Carbohydrate Research, 127 (1984) 211–216
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

SULFATIDES OF *Mycobacterium tuberculosis*. SYNTHESIS OF THE CORE α,α-TREHALOSE 2-SULFATE*

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(Received July 25th, 1983; accepted for publication, September 5th, 1983)

ABSTRACT

α,α-Trehalose 2-sulfate, the core carbohydrate of sulfatides of *Mycobacterium tuberculosis*, and the 3-sulfate isomer were synthesized by sulfation of 4,6:4′,6′-di-*O*-benzylidene-α,α-trehalose with pyridine–sulfur trioxide complex to give the 2- and 3-sulfates, which were separated by column chromatography. The ammonium 2-sulfate salt was identical with the natural product obtained from the principal sulfatide (SL-I) of *M. tuberculosis*.

INTRODUCTION

The sulfatides (SL) of *Mycobacterium tuberculosis*[1–3] have been convincingly implicated in the virulence of this pathogenic microorganism. Elaboration of these glycolipids was correlated[4,5] with the relative order of virulence amongst a spectrum of patient isolates of phage types A and I, (but not[6] of phage types B). Recognition of their behavior as antagonists of phagosome–lysosome fusion in cultured macrophages[7] documented, for the first time, this activity in a natural product derived from a pathogen whose intracellular survival may be promoted by a similar phenomenon[8].

The purified sulfatides have not been found to be antigenic[9], but complexes of SL-I (the principal sulfatide of *M. tuberculosis*) with methylated bovine serum albumin appear to elicit a humoral antibody response in rabbits[10,11]. More recently, Khuller *et al.*[12,13] reported that sera from tuberculosis patients and also from leprosy patients are similarly reactive for SL-I. However, in the absence of in-

*This investigation was supported by Grant AI 08401 from the U.S.–Japan Cooperative Medical Science Program, administered by the National Institute of Allergy and Infectious diseases. MBG is the Margaret Regan Investigator in Chemical Pathology, NJHRC/NAC.

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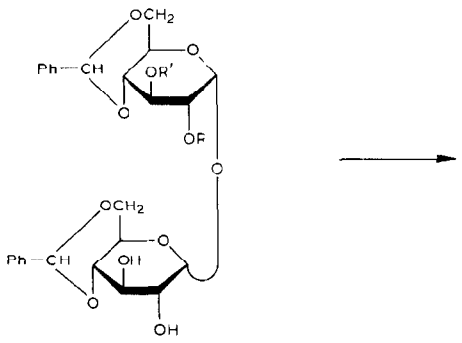
0008-6215/84/$ 03.00

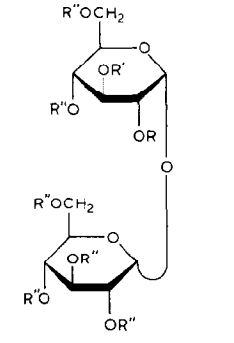
hibition studies with rigorously pure substances, unquestionably free of any mycobacteria-derived impurities, such as proteins and polysaccharides, the foregoing results must still be viewed as only tentative. For this reason, it was desirable to have available pure trehalose 2-sulfate, preferably synthetic. This communication describes the synthesis of this specific substance and of the isomeric 3-sulfate, as well as of intermediates that may be useful in serological tests as substitutes for naturally derived sulfatides.

RESULTS AND DISCUSSION

Treatment of 4,6:4′,6′-di-*O*-benzylidene-α,α-trehalose[14] (**1**) with pyridine–sulfur trioxide complex gave a mixture of two monosulfates, which were separated by column chromatography. The 3-sulfate derivative was isolated as the ammonium salt (**2**) in 8% yield, and the 2-sulfate as the ammonium salt (**3**) in 40% yield. That the major product was the 2-sulfate was anticipated on the basis of the higher reactivity[14] of OH-2. This structure assignment was confirmed by converting **3** into the known ammonium α,α-trehalose 2-sulfate[15] (**4**). The ^{1}H-n.m.r. spectrum of **3** was rather complex, owing to the lack of symmetry of the two trehalose rings. The removal of the benzylidene groups from **2** and **3** was studied under various conditions, in order to minimize the undesired desulfation reactions[16]. The best results were obtained with 1% aqueous sulfuric acid at room temperature to give **4** and **5**, respectively, only a trace amount of trehalose being detected. The purification of the final product was attempted first by ion-exchange chromatography. The crude product was applied to a column of AG 2-X8 (OH^-) ion-exchange resin, which did not adsorb trehalose, and the homogeneous sulfated product was eluted with 10% ammonium acetate. However, the prolonged, high-vacuum evaporation of ammonium acetate (which was present in abundance in the effluent) resulted in



1 R = R′ = H
2 R = H, R′ = SO_3NH_4
3 R = SO_3NH_4, R′ = H



4 R = SO_3NH_4, R′ = R″ = H
5 R = R″ = H, R′ = SO_3NH_4
6 R = SO_3·Py, R′ = R″ = Ac
7 R = R″ = Ac, R′ = SO_3·Py

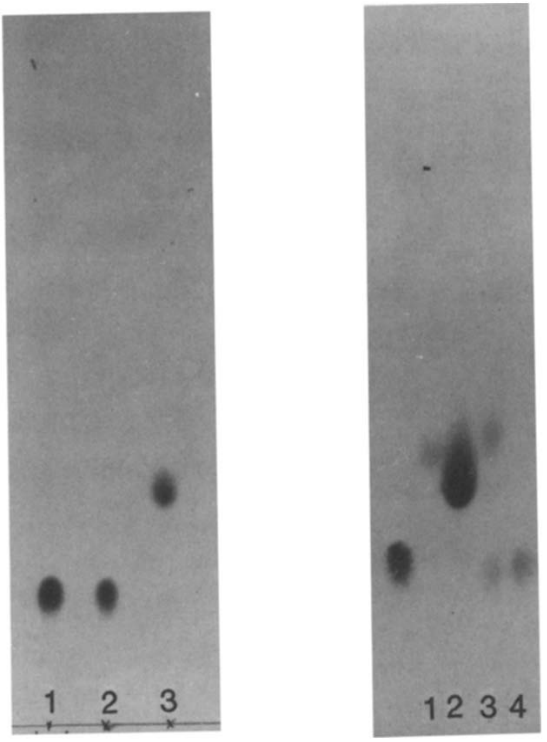


Fig. 1. Thin-layer chromatogram developed in 1:3:1 (v/v) chloroform–methanol–conc. ammonium hydroxide: (1) α,α-trehalose 2-sulfate from SL-I, (2) synthetic α,α-trehalose 2-sulfate (**4**), and (3) synthetic α,α-trehalose 3-sulfate (**5**).

Fig. 2. Desulfation of α,α-trehalose 2-sulfate (**4**) in 1,4-dioxane in presence of sulfuric acid (1 equiv.) and water (trace). Extreme left: trehalose; (1) **4** at beginning of desulfation; (2) trehalose 2-sulfate (**4**); (3) (**4**) after 1 h of desulfation; and (4) after 2 h. Solvent system as in legend to Fig. 1.

partial desulfation (attributed to the formation of some acetic acid during evaporation).

In a more satisfactory approach, crude **4** and **5** were converted into the peracetate derivatives, which were then chromatographed (probably as pyridinium salts **6** and **7**, respectively) on silica gel. Deacetylation of pure **6** and **7** (pyridinium salts) and subsequent crystallization gave analytically pure ammonium α,α-trehalose 2-sulfate (**4**) and ammonium α,α-trehalose 3-sulfate (**5**), respectively.

The identity of synthetic **4** with the product obtained by solvolysis of the principal mycobacterial sulfatide was established by a comparison of melting points, optical rotations, and t.l.c. mobilities (Fig. 4), and by elemental analysis. Ammonium trehalose 3-sulfate (**5**), which hitherto had not been described, differs from the 2-sulfate (**4**) in melting point, optical rotation, and t.l.c. mobility (Fig. 1). A convenient desulfation of synthetic **4** was achieved at room temperature; it was ~40% complete within 1 h, and complete after 2 h, only trehalose being present

(Fig. 2). The trace of water was required absolutely.

The trehalose sulfates may be used as competitive inhibitors in either ELISA- or kaolin-agglutination tests with the mycobacterial sulfatides as antigen, in order to determine whether the immunoglobulins present in sera of patients with mycobacterial disease recognized the trehalose sulfate determinant, and to assess the specificity of this recognition. In a previous report, Khuller *et al.*[11] have described the inhibition of serological activity by "trehalose 2-sulfate". However, the conditions under which the sulfolipid had been deacylated to yield trehalose 2-sulfate (heating for 1 h in benzene–methanol) had no effect on SL-I in our hands. Thus the inhibition described by Khuller *et al.*[11] was probably not due to trehalose sulfate.

We have described earlier[15] the very facile deacylation of SL-I at room temperature in ethanol solution, following addition of a small amount of concentrated sodium hydroxide. Sodium trehalose sulfate began to crystallize from this reaction mixture in a very short time, and the deacylation was rapidly completed. On the other hand, the sulfate ester **4** was entirely stable under these conditions. The SL-derived trehalose sulfate may contain unsuspected contaminants of mycobacterial origin, but the synthetic carbohydrate sulfate **4** is clearly free from these.

As a side-product of this synthesis, the sulfated dibenzylidenetrehalose **3** may be useful as a substitute for natural SL-I in ELISA- or kaolin-agglutination, or immunodiffusion analyses, as it bears the trehalose 2-sulfate determinant. Appropriate derivatives of the synthetic carbohydrate sulfate (*i.e.*, hemisuccinates) also may be used for the preparation of protein conjugates for immunization and of affinity columns for isolating immunoglobulins specific for the SL-I core (if they indeed exist).

EXPERIMENTAL

General methods. — Melting points were determined with a Fisher–Johns apparatus and are not corrected. Optical rotations were recorded with a Jasco Dip-140 polarimeter. Thin-layer chromatography was performed on Eastman-Kodak silica gel plates. Chromatography columns were packed with silica gel (Baker No. 3405). Microanalyses were performed by Galbraith Laboratories Inc., Knoxville, TN 37921.

Ammonium 4,6:4′,6′-di-O-benzylidene-α,α-trehalose 3-sulfate (**2**) *and ammonium 4,6:4′,6′-di-O-benzylidene-α,α-trehalose 2-sulfate* (**3**). — To a solution of 4,6:4′,6′-di-*O*-benzylidene-α,α-trehalose[14] (**1**) (580 mg, 1.12 mmol) in pyridine (6 mL) was added pyridine–sulfur trioxide complex (Aldrich, 460 mg, 2.80 mmol), and the mixture was stirred at room temperature. More pyridine–sulfur trioxide complex (240 mg, 1.5 mmol) was added after 17 h. After 24 h, the mixture was evaporated and the residue chromatographed on silica gel. Elution with 20:1 (v/v) chloroform–methanol gave unreacted **1** (76 mg, 13%), and with 10:1 (v/v) chloroform–methanol 4,6:4′,6′-di-*O*-benzylidene-α,α-trehalose 3-sulfate which was

characterized as the ammonium salt **2** (yield 54 mg, 8%); $[\alpha]_D^{22}$ +49° (*c* 0.7, methanol).

Anal. Calc. for $C_{26}H_{33}NO_{14}S \cdot 2\,H_2O$: C, 47.92; H, 5.72; S, 4.92. Found: C, 48.24; H, 5.94; S, 4.63.

Continued elution with the same solvent system, followed by 8:1 (v/v) chloroform–methanol gave the major product, 4,6:4′,6′-di-*O*-benzylidene-α,α-trehalose 2-sulfate which was isolated as the ammonium salt **3** (yield 276 mg, 40%); $[\alpha]_D^{22}$ +66° (*c* 0.8, methanol).

Anal. Calc. for $C_{26}H_{33}NO_{14}S \cdot 2\,H_2O$: C, 47.92; H, 5.72; S, 4.92. Found: C, 48.00; H, 5.70; S, 4.92.

Ammonium α,α-trehalose 2-sulfate (**4**). — The dibenzylidene derivative **3** (274 mg) was treated with 1% aqueous sulfuric acid (3 mL) in methanol (15 mL). The mixture was kept overnight at room temperature and made neutral with a saturated barium hydroxide solution. The salts were removed by centrifugation, and the supernatant solution was evaporated to give an amorphous residue which was homogenous on t.l.c. but still contained traces of salts. This product was acetylated in the usual way with acetic anhydride and pyridine. The peracetate was applied to a column of silica gel and the pure product (**6**) eluted with 10:1 (v/v) ethyl acetate–methanol. The product was deacetylated by treatment with conc. ammonium hydroxide solution (5 mL) in methanol (5 mL). The mixture was kept overnight at room temperature and evaporated. Ammonium acetate was removed by high-vacuum evaporation, and the product crystallized by dissolving it in methanol and allowing the solution to equilibrate with absolute alcohol in a closed flask. The crystalline product was isolated by decantation of the solvent and was washed with 9:1 (v/v) ethanol–methanol. The product was very hygroscopic, which made the isolation by filtration almost impossible (yield 98 mg, 50%); m.p. 150° (browning), 192–195° (dec.)*; $[\alpha]_D^{22}$ +151° (*c* 0.6, methanol); lit.[15] $[\alpha]_D^{22}$ +154 ±5.2°; t.l.c. (3:1:1, v/v, methanol–conc. ammonium hydroxide–chloroform) R_F 0.17 and same mobility as the natural product (Fig. 1).

Anal. Calc. for $C_{12}H_{25}NO_{14}S \cdot H_2O$: C, 31.51; H, 5.95; S, 7.01. Found: C, 31.47; H, 5.73; S, 6.87.

Compound **4** (2 mg) was desulfated at room temperature in a stirred solution in 1,4-dioxane (2 mL) containing sulfuric acid (1 equiv.) in water (10 μL) (see Fig. 2).

Ammonium α,α-trehalose 3 sulfate (**5**). — The dibenzylidene derivative **2** (74 mg) was treated with 1% aqueous sulfuric acid as described for the derivative **3**. The crude α,α-trehalose 3-sulfate was purified by conversion into the peracetate derivative **7**, which was then chromatographed and deacetylated with ammonium hydroxide. Compound **5** was crystallized from methanol as described for **4** (yield 24 mg, 45%); m.p. 200° (browning) and gradual dec.; $[\alpha]_D^{22}$ +113° (*c* 0.7, methanol);

*In ref. 15, the m.p. value is given for the barium salt, but re-examination of the melting point of natural ammonium trehalose 2-sulfate showed it to be identical with the synthetic product.

t.l.c. (3:1:1, v/v, methanol–conc. ammonium hydroxide–chloroform) R_F 0.31 (Fig. 1).

Anal. Calc. for $C_{12}H_{25}NO_{14}S \cdot H_2O$: C, 31.51; H, 5.95; S, 7.01. Found: C, 31.80; H, 5.74; S, 6.84.

ACKNOWLEDGMENTS

The authors thank Juanita Derry and Carol Breibart for preparation of the manuscript, and Nadia de Stackelburg for illustrations.

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