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Efficient and Reproducible Synthesis of an Fmoc-protected Tn Antigen

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This concise total synthesis of the Thomsen-Nouveau (Tn) glycoconjugate was accomplished using a palladium-catalyzed coupling between the glycosyl donor and Fmoc-protected serine acceptor. This is, to the best of our knowledge, the shortest synthesis reported from galactose for preparing this essential building block for large-scale solid phase peptide synthesis.

Cancer is a leading cause of death globally. Despite constant advances in treatment, the percentage of cancer-related deaths is expected to increase in the developed world as other fatal conditions are continuously becoming better controlled. Although there is ever accelerating innovation in the treatment of cancer, progress remains agonizingly slow due to the enormous diversity of mutations responsible for causing cancer. The current primary triad of surgery and/or radiation/chemotherapy both in tandem or individually can be highly effective; but success is greatly dependent on a series of variables including (but not limited to): the location of the disease, metastasis status, types of mutations present, and stage at detection. Therapeutic options are particularly lacking for late-stage cancers. Despite a few targeted therapies recently becoming available, especially for certain subtypes of breast and prostate cancer, in many cases these pharmaceutical interventions still often result in short term improvement, with disease recurrence emerging as the cancer mutates to resist the treatment. We consequently need completely orthogonal therapeutic modalities to complement this therapeutic triad. Cancer vaccines are a promising avenue as they could re-engage the body’s immune system to the battle. For many purposes, cancer can be defined as uncontrolled cell growth that has evaded the immune system, and will eventually progress to the death of the host: the vast majority of precancerous clusters are hypothesized to be cleared by the immune system long before they can develop into a tumor. When looking to activate the immune systems, vaccines are particularly attractive; however, they require the identification of biomarkers correlated with cancer. Unfortunately, many oncotargets are simply upregulated in cancer. As they are also present on healthy cells, they are inappropriate vaccine targets as stimulation would initiate a systemic and very dangerous immune response. Tumor-Associated Carbohydrate Antigens (TACAs) are different as they are not found on healthy adult tissue, yet are found on over 90% of biopsied carcinomas (i.e. breast, ovarian, colon, lung, and prostate cancers). This discrepancy likely arises because these TACAs are found at the reducing-end of surface glycans, and are anchored to the amino acids or lipids of the membranes. In healthy tissue they are always further elaborated into larger glycostructures. However in cancer, the misregulation of glycosyl transferases responsible for this elaboration ensures that these TACA scaffolds remain naked, missing the additional residues. Their precise role in oncogenesis is unclear, but overexpression is generally correlated with a poor prognosis. These carbohydrate antigens have, when incorporated into glycopeptides or other immunogenic scaffolds, formed the basis of the development of anti-tumor immunotherapies through the induction of a specific immune response against cancer cells. However, ready access to these materials remains a significant challenge, and a simple scalable synthesis is required. As part of an extensive carbohydrate vaccine effort, we needed a route to produce the simplest TACA building block appropriate for solid phase peptide synthesis on multigram, and up to 100g scale: the Fmoc-protected peracetylated Tn antigen (1), α-2-deoxy-2-N-acetylamino-d-galactose (GalNAc) O-linked to a serine residue. Although this molecule has been prepared repeatedly in the past, we found many of the simple published routes were 1: A)
inconsistent batch-to-batch and couldn’t always be reproduced

if the chemist doing the reactions changed. Furthermore, published routes often required complex protecting group strategies that increased the step and purification count, decreasing yield (Figure 1). We wish to report the route we are using for this scale-up and that can be readily executed by a junior undergraduate student.

Our synthetic approach begins with a series of well-precedented steps as outlined in Scheme 1. A medium scale peracetylation of d-Galactose (100g scale) providing a 78% yield of the β anomer after recrystallization from methanol. Subsequent bromination in strong acid yielded the galactosyl bromide in good yield after precipitation. Treatment of the unstable bromosugar with zinc metal in acetonitrile at reflux, provides bench stable galactal in high yield. Although other approaches were explored, this route proved to be the most reliable access to this material, with no chromatography needed.

To introduce the C2-amine, we found that Lemieux’s ceric ammonium nitrate (CAN)-mediated azidonation was highly effective and robust providing crude that readily hydrolyses the anomeric nitrate to provide reducing sugar. This transformation, although effective, does result in the formation of inseparable byproducts and although it is crystallizable, this is very slow and inefficient, and so chromatography must be performed. This hemiacetal was converted to a large number of different activating groups (see references above), but yields for introducing the activating group were highly variable and the subsequent glycosylations inevitably inconsistent. Schmidt’s trichloroacetamidates (TCA) were the most promising.

General protocols call for the use of potassium carbonate however, we found that the more soluble cesium carbonate reduced reaction times from several hours to mere minutes with a concomitant reduction in product decomposition, leading to higher yields. This intermediate should be used immediately upon isolation to minimize background hydrolysis.

TACAs are generally made to be incorporated into peptides. This means that the resulting product should have an Fmoc-protected amino group and a free acid. Fmoc amino acids are notoriously challenging to couple and manipulate compared to Boc-protected analogues, and a common approach involves making the glycoconjugate, deprotecting the Boc group, and installing the Fmoc. This is inefficient especially as the price difference between Boc and Fmoc amino acids continues to close, and as Fmoc-installation is non-trivial. Similarly, we wanted to avoid conditions that required the carboxylate group on the amino acid to be protected; minimizing steps on the installation is non-trivial. Similarly, we wanted to avoid conditions that required the carboxylate group on the amino acid to be protected; minimizing steps on the installation is non-trivial. Consequently we only focused on published methods that used Fmoc-Ser(OH)-OH as the partner. All these routes use one of only a handful of Lewis acids as promoter. These proved unsuccessful (Table 1). The best result, 17%, was obtained with TMSCOTf with a 24 hour reaction time with moderate conversion. Leaving the reaction, sealed, for longer time reduced reaction times from several hours to mere minutes with a concomitant reduction in product decomposition, leading to higher yields. This intermediate should be used immediately upon isolation to minimize background hydrolysis.

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Figure 2. Proposed mechanisms of the Nguyen glycosylation in the presence or absence of a cis-C-2 ether. Proposed mechanism in the presence of a cis-C-2 azide slow consumption of starting material and then product decomposition under the reaction conditions. All Lewis acids were freshly distilled under Schlenk technique, all solvents were obtained using air-free techniques directly from ketal stills, or a solvent purification system. All transfers were made using air-free techniques. Advantageous water was not a serious challenge explaining the limited conversions.

Before abandoning TCAs as a viable system and returning to glycosyl sulfoxides, we considered Nguyen’s unusual palladium (II) catalyst, Pd(CH$_2$CN)$_2$(BF$_4$)$_2$, as a promoter. Nguyen used it as a promoter for the formation of β-glycosides through a proposed modified anchimeric effect where the Pd chelates to a C2 ether as well as the nitrogen of the TCA, keeping the TCA localized to the bottom face of the oxocarbenium, forcing β-attack. In the case of mannose or rhamnose where the C-2 ether is axial, this chelation is impossible, and an oxocarbenium intermediate followed by anomic-effect-driven α-glycosylation was observed (Figure 2). This meant that this chemistry was incapable of providing α-anomers in the presence of an equatorial substituent at C2; however, we could find no examples where galactose was evaluated, nor any examples with a C-2 azide. As the azide should not complex to the Pd in the same fashion as the etheric oxygen, we believed that we would pass through an oxocarbenium instead, and the anomic effect would preferentially drive the formation of the desired α-product. Some reaction screening provided a new set of conditions that reliably formed 8 from 7 using the Nguyen glycosylation in up to 38% yield of the desired α-product of the with an initial α to β ratio of approximately 3:1 (total combined isolated yields of both anomers up to 50%). Importantly, the starting materials can be recovered from the reaction mixture allowing for resubmission to conditions (longer reaction times started leading to product decomposition; yield based on recovered starting material is far higher, 68-91% combined anomer yield, making this cost effective as the material produced to that point need not be unnecessarily yeted in this penultimate step). The reaction was conducted by four different chemists (undergraduate, graduate, and postdoctoral) with just the written protocol, with the obtained yields being consistent.

This result remains far from ideal, and reaction optimization and the search for alternatives to Pd$^{2+}$ salts is currently underway in the group, but this was certainly the most robust of the dozens of conditions we screened to access this material.

The final step before global deprotection is a one pot reduction and acetylation to form the NHAc functionality at C-2. Standard reducing conditions afforded 1 in high yield, and ready for incorporation into glycopeptides. Our design-of-experiment study on the Nguyen glycosylation and screening of alternate metal catalysts will be reported on in due course; samples of the Fmoc-protected Tn antigen are available for collaboration or at cost.

Conclusions

This is the first readily scalable total synthesis of a suitably protected Tn antigen for SPPS, with a lower step-count than any other report in the literature and incorporating a robust and reproducible Nguyen glycosylation as the key step. The overall yield for the process is 7% from Galactose, with only two chromatography steps, and the process can be conducted on scale in under two weeks by a practitioner skilled in the art. Or by a skilled undergraduate student while carrying out a full course-load in about 6 weeks. The intermediates are, for the most part, bench stable making this a very simple way to access this extremely important building block for glycopeptide chemistry.

Author Contributions

Conceptualization, JFT; Funding acquisition JFT; Investigation, SP, JC, MRR; Methodology, SP, MRR; Project administration, JFT; Supervision, JFT; Writing original draft, SP, MRR; Writing – review and editing, SP, MRR, JFT.

Conflicts of interest

There are no conflicts to declare

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for sharing J. Chiaramonte with us for too short a time, we share their grief, and miss his participation in our lives every day.

References

23. This molecule is stable in the freezer under argon, but rapidly decomposes under ambient conditions and should not be stored on the bench overnight or longer.