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Synthesis of the Melanoma-associated Ganglioside 9-*O*-Acetyl GD3 Through Regioselective Enzymatic Acetylation of GD3 Using Subtilisin

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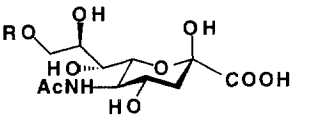
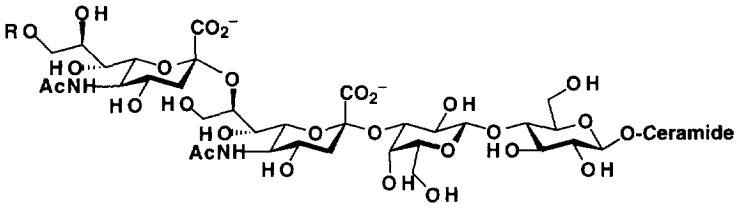
Abstract: The melanoma-associated disialoganglioside 9-*O*-acetyl GD3 has been synthesized for the first time through regioselective enzymatic acetylation of GD3 using subtilisin as the biocatalyst and vinyl acetate as the acetyl donor. Copyright © 1996 Elsevier Science Ltd

9-*O*-Acetyl *N*-acetylneuraminic acid (9-*O*-acetyl Neu5Ac, **2**) containing glycolipids and glycoproteins found on mammalian cells are important recognition elements involved in numerous biological events. In humans, 9-*O*-acetyl Neu5Ac (**2**) itself is known as the recognition element that mediates influenza C virus attachment to cells.[1] Recently, the disialoganglioside 9-*O*-acetyl GD3 (**4**) which also contains a terminal 9-*O*-acetyl Neu5Ac moiety, has been reported as a malignant melanoma cell-specific antigen that is attractive as a target for immune intervention.[2, 3]



1 R = H (*N*-Acetylneuraminic acid)

2 R = Ac (9-*O*-Acetyl*N*-acetylneuraminic acid)

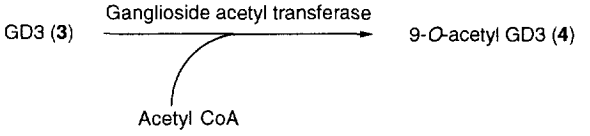


3 R = H (GD3)

4 R = Ac (9-*O*-Acetyl GD3)

The incidence of malignant melanoma has increased rapidly over the last decade. In 1996, in the United States alone, an estimated 38,300 individuals will be diagnosed with melanoma and approximately 7300 deaths from melanoma will occur.[4] Though surgical treatment is fairly effective for small tumors in the early stages of this cancer, the 5-year survival rate after elective or therapeutic dissection drops to 25-35% for stage III patients.[5] Recently, the importance of gangliosides as targets for passive and active specific immunotherapy has been documented by the clinical responses seen after treatment with anti-GD2,[6-8] anti-GD3[9] and anti-GM2[10] mAb, and by the correlation between antibody induction and improved prognosis after immunization with GM2 vaccines.[11, 12] Among the various gangliosides expressed by malignant melanoma cells, 9-*O*-acetyl GD3 is interesting in that this antigen is found almost exclusively on malignant melanoma cells in adult humans and exhibits excellent availability for recognition by antibodies. Therefore, 9-*O*-acetyl GD3 is an especially attractive target for immune intervention.[2,3, 13-14]

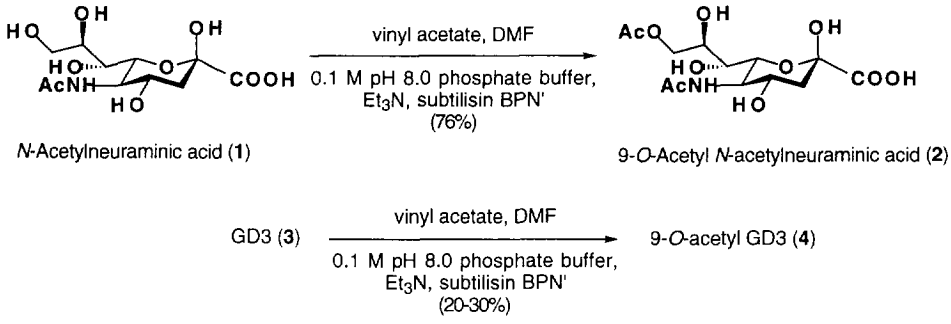
As such, procurement of sufficient amounts of pure 9-*O*-acetyl GD3 for the construction of vaccines and for further biological studies is very important. However, the 9-*O*-acetyl GD3 content of human melanoma tissues is low and there are no convenient natural sources for this acetylated disialoganglioside. Chemical acetylation of the more readily available GD3 has also been unsuccessful in providing the desired 9-*O*-acetyl GD3. Acetylation using *N*-acetyl imidazole and pyridine only provided GD3 acetylated at the internal sialic acid instead of at the desired terminal sialic acid.[15]



Scheme 1. Synthesis of 9-*O*-acetyl GD3 (**4**)*in vivo*.

We decided to investigate enzymatic methods for converting GD3 to 9-*O*-acetyl GD3. *In vivo*, 9-*O*-acetyl GD3 is produced through enzymatic acetylation of GD3 by ganglioside *O*-acetyl transferase using acetyl-coenzyme A as the acetyl donor (Scheme 1).[16, 17] This acetyl transferase, however, is very labile and has not been well characterized. We decided to investigate an alternative enzymatic synthesis using the readily available serine protease, subtilisin for the regioselective*O*-acetylation of GD3. Serine esterases and proteases in conjunction with enol esters have been usefully employed to achieve regioselective carbohydrate acylations that are difficult to perform chemically.[18, 19] Subtilisin together with DMF as solvent has been particularly useful for acylation of oligosaccharides.[20, 21] We first examined the enzymatic acetylation of Neu5Ac (**3**), which is the terminal saccharide unit in GD3 and itself a very important recognition element on cell surfaces.[1] Optimal reactions were obtained in DMF containing small amounts of buffer and triethylamine at 37 °C (Scheme 2). Such conditions have previously been observed to increase the reaction rate of subtilisin catalyzed reactions in organic media.[22] This was also important for achieving good yields and increased reaction rates in the acetylation of Neu5Ac as well. Next, GD3[23] was submitted to similar reaction conditions. This disialoganglioside was also found to be a substrate for subtilisin catalyzed acetylation providing the desired 9-*O*-acetyl GD3 along with smaller amounts of some GD3 acetylated at a non-terminal carbohydrate moiety (Scheme 2). The solvent system had a large effect on the acetylation of GD3. Too little triethylamine resulted in slow

reactions and low yield and too much resulted in polyacetylation of GD3, presumably from acetylation of more than one primary hydroxyl group. A more surprising effect was the effect of omitting aqueous buffer, which resulted in exclusive acetylation at an internal carbohydrate moiety.



Scheme 2.[24] Enzymatic acetylation of *N*-acetylneuraminic acid and GD3 using subtilisin BPN'.

We have described the enzymatic acetylation of Neu5Ac and the disialoganglioside GD3 to give 9-*O*-acetyl Neu5Ac and 9-*O*-acetyl GD3, respectively. This is the first report of a regioselective acetylation of GD3 at the 9-position of the terminal sialic acid moiety, as well as the first regioselective enzymatic acetylation of a disialoganglioside by a serine protease. This method provides access to *O*-acylated disialogangliosides as well as other *O*-acylated sialic acid compounds which are biologically and medicinally very important and difficult to obtain from nature or by chemical acylations. Now that sufficient amounts of 9-*O*-acetyl GD3 are available, the construction of 9-*O*-acetyl GD3 vaccines is underway and these will be tested for immunogenicity in melanoma patients.

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23. Purchased from Matreya Inc., Pleasant Gap, PA, USA. This GD3 is a mixture of compounds with different chain lengths in the ceramide portion.
24. ***Enzymatic acetylation of Neu5Ac (1)*:** A mixture of *N*-acetylneuraminic acid (**1**) (30 mg, 0.10 mmol), DMF (0.27 mL), vinyl acetate (0.16 mL), 0.1 M pH 8.0 potassium phosphate buffer (0.01 mL), triethylamine (0.012 mL), and subtilisin BPN' (2 X 10 mg , 200 U total, 10 mg at the beginning and after 24 h) was stirred at 37 °C for 48 h. The reaction was stopped by filtering through Celite®. After concentration *in vacuo* the residue was adsorbed onto silica gel and chromatographed (SiO_2, EtOAc/MeOH/0.02% $CaCl_2$ aq, 5/2/1) to afford 9-*O*-acetyl Neu5Ac (**2**) (26 mg, 76%). Its physical data were consistent with the reported data: Hauerkamp, J.; VanHalbeek, H.; Dorland, L.; Vliegenthart, J. F. G.; Pfeil, R.; Schauer, R. *Eur. J. Biochem.* **1982**, *122*, 305-311.

***Enzymatic acetylation of GD_3 (3)*:** A mixture of GD_3 (**3**) (20 mg, 0.0018 mmol), DMF (0.23 mL), vinyl acetate (0.16 mL), H_2O (0.008 mL), triethylamine (0.004 mL), and subtilisin BPN' (5 X 4 mg, 200 U total, 4 mg portions added at 0 h, 24 h, 48 h, 72 h, 96 h) was stirred at 37 °C for 106 h. The reaction was stopped by filtering through Celite®. After concentration *in vacuo* the residue was adsorbed onto silica gel and chromatographed (SiO_2, EtOAc/MeOH/0.02% $CaCl_2$ aq, 5/2/1) to afford 9-*O*-acetyl GD3 (**4**) (0.7 mg, 23%) and a mixture of unreacted GD_3 (**3**) and a small amount of internally monoacetylated GD3 (1.9 mg). The physical data of **4** were consistent with the reported data.[13]

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