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Note

An improved synthesis of 6-*O*-mycoloyl- and 6-*O*-corynomycoloyl-α,α-trehalose with observations on the permethylation analysis of trehalose glycolipids*

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In a recent report[1], we described the synthesis of 6-*O*-mycoloyl- and 6-*O*-corynomycoloyl-α,α-trehalose (**6** and **7**, respectively), the isolation of which from various bacterial sources has been described[2–5]. In this synthesis, the monomycolate and monocorynomycolate esters were obtained from 6-*O*-acetyl-2,3,4,2′,3′,4′-hexa-*O*-benzyl-α,α-trehalose, isolated as a byproduct in the acetic acid-catalyzed hydrolysis of the corresponding ditrityl derivative. However, since the starting material in the already described synthesis is a by-product which can only be obtained in a limited amount (maximum yield 24%), the synthesis was not suitable for the larger quantities we sought for biological and serological evaluations.

Both trehalose monoesters **6** and **7** described herein and the 6-acetate-6′-mycolate ("MAT", **9**) (but *not* cord factor, 6,6′-di-*O*-mycoloyltrehalose) behave as equally potent serologically active antigens for detecting humoral antibody against the late Reggiardo's "SAG" antigen A_1 (ref. 6). In her studies, this glycolipid was recovered from *Mycobacterium bovis BCG* and found reactive for sera from patients with a variety of mycobacterioses[7,8]. Only recently it was found in her collaborative (unpublished) studies with J. Polonsky and E. Lederer that antigen A_1 is in fact trehalose monomycolate[9]. These considerations underlie our interest in these trehalose glycolipids. We describe herein an improved synthesis of (principally) 6-*O*-mycoloyl- and 6-*O*-corynomycoloyl-α,α-trehalose.

Selective tosylation of 2,3,2′,3′-tetra-*O*-benzyl-α,α-trehalose[10] (**1**) gave a mixture from which three components were separated by column chromatography: the ditosyl derivative **2** (27%), the desired monotosyl derivative **3** (51%), and un-

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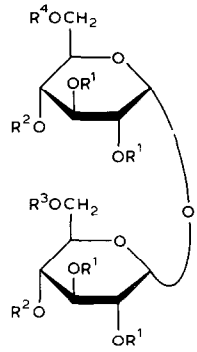
changed starting material (20%). Treatment of the monotosyl derivative **3** with potassium mycolate[1] or potassium corynomycolate[1] gave the corresponding 6-*O*-mycoloyl and 6-*O*-corynomycoloyl derivatives **4** and **5**, respectively. Catalytic hydrogenolysis of **4** and **5** afforded 6-*O*-mycoloyl- and 6-*O*-corynomycoloyl-α,α-trehalose (**6** and **7**, respectively). Compounds **6** and **7** were identical with the monomycolate and monocorynomycolate described earlier[1].

Our interest in 6-*O*-acetyl-6′-*O*-mycoloyl-α,α-trehalose (**9**) led us to the synthesis of this compound from **4**. This glycolipid was synthesized earlier by Polonsky *et al.*[11]. It is noteworthy that **9** was implicated as an early product in biosynthesis of mycolic acids and as a possible intermediate for delivering mycolate for incorporation into mycobacterial cell wall[12]. Since all mycobacteria so far examined synthesize mycolic acids, it is probably the relative "prominence" and ubiquity (see below) of the trehalose monomycolates and possibly **9** that elicits the antibody response to "SAG" A_1 in almost any mycobacterial infection.

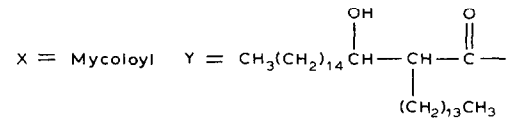
Treatment of **4** with 80% aqueous acetic acid, as described previously[1], followed by chromatography gave the 6-*O*-acetyl-2,3,2′,3′-tetra-*O*-benzyl derivative **8** in 11% yield. Hydrogenolysis of **8** gave 6-*O*-acetyl-6′-*O*-mycoloyl-α,α-trehalose (**9**). The meager yield of **8** probably reflects the sluggishness with which a second acetyl group is inserted by the 80% acetic acid reagent. It should be recalled that acetylation of 2,3,4,2′,3′,4′-hexa-*O*-benzyltrehalose with this reagent afforded exclusively the 6-monoacetate, but in only 24% yield.

The structure of compound **6** was confirmed by permethylation and subsequent alkaline hydrolysis, followed by g.l.c. comparison of the carbohydrate product with an authentic sample of 2,3,4,2′,3′,4′,6′-hepta-*O*-methyl-α,α-trehalose[3] (**10**). In a similar manner, compound **9** was converted into 2,3,4,2′,3′,4′-hexa-*O*-methyl-α,α-trehalose[13] (**11**). Compounds **6** and **9** were permethylated with methyl trifluoromethanesulfonate in the presence of 2,6-di-(*tert*-butyl)pyridine[14]. In our experience, this procedure has been the most useful and reliable one for permethylating these glycolipids as no evidence for hydrolysis or acyl migrations was observed. This procedure is preferable to our own method described earlier[15], in which report we also recapitulated difficulties encountered in permethylating trehalose glycolipids. At that time milligram quantities of acylated trehalose products from mycobacterial sulfatides, and trehalose monomycolate of *M. tuberculosis*[3] had been successfully permethylated with diazomethane and boron trifluoride etherate[16]. Still, in our hands this procedure was always tedious and curiously unpredictable. Our efforts at permethylating 1–3-mg samples of cord factor from *M. tuberculosis* by this method, or with sodium hydride and methyl iodide in *N,N*-dimethylformamide[17], or by variants of the Hakomori technique[18], have been unsuccessful. With larger quantities of cord factor "Peurois" and methyl iodide–sodium hydride–*N,N*-dimethylformamide, only a meager conversion to the fully methylated products was achieved[15]. Examination of a recently-described simple and rapid method for permethylating carbohydrates with methyl iodide and powdered NaOH in dimethyl sulfoxide[19] gave excellent results with both cord factor and trehalose

monomycolate, and in a very short time. Indeed, the correct methylated trehalose was obtained in both instances. However, it was never certain that this method would not induce acyl migrations. When 2,3,4,2′,3′,4′-hexa-*O*-acetyltrehalose[20] was permethylated, essentially the sole product obtained was octa-*O*-methyltrehalose. Thus, low-molecular-weight acyl substituents are cleaved. On the other hand, when the hexaacetate was methylated with trifluoromethanesulfonate and 2,6-di-(*tert*-butyl)pyridine, the sole product, obtained in high yield, was found by ^{1}H-n.m.r. spectroscopy to be the desired 2,3,4,2′,3′,4′-hexa-*O*-acetyl-6,6′-di-*O*-methyl-α,α-trehalose (**12**). This constitutes a useful route for the synthesis of 6,6′-di-*O*-methyl-α,α-trehalose and, thus, for 6-*O*-methyl-D-glucose. Dhariwal *et al.*[21] employed this method for permethylating ~200 μg of a glycolipid recovered from armadillo-grown *Mycobacterium leprae* and proved, by ^{252}Cf-plasma desorption mass spectrometry, the structure to be a trehalose monomycolate. The permethylation analysis showed it to be 6-*O*-mycoloyl-α,α-trehalose and, thus, establishes the first unequivocal isolation of this glycolipid from *M. leprae*.



	R^1	R^2	R^3	R^4
1	Bn	H	H	H
2	Bn	H	Ts	Ts
3	Bn	H	Ts	H
4	Bn	H	X	H
5	Bn	H	Y	H
6	H	H	X	H
7	H	H	Y	H
8	Bn	H	X	Ac
9	H	H	X	Ac
10	Me	Me	H	Me
11	Me	Me	H	H
12	Ac	Ac	Me	Me



EXPERIMENTAL

General methods. — Melting points were determined with a Fisher–Johns apparatus and are not corrected. Optical rotations were determined with a Jasco Dip-140 polarimeter. ^{1}H-N.m.r. spectra were recorded with an EM360A Varian spectrometer with tetramethylsilane as the internal standard and (^{2}H)$CHCl_3$ as the solvent. Eastman–Kodak plates were used for t.l.c. Chromatography columns were packed with silica gel (Baker No. 3405). For g.l.c. analysis of the permethylated

carbohydrates, columns (1.8 m) of SE 30 maintained isothermally at 200° were used. Organic solutions were dried over Na_2SO_4. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tennessee.

2,3,2',3'-Tetra-O-benzyl-6,6'-di-O-p-tolylsulfonyl-α,α-trehalose (**2**) *and 2,3,2',3'-tetra-O-benzyl-6-O-p-tolylsulfonyl-α,α-trehalose* (**3**). — 2,3,2',3'-Tetra-*O*-benzyl-α,α-trehalose[10] (**1**) (280 mg) was treated with *p*-tolylsulfonyl chloride (330 mg) in pyridine (5 mL). The mixture was stirred for 80 min at room temperature. Ethyl acetate (10 mL) and 2M HCl (10 mL) were added, and the organic phase was separated and washed with water, a saturated $NaHCO_3$ solution, and water again. It was dried and evaporated and the residue chromatographed. Elution with chloroform gave the syrupy di-*O*-tosyl derivative **2** (150 mg, 27%), $[\alpha]_D^{22}$ +70° (*c* 1.2, chloroform); R_F 0.90 (3:1 benzene–methanol); ^{1}H-n.m.r.: δ 7.90–7.32 (m, 28 H, aryl) and 2.42 (s, 6 H, CH_3Ts).

Anal. Calc. for $C_{54}H_{58}O_{15}S_2$: C, 64.14; H, 5.78; S, 6.34. Found: C, 63.93; H, 6.01; S, 6.23.

Continued elution with chloroform gave, as the major product, the syrupy monotosyl derivative **3** (239 mg, 51%); $[\alpha]_D^{22}$ +77° (*c* 0.9, chloroform); R_F 0.80 (3:1 benzene–methanol); ^{1}H-n.m.r.: δ 7.82–7.30 (m, 24 H, aryl) and 2.40 (s, 3 H, CH_3Ts).

Anal. Calc. for $C_{47}H_{52}O_{13}S$: C, 65.87; H, 6.11; S, 3.74. Found: C, 66.23; H, 6.15; S, 3.82.

Finally, elution with 20:1 chloroform–methanol yielded the unchanged starting material (78 mg, 20%), R_F 0.70 (3:1 benzene–methanol).

2,3,2',3'-Tetra-O-benzyl-6-O-mycoloyl-α,α-trehalose (**4**). — The monotosyl derivative **3** (85 mg) was treated with potassium mycolate[1] in *N,N,N',N',N'',N''*-hexamethylphosphoric triamide (2 mL) at 100° for 5 h. The mixture was cooled and 2M HCl (2 mL) was added. The product was filtered off and washed with water. The precipitate was dissolved in chloroform and the solution evaporated. The residue was dissolved in 1:1 chloroform–methanol and the solution was stirred with AG-MP (OH^-) anion-exchange resin to remove the excess of mycolic acid, and the product was then purified by column chromatography. Elution with 2:1 hexane–ethyl acetate removed fast-moving impurities, and with 1:1 hexane–ethyl acetate gave the homogeneous product **4** (134 mg, 73%), wax, $[\alpha]_D^{22}$ +34.5° (*c* 1.0, chloroform); R_F 0.71 (3:1 ethyl acetate–hexane).

Anal. Calc. for $C_{119}H_{200}O_{14}$: C, 77.05; H, 10.87. Found: C, 77.38; H, 11.06.

2,3,2',3'-Tetra-O-benzyl-6-O-corynomycoloyl-α,α-trehalose (**5**). — Compound **3** (128 mg) was treated with potassium corynomycolate[1] (230 mg) *N,N,N',N',N'',N''*-hexamethylphosphoric triamide (2 mL) as described for the preparation of **4**. The product was purified by column chromatography. Elution with 1:1 ethyl acetate–hexane, followed by 2:1 ethyl acetate–hexane gave **5** (149 mg, 86%), wax, $[\alpha]_D^{22}$ +66.5° (*c* 1.2, chloroform); R_F 0.62 (3:1 ethyl acetate–hexane).

Anal. Calc. for $C_{72}H_{108}O_{13}$: C, 73.18; H, 9.21. Found: C, 72.96; H, 9.43.

6-O-Mycoloyl-α,α-trehalose (**6**). — Compound **4** (100 mg), dissolved in ethyl

acetate (40 mL) and ethyl alcohol (25 mL), was hydrogenolyzed in the presence of 10% Pd–C catalyst (100 mg) at 340 pKa for 16 h. The catalyst was filtered off and washed with chloroform, and the filtrate evaporated. The residue was triturated with methanol to give a pure solid (55 mg, 69%), which was found to be identical with 6-*O*-mycoloyl-α,α-trehalose described earlier on the basis of optical rotation, t.l.c. comparison with the authentic sample (R_F 0.40, 14:6:1 chloroform–methanol–water), early charring to a blue-gray definitive color, and by the highly characteristic i.r. spectrum[3]. As described before, ^{1}H-n.m.r. spectra of the unsymmetrical trehalose 6-monoesters are essentially uninformative partly because of complexity and also because, as noted before, even for the symmetrical but otherwise unsubstituted 6,6′-di-*O*-acyltrehaloses, the carbohydrate protons are unrelaxed[22] and give broad, clumped signals at δ 5.18 to 3.38.

6-O-Corynomycoloyl-α,α-trehalose (**7**). — Compound **5** (126 mg), dissolved in 1:1 ethyl acetate–ethyl alcohol (50 mL), was hydrogenolyzed in the presence of 10% Pd–C catalyst as described for the preparation of **6**. The crude product was purified by column chromatography. Elution with 10:1 chloroform–methanol removed fast-moving impurities. Continued elution with 7:1 chloroform–methanol, followed by 5:1 chloroform–methanol gave **7** (50 mg, 57%) as a wax. It was identical with 6-*O*-corynomycoloyl-α,α-trehalose described previously on the basis of optical rotation, t.l.c. comparison (R_F 0.46, 4:6:1 chloroform–methanol–water), and its i.r. spectrum.

6′-O-Acetyl-2,3,2′,3′-tetra-O-benzyl-6-O-mycoloyl-α,α-trehalose (**8**). — 2,3,2′,3′-Tetra-*O*-benzyl-6-*O*-mycoloyl-α,α-trehalose (**4**, 267 mg) was treated with 80% aqueous acetic acid (15 mL) at 85° for 15 h. The mixture was evaporated and the residue chromatographed. Elution with 3:1 hexane–ethyl acetate gave the monoacetate **8** (31 mg, 11%), $[\alpha]_D^{22}$ +41.5° (*c* 0.9, chloroform); R_F 0.74 (1:1 ethyl acetate–hexane); ^{1}H-n.m.r.: δ 7.50–7.38 (m, 20 H, aryl), 2.10 (s, 3 H, CH_3CO), and 1.60–0.60 (m, mycoloyl).

Anal. Calc. for $C_{121}H_{202}O_{15}$: C, 76.61; H, 10.73. Found: C, 76.63; H, 10.84.

Continued elution with 3:1 hexane–ethyl acetate removed a trace amount of a by-product. Elution with 2:1 hexane–ethyl acetate gave the unchanged starting material (183 mg).

6′-O-Acetyl-6-O-mycoloyl-α,α-trehalose (**9**). — Compound **8** (29 mg), dissolved in 1:1 ethyl acetate–ethyl alcohol (30 mL), was hydrogenolyzed in the presence of 10% Pd–C catalyst (60 mg) as described for the preparation of **6**. The product was purified by column chromatography. Elution with 7:1 chloroform–methanol removed a trace amount of impurities. Continued elution with the same solvent gave pure **9** (13 mg, 55%), wax, $[\alpha]_D^{22}$ +41.5° (*c* 0.8, chloroform); lit.[11,12] $[\alpha]_D$ +44.8°, 46.6°; R_F 0.72 (14:6:1 chloroform–methanol–water).

Anal. Calc. for $C_{93}H_{178}O_{15}$: C, 72.70; H, 11.68. Found: C, 72.66; H, 11.56.

2,3,4,2′,3′,4′-Hexa-O-acetyl-6,6′-di-O-methyl-α,α-trehalose (**12**). — 2,3,4,2′,3′,4′-Hexa-*O*-acetyl-α,α-trehalose[20] (52 mg) was treated with methyl trifluoromethanesulfonate (0.2 mL) and 2,6-di-(*tert*-butyl)pyridine (0.4 mL) in

dichloromethane (1 mL). The mixture was stirred at 60° for 16 h and evaporated. Ethyl acetate (10 mL) and 2M HCl (10 mL) were added to the residue. The organic phase was washed with water, a saturated $NaHCO_3$ solution, and water, dried, and evaporated. The residue was chromatographed. Elution with 3:1 ethyl acetate–hexane gave crystalline **12** (49 mg, 90%). It was recrystallized from acetone–hexane to give fine needles, m.p. 156–157°, $[\alpha]_D^{22}$ +145° (*c* 0.9, chloroform); ^{1}H-n.m.r.: δ 5.70 (2 H, $J_{2,3}$ 9.5, $J_{3,4}$ 10.0 Hz, H-3,3'), 5.30 (2 H, $J_{1,2}$ 4.0 Hz, H-1,1'), 5.08 (2 H, $J_{4,5}$ 10.0 Hz, H-4,4'), 5.05 (2 H, H-2,2'), 3.35 (s, 6 H, 2 CH_3), 2.04, 2.02, and 2.0 (3 s, 18 H, 6 CH_3CO).

Anal. Calc. for $C_{26}H_{38}O_{17}$: C, 50.16; H, 6.15. Found: C, 49.70; H, 6.14.

Permethylation studies. — To a solution of **6** (11 mg) in dichloromethane (0.5 mL) and 2,6-di-(*tert*-butyl)pyridine (0.2 mL) was added methyl trifluoromethanesulfonate (0.1 mL). The mixture was stirred at 65° for 16 h and evaporated. The residue was extracted with ethyl acetate and the extract washed with 2M HCl, water, a saturated $NaHCO_3$ solution, and water. The organic solution was evaporated and the residue was treated with 10% ethanolic KOH (0.5 mL) in 1,4-dioxane (0.5 mL). The mixture was boiled under reflux for 90 min and evaporated. T.l.c. indicated the presence of one major spot having the same mobility as that of 2,3,4,6,2',3',4'-hepta-*O*-methyl-α,α-trehalose[3] (**10**)*. The crude product was purified by column chromatography. Elution with chloroform removed methyl mycolate. Elution with 20:1 chloroform–methanol gave a product that was still contaminated with potassium mycolate. Methanol (1 mL) was added, the insoluble potassium mycolate filtered off and washed with a minimal amount of methanol, and the filtrate evaporated. G.l.c. examination of the residue showed it to be homogeneous and to have the same retention time as an authentic sample of 2,3,4,6,2',3',4'-hepta-*O*-methyl-α,α-trehalose (**10**). Under the same conditions, the mono-*O*-acetyl derivative **9** was converted into 2,3,4,2',3',4'-hexa-*O*-methyl-α,α-trehalose[13] (**11**).

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*Permethylation of the original sample of trehalose monomycolate isolated by Kato and Maeda[3] was carried out in this laboratory (by J. Maeda and M. Goren) under nonisomerizing conditions with diazomethane and borontrifluoride[3,16] to give authentic hepta-*O*-methyltrehalose for comparison.

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