Electronic Supplementary Information for

Phytosulfokine Peptide Library: Chemical Synthesis and Biological Evaluation on Protoplast Regeneration

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Experimental

General procedures

Starting materials, reagents, and solvents were purchased from commercial vendors and used as received unless stated otherwise. 4 M HCl in dioxane, allyl chloroformate, ammonium acetate (NH_4OAc), ammonium chloride (NH_4Cl), copper sulfate ($CuSO_4 \cdot H_2O$), palladium on carbon (Pd/C), potassium bisulfate ($KHSO_4$), sodium sulfate (Na_2SO_4), and sodium thiosulfate ($Na_2S_2O_3$) were purchased from Acros Organics. Acetic acid (AcOH, glacial, 99+%), acetic anhydride, and benzyl bromide were purchased from ALFA Aesar. Fmoc-*allo*-Ile-OH, Fmoc-*allo*-Thr(*t*Bu)-OH, and Fmoc-Tyr(SO_2ONp)-OH were purchased from Bachem. *N,N*-dimethylformamide (DMF, peptide synthesis grade), ethanol (absolute, HPLC grade), *N*-methyl-2-pyrrolidone (NMP, peptide synthesis grade), tetrahydrofuran (THF), and trifluoroacetic acid (TFA), were purchased from Biosolve Chimie. *N,N*-diisopropylethylamine (DIPEA, peptide synthesis grade) was purchased from Fisher Bioreagents. (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) and cesium carbonate (Cs_2CO_3) were purchased from Fluorochem. Hexane was purchased from Honeywell. Acetonitrile (MeCN), dichloromethane (DCM), and diethyl ether were purchased from Honeywell Riedel-de Haën. Piperidine was purchased from Honeywell Burdich & Jackson. Methanol (MeOH, anhydrous) was purchased from Macron Fine Chemicals. 1-hydroxybenzotriazole (HOBt), 4-pentynoic acid, hydrochloric acid 37%, sodium hydroxide (NaOH), and thiosalicylic acid were purchased from Sigma-Aldrich. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Boc-Ile-OH, Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Thr(*t*Bu)-OH, H_2N-Thr(*t*Bu)-OH, and Fmoc-Tyr(*t*Bu)-OH were purchased from Novabiochem®, Sigma-Aldrich. Sodium hydride and triisopropylsilane (TIS) were purchased from TCI Europe. Chloroform ($CHCl_3$), deuterated chloroform ($CDCl_3$), deuterated water (D_2O), magnesium sulfate ($MgSO_4$), and methyl iodide were purchased from Thermo Scientific. Ethyl acetate (EtOAc) and formic acid (FA) were purchased from VWR Chemicals. Anhydrous CH_2Cl_2 and anhydrous THF were obtained from a PureSolv Solvent Purification System. Milli-Q (deionized water) was obtained from a Milli-Q Integral 3 system; Millipore, Molsheim/France). Traces of water from reagents were removed by co-evaporation with toluene in reactions that required anhydrous conditions. Reactions were monitored by thin-layer chromatography (TLC) using Merck aluminum sheets (Silica gel 60 F_{254}) with detection by UV absorption (254 nm), by spraying with a solution of 2% ninhydrin in *n*-butanol, or a solution of $KMnO_4$ (10 g/L) and K_2CO_3 (50 g/L) in water, followed by charring at ~150 °C. Organic solvents were removed under reduced pressure at 40 °C. Flash column chromatography was performed using SiliaFlash® P60 silica gel (particle size of 40–63 µm, pore diameter of 60 Å) with the indicated eluents. ^{1}H NMR and ^{13}C NMR spectra were recorded using a Brüker AV-400 (400 and 101 MHz, respectively) spectrometer in the given deuterated

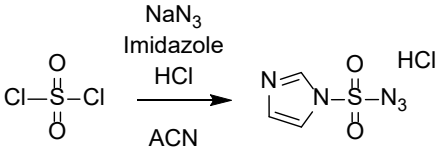
solvent. Chemical shifts are given in ppm (δ) relative to the residual solvent peak or tetramethylsilane (0 ppm) as internal standard and coupling constants are given in Hz. Multiplicity is reported as s: singlet, d: doublet, dd: doublet of doublets, td: triplet of doublets, qd: quartet of doublets, ddd: doublet of doublet of doublets, t: triplet, tt: triplet of triplets, ddt: doublet of doublet of triplets, q: quartet, m: multiplet. Assignments were made by standard COSY and HSQC analysis. High-resolution mass spectrometry (HRMS) analysis was performed with an Exactive Plus Orbitrap Mass Spectrometer (Thermo Fisher), equipped with an electrospray ion source (ESI) in positive mode. MS-grade methanol was used as eluent. The high-resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Lyophilization was done with a Labconco FreeZone lyophilizer (2.5 L, -84 °C, connected to a 35_i xDS Edwards Oil-Free Dry Scroll Pump). Peptide purification was done with an Agilent 1260 Preparative high-performance liquid chromatography (HPLC) with a DAD G7115A and MSD) using a preparative Grace Alltima column (C18, 22 x 250 mm, 5-Micron) or a semi-preparative Zorbax Eclipse column (XDB-C18, 9.4 x 250 mm, 5-Micron). Peptide analysis was done with HPLC coupled to mass spectrometry (ESI-MS, measuring both positive and negative in a switch mode) using a Q Exactive Focus Agilent 1290 Infinity UHPLC-MS system. The HPLC system is equipped with a diode array detector (DAD G4212A, at 415 and 454 nm) and a Dr. Maisch ReproSil Gold 120 C18, 3 µm, 200 x 3 mm column containing a 10 mm guard with a flow rate of 0.4 mL/min. For washing or extractions we typically used equivolume amounts of the washing/extracting solvent.

General procedure for Phytosulfokine synthesis

Peptides were synthesized following Fmoc/*t*Bu Solid-Phase Peptide Synthesis (SPPS) strategy. Chain elongation was initiated from Fmoc-Gln(Trt)-Wang resin 100–200 mesh Novabiochem®, a *p*-alkoxy-benzyl alcohol polymer-bound (polystyerene-1%, DVB) amino acid (loading capacity 0.4-0.9 mmol/g). Amino acids were added as follows: the resin was pre-swollen with NMP or DCM. The Fmoc-protecting group was removed using 20% piperidine in DMF (2 x 8 min). The resin was then washed with DMF (3 x 2 min). Fmoc-Thr(tBu)-OH was treated with HBTU, HOBt, and DIPEA in DMF for 2 min before being added to the resin. The reaction mixture was allowed to couple for 2 h, and reaction completion was monitored by resin staining using ninhydrin (15 g/L, supplemented with 30 mL/L acetic acid in *n*-butanol) for primary amines, or performing a chloranil test (20 mL/L acetaldehyde in DMF as reagent A and 20 g/L *p*-chloranil in DMF as reagent B) for secondary amines. Upon reaction completion, the resin was washed with DMF (3 x 2 min) and deprotected by 20% piperidine in DMF (2 x 8 min). After washing again with DMF (3 x 2 min), Fmoc-Tyr(SO_2ONp)-OH was activated with HBTU, HOBt, DIPEA, and subsequently coupled the resin. Fmoc-Ile-OH and Fmoc-Tyr(SO_2ONp)-OH were subsequently coupled. After installation of the second tyrosine residue, Fmoc was removed and the peptide was

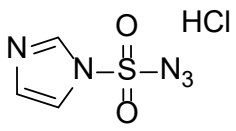
cleaved from the resin by treatment with a cocktail of 95% TFA, 2.5% TIS, 2.5% Milli-Q for 2 h (10 mL TFA cocktail/1 g initial resin). The cleaved peptide was precipitated by dropwise addition of the TFA cocktail-peptide mixture to ice-cold diethyl ether (1:1 ether:hexane, 10x initial cocktail volume) and the cleaved peptide resin was washed once with fresh cleavage cocktail. The precipitate was centrifuged for 10 min at 6000 rpm. The supernatant was discarded and the precipitate was washed with ice-cold diethyl ether and again centrifuged for 10 min at 6000 rpm. This washing step was repeated once more. The resulting precipitate was then dried in a light stream of N_2, redissolved in MeCN:Milli-Q (4:6) and then lyophilized. The lyophilized peptide was purified by preparative reverse phase HPLC. The eluents for purification contained 0.1% FA in H_2O and 0.1% FA in MeCN with a gradient of 5→5→95→95→5→5% (percentage CH_3CN, 0→5→25→30→35→40 min). The obtained purified peptide was then lyophilized. The pure neopentyl-protected disulfated peptide was treated with 2M NH_4OAc to remove neopentyl from the sulfated tyrosine residues at 45 °C for 40 h. Obtained deprotected peptides were purified by (semi)preparative reverse phase HPLC. The eluents for Np-deprotected peptide purification contained 10 mM NH_4OAc in H_2O and MeCN with a gradient of 5→5→60→60→5→5% (percentage CH_3CN, 0→5→25→30→35→40 min). The purified peptide fractions were subsequently lyophilized. The purity of synthesized peptides was analyzed by HPLC ESI-MS. The eluents contained 10 mM NH_4OAc in H_2O and MeCN with a gradient of 5→5→60→60→5→5% (percentage CH_3CN, 0→5→25→30→35→40 min).

Diazotransfer reagent for N_3-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH (4)



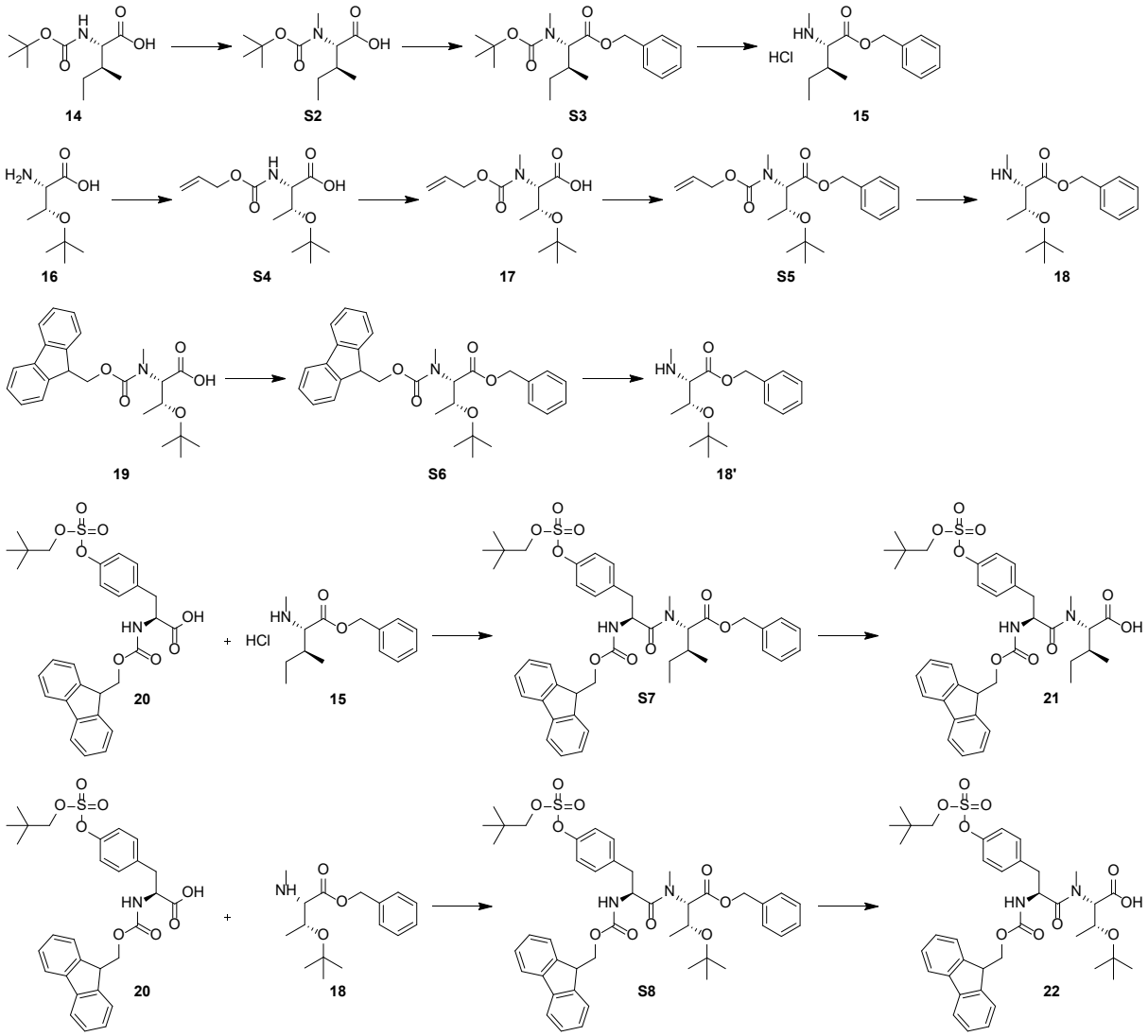
S1

Imidazole-1-sulfonyl azide hydrochloride (S1)



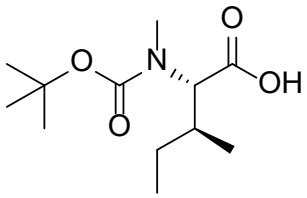
Diazotransfer reagent was synthesized as previously described.[1] Sulfuryl chloride (8.1 mL, 100 mmol) was added dropwise to an ice-cooled suspension of sodium azide (6.5 g, 100 mmol) in anhydrous acetonitrile (100 mL) and the mixture was stirred at rt for 20 h. Imidazole (13.6 g, 200 mmol) was added portion wise to the ice-cooled mixture and was stirred at rt for 5 h. The reaction mixture was diluted with ethyl acetate (200 mL) and H_2O was added (200 mL). The organic layer was washed with H_2O (2 x 200 mL), saturated $NaHCO_3$ (2 x 200 mL), and brine (200 mL). The organic layer was dried over Na_2SO_4. Ethanol (37.5 mL) was cooled on ice and acetylchloride (10.7 mL) was added dropwise to obtain a solution of HCl/EtOH. The HCl/EtOH solution was added dropwise to the obtained filtrate while stirring, which was dried to obtain **S1** as a white powder (14.7 g, 85 mmol, 85%). ^{1}H NMR (400 MHz, D_2O) δ 9.42 (t, *J* = 1.4 Hz, 1H), 8.05 (t, *J* = 1.9 Hz, 1H), 7.64 (dd, *J* = 2.3, 1.3 Hz, 1H). ^{13}C NMR (101 MHz, D_2O) δ 137.7, 123.3, 120.1. HRMS (ESI): *m/z* = $[M+H]^+$ calc for $C_3H_4N_5O_2S$ 174.0080, found 174.0077.

N-methylated building blocks for peptides 12 and 13



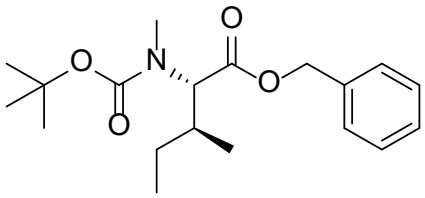
Scheme S1. Overview of Solution-phase synthesis of dipeptides **21** and **22**.

Boc-*N*me-Ile-OH (S2)



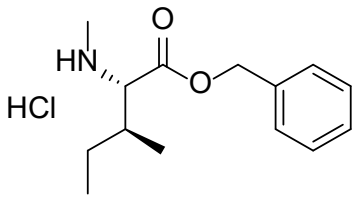
N-(*tert*-butoxycarbonyl)-L-isoleucine (**14**, 5.0 g, 21.6 mmol) was dissolved in THF (144 mL) and cooled to 0 °C. The solution was slowly treated with sodium hydride (60% in mineral oil, 2.6 g, 64.9 mmol). After 30 min, methyl iodide (10.7 mL, 173 mmol) was added and the reaction was stirred for 20 h at room temperature. Diethyl ether was added and the organic layer was washed with H_2O twice. The combined aqueous layers were acidified with 1 M $KHSO_4$ and extracted with ethyl acetate twice. The combined organic phases were washed with $Na_2S_2O_3$ solution and brine and dried over Na_2SO_4. The product was concentrated under reduced pressure to afford title compound **S2** as a grey oil (4.7 g, 19 mmol, 89%). The NMR reported are for a mixture of cis/trans rotamers originating from the N-methylated carbamate: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.28–4.12 (m, 1H), 2.87 (s, 3H), 2.20–1.92 (m, 1H), 1.47 (s, 10H), 1.09 (ddt, *J* = 13.9, 8.7, 7.0 Hz, 1H), 0.98 (d, *J* = 6.7 Hz, 3H), 0.90 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.7, 157.3, 81.4, 80.7, 65.6, 63.5, 33.6, 33.4, 30.8, 28.5, 25.3, 16.1, 15.9, 10.8. HRMS (ESI): *m/z* = $[M+Na]^+$ calc for $C_{12}H_{23}NO_4Na$ 268.1519, found 268.1512; *m/z* = $[2M+Na]^+$ calc for $C_{12}H_{23}NO_4Na$ 513.3146, found 513.3138.

Boc-*N*me-Ile-OBn (S3)



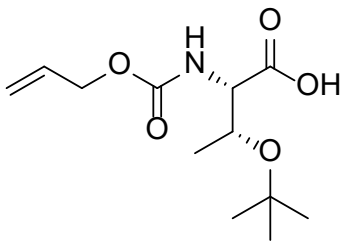
Boc-*N*me-Ile-OH (**S2**, 4.7 g, 19 mmol) was dissolved in MeOH (51 mL) and Cs_2CO_3 (3.1 g, 9.5 mmol) was added. The mixture was stirred for 45 min before being concentrated *in vacuo*. The resulting pale yellow foam was dissolved in acetonitrile (95 mL) and benzyl bromide (4.5 mL, 38 mmol) was added. The reaction mixture was stirred at room temperature for 16 h. Upon reaction completion, the mixture was diluted with H_2O and ethyl acetate. The water layer was extracted twice with ethyl acetate. The organic layer was then washed with $KHSO_4$, H_2O (twice), saturated $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated. The crude product was purified by silica gel column chromatography (petroleum ether : ethyl acetate; 20:1) to afford the title compound **S3** as a colorless oil (5.4 g, 16 mmol, 84%). The NMR reported are for a mixture of cis/trans rotamers originating from the N-methylated carbamate: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39–7.28 (m, 5H), 5.20 – 5.09 (m, 2H), 4.60 (d, *J* = 10.5 Hz, 0.5H), 4.29 (d, *J* = 10.7 Hz, 0.5H), 2.85 – 2.74 (m, 3H), 2.07 – 1.89 (m, 1H), 1.56 (s, 1H), 1.43 (d, *J* = 14.5 Hz, 10H), 1.16 – 1.01 (m, 1H), 0.93 – 0.84 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 128.6, 128.4, 128.2, 66.5, 66.3, 63.5, 33.6, 28.5, 25.1, 16.0, 10.9, 10.5. HRMS (ESI): *m/z* = $[M+Na]^+$ calc for $C_{19}H_{29}NO_4Na$ 358.1989, found 358.1981; *m/z* = $[2M+Na]^+$ calc for $C_{19}H_{29}NO_4Na$ 693.4086, found 693.4077.

_N_me-Ile-OBn hydrochloride (15)



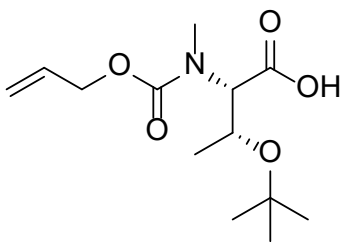
Boc-_N_me-Ile-OBn (**S3**, 4.0 g, 11.9 mmol) was dissolved in 4 M HCl in dioxane (20 mL) and the mixture was stirred for 2 h. Upon reaction completion, the mixture was concentrated *in vacuo* to afford title compound **15** as a pale yellow solid (3.2 g, 11.8 mmol, 99%). ^{1}H NMR (400 MHz, D_2O) δ 7.55–7.42 (m, 5H), 5.36 (q, 2H), 4.08 (d, *J* = 3.6 Hz, 1H), 2.77 (d, *J* = 1.5 Hz, 3H), 2.24 (d, *J* = 1.4 Hz, 3H), 2.13–2.04 (m, 1H), 1.53–1.26 (m, 2H), 0.99–0.87 (m, 6H). ^{13}C NMR (101 MHz, D_2O) δ 168.5, 134.5, 129.1, 129.0, 128.9, 68.6, 65.3, 35.9, 32.4, 30.3, 25.6, 13.6, 10.9. HRMS (ESI): *m/z* = $[M+H]^+$ calc for $C_{14}H_{22}NO_2$ 236.1645, found 236.1640.

Alloc-Thr(*t*Bu)-OH (S4)



O-tert-butyl-L-threonine (**16**, 5.0 g, 28.53 mmol) was dissolved in anhydrous THF (7 mL) and saturated $NaHCO_3$ solution (29 mL) was added. The mixture was cooled down to 0 °C and allyl chloroformate (4.6 mL, 42.8 mmol) was added. The reaction mixture was stirred for 16 h at room temperature. The mixture was diluted with ethyl acetate and the reaction was quenched with some droplets of 1 M HCl. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The crude product was purified by silica column chromatography (Hexane + 2% AcOH → 10% ethyl acetate in hexane + 2% AcOH). Last traces of AcOH were removed by co-evaporation with cyclohexane (3 times) to obtain title compound **S4** as a colorless oil (6.8 g, 26.4 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.99–5.85 (m, 1H), 5.68 (d, *J* = 5.4 Hz, 1H), 5.37–5.21 (m, 2H), 4.62–4.55 (m, 2H), 4.39–4.26 (m, 2H), 1.31 (s, 9H), 1.16 (d, *J* = 6.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.5, 156.2, 132.5, 118.1, 66.8, 66.1, 58.6, 28.6, 28.2, 27.1, 20.7, 18.1. HRMS (ESI): *m/z* = $[M+Na]^+$ calc for $C_{12}H_{21}NO_5Na$ 282.1312, found 282.1308; *m/z* = $[2M+Na]^+$ calc for $C_{12}H_{21}NO_5Na$ 541.2732, found 541.2729.

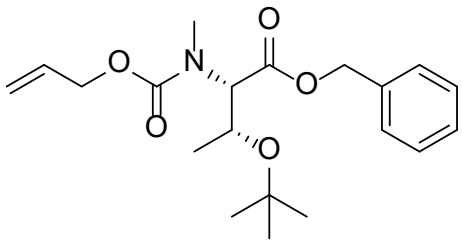
Alloc-_N_me-Thr(*t*Bu)-OH (17)



Alloc-Thr(*t*Bu)-OH (**S4**, 1.95 g, 7.55 mmol) was dissolved in THF (50 mL) and cooled to 0 °C. The solution was slowly treated with sodium hydride (60% in mineral oil, 906 mg, 22.65 mmol). After 30 min, methyl iodide (3.8 mL, 61 mmol) was added and the reaction was stirred for 20 h at room temperature. Diethyl ether was added and the organic layer was washed with H_2O twice. The combined aqueous layers were acidified with 1 M $KHSO_4$ and extracted with ethyl acetate twice. The combined organic layers were washed with $Na_2S_2O_3$ solution

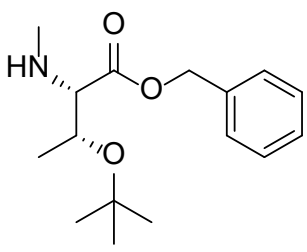
and brine and dried over $MgSO_4$. The product was concentrated under reduced pressure to afford title compound **17** as a pale yellow oil (1.72 g, 6.30 mmol, 83%). The NMR reported are for a mixture of cis/trans rotamers originating from the N-methylated carbamate: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.99–5.84 (m, 1H), 5.33–5.14 (m, 2H), 4.81 (d, *J* = 4.5 Hz, 1H), 4.62–4.57 (m, 2H), 4.40–4.26 (m, 1H), 3.07 (d, *J* = 2.9 Hz, 3H), 1.22–1.14 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.0, 157.7, 132.8, 132.7, 117.8, 117.4, 74.7, 68.2, 68.1, 66.6, 64.3, 64.1, 34.1, 34.0, 28.7, 20.9, 20.6, 20.4. HRMS (ESI): *m/z* = $[M+Na]^+$ calc for $C_{13}H_{23}NO_5Na$ 296.1468, found 296.1470; *m/z* = $[2M+Na]^+$ calc for $C_{13}H_{23}NO_5Na$ 569.3044, found 569.3040.

Alloc-*N*me-Thr(*t*Bu)-OBn (S5)



Alloc-*N*me-Thr(*t*Bu)-OH (**17**, 424 mg, 1.56 mmol) was dissolved in methanol (4.2 mL) and Cs_2CO_3 (254 mg, 0.78 mmol) was added. The reaction was stirred for 60 min and then concentrated *in vacuo*. The residue was redissolved in acetonitrile (7.8 mL) and benzyl bromide (276 µL, 2.34 mmol) was added. The reaction mixture was stirred for 16 h before addition of H_2O and ethyl acetate. The water layer was extracted with ethyl acetate twice. The combined organic layers were washed with $KHSO_4$, H_2O (twice), saturated $NaHCO_3$, brine, dried over $MgSO_4$, and concentrated. Flash column chromatography was used to purify the crude product. First hexane was used to flush excess of benzyl bromide from the column, then 10% ethyl acetate in hexane was used to obtain title compound **S5** (451 mg, 1.24 mmol, 80%). The NMR reported are for a mixture of cis/trans rotamers originating from the N-methylated carbamate: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36–7.34 (m, 5H), 6.01–5.78 (m, 1H), 5.34–5.23 (m, 2H), 5.23–5.12 (m, 1H), 5.03 (d, *J* = 12.2 Hz, 0.3H), 5.00 (d, *J* = 12.3 Hz, 0.7H), 4.85 (d, *J* = 4.2 Hz, 1H), 4.63–4.52 (m, 2H), 4.44–4.26 (m, 1H), 3.12 (d, *J* = 13.0 Hz, 3H), 1.18 (dd, *J* = 6.2, 3.0 Hz, 3H), 1.10 (d, *J* = 1.7 Hz, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3, 157.6, 135.6, 133.2, 128.7, 128.7, 128.6, 128.5, 128.5, 117.6, 117.2, 74.1, 68.6, 68.0, 66.9, 66.4, 66.0, 64.7, 64.3, 34.3, 33.8, 28.8, 28.5, 20.9, 20.6. HRMS (ESI): *m/z* = $[M+Na]^+$ calc for $C_{20}H_{29}NO_5Na$ 386.1938, found 386.1936; *m/z* = $[2M+Na]^+$ calc for $C_{20}H_{29}NO_5Na$ 749.3984, found 749.3978.

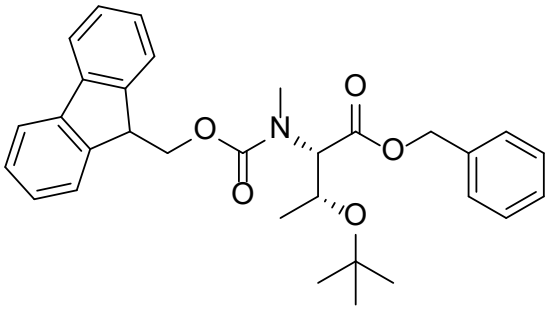
***N*me-Thr(*t*Bu)-OBn synthesized with S5 (18)**



To Alloc-*N*me-Thr(*t*Bu)-OBn (**S5**, 237 mg, 0.65 mmol), dissolved in anhydrous DCM (3.3 mL), was added thiosalicylic acid (201 mg, 1.3 mmol). To this mixture, tetrakis(triphenylphosphine)palladium(0) (75 mg, 0.065 mmol) was added. The reaction mixture was stirred at room temperature for 1 h before addition of saturated aqueous $NaHCO_3$ (20 mL). The mixture

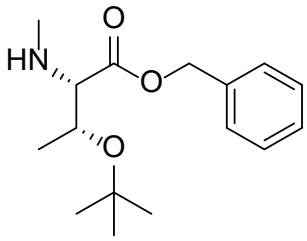
was further diluted with DCM (10 mL) and both layers were separated. The water layer was extracted with DCM (2 x 10 mL). the combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The crude product was purified with silica column chromatography (0 → 20% ethyl acetate in hexane) to afford title compound **18** (150 mg, 0.54 mmol, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40–7.31 (m, 5H), 5.16 (q, 2H), 3.94 (qd, *J* = 6.2, 4.2 Hz, 1H), 3.09 (d, *J* = 4.2 Hz, 1H), 2.38 (s, 3H), 1.20 (d, *J* = 6.2 Hz, 3H), 1.12 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.6, 135.9, 128.7, 128.5, 128.4, 73.9, 69.4, 68.5, 66.6, 35.5, 28.5, 20.4. HRMS (ESI): *m/z* = $[M+H]^+$ calc for $C_{16}H_{26}NO_3$ 280.1907, found 280.1909; *m/z* = $[M+Na]^+$ calc for $C_{16}H_{25}NO_3Na$ 302.1726, found 302.1726; *m/z* = $[2M+Na]^+$ calc for $C_{16}H_{25}NO_3Na$ 581.3560 found 581.3569.

Fmoc-*N*me-Thr(*t*Bu)-OBn (S6)



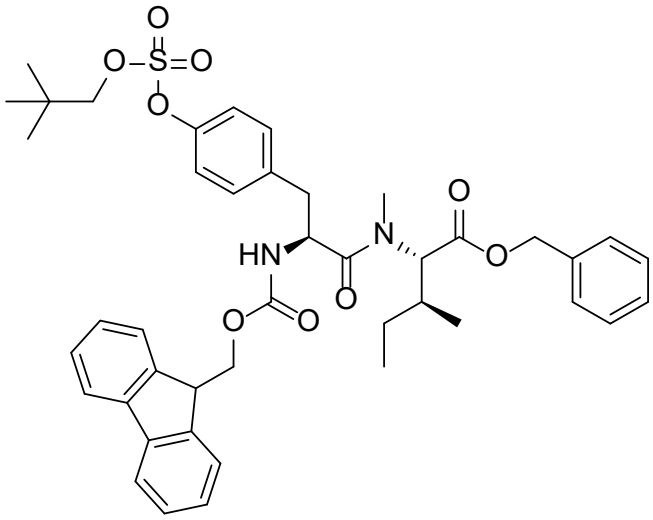
Fmoc-*N*-methyl-*O*-*tert*-butyl-L-threonine (**19**, 370 mg, 0.90 mmol) was dissolved in methanol (2.4 mL) and Cs_2CO_3 (146.5 mg, 0.45 mmol) was added. The mixture was stirred for 60 min before concentration. Afterwards, the concentrated crude was dissolved in acetonitrile (4.5 mL) and benzyl bromide (160 µL, 1.35 mmol) was added. The reaction mixture was stirred for 16 h and subsequently diluted with ethyl acetate and H_2O. The water layer was extracted with ethyl acetate twice. The combined organic layers were washed with $KHSO_4$, H_2O (twice), saturated $NaHCO_3$, brine, dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by flash column chromatography. Excess of benzyl bromide was flushed from the column by extensively eluting with hexane. The product was obtained with 10% ethyl acetate in hexane to afford title compound **S6** (441 mg, 0.88 mmol, 98%). The NMR reported are for a mixture of cis/trans rotamers originating from the N-methylated carbamate: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81–7.70 (m, 2H), 7.66–7.48 (m, 2H), 7.44–7.19 (m, 9H), 5.30 (d, *J* = 12.2 Hz, 0.7H), 5.21 (d, *J* = 12.3 Hz, 0.3H), 5.01 (dd, *J* = 12.3, 3.2 Hz, 1H), 4.88 (d, *J* = 4.2 Hz, 0.7H), 4.55 (d, *J* = 5.1 Hz, 0.3H), 4.47–4.34 (m, 2H), 4.31–4.14 (m, 1H), 3.20 (s, 2H), 3.09 (s, 1H), 1.19 (d, *J* = 6.2 Hz, 3H), 1.10 (d, *J* = 11.0 Hz, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3, 157.7, 144.3, 144.1, 141.5, 135.6, 128.74, 128.70, 128.6, 128.53, 128.47, 127.8, 127.2, 125.28, 125.26, 125.1, 125.0, 120.10, 120.07, 74.2, 74.1, 68.6, 67.9, 67.8, 67.7, 66.9, 64.3, 47.4, 33.9, 33.7, 28.9, 20.7, 20.6. HRMS (ESI): *m/z* = $[M+Na]^+$ calc for $C_{31}H_{35}NO_5Na$ 524.2407, found 524.2406.

_N_me-Thr(_t_Bu)-OBn synthesized with S6 (18')



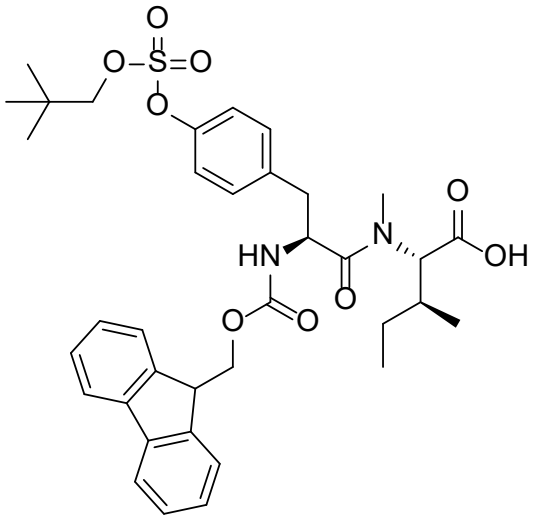
Fmoc-_N_me-Thr(_t_Bu)-OBn (**S6**, 485 mg, 0.97 mmol) was dissolved in anhydrous DCM (5 mL) and piperidine (250 µL, 2.5 mmol) was added. The reaction mixture was stirred for 24 h. Upon reaction completion, the mixture was concentrated *in vacuo* and the crude product was purified by column chromatography (5→20% ethyl acetate in hexane) to obtain title compound **18'** (225 mg, 0.81 mmol, 83%). Analytical data is in accordance with compound **18**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42–7.28 (m, 5H), 5.16 (q, 2H), 3.94 (qd, *J* = 6.2, 4.2 Hz, 1H), 3.09 (d, *J* = 4.2 Hz, 1H), 2.38 (s, 3H), 1.19 (d, *J* = 6.2 Hz, 3H), 1.12 (s, 9H). HRMS (ESI): *m/z* = $[M+H]^+$ calc for $C_{16}H_{30}NO_5$ 280.1907, found 280.1903.

Fmoc-Tyr(SO_2ONp)-_N_me-Ile-OBn (S7)



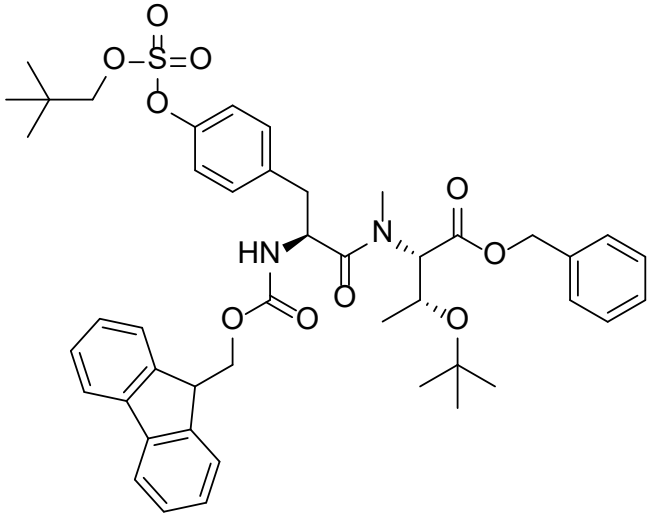
Fmoc-Tyr(SO_2ONp)-OH (**20**, 407 mg, 0.74 mmol) was dissolved in anhydrous THF (8 mL) and HATU (420 mg, 1.11 mmol) and DIPEA (384 µL, 2.22 mmol) were added. The mixture was stirred, and _N_me-Ile-OBn hydrochloride (**15**, 300 mg, 1.11 mmol) was added. After 16 h of stirring, the reaction mixture was quenched with a saturated aqueous solution of NH_4Cl. The water layer was extracted with DCM (3 x 10 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtrated, and concentrated. The residue was purified by flash chromatography on silica gel (0 → 20% ethyl acetate in hexane) to afford title compound **S7** as a white solid (586 mg, 0.74 mmol, quantitative). The NMR reported are for a mixture of cis/trans rotamers originating from the N-methylated carbamate: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.6 Hz, 2H), 7.60–7.50 (m, 2H), 7.46–7.29 (m, 10H), 7.12 (s, 3H), 5.59 (d, *J* = 8.7 Hz, 1H), 5.22–5.10 (m, 2H), 5.02 (d, *J* = 10.4 Hz, 1H), 4.92–4.83 (m, 1H), 4.41–4.28 (m, 2H), 4.18 (t, *J* = 7.1 Hz, 1H), 4.11 (t, *J* = 7.1 Hz, 1H), 4.05 (d, *J* = 3.4 Hz, 2H), 2.99 (dd, *J* = 13.6, 7.0 Hz, 1H), 2.84 (s, 3H), 2.04 (s, 2H), 1.26 (t, *J* = 7.1 Hz, 3H), 0.98 (d, *J* = 1.3 Hz, 9H), 0.92 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.0, 170.5, 155.7, 149.4, 143.9, 141.5, 135.6, 135.2, 131.1, 128.8, 128.7, 128.6, 128.3, 127.9, 127.2, 125.2, 121.1, 120.2, 83.6, 67.2, 66.8, 60.8, 60.5, 52.0, 47.3, 38.3, 33.5, 32.1, 31.3, 29.8, 26.1, 25.1, 21.2, 15.8, 14.4, 10.9. HRMS (ESI): *m/z* = $[M+Na]^+$ calc for $C_{43}H_{50}N_2O_9SNa$ 793.3129, found 793.3122.

Fmoc-Tyr(SO_2ONp)-*N*me-Ile-OH (21)



Fmoc-Tyr(SO_2ONp)-*N*me-Ile-OBn (**S7**, 550 mg, 0.71 mmol) was dissolved in ethanol (25 mL) and chloroform (2 mL). The solution was bubbled with H_2 gas. Subsequently, Pd/C (10 wt. % loading, 100 mg) was added and H_2 was bubbled in the solution for another 10 min. Afterwards, the H_2 balloon was placed above the solution inside the flask and the reaction mixture was stirred for 16 h. Upon reaction completion, the mixture was filtered over Celite and concentrated *in vacuo* to afford title compound **21** as a white solid (458 mg, 0.67 mmol, 94%). The NMR reported are for a mixture of cis/trans rotamers originating from the N-methylated carbamate: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.57 (dd, *J* = 7.7, 3.4 Hz, 2H), 7.43–7.37 (m, 2H), 7.31 (tt, *J* = 7.4, 1.5 Hz, 2H), 7.26–7.18 (m, 4H), 5.96 (d, *J* = 8.9 Hz, 1H), 4.99–4.88 (m, 2H), 4.36–4.31 (m, 2H), 4.19 (t, *J* = 7.1 Hz, 1H), 4.06 (s, 2H), 3.11–2.97 (m, 2H), 2.83 (s, 3H), 2.02–1.89 (m, 1H), 1.26 (s, 1H), 1.03–0.94 (m, 13H), 0.81 (t, *J* = 7.4 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.8, 172.1, 155.9, 149.3, 143.9, 143.8, 141.4, 135.3, 131.2, 127.9, 127.2, 125.2, 121.4, 120.2, 83.8, 67.3, 61.3, 52.1, 47.2, 38.7, 33.0, 32.1, 31.9, 26.1, 24.9, 15.8, 10.8. HRMS (ESI): *m/z* = $[M+Na]^+$ calc for $C_{36}H_{44}N_2O_9SNa$ 703.2660, found 703.2654.

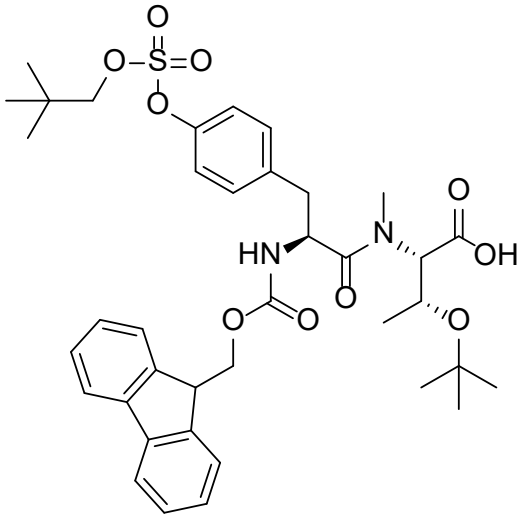
Fmoc-Tyr(SO_2ONp)-*N*me-Thr(*t*Bu)-OBn (S8)



Fmoc-Tyr(SO_2ONp)-OH (**20**, 659 mg, 1.19 mmol) was dissolved in anhydrous THF (8 mL) and HATU (453 mg, 1.19 mmol), and DIPEA (415 µL, 2.38 mmol) were added. The mixture was stirred, and *N*me-Thr(*t*Bu)-OBn (**18**, 222 mg, 0.79 mmol) was added. After 16 h of stirring, the reaction mixture was diluted with DCM. The organic layer was washed with 1 M $KHSO_4$, H_2O, saturated $NaHCO_3$, brine, dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by flash column chromatography on silica gel (0 → 20% ethyl acetate in hexane) to obtain title compound **S8** as a white solid (651 mg, 0.79 mmol, quantitative).

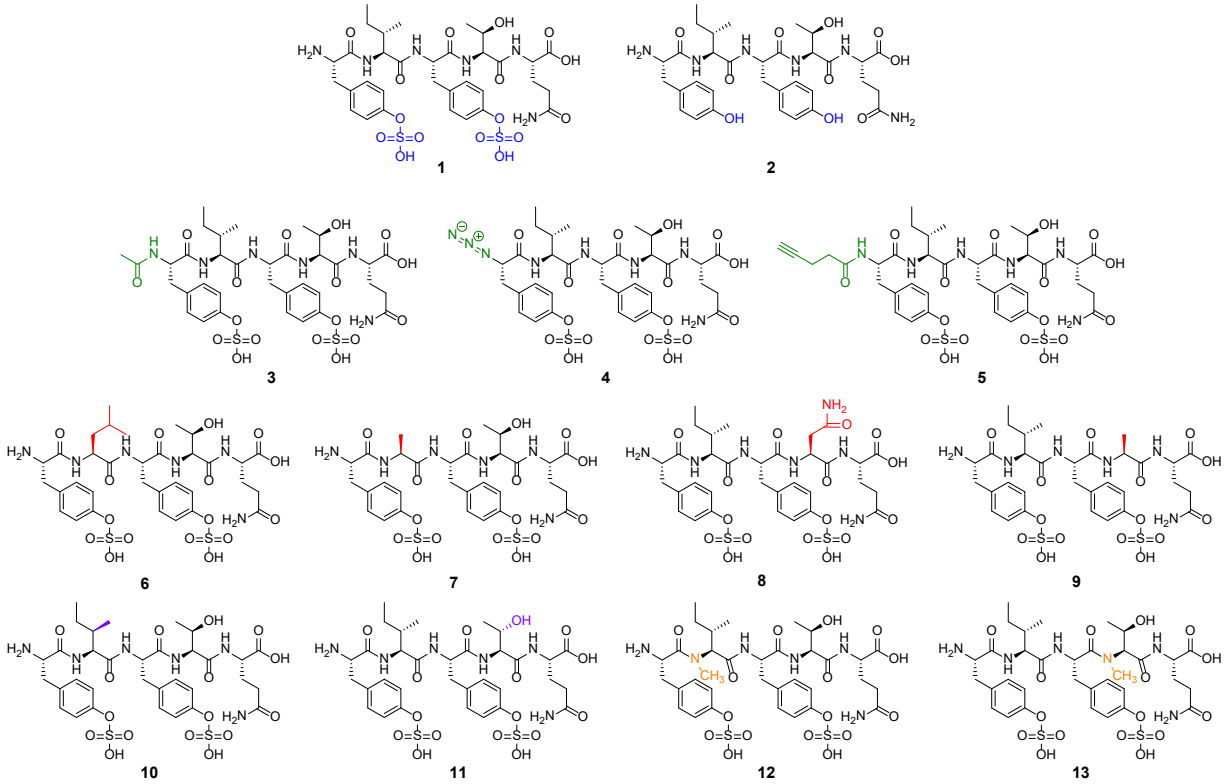
The NMR reported are for a mixture of cis/trans rotamers originating from the N-methylated carbamate: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.56 (dd, *J* = 7.8, 3.0 Hz, 2H), 7.42–7.13 (m, 13H), 5.55 (dd, *J* = 24.2, 8.7 Hz, 1H), 5.34–5.25 (m, 1H), 5.05–4.95 (m, 2H), 4.47–4.26 (m, 3H), 4.20–4.15 (m, 1H), 4.04 (s, 2H), 3.22 (d, *J* = 16.1 Hz, 2H), 3.08 (dd, *J* = 13.8, 6.2 Hz, 1H), 2.94–2.86 (m, 1H), 1.59 (s, 3H), 1.13–1.05 (m, 11H), 0.98 (d, *J* = 2.7 Hz, 10H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.7, 171.3, 169.6, 155.7, 149.4, 144.0, 143.91, 143.89, 141.5, 135.41, 135.37, 131.2, 129.0, 128.82, 128.77, 128.7, 128.62, 128.57, 127.9, 127.2, 125.2, 121.2, 121.0, 120.1, 83.6, 74.3, 68.6, 68.1, 67.14, 67.07, 61.8, 60.5, 51.8, 47.3, 37.9, 35.2, 34.5, 32.0, 29.0, 28.9, 28.8, 28.7, 26.1, 21.2, 20.9, 20.5, 14.3. HRMS (ESI): *m/z* = $[M+Na]^+$ calc for $C_{45}H_{54}N_2O_{10}SNa$ 837.3391, found 839.3391.

Fmoc-Tyr(SO_2ONp)-*N*me-Thr(*t*Bu)-OH (22)



Fmoc-Tyr(SO_2ONp)-*N*me-Thr(*t*Bu)-OBn (**S8**, 647 mg, 0.79 mmol) was dissolved in ethanol (25 mL) and chloroform (2 mL). The solution was bubbled with H_2 gas. Subsequently, Pd/C (10 wt. % loading, 100 mg) was added and H_2 was bubbled in the solution for another 10 min. Afterwards, the H_2 balloon was placed above the solution inside the flask and the reaction mixture was stirred for 16 h. Upon reaction completion, the mixture was filtered over Celite and concentrated *in vacuo*. Excess benzyl was removed by flash column chromatography on silica gel, flushing with 1:1 ethyl acetate:hexane, and subsequently product purification by eluting with 1:1 ethyl acetate:hexane supplemented with 1% AcOH. Concentration of purified fractions afforded title compound **22** as a white solid (354 mg, 0.49 mmol, 62%). The NMR reported are for a mixture of cis/trans rotamers originating from the N-methylated carbamate: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (dd, *J* = 7.7, 3.0 Hz, 2H), 7.57 (d, *J* = 7.3 Hz, 2H), 7.43–7.36 (m, 2H), 7.34–7.27 (m, 2H), 7.27–7.15 (m, 4H), 5.90 (dd, *J* = 19.2, 8.8 Hz, 1H), 5.32 (d, *J* = 4.0 Hz, 1H), 5.07–4.95 (m, 1H), 4.41–4.22 (m, 3H), 4.22–4.15 (m, 1H), 4.09–4.03 (m, 2H), 3.18–3.06 (m, 3H), 3.02–2.93 (m, 1H), 2.11 (s, 3H), 1.17 (d, *J* = 4.3 Hz, 11H), 1.02–0.93 (m, 10H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.7, 173.4, 155.9, 155.8, 149.4, 143.94, 143.88, 141.5, 141.4, 135.5, 135.3, 131.20, 131.17, 127.88, 127.86, 127.2, 125.3, 125.2, 121.4, 121.3, 121.2, 120.1, 83.6, 75.3, 68.1, 67.6, 67.3, 67.2, 61.5, 51.9, 51.8, 47.2, 39.0, 38.0, 34.7, 32.0, 29.0, 28.7, 28.6, 26.1, 20.6, 20.3. HRMS (ESI): *m/z* = $[M+Na]^+$ calc for $C_{38}H_{48}N_2O_{10}SNa$ 747.2922, found 747.2926; *m/z* = $[2M+Na]^+$ calc for $C_{38}H_{48}N_2O_{10}SNa$ 1471.5952 found 1471.5952.

Synthesis and HPLC/MS data of PSK-like peptides (1-13)



Scheme S2. Overview of PSK and PSK analogues (**1-13**).

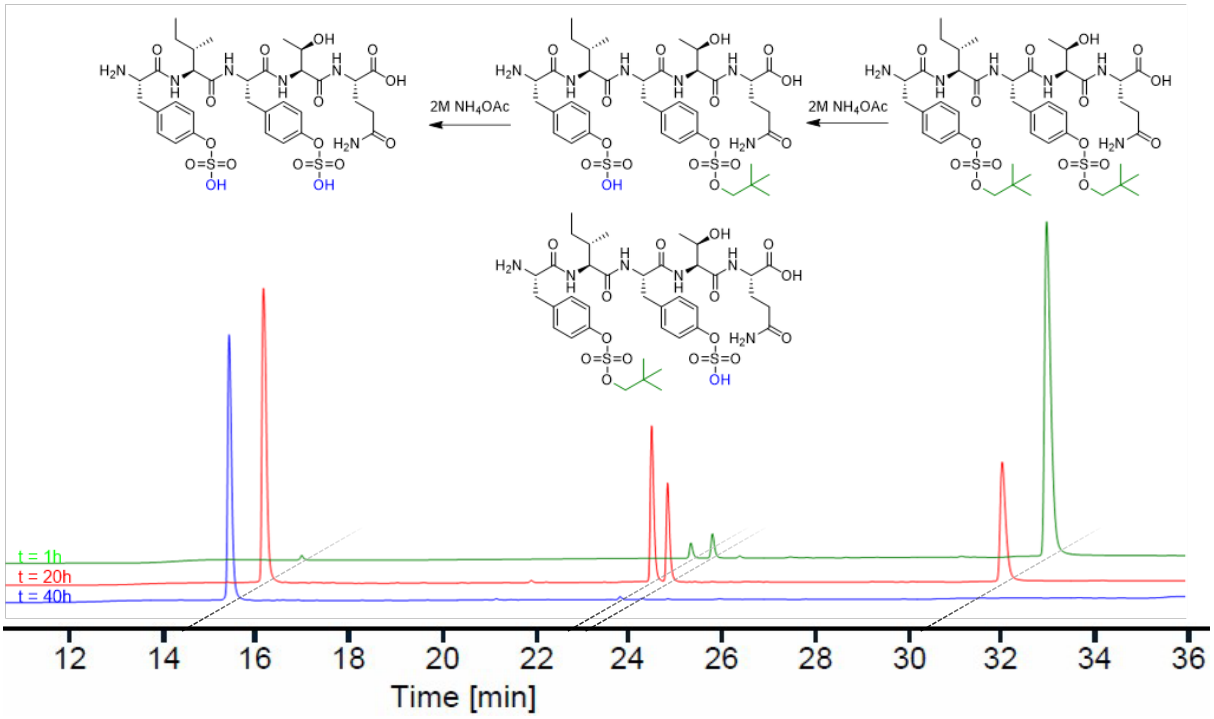


Figure S1. RP-HPLC chromatograms (absorption at 214 nm; eluent: 95% H_2O, 5% MeCN, 10mM NH_4OAc to 40% H_2O, 60% MeCN, 10mM NH_4OAc) of Np-removal from the purified Np-protected sulfotyrosine PSK peptide (right) with 2 M NH_4OAc, initially leading to removal of Np group (middle), and ultimately resulting in full deprotected peptide (left). HPLC analysis was performed after 1 h treatment (green), 20 h treatment (red), and 40 h treatment (blue). Structure of the peptide is displayed in neutral form.

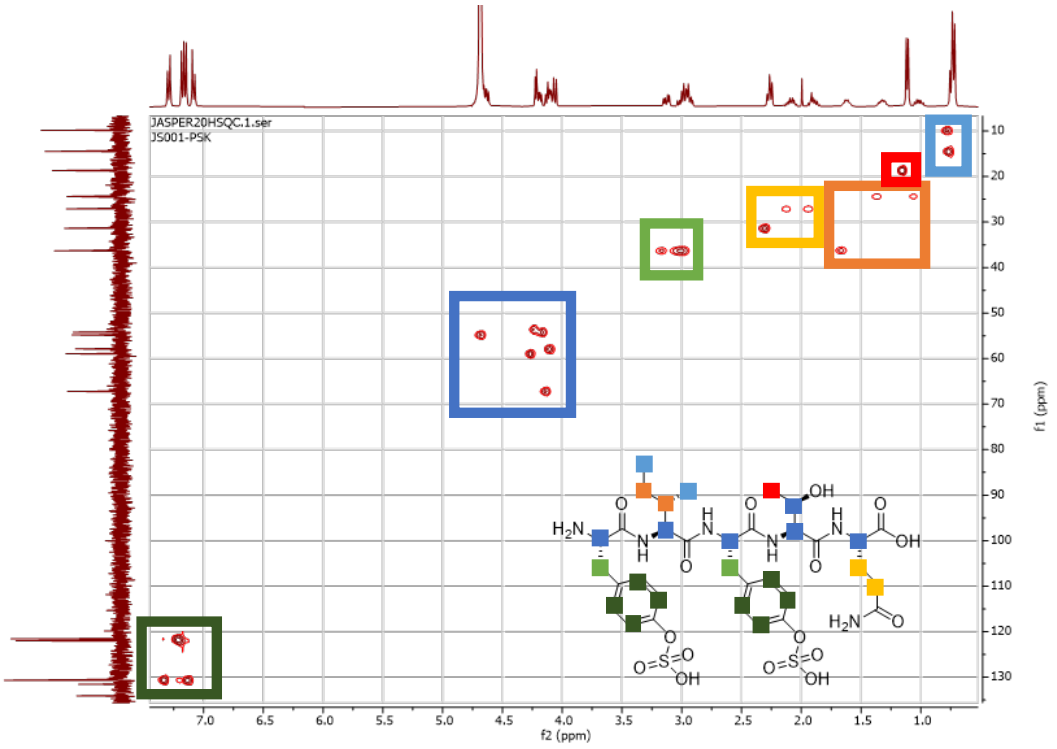


Figure S2. Phytosulfokine (PSK, **1**) peak assignment with heteronuclear single quantum coherence spectroscopy (HSQC). The 2D spectrum uses the ^{1}H-spectrum on the X-axis and the ^{13}C-spectrum on the Y-axis.

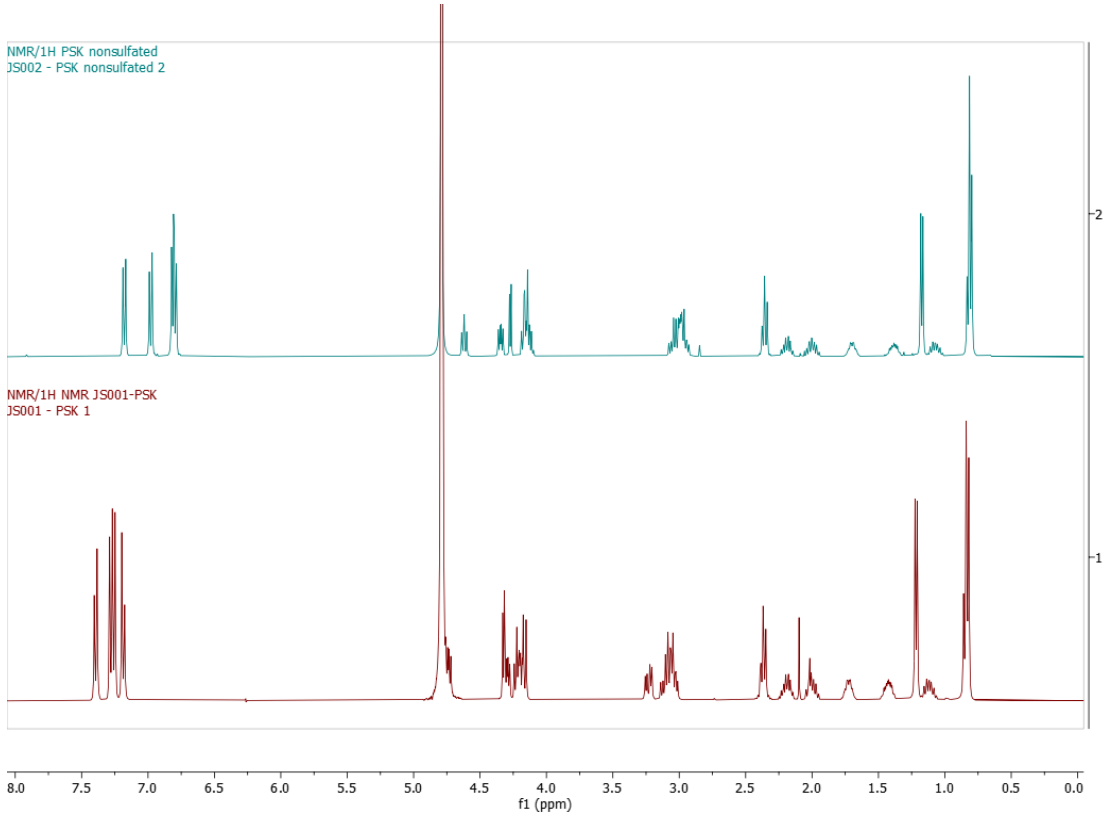


Figure S3. ^{1}H NMR of PSK **1** (bottom), and nonsulfated PSK **2** (top). In the bottom spectrum, an additional peak was observed at 2.1 ppm. This peak indicates the presence of acetate, the remainder of the NH_4OAc ammonium salt. The proton peak integrates for 0.32 protons for a CH_3 group, which indicates the presence of 10% ammonium salt adduct on the dry peptide.

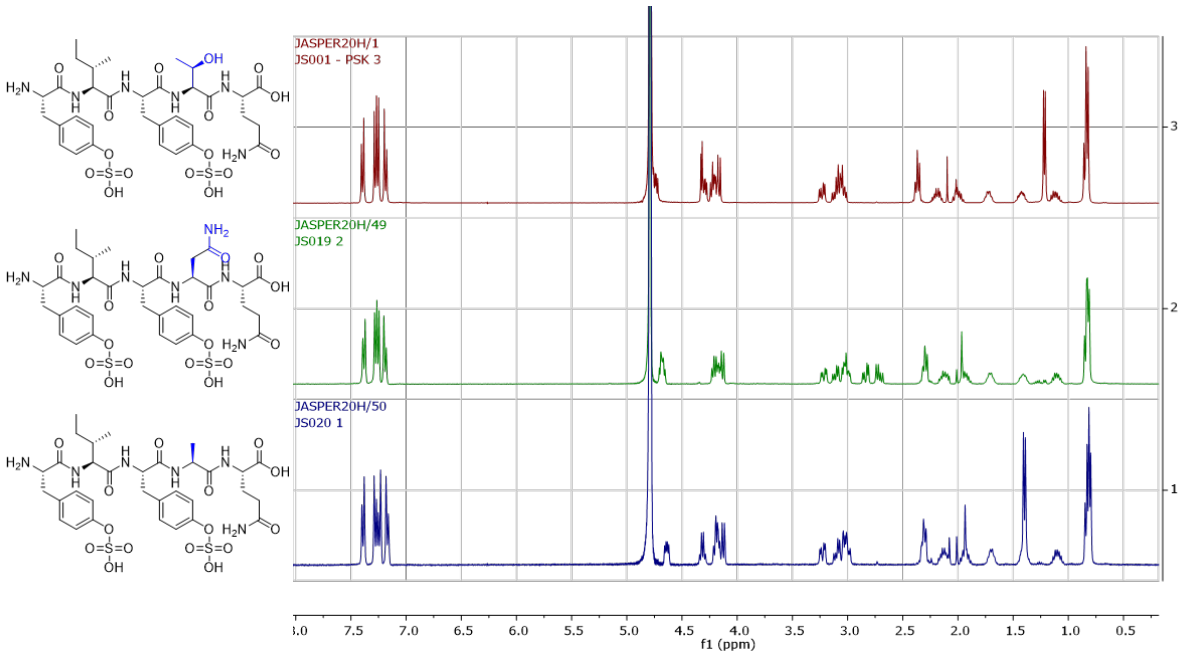
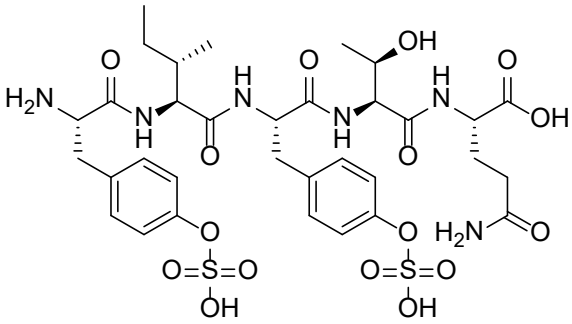


Figure S4. ^{1}H NMR comparison of PSK **1** (top), 4-Asn PSK **8** (middle), and 4-Ala PSK **9** (bottom). Distinct peaks for different used amino acids Thr/Asn/Ala are highlighted.

H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH (PSK, 1)



Synthesis of PSK was performed as described under 'General procedure for Phytosulfokine synthesis'. After the first purification step, neopentyl-protected disulfated peptide was obtained. MS analysis was done in Milli-Q : acetonitrile 1:1 (**Figure S5**). Np-protected PSK was treated with 2 M NH_4OAc for 40 h and afterwards purified by preparative-HPLC and lyophilized to yield pure phytosulfokine (**PSK, 1**, **Figure S6**). ^{1}H NMR (400 MHz, D_2O) δ 7.42 – 7.36 (m, 2H), 7.31 – 7.22 (m, 4H), 7.21 – 7.15 (m, 2H), 4.75 – 4.70 (m, 1H), 4.34 – 4.26 (m, 2H), 4.25 – 4.14 (m, 3H), 3.23 (dd, *J* = 14.0, 5.7 Hz, 1H), 3.15 – 2.99 (m, 3H), 2.43 – 2.31 (m, 2H), 2.24 – 2.14 (m, 1H), 2.04 – 1.96 (m, 1H), 1.78 – 1.66 (m, 1H), 1.47 – 1.38 (m, 1H), 1.22 (d, *J* = 6.4 Hz, 3H), 1.17 – 1.05 (m, 1H), 0.84 (dd, *J* = 8.6, 6.9 Hz, 6H). ^{13}C NMR (101 MHz, D_2O) δ 178.2, 172.9, 172.2, 170.9, 168.4, 150.7, 150.2, 134.1, 131.6, 130.7, 130.6, 122.0, 121.6, 67.2, 59.0, 57.9, 54.8, 54.2, 36.3, 36.2, 31.4, 27.1, 24.4, 18.7, 14.5, 9.9.

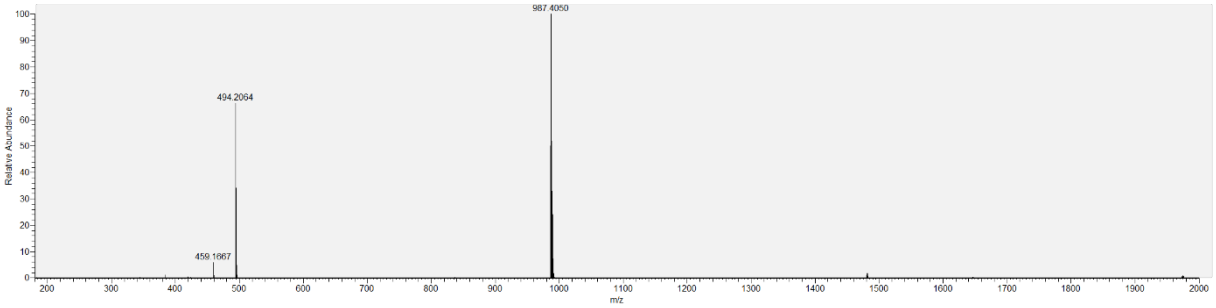


Figure S5. MS spectrum of neopentyl-protected PSK. The peaks are assigned as follows: HRMS (ESI): *m/z* = $[M+2H-1Np]^{2+}$ calc for $C_{38}H_{58}N_6O_{16}S_2$ 459.1670, found 459.1667 due to in-line fragmentation; *m/z* = $[M+2H]^{2+}$ calc for $C_{43}H_{68}N_6O_{16}S_2$ 494.2061, found 494.2065; *m/z* = $[M+H]^+$ calc for $C_{43}H_{67}N_6O_{16}S_2$ 987.4050, found 987.4051.

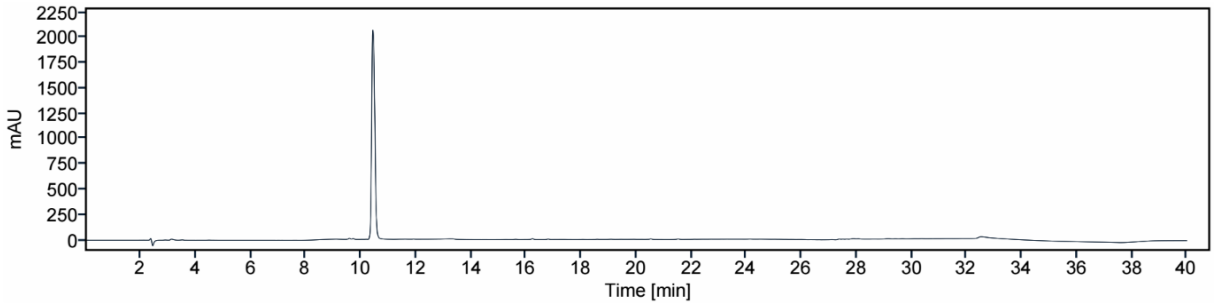


Figure S6.1. HPLC trace of peptide PSK **1**. The t_R of the product is 10.4 min.

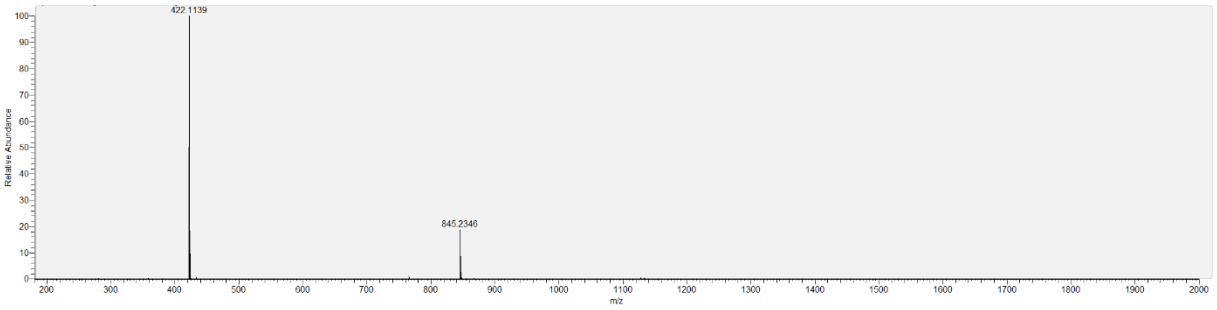
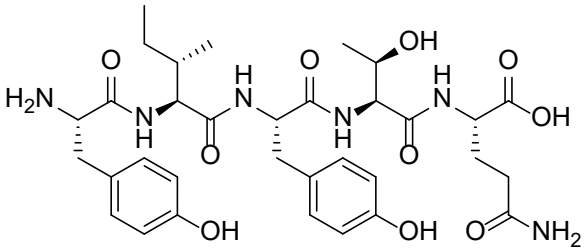


Figure S6.2. MS spectrum of peptide PSK **1** in negative ion mode. Spectrum belongs to the LC peak with t_R 10.4 min (shown in Figure **S6.1**). The peaks are assigned as follows: HRMS (ESI): *m/z* = $[M\text{-}H]^-$ calc for $C_{33}H_{45}N_6O_{16}S_2$ 845.2339, found 845.2346; *m/z* = $[M\text{-}2H]^{2-}$ calc for $C_{33}H_{44}N_6O_{16}S_2$ 422.1133, found 422.1139.

H-Tyr-Ile-Tyr-Thr-Gln-OH (nonsulfated PSK, 2)



Peptide **2** was obtained by following the 'General procedure for Phytosulfokine synthesis'. For the two tyrosine residues, commercially available Fmoc-Tyr(*t*Bu)-OH was used. Final purification resulted in pure title peptide **2** (**Figure S7**). ^{1}H NMR (400 MHz, D_2O) δ 7.22 – 7.14 (m, 2H), 7.02 – 6.94 (m, 2H), 6.86 – 6.75 (m, 4H), 4.62 (dd, *J* = 8.1, 7.0 Hz, 1H), 4.34 (dd, *J* = 8.8, 5.3 Hz, 1H), 4.27 (d, *J* = 5.0 Hz, 1H), 4.21 – 4.07 (m, 3H), 3.05 (dd, *J* = 14.0, 7.0 Hz, 1H), 3.02 – 2.91 (m, 3H), 2.36 (t, *J* = 7.9 Hz, 2H), 2.24 – 2.13 (m, 1H), 2.07 – 1.94 (m, 1H), 1.76 – 1.63 (m, 1H), 1.44 – 1.32 (m, 1H), 1.17 (d, *J* = 6.4 Hz, 3H), 1.07 (ddd, *J* = 13.3, 8.8, 7.0 Hz, 1H), 0.81 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, D_2O) δ 177.8, 174.4, 172.9, 171.9, 171.0, 168.6, 163.1, 162.8, 155.0, 154.5, 130.7, 130.6, 127.8, 125.3, 117.7, 115.8, 115.5, 114.9, 67.2, 58.7, 57.8, 55.1, 54.3, 52.1, 36.4, 36.1, 31.1, 26.3, 24.3, 18.7, 14.4, 9.9.

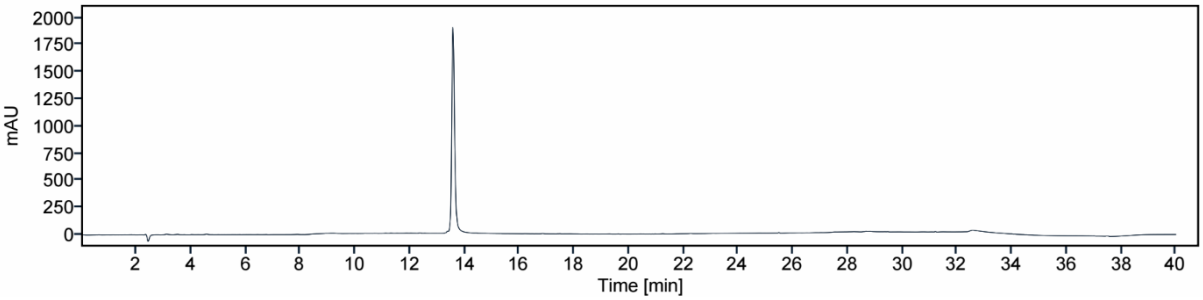


Figure S7.1. HPLC trace of peptide nonsulfated PSK **2**. The t_R of the product is 13.6 min.

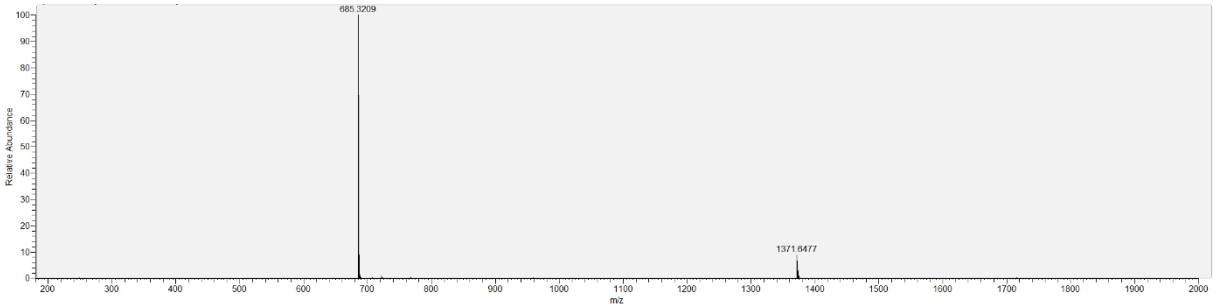
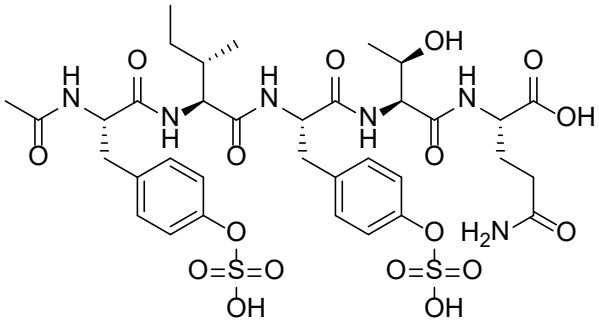


Figure S7.2. MS spectrum of peptide nonsulfated PSK **2** in negative ion mode. Spectrum belongs to the LC peak with t_R 13.6 min (shown in Figure **S7.1**). The peaks are assigned as follows: HRMS (ESI): *m/z* = $[M-H]^-$ calc for $C_{33}H_{45}N_6O_{10}$ 685.3202, found 685.3209; *m/z* = $[2M-H]^-$ calc for $C_{33}H_{45}N_6O_{10}$ 1371.6477, found 1371.6477.

Ac-Tyr(SO$_3$H)-Ile-Tyr(SO$_3$H)-Thr-Gln-OH (Ac-PSK, 3)



Synthesis was performed as described under the 'General procedure of Phytosulfokine synthesis'. Before acidic cleavage, the *N*-terminal amine was acetylated using acetic anhydride and DIPEA. After final purification and lyophilization, pure title peptide **3** was obtained (**Figure S8**).

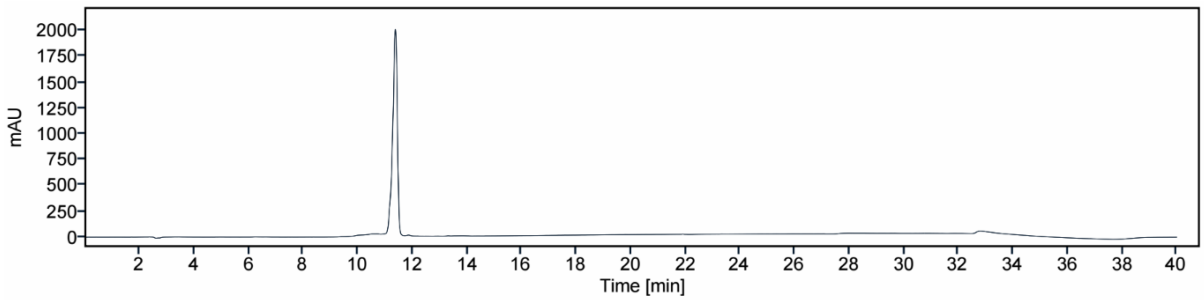


Figure S8.1. HPLC trace of peptide Ac-PSK **3**. The t_R of the product is 11.4 min.

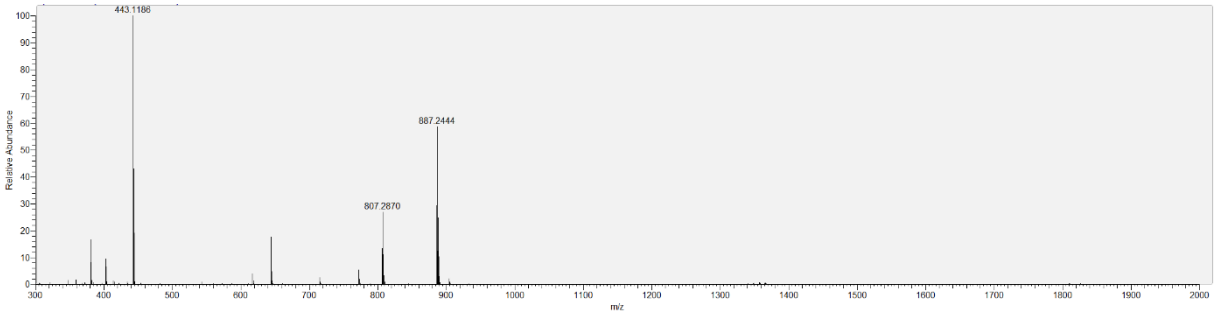
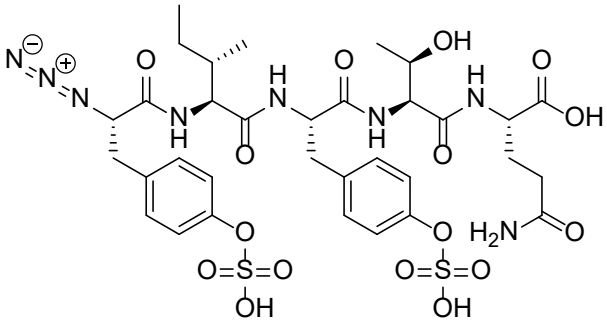


Figure S8.2. MS spectrum of peptide Ac-PSK **3** in negative ion mode. Spectrum belongs to the LC peak with t_R 11.4 min (shown in Figure **S8.1**). The peaks are assigned as follows: HRMS (ESI): *m/z* = $[M-H]^-$ calc for $C_{35}H_{47}N_6O_{17}S_2$ 887.2444, found 887.2444; *m/z* = $[M-SO_3H]^-$ calc for $C_{35}H_{47}N_6O_{14}S$ 807.2876, found 807.2870; *m/z* = $[M-2H]^{2-}$ calc for $C_{35}H_{46}N_6O_{17}S_2$ 443.1186, found 443.1186.

N_3-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH (N_3-PSK, 4)



Synthesis was performed as described under the 'General procedure of Phytosulfokine synthesis'. Purified PSK (**1**, 10 mg, 11.8 µmol) was used to undergo diazotransfer. The peptide was dissolved in Milli-Q (2 mL), and diazotransfer reagent (**S1**, 3.6 mg, 20.7 µmol) in 1 M NaOH (21 µL) was added, together with Na_2CO_3 (590 µL of 10 mg/mL stock solution), and $CuSO_4 \cdot H_2O$ (500 µL of 1 mg/mL stock solution). The reaction mixture was stirred for 40 h, after which the mixture was further diluted with Milli-Q and subsequently lyophilized to afford pure title peptide **4** (**Figure S9**).

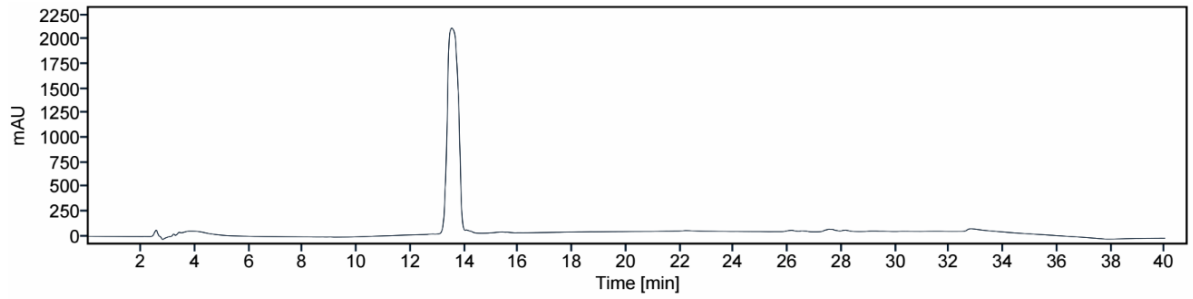


Figure S9.1. HPLC trace of peptide N_3-PSK **4**. The t_R of the product is 13.5 min.

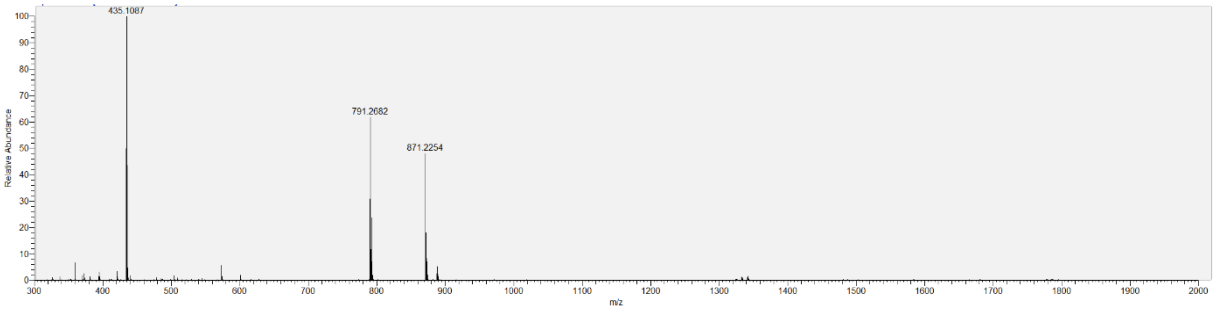
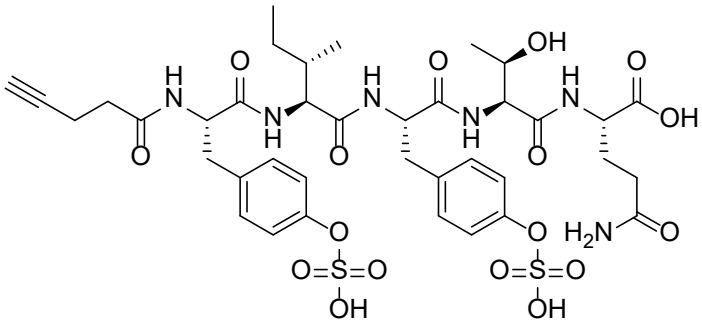


Figure S9.2. MS spectrum of peptide N_3-PSK **4** in negative ion mode. Spectrum belongs to the LC peak with t_R 13.5 min (shown in Figure **S9.1**). The peaks are assigned as follows: HRMS (ESI): *m/z* = $[M-H]^-$ calc for $C_{33}H_{43}N_8O_{16}S_2$ 871.2244, found 871.2254; *m/z* = $[M-SO_3H]^-$ calc for $C_{33}H_{43}N_8O_{11}S$ 791.2676, found 791.2682; *m/z* = $[M-2H]^{2-}$ calc for $C_{33}H_{42}N_8O_{16}S_2$ 435.1086, found 435.1087.

pentyne-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH (pentyne-PSK, 5)



Synthesis was performed as described under the 'General procedure of Phytosulfokine synthesis'. Before acidic cleavage, the *N*-terminal amine was treated with 4-pentynoic acid, activated with HBTU/HOBt and DIPEA, in a similar way as the other amino acid couplings were performed. After final purification and lyophilization, pure title peptide **5** was obtained (**Figure S10**).

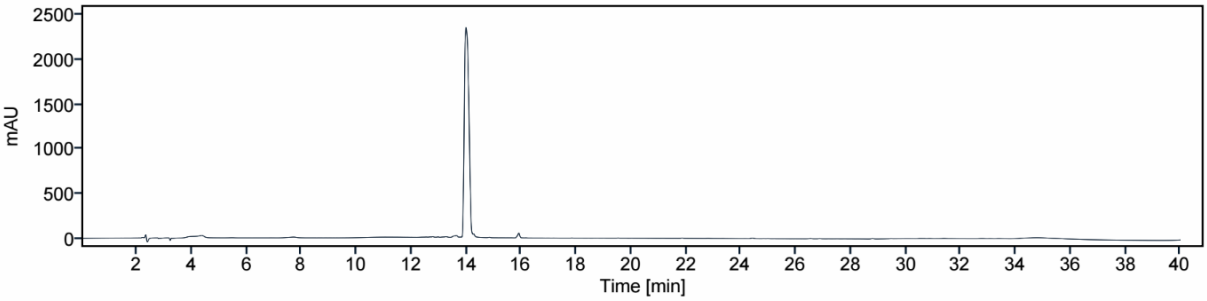


Figure S10.1. HPLC trace of peptide pentyne-PSK **5**. The t_R of the product is 14.0 min.

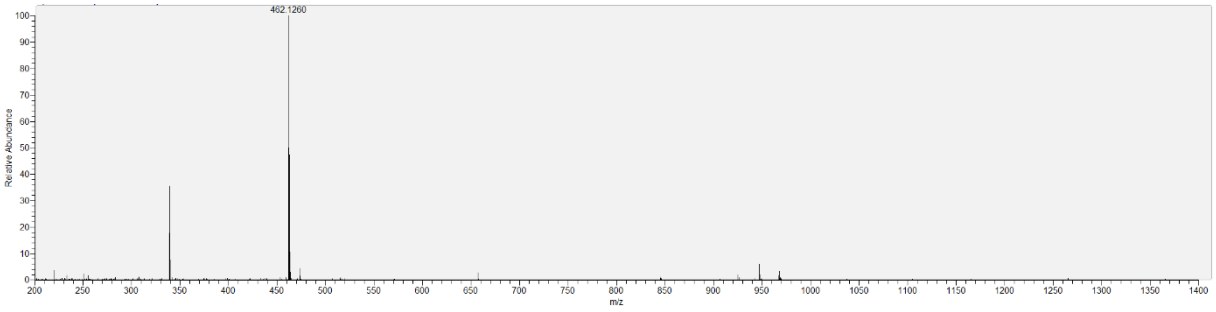
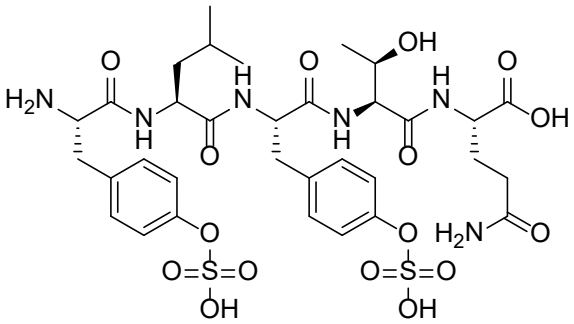


Figure S10.2. MS spectrum of peptide pentyne-PSK **5** in negative ion mode. Spectrum belongs to the LC peak with t_R 14.0 min (shown in Figure **S10.1**). The peak is assigned as follows: HRMS (ESI): *m/z* = $[M-2H]^{2-}$ calc for $C_{38}H_{48}N_6O_{17}S_2$ 462.1264, found 462.1260.

H-Tyr(SO_3H)-Leu-Tyr(SO_3H)-Thr-Gln-OH (2-Leu PSK, 6)



Synthesis was performed as described under the 'General procedure of Phytosulfokine synthesis'. Instead of Fmoc-Ile-OH for the coupling of the 4th amino acid, Fmoc-Leu-OH was used. After final purification and lyophilization, pure title peptide **6** was obtained (**Figure S11**).

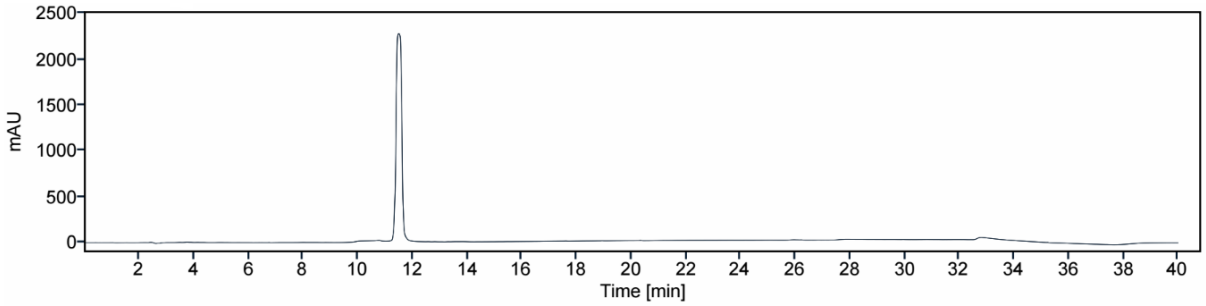


Figure S11.1. HPLC trace of peptide 2-Leu PSK **6**. The t_R of the product is 11.5 min.

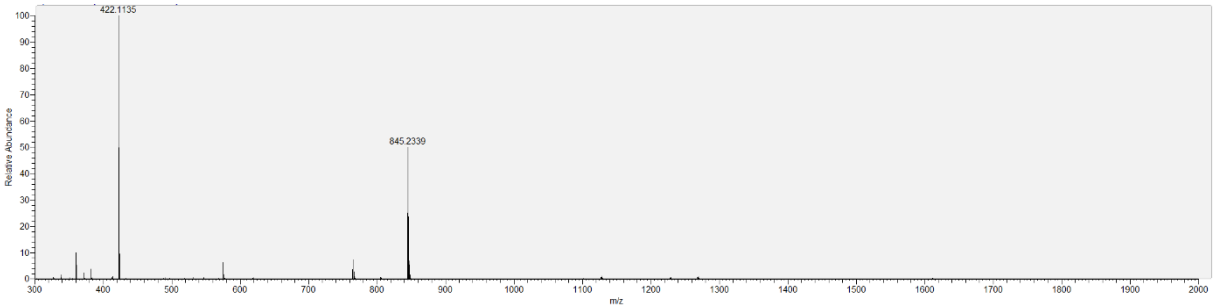
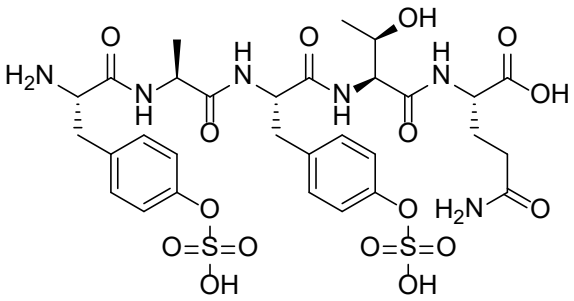


Figure S11.2. MS spectrum of peptide 2-Leu PSK **6** in negative ion mode. Spectrum belongs to the LC peak with t_R 11.5 min (shown in Figure **S11.1**). The peaks were assigned as follows: HRMS (ESI): *m/z* = $[M-H]^-$ calc for $C_{33}H_{45}N_6O_{16}S_2$ 845.2339, found 845.2339; *m/z* = $[M-2H]^{2-}$ calc for $C_{33}H_{44}N_6O_{16}S_2$ 422.1133, found 422.1135.

H-Tyr(SO_3H)-Ala-Tyr(SO_3H)-Thr-Gln-OH (2-Ala PSK, 7)



Synthesis was performed as described under the 'General procedure of Phytosulfokine synthesis'. Instead of Fmoc-Ile-OH for the coupling of the 4th amino acid, Fmoc-Ala-OH was used. After final purification and lyophilization, pure title peptide **7** was obtained (**Figure S12**).

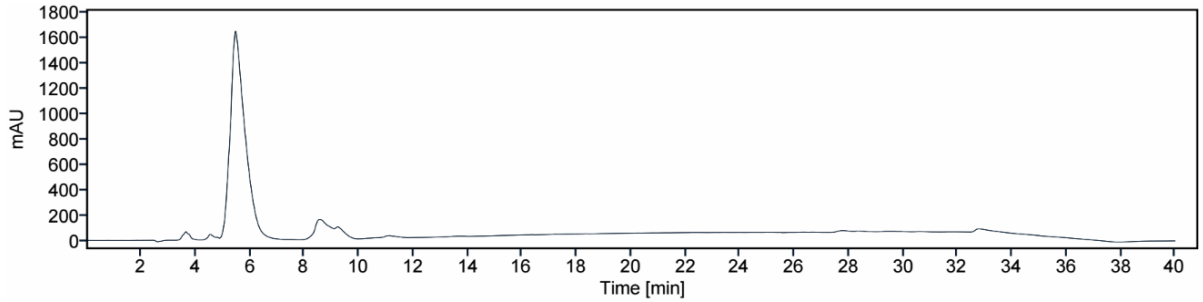


Figure S12.1. HPLC trace of peptide 2-Ala PSK **7**. The t_R of the product is 5.5 min.

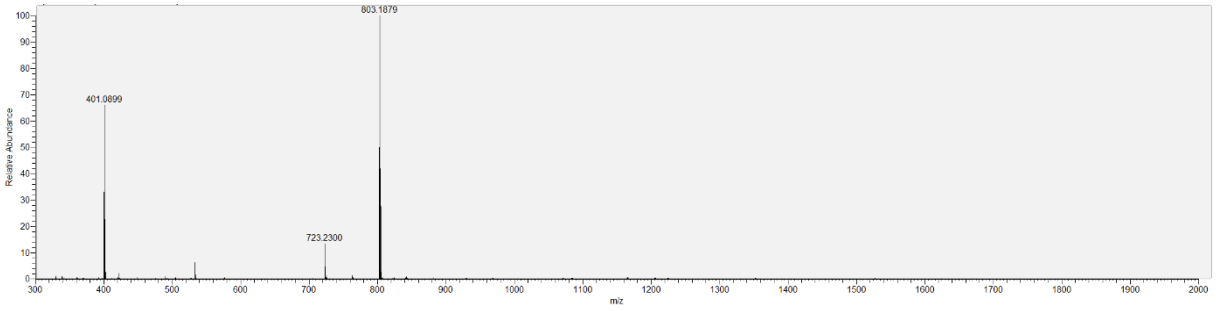
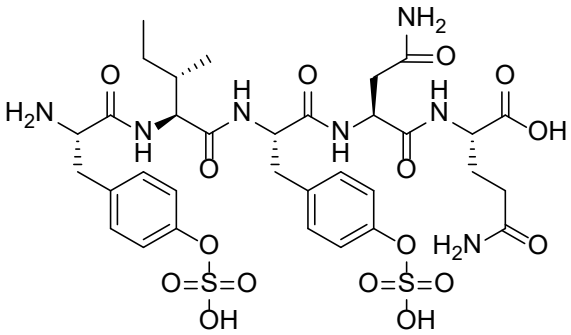


Figure S12.2. MS spectrum of peptide 2-Ala PSK **7** in negative ion mode. Spectrum belongs to the LC peak with t_R 5.5 min (shown in Figure **S12.1**). The peaks were assigned as follows: HRMS (ESI): *m/z* = $[M-H]^-$ calc for $C_{30}H_{39}N_6O_{16}S_2$ 803.1869, found 803.1879; *m/z* = $[M-SO_3H]^-$ calc for $C_{30}H_{39}N_6O_{13}S$ 723.2301, found 723.2300; *m/z* = $[M-2H]^{2-}$ calc for $C_{30}H_{38}N_6O_{16}S_2$ 401.0898, found 401.0899.

H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Asn-Gln-OH (4-Asn PSK, 8)



Synthesis was performed as described under the 'General procedure of Phytosulfokine synthesis'. Instead of Fmoc-Thr(*t*Bu)-OH for the coupling of the 2nd amino acid, Fmoc-Asn(Trt)-OH was used. After final purification and lyophilization, pure title peptide **8** was obtained (**Figure S13**).

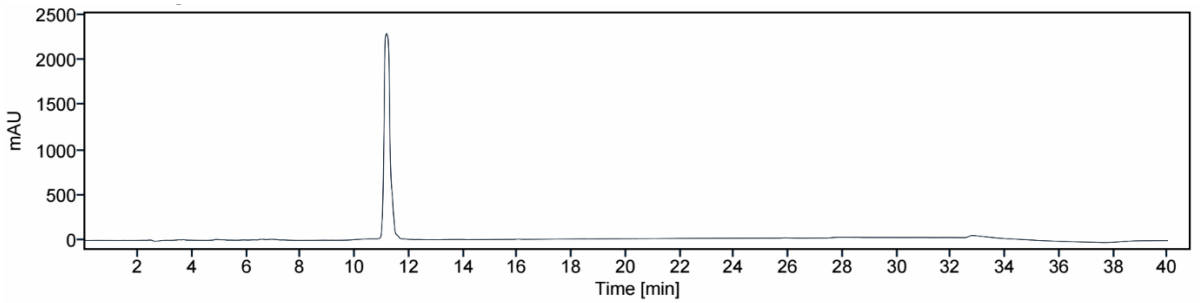


Figure S13.1. HPLC trace of peptide 4-Asn PSK **8**. The t_R of the product is 11.2 min.

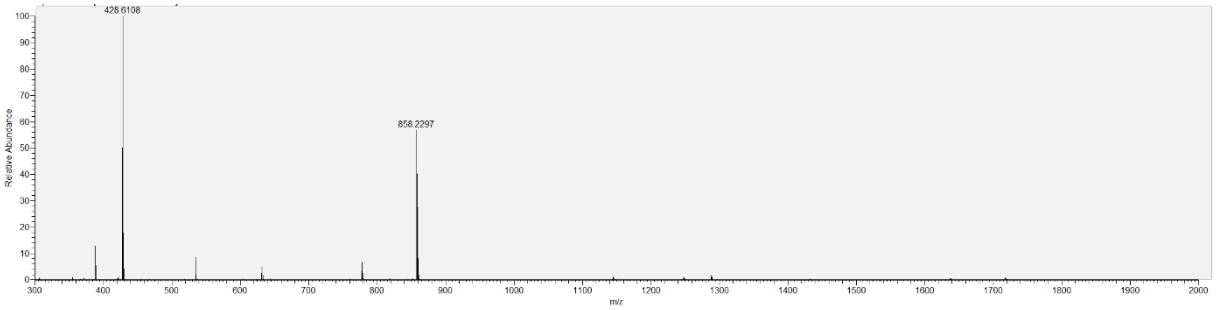
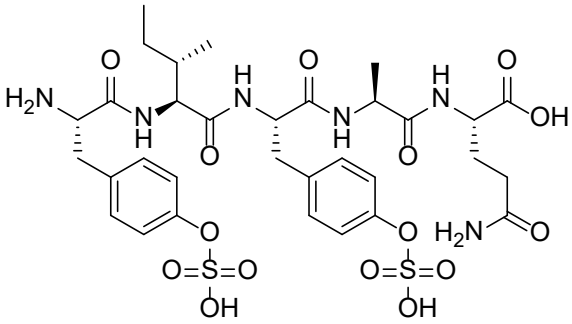


Figure S13.2. MS spectrum of peptide 4-Asn PSK **8** in negative ion mode. Spectrum belongs to the LC peak with t_R 11.2 min (shown in Figure **S13.1**). The peaks were assigned as follows: HRMS (ESI): *m/z* = $[M-H]^-$ calc for $C_{33}H_{44}N_7O_{16}S_2$ 858.2291, found 858.2297; *m/z* = $[M-2H]^{2-}$ calc for $C_{33}H_{43}N_7O_{16}S_2$ 428.6109, found 426.6108.

H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Ala-Gln-OH (4-Ala PSK, 9)



Synthesis was performed as described under the 'General procedure of Phytosulfokine synthesis'. Instead of Fmoc-Thr(*t*Bu)-OH for the coupling of the 2nd amino acid, Fmoc-Ala-OH was used. After final purification and lyophilization, pure title peptide **9** was obtained (**Figure S14**).

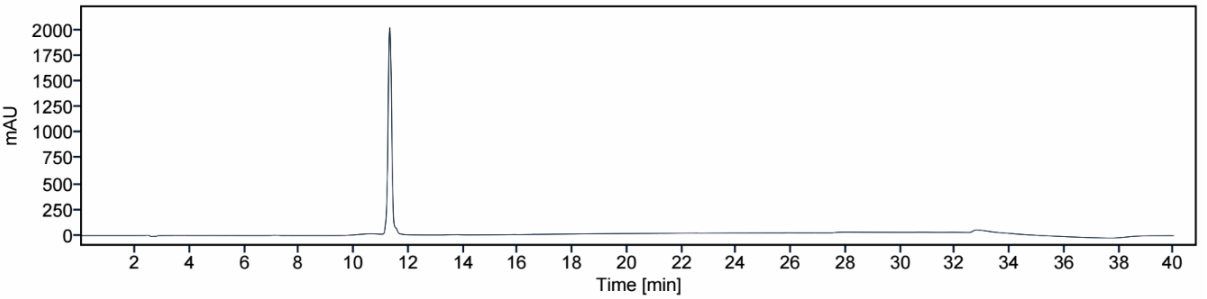


Figure S14.1. HPLC trace of peptide 4-Ala PSK **9**. The t_R of the product is 11.3 min.

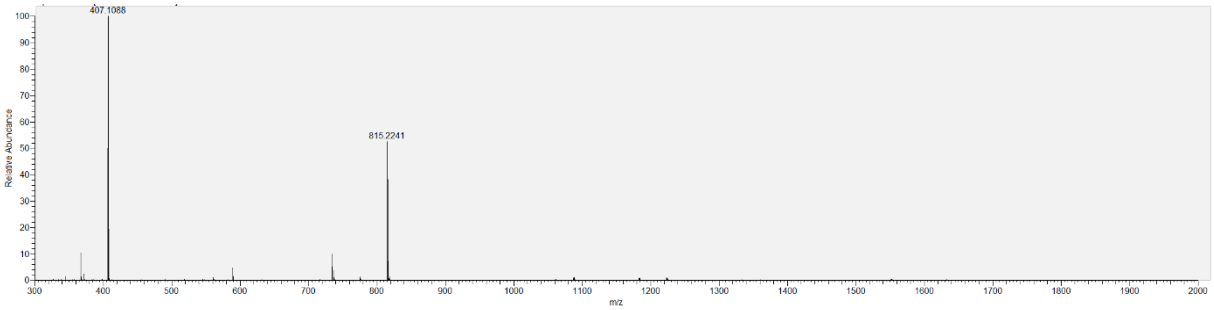
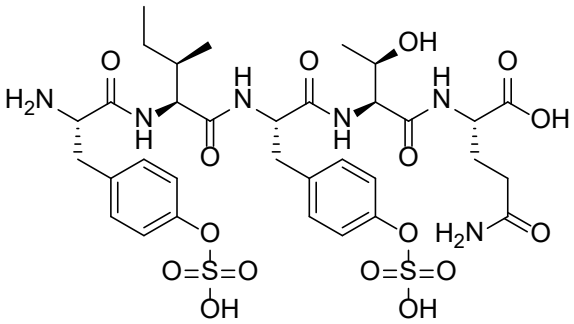


Figure S14.2. MS spectrum of peptide 4-Ala PSK **9** in negative ion mode. Spectrum belongs to the LC peak with t_R 11.3 min (shown in Figure **S14.1**). The peaks were assigned as follows: HRMS (ESI): *m/z* = $[M-H]^-$ calc for $C_{32}H_{43}N_6O_{15}S_2$ 815.2233, found 815.2241; *m/z* = $[M-2H]^{2-}$ calc for $C_{32}H_{42}N_6O_{15}S_2$ 407.1080, found 407.1088.

H-Tyr(SO_3H)-*allo*Ile-Tyr(SO_3H)-Thr-Gln-OH (2-*allo*-Ile PSK, 10)



Synthesis was performed as described under the 'General procedure of Phytosulfokine synthesis'. Instead of Fmoc-Ile-OH for the coupling of the 4th amino acid, Fmoc-*allo*Ile-OH was used. After final purification and lyophilization, pure title peptide **10** was obtained (**Figure S15**).

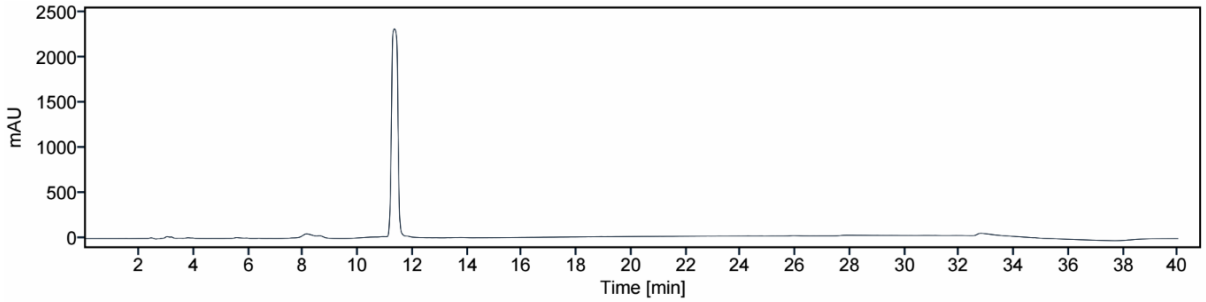


Figure S15.1. HPLC trace of peptide 2-*allo*Ile PSK **10**. The t_R of the product is 11.3 min.

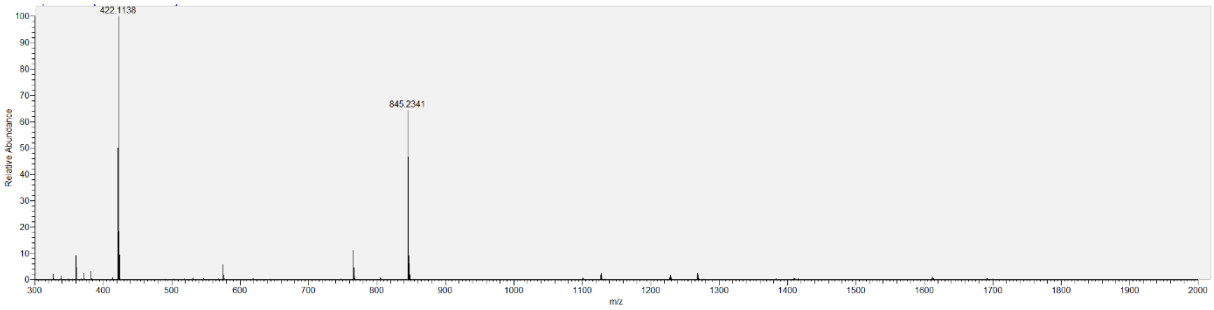
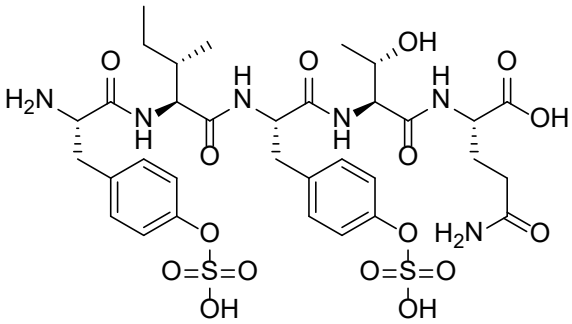


Figure S15.2. MS spectrum of peptide 2-*allo*Ile PSK **10** in negative ion mode. Spectrum belongs to the LC peak with t_R 11.3 min (shown in Figure **S15.1**). The peaks were assigned as follows: HRMS (ESI): *m/z* = $[M-H]^-$ calc for $C_{33}H_{45}N_6O_{16}S_2$ 845.2339, found 845.2341; *m/z* = $[M-2H]^{2-}$ calc for $C_{33}H_{44}N_6O_{16}S_2$ 422.1133, found 422.1138.

H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-*allo*Thr-Gln-OH (4-*allo*-Thr PSK, 11)



Synthesis was performed as described under the 'General procedure of Phytosulfokine synthesis'. Instead of Fmoc-Thr(*t*Bu)-OH for the coupling of the 2nd amino acid, Fmoc-*allo*Thr(*t*Bu)-OH was used. After final purification and lyophilization, pure title peptide **11** was obtained (**Figure S16**).

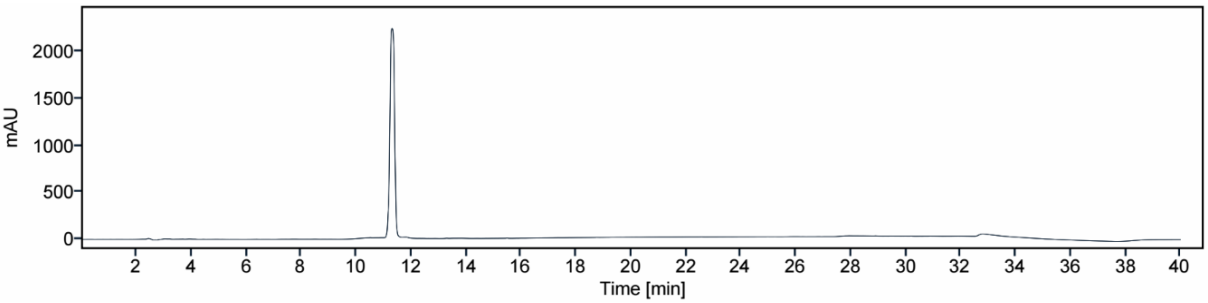


Figure S16.1. HPLC trace of peptide 4-*allo*Thr PSK **11**. The t_R of the product is 11.3 min.

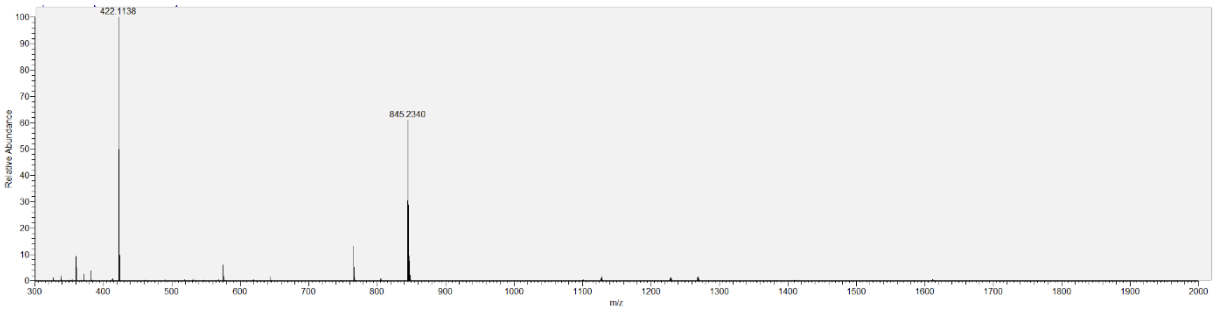
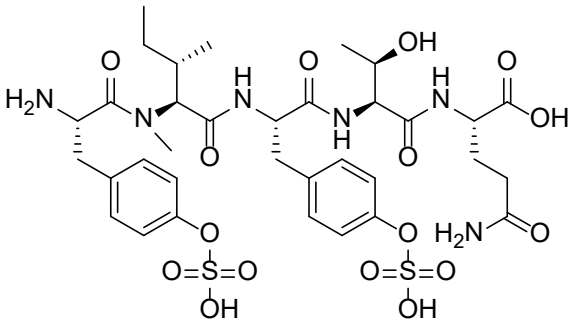


Figure S16.2. MS spectrum of peptide 4-*allo*Thr PSK **11** in negative ion mode. Spectrum belongs to the LC peak with t_R 11.3 min (shown in Figure **S16.1**). The peaks are assigned as follows: HRMS (ESI): *m/z* = $[M-H]^-$ calc for $C_{33}H_{45}N_6O_{16}S_2$ 845.2339, found 845.2340; *m/z* = $[M-2H]^{2-}$ calc for $C_{33}H_{44}N_6O_{16}S_2$ 422.1133, found 422.1138.

H-Tyr(SO_3H)-*N*me-Ile-Tyr(SO_3H)-Thr-Gln-OH (2-*Nme*-Ile PSK, 12)



Synthesis on solid-phase was performed as described under the 'General procedure of Phytosulfokine synthesis' up until H_2N-Tyr(SO_2ONp)-Thr(*t*Bu)-Gln(Trt)-Wang resin is formed. The resin was then treated with Fmoc-Tyr(SO_2ONp)-*N*me-Ile-OH (**21**, 2 equivalents compared to resin loading capacity), activated with HATU (2 equivalents) and treated with DIPEA (4 equivalents) in DMF. The coupling was reacted for 3 h before washing the syringe three times with DMF, followed by Fmoc removal with 20% piperidine, and subsequent 3 x DMF and 3 x DCM wash steps. The formed peptide was then cleaved and further processed as described in the general procedure to eventually obtain pure title peptide **12** (**Figure S17**).

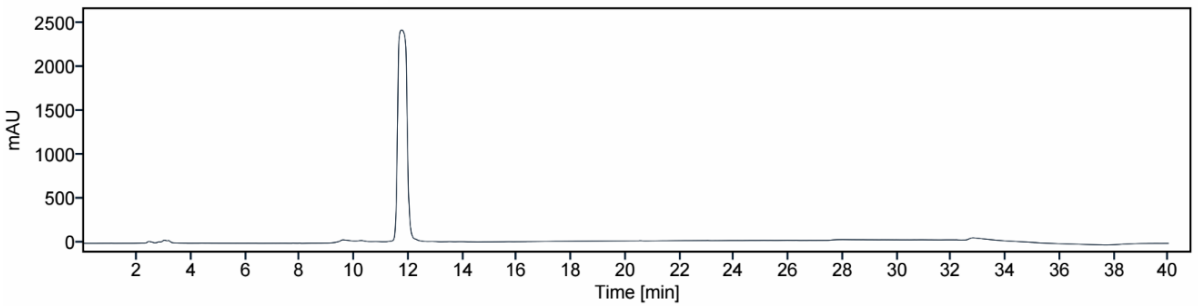


Figure S17.1. HPLC trace of peptide 2-*Nme*-Ile PSK **12**. The t_R of the product is 11.7 min.

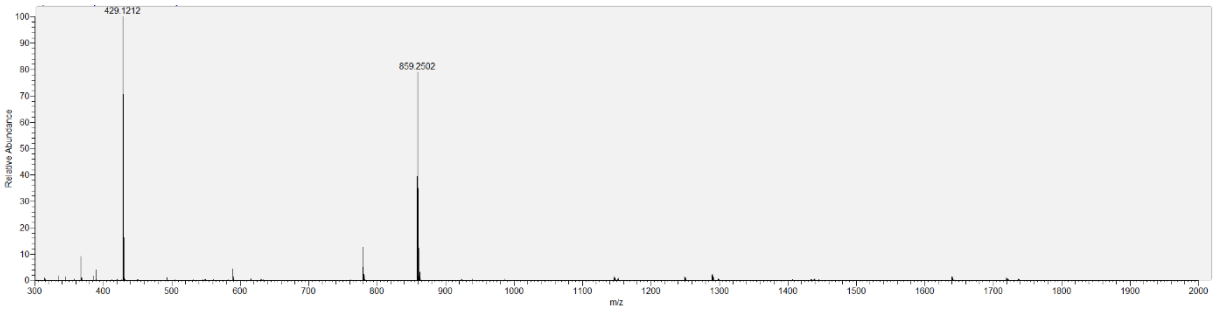
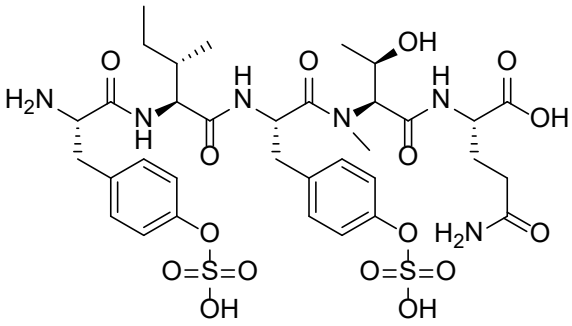


Figure S17.2. MS spectrum of peptide 2-*Nme*-Ile PSK **12** in negative ion mode. Spectrum belongs to the LC peak with t_R 11.7 min (shown in Figure **S17.1**). The peaks are assigned as follows: HRMS (ESI): *m/z* = $[M-H]^-$ calc for $C_{34}H_{47}N_6O_{16}S_2$ 859.2495, found 859.2502; *m/z* = $[M-2H]^{2-}$ calc for $C_{34}H_{46}N_6O_{16}S_2$ 429.1211, found 429.1212.

H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-*N*me-Thr-Gln-OH (4-*Nme*-Thr PSK, 13)



Fmoc-Gln(Trt)-Wang resin was first treated with 20% piperidine and washed 3x with DMF. The resin was then treated with Fmoc-Tyr(SO_2ONp)-*N*me-Thr(*t*Bu)-OH (**22**, 2 equivalents compared to resin loading capacity), activated with HATU (2 equivalents), and treated with DIPEA (4 equivalents) in DMF. The coupling was allowed to react for 3 h, after which the remaining synthesis was performed as described under 'General procedure of Phytosulfokine synthesis'. After final purification and lyophilization, pure title peptide **13** was obtained (**Figure S18**).

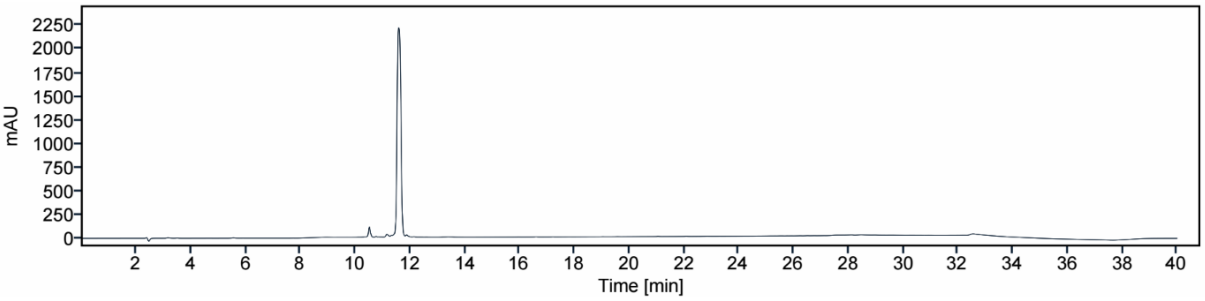


Figure S18.1. HPLC trace of peptide 4-*Nme*-Thr PSK **13**. The t_R of the product is 11.6 min.

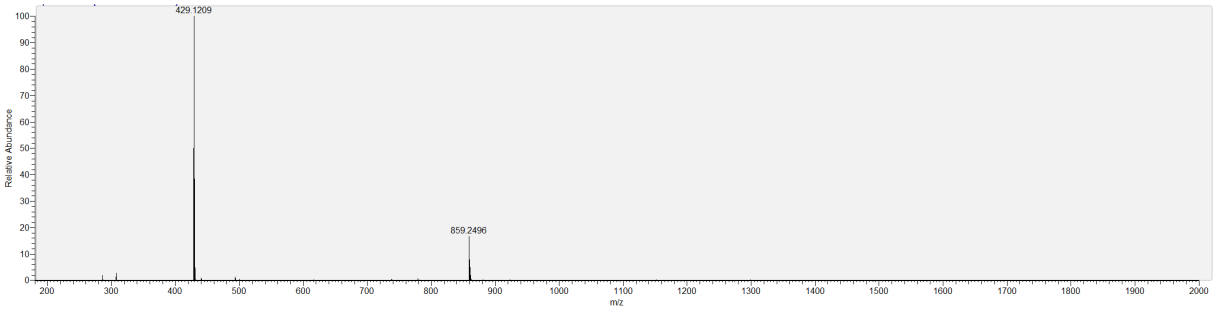


Figure S18.2. MS spectrum of peptide 4-*Nme*-Thr PSK **13** in negative ion mode. Spectrum belongs to the LC peak with t_R 11.6 min (shown in Figure **S18.1**). The peaks are assigned as follows: HRMS (ESI): *m/z* = $[M-H]^-$ calc for $C_{34}H_{47}N_6O_{16}S_2$ 859.2495, found 859.2496; *m/z* = $[M-2H]^{2-}$ calc for $C_{34}H_{46}N_6O_{16}S_2$ 429.1211, found 429.1209.

Plant proliferation assay

Protoplast isolation and incubation

Brassica oleracea plants were grown *in-vitro* on ½ Murashige and Skoog (MS) media with 1% sucrose and 1% agar (pH 5.6) under 16 h light at 25 °C. One gram of young leaves was used for protoplast isolation. Leaves were cut into small pieces and incubated at 25 °C in plasmolysis solution (54.6 g/L sorbitol, 7.4 g/L CaCl, 0.5 g/L MES, pH 5.8) and then incubated for 20 h at 25 °C in 15 mL Hazel media (2% glucose, 7% mannitol, 3.6 g/L KAO & MICHAYLUK MEDIUM basal salt mixture, 0.5 g/L MES (pH 5.8), 250 mg/L casein hydrolysate, 27.8 mg/L $FeSO_4 \cdot 7H_2O$, 37.3 mg/L Na_2 EDTA, 100 mg/L myo-Inositol, 1 mg/L nicotinic acid, 1 mg/L pyridoxine·HCl, 10 mg/L thiamine, 1 mg/L 2,4-D, 0.2 mg/L zeatin, 200 µg/L cefotaxime) with 0.5% cellulase and 0.1% macerozyme R-10. The digested leaves were filtered over a 100 µM mesh nylon filter and the flow through was centrifugated for 5 min at 100g. The pellet was resuspended in 8 mL of SAH/MES (171.15 g/L sucrose, 0.5 g/L MES pH 5.8) and overlayed with 2 mL of washing solution (8.4 g/L $CaCl_2 \cdot 2H_2O$, 9 g/L NaCl, 0.1 % glucose, 0.5 g/L KCl, 0.5 g/L MES pH 5.8) and centrifugated at 100g. The interphase was collected and washed with 10 mL of washing solution and centrifugated at 100g for 5 min. The pellet was resuspended in 10 mL of Hazel media and centrifugated at 100g for 5 min, after which the pellet was resuspended in 2 mL of Hazel media. The concentration of protoplasts was determined and diluted to 1.10^5 protoplasts/mL. 0.75 mL of protoplasts were mixed with 0.75 mL of alginate (0.4 M mannitol, 2% low viscosity alginate pH 5.8) and poured on a 6 cm petri dish with 1% agar, 30 mM CaCl, 0.4 M mannitol (pH 5.8) and left to harden for 1h. The disc was cut in two and transferred to a 6cm petri dish with 4 mL of Hazel media with the appropriate peptide. The protoplasts were incubated in the dark for 2 weeks at 25 °C after which the media was refreshed with 4 mL of Hazel media without any peptides. The newly formed calli were counted after 5 weeks of growth. Protoplast proliferation experiments were performed in three biological replicates.

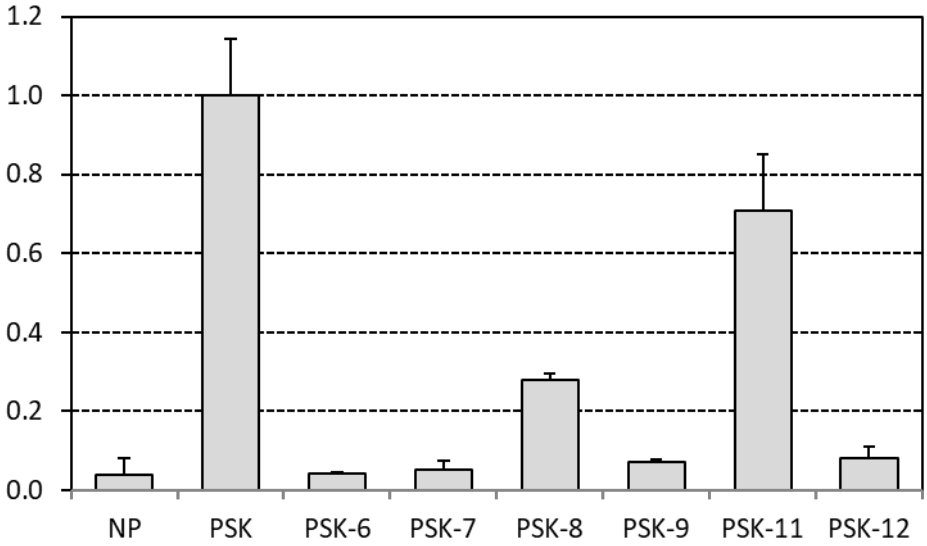


Figure S19. Testing of mutated versions of PSK for bioactivity. Different PSK analogues were tested at 100 nM and compared relative to the activity of native PSK for their ability to induce cell division, resulting in callus, in protoplasts from white cabbage. The number of calli formed was counted and compared relative to the number of calli formed with native PSK (set at 1). PSK-6, 7, 9 and 12 were not bioactive and not different from no peptide treatment ($P<0.01$). Only PSK-8 and PSK-11 were bioactive but weaker than native PSK. Error bars indicate the standard deviation over the number of calli in three biological replicates. NP is no peptide.

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

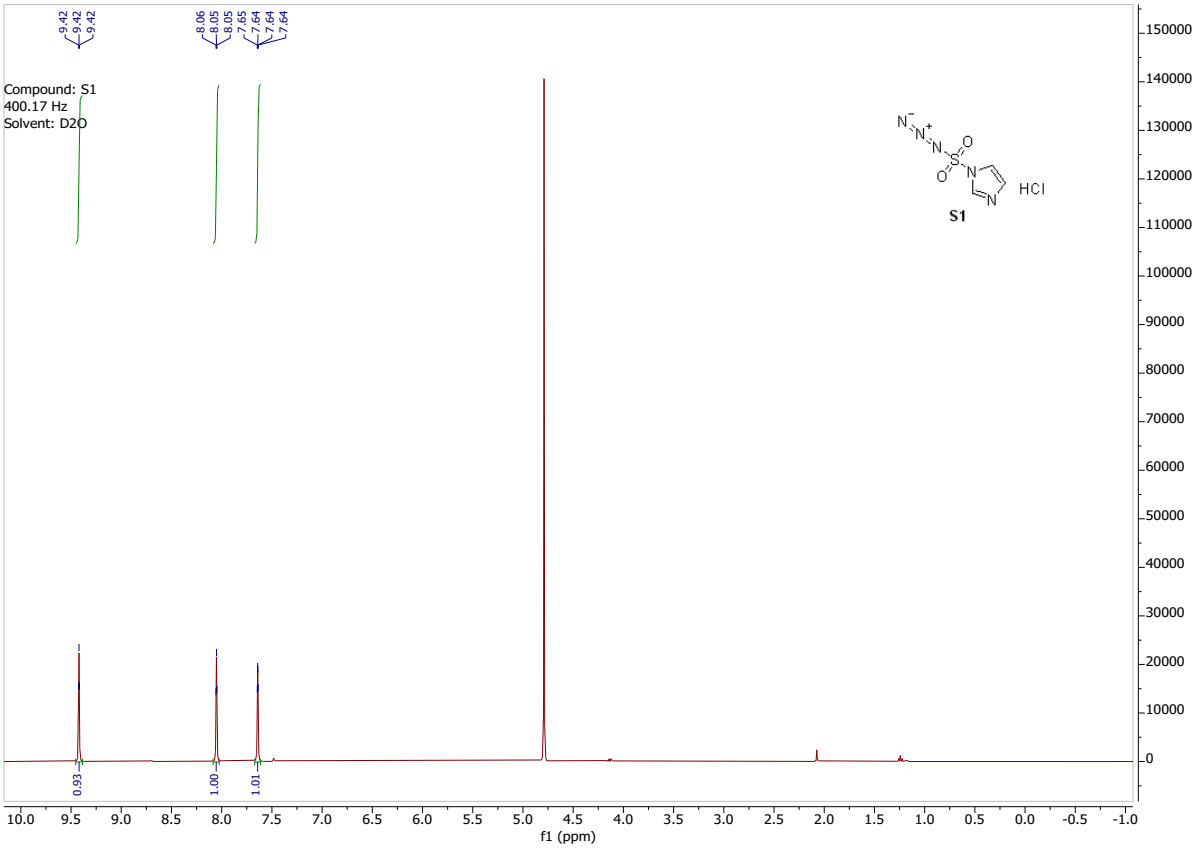


Figure S20. ^{1}H spectrum of imidazole-1-sulfonyl azide hydrochloride (**S1**).

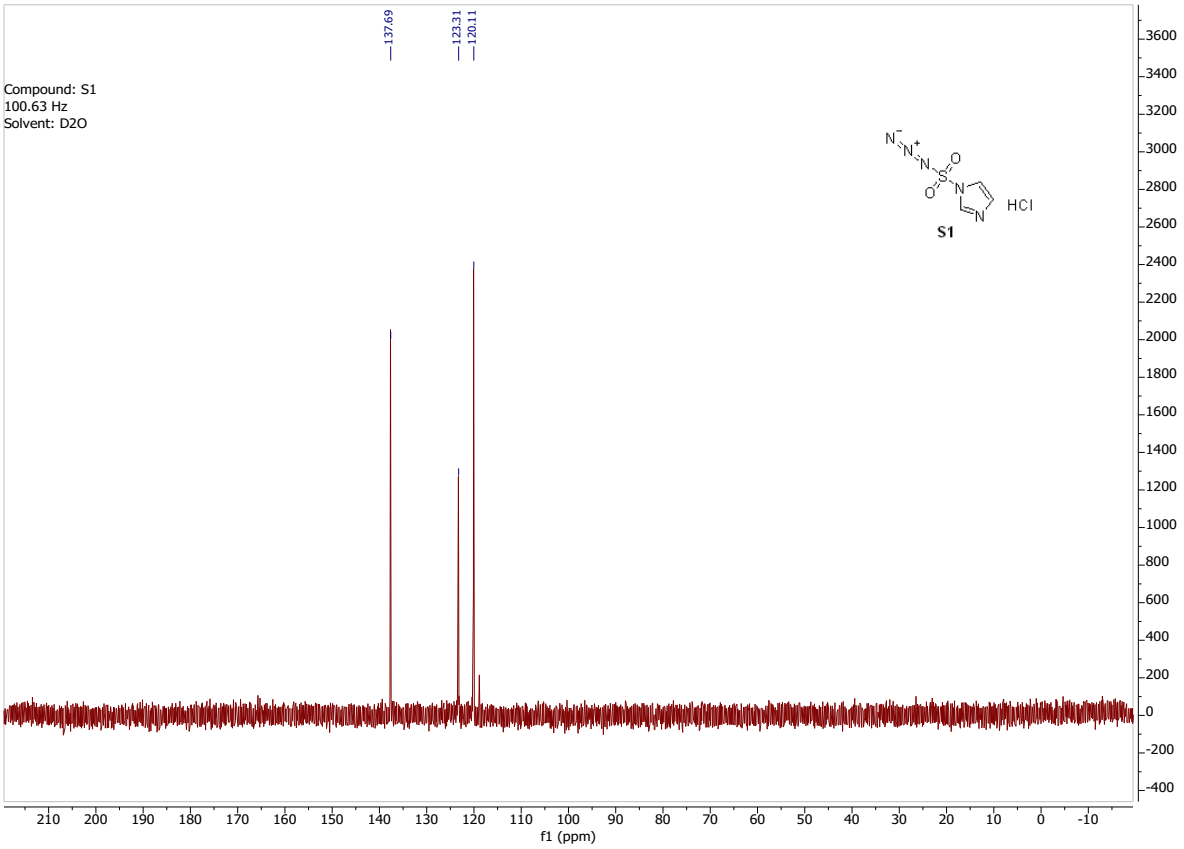


Figure S21. ^{13}C spectrum of imidazole-1-sulfonyl azide hydrochloride (**S1**).

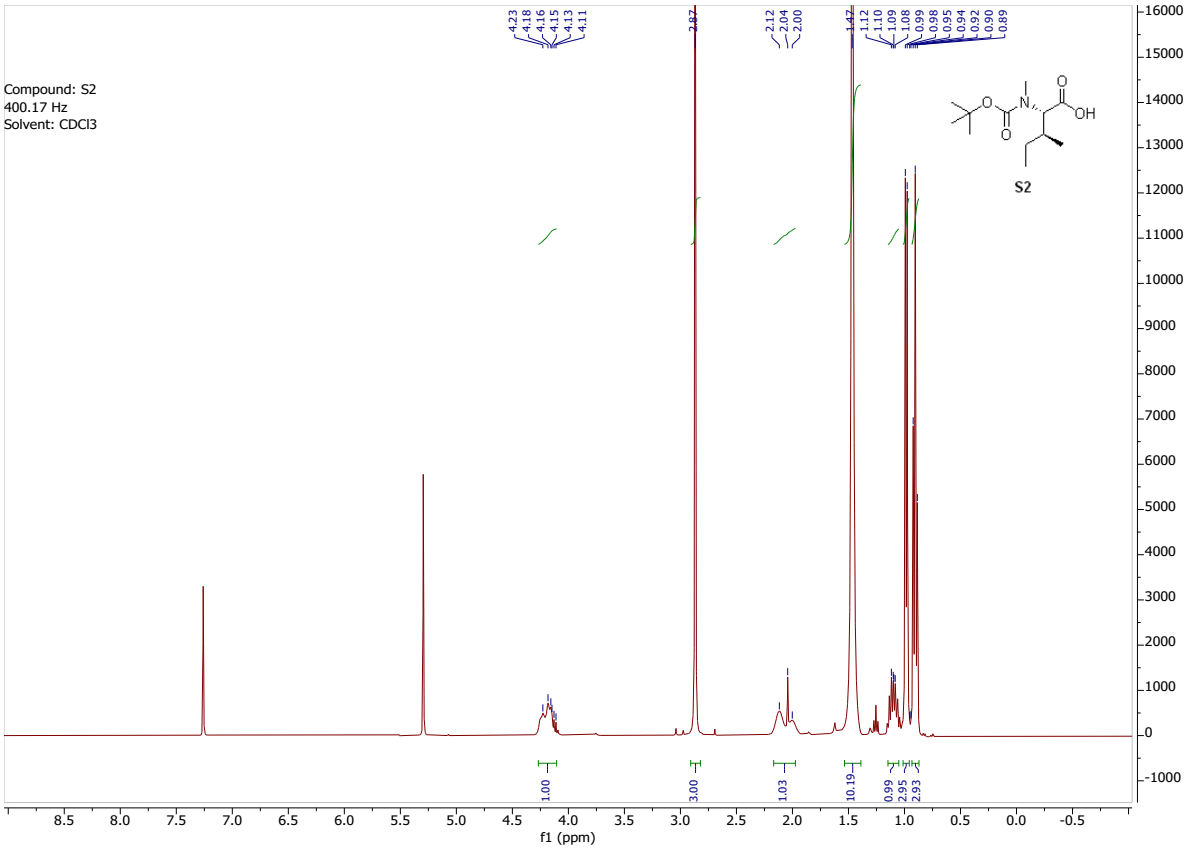


Figure S22. ^{1}H spectrum of Boc-*N*me-Ile-OH (**S2**). Due to the presence of cis-trans rotamers of the N-methylated carbamate, several peaks are split into subsets.

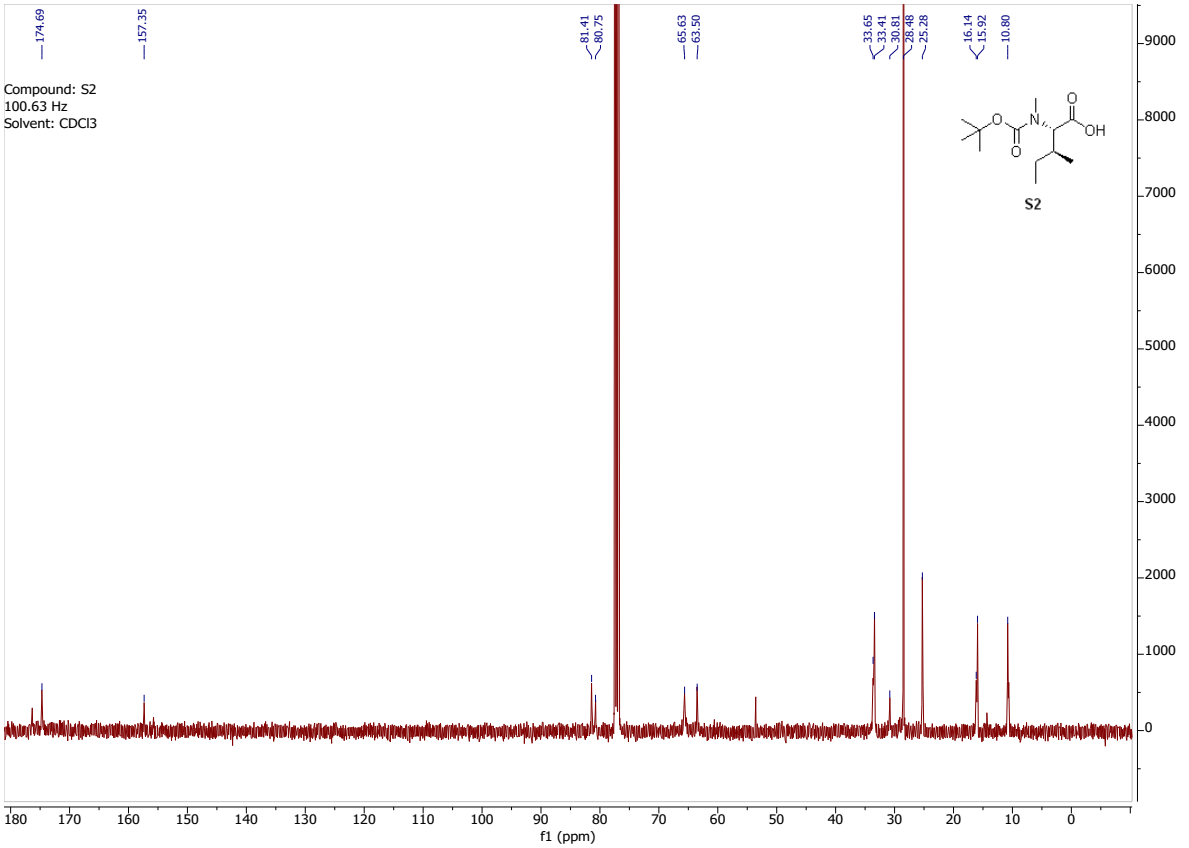


Figure S23. ^{13}C spectrum of Boc-*N*me-Ile-OH (**S2**). Due to the presence of cis-trans rotamers of the N-methylated carbamate, several peaks are split into subsets.

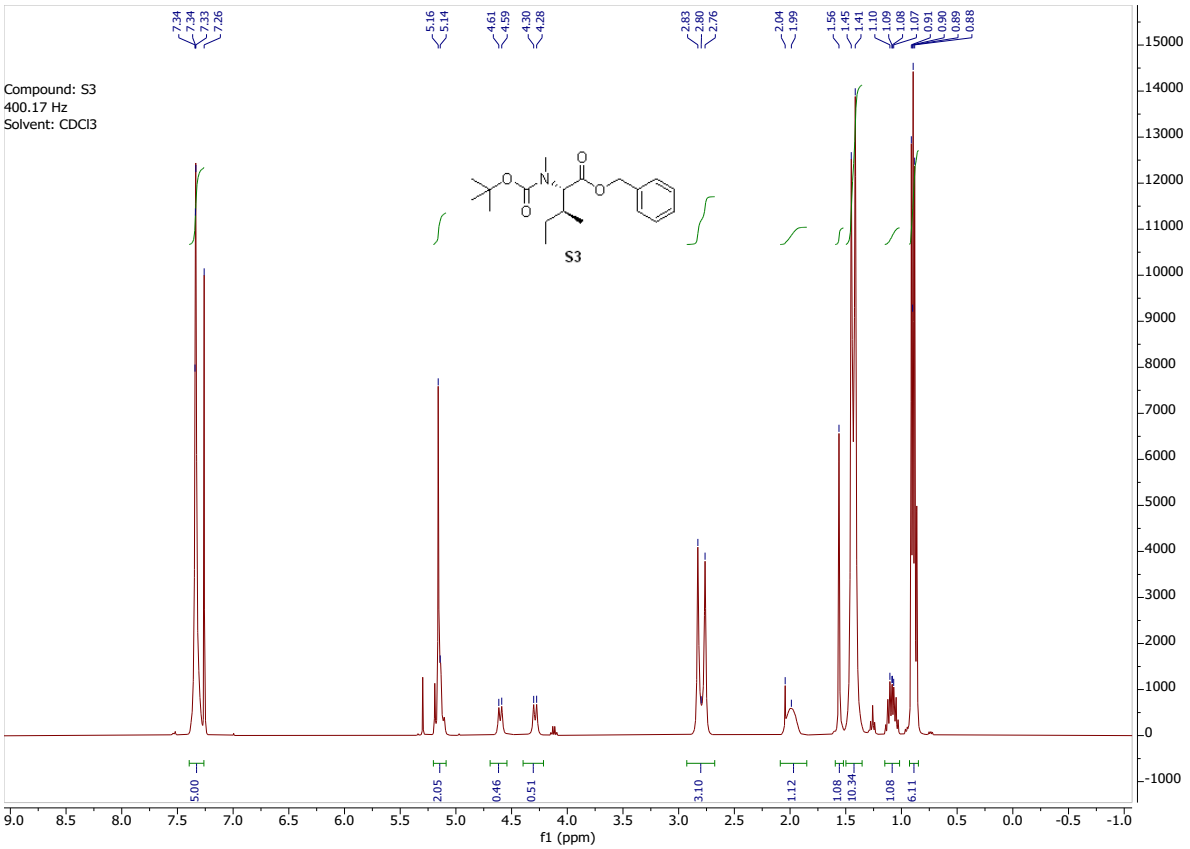


Figure S24. ^{1}H spectrum of Boc-*N*me-Ile-OBn (**S3**). Due to the presence of cis-trans rotamers of the N-methylated carbamate, several peaks are split into subsets.

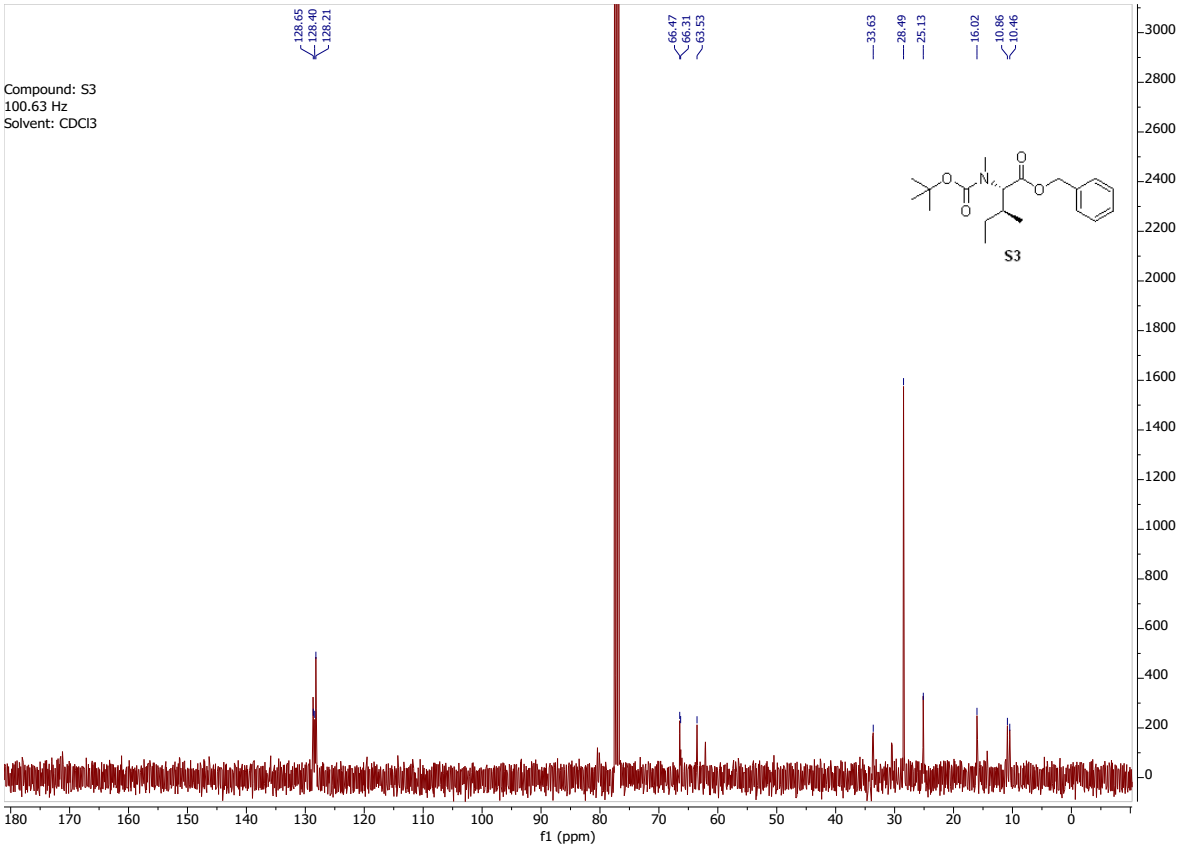


Figure S25. ^{13}C spectrum of Boc-*N*me-Ile-OBn (**S3**). Due to the presence of cis-trans rotamers of the N-methylated carbamate, several peaks are split into subsets.

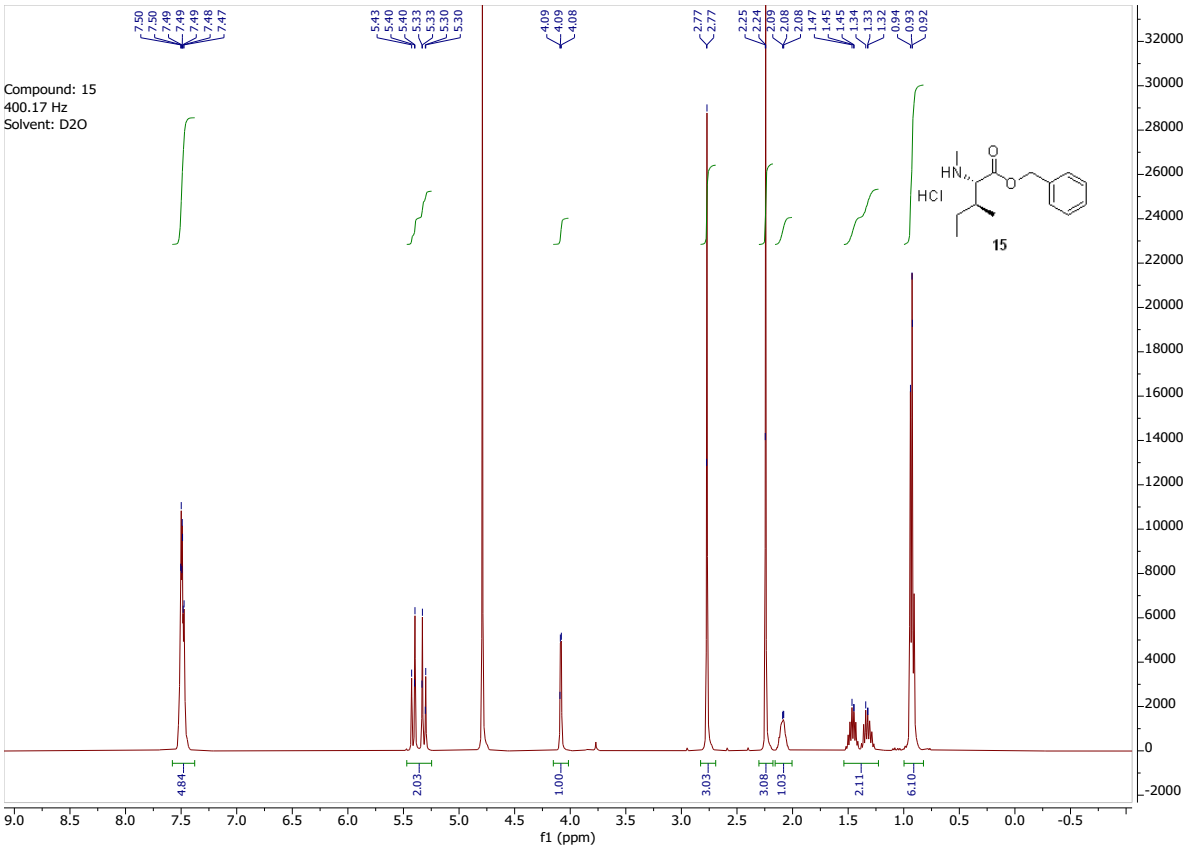


Figure S26. ^{1}H spectrum of *N*me-Ile-OBn hydrochloride (**15**).

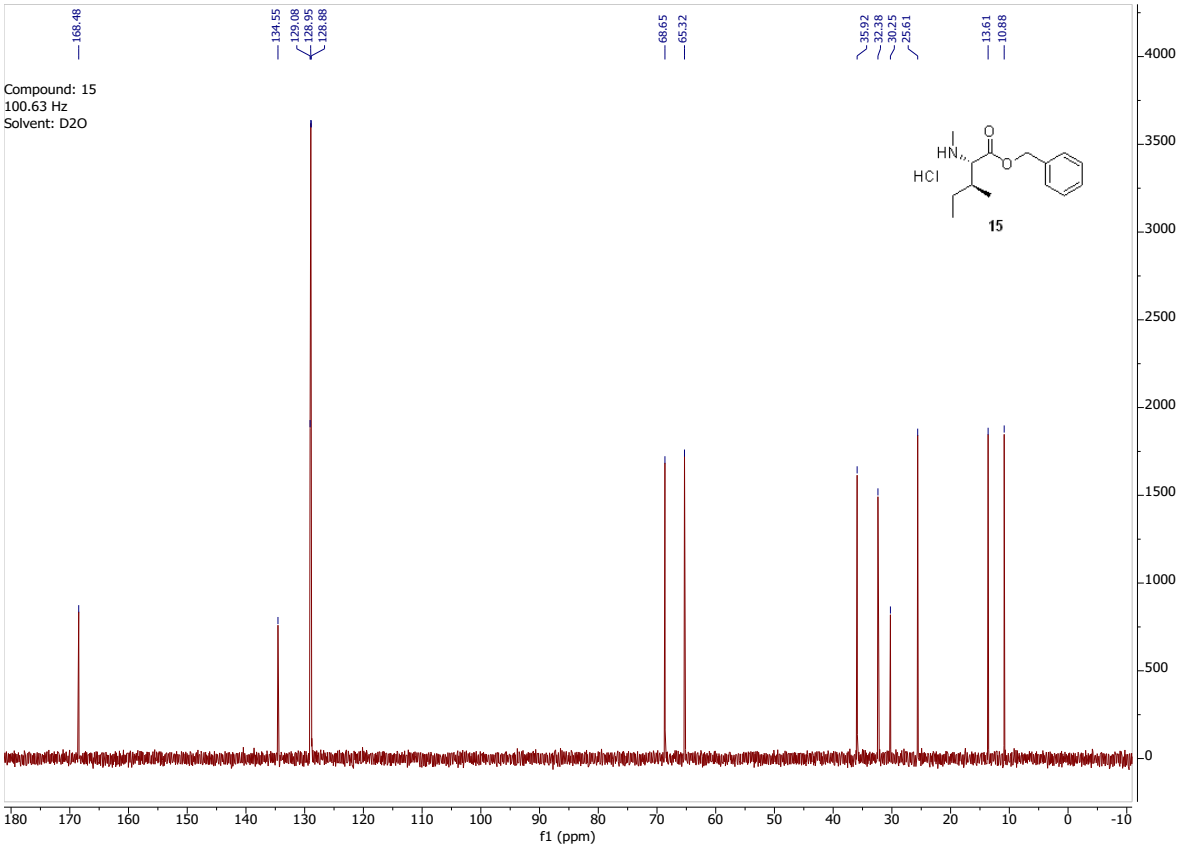


Figure S27. ^{13}C spectrum of *N*me-Ile-OBn hydrochloride (**15**).

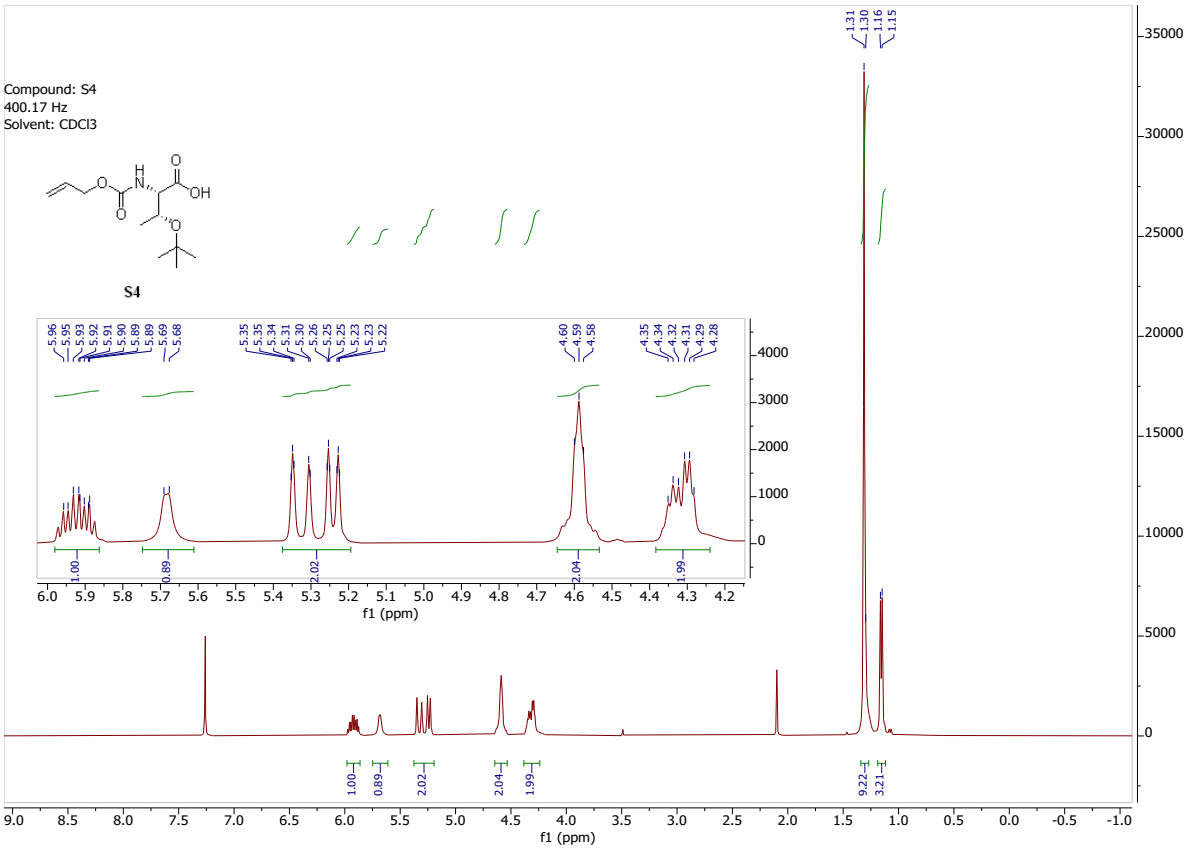


Figure S28. ^{1}H spectrum of Alloc-Thr(*t*Bu)-OH (**S4**).

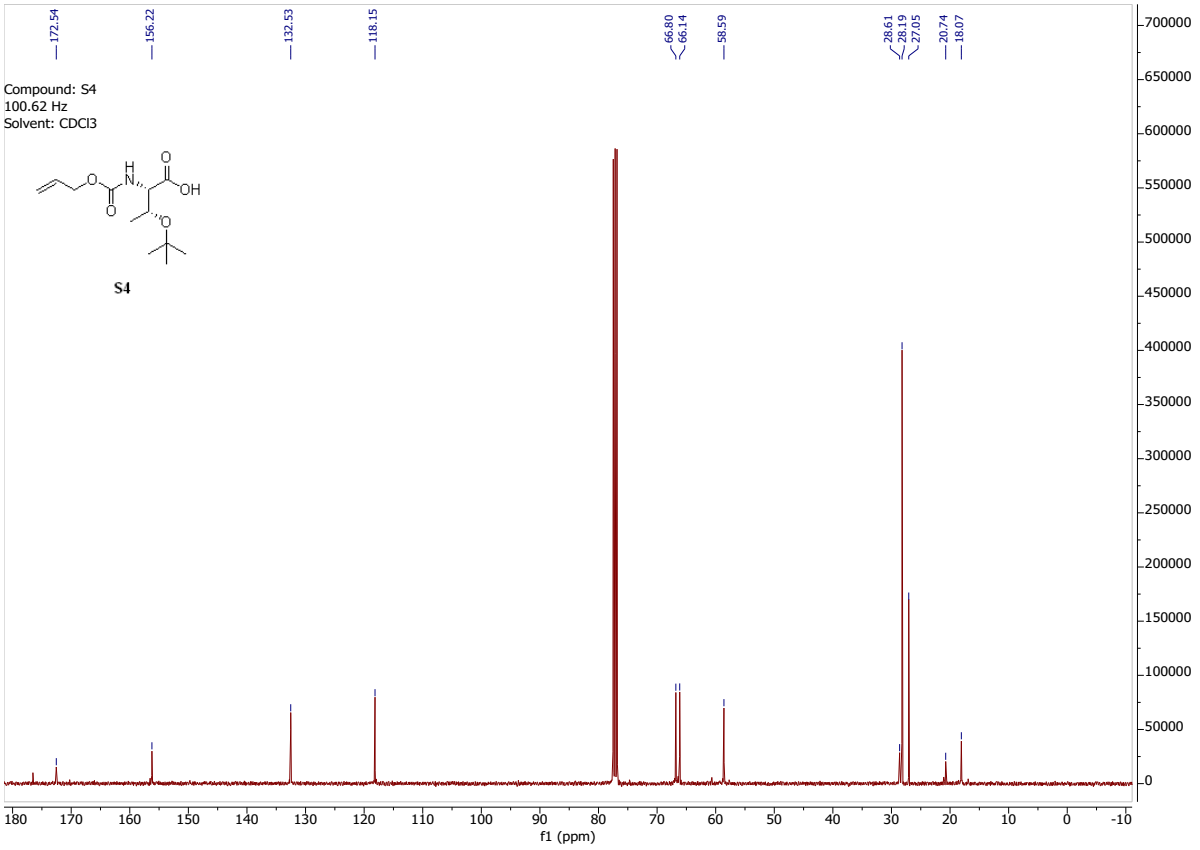


Figure S29. ^{13}C spectrum of Alloc-Thr(*t*Bu)-OH (**S4**).

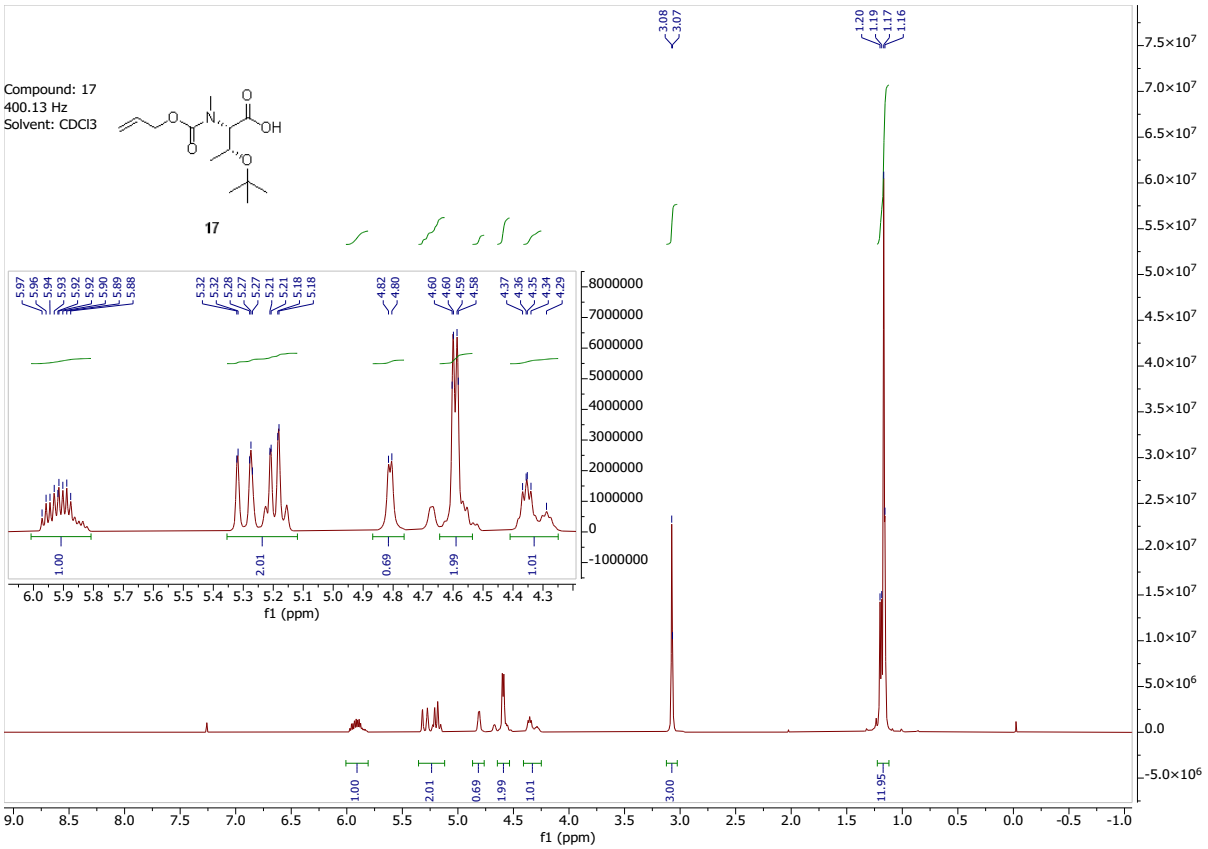


Figure S30. ^{1}H spectrum of Alloc-*N*me-Thr(*t*Bu)-OH (**17**). Due to the presence of cis-trans rotamers of the N-methylated carbamate, several peaks are split into subsets.

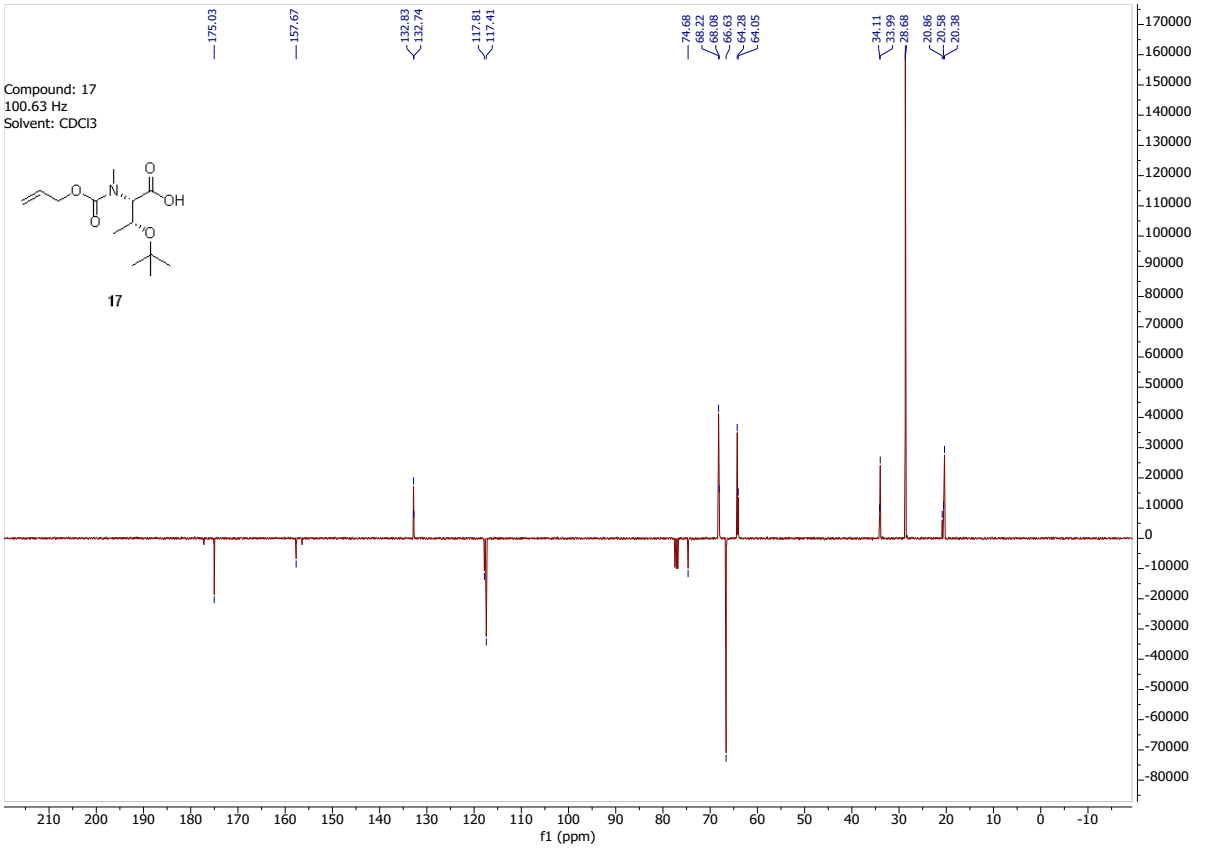


Figure S31. ^{13}C APT spectrum of Alloc-*N*me-Thr(*t*Bu)-OH (**17**). Due to the presence of cis-trans rotamers of the N-methylated carbamate, several peaks are split into subsets.

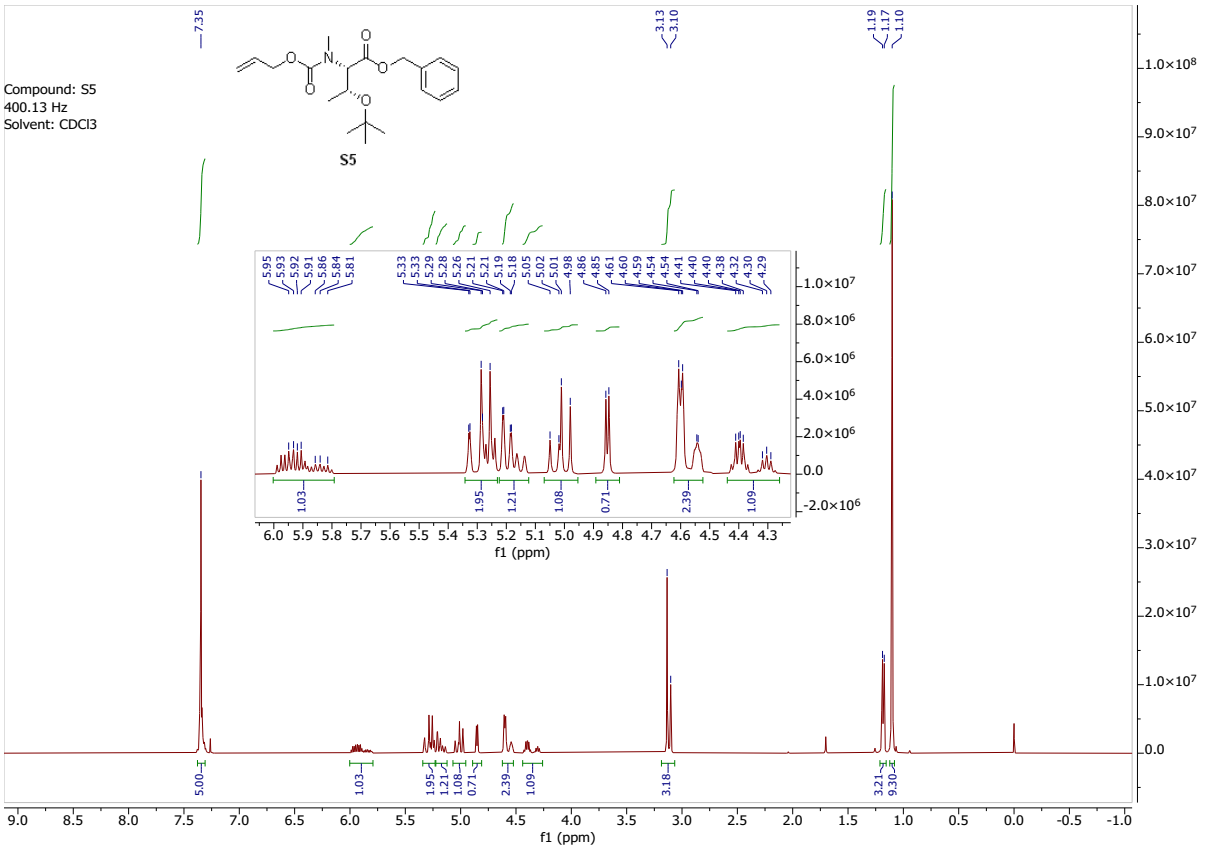


Figure S32. ^{1}H spectrum of Alloc-*N*me-Thr(*t*Bu)-OBn (**S5**). Due to the presence of cis-trans rotamers of the N-methylated carbamate, several peaks are split into subsets.

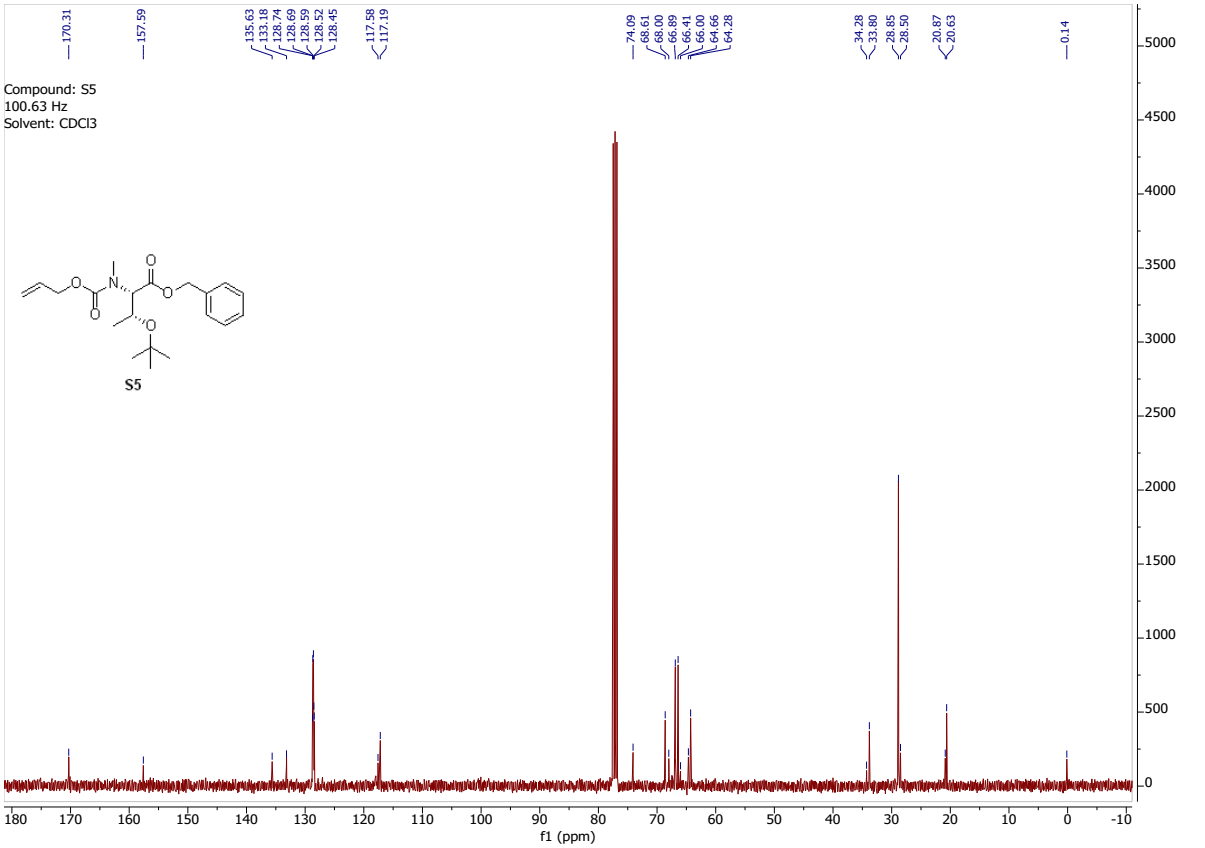


Figure S33. ^{13}C spectrum of Alloc-*N*me-Thr(*t*Bu)-OBn (**S5**). Due to the presence of cis-trans rotamers of the N-methylated carbamate, several peaks are split into subsets.

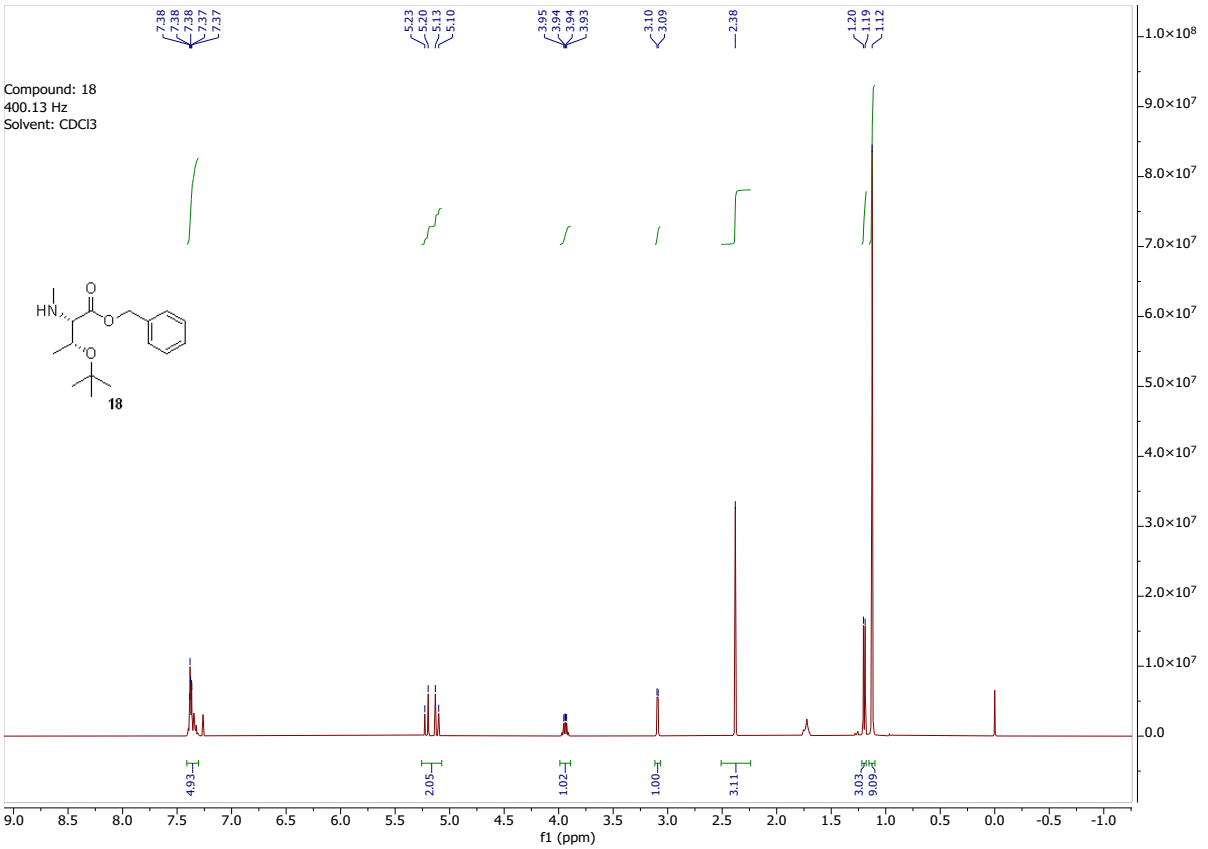


Figure S34. ^{1}H spectrum of *N*me-Thr(*t*Bu)-OBn synthesized with S5 (**18**).

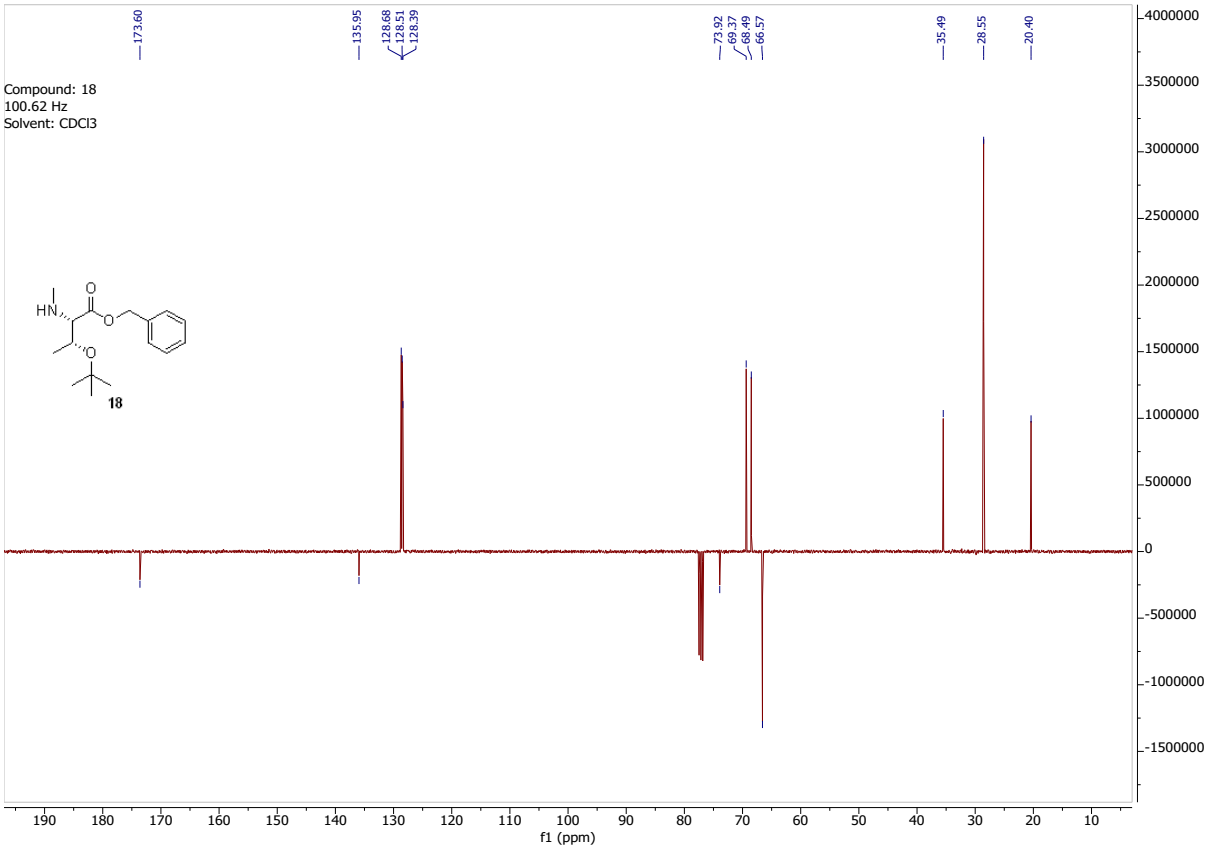


Figure S35. ^{13}C APT spectrum of *N*me-Thr(*t*Bu)-OBn synthesized with S5 (**18**).

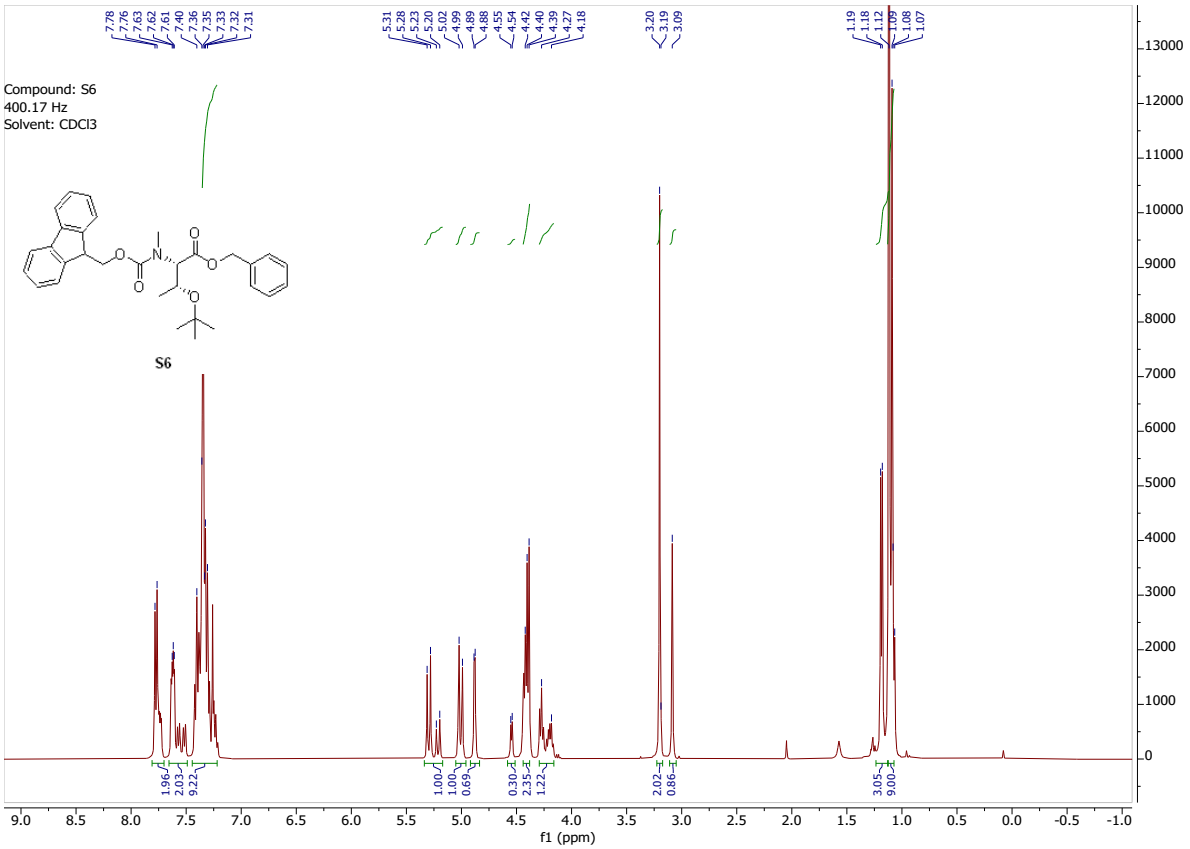


Figure S36. ^{1}H spectrum of Fmoc-*N*me-Thr(*t*Bu)-OBn (**S6**). Due to the presence of cis-trans rotamers of the N-methylated carbamate, several peaks are split into subsets.

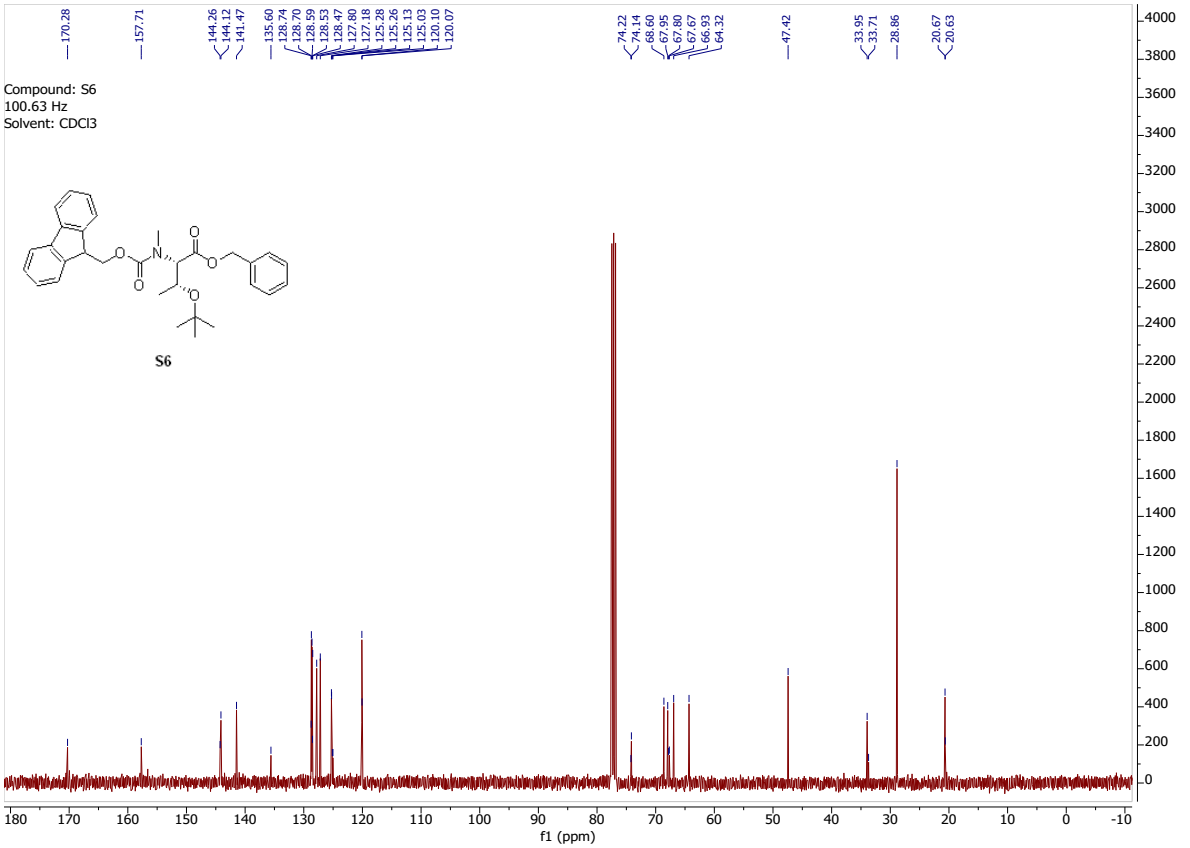


Figure S37. ^{13}C spectrum of Fmoc-*N*me-Thr(*t*Bu)-OBn (**S6**). Due to the presence of cis-trans rotamers of the N-methylated carbamate, several peaks are split into subsets.

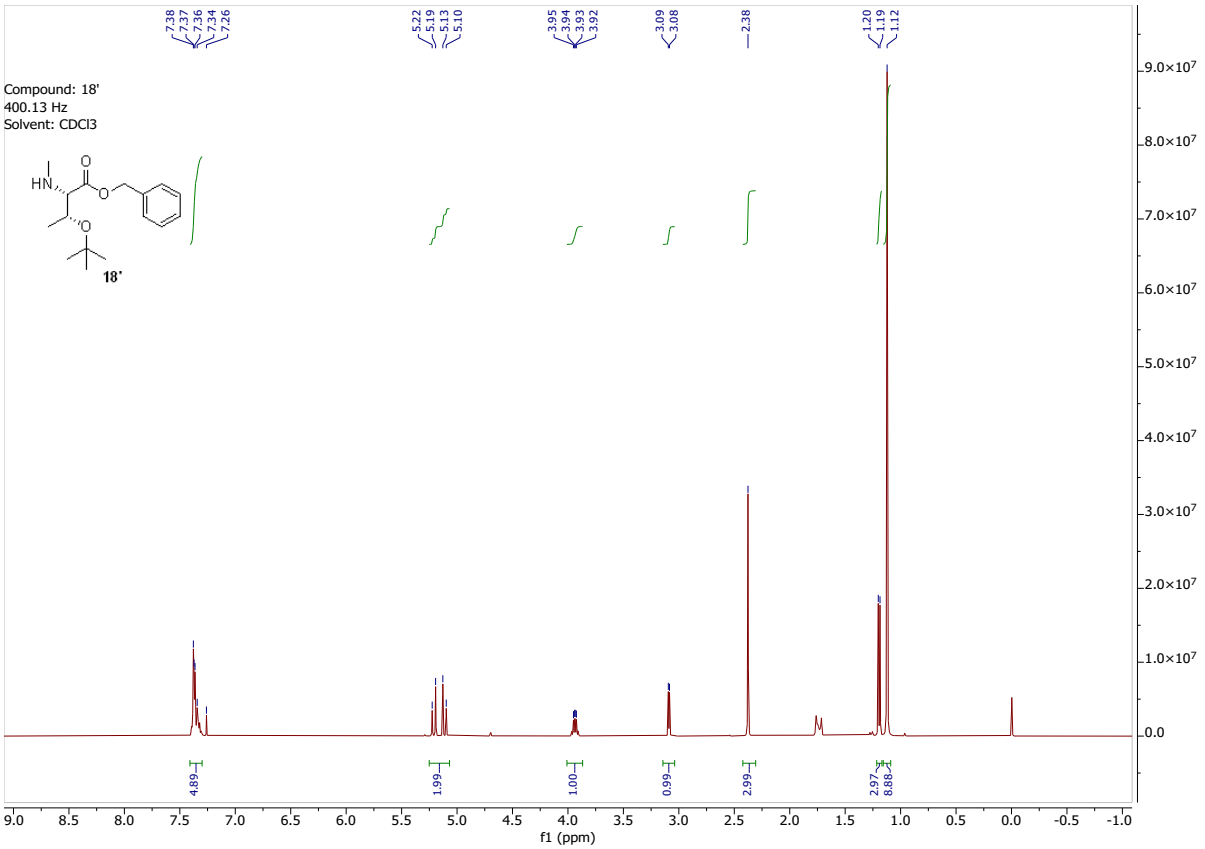


Figure S38. ^{1}H spectrum of *N*me-Thr(*t*Bu)-OBn synthesized with **S6** (**18'**).

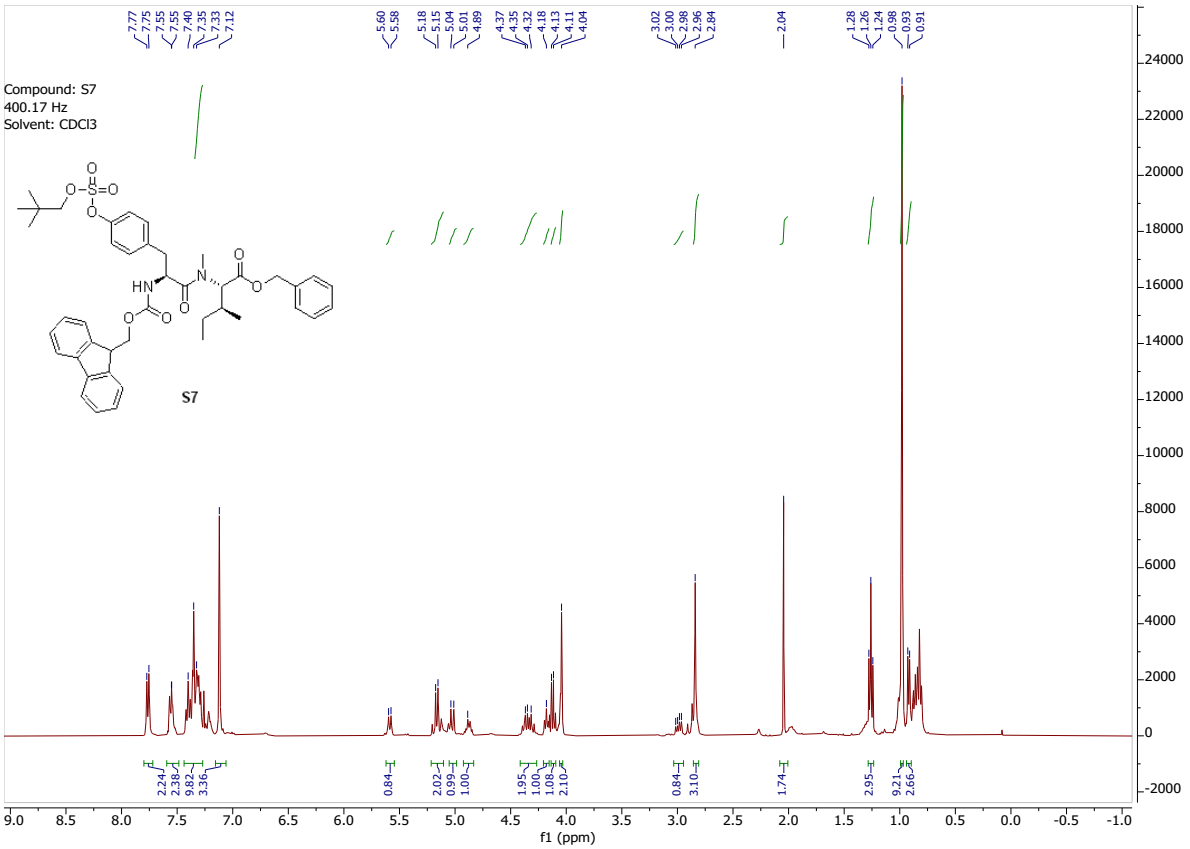


Figure S39. ^{1}H spectrum of Fmoc-Tyr(SO_2ONp)-*N*me-Ile-OBn (**S7**). Due to the presence of cis-trans rotamers of the N-methylated peptide bond, several peaks are split into subsets.

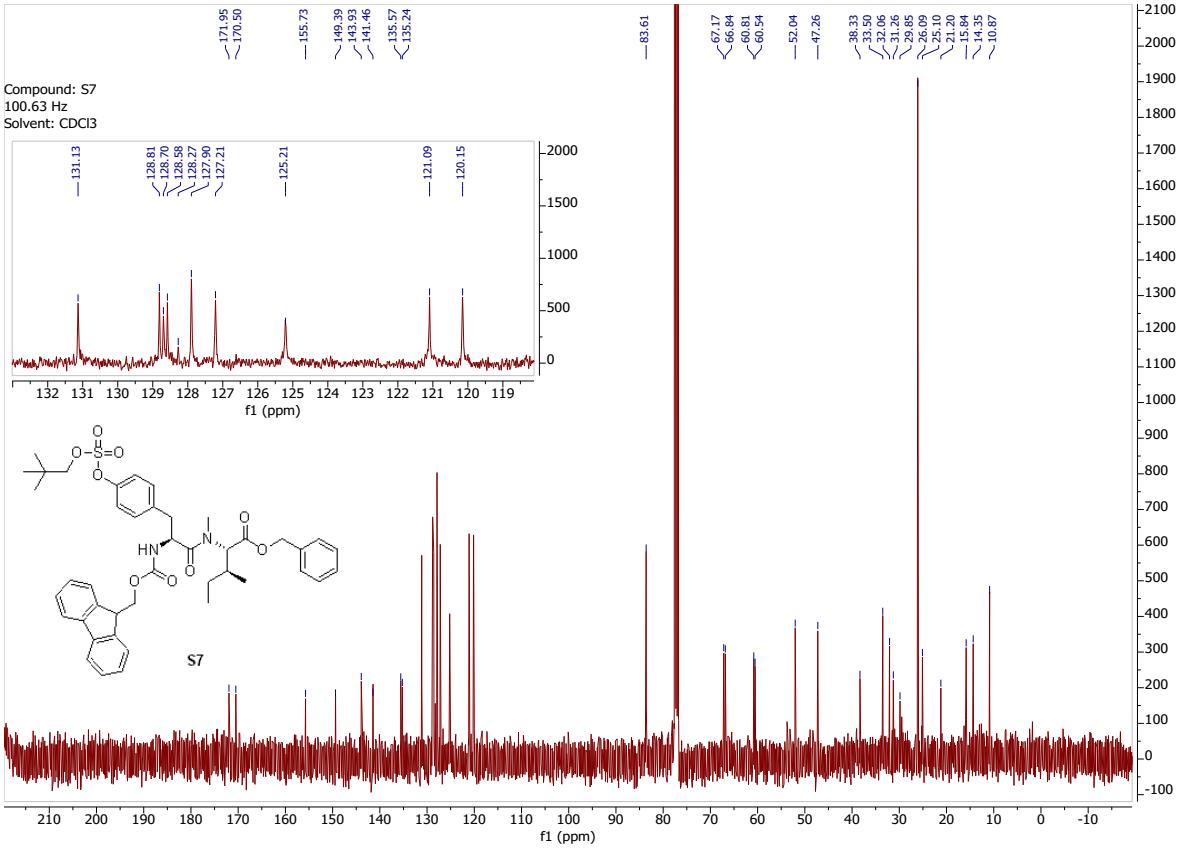


Figure S40. ^{13}C spectrum of Fmoc-Tyr(SO_2ONp)-*N*me-Ile-OBn (**S7**). Due to the presence of cis-trans rotamers of the N-methylated peptide bond, several peaks are split into subsets.

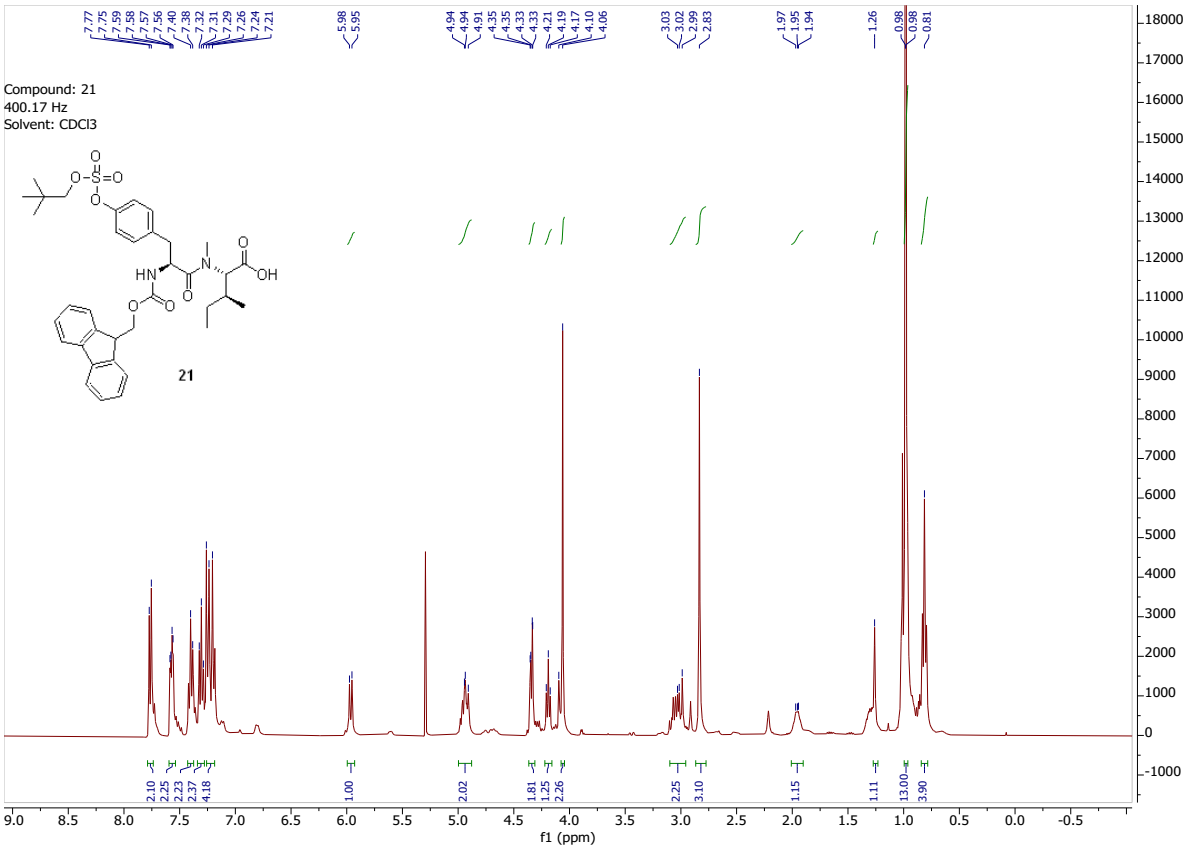


Figure S41. ^{1}H spectrum of Fmoc-Tyr(SO_2ONp)-*N*me-Ile-OH (**21**). Due to the presence of cis-trans rotamers of the N-methylated peptide bond, several peaks are split into subsets.

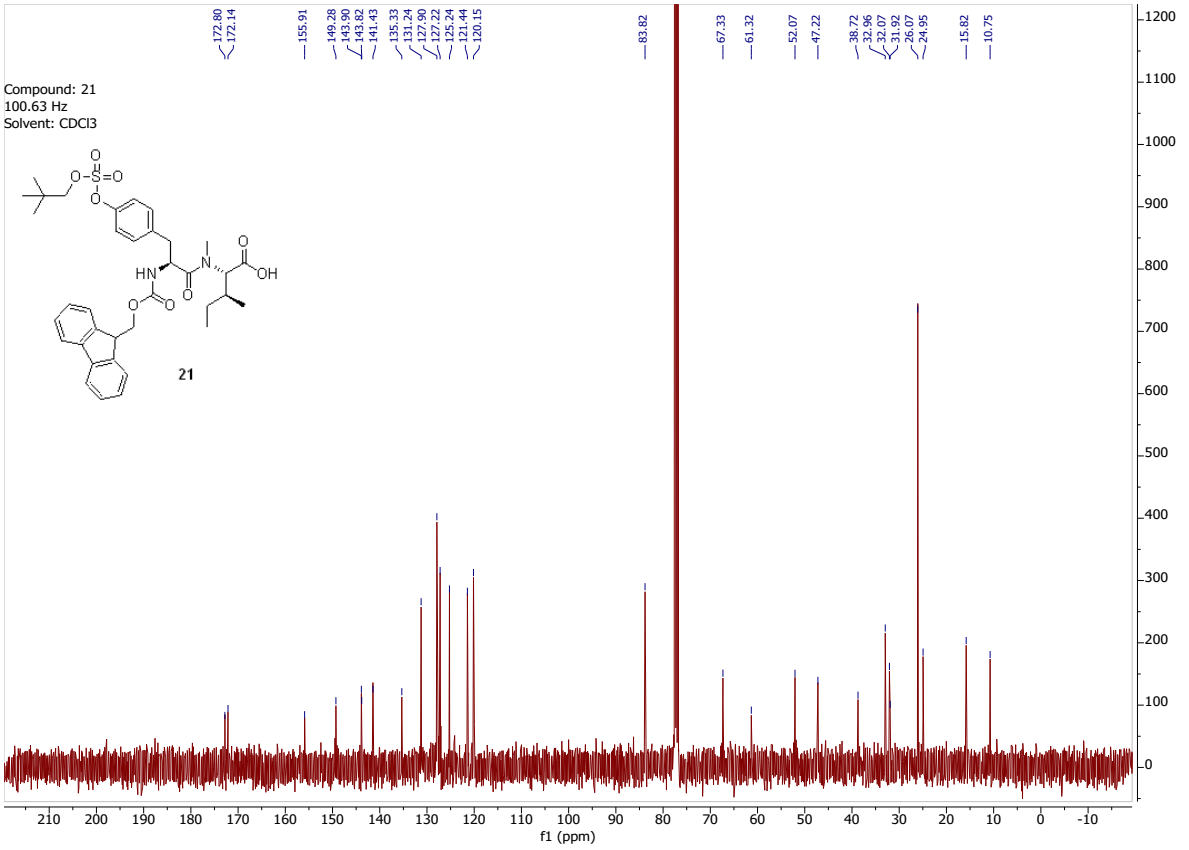


Figure S42. ^{13}C spectrum of Fmoc-Tyr(SO_2ONp)-*N*me-Ile-OH (**21**). Due to the presence of cis-trans rotamers of the N-methylated peptide bond, several peaks are split into subsets.

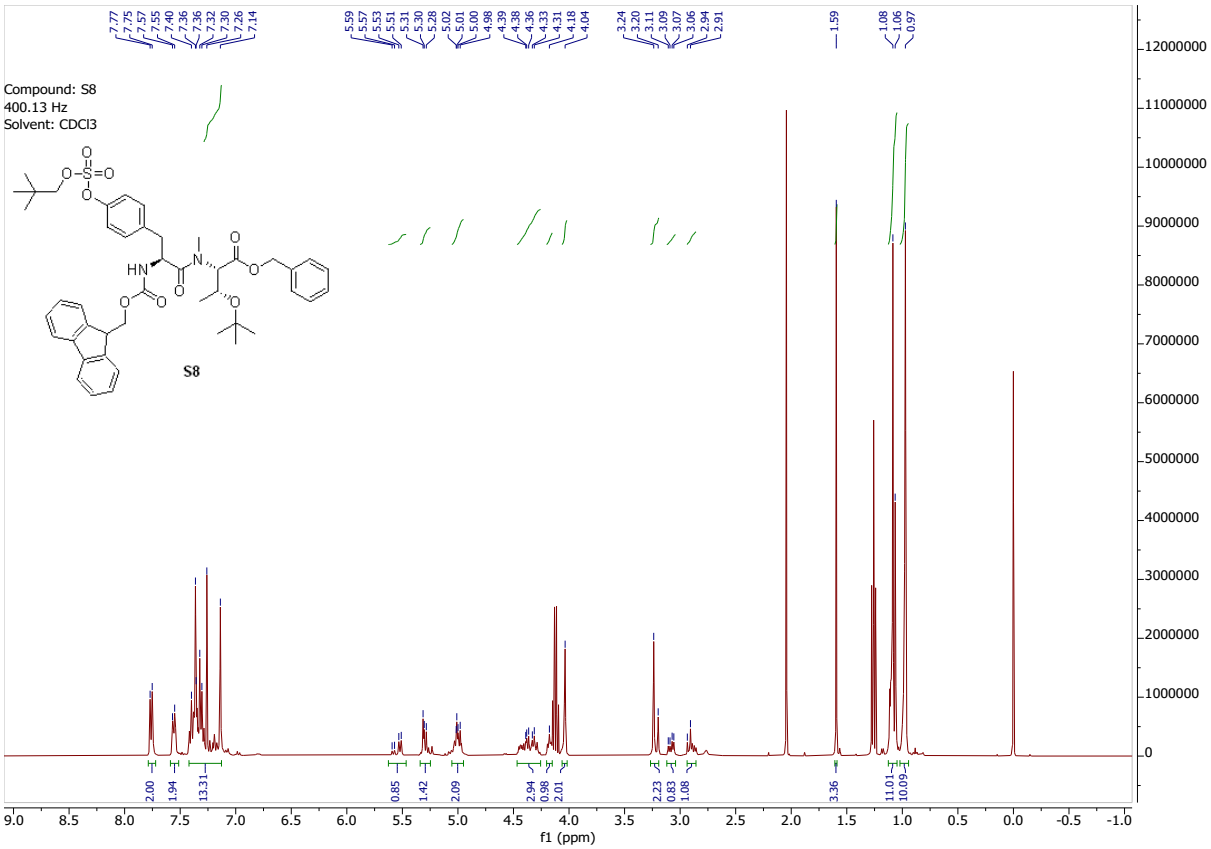


Figure S43. ^{1}H spectrum of Fmoc-Tyr(SO_2ONp)-*N*me-Thr(*t*Bu)-OBn (**S8**). Due to the presence of cis-trans rotamers of the N-methylated peptide bond, several peaks are split into subsets.

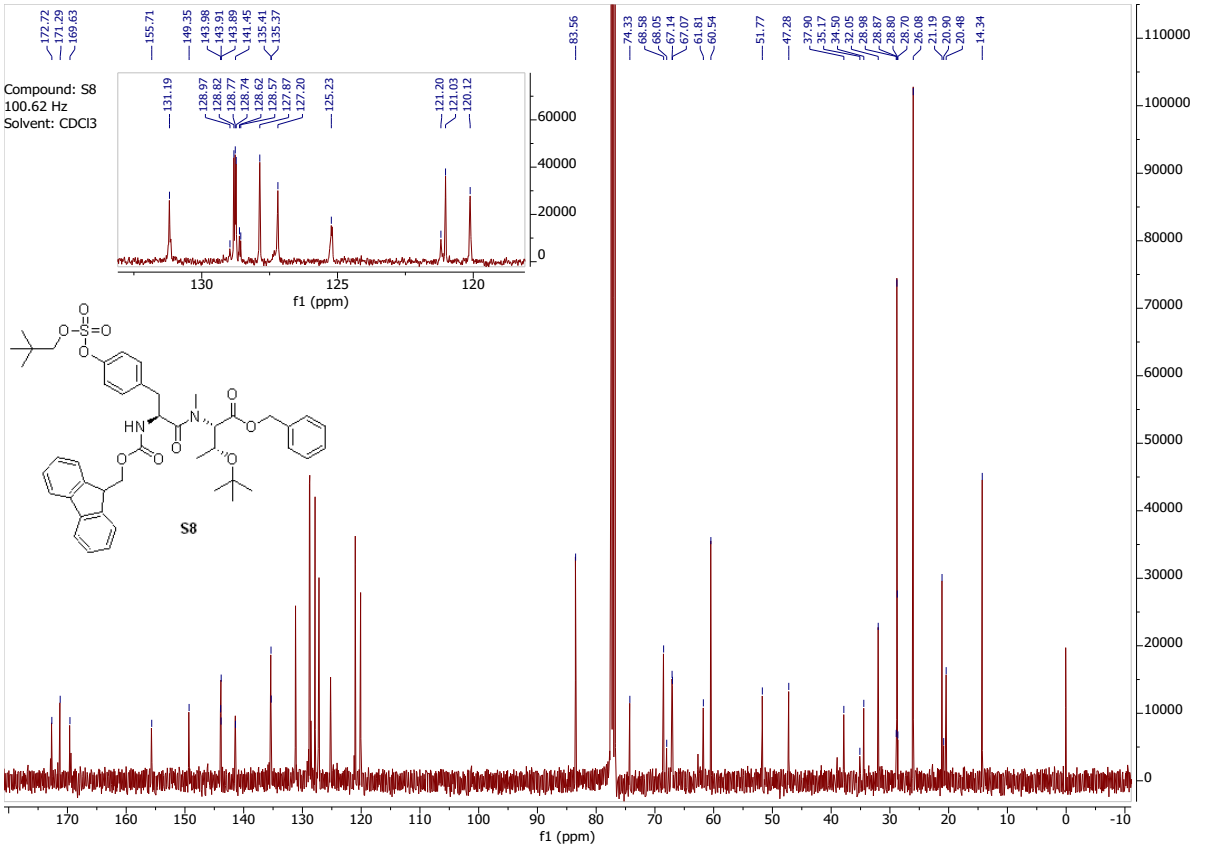


Figure S44. ^{13}C spectrum of Fmoc-Tyr(SO_2ONp)-*N*me-Thr(*t*Bu)-OBn (**S8**). Due to the presence of cis-trans rotamers of the N-methylated peptide bond, several peaks are split into subsets.

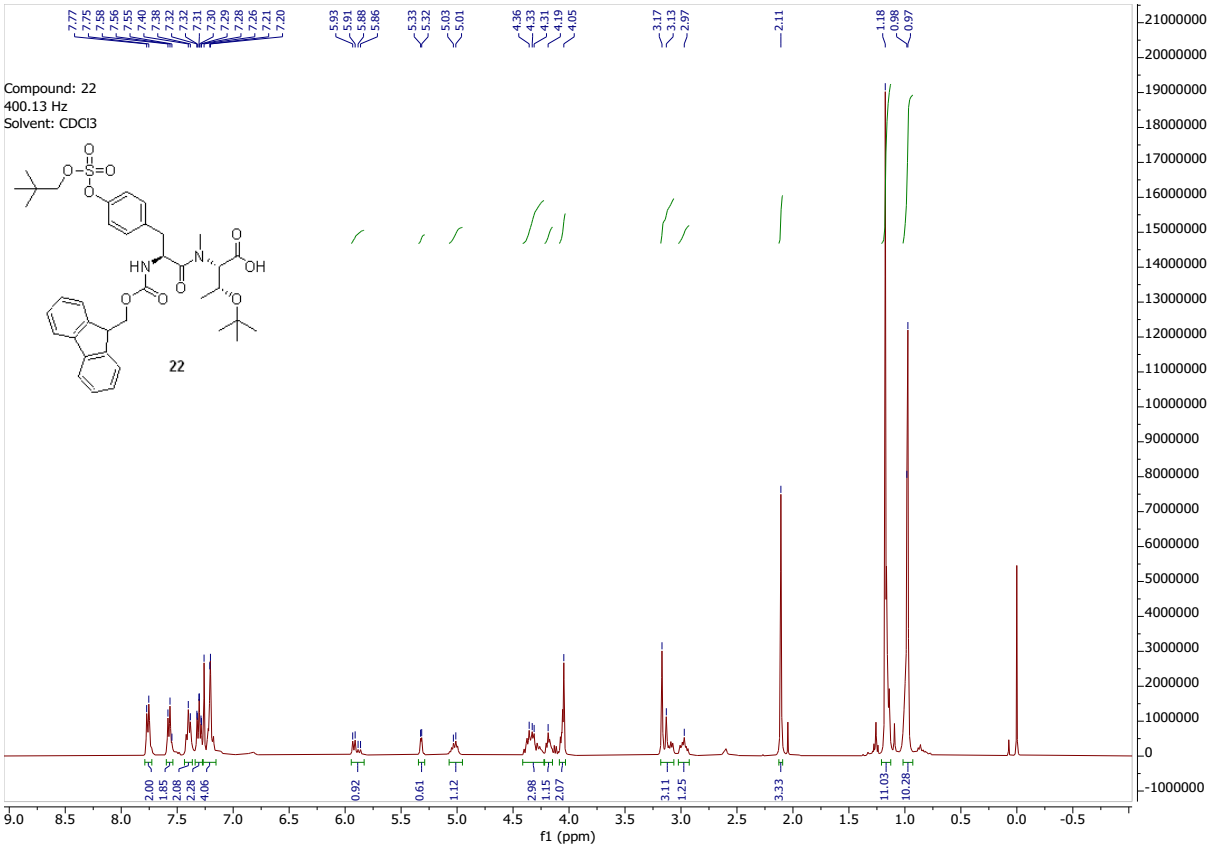


Figure S45. ^{1}H spectrum of Fmoc-Tyr(SO_2ONp)-*N*me-Thr(*t*Bu)-OH (**22**). Due to the presence of cis-trans rotamers of the N-methylated peptide bond, several peaks are split into subsets.

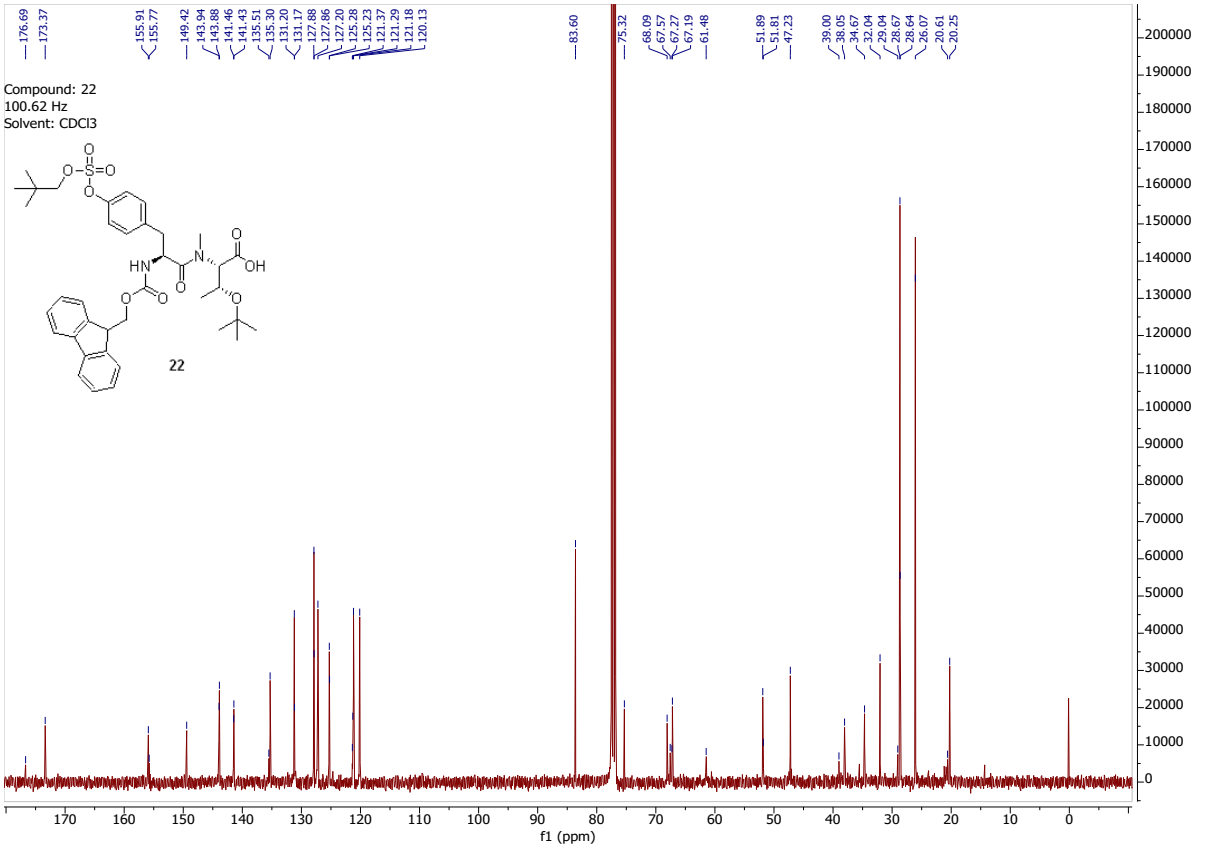


Figure S46. ^{13}C spectrum of Fmoc-Tyr(SO_2ONp)-*N*me-Thr(*t*Bu)-OH (**22**). Due to the presence of cis-trans rotamers of the N-methylated peptide bond, several peaks are split into subsets.

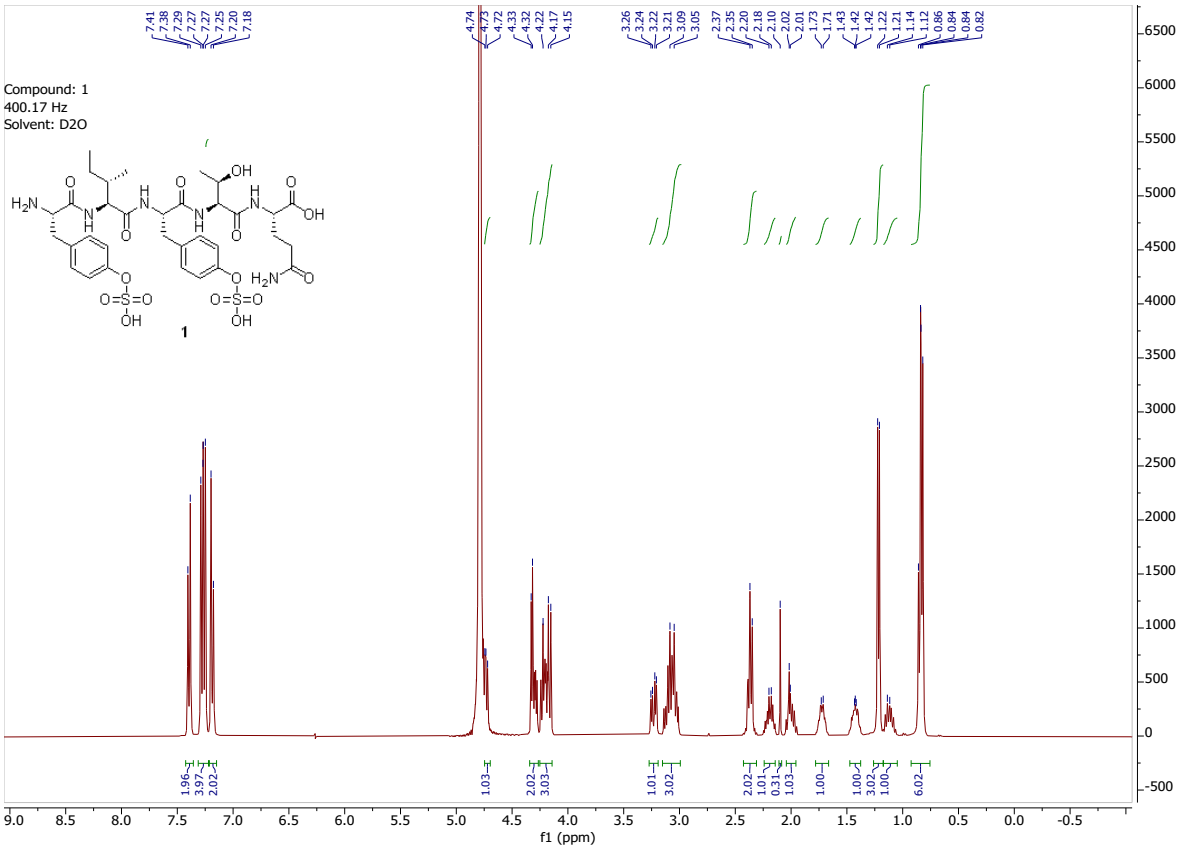


Figure S47. ^{1}H spectrum of H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH **(PSK, 1)**.

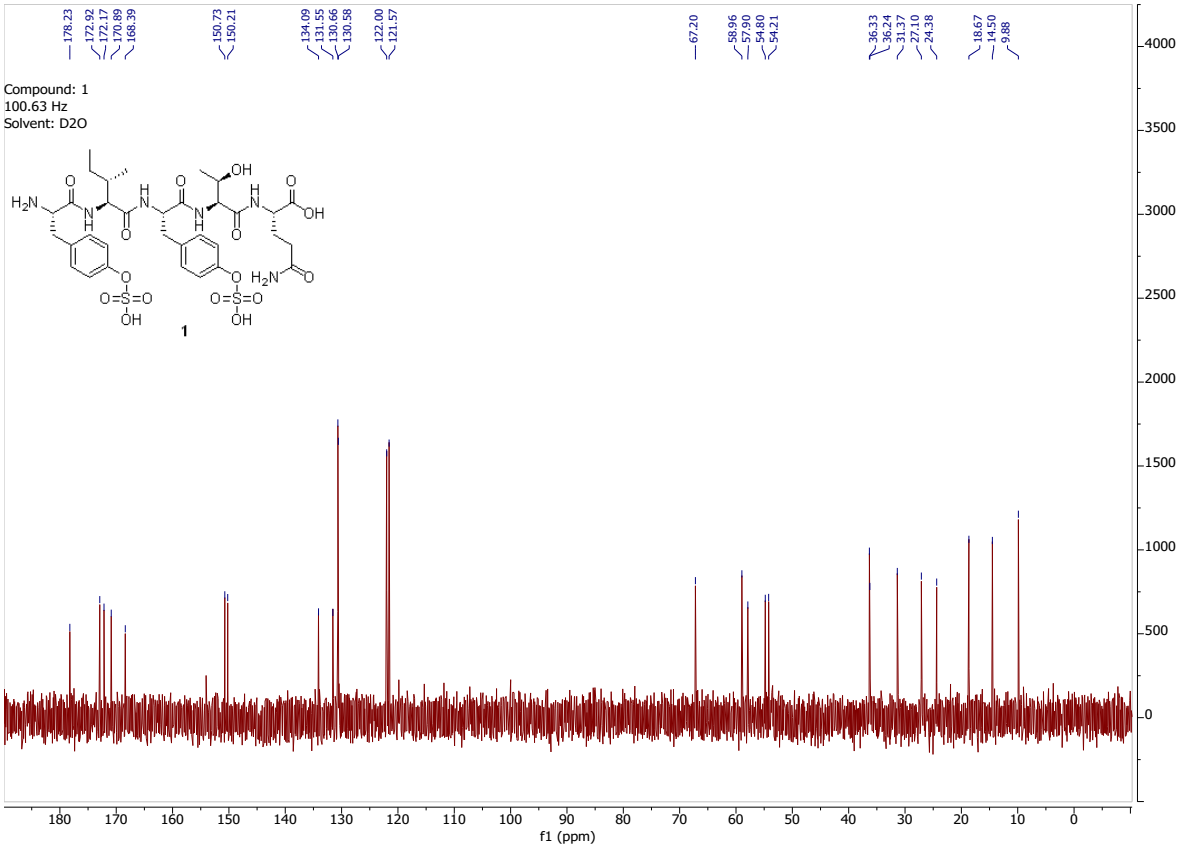


Figure S48. ^{13}C spectrum of H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH **(PSK, 1)**.

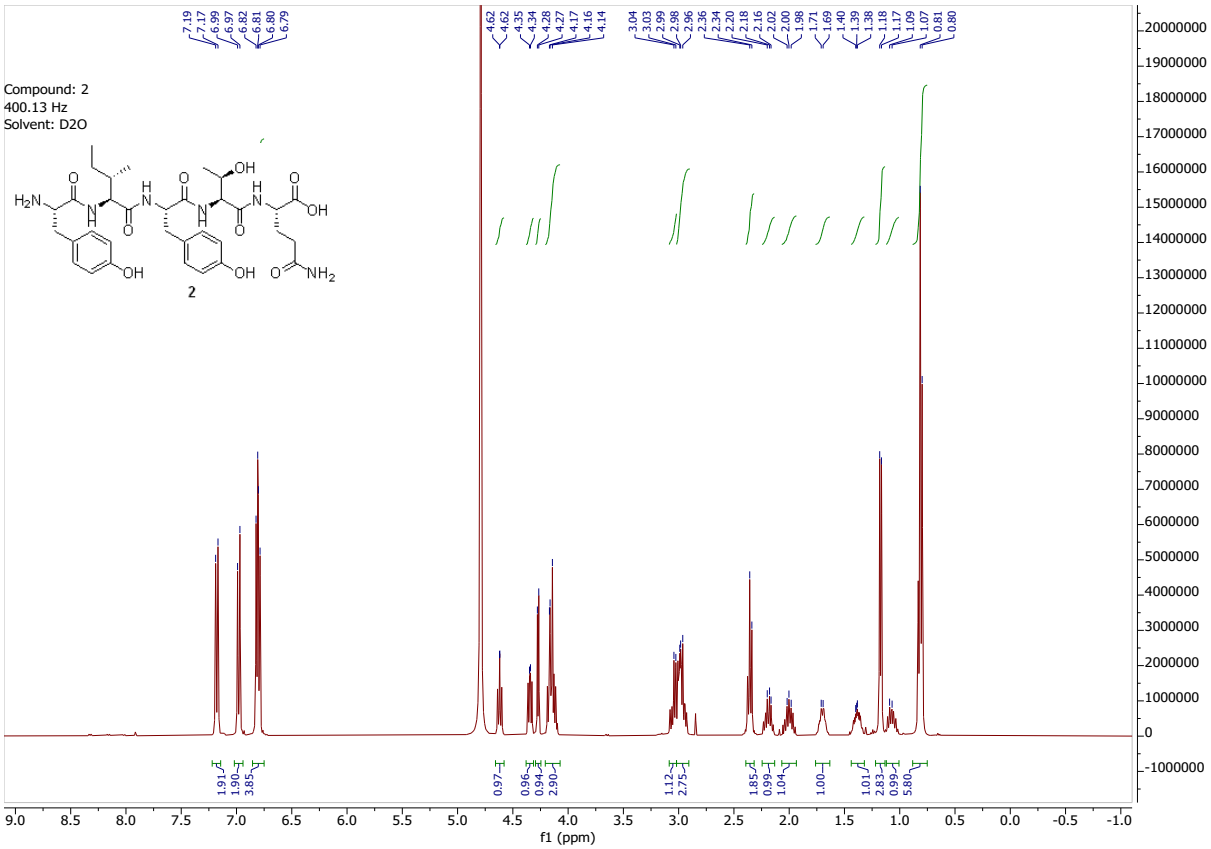


Figure S49. ^{1}H spectrum of H-Tyr-Ile-Tyr-Thr-Gln-OH **(nonsulfated PSK, 2)**.

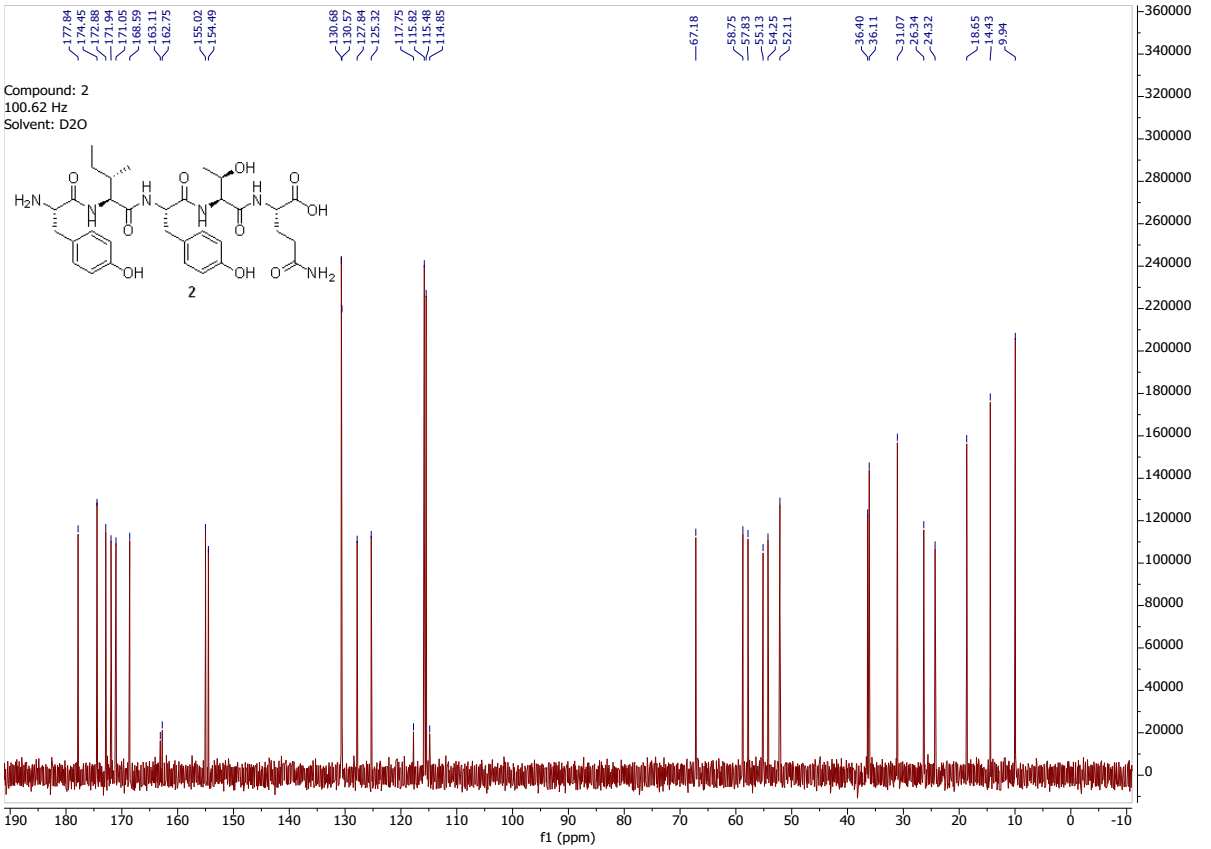


Figure S50. ^{13}C spectrum of H-Tyr-Ile-Tyr-Thr-Gln-OH **(nonsulfated PSK, 2)**.

References

1. E. D. Goddard-Borger and R. V. Stick, An Efficient, Inexpensive, and Shelf-Stable Diazotransfer Reagent: Imidazole-1-sulfonyl Azide Hydrochloride, *Organic Letters*, 2007, **9**, 3797-3800.