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Hydroxynorleucine as a glycosyl acceptor is an efficient means for introducing amino acid functionality into complex carbohydrates

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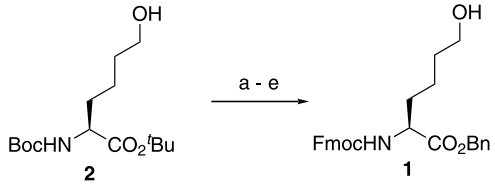
Abstract—A new approach to the synthesis of biologically relevant glycosyl amino acids using a non-natural amino acid as the glycosyl acceptor is described. The procedure involves a glycosylation reaction of a suitable carbohydrate donor with Fmoc-L-hydroxynorleucine benzyl ester. This reaction results in the direct incorporation of the amino acid moiety. The acceptor can be used for the preparation of α- or β-*O*-linked glycosides depending upon the nature of the glycosyl donor. This method has been applied in the synthesis of six different tumor-associated carbohydrate antigens.

It is known that certain types of glycoconjugates found in normal tissue types are often significantly overexpressed in tumors of that tissue.[1] Given effective immunosurveillance, a host would generate an immune response directed to these tumor-associated antigens. Unfortunately, initial antibody response to tumor antigens is apparently inefficient in eradicating the disease, thus tumor progression ensues. The idea of using synthetically derived and cell-free glycoconjugates of tumor-associated antigens in the development of antitumor vaccines to generate an increased immune response is attractive. One of the main drawbacks in exploring the viability of this approach is the limited availability of purified tumor-associated carbohydrate antigens. Therefore, total chemical synthesis has become increasingly important for providing probe structures to be evaluated in such a program. Our involvement in numerous synthetic and subsequent preclinical and clinical studies with many different tumor-associated carbohydrate antigens has intensified our quest for optimizing the process of vaccine design and synthesis.[2]



Scheme 1. Synthesis of Fmoc-L-hydroxynorleucine benzyl ester. *Reagents*: (a) 2,2,2-trichloroethyl chloroformate, pyr., CH_2Cl_2; (b) 4N HCl/dioxane; (c) Fmoc-OSu, $NaHCO_3$, acetone/H_2O; (d) BnBr, $NaHCO_3$, DMF; (e) Zn, AcOH/THF.

Based on our earlier investigations, we have come to prefer the display of tumor antigens on a peptide backbone. Efforts in our laboratory for introducing amino acid functionality to tumor-associated carbohydrate antigens have included glycosylations with serine or threonine to form the core-mucin structure,[3] transformation of *O*-pentenyl glycosides via an ozonolysis–Wittig–asymmetric hydrogenation protocol[4] and cross-metathesis of *O*-allyl glycosides with allylglycine.[5] The first of these methods introduces amino acids of natural origin while the latter two result in non-natural amino acid side chains.

Our ongoing efforts in the total synthesis of carbohydrate-based antitumor vaccines have led us to favor the non-natural amino acids as components of our peptide-linked vaccines.[6] The use of vaccines incorporating non-natural amino acids as linkers to carbohydrate

Keywords: hydroxynorleucine; glycosylation; glycosylamino acid; carbohydrate-based antitumor vaccines.

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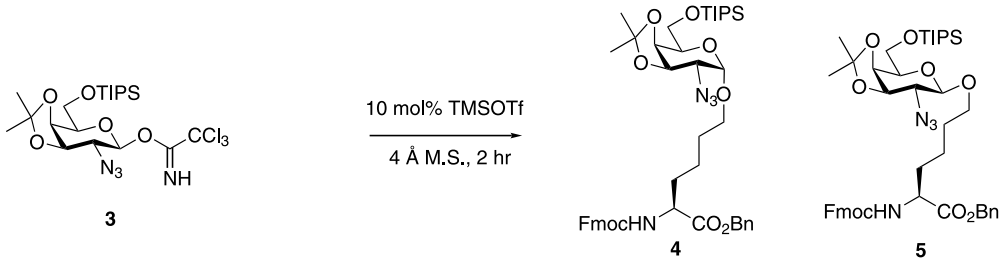
Table 1. Comparison of glycosylations of donor **4** with three different acceptors



Entry	Acceptor	Temperature (°C)	Solvent	Yield (%)	α:β[a]
1	4-Penten-1-ol	−10	THF	90	1:1
2	**1**	−10	THF	39	2:1
3	**1**	−40	THF	47	2.5:1
4	**1**	−78	THF	88	4:1
5	**1**	−78	CH_2Cl_2	80	1:9
6	Fmoc-Ser-OBn	−78	THF	86	1:0

[a] α:β Ratios were determined by ^{1}H NMR of the crude material.

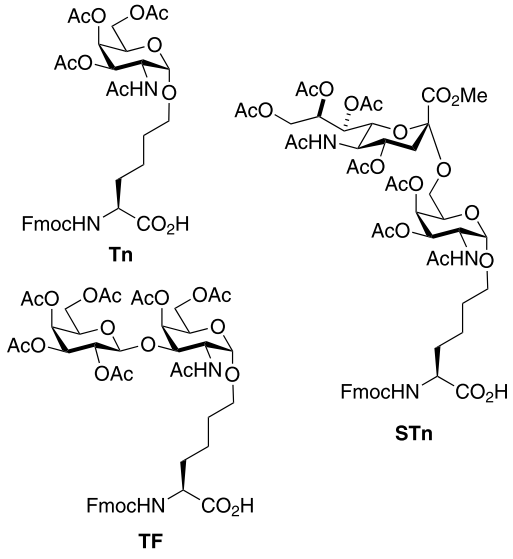
domains is under continuing investigation. Thus far, at least, there is no indication that constructs featuring non-natural linkages are disadvantageous with respect to immunogenicity. Indeed, in principle such unnatural linkages might result in an enhanced immune response.

Introduction of non-natural linkages utilizes well-established principles of glycosylation. Thus, α-1,2 oxido donors (glycal epoxides) result in primarily β-linked products as seen in our syntheses of Globo-H and Lewis^y (Le^y) pentenyl glycosides.[2] However, in the case of the glycophorins, the α-anomer is desired. The use of a non-participating azido group at C-2 allows for α-selectivity with serine or threonine acceptors.[3a] However, relatively no selectivity was realized with 4-penten-1-ol as the glycosyl acceptor.[4] The mixture of pentenyl anomers was inseparable and although the undesired β-anomer could eventually be removed, it was realized that an alternative and perhaps diastereoselective method for introduction of the desired non-natural amino acid functionality would be useful. Building on the successful results of using serine and threonine for α-*O*-linked glycosylamino acids we chose to investigate the viability of hydroxynorleucine as an acceptor.

Boc-L-hydroxynorleucine *tert*-butyl ester **2** was synthesized from L-lysine according to literature procedures.[7] Protecting group manipulations (Scheme 1) provided the desired suitably protected hydroxynorleucine **1** for use in the glycosylation reaction.

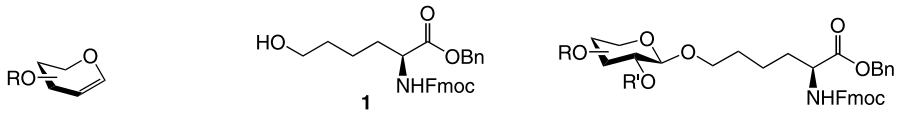
Reaction of trichloroacetimidate donor **3**,[3a] derived from tri-*O*-acetyl-D-galactose, with hydroxy norleucine acceptor **1** (1.5 equiv.) in the presence of TMSOTf in THF provided a 2:1 mixture of anomeric products with the desired α-anomer **4** as the major product (Table 1, entry 2). Decreasing the temperature increased the yield and selectivity (88%, 4:1, entry 4) while changing the solvent to dichloromethane caused a reversal with the β-anomer **5** predominating (1:9, entry 5). Although the anomeric selectivity was not as good as what was seen with serine (1:0, entry 6), the results were significantly better than 4-penten-1-ol (1:1, entry 1). Additionally, the undesired β-anomer **5** could be removed by silica gel chromatography. This successful diastereoselective glycosylation allowed for the efficient transformation of **4** into three different glycosylamino acids, which contain the tumor-associated carbohydrate antigens Tn, TF and STn (Scheme 2).

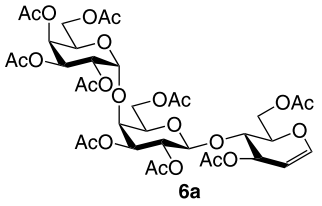
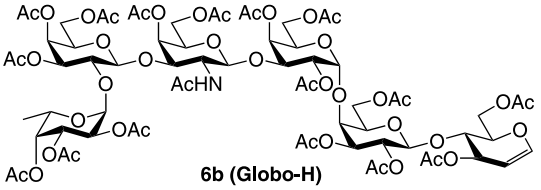
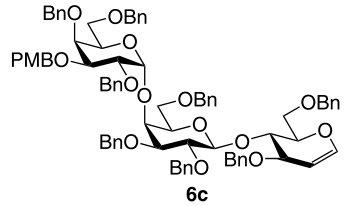
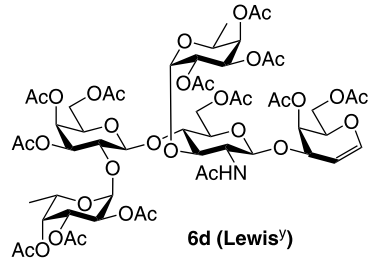
We then explored the utility of a suitably protected hydroxynorleucine as an acceptor in the glycosylations of more complex carbohydrates. Four different carbohydrate donors were chosen: Gb3 glycal **6a**, Globo-H hexasaccharide glycal **6b**, ABC trisaccharide glycal fragment of Globo-H **6c**, and Le^y pentasaccharide glycal **6d** (Table 2). The amino acid functionality was



Scheme 2. Glycosylamino acid **4** was used in the synthesis Tn, TF and STn glycosylamino acids.

Table 2. β-selective glycosylations of complex glycals with acceptor **1**



Entry	Carbohydrate Glycal	Procedure[a]	Overall yield (%)
1.	 **6a**	I	26
2.	 **6b (Globo-H)**	I II	20 49
3.	 **6c**	II	70
4.	 **6d (Lewis^y)**	II	65

[a] I. a) DMDO, CH_2Cl_2; b) EtSH, TFAA, CH_2Cl_2; c) BzCl, TEA, CH_2Cl_2; d) **1**, NIS, TfOH, CH_2Cl_2. R' = Bz.
II. a) DMDO, CH_2Cl_2; b) **1**, $ZnCl_2$, THF, 4 Å M.S. R' = H.
[b] No α-linked products were isolated, however, some side products due to β-epoxide formation were observed (see ref. 8) as well as ortho-ester formation in Method 1 (< 10%).

introduced in two different ways. The first method involved four steps in which the glycal was oxidized using dimethyldioxirane to the glycal epoxide[8] and subsequently opened with ethanethiol to provide the ethyl sulfide. The C-2 hydroxyl was protected as a benzoate ester, which acted as a participating group during glycosylations with hydroxynorleucine acceptor **1**. This reaction sequence is very reliable and generates the desired β-linkage in good yield for Gb3 glycal (26%, entry 1) and Globo-H (20%, entry 2). Surprisingly, we also found the glycosyl acceptor could be introduced selectively in a simpler, two-step procedure. In this case the glycosyl acceptor was used to open the glycal epoxide directly. The yields are better for the two-step compared with the four-step procedure with generation of the necessary β-linkage. In both methods an excess of the amino acid acceptor was used, typically 2–10 equiv. Using the shorter synthetic sequence we were able to make the differentially protected ABC unit of Globo-H (70%, entry 3) and Le^y glycosylamino acids (65%, entry 4) in high yield of the β-linked anomer.

In conclusion, we describe the use of a new glycosyl acceptor, hydroxynorleucine, for the incorporation of amino acid functionality in the context of complex carbohydrates. This procedure directly affords the *N*-

and *C*-protected glycosylamino acid for further functionalization of the carbohydrate domain or for deprotection and use in the preparation of glycopeptides. We have synthesized hydroxynorleucine acceptor **1** in large quantities and successfully generated sufficient amounts of many glycosylamino acids for incorporation into vaccine constructs for immunological investigation.

Acknowledgements

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8. The epoxidation of the peracetylated glycals resulted not only in the desired α-epoxides, but also β-epoxides (5–29%) that gave β-mannoside products upon epoxide opening. Upon changing the hydroxyl protecting groups from acetates to benzyl ethers, the DMDO epoxidation resulted in exclusively α-epoxide formation.