Synthesis and Pharmacological Activity of Deltorphin and Dermorphin-Related Glycopeptides

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The solid phase procedure, based on the Fmoc chemistry, was used to prepare some opioid deltorphin (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2, DEL C) and dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2, DER) analogues in which a D-glucopyranosyl moiety is β-O-glycosidically linked to a Thr^4 or Thr^7 side chain. Their activities were determined in binding studies based on displacement of μ- and δ-receptor selective radiolabels from rat brain membrane synaptosomes, in guinea pig ileum and rabbit jejenum bioassays, and, *in vivo*, by a mouse tail-flick test after intracerebroventricular (icv) and subcutaneous (sc) administrations. The glyco analogues modified at position 4 displayed low opioid properties, while Thr^7-glycosylated peptides retained high δ- or μ-selectivity and remarkable activity *in vivo*. In particular, as systemic antinociceptive agents, the latter glucoside-bearing compounds were more potent than the parent unglycosylated peptide counterparts, showing a high blood to brain rate of influx which may be due to the glucose transporter GLUT-1.

Introduction[1]

Phyllomedusa amphibians secrete two major classes of opioid peptides from their skin glands.[2] These heptapeptides,[2,3] called dermorphins (dermal morphine-like substances) and deltorphins (delta-specific opioids), are potent and selective μ- or δ-agonists, respectively. Dermophin[4] (DER, H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and deltorphin C[5] (DEL C, H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2, originally designated as [D-Ala^2]-deltorphin I[2]) are the prototypes of these two peptide families. More than 200 analogues of DER and DEL C were synthesized, and the role of each amino acid in binding to μ- and δ-receptors and bioactivity has been defined.[6−16] Both natural heptapeptides and their synthetic analogues are generally resistant to enzymatic degradation,[17,18] and showed potent antinociceptive effects following intracerebroventricular administration, but only moderate or negligible central actions after systemic administration in mice and rats.[8,12,19−21] It was suggested that inadequate blood−brain barrier (BBB) permeation was responsible for the different analgesic potencies.

Since the quantities of peptide opioids normally transported into the CNS are relatively low,[22] one major avenue of investigation involves the development of opioid analogues that penetrate the BBB. Modification of opioid physicochemical properties, such as potentiating lipophilicity[23,24] or glucosylation[25,26] in order to take advantage of the glucose transporters,[27] represented efforts to increase the BBB permeation. Following the latter strategy, Polt et al.[25] designed and prepared glycopeptide enkephalin analogues able to cross the BBB and to produce prolonged analgesia in mice.[25] These considerations have prompted us to prepare and test DER and DEL C analogues in which D-glucopyranosyl units are β-O-glycosidically linked to Thr^4 or Thr^7 side chains (Table 1 and Scheme 1). We have previously found that some substitutions at position 4 or 7 in both heptapeptides, as well as the C-terminal extension in DER, are tolerated or slightly advantageous for bioactivity,[5,8,10,16] and this is also expected to be the case of O-glycosylated analogues **1**−**8**.

Results and Discussion

Synthesis. Glycopeptides **1**−**8** were synthesized by solid phase method (Scheme 1) as C-terminal amides on a 4-methylbenzhydrilamine polystyrene resin (MBHA resin) functionalized with the linker 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamidonorleucine,[28] using DMF as solvent. The stepwise synthesis, by a fully automated continuous-flow peptide synthesizer, was carried out by Fmoc chemistry, and no special efforts were made to optimize the repetitive steps. The N^{α}-Fmoc amino acids carrying standard side chain protective groups and the building block Fmoc-Thr[β-Glc$(OAc)_4$]-OH were converted to benzotriazolyl esters with HOBt and DIPCDI in the synthesizer. The protected monosaccharide derivative of Fmoc-threonine was prepared from Z-Thr-OBzl by a procedure described and discussed previously.[29] The Fmoc group was cleaved with 20% piperidine−DMF solution. After completion of the synthesis the protected β-glucosylated peptides were cleaved from the resin, and the amino acid side chains were simultaneously deprotected by treatment with TFA/H_2O/Et_3SiH (88:5:7) mixture. The resulting acetylated glycopeptides were purified by preparative HPLC. Acetyl groups were removed from carbohydrate unit using hydrazine in methanol[30] (Scheme 1). No particular base-induced side reactions[31] during Fmoc deblocking and carbohydrate deacetylation of glycopeptides were observed. Purification of target glycopeptides

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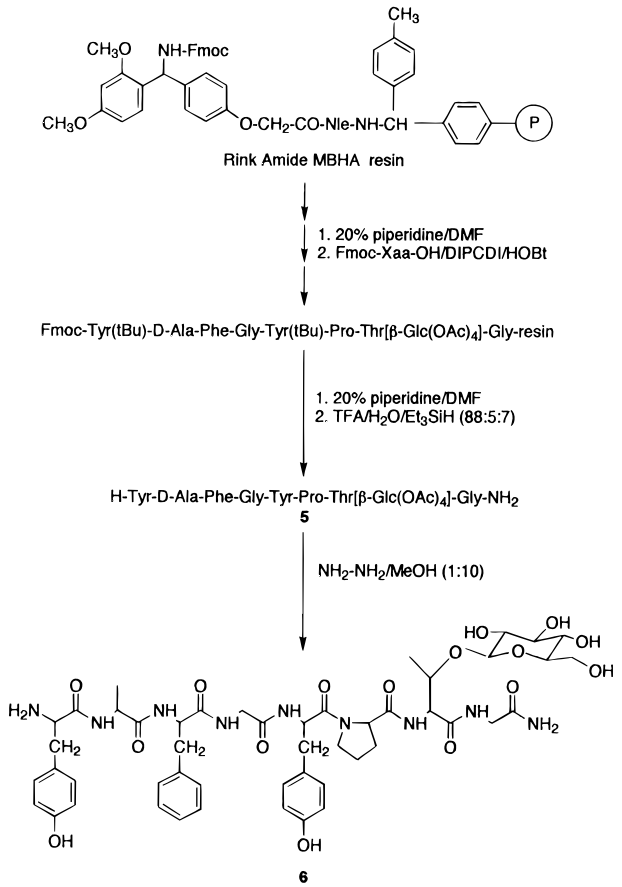
Table 1. Receptor Binding Properties of Glycopeptides

no.	peptide ligand	$K_i\delta$ (nM)	$K_i\mu$ (nM)	$K_i\delta/K_i\mu$
1	Tyr-D-Ala-Phe-Asp-Val-Val-Thr[β-D-Glc(OAc)$_4$]-Gly-NH$_2$	1.30 ± 0.3	634.9 ± 94	0.002
2	Tyr-D-Ala-Phe-Asp-Val-Val-Thr(β-D-Glc)-Gly-NH$_2$	1.55 ± 0.5	578.9 ± 38	0.002
3	Tyr-D-Ala-Phe-Thr[β-D-Glc(OAc)$_4$]-Val-Val-Gly-NH$_2$	2,110	1,520	1.3
4	Tyr-D-Ala-Phe-Thr(β-D-Glc)-Val-Val-Gly-NH$_2$	9,500	1,993	4.8
	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH$_2$ (deltorphin C)	0.15 ± 0.03	147 ± 29	0.001
5	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Thr[β-D-Glc(OAc)$_4$]-Gly-NH$_2$	86.8 ± 14.7	0.46 ± 0.06	190
6	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Thr(β-D-Glc)-Gly-NH$_2$	67.0 ± 22	0.29 ± 0.02	231
7	Tyr-D-Ala-Phe-Thr[β-D-Glc(OAc)$_4$]-Tyr-Pro-Ser-NH$_2$	22,600	4,400	5
8	Tyr-D-Ala-Phe-Thr(β-D-Glc)-Tyr-Pro-Ser-NH$_2$	1,760	1,700	1
	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH$_2$ (dermorphin)	82.5 ± 16.1	0.28 ± 0.03	295

[a] K_i is the affinity constant derived from the IC_{50} values according to Cheng and Prusoff[37] given as the mean ± standard error (n = 3−9).

Scheme 1. Synthesis of Glycopeptides **5** and **6**



Xaa = Gly, Thr[β-Glc(OAc)$_4$], Pro, Tyr(tBu), Phe, D-Ala.

was achieved by preparative HPLC, and homogeneity of the purified products was assessed by analytical HPLC. Structure verification was achieved by amino acid analysis, mass spectrometry, and NMR spectroscopy.

Binding Property and Biological Activity. Opioid receptor affinities of glycopeptides were determined by displacement of selective radioligands from rat brain membrane synaptosomes. The δ- and μ-opioid receptor binding sites were specifically labeled using [^{3}H][D-Pen^{2,5}]enkephalin (DPDPE) and [^{3}H][DAla^2,MePhe^4,Gly-ol^5]enkephalin (DAGO), respectively (Table 1). Furthermore, the bioactivity of compounds listed in Table 1 were tested *in vitro* in two isolated organ preparations, guinea pig ileum (GPI) and rabbit jejunum (RJ), run as described previously[14,32] (Table 2).

Competitive-binding experiments (Table 1) indicate that in the DEL C peptides the presence of a D-glucopyranosyl moyety linked to Thr side chains differentially affects affinities (K_i) and selectivity ($K_{i\delta}/K_{i\mu}$). In fact, the O-glycosylation at Thr^7 (compounds **1** and **2**) produces a moderate decrease in δ- and in μ-receptor affinities, while the introduction of the identical carbohydrate unit at Thr^4 (compounds **3** and **4**) is very detrimental, particularly at δ-sites. A similar trend was also observed for DER-related glycopeptides **5**−**8**. In fact, analogues **5** and **6** modified at position 7 possess affinity and selectivity characteristics close to those of DER, whereas compounds **7** and **8** modified at position 4 display low affinities.

Table 2. *In Vitro* and *in Vivo* Activities of Glycopeptides

	IC_{50} (nM)		tail-flick test[b]	
compound	GPI[a]	RJ[a]	icv ED_{50} (nM)	sc ED_{50} (μmol/kg)
1		890 ± 81	10.5 (6.6−23.2)	−[c]
2		995 ± 89	11.3 (7.1−22.9)	96.4 (51.8−168)
3	nt	>4000	nt	
4	nt	>4000	nt	
5	22.1 ± 2.4		2.8 (1.9−3.8)	88.9 (46.5−151)
6	3.1 ± 0.2		1.3 (0.9−2.7)	0.53 (0.38−1.31)
7	>4000		nt	nt
8	3400		nt	nt
DEL C		510 ± 62	5.0 (4.3−6.1)	−[c]
DER	2.1 ± 0.18		0.015 (0.009−0.030)	3.1 (1.9−5.9)
morphine			3.2 (2.9−3.7)	10.9 (7.0−18.8)

[a] The values are the means of six experiments ± SEM. [b] Analgesia of at least four doses of each compound was investigated. Each dose was tested for at least 10 animals. [c] Highest doses tested, from 80 to 150 μmol/kg, were unable to produce a significant analgesia. [d] nt = not tested.

These results reinforced earlier observations indicating that modifications at the C-terminal part in DEL C and DER permit expression of high δ- and/or μ-receptor affinities, whereas the changes accompanying modifications at position 4 depend on the chemical nature or bulkiness of the side chain.[5,10,16] The present data indicate that the loss of backbone flexibility imposed by the bulky glycosylated Thr^4 in DER and DEL C peptides may presumably limit the opioid binding.[16] The binding properties for μ- and δ-opioid receptors for all of glycopeptides were consistent with the biological activity as tested by GPI and RJ bioassays. These experiments indicated that DER glycopeptides **5** and **6** reduced the electrically induced contractions of guinea pig ileum while DEL C glyco derivatives **1** and **2** failed to cause significant inhibition up to micromolar concentrations (Table 2). Since naloxone at a concentration of 0.05 nM reversed the inhibition effect of **5** and **6**, the results are entirely consistent with an interaction of μ-opioid receptors. On the other, the inhibition of the spontaneous contractions of rabbit jejenum by glyco compounds **1** and

2 indicated an interaction with δ-opioid receptors since these effects were totally blocked by naltrindole,[33] an established δ-opioid receptor antagonist, at an equimolecular concentration.

Analgesic Assay. *In vitro* active glycopeptides were also tested *in vivo* by a mouse tail-flick assay[34] after intracerebroventricular (icv) and subcutaneous (sc) injections as described previously.[35] In this test, icv administration of DEL C analogues **1** and **2** displayed significant antinociceptive action (Table 2) comparable to that of the parent DEL C. The analgesic profile of the latter compound is in agreement with the trend previously observed for [D-Ala^2]deltorphin II.[20] DER-related glycopeptides **5** and **6** were about 5-fold more analgesically potent than **1** and **2** but were also 2 orders of magnitude less active than DER. This is quite unexpected since glycopeptides **5** and **6** showed binding properties and *in vitro* activities (Table 1 and 2) rather similar to that of DER. The discrepancies between binding or bioassay data and icv analgesia may be due to a different degree of nonspecific adsorption of peptides in various tissues or to reduced intrinsic activity of glycopeptides at the central opioid receptors. Since DER and Tyr-D-Ala-Phe-Gly-Tyr-Pro-Thr-Gly-NH_2 possess enzymatic stability[8,17] and since a glycopeptide has generally enhanced resistance to brain enzymes,[25,26] all examined compounds can be expected to be equally stable against enzymolysis under the conditions of the tests, and thus, a different extent of peptide degradation in the various tissues can be ruled out as a factor explaining the differences between different bioassay data. Naltrindole (0.05 nM, icv) antagonized the antinociceptive action during coadministration of DEL C peptides, while pretreatment with naloxone (0.05 nM, icv) antagonized the activity of DER derivatives (data not shown). Evaluation of the antinociceptive effects of glycopeptides after sc injection in mice (Table 2) revealed additional interesting differences due to structural modifications to DEL C and DER peptides. The fully acetylated glycopeptide **1**, like DEL C, was unable to produce antinociception up to 150 μM/kg, while deacetylated glycopeptide **2** showed low but significant analgesia. Apparently this result can be interpreted to be in agreement with the predictions made on the basis of the glucose transporter(s) concept[27] and with the trend previously observed for glycoenkephalins.[25] Such an interpretation seems to be supported by the comparison of analgesic characteristics that pertain to DER analogues. In fact, glycopeptide **6** displayed remarkable sc analgesic activity: it was 20 times as potent as morphine and, particularly, 170 and 6 times more potent than its parent acetylated glycoanalogue **5** and dermorphin, respectively. This finding is even more remarkable when one takes into account that unglycosylated peptide Tyr-D-Ala-Phe-Gly-Tyr-Pro-Thr-Gly-NH_2 retains only 20−30% of DER sc analgesia,[8] and considering that icv antinociception of glycopeptide **6** was significantly much lower than that of DER (Table 2).

In summary, the present results showed that an appropriate attachment of β-D-glucose unit in deltorphin or dermorphin peptides give rise to glyco analogues retaining high δ- or μ-selectivity and remarkable activity *in vivo*. In particular, as systemic antinociceptive agents, the glucoside-bearing compounds **2** and **6** are more potent than the parent unglycosylated peptide counterparts. This probably indicates that peripheral glycopeptides possess (a) an adeguate diffusion, from subcutaneous sites into the bloodstream, which can be attributed to their evident water solubility, and (b) a high blood to brain rate of influx which may be due, at least in part, to the glucose transporter GLUT-1.[27]

Experimental Procedures

General. Melting points were determined by a Kofler apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter with a 10 cm water jacketted cell. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) were obtained using the HPG2025A mass spectra. HPLC analysis was performed on Bruker liquid chromatograph LC21-C instrument using a Vydac C 18 218 TP 5415 column (175 × 4.5 mm, 5 μm particle size) equipped with a Bruker LC 313 UV variable-wavelength detector; recording and quantification were accomplished with a chromatographic data processer (Epson computer FX80X7). Analytical determinations were carried out by two solvent systems: (1) A: 10% (v/v) acetonitrile in water, B: 60% (v/v) acetonitrile in water, both containing 0.1% TFA, linear gradient from 0 to 100% B in 25 min at a flow of 1 mL/min; (2) A: NaH_2PO_4 buffer (0.05 M), B: acetonitrile, linear gradient from 0 to 50% B in 25 min at a flow of 1 mL/min. All analogues showed purity greater than 99% following analytical HPLC monitored at 220 nm. Preparative reversed phase HPLC was carried out with a Water Delta Prep 3000 using a Delta Pack C 18-300 A column (30 mm × 30 cm, 15 μm, spherical). The mobile phase and the gradient used were identical to that of analytical determinations. Chromatography was performed at a flow rate of 30 mL/min. ^{1}H-NMR spectra were obtained on a Bruker spectrometer (Bruker WM 500 MHz). Amino acid analyses were carried out using PITC methodology as the amino acid derivatization reagent (Pico-Tag, Waters-Millipore, Waltham, MA). Lyophilized samples of peptides (50−100 pmol) were placed in heat-treated borosilicate tubes (50 × 4 mm), sealed, and hydrolyzed using 200 μL of 6 N HCl containing 1% phenol in the Pico-Tag work station for 1 h at 150 °C. A Hypersil ODS column (250 × 4.6 mm, 5 μm particle size) was employed to separate the PITC-amino acid derivatives. TLC used precoated plates of silica gel F254 (E. Merk, Darmstadt, FRG) in the following solvent system: (A) 1-butanol/acetic acid/H_2O (3:1:1), (B) EtOAc/pyridine/acetic acid/H_2O (12:4:1.2:2.2), (C) CH_2Cl_2/MeOH/toluene (8.5:1:0.5), (D) $CHCl_3$/MeOH/benzene/H_2O (8:8:8:1). Ninhydrin 1%, fluorescamine, and chlorine spray reagents were employed to detect the peptides.

General Procedure for Solid Phase Glycopeptide Synthesis. Glycopeptides **1**−**8** (Tables 1 and 2) were prepared by solid phase method (Scheme 1) with a continuous-flow instrument with on-line UV monitoring (Milligen/Biosearch 9050). The stepwise syntheses were carried out by Fmoc chemistry, and no special efforts were made to optimize the repetitive steps. For each glycopeptide, 0.5 g (0.06 mequiv) of MBHA resin (Novabiochem, Läufelfingen, Switzerland) functionalized with the linker 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamidonorleucine[28] (Novabiochem) was used. The functionalized resin was swelled in DMF for 1 h and packed in the reaction column. *tert*-Butyl was used as a side chain protecting group for serine, aspartic acid, and tyrosine. N^{α}-Fmoc amino acids as well as Fmoc-Thr[β-Glc-$(OAc)_4$]-OH in a 4-fold excess using DIPCDI in the presence of HOBt always in 4-fold excess for 1 h. The Fmoc group was cleaved with a flow of 20% piperidine in DMF for 25 min. After completion of the synthesis, each protected β-glucosylated peptide was cleaved from the resin, and the amino acid side chains were simultaneously deprotected by treatment with modified reagent B (88% TFA, 5% H_2O, 7% Et_3SiH) 7 mL for 1 h at room temperature. The resin was removed by filtration and washed with TFA (2 × 1 mL), the filtrate and washing were combined and evaporated at 25 °C, and the oily residue was triturated with ethyl ether (10 mL). The resulting solid

acetylated glycopeptide was collected by centrifugation and purified by preparative HPLC (purification yield, 79−88%). Acetyl groups were removed from carbohydrate moiety using hydrazine in methanol.[31] Concentration of the solution and purification of the residue by preparative HPLC then gave the target glycopeptides. The homogeneity of the purified products was accessed by analytical HPLC. Structural verification was achieved by amino acid analysis, mass spectrometry, and ^{1}H-NMR spectroscopy.

H-Tyr-D-Ala-Phe-Asp-Val-Val-Thr[β-D-Glc(OAc)$_4$]-Gly-NH$_2$ (1). Synthesis, cleavage of the resin-bound glycopeptide, and purification by HPLC, according to the general procedure, gave O-acetylated **1** (79%): HPLC$_{K'}$ 5.43 (1), 6.71 (2); mp 180−182 °C; $[\alpha]^{21}_D$ −33.5 (*c* 1.0, MeOH); ^{1}H-NMR (DMSO) of the carbohydrate part of the glycopeptide δ 4.14 (H-1), 3.27 (H-2), 3.33 (H-3), 3.80 (H-4), 3.42 (H-5), 3.69 (H-6); ^{1}H-NMR (DMSO) of the peptide part of glycocompound δ 1.08−1.37 (γ, Val), 1.13−1.43 (γ, Val), 1.18 (β, D-Ala), 1.45 (γ, Thr), 1.75 (β, Val), 2.30−2.54 (β, Asp), 2.85 (β, Phe), 2.93−2.95 (β, Tyr), 3.38−3.49 (β, Thr), 3.60−3.68 (α, Gly), 3.73 (α, Asp), 4.08 (α, Val), 4.11 (α, Val), 4.15 (α, Thr), 4.20 (α, D-Ala), 4.31 (α, Phe), 4.42 (α, Tyr), 6.68−7.05 (arom, Tyr), 7.12 (α-CONH$_2$, Gly), 7.23 (arom, Phe), 7.82 (NH, Val), 7.85 (NH, Val), 7.95 (NH, Thr), 7.99 (NH, Asp), 8.10 (NH, Phe), 8.28 (NH, D-Ala), 8.33 (NH, Gly), 9.20 (OH, Tyr); MS (M + H)$^+$ 1200 (calcd 1200). Amino acid analysis: Tyr 0.96 (1), Ala 0.97 (1), Phe 1.00 (1), Asp 1.05 (1), Val 1.95 (2), Thr 0.96 (1), Gly 1.03 (1).

H-Tyr-D-Ala-Phe-Asp-Val-Val-Thr(β-D-Glc)-Gly-NH$_2$ (2). The title glycopeptide was obtained by deacetylation of **1** and subsequent purification according to the general procedure (87%): HPLC$_{K'}$ 3.19 (1), 4.52 (2); mp 199−203 °C; $[\alpha]^{21}_D$ −41.7 (*c* 1.0, MeOH); MS (M + H)$^+$ 1033 (calcd 1033). Amino acid analysis: Tyr 0.97 (1), Ala 0.98 (1), Phe 1.00 (1), Asp 1.03 (1), Val 1.97 (2), Thr 0.96 (1), Gly 1.02 (1).

H-Tyr-D-Ala-Phe-Thr[β-D-Glc(OAc)$_4$]-Val-Val-Gly-NH$_2$ (3). It was prepared and purified as for **1** according to general procedure (purification yield 86%): HPLC$_{K'}$ 6.69 (1), 7.21 (2); mp 166−170 °C; $[\alpha]^{21}_D$ −18.8 (*c* 1.0, MeOH); MS (M + H)$^+$ 1085 (calcd 1085). Amino acid analysis: Tyr 0.95 (1), Ala 1.04 (1), Phe 1.00 (1), Val 1.86 (2), Thr 0.97 (1), Gly 1.05 (1).

H-Tyr-D-Ala-Phe-Thr(β-D-Glc)-Val-Val-Gly-NH$_2$ (4). It was obtained by the deacetylation of **3** (87%): HPLC$_{K'}$ 4.69 (1), 5.27 (2); mp 190−193 °C; $[\alpha]^{22}_D$ −16.6 (*c* 1.0, MeOH); MS (M + H)$^+$ 917.5 (calcd 917.5). Amino acid analysis: Tyr 1.01 (1), Ala 0.99 (1), Phe 1.00 (1), Val 1.96 (2), Thr 0.95 (1), Gly 1.02 (1).

H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Thr[β-D-Glc(OAc)$_4$]-Gly-NH$_2$ (5). The title compound was prepared and purified according to the general procedure (84%): HPLC$_{K'}$ 3.94 (1), 4.51 (2); mp 184−187 °C; $[\alpha]^{21}_D$ −21.8 (*c* 1.0, MeOH); MS (M + H)$^+$ 1035 (calcd 1035). Amino acid analysis: Tyr 1.94 (2), Ala 1.01 (1), Phe 1.00 (1), Pro 0.96 (1), Thr 0.97 (1), Gly 2.03 (2).

H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Thr(β-D-Glc)-Gly-NH$_2$ (6). It was obtained by deacetylation of **5** (88%): HPLC$_{K'}$ 2.62 (1), 3.34 (2); mp 202−205 °C; $[\alpha]^{21}_D$ −35.4 (*c* 1.0, MeOH); ^{1}H-NMR (DMSO) of the carbohydrate part of glycopeptide δ 4.17 (H-1), 3.40 (H-2), 3.31 (H-3), 3.72 (H-4), 3.42 (H-5), 3.62 (H-6); ^{1}H-NMR (DMSO) of the peptide part of glycocompound δ 1.15 (β, D-Ala), 1.47 (γ, Thr), 1.63−1.69 (γ, Pro), 1.84−1.87 (β, Pro), 2.58 (β, Phe), 2.73−2.75 (β, Tyr), 2.77−2.79 (β, Tyr), 2.94−2.97 (δ, Pro), 3.29−3.37 (β, Thr), 3.50−3.55 (α, Gly), 3.60−3.66 (α, Gly), 4.13 (α, Thr), 4.17 (α, Pro), 4.22 (α, D-Ala), 4.33 (α, Phe), 4.43 (α, Tyr), 4.48 (α, Tyr), 6.60−7.10 (2 arom, Tyr), 7.17 (α-CONH$_2$, Gly), 7.26 (arom, Phe), 7.92 (NH, Thr), 8.09 (NH, Phe), 8.21 (NH, Tyr), 8.32 (NH, D-Ala), 8.35 (NH, Gly), 8.40 (NH, Gly), 9.18−9.24 (2 OH, Tyr); MS (M + H)$^+$ 868 (calcd 868). Amino acid analysis: Tyr 1.81 (2), Ala 1.02 (1), Phe 1.00 (1), Pro 0.97 (1), Thr 0.94 (1), Gly 2.04 (2).

H-Tyr-D-Ala-Phe-Thr[β-D-Glc(OAc)$_4$]-Tyr-Pro-Ser-NH$_2$ (7). The title glycopeptide was synthesized and purified as described in the general procedure (81%): HPLC$_{K'}$ 4.12 (1), 5.23 (2); mp 179−181 °C; $[\alpha]^{21}_D$ −27.8 (*c* 1.0, MeOH); MS (M + H)$^+$ 1008 (calcd 1008). Amino acid analysis: Tyr 1.91 (2), Ala 1.05 (1), Phe 1.00 (1), Pro 0.97 (1), Thr 0.96 (1), Ser 0.97 (1).

H-Tyr-D-Ala-Phe-Thr(β-D-Glc)-Tyr-Pro-Ser-NH$_2$ (8). It was obtained by deacetylation of **7** (84%): HPLC$_{K'}$ 2.75 (1), 3.93 (2); mp 201−203 °C; $[\alpha]^{21}_D$ −21.4 (*c* 1.0, MeOH); MS (M + H)$^+$ 841 (calcd 841). Amino acid analysis: Tyr 1.92 (2), Ala 1.03 (1), Phe 1.00 (1), Pro 1.01 (1), Thr 0.92 (1), Ser 0.95 (1).

Receptor Binding Assays. Synaptosomal preparations (P$_2$) were prepared from Sprague−Dawley CD male rats as described.[5,36] The synaptosomes were preincubated to remove endogenous opioids and washed thrice by centrifugation before storage in buffer (50 mM HEPES, pH 7.5 containing 20% glycerol, 50 μL/mL aliquots at −80 °C.[36] Under these conditions, synaptosomes could be thawed and frozen without deleterious effects on binding.[36] Receptor binding assays were measured by filtration through Whatman glass fiber filters (GC/C) and washed 3 × 2 mL with ice-cold buffered BSA within 5 s. Steady-state binding was conducted at 22 °C for 120 min in duplicate assays containing 1.6 mg of protein using 0.62 nM [^{3}H]DPDPE (34.3 Ci/mmol, New England Nuclear-DuPont, Boston, MA) to label δ-sites and 0.68 nM [^{3}H]DAGO (60 Ci/mmol, Amersham, Arlington, IL) for μ-sites as detailed previously.[36] Each peptide was assayed with five to seven concentrations (three to nine repetitions for each concentration) with three to five synaptosomal preparations on consecutive days. The difference in radioligand bound to that displaced by either 1−2 μM DPDPE or DAGO is defined as specific binding. Filters were dried at 80 °C and bound radioactivity measured by liquid scintillation spectrometry using CytoScint (ICN, Irvine, CA). The K_i values were calculated from the IC$_{50}$ according to Cheng and Prusoff.[37]

Guinea Pig Ileum (GPI) and Rabbit Jejunum (RJ) Bioassays. Bioassays were conducted according to *Guerrini et al.*[35] using a 2−3 cm portion of guinea pig ileum (GPI) in a 20 mL organ bath containing 70 μM hexamethonium bromide and 0.125 μM mepyramine maleate aerated with 95% O$_2$/5% CO$_2$ at 36 °C for μ-receptors as follows: GPI was stimulated transmurally with 0.5 ms square-wave pulses at 0.1 Hz in which the stimulus was 1.5 times that necessary to produce a maximal twitch (∼30 V) and recorded at a magnification ratio of 1:15. The IC$_{50}$ is the concentration of compound necessary to inhibit the amplitude of the electrically induced twitch by 50%. For δ receptors a 3 cm segment of rabbit jejunum (RJ) was used suspended in a 10 mL organ bath containing tyrode solution of the following composition (nM): NaCl 137, KCl 2.7, CaCl$_2$ 1.8, MgCl$_2$ 1.05, NaH$_2$PO$_4$ 0.4, NaHCO$_3$ 11.9, and glucose 5, gassed with 95% O$_2$ and 5% CO$_2$ and maintained at 37 °C. The tissue was connected to an isotonic transducer (Ugo Basile, Italy) counterbalanced by 1 g loading and allowed to equilibrate for 45 min; during this period regular spontaneous activity was recorded without washing. All experiments were repeated on at least four separate jejunum preparations obtained from different rabbits. Dose−response curves were prepared for each compound in comparison to known compounds for each tissue preparation (dermorphin for GPI, and deltorphin C for RJ). The K_e values for the antagonists were determined from the ratio of IC$_{50}$ values obtained in the presence and absence of a fixed antagonist concentration as detailed previously.[32,35]

Antinociception. The analgesic potency of glycopeptides and reference compounds was estimated in Swiss-Webster mice weighing 23−25 g. The tail-flick test was essentially that described by Janssen,[34] using water at 55 °C as nociceptive stimulus. Tests were made prior to and at various times after icv and sc administration of each compound in saline (4 μL). The average reaction time in control animals was 4 s. Complete analgesia was assumed to be present when no reaction appeared 12 s after application of noxious stimulus. Percent analgesia was calculated according to the formula $[(T - T_0)/(12 - T_0)] \times 100$ (T = reaction time (seconds) after administration of compound; T_0 = "normal" reaction time before injection of compound; 12 = cutoff time). The specificity of the effects was tested by pretreating the animals with naloxone or naltrindole (1−5 mg/kg sc). In all cases, the appropriate antagonist prevented any analgesic effect. The median antinociceptive dose (ED$_{50}$) and 95% confidence limits were calculated according to the method of Litchfield and Wilcox.[38]

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References

(1) The abbreviations used are in accord with the recommendations of the IUPAC−IUB Commission on Nomenclature (*J. Biol. Chem*. **1972**, *247*, 977). Other abbreviations: Ac, acetyl; tBu, *tert*-butyl; Bzl, benzyl; DMF, dimethylformamide; DIPCDI, 1,3-diisopropylcarbodiimide; DMSO, dimethyl sulfoxide; EtOAc, ethyl acetate; Et_3SiH, triethylsilane; Fmoc, fluoren-9-ylmethoxycarbonyl; Glc, D-glucopyranosyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption-time of flight; MeOH, methyl alcohol; MS, mass spectrometry; NMM, *N*-methylmorpholine; PITC, phenyl isothiocyanate; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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