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MULTIPLE COLUMN SOLID PHASE GLYCOPEPTIDE SYNTHESIS

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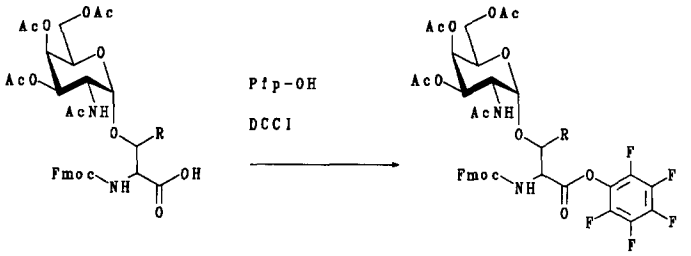
Abstract: The simultaneous synthesis of 40 different α-linked O-glycopeptides from human intestinal mucin and porcine submaxillary gland mucin in a manual multiple column peptide synthesizer using the new glycosylamino acid building blocks N^{α}-Fmoc-Ser(Ac_3-α-D-GalpNAc)-OPfp (3) and N^{α}-Fmoc-Thr(Ac_3-α-D-GalpNAc)-OPfp (4) is described.

The important role in biological systems played by the surface carbohydrates on glycoproteins and cell membranes in recognition and transport processes has increased the interest and demand for a variety of glycopeptides as model compounds[1]. For the study of the biosynthesis[2] of mucin type glycoproteins synthetic O-glycopeptides, which carry an α-linked GalNAc-sugar on serine or threonine, are required. Recently, Brockhausen[3] has shown that the enzymatic synthesis of O-glycan core structures is controlled by the peptide moiety. The activity of the β3-Galactosyltransferase, which glycosylates the 3-OH position in the GalNAc unit, is dependent of the peptide sequence, the chain length, the attachment site of the GalNAc and the number of GalNAc-residues in the substrate. N-terminally acetylated glycopeptide amides were much better substrates than the corresponding molecules with free amino and carboxy termini especially in the case of partially purified enzymes. For a more detailed enzymatic investigation a large number of synthetic glycopeptides[4] is needed. The concept to synthesize O-glycopeptides by using glycosylated hydroxy amino acids as building blocks for solid phase synthesis has been used successfully several times[5].

In this paper the application of the two new building blocks **3** and **4** is reported. These key compounds were obtained by esterification of the acids **1** and **2**[6] using pentafluorophenol (Pfp-OH) and dicyclohexylcarbodiimide (DCCI) in dry ethyl acetate at 0°C. After chromatographic purification on silica gel under dry conditions[5d] and precipitation from diethyl ether the activated ester **3** and **4** were both obtained in 75% yield as stable solid materials (~ 24% of the valuable starting material could be recovered from each separation). The active esters **3** and **4** were characterized by 1D-and 2D-^{1}H-NMR spectroscopy and FAB-MS[7].



1 R - H
2 R - CH_3

3 R - H
4 R - CH_3

The protecting group pattern of these building blocks is very suitable for solid phase glycopeptide synthesis. Pentafluorophenyl ester show very fast coupling rates in the presence of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) and the progress of the acylation reaction could be followed visually by the displacement of the yellow ion pair formed between Dhbt-OH and resin bound amino groups[5d,e;8]. Application of the fluoren-9-yl-methoxycarbonyl (Fmoc) group allows an α-amino deprotection under mild conditions with morpholine[9]. The O-acetyl groups of the carbohydrate part can easily be removed at the end of the synthesis with catalytic amounts of sodium methoxide in methanol[10]. The two octapeptides PTTTPIST (**5**) and GSSSGSPG (**43**) which represent parts of the repeating units from human intestinal mucin[11] and porcine submaxillary gland mucin[12] were selected as target molecules. Based on these two sequences a series of N-terminally acetylated O-glycopeptide amides were designed and synthesized as shown in figure 1.

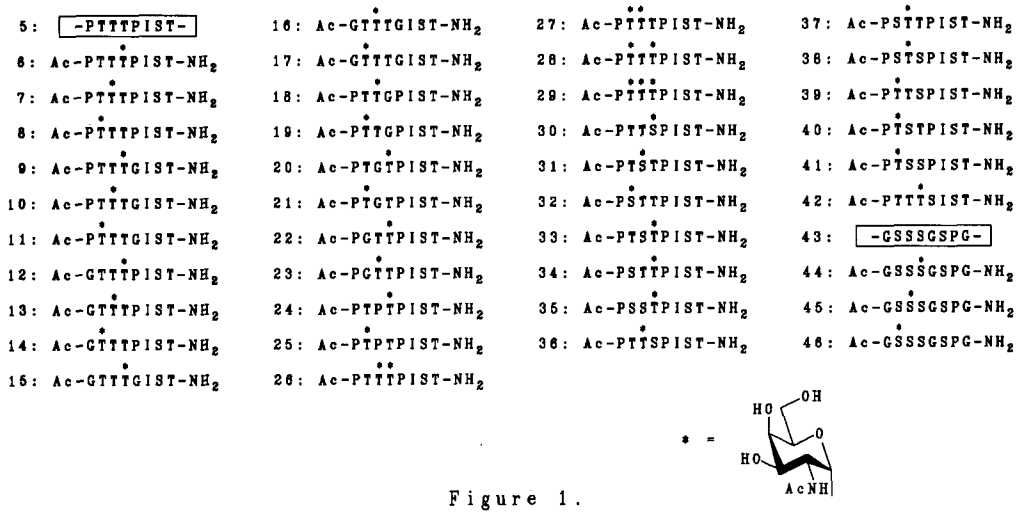


Figure 1.

The multiple column peptide synthesizer[13] used for simultaneous assembly of 40 glycopeptides consist of a steel frame equipped with two parallel 96 channel dispensers, one connected to a dispensing bottle with DMF and one to a dispensing bottle with deprotection reagent. The synthesis is carried out in a block of teflon with 96 wells in a ELISA type of arrangement all connected via a filter to a pressure/vacuum regulated chamber beneath. Resin columns (25 mg each) are packed to the wells and addition of DMF or deprotection reagent from dispensing bottles can be carried out by mounting the block on elevator tables under the washers. The acylating reagents are dissolved in a ELISA plate of teflon and added to the wells by a 8 channel multi pipette. The resin was washed 4 times after acylations and 5 times after deprotections.

The 5-(4-aminomethyl-3,5-dimethoxyphenoxy)-valeric acid (PAL)[14], which has successfully been applied in glycopeptide synthesis of morphiceptin analogues[5f] and glycosyl enkephalinamides[5g], was used as acid labile amide linker.

The Fmoc protected PAL group was coupled to a kieselguhr supported polydimethyl acrylamide resin carrying norleucine as reference amino acid by activation with O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU)[15] and 4-ethylmorpholine. After removing the Fmoc group with 20% piperidine in DMF

the first amino acid was coupled as Dhbt-ester to the linker in a quantitative yield as shown by amino acid analysis. All the following Fmoc deprotections were effected under mild conditions by treatment of the resin with 50% morpholine in DMF. The Fmoc amino acids were introduced into the peptide chain as Dhbt-esters. The hydroxy groups of *non* glycosylated serine and threonine were protected as tBu ether. In case of the glycosylated amino acids the pentafluorophenyl ester building blocks **3** and **4** were used with the addition of Dhbt-OH as auxiliary nucleophile. In order to assure complete acylation reactions the activated esters were allowed to react overnight. The progress of the peptide bond formation was indicated by the decrease in coloration of the solid support. After attachment of the last amino acid and Fmoc removal the terminal amino groups were acetylated with acetic anhydride in DMF (1:7). The resins were simultaneously transferred into small glass vials. All 40 glycopetides were cleaved off the resin by treatment with 95% aqueous TFA with concurrent removal of the O-tBu groups. Finally, the deacetylation of the carbohydrate unit was performed with sodium methoxide in methanol at pH 8.5 for 3 hours. Racemization or β-elimination of the glycosyl moiety was not observed.
The crude products were purified by preparative reversed phase HPLC. The pure N^{α}-acetyl-O-glycopeptide amides were obtained in yields of 4 to 6 mg after lyophilization. The characterization of all compounds was performed by 1D- and 2D-^{1}H-NMR spectroscopy and all proton signals were assigned as exemplified for compound **10**[16]. The amino acid analysis were all correct within 8%. The full experimental data will be published elsewhere.
This paper describes an efficient strategy for the synthesis of many different O-glycopeptides of biological interest in a short period of time by multiple column peptide synthesis.

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7. Compound **3**: m.p. 96 C (not corr.); $[\alpha]_D^{23} = +43.8$ (c = 1.0, $CHCl_3$);
FAB-MS found MH^+: 823.0; calc. for $C_{38}H_{35}F_5N_2O_{13}$: 822.2
Compound **4**: m.p. 110 C (not corr.); $[\alpha]_D^{23} = +34.0$ (c = 1.0, $CHCl_3$);
FAB-MS found MH^+: 837.3; calc. for $C_{39}H_{37}F_5N_2O_{13}$: 836.7.

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16. Compound **10** (Ac-P-T-T(α-D-GalNAc)-T-G-I-S-T-NH_2). 400 MHz ^{1}H-NMR; δ, ppm (in D_2O, ref DOH= 4.80 ppm at 300 K) J, Hz: α-D-GalNAc 4.98 (d, 3.8 Hz, H-1); 4.17 (dd, 11.0 Hz, H-2); 4.08 (H-5); 4.03 (dd, 0.8 Hz, H-4); 3.95 (dd, 3.2 Hz, H-3); 3.83-3.77 (H-6, H-6'); 2.18[a] (Ac); Thr.s 4.73 (d, 2.0 Hz, H-α1); 4.57 (d, 5.0 Hz, H-α2);4.43(d, 4.4 Hz, H-α3); 4.43 (H-β1); 4.39 (H-α4); 4.37 (H-β4); 4.31 (H-β2); 4.26 (H-β3); 1.34 (d, 6.6 Hz, H-γ1); 1.31 (d, 6.6 Hz, H-γ2); 1.29 (d, 6.8 Hz, H-γ3); 1.27 (d, 6.8 Hz, H-γ4); Ser 4.62 (t, H-α); 3.97 (dd, 6.0 and 11.4 Hz, H-β); 3.92 (dd, 6.0 Hz, H-β'); Ile 4.30 (d, 7.6 Hz, H-α); 1.99-1.89 (H-β); 1.59-1.46 (H-γ); 1.33-1.18 (H-γ'); 0.98 (d, 6.8 Hz, H-γ''); 0.93 (t, 7.4 Hz, H-δ); Gly 4.10 (d, 16.8 Hz, H-α); 4.01 (d, H-α'); Pro 4.54 (dd, 4.0 and 8.0 Hz, H-α); 3.83-3.61 (2 Hδ); 2.43-2.32 (H-β); 2.12-1.99 (H-β', 2 H-γ); 2.08[a] (Ac); a: The assignment can be interchanged.

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