Supporting Information

Synthesis and Evaluation of New Endomorphin-2 Analogues Containing (Z)-α,β-Didehydrophenylalanine (Δ^{Z}Phe) Residues

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Contents:

A) Synthetic procedure for the intermediates and ^{1}H NMR data

Boc-Pro-(*β*-OH)Phe-OH (4): To a precooled solution (-10°) of Boc-Pro-OH (2 g, 9.3 mmol) in THF, NMM (9.3 mmol) and IBCF (9.3 mmol) were added. After 10 min of stirring, a solution of DL-(*β*-OH)Phe-OH (2.22g, 11.16 mmol) in 1N NaOH (11.1 ml) was added and the mixture stirred at 0°C for 2 h and at room temperature overnight. The organic solvent was removed under reduced pressure and the acqueous phase was acidified with solid citric acid to pH 3 and extracted with EtOAc (3 x 15ml). EtOAc layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give **4** as mixture of the two pure expected diastereomers (colourless oil; 82% yield).

Boc-Pro-Phe-azlactone (5): To a solution of **4** (2.89 g, 7.65 mmol) in acetic anhydride (11.7 ml) was added freshly fused sodium acetate (0.627 g, 7.65 mmol) and the mixture was left overnight at room temperature. The reaction mixture was poured over crushed ice and stirred. Then was extracted with EtOAc and organic layer was washed with 10% $NaHCO_3$, water and dried under reduced pressure. The azlactone **5** was used for preparation of **6** without further purification.

Boc-Pro-Δ^ZPhe-PheOMe (6): To a stirred solution of azlactone **5** (2.48 g, 7.24 mmol) and HCl·Phe-OMe in DCM (20 ml), DIEA (1.24 ml, 7.24 mmoL) and DMAP (0.035 g, 0.04 mmoL, 4 mol %) were added at 0 °C and the reaction mixture was stirred overnight at room temperature. Then the reaction mixture was diluted with DCM and the organic layer was washed by saturated acqueous $NaHCO_3$, water and brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give TLC pure title product **6** as white foam (86% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.41 [9H, s, $C(CH_3)_3$], 1.70-2.19 (4H, m, Pro C^3H_2 and Pro C^4H_2), 3.05-3.10 (2H, m, Phe $C^\beta H_2$), 3.56 (3H, s, $COOCH_3$), 3.4-3.6 (2H, m, Pro C^5H_2), 4.27 (1H, m, Pro

$C^{\alpha}H$), 4.54 (1H, m, Phe $C^{\alpha}H$), 7.18-7.61 (11H, m, aromatics and Δ^{Z}Phe $C^{\beta}H$), 8.02 (1H, d, *J* = 7.2 Hz, Phe NH), 9.69 (1H, s, Δ^{Z}Phe NH).

Boc-Tyr-Pro-Δ^{z}Phe-Phe-OMe (7): The Boc group of **6** was removed by treatment with TFA in DCM (1:1) for 1h at room temperature. Removal of solvent and precipitation of the residue with ether gave a quantitative yield of TFA·Pro-Δ^{Z}Phe-Phe-OMe. To an ice-cooled solution of *N*-Boc-Tyr-OH (0.924 g, 3.28 mmol) in DCM (20 ml), HOBt (0.510 g, 3.28 mmol) and EDC (0.630 g, 3.28 mmol) were added. After 10 min TFA·Pro-Δ^{Z}Phe-Phe-OMe (1.597 g, 2.98 mmol) in DCM (15 ml) and NMM (0.667 g, 6.57 mmol) were added and the reaction mixture was allowed to warm to room temperature. After 12 h the solvent was evaporated and the residue was diluted with EtOAc (20 ml) and washed with citric acid 5% (3 x 15 ml), saturated aqueous $NaHCO_3$ (3 x 15 ml) and brine (2 x 15 ml). The organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. Silica gel chromatography (DCM/MeOH 98:2) gave a pure product **7** as a white foam (40% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.3 [9H, s, $C(CH_3)_3$], 1.61-2.15 (4H, m, Pro $C^{3}H_2$ and Pro $C^{4}H_2$), 2.8-3.2 (4H, m, Phe $C^{\beta}H_2$ and Tyr $C^{\beta}H_2$), 3.3 and 3.75 (2H, m, Pro $C^{5}H_2$), 3.71 (3H, m, $COOCH_3$), 4.57 (1H, m, Pro $C^{\alpha}H$), 4.70 (1H, m, Tyr $C^{\alpha}H$), 4.89 (1H, m, Phe $C^{\alpha}H$), 5.22 (1H, d, *J* = 8.8 Hz, Tyr NH), 6.6-7.55 (16H, m, aromatics, Δ^{Z}Phe $C^{\beta}H$ and Δ^{Z}Phe NH), 7.09 (1H, d, *J* = 4.4 Hz, Phe NH).

Boc-Pro-Δ^{z}Phe-Phe-azlactone (9). Prepared as above described for compound **5**. The azlactone **9** (95% yield) was used for preparation of **10** without further purification.

Boc-Pro-Δ^{z}Phe-Δ^{z}Phe-OMe (10): To a stirred solution of azlactone **9** (0.28 g, 0.70 mmol) in MeOH (10 ml), DMAP (0.085 g, 0.70 mmol) was added at room temperature and the reaction mixture was stirred at room temperature overnight. Then the reaction mixture was acidified with

saturated aqueous citric acid to pH 4, the organic solvent was evaporated and the acqueous phase was extracted with Et_2O (3 x15 ml). Et_2O layer was washed with citric acid 5% and brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. Silica gel chromatography (DCM/EtOAc 8:2) gave a pure product **10** as a white foam (0.720 g, 60%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.31 [9H, s, $C(CH_3)_3$], 1.70-2.35 (4H, m, Pro C^3H_2 and Pro C^4H_2), 3.44 (2H, m, Pro C^5H_2), 3.88 (3H, s, $COOCH_3$), 4.30 (1H, m, Pro $C^{\alpha}H$), 7.2-7.68 (12H, m, aromatics, Δ^ZPhe^3 $C^{\beta}H$ and Δ^ZPhe^4 $C^{\beta}H$), 7.84 (1H, s, Δ^ZPhe^4 NH), 8.54 (1H, s, Δ^ZPhe^3 NH).

Boc-Tyr-Pro-Δ^ZPhe-Δ^ZPhe-OMe: Prepared as above described for compound **7**. Silica gel chromatography (DCM/EtOAc 7:3) gave a pure product as a white foam (44% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.4 [9H, s, $C(CH_3)_3$], 1.6-1.93 (4H, m, Pro C^3H_2 and Pro C^4H_2), 2.62-2.69 (2H, m, Tyr $C^{\beta}H_2$), 3.12 and 3.68 (2H, m, Pro C^5H_2), 3.85 (3H, s, $COOCH_3$), 4.44 (1H, m, Pro $C^{\alpha}H$), 4.65 (1H, m, Tyr $C^{\alpha}H$), 5.13 (1H, d, J = 8 Hz, Tyr NH), 6.5-7.63 (16H, m, aromatics, Δ^ZPhe^3 $C^{\beta}H$ and Δ^ZPhe^4 $C^{\beta}H$), 7.68 (1H, s, Δ^ZPhe^4 NH), 8.37 (1H, s, Δ^ZPhe^3 NH).

Boc-Phe-(*β*-OH)Phe-OH (12): Prepared as above described for compound **4** and obtained as pure mixture of the two expected diastereomers (quantitative yield).

Boc-Phe-Phe-azlactone (13): Prepared as above described for compound **5**. The azlactone **13** (75% yield) was used for preparation of **14** without further purification.

Boc-Phe-Δ^ZPhe-OMe (14): Prepared as above described for compound **10**. Pure on TLC (95% yield). ^{1}H NMR ($CDCl_3$): δ 1.37 [9H, s, $C(CH_3)_3$], 3.02 and 3.15 (2H, m, Phe $C^{\beta}H_2$), 3.77 (3H, s, $COOCH_3$), 4.46 (1H, m, Phe $C^{\alpha}H$), 4.93 (1H, d, J = 8.4 Hz, Phe NH), 7-7.4 (11H, m, aromatics and Δ^ZPhe^4 $C^{\beta}H$), 7.61 (1H, s, Δ^ZPhe^4 NH).

Boc-Pro-Phe-Δ^{z}Phe-OMe (15): Prepared as above described for compound **7**. Silica gel chromatography (DCM/EtOAc 9:1) gave a pure product **15** as a white foam (44% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.37 [9H, s, $C(CH_3)_3$], 1.6-2.1 (4H, m, Pro C^3H_2 and Pro C^4H_2), 3.2 (4H, m, Phe $C^{\beta}H_2$ and Pro C^5H_2), 3.83 (3H, s, $COOCH_3$), 4.14 (1H, m, Pro $C^{\alpha}H$), 4.86 (1H, m, Phe $C^{\alpha}H$), 6.67 (1H, d, *J* = 8 Hz, Phe NH), 7.19-7.51 (11H, m, aromatics and $\Delta^{Z}Phe^4$ $C^{\beta}H$), 8.25 (1H, s, $\Delta^{Z}Phe^4$ NH).

Boc-Tyr-Pro-Phe-Δ^{z}Phe-OMe: Prepared as above described for compound **7**. Silica gel chromatography (DCM/EtOAc 3:2) gave a pure product as a white foam (40% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.37 [9H, s, $C(CH_3)_3$], 1.6-1.9 (4H, m, Pro C^3H_2 and Pro C^4H_2), 2.71-3.62 (6H, m, Phe $C^{\beta}H_2$, Tyr $C^{\beta}H_2$ and Pro C^5H_2), 3.83 (3H, s, $COOCH_3$), 4.48 (1H, m, Pro $C^{\alpha}H$), 4.5 (1H, m, Tyr $C^{\alpha}H$), 4.71 (1H, m, Phe $C^{\alpha}H$), 5.10 (1H, d, *J* = 8.8 Hz, Tyr NH), 6.08 (1H, d, *J* = 7.6 Hz, Phe NH), 6.5-7.51 (15H, m, aromatics and $\Delta^{Z}Phe^4$ $C^{\beta}H$), 8.12 (1H, s, Tyr OH), 8.51 (1H, s, $\Delta^{Z}Phe^4$ NH).

B) X-ray crystallographic data for 8

Table 1S. Main backbone and side chains torsion angles (°) of the X-ray crystal structure of **8**[a]

Backbone		
O_0-C_0'-$N_1C_1^{\alpha}$	ω_0	177.5(4)
C_0'-N_1-C_1^{α}-C_1'	φ_1	-62.7(6)
N_1-C_1^{α}-C_1'-N_2	ψ_1	150.3(4)
C_1^{α}-C_1'-N_2-C_2^{α}	ω_1	175.5(5)
C_1'-N_2-C_2^{α}-C_2'	φ_2	-59.4(6)
N_2-C_2^{α}-C_2'-N_3	ψ_2	127.9(4)
C_2^{α}-C_2'-N_3-C_3^{α}	ω_2	-175.5(4)
C_2'-N_3-C_3^{α}-C_3'	φ_3	113.8(5)
N_3-C_3^{α}-C_3'-N_4	ψ_3	-14.2(7)
C_3^{α}-C_3'-N_4-C_4^{α}	ω_3	-179.3(5)
C_3'-N_4-C_4^{α}-C_4'	φ_4	-78.1(6)
N_4-C_4^{α}-C_4'-N_5	ψ_4	-19.3(6)
Side chains		
N_1-C_1^{α}-C_1^{β}-C_1^{γ} (Tyr)	$\chi_1^{1,1}$	-156.2(4)
N_2-C_2^{α}-C_2^{β}-C_2^{γ} (Pro)	$\chi_2^{1,1}$	18.2(6)
N_3-C_3^{α}-C_3^{β}-C_3 (Δ^ZPhe)	$\chi_3^{1,1}$	-8.0(11)
N_4-C_4^{α}-C_4^{β}-C_4^{γ} (Phe)	$\chi_4^{1,1}$	-72.6(5)

[a] Numbers in parenthesis are e.s.d. values.

Crystal Data

$C_{37}H_{43}N_5O_7$, FW = 669.76. Monoclinic, space group $P2_1$, a = 13.401(4), b = 8.9611(15), c = 15.027(4) Å, β = 103.31(3)°, V = 1756.0(8) Å^3, Z = 2, ρ_{calc} = 1.267 Mg m^{-3}, λ = Mo $K\alpha$, μ = 0.09 mm^{-1}.

Data Collection and Refinement

A clear colourless 0.15x0.10x0.10 mm crystal was mounted on an Oxford Diffraction Xcalibur diffractometer equipped with a CCD detector. Lattice parameters were obtained using CrysAlis CCD from 1162 reflections in the range 3 < θ < 29°. 2785 reflections (1959 independent) were collected at room temperature and corrected for Lorentz, polarization and absorption effects. The structure was solved with SIR97 and refined with the aid of SHELXL-97. In the final full-matrix least-squares calculations 455 parameters were refined. H atoms were included using a riding model [C-H bond distances in the range 0.93 - 0.97 Å, U_{iso}(H) equal to 1.2 or 1.5 U_{eq}(C)]. Final

residuals were $R1 = 0.036$ for the 1372 reflections having $F_o > 4\sigma(F_o)$ and 0.060 for all data. Final difference Fourier excursions were 0.10 and -0.12 eÅ^3.

CCDC 747923 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

C) C, H, N analysis of final products (table)

Compound	Molecular formula	Calculated	Found
1	$C_{34}H_{36}F_3N_5O_7$	C: 59.73 H: 5.31 N: 10.24	C: 59.96 H: 5.33 N: 10.28
2	$C_{34}H_{36}F_3N_5O_7$	C: 59.73 H: 5.31 N: 10.24	C: 59.52 H: 5.28 N: 10.19
3	$C_{34}H_{34}F_3N_5O_7$	C: 59.91 H: 5.03 N: 10.27	C: 60.03 H: 5.01 N: 10.22