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Fmoc-protected, glycosylated asparagines potentially useful as reagents in the solid-phase synthesis of *N*-glycopeptides

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ABSTRACT

1-Amino 1-deoxy derivatives of unprotected *O*-β-D-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-β-D-glucopyranose, 2-acetamido-2-deoxy-D-galactose, D-galactose, lactose, D-fucose, D-mannose, and 2-deoxy-D-*arabino*-hexose were prepared and acylated with *N*-fluorenylmethoxycarbonylaspartic acid α-*tert*-butyl ester. The anomeric configuration of the *N*-glycosyl bond (including that of the mannose derivative) in each of the purified compounds was shown to be β. The probable stability of the *N*-glycosyl and glycosidic bonds during the conditions of solid-phase peptide synthesis was investigated by treatment of the glycosylated asparagine derivatives with different concentrations of trifluoroacetic acid. Based on their stability, we found that Fmoc-Asn(sugar)-OH derivatives are excellent candidates for automated synthesis of biologically active glycopeptides.

INTRODUCTION

During the last decade it has become known that many eukaryotic proteins carry covalently linked oligosaccharides[1,2]. The saccharide side chains can strongly influence the conformation[3,4] and the biological function of the native proteins[5,6]. Since the role of carbohydrates in these processes cannot be understood without exploring glycopeptide models, the development of efficient and widely applicable techniques for solid-phase synthesis of them is of considerable interest. Earlier we described the synthesis and application in solid-phase peptide synthesis (SPPS) of N^{α}-fluorenylmethoxycarbonyl-L-asparagines glycosylated with mono- and di-saccharide units of 2-acetamido-2-deoxy-D-glucose[7–9] or D-[illegible]cose[9]. The common

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features of these derivatives were the β-configuration of the sugar attached to the amide N of asparagine, the presence of an acetamido or hydroxyl group in an equatorial position at C-2 of the glucopyranose ring(s), and a 1 → 4 glycosidic bond in the oligosaccharides.

It was found that the ammonium hydrogencarbonate method, described first by Kotchetkov and coworkers[10] for the preparation of glycosylamines of 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactose, can also be used for 1-amination of mono- and oligo-saccharides which, instead of the acetamido group, contain a hydroxyl function at C-2 of the reducing sugar residue[11]. In the resulting Fmoc-Asn(sugar)-O-*t*-Bu(OH) derivatives, the high value (~ 9 Hz) of the H-1–H-2 coupling constant indicated the β anomeric configuration[9]. The epimeric purity of the final products was also demonstrated[10,11]. Fmoc-Asn(sugar)-OH derivatives with free hydroxyl functions on their sugar moieties showed higher coupling efficiencies in SPPS than those with acetyl-protected OH groups[7–9]. Furthermore, neither the symmetrical anhydrides nor the pentafluorophenyl esters of Boc- and Fmoc-amino acids were observed to acylate the free OH groups of the sugar residue(s).

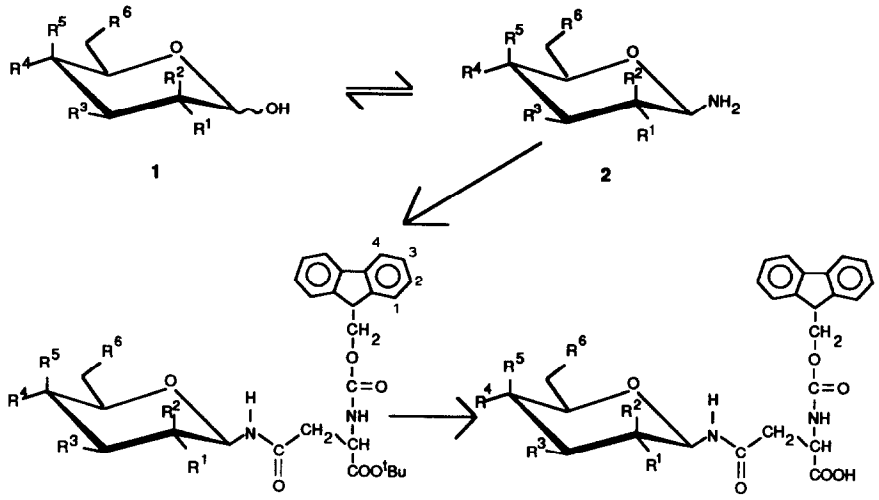
This paper reports the synthesis and characterization of additional N^{β}-glycosylated Fmoc-Asn-O-*t*-Bu(OH) derivatives and the stereochemistry of the amination reaction, as well as studies on the stability of the derivatives to the conditions of SPPS. The new reagents, together with those described earlier[7–9], will facilitate the exploration of the effect of *N*-glycosylation on the conformation and biological activity of peptides. Scheme 1 shows the reaction sequence for the preparation of Fmoc-Asn(sugar)-OH derivatives.

RESULTS AND DISCUSSION

Synthesis of 1-amino 1-deoxy sugars.—1-Amino 1-deoxy sugars were prepared by a new and simple approach reported earlier[10] for the synthesis of glycosylamines containing an equatorial acetamido group at the C-2 atom of the pyranose ring. Earlier we extended the applicability of this method to glucose, cellobiose, and maltose. To explore whether amination with ammonium hydrogencarbonate can be used as a general method for preparing 1-amino 1-deoxy hexopyranoses, we applied the reaction protocol for some other sugars. Our main concern was to see whether the method could be applied to saccharides in which the C-2 carries other functional groups instead of the acetamido present in natural glycoproteins.

The formation of *O*-β-D-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-β-D-glucopyranosylamine [**2a**, Gal(β1-3)GlcNAc(β)-NH_2] can be achieved similarly to that reported earlier for the preparation of 2-acetamido-2-deoxy-β-D-glucopyranosylamine[10]. It appears that the saccharide group attached at the C-3 atom of the pyranose ring has no influence on the amination reaction.

2-Acetamido-2-deoxy-β-D-galactopyranosylamine (**2b**) was obtained as described earlier[9,10]. The side-product of the amination reaction[10,12], after separation by



Compound	Abbreviated name of the sugar moiety	R^1	R^2	R^3	R^4	R^5	R^6
1a, 2a, 3a, 4a	Gal(β1-3)GlcNAc	NHAc	H	Galβ(1 → O)	OH	H	OH
1b, 2b, 3b, 4b	D-Gal*p*NAc	NHAc	H	OH	H	OH	OH
1c, 2c, 3c, 4c	D-Gal*p*	OH	H	OH	H	OH	OH
1d, 2d, 3d, 4d	Gal(β1-4)Glc	OH	H	OH	Galβ(1 → O)	H	OH
1e, 2e, 3e, 4e	D-Fuc*p*	OH	H	OH	H	OH	H
1f, 2f, 3f, 4f	D-Man*p*	H	OH	OH	OH	H	OH
1g, 2g, 3g, 4g	2dGlc*p*	H	H	OH	OH	H	OH

Scheme 1

column chromatography on silica gel, was identified by positive fast atom bombardment mass spectroscopy (FABMS) as $(GalNAc)_2NH$ $[M + H^+ = 424]$. The common feature of galactose (**1c**), lactose (**1d**), and fucose (**1e**) is that they contain an OH group in an equatorial position at the C-2 atom of the first pyranose ring. No significant difference was found in the results of the amination of these sugars. Thus variation of the groups at C-4 (galactose, lactose) and at C-4 and C-6 (fucose) has no particular influence on the formation of glycosylamines. The amination reaction yielded crude glycosylamines (**2c–2e**) containing 20–40% of the starting sugars after 8 days by TLC. Elongation of the reaction time did not increase the conversion. Due to the higher lability of these 1-amino sugars, attempts to separate them from the starting sugars by using Amberlist 15 cation-exchange resin[10] remained unsuccessful. Products obtained after resin treatment were always contaminated with some reducing sugar. Amination of mannose (**1f**) (axial OH group

on C-2) resulted in 1-amino product (**2f**) with a yield compatible to that of the glucosyl, galactosyl, and fucosyl derivatives. However, for 2-acetamido-2-deoxy-D-mannopyranose (the acetamido group linked to the C-2 atom in the axial position), considerable amounts of a side-product (40–50%) was always detected on TLC. This side-product was characterized by positive FABMS as $(ManNAc)_2NH$ (ManNAc-NH_2, $M + H^+ = 221$; bis product, $M + H^+ = 424$, $M + Na^+ = 446$) and was present even if the concentration of the sugar or the temperature of the reaction was decreased[10,11].

Less than 50% amination occurred after 3 weeks for 2-deoxy-D-*arabino*-hexose (2-deoxy-D-glucose) (**1g**). The decomposition during the repeated evaporations required for removal of the excess ammonium hydrogen carbonate (see Experimental) was followed by TLC. After 2 h standing following the last distillation, practically no glycosylamine could be detected. Purification of the product on cation-exchange resin was also unsuccessful because the 1-amino derivative disappeared soon after it was washed from the resin. Due to the surprisingly high lability of this compound, we began acylation before complete removal of the ammonium hydrogencarbonate (see Experimental).

The stability of glycosylamines in aqueous solutions was also investigated. We have found on TLC that compounds **2a** and **2b** (equatorial NHAc group at C-2) can be kept in water for 24 h without significant increase of the amount of the breakdown products **1a** and **1b**, respectively. In the cases of **2c–2f** ((equatorial or axial OH group at C-2) after 24 h the amount of **1c–1f** increased considerably, while **2g** (only H at C-2) could not be detected on TLC after a few hours. This finding is in agreement with the existence of an equilibrium between the glycosylamine and its precursor.

As a consequence, as suggested earlier[9–11], ammonium hydrogencarbonate can be used extensively for fast and simple preparation of 1-amino 1-deoxy hexopyranoses. However, we found that the purity and stability of the afforded 1-amino hexopyranoses (**2a–2g**) depended widely upon the structure of the reducing sugar moiety and the steric position of the group attached to the C-2 atom of the pyranose ring. The glycosylamines investigated are likely stabilized by a hydrogen bond between the amino group and the group attached to the C-2 atom.

Acylation with Fmoc-Asp-O-t-Bu.—Crude glycosylamines were the starting materials for the synthesis of N^{α}-fluorenylmethoxycarbonyl-N^{β}-glycosyl-L-asparagine *tert*-butyl esters (**3a–3g**), for use as building blocks in the synthesis of glycopeptides. To the solution of Fmoc-Asp(OPfp)-O-*t*-Bu in *N*,*N*-dimethylformamide (DMF), an excess of crude glycosylamines (**2a–2f**) was added in a DMF–water mixture. Water is required to dissolve the 1-amino 1-deoxy sugars but a water content higher than 15% of the final solution may lead to precipitation of the active ester. (Compound **2g** was treated exceptionally due to its high lability; see Experimental.)

Our findings are as follows: (*i*) 1-Amino 1-deoxy sugars (**2a–2g**) can be selectively *N*-acylated with N^{α}-fluorenylmethoxycarbonylaspartic acid α-*tert*-butyl β-

pentafluorophenyl ester (Fmoc-Asp(OPfp)-O-*t*-Bu). Similar to the previously reported selectivity of pentafluorophenyl esters[13] toward acylation, TLC, reversed-phase high performance liquid chromatography (RP-HPLC), FABMS, and ^{1}H NMR did not indicate *O*-acylation. (*ii*) Reducing sugar contaminants had no particular influence on the yield of *N*-acylation (in the case of compounds **2a–2f**), while their removal on a cation-exchange resin led to a 30–50% loss of the 1-amino 1-deoxy derivative, or the separation simply remained unsuccessful.

Stability of glycosidic bonds to trifluoroacetic acid.—The α-*tert*-butyl ester groups of **3a–3g** were cleaved with 100% trifluoroacetic acid (TFA) affording compounds **4a–4g**. After 20 min, TFA was removed in vacuo. Alternatively, the TFA solution can be diluted with water, immediately frozen, and lyophylized. The products were obtained by trituration with dry ether. Analytical RP-HPLC (chromatographic condition II) indicated < 93% purity for **4a–4f** and 83% purity for **4g**. Preparative RP – HPLC purification (chromatographic condition III) was used for sample preparation for ^{1}H NMR studies.

The applicability of unprotected sugar derivatives in SPPS depends upon the stability of the glycosidic and *N*-glycosyl bonds in an acidic environment. Thus small amounts of compounds **4a–4g** were kept for an additional 2 h in TFA. Peaks detected in the RP-HPLC profile after 2 h did not represent products of glycosidic or glycosylamine bond cleavage, such as Fmoc-Asn(GlcNAc)-OH and Fmoc-Asn(glucose)-OH (retention times 55.8 and 55.2 min, respectively; chromatographic condition II) from disaccharide derivatives **4a** and **4d**, or Fmoc-Asn-OH and Fmoc-Asp-OH (retention times 63.0 and 68.1 min, respectively) from either the disaccharide or monosaccharide (**4b**, **4c**, **4e**, and **4f**) derivatives. The only exception was the 2-deoxy-D-*arabino*-hexose derivative (**4g**), where considerable decomposition was observed. Recently we reported[14] the stability of Fmoc-Asn(chitobiose)-OH, Fmoc-Asn(chitotriose)-OH, and Fmoc-Asn(cellobiose)-OH during the solid-phase synthesis of glycopeptides. TLC, positive FABMS and ^{1}H NMR verified the RP-HPLC data. After standing for 24 h in TFA, the following extents of decomposition was found by RP-HPLC using a combination of peak heights and peak area integration values[7]: < 10% for **4b**, **4c**, **4e** and **4f**; 20% for **4d**; 32% for **4a**; and 44% for **4g**. The corresponding values for Fmoc-Asn(Chitobiose)-OH and Fmoc-Asn(cellobiose)-OH are 12 and 20%, respectively[9,14]. Our results indicate the following stability order of glycosidic bonds in TFA: Gal(β1-3) < Gal(β1-4), Glc(β1-4) < GlcNAc(β1-4) < *N*-glycosyl bond. When the C-2 atom has no substituents other than H (**4g**), the *N*-glycosyl bond becomes highly acid labile.

It was reported that the fucosyl (1 → 6) bond is highly acid labile[15]. It is thus predictable that unprotected saccharides with *O*-fucosidic bonds are potentially acid-sensitive. In order to find an applicable TFA concentration for the synthesis of glycopeptides containing unprotected, fucose-containing saccharides, we studied the acid stability of the Y-antigen [Fucα1-2Galβ1-4(Fucα1-3)GlcNAc(β)-O-$(CH_2)_8$-COOH]. The Y-antigen is a gastrointestinal tumor-associated hapten, which contains two L-fucosidic bonds. A long apolar chain is attached to the sugar

for biotechnology purposes (the apolar side chain also plays a major role in binding to the C_{18} column). According to RP-HPLC, 15% decomposition was detected after 15 min and 80% after standing in 100% TFA for 1 h. On isolation of the decomposed product (the retention time of the Y-antigen is 30.5 min and of the decomposed compound, 31.3 min, chromatographic condition I; see Experimental), the lack of one fucose residue was evidenced by positive FABMS (Y-antigen: $M + Na^+ = 854$, decomposed product $M + Na^+ = 708$). (We did not identify which fucose was removed.) In contrast, in 1 : 1 (v/v) TFA–DMF solution, no decomposition occurred after 1 h, which suggests that the Fuc(α1-2) and Fuc(α1-3) glycosidic bonds would be stable during the conditions of deprotection and cleavage of peptides from the solid support.

These results indicate that glycopeptides with unprotected oligosaccharide moieties featuring Gal(β1-3), Gal(β1-4), Glc(α1-4), Glc(β1-4), GlcNAc(β1-4), and *N*-glycosyl bonds (except that in **4g**) can be synthesized by the standard Fmoc protocol. The method can, in principle, also be applied to more sensitive, branched oligosaccharides, provided the TFA concentration in any step never exceeds 50% in DMF. The 50% TFA concentration and the 2 h cleavage time are sufficient to remove side-chain protecting groups of other, non-glycosylated amino acids and to cleave the peptide from the *p*-alkoxybenzylalcohol or other resins typically employed in SPPS utilizing Fmoc chemistry.

Stereochemistry of the N-*glycosyl bond.*—^{1}H NMR was used to confirm the structure and the anomeric configuration of the *N*-glycosyl linkages of the afforded Fmoc-Asn(sugar)-O-*t*-Bu(OH) derivatives. The high values of the H-1–H-2 coupling constants (Tables I and II) indicate the β anomeric configuration[16] (except **3f** and **4f**). The β anomeric form of **3f** was also evidenced; although the dihedral angle between H-1 and H-2 is the same in both α and in β configurations, the distances between H-1, H-3, and H-5 are short enough to observe a nuclear Overhauser effect. Also, in all β anomeric forms of Fmoc-Asn(sugar)-O-*t*-Bu(OH) derivatives, the NH–H-1 coupling constants are around 8–9 Hz (8.7 Hz for **3f**). This value may be diagnostic for the β anomeric forms of glycosylamines.

Our preliminary results show that the Fmoc-Asn(sugar)-OPfp derivatives couple to resin-bound amino acids and peptides with high efficiency[9,17,18]. This coupling efficiency can be further increased by using polyamide- or polyepoxy-based resins. The stability of the glycosidic and *N*-glycosyl bonds during TFA treatment and the high coupling efficiencies show the widespread applicability of the Fmoc-Asn(sugar)-OH reagents for custom synthesis of *N*-glycosylated peptides by standard Fmoc-based SPPS protocol[17,18]. More generally, free amino groups can be acylated by Fmoc-Asn(sugar)-OPfp derivatives and sugar can be attached through *N*-glycosyl linkages to the carboxyl functions of biologically important molecules.

EXPERIMENTAL

General methods.—^{1}H NMR studies were performed on a Bruker 500 spectrometer at 313 K. All NMR spectra were recorded in $(CD_3)_2SO$ containing some

TABLE I

^{1}H NMR signals of Fmoc-Asn(sugar)-O-*t*-Bu derivatives

Hydrogen atom		Chemical shift (ppm) or coupling constants (Hz)			
		3c	**3e**	**3f**	**3g**
Fmoc group	H-1	7.90	7.89	7.87	7.85
	H-2	7.40	7.41	7.39	7.40
	H-3	7.32	7.32	7.30	7.32
	H-4	7.70	7.62	7.67	7.65
	CH	6.32	6.33	6.32	6.31
	CH_2	1.20	1.00	1.17	1.16
Asn residue	*t*Bu	1.40	1.40	1.30	1.39
	αCH	4.32	4.26	4.28	4.25
	βCHa	2.50	2.50	2.48	2.50
	βCHb	2.60	2.61	2.59	2.60
	βNH	8.50	8.51	8.18	8.54
Sugar residue	H-1	4.70	4.65	4.97	4.98
	H-2	3.33	3.33	3.64	1.80 (*ax*)
					1.41 (*eq*)
	H-3	3.40	3.40	3.35	3.40
	H-4	3.50	3.51	3.39	3.00
	H-5	3.26	3.27	3.30	3.70
	H-6a	3.37	1.39	3.35	3.37
	H-6b	3.55		3.55	3.64
	$J_{1,NH}$	9.7	9.3	8.7	8.8
	$J_{1,2}$	11.0	11.0		11.3 (*ax*)
					1.9 (*eq*)

drops of D_2O. Liquid secondary ion mass spectrometry experiments were carried out in the positive ion mode using a VG Analytical ZAB-E mass spectrometer equipped with a cesium ion source and a high field magnet. Thioglycerol in aq 1% TFA was used as liquid matrix. The HPLC system consisted of two 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 aq 0.1% TFA; solvent B was acetonitrile containing 0.1% TFA. Chromatographic conditions were: I, column 7.8 × 300 mm, initial solvent composition was 95% A and 5% B. A linearly increasing proportion of solvent B was added at the rate of 1.33%/min starting at 6.7 min of the run. Flow rate was 2 mL/min. II, column 10 × 250 mm, a linear gradient of 0.66%/min solvent B starting from 5% at 6.7 min, flow rate 3 mL/min. III, preparative column 21.2 × 150 mm, a linear gradient of 0.2%/min solvent B, starting at 15%. The flow rate was 8 mL/min. TLC was conducted on Merck aluminum-backed Silica Gel 60, and 60 F_{254} plates. Detection was by UV at 254 nm, and spots were visualized with color reagents using KI/tolidine or 0.2% ninhidrin in acetone. Spots were also developed by heating with 10% H_2SO_4 in EtOH. Eluents for TLC were as follows (all proportions v/v): buffer, 20:6:11 pyridine–acetic acid–water; eluent

TABLE II

^{1}H NMR signals of Fmoc-Asn(sugar)-OH derivatives

Hydrogen	Atom	Chemical shift (ppm) or coupling constants (Hz)					
		4b [a]	**4c**	**4d**	**4e**	**4f**	**4g**
Fmoc group	H-1	7.90	7.89	7.89	7.89	7.87	7.90
	H-2	7.43	7.42	7.42	7.41	7.41	7.43
	H-3	7.33	7.35	7.33	7.33	7.32	7.34
	H-4	7.76	7.71	7.70	7.71	7.68	7.70
	CH	6.33	6.30	6.29	6.27	6.09	6.33
	CH_2	1.22	1.17	1.19	1.08	1.20	1.19
Asn residue	αCH	4.25	4.30	4.32	4.37	4.34	4.28
	βCHa	2.48	2.49	2.49	2.55	2.50	2.48
	βCHb	2.60	2.56	2.61	2.63	2.63	2.60
	βNH	8.22	8.72	8.54	8.44	8.18	8.22
Sugar residue	H-1	4.67	4.67	4.78 [b] 4.68	4.65	5.04	4.99
	H-2	3.75	3.33	3.77 3.66	3.34	3.74	1.81 (*ax*) 1.42 (*eq*)
	H-3	3.53	3.42	3.69 3.57	3.44	3.35	3.42
	H-4	3.42	3.50	3.50 3.44	3.56	3.40	2.99
	H-5	3.40	3.27	3.47 3.52	3.32	3.30	3.80
	H-6a	3.69	3.38	3.69 3.57	0.993	3.35	3.36
	H-6b	3.77	3.55	3.81 3.76		3.55	3.49
	$J_{1,NH}$	8.2	8.7	8.9	8.9	9.0	8.8
	$J_{1,2}$	7.7	10.8		11.0	0.9	11.1 (*ax*) 1.9 (*eq*)

[a] CH_3CO, δ 2.56. [b] The first value in each pair is for the inner unit (Glc), the second is for the outer unit (Gal).

(a) 1:1 $CHCl_3$–MeOH; eluent (b) 3:2 buffer–EtOAc; eluent (c) 2:3 buffer–EtOAc; eluent (d) 1:3 buffer–EtOAc; eluent (e) 1:5 buffer–EtOAc. Sugars were purchased from Aldrich and Sigma.

Preparation of glycosylamines.—Sugar solution (0.1 M) in water was saturated with NH_4HCO_3 and kept at 30° for 7–8 days. The solution was diluted with water and the excess of NH_4HCO_3 was removed either by lyophylization a few times to constant weight or by dilution with water and evaporation in vacuo 8 times (bath temperature $< 30°$). The amination reaction was monitored by TLC.

The following products were identified: *O*-β-D-Galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-β-D-glucopyranosylamine (**2a**); after 7 days in NH_4HCO_3 90% conversion to **2a** as determined by semiquantitative TLC (R_F, 0.14, eluent a) without side-products. Yellowish solid.

2-Acetamido-2-deoxy-β-D-galactopyranosylamine (**2b**); amorphous solid containing 10% *bis*-product and less than 5% **1b** after 7 days (R_F 0.25 for **2b**, 0.20 for the *bis*-product, eluent a).

β-D-Galactopyranosylamine (**2c**); 60–70% conversion after 8 days (R_F, 0.18, eluent b). No *bis*-product was detected. Amorphous solid.

O-β-D-Galactopyranosyl-(1 → 4)-β-D-glucopyranosylamine (**2d**); conversion of **1d** to **2d** was 60% after 8 days (R_F, 0.1 eluent b). Amorphous solid.

β-D-Fucosylamine (**2e**); 70–80% conversion after 7 days (R_F, 0.28, eluent b). Yellowish solid.

β-D-Mannopyranosylamine (**2f**); 80% conversion after 7 days (R_F, 0.19 eluent b). Amorphous solid.

2-Deoxy-β-D-*arabino*-hexopyranosylamine (**2g**); 50% conversion after 21 days (R_F, 0.28, eluent a). **2g** was unstable and could not be isolated.

Preparation of N^{α}-*fluorenylmethoxycarbonyl*-N^{β}-*glycosyl*-L-*asparagine* α-*tert-butyl esters.*—Fmoc-Asp-O-*t*-Bu (432 mg, 1.05 mmol) and pentafluorophenol (193 mg, 1.05 mmol) were dissolved in 2 mL DMF and *N*,*N*′-diisopropylcarbodiimide (DIC) (216 mg, 1.05 mmol) was added. The mixture was stirred at room temperature for 1 h, then 2 mmol crude glycosylamine (**2a–2f**) was added in 2–3 mL DMF–water (3:1, v/v) and stirring was continued until no more Fmoc-Asp(OPfp)-O-*t*-Bu was detected on TLC (24–48 h). The insoluble material was filtered off and the solvent was removed in vacuo. The crude products were triturated with ether several times, then with water. The products were dried in a vacuum desiccator over P_2O_5. The R_F values, RP-HPLC retention times and FABMS data are listed in Table III. ^{1}H NMR data are listed in Table I.

N^{α}-Fluorenylmethoxycarbonyl-N^{β}-[*O*-β-D-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-L-asparagine *tert*-butyl ester (**3a**); N^{α}-fluorenylmethoxycarbonyl-N^{β}-β-D-fucopyranosyl-L-asparagine *tert*-butyl ester (**3e**); N^{α}-fluorenylmethoxycarbonyl-N^{β}-β-D-mannopyranosyl-L-asparagine *tert*-butyl ester (**3f**);

TABLE III

Characterization of Fmoc-Asn(sugar)-O-*t*-Bu derivatives

Compound	Abbreviated name	R_F (eluent)	RP-HPLC [a] ret. time system II (min)	FABMS	
				$(M+H)^+$	$(M+Na)^+$
3a	Fmoc-Asn[Gal(β1-3)GlcNAc]-O-*t*-Bu	0.31 (c)	66.8	776	798
3b	Fmoc-Asn(GalNAc)-O-*t*-Bu	0.30 (d)	69.8	614	636
3c	Fmoc-Asn(Gal)-O-*t*-Bu	0.29 (d)	69.2	573	595
3d	Fmoc-Asn[Gal(β1-4)Glc]-O-*t*-Bu	0.30 (c)	66.8	735	757
3e	Fmoc-Asn(Fuc)-O-*t*-Bu	0.50 (d)	73.3		579
3f	Fmoc-Asn(Man)-O-*t*-Bu	0.29 (d)	69.8	573	595
3g	Fmoc-Asn(dGlc)-O-*t*-Bu [b]	0.32 (e)	72.0	557	579

[a] RP-HPLC: reversed-phase high performance liquid chromatography. [b] dGlc: "2-deoxy-D-glucose".

the products were free from impurities by HPLC and TLC. Yields were 29, 37, and 35%, respectively.

N^{α}-Fluorenylmethoxycarbonyl-N^{β}-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-L-asparagine *tert*-butyl ester (**3b**); due to the presence of *bis*(galactosylamine) in **2b**, the product **3b** was contaminated (R_F of the impurity 0.27). Purification in open column chromatography using eluent d resulted in pure **3b**. Yield was 30%.

N^{α}-Fluorenylmethoxycarbonyl-N^{β}-β-D-galactopyranosyl-L-asparagine *tert*-butyl ester (**3c**); N^{α}-fluorenylmethoxycarbonyl-N^{β}-[*O*-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl]-L-asparagine *tert*-butyl ester (**3d**); small amounts of **1c** and **1d** contaminants were removed by open column flash chromatography using eluents d and c, respectively. Yield was 28% for both.

N^{α}-Fluorenylmethoxycarbonyl-N^{β}-(2-deoxy-β-D-*arabino*-hexopyranosyl)-L-asparagine *tert*-butyl ester (**3g**); to the solution of 1 mmol Fmoc-Asp(OPfp)-O-*t*-Bu prepared by the above method, 6 mmol crude **2g** (contaminated with some NH_4HCO_3 and ~ 50% **1g** based on TLC) was added in 4 mL DMF. After stirring overnight, the reaction was continued as described above. Fmoc-Asn-O-*t*-Bu that resulted from the reaction of the active ester with NH_4HCO_3, was removed by column chromatography with eluent e. Yield was 8%.

Cleavage of α-tert-butyl ester group by TFA.—1 mmol Fmoc-Asn(sugar)-O-*t*-Bu was dissolved in 5 mL TFA. After 20 min the TFA was removed and the product was triturated with ether. Yield was > 95%. Table IV lists the R_F values, RP-HPLC retention times, and the FABMS data. The purity of the products was determined by RP-HPLC (chromatographic condition II). Preparative RP-HPLC was used (chromatographic condition III) to yield pure products for ^1H NMR studies (data are listed in Table II). Unpurified products (**4a–4f**) were used for solid-phase synthesis of glycopeptides[17,18].

N^{α}-Fluorenylmethoxycarbonyl-N^{β}-[*O*-β-D-galactopyranosyl-(1 → 3)-(2-acetamido-2-deoxy-β-D-glucopyranosyl)]-L-asparagine (**4a**, purity 94%).

TABLE IV

Characterization of Fmoc-Asn(sugar)-OH derivatives

Compound	Abbreviated name	R_F (eluent)	RP-HPLC [a] ret. time system II (min)	FABMS	
				$(M+H)^+$	$(M+Na)^+$
4a	Fmoc-Asn[Gal(β1-3)GlcNAc]-OH	0.40 (b), 0.12 (c)	53.8	720	
4b	Fmoc-Asn(GalNAc)-OH	0.25 (c), 0.10 (d)	55.8	558	
4c	Fmoc-Asn(Gal)-OH	0.25 (c), 0.10 (d)	55.2	517	
4d	Fmoc-Asn[Gal(β1-4)Glc]-OH	0.40 (b), 0.12 (c)	53.8		701
4e	Fmoc-Asn(Fuc)-OH	0.31 (c)	58.2	501	523
4f	Fmoc-Asn(Man)-OH	0.25 (c), 0.10 (d)	55.4	517	539
4g	Fmoc-Asn(dGlc)-OH	0.28 (c)	56.6		523

[a] RP-HPLC: reversed-phase high performance liquid chromatography.

N^{α}-Fluorenylmethoxycarbonyl-N^{β}-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-L-asparagine (**4b**, purity 95%).

N^{α}-Fluorenylmethoxycarbonyl-N^{β}-β-D-galactopyranosyl-L-asparagine (**4c**, purity 94%).

N^{α}-Fluorenylmethoxycarbonyl-N^{β}-[*O*-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl]-L-asparagine (**4d**, purity 95%).

N^{α}-Fluorenylmethoxycarbonyl-N^{β}-β-D-fucopyranosyl-L-asparagine (**4e**, purity 97%).

N^{α}-Fluorenylmethoxycarbonyl-N^{β}-β-D-mannopyranosyl-L-asparagine (**4f**, purity 94%).

N^{α}-Fluorenylmethoxycarbonyl-N^{β}-(2-deoxy-β-D-*arabino*-hexopyranosyl)-L-asparagine (**4g**, purity 83%).

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