Electronic Supplementary Information

4-Aminoproline-Based Arginine-Glycine-Aspartate Integrin Binders with Exposed Ligation Points: Practical in-Solution Synthesis, Conjugation and Binding Affinity Evaluation

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General methods

All chemicals were of reagent grade and were used as supplied without further purification. All organic solvents were dried and freshly distilled before use according to literature procedures. Coupling reagents were from either Bachem or Aldrich. All moisture sensitive reactions were carried out under a positive pressure of nitrogen or argon.

Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} precoated plates (Merck) with visualization under short-wavelength UV light or by dipping the plates with molybdate reagent (aqueous H_2SO_4 solution of cerium sulfate/ammonium molybdate) followed by heating. Flash chromatography was performed on 40-63 μm silica gel (Merck) using the indicated solvent mixtures. Direct infusion ESI-MS spectra were recorded on API 150EX apparatus (Applied Biosystems).

Optical rotations were measured using a Perkin-Elmer model 341 polarimeter at ambient temperature using a 100-mm cell with a 1-mL capacity and are given in units of 10^{-1} deg cm^2 g^{-1}. Elemental analyses were performed by the Microanalytical Laboratory of the University of Parma.

NMR spectra were recorded on Avance 300 (Bruker) or Mercury Plus MP-400 (Varian) or 600 INOVA (Varian) NMR spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) with TMS ($CDCl_3$), CD_2HOD, and HOD resonance peaks set at 0, 3.31, and 4.80 ppm, respectively. Peak assignments were performed using conventional 1D and 2D NMR experiments, such as COSY, TOCSY, HSQC and DEPT sequences.

Trans-4-hydroxy-L-proline (**1**) was purchased from Fluka.

Synthetic procedures

(2*S*,4*R*)-1-(*tert*-Butoxycarbonyl)-4-hydroxyproline benzyl ester (2). To a solution of triethylamine (4 mL) and methanol (36 mL) was added *trans*-4-hydroxy-L-proline (**1**) (2.0 g, 15.25 mmol) and di-*tert*-butyl dicarbonate (6.65 g, 30.5 mmol). After being refluxed for 2 h, the reaction mixture was allowed to cool to room temperature and the solvent removed in vacuo. The residue was dissolved in 40 mL of water and treated with NaH_2PO_4 (200 mg); the resulting solution was cooled to 0 °C and acidified with diluted HCl to pH 2. After being stirred for 30 min at 0 °C, the mixture was extracted with EtOAc (4 ×), and the combined organic layers were collected, dried ($MgSO_4$), and filtered. Evaporating off the solvent under reduced pressure afforded *trans*-*N*-(*tert*-butoxycarbonyl)-4-hydroxy-L-proline (3.03 g, 86%) which was used as such in the following step. A small portion of crude was subjected to flash chromatographic purification (EtOAc/MeOH, 90:10) to fully characterize *N*-Boc intermediate: a colourless foam; $[\alpha]^{25}_D$ –77.0 (*c* 0.8, H_2O); ^{1}H NMR (300 MHz, CD_3OD) δ 4.40 (m, 1H), 4.32 (dd, *J* = 8.0, 8.0 Hz, 1H), 3.56 (dd, *J* = 11.5, 4.1 Hz, 1H), 3.45 (m, 1H), 2.28 (m, 1H), 2.07 (m, 1H), 1.48 and 1.45 (2s, 9H); ^{13}C NMR (75 MHz, CD_3OD) δ 176.0, 147.9, 81.7, 70.1, 59.4, 55.6, 40.1, 28.5 (3C). The spectral and chiro-optical characteristics of this compound fully matched those reported for the

commercially available material (Fluka).
N-Boc-4-hydroxyproline intermediate (3.0 g, 13.0 mmol) was dissolved in methanol (50 mL) and the solution was cooled to 0 °C. Aqueous caesium carbonate (2.12 g, in 34 mL H_2O, 6.5 mmol) was added. The solution was concentrated and sufficient DMF was added to azeotrope the water, leaving a white solid which was dissolved in DMF (42 mL). Benzyl bromide (1.5 mL, 13.0 mmol) was added at 0 °C and the mixture was stirred vigorously at room temperature for 20 h. The reaction mixture was concentrated in vacuo, dissolved in EtOAc (40 mL), and washed with water (2 ×) and saturated aq NaCl solution (2 ×). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo to produce a residue which was purified by silica gel flash chromatography (hexanes/EtOAc, 50:50) affording benzylproline **2** as a colourless oil (4.16 g, 100%): $[\alpha]^{25}_D$ –61.3 (*c* 4.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, mixture of rotamers) δ 7.30 (m, 5H, Ph), 5.15 (m, 2H, C*H*$_2$Ph), 4.45 (dd, *J* = 8.8, 1.7 Hz, 1H, H2), 4.37 (m, 1H, H4), 3.74 (bs, 1H, OH), 3.55 (dd, *J* = 11.6, 4.3 Hz, 1H, H5a), 3.42 (m, 1H, H5b), 2.24 (m, 1H, H3a), 1.97 (m, 1H, H3b), 1.40 and 1.24 (2s, 9H, *t*-Bu); ^{13}C NMR (75 MHz, $CDCl_3$, mixture of rotamers, major isomer) δ 173.0, 154.1, 135.5, 128.8 (2C), 128.4 (2C), 128.1, 80.4, 69.1, 66.8, 58.1, 54.7, 39.1, 28.4 (3C). MS (ESI) calcd for $C_{17}H_{24}NO_5$ $[M+H]^+$: 322.16; found: 322.4. Anal. calcd for $C_{17}H_{23}NO_5$: C, 63.53; H, 7.21; N, 4.36; found: C, 63.35; H, 7.29; N, 4.41.

(2*S*,4*S*)-4-Azidoproline benzyl ester (3). To an ice-cooled solution of proline **2** (4.16 g, 12.96 mmol) and PPh_3 (5.1 g, 19.44 mmol) in dry THF (100 mL) under stirring, 8.9 mL of DEAD (40% solution in toluene, 19.44 mmol) were added dropwise over 30 min, under argon atmosphere. After 10 min, DPPA (4.2 mL, 19.44 mmol) was added dropwise and the reaction was allowed to rise to room temperature. After 24 h, the mixture was concentrated in vacuo and the residue was purified by silica gel flash chromatography (hexanes/EtOAc, 80:20) to afford an azidoproline intermediate (4.40 g, 98%) as a yellowish oil: $[\alpha]^{25}_D$ –40.1 (*c* 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$, mixture of rotamers) δ 7.31 (m, 5H, Ph), 5.21 (m, 2H, C*H*$_2$Ph), 4.50 (dd, *J* = 8.9, 3.5 Hz, 0.4H, H2), 4.38 (dd, *J* = 8.9, 3.9 Hz, 0.6H, H2), 4.16 (m, 1H, H4), 3.73 (ddd, *J* = 18.0, 11.7, 6.1 Hz, 1H, H5a), 3.50 (ddd, *J* = 17.8, 11.7, 3.7 Hz, 1H, H5b), 2.46 (m, 1H, H3a), 2.20 (ddd, *J* = 13.5, 3.8, 3.8 Hz, 1H, H3b), 1.48 and 1.36 (2s, 9H, *t*-Bu); ^{13}C NMR (75 MHz, $CDCl_3$, mixture of rotamers, major isomer) δ 171.4, 153.7, 135.6, 128.6 (2C), 128.5 (2C), 128.2, 80.6, 67.1, 58.3, 57.8, 51.4, 36.1, 28.1 (3C).
A solution of *N*-Boc-4-azidoproline intermediate (4.40 g, 12.7 mmol) in dry DCM (90 mL) was cooled to 0 °C and treated with anhydrous TFA (18 mL). After the mixture was stirred at room temperature for 4 h, saturated aq $NaHCO_3$ solution and solid Na_2CO_3 were sequentially added until pH 9. The mixture was extracted with EtOAc (4 ×) and the organic layers were collected, dried over $MgSO_4$, filtered, and concentrated to afford a crude residue which was purified by silica gel flash chromatography (EtOAc/MeOH, 96:4) to afford the N^{α}-deprotected azidoproline **3** (2.72 g, 87%) as a colorless oil: $[\alpha]^{25}_D$ –42.5 (*c* 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34 (m, 5H, Ph), 5.21 (1/2 ABq, *J* = 12.2 Hz, 1H, C*H*$_2$Ph), 5.16 (1/2 ABq, *J* = 12.2 Hz, 1H, C*H*$_2$Ph), 4.05 (dddd, *J* = 6.0, 4.8, 2.5, 2.5 Hz, 1H, H4), 3.83 (dd, *J* = 9.5, 4.3 Hz, 1H, H2), 3.13 (ddd, *J* = 12.0, 2.3, 1.4 Hz, 1H, H5a), 2.96 (dd, *J* = 12.0, 4.8 Hz, 1H, H5b), 2.64 (bs, 1H, NH), 2.33 (ddd, *J* = 14.0, 9.5, 6.0 Hz, 1H, H3a), 2.11 (dddd, *J* = 14.0, 4.2, 2.7, 1.4 Hz, 1H, H3b); ^{13}C NMR (75

MHz, $CDCl_3$) δ 173.9, 135.5, 128.6 (2C), 128.5, 128.3 (2C), 67.1, 61.3, 58.9, 52.6, 36.0. MS (ESI) calcd for $C_{12}H_{15}N_4O_2$ $[M+H]^+$: 247.12; found: 247.2. Anal. calcd for $C_{12}H_{14}N_4O_2$: C, 58.53; H, 5.73; N, 22.75; found: C, 58.69; H, 5.83; N, 22.62.

(2*S*,4*S*)-1-(*tert*-Butyldimethylsilyloxyethyl)-4-Fmoc-4-aminoproline (4). To a solution of azidoproline **3** (2.72 g, 11.04 mmol) in DCE (40 mL) were added a solution of (*tert*-butyldimethylsilyloxy)acetaldehyde (2.3 mL, 12.14 mmol) in 20 mL of DCE and $NaBH(OAc)_3$ (3.28 g, 15.46 mmol). The resulting mixture was stirred at room temperature for 1 h, and quenched with saturated aq $NaHCO_3$ solution. Extraction with EtOAc (4 ×), drying of the organic layers over $MgSO_4$, filtration, and evaporation of the solvent in vacuo gave a crude residue, which was purified by silica gel flash chromatography (hexanes/EtOAc, 90:10) to yield N-silyloxyethyl azidoproline intermediate (3.93 g, 88%) as an oil: $[\alpha]^{25}_D$ –36.1 (*c* 12.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.32 (m, 5H, Ph), 5.18 (1/2 ABq, *J* = 12.6 Hz, 1H, C*H*$_2$Ph), 5.11 (1/2 ABq, *J* = 12.0 Hz, 1H, C*H*$_2$Ph), 3.98 (m, 1H, H4), 3.7-3.8 (m, 2H, H2'a, H2'b), 3.44 (dd, *J* = 9.6, 6.0 Hz, 1H, H2), 3.24 (bdd, *J* = 10.2, 1.8 Hz, 1H, H5a), 2.88 (ddd, *J* = 12.6, 6.0, 6.0 Hz, 1H, H1'a), 2.80 (dd, *J* = 10.2, 5.4 Hz, 1H, H5b), 2.66 (ddd, *J* = 12.6, 6.6, 6.6 Hz, 1H, H1'b), 2.44 (ddd, *J* = 13.8, 9.0, 7.2 Hz, 1H, H3a), 2.05 (dddd, *J* = 14.4, 5.4, 3.0, 1.2 Hz, 1H, H3b), 0.85 (s, 9H, *t*-Bu), 0.02 (s, 3H, CH_3), 0.01 (s, 3H, CH_3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.5 (Cq, CO_2R), 135.6 (Cq, Ph), 128.5 (2C, CH, Ph), 128.2 (3C, CH, Ph), 66.5 (CH_2, *C*H_2Ph), 64.5 (CH, C2), 62.0 (CH_2, C2'), 59.0 (CH_2, C5), 58.9 (CH, C4), 55.6 (CH_2, C1'), 35.3 (CH_2, C3). 25.8 (3C, CH_3, *t*-Bu), 18.1 (Cq, *t*-Bu), –5.5 (2C, CH_3).

Azidoproline intermediate (3.93 g, 9.72 mmol) was dissolved in EtOH (120 mL) and a catalytic amount of 10% palladium on carbon (300 mg) was added. The reaction vessel was evacuated by aspirator and thoroughly purged with hydrogen (three times), and the resulting heterogeneous mixture was stirred under hydrogen atmosphere for 8 h at room temperature. The catalyst was filtered off and the filtrate was concentrated in vacuo to give crude aminoproline intermediate (2.66 g, 95%) which was used as such in the following step: a pale yellow oil; $[\alpha]^{25}_D$ –22.0 (*c* 3.2, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 3.78 (m, 3H, H2'a, H2'b and H4), 3.33 (dd, *J* = 9.6, 6.6 Hz, 1H, H2), 3.28 (m, 1H, H5a), 2.9-3.0 (m, 2H, H1'a and H5b), 2.74 (ddd, *J* = 12.8, 6.4, 6.4 Hz, 1H, H1'b), 2.50 (ddd, *J* = 14.0, 9.6, 7.6 Hz, 1H, H3a), 1.86 (ddd, *J* = 14.0, 6.0, 1.6 Hz, 1H, H3b), 0.86 (s, 9H, *t*-Bu), 0.04 (s, 6H, CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 173.3 (Cq), 67.9 (CH), 60.8 (CH_2), 56.9 (CH_2), 56.0 (CH_2), 49.3 (CH), 33.8 (CH_2), 25.1 (3C, CH_3), 17.8 (Cq), –6.4 (CH_3), –6.5 (CH_3).

4-Aminoproline intermediate (2.66 g, 9.24 mmol) was dissolved in THF (30 mL), and 10% aqueous Na_2CO_3 solution (25 mL) was added. FmocOSu (3.17 g, 9.24 mmol) dissolved in THF (25 mL) was then added to the solution pre-cooled to 0 °C. The reaction mixture was stirred for 3 h at room temperature and concentrated in vacuo to leave a residue which was dissolved in EtOAc (10 mL) and treated with saturated aq NH_4Cl solution. The mixture was extracted with EtOAc (3 ×) and the organic layers were collected, dried, filtered, and concentrated to afford a crude residue which was purified by silica gel flash chromatography (EtOAc/MeOH, 70:30) to afford aminoproline **4** (3.68 g, 78%) as an amber glassy solid: $[\alpha]^{25}_D$ –25.0 (*c* 2.0, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 7.67 (d, *J* = 7.6 Hz, 2H, Fmoc), 7.53 (d, *J* = 7.2 Hz, 2H, Fmoc), 7.28

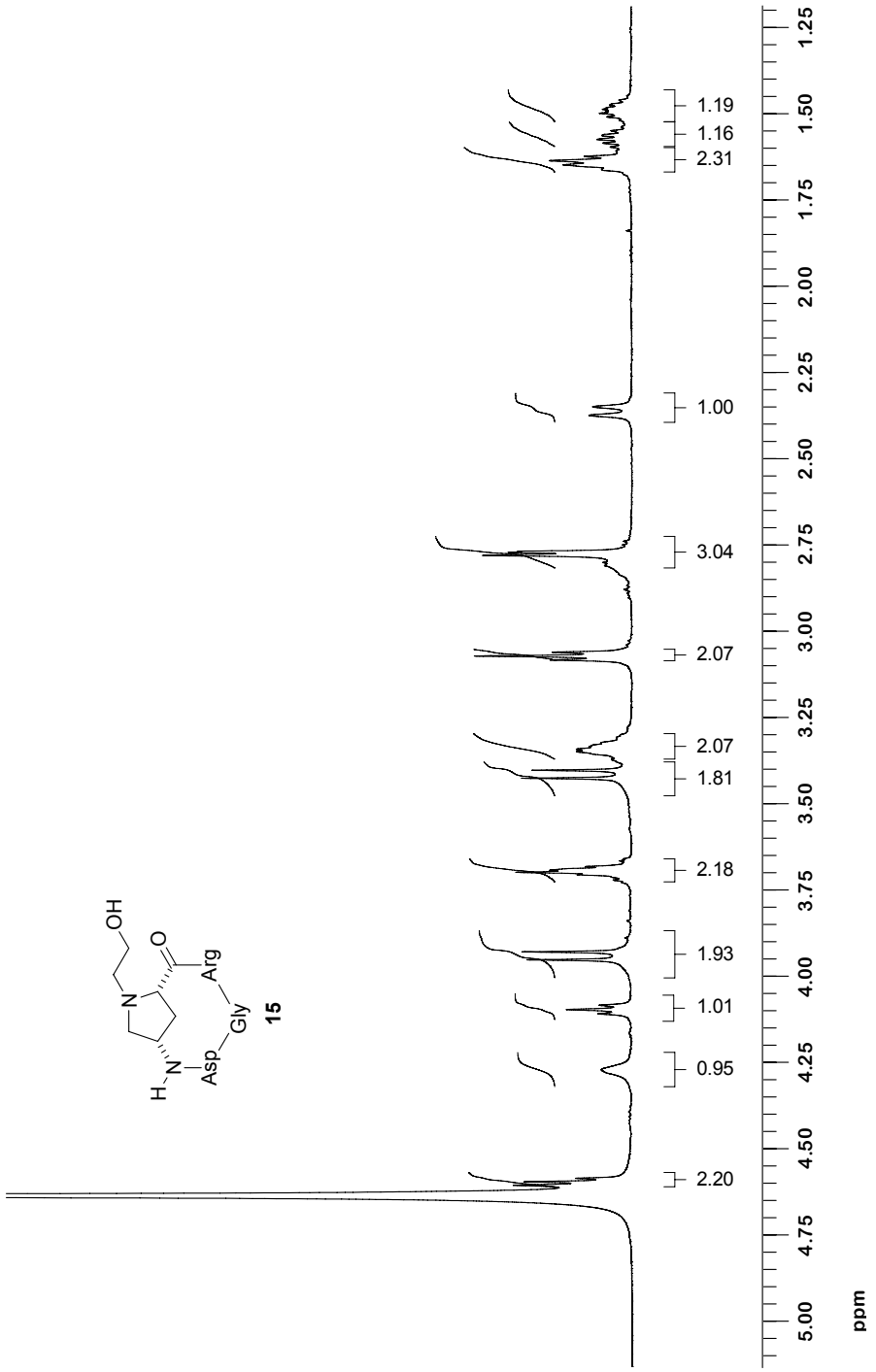
(t, J = 7.6 Hz, 2H, Fmoc), 7.20 (ddd, J = 7.6, 7.6, 1.2 Hz, 2H, Fmoc), 4.37 (m, 2H, CH_2Fmoc), 4.16 (m, 2H, H4 and C*H*Fmoc), 3.85 (m, 2H, H2'), 3.4-3.5 (m, 2H, H2 and H5a), 3.23 (ddd, J = 12.0, 6.0, 6.0 Hz, 1H, H5b), 3.02 (m, 1H H1'a), 2.78 (m, 1H, H1'b), 2.63 (m, 1H, H3a), 1.19 (m, 1H, H3b), 0.82 (s, 9H, *t*-Bu), 0.01 (s, 3H, CH_3), –0.01 (s, 3H, CH3); ^{13}C NMR (100 MHz, CD_3OD) δ 180.0 (Cq), 156.3 (Cq), 143.8 (2C, Cq), 141.2 (2C, Cq), 127.4 (2C, CH), 126.8 (2C, CH), 124.7 (2C, CH), 119.6 (2C, CH), 69.1 (CH), 66.2 (CH_2), 59.9 (CH_2), 59.7 (CH_2), 56.5 (CH_2), 49.5 (CH), 46.7 (CH), 35.1 (CH_2), 25.0 (3C, CH_3), 17.7 (Cq), –6.6 (2C, CH_3). MS (ESI) calcd for $C_{28}H_{39}N_2O_5Si$ $[M+H]^+$: 511.26; found: 511.4. Anal. calcd for $C_{28}H_{38}N_2O_5Si$: C, 65.85; H, 7.50; N, 5.48; found: C, 65.98; H, 7.37; N, 5.37.

Boc-Arg(Mtr)-Gly-OBn (7). To a solution of Boc-Arg(Mtr)-OH (**5**) (4.87 g, 10.0 mmol) in dry THF (80 ml) cooled to –20 °C, NMM (4.4 mL, 40.0 mmol) and isobutyl chloroformate (1.56 mL, 12.0 mmol) were added under nitrogen atmosphere. After 20 min H-Gly-OBn (**6**) (2.42 g, 12.0 mmol) was added; the mixture was allowed to warm to room temperature and stirred for 48 h. The suspension was then filtered through a Celite pad and washed with THF. The filtrate was evaporated under reduced pressure and the crude, dissolved in water, was extracted with EtOAc (3 ×). The combined organic layers were washed with 1N HCl (2 ×), saturated aq $NaHCO_3$ solution (2 ×), dried and evaporated under reduced pressure affording dipeptide **7** (6.33 g, 100%) which was used as such in the next step: white foam; $[\alpha]^{25}_D$ –5.4 (*c* 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.57 (dd, J = 5.5, 5.5 Hz, 1H, NH Gly), 7.32 (m, 5H, CH_2*Ph*), 6.53 (s, 1H, C*H*Mtr), 6.37 (m, 3H, NHε Arg), 5.62 (d, J = 7.5 Hz, 1H, NH Arg), 5.11 (m, 2H, CH_2Ph), 4.28 (m, 1H, Hα Arg), 4.11 (dd, J = 17.8, 5.8 Hz, 1H, Hα Gly), 3.95 (dd, J = 17.8, 5.4 Hz, 1H, Hα Gly), 3.83 (s, 3H, OMe Mtr), 3.23 (m, 2H, Hδ Arg), 2.68 (s, 3H, Me Mtr), 2.62 (s, 3H, Me Mtr), 2.13 (s, 3H, Me Mtr), 1.85 (m, 1H, Hβ Arg), 1.5-1.7 (m, 3H, Hβ and Hγ Arg), 1.41 (s, 9H, *t*-Bu); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.3 (Cq, Arg), 169.9 (Cq, Gly), 158.5 (Cq, Mtr), 156.6 (Cq, Cα Arg), 155.9 (Cq, Boc), 138.5 (Cq, Mtr), 135.2 (Cq, Mtr), 133.2 (Cq, Ph), 128.6 (2C, CH, Ph), 128.4 (CH, Ph), 128.2 (2C, CH, Ph), 124.8 (Cq, Mtr), 111.7 (CH, Mtr), 79.9 (Cq, Boc), 67.1 (CH_2, *C*H_2Ph), 55.4 (CH_3, Mtr), 53.7 (CH, Cα Arg), 41.2 (CH_2, Cα Gly), 40.4 (CH_2, Cδ Arg), 30.9 (CH_2, Cβ Arg), 28.3 (3C, CH_3, Boc), 25.3 (CH_2, Cγ Arg), 24.1 (CH_3, Mtr), 18.3 (CH_3, Mtr), 11.9 (CH_3, Mtr). MS (ESI) calcd for $C_{30}H_{44}N_5O_8S$ $[M+H]^+$: 634.29; found: 634.1. Anal. calcd for $C_{30}H_{43}N_5O_8S$: C, 56.85; H, 6.84; N, 11.05; found: C, 56.71; H, 6.99; N, 10.96.

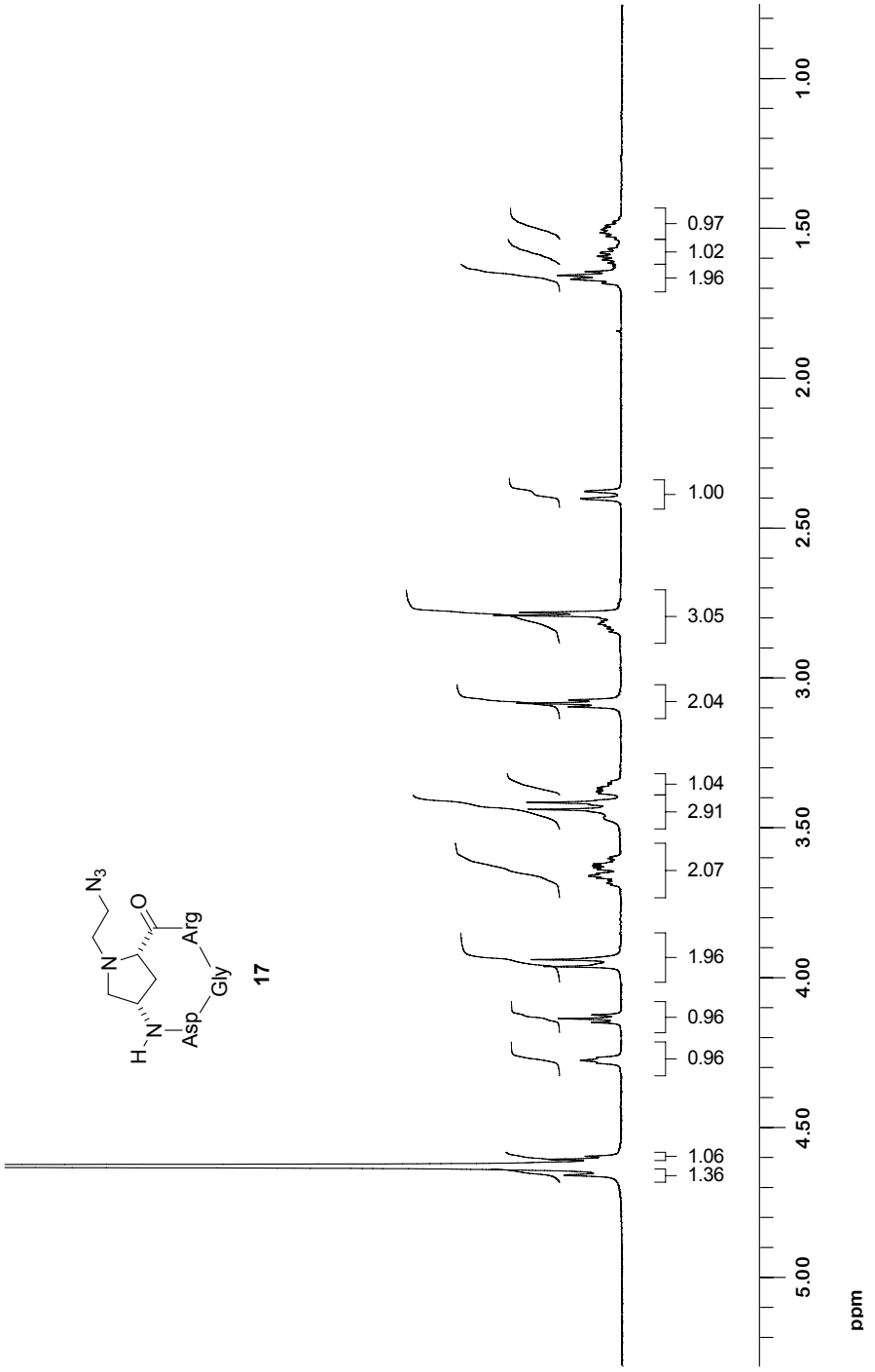
H-Arg(Mtr)-Gly-OBn (8). To a solution of protected dipeptide **7** (6.33 g, 10.0 mmol) in anhydrous DCM (100 mL) cooled to 0° C under argon atmosphere, $Sn(OTf)_2$ (4.17 g, 10.0 mmol) was added and the resulting suspension was stirred for 24 h at room temperature, meanwhile an additional portion of $Sn(OTf)_2$ (10.0 mmol) was added. After reaction completion, the solution was neutralized with saturated aq $NaHCO_3$ solution and treated with solid Na_2CO_3 until pH 9. The mixture was extracted with EtOAc (3 ×) and the collected organic layers were dried, filtered and concentrated furnishing dipeptide **8** (5.12 g, 96%) which was used as such in the next step: white foam; $[\alpha]^{25}_D$ +3.9 (*c* 0.9, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.96 (m, 1H, NH Gly), 7.29 (m, 5H, Ph), 6.49 (s, 1H, C*H*Mtr), 6.42 (m, 3H, NHε Arg), 5.13 (m, 2H, CH_2Ph), 3.98 (m, 2H, Hα Gly), 3.77 (s, 3H, CH_3 Mtr), 3.45 (m, 1H, Hα Arg), 3.14 (m, 2H, Hδ Arg), 2.64 (s, 3H,

CH_3 Mtr), 2.57 (s, 3H, CH_3 Mtr), 2.49 (m, 2H, NH_2 Arg), 2.09 (s, 3H, CH_3 Mtr), 1.75 (m, 1H, Hβ Arg), 1.4-1.6 (m, 3H, Hβ and Hγ Arg); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.5 (Cq), 170.0 (Cq), 158.5 (Cq), 156.6 (Cq), 138.4 (Cq), 136.6 (Cq), 135.1 (Cq), 133.1 (Cq), 128.6 (2C, CH), 128.4 (CH), 128.3 (2C, CH), 124.9 (Cq), 111.8 (CH), 67.2 (CH_2), 55.4 (CH_3), 53.9 (CH), 41.2 (CH_2), 40.5 (CH_2), 31.0 (CH_2), 24.9 (CH_2), 24.1 (CH_3), 18.3 (CH_3), 11.9 (CH_3). MS (ESI) calcd for $C_{25}H_{36}N_5O_6S$ $[M+H]^+$: 534.24; found: 534.0. Anal. calcd for $C_{25}H_{35}N_5O_6S$: C, 56.27; H, 6.61; N, 13.12; found: C, 56.33; H, 6.85; N, 13.06.

^{1}H NMR spectrum (600 MHz, D_2O) of cyclopeptide **15**



^{1}H NMR spectrum (600 MHz, D_2O) of cyclopeptide **17**



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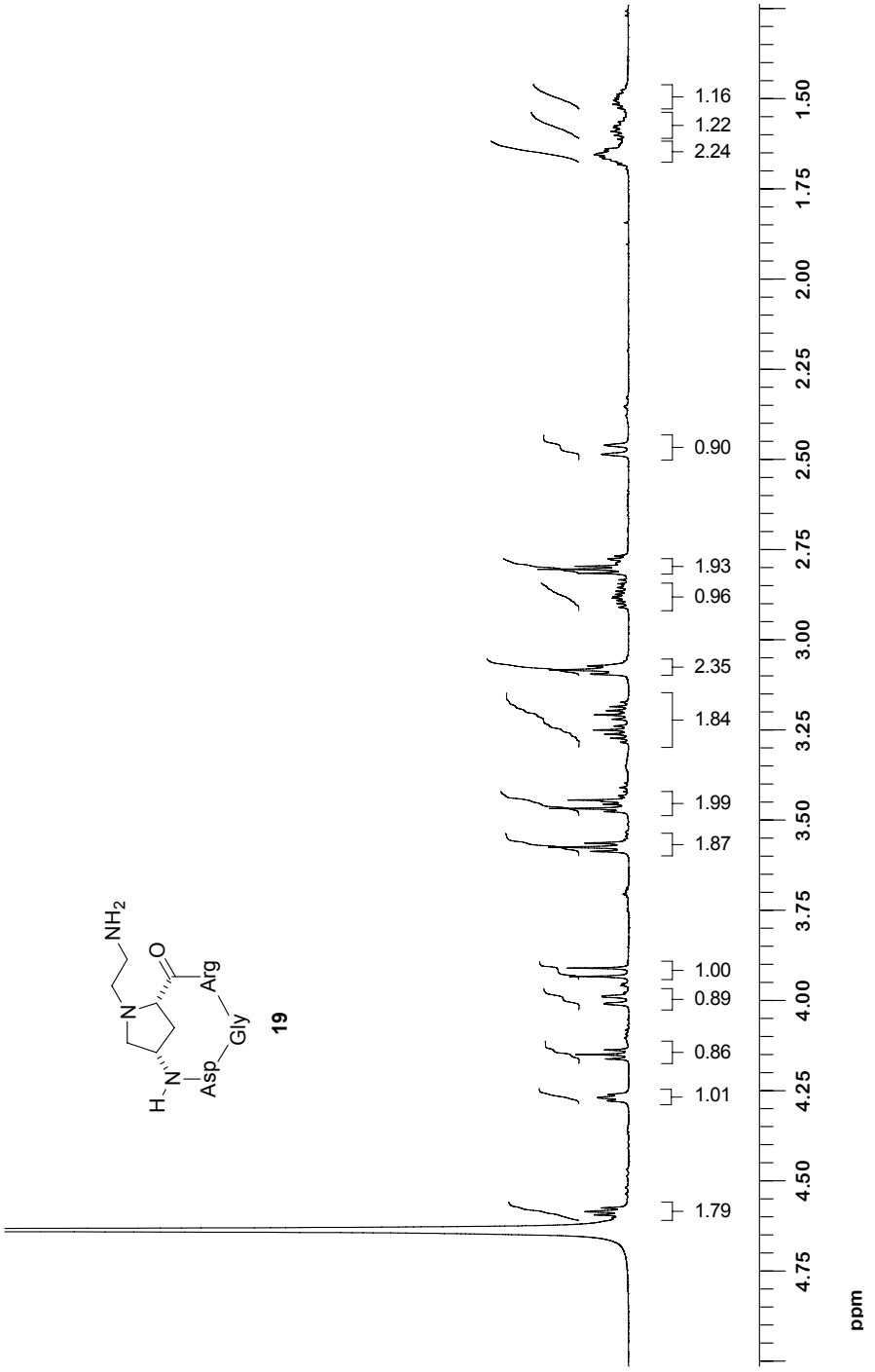

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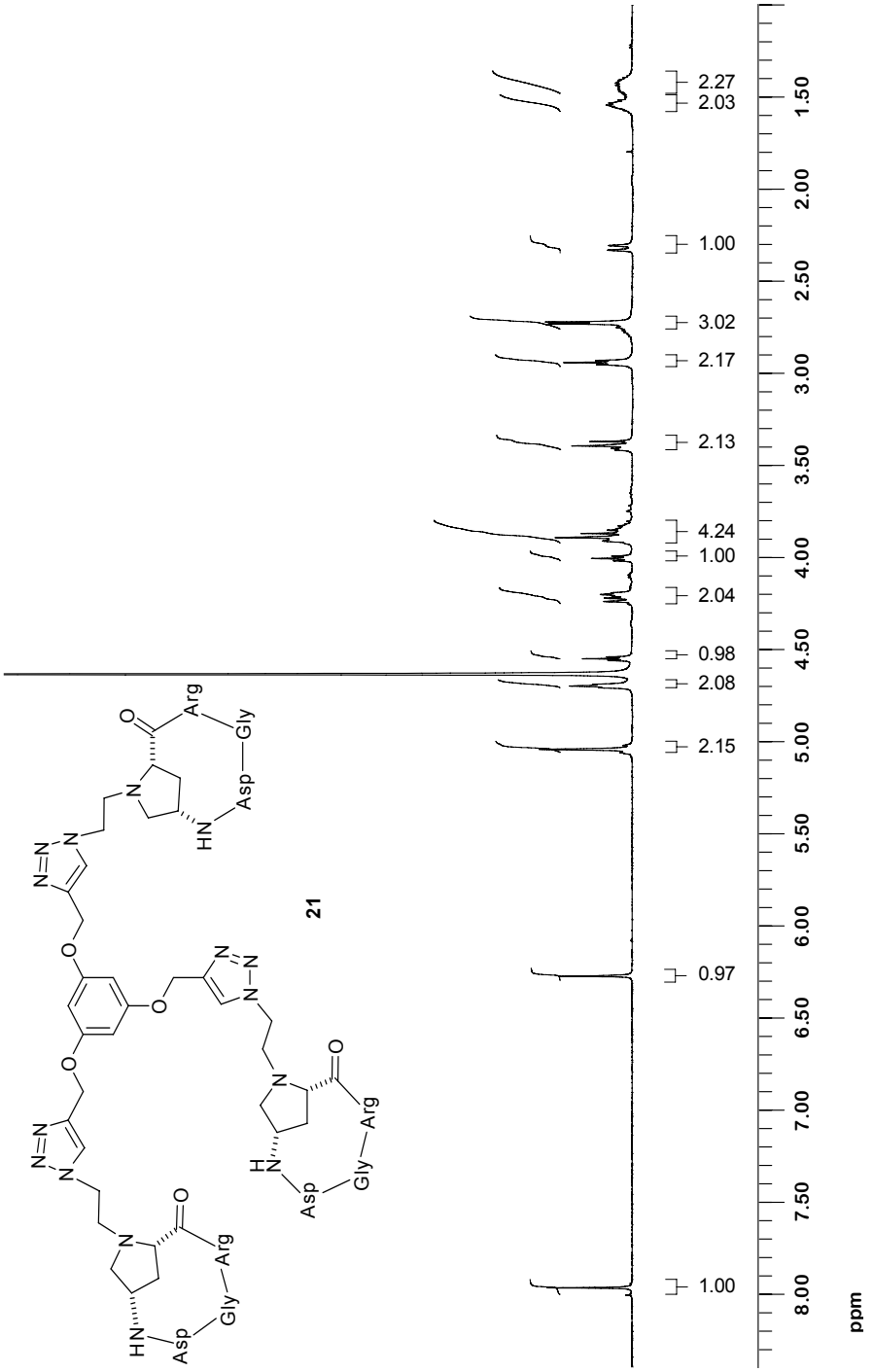

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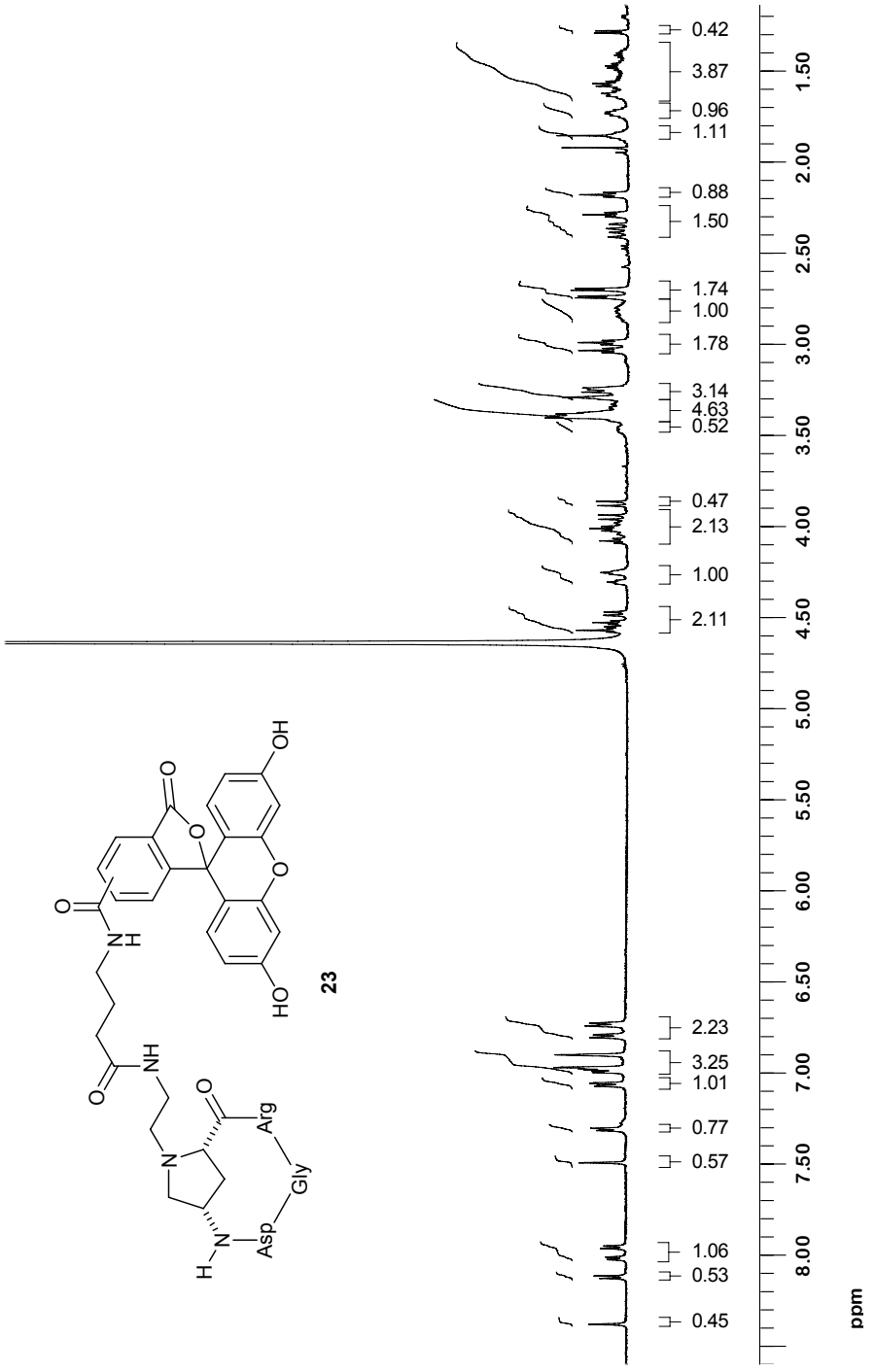

^{1}H NMR spectrum (600 MHz, D_2O) of cyclopeptide **25**