# notes on methodology

## Notes on improved procedures for the chemical modification and degradation of glycosphingolipids

D. L. MacDonald,[1] L. M. Patt,[2] and S. Hakomori

*Division of Biochemical Oncology, Fred Hutchinson Cancer Research Center and Departments of Pathobiology and Microbiology, University of Washington, Seattle, WA 98104*

**Summary** Some simplified and efficient procedures are described for the following chemical modifications of glycosphingolipids. 1) The olefinic bond of the ceramide moiety of the acetylated glycolipid was quantitatively oxidized with osmium tetroxide and periodic acid. Treatment of the resulting glycolipid aldehyde with sodium methodixe resulted in the release of the intact oligosaccharide. The yield of oligosaccharides under the new condition was much higher than previously found. 2) The olefinic bond was also oxidized to a carboxyl function by either of two methods: *a*) the aldehyde group resulting from the above oxidation was further oxidized with performic acid; and *b*) the olefinic bond of the fully acetylated glycolipid was oxidized directly to the acid by potassium permanganate in acetone. 3) The methyl ester of the carboxyl group of the sialic acid in gangliosides can be formed with diazomethane in methanol-ether after treatment of the gangliosides with Dowex-50 ($H^+$ form). Possible uses of these glycolipid modifications are discussed.—**MacDonald, D. L., L. M. Patt, and S. Hakomori.** Notes on improved procedures for the chemical modification and degradation of glycosphingolipids. *J. Lipid Res.* 1980. **21:** 642–645.

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Chemical modification of glycosphingolipids has been explored for various purposes. One chemical modification that is of considerable interest is the cleavage of the glycosidic bond linked to the ceramide to obtain an intact oligosaccharide. This can be accomplished by oxidation of the olefinic double bond of sphingosine to an aldehyde group by ozone (1, 2) or with osmium tetroxide and sodium metaperiodate (3, 4); the oligosaccharide is released from the resulting aldehyde by treatment with alkali, presumably through the migration of the carbonyl double bond and $\beta$-elimination. Alternatively, the allylic hydroxyl group (secondary hydroxyl function adjacent to the double bond of sphingosine) can be oxidized to a ketone by dichlorodicyanobenzoquinone (Kishimoto's oxidation) (5) followed by exposure to alkali resulting in the release of the oligosaccharide by $\beta$-elimination (6). However, none of these procedures result in the quantitative release of oligosaccharide, and the yield of the oligosaccharide can be highly variable. We have studied the conditions for oxidation with osmium tetroxide and sodium metaperiodate (3) and found that a slight modification can greatly improve the yield of oligosaccharide. This modification is described in the first part of this communication.

Another chemical modification has been designed to introduce functional groups that will facilitate coupling of a glycolipid to a suitable solid support. For this purpose the olefinic double bond of the sphingosine has been oxidized to a carboxyl function which can then be coupled to amino groups in the supporting structures (7–9). Simplified procedures for this oxidation will be described in the second part of this paper.

The third chemical modification involves the introduction of a protective methyl ester group to the sialic acid carboxyl of gangliosides. The usual procedure for sialic acid esterification which involves stirring with Dowex-50W in methanol (10–12) is not quantitative and requires several days. The methyl-esterification of gangliosides with diazomethane in ether usually is not successful. However, if a methanolic solution of ganglioside is first passed through a small column of Dowex 50W × 8, subsequent esterification with diazomethane is then rapid and essentially quantitative. This simplified procedure is described in the third part of this paper.

## MATERIALS AND METHODS

The glycolipids used were prepared in this laboratory using established procedures (13–15). Ceramide dihexoside and globoside were from human erythro-

Abbreviations: glycolipid aldehyde, 2-hydroxy-3-(*N*-fatty acylamido)-4-glycosyl-butyraldehyde; glycolipid acid, the corresponding butyric acid derivative; ceramide dihexoside, Gal($\beta$1 → 4)Glc → Cer; ceramide trihexoside, Gal($\alpha$1 → 4)Gal($\beta$1 → 4)Glc → Cer; globoside, GalNAc($\beta^1$ → 3)Gal$\alpha$(1 → 4)Gal($\beta$1 → 4)Glc → Cer; *N*-acetylhematoside, NeuNAc($\alpha$2 → 3)Gal($\beta$1 → 4)Glc → Cer; $GM_1$, Gal($\beta$1 → 3)GalNAc($\beta$1 → 4)[NeuNAc($\alpha$2 → 3)]Gal($\beta$1 → 4)Glc → Cer. TLC: thin-layer chromatography.

[1] On sabbatical leave, 1978–1979, from the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331.

[2] Present address: Virginia Mason Research Center, 1000 Seneca St., Seattle, WA 98101.

cytes, *N*-acetylhematoside from dog erythrocytes, Forssman glycolipid from goat erythrocytes and $GM_1$-ganglioside from bovine brain. Gangliosides were separated by DEAE-Sephadex chromatography with an ammonium acetate gradient (16). Specific reagents, solvents, and chromatography absorbent are indicated under each procedure described below. Globoside and CDH were labeled with tritium in the terminal galactose residue by the method of Suzuki and Suzuki (17).

### Preparation of glycolipid aldehyde

A sample of glycolipid or ganglioside methyl ester (1–3 mg) was acetylated in a mixture of pyridine (1 ml) and acetic anhydride (0.5 ml) at 37°C overnight. The mixture was taken to dryness in a stream of nitrogen, and added toluene was evaporated to remove all acetylating reagents. The product was dissolved in 0.5 ml of methanol, and to this was added 0.2 ml of freshly prepared 0.2 M periodic acid ($HIO_4$ reagent grade) in methanol, followed by 25 $\mu$l of freshly prepared 5% osmium tetroxide (obtained through Sigma Chemical Co.) in ether, and the solution was left at 5°C overnight. Thin-layer chromatography of a sample on silica gel G using dichloroethane–acetone 55:45, followed by spraying with orcinol revealed a complex mixture in which only traces of unoxidized starting material remained.

A drop of glycerol was added to the oxidation mixture, followed by 6 ml of chloroform–methanol 2:1 and 1.5 ml of water. After mixing and separation of the layers (centrifugation may be necessary), the organic phase was washed four times with 2.5 ml portions of chloroform–methanol–water 1:10:10. The organic solvent was then removed in a stream of nitrogen and the residue dried by addition and subsequent evaporation of toluene.

### Release of oligosaccharides from glycolipid aldehyde

The acetylated glycolipid aldehyde was dissolved in 0.3 ml of methanol and 50 $\mu$l of 0.2 M sodium methoxide in methanol was added. The basic solution was left at room temperature for 1 hr. To saponify the sialyl methyl ester of gangliosides a drop of water was added at the point and the basic solution left 1 hr at room temperature. A drop of glacial acetic acid was added and the solvents were removed in a stream of nitrogen. The residue was dissolved in 0.5 ml of chloroform–methanol–water 1:10:10 and the solution washed five times with 0.5 ml portions of chloroform–methanol–water 60:35:8; in each instance, centrifugation was usually necessary prior to removal of the organic layer by aspiration. The aqueous layer contained essentially pure oligosaccharide. It was examined by paper chromatography using ethyl acetate–pyridine–water 12:5:4 as solvent and silver nitrate-sodium hydroxide as detecting agent, or by thin-layer chromatography on silica gel G, using n-butanol–ethanol–water 50:30:20 as solvent and orcinol as spray reagent.

The yield of oligosaccharide was as follows: ceramide dihexoside 44%, ceramide trihexoside 70%, globoside 69%, *N*-acetylhematoside 61%, and $GM_1$ 53%.

### Oxidation of "glycolipid aldehyde" to the corresponding acid

A performic acid solution was prepared by mixing 50 $\mu$l of 30% hydrogen peroxide with 1 ml of 88% formic acid and allowing the mixture to stand at room temperature for 2 hr. The acetylated glycolipid aldehyde described above was dissolved in 0.2 ml of freshly-prepared performic acid solution and left at room temperature 20 hr. A stream of nitrogen was used to remove solvent and residual traces were removed by addition of toluene and evaporation with nitrogen. The product, dissolved in benzene, was purified by chromatography on 3 g of BioSil A (Bio-rad Chemical Co., Richmond, CA, 100–200 mesh); elution was accomplished using benzene containing increasing concentrations of acetone. Thin-layer chromatography on Silica gel G with dichlorethane–methanol 8:2 was used to monitor the separation. The solvent composition which eluted the product varied with the starting glycolipid; that from CDH was eluted with benzene containing 30% acetone, while 50% acetone was required for the product from globoside. The yields obtained were: ceramide dihexoside 66%, globoside 63%, *N*-acetylhematoside 28% and $GM_1$ 56%.

### Globoside oxidation by permanganate in acetone

Two mg of globoside containing approx $4 \times 10^6$ cpm of [$^3$H]globoside was acetylated with 1 ml of acetic anhydride and 2 ml of pyridine overnight at 37°C. The acetic anhydride and pyridine were removed by repeated evaporation to dryness in the presence of excess toluene. The fully acetylated glycolipid was dissolved in 0.2 ml of acetone and 0.3 ml of a 10 mg/ml solution of $KMnO_4$ in acetone was added. The reaction mixture was allowed to stand at room temperature overnight. The reaction was partitioned by the addition of 3 ml of chloroform–methanol 2:1 and 0.5 ml of 0.1N $H_2SO_4$. The pooled organic layers were concentrated and dissolved in a small volume of benzene. The glycolipid acid was separated on a Bio-Sil A column in benzene using increasing concentrations of acetone in benzene. The yield of acetylated oxidized globoside was 73% plus an addi-

tional 11% obtained by re-oxidation of the acetylated globoside that was removed from the Bio-Sil chromatography. The yield is based on the acetate and is better than that described in the previous method (9).

The oxidized globoside was deacetylated by treatment with sodium methoxide in methanol, followed by removal of cations with Dowex-50W × 8 ($H^+$ form). In chloroform–methanol solution this oxidized globoside was converted slowly to a less polar substance as indicated by the change of TLC mobility. The process was reversed with mild alkali, suggesting that lactone formation had occurred.

**Methylesterification of the sialic acid carboxyl group of gangliosides**

Esterification of gangliosides was accomplished by first passing a methanolic solution of the material (1–3 mg) through a small column (*ca* 1 ml) of Dowex-50W × 8 (100–200 mesh $H^+$ form) in methanol and washing with methanol. The effluent was concentrated in a stream of nitrogen and dissolved in 0.1 ml of methanol; then, an excess of distilled diazomethane in ether was added and it was kept in a dark place. Diazomethane was conveniently prepared from *N*-methyl-*N'*-nitrosoguanidine in a simple special tube obtained from Aldrich Chemical Company (The Aldrich-Diazald Kit, catalogue #Z10,025-0, Milwaukee, WI). After about 30 min, the reaction mixture was concentrated in a stream of nitrogen and the residue dried by addition and evaporation of toluene. This product, chromatographed on Silica gel G in chloroform–methanol–water 60:35:8, and detected using resorcinol, was essentially free of starting material.

Esterification of $GM_3$ with Dowex 50 in methanol usually resulted in several components, two major and a few minor, in addition to unreacted starting material. A band with $R_f$ 0.4–0.45 in chloroform–methanol–water 65:25:4 was identified as $GM_3$ having a methylester sialic acid and a band with $R_f$ 0.33 in the same solvent was identified as $GM_3$ with lactone structure.[3] The unreacted starting material had a band with $R_f$ 0.1–0.15. The proportion of these components varied depending on a subtle difference of the condition, however it was impossible to convert all $GM_3$ into esters even after 3–5 days reaction. Esterification of $GM_1$ under the same condition as above also resulted in several minor components and a major component that was identified as the ester ($R_f$ 0.05 in chloroform–methanol–water 65:25:4). Minor components could be a degradation product.

[3] Identified by direct probe mass spectrometry as the trimethylsilyl derivative. Yoshino, T., and S. Hakomori. Unpublished data.

A pure sample of $GM_3$ and $GM_1$ was respectively esterified by the present method with diazomethane. In both cases, the reaction was quantitative to give a single ester component and the whole procedure, including the preparation of diazomethane, can be finished within an hour.

## DISCUSSION

The release of oligosaccharides from glycosphingolipids has been based on the oxidation of the olefinic double bond to an aldehyde or of the allylic hydroxyl group to a ketone followed by base-induced double bond migration and $\beta$-elimination (1–4, 6). The drawback to these reactions is the variable yield of oligosaccharide, possibly due to incomplete oxidation of the olefinic double bond. The oxidation reaction with osmium tetroxide and sodium metaperiodate has also given variable results, possibly due to subtle variations in the reagents used. We have studied the conditions of the reaction and have found that the reaction could be quantitative and consistent if *a*) the concentration of osmium tetraoxide is increased and *b*) sodium periodate in aqueous methanol is replaced with periodic acid in methanol. The method can be applied to a microgram quantity of glycolipids and is useful for structural and biological studies of glycosphingolipids. The liberated oligosaccharide can be utilized for structural studies and for the inhibition of immunological or biological reactions thought to involve glycolipids. Recently, Nilsson and Svensson (18, 19) reported that oligosaccharides can be liberated from sphingosine by trifluoroacetolysis with trifluoroacetic acid and trifluoroacetic anhydride. However, the method will destroy sialic acids and partially destroy amino sugars. Therefore, the method described in this paper is still valuable to obtain intact oligosaccharides from glycosphingolipids.

The oxidation of the olefinic double bond of sphingosine in glycosphingolipids to a carboxyl function has been greatly simplified by permanganate oxidation in acetone. The procedure is simpler when compared to the method previously described (7–9) and does not utilize the expensive "crown ether." The glycolipid acid thus prepared will be a useful intermediate for covalent attachment of glycolipids to solid supports and to the water soluble macromolecules. The glycolipid-solid support conjugates are useful for purification of antibodies (7, 8), hormones, growth factors and other ligands that interact with glycolipids (20), and perhaps for affinity purification of glycosyl hydrolases and transferases. A water-soluble glycolipid-macromolecule complex is useful as a potent

glycolipid antigen and in the radioimmunoassay of glycosphingolipids (21).

In the coupling of gangliosides to solid supports or macromolecules, the sialic acid carboxyl group has been protected by methyl esterification (7, 8). This modification is important to avoid the linkage between the sialic acid carboxyl and amino groups of the support. The same esterification of the carboxyl group of sialic acids can be used to obtain a ganglioside derivative whose sialic acid carboxyl function is reduced to a primary alcohol in 50% aqueous methanol containing 1% $NaBH_4$ or $KBH_4$ at room temperature for 5–6 hr. The reduced compounds were characterized by yellow color development with resorcinol · HCl reaction in which normal gangliosides and their esters showed a violet color ('gangliosidol and hematosidol").[4] The method proposed in this paper, based on diazomethane treatment after cation elimination has been found to be quantitative and instantaneous. This reaction, if combined with permanganate oxidation in acetone, will greatly improve the efficiency of ganglioside coupling to various solid supports and macromolecules.

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