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PENTAFLUOROPHENYL ESTERS FOR TEMPORARY CARBOXYL GROUP PROTECTION IN SOLID PHASE SYNTHESIS OF N-LINKED GLYCOPEPTIDES.

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Abstract: The compound Ac_3GlcNAcβ1-NH-Fmoc (3) was synthesized and transformed into the β-glucosyl amine (1) which was subsequently acylated with Fmoc-Asp(Cl)-O-Pfp (5) prepared from the readily available Fmoc-Asp(O-tBu)-O-Pfp (4). The resulting Fmoc-Asn(Ac_3GlcNAcβ1-N-)-O-Pfp (6) was used as a building block in the solid phase synthesis of an 11 residue glycopeptide fragment of the enzyme glucoamylase (AMG).

Transport of proteins and a variety of important recognition phenomena in the eukariotic cellular compartment and on the cell membrane are controlled by signals mediated e. g. via oligosaccharides N-linked to asparagine sidechains of glycoproteins. In contrast to other proteins, the glycosylated proteins are not easily available by genetechnology since they are posttranslational products resulting from the activity of trimming glycosyl hydrolases and transferases. Therefore, organic synthesis provide a valuable alternative.

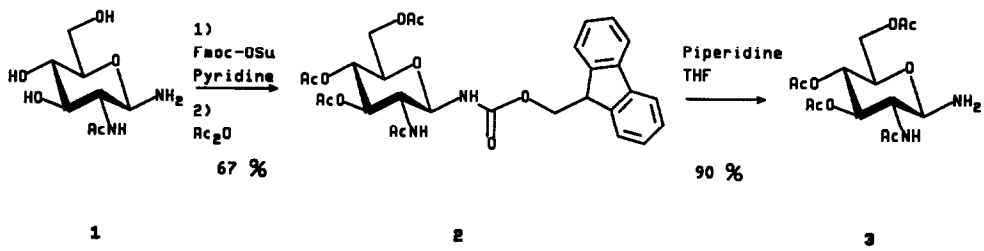
The core structure of the N-glycosylated glycoproteins is a trisaccharide, Manβ1-4GlcNAcβ1-4GlcNAcβ, the terminal N-acetylglucosamine residue is connected to the amide of an asparagine residue of the glycoprotein. Access to partial structures of glycopeptides consisting of longer peptides with e. g. GlcNAc or its dimer chitobiose attached will make it possible to study the structure-function relationship e. g. the conformational preference (the interaction between the sugar and the peptide) and the application of glycosyl transferases for the elongation to more complex glycopeptides.

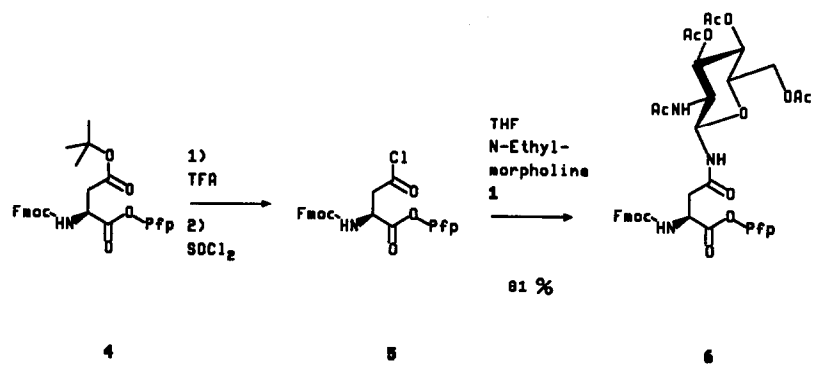
The N-glycosylated asparagine molecule with more or less permanent protection of the α-amino and the α-carboxyl group has been reported previously.[1-7] The permanent character of these protection groups has, however, prevented the application of these derivatives for the solid phase synthesis of larger N-glycosylated peptides. Recently Kunz reported strategies using Boc- or Aloc-protection of the α-amino group and allyl- or t-butyl ester for the α-carboxyl group for the solution synthesis of small N-linked glycosylated peptides.[8,9]

Recently we have reported the application of the pentafluorophenyl (Pfp) group for the temporary protection of the α-carboxyl group in the O-glycosylation of serine and threonine.[10] In the present communication we demonstrate that the Pfp ester also can be used as a general temporary protection group in the solid phase synthesis of N-glycopeptides. During degradation studies on the glycohydrolase, glucoamylase, in this laboratory[11] a glycosylated undeca-peptide (residue 388-398) H-Thr-His-Ala-Ala-Ser-Asn(glycoside)-Gly-Ser-Met-Ser-Glu-OH was isolated and subjected to further characterization. We have therefore selected this glycopeptide as a synthetic target for the demonstration of the new methodology. As a glycosyl model 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl amine (3) was prepared by a new approach from N-acetyl-glucosamine.[12] Thus reaction of N-acetylglucosamine with ammonium carbonate and lyophilization to constant weight afforded a mixture which according to ^{1}H-NMR was 85% of the β-glycosyl amine (1) and 15% starting material. This mixture was dissolved in pyridine and treated with Fmoc-OSu for 3 h followed by addition of pyridine and acetic anhydride

and after 7 h excess anhydride was hydrolyzed. The crystalline compound **2** could be isolated in 67% yield by dilution with water and was recrystallized from THF and hexane.[13] The Fmoc group was removed with 20% piperidine in THF and crystalline **3**[14] was isolated in a 90% yield by dilution with hexane.

The active ester Fmoc-Asp(O-tBu)-O-Pfp **4** is a major reagent in Fmoc based solid phase synthesis and as such readily available.[15] Treatment of **4** with TFA for 30 min cleaved the t-butyl ester quantitatively and all volatile material could be removed by repeated evaporation with dry THF. The free acid was suspended in distilled thionyl chloride and after 1h all excess reagent was removed by evaporation at .1 mm Hg. The acid chloride **5** was immediately treated with a mixture of compound **3** and N-ethylmorpholine in dry THF. The hydrochloride was removed and Fmoc-Asn(Ac_3GlcNAcβ1-N-)-O-Pfp[16] (**6**) was isolated in a 81% yield by crystallization from THF/hexane.

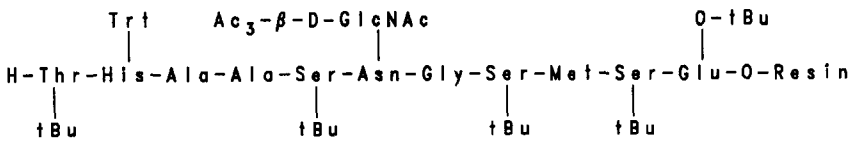




Compound **6** was used in a solid phase assembly of the N-linked glycopeptide **7** employing Dhbt esters in the continuous flow version of the polyamide method.[17] The assembly was conducted on a fully automatic peptide synthesizer constructed in this laboratory. The first amino acid was added as the symmetrical anhydride with DMAP catalysis. Reaction times for the acylations with Fmoc-amino acids-O-Dhbt esters were determined with a solid phase spectrophotometer[18] except for histidine. This was coupled as Fmoc-His(Trt)-OH with the TBTU reagent[19] in the presence of diisopropylethylamine. The acylation with **6** was catalyzed by Dhbt-OH as a auxiliary nucleophile and a reaction time of 96 min was observed. Ala-4

also had a prolonged reaction time of 180 min, whereas all other acylations were complete within 20 min.

Peptide 7 was cleaved off the resin with 95% aqueous TFA to yield 25 mg of crude peptide which was purified by HPLC to yield 11.5 mg. The O-acetyl groups were then removed by a 1 h treatment with sodium methoxide in methanol (pH 12.5 at moist pH paper) followed by neutralization with carbon dioxide. Purification by HPLC yielded 10 mg of the fully deprotected peptide 8, which was characterized by 1D- and 2D-NMR spectroscopy.[20]



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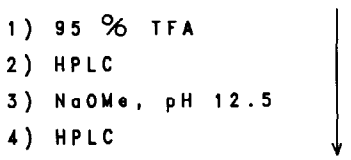

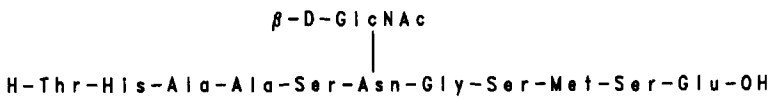

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In this report a new and efficient method for the synthesis of N-glycosylated peptides by solid phase techniques has been described. During the acylation of glycosyl amines the α-carboxyl group of aspartic acid is protected as a Pfp ester. The method employs N-glycosylated Fmoc-Asn-O-Pfp as a standard building block in the active ester method.

Notes and references

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13. Compound **2**; 500 MHz ^{1}H-NMR δ, ppm (in DMSO-d6=2.50 ppm at 300 K), J, Hz: H1 5.045, 9.6, 9.6; H2 3.881, 9.6, 9.8, 9.0; H3 5.153, 9.8, 9.8; H4 4.805, 9.8, 9.8; H5 3.812, 3.8, 2.1, 9.8; H6 3.963, 12.5, 2.1; H6' 4.177, 12.5, 3.8; N2H 7.968, 9.0; N1H 8.205, 9.6; Methyl groups 2.00, 1.99, 1.93 and 1.78; Fmoc: CH_2 4.362 and 4.230; CH 4.230; H1 7.718, 7.5 and 7.683, 7.5; H2 7.421, 7.5, 7.5; H3 7.322, 7.5, 7.5; H4 7.892, 7.5.
14. Compound **3**; 500 MHz ^{1}H-NMR δ, ppm (in DMSO-d6=2.50 ppm at 300 K), J, Hz: H1 4.162, 9.6; H2 3.616, 9.6, 9.6, 9.8; H3 5.036, 9.8, 9.8; H4 4.768, 9.8, 9.8; H5 3.708 4.9, 2.4, 9.8; H6 4.108, 4.9, 12.1; H6' 3.967, 2.4, 12.1; N2H 7.83, 9.6; Methyl groups 2.01, 1.97, 1.91 and 1.76.
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16. Compound **6**; 500 MHz ^{1}H-NMR δ, ppm (in DMSO-d6=2.50 ppm at 300 K), J, Hz: GlcNAc H1 5.182, 9.5, 9.3; H2 3.882, 9.5, 9.4, 9.8; H3 5.108, 9.8, 9.8; H4 4.185, 9.8, 9.8; H5 3.826, 9.8, 3.8, 2.5; H6 3.917, 12.5, 2.5; H6' 4.163, 12.5, 3.8; N2H 7.903, 9.4; Methyl groups 1.980, 1.965, 1.905 and 1.715; Asn Hα 4.847, 6.9, 6.5, 8.0; Hβ 2.707, 16.2, 6.9; Hβ' 2.872, 16.2, 6.5; NαH 8.118, 8.0; NδH 8.772, 9.3; Fmoc CH_2 4.325, 10.3, 7.0; CH_2' 4.386, 10.3, 7.0; CH 4.230, 7.0, 7.0; H1 7.696, 7.5; H2 7.396, 7.5, 7.5, H2' 7.407, 7.5, 7.5; H3 7.303, 7.5, 7.5; H4 7.885, 7.5.
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20. Compound **8**: 500 MHz ^{1}H-NMR δ, ppm (in D_2O=4.63 ppm at 310 K, and pH 3.0), J, Hz: GlcNAc H1 5.013, 9.4; H2 4.783, 9.4, 9.8; H3 3.581, 9.8, 8.8; H4 3.448, 8.8, 10.0; H5 3.474, 10.0, 4.4, 2.5, H6 3.728, 4.4, 12.5; H6' 4.859, 2.5, 12.5; Thr Hα 3.901, 5.6; Hβ 4.146, 5.6, 6.3; Hγ 1.261, 6.3; His Hα 4.723, 6.8, 7.2; Hβ 3.262, 6.8, 15.6; Hβ' 3.184, 7.2, 15.6; H2 7.315; H5 8.110; Ala Hα 4.338, 7.6; Hβ 1.388, 7.6; Ala Hα 4.308, 7.6; Hβ 1.349, 7.6; Serines Hα 4.430-4.441; Hβ 3.800-3.891; Asn Hα 4.746, 6.0; Hβ 2.853, 6.0, 16.2; Hβ' 2.818, 6.0, 16.2; Gly Hα 3.992, 17.2; Hα' 3.922, 17.2; Met Hα 4.411, 5.2, 9.6; Hβ 2.191, 7.4, 5.2, 16.5; Hβ' 1.979, 7.4, 9.6, 16.5; Hγ 2.468, 7.4, 7.4; CH_3 2.078; Glu Hα 4.532, 5.3, 9.2; Hβ 2.116, 5.4, 8.8, 14.0; Hβ' 2.008, 9.2, 8.4, 14.0; Hγ 2.606, 8.4, 5.4, 15.6; Hγ' 2.528, 8.8, 9.2, 15.6. 125.77 MHz ^{13}C-NMR δ, ppm (in D_2O, Dioxane = 67.40 ppm at 310 K and pH 3.0): Carbonyls 177.94, 175.82, 175.56, 175.30, 174.05, 173.66, 173.31, 172.70, 172.51, 172.40, 172.11, 171.54, 168.65, GlcNAc C1 79.15; C2 54.99; C3 75.05; C4 70.27; C5 78.39; C6 61.32; Me 22.89; Thr Cα 59.09; Cβ 66.93; Cγ 19.39; His Cα 53.29; Cβ 27.25; C2 134.45; C4 128.72; C5 118.25; Ala Cα 50.38; Cβ 17.44; Ala Cα 50.33; Cβ 17.34; Serines Cα 56.55, 56.37, 56.19; Cβ 61.90, 61.78, 61.78; Asn Cα 50.86; Cβ 37.23; Met Cα 53.16; Cβ 26.78; Cγ 30.77; Me 14.95; Glu Cα 53.58; Cβ 30.90; Cγ 30.09.

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