Supporting Information

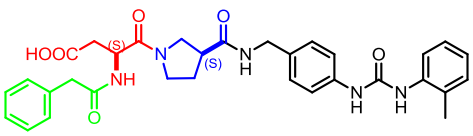
Synthesis and assay of retro-$\alpha_4\beta_1$ integrin-targeting motifs

Samantha D. Dattoli,[b] Rossella De Marco,[¥,a] Monica Baiula,[b] Santi Spampinato,[§,b] Arianna Greco,[a] Alessandra Tolomelli,[a] Luca Gentilucci.*[,a]

[a] Dept. of Chemistry "G. Ciamician", University of Bologna, via Selmi 2, 40126 Bologna, Italy.
[b] Dept. of Pharmacy and BioTechnology, University of Bologna, via Irnerio 48, 40126 Bologna, Italy.

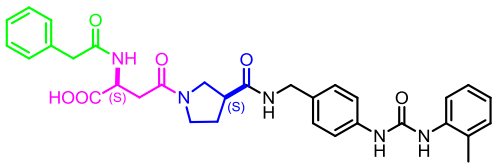
* Fax: +39 051 2099456, Tel: +39 051 2099570, E-mail: luca.gentilucci@unibo.it
[¥] E-mail: rossella.demarco2@unibo.it
[§] E-mail: santi.spampinato@unibo.it

KEYWORDS: VLA-4 ligands; scintillation proximity assay; cell adhesion inhibition; peptidomimetics; retro sequences; β^2-proline; diphenylurea; isoaspartate



BnCO-Asp-β^2-Pro-AMPUMP

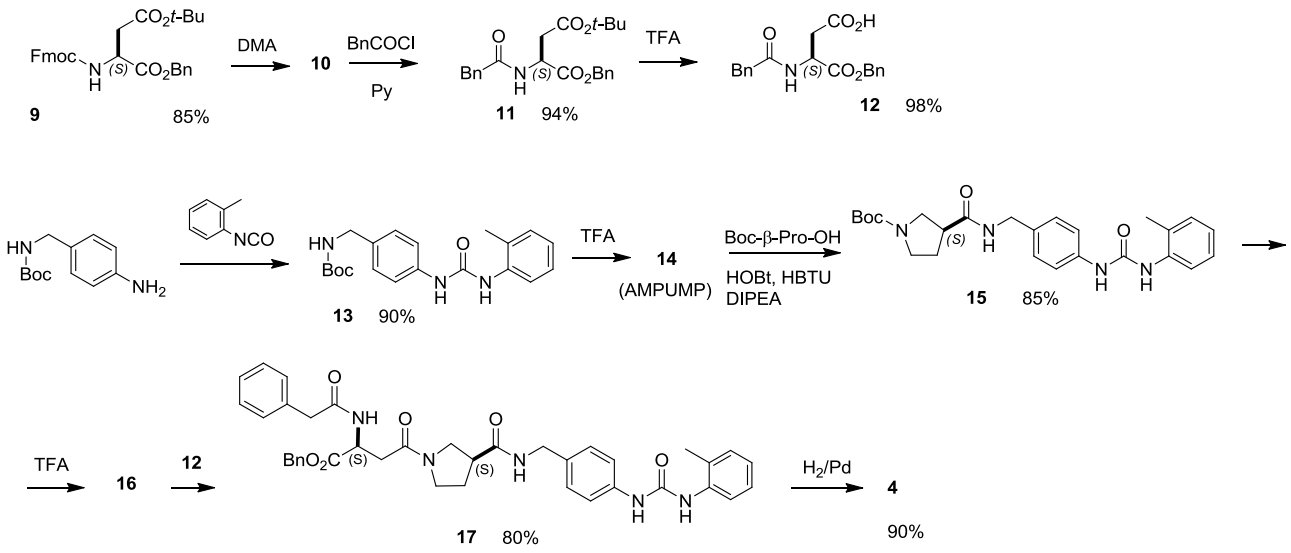
SPA IC_{50}: 1.0×10^{-7} M
$\alpha4\beta1$/VCAM-1 IC_{50}: 8.0×10^{-8} M



BnCO-*iso*Asp-β^2-Pro-AMPUMP

SPA IC_{50}: 2.9×10^{-7} M
$\alpha4\beta1$/VCAM-1 IC_{50}: 9.3×10^{-8} M

Content summary



Scheme S1. Synthesis of **4**.

*Fmoc-Asp(OtBu)-OBn (**9**).* A mixture of Fmoc-Asp(O*t*Bu)-OH (1.50 g, 3.65 mmol), BnBr (1.7 mL, 14.6 mmol) $NaHCO_3$ (0.92 g, 10.9 mmol) in DMF (6 mL), was stirred for 8 h. Then the mixture was diluted with water (10 mL) and extracted three times with EtOAc (20 mL). The combined organic layers were dried over Na_2SO_4, and solvent was distilled at reduced pressure. The oily residue was purified by flash chromatography over silica-gel (eluant EtOAc/cyclohexane 1:9) giving **7** (1.58 g, 85%). The analyses were in agreement with the literature [1].

*BzCO-Asp(OtBu)-OBn (**11**).* **9** (0.89 g, 1.71 mmol) was treated with 2M dimethylamine in THF (4 mL) under inert atmosphere while stirring. After 10 min, the solvent was distilled at reduced pressure, and the residue was treated as before for additional 40 min. After solvent evaporation, the residue was triturated with Et_2O, and the precipitate **10** (quantitative yield) was utilized without purification. ESI-MS *m/z* 293.1 $(M+H)^+$, calcd 293.1. Crude **10** was dissolved in DCM (10 mL) and treated with BnCOCl (0.250 mL, 1.71 mmol) and pyridine (0.21 mL, 2.6 mmol) under inert atmosphere. After 4 h the reaction was diluted with EtOAc (30 mL) and washed with 0.5 M HCl (5 mL), sat. $NaHCO_3$ (5 mL), and brine (5 mL), and dried over Na_2SO_4. Solvent was distilled at reduced pressure, and the residue was purified by flash chromatography over silica-gel (eluant EtOAc/cyclohexane 2:8) giving **11** (0.64 g, 94%). ^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): 1.40 (s, 9H, *t*-Bu), 2.73 (dd, J = 4.2, 17.1 Hz, 1H, AspHβ), 2.95 (dd, J= 4.0, 17.1 Hz, 1H, AspHβ), 3.60 (s, 2H, CH_2Ph), 4.86 (q, J = 8.2 Hz, 1H, AspHα), 5.10 (d, J = 13.0 Hz, 1H, CH_2Ph), 5.22 (d, J = 13.0 Hz, 1H, CH_2Ph), 6.51 (br.d, J = 7.2 Hz, 1H, AspNH), 7.40-7.80 (m, 10H, ArH); ESI-MS *m/z* 398.1 $(M+H)^+$, calcd 398.2.

*BzCO-Asp-OBn (**12**).* **11** (0.64 g, 1.61 mmol) was stirred in 1:3 TFA/DCM (4 mL). After 30 min the mixture was concentrated at reduced pressure, and the procedure was repeated. The oily residue was triturated in Et_2O, and the crude **12** which precipitated (0.54 g, 98%), was utilized without purification. ESI-MS *m/z* 342.1 $(M+H)^+$, calcd 342.1

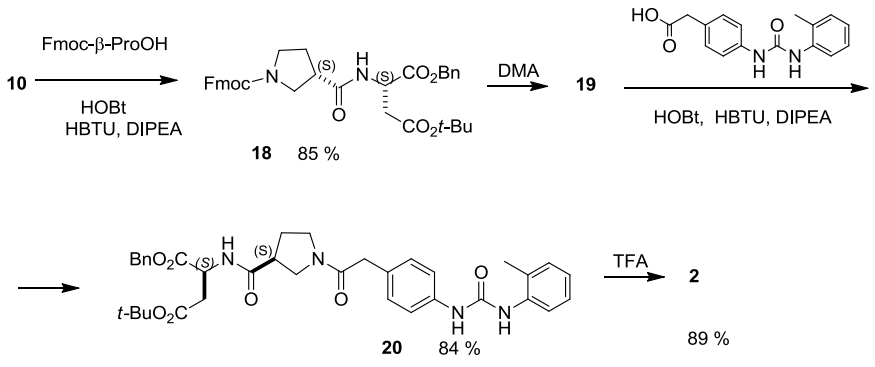
*Synthesis of Boc-AMPUMP **13**.* 2-Methylphenyl isocyanate (0.56 mL, 4.50 mmol) was added to a stirred solution of *t-Bu*-4-aminobenzylcarbamate (1.0 g, 4.50 mmol) in DMF (8 mL) at r.t. under inert atmosphere.

After 2 h the reaction was diluted with EtOAc (30 mL), giving the precipitate **13** (1.44 g, 90%) collected by filtration. ^{1}H NMR (DMSO-d6, 200 MHz) δ (ppm): 1.38 (s, 9H, *t*-Bu), 2.22 (s, 3H, CH_3Ph), 4.06 (d, J = 5.8 Hz, 2H, CH_2Ph), 4.24 (d, J = 5.4 Hz, 1H, NH), 6.86 (dd, J = 6.2, 13.2 Hz, 2H, ArH), 6.91-7.22 (m, 4H, ArH), 7.35 (d, J = 8.6 Hz, 1H, ArH), 7.76 (s, 1H, NH), 7.81 (d, J = 8.0 Hz, 1H, ArH), 8.82 (s, 1H, NH); ESI-MS *m/z* 356.1 $(M+H)^+$, calcd .356.2.

*Synthesis of **15**.* Boc deprotection of **13** (1.44 g, 4.05 mmol) was performed as described for the preparation of **12**. The residue was suspended in Et_2O (20 mL). The TFA-AMPUMP salt **14** which precipitated in quantitative yield was used for the next coupling without further purifications. ESI-MS *m/z* 256.1 $(M+H)^+$, calcd 256.1. The TFA salt **14** was coupled with Boc-(*S*)-β-*iso*Pro-OH (1.13 g, 5.0 mmol) under MW irradiation as described in the main text (Peptide coupling, general procedure). The residue was purified by flash chromatography over silica-gel (eluant EtOAc) giving **15** as a waxy solid (1.55 g, 85%). ^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): 1.44 (s, 9H, *t*-Bu),1.50-1.63 (m, 1H, PyrrolidineH-4), 1.90-2.01 (m, 2H, PyrrolidineH-4+H-3), 2.13 (s, 3H, CH_3Ph), 2.76-2.90 (m, 1H, PyrrolidineH-5), 3.10-3.22 (m, 1H, PyrrolidineH-5), 3.38-3.58 (m, 2H, PyrrolidineH-2), 4.06 (d, J = 5.8 Hz, 2H, CH_2Ph), 4.24 (d, J = 5.4 Hz, 1H, NH), 6.86 (dd, J = 6.2, 13.2 Hz, 2H, ArH), 6.91-7.22 (m, 4H, ArH), 7.35 (d, J = 8.6 Hz, 1H, ArH), 7.76 (s, 1H, NH), 7.81 (d, J = 8.0 Hz, 1H, ArH), 8.82 (s, 1H, NH); ESI-MS *m/z* 475.1 $(M+Na)^+$, calcd 475.2.

*Synthesis of **17**.* **15** (1.55 g, 3.4 mmol) was deprotected as described for **14**; the TFA salt **16** (quantitative yield) was used without further purifications. ESI-MS *m/z* 353.1 $(M+H)^+$, calcd 353.2. Crude **16** (0.26 g, 0.73 mmol) was coupled with **12** (0.25 g, 0.73 mmol) under MW irradiation as described in the main text (Peptide coupling, general procedure). The residue was purified by flash chromatography over silica-gel (eluant EtOAc/cyclohexane 2:8) giving **17** (0.39 g, 80%). ^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): 1.84-2.19 (m, 2H, PyrrolidineH-4), 2.19 (s, 3H, CH_3), 2.58 (m, 1H, AspHβ), 2.70-2.81 (m, 1H, PyrrolidineH-3), 2.94 (m, 1H, AspHβ), 3.20-3.30 (m, 2H, PyrrolidineH-5$_<$), 3.41-3.51 (m, 4H, CH_2Ph+PyrrolidineH-2$_>$), 3.51-3.62 (m, 2H, PyrrolidineH-5$_>$), 4.23-4.40 (m, 4H, CH_2Ph+PyrrolidineH-2$_<$), 4.82 (m, 1H, AspHα), 5.25 (m, 2H, CH_2Ph), 6.30 (br.t, 1H, NH), 6.40 (s, 1H, NH), 6.60 (br.d, 1H, AspNH), 6.70 (s, 1H, NH), 6.90 (t, J = 7.4 Hz, 1H, ArH), 6.94-7.20 (m, 9H, ArH), 7.20-7.25 (m, 5H, ArH), 7.25-7.36 (m, 2H, ArH), 7.59 (d, J = 8.0 Hz, 1H, ArH); ESI-MS *m/z* 676.2 $(M+H)^+$, calcd 676.3.

Synthesis of ***4***. **17** (0.39 g, 0.58 mmol) was treated with H_2 and catalytic Pd/C in EtOH (20 mL). After 6 h, the mixture was filtered over Celite, and the solvent was evaporated at reduce pressure. The residue was purified by semi-preparative RP-HPLC (general methods) giving **4** (0.27 g, 80 %).

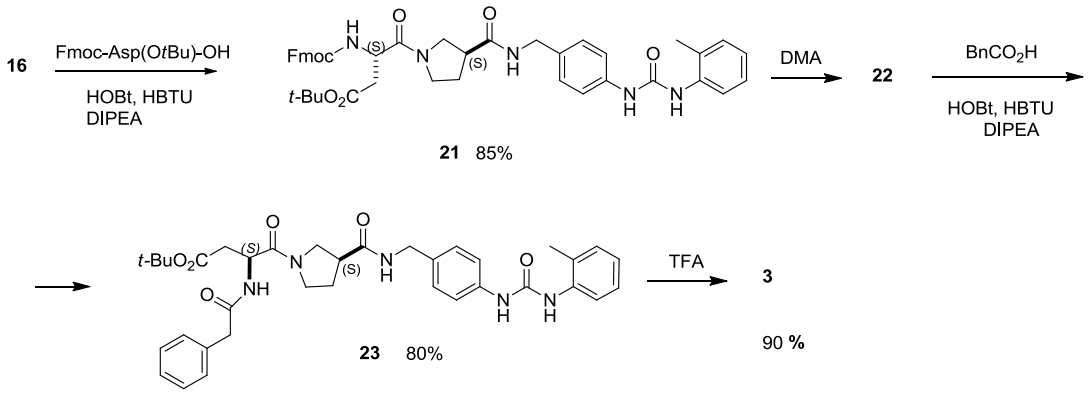


Scheme S2. Synthesis of **2**.

*Synthesis of **18**.* Fmoc-(*S*)-β-*iso*Pro-OH (0.13 g, 0.38 mmol) was coupled with **10** (0.10 g, 0.34 mmol) under MW irradiation as described in the main text (Peptide coupling, general procedure). The residue was purified by flash chromatography over silica-gel (eluant EtOAc/cyclohexane 1:1) giving **18** (0.20 g, 85%). ESI-MS *m/z* 621.2 $(M+Na)^+$, calcd 621.3.

*Synthesis of **20***. Fmoc deprotection of **18** (0.20 g, 0.32 mmol) was performed as reported for the synthesis of **10**; after solvent evaporation, the residue containing **19** (quantitative yield) was utilized without purification. ESI-MS *m/z* 377.1 $(M+H)^+$, calcd 377.2. Crude **19** was coupled with 4-[N-(2-methylphenyl)ureido]phenylacetyl [2] (0.14 g, 0.50 mmol) as described in the main text (Peptide coupling, general procedure). The residue was purified by flash chromatography over silica-gel (eluant EtOAc/ cyclohexane 1:1) giving **20** (0.18 g, 84%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): (two sets of resonances: > = major conformer; < = minor conformer) 1.36 (s, 9H, *t*-Bu), 2.03 (q, J = 7.4 Hz, 1H, PyrrolidineH-4), 2.16 (s, 4H, CH_3+PyrrolidineH-4), 2.72 (dd, J = 4.6, 17.0 Hz, 1H, AspHβ), 2.82-2.98 (m, 2H, PyrrolidineH-3+AspHβ), 3.38 (m, 1H, PyrrolidineH-$5_<$), 3.40-3.50 (m, 3H, CH_2Ph+PyrrolidineH-$2_>$+PyrrolidineH-$5_<$), 3.51-3.60 (m, 6H, PyrrolidineH-$2_<$+PyrrolidineH-$2_>$+CH_2Ph+PyrrolidineH-$5_>$), 4.80 (m, 1H, AspHα), 5.10 (d, J = 12.4 Hz, 1H, CH_2Ph$_<$), 5.13 (d, J = 12.0 Hz, 1H, CH_2Ph$_>$), 5.15 (d, J = 12.4 Hz, 1H, CH_2Ph$_<$), 5.18 (d, J = 12.0 Hz, 1H, CH_2Ph$_>$), 6.76 (d, J = 8.0 Hz, 1H, AspNH$_<$), 6.87 (d, J = 8.4 Hz, 1H, AspNH$_>$), 6.93-7.04 (m, 4H, ArH), 7.04-7.14 (m, 5H, ArH), 7.21-7.33 (m, 4H, ArH+NH), 7.63 (d, J = 7.6 Hz, 1H, ArH), 7.68 (s, 1H, NH); ESI-MS *m/z* 665.2 $(M+Na)^+$, calcd 665.3

*Synthesis of **2**.* The deprotection of **20** (0.18 g, 0.27mmol) was performed as described for **12** . The residue was purified by semi-preparative RP-HPLC (general methods) giving **2** (0.14 g, 89%).



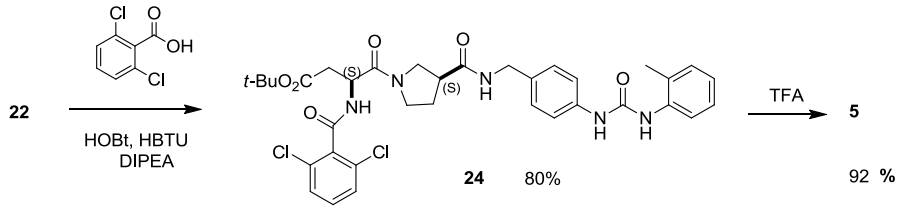
Scheme S3. Synthesis of **3**.

*Synthesis of **21**.* Fmoc-Asp(O*t*Bu)OH (0.321 g, 0.78 mmol) was coupled with **16** (0.25 g, 0.71 mmol) as described in the main text (Peptide coupling, general procedure). The residue was purified by flash chromatography over silica-gel (eluant EtOAc/cyclohexane 8:2) giving **21** (0.45 g, 85%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 1:1 mixture of two conformers: 1.31+1.33 (s, 9H, *t*-Bu), 1.90 (q, J = 7.2 Hz, 1H,

PyrrolidineH-4), 1.98 (m, 1H, PyrrolidineH-4), 2.04+2.05 (s, 3H, CH_3), 2.45 (m, 1H, AspHβ), 2.62-2.73 (m, 2H, PyrrolidineH-3+AspHβ), 3.22 (m, 1H, PyrrolidineH-5), 3.36-3.49 (m, 2H, PyrrolidineH-2+PyrrolidineH-5), 3.58 (dd, J = 7.0, 9.0 Hz, 1H, PyrrolidineH-2), 3.65-3.76 (m, 2H, PyrrolidineH-2+PyrrolidineH-5), 4.09, (d, J = 6.4 Hz, 2H, CH_2Ph), 4.14-4.30 (m, 3H, Fmoc), 4.78 (br.q, 1H, AspHα), 5.85+5.90 (d, J = 9.2 Hz, 1H, AspNH), 6.80 (d, 1H, J = 8.8 Hz, 1H, ArH), 6.85-6.90 (m, 2H, ArH), 6.94-7.09 (m, 5H, ArH), 7.12-7.22 (m, 2H, ArH), 7.22-7.35 (m, 3H, ArH+NH), 7.42-7.54 (m, 3H, ArH+NH), 7.64 (d, J = 8.0 Hz, 2H, ArH), 7.92 (s, 1H, NH); ESI-MS *m/z* 746.3 $(M+H)^+$, calcd 746.4.

*Synthesis of **23**.* Fmoc deprotection of **21** (0.32 g, 0.43 mmol) was performed as described for the preparation of **10** giving crude **22** in quantitative yield. ESI-MS *m/z* 524.3 $(M+H)^+$, calcd 524.4. The resulting residue was coupled with phenylacetic acid (0.065 g, 0.48 mmol) as described in the main text (Peptide coupling, general procedure). The residue was purified by flash chromatography over silica-gel (eluant EtOAc/cyclohexane 8:2) giving **23** (0.22 g, 80%). ^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): (two sets of resonances: > = major conformer; < = minor conformer) 1.39 (s, 9H, *t*-Bu), 1.95-2.05 (m, 2H, PyrrolidineH-4), 2.28 (s, 3H, CH_3), 2.50 (m, 1H, AspHβ), 2.75-2.90 (m, 2H, AspHβ+PyrrolidineH-3), 3.22 (m, 1H, PyrrolidineH-$5_<$), 3.37-3.52 (m, 6H, PyrrolidineH-$2_>$+CH_2Ph+PyrrolidineH-$5_>$+PyrrolidineH-$5_<$), 3.61-3.80 (m, 3H, PyrrolidineH-$2_<$+PyrrolidineH-$5_>$), 4.15-4.25 (m, 2H, CH_2Ph), 4.99 (m, 1H, AspHα), 6.82-7.12-7.20 (m, 10H, ArH), 7.23-7.40 (m, 4H, ArH+AspNH+NH), 7.48-7.55 (m, 2H, NH), 7.65 (br.d, 1H, ArH); ESI-MS *m/z* 642.2 $(M+H)^+$, calcd 642.3.

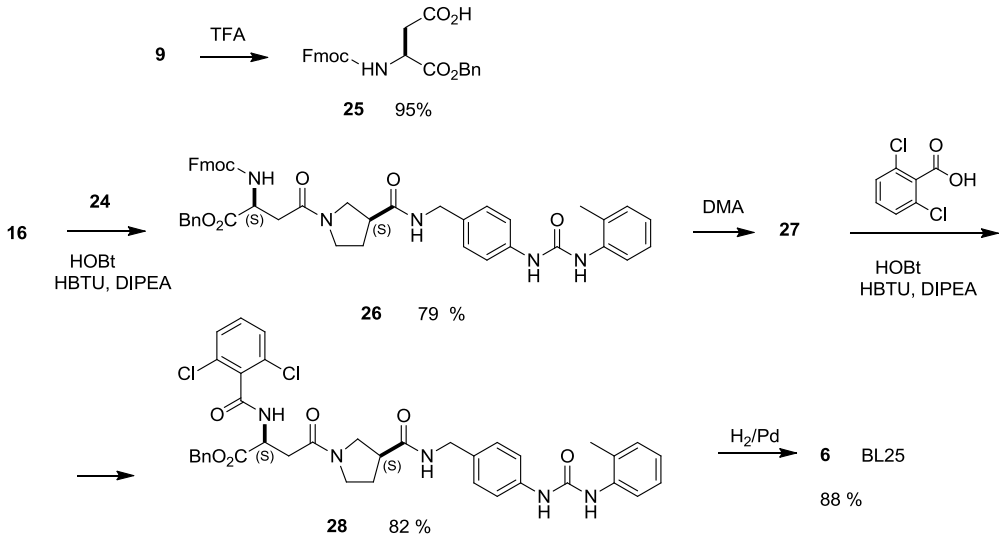
*Synthesis of **3***. **23** (0.22 g, 0.34 mmol) was deprotected as described for **12**. The residue was purified by semi-preparative RP-HPLC (general methods) giving **3** (0.18 g, 90%).



Scheme S4. Synthesis of **5**.

*Synthesis of **24**.* **22** was prepared as described above and in the same amount, and it was coupled with 2,6-dichlorobenzoic acid (0.090 g, 0.48 mmol) under MW irradiation as described in the main text (Peptide coupling, general procedure). The residue was purified by flash chromatography over silica-gel (eluant EtOAc/cyclohexane 8:2) giving **24** (0.24 g, 80 %). ^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): (two sets of resonances: > = major conformer; < = minor conformer) 1.35 (s, 9H, *t*-Bu), 2.05-2.20 (m, 2H, PyrrolidineH-4), 2.39 (s, 3H, CH_3Ph), 2.52 (m, 1H, AspHβ), 2.89-2.99 (m, 2H, PyrrolidineH-$3_>$+AspHβ), 3.10 (m, 1H, PyrrolidineH-$3_<$), 3.25 (m, 1H, PyrrolidineH-$5_<$), 3.45-3.63 (m, 2H, PyrrolidineH-$2_>$+PyrrolidineH-$5_<$+PyrrolidineH-$5_>$), 3.69-3.78 (m, 2H, PyrrolidineH-$2_>$+PyrrolidineH-$2_<$), 4.09-4.10 (m, 2H, PyrrolidineH-$5_>$+PyrrolidineH-$2_<$), 4.26 (d, J = 5.7 Hz, 2H, CH_2Ph), 4.99 (m, 1H, AspHα), 6.84 (br.t, 1H, ArH), 7.05-7.15 (m, 5H, ArH+NH), 7.15-7.26 (m, 5H, ArH+AspNH+NH), 7.25-7.33 (m, 3H, ArH+NH), 7.75-7.87 (m, 2H, ArH+NH). ESI-MS *m/z* 696.1 $(M+H)^+$, calcd 696.2

*Synthesis of **5**.* **24** (0.24 g, 0.34 mmol) was deprotected as described for **12**. The residue was purified by semi-preparative RP-HPLC (general methods) giving **5** (0.20 g, 92%).



Scheme S5. Synthesis of **6**.

*Synthesis of **26***. *Fmoc-Asp(Ot-Bu)-OBn* (**9**) (0.20 g, 0.39 mmol) was deprotected as described for **12**, giving **25** in quantitative yield. ESI-MS *m/z* 640.1 $(M+H)^+$, calcd 640.2. Crude **25** was coupled with **16** (0.13 g, 0.38 mmol) as described in the main text (Peptide coupling, general procedure). The residue was purified by flash chromatography over silica-gel (eluant EtOAc/cyclohexane 8:2) giving **26** (0.23 g, 79 %). ^{1}H NMR (95:5 $CDCl_3$/DMSO-d6, 400 MHz) δ (ppm): (two sets of resonances: > = major conformer; < = minor conformer) 1.99 (m, 2H, PyrrolidineH-4$_<$), 2.10 (m, 2H, PyrrolidineH-4$_>$), 2.20 (s, 3H, CH_3Ph), 2.66 (m, 1H, AspHβ), 2.90 (m, 1H, PyrrolidineH-3), 2.99 (m, 1H, AspHβ), 3.30 (m, 1H, PyrrolidineH-5$_<$), 3.42-3.60 (m, 4H, PyrrolidineH-2$_>$+PyrrolidineH-5$_<$+PyrrolidineH-5$_>$), 3.64 (m, 1H, PyrrolidineH-2$_<$), 4.00-4.13 (m, 2H, PyrrolidineH-5$_>$+PyrrolidineH-2$_<$), 4.20-4.40 (m, 5H, CH_2Ph+Fmoc), 4.61 (m, 1H, AspHα), 5.10 (br.s, 2H, CH_2Ph), 6.47 (br.d, 1H, AspNH$_>$), 6.52 (br.d, 1H, AspNH$_<$), 6.89 (br.t, 1H, ArH), 7.00-7.20 (m, 4H, ArH), 7.20-7.30 (m, 7H, ArH+NH), 7.30-7.40 (m, 4H, ArH), 7.45-7.55 (m, 3H, ArH), 7.70 (br.d, 2H, ArH), 7.75-7.82 (m, 2H, ArH+NH), 8.59 (s, 1H, NH); ESI-MS *m/z* 780.2 $(M+H)^+$, calcd 780.3

*Synthesis of **28**.* Fmoc deprotection of **26** (0.23 g, 0.30 mmol) was performed for the preparation of **10,** giving **27** in quantitative yield. ESI-MS *m/z* 558.2 $(M+H)^+$, calcd 558.3. The resulting crude **27** was coupled with 2,6-dichlorobenzoic acid (0.057 g, 0.30 mmol) as described in the main text (Peptide coupling, general procedure). The residue was purified by flash chromatography over silica-gel (eluant EtOAc) giving **28** (0.18 g, 82 %). ^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm): (two sets of resonances: > = major conformer; < = minor conformer) 1.90 (m, 2H, PyrrolidineH-4$_<$), 2.10 (m, 2H, PyrrolidineH-4$_>$), 2.15 (s, 3H, CH_3Ph), 2.55 (m, 1H, AspHβ), 2.90-3.00 (m, 2H, PyrrolidineH-3+AspHβ), 3.20 (m, 1H, PyrrolidineH-5$_<$), 3.37-3.58 (m, 3H, PyrrolidineH-2$_>$+PyrrolidineH-5$_<$+PyrrolidineH-5$_>$), 3.59-3.89 (m, 3H, PyrrolidineH-2$_>$+PyrrolidineH-2$_<$+PyrrolidineH-5$_>$), 4.10-4.27 (m, 3H, PyrrolidineH-2$_<$+CH_2Ph), 4.98-5.18 (m, 3H, CH_2Ph+AspHα), 6.90

(br.t, 1H, ArH), 7.01-7.14 (m, 8H, ArH+NH), 7.15-7.26 (m, 6H, ArH+AspNH), 7.26-7.34 (m, 3H, ArH+NH), 7.70 (m, 2H, ArH+NH); ESI-MS *m/z* 730.1. $(M+H)^+$, calcd 730.2

Synthesis of ***6.*** **28** (0.18 g, 0.24 mmol) was deprotected as described for **4**. The residue was purified by semi-preparative RP-HPLC (general methods) giving **6** (0.14 g, 88 %).

Description of biological assays

Materials employed for Scintillation proximity-binding assay (SPA) and cell adhesion assays.

Cell culture media, phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were from Lonza (Euroclone S.p.A, Milan, Italy); HBSS and chloromethylfluorescein diacetate (CMFDA) were from Invitrogen (Carlsbad, CA, USA). Lectin from Triticus vulgaris, soluble fibronectin (FN) from human plasma were purchased from Sigma-Aldrich (Steinheim, Germany). Jurkat clone E6.1 was obtained from the European Cell Culture Collection (ECACC, Wiltshire, UK); SK-MEL-24 cell line was from American Type Culture Collection (ATCC, Manassas, VA, USA).

Black 96-well clear-bottom plates were purchased from Corning Costar (Celbio, Milan, Italy). Soluble human VCAM-1 was purchased from R&D Systems (Minneapolis, MN, USA). BIO-1211 (N-[[4-[[[(2-methylphenyl)amino]carbonyl]amino]-phenyl]acetyl]-L-leucyl-L-aspartyl-L-valyl-L-proline) was purchased from Bachem (Weil am Rhein, Germany). Tissue Protein Extraction Reagent (TPER1) and BCA1 protein assay were purchased from Pierce (Rockford, IL, USA).

Rabbit anti-human monoclonal antibodies against the α4 subunit of $\alpha4\beta1$ integrin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse anti-human monoclonal antibody against β1 subunit (MAB 2000) was obtained from Chemicon (Millipore, Billerica, MA, USA). Secondary antibody (goat anti-mouse or goat anti-rabbit) were purchased from Santa Cruz Biotechnology Inc. Polyacrylamide gel, N,N,N0,N0-tetramethy-lethylenediamine (TEMED), ammonium persulfate (PSA) and SDS were purchased from Sigma-Aldrich. Hybond-ECL Nitrocellulose Membrane was from Amersham Biosciences (GE Healthcare Europe, Milan, Italy). $Na^{125}I$ was obtained from PerkinElmer Inc., Waltham, Massachussets, USA). Polyvinyltoluene (PVT) anti-rabbit binding beads were supplied by Amersham Biosciences as a powder and reconstituted in distilled water. Plastic disposables were from Sarstedt (Verona, Italy). All other reagents were of analytical grade or the highest purity available, purchased from Sigma.

Scintillation proximity-binding assay (SPA).

The radioligand binds to the $\alpha4\beta1$ integrin and the close proximity of the isotope to the scintillant incorporated in the beads allows the radiation energy to transfer to the scintillant where it can be detected as counts per min (cpm). FN was labeled with Na[^{125}I] using the IodogenKit, as specified by the manufacturer (Pierce). ^{125}I-FN was purified from unincorporated iodine by gel filtration chromatography on PD-10 columns; trichloroacetic acid-precipitable radioactivity was $6.30x10^{10}$ μCi/mol. The experiments were carried out in scintillation vials; in each vial 1mg/50 μl anti-rabbit-coated beads, 400 ng of rabbit anti-α4 integrin antibody and a portion of cell eluate (containing approximately 100 μg of $\alpha4\beta1$ integrin) were added. The $\alpha4\beta1$integrin

was extracted from Jurkat cells (cultured in RPMI 1640 medium, supplemented with L-glutamine, 10% FBS, antibiotic-antimicotycotic solution and kept in a humidified incubator at 37°C in a 5% CO_2 atmosphere). The cells were collected and then cytoplasmic proteins were extracted with the T-PER extraction buffer and $\alpha 4\beta 1$ integrin was purified by affinity chromatography, as described [3]. For Western blot experiments on extracts we used antibodies against $\alpha 4$ and $\beta 1$ integrin subunits to confirm that both integrins partitioned to the cell lysate collected from the affinity column. The binding buffer contained Tris-HCl 25 mM pH 7,5; $CaCl_2$ 1 mM; $MgCl_2$ 1 mM; $MnCl_2$ 1mM; BSA 2% (w/v); phenylmethanesulfonyl fluoride (PMSF) 1 mM; aprotinin 1 mg/ml; leupeptin 50 mM.

First, we allowed for the slow interaction between the $\alpha 4$ integrin protein and the compound under examination by incubating together for 1,5 hr at room temperature. Then we added the rabbit anti-human $\alpha 4$ integrin antibody, followed by an incubation time of 2 hours at 4°C. From this point on, all incubations were conducted at room temperature Then the anti-rabbit antibody binding beads were added, and the solution containing the four components was incubated for 2 h at room in the dark. ^{125}I-FN was added to the vials, which were then incubated overnight on a shaker in the dark.

The samples were read using a LS 6500 multipurpose scintillation counter (Beckham Coulter, Fullerton, CA, USA). The SPA procedure was optimized in preliminary experiments, as described [4]. As shown in Figure S1, western blot analysis of cell lysates, purified by a chromatographic technique already reported [3], confirmed that both $\alpha 4$ and $\beta 1$ integrin subunits were present in the eluate employed for the SPA. Specific binding of ^{125}I-FN to an antibody-captured $\alpha 4\beta 1$ integrin was time-dependent; the signal increasing during the first 10 h, then reaching a plateau and remaining constant for the remainder of the 24 h incubation (data not shown). The relatively slow kinetics of the SPA may require the establishment of equilibrium between the different components [5,6].

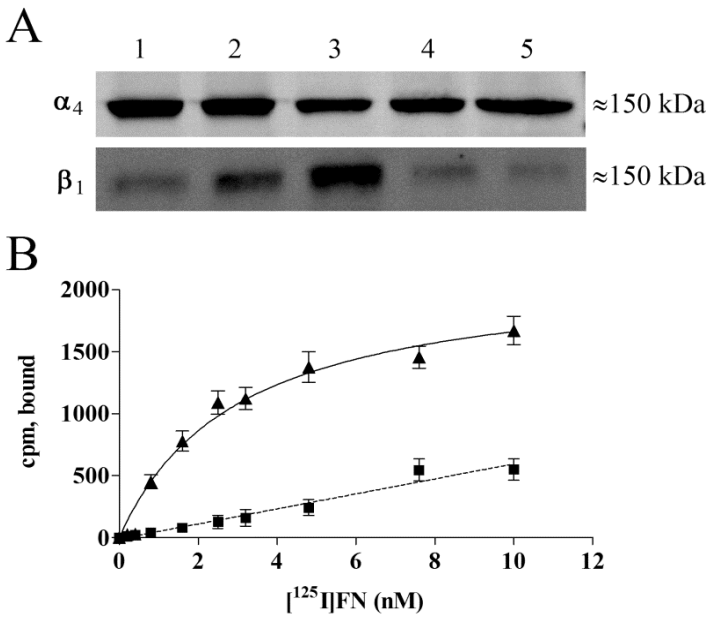


Figure S1. A. Representative autoradiogram of Western blot experiments to evaluate the α4 and β1 integrin subunits in cell lysates of Jurkat E6.1 cells endogenously expressing α4β1 integrin. The lysates were purified by affinity chromatography and fractions of approximately 0.5 ml were collected; the presence of the α4 or β1 integrin subunit was evaluated by Western blotting. **1**: total lysate; **2**-**5**: four eluted fractions. Fractions 2-5 were used for SPA. B. Saturation

SPA binding of ^{125}I-FN to the purified α4β1 integrin extracted from Jurkat E6.1 cells and incubated with increasing concentrations of the radioligand (cpm) as described in Materials and Methods section. Nonspecific binding was determined in the presence of 100 mM unlabeled fibronectin. (■) Non-specific binding; (▲) specific binding obtained by subtraction of the non-specific binding from total binding counts. Mean ± S.E.M. of three experiments done in triplicate.

Cell adhesion assays.

Jurkat cell adhesion assays were done was as described [3,7] Briefly, 96-well plates were coated at 4°C overnight with 2 μg/ml of VCAM-1 and a saturation curve for the ligand was plotted to establish the best signal-to-noise ratio. Non-specific hydrophobic binding sites were blocked by incubation with a BSA 1%/HBSS (w/v) solution for 30 min at 37°C. The day of the assay, the cells were counted and stained with 12.5 μM CMFDA (30 min at 37°C). After three rinses with BSA/HBSS to wash away the excess dye, aliquots of 500,000 (Jurkat) cells were divided among a number of tubes corresponding to the number of treatments. For inhibition experiments, cells were mixed with the drug and pre-incubated at 37°C for 30 min to reach equilibrium before being plated. After 30 min incubation at 37°C in the coated wells, the non-specifically bound cells were washed away with BSA/HBSS solution. Adherent cells were lysed by the addition of 0.5% Triton-X-100 in PBS (30 min at 4°C). Released CMFDA was quantified by fluorescence imaging at Ex485 nm/Em535 nm (Wallac ARVO 1420 multilabel counter) and adherent cells was counted by interpolation on a standard curve. The fluorescence intensity with and without VCAM-I was taken as respectively 100% and 0%. Alternatively, the number of adherent cells was calculated by comparison with a standard curve prepared in the same plate using known concentrations of labeled cells.

SK-MEL-24 cell adhesion assays were carried out as described [8]. 96-well plates were coated by passive adsorption with FN (10 μg/ml) or nonspecific substrate poly-l-lysine (0.002 %) overnight at 4°C. Cells (routinely grown in minimum essential medium supplemented with 10% FBS, nonessential amino acids, and sodium pyruvate) were counted with a hemocytometer and pre-incubated with various concentrations of each compound for 30 min at room temperature to reach a ligand–receptor equilibrium. At the end of the incubation time, the cells were plated (50000 cells per well) and incubated at room temperature for 1 h. All the wells were then washed with PBS to remove nonadherent cells, and 50 μL hexosaminidase [4-nitrophenyl-N-acetyl-β-d-glucosaminide dissolved at a concentration of 7.5 mM in 0.09 M citrate buffer (pH 5) and mixed with an equal volume of 0.5% Triton X-100 in H_2O] was added. This product is a chromogenic substrate for β-N-acetylglucosaminidase, whereby it is transformed into 4-nitrophenol; absorbance was measured at 405 nm. The reaction was blocked by the addition of 100 μL of a stopping solution [50 mM glycine and 5 mM EDTA (pH 10.4)], and the plates were read in a Victor2 Multilabel Counter (PerkinElmer, Waltham, MA, USA).

The efficacy of putative antagonists (at least eight different concentrations were used) was determined by the reduction in adherent cells compared to the untreated control. Each experiment was conducted in quadruplicate and the data are presented as the mean ± S.E.M. of at least three independent assays.

Conformational analysis.

NMR analysis.

^{1}H-NMR spectra were recorded at 400 MHz or 200 MHz in 5 mm tubes using 0.01 M peptide at r.t. ^{1}H NMR spectra of the peptides in $CDCl_3$ at 298 K were poorly resolved with very broad resonances, so the experiments were recorded in mixtures of $CDCl_3$ and DMSO-d_6; in particular, the NMR experiments on **3** and **4** were performed in the biomimetic 8:2 DMSO-d_6/H_2O. The unambiguous assignment of ^{1}H-NMR resonances was done by 2D gCOSY, conducted with a proton spectral width of 3103 Hz. 2D spectra were recorded in the phase sensitive mode and processed using a 90°-shifted, squared sine-bell apodization. 2D ROESY experiments of **3** and **4** were recorded in 8:2 DMSO-d_6/H_2O, with a 250 ms mixing time with a proton spectral width of 3088 Hz. Peaks were calibrated on DMSO. The ^{1}H-NMR of both **3** and **4** revealed two sets of resonances, in about 1:1 ratio, suggestive of a slow equilibrium between the cis and trans conformers of the peptide bond between Asp or *iso*Asp and β-Pro. The cis conformations were assigned by detection of the NOE cross peaks between AspHα (**3**) or *iso*AspHβ (**4**) and β-PyrrolidineH-2, while the trans conformations were deduced by detection of the NOE cross peaks between AspHα (**3**) or *iso*AspHβ (**4**) and β-*iso*PyrrolidineH-5.

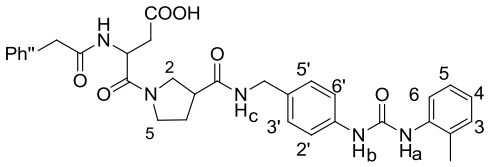


Table S1. Non-obvious ROESY cross-peaks observed for cis-**3**.[a,b,c]

Cross peak[b]	Intensity[c]	Cross peak[b]	Intensity[c]
NHb-NHa	vs	NHb-Ar$H_{2',6'}$	vs
NHb-Ar$H_{3',5'}$	w	NHb-Me	w
AspNH-AspHα	m	AspNH-CH_2Ph''	s
AspNH-AspHβ_{dw}	m	AspNH-AspHβ_{up}	m
NHc-Ar$H_{3',5'}$	m	NHc-CH_2Ph'	s
NHc-PyrrolidineH-$2_{up,un}$	w	NHc-PyrrolidineH-3	s
NHc-PyrrolidineH-$4_{dw,on}$	w	NHc-PyrrolidineH-$4_{up,un}$	w
NHa-Me	s	Ph''-AspHα	w
Ar$H_{3',5'}$-PyrrolidineH-$2_{up,un}$	w	Ar$H_{3',5'}$-PyrrolidineH-$4_{dw,on}$	w
AspHα-PyrrolidineH-$2_{dw,on}$	vs	AspHα-PyrrolidineH-$2_{up,un}$	s
AspHα-AspHβ_{dw}	s	AspHα-AspHβ_{up}	vs
CH_2Ph'-PyrrolidineH-$4_{dw,on}$	w	AspHα-CH_2Ph''	m
CH_2Ph'-AspHβ_{dw}	w	CH_2Ph'-PyrrolidineH-$4_{up,un}$	w

β-PyrrolidineH-$2_{dw,on}$-AspHβ$_{up}$	w	β-PyrrolidineH-$2_{dw,on}$-AspHβ$_{dw}$	w

[a] up = upfield; dw = downfield; [b] on = same side of Hβ; un = opposite side of Hβ; [c] vs = very strong, s = strong, m = medium, w =weak.

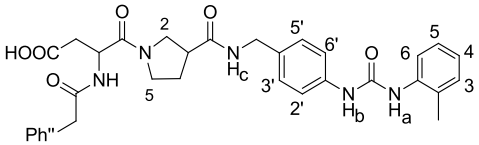


Table S2. Non-obvious ROESY cross-peaks observed for all-trans-**3**.[a,b]

Cross peak[a,b]	Intensity[c]	Cross peak[b]	Intensity[c]
NHb-NHa	vs	NHb-Ar$H_{6'}$	vs
NHb-Ar$H_{5'}$	w	NHb-Me	w
AspNH-AspHα	m	AspNH-CH_2Ph''	s
AspNH-PyrrolidineH-$2_{up,un}$	w	AspNH-AspHβ$_{dw}$	m
AspNH-AspHβ$_{up}$	m	NHc-Ar$H_{3',5'}$	m
NHc-CH_2Ph'	s	NHc-PyrrolidineH-$2_{up,un}$	w
NHc-PyrrolidineH-3	s	NHc-PyrrolidineH-$4_{dw,on}$	w
NHc-PyrrolidineH-$4_{up,un}$	w	NHa-Me	s
Ph''-AspHα	w	Ar$H_{3',5'}$-PyrrolidineH-3	w
Ar$H_{3',5'}$-PyrrolidineH-$4_{up,un}$	w	AspHα-PyrrolidineH-$5_{dw,un}$	s
AspHα-PyrrolidineH-$5_{up,on}$	s	AspHα-PyrrolidineH-$2_{up,un}$	w
AspHα-AspHβ$_{dw}$	s	AspHα-AspHβ$_{up}$	s
CH_2Ph'-PyrrolidineH-$4_{dw,on}$	w	AspHα-CH_2Ph''	m
CH_2Ph'-AspHβ$_{dw}$	w	CH_2Ph'-PyrrolidineH-$4_{up,un}$	w
β-PyrrolidineH-$5_{dw,un}$-AspHβ$_{up}$	w	CH_2Ph'-PyrrolidineH-3	w

[a] up = upfield; dw = downfield; [b] on = same side of Hβ; un = opposite side of Hβ; [c] vs = very strong, s = strong, m = medium, w =weak.

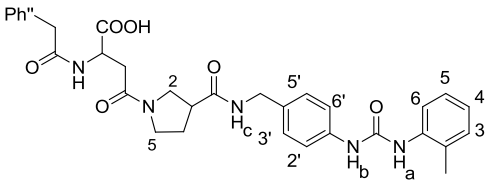


Table S3. Non-obvious ROESY cross-peaks observed for cis-**4**.[a,b]

Cross peak[a,b]	Intensity[c]	Cross peak[b]	Intensity[c]
NHb-NHa	vs	NHb-Ar$H_{6'}$	vs

NHb-$ArH_{5'}$	w	NHb-Me	w
AspNH-AspHα	m	AspNH-CH_2Ph''	s
AspNH-AspHβ_{dw}	m	AspNH-AspHβ_{up}	m
NHc-$ArH_{3',5'}$	m	NHc-CH_2Ph'	s
NHc-PyrrolidineH-$2_{up,un}$	m	NHc-PyrrolidineH-3	vs
NHc-PyrrolidineH-$4_{dw,on}$	w	NHa-Me	vs
Ph''-AspHα	w	$ArH_{3',5'}$-PyrrolidineH-3	w
$ArH_{3',5'}$-PyrrolidineH-$4_{up,un}$	w	AspHα-AspHβ_{dw}	s
AspHα-AspHβ_{up}	s	AspHα-CH_2Ph''	m
CH_2Ph'-PyrrolidineH-$4_{dw,on}$	w	CH_2Ph'-PyrrolidineH-$4_{up,un}$	w
CH_2Ph'-AspHβ_{dw}	w	CH_2Ph'-PyrrolidineH-3	w
β-PyrrolidineH-$5_{dw,un}$-AspHβ_{up}	w	AspHβ_{dw}-PyrrolidineH-$2_{dw,on}$	s
AspHβ_{dw}-PyrrolidineH-$2_{up,un}$	s	AspHβ_{up}-PyrrolidineH-$2_{dw,on}$	s
AspHβ_{up}-PyrrolidineH-$2_{up,un}$	s		

[a] up = upfield; dw = downfield; [b] on = same side of Hβ; un = opposite side of Hβ; [c] vs = very strong, s = strong, m = medium, w =weak.

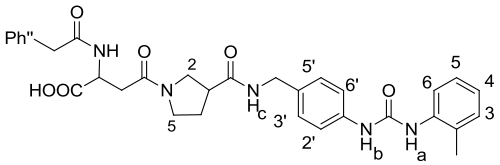


Table S3. Non-obvious ROESY cross-peaks observed for trans-**4**.[a,b]

Cross peak[a,b]	Intensity[c]	Cross peak[b]	Intensity[c]
NHb-NHa	vs	NHb-$ArH_{6'}$	vs
NHb-$ArH_{5'}$	w	NHb-Me	w
AspNH-AspHα	m	AspNH-CH_2Ph''	s
AspNH-AspHβ_{dw}	s	AspNH-AspHβ_{up}	s
NHc-$ArH_{3',5'}$	m	NHc-CH_2Ph'	s
NHc-PyrrolidineH-$2_{up,un}$	m	NHc-PyrrolidineH-3	vs
NHc-PyrrolidineH-$4_{dw,on}$	w	NHa-Me	vs
Ph''-AspHα	w	$ArH_{3',5'}$-PyrrolidineH-3	w
$ArH_{3',5'}$-PyrrolidineH-$4_{up,un}$	w	AspHα-AspHβ_{dw}	s
AspHα-AspHβ_{up}	s	AspHα-CH_2Ph''	s

CH_2Ph'-PyrrolidineH-$4_{dw,on}$	w	CH_2Ph'-PyrrolidineH-$4_{up,un}$	w
CH_2Ph'-Asp$H\beta_{dw}$	w	CH_2Ph'-PyrrolidineH-3	w
Asp$H\beta_{dw}$-PyrrolidineH-$5_{dw,un}$	s	Asp$H\beta_{dw}$-PyrrolidineH-$2_{up,on}$	s
Asp$H\beta_{up}$-PyrrolidineH-$5_{dw,un}$	s	Asp$H\beta_{up}$-PyrrolidineH-$5_{up,on}$	s

[a] up = upfield; dw = downfield; [b] on = same side of Hβ; un = opposite side of Hβ; [c] vs = very strong, s = strong, m = medium, w =weak.

Restrained molecular dynamics.

Molecular conformations were investigated by 2D ROESY and restrained molecular dynamics. The intensities of the cross-peak were ranked to infer plausible inter-proton distances as restraints (Tables S1-S4). Only ROESY-derived constraints were included in the restrained molecular dynamics. Cross-peak intensities were classified very strong, strong, medium, and weak, and were associated with distances of 2.2, 2.6, 3.0, and 4.5 Å, respectively. Geminal couplings and other obvious correlations were discarded. The ω bonds were set at 180° (force constant: 16 kcal $mol^{-1}Å^{-2}$).

The restrained MD simulations [9] were conducted using the AMBER [10] force field in a 45×45×45 Å box of standard TIP3P models of equilibrated water [11]. All water molecules with atoms that come closer than 2.3 Å to a solute atom were deleted. A 100 ps simulation at 1200 °K was used for generating 50 random structures that were subsequently subjected to a 50 ps restrained MD with a 50% scaled force field at the same temperature, followed by 50 ps with full restraints (distance force constant of 7 kcal mol^{-1} $Å^{-2}$), after which the system was cooled in 20 ps to 50°K. H-bond interactions were not included, nor were torsion angle restraints. The resulting structures were minimized with 3000 cycles of steepest descent and 3000 cycles of conjugated gradient (convergence of 0.01 kcal $Å^{-1}$ mol^{-1}). The backbones of the structures were clustered by the rmsd analysis module [9].

To investigate the dynamic behavior, the ROESY-restrained structures were analyzed by unrestrained MD for 10 ns in a 45×45×45 Å box of explicit, equilibrated water molecules, at 298 °K, constant temperature and pressure (Berendsen scheme [12], bath relaxation constant of 0.2). For 1-4 scale factors, van der Waals and electrostatic interactions are scaled in AMBER to half their nominal value. The integration time step was set to 0.1 fs.

References.

[1] A.L. Larroque, J. Dubois, S. Thoret, G. Aubert, A. Chiaroni, F. Guèritte, D. Guènard, Novel C2-C30 N-peptide linked macrocyclic taxoids. Part 2: Synthesis and biological activities of docetaxel analogues with a peptide side chain at C2 and their macrocyclic derivatives. Bioorg. Med. Chem. 15 (2007) 563-574.

[2] K. Lin, H.S. Ateeq, S.H. Hsiung, L.T. Ching, C.N. Zimmerman, A. Castro, W.C. Lee, C.E. Hammond, S. Kalkunte, L.L. Chen, R.B. Pepinsky, D.R. Leone, A.G. Sprague, W.M. Abraham, A. Gill, R.R. Lobb, S.P. Adams, Selective, tight-binding inhibitors of integrin α4β1 that inhibit allergic airway responses. J. Med. Chem. 42 (1999) 920-934.

[3] A.R. Qasem, C. Bucolo, M. Baiula, A. Spartà, P. Govoni, A. Bedini, D. Fascì, S. Spampinato, Contribution of α4β1 integrin to the antiallergic effect of levocabastine. Biochem. Pharmacol. 76 (2008) 751-762.

[4] S. Sun, J. Almaden, T.J. Carlson, J. Barker, M.R. Gehring, Assay development and data analysis of receptor-ligand binding based on scintillation proximity assay. Metab. Eng. 7 (2005) 38-44.

[5] J.A. Pachter, R. Zhang, R. Mayer-Ezell, Scintillation proximity assay to measure binding of soluble fibronectin to antibody-captured α5β1 integrin. Anal. Biochem. 230 (1995)101-107.

[6] Y. Takada, M.J. Elices, C. Crouse, M.E. Hemler, The primary structure of the alpha 4 subunit of VLA-4: homology to other integrins and a possible cell-cell adhesion function. EMBO J. 8 (1989)1361-1368.

[7] C. Marcinkiewicz, J.J. Calvete, M.M. Marcinkiewicz, M. Raida, S. Vijay-Kumar, Z. Huang, R.R. Lobb, S. Niewiarowski, EC3, a novel heterodimeric disintegrin from Echis carinatus venom, inhibits α4 and α5 integrins in an RGD-independent manner. J. Biol. Chem. 274 (1999) 12468-12473.

[8] A. Tolomelli, L. Gentilucci, E. Mosconi, A. Viola, S.D. Dattoli, M. Baiula, S. Spampinato, L. Belvisi, M. Civera, Development of isoxazoline-containing peptidomimetics as dual αVβ3 and α5β1 integrin ligands. Chem. Med. Chem. 6 (2011) 2264-2272.

[9] HyperChem Release 8.0.3, 2012, Hypercube Inc. 1115 NW 4th St. Gainesville, FL 32608 (USA).

[10] W.D. Cornell, P. Cieplak, C.I. Bayly, I.R. Gould, K.M. Merz, D.M. Ferguson, D.C. Spellmeyer, T. Fox, J.W. Caldwell, P.A. Kollman. A second generation force field for the simulation of proteins, nucleic acids, and organic molecoles. J. Am. Chem. Soc. 117 (1995) 5179-5197.

[11] W.L. Jorgensen, J. Chandrasekhar, J. Madura, R.W. Impey, M.L. Klein, Comparison of simple potential functions for simulating liquid water. J. Chem. Phys. 79 (1983) 926-935.

[12] H.J.C. Berendsen, J.P.M. Postma, W.F. van Gunsteren, A. Di Nola, J.R. Haak, Molecular dynamics with coupling to an external bath. J. Chem. Phys. 81 (1984) 3684-8690.