Acid-Labile Cys-Protecting Groups for the Fmoc/*t*Bu Strategy: Filling the Gap

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Abbreviations

2MeOBn – 2-methoxybenzyl; 2,4diMeOBn – 2,4-dimethoxybenzyl; 2,6diMeOBn – 2,6-dimethoxybenzyl; 2,6diMeO-4MeBn – 2,6-dimethoxy4-methylbenzyl; 2,6diMe-4MeOBn – 4-methoxy2,6-dimethylbenzyl; 4,4'diMeODpm – 4,4'-dimethoxydiphenylmethyl; 4,4'diMeDpm – 4,4'-dimethyldiphenylmethyl; 9F – 9*H*-fluorenyl; Alloc – allyloxycarbonyl; Boc – *tert*-butoxycarbonyl; Bpm – biphenylmethyl; DIEA – *N,N'*-diisopropylethylamine, DMF – *N,N'*-dimethylformamide, DMSO - dimethyl sulfoxide; DIPCDI – *N,N'*-diisopropylcarbodiimide; Dpm – diphenylmethyl; Fmoc – 9-fluorenylmethyl carbamate; Mob – 4-methoxybenzyl; Oxyma Pure – ethyl 2-cyano-2-(hydroxyimino)acetate; Pbf – 2,2,4,6,7- pentamethyldihydrobenzofurane-5-sulfonyl; PG – protecting group; *t*Bu – *tert*-butyl; TFA – trifluoroacetic acid; TMeb – 2,4,6-trimethylbenzyl.

General information

Benzhydrol, 4-biphenylmethanol, 4,4'-dimethylbenzophenone, 4,4'-dimethoxybenzhydrol, 2,4,6-trimethylbenzaldehyde and 2,6-dimethoxy-4-methylbenzaldehyde were purchased from Alfa Aesar (Karlsruhe, Germany), 9-hydroxyfluorene, 2,4-dimethoxybenzyl alcohol and 2-methoxybenzyl alcohol were procured from Sigma-Aldrich (St Louis, MO), 4-methoxy-2-methylbenzaldehyde from Acros (Geel, Belgium), 2,6-dimetoxybenzaldehy from TCI (Zwijndrecht, Belgium), and 2,6-dimethyl-4-hydroxybenzaldehyde from Fluorochem (Hadfield, UK). Commercial available Fmoc-amino acid derivatives and Fmoc-Rink amide polystyrene resin were acquired from IRIS Biotech (Marktredwitz, Germany), Fmoc-Sieber amide resin was obtained from Merck (Darmstadt, Germany), DIEA, DIPCDI and TFA were procured from Aldrich (Milwaukee, WI), Oxyma Pure from Luxembourg Industries Ltd. (Tel Aviv, Israel), and $NaBH_4$, $(CH_3)_2SO_4$, NH_4HCO_3, KH_2PO_4 and $K_2HPO_4{\cdot}3H_2O$ from Sigma-Aldrich (St Louis, MO). DMF, CH_2Cl_2, Et_2O, DMSO, piperidine and ACN (HPLC grade) were purchased from SDS (Peypin, France), anisole from Fluka Chemika (Buchs, Switzerland), and NH_3 aqueous solution (32%, w/w) from Scharlau (Barcelona, Spain). All commercial reagents and solvents were used as received.

Solid-phase peptide synthesis (SPPS)

Solid-phase syntheses were carried out manually in polypropylene syringes fitted with a polyethylene porous disc. Solvents and soluble reagents were removed by suction. The Fmoc group was removed with piperidine-DMF (1:4, v/v) (1 × 1 min, 2 × 5 min). Washings between deprotection, coupling, and final deprotection steps were carried out with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Peptide synthesis transformations and washes were performed at 25 °C.

Reverse phase-high performance liquid chromatography (RP-HPLC) analysis

XBridge™ BEH130 C18 reversed-phase HPLC analytical column (4.6 mm x 100 mm, 3.5 μm) was obtained from Waters (Ireland). Analytical RP-HPLC was performed on a Waters instrument comprising a separation module (Waters 2695), an automatic injector (Waters 717 autosampler), a photodiode array detector (Waters 2998), and a software system controller (Empower). UV detection was at 220 nm, and linear gradients of ACN (+0.036% TFA) into H_2O (+0.045% TFA) were run at a flow rate of 1.0 mL·min^{-1} over 8 min.

Reverse phase-high performance liquid chromatography-electrospray mass spectrometry (RP-HPLC-ESMS) analysis

SunFire™ C18 reversed-phase HPLC analytical column (2.1 mm x 100 mm, 5 μm) was procured from Waters (Ireland). Analytical RP-HPLC-ESMS was performed on a Waters Micromass ZQ spectrometer comprising a separation module (Waters 2695), an automatic injector (Waters 717 autosampler), a photodiode array detector (Waters 2998), and a software system controller MassLynx v. 4.1). UV detection was at 220 nm, mass scans were acquired in positive ion mode, and linear gradients of ACN (+0.07% formic acid) into H_2O (+0.1% formic acid) were run at a flow rate of 0.3 mL·min^{-1} over 8 min.

Semi-preparative RP-HPLC

SunFire™ Prep C18 OBD™ reversed-phase HPLC analytical column (19 x 100 mm, 5 μm) was acquired from Waters (Ireland). Semi-preparative RP-HPLC was performed on a Waters Delta 600 system comprising a sample manager (Waters 2700), a controller (Waters 600), a dual λ absorbance detector (Waters 2487), a fraction collector II, and a software system controller (MassLynx). UV detection was at 220 and 254 nm, and linear gradients of ACN (+0.1% TFA) into H_2O (+0.1% TFA) were run at a flow rate of 16 mL·min^{-1} over 30 min.

CombiFlash Rf 200 system

Normal mode: crude residue and silica media (1.5 mL media/200 mg crude reside) were dissolved in CH_2Cl_2, concentrated, and the resultant solid samples were eluded on a 40 g Redi*Sep* Rf GOLD silica column (Teledyne Isco) on a CombiFlash Rf 200 system (Teledyne Isco, Lincoln). Prior to each run, the column was equilibrated with 5 column volume of hexane. UV detection was at 254 nm, and gradients of ethyl acetate into hexane were run at a flow rate of 40 mL·min^{-1}.

Reverse mode: crude residues and C18 media (1.5 mL media/200 mg crude residue) were dissolved in CH_2Cl_2, concentrated, and the resultant solid samples were eluded on a 13 g Redi*Sep* Rf GOLD C18 column (Teledyne Isco, Lincoln) on a Combi*Flash* Rf 200 system (Teledyne Isco, Lincoln). Prior to each run, the column was equilibrated with 3 column volume of H_2O (+0.045% TFA). UV

detection was at 220 and 254 nm, and gradients of ACN (+0.036% TFA) into H_2O (+0.045% TFA) were run at a flow rate of 30 $mL \cdot min^{-1}$.

High-resolution mass spectrometry (HRMS) analysis

HRMS analyses were performed on a LTQ-FT Ultra Mass Spectrometer (Thermo Scientific) equipped with an automated nanoelectrospray NanoMate (Advion BioSciences, Ithaca, NY, USA). Spray voltage was 1.75 kV and delivery pressure was 0.50 psi. MS conditions: NanoESI, positive ionization, capillary temperature 200 °C, tube lens 100 V and *m/z* 450-1200 a.m.u. Samples were acquired with Xcalibur software (vs. 2.0SR2), elemental compositions from experimental exact mass monoisotopic values were obtained with Xaclibur software (vs. 2.1.0 SP1), and ion deconvolution to zero charged monoisotopic masses was performed with Xract algorithm in Xcalibur software (vs. 2.1.0 SP1).

Fourier transform Infrared (FTIR) spectrometry

FTIR spectra were acquired on a Thermo Nicolet NEXUS™ 670 Series FTIR spectrometer (Thermo Scientific, Waltham, MA) equipped with OMNIC 6.0 software. Samples were dissolved in CH_2Cl_2 and deposited on a KBr window, the solutions were then evaporated to dryness and the thin films formed on the window were analyzed directly.

Nuclear magnetic resonance (NMR) spectrometry

NMR spectra were acquired on a Varian Mercury 400 MHz spectrometer, operating at 400 and 101 MHz for ^1H and ^{13}C, respectively. Chemical shifts (δ) are reported in ppm relative to residual signals ($CDCl_3$: 7.26 ppm for ^1H NMR and 77.16 ppm for ^{13}C NMR; DMSO-d_6 : 2.50 ppm for ^1H NMR and 39.52 ppm for ^{13}C NMR). Multiplicities are indicated using the following abbreviations: s = singlet, d = doublet, t = triplet, p = pentet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, qd = quartet of doublets, ddd = doublet of doublet of doublets, tdd = triplet of doublet of doublets, and m = multiplet.

Experimental section

Fmoc-Cys(PG)-OH derivatives (1a-l)

All Fmoc-Cys(PG)-OH compounds were prepared from the Fmoc-Cys-OH **1** and the corresponding alcohol, except for the 4-biphenylmethyl and 2-methoxybenzyl derivatives that were synthesized from the corresponding bromide compound. In any case, the reaction conditions of the protection of the β-thiol function of any Fmoc-Cys(PG)-OH derivative were optimized.

General procedure for the reduction of aldehydes and ketones

To a suspension of aldehyde (6 mmol) in 2-propanol (30 mL) was added $NaBH_4$ (1.6 mmol) and the mixture was stirred at 25 °C for 16 h. After addition of H_2O (2 mL), the mixture was evaporated under reduced pressure and the crude residue was extracted with Et_2O (2 × 20 mL). The organic layer was then washed with H_2O (3 × 10 mL) and brine (3 × 10 mL), dried over $MgSO_4$, filtered and finally the Et_2O was removed under reduced pressure to obtain the desired alcohols.

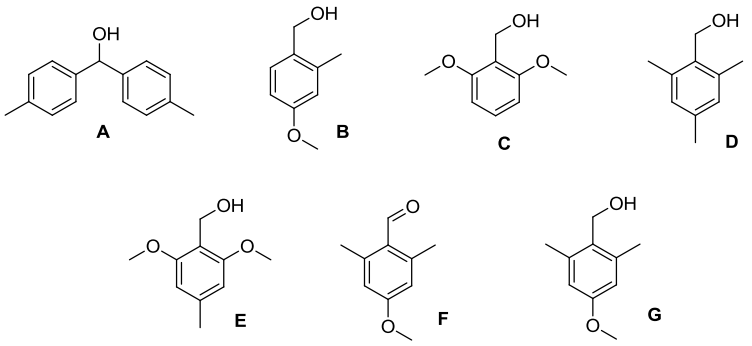
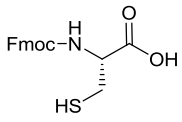


Figure S1. Intermediates involved in the synthesis of the Fmoc-Cys(PG)-OH derivatives.

Preparation of Fmoc-Cys-OH (1)

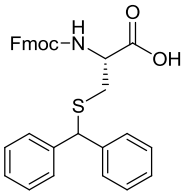


To a suspension of Fmoc-Cys(Trt)-OH (5.0 g, 8.5 mmol) in CH_2Cl_2 (340 mL) was added TIS (10 mL) and TFA (40 mL). The reaction mixture was stirred at 25 °C for 30 min, and the solvent was removed under reduced pressure. The crude was then washed with hexane (120 mL) and centrifuged (× 5) to afford Fmoc-Cys-OH **1** (2.9 g, 98% yield) as a white powder.

^1H NMR (400 MHz, DMSO-d_6): δ 12.79 (s, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.73 (d, *J* = 7.3 Hz, 2H), 7.68 (d, *J* = 8.3 Hz, 2H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.32 (td, *J* = 7.4, 1.0 Hz, 2H), 4.31 (d, *J* = 6.5 Hz, 2H), 4.23 (t, *J* = 6.9 Hz, 1H), 4.12 (td, *J* = 8.4, 4.4 Hz, 1H), 2.89 (ddd, *J* = 13.1,

8.4, 4.4 Hz, 1H), 2.73 (dt, *J* = 13.6, 8.5 Hz, 1H), 2.49 (t, *J* = 8.4 Hz, 1H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 171.9, 156.1, 143.8, 140.7, 127.6, 127.1, 125.3, 120.1, 65.7, 56.6, 46.6, 25.4. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3322, 3065, 1717, 1520, 1449, 1335, 1215, 1073, 758, 739. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 3.1 min). RP-HPLC-ESMS showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.5 min; *m/z* calcd for $C_{18}H_{17}NO_4S$, 343.4; found, 344.5 $[M+H]^+$, where M is the MW of Fmoc-Cys-OH **1**).

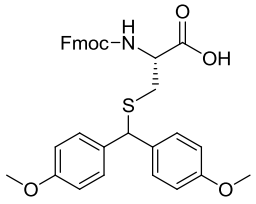
Preparation of Fmoc-Cys(Dpm)-OH (1a)



Fmoc-Cys-OH **1** (2.9 g, 8.4 mmol) and benzhydrol (1.6 g, 8.4 mmol) were placed in a round-bottom flask equipped with a stir bar and then neat TFA (25 mL) was added, and the mixture was stirred for 1 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was washed with Et_2O (3 × 25 mL). The crude residue was purified on a 40 g Redi*Sep* Rf GOLD silica column on a Combi*Flash* Rf 200 system following by concentration under reduced pressure to afford Fmoc-Cys(Dpm)-OH **1a** (3.36 g, 78% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, *J* = 7.4 Hz, 2H), 7.59 (t, *J* = 6.6 Hz, 2H), 7.37 (d, *J* = 7.0 Hz, 6H), 7.28 (dt, *J* = 7.9, 6.7 Hz, 6H), 7.21 (dd, *J* = 10.5, 3.5 Hz, 2H), 5.54 (d, *J* = 7.9 Hz, 1H), 5.21 (s, 1H), 4.56 (dd, *J* = 12.1, 5.3 Hz, 1H), 4.40 (d, *J* = 6.8 Hz, 2H), 4.22 (t, *J* = 6.7 Hz, 1H), 2.87 (qd, *J* = 13.9, 5.5 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.0, 156.1, 143.8, 141.5, 140.6, 128.8, 128.4, 127.9, 127.6, 127.3, 125.2, 120.2, 67.5, 54.7, 53.4, 47.2, 34.2. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3062, 3011, 1718, 1515, 1449, 1418, 1336, 1243, 1104, 1077, 1052, 758, 739, 701, 621. **HRMS** calcd for $C_{31}H_{27}NNaO_4S$, 532.15530; found, 532.15646 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.9 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 8.3 min; *m/z* calcd for $C_{31}H_{27}NO_4S$, 509.6; found, 510.3 $[M+H]^+$, where M is the MW of the Fmoc-Cys(Dpm)-OH **1a**).

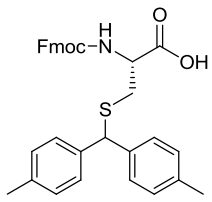
Preparation of Fmoc-Cys(4,4'diMeODpm)-OH (1b)



Fmoc-Cys-OH **1** (1 g, 2.9 mmol) and 4,4'-dimethoxybenzhydrol (700 mg, 2.9 mmol) were placed in a round-bottom flask equipped with a stir bar and then neat TFA (10 mL) was added, and the mixture was stirred for 1 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC (linear gradient from 80% to 90% ACN) following by lyophilization to afford Fmoc-Cys(4,4'diMeODpm)-OH **1b** (353 mg, 21% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, *J* = 7.3 Hz, 2H), 7.60 (t, *J* = 7.4 Hz, 2H), 7.38 (t, *J* = 7.2 Hz, 2H), 7.31 – 7.22 (m, 6H), 6.80 (dd, *J* = 8.3, 3.9 Hz, 4H), 5.56 (d, *J* = 7.9 Hz, 1H), 5.14 (s, 1H), 4.57 (dd, *J* = 12.6, 5.7 Hz, 1H), 4.48 – 4.28 (m, 2H), 4.23 (t, *J* = 6.9 Hz, 1H), 3.74 (s, 3H), 3.73 (s, 3H), 2.85 (qd, *J* = 14.1, 5.6 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.3, 158.9, 156.1, 143.9, 141.4, 132.9, 129.5, 129.5, 127.9, 127.3, 125.2, 120.1, 114.2, 67.6, 55.4, 53.5, 47.2, 34.1. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3065, 2954, 1718, 1607, 1508, 1450, 1301, 1247, 1175, 1033, 759, 740. **HRMS** calcd for $C_{33}H_{31}NNaO_6S$, 592.17643; found, 592.17722 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.6 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 8.1 min; *m/z* calcd for $C_{33}H_{31}NO_6S$, 569.67; found, 797.2 $[M+C_{15}H_{15}O_2]^+$, where M is the MW of the Fmoc-Cys(4,4'diMeODpm)-OH **1b**).

Preparation of Fmoc-Cys(4,4'diMeDpm)-OH (1c)



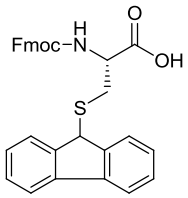
Fmoc-Cys-OH **1** (1.3 g, 3.7 mmol) and the previously prepared 4,4'-dimethybenzhydrol **A** (800 mg, 3.7 mmol) were placed in a round-bottom flask equipped with a stir bar and then neat TFA (10 mL) was added, and the mixture was stirred for 1 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure. The solid crude was then dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC (linear gradient from 85% to 95% ACN) following by lyophilization to afford Fmoc-Cys(4,4'diMeDpm)-OH **1c** (1.0 g, 52% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.59 (t, *J* = 6.5 Hz, 2H), 7.38 (t, *J* = 7.1 Hz, 2H), 7.33 – 7.20 (m, 6H), 7.07 (d, *J* = 7.6 Hz, 2H), 5.15 (s, 1H), 4.57 (dd, *J* = 12.2, 5.2 Hz, 1H), 4.40 (p, *J* = 10.3 Hz, 2H), 4.22 (t, *J* = 6.8 Hz, 1H), 2.95 – 2.80 (m, 2H), 2.27 (s, 3H), 2.27 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.5, 156.2, 143.9, 141.4, 137.8, 137.3, 129.5, 128.2, 127.9, 127.3, 125.2, 120.2, 67.6, 54.2, 53.4, 47.2, 34.1, 21.2. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3020, 2920, 1719, 1509, 1449, 1418, 1336, 1212, 1106, 1051, 758, 739.

HRMS calcd for $C_{33}H_{31}NNaO_4S$, 560.18660; found, 560.18815 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.1 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 9.8 min; *m/z* calcd for $C_{33}H_{31}NO_4S$, 537.67; found, 733.0 $[M+C_{15}H_{15}]^+$, where M is the MW of the Fmoc-Cys(4,4'diMeDpm)-OH **1c**).

The 4,4'-dimethylbenzhydrol **A** (*Figure S1*) was prepared in 87% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 7.4 min), and RP-HPLC-ESMS analysis showed the target alcohol (linear gradient from 5% to 100% ACN over 8 min; t_R : 9.5 min; *m/z* calcd for $C_{15}H_{16}O$, 212.29; found, 195.3 $[M\text{-}OH]^+$, where M is the MW of alcohol **A**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.24 (d, *J* = 8.0 Hz, 4H), 7.13 (d, *J* = 7.9 Hz, 4H), 5.76 (s, 1H), 2.32 (s, 6H), 2.16 (s, *J* = 3.8 Hz, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 141.3, 137.3, 129.3, 126.6, 76.1, 21.2.

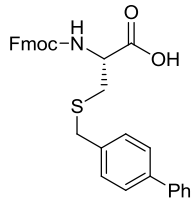
Preparation of Fmoc-Cys(9F)-OH (1d)



Fmoc-Cys-OH **1** (500 mg, 1.4 mmol) and 9-hydroxyfluorene (263 mg, 1.4 mmol) were placed in a round-bottom flask equipped with a stir bar and then neat TFA (5 mL) was added, and the mixture was stirred for 1 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC following by lyophilization to afford Fmoc-Cys(9F)-OH **1d** (256 mg, 36% yield) as white powder.

^{1}H NMR (400 MHz, DMSO-d_6): δ 7.69 (t, *J* = 8.5 Hz, 4H), 7.53 (d, *J* = 7.6 Hz, 2H), 7.50 (d, *J* = 8.5 Hz, 1H), 7.45 (t, *J* = 6.7 Hz, 2H), 7.22 (t, *J* = 7.1 Hz, 4H), 7.19 – 7.09 (m, 4H), 5.02 (s, 1H), 4.15 – 4.01 (m, 3H), 3.72 (dd, *J* = 15.5, 7.3 Hz, 1H), 2.36 – 2.32 (m, 2H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 174.2, 156.0, 143.7, 141.5, 140.7, 134.9, 129.2, 128.5, 128.0, 127.9, 127.3, 125.6, 124.5, 120.2, 67.4, 53.0, 48.7, 47.2, 29.7. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3063, 1716, 1507, 1449, 1419, 1335, 1213, 1050, 736. **HRMS** calcd for $C_{31}H_{25}NNaO_4S$, 530.13965; found, 530.14081 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.0 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 8.6 min; *m/z* calcd for $C_{31}H_{25}NO_4S$, 507.60; found, 508.2 $[M+H]^+$, where M is the MW of the Fmoc-Cys(9F)-OH **1d**).

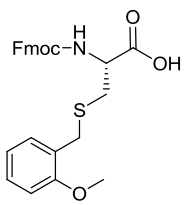
Preparation of Fmoc-Cys(Bpm)-OH (1e)



Fmoc-Cys-OH **1** (747 mg, 2.1 mmol) and 4-(bromomethyl)biphenyl (522 mg, 2.1 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (50 mL). Then, DIEA (2.5 mL, 5%) was added, and the mixture was stirred for 16 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC following by lyophilization to afford Fmoc-Cys(Bpm)-OH **1e** (418 mg, 39% yield) as white powder.

^{1}H NMR (400 MHz, DMSO-d_6): δ 12.80 (s, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.78 (d, *J* = 8.4 Hz, 1H), 7.74 (d, *J* = 7.4 Hz, 2H), 7.63 (d, *J* = 7.3 Hz, 2H), 7.59 (d, *J* = 8.2 Hz, 2H), 7.47 – 7.29 (m, 9H), 4.36 – 4.16 (m, 4H), 3.81 (s, 2H), 2.86 (dd, *J* = 13.6, 4.6 Hz, 1H), 2.72 (dd, *J* = 13.6, 9.4 Hz, 1H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 172.3, 156.0, 143.8, 140.7, 139.8, 138.7, 137.6, 129.5, 128.9, 127.6, 127.4, 127.1, 126.6, 126.5, 125.3, 120.1, 65.8, 53.7, 46.6, 34.9, 32.3. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3028, 1720, 1516, 1487, 1449.69, 1234.68, 1052.15, 759.66, 738.98, 698.31. **HRMS** calcd for $C_{31}H_{27}NNaO_4S$, 532.15530; found, 532.15729 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.2 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 8.6 min; *m/z* calcd for $C_{31}H_{27}NO_4S$, 509.62; found, 510.7 $[M+H]^+$, where M is the MW of the Fmoc-Cys(Bpm)-OH **1e**).

Preparation of Fmoc-Cys(2MeOBn)-OH (1f)

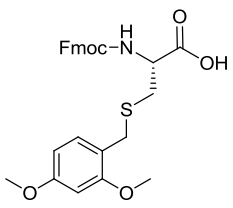


Fmoc-Cys-OH **1** (1.2 g, 3.5 mmol) and 1-(bromomethyl)-2-methoxybenzene (1.1 g, 5.4 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (100 mL). Then, DIEA (5 mL, 5%) was added, and the mixture was stirred for 16 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC (linear gradient from 65% to 80% ACN) following by lyophilization to afford Fmoc-Cys(2MeOBn)-OH **1f** (345 mg, 21% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (d, *J* = 7.5 Hz, 2H), 7.62 – 7.54 (m, 2H), 7.37 (t, *J* = 7.3 Hz, 2H), 7.28 (t, *J* = 7.4 Hz, 2H), 7.21 (t, *J* = 7.1 Hz, 2H), 6.89 (t, *J* = 7.3 Hz, 1H), 6.83 (d, *J* = 8.4 Hz, 1H), 5.73 (d, *J* = 7.6 Hz, 1H), 4.62 (d, *J* = 5.5 Hz, 1H), 4.44 (d, *J* = 6.9 Hz, 2H),

4.22 (t, *J* = 6.8 Hz, 1H), 3.77 (s, *J* = 7.1 Hz, 3H), 3.75 (s, 2H), 2.94 (qd, *J* = 14.3, 5.3 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.4, 157.3, 156.3, 143.8, 141.5, 130.6, 128.9, 127.9, 127.2, 126.1, 125.2, 120.9, 120.1, 110.9, 67.3, 55.6, 53.9, 47.3, 33.5, 31.2. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3322, 3064, 2938, 2938, 1719, 1513, 1493, 1450, 1290, 1245, 1103, 1049, 740. **HRMS** calcd for $C_{26}H_{25}KNO_5S$, 502.10850; found, 502.10898 $[M+K]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.9 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.9 min; *m/z* calcd for $C_{26}H_{25}NO_5S$, 463.55; found, 464.6 $[M+H]^+$, where M is the MW of the Fmoc-Cys(2MeOBn)-OH **1f**).

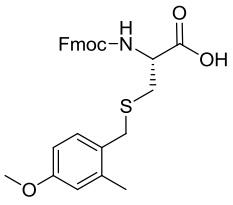
Preparation of Fmoc-Cys(2,4diMeOBn)-OH (1g)



Fmoc-Cys-OH **1** (1.1 g, 3.2 mmol) and 2,4-dimethoxybenzyl alcohol (1.2 g, 7.1 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (25 mL). TFA (50 μL, 0.2%) was then added, and the mixture was stirred for 2 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was dissolved in H_2O-ACN (1:1) and purified by semi-preparative RP-HPLC following by lyophilization to afford Fmoc-Cys(2,4diMeOBn)-OH **1g** (300 mg, 19% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (d, *J* = 7.5 Hz, 2H), 7.63 – 7.57 (m, 2H), 7.40 – 7.35 (m, 2H), 7.28 (t, *J* = 7.4 Hz, 2H), 7.11 (d, *J* = 8.8 Hz, 1H), 6.42 (d, *J* = 2.1 Hz, 2H), 6.41 (d, *J* = 2.3 Hz, 1H), 5.74 (d, *J* = 7.9 Hz, 1H), 4.63 (dd, *J* = 12.6, 5.8 Hz, 1H), 4.43 (d, *J* = 7.0 Hz, 2H), 4.23 (t, *J* = 6.9 Hz, 1H), 3.76 (s, 3H), 3.76 (s, 3H), 3.71 (d, *J* = 1.7 Hz, 2H), 2.93 (qd, *J* = 14.4, 5.7 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.4, 160.5, 158.3, 156.3, 143.8, 141.4, 131.1, 127.9, 127.2, 125.2, 120.1, 118.4, 104.6, 98.9, 67.4, 55.6, 55.5, 53.8, 47.3, 33.4, 30.9. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3065, 2937, 1718, 1611, 1506, 1450, 1329, 1208, 1156, 1040, 740. **HRMS** calcd for $C_{27}H_{27}NNaO_6S$, 516.14513; found, 516.14643 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.8 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.3 min; *m/z* calcd for $C_{27}H_{27}NO_6S$, 493.57; found, 494.7 $[M+H]^+$, where M is the MW of the Fmoc-Cys(2,4diMeOBn)-OH **1g**).

Preparation of Fmoc-Cys(4MeO-2MeBn)-OH (1h)

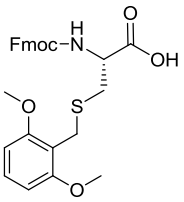


Fmoc-Cys-OH **1** (160 mg, 0.6 mmol) and the previously prepared 4-methoxy2-methylbenzyl alcohol **B** (70 mg, 0.46 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (5 mL). Then TFA (50 μL, 1%) was added, and the mixture was then stirred for 2 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under vacuum and the solid crude was washed with Et_2O (3 × 25 mL). The crude residue was purified on a 13 g Redi*Sep* Rf GOLD C18 column on a Combi*Flash* Rf 200 system following by lyophilization to afford Fmoc-Cys(4MeO-2MeBn)-OH **1h** (135 mg, 61% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (dd, *J* = 7.4, 3.0 Hz, 2H), 7.63 – 7.54 (m, 2H), 7.41 – 7.34 (m, 2H), 7.32 – 7.26 (m, 2H), 7.07 (d, *J* = 8.3 Hz, 1H), 6.70 (d, *J* = 2.1 Hz, 1H), 6.64 (dd, *J* = 8.2, 1.8 Hz, 1H), 5.58 (d, *J* = 7.8 Hz, 1H), 4.61 (dd, *J* = 12.2, 5.6 Hz, 1H), 4.42 (d, *J* = 7.0 Hz, 2H), 4.22 (t, *J* = 6.9 Hz, 1H), 3.74 (s, *J* = 21.7 Hz, 3H), 3.68 (s, 2H), 2.96 (qd, *J* = 14.1, 5.3 Hz, 2H), 2.32 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.2, 159.1, 156.2, 143.7, 141.4, 138.4, 131.1, 127.9, 127.3, 127.3, 125.2, 120.2, 116.7, 111.0, 67.5, 55.3, 53.6, 47.2, 34.7, 33.7, 19.5. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3320, 3065, 2950, 1718, 1608, 1501, 1449, 1256, 1202, 1049, 759, 739. **HRMS** calcd for $C_{27}H_{27}NNaO_5S$, 500.15021; found, 500.15121 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.1 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.7 min; *m/z* calcd for $C_{27}H_{27}NO_5S$, 477.57; found, 478.6 $[M+H]^+$, where M is the MW of the Fmoc-Cys(4MeO-2MeBn)-OH **1h**).

The 4-methoxy2-methylbenzyl alcohol **B** (*Figure S1*) was prepared in 82% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 4.9 min). RP-HPLC-ESMS showed the target alcohol (linear gradient from 5% to 100% ACN over 8 min; t_R : 6.6 min; *m/z* calcd for $C_9H_{12}O_2$, 152.19; found, 135.2 $[M-OH]^+$, where M is the MW of alcohol **B**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.22 (d, *J* = 8.2 Hz, 1H), 6.74 (d, *J* = 2.6 Hz, 1H), 6.71 (dd, *J* = 8.2, 2.6 Hz, 1H), 4.62 (s, 2H), 3.79 (s, *J* = 3.0 Hz, 3H), 2.36 (s, *J* = 18.9 Hz, 3H), 1.55 (s, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 159.4, 138.2, 131.3, 129.7, 116.4, 110.9, 63.4, 55.4, 19.1.

Preparation of Fmoc-Cys(2,6diMeOBn)-OH (1i)

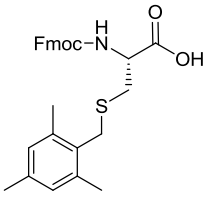


Fmoc-Cys-OH **1** (300 mg, 0.86 mmol) and the previously prepared 2,6-dimethoxybenzyl alcohol **C** (178 mg, 1.0 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (10 mL). TFA (200 μL, 2%) was then added, and the mixture was stirred for 6 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under vacuum and the solid crude was washed with Et_2O (3 × 25 mL). The crude residue was purified on a 13 g Redi*Sep* Rf Gold C18 column on a Combi*Flash* Rf 200 system following by lyophilization to afford Fmoc-Cys(2,6diMeOBn)-OH **1i** (141 mg, 33% yield) as white powder.

^{1}H NMR (400 MHz, DMSO-d_6): δ 7.88 (d, *J* = 7.5 Hz, 2H), 7.72 (d, *J* = 7.5 Hz, 2H), 7.63 (d, *J* = 8.5 Hz, 1H), 7.40 (t, *J* = 7.3 Hz, 2H), 7.30 (tdd, *J* = 7.4, 2.7, 1.0 Hz, 2H), 7.18 (t, *J* = 8.3 Hz, 1H), 6.61 (d, *J* = 8.4 Hz, 2H), 4.35 – 4.25 (m, 2H), 4.26 – 4.20 (m, 2H), 3.73 (d, *J* = 12.7 Hz, 1H), 3.73 (s, 6H), 3.60 (d, *J* = 12.7 Hz, 1H), 2.85 (dd, *J* = 13.8, 4.8 Hz, 1H), 2.64 (dd, *J* = 13.8, 9.6 Hz, 1H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 172.6, 157.6, 156.0, 143.8, 140.7, 128.3, 127.6, 127.1, 125.3, 120.1, 114.5, 103.9, 65.7, 55.7, 53.8, 46.6, 39.5, 32.8, 23.0. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3322, 2938, 2836, 1715, 1595, 1521, 1475, 1257, 1100, 739. **HRMS** calcd for $C_{27}H_{27}NNaO_6S$, 516.14513; found, 516.14612 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.1 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.1 min; *m/z* calcd for $C_{27}H_{27}NO_6S$, 493.57; found, 494.8 $[M+H]^+$, where M is the MW of the Fmoc-Cys(2,6diMeOBn)-OH **1i**).

The 2,6-dimethoxybenzyl alcohol **C** (*Figure S1*) was prepared in 68% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 4.9 min). RP-HPLC-ESMS showed the target alcohol (linear gradient from 5% to 100% ACN over 8 min; t_R : 6.6 min; *m/z* calcd for $C_9H_{12}O_3$, 168.19; found, 151.2 $[M-OH]^+$, where M is the MW of the alcohol **C**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.22 (t, *J* = 8.4 Hz, 1H), 6.56 (d, *J* = 8.4 Hz, 2H), 4.79 (s, 2H), 3.84 (s, 6H), 2.47 (s, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 158.5, 129.3, 117.2, 103.9, 55.9, 54.8.

Preparation of Fmoc-Cys(TMeb)-OH (1j)

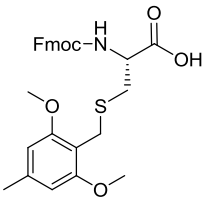


Fmoc-Cys-OH **1** (270 mg, 0.78 mmol) and the previously prepared 2,4,6-trimethylbenzyl alcohol **D** (118 mg, 0.78 mmol) were placed in a round-bottom flask equipped with a stir bar and then neat TFA (10 mL) was added, and the mixture was stirred for 2 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was washed with Et_2O (25 mL x 3). The crude residue was purified on a 13 g Redi*Sep* Rf GOLD C18 column on a Combi*Flash* Rf 200 system following by lyophilization to afford Fmoc-Cys(TMeb)-OH **1j** (191 mg, 51% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (t, *J* = 6.9 Hz, 2H), 7.62 – 7.54 (m, 2H), 7.37 (dd, *J* = 15.6, 7.9 Hz, 2H), 7.28 (tdd, *J* = 7.5, 2.6, 1.2 Hz, 2H), 6.79 (s, 2H), 5.63 (d, *J* = 7.4 Hz, 1H), 4.67 (d, *J* = 5.8 Hz, 1H), 4.43 (d, *J* = 6.5 Hz, 2H), 4.21 (t, *J* = 6.8 Hz, 1H), 3.76 (s, 2H), 3.08 (qd, *J* = 14.2, 4.7 Hz, 2H), 2.31 (s, 6H), 2.22 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.4, 156.1, 143.7, 141.5, 137.1, 137.0, 130.2, 129.2, 127.9, 127.3, 125.2, 120.1, 67.4, 53.8, 47.2, 35.0, 32.1, 21.1, 19.7. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3319, 2948, 1718, 1513, 1449, 1335, 1202, 1051, 853, 739. **HRMS** calcd for $C_{28}H_{29}NNaO_4S$, 498.17095; found, 498.17139 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.4 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 9.8 min; *m/z* calcd for $C_{28}H_{29}NO_4S$, 475.60; found, 476.6 $[M+H]^+$, where M is the MW of the Fmoc-Cys(TMeb)-OH **1j**).

The 2,4,6-trimethylbenzyl alcohol **D** (*Figure S1*) was prepared in 65% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 6.1 min). RP-HPLC-ESMS showed the target alcohol (linear gradient from 5% to 100% ACN over 8 min; t_R : 8.0 min; *m/z* calcd for $C_{10}H_{14}O$, 150.22; found, 133.2 $[M-OH]^+$, where M is the MW of the alcohol **D**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 6.86 (s, 2H), 4.68 (s, 2H), 2.38 (s, 6H), 2.26 (s, *J* = 10.6 Hz, 3H), 1.34 (s, 1H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 137.8, 137.4, 133.9, 129.3, 59.3, 21.1, 19.5.

Preparation of Fmoc-Cys(2,6diMeO-4MeBn)-OH (1k)

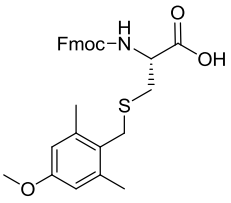


Fmoc-Cys-OH **1** (300 mg, 0.86 mmol) and the previously prepared 2,6-dimethoxy4-dimethylbenzyl alcohol **E** (188 mg, 0.86 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (10 mL). TFA (20 μL, 0.2%) was then added, and the mixture was stirred for 2 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was washed with Et_2O (25 mL × 3). The crude residue was purified on a 13 g Redi*Sep* Rf GOLD C18 column on a Combi*Flash* Rf 200 system following by lyophilization to afford Fmoc-Cys(2,6diMeO-4MeBn)-OH **1k** (175 mg, 40% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (d, *J* = 7.6 Hz, 2H), 7.65 – 7.55 (m, 2H), 7.37 (t, *J* = 7.5 Hz, 2H), 7.32 – 7.25 (m, 2H), 6.35 (s, 2H), 5.88 (d, *J* = 7.8 Hz, 1H), 4.64 (dd, *J* = 11.5, 7.0 Hz, 1H), 4.44 (d, *J* = 6.9 Hz, 2H), 4.23 (t, *J* = 6.9 Hz, 1H), 3.85 (d, *J* = 12.6 Hz, 1H), 3.77 (s, *J* = 25.7 Hz, 6H), 3.74 (s, *J* = 8.2 Hz, 1H), 2.99 (dd, *J* = 14.4, 4.1 Hz, 1H), 2.86 (dd, *J* = 14.1, 7.2 Hz, 1H), 2.31 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 175.6, 158.0, 156.5, 143.8, 141.4, 139.0, 127.8, 127.2, 125.2, 120.1, 111.6, 104.9, 67.3, 55.9, 54.3, 47.3, 33.2, 24.5, 22.3. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3345, 2938, 1721, 1609, 1588, 1450, 1413, 1243, 1186, 1107, 1049, 815, 739. **HRMS** calcd for $C_{28}H_{29}NNaO_6S$, 530.16078; found, 530.16193 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.5 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.8 min; *m/z* calcd for $C_{28}H_{29}NO_6S$, 507.60; found, 508.7 $[M+H]^+$, where M is the MW of the Fmoc-Cys(2,6diMeO-4MeBn)-OH **1k**).

The 2,6-dimethoxy4-methylbenzyl alcohol **E** (*Figure S1*) was prepared in 81.2% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 5.5 min), and RP-HPLC-ESMS analysis showed the target alcohol (linear gradient from 5% to 100% ACN over 8 min; t_R : 7.3 min; *m/z* calcd for $C_{10}H_{14}O_3$, 182.22; found, 165.2 $[M-OH]^+$, where M is the MW of the alcohol **E**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 6.38 (s, 2H), 4.74 (s, 2H), 3.82 (s, 6H), 2.34 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 158.3, 139.6, 114.3, 104.8, 55.8, 54.7, 22.3.

Preparation of Fmoc-Cys(4MeO-2,6diMeBn)-OH (1l)



Fmoc-Cys-OH **1** (400 mg, 1.2 mmol) and the previously prepared 4-methoxy2,6-dimethylbenzyl alcohol **G** (194 mg, 1.2 mmol) were placed in a round-bottom flask equipped with a stir bar and dissolved in CH_2Cl_2 (10 mL). TFA (30 μL, 0.3%) was then added, and the mixture was stirred for 6 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the solvent was removed under reduced pressure and the solid crude was washed with Et_2O (25 mL x 3). The crude residue was purified on a 13 g Redi*Sep* Rf GOLD C18 column on a Combi*Flash* Rf 200 system following by lyophilization to afford Fmoc-Cys(4MeO-2,6diMeBn)-OH **1l** (400.4 mg, 67% yield) as white powder.

^{1}H NMR (400 MHz, DMSO-d_6): δ 12.94 (s, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.77 (d, *J* = 8.5 Hz, 1H), 7.73 (d, *J* = 7.5 Hz, 2H), 7.41 (t, *J* = 7.5 Hz, 2H), 7.30 (qd, *J* = 7.6, 1.0 Hz, 2H), 6.57 (s, 2H), 4.35 – 4.28 (m, 2H), 4.28 – 4.21 (m, 2H), 3.77 (s, 2H), 3.68 (s, 3H), 3.00 (dd, *J* = 13.8, 4.6 Hz, 1H), 2.81 (dd, *J* = 13.8, 9.7 Hz, 1H), 2.30 (s, 6H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 172.4, 157.7, 156.0, 143.8, 140.7, 138.2, 127.6, 127.1, 125.8, 125.2, 120.1, 113.3, 65.7, 54.8, 54.1, 46.6, 39.5, 33.8, 30.5, 19.4. **FTIR** (KBr film): υ_{max} (cm^{-1}) 3321, 2948, 1718, 1603, 1486, 1321, 1197, 1143, 1062, 740. **HRMS** calcd for $C_{28}H_{29}NNaO_5S$, 514.16586; found, 514.16705 $[M+Na]^+$. Analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.4 min), and RP-HPLC-ESMS analysis showed the target compound (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.7 min; *m/z* calcd for $C_{28}H_{29}NO_5S$, 491.60; found, 492.7 $[M+H]^+$, where M is the MW of the Fmoc-Cys(4MeO-2,6diMeBn)-OH **1l**).

To a suspension of 2,6-dimethyl-4-hydroxybenzaldehyde (1 g, 6.7 mmol) and K_2CO_3 (1.1 g, 8.0 mmol) in ACN (15 mL) was added $(CH_3)_2SO_4$ (940 μL, 9.9 mmol) and the mixture was stirred for 16 h at 25 °C. At this time, RP-HPLC analysis showed complete consumption of the starting material and the product was extracted with ethyl acetate (100 mL). The organic layer was washed with H_2O (2 × 25 mL) and brine (25 mL), dried over $MgSO_4$, filtered and the ethyl acetate was then removed under reduced pressure to render the 4-methoxy-2,6-dimethylbenzylaldehyde **F** (*Figure S1*) (1.1 g, 100% yield). Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 6.7 min), and RP-HPLC-ESMS analysis showed the target aldehyde (linear gradient from 50% to 100% ACN over 8 min; t_R : 9.2 min; *m/z* calcd for $C_{10}H_{12}O_2$, 164.20; found, 165.2 $[M+H]^+$, where M is the MW of the 2,6-diMe4MeOBnCHO **F**). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 10.48 (s, 1H), 6.59 (s, 2H), 3.84 (s, 3H), 2.61 (s, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 191.7, 162.9, 144.6, 126.1, 115.0, 55.4, 21.2. Next, the 4-methoxy-2,6-dimethylbenzyl alcohol **G** (*Figure S1*) was prepared in 81% yield according to the general procedure for reduction of aldehydes and ketones. Analytical RP-HPLC (linear gradient from 5% to 100% ACN over 8 min; t_R : 5.3 min), and RP-HPLC-ESMS analysis showed the target alcohol (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.1 min; *m/z* calcd for $C_{10}H_{14}O_2$, 166.22; found, 149.2 $[M-OH]^+$, where M is the MW of the 2,6-diMe4-MeOBnOH **G**).

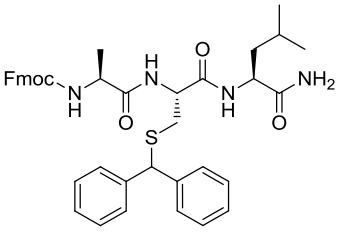
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.58 (s, 2H), 4.66 (s, 2H), 3.77 (s, 3H), 2.39 (s, *J* = 0.5 Hz, 6H), 1.32 (s, 1H). **^{13}C NMR** (101 MHz, $DCDl_3$): δ 159.0, 139.1, 129.4, 113.7, 59.0, 55.2, 19.8.

Fmoc-Ala-Cys(PG)-Leu-NH_2 tripeptides (2a-m): syntheses and TFA-lability studies

All tripeptides were synthesized manually on a Fmoc-Sieber-amide AM-polystyrene resin (0.69 mmol/g). Before peptide elongation, the resin was washed with DMF (3 × 5 mL × 1 min) and CH_2Cl_2 (3 × 5 mL × 1 min), and the Fmoc group was cleaved by treatments with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Based on the resin loading, the homemade protected Cys residues (2 equiv.) were incorporated with DIPCDI (2 equiv.) and Oxyma Pure (2 equiv.) in DMF, with a 5-min pre-activation, for 2 h at 25 °C; while protected Cys(Mob) (3 equiv.), DCys(Trt) (3 equiv.), Ala (3 equiv.) and Leu (3 equiv.) residues were incorporated with DIPCDI (3 equiv.) and Oxyma Pure (3 equiv.) in DMF, with a 5-min pre-activation, for 1 h at 25 °C. These conditions assured the absence of racemization.[1] Cleavage of the Fmoc group was achieved by treatment with piepridine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Washes between couplings and deprotections were performed with DMF (5 × 3 mL × 1 min) and CH_2Cl_2 (5 × 3 mL × 1 min). After completing the elongation, the tripeptides were cleaved from the resin by washes with a solution of TFA-CH_2Cl_2 (2:98) (5 × 3 mL × 1 min) at 25 °C, and the filtered washes were poured over H_2O (3 mL). The final mixtures were evaporated and the crude peptides were directly dissolved in H_2O-ACN (1:2) (20 mL) and lyophilized.

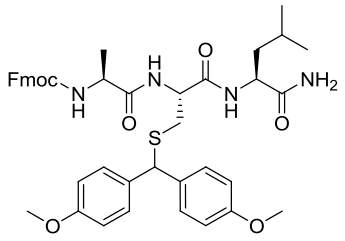
All TFA-lability studies were carried out with the lyophilized tripeptide crudes (1 mg) in glass tubes. To the acidic mixture were added H_2O (2.5%) and TIS (2.5%) as a scavengers, and CH_2Cl_2 was used as a solvent when it was required. After the acidic treatment, the mixtures were completely evaporated by bubbling N_2 (g) and the resultant residue was re-dissolved in H_2O-ACN (3:7, 2mL) and analyzed by RP-HPLC to determine the % of deprotected tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 3.6 min; *m/z* calcd for $C_{27}H_{34}N_4O_5S$, 526.7; found, 527.2 $[M+H]^+$, where M is the MW of the free-thiol tripeptide Fmoc-Ala-Cys-Leu-NH_2).

Dpm tripeptide (2a)



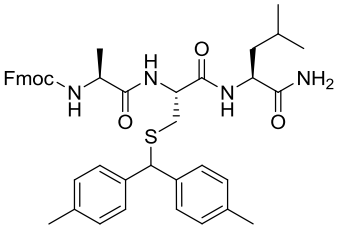
Fmoc-Ala-Cys(Dpm)-Leu-NH_2 tripeptide was obtained in 83% yield and 98% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.5 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.2 min; *m/z* calcd for $C_{40}H_{44}N_4O_5S$, 692.9; found, 694.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{40}H_{45}N_4O_5S$, 693.31052; found, 693.31138 $[M+H]^+$.

4,4'diMeODpm tripeptide (2b)



Fmoc-Ala-Cys(4,4'diMeODpm)-Leu-NH_2 tripeptide was obtained in 29% yield and 94% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.2 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.9 min; m/z calcd for $C_{42}H_{48}N_4O_7S$, 752.9; found, 754.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{42}H_{49}N_4O_7S$, 753.33165; found, 753.33182 $[M+H]^+$.

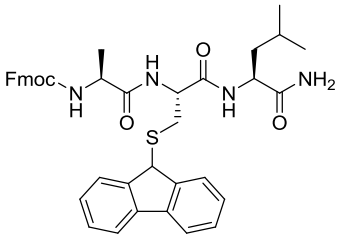
4,4'diMeDpm tripeptide (2c)



Fmoc-Ala-Cys(4,4'diMeDpm)-Leu-NH_2 tripeptide was obtained in 61% yield and 94% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.5 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 8.5 min; *m/z* calcd for $C_{42}H_{48}N_4O_5S$, 720.9; found, 722.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{42}H_{49}N_4O_5S$, 721.34182; found, 721.34279 $[M+H]^+$.

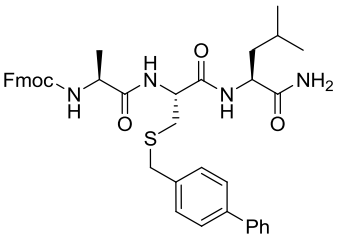
1 (a) Han, Y; Albericio, F.; Barany, G. *J. Org. Chem.* **1997**, *62*, 4307-4312; (b) Angell, Y.M.; Alsina, J.; Albericio, F.; Barany, G. *J. Peptide Res.* **2002**, *60*, 292-299.

9F tripeptide (2d)



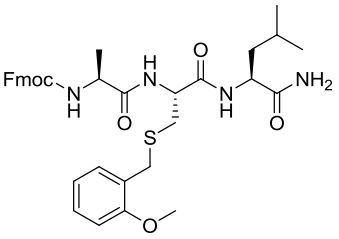
Fmoc-Ala-Cys(9F)-Leu-NH_2 tripeptide was obtained in 57% yield and 94% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.5 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.5 min; *m/z* calcd for $C_{40}H_{42}N_4O_5S$, 690.9; found, 691.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{40}H_{43}N_4O_5S$, 691.29487; found, 691.29548 $[M+H]^+$.

Bpm tripeptide (2e)



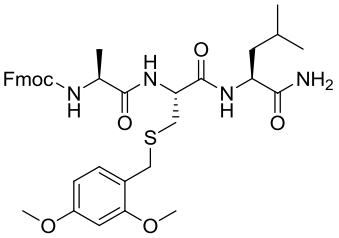
Fmoc-Ala-Cys(Bpm)-Leu-NH_2 tripeptide was obtained in 76% yield and 94% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.6 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.6 min; *m/z* calcd for $C_{40}H_{44}N_4O_5S$, 692.9; found, 694.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{40}H_{45}N_4O_5S$, 693.31052; found, 693.31111 $[M+H]^+$.

2MeOBn tripeptide (2f)



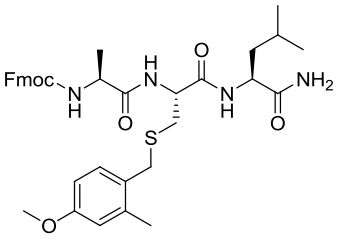
Fmoc-Ala-Cys(2MeOBn)-Leu-NH_2 tripeptide was obtained in 25% yield and 94% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.5 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.9 min; *m/z* calcd for $C_{35}H_{42}N_4O_6S$, 646.8; found, 647.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{35}H_{43}N_4O_6S$, 647.28978; found, 647.29035 $[M+H]^+$.

2,4diMeOBn tripeptide (2g)



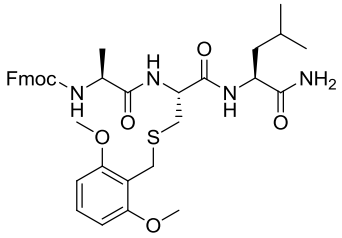
Fmoc-Ala-Cys(2,4diMeOBn)-Leu-NH_2 tripeptide was obtained in 32% yield and 99% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.4 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.3 min; *m/z* calcd for $C_{36}H_{44}N_4O_7S$, 676.8; found, 678.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{36}H_{45}N_4O_7S$, 677.30035; found, 677.30131 $[M+H]^+$.

4MeO-2MeBn tripeptide (2h)



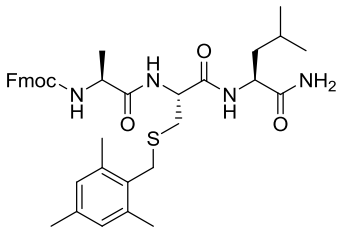
Fmoc-Ala-Cys(4MeO-2MeBn)-Leu-NH_2 tripeptide was obtained in 64% yield and 96% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.6 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.0 min; *m/z* calcd for $C_{36}H_{44}N_4O_6S$, 660.8; found, 661.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{36}H_{45}N_4O_6S$, 661.30543; found, 661.30588 $[M+H]^+$.

2,6diMeOBn tripeptide (2i)



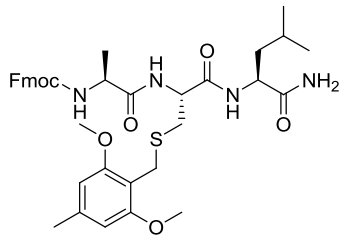
Fmoc-Ala-Cys(2,6diMeOBn)-Leu-NH_2 tripeptide was obtained in 58% yield and 96% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.6 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.8 min; *m/z* calcd for $C_{36}H_{44}N_4O_7S$, 676.8; found, 677.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{36}H_{45}N_4O_7S$, 677.30035; found, 677.30104 $[M+H]^+$.

TMeb tripeptide (2j)



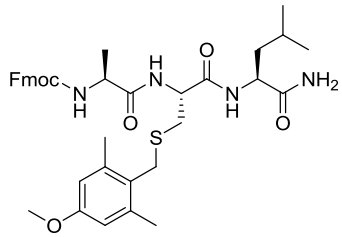
Fmoc-Ala-Cys(TMeb)-Leu-NH_2 tripeptide was obtained in 69% yield and 95% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.7 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 7.8 min; *m/z* calcd for $C_{37}H_{46}N_4O_5S$, 658.9; found, 659.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{37}H_{47}N_4O_5S$, 659.32617; found, 659.32666 $[M+H]^+$.

2,6diMeO-4MeBn tripeptide (2k)



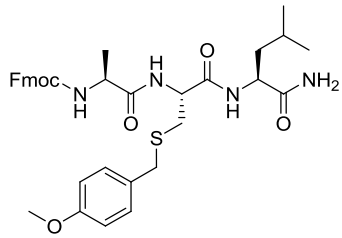
Fmoc-Ala-Cys(2,6diMeO-4MeBn)-Leu-NH_2 tripeptide was obtained in 59% yield and 95% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.0 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.8 min; *m/z* calcd for $C_{37}H_{46}N_4O_7S$, 690.9; found, 692.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{37}H_{47}N_4O_7S$, 691.31600; found, 691.31685 $[M+H]^+$.

4MeO-2,6diMeBn tripeptide (2l)



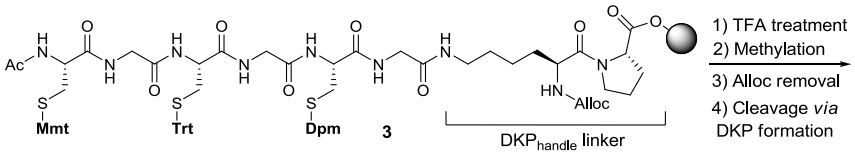
Fmoc-Ala-Cys(4MeO-2,6diMeBn)-Leu-NH_2 tripeptide was obtained in 69% yield and 99% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.8 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.0 min; *m/z* calcd for $C_{37}H_{46}N_4O_6S$, 674.9; found, 676.0 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{37}H_{47}N_4O_6S$, 675.32108; found, 675.32171 $[M+H]^+$.

Mob tripeptide (2m)



Fmoc-Ala-Cys(Mob)-Leu-NH_2 tripeptide was obtained in 36% yield and 96% purity, as determined by analytical RP-HPLC (linear gradient from 50% to 100% ACN over 8 min; t_R : 4.3 min). RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 5.9 min; *m/z* calcd for $C_{35}H_{42}N_4O_6S$, 646,8 ; found, 647.9 $[M+H]^+$, where M is the MW of the target tripeptide). **HRMS** calcd for $C_{35}H_{43}N_4O_6S$, 647.28978; found, 647.29037 $[M+H]^+$.

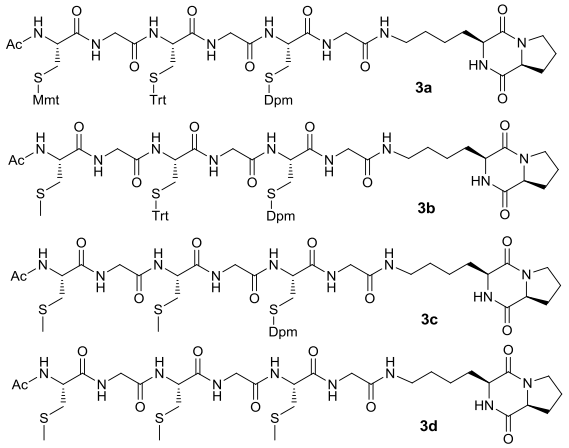
DKP$_{handle}$ experiment (3)



Dipeptydil linker was constructed manually on a HMPS resin (150 mg, 0.98 mmol/g). After washing the resin with DMF (3 × 5 mL × 1 min) and CH_2Cl_2 (3 × 5 mL × 1 min), a mixture of Fmoc-D-Pro-OH (199 mg, 4 equiv.) and DIPCDI (45.7 μL, 2 equiv.) in CH_2Cl_2-DMF (15:1 (v/v), 800 μL) was added to the resin. DMAP (7.3 mg, 0.4 equiv.) in CH_2Cl_2 (200 μL) was then added, and the mixture was left to stand for 2 h at 25 °C. Consecutively, Fmoc-D-Pro-OH was re-coupled following the same procedure for 16 h at 25 °C. The resin was washed with CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min) and successively capped using acetic anhydride (150 μL, 10 equiv.) and DIEA (256 μL, 10 equiv.) in DMF (500 μL) for 30 min at 25 °C. After capping, the resin was washed with CH_2Cl_2 (5 × 1 min) and DMF (5 × 1min), and the Fmoc group was then removed and a 0.98 mmol/g resin loading was determined by UV quantification. To introduce the second amino acid of the DKP$_{handle}$ linker, a mixture of Alloc-Lys(Fmoc)-OH (200 mg, 3 equiv.), Oxyma Pure (63 mg, 3 equiv.) and DIPCDI (68.5 mL, 3 equiv.) in DMF (1 mL) was shaken for 5 min at 25 °C, and then added to the resin. The mixture was left to stand for 16 h at 25 °C and the resin was then washed with CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min), and the Fmoc group was removed.

On the previously prepared resin was elongated the hexapeptide. Based on the resin loading, the protected residues (3 equiv.) were incorporated with DIPCDI (3 equiv.) and Oxyma Pure (3 equiv.) in DMF, with a 5-min pre-activation, for 1 h at 25 °C. Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Washes between couplings and deprotections were performed with DMF (5 × 3 mL × 1 min) and CH_2Cl_2 (5 × 3 mL × 1 min). Finally, the *N*-terminus was acetylated using acetic anhydride (10 equiv.) and DIEA (10 equiv.) in DMF (1 mL) for 30 min at 25 °C (× 2).

After completing the elongation by SPPS, the TFA-lability study on solid phase was carried out. Thus, different peptidyl-resins (20 mg) were treated with diverse solutions of TFA in CH_2Cl_2 with or without the presence of TIS as scavenger, following by methylation using CH_3I (7.5 μL, 5 equiv.) and DIEA (7 μL, 5%) in CH_2Cl_2 (135 μL) for 2 h at 25 °C. Ellman's test for detection of thiol was then performed; if the test was positive an extra treatment with CH_3I in basic conditions was carried out. Subsequently, peptides were cleaved from the resin by Alloc removal from the L-Lys residue of the dipeptidyl linker by treatment with $Pd(Ph_3)_4$ (1 mg, 0.1 equiv.), $PhSiH_3$ (10 μL, 10 equiv.) in CH_2Cl_2 (3 × 15 min), and posterior DKP formation by treatment with piperidine-THF (5:95, 2 × 5 min). THF was removed by evaporation under reduced pressure, and the residual crude peptides comprising the DKP *C*-terminal protecting moiety were analyzed by RP-HPLC.



Ac-Cys(Mmt)-Gly-Cys(Trt)-Gly-Cys(Dpm)-Gly-DKP$_{handle}$ (**3a**)

RP-HPLC analysis: linear gradient from 50% to 100% ACN over 8 min; t_R : 6.7 min. RP-HPLC-ESMS analysis: linear gradient from 50% to 100% ACN over 8 min; t_R : 11.4 min; *m/z* calcd for $C_{80}H_{85}N_9O_{10}S_3$, 1428.9; found, 1431.0 $[M+H]^+$, where M is the MW of the fully protected peptide.

Ac-Cys(Me)-Gly-Cys(Trt)-Gly-Cys(Dpm)-Gly-DKP$_{handle}$ (**3b**)

RP-HPLC analysis: linear gradient from 50% to 100% ACN over 8 min; t_R : 3.7 min. RP-HPLC-ESMS analysis: linear gradient from 50% to 100% ACN over 8 min; t_R : 4.9 min; *m/z* calcd for $C_{61}H_{71}N_9O_9S_3$, 1170.4; found, 1171.5 $[M+H]^+$, where M is the MW of the target peptide.

Ac-Cys(Me)-Gly-Cys(Me)-Gly-Cys(Dpm)-Gly-DKP$_{handle}$ (**3c**)

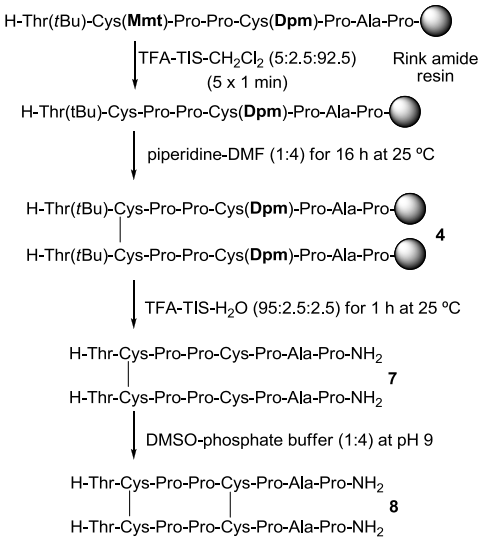
RP-HPLC analysis: linear gradient from 5% to 100% ACN over 8 min; t_R : 5.3 min. RP-HPLC-ESMS analysis: linear gradient from 5% to 100% ACN over 8 min; t_R : 7.3 min; *m/z* calcd for $C_{43}H_{59}N_9O_9S_3$, 942.2; found, 943.1 $[M+H]^+$, where M is the MW of the target peptide.

Ac-Cys(Me)-Gly-Cys(Me)-Gly-Cys(Me)-Gly-DKP$_{handle}$ (**3d**)

RP-HPLC analysis: linear gradient from 5% to 100% ACN over 8 min; t_R : 3.6 min. RP-HPLC-ESMS analysis: linear gradient from 5% to 100% ACN over 8 min; t_R : 5.7 min; *m/z* calcd for $C_{31}H_{51}N_9O_9S_3$, 790.0; found, 790.9 $[M+H]^+$, where M is the MW of the target peptide.

Regioselective synthesis of the hinge fragment of human IgG1 (8)

Rink-amide approach



Linear peptide was synthesized manually on Fmoc-Rink-amide AM-polystyrene resin (300 mg, 0.45 mmol/g). Before peptide elongation, the resin was washed with DMF (3 × 5 mL × 1 min) and CH_2Cl_2 (3 × 5 mL × 1 min), and the Fmoc group was cleaved by treating with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Based on the resin loading, the protected residues (3 equiv.) were incorporated with DIPCDI (3 equiv.) and Oxyma Pure (3 equiv.) in DMF, with a 5-min pre-activation, for 1 h at 25 °C. Cleavage of the Fmoc group was achieved by treatment with piepridine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Washes between couplings and deprotections were performed with DMF (5 × 3 mL × 1 min) and CH_2Cl_2 (5 × 3 mL × 1 min).

After completing the elongation by SPPS, a portion of peptidyl-resin (1 mg) was cleaved from the resin by treating with TFA-TIS-H_2O (95:2.5:2.5) (1 mL) for 1 h at 25 °C and the crude was precipitated with pre-cooled Et_2O and analyzed by RP-HPLC. H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-NH_2 was obtained in 91% purity, as determined by analytical RP-HPLC (linear gradient from 5% to 50% ACN over 8 min at 60 °C; t_R : 4.3 min). RP-HPLC-ESMS showed the fully deprotected target peptide (linear gradient from 0% to 30% ACN over 8 min; t_R : 7.2 min; *m/z* calcd for $C_{33}H_{53}N_9O_9S_2$, 783.9; found, 785.0 $[M+H]^+$, where M is the MW of the target peptide). The RP-HPLC analysis showed two peaks when it was performed at 25 °C due to the existing conformational species of the free linear monomer (linear gradient from 5% to 50% ACN over 8 min; t_R : 3.5 and 3.7 min).

S-Mmt removal and posterior disulfide bond construction on solid phase (**7**)

A portion of peptidyl-resin was treated with TFA-TIS-CH_2Cl_2 (5:2.5:92.5) (5 × 1 min) and then washed with CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Subsequently, the peptidyl-resin was treated with piperidine-DMF (1:4) at 25 °C and the oxidation was monitored by Ellman's test and RP-HPLC analysis until completion, when the crude peptide was cleaved from the resin with TFA-TIS-H_2O (95:2.5:2.5) for 1 h at 25 °C. The acidic mixture was evaporated, the crude peptide precipitated with pre-cooled Et_2O, centrifuged and finally lyophilized, and analyzed by RP-HPLC (linear gradient from 5% to 80% ACN over 8 min; t_R : 3.7 min). RP-HPLC-ESMS showed the fully deprotected mono-disulfide bond dimer (linear gradient from 0% to 30% ACN over 8 min; t_R : 7.6 min; m/z calcd for $C_{66}H_{104}N_{18}O_{18}S_4$, 1565.9; found, 784.1 $[M+2H/2]^{2+}$, where M is the MW of the target intermediate).

A one-pot approach for the total deprotection and second disulfide bond formation was assayed. Thus, after first disulfide bond formation the peptidyl-resin was treated with TFA-DMSO-TIS (89:10:1). However, although, the fully *S*-Dpm removal was achieved after 1 h, the subsequent disulfide oxidation was not complete under acidic conditions.

2nd disulfide bond construction in solution (**8**)

The resultant mono-disulfide bond intermediate **7** was then dissolved in DMSO-phosphate buffer (20 mM) (1:4) at pH 9 and 25 °C to render the totally oxidized parallel dimer in 95% purity, as determined by RP-HPLC analysis (linear gradient from 5% to 80% ACN over 8 min; t_R : 3.3 min). RP-HPLC-ESMS showed the parallel dimer (linear gradient from 0% to 30% ACN over 8 min; t_R : 6.8 min; m/z calcd for $C_{66}H_{102}N_{18}O_{18}S_4$, 1563.9; found, 783.2 $[M+2H/2]^{2+}$, where M is the MW of the target peptide).

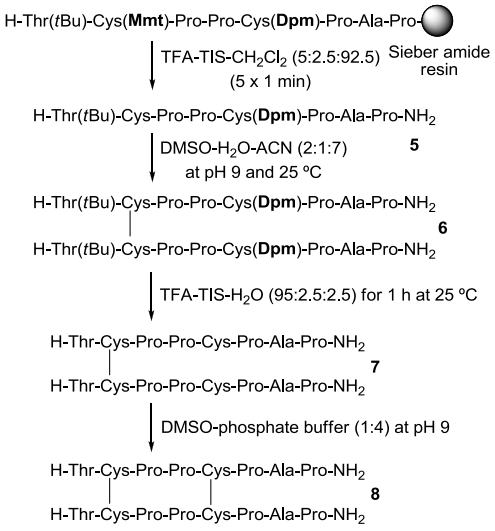
Random approach

A portion of peptidyl-resin from the Rink-amide approach (62.5 mg) was treated with TFA-TIS-H_2O (95:2.5:2.5) (3 mL) for 1 h at 25 °C, and the cleavage mixture was then evaporated and the crude peptide was precipitated with pre-cooled Et_2O (2 × 5 mL), centrifuged and lyophilized to render the fully deprotected linear peptide H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-NH_2 (10.5 mg, 74%

yield) in 91% purity, as determined by RP-HPLC analysis (linear gradient from 5% to 80% ACN over 8 min; t_R : 3.5 and 3.7 min). **HRMS** analysis m/z calcd for $C_{33}H_{53}N_9O_9S_2$, 784.34804; found, 784.34965 $[M+H]^+$.

A portion of the linear monomer (1 mg) was dissolved in a 20 mM phosphate buffer (425 μL, 3 mM) at pH 6.8 and 25 °C and the oxidative folding was monitored by RP-HPLC analysis (linear gradient from 5% to 80% ACN over 8 min; t_R : 3.3 min). The main peak corresponded to the parallel cyclic dimer, as determined by RP-HPLC-ESMS (linear gradient from 0% to 30% ACN over 8 min; t_R : 6.7 min; m/z calcd for $C_{66}H_{102}N_{18}O_{18}S_4$, 1563.9; found, 783.2 $[M+2H/2]^{+2}$, 1565.0 $[M+H]^+$, where M is the MW of the target peptide). **HRMS** analysis m/z calcd for $C_{66}H_{102}N_{18}O_{18}S_4$, 1562.64968; found, 1562.62109 $[M+H]^+$.

Sieber-amide approach



Linear peptide was synthesized manually on a Fmoc-Sieber-amide AM-polystyrene resin (200 mg, 0.69 mmol/g) following the same procedure described in the Fmoc-Rink-amide approach.

After completing the elongation by SPPS, a portion of peptidyl-resin (1 mg) was cleaved from the resin by treating with TFA-TIS-H_2O (95:2.5:2.5) (1 mL) for 1 h at 25 °C and the crude was precipitated with pre-cooled Et_2O and analyzed by RP-HPLC.

H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-NH_2 was obtained in 91% purity, as determined by analytical RP-HPLC (linear gradient from 5% to 50% ACN over 8 min at 60 °C; t_R : 4.3 min). RP-HPLC-ESMS showed the fully deprotected target peptide (linear gradient from 0% to 30% ACN over 8 min; t_R : 7.2 min; *m/z* calcd for $C_{33}H_{53}N_9O_9S_2$, 783.9; found, 785.0 $[M+H]^+$, where M is the MW of the target peptide).

Cleavage from the resin and concomitant S-Mmt removal (**5**)

A portion of peptidyl-resin (122 mg) was treated with TFA-TIS-H_2O-CH_2Cl_2 (5:2.5:2.5:90) (1 × 30 min, 3 × 1 min; 6 mL each) at 25 °C and the resultant resin was then washed with CH_2Cl_2 (3 × 1 min). The filtered solutions were poured over H_2O (15 mL) and the organic solvents were then concentrated under reduced pressure. To the final aqueous solution was added ACN (15 mL) and the mixture was then lyophilized. The resultant oil was precipitated with pre-cooled Et_2O (1 × 10 mL, 1 × 5 mL) and centrifuged to render the desired *S*-Dpm protected monomer intermediate **5** (50 mg, 89% yield) in 95% purity, as determined by analytical RP-HPLC (linear gradient from 5% to 80% ACN over 8 min; t_R : 6.1 min). RP-HPLC-ESMS showed the target *S*-Dpm protected monomer intermediate **5** (linear gradient from 5% to 80% ACN over 8 min; t_R : 7.3 min; *m/z* calcd for $C_{50}H_{71}N_9O_9S_2$, 1006.3; found, 1007.4 $[M+H]^+$, where M is the MW of the target intermediate).

1st disulfide bond construction in solution (**6** and **7**)

The partial protected monomer was dissolved in H_2O-ACN (1:9) (2 mL), and DMSO (500 μL, 20%) was added as an oxidant. The pH was adjusted to 9 using DIEA (45 μL) and the mixture was stirred at 25 °C. The disulfide bond formation was monitored by analytical RP-HPLC until completion to render the *S*-Dpm protected dimer **6** in 81% purity, as determined by RP-HPLC HPLC (linear gradient from 5% to 80% ACN over 8 min; t_R : 6.6 min). RP-HPLC-ESMS showed the target *S*-Dpm protected dimer intermediate **6** (linear gradient from 5% to 80% ACN over 8 min; t_R : 6.7 min; *m/z* calcd for $C_{100}H_{140}N_{18}O_{18}S_4$, 2010.6; found, 1006.9 $[M+2H/2]^{2+}$, where M is the MW of the target intermediate). Next, the solvent was removed by lyophilization and the total deprotection of the dimer intermediate **6** was then achieved by treating the crude peptide with TFA-TIS-H_2O (95:2.5:2.5) for 2 h at 25 °C. The mixture was evaporated by bubbling N_2 (g) and the crude peptide was then precipitated with pre-cooled Et_2O, centrifuged (× 3) and lyophilized to render the mono-disulfide bond dimer **7** in 85% purity, as determined by RP-HPLC analysis HPLC (linear gradient from 5% to 80% ACN over 8 min; t_R : 3.6 min) and RP-HPLC-ESMS (linear gradient from 5% to 80% ACN over 8 min; t_R : 7.1 min; *m/z* calcd for $C_{66}H_{104}N_{18}O_{18}S_4$, 1565.9; found, 1567.8 $[M+H]^+$, 784.9 $[M+2H/2]^{2+}$, where M is the MW of the target intermediate).

2nd disulfide bond construction in solution (**8**)

The formation of the 2nd disulfide bond in solution was accomplished following the same procedure indicated for the Rink-amide approach. **HRMS** analysis m/z calcd for $C_{66}H_{102}N_{18}O_{18}S_4$, 1562.64968; found, 1562.65136 $[M+H]^+$.

Regioselective synthesis of α-conotoxin ImI (11)

Linear peptide was synthesized manually on a Fmoc-Sieber-amide AM-polystyrene resin (202 mg, 0.69 mmol/g). Before peptide elongation, the resin was washed with DMF (3 × 5 mL × 1 min) and CH_2Cl_2 (3 × 5 mL × 1 min), and the Fmoc group was cleaved by treating with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Based on the resin loading, the protected residues (3 equiv.) were incorporated with DIPCDI (3 equiv.) and Oxyma Pure (3 equiv.) in DMF, with a 5-min pre-activation, for 1 h at 25 °C. Cleavage of the Fmoc group was achieved by treatment with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min). Washes between couplings and deprotections were performed with DMF (5 × 3 mL × 1 min) and CH_2Cl_2 (5 × 3 mL × 1 min).
After completing the elongation by SPPS, a portion of peptidyl-resin (5 mg) was cleaved from the resin by treating with TFA-CH_2Cl_2 (1:99) (1 mL) for 1 min at 25 °C and the crude was precipitated with pre-cooled Et_2O and analyzed by RP-HPLC. The fully protected peptide H-Gly-Cys(Trt)-Cys(Dpm)-Ser(*t*Bu)-Asp(O*t*Bu)-Pro-Arg(Pbf)-Cys(Trt)-Ala-Trp(Boc)-Arg(Pbf)-Cys(Dpm)-NH_2 was obtained as determined by analytical RP-HPLC (linear gradient from 80% to 100% ACN over 8 min; t_R : 6.7 min), and RP-HPLC-ESMS analysis (linear gradient from 70% to 100% ACN over 8 min; t_R : 8.69 min; *m/z* calcd for $C_{155}H_{186}N_{20}O_{23}S_6$, 2889.6; found, 1447.1 $[M+2H/2]^{2+}$, where M is the MW of the target peptide).

Cleavage from the resin and selective S-Trt removal (**9**)
A portion of peptidyl-resin (95 mg) was treated with TFA-TIS-CH_2Cl_2 (5:5:90) (3 min × 5 mL, 2 × 1 min × 5 mL) and washed with CH_2Cl_2 (3 × 1 min × 3 mL). The filtered mixtures were poured over H_2O (5 mL) and evaporated and the crude peptide was then lyophilized to render de partial *S*-Dpm protected intermediate **9** (40.8 mg, 80% yield) in 80% purity, as determined by analytical RP-HPLC (linear gradient from 70% to 100% ACN over 8 min; t_R : 4.8 min). RP-HPLC-ESMS showed the linear 2 *S*-Dpm deprotected peptide **9** (linear gradient from 70% to 100% ACN over 8 min; t_R : 5.3 min; *m/z* calcd for $C_{117}H_{158}N_{20}O_{23}S_6$, 2405.0; found, 1204.5$[M+2H/2]^{2+}$, where M is the MW of the target peptide).

1st disulfide bond construction in solution (**10**)
A portion of crude peptide (1 mg) was dissolved in H_2O-ACN (1:1) (7 mL) and the pH was adjusted up to 8.5 with an aqueous NH_3 solution (1 M) and the mixture was left to stand for 24 h at 25 °C and lyophilized to afford the oxidized 2 *S*-Dpm intermediate **10** in 80% purity, as determined by analytical RP-HPLC (linear gradient from 70% to 100% ACN over 8 min; t_R : 5.7 min) and RP-HPLC-ESMS analysis (linear gradient from 50% to 100% ACN over 8 min; t_R : 6.2 min; *m/z* calcd for $C_{117}H_{156}N_{20}O_{23}S_6$, 2403.0; found, 1203.4$[M+2H/2]^{2+}$, where M is the MW of the target peptide).

2nd disulfide bond construction in solution (**11**)
The cyclic 2 *S*-Dpm intermediate **10** was then treated with TFA-DMSO-anisole (89:10:1) (2 mL) for 3 h at 25 °C and the mixture was evaporated under reduced pressure and lyophilized to render the α-conotoxin ImI in 80% purity, as determined by analytical RP-HPLC (linear gradient from 10% to 35% ACN over 8 min; t_R : 5.2 min). RP-HPLC-ESMS showed the fully deprotected target peptide **11** (linear gradient from 0% to 30% ACN over 8 min; t_R : 7.6 min; *m/z* calcd for $C_{52}H_{78}N_{20}O_{15}S_4$, 1351.6; found, 1352.8 $[M+H]^+$, 677.2 $[M+2H/2]^{2+}$, where M is the MW of the target peptide). **HRMS** calcd for $C_{52}H_{78}N_{20}O_{15}S_4$, 1350.48329; found, 1350.48492 [M].

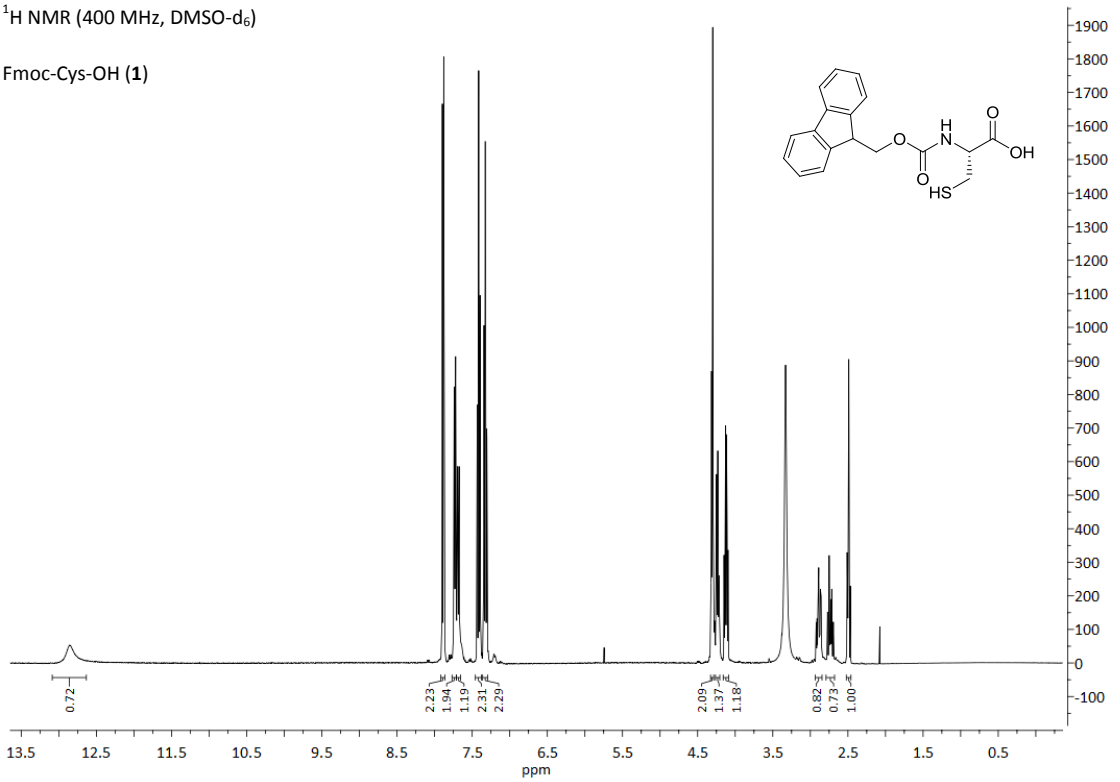
Random approach
A portion of peptidyl-resin (54 mg) was treated with a solution of TFA-TIS-H_2O (95:2.5:2.5) (5 mL) for 1 h at 25 °C and the mixture was then evaporated by bubbling N_2 (g) and the crude peptide was precipitated with pre-cooled Et_2O (10 mL) and centrifuged (×3) to render the fully deprotected linear peptide H-Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys-NH_2 (17 mg, 98% yield).

a) A portion of the resultant crude (1 mg) was re-dissolved in H_2O (2 mL) and the pH was adjusted up to 8 with an aqueous NH_3 solution (1 M) and the oxidative folding was monitored by RP-HPLC analysis. After 18 h, two main peaks corresponded to the structural isomers were observed (linear gradient from 10% to 35% ACN over 8 min; t_R : 4.7 and 5.2 min).
b) A portion of the resultant crude (1 mg) was re-dissolved in H_2O-2-propanol (1:1) (2 mL) and the pH was adjusted up to 10 with DIEA and the oxidative folding was monitored by RP-HPLC analysis. After 18 h, two main peaks corresponded to the structural isomers were observed (linear gradient from 10% to 35% ACN over 8 min; t_R : 4.7 and 5.2 min).

Spectral data of prepared Fmoc-Cys(PG)-OH

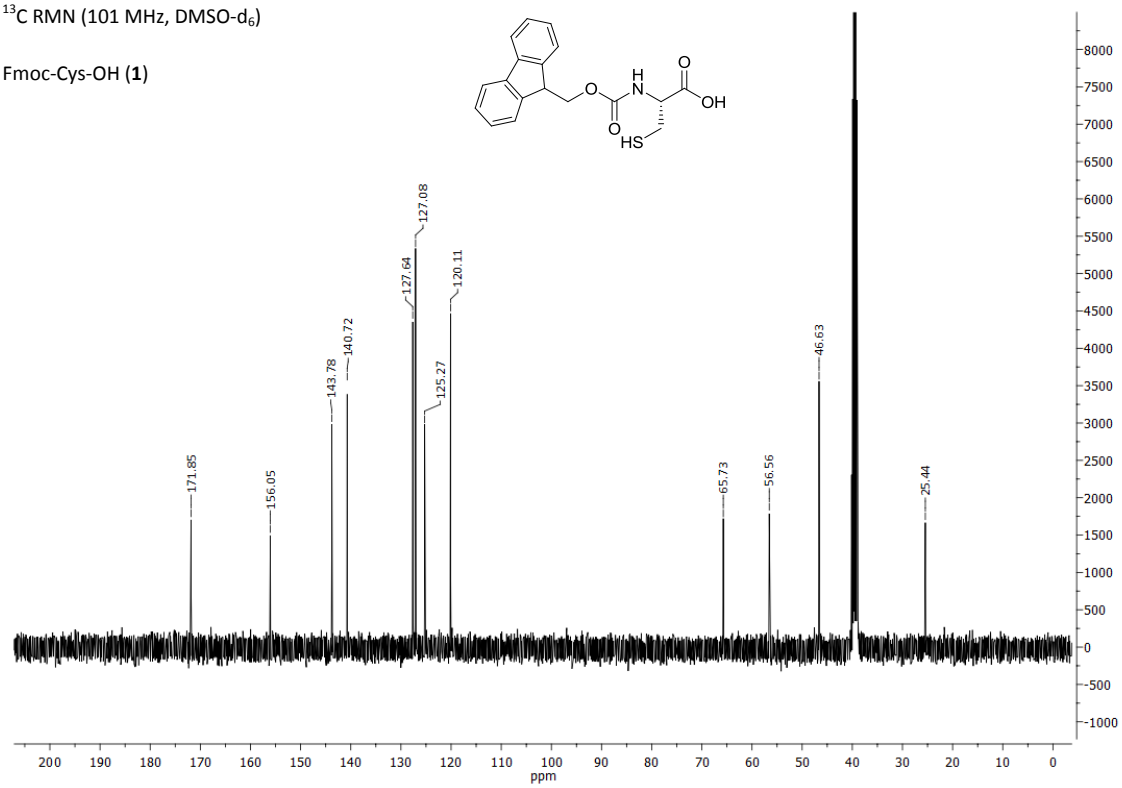
^{1}H NMR (400 MHz, DMSO-d_6)

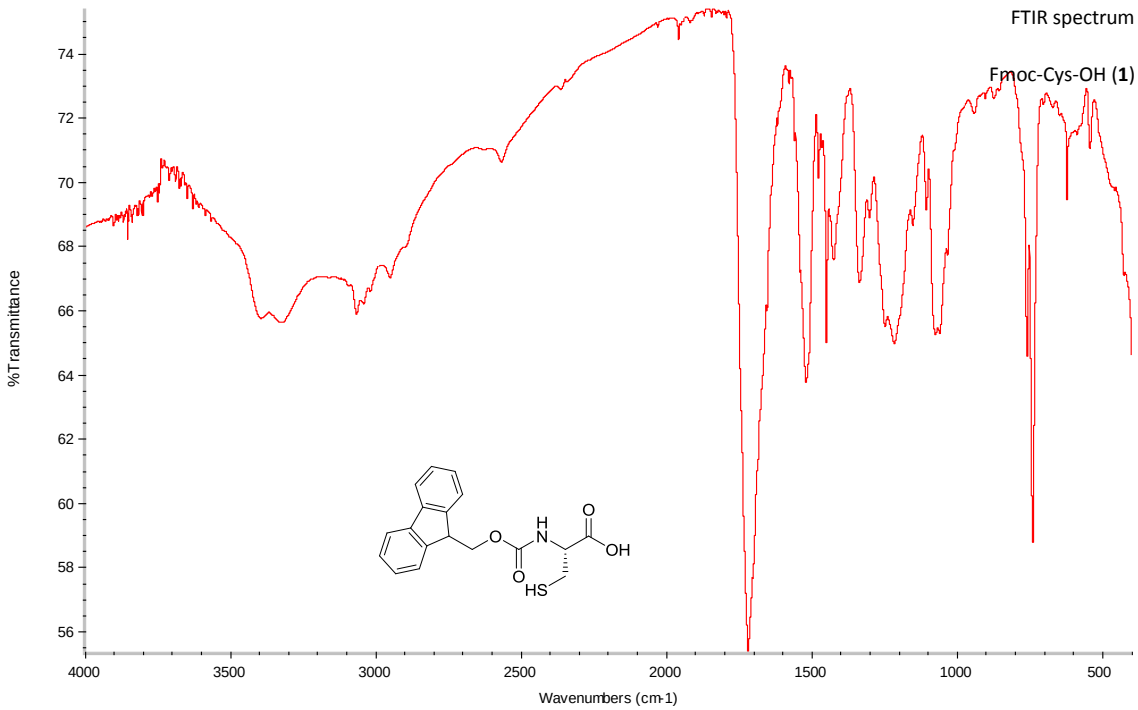
Fmoc-Cys-OH (**1**)



^{13}C RMN (101 MHz, DMSO-d_6)

Fmoc-Cys-OH (**1**)

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%Transmittance
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66
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62
60
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3500
3000
2500
2000
1500
1000
500
Wavenumbers (cm-1)
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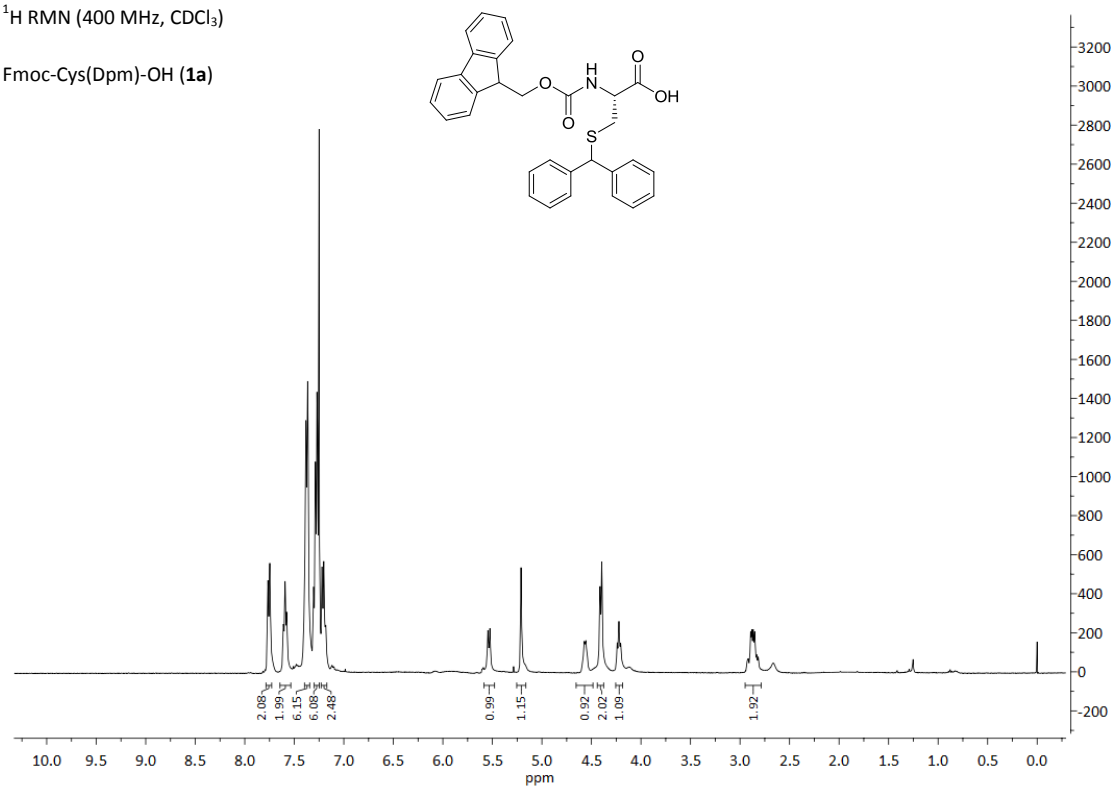
O
OH
O
HS

^{1}H RMN (400 MHz, $CDCl_3$)

Fmoc-Cys(Dpm)-OH (**1a**)



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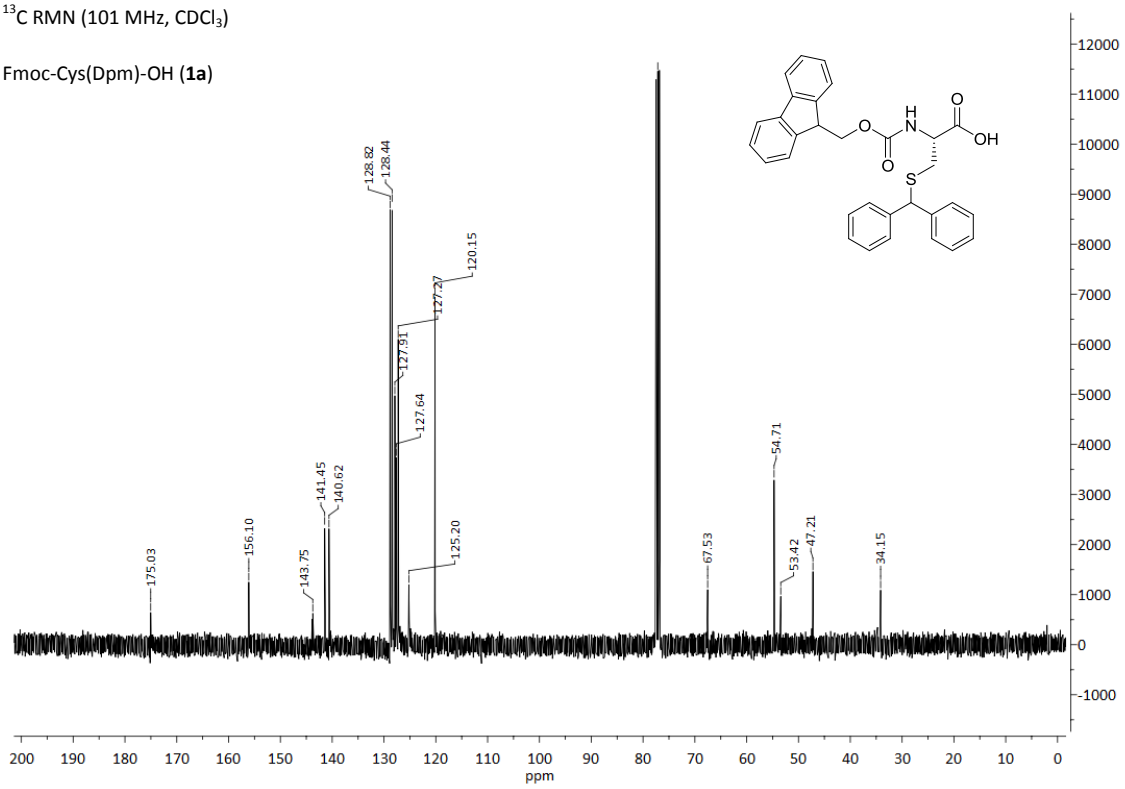
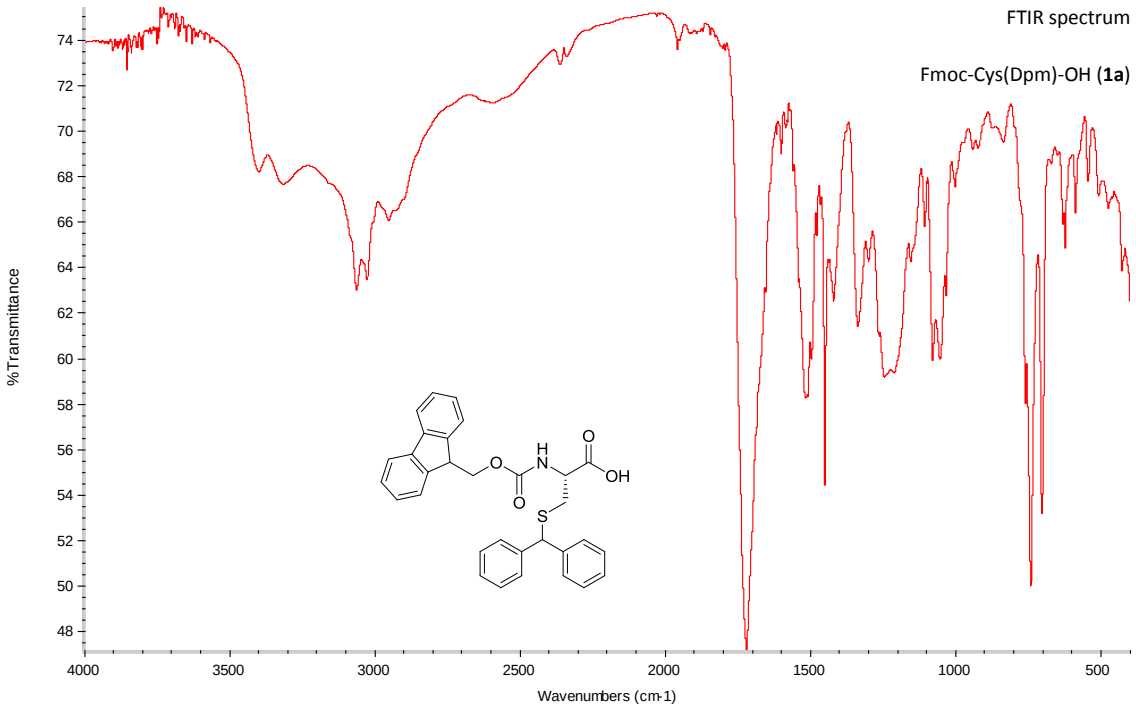
Fmoc-Cys(Dpm)-OH (**1a**)




FTIR spectrum
Fmoc-Cys(Dpm)-OH (1a)
%Transmittance
Wavenumbers (cm-1)
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3500
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1500
1000
500
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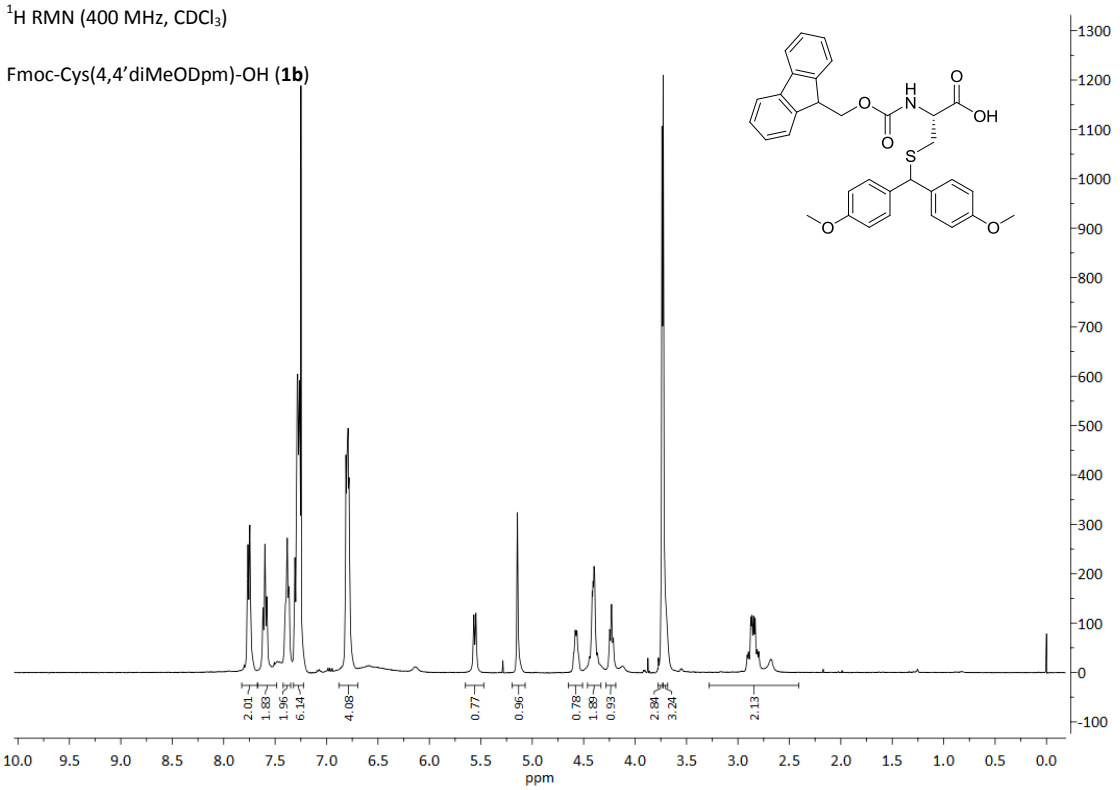
S

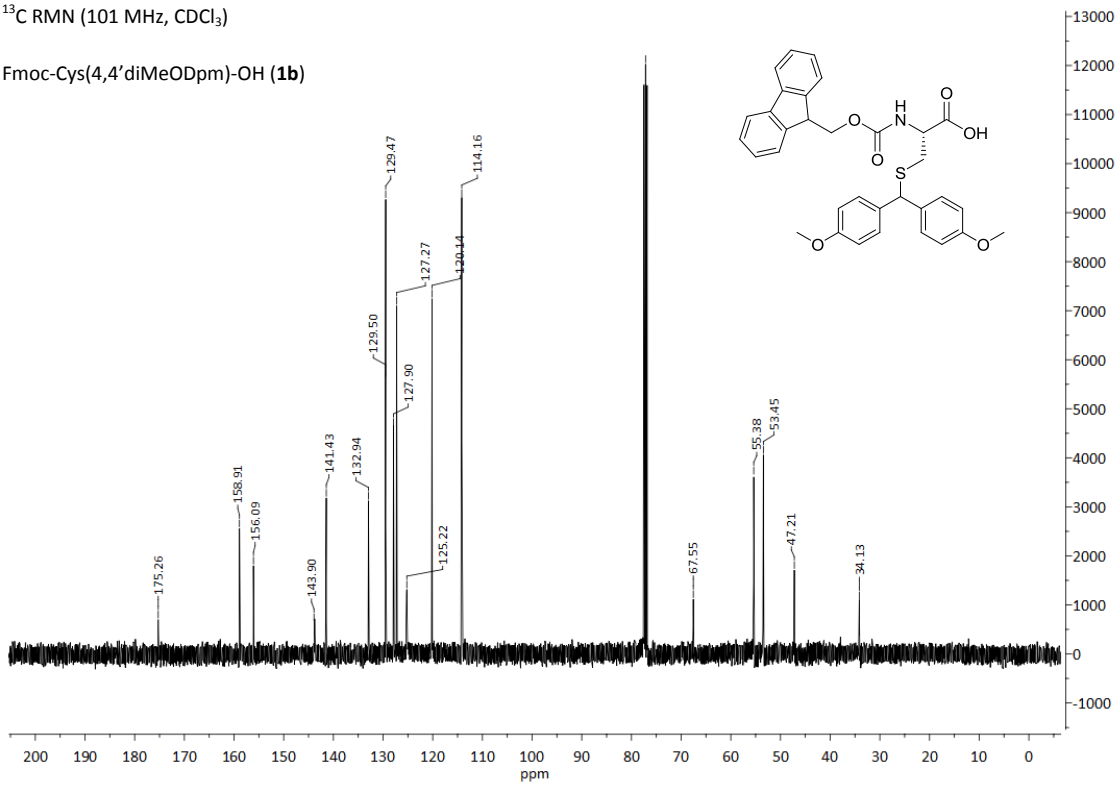
^{1}H RMN (400 MHz, $CDCl_3$)

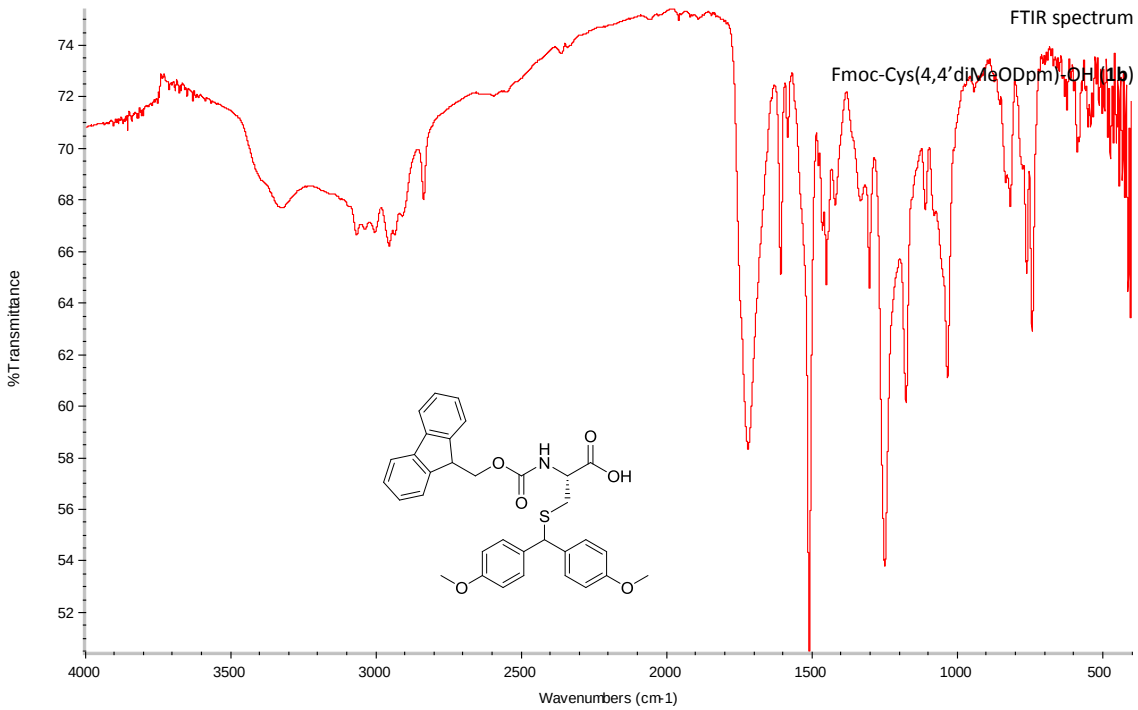
Fmoc-Cys(4,4'diMeODpm)-OH (**1b**)



^{13}C RMN (101 MHz, $CDCl_3$)

Fmoc-Cys(4,4'diMeODpm)-OH (**1b**)

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Fmoc-Cys(4,4'diMeODpm)-OH (1b)
%Transmittance
Wavenumbers (cm-1)
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2500
2000
1500
1000
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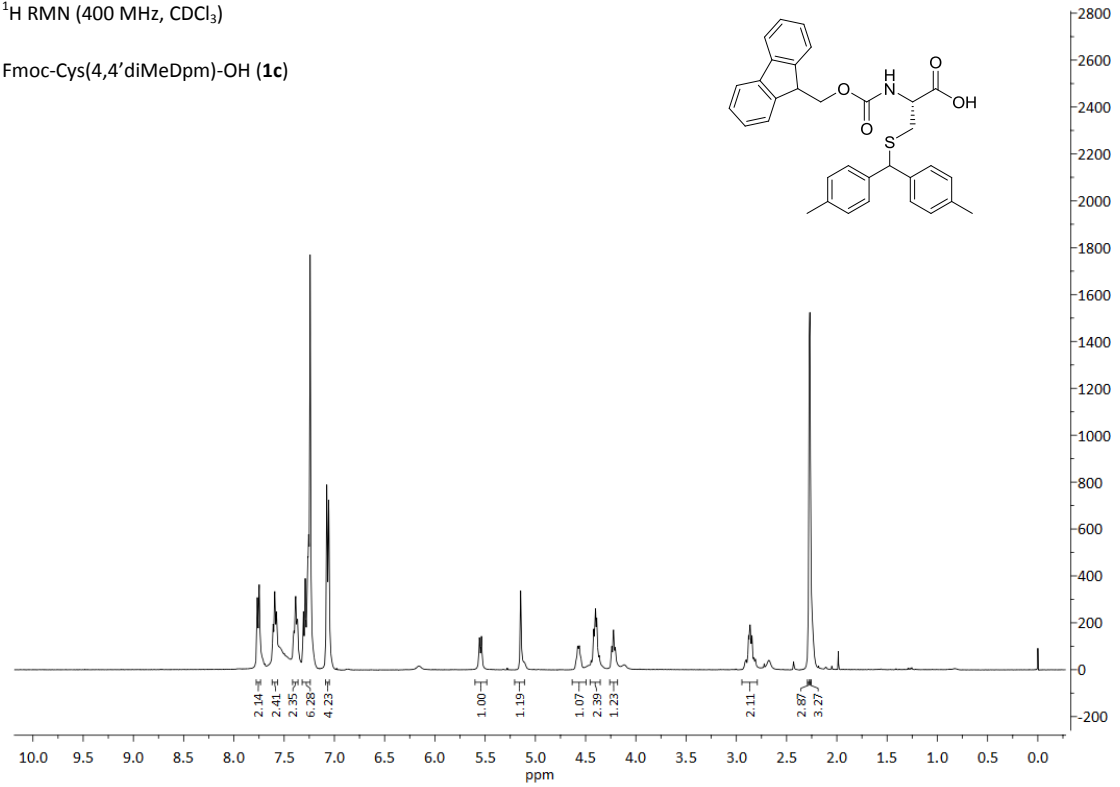
S
O

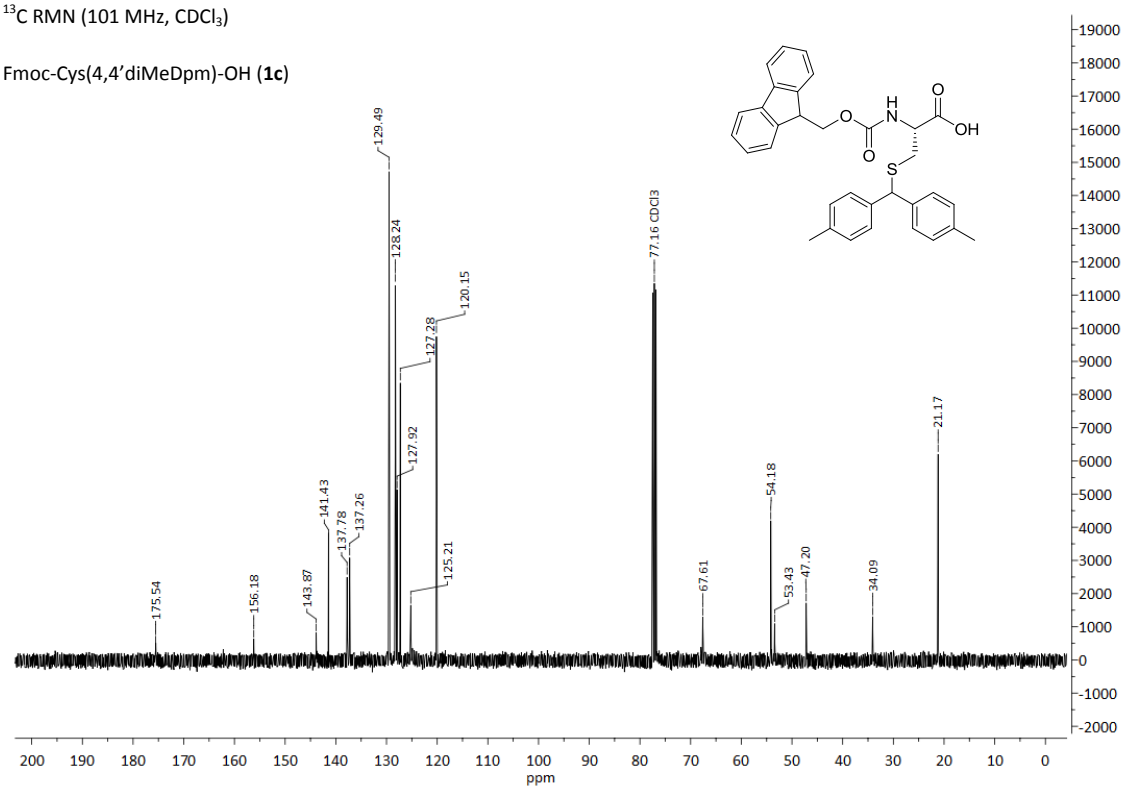
^{1}H RMN (400 MHz, $CDCl_3$)

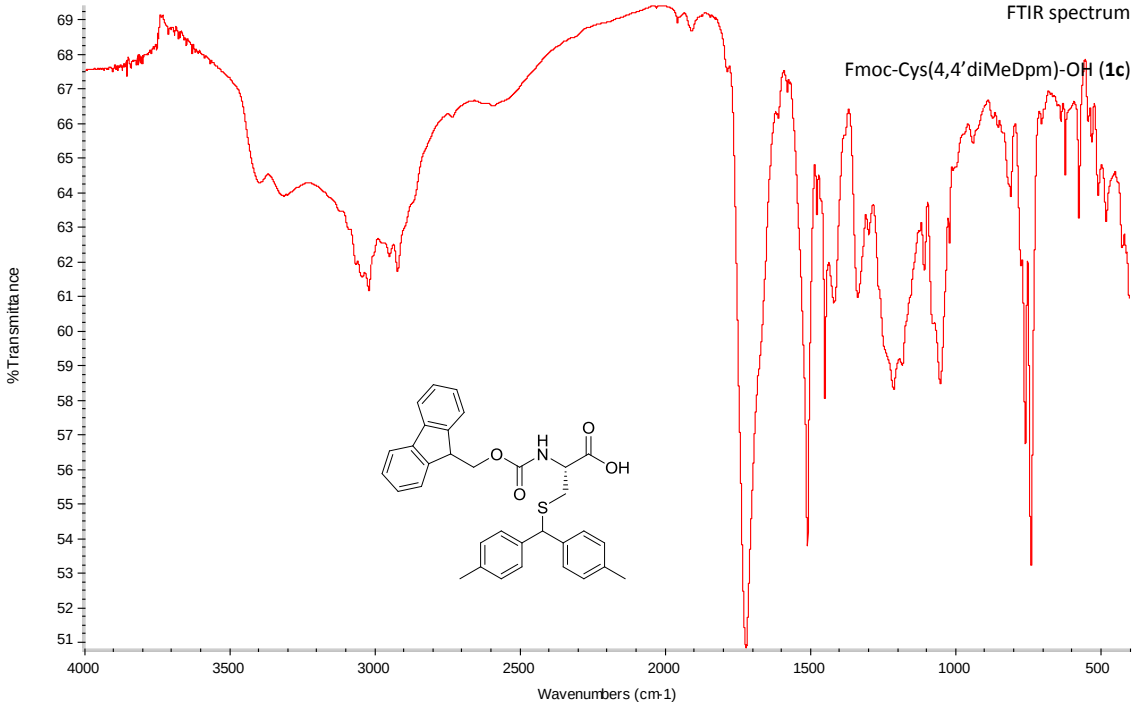
Fmoc-Cys(4,4'diMeDpm)-OH (**1c**)



^{13}C RMN (101 MHz, $CDCl_3$)

Fmoc-Cys(4,4'diMeDpm)-OH (**1c**)

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Fmoc-Cys(4,4'diMeDpm)-OH (1c)
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58
57
56
55
54
53
52
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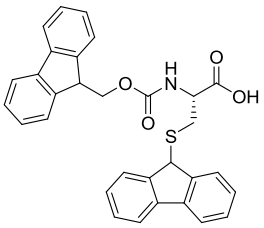
O
H
N
O
OH
O
S

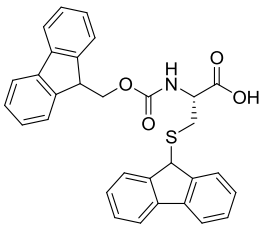
^{1}H RMN (400 MHz, $CDCl_3$)

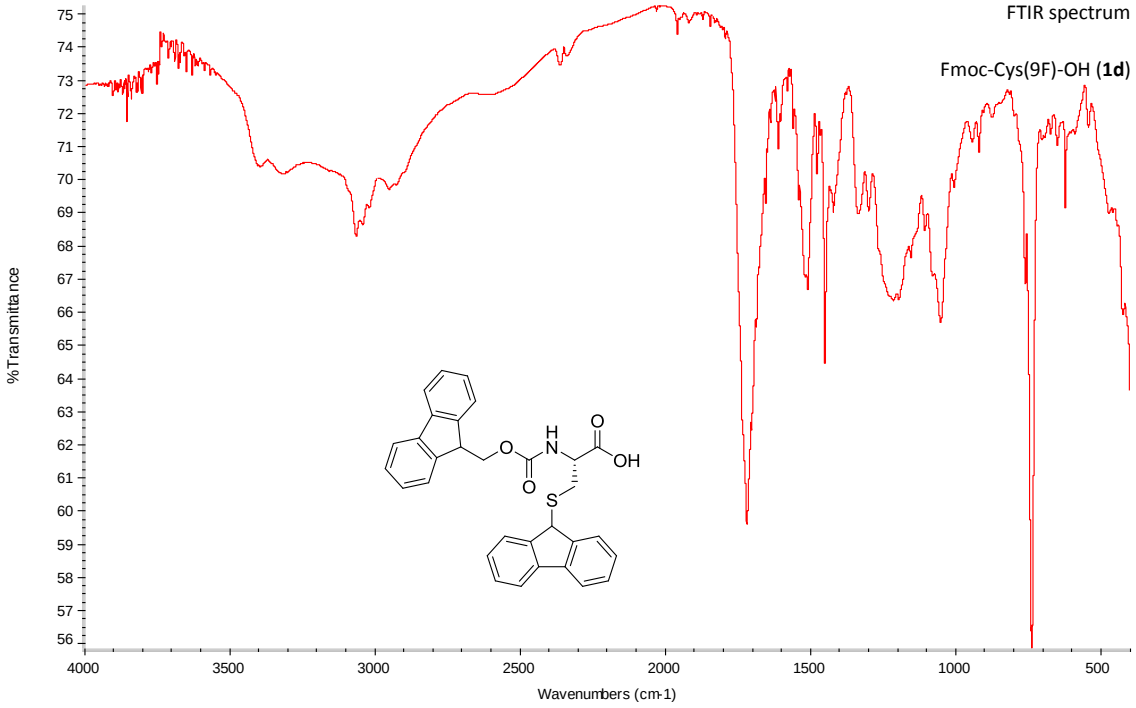
Fmoc-Cys(9F)-OH (**1d**)



^{13}C RMN (101 MHz, $CDCl_3$)

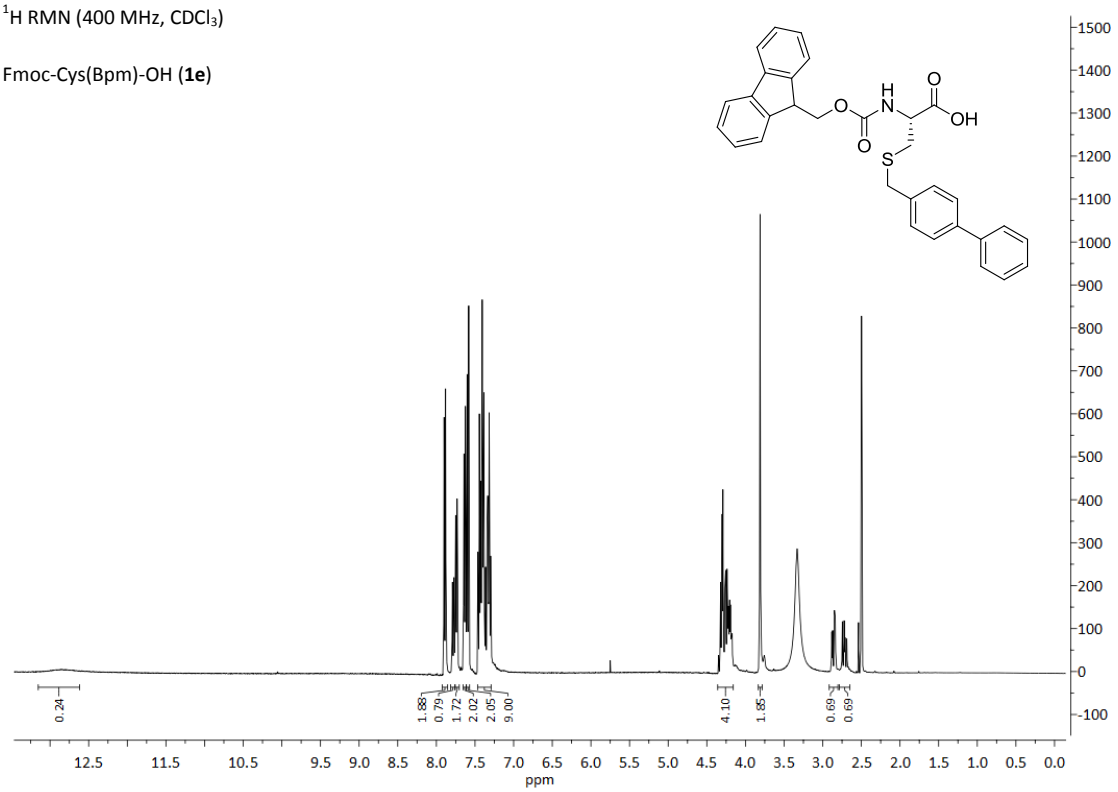
Fmoc-Cys(9F)-OH (**1d**)




FTIR spectrum
Fmoc-Cys(9F)-OH (1d)
%Transmittance
Wavenumbers (cm-1)
4000
3500
3000
2500
2000
1500
1000
500
O
H
N
O
OH
O
S

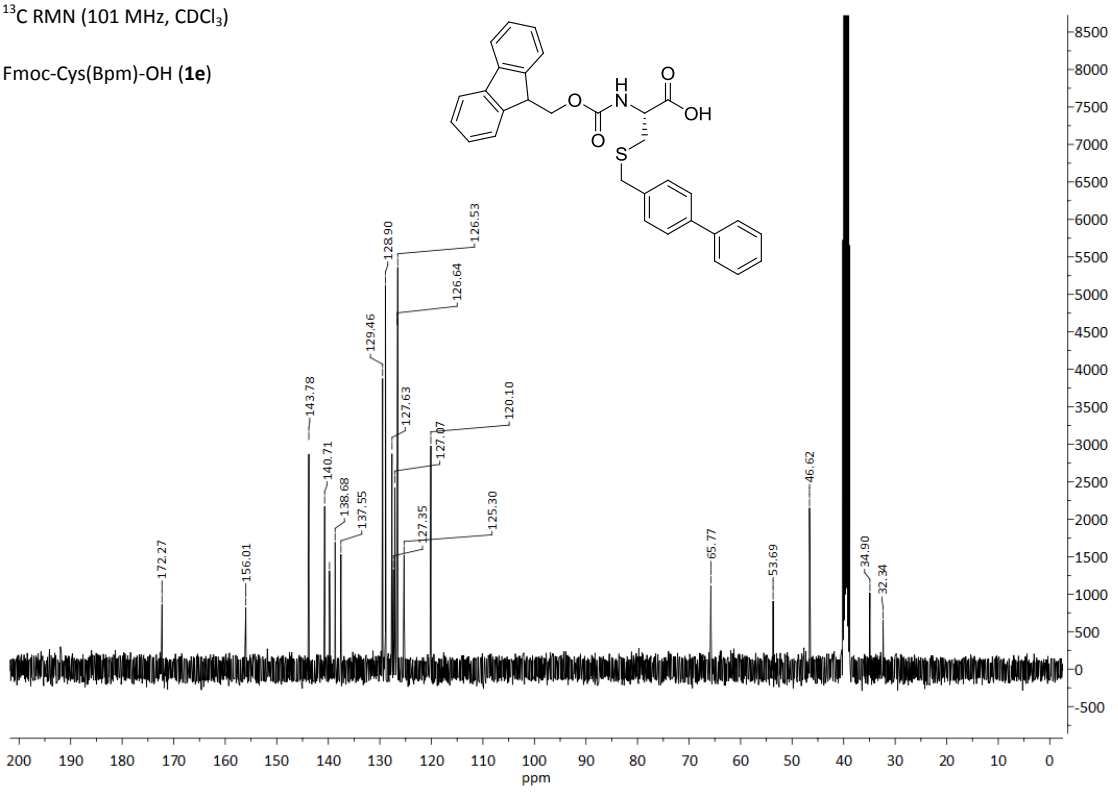
^{1}H RMN (400 MHz, $CDCl_3$)

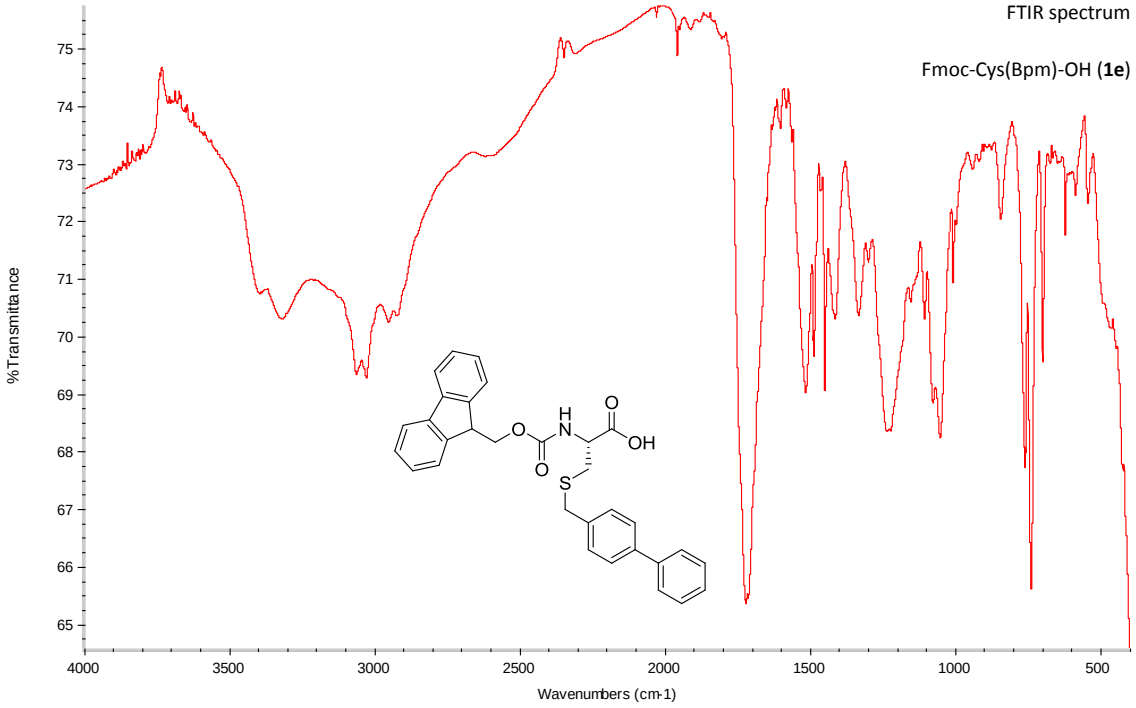
Fmoc-Cys(Bpm)-OH (**1e**)



^{13}C RMN (101 MHz, $CDCl_3$)

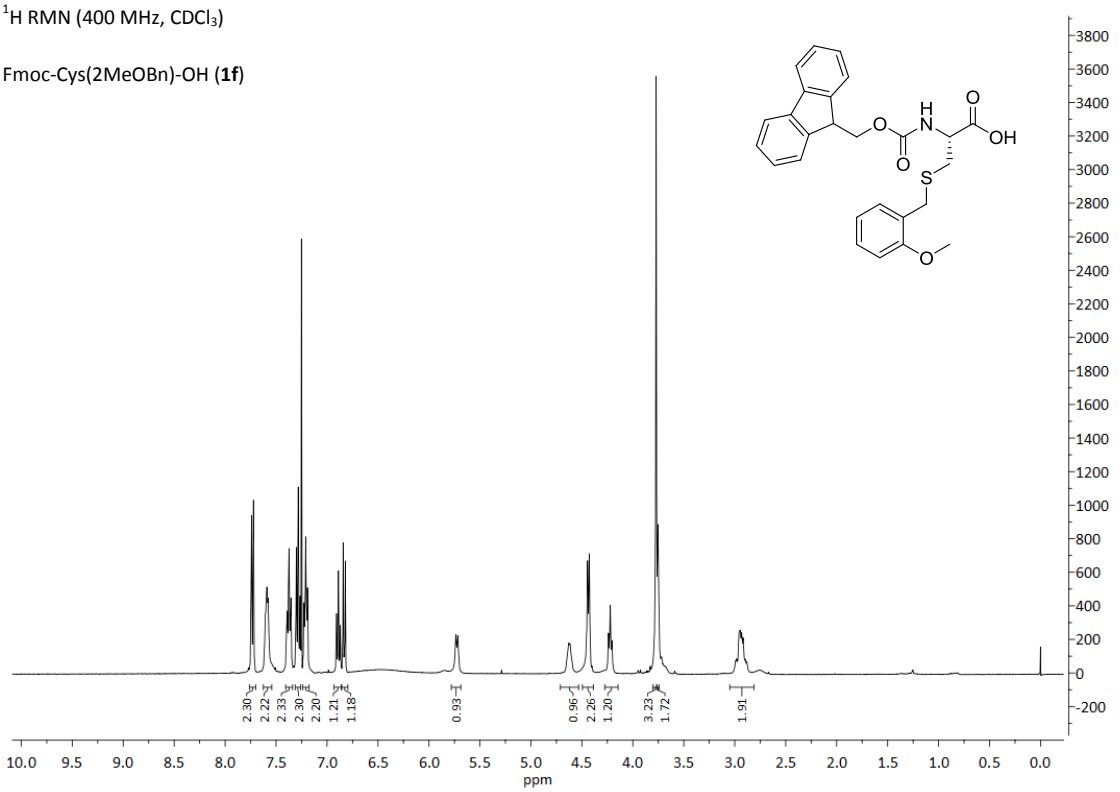
Fmoc-Cys(Bpm)-OH (**1e**)




FTIR spectrum
Fmoc-Cys(Bpm)-OH (1e)
%Transmittance
75
74
73
72
71
70
69
68
67
66
65
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers (cm-1)
O
H
N
O
OH
O
S

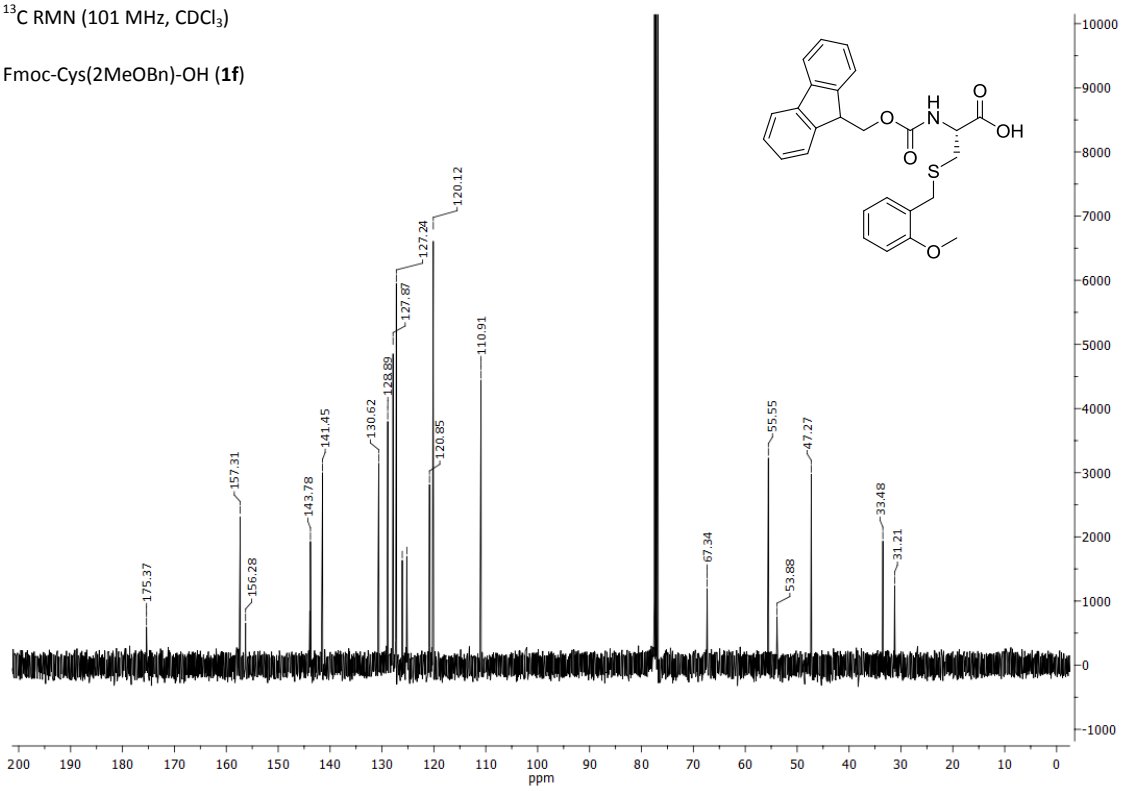
^{1}H RMN (400 MHz, $CDCl_3$)

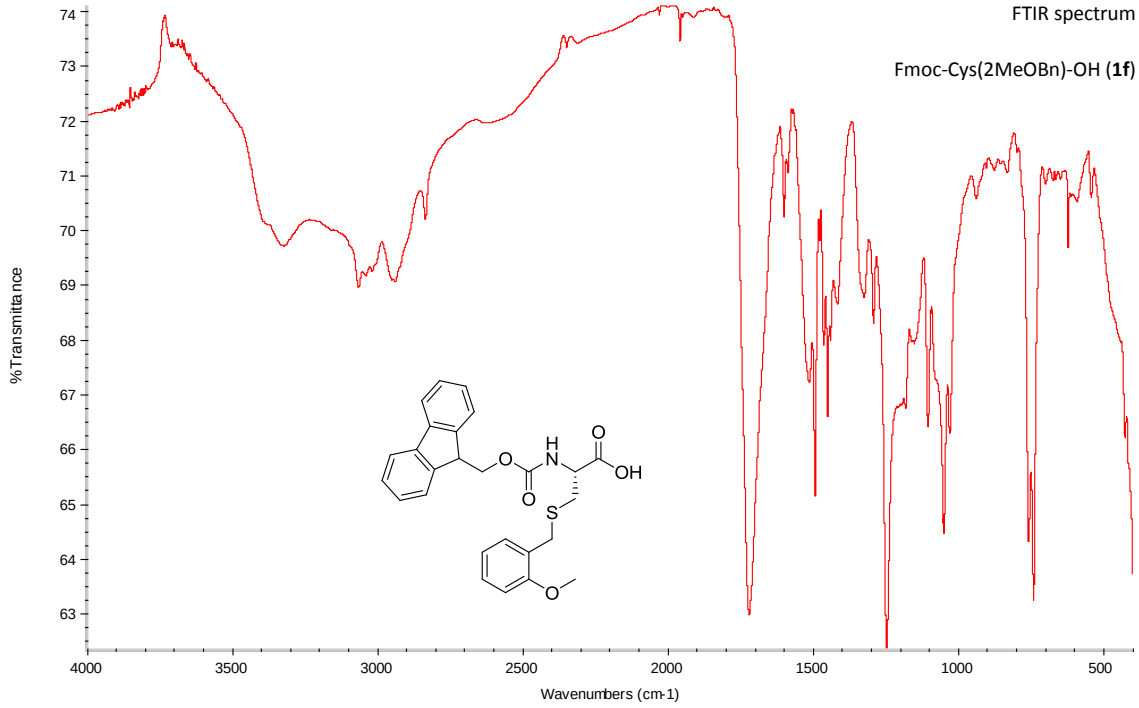
Fmoc-Cys(2MeOBn)-OH (**1f**)



^{13}C RMN (101 MHz, $CDCl_3$)

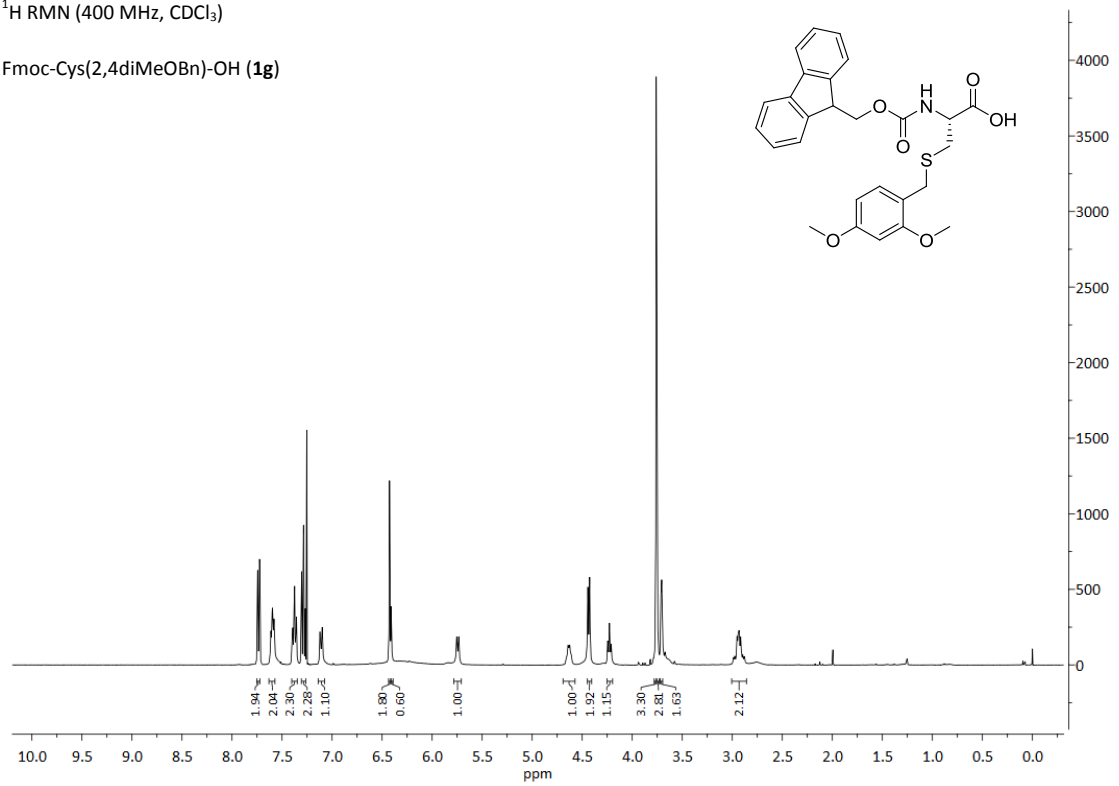
Fmoc-Cys(2MeOBn)-OH (**1f**)




FTIR spectrum
Fmoc-Cys(2MeOBn)-OH (1f)
%Transmittance
74
73
72
71
70
69
68
67
66
65
64
63
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers (cm-1)
H
N
O
OH
O
O
S
O

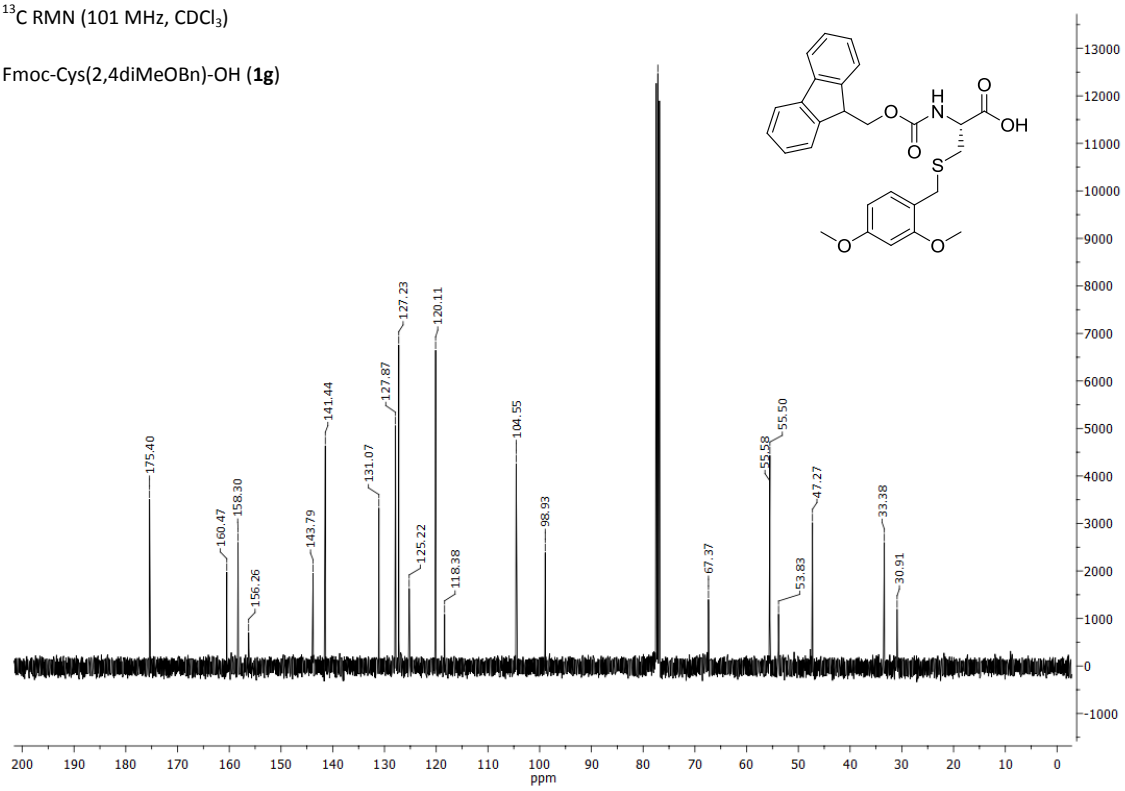
^{1}H RMN (400 MHz, $CDCl_3$)

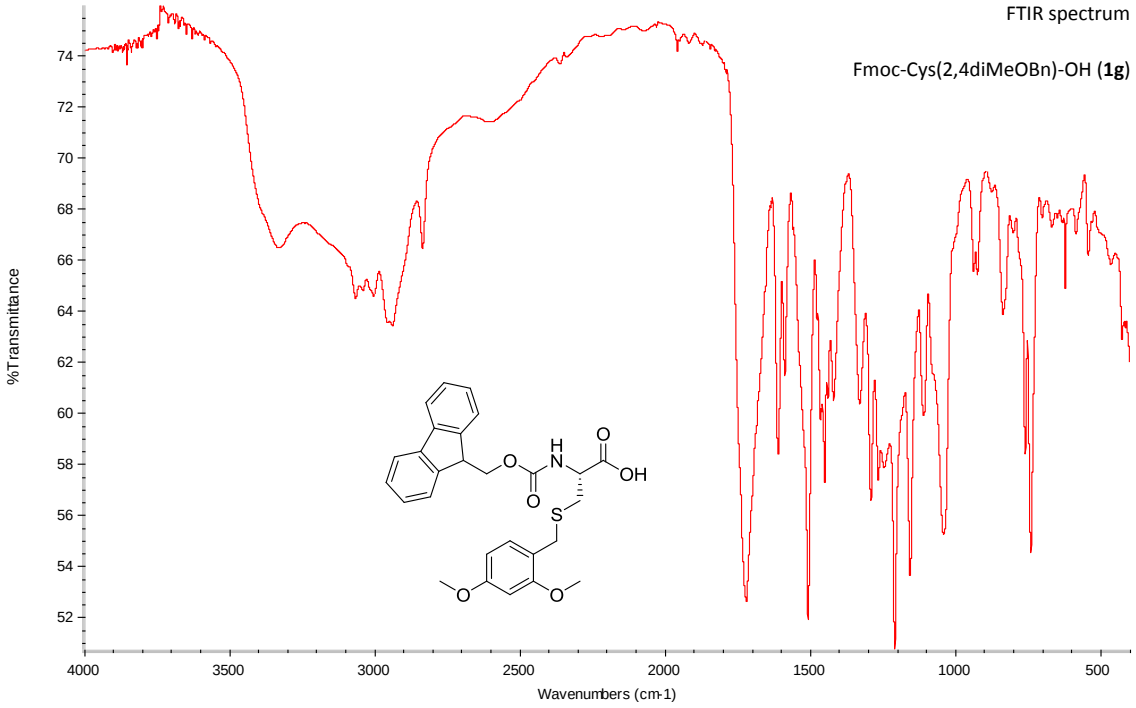
Fmoc-Cys(2,4diMeOBn)-OH (**1g**)



^{13}C RMN (101 MHz, $CDCl_3$)

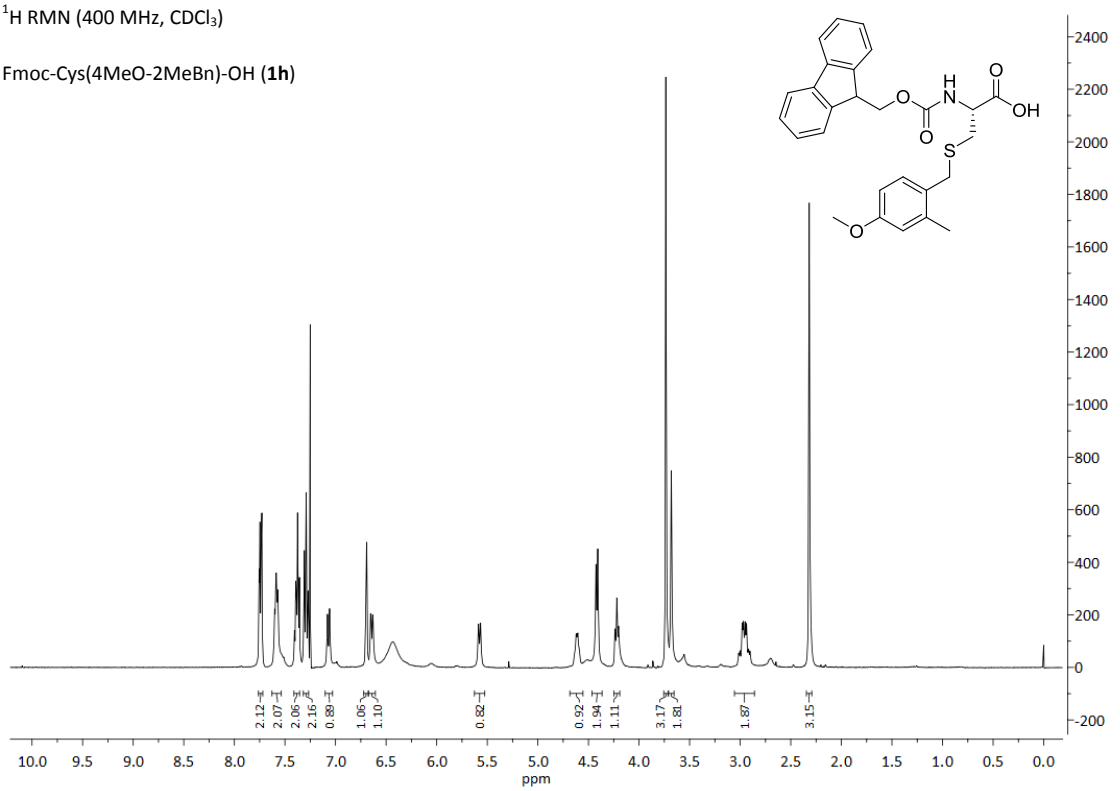
Fmoc-Cys(2,4diMeOBn)-OH (**1g**)




FTIR spectrum
Fmoc-Cys(2,4diMeOBn)-OH (1g)
%Transmittance
74
72
70
68
66
64
62
60
58
56
54
52
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers (cm-1)
O
H
N
O
OH
O
S
O
O

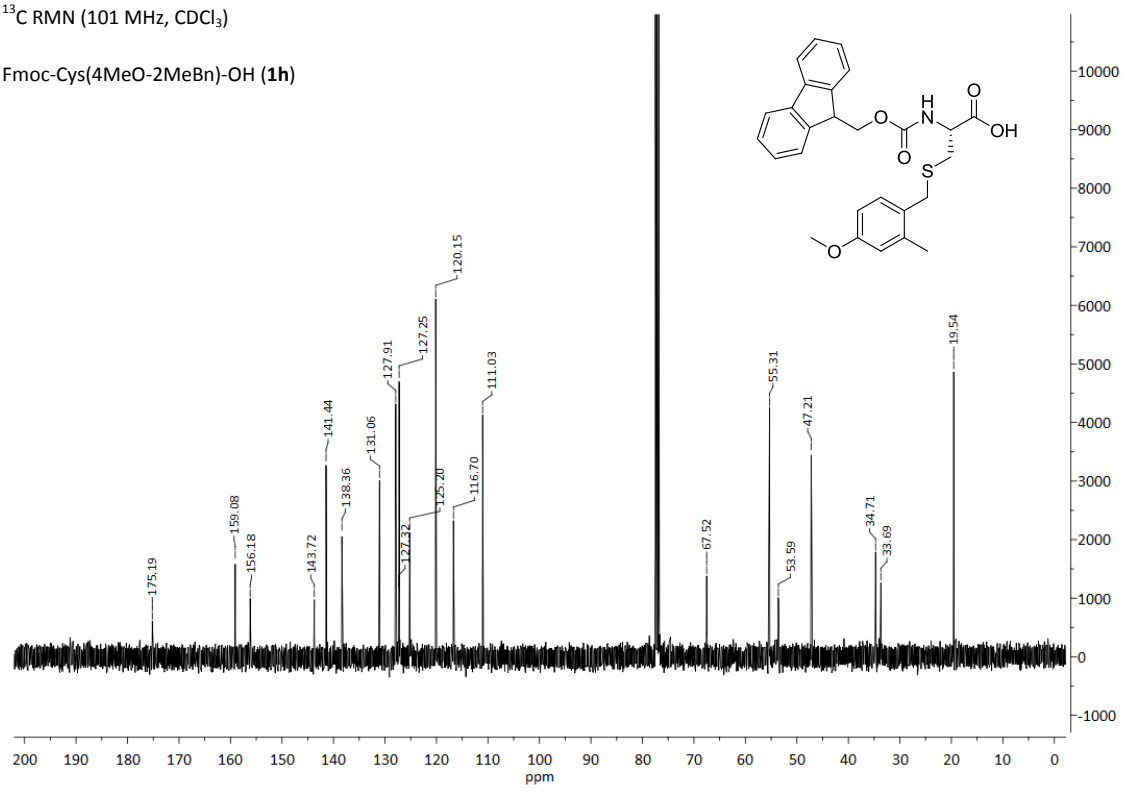
^{1}H RMN (400 MHz, $CDCl_3$)

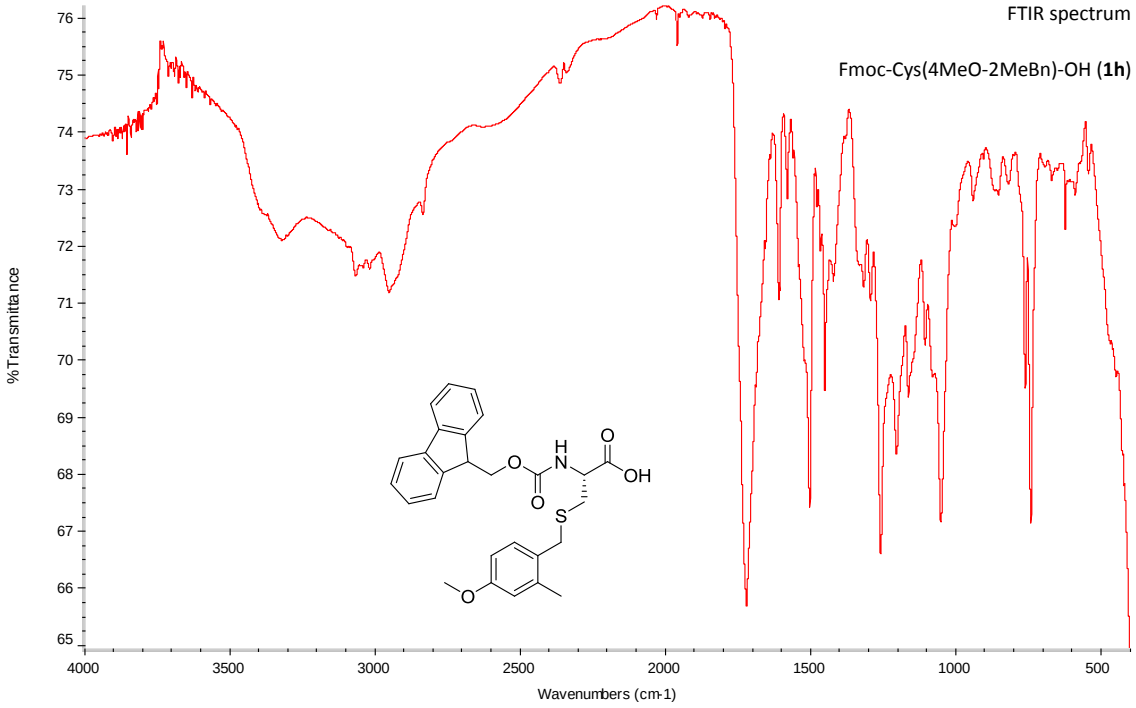
Fmoc-Cys(4MeO-2MeBn)-OH (**1h**)



^{13}C RMN (101 MHz, $CDCl_3$)

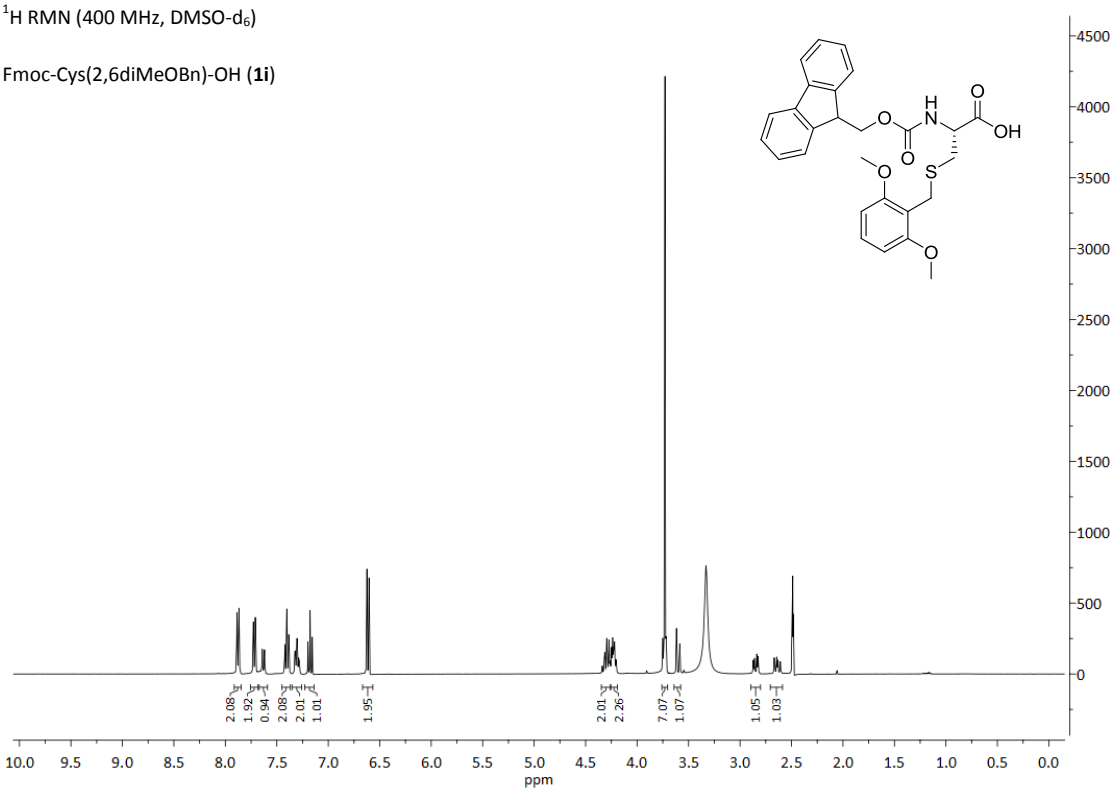
Fmoc-Cys(4MeO-2MeBn)-OH (**1h**)




FTIR spectrum
Fmoc-Cys(4MeO-2MeBn)-OH (1h)
%Transmittance
Wavenumbers (cm-1)
4000
3500
3000
2500
2000
1500
1000
500
76
75
74
73
72
71
70
69
68
67
66
65
O
H
N
O
OH
O
S
O

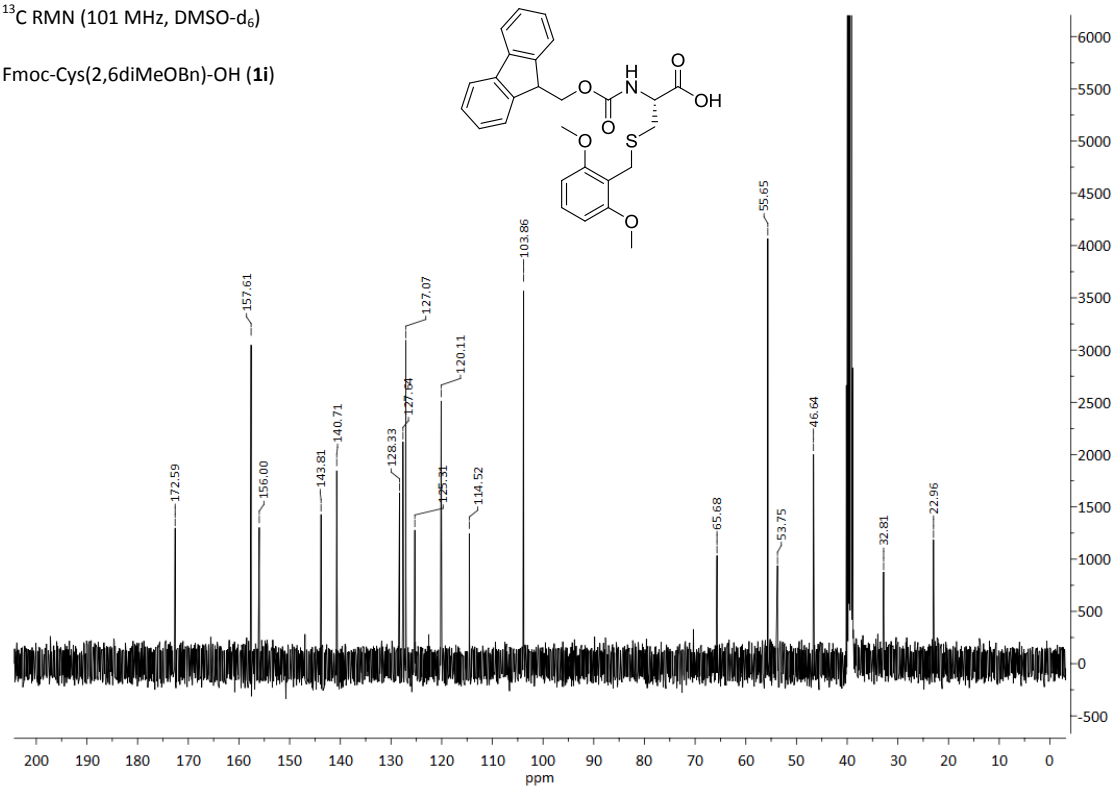
^{1}H RMN (400 MHz, DMSO-d_6)

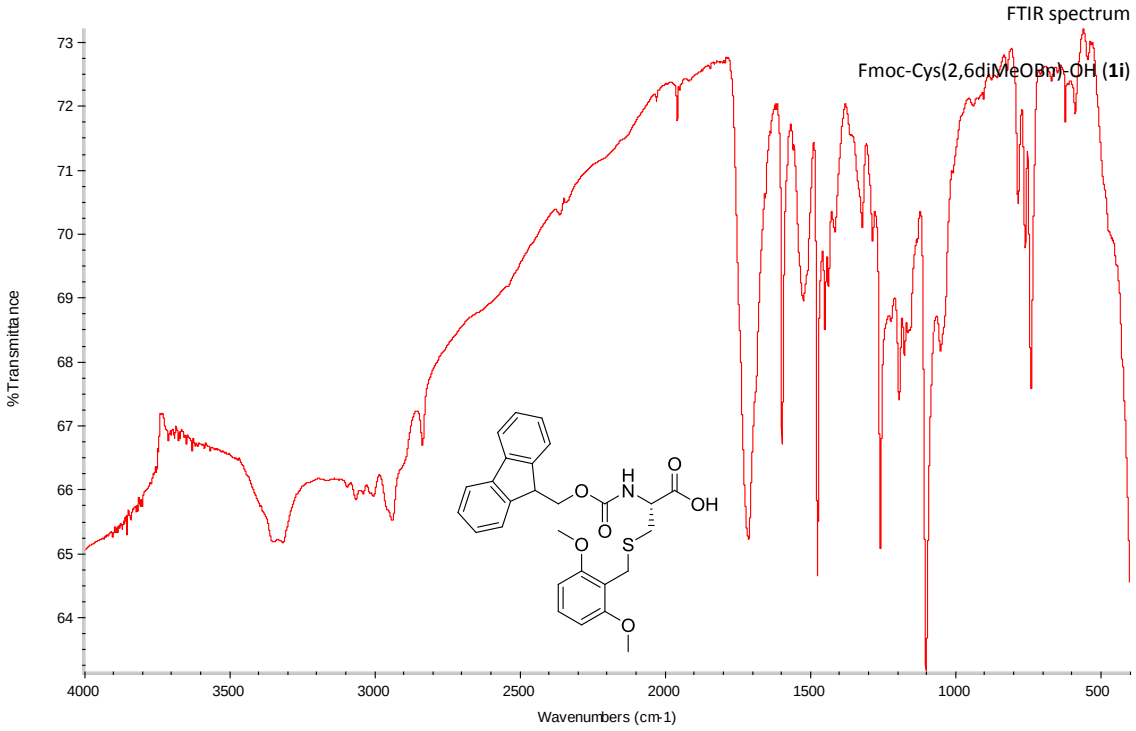
Fmoc-Cys(2,6diMeOBn)-OH (**1i**)



^{13}C RMN (101 MHz, DMSO-d_6)

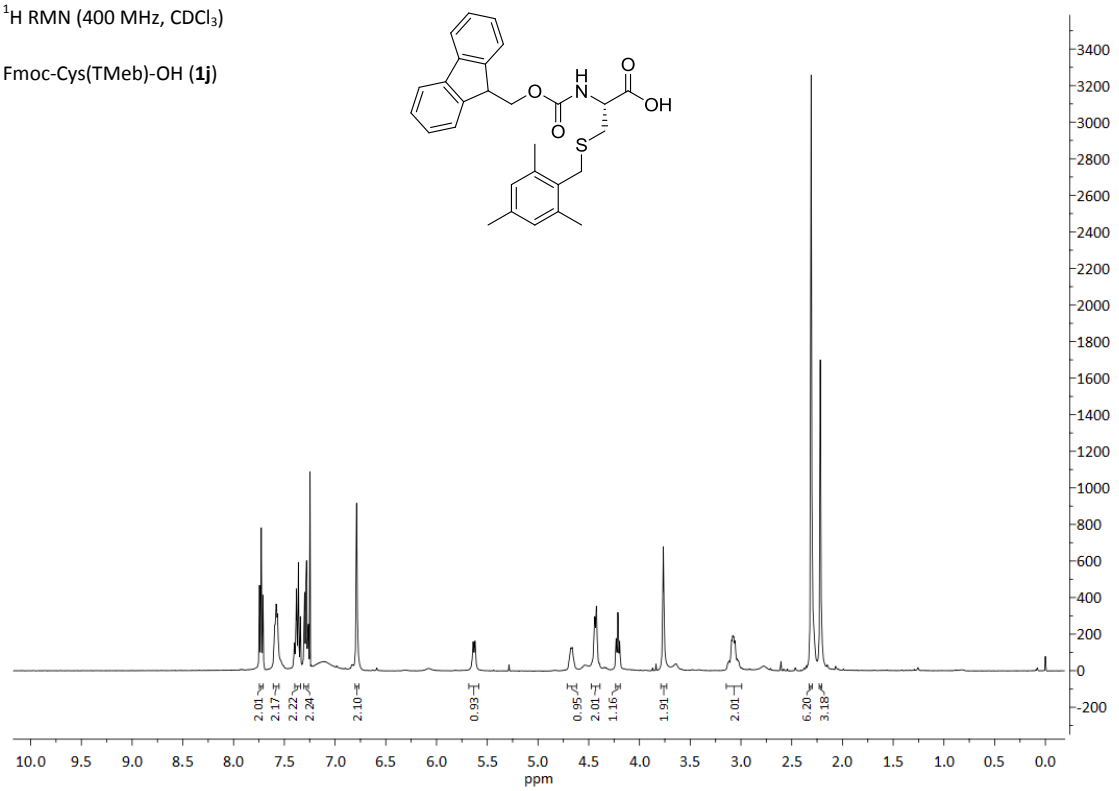
Fmoc-Cys(2,6diMeOBn)-OH (**1i**)




FTIR spectrum
Fmoc-Cys(2,6diMeOBn)-OH (1i)
%Transmittance
Wavenumbers (cm-1)
4000
3500
3000
2500
2000
1500
1000
500
73
72
71
70
69
68
67
66
65
64
H
N
O
OH
S

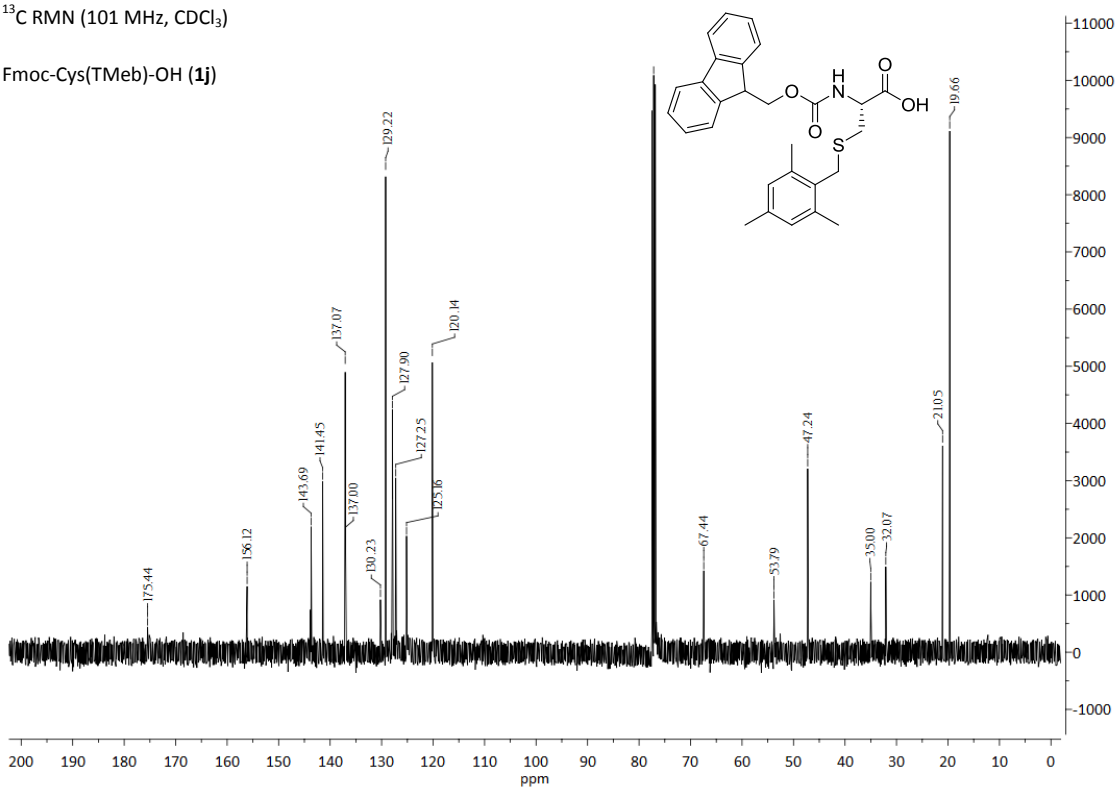
^{1}H RMN (400 MHz, $CDCl_3$)

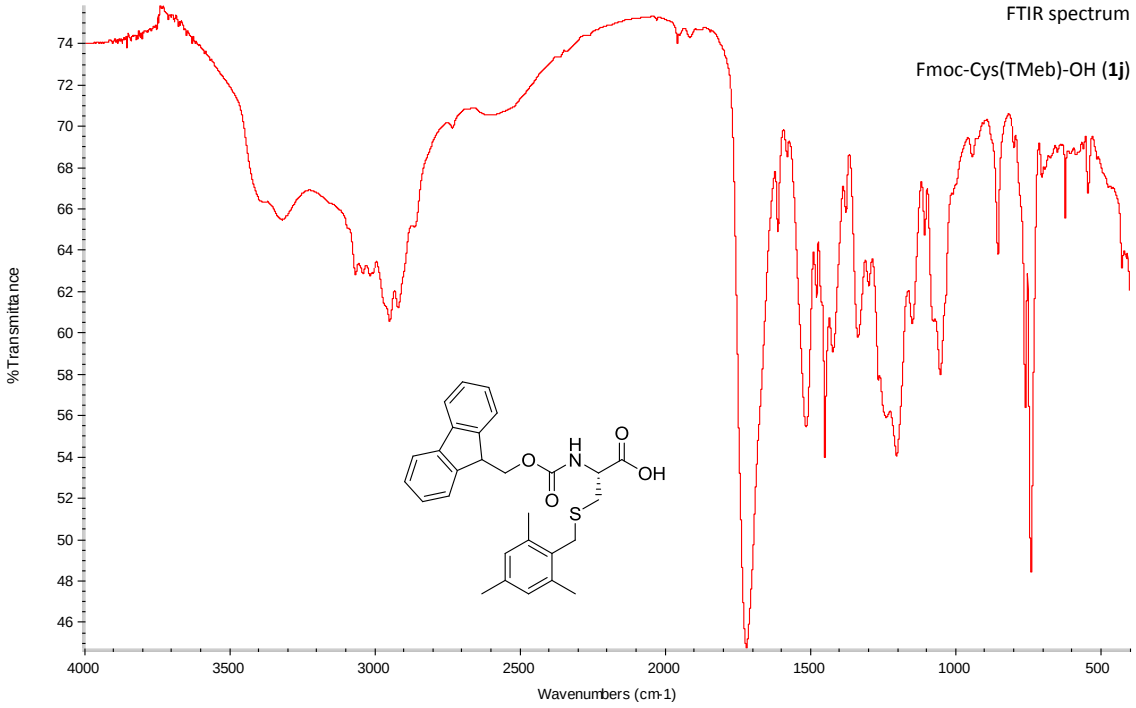
Fmoc-Cys(TMeb)-OH (**1j**)



^{13}C RMN (101 MHz, $CDCl_3$)

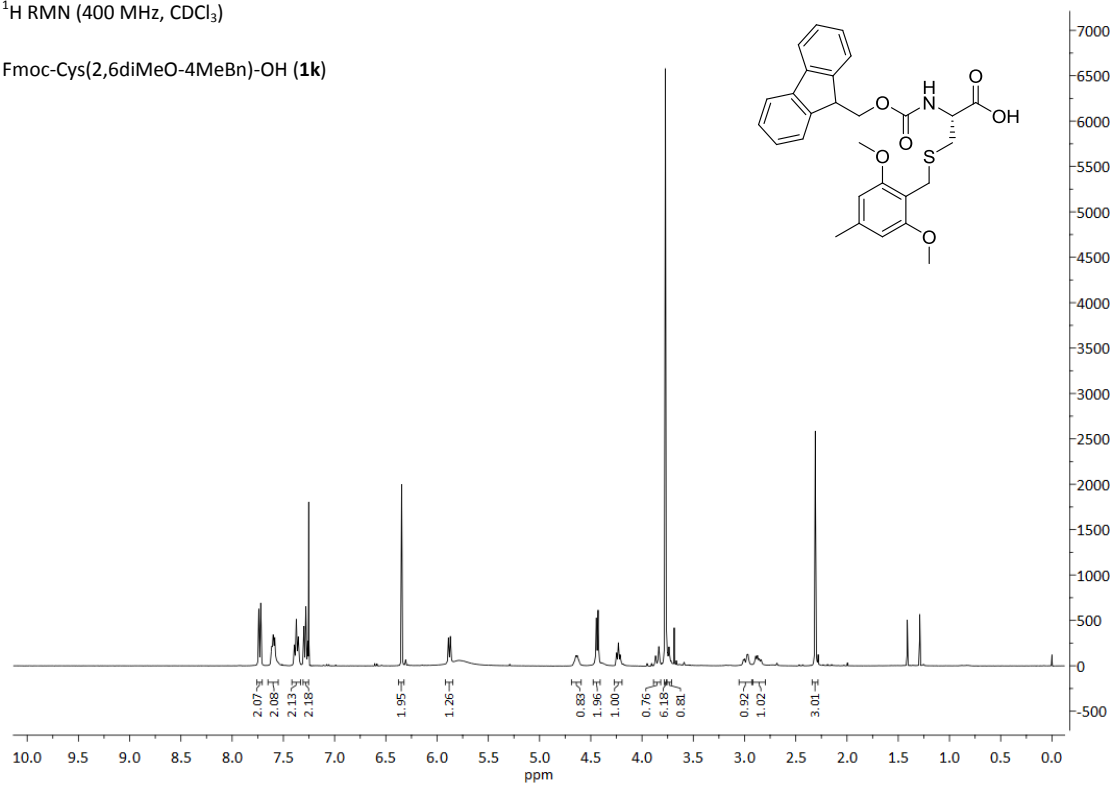
Fmoc-Cys(TMeb)-OH (**1j**)




FTIR spectrum
Fmoc-Cys(TMeb)-OH (1j)
%Transmittance
74
72
70
68
66
64
62
60
58
56
54
52
50
48
46
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers (cm-1)
O
H
N
O
OH
O
S

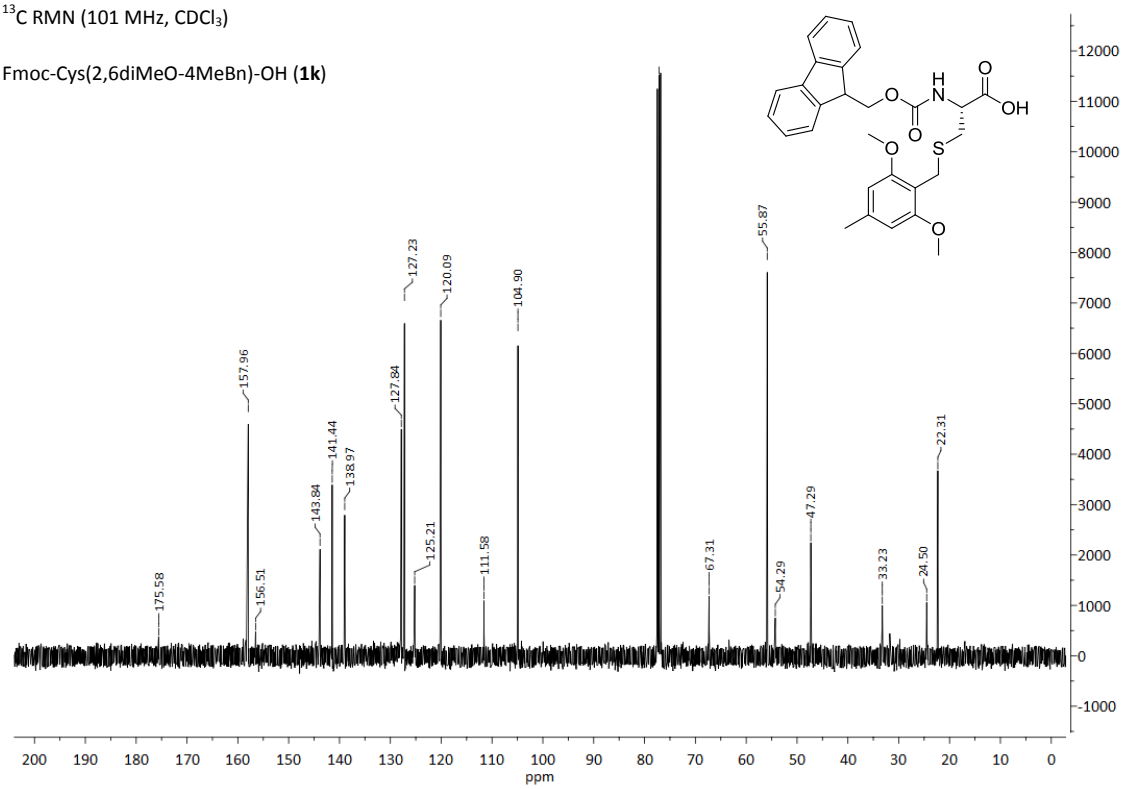
^{1}H RMN (400 MHz, $CDCl_3$)

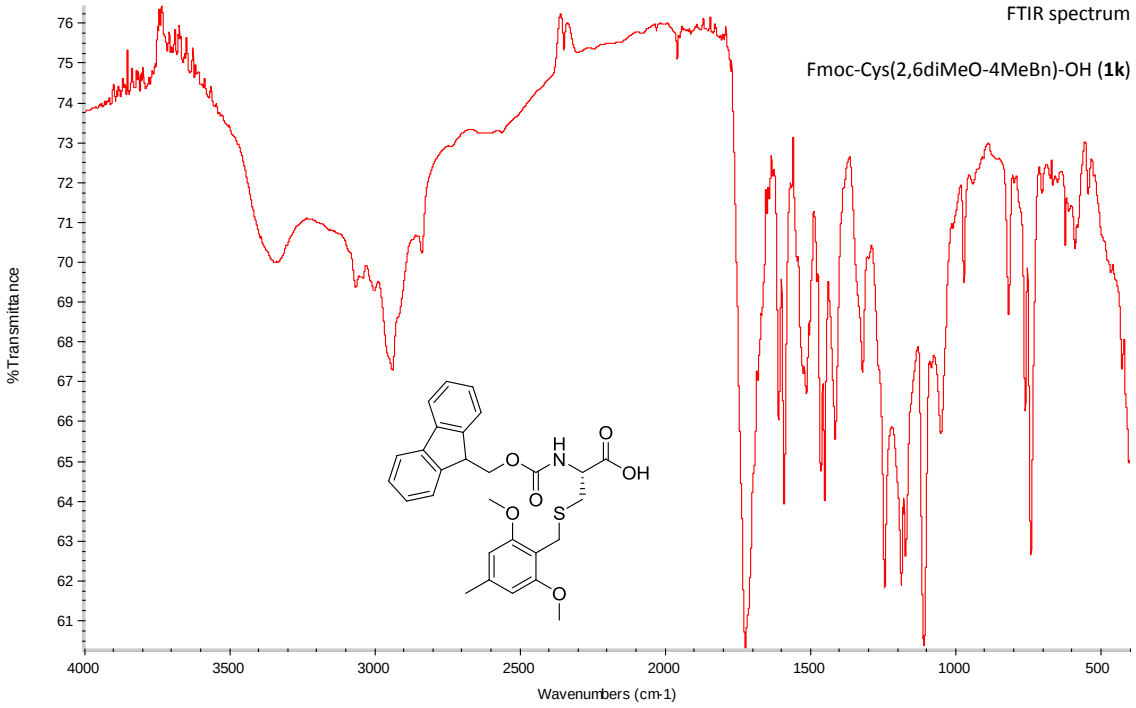
Fmoc-Cys(2,6diMeO-4MeBn)-OH (**1k**)



^{13}C RMN (101 MHz, $CDCl_3$)

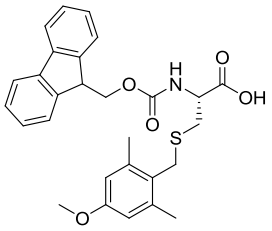
Fmoc-Cys(2,6diMeO-4MeBn)-OH (**1k**)




FTIR spectrum
Fmoc-Cys(2,6diMeO-4MeBn)-OH (**1k**)
%Transmittance
Wavenumbers (cm-1)
4000
3500
3000
2500
2000
1500
1000
500
76
75
74
73
72
71
70
69
68
67
66
65
64
63
62
61
H
N
O
OH
S

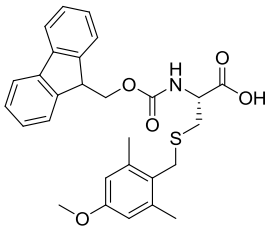
^{1}H RMN (400 MHz, DMSO-d_6)

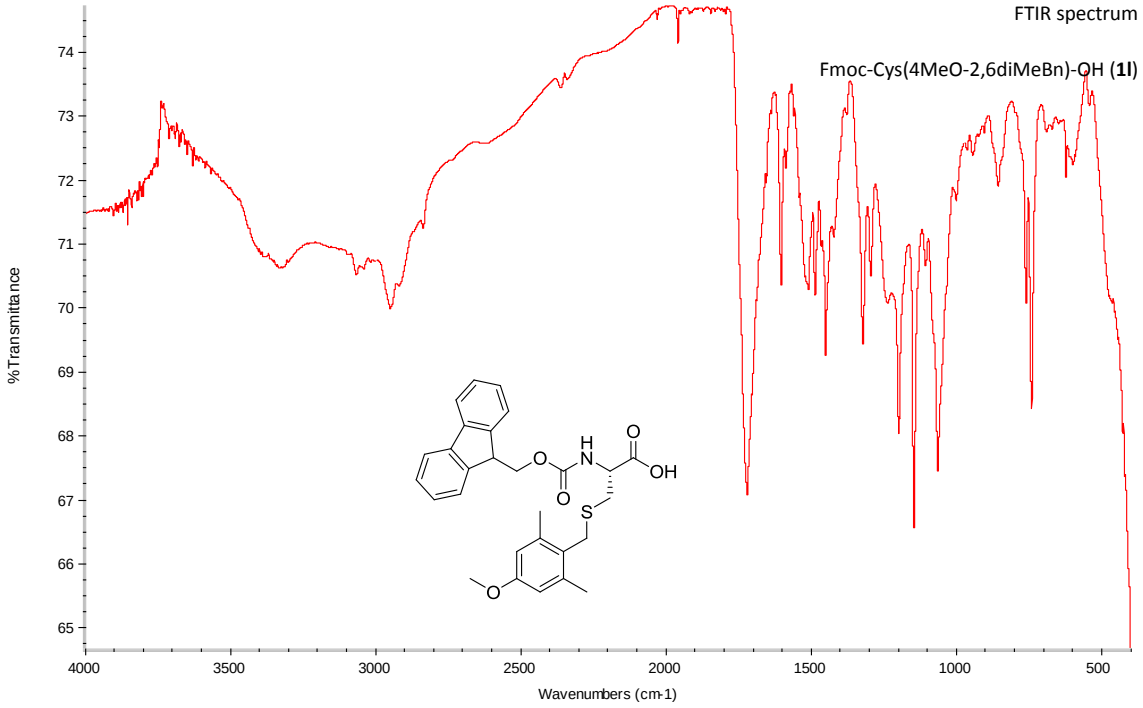
Fmoc-Cys(4MeO-2,6diMeBn)-OH (**1l**)



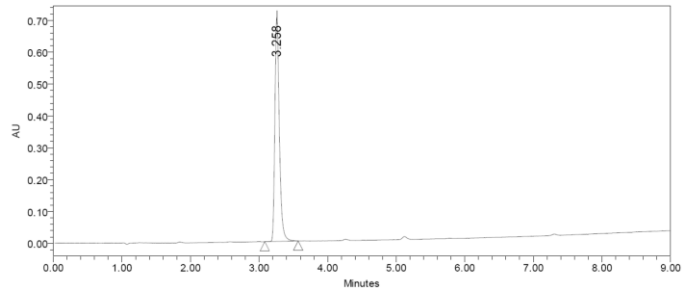
^{13}C RMN (101 MHz, DMSO-d_6)

Fmoc-Cys(4MeO-2,6diMeBn)-OH (**1l**)



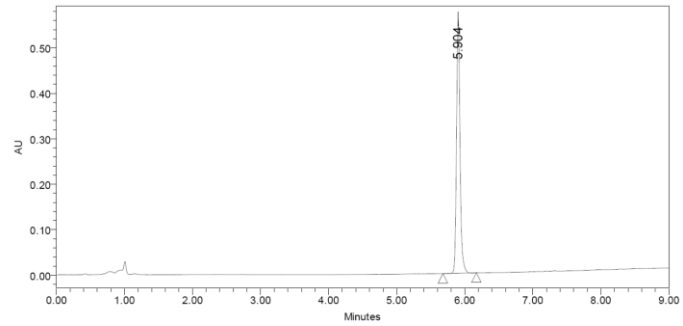

FTIR spectrum
Fmoc-Cys(4MeO-2,6diMeBn)-OH (1l)
%Transmittance
Wavenumbers (cm-1)
74
73
72
71
70
69
68
67
66
65
4000
3500
3000
2500
2000
1500
1000
500
H
N
O
OH
S
O

RP-HPLC analysis of the Fmoc-Cys(PG)-OH derivatives (1a-l)



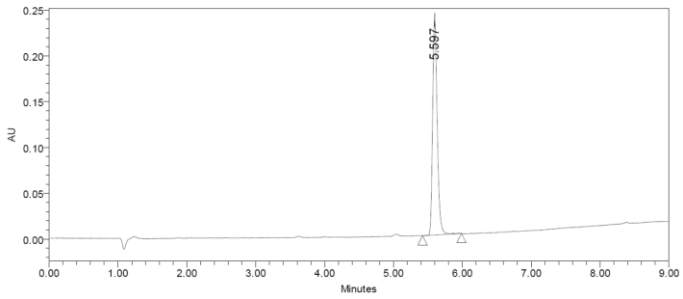
Fmoc-Cys-OH **1**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



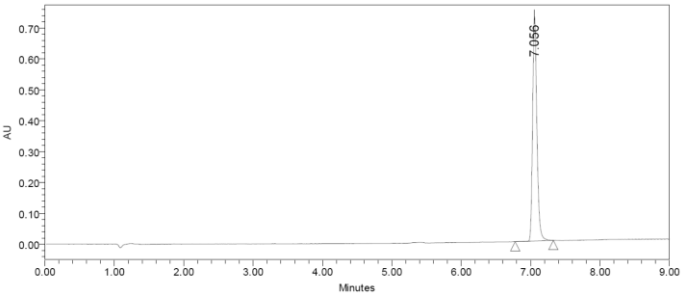
Fmoc-Cys(Dpm)-OH **1a**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



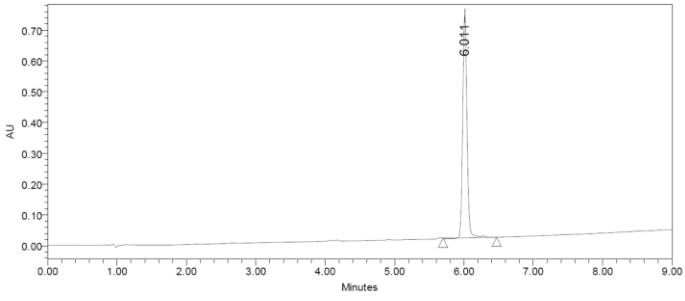
Fmoc-(4,4'diMeODpm)-OH **1b**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



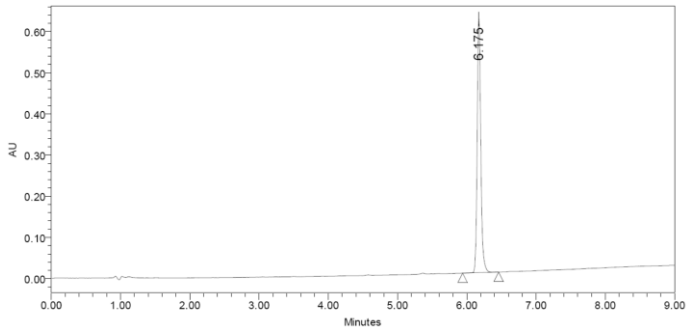
Fmoc-Cys(4,4'diMeDpm)-OH **1c**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



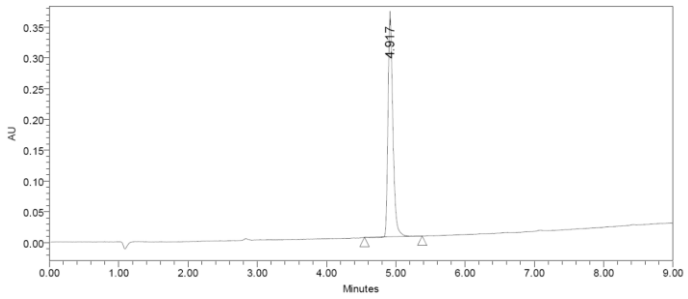
Fmoc-Cys(9F)-OH **1d**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



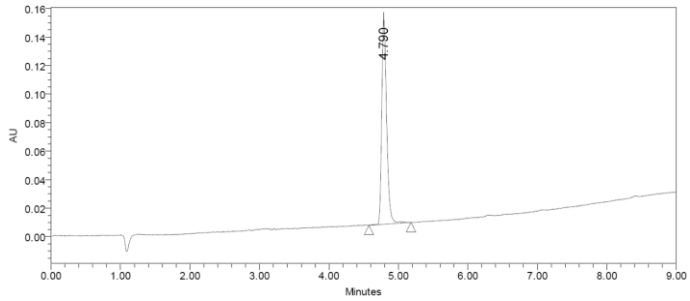
Fmoc-Cys(Bpm)-OH **1e**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



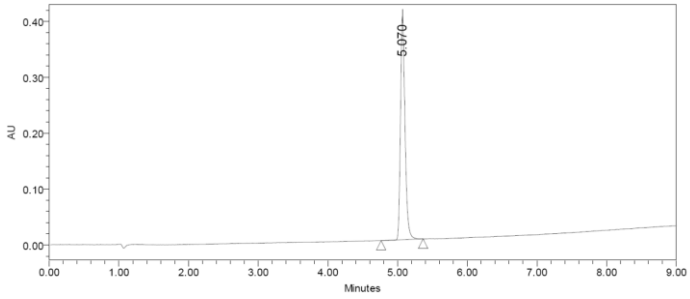
Fmoc-Cys(2MeOBn)-OH **1f**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



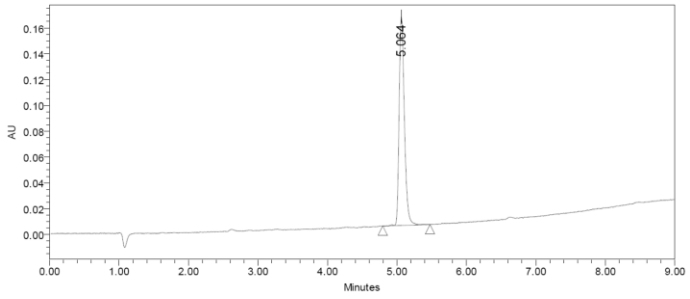
Fmoc-Cys(2,4diMeOBn)-OH **1g**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



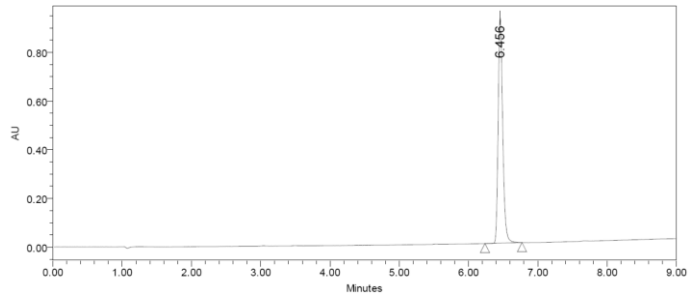
Fmoc-Cys(4MeO-2MeBn)-OH **1h**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



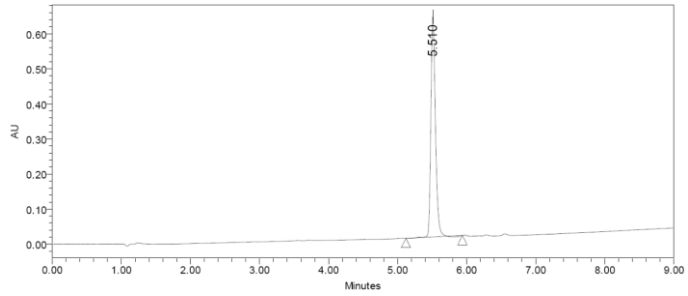
FmocCys(2,6diMeOBn)-OH **1i**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



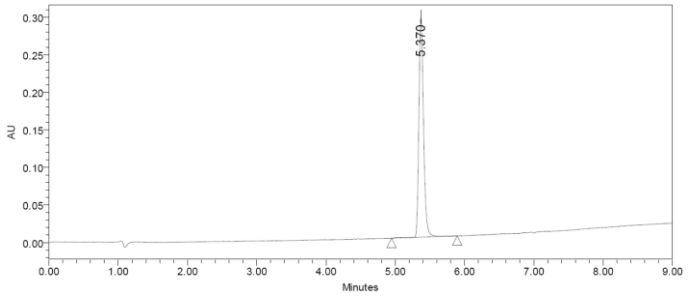
Fmoc-Cys(TMeb)-OH **1j**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



Fmoc-Cys(2,6diMeO-4MeBn)-OH **1k**

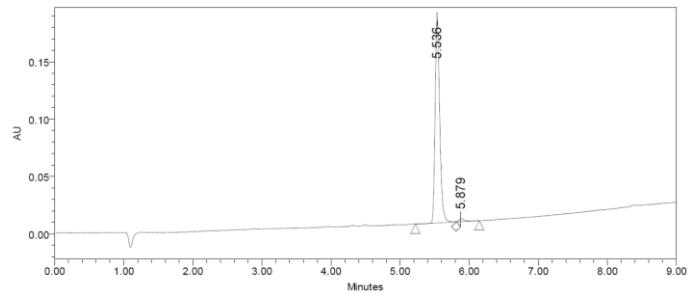
Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



Fmoc-Cys(4MeO-2,6diMeBn)-OH **1l**

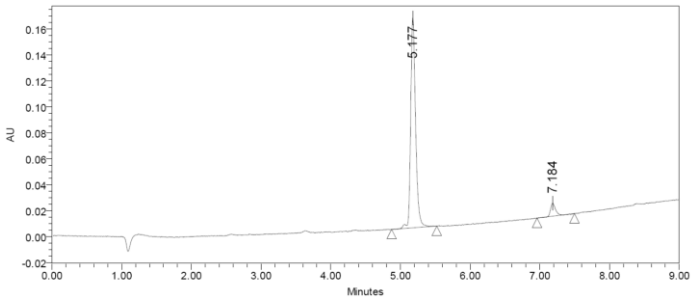
Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm

RP-HPLC analysis of the Fmoc-Ala-Cys(PG)-Leu-NH_2 tripeptides (2a-m)



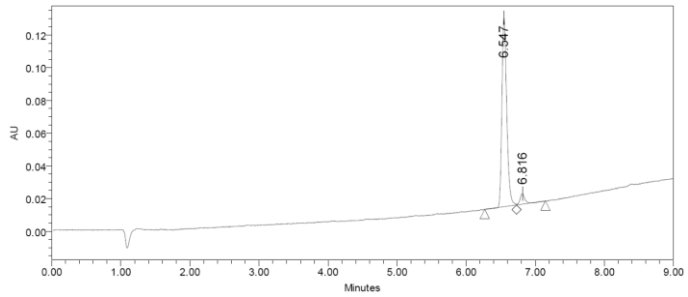
Cys(Dpm) tripeptide **2a**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



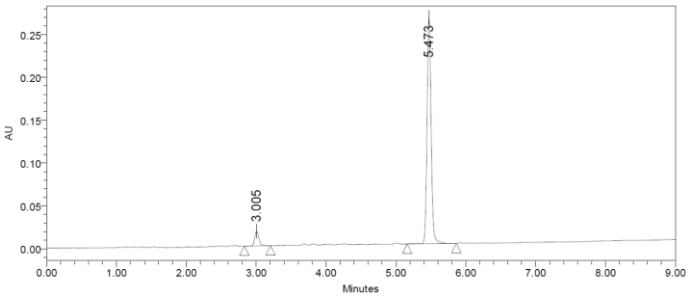
Cys(4,4'diMeODpm) tripeptide **2b**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



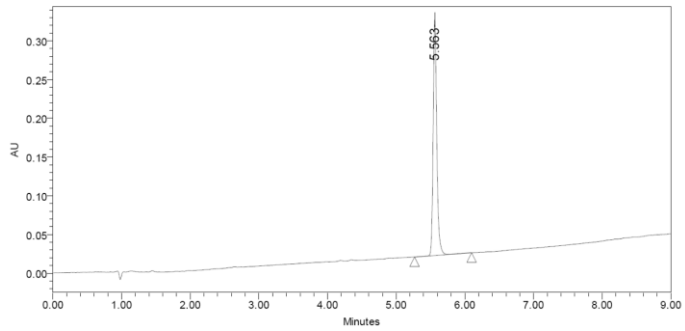
Cys(4,4'diMeDpm) tripeptide **2c**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



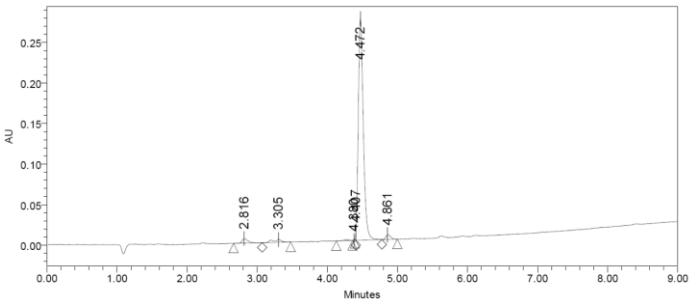
Cys(9F) tripeptide **2d**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



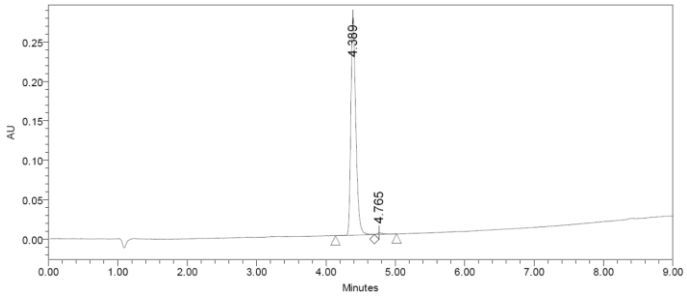
Cys(Bpm) tripeptide **2e**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



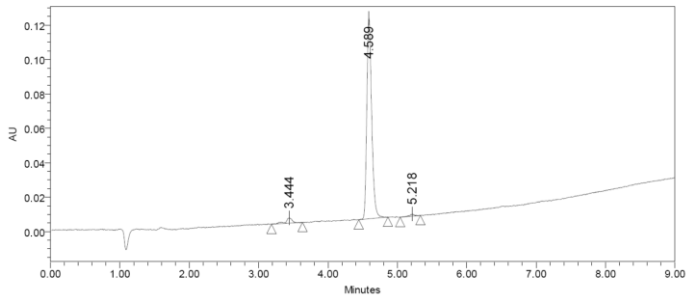
Cys(2MeOBn) tripeptide **2f**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



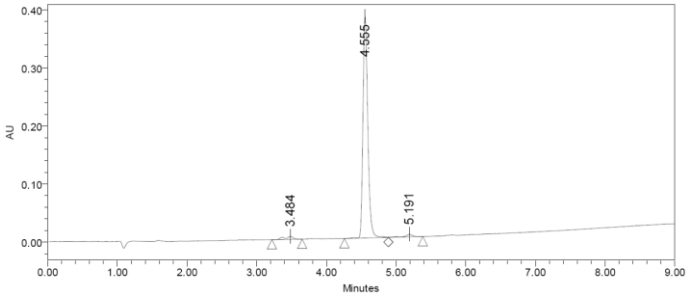
Cys(2,4diMeOBn) tripeptide **2g**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



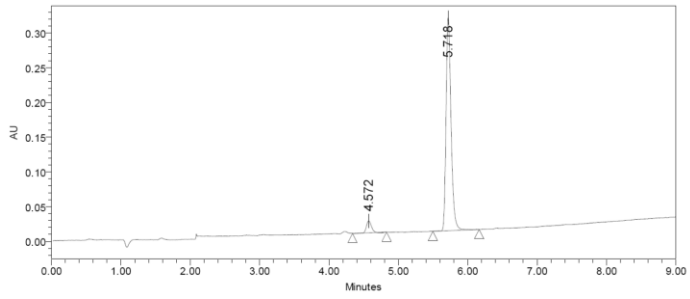
Cys(4MeO-2MeBn) tripeptide **2h**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



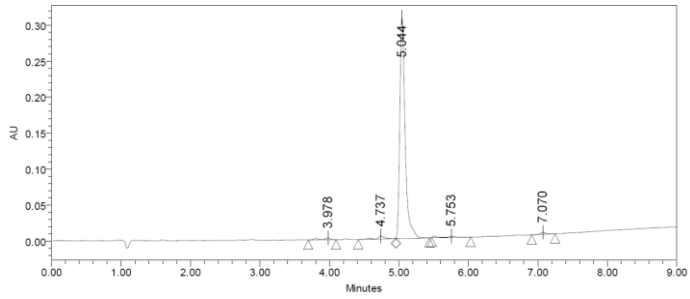
Cys(2,6diMeOBn) tripeptide **2i**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



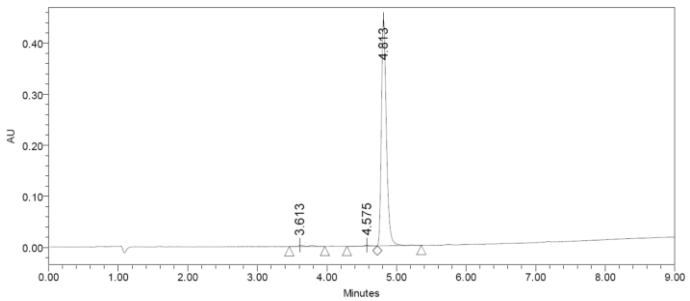
Cys(TMeb) tripeptide **2j**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



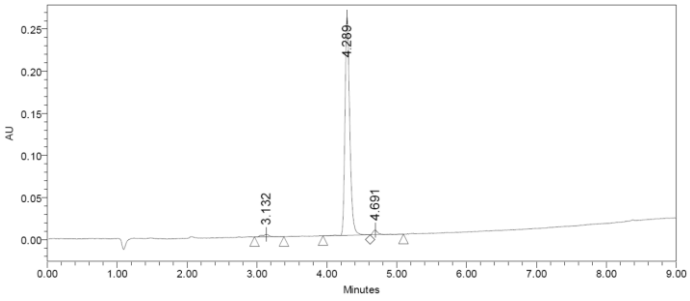
Cys(2,6diMeO-4MeBn) tripeptide **2k**

Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



Cys(2,6diMe-4MeOBn) tripeptide **2l**

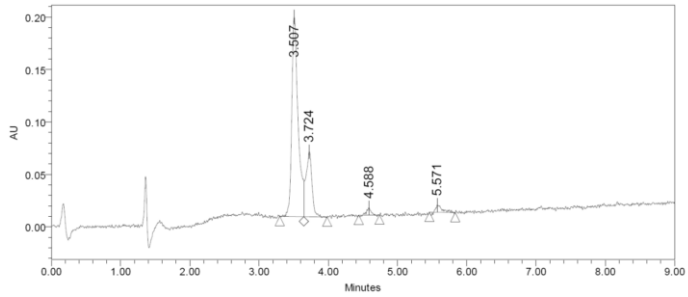
Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm



Cys(Mob) tripeptide **2m**

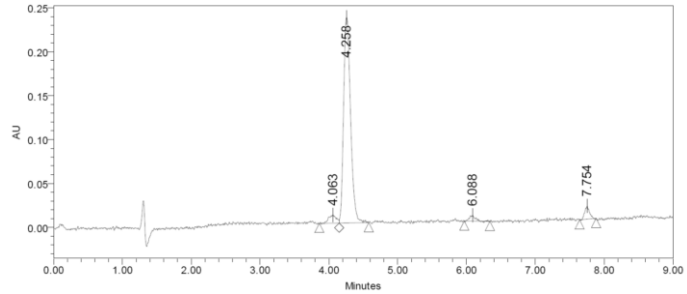
Gradient: 50% to 100% ACN at 25 °C
Wavelength: 220 nm

RP-HPLC analysis of the hinge fragment of human IgG1 (8) and intermediates



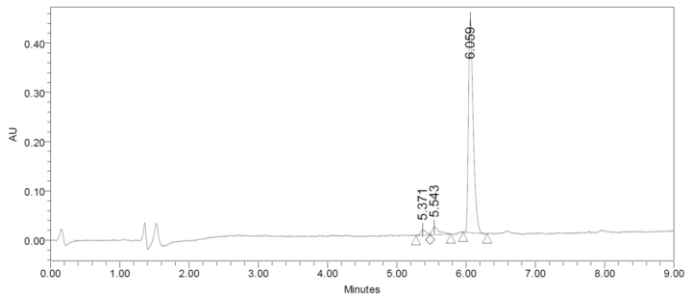
Fully deprotected linear monomer
H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-NH_2
(Rink-amide approach)

Gradient: 5% to 80% ACN at 25 °C
Wavelength: 220 nm



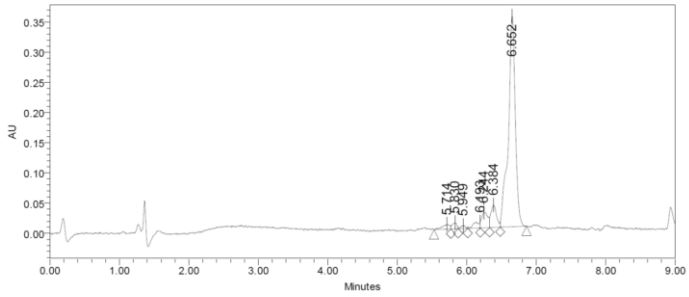
Fully deprotected linear monomer
H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-NH_2
(Rink-amide approach)

Gradient: 5% to 80% ACN at 60 °C
Wavelength: 220 nm



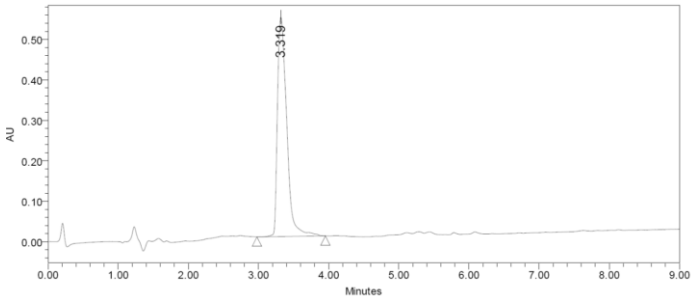
Partial deprotected linear monomer **5**
H-Thr(*t*Bu)-Cys-Pro-Pro-Cys(Dpm)-Pro-Ala-Pro-NH_2
(Sieber-amide approach)

Gradient: 5% to 80% ACN at 25 °C
Wavelength: 220 nm



Protected dimer **6**
[H-Thr(*t*Bu)-Cys-Pro-Pro-Cys(Dpm)-Pro-Ala-Pro-NH_2]$_2$
(Sieber-amide approach)

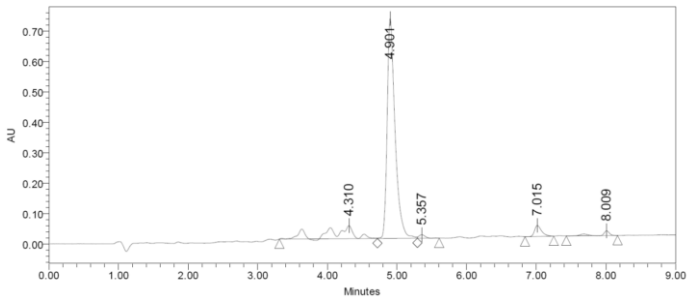
Gradient: 5% to 80% ACN at 25 °C
Wavelength: 220 nm



Final parallel dimer **8**
(Random approach)

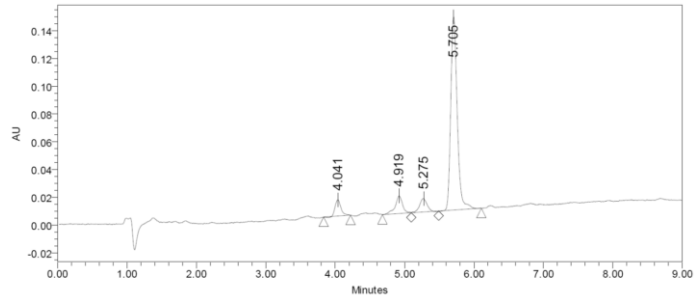
Gradient: 5% to 80% ACN at 25 °C
Wavelength: 220 nm

RP-HPLC analysis of α-conotoxin ImI and intermediates



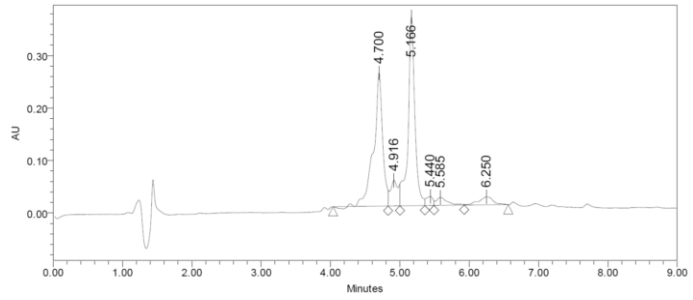
Reduced *S*-Dpm-protected intermediate **9**

Gradient: 70% to 100% ACN at 25 °C
Wavelength: 220 nm



Oxidized *S*-Dpm-protected intermediate **10**

Gradient: 70% to 100% ACN at 25 °C
Wavelength: 220 nm

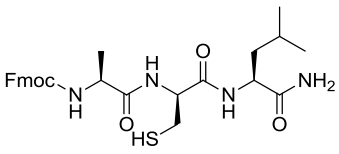


Final bicylic α-conotoxin ImI **11**
(Random strategy a)

Gradient: 10% to 35% ACN at 25 °C
Wavelength: 220 nm

Racemization study of Cys(Dpm)

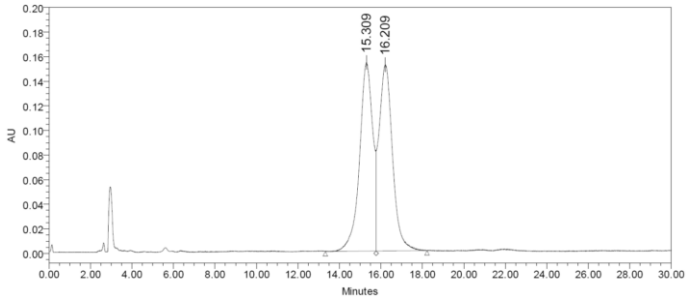
Fmoc-Ala-DCys-Leu-NH_2



Fmoc-Ala-DCys-Leu-NH_2 was synthesized as described for all tripeptides. After elongation, a portion of peptidyl-resin (5 mg) was cleaved and deprotected by using TFA-TIS-H_2O (95:2.5:2.5) for 10 min at 25 °C. RP-HPLC-ESMS showed the target tripeptide (linear gradient from 50% to 100% ACN over 8 min; t_R : 3.6 min; *m/z* calcd for $C_{27}H_{34}N_4O_5S$, 526.7; found, 527.2 $[M+H]^+$, where M is the MW of the target tripeptide).

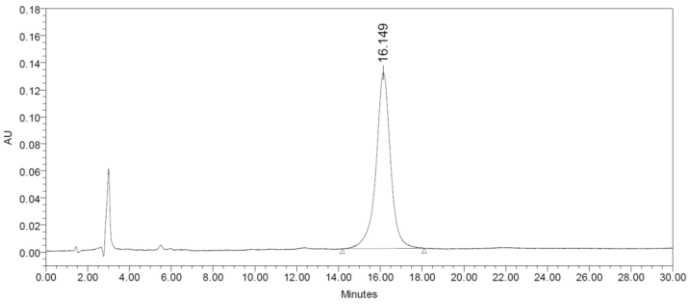
HPLC analysis of Fmoc-protected tripeptides: Fmoc-Ala-DCys-Leu-NH_2 and Fmoc-Ala-Cys-Leu-NH_2

Fmoc-Ala-DCys-Leu-NH_2 was prepared as described above and Fmoc-Ala-Cys-Leu-NH_2 was obtained by treating the Cys(Dpm) tripeptide with TFA-TIS-H_2O (95:2.5:2.5) for 1 h at 25 °C. Both thiol-free tripeptides were analyzed and compared with each other by RP-HPLC analysis on a PLRP-S reversed-phase analytical column (4.6 × 250 mm, 10 μm) from Varian.



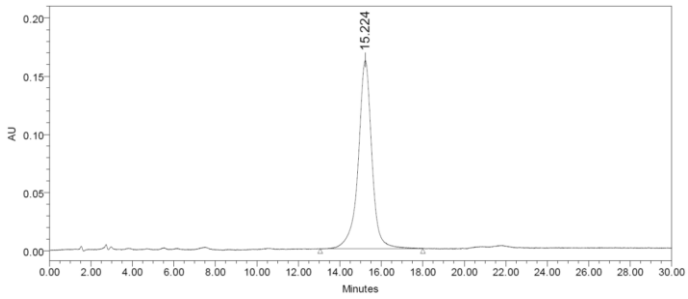
DCys and Cys
Fmoc-protected tripeptides

Gradient: 40% to 45% ACN over 30 min at 25 °C
Wavelength: 220 nm



Fmoc-Ala-DCys-Leu-NH_2

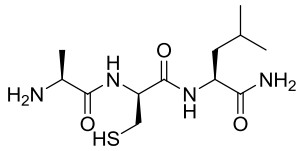
Gradient: 40% to 45% ACN over 30 min at 25 °C
Wavelength: 220 nm

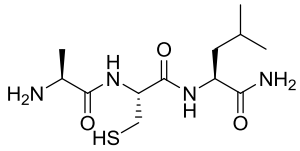


Fmoc-Ala-Cys-Leu-NH_2
from Cys(Dpm) tripeptide

Gradient: 40% to 45% ACN over 30 min at 25 °C
Wavelength: 220 nm

H-Ala-DCys-Leu-NH_2 and H-Ala-Cys-Leu-NH_2

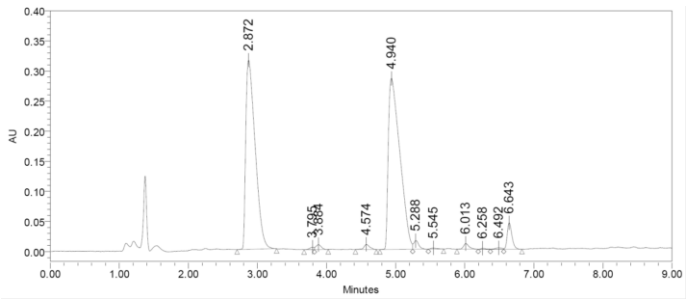




Because a clear separation was not achieved for these Fmoc-protected tripeptides, we decided to remove the Fmoc group first. Thus, a portion of DCys tripeptide anchored on the resin (5 mg) was treated with piperidine-DMF (1:4) (1 × 5 mL × 1 min, 2 × 5 mL × 5 min) and consecutively cleaved and deprotected by using TFA-TIS-H_2O (95:2.5:2.5) for 10 min at 25 °C to render the H-Ala-DCys-Leu-NH_2 tripeptide. RP-HPLC-ESMS showed the target tripeptide (linear gradient from 0% to 50% ACN over 8 min; t_R : 1.2 min; *m/z* calcd for $C_{12}H_{24}N_4O_3S$, 304.4; found, 305.1 $[M+H]^+$, where M is the MW of the target tripeptide). On the other hand, the Fmoc group form the Cys(Dpm) tripeptide was removed in solution. Therefore, the solid tripeptide (2 mg) was dissolved in piperidine-ACN (1:40) (2 mL) and left to stand for 30 min at 25 °C. Then, the solvent was removed under vacuum and the crude residue was treated with TFA-TIS-H_2O (95:2.5:2.5) for 1 h at 25 °C to render the H-Ala-Cys-Leu-NH_2 tripeptide. RP-HPLC-ESMS showed the target tripeptide (linear gradient from 0% to 50% ACN over 8 min; t_R : 1.2 min; *m/z* calcd for $C_{12}H_{24}N_4O_3S$, 304.4; found, 305.1 $[M+H]^+$, where M is the MW of the target tripeptide).

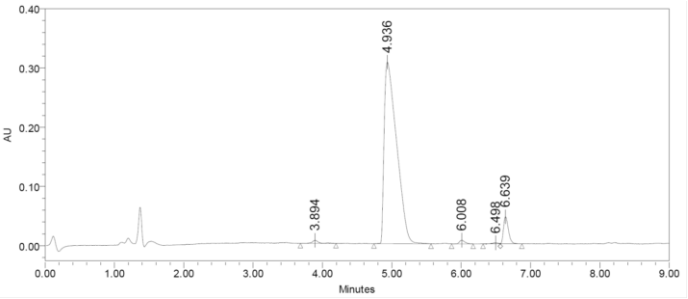
HPLC analysis of Fmoc-deprotected tripeptides: H-Ala-DCys-Leu-NH_2 and H-Ala-Cys-Leu-NH_2

Both thiol-free tripeptides were analyzed and compared with each other by RP-HPLC analysis on a XBridge™ BEH130 C18 reversed-phase HPLC analytical column (4.6 mm x 100 mm, 3.5 μm) from Waters.



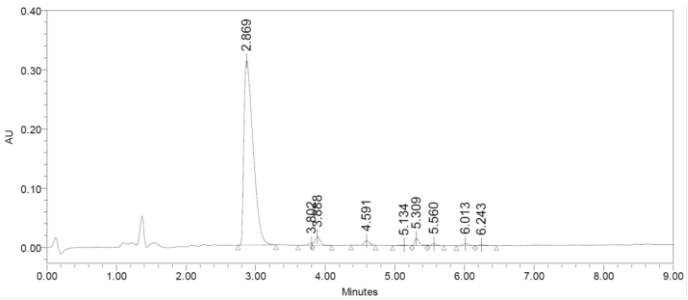
DCys and Cys
Fmoc-deprotected tripeptides

Gradient: 10% to 30% ACN over 8 min at 25 °C
Wavelength: 220 nm



H-Ala-DCys-Leu-NH_2

Gradient: 10% to 30% ACN over 8 min at 25 °C
Wavelength: 220 nm



H-Ala-Cys-Leu-NH_2
from Cys(Dpm) tripeptide

Gradient: 10% to 30% ACN over 8 min at 25 °C
Wavelength: 220 nm

No racemization occurred during Fmoc-Cys(Dpm)-OH coupling under neutral conditions, as determined by HPLC analysis.