Preparative scale isolation of sphingosine

Norman S. Radin

Mental Health Research Institute, The University of Michigan, Ann Arbor, MI 48109

Summary A high-yield method is described for the preparation of sphingenine from galactosylceramide. Two successive cleavage reactions are used, alkaline butanol to form galactosylsphingosine, then acidic acetonitrile to form the sphingenine. A column purification procedure utilizing silica gel is described. The method is superior to previously described methods in its rapidity and avoidance of isomerization and contamination by racemization and rearrangement products. —**Radin, N. S.** Preparative scale isolation of sphingosine. *J. Lipid Res.* 1990. **31:** 2291–2293.

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A major obstacle in the preparative isolation of sphingosine is its tendency to undergo isomerization and degradation during acid-catalyzed release from sphingolipids. This results from the allylic alcohol group, which readily enters into racemizing allylic rearrangements or substitution by methoxy groups, since the usual solvent is methanol (1, 2). Aqueous acid cleavage methods, including those using acetonitrile and aqueous HCl (3), do not produce methyl ethers but racemization and rearrangement nevertheless occur. The yield of D-*erythro*-sphingosine by these procedures is thus rather low and the requisite chromatographic purification is difficult, particularly because of the problem of removing the closely eluting *threo*-sphingosine (1, 4). One of the products, which probably is not removable by silica gel chromatography, is *erythro*-1,5-dihydroxy-2-amino-3-octadecene, a rearrangement product formed by movement of the C-3 hydroxyl group to carbon-5 (5, 6).

Recent reports state that different commercial batches of sphingosine showed differing degrees of inhibitory action against protein kinases (7, 8). While several contaminants were detected by TLC, it is also possible that the differences were due to differing degrees of contamination with the C-5 isomer. Additionally, some formation of N,N-dimethylsphingosine, a strong inhibitor of protein kinase C (7, 8), can be expected due to the alkylating properties of anhydrous methanol and sulfuric or hydrochloric acid. (For example, an industrial process for converting aniline to dimethylaniline involves heating with methanolic sulfuric acid.) There is thus a need for a more reliable, higher-yield method of preparing pure sphingosine other than total synthesis, which is not readily carried out.

Gaver and Sweeley (9), in studying the problem of reducing the formation of byproducts, noted that free sphingosine did not produce ethers when heated in methanolic HCl. N-Acetyl sphingosine, like the more complex sphingolipids, did form byproducts. A similar discovery by Taketomi and Kawamura (10) pointed to a solution to this problem. They noted that lysosphingolipids (sphingolipids whose fatty acid moiety had been removed by alkaline hydrolysis) did not produce the methyl ethers or rearrangement products when cleaved with methanolic HCl. They suggested that the N-acyl group in intact sphingolipids is converted by acid to a cyclic product involving the 3-OH group (an oxazolidine). The acyl group of this intermediate then rearranges to form the free (protonated) amine group and the 3-O-acyl ester. It is the ester that undergoes the allylic rearrangements. Whatever the mechanism, it seemed likely that a two-stage cleavage procedure could give higher yields of sphingosine and make it possible to prepare the sphingol (11) free of isomers. This report describes a relatively simple procedure which can be run on any scale, limited primarily by the size of the column available for the final purification step. By starting with galactosylceramide from brain, one can eliminate contamination by the C_{20} homolog of sphingosine, a constitutent of gangliosides. The original alkaline hydrolysis method has been used to prepare galactosylsphingosine in ~70% yield (12). The present report describes a column purification procedure that could be used to purify the psychosine.

METHOD

Alkaline cleavage

The procedure of Taketomi and Yamakawa was followed (13), as adapted by Radin (14) and modified slightly here. The method consists of refluxing a solution of 2.8 g KOH in 4 ml water and 36 ml of n-butanol with 2 g cerebroside (the KOH is dissolved in the water first, before adding the other components). The mixture, which contains two liquid phases, is stirred magnetically in a 250-ml flat-bottom flask while being refluxed 3 h in a 125°C oil bath. The brownish mixture is diluted with 40 ml methanol + 40 ml isopropyl alcohol (containing 0.5 mg of bromphenol blue) + 20 ml chloroform. The indicator gives the liquid a greenish-blue color.

The mixture is acidified in ice to pH ~4 with enough (~8.5 ml) of a sulfuric acid–water mixture (1:5 v/v) to change the indicator to a distinct yellow. Precipitated K_2SO_4 is removed by filtration. Some time can be saved

Abbreviation: TLC, thin-layer chromatography.

if the crystals are allowed to settle first and the clear liquid is decanted. Three grams of Celite Analytical Filter Aid (Fisher Scientific) are added to the suspension before it is filtered, preferably with a pressure funnel. The glassware and K_2SO_4 are rinsed with 20 ml of chloroform–methanol 2:1 to complete the transfer.

Toluene (50 ml) is added to the filtrate, which is then cooled in ice and rotoevaporated in a 1-liter flask with a 35°C water bath to the point where evaporation becomes slow; 10–20 ml is left. The crude psychosine solution is now transferred to a separatory funnel with 50 ml each methanol and hexane and 20 ml of water. The lower phase, after phase separation, is washed with 3 × 50 ml of hexane to remove more fatty acid products. The hexane-rich layer is a clear tan liquid, while the lower layer is turbid.

Next the lower layer, which contains mainly the sulfate salt of psychosine, is cooled and rotoevaporated to a small volume (10–20 ml) with 50 ml of toluene as above.

Acid hydrolysis of psychosine

The solution is transferred to a 250-ml flat-bottom flask with rinses of acetonitrile (80 ml total). This leaves some sticky residue in the flask and transfer is completed with 6.4 ml of conc. HCl-water 1:3 (v/v). The flask is stirred magnetically under active reflux for 3 h in a ~95°C oil bath. Magnetic stirring is used because some psychosine hydrochloride precipitates in the reflux flask; this dissolves soon. **CAUTION**: Acetonitrile should be handled in a hood.

The sphingosine is extracted from the reaction mixture by adding 320 ml of chloroform-methanol 2:1 and 80 ml of 2 N ammonium hydroxide, which forms a blue upper layer (NH_4Cl) and a light greenish-gray lower layer (sphingosine). The lower layer is washed with 50 ml of a concentrated NaCl solution (100 g + 290 ml water) and the slightly cloudy, almost colorless lower layer is rotoevaporated to dryness with toluene as above. A few milligrams of sphingosine hydrochloride are lost on the walls of the separatory funnel. The yield of dry light colored solid is ~630 mg. The theoretical yield is ~750 mg, assuming an average molecular weight of 800 for brain cerebrosides.

Column purification of sphingosine

The column procedure is a modification of the ones previously described (4, 15). A glass column 1.35 × 102 cm is packed with 75 g of silica gel 60 (E. Merck #7734, 70/230 mesh) in hexane. The packing is washed with 50 ml each of deaired hexane–chloroform 80:20, hexane–chloroform 50:50, and chloroform, then 75 ml of chloroform–methanol 90:10.

The sphingosine sample is dissolved in 75 ml of chloroform–methanol–2 N NH_4OH 90:10:1 and filtered to remove salt. It is added to the column, followed by 1500 ml of the same solvent. This solvent is followed by chloroform–methanol–2 N NH_4OH 85:15:1.5 (383:68:6.8 ml) and 70:30:3 (875:375:37.5 ml). The last solvent is useful only to salvage small amounts of psychosine and dihydrosphingosine. The solvents are deaired by evacuation just before adding the ammonium hydroxide and the mixtures are driven through the column by helium pressure.

The first fraction collected is 600 ml; thereafter 65 to 75-ml fractions are collected. Each fraction is analyzed by TLC, using a 6-μl or 10-μl streak on a silica gel 60 plate (E. Merck, 20 × 20 cm) and the separating solvent, chloroform–methanol–water–conc. NH_4OH 70:30:4:1. While awaiting the results, the fractions are stored in the cold room. The developed plates are air-dried, then charred with the copper sulfate/phosphoric acid spray (16). Care should be taken to avoid excessive spraying as the sphingosine spots can form a halo, due to diffusion in the polar spray. It is better to spray lightly, twice, heating each time at 150°C.

The yield of sphingosine in the pooled purified fractions is ~400 mg (53% of theor.).

RESULTS AND DISCUSSION

Examination of the pooled hexane extracts after alkaline cleavage, by TLC with hexane–ether–acetic acid 70:30:1, showed that it consisted mainly of unesterified fatty acids of the 2-hydroxy and nonhydroxy types. However a significant amount of material migrating a trifle faster than methyl fatty acid esters could also be seen; evidently the butanol in the alkaline mixture produced some transesterification despite the presence of water. Some psychosine was also present.

The lower layer from the hexane extraction, examined by TLC with chloroform–methanol–NH_4OH as used for sphingosine (above), contained mainly psychosine (hR_f = 29) with small amounts of free fatty acid (hR_f 53), fatty acid esters, and sphingosine (hR_f 67). Evidently the alkali also catalyzed some removal of galactose. The crude sphingosine, before column purification, exhibited small amounts of dihydrosphingosine (hR_f 59) and psychosine as its major impurities. Spots corresponding to fatty acids, free and esterified, and a spot (hR_f 83) between the esters and sphingosine were also present at trace levels. The faster spot could be due to the previously observed dehydration product, 1-hydroxy-2-amino-octadeca-3,5-diene (6, 17).

Acetonitrile and aqueous HCl have been used for ganglioside hydrolysis (3). Acting on the suggestion of Dr. Robert McCluer, we tried various conditions to see what might be suitable for psychosine. The conditions described here (~0.22 M HCl, ~3.2 M water) seem to consti-

tute a reasonable compromise: a 2-h hydrolysis left more psychosine unhydrolyzed while longer heating increased the amount of the fast-moving impurity, the hypothetical diene. An important point is that no *threo*-sphingosine could be seen on the TLC plates; it normally migrates just below sphingosine, just above sphinganine. Thus it is probably safe to assume that no other allylic rearrangement products formed. (Because of the need for careful control of TLC technology, not all published photos of plates have shown the presence of *threo*-sphingosine in sphingosine preparations.) When the psychosine was methanolyzed with sulfuric acid in methanol, no *threo*-sphingosine was formed but a pair of faster-moving ninhydrin-positive spots was seen, possibly the *cis*- and *trans*-diene dehydration products.

It is important to apply caution if one wishes to change the volumes and compositions of the various solvents given above as we found difficult emulsions formed readily with several other ratios that were tried.

When HCl, instead of sulfuric acid, was used to acidify the alkaline mixture, after cerebroside cleavage, a serious foaming problem occurred during the evaporation step. Possibly psychosine hydrochloride is a foaming agent, or the HCl evaporated, leaving some fatty acid salts of psychosine, which could be a foaming agent. The use of cold toluene as an evaporation aid is highly recommended provided the rotoevaporation seal allows maintenance of a good vacuum.

The yield of psychosine is reduced by a side reaction during the alkaline cleavage reaction, which causes some kind of degradation of the sphingosine moiety (13). In previous studies of alkaline cleavage methods at higher temperatures, we observed degradation of both sphingosine and psychosine (R. J. Metz and N. S. Radin, unpublished work).

The starting material, cerebroside, can be isolated by a variety of methods (see, for example, 14, 18). The entire hydrolysis and purification can be carried out in 3 days.

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