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# Chemoenzymatic synthesis of a trimeric ganglioside $GM_3$ analogue

Marion A. Earle [a], Sukhdev Manku [a], Philip G. Hultin [a,*], Hong Li [b], Monica M. Palcic [b,*]

[a] *Department of Chemistry, University of Manitoba, Winnipeg, MB, Canada R3T 2N2*
[b] *Department of Chemistry, University of Alberta, Edmonton, AB, Canada T6G 2G2*

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**Abstract**

A trimeric β-lactosyl cluster based on 2-nitro-2-(hydroxymethyl)propane-1,3-diol was prepared using the trichloroacetimidate method. Kinetic studies showed that this cluster was an effective acceptor for rat-liver α-(2 → 3)-sialyltransferase. Its $K_M$ was comparable to those for monomeric lactose and *N*-acetyllactosamine acceptors, and its $V_{max}$ was 1% of that measured for the LacNAc acceptor. Preparative-scale sialylation using this enzyme afforded a trimeric cluster of the ganglioside $GM_3$ oligosaccharide in good yield. The NMR spectra of the trimeric $GM_3$ analogue suggest that the oligosaccharide conformation is not significantly perturbed by this level of clustering. © 1997 Elsevier Science Ltd.

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The recognition and binding of carbohydrate ligands by biological receptors is often enhanced when the sugars are presented in a multivalent, clustered format [1]. Synthetic clusters of biologically important carbohydrates have therefore attracted recent interest as enzyme inhibitors [2–5]. The immunogenicity of oligosaccharides can also be improved by multivalent clustering, a fact that has been exploited in synthetic vaccines [6].

Ganglioside $GM_3$ [α-NeuAc-(2 → 3)-β-D-Gal-(1 → 4)-β-D-Glc-(1 → O)-Cer] is a characteristic antigen of murine B16 melanoma [7] and is also found on human melanoma cells [8]. $GM_3$ would be a useful antigen in a therapeutic vaccine for melanoma if the relatively weak immunogenicity of the native ganglioside could be enhanced. The cluster effect offers an attractive approach to this goal, but the optimal structural parameters to exploit this effect have not been clearly determined. As our first foray into the synthesis of clustered carbohydrate antigens, we now report our straightforward preparation of a trimeric cluster of the $GM_3$ trisaccharide, using an efficient combination of chemical and enzymatic glycosidation methods.

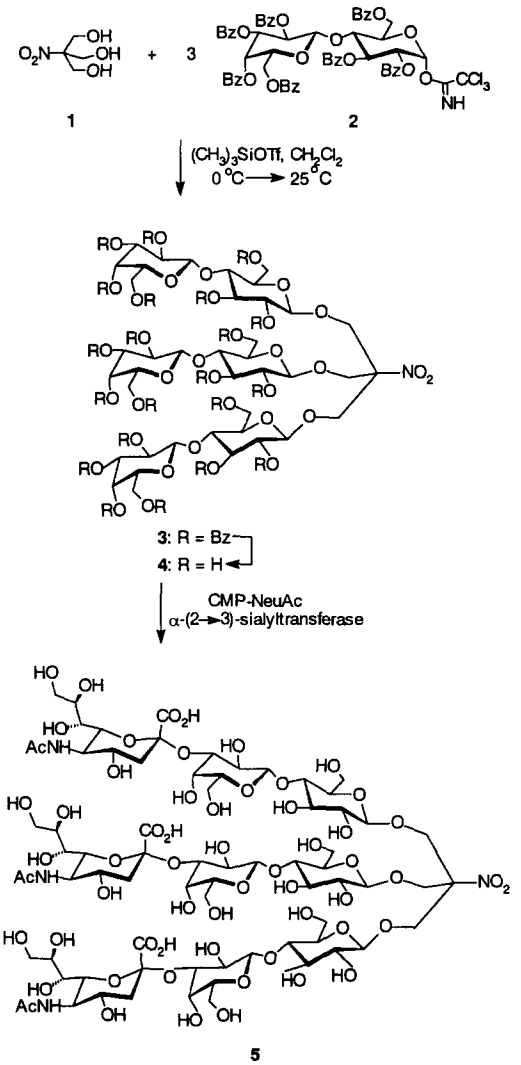
The chemical synthesis of sialyl glycosides is especially difficult and is frequently low yielding [9]. To avoid such problems, we planned to employ an enzyme-catalysed transfer of *N*-acetylneuraminic acid (NeuAc) to a pre-formed trimeric lactosyl acceptor. This strategy avoids tedious multistep protecting-group manipulations as well. Multiple enzyme-promoted sialyl transfers to some polyvalent *N*-acetyl-

* Corresponding authors.

lactosamine acceptors have been demonstrated [10–13]. The enzymatic transfer of a single sialic acid to monomeric lactosyl acceptors is also known [14,15], but the enzyme-catalysed transfer of several sialyl groups to a polyvalent lactosyl acceptor has not been described before.

Our first synthetic target was the trilactosyl cluster **4**, which would then serve as a sialyl acceptor. Lee and Lee have made related lactosyl clusters based on tris(hydroxymethyl)aminomethane [16]. We chose the commercially available 2-nitro-2-(hydroxymethyl)propane-1,3-diol (**1**) to be the backbone for our cluster for several reasons. First, the resulting clusters would be symmetrical, thus providing comparatively simple NMR spectra. We also envisaged that the eventual reduction of the nitro group to an amine would permit further elaboration into larger dendrimeric clusters [3,5,17–19].

Triol **1** turned out to be a weak glycosyl acceptor, however, and β-selective coupling of three lactosyl groups was difficult to achieve. After considerable experimentation with a variety of glycosidation protocols, we found that reaction of heptabenzoyl lactosyl trichloroacetimidate **2** [20] with **1** in dry dichloromethane, in the presence of trimethylsilyl trifluoromethanesulfonate, afforded the benzoylated trilactosyl cluster **3** [1] in 45% yield after HPLC purification (see Scheme 1). The benzoate protecting groups of **3** were removed under Zemplén conditions. The reaction mixture was then concentrated, and methyl benzoate was separated from the highly polar **4** by washing the solid residue with anhydrous ether until no further aromatic material could be detected in the washings by thin-layer chromatography. Cluster **4** prepared in this way (93%) was sufficiently pure, as judged by $^1$H and $^{13}$C NMR spectrometry, to be used as an acceptor for the sialyltransferase.




Scheme 1.

We examined the kinetics of sialylation using rat-liver α-(2 → 3)-sialyltransferase (EC 2.4.99.6) and $^3$H-labelled cytidine 5′-monophospho-*N*-acetylneuraminic acid sodium salt (CMP-NeuAc) [21]. The radiolabelled reaction products were isolated by adsorption onto Waters Sep-Pak™ $C_{18}$ cartridges, washing with dilute trifluoroacetic acid solution (pH 1.4) and eluting with methanol. This study showed that **4** was effectively bound by the enzyme with a $K_M = 0.60 \pm 0.07$ mM. This binding is about as tight as we observed for the monomeric acceptor β-LacO$(CH_2)_8CO_2CH_3$ ($K_M = 0.75 \pm 0.15$ mM), and almost twice as good as for β-LacNAcO$(CH_2)_8CO_2CH_3$ ($K_M = 1.1 \pm 0.15$ mM), available from previous work. On the other hand, **4** was turned over relatively slowly, having a $V_{max}$ of only 0.012 compared with the LacNAc acceptor (1.0) and the lactose acceptor (1.1). While the relative $V_{max}$ is low, nevertheless **4** is a sufficiently competent acceptor for preparative-scale reactions using the sialyltransferase.

Enzymatic sialylation of **4** (4.0 mg, 3.6 μmol) was carried out at 37 °C in 25 mM aqueous sodium cacodylate buffer (pH 6.5) containing 25 mM $MnCl_2$ (1.0 mL), using 100 milliunits of sialyltransferase, 2 units of alkaline phosphatase (EC 3.1.3.1) and CMP-NeuAc (5.0 mg, 7.9 μmol). During the first 24 h of the reaction, three additional portions of CMP-NeuAc

[1] All compounds were chromatographically homogeneous and afforded $^1$H and $^{13}$C NMR spectra in full agreement with the proposed structures.

Table 1
$^1$H NMR data for $GM_3$ analogue cluster **5** [a,b]

| Residue | Proton | Chemical shift [c] (ppm) | Coupling constants (Hz) |
|---|---|---|---|
| β-Glc | H-1 | 4.51 (d) | 7.9 |
| | H-2 | 3.32 (dd) | 7.9, 9.4 |
| | H-3,4 | 3.61–3.68 (m) | – [d] |
| | H-5 | 3.58 (m) | – [d] |
| | H-6a | 3.82 (dd) | 5.0, −12.5 |
| | H-6b | 3.98 (dd) | 2.0, −12.5 |
| β-Gal | H-1 | 4.52 (d) | 7.8 |
| | H-2 | 3.56 (dd) | 7.8, 9.9 |
| | H-3 | 4.11 (dd) | 3.1, 9.9 |
| | H-4 | 3.95 (d) | 2.7 |
| | H-5 | 3.71 (m) | – [d] |
| | H-6a,6b | 3.71–3.78 (m) | – [d] |
| α-Neu-5-Ac | H-3ax | 1.79 (dd) | 11.9, −12.4 |
| | H-3eq | 2.75 (dd) | 4.6, −12.4 |
| | H-4 | 3.69 (ddd) | 4.6, 10.2, 11.9 |
| | H-5 | 3.85 (dd) | 10.0, 10.2 |
| | H-6 | 3.63 (dd) | 1.9, 10.0 |
| | H-7 | 3.59 (dd) | 1.9, 9.1 |
| | H-8 | 3.89 (m) | – [d] |
| | H-9a | 3.65 (dd) | 7.0, −11.3 |
| | H-9b | 3.87 (m) | – [d] |
| | $CH_3$ | 2.03 (s) | – [d] |
| "$NO_2$-Tris" [e] | $H_a$ | 4.20 (d) | −11.4 |
| | $H_b$ | 4.45 (d) | −11.4 |

[a] Spectra were acquired at 500 MHz in $D_2O$ at 300 K. Chemical shifts were referenced to external acetone at δ 2.225 ppm downfield from tetramethylsilane.
[b] Peak assignments are based on DQF–COSY, HSQC, and 1D TOCSY experiments.
[c] Signal multiplicities are indicated in parentheses: s = singlet, d = doublet, dd = double doublet, ddd = double double doublet, m = multiplet.
[d] Indicated couplings could not be determined.
[e] $O_2N$-$C(CH_aH_bO)_3$–.

(1.8 mg, 2.8 μmol each) were added, and three further portions (1.0 mg, 1.6 μmol each) were added during the second 24 h. The progress of the conversion of **4** into **5** was monitored by MALDI-TOF mass spectrometry on aliquots of the reaction mixture. The reaction was allowed to proceed for a total of 60 h. Chromatography of the crude reaction mixture on a Bio-Gel P-4 column (1.5 × 120 cm), eluting with 10% ethanol in water, afforded pure **5** (4.6 mg, 64%) and mixed fractions (1.6 mg) containing **5** and some of the bis-sialylated compound.

We identified and characterised the sialyllactose cluster **5** by $^1$H and $^{13}$C NMR spectrometry and electrospray mass spectrometry. The complete $^1$H NMR assignments are summarised in Table 1. These data are in good agreement with those obtained by other workers for the native ganglioside $GM_3$ and for 3′-sialyllactose, [22,23]. This agreement suggests that the trisaccharide portions of **5** likely adopt a similar conformation in solution to that found in $GM_3$ and 3′-sialyllactose. We therefore expect that **5** will be a good mimic of the $GM_3$ melanoma antigen. Our study of these compounds is continuing. We will describe the preparation of larger clusters derived from **5**, as well as their immunological properties, in forthcoming papers.

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