

# Solution-Phase Synthesis with Solid-State Workup of an *O*-Glycopeptide with a Cluster of Cancer-Related T Antigens

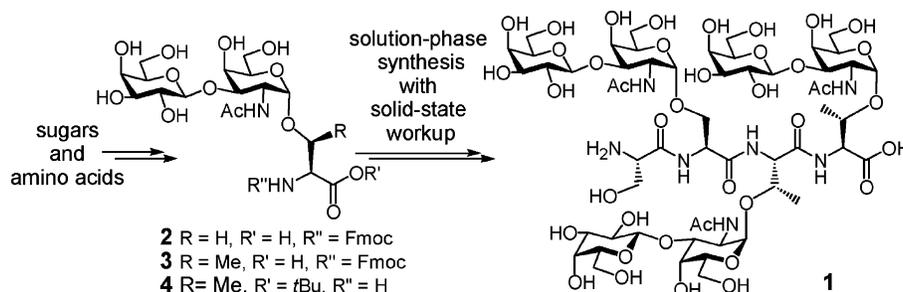
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## ABSTRACT



An *N*-terminal glycopeptide of asialoglycophorin AM with three *O*-linked T antigens was prepared by “solution-phase synthesis with solid-state workup” using unprotected glycosyl amino acids as building blocks. For the glycopeptide assembly, all reactions were conducted in homogeneous NMP solutions, while the product of each reaction was readily isolated as solid precipitates upon addition of diethyl ether. In the preparation of building blocks, a robust approach was established to selectively  $\alpha$ -glycosylate Ser and Thr derivatives.

The chemical synthesis of complex glycopeptides is a significant challenge in organic chemistry.<sup>1</sup> To confront it, we have recently explored a new synthetic strategy known as “solution-phase synthesis with solid-state workup” using glycosyl amino acids with unprotected glycans as the key building blocks.<sup>2,3</sup> This strategy has several useful features. For example, while all reactions to construct glycopeptides were achieved in homogeneous solutions in a polar solvent, the product of each step could be precipitated and separated easily in the solid state, as glycopeptides with free glycans are insoluble in most organic solvents. The use of unprotected glycosyl amino acids could also eliminate the need to

deprotect carbohydrates at the final stage and overcome the problem of chemical incompatibility of some peptides to carbohydrate deprotection reactions.<sup>4</sup> Additionally, the free carbohydrate moieties are more stable to acids than the corresponding *O*-benzylated forms,<sup>5</sup> which will help acidic deprotection of peptide chains, as well as the preparation of glycopeptides with acid-labile glycans.

Even though this strategy has been utilized successfully to synthesize a number of *N*-glycopeptides of considerable complexity,<sup>2,3,6</sup> its applicability to *O*-glycopeptides has yet to be explored. Nonetheless, we anticipate that, because of the high efficiency and simple intermediate purification of

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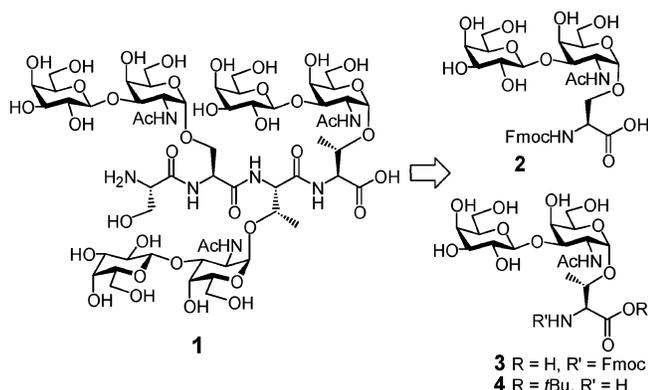
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the new strategy, it should be especially applicable to *O*-linked glycopeptides that often contain glycan clusters. Moreover, homogeneous reaction conditions can avoid the decrease of reaction rate and yield associated with solid-phase synthesis for these compounds. To test the hypothesis, the strategy was used to synthesize *N*-terminal glycopeptide **1** of asialoglycophorin AM containing three *O*-linked cancer-related T antigens (Scheme 1).

Scheme 1



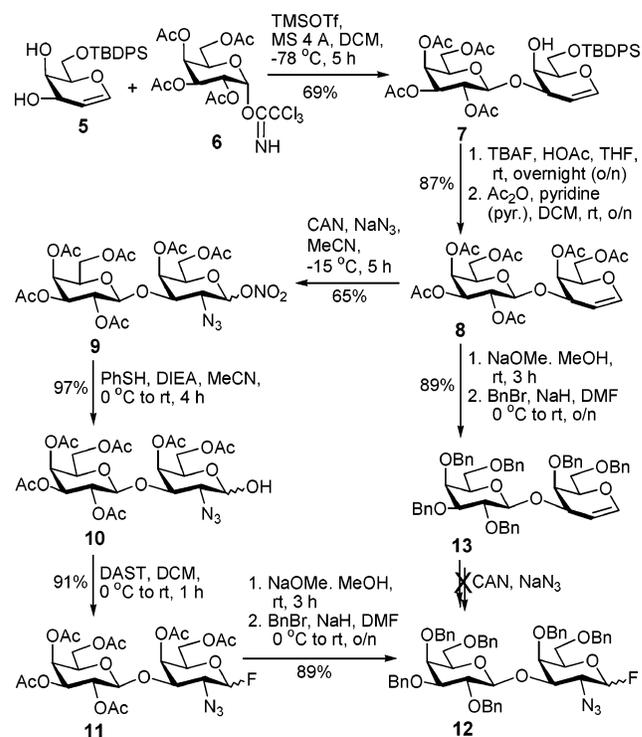
Asialoglycophorin AM is the desialylated structure of glycophorin AM, a major transmembrane sialoglycoprotein of the human erythrocyte.<sup>7</sup> Their *N*-terminal sequence contains numerous Ser and Thr residues that are modified by T and sialylated T antigens, respectively. Because asialoglycophorin AM is relevant to oncological changes,<sup>8–10</sup> its structure has gained significant interest as a research target for cancer therapy. For example, short glycopeptide segments of asialoglycophorin AM have been coupled with proteins, and the resultant conjugates were investigated as potential cancer vaccines.<sup>11–14</sup> Indeed, chemical synthesis of glycophorin A or asialoglycophorin A glycopeptides by itself is an important subject.<sup>15,16</sup>

As shown in Scheme 1, the synthesis of glycopeptide **1** required three key building blocks, i.e., the glycosylated Ser

and Thr derivatives **2**, **3**, and **4** that contain a free  $\alpha$ -linked T antigen. The preparation of  $\alpha$ -linked glycosyl amino acids is one of the critical issues in *O*-glycopeptide synthesis. Many studies have been directed to solve this problem,<sup>17–20</sup> but there is still no general method to realize the reaction with predictable stereochemistry, especially when oligosaccharide donors are used. Consequently, our first task was to develop an approach for the preparation of free glycosyl amino acids **2**, **3**, and **4**.

To obtain the desired  $\alpha$ -linkage during the glycosylation of Ser and Thr, the amino group of galactosamine had to be protected by a nonparticipating group. For this purpose, an azido group was used as a latent amino group.<sup>17</sup> A partially protected derivative **5** of D-galactal was used as the starting material to prepare the disaccharide donor **12** (Scheme 2),

Scheme 2



as the transformation of glycols to corresponding 2-azido sugars is well documented.<sup>13,21</sup>

The equatorial 3-OH of **5** was more reactive than 4-OH, so it was possible to regioselectively glycosylate **5** at  $-78$   $^\circ\text{C}$  with an  $\alpha$ -trichloroacetimidate (**6**) as the glycosyl donor<sup>22</sup> and a catalytic amount of trimethylsilyl triflate (TMSOTf,

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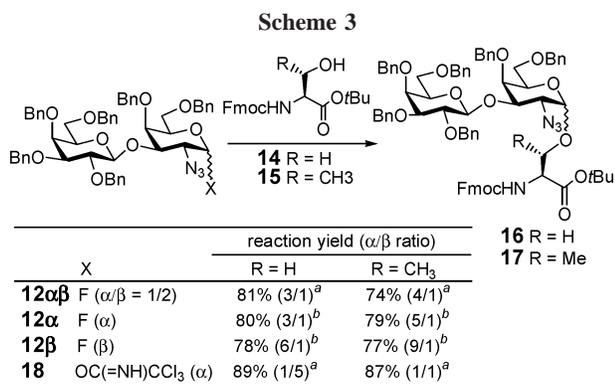
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0.05 equiv) as the promoter. The desired disaccharide **7** was obtained in 69% yield with an ortho ester (10%) being identified as the major side product. No *O*-4 glycosylation was observed. However, it is worth noting that the use of 0.1 equiv of TMSOTf under the same condition resulted in degradation of galactal, probably as a result of the relatively poor stability of the enol ether under stronger acidic conditions. After the *tert*-butyldiphenylsilyl (TBDPS) group was detached, the diol was acetylated to afford **8**. Azidonitration of **8** was realized by the reported process<sup>21</sup> to form nitrate **9** (65%). Then, the nitrate was selectively removed, and the resultant hemiacetal **10** was transformed smoothly into the glycosyl fluoride **11** using diethylaminosulfur trifluoride (DAST). Thereafter, all hydroxyl groups were benzylated following deacetylation to produce the disaccharide **12** that was ready as a glycosyl donor for being coupled with amino acids. Azidonitration of the perbenzylated disaccharide **13** was also investigated, but we found that the reaction was very complex and no desired product could be isolated. It well indicates the severe influence of protecting groups on the reactivity of substrates. Overall, the reactions involved in the synthesis of **12** (Scheme 2) afforded good to excellent yields, with glycosylation and azidonitration being the key transformations.

Next, we carefully studied the glycosylation of Ser and Thr by **12**. The reactions were carried out at  $-78$  to  $0$  °C in dichloromethane (DCM) with  $\text{Cp}_2\text{ZrCl}_2/\text{AgClO}_4$  as the promoter.<sup>23,24</sup> As shown in Scheme 3, these reactions gave



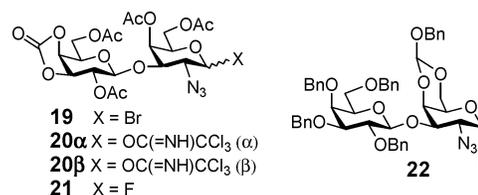
<sup>a</sup> Ratio of separated products. <sup>b</sup> Ratio determined by NMR.

the desired  $\alpha$ -glycosyl amino acids (**16 $\alpha$** , **17 $\alpha$** ) as the major products with good stereoselectivity ( $\alpha/\beta$  3/1 to 9/1). The  $\alpha$ - and  $\beta$ -glycosylation products were readily separable by silica gel column chromatography. Since the glycosylation of **14** and **15** by **12 $\alpha$** , **12 $\beta$**  or their mixture offered similar results, these reactions must have involved the same intermediate, and the results suggest an  $\text{S}_{\text{N}}1$  or solvent-separated

ionic reaction mechanism, while the preferential formation of  $\alpha$ -anomers may result from anomeric effect.

Intriguingly, when  $\alpha$ -trichloroimidate **18** was used as the donor, the glycosylation of **14** gave **17 $\beta$**  predominantly, whereas the reaction of **15** showed no stereoselectivity. These reactions must have gone through a different mechanism from that of **12**.

It was even more interesting to compare our results with those of the literature.<sup>13,25</sup> Danishefsky and co-workers<sup>13</sup> reported that when peracetylated disaccharides **19–21** were used as glycosyl donors, the glycosylation of Ser and Thr derivatives under a variety of conditions did not offer meaningful stereoselectivity, although the chemical yields were generally good. Some reactions were even in favor of the  $\beta$ -anomer. It is particularly worth noting that under conditions similar to ours the reaction of glycosyl fluoride **21** did not show preference for  $\alpha$ -anomeric products either. On the other hand, Ogawa and co-workers<sup>24</sup> did find that the glycosylation of Ser by a benzylated disaccharide **22** under these conditions gave mainly  $\alpha$ -anomers ( $\alpha/\beta$ : 3/1).



These findings clearly indicated that in addition to the leaving groups at the anomeric center of the donor, the protecting groups also had a decisive influence on the stereochemistry of a glycosylation reaction. We initially suspected that the cyclic acetal in **22** might play a significant role in fostering its stereoselective reactions. However, after comparing our results with those of literature, we think that the electronic effects may be a major factor affecting the stereochemical outcome, instead of the molecular geometry. It seems that electron-withdrawing protecting groups favor the formation of the  $\beta$ -anomer and electron-donating groups favor the  $\alpha$ -anomer.

Once the azido group in the carbohydrate chain fulfilled its missions, it was converted to an acetamino group via a one-pot reduction-acylation protocol employing thioacetic acid<sup>26–28</sup> (Scheme 4). Catalytic debenzoylation of the glycans of **23** and **24** proceeded well, but a fraction of the product had its Fmoc group removed as shown by TLC, which may be caused by the catalyst, i.e.,  $\text{Pd}(\text{OH})_2$ . Nevertheless, upon addition of  $\text{H}_2\text{O}$  to the reaction mixture, the reactions gave **25** and **4** as the only products (99%). Compound **4** was one of the desired building blocks. To obtain **2** and **3**, **25** and a part of **4** were subjected to *N*-acylation and acid-catalyzed C-terminal debutylolation. To our surprise, the reaction of **25**

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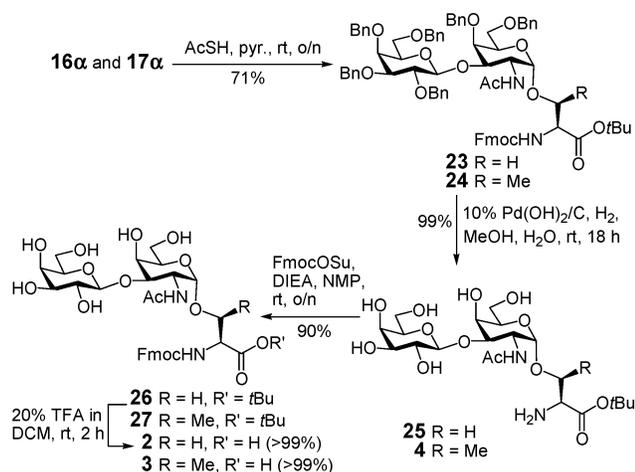
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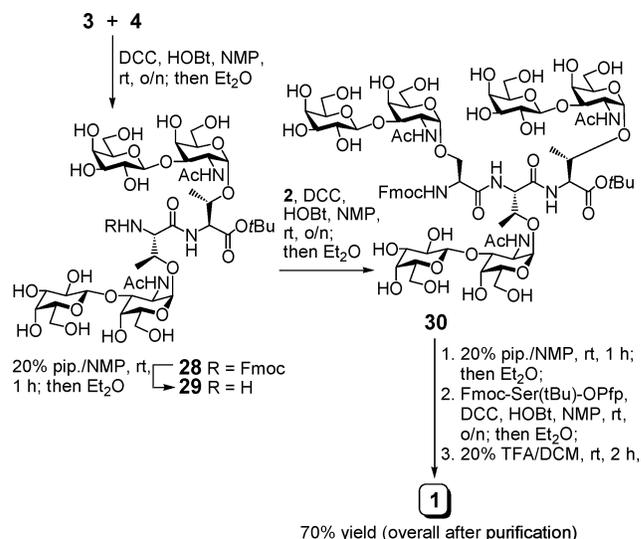
Scheme 4



and **4** with Fmoc-OSu formed two types of products: the acylation products **26** and **27** and the desired free acids **2** and **3** resulting from removal of the *tert*-butyl group. As the reactions were performed under almost neutral conditions and *tert*-butyl esters proved to be stable to the byproduct *N*-hydroxysuccinimide (HOSu) in the synthesis of *N*-linked glycopeptides,<sup>2,3</sup> it was not clear how this debutylation occurred. However, this reaction did not bother us, since both **26** and **27** were eventually converted into free acids **2** and **3** following treatment with 20% trifluoroacetic acid (TFA) in DCM. Overall, **2** and **3** were obtained from **25** and **4** in an excellent yield (90%).

The assemblies of amino acids and glycosyl amino acids to build the target glycopeptide **1** followed the principles of solution-phase synthesis with solid-state workup with **2**, **3** and **4** as the key building blocks (Scheme 5). *N*-Methylpyrrolidinone (NMP) was the principal solvent to perform homogeneous reactions, and diethyl ether was the precipitation solvent to isolate products. For the coupling reaction between **3** and **4**, because both substrates contain a free carbohydrate chain, neither should be used in excess. Otherwise, the excess substrate would be coprecipitated with the product. Thus, after **3** was dissolved in NMP and treated with *N,N'*-dicyclohexylcarbodiimide (DCC) and *N*-hydroxybenzotriazole (HOBT) to form the activated ester in situ, 1 equiv of **4** was introduced. The solution was stirred at room temperature overnight, and TLC showed that it gave only one product. Diethyl ether was then added to the reaction mixture, and the precipitates were isolated through filtration and further washed with ether to afford **28** in 90% yield. The <sup>1</sup>H NMR spectra of the crude product suggested that it was quite homogeneous. It was thereby dissolved in 20% piperidine in NMP to remove the Fmoc group. Again the product was isolated by precipitation and washed with diethyl

Scheme 5



ether. Similar protocols were used for the coupling of **2** and **29** to obtain **30** and for removing the Fmoc group from **30**, as well as for the introduction of a Ser residue to the *N*-terminus of the resultant glycopeptide using its commercial pentafluorophenyl (Pfp) ester. Finally, after purification by size exclusion chromatography, the target glycopeptide **1** was obtained in a 70% overall yield based on **4**.

In brief, a glycopeptide segment (**1**) of asialoglycophorin AM containing three *O*-linked T antigens was synthesized from **2**, **3**, and **4** via solution-phase synthesis with solid-state workup. In the preparation of key building blocks **2**, **3**, and **4**, a robust method was developed for  $\alpha$ -selective glycosylation of amino acids. It was observed that both the leaving groups and protecting groups of glycosyl donors could affect the stereochemical outcome of glycosylation reactions, with electron-donating protecting groups in favor of producing the  $\alpha$ -anomer. The new strategy proved to be convenient and efficient for the synthesis of *O*-linked glycopeptides with glycan clusters. The synthetic target **1** is ready for being linked to various carrier molecules through its *N*- or *C*-terminus to form multivalent glycoconjugates of biological importance, e.g., as cancer vaccines.

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**Supporting Information Available:** Experimental data and selected NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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