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Neoglycopeptide Synthesis and Purification in Multi-gram Scale: Preparation of *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-N^{α}-fluoren-9-yl-methoxycarbonyl-hydroxyproline and Its Use in the Pilot-scale Synthesis of the Potent Analgesic Glycopeptide $O^{1.5}$-β-D-galactopyranosyl [DMet^2, Hyp^5]enkephalinamide

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Abstract: The preparation of a β-galactosylated hydroxyproline derivative and its use in the multi-gram solid-phase synthesis of the potent analgesic neoglycopeptide $O^{1.5}$-β-D-galactopyranosyl [D-Met^2, Hyp^5]enkephalinamide is described in this paper. The most closely related impurities have been identified, isolated and characterized. Significant aspects of the synthesis and purification affecting yields and purity of both the building block and the target neoglycopeptide are discussed. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: neoglycopeptides; solid-phase peptide synthesis; analgesic glycopeptides; pilot scale; preparative purification

INTRODUCTION

Glycosylation of peptides and other potential therapeutic agents is a promising approach in rational drug design. The presence of a sugar moiety is believed to influence the transport of the drug through biological membranes as well as to increase its resistance to hydrolytic enzymes. We have described the synthesis and antinociceptive activity of *O*-glycosylated conjugates [1, 2] of a potent analgesic pentapeptide [3]. The glycopeptides exerted a long-lasting effect and *in vivo* potency was dramatically increased with respect to the unglycosylated parent compound [1]. More recently, Hruby and co-workers synthesized and evaluated a series of glucosyl derivatives of cyclic enkephalin analogues that appear to cross the blood–brain barrier by active glucose transport [4]. Glycosylation of enkephalin-related peptides appears to yield conjugates with long-lasting effects and improved transport. This makes glycosylenkephalins promising candidates for novel drugs in pain treatment.

Since *in vivo* pre-clinical trials require the use of multi-gram amounts of any bioactive molecule, the scale-up of the synthesis is a key step that may determine the molecule's future as therapeutic drug. The preparation of such amounts of *O*-glycopeptides is especially complicated mainly due to the low

Abbreviations: aufs, absorbance units full scale; CZE, capillary zone electrophoresis; DEPT NMR, distorsionless enhancement by polarization transfer nuclear magnetic resonance; DIC, diisopropylcarbodiimide; EDT, ethanedithiol; ES-MS, electrospray mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; TEAP, triethylamine phosphate.

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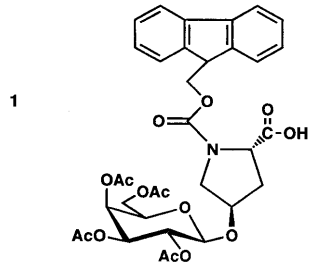
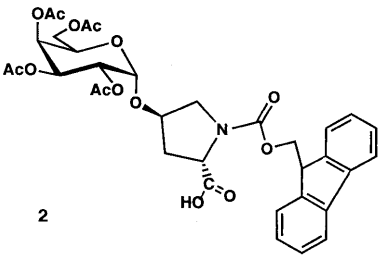
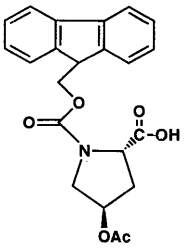
efficiency of the glycosylation methods available, compared to the formation of the peptide bond.

The technique of solid-phase peptide synthesis [5] was successfully applied to glycopeptides using the Fmoc approach [6, 7]. The synthesis of glycosylated Fmoc–amino acids and their incorporation into the resin–bound growing peptide chain has been the strategy most widely used for the solid–phase synthesis of glycopeptides [8]. Of all the steps involved, the bottle-neck is the preparation of the glycosylated building block [8–10]. While repetitive acylation and protection–deprotection reactions involved in solid-phase synthesis are almost quantative, the glycosylation yields are much lower. Moreover, anomerization and other side reactions [11] lead to the formation of closely related impurities that dramatically lower the yield after the purification step. While this may not be a big problem at a small scale (hundreds of milligrams), it becomes a major obstacle when it comes to preparing larger amounts of glycosylated amino acids in an efficient way.

There are a number of methods for the formation of the *O*-glycosidic linkage between the sugar and the amino acid [12]. Different combinations of protecting groups on the carboxyl and amino function of the amino acid have been used [8]. A very attractive approach for the preparation of high amounts of *β*-*O*-glycosylated Fmoc–amino acids from per-acetylated monosaccharides has been proposed by Kihlberg and co-workers [13, 14]. The authors describe the use of the Lewis acid $BF_3.Et_2O$ as catalyst for the glycosylation of Fmoc–amino acids by per-acetylated galactose. The Fmoc–AA are used without C-protection and per-acetylated sugars are commercially available and cheaper than other activated sugars. The resulting conjugate can be used directly in solid-phase glycopeptide synthesis. Another convenient strategy uses the pentafluorophenyl (Pfp) group as C-protection during glycosylation [15]. Then Pfp acts as an activating group in the subsequent glycopeptide chain extension. In our case, we chose the low-protection scheme because it appeared to be the simplest and cheapest method. We describe here the preparation of *O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-N^{α}-fluoren-9-yl-methoxycarbonyl-hydroxyproline **1** and its use for the solid-phase synthesis of multigram quantities of $O^{1,5}$-*β*-D-galactopyranosyl[D-Met^2, Hyp^5]enkepalinamide **6** of the highest purity by both RP-HPLC and CZE. The most closely related impurifying structures detected for both the building block and the glycopeptide were the *α*-galactose-







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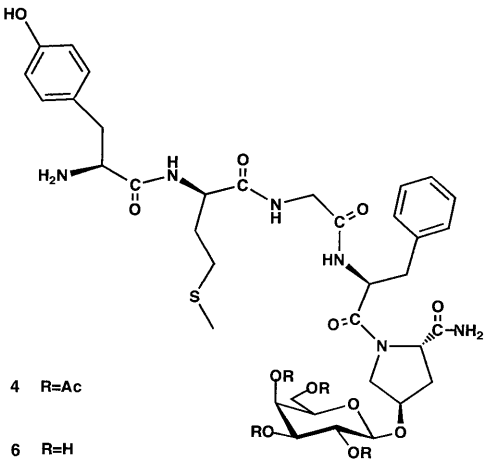
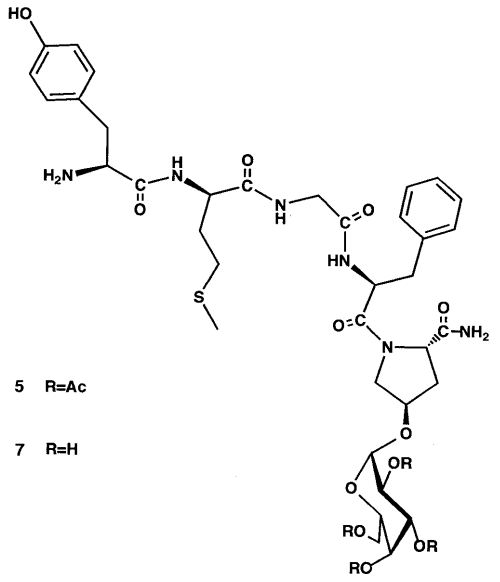


containing compounds **2** and **5**. They have been isolated and characterized along the way. The critical aspects in the synthesis and purification of the glycosylated amino acid are discussed in relation to its use as a building block for the multi-gram solid-phase synthesis of pharmacologically active *β*-*O*-glycopeptides.



5 R=Ac

7 R=H

MATERIALS AND METHODS

4-(2′,4′–Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink resin) (Novabiochem, Läufelfingen, Switzerland) was used for the solid-phase glycopeptide synthesis. Amino acids used include *O*-*t*-butyl-Fmoc-L-Tyr-OH, Fmoc-D-Met-OH, Fmoc-Gly-OH and Fmoc-Phe-OH (Saxon Biochemicals, Hannover, Germany). *O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-N$^{\alpha}$-Fmoc-Hyp-OH was synthesized and purified as described below from Fmoc-L-Hyp-OH (4-trans, Novabiochem, Läufelfingen, Switzerland) and *β*-D-galactose-pentaacetate (Aldrich, Steinheim, Germany). The Lewis acid catalyst $BF_3.Et_2O$ was purchased from Fluka (Buchs, Switzerland). Other reagents include DIC, EDT, Anisole (Aldrich) and HOBt (Fluka). Phosphoric acid, triethylamine buffer grade and TFA were from Merck (Darmstad, Germany). TFA used as mobile phase modifier was distilled in house.

Acetonitrile (CH_3CN) and methylene chloride (CH_2Cl_2) used for glycosylation were previously dried with CaH_2 and P_2O_5, respectively, and distilled under argon over the same drying agents. All solvents for solid-phase synthesis were of analytical grate. Dimethylformamide (DMF) was dried and stored over 3 Å molecular sieves and, immediately before use, was also freed of volatile amines by nitrogen steam. CH_3CN used for purification of the building block and TEAP runs was of preparative grade (Scharlau, Barcelona, Spain). Desalting runs were done with HPLC grade acetonitrile (Baker, Deventer, Holland). Deionized and Milli-Q grade water were used for preparative and analytical runs, respectively.

Analytical HPLC was performed on a Kontron Analytical system or a Merck-Hitachi (Darmstad, Germany) fitted with a VYDAC (The Separations Group, Hesperia, CA) C_{18}, 5 μm column. Preparative HPLC runs were performed on a Waters (Milford, MA) Prep LC 4000 pumping system and a Waters PrepPack 1000 module fitted with either a Delta Pak (Waters) C_4, 300 Å, 15 μm column or a PrepPack (Waters) column, filled with VYDAC C_{18}, 300 Å, 15–20 μm stationary phase.

Mass spectrometry analyses were performed at the *Servei d'Espectrometria de masses de la Universitat de Barcelona*. FAB-MS and ES-MS analyses were recorded on a VG-Quattro system from Fisons Instruments (Altrincham, UK). FAB-MS: positive ions produced by caesium bombardment with an accelerating of 10 kV, source temperature 25 °C; matrix, *p*-nitrobenzyl alcohol (NBA). ES-MS: (a) interphase, electrospray (pneumatically assisted), nebulizing nitrogen 10 l/h, drying nitrogen 450 l/h, source temperature 80 °C with a capillary voltage of 3.5 kV and focus voltage of 55 V; (b) pumping system: Phoenix 20 (Carlo Erba, Milan, Italy), carrier solution water/CH_3CN (1:1) containing 1% formic acid, injection at a flow rate of 7 μl/min. NMR analyses were carried out at the Department of Biological Organic Chemistry, CID-CSIC. ^1H, ^{13}C and DEPT NMR spectra were observed with either a Unity-300 or a Gemini-200 spectrometer from Varian (Palo Alto, CA) for solutions in $CDC1_3$ or d_6-DMSO. Chemical shifts are reported as *δ*-values downfield from tetramethylsilane as reference. Amino acid analysis was done at the *Servei de Microanàlisi de la Universitat de Barcelona*.

O-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-N$^{\alpha}$-fluoren-9-yl-methyoxycarbonyl-hydroxyproline 1

Batch BB-1. $BF_3.Et_2O$ (15.6 ml, 125 mmol) was added dropwise at room temperature to a suspension of Fmoc-Hyp-OH (17.7 g, 50.0 mmol) and penta-*O*-acetyl-*β*-D-galactopyranose (16.3 g, 41.8 mmol) in freshly distilled CH_2C1_2 (500 ml). After 4 h the suspension became a clear pink solution that was allowed to react for another 20 h. Figure 1 shows the analytical HPLC profile of the reaction mixture at different times. The reaction was stopped by addition of 15% citric acid (200 ml). The organic layer was washed with 15% citric acid (3 × 100 ml), dried (Na_2SO_4) and concentrated. The residue was

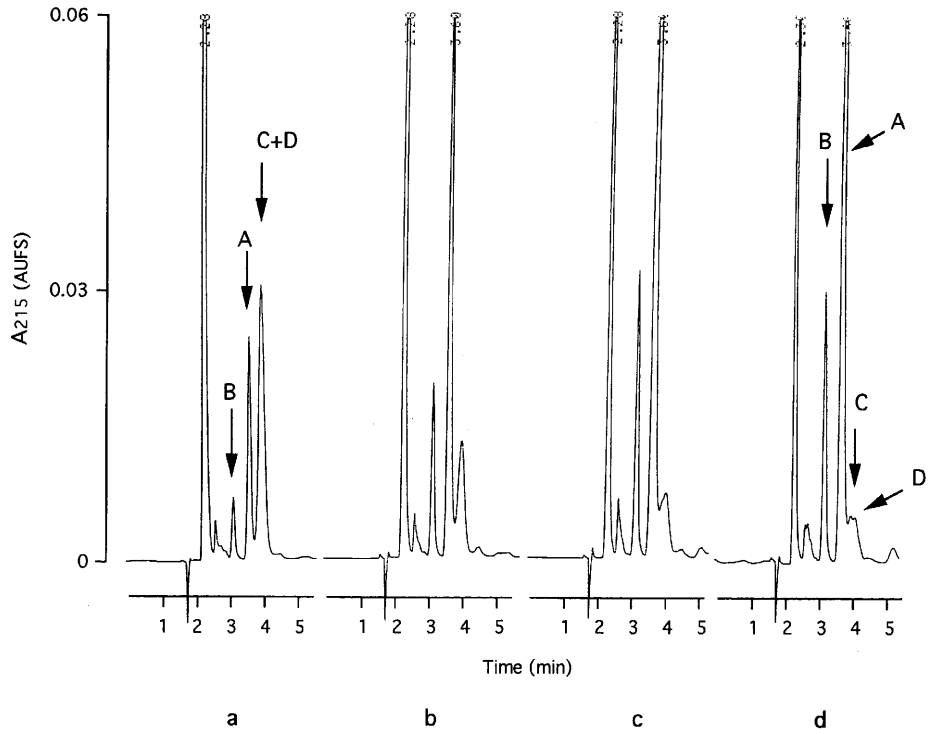


Figure 1 Analysis of the glycosylation reaction performed in methylene chloride. HPLC profile of the reaction mixture during the synthesis of **1**. Samples taken after: (a) 0.5 h; (b) 1.5 h; (c) 5 h; (d) 24 h. Sample preparation: CH_2CI_2 suspensions diluted 1:100 with water/CH_3CN (1:1). Loads: 5 μl, 5 μg. Column: VYDAC C_{18}, 5 μm, 4.6 × 250 mm. Elution: (A) 0.10% TFA, (B) 0.08% TFA in water/CH_3CN 1 : 4, isocratic conditions 66.6% B, at flow rate of 1.5 ml/min. Detection 215 nm, 0.06 aufs.

suspended in water/CH_3CN (1 : 1) (200 ml) and the remaining CH_2C1_2 evaporated under vacuum. Then the resulting suspension was lyophilized to a white solid (33 g).

The target building block was purified from this crude by preparative HPLC on a DeltaPack C_4, 47 × 300 mm, 300 Å, 15 μm column. Portions of 1.5 grams of crude solid were suspended in water/CH_3CN (5 : 2) and loaded onto the column through the pump. The mixture formed a colloidal suspension that passed through filters. No pressure build-up was detected during loading. The building block was eluted using a programmed gradient of CH_3CN (30 to 47% over 30 min) in water, with no mobile phase modifier, at a flow rate of 100 ml/min and detection at 230 nm. Analysis of the fractions was accomplished on a VYDAC C_{18}, 5 μm, 4.6 × 250 mm column eluted with a binary system, (A) 0.10% TFA, (B) 0.08% TFA in water/CH_3CN 1 : 4 under isocratic conditions 66.6% B, at a flow rate of 1.5 ml/min and detection at 215 nm, 0.016 aufs. The pure (> 99.5% by HPLC) fractions were pooled and lyophilized. Approximately 500 mg of pure **1** were obtained after each run. The lyophilized solids resulting from 20 runs were pooled, suspended in water/CH_3CN (5 : 2) and lyophilized. Finally, 10 grams of pure (Figure 2) *O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-N^{α}-fluoren-9-yl-methoxycarbonyl-hydroxyproline **1** were obtained. Yield 38%. ES-MS *m/z* 684.3 (*M* + 1), $C_{34}H_{37}N_1O_{14}$ requires 683.2. ^{1}H-NMR ($CDCl_3$, 300 MHz, selected signals) 2.01–2.18 (12H, m, Ac), 2.22–2.43 (2H, m, *β*-Hyp), 3.63–3.77 (2H, m, *δ*-Hyp), 7.26–7.79 (8H, m, arom Fmoc). ^{13}C-NMR ($CDCl_3$, 75.4 MHz) 100.07, 100.57 anomeric carbon of galactose, *β*-anomer. Most of the signals for both ^{1}H and ^{13}C-NMR appear as doublets, probably due to the existence of a slow inversion equilibrium centred on the nitrogen atom of the hydroxyproline ring [16].

Batch BB-2. Previously, another batch of the same building block had been prepared using freshly distilled CH_3CN as solvent and conditions similar to those described above. The white suspension turned into a pink solution immediately after addition of $BF_3.Et_2O$. Under these conditions the formation of *O*-acetyl-N^{α}-fluoren-9-yl-methoxycarbonyl-hydroxyproline **3** (same as peak B, Figure 1) was

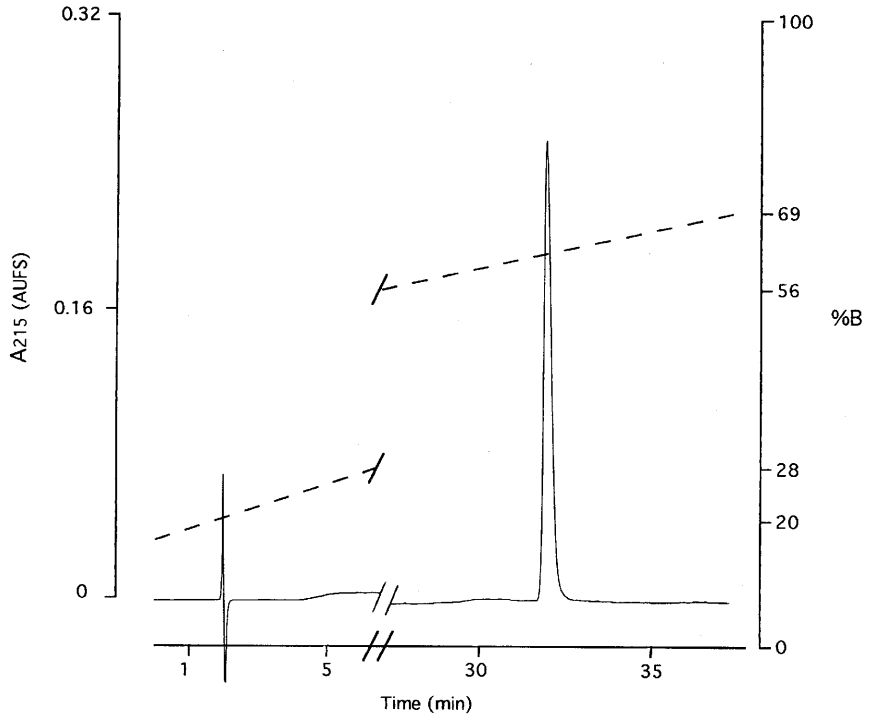


Figure 2 Analytical HPLC profile of pure *O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-N^{α}-fluoren-9-yl-methoxycarbonyl-hydroxyproline **1**. Load: Compound **1** (10 μl, 3 μg) coming from the pool of pure fractions collected over 20 preparative runs and lyophilized. Column: VYDAC C_{18}, 5 μm, 4.6 × 250 mm. Elution: (A) 0.10% TFA, (B) 0.08% TFA in water/CH_3CN 1 : 4, gradient 20–80% B over 45 min at a flow rate of 1.5 ml/min. Detection 215 nm, 0.32 aufs.

favoured. When the reaction was completed, aliquots of the crude solution were diluted (1 : 2) with water and directly loaded onto the preparative cartridge with no further work-up. In this case the best fractions collected showed a purity lower than 95%. They included impurities B, C and D.

O-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-*N*-$^{\alpha}$-fluoren-9-yl-methoxycarbonyl-hydroxyproline 2

The galactosylated hydroxyproline bearing the α anomer (compound **2**, peak C in Figure 1) was purified from a hydrophobic fraction during the preparative purification of **1**, batch BB-1. The fractions from repetitive runs were pooled and lyophilized. This lyophilized mixture (420 mg) was suspended in water/CH_3CN (5 : 2) and loaded onto the same DeltaPack C_4 cartridge. The product was eluted by a CH_3CN gradient (36 to 52% over 40 min) in 0.10% aqueous TFA. Fractions were analysed as stated for the *β*-anomer. The best fractions were pooled and lyophilized. 140 mg of *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-N^{α}-fluoren-9-yl-methoxycarbonyl-hydroxyproline **2** were obtained. Yield 0.5%. ES-MS *m/z* 684.4 ($M+1$) $C_{34}H_{37}N_1O_{14}$ requires 683.2. ^{1}H-NMR ($CDC1_3$, 300 MHz, selected signals) 1.97–2.16 (12H, m, Ac), 2.18–2.36 (1H, m, β_1-Hyp), 2.46–2.58 (1H, m, β_2-Hyp), 3.57–3.77 (2H, m, *δ*-Hyp), 7.25–7.78 (8H, m, arom Fmoc). ^{13}C-NMR ($CDCl_3$, 75.4 MHz) 95.68 anomeric carbon of galactose, α-anomer.

$O^{1.5}$-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-[DMet^2, Hyp^5]enkephalinamide 4

Batch GP-1. Stepwise build-up of the peptide was done manually from 17.4 g (6–7 meq) of resin. N^{α}-fluorenyl-9-methoxycarbonyl temporary protection was used and was removed by treatment with piperidine/DMF (1 : 5), (1 × 1 min, 1 × 10 min). Fmoc elimination was followed by washing with DMF (five times) and CH_2C1_2 (five times), successively. Chain extension was accomplished by stepwise incorporation of a three-fold molar excess of Fmoc amino acid, except for Fmoc-D-Met-OH and *O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-N^{α}-Fmoc-Hyp-OH attachment, when only 1.5 equivalents

were used. Couplings (60–120 min) were mediated by DIC in the presence of 0.75 equivalents of 1-hydroxybenzotriazole (HOBt) per equivalent of DIC. Capping by 40% acetic anhydride in CH_2C1_2 (1 × 10 min) was performed after each acylation. CH_2Cl_2-DMF (1 : 1) was used as solvent mixture throughout the synthesis. Acylation was monitored by the ninhydrin test [17]. Prior to deprotection, the resin was washed with DMF (three times), methanol (two times) and CH_2Cl_2 (three times). A weight gain of 5.6 g was obtained. The glycopeptide was cleaved from the resin while the galactose moiety was still fully acetylated. Optimal cleavage conditions were determined on 5 mg portions of resin treated with different cleavage mixtures. The final conditions used for the cleavage and partial deprotection of the glycopeptide-resin (23 g) were TFA/anisole/EDT/CH_2C1_2 (75 : 5 : 3 : 17) (230 ml, 10 ml reaction mixture/g resin) for 1.5 h at 37 °C. When the reaction was completed, the mixture was extracted with cold tert-butyl methyl ether (2 l), filtered through a no. 4 filtering funnel, and the residue was washed with cold *tert*-butyl methyl ether (3 × 400 ml). The remaining pellet was suspended in 0.10% aqueous TFA (40 ml) and the resin filtered off. After washing the resin with 0.10% aqueous TFA (2 × 20 ml) and 0.10% TFA in water/CH_3CN (4 : 1) (3 × 20 ml), all fractions were pooled and partially evaporated under vacuum. The crude was analysed by HPLC on a VYDAC C_{18}, 5 μm, 4.6 × 250 mm column, eluted with a binary system, (A) 0.10% aqueous TFA, (B) 0.08% TFA in water CH_3CN 2 : 3, under gradient conditions from 10 to 70% B over 30 min, at a flow rate of 1.5 ml/min, with detection at 215 nm, 0.016 aufs. After lyophilization, the crude (5.8 g) was loaded in portions of 1.2 g onto a preparative VYDAC C_{18}, 15–20 μm, 50 × 300 mm, cartridge and eluted using a gradient of CH_3CN (15 to 27% over 60 min) in TEAP pH 2.35 buffer, at a flow rate of 100 ml/min, with detection at 230 nm. Analysis of the fractions was accomplished under isocratic conditions in 0.10% aqueous TFA/CH_3CN using the same column, flow rate and detection described above. The pure fractions were pooled and desalted, in two portions of approximately 2 g each, on the same cartridge, by a CH_3CN gradient in 0.10% aqueous TFA. After combining the eluates and lyophilization, pure $O^{1.5}$-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl) [D-Met^2, Hyp^5] enkephalinamide **4** (4.2 g) was obtained as the trifluoroacetate. FAB-MS m/z 959.5 ($M+1$) $C_{44}H_{58}N_6O_{16}S_1$ requires 958.4. ^{13}C-NMR (d_6-DMSO, 75.4 MHz) 99.23 anomeric carbon of galactose, β-anomer.

Batch GP-2. Another batch of the acetylated glycopeptide was obtained using the building block **1**, batch BB-2, and essentially the same procedures described above at 0.5 mmolar scale. 230 mg of $O^{1.5}$-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl) [D-Met^2, Hyp^5] enkephalinamide **4** were obtained as the trifluoroacetate salt.

$O^{1.5}$-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl) [D-Met^2, Hyp^5]enkephalinamide 5

The crude corresponding to batch GP-2 contained a phobic minor impurity that was purified (TEAP and TFA) and characterized. The product resulted to be $O^{1.5}$-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-[D-Met^2, Hyp^5] enkephalinamide **5** that was obtained as the trifluoroacetate salt (5 mg). FAB-MS *m/z* 959.4 (M + 1) $C_{44}H_{58}N_6O_{16}S_1$ requires 958.4. DEPT-NMR (d_6-DMSO, 75.4 MHz) 94.59 anomeric carbon of galactose, α-anomer.

$O^{1.5}$-β-D-galactopyranosyl[D-Met^2, Hyp^5] enkephalinamide 6

$O^{1.5}$-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-[D-Met^2, Hyp^5]enkephalinamide (3.0 g, 3.1 mmol) was treated with a solution of $NaCH_3O$ (1.08 g, 20.0 mmol) in dry methanol for 2 h at room temperature. The reaction was stopped by addition of previously degassed cold 0.10% aqueous TFA (200 ml). TFA (3 ml) was added to bring the solution to pH between 2 and 3 and methanol was evaporated under vacuum. The remaining solution was diluted to 1.8 l with water, loaded onto the VYDAC C_{18} cartridge, and eluted with a gradient of CH_3CN (6 to 36% over 20 min) in 0.10% aqueous TFA. After lyophilization, pure (Figures 3 and 4) $O^{1.5}$–β-D-galactopyranosyl [D-Met^2, Hyp^5]enkephalinamide **6** (2.5 g) was obtained as the trifluoroacetate. FAB-MS *m/z* 791.5 ($M+1$), C_{36},$H_{50}N_6O_{12}S_1$ requires 790.3. ^{13}C-NMR (d_6-DMSO, 75.4 MHz) 102.86 anomeric carbon of galactose, β-anomer. AA analysis: Tyr, 0.95; D-Met, 0.99; Gly, 1.00; Phe, 0.98.

RESULTS

Synthesis and Purification of the Glycosylated Building Block

Galactosylation of Fmoc-Hyp-OH was performed in either methylene chloride or acetonitrile (batches BB-1 and BB-2, respectively). Glycosylation in

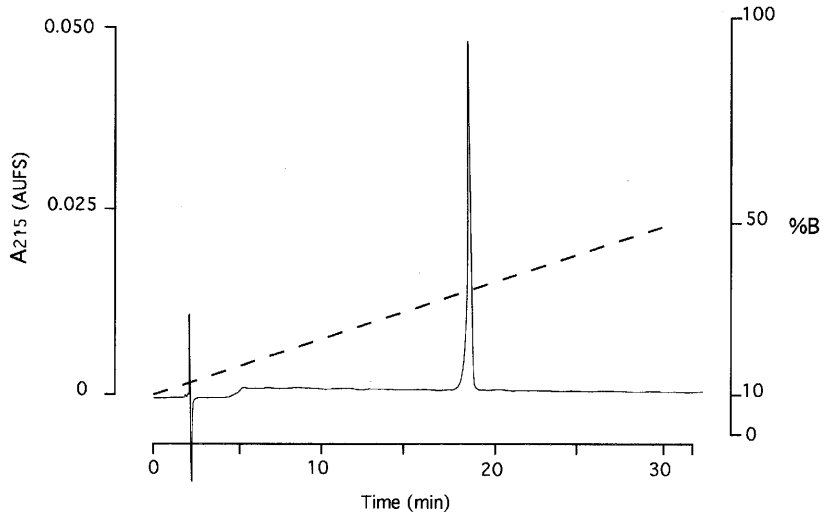


Figure 3 Analytical HPLC profile of $O^{1.5}$-β-D-galactopyranosyl) [D-Met^2, Hyp^5] enkephalinamide **6**. Load: Compound **6**(10 μl, 1 μg). Column VYDAC C_{18}, 5 µm, 4.6 × 250 mm. Elution: (A) 0.10% TFA, (B) 0.08% TFA in water/CH_3CN 2 : 3, gradient 10–70% B over 45 min at a flow rate of 1.5 ml/min. Detection 215 nm, 0.05 aufs.

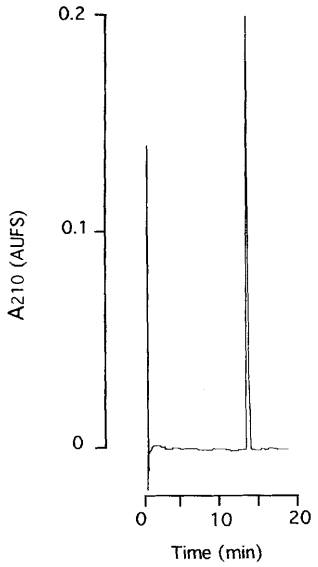


Figure 4 Capillary zone electrophoresis profile of $O^{1.5}$-β-D-galactopyranosyl) [D-Met^2, Hyp^5]enkephalinamide **6**. Capillary: 75 cm × 50 µm i.d. fused silica. Buffer: citric acid 0.05 M, pH 2.78. Voltage and current: 20 kV, 22–27 µA. Detection 210 nm, 0.2 aufs. Temperature: 30 °C.

CH_3CN was faster but led to mixtures with lower purity. The scaled-up glycosylation reaction (batch BB-1) was performed in CH_2Cl_2 at 50 millimolar scale. Figure 1 shows the analytical HPLC profile of the reaction mixture at different times. After 30 min, A/C + D ratio (measured as integration ration on the HPLC at 215 nm) was about 1 : 2 (Figure 1(a)). Then peak D decreased progressively while peak A (compound **1**) increased. Eventually, peak C (compound **2**) appeared on the chromatograms (Figure 1(b)–(d)). After 5 h the consumption rate of Fmoc-Hyp-OH decreased and the reaction was allowed to proceed overnight.

After work-up, 33 grams of crude lyophilized building block were obtained. Building block **1** was purified following the preparative conditions described in [18]. Briefly, loads of 1.5 g were made on a C_4 cartridge and more than 0.5 g of over 99% pure building block were obtained per run using a gradient of CH_3CN in plain de-ionized water. After processing all the crude material, 10 g (38% yield) of pure (Figure 2) *O*-(2,3,4,6-tetra-*O*-acetyl-*β*-*D*-galactopyranosyl)-N^{α}-fluoren-9-yl-methoxycarbonyl-hydroxyproline **1** were obtained. The phylic impurity (peak B, Figure 1) was identified as *O*-acetyl-N^{α}-fluoren-9-ylmethoxycarbonyl-Hyp-OH **3** [14, 18]. Working at this scale also allowed the isolation and characterization of the very minute impurity corresponding to peak C in Figure 1. It resulted to be *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-N^{α}-fluoren-9-yl-methoxycarbonyl-hydroxyproline (com-

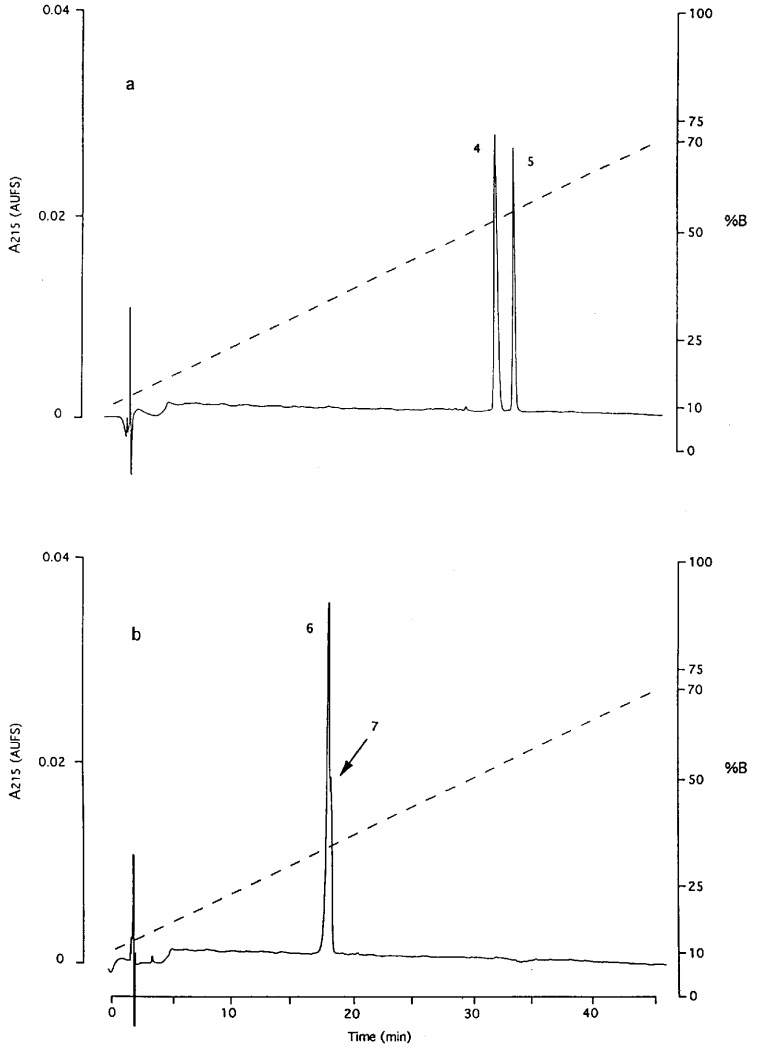


Figure 5 Analytical HPLC behaviour of glycopeptides bearing β and α anomers of galactose. Column: VYDAC C_{18}, 5 μm, 4.6 × 250 mm. Loads: 10 μ1, 1 μg total amount approximately. Elution: (A) 0.10% TFA, (B) 0.08% TFA in water/CH_3CN 2 : 3, gradient 10 to 70% B over 45 min at a flow rate of 1.5 ml/min. Detection 215 nm, 0.04 aufs. (a) Mixture of compounds **4** and **5** (molar ration 2 : 1 approximately). (b) Mixture of compounds **6** and **7** generated from the sample analysed in (a) by successive lyophilization, treatment with $NaCH_3O$/methanol, and dilution in 0.10% TFA.

pound **2**, 140 mg, 0.5% yield), formed by anomerization during glycosylation.

Glycopeptide Synthesis and Purification

Two batches (GP-1, GP-2) of **6** were obtained using building block **1**, batches BB-1 and BB-2, respectively. In both cases compound **1** was linked to a polystyrene resin bearing a TFA labile handle that yields terminal amides [19]. The rest of the amino acids were incorporated stepwise and the final cleavage of the acetylated glycopeptide **4** from the resin was accomplished by a cocktail that contained TFA, anisole, EDT and CH_2C1_2 for 1.5 h at 37 °C. From these two batches both the protected and unprotected neoglycopepetides (compounds **4** and **6**, respectively) as well as their α-anomers (compounds **5** and **7**) were isolated. Figure 5 compares the chromatographic behaviour of **4, 5, 6** and **7** on reversed-phase HPLC.

The scaled-up synthesis of **4** and **6** (batch GP-1) was done at 7millimolar scale using pure (Figure 2) building block **1** (batch BB-1). After cleavage and work-up, 5.8 g of crude were obtained. The partially protected glycopeptide **4** was purified by preparative HPLC on a VYDAC C_{18} column using TEAP buffer at

pH 2.35 in five repetitive runs of 1.1 to 1.2 g loads per run. The glycopeptide was desalted with a 0.10% TFA/CH_3CH solvent system in two runs of over 2 g loads per run. After lyophilization, 4.2 g of $O^{1.5}$-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl) [D-Met^2, Hyp^5] enkephalinamide **4** were obtained.

Three grams of acetylated glycopeptide were treated with sodium methoxide in methanol and desalted on the same preparative column in a single run using a steep gradient of 0.10% aqueous TFA in CH_3CN. After lyophilization, 2.5 g of the target glycopeptide $O^{1.5}$-β-D-galactopyranosyl[D-Met^2, Hyp^5] enkephalinamide **6** were obtained. Figures 3 and 4 show the analytical profile of **6** by reverse-phase HPLC and capillary zone eletrophoesis, respectively.

DISCUSSION

The goal of a scale-up procedure like the one described here is to get the highest yields and purity of the target drug at the lowest cost. The purity of the glycosylated building block used in the glycopeptide assembly will determine the quality of the crude and, subsequently, the efficiency of the whole process. There follows a discussion about critical aspects of the synthesis and purification of the building block **1** and the influence of its purity on the glycopeptide synthesis process.

The impurities generated during glycosylation included the acetylated amino acid **3** (Figure 1, peak B) [14, 18], the α-anomer **2** (Figure 1, peak C) and the compound(s) corresponding to peak D. The reaction rate and the quality of the crudes depended on the solvent used. Fmoc-Hyp-OH was poorly soluble in CH_2C1_2 and this was probably the reason why the reaction proceeded slowly compared with the case when CH_3CN was the glycosylation solvent. Interestingly, dichloromethane led to the crude with less *O*-acetyl-N^{α}-fluoren-9-yl-methoxycarbonyl-Hyp-OH **3**. The polarity of the solvent may influence the acetylation of hydroxyproline. Scale-up of the reaction and purification yielded enough amount of the impurity corresponding to peak C to fully characterize it as compound **2**. The isomer is formed by anomerization during glycosylation. The impurity(s) corresponding to peak D could not be purified in enough quantity to allow its unequivocal identification. From mass spectrometry results obtained after micropurification on analytical HPLC, we suggest that this fraction might contain adducts glycosylated on both the side-chain hydroxyl and the α-carboxylate function. The latter observation, together with the disappearance of D depicted in Figure 1 are in agreement with the comments of Kihlberg and coworkers [13, 14] about glycosylated compounds on the unprotected carboxyl group being formed and rearranged to give the desired molecule. The remaining isolated by-products would correspond to species, already glycosylated on the side chain, unable to rearrange.

The three main impurities detected after glycosylation were expected to add different degrees of difficulty to the final purification of the glycopeptides. A small amount of acetylated Fmoc-hydroxyproline would result in an unglycosylated peptide chain, with differentiated chromatographic behaviour. The purative di-glycosylated Fmoc-hydroxyproline would be washed away after the first acylation step on the solid support. These two kinds of compounds were not supposed to generate interfering impurities after glycopeptide chain assembly. On the other hand, the presence of α-anomer **2** (peak C, Figure 1) was expected to be a major problem. The final glycopeptide would be impure with something very similar from the structural standpoint that might show significant differences in biological activity. In accordance to the above predictions, the presence of the α-anomer of galactose happened to give the closest impurity after glycopeptide synthesis (batch GP-2, 0.5 millimolar scale) when an impure building block preparation (batch BB-2) was used. After cleavage from the resin, the HPLC profile showed a major peak corresponding to the acetylated glycopeptide **4** and a main hydrophobic impurity. This impurity was easily separated by preparative chromatography and shown to be the glycopeptide containing the α anomer of the galactosyl moiety (compound **5**). Figure 5(a) shows the analytical profile of a mixture of purified **4** and **5** (sugar-protected glycopeptides bearing anomers β and α, respectively). The mixture was de-acetylated by treatment with sodium methoxide in methanol. Figure 5(b) shows the chromatographic profile corresponding to the fully deprotected glycopeptides (**6** and **7**). The two profiles depicted in Figure 5 explain clearly that while preparative separation of per-acetylated species was relatively easy, processing the deprotected glycopeptide would have been completely unsatisfactory.

We have found that an amount of 1–2% α-anomer in a preparation of the target glycopeptide appears as a little unresolved shoulder on the analytical HPLC even using very shallow gradients. We previously proposed the convenient deprotection of the

sugar moiety while the peptide chain remained anchored to the resin [7] based on the hydrazinolysis method used by Schultheiss-Reiman and Kunz [20] in solution. When following this strategy, one has to make absolutely sure that the glycosylated building block is free of the α-anomer. Otherwise the target glycopeptide must be purified before sugar deprotection.

Care should be taken not to generalize the results described in this paper concerning the separation of anomeric mixtures. The chromatographic behaviour of glycopeptides bearing either sugar anomer appears to depend strongly on the peptide sequence and the position of the sugar moiety. Results obtained by other authors show that glycopeptides bearing protected α and β anomers are poorly resolved or not resolved at all on RP-HPLC [21–23]. In contrast, hydroxyl-free species are completely resolved in some instances [23]. Accordingly, in order to devise the best scale-up glycopeptide purification strategy it is crucial to study the chromatographic behaviour of both the protected and hydroxyl-free conjugates bearing both anomers for any new synthetic glycopeptide.

The above discussion on anomeric mixtures is applicable to those occasions when a compromise between yield and purity of the precursor is inevitable. In any case, the best way to the purest glycopeptides lies in the use of the purest building blocks. Our separation conditions allow the preparation of the multi-gram amounts of Fmoc-β-glycosylated hydroxyproline totally free of α-anomer and the efficient scaled-up synthesis of neoglycopeptides containing the galactosyl-hydroxyproline moiety. This and other successful multi-gram syntheses of biologically active neoglycopeptides will facilitate their exhaustive evaluation and eventually help define their future as therapeutic drugs.

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REFERENCES

1. R. E. Rodríguez, F. E. Rodríguez, M. P. Sacristán, J. L. Torres, G. Valencia and J. M. García-Anton (1989). New glycosyl peptides with high analgesic activity. *Neurosci. Lett. 101*, 89–94.
2. J. L. Torres, E. Bardají and G. Valencia in: *Methods in Neurosci.*, vol. 6, P. M. Conn, Ed., p. 35–49, Academic Press, San Diego 1991.
3. J. L. Székely, A. Z. Rónai, Z. Dunai-Kovács, E. Miglécz, I. Berzétri, S. Bajusz and L. Gráf (1977). [D-Met^2, Pro^5] enkephalinamide: a potent morphine-like analgesic. *Eur. J. Pharmacol. 43*, 293–294.
4. R. Polt, F. Porreca, L. Z. Szabò, E. J. Bilsky, P. Davis, T. J. Abbruscato, T. P. Davis, R. Horvath, H. I. Yamamura and V. J. Hruby (1994). Glycopeptide enkephalin analogues produce analgesia in mice: evidence for penetration of the blood–brain barrier. *Proc. Natl. Acad. Sci. USA 91*, 7117–7118.
5. G. Barany and B. R. Merrifield in: *The Peptides: Analysis, Synthesis, Biology*, vol. 2, E. Gross and J. Meienhofer, Eds., p. 1–284, Academic Press, New York 1979.
6. H. Paulsen, G. Merz and U. Weichert (1988). Solid-phase synthesis of *O*-glycopeptide sequences. *Angew. Chem. Int. Ed. Engl. 27*, 1365–1367.
7. E. Bardají, J. L. Torres, P. Clapeś, F. Albericio, G. Barany and G. Valencia (1990). Solid-phase synthesis of glycopeptide amides under mild conditions: Morphiceptin Analogues. *Angew. Chem. Int. Ed. Engl. 29*, 291–292.
8. M. Meldal in: *Glycopeptide Synthesis. In Neoglycoconjugates: Preparation and Application*, Y. C. Lee and R. T. Lee, Eds., p. 144–198, Academic Press, Orlando 1994.
9. H. Kunz (1987). Synthesis of glycopeptides, partial structures of biological recognition components. *Angew. Chem. Int. Ed. Engl. 26*, 294–308.
10. H. Paulsen (1990). Syntheses, conformations and X-ray analyses of the saccharide chains from the core regions of glycoproteins. *Angew. Chem. Int. Ed. Engl. 29*, 823–839.
11. K. Wakabayashi and W. Pigman (1974). Synthesis of some glycopeptides containing hydroxyamino acids, and their stabilities to acids and bases. *Carbohydrate Res. 35*, 3–14.
12. H. Paulsen (1982). Advances in selective chemical syntheses of complex oligosaccharides. *Angew. Chem. Int. Ed. Engl. 21*, 155–224.
13. M. Elofsson, B. Walse and J. Kihlberg (1991). Building blocks for glycopeptide synthesis: Glycosylation of 3-mercaptopropionic acid and Fmoc amino acids with unprotected carboxyl groups. *Tetrahedron Lett. 32*, 7613–7616.
14. L. A. Salvador, M. Elofsson and J. Kihlberg (1995). Preparation of building blocks for glycopeptide synthesis by glycosylation of Fmoc amino acids having unprotected carboxyl groups. *Tetrahedron 51*, 5643–5656.
15. M. Meldal and K. J. Jensen (1990). Pentafluorophenyl esters for the temporary protection of the α-carboxyl group in solid phase glycopeptide synthesis. *J. Chem. Soc. Chem. Commun.*, 483–485.

16. J. M. Lacombe, A. A. Pavia and M. Rocheville (1981). Un nouvel agent de glycosylation: l'Anhydride trifluoromethanesulphonique. Synthèse des α et β *O*-glycosyl-L-Sérine,-L-Thrénonine et-L-Hydroxyproline. *Can. J. Chem. 59*, 473–481.
17. J. M. Steward and J. D. Young: *Solid Phase Peptide Synthesis*, Pierce Chemical Company, Rockford, Illinois 1984.
18. J. L. Torres, E. Pagans and P. Clapés (1996). Rapid and efficient preparative purification of building blocks suitable for solid-phase synthesis of neoglycopeptides. Synthesis and purification of *O*-(2, 3, 4, 6-tetra-*O*-acetyl-β-D-galactopyranosyl/glucopyranosyl)-N^{α}-fluoren-9-yl-methoxycarbonyl-hydroxyproline. *Lett. Peptide. Sci. 3*, 61–68.
19. H. Rink (1987). Solid-phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methylester resin. *Tetrahedron Lett. 28*, 3787–3790.
20. P. Schultheiss-Reiman and H. Kunz (1983). *O*-Glycopeptide synthesis using 9-fluorenylmethoxycarbonyl (Fmoc)-protected synthetic units. *Angew. Chem. Int. Ed. Engl. 22*, 62–63.
21. L. Varga-Defterdarović, S. Horvat, M. Skurić and J. Horvat (1994). Correlation of structure and retention behaviour in reversed-phase high-performance liquid chromatography I. Leucine-enkephalin-related glycoconjugates. *J. Chromatogr. 687*, 101–106.
22. L. Varga-Defterdarovidć, S. Horvat, M. Skurić and J. Horvat (1994). Correlation of structure and retention behaviour in reversed-phase high-performance liquid chromatography II. Methionine-enkephalin-related glycoconjugates. *J. Chromatogr. 687*, 107–112.
23. H. Paulsen, S. Peters, T. Bielfeldt, M. Meldal and K. Bock (1995). Synthesis of the glycosyl amino acids N^{α}-Fmoc-Ser[Ac_4-β-D-Gal*p*-(1 → 3)-Ac_2-α-D-Ga1N_3*p*]-OPfp and N^{α}-Fmoc-Thr[Ac_4-β-D-Gal*p*-(1 → 3)-Ac_2-α-D-Ga1N_3*p*]-OPfp and the application in the solid-phase peptide synthesis of multiply glycosylated mucin peptides with T^n and T antigenic structures. *Carbohydrate Res. 268*, 17–34.

