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Solid Phase Synthesis of *C*-Terminal Carbohydrate Modified Enkephalins

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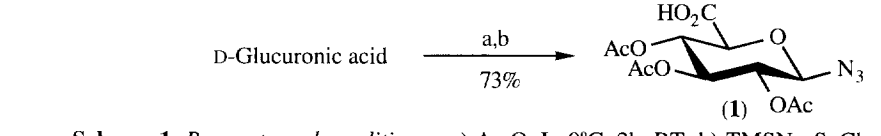
Abstract. 1-Azido-2,3,4-tri-*O*-acetyl-β-D-glucuronic acid (**1**), immobilised on 2-chlorotrityl and NovaSyn TGR resin, was efficiently reduced to its corresponding resin bound glycosyl amine (**3**) using propane-1,3-dithiol and triethylamine. Subsequent acylation of (**3**) with (**1**), generated the carbohydrate dimer (**4**). In addition, Fmoc based peptide syntheses performed on (**3**) and (**4**) afforded the *C*-terminal modified Leu and Met-enkephalins (**7-10**). Preliminary pharmacological evaluation of these compounds identified glycopeptide (**7**) as a potent and selective δ-opioid receptor agonist. © 1997 Elsevier Science Ltd.

The rapidly developing field of combinatorial chemistry has resulted in a plethora of novel solid phase methodologies in an attempt to meet the demands for lead molecule discovery.[1] Despite numerous and significant advances,[2] carbohydrate based syntheses using solid phase techniques have not yet become routine. However, glycosylated peptide and non-peptide entities still remain a most attractive target for drug development. One particular clinical area that would potentially benefit from this technology is the development of more potent and selective analgesics; of which the endogenous enkephalins[3] make an ideal template. The identification of multiple opioid receptors[4] has tended to focus research towards the design and synthesis of selective and conformationally restricted enkephalin analogues,[5] which do not display the numerous undesirable side effects associated with opioids currently exploited in the clinic. Reports have suggested that introduction of a sugar moiety into the enkephalin molecule does invoke such receptor specificity.[6] Most cases examined the attachment of β-D-glucose, and analogues thereof, to the active peptide in an attempt to increase both receptor selectivity and to increase blood brain barrier (BBB) permeability via the glucose transporters located in the endothelial cell membranes.[7]

However, it has recently been demonstrated that morphine-6-glucuronide is 10-50 times more potent than morphine itself in producing analgesia,[8] and therefore we felt introduction of a glucuronic acid analogue onto the *C*-terminal of Leu and Met enkephalin would be a desirable objective. In addition, we reasoned that a solid phase strategy to achieve this manipulation would allow future library development. Herein, we report the synthesis of a novel glycosylamino acid precursor (**1**) and its exploitation in the solid phase synthesis of *C*-terminally modified opioid peptides.

The facile synthesis of sugar azide (**1**) was achieved *via* a two step process, **Scheme 1**. Glucuronic acid was acetylated with a mixture of acetic anhydride and iodine,[9] and without protection of the carboxyl function the product converted to the β-anomeric azide (**1**) by reaction with an excess of trimethylsilyl azide and tin (IV) chloride in dichloromethane.[10,11]



Scheme 1. *Reagents and conditions:* a) Ac_2O, I_2, 0°C, 2h, RT; b) $TMSN_3$, $SnCl_4$, CH_2Cl_2, 16h.

The monosaccharide was immobilised on a polystyrene based resin support via a 2-chlorotrityl linker (loading 1.05 mmol/g) with methanol capping to afford the anchored compound (**2**), **Scheme 2**. Reduction of

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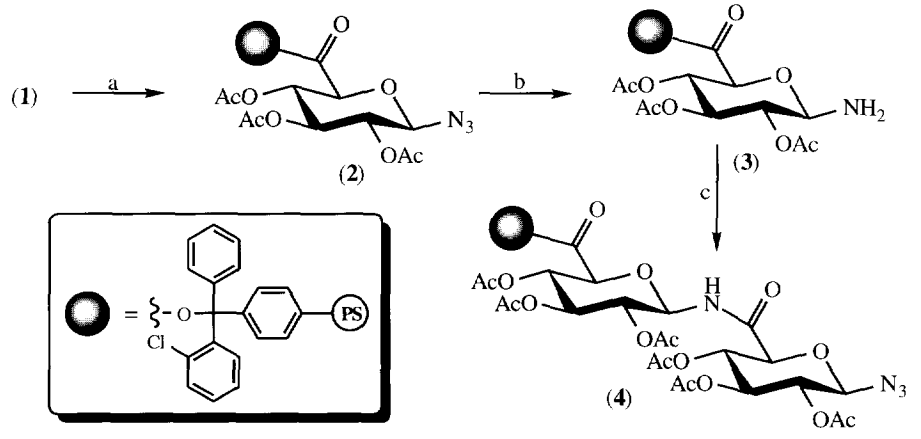
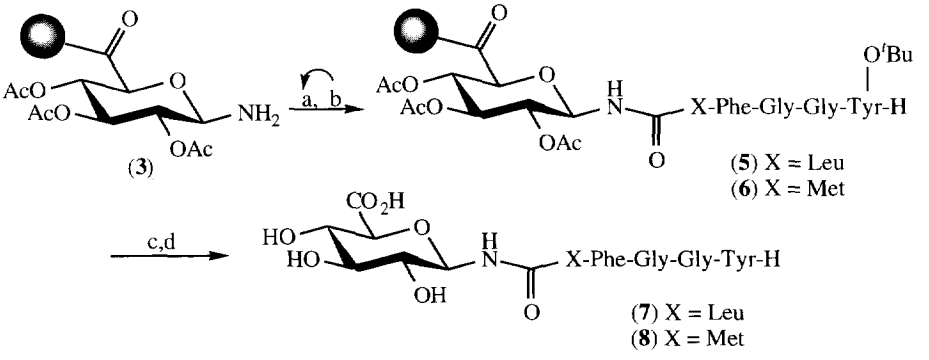
the resin bound sugar-azide was achieved by treatment with a mixture of triethylamine and propane-1,3-dithiol (25 eq.) to generate the free amine *in situ.*[12] Reaction progress was monitored by periodic resin cleavage and analysis of the product by TLC and FAB-MS. The resin bound glycosylamino sugar (**3**) was washed several times with DMF and immediately coupled with monosaccharide (**1**) (3.6 eq.) using a HBTU/HOBt/DIEA strategy[13] with acetic anhydride capping, affording the immobilised dimer (**4**) in almost quantitative yield. Cleavage of (**4**) with 0.5% TFA released the acetate protected dimer which was subsequently characterised by MS and ^{1}H NMR.[14]



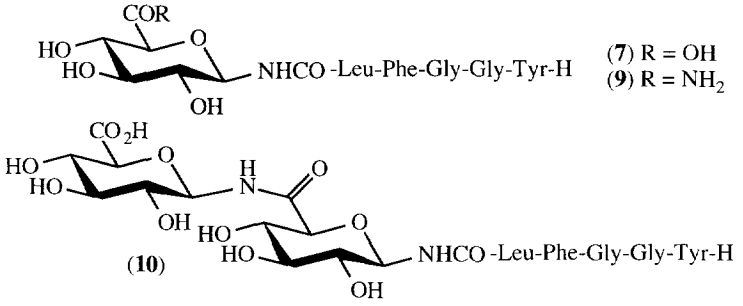
Scheme 2. *Reagents and conditions:* a) 2-Clt resin, DIEA, CH_2Cl_2, 1h, then MeOH; b) $HS(CH_2)_2SH$, NEt_3 10h; c) (**1**), HBTU, HOBt, DIEA, DMF, 1h.

Having demonstrated the ability to acylate the resin bound glycosyl amine in high yield, we embarked upon the solid phase synthesis of both the Leu- and Met-enkephalin conjugates *via* standard Fmoc based protocols, **Scheme 3**. Following reduction of the immobilised azido sugar (**2**) the peptide was constructed using an HBTU/HOBt/DIEA coupling strategy and the progress of the individual coupling reactions monitored by the TNBS amine test.[15]



Scheme 3 *Reagents and conditions:* a) *N*-Fmoc protected amino acids - Leu or Met, Phe, Gly, Gly and Tyr(O^{t}Bu), HBTU, HOBt, DIEA; b) 20% piperidine v/v in DMF; c) $NH_2NH_2.H_2O$:MeOH (1:7); d) (**5**): TFA:H_2O:TIPS (95:2.5:2.5), 2 h, (**6**): TFA:H_2O:EDT:TIPS (92.5:2.5:2.5:2.5) 2 h.

Upon completion of the syntheses, the *N*-terminal Fmoc group was removed, followed by hydrazine mediated deprotection of the sugar acetates. Acidolytic resin cleavage and concomitant side chain deprotection relinquished the carbohydrate modified Leu- (**7**) and Met-enkephalins (**8**) with an overall purity of 55% and 60% respectively, as demonstrated by analytical RPHPLC. MALDI-TOF MS of the two purified glycopeptides exhibited the correct molecular ion peaks for both compounds.[16] The generality of the process was further demonstrated by the synthesis of the sugar dimer modified Leu-enkephalin (**10**) by addition of the relevant amino acids to the reduced resin bound disaccharide mimetic (**4**). The carboxamide analogue (**9**) of glycoconjugate (**7**) was also synthesised by initial immobilisation of the glycosyl azide onto NovaSyn TGR resin, modified with the Rink linker.[17]



Acidolytic cleavage and side-chain deprotection afforded the glycopeptides (**9**) and (**10**) in 95% and 90% overall purity as demonstrated by analytical RPHPLC. Purification and subsequent MALDI-TOF MS once again displayed the correct molecular ion peaks for both compounds.[18]

Preliminary *in vitro* pharmacological evaluation, using both a guinea pig ileum (GPI) and mouse vas deferens (MVD) assay revealed that the sugar conjugated enkephalin analogue (**7**) was 3 times more potent than Leu-enkephalinamide at inhibiting electrically stimulated muscle contractions in the GPI model, and 40 times more potent in the MVD example, **Table 1**. This result suggested a strong selectivity towards the δ-receptor, which was confirmed by the total obliteration of glycoconjugate (**7**) activity when the tissue was treated with the δ-selective antagonist naltrindole. The Met-enkephalin analogue (**8**) however was less active, exhibiting only a 4 fold increase in relative potency in both the MVD and GPI assays when compared to that of Leu-enkephalinamide.

Compound No.	GPI		MVD	
	IC_{50} [μM][a]	Rel. Potency	IC_{50} [μM][a]	Rel. Potency
(**7**)	0.20 ± 0.03	2.74 ± 0.37	0.008 ± 0.001	35.39 ± 5.45
(**8**)	0.11 ± 0.02	4.54 ± 0.80	0.07 ± 0.01	3.80 ± 0.73
(**9**)	3.86 ± 0.28	0.14 ± 0.01	0.11 ± 0.03	0.51 ± 0.03
(**10**)	1.04 ± 0.16	0.52 ± 0.08	0.55 ± 0.17	2.56 ± 0.17
Leu-enkephalinamide	0.54 ± 0.16	1	0.28 ± 0.07	1

[a] Mean of three determinations ± SEM

Table 1. Guinea pig ileum (GPI) and mouse vas deferens (MVD) assay of *C*-terminal glycoconjugates of Leu- and Met-enkephalin.

It was further demonstrated that the carboxamide analogue (**9**) displayed a 50% reduction in activity in the MVD assay and an 86% reduction in the GPI when compared to the activity of leu-enkephalinamide. The sugar dimer (**10**) however, displayed a 50% reduction in activity in the GPI model, yet a 2.5 fold increase in activity in the MVD assay, suggesting a retained δ-receptor selectivity.

In summary, we have demonstrated a facile and high yielding synthesis of a glycosyl azide building block (**1**) and illustrated its application in solid phase synthesis by its immobilisation to both a carboxylic acid and carboxamide generating resin. Ensuing reduction to, and acylation of, the corresponding glycosyl amine has allowed the construction of *C*-terminal modified glycoconjugates of both Leu- and Met-enkephalin, which in the case of glycopeptide (**7**) resulted in a highly potent and δ-receptor selective opioid agonist. We are now exploring the synthesis and evaluation of various carbohydrate modified enkephalin, and alternative bioactive peptide libraries.

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References and Notes

Abbreviations: DIEA, diisopropylethylamine; DMF, *N,N'*-dimethylformamide; EDT, 1,2-ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MALDI-TOF MS, matrix assisted laser desorption time of flight mass spectrometry; RPHPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

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11. **1-Azido-2,3,4-tri-*O*-acetyl-β-D-glucuronic acid (1)**: m.p. = 70-73°C (dec); 1H NMR (500 MHz, $CDCl_3$) δ 2.03, 2.05, 2.08 (3s, 3 x 3H, 3 x $COCH_3$), 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 & H-4)
m/z (FAB) (%): 391 $[M + 2Na]^+$ (20), 368 $[M + Na]^+$ (73), 346 $[M + H]^+$ (20), 303 $[M - N_3]^+$ (100).
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14. Selected spectral data; ^1H NMR (500 MHz, $CDCl_3$) δ 1.91-2.16 (6s, 18H, 6 x $COCH_3$), 3.98 (d, $J_{4',5'}$ = 10Hz, C[*H*-5']CONH), 4.17 (d, $J_{4,5}$ = 10Hz, 1H, C[*H*-5]CO_2H), 4.69 (d, $J_{1,2}$ = 9Hz, 1H, C[H-1]N_3), 4.92-5.02 (m, 3H, C[*H*-1]NHCO, H-2 & H-2'), 5.17-5.29 (m, 3H, H-3, H-3' & H-4), 5.39 (dd, $J_{3',4'}$ = 9.5Hz, H-4), 7.44 (d, *J* = 9Hz, CONH)
m/z (FAB) (%): 691 $[M + 2Na]^+$ (25), 669 $[M + Na]^+$ (55), 647 $[M + H]^+$ (15).
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16. MALDI-TOF MS (%) (**7**): 753 $[M + Na]^+$ (90), 731 $[M + H]^+$ (100). (**8**): 771 $[M + Na]^+$ (100), 749 $[M + H]^+$ (64)
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18. MALDI-TOF MS (%) (**9**): 752 $[M + Na]^+$ (100), 730 $[M + H]^+$ (38). (**10**): 928 $[M + Na]^+$ (100), 905 $[M + H]^+$ (46).

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