Sialylglycopeptide Synthesis

Solid-Phase Synthesis of Sialylglycopeptides through Selective Esterification of the Sialic Acid Residues of an Asn-Linked Complex-Type Sialyloligosaccharide**

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Glycoproteins and glycopeptides contain O- or N-linked oligosaccharides on their peptide backbones and play a central role in several biological events.[1] Thus, it is essential to be able to synthesize glycoproteins and glycopeptides for use as probes to study their biological roles.[2–4]

A chemoenzymatic method based on the solid-phase chemical synthesis of peptides and the enzymatic attachment of a sialyloligosaccharide to the peptide by the endoglycosidase enzyme Endo M has been reported for the synthesis of N-linked sialylglycopeptides.[3a,4] However, as the enzyme used is not commercially available and as the enzymatic method often requires substrate specificity,[5] chemical synthesis is essential for the preparation of pure sialyloligosaccharides. To date there has been no solid-phase chemical synthesis of glycopeptides with the N-linked complex-type biantennary sialyloligosaccharide structure that is commonly found on glycoproteins reported, as there are two major problems concerning the solid-phase synthesis of these compounds.

The first of these problems is the tendency of the sialyl linkage of glycopeptides to undergo acid-catalyzed hydrolysis in the presence of 95% trifluoroacetic acid (TFA) during the final cleavage step in solid-phase glycopeptide synthesis.[6] Both α-2,6 and α-2,3 sialyl linkages are very labile, but particularly the former. If α-2,6 sialyloligosaccharides are to resist acid hydrolysis, an electron-withdrawing group such as an acetyl group should be used as a protecting group.[3g] Another way to avoid hydrolysis of the sialyl linkage is to use a sophisticated linker such as HYCRON, which can be cleaved under mild conditions.[7]

The second problem is the quantity of complex-type sialyloligosaccharide required. If 10 mg of a decapeptide (M_W ca. 1000) is to be attached to a complex-type biantennary sialyloligosaccharide (M_W ca. 2000) by solid-phase peptide synthesis, at least 20 mg of the oligosaccharide must be used. Unverzagt and Prahl have developed a highly efficient chemoenzymatic method for the synthesis of such large oligosaccharides.[3j,8] However, 20 mg of a complex-type biantennary sialyloligosaccharide would be a very valuable sample, which would take several months to prepare by a chemical or chemoenzymatic method. The sialylglycopeptide **1** is available in multigram quantities from eggs.[9] If the sialyloligosaccharide part of **1** could be used for the solid-phase chemical synthesis of sialylglycopeptides, research on the biological role of sialylglycopeptides could be advanced.

The sialylglycopeptide **1** can be converted into the asparaginyl sialyloligosaccharide **2** upon treatment with the enzyme Actinase E (Scheme 1).[4a] However, as this Asn-linked oligosaccharide **2** contains three carboxylic acid groups, selective protection of the carboxylic acid groups on the sialic acid residues would be essential for solid-phase glycopeptide synthesis, in which undesired amide condensations must be avoided. Herein we describe solutions to these problems, and the resulting efficient solid-phase synthesis of an N-linked sialylglycopeptide via the sialyloligosaccharide **4**.

We investigated various conditions in our search for a selective esterification of the sialic acid carboxylic acid groups and found that the use of Cs_2CO_3 and BnBr[10] afforded the desired disialyloligosaccharide dibenzyl ester **4** in 85% yield from **3** (Scheme 1).[11,12] Compound **3** was in turn prepared by adding an Fmoc group to the asparagine residue of **2**. The ^{1}H NMR spectrum of **3** showed enhanced broadening of the AcN-H and C_1-H signals of the Asn-linked 2-acetamido-2-deoxy-β-D-glucopyranosyl residue and of the β-methylene proton signals of the asparagine residue, relative that of **2**. This observation may indicate that clustering of the Fmoc groups occurs, as a result of the hydrophobic nature of the aqueous solution. Selective neutralization by Cs_2CO_3 in water and subsequent selective benzyl esterification of the sialic acid residues might then take place because of the steric bulk of the clustered Fmoc groups. Even though three equivalents of Cs_2CO_3 (3 equiv of Cs to each sialic acid residue) were used, the triply esterified product was not obtained. We also found that compound **4** was stable in 95% aqueous TFA for three hours;[13] that is, under typical conditions for the cleavage of products from peptides in solid-phase synthesis. This resistance to acid may be a result of the steric bulk of the benzyl esters around the sialyl linkages.

We took advantage of this new finding and attempted the stepwise, solid-phase assembly of an N-linked sialylglycopeptide. As a target compound we chose the sequence ALLVNSS (79–85), which is part of the pharmaceutically important glycoprotein hormone erythropoietin (EPO).[14]

The N-linked sialylglycopeptide synthesis was performed on the poly(ethylene glycol)–poly(dimethylacrylamide) copolymer (PEGA) resin, which contains the acid-labile linker hydroxymethylphenoxyacetic acid (HMPA)[15] (Scheme 2). The first amino acid, serine, was coupled as Fmoc-Ser(*t*Bu)-OH by activation with 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT) and *N*-methylimidazole. The second serine residue was coupled in the presence of 1-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIPCDI). At this point, the oligosaccharide was introduced through reaction with glycosylated Fmoc-Asn-OH **4** in the

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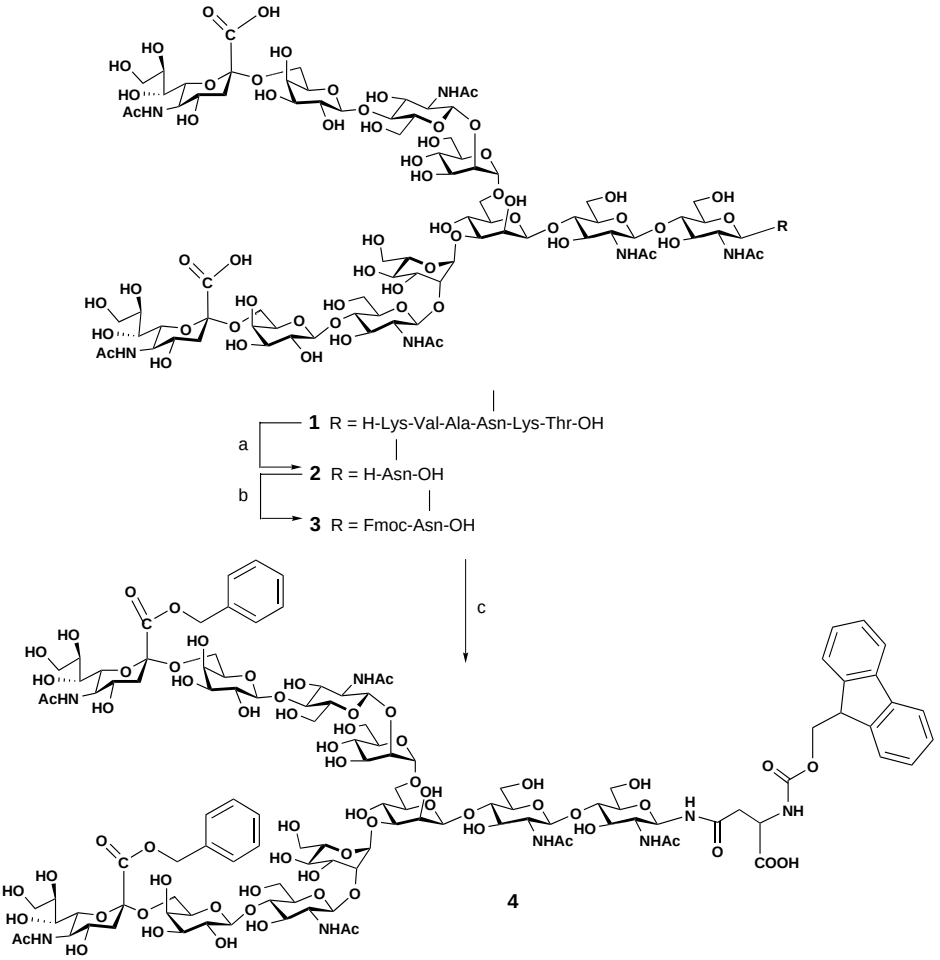
[**] We thank Dr. Michio Sasaoka (Otsuka Chemical Co., LTD) for supporting this work.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

 DOI: 10.1002/ange.200250572



Scheme 1. Reagents: a) Actinase E, Tris-HCl buffer, NaN_3, pH 7.5, 86%; b) Fmoc-OSucc, $NaHCO_3$, acetone, H_2O, 68%; c) Cs_2CO_3, H_2O, then BnBr, DMF, 85%. Tris = tris(hydroxymethyl)aminomethane, Fmoc-OSucc = 9-fluorenylmethyl *N*-succinimidyl carbonate.

presence of the coupling reagent 2-(1*H*-9-azobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphonate (HATU) and diisopropylethylamine (DIPEA) in DMF/DMSO (1:1) for 24 hours. The yield for this coupling step was 38%, based on the Fmoc-fullvene estimation method.[15] The other amino acids of the peptide in **5** were coupled as activated pentafluorophenol (OPfp) esters, in the presence of the catalyst 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH).[15] However, the coupling of Fmoc-Val-OPfp was monitored based on the disappearance of the yellow Dhbt-OH color and stopped at 85% conversion. If coupling with Val was continued, esterification of the hydroxyl groups on the oligosaccharide occurred.[16]. The coupling of the other amides (Leu, Leu, Ala) by the OPfp method took place quantitatively and the reactions were monitored in the same way, based on the disappearance of the Dhbt-OH color. In these cases no esterification of the hydroxy groups of the oligosaccharide was observed. After construction of the glycopeptide, the sialylglycopeptide was deprotected and cleaved from the resin by treatment with TFA/water (95:5) for three hours. As expected, the sialyl linkage was resistant to this acid treatment. Hydrolysis of the benzyl esters was carried out with aqueous sodium hydroxide at pH 11. This reaction was carefully monitored by ^{1}H NMR spectroscopy, and we could confirm that no epimerization of any of the amino acid α-carbon centers occurred. The crude product was then subjected to reversed-phase HPLC purification (Figure 1), and the pure sialylglycopeptide **5** thus obtained was characterized by ^{1}H NMR spectroscopy (2D-TOCSY, 2D-NOESY).[17]

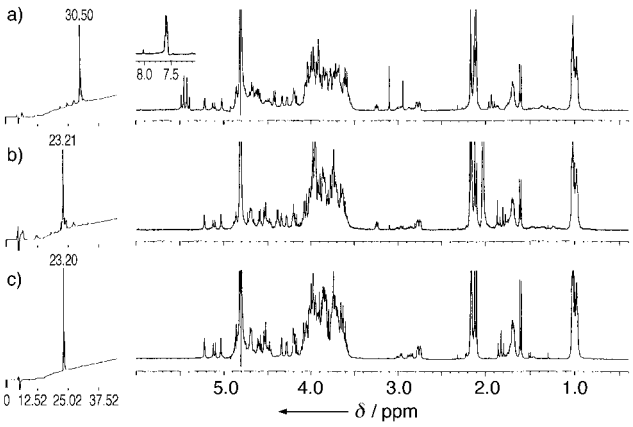
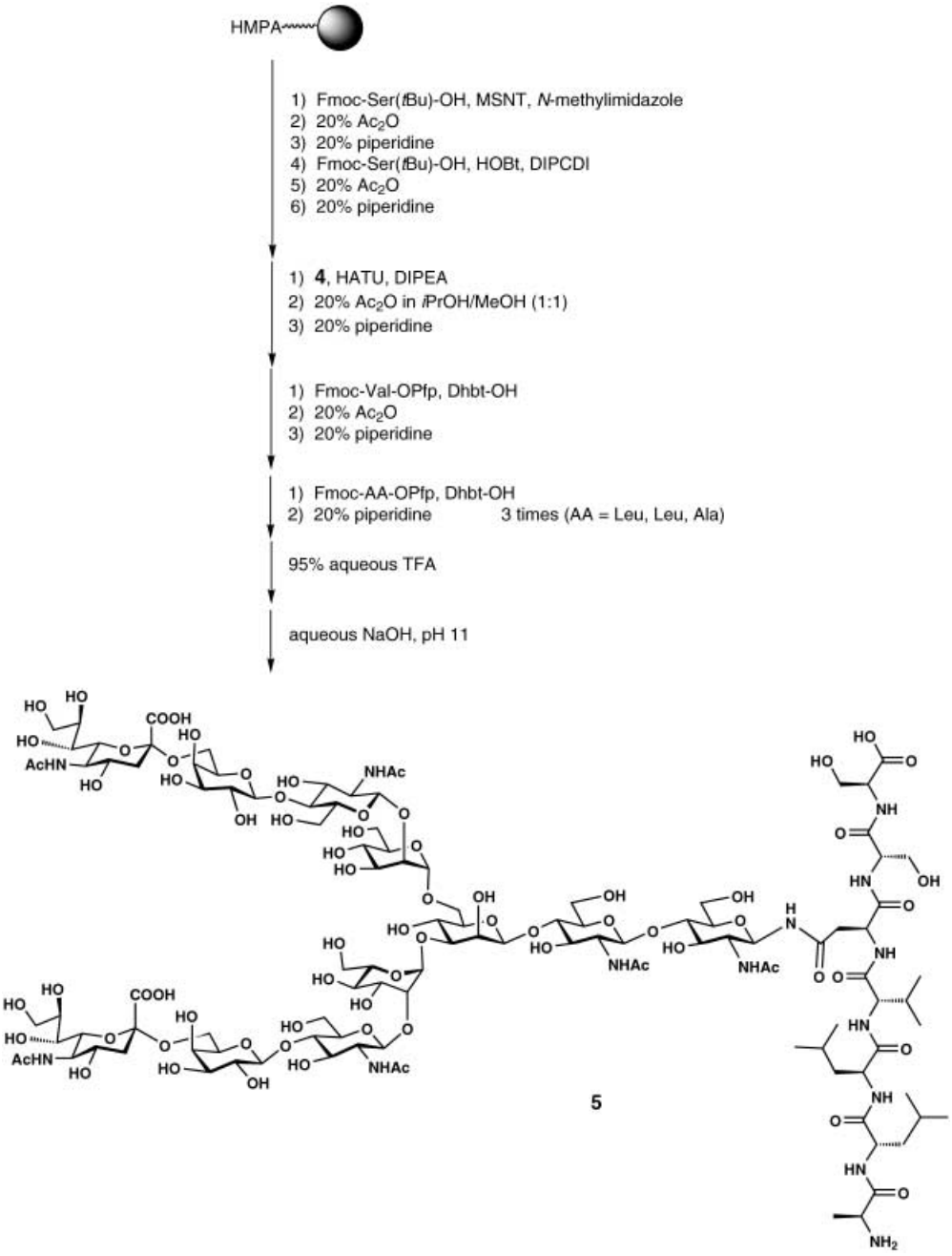


Figure 1. HPLC traces with corresponding ^{1}H NMR spectra: a) crude sample after cleavage from the resin with 95% TFA; b) crude sample after NaOH treatment of (a); c) sialylglycopeptide **5** after purification by HPLC.



Scheme 2. Solid-phase synthesis of the sialylglycopeptide **5**.

In conclusion, we have developed a selective esterification method for the sialic acid residues of asparagine-linked sialyloligosaccharides, which has enabled us to perform the first solid-phase chemical synthesis of an Asn-linked sialylglycopeptide. This method should be applicable to the synthesis of large sialylglycopeptides and sialylglycoproteins by native chemical ligation and intein methods,[18] and ultimately allow the investigation of the roles of sialyloligosaccharides in biological systems.

Received: November 18, 2002 [Z50572]

Keywords: glycopeptides · oligosaccharides · sialic acids · solid-phase synthesis

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[5] Another method (not on a solid phase) involves the use of sialyltransferases: Following the synthesis of an asialoglycopeptide, sialic acid can be attached by using sialyltransferase.[3j] However, this enzymatic reaction is not quantitative and the incomplete sialylation results in a time-consuming purification. See: a) T. Miyazaki, H. Sato, T. Sakakibara, Y. Kajihara, *J. Am. Chem. Soc.* **2000**, *122*, 5678 – 5694; b) T. Miyazaki, T. Sakakibara, H. Sato, Y. Kajihara, *J. Am. Chem. Soc.* **1999**, *121*, 1411 – 1412; c) S. Sabesan, J. O. Duus, P. Domaille, S. Kelm, J. C. Paulson, *J. Am. Chem. Soc.* **1991**, *113*, 5865 – 5866; d) D. H. Joziasse, W. E. C. M. Schiphorst, D. H. Van den Eijnden, J. A. Van Kuik, H. Van Halbeek, J. F. G. Vliegenthart, *J. Biol. Chem.* **1987**, *262*, 2025 – 2033.

[6] For the α-2,3 sialyl linkage in the glycopeptide, Kunz and coworkers (N. Bézay, G. Dudziak, A. Liese, H. Kunz, *Angew. Chem.* **2001**, *113*, 2350 – 2353; *Angew. Chem. Int. Ed.* **2001**, *40*, 2292 – 2295) and Nakahara et. al[3e] showed that esterified and lactonized O-linked sialyltrisaccharides, respectively, are resistant toward acid treatment in the final step of solid-phase peptide synthesis.

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[11] This structure was confirmed by ^{1}H NMR spectroscopic analysis and HRMS analysis. The signals for the anomeric protons of the two galactose residues were shifted upfield by 0.12 ppm and the two axial proton signals of the deoxygenated carbon atom (C-3) of the two sialic acid residues were shifted downfield by 0.13 ppm relative to those of **3**. No other proton signals were shifted relative to **3**.

[12] For the benzyl esterification of sialic acid in the synthesis of a sialyldisaccharylpeptide, see: S. Keil, C. Claus, W. Dippold, H. Kunz, *Angew. Chem.* **2001**, *113*, 379 – 382; *Angew. Chem. Int. Ed.* **2001**, *40*, 366 – 369.

[13] ^{1}H NMR spectroscopic data showed that the sialyl linkage of the benzyl diester **4** did not undergo acid-catalyzed hydrolysis in 95 % TFA solution (see the Supporting Information).

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[16] This undesired esterification was observed by HPLC after the release of the glycopeptide from the solid phase. When this sample was treated with aqueous NaOH (pH 11), many peaks were simplified in the HPLC trace.

[17] As seen in the Supporting Information, the NOESY data show an NOE interaction between Asn-N-H and Val-C_α-H. This interaction indicates that Asn is linked to Val in the sialylglycopeptide **5**. Furthermore, acid treatment (40 mM HCl, 80 °C, 1 h) of the sialylglycopeptide **5** afforded the corresponding asialoglycopeptide quantitatively, which indicates that the carboxylic acid moieties of the sialic acid residues are not linked to the amino acid during the solid-phase synthesis, because of the benzyl ester protecting groups.

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