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Stereoselective Synthesis of Natural and Non-natural Thomsen-nouveau Antigens and Hydrazide Derivatives

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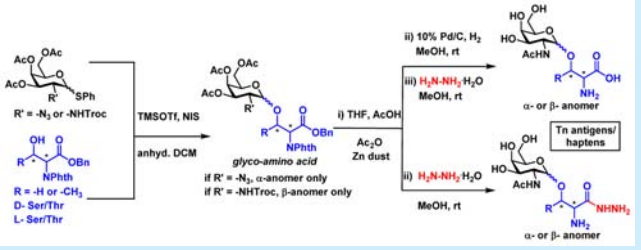
Supporting Information

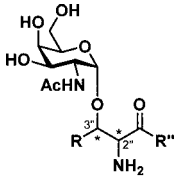
ABSTRACT: A selective glycosylation strategy enabling access to all stereochemical combinations of tumor associated Thomsen-nouveau (Tn) antigen, D-GalNAc-*O*-Ser/Thr, has been developed. The key component for selectivity is the phthalimide-protected D- or L-amino acid acceptors which allow access to α- or β-anomers in excellent yields (72−96%) and selectivity (~100%) when appropriate C-2 substitution is installed. The glycoamino acid intermediates were divergently converted to Tn-based carboxylates or to hydrazides by tandem Pd−C debenzylation followed by treatment with hydrazine hydrate or hydrazine hydrate treatment alone.



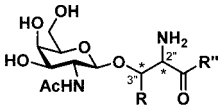
Defects in cellular glycosylation machinery of malignant cells give rise to aberrant changes on cell surface carbohydrates leading to overexpression of tumor-associated carbohydrate antigens (TACAs).[1] These TACAs are found on epithelial cells containing the commencing amino acid linked glycoside α-D-GalNAc-*O*-Ser/Thr, otherwise known as the Thomsen-nouveau (Tn) antigen.[2] The Tn antigen has been identified in high density on breast carcinomas and evidenced on ~90% of tumors including colon, prostate, lung, ovary, bladder, and stomach.[3] TACAs have been conjugated to immunogenic proteins,[4] viruses,[5] nanoparticles[6] and recently to zwitterionic polysaccharides[7] for vaccine development. For the most part, vaccine strategies have overlooked the importance of linkers. Immune responses toward linkers can suppress the immunogenicity of the target antigen.[8] In order to overcome some of the current linker shortcomings in directing specific immune responses toward a desired antigen, we have elected to modify the Tn antigens with a hydrazide functionality that can be directly conjugated to an appropriate partner (Table 1). Hydrazides have been used in conjugation strategies including antibody−drug conjugates,[9] vaccine constructs,[10] and drug delivery systems.[11] In pursuing hydrazides as a functional group linker, we aim to balance the rationalized chemical modifications while retaining activity of the construct.[12] Taking these aspects into consideration, as well as noted conjugation/linker limitations, we have developed an approach to hydrazides that can be utilized for direct coupling to aldehyde-containing molecules.

Further problems worth addressing are the relative immunological contributions of the sugar portion versus amino acid portion of the Tn antigen and whether stereochemistry of the amino acid or the α-linkage plays a role in immune recognition. To begin to answer these questions, the syntheses of the complete set of D-GalNAc-*O*-Ser/Thr (Tn) stereoisomers was undertaken by routes that control the α/β selectivities as well as avoid unwanted amino acid epimerization. Published procedures note utilizing nonparticipating azido groups at the C-2 of D-galactose (e.g., D-GalN$_3$) allowing for high α-selectivity.[13] Other studies have examined structural aspects of sugar donors including the nature of protecting groups,[14] leaving groups,[15] and reaction conditions.[16] Electronics of glycosyl acceptors are also a major contributing factor dictating stereoselectivity.[17] Although a number of amino acid protecting groups have been explored in peptide synthesis,[18] glycopeptide conjugation has largely been limited to Fmoc and Cbz,[19] yet even after extensive optimization of reaction conditions, the glycosylation of D-GalN$_3$ can often yield a mixture of α/β anomers.[20] We chose to employ

Table 1. Target Tn Antigens (1a and 1c) Including Non-natural Moieties 1b,d and 2−4



1a-d, if R" = -OH
3a-d, if R" = -NHNH$_2$



2a-d, if R" = -OH
4a-d, if R" = -NHNH$_2$

R	stereoconfiguration	amino acid	compd[a]
Me	2″*S*,3″*R*	L-threonine	*X*a
Me	2″*R*,3″*S*	D-threonine	*X*b
H	2″*S*	L-serine	*X*c
H	2″*R*	D-serine	*X*d

[a]Where *X* = 1, 2, 3, or 4.

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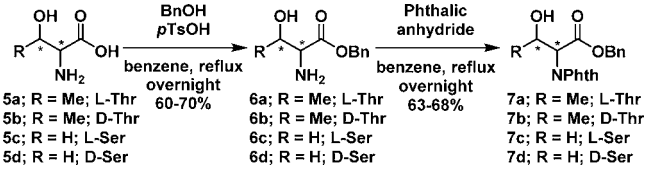
a readily removable and bulky phthalimide group on the amino acid acceptor in search of achieving the desired α-stereoselectivity while maintaining inherent stability when using varying reagents and reaction conditions.[21]

We began with the synthesis of *N*-phthaloylamino acid benzyl ester glycosyl acceptors **7a−d**. This was achieved by starting from commercially available amino acids **5a−d** using a two-step synthetic protocol (Scheme 1).[22] In short, amino acids were

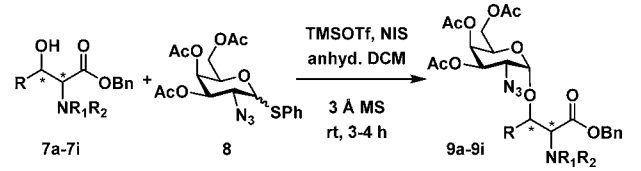
Scheme 1. Synthesis of *N*-Phthaloylamino Acid Benzyl Esters

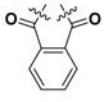


condensed with benzyl alcohol using Fischer esterification conditions followed by *N*-protection with phthalic anhydride affording **7a−d** in moderate to good yields. To the best of our knowledge, there are no reports describing *N*-phthaloylamino acid benzyl esters employed as glycosyl acceptors for synthesizing the Tn antigen.

Glycosyl donor 2-azido-2-deoxy-1-(phenylthio)peracetyl-D-galactopyranose (**8**) was synthesized following literature procedures.[23] The glycosylation using donor **8** (nonparticipating C-2 azido) and protected amino acid acceptors **7a−d** was accomplished using *N*-iodosuccinimide (NIS) and trimethylsilyl triflate (TMSOTf) in anhydrous dichloromethane (DCM) at room temperature in the presence of 3 Å molecular sieves (Table 2). These conditions, using acceptors **7a−d** and donor **8**, resulted in complete α-selectivity and excellent overall yields. To ensure reproducibility, multiple glycosylation reactions employing *N*-phthaloyl-D-threonine benzyl ester (**7b**) were conducted, including those run in the absence of molecular sieves under Schlenk conditions. It was noted that molecular sieves did not affect the stereoselectivity and that the exact outcome, as noted

Table 2. General Tethering Strategy for Peracetyl D-GalN$_3$ Thioacetal Donor with *N*-Protected Amino Acid Benzyl Ester Acceptors



cmpd	R	R$_1$	R$_2$	α:β[a]	J(α-H$_1$) (Hz)	δ(α-H$_1$)	J(β-H$_1$) (Hz)	δ(β-H$_1$)	yield (%)[c]
9a-b	Me			99:1	**9a**: 3.90	4.93	NA[b]	NA[b]	96
					9b: 3.42	5.11			90
9c-d	H			99:1	**9c**: 3.75	4.94	NA[b]	NA[b]	80
					9d: 3.60	5.08			72
9e	Me	H	Fmoc	73:27	3.64	4.93	7.97	4.32	50
9f	Me	H	Cbz	66:33	3.60	5.10	8.11	4.04	45
9g	H	H	Fmoc	70:30	3.42	4.87	7.92	4.34	45
9h	H	H	Cbz	75:25	3.28	4.91	8.00	4.31	44
9i	Me	Bn	Bn	99:1	3.63	5.05	NA[b]	NA[b]	20

[a]Based on ^{1}H NMR of the nonpurified reaction mixture. [b]NA: not observed. [c]Isolated yield for α-anomer only.

above, was obtained in all cases (presence or absence of 3 Å molecular sieves).

A small study was then conducted to further evaluate the *N*-protecting group of the acceptor keeping the benzyl ester constant. In this context, we screened benzyl esters of *N*-Fmoc- and *N*-Cbz-protected amino acid acceptors **7e−h** that are routinely used in glycopeptide synthesis. When we conducted our glycosylation reactions, we noted that the couplings with Fmoc- and Cbz-protected amino acids gave moderate anomeric α-selectivities (Table 2). To justify the lack of selectivity in the aforementioned cases whether it be as a result of initial nucleophilic attack or for in situ anomerization, we subjected the purified α-anomer of compounds **9e−h** to the conditions delineated in Table 2 and found no change/no reaction had occurred. In contrast, the *N*-phthaloyl-protected threonine or serine acceptors gave essentially all α-product.

For the α-selective glycosylation reactions, we suggest that the reaction proceeds through an S_N1 pathway involving a half-chair intermediate when a nonparticipating C-2 substituent is present. Our rational is based on evidence that glycosyl triflates, which are noted in contact-ion pair oxocarbenium intermediates of glycosylation, decompose at higher temperatures,[24] and therefore, we exclude the S_N2 pathway from our rational. With two possible half-chair conformations, as shown in Figure 1, conformer $^4H_3$ has been reported to be more stable than $^3H_4$ (Figure 1).[25]

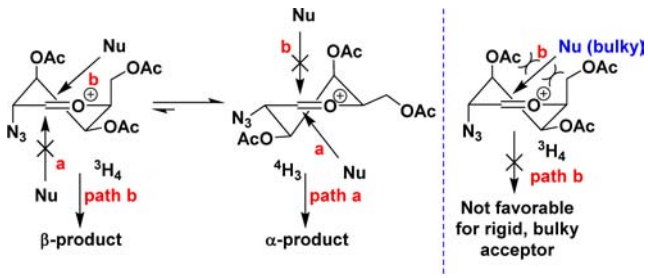


Figure 1. Facial selectivity for nucleophilic attack.

A nucleophile will approach the half-chair oxocarbenium ion conformer following a pseudoaxial trajection that allows for the formation of a more stable chair-oriented product rather than the less stable twist boat conformer.[26] Therefore, attack through b on conformer $^3H_4$ is more favorable and will give the β-product, whereas attack through a on $^4H_3$ will result in the α-product. In the case of the $^3H_4$ conformer, the nucleophilic attack of an acceptor containing a rigid bulky group will be hindered due to 1,3-diaxial interactions.[26] The more rigid/bulky phthaloyl-protected acceptor will preferentially attack the $^4H_3$ conformer resulting in an exclusive α-product. The absence of rigid/bulky groups in the acceptors (Fmoc and Cbz protected) results in a mixture of anomers. To further examine the effect of bulky substituents, we installed dibenzyl groups on the amine **7i**[27] and conducted glycosylation. We noted exclusive α-selectivity of compound **9i** albeit with a moderate overall yield (Table 2).

In the cases of Fmoc and Cbz amino acid protection (Table 2; **7e−h**), there remains a lone hydrogen atom attached to the nitrogen which is prone to intramolecular hydrogen bonding with the oxygen atom of threonine or serine leading to the formation of a five-membered ring (Figure 2). This internal hydrogen bonding will decrease the nucleophilicity[28] of the hydroxyl group and affect the overall shape of the glycosyl

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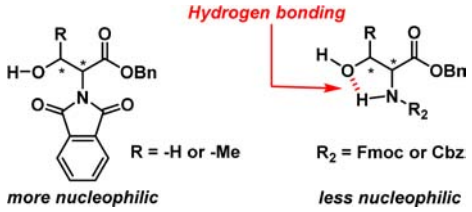
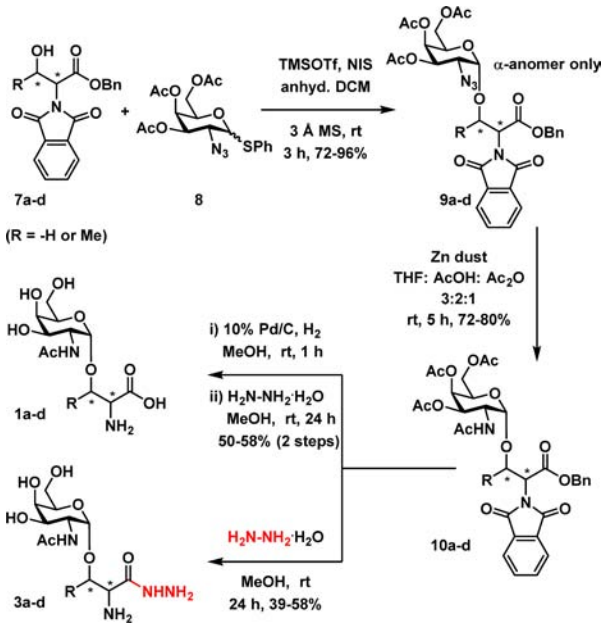


Figure 2. Hydrogen bonding leads to a decrease in hydroxyl nucleophilicity and a proposed decrease in α-/β-selectivity for glyco-amino acid coupling.

acceptor factors, which could influence the stereochemical outcome of the glycosylation.

Selective azide reduction of compounds **9a−d** followed by *N*-acetylation (Scheme 2) was carried out in a one-pot protocol

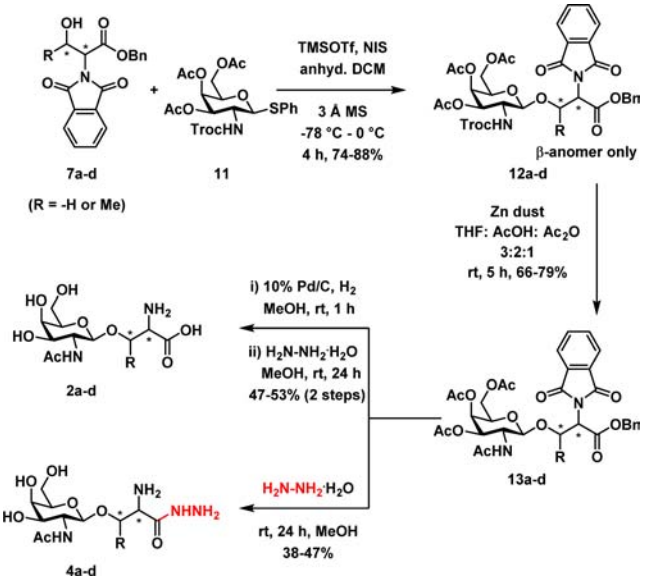
Scheme 2. Synthesis of Tn Antigen α-Anomers and Corresponding Hydrazides



using Zn dust−AcOH/Ac_2O in THF at room temperature affording suitably protected Tn antigens in moderate to good yields. Natural Tn antigens **1a** and **1c** and unnatural Tn antigens **1b** and **1d** were obtained from compounds **10a−d** employing a two-step procedure. The first step involved hydrogenolysis of the benzyl ester using 10% Pd−C to reveal the carboxylic acid followed by deacetylation and *N*-phthaloyl deprotection using hydrazine hydrate. The debenzylation followed by global deprotection gave a relatively clean conversion; ^{1}H NMR of the unpurified reaction mixture did not show any starting material. The syntheses of hydrazides **3a−d** were carried out in a single step from the benzyl ester of compounds **10a−d** using 20-fold excess of hydrazine hydrate in methanol at room temperature for 36 h. The ^{1}H NMR of the unpurified reaction mixture also showed a quantitative conversion of the desired hydrazides plus the known byproducts phthalhydrazide and acetyl hydrazide.

In order to selectively synthesize the β-anomers of D-GalNAc-*O*-Ser/Thr, we opted for a neighboring group participation strategy. Although there are numerous well-known sugar protecting groups that lead to anchimeric assistance such as acetyl, 2-pyridylmethyl, dialkyl phosphates, 4-acetoxy-2.2-dimethylbutanoyl, pivaloyl, and phthaloyl, we chose 2,2,2-trichloroethoxycarbonyl (Troc) mainly due to its favorable stability under our chosen reaction conditions, ease of deprotection, and synthetic continuity; a similar reaction sequence is shown in Scheme 2. *N*-Troc-protected thioglycoside donor **11** was synthesized from readily available D-galactosamine in a three-step sequence and purified in a fashion similar to that from a previously reported literature procedure.[29] Glycosylations of thioglycoside donor **11** with protected amino acid acceptors **7a−d** were carried out using *N*-iodosuccinimide (NIS) and trimethylsilyl triflate (TMSOTf) at −78 to 0 °C giving exclusively β-anomeric glycosylated products **12a−d** in high yields (Scheme 3). Troc deprotection followed by *N*-acetylation

Scheme 3. Synthesis of Tn Antigen β-Anomers and Corresponding Hydrazides



and then conversion to unprotected Tn antigens and corresponding hydrazides were accomplished utilizing the same reaction conditions as mentioned in Scheme 2.

In summary, we have developed an effective and highly efficient stereoselective strategy for the synthesis of Tn antigens and the complete complement of stereoisomers. Utilization of thermodynamic reaction conditions with nonparticipating C-2 substitution in the donor moiety as well as the rigid and bulky *N*-phthaloyl protecting group on the amino acid acceptor seem to be required features in obtaining high α-selectivity. The use of this efficient, under-explored, and readily removable protecting group can facilitate glycopeptide coupling. Our work represents the first examples by which all the stereoisomers (α-/β-) of the Tn antigen were accessed in high stereoselectivity. We believe this work will have important, general implications for the syntheses of other peptide-related carbohydrate antigens. Apart from developing this stereoselective methodology, we have also commenced chemistry for conjugating peptide-based hydrazides to carbonyl functionality, which will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Characterization data including optical rotations, experimental procedures, and ^{1}H (1D and 2D) and ^{13}C NMR spectra. The

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Notes

The authors declare no competing financial interest.

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