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Novel glycosylated (Lys^7)-dermorphin analogues: synthesis, biological activity and conformational investigations‡

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Abstract: Syntheses of the [Lys^7]- and [Hyp^6,Lys^7]-dermorphin analogues in which either Tyr^5 or Hyp^6 are *O*-glucosylated are described. For comparison, the carbohydrate-free peptides have also been prepared. Structural investigations by FT-IR and CD measurements were carried out on the synthetic analogues and some preliminary pharmacological experiments were also performed.

The biological potency of the glucosylated analogues was compared with that of the μ-opioid receptor agonist dermorphin in GPI preparations. Glucosylation of either Tyr^5 or Hyp^6 reduces the potency of both [Lys^7]-dermorphin and [Hyp^6,Lys^7]-dermorphin. The effect induced by the Tyr^5 glucosylation is quite strong and the potency of both peptides is reduced by about 150 times. A similar but less dramatic effect is induced by the glucosylation of the Hyp^6 residue, and the potency of the parent peptide is reduced by about 15 times. The presence of acetyl groups on the sugar hydroxyl functions further reduces the agonistic potency of the glucosylated analogues. The analgesic potency of [Hyp^6,Lys^7]-, [Hyp(βGlc)^6,Lys^7]- and [Tyr(βGlc)^5,Lys^7]-dermorphin were also tested *in vivo* by the tail-flick test. The glucosylated hydroxyproline-containing analogue is 8–10 times less active than the parent peptide, but its analgesic effect lasts significantly longer. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: biological activity; conformational investigations; dermorphin; opioid peptides; glycopeptides; peptide synthesis

INTRODUCTION

Dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and its variant form containing hydroxyproline instead of proline are opioid peptides isolated from the skin of the South American frogs *Phyllomedusa sauvagei* [1] and *Phyllomedusa rohdei*, and *Phyllomedusa burneisteri* [2,3], respectively. They have high affinity and selectivity for μ-opioid receptors and produce analgesia in laboratory animals and humans [4]. As an antinociceptive agent, dermorphin is 40 000-fold less potent after subcutaneous (s.c.) administration than after intracerebroventricular (i.c.v.) administration [5].

Screening of a cDNA library prepared from the skin of *Phyllomedusa bicolor* predicted the amino acid sequence of three dermorphin-like peptides (without amidation at the carboxyl terminus) [6]. Two of the three predicted dermorphin analogues have been later isolated from methanol extracts of the skin from this frog and their amino acid sequence have been confirmed [7]. These were found to be H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-OH and H-Tyr-D-Ala-Phe-Trp-Tyr-Pro-Asn-OH and the novel peptides were named [Lys^7]-dermorphin-OH and [Trp^4, Asn^7]-dermorphin-OH, respectively [7]. These naturally occurring dermorphin-like peptides and the related carboxyl-terminal amides were synthesized, their binding profiles to opioid receptors were determined and their biological activities were studied in isolated organ preparations and intact animals [7]. Extensive *in vitro* and *in vivo* studies demonstrated that dermorphins are the most potent and selective μ-opioid agonists among the naturally occurring opioids and that [Lys^7]-dermorphin is the most potent μ-opioid agonist even when injected peripherally [5,8]. Among the naturally occurring dermorphins, those with a free terminal carboxyl group have a μ-affinity 30–100 times lower than that of their amidated analogues and are less active on isolated organ preparations.

Abbreviations: Except where stated otherwise, the amino acid residues are of the 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. Abbreviations listed in the guide published in *J. Peptide Sci.* 2006; **12**: 1–12 are used without explanation. For representing the glucosylated α-amino acid residue, we indicate the substituent in brackets after the three-letter symbol. Other abbreviations are as follows: EDC, *N*-(3-dimethylamino-isopropyl)-*N*-ethyl-carbodiimide; ESI-MS, electrospray ionization-mass spectroscopy; Glc, D-glucopyranosyl; Glc(Ac)$_4$, 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl; NMP, *N*-methylpyrrolidone; Rink amide MBHA resin, [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-4-methyl-4-benzydrylamine polystyrene]; TDM, 4.4′-tetramethyldiphenylmethane; TIS, tri-isopropyl silane.

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Table 1 Amino Acid Sequence of [Lys^7]-dermorphin Analogues

I	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-NH_2	[Lys^7]-dermorphin
II	H-Tyr-D-Ala-Phe-Gly-Tyr-Hyp-Lys-NH_2	[Hyp^6, Lys7]-dermorphin
III	H-Tyr-D-Ala-Phe-Gly-Tyr[βGlc(Ac)$_4$]-Pro-Lys-NH_2	{Tyr[βGlc(Ac)$_4$]^5, Lys^7}-dermorphin
IV	H-Tyr-D-Ala-Phe-Gly-Tyr(βGlc)-Pro-Lys-NH_2	[Tyr(βGlc)^5, Lys^7]-dermorphin
V	H-Tyr-D-Ala-Phe-Gly-Tyr[βGlc(Ac)$_4$]-Hyp-Lys-NH_2	{Tyr[βGlc(Ac)$_4$]^5, Hyp^6, Lys^7}-dermorphin
VI	H-Tyr-D-Ala-Phe-Gly-Tyr(βGlc)-Hyp-Lys-NH_2	[Tyr(βGlc)^5, Hyp^6, Lys^7]-dermorphin
VII	H-Tyr-D-Ala-Phe-Gly-Tyr-Hyp[βGlc(Ac)$_4$]-Lys-NH_2	{Hyp [βGlc(Ac)$_4$]^6, Lys^7}-dermorphin
VIII	H-Tyr-D-Ala-Phe-Gly-Tyr-Hyp(βGlc)-Lys-NH_2	[Hyp (βGlc)^6, Lys^7]-dermorphin

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, resistance 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. In previous papers [9,10] 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 s.c. injection in mice.

In continuation of our investigations on the effect of glycosylation on the pharmacological properties of dermorphin and dermorphin-like peptides, we report in this communication the synthesis of [Lys^7]- and [Hyp^6, Lys^7]-dermorphin analogues in which either Tyr^5 or Hyp^6 are *O*-glucosylated (Table 1). For comparison, the carbohydrate-free peptides have also been prepared. Structural investigations by FT-IR and CD measurements were carried out on the synthetic analogues and some preliminary pharmacological experiments were also performed.

MATERIALS AND METHODS

General

All chemicals were commercial products of the best grade available. Fmoc-Tyr(OBu^t)-OH, Fmoc-D-Ala-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, H-Pro-OBzl.HCl, H-Hyp-OBzl.HCl, H-Pro-OBu^t.HCl, Z-Tyr-OH, Z-Tyr-OBzl and Rink amide MBHA resin were obtained from Novabiochem. H-Hyp(tBu)-OtBu.HCl was obtained from Senn Chemicals. Dichloromethane, acetonitrile (HPLC grade), 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose and HATU were supplied by Fluka. All other chemicals for the solid phase synthesis (TFA, EDC, HOBt, DIEA, HBTU, DMF, piperidine) were supplied by Applied Biosystems. Fmoc-Tyr[βGlc(Ac$_4$)]-OH and H-Hyp[βGlc(Ac$_4$)]-OBzl were prepared as described below. Ascending thin-layer chromatographies were routinely performed on TLC plates of Silica Gel 60, UV_{254}, and Machery-Nagel using the following solvent systems: E1, butan-1-ol–acetic acid–water (6 : 2 : 2 by vol); E1bis, ethyl acetate–butan-1-ol–acetic acid–water (10 : 6 : 2 : 2 by vol); E2, dichloromethane–acetone (15 : 1 v/v); E2 bis, dichloromethane–acetone (6 : 1 v/v); E3, chloroform–acetic acid–*n*-hexane (85 : 10 : 5 by vol.); E4, light petroleum–ethyl ether (20 : 1); E6, chloroform–methanol–acetic acid (90 : 8 : 2 by vol.); E7, chloroform–methanol (85 : 15 v/v). Amino acid derivatives, peptides and sugar containing products were visualized by one or more of the following procedures: ninhydrin, TDM reagent [11], UV light and 10% sulfuric acid in ethanol, followed by heating for 10 min at 100 °C. Optical rotations were determined at 25 °C in the indicated solvent with a Perkin Elmer model 241 polarimeter. Analytical HPLC separations were performed on a Vydac C 18 column (0.4 × 25 cm, 5 μm, flow rate 1.5 ml/min) using a Dionex liquid chromatograph P680 HPLC Pump equipped with a UVD 170U detector and Chromeleon Chromatography Management system. Eluants A (0.1% TFA in 90% aqueous acetonitrile) and B (aqueous 0.1% TFA) were used for preparing binary gradients (explained in text). Semipreparative HPLC separations (Vydac RP-C18 column, 250 × 22 mm, 10 μm; flow rate 15 ml/min) were performed on a Shimadzu series LC-6A chromatograph equipped with two independent pump units model LC-8A, a SPD-6A detector and a C-R6A integrator (eluants were those used for the analytical separations; see text for the elution conditions).

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 Nuclear Magnetic Resonance

Proton NMR spectra at 400 MHz were recorded at 298 K in $CDCl_3$ or DMSO-d_6 (99.996%) on a Bruker spectrometer (DRX 400). Sample concentrations were in the range 8–10 mg/ml and 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 assignments were determined by DQF-COSY and TOCSY experiments.

Mass Spectra

Electrospray ionization-mass spectrometry (ESI-MS) was performed on a PerSeptive Biosystem Mariner API-TOF instrument (Ionization potential 4200 V, acceleration potential 100 V).

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Infrared Absorption

Solid state FT-IR absorption spectra (KBr disk technique, 1% w/w) and solution spectra (TFE, sample concentration about 3 mM) were recorded at room temperature using a nitrogen flushed Perkin Elmer Model 1720X FT-IR spectrophotometer connected to a PC IBM P/2 model 50Z. Elaboration of the spectra by baseline subtraction and second derivative formation was achieved using the Spectra Calculation program (Galactic, Salem, USA). Before measurements, peptides and glycopeptides were lyophilized several times from 0.05 M HCl.

Circular Dichroism

Circular dichroism spectra were recorded in the indicated solvents at 298 K over 190–250 nm with a Jasco 715 spectropolarimeter connected with a PC IBM PS/2 Model 40 SIC for the spectra elaboration (Spectra Manager program). Hellma Suprasil quartz cells of 0.1 and 0.05 cm path length were used and six scans were accumulated for all spectra. Solutions of the desired concentration ($\sim 1.5 \times 10^{-4}$ M) were prepared by dissolving the different samples in minimum amount of water. The peptide concentration in aqueous solution was determined by the molar extinction coefficients of the tyrosine residues (ε_{tyr} 1420 at 274.6 nm) [12]. Aliquots of the mother solution were diluted with the appropriate volumes of 5 mM Tris buffer (pH 7.4), 35 mM aqueous SDS or TFE. Final solutions were 30 mM SDS and 97% TFE (v/v). The spectra reported are original computer-drawn CD curves: $[\Theta]_R$ represents the molar ellipticity (deg cm^2/dmol).

Pharmacological Tests

Activity on isolated organ preparations. Preparations of the myenteric plexus-longitudinal muscle obtained from male guinea pig ileum (GPI, rich in μ-opioid receptors) were used for field stimulation with bipolar rectangular pulses of supramaximal voltage [13]. Agonists were evaluated for their ability to inhibit the electrically evoked twitch. Results are expressed as the IC_{50} values obtained from concentration–response curves (Prism). IC_{50} values represent the mean of not less than six tissue samples ± SEM.

Antinociception studies. Adult male Sprague-Dawley rats (280–350 g, b.w.) were used for the experiments under protocols approved by the Animal Care of the Italian Ministry of Health according to European Community directives. Chronic lumbar intrathecal catheters were implanted in rats under ketamine-xylazine anaesthesia [14]. Compounds were administered to rats intrathecally in a volume of 10 μl. Each animal received one injection only. Every dose of each compound was evaluated in groups of five animals. Antinociception was measured by the tail-flick test [15] and expressed as percentage of the maximum possible effect (MPE): %MPE = 100 × (test latency – control latency)/(15 sec – control latency). The AD_{50} value of each peptide was defined as the dose producing 50% of the MPE.

Building Block Syntheses

As described below, assemblies of the [Lys^7]-dermorphin heptapeptide analogues were performed by the solid phase procedure. To prevent the possible intrachain aminolysis at the dipeptide stage [16] and the consequent chain loss in the form of diketopiperazine, the dipeptides Fmoc-Tyr-Pro-OH, Fmoc-Tyr-Hyp-OH, Fmoc-Tyr[βGlc(Ac)$_4$]-Pro-OH, Fmoc-Tyr[βGlc(Ac)$_4$]-Hyp-OH and Fmoc-Tyr-Hyp[βGlc(Ac)$_4$]-OH (positions 5 and 6) were prepared by solution synthesis (Figures 1 and 2) and used for acylating the H-Lys(Boc)-Rink amide resin during the synthesis of the [Lys^7]-dermorphin analogues **I, II, III, V** and **VII.**

Z-Tyr-Pro-OBzl. NMM (1.63 ml, 14.8 mmol) was added to a stirred, ice-cold suspension of H-Pro-OBzl hydrochloride (3.58 g, 14.8 mmol) in dichloromethane (60 ml). After 20 min, a solution of Z-Tyr-OH (4.67 g, 14.8 mmol) and HOBt (2.28 g, 14.8 mmol) in dichloromethane (20 ml) was added and the reaction mixture was kept under stirring at 0 °C until almost complete dissolution occurred. A dichloromethane solution of EDC (2.84 g, 14.8 mmol in 20 ml) was added and the mixture was kept at room temperature for 5 h and EDC (0.2 g, 1.0 mmol) and DIEA (0.24 ml, 1.4 mmol) were again added. After stirring overnight, the dichloromethane solution was washed with 0.5 M $KHSO_4$ (2 × 50 ml), aqueous 5% $NaHCO_3$ (3 × 50 ml) and saturated aqueous NaCl (2 × 50 ml), and was then dried and evaporated to dryness. Yield: 6.97 g (93.6%). Single spot by TLC in E7 (Rf 0.81), single peak (18.5 min) by analytical HPLC (elution conditions: isocratic 20% A for 3 min, linear gradient 20–90% A in 20 min.), $[M + H]^+$ 503.2 (calcd for $C_{29}H_{30}N_2O_6$ 502.55), $[\alpha]_D$ −40.98° (c 1.02, methanol); ^{1}H-NMR, 400 MHz, $CDCl_3$: 7.3 (m, 10H, aromatics benzyl), 7.1–6.6 (dd, 4H, aromatics Tyr), 5.5 (d, 1H NH), 5.2–5.0 (m, 4H, $2CH_2$ benzyl), 4.66 (m, 1H, CHα Tyr), 4.55 (m, 1H, CHα Pro), 3.7–3.2 (2m, 2H, $CH_2\delta$ Pro), 3.05–2,75 (2m, 2H, $CH_2\beta$ Tyr), 2.15 (m, 2H, $CH_2\beta$ Pro), 1.90 (m, 2H, $CH_2\gamma$ Pro).

Fmoc-Tyr-Pro-OH. Z-Tyr-Pro-OBzl (3.0 g, 5.96 mmol) was dissolved in a methanol–acetic acid mixture (35 ml, 6 : 1 v/v) and catalytically hydrogenated over 10% Pd/C. The reaction was monitored by TLC in E7 and after 4 h the catalyst was removed by filtration and the filtrate was evaporated to dryness. The oily residue was triturated several times with ethyl ether and yielded a white solid (1.90 g, 95%), single spot by TLC (E1, Rf 0.11), $[M + H]^+$ 279.16 (calcd for $C_{14}H_{18}N_2O_4$ 278.30). The crude dipeptide acetate was taken up with water (20 ml) and the pH value was adjusted to 8.0 with 1 M NaOH. A small amount of insoluble material was filtered off. A solution of Fmoc-OSu (1.59 g, 4.7 mmol) in acetonitrile (50 ml) was added under stirring to the filtrate and the reaction mixture

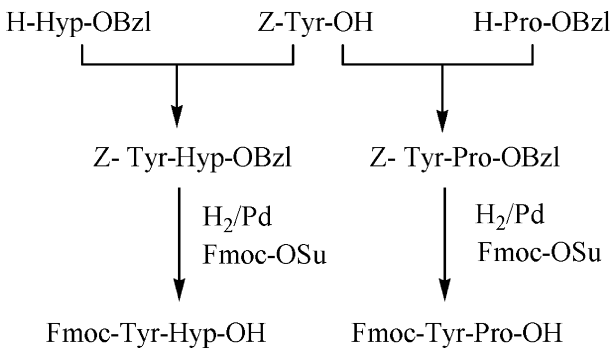

Figure 1 Synthesis of the dipeptides corresponding to residues 5–6 of the dermorphin sequence.

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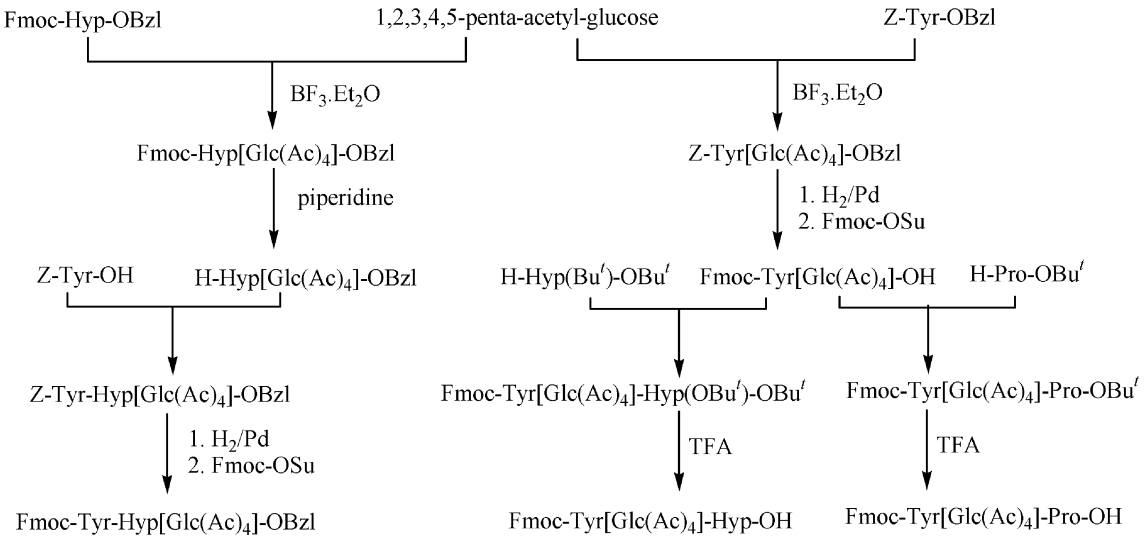

Figure 2 Synthesis of the glycosylated dipeptides corresponding to residues 5–6 of the dermorphin sequence.

was stirred overnight at room temperature. The organic solvent was removed *in vacuo* and the pH of the aqueous solution was re-adjusted to 8.0 with TEA followed by extraction with ethyl ether (2 × 20 ml). Acidification with 1 N HCl yielded the separation of an oily product, which was collected, triturated several times with water and dried. Yield: 2.0 g (85%). Single spot by TLC in E6 (Rf 0.39), single peak (16.4 min) by analytical HPLC (elution conditions: isocratic 20% A for 2 min, linear gradient 20–90% A in 20 min.), $[M+H]^+$ 501.16 (calcd for $C_{29}H_{28}N_2O_6$ 500.53), $[\alpha]_D$ −31.55° (c 1.0, methanol); ^{1}H-NMR, 400 MHz, $CDCl_3$: 7.3–7.8 (m, 8H, aromatics Fmoc), 6.9–6.65 (dd, 4H, aromatics Tyr), 5.75 (d, 1H, NH), 4.8 (m, 1H, CHα Tyr), 4.6 (t, 1H, CHα Pro), 4.4 (d, 2H, CH_2 Fmoc), 4.2 (t, 1H, CH Fmoc), 3.7–3.4 (2m, 2H, $CH_2\delta$ Pro), 3.1 (m, 2H, $CH_2\beta$ Tyr), 2.2 (m, 2H, $CH_2\beta$ Pro), 2.0 (m, 2H $CH_2\gamma$ Pro).

Z-Tyr-Hyp-OBzl. H-Hyp-OBzl hydrochloride (1.3 g, 5.04 mmol) and DIEA (1.74 ml, 10 mmol) were added to a stirred DMF solution (20 ml) of Z-Tyr-OH (1.6 g, 5.07 mmol) and HBTU (1.9 g, 5.0 mmol). The reaction was monitored by TLC in E7 and the mixture was kept overnight under stirring at room temperature. The solvent was removed and the oily residue was taken up with ethyl acetate, washed with water (3 × 20 ml), 0.5 M $KHSO_4$ (2 × 20 ml), 5% aqueous $NaHCO_3$ (2 × 20 ml) and saturated aqueous NaCl, dried and evaporated to dryness. Yield: 2.57 g (98%, oil). TLC in E7 (main product Rf 0.57) and analytical HPLC (main peak at 17 min, elution conditions: isocratic 20% A for 2 min, linear gradient 20–90% A in 30 min) showed the presence of some very minor contaminants. $[M+H]^+$ 519.50 (calcd for $C_{29}H_{30}N_2O_7$ 518.50); ^{1}H-NMR, 400 MHz, $CDCl_3$: 7.3 (m, 10H, aromatics Z and benzyl), 7.08–6.7 (dd, 4H, aromatics Tyr), 5.57 (t, 1H, NH), 5.2 (m, 2H, CH_2 Z), 5.05 (m, 2H, *O*–CH_2–C_6H_5), 4.67 (m 1H, CHα Hyp), 4.59 (m, 1H, CHα Tyr), 4.40 (m, 1H, CHγ Hyp), 3.75–3.1 (2m, 2H, $CH_2\delta$ Hyp), 2.98–2.80 (2m, 2H, $CH_2\beta$ Tyr), 2.25 (m, 2H, $CH_2\beta$ Hyp).

Fmoc-Tyr-Hyp-OH. Z-Tyr-Hyp-OBzl (2.57 g, 4.96 mmol) was dissolved in a methanol–acetic acid mixture (55 ml, 6 : 1 v/v) and catalytically hydrogenated over 10% Pd/C. The reaction was monitored by TLC in E7 and after 2.5 h the catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was triturated several times with ethyl ether and yielded a white solid (1.37 g, 95%), $[M+H]^+$ 295.12 (calcd for $C_{14}H_{18}N_2O_5$ 294.29). The crude dipeptide was taken up with water (70 ml), the pH value of the resulting solution was adjusted to 8.0 with 1 M NaOH, a solution of Fmoc-OSu (1.60 g, 4.72 mmol) in acetonitrile (70 ml) was added and the reaction mixture was stirred overnight at room temperature. The organic solvent was removed *in vacuo* and the pH of the aqueous solution was re-adjusted to 8.0 with TEA followed by extraction with ethyl ether (2 × 25 ml). Acidification with 1 N HCl yielded the separation of an oily product, which was re-extracted with ethyl acetate (3 × 20 ml). The extracts were combined, washed several times with ice-cold water, dried and evaporated to dryness. Yield: 1.56 g (61%). Single spot by TLC (E1bis, Rf 0.39), single peak (15.34 min) by analytical HPLC (elution conditions: isocratic 20% A for 3 min, linear gradient 20–90% A in 30 min.), $[M+H]^+$ 517.2 (calcd for $C_{29}H_{28}N_2O_6$ 516.5); $[\alpha]_D$ −33.8° (*c* 1.0, methanol); ^{1}H-NMR, 400 MHz, DMSO-d_6: 7.87–7.30 (m, 8H, aromatics Fmoc), 7.11–6.63 (dd, 4H, aromatics Tyr), 5.16 (d, 1H, NH), 4.33 (m, 1H, CHα Tyr), 4.31 (d, 2H, CH_2 Fmoc), 4.3 (t, 1H, CHα Hyp), 4.3 (t, 1H, CHγ Hyp), 4.15 (t, 1H, CH Fmoc), 3.6 (m, 2H, $CH_2\beta$ Tyr), 2.78–2.66 (m, 2H, $CH_2\delta$ Hyp), 2.07–1.88 (m, 2H, $CH_2\beta$ Hyp).

Z-Tyr(β Glc(Ac$_4$))-OBzl. Glucosylation of Z-Tyr-OBzl was carried out according to the procedure described for the *O*-glycosylation of phenols [17]. $BF_3{\cdot}Et_2O$ (5.8 ml, 46.25 mmol) in anhydrous dichloromethane (20 ml) was added dropwise in 60 min to a stirred solution of TEA (1.29 ml, 9.25 mmol), Z-Tyr-OBzl (7.5 g, 18.5 mmol) and 1,2,3.4.6-penta-*O*-acetyl-β-D-glucopyranose (10.85 g, 27.75 mmol) in ice-cold anhydrous dichloromethane (50 ml). The reaction mixture was kept at room temperature for 24 h, washed with aqueous 2.5% $NaHCO_3$ (3 × 60 ml) and saturated aqueous NaCl (3 × 60 ml) and evaporated to dryness. The oily residue was triturated several times with ethyl ether, suspended in ethyl ether and collected by filtration. Yield: 9.8 g (72%). A second crop of Z-[βGlc(AC$_4$)]Tyr–OBzl (2.5 g) was obtained by evaporation of the ethyl ether used for trituration. Total yield: 11.3 g (90%).

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Single spot by TLC (E2, Rf 0.59), single peak (20.67 min) by analytical HPLC (elution conditions: isocratic 40% A for 3 min, linear gradient 40–90% A in 30 min), $[M+H]^+$ 736,26 (calcd for $C_{38}H_{41}NO_{14}$ 735,74), $[\alpha]_D -11.96^\circ$ (c 1.12, DMF); ^{1}H-NMR, 400 MHz, $CDCl_3$: 7.35 (m, 10H, aromatics Z and benzyl), 6.93–6.81 (dd, 4H aromatics Tyr), 5.71 (d, 1H, NH), 5.29 (m 2H, CH_2 Z), 5.25–5.10 (m 3H, H2, H3, H4), 5.09 (s, 2H, *O*–CH_2–C_6H_5), 5.01 (d, 1H, H1, J H1-H2 7.18 Hz), 4.65 (m, 1H, CHα), 4.27 (dd, 1H, H6), 4.16 (t, 1H, H6′), 3.85 (m, 1H, H5), 3.06 (m, 2H, $CH_2\beta$), 2.12–2.01 (m, 12H, $4CH_3CO$–).

Fmoc-Tyr(βGlc(Ac)$_4$)-OH. Z-Tyr[βGlc(Ac)$_4$]-OBzl (10.2 g, 13.86 mmol) was dissolved in a mixture of methanol (110 ml) and acetic acid (120 ml) previously flushed with nitrogen, and catalytically hydrogenated over 10% Pd/C. The reaction was monitored by TLC in E2 and after 180 min the catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was suspended in methanol (150 ml), stirred for 30 min, recovered by filtration and dried. Yield: 5.45 g (69% as acetate). Single spot by TLC (E1bis, Rf 0.10), single peak (10.03 min) by analytical HPLC (elution conditions: isocratic 20% A for 3 min, linear gradient 20–90% A in 30 min), $[M+H]^+$ 512.14 (calcd for $C_{23}H_{29}NO_{12}$ 511.46). TEA (1.31 ml, 9.41 mmol) and a solution of Fmoc-OSu (3.17 g, 9.40 mmol) in acetonitrile (40 ml) were added to a suspension of H-[βGlc(Ac)$_4$]Tyr-OH acetate (5.38 g, 9.41 mmol) in acetonitrile (70 ml). The mixture was diluted by adding 60% aqueous acetonitrile (100 ml), the pH of the resulting solution was adjusted to 8.0 with TEA and Fmoc-OSu (0.31 g, 0.92 mmol) was further added after 6 h. After stirring overnight, the organic solvent was removed *in vacuo*, the aqueous solution was acidified with 0.5 M $KHSO_4$ and the desired product was extracted with ethyl acetate (6 × 30 ml). The organic extracts were combined, washed with water, dried and evaporated to dryness. Yield: 6.61 g (96%). single spot by TLC (E6, Rf 0.56), single peak (10.4 min) by analytical HPLC (elution conditions: isocratic 40% A for 3 min, linear gradient 40–90% A in 30 min), $[\alpha]_D -28.2^\circ$ (c 0.9, methanol), $[M+H]^+$ 734,20 (calcd for $C_{38}H_{39}NO_{14}$ 733,71); ^{1}H-NMR, 400 MHz, $CDCl_3$: 7.8–7.3 (m, 8H, aromatics Fmoc), 7.09–6.85 (dd, 4H aromatics Tyr), 5.30–5.20 (m, 3H, H3 NH H2), 5.15 (m 1H, H4), 4.98 (d 1H, H1 J H1-H2 7.27 Hz), 4.65 (m, 1H, CHα), 4.45 (m, 1H, CH_2 Fmoc), 4.35 (m, 1H, CH_2 Fmoc), 4.23 (dd, 1H, H6), 4.19 (m, 1H, CH Fmoc), 4.09 (m, 1H, H6′), 3.75 (m, 1H, H5), 3.20–3.07 (m, 2H, $CH_2\beta$), 2.04 (s, 12H, $4CH_3CO$–).

Fmoc-Tyr(βGlc(Ac)$_4$)-Pro-OtBu. Fmoc-Tyr[βGlc(Ac)$_4$]-OH (3.0 g, 4.09 mmol), HBTU (1.55 g, 4.09 mmol), HOBt (0.63 g, 4.09 mmol) and DIEA (0.7 ml, 4.09 mmol) were dissolved under stirring in anhydrous DMF (40 ml). After 10 min, further DIEA (0.7 ml, 4.09 mmol) and H-Pro-OtBu hydrochloride (0.85 g, 4.09 mmol) were added and the yellow reaction mixture was kept overnight at room temperature. The solvent was removed *in vacuo* and the oily residue was taken up with ethyl acetate (40 ml), washed with water (3 × 30 ml), aqueous 5% $NaHCO_3$ (2 × 30 ml) and water (30 ml) and dried. Evaporation of the solvent gave a solid. Yield: 3.43 g (95%). Single spot by TLC (E6, Rf 0.81), single peak (22.45 min) by analytical HPLC (elution conditions: isocratic 40% A for 3 min, linear gradient 40–90% A in 30 min), $[M+H]^+$ 887.31 (calcd for $C_{47}H_{54}N_2O_{15}$ 886.93); ^{1}H-NMR, 400 MHz, $CDCl_3$: 8.7–7.3 (mm, 8H, aromatics Fmoc), 7.20–6.87 (dd, 4H aromatics Tyr), 5.61 (d, 1H, NH), 5.3–5.1 (m 4H, H1, H2, H3, H4), 4.89 (dd 1H, CHα Pro), 4.7 (m, 1H, CHα Tyr), 4.35 (dd, 1H, H6) 4.2–4.0 (m, 4H, CH_2 Fmoc, CH Fmoc, H6′), 3.66 (m, 2H $CH_2\delta$ Pro), 3.42 (m, 1H, H5), 3.15 (dd, 1H, $CH_2\beta$ Tyr), 2.9 (m, 1H, $CH_2\beta$ Tyr), 2.03 (m, 16H, $4CH_3CO$–, $CH_2\beta$ Pro, $CH_2\gamma$ Pro), 1.49 (s, 9H, CH_3 tBu).

Fmoc-Tyr(βGlc(Ac)$_4$)-Pro-OH. Fmoc-[βGlc(Ac)$_4$]Tyr-Pro-OtBu (2.0 g, 2.25 mmol) was dissolved under stirring in a mixture of TFA (38 ml), H_2O (1 ml) and TIS (1 ml) and the reaction was monitored by TLC in E6. After 180 min, the light yellow solution was evaporated to dryness, the oily residue was taken up with ethyl acetate (80 ml), washed with water (6 × 40 ml), 0.5% aqueous $NaHCO_3$ (3 × 30 ml) and saturated aqueous NaCl (3 × 30 ml) and dried. Evaporation of the solvent gave an oil that gave a light yellow solid by standing *in vacuo*. Yield: 1.78 g (95%). TLC in E6 (main product Rf 0.64) and analytical HPLC (main peak 21.95 min, elution conditions: isocratic 20% A for 3 min, linear gradient 20–90% A in 30 min) showed the presence of some very minor contaminants; $[\alpha]_D -41.1^\circ$ (c 1.02, methanol), $[M+H]^+$ 831.37 (calcd for $C_{43}H_{46}N_2O_{15}$ 830.82); ^{1}H-NMR, 400 MHz, $CDCl_3$: 7.76–7.29 (m, 8H, aromatics Fmoc), 7.17–6.89 (dd, 4H aromatics Tyr), 5.92 (d, 1H, NH), 5.22–5.12 (m, 3H, H2, H3, H4), 4.90 (d, 1H, H1, J H1-H2 7 Hz), 4.73 (m, 1H, CHα Tyr), 4.56 (t 1H, CHα Pro), 4.22 (d, 2H, CH_2 Fmoc), 4.22–4.16 (m, 2H, 2H6), 4.14 (t, 1H, CH Fmoc), 3.69 (m, 1H, H5), 3.67 (m, 2H, $CH_2\gamma$ Pro), 3.22 (m, 2H, $CH_2\delta$ Pro), 3.08–2.97 (m, 2H, $CH_2\beta$ Tyr), 2.14–1.93 (m, 2H, $CH_2\beta$ Pro), 2.00 (s, 12H, $4CH_3CO$–).

Fmoc-Tyr(βGlc(Ac)$_4$)-Hyp(tBu)-OBu^t. H-Hyp(Bu^t)-OtBu hydrochloride (1.35 g, 4.84 mml) and DIEA (0,83 ml, 4.84 mmol) were added under stirring to a solution of Fmoc-Tyr[βGlc(Ac)$_4$]-OH (3.55 g, 4.84 mmol), HATU (1.84 g, 4.84 mmol) and DIEA (0,83 ml, 4.84 mmol) in anhydrous DMF (50 ml) and the reaction was monitored by TLC in E6. After 4 h the solvent was removed *in vacuo* and the oily residue was taken up with ethyl acetate (60 ml), washed with water (3 × 40 ml), aqueous 5% $NaHCO_3$ (2 × 40 ml) and saturated aqueous NaCl (3 × 30 ml) and dried. Evaporation of the solvent gave an oil that gave a light yellow solid by standing *in vacuo*. Yield: 4.28 g (92%). Single peak (26.85 min) by analytical HPLC (elution conditions: isocratic 40% A for 3 min, linear gradient 40–90% A in 30 min), $[M+H]^+$ 959.36 (calcd for $C_{51}H_{62}N_2O_{16}$ 958.32).

Fmoc-Tyr(βGlc(Ac)$_4$)-Hyp-OH. Fmoc-Tyr[βGlc(Ac)$_4$]-Hyp (tBu)-OtBu (2.05 g, 2.14 mmol) was dissolved under stirring in a mixture of TFA (38 ml), water (1.0 ml) and TIS (1.0 ml). The reaction was monitored by TLC in E6 and after 3 h the solvent was removed *in vacuo* and the oily residue was taken up with ethyl acetate (50 ml). The resulting green solution was washed with water (3 × 40 ml), aqueous 5% $NaHCO_3$ (20 ml) and saturated aqueous NaCl, dried and evaporated to dryness. Yield: 1.74 g (96%). Single spot by TLC (E6, Rf 0.46), single peak (17.37 min) by analytical HPLC (elution conditions: isocratic 10% A for 3 min, linear gradient 10–90%A in 30 min), $[\alpha]_D -43.3^\circ$ (c 1.0.01, methanol), $[M+H]^+$ 847.23 (calcd for $C_{43}H_{46}N_2O_{16}$ 846.84); ^{1}H-NMR, 400 MHz, $CDCl_3$: 7.72–7.25 (m, 8H, aromatics Fmoc), 7.15–6.86 (dd, 4H aromatics Tyr), 6.10 (d, 1H, NH), 5.25–5.07 (m, 3H, H2, H3, H4), 4.90 (d, 1H, H1, JH1-H2 7 Hz), 4.68 (m, 1H, CHα Tyr), 4.65 (t, 1H, CHα Hyp), 4.39 (m, 1H, CHγ Hyp), 4.32 (d, 2H, CH_2 Fmoc), 4.18–4.08 (m, 2H, 2H6), 4.07 (t, 1H, CH Fmoc), 3.77 (m, 1H, $CH_2\delta$ Hyp), 3.65 (m, 1H, H5), 3.24 (m, 1H, $CH_2\delta$ Hyp), 3.00 (m,

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2H, $CH_2\beta$ Tyr), 2.30–2.15 (m, 2H, $CH_2\beta$ Hyp), 2.00 (s, 12H, $4CH_3CO$–).

Fmoc-Hyp(βGlc(Ac)$_4$)-OBzl. Fmoc-OSu (6.5 g, 19.1 mmol) was dissolved in acetonitrile (60 ml), added to a solution of H-Hyp-OBzl hydrochloride (5.0 g, 19.4 mmol) in water (35 ml) and the pH value was adjusted to 8.0 with TEA. After 45 min the acetonitrile was removed *in vacuo*, the aqueous solution was extracted with ethyl ether (3 × 30 ml) and the extracts were combined, dried and evaporated to dryness. The residue was added to a stirred ice-cold solution of 1,2,3.4.6-penta-*O*-acetyl-β-D-glucopyranose (15.5 g, 39 mmol) in anhydrous dichloromethane (50 ml), and $BF_3{\cdot}Et_2O$ (47 mmol) in dichloromethane (20 ml) was added dropwise in 50 min. The reaction mixture was monitored by TLC in E2 bis and kept at room temperature for 5 h, washed with aqueous 5% $NaHCO_3$ (2 × 80 ml) and saturated aqueous NaCl (3 × 60 ml), dried and evaporated to dryness. The residue was purified in portions by low-pressure liquid chromatography on a Buchi 688 Chromatographic Pump equipped with a detector Buchi UV/Vis filter Photometer (254 nm) and a 2210 LKB recorder (Silica Gel F 60, 0.04–0.06 mm, 470 × 36, 9 mm column, flow rate 40 ml/min, eluant: dichloromethane–acetone 99 : 1 v/v). Yield: 11.25 g (75%). Single spot by TLC (E4, Rf 0.52), single peak (23.7 min) by analytical HPLC (elution conditions: isocratic 40% A for 3 min, linear gradient 40–90% A in 30 min), $[M + H]^+$ 774.24 (calcd for $C_{41}H_{43}NO_{14}$ 773.48); ^{1}H-NMR, 400 MHz, $CDCl_3$: 7.8–7.3 (mm, 8H, aromatics Fmoc), 7.4 (m, 5H aromatics benzyl), 5.2 (s, 2H, CH_2–*O*–), 5.19 (m 1H, H1), 5.08 (m 1H, H2), 4.95 (m, 1H, H3), 4.57 (m, 1H, H4), 4.43 (m, 1H, CHα), 4.40 (m, 2H, CH_2 Fmoc), 4.36 (m, 1H, CHγ), 4.25 (m, 1H, CH Fmoc), 4.24 (m, 1H, H6), 4.12 (m, 1H, H6′), 3.85 (m, 1H, $CH_2\delta$), 3.75 (m, 1H, $CH_2\delta$), 3.68 (m, 1H, H5), 2.34 (m, 1H, $CH_2\beta$), 2.14 (m, 1H, $CH_2\beta$), 2.08–1.95 (m, 12H, $4CH_3CO$–).

Z-Tyr-Hyp(βGlc(Ac)$_4$)-OBzl. Fmoc-Hyp[βGlc(Ac)$_4$]-OBzl (1.61 g, 2.08 mmol) was dissolved in a 4% solution of piperidine in DMF (20 ml), the reaction mixture was stirred at room temperature and after 2.5 h the solvent was removed *in vacuo*. The residue was taken up with ethyl acetate (50 ml), washed with water (4 × 30 ml) and saturated aqueous NaCl, dried and evaporated to dryness. The crude H-Hyp[βGlc(Ac)$_4$]-OBzl (main spot by TLC in E6, Rf 0.63, main peak by analytical HPLC rt. 13.07 min), $[M + H]^+$ 552.17 (calcd for $C_{26}H_{33}NO_{12}$ 551.54), was dissolved in DMF (30 ml) and combined with a DMF solution (20 ml) containing Z-Tyr-OH (0.66 g, 2.1 mmol), HATU (0.80 g, 2.1 mmol) and DIEA (0.72 ml, 4.2 mmol). The reaction mixture was kept under stirring for 4 h, the solvent was evaporated *in vacuo* and the oily residue was taken up with ethyl acetate (70 ml), washed with water (2 × 50 ml), aqueous 2.5% $NaHCO_3$ (50 ml), 0.1 M $KHSO_4$ and saturated aqueous NaCl, dried and evaporated to dryness. The crude product (1.86 g) was purified in portions by liquid chromatography on a 70 × 28 mm column (Silica Gel F 60, 0.04–0.06 mm), eluants: dichloromethane (90 ml) followed by ethyl acetate (40 ml). The peptide-containing fractions were pooled and evaporated to dryness. Yield: 1.45 g (82%). The yield was practically homogeneous by TLC in E3 (main spot Rf 0.10) and analytical HPLC (main peak at 18.94 min) (elution conditions: isocratic 10% A for 3 min, linear gradient 10–90% A in 20 min), $[M + H]^+$ 849.25 (calcd for $C_{43}H_{48}N_2O_{16}$ 848.82).

Fmoc-Tyr-Hyp(βGlc(Ac)$_4$)-OH. Z-Tyr-Hyp [βGlc(Ac)$_4$]-OBzl (1.43 g, 1.69 mmol) was dissolved in a methanol–acetic acid mixture (100 ml, 3 : 2 v/v) previously flushed with nitrogen, and catalitically hydrogenated over 10% Pd/C. The reaction was monitored by TLC in E7 and after 180 min the catalyst was removed by filtration, the filtrate was evaporated to dryness and the residue was triturated several times with ethyl ether, collected and dried. Yield: 0.86 g (81.5%) $[M + H]^+$ 625.2 (calcd for $C_{28}H_{36}N_2O_{14}$ 624.6). TLC in E7 (main product Rf 0.14) and analytical HPLC (main peak 10.37 min, elution conditions: isocratic 10% A for 3 min, linear gradient 10–90% A in 20 min) showed the presence of a very minor contaminant. The crude product (0.84 g, 1.34 mmol) was dissolved in water (50 ml), the pH was adjusted to 8.0 with 0.5 N NaOH, the solution was extracted with ethyl acetate (5 × 50 ml) and Fmoc-OSu (0.46 g, 1.34 mmol) in acetonitrile (50 ml) was added. After stirring overnight at room temperature, the organic solvent was removed *in vacuo*, the pH of the reaction mixture was re-adjusted to 8.0 with TEA and the aqueous solution was extracted with ethyl ether (2 × 40 ml). Acidification to pH 2.0 with 1 N HCl yielded the separation of an oily product, which was extracted with ethyl acetate (3 × 50 ml). The extracts were combined, dried and evaporated to dryness. Yield: 0.93 g (82%). Single spot by TLC (E6, Rf 0.41), single peak (15.34 min) by analytical HPLC (elution conditions: 10% A for 3 min, linear gradient 10–90% A in 20 min), $[M + H]^+$ 847.22 (calcd for $C_{43}H_{46}N_2O_{16}$ 846.8), ^{1}H-NMR, 400 MHz, $CDCl_3$: 7.76–7.33 (m, 8H, aromatics Fmoc), 6.98–6.67 (dd, 4H, aromatics Tyr), 5.80 (d, 1H, NH), 5.17.4.90 (m, H2, H3, H4), 4.72 (m, 1H, CHα Tyr), 4.59 (m, 1H, CHγ Hyp), 4.55 (d, 1H, H1, J H1-H2 8 Hz), 4.38 (t, 1H, CHα Hyp), 4.3 (d, 2H, CH_2 Fmoc), 4.25–4.15 (m, 2H, 2H6), 4.15 (t, 1H, CH Fmoc), 3.76 (m, 1H, $CH_2\delta$ Hyp), 3.66 (s, 1H, H5), 3.4 (m, 1H, $CH_2\delta$ Hyp), 2.98 (m, 2H, $CH_2\beta$ Tyr), 2.27 (m, 2H, $CH_2\beta$ Hyp), 2.1–1.5 (s, 12H, 4 CH_3–CO–).

Solid Phase Synthesis

Assemblies of the [Lys^7]-dermorphin analogues on the Advance ChemTech mod.348Ω Peptide Synthesizer were performed on a 0.06 mmol scale, starting with Rink amide MBHA resin (0.085 g, substitution 0.73 mmol/g). The Fmoc strategy and a single coupling protocol with HBTU/HOBt in DMF (coupling time 45 min) was normally used through the syntheses. NMP was only used for dissolving Fmoc-Phe-OH. As already pointed out, the dipeptides Fmoc-Tyr-Pro-OH, Fmoc-Tyr-Hyp-OH, Fmoc-Tyr[βGlc(Ac)$_4$]-Pro-OH, Fmoc-Tyr[βGlc(Ac)$_4$]-Hyp-OH and Fmoc-Tyr-Hyp[βGlc(Ac)$_4$]-OH (residues 5 and 6) were used as building blocks during the synthesis of the dermorphin analogues **I**, **II**, **III**, **V** and **VII**. For the synthesis of {Tyr[βGlc(Ac)$_4$]^5, Lys^7}-dermorphin, {Tyr[βGlc(Ac)$_4$]^5, Hyp^6, Lys^7}-dermorphin and {Hyp[βGlc(Ac)$_4$]^6, Lys^7}-dermorphin, activation of the carboxyl function of the glycosylated building blocks and of Fmoc-Gly-OH was achieved by adding HATU. The final peptide resin was N^α-deprotected with 20% piperidine in DMF, thoroughly washed with dichloromethane and dried. Cleavage from the resin and removal of the side chain protecting groups were simultaneously achieved by treatment with a mixture of TFA : H_2O : TIS (95 : 2.5 : 2.5 by vol, 4 ml/100 mg of peptide resin, 3.5 h at room temperature). The acid solution was concentrated *in vacuo* and the peptide analogue was precipitated with excess cold ethyl ether, collected by centrifugation, washed twice with cold ethyl ether and dried.

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Deacetylation of the carbohydrate moieties was achieved by adding 1% $NaOCH_3$ in methanol to a methanolic solution of the synthetic glycopeptides. The pH value was 8.5–9.0 and the reaction was monitored by analytical HPLC (elution conditions: isocratic 10% A for 3 min, linear gradient 10–50% A in 20 min). After 7 days at room temperature the solution was neutralized with aqueous 1% acetic acid, evaporated to dryness and the residue was lyophilized from water. Peptides and glycopeptides were further purified by semipreparative HPLC (elution conditions: for **I**, **II**, **IV**, **VI** and **VIII**, isocratic 15% A for 5 min, linear gradient 15–50% A in 25 min; for **III**, **V** and **VII**, isocratic 25% A for 5 min, linear gradient 25–50% A in 15 min). All products were characterized by reverse phase analytical HPLC (elution conditions: isocratic 10% A for 3 min, linear gradient 10–50% A in 20 min) and molecular weight determination.

(Lys^7)-dermorphin, I. The synthesis started with Rink amide resin and the peptide was cleaved from the resin and worked up as described previously. Yield: 15.9 mg (24.0%), homogeneous by analytical HPLC; $[M+H]^+$ 844.40, calculated for $C_{43}H_{57}N_9O_9$ 843.97.

(Hyp^6,Lys^7)-dermorphin, II. The synthesis was carried out as described for **I**. Yield: 16.3 mg (24.2%), homogeneous by analytical HPLC; $[M+H]^+$ 860.40, calculated for $C_{43}H_{57}N_9O_{10}$ 859.65.

{Tyr (βGlc(Ac)$_4$)^5,Lys^7}-dermorphin, III. The synthesis was carried out as described previously. Yield: 17.5 mg (20.1%), homogeneous by analytical HPLC; $[M+H]^+$ 1174.56, calculated for $C_{57}H_{75}N_9O_{18}$ 1173.52.

(Tyr (βGlc)^5,Lys^7)-dermorphin, IV. Deacetylation of **III** (42.7 mg) was carried out as described previously. Yield: 24.9 mg (68%), homogeneous by analytical HPLC; $[M+H]^+$ 1006.51, calculated for $C_{49}H_{67}N_9O_{14}$ 1005.47.

{Tyr (βGlc(Ac)$_4$)^5,Hyp^6,Lys^7}-dermorphin, V. The synthesis was carried out as described for **III**. Yield, 26.7 mg (30.3%), homogeneous by analytical HPLC, $[M+H]^+$ 1190.50, calculated for $C_{57}H_{75}N_9O_{19}$ 1189.49.

(Tyr (βGlc)^5,Hyp^6,Lys^7)-dermorphin, VI. Deacetylation of **V** (33 mg) was carried out as described previously. Yield: 14.0 mg (51%), homogeneous by analytical HPLC; $[M+H]^+$ 1022.45, calculated for $C_{49}H_{67}N_9O_{15}$ 1021.45.

{Hyp (βGlc(Ac)$_4$)^6,Lys^7}-dermorphin, VII. The synthesis was carried out as described for **I**. Yield: 28.6 mg (32.5%), homogeneous by analytical HPLC; $[M+H]^+$ 1190.50, calculated for $C_{57}H_{75}N_9O_{19}$ 1189.49.

(Hyp (βGlc)^6,Lys^7)-dermorphin, VIII. Deacetylation of **VII** (29 mg) was carried out as described previously. Yield: 16.1 mg (56%), homogeneous by analytical HPLC; $[M+H]^+$ 1022.45, calculated for $C_{49}H_{67}N_9O_{15}$ 1021.45.

RESULTS AND DISCUSSION

Circular Dichroism

The far UV region, below 250 nm, is of utmost concern in the application of CD to the conformational analysis of peptides and proteins and is apparently dominated by the peptide contribution. However, in small tyrosine-containing peptides, the low-energy transitions of the phenolic side chain tend to dominate the CD spectrum. The contribution from aromatic side chains to the CD spectrum of peptides and proteins has been acknowledged in both experimental and theoretical studies [18,19,] being more prominent in systems where several aromatic groups are in close proximity [20]. Tyrosine residues have been reported to contribute to a CD spectrum with four additional transitions: the L*b* band at 275 nm, the L*a* band at 227 nm and the B*a* and B*b* bands near 190 nm. The CD spectra of [Lys^7]-dermorphin in TRIS buffer, TFE and SDS solutions are shown in Figure 3. The far UV CD spectrum in buffer solution is characterized by two negative minima below 190 nm and at 196 nm, two positive bands at 205 and 227 nm and a positive minimum near 215 nm. In TFE, the minimum at 215 nm is negative and the absorption at lower wavelengths is characterized by a broad positive band. In SDS the spectrum shows two positive bands at 192 and 232 nm, a negative maximum at 207 nm and two negative minima at 198 and 215 nm.

The maximum in the 227–232 nm region shown in all the solvents by the [Lys^7]-dermorphin CD spectra could be essentially attributed to the Tyr^1 and Tyr^5 residues. The assignment of the bands near and below 200 nm in buffer solution and in TFE is rather questionable, owing to the presence of contributions arising from the peptide chromophores and from the B transitions of both Tyr and Phe chromophores. The CD spectra could reflect the contribution of both folded and unordered structures. In SDS solution, the [Lys^7]-dermorphin shows a class C CD spectrum characteristic of type I (III) β-turns [21]: the spectrum exhibits a negative minimum below 190 nm, a positive band at 192 nm, a negative band at 199 nm, a negative maximum at 207 nm and a second negative minimum at 215 nm, suggesting that SDS may preferentially contribute to stabilize a β-turn structure. Possibly, the ionic interaction between the

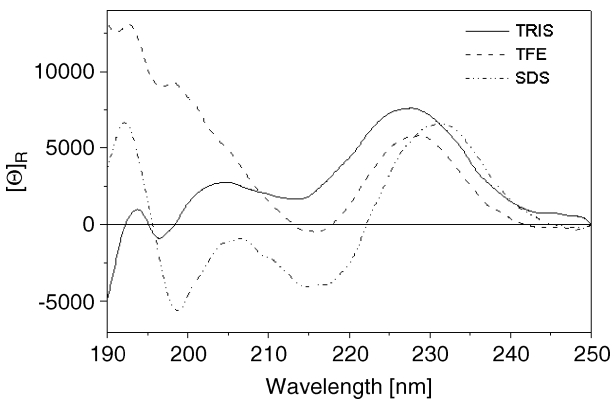

Figure 3 CD Spectra of [Lys^7]-dermorphin (**I**) in the indicated solvents.

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cationic peptide and the negatively charged micelles promotes the incorporation of the peptide hydrophobic portion with a conformational transition from a mostly random coil to a predominantly β-turn structure [22]. This feature could agree with the average structure of dermorphin described by NMR measurements [23] in phospholipid micelles.

The CD spectra of glycopeptides **III** and **IV**, in which D-glucose has been linked to the hydroxyl side chain function of the Tyr^5 residue, are shown in Figures 4 and 5, respectively. In TRIS buffer and in SDS solution, the spectra of both peptides are similar to those of peptide **I**, although minor intensity changes may reflect a shift in the populations of the systems with different forms. In TFE solution, the spectra of **III** and **IV** show a negative band at 215 nm, a strong positive one at about 200 nm with a negative minimum below 190 nm, and could agree with the CD spectra of peptides folded in type II β-turns [24]. It seems that glucosylation contributes to stabilize some preferential structures in low dielectric constant solvents. Moreover, [Lys^7] dermorphin contains D-Ala and Pro residues that are β-turn inducers, and β-turn-like structures have been determined in NMR studies on dermorphin [25].

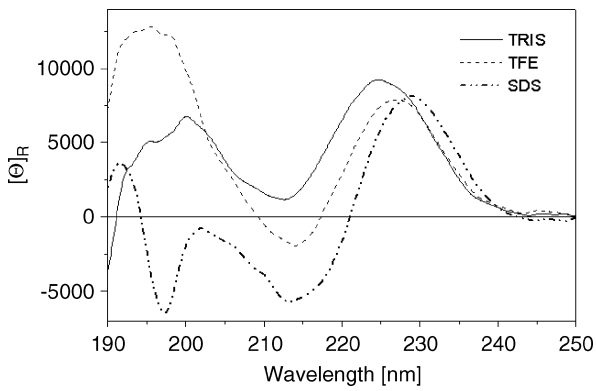

Figure 4 CD Spectra of {Tyr [βGlc(Ac)$_4$]^5,Lys^7}-dermorphin (**III**) in the indicated solvents.

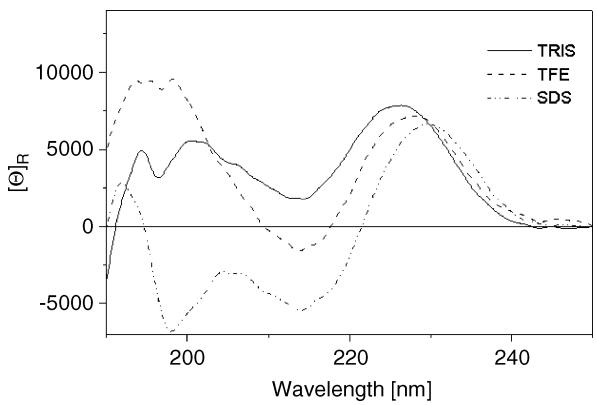

Figure 5 CD Spectra of [Tyr (βGlc)]^5,Lys^7}-dermorphin (**IV**) in the indicated solvents.

In all solvents, the CD spectra of [Hyp^6, Lys^7]-dermorphin **II** and the glucosylated analogues **V**, **VI**, **VII** and **VIII** are similar, with minor differences, to those of [Lys^7]-dermorphin **I** and the glycopeptides **III** and **IV**, respectively. Apparently, the Pro/Hyp substitution does not significantly affect the overall peptide conformation, and the presence of the D-glucose moiety *O*-glycosidically linked to the side chain hydroxyl function of either Tyr^5 or Hyp^6 induces rather similar conformational features.

Infrared Absorption

The conformational preferences of the synthetic peptides and glycopeptides were also examined by FT-IR absorption in the solid state as well as in TFE solution. In the solid state, the spectra of all peptides in the 1880–1500 cm^{-1} region (C=O stretching mode) are quite similar. As an example, the FT-IR spectra of **I**, **III** and **IV** are shown in Figure 6. The glycosylated peptide **III** and the analogues **V** and **VII** show the acetyl function bands at about 1755 and 1730 cm^{-1}. The bands at about 1515 and 1613 cm^{-1} arise from the aromatic side chains of Tyr^1 and Tyr^5 and the bands at about 1495 and 1593 cm^{-1} may be assigned to the aromatic side chain of Phe^3. The region of the amide I modes is characterized by two main bands: 1633–1635 cm^{-1} and 1661–1667 cm^{-1} with a shoulder at 1685–1690 cm^{-1}. The amide II shows a band at 1535–1539 cm^{-1} with a broad shoulder at about 1550 cm^{-1}. Generally, the infrared bands appearing between 1660 and 1690 cm^{-1} have been assigned to weakly solvated or shielded amide carbonyls not involved in H-bonds. The amide I component bands near or below 1640 cm^{-1} have been associated with the acceptor amide C=O of both type I and II 1←4 H-bonded β-turns [26,27]. Therefore the IR spectra of the examined peptides could indicate the presence of some β-turn population and it seems that glycosylation of either Tyr^5 or Hyp^6 with acetylated or de-acetylated D-glucose moiety does not influence the peptide backbone conformation.

Peptides **I**, **II** and **V**–**VIII** show quite similar FT-IR spectra in TFE. Two main bands at 1632–1634 cm^{-1} and 1668–1670 cm^{-1}, with a shoulder at 1685–1686 cm^{-1}, characterize the region of the amide I modes, and the amide II shows a broadband at 1535–1550 cm^{-1}. Comparison of the amide I component bands in TFE solution with the amide I bands in the solid state shows significant differences in the relative intensities. In TFE, the component band at 1632–1634 cm^{-1} is generally more intense than in the solid state, suggesting that peptides in solution adopt a larger proportion of secondary structure. The peptides **III** and **IV** show two amide I component bands at 1630 cm^{-1} and 1656–1658 cm^{-1} (the more intense). The position of the spectrum, shifted to lower

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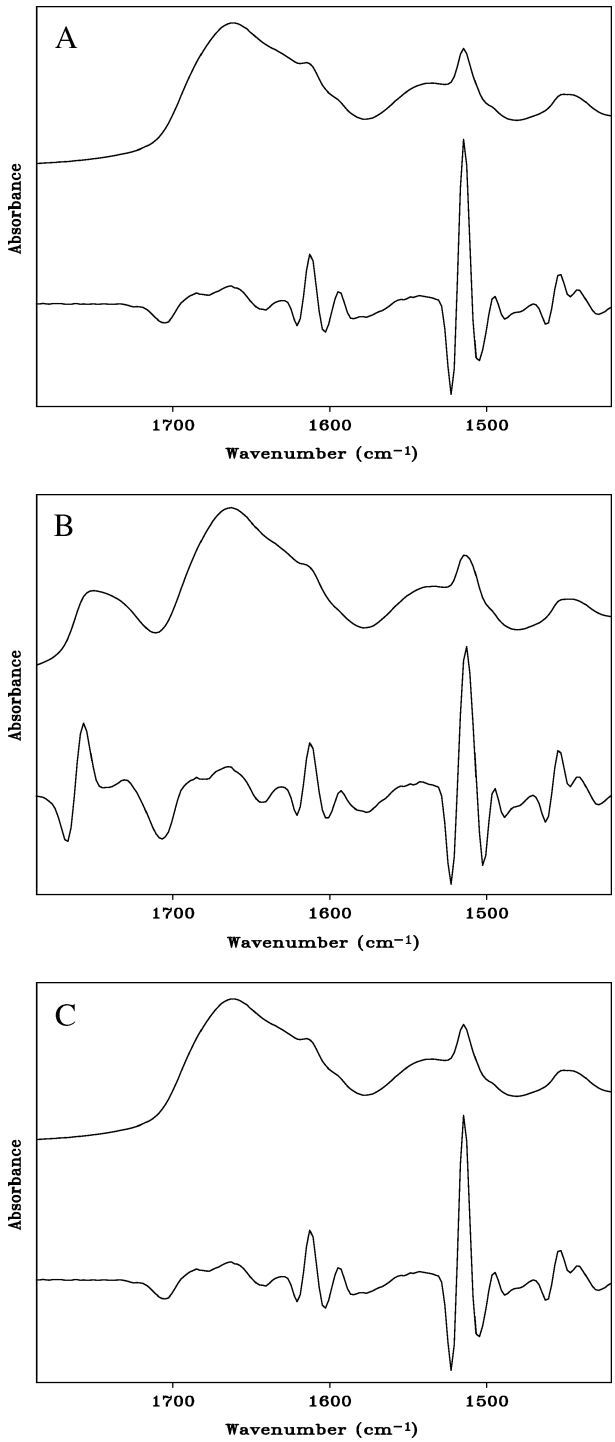

Figure 6 IR Spectra of **I** (A), **III** (B) and **IV** (C) in the 1440–1800 cm^{-1} region.

wavenumbers, could be associated with stronger hydrogen bonded amide groups. TFE is known for shifting the conformational equilibrium of peptides towards the adoption of folded conformers stabilized by intramolecular H-bonding. It can be concluded that the peptide conformational features estimated by the FT-IR spectroscopy measurements are in agreement with those suggested by the CD measurements.

Pharmacological Screenings

As already demonstrated, [Lys^7]-dermorphin is a highly selective μ-opioid agonist, and the new glycosylated [Lys^7]-dermorphin analogues were first tested *in vitro* on GPI smooth muscle preparations. The biological potencies of [Lys^7]-dermorphin, [Hyp^6, Lys^7]-dermorphin and the glucosylated analogues, evaluated for their ability to inhibit the electrically evoked twitch on isolated organ preparations, are reported in Table 2. [Hyp^6, Lys^7]-dermorphin displays comparable potency as [Lys^7]-dermorphin. Glucosylation of Tyr^5 reduces the potency of both [Lys^7]-dermorphin and [Hyp^6, Lys^7]-dermorphin by about 150 times, and the presence of the acetyl groups on the sugar hydroxyl functions strongly reduces (about 2000–3000 times) the agonistic potency of both glycosylated peptides. Similar but less dramatic effects are induced by the glucosylation of the Hyp^6 residue. The potency of the parent peptide is reduced by about 18–20 times (IC_{50} = 84 nM *vs* 4.6 nM), but the acetylation of the sugar moiety further reduces (by about 1000 times) the potency of [Hyp^6,Lys^7]-dermorphin.

Compounds **II**, **IV** and **VIII** were also tested in rats, after intrathecal injection, for their analgesic potencies. *In vivo*, compound **IV** is 60–80 times less potent than compound **II** and compound **VIII** is 8–10 times less potent than compound **II**, but its analgesic effect is longer-lasting (Figure 7).

These data showed that glucosylation of amino acid residues in position 6 (compound **VIII**) or, worse, in position 5 (compound **IV**) impairs the peptide's ability to bind and activate the μ-opioid receptors. However, the glucosylation-induced decrease in the antinociceptive potency is significantly lower than that induced in the μ-opioid receptor affinity, as we could assume from the GPI results. Such a difference might depend on the higher resistance to enzymatic degradation of the glucosylated peptides with respect to the parent peptide, as also indicated by the longer-lasting analgesic effect of the peptide **VIII**.

A similar significantly prolonged antinociceptive activity was shown by some synthetic dermorphin

Table 2 Biological Activity of Glycosylated [Lys^7]-dermorphin Analogues on GPI Preparations

Peptides	IC_{50} (nM)
I [Lys^7]-dermorphin	1.2 ± 0.2
II [Hyp^6,Lys^7]-dermorphin	4.6 ± 0.5
III {Tyr [β-Glc(Ac)$_4$]^5,Lys^7}-dermorphin	2215 ± 190
IV [Tyr (β-Glc)^{5}Lys^7]-dermorphin	179 ± 20
V {Tyr [β-Glc(Ac)$_4$]^5,Hyp^6,Lys^7}-dermorphin	13 269 ± 1500
VI [Tyr (β-Glc)^5,Hyp^6,Lys^7]-dermorphin	800 ± 79
VII {Hyp [β-Glc(Ac)$_4$]^6,Lys^7}-dermorphin	5728 ± 650
VIII [Hyp (β-Glc)^6,Lys^7]-dermorphin	84 ± 10

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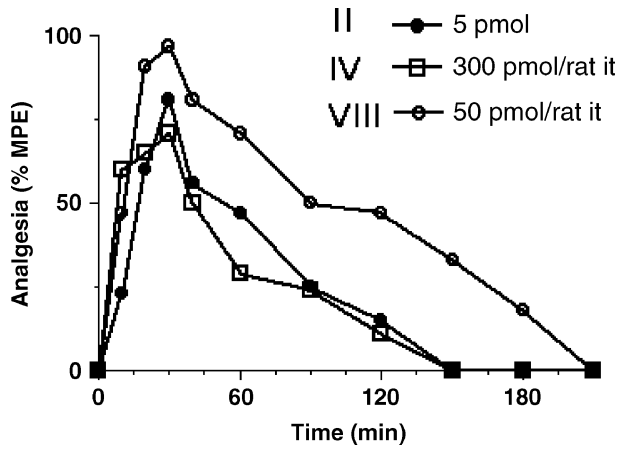

Figure 7 Time course of the antinociceptive effect of glycopeptides **II**, **IV** and **VIII** in rats, measured by the tail-flick test [15].

analogues, either *O*-β-glucosylated or α-galactosylated, on the *C*-terminal residue, [Ser(β-Glc)]^7-dermorphin and [Ala(α-Gal)]^7-dermorphin, respectively [9]. It is worth noting that glycosylation of the dermorphin *C*-terminal residue, either Ser^7 or Ala^7, slightly lowered the peptide receptor affinity and the biological potency on GPI; however, when administered i.c.v. and s.c. in rats, the glycosylated analogues were about 2 times more potent than dermorphin in reducing the nociceptive response to radiant heat. Acetylation of sugar hydroxyl groups reduces, 5–10 times, both biological activity on GPI and μ-receptor affinity, whereas the antinociceptive potency remains equal to (i.c.v.) or only 2–3 times (s.c.) lower than the dermorphin potency.

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