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SYNTHESIS OF SIALIC ACID-LIPID CONJUGATES AND THEIR NEURITOGENIC EFFECTS ON N1E.115 NEUROBLASTOMA CELLS

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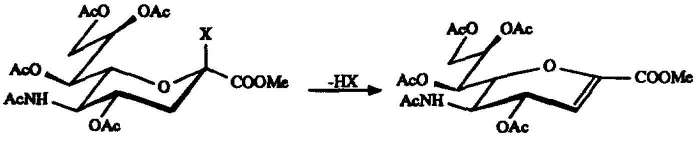
Abstract. Several sialic acid-lipid conjugates having one or more sialic acid residues were prepared *via* phosphite and methylthiomethyl intermediates. The synthetic compounds were found to promote neurite outgrowth on N1E.115 neuroblastoma cells. In particular, the trisialocholesterol derivative **19** exhibited potent neuritogenic activity.

Introduction

The last decade, many morphological, developmental, biochemical and behavioral studies have uncovered that endogenous gangliosides participate in a variety of neurobiological functions related to the maturation and repair of neural tissue[1,2]. It now has become apparent that application of exogenous gangliosides tend to increase cell survival and enhance neurite outgrowth, promote regeneration of central and peripheral nerves and facilitate behavioral recovery. Therefore, gangliosides have been recommended as useful pharmacological agents to reinforce the plasticity of the nervous system and to counterbalance the mechanisms leading to neuronal degeneration or death[3]. The precise mode of action by which the glycolipids exert their propitious effects still remains speculative. Thusfar, no systematic structure-activity study with the individual glycolipids have been performed, presumably due to difficulties encountered in their isolation. In an attempt to obtain insight into structural requirements for neurotrophic activity of glycolipids, we and others have focussed attention on the design of synthetic ganglioside mimetics. It was established that the presence of both a sialic acid residue and the lipid moiety in gangliosides are essential features for neurotrophic activity. Tsuji *et al.* reported[4] that α-sialocholesterol[5] (αSC) induced neuritogenesis in the mouse neuroblastoma cell line Neuro2A. Interestingly, the neuritogenic aptitude of the latter compound was significant higher than those of natural gangliosides. Recently, we have disclosed that αSC also protects against a cytostatic drug-induced decrease of laminin production by Schwann cells[6]. Using new sialic acid donors, we have synthesized several sialic acid-lipid conjugates having one or more sialic acid residues. Furthermore, we have investigated the neurotrophic potential of the synthetic compounds on N1E.115 neuroblastoma cells.

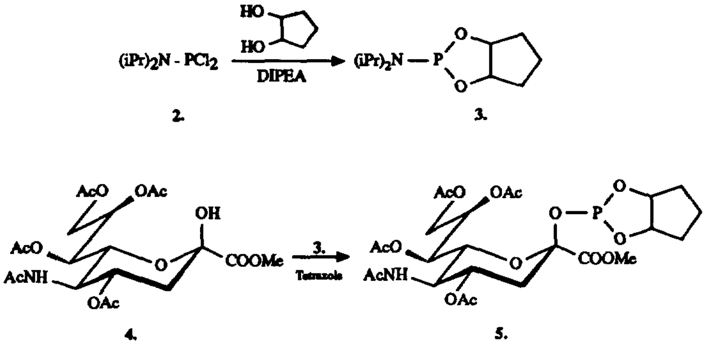
Results and discussion

Glycosidation strategies with sialic acid derivatives have made significant progress in recent years[7].

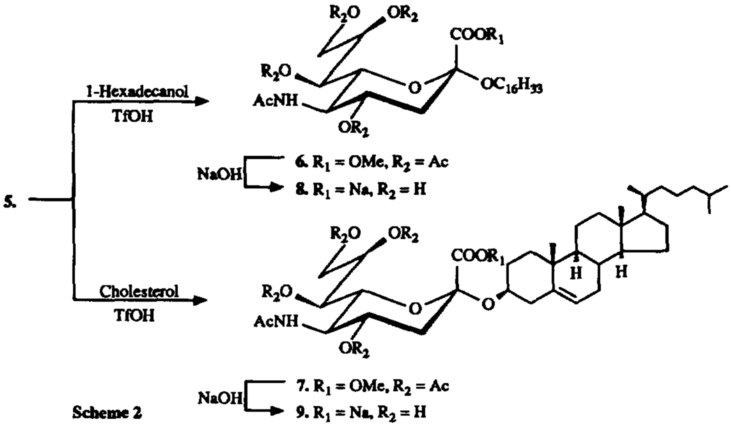


1.

New methods have emerged which reduces the competing elimination reaction that gives rise to glycal **1**. We have utilized the phosphite methodology, originally introduced by Schmidt[8] and Wong[9], for the synthesis of sialic acid-lipid conjugates. In our approach, we have explored the use of the bicyclic phosphite **5**. Amidite **3** (^{31}P-NMR: δ 155.4) was readily obtained by reacting dichloridite **2** with cis-1,2-cyclopentanediol in the presence of DIPEA. Condensation of bicyclic amidite **3** with hemiketal **4** in the presence of tetrazole gave phosphite **5** (^{31}P-NMR: δ 140.3; 134.6) in almost quantitative yield. Compound



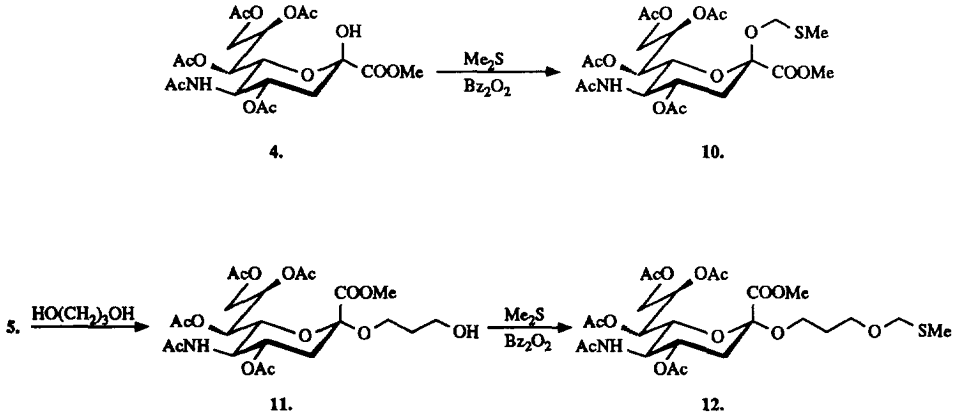
Scheme 1



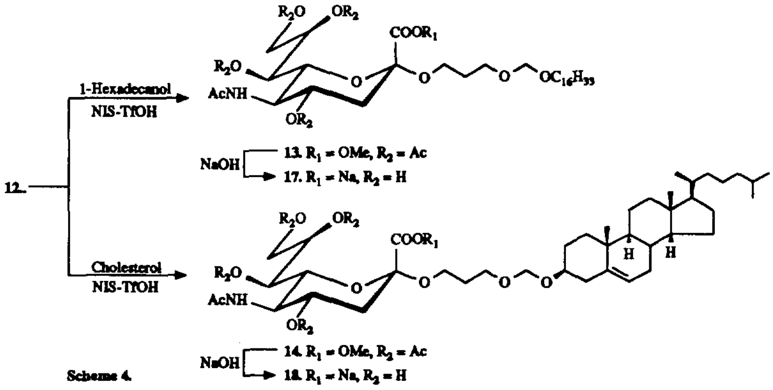
Scheme 2

5 was found to be fairly stable which allowed chromatographic purification. Phosphite **5** was condensed with 1-hexadecanol in the presence of catalytic TfOH to give α-sialohexadecanol **6** as the major product (63%, α/β 85:15). In a similar fashion, condensation of phosphite **5** with cholesterol furnished α-sialocholesterol **7** (60%, α/β 80:20). These results show that bicyclic phosphite **5** is a very convenient sialic acid donor. Removal of the base-labile protecting groups from **6** and **7** afforded **8** and **9**, respectively[18].

Although the yields and α/β ratios of the above sialidations are quite acceptable, we found this and other sialidation procedures currently available less suitable for simultaneous introduction of two ore more sialic acid residues into polyols due to the occurrence of inseparable anomeric mixtures. To circumvent this problem we have searched for an alternative approach. Recently, it was shown[10] that a methylthiomethyl (MTM) functionality in nucleosides could be readily activated with *N*-iodosuccinimide (NIS) and catalytic trifluoromethanesulphonic acid (TfOH) to give, in the presence of alcohols, formacetals in excellent yield. We reasoned that introduction of a methylthiomethyl function at the reducing end of properly protected sialic acid derivatives would engender an iodonium ion activatable sialic acid donor suitable for the preparation of formacetal linked sialosides having a similar anomeric configuration as the starting MTM derivative. Initially we have investigated the features of compound **10** having a MTM function attached directly to the ketosidic hydroxyl function of sialic acid. Satisfactory results were obtained by reacting hemiketal **4** with benzoylperoxide and dimethylsulphide[11] to give **10** in 60% yield. Under these conditions, exclusively the β-anomer was formed. Unfortunately, no reaction was observed in NIS-TfOH mediated condensation with alcohols. The latter result may be due to a strong deactivating effect of the C-1 carboxyl group. To overcome this impediment we anticipated that introduction of a propyloxy spacer between the sialic acid residue and the MTM function would decrease the deactivating nature of the carboxyl group. Therefore, the synthesis of derivative **12** was undertaken. Condensation of phosphite **5** with 1,3-propanediol (5 eq.) was effected at low temperature with catalytic TfOH (0.01 eq.) to give α-linked 3-hydroxypropyl sialoside **11** in 65% yield. Introduction of the MTM function in **11** was accomplished by reaction with benzoylperoxide and methylsulphide to provide target MTM compound **12** in 90% yield. Compound **12** could now readily be activated by the iodonium ion source NIS-TfOH to give, in the presence of cholesterol and cetyl alcohol, formacetal linked sialosides **13** and **14** in 80 and

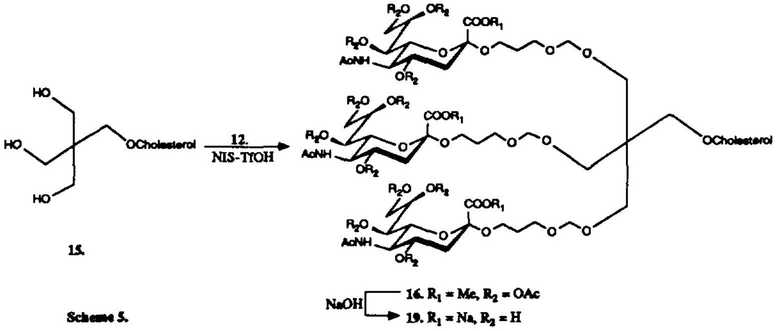


Scheme 3



Scheme 4.

85% yield,respectively. Thus, the incorporation of a propyl spacer between the sialic acid residue and the MTM function in **12** effectively circumvents the deactivating nature of the carboxyl group. We then turned our attention to the synthesis of trisialolipid **16**. 2,2-Bis-hydroxymethyl-3-hydroxypropyl-cholesterol (**15**) was obtained by condensing[12] tosyl cholesterol with pentaerythrol. NIS-TfOH mediated reaction of excess (3.3 eq.) MTM-derivative **12** with triol **15** proceeded smoothly to produce tris-sialoside **16** in 70% yield. The latter result clearly indicates that MTM derivative **12** is indeed very suitable for simultaneous introduction of several sialic acid residues into polyols. Zemplèn deacetylation and subsequent saponification of the methyl esters in compounds **13**, **14** and **16** furnished sialosides **17**, **18** and **19** which were isolated as the corresponding sodium salts[18].



Scheme 5.

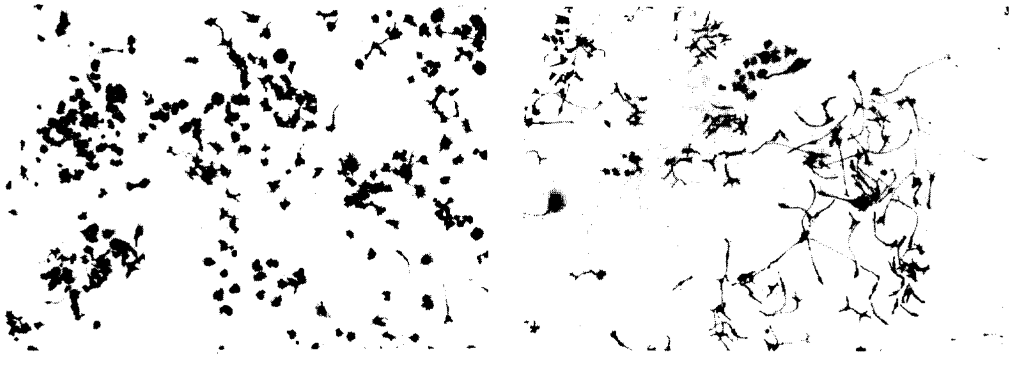
Neuritogenic activity of the sialic acid-lipid conjugates on N1E.115 neuroblastoma cells.

The synthetic compounds **8**, **9**, **17**, **18** and **19** were tested for their capacity to induce neurite outgrowth on N1E.115 neuroblastoma cells which is a clone of the murine neuroblastoma cell line C1300. In vitro these cells can be induced to differentiate into neurite forming cells. We have measured the effect of sialic acid-lipid conjugates on neurite outgrowth by measuring the presence of cytoskeletal components by a neurofilament (NF) ELISA[13]. By performing a neutral red (NR) assay the cell number relative to control was defined. By calculating the NF/NR ratio the average amount of neurofilament per cell could be determined. Preliminary experiments with a more time consuming morphometric analysis indicated that the NF/NR assay gives reliable information on neurotrophic activity. For comparison, sodium cholesterylmethylenecarboxylate (CA) and ganglioside GM1 were also tested.

The results are shown in *Table 1.* It can be concluded that the synthetic compounds exhibit neuritogenic activity on the N1E.115 cell line. We found the concentration range to be rather narrow. Maximum effects were observed close to 3 μM. At lower concentration the effects tend to decrease rapidly. On the other hand, at concentrations above 10 μM cytotoxic effects predominate. The latter finding is in agreement with results obtained by Tsuji *et al.* using the Neuro2A cell line[4]. Compounds **8** and **9**, having the lipid moiety attached directly to the anomeric center, of sialic acid exhibited moderate increase in neurofilament production. Introduction of a spacer between the sialic acid and cholesterol units in compound **18** significantly enhances neurite outgrowth. However, a similar effect is not seen with **17** in which cholesterol is replaced by a hexadecyl unit, indicating that the nature of the lipid contributes significantly to the desired activity. The most active compound detected thusfar appears to be trisialocholesterol **19**. Thus, the number of sialic acid residues strongly influences the neurite inducing capacity (*Fig.1*). This remarkable result is in good agreement with findings of Tsuji[14] in which the ganglioside GQ1b, having four sialic acid residues, showed the highest neuritogenic activity in the human cell line GOTO of a variety of gangliosides tested. In our N1E.115-assay we did not observe any activity of ganglioside GM1, however. It is likely, therefore, that the mechanism of action of gangliosides and synthetic sialic acid-lipid conjugates is different[15]. Furthermore,

	1 μM				3 μM			
Compound	**NF**	**NR**	**NF/NR**	**Neurites**	**NF**	**NR**	**NF/NR**	**Neurites**
8	**0**	--	+	+	**0**	--	+	+
9	**0**	**0**	**0**	**0**	--	---	+	+
17	**0**	**0**	**0**	**0**	**0**	-	+	+
18	**0**	--	+	+	**0**	--	++	++
19	**0**	-	+	+	**0**	--	+++	+++
CA	**0**	-	**0**	**0**	**0**	-	**0**	**0**
GM1	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

***Tabel 1.** Semi-quantitative evaluation of drug treatment on neurofilament content (NF), neutral red absorbance (NR), NF/NR-ratio (NF/NR) and neurite outgrowth as seen through the microscope (neurites). (control cells = 100%). "---" 65-75%; "--" 75-85%; "0" 95-105%; "+" 105-115%; "++" 115-125%; "+++" 125-135%.*



Control

Compound 19 (3 μM)

***Figure 1.** Effect of 19 on neurite outgrowth in N1E.115 cells. Cells and neurites are stained with Coomassie Brilliant Blue*

cholesteryl-methylenecarboxylate (CA) did not show significant activity, indicating that the neuritogenic activity is depending on structural features of the sialic acid residue. In conclusion, the results obtained in this study indicate that MTM-derivative **12**, readily obtained by the phosphite approach, is a very convenient sialic acid donor for the synthesis of sialic acid-lipid conjugates having one or more sialic acid residues[16]. Furthermore, the synthetic sialic acid-lipid conjugates exhibit interesting neurotrophic activity denoting that this class of molecules may be useful to alleviate neuropathological syndromes. In particular, the trisialocholesterol derivative **19** appeared to be very promising. Further structure-activity studies are required to obtain more insight into the mechanism of action of these glycolipids.

References and notes

\# Part of this work was presented at the "Glycotechnology" symposium in Washington DC, U.S.A., February 28 - March 2, 1994.

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13. **Cell culture:** The mouse neuroblastoma cell line, N1E.115, was cultures in M505 medium (Hyclone) with 10% bovine calf serum and 1% non-essential amino acids (NEAA, Flow laboratories). The N1E.115-cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. N1E.115 cells were plated at a density of 5,000 cells in 100 µL per well in M505 + 10% BCS + 1% NEAA in flatbottom 96-wells plates which were coated in advance with poly-L-lysine (10 µg/mL, 1 h). After one day in culture the medium was aspirated off and test compounds dissolved in fresh medium (100 µL) were added to the wells and incubated for 48 h. Next, the plates were examined microscopically and a neutral red assay and neurofilament assay were performed in parallel. **Neurofilament assay:** 100 µL of 8% paraformaldehyde in phosphate buffered saline (PBS) was added to each well for 15 min and were washed with PBS. The wells were preincubated for 30 min with 100 µL PBS containing 0.2% Triton X-100 and 10% horse serum, pH 7.4, and incubated overnight at 37°C with 50 µL mouse-anti-neurofilament (RT 97, Boehringer Mannheim). Next the wells were rinsed and incubated with 50 µL horse-radish peroxidase conjugated sheep-anti-mouse IgG (ShAM-PO, Sigma). The wells were rinsed with PBS and stained with tetramethylbenzidine substrate. The reaction was quenched by adding 50 µL 0.18M H_2SO_4 and plates were read in an ELISA plate reader at 450 nm against 620 nm. **Neutral red assay:** The effect of the test compounds on the number of viable cells was assessed by the incorporation of neutral red into the lysosomes of viable cells[17]. 50 µL Neutral red (0.5 mg in 0.9% NaCl) was added to each well and incubated for 1.5 h in a CO_2-incubator at 37°C. The wells were washed with PBS and 50 µL of lyase buffer (0.05M NaH_2PO_4 in 50% ethanol) was added to extract the dye from the cells. Optical density was then red at 540 nm against 620 nm using a 7520 Microplate Reader (Cambridge Technology).
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15. Recently, Tsuji [*J. Neurochem.* **1992**, *58*, 1360] demonstrated that αSC is readily taken up by Neuro2A neuroblastoma cells and influences *de novo* RNA-synthesis. In contrast, gangliosides are not significantly taken up and have no effect on gene expression. Thus, it may be hypothesized that gangliosides act extracellularly (perhaps receptor mediated) whereas small sialic acid-lipid conjugates perform their activity intracellularly. It is not clear whether the N1E.115 cell line expresses ganglioside receptors.
16. Compound **12** or its analogues may also be useful for the preparation of cluster sialoside inhibitors for Influenza Virus (e.g. see Sabesan, S. *J. Am. Chem. Soc.* **1992**, *114*, 8363).
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18. Relevant NMR data: ^1H NMR ($CDCl_3$, δ-values): **6**: 2.58, dd, 1H, H-3_{eq}, $J_{3,3'}$ 12.4 Hz, $J_{3,4}$ 4.4 Hz; **7**: 2.60, dd, 1H, H-3_{eq}, $J_{3,3'}$ 12.5 Hz, $J_{3,4}$ 4.6 Hz; **8**: 2.68, dd, 1H, H-3_{eq}; **9**: 2.81, dd, 1H, H-3_{eq}; **10**: 4.66, AB, 2H, OCH_2S; **11**: 2.58, dd, 1H, H-3_{eq}, $J_{3,3'}$ 12.4 Hz, $J_{3,4}$ 4.4 Hz; **12**: 4.66, s, 2H, OCH_2S; **13**: 4.65, s, 2H, OCH_2O; **14**: 4.73, s, 2H, OCH_2O; **16**: 4.62, s, 6H, 3x OCH_2O; **17**: 1.99, t, 1H, H-3_{ax}; 2.98, dd, 1H, H-3_{eq}; 4.82, s, 2H, OCH_2O; **18**: 2.81, dd, 1H, H-3_{eq}; 4.72, s, 2H, OCH_2O; **19**: 2.80, d, 3H, 3x H-3_{eq}; 4.63, s, 6H, 3x OCH_2O.

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