Supporting info

Novel cyclic biphalin analogue with improved antinociceptive properties

Adriano Mollica,[£,*] Alfonso Carotenuto,[#,*] Ettore Novellino,[#] Antonio Limatola,[#] Roberto Costante,[£] Francesco Pinnen,[£] Azzurra Stefanucci,[¥] Stefano Pieretti,[§] Anna Borsodi,[$] Reza Samavati,[$] Ferenc Zador,[$] Sándor Benyhe,[$] Peg Davis,[±] Frank Porreca,[±] Victor J. Hruby[ζ]

[£] Dipartimento di Farmacia, Università di Chieti-Pescara "G. d'Annunzio", Via dei Vestini 31, 66100, Chieti, Italy

[#] Dipartimento di Farmacia, Università di Napoli "Federico II", Via D. Montesano, 49, 80131, Naples, Italy

[¥] Dipartimento di Chimica, Sapienza, Università di Roma., P.le A. Moro, 5, 00187, Rome, Italy;

[§] Department of Therapeutic Research and Medicine Evaluation, Istituto Superiore di Sanità, V.le Regina Elena 299, 00161, Rome, Italy;

[$] Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, 6726, Szeged, Hungary;

[±] Department of Pharmacology, University of Arizona, Tucson, AZ, 85721, USA;

[ζ] Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, 85721, USA

Contents

S1 …….Title, affiliation and contents.
S2-5 ……..Synthesis and spectroscopic data for all intermediate products
S7-9……...*In vitro* biological assays
S10-11……...*In vivo* biological assays
S11-13……...Conformational Analysis and Structure calculations
S14-S17…….Figures
S18-S22…….Tables
S23-24…..……..……...……References

Synthesis and Spectroscopic data for all intermediate products.

Chemistry

General

All products were synthesized in solution using the EDC/HOBt/DIPEA coupling method. The N^{α} terminal Boc-protected peptides were all deprotected by a mixture of TFA in DCM 1:1 at r.t. The intermediate TFA salts were used for subsequent reactions without further purification. Boc protected intermediate products were purified by silica gel column chromatography, or in case of scarcely soluble products, the purification was performed by trituration in EtOAc. The disulfide bond formation was achieved oxydizing the intermetiates by I_2 in MeOH for 3h at room temperature. Final products were purified by RP-HPLC using a Waters XBridge^{TM} Prep BEH130 C_{18}, 5.0 μm, 250 mm x 10 mm column at a flow rate of 4 mL/min on a Waters Binary pump 1525, using as eluent a linear gradient of H_2O/acetonitrile 0.1% TFA starting from 5% acetonitrile to 90 % acetonitrile in 50 min. The purity of the N^{α}-Boc-protected products was confirmed by NMR analysis on a Varian VXR 300 MHz instrument and mass spectrometry ESI-HRMS (Thermo Finnigan). The purity of all final TFA salts **9** and **10** was confirmed by NMR analysis, ESI-HRMS and by analytical RP-HPLC (C_{18}-bonded 4.6 x 150 mm) at a flow rate of 1 mL/min using as eluent a gradient of H_2O/acetonitrile 0.1% TFA ranging from 5% acetonitrile to 95% acetonitrile in 50 min, and was found to be ≥ 95%.

Boc-D-Pen-OH. Boc_2O (1.1 eq.) was added to a solution of D-Pen-OH (1 eq.) in THF/H_2O 1:1 (80 mL), pH was adjusted at 8 by adding a solution of 1N NaOH. After 24h the mixture was concentrated under vacuum. The alkaline solution was extracted by two portions of Et_2O. Then the aqueous layer was acidified to pH 3 by adding 2N HCl. The precipitate was extracted by EtOAc and dried under reduced pressure to give the desired product as a pure white solid (68%). R_f = 0.17 (EtOAc). ^{1}H NMR (DMSO-*d6*) δ: 1.36-1.55 (9H, s, Boc and 6H $2CH_3$), 4.07 (1H, d, Pen αCH), 6.99 (1H, d, Pen NH), 12.78 (1H, s, Pen OH). ESI-HRMS calcd for C10H19NO4S *m/z*: 249.1035; $[M+H]^+$; found 249.1036.

Boc-L-Pen-OH. Boc_2O (1.1 eq.) was added to a solution of L-Pen-OH (1 eq.) in THF/H_2O 1:1 (80 mL), pH was adjusted at 8 by adding a solution of 1N NaOH. After 24h the mixture was concentrated under vacuum. The alkaline solution was extracted by two portions of Et_2O. Then the aqueous layer was acidified to pH 3 by adding 2N HCl. The precipitate was extracted by EtOAc and dried under reduced pressure to give the desired product as a pure white solid (64%). R_f = 0.15 (EtOAc). ^{1}H NMR (DMSO-*d6*) δ: 1.38-1.51 (9H, s, Boc and 6H CH_3), 4.05 (1H, d, Pen αCH), 6.97 (1H, d, Pen NH), 12.76 (1H, s, Pen OH). ESI-HRMS calcd for $C_{10}H_{19}NO_4S$ *m/z*: 249.1035; $[M+H]^+$; found 249.1036.

(Boc-Phe)$_2$-hydrazine. EDC·HCl (2.2 eq.), HOBt (2.2 eq.) and DIPEA (6.6 eq.) were added to a solution of Boc-Phe-OH (2.2 eq.) in DMF at 0°C. The reaction mixture was stirred for 10 min, hydrazine (1 eq.) was added and the reaction was stirred for an additional 10 min at 0°C and then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue

was precipitated with EtOAc and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, $NaHCO_3$ s.s., brine and distilled water. The solid was dried under reduced pressure and triturated with diethyl ether to give the desired product as a crude white solid, in 83% yield. R_f 0.48 (CH_2Cl_2/AcOEt 7:3). The product was used for the next step without further purification. ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.29 (18H, s, Boc), 2.78-2.70 (4H, m, Phe βCH_2), 4.24 (2H, m, Phe αCH), 7.19-7.35 (10H, m, Phe Ar); 7.17 (2H, d, Phe NH), 10.21 (2H, s, NH). HRMS (ESI) calcd. for $C_{28}H_{38}N_4O_6$ m/z: 526.2791; $[M + H]^+$ found 526.2793.

(Boc-Gly-Phe)$_2$-hydrazine (2). (Boc-Phe)$_2$-hydrazine was deprotected at the N^{α} terminal by TFA in DCM 1:1 using 1 mL of mixture per 100 mg of Boc-protected product for 1.5 h at r.t. The mixture was then evaporated under high vacuum and the TFA salt was used for the next step without further purification. EDC·HCl (2.2 eq.), HOBt (2.2 eq.) and DIPEA (6.6 eq.) were added to a solution of Boc-Gly-OH (2.2 eq.) in DMF at 0°C. The reaction mixture was stirred for 10 min, TFA·Phe-NH-NH-Phe·TFA (1 eq.) was added, the reaction was stirred for an additional 10 min at 0°C then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated with EtOAc and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, $NaHCO_3$ s.s., brine and distilled water. The solid was dried under reduced pressure was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 3:7 to EtOAc/MeOH 9:1) to obtain the pure product (84%). R_f = 0.67 (EtOAc). ^{1}H NMR (DMSO-d_6, 300 MHz) (δ, ppm): 1.33 (18H, s, Boc), 2.47–3.03 (H, m, Phe βCH_2), 3.32-3.58 (4H, m, Gly CH_2), 4.57 (2H, m, Phe αCH), 6.87 (2H br, Gly NH), 7.15–7.24 (10H, m, Phe Ar), 8.06 (2H, d, Phe NH), 10.18 (2H, s, NH). HRMS (ESI) calcd. for $C_{32}H_{44}N_6O_8$ m/z: 640.3221 $[M + H]^+$; found 640.3222.

(Boc-D-Pen-Gly-Phe)$_2$-hydrazine (3). (Boc-Gly-Phe)$_2$-hydrazine was deprotected by N^{α} terminal by TFA in CH_2Cl_2 using 1 mL of mixture per 100 mg of Boc-protected product for 1h at r.t. The mixture was than evaporated under vacuum and TFA salt was used for the next step without further purification. EDC·HCl (2.2 eq.), HOBt (2.2 eq.), and DIPEA (6.6 eq.) were added to a solution of Boc-D-Pen-OH (2.2 eq.) in DMF at 0 °C. The reaction mixture was stirred for 10 min, 2 TFA·(Gly-Phe-NH)$_2$ (1 eq.) was added and the reaction was stirred for additional 10 min at 0 °C and then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated in EtOAc and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, $NaHCO_3$ s.s., brine and distillated water. The solid was dried under reduced pressure and triturated with diethyl ether to give the desired product as a pure white solid (85%). R_f = 0.36 (EtOAc/MeOH 9:1). ^{1}H NMR (DMSO-*d6*) δ: 1.17 (6H, d, D-Pen CH_3), 1.38 (18H, s, Boc), 2.65–2.89 (4H, m, Phe βCH_2), 3.44–3.67 (4H, m, Gly αCH_2), 4.21 (2H, m, Phe αCH), 4.09 (2H, d, D-Pen αCH), 6.59 (2H, d, D-Pen NH), 7.15–7.27 (10H, m, Phe Ar), 7.74 (2H, d, Phe NH), 7.94 (2H, t, Gly NH), 9.85 (2H, s, NH-NH). ESI-HRMS calcd for $C_{42}H_{62}N_8O_{10}S_2$ *m/z*: 902.4030 $[M+H]^+$; found 902.4034.

(Boc-L-Pen-Gly-Phe)$_2$-hydrazine (4). (Boc-Gly-Phe)$_2$-hydrazine was deprotected by N^{α} terminal by TFA in CH_2Cl_2 using 1 mL of mixture per 100 mg of Boc-protected product for 1h at r.t. The mixture was than evaporated under vacuum and TFA salt was used for the next step without further

purification. EDC·HCl (2.2 eq.), HOBt (2.2 eq.), and DIPEA (6.6 eq.) were added to a solution of Boc-L-Pen-OH (2.2 eq.) in DMF at 0 °C. The reaction mixture was stirred for 10 min, 2 TFA·(Gly-Phe-NH)$_2$ (1 eq.) was added and the reaction was stirred for additional 10 min at 0 °C and then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated in EtOAc and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, $NaHCO_3$ s.s., brine and distillated water. The solid was dried under reduced pressure and triturated with diethyl ether to give the desired product as a pure white solid (87%). R_f = 0.34 (EtOAc/MeOH 9:1). ^{1}H NMR (DMSO-*d6*) δ: 1.15 (6H, d, Pen CH_3), 1.37 (18H, s, Boc), 2.62–2.86 (4H, m, Phe βCH_2), 3.44–3.62 (4H, m, Gly αCH_2), 4.22 (2H, m, Phe αCH), 4.07 (1H, d, Pen αCH), 6.57 (1H, d, Pen NH), 7.16–7.27 (10H, m, Phe Ar), 7.75 (2H, d, Phe NH), 7.90 (2H, t, Gly NH), 9.83 (2H, s, NH-NH). ESI-HRMS calcd for $C_{42}H_{62}N_8O_{10}S_2$ *m/z*: 902.4030 $[M+H]^+$; found 902.4036.

***c*(Boc-D-Pen-Gly-Phe)$_2$-hydrazine (5).** To a stirred solution of I_2 (2.2 eq.) in MeOH (6.5 mL), (Boc-D-Pen-Gly-Phe)$_2$-hydrazine (1 eq.) in MeOH (22 mL) was added portionwise at room temperature during 45 min. After 4h under stirring reaction mixture was cooled at 0°C and decolourized with 1N $Na_2S_2O_3$. The residue obtained after removal of the solvent was extracted with EtOAc and the organic layer washed with 1N $Na_2S_2O_3$ and H_2O. Drying and evaporation followed by purification with silica gel column chromatography (CH_2Cl_2/EtOAc 3:7 to CH_2Cl_2/EtOAc 2:8) of the resulting crude product, afforded pure disulfide product as a with powder (86%). R_f = 0.8 (EtOAc/MeOH 9.5:0.5). ^{1}H NMR (DMSO-*d6*) δ: 1.17 (12H, d, D-Pen CH_3), 1.39 (18H, s, Boc), 2.62–2.84 (4H, m, Phe βCH_2), 3.45–3.62 (4H, m, Gly αCH_2), 4.26 (2H, m, Phe αCH), 4.13 (2H, d, D-Pen αCH), 6.55 (2H, d, D-Pen NH), 7.17–7.25 (10H, m, Phe Ar), 7.75 (2H, d, Phe NH), 7.87 (2H, t, Gly NH), 9.83 (2H, s, NH-NH). ESI-HRMS calcd for $C_{42}H_{60}N_8O_{10}S_2$ *m/z*: 900.3874 $[M+H]^+$; found 900.3878.

***c*(Boc-L-Pen-Gly-Phe)$_2$-hydrazine (6).** To a stirred solution of I_2 (2.2 eq.) in MeOH (6.5 mL), (Boc-L-Pen-Gly-Phe)$_2$-hydrazine (1 eq.) in MeOH (22 mL) was added portionwise at room temperature during 45 min. After 4h under stirring reaction mixture was cooled at 0°C and decolourized with 1N $Na_2S_2O_3$. The residue obtained after removal of the solvent was extracted with EtOAc and the organic layer washed with 1N $Na_2S_2O_3$ and H_2O. Drying and evaporation followed by purification with silica gel column chromatography (CH_2Cl_2/EtOAc 3:7 to CH_2Cl_2/EtOAc 2:8) of the resulting crude product, afforded pure disulfide product as a solid powder (80%). R_f = 0.78 (EtOAc/MeOH 9.5:0.5). ^{1}H NMR (DMSO-*d6*) δ: 1.15 (6H, d, Pen CH_3), 1.37 (18H, s, Boc), 2.62–2.86 (4H, m, Phe βCH_2), 3.44–3.62 (4H, m, Gly αCH_2), 4.22 (2H, m, Phe αCH), 4.10 (2H, d, Pen αCH), 6.57 (2H, d, Pen NH), 7.16–7.27 (10H, m, Ar), 7.75 (2H, d, Phe NH), 7.90 (2H, t, Gly NH), 9.83 (2H, s, NH-NH). ESI-HRMS calcd for $C_{42}H_{60}N_8O_{10}S_2$ *m/z*: 900.3874 $[M+H]^+$; found 900.3875.

(Boc-Tyr-*c*(D-Pen-Gly-Phe))$_2$-hydrazine (7). (Boc-D-Pen-Gly-Phe-NH)$_2$ was deprotected by N^{α} terminal by TFA in CH_2Cl_2 using 1 mL of mixture per 100 mg of Boc-protected product for 1h at r.t. The mixture was evaporated under vacuum and TFA salt was used for the next step without further purification. EDC·HCl (2.2 eq.), HOBt (2.2 eq.), and DIPEA (6.6 eq.) were added to a

solution of Boc-Tyr-OH (2.2 eq.) in DMF at 0 °C. The reaction mixture was stirred for 10 min, 2 TFA·(D-Pen-Gly-Phe-NH)$_2$ (1 eq.) was added and the reaction was stirred for additional 10 min at 0°C and then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated in EtOAc and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, $NaHCO_3$ s.s., brine and distillated water. The solid was dried under reduced pressure and triturated with diethyl ether to give the desired product as a crude white solid. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 3:7 to CH_2Cl_2/EtOAc 1:9) to obtain the pure product (26%). R_f = 0.69 (EtOAc/MeOH 9.5:0.5). ^{1}H NMR (DMSO-*d6*) δ: 1.28-1.57 (18H, s, Boc and 12H, s, D-Pen CH_3), 2.62–2.74 (4H, m, Tyr βCH_2), 2.74–2.72 (4H, m, Phe βCH_2), 3.48–3.59 (4H, m, Gly αCH_2), 4.58 (2H, t, Tyr αCH), 4.48 (2H, t, D-Pen αCH), 4.2 (2H, m, Phe αCH), 6.57 (2H, d, D-Pen NH), 6.63 (2H, d, Tyr NH), 6.94–7.05 (8H, m, Tyr Ar), 7.14–7.27 (10H, m, Phe Ar), 7.75 (2H, d, Phe NH), 7.99 (2H, t, Gly NH), 9.16 (2H, s, OH), 10.08 (2H, s, NHNH). ESI-HRMS calcd for $C_{60}H_{78}N_{10}O_{14}S_2$ *m/z*: 1226.5140 $[M+H]^+$; found 1226.5145.

(Boc-Tyr-*c(*L-Pen-Gly-Phe))$_2$-hydrazine (8). (Boc-L-Pen-Gly-Phe-NH)$_2$ was deprotected by N^α terminal by TFA in CH_2Cl_2 using 1 mL of mixture per 100 mg of Boc-protected product for 1h at r.t. The mixture was evaporated under vacuum and TFA salt was used for the next step without further purification. EDC·HCl (2.2 eq.), HOBt (2.2 eq.), and DIPEA (6.6 eq.) were added to a solution of Boc-Tyr-OH (2.2 eq.) in DMF at 0 °C. The reaction mixture was stirred for 10 min, 2 TFA·(L-Pen-Gly-Phe-NH)$_2$ (1 eq.) was added and the reaction was stirred for additional 10 min at 0 °C and then allowed to warm at r.t. overnight. The solvent was evaporated under reduced pressure, the residue was precipitated in EtOAc and the suspension was filtered through a Buchner funnel under reduced pressure. The solid residue was washed with three portions of 5% citric acid, $NaHCO_3$ s.s., brine and distillated water. The solid was dried under reduced pressure and triturated with diethyl ether to give the desired product as a crude white solid. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 3:7 to CH_2Cl_2/EtOAc 1:9) to obtain the pure product (24%). R_f = 0.67 (EtOAc/MeOH 9.5:0.5). ^{1}H NMR (DMSO-*d6*) δ: 1.28-1.57 (18H, s, Boc and 12H, s, Pen CH_3), 2.62–2.71 (4H, m, Tyr βCH_2), 2.74–2.76 (4H, m, Phe βCH_2), 3.48–3.59 (4H, m, Gly αCH_2), 4.04 (2H, t, Tyr αCH), 4.08 (2H, t, Pen αCH), 4.15 (2H, m, Phe αCH), 6.57 (2H, d, Pen NH), 6.61 (2H, d, Tyr NH), 6.90–7.01 (8H, m, Tyr Ar), 7.16–7.26 (10H, m, Phe Ar), 7.78 (2H, d, Phe NH), 7.99 (2H, t, Gly NH), 9.16 (2H, s, OH), 10.08 (2H, s, NHNH). ESI-HRMS calcd for $C_{60}H_{78}N_{10}O_{14}S_2$ *m/z*: 1226.5140 $[M+H]^+$; found 1226.5141.

2 TFA·Tyr-c(D-Pen-Gly-Phe))$_2$-hydrazine (9). (Boc-Tyr-c(D-Pen-Gly-Phe))$_2$-hydrazine was deprotected by N^α terminal by TFA in CH_2Cl_2 using 1 mL of mixture per 100 mg of Boc-protected product for 1h at r.t. The mixture was evaporated under vacuum and TFA salt and purified by RP-HPLC. Rt = 17.60 min. ^{1}HNMR (H_2O/D_2O 9:1, 5 °C) δ: 1.06 -1.15 (12H, s, Pen CH_3); 3.00 -3.28 (4H, m, Phe βCH_2); 3.09 (4H, m, Tyr βCH_2); 3.55-3.95 (4H, m, Gly αCH_2); 4.30 (2H, t, Tyr αCH); 4.32 (2H, d, Pen αCH); 4.69 (2H, m, Phe αCH); 6.84 (4H, d, Tyr Ar); 7.12 (4H, d, Tyr Ar); 7.27 (10H, m, Phe Ar); 7.31 (2H, d, Phe Ar); 7.36 (2H, d, Phe Ar); 8.45 (2H, d, Pen NH); 8.52(2H, t, Tyr NH); 8.83(2H, t, Gly NH). ESI-HRMS calcd for $C_{50}H_{62}N_{10}O_{10}S_2$ *m/z*: 1027.4170 $[M+H]^+$; found 1027.4176.

2 TFA·Tyr-c(L-Pen-Gly-Phe))$_2$-hydrazine (10). (Boc-Tyr-c(L-Pen-Gly-Phe))$_2$-hydrazine was deprotected by N^{α} terminal by TFA in CH_2Cl_2 using 1 mL of mixture per 100 mg of Boc-protected product for 1h at r.t. The mixture was evaporated under vacuum and TFA salt and purified by RP-HPLC. Rt = 17.84 min. ^{1}HNMR (H_2O/D_2O 9:1, 5 °C) δ: 1.26 (12H, s, Pen CH_3); 3.00-3.13 (4H, m, Phe βCH_2); 3.02-3.14- (4H, m, Tyr βCH_2); 3.72-3.88 (4H, m, Gly αCH_2); 4.24 (2H, t, Tyr αCH); 4.40 (2H, d, Pen αCH); 4.65 (2H, m, Phe αCH); 6.80 (4H, d, Tyr Ar); 7.08 (4H, d, Tyr Ar); 7.23 (10H, m, Phe Ar); 7.32 (2H, d, Phe Ar); 7.37 (2H, d, Phe Ar); 8.10 (2H, t, Tyr NH); 8.47 (2H, d, Pen NH); 8.59(2H, t, Gly NH). ESI-HRMS calcd for $C_{50}H_{62}N_{10}O_{10}S_2$ *m/z*: 1027.4170 $[M+H]^+$; found 1027.4174.

***In vitro* biological assays**

Binding Assays

Chemicals. Tris-HCl, EGTA, NaCl, $MgCl_2$ x $6H_2O$, GDP, the GTP analogue GTPγS, and the κ opioid receptor specific agonist U69593 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The radiolabelled GTP analogue, [^{35}S]GTPγS (specific activity: >1000 Ci/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary). The μ opioid receptor specific enkephalin analogue Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) was obtained from Bachem Holding AG (Bubendorf, Switzerland). The modified δ opioid receptor specific deltorphin II derivative, Ile^{5,6}deltorphin II was synthesized and tritiated ([^{3}H]Ile^{5,6}deltorphin II; specific activity: 28 Ci/mmol[1]) in the Isotope Laboratory of BRC (Szeged, Hungary) together with the tritiated DAMGO ([^{3}H]DAMGO; specific activity: 41 Ci/mmol[2]). Tritiated U69593 ([^{3}H]U69593; specific activity: 43,7 Ci/mmol[3]) was purchased from PerkinElmer (Waltham, USA). The opioid antagonist naloxone was kindly provided by the company Endo Laboratories DuPont de Nemours (Wilmington, DE, USA). All applied receptor ligands were stored in 1 mM stock solution at -20 ˚C.

Animals. Male Wistar rats (250–300g body weight) were housed in the local animal house of the Biological Research Center (BRC, Szeged, Hungary) in groups of 4 or 8 animals and were maintained on a 12:12 hour of light/dark cycle. The animals were handled in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§).

Rat brain membrane preparation. Brain membrane fractions from male Winstar rat brains were prepared according to the method previously describe.[4] Briefly, rats were decapitated and the brain was quickly removed, and homogenized on ice in 50 mM Tris-HCl buffer (pH 7.4) with a Teflon-glass homogenizer. The homogenate was centrifuged at 40,000 x g for 20min at 4°C and the pellet was resuspended in fresh buffer and incubated for 30 min at 37°C. This centrifugation step was repeated, and the final pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose and stored at -80°C until use.

Cell culture and cell membrane preparations. Chinese hamster ovary cells (CHO) overexpressing the appropriate opioid receptors, such as mouse δ and rat κ and μ opioid receptors were provided by Dr. Zvi Vogel (Rehovot, Israel).[5] The growing of the cells was performed as we previously described.[6] Briefly the cells were grown in Dulbecco's modified Eagle's medium and in α-minimum essential medium, respectively. Both media were supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 25 mg/ml fungizone and 0.5 mg/ml geneticin. Cells were kept in culture at 37˚C in a humidified atmosphere consisting of 5% CO_2 and 95% air. CHO cell membranes overexpressed with opioid receptors were prepared for the [^{35}S]GTPγS binding assays similarly as we previously described.[7]

Competition binding experiments. Aliquots of frozen rat brain membranes were first centrifuged (40000 × g, 20 min, 4˚C) to remove sucrose and the pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4). Membranes were incubated with gentle shaking with the appropriate incubation

conditions depending on the opioid radioligand ([^{3}H]DAMGO and [^{3}H]Ile^{5,6}deltorphin II: 35˚C for 45 min; [^{3}H]U69593: 30˚C for 60 min) in a final volume of 1 ml. The incubation compound also contained 10^{-10} – 10^{-5} M concentration interval of unlabeled compound **9** and **10** together with DAMGO or Ile^{5,6}deltorphin II or U69593 and finally biphalin for control (Figure S1). The corresponding tritiated opioid receptor specific ligand was added in approximately in 1 nM concentrations. Total binding was measured in the presence of radioligand, in the absence of the competitor ligands. The non-specific binding was determined in the presence of 10 μM unlabeled naloxone. The reaction was terminated by rapid filtration under vacuum (Brandel M24R Cell Harvester), and washed three times with 5 ml ice-cold 50 mM Tris-HCl (pH 7.4) buffer through Whatman GF/C ([^{3}H]DAMGO and [^{3}H]Ile^{5,6}deltorphin II) or GF/B glass fiber filters ([^{3}H]U69593, the filter was also pretreated in 3% polyethyleneimine for 60 min to reduce non-specific binding) . The radioactivity of the filters was detected in UltimaGold^{TM} MV aqueous scintillation cocktail with Packard Tricarb 2300TR liquid scintillation counter. The competition binding assays were performed in duplicate and repeated at least three times.

Functional [^{35}S]GTPγS binding assays.

The G-protein activation of the opioid receptors were measured in functional [^{35}S]GTPγS binding experiments, which monitors the nucleotide exchange process of the G_α-protein using a non-hydrolysable radiolabeled GTP analog, ^{35}[S]GTPγS in the presence of increasing concentrations of the observed ligand (Figure S2).
The assays were performed as previously described,[8] with slight modifications. Membrane fractions of CHO cell lines overexpressed with the corresponding opioid receptors were incubated in a final volume of 1 ml at 30˚C for 60 min in Tris-EGTA buffer (pH 7.4; 50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl). [^{35}S]GTPγS (20 MBq/0.05 cm^3) was added in 0.05 nM concentrations together with compounds **9** and **10** and DAMGO, Ile^{5,6}deltorphin II, U69593 biphalin for control in increasing concentrations (10^{-10} – 10^{-5} M). Total binding (T) was measured in the absence of the ligands, non-specific binding (NS) was determined in the presence of 10 μM unlabeled GTPγS and subtracted from total binding. The difference (T–NS) represents basal activity. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filters with Brandel M24R Cell harvester. Filters were washed three times with 5 ml ice-cold buffer (pH 7.4), and the radioactivity of the dried filters was detected in UltimaGold^{TM} MV scintillation cocktail with Packard Tricarb 2300TR liquid scintillation counter. The [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times.

Data analysis. Experimental data were presented as means ± S.E.M. and were fitted using non-linear regression with the curve fitting program, GraphPad Prism 5.0 (GraphPad Prism Software Inc., San Diego, CA),. During the competition binding assays the 'One-site competition' fitting equation was applied to determine the inhibition constant (K_i). The inhibition of the specifically bound tritiated opioid receptor specific ligand was given in percentage, the total specific binding and the non-specific binding was defined as 100% and 0% respectively. In the [^{35}S]GTPγS binding assays the 'Sigmoid dose-response' fitting was used to establish the maximal stimulation or efficacy (E_{max}) of the receptor, and the potency (EC_{50}) of the stimulator ligand. The receptor stimulation was given as percent of the specific [^{35}S]GTPγS binding observed over the basal activity, which was

settled as 100%. In case of three or more data sets One-way ANOVA with Bonferroni's Multiple Comparison post hoc test was performed to determine the significance level, using GraphPad Prism 5.0. Significance was accepted at the $P < 0.05$ level.

GPI and MVD in vitro bioassays

Electrically induced smooth muscle contractions of mouse vas deferens and strips of guinea pig ileum longitudinal muscle myenteric plexus were used. Tissues came from male ICR mice weighing 25-30g and from male Hartley guinea pigs weighing 150-400 g. The tissues were first tied to gold chains with suture silk, suspended in 20 mL baths containing 37 °C oxygenated (95% O_2, 5% CO_2), Krebs bicarbonate solution (magnesium-free for the MVD), and allowed to equilibrate for 15 min. Tissues were then stretched to optimal length previously determined to be 1g tension (0.5 g for MVD), allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum wire electrodes at 0.1 Hz, 0.4 ms pulses (2.0 ms pulses for MVD) at supramaximal voltage. Biphalin and analogues **9** and **10** were added to the baths in 20-60 mL volumes at five to seven different concentrations to produce cumulative dose-response curves. Percent inhibition was calculated by using an average contraction height for 1 min preceding the addition of the peptide divided by contraction height 3 min after the exposure to the peptide. IC_{50} values are the mean of not less than four separate assays. IC_{50} estimates and their associated standard errors were determined by fitting the mean data to the Hill equation using a computerized least-squares method. All studies in the U.S. were part of protocols approved by the Institutional Care and Use Committee.

***In vivo* Nociception Test.**

Animals. Male CD-1 mice (Harlan, Italy) weighing 25-30 g were used for all experiments. Mice were housed for at least 1 week before experimental sessions in colony cages (7 mice in each cage) under standard light (light on from 7.00 a.m. to 7.00 p.m.), temperature (21 ± 1°C), relative humidity (60 ± 10%) with food and water available *ad libitum*. The experiments conformed to the guidelines for pain research with laboratory animals. The research protocol was approved by the Service for Biotechnology and Animal Welfare of the Istituto Superiore di Sanità and authorized by the Italian Ministry of Health, according to Legislative Decree 116/92, which implemented the European Directive 86/609/EEC on laboratory animal protection in Italy. Animal welfare was routinely checked by veterinarians from the Service for Biotechnology and Animal Welfare.

Drugs and treatment procedure. DMSO was purchased from Merck (Italy). Morphine sulphate was purchased from SALARS (Italy). On each test day, morphine or peptide solutions were freshly prepared using DMSO: 0.9% saline 1:5 v/v. These solutions were injected at a volume of 5 μL/mouse for intracerebroventricular (i.c.v.) and 5 mL/Kg for intravenous (i.v.) administrations.

Surgery for i.c.v. injections. For i.c.v. injections, mice were lightly anesthetized with isoflurane, and an incision was made in the scalp. Injections were performed using a 10 μl Hamilton microsyringe at a point 2-mm caudal and 2-mm lateral from the bregma at a depth of 3 mm in a volume of 5 μL.

Surgery for i.v. injections. For i.v. injections, mice were lightly anesthetized with isoflurane, gentle constrained by using a glove and intravenous injection performed directly into the tail vein using a 500 μl Hamilton syringe equipped with a 30 gauge needle in a volume of 5 ml/kg.

Hot plate and tail flick tests. Thermal nociception (hot plate test) was assessed with a commercially available apparatus consisting of a metal plate 25x25 cm (Ugo Basile, Italy) heated to a constant temperature of 55.0 ± 0.1°C, on which a plastic cylinder (20 cm diameter, 18 cm high) was placed. The time of latency (s) was recorded from the moment the animal was placed in the cylinder on the hot plate until it licked its paws or jumped; the cut-off time was 60 s. The baseline was calculated as mean of three readings recorded before testing at intervals of 15 min, and was in the same order of magnitude in all experimental groups (mean 9.8±1.2 s, N=8-10). The time course of latency was then determined at 15, 30, 45, 60, 90 and 120 min after compound treatment (for i.v. administration until to 180 min). The tail-flick latency was obtained using a commercial unit (Ugo Basile, Italy), consisting of an infrared radiant light source (100 W, 15 V bulb) focused onto a photocell utilizing an aluminium parabolic mirror. During the trials the mice were gently hand-restrained with a glove. Radiant heat was focused 3-4 cm from the tip of the tail, and the latency (s) of the tail withdrawal recorded. The measurement was interrupted if the latency exceeded the cut off time (15 s at 15 V). Also in this case, the baseline was calculated as mean of three readings recorded before testing at intervals of 15 min. Baseline was in the same order of magnitude in all groups (mean 4.3±0.9 s, N=8-10). The time course of latency was then determined at 15, 30, 45, 60, 90 and 120 min after compound treatment (for i.v. administration until to 180 min). In both the hot plate and tail flick tests, data were expressed as time course of the percentage of maximum effect (%MPE) = (post drug latency – baseline latency) / (cut-off time – baseline latency) x 100.

Data analysis and statistics. Experimental data were expressed as mean ± S.E.M. The significance among groups was evaluated with the analysis of variance (two-way ANOVA test) followed by Bonferroni's post-hoc comparisons using the statistical software SPSS. Statistical significance was assumed at $P<0.05$ (*$P<0.05$; ***$P<0.005$)

NMR Spectroscopy.

99.9% ^2H_2O were obtained from Aldrich (Milwaukee, USA), 98% DPC-d_{38} was obtained from Cambridge Isotope Laboratories, Inc. (Andover, USA), [(2,2,3,3-tetradeuterio-3-(trimethylsilanyl)]propionic acid (TSP) from MSD Isotopes (Montreal, Canada). The samples for NMR spectroscopy were prepared by dissolving the appropriate amount of peptide in 0.54 ml of ^1H_2O (pH 5.5), 0.06 ml of ^2H_2O to obtain a concentration 2 mM and 200 mM of DPC-d_{38}. NH exchange studies were performed dissolving peptide in 0.60 ml of ^2H_2O and 200 mM of DPC-d_{38}. NMR spectra were recorded on a Varian INOVA 700 MHz spectrometer equipped with a z-gradient 5 mm triple-resonance probe head. All the spectra were recorded at a temperature of 25 °C. The spectra were calibrated relative to TSP (0.00 ppm) as internal standard. One-dimensional (1D) NMR spectra were recorded in the Fourier mode with quadrature detection. The water signal was suppressed by gradient echo.[9] 2D DQF-COSY,[10] TOCSY,[11] and NOESY[12] spectra were recorded in the phase-sensitive mode using the method from States.[13] Data block sizes were 2048 addresses in t_2 and 512 equidistant t_1 values. Before Fourier transformation, the time domain data matrices were multiplied by shifted $\sin^2$ functions in both dimensions. A mixing time of 70 ms was used for the TOCSY experiments. NOESY experiments were run with mixing times in the range of 50-200 ms. The qualitative and quantitative analyses of DQF-COSY, TOCSY, and NOESY spectra, were obtained using the interactive program package XEASY.[14] The temperature coefficients of the amide proton chemical shifts were calculated from 1D ^1H NMR and 2D TOCSY experiments performed at different temperatures in the range 25-40°C by means of linear regression.

Spectra Analysis.

The NOESY spectra of peptides **9** and **10** are shown in Figure S3. As a consequence of the conformational restrictions imposed by the D-Pen2,2' bridge, peptide **9** showed three slowly interchangeable conformational states, labeled in Figure S3-a as I, II, and III. Differently, a single signal system could be observed for peptide **10** (Figure S3-b). Population ratios of the three states of **9** is 2:1:0.3 (I:II:III) from NMR integration. Complete ^1H NMR chemical shift assignments were effectively achieved for peptide **9** (states I and II, only partial assignments for state III) and peptide **10** according to the Wüthrich[15] procedure via the usual systematic application of DQF-COSY, TOCSY, and NOESY experiments with the support of the XEASY software package (Tables S1-S2). Since only intra-residue or sequential NOEs could be unambiguously assigned for states II and III of peptide **9** indicating extended or random conformations associated with those signals, only state I was considered for structure calculation.

Structure Calculation

The NOE-based distance restraints were obtained from NOESY spectra of peptide **9** (state I) and **10** collected with a mixing time of 50 ms. The NOE cross peaks were integrated with the XEASY program and were converted into upper distance bounds using the CALIBA program incorporated into the program package DYANA.[16] Only NOE derived constraints were considered in the annealing procedures. The restraints applied during the calculations are reported in Tables S3 and S4. NMR-derived upper bounds were imposed as semiparabolic penalty functions with force constants of 16 Kcal mol^{-1} $Å^{-2}$. A distance maximum force constant of 250 Kcal/ mol^{-1} $Å^{-2}$ was used. Considering the multiple conformation state of peptide **9** demonstrated by its NMR spectra, restraints were imposed only to one side of the palindromic sequence (residues 1-4) leaving the other side (residues 1'-4') unrestrained. For comparison purposes, the same strategy was applied to

peptide **10**. Cyclic peptides **9** and **10** were built using the Insight Builder module (Accelrys Software Inc., San Diego). Atomic potentials and charges were assigned using the consistent valence force field (CVFF).[17] The conformational space of compounds was sampled through 100 cycles of restrained Simulated Annealing (ε = 1r). In Simulated Annealing, the temperature is altered in time increments from an initial temperature to a final temperature by adjusting the kinetic energy of the structure (by rescaling the velocities of the atoms). The following protocol was applied: the system was heated up to 1500 K over 2000 fs (time step = 1.0 fs); the temperature of 1500 K was applied to the system for 2000 fs (time step = 1.0 fs) with the aim of surmounting torsional barriers; successively, temperature was linearly reduced to 300 K in 1000 fs (time step = 1.0 fs). Resulting conformations were then subjected to restrained Molecular Mechanics (MM) energy minimization within Insight Discover module (ε = 1r) until the maximum RMS derivative was less than 0.001 kcal/Å, using Conjugate Gradient as minimization algorithm. Finally, conformations were subjected to 1000 steps of unrestrained MM Conjugate Gradient energy minimization. From the produced 100 conformations, 10 structures, whose interprotonic distances best fitted NOE derived distances, were chosen for statistical analysis (Table S5).

The lowest energy conformer of each peptide was then subjected to 60 ns of molecular dynamics calculations after an equilibration period of 30 ps using a temperature of 300 K, applying the same restraints mentioned above. During molecular dynamics, frame structures were saved every 1 ps. Distances between pharmacophoric points are reported in Figure S4.

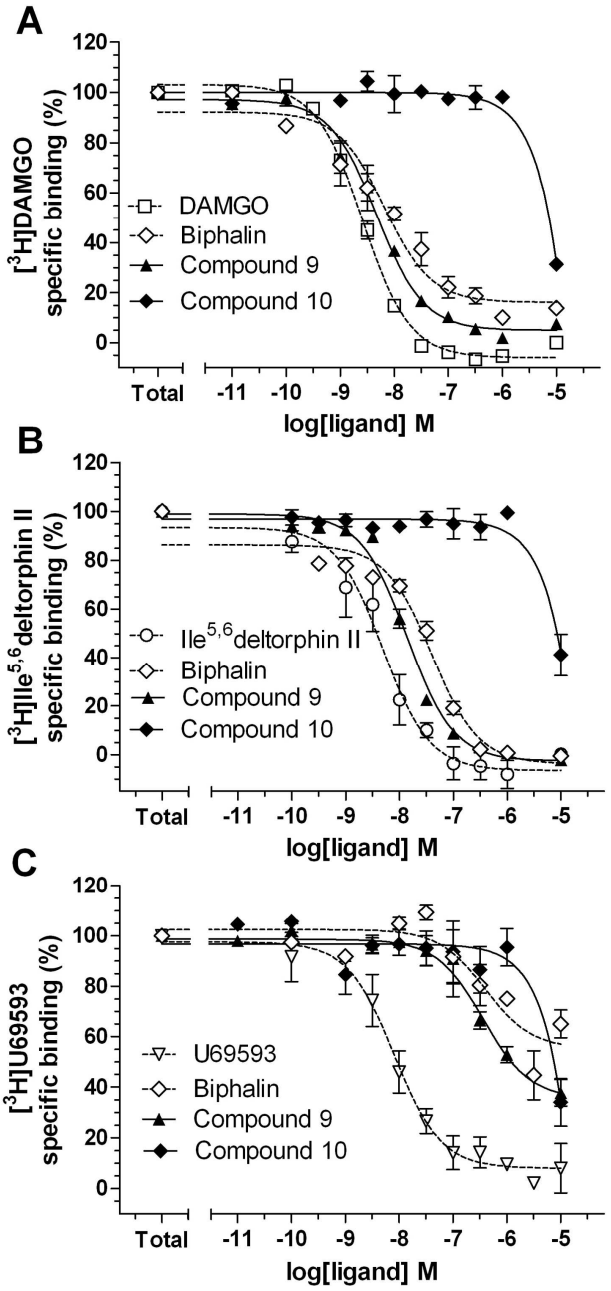

Figure S1. Competition binding curves of compounds **9** and **10** in the presence of μ (A), δ (B) and κ opioid receptor (C) specific tritiated ligands in rat brain membranes. The unlabeled opioid ligands were also tested for comparison together with biphalin. Results are the percentage of specific radiolabeled opioid ligand bound to the receptor in fixed concentrations observed in the presence of increasing (10^{-10}-10^{-5} M) concentrations of unlabeled compounds **9** and **10**, and unlabeled DAMGO, $Ile^{5,6}$deltorphin II or U69593. Points represent means ± S.E.M. for at least 3 experiments performed twice. "Total" (=100%) indicates the points which do not contain competitor ligands.

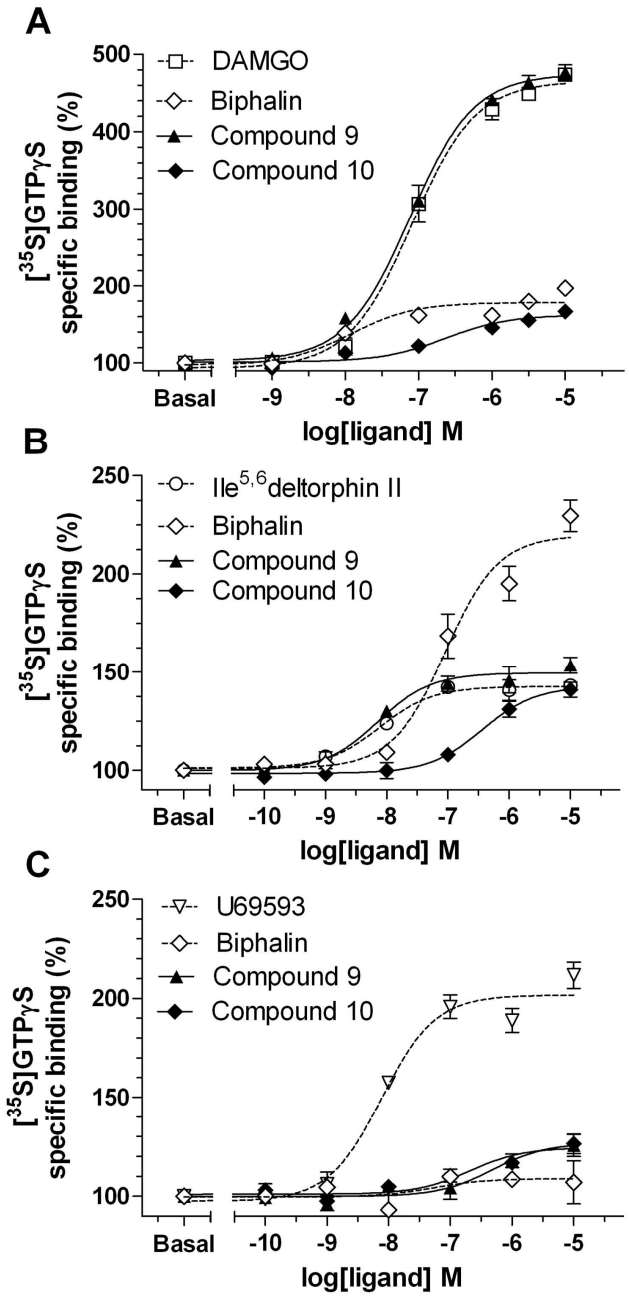

Figure S2. Sigmoid dose-response curves of [^{35}S]GTPγS specific binding in ligand-modulated [^{35}S]GTPγS binding assays in CHO cell membranes over expressed with μ (A), δ (B) or κ opioid receptors (C). Results are the percentage of the specifically bound [^{35}S]GTPγS in the presence of increasing concentrations (10^{-10}-10^{-5} M) of compounds **9** and **10** or one of the corresponding opioid receptor specific ligand or biphalin for control (μ: DAMGO; δ: Ile^{5,6} deltorphine II; κ: U69593). Points and columns represent means ± S.E.M. for at least 3 experiments performed in 3 times each. "Basal" indicates basal activity level (=100%).

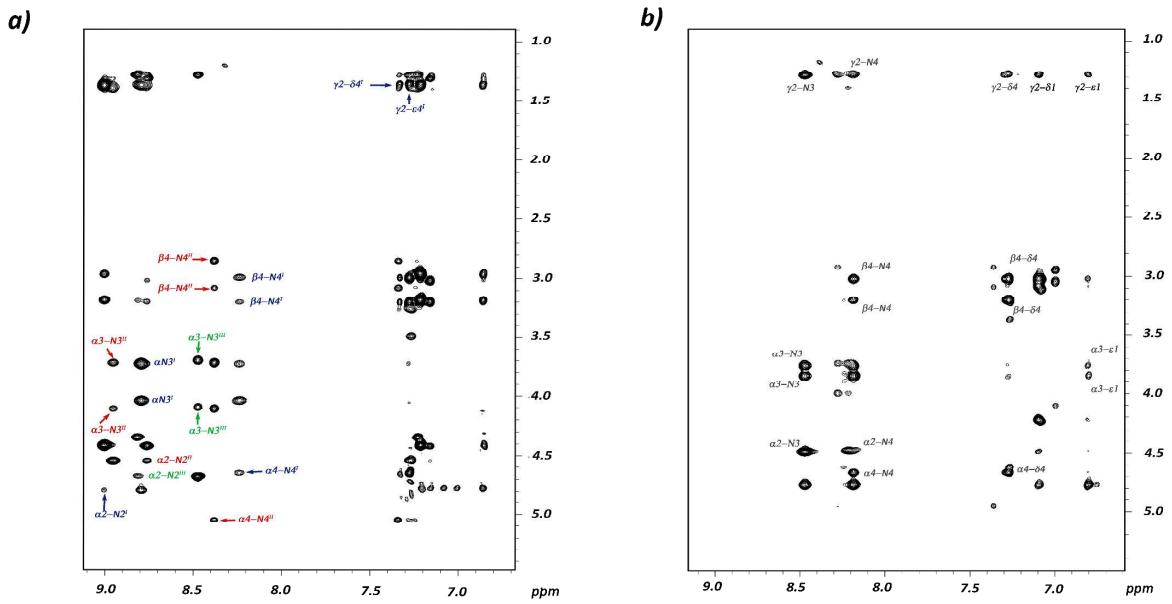

Figure S3. Extended amide and aromatic region of the NOESY spectrum of peptide **9** (a) and **10** (b).

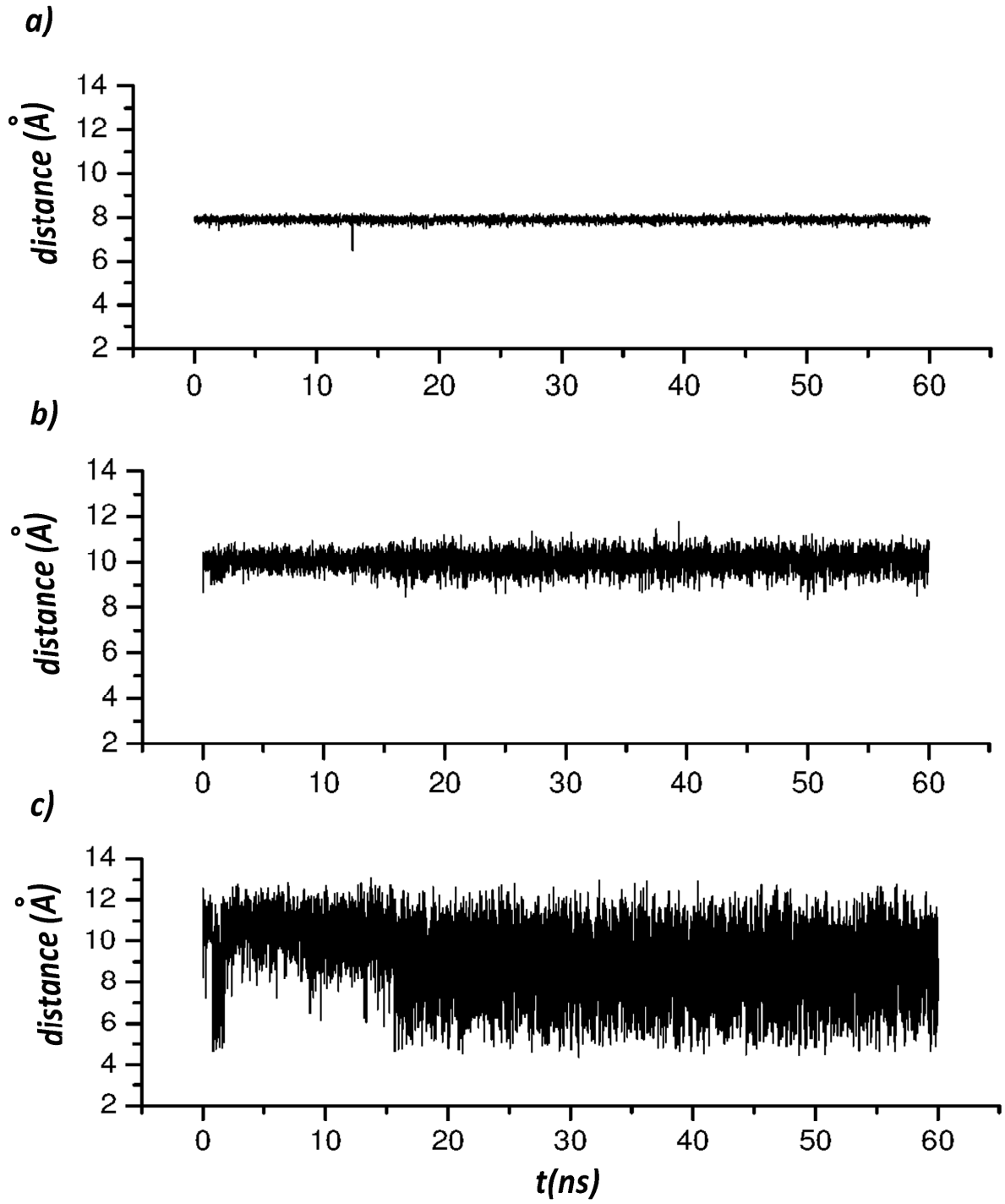

Figure S4. Distances between pharmacophoric points in peptide **9**. (a) N-terminus - Tyrosine OH; (b) N-terminus - Phenylalanine aromatic centroid; (c) Tyrosine OH - Phenylalanine aromatic centroid.

Table S1. ^{1}H NMR assignment of peptide **9**.

residue	NH (Δδ/ΔT)	$C^{\alpha}H$	$C^{\beta}H$	Others
		State I		
Tyr[1]		4.40	3.17, 2.96	7.21(δ);6.85(ε)
DPen[2]	8.99 (-8.4)	4.77		1.35(γ)
Gly[3]	8.78 (-7.0)	4.03, 3.71		
Phe[4]	8.23(-5.5)	4.63	3.20, 2.99	7.33(δ);7.27(ε)
HB	9.89			
		State II		
Tyr[1]		4.41	3.19, 3.01	7.15(δ);6.85(ε)
DPen[2]	8.75 (-6.0)	4.53		1.39, 1.30 (γ)
Gly[3]	8.94 (-10.0)	4.10, 3.71		
Phe[4]	8.37(-6.1)	5.03	2.84, 3.07	7.33(δ);7.23(ε)
HB	10.48			
		State III		
Tyr[1]		4.33	2.96, 3.17	7.22(δ);6.84(ε)
DPen[2]	8.80 (-6.8)	4.66		1.27 (γ)
Gly[3]	8.46 (-6.5)	3.68, 4.09		
Phe[4]	*	*	*	*
HB	*			

* Not assigned resonances. [a]: HB: Hydrazine bridge.

Table S2. ^{1}H NMR assignment of peptide **10**.

residue	NH (Δδ/ΔT)	$C^{\alpha}H$	$C^{\beta}H$	Others
Tyr[1]		4.22	3.08-3.01	7.10(δ);6.80(ε)
DPen[2]	8.95 (-5.0)	4.48		1.40, 1.28(γ)
Gly[3]	8.47 (-5.5)	3.85, 3.76		
Phe[4]	8.19 (-7.2)	4.66	3.20, 3.02	7.31(δ);7.28(ε);7.36(ζ)
HB	10.24, 9.33			

* Not assigned resonances. HB: Hydrazine bridge.

Table S3. Lists of upper limits used in the structure calculations of peptide **9.**

1	TYR	HA	1	TYR	QD	4.54
1	TYR	HA	2	DPEN	HN	2.40
1	TYR	HA	2	DPEN	QQG	5.72
1	TYR	QD	2	DPEN	QQG	6.51
1	TYR	QE	2	DPEN	QQG	7.54
2	DPEN	HN	2	DPEN	QQG	4.56
2	DPEN	HN	3	GLY	HN	2.74
2	DPEN	HA	3	GLY	HN	2.50
2	DPEN	QQG	3	GLY	HN	5.08
2	DPEN	QQG	3	GLY	HA1	6.44
2	DPEN	QQG	3	GLY	HA2	6.03
2	DPEN	QQG	4	PHE	HN	5.50
2	DPEN	QQG	4	PHE	QD	6.50
2	DPEN	QQG	4	PHE	QE	6.50
3	GLY	HN	4	PHE	HN	3.80
3	GLY	HA1	4	PHE	HN	3.60
3	GLY	HA2	4	PHE	HN	2.66
4	PHE	HA	4	PHE	QD	4.94

Table S4. Lists of upper limits used in the structure calculations of peptide **10.**

1	TYR	HA	2	PEN	QQG	5.77
1	TYR	QD	2	PEN	HA	6.64
1	TYR	QD	2	PEN	QQG	6.52
1	TYR	QE	2	PEN	QQG	7.51
1	TYR	QE	3	GLY	HA1	6.63
1	TYR	QE	3	GLY	HA2	6.63
2	PEN	HA	3	GLY	HN	2.50
2	PEN	HA	4	PHE	HN	3.30
2	PEN	QQG	3	GLY	HN	5.09
2	PEN	QQG	3	GLY	HA1	5.47
2	PEN	QQG	3	GLY	HA2	5.47
2	PEN	QQG	4	PHE	HN	5.47
2	PEN	QQG	4	PHE	QD	6.50
3	GLY	HN	4	PHE	HN	3.00
3	GLY	HA1	4	PHE	HN	3.53
3	GLY	HA2	4	PHE	HN	2.60

Table S5. Torsion angles of described compounds.[a]

Angle	**9** [b]	**10** [b]
ψ^1	107 ± 9	142 ± 5
χ_1^1	-174 ± 3	-178 ± 3
ω^1	160 ± 6	-174 ± 2
ϕ^2	126 ± 10	-91 ± 11
ψ^2	67 ± 7	120 ± 5
χ_1^2	-61 ± 4	-73 ± 8
ω^2	-170 ± 4	168 ± 2
ϕ^3	80 ± 6	-83 ± 4
ψ^3	-69 ± 7[d]	-38 ± 4
ω^3	-174 ± 5	-174 ± 4
ϕ^4	-72 ± 5	-77 ± 9
ψ^4	12 ± 67	86 ± 73
χ_1^4	65 ± 4[c]	58 ± 4

[a]Angles are in degrees. Only angles of the fragment 1-4 (restrained) are reported. [b]Angles value of the mean structure of the 10 lowest energy conformers are reported (± standard deviation). [c]Calculated on 9/10 conformers, the other has χ_1^4 = -59°.

References

(1) Nevin, S. T.; Kabasakal, L.; Otvös, F.; Töth, G.; Borsodi, A. Binding characteristics of the novel highly selective delta agonist, [^{3}H]IIe5,6deltorphin II. *Neuropeptides* **1994**, *26*, 261–265.

(2) Rónai, A. Z.; Magyar, A.; Orosz, G.; Borsodi, A.; Benyhe, S.; Tóth, G.; Makó, E.; Kátay, E.; Babka, E.; Medzihradszky, K.. Opioid antagonist properties of the highly delta-receptor-selective BOC-Tyr-Pro-Gly-Phe-Leu-Thr (OtBu) peptide and of its Phe^1 and Mel1 analogues. *Arch. Int. Pharmacodyn. Ther.* **1995**, *330*, 361–369.

(3) Benyhe, S.; Szücs, M.; Borsodi, A.; Wollemann, M. Species differences in the stereoselectivity of kappa opioid binding sites for [^{3}H]U-69593 and [^{3}H]ethylketocyclazocine. *Life Sci.* **1992**, *51*, 1647–1655.

(4) Benyhe, S.; Farkas, J.; Tóth, G.; Wollemann, M.. Met5-enkephalin-Arg6-Phe7, an endogenous neuropeptide, binds to multiple opioid and nonopioid sites in rat brain. *J. Neurosci. Res.* **1997**, *48*, 249–258.

(5) (a) Befort, K.; Tabbara, L.; Kieffer, B. L. [^{35}S]GTP gamma S binding: a tool to evaluate functional activity of a cloned opioid receptor transiently expressed in COS cells. Neurochemical research 1996, 21, 1301–1307. (b) Carboni, L., Campana, G., Cacciaguerra, S., Murari, G., Speroni, E., Pappalardo, M.S., Ronsisvalle, G., Spampinato, S. Agonist binding properties for recombinant kappa opioid receptors expressed in CHO-K1 cells. *Ann. NY Acad. Sci.* **1997**, *812*, 203–204.

(6) Ioja, E.; Tourwé, D.; Kertész, I.; Tóth, G.; Borsodi, A.; Benyhe, S. Novel diastereomeric opioid tetrapeptides exhibit differing pharmacological activity profiles. Brain Res. Bull. **2007**, *74*, 119–29.

(7) Zádor, F.; Otvös, F.; Benyhe, S.; Zimmer, A.; Páldy, E. Inhibition of forebrain μ-opioid receptor signaling by low concentrations of rimonabant does not require cannabinoid receptors and directly involves μ-opioid receptors. *Neurochem. Int.* **2012**, *61*, 378–88.

(8) (a) Sim, L. J.; Selley, D. E.; Childers, S. R. *In vitro* autoradiography of receptor-activated G proteins in rat triphosphate binding **1995**, *92*, 7242–7246. (b) Traynor, R.; Nahorski, R. Modulation by mu-opioid agonists of guanosine-5'-O-(3-[35S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol. Pharmacol.* **1995**, *47*, 848–54.

(9) Hwang, T. L.; Shaka, A. J. Water Suppression that Works. Excitation Sculpting Using Arbitrary Wave-Forms and Pulsed-Field Gradients. *J. Magn. Res.* **1995**, *112*, 275-279.

(10) (a) Piantini, U.; Sorensen, O. W.; Ernst, R. R. Multiple Quantum Filters for Elucidating NMR Coupling Network. *J. Am. Chem. Soc.* **1982**, *104*, 6800-6801. (b) Marion. D.; Wüthrich, K. Application of Phase Sensitive Two-Dimensional Correlated Spectroscopy (COSY) for Measurements of 1H-1H Spin-Spin Coupling Constants in Proteins. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967-974.

(11) Braunschweiler, L.; Ernst, R. R. Coherence Transfer by Isotropic Mixing: Application to Proton Correlation Spectroscopy. *J. Magn. Reson.* **1983**, *53*, 521–528.

(12) Jenner, J.; Meyer, B. H.; Bachman, P.; Ernst, R. R. Investigation of Exchange Processes by Two-Dimensional NMR Spectroscopy. *J. Chem. Phys.* **1979**, *71*, 4546-4553.

(13) States, D. J.; Haberkorn, R. A.; Ruben, D. J. A Two-Dimensional Nuclear Overhauser Experiment with Pure Absorption Phase in Four Quadrants. *J. Magn. Reson.* **1982**, *48*, 286-292.

(14) Bartels, C.; Xia, T.; Billeter, M.; Guentert, P.; Wüthrich, K. The Program XEASY for Computer-Supported NMR Spectral Analysis of Biological Macromolecules. *J. Biomol.*

NMR **1995**, *6*, 1-10.
(15) Wüthrich, K. In NMR of Proteins and Nucleic Acids; John Wiley & Sons: New York, **1986**.
(16) Güntert P.; Mumenthaler C.; Wüthrich, K.; Torsion Angle Dynamics for NMR Structure Calculation with the New Program DYANA. *J. Mol. Biol.* **1997**, *273*, 283-298.
(17) Maple, J.; Dinur, U.; Hagler, A. T. Derivation of Force Fields for Molecular Mechanics and Dynamics from Ab Initio Energy Surface. *Proc. Natl. Acad. Sci.* U.S.A. **1988**, *85*, 5350-5354.