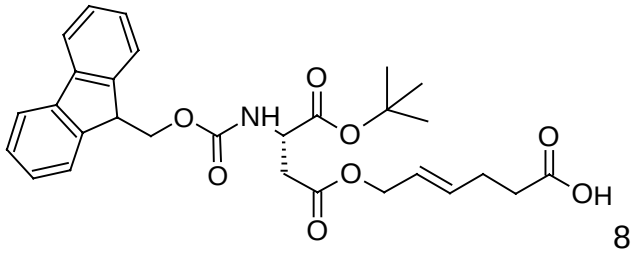
A solution of 6-bromo-hex-4-enoic acid *tert*-butyl ester **5** (9.18 g, 37 mmol) in trifluoroacetic acid was stirred at room temperature for 1h, then the trifluoroacetic acid was removed under vacuum and the residue was dissolved in dichloromethane. The organics were washed with 0.1 M hydrochloric acid (100 mL) and dried ($MgSO_4$). The solvent was removed and the crude acid product **6** was dissolved in DMF (38 mL). This solution was added to a suspension of potassium fluoride (6.10 g, 104.5 mmol) and phenacylbromide (8.55 g, 42 mmol) in DMF (95 mL) and stirred for 50 minutes at rt. Ethyl acetate (100 mL) was added and then the solution washed with saturated sodium hydrogen carbonate solution (2 x 50 mL). The crude material was purified by column chromatography (SiO_2, eluting with cyclohexane / ethyl acetate (80:20) to give **7** (6.02 g, 19.53 mmol, 52%).

^{1}H NMR ($CDCl_3$); δ 2. 39-2. 72 (m, 4H), 4.05 (d, 2H, *J* = 5.6 Hz), 5.38 (s, 1H), 5.81 -5. 92 (m, 2H), 7.65 (t, 1H, *J* = 7.3 Hz), 7.53 (t, 2H, *J* = 7.3 Hz), 7.95 (d, 2H, *J* = 7.2 Hz).

^{13}C NMR ($CDCl_3$); δ 27.63, 33.19, 33.47, 66.39, 128.15, 129.26, 134.16, 134.26, 134.66, 172.48, 192.46

Calculated $C_{14}H_{15}BrO_3$: C 54.02%, H 4.86%; found: C 54.35%, H 4.75%.

2-(9H-Fluoren-9-ylmethoxycarbonylamino)-succinic acid 1-tert-butyl ester 4-[5-(2-oxo-2-phenyl-ethoxycarbonyl)-pent-2-enyl]ester 8



8

To a stirred solution of Fmoc-Asp-O^{t}Bu (3.57 g, 8.67 mmol) in methanol (40 mL) was added potassium carbonate (0.60 g, 4.34 mmol) and the reaction stirred at rt for 45 min. The solvent was removed under vacuum and the residue dried by co-evaporating with toluene (3 x 20 mL). To the residue was added DMF (50 mL) followed by 6-bromo-hex-4-enoic acid 2-oxo-2-phenyl-ethyl ester **7** (2.46 g, 7.9 mmol) and the mixture was stirred at room temperature for 15 h. The solvent was than removed under vacuum and the residue was dissolved in dichloromethane (50 mL) and the mixture washed with a saturated solution of sodium hydrogen carbonate (2 x 30 mL). The organic solution was dried over sodium sulphate and the crude product purified by short column chromatography (SiO_2); eluting with dichloromethane / methanol (97/3) to give **8** (3.99 g, 6.23 mmol, 78.8%).

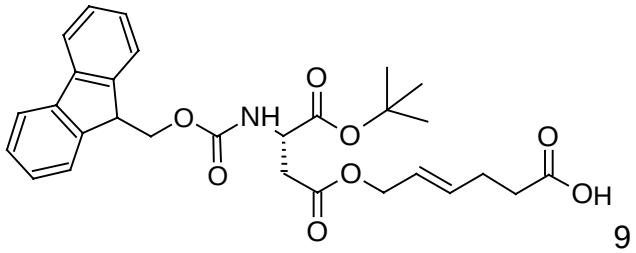
^{1}H NMR ($CDCl_3$); δ 1.46 (s, 9H), 2.41-2.48 (m, 2H), 2.55-2.60 (m, 2H), 2.81-3.03 (m, 2H), 4.21 (t, 1H, *J* = 7.0 Hz), 4.31-4.3 (m, 2H), 4.56 (d, 3H, *J* = 5.6 Hz), 5.59-5.69 (m, 1H), 5.79-5.89 (m, 2H), 7.25-7.47 (m, 6H), 7.58-7.60 (m, 3H), 7.74 (d, 2H, *J* = 7.4 Hz), 7.88 (d, 2H, *J* = 8.1 Hz).

^{13}C NMR ($CDCl_3$); δ 28.3, 33.49, 37.33, 47.58, 51.44, 53.85, 65.72, 66.39, 67.59, 82.98, 120.36, 125.29, 125.56, 127.48, 128.10, 129.24, 134.24, 134.51, 134.65, 141.69, 144.23, 144.33, 156.35, 169.98, 170.51, 172.51, 192.45.

MS (ES) *m/z* 642 (M+H).

Calculated $C_{37}H_{39}NO_9$: C 69.25%, H 6.13%, N 2.18%, found C69.40%, H 6.35%, N 1.99%

(E)-6-[3-tert-butoxycarbonyl-3-(2-9H-fluoren-9-yl-acetylamino)-propoxy]-hex-4-enoic acid 9



9

Zinc powder (5.2 g, 10 eq) was washed with 6 M hydrochloric acid (10 mL) and water. The zinc was then suspended in glacial acetic acid (20 mL) and a suspension of 2-(9H-fluoren-9-ylmethoxycarbonylamino)-succinic acid 1-tert-butyl ester 4-[5-(2-oxo-2-phenyl-ethoxycarbonyl)-pent-2-enyl]ester **8** (2.56 g, 4.0 mmol) in glacial acetic acid (20 mL) was added and the reaction mixture stirred at rt for 4 h. The zinc was filtered off and washed with glacial acetic acid (3 x 10 mL). The solvent was than removed under vacuum and the residue was purified by short column chromatography

(SiO_2); eluting with dichloromethane / methanol (95/5) to give **9** (1.407 g, 2.68 mmol, 67%) as a waxy hygroscopic solid, which was used directly for the next step.

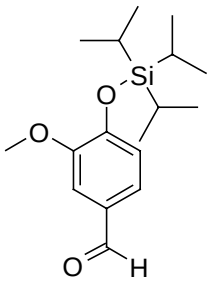
^{1}H NMR ($CDCl_3$); δ 167 (s, 9H), 2.37-2.48 (m, 4H), , 275-3.01 (m, 2H), 4.23 (t, 1H, J = 7.0 Hz), 4.30-4.3 (m, 2H), 4.56 (d, 3H, J = 5.6 Hz), 5.56-5.66 (m, 1H), 5.74-5.86 (m, 2H), 7.25-7.50 (m, 4H), 7.60 (d, 2H, J = 7.2 Hz), 7.75 (d, 2H, J = 7.4 Hz).

^{13} C NMR ($CDCl_3$); δ 27.89, 30.91,33.05, 36.97, 47.14, 50.99, 65.29, 67.25, 82.75, 119.98, 125.019, 125.17, 127.08, 127.72, 134.06, 141.30, 143.78, 156.03, 169.69, 170.57.

MS (ES) *m/z* 524 (M+H).

Preparation of arvanil precursor (see scheme 2 of manuscript)

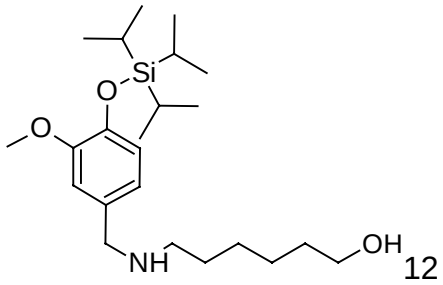
3-Methoxy-4-triisopropylsilanyloxy-benzaldehyde



Chloro-triisopropyl-silane (8.23 g, 9.1 mL, 42.72 mmol) was added to a stirred solution of 4-hydroxy-3-methoxy-benzaldehyde (5.00 g, 32.86 mmol) and imidazole (8.70 g, 131.44 mmol, 4eq) in THF (40 mL) at rt. After 16 h water (5 mL) was added, with further stirring for 10 min, and the solvent was removed to dryness. The residue was dissolved in dichloromethane (100 mL), and washed with 0.1 M hydrochloric acid (2 x 30mL) and brine (3 x 50 mL). The organic layer was dried ($MgSO_4$) and the solvent removed under reduced pressure. The product was purified by silica gel chromatography, eluting with cyclohexane / diethyl ether (50:50) to give 3-Methoxy-4-triisopropylsilanyloxy-benzaldehyde (9.80 g, 32.1 mmol, 98%) and used directly in the next step.

^{1}H NMR ($CDCl_3$); δ 1.34-1.24 (m, 18H), 1.69-1.49 (m, 3H), 4.14 (s, 3H), 7.29(d, 1H, *J* = 8.5 Hz), 7.65-7.59(m, 2H).

6-(3-Methoxy-4-tri-*tert*-butysilanyloxy-benzylamino)-hexan-1-ol, 12



12

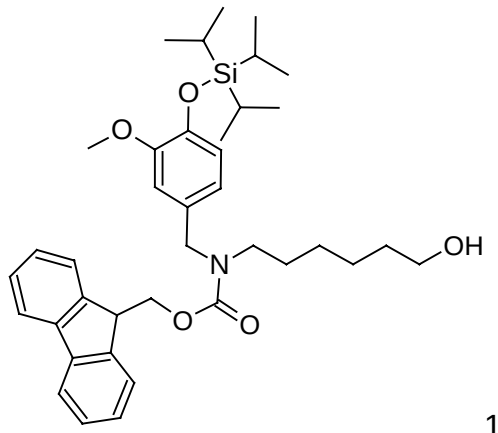
3-Methoxy-4-triisopropylsilanyloxy-benzaldehyde (5.00 g, 16.20 mmol) and 1-aminohexan-6-ol (1.90 g, 16.2 mmol) were dissolved in toluene (50 mL) and heated under reflux in a Dean-Stark apparatus for 1h. The reaction mixture was cooled to rt, the solvent removed and the residue dissolved in methanol (50 mL), sodium borohydride (1.50 g, 32.4 mmol) was added and the reaction mixture stirred at rt for 1 h. Dichloromethane (100 mL) was added and the organic layer was washed with water (3 x 50 mL) and then dried ($MgSO_4$). The product was purified by column chromatography (SiO_2), eluting with dichloromethane / methanol (gradient 0 to 3% methanol) to give **12** (4.31 g, 10.53 mmol, 65%).

^{1}H NMR ($CDCl_3$); δ 1.30-1.28 (m, 18H), 1.47-1.1.40 (m, 3H), 1.57-1.55 (m, 4H), 1.78-1.58 (m, 4H), 2.83 (t, 3H, *J* = 7.1 Hz), 3.81 (t, 3H, *J* = 6.6 Hz), 4.14 (s, 3H), 3.93 (s, 2H), 6.82-6.65(m, 3H).

^{13}C NMR ($CDCl_3$); δ 13.27, 18.27, 25.94, 27.39, 29.99, 33.01, 49.34, 51.10, 54.06, 55.91, 63.15, 112.80, 120.53, 120.86, 133.10, 145.04, 151.25.
Theoretical Mass: (M+H) 409.30120. Measured Mass: (M+H) 409.30140

MS (EI) m/z 410 $[M]^+$

(6-Hydroxy-hexyl)-(3-methoxy-4-tri-tert-butylsilanyloxy-benzyl)-carbamic acid 9H-fluoren-9-ylmethyl ester, 13

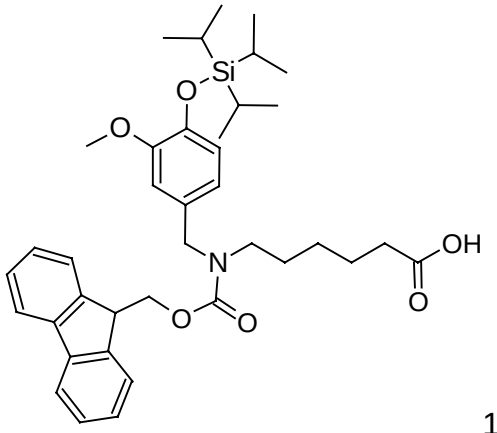


6-(3-Methoxy-4-tri-*tert*-butysilanyloxy-benzylamino)-hexan-1-ol, **12** (4.00 g, 9.77 mmol) was dissolved in a mixture of acetone (50 mL) and water (20 mL). Sodium hydrogen carbonate (1.63g, 19.54 mmol) was added and stirred at rt for 15 min then 9-fluorenylmethyl chloroformate (2.78 g, 10.74 mmol) was added and the reaction mixture stirred at room temperature for 20 h. The solvent was removed under vacuum and the residue was dissolved in ethyl acetate (100 mL). The organics were washed with water (3 x 50 mL) and then dried ($MgSO_4$) and the solvent removed under reduced pressure. The product was purified by column chromatography (SiO_2), eluting with dichloromethane / methanol (gradient 0 to 3% methanol) to give **13** as dark yellow oil (5.00 g, 7.90 mmol, 81%).

^{1}H NMR ($CDCl_3$); δ 1.11 (m, 18H), 1.49-1.1.23 (m, 9H), 1.55-1.49 (m, 4H), 3.11 (bs, 2H), 3.59 (t, 3H, *J* = 6.5 Hz), 3.93 (s, 3H), 4.24 (t, 1H, *J* = 6.1 Hz), 4.44 (s, 2H), 4.55 (d, 2H, *J* = 6.1 Hz), 6.59 (d, 1H, *J* = 7.5 Hz), 6.7 (s, 1H), 7.27 (t, 2H, *J* = 7.3 Hz), 7.38 (t, 2H, *J* = 7.3 Hz), 7.56 (d, 2H, *J* = 7.3 Hz), 76.81 (d, 1H, *J* = 8 Hz), 7.73 (d, 2H, *J* = 7.5 Hz),

^{13}C NMR ($CDCl_3$); δ 12.98, 17.86, 25.37, 26.54, 27.85, 32.68, 47.68, 50.31, 55.631, 62.69, 67.08, 119.88, 120.29, 124.83, 126.98, 127.57, 130.96, 141.47, 144.23, 145.15, 151.08, 156.48.
Theoretical Mass: (M+Na) 654.35905 Measured Mass: (M+Na) 654.36080

6-[(9H-Fluoren-9-ylmethoxycarbonyl)-(3methoxy-4-tri-*tert*-butylsilanoxy)-amine] hexanoic acid 14



14

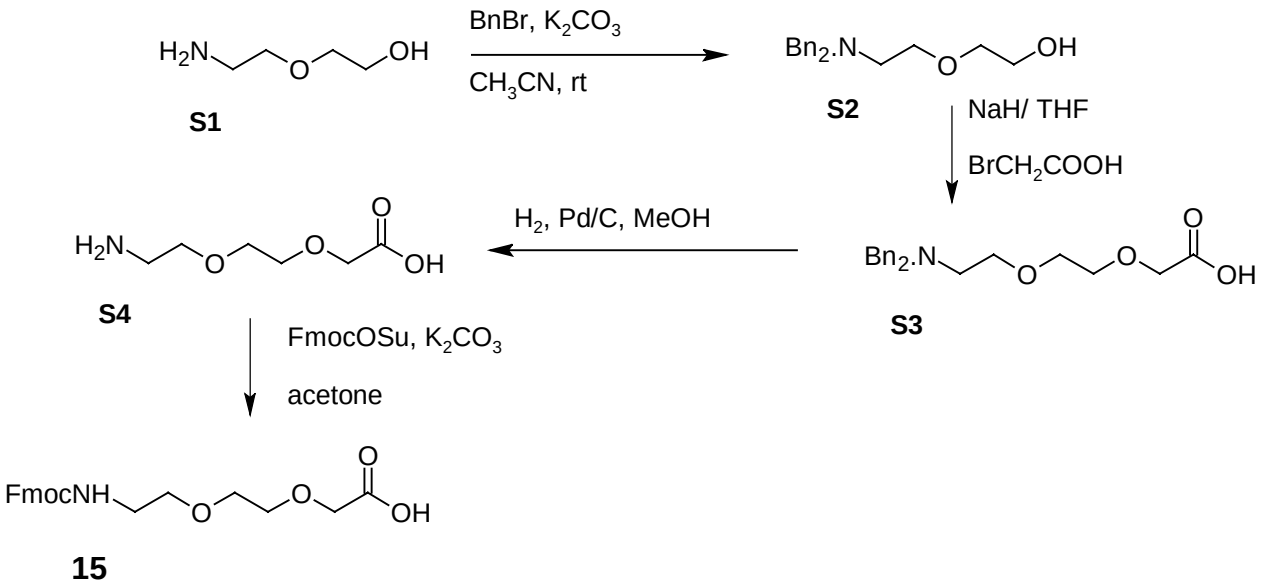
(6-Hydroxy-hexyl)-(3-methoxy-4-tri-*tert*-butylsilanyloxy-benzyl)-carbamic acid 9H-fluoren-9-ylmethyl ester **13** (4.67 g 7.38 mmol) was dissolved in a mixture of carbon tetrachloride, acetonitrile, water (30 mL, 30 mL, 45 mL). Sodium periodate (3.9 g, 22.14 mmol) and ruthenium trichloride monohydrate (0.090 g) were added and the reaction mixture was stirred at rt for 1 h. Ethyl acetate (100 mL) was added and the organic layer washed with water (3 x 50 mL), dried ($MgSO_4$) and the solvent removed under vacuum. The product was purified by column chromatography (SiO_2), eluting with

dichloromethane / methanol (gradient 0 to 1% methanol) to give **14** as a yellow oil (2.73g, 4.20 mmol, 57%).

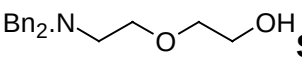
^{1}H NMR ($CDCl_3$); δ 1.11 (m, 18H), 1.30-1.1.20 (m, 9H), 1.55-1.40 (m, 4H), 2.30-2.26 (m, 2H), 3.10 (bs, 2H), 3.73 (s, 3H), 4.23 (t, 1H, *J* = 6.0 Hz), 4.34 (s, 2H), 4.55-4.50 (m, 2H), 6.56 (d, 1H, *J* = 7.5 Hz), 6.68 (s, 1H), 6.78 (d, 1H, *J* = 8 Hz), 7.27 (t, 2H, *J* = 7.3 Hz), 7.38 (t, 2H, *J* = 7.3 Hz), 7.56 (d, 2H, *J* = 7.3 Hz), 7.73 (d, 2H, *J* = 7.5 Hz).

^{13}C NMR ($CDCl_3$); δ 13.0, 17.89, 21.77, 24.44, 26.33, 27.61, 33.50, 47.70, 50.41, 55.65, 62.76, 119.91, 120.32, 124.84, 127.03, 127.61, 130.87, 141.51, 144.23, 145.20, 151.12, 201.8.
Theoretical Mass: (M+Na) 668.33832 Measured Mass: (M+Na) 668.33854

Scheme S1. Synthesis of the spacer {2-[3-(9H-fluoren-9-ylmethoxy)-3-oxopropoxy]ethoxy}acetic acid **15**



2-[(Dibenzylamino)ethoxy]ethanol, S2



S2

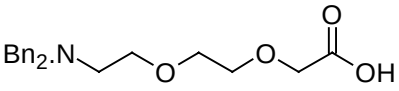
A mixture of 2-(aminoethoxy)ethanol (10.0 g, 95 mmol) , potassium carbonate (32.0 g, 237.5 mmol) and benzyl bromide (27.2 mL, 190 mmol) in acetonitrile (500 mL) was stirred at 50^{o}C for 20 h. The solid was filtered and the solvent was removed from the filtrate. The residue was dissolved in hydrochloric acid (0.1 M) and washed with ethyl acetate (2 x 50 mL). The aqueous layer was then made

basic with sodium hydroxide (1 M) and extracted with dichloromethane (4 x 50 mL). The solvent was dried ($MgSO_4$) and removed under vacuum, to give **S2**, the product was used in the next reaction without further purification (26.86 g, 94,1 mmol, 99%).

^{1}H NMR ($CDCl_3$); δ 2.85 (t, 2H, *J* = 5.9 Hz), 3.63 (t, 2H, *J* = 4.5 Hz), 3.73 (t, 2H, *J* = 5.9 Hz), 3.90-3.94 (m, 6H), 7.40-7.75 (m, 10H).

^{13}C NMR ($CDCl_3$); δ 53.54, 59.55, 70.16, 72.44, 127.28, 128.58, 128.98, 129.20, 139.95.

[2-(Dibenzylaminoethoxy)ethoxy]acetic acid S3



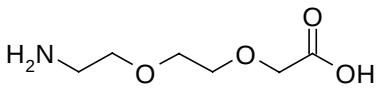
S3

S2 (26.82 g, 93 mmol) was dissolved in dry THF (200 mL) and cooled at 0^{o}C, then sodium hydride (12.5 g. 372 mmol; 60% dispersion in oil) was added in portions followed by α bromo acetic acid (19.45 g, 140 mmol). The suspension was refluxed under nitrogen overnight. Water (7 mL) was added carefully and then stirred for 5 min, then more water (200 mL) was added and the aqueous layer washed with a mixture of hexane/diethyl ether 1:1, (2 x 100 mL). The aqueous solution was acidified to pH 2-3 with hydrochloric acid (1M) and washed with diethyl ether (3 x 100 mL), then neutralized to pH 6/7 with sodium hydroxide (1M). Solid sodium chloride was added and the mixture was extracted with dichloromethane (5 x 150 mL). The solvent was dried and removed under vacuum, the crude product **S3** was used in the next reaction without further purification (25.12 g, 73 mmol, 79%).

^{1}H NMR ($CDCl_3$); δ 2.62 (t, 2H, *J* = 5.8 Hz), 3.22-3.32 (m, 2H), 3.34-3.61 (m, 4H), 3.68 (s, 4H), 3.88 (s, 2H), 7.040-7.36 (m, 10H).

^{13}C NMR ($CDCl_3$); δ 52.63, 59.14, 69.34, 69.69, 70.33, 71.39, 127.93, 128.78, 129.84, 137.53.

[2-(2Aminoethoxy)ethoxy]acetic acid



S4

S3 (25.0 g, 73 mmol) was dissolved in methanol (300 mL), palladium (12.5 g, 5% on carbon) was added and the mixture was stirred under hydrogen atmosphere (760 mm Hg) at 45^{o}C for 20 h. The

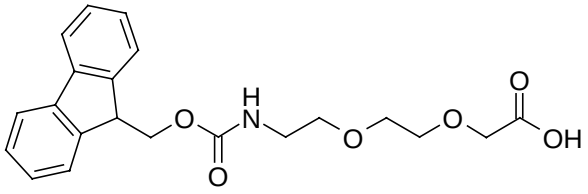
catalyst was eliminated by filtration through a celite pad and the solvent removed under vacuum. The residue was triturated with diethyl ether (200 mL) to give **S4** (10.70 g, 65.5 mmol, 90%).

^{1}H NMR (MeOD); δ 3.19-3.22 (m, 2H), 3.72-3.89 (m, 6H), 3.98 (s, 2H).

^{13}C NMR (MeOD); δ 40.85, 68.36, 71.53, 71.69, 72.07.

Theoretical Mass: (M+H) 164.09228 Measured Mass: (M+H) 164.09275

{2-[(9H-Fluoren-9-ylmethoxycarbonylamino)-methoxy]-ethoxy}-acetic acid, 15



15

The crude [2-(2Aminoethoxy)ethoxy]acetic acid (10.45 g, 65 mmol) was suspended in water (100 mL) and potassium carbonate (17.8 g, 129 mmol) and stirred at room temperature for 10 minutes. To the mixture N-(9-fluorenylmethoxycarbonyl) succinimide (21.9 g) was added and stirred for 16 h. If necessary the pH of the mixture was adjusted to pH 9 and then washed with diethyl ether (2 x 30 mL). The aqueous solution was acidified to pH 1-2 with hydrochloric acid, concentrated and extracted with dichloromethane (5 x 30 mL). The solvent was dried and removed under vacuum, the obtained product was crystallized from acetonitrile to give {2-[3-(9H-fluoren-9-ylmethoxy)-3-oxopropoxy]ethoxy}acetic acid **15** (16.3 g 42.3 mmol, 65%).

δ(^{1}H)($CDCl_3$); δ 3.25 (m, 2H), 3.60-3.74 (m, 3H), 4.15 (s, 1H), 4.17-4.24 (m, 1H), 4.40 (d, 2H, *J* = 6.5 Hz), 5.22 (m, 1H), 7.28-7.41 (m, 4H), 7.59 (d, 2H, *J* = 7.3 Hz), 7.75 (t, 2H, *J* = 7.3 Hz).

^{13}C NMR (MeOD); δ 42.16, 68.11, 69.51, 71.35, 71.719, 72.22.

Calculated $C_{21}H_{23}NO_6 \cdot 1/4H_2O$: C 64.69%, H 5.95%, N 3.59%; found: C 64.85%, H 6.11%, N 3.59%.

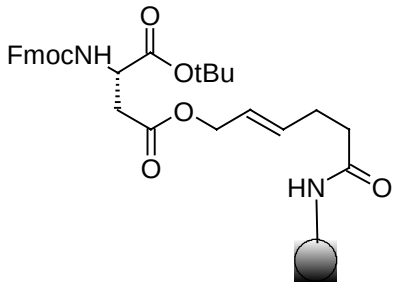
Theoretical Mass: (M+H) 386.16035 Measured Mass: (M+H) 386.16097

MS (EI) m/z 386 $[M]^+$

General conditions for the solid-phase synthesis:

All the coupling reactions were carried out at room temperature if no specifications are given. Solid-phase synthesis was performed manually using Isolute filtration reservoirs as the reaction vessel, fitted with polyethylene frits (Argonaut Technologies Inc). Each coupling step of the synthesis was assessed for completion using three different assays: the Kaiser test for primary and secondary amines, the TNBS test for primary amines and the chloranil test for secondary amines.[2]

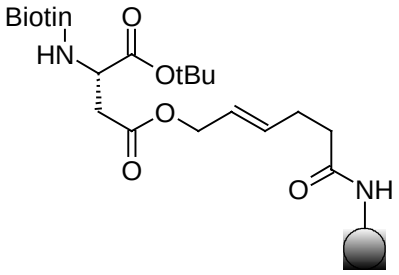
Coupling the linker 9 to the resin.



10

Argogel NH_2 resin (loading 0.43 mmol/g, 1.78 g, 0.767 mmol) in a 50 mL reaction vessel was swollen for 2 h in DMF than washed with DMF (3 x 10 mL). To a solution of acid **9** (1.40 g, 2.68 mmol) in DMF (20 mL), PyBop (1.196 g, 2.30 mmol) followed by *N* methyl morpholine (0.50 mL, 4.60 mmol) were added, and this mixture was added to the resin. The reaction mixture was shaken for 2 h and then the resin was filtered and washed with DMF (6 x 10 mL) followed by dichloromethane (6 x 10 mL). The resin (2.163 g, ~0.7 mmol) was dried under vacuum and stored at 4^oC. In this case both the tests Kaiser and TNBS were negative.

Attachment of the biotin to the linker10



16

Resin **10** (2.08 g, ~0.7 mmol) in a 50 mL reaction vessel was swollen for 2 h in DMF than washed with DMF (3 x 10 mL). A solution of piperidine (20%) in DMF (15 mL) was added, the mixture was shaken

[2] Kaiser, E., Colescott, R.L., Bossinger, C.D., Cook, P. *Anal. Biochem*. **1970**, *34*, 595-598,W. S. Hancock and J. E. Battersby, *Anal. Biochem*., **1976**, *71*, 260, Christensen, T.C. (**1979**) A chloroanil color test for monitoring coupling completeness in solid phase peptide synthesis. In *Peptides, Structure and*

for 20 min and then the resin filtered and washed with DMF (6 x 10 mL). To a solution of biotin (0.81 g, 3.34 mmol) in DMF (20 mL), was added PyBop (1.52 g, 3.04 mmol) followed by DIPEA (1.06 mL, mmol), and this mixture was added to the resin. The reaction mixture was shaken for 2 h and then the resin was filtered and washed with DMF (6 x 10 mL) followed by dichloromethane (6 x 10 mL). The resin **16** (2.163 g, ~0.7 mmol) was dried under vacuum and stored at 4^{o}C. Kaiser and TNBS test were negative.

General method for ^{t}butyl ester removal

Reaction vessels containing 50 μmol of resin each, were treated with 50% trifluoro-acetic acid in dichloromethane (2 mL), containing 2.5% of tri-n- propylsilane, and shaken for 1.5 h, followed by filtration and washing first with DCM (3 x 3mL), then DMF (6 x 3mL).

General method for PyBop amide couplings

PyBop (51 mg, 0.1 mmol) was dissolved in DMF (2 mL) and added to the resin (50 μmol) followed by *N*-methyl morpholine (22 μL, 0.2 mmol) and 4,7,10 trioxa-1,13-trideanediamine (22 μL, 0.1 mmol). The mixture was shaken for 1 h then the reactants were removed by filtration. The support was washed with DMF (6 x 3mL).

General method for Fmoc removal

The vessel containing 50 mmol of resin was shaken in a solution of piperidine (20%) in DMF (3 mL) for 20 min then the resin was filtered and washed with DMF (6 x 3 mL).

General procedure for coupling of arachidonic acid (double coupling)

A solution of arachidonic acid (0.076 g, 0.25 mmol) in DMF (1.3 mL), HATU (0.09 g, 0.237 mmol) followed by DIPEA (75 μL, 0.5 mmol) was stirred for 15 minutes and then added to the deprotected resin (50 μmol) (manuscript schemes 3 and 4). The reaction mixture was shaken for 1.5 h and then the

resin was filtered and washed with DMF (3 x 3 mL). This procedure was then repeated (double coupling). After the second coupling the chloranil test was negative.

General procedure for Tips-removal: Resin (50 μmol) was washed with THF (6 x 3 mL) and then shaken in a solution of triethylaminetrihydrofluoride in THF (0.16 mL of TATF in 1 mL of THF). The resin was washed with THF (3 x 3 mL) and DMF which was previously degassed (6 x 3 mL), using N_2 for the filtration.

General procedure for cleavage of the allyl linker: Resin (50 μmol) in a dark glass vessel with DMF (N_2 degassed 1 mL) and morpholine (22 μL, 0.25 mmol) and the mixture was degassed for another 5 min with N_2. Tetrakis(triphenylphosphine)palladium(0) (0.0164 g, 0.015 mmol) was added and the mixture was shaken overnight in the absence of light. The resin was filtered and washed with DMF (6 x 3 mL), and the filtrate was collected and the solvent removed under vacuum (Genevac). The residue was washed with diethyl ether and the products were purified by reverse phase HPLC chromatography.

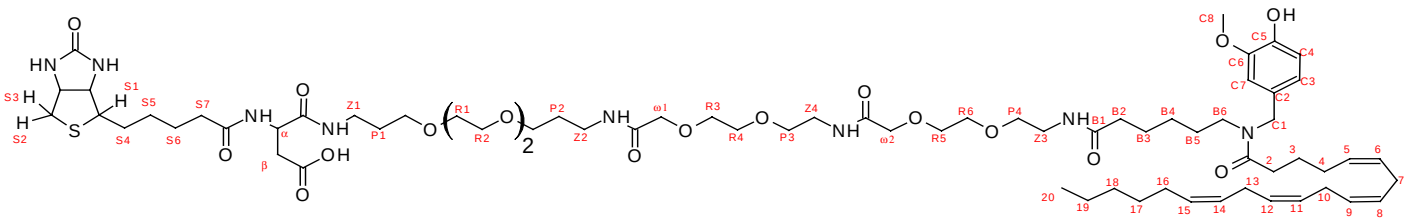
25

The compound was purified by HPLC chromatography using a gradient elution of 50-97% acetonitrile in 0.1% aqueous TFA solution over 22 min at a flow rate of 10 mL/min. Retention time 7.9 min.

LCMS (ES +ve) m/z 1243.6 $[M+H]^+$ (Retention time 13.30min.)

26

The compound was purified by HPLC chromatography using a gradient elution of 40-97% acetonitrile in 0.1 TFA aqueous solution over 22 min at a flow rate of 10 mL/min. Retention time 8.2 min.



The NMR spectrum of this analogue was completely assigned with the use of ^{1}H NMR and COSY.

^{1}H NMR ($CDCl_3$); δ 0.85 (m, 3H, ($CH_3$20)), 1.08-1.25 (8H, m, ($CH_2$17, 18) (CH_2B4, B5)), 1.2-1.6 (8H, m, (CH_2 P1, P2, P3, P4)), 1.25-1.5 (6H, (CH_2S4, S5, S6)),1.60 (2H, m, ($CH_2$3)), 1.77(2H, m, (CH_2B3)), 2.00-2.04 (4H, m, ($CH_2$4, 16)),2.08 (2H, m, (CH_2S7)), 2.34 (2H, m, (CH_2B2)), 2,36 (2H, m, ($CH_2$2)), 2.63 (6H, m, ($CH_2$7, 10, 13)), 2.74-2.94 (4H, m, (CH_2S2, S3)), 2.80 (2H, bs, b), 3.16 (2H, (CH_2 B6)), 3.17(1H, m, (CHS1)), 3.36 (8H, m, (CH_2 Z1, Z2, Z3, Z4)), 3.4-3.7 (12H, m, (CH_2 R1, R2, R3, R4, R5, R6)), 3.84-3.86 (3H, 1bs, (CH_3 C8)), 4.01 (4H, bs, ω1, ω2), 4.34 (1H, b, bridge), 4.42 (2H, b, (CH_2C1)), 4.53 (1H, b, bridge), 4.77 (1H, b, α), 5.28-5.42 (8H, m, (CH 5, 6, 8, 9, 11, 12, 14, 15)),6.63-6.69 (1H, bd, *J* 8Hz, Ar C2) 6.8-6.61 (1H, 2bs, Ar C3), 6.81-6.88(1H, d, *J* 8Hz, Ar C1), 7.2-7.66 (7H, b, NHCO).

LCMS (ES +ve) m/z 1388.8 $[M+H]^+$ (Retention time 12.76min.)

27

The compound was purified by HPLC chromatography using a gradient elution of 40-97% acetonitrile in 0.1 TFA aqueous solution over 22 min at a flow rate of 10 mL/min. Retention time 8.0 min.

LCMS (ES +ve) m/z 1534.1 $[M+H]^+$ (Retention time 12.11min.)

34

The compound was purified by HPLC chromatography using a gradient elution of 55-97% acetonitrile in 0.1 TFA aqueous solution over 22 min at a flow rate of 10 mL/min. Retention time 10.2 min.

LCMS (ES +ve) m/z 1495.0 $[M+H]^+$ (Retention time 16.72min.)

35

The compound was purified by HPLC chromatography using a gradient elution of 55-97% acetonitrile in 0.1 TFA aqueous solution over 22 min at a flow rate of 10 mL/min. Retention time 10.8 min.

MALDI m/z 1639.1 $[M]^+$

General procedure for coupling reactions to attach the crosslinking group

To a solution of the appropriate Fmoc amino acid (0.2 mmol) in DMF (1.3 mL), was added PyBop (0.076 g, 0.15 mmol) followed by *N*-methyl morpholine (44 μL, 0.4 mmol). This mixture was then added to the resin (50 μmol). The reaction mixture was shaken for 1 h and then the resin was filtered and washed with DMF (6 x 3 mL). Kaiser test and TNBS test were negative.

36

The compound was purified by HPLC chromatography using a gradient elution of 65-97% acetonitrile in 0.1 TFA aqueous solution over 22 min at a flow rate of 10 mL/min. Retention time 8.2 min.

LCMS (ES +ve) m/z 1371.7 $[M+H]^+$ (Retention time 16.43min.)

37

The compound was purified by HPLC chromatography using a gradient elution of 65-97% acetonitrile in 0.1 TFA aqueous solution over 22 min at a flow rate of 10 mL/min. Retention time 7.3 min.

LCMS (ES +ve) m/z 1517.1 $[M+H]^+$ (Retention time 15.28min.)

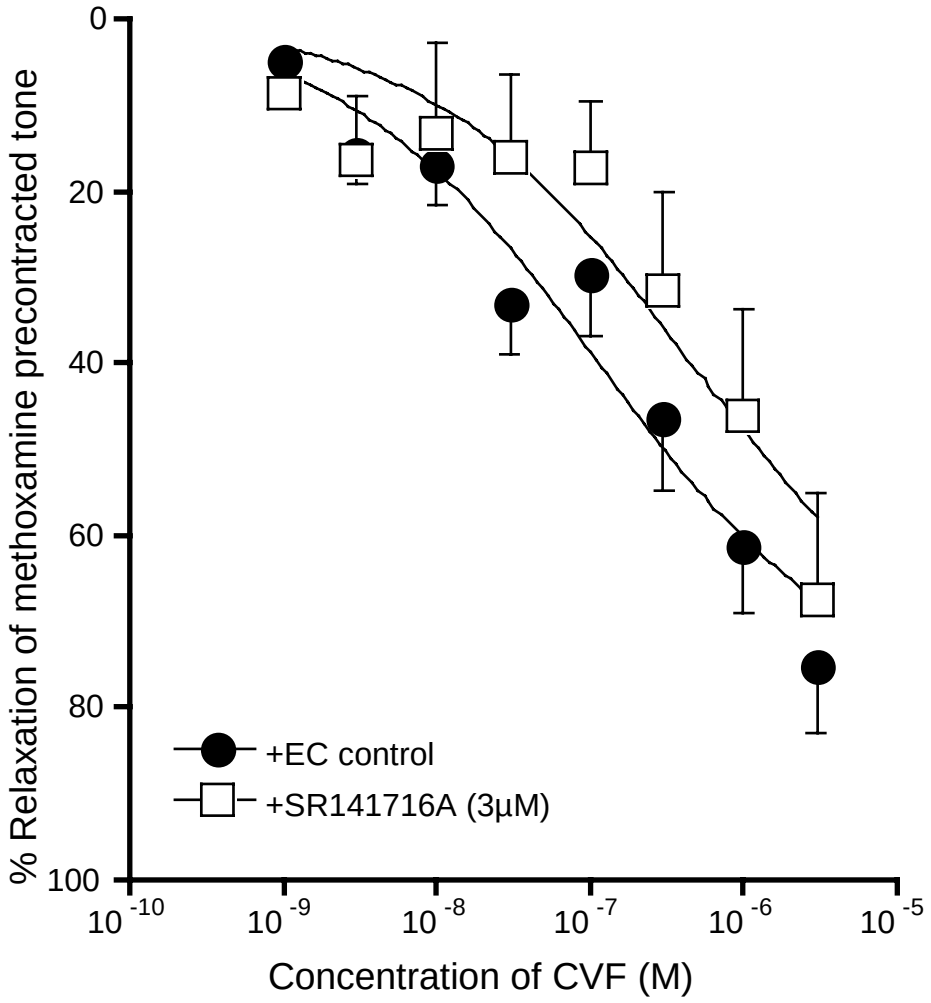
Methodology for binding of [^{3}H] SR141716A.

Preparation of rat cerebellar homogenates-Experiments were performed using cerebellum from Male Wistar rats weighing 175-250g. All efforts were made to reduce the number of animals used and all experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and the European Community Council Directive of 24 November 1986 (86/609/EEC). Following killing of

animals by stunning and decapitation the cerebellum was dissected into ice-cold 0.25 M sucrose. Cerebella were transferred to tubes containing 10 volumes of ice-cold 50 mM pH 7.4 HEPES buffer and homogenised for approximately 10 seconds using an Ultra-TurraxTM homogeniser. The resulting homogenate was centrifuged at 45,000 x g at 4° for 15 min and the pellet obtained following centrifugation resuspended in fresh ice-cold 50 mM pH 7.4 HEPES buffer as above. Following a further centrifugation step the final washed pellet was resuspended as above at a concentration of 2 mg/mL of original wet of cerebellum.

Binding of [^{3}H] SR141716A- binding of [3H] SR141716A.was carried out using 14 mL polypropylene test tubes. Assays were carried out in a final volume of 0.5 mL containing the compound under test (usually in DMSO to give a final concentration not exceeding 1% v/v), 100µL [3H] SR141716A at a final concentration of approximately 0.5 nM in 50 mM pH 7.4 HEPES buffer containing 0.1% v/v Tris(2-butoxyethyl) phosphate and 50 mM pH 7.4 HEPES buffer containing BSA such that the final assay concentration of BSA was 0.2% w/v. Incubations were initiated by the addition of cerebellar homogenate to give a final concentration of 0.8 mg/ mL original wet weight per tube. Samples were mixed and incubated @ 37° for 60 minutes and binding terminated by the addition of 5 mL ice-cold 50 mM pH 7.4 HEPES buffer followed immediately by vacuum filtration through Whatman GF/C glass fibre filters using a BrandelTM cell harvester. A further 3 x 5 mL of ice-cold wash buffer was added to each tube and the vacuum filtration step repeated. The GF/C glass fibre filters, containing bound [^{3}H] SR141617A, were transferred to minivials and 4 mL Picofluor40 liquid scintillant added using a BrandelTM deposit/dispense system. Radioactivity was measured using a Beckman Liquid Scintillation Counter and cpm converted directly to dpm via reference to appropriate quench parameters.

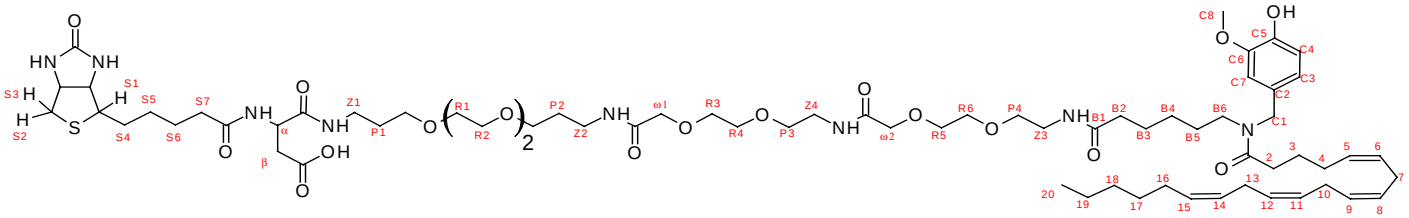
Figure S1. Effect of **36** on pre-contracted rat small mesenteric artery

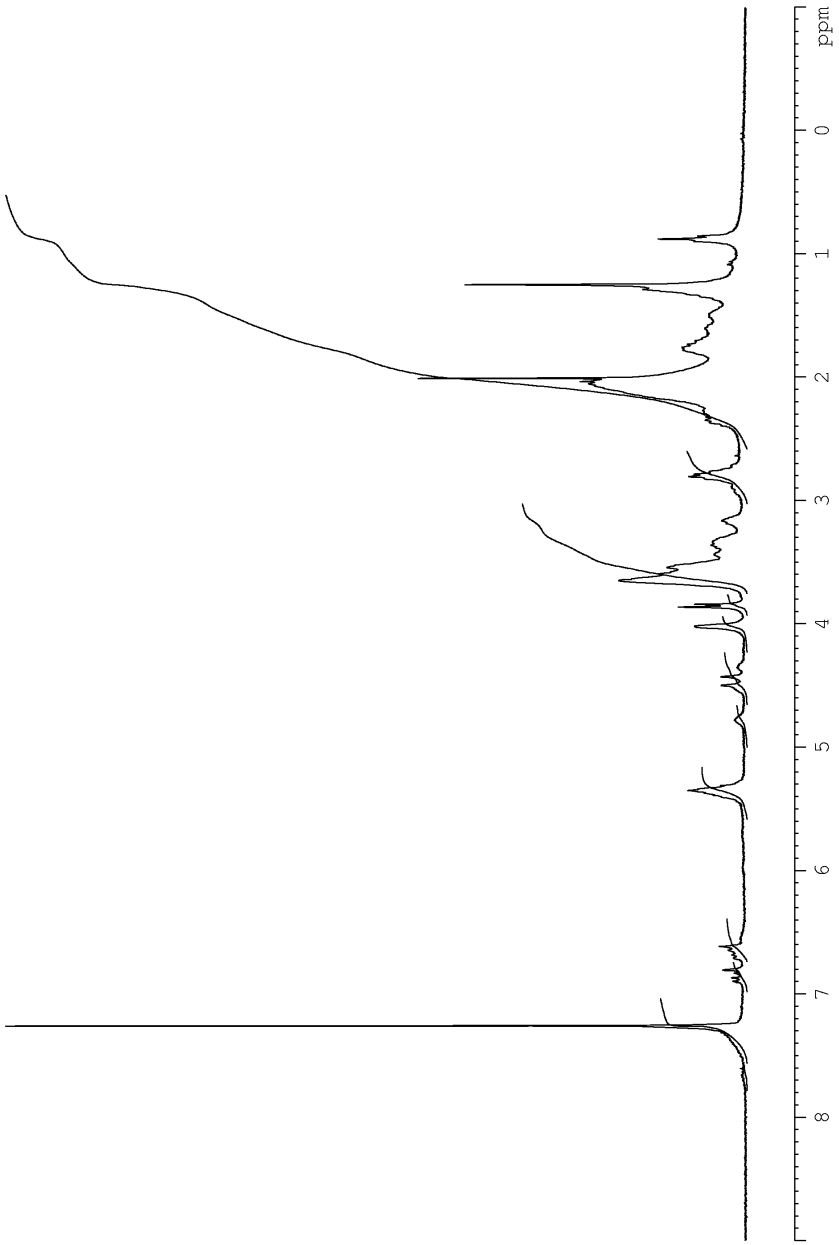


Paired experiments for CVF (36) control and after SR141716A inhibition. Experiments were carried out as described[3]. Preparation was rat third generation mesenteric artery with an intact endothelium; $n = 5$
Control: $EC_{50\%}$ $3x10^{-7}$M; +SR141716A: $EC_{50\%}$ 10^{-6}M
The shift of the curve is significant with $P < 0.05$.

[3] White, R.; Ho, W. S.; Bottrill, F. E.; Ford, W. R.; Hiley, C. R. *Br.J.Pharmacol.* **2001**, *134*, 921-929.

^{1}H NMR ($CDCl_3$) compound **26**





Two dimensional COSY ($CDCl_3$) compound **26**