Sugar Synthesis and the Effect of Glycosylation on Chemotaxis

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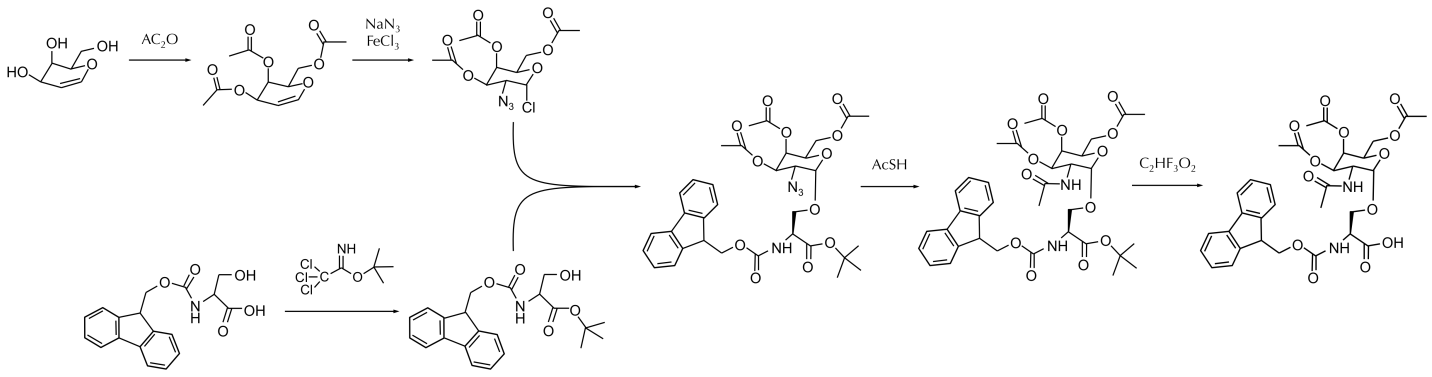

ABSTRACT: The protein chemokine ligand-5 (CCL5) is produced in the human body and is responsible for the chemotaxis (movement of responsive cells to certain environments) of white blood cells to inflammatory sites. Glycosylation is the process of synthesizing a peptide chain with sugars on designated amino acids so as to influence the folding pattern and consequently the structure and function of the final protein. Successfully glycosylated CLL5 proteins have the potential to alter the level of chemotaxis and therefore offer a solution to conditions like inflammatory diseases. Here, I synthesized a glycosylated amino acid used in protein synthesis called N-acetylgalactoseamine-serine (GalNac-serine). I prepared the glucose ring first by protecting the exposed hydroxyl groups and activating the sugar. The carboxylate group of the Fmoc-Ser was protected with a tert-butyl (tbu) group. I coupled the activated sugar with Fmoc-Ser-Otbu and reduced the azide. The final step was removing the tbu group. The organic synthesis of GalNac-Serine had a 65% average yield and produced 1.208 g, 20.8% more than the target mass. Nuclear magnetic resonance (NMR) revealed a 96% pure final product.

INTRODUCTION

A process called chemotaxis is responsible for the movement of white blood cells to inflammatory sites.[1,4] The chemokine ligand-5 (CCL5), also known as RANTES, is a protein produced in the human body that is sometimes naturally glycosylated and plays an active role in chemotaxis.[8] Previous studies have shown that proteins like CCL5 may behave differently depending on their folding patterns and thus, structure.[1,4,10,12-15] Sugars can be attached at predetermined sites on a peptide chain by glycosylation in order to manipulate the protein structure. The attached sugars inhibit the protein from folding on itself by getting in the way, thereby causing an adjusted folding pattern. An effectively adjusted CCL5 can then affect chemotaxis and fundamentally, inflammation or immune system functions in the body.[9] Currently, glycosylation is also popular in the chemokine receptor-5 inhibitors (CCR5) because it can block one of the two Human Immunodeficiency Virus (HIV) coreceptors and ultimately protect against it.[15]

The initial step in testing for the most effective glycoform of a peptide is to synthesize the sugars. These sugars are then attached to amino acids in a peptide chain that is incorporated into a protein. Hence, the purpose of this project is to synthesize N-acetylgalactoseamine-serine (GalNac-serine), a glycosylated peptide chain. Ultimately, the purpose is to utilize glycosylation to manipulate protein function.

MATERIALS AND METHODS

There are 6 total reactions in the synthesis of the final sugar product.

First, I will start with D-Galactal and protect its hydroxyl groups using acetic anhydride in pyridine. The work up consists of reacting the mixture with 1 M hydrochloric acid (HCl), sodium bicarbonate, and brine. It is then dried over

magnesium sulfate. It will then be purified using a silica column, and removed extracted in fragments of 100 mL. The product will be taken to a nuclear magnetic resonance (NMR) spectrum to verify the structure and purity.

The second step is to activate the sugar using sodium azide, iron trichloride, and hydrogen peroxide at -30°C. Again I will work up the reaction with 1 M HCl, sodium bicarbonate, and brine, and dry over magnesium sulfate. The solvent will then be removed and taken crude to the NMR.

Thirdly, the carboxylate group of the Fmoc-Ser will then be protected with a tert-butyl (tbu) group using trichloroethane (TCA) in ethylacetate and cyclohexane. I will purify the product by silica column and verify by NMR.

My fourth step will be coupling the activated sugar with Fmoc-Ser-Otbu using silver chlorate and silver carbonate in dichloromethane (DCM) and toluene starting at 0°C and gradually warming to room temperature overnight. After the workup, I will purify on silica and again verify purity with NMR.

The fifth step is to reduce the azide by stirring in AFEX-pretreated corn stover hydrosylate (AcSH) and pyridine overnight. The solvent will be removed and checked in its crude state with NMR.

The sixth and last step is to remove the tbu group with 95% trifluoroacetic acid (TFA) for 1.5 hours. I will purify twice on silica and lyophilize it several times to remove the TFA. Then an NMR spectrum will be used to thoroughly analyze and verify the final product.

This final product will eventually be utilized to synthesize glycosylated CCL5 for biological studies such as analyzing the behavior of the protein in an eosinophil (white blood cell) environment. The density of white blood cells can then be measured within a certain radius of the protein for discrete CCL5 proteins.

RESULTS

The average percent yield of the reactions (individual values below in Table 1) was 65%.

Table 1: Percent yields of each reaction step.

Step	% Yield	Product Mass (g)
1	95	1.425
2	78	1.149
3	49	2.205
4	54	2.392
5	57	2.480
6	56	1.208

The target mass was 1.000 g. The final mass was 1.208 g as in Table 1, 20.8% greater than expected. All impure products in individual reactions were collected separately for further purification or alternate analysis.

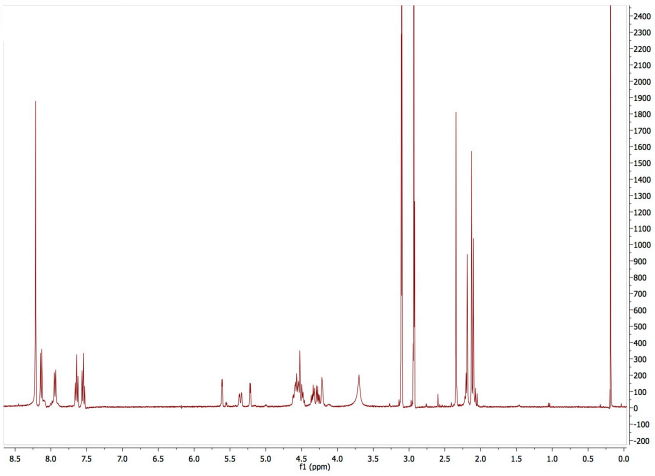

Figure 1: Nuclear magnetic resonance spectrum for the final product, GalNac-serine.

Three of the tallest peaks in Figure 1 at roughly 8.25 ppm, 3.1 ppm, and 2.9 ppm correspond to the solvent used in the NMR, dimethylflormamide. The four distinct peaks towards the left-hand side of the spectrum are representative of the aromatic Fmoc group of the glycosylated amino acid.

Reaction step four yields two different products: α and β. The desired product for the rest of the reaction is the α product, and can be identified in Figure 1 by peak a in the NMR spectrum, whose J value (coupling constant) is 3.6 Hz. The β product would have a J value in the range of 8 and 11 Hz. Therefore because the coupling constant is so small and the peaks are virtually indistinguishable from one another, I know that the final product is pure α. The final product was 96% pure, with minor impurities caused by the β byproduct and potential leftover trifluroacetic acid.

DISCUSSION

In my experiment, I achieved my purpose of synthesizing N-acetylgalactoseamine-serine (GalNac-serine), a glycosylated peptide chain. This achievement was notable because the target mass of the GalNac-serine is 1.000 g, and there was a mass yield of 1.208 g of the pure product. A nuclear magnetic resonance (NMR) spectrum was taken of the final product. The three tallest peaks on the spectrum (~8.25 ppm, 3.17 ppm, and 2.85 ppm) correspond to the solvent that was used to dissolve the product, dimethylformamide (dmf). The cluster of relatively high peaks on the right (~2.25 ppm) indicates the acetyl groups surrounding the glucose ring.

Cells use glycosylation of cytokines (a subdivision of a chemokine) to alter their functions by targeting different populations of responsive cells, such as eosinophils.[9]

CCL5 plays the largest role in chemotaxis of eosinophils due to its wide spectrum of immune cells it can influence.

Inflammatory diseases including some immunodeficiencies and cancers are generally characterized by the buildup of responsive cells.[10,12] It was found that in breast cancer, for example, CCL5 and CCL2 (chemokine ligand-2) expression is acquired in the course of malignant transformation, suggesting that they both contribute to development or progression of the growth of the tumor cells.[14] Therefore, if the magnitude of chemotaxis caused by CCL5 can be deliberately lessened using glycosylation, a solution to combating or preventing malignant inflammation may be discovered.[14,15,17]

The glycosylation of CCR5 with the intent to synthesize a protein that will occupy one of the binding sites of an HIV is also being largely studied. Successful glycosylation of the receptor has the potential to ultimately inhibit the virus to infect other cells. Several studies support that the incorporation of CCR5 can actually eliminate viremia, where viruses enter the bloodstream and gain access to the rest of the body, such as the studies conducted by Ronald S. Veazy, where he concluded that the CCR5 fully protects against infection from the HIV in the rhesus vaginal challenge model.[15] Internalization of CCR5 may protect target cells form infection, however CCR5 activation is also shown to possibly induce inflammation, a risk for HIV transmission.[15] Glycosylation and protein-structure adjustment are furthermore being investigated to improve CCR5's effects.

CONCLUSION

The next step in the investigation is to attach GalNac-serine to additional amino acids to form a peptide chain. Peptides are generally synthesized using an Applied Biosystems Synthesize model by the solid-phase method, which is essentially stringing amino acids together individually.[8] After complete synthesis, peptide folding takes place until the structure is established, at which point the relationship between structure and behavior can be further analyzed.

The specific protein that will be made using the GalNac-serine amino acid is CCL5. Three different structures of CCL5, two of which are glycosylated and the other not, will be analyzed in terms of the chemotaxis effect. The synthesis of CCL5 containing GalNac-serine is significant because the naturally produced CCL5 proteins in the human body are difficult and costly to extract and separate into isolated samples of glycosylated and non-glycosylated proteins. Therefore, by synthesizing it with known structures, I can observe if glycosylation of CCL5 has an effect on chemotaxis.

SUPPORTING INFORMATION

Nuclear magnetic resonance spectrum analysis performed on a 400 Hz frequency NMR instrument. This material is available free of charge via the Internet at http://pubs.acs.org.

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