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1 **α-1,4-Galactosyltransferase-catalyzed glycosylation of sugar and lipid modified**
2 **Leu-enkephalins**

3

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22

23 **Abstract**
24 Glycosylation of therapeutic peptides has been reported to improve delivery and
25 targeting of various vaccines and drugs to specific cells/tissues. However, chemical

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1 synthesis of complex oligosaccharide derivatives via conventional methods can be
2 challenging due to the need for several orthogonal hydroxyl group protections.
3 Liposaccharyl galactosyltransferase C, a naturally occurring glycosyltransferase
4 enzyme from *Neisseria meningitidis,* was found to have the ability to transfer a
5 galactosyl moiety to glyco(lipo)peptides. An enzymatic glycosylation of Leu-
6 enkephalin glyco(lipo)peptides was developed and optimized in this study in order to
7 prepare pain regulating peptides with potentially improved central nervous system
8 delivery.
9

10 **Keywords**
11 enzymatic synthesis, glycopeptide, Leu-enkephalin, galactosyltransferase,
12 glycosylation
13

14 **1. Introduction**
15

16 Opioid receptors on neuronal cell membranes in the brain and spinal cord have ligand
17 specificity for endogenous opioid peptides. These peptides have been shown to
18 possess potent pain-reducing effects and have the potential to be used as pain
19 regulating therapeutics.[1, 2] Leu-enkephalin (Enk) was chosen as a model central
20 nervous system (CNS)-targeted peptide because of its high binding affinity for μ- and
21 δ-type opioid receptors and analgesic effects *in vivo*.
22 Peptides have been used as therapeutics for decades, however their delivery *in vivo*
23 remains challenging predominantly because of poor enzymatic stability and limited
24 bioavailability. Various strategies involving peptide production, chemical
25 modification and formulation have been attempted to overcome these issues.[3-7]

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Glycosylation and lipidation have been studied to increase the enzymatic stability, membrane permeability, activity and/or affinity of Enk to opioid receptors.[8-10] Lipidation was also found to improve transport across biological barriers[11] and the lipoamino acids[12] have previously been used for peptide drug delivery.[1, 8] While many advantages have been described, the drawback of the increased lipophilicity, poor water solubility, can be a limiting factor in drug delivery. Lipoamino acids in combination with carbohydrates have not only improved stability and uptake, but also enhanced water solubility and the targeting of drug candidates to specific sites.[13, 14] Carbohydrates have useful structural properties for which they have been utilized as components of peptide drugs [4, 6, 15-17] and vaccines.[18-23]

We describe the chemo-enzymatic glycosylation of Enk with mono-, di- and trisaccharide sugar moieties terminating with a galactose residue. It is expected that these modifications would not only improve biological transport, stability and retention time in the body but also enable targeting to the asialoglycoprotein receptor. Asialoglycoprotein receptors are not only highly distributed in hepatic cells but are also expressed in discrete areas of the brain,[24] and have specificity for terminal galactose.[25, 26] An enzymatic strategy[27-29] can circumvent the challenges associated with the manipulation of protecting groups on multiple hydroxyl groups during the chemical synthesis of oligosaccharides.[30, 31] Here we utilized a galactosyltransferase, lipopolysaccharyl α-1,4- galactosyltransferase (EC 2.4.1.; LgtC) derived from *Neisseria meningitides.*[32, 33] The natural function of LgtC is to transfer an α-D-galactosyl moiety from uridine-5'-diphosphate-α-D-galactose (UDP-Gal) donor to the terminal lactose of lipooligosaccharides, while retaining the anomeric configuration of the donor's glycosidic bond, and forming an α-1,4 linkage.[34, 35] We expressed LgtC in *Escherichia coli* and employed it to selectively

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1 attach a galactose unit onto chemically synthesized (lipo)saccharide peptide scaffolds.
2 The combination of chemical and enzymatic synthesis allowed us to produce
3 compounds, which may permit targeted delivery to the CNS.
4
5 **2. Experimental**
6
7 *2.1. Materials*
8
9 Unless otherwise stated, all chemicals used in this project were of analytical grade or
10 equivalent. *N,N*-Dimethylformamide (DMF) was obtained from Emanuel Merck,
11 Darmstadt (EMD, Darmstadt, Germany), while *O*-benzotriazole-*N,N,N',N'*-
12 tetramethyl-uronium-hexafluoro-phosphate (HBTU) and Fmoc-L-amino acids were
13 obtained from Mimotopes (Clayton, VIC, Australia). *N,N*-Diisopropylethylamine
14 (DIPEA), dichlormethane (DCM), HCl, trifluoroacetic acid (TFA), and diethyl ether
15 were supplied by Merck (Kilsyth, VIC, Australia). Piperidine and triisopropylsaline
16 (TIPS) scavenger were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).
17 Toluene was supplied from Lab-Scan Pty Ltd (Dublin, Ireland). For carbohydrate
18 synthesis, Ac_2O was supplied by Univar (Ingleburn, NSW, Australia), and
19 dimethaminopyridine (DMAP), tetrahydrofuran (THF) and triethylamine (TEA) from
20 Merck (Kilsyth, VIC, Australia). Succinic anhydride and $LiClO_4$ were purchased from
21 Sigma-Aldrich (Castle Hill, NSW, Australia). High performance liquid
22 chromatography (HPLC) and MS grade acetonitrile (ACN) and MeOH were supplied
23 by Scharlau (Port Adelaide, SA, Australia), while acetic acid (AcOH) was supplied
24 from Merck (Kilsyth, VIC, Australia). TLC grade ammonia solution 25% was
25 purchased from Sigma-Aldrich (Castle Hill, NSW, Australia), isopropanol from Lab-

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1 Scan Pty Ltd (Dublin, Ireland), and $CHCl_3$ from Merck (Kilsyth, VIC, Australia). For
2 enzymatic experiments, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
3 (HEPES) was obtained from Grand Island Biological Company (GIBCO, Mulgrave
4 VIC, Australia), and UDP-Gal disodium salt from CalBioChem (Darmstadt,
5 Germany).
6
7 *2.2. Methods*
8
9 Thin layer chromatography (TLC) was carried out on Kieselgel $60F_{254}$ silica gel
10 coated aluminum plates from Merck (Darmstadt, Germany). All TLCs were
11 developed using eluent $CHCl_3$:MeOH:H_2O, 7:5:1, v/v and visualized by 20%
12 H_2SO_4:EtOH solution or an anisaldehyde reagent followed by heating, unless stated
13 otherwise. THF was dried over sodium and benzophenone; DCM over calcium
14 hydride; and $CHCl_3$ over calcium chloride. Analytical reversed phase HPLC (RP-
15 HPLC) was performed using Shimadzu (Kyoto, Japan) Instrumentation (LabSolutions
16 software, SIL-20AC HT autosampler, LC-20AB pump, SPD-M10A detector, DGU-
17 20A5 degasser). Analysis was achieved using a linear 0-100%, 20-35% and/or 30-
18 80% gradient of solvent B (solvent A: 0.1% TFA in H_2O; solvent B: 90%
19 ACN:H_2O:0.1% TFA) for 30 min with a 1 mL/min flow rate and detection at 214 nm,
20 unless stated otherwise.
21 Analytical separations were achieved on Grace Vydac® (Columbia, Maryland, USA)
22 columns (10 µm, 4.6 mm i.d. × 250 mm; Hesperia, CA) - either C8 (208TP104) or
23 C18 (218TP104) column; or Alltima® C18 (5 µm 4.6 mm x 250 mm) column
24 depending on the hydrophobicity of the compounds. Preparative RP-HPLC was
25 performed using a 30-80% gradient of solvent B for 60 min on a Waters Delta 600

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1 system (Milford, Massachusetts, USA) with a 10-20 mL/min flow rate, and detection
2 at 230 nm using PicoLog software. Separations were achieved from a Vydac® C8 or
3 Alltima® C18 preparative column (10 µm, 22 mm i.d. × 250 mm). Fractions
4 containing pure compound were pooled and lyophilized overnight. Electrospray
5 ionization mass spectrometry (ES-MS) and liquid chromatography mass spectrometry
6 (LC-MS) analyses were performed on a Perkin-Elmer-Sciex API 3000 using Analyst
7 1.4 (Applied Biosystems/ MDS Sciex, Toronto, Canada) software. High resolution
8 mass spectrometry (HR-MS) was performed using ABSCIEX 5600 Triple TOF,
9 positive ion mode and approximately 30,000-35,000 mass resolution, 10 uL
10 injections. Samples from the enzymatic reactions were filtered through Vydac®
11 BioSelect SPE Columns (C4, 1 mL, 214SPE1000 and C18, 1 mL, 218SPE1000) prior
12 to ES-MS. Columns used for LC-MS were either Vydac® C8 208TP5205 5µm (2.1
13 mm i.d. × 50 mm) or Phenomenex® Luna® C18 5 µm (2 mm i.d. × 50 mm). Solvent
14 A used for MS consisted of 100% purified Millipore H_2O and 0.1% AcOH, while
15 solvent B consisted of 90% MeOH, 10% purified Millipore H_2O, and 0.1% AcOH.
16 NMR spectroscopy was recorded on a Bruker AM 300 MHz instrument with the
17 chemical shifts given in parts per million with reference to tetramethylsilane as an
18 internal standard.
19
20 *2.3. Chemical synthesis of substrates*
21
22 2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl azide (**1a**) and 2,3,6-tri-*O*-acetyl-4-*O*-
23 (2',3',4',6'-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranosyl azide (**1b**) were
24 obtained after the reaction of the bromides with sodium azide in acetone in 67% and
25 80% yields, respectively (Supporting Information).[36-38] The galactose and lactose

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1 azides (**1a-b**) were reduced to amines through hydrogenation and immediately treated
2 with succinic anhydride in dry THF (galactose derivative) or pyridine (lactose
3 derivative) with catalytic amounts of DMAP to form N^1-(2,3,4,6-tetra-*O*-acetyl-β-D-
4 galactopyranosyl)succinamic acid **2a** (67% yield) and N^1-(2,3,6-tri-*O*-acetyl-4-*O*-
5 (2',3',4',6'-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranosyl)succinamic
6 acid **2b** (46% yield).[39, 40]
7 To synthesize the lipid moiety, 1-bromotetradecane and diethyl acetamidomalonate
8 were reacted under a reflux with HCl to produce 2-amino-D,L-hexadecanoic acid
9 hydrochloride in 54% yield. A stirred solution of 5,5-dimethyl-1,3-cyclohexanedione,
10 TEA and Ac_2O yielded (72%) 2-(1-hydroxyethylidene)-5,5-dimethylcyclohexane-1,3-
11 dione, which was further conjugated to 2-amino-D,L-hexadecanoic acid
12 hydrochloride.[41] The 2-((1-(4,4-dimethyl-2,6-
13 dioxocyclohexylidene)ethyl)amino)hexadecanoic acid (Dde-C_{16}), produced with 55%
14 yield, was used as a lipid building block in the synthesis of glycolipopeptides **3b** and
15 **3d**.
16
17 *2.4. Chemical glycosylation and peptide/lipopeptide solid phase synthesis*
18
19 All peptides, lipopeptides and glycolipopeptides were synthesized using standard *in*
20 *situ* neutralization stepwise solid-phase synthesis protocol for Fmoc chemistry.[42]
21 Peptides and lipopeptides were synthesized using Rink Amide-4-methylbenzhydryl
22 amine (Rink Amide-MBHA) resin (100-200 mesh, 0.59 mmol/g; Peptides
23 International, USA). The resin was swollen in 10% DIPEA/DMF for a minimum of 2
24 h. Fmoc-L-amino acids (4 eq) were activated with 4 eq 0.5 M HBTU in DMF, and 5
25 eq DIPEA, then reacted with amino groups on Rink Amide-MBHA resin (2 x 1 h).

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1 The Fmoc protecting group was removed using 20% piperidine/DMF (2 x 15 min).
2 The Dde protecting group was removed by treating the resin with 5% hydrazine/DMF
3 (2 x 15 min). After each manipulation, the resin was washed with DMF.
4 Carbohydrate couplings to the N-terminus of the peptide or lipopeptide were carried
5 out overnight using 2 eq of glycosyl (lactose or galactose) succinates activated with
6 1.9 eq of HBTU in DMF and 2.5 eq equivalents of DIPEA. The glycopeptides were
7 de-acetylated using 75% (v/v) hydrazine hydrate in methanol (2 x 20 min). The resin
8 was washed consecutively with DMF, DCM, and MeOH and left to dry under vacuum
9 overnight. The crude glycopeptides were cleaved from the resin using a 3 h
10 incubation with TFA:TIPS:H_2O (95:2.5:2.5) and precipitation from cold diethyl ether.
11 The precipitated compounds were dissolved in ACN:H_2O:TFA (for
12 glycolipopeptides; 90:10:0.1) or H_2O:TFA (for glycopeptides; 100:0.1) and
13 lyophilized overnight. All crude peptides were purified using RP-HPLC as described
14 above.
15

16 *N^1-Leu-enkephalin-N^4-(β-D-galactopyranosyl)succinamide (GalEnk, **3a**)*
17 Purified yield: 70%. ES-MS ($C_{38}H_{53}N_7O_{13}$, 815.4) m/z: 816.4 $[M+H]^+$ (calcd. 816.4);
18 HR-MS ($C_{38}H_{53}N_7O_{13}$, 815.3701) m/z: 816.3774 $[M+H]^+$ (calcd. 816.3779); RP-
19 HPLC (C18 Vydac®, 20-35% B) 12.1 min; (C18 Alltima®, 0-70% B) t_R =17.9 min.
20

21 *N^1-Leu-enkephalin-C_{16}-N^4-(β-D-galactopyranosyl)succinamide (GalEnkC$_{16}$, **3b**)*
22 Purified yield: 16%. ES-MS ($C_{54}H_{84}N_8O_{14}$, 1068.6) m/z: 1070.1 $[M+H]^+$ (calcd.
23 1069.6); HR-MS ($C_{54}H_{84}N_8O_{14}$, 1068.6107) m/z: 1069.6124 $[M+H]^+$ (calcd.
24 1069.6185); RP-HPLC (C8 Vydac®, 60-100% B) t_R = 14.3 and 15.5 min
25 (diastereomers).

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1

2 *N^1-Leu-enkephalin-N^4-(β-D-galactopyranosyl-(1→4)-β-D-*

3 *glucopyranosyl)succinamide (LacEnk, **3c**)*

4 Purified yield: 34%. ES-MS ($C_{44}H_{63}N_7O_{18}$, 977.4) m/z: 978.5 $[M+H]^+$ (calcd. 978.4);

5 HR-MS ($C_{44}H_{63}N_7O_{18}$, 977.4230) m/z: 978.4284 $[M+H]^+$ (calcd. 978.4308); RP-

6 HPLC (C18 Vydac®, 20-35% B) 11.5 min; (C18 Alltima®, 0-70% B) t_R = 17.5 min.

7

8 *N^1-Leu-enkephalin-C_{16}-N^4-(β-D-galactopyranosyl-(1→4)-β-D-*

9 *glucopyranosyl)succinamide (LacEnkC$_{16}$, **3d**)*

10 Purified yield: 49%. ES-MS ($C_{60}H_{94}N_8O_{19}$, 1230.7) m/z: 1231.8 $[M+H]^+$ (calcd.

11 1231.7), 1254.0 $[M+Na]^+$ (calcd. 1253.7); HR-MS ($C_{60}H_{94}N_8O_{19}$, 1230.6635) m/z:

12 1231.6675 $[M+H]^+$ (calcd. 1231.6713); RP-HPLC (C8 Vydac®, 60-100% B) t_R = 13.7

13 and 14.8 min (diastereomers).

14

15 *2.5. Enzyme expression and purification*

16

17 Recombinant LgtC was expressed and purified according to Johnstone et al..[27] *E.*

18 *coli* AD202 harboring pCWLgtC-25 and containing a His-tag[32, 43] was grown to

19 an A_{600} of 0.4 in 2L of shaken 2xYT broth containing 100 μg/mL of ampicillin at 37

20 °C. LgtC expression was induced by addition of 0.5 mM isopropyl 1-thio-β-D-

21 galactospyranoside and growth of culture was continued for 4 hours at 37 °C. Cells

22 were harvested by centrifugation and re-suspended in 10 mL buffer A (20 mM

23 Na_2HPO_4, 0.5 M NaCl, 10 mM imidazole pH 7.5). Complete EDTA-free Protease

24 Inhibitor Cocktail (Roche) was added according to product instructions. Cells were

25 then lysed by 5 passages through a French press at 1000 psi as indicated by gauge

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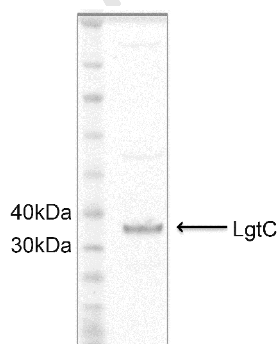
1 pressure. The lysate was centrifuged at 18 000 g for 10 min at 4 °C. For histidine-
2 tagged protein purification, the supernatant was collected and applied to an
3 immobilized metal affinity column containing TALON™ Metal Affinity Resin (BD
4 Biosciences).[27] After the sample passed through the column, the column was
5 washed with 10 column volumes of buffer A and 5 column volumes of buffer A with
6 50 mM imidazole added. The protein was eluted by collecting 1 mL fractions over 10
7 column volumes of buffer A with 150 mM imidazole. Fractions were analyzed by 4-
8 12% SDS-PAGE (Invitrogen). Fractions containing purified LgtC were collected and
9 pooled. From 8 g of cells, 8.4 mg of purified LgtC protein was dialyzed in 10 kDa
10 molecular mass cut-off Snakeskin™ (Pierce) dialysis tubing against 5 L of 20 mM
11 Na_2HPO_4, pH 7.5 at 4 °C overnight. The dialyzed sample was concentrated using a 10
12 kDa molecular mass cut-off Macrosep® Device (Pall Corporation). The concentration
13 of purified LgtC yield was estimated by BCA (Bicinchoninic acid) protein assay kit
14 (Pierce). The LgtC protein (1 μg) was analyzed by sodium dodecyl sulfate
15 polyacrylamide gel electrophoresis (SDS-PAGE) using a 4-12% NuPAGE® Novex
16 Bis-Tris Gels (Invitrogen) stained with Coomassie Blue to confirm its homogeneity
17 and molecular mass of 35.7 kDa (Fig. 1). To maintain the enzyme activity, glycerol
18 (50% v/v) was added to the LgtC enzyme mix (2 mg/mL in 20 mM Na_2HPO_4, pH 7.5
19 buffer), which was then stored at -20°C.
20



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1 **Figure 1.** SDS-PAGE gel analysis of the purified LgtC protein stained with
2 Coomassie Blue. Lane 1: pre-stained protein marker (New England Biolabs); lane 2: 1
3 μg of purified LgtC protein.
4
5 *2.6. Enzymatic glycosylation of glycopeptides and glycolipopetides*
6
7 *2.6.1. Enzyme activity with lactose as acceptor*
8 Donor UDP-Gal (20 μL, 12 mM) and acceptor β-D-galactopyranosyl-(1→4)-D-
9 glucose (20 μL, 10 mM) in 50 mM HEPES buffer, pH 7.0 containing 10 mM $MnCl_2$,
10 were reacted under catalysis with LgtC enzyme mixture (10 μL, 1 mg/mL, 50%
11 glycerol). The reaction mixture was incubated at 37 °C overnight. The product (α-D-
12 Gal-(1→4)-ß-D-Gal-(1→4)-D-Glc) formation was observed by TLC
13 (isopropanol:NH_4OH:H_2O 7:3:2, v/v): R_F (GalLac) = 0.16; R_F (Lac) = 0.25 and
14 confirmed by ES-MS ($C_{18}H_{32}O_{11}$, 424.2) m/z: 425 $[M+H]^+$ (calcd. 425.2).
15
16 *2.6.2. Analytical enzymatic reactions and optimizations*
17 Donor UDP-Gal (20 μL, 6 mM) and GalEnk or LacEnk (20 μL, 2 mM) in 50 mM
18 HEPES buffer, pH 7.0 containing 10 mM $MnCl_2$, were reacted under catalysis by
19 LgtC enzyme mixture (0.5 μL, 2 mg/mL, 50% glycerol). For the reactions with
20 $GalEnkC_{16}$ and $LacEnkC_{16}$, 1 μL of dimethyl sulfoxide (DMSO) was added to the
21 reaction mixture. The reactions were incubated at 37 °C overnight. The reaction
22 mixtures were filtered (SPE Vydac® columns: C18 for glycopeptides or C4 for
23 lipidated glycopeptides) to remove salt prior to monitoring with RP-HPLC and/or MS
24 (0, 2, 4, 6 and 24 h). Control reactions were set up as described above replacing LgtC
25 enzyme with HEPES/$MnCl_2$ buffer.

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2 *2.6.3. Semi-preparative enzymatic reactions*

3 UDP-Gal and an acceptor (Table 1) were dissolved in 0.4 mL of 50 mM HEPES
4 buffer, pH 7.0, 10 mM $MnCl_2$. The reaction mixture used for the synthesis of the
5 lipidated substrates **4b** and **4d** contained 5% DMSO. LgtC enzyme (2 mg/mL, 50%
6 glycerol) was added and the reaction mixture was incubated at 37 °C. Further UDP-
7 Gal and LgtC were added to the reaction mixture portion-wise (Table 1). The reaction
8 mixture was lyophilized and re-dissolved in 120 μL of solvent A (**4a** and **4c**) or in
9 40% DMSO in solvent B (**4b** and **4d**) and purified by RP-HPLC using C18 Alltima®
10 column and 10-35% gradient of solvent B (**4a** and **4c**) or C8 Vydac® column and 0-
11 70% gradient of solvent B (**4b** and **4d**).

12

13 **Table 1.** The reaction conditions for the enzymatic synthesis of **4a-d**.

Product	UDP-Gal (mg/μmol)	Acceptor (mg/μmol)	LgtC (μL)	Reaction time (days)
4a	3.7/6.0 + 4.3/7.0	**3a**: 1.6/2.0	40 + 50	4
4b	3.0/5.0 + 1.2/2.0	**3b**: 1.3/1.2	50 + 90	7
4c	6.8/11.0	**3c**: 3.5/3.6	75	1
4d	2.0/3.0 + 3.0/5.0	**3d:** 2.0/1.5	25 + 200	7

14

15 *N^1-Leu-enkephalin-N^4-(α-D-galactopyranosyl-(1→4)-β-D-*
16 *galactopyranosyl)succinamide (GalGalEnk)* ***(4a)***

17 Purified yield: 45% (0.9 mg, 0.9 μmol); ES-MS ($C_{44}H_{63}N_7O_{18}$, 977.4) m/z: 978.6
18 $[M+H]^+$ (calcd. 978.4); HR-MS ($C_{44}H_{63}N_7O_{18}$, 977.4230) m/z: 978.4283 $[M+H]^+$
19 (calcd. 978.4308); RP-HPLC (C18 Alltima®, 0-70% B) t_R = 17.6 min.

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2 *N^1-Leu-enkephalin-C_{16}-N^4-(α-D-galactopyranosyl-(1→4)-β-D-*

3 *galactopyranosyl)succinamide (GalGalEnkC$_{16}$)(**4b**)*

4 Purified yield: 13% (0.2 mg, 0.16 μmol); ES-MS ($C_{60}H_{94}N_8O_{19}$, 1230.6) m/z: 1231.8

5 $[M+H]^+$ (calcd. 1231.6). RP-HPLC (C8, Vydac® 60-100% B) t_R = 15.2 min (further

6 galactosylated products t_R = 11.8, 13.0, 14.0). LC-MS (C8, Vydac®, 0-100% B), 7.6

7 min, GalGalEnkC$_{16}$ 1232 m/z: 5.23 min and GalEnkC$_{16}$ 1069 m/z: 5.31 min; UDP-

8 Gal 610 m/z: 1.10 min.

9

10 *N^1-Leu-enkephalin-N^4-(α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-*

11 *β-D-glucopyranosyl)succinamide (GalLacEnk) (**4c**)*

12 Purified yield: 71% (2.9 mg, 2.6 μmol); ES-MS ($C_{50}H_{73}N_7O_{23}$, 1139.5) m/z: 1140.7

13 $[M+H]^+$ (calcd. 1140.5); HR-MS ($C_{50}H_{73}N_7O_{23}$, 1139.4758) m/z: 1140.4691 $[M+H]^+$

14 (calcd. 1140.4836); RP-HPLC (C18 Alltima®, 0-70% B) t_R = 17.2 min.

15

16 *N^1-Leu-enkephalin-C_{16}-N^4-(α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-*

17 *(1→4)-β-D-glucopyranosyl)succinamide (GalLacEnkC$_{16}$) (**4d**)*

18 Purified yield: 20% (0.4 mg, 0.3 μmol); ES-MS ($C_{66}H_{104}N_8O_{24}$, 1392.7) m/z: 1394.3

19 $[M+H]^+$ (calcd. 1393.7); HR-MS ($C_{66}H_{104}N_8O_{24}$, 1392.7163) m/z: 1393.7209 $[M+H]^+$

20 (calcd. 1393.7241); RP-HPLC (C8, Vydac® 60-100% B) t_R = 13.0, 14.0 min

21 (diastereomers). LC-MS (C8, Vydac®, 0-100% B, 7.6 min, GalLacEnkC$_{16}$ 1394 m/z:

22 5.06 min and LacEnkC$_{16}$ 1232 m/z: 5.09 min; UDP-Gal 610 m/z: 1.10 min.

23

24 **3. Results and discussion**

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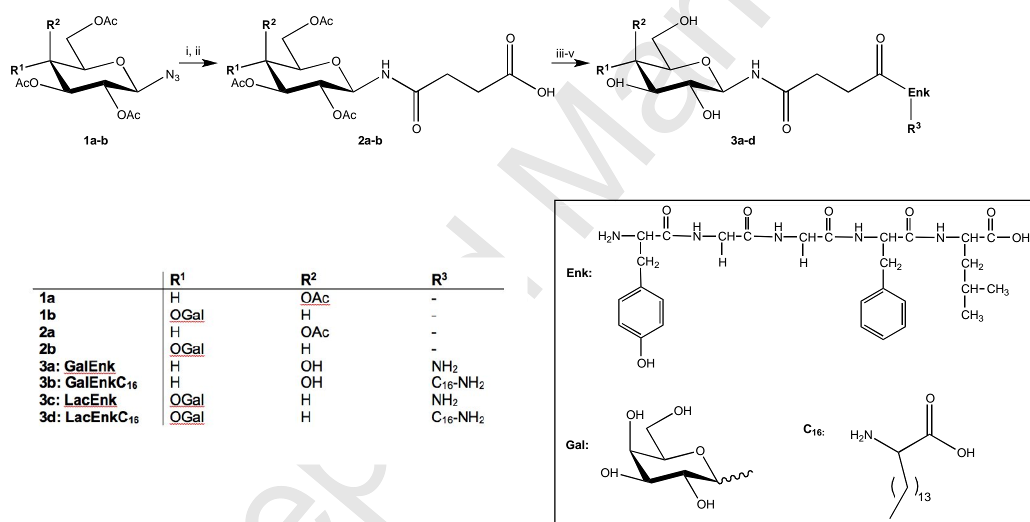
3.1. Chemical synthesis of Leu-enkephalin and its lipid- and/or sugar-modified derivatives

In this study, we investigated the effect of N-terminal carbohydrate and C-terminal lipid attachments to the Enk peptide on *N. meningitidis* LgtC specificity. Conventional stepwise solid phase peptide synthetic methodology[42, 44] was used for the production of simple carbohydrate and lipid Enk conjugates (**3a-d**) (Scheme 1), which served as substrates of LgtC.



	R^1	R^2	R^3
1a	H	OAc	-
1b	OGal	H	-
2a	H	OAc	-
2b	OGal	H	-
3a: GalEnk	H	OH	NH_2
3b: GalEnkC$_{16}$	H	OH	C_{16}-NH_2
3c: LacEnk	OGal	H	NH_2
3d: LacEnkC$_{16}$	OGal	H	C_{16}-NH_2

Scheme 1. Chemical synthesis of glyco(lipo)peptides **3a-3d**. i+ii (for **2a**): dry THF, H_2, 10% Pd/C, DMAP (cat.), succinic anhydride, RT, overnight; i (for **2b**): DCM, H-cube hydrogenation (H_2, Pd/C, 4 h, 40 °C, flow rate 2 mL/min); ii (for **2b**): dry pyridine, DMAP (cat.), succinic anhydride, 24 h, N_2; iii: stepwise solid phase peptide synthesis of Enk-NH-MBHA resin (for **3a** and **3c**) or Enk-C_{16}-NH-MBHA resin (for **3b** and **3d**), DMF, HBTU, DIPEA, overnight; iv: 75% (v/v) hydrazine hydrate in MeOH; v: TFA:TIPS:H_2O (95:2.5:2.5), 3 h, cold diethyl ether.

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2 The Dde-C_{16} was coupled on the Rink Amide-MBHA resin followed by couplings of
3 Enk peptide sequence (Tyr-Gly-Gly-Phe-Leu) using activated Fmoc-L-amino acids
4 with HBTU in DMF and DIPEA. The carboxylic acid functionalized carbohydrate
5 derivatives, **2a-b**, were coupled to the N-terminus of the Enk peptide or lipopeptide
6 under similar conditions. However, only 2 equivalents (instead of 4) of glycosyl
7 succinates, **2a-b**, and a prolonged coupling time (overnight instead of 2 x 1 h) were
8 used to achieve higher yields with less starting material (data not shown). After de-
9 acetylation with hydrazine hydrate in MeOH, the crude products were cleaved from
10 the resin, precipitated from cold diethyl ether, dissolved in ACN:H_2O:TFA and
11 lyophilized overnight.
12 Following the RP-HPLC purification with gradient of either 0-70% of solvent B using
13 C18 Alltima® column (for glycopeptides **3a** and **3c**), or 60-100% gradient of solvent
14 B and C8 Vydac® column (for glycolipopeptides **3b** and **3d**), GalEnk (**3a**) was
15 obtained in 70% yield, GalEnkC_{16} (**3b**) in 16%, LacEnk (**3c**) in 34% and LacEnkC_{16}
16 (**3d**) in 49% yields. The lipid-containing glycolipopeptides, **3b** and **3d**, were observed
17 as two peaks on RP-HPLC, as they were diastereomers synthesized from a racemic
18 mixture of D- and L-lipoamino acid (Fig. 2). Structure elucidation and purity of all
19 products was confirmed by ES/HR-MS and RP-HPLC, commonly used methods for
20 peptide containing products.[8, 9]

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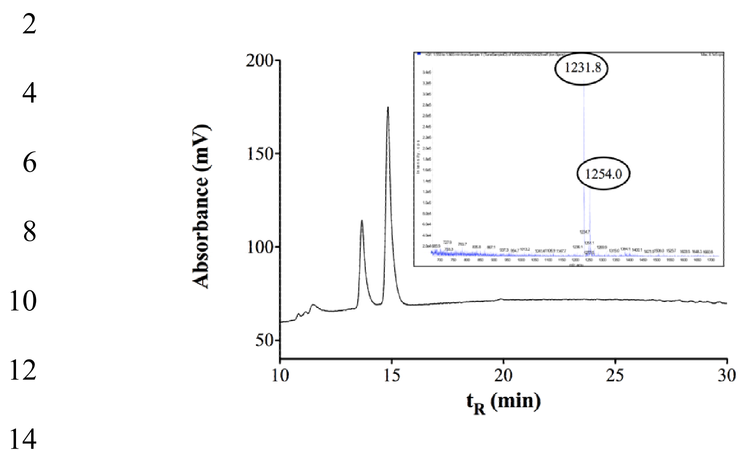

14

15 **Figure 2.** RP-HPLC and ES-MS of the LacEnkC$_{16}$ (**3d**) acceptor using C8 column

16 and 60-100% solvent B.

17

18 *3.2. Enzymatic synthesis of Leu-enkephalin glycolipopeptides*

19

20 The application of *N. meningitidis* LgtC α-1,4-galactosyltransferase[32, 45] was

21 previously reported by Antoine et al. and Zhang et al.[35, 46] In our study, we used

22 LgtC galactosyltransferase to add a galactose unit from UDP-Gal to

23 glyco(lipo)peptides, **3a-3d**, to investigate LgtC specificity and reactivity towards

24 these substrates.

25 The LgtC enzyme is known to transfer a galactosyl unit to the D-lactosyl glycolipid

26 acceptor via an α-1,4 linkage.[35] Therefore, D-lactose was chosen as a first acceptor

27 to test the enzyme activity in HEPES buffer. Binding interaction with metal ions (e.g.

28 Mn^{2+}) has been reported as vital for the stability of the enzyme fold and overall LgtC

29 activity[32, 47] thus, 10 mM $MnCl_2$ was added to all LgtC catalyzed reactions. The

30 formation of globotriose (α-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc) was observed by

31 thin layer chromatography (TLC): R_F of the product GalLac = 0.16 (R_F of Lac = 0.25;

32 isopropanol:NH_4OH:H_2O 7:3:2, v/v) and confirmed by MS.

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1 The four glyco(lipo)peptides, **3a-d**, and UDP-Gal were dissolved in HEPES/$MnCl_2$
2 buffer and reacted under catalysis of LgtC at 37 °C (Scheme 2). The progress of
3 glycosylation of the lipid modified Enk was monitored by LC-MS, ES-MS and RP-
4 HPLC (with samples filtered through SPE Vydac® columns prior to analysis to
5 remove the salt). Interestingly, the products were eluted via LC-MS at very similar
6 times to the starting materials: GalGalEnkC_{16} (**4b**) 5.23 min and GalEnkC_{16} 5.31 min;
7 GalLacEnkC_{16} (**4d**) 5.06 min and LacEnkC_{16} 5.09 min. Therefore, RP-HPLC
8 conditions were optimized for better separation of the starting materials and products
9 (see below). To further confirm that the peaks observed by RP-HPLC corresponded to
10 products containing Enk, absorbance at 280 nm (for Tyr) and absorbance at 260 nm
11 (for Phe) were measured and the 260/280 ratio was calculated. The 260/280 ratio was
12 <1, confirming the RP- HPLC additional peak corresponded to peptide.
13

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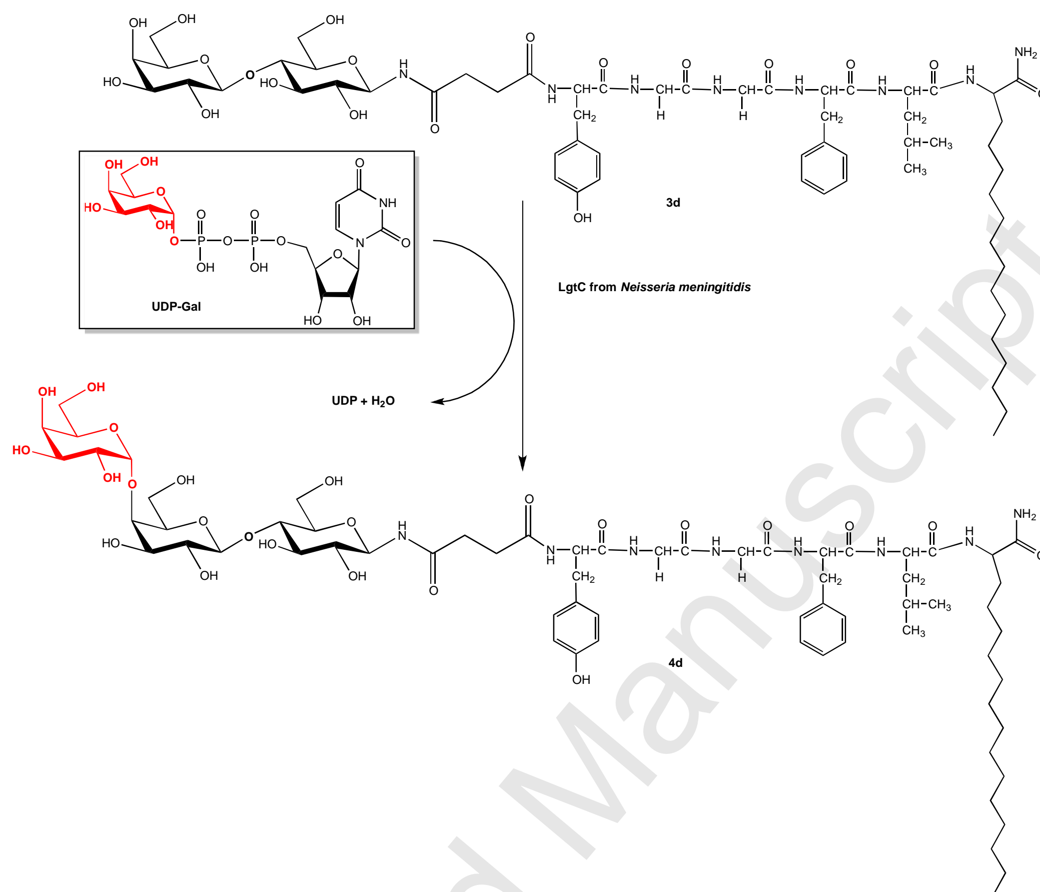

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2

3 **Scheme 2.** Enzymatic glycosylation of LacEnkC$_{16}$ (**3d**). The reaction was performed

4 using *N. meningitidis* LgtC galactosyltransferase, UDP-Gal in HEPES/MnCl$_2$ buffer

5 and 5% DMSO at 37 °C for 7 days to give GalLacEnkC$_{16}$ (**4d**) in 20% yield.

6

7 LacEnk (**3c**) was completely converted into GalLacEnk (**4c**) after 24 h (Fig. 3).

8 Reaction with the GalEnk (**3a**) acceptor was not completed after 24 h, therefore it was

9 reacted for an additional 3 days, when the highest amount of the GalGalEnk (**4a**) was

10 observed and by-product formation was detected. The lipid modified Enk peptides

11 (**3b** and **3d**) were also allowed to react for longer (7 days) to achieve higher yields (13

12 and 20%, respectively). UDP-Gal and LgtC were added to the reaction mixtures

13 portion-wise to overcome problems with their instability. All peptides **4a-d** were

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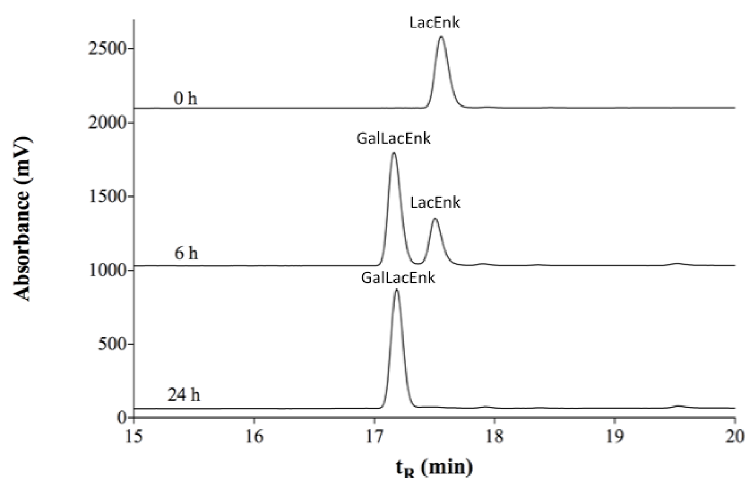
1 purified by RP-HPLC using C18 Alltima® column and 10-35% gradient of solvent B
2 (**4a** and **4c**) or 40% DMSO addition, C8 column and 0-70% gradient of solvent B (**4b**
3 and **4d**). Even though glycopeptide **3c** was converted to compound **4c** quantitatively
4 (Fig. 3), the isolated yield was 71%. The pure glycopeptide **4a** was obtained in 45%
5 yield and glycolipopeptides **4b** and **4d** in 13% and 20% yields, respectively (Table 2).
6 Blank reactions omitting the enzyme did not yield any products.
7



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9 **Figure 3.** Reaction progress of the enzymatic synthesis of GalLacEnk (**4c**) using LgtC
10 galactosyltransferase monitored by analytical RP-HPLC on an Alltima® C18 column,
11 0-70% B.
12

13 Substrate specificity of LgtC was studied previously and showed 667-fold higher
14 relative LgtC activity,[35] 500 to 1200-fold higher second-order rate,[34] or 5-fold
15 higher reaction rate[48] towards D-lactosyl acceptors when compared with those of D-
16 galactosyl. We also noticed higher activity of the LgtC enzyme towards D-lactosyl
17 terminated acceptors, **3c** and **3d**, although, the difference in activities (towards D-
18 lactosyl and D-galactosyl acceptors) was not as high as those published previously.
19 Remarkably, we were not only able to synthesize GalLacEnk (**4c)** in 71% yield, but

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1 also GalGalEnk (**4a**) in 45% yield which is a 9x higher yield than that reported by
2 Zhang et al. when using similar substrates.[35] The synthesis of **4a** required the use of
3 over 2x higher concentration of UDP-Gal and LgtC enzyme as well as a longer
4 reaction time (4 days compared to 24 h) than that used for the synthesis of **4c** (Table
5 2). The formation of several additional galactosylated by-products was also observed
6 by MS when using GalEnk (**3a**) and GalEnkC_{16} (**3b**) as acceptors in the enzymatic
7 glycosylation reactions (data not shown).
8
9 **Table 2.** Substrate specificity of LgtC towards modified Enk acceptors (1 μmol).

Acceptor (1 μmol)	**UDP-Gal (μmol)**	**LgtC (μL)**	**Purified Yield (%)**
GalEnk (**3a**)	6.5	45	45
GalEnkC_{16} (**3b**)	5.8	117	13
LacEnk (**3c**)	3.0	21	71
LacEnkC_{16} (**3d**)	5.3	150	20

10
11 The natural substrate of LgtC enzyme was reported to be lipooligosaccharide.[34]
12 Nevertheless, our results showed lower yields when facilitating reactions with
13 lipidated constructs **3b** and **3d** (13% and 20%, respectively) compared with non-
14 lipidated constructs **3a** and **3c** (45% and 71%, respectively). This was due to poor
15 solubility resulting from the long alkyl side chain of the lipoamino acid. Therefore, it
16 was necessary to include DMSO in reaction mixtures that contained lipidated peptides
17 (no products were formed in the reactions without DMSO). Varied amounts of DMSO
18 were tested (0-10%) to increase the solubility of **3b** and **3d**. Five percent DMSO was

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found to be optimal to balance the substrate solubility (increasing with DMSO additions) and enzymatic activity (decreasing with DMSO additions).

4. Conclusion

We successfully designed and developed the enzymatic synthesis of modified Enk derivatives. Our results demonstrate the prospective use of glycosyltransferases for the glycosylation of glyco(lipo)peptides. The enzymatic reactions catalyzed by LgtC galactosyltransferase from *N. meningitidis* were performed on an analytical scale giving purified yields of between 13 and 71%. Moreover, the LgtC enzyme had broader substrate specificity than previously reported (also active towards galactosyl terminated glycopeptides and glycolipopeptides). The glyco(lipo)peptides described in this study could potentially improve delivery of Enk to the CNS and will therefore be tested in the future *in vitro* and *in vivo* experiments.

Acknowledgements

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1 **Highlights**

2 - Sugar and lipid modified enkephalin peptides were chemically synthesized.
3 - LgtC galactosyltransferase catalyzed conversion of enkephalin derivatives.
4 - Galactosylated products were purified in 13-71% yields.
5 - 5% DMSO increased solubility and enzymatic conversion of lipidated
6 enkephalins.
7 - LgtC displayed unexpectedly high substrate specificity towards galactosyl
8 moieties.
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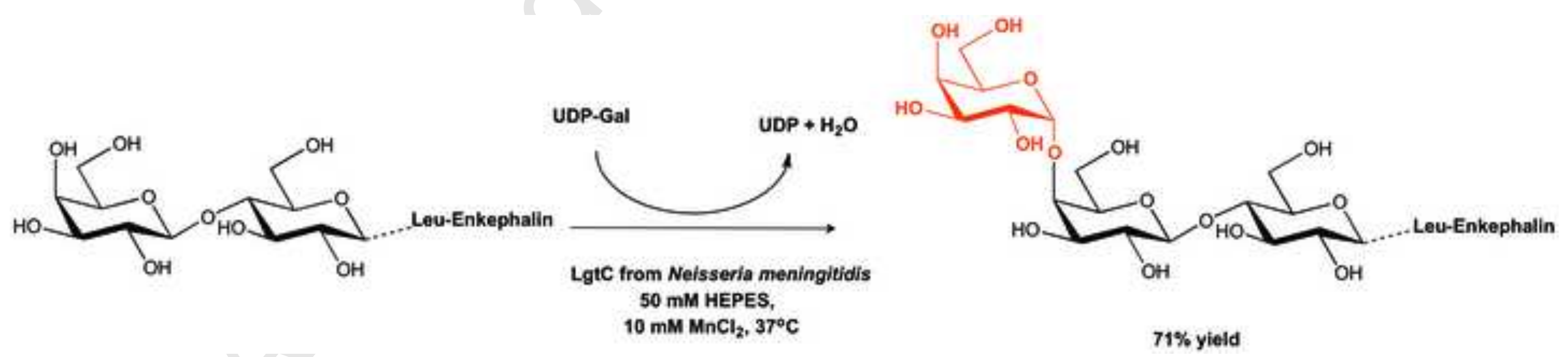

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