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SOLID-PHASE SYNTHESIS OF GLYCOPEPTIDES: SYNTHESIS OF N^{α}-FLUORENYLMETHOXYCARBONYL L-ASPARAGINE N^{β}-GLYCOSIDES

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Abstract: It was found that crude 1-glycosylamines, prepared with saturated aqueous NH_4HCO_3 from reducing sugars, can be coupled with Fmoc-Asp(OPfp)-O^{t}Bu in N,N-dimethylformamide-water mixtures. Purification and removal of the ^{t}Bu group results in N^{β}-glycosides of Fmoc-Asn-OH which can be used for the solid phase synthesis of glycopeptides.

In contrast to the structural diversity of oligosaccharides found in O-glycosides, the common feature of the major type carbohydrate antennae of N-glycosides is a mannotriosido-di-N-acetyl-chitobiose core[1] linked through a $\beta(1\rightarrow N^{\beta})$ linkage to asparagine The role of carbohydrates in biological recognition has been reviewed extensively in the last decade [2,3] Carbohydrates are hydrophilic. However, the acetamido (-NH-CO-CH_3) group, attached at C2 of the first and second glucopyranose ring of most N-glycosides, has an ambivalent character: its methyl group is hydrophobic but, similar to the proton-donating and accepting peptide bond, the acetamido group has the potential to form strong intra- or intermolecular H-bonds. Hydrogen-bonds and/or hydrophobic interactions between the peptide backbone (or side chains) and the adjacent monosaccharides of the core may induce the adoption of specific glyco-turns[4] or glyco-loops, which can act as highly specific recognition sites in a variety of biological processes

A simple approach to studying the role of the acetamido group in recognition of other molecules is to synthesize biologically active peptides bearing N-linked mono- or oligosaccharides with or without an acetamido group at C2 of the first and second pyranose ring. Earlier we reported the preparation of Boc-Asn(Ac_3GlcNAc)-OH, Fmoc-Asn(Ac_3GlcNAc)-OH and Fmoc-Asn(GlcNAc)-OH (*3a*), as well as their use in the solid phase synthesis of peptides.[5,6] 2-Acetamido-2-deoxy-β-D-glucopyranosylamine (GlcNAc-NH_2, 2a) was obtained by amination with NH_4HCO_3 as described earlier.[7] This paper reports the synthesis of oligomeric $\beta(1\rightarrow N^{\beta})$-glycosides [$(GlcNAc)_2$ and $(GlcNAc)_3$] of Fmoc-Asn-OH and extends the use of NH_4HCO_3 to amination of mono- and oligosaccharides which do not contain an acetamido group at C2 of the reducing end.

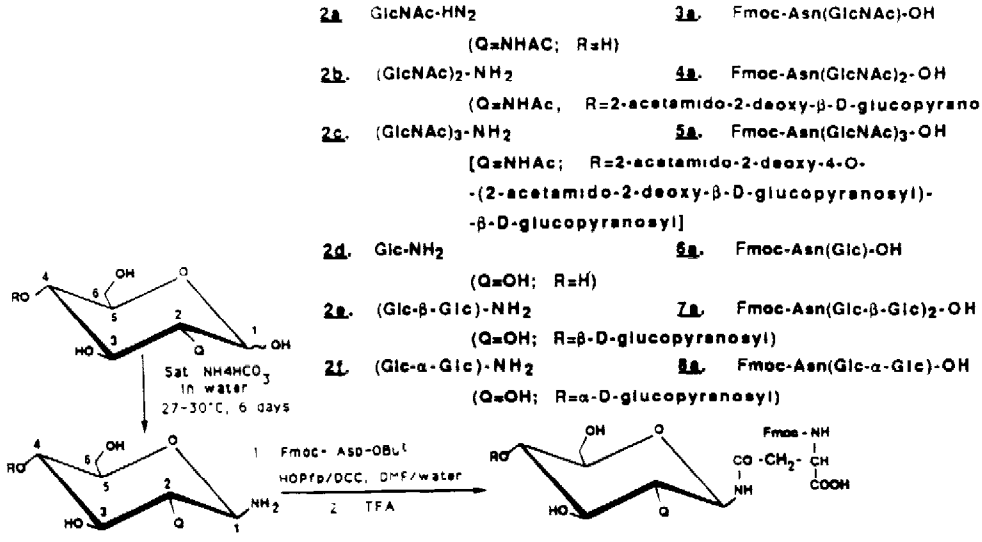
1-Glycosylamines (*2a* and *2b*), prepared by the NH_4HCO_3 method and purified by Amberlist 15 (H^+) ion-exchange resin chromatography using 0.5 molar NH_3/methanol eluent,[7] were found to contain some (≥5%) starting sugar. We observed, however, that crude glycosylamines, free of NH_4HCO_3 but containing considerable amounts of the starting sugar, can be coupled with

Boc-Asp-OBzl or Fmoc-Asp-O^{t}Bu using pentafluorophenol (HOPfp)[8] and dicyclohexyl- or diisopropyl-carbodiimide (DCC or DIC) in dimethylformamide (DMF)-water mixtures.

In a typical amination experiment, 2.5 mmole (550 mg) of 2-acetamido-2-deoxy-D-glucose was reacted with 20 ml saturated aqueous NH_4HCO_3 for 6 days at 30°C To remove the excess of NH_4HCO_3, the reaction mixture was diluted with water (10 ml) and concentrated *in vacuo* to half of its original volume. This procedure was repeated 7 to 8 times.[7] Finally, the water was removed by distillation *in vacuo* (bath temperature ≤30°C).

Peracetyl chitotriose was obtained, together with peracetyl chitobiose, from chitin.[9] When started from peracetyl sugars, the reagent (NH_4HCO_3) caused complete O-deacetylation in addition to amination at C1. Deacetylation of these carbohydrates (0.6 mmol) was achieved by dissolving them in a mixture of 180 ml methanol and 120 ml water, followed by saturation with NH_4HCO_3 at room temperature After standing for one day and distillation of the reagent *in vacuo*, amination was done as described above. The completion of the smooth and effective deacetylation reaction was followed by thin layer chromatography (t.l.c.) (Kieselgel DC Alufolien, HF_{254}, Merck) using eluent chloroform-methanol (1:1) with detection by 10% sulfuric acid in ethanol (5 min at 120°C). The crude 1-amino-chitotriose (*2c*) containing ~20% starting sugar, some acetamide, and ~10% of a minor product [probably *bis*(glycosylamine), cf. Ref. 10], gave pure Fmoc-Asn(GlcNAc)$_3$-OH (*5a*) after coupling with Fmoc-Asp(OPfp)-O^{t}Bu and removal of the ^{t}Bu group with trifluoroacetic acid (TFA) According to ^{1}H and ^{13}C NMR[6,7] as well as circular dichroism spectroscopic studies, amination of 2-acetamido-2-deoxy-D-glucose, chitobiose, and peracetyl chitobiose resulted in β-glycosylamines and caused no epimerization at C2.

We applied the amination reaction also for D-glucose and the disaccharides cellobiose and maltose, the common feature of which is that neither contain an acetamido group at C2 of the pyranose ring to be aminated at C1. In these cases, the amination yielded crude 1-glycosylamines containing 20-60% starting sugar. Due to the higher lability of these 1-amino sugars, attempts to separate them from the starting reducing sugars by using Amberlist 15 (H^+) ion-exchange resin remained unsuccessful. Products obtained after the resin treatment were contaminated with roughly the same amount of starting sugar as before. Crude amino sugars (containing ≥20% starting sugar and possibly other impurities) were used without further purification for the synthesis of Fmoc-Asn(sugar)-O^{t}Bu derivatives In a typical coupling reaction 1 mmol (411 mg) Fmoc-Asp-OBu^t and 1 mmol (184 mg) pentafluorophenol were dissolved in 3 ml DMF and 1 mmol (206 mg) DCC (or DIC) was added. After stirring at room temperature for 30 min, 2.0-2.5 mmol crude 1-amino sugar was added to the active ester in 2 ml DMF-H_2O (2:1; v/v) overnight. The higher amount of nonaminated sugars had no unfavorable influence on the purity of the N-glycosides of Fmoc-Asn-O^{t}Bu. The formation of O-acyl derivatives could not be detected by t.l.c. After the insoluble material was filtered off and the solvent removed *in vacuo* (≤40°C), the product was washed with cold ether and cold water. Its purity was evaluated by t.l.c. using ethyl acetate-buffer 1:3 and 2:3 mixtures (v/v) as eluents [buffer:pyridine-acetic acid-water (20:6:11; v/v/v)]. Crude products (*4c-8c*) containing >10% impurities according to t l.c. were purified by open column flash chromatography[4] on Kieselgel 60 (Merck), using the above buffers as eluents.



^{t}Bu esters *3c-8c* contained <5% impurities according to RP-HPLC. The ^{t}Bu group was cleaved by TFA (15 min at room temperature). T.l.c. and RP-HPLC did not indicate the cleavage of the N-glycosidic or, in the case of disaccharides, N- and/or O-glycosidic linkages. Acids *3a-8a* were isolated by triturating and washing with anhydrous ether and drying in a vacuum desiccator over P_2O_5. ^{t}Bu and free acid derivatives of Fmoc-Asn N$^\beta$-glycosides were characterized by RP-HPLC,[12] +FAB-MS,[12] and 500 MHz ^{1}H-NMR spectroscopy. In the ^{1}H-NMR spectra[13] of Fmoc-Asn(Glc)-OH (*6a*), Fmoc-Asn(Glc-β-Glc)-OBu^t (*7c*) and Fmoc-Asn(Glc-α-Glc)-OBu^t (*8c*) in DMSO-d6 only one set of resonance signals was present. The C1 protons gave asymmetric triplets near 4.7 p p m. due to the coupling (J~9Hz) with the C2H and NH (asparagine) protons. The Asn NH$^\beta$ protons gave doublets with high $^3J_{C1NH}$ coupling constants (*6a*, 8.44 p p m., 8.9 Hz; *7c*, 8.71 p.p.m., 8.7 Hz; and *8c*, 8 52 p.p.m., 8 9 Hz). Addition of some drops of D_2O brought about a deuterium exchange of the NH and OH protons. In the resulting spectra the C1H signals appeared as doublets, due to coupling with C2H only. The high value (~8.9 Hz) of the J_{C1C2} coupling constant indicated the β configuration of the anomeric linkage[7,11] and the epimeric purity even for derivatives of reducing sugars lacking an acetamido group at C2.

The coupling efficiencies were tested by RP-HPLC as earlier,[5] on the synthesis of N-glycosides of H-Ile-Met-Met-Asn*-Gly-NH_2. A combination of peak area integration values and peak heights of the cleaved products after single couplings showed 100% incorporation of the Asn(Glc) residue and ~65% incorporation of Asn(GlcNAc)$_3$ and Asn(Glc-β-Glc), which suggests a widespread applicability of Fmoc-Asn(sugar)-OH derivatives with unprotected hydroxyl groups in the solid-phase glycopeptide synthesis. More generally, free amino functions can be acylated by Fmoc-Asn(sugar)-OH derivatives, pentafluorophenol, and DCC or DIC as coupling reagents.

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12. Characterization of Fmoc-Asn(sugar) derivatives.

No.	Abbreviated Name	RP-HPLC[14] (Retention time, min)	$^{+}$FAB-MS[15] (MH^{+})
3a	Fmoc-Asn(GlcNAc)-OH	36.6	558[5]
3c	Fmoc-Asn(GlcNAc)-OBu^{t}	43.6	614[5]
4a	Fmoc-Asn(GlcNAc)$_2$-OH	35.8	761,783 (M+Na)[6]
4c	Fmoc-Asn(GlcNAc)$_2$-OBu^{t}	43.6	839 (M+Na)[6]
5a	Fmoc-Asn(GlcNAc)3-OH	35.2	964
5c	Fmoc-Asn(GlcNAc)3-OBu^{t}	41.5	1020.4; 1042.4 (M+Na)*
6a	Fmoc-Asn(Glc)-OH	36.8	517.2; 539.2 (M+Na)*
6c	Fmoc-Asn(Glc)-OBu^{t}	43.7	595.2 (M+Na)*
7a	Fmoc-Asn(Glc-β-Glc)-OH	35.9	679
7c	Fmoc-Asn(Glc-β-Glc)-OBu^{t}	42.8	735, 757 (M+Na)
8a	Fmoc-Asn(Glc-α–Glc)$_2$-OH	35.3	679
8c	Fmoc-Asn(Glc-α-Glc)-OBu^{t}	41.5	735, 757 (M+Na)

13. DQF Cosy ^{1}H-NMR studies were performed on a Bruker 500 spectrometer at 313°K.
14. The high performance liquid chromatographic system consisted of 2 Beckman 110A pumps, an Altex Ultrasphere ODS column, a Beckman 160 fixed-wavelength detector operating at 214 nm, 0.1 a.u.f.s., and a Shimadzu C-R6A integrator. Solvent A was 0.1% aqueous TFA; solvent B was 0.1% TFA in acetonitrile. A linear gradient of 1.33%/min of solvent B was used starting at 5%. The flow-rate was 3 ml/min.
15. Liquid secondary ion mass spectrometry experiments were carried out in the positive ion mode using a VG Analytical ZAB-E and a Kratos MS 505 double focusing mass spectrometer(*), equipped with a cesium ion source and a high field magnet. Glycerol:thioglycerol = 1:1 (v/v) in 1% aqueous TFA was applied as liquid matrix.

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