THE LARGE SCALE DEUTERIUM LABELING OF CEREBROSIDE AT THE
3-SPHINGOSINE POSITION

by

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THESIS
FOR THE
DEGREE OF BACHELOR OF SCIENCE
IN
CHEMISTRY

College of Liberal Arts and Sciences
University of Illinois
Urbana, Illinois
1979
UNIVERSITY OF ILLINOIS

May 9, 1979

THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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ENTITLED: The Large Scale Deuterium Labeling of Cerebroside at the 3-Sphingosine Position

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science in the Chemical Sciences

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Instructor in Charge

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HEAD OF DEPARTMENT OF Chemistry
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I. Introduction

Until recently, one of the least studied and poorest understood organs of the body has been the animal brain. Chemical investigation of organs of the body developed around 1800.¹ At that time the gross anatomy of the central nervous system was already known, but its physiology and pathology was unknown.² Those studies became commonplace during the 19th century, but biochemical and electrophysiological studies were uncommon until the 1920's.³ Studies of brain functions and specifically the functions of chemicals within the brain are just in their pioneer days.

One type of brain chemical, cerebrosides, was discovered and characterised as early as 1884 by Thudichum.⁴ Although cerebrosides are found primarily in the brain, they have been isolated from adrenal glands, kidneys, spleen, liver, and atherosclerotic vessels.⁵

Cerebral white matter of many animals is one of the richest sources of cerebrosides.⁶ By weight, 5.3% of the spinal cord of adult mammals is cerebroside and 2.4% of the brain as a whole is cerebroside.⁷ Cerebrosides are absent from many but not all invertebrates and have been isolated in fungi.⁸

Chemically, cerebrosides consist of a base (sphingosine) bound to a sugar (galactose) and a long-chained fatty acid (these vary in length from fifteen to twenty-four carbons). (See Fig. 1)

Dr. Eric Oldfield (associate professor, University of Illinois) and Robert Skarjune (graduate student, University of Illinois) have
**Figure 1**

*erythro-N-palmitoyl galactosyl cerebroside*
performed deuterium Fourier transform nuclear magnetic spectra on a series of N-palmitoylgalactosylceramides (cerebrosides) labeled with deuterium at the 7, 6, 10, and 16 positions of the acyl chain or in the C-6 hydroxymethyl group of the galactose portion. These were obtained using a spin-echo technique at 34.1 MHz with a home-built superconducting magnet spectrometer. 9

Until now, no attempts had been made by the Oldfield group to deuterium label the sphingosine portion of cerebroside. The purpose of this study is to investigate the utility of labeling N-palmitoylgalactosylceramides with deuterium at the 3-sphingosine position at a scale large enough to enable Oldfield et al. to perform NMR studies on the resulting product (250 milligrams are needed for each spectrum).
Footnotes


6 McIlwain and Bachelard, *op. cit.*, p. 344.


II. Experimental Summary

The labeling of erythro N-Palmitoylgalactosylcerabroside (obtained at the 3-sphingosine position is done as follows:

The alcohol at the third position of the base is oxidized to the corresponding ketone. This is done using a 3% solution of 2,3-dichloro-5,6-dicyanobenzoquinone in pre-distilled dioxane at 37°C which is allowed to react with the starting material for 72 hours.

Buren, Petrow, and Weston (1960) found that a slight excess of dichlorodicyanobenzoquinone (DDQ) dissolved in dry dioxane or benzene oxidizes steroidal allylic alcohols to the corresponding 3-ununsaturated ketone in excellent yield. Under these conditions no other functional groups were altered by the reagent.

The 3-sphingosine position of cerebroside conforms excellently to the conditions needed for its selective oxidation by DDQ, but cerebroside is not soluble in dioxane at room temperature. Kishimoto and Mitry (1974) found that the material would gradually dissolve in dioxane at 37°C. Nevertheless a relatively large amount of dioxane was required.

Although a 3% solution of the oxidizing agent creates a large excess of DDQ, Kishimoto and Mitry found that with a solution of 1.5% or less concentration, varying amounts of starting material were recovered and that 3% worked best.
Tsuchimi, Moeer, McCluer, and Kishimoto (1975) tested various reaction times to determine which length was best. They found that the oxidation reached completion after 48 hours and that another 24 hours produced no change. They were working with only 40 micrograms of starting material, so we upped the reaction time to 72 hours for 1 gram prep.

At the end of the reaction the solvent is removed. The solid residue is taken up in chloroform-methanol (2:1) and then washed with enough 0.1M NaOH to remove the excess reagent. The lower phase is further washed with portions of 0.1M NaOH-Methanol (1:1) until the upper phase is colorless. The lower phase is finally washed repeatedly with Methanol-water (1:1) until the pH is neutral.

The final lower phase is evaporated to dryness and a fairly pure ketone is isolated by column chromatography using silica gel and eluting first with chloroform-methanol (98:2) to rid of remaining reagent, and then with chloroform-methanol (2:1) to obtain ketone containing fractions.

The ketone fractions are pooled and the solvent is removed. The relatively pure ketone is dissolved in pre-distilled tetrahydrofuran. It is then mixed with a solution of 0.1M NaOH containing a slight excess of sodium borodeuteride. The NaBD4 should re-reduce the ketone while introducing deuterium to the 3-sphingosine position.
The solution is allowed to stand for 30 minutes (up from 15 in the smaller scale preps). Another equal portion of the reducing solution is added and the mixture is allowed to stand another 30 minutes.

The mixture is then chilled in ice and the excess reducing agent is destroyed by adding 0.1M acetic acid. The product is extracted with chloroform-methanol (2:1) and then washed.

The resulting deuterated cerebrosides are saponified with methanolic KOH so there are no fatty acid mixtures among the products.

The product is column purified by eluting with fractions of chloroform, chloroform-methanol (4:1), chloroform-methanol (2:1), chloroform-methanol (1:4), and methanol.

The resulting erythro-threo mixture of psychosines, the reduction is stereoselective in a ratio of 84:16 erythro-threo, is separated into erythro and threo components using a series of preparatory TLC plates and eluting with chloroform-methanol-concentrated ammonia (14:5:1).

The resulting psychosine is reacted with palmitoyl chloride to produce erythro-N-palmitoyl galactosyl cerebroside labeled with deuterium at the 3-sphingosine position.
Footnotes


III. The First Prep

Our first attempt at a 3-sphingosine labeling was on a 200 milligram scale. We decided to attempt the labeling on psychosine (cerebroside with the fatty acid stripped off). This was done to increase the solubility of the starting material in dioxane, and if successful it would allow us to skip the saponification step later in the reaction scheme.

116 milligrams of the psychosine (equivalent in moles to 200 milligrams of cerebroside) was dissolved in a 200 milliliter solution of pre-distilled dioxane containing 6 grams of DDQ.

The mixture was stirred magnetically in a round bottom flask at 37°C for 48 hours. The 37 degree temperature was maintained by having the flask very slightly elevated above a dual stirrer-heater. The heater was set at the lowest setting.

As the reaction proceeded, the mixture maintained a deep orange-red color. This was contrary to earlier reports that the reaction mixture would initially be orange and darken to red as the reaction proceeded.

After 48 hours, the dioxane was removed on a rotary evaporator. The residue was taken up in 200 milliliters of chloroform-methanol (2:1) and washed with 40 milliliters of 0.1M NaOH to remove excess reagent.

The lower phase was washed with 100 ml portions of methanol—
0.1M NaOH until the upper phase was virtually colorless. This involved 13 washings.

During the washings, there was a decrease in the volume of the organic phase and the washings cleared up fairly slowly. After the tenth washing, a small amount of second phase was found to have separated from the combined aqueous washings. This was added to the organic phase for the final three washings.

There were major problems with the methanol-water (1:1) washings. The first addition of 100 milliliters of methanol-water produced no phase separation. Addition of 25 milliliters of chloroform produced an emulsion. 50 additional milliliters of chloroform produced little change. A trace of potassium chloride caused the solution to clear up within 30 minutes. The pH at this point was 9.0 so a second washing was attempted. Chloroform addition helped cause the resulting emulsion to clear up. With a pH of 8.5, the organic phase was TLC analyzed and was found to contain no ketone. TLC's were performed on silica gel and developed in chloroform-methanol-concentrated ammonium hydroxide (85:15:1). The visualizing agent was 2,4-dinitrophenylhydrazine sulfate.
Footnotes


IV. Prep Number Two

In this prep the experimental conditions and procedures were identical to the first prep, except 200 milligrams of cerebroside were used instead of psychosine. The purpose of this prep was to compare psychosine results to cerebroside results in addition to ironing out the ketone procedure.

Notable results are as follows: It took three hours for the cerebroside to dissolve in the dioxane solution. There were only 3 NaOH-methanol washings. During them there was a steady decrease in the volume of the organic phase. 50 milliliter portions of chloroform were added to the solution at each washing to attempt to leach out any product we were losing to the aqueous phases.

There were two methanol-water washings. Emulsions again were occurring, but this time addition of water helped clear them up.

TLC's indicated presence of ketone and unreacted cerebroside so a column was run to recover those compounds. The column consisted of 6 grams of silica gel in chloroform-methanol (98:2) upon which rested the reaction product. The product was dissolved in chloroform-methanol (90:10), added to 4 grams silica gel to form a slurry, this solution was rotovapped to complete dryness. This produced a uniform silica gel-product mixture. A slurry was formed by mixing the solid with chloroform-methanol (98:2). This slurry was added to the column.
Any remaining dichlorodicyanobenzoquinone was to be removed by eluting with 100 milliliters of chloroform-methanol (98:2) at a flow rate of 5 milliliters per minute. The product was to be recovered by eluting with chloroform-methanol (90:10) and collecting 10 milliliter fractions.

Fractions 2–4 contained ketone and cerebroside contaminated with reagent. The total weight of the solids within the three fractions was 113.4 milligrams. The low yield and high impurity of the product prompted us to start a new prep in hopes of gaining enough ketone to produce the 250 milligrams of labeled cerebroside required for a spectrum.
V. Prep Numbers Three and Four

The third prep was another 200 milligram cerebroside prep. In this prep all solvent volumes were greatly increased. In particular, the 0.1M NaOH volume was increased to 300 milliliters when it was discovered that 40 milliliters would not destroy all of the excess 2,3-dichloro-5,6-dicyanobenzenoquinone.

Other solvent volumes were as follows: the reaction mixture was taken up in 250 milliliters of chloroform-methanol (2:1) and the methanol-sodium hydroxide and the methanol-water washings were 200 milliliters apiece.

Prior to the full scale washings four, five milliliter samples of the reaction solution were pulled to investigate the utility of centrifugation to cause clear and complete separation of the washing solutions after mixing.

Along with 5 milliliters of the ketone solution, 6 milliliters of 0.1M NaOH was used in each tube for the first washing and 4 milliliters was the volume used for the sodium hydroxide-methanol and water-methanol washings. These volumes were in direct proportion to the volumes to be used in the full scale washings. For detailed washing results see table 1 (next page).

The large loss in organic volume during the washings was quite disturbing, but TLC analysis showed good ketone conversion and little loss of product to the aqueous layer.
<table>
<thead>
<tr>
<th>Washing</th>
<th>Initial Volume (ml)</th>
<th>Final Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M NaOH</td>
<td>5.2</td>
<td>3-3.5</td>
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<tr>
<td>0.1M NaOH-methanol (1:1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.0-3.5</td>
<td>2.9-3.3</td>
</tr>
<tr>
<td>2</td>
<td>2.9-3.3</td>
<td>2.6-3.0</td>
</tr>
<tr>
<td>3</td>
<td>2.6-3.0</td>
<td>2.2-2.6</td>
</tr>
<tr>
<td>4</td>
<td>2.2-2.6</td>
<td>1.9-2.4</td>
</tr>
<tr>
<td>5</td>
<td>1.9-2.4</td>
<td>1.5-1.9</td>
</tr>
<tr>
<td>6</td>
<td>1.5-1.9</td>
<td>1.3-1.8</td>
</tr>
<tr>
<td>7</td>
<td>1.3-1.8</td>
<td>0.9-1.4</td>
</tr>
<tr>
<td>8</td>
<td>0.9-1.4</td>
<td>0.6-1.1</td>
</tr>
<tr>
<td>methanol-water (1:1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.6-1.1</td>
<td>0.2-0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH after washing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>washing</td>
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<td></td>
<td></td>
<td>7.2</td>
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Centrifuging appeared promising, so we began washing and centrifuging of the 200 milligram prep and at the same time began the attempted conversion of 1 gram of cerebroside to its keto-derivative. We allowed 72 hours for the full gram to react.

Volumes for the 1 gram washings were: 1250 milliliters of chloroform-methanol (2:1), 1500 milliliters of 0.1M NaOH, and one liter of methanol-sodium hydroxide and methanol-water.

The centrifuge bottles used were conventional 250 milliliter-Nalgene polypropylene bottles. In the 200 milligram prep we used the black rubber centrifuge caps for a tight seal. The degradation of the caps in that prep prompted us to use the polypropylene screw caps that come with the bottles for the 1 gram prep. In the 1 gram prep we discovered that the chloroform was also destroying the plastic bottles. The plastic and rubber totally contaminated the products in both preps and probably caused poor recovery from the columns used to obtain products.

Noteworthy results are as follows: It took 4 to 6 methanol-sodium hydroxide (1:1) washings per bottle. Loss of organic phase was minimal, and any volume loss was replaced by addition of chloroform-methanol (2:1).

It took 1 to 3 methanol-water (1:1) washings to neutralize the pH. Centrifuging caused two clear layers and little loss of volume. Rubber and plastic were visible within the liquids, though.
The column chromatography procedure for the 200 milligram prep was identical to the column in the first 200 milligram prep. In the 1 gram prep 500 milliliters of chloroform–methanol (98:2) were used and 50 milliliter fractions of chloroform–methanol (90:10) were collected.

Yields were 50 milligrams of ketone and rubber in the 200 milligram prep and 1.155 grams of ketone, cerebroside, and plastic in the 1 gram prep. Most of the gram of product was plastic. Attempts to re-extract product from the aqueous washings of the 1 gram prep yielded plastic with some traces of ketone and cerebroside.

Lack of time and funds to obtain bottles that would not decompose in chloroform prompted us to attempt one more method to obtain a good yield of pure ketone.
VI. The Fifth Prep

In this oxidation attempt we used two ten liter bottles. Six liters of chloroform-methanol (2:1) were used to take up the product residue, 1.5 liters of 0.1M sodium hydroxide was used, and three liters of methanol-water and methanol-sodium hydroxide were used for each washing. We started with one gram of cerebroside.

The first bottle was used for the washings according to procedure. The second bottle was a type of safety bottle. Its purpose was to trap any product that was left in the aqueous layer from the first bottle. Six liters of chloroform-methanol (2:1) were placed in the second bottle. Aqueous layers from the first bottle were siphoned into the second bottle and the two layers were mixed together. This bottle and washings in the first bottle were allowed to separate overnight.

Volume and pH data can be found on table 2 (next page). Other results and observations are as follows: The methanol: 0.1M NaOH (1:1) washings went very smoothly although lower layer volumes did decrease somewhat. The methanol:water (1:1) washings caused several problems. Loss of organic volume increased at an alarming rate. This prompted us to stop the washings at pH-8.25. In addition, we took two liters of the lower layer in the second bottle and placed it in the first bottle to replenish some of the lost organic volume prior to the fourth methanol-water washing.
<table>
<thead>
<tr>
<th>Washing</th>
<th>Bottle 1 organic vol. (liters)</th>
<th>Bottle 2 organic vol. (liters)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M NaOH</td>
<td>4.3</td>
<td>5.7</td>
</tr>
<tr>
<td>0.1M NaOH–methanol (1:1) washings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.1</td>
<td>4.5</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>3.8</td>
<td>3.9</td>
</tr>
<tr>
<td>4</td>
<td>3.5</td>
<td>3.9</td>
</tr>
<tr>
<td>Methanol–water (1:1) washings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.1</td>
<td>11.35</td>
</tr>
<tr>
<td>2&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.1</td>
<td>9.25</td>
</tr>
<tr>
<td>3&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.8</td>
<td>9.20</td>
</tr>
<tr>
<td>4&lt;sup&gt;C&lt;/sup&gt;</td>
<td>2.9</td>
<td>8.45</td>
</tr>
<tr>
<td>5&lt;sup&gt;D&lt;/sup&gt;</td>
<td>2.9</td>
<td>8.45</td>
</tr>
<tr>
<td>6</td>
<td>1.3</td>
<td>8.26</td>
</tr>
</tbody>
</table>

A - 1.5 liters of methanol were added to the first bottle.

B - 750 milliliters of methanol were added to the first bottle.

C - 2 liters of the lower layer in the second bottle were added to the first bottle and 600 milliliters of methanol were added to the second bottle.

D - In this washing the ratio of methanol to water was 2:1.
During the methanol-water washings emulsions began to occur. The methanol added to clear them up prompted us to attempt using methanol-water (2:1) rather than 1:1. Unfortunately, no phase separation occurred at the final washing of bottle 1, so 800 milliliters of water were added to that washing to virtually restore the 1:1 ratio.

One change in the column chromatography procedure occurred when we obtained 49 additional milligrams of product by eluting with 200 milliliters of chloroform-methanol (2:1) following chloroform-methanol (90:10) elution. Our fractions for the bottle two column were obtained eluting with chloroform-methanol (2:1).

From bottle one a total of 143 milligrams of product were obtained.

From bottle two 71 milligrams were obtained.

TLC analysis showed that less than half of the product was ketone. The rest of the product was unreacted cerebroside.

These discouraging results prompted us to search for an alternate method in labeling erythro-N-palmitoyl galactosyl cerebroside at the 3-sphingosine position.
VII. A Method For Oxidizing Sphingosine To Its 3-Keto Derivative

Mancuso, Huang, and Swern have developed a useful method of oxidizing long-chained alcohols to carboxyls using dimethylsulfoxide and oxalyl chloride. This oxidation method should be applicable to a protected form of sphingosine. The reaction scheme is presented in Figure 2 (next page).

N-protection is performed by refluxing 4 grams of sphingosine and 5 milliliters of methylidichloroacetate in 30 milliliters of tetrahydrofuran for two hours. The solution is evaporated at 40°C and the residue is purified on a 60 gram silicic acid column. Impurities are removed by eluting with chloroform containing 1% methanol. The product (B) can be seen as an opalescent band on the column. It is eluted with chloroform containing 2.3% methanol. The expected yield is 4.95 grams or 90%.

4.0 grams of compound B and 4.0 grams of trityl chloride are placed in 20 milliliters of pyridine and 30 milliliters of tetrahydrofuran. The solution is allowed to stand at room temperature for 48 hours. Evaporation of solvent should reveal a pure enough compound (C) for oxidation.

Prior to oxidation, all solvents and compounds used in the reaction are thoroughly dried. 25 milliliters of methylene
Figure 2. Oxidation of sphingosine to its 3-keto derivative.
chloride and 2 milliliters of oxalyl chloride are placed in a
100 milliliter four neck round bottom flask. A calcium sulfate
drying tube is placed at one of the necks and a mechanical stirrer
is placed at a second neck. Pressure-equalizing dropping funnels
are placed at the other two necks.

In one of the dropping funnels 1.7 milliliters of dimethyl-
fulfoxide is dissolved in 5 milliliters of methylene chloride.
In the other dropping funnel 4 grams of protected sphingosine is
dissolved in 10 milliliters of a methylene chloride solution con-
taining 1.7 milliliters of dimethylsulfoxide.\(^5\)

The dimethylsulfoxide is added to the stirred oxalyl chloride
solution at \(-10^\circ\) C. The reaction mixture is stirred for 2 minutes
and the sphingosine containing solution is added over 5 minutes.
7.0 milliliters of triethylamine is added and the mixture is stirred
for an additional 15 minutes. The mixture is then allowed to warm
to room temperature.\(^6\)

50 milliliters of water is added and the resulting aqueous layer
is re-extracted with 50 milliliters of methylene chloride. The
combined organic layers are washed with 100 milliliters of saturated
NaCl and dried over anhydrous magnesium sulfate. The filtered
solution is evaporated down to 25 milliliters. This solution is
washed with 1% HCl, water, then 5% Na\(_2\)CO\(_3\), and finally water. The
evaporated solution should give a pure enough ketone (compound D)
for the next step.

The crude ketone is warmed in 50 milliliters of 90% acetic acid on a boiling water bath for 30 minutes. The solution is cooled, diluted with 200 milliliters of water and extracted with 200 milliliters of ether. The ether layer is washed with 2.5% sodium bicarbonate and water and dried over anhydrous magnesium sulphate. The residue is suspended in 75 milliliters of hexane at 60°C and cooled to 0°C. At this point precipitated triphenyl carbinol is filtered off. The remaining liquid is evaporated to dryness and the resulting oil is purified on a 50 gram silicic acid column. 250 milliliter fractions of chloroform and chloroform containing 0.5, 1, 2, and 10% are used for elution. A crude compound E is obtained from the 12 methanol fraction. It is further purified on a silicic acid column by eluting with 5-10% methylacetate in hexane. 2.5-3.0 grams of pure E should be obtained as a colorless viscous oil.

Compound E is carefully dried and 1.27 grams of E is dissolved in a mixture of 35 milliliters of dry benzene and 35 milliliters of nitromethane. This solution is heated under anhydrous conditions until approximately 15 milliliters of solvent has boiled off. At this time 0.8 grams of acetobromogalactose and 0.5 grams of Mg(CN)₂ are added. An additional 0.4 grams of acetobromogalactose and 0.25 grams of Mg(CN)₂ are added after 2 and 3 hours respectively. During
this time the solution is heating at 80° C and remains at that temperature for 4½ hours. The solution is then cooled and diluted with 100 milliliters of ether. Precipitated mercuric salts are filtered out and the resulting filtrate is washed with water and dried over anhydrous magnesium sulphate.  

The oily residue from above (Compound F) is dissolved in 150 milliliters of 0.2M KOH in 90% methanol and allowed to stand at room temperature for 2 hours. 270 milliliters of chloroform and 85 milliliters of water are added to give a partitioned mixture of chloroform-methanol-water (8:4:3). The lower phase is separated and the upper phase is washed three times with pure lower phase. The combined lower phases are evaporated down and the residue is column purified on silicic acid eluting with chloroform-methanol. Compound G is obtained in chloroform containing 10-20% methanol. It can be recrystallized from 95% ethanol or chloroform-methanol (97:3) to yield approximately 700 milligrams of 3-ketopsycosine (compound G).  

Compound G can be reduced and purified according to the procedure outlined earlier in this paper to obtain erythro-N-palmitoyl-galactosyl cerebroside labeled with deuterium at the 3-sphingosine position.
Footnotes


VIII. Conclusion

Problems existed in all aspects of the original cerebroside oxidation scheme. Loss of product into aqueous layers was unavoidable. Washings were very tedious. The amount of cerebroside that fully reacted with 2,3-dichloro-5,6-dicyanobenzquinone was quite inconsistent. Column purification was often difficult due to large amounts of DDQ remaining in the product solution.

The alternate procedure for sphingosine oxidation appears to have much greater utility. Even though the reaction involves several time consuming steps, each step has been performed on the type of quantities we want to work with and the yield seems fairly reasonable. For this reason, further investigation into large scale oxidation of cerebroside by DBQ seems unnecessary, whereas the oxidation of protected sphingosine by activated dimethyl sulfoxide appears to have great potential.
Bibliography


4. Robert Shajun, A Deuterium NMR Study of Glycosphingolipids, co. 1979 at University of Illinois - Urbana-Champaign.


