Mixed-phase synthesis of glycopeptides using *N*-peptidyl-2,4-dinitrobenzenesulfonamide-thioacid ligation strategy†

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SUPPORTING INFORMATION

Table of Contents

General Information

Materials. Only L-amino acids were used. Fmoc Ala-preloaded Wang resin and H_2N-Ala 2-chlorotrityl resin (Loading: varying from 0.6 to 0.8 mmol/g), amino acids and HOBt were purchased from Chem-Impex International and PyBOP was acquired from Acros Organics. Fmoc-Ac_3-Tn-α-Thr-OH was obtained from Sussex Research. All other fine chemicals were sourced from the following commercial suppliers: Acros Organics, Alfa Aesar, Fisher Scientific and Sigma-Aldrich. Pyridine, and peptide grade DMF and DIEA were dried over 3Å and 4Å molecular sieves, respectively. Anhydrous CH_2Cl_2 was freshly distilled over CaH_2. Extra dry MeOH was used as received.

General Procedures. RP-HPLC analyses were carried out on a Shimadzu LC-20AT prominence liquid chromatograph equipped with DGU-$20A_3$ prominence degasser. Data was processed with Shimadzu LC solution software. Analytical RP-HPLC was performed on a Premier C8 column (150 x 4.6 mm, 5 μm) with a flow rate of 1.0 mL/min, whereas semi-preparative RP-HPLC was accomplished on a Restek UltraC8 column (150 x 10.0 mm, 5 μm) with a flow rate of 5.0 mL/min. Samples were eluted with a gradient of either water (0.1% TFA) and MeOH (0.1% TFA) or water (0.1% TFA) and acetonitrile and UV detection at 254 nm. Proton and carbon nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR) were recorded on either INOVA-600 (^{1}H NMR 600 MHz; ^{13}C NMR 150 MHz) or Varian VXR-400 (^{1}H NMR 400 MHz; ^{13}C NMR 100 MHz) spectrometers with solvent resonance as internal standard (MeOH-d_4: ^{1}H NMR at δ 3.31, ^{13}C NMR at δ 49.15; CD_3CN: ^{1}H NMR at δ 1.94). The ^{1}H NMR data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet,

q = quartet, m = multiplet), coupling constants in Hertz, integration, and assignments. ^{1}H-^{1}H gCOSY was performed on the INOVA-600 spectrometer. Low resolution mass spectra were taken on Esquire-LC electrospray ionization (ESI) mass spectrometer operated in the positive ion mode.

Solid-Phase Peptide Synthesis for dNBS capped fragments. Peptides were manually assembled on Fmoc-Ala-preloaded Wang resin (100 mg to 150 mg) using Fmoc strategy. The reactions were performed in a 20 mL syringe reactor cartridge with agitation provided by a stream of N_2. The following amino acids were used: Fmoc-Ser(O*t*Bu)-OH, Fmoc-Thr(O*t*Bu)-OH, Fmoc-Val-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Pro-OH and Fmoc-Ac_3-Tn-α-Thr-OH. The synthesis involved the following steps: (i) Fmoc deprotection with 20% piperidine in DMF (3 mL) for 25 min; (ii) Kaiser test; (iii) washing with DMF (3 x 3 mL, 5 min/wash); (iv) coupling of Fmoc amino acid (2 eq) for 3-4 h with pre-activation (2 min) in PyBOP (2 eq), HOBt (2 eq), DIEA (4 eq) and DMF; (vi) Kaiser test; (vii) washing with DMF (3 x 3 mL, 5 min/wash). Double couplings were performed sometimes as indicated by the Kaiser test. Coupling involving Fmoc-Ac_3-Tn-α-Thr-OH was carried out for 6 h using the same amount of reagents as mentioned above. After assembly, the Fmoc group was removed, the resin was washed with DMF (3 x 3 mL, 5 minutes/wash), followed by CH_2Cl_2 (3 x 3 mL, 5 minutes/wash). The resin was then reacted with 2,4-dinitrobenzenesulfonyl chloride as described below. The peptide was released from the resin following treatment with TFA/H_2O/TIPS (95:2.5:2.5), for 4 h under N_2 atmosphere. The mixture was filtered, washed with the cleavage cocktail (1-2 mL), followed by a liberal amount of CH_2Cl_2. The filtrate was concentrated to dryness *in vacuo* and the crude peptide was purified. Yields are based on the resin loading provided by the manufacturer.

General Procedure for N-Terminal Sulfonation. 2,4-dinitrobenzenesulfonyl chloride (0.096 g, 0.36 mmol), CH_2Cl_2 (0.5 mL) and pyridine (0.12 mL, 1.44 mmol) were added to the pre-swollen peptidyl resin in CH_2Cl_2 taken in a 20 mL syringe reactor cartridge. Agitation was provided at room temperature with continuous N_2 stream and CH_2Cl_2 (0.5 mL) was occasionally added whenever the reaction mixture tended to dryness. After 4 h, the mixture was washed with CH_2Cl_2 (1 x 6 mL, 5 min/wash), DMF (3 x 3 mL, 5 min/wash) and CH_2Cl_2 (3 x 3 mL, 5 min/wash). The above coupling procedure sometimes required to be performed twice. Kaiser test was used to monitor the completion of the reaction.

dNBS-Alanine. After N-terminal capping with dNBS following the general procedure described earlier, the sulfonamide was cleaved from the resin (140 mg) upon treatment with the cleavage cocktail (1.4 mL). The crude sulfonamide was purified by flash column chromatography on silica gel (230-400 mesh, 6 x 1.3 cm) using MeOH/CH_2Cl_2/CH_3COOH (5:95:0.6) to give a yellow solid (11.6 mg, 40%). ^{1}H NMR (600 MHz, CD_3OD) δ 8.72 (d, J = 2.4 Hz, 1H), 8.59 (dd, J = 2.4, 8.4 Hz, 1H), 8.34 (d, J = 8.4 Hz, 1H), 4.19 (q, J = 7.2 Hz, 1H), 1.45 (d, J = 7.2 Hz, 3H); ^{13}C NMR (400 MHz, CD_3OD) δ 174.92, 151.42, 149.41, 140.77, 133.51, 127.92, 121.37, 53.58, 19.53; mass spectrum (ESI-MS), m/z = 342.0 $[M+Na]^+$, ($C_9H_9N_3NaO_8S$ requires 342.0). This compound was reported in the literature. [‡]

dNBS-Ser-Ala-OH (1). After N-terminal capping with dNBS following the general procedure described earlier, a portion (18.4 mg) of the recovered resin (147 mg) was treated with the

cleavage cocktail (0.2 mL) to afford the crude peptide. The crude peptide was purified by reverse-phase flash column chromatography on a PrepSep C18 plug (2 x 1.5 cm) using 60 mL each of the following solvents in the order indicated: MeOH/H_2O (1:3), MeOH/H_2O (1:1), MeOH/H_2O (3:1) and MeOH to afford a white solid (3.9 mg, 85%). ^{1}H NMR (600 MHz, MeOH-d_4) δ 8.75 (d, J = 2.4 Hz, 1H), 8.59 (dd, J = 2.4, 9.0 Hz, 1H), 8.35 (d, J = 8.4 Hz, 1H), 4.16 (t, J = 6.0 Hz, IH, Ser-β-CH), 4.13 (q, J = 7.2 Hz, 1H, Ala-α-CH), 3.81-3.75 (m, 2H, Ser-α-CH, and Ser-β-CH), 1.28 (d, J = 7.2 Hz, 3H, Ala-CH_3); mass spectrum (ESI-MS), m/z = 429.2 $[M+Na]^+$ ($C_{12}H_{14}N_4NaO_{10}S$ requires 429.03).

‡ Ponomareva, E.A.; Sogulyaeva, V.M.; Serebryanyi, S.B. *Ukr. Khim. Zh. (Russ. Ed.)*. 1963, *29*, 67-72.

dNBS-Val-Thr-Ser-Ala-OH (2). After N-terminal capping with dNBS following the general procedure described earlier, a portion (20.4 mg) of the recovered resin (158 mg) was treated with the cleavage cocktail (0.2 mL) to give the crude peptide. The crude peptide was purified by semi-preparative RP-HPLC and freeze-dried to afford a white fluffy hygroscopic solid (5.4 mg, 77%). ^{1}H NMR (600 MHz, MeOH-d_4) δ 8.74 (d, J = 2.4 Hz, 1H), 8.59 (dd, J = 2.4, 9.0 Hz, 1H), 8.35 (d, J = 9.0, 1H), 4.40 (t, J = 5.4 Hz, 1H, Ser-α-CH), 4.38-4.32 (m, 1H, Ala-α-CH), 4.17 (d, J = 4.2 Hz, 1H, Thr-α-CH), 3.97 (d, J = 6.6 Hz, 1H, Val-α-CH), 3.94-3.93 (m, 1H, Thr-β-CH), 3.81 (dd, J = 6.0, 11.4 Hz, 1H, Ser-β-CH), 3.74 (dd, J = 4.8, 11.4 Hz, 1H, Ser-β-CH), 2.11 (sextet, J = 6.6 Hz, 1H, Val-β-CH), 1.39 (d, J = 7.2 Hz, 3H, Ala-CH_3), 1.00 (d, J = 6.0 Hz, 3H, Thr-CH_3), 0.99 (d, J = 6.6 Hz, 3H, Val-CH_3), 0.93 (d, J = 7.2 Hz, 3H, Val-CH_3); mass spectrum (ESI-MS), m/z = 629.3 $[M+Na]^+$ ($C_{21}H_{30}N_6NaO_{13}S$ requires 629.15).

dNBS-Gly-Val-Thr-Ser-Ala-OH (3). After N-terminal capping following the improved general procedure (Method B) described earlier, a portion (20.3 mg) of the recovered resin (170 mg) was

treated with the cleavage cocktail (0.2 mL) to give the crude peptide. The crude peptide was purified by semi-preparative RP-HPLC and freeze-dried to afford a white fluffy solid (4.4 mg, 62%). ^{1}H NMR (600 MHz, CD_3CN) δ 8.65 (d, J = 2.4 Hz, 1H), 8.51 (dd, J = 2.4, 8.4 Hz, 1H), 8.26 (d, J = 8.4 Hz, 1H), 7.26 (d, J = 6.6 Hz, 1H, NH), 7.21-7.14 (m, 3H, Val-NH, Thr-NH, and Ser-OH), 4.34-4.28 (m, 2H, Ser-α-CH, and Ala-α-CH), 4.26 (dd, J = 3.0, 7.2 Hz, 1H, Thr-α-CH), 4.18-4.14 (m, 1H, Thr-β-CH), 4.05 (t, J = 7.2 Hz, 1H, Val-α-CH), 3.96 (d, J = 17.4 Hz, 1H, Gly-α-CH), 3.92 (d, J = 17.4 Hz, 1H, Gly-α-CH), 3.80 (dd, J = 4.2, 12.0 Hz, 1H, Ser-β-CH), 3.67 (dd, J = 4.2, 11.4 Hz, 1H, Ser-β-CH), 2.09-2.04 (m, 1H, Val-β-CH), 1.34 (d, J = 7.2 Hz, 3H, Ala-CH_3), 1.09 (d, J = 6.6 Hz, 3H, Thr-CH_3), 0.88 (d, J = 3.6 Hz, 3H, Val-CH_3), 0.87 (d, J = 3.6 Hz, 3H, Val-CH_3); mass spectrum (ESI-MS), *m/z* = 686.3 $[M+Na]^+$ ($C_{23}H_{33}N_7NaO_{14}S$ requires 686.17).

dNBS-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (4). After N-terminal capping with dNBS following the general procedure described earlier, a portion (20.7 mg) of the recovered resin (205 mg) was treated with TFA/TIPS/H_2O (95:2.5:2.5) (0.2 mL) for 3 h to give the crude glycosylpeptide. The crude material was purified by semi-preparative RP-HPLC and freeze-dried to afford a white fluffy solid (4.4 mg, 49%). ^{1}H NMR (600 MHz, CD_3CN) δ 8.66 (d, J = 2.4 Hz, 1H), 8.51 (dd, J = 2.4, 8.4 Hz, 1H), 8.28 (d, J = 7.8 Hz, 1H), 7.25 (d, J = 7.8 Hz, 1H, Ser-NH), 7.17-7.15 (m, 3H, Ala-NH, Thr-NH, Val-NH), 6.82 (d, J = 9.0 Hz, 1H, N*H*Ac), 5.33 (d, J = 1.8 Hz, 1H, H-4), 5.06 (dd, J = 3.6, 11.4 Hz, 1H, H-3), 4.99 (d, J = 3.0 Hz, 1H, H-1), 4.43 (dd, J = 2.4, 8.4 Hz, 1H, Thr-α-CH), 4.39-4.36 (m, 1H, Ser-α-CH), 4.33-4.27 (m, 3H, Ala-α-CH, H-2, and H-5), 4.27-4.22 (m, 2H, Thr-β-CH and Ser-OH), 4.16 (dd, J = 6.6, 7.8 Hz, Val-α-CH), 4.06 (s, 1H, H-6'), 4.05 (d, J = 1.2 Hz, 1H, H-6), 3.97 (d, J =17.4 Hz, 1H, Gly-α-CH), 3.91 (d, J

=17.4 Hz, 1H, Gly-α-CH), 3.74 (dd, *J* = 5.4, 11.4 Hz, Ser-β-CH), 3.69 (dd, *J* = 4.8, 11.4 Hz, Ser-β-CH), 2.09 (s, 3H, CH_3CO), 2.08-2.04 (m, 1H, Val-β-CH), 1.97 (s, 3H, CH_3CO), 1.90 (s, 3H, CH_3CO), 1.87 (s, 3H, CH_3CO), 1.36 (d, *J* = 7.2 Hz, 3H, Ala-CH_3), 1.18 (d, *J* = 6.6 Hz, 3H, Thr-CH_3), 0.90 (d, *J* = 6.6 Hz, 3H, Val-CH_3), 0.88 (d, *J* = 7.2 Hz, 3H, Val-CH_3); mass spectrum (ESI-MS), *m/z* = 1015.5 $[M+Na]^+$ ($C_{37}H_{52}N_8NaO_{22}S$ requires 1015.28).

dNBS-His-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (5). After N-terminal capping with dNBS following the general procedure described earlier, a portion (70 mg) of the recovered resin (150 mg) was treated with TFA/TIPS/H_2O (95:2.5:2.5) (0.2 mL) for 3 h to give the crude glycosylpeptide. The crude material was purified by semi-preparative RP-HPLC and freeze-dried to afford a white fluffy solid (20 mg, 56%). ^{1}H NMR (600 MHz, CD_3CN) δ 8.98 (s, 1H, imidazole-NH), 8.62 (d, *J* = 1.8 Hz, 1H, aromatic), 8.53 (dd, *J* = 3.0, 8.4 Hz, 1H, aromatic), 8.41 (s, 1H, imidazole CH), 8.20 (d, *J* = 8.4 Hz, 1H aromatic), 8.05 (t, *J* = 6 Hz, 1H, Gly-NH), 7.88 (d, *J* = 8.4 Hz, 1H, NH), 7.81 (d, *J* = 6.6 Hz, 1H, NH), 7.77-7.71 (m, 1H, NH), 7.61-7.58 (m, 1H, NH), 7.23 (s, 1H, imidazole CH), 7.12 (d, *J* = 12 Hz, 1H, AcNH), 7.01 (d, *J* = 9.6 Hz, 1H, NH), 5.30 (d, *J* = 3.0 Hz, 1H, H-4), 5.07 (dd, *J* = 3, 11.4 Hz, 1H, H-3), 5.00 (d, *J* = 3.6 Hz, 1H, H-1), 4.54-4.50 (m, 1H, Thr-α-CH), 4.43-4.37 (m, 1H, Ser-α-CH), 4.29-4.22 (m, 3H, Ala-α-CH, H-2 and H-5), 4.05 (d, *J* = 6.00, 1H, His-α-CH), 3.90-3.85 (m, 2H, Thr-β-CH, Ser-OH), 3.78-3.76 (m, 1H, Val-α-CH), 3.71-3.70 (m, 2H, H-6), 3.26-3.23(m, 2H, Ser-β-CH), 3.16-3.12 (m, 2H, His-β-CH), 2.10 (s, 3H, CH_3CO), 1.97-1.94 (m, 7H, Val-β-CH, 2 CH_3CO), 1.90 (s, 3H, CH_3CO), 1.88 (d, *J* = 5.4, 3H, Ala-CH_3), 1.34 (d, *J* = 7.2, 3H, Thr-CH_3), 1.22 (dd, *J* = 6.6, 15.6 Hz, 3H, Val-CH_3), 0.89 (dd, *J* = 6.6, 22.2, 3H, Val-CH_3); mass spectrum (ESI-MS), *m/z* = 1130.5 $[M+H]^+$ ($C_{43}H_{60}N_{11}O_{23}S$ requires 1130.35).

***N*-α-*N*-Fmoc-*N*-im-Trityl-Protected L-Histidine Trityl Thioester.** Fmoc protected *N*(trityl)-histidine (0.177 g, 0.29 mmol), HATU (0.166 g, 0.44mmol) and tritylmercaptan (0.087 g, 0.31 mmol) were dried in vacuum for 30 minutes and to this dried mixture dry DMF (1.0 mL) was added followed by DIEA (0.20 mL, 1.26 mmol). The resulting solution was stirred for 2 h and completion of the reaction was confirmed by TLC. The reaction mixture was diluted with ethyl acetate (20 mL), washed with water (2 x 10 mL) and NaCl (1 x 10 mL). The organic layer was dried over sodium sulfate and concentrated under reduced pressure. The crude material was purified by flash column chromatography on SiO_2 using stepwise gradient of acetone-ethyl acetate-hexane (5:5:90 and 10:10:80) to provide a colorless powder. Yield: 0.231 g (92%); TLC R_f = 0.09 (acetone-ethyl acetate-hexane =7.5:7.5:85); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.73 (d, J = 7.8 Hz, 2H, aromatic H), 7.26 (t, J = 9.0, 2H, aromatic H), 7.39 (s, 1H, imidazole H), 7.35 (t, J = 7.8 Hz, 2H, aromatic H), 7.31-7.25 (m, 8H, aromatic H), 7.22-7.08 (m, 24H, aromatic H), 7.02 (d, J = 7.8 Hz, 1H, NH), 6.61 (s, 1H, imidazole H), 4.58 (q, J = 7.8 Hz, 1H, α-CH), 4.45 (dd, J = 6.0, 9.0 Hz, 1H, Fmoc CH), 4.55-4.40 (m, 2H, Fmoc CH_2), 2.95 (d, J = 5.4, 2H, β-CH); ^{13}C NMR (150 MHz, $CDCl_3$): δ 198.43 (SC=O), 156.23,144.17,144.03,143.88, 142.48, 141.38, 141.36, 138.96,136.19, 130.04, 129.96, 128.27, 128.25, 127.83, 127.77, 127.75, 127.24, 127.20, 127.10, 125.62, 125.46, 120.04, 119.59, 75.50, 70.11, 67.48, 61.25, 47.28, 29.88; mass spectrum (ESI-MS), *m/z* = 878.0 $[M+H]^+$ ($C_{59}H_{49}N_3O_3S$ requires 878.3).

Fmoc-His-SH (6). The *N*-α-*N*-Fmoc-*N*-im-Trityl-Protected L-Histidine Trityl Thioester (0.2 g) was treated with cleavage cocktail of TFA-TIPS-CH_2Cl_2 (50:5:45) for 15 minutes under N_2 atmosphere. The mixture was concentrated to dryness under reduced pressure and the crude

material was kept under high vacuum for an hour. The desired thioacid was detected by ESI-MS and the crude was directly taken to the thioacid-dNBS ligation reaction without any further purification.

Solid Phase Synthesis of Fmoc-Pro-Ala-OH. The Fmoc-Pro-OH was manually assembled on a H_2N-Ala preloaded 2-chlorotrityl resin (0.1 g) using Fmoc strategy. The reaction was performed in a 20 mL syringe reactor cartridge with agitation provided by a stream of N_2. The synthesis involved the following steps: (i) coupling of Fmoc-Pro-OH (4 eq) for 3 h with pre-activation (2 min) in PyBOP (4 eq), HOBt (4 eq), DIEA (8 eq) and DMF (0.5 mL); (ii) Kaiser test; (iii) washing with DMF (3 x 3 mL, 5 min/wash). (iv) washing with DMF (3 x 3 mL, 5 min/wash followed by CH_2Cl_2 (3 x 3 mL, 5 min/wash) Double coupling was performed to achieve a complete reaction. The resin was treated with cleavage cocktail of TFE-AcOH-CH_2Cl_2 (2:2:6) (3 mL) for 4 h at room temperature under N_2 atmosphere followed by washing the resin with a liberal amount of CH_2Cl_2. Evaporation of the solvents from the washings gave a thick oil which after a couple of co-evaporation with toluene afforded the desired dipeptide as a white solid. The purity of the product was satisfactory enough according to TLC, ESI-MS and ^{1}HNMR and it was directly used for the next step without further purification. Yield: 0.026 g (91%). TLC R_f = 0.3 (methanol-dichloromethane = 1:9). This compound was reported in the literature. ‡

***N*-Fmoc-L-prolyl-L-alanine Trityl Thioester (9).** The peptide (19.7 mg, 0.048 mmol) was reacted with HATU (62.9 mg, 0.17 mmol), tritylmercaptan (32.6 mg, 0.12 mmol), DIPEA (0.035 mL, 0.21mmol) in DMF (0.75 mL) to furnish the thioester after 3 h following the General Procedure given for amino acid thioesters. The crude material was purified by flash column

‡Basak A.; Bag S. S.; Basak A.; *Bioorg.Med.Chem.,* 2005,

chromatography on SiO_2 (1.6 x 14 cm) using a step-wise gradient elution with MeOH-CH_2Cl_2 ‡Basak A.; Bag S. S.; Basak A.; *Bioorg.Med.Chem.,* 2005, **13**, 4096-4102. (0:100 and 0.5:99.5) to afford the thioester as a yellowish film. Yield: 11.7 mg (36%); silica gel TLC R_f = 0.09 (MeOH-$CHCl_3$ = 1:99); ESI-MS: *m/z* calcd for $C_{42}H_{38}NaN_2O_4S$ 689.2, found: 689.1 $[M+Na]^+$.

Fmoc-Pro-Ala-SH (10). To a solution of CDI (0.025 g, 0.159 mmol) in CH_2Cl_2 (1 mL) a solution of Fmoc-Pro-Ala-OH (0.013 g, 0.0318 mmol) in CH_2Cl_2 (1.5 mL) was added and stirred for 30 min under N_2 atmosphere. Then NaHS was added to the mixture and the mixture was stirred for another 3 h under N2 atmosphere. The completion of the reaction was confirmed by ESI-MS monitoring. The reaction mixture was diluted with CH_2Cl_2 (6 mL) and the pH was brought to 3 by adding ice cold 1(N) HCl. The organic layer was immediately separated, washed with brine and dried over sodium sulfate. Evaporation of the solvent afforded the desired crude product as yellowish white semisolid (0.010 g, 74%). It was directly used in the thioacid-dNBS ligation reaction without further purification. Mass spectrum (ESI-MS), *m/z* = 447.4 $[M+Na]^+$ ($C_{23}H_{24}N_2O_4SNa$ requires 447.5).

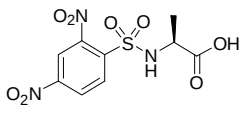
General Procedure for Thioacid-dNBS ligation: To a DMF solution of peptidyl thioacid (1.2 eq), Cs_2CO_3 (2 eq) was added followed by DMF solution of the dNBS-peptide (1 eq). the reaction mixture is stirred for 15 minutes under N_2 atmosphere at room temperature. The completion of the reaction was monitored by ESI-MS. The DMF was co-evaporated with toluene under reduced pressure to get the crude peptide as yellowish semisolid. The crude material was purified by semi-preparative RP-HPLC and freeze-dried to afford the pure product.

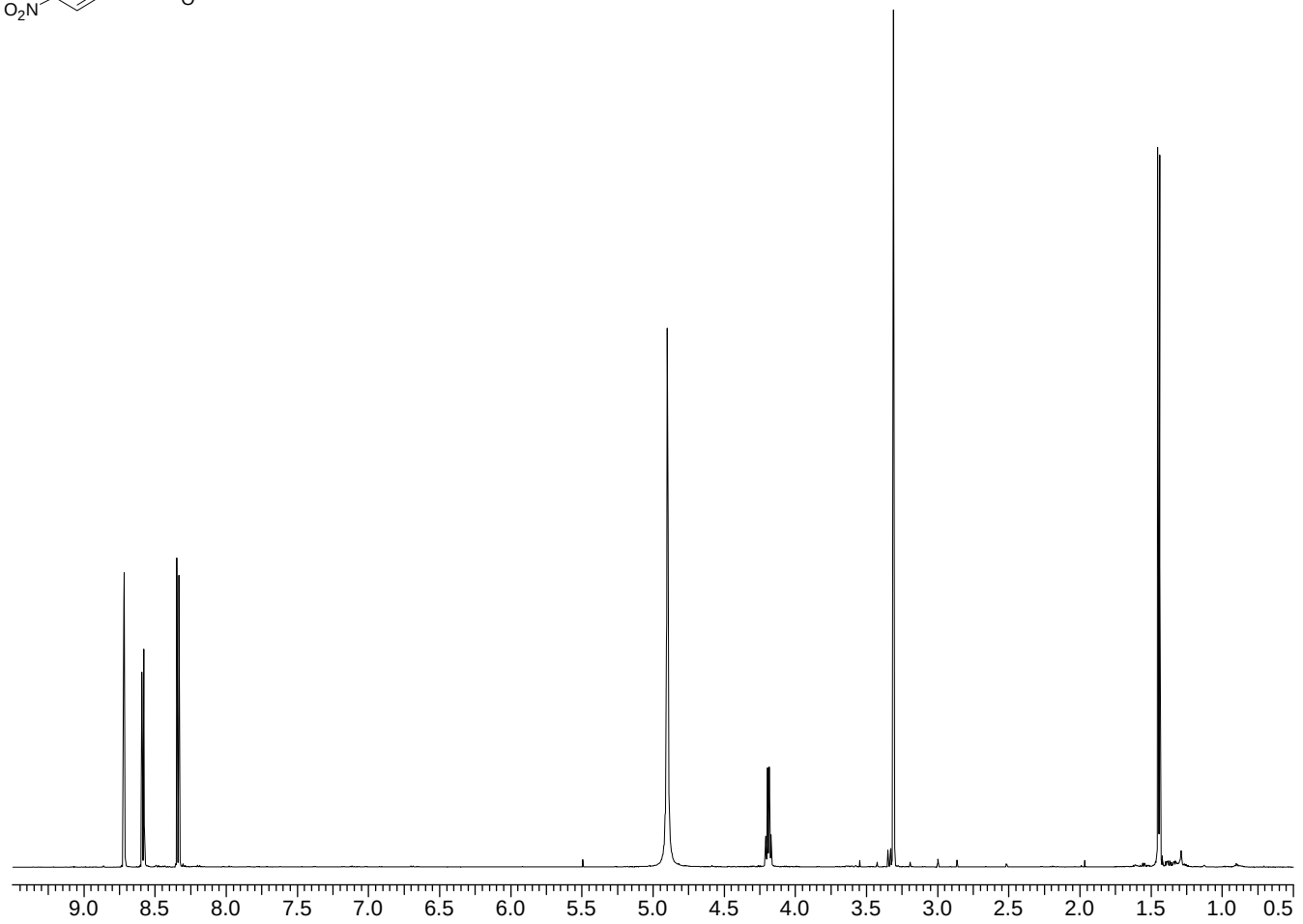
Fmoc-His-Gly-Val-(Ac_3-Tn-α-Thr)-Ser-Ala-OH (7): To a DMF(0.1 mL) solution of Fmoc-His-SH (0.84 mg, 0.0020 mmol) Cs_2CO_3 (1.1 mg, 0.0034 mmol) was added followed by DMF solution of the dNBS-Gly-Val-(Ac_3-Tn-α-Thr)-Ser-Ala-OH (1.7 mg, .0017 mmol). The reaction mixture was stirred for 15 minutes under N_2 atmosphere at room temperature. The completion of the reaction was monitored by ESI-MS. The DMF was co-evaporated with toluene under reduced pressure to get the crude peptide as yellowish semisolid. The crude material was purified by semi-preparative RP-HPLC and freeze-dried to afford the pure product. Yield: 1.4 mg (71%). ^{1}H NMR (600 Mhz, CD_3OD): δ 8.71 (s, 1H, imidazole H), 7.81 (d, J = 7.2 Hz, 2H), 7.62 (d, J = 7.8 Hz, 2H), 7.40 (t, J = 7.2 Hz, 2H), 7.33-7.30 (m, 2H), 7.22 (s, 1H, imidazole H), 7.02 (d, J = 9.0 Hz,1H, NH), 6.65 (d, J = 8.4 Hz, 1H, NH), 5.34 (s, 1H, H-4), 5.14 (dd, J = 3.0, 11.4 Hz, 1H, H-3), 5.11 (d, J = 4.2 Hz, 1H,H-1), 4.63 (s, 1H, Thr-α-CH), 4.50 (t, J = 4.8 Hz, 1H, Ser-α-CH), 4.48-4.40 (m, 3H, His-α-CH & Fmoc CH_2), 4.39-4.34 (m, 2H, H-2, Ala-α-CH), 4.30-4.28 (m, 3H, Thr-β-CH & 2 other protons), 4.20 (t, J = 6.0 Hz, 1H, Fmoc CH), 4.09-4.03 (m, 2H), 3.97 (d, J = 5.4, 2H, Gly-CH_2), 3.82 (dd, J = 4.8, 11.4 Hz,1H, Ser-β-CH), 3.14-3.09(m, 2H, His-β-CH), 2.19-2.17 (m, 1H, Val-β-CH), 2.14(s, 3H, CH_3CO), 2.00(s, 3H, CH_3CO), 1.97 (s, 3H, CH3CO), 1.93 (s, 3H, CH_3CO), 1.42 (d, J = 7.8 Hz, Ala-CH_3), 1.28 (d, J = 6.6 Hz, Thr-CH_3), 1.00 (d, J = 7.2 Hz, 3H, Val-CH_3), 0.99 (d, J = 7.2 Hz, 2H, Val-CH_3); Mass spectrum (ESI-MS), *m/z* = 1122.3 $[M+H]^+$($C_{52}H_{68}N_9O_{19}$ requires 1122.5).

Fmoc-Pro-Ala-His-Gly-Val-(Ac_3-Tn-α-Thr)-Ser-Ala-OH (11): To a DMF(0.1 mL) solution of Fmoc-Pro-Ala-SH (1.53 mg, 0.0036 mmol) Cs_2CO_3 (1.95 mg, 0.006 mmol) was added followed by DMF solution of the dNBS-His-Gly-Val-(Ac_3-Tn-α-Thr)-Ser-Ala-OH (3.5 mg, .003 mmol).

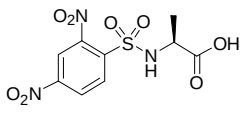
The reaction mixture was stirred for 15 minutes under N_2 atmosphere at room temperature. The completion of the reaction was monitored by ESI-MS. The DMF was co-evaporated with toluene under reduced pressure to get the crude peptide as yellowish semisolid. The crude material was purified by semi-preparative RP-HPLC and freeze-dried to afford the pure product. Yield: 2.05 mg (67%). Mass spectrum (ESI-MS), *m/z* = 1291.1 $[M+H]^+$ ($C_{60}H_{80}N_{11}O_{21}$ requires 1290.55).

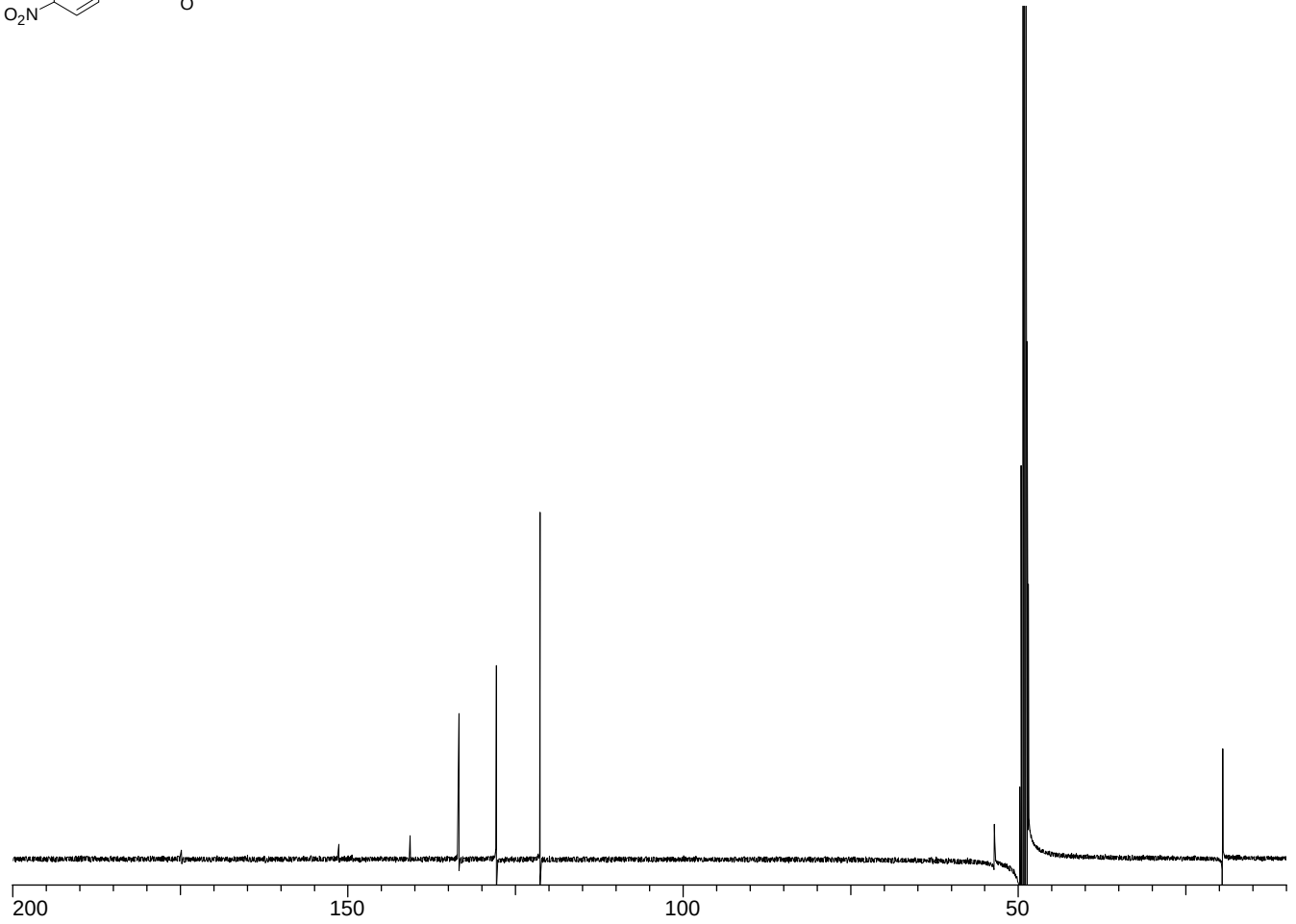
^{1}H NMR of dNBS-Alanine



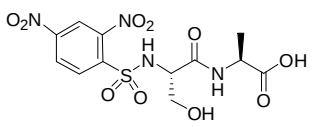


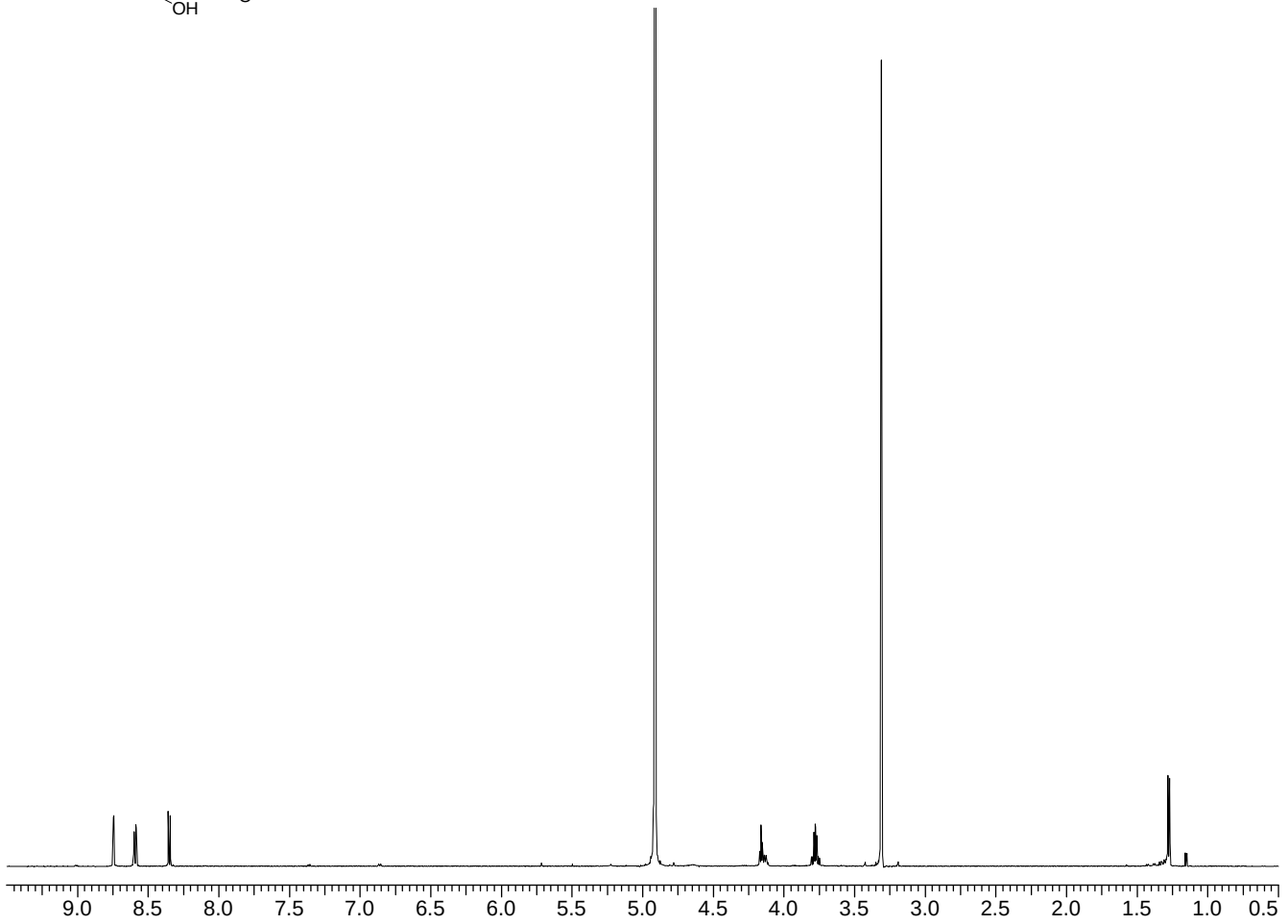
^{13}C NMR of dNBS-Alanine



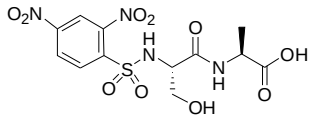


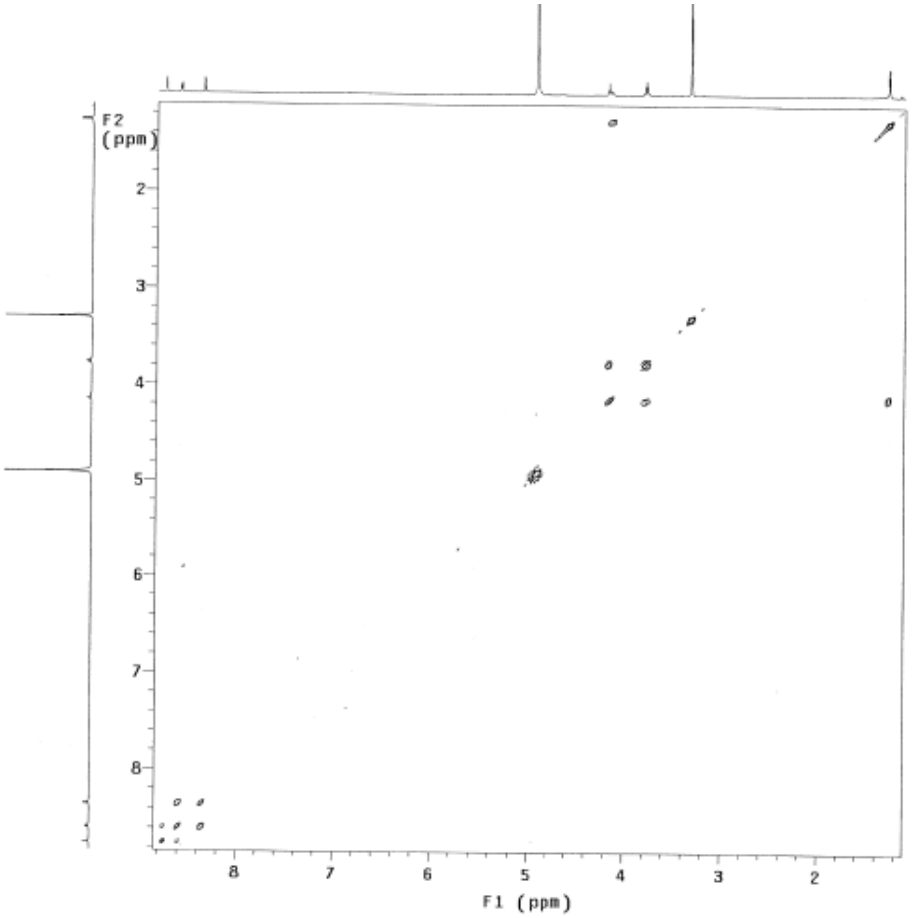
^{1}H NMR of dNBS-Ser-Ala-OH (**1**)



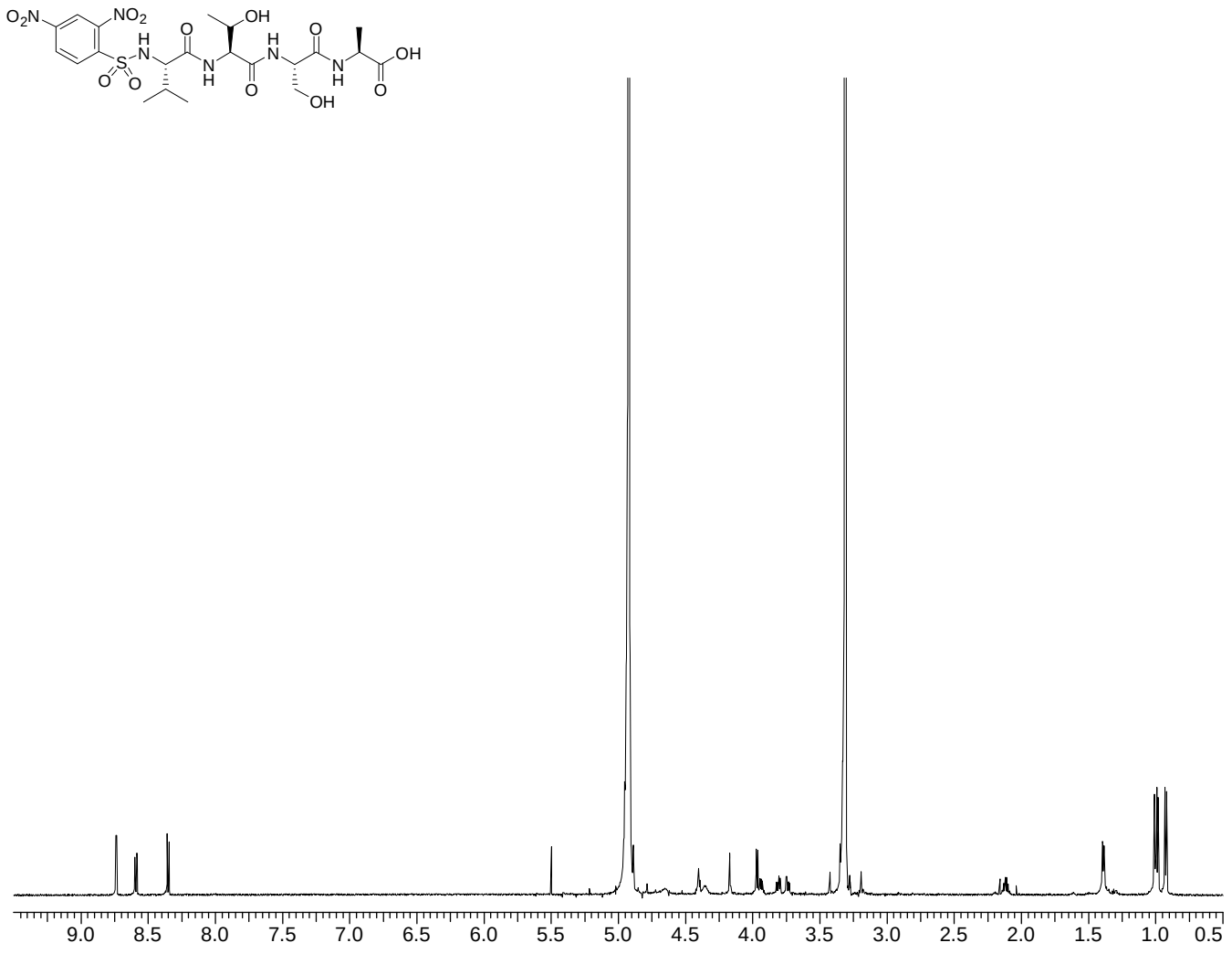


^{1}H-^{1}H gCOSY NMR of dNBS-Ser-Ala-OH (**1**)

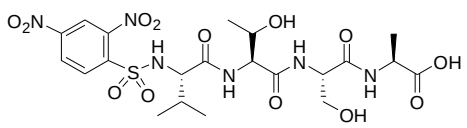


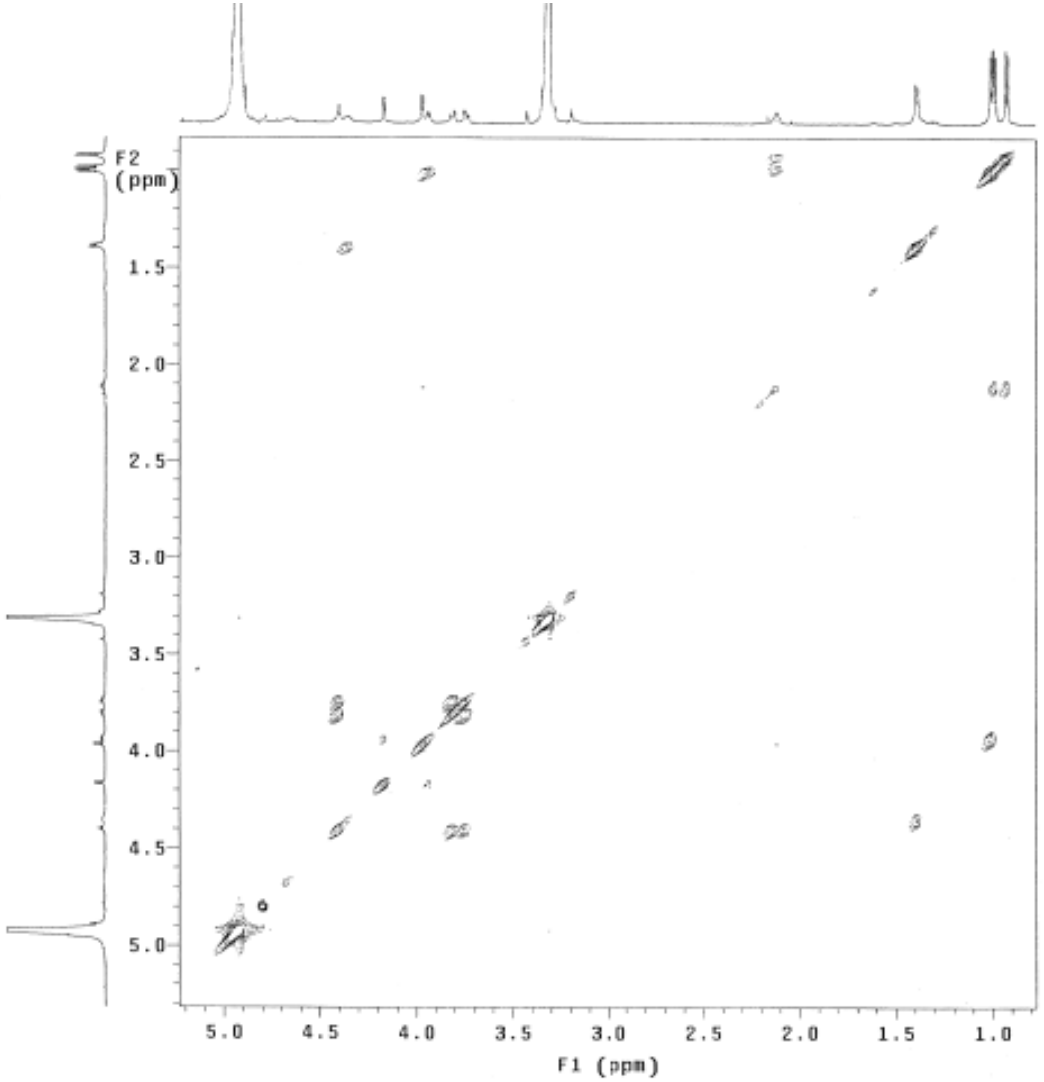


^{1}H NMR of dNBS-Val-Thr-Ser-Ala-OH (**2**)

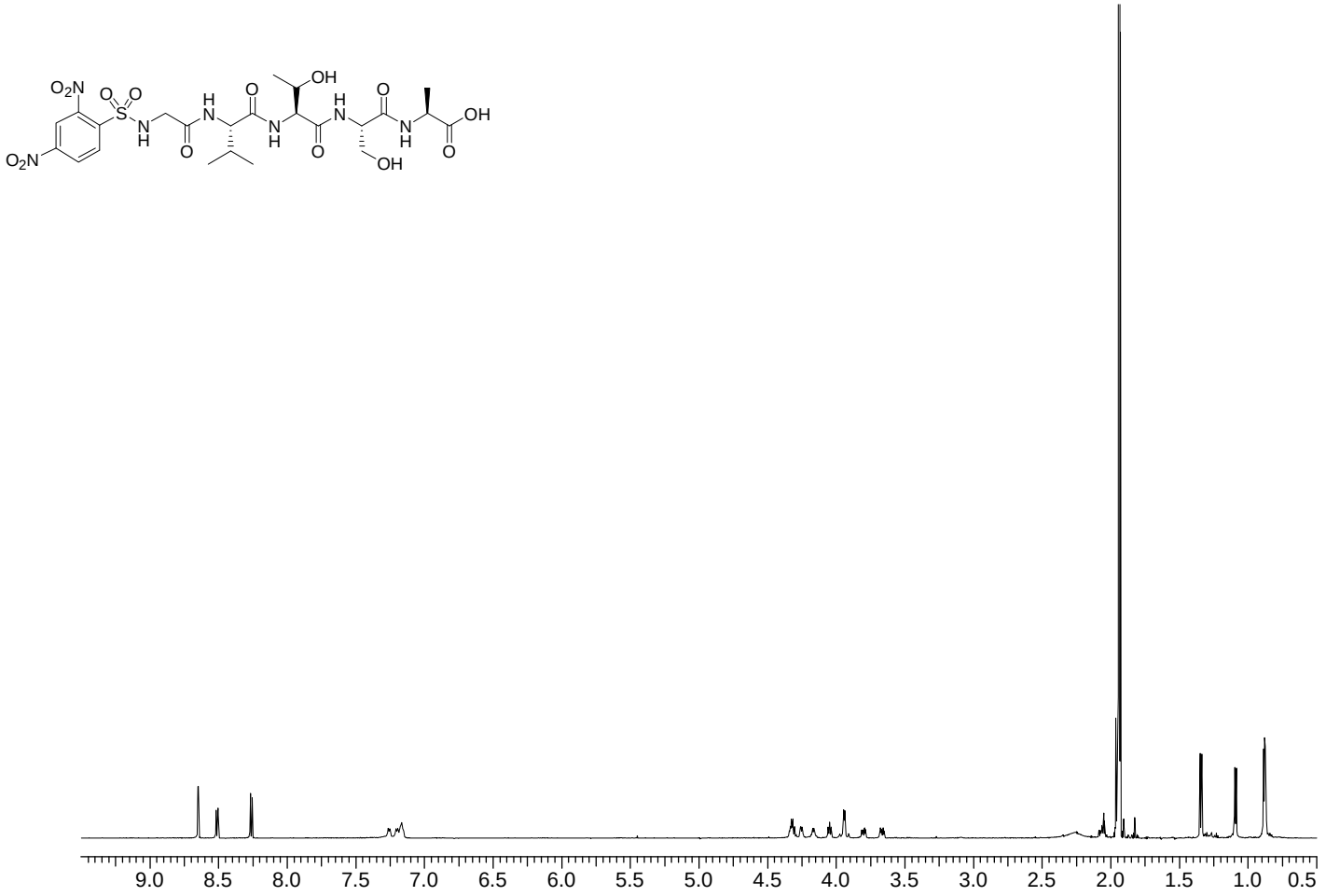


Characteristic region of ^{1}H-^{1}H gCOSY NMR of dNBS-Val-Thr-Ser-Ala-OH (**2**)

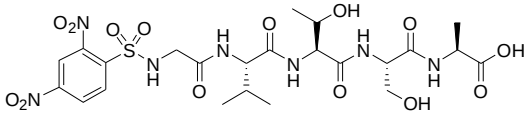


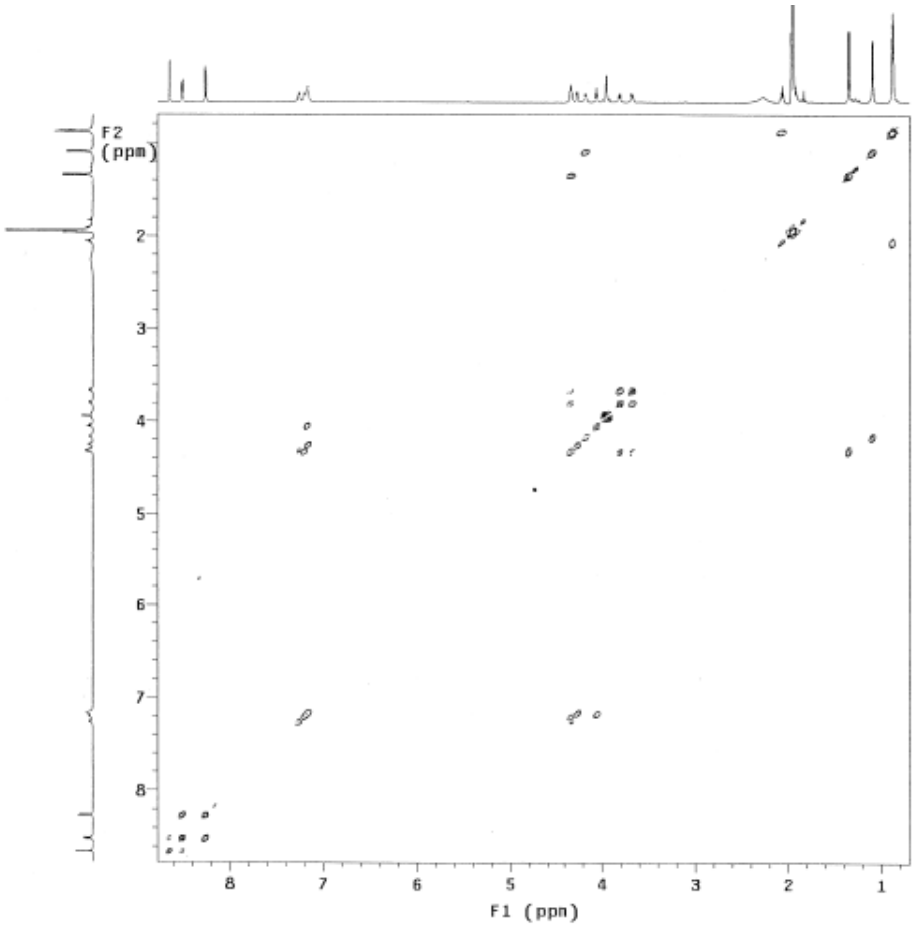


^{1}H NMR of dNBS-Gly-Val-Thr-Ser-Ala-OH (**3**)

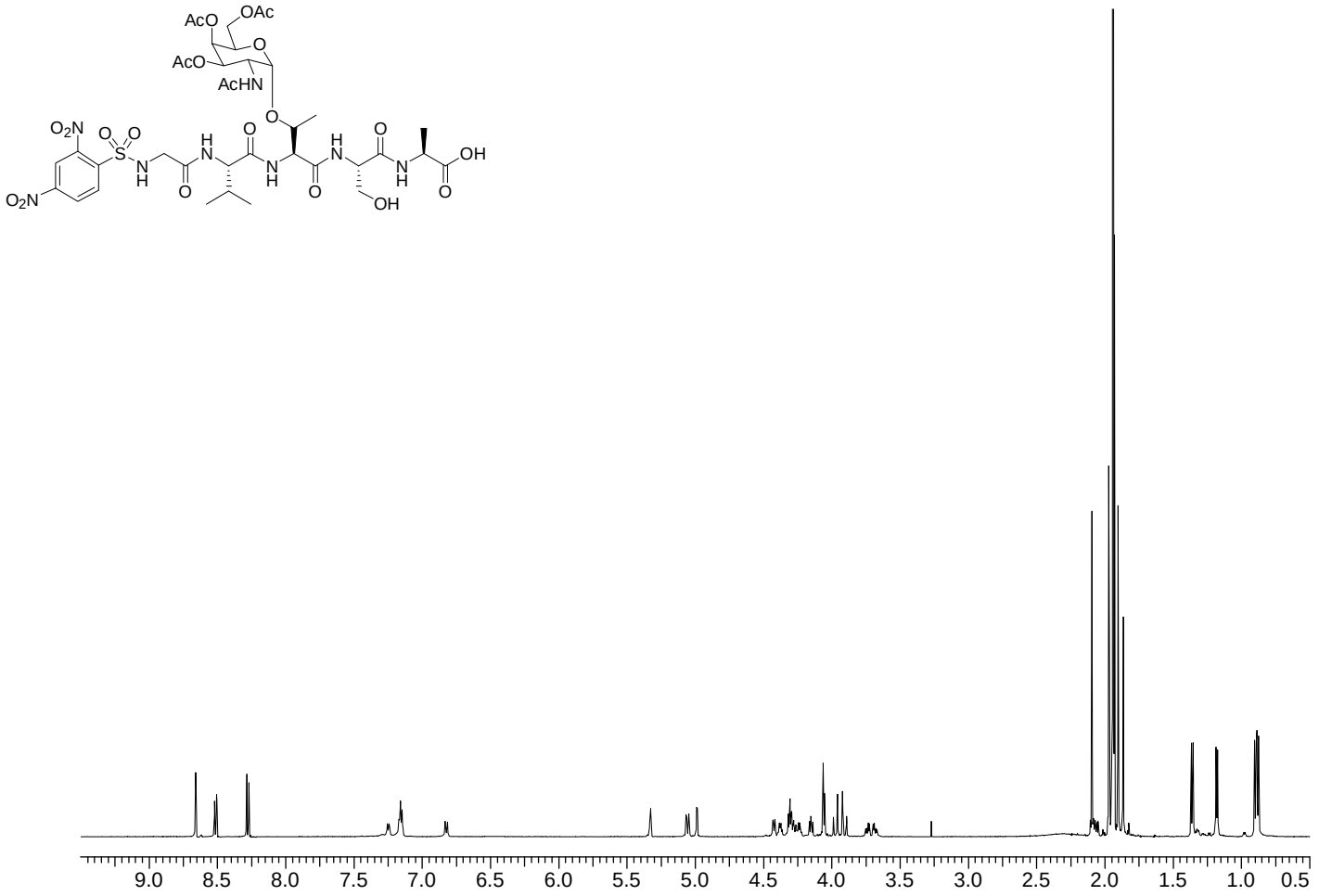


^{1}H-^{1}H gCOSY NMR of dNBS-Gly-Val-Thr-Ser-Ala-OH (**3**)

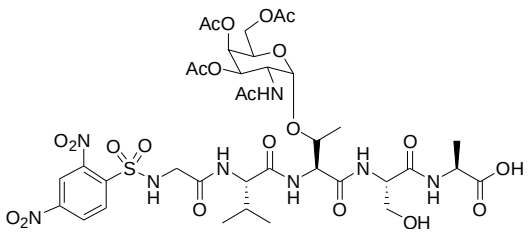


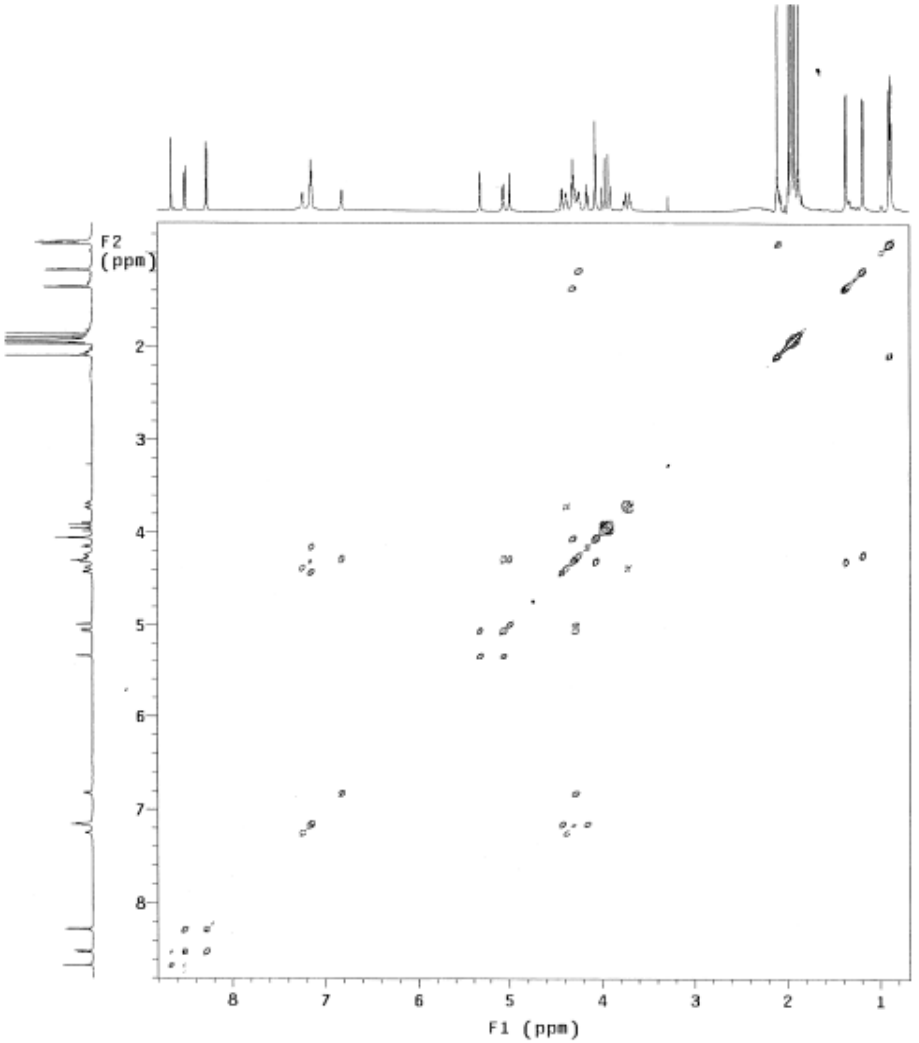


^{1}H NMR of dNBS-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (**4**)

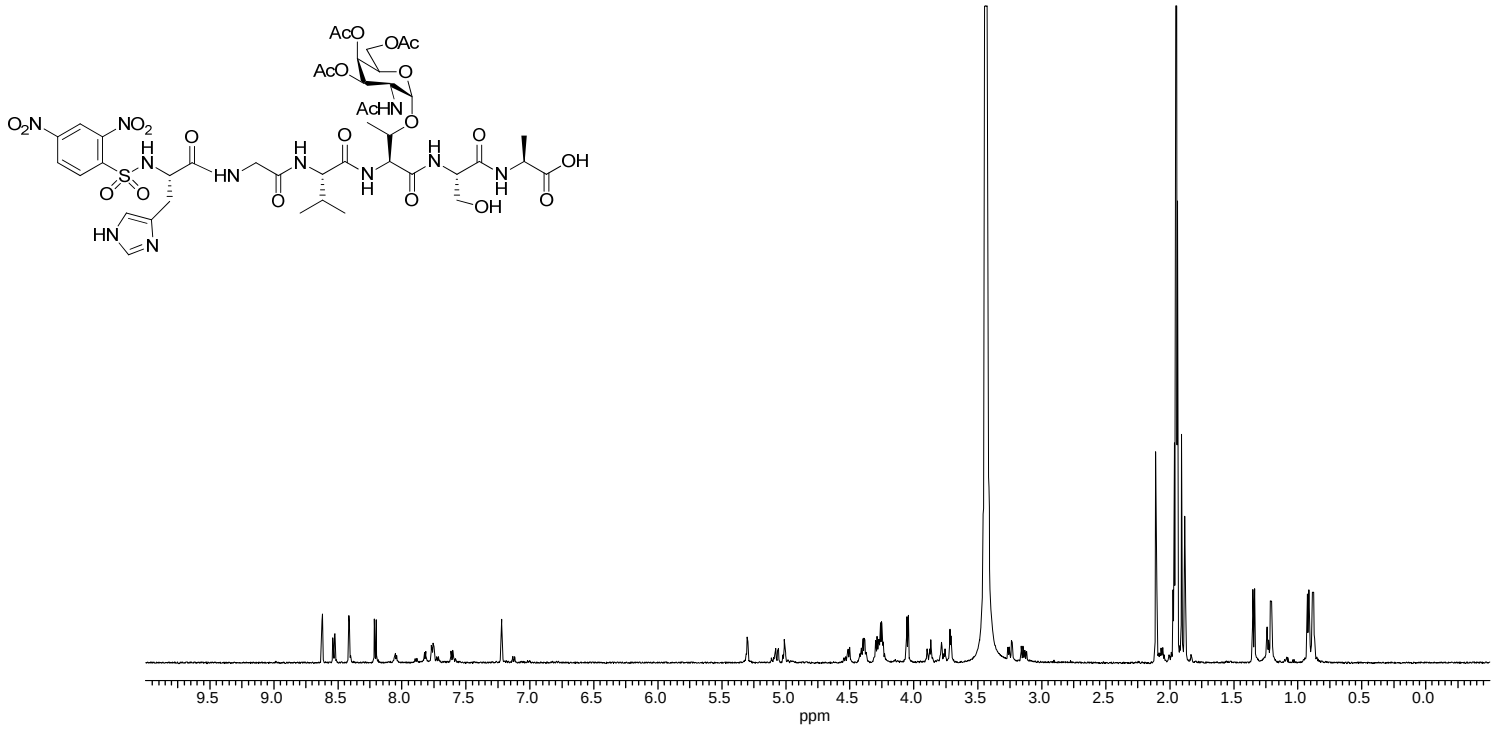


^{1}H-^{1}H gCOSY NMR of dNBS-Gly-Val-(Ac$_3$-α-Tn-Thr)-Ser-Ala-OH (**4**)

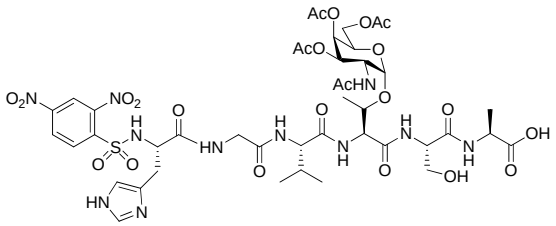


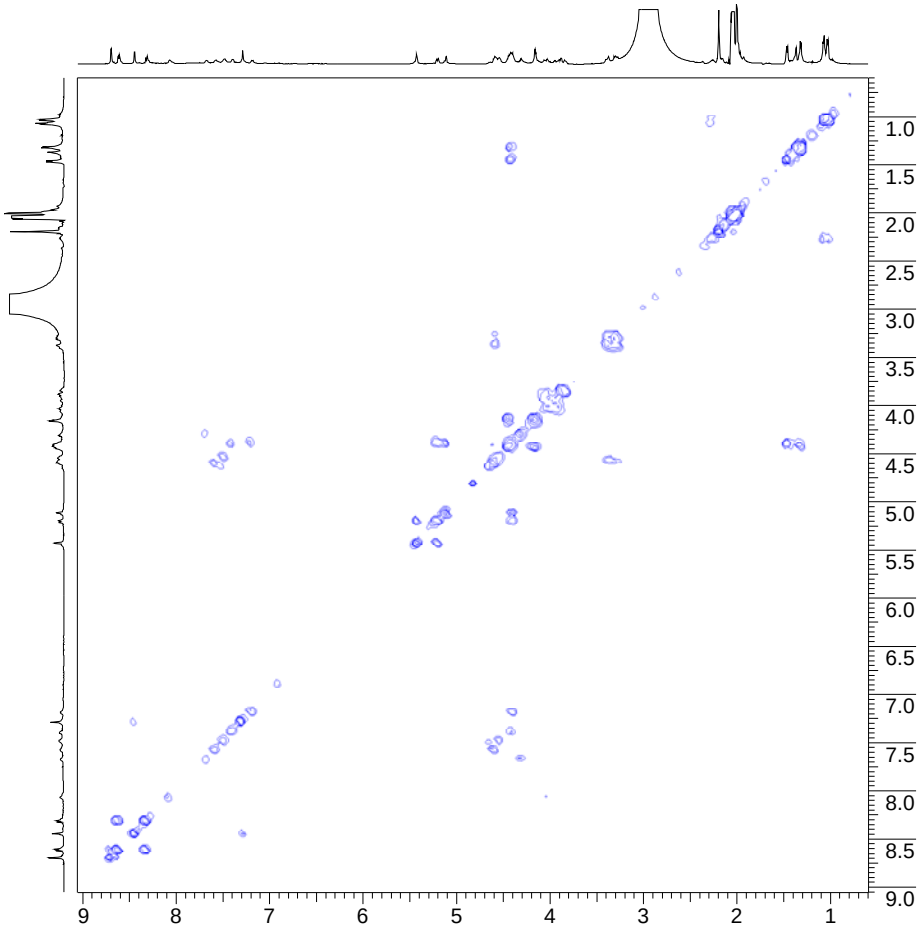


^{1}H NMR of dNBS-His-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (**5**).



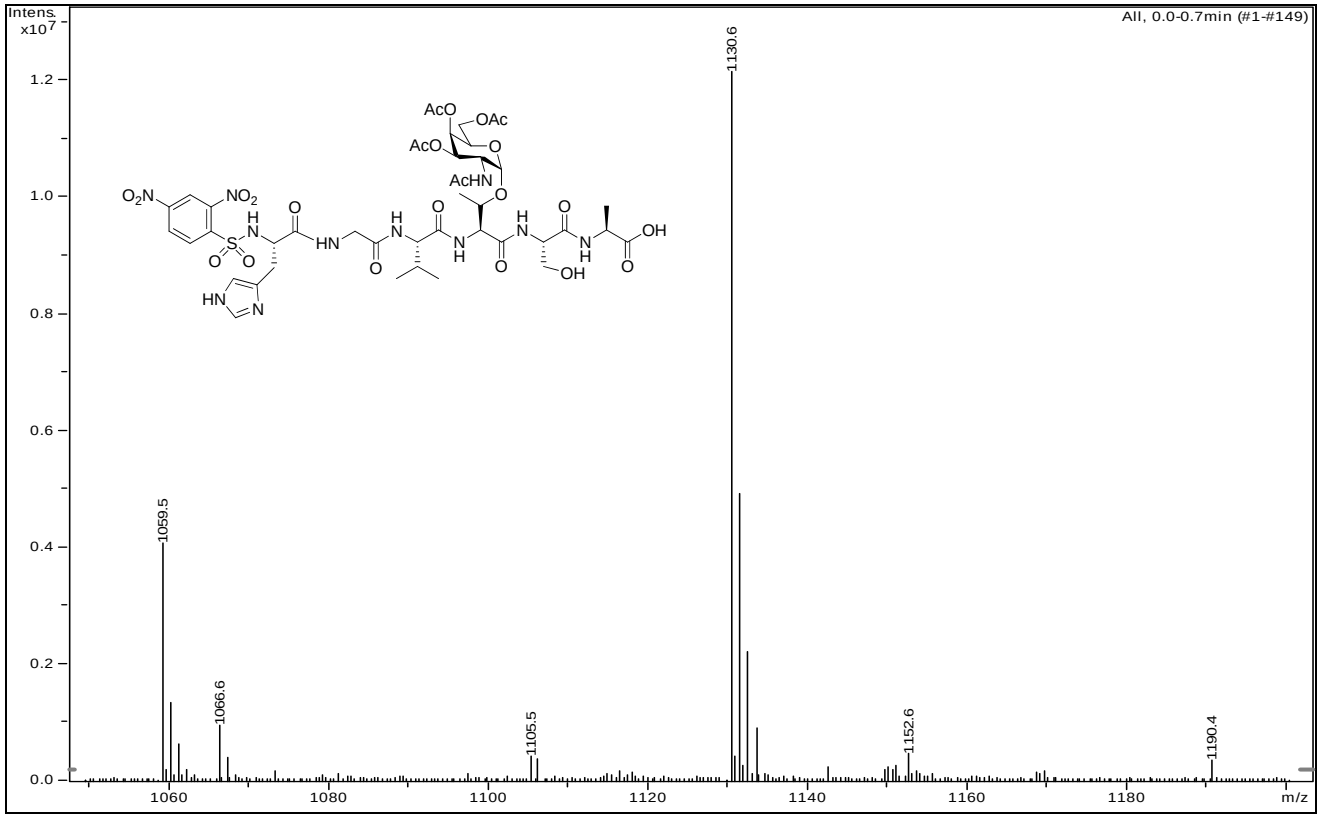
^{1}H-^{1}H gCOSY NMR of dNBS-His-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (**5**).



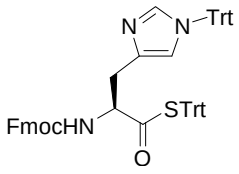


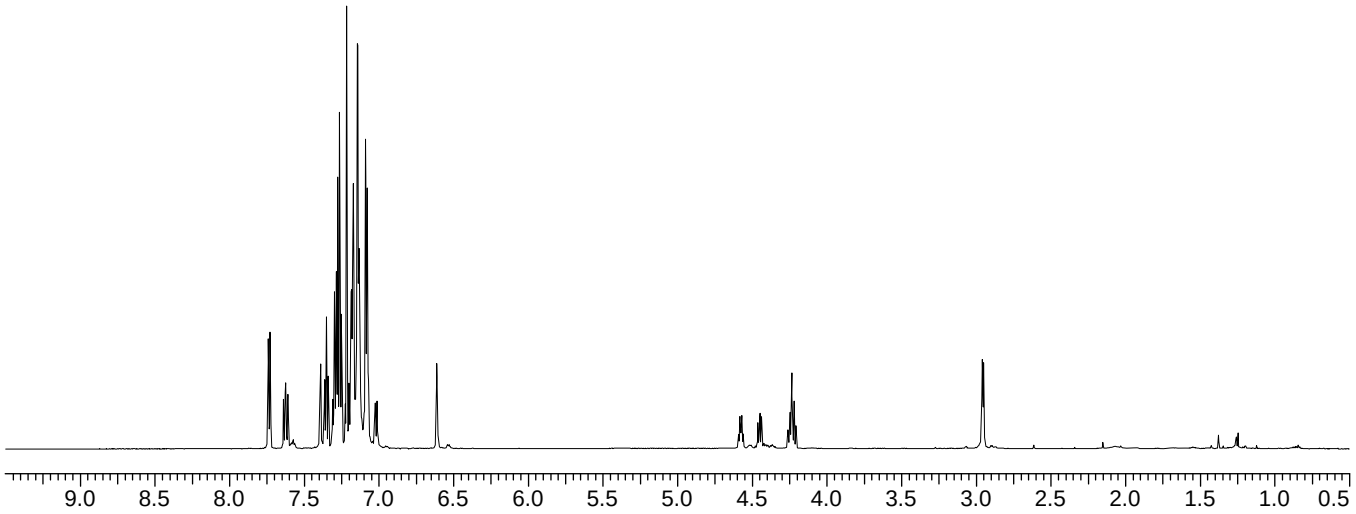
Note: The peak at δ = 3.43 for residual water was deemphasized by apodization.

ESI-MS of dNBS-His-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (**5**).

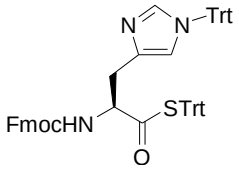


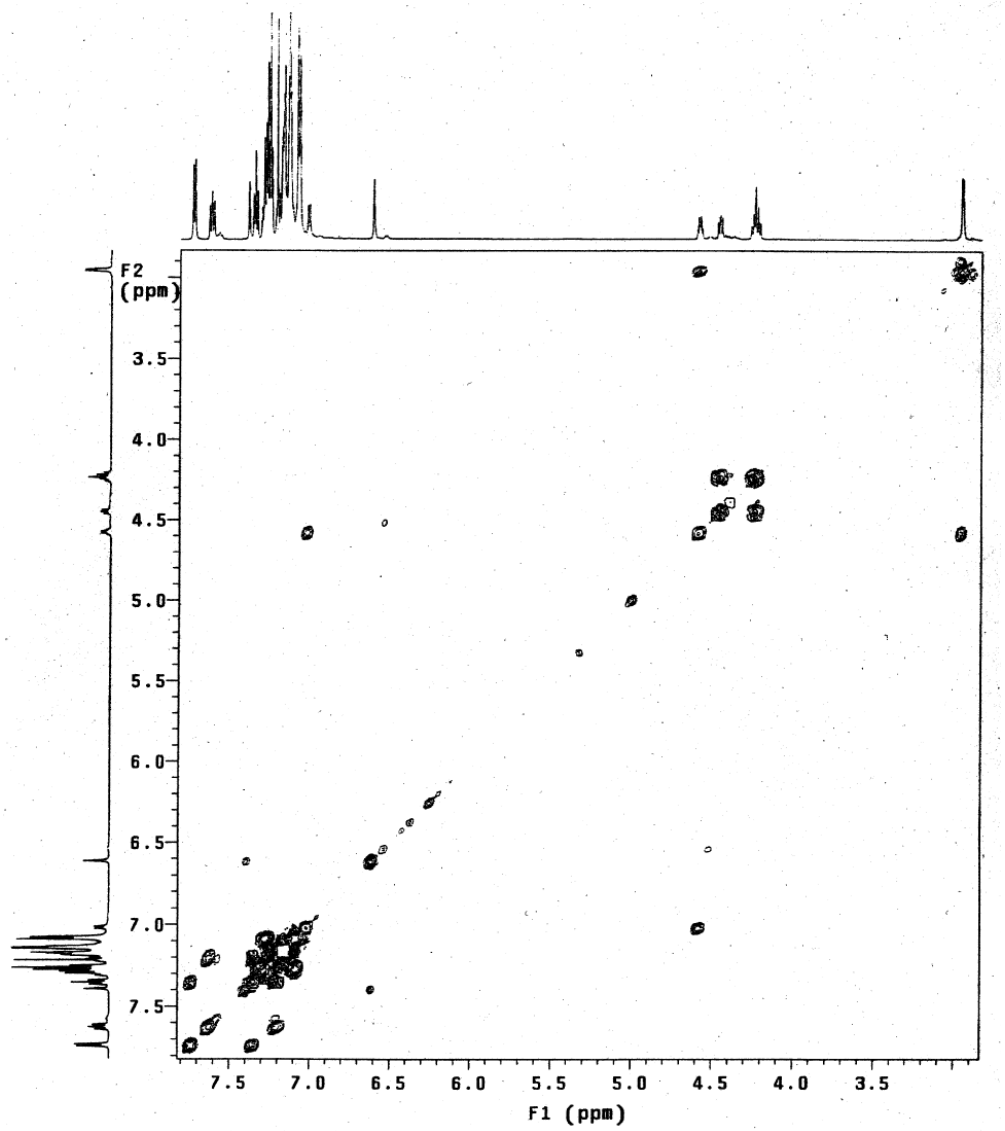
^{1}H NMR of *N*-α-Fmoc-*N*-im-Trityl-Protected L-Histidine Trityl Thioester:



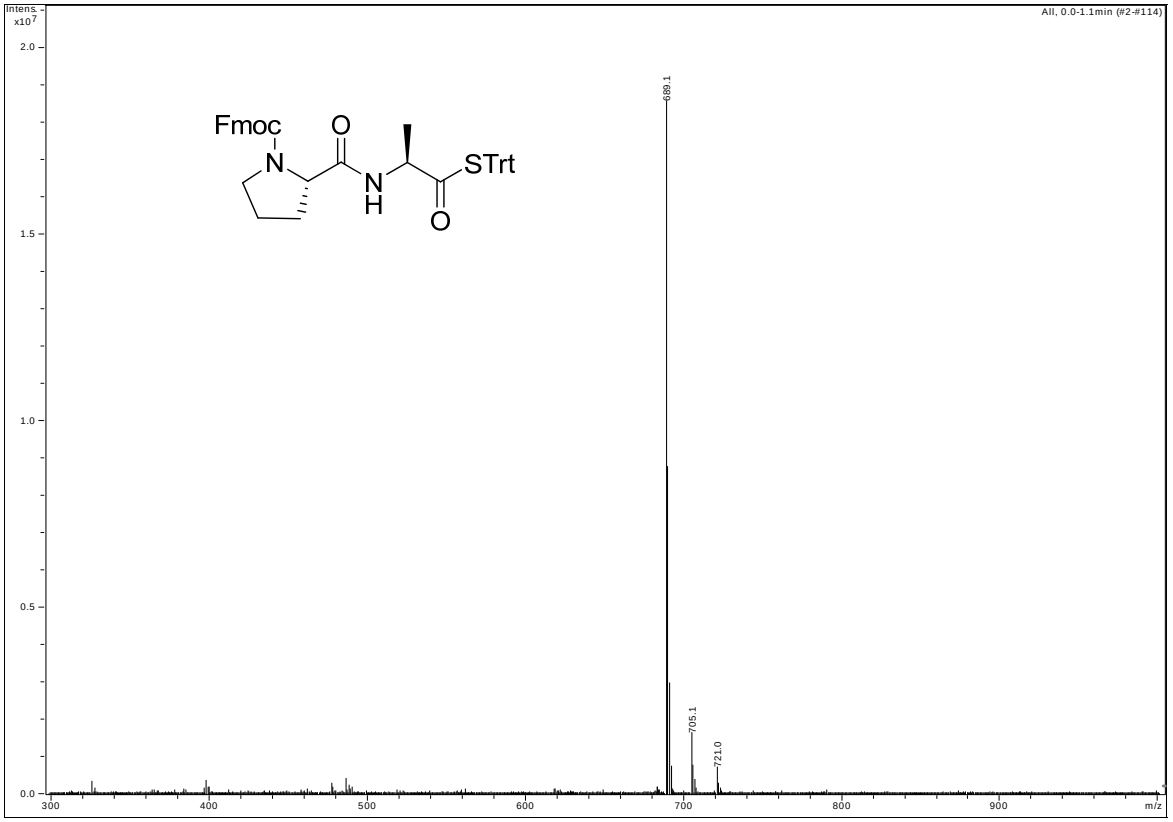


^{1}H-^{1}H gCOSY NMR of ***N***-α-Fmoc-*N*-im-Trityl-Protected L-Histidine Trityl Thioester:

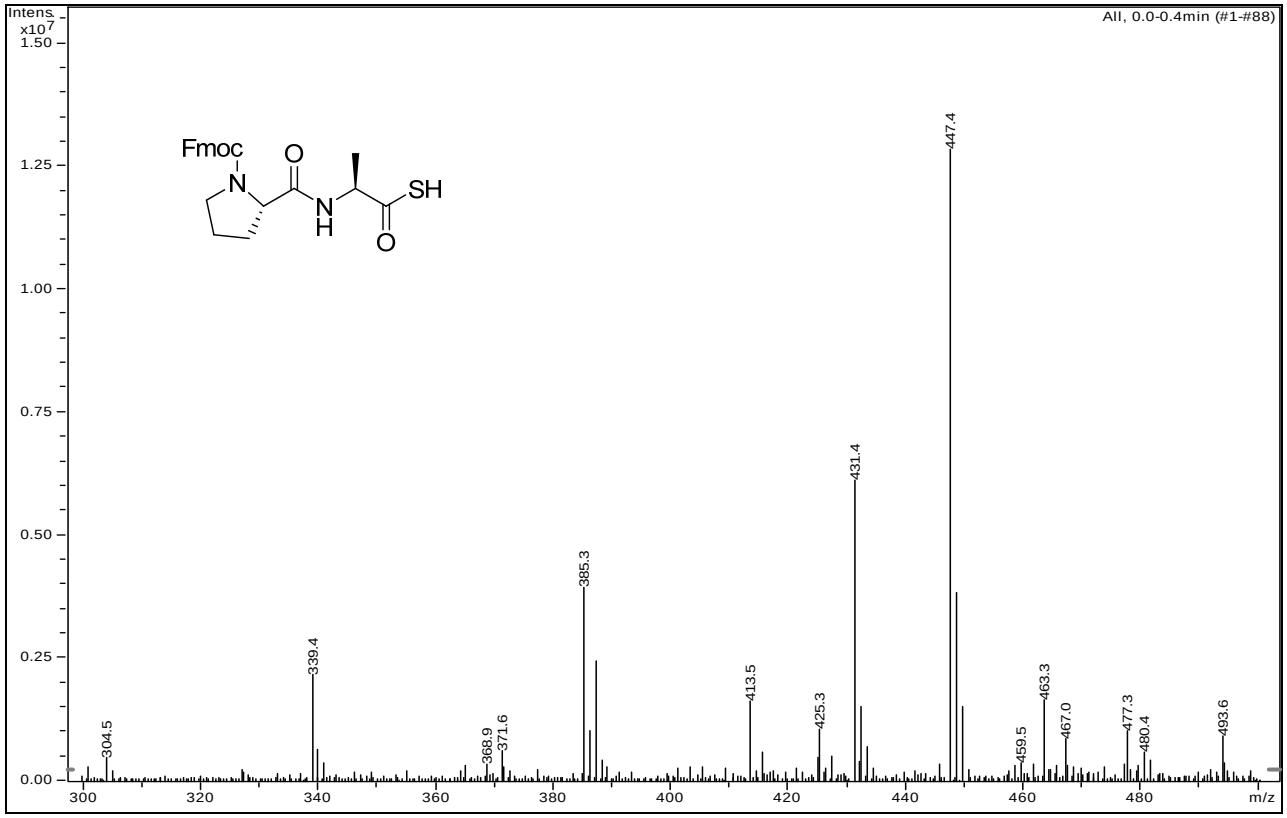




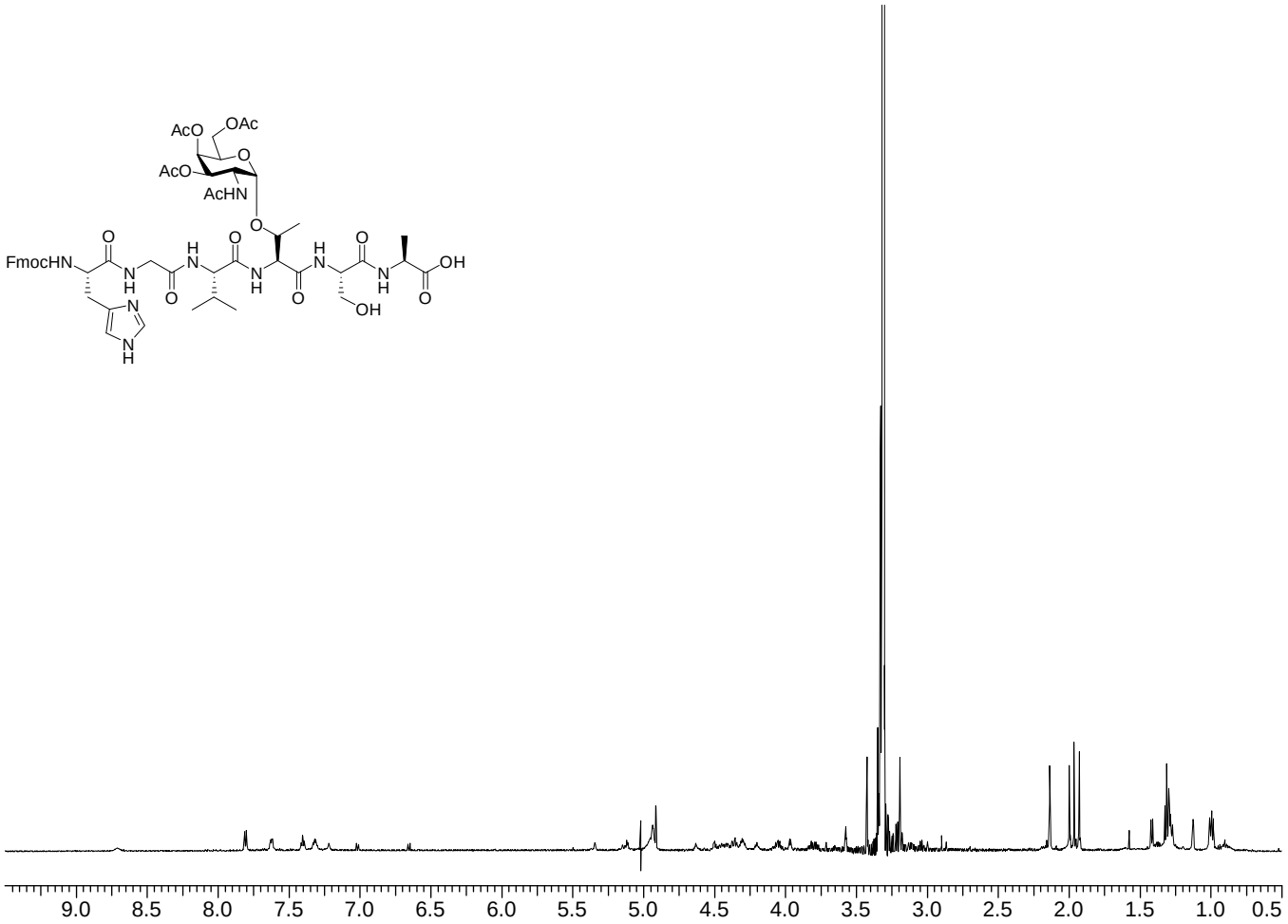
ESI-MS of *N*-Fmoc-L-prolyl-L-alanine Trityl Thioester (**9**).



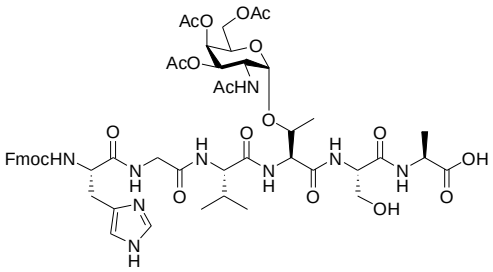
ESI-MS of Fmoc-Pro-Ala-SH (**10**).

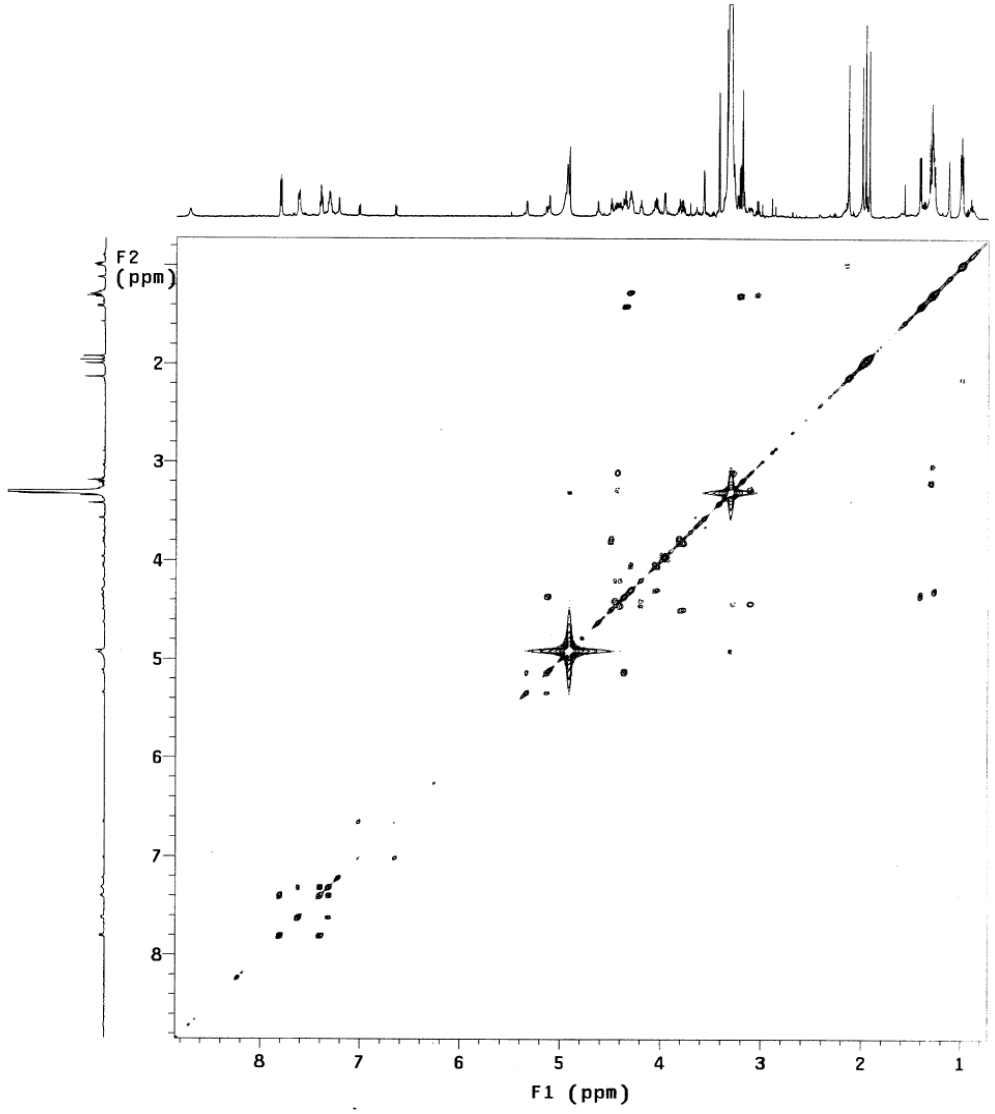


^{1}H NMR of Fmoc-His-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (**7**):

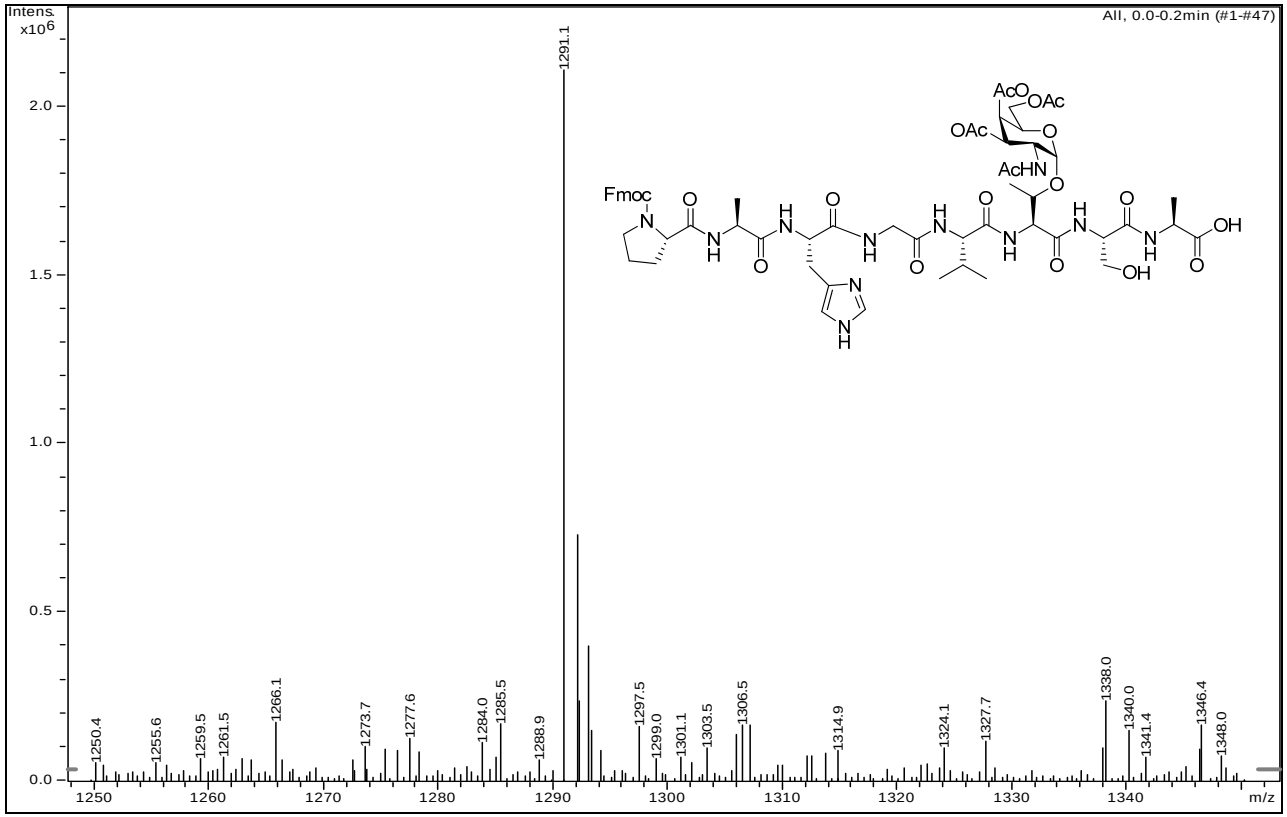


^{1}H-^{1}H gCOSY NMR of Fmoc-His-Gly-Val-(Ac$_3$-Tn-α-Thr)-Ser-Ala-OH (**7**):





ESI-MS of Fmoc-Pro-Ala-His-Gly-Val-(Ac_3-Tn-α-Thr)-Ser-Ala-OH (**11**):



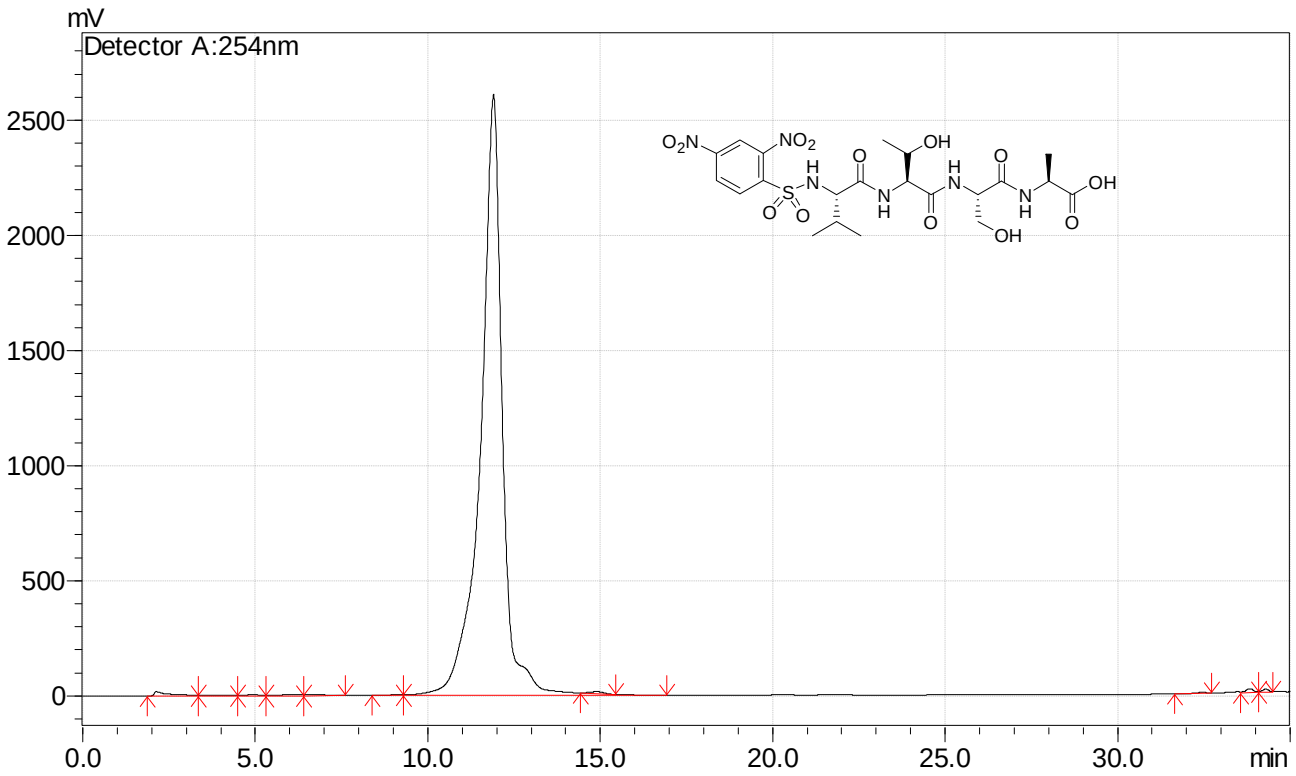


Figure 1. Analytical RP-HPLC of purified dNBS-Val-Thr-Ser-Ala-OH (**2**) eluting with 35-90% gradient of H_2O (0.1%TFA) and MeOH (0.1% TFA) over a period of 35 minutes, UV detection at 254 nm.

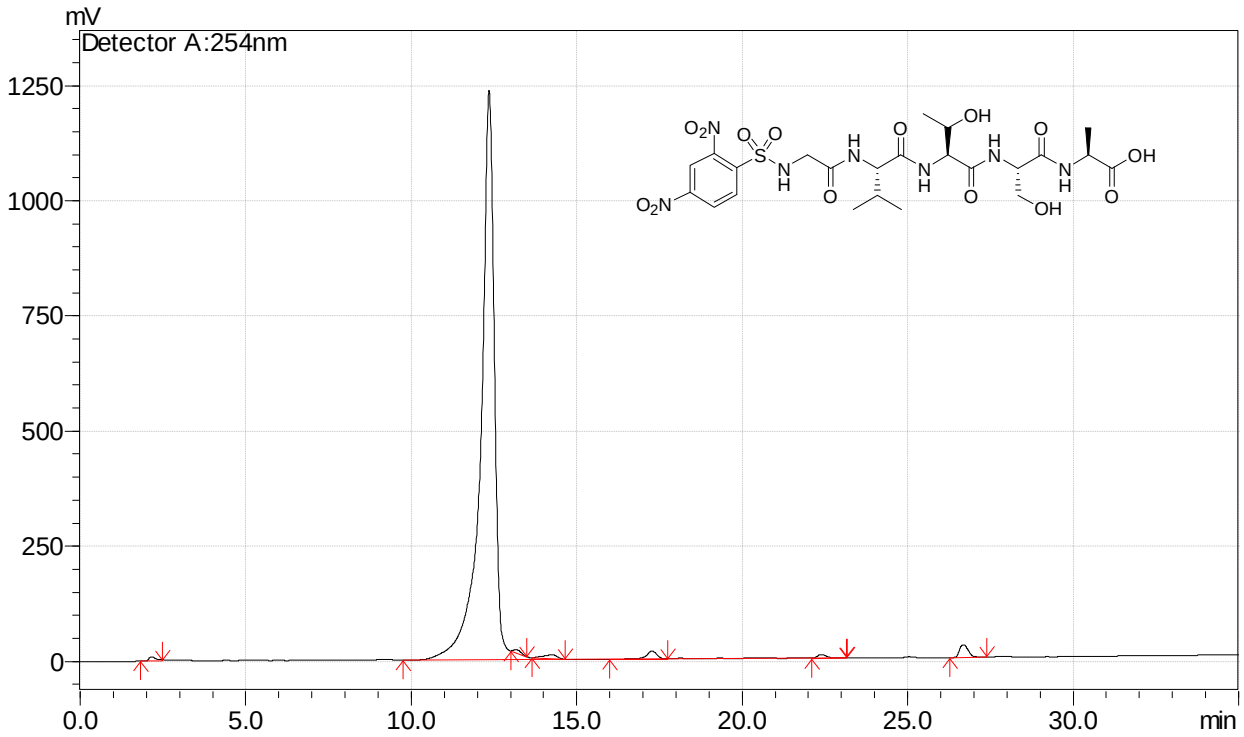
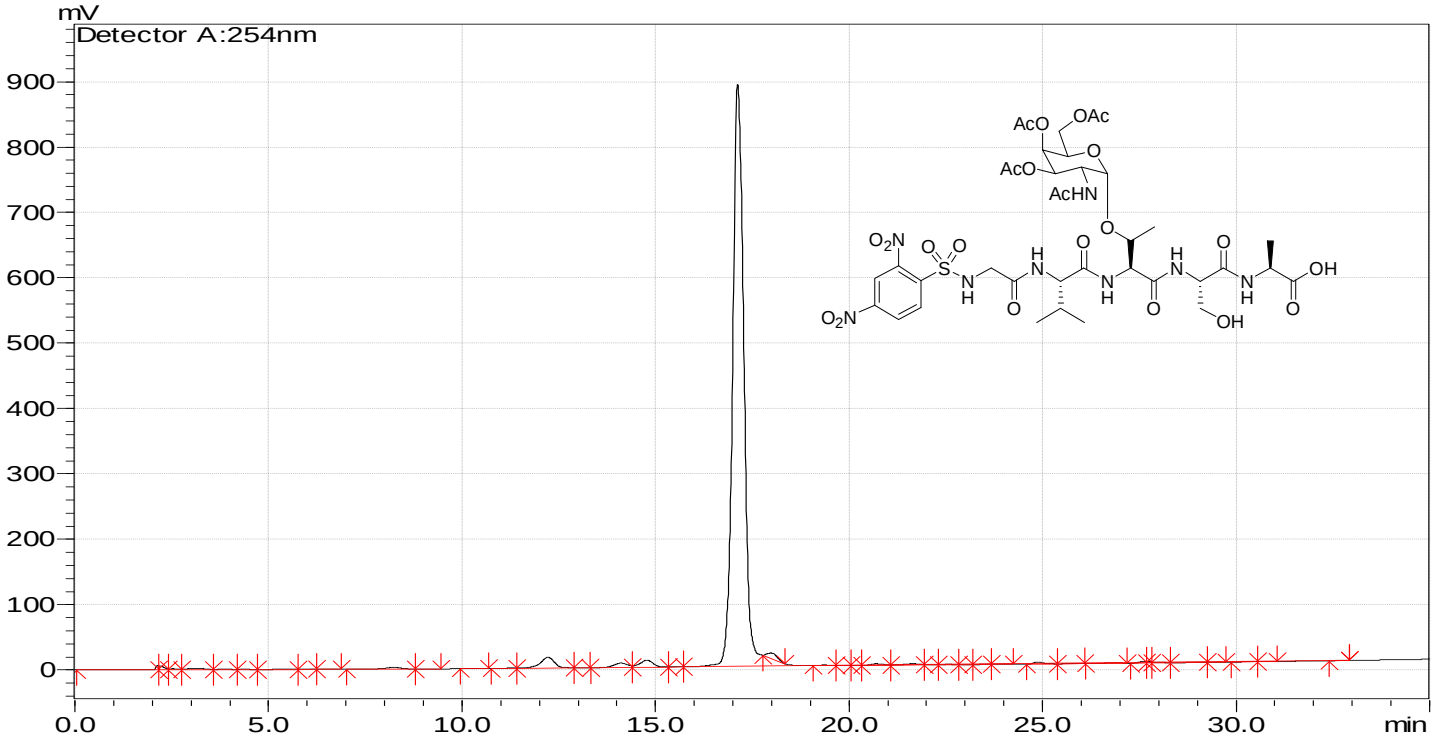
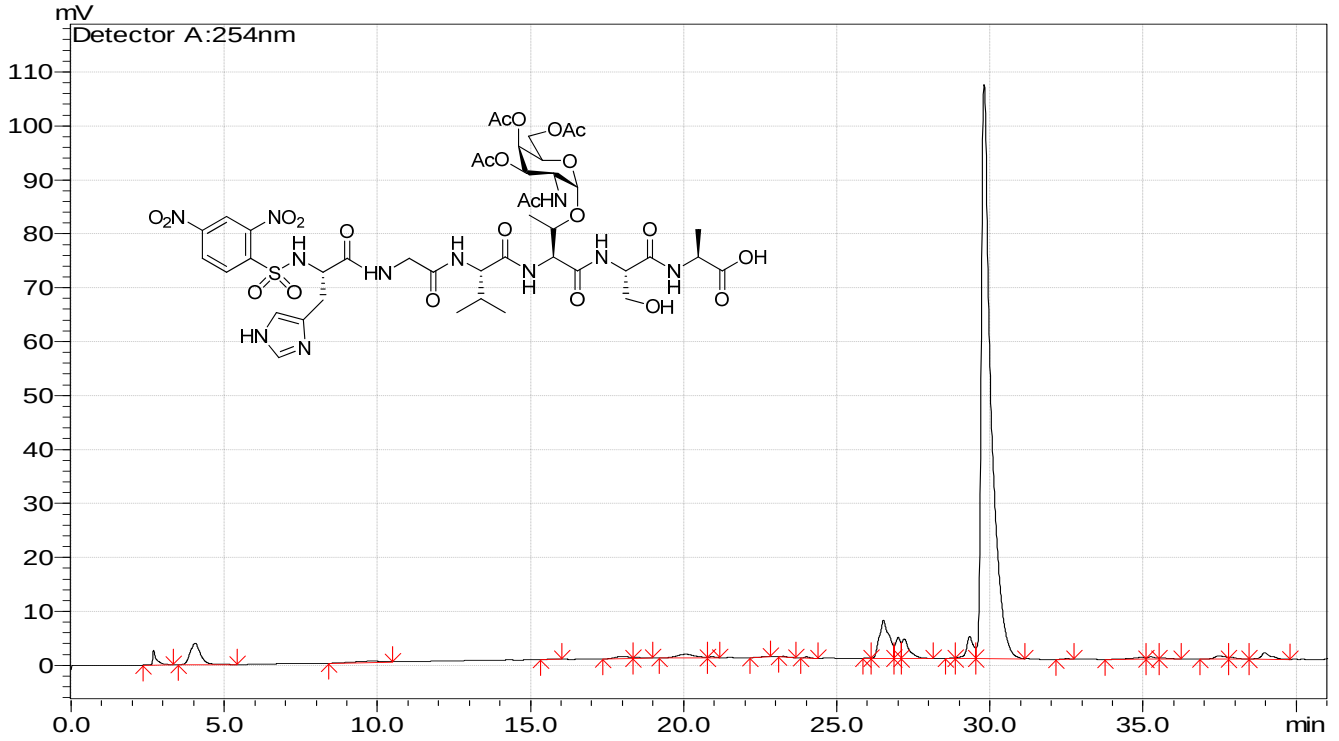


Figure 2. Analytical RP-HPLC of purified dNBS-Gly-Val-Thr-Ser-Ala-OH (**3**) eluting with 30-90% gradient of H_2O (0.1%TFA) and MeOH (0.1% TFA) over a period of 35 minutes, UV detection at 254 nm.



Retention time (minute)

Figure 3. Analytical RP-HPLC of purified and dNBS-Gly-Val-(Ac_3-Tn-α-Thr)-Ser-Ala-OH (**4**) eluting with 30-90% gradient of H_2O (0.1%TFA) and MeOH (0.1% TFA) over a period of 35 minutes, UV detection at 254 nm.



Retention time (minute)

Figure 4. Analytical RP-HPLC of purified dNBS-His-Gly-Val-(Ac_3-Tn-α-Thr)-Ser-Ala-OH (**5**), eluting with 3-55% gradient of H_2O (0.1%TFA) and ACN over a period of 50 minutes, UV detection at 254 nm.

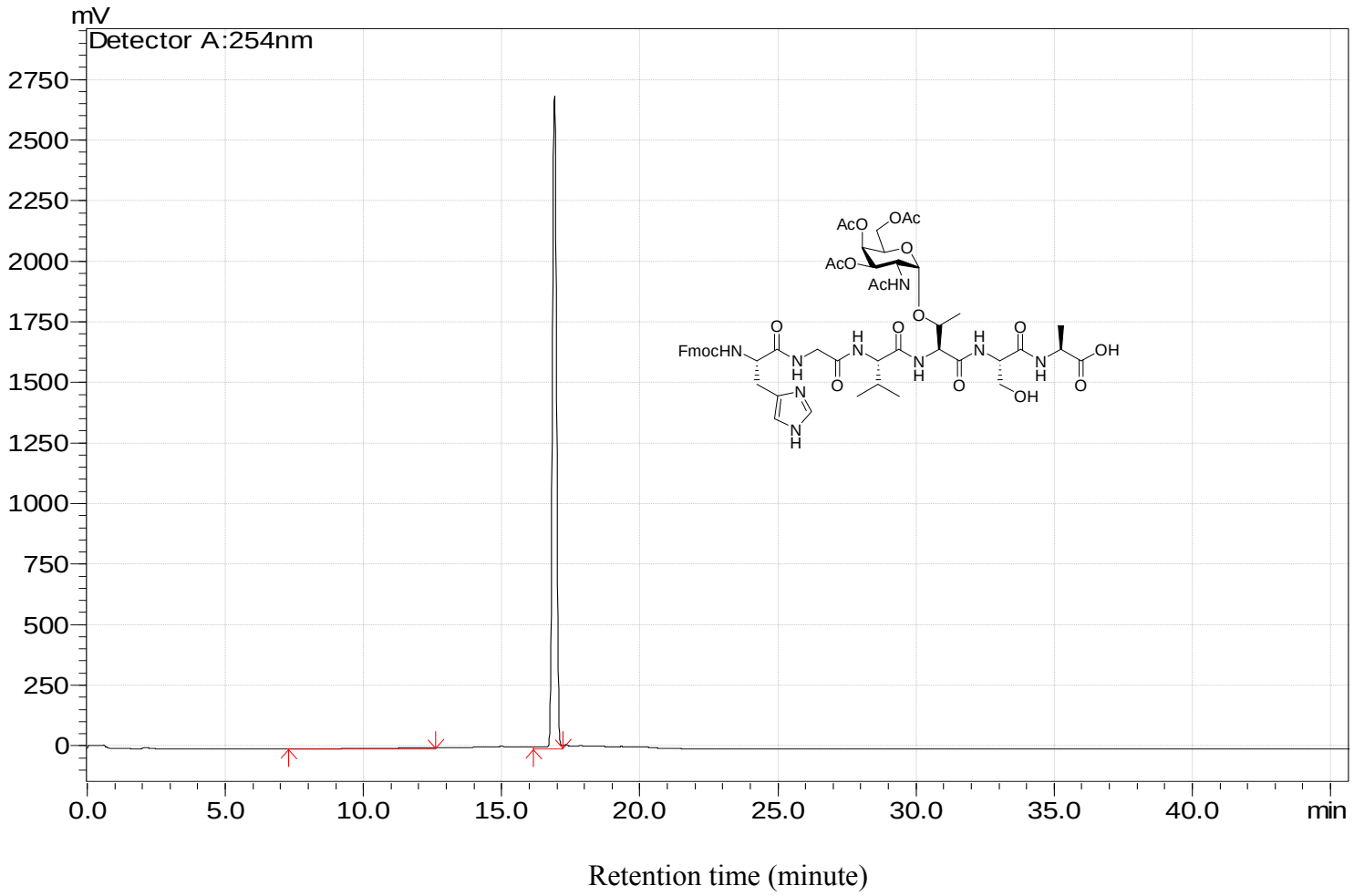
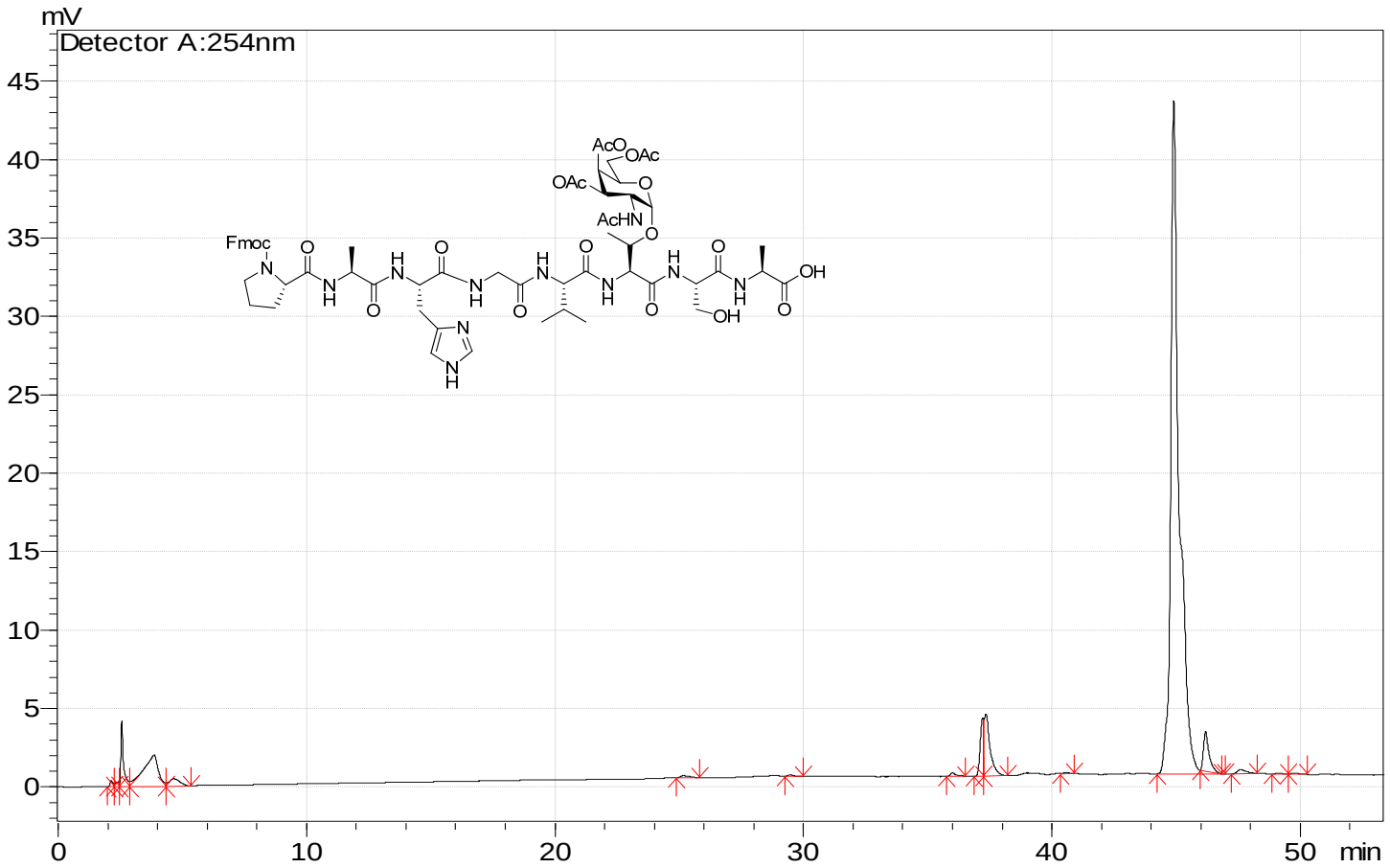


Figure 5. Analytical RP-HPLC of purified Fmoc-His-Gly-Val-(Ac_3-Tn-α-Thr)-Ser-Ala-OH (**7**), eluting with 30-90% gradient of H_2O (0.1%TFA) and MeOH (0.1% TFA) over a period of 35 minutes, UV detection at 254 nm.



Retention time (minute)

Figure 6. Analytical RP-HPLC of purified Fmoc-Pro-Ala-His-Gly-Val-(Ac_3-Tn-α-Thr)-Ser-Ala-OH (**11**), eluting with 3-55% gradient of H_2O (0.1%TFA) and ACN over a period of 50 minutes, UV detection at 254 nm.