Supporting Information

Biological active analogues of the opioid peptide biphalin: mixed α/β^3-peptides.

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Contents:

Chemical synthesis and spectroscopic data for all intermediates.

Chemistry. General. All products were synthesized in solution using the EDC/HOBt/NMM 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. Final products **1-4** were purified by RP-HPLC using a Waters XBridge™ Prep BEH130 C_{18}, 5.0 μm, 250 mm x 10 mm column at a flow rate of 1 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 45 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).

(Boc-hβ^{3}Phe-NH)$_2$. EDC·HCl (2.2 eq.), HOBt (2.2 eq.), and NMM (6.6 eq.) were added to a solution of Boc-hβ^{3}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 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 white crude solid. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 8:2 to CH_2Cl_2/EtOAc 1:1) to obtain the pure product (84%) R_f = 0.71 (CH_2Cl_2/EtOAc 1:1). ^{1}HNMR ($CDCl_3$) δ: 1.21 (18H, s, Boc) 2.25 (4H, m, hβ^{3}Phe hβCH_2), 2.58–2.80 (4H, m, hβ^{3}Phe βCH_2), 4.20 (2H, m, hβ^{3}Phe αCH), 6.65 (2H, d, hβ^{3}Phe NH), 7.17–7.26 (10H, m, Ar), 9.82 (2H, s, NH-NH). ESI-HRMS calcd for $C_{30}H_{42}N_6O_6$ *m/z*: 555.3183 $[M+H]^+$; found 555.3185.

(Boc-Gly-hβ^{3}Phe-NH)$_2$. (Boc-hβ^{3}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 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 NMM (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, 2 TFA·(hβ^{3}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 (92%). R_f = 0.39 (EtOAc/MeOH 95:5). ^{1}HNMR (DMSO-*d$_6$*) δ: 1.35 (18H, s, Boc), 2.26 (4H, t, hβ^{3}Phe hβCH_2), 2.63–2.83 (4H, m, hβ^{3}Phe βCH_2), 3.48–3.51(4H, m, Gly αCH_2), 4.21 (2H, m, hβ^{3}Phe αCH), 6.84 (2H, t, Gly NH), 7.15–7.22 (10H, m, Ar), 7.75 (2H, d, hβ^{3}Phe NH), 9.84 (2H, s, NH-NH). ESI-HRMS calcd for $C_{34}H_{48}N_6O_8$ *m/z*: 669.3612 $[M+H]^+$; found 669.3613.

(Boc-DAla-Gly-hβ^{3}Phe-NH)$_2$. (Boc-Gly-hβ^{3}Phe)$_2$ 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 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 NMM (6.6 eq.) were added to a solution of Boc-DAla-OH (2.2 eq.) in DMF at 0 °C. The reaction mixture was stirred for 10 min, 2 TFA·(Gly-hβ^{3}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 (27%). R_f = 0.70 (EtOAc/MeOH 9:1). ^{1}HNMR (DMSO-d_6) δ: 1.15 (3H, d, DAla CH_3), 1.37 (18H, s, Boc), 2.27 (4H, t, hβ^{3}Phe hβCH_2), 2.62–2.86 (4H, m, hβ^{3}Phe βCH_2), 3.44–3.62 (4H, m, Gly αCH_2), 3.93 (2H, t, DAla αCH), 4.22 (2H, m, hβ^{3}Phe αCH), 7.00 (2H, d, DAla NH), 7.16–7.27 (10H, m, Ar), 7.75 (2H, d, hβ^{3}Phe NH), 7.90 (2H, t, Gly NH), 9.83 (2H, s, NH-NH). ESI-HRMS calcd for $C_{40}H_{58}N_8O_{10}$ *m/z*: 811.4354 $[M+H]^+$; found 811.4358.

(Boc-Tyr-DAla-Gly-hβ^{3}Phe-NH)$_2$. (Boc-DAla-Gly- hβ^{3}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 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 NMM (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·(DAla-Gly-hβ^{3}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 2:8 to EtOAc/MeOH 9:1) to obtain the pure product (24 %). R_f = 0.67 (EtOAc/MeOH 9:1). ^{1}HNMR (DMSO-d_6) δ: 1.20 (3H, d, DAla CH_3), 1.28 (18H, s, Boc), 2.28 (4H, t, hβ^{3}Phe hβCH_2), 2.62–2.71 (4H, m, Tyr βCH_2), 2.74–2.76 (4H, m, hβ^{3}Phe βCH_2), 3.48–3.59 (4H, m, Gly αCH_2), 4.04 (2H, t, Tyr αCH), 4.08 (2H, t, DAla αCH), 4.2 (2H, m, hβ^{3}Phe αCH), 6.61 (2H, d, Tyr NH), 6.90–7.01 (8H, m, Tyr Ar), 7.16–7.26 (10H, m, hβ^{3}Phe Ar), 7.78 (2H, d, hβ^{3}Phe NH), 7.99 (2H, t, Gly NH), 8.10 (2H, d, DAla NH), 9.16 (2H, s, OH), 9.84 (2H, s, NH-NH). ESI-HRMS calcd for $C_{58}H_{76}N_{10}O_{14}$ *m/z*: 1137.5621 $[M+H]^+$; found 1137.5624.

(Boc-Phe-NH)$_2$. EDC·HCl (2.2 eq.), HOBt (2.2 eq.), and NMM (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 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 white crude solid. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 8:2 to CH_2Cl_2/EtOAc 3:7) to obtain the pure product (91%) R_f = 0.35 (CH_2Cl_2/EtOAc 8:2). ^{1}HNMR ($CDCl_3$) δ: 1.27 (18H, s, Boc), 2.76–3.05 (4H, m, Phe βCH_2), 4.26 (2H, m, Phe αCH), 6.95 (2H, d, Phe NH), 7.19–7.38 (10H, m, Ar), 10.15 (2H, s, NH–NH). ESI-HRMS calcd for $C_{28}H_{38}N_4O_6$ *m/z*: 527.2870 $[M+H]^+$; found 527.2872.

(Boc-βAla-Phe-NH)$_2$. (Boc-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 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 NMM (6.6 eq.) were added to a solution of Boc-βAla-OH (2.2 eq.) in DMF at 0 °C. The reaction mixture was stirred for 10 min, 2 TFA·(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 1:1 to EtOAc/MeOH 9:1) to obtain the pure product (62 %). R_f = 0.31 (EtOAc).[1]HNMR (DMSO-d_6) δ: 1.25 (18H, s, Boc), 2.06–2.25 (4H, m, βAla CH_2-CO), 2.70–3.06 (4H, m, βAla CH_2-N), 2.93–3.06 (4H, m, Phe βCH_2), 4.52–4.60 (2H, m, Phe αCH), 6.60 (2H, m, βAla NH), 7.13–7.26 (10H, m, Ar), 8.22 (2H, d, Phe NH), 10.13 (2H, s, NH-NH). ESI-HRMS calcd for $C_{34}H_{48}N_6O_8$ *m/z*: 669.3612 $[M+H]^+$; found 669.3615.

Boc(DAla-βAla-Phe-NH)$_2$. (Boc-βAla-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 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 NMM (6.6 eq.) were added to a solution of Boc-DAla-OH (2.2 eq.) in DMF at 0 °C. The reaction mixture was stirred for 10 min, 2 TFA·(βAla-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 (57%). R_f = 0.23 (EtOAc). [1]HNMR (DMSO-d_6) δ: 1.09 (6H, d, DAla βCH_3), 1.26 (18H, s, Boc), 2.07–2.24 (4H, m, βAla CH_2-CO), 2.69–3.08 (4H, m, Phe βCH_2), 3.02-3.08 (4H, m, βAla NH), 3.84 (2H, t, DAla αCH), 4.53 (2H, t, Phe αCH), 6.83 (2H, d, DAla NH), 7.16–7.25 (10H, m, Ar), 7.74 (2H, d, βAla NH), 8.22 (2H, d, Phe NH), 10.15 (2H, s, NH-NH). ESI-HRMS calcd for $C_{40}H_{58}N_8O_{10}$ *m/z*: 811.4354 $[M+H]^+$; found 811.4355.

(Boc-Tyr-DAla-βAla-Phe-NH)$_2$. Boc(DAla-bAla-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 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 NMM (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·(DAla-βAla-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 (EtOAc to EtOAc/MeOH 7:3) to obtain the pure product (67 %). R_f = 0.43 (EtOAc/MeOH 95:5). [1]HNMR (DMSO-d_6) δ: 1.05 (6H, d, DAla CH_3), 1.31 (18H, s, Boc), 2.19–2.40 (4H, m, βAla CH_2-CO), 2.48–2.63 (4H, m, Tyr βCH_2), 2.67–3.04 (4H, m, Phe βCH_2), 3.06–3.27 (4H, m, βAla NH), 3.99 (2H, t, Tyr αCH), 4.12 (2H, t, DAla αCH), 4.55 (2H, t, Phe αCH), 6.61 (2H, d, Tyr NH), 6.93–7.00 (8H, m, Tyr Ar), 7.20–7.25 (10H, m, Phe Ar), 7.99 (2H, d, DAla NH), 8.23 (2H, d, Phe NH), 9.15 (2H, s, OH), 10.16 (2H, s, NH-NH). ESI-HRMS calcd for $C_{58}H_{76}N_{10}O_{14}$ *m/z*: 1137.5621 $[M+H]^+$; found 1137.5624.

(Boc-Gly-Phe-NH)$_2$. (Boc-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 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 NMM (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, 2 TFA·(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 (82 %). R_f = 0.56 (CH_2Cl_2/EtOAc 1:1). ^{1}HNMR (DMSO-d_6) δ: 1.40 (18H, s, Boc), 2.80–3.05 (4H, m, Phe βCH_2), 3.44–3.59 (4H, m, Gly CH_2), 4.61 (2H, m, Phe αCH), 6.86 (2H, m, Gly NH), 7.18–7.27 (10H, m, Ar), 8.03 (2H, d, Phe NH), 10.18 (2H, s, NH–NH). ESI-HRMS calcd for $C_{32}H_{44}N_6O_8$ *m/z*: 641.3299 $[M+H]^+$; found 641.3300.

(Boc-βAla-Gly-Phe-NH)$_2$. (Boc-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 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 NMM (6.6 eq.) were added to a solution of Boc-βAla-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 (67 %). R_f = 0.28 (EtOAc). ^{1}HNMR (DMSO-d_6) δ: 1.34 (18H, s, Boc), 2.22 (4H, m, βAla CH_2-CO), 2.70–3.21 (4H, m, βAla CH_2-NH), 2.71–2.86 (4H, m, Phe βCH_2), 3.48–3.76 (4H, m, Gly CH_2), 4.58 (2H, t, Phe αCH), 6.71 (2H, t, βAla NH), 7.16–7.25 (10H, m, Ar), 7.86 (2H, t, Gly NH), 8.16 (2H, d, Phe NH), 10.19 (2H, s, NH-NH). ESI-HRMS calcd for $C_{38}H_{54}N_8O_{10}$ *m/z*: 783.4041 $[M+H]^+$; found 783.4044.

(Boc-Tyr-βAla-Gly-Phe-NH)$_2$. (Boc-βAla-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 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 NMM (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·(βAla-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 (EtOAc to EtOAc/MeOH 8:2) to obtain the pure product (33 %). R_f = 0.42 (EtOAc/MeOH 9:1). ^{1}HNMR (DMSO-d_6) δ: 1.27 (18H, s, Boc), 2.23 (4H, m, βAla CH_2-CO), 2.53–2.71 (4H, m, Tyr βCH_2), 2.78–3.18 (4H, m, βAla CH_2-NH), 2.79–3.05 (4H, m, Phe βCH_2), 3.48–3.76 (4H, m, Gly CH_2), 3.95 (2H, t, Tyr αCH), 4.58 (2H, Phe αCH), 6.61 (2H, d, Tyr NH), 6.76–6.99 (8H, m, Tyr Ar), 7.20–7.25 (10H, m, Phe Ar), 7.86 (2H, t, Gly NH) 8.02 (2H, t, βAla NH), 8.18 (2H, d, Phe NH), 9.19 (2H, s, OH), 10.19 (2H, s, NH-NH). ESI-HRMS calcd for $C_{65}H_{72}N_{10}O_{14}$ *m/z*: 1109.5308 $[M+H]^+$; found 1109.5310. ESI-HRMS calcd for $C_{58}H_{76}N_{10}O_{14}$ *m/z*: 1137.5621 $[M+H]^+$; found 1137.5622.

(Boc-DAla-Gly-Phe-NH)$_2$. (Boc-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 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 NMM (6.6 eq.) were added to a solution of Boc-DAla-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 (67 %). R_f = 0.19 (EtOAc). ^{1}HNMR (DMSO-d_6) δ: 1.10 (6H, d, DAla CH_3), 1.39 (18H, s, Boc), 2.82–3.09 (4H, m, Phe βCH_2), 3.52–3.64 (4H, m, Gly CH_2), 3.90 (2H, t, DAla αCH), 4.61 (2H, m, Phe αCH), 6.94 (2H, d, DAla NH), 7.21–7.38 (10H, m, Ar), 8.05 (2H, t, Gly NH), 8.12 (2H, d, Phe NH), 10.17 (2H, s, NH-NH). ESI-HRMS calcd for $C_{38}H_{54}N_8O_{10}$ *m/z*: 783.4041 $[M+H]^+$; found 783.4044.

Boc-hβ^{3}Tyr-OH. A solution of HCl·hβ^{3}Tyr-OH (1 eq.) was stirred in a 1:1 mixture of dioxane/water at 0 °C. TEA (1.5 eq.) and di-*tert*-butyl dicarbonate (1.1 eq.) was added and the reaction was stirred for additional 1h at 0 °C and then allowed to warm at r.t. overnight. The solution was concentrated under reduced pressure and acidified to pH 2-3 adding 2N HCl solution and the aqueous layer was extracted with EtOAc. The organic layers were washed with brine, dried over sodium sulphate and the solvent was evaporated to give the desired product as a crude white solid. The product was purified by silica gel column chromatography (CH_2Cl_2/EtOAc 1:1 to CH_2Cl_2/EtOAc 2:3) to obtain the pure product (72 %). R_f = 0.28 (CH_2Cl_2/EtOAc 2:3). ^{1}HNMR (DMSO-d_6) δ: 1.36 (9H, s, Boc), 2.47–2.65 (2H, m, βCH_2), 2.59 (2H, t, hβCH_2), 3.90 (1H, m, αCH), 6.68 (1H, d, NH), 6.85–7.05 (4H, m, Ar), 9.16 (1H, s, Ar-OH), 11.15 (1H, s, COOH). ESI-HRMS calcd for $C_{15}H_{21}NO_5$ *m/z*: 296.1498 $[M+H]^+$; found 296.1499.

(Boc-hβ^{3}Tyr-DAla-Gly-Phe-NH)$_2$. (Boc-DAla-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 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 NMM (6.6 eq.) were added to a solution of Boc-hβ^{3}Tyr-OH (2.2 eq.) in DMF at 0 °C. The reaction mixture was stirred for 10 min, 2 TFA·(DAla-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 (EtOAc to EtOAc/MeOH 7:3) to obtain the pure product (32 %). R_f = 0.81 (EtOAc/MeOH 9:1). ^{1}HNMR (DMSO-d_6) δ: 1.18 (6H, d, DAla βCH_3), 1.38 (18H, s, Boc), 2.24 (4H, m, hβ^{3}Tyr βCH_2), 2.60 (4H, t, hβ^{3}Tyr hβCH_2), 2.80–3.10 (4H, m, Phe βCH_2), 3.54–3.60 (4H, m, Gly αCH_2), 3.88 (2H, m, hβ^{3}Tyr αCH), 4.26–4.31 (2H, m, DAla αCH), 4.62 (2H, m, Phe αCH), 6.66 (2H, d, hβ^{3}Tyr NH), 6.80–7.05 (8H, m, hβ^{3}Tyr Ar), 7.22–7.35 (10H, m, Phe Ar), 7.98 (2H, d, DAla NH), 8.07 (2H, t, Gly NH), 8.14 (2H, d, Phe NH), 9.17 (2H, s, OH), 10.18 (2H, s, NH-NH). ESI-HRMS calcd for $C_{58}H_{76}N_{10}O_{14}$ *m/z*: 1137.5621 $[M+H]^+$; found 1137.5623.

Biological assays

Radioligand Binding Assay. Adult male Sprague-Dawley rats (200-300 g) were sacrificed and their brains immediately removed and placed on ice. Whole brains were homogenized in 20 volumes of 50 mM Tris-HCl stock buffer (pH = 7.4) with a glass-teflon homogenizer. The homogenate was centrifuged (48,000 x g for 15 min), re-suspended and pre-incubated (25 °C for 30 min) to remove endogenous opioids. The homogenate was centrifuged and re-suspended again (0.5% final cont.). Binding affinities of the compounds at six to seven different concentrations were measured against [^{3}H]Deltorphin (42.7 Ci/mmol) and [^{3}H]DAMGO (64.1 Ci/mmol) (New England Nuclear, Boston, MA) by a rapid filtration technique. A 100 μL aliquot of the rat brain homogenate was incubated at 25 °C for 180 min with either 0.75 nM [^{3}H]Deltorphin or 0.5 nM [^{3}H]DAMGO in a total volume of 1 mL of 50 mM Tris-HCl pH (pH = 7.4) containing bovine serum albumin (1 μg/mL), bacitracin (50 μg/mL), bestatin (30 μM), and captopril (l0 μM), and were done in duplicate. Naltrexone hydrochloride (10 μM) was used to define non-specific binding to tissue. The binding reaction was terminated by rapid filtration through presoaked (0.5% polyethylenimine solution) GF/B Whatman glass fiber strips with a Brandel Cell Harvester followed immediately by three rapid washes with 4 mL aliquots of ice-cold saline solution. The filters were removed and soaked in 10 mL scintillation fluid at 4 °C for at least 6 h before bound radioactivity was measured. At least two assays run in duplicate were performed. The data were analyzed by a non-linear least square regression analysis computer program.

GPI and MVD *in vitro* Bioassays. The bioassays of all of the analogues were based on inhibition of electrically induced smooth muscle contractions of mouse vas deferens and strips of guinea pig ileum longitudinal muscle myenteric plexus. Tissues came from male ICR mice weighing 25-30 g 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. The tissues were then stretched and allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum plate electrodes at 0.1 Hz, 0.4 ms pulses (2.0 ms pulses for MVD) at suprarmaximal voltage. Drugs at five to seven different concentrations were added to the baths in 14-60 μL volumes 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.

***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. 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 and the time course of latency determined at 15, 30, 45, 60, 90 and 120 min after 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 (see Tables 1-2)
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 $P<0.05$ (*$P<0.05$; ***$P<0.005$).

Table 1. Antinociceptive effect of Biphalin and Derivative **1** in Hot Plate test, following i.v. administration.

Time (min)	% MPE[a]		Δ %[b]
	Compound 1	Biphalin	
15	43,4 ± 7,1	25,3 ± 2,9	71,6
30	52,4 ± 8,5	36,9 ± 3,2	42,1
45	49,2 ± 4,6	30,4 ± 2,9	61,8
60	39,3 ± 4,9	16,4 ± 2,0	140,2
90	29,9 ± 5,4	12,0 ± 1,5	148,0
120	20,0 ± 3,3	4,3 ± 0,4	369,8
180	13,7 ± 1,9	1,8 ± 0,6	676,7

[a] ± S.E.M. [b] Δ % was calculated using mean values as follow: Δ % = [MPE (Compound **1**) – MPE (Biphalin)]/MPE (Biphalin) x 100.

Table 2. Antinociceptive effect of Biphalin and Derivative **1** in Tail Flick test, following i.v. administration.

Time (min)	% MPE		Δ %
	Compound 1	Biphalin	
15	33,8 ± 4,4	10,8 ± 2,4	213,4
30	51,3 ± 7,3	35,4 ± 7,4	44,9
45	53,0 ± 9,7	31,2 ± 6,3	69,7
60	41,9 ± 8,3	20,4 ± 3,4	105,5
90	38,0 ± 7,5	9,9 ± 1,2	283,7
120	25,1 ± 4,4	5,5 ± 1,2	354,4
180	12,1 ± 2,3	2,6 ± 0,5	358,7

[a] ± S.E.M. [b] Δ % was calculated using mean values as follow: Δ % = [MPE (Compound **1**) – MPE (Biphalin)]/MPE (Biphalin) x 100.

Metabolic stability in Human Plasma

Sample preparation. 495 μL of thawed human plasma (previously frozen at −70 °C) was spiked with 5 μL biphalin and biphalin derivative **1** in DMSO to achieve a final concentration of 100 μg/mL, and then incubated at 37° C (± 1° C).

Prepared samples were removed at several designated time points and incubation was stopped by adding an equal volume of the blocking solution 5% aqueous $ZnSO_4$ solution, MeOH, and ACN (5:3:2) which precipitated proteins. The mixture was vortexed and centrifuged at 12.000 x g for 5 min, then 20 μL of clear supernatant was directly injected into the HPLC system (Waters model 600 solvent pump and 2996 photodiode array detector, with XBridge BEH 130 C18, 4.6 × 250 mm, 5 μm). The samples were tested in three independents experiments (n = 3) and reported values represent the mean ± the Standard Error (SEM). 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 GraphPad Prism v.4. Statistical significance was assumed at $P<0.05$ (**$P<0.01$; ***$P<0.001$); times from t_0 to t_{45} produced no statistical significance ($P>0.05$).

Analytical supporting information. The chemical standards stock solutions were made at the concentration of 1 μg/mL in a final volume of 10 mL of DMSO. Five calibration standards were injected into the HPLC-UV/Vis system.

HPLC analyses were performed on a Waters liquid chromatograph equipped with a model 600 solvent pump and a 2996 photodiode array detector, and the mobile phase was degassed directly on-line by using Degassex, mod. DG-4400 (Phenomenex, Torrance, CA, USA). Empower v.2 Software (Waters Spa, Milford, MA, USA) was used for data acquisition and elaboration.

A reversed-phase packing column (XBridge BEH 130 C18, 4.6 × 250 mm, 5 μm; Waters Spa, Milford, MA, USA) was employed for the separation and the column was held at room temperature (25 ± 1 °C) using a Jetstream2 Plus column oven.

Biphalin and biphalin derivative **1** remaining concentrations at different times are reported in Table 3. For quantitative analyses, selective detection was performed at 276 nm. Gradient elution mode was performed using a mobile phase containing a 95:5 water-methanol ratio as starting conditions and gradient program as reported in Table 4.

Table 3. Stability data.

Time (minutes)	Compound **1** Concentration (μg/mL)	Biphalin Concentration (μg/mL)
0	88,2 ± 9.26	90,9 ± 9.01
15	66,8 ± 7.23	76,3 ± 7.87
30	59,3 ± 6.83	56,1 ± 5.97
45	54,5 ± 5.91	43,1 ± 4.26
60	53,2 ± 5.34	35,1 ± 3.84
90	51,7 ± 4.92	23,5 ± 3.59
120	53,5 ± 5.29	4,87 * ± 5.41
180	42,5 ± 3.98	ND[a]
240	41,8 ± 4.42	ND[a]
720	22,9 ± 2.89	ND[a]

[a] ND: Not detected. *: between LOD and LOQ; reported values are mean ± S.E.M. (n=3).

Table 4. Chromatographic gradient elution mode.

Time (minutes)	%A (Water)	%B (MeOH)
0	95	5
5	95	5
20	20	80
25	20	80
26	5	95
31	95	5
38	95	5

All the sample solutions were previously centrifuged and 20 mL of the supernatant was injected into the HPLC-UV/Vis system.

Calibration curves from 10 to 200 μg/mL were calculated by analyzing five non-zero concentration standards prepared in freshly spiked plasma solution in triplicate and extracted.

All quantitative analyses were performed at 276 nm. Calibration curves were linear with r^2 values always greater than 0.9913 (n = 3). LOD can be set, under previously reported conditions, at 3.5 μg/mL, while LOQ was set at 10 μg/mL. Variability of Biphalin and Biphalin derivative **1** quality control samples was less than 9%. Thus, the lower limit of quantification was at least 10 μg/mL (10% of the starting concentration). Recovery for the precipitation procedure was quantitatively.

After establishing the linearity and recovery of the assay, stability tests were performed without calibration curves, as long as the signal of the starting concentration (t = 0) was in agreement with values determined during the validation. In reported chromatographic conditions, the retention times for Bihalin derivatives (Figure 1) and Biphalin (Figure 2) were 17.33 minutes (± 0.3, n = 20) minutes and 17.73 (± 0.4, n = 20), respectively.

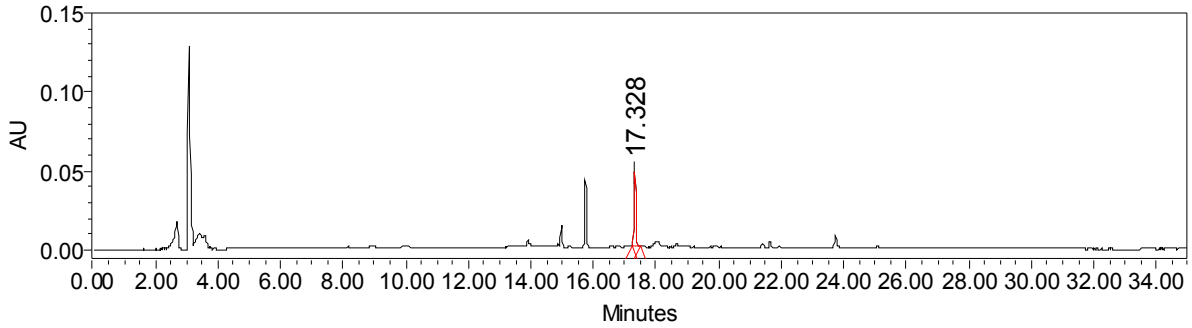

Figure 1. Calibration chromatogram for Biphalin derivatives at 200 µg/mL.

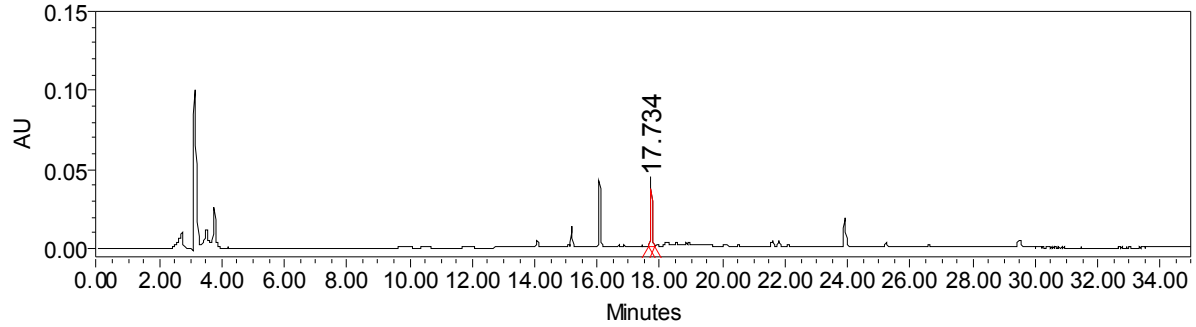

Figure 2. Calibration chromatogram for Biphalin at 200 µg/mL.

In figures 3 and 4 the chromatogram obtained analyzing real plasma samples was incubated at 37° C (± 1° C) for 45 minutes.

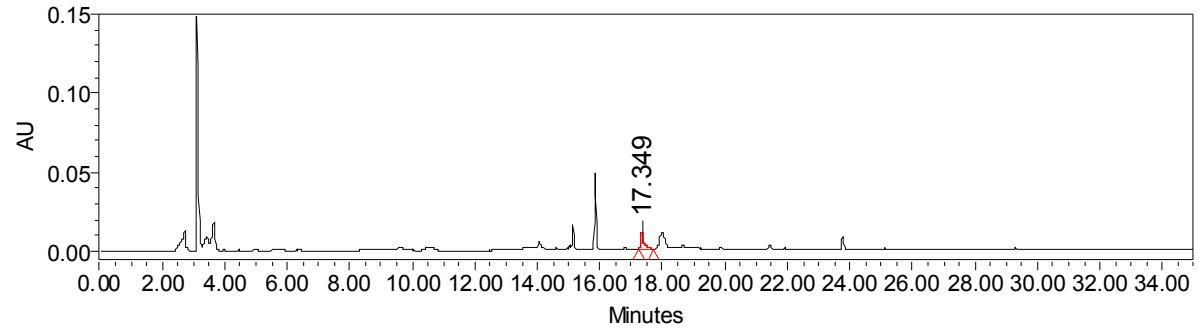

Figure 3. Chromatogram for Biphalin in incubated plasma at 37° C (± 1° C) post 45 minutes.

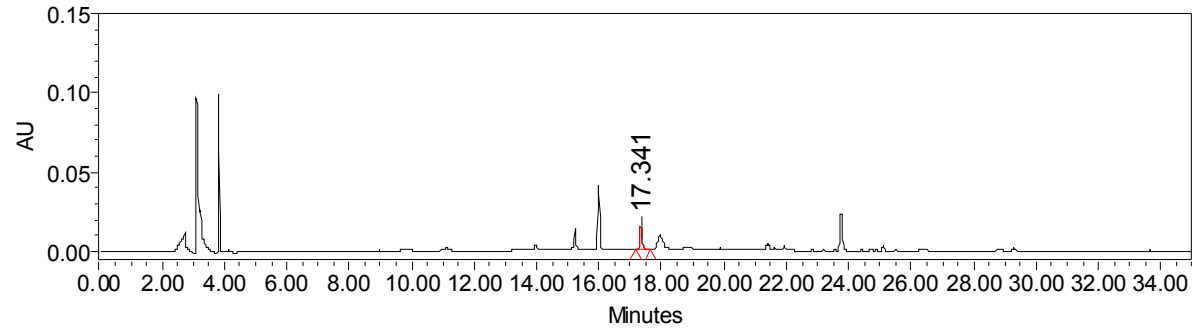

Figure 4. Chromatogram for Biphalin derivatives in incubated plasma at 37° C (± 1° C) post 45 minutes.