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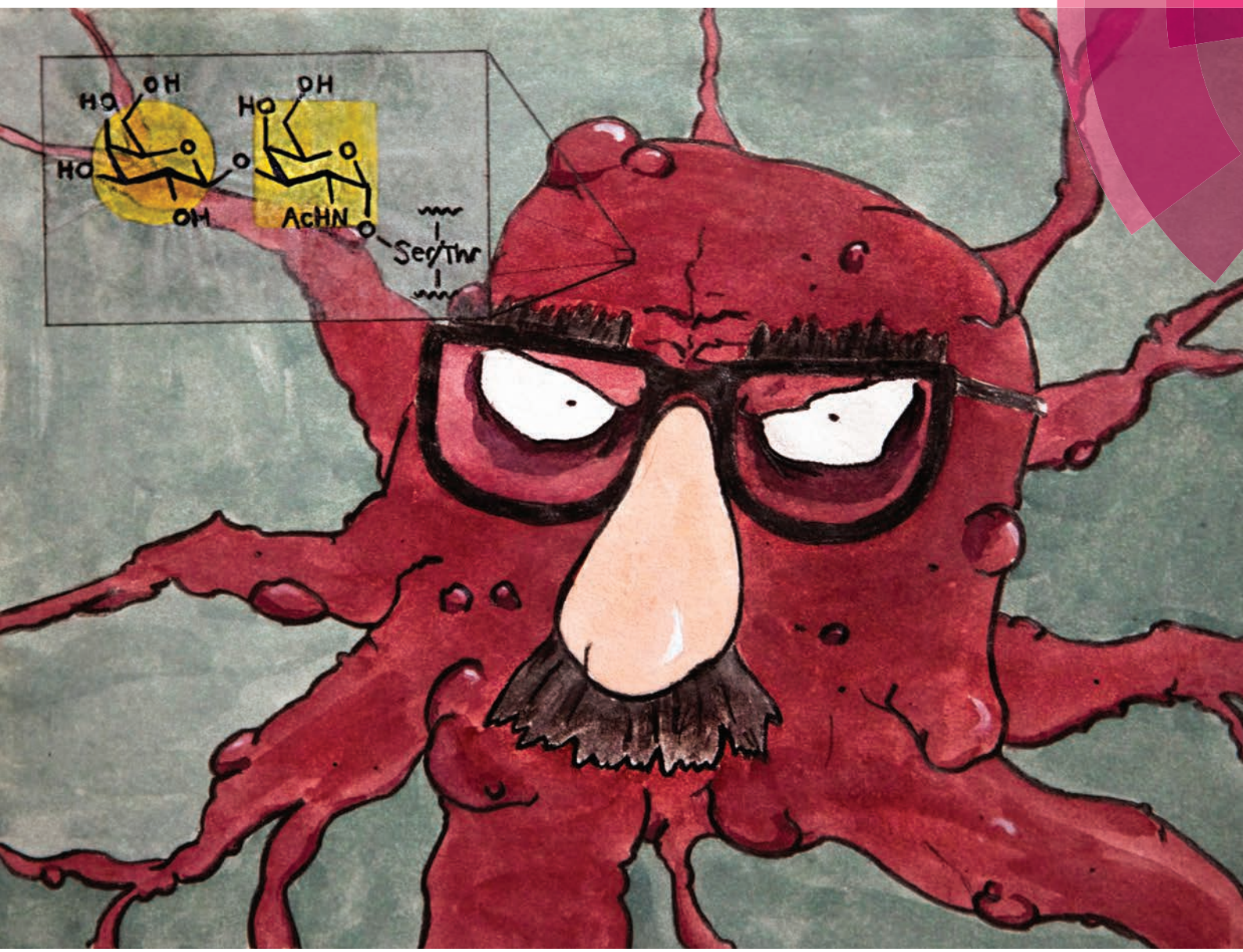

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Synthesis of the tumor associative α-aminooxy disaccharide of the TF antigen and its conjugation to a polysaccharide immune stimulant†

Jean Paul Bourgault, Kevin R. Trabbic, Mengchao Shi and Peter R. Andreana*

The α-aminooxy derivative of the Thomsen–Friedenriech tumor associated carbohydrate antigen has been synthesized in 11 steps utilizing a D-GalN$_3$ acceptor carrying a pre-installed α-*N*-hydroxysuccinimidyl moiety. The natural α linkage was prepared in high selectivity employing a suitably protected D-GalN$_3$-thioglycoside donor with *N*-hydroxysuccinimide. With access to α-TF-ONH$_2$, the preparation of the TF-PS A1 vaccine candidate ensued smoothly through oxime bond formation.

The Thomsen–Friedenriech (TF or T) antigen [β-D-Gal-(1,3)-α-D-GalNAc-Ser/Thr], (**1a**/**1b**) (Fig. 1), is an important cancer antigen/biomarker found in high density on human breast, colon, stomach, bladder, prostate, and liver tumor cells.[1] TF can be found on the large transmembrane mucin protein MUC1, which is overexpressed in epithelial cancers.[2] Modifications of TF include non-reducing end sialylation, *N*-acetylglucosaminylation or fucosylation creating a host of *O*-glycans on healthy tissues.[3] Under normal metabolic conditions with saccharide "caps" the TF is "masked" from immune surveillance, however in various carcinoma cells this *O*-linked tumor disaccharide is revealed. The presence of TF could occur through increased T synthase activity or through decreased subsequent modification.[4] Alternatively increased cellular concentrations of UDP-Gal and the TF precursor, the Thomsen-nouveau antigen (Tn antigen, α-D-GalNAc-Ser/Thr) could also play a role.[1] TF was discovered by the combined efforts of Thomsen and Friedenriech.[5] The initial observation occurred during an attempt to determine the ABO blood type of bacterial contaminated red blood cells. The bacteria neuraminidases exposed the disaccharide and the resulting desialylated TF caused agglutination on addition of all human sera (ABO). Two significant facts were determined from the discovery namely: (1) that antibodies against the TF antigen were already present in normal human sera and (2) humans are intolerant to this antigen and hence application thereof in vaccine design and development could prove promising.[1]

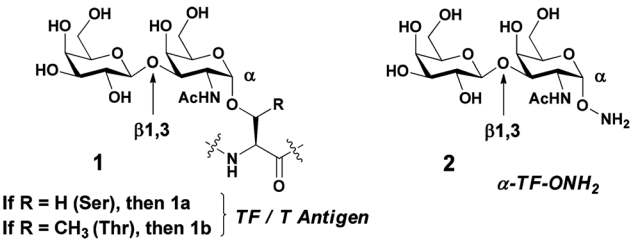

Fig. 1 TF antigen is found α-linked to Ser/Thr on cancer mucins such as MUC1 (**1a**/**1b**). α-TF-ONH$_2$ (**2**).

The TF disaccharide has attracted considerable attention from the scientific community[1,6] due to expression on numerous carcinomas. TF is also believed to play a direct role in adhesion and metastasis through interaction with galectin-3.[2,7] Verification of the tumor relevance for TF has encouraged our group to investigate the preparation of an aminooxy derivative of the TF antigen (**2**) (Fig. 1) for subsequent conjugation to the immunogenic zwitterionic polysaccharide PS A1 (**15**).[8] This capsular polysaccharide is isolated from the commensal anaerobic bacteria *B. fragilis* and has challenged the paradigm concerning carbohydrates only eliciting a T-cell independent immune response. PS A1 is a tetrasaccharide repeating unit, has a MW of approximately 110 kD and consists of a right handed helical structure with two repeating units per turn. The T-cell dependent activity of this polysaccharide has been shown to arise from the zwitterionic nature of the polymer. Previous work has revealed that when this alternating charge character is chemically neutralized the T-cell dependent response is as well.[8d]

An important component of this conjugation strategy, in the same fashion as our earlier report with the conjugation of Tn,[8b] involves the use of anomeric aminooxy sugars first prepared by Roy and then later by Dumy and Bertozzi.[9] To the best of our knowledge the α-aminooxy TF antigen (**2**) has been

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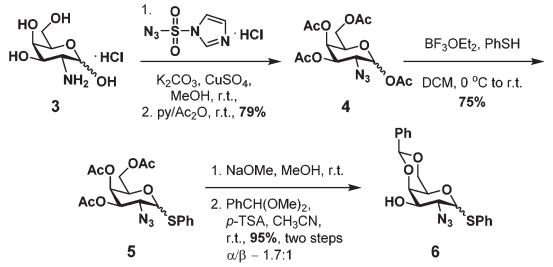
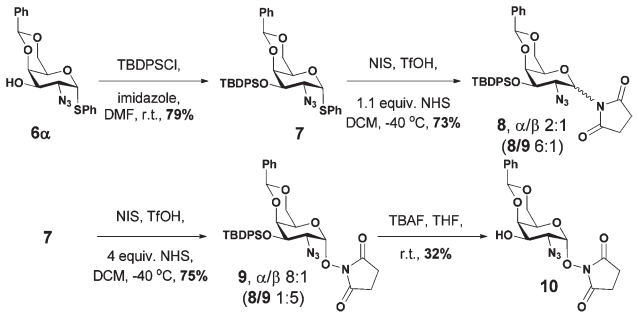
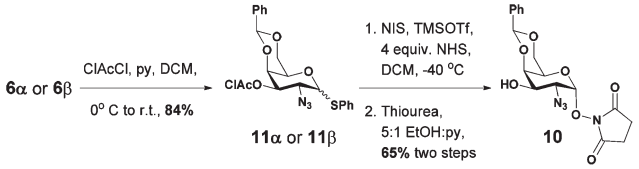


Scheme 1 Synthesis of thioglycoside **6**.



Scheme 2 Initial attempt at preparing NHS acceptor **10**.



Scheme 3 Preparation of α-NHS acceptor **10** from chloroacetyl thioglycoside **11α** or **11β**.

synthesized previously by only one other group.[10] TF linked to Ser/Thr has also been prepared by various methods as a building block for solid phase peptide synthesis.[6a,11]

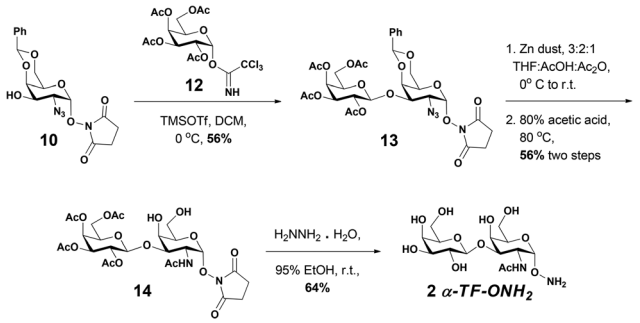
Alternative to the previous synthesis of α-TF-ONH_2,[10] D-galactosamine·HCl (**3**) was chosen as a suitable starting material (Scheme 1). First, **3** was treated with imidazole-1-sulfonyl azide hydrochloride, followed by acetic anhydride to provide peracetylated azide **4**.[12] Phenyl thioglycoside **5** was then prepared through treatment of **4** with $BF_3{\cdot}OEt_2$ and PhSH. Next the acetyl groups of **5** were removed using the Zemplén method and the benzylidene acetal was installed to provide thioglycoside acceptor **6** in an α/β ratio of 1.7 : 1 (readily separable by silica gel column chromatography). Inspired by previous uses of alkoxy protection[10,11] at the anomeric position during the required glycosylation, an acceptor was envisioned that would carry a preinstalled α-NHS group for the duration of the synthetic strategy. There are numerous reports of reductions,[9a,b,10,13] and also a single example of TBS protection[14] being conducted in the presence of NHS glycosides. The only previous example found of a glycosylation involving a succinimidyl protecting group was reported by Bertozzi and co-workers[13] for the preparation of the aminooxy STn antigen. In order for the Bertozzi approach to be realized, a suitable protecting group was required; removal of which would occur under mild conditions, leaving the α-NHS moiety intact at the anomeric position.

In our initial attempts, along the lines of the aforementioned work,[13] we elected to pursue TBDPS for 3-OH protection. Compound **7** was prepared in 79% yield by treatment of **6α** with excess TBDPSCl and imidazole in DMF (Scheme 2). The protected donor **7** was then activated with NIS–TfOH in the presence of 1.1 eq. of NHS. Unfortunately under these conditions, the succinimidyl anion, the by-product from the NIS activation, out competed NHS for the donor. This unexpected reaction pathway gave a 2 : 1 – α/β mixture of compound **8** in 73% yield. However we were able to isolate α-NHS glycoside **9** in low yield. In an attempt to avoid the unwanted C–N bond formation, the reaction was conducted with excess (4 equiv.) NHS. These conditions proved sufficient for the formation of the α-NHS product **9** in 75% yield, 8 : 1 – α/β).[15]

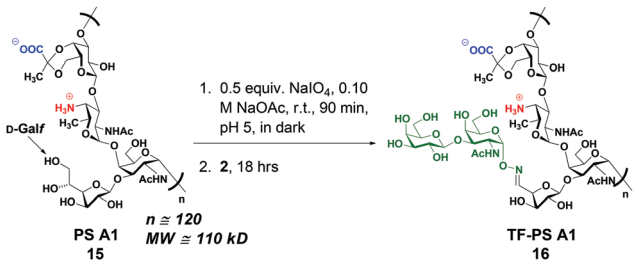
With protected NHS acceptor **9** in hand, we next turned our attention to the removal of the TBDPS group. Compound **9** was treated with TBAF in THF at r.t. which completely converted **9** to **10** as observed by thin layer chromatography, however, we were only able to isolate **10** in <40% yield. The low yield was attributed to opening of the NHS ring by hydroxide, forming the glycosyl carboxylic acid as observed by Andersson and Roy.[9a,16] The presence of hydroxide is believed to arise from the strong basicity of TBAF and that moisture entered our reaction vessel. This alternate reaction pathway might be avoided by adjusting the pH of the commercial TBAF before addition.[13,17] This strategy could provide a viable pathway to **10**, but reported deprotections utilizing this method in the presence of NHS continually suffer from low yields.[13] In light of the fact that excess reagent and a subsequent laborious purification step were needed we decided to pursue an alternative strategy to prepare compound **10**.[18]

In a similar manner as reported in a synthesis of TF-*O*-Ser/Thr,[11c] the chloroacetyl group (ClAc) was examined as an alternative to TBDPS. Starting from **6α** or **6β** (Scheme 3) the chloroester **11** was prepared in 84% yield by treating the respective secondary alcohol with ClAcCl/pyr.[19] The masked thioglycoside **11α** or **11β** was treated with NIS–TfOH and 4 equiv. NHS providing the α-NHS glycoside as the major product.[20] Determining the α/β ratio for this reaction was complicated by the formation of the aforementioned succinimidyl by-product; our reported yield is based solely on the isolated α-NHS. The NHS group proved to be base liable with decomposition occurring under both NEt_3–MeOH[19] and saturated $NaHCO_3$ 5 : 1 MeOH–H_2O[21] conditions. The successful deprotection was achieved through the use of the non-basic nucleophile, thiourea, in 5 : 1 EtOH–pyr[22] at room temperature giving rise to the deprotected NHS acceptor **10** in 65% yield over two steps.

With access to appreciable quantities of our desired α-NHS acceptor **10**, we attempted the glycosylation using trichloroacetimidate donor **12**[23] (Scheme 4). Recent reports noted such



Scheme 4 Preparation of α-aminooxy TF (**2**).



Scheme 5 Synthesis of TF-PS A1 conjugate (**16**).

reactions as being hampered by competing orthoester formation.[11b,24] Fortunately the major product of this pivotal glycosylation had the required β 1,3 linkage found in the TF antigen (Fig. 1) and provided disaccharide **13**, which was isolated in 56% yield. The characteristic anomeric doublet observed at 4.80 ppm with a J_{H1-H2} of 8.00 Hz confirmed that the desired β linkage was formed. This conclusion was further supported by COSY and HMQC.

With protected disaccharide **13** in hand, subsequent steps focused on removal of the benzylidene acetal and reduction of the azide. First, activated zinc dust, in 3 : 2 : 1 THF–AcOH–Ac_2O, was used to reveal the *N*-acetyl[11a,25] moiety at the 2-position. The crude reaction mixture, obtained from the azide reduction, was then treated with 80% aqueous acetic acid at 80 °C to remove the benzylidene acetal and provide diol **14** in 56% yield over two steps.[24] The product exhibited no overlap of signals in the ^{13}C NMR, and therefore all of the carbonyls were precisely accounted for.[26] This spectrum confirmed that the β 1,3 linkage had been made and that **14** was not the unwanted orthoester.

In the final deprotection step, hydrazine hydrate was utilized to remove the remaining acetyl groups as well as to displace the succinimidyl protecting group to reveal the α-aminooxy TF antigen **2**. As earlier reports noted,[9a,10] the two byproducts from this reaction, acetohydrazide and tetrahydropyridazine-3,6-dione, complicate the purification of the respective aminooxy sugar. After several attempts at crystallizing the crude reaction mixture from methanol, we turned our attention to size exclusion chromatography. The disparity in MW allowed excellent separation on a Bio-gel P-2 column using water as the eluent giving rise to pure **2** in 64% yield from protected disaccharide **14**; NMR data matched the previously reported values.[10]

Following the preparation of **2**, the key one pot oxidation and conjugation between PS A1 and the α-aminooxy TF was conducted (Scheme 5). This was accomplished by cleaving the vicinal diols of galactofuranose residues present on PS A1 with $NaIO_4$, creating strategically placed aldehydes along the tetrasaccharide repeating unit.[8a,27] After 90 min of PS A1 (**15**) exposure to $NaIO_4$, KCl was introduced to precipitate any remaining periodate as KIO_4.[28] Finally **2** was added to oxidized PS A1 and the reaction mixture was allowed to stir overnight in the dark at r.t. to provide the TF-PS A1 oxime conjugate, with a percent loading assumed to be similar to our previous report with conjugated Tn.[8b] The ^1H NMR spectrum of **16** showed a distinct oxime doublet centered at 8.00. In addition a new singlet could be observed at 2.29 (*N*-acetyl of TF) and 1D TOCSY of anomeric protons at 4.61 and 5.46 displayed spin systems comparable to the TF-ONH_2 (**2**) proton spectra.

Conclusions

In summary, the synthetic preparation of the α-aminooxy TF antigen and its conjugation to PS A1 has been described. The synthesis of **2** is highlighted by successful α-selective glycosylation and deprotection in the presence of the succinimidyl protecting group. New evidence is presented that the succinimidyl group can be carried through a number of transformations provided that basic conditions be avoided.[9a,13,16] This study further establishes the utility of NHS glycosides in the synthesis of aminooxy sugars, thereby making conjugations of nucleophilic sugars to appropriate carbonyls possible through physiologically stable oxime formation.[29] Future studies are concomitantly underway for the evaluation of the TF-PS A1 conjugate as a potential cancer vaccine candidate utilizing murine models.

Acknowledgements

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