Electronic Supplementary Information

Simplifying native chemical ligation with an *N*-acylsulfonamide linker

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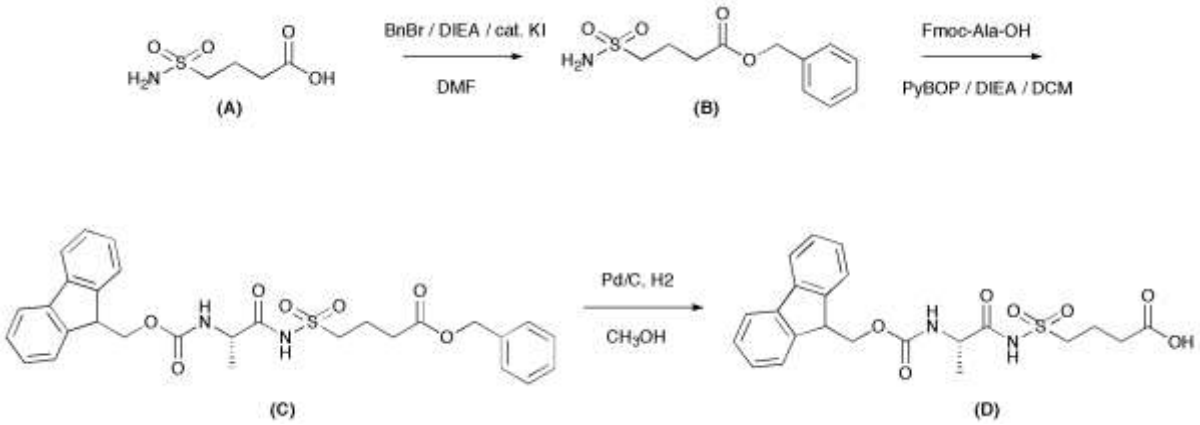
Abbreviations

ACN: acetonitrile. BPTI: bovine pancreatic trypsin inhibitor. CHCA: α-cyano-4-hydroxycinnamic acid. DCM: dichloromethane. DIC: 1,3-diisopropylcarbodiimide. DIEA: *N*,*N*-diisopropylethylamine. DMF: *N*,*N*-dimethylformamide. EDTA: ethylenediamine tetraacetic acid. Fmoc: 9-fluorenylmethoxycarbonyl. Gn.HCl: guanidine.HCl. HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide. HOBt: 1-hydroxybenzotriazole. MALDI-TOF MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry. MPAA: 4-mercaptophenylacetic acid. PyBOP: benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate. TCEP: tris(2-carboxyethyl)phosphine. TFA: trifluoroacetic acid.



Scheme S1.

Sulfamylbutyric acid benzylester (B)

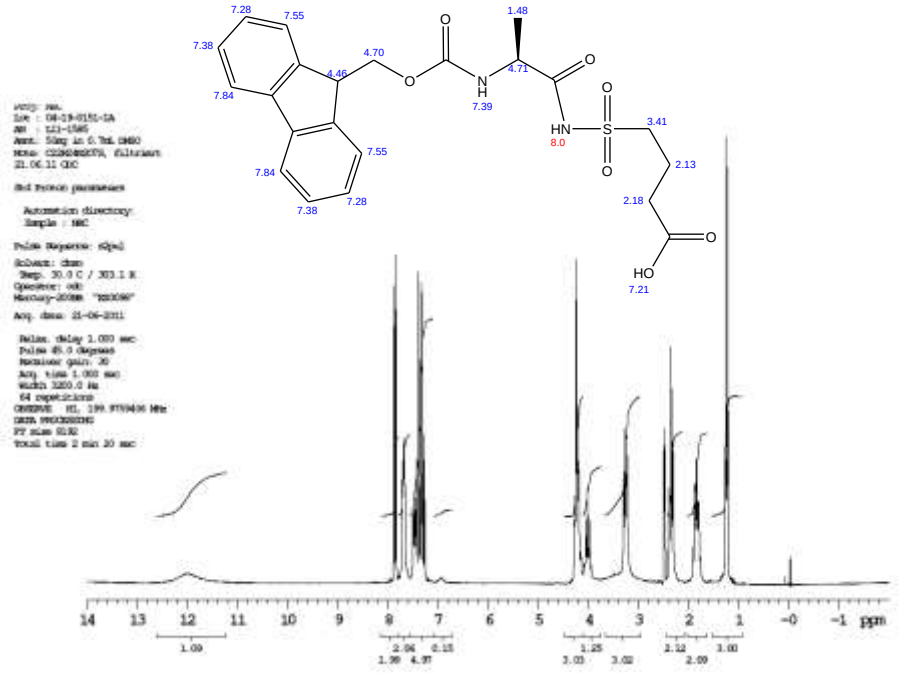
3-carboxypropane sulfonamide **(A)** (83.5 g, 500 mmol), benzylbromide (59.0 mL, 500 mmol), DIEA (86 mL, 500 mmol) and catalytic amounts of potassium iodide (25 g, 150 mmol) were added to DMF (1 L). After completion of the reaction the mixture was diluted (EtOAc, 2 L), washed with H_2O (2x3 L), HCl (1 M; 2 x 500 mL), H_2O (1 x 500 mL) and saturated NaCl (2 x 500 mL), dried (Na_2SO_4) and evaporated. The solid was crystallized from toluene/hexane yielding a homogenous product (95.9 g, 75 % of the theory) (TLC- purity > 99%, HPLC- purity > 98 area %). The product was characterized by ESI mass spectrometry (m/z = 258.2 $[M+H]^+$, calc. M = 257.1 for $C_{11}H_{15}NO_4S$) and melting point (83°C).

N-Fmoc-Ala-sulfamylbutyric acid (D)

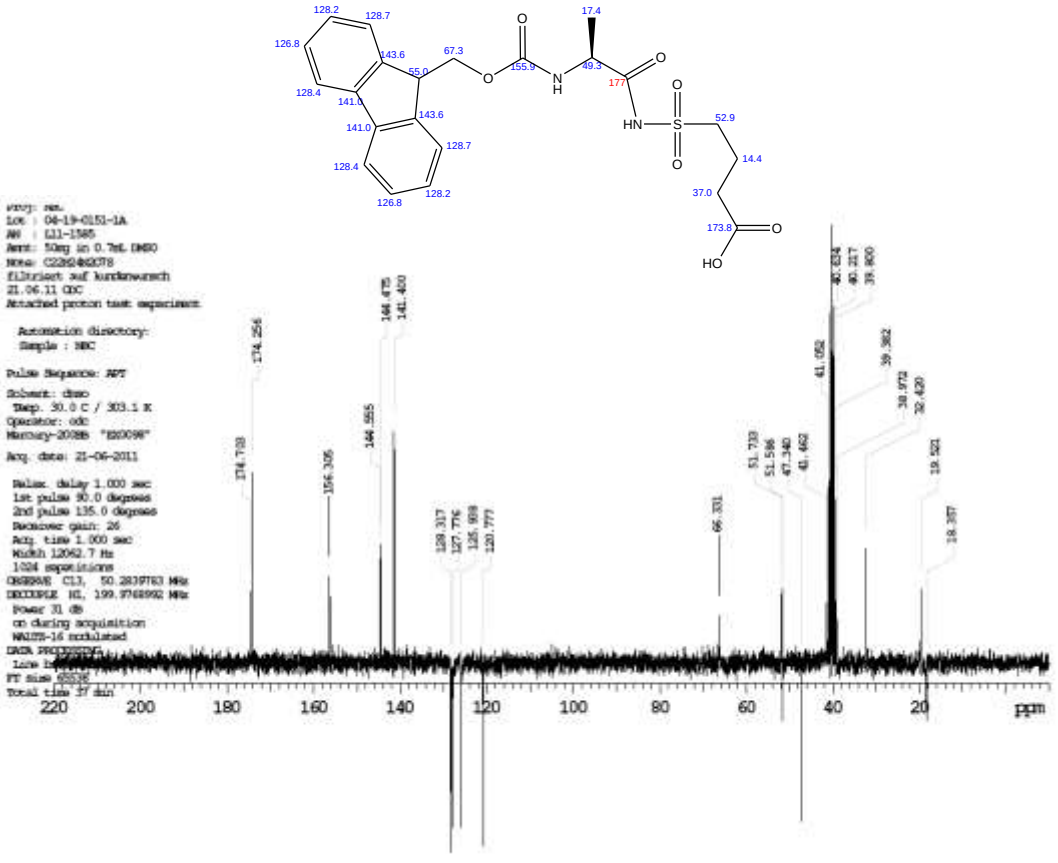
Fmoc-Ala-OH (6.0 g, 19 mmol) was dissolved in DCM (300 mL) and coupled to sulfamylbutyric acid benzylester **(B)** (5.0 g, 19 mmol) using PyBOP (9.9 g, 19 mmol) and DIEA (9.8 mL, 57 mmol). The mixture was evaporated, the residual oil dissolved (EtOAc), washed with dilute acid and extracted. The organic volatiles were evaporated and the resulting foam used as such in the next step (HPLC- purity > 90 %). Hydrogenolysis of the benzylester **(C)** (10.5 g, 19 mmol) with H_2, 5 % Pd/C (Fluka) in methanol gave the acid. The mixture was filtered and the resulting filtrate evaporated. The residue was dissolved in aq. $NaHCO_3$, washed with *t*-butylmethyl ether (2 x 100 mL). The aqueous layer was adjusted to pH 3 employing cooling to precipitate the product. The precipitate was filtered, washed with HCl (1 M), H_2O and Et_2O and dried under vacuum to yield 9.1 g (85 % of the theory). The compound was characterized by ESI mass-spectrometry (m/z = 483.1 $[M+Na]^+$ calc. M = 460.1 for $C_{22}H_{24}N_2O_7S$) and ^{1}H-NMR (200 MHz, d_6-DMSO) δ 7.9 -7.2 (m,

5H), δ 4.4 - 4.2 (m, 3H), δ 4.2 – 3.9 (t, 1H), δ 3.5 – 3.1 (m, 2H), δ 2.4 - 2.1 (m, 2H), δ 2.0 – 1.6 (m, 2H), δ 1.4 - 1.1 (d, 3H) and ^{13}C APT-NMR (50 MHz, d_6-DMSO) δ 174.7, 174.3, 156.4, 144.6, 141.5, 141.4, 128.3, 127.8, 125.9, 120.8, 66.3, 51.7, 47.3, 32.4, 19.5, 18.4. The optical purity of Alanine was proved by GC-MS on CHIRASIL-VAL by C.A.T. The content of D-Ala enantiomer was determined to 0.2 %.

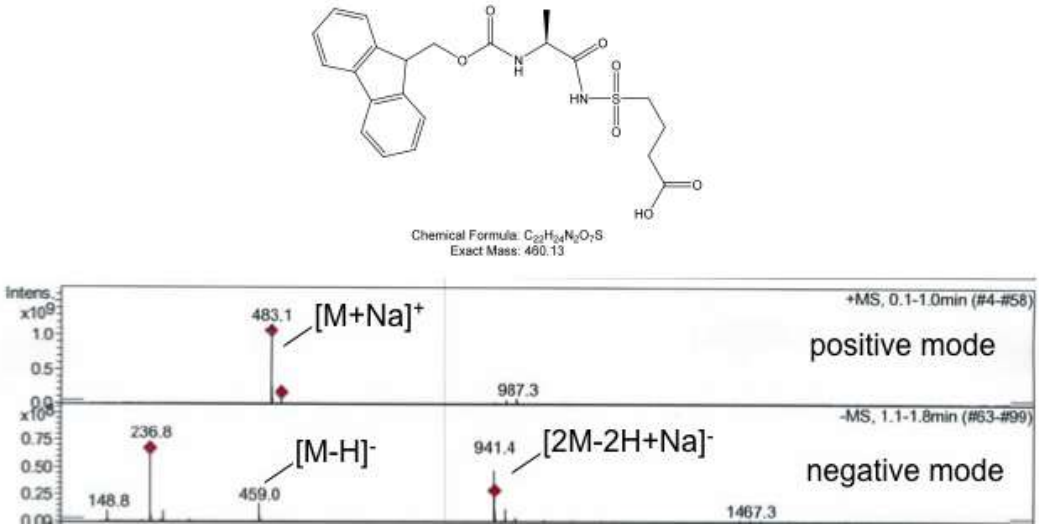
^{1}H-NMR of Fmoc-Ala-sulfamylbutyric acid



^{13}C-NMR of Fmoc-Ala-sulfamylbutyric acid



ESI-MS of Fmoc-Ala-sulfamylbutyric acid



N-Fmoc-Ala-sulfamylbutyryl Sieber Amide Resin

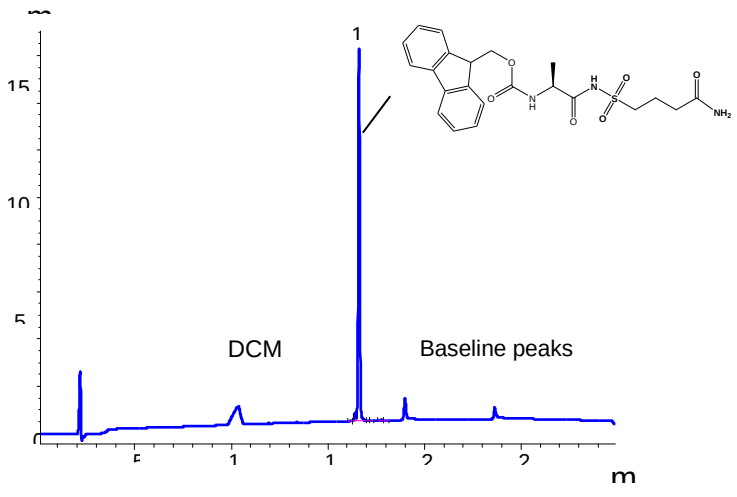
The Fmoc group was removed from the Sieber amide resin (10 g, 4.4 mmol) by treatment with 20 % piperidine in DMF (1 x 5 min, 1 x 10 min). Afterwards the resin was washed alternately with DMF and isopropanol three times. *N*-Fmoc-alanyl-sulfamylbutyric acid (**D**) (6.1 g, 13.2 mmol) was loaded onto the resulting resin by adding HBTU (4.5 g, 11.9 mmol) and DIEA (3.4 mL, 18.9 mmol) in DMF. The reaction was monitored by TNBS and chloranil colour tests. Upon negative colour tests, the resin was washed alternately with DMF and isopropanol three times. Final washing was performed with methanol and diisopropyl ether. The resin was dried under vacuum to constancy of weight yielding 11.9 g (theory 12.3 g).

The loading was determined to 0.32 mmol/g by UV absorption at 304 nm detecting the Fmoc cleavage product. For additional characterization a small sample of the resin was treated with 5 % TFA in DCM for 30 min to cleave off *N*-Fmoc-Ala-sulfamylbutyramide which was subjected to ESI mass spectrometry (m/z = 460.1 $[M+H]^+$, calc. M = 459.2 for $C_{22}H_{25}N_3O_6S$) and HPLC-chromatography (> 97 area %).

HPLC profile of cleaved Fmoc-Ala-sulfamylbutyramide

Linear gradient 0% B to 100 % B in 30‘
Eluent A = 0.1% TFA + 2 % ACN in H_2O
Eluent B = 0.1% TFA in ACN,
Column = ACE C18-AR, 5 um, 150x4,6 mm
Detection = 215 nm

$[M+H]^+$ = 460.1; M = 459.15 calc. for $C_{22}H_{25}N_3O_6S$
Area % > 97.0 %

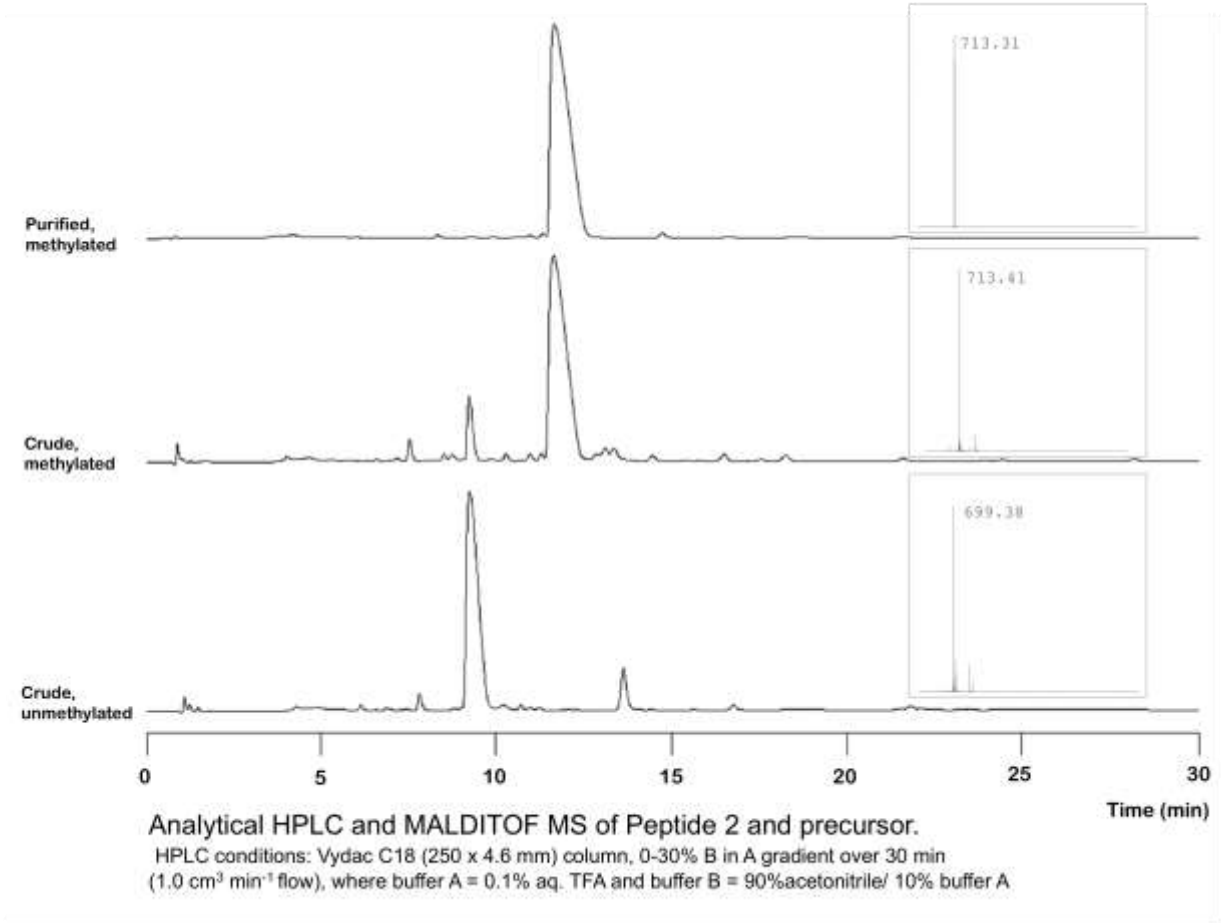


Synthesis and characterization of the peptides used in the ligation reaction[1]

Peptides were prepared by standard automated solid-phase synthesis using DIC/HOBt activation for Fmoc/t-Bu chemistry (CS Bio 336 automated synthesizer). Couplings were carried out with a 5-fold excess of activated amino acid for a minimum of 45 min.

BPTI(38-58) was synthesized on Fmoc-Ala-Wang resin (Merck Chemicals UK), peptide **3** on Rink Amide AM resin (Merck Chemicals UK).
Peptides **1**, **2** and BPTI(1-37) were assembled on the appropriate preloaded (Fmoc-Gly- or Fmoc-Ala-) sulfamylbutyryl Sieber amide resin on a 0.1 mmol scale (resin loading 0.32 $mmol.g^{-1}$; 329 mg resin). Alkylation of the *N*-acylsulfonamides was performed by overnight treatment of the peptide-resin with trimethylsilyl diazomethane (TMS-CHN_2) in hexane/DCM (1:1).
Cleavage was performed by treatment of the peptide-resin with a cleavage cocktail containing TFA and scavengers, triethylsilane, H_2O, ethanedithiol in the ratio 94.5:2.5:2.5:0.5 (v/v) for 1.5 h. The cleavage cocktail was filtered, the filtrate sparged (N_2) and peptides precipitated (Et_2O, Na- dried, 4 ^oC) and freeze-dried. Peptides were purified by semi-preparative HPLC on a RP-C18 column (22 x 250 mm, Vydac) using linear gradients of CH_3CN and 0.1%TFA/H_2O.
Peptides were characterized by MALDI-TOF MS on a BRUKER microflex using CHCA matrix (10 $mg.mL^{-1}$ in CH_3CN/H_2O/TFA, 50:50:0.1). The ion positive reflector mode was used for peptides with m/z < 4000, the ion positive linear mode was used for peptides with m/z > 4000.

Peptide 1 = *H*-AYRAG-N(Me)SO_2-CH_2-CH_2-CH_2-$CONH_2$, m/z = 699.4 $[M+H]^+$ (first isotope), calc.: 699.3. 25 mg of peptide were obtained after HPLC purification (purity > 98 %), 27 % yield
Peptide 2 = *H*-AYRAA-N(Me)SO_2-CH_2-CH_2-CH_2-$CONH_2$, m/z = 713.3 $[M+H]^+$ (first isotope), calc.: 713.3. 26.8 mg of peptide were obtained after HPLC purification (purity > 98 %), 28.5 % yield.

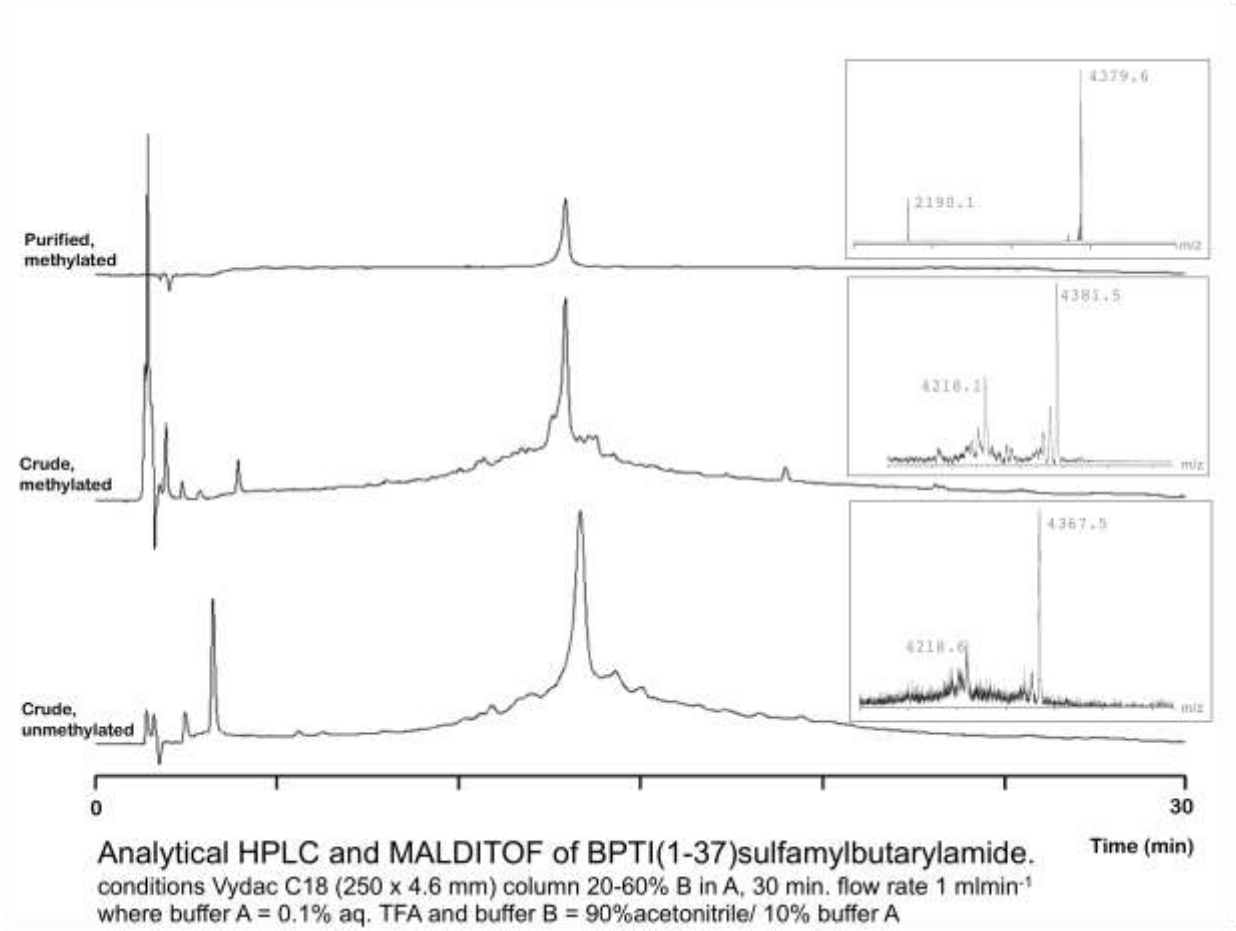


Analytical HPLC and MALDITOF MS of Peptide 2 and precursor.
HPLC conditions: Vydac C18 (250 x 4.6 mm) column, 0-30% B in A gradient over 30 min (1.0 cm^3 min^{-1} flow), where buffer A = 0.1% aq. TFA and buffer B = 90%acetonitrile/ 10% buffer A

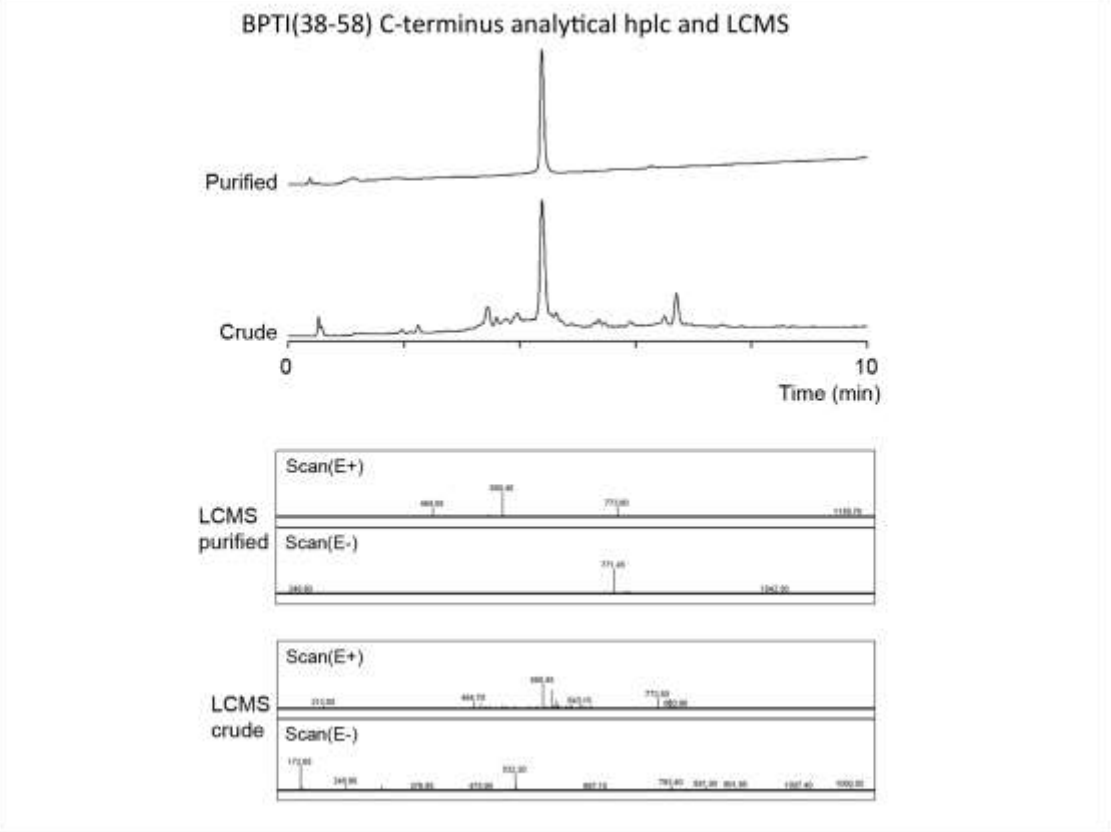
Peptide 3 = *H*-CFALRGWR-*NH*$_2$, m/z = 1007.5 $[M+H]_+$ (first isotope), calc.: 1007.5.

BPTI(1-37) = *H*-RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGG-*N*(Me)SO_2-CH_2-CH_2-CH_2-$CONH_2$, m/z = 4379.6 $[M+H]^+$ (average isotope composition), calc.: 4376.1. HPLC purification using a Vydac C18 column (25 x 2.5 cm) flow 15 mL.min^{-1} gradient 15-35% B in A over 5 min then 35-50% B in A over 30 min yielded 45 mg peptide (purity > 95 %), 9 % yield.

BPTI(38-58) = *H*-CRAKRNNFKSAEDCMRTCGGA-*OH*, m/z = 2318.03 $[M+H]^+$ (first isotope), calc.: 2317.3 Analytical HPLC was performed with a Merck Chromolith RP-8 (100 x 4.6 mm) using gradients 0-40 % B in A over 10 min. flow rate 3 mL.min.$^{-1}$ HPLC purification using a Vydac C18 column (25 x 2.5 cm) flow 15 mL.min^{-1} gradient 10-50% B in A over 30 min yielded 43 mg; 14 % yield.



Analytical HPLC and MALDITOF of BPTI(1-37)sulfamylbutarylamide.
conditions Vydac C18 (250 x 4.6 mm) column 20-60% B in A, 30 min. flow rate 1 mlmin^{-1}
where buffer A = 0.1% aq. TFA and buffer B = 90%acetonitrile/ 10% buffer A



Ligation of small model peptides[1]

Peptide **2** (1.06 mg, 1.13 µmol; final concentration 11.3 mM) and peptide **3** (1.25 mg, 0.93 µmol; 9.3 mM) were dissolved in 100 µL degassed 0.2 M sodium phosphate buffer, 1 M guanidine.HCl, 2 mM EDTA, 50 mM TCEP, 60 mM MPAA, final pH 7.5. The reaction mixture was heated at 40 °C. The reaction was monitored at 214 nm using analytical HPLC on a Chromolith column (4.6 x 100 mm, RP-8e, Merck) using a gradient of 0 to 30 % B in 10 min with a flow rate of 3 mL.min^{-1} (B = 0.1% TFA, 10% H_2O, 90% CH_3CN). The ligation product (*H*-AYRAACFALRGWR-*NH_2*) was collected and analysed by MALDI-TOF MS in positive reflector mode using CHCA matrix. m/z = 1539.7 $[M+H]^+$ (first isotope) (observed), calc.: 1539.8.

The same procedure was used to ligate peptides **1** and **3**. The ligation product (*H*-AYRAGCFALRGWR-*NH_2*) was characterized by MALDI-TOF MS in positive reflector mode using CHCA matrix. m/z = 1525.8 $[M+H]^+$ (first isotope) (observed), calc.: 1525.8.

BPTI Ligation[1]

BPTI(1-37) (0.36 mg, 0.07 µmol; final concentration 0.65 mM) and BPTI(38-58) (0.32 mg, 0.11 µmol; final concentration 1 mM) were dissolved in 110 µL of degassed 0.2 M sodium phosphate buffer pH 7.5, containing 6 M guanidine.HCl, 2 mM EDTA, 50 mM TCEP, 60 mM MPAA. The solution was heated at 40 °C. The reaction was monitored by analytical HPLC (detection 214 nm) on a RP-C18 column (4.6 x 100 mm, Vydac) using a linear gradient of 0 to 70 % B in 25 min with a flow rate of 1 mL.min^{-1} (B =0.1 % TFA, 10% H_2O, 90 % CH_3CN). The ligation product was collected and characterized by MALDI-TOF MS in positive linear mode using CHCA matrix: m/z= 6517.9 $[M+H]^+$ (average isotope composition), calc: 6517.6.

The removal of aliquots to monitor the above reaction depleted the yield therefore to obtain a representative yield the ligation was repeated on a larger scale but at the same concentration and pH. BPTI(1-37) (10.0 mg, 2.0 µmol) and BPTI(38-58) (5.9 mg, 2.0 µmol) were dissolved in degassed ligation buffer as detailed above and left under argon for 8 h. HPLC purification using a Vydac C18 column (25 x 2.5 cm) flow 15 mL.min^{-1} gradient 0-20% B in A over 5 min; 20-65% B in A over 30 min yielded full-length BPTI (2.4 mg, 16 %). The preparative column available for

this purification was too large for the small amount of material and therefore handling losses were large resulting in a low isolated yield. No significant side reactions were observed during ligation.

Note

(1) Molecular weights used to calculate yields and concentrations include the CF_3COO^- counter-ions.