

PREPARATION OF 6-³H GLUCOCEREBROSIDE

Marvin C. McMaster, Jr. and Norman S. Radin
Mental Health Research Institute and Department of Biological
Chemistry, University of Michigan, Ann Arbor, MI 48109, U.S.A.

Received June 7, 1976

Revised July 12, 1976

SUMMARY

Glucocerebroside (1-O- β -glucosyl ceramide) can be labeled with ³H-borohydride at the 6-position of the glucose moiety. The 6-trityl ether of cerebroside is formed first, the remaining hydroxyl groups are acetylated, the trityl group is removed, and the free 6-hydroxyl group is oxidized to an aldehyde. The carbonyl group is then reduced with borohydride and the acetyl groups are removed, regenerating the original glycolipid.

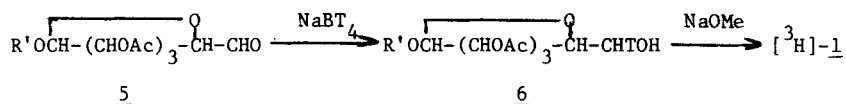
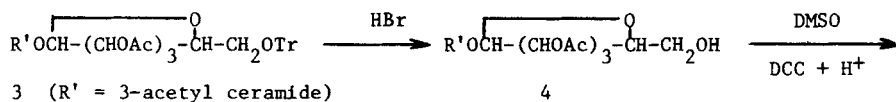
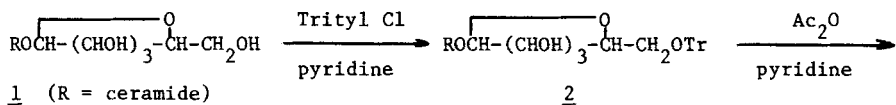
Key Words: Glucocerebroside, ³H-glucose labeled; Glucosyl ceramide, glucose labeled; Glucosyl ceramide, 6-trityl ether; Glucosyl ceramide, acetate esters

INTRODUCTION

Glucocerebroside (1) is a naturally occurring sphingoglycolipid that accumulates greatly in the tissues of humans suffering from a genetic lack of β -glucosidase (Gaucher's disease). It is useful to assay the enzyme with radioactive substrate and methods have been devised for labeling 1 in the glucose moiety (1), the fatty acid moiety (2), the ceramide moiety (3,4) and the long chain base moiety (5). The enzyme possesses hydrolytic and transglucosidic activities which can be conveniently distinguished by the use of substrate labeled in both the glucose and lipid moieties. This paper describes an improved procedure for preparing glucose-labeled 1. The new procedure is more economical, involves fewer steps, requires less handling of radioactive material, and can be used (if desired) entirely with commercially available raw materials (1, contain-

ing DL-dihydrosphingosine, is available from Miles Laboratories, Elkhart, Indiana).

The chemical reactions in our synthesis are as follows:



EXPERIMENTAL SECTION

Materials 1 was prepared from human Gaucher spleen (6) and dried in vacuo at 100° over P₂O₅ for 2 hr. All other products were dried similarly but at room temperature. Solvents were dried by refluxing with desiccants (7). Anhydrous HBr was obtained from Air Products and Chemicals, Inc. Thin layer chromatography (tlc) was performed with 20x20 cm plates coated with silica gel 60 PF-254 (E. Merck), washed with CHCl₃-CH₃OH-water (100:42:6) and dried 30 min at 100° before use.

Experimental A solution of 110 mg of 1 (about 0.12 mmole) and 64 mg of triphenylmethyl chloride in 1.5 ml of pyridine was heated at 40° for 18 hr. The sample was cooled to room temperature, 0.11 ml of Ac₂O (1.15 mmole) was added, and the mixture was left overnight. Excess reagents were hydrolyzed by stirring 30 min with 15 ml of water and the ether ester, 3, was isolated by extraction with 3 x 5 ml of CHCl₃. The pooled lower layers were washed with 5 ml water and evaporated under vacuum with added benzene, yielding an oily brown residue.

The trityl group in 3 was removed by dissolving the residue in 16 ml of HOAc-CHCl₃ (1:1), cooling to -10° in a stirred ice-salt bath, and adding 0.3 ml of HBr in HOAc (302 mg/ml acid). This was stirred 2.5 min, then poured into 250 ml of

cold water. The tetraacetate, 4, was extracted with 3 x 25 ml of CHCl₃, the extract was washed with 25 ml of water, and the lipid was isolated by vacuum evaporation with benzene and hexane. The white residue weighed 211 mg.

The hydroxyl group of 4 was oxidized by the Pfitzner-Moffat reaction (8), carried out by dissolving 4 and 384 mg of N,N'-dicyclohexylcarbodiimide (1.86 mmole) in 7 ml of dimethylsulfoxide. To this was added 0.075 ml of 1.12 M phosphoric acid in DMSO, the solution was covered with dry nitrogen, and the sealed flask was stirred overnight. Excess DCC was decomposed by stirring 15 min with 0.8 ml of HOAc, the mixture was poured into 110 ml of water, and the aldehyde, 5, was isolated by extraction with 75 ml and 3 x 50 ml of CHCl₃. The pooled extracts were washed with 3 x 75 ml of CH₃OH-aqueous NaCl (1:1) and then evaporated to dryness in vacuum and with a nitrogen stream. Dicyclohexylurea was removed by sonicating the residue with 50 ml of hexane and filtering; evaporation of the filtrate yielded a gummy yellowish solid (235 mg).

The aldehyde was reduced in a 100 ml screw cap centrifuge tube by dissolving it in 15 ml of tetrahydrofuran (freshly distilled from KOH) and adding a solution of 4.6 mg (25 mCi) of NaB³H₄ in 1.25 ml of 1 mM NaOH. The mixture was stirred overnight, then stirred 2 hr with an additional 30 mg of unlabeled NaBH₄. Excess borohydride was decomposed by stirring the mixture with 14 ml of 1 M HOAc for 15 min. The acetylated cerebroside, 6, was isolated by mixing the solution with 50 ml of CHCl₃-CH₃OH (2:1), centrifuging at low speed, and sucking off the upper layer. The lower layer was washed with 3 x 34 ml of CH₃OH-aqueous salt (1:1) and evaporated to dryness with a nitrogen stream. Benzene-ethanol (1:1) was added near the end of the evaporation to aid in water removal. (CAUTION: Do not use vacuum for evaporation to avoid risk of spattering.) Yield: 230 mg of white solid.

Deacetylation of 6 was performed by dissolving the lipid in the same centrifuge tube in 7.5 ml of NaOCH₃ solution (20 mg Na/ml CH₃OH) under dry N₂ and leaving the solution overnight. The glucocerebroside was recovered by partitioning as above, first adding 24 ml CHCl₃, 4.5 ml CH₃OH, and 9 ml water. The crude lipid weighed 112 mg.

The lipid was purified with a 7 x 500 mm column containing 8 g of silica gel

60 (230/400 mesh, E. Merck) packed in CHCl_3 - CH_3OH (97:3). The sample was added in small portions of the same solvent mixture, in the ratio 96:4, then eluted with 70 ml of the same mixture and 300 ml of a 95:5 mixture. Pooling the fractions identified by tlc with CHCl_3 - CH_3OH -water (24:7:1) and carrier 1 yielded 52 mg (47% yield) of 1 with an activity of 1.2×10^9 cpm (6% of the starting activity). The radiopurity, estimated by scanning a tlc plate with a recording windowless scanner, was over 99%. The product was stored in CHCl_3 - CH_3OH (2:1) at -20° .

The above reactions were validated additionally by carrying them out with a more homogeneous 1: N-stearoyl-0-glucosyl sphingosine (9). This allowed us to crystallize the intermediates and demonstrate their composition by elemental analysis. This lipid also yielded clearer tlc spots, since 1 from spleen contains a variety of fatty acids that cause partial separation by tlc. The R_f values of the various intermediates, relative to that of cholesterol with the solvent CHCl_3 - CH_3OH (30:1), were as follows: 2 - 0.22, 3 - 1.40, 4 - 1.13, 5 - 1.27. Other R_f values for related compounds (relative to cholesterol) were: 1 Ac_5 - 1.41 and 1 Ac_3 - about 0.77. Spraying the tlc plate with 10% sulfuric acid, then with 1% anthrone in toluene, and heating to 100° quickly yielded bright yellow spots with trityl compounds, then purple to brown spots with glycolipids. Thus it was helpful to follow the color development with a glass door oven.

The most difficult step to work out was the detritylation. The techniques described in the literature removed acetyl groups as well as the trityl group. A low temperature and brief exposure to HBr solved the problem.

ACKNOWLEDGMENTS

This work was supported by NIH grant NS03192 from the National Institute of Neurological and Communicative Disorders & Stroke. We are indebted to Dan del Vecchio for technical assistance and to Dr. Ranga R. Vunnam for his valuable suggestions on the synthesis.

REFERENCES

1. Brady R.O., Kanfer J.N., and Shapiro D. - *J. Biol. Chem.* 240: 39 (1965)
2. Erickson J.S. and Radin N.S. - *J. Lipid Res.* 14: 133 (1973)
3. Di Cesare J.L. and Rapport M.M. - *Chem. Physics Lipids* 13: 447 (1974)

4. Barton N.W. and Rosenberg A. - J. Biol. Chem. 250: 3966 (1975)
5. Iwamori M., Moser H.W., and Kishimoto Y. - J. Lipid Res. 16: 332 (1975)
6. Radin N.S. - J. Lipid Res. 17: 290 (1976)
7. Perrin, D.D., Armarego W.L., and Perrin D.R. - Purification of Laboratory Chemicals, Pergamon Press, New York, 1966
8. Butterworth R. and Hanessian S. - Synthesis 1971: 70
9. Radin N.S. - Methods Enz. 28: 300 (1972)