

Short Note

## Luteinizing Hormone Releasing Hormone/Galactose Core/Lipopeptide

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**Abstract:** A self-adjuvanting vaccine candidate comprising four copies of luteinizing hormone releasing hormone (LHRH), a galactose carrier/core and lipoamino acid based adjuvant was synthesized in 21% yield by a solid phase peptide synthesis, carbohydrate and Boc-chemistry methods.

**Keywords:** self-adjuvanting vaccine; carbohydrate carrier; luteinizing hormone releasing hormone

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### 1. Introduction

Prostate cancer has been identified as one of the most high-risk cancers in males [1]. The aim of our work was to develop a vaccine candidate for treatment of prostate cancer as an effective treatment is highly needed [2–4]. A vaccine candidate containing LHRH decapeptide (EHWSYGLRPG) antigen was used to generate anti-LHRH antibodies, which helped to control the growth of cancerous cells [5].

The Lipid Core Peptide (LCP) system [6] is a self-adjuvanting vaccine delivery system based on lipidic amino acids, a carrier and peptide antigens. Several LCP-based vaccine candidates have elicited high immune response *in vivo* [7,8].

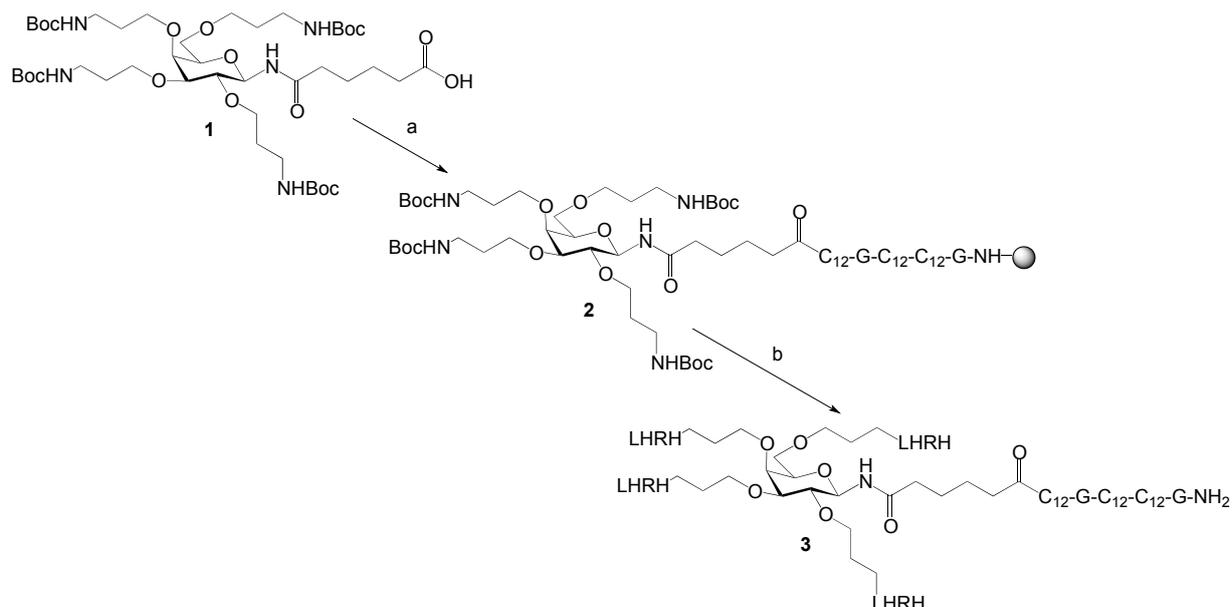
One of the applications of carbohydrates in synthetic peptide vaccines [9] is their use as a carrier of peptide antigens, as they provide, after a modification of their functional groups, a number of attachment sites for conjugation of multiple epitopes. Different sugar entities are commercially available and give different spatial arrangements to the attached peptide epitopes, which may result in better recognition by immune cells. Besides that, the stability of peptide antigens in glycopeptide conjugates is improved [10]. Immune responses have been detected when carbohydrates were used as a branching core in vaccine constructs [8].

In this study, an LHRH vaccine candidate was synthesized using a combination of LCP system with three 12-carbon lipoamino acids and a galactose core as carrier of four copies of LHRH peptide antigen. A lipopeptide was attached to the anomeric carbone of the galactose core through a succinic acid linker.

## 2. Results and Discussion

From D-galactose, a series of reactions (acetylation, bromination, azide formation followed by the removal of acetyl protecting group) were carried out to form  $\beta$ -D-galactopyranosyl azide [11].  $\beta$ -D-Galactopyranosyl azide was cyanoethylated, and the azide was reduced to amine prior its coupling to monobenzyl adipate [8]. The reduction of cyanoethyl groups was achieved by addition of sodium borohydride and cobalt chloride hexahydrate into the reaction. The final reaction step for the carbohydrate core synthesis involved cleavage of benzyl group from the monobenzyl adipate using hydrogenator and Pd catalyst [8].

After the synthesis of the carbohydrate core **1**, the lipidic adjuvanting moiety consisting of three 2-amino-dodecanoic acids (Boc-C<sub>12</sub>-OH) and two glycine spacers was synthesized [12]. Subsequently, the carbohydrate core was coupled onto the lipidic adjuvant to give **2**, followed by the stepwise solid phase peptide synthesis of four copies of LHRH peptide (Scheme 1). For each coupling, 4.4 equiv. of amino acid, 4 equiv. of HBTU in DMF and 6 equiv. DIPEA was used. The crude product was cleaved from the resin by HF and purified by Reverse Phase High Performance Liquid Chromatography (RP-HPLC; Table 1). The pure fractions were lyophilized to give compound **3**, (LHRH)<sub>4</sub>-Gal-LCP, as a white powder (4.7 mg) in 21% yield. Using analytical RP-HPLC with C4 Vydac column, 0%–100% gradient over 30 min, and 1 mL/min flow rate, the retention time of (LHRH)<sub>4</sub>-Gal-LCP was 21 and 21.3 min (Figure S1, Supplementary materials). Generally, when using racemic lipoamino acids, the multiple peaks in RP-HPLC chromatogram are assigned to diastereomeric mixture of products including both D- and L-isoforms [13]. The mass of **3** (C<sub>284</sub>H<sub>413</sub>N<sub>75</sub>O<sub>64</sub>, 5901.8) was confirmed by Electrospray Ionisation Mass Spectrometry (ESI-MS) (Figure S2, Supplementary materials).



**Scheme 1.** Synthetic scheme of (LHRH)<sub>4</sub>-Gal-LCP (**3**); (a) C<sub>12</sub>-G-C<sub>12</sub>-C<sub>12</sub>-G-NH-MBHA resin; C<sub>12</sub>=2-amino-D,L-dodecanoic acid; (b) solid phase peptide synthesis with 4.4 equiv. LHRH (EHWSYGLRPG), 4 equiv. HBTU, 6 equiv. DIPEA, HF cleavage of **3** from the resin.

**Table 1.** RP-HPLC purification method for **3**, (LHRH)<sub>4</sub>-Gal-LCP. Mobile phases were solvent A (water containing 0.1% TFA); solvent B (90% CH<sub>3</sub>CN, 10% water and 0.1% TFA), C4 Vydac column (10 μm, 22 mm × 250 mm), and flow rate 10 mL/min.

Time (min)	Solvent B (%)
0–10	0–40
10–60	40–80
60–80	80–100
80–90	100

### 3. Experimental Section

The (LHRH)<sub>4</sub>-Gal-LCP peptide **3** was synthesised using standard manual Solid Phase Peptide Synthetic (SPPS) protocols, Boc chemistry and *p*-4-methyl benzhydryl amine (*p*-MBHA) resin (Peptides International, Louisville, KY, USA) [14]. The *p*MBHA resin (substitution 0.45 mmol·g<sup>-1</sup>) was swollen in DMF overnight, the hydrochloride salt of the resin-bound amine was neutralized by treating the resin with 10% (*v/v*) DIPEA in DMF (3 × 5 mL × 15 min). The peptides were coupled using 4.4 equiv. amino acids preactivated with 4 equiv. of 0.5 M HBTU in DMF and 6 equiv. DIPEA, then mixed with the resin for 30–60 min at r.t. The Boc protecting groups were removed from the amino acids using neat TFA.

The building blocks, lipoamino acid Boc-C<sub>12</sub>-OH and galactose core were synthesized according to previously published procedures [8]. For the synthesis of LCP moiety, *p*MBHA resin (0.25 mmol scale) was used onto which Boc-Gly-OH, 2 × Boc-C<sub>12</sub>-OH, Boc-Gly-OH and Boc-C<sub>12</sub>-OH were coupled. The activated galactose core (**1**, 2equiv., 0.6mmol, 56 mg) was coupled onto the LCP moiety on the resin (0.37 mmol·g<sup>-1</sup>; 0.03mmol scale) for 8 hours using the standard SPPS procedure. Four copies of LHRH peptide (EHWSYGLRPG) were coupled onto the Gal-LCP-resin **2** (Scheme 1). Upon

the completion of coupling of all amino acids, the formyl protecting group on tryptophan was removed by treating the peptide with 20% (v/v) piperidine in DMF ( $2 \times 10 \text{ mL} \times 15 \text{ min}$ ) and the 2,4-dinitrophenyl protecting group was removed by adding 20% 2-mercaptoethanol/10% DIPEA in DMF ( $2 \times 10 \text{ mL} \times 20 \text{ min}$ ). After the complete removal of all protecting groups including Boc deprotection, the peptidyl resin was washed with DMF, DCM and MeOH and dried under vacuum overnight.

HF cleavage of peptidyl-resin was carried out at  $0^\circ\text{C}$  for 2 hours, by treating the peptidyl-resin with HF ( $10 \text{ mL}\cdot\text{g}^{-1}$ ) and 5% (v/v) *p*-cresol. HF was removed under vacuum, the peptides were precipitated in ice-cold diethyl ether, filtered and dissolved in 50% MeCN/H<sub>2</sub>O + 0.1% TFA and lyophilized. The crude product **3** was purified on a C4 Vydac column ( $10 \mu\text{m}$ ,  $22 \text{ mm} \times 250 \text{ mm}$ ) using preparative RP-HPLC, 10 mL/min flow rate, gradient of solvents as listed in Table 1 with detection at 230 nm. The collected fractions were then characterized with analytical RP-HPLC and ESI-MS. Peptide purity of **3**, (LHRH)<sub>4</sub>-Gal-LCP, was checked using analytical RP-HPLC on a Vydac C4 ( $5 \mu\text{m}$ ,  $4.6 \text{ mm} \times 250 \text{ mm}$ ) with a gradient of 0 to 100% B over 30 min at a flow rate of 1 mL/min. ESI-MS of **3**, (LHRH)<sub>4</sub>-Gal-LCP, was performed on a PE Sciex AP13000 triple quadrupole mass spectrometer (a constant flow of 1:1 mixture of water containing 0.1% acetic acid and 90% acetonitrile/10% water/0.1% acetic acid at a rate of 0.5 mL/min).

(LHRH)<sub>4</sub>-Gal-LCP (**3**): white powder; purification yield: 4.7 mg (21%); RP-HPLC (C4 column; 0%–100% solvent B (90% ACN + 10% H<sub>2</sub>O + 0.1% TFA) over 30 min, 1 mL/min flow rate):  $t_{\text{R}} = 21.0 \text{ min}$ ,  $21.3 \text{ min}$ . ESI-MS (C<sub>284</sub>H<sub>413</sub>N<sub>75</sub>O<sub>64</sub>, 5901.8):  $m/z = 846.4$  [M + 7H]<sup>7+</sup> (calcd, 844.1), 984.9 [M + 6H]<sup>6+</sup> (calcd, 984.5), 1181.2 [M + 5H]<sup>5+</sup> (calcd, 1181.2).

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## Author Contributions

PS and IT designed and wrote the manuscript. PS and HLL performed synthesis, purification and characterization of the vaccine candidate.

## Conflicts of Interest

The authors declare no conflict of interest.

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