The role of disulfide bond replacements in analogues of the tarantula toxin ProTx-II and their effects on inhibition of the voltage-gated sodium ion channel $Na_v1.7$

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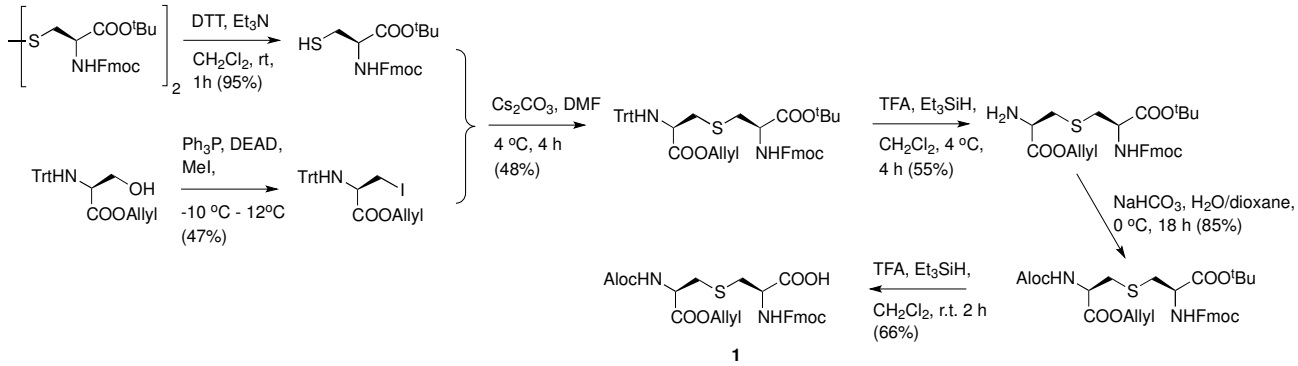
Supporting Information

Synthesis of (*2R, 6R*)(Aloc, Allyl/Fmoc) lanthionine 1



General Experimental

Unless otherwise indicated, all reagents were obtained from chemical suppliers with no further purification. Medium-scale preparation of (*S*)-Trt-Ser-OAllyl and (Fmoc-Cys-O^{t}Bu)$_2$ was outsourced to Oxygen Healthcare, Cambridge, UK, which is part of Piramal Enterprises Ltd, Mumbai, India, who produced 100 g of each compound following previously published procedures.[1,2]

Dry DMF, THF and CH_2Cl_2 were dried over anhydrous alumina columns,[3] with moisture levels typically below 15 ppm by Karl Fischer titration. Brine refers to a saturated solution of sodium chloride in water and sodium bicarbonate refers to a saturated solution of sodium hydrogen carbonate in water. Ether refers to diethyl ether and petrol to petroleum ether fractions boiling between 40 and 60 °C. All water used was either distilled using an Elga Purelab Option R 7 water purifier or used directly from a bottle of HPLC-grade water. All reactions were carried out in closed systems under Argon.

NMR spectra were recorded using a Bruker AC300, AV400, AC500 or AC600 spectrometer (300 MHz, 400 MHz, 500 MHz and 600 MHz respectively) and all samples were dissolved in deuterated chloroform unless otherwise stated. Chemical shifts (δ) are given in ppm units relative to tetramethylsilane and coupling constants (J) are measured in Hertz. Proton (^{1}H) NMR multiplicities are shown as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (double doublet), dt (double triplet), dq (doublet of quartets), dt (doublet of triplets), tt (triplet of triplets), br s (broad singlet), br d (broad doublet), ddt (doublet of doublet of triplets), dddt (doublet of doublet of doublet of triplets), dddd (doublet of doublet of doublet of doublets). ^{1}H and ^{13}C NMR were assigned using COSY, DEPT, HMBC and HSQC spectra.

MS refers to low resolution mass spectrometry and HRMS refers to high resolution mass spectrometry. MS was performed on a Waters Acquity UPLC SQD using HPLC grade water and acetonitrile (both with 0.1% formic acid) as the solvents. Electrospray ionization (ESI) accurate mass was determined using a Waters LCT Premier XE.

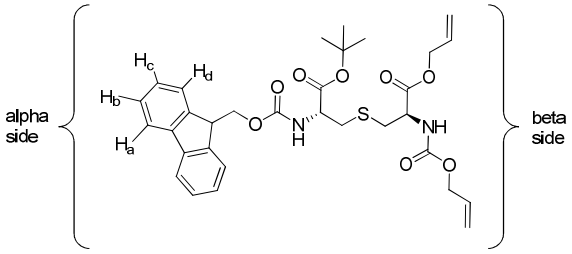
Infrared spectra were recorded using a Perkin Elmer 100 FT-IR spectrometer and Optical Rotations were recorded using a Perkin Elmer 343 polarimeter and are reported in 10^{-1} deg cm^2 g^{-1}. Melting points were recorded on a Gallenkamp Hot Stage apparatus and are uncorrected.

Flash column chromatography was carried out using silica gel with particle size <60 μm and reverse phase column chromatography was carried out using silica gel 60 silanized (53-200 μm). Thin layer chromatography (TLC) was performed on aluminium backed Sigma-Aldrich TLC plates with F254 fluorescent indicator. Developed plates were air dried and analysed under a UV light or by staining with the appropriate indicator.

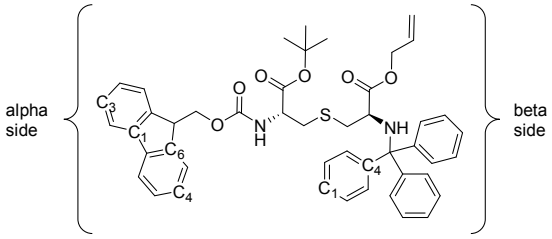
Large scale column chromatography was carried out using a Biotage Isolera 4 with either Biotage 340 g KP-Sil Snap Cartridges filled with 50 μm silica or Biotage 375 g KP-NH Snap Cartridges filled with amine-bonded silica.

The following nomenclature is used throughout when assigning NMRs:

^{1}H NMR:



^{13}C NMR:

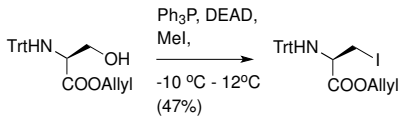


Safety Note

The large scale synthesis was carried out at the European Knowledge Centre of Eisai Ltd. in Hatfield, UK. When scaling up reactions, a number of problems can be encountered,[4,5] including localised exotherms and higher concentrations of reactants. This was particularly important for the Mitsunobu step to form (*R*)-Allyl 3-iodo-2-(trimethylphenylamino)propanoate, because the use of DEAD carries with it a risk of explosion. Although the replacement of DEAD with DIAD was investigated, none of the desired product formed, possibly due to the increased size of the ligand

preventing reaction occurring with the starting material. During optimization of this reaction it was observed that large temperature spikes could occur during the addition of DEAD and again during the addition of MeI, and in the large scale procedure both of these reagents were slowly added in CH_2Cl_2 solution with careful monitoring of the temperature. All other steps in the reaction sequence could be carried out in a similar manner to the small-scale synthesis previously reported.[1,2]

(R)-Allyl 3-iodo-2-(trimethylphenylamino)propanoate



(*S*)-Trt-Ser-OAllyl[1] (30.0 g, 77.4 mmol, 1 eq.) was dissolved in dry CH_2Cl_2 (66 mL) at room temperature in the presence of triphenylphosphine (30.5 g, 116.1 mmol, 1.5 eq.). The solution was cooled to -10 °C using an acetone/ice/water bath. The temperature was monitored throughout the reaction by the presence of a thermometer within the reaction vessel. Diethylazodicarboxylate (18.3 mL, 116 mmol, 1.5 eq.) in CH_2Cl_2 (20 mL) was then added dropwise over 45 min, ensuring the temperature did not exceed 12 °C. The reaction was left for 5 min before the addition of methyl iodide over 5 min (7.20 mL, 116 mmol, 1.5 eq.) in CH_2Cl_2 (10 mL). The reaction was left to stir for a further 3 h and then purified directly by column chromatography (Biotage KP-SIL 340 g snap column, 0 – 50% ethyl acetate in hexane over 15 column volumes) to yield the product as a pale yellow viscous oil (18.1 g, 36.4 mmol, 47%). R*f* = 0.35 (hexane : ethyl acetate 20 : 1).

NMR: δ_H (400 MHz, $CDCl_3$) 7.50 (6H, m, trityl) 7.27 (9H, m, trityl), 5.77 (1H, ddt, *J* 17.2, 10.5, 6.0 Hz, $CH_2CH{=}CH_2$), 5.22 (2H, m, $CH_2CH{=}CH_2$), 4.27 (1H, dddd, *J* 13.0, 6.0, 1.5, 1.2 Hz, $CHH'CH{=}CH_2$), 4.13 (1H, dddd, *J* 13.0, 6.0, 1.5, 1.2 Hz, $CHH'CH{=}CH_2$), 3.54 (1H, m, $CHCH_2I$), 3.35 (1H, dd, *J* 9.8, 3.5 Hz, $CHCHH'I$), 3.23 (1H, dd, *J* 9.8, 6.0 Hz, $CHCHH'I$), 2.92 (1H, d, *J* 9.8 Hz, NH).

δ_C (100 MHz $CDCl_3$) 171.9 (*C*OOallyl), 145.6 (*C4* trityl), 131.7 ($CH_2CH{=}CH_2$), 128.7 (*C1* trityl), 128.1 (*C2* trityl), 126.7 (*C3* trityl), 118.7 ($CH_2CH{=}CH_2$), 71.2 (CPh_3), 66.0 ($CH_2CH{=}CH_2$), 56.3 ($CHCH_2I$), 9.8 ($CHCH_2I$).

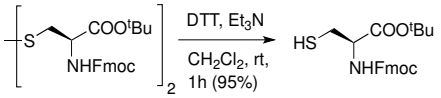
MS: m/z (CI^+) 498 ($[M+H]^+$, 15%), 420 ($[M-Ph]^+$, 60%), 243 ($[CPh_3]^+$, 100%);

HRMS: $C_{25}H_{25}NO_2I$ ($[M+H]^+$) requires 498.0930, found 498.0925.

ν_{max} ($CHCl_3$) 3057 (NH stretch, broad), 1735 (C=O stretch).

$[\alpha]_D^{20}$ +0.8 ° (c 1.0, CH_3OH).

(*R*)-Fmoc-Cys-O^{t}Bu[6]



Dithiothreitol (8.71 g, 56.5 mmol, 1.5 eq.) was added to a stirred solution of (Fmoc-Cys-O^{t}Bu)$_2$[2] (30.0 g, 37.6 mmol, 1 eq.) in dry CH_2Cl_2 (450 mL). To this, triethylamine (7.90 mL, 56.5 mmol, 1.5 eq.) was added and the solution was left to stir for 1 h. The solution was then washed with sodium bicarbonate (2 x 400 mL) and water (2 x 400 mL), dried ($MgSO_4$) and concentrated *in vacuo* to yield the product as a colourless oil (28.7 g, 71.9 mmol, 95%).

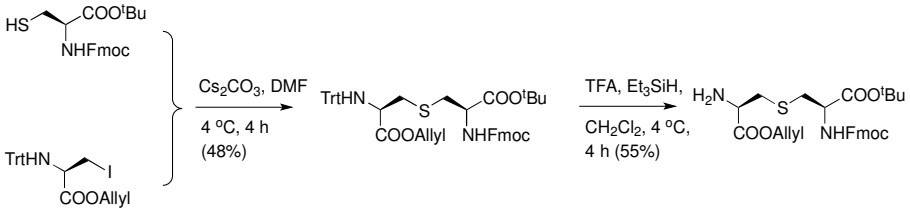
NMR: δ_H (600 MHz $CDCl_3$) 7.77 (2H, d, *J* 7.4 Hz, Fmoc H_a) 7.62 (2H, d, *J* 7.4 Hz, Fmoc H_d), 7.41 (2H, t, *J* 7.4 Hz, Fmoc H_b), 7.33 (2H, t, *J* 7.4 Hz, Fmoc H_c) 5.76 (1H, d, *J* 7.2 Hz, N*H*), 4.56 (1H, m, SCH_2CH), 4.41 (2H, d, *J* 6.9 Hz, CH_2Fmoc), 4.24 (1H, t, *J* 6.9 Hz, C*H*Fmoc), 3.00 (2H, td, *J* 8.6, 4.1 Hz, SCH_2CH), 1.51 (9H, s, *t*Bu).

δ_C (125 MHz $CDCl_3$) 169.1 (*C*OO*t*Bu), 155.8 (NH*C*OO), 144.0 (*C1* Fmoc), 141.5 (*C6* Fmoc), 127.9 (*C2* Fmoc), 127.2 (*C5* Fmoc), 125.3 (*C4* Fmoc), 120.2 (*C3* Fmoc), 83.2 ($C(CH_3)_3$), 67.2 (CH_2 Fmoc), 55.6 ($CHCH_2SH$), 47.3 (*C*H Fmoc), 28.2 ($C(CH_3)_3$), 27.5 (CH_2SH).

MS: m/z (CI$^+$) 400.2 ([M+H]$^+$, 10%), 313 ([M-OtBu]$^+$, 50%), 179 ([FmocNHC]$^+$, 100%); HRMS $C_{22}H_{26}O_2NSH$ ([M+H]+) requires 400.1583, found 400.1587.

$[\alpha]_D^{20}$ -16.9° (c 1.0, CH_3OH)

(2R, 6R)-(Allyl/Fmoc, ^{t}Bu) lanthionine



(*R*)-Allyl 3-iodo-2-(trimethylphenylamino)propanoate (18.1 g, 36.4 mmol, 1 eq.) and (*R*)-Fmoc-Cys-O^{t}Bu (14.5 g, 36.4 mmol, 1 eq.) were dissolved in DMF (300 mL) at 4 °C. Dry caesium carbonate (5.95 g, 18.2 mmol, 0.5 eq.) was added and the reaction stirred for 2 h before the addition of a second portion of caesium carbonate (5.95 g, 18.2 mmol, 0.5 eq.). The reaction was left to stir for a further 2 h and then excess ethyl acetate (250 mL) was added and the mixture washed with cold citric acid (4 °C, 5% aq. w/v, 3 x 250 mL). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. Partial

purification by column chromatography (Biotage KP-SIL 340 g snap column, 0 – 50% ethyl acetate in hexane over 15 column volumes) yielded *(2R, 6R)-(Trt, Allyl/Fmoc, ^{t}Bu) lanthionine* as a white solid (13.5 g, 17.6 mmol, 48%):

R_f = 0.4 (hexane : ethyl acetate 4 : 1)

NMR: δ_H (600 MHz $CDCl_3$) 7.75 (2H, m, Fmoc H_a) 7.60 (2H, m, Fmoc H_d), 7.49 (6H, d, *J* 7.6 Hz, trityl), 7.39 (2H, m, Fmoc H_c), 7.29 (2H, m, Fmoc H_b), 7.24 (6H, t, *J* 7.7 Hz, trityl), 7.17 (3H, t, *J* 7.3 Hz, trityl), 5.70 (1H, ddt, *J* 17.0, 10.7, 7.1 Hz, CH_2CH=CH_2), 5.63 (1H, d, *J* 7.9 Hz, N*H*), 5.20 (2H, m, CH_2CH=CH_2), 4.49 (1H, m, SCH_2CH α side), 4.35 (2H, m, CH_2Fmoc), 4.21 (1H, t, *J* 7.1 Hz, C*H*Fmoc), 4.14 (1H, dd, *J* 13.1, 7.4 Hz, C*H*H'CH=CH_2), 3.99 (1H, dd, *J* 13.1, 7.1 Hz, CH*H'*CH=CH_2), 3.55 (1H, m, SCH_2CH β side), 2.97 (1H, d, *J* 7.4 Hz, SCH_2CH α side), 2.81 (1H, m, SCH_2CH β side), 1.59 (1H, br s, N*H*), 1.48 (9H, s, *t*Bu).

δ_C (150 MHz $CDCl_3$) 173.1 (*C*OO*t*Bu), 169.7 (*C*OOFmoc), 155.8 (*C*OOallyl), 145.9 (*C4* trityl), 143.9 (*C1* Fmoc), 141.4 (*C6* Fmoc), 131.8 (CH_2CH=CH_2), 128.9 (trityl), 128.1 (trityl), 127.8 (Fmoc), 127.4 (trityl) 127.2 (Fmoc), 126.7 (trityl), 125.3 (Fmoc), 120.1 (*C5* Fmoc), 118.8 (CH_2CH=CH_2), 83.0 (C*t*Bu), 71.3 (*C*Ph_3), 67.2 (*C*H_2 Fmoc), 65.9 (*C*H_2CH=CH_2), 56.6 (*C*HCOOallyl), 54.4 (*C*HCOO*t*Bu), 47.2 (*C*H Fmoc), 38.4 (S*C*H_2 Fmoc side), 36.1 (S*C*H_2 trityl side), 28.1 (*C*H_3).

MS: m/z (ES^+) 791.3 ($[M+Na]^+$, 10%), 243.1 ($[CPh_3]^+$, 100%); HRMS $C_{47}H_{48}N_2O_6S$ ($[M+Na]^+$) requires 791.3131, found 791.3148.

ν_{max} ($CHCl_3$) 3404 (NH stretch, broad), 2978 (CH stretch, broad), 1724 (C=O stretch).

Melting point: 57-59 °C.

$[\alpha]_D^{20}$ +8.0 (c 1.0, CH_3OH).

A solution of trifluoroacetic acid (20.4 mL, 265 mmol, 10 eq.) in CH_2Cl_2 (50 mL) was added to a stirred solution of triethylsilane (42.2 mL, 265 mmol, 10 eq.) and (*2R, 6R*)-(Trt, Allyl/Fmoc, ^{t}Bu) lanthionine (20.3 g, 26.5 mmol, 1 eq.) in CH_2Cl_2 (150 mL) on ice. The temperature inside the reaction vessel was maintained at 20 °C throughout the addition. The temperature was monitored throughout the reaction by the presence of a thermometer within the reaction vessel. Once addition was complete, the ice bath was removed and the reaction was left to stir at room temperature for 4 h. After this time, excess CH_2Cl_2 (200 mL) was added and the organic layer was washed with sodium bicarbonate (2 x 200 mL) and brine (200 mL) before being dried over $MgSO_4$ and concentrated *in vacuo*. The product was then purified by column chromatography (Biotage KP-NH 375 g snap column, 20 – 80% ethyl acetate in hexane over 10 column volumes) to yield the title compound as a colourless oil (7.61 g, 14.5 mmol, 55%).

R_f = 0.25 (2% MeOH in CH_2Cl_2).

NMR: δ_H (600 MHz $CDCl_3$) 7.76 (2H, m, Fmoc H_a) 7.61 (2H, m, Fmoc H_d), 7.40 (2H, t, *J* 7.4 Hz, Fmoc H_b), 7.31 (2H, t, *J* 7.4 Hz, Fmoc H_c), 6.09 (1H, m, N*H*), 5.88 (1H, ddt, *J* 17.2, 10.9, 5.7 Hz, CH_2CH=CH_2), 5.28 (2H, m, CH_2CH=CH_2), 4.61 (2H, m, CH_2CH=CH_2), 4.51 (1H, m, C*H*NH_2), 4.38 (2H, d, *J* 7.1 Hz, CH_2Fmoc), 4.23 (1H, t, *J* 7.1, C*H*Fmoc), 3.71 (1H, m, SCH_2CH β side), 3.02 (3H, m, SCH*H'*CH α side and SCH_2CH β side), 2.85 (1H, m, SC*H*H'CH α side), 1.49 (9H, s, *t*Bu).

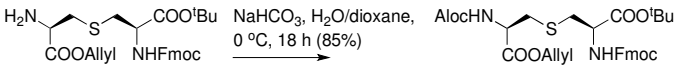
δ_C (150 MHz $CDCl_3$) 173.0 (*C*OO*t*Bu), 169.5 (*C*OOFmoc), 155.8 (*C*OOallyl), 143.9 (*C1* Fmoc), 141.4 (*C6* Fmoc), 131.5 (CH_2CH=CH_2), 127.9 (*C4* Fmoc), 127.7 (*C3* Fmoc), 125.1 (*C2* Fmoc), 120.0 (*C5* Fmoc), 119.1 (CH_2CH=CH_2), 82.3 (C*t*Bu), 67.1 (*C*H_2 Fmoc), 66.1 (*C*H_2CH=CH_2), 54.6 (*C*HNH2), 50.1 (*C*HNHFmoc), 47.1 (*C*H Fmoc), 37.8 & 35.6 (SCH_2 α and β sides), 28.0 (*C*H_3).

MS: m/z (ES^+) 527.2 ($[M+H]^+$, 85%), 471.2 ($[M+H\text{-}tBu]^+$, 100%); HRMS $C_{28}H_{35}N_2O_6S$ ($[M+H]^+$) requires 527.2216, found 527.2216.

ν_{max} ($CHCl_3$) 3347 (NH stretch, broad), 2980 (CH stretch, broad), 1723 (C=O stretch).

$[\alpha]_D^{20}$ -11.0 (c 1.0, CH_3OH).

(2R, 6R)-(Aloc, Allyl/Fmoc, ^{t}Bu) lanthionine



Sodium bicarbonate (6.68 g, 39.8 mmol, 4 eq.) was added to a solution of (*2R, 6R*)-(Allyl/Fmoc, ^{t}Bu) lanthionine (10.5 g, 19.9 mmol, 1 eq.) in 1,4-dioxane (120 mL). The reaction was cooled to 0 °C and allyl chloroformate (4.24 mL, 39.8 mmol, 2 eq.) was added. The reaction was left to stir for 18 h before the addition of excess CH_2Cl_2 (250 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (2 x 150 mL) and brine (150 mL), dried over $MgSO_4$ and concentrated *in vacuo* to yield the title product as a colourless oil (10.3 g, 16.9 mmol, 85%).

NMR: δ_H (500 MHz $CDCl_3$) 7.77 (2H, d, *J* 7.5 Hz, Fmoc H_a) 7.61 (2H, d, *J* 7.5 Hz, Fmoc H_d), 7.40 (2H, t, *J* 7.5 Hz, Fmoc H_b), 7.31 (2H, t, *J* 7.5 Hz, Fmoc H_c), 5.89 (2H, m, CH_2CH=CH_2 allyl and Alloc), 5.64 (1H, m, N*H*), 5.32 (1H, m, CH_2CH=CH*H'* allyl), 5.29 (1H, m, CH_2CH=CH*H'* Alloc), 5.24 (1H, m, CH_2CH=C*H*H' allyl), 5.19 (1H, m, CH_2CH=C*H*H' Alloc), 4.64 (2H, d, *J* 5.7 Hz, CH_2CH=CH_2 allyl), 4.57-4.59 (3H, m, C*H*NH Alloc and CH_2CH=CH_2 Alloc), 4.48 (1H, m,

C*H*NH Fmoc), 4.40 (2H, m, CH_2 Fmoc), 4.24 (1H, t, *J* 7.5 Hz, C*H* Fmoc), 2.93-3.04 (4H, m, SCH_2CH both sides), 1.48 (9H, s, *t*Bu).

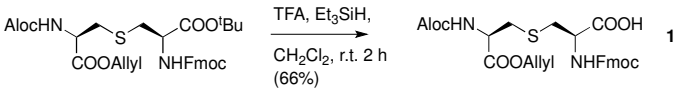
δ_C (125 MHz $CDCl_3$) 170.3 (*C*OOAlloc), 169.5 (*C*OOFmoc), 155.9 (*C*OO^{t}Bu), 155.8 (*C*OOallyl), 144.0 (*C1* Fmoc), 141.4 (*C6* Fmoc), 132.6 (CH_2CH=CH_2 allyl), 131.4 (CH_2CH=CH_2 Alloc), 128.4 (*C3* Fmoc), 127.9 (*C4* Fmoc), 125.3 (*C5* Fmoc), 120.1 (*C2* Fmoc), 119.5 & 118.1 (CH_2CH=CH_2 allyl and Alloc), 83.3 (*C*^{t}Bu), 67.3 (*C*H_2 Fmoc), 66.6 & 66.1 (*C*H_2CH=CH_2 allyl and Alloc), 54.4 (*C*HNH Fmoc), 53.9 (*C*HNH Alloc), 47.2 (*C*H Fmoc), 35.7 & 35.5 (S*C*H_2 both sides), 28.1 (*C*H_3).

MS: m/z (ES^+) 633.2 ([M+Na]$^+$, 100%), 577.2 ([M+Na-O^{t}Bu]$^+$, 50%); HRMS $C_{32}H_{38}N_2O_8SNa$ ([M+Na]$^+$) requires 633.2247, found 633.2249.

ν_{max} ($CHCl_3$) 3336 (NH stretch, broad), 2980 (CH stretch, broad), 1714 (C=O stretch).

$[\alpha]_D^{20}$ -27.6 (c 1.0, CH_3OH).

(2R, 6R)-(Aloc, Allyl/Fmoc) lanthionine 1



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(*2R, 6R*)-(Aloc, Allyl/Fmoc, ^{t}Bu) lanthionine (5.00 g, 8.19 mmol. 1 eq.) was dissolved in a solution of trifluoroacetic acid (50 mL, 650 mmol, 79 eq.), triethylsilane (1.31 mL, 8.19 mmol, 1 eq.) and CH_2Cl_2 (50 mL) and stirred for 2 h before concentrating *in vacuo*. Removal of trifluoroacetic acid was aided by the addition of toluene (20 mL). The product was then re-dissolved in toluene (50 mL) with heating and then left in the freezer for 72 h. After this time, the toluene was decanted off to give the product as a white solid. The filtrate was concentrated and recrystallised for a second time. The solid was filtered and combined with the previous crystallisation to afford the desired product as a white solid (3.00 g, 5.42 mmol, 66%).

NMR: δ_H (600 MHz $CDCl_3$) 9.40 (1H, br s, COO*H*), 7.75 (2H, m, H_a) 7.56 (2H, m, H_d), 7.38 (2H, m, H_b), 7.30 (2H, t, *J* 7.4, H_c), 6.86 (1H, d, *J* 6.9, N*H*), 5.97 (1H, d, *J* 7.3, N*H* Fmoc), 5.79- 5.89 (2H, m, CH_2CH=CH_2 allyl and Alloc), 5.16-5.35 (4H, m, CH_2CH=CH_2 allyl and Alloc), 4.60- 4.63 (2H, m, SCH_2CH both sides), 4.53-4.58 (4H, m, CH_2CH=CH_2 allyl and Alloc), 4.39 (2H, m, CH_2Fmoc), 4.22 (1H, t, *J* 6.8 C*H*Fmoc), 2.98-3.14 (4H, m, SCH_2CH both sides).

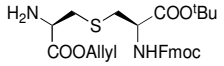
δ_C (150 MHz $CDCl_3$) 173.8 (*C*OOAlloc), 170.4 (*C*OOFmoc), 157.6 (*C*OOH), 156.2 (*C*OOallyl), 143.8 (*C6* Fmoc), 141.4 (*C1* Fmoc), 132.4 (CH_2*C*H=CH_2 allyl), 131.3 (CH_2*C*H=CH_2 Alloc), 127.9 (*C3* Fmoc), 127.2 (*C4* Fmoc), 125.3 (*C5* Fmoc), 120.1 (*C2* Fmoc), 119.5 (CH_2CH=*C*H_2 allyl), 118.3 (CH_2CH=*C*H_2 Alloc), 67.7 (*C*H_2 Fmoc), 66.7 (*C*H_2CH=CH_2 allyl), 66.2 (*C*H_2CH=CH_2 Alloc), 54.5 (*C*HNHFmoc), 53.8 (*C*HNHAlloc), 47.2 (*C*H Fmoc), 35.2 & 35.0 (S*C*H_2 both sides).

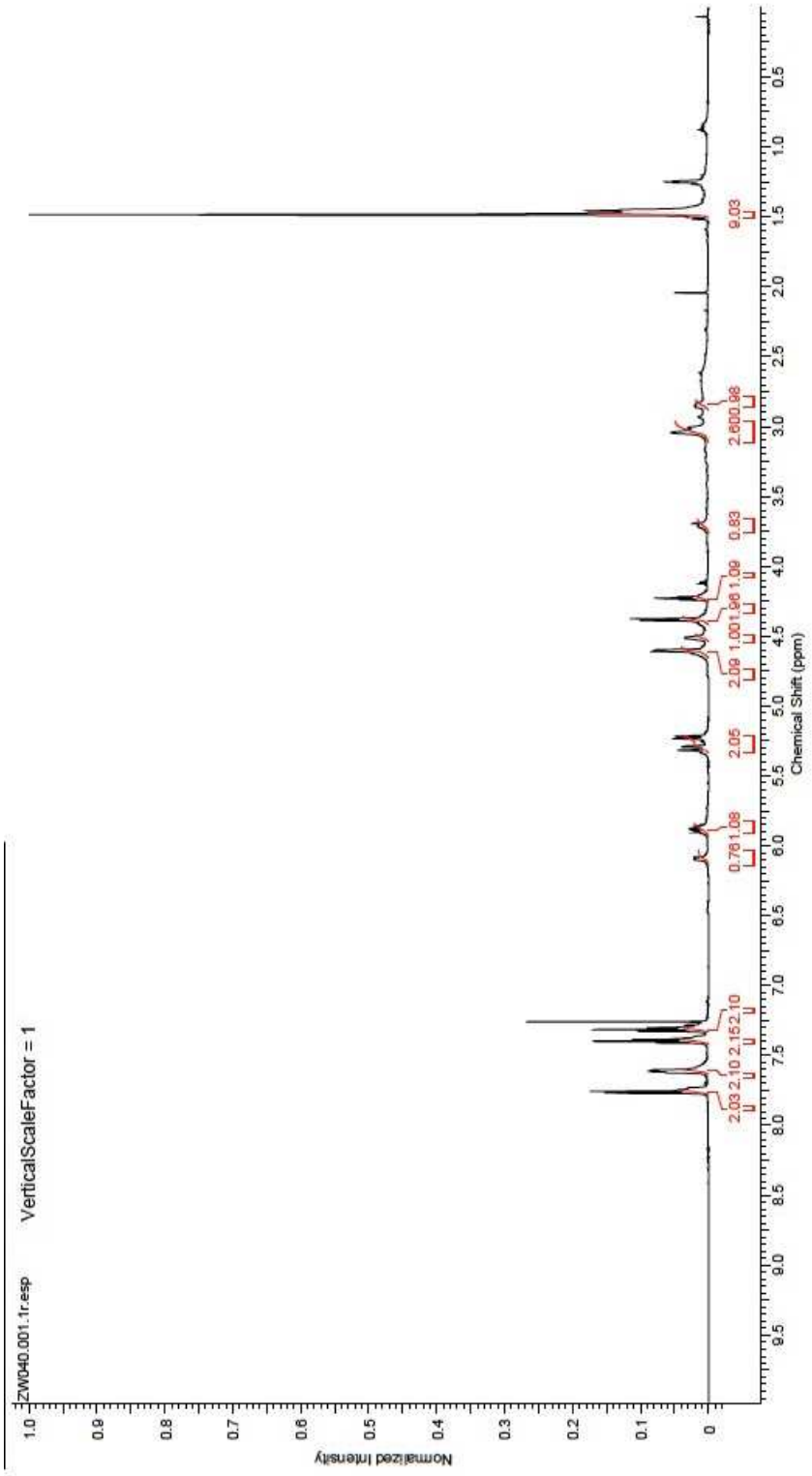
MS: m/z (ES^+) 577.2 ($[M+Na]^+$, 100%); HRMS $C_{28}H_{30}N_2O_8SNa$ ($[M+Na]^+$) requires 577.1621, found 577.1613.

ν_{max} ($CHCl_3$) 3320 (NH stretch, broad), 2950 (OH stretch, broad), 1710 (C=O stretch, broad), 1515 (C-N amide bend).

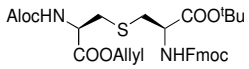
$[\alpha]_D^{25}$ -19.0 (c 1.0, CH_3OH).

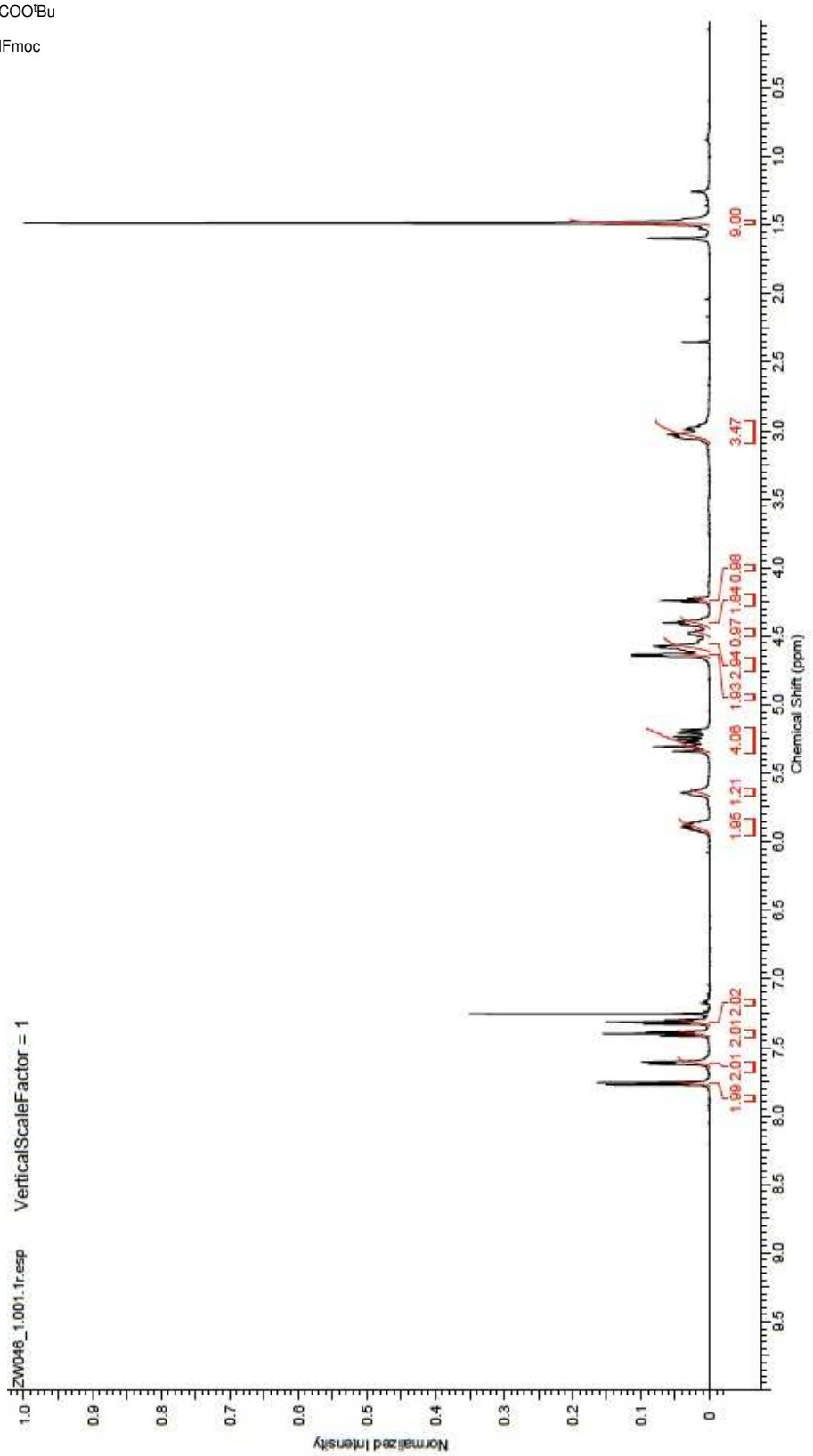
^{1}H NMR of *(2R, 6R)-(Allyl/Fmoc, ^{t}Bu) lanthionine*



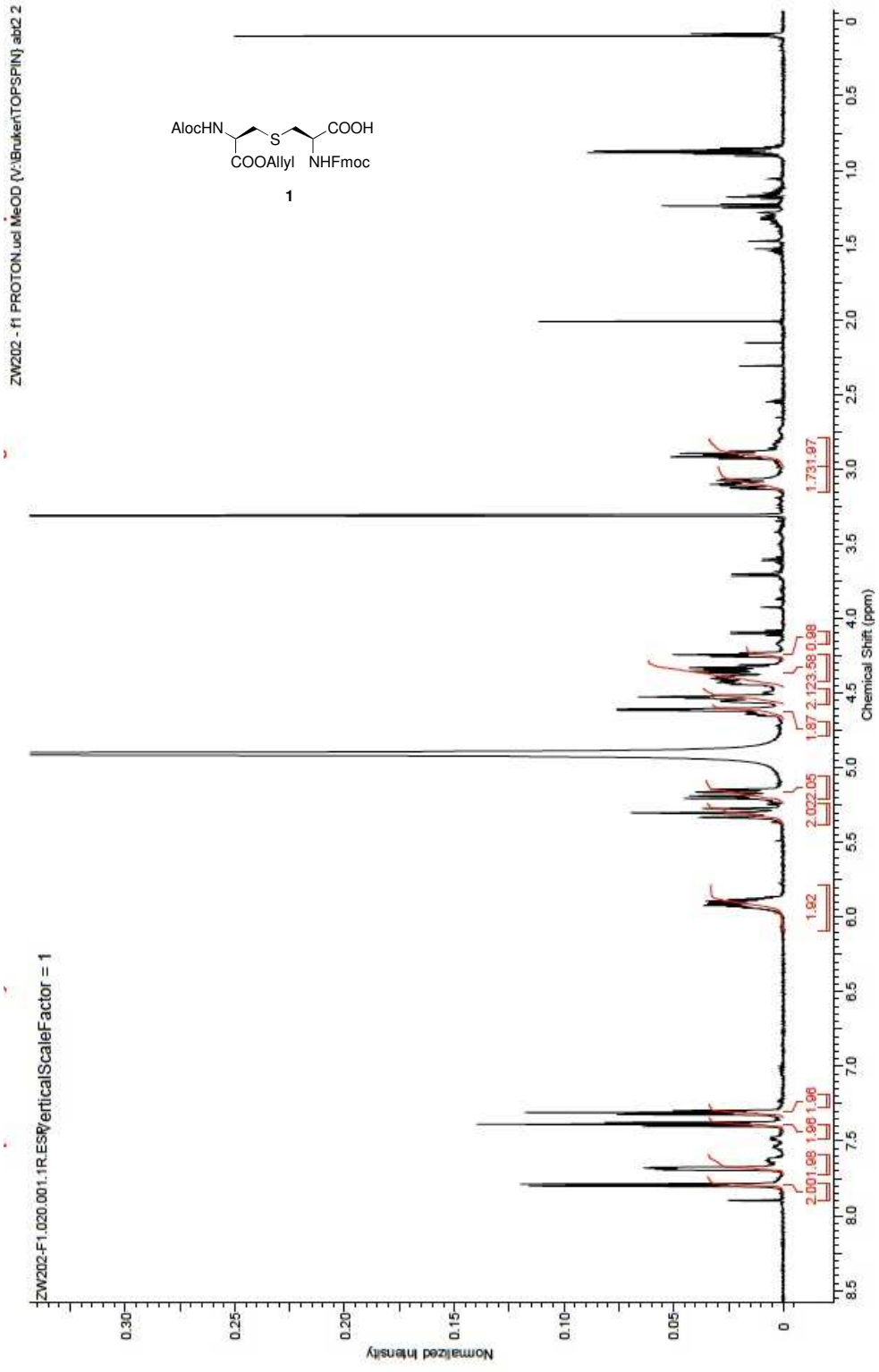


^{1}H NMR of *(2R, 6R)-(Aloc, Allyl/Fmoc, ^{t}Bu) lanthionine*





^{1}H NMR of *(2R, 6R)-(Aloc, Allyl/Fmoc) lanthionine 1*



Peptide Synthesis

General Experimental Details: Protected amino acids and coupling reagents were purchased from Novabiochem, coupling reagents, bases and solvents were purchased from Sigma-Aldrich. All water used was either distilled using an Elga Purelab Option R 7 water purifier or used directly from a bottle of HPLC-grade water. The peptides were synthesised on a MultiSynTech Syro Peptide Synthesiser (Model MP-60). Amino acid and reagent concentrations were calculated based on the quantity and loading of the resin. The total volume of all reagents in each step was 1.5 mL. All reagents were dissolved in HPLC grade DMF. Peptides were centrifuged using an Eppendorf Centrifuge, model 5810R and were lyophilised using a Thermo Scientific Heto PowerDry LL1500 freeze-dryer.

Fmoc Deprotection: For the automated peptide syntheses, all Fmoc deprotections were carried out using a solution of 40% piperidine in DMF, which was added to the syringe containing the resin. The mixture was agitated for 20 sec every minute for a total of 3 min before removal of reagent by filtration under vacuum. The resin was washed with DMF (4 x 1.5 mL) before addition of a second portion of piperidine in DMF solution (40% v/v, 0.75 mL), followed by DMF (0.75 mL) to make an overall 20% v/v solution of piperidine in DMF. This mixture was agitated for 20 sec every minute for a total of 10 min. The reagents were removed by filtration under vacuum and the resin washed with DMF (6 x 1.5 mL).

Amino Acid Coupling: Fmoc-protected amino acid (4 eq.) was added to the reaction syringe with HBTU (4 eq.) and DIPEA (8 eq.). The mixture was agitated for 20 sec every 3 min for a total of 40 min. The reagents were removed by filtration under vacuum and the resin washed with DMF (4 x 1.5 mL).

Cleavage from the Resin: Peptides were first washed with DMF (4 x 1.5 mL), CH_2Cl_2 (4 x 1.5 mL), methanol (4 x 1.5 mL) and diethyl ether (4 x 1.5 mL) before drying in a desiccator for 45 min. A solution of 94% TFA, 2.5% water, 2.5% EDT and 1% TIPS (2.5 mL) was then added to the resin and left to agitate for 30 min on the platform shaker. After this time, the entire solution was transferred to a Falcon tube and 12 mL of diethyl ether was added to precipitate the peptide. The cleavage procedure was then repeated with fresh cleavage solution (2.5 mL containing 94% TFA, 2.5% water, 2.5% EDT and 1% TIPS) and left to agitate for a further 40 min. The entire solution was again transferred to a Falcon tube before addition of 12 mL of diethyl ether to precipitate the peptide.

The Falcon tubes were then centrifuged for 10 min at 4000 rpm and 4 °C before decanting off the diethyl ether solution. This process was performed 3 times in total before re-dissolving the peptide in water and lyophilising.

HPLC Purification: The peptides were analysed and purified *via* reverse phase HPLC using either a Varian ProStar system with a Model 210 solvent delivery module and a Model 320 UV detector or a Dionex system with a PDA-100

photodiode array detector and a model ASI-100 automated sample injector. The preparative purification was performed using an ACE C8-300 semi-preparative column (150 x 10 mm, flow rate of 8.0 mL/min), with UV detection at 215 and 254 nm, loaded with 200 – 1850 μL aliquots of a 10-20 mg/mL solution of peptide dissolved in water. Gradient conditions are reported for each peptide. The fractions containing the correct peak were pooled, the solvent removed under reduced pressure to approximately 2 mL and the solution freeze-dried.

HPLC Analysis: All peptides were analysed using UV detection at 215 and 254 nm, using the conditions shown below. Retention times are reported for each peptide.

HPLC Method A: ACE C8-300 analytical column (150 x 10 mm, flow rate of 1.0 mL/min). Gradient: 2 – 98% B over 20 min (A = water, B = acetonitrile, 0.1% TFA).

HPLC Method B: Dr Maisch C8 column (Reprosil Gold 200 C8, 5 μm, 250 x 4.6 mm, flow rate of 1.0 mL min^{-1}). Gradient: 0-2 min on 95% A; then 5 - 95% B over 48 min; then 95% B over 2 min; then 95 – 5% B over 3 min; then 5% B over 5 min (A = water, B = acetonitrile, 0.1% TFA).

ESI-MS analysis: This was performed on a Waters Acquity Ultra Performance LC/MS machine using a linear gradient of 5 – 95% B over 5 min (A = water, B = acetonitrile, 0.1% TFA). The analysis of the chromatograms was conducted using MassLynx software version 4.0.

Synthesis of S-S bridged single ring peptides

*Single ring analog **2b**:* The synthesis was carried out on a 100 mg scale using pre-loaded H-Lys(Boc)-2-Cl-Trt resin (loading = 0.75 mmol/g). The following protected amino acids were used: Fmoc-Cys(Trt)-OH; Fmoc-Glu(O^{t}Bu)-OH; Fmoc-Gly-OH; Fmoc-Lys(Boc)-OH; Fmoc-Leu-OH; Fmoc-Met-OH: Fmoc-Arg(Pbf)-OH; Fmoc-Val-OH; Fmoc-Trp(Boc)-OH. Amino acid coupling steps and Fmoc deprotection were all carried out according to the general procedures above. The peptide was cleaved under standard conditions, washed with ether and lyophilised. The peptide was purified by semipreparative HPLC using a gradient of 40 – 80 % B over 15 min (A = water, B = acetonitrile) and left to cyclise for 10 days in pure water at 4 °C to yield 13 mg of product (10 %).

m/z (ES^+) 871.34 ($[M + 2H]^{2+}$), 581.41 ($[M + 3H]^{3+}$). *HPLC Method A* RT 2.46 min.

*Single ring analog **3b**:* The synthesis was carried out on a 100 mg scale using pre-loaded Fmoc-Trp(Boc)-NovaSyn® TGT resin (loading 0.20 mmol/g). The following protected amino acids were used: Fmoc-Cys(Trt)-OH; Fmoc-Glu(O^{t}Bu)-OH;

Fmoc-Gly-OH; Fmoc-Lys(Boc)-OH; Fmoc-Leu-OH; Fmoc-Met-OH: Fmoc-Arg(Pbf)-OH; Fmoc-Val-OH; Fmoc-Trp(Boc)-OH. Amino acid coupling steps and Fmoc deprotection were all carried out according to the general procedures above. The peptide was cleaved under standard conditions, washed with ether and lyophilised. The peptide was purified by semipreparative HPLC using a gradient of 40 – 80 % B over 15 min (A = water, B = acetonitrile) and left to cyclise for 10 days in pure water at 4 °C to yield 7 mg of product (16 %).

m/z (ES^+) 1086.60 ($[M + 2H]^{2+}$), 724.62 ($[M + 3H]^{3+}$), 543.80 ($[M + 4H]^{4+}$). *HPLC Method A* RT 2.11 min.

Single ring analog ***4b***: The synthesis was carried out on a 100 mg scale using pre-loaded Fmoc-Trp(Boc)-NovaSyn® TGT resin (loading 0.20 mmol/g). The following protected amino acids were used: Fmoc-Cys(Trt)-OH; Fmoc-Asp(O^tBu)-OH; Fmoc-Glu(O^tBu)-OH; Fmoc-Gly-OH; Fmoc-Lys(Boc)-OH; Fmoc-Leu-OH; Fmoc-Met-OH: Fmoc-Arg(Pbf)-OH; Fmoc-Ser(^tBu)-OH; Fmoc-Thr(^tBu)-OH; Fmoc-Val-OH; Fmoc-Trp(Boc)-OH. Amino acid coupling steps and Fmoc deprotection were all carried out according to the general procedures above. The peptide was cleaved under standard conditions, washed with ether and lyophilised. The peptide was purified by semipreparative HPLC using a gradient of 40 – 80 % B over 15 min (A = water, B = acetonitrile) and left to cyclise for 10 days in pure water at 4 °C to yield 1.5 mg of product (4 %).

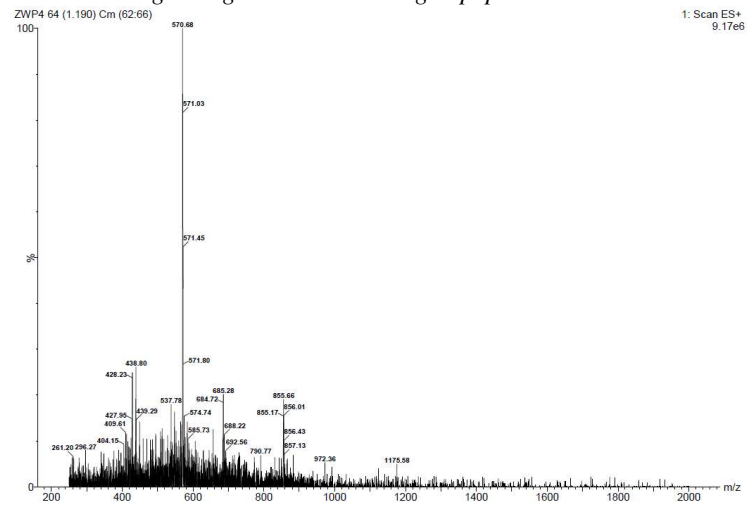
Analysis: m/z (ES^+) 1037.88 ($[M + 2H]^{2+}$), 692.35 ($[M + 3H]3^+$), 519.44 ($[M + 4H]^{4+}$). *HPLC Method A* RT 2.42 min.

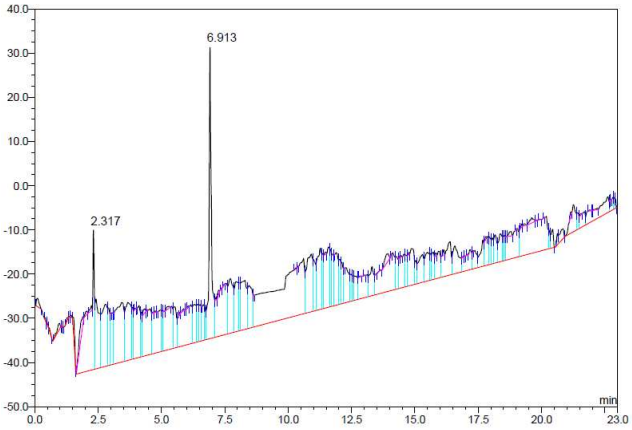
Single ring analog ***5b***: The synthesis was carried out on a 100 mg scale using pre-loaded Fmoc-Gly-NovaSyn® TGT resin (loading 0.20 mmol/g). The following protected amino acids were used: Fmoc-Cys(Trt)-OH; Fmoc-Asp(O^tBu)-OH; Fmoc-Glu(O^tBu)-OH; Fmoc-Gly-OH; Fmoc-Lys(Boc)-OH; Fmoc-Met-OH: Fmoc-Gln(Trt)-OH; Fmoc-Arg(Pbf)-OH; Fmoc-Ser(^tBu)-OH; Fmoc-Thr(^tBu)-OH; Fmoc-Tyr(^tBu)-OH; Fmoc-Trp(Boc)-OH. Amino acid coupling steps and Fmoc deprotection were all carried out according to the general procedures above. The peptide was cleaved under standard conditions, washed with ether and lyophilised. The peptide was purified by semipreparative HPLC using a gradient of 10 – 60 % B over 30 min (A = water, B = acetonitrile) and left to cyclise for 10 days in pure water at 4 °C to yield 12 mg of product (26 %).

Analysis: m/z (ES^+) 1156.45 ($[M + 2H]^{2+}$), 771.48 ($[M + 3H]^{3+}$) 578.57 ($[M + 4H]^{4+}$). *HPLC Method A* RT 11.02 min.

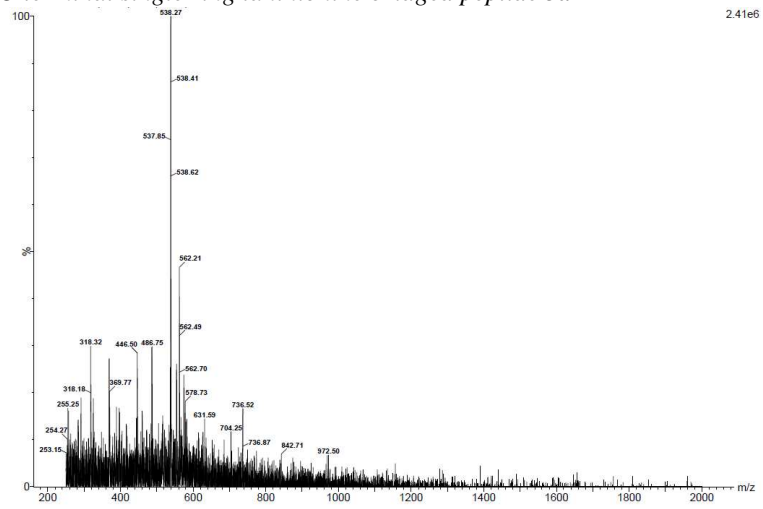
Mass spectra and HPLC data for peptides

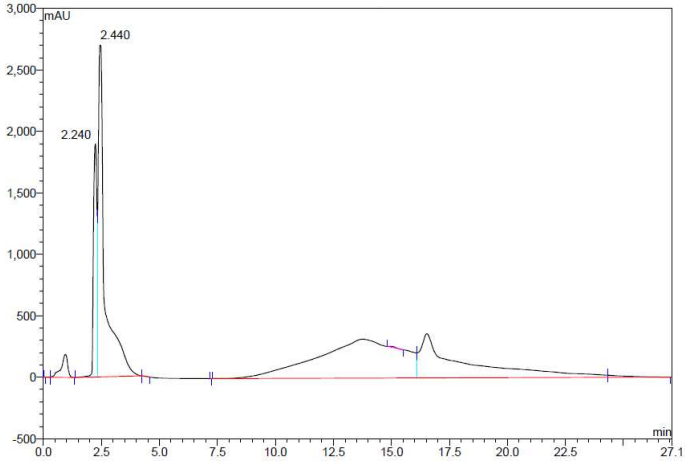
C-terminal single ring lanthionine bridged peptide ***2a***



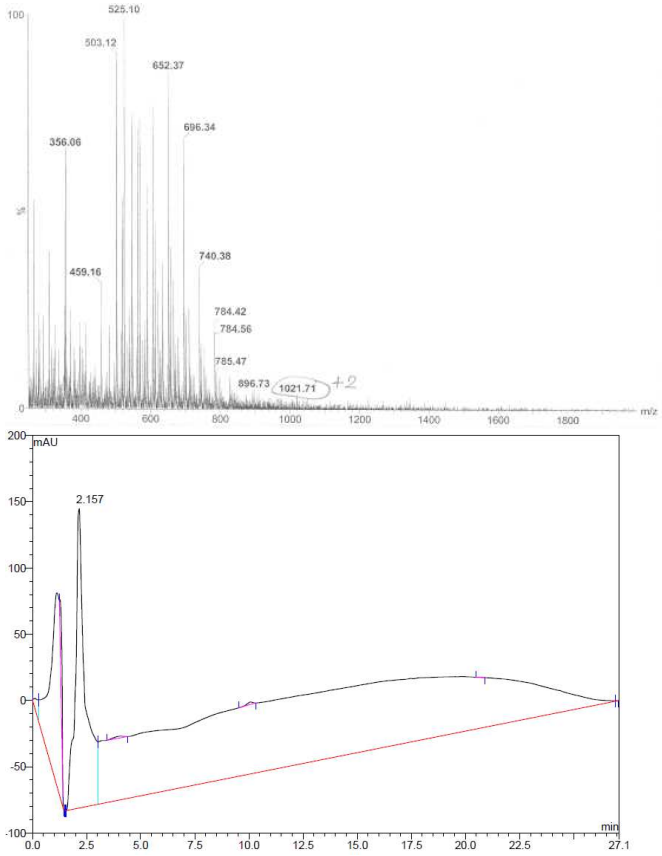


C-terminal single ring lanthionine bridged peptide ***3a***

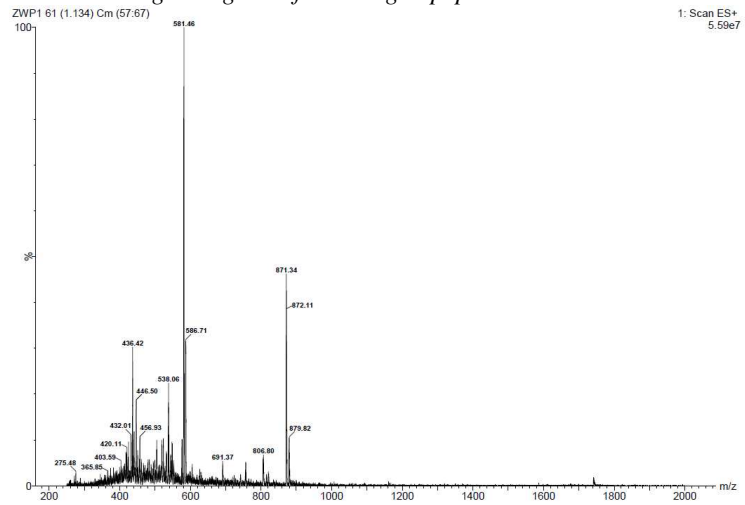


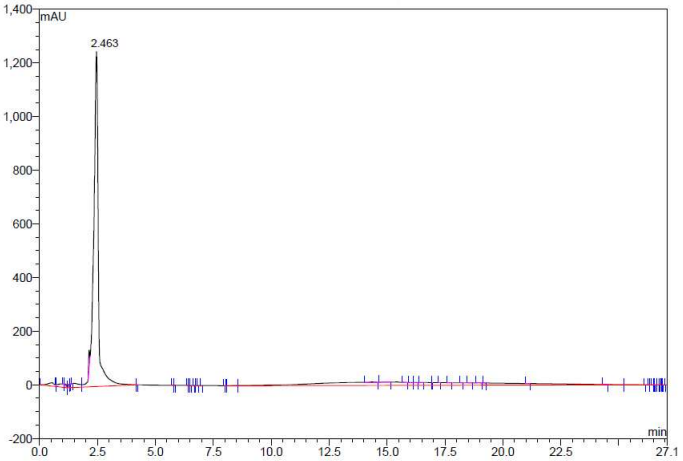


Middle single ring lanthionine bridged peptide ***4a***

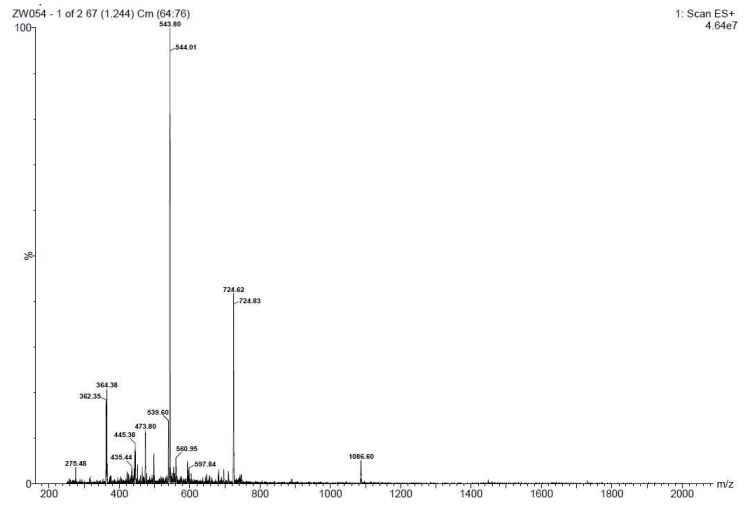


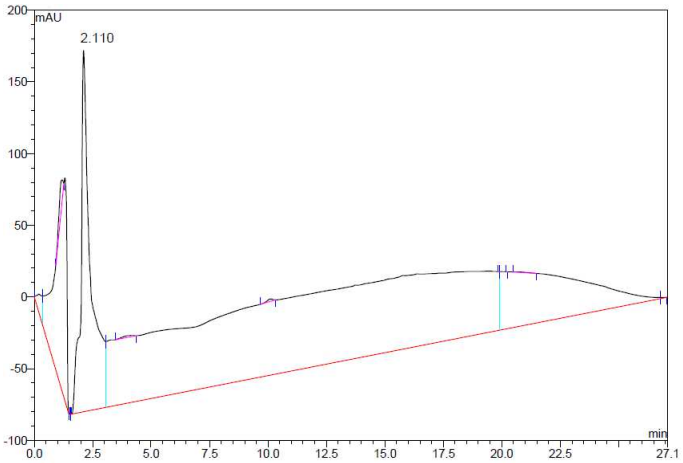
C-terminal single ring disulfide bridged peptide ***2b***



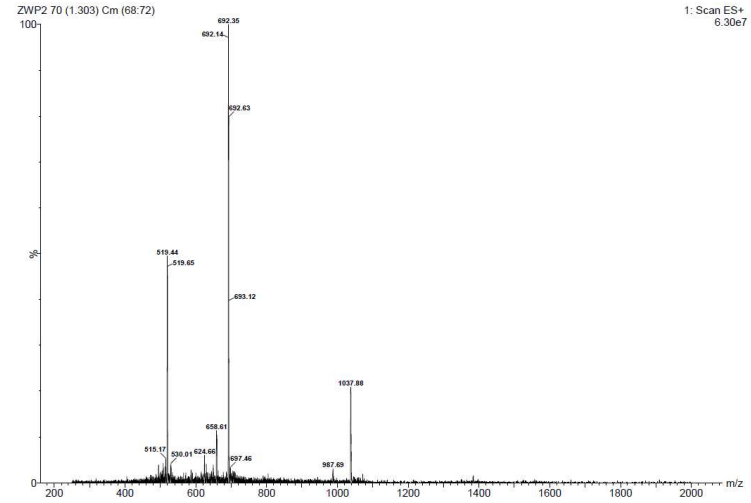


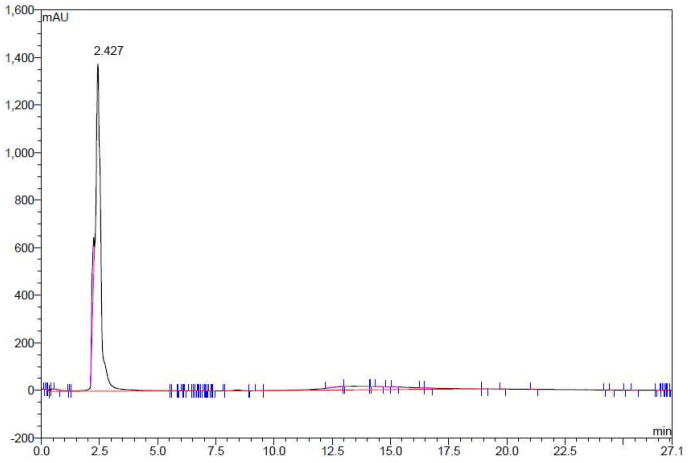
C-terminal single ring disulfide bridged peptide ***3b***



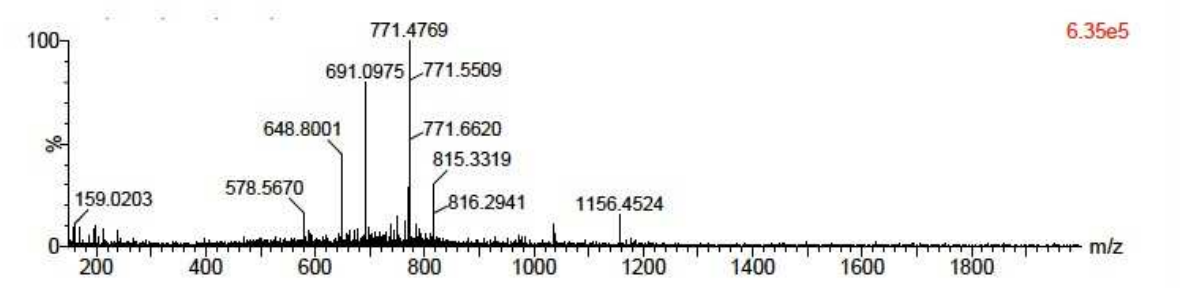


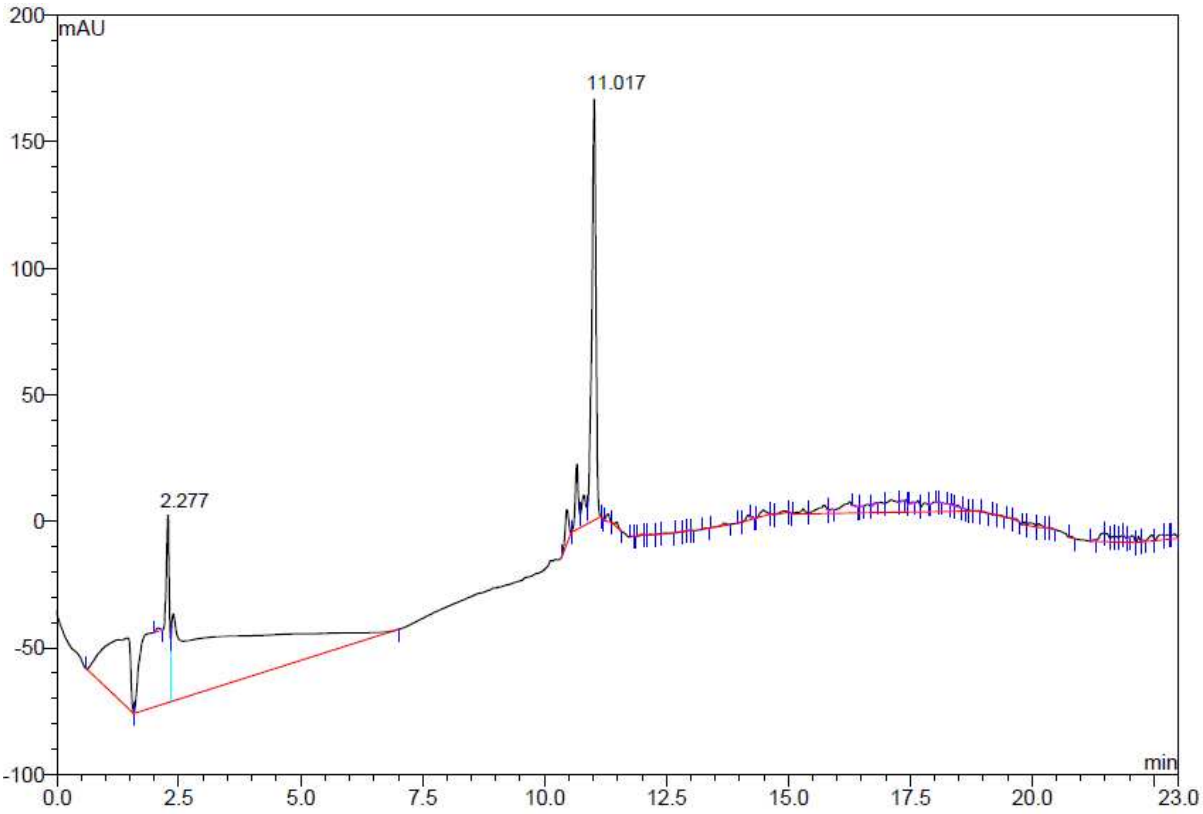
Middle single ring disulfide bridged peptide ***4b***



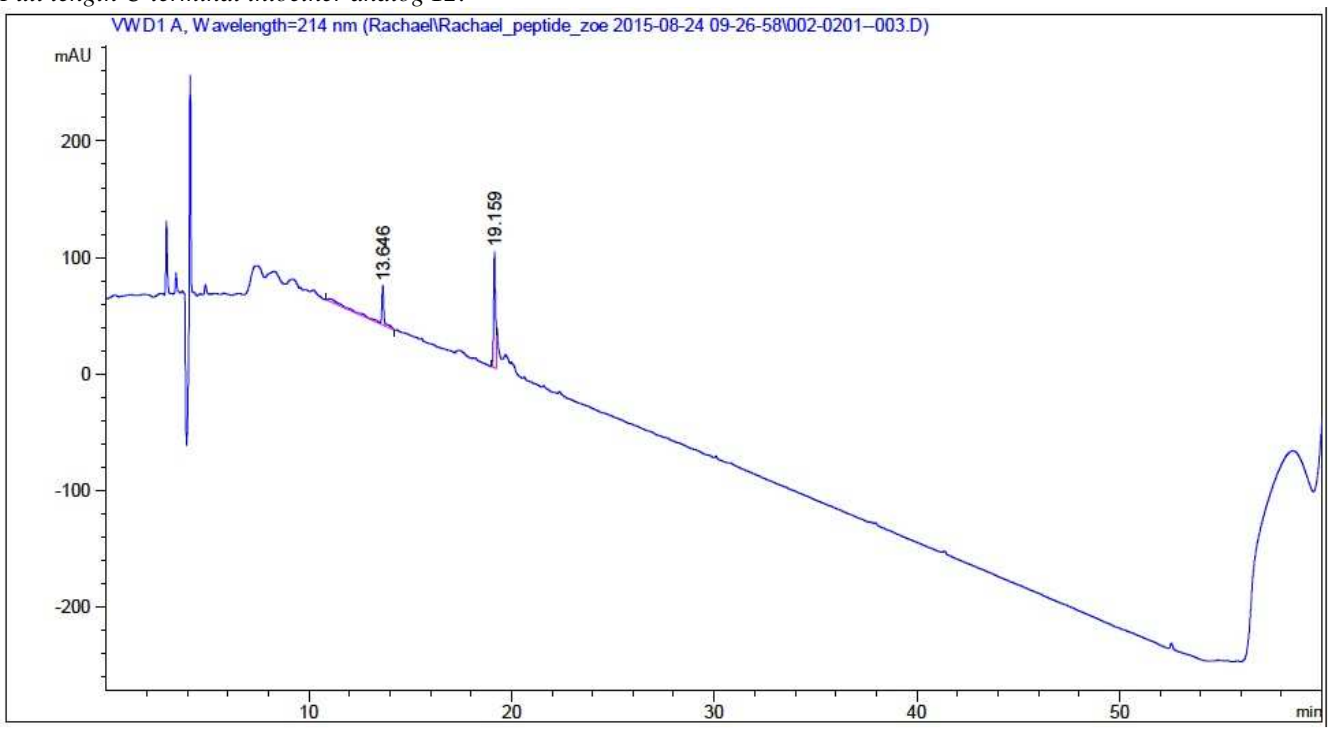


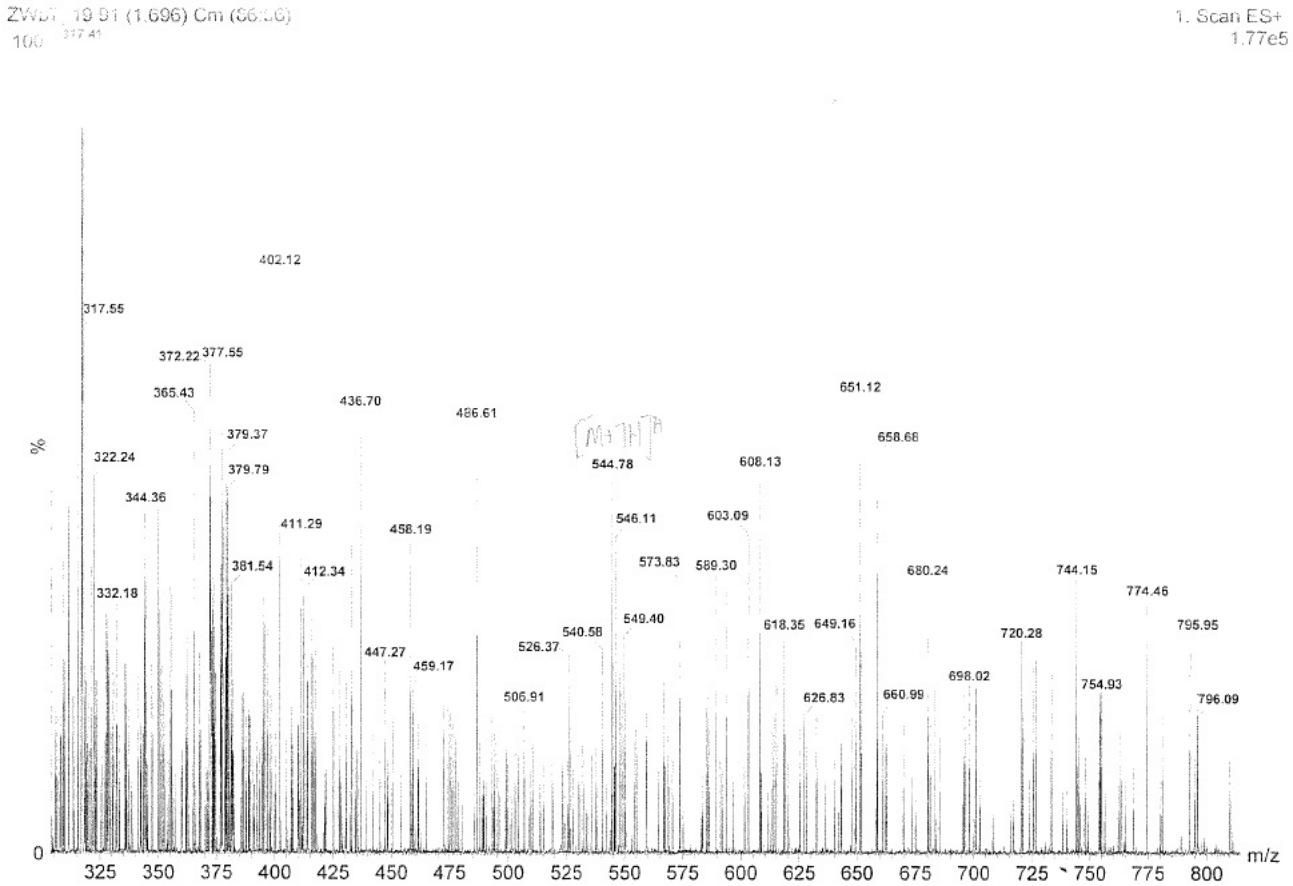
N-terminal single ring disulfide bridged peptide ***5b***



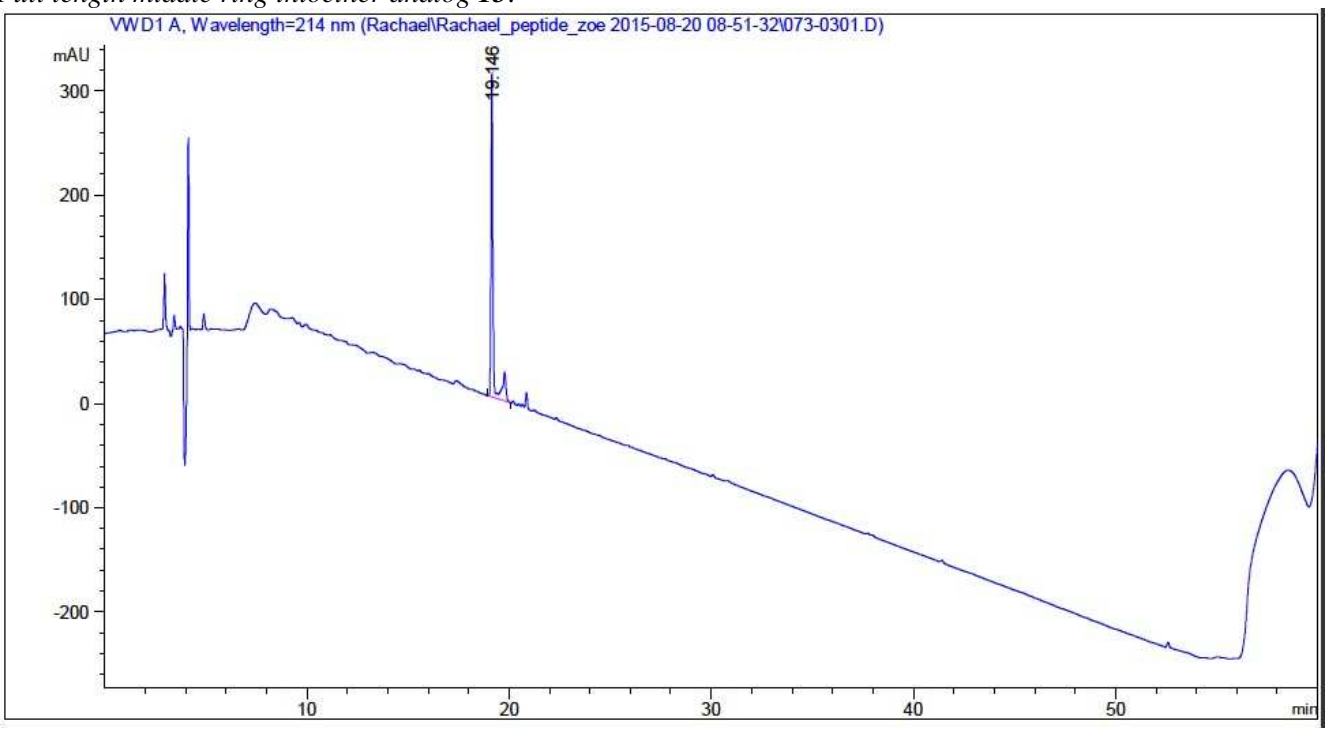


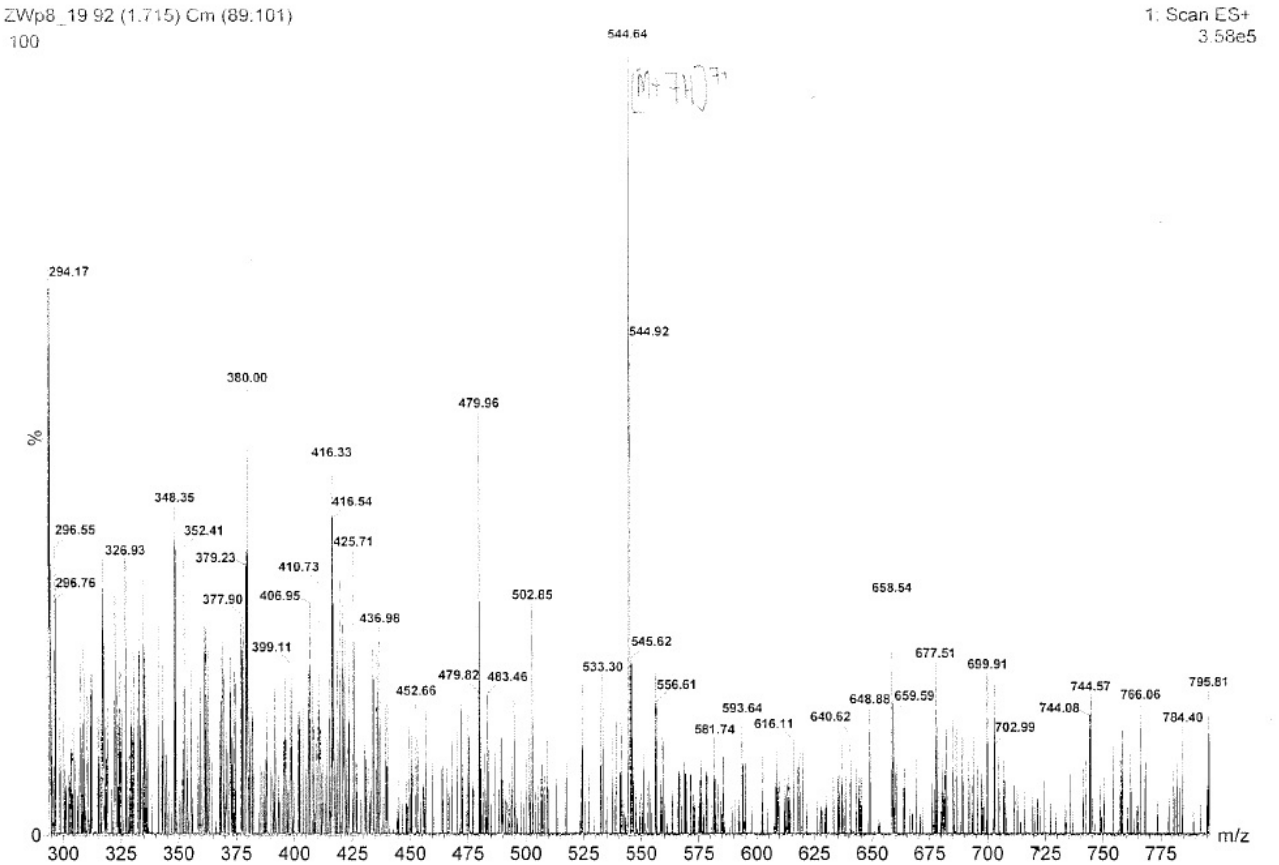
Full length C-terminal thioether analog ***12****:*



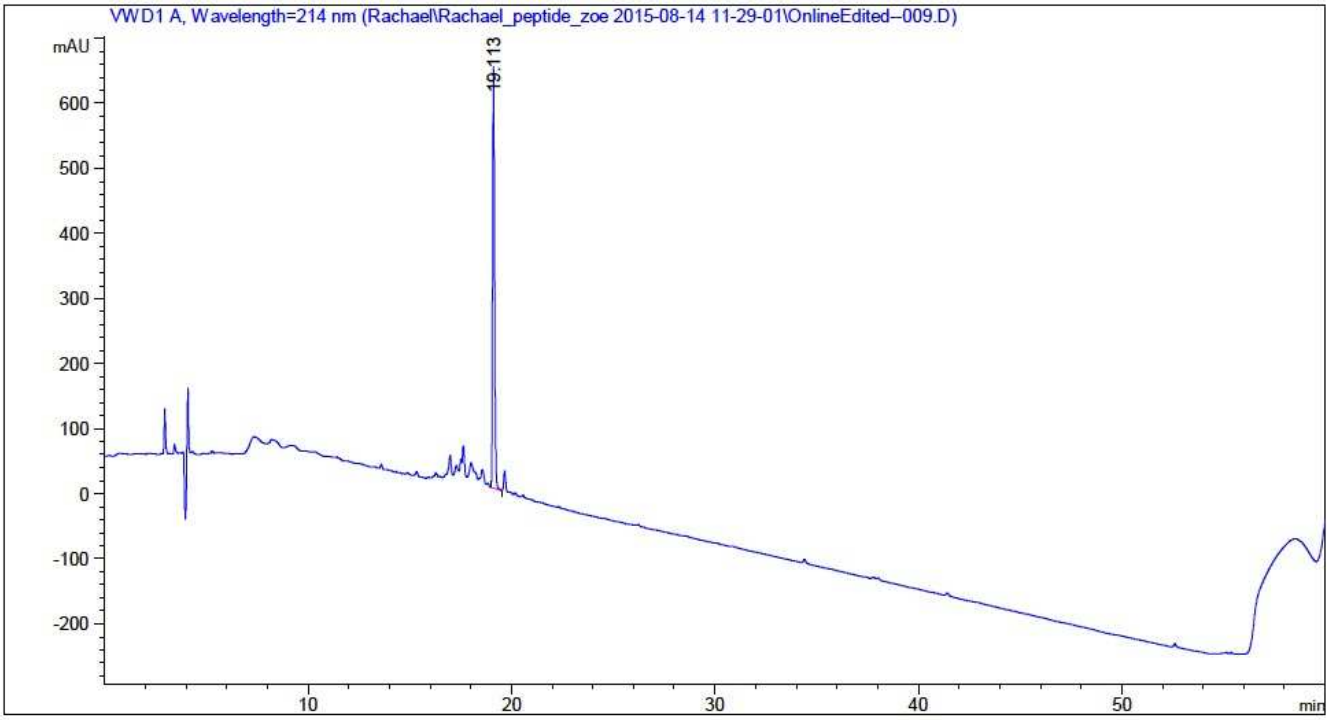


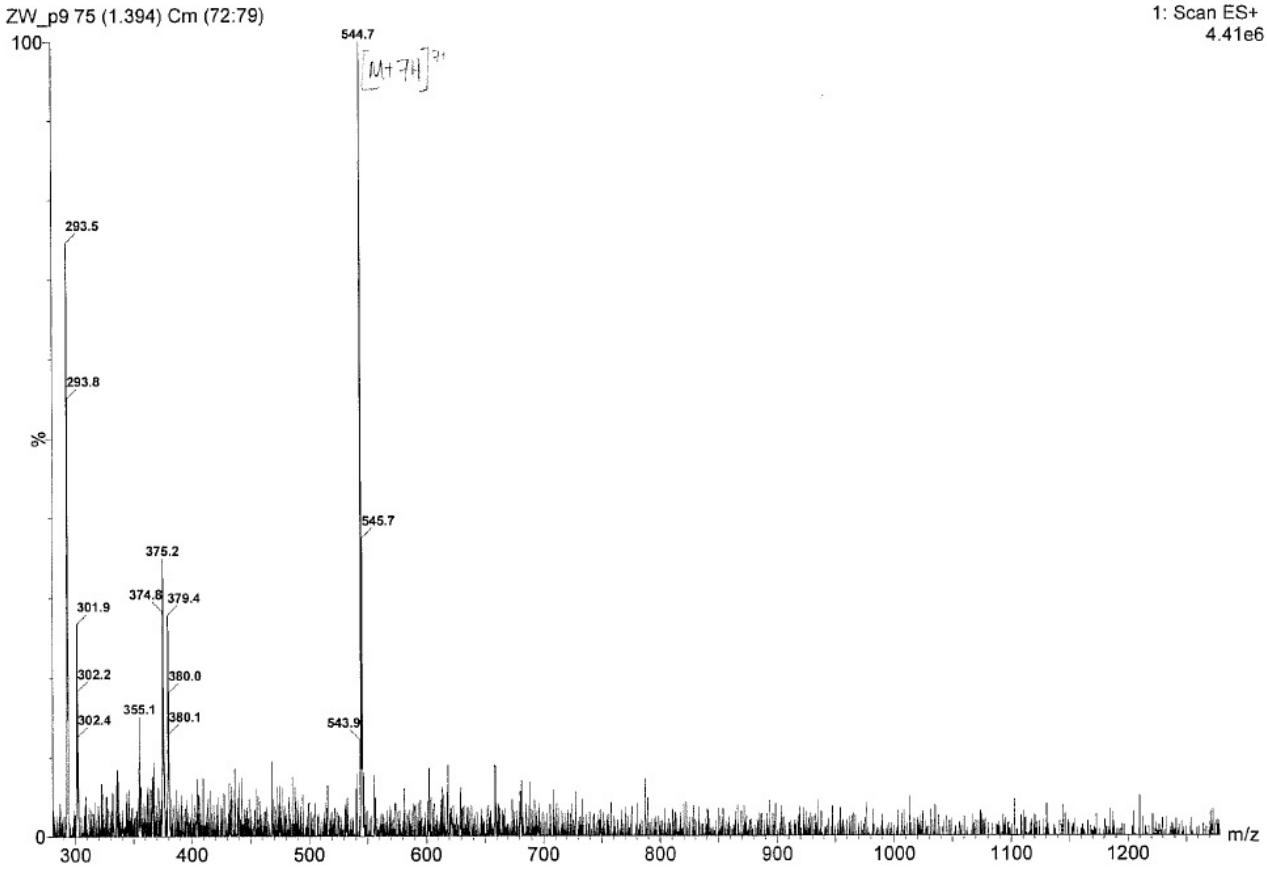
*Full length middle ring thioether analog **13**:*



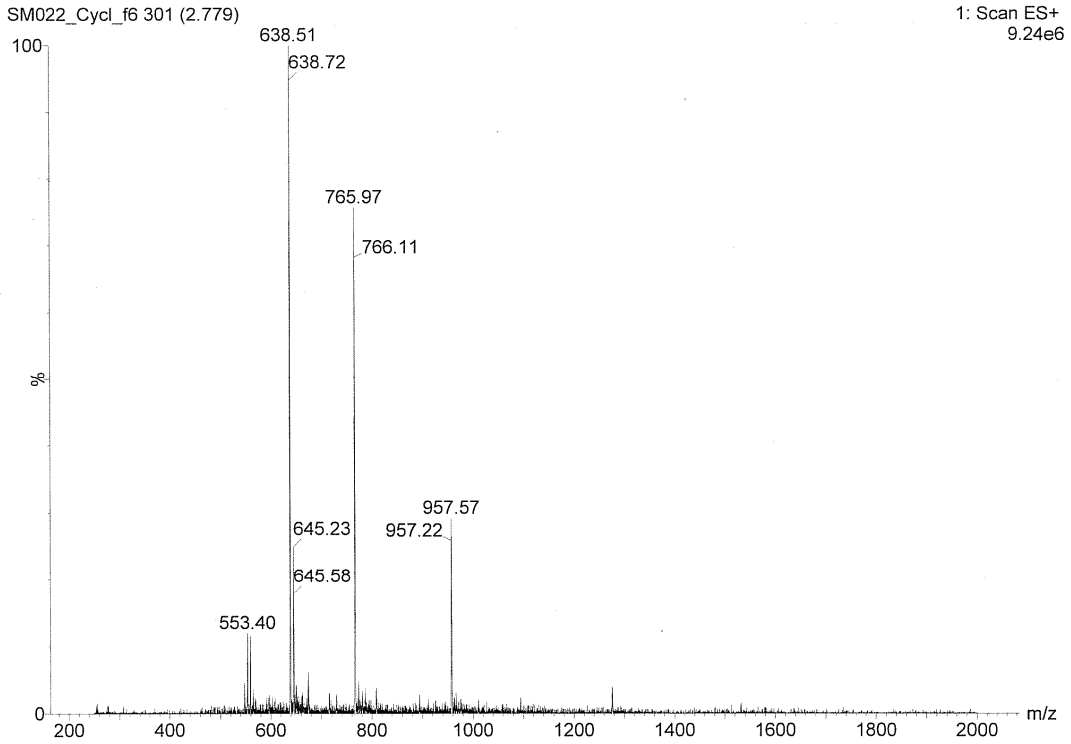


*Full length N-terminal thioether analog **14**:*

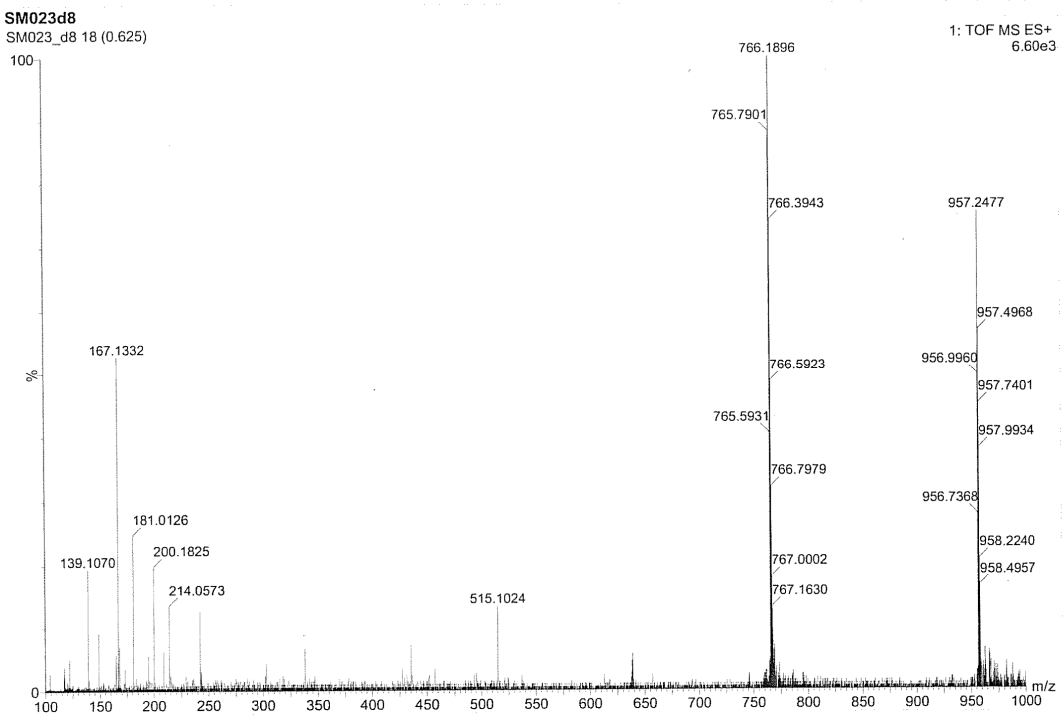




ProTx-II/7d



ProTx-II/24h



Crystallization

A sample of ProTx-II was obtained from Smartox Biotechnology (570 rue de la chimie, 38400 Saint Martin d'Hères, France). The sample was dissolved in water to a concentration of 10 mg/mL, and the solution was passed by centrifuge down a pre-washed desalting column (Spin-OUT GT100, 2 min at 3750 rpm). Hanging-drop vapour-diffusion crystallisation trials were performed using 96-well plates with a well volume of 100 μL and drop volume of 100 nL. Trays were made using a Labtech Mosquito nanodrop robot, and incubated at room temperature in a Rock Imager (Formulatrix Inc). Crystal growth was monitored daily using Rock Maker software (Formulatrix Inc).

Hits were scaled up in 24-well plates with a well volume of 500 μL and drop volume of 2 μL. Crystals were grown in a buffer containing 2M Li_2SO_4, 100 mM Tris/HCl (pH 8.5), and 2% v/v PEG 400, and appeared within 48 h.

Diffraction

Crystals were flash cooled in liquid nitrogen without additional cryoprotection. A heavy atom derivative crystal was prepared by adding 2 μL of 500 mM potassium iodide to the crystallization drop and incubating at room temperature for 30 minutes. The native crystal was obtained from the same drop prior to heavy atom derivatization. Both datasets were collected collected using a Rigaku Micromax-007HF source equipped with Varimax HF optics, a Saturn 944 HG CCD detector, and a 4-circle AFC11 goniometer. High-resolution diffraction data on a native crystal was collected at beamline 5.0.2. at the Advanced Light Source (Berkeley, CA, USA).

Processing and Experimental Phasing

All diffraction data was indexed, integrated, merged and scaled using autoPROC.[7] Key statistics from the data processing are listed in **Table S1**.

	Native crystal (synchrotron source)	Native crystal (home source)	Derivative crystal (home source)
Data collection			
Space group	H 3 2	H 3 2	H 3 2
Cell dimensions□□			
a, *b*, *c* (Å)	*a*=*b*=37.67, *c*=103.94	*a*=*b*=37.60, *c*=104.05	*a*=*b*=37.70, *c*=103.84
□□□□□□□□□□□□□ (°)	□□□□□□□□□□□□□□□□□□□□	□□□□□□□□□□□□□□□□□□□□	□□□□□□□□□□□□□□□□□□□□
Resolution (Å)	34.65-0.992 (1.010-0.992)[a]	34.69-1.651 (1.710-1.651)	34.62-1.088 (1.130-1.088)
R_{merge}, R_{meas}, and R_{pim}	0.062 (0.496)	0.034 (0.462)	0.023 (0.258)
	0.046 (0.222)	0.048 (0.653)	0.033 (0.365)
	0.020 (0.361)	0.034 (0.462)	0.023 (0.258)
$I/\sigma(I)$	25.0 (2.1)	15.5 (0.7)	19.9 (2.6)
$CC_{1/2}$	0.999 (0.700)	0.998 (0.179)	0.999 (0.679)
Completeness (%)	92.2 (54.1)	89.0 (21.7)	98.0 (99.5)
Redundancy	8.7 (1.7)	1.9 (1.2)	2.0 (2.0)
Refinement			
Resolution (Å)	0.992		
No. reflections	14960		
R_{work} / R_{free}	0.133 / 0.151		
No. atoms	329		
Protein	265		
Ligand/ion	10		
Water	54		
B factors			
Protein	9.98		
Ligand/ion	28.67		
Water	40.03		
R.m.s deviations			
Bond lengths (Å)	0.0174		
Bond angles (°)	2.189		

[a] Values in parentheses are for highest-resolution shell.

Table S1 Data collection and refinement statistics for the three crystal diffraction datasets collected in this study.
Initial phases were solved using single isomorphous replacement with anomalous signal (SIRAS) using the native and derivative datasets collected on the home source, followed by solvent flattening and electron density modification, all implemented in autoSHARP.[8] Manual building of the structure was carried out in Coot. A higher resolution native dataset was collected using synchrotron radiation. The model built using SIRAS phases was rigid body refined into the new dataset and least-squares restrained refinement continued within SHELXL[9] and Refmac.[10] Inspection of the electron density maps allowed solvent molecules and some alternative sidechain conformations to be modelled. Some sidechain conformations could not be determined from the electron density maps and were truncated in the final model. Refinement statistics are shown in Table 1.

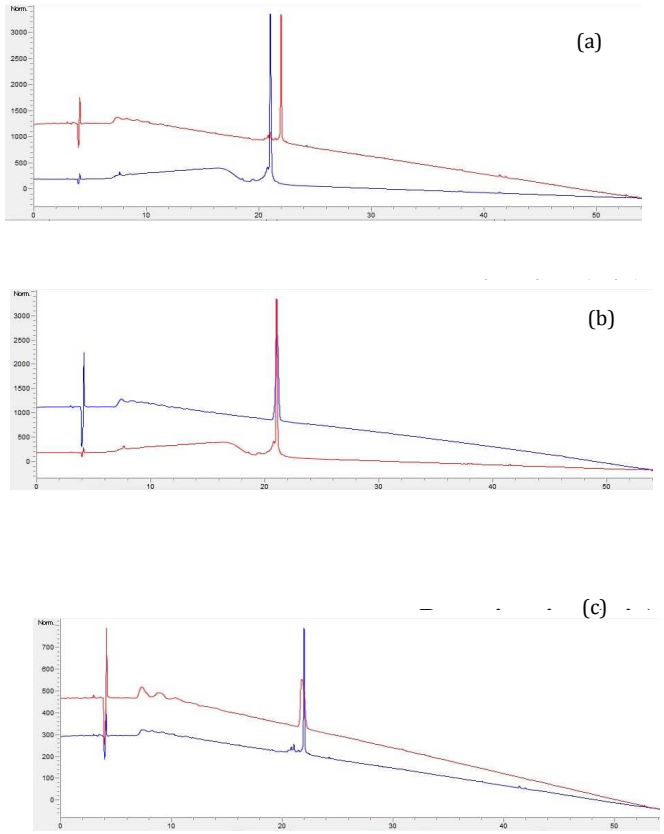


Figure S1. HPLC of ProTx-II samples. (a) Overlay of Sigma and Smartox samples; (b) Overlay of ProTx-II/24h and Smartox samples; (c) Overlay of ProTx-II/7d and Sigma samples. All experiments were run using Analytical *HPLC Method B*.

Patch clamp data

Peptides were tested against stably transfected cell lines expressing the hNav1.7 receptor, using the hNav1.7-HEK cell IonWorks Population Patch Clamp Assay (Essen) and the QPatch™ Patch Clamp Assay (B'SYS GmbH). The Study Reports from the two companies are included below. As the testing was carried out blind, without knowledge of the structure and sequence of the peptides, **Table S2** shows the codes used by B'Sys and correlates these to the numbering used in the present paper.

Table S2

B'Sys code	**Peptide**
ZWP1	**2a**
ZWP2	**4a**
ZWP3	**5a**
ZWP4	**2b**
ZWP5	**4b**
ZWP6	**ProTx-II/7d**
ZWP7	**12**
ZWP8	**13**
ZWP9	**14**

ESSEN BioScience
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Ion Channel Discovery Services

Customer:	UCL	**Customer contact:**	Zoe Wright
Customer PO:		**Essen Quote No:**	2014/UCL_0002A
Essen contact:	Srini Kanumilli	**Lab Notebook Ref:**	LNB-2650
Date of assay:	01 July 2014	**Date of Report:**	01 July 2014

Experiment overview

Nine Protoxin-II analogues and Protoxin-II purchased from Sigma (Cat # P0033) were tested for inhibition of the human voltage-gated sodium channel $Na_V1.7$ using IonWorks patch clamp electrophysiology. 8-Point concentration-response curves were generated using 3-fold serial dilutions from the maximum final assay concentration shown in the table below.

Results summary

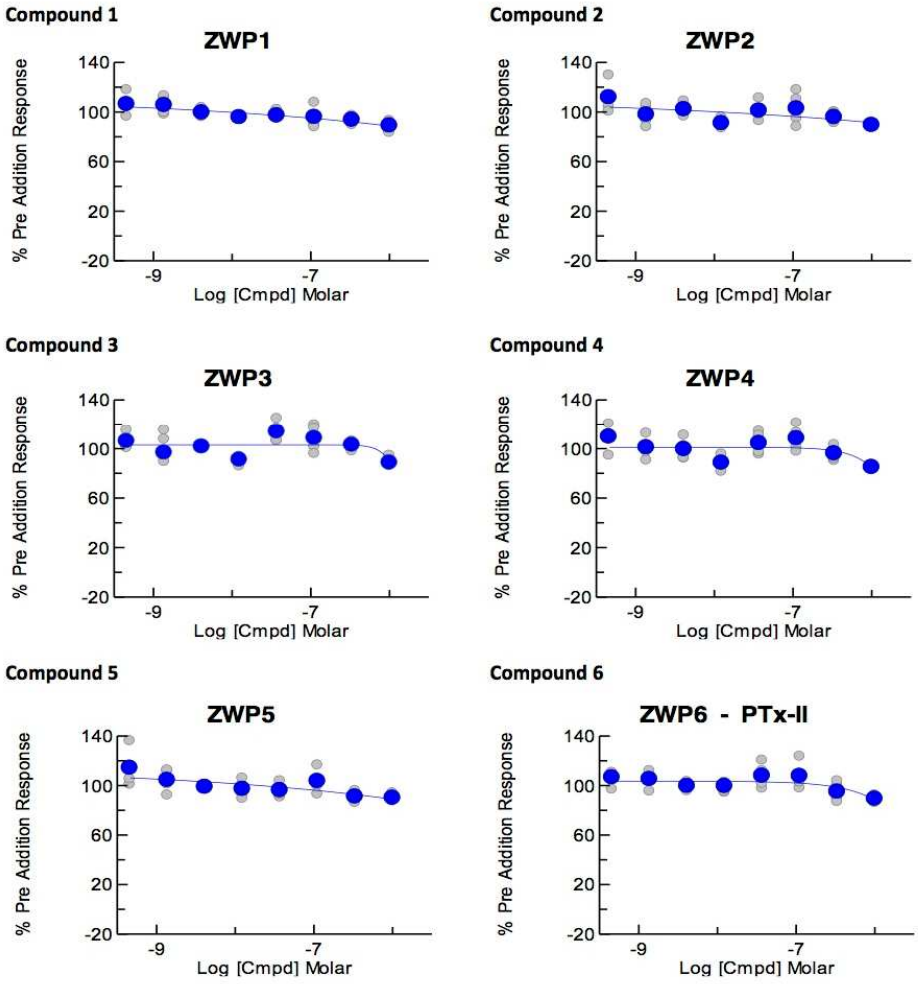
Compound		pIC_{50}		IC_{50} (μM)		Curve Profile				
ID	No	Value[1]	95% CI[3]	Value[2]	95% CI[4]	Slope	[Max] μM	Well count[5]	QC	Notes[6]
ZWP1	1	**<6.0**	-	>1.0	-	-	1	31	PASS	11 ± 2% inhibition at 1 μM
ZWP2	2	**<6.0**	-	>1.0	-	-	1	32	PASS	11 ± 0% inhibition at 1 μM
ZWP3	3	**<6.0**	-	>1.0	-	-	1	31	PASS	11 ± 2% inhibition at 1 μM
ZWP4	4	**<6.0**	-	>1.0	-	-	1	32	PASS	15 ± 1% inhibition at 1 μM
ZWP5	5	**<6.0**	-	>1.0	-	-	1	32	PASS	10 ± 2% inhibition at 1 μM
ZWP6 - PTx-II	6	**<6.0**	-	>1.0	-	-	1	31	PASS	11 ± 1% inhibition at 1 μM
ZWP7	7	**<6.0**	-	>1.0	-	-	1	32	PASS	13 ± 2% inhibition at 1 μM
ZWP8	8	**<6.0**	-	>1.0	-	-	1	32	PASS	9 ± 2% inhibition at 1 μM
ZWP9	9	**<6.0**	-	>1.0	-	-	1	32	PASS	12 ± 1% inhibition at 1 μM
PTx-II	10	**5.9**	0.09	1.23	[1.0 - 1.4]	1.3	11	32	PASS	
Tetracaine	11	**6.9**	0.08	0.1	[0.1 - 0.1]	1.3	33	32	PASS	

[1]pIC_{50} (i.e. the negative logarithm of the IC_{50}) and [2]IC_{50} values were obtained from a 4 parameter logistic fit of the concentration-response data. Greater or less than values indicate where the potency estimates could not be fully resolved due to the limited concentration range tested, or insolubility. In such cases % maximum inhibition values are returned in the notes column.

[3]95% Confidence limits for the pIC_{50} and [4]IC_{50} value from the curve fit. [3]Values <0.5 indicate that the pIC_{50} is resolved within ±0.5 of a log unit with 95% probability.

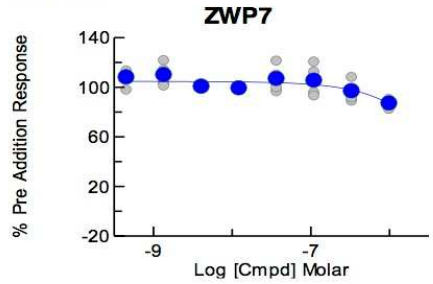
[5]Denotes the number of data points (wells) included in the curve fit. Up to 4 wells per test concentration may pass the well QC criteria. Thus, for an 8 point concentration response curve a maximum of 32 cells may be obtained.

[6]Solubility observations record the visual presence of precipitate in the assay ready compound plate where samples are prepared at 3x the final test concentration. Solubility observations are reported with reference to the final assay concentration in order to flag the possibility of compromised data points.



In each graph the log of molar concentration of test peptide is plotted against the effect of the compound on the $hNa_V1.7$ current, expressed as a percentage of the pre-compound signal. In this experiment an average of 6±3% signal decay was observed in the time- and vehicle (0.1% BSA) control. Data are normalised for this vehicle-response such that a value of 100% = no drug effect. The smaller grey symbols represent data from individual wells. The larger blue points show the mean data at each test concentration. The line of best fit (4 parameter logistic equation) is generated from the individual well data points. Outlier data points (boxed symbols) were manually excluded from the curve fit. No more than two per curve were excluded, other than where compounds were visibly out of solution.

Compound 7



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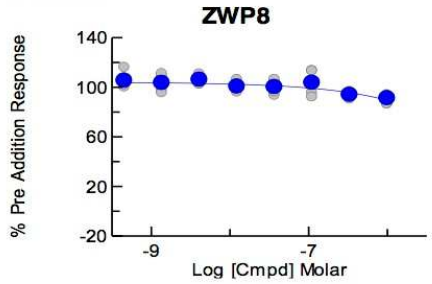

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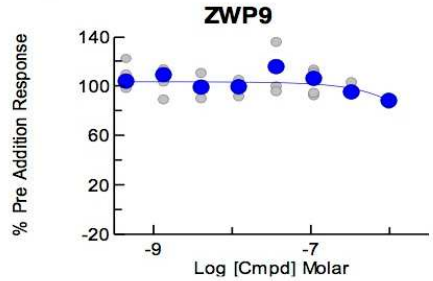
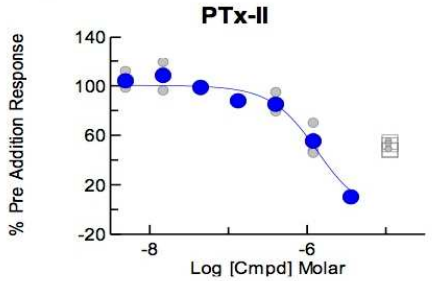
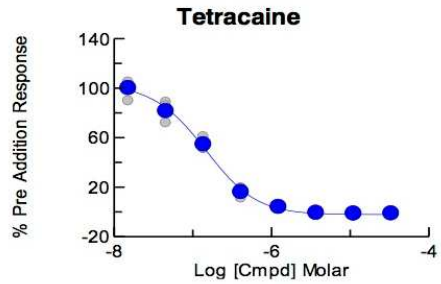


Compound 10



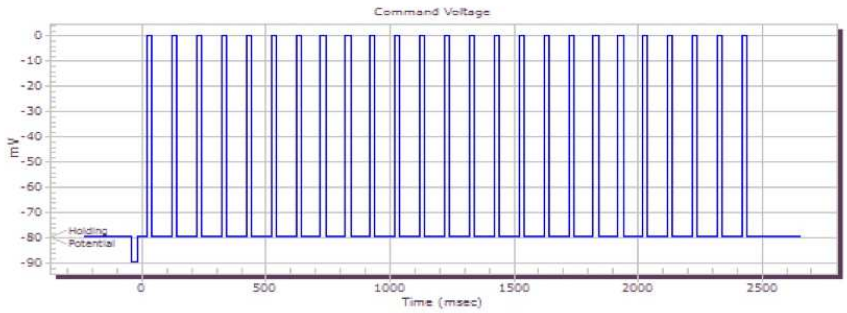
Compound 11



In each graph the log of molar concentration of test peptide is plotted against the effect of the compound on the $hNa_V1.7$ current, expressed as a percentage of the pre-compound signal. In this experiment an average of 6±3% signal decay was observed in the time- and vehicle (0.1% BSA) control. Data are normalised for this vehicle-response such that a value of 100% = no drug effect. The smaller grey symbols represent data from individual wells. The larger blue points show the mean data at each test concentration. The line of best fit (4 parameter logistic equation) is generated from the individual well data points. Outlier data points (boxed symbols) were manually excluded from the curve fit. No more than two per curve were excluded, other than where compounds were visibly out of solution.

Methods summary

Electrophysiological recordings were made from human embryonic kidney (HEK) cell lines stably expressing the full length $Na_V1.7$. Population patch clamp measurements were made in the perforated patch clamp configuration (200 µg ml^{-1} amphotericin) at room temperature (21-23°C) using an IonWorks Quattro instrument. The internal solution contained (mM): 90 K gluconate, 40 KCl, 10 NaCl, 3.2 $MgCl_2$, 3.2 EGTA, 5 HEPES and was buffered to pH 7.3. The external solution contained (mM): 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES also buffered to pH 7.3. The voltage protocol is illustrated below. Currents were first measured under control (pre-compound addition) conditions. Compounds were then incubated for 6-7 minutes prior to a second measurement of the signal using an identical pulse train. Currents were measured from the depolarising step at the 25th pulse, and referenced to the holding current. Compounds were then incubated for 5-7 minutes prior to a second measurement of the $hNa_V1.7$ signal using an identical pulse train.



Quality control

The following QC conditions were applied:

(1) Individual cells with any of the following properties were excluded from subsequent analysis (1) seal resistances <20MΩ (2) hNa_V 1.7 currents <400pA (3) seal resistances that changed by >50% during the experiment

(2) A minimum of 17 wells were required for each pIC_{50} curve fits

(3) pIC_{50} curve fits with a 95% confidence limit of > ±0.5 log were failed

(4) Entire assay plates in which the pIC_{50} of the standard compound (tetracaine) was outside of the normal range [6.2-7.2] were failed.

NON- GLP STUDY REPORT

ProTx-II: Effect on $Na_V1.7$ channel Peak Currents recorded from Stably Transfected CHO cells

B'SYS Study No.: A1233

Sponsor: University College London

TABLE OF CONTENTS

1. SIGNATURE

Study Director: Dr. Simon Hebeisen

.....
Date:

2. PREFACE

2.1. GENERAL

Title	ProTx-II: Effect on $Na_V1.7$ channel Peak Currents recorded from Stably Transfected CHO cells
Sponsor	University College London Department of Chemistry 20 Gordon Street London WC1H 0AJ / United Kingdom
Study Monitor	Dr. Alethea Tabor
Test Facility	B'SYS GmbH Analytics Benkenstrasse 254 CH-4108 Witterswil / Switzerland

2.2. RESPONSIBILITIES

Study Director	Dr. Simon Hebeisen B'SYS GmbH Analytics Benkenstrasse 254 CH-4108 Witterswil / Switzerland

2.3. SCHEDULE

Experimental Starting Date	17-Sep-2014
Experimental Completion Date	18-Apr-2017
Report	(see page 3)

2.4. ARCHIVING

B'SYS GmbH, CH-4108 Witterswil, Switzerland, will retain the study plan, raw data and the final report of the present study for at least ten years. At the end of the archive period, the Study Sponsor will be contacted and asked if they wish the testing facility to continue archival storage (at their expense) or have the materials returned to them.

3. PURPOSE

First, $Na_V1.7$ peak currents were recorded from stably transfected CHO cells and the effect of one or two concentrations of one test item on three different channel states (resting, fast inactivated and slow inactivated) was investigated.

Second, $Na_V1.7$ peak currents elicited by a single pulse protocol were recorded from stably transfected CHO cells and the effect of one or two concentrations of one test item was investigated.

4. MATERIALS AND METHODS

General Remark: Details of the materials and methods that are not specified in the subsequent sections of this study report are contained in the appropriate B'SYS standard operating procedures.

4.1. TEST SYSTEM

In this study CHO cells stably transfected with $hNa_V1.7$ sodium channels were used.

Test system	CHO cells stably expressing the $Na_V1.7$ channel
Source	B'SYS GmbH, 4108 Witterswil, Switzerland
Storage	Maintenance in culture or cryogenic storage
Passage number	The passage number of the cells used in the study are documented in the raw data

4.2. EQUIPMENT

Amplifier	EPC-10, HEKA Electronics
Headstage	Preamplifier EPC-10, HEKA Electronics
Software	PatchMaster, HEKA Electronics
Amplifier	WPC-100, E.S.F. Electronic
Headstage	WPC-100, E.S.F. Electronic
Software	PatchMaster, HEKA Electronics

4.3. CELL CULTURE

In general, cells were passaged at a confluence of about 50-80%. For electrophysiological measurements cells were seeded onto 35 mm sterile culture dishes containing 2 mL culture medium without antibiotics. Confluent clusters of cells are electrically coupled. Because responses in distant cells are not adequately voltage clamped and because of uncertainties about the extent of coupling, cells were cultivated at a density that enabled single cells (without visible connections to neighbouring cells).

Conditions	Standard Laboratory Conditions. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 (rel. humidity about 95%)
Culture media	The cells were continuously maintained and passaged in sterile culture flasks containing nutrient mixture HAM/F-12 (1x, liquid, with L-Glutamine) supplemented with 10% foetal bovine serum and 1.0% Penicillin/Streptomycin solution
Antibiotics	The complete medium as indicated above is supplemented with 100 µg/ml Hygromycin B.

4.4. TEST ITEMS

The test items were supplied by University College London. The precautions necessary when handling the test items are based on information supplied by the sponsor.

(Information as provided by the sponsor)

Substance ID	Molecular weight (g/mol)	Stock concentrations	Concentrations to test
ProTx-II	3826	100 µM / 10 µM	1.0 and 10 nM
ProTx-II/7d	3826	100 µM / 10 µM	10 and 100 nM
linear ProTx-II	3832	100 µM / 10 µM	10 nM
ProTx-II Sigma Aldrich	3826	100 µM	100 nM
ProTx-II peptide 14	2794	100 µM	100 nM
ProTx-II peptide 3a	2139	100 µM	100 nM
ProTx-II peptide 3b	2171	100 µM	100 nM
ProTx-II Smartox	3826	100 µM	100 nM
ProTx-II/24h	3826	100 µM	100 nM

4.5. REFERENCE ITEM

The reference item was supplied by Sigma. The precautions necessary when handling the test items were based on information supplied by Sigma.

Lidocaine

Identity	Lidocaine hydrochloride monohydrate
Physical State	solid
Physical form	white powder
Solubility	soluble in water
Source	supplied by Sigma
Batch Number	069K0002
Composition	$C_{14}H_{22}N_2O \cdot HCl \cdot H_2O$
Purity	100%
Molecular weight (salt)	288.81 g/mol
Storage conditions	Room temperature (19°C to 30°C)
Expiry date	14-Oct-2015
Safety precautions	routine hygienic procedures (gloves, goggles etc.)

4.6. BATH SOLUTION

The 1x bath solution were prepared by diluting 10x bath solution without Glucose and 100x Glucose solution with water at least every 7 days. Both stock solutions had been prepared prior to the experimental start of the present study and stored at 1°C to 9°C (10x bath solution) or -10°C to -30° (100x Glucose solution). The batch numbers of the bath solutions used in the experiments are documented in the raw data. When in use, the 1x bath solution was kept at room temperature (19°C - 30°C). When not in use, the 1x bath solution was stored at 1°C to 9°C. The final bath solution included the components outlined below:

Sodium Chloride	137 mM
Potassium Chloride	4 mM
Calcium Chloride	1.8 mM
Magnesium Chloride	1 mM
HEPES	10 mM
D-Glucose	10 mM
pH (NaOH)	7.4

4.7. PIPETTE SOLUTION

The 1x pipette solution was thawed every day out of a frozen 1x pipette solution, which had been prepared prior to the experimental start of the present study, aliquoted and stored at −10°C to −30°C. When in use, the 1x pipette solution was filled into the patch-pipettes and kept at room temperature (19°C to 30°C). The 1x pipette solution included the components outlined below:

Cesium Fluoride	135 mM
Sodium Chloride	10 mM
HEPES	10 mM
EGTA	5 mM
pH (KOH)	7.3

4.8. DOSE FORMULATION

4.8.1. STOCK SOLUTION

Dose levels were in terms of test items as supplied. The test item -pre-weighed by the sponsor – was dissolved by adding bath solution (see section 4.6) to achieve stock a concentration of 100 µM. If two concentrations were tested, a 10 µM stock solution was prepared by diluting the 100 µM solution with bath solution. Details of stock solution usage (thawing, dose formulations) are documented in the raw data. The time period of stock solution usage are detailed in the report.

4.8.2. TEST ITEM CONCENTRATIONS

The concentrations used for ProTx-II were listed in 4.4. They were diluted from the 1000x stock solutions using bath solution shortly prior to the electrophysiological experiments and kept at room temperature (19°C to 30°C) when in use. Details of all formulations were documented in the raw data.

The reference compound Lidocaine was weighed into a container on a calibrated precision balance and was dissolved by adding Bath solution to achieve concentration of 10.0 mM. Concentrations of 0.01, 0.1 and 1.0 were prepared by serial dilution.

Frequency of preparation	daily
Stability of dose formulation	All preparation times were documented in the raw data. Any observation regarding instability of test items were mentioned in the raw data and a separate section discusses this issue in this report.
Storage of dose formulation	On the day of experimentation dose formulations were maintained at room temperature (19 - 30°C) when in use.
Analysis of dose formulations	The identity and concentration of the test items in the dose formulations were not further analyzed.

4.9. PROCEDURE AND OBSERVATIONS

4.9.1. REASONS FOR THE CHOICE OF CONCENTRATIONS

According to sponsor's wishes one or two test item concentrations (see section 4.4) chosen by the sponsor were tested to investigate the effects of the test items on $Na_V1.7$ channel activity.

4.9.2. EXPERIMENTAL PROCEDURE

The 35 mm culture dishes upon which cells were seeded at a density allowing single cells to be recorded were placed on the dish holder of the microscope and continuously perfused (at approximately 1.5 ml/min) with the bath solution described in section 4.6. All solutions applied to cells including the pipette solution were maintained at room temperature (19°C - 30°C). After formation of a Gigaohm seal between the patch electrodes and individual cells (pipette resistance range: 2.0 MΩ - 7.0 MΩ; seal resistance range: > 1 GΩ) the cell membrane across the pipette tip was ruptured to assure electrical access to the cell interior (whole-cell patch-configuration). Afterwards fast and slow capacitances and serial resistance (70%) were compensated. In case the quality of the seal was poor, the process of seal formation was repeated with a different cell and a new pipette.

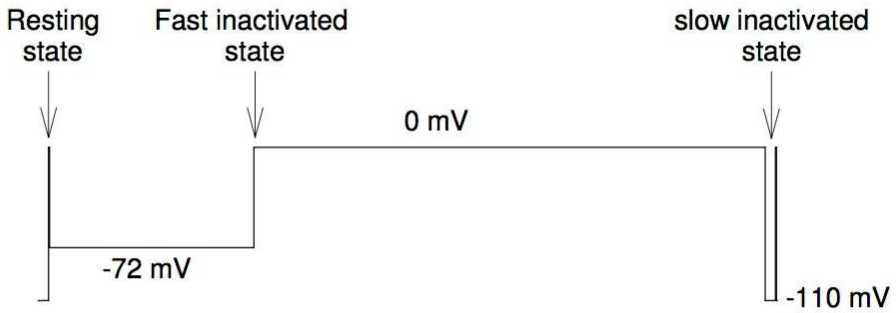
The voltage protocol applied during experiments was as follows:

For the results presented in section 5.1 and 5.2, a group of three inward peak currents were measured within one sequence of holding potential niveaus (= one "sweep"). The peak currents

was elicited by changes to a holding potential of 0 mV starting from conditioning potentials of −110 mV, −72 mV and 0 mV. A short step to −110 mV for 100 ms was performed prior to the last test pulse.

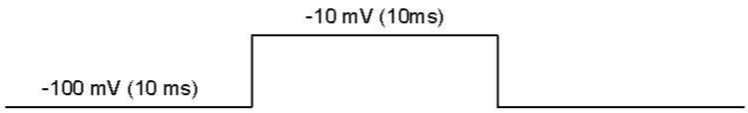
Table I: Sweep segment durations and holding potentials.

Start time [ms]	Duration [ms]	Holding potential [mV]
0	100	-110
100	10	0
110	2000	-72
2110	5000	0
7110	100	-110
7210	10	0
7220	10	-110



This voltage protocol was applied three times for each test concentration (30 s inter-sweep interval), after an incubation time of 90 s. The application protocol consisted of three (ProTx-II) or five periods (Lidocaine), during which bath solution and the test item or reference item concentrations were applied with increasing order. As negative control one set of cells were treated with vehicle.

For all other experiments single pulse protocol was used to test the effect of different ProTX-II samples on $Na_V1.7$ channel activity. Cells were clamped to -100 mV and depolarized to -10 mV for 10 ms every 10 s:



4.9.3. DATA COMPILATION AND STATISTICAL ANALYSIS

The data generated during recording were initially stored on the computer hard disk. Values (in pA) of the peak current amplitudes were generated for each sweep and channel state and printed.

For all states, the first and the last (third) repetition within an application period was used for evaluation of relative currents. These current amplitudes were compared to those from control conditions of the same cell. The amount of current block was calculated as percentage of control. Data from at least two individual experiments per test item was collected and the corresponding mean values and standard errors calculated.

4.10. IC_{50} DETERMINATION

As $Na_V1.7$ peak currents were inhibited by the reference substance Lidocaine, a concentration-response curve was determined and the IC_{50} was calculated.

SigmaPlot 11.0 /Excel 2003 was used to calculate the means ± SD of remaining relative peak current for each concentration of test item. Vehicle control was used as basis (=100%).

In case of Lidocaine application, the remaining current amplitude at the highest tested concentration was lower than 70%. Therefore, the dose response curve was constructed with a sigmoidal two parameter equation:

$$current_{peak,relatve} = 100 / 1 + (X / IC_{50})^{H}$$

where X is the drug concentration, IC_{50} is the concentration of drug at half maximal inhibition and H is the Hill coefficient. SigmaPlot 11.0 was used for construction of the curve.

In case of ProTx-II application, the remaining current amplitude at the highest tested concentration was higher than 70%. Therefore, no dose response curve was constructed and no IC_{50} value was determined.

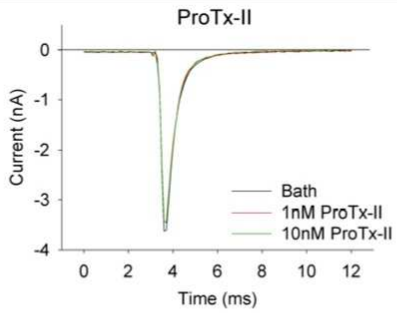
5. EXPERIMENTAL RESULTS

5.1. ProTx-II

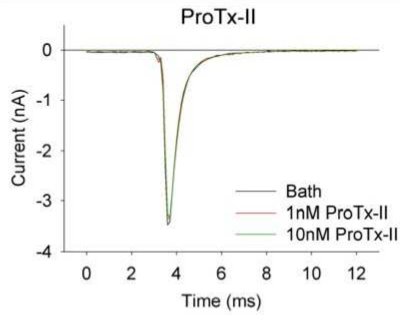
Application	Resting state Mean ± SD		Fast inactivated state Mean ± SD	Slow inactivated state Mean ± SD
	1st Pulse	3rd Pulse		
1 nM ProTx-II	98.38 ± 3.12% (n=3)	100.54 ± 3.90% (n=3)	88.65 ± 5.16% (n=3)	91.36 ± 6.96% (n=3)
10 nM ProTx-II	99.08 ± 3.81% (n=3)	98.70 ± 1.91% (n=3)	80.17 ± 8.19% (n=3)	87.77 ± 9.87% (n=3)

Resting state

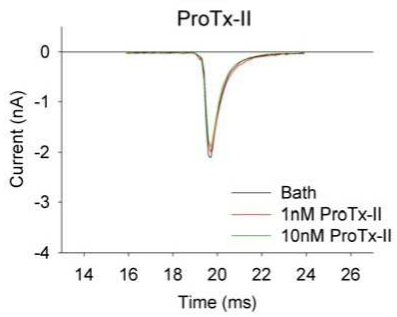
1st Pulse



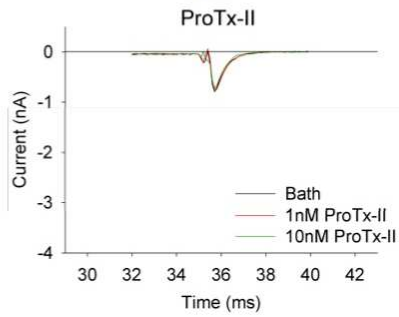
3rd Pulse



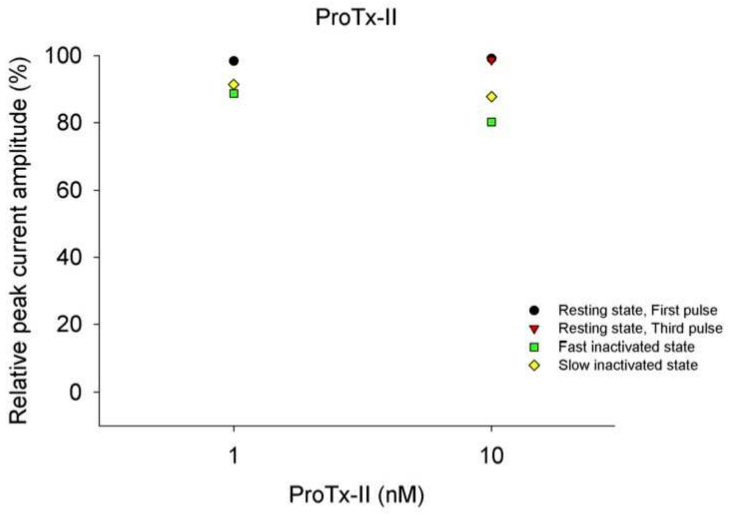
Fast inactivated state



Slow inactivated state



Application	Resting state (IC_{50}, Hill)		Fast inactivated state (IC_{50}, Hill)	Slow inactivated state (IC_{50}, Hill)
	1st Pulse	3rd Pulse		
ProTx-II	remaining current at highest concentration >70%, no dose-response curve fitted			

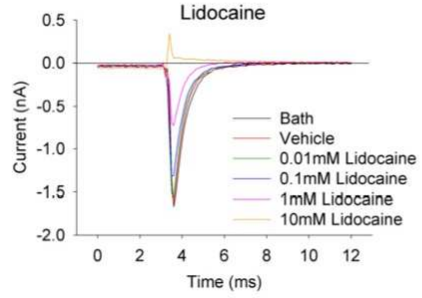


5.2. Lidocaine

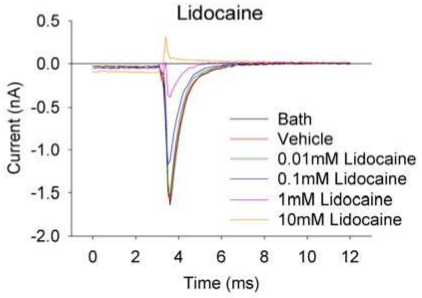
Application	Resting state Mean ± SD		Fast inactivated state Mean ± SD	Slow inactivated state Mean ± SD
	1st Pulse	3rd Pulse		
Vehicle (0.1% DMSO)	98.38 ± 0.64% (n=2)	97.37 ± 0.61% (n=2)	78.14 ± 4.67% (n=2)	82.08 ± 1.16% (n=2)
0.01 mM Lidocaine	92.54 ± 1.30% (n=2)	91.47 ± 2.91% (n=2)	47.19 ± 1.35% (n=2)	73.27 ± 6.46% (n=2)
0.1 mM Lidocaine	72.64 ± 9.21% (n=2)	66.81 ± 6.21% (n=2)	9.24 ± 3.25% (n=2)	41.07 ± 3.90% (n=2)
1 mM Lidocaine	38.67 ± 7.06% (n=2)	22.91 ± 1.90% (n=2)	-0.22 ± 0.21% (n=2)	1.66 ± 2.06% (n=2)
10 mM Lidocaine	-0.66 ± 0.35% (n=2)	-0.85 ± 0.54% (n=2)	-0.99 ± 0.42% (n=2)	-5.11 ± 3.48% (n=2)

Resting state

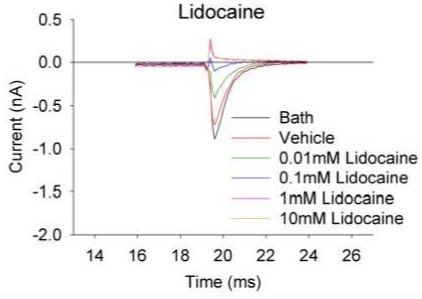
1st Pulse



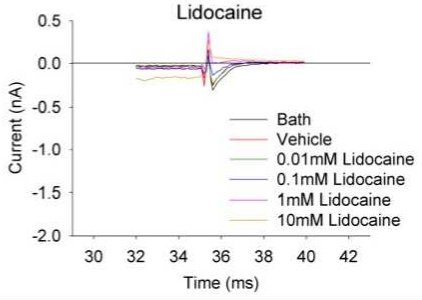
3rd Pulse



Fast inactivated state



Slow inactivated state

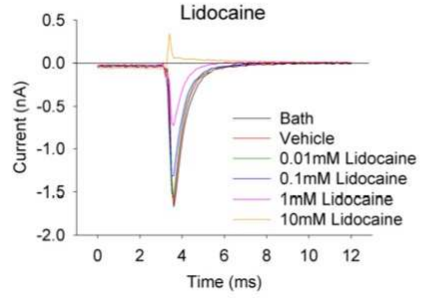


5.2. Lidocaine

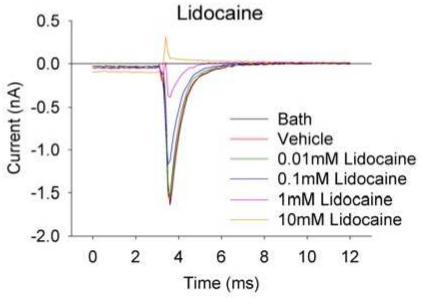
Application	Resting state Mean ± SD		Fast inactivated state Mean ± SD	Slow inactivated state Mean ± SD
	1st Pulse	3rd Pulse		
Vehicle (0.1% DMSO)	98.38 ± 0.64% (n=2)	97.37 ± 0.61% (n=2)	78.14 ± 4.67% (n=2)	82.08 ± 1.16% (n=2)
0.01 mM Lidocaine	92.54 ± 1.30% (n=2)	91.47 ± 2.91% (n=2)	47.19 ± 1.35% (n=2)	73.27 ± 6.46% (n=2)
0.1 mM Lidocaine	72.64 ± 9.21% (n=2)	66.81 ± 6.21% (n=2)	9.24 ± 3.25% (n=2)	41.07 ± 3.90% (n=2)
1 mM Lidocaine	38.67 ± 7.06% (n=2)	22.91 ± 1.90% (n=2)	-0.22 ± 0.21% (n=2)	1.66 ± 2.06% (n=2)
10 mM Lidocaine	-0.66 ± 0.35% (n=2)	-0.85 ± 0.54% (n=2)	-0.99 ± 0.42% (n=2)	-5.11 ± 3.48% (n=2)

Resting state

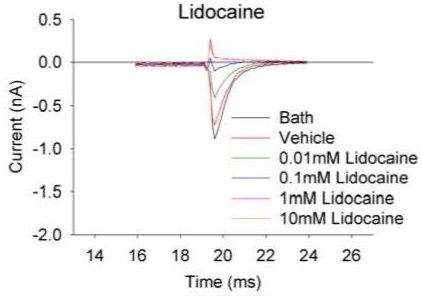
1st Pulse



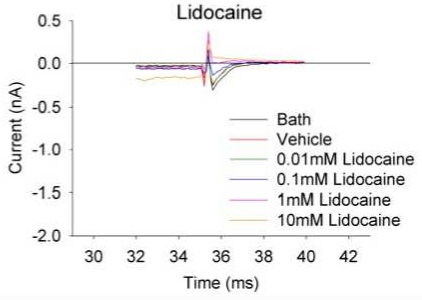
3rd Pulse



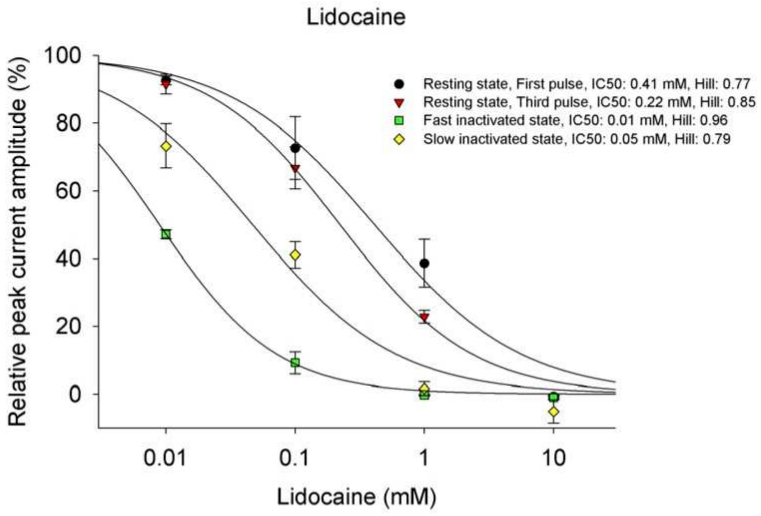
Fast inactivated state



Slow inactivated state



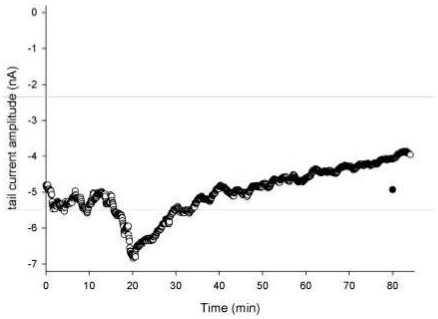
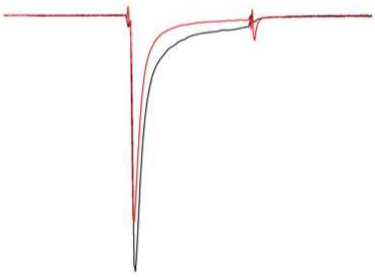
Application	Resting state (IC_{50}, Hill)		Fast inactivated state (IC_{50}, Hill)	Slow inactivated state (IC_{50}, Hill)
	1st Pulse	3rd Pulse		
Lidocaine	0.41 mM, 0.77	0.22 mM, 0.85	0.01 mM, 0.96	0.05 mM, 0.79

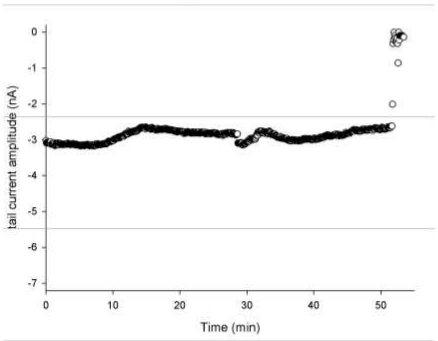
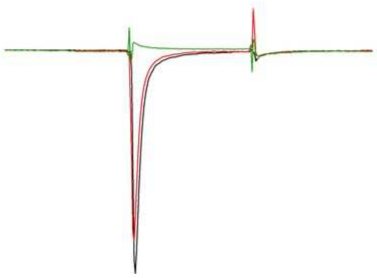


5.3. Single Pulse Experiments and incubation overnight

1 nM ProTx-II was superfused for at least 25 min to determine compound effect on hNa$_V$1.7 sodium channels stably expressed in CHO cells. Furthermore, CHO cells were treated with 1 nM ProTx-II overnight. The results can be summarized as follows:

Application	Peak Current Amplitude	
	[pA]	[%]
Bath	4924	100
1 nM ProTx-II	3950	80.22
Bath	3096	100
Vehicle (Bath Solution)	2693	86.98
10 mM Lidocaine	125	4.04

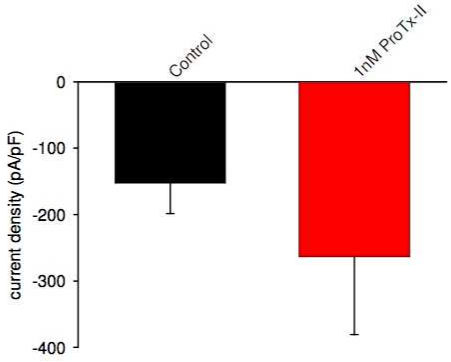
Single Measurement (Bath, 1 nM ProTx-II)	
Long time course	**Bath: black / ProTx-II: red**
	

Single Measurement (Bath, 0.1% DMSO, 10 mM Lidocaine)	
Long time course	**Bath: black / Vehicle: red / Lidocaine: green**
	

To quantify an effect of ProTx-II after overnight induction, the current densities of six individual experiments were compared to control cells:

Application	Current density (Mean ± SD [pA/pF]) *
Not treated	-152.51 ± 45.93 (n=6)
1 nM ProTx-II	-263.71 ± 116.94 (n=6)

- unpaired t-test: $p = 0.0553$



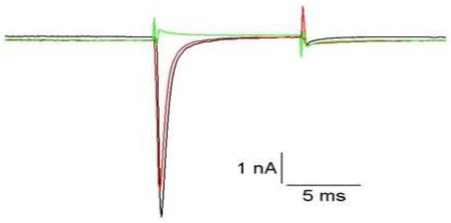
5.4. ProTx-II samples

Different ProTx-II samples were applied to cells stably expressing $Na_V1.7$ channels activated by single depolarizing voltage pulses to -10 mV. The results can be summarized as follows:

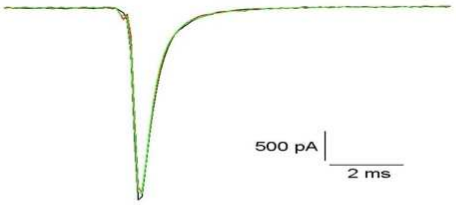
Cell number	Control current (pA)	Test con-centration	Steady state current (pA)	Relative current	Remaining relative peak current amplitude Mean ± SD
ProTx-II Bath Solution Cell1	-3095.83	10 nM	-2692.87	86.98%	86.98% (n=1)
ProTx-II 10 mM Lidocaine Cell1	-3095.83	10 nM	-125.10	4.04%	4.04% (n=1)
ProTx-II/7d Cell1	-3531.67	10 nM	-3386.67	95.89%	98.16 ± 3.21% (n=2)
ProTx-II/7d Cell2	-2394.30	10 nM	-2404.60	100.43%	
ProTx-II/7d Cell1	-3531.67	100 nM	-3388.67	95.95%	97.53 ± 2.24% (n=2)
ProTx-II/7d Cell2	-2394.30	100 nM	-2373.13	99.12%	
linear ProTx-II Cell1	-618.92	10 nM	-1235.63	199.64%	150.41 ± 69.63% (n=2)
linear ProTx-II Cell2	-3816.13	10 nM	-3860.87	101.17%	
ProTx-II Sigma Aldrich Cell1	-3051.37	100 nM	-3085.33	101.11%	98.87 ± 3.17% (n=2)
ProTx-II Sigma Aldrich Cell2	-5902.17	100 nM	-5703.47	96.63%	

Cell number	Control current (pA)	Test con-centration	Steady state current (pA)	Relative current	Remaining relative peak current amplitude Mean ± SD
ProTx-II peptide 14 Cell1	-3979.47	100 nM	-5118.00	128.61%	108.79 ± 17.31% (n=3)
ProTx-II peptide 14 Cell2	-3051.37	100 nM	-3085.33	101.11%	
ProTx-II peptide 14 Cell3	-5902.17	100 nM	-5703.47	96.63%	
ProTx-II peptide 3a Cell1	-3234.47	100 nM	-2295.47	70.97%	87.88 ± 23.91% (n=2)
ProTx-II peptide 3a Cell2	-3555.93	100 nM	-3725.97	104.78%	
ProTx-II peptide 3b Cell1	-11618.00	100 nM	-11904.33	102.46%	103.02 ± 0.79% (n=2)
ProTx-II peptide 3b Cell2	-4608.83	100 nM	-4773.67	103.58%	
ProTx-II Smartox Cell1	-5477.87	100 nM	-77.28	1.41%	2.22 ± 0.99% (n=3)
ProTx-II Smartox Cell2	-4575.60	100 nM	-151.96	3.32%	
ProTx-II Smartox Cell3	-2797.73	100 nM	-53.95	1.93%	
ProTx-II/24h Cell1	-1626.00	100 nM	-42.00	2.58%	3.96 ± 1.20% (n=3)
ProTx-II/24h Cell1	-1458.00	100 nM	-67.00	4.60%	
ProTx-II/24h Cell1	-3479.00	100 nM	-164.00	4.71%	

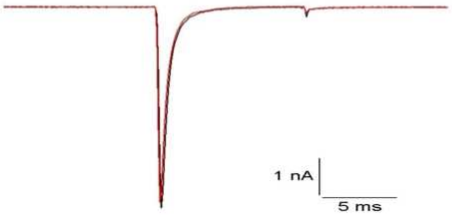
Representative current traces



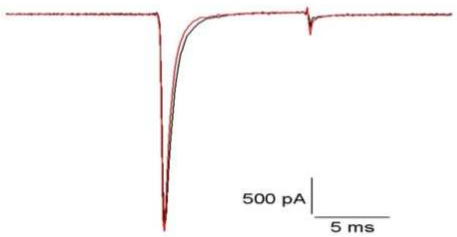
**Control current level (black),
Bath Solution (red),
10 mM Lidocaine (green)**



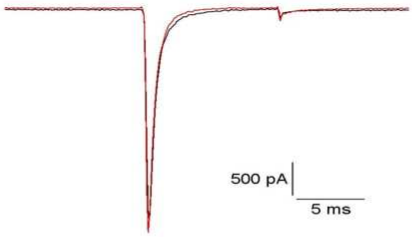
**Control current level (black),
10 nM ProTx-II/7d (red),
100 nM ProTx-II/7d (green)**



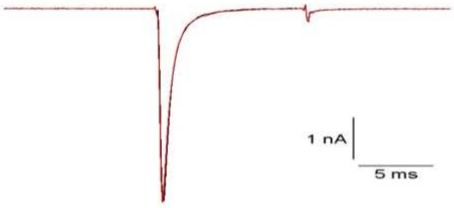
**Control current level (black),
100 nM ProTx-II Sigma Aldrich
(red)**



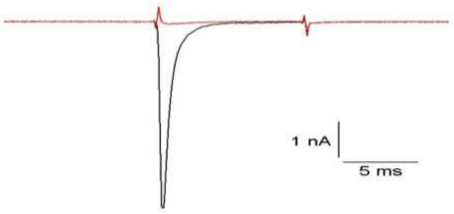
Control current level (black), 10 nM ProTx-II peptide 14 (red)



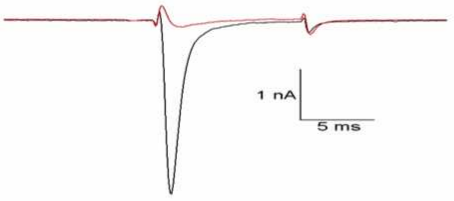
Control current level (black), 100 nM ProTx-II peptide 3a (red)



Control current level (black), 100 nM ProTx-II peptide 3b (red)



Control current level (black), 100 nM ProTx-II Smartox (red)



Control current level (black), 100 nM ProTx-II/24h (red)

6. SUMMARY AND CONCLUSIONS

In this study $hNa_V1.7$ inward peak currents were recorded in CHO cells stably transfected with cDNA encoding this sodium channel in the resting, fast and slow inactivated state.

The test item ProTx-II had no or little effect on the peak current amplitudes of the resting, fast or slow inactivated states investigated. The remaining current amplitudes at the highest tested concentration were higher than 70%. Therefore, no dose response curve was constructed and no IC_{50} value was determined.

For the reference compound Lidocaine IC_{50} values for all four tested states could be determined. None of these effects could be explained by any cytotoxicity effect.

After superfusion of the test item ProTx-II for more than 25 min there was no or only little effect on the peak current amplitude investigated. The amplitude was still efficiently reduced by 10 mM of the reference compound Lidocaine (4.04% remaining tail current amplitude).

The peak current amplitude reduction in the control group as well as the test item group is most likely due to a series resistance raise and not due to an actual compound effect (in case of the test item group).

Additionally, cells were treated with 1 nM ProTx-II overnight. Still, there was no significant difference between treated and untreated cells ($p = 0.0553$).

Application of the different test item samples of ProTx-II (Sample1, Sigma Aldrich, peptide 14, peptide 3a, and peptide 3b) had no or little effect on the peak current amplitudes investigated by application of single pulses.

The test item ProTx-II provided by Smartox (two different batches), as well as ProTx-II/24h blocked the $Na_V1.7$ channels by 97.78%, and 96.04% (ProTx-II/24h) (residual currents: 2.22 ± 0.99%, n=3 (Smartox), 3.96 ± 1.20% (ProTx-II/24h), n=3.

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