Synthesis of Mycolic Acid Biosurfactants and Their Physical and Surface-Active Properties

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ABSTRACT: Five mycolic acids [2-alkyl-3-hydroxy FA: $R_1C^*H(OH)C^*HR_2COOH$] were synthesized using acyl chlorides with alkyl chains of different lengths (total carbon numbers of mycolic acids, 12, 16, 20, 24, 36). The relationship between the chemical structures of the mycolic acids and their surface-active properties was determined. The acids were synthesized in three steps: (i) dimerization of acyl chloride into alkyl ketene dimer, (ii) selective reduction of C=C to C–C by hydrogenation, and (iii) β-lactone ring cleavage under alkaline conditions. The yields of C_{12}-, C_{16}-, C_{20}-, C_{24}-, and C_{36}-mycolic acid were 72, 73, 73, 73, and 73%, respectively. The critical micelle concentrations (CMC) of C_{12}-, C_{16}-, and C_{20}-mycolic acid were 2.2×10^{-4}, 1.36×10^{-4}, and 7.4×10^{-5} M, respectively. As the carbon number increased, the surface tension at the CMC value was also lower; the values for C_{12}-, C_{16}-, and C_{20}-mycolic acid were 46.54, 43.59, and 41.57 dyn/cm, respectively. The emulsifying activities of mycolic acids were determined for *n*-tetradecane, *n*-hexadecane, cyclohexane, and diesel oil. The results showed that C_{12}-mycolic acid was the best emulsifier for diesel oil, C_{16}-mycolic acid was the best emulsifier for *n*-tetradecane and *n*-hexadecane, and C_{20}-mycolic acid was the best emulsifier for cyclohexane. This study showed that mycolic acids having surface-active properties can be chemically synthesized for potential applications in the detergent/cleaning material industries, for example, in oil spill cleanup, oil recovery, textiles, pharmaceuticals, and cosmetics.

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Biosurfactants are amphiphilic molecules produced by microorganisms with both hydrophobic and hydrophilic domains. They are frequently produced in nature by microorganisms growing on a water-immiscible substrate. By synthesizing a surface-active agent, they can reduce the interfacial energy/tension through emulsification or solubilization of hydrophobic substrates that otherwise are not bioavailable to them (1). Biosurfactants have a wide range of applications in various industries (2–4), including the environmental remediation of hydrophobic pollutants (5–9).

Biosurfactants may be divided into four different groups: (i) glycolipids, (ii) phospholipids, (iii) lipoproteins or lipopeptides, and (iv) polymeric biosurfactants. Mycolic acids are long-chain, β-hydroxy FA with a moderately long aliphatic chain at the α-carbon atom (10). The total number of carbon atoms varies from 30 to 86. They are produced by bacterial species belonging to the genus *Mycobacterium* and to genera of *Nocardia*, *Rhodococcus*, *Corynebacterium*, as well as other species in the minor genera (e.g., *Gordonia*, *Bacterionema*, *Micropolyspora*, and *Brevibacterium*) (11). Mycolic acids usually form part of the cell wall complex and are linked to the arabinogalactan–peptidoglycan matrix (12). However, mycolic acids are also found as a lipid component of certain extracellular glycolipids produced by these bacteria, particularly following growth on alkanes or related substrates. In these cases, the mycolic acid usually is esterified with the hydroxyl groups of a trehalose unit (13).

There are considerable variations in the FA chain. They may contain a keto-, methoxy-, or methyl-group, and/or cyclopropane rings. Both *cis* and *trans* double bonds may occur within the alkyl backbone chain itself, and the side-chain FA also may contain a double bond.

Mycolic acids have previously been produced through different synthetic routes (14,15). In this paper, we report a new synthesis pathway to obtain a high yield of mycolic acids using various acyl chlorides as starting materials. The present study also describes the yield of each step, characterization of each mycolic acid, and determination of the properties of mycolic acids as surface-active agents.

EXPERIMENTAL PROCEDURES

Mycolic acid synthesis and characterization. The mycolic acids were synthesized in three steps from acyl chlorides with alkyl chain lengths of 6, 8, 10, 12, and 18, respectively, as the starting material.

The first step was the dimerization of acyl chloride to make alkyl ketene dimer (16). This was accomplished by adding 100 g of acyl chloride to 800 mL of toluene and cooling to a temperature below 10°C in an ice bath. Triethylamine (TEA, 1.1 molar equiv) was then slowly added from a dropping funnel while stirring. After 1 h in the ice bath, the mixture was stirred for about 3 to 4 h at room temperature. Water (250 mL) was then added to the reaction mixture and the mixture was extracted with 1,000 mL of CH_2Cl_2, filtered using Na_2SO_4 to remove the water, and then dried at reduced pressure using a rotary evaporator. It was then purified by flash column chromatography on silica gel eluting with ethyl acetate/*n*-hexane (1:30 vol/vol) and dried at reduced pressure. In the end, the alkyl

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ketene dimer was obtained in high yield. The physical properties of the alkyl ketene dimer were confirmed by TLC (ethyl acetate/*n*-hexane = 1:20 vol/vol), FTIR spectrometry, MS, and ^{1}H NMR spectrometry. The ^{1}H NMR spectra were recorded in $CDCl_3$ with a Fourier transform-NMR spectrometer using tetramethylsilane as an internal standard.

The second step was selective reduction of the alkyl ketene dimer by hydrogenation by adding 100 g of the dimer and 0.2 g of 10% palladium/charcoal (Sigma, St. Louis, MO) to 800 mL of an ethyl acetate/ethanol (3:1 vol/vol) mixture. This mixture was stirred for 3 or 4 h under H_2 and then purified using a column packed with silica gel (Silica gel 60; Merck, Darmstadt, Germany). This step produced the β-lactone in high yield. The physical properties of the β-lactone were confirmed by analyses using TLC (ethyl acetate/hexane = 1:20), FTIR spectrometry, MS, and ^{1}H NMR spectrometry.

The final step was the cleavage of β-lactone to make mycolic acids. This was done by adding 50 g of the β-lactone and NaOH (1.5 molar equiv) to 600 mL of an ethanol/water (3:1 vol/vol) mixture. This mixture was stirred for 3 or 4 h and acidified with 6 N HCl, and then purified using a column packed with silica gel. The mycolic acids were obtained in high yields. The physical properties of the final products were confirmed by analyses using TLC (methylene chloride/ethanol = 45:1 vol/vol), FTIR spectrometry, MS, and ^{1}H NMR spectrometry. Table 1 summarizes the properties and yields (both in grams and percent yield) of the intermediates and products obtained for the five mycolic acids synthesized.

HPLC analysis. All the products obtained were analyzed by HPLC before structural analysis. The HPLC analyses were performed with an HPLC system (consisting of a processor, pump, UV detector, column oven, and an autosampler), equipped with a Waters Atlantis dC_{18} reversed-phase column (150 × 3.9 mm, 5 μm; Waters, Milford, MA). Five hundred microliters of the properly diluted reaction mixture was injected. Methanol (100%) was used as eluent at 30°C with a flow rate of 0.5 mL/min. Products were detected using a UV detector (215 nm).

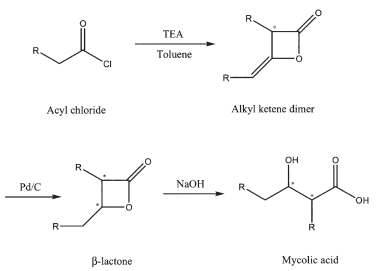
Surface tension measurement of mycolic acids. The surface tension properties of the synthetic mycolic acids were measured in an alkaline solution (pH 9.5) at various concentrations. The solution was prepared by dissolving mycolic acid in 0.01 M NaOH. It was then titrated with 0.001 M NaOH to attain a pH value between 10 and 12. Surface tension was determined at 20°C with a Thermo Cahn tensiometer supported by the WinDCA32 software suite.

Measurement of emulsification activity. To estimate the emulsification activity, 5 mL of *n*-tetradecane, *n*-hexadecane, cyclohexane, or diesel oil was added to 5 mL of alkaline water (pH 9.5) containing the synthetic mycolic acids at various concentrations (10, 100, and 1,000 mg/L) in graduated test tubes. These tubes were then vortexed for 2 min and allowed to stand at 30°C. The emulsion stability was measured after incubation for 24 h. The emulsification index (E_{24}%) was calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying by 100.

TABLE 1
Volumes, Yields, and Properties of the Intermediates and Products for Five Mycolic Acids

Starting material		Hexanoyl chloride	Octanoyl chloride	Decanoyl chloride	Lauroyl chloride	Stearoyl chloride
Step 1	IUPAC[a]	(*E*)-3-Butyl-4-pentylidene-oxetan-2-one	(*E*)-4-Heptylidene-3-hexyl-oxetan-2-one	(*E*)-4-Nonylidene-3-octyl-oxetan-2-one	(*E*)-3-Decyl-4-undecylidene-oxetan-2-one	(*E*)-3-Heptadecylene-3-hexadecyl-oxetan-2-one
	Initial	100 g (0.74 mol)	100 g (0.61 mol)	100 g (0.52 mol)	100 g (0.45 mol)	100 g (0.30 mol)
	Final	58 g	69 g	69 g	74 g	90 g
	Yield	72%	90%	90%	90%	90%
	R_f value[b]	0.70	0.70	0.70	0.70	0.70
	Phase	Yellowish-white liquid	Yellowish-white liquid	White solid	White solid	White solid
Step 2	IUPAC[a]	2-Butyl-octanoic β-lactone	2-Hexyl-decanoic β-lactone	2-Octyl-dodecanoic β-lactone	2-Decyl-tetradecanoic β-lactone	2-Hexadecyl-eicosanoic β-lactone
	Initial	100 g (0.51 mol)	100 g (0.39 mol)	100 g (0.32 mol)	100 g (0.27 mol)	100 g (0.19 mol)
	Final	95 g	90 g	90 g	90 g	90 g
	Yield	95%	90%	90%	90%	90%
	R_f value[b]	0.58	0.58	0.58	0.58	0.58
	Phase	Yellowish-white wax	Yellowish-white wax	White solid	White solid	White solid
Step 3	IUPAC[a]	2-Butyl-3-hydroxyoctanoic acid	2-Hexyl-3-hydroxydecanoic acid	2-Octyl-3-hydroxydodecanoic acid	2-Decyl-3-hydroxytetradecanoic acid	2-Hexadecyl-3-hydroxyeicosanoic acid
	Initial	50 g (0.25 mol)	50 g (0.20 mol)	50 g (0.16 mol)	50 g (0.125 mol)	50 g (0.09 mol)
	Final	51 g	48 g	47 g	43.3 g	45 g
	Yield	95%	90%	90%	90%	90%
	R_f value[b]	0.48	0.48	0.48	0.48	0.48
	Phase	Yellowish-white wax	Yellowish-white wax	White solid	White solid	White solid

[a]IUPAC, IUPAC names of the intermediates and products for five mycolic acids; Initial, the reaction volumes of starting materials and intermediates; Final, the final volumes of intermediates and products.
[b]The R_f values of intermediates and products were confirmed by TLC (ethyl acetate/*n*-hexane = 1:20 vol/vol).



SCHEME 1

RESULTS AND DISCUSSION

Synthesis and characterization of mycolic acids. The mycolic acid containing 16 total carbons was synthesized in three steps with octanoyl chloride as the starting material. The first step was the dimerization of octanoyl chloride to make (*E*)-4-heptylidene-3-hexyloxetan-2-one. The second step was selective reduction of the (*E*)-4-heptylidene-3-hexyloxetan-2-one to produce 2-hexyl-decanoic β-lactone. The third step was the cleavage of the 2-hexyl-decanoic β-lactone to make 3-hydroxy-2-hexyldecanoic acid. Scheme 1 shows the scheme for the synthesis of mycolic acids (where C* indicates chiral carbons). The mycolic acids thus obtained consist of the mixture of approximately equal amounts of *syn*-type and *anti*-type isomers. With regard to the stereochemistry of natural mycolic acid, on the other hand, there are four different possible arrangements at two chiral carbon atoms of the mycolic acids, i.e., 2*R*/3*R*, 2*R*/3*S*, 2*S*/3*R*, and 2*S*/3*S*. Natural mycolic acids usually show the same stereochemistry as 2*R*/3*R* formed at the chiral carbon positions. Therefore, it can be postulated that natural mycolic acids consist of only the *anti*-type isomer, although this was not demonstrated by our experiments.

The product structure in each step was analyzed and determined by the ^{1}H NMR spectra. In the first synthesis step, the ^{1}H NMR ($CDCl_3$) signals of (*E*)-4-heptylidene-3-hexyloxetan-2-one observed included the hydrogens of the methyl carbons of (*E*)-4-heptylidene-3-hexyloxetan-2-one (*t*, 6H, $C\underline{H}_3$: 0.90 ppm), the hydrogens of the alkyl chain carbons of (*E*)-4-heptylidene-3-hexyloxetan-2-one (*br*, 16H, $C\underline{H}_2$: 1.29–1.44 ppm), the hydrogen of the carbon next to the α-carbon of (*E*)-4-heptylidene-3-hexyloxetan-2-one (*q*, 2H, $CH_2C\underline{H}_2CHC{=}O$: 1.76 ppm), the hydrogens of the carbon next to the double bonded γ-carbon (*q*, 2H, $CH_2C\underline{H}_2CH{=}C$: 2.12 ppm), the hydrogen of the α-carbon next to the carbonyl group (*dd*, 1H, $CH_2C\underline{H}CO$: 3.93 ppm), and the hydrogen of the double bonded γ-carbon of (*E*)-4-heptylidene-3-hexyloxetan-2-one (*t*, 1H, $CH_2C\underline{H}{=}C$: 4.69 ppm) (data not shown). In the second synthesis step, the ^{1}H NMR ($CDCl_3$) signals of 2-hexyl-decanoic β-lactone included the hydrogens (*t*, 6H, $C\underline{H}_3$) of the methyl carbons of 2-hexyl-decanoic β-lactone (0.87 ppm), the hydrogens (*br*, 22H, $C\underline{H}_2$) of the alkyl-chain carbons of 2-hexyl-decanoic β-lactone (1.12–1.91 ppm), the hydrogen (*m*, 1H, $CH_2C\underline{H}C{=}O$) of the α-carbon (3.60 ppm), and the hydrogen (*m*, 1H, $CHC\underline{H}$-O) of the β-carbon of 2-hexyl-decanoic β-lactone (4.51 ppm) (data not shown). Finally, the ^{1}H NMR ($CDCl_3$) signals of the mycolic acid 3-hydroxy-2-hexyldecanoic acid include the hydrogens (*t*, 6H, $C\underline{H}_3$) of the methyl carbons (0.87 ppm), the hydrogens (*br*, 28H, $C\underline{H}_2$) of the alkyl-chain carbons (0.880–1.733 ppm), the hydrogen (*m*, 1H, $CHC\underline{H}C{=}O$) of the α-carbon (2.476 ppm), and the hydrogen (*m*, 1H, $CH_2C\underline{H}$(OH)CH) of the β-carbon (3.860 ppm) of the mycolic acid (Fig. 1). However, ^{1}H NMR spectral data did not show that the *syn*- and *anti*-diastereomers of C_{16} mycolic acid had slightly different shifts.

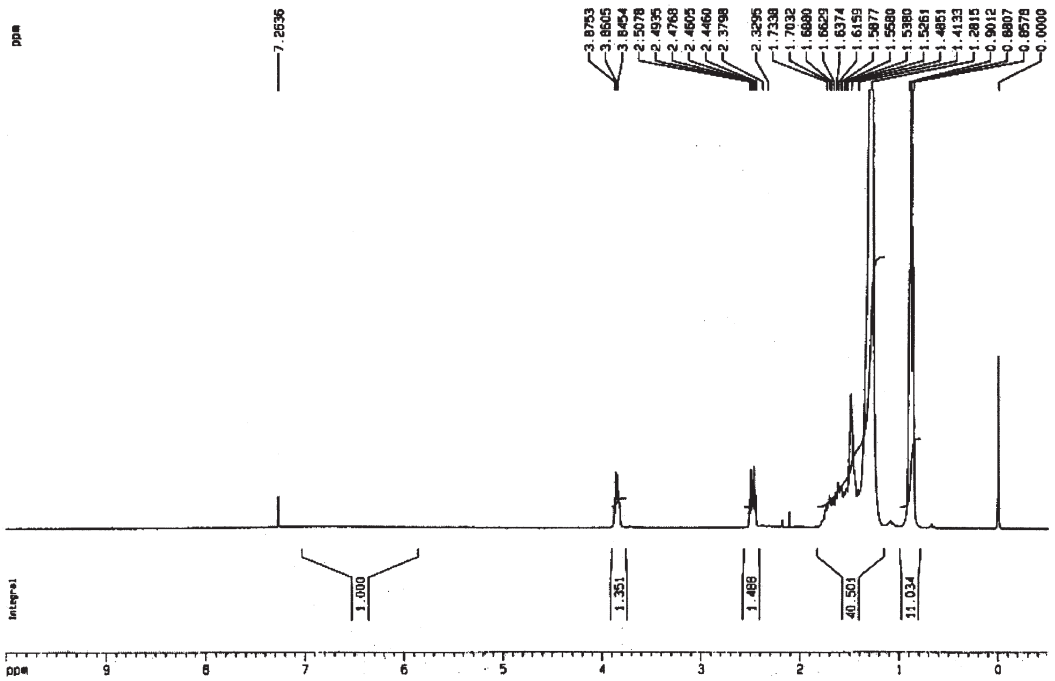


FIG. 1. ^{1}H NMR spectrum of 2-hexyl-3-hydroxydecanoic acid. A sample of 10 mg of the material in 0.6 mL of $CDCl_3$ was used. Frequency: 300 MHz; internal standard: tetramethylsilane; 0.87 ppm: *t*, 6H, $C\underline{H}_3$; 0.880–1.733 ppm: *br*, 28H, $C\underline{H}_2$; 2.476 ppm: *m*, 1H, $CHC\underline{H}C{=}O$; 3.860 ppm: *m*, 1H, $CH_2C\underline{H}$(OH)CH.

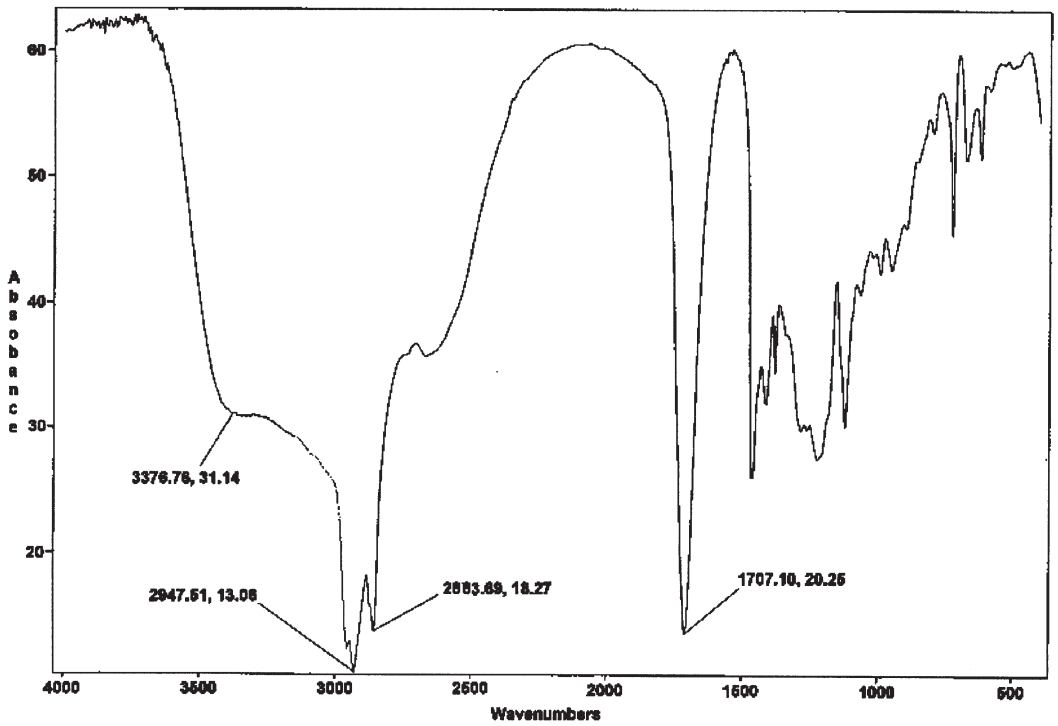


FIG. 2. FTIR spectrum of 2-hexyl-3-hydroxydecanoic acid. The FTIR spectrum of 2-hexyl-3-hydroxydecanoic acid was measured in a liquid state (chloroform). Absorption band at 3,376 cm^{-1}: O–H stretching vibration; absorption band at 3000–2850 cm^{-1}: C–H stretching in CH_3 and CH_2; absorption band at 1,707 cm^{-1}: vibration of the keto group.

Figure 2 is an FTIR spectrum of 2-hexyl-3-hydroxydecanoic acid in the liquid state. Absorption bands were observed at 3,376 cm^{-1} (O–H stretching vibration) and 1,707 cm^{-1} (the vibration of the keto group), which determined the existence of a hydroxyl group and a keto group in the structure of the mycolic acid.

Up to this point, ^{1}H NMR and FTIR spectra for 2-hexyl-3-hydroxydecanoic acid have been described. The ^{1}H NMR and FTIR spectra of the other mycolic acids, i.e., 2-butyl-3-hydroxyoctanoic acid (C_{12}-mycolic acid), 2-octyl-3-hydroxydodecanoic acid (C_{20}-mycolic acid), 2-decyl-3-hydroxytetradecanoic acid (C_{24}-mycolic acid), and 2-hexadecyl-3-hydroxyeicosanoic acid (C_{36}-mycolic acid), showed values similar to those of 2-hexyl-3-hydroxydecanoic acid (C_{16}-mycolic acid) because they had the same chemical structures and functional groups (hydroxyl and carboxyl groups) except for the lengths of the carbon chains. The ^{1}H NMR and FTIR spectral data of the other mycolic acids are tabulated in Table 2. There were no unusual observations in the ^{1}H NMR and FTIR spectra of the other mycolic acids compared with those of 2-hexyl-3-hydroxydecanoic acid.

Table 2 presents yields, m.p., and spectral data, i.e., FTIR, ^{1}H NMR, UV, and mass spectra of the five mycolic acids. The m.p. were 45–46°C for C_{20}-, 54–55°C for C_{24}-, and 68–70°C for C_{36}-mycolic acid. The C_{12}- and C_{16}-mycolic acids were waxes at room temperature. The acids were analyzed by a mass spectrophotometer. Table 2 also presents the extinction coefficients (ε) of the mycolic acids using a UV spectrophotometer (Cary 1 Bio; Varian, Palo Alto, CA): ε = 88.68 $M^{-1}cm^{-1}$ at 211 nm for C_{12}-, 249.02 $M^{-1}cm^{-1}$ at 215 nm for C_{16}-, 161.51 $M^{-1}cm^{-1}$ at 212 nm for C_{20}-, 231.88 $M^{-1}cm^{-1}$ at 214 nm for C_{24}-, and 256.37 $M^{-1}cm^{-1}$ at 202 nm for C_{36}-mycolic acid. Figure 3 shows the fragmentation pattern of C_{16}-mycolic acid. It was identified by comparing the fragmentation pattern and the M.W. (MS 254 M − H_2O and MS 272). Figure 4 shows the peak for C_{16}-mycolic acid obtained by HPLC, which appeared at 3.40 min. However, it did not show separate peaks for the *syn*- and *anti*-diastereomers of C_{16}-mycolic acid.

These mycolic acids can be obtained in high purity and potentially at a relatively low cost. Since the synthesis of mycolic acids is relatively simple, the yields were also high, at approximately 72 to 73% of the starting material (Table 2).

Surface-active properties of synthetic mycolic acids. The surface tension of alkaline solutions (pH 9.5) containing C_{12}-, C_{16}-, or C_{20}-mycolic acid was measured at various concentrations using a Thermo Cahn surface tensiometer at 20°C. The C_{24}- and C_{36}-mycolic acids were partially soluble/dispersible and insoluble in alkaline solution, respectively (Table 2). This method allows a comparison of the surface-active properties of different mycolic acids at their critical micelle concentration (CMC) values. The surface tension–concentration plot is shown in Figure 5. The surface tension of mycolic acids decreased with increasing concentrations and showed a well-defined CMC (Fig. 5). The CMC values of C_{12}-, C_{16}-, and

TABLE 2
Yields, Physical Properties, and Spectral Data[a]

	A	B	C	D	E
Yield (%) (step)	72	73	73	73	73
	(80, 95, 95)	(90, 90, 90)	(90, 90, 90)	(90, 90, 90)	(90, 90, 90)
m.p. (°C)					
	Wax	Wax	45–46	54–55	68–70
IR (cm^{-1})	3391 (OH, *br*)	3376 (OH, *br*)	3368 (OH, *br*)	3325 (OH, *br*)	3301 (OH, *br*)
	1707 (C=O)	1707 (C=O)	1690 (C=O)	1696 (C=O)	1693 (C=O)
NMR ($CDCl_3$)	0.872–1.609 (*m*, 20 H)	0.880–1.733 (*m*, 28 H)	0.880–1.697 (*m*, 36 H)	0.880–1.705 (*m*, 44 H)	0.860–1.911 (*m*, 68 H
	2.483 (*m*, 1 H)	2.476 (*m*, 1 H)	2.466 (*m*, 1 H)	2.487 (*m*, 1 H)	2.465 (*m*, 1 H)
	3.869 (*m*, 1 H)	3.860 (*m*, 1 H)	3.859 (*m*, 1 H)	3.864 (*m*, 1 H)	3.858 (*m*, 1 H)
MS	198 ($M - H_2O$)	254 ($M - H_2O$)	310 ($M - H_2O$)	366 ($M - H_2O$)	534 ($M - H_2O$)
ε ($M^{-1}cm^{-1}$)	88.68	249.02	161.51	231,88	256.37
	at 211 nm	at 215 nm	at 212 nm	at 214 nm	at 202 nm
Water solubility[b]	100%	100%	100%	Partially soluble or dispersible	Insoluble

[a]A, 2-butyl-3-hydroxyoctanoic acid; B, 2-hexyl-3-hydroxydecanoic acid; C, 2-octyl-3-hydroxydodecanoic acid; D, 2-decyl-3-hydroxytetradecanoic acid; E, 2-hexadecyl-3-hydroxyeicosanoic acid; ε, extinction coefficient.
[b]Water solubility, 1 g mycolic acid was solubilized in 1,000 mL alkaline solution (pH 9.5) for water solubility and critical micelle concentration measurements.

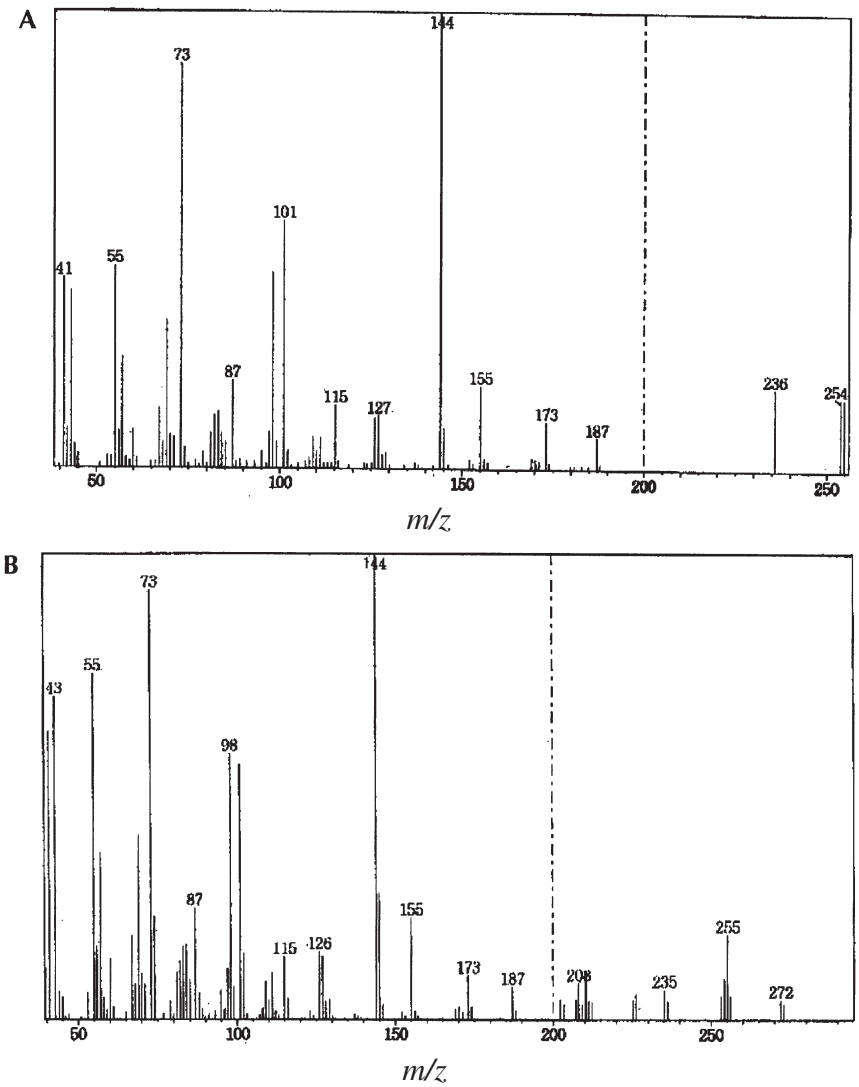


FIG. 3. Mass of 2-hexyl-3-hydroxydecanoic acid. M.W. of 2-hexyl-3-hydroxydecanoic acid: (A) MS 254 – H_2O and (B) MS 272; ionization mode: EI; ionization voltage: 70 eV; ion source temperature: 200°C.

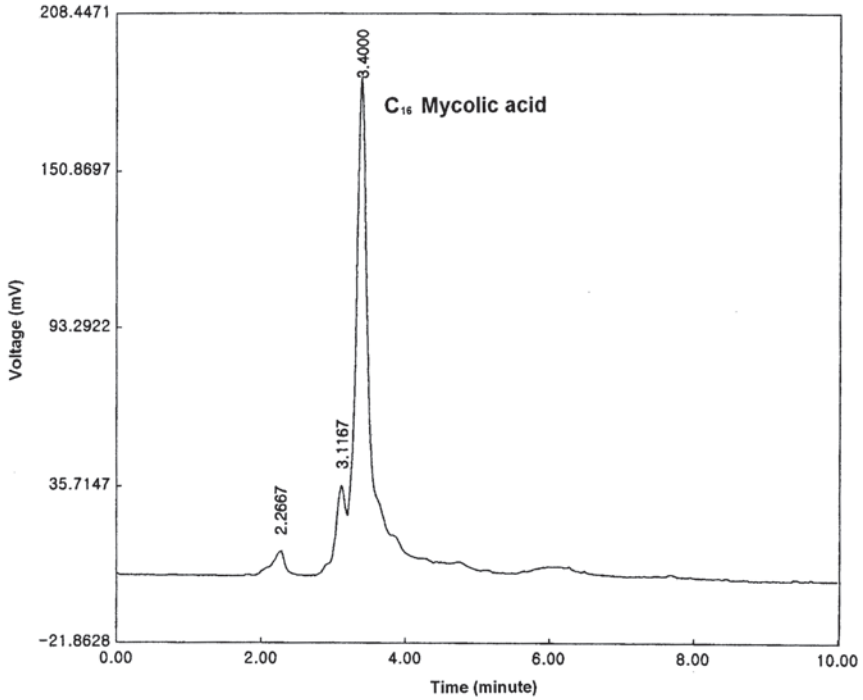


FIG. 4. HPLC chromatogram of 2-hexyl-3-hydroxydecanoic acid. The peak for 2-hexyl-3-hydroxydecanoic acid appeared at 3.40 min (retention time). Injection volume: 500 µL; eluent: 100% methanol; temperature: 25°C; flow rate: 10 mL/min; HPLC column: Waters Atlantis dC_{18} reversed-phase column (150 × 3.9 mm, 5 µm; Waters, Milford, MA); UV detector: 215 nm.

C_{20}-mycolic acids were 2.2×10^{-4}, 1.36×10^{-4}, and 7.4×10^{-5} M, respectively. As the carbon number increased, the CMC value decreased. The surface tension at CMC also decreased (46.54, 43.59, and 41.57 dyn/cm for C_{12}-, C_{16}-, and C_{20}-mycolic acid, respectively).

Emulsification activity. Low CMC values of C_{12}-, C_{16}-, and C_{20}-mycolic acids indicate that they can effectively reduce the surface tension. Therefore, as a measurement of their emulsification activities, the E_{24}% of *n*-tetradecane, *n*-hexadecane, cyclohexane, and diesel oil was determined for each mycolic

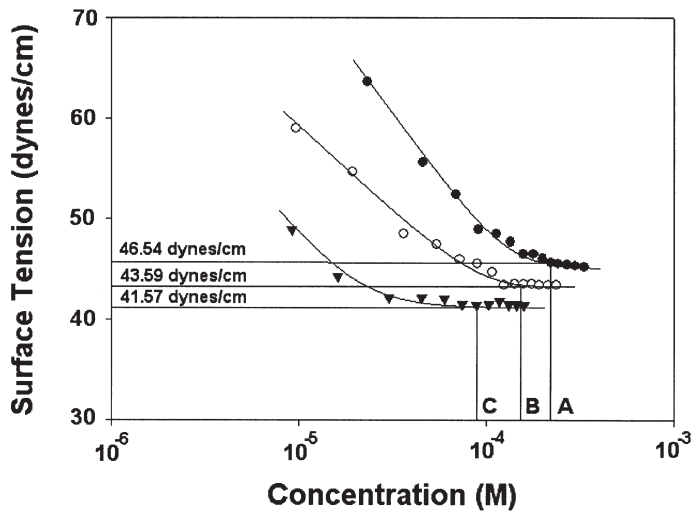


FIG. 5. Surface tension vs. concentration curves of synthetic mycolic acids at 30°C. (●) 2-Butyl-3-hydroxyoctanoic acid, (○) 2-hexyl-3-hydroxydecanoic acid, and (▼) 2-octyl-3-hydroxydodecanoic acid. Critical micelle concentration values were: A (2-butyl-3-hydroxyoctanoic acid), 2.2×10^{-4} M; B (2-hexyl-3-hydroxydecanoic acid), 1.36×10^{-4} M; and C (2-octyl-3-hydroxydodecanoic acid), 7.4×10^{-5} M.

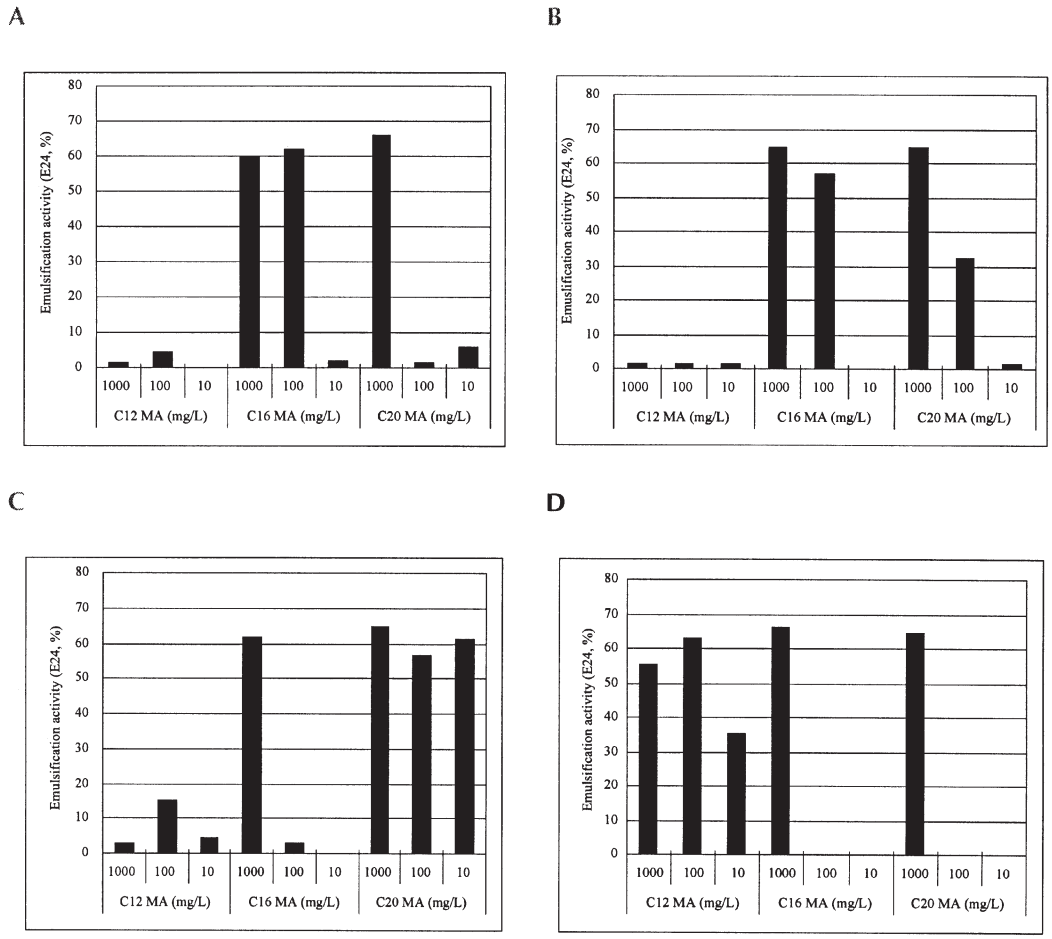


FIG. 6. Comparison of emulsification activity (E_{24}%) of the synthetic mycolic acids (MA; at 10, 100, and 1,000 mg/L) toward (A) *n*-tetradecane; (B) *n*-hexadecane; (C) cyclohexane; (D) diesel oil.

acid. Figure 6 shows the comparison of their E_{24}% at 10, 100, and 1,000 mg/L of each mycolic acid, respectively. The C_{16}-mycolic acid showed higher emulsifying activity for *n*-tetradecane and *n*-hexadecane than did the C_{12}- and C_{20}-mycolic acids at 10, 100, and 1,000 mg/L, while exhibiting a lower emulsifying activity for cyclohexane and diesel oil at 10 and 100 mg/L. The C_{12}-mycolic acid showed higher emulsifying activity for diesel oil than did the C_{16}- and C_{20}-mycolic acids at 10, 100, and 1,000 mg/L, but it showed a lower emulsifying activity for *n*-tetradecane, *n*-hexadecane, and cyclohexane than did the others at 10, 100, and 1,000 mg/L. The C_{20}-mycolic acid showed higher emulsifying activity for cyclohexane than did the C_{12}- and C_{16}-mycolic acids at 10, 100, and 1,000 mg/L, and it also showed high emulsifying activity for *n*-tetradecane, *n*-hexadecane, and cyclohexane at 1,000 mg/L.

The CMC and emulsification activities of the synthetic mycolic acids are sensitive to the length of the alkyl chains (Figs. 5,6). This may be explained by the hydrophilic-lipophilic balance (HLB) of the acids. Longer mycolic acids tended to be lipophilic and shorter mycolic acids hydrophilic. Mycolic acids with a chain length of 12 to 20 appeared to be the best surfactants because of their favorable HLB for the surface-active properties. As the acids became longer and more lipophilic, they became less soluble or insoluble in water and were poorer surfactants (Table 2). Being hydrophilic in nature, the hydroxyl functional group close to the carboxyl group renders the molecule more hydrophilic, resulting in a more favorable HLB with the large alkyl chains (17).

Since mycolic acids have two hydrophilic functional groups in the water and two hydrophobic chains, they can create lamellar-type surfactant aggregations. For this reason, they are structurally somewhat more complex than a linear amphiphile, and their packing orientations at the aqueous interface may be complicated (18). This is analogous to the surface packing in some phospholiplids, which have good surfactant properties (19,20). Such a monomolecular-layer orientation at the air/water interface reduces the surface tension to less than 50 dyn/cm at the CMC, probably because of the hindrance and repulsion between the hydrocarbon chain of one molecule and that of the adjoining molecule.

The new synthetic route to the mycolic acids presented here is relatively simple. They can be obtained in high purity and potentially at a relatively low cost, and they show high emulsification properties toward several oils. These synthetic mycolic acids thus have significant potential as new materials for use in the removal of hydrocarbons in oil-contaminated environments, such as oil spills in the sea or soil. They also can be used for *in situ* flushing of hydrocarbon contaminants in the soil and for enhanced recovery of oil from oil fields.

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