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Opioid peptides: synthesis and biological properties of [(N^{γ}-glucosyl,N^{γ}-methoxy)-α,γ-diamino-(*S*)-butanoyl]^4-deltorphin-1-neoglycopeptide and related analogues †

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The [D-Ala^2]deltorphin I sequence in which the aspartic acid residue is replaced by the N$^{\gamma}$-OCH$_3$-α,γ-diamino (*S*) butanoyl residue was synthesized using the Fmoc-chemistry-based solid phase procedure. The resulting deltorphin analogue was chemoselectively glucosylated by reaction with unprotected D-glucose (Glc). The Asn^4-, (2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-Asn^4- and the (2-acetamido-2-deoxy-D-galactopyranosyl)-Asn^4-deltorphin I were also prepared for comparison. The affinity of the new compounds for the δ-opioid receptor was expressed by the inhibition constant (K_i) of the binding of the δ-receptor selective ligand [^{3}H]naltrindole (NTI) to rat brain membrane preparations. The *in vitro* biological activity of the synthetic peptides was compared with that of the μ-opioid receptor agonist dermorphin in guinea pig ileum (GPI) preparations and with that of the δ-opioid receptor agonist deltorphin I in mouse vas deferens (MVD) preparations. The substitution of Asp^4 with Asn failed to affect drastically the K_i and IC_{50} values for δ-sites, suggesting that an electrostatic interaction does not play an essential role in the binding to δ-opioid sites. The steric hindrance of the side chain of the residue in position 4 affects binding to δ-sites. The increase of the K_i value is smaller when the sugar-peptide linkage involves the γ-nitrogen of the Dab residue in comparison with the Asn amide side chain.

Introduction

Deltorphins are opioid heptapeptides isolated from frog skin and are highly selective ligands at δ-opioid receptors.[1] When injected into the brain of mice and rats, deltorphin I and deltorphin II produce analgesia,[2,3] locomotor stimulation[4] and motivational rewarding[5] without development of physical dependence[6] or respiratory depression.[7] It is known that the use of peptides as therapeutic agents meets with some severe limitations. Intensive efforts have been made in recent years to develop peptidomimetics displaying pharmacological properties more favorable than the prototypes with regard to specificity of action, resistence towards enzymatic degradation, pharmacokinetics and bioavailability. Glycosylation of peptides and other potential therapeutic agents is a promising approach in rational drug design and a number of elegant approaches for glycosylation of bioactive peptides have been described. The synthesis of glycopeptides and neoglycopeptides has been extensively reviewed and excellent articles and reviews summarize the development in the solution and solid phase methodologies.[8–16]

A new type of chemoselective glycosylation by reaction of unprotected reducing sugars with any derivatized peptide containing a *N*,*O*-di-substituted hydroxylamine side chain function (R_1–NH–O–R_2) has been described.[17,18]

In previous papers[19,20] we described the synthesis of some dermorphin and deltorphin analogues β-*O*- and α-*C*-glycosylated on the C-terminal amino acid residue and reported their opioid receptor affinity and their analgesic potency after subcutaneous injection in mice. We also reported[21,22] the development of the oxyamino derivatives of α,β-diamino-(*S*)-propanoic acid and α,γ-diamino-(*S*)-butanoic acid (Dab) for the preparation of analogues of *O*-glycosylated serine, or homoserine (Hse), containing peptides.

In particular we described the solution synthesis of the model neoglycopeptides N^{α}-Boc,N^{β}-(β-D-Glc)-N^{β}-*O*Bzl-α,β-diamino-(*S*)-propanoylserine methyl ester[22] and of N^{α}-Fmoc-phenylalanyl-N^{γ}–(β-D-Glc)-N^{γ}-OMe-α,β-diamino-(*S*)-butanoylvaline,[23] which corresponds to the 3–5 sequence of deltorphin I in which Asp 4 was replaced by the N^{γ}-glycosylated Dab residue. The preparation of another model neoglycopeptide by the chemoselective ligation methodology based on the oxyamino chemistry has also been recently described.[24] This paper will focus on the synthesis of N^{α}–Fmoc,N^{γ}-Boc,N^{γ}-OCH$_3$-α,γ-diamino-(*S*)-butanoic acid (**7**) from homoserine and its incorporation into the [D-Ala^2]deltorphin I sequence (position 4) by using the Fmoc-chemistry-based solid phase peptide synthesis. Chemoselective glucosylation[17] of the resulting deltorphin analogue **C** yielded the [N^{γ}-OCH$_3$,N^{γ}-(βGlc)-Dab^4]-deltorphin I (**F**). The Asn^4-deltorphin I (**B**), the (2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-Asn^4-deltorphin I (**D**), and the (2-acetamido-2-deoxy-β-D-glucopyranosyl)-Asn^4-deltorphin I (**E**) were also prepared for comparison. The amino acid sequences of the synthetic deltorphin I analogues are shown in Table 1.

Table 1 Deltorphin I analogues

A	Tyr–D–Ala–Phe–Asp–Val–Val–Gly–NH_2
B	Tyr–D–Ala–Phe–Asn-Val–Val–Gly–NH_2
C	Tyr–D–Ala–Phe–(N^{γ}–OCH$_3$)Dab–Val–Val–Gly–NH_2
D	Tyr–D–Ala–Phe–[β-GlcNAc(Ac)$_3$]Asn–Val–Val–Gly–NH_2
E	Tyr–D–Ala–Phe–(β-GlcNAc)Asn–Val–Val–Gly–NH_2
F	Tyr–D–Ala–Phe–[N^{γ}–OCH$_3$, N^{γ}-(β-Glc)–]Dab–Val–Val–Gly–NH_2

† With the exception of D-Ala the amino acid residues are of L-configuration. Standard abbreviations for amino acid derivatives and peptides are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature, *Eur. J. Biochem.*, 1984, **138**, 9–37 and of *J. Pept. Sci.* 2003, **9**, 1–8.

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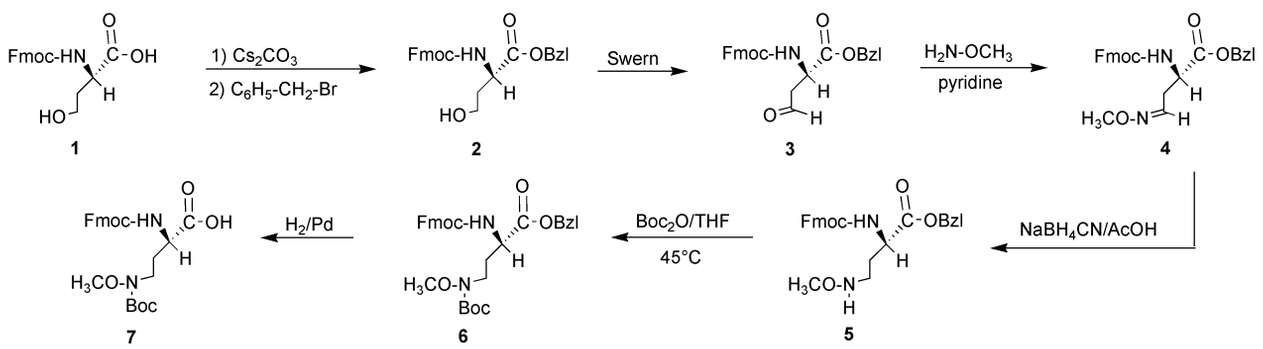
Table 2 δ-Opioid receptors affinities and biological activities of synthetic peptides

Peptide	K_i,δ/nM, [^{3}H]NTL	GPI (IC_{50}/nM)	MVD (IC_{50}/nM)
Deltorphin, **A**	4.7 ± 0.6	—	1.9 ± 0.08
[Asn^4]-deltorphin, **B**	44.6 ± 5.2	4688 ± 410	16.9 ± 1.23
[N^{γ}-(OCH_3)-Dab^4]-deltorphin, **C**	113 ± 10.3	5102 ± 360	38 ± 0.29
[β-GlcNAc($Ac)_3$-Asn^4]-deltorphin, **D**	1474 ± 130	4557 ± 320	1321 ± 98
[β-GlcNAc-Asn^4]-deltorphin, **E**	1120 ± 105	18535 ± 1500	329 ± 27
[N^{γ}-OCH_3,N^{γ}-(β-Glc)-Dab^4]-deltorphin, **F**	280.2 ± 23	31790 ± 3200	89 ± 6.5

K_i, dissociation constant; the values are the mean of three experiments ± SEM. δ-Opioid receptors were labeled with [^{3}H]NLT, 0.1 nM; IC_{50} agonist concentration that produced 50% inhibition of the electrically evoked twitch; the values are the mean of not less than six experiments ± SEM.



Scheme 1 Synthesis of N^{α}–Fmoc,N^{γ}–Boc,N^{γ}–OCH_3–Dab–OH.

Results and discussion

Peptide synthesis

The amino acid derivative containing the *O*-methyl hydroxylamine side chain function, used for preparing the deltorphin analogue **C**, was synthesized as depicted in Scheme 1. Fmoc-Hse-OH (**1**) was converted into the corresponding benzyl ester (**2**) and was oxidized to benzyl 2-fluorenylmethyloxycarbonyl-3-formylpropanoate (**3**) by the Swern procedure.[25] Reaction of **3** with *O*-methyl hydroxylamine, followed by reduction, *tert*-butyloxycarbonylation and catalytic hydrogenation yielded the N^{α}–Fmoc-N^{γ}-Boc-N^{γ}-OCH_3-Dab-OH (**7**) which was used for peptide elongation by the solid phase procedure. Cleavage of the heptapeptide from the resin and simultaneous removal of the Boc group were carried out by treatment with aqueous 95% trifluoroacetic acid (TFA). The crude peptide **C** was glycosylated[17] and purified by semipreparative HPLC yielding **F**. Deltorphin analogues **A**, **B**, and **D** were also prepared by the solid phase procedure. Fmoc-[β-GlcNAc($Ac)_3$]-Asn-OH was used for preparing peptide **D** which was converted into the (β-GlcNAc)-Asn^4-deltorphin I (**E**) by treatment with hydrazine hydrate. Analytical characterization of the deltorphin analogues was carried out by reverse phase HPLC, amino acid analysis and mass spectrometry.

Pharmacological tests

The *in vitro* biological activity of the synthesized peptides was tested in two isolated smooth muscle preparations: guinea pig ileum (GPI) and mouse vas deferens (MVD) rich in μ- and δ-opioid receptors, respectively.[26] δ-Opioid receptor affinities are expressed by the inhibition constant (K_i) of the binding of selective radio ligands to mice brain membrane preparations. The opioid receptor binding sites were selectively labelled using [^{3}H]naltrindole (NTI) (Table 2). A previous investigation[26] showed that the *N*-terminal tripeptide appears to be essential for the best fitting of both deltorphin and dermorphin to their respective receptor sites and that the C-terminal tripeptide sequence of [D-Ala^2]-deltorphins appears to be responsible for their high δ-opioid receptor affinity. Negatively charged side chains of Asp^4 (or Glu^4) could be involved in an electrostatic ligand repulsion with a negatively charged μ-receptor site or with a negatively charged membrane compartment that contains μ-receptors. However, since the substitution of Asp^4 with Asn failed to affect drastically the K_i and IC_{50} values for δ-sites, such electrostatic interactions apparently do not play an essential role in the binding to δ-opioid sites. In addition to charge, changes in the steric hindrance might affect binding and peptides **C, D, E** and **F** show a significant increase in the K_i and IC_{50} values for δ-sites in comparison with **A**. The increase in the K_i value is smaller when the sugar-peptide linkage involves the γ-nitrogen of the Dab residue (peptide **F**) in comparison with the Asn amide side chain (peptide **E**).

Experimental

General methods

All chemicals were commercial products of the best grade available. TFA, *N*-(3-dimethylaminoisopropyl)-*N*-ethylcarbodiimide (EDC) and *N*-methylpyrrolidone (NMP) were Fluka products; acetonitrile (HPLC grade) was supplied by Carlo Erba. Rink amide MBHA resin, [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamidonorleucyl-4-methyl-4-benzhydrylamine polystyrene] and Fmoc-[β-GlcNAc($Ac)_3$]Asn-OH were obtained from Novabiochem. All other chemicals for the solid phase synthesis were supplied by Advance Chemtech. Melting points were taken on a Buchi model 150 melting point apparatus in open capillaries and are not corrected. Optical rotations were determined, at 25 °C, with a Perkin Elmer model 241 polarimeter and $[\alpha]_D$ values are given in 10^{-1}deg cm^2 g^{-1}. Amino acid analyses were made with a Carlo Erba model 3A 29 amino acid analyzer equipped with a Perkin Elmer Signa 10 chromatography data station following hydrolysis for 22 h (46 h for Val) at 110 °C, in sealed, evacuated vials in constant boiling hydrochloric acid. Ascending thin-layer chromatographies were routinely performed on TLC plates Silica Gel 60, UV_{254}, Machery-Nagel, using the following solvent systems: E1: butan-1-ol–acetic acid–water–acetonitrile (3 : 1 : 1 : 5 by vol.); E2: dichloromethane–methanol (25 : 1 v/v); E3: light petroleum–ethyl acetate (2 : 1 v/v); E4: light petroleum : ethyl acetate (1 : 1 v/v); E5: ethyl acetate–butan-1-ol–acetic acid–water (15 : 3 : 1 : 1 by vol.). Amino acid derivatives and peptides were visualized by one or more of the following procedures: ninhydrin, 4,4′-tetramethyldiphenylmethane (TDM) reagent[27] and UV light. Sugars and sugar-containing products were

located by spraying the plates with 10% sulfuric acid in ethanol, followed by heating for 10 min at 100 °C. Low pressure gel chromatographies (Silica Gel 60, 0.040–0.063 mm, Machery-Nagel, column 260 × 26 mm, flow rate 10 cm^3 min^{-1}, fraction volume 30 cm^3) were performed with a Buchi 688 chromatographic pump equipped with a Buchi UV/VIS filter photometer (254 nm) detector (see Experimental for elution conditions). Analytical HPLC separations were performed on an Aquapore RP 300 column (220 × 4.6 mm, 7 μm, Brownlee Labs., flow rate 1.5 cm^3 min^{-1}) using a Perkin Elmer series 3B liquid chromatograph equipped with a LC-90 UV detector and LCI-100 integrator. Eluents A (0.1% TFA in 90% aqueous acetonitrile) and B (0.1% aqueous TFA) were used for preparing binary gradients (elution conditions: isocratic 10% A for 2 min, linear gradient 10–90% A in 30 min). TFA-Containing eluents induced progressive de-glycosylation of peptide **F**. To prevent this inconvenience, TFA was replaced by acetic acid in the eluents used for HPLC separations (eluent A′ and B′). Semi-preparative HPLC separations (Vydac 300 column, 250 × 20 mm, 10 μm, flow rate 15 cm^3 min^{-1}) was performed on a Shimadzu series SLC-6B chromatograph equipped with two independent pump units model LC-8, a SPD-6A detector and a C-R6A integrator (eluents and elution conditions as those used for the analytical separations). Solvents were dried and freshly distilled and evaporations were carried out under reduced pressure at 40–45 °C, using a rotary evaporator. Sodium sulfate was used for drying purposes. Yields are based on the weight of vacuum-dried products.

Proton NMR spectra at 200 and 250 MHz were recorded at 298 K, unless stated otherwise, on Bruker spectrometers (WP 200 SY and AC-250-F, respectively). Sample concentrations were in the range 8–10 mg cm^{-3} in $CDCl_3$ or DMSO-d_6 (99.996%). Chemical shifts (δ) are expressed relative to the residual signals at 7.26 ppm in $CDCl_3$, and 2.49 ppm in DMSO-d_6. Proton assignements were determined by selective homo-spin decoupling. Electrospray ionization mass spectrometry (ESI-MS) was performed on a PerSeptive Biosystem Mariner instrument. Ionization potential 4200 V, acceleration potential 100 V.

Chemical synthesis

Assembly of peptides **A–D** on the Advance Chemtech 348Ω Peptide Synthesizer was performed on a 0.06 mmol scale, by the FastMoc methodology (HBTU–HOBt–DIEA, single acylation protocol, 180 min coupling time, NMP as the solvent), starting with Rink Amide MBHA resin (0.085 g, substitution 0,73 mmol g^{-1}). The final peptide resin was N^{α}-deprotected with 20% piperidine in NMP, thoroughly washed with NMP and dried. Cleavage from the resin and removal of the side chain protecting groups were simultaneously achieved on the peptide resin by treatment with a mixture of trifluoroacetic acid–water–triisopropylsilane (95: 2.5 : 2.5 by vol) (10 cm^3, 3.5 h at room temperature). The acid solution was concentrated *in vacuo* and the peptide was precipitated twice with excess *tert*-butylmethyl ether, collected, dried and lyophylized from water. Peptide **E** was obtained from peptide **D** by deacetylation of the sugar moiety and peptide **C** was converted into **F** by selective glycosylation.[17] All products were characterized by reverse phase analytical HPLC (elution: isocratic 15% A for 3 min, linear gradient 15–45% A in 25 min), amino acid composition and molecular weight determination.

Fmoc-Hse-OH, 1

Fmoc-OSu (5.7 g, 16.9 mmol) was dissolved in acetonitrile (40 cm^3) and added to a stirred solution of Hse (2.0 g, 16.8 mmol) in aqueous 5% sodium hydrogen carbonate (55 cm^3) (TLC monitoring in E1). The organic solvent was evaporated *in vacuo* and the mixture was diluted with aqueous 5% sodium hydrogen carbonate (50 cm^3) and extracted with ethyl acetate (3 × 60 cm^3). The pH of the aqueous layer was adjusted to 2–3 with 1 M hydrochloric acid and re-extracted with ethyl acetate (3 × 50 cm^3). The organic extracts were combined, washed with saturated aqueous sodium chloride, dried and evaporated to dryness. The residue was dissolved in the minimum amount of ethyl acetate and precipitated with light petroleum. Yield 4.9 g (85%), single spot by TLC in E1, mp 124–126 °C; $[\alpha]_D$ −19.6 (*c* 1.0, ethanol). Found: C, 67.06; H, 5.87; N, 4.11. $C_{19}H_{19}NO_5$ requires: C, 66.85; H, 5.61; N, 4.12%. ^{1}H NMR (250 MHz, DMSO-d_6): 1.94–1.62 (m, 2H, βCH_2 Hse); 3.55–3.35 (m, 2H, γCH_2 Hse); 4.14–4.02 (m, 1H, αCH Hse); 4.35–4.15 (m, 3H, CH–CH_2 Fmoc); 7.48–7.25 (m, 4H aromatics Fmoc); 7.61 (d, 1H, NH Hse, *J* 8.0 Hz); 7.92–7.67 (m, 4H, aromatics Fmoc). ^{1}H NMR (250 MHz, $CDCl_3$, at 50 °C owing to the low solubility of the compound): 2.17 (m, 2H, βCH_2 Hse); 3.76 (m, 2H, γCH_2 Hse); 4.22 (t, CH Fmoc, *J* 6.8 Hz); 4.70–4.43 (m, 3H, αCH Hse and CH_2 Fmoc); 5.77 (d, 1H, NH Hse, *J* 7.3 Hz); 7.80–7.27 (m, 8H, aromatics Fmoc). Lactone formation, evidentiated by the NMR spectra, occurs slowly on standing in cloroform solution: 58% after 32 h and 95% after 197 h.

Fmoc-Hse-OBzl, 2

An aqueous 20% solution of cesium carbonate (1.7 cm^3, 0.34 g, 1.04 mmol) was added to Fmoc-Hse-OH (0.50 g, 1.46 mmol) dissolved in a mixture of methanol (6.2 cm^3) and water (0.6 cm^3). The solvent was removed and the reaction mixture was taken up (twice) with *N*,*N*-dimethylformamide (DMF)(3.7 cm^3) and evaporated to dryness. The cesium salt was taken up once more with DMF (3.7 cm^3) and benzyl bromide (0.2 cm^3, 1.7 mmol) was added. After 6 h reaction the solvent was evaporated *in vacuo* and the residue taken up with water (25 cm^3) and extracted with ethyl acetate (2 × 25 cm^3). The organic layers were combined, dried for less than one hour to prevent lactone formation, and concentrated to a small volume. Addition of light petroleum gave a precipitate which was collected and dried. Yield 0.3 g (48%); single spot by TLC in E2; mp 107–110 °C; $[\alpha]_D$ −6.2 (*c* 0.97, chloroform). Found: C, 72.06; H, 5.87; N, 3.11. $C_{26}H_{25}NO_5$ requires: C, 72.37; H, 5.84; N, 3.25%. ^{1}H NMR (200 MHz, $CDCl_3$): 2.25–2.10 (m, 2H, βCH_2 Hse); 3.80–3.45 (m, 2H, γCH_2 Hse); 4.30–4.10 (t, CH Fmoc, *J* 6.7 Hz); 4.70–4.30 (m, 3H, αCH Hse and CH_2 Fmoc); 5.20 (s, 2H, CH_2 benzyl); 5.68 (d, 1H, NH Hse, *J* 7.7 Hz); 7.80–7.20 (m, 13H, aromatics Fmoc and benzyl). Lactone formation, evidentiated by the NMR spectra, occurs on standing in cloroform solution: 50% after 6 h.

N-Fmoc-(2-amino-3-formyl)propanoate-*O*Bzl, 3

Anhydrous dimethylsulfoxide (0.62 cm^3) was diluted with anhydrous dichloromethane (DCM) (2.0 cm^3) and added in 25 min to a solution of oxalyl chloride (0.37 cm^3, 4.37 mmol) in anhydrous DCM (7.0 cm^3), previously cooled at −78 °C and kept under nitrogen. The temperature of the reaction mixture was raised to −60 °C, in 20 min, and a solution of Fmoc-Hse-OBzl (1.25 g, 2.9 mmol) in anhydrous DCM (5.0 cm^3) was slowly added (50 min). The temperature was adjusted to −45 °C in 30 min. *N*,*N*-diisopropylethylamine (3.0 cm^3) and anhydrous DCM (0.5 cm^3) were slowly added and the temperature was raised to 0 °C. Precooled (0 °C) 1 M hydrochloric acid (12 cm^3) was added, two phases separated and the aqueous phase was extracted with DCM (3 × 8 cm^3). The combined organic layers were washed with a phosphate buffer (pH 7.0, 3 × 15 cm^3), dried and evaporated to dryness. The resulting oily residue (1.2 g) was purified by gel chromatography (eluant: *n*-hexane–diethyl ether 2 : 1 v/v) . Yield 0.78 g (63%) single spot by TLC in E3; mp 100–101 °C; $[\alpha]_D$ +9.3 (*c* 1.15, chloroform). Found: C, 72.04; H, 5.77; N, 3.11. $C_{26}H_{23}NO_5$ requires: C, 72.71; H, 5.40; N, 3.26%. ^{1}H NMR (200 MHz, $CDCl_3$,): 3.20–3.10 (m, 2H, βCH_2 Hse); 4.20 (t, 1H, CH Fmoc, *J* 6.9 Hz); 4.40 (m, 2H, CH_2 Fmoc); 4.65 (m, 1H, αCH); 5.20 (s, 2H, CH_2 benzyl); 5.72 (d,

1H, NH, *J* 8.0 Hz); 7.85–7.20 (m, 13H, aromatics Fmoc and benzyl); 9.7 (s, 1H, H–C=O).

Benzyl N^{α}-Fmoc-N^{γ}-(methoxy)imino-α-amino-L-butanoate, 4

O-Methyl hydroxylamine hydrochloride (0.57 g, 6.84 mmol), which is scarcely soluble in pyridine, was slowly added, in 5 hours, to a pyridine solution (11 cm^3) containing benzyl *N*-Fmoc-(2-amino-3-formyl)propanoate (2.88 g, 6.71 mmol). Molecular sieves (4 Å) were added, the reaction mixture was stirred overnight and evaporated to dryness. The residue was taken up with ether (50 cm^3), washed with water (2 × 40 cm^3), 0.1 M potassium hydrogen sulfate (3 × 35 cm^3) and saturated aqueous sodium chloride and dried . Evaporation of the solvent yielded the title product (2.91 g, 95%); single spot by TLC in E3; mp 80–81 °C; $[\alpha]_D$ +4.3 (*c* 0.97, chloroform). Found: C, 70.32; H, 5.87; N, 5.98. $C_{27}H_{26}N_2O_5$ requires: C, 70.73; H, 5.71; N, 6.11%. ^{1}H NMR (200 MHz, $CDCl_3$,): 2.80 (m, 2H, βCH_2 Hse); 3.78 (d, 3H, OCH_3); 4.70–4.10 (m, 4H, αCH, CH–CH_2 Fmoc); 5.20 (s, 2H, CH_2 benzyl); 5.50 (dd, 1H, NH); 6.65 (m, 1H, H-vinyl); 7.85–7.20 (m, 13H, aromatics Fmoc and benzyl).

Benzyl N^{α}-Fmoc-N^{γ}-methoxy-α,γ-diamino-L-butanoate, 5

Sodium cyanoborohydride (0.69 g, 10.98 mmol) was slowly added, in portions, to a solution of **4** (2.90 g, 6.33 mmol) in glacial acetic acid (40 cm^3). The reaction was monitored by TLC in E3 and after 3 h the mixture was diluted with water (20 cm^3), the pH value was adjusted to 8.0 with 1.0 M acetic acid and the solution was extracted with ethyl acetate (3 × 40 cm^3). The combined organic layers were washed with 0.1 M potassium hydrogen sulfate (40 cm^3), 5% sodium hydrogen carbonate (40 cm^3) and saturated aqueous sodium chloride, dried and evaporated to dryness. Yield 2.90 g (99%); single spot by TLC in E3; mp 79–80 °C. Found: C, 70.06; H, 6.07; N, 6.14. C_{27} H_{28} N_2O_5 requires: C, 70.42; H, 6.13; N, 6.08%. ^{1}H NMR (250 MHz, $CDCl_3$,): 2.31–1.81 (m, 2H, βCH_2 Hse); 3.14–2.85 (m, 2H, γCH_2); 3.60 (s, 3H, OCH_3); 4.21 (t, 1H, CH Fmoc, *J* 7.7 Hz); 4.40 (d, 2H, CH_2 Fmoc); 4.47–4.45 (m, 1H, αCH); 5.19 (s, 2H, CH_2 benzyl); 5.96 (d, 1H, NH Fmoc, *J* 7.5 Hz); 7.81–7.27 (m, 8H, aromatics Fmoc).

Benzyl N^{α}-Fmoc-N^{γ}-Boc-N^{γ}-methoxy-α,γ-diamino-L-butanoate, 6

Di-*tert*-butyl dicarbonate (1.37 g, 6.28 mmol) was added, at 40°, to a solution of **5** (2.89 g, 6.28 mmol) in tetrahydrofuran (25 cm^3) and the reaction was monitored by TLC in E3. Further di-*tert*-butyl dicarbonate (1.37 g, 6.28 mmol) was added twice, after 4.5 h and 6.5 h, respectively, and the reaction mixture was stirred overnight at 45 °C. The solvent was evaporated *in vacuo* and the residue was taken up with ethyl acetate (50 cm^3) and washed with 0.1 M potassium hydrogen sulfate (2 × 40 cm^3), 5% sodium hydrogen carbonate (2 × 40 cm^3) and saturated aqueous sodium chloride, dried and evaporated to dryness. The crude product was purified by chromatography on silica gel (260 × 26 mm column, *n*-hexane/diethyl ether 3:1 v/v as the eluent). Yield 2.97 g (84%, oil); single spot by TLC in E4; $[\alpha]_D$ −0.9° (c 1.11, chloroform). Found: C, 68.06; H, 6.77; N, 4.81. $C_{32}H_{36}N_2O_7$ requires: C, 68.55; H, 6.47; N, 4.99. ^{1}H NMR (250 MHz, $CDCl_3$,): 1.48 (s, 9H, Boc); 2.27–1.94 (m, 2H, βCH_2 Hse); 3.67–3.40 (m, 2H, γCH_2); 3.64 (s, 3H, OCH_3); 4.22 (t, 1H, CH Fmoc, *J* 7.1 Hz); 4.53–4.28 (m, 3H, CH_2 Fmoc, αCH); 5.18 (s, 2H, CH_2 benzyl); 5.57 (d, 1H, NH Fmoc, *J* 8.4 Hz); 7.81–7.20 (m, 8H, aromatics Fmoc).

N^{α}-Fmoc-N^{γ}-Boc-N^{γ}-methoxy-α,γ-diamino-L-butanoic acid, 7

Benzyl N^{α}-Fmoc-N^{γ}-Boc-N^{γ}-methoxy-α,γ-diamino-L-butanoate (1.96 g, 3.5 mmol) was dissolved in methanol (20 cm^3), previously flushed with nitrogen, and catalytically hydrogenated over 10% Pd/C . The reaction was monitored by TLC in E5 and after 90 min the catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was taken up with ethyl acetate (70 cm^3) and washed with 5% sodium hydrogen carbonate (5 × 40 cm^3). The aqueous phases were combined, acidified to pH ~1.0 by adding solid potassium hydrogen sulfate and re-extracted with ethyl acetate (2 × 40 cm^3). The organic layers were washed with saturated aqueous sodium chloride, dried and evaporated to dryness. Yield 1.47 g (89%); mp 49–50 °C; single spot by TLC in E5; $[\alpha]_D$ +10.9 (*c* 1.07, chloroform). Found: C, 63.66; H, 6.27; N, 5.85. $C_{25}H_{30}N_2O_7$ requires: C, 63.82; H, 6.43; N, 5.95%. ^{1}H NMR (250 MHz, $CDCl_3$,): 1.49 (s, 9H, Boc); 2.23–2.05 (m, 2H, βCH_2 Hse); 3.76–3.45 (m, 2H, γCH_2); 3.69 (s, 3H, OCH_3); 4.22 (t, 1H, CH Fmoc, *J* 6.9 Hz); 4.51–4.30 (m, 3H, αCH, CH_2 Fmoc,); 5.70 (d, 1H, NH Fmoc, *J* 8.0 Hz); 7.81–7.24 (m, 8H, aromatics Fmoc).

H–Tyr–D–Ala–Phe–Asp–Val–Val–Gly–NH_2, A. Yield: 34 mg (74%); $[M + H]^+$ found 769.3799; $C_{37}H_{52}N_8O_{10}$ requires 768.87. Amino acid ratios: Asp, 1.00; Gly, 1.01; Ala, 0.97; Val, 1.89; Tyr, 0.97; Phe, 0.98.

H–Tyr–D–Ala–Phe–Asn-Val–Val–Gly–NH_2, B. Yield: 31 mg (67%), $[M + H]^+$ found 768.4906; $C_{37}H_{53}N_9O_9$ requires 767.89. Amino acid ratios: Asp, 0.98; Gly, 1.00; Ala, 0.99; Val, 1.85; Tyr, 0.98; Phe, 1.00.

H–Tyr–D–Ala–Phe–(N^{γ}–OCH_3)Dab–Val–Val–Gly–NH_2, C. Yield: 40 mg (85%), $[M + H]^+$ found 784.4928; $C_{38}H_{57}N_9O_9$ requires 783.94. Amino acid ratios: Gly, 0.98; Ala, 0.98; Val, 1.87; Tyr, 0.97; Phe, 1.02, Dab, not determined.

H–Tyr–D–Ala–Phe–[β-GlcNAc$(Ac)_3$]Asn–Val–Val–Gly–NH_2, D. Yield: 57 mg (86%), $[M + H]^+$ found 1097.58; $C_{51}H_{71}N_{10}O_{17}$ requires 1096.18. Amino acid ratios: Asp, 0.99; Gly, 1.02; Ala, 0.99; Val, 1.91; Tyr, 0.99; Phe, 1.02.

H–Tyr–D–Ala–Phe–(β-GlcNAc)Asn–Val–Val–Gly–NH_2, E. Hydrazine hydrate (0.05 cm^3, 1.029 mmol) was added to a methanolic solution (2 cm^3) of **D** (50 mg, 0.046 mmol). Further hydrazine was added in portions (0.03 cm^3, 0.617 mmol each) after 5, 20, 30, 45 and 50 h, and the removal of the acetyl protecting groups was monitored by analytical HPLC (elution: isocratic 10% A for 2 min, linear gradient 10–90% A in 30 min.). After 52 h the reaction mixture was diluted with diethyl ether (15 cm^3) and the resulting precipitate was collected by centrifugation, washed with diethyl ether (15 cm^3), dissolved in water and lyophilized. Yield 39 mg (87%); single peak by analytical HPLC; $[M + H]^+$ found 971.4353; $C_{45}H_{65}N_{10}O_{14}$ requires 970.07. Amino acid ratios: Asp, 0.97; Gly, 1.00; Ala, 0.99; Val, 1.94; Tyr, 0.98; Phe, 1.01.

H–Tyr–D–Ala–Phe–(N^{γ}–OCH_3, N^{γ}–β-Glc)Dab–Val–Val–Gly–NH_2, F. The heptapeptide analogue **C** (18 mg, 0.023 mmol) was dissolved in 2 cm^3 of DMF–acetic acid mixture (1 : 1 v/v) and D-glucose (5 mg, 0.03 mmol) was added. The solution was stirred at 45 °C and the reaction was monitored by analytical HPLC (elution: isocratic 15% A′ for 3 min, linear gradient 15–45% A′ in 25 min). Further D-glucose (5 mg, 0.03 mmol) was added after 3.5 h and the reaction mixture was kept under stirring for 6 h. The solvent was removed and the crude product was dissolved in a water–acetonitrile mixture (8 : 2 v/v) and purified by semipreparative HPLC (eluent: isocratic 25% A′ for 5 min, linear gradient 25–50% A′ in 25 min). Lyophilization of the product containing peak yielded 5 mg (23%) of a white product; single peak by analytical HPLC; $[M + H]^+$ found 946.51; $C_{44}H_{67}N_9O_{14}$ requires 945.96. Amino acid ratios: Asp, 0.99; Gly, 0.98; Ala, 1.01; Val, 1.92; Tyr, 0.98; Phe, 1.00.

Receptor binding assays

The binding of deltorphin analogues to δ-opioid receptors was tested on crude membrane preparations from adult male rats (Wistar, 250–300 g) as previously described.[26] The brains were homogenized in 50 volumes of Tris-HCl buffer (50 mM, pH 7.4, 4 °C) using a Kinematica PT 3000 polytron (20 s, speed 16000 rpm). The homogenate was centrifuged at 41000 × g for 20 min at 4 °C, pellets were resuspended in buffer (50 volumes) and incubated at 25 °C for 30 min to remove endogenous opioids. After centrifugation, pellets were resuspended in buffer, containing 5% glycerol, to give a final w/v of 20 mg cm^{-3} fresh tissue. The affinity to opioid receptors was determined by competition of unlabelled compounds with the δ-receptor selective ligand [^{3}H]NTI (0.1 nM; 35 Ci $mmol^{-1}$). Drugs were assayed in a final volume of 2 cm^3 Tris-HCl buffer (50 mM, pH 7.4), at 35 °C for 90 min. All experiments were performed in triplicate and total binding, nonspecific binding (2.5 μM [^{3}H]NTI) and 14 inhibitor concentrations were determined. Reagents and membranes were distributed with a robotic sample processor (Tecan RSP 5000-series). Using a Brandel M-24 cell harvester, assays were terminated by filtration through Whatman GF/B filter strips previously soaked in 0.5% poly(ethylenimine) for one hour. Filters were washed with ice-cold buffer (3 × 4 cm^3) and radioactivity was counted in a liquid scintillation spectrometer (Betamatic, Kintron). The inhibition constant (K_i) of the drugs was calculated from competitive binding curves with the computer program Prism. Data are presented as the arithmetic mean ±SEM of four independent measurements (Table 2).

Activity on isolated organ preparations

Preparations of the myoenteric plexus-longitudinal muscle obtained from male guinea pig ileum (GPI, rich in μ-opioid receptors) and preparations of mouse vas deferens (MVD, rich in δ-opioid receptors) were used for field stimulation with bipolar rectangular pulses of supramaximal voltage.[26] Agonists were evaluated for their ability to inhibit the electrically-evoked twitch. The biological potency of peptoid analogues was compared with that of the μ-opioid receptor agonist dermorphin in GPI preparations and with that of the δ-opioid receptor agonist deltorphin-I in MVD preparations. The results are expressed as the IC_{50} values obtained from concentration-response curves (Prism) (Table 2).

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