Carbohydrate Research, 245 (1993) 151–158
Elsevier Science Publishers B.V., Amsterdam

Note

Isolation and purification of trehalose 6-mono- and 6,6′-di-corynomycolates from *Corynebacterium matruchotii*. Structural characterization by ^{1}H NMR

Arun K. Datta * and Kuni Takayama †

Mycobacteriology Research Laboratory, William S. Middleton Memorial Veterans Hospital, and Department of Bacteriology, College of Agricultural and Life Sciences, University of Wisconsin, Madison, WI 53706 (USA)

(Received October 21st, 1991; accepted in revised form July 10th, 1992)

Trehalose esters of mycolic acids (2-alkyl long chain hydroxy fatty acids) and their analogs are biologically important components of Corynebacteriae, Rhodococci, Nocardiae, and Mycobacteriae (order *Actinomycetales*; for reviews, see refs 1 and 2). These glycolipids have been studied by several groups (for review, see ref 2). The isolation and partial characterization of trehalose dicorynomycolate (**1**) from an acetone-insoluble fraction of *Corynebacterium diphtheriae* was first reported by Ioneda et al.[3]. This corynomycolate was later found to be a mixture of three components[4–7], which could be separated as their trimethylsilyl derivatives[7]. These compounds, termed B1, B2, and B3, were characterized by Puzo et al.[7] using electron-impact mass spectrometry (EIMS), as the dicorynomycolate, the monocorynomycolate-mono(dehydrocorynomycolate), and the bis(dehydrocorynomycolate) **. A similar study was done by Puzo and Prome[8] on the 6-monocorynomycolate of α,α-trehalose (**2**) from the same organism.

Corynebacterium matruchotii also contain mycolate ester glycolipids. Shimakata et al.[9] tentatively identified trehalose monocorynomycolate as a product of synthesis of corynomycolic acid by a cell-free system of *C. matruchotii*. In our work with *C. matruchotii*, we have found that the composition of the mycolate ester glycolipid fraction is relatively simple as compared to that of *C. diphtheriae*. Thus, it appears

† Corresponding author.
* Present address: Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235 (USA).
** Dehydrocorynomycolate refers to 3-oxo-2-tetradecyloctadecanoate.

to be a better system for studying the biosynthesis of corynomycolic acids. For this reason, we decided to complete the structural determination of the corynomycolate esters in *C. matruchotii*.

The dicorynomycolate fraction was isolated from cells of *C. matruchotii*, purified by column chromatography, and analyzed by TLC as the Me_3Si derivative by the method of Puzo et al.[7]. A single major band (~95%) was observed that cochromatographed with the Me_3Si derivative of synthetic trehalose 6,6′-dicorynomycolate[10]. It was equivalent to Puzo's B1 component[7]. A minor band (<5%), which could represent Puzo's B2 component[7], was observed at R_f 0.70 in 19:1 hexane–Et_2O. The B3-like component observed by Puzo et al.[7] was absent in our preparation. Thus, mycolate esters of *C. matruchotii* appear to contain only small amounts of 3-oxo fatty acyl groups, in contrast to those of *C. diphtheriae* where they are a major component (data not shown). Cf plasma desorption MS of the purified dicorynomycolate from *C. matruchotii* showed molecular ions $M + Na^+$ at m/z 1322 as the major component and m/z 1349 as the minor component. These results indicated that the major fatty acyl group of this glycolipid is a C_{32}-corynomycolic acid. This was confirmed by EIMS of the methyl ester of the isolated fatty acid. A major $M - 18$ ion at m/z 492 and a characteristic pyrolysis fragment (methyl palmitate) at m/z 270 were observed. The major fatty acyl group of the dicorynomycolate from *C. matruchotii* is thus determined as 3-hydroxy-2-tetradecyloctadecanoyl. Saponification of the dicorynomycolate yielded a water-soluble product identified to be trehalose. Acid hydrolysis yielded 2 mol of glucose per mol of dicorynomycolate.

Puzo et al.[7] determined the position of acyl groups on trehalose by comparing the mass spectrum of the Me_3Si derivative of synthetic trehalose dicorynomycolate with that of the natural isolate from *C. diphtheriae*. These authors noted that the unsaturation in the acyl groups made the interpretation difficult because of the superimposition of different ion structures[7]. We have found that ^1H NMR gives a spectrum clearly indicating the position of the acyl groups of trehalose dicorynomycolate without the need for derivatization[10]. The synthetic 6,6′-di-*O*-[(2*RS*,3*RS*)-3-hydroxy-2-tetradecyloctadecanoyl]-α,α-trehalose and its natural isomer isolated from *C. matruchotii* gave similar ^1H NMR spectra. The spectrum for the natural isomer was simpler because of the presence of only one isomeric corynomycolic acid (probably the 2*R*, 3*R* isomer, see below), unlike the synthetic analog which contains a mixture of both (2*R*,3*R*)- and (2*S*,3*S*)-3-hydroxy-2-tetradecyloctadecanoic acids[10]. This was evident by comparing the spectrum with that of trehalose dipalmitate. As expected, both spectra were similar (Fig. 1) in the region from δ 5.0–3.0, which contains the sugar C*H* and CH_2 signals. Downfield shift of equivalent H-6a and H-6′a resonances to δ 4.26 is consistent with the presence of acyl groups at O-6 and O-6′ of trehalose.

The purified monocorynomycolate fraction from *C. matruchotii* was examined by TLC after Me_3Si derivatization. It gave a single band that comigrated with the Me_3Si derivative of synthetic trehalose monocorynomycolate. The Cf plasma-de-

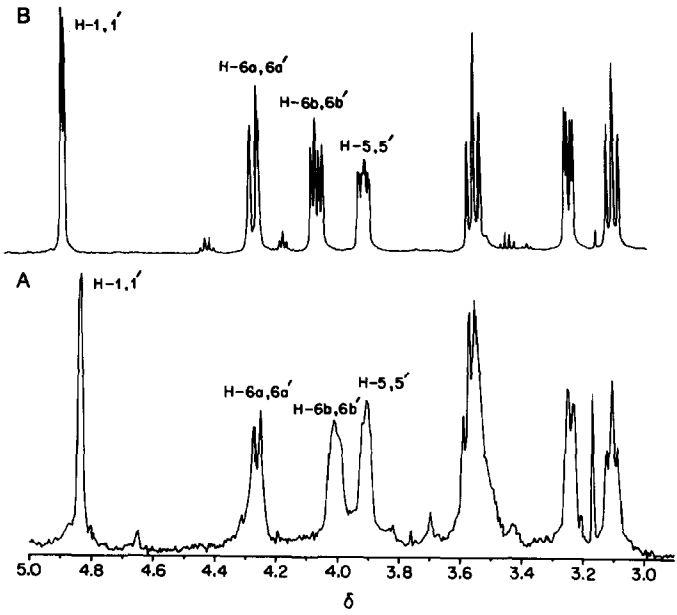
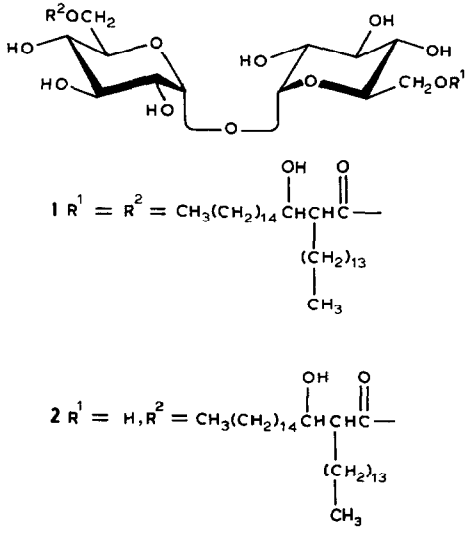


Fig. 1. Partial ^{1}H NMR spectra of: (A) purified trehalose dicorynomycolate from *C. matruchotii*, and (B) synthetic trehalose 6,6'-dipalmitate[10].

sorption MS of this natural isolate showed a major $M + Na^+$ ion peak at m/z 843, indicating the presence of 3-hydroxy-2-tetradecyloctadecanoyl-α,α-trehalose (also see ref 10). When the fatty acyl group was liberated from this compound and analyzed by EIMS as its methyl ester, the spectrum was similar to that observed for



Scheme 1.

the fatty acyl group of trehalose dicorynomycolate (see above). A M − 18 peak at m/z 492 was observed for the fatty acyl group of this natural compound.

The position of acyl group on trehalose of trehalose monocorynomycolate was determined by Shimakata et al.[9] following the method of Puzo and Prome[8]. However, Me_3Si derivatization was required. We obtained a clear and interpretable ^1H NMR spectrum by use of the procedure described earlier[10]. When the spectrum of this natural isolate was compared with that of its synthetic product, they were identical, indicating O-6 as the position of the (2*R*, 3*R*)-corynomycoyl group on trehalose. The H-6a resonance was at δ 4.30.

In conclusion, this study showed that *C. matruchotii* synthesizes both trehalose 6-mono- and 6,6′-di-corynomycolates. Chromatographic evidence suggested that, unlike those of *C. diphtheriae*, the trehalose esters of *C. matruchotii* contain only small amounts of 3-oxo fatty acyl groups. Thus, *C. matruchotii* appears to be a better system to use for the study of the biosynthesis of corynomycolic acids than *C. diphtheriae*. We have utilized ^1H NMR to determine the position of the fatty acyl groups on the trehalose corynomycolates isolated from *C. matruchotii*. This method is direct and the results are unambiguous. It offers certain advantages over the method involving EIMS of the Me_3Si derivative.

EXPERIMENTAL

General methods.—Melting points were measured with a Fisher–Johns melting point apparatus and are uncorrected. Optical rotations were determined with a Perkin–Elmer Model 141 polarimeter. NMR spectra were determined in the National Magnetic Resonance Facility at Madison by use of a Bruker AM-400 or Bruker AM-500 spectrometer at ambient temperature. Samples were dissolved in $(CD_3)_2SO$ with an added drop of trifluoroacetic acid. ^1H Chemical shifts, quoted from the signal of Me_4Si, were measured from internal $CHCl_3$ signal at δ 8.31 for solutions in $(CD_3)_2SO$. EIMS were recorded with an A.E.I. MS DS-50 instrument. Cf plasma desorption MS was performed at the Middle Atlantic Mass Spectrometry Laboratory, Dept. of Pharmacology and Molecular Science, The Johns Hopkins University School of Medicine, Baltimore, MD. Separations were accomplished by open column chromatography on Silica Gel 60 (70–230 mesh, Merck) and DEAE-cellulose (AcO^-, Bio-Rad). TLC was performed on silica gel plates (250 μm, Merck). The glycolipids on the plates were detected by spraying with orcinol–H_2SO_4 (ref 11) and heating (for the detection of sugars) with K_2CrO_7–H_2SO_4 (ref 12) for nonspecific charring, and with an Mo reagent[13] (for the detection of organic phosphates).

Growth of organisms.—Cells of *C. matruchotii* (ATCC 14266) were grown in brain heart infusion broth with 2% yeast extract (Difco) at 37°C with shaking for 72 h to late-log growth and were harvested by centrifugation.

Isolation and purification of trehalose mono- and di-corynomycolates from C. matruchotii.—Cells (45 g wet weight) were suspended in 2:1 $CHCl_3$–MeOH (1 L),

and the lipids were extracted by the method of Folch et al.[14]. The extraction was repeated twice, and the pooled extracts were dried (6.85 g) and dissolved in 2:1 $CHCl_3$–MeOH (15 mL). This solution was added to cold acetone (1.5 L) and stirred, and the acetone-insoluble material was recovered by filtration (1.3 g). It contained the glycolipids including trehalose mono- and di-corynomycolates along with contaminating phospholipids. This sample was passed through a DEAE-cellulose column to remove the phospholipids as follows: the acetone-insoluble materials (1.26 g) were dissolved in 4:1 $CHCl_3$–MeOH, converted to the free acid form by passage through a Dowex 50 (H^+) column in the same solvent, and applied to a column (4 × 35 cm) of DEAE-cellulose (AcO^-). The column was washed with $CHCl_3$ (1.2 L), 2:3:1 $CHCl_3$–MeOH–H_2O (1.2 L), and then 0.1 M NH_4OAc (1.2 L) in 2:3:1 $CHCl_3$–MeOH–H_2O, each eluting solvent being collected separately. The three eluates were evaporated to dryness, and the residues were examined by TLC. The 2:3:1 $CHCl_3$–MeOH–H_2O eluate contained trehalose esters (321 mg dry weight) as judged by TLC in 39:11:1 $CHCl_3$–MeOH–H_2O. This fraction was completely free of phospholipids since the Mo reagent[13] did not reveal organic phosphate on the TLC plates. The trehalose esters (entire sample) were further fractionated on a 3.4 × 28 cm Silica Gel 60 column using a stepwise gradient of $CHCl_3$ (300 mL), 9:1 $CHCl_3$–MeOH (400 mL), 4:1 $CHCl_3$–MeOH (400 mL), and 3:1 $CHCl_3$–MeOH (400 mL). Fractions were collected and analyzed by TLC using the solvent system of 39:11:1 $CHCl_3$–MeOH–H_2O. The trehalose dicorynomycolate fraction eluted from the column in 19:1 $CHCl_3$–MeOH was pooled and dried (59.3 mg). TLC of this sample in 39:11:1 $CHCl_3$–MeOH–H_2O showed a single band at R_f 0.78 that co-chromatographed with synthetic 6,6′-di-*O*-[(2*RS*,3*RS*)-3-hydroxy-2-tetradecyloctadecanoyl]-α,α-trehalose[10]. It was a white solid, mp (deposited from MeOH) 146–147°C; $[\alpha]_D^{20}$ +82° (*c* 0.3, $CHCl_3$) *; ^1H NMR [$(CD_3)_2SO + CF_3CO_2H$]: δ 4.84 (br 2 H, H-1,1′), 4.26 (br d., 2 H, *J* 10.5 Hz, H-6a,6′a), 4.01 (m, 2 H, H-6b,6′b), 3.90 (m, 2 H, H-5,5′), 3.60–3.05 (series of m, sugar CH and CH_2, H-3 of acyl), 2.28 (m, 2 H, H-2 of acyl), 1.94 (m), 1.55–1.05 (series of m, acyl CH_2), 0.80 (two superimposed t, terminal CH_3). Cf plasma-desorption MS: positive ion, molecular ion peaks at *m/z* 1322 and 1349 (Calcd M + Na^+ where $R^1 = R^2 = C_{32}$, *m/z* 1322; Calcd M + Na^+ where $R^1 = C_{32}$, $R^2 = C_{34}$, *m/z* 1350).

A part (300 μg) of this natural isolate was converted to its Me_3Si derivative by the scaled-down procedure of Puzo et al.[7] TLC of this product in 19:1 hexane–Et_2O gave a single major band at R_f 0.87, cochromatographing with the Me_3Si derivative of the synthetic trehalose dicorynomycolate.

The 4:1 $CHCl_3$–MeOH eluate of the Silica Gel 60 column fractionation of the trehalose esters (see above) contained the trehalose monocorynomycolates. The

* The earlier isolate[3] from *C. diphtheriae* had lower mp (110–115°C) and $[\alpha]_D$ +64°, presumably because of the impurities[4,5], and the synthetic analog, which was a mixture of diastereoisomers (with respect to corynomycoloyl groups), had mp 150–151°C and $[\alpha]_D^{20}$ +42° (ref 10).

appropriate fractions (based on TLC) were pooled and evaporated to dryness (15.9 mg); TLC in 39:11:1 $CHCl_3$–MeOH-H_2O showed a single band at R_f 0.41 that cochromatographed with synthetic 6-*O*-[(2*R*,3*R*)-3-hydroxy-2-tetradecyloctadecanoyl]-α,α-trehalose[10]. After deposition from MeOH, a white solid, it had mp 194–196°C with softening at about 160–165°C (lit.[10] 195–196°C) and $[\alpha]_D^{20}$ +70° (*c* 0.2, $CHCl_3$) (lit.[10] + 68°); ^1H NMR [$(CD_3)_2$ + CF_3CO_2H] identical to that of synthetic 6-*O*-[(2*R*,3*R*)-3-hydroxy-2-tetradecyloctadecanoyl]-α,α-trehalose[10]. Cf plasma desorption MS: positive ion, major peak at *m/z* 843 for M + Na^+.

After conversion of this natural isolate (~ 250 μg) to its Me_3Si derivative as described above, TLC in 19:1 hexane–Et_2O showed a single band at R_f 0.53, which cochromatographed with the Me_3Si derivative of synthetic 6-*O*-[(2*R*, 3*R*)-3-hydroxy-2-tetradecyloctadecanoyl]-α,α-trehalose[10] (trehalose monocorynomycolate).

Characterization of the sugar.—The purified mono- and di-corynomycolates (2 mg of each) were suspended in 2 M CF_3CO_2H (200 μL) and incubated at 100°C for 6 h. The lipids were removed from the hydrolyzates by extraction with petroleum ether. The aqueous layer was evaporated to dryness, the residue was dissolved in of 1:1 EtOH–H_2O (100 μL), and the solution (5 μL) was spotted on Whatman 3 M paper (12 × 42 cm), along with D-glucose. Two solvent systems of 12:5:4 EtOAc–pyridine–H_2O and 7:1:2 2-propanol–EtOAc–H_2O were used. The spots were visualized using silver nitrate reagent[15]. In both solvents, the monosaccharide derived from the purified corynomycolate esters comigrated with the glucose standard.

The presence of trehalose in both samples of corynomycolate esters was demonstrated as follows: samples (2 mg each) were incubated overnight in 5% KOH in 1:1 EtOH–H_2O at 37°C and acidified with AcOH, and the fatty acids were removed by extraction with petroleum ether. The remaining aqueous phases were desalted by passage through a mixed-bed resin column. The residue was analyzed by PC using the two solvent systems mentioned above. After spraying the developed chromatogram with HIO_4–benzidine reagent[16], a single spot appeared which comigrated with trehalose. Both trehalose and sample spots were negative to the $AgNO_3$ dip reagent[15].

Quantitative estimation of sugar.—The solutions obtained by CF_3CO_2H hydrolysis of the purified trehalose mono- and di-corynomycolates (see above) were used for quantitation of the glucose content with the cysteine–H_2SO_4 reagent[17]. Standard D-glucose and synthetic 6-*O*-[(2*RS*,3*RS*)-3-hydroxy-2-tetradecyloctadecanoyl]-α,α-trehalose[10] were used as controls. Based on 821 and 1300 as the average mol wt for trehalose mono- and di-corynomycolates from *C. matruchotii*, the molar ratios of trehalose to glycolipid were 1.03:1 and 0.98:1, respectively.

Analysis of fatty acids.—The fatty acids obtained from trehalose mono- and di-corynomycolates by saponification–acidification (see above) were examined by TLC. These samples cochromatographed with synthetic (2*R*,3*R*)-3-hydroxy-2-tetradecyloctadecanoic acid; R_f (19:1 $CHCl_3$–MeOH) 0.34, (20:20:1 petroleum

ether–Et_2O–AcOH) 0.29. When examined by TLC, the methylated samples (by diazomethane) also cochromatographed with synthetic methyl (2*RS*,3*RS*)-3-hydroxy-2-tetradecyloctadecanoate[10] in $CHCl_3$ (R_f 0.42) and 4:1 petroleum ether–Et_2O (R_f 0.38); EIMS of the methylated samples: m/z 492 (major) for M − 18, 299 for M − $C_{15}H_{31}$, and 270 for methyl palmitate. As reported earlier[18,19], a homologous series of M − 18 were also observed at m/z 506 and 518 (minor).

ACKNOWLEDGMENTS

This work was supported, in part, by the Veterans Administration and by the National Institutes of Health Grant GM-36054 (to K.T.). The authors are grateful for the collaboration of Dr. Robert Cotter and the staff of the Middle Atlantic Mass Spectrometry Laboratory, a National Science Foundation Shared Instrumentation Facility. Efficient services were also provided by the staff of the National Magnetic Resonance Facility at Madison, which is supported, in part, by NIH grant RR02301 from the Biomedical Research Technology Program, Division of Research Resources. Equipment in the facility was purchased with funds from the University of Wisconsin, the NSF Biological Instrumentation Program (grant PCM-8415048), the NIH Biomedical Research Technology Program (grant RR02031), the NIH Shared Instrumentation Program (grant RR02781), and the U.S. Department of Agriculture. The authors are thankful to Dr. Laurens Anderson for his critical review of this manuscript.

REFERENCES

1 E. Lederer, in G.P. Kubica and L.G. Wayne (Eds.), *The Mycobacteria — A Sourcebook*, Part A, Marcel Dekker, New York, 1984, pp 361–378.
2 C. Asselineau and J. Asselineau, *Prog. Chem. Fats Other Lipids*, 16 (1978) 59–99.
3 T. Ioneda, M. Lenz, and J. Pudles, *Biochem. Biophys. Res. Commun.*, 13 (1963) 110–114.
4 M. Senn, T. Ioneda, J. Pudles, and E. Lederer, *Eur. J. Biochem.*, 1 (1967) 353–356.
5 D.W. Thomas, A.K. Matida, C.L. Silva, and T. Ioneda, *Chem. Phys. Lipids*, 23 (1979) 267–282.
6 G. Puzo, G. Tissie, C. Lacave, H. Aurelle, and J.C. Prome, *Biomed. Mass Spectrom.*, 5 (1978) 699–703.
7 G. Puzo, G. Tissie, H. Aurelle, C. Lacave, and J.C. Prome, *Eur. J. Biochem.*, 98 (1979) 99–105.
8 G. Puzo and J.C. Prome, *Biomed. Mass Spectrom.*, 5 (1978) 146–152.
9 T. Shimakata, K. Tsubokura, T. Kusaka, and K.-I. Shizukuishi, *Arch. Biochem. Biophys.*, 238 (1985) 497–508.
10 A.K. Datta, K. Takayama, M.A. Nashed, and L. Anderson, *Carbohydr. Res.*, 218 (1991) 95–105.
11 A.K. Datta, V.M. Katoch, K. Katoch, V.D. Sharma, C.T. Shivannavar, and J.J. Ghosh, *Indian J. Med. Microbiol.*, 6 (1988) 33–39.
12 A.K. Datta, V.M. Katoch, V.D. Sharma, and K. Katoch, *Int. J. Lepr.*, 53 (1985) 45–51.
13 J.C. Dittmer and R.L. Lester, *J. Lipid Res.*, 5 (1964) 126–127.
14 J. Folch, M. Lees, and G.H. Sloane Stanley, *J. Biol. Chem.*, 226 (1957) 497–509.
15 I. Smith, in I. Smith (Ed.), *Chromatographic and Electrophoretic Techniques*, Vol. I, Interscience, New York, 1960, pp 246–260.
16 H.T. Gordon, W. Thornburg, and L.N. Werum, *Anal. Chem.*, 28 (1956) 849–855.

17 Z. Dische, *Methods Carbohydr. Chem.*, 1 (1962) 488–494.
18 L. Alshamaony, M. Goodfellow, D.E. Minnikin, G.H. Bowden, and J.H. Hardie, *J. Gen. Microbiol.*, 98 (1977) 205–213.
19 H. Wada, H. Okada, H. Suginaka, I. Tomiyasu, and I. Yano, *FEMS Microbiol. Lett.*, 11 (1981) 187–192.