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Synthesis and in vitro evaluation of lipoamino acid and carbohydrate-modified enkephalins as potential antinociceptive agents

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Abstract

The principal hindrance to drug uptake into central nervous tissue is the blood–brain barrier (BBB). In addition, potential peptide-based neuropharmaceuticals are rapidly destroyed by intra- and extracellular peptidases. In an attempt to address these biological hurdles, a series of lipo-, glyco- and glycolipo-conjugates of Leu-enkephalin have been synthesised via novel solid-phase strategies, and their in vitro activity assessed using mouse vas deferens (MVD) and guinea pig ileum (GPI) assays. Conjugation of a single lipoamino acid onto the C-terminal of the Leu-enkephalin molecule retains biological activity whilst increasing the molecule's overall lipophilicity. Conjugation of a glucuronic acid analogue in an analogous position, however, increases activity 40-fold when compared to the native peptide and induces a high degree of δ-opioid receptor selectivity. © 1998 Elsevier Science B.V.

Keywords: Blood–brain barrier; Drug delivery; Enkephalins; Solid-phase peptide synthesis; Lipoamino acids; Glycopeptides

1. Introduction

The blood–brain barrier (BBB) is a crucial element in the regulation and constancy of the brain's internal environment. The presence of tight junctions and lack of aqueous pathways between cells greatly retards the movement of polar solutes between the capillary lumen and the cerebral tissue (Zlokovic, 1995; Begley, 1996; Pardridge, 1996, and references cited therein). Peptides provide excellent examples of such polar solutes which have also been demonstrated to

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exert multiple biological actions within the CNS, and are therefore extremely attractive targets for neuropharmaceuticals (Pardridge et al., 1991). However, their lack of penetration through the BBB, and rapid degradation by cellular and extracellular peptidases (Brownson et al., 1994), remain major obstacles which must be addressed before their clinical application becomes tangible.

One of the most extensively studied biologically active family of peptides is the enkephalins (Hughes et al., 1975). These centrally acting antinociceptive agents are ideal molecular templates for the development of novel analgesics which may not produce the undesired side effects exhibited by opioids currently utilised in the clinic. However, as with most other peptides, the native compounds are rapidly catabolised via enzymatic degradation following administration, and any which remains within the blood stream displays poor BBB transport due to its highly polar nature. Many groups have attempted to address these two issue by either synthesising atypical peptidomimetics that are converted to a lipophilic analogue to increase the molecule's membrane permeability (Tsuzuki et al., 1991) or conjugating the native peptide to a molecular chaperone in an attempt to 'piggy-back' the molecule across the blood–brain barrier (Polt et al., 1994). In both cases the transformation of the overall molecular architecture was envisaged to bestow a degree of peptidase resistance to the active peptide.

In the case of molecular chaperones, a commonly exploited system was that of glucose uptake, and many groups have conjugated glucose mimetics to both Leu- and Met-enkephalins in an attempt to increase BBB permeability and receptor subtype selectivity (Varga-Defterdarovic et al., 1992; Horvat et al., 1993). However, in both the case of the latter and the former examples, the synthetic methodology involved to produce the required conjugates is protracted in nature and often poorly yielding.

We now report a facile, high yielding and reproducible methodology with which to generate lipid-modified, glycosylated and glyco-lipid-modified enkephalins via solid-phase peptide synthetic techniques in an attempt to deliver potent and efficacious antinociceptive agents to the CNS.

2. Materials and methods

2.1. General experimental

^{1}H NMR spectra were obtained on Varian XL-300 and Bruker AM500 instruments operating at fields of 300 and 500 MHz, respectively. Chemical shifts are reported in ppm downfield from an internal tetramethylsilane standard. Mass spectra were recorded using either a VG Analytical ZAB-SE instrument using fast atom bombardment (FAB) ionisation, or a Fisons VG-TOF spectrometer using matrix-assisted laser desorption ionisation (MALDI).

2-*tert*-Butoxycarbonylaminoalkanoic acids were synthesised from their corresponding 1-bromoalkanes and diethylacetamido malonate (Gibbons et al., 1990).

2.2. Peptide synthesis

Peptide **1a**: 4-methyl benzhydrylamine (MBHA) resin (substitution ratio, 0.48 mmol/g) (500 mg) was swelled in *N*,*N*′-dimethylformamide (DMF) for 60 min. Using a sintered glass peptide synthesis reaction vessel, the following manipulations were performed on the resin.

Operation		Time
(1)	DMF wash	5 min
(2)	Boc-C_{10}-OH (275 mg, 0.96 mmol) HBTU 364 mg, 0.96 mmol), HOBt (73 mg, 0.48 mmol), DIEA (334 μl, 1.9 mmol) in DMF	
	Shake	20 min
(3)	Ninhydrin test.	
(4)	DMF wash	5 min
(5)	TFA (100%)	2×1 min
(6)	DMF wash	5 min

Abbreviations: DIEA, diisopropylethylamine; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; TFA, trifluoroacetic acid.

The above procedures were then applied to the remaining amino acid residues; Boc-Leu-OH (239 mg, 0.96 mmol), Boc-Phe-OH (254 mg, 0.96 mmol), Boc-Gly-OH (168 mg, 0.96 mmol), Boc-Gly-OH (168 mg, 0.96 mmol) and Boc-Tyr(2-Br-Z)-OH (475 mg, 0.96 mmol).

Upon completion of the synthesis and removal of the terminal Boc group, the resin was washed with DMF (3×20 ml), dichloromethane (DCM) (3×20 ml), methanol (3×20 ml) and finally DCM (2×20 ml). The resin was then dried in vacuo over potassium hydroxide (KOH) overnight. The peptides were cleaved from the resin with concomitant side chain deprotection using a high HF method (600 mg *p*-cresol, 10 ml HF). The cleaved peptide was precipitated using HPLC-grade diethyl ether, redissolved in 50% acetic $acid_{(aq)}$ and lyophilised to afford the peptide as a white amorphous powder. Peptides **1b** to **2f** were synthesised in an analogous manner.

2.3. *N*-(2,3,4,5-Tetra-*O*-acetyl-β-D-gluco-pyranosyl)-succinate

2,3,4,5 - Tetra - *O* - acetyl - β - D - glucopyranosylamine (26.72 g, 77 mmol) was dissolved in DCM (130 ml) containing pyridine (30 ml) and *N*,*N*′-dimethylaminopyridine (0.50 g). The solution was cooled to 0°C and succinic anhydride (15.41 g, 154 mmol) was added over 1 h. The reaction mixture was allowed to slowly warm to room temperature and stirred for an additional 3 h. The solution was then diluted with DCM (100 ml) and washed with 5% $HCl_{(aq)}$ (300 ml). The organic layer was dried ($MgSO_4$) and evaporated to dryness in vacuo. The crude compound was recrystallised from ethyl acetate to afford the title compound as a white crystalline solid (29 g, 84%), m.p = 142–143°C. ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.03, 2.05, 2.06, 2.08 (4s, 4×3H, $4 \times COCH_3$), 2.49 (t, $J = 6$ Hz, 2H, CH_2CO), 2.70 (m, 2H, CH2CO), 3.80 (m, 1H, H_5), 4.05 (dd, $J = 13$ and 3 Hz, 1H, H_6'), 4.28 ((dd, $J = 13$ and 3 Hz, 1H, H_6), 4.92 (dd, $J = 9$ and 9 Hz, H_2), 5.05 (dd, $J = 9$ and 9 Hz, H_4), 5.24 (dd, $J = 9$ and 9 Hz, H_1), 5.28 (dd, $J = 9$ and 9 Hz, H_3), 6.52 (d, $J = 9$ Hz, NH), 8.01 (br, s, 1H, CO_2H). FAB m/z (%): 470 $[M + Na]^+$ (100), 448 $[M + H]^+$ (45).

2.4. Glycolipopeptide synthesis

Glycolipopeptide **6a**: MBHA resin (substitution ratio, 0.48 mmol/g) (500 mg) was swelled in DMF in a sintered glass peptide synthesis vessel for 60 min. An activation mixture consisting of Boc-Lys(Fmoc)-OH (450 mg, 0.96 mmol), HBTU (364 mg, 0.96 mmol), HOBt (73 mg, 0.48 mmol) and DIEA (334 μl, 1.9 mmol) in DMF (5 ml) was added to the resin and the reaction vessel shaken for 30 min. The ninhydrin test was demonstrated to be negative after this time and the side chain Fmoc protecting group was then removed using 20% piperidine in DMF. Acylation of the lysine side chain was achieved using *N*-(2,3,4,5-tetra-*O*-acetyl-β-D-glucopyranosyl) succinate (429 mg, 0.96 mmol), HBTU (364 mg, 0.96 mmol), HOBt (73 mg, 0.48 mmol) and DIEA (334 μl, 1.9 mmol) in DMF (5 ml) and the reaction mixture shaken. The ninhydrin test was demonstrated to be negative after 30 min and the Boc protecting group was subsequently removed using 100% TFA. The following manipulations were then performed on the resin.

Operation		Time
(1)	DMF wash	5 min
(2)	Boc-C_{14}-OH (329 mg, 0.96 mmol) HBTU (364 mg, 0.96 mmol), HOBt (73 mg, 0.48 mmol), DIEA (334 μl, 1.9 mmol) in DMF	
	Shake	20 min
(3)	Ninhydrin test	
(4)	DMF wash	5 min
(5)	TFA (100%)	2×1 min
(6)	DMF wash	5 min

The above procedures were then applied to the remaining amino acid residues; Boc-Leu-OH (239 mg, 0.96 mmol), Boc-Phe-OH (254 mg, 0.96 mmol), Boc-Gly-OH (168 mg, 0.96 mmol), Boc-

Gly-OH (168 mg, 0.96 mmol) and Boc-Tyr(2-Br-Z)-OH (475 mg, 0.96 mmol). Following hydrazine:methanol (1:7) removal of the sugar acetates, the resin-bound construct was washed and dried as described for peptide **1a**. Glycolipopeptide **6a** was cleaved and worked up in an analogous manner to peptide **1a**. Glycolipopeptide **6b** was constructed in an identical manner to that of **6a** with the inclusion of one extra C_{14} lipoamino acid residue into the sequence.

2.5. 2-(4,4-Dimethyl-2,6-dioxocyclohex-1-ylidene)ethylaminohexadecanoic acid

2-Aminohexadecanoic acid (1.36 g, 5 mmol) was suspended in a solution of 2-acetyldimeone (Bycroft et al., 1993) (3.64 g, 46 mmol) in dry ethanol (30 ml) and the mixture refluxed overnight. The clear solution was cooled and evaporated to dryness and the oily residue dissolved in ethyl acetate (30 ml) and washed with 1 M $KHSO_{4(aq)}$. The organic layer was then dried ($MgSO_4$) and evaporated to dryness to produce a yellow oil. Hexane trituration produced a white amorphous powder which, following recrystallisation from acetonitrile, afforded the title compound as a white crystalline solid, 1.49 g (68%), m.p. = 115–117°C.

^{1}H NMR: ($CDCl_3$); δ 0.85 (3H, t, $-CH_2CH_3$), 1.00 (6H, s, $2\times$ CH_3), 1.22 (24H, m $(CH_2)_{12}$), 1.85 (2H, m, $-CHCH_2CH_2-$), 2.38 (4H, s, $2\times$ $-CH_2-CO-$), 2.51 (3H, s, $-C{=}C(NH)CH_3$), 4.45 (1H, m, α-CH), 9.45 (1H, s, br, $-CO_2H$), 13.65 (1H, d, NH). FAB-MS m/z (%): 436 (100) [M + H], 391 (45) [M − CO_2H].

2.6. Tetra-O-acetyl-β-D-glucopyranuronic acid

D-Glucuronic acid (6 g, 31 mmol) was suspended in acetic anhydride (85 ml) and iodine (0.425 g, 2 mmol) was added slowly at 0°C. The resulting solution was stirred at that temperature for 2 h and at room temperature for a further 1 h. The solution was cooled again to 0°C and methanol (30 ml) was added slowly to remove any unreacted acetic anhydride. After standing overnight, solvents were azeotropically removed with toluene under reduced pressure. The residual solid was recrystallised from a mixture of ethyl acetate and hexane to afford the title compound as a white solid (10.3 g, 92%) which was utilised for the following procedure without further purification.

2.7. (2,3,4-Tri-O-acetyl-β-D-glucopyranosylazide)-uronic acid

Tetra-*O*-acetyl-D-glucopyranuronic acid (10 g, 28 mmol) was dissolved in dry dichloromethane (DCM) (130 ml) and maintained under a nitrogen atmosphere. Trimethylsilyl azide (9.13 ml, 69 mmol) and tin(IV) chloride (1.61 ml, 14 mmol) were added to the solution and the reaction mixture stirred overnight. Dichloromethane (100 ml) was added and the resulting solution washed with cold 1 M $KHSO_{4(aq)}$ (2×50 ml), brine (50 ml) and dried ($MgSO_4$). Following removal of volatiles, the crude product was purified by flash chromatography on silica gel (DCM/methanol/acetic acid, 20:1.6:0.08) to yield the title compound as a white crystalline solid (7.53 g, 79%) m.p. = 70–73°C (dec). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.03, 2.05, 2.08 (3s, $3\times$ 3H, $3\times$ $COCH_3$), 4.18 (d, $J_{4,5}=8.5$ Hz, 1H, H-5), 4.75 (d, $J_{1,2}=9.5$ Hz, 1H, H-1), 4.97 (dd, $J_{2,3}=9$ Hz, 1H, H-2), 5.29 (m, 2H, H-3 and H-4). FAB m/z (%): 391 $[M+2Na]^+$ (20), 368 $[M+Na]^+$ (73), 346 $[M+H]^+$ (20), 303 $[M-N_3]^+$ (100).

2.8. Immobilisation of (2,3,4-tri-O-acetyl-β-D-glucopyranosylazide)-uronic acid onto 2-Cl-trityl resin

2-Cl-trityl resin (1 g; subst. ratio, 1.05 mmol/g) was swelled in dry DCM (10 ml) for 20 min, then (2,3,4 - tri - *O* - acetyl - β - D - glucopyranosylazide) uronic acid (340 mg, 1 mmol) and diisopropylethylamine (DIEA) (0.15 ml, 0.1 mmol) were added and the suspension stirred for 5 min. DIEA/DCM (0.60 ml, 1:1 v/v) was then added and the resulting suspension stirred for 2 h. Methanol (0.8 ml) was then added to the mixture

and stirred for a further 5 min. The resin was finally washed with dry DCM (3×20 ml) and dried over phosphorous pentoxide overnight.

2.9. Reduction of the resin-bound (2,3,4-tri-O-acetyl-β-D-glucopyranosylazide) uronic acid

(2,3,4-Tri-*O*-acetyl-β-D-glucopyranosylazide) uronyl–2-Cl-trityl resin (600 mg) was stirred for 16 h in a mixture of triethylamine (2.04 ml, 15 mmol) and propane-1,3-dithiol (1.47 ml, 15 mmol). The resin was washed with dimethylformamide (DMF) and the glycosyl amine-charged support employed straight away for acylation with a second monosaccharide unit or for solid-phase peptide synthesis.

2.10. Glycopeptide synthesis

Glycopeptide **3**: using a Novasyn Gem semi-automatic peptide synthesiser, the following operations were performed on the glycosyl amine-charged resin (600 mg) under continuous flow conditions (Drouillat et al., 1997). A constant flow rate of 3.0 ml/min was maintained, with post-column eluent monitored at 290 nm.

Operation		Time
(1)	DMF wash	10 min
(2)	Fmoc-Leu-OH (848 mg, 2.4 mmol) HBTU (910 mg, 2.4 mmol), HOBt (184 mg, 1.2 mmol), DIEA (836 μl, 4.8 mmol) in DMF, load	
(3)	Recycle	45 min
(4)	DMF wash	2 min
(5)	Load port wash	
(6)	Picrylsulphonic acid test	
(7)	20% piperidine in DMF deprotection	10 min

The above operation scheme was then followed for the remaining amino acid residues; Fmoc-Phe-OH (929 mg, 2.4 mmol), Fmoc-Gly-OH (713 mg, 2.4 mmol), Fmoc-Gly-OH (713 mg, 2.4 mmol) and Fmoc-Tyr(tBu)-OH (1.103 g, 2.4 mmol). Following hydrazine:methanol (1:7) removal of the sugar acetates, the resin was transferred to a sintered glass funnel and sequentially washed with DMF (30 ml), methanol (30 ml) and finally ether (30 ml). The resin was then dried in vacuo over KOH overnight. The glycopeptide was cleaved from the resin with concomitant side chain deprotection using TFA–triisopropylsilane–water (95:2.5:2.5). The cleaved glycopeptide was precipitated using HPLC-grade diethyl ether, redissolved in water and lyophilised to afford the product as a white amorphous powder. Glycopeptides **4**, **5** and **7** were synthesised in an analogous manner, except in the case of **4**, which was constructed using NovaSyn TGR resin.

2.11. Peptide analysis and purification

Peptide and glycopeptide purification: analytical RP-HPLC was carried out on a Vydac C_4 column (4.6×150 mm) at a constant flow rate of 1.2 ml min^{-1}. Mobile phases employed were: (A) 0.06% $TFA_{(aq)}$ and (B) 0.06% TFA in 90% $MeCN_{(aq)}$. Analytical RP-HPLC was performed using a Waters 616/600S twin pump system and 486 tuneable absorbance detector controlled via a Millennium software package. Post-column eluent was monitored by UV absorbance at 214 nm. The gradient employed for separation was 0–100% (B) in 30 min linearly. Semi-preparative RP-HPLC was carried out on a Vydac C_4 or a Vydac C_6 column (8×150 mm) at constant flow rate of 2.5 ml min^{-1}. Mobile phases were as above and RP-HPLC was performed using two microprocessor-controlled Gilson 302 single-piston pumps. The gradient employed for separation was 0–100% (B) in 60 min linearly. Compounds were detected with a Holochrome UV-Vis detector at 220 nm and chromatographs were recorded using an LKB 2210 single-channel recorder. All lipopeptide, glycopeptide and lipoglycopeptide structures were confirmed by MALDI-TOF mass spectrometry (Table 1).

2.12. Pharmacological evaluation

Male albino mice (A.R. Tuck) weighing 25–30 g, and male guinea pigs (bantin and Kingman) weighing 350–500 g were used for these experiments. Animals were killed using a recommended humane procedure. Mouse vasa deferentia (whole) and segments of guinea pig terminal ileum (approximately 1 cm) were dissected free into Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.1. A platinum electrode was tied into the lumen of each ileal segment, and a second platinum electrode was placed outside and parallel to the tissue, at a distance of 0.5 cm. The whole assembly was placed into Krebs solution in a 20-ml organ bath, held at 37°C and gassed with 5% CO_2 in oxygen. Each vas deferens was positioned between two platinum electrodes, held 0.5 cm apart, and the tissues suspended in 20-ml organ baths as above. The free ends of all tissues were attached to transducers by means of a thread, and held under a tension of 1 g (ileum) or 0.5 g (vas deferens).

The electrodes were attached to stimulators (Bioscience). Trains of square-wave electrical pulses were applied by transmural stimulation to contract ileal smooth muscle (2 ms pulse width, 3 V strength, 5 Hz delivered for 1 s every 30 s), and by electrical field stimulation to contract vasa deferentia (2 ms pulse width, 10 V strength, 10 Hz delivered for 1 s every 30 s). Contractions were recorded on an oscillograph (Harvard).

Control doses of leucine-enkephalinamide (Leu-Enk) were applied to the tissues in order to produce 30–80% inhibition of electrically evoked contractions. The peptides and glycopeptides described above were then investigated for their inhibitory efficacy over a range of doses. Dose–response curves were then constructed of inhibition of contraction (%) versus log drug concentration (μM), and ED_{50} values corresponding to 50% inhibition were read off.

Table 1
MALDI-TOF mass spectral analysis of the lipo-, glyco- and glycolipopeptides synthesised

Compound no.	Calculated mass	MALDI-TOF MS peak(s) observed
1a	723	746 [M+Na], 724 [M+H]
1b	892	915 [M+Na], 893 [M+H]
1c	751	774 [M+Na], 752 [M+H]
1d	948	971 [M+Na]
1e	779	802 [M+Na], 780 [M+H]
1f	1004	1027 [M+Na], 1005 [M+H]
1g	835	858 [M+Na]
1h	1116	1139 [M+Na]
2a	723	746 [M+Na], 724 [M+H]
2b	751	774 [M+Na], 752 [M+H]
2c	779	802 [M+Na]
2d	1004	1027 [M+Na], 1005 [M+H]
2e	1060	1083 [M+Na], 1061 [M+H]
2f	863	886 [M+Na]
3	730	753 [M+Na], 731 [M+H]
4	729	771 [M+Na], 730 [M+H]
5	904	928 [M+Na], 905 [M+H]
6a	1167	1191 [M+Na+H]
6b	1393	1416 [M+Na]
7	1040	1063 [M+Na]

2.13. Data analysis

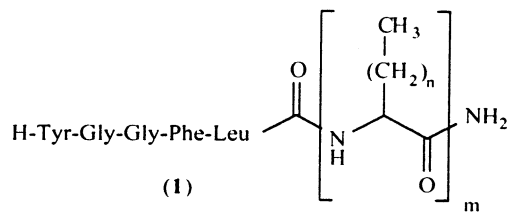
Ratios of ED_{50} values were used to give an estimate of the relative inhibitory potency of each peptide or glycopeptide derivative compared to the native Leu-enkephalinamide. Each experiment was repeated three to six times and the results analysed by Student's *t*-test.

3. Results and discussion

To investigate the effect of increasing lipophilicity on biological activity, the following series of C-terminal diastereomeric lipoamino acid (LAA)-modified Leu-enkephalinamides **1a**–**1h** were synthesised via standard Boc/benzyl SPPS conditions

Table 2
C-Terminal (**1**)- and N-terminal (**2**)-modified Leu-enkephalinamides synthesised

Compound no.	*m*	*n*
1a	1	7
1b	2	7
1c	1	9
1d	2	9
1e	1	11
1f	2	11
1g	1	15
1h	2	15
2a	1	7
2b	1	9
2c	1	11
2d	2	11
2e	2	13
2f	1	17



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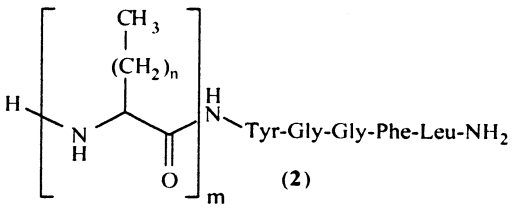

(2)

(Merrifield, 1963) (Table 2). To further investigate the observation that N-terminal modifications are poorly tolerated within the enkephalin family (Tsuzuki et al., 1991), the following series of N-terminal diastereomeric (LAA)-modified Leu-enkephalinamides **2a**–**2f** were also synthesised (Table 2).

To investigate the effect of a glucose or glucuronic acid analogue on overall activity (Mulder, 1992) the C-terminal-modified glycopeptides **3**, **4** and **5**, and the glyco-lipopeptides **5a**, **5b** and **6** were synthesised via either an Fmoc/′Boc or Boc/benzyl solid-phase strategy, respectively (Fig. 1).

Following RP-HPLC purification, the individual diastereomeric mixtures of lipopeptides **1a**–**2f**, glycolipopeptide conjugates **6a** and **6b** and the chirally pure glycopeptides **3**–**5** were investigated for intrinsic activity using both a guinea pig ileum (GPI) and mouse vas deferens (MVD) assay (Table 3).

The in vitro data indicated that C-terminal modification (peptides **1a**–**1h**) of the Leu-enkephalin structure is more favourably tolerated than the N-terminal (peptides **2a**–**2f**) conjugation. From the series of C-terminal analogues, the LAA–peptide conjugates containing one lipoamino acid (**1a**, **1c**, **1e** and **1g**) displayed activity whilst the dimers (**1b**, **1d**, **1f** and **1h**) were inactive. The most promising candidate, **1a**, displayed a 1.5-fold increase in activity in the MVD assay and almost equipotency in the GPI when compared with the native peptide. The remaining LAA–peptide conjugates, whilst displaying reduced overall activity when compared to that of Leu-enkephalinamide, demonstrated a similar tissue trend suggesting an overall selectivity towards the δ-receptor (Fries, 1995).

The glycolipopeptide conjugates **6a**, **6b** and **7** displayed low activities in both the GPI and MVD assays. However, the glycopeptides **3**–**5**, synthesised via a novel solid-phase route (Drouillat et al., 1997) provided the most encouraging results. The glucuronide-modified Leu-enkephalin possessed a 3-fold increase in activity in the GPI assay and was 40 times more potent in the case of the MVD experiments when compared to Leu-enkephalinamide. This result suggested a strong receptor selectivity towards the δ-receptor, and this hypothesis was confirmed by the total cessation of activity when the tissue was treated with the δ-selective antagonist naltrindole. The sugar dimer conjugate **5**, however, displayed a 50% reduction in activity in the GPI model, yet a 2.5-fold increase in activity in the MVD assay when compared to the native peptide, thereby suggesting a continued selectivity towards the δ-receptor.

To investigate the importance of the carboxylic acid inherent within the glycopeptide structure **3**, the carboxamide analogue **4** was synthesised using NovaSyn TGR resin modified with the Rink

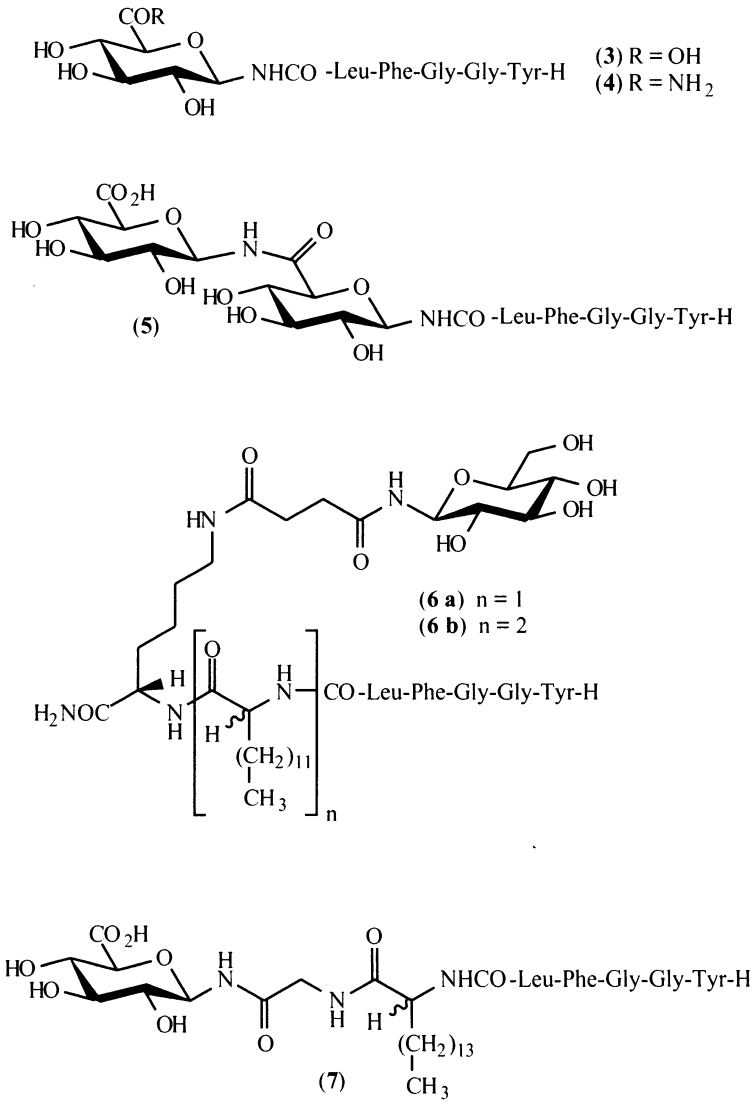

Fig. 1. Glyco- and glycolipopeptide conjugates of Leu-enkephalin.

Linker (Rink, 1987). The in vitro data demonstrated a decrease in activity with both assays, the most pronounced being in the MVD. This agrees with the observation that amidation of the C-terminal carboxyl group increased μ-receptor selectivity (DiMaio et al., 1982).

4. Conclusion

We have described the synthesis and in vitro biological activity of a number of lipopeptide, glycopeptide and glycolipopeptide conjugates of Leu-enkephalin. The in vitro activities of the peptide conjugates was established using MVD and GPI assays. These experiments revealed that attachment of one lipoamino acid to the C-terminal of Leu-enkephalin maintained biological activity and, in the case of conjugate **1a**, produced a compound of higher activity than that of the parent peptide. Inclusion of a glucose or glucuronic acid moiety into the lipopeptide structure,

Table 3
Guinea pig ileum (GPI) and mouse vas deferens (MVD) assay results of the active lipo-, glyco- and glycolipopeptide conjugates of Leu-enkephalinamide

Compound no.	GPI		MVD	
	IC_{50} (mM)[a]	Rel. potency	IC_{50} (mM)[a]	Rel. potency
1a	1.8 ± 0.41	0.97 ± 0.22	0.18 ± 0.01	1.58 ± 0.10
1b	not active	not active	7.0 ± 0.35	0.04 ± 0.002
1c	3.0 ± 0.67	0.18 ± 0.04	2.3 ± 0.35	0.12 ± 0.01
1e	6.0 ± 1.30	0.09 ± 0.02	2.55 ± 0.23	0.11 ± 0.01
1g	60.0 ± 4.0	0.009 ± 0.0006	14.0 ± 2.1	0.02 ± 0.003
2a	0.92 ± 0.12	0.60 ± 0.08	0.50 ± 0.02	0.55 ± 0.02
2b	2.16 ± 0.52	0.25 ± 0.06	1.08 ± 0.25	0.26 ± 0.06
2c	7.71 ± 1.10	0.07 ± 0.01	2.15 ± 0.66	0.13 ± 0.04
3	0.20 ± 0.03	2.74 ± 0.37	0.008 ± 0.001	35.39 ± 5.45
4	3.86 ± 0.28	0.14 ± 0.01	0.55 ± 0.06	0.51 ± 0.03
5	1.04 ± 0.16	0.52 ± 0.08	0.11 ± 0.01	2.56 ± 0.17
7	46.2 ± 5.90	0.012 ± 0.001	64.0 ± 4.10	0.004 ± 0.0003
Leu-enkephalinamide	0.54 ± 0.16	1.0	0.26 ± 0.07	1.0

[a] Mean of three determinations $\pm$ S.E.M.

conjugates **6a**, **6b** and **7**, decreased activity. However, C-terminal conjugation of one or two glucuronic acid analogues to the native peptide via amide linkage(s) (conjugates **3** to **5**) led to a dramatic increase in activity, which in the case of compound **3** was 40-fold greater than Leu-enkephalinamide and displayed a high selectivity towards the δ-opioid receptor.

We are currently assessing the most promising candidates for their ability to cross the blood–brain barrier and thereby illicit an antinociceptive effect in vivo, and exploring the construction of glycopeptide libraries in an attempt to identify the best sugar and amino acid composition for potent δ-opioid receptor agonists.

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