ORGANIC LETTERS

2004
Vol. 6, No. 22
4001−4004

An Efficient Synthetic Route to Glycoamino Acid Building Blocks for Glycopeptide Synthesis

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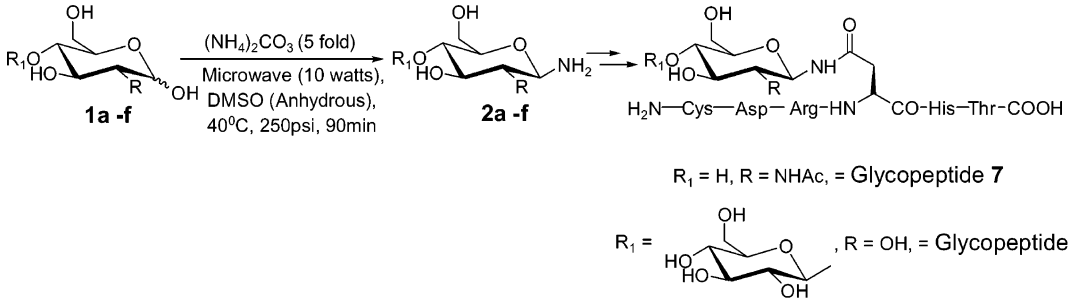
Received August 20, 2004

ABSTRACT



Chemical glycopeptide synthesis requires access to gram quantities of glycosylated amino acid building blocks. Hence, the efficiency of synthesis of such building blocks is of great importance. Here, we report a fast and highly efficient synthetic route to Fmoc-protected asparaginyl glycosides from unprotected sugars in three steps with high yields. The glycosylated amino acids were successfully incorporated into target glycopeptides 7 and 8 by standard Fmoc solid-phase peptide synthesis.

It is estimated that more than half of all proteins carry carbohydrate side chains, with the majority being *N*-glycans linked to asparagine residues that are part of the Asn-X-Ser/Thr tripeptide codon for *N*-glycosylation.[1] Very little is understood about how the glycan chains specifically modulate stability and activity of glycoproteins because of the complexity and heterogeneity of glycoprotein samples isolated from biological sources. Glycopeptides are therefore important targets for in vitro synthesis,[2] and with the advent of the native chemical ligation of peptides even small- and medium-sized glycoproteins are now targeted by chemical synthesis.[3]

N-Glycans can influence glycoprotein biosynthesis, structure, and activity in many ways. One intriguing question is that of the role of the highly conserved core pentasaccharide of *N*-glycans. Although *N*-glycosylation is diverse across tissues and species, the pentasaccharide core which is linked through the reducing *N*-acetylglucosamine to an asparaginyl side chain of the polypeptide is conserved in almost all higher organisms. Recent crystallographic and NMR data has shown that the core, especially the first two *N*-acetylglucosamine residues, can have important interactions with the protein and be responsible for stabilization of the active protein structure.[4]

To address questions of the structure−activity relationship of *N*-glycans, we are seeking to develop synthetic methods that would give us good access to glycopeptides carrying

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10.1021/ol048342n CCC: $27.50
Published on Web 09/30/2004

natural *N*-glycan core structures and a number of specific analogues.

During previous work on the synthesis of glycopeptides, two strategies have been employed. One approach introduces the carbohydrate as part of a glycoamino acid building block during solid-phase synthesis of the polypeptide chain.[5] Alternatively, the carbohydrate can be attached to a selectively deprotected aspartic acid residue once the polypeptide has been formed using the method developed by Lansbury.[6] By a similar route, unnatural glycopeptides have also been generated using selective alkylation of free cysteine residues.[7]

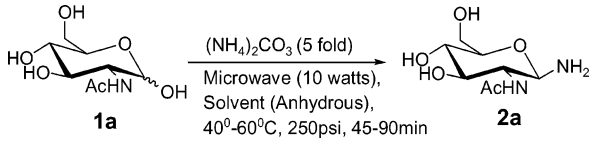
Both of the routes to peptides bearing *N*-glycans generally use β-glycosylamines as key intermediates,[8] with subsequent acylation by a suitably protected amino acid or a polypeptide side chain depending on the approach taken. Such glycosylamines are accessible by two routes: from suitably protected glycosyl azides requiring multiple protection and deprotection steps (generally five steps) or by direct amination (one step) using the Kochetkov reaction.[8] The latter method starts with a fully deprotected reducing sugar which is treated with 40−50 times excess ammonium bicarbonate for 6 days.[8] The longer reaction times and difficulties in removing ammonium bicarbonate effectively by evaporation (a process that takes several days) have been a major drawback of the Kochetkov amination reaction.[9,10]

Prompted by reports that imine formation can be accelerated by microwave irradiation,[11] we decided to study the Kochetkov reaction using microwave irradiation. Our aim was to develop methodology that would overcome the substantial practical drawbacks of this key reaction for glycopeptide synthesis, in particular, shorten reaction times and reduce the amount of bicarbonate needed.

Initially, the reaction conditions were optimized for *N*-acetyl glucosamine (**1a**), which is conserved as the reducing monosaccharide unit of the pentasaccharide core (Table 1). Of the various solvents tested, only DMSO resulted in good yields of **2a** (Table 1, compare the first four entries). Gratifyingly, the reaction was found to be efficient with only 5-fold excess (w/w) of ammonium carbonate over sugar compared to the 40−50-fold excess needed under thermal conditions.[8] Reactions appeared to be complete after 90 min of microwave irradiation (10 W), while maintaining the vessel temperature at 40 °C and maximum pressure at 250 psi.

In addition to the product **2a**, a small amount of side product, diglycosylamine, was also observed by mass spectrometry. Such a dimer is formed by further condensation of **2a** to starting sugar. The formation of this dimer in up to 10% yield has also been observed in thermal reactions[8b] and has been shown not to interfere with subsequent acylations. The formation of dimer was significantly increased at higher temperatures such as 50 and 60 °C (Table 1), and reaction temperatures were therefore generally kept at 40 °C.

Table 1. Optimization of Microwave-Assisted Kochetkov Amination

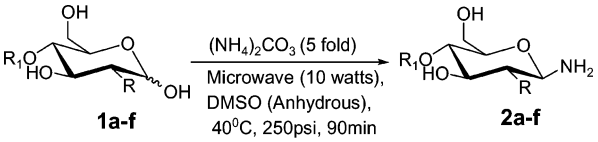


solvent	*T* (°C)	time (min)	product	dimer[a] (%)
MeOH	40	90	no product	ND
CH_3CN	40	90	no product	ND
DMF	40	90	trace	ND
DMSO	40	90	80−90	5
DMSO	50	90	60−70	15
DMSO	60	90	50−60	20
DMSO	40	45	60−70	5

[a] ND (not determined). Dimerization of the amine increased with temperature from 40 to 60 °C.

The optimized reaction conditions were used successfully to afford glycosylamines **2a**−**f** from mono-, di-, and trisaccharides **1a**−**f** in excellent yields (Table 2). In all cases, the small excess of ammonium carbonate and DMSO was easily removed by freeze-drying the reaction mixture overnight to yield a colorless hygroscopic solid, which could used for further acylation studies without any purification. Selective formation of the β-glycosylamine was demonstrated by NMR.

Table 2. Microwave-Assisted Kochetkov Amination



substrate	R_1	R	product (yield, %)[a]
1a	H	NHAc	**2a** (87)
1b	Glc (β1-	OH	**2b** (86)
1c	Gal (β1-	OH	**2c** (75)
1d	GlcNAc (β1-	NHAc	**2d** (35)
1e	Glc(α1−4)Glc (α1-	OH	**2e** (75)
1f	Glc (α1-	OH	**2f** (70)

[a] Yields were calculated from ^{1}H NMR spectra in D_2O and are based on the integration of the anomeric proton of crude glycosylamine and substrate.

Formation of glycosylamines was also attempted starting from peracetylated chitobiose and lactose to determine if

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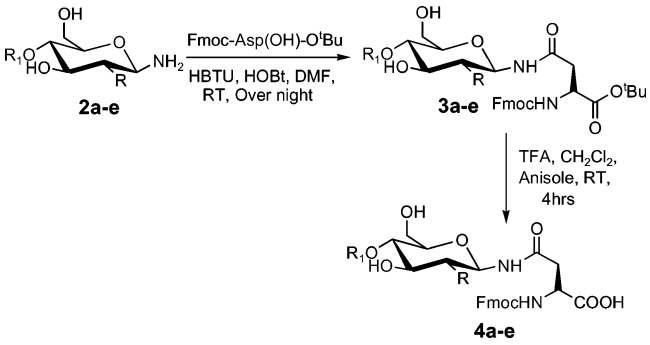
deacetylation and amination could be achieved at the same time. However, under our reaction conditions only 1-*O*-deacetylated glycosides together with trace amounts of glycosylamines were observed.

The crude glycosylamines **2a**−**e** were then used for the preparation of glycoamino acid building blocks (Table 3).

Table 3. Synthesis of Fmoc-Protected Glycoamino Acids from Crude Glycosylamines



substrate	R_1	R	product (yield, %)[b]
2a	H	NHAc	**4a** (89)
2b	Glc (β1-	OH	**4b** (86)
2c	Gal (β1-	OH	**4c** (79)
2d	GlcNAc (β1-	NHAc	**4d** (74)
2e	Glc(α1−4) Glc (α1-	OH	**4e** (81)

[b] Overall yields from **2a**−**e.**

Coupling of the amines (**2a**−**e**) with N-α-Fmoc-protected L-aspartic acid α-*tert*-butyl ester using HOBt/HBTU as coupling agent and subsequent acidic deprotection resulted in the corresponding Fmoc-protected glyco amino acids **4a**−**e** in good overall yields. Optimized yields were obtained with only 1.2-fold excess of amino acid over crude amine, which shows that most of the ammonium carbonate had been successfully removed during workup from the previous reaction mixture.

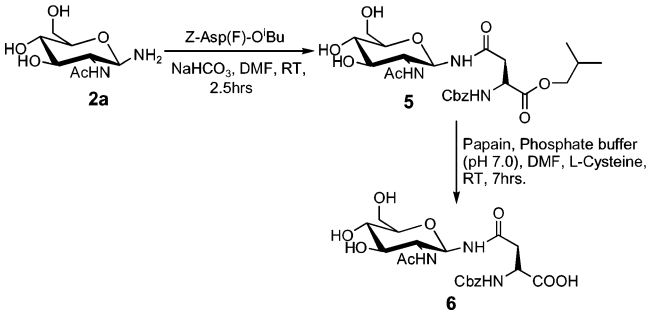
Alternatively, the Cbz-protected glycoamino acid **6** was prepared via acylation of **2a** with the β-acyl fluoride of *N*-α-Cbz protected L-aspartic acid α-isobutyl ester, followed by enzymatic hydrolysis of the isobutyl ester with papain (Scheme 1).[10]

One of the future aims of our studies is the chemical synthesis of the measles binding domains of the CD46 complement cofactor in humans. Structural studies have shown that the core saccharides (in particular, the first two GlcNAc residues) interact strongly with the polypeptide backbone, and functional studies have highlighted that glycosylation is essential for measles virus recognition.[12]

Our aim is the total synthesis of such receptor modules bearing defined natural and unnatural carbohydrate chains using native chemical ligation. Such synthesis requires access to glycosylated peptides such as **7** and **8**.

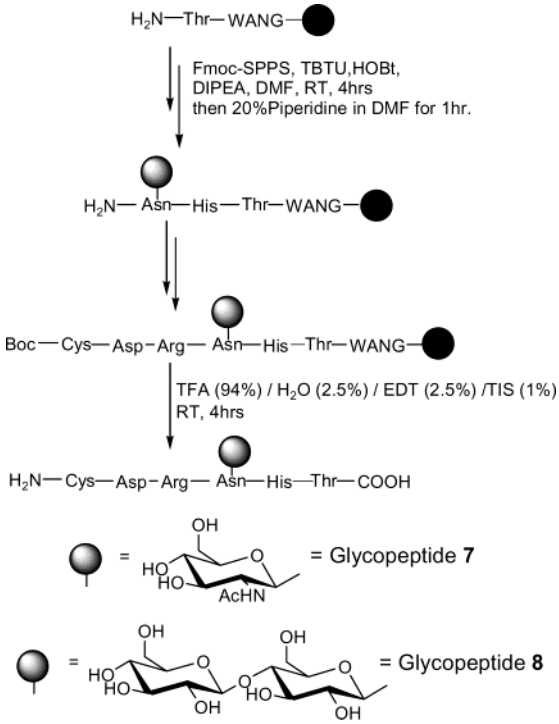
(12) Casasnovas, J. M.; Larvie, M.; Stehle, T. *EMBO J*. **1999**, *18*, 2911.

Scheme 1. Synthesis of Cbz-Protected Glycoamino Acid **6**



Thus, the glycosylated asparagine building blocks **4a** and **4b** were incorporated into target glycopeptides **7** and **8** using Fmoc-based solid-phase peptide synthesis on the Wang resin (Scheme 2).

Scheme 2. Fmoc-SPPS of Glycopeptide **7** and **8**



Reaction conditions for each coupling step were 4-fold excess of amino acid with TBTU, HOBt, and DIPEA in DMF for 4 h, followed by Fmoc deprotection using 20% piperidine in DMF for 1 h. All amino acid coupling steps were single couplings except for coupling of the glycoamino acids **4a** and **4b**, which were incorporated into peptide using double coupling before Fmoc deprotection to ensure completion.

The crude glycopeptides were cleaved from the resin using a mixture of TFA (94%)/H_2O (2.5%)/EDT (2.5%)/TIS (1%) for 4 h, in good overall yields (67% for **7** and 54% for **8** based on a loading of 0.56 mmol/gram of resin). Analysis by LC−MS(ESI) confirmed their structure.

In summary, we have developed a short (three-step) synthetic route from unprotected sugar to glycosylated asparagine building blocks for glycopeptide synthesis by using microwave-assisted Kochetkov amination as a key reaction. This route can be applied to mono- and oligosaccharides and yields glycosylated asparagine building blocks in a short period of time with minimal workup. The overall yields of the microwave-assisted aminations appear similar to that reported for the thermal conversion and suggest that this methodology will be very useful for other applications such as the preparation of glycoconjugates for subsequent immobilization.[8b] The application of the glycoamino acid building blocks in glycopeptide synthesis of **7** and **8**, which are key intermediates for the synthesis of glycoproteins by native chemical ligation, was successfully demonstrated.

Acknowledgment. We thank Drs. Ashley Causton, Alison Daines, and Beatrice Maltman for assistance and helpful discussions and John Miller and Robert Smith for analytical support. We thank ORS, BBSRC, Wellcome Trust, and University of Edinburgh for financial support.

Supporting Information Available: Experimental procedures and characterization data [^{1}H NMR, ^{13}C NMR, HRMS (FAB)] for all new compounds and LC−MS data for target glycopeptide **7** and glycopeptide **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL048342N