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 F254 precoated plates (Merck) with visualization under short-wavelength UV light or by dipping the plates with molybdate reagent (aqueous H2SO4 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 (CDCl3), CD2HOD, 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

(2S,4R)-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 NaH2PO4 (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 (MgSO4), 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; [α]^25_D = −77.0 (c 0.8, H2O); 1H NMR (300 MHz, CD3OD) 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); 13C NMR (75 MHz, CD3OD) 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
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 H2O, 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 (MgSO4), 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%): [α]25D –61.3 (c 4.0, CHCl3); 1H NMR (300 MHz, CDCl3, mixture of rotamers) δ 7.30 (m, 5H, Ph), 5.15 (m, 2H, C6H2Ph), 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); 13C NMR (75 MHz, CDCl3, 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 C17H24NO5 [M+H]+: 322.16; found: 322.4. Anal. calcd for C17H23NO5: C, 63.53; H, 7.21; N, 4.36; found: C, 63.35; H, 7.29; N, 4.41.

(2S,4S)-4-Azidoproline benzyl ester (3). To an ice-cooled solution of proline 2 (4.16 g, 12.96 mmol) and PPh3 (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: [α]25D –40.1 (c 1.0, CHCl3); 1H NMR (300 MHz, CDCl3, mixture of rotamers) δ 7.31 (m, 5H, Ph), 5.21 (m, 2H, C6H2Ph), 4.50 (dd, J = 8.9, 3.5 Hz, 0.4H, H2), 4.38 (dd, J = 8.9, 3.9 Hz, 1H, H5a), 3.42 (m, 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); 13C NMR (75 MHz, CDCl3, mixture of rotamers, major isomer) δ 171.4, 153.7, 135.6, 128.6 (2C), 128.4 (2C), 128.1, 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 NaHCO3 solution and solid Na2CO3 were sequentially added until pH 9. The mixture was extracted with EtOAc (4 ×) and the organic layers were collected, dried over MgSO4, filtered, and concentrated to afford a crude residue which was purified by silica gel flash chromatography (EtOAc/MeOH, 96:4) to afford the Nα-deprotected azidoproline 3 (2.72 g, 87%) as a colorless oil: [α]25D –42.5 (c 1.0, CHCl3); 1H NMR (300 MHz, CDCl3) δ 7.34 (m, 5H, Ph), 5.21 (1/2 ABq, J = 12.2 Hz, 1H, C6H2Ph), 5.16 (1/2 ABq, J = 12.2 Hz, 1H, C6H2Ph), 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 (dd, 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); 13C NMR (75 Hz, DMSO-d6) δ...
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 NaHCO₃ solution. Extraction with EtOAc (4 ×), drying of the organic layers over MgSO₄, 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: [α]₂⁵_D –36.1 (c 12.0, CHCl₃); ¹H NMR (600 MHz, CDCl₃) δ 7.32 (m, 5H, Ph), 5.18 (1/2 ABq, J = 12.6 Hz, 1H, CH₂Ph), 5.11 (1/2 ABq, J = 12.0 Hz, 1H, CH₂Ph), 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 (dd, 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 (ddddd, J = 14.4, 3.2, 1.2, 1.0 Hz, 1H, H₃b), 0.85 (s, 9H, tert-Bu), 0.02 (s, 3H, CH₃), 0.01 (s, 3H, CH₃); ¹³C NMR (150 MHz, CDCl₃) δ 172.5 (Cq, CO₂R), 135.6 (Cq, Ph), 128.5 (2C, CH₂Ph), 128.2 (3C, CH, Ph), 66.5 (CH₂, CH₂Ph), 64.5 (CH, C2), 62.0 (CH₂, C2'), 59.0 (CH₂, C5), 58.9 (CH, C4), 55.6 (CH₂, C1'), 35.3 (CH₂, C3). 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; [α]₂⁵_D –22.0 (c 3.2, MeOH); ¹H NMR (400 MHz, CD₃OD) 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, tert-Bu), 0.04 (s, 6H, CH₃); ¹³C NMR (100 MHz, CD₃OD) δ 173.3 (Cq), 67.9 (CH), 60.8 (CH₂), 56.9 (CH₂), 56.0 (CH₂), 49.3 (CH), 33.8 (CH₂), 25.1 (3C, CH₃), 17.8 (Cq), –6.4 (CH₃), –6.5 (CH₃).
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 NaHCO₃ 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; [α]²⁵D −5.4 (c 1.0, CHCl₃); ¹H NMR (300 MHz, CDCl₃) δ 7.57 (dd, J = 5.5, 5.5 Hz, 1H, NH Gly), 7.32 (m, 5H, CH₂Ph), 6.53 (s, 1H, CH₂Mtr), 6.37 (m, 3H, NH ε Arg), 5.62 (d, J = 7.5 Hz, 1H, NH Arg), 5.11 (m, 2H, CH₂ Ph), 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, H δ 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); ¹³C NMR (75 MHz, CDCl₃) 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 (CH₂, CH₂Ph), 55.4 (CH₃, Mtr), 53.7 (CH, Cα Arg), 41.2 (CH₂, Cγ Gly), 40.4 (CH₂, Cδ Arg), 30.9 (CH₂, Cβ Arg), 28.3 (3C, CH₃, Boc), 25.3 (CH₂, Cγ Arg), 24.1 (CH₃, Mtr), 18.3 (CH₃, Mtr), 11.9 (CH₃, Mtr). MS (ESI) calcld for C₂₈H₃₉N₂O₅Si [M+H]⁺: 511.26; found: 511.4. Anal. calcld for C₂₈H₃₈N₂O₅Si: C, 65.85; H, 7.50; N, 5.48; found: C, 65.98; H, 7.37; N, 5.37.

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 NaHCO₃ solution and treated with solid Na₂CO₃ 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; [α]²⁵D +3.9 (c 0.9, CHCl₃); ¹H NMR (300 MHz, CDCl₃) δ 7.96 (m, 1H, NH Gly), 7.29 (m, 5H, Ph), 6.49 (s, 1H, CH/Mtr), 6.42 (m, 3H, NHε Arg), 5.13 (m, 2H, CH₂Ph), 3.98 (m, 2H, Hδ Gly), 3.77 (s, 3H, CH₃ Mtr), 3.45 (m, 1H, Hα Arg), 3.14 (m, 2H, Hδ Arg), 2.64 (s, 3H, Hγ Arg), 2.59 (s, 3H, Hβ Arg), 2.22-2.38 (m, 3H, Hβ and Hγ Arg). ¹³C NMR (100 MHz, CDCl₃) 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 (CH₂), 59.9 (CH₂), 59.7 (CH₂), 56.5 (CH₂), 49.5 (CH), 46.7 (CH), 35.1 (CH₂), 25.0 (3C, CH₃), 17.7 (Cq), –6.6 (2C, CH₃). MS (ESI) calcld for C₂₈H₃₈N₂O₅Si [M+H]⁺: 511.26; found: 511.4. Anal. calcld for C₂₈H₃₈N₂O₅Si: C, 65.85; H, 7.50; N, 5.48; found: C, 65.98; H, 7.37; N, 5.37.
CH₃ Mtr), 2.57 (s, 3H, CH₃ Mtr), 2.49 (m, 2H, NH₂ Arg), 2.09 (s, 3H, CH₃ Mtr), 1.75 (m, 1H, Hβ Arg), 1.4-1.6 (m, 3H, Hβ and Hγ Arg); \(^{13}\)C NMR (75 MHz, CDCl₃) \(δ\) 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 (CH₂), 55.4 (CH₃), 53.9 (CH), 41.2 (CH₂), 40.5 (CH₂), 31.0 (CH₂), 24.9 (CH₂), 24.1 (CH₃), 18.3 (CH₃), 11.9 (CH₃). MS (ESI) calcd for C₂₅H₃₆N₅O₆S [M+H]⁺: 534.24; found: 534.0. Anal. calcd for C₂₅H₃₅N₅O₆S: 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$_2$O) of cyclopeptide 15
$^1$H NMR spectrum (600 MHz, D$_2$O) of cyclopeptide 17
$^1$H NMR spectrum (600 MHz, D$_2$O) of cyclopeptide 19
$^1$H NMR spectrum (600 MHz, D$_2$O) of cyclopeptide 21
$^1$H NMR spectrum (600 MHz, D$_2$O) of cyclopeptide 23
$^1$H NMR spectrum (600 MHz, D$_2$O) of cyclopeptide 25