Pergamon

Tetrahedron Letters 43 (2002) 1599–1602

TETRAHEDRON LETTERS

Neoglycoprotein cancer vaccines: synthesis of an azido derivative of GM3 and its efficient coupling to proteins through a new linker

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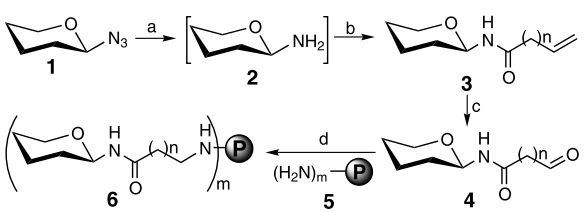
Received 31 October 2001; accepted 2 January 2002

Abstract—A GM3 derivative having an azido group on its reducing end was synthesized in nine separate steps, and it was then conjugated to proteins by a new linker. Therefore, the azido group was reduced to a free amino group, which was followed by the introduction of a 4-pentenoyl group. The carbon–carbon double bond of the 4-pentenoyl group was ozonolyzed to form a carbonyl group, and GM3 was thus linked to proteins via the carbonyl group by reductive amination. © 2002 Elsevier Science Ltd. All rights reserved.

It was revealed more than three decades ago that oncogenic transformation was associated with dramatic change in cell surface carbohydrates.[1] Since then, numerous carbohydrate antigens that are either specific or markedly overexpressed on cancer cells have been characterized, and the structural motifs are therefore called tumor-associated carbohydrate antigens (TACAs).[2–8]

TACAs are the molecular basis of a new and very attractive antitumor development, i.e. use of cancer vaccines to treat cancer patients.[7,9–15] Many TACAs have been exploited in this connection, and it has been recognized that, to induce specific antitumor immune responses in patients, TACAs have to be conjugated to an immunostimulatory agent for overcoming immunotolerance problems. Nevertheless, the concept of TACA-based therapeutic cancer vaccines has been well established, and several synthetic glycoconjugate vaccines are now in clinical trials.[6,9,16–20]



Scheme 1. *Reagents and conditions:* (a) selective reduction of the azido group; (b) $(CH_2{=}CH(CH_2)_nCO)_2O$; (c) oxidative cleavage of C=C; (d) reductive amination using $NaBH_3CN$.

The glycoconjugates that are most commonly employed for vaccine designs are neoglycoproteins with the carbohydrate antigens covalently linked to a carrier protein,[21] e.g. tetanus toxoid[21] or keyhole limpet hemocyanin (KLH).[18,19,22,23] In regard to the preparation of neoglycoproteins, one major concern is the coupling of TACAs to carrier proteins. This problem is more obvious and significant if we combine it with the complexity of carbohydrate syntheses,[24] since a good coupling method is not necessarily compatible to the strategies, including protection and glycosylation, used in carbohydrate syntheses. For instance, reductive amination is a simple and reliable conjugation method that has been widely used for carbohydrate–protein couplings,[21] and thus, many linkers containing carbonyl groups or functionalities that can be converted to carbonyl groups are designed for this purpose.[9,16,21,25,26] However, if the linkers are attached to the oligosaccharide reducing ends at an early stage of the syntheses, they may make it difficult to design a compatible protection–deprotection strategy. Introduction of linkers to finished oligosaccharides at the final stage may circumvent this problem, but the established linkers and attachment methods usually require multiple transformations of the precious oligosaccharides, such as the removal of original protecting groups and then introduction of a new protection that is compatible with the linker, attach-

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PII: S0040-4039(02)00071-0

ment of the linker via glycosylation and final deprotection. Moreover, some of the potential linkers, e.g. unsaturated acyls, cannot be easily applied even under this circumstance.

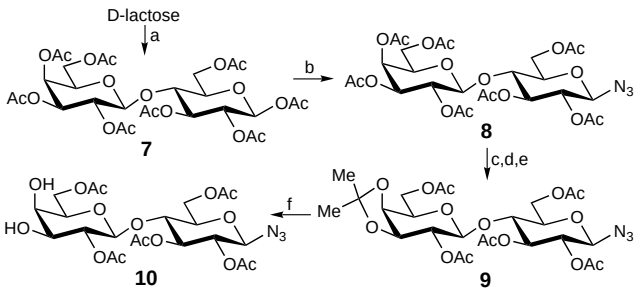
As a part of our campaign to develop new cancer vaccines, we are interested in finding new and efficient methods for TACA synthesis and oligosaccharide-protein conjugation. We describe herein a new coupling strategy that introduces the linker to TACAs after their syntheses are completed and an efficient method to prepare the GM3 derivative that can be used for this new strategy.

Our general design is shown in Scheme 1. First, deprotected TACAs with an azido group on their reducing ends (**1**) will be prepared. As an azido group is stable to most conditions involved in carbohydrate synthesis, it may be used as a protecting group of the anomeric center in the preparation of TACAs. On the other hand, the azido group can be very easily and selectively reduced to give the glycosyl amine **2** that will facilitate the attachment of an acyl group. Thus, after deprotection of carbohydrates and azido reduction, an acyl linker can be introduced. Even if benzyl is employed as the permanent protection of the oligosaccharides, after reductive debenzylation and concomitant azido reduction, an acyl group can still be selectively attached to the amino group, which was shown in the syntheses of *N*-linked glycopeptides.[27] Therefore, introduction of an unsaturated acyl group to the free amino group in **2** will afford the glycosyl amide **3**. Then, the carbon–carbon double bond in **3** can be ozonolyzed to give an aldehyde **4** which will be finally coupled to the free amino groups of a carrier protein (**5**) by well-established reductive amination and produce the glycoconjugate vaccine **6**.

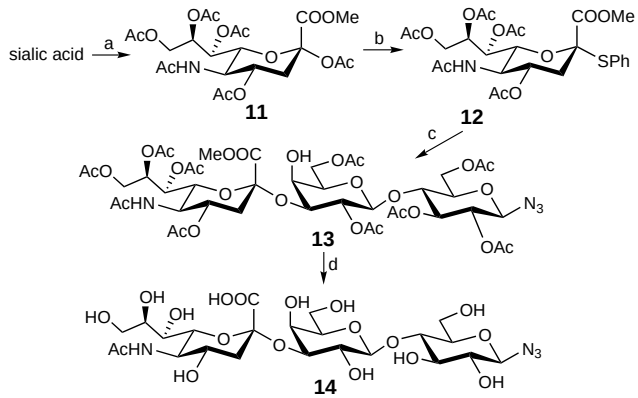
To test the design, we chose GM3 and its conjugates as the synthetic targets with 4-pentenoyl as the linker. GM3 is a TACA that is abundant on melanoma, breast cancer and other tumors.[2,6,7] GM3 and its conjugates are the focus of many biological and synthetic studies.[2,6,7,28–33] Moreover, GM3 contains a sialosyl residue that often creates problems to the coupling between carbohydrates and proteins. Thus, a strategy developed on GM3 may be of general significance. 4-Pentenoyl was employed as the linker due to its proper chain length. We have studied the coupling of GM3 to two proteins, KLH and human serum albumin (HSA), by the new linker. KLH is a favorite carrier protein for cancer vaccines due to its excellent immunological properties[22,23] and rich lysine residues for the attachment of TACAs. For instance, Danishefsky and his coworkers[9,18,34,35] used KLH in several cancer vaccines that are now in clinical trials. On the other hand, HSA conjugates are widely accepted as the coating antigens in immunological evaluations of vaccines, though HSA is not often used as a carrier protein.

The synthesis of the azido derivative of GM3 started from the modification of lactose (Scheme 2). Thus, when lactose was refluxed in acetic anhydride in the presence of sodium acetate, the expected α-anomer **7** (H-1: δ 5.68, 4.47; $^3J_{1,2}$ 8.2, 7.7 Hz) was obtained as the only product that was purified by recrystallization from toluene–hexane. Treating **7** with azidotrimethylsilane (N_3TMS) and boron trifluoride diethyl etherate ($BF_3{\cdot}OEt_2$) in methylene dichloride afforded the glycosyl azide **8** (H-1: δ 4.63, 4.48; $^3J_{1,2}$ 8.8, 7.4 Hz) in an excellent yield (93%). Then, deacetylation of **8** under basic condition, followed by 2,2-dimethoxypropane and acetic anhydride treatments, gave the acetonide **9**. Glycosyl acceptor **10** was finally obtained on the removal of the isopropylidene group in **9** under acidic condition in THF aqueous solution.

The sialosyl donor **12** was prepared in an excellent overall yield (76%) following a reported procedure[36] (Scheme 3). When sialic acid was treated by an acidic resin, amblyst-H^+, in methanol and then by acetic anhydride in pyridine, a fully protected derivative of sialic acid (**11**) was obtained. Compound **11** was transformed to the glycosyl donor **12** by reaction with thiophenol using $BF_3{\cdot}OEt_2$ as promoter.



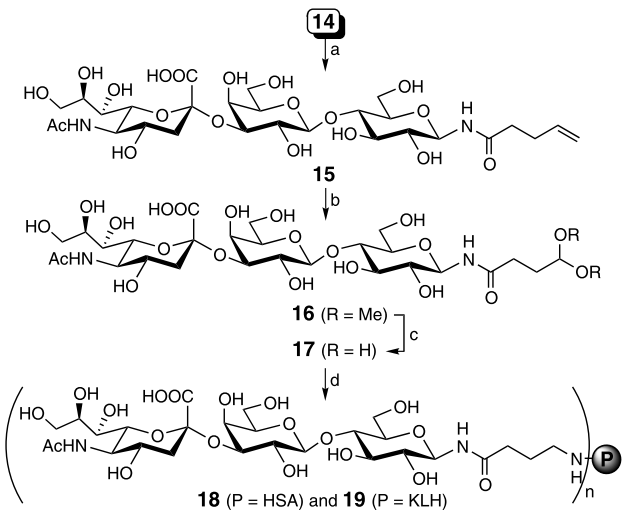
Scheme 2. *Reagents and conditions:* (a) Ac_2O, NaOAc, reflux, 6 h, 88%; (b) N_3TMS, $BF_3{\cdot}OEt_2$, CH_2Cl_2, 0°C to rt, overnight, 93%; (c) NaOMe, MeOH, rt, 4 h, quant.; (d) $Me_2C(OMe)_2$, TsOH, DMF, 40°C, 2 days, 92%; (e) Ac_2O, Pyr., rt, overnight, 80%; (f) HCl, THF, H_2O, rt, overnight, 40%.



Scheme 3. *Reagents and conditions:* (a) i. Amblyst-H^+, MeOH, 45–50°C, 6 h; ii. Ac_2O, Pyr., rt, overnight, 88%; (b) PhSH, $BF_3{\cdot}OEt_2$, MS 4 Å, CH_2Cl_2, 0°C to rt, overnight, 86%; (c) **10**, NIS, TfOH, MS 3 Å, MeCN, –35°C, 2 days, 26%; (d) 1.0 M NaOH, MeOH–H_2O (1:1), quant.

The glycosylation of **10** by **12** was achieved by Hasegawa method,[29,37,38] i.e. sialosylation in acetonitrile with glycosyl sulfide as donor and *N*-iodosuccinimide (NIS)–triflic acid (TfOH) as promoter. As expected, the desired trisaccharide **13** (H-1 of lactose: δ 4.61, 4.54; $^3J_{1,2}$ 8.5, 7.5 Hz; H-3's of sialic acid: δ 2.76, ^3J 12.5, 4.5 Hz; δ 1.83; ^3J 12.5, 12.0 Hz) was obtained as a major product (26% isolated yield), and the β-anomer (H-3s of sialic acid: δ 2.46, ^3J 12.5, 4.5 Hz; δ 1.81; ^3J 12.5, 11.5 Hz) as a minor one (8%). The anomeric isomers were separated by column chromatography, and their configurations were derived by comparing their ^{1}H NMR data to that of the similar structures reported.[29,30,37,38] Finally, deprotection of **13** using 1.0 M NaOH in water and methanol (1:1) afforded the azido derivative of GM3 (**14**, H-1: δ 4.78, 4.54; $^3J_{1,2}$ 8.5, 8.0 Hz; H-3's of sialic acid: δ 2.77, ^3J 12.5, 4.5 Hz; δ 1.84; ^3J 12.0, 12.0 Hz; Ac: δ 2.04).

The procedure to link GM3 to proteins is shown in Scheme 4. The azido group was reduced to a free amino group in a H_2 atmosphere using 10% Pd/C as catalyst. After removal of the catalyst through filtration, 4-pentenoic anhydride was added, and the mixture was stirred at room temperature overnight. When ninhydrin test showed no remaining free amine, the solution was condensed to dryness. The residual product was dissolved in water and washed carefully with ethyl ester and methylene dichloride. The water solution was lyophilized to give **15** as a white powder (Pent: δ 5.85, 5.13, 5.07, 2.53, 2.47, 2.40, 2.39; NH: 5.24, ^3J 2.5 Hz; H-1: δ 4.57; ^3J 8.0, 2.5 Hz). The product was practically pure (^{1}H NMR). Then, ozone was bubbled into a solution of **15** in methanol at −78°C until it turned to blue, when dimethyl sulfide was added. The mixture was warmed up slowly to room temperature and finally concentrated under vacuum. The reaction offered a dimethyl acetal **16** (OMe: δ 3.35) instead of the aldehyde. Thus, the product was dissolved in acetone and water (1:9) and treated with trifluoroacetic acid at room temperature to afford the hydrated aldehyde **17** (H: δ 5.15). Coupling reactions between **17** and proteins were realized in a 0.1 M aqueous $NaHCO_3$ buffer (pH 7.6–8.0).[39] Thus, a solution of **17**, $NaBH_3CN$ and the protein, HSA or KLH, in buffer was kept at 37°C in the dark for 3 days with occasional shaking. The reaction mixture was then dialyzed against distilled water (with frequent change of water) and finally lyophilized to afford **18** and **19**, respectively.



Scheme 4. *Reagents and conditions:* (a) i. 10% Pd/C, H_2, MeOH, rt, 4 h; ii. 4-pentenoic anhydride, MeOH, rt, 12 h, >95%; (b) O_3, MeOH, −78°C to rt, ca. 2–3 h, quant.; (c) TFA, acetone–H_2O (1:9), rt, overnight, quant.; (d) HSA or KLH, $NaBH_3CN$, 0.1 M $NaHCO_3$ aq. buffer, 37°C, 3 days.

The carbohydrate loading ($W_{\text{carbohydrate}}/W_{\text{conjugate}}\times$ 100%) of glycoconjugates **18** and **19** was determined by examining their sialic acid contents using well-established resorcinol method.[40] It turned out that **18** and **19** contained 13.4 and 15.9% of carbohydrate, respectively. They are in the ideal loading range for glycoconjugate vaccines.

Therefore, we describe herein a convergent synthesis of the azido derivative of GM3, as well as its convenient coupling to proteins by a new linker. This new coupling strategy was proved to be very efficient, and it may also be utilized to prepare other useful neoglycoproteins. The immunological properties of GM3-KLH conjugate **18** as a cancer vaccine is now under investigation, and the results will be reported elsewhere.

Acknowledgements

We thank Mr. Jim Faulk for the MS measurements. This project was supported by American Cancer Society (IRG-91-022-06), CWRU Ireland Cancer Center and a Research Innovation Award (RI0663) of Research Corporation.

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