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Note

A basidiomycetous yeast, *Pseudozyma tsukubaensis*, efficiently produces a novel glycolipid biosurfactant. The identification of a new diastereomer of mannosylerythritol lipid-B

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Abstract—Mannosylerythritol lipids (MELs) are glycolipid biosurfactants produced by the yeast strains of the genus *Pseudozyma*. These compounds show not only excellent surface-active properties but also versatile biochemical activities. In the course of MEL production by *Pseudozyma tsukubaensis*, we found an unusual MEL that had a different carbohydrate structure from that of conventional MELs. The carbohydrate structure was identified as 1-*O*-β-D-mannopyranosyl-D-erythritol, and the MEL was confirmed to be 1-*O*-β-(2′,3′-di-*O*-alka(e)noyl-6′-*O*-acetyl-D-mannopyranosyl)-D-erythritol. Interestingly, the configuration of the erythritol moiety in the present MEL was opposite to that of the known MEL-B, 4-*O*-β-(2′,3′-di-*O*-alka(e)noyl-6′-*O*-acetyl-D-mannopyranosyl)-D-erythritol, and to that of all MELs hitherto reported. The present MEL should thus provide different interfacial and biochemical properties compared to conventional MELs.

Keywords: Biosurfactant; Glycolipid; Mannosylerythritol lipid; *Pseudozyma* yeasts; *Pseudozyma tsukubaensis*

Mannosylerythritol lipids (MELs, Fig. 1) are one of the most promising biosurfactants,[1–3] which are surface-active compounds secreted by various microorganisms. MELs are produced in large amounts (over 100 g/L) from vegetable oils by the yeast strains belonging to the genus *Pseudozyma*.[4–6] These compounds exhibit not only excellent surface-active properties,[7] but also versatile biochemical activity,[2,8–14] including the induction of cell-differentiation of different mammalian cells[11] as well as affinity binding toward different immunoglobulins.[8,13,14] MELs have thus great potential as environmentally friendly and advanced materials that can be manufactured from renewable resources.

All hitherto known MELs, namely MEL-A, -B, and -C, consist of 4-*O*-β-D-mannopyranosyl-D-erythritol as the hydrophilic part and two fatty acyl groups as the hydrophobic part (Fig. 1). Each homolog has one or two acetyl groups at C-4′ and/or C-6′ in the mannose moiety. Interestingly, these compounds show specific phase behavior and different self-assembled structures

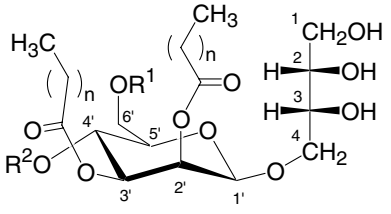

Figure 1. Chemical structures of conventional mannosylerythritol lipids.

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in aqueous solution,[15–18] although the difference in the chemical structure is very small. For instance, MEL-A spontaneously forms an L_3 (sponge) phase in a wide range of concentrations, while MEL-B forms L_α (lamellar) and myelin structures.

As described above, even a slight difference in chemical structure brings about drastic changes in the interfacial properties of MELs. We thus have focused our attention on the identification of structural variants of MELs, and have studied the products of different strains of the genus *Pseudozyma*. Very recently, we found novel types of MELs, namely the mono-acylated[19] and tri-acylated MEL,[20] which have different hydrophobic structures and show different hydrophilicities compared to the conventional di-acylated MELs. Moreover, we have reported that newly isolated *Pseudozyma* strains selectively produce each MEL homolog, MEL-A, -B, and -C, respectively.[21] These studies will facilitate the research and development of MELs because selective production will make the downstream process more simple and cost-effective.

Among MEL-producing yeasts, we have concentrated on *Pseudozyma tsukubaensis*[21,22] due to its unique production features. Interestingly, it efficiently produces only a glycolipid similar to MEL-B, while many other *Pseudozyma* strains produce a mixture of different MEL homologs. MEL-B shows higher hydrophilicity and critical micelle concentration than MEL-A, and thus is advantageous for the use as water-in-oil type emulsifiers and/or washing detergents.[17] Therefore, we investigated the production conditions and structure of the glycolipid produced by *P. tsukubaensis*. Surprisingly, the structure of the carbohydrate moiety was different from that of the hitherto known MELs. Here we describe for the first time a new diastereomer of the known MEL-B.

In the known high-level MEL producers such as *Pseudozyma antarctica*,[4] *Pseudozyma aphidis*,[5] and *Pseudozyma rugulosa*,[6] MEL-A is produced in the largest amount and comprises more than 70% of the total MELs. It is thus difficult to isolate MEL-B in abundance from the culture broth of these yeasts, because the polarity of MEL-B is intermediate among the three MEL homologs and is present only in very small amount. In contrast, *P. tsukubaensis* JCM10324^T and *Pseudozyma* sp. KM-160 selectively produce only a single glycolipid product, which corresponds to MEL-B, in high yield (more than 25 g/L from 4% (w/v) of soybean oil).[21,22] These strains should thus be potential MEL-B producers.

In our previous study, the glycolipid produced by *P. tsukubaensis* was tentatively identified as MEL-B, based on ^{1}H and ^{13}C NMR data that showed very similar spectra to those of purified MEL-B prepared from soybean oil by *P. antarctica*. However, when the spectra were compared more in detail, the glycolipid had different peak patterns in the part of the carbohydrate moiety (Fig. 2). For example, the two resonances arising from the H-4a and H-4b in the erythritol (~3.8–4.1 ppm) were widely separated in the product produced from

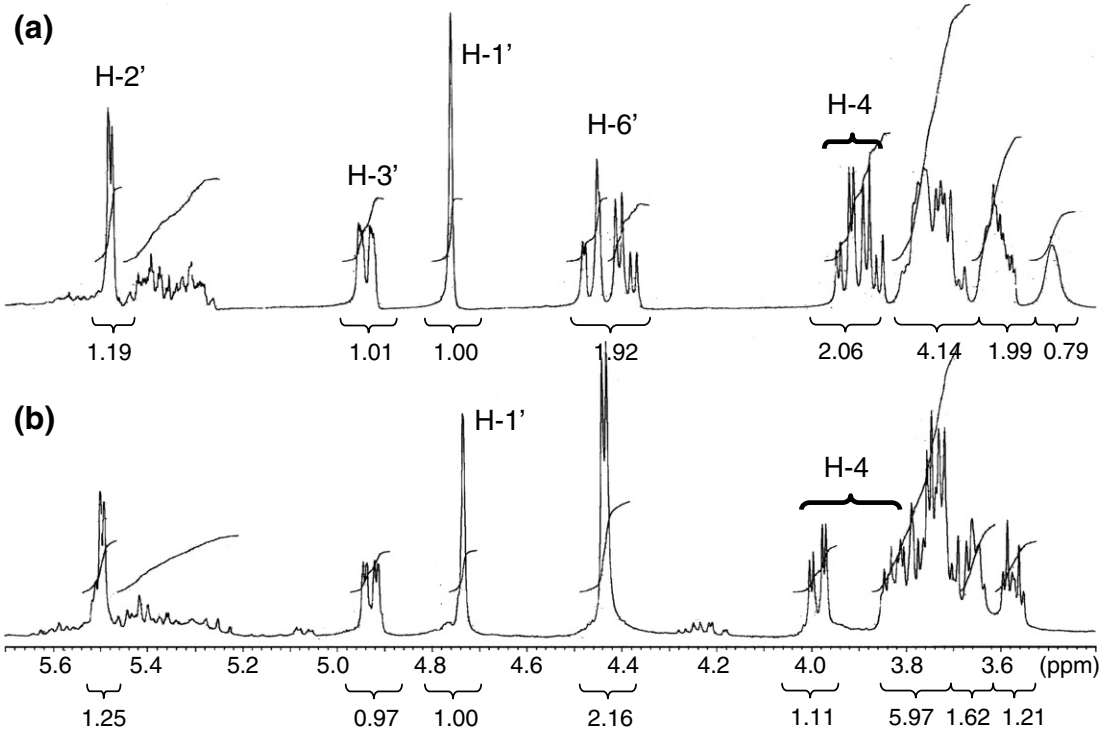

Figure 2. Partial ^{1}H NMR spectra of: (a) the glycolipid produced by *P. tsukubaensis*, and (b) MEL-B produced by *P. antarctica*.

P. antarctica, while these peaks overlapped in the product produced from *P. tsukubaensis*. Moreover, in the product from *P. tsukubaensis*, the resonance from the mannose anomeric hydrogen (H-1′) was shifted to lower field, from 4.73 to 4.76 ppm. On the other hand, in all conventional MELs produced by other *Pseudozyma* strains, the NMR spectra showed the patterns same as those from isolated from *P. antarctica*. The chemical shifts of the glycolipid produced by *P. tsukubaensis* are summarized in Table 1.

Crich and co-workers had previously reported the total synthesis of the two diastereomers of MEL-A.[23] Based on their data, the carbohydrate configuration of MEL-A produced by *P. antarctica* is 4-*O*-β-D-mannopyranosyl-D-erythritol, and another diastereomer bearing 1-*O*-β-D-mannopyranosyl-D-erythritol shows different peak patterns in the ^{1}H NMR spectra from the microbial MEL-A. In the diastereomer, H-1′ was shifted significantly to lower field, from 4.71 to 4.75 ppm, and H-4a and H-4b were not clearly split and partially overlapped. This corresponds well with the observed difference between the present glycolipid and known MEL-B. Accordingly, we propose that the glycolipid produced by *P. tsukubaensis* is 1-*O*-(6′-*O*-acetyl-2′,3′-di-*O*-alka(e)noyl-β-D-mannopyranosyl)-D-erythritol (Fig. 3).

The $[\alpha]_D$ value of the present MEL at 27 °C was −48.0 (*c* 1.0, $CHCl_3$), and there was no significant difference between this value and that obtained from MEL-B produced by *P. antarctica* [−48.1 (*c* 1.2, $CHCl_3$)].[24] The major fatty acids of the present MEL were C_{12} and C_{14} species (more than 54%),[22] while those of MELs produced by *P. antarctica* were C_8 and C_{10} acids. Indeed, the molecular weight of the present MEL (683.8 or 657.8) determined by the main peak ($[M+Na]^+$) was found to be slightly greater than conventional MELs. Thus, it may not be meaningful to discuss small differences in $[\alpha]_D$ value. To clarify the difference of the carbohydrate configuration between the present MEL and conventional MEL-B, we prepared mannosylerythritol (ME) by alkaline hydrolysis.

The purified ME from *P. antarctica* was obtained as a white needle-like crystals by recrystallization from 90% ethanol. The melting point of this material was 155.4–158.3 °C and the $[\alpha]_D$ value at 27 °C was −35.2 (*c* 1.0, H_2O). In contrast, the ME from *P. tsukubaensis* was obtained not as a solid but as a colorless syrup. Its $[\alpha]_D$ value at 27 °C was −39.6 (*c* 1.0, H_2O). This result showed that these two MEs have different chirality.

Figures 4 and 5 show the partial ^{1}H and ^{13}C NMR spectra of both MEs, and the chemical shifts of MEs are summarized in Table 2. Both peak patterns almost correspond. However, similar to the spectra of two MELs described above, the peaks at H-4a and H-4b were separated and the chemical shifts of H-1′ were also slightly different. Although the absolute configuration has not yet been confirmed by X-ray, these results are most likely attributed to a difference in the configuration of erythritol in this MEL.

As mentioned above, *P. tsukubaensis* is a promising MEL producer, because it geneartes not only MEL-B exclusively, but also a novel MEL diastereomer having a chirality different from that of conventional MELs. The present diastereomer was identified as 1-*O*-(6′-*O*-acetyl-2′,3′-di-*O*-alka(e)noyl-β-D-mannopyranosyl)-D-erythritol (Fig. 3), indicating that erythritol is bound to mannose in a manner different from all known MELs. To the best of our knowledge, this is the first report on a new diastereomer of MEL from microbial products. This fact implies that *P. tsukubaensis* possesses a different mannosyltransferase from other *Pseudozyma* yeasts to give a different diastereomer of MEL. The investigation of biosynthesis of the new MEL will provide us much useful information on the development of various glycolipid biosurfactants.

Another interesting feature of the present MEL may be the hygroscopic property of the carbohydrate moiety, which is suggested from the fact that the present ME is difficult to crystallize. We have recently reported that conventional MELs show excellent moisturizing properties, equivalent to those of natural ceramides, toward human skin.[25–28] Thus, the present new diastereomer might exhibit superior skin care properties compared to conventional MELs, due to the unique configuration

Table 1. NMR data for the glycolipid produced by *P. tsukubaensis* ($CDCl_3$, 400 MHz)

	^{13}C NMR δ (ppm)		^{1}H NMR δ (ppm)
Mannose			
C-1′	99.3	H-1′	4.76 d
C-2′	69.1	H-2′	5.48 dd
C-3′	73.4	H-3′	4.94 dd
C-4′	65.9	H-4′	3.70–3.82 m
C-5′	74.7	H-5′	3.57–3.64 m
C-6′	63.5	H-6′	4.36–4.49 m
Erythritol			
C-1	63.9	H-1	3.57–3.82 m
C-2	72.4	H-2	3.57–3.64 m
C-3	71.5	H-3	3.70–3.82 m
C-4	72.1	H-4	3.84–3.95 m
Acetyl groups			
–C=O	171.8		
$-CH_3$	21.0		2.13 s
Acyl groups			
–C=O (C-2′)	173.8		
(C-3′)	173.6		
–CO–CH_2– (C-2′)	34.3		2.40 m
(C-3′)	34.3		2.30 m
–CO–CH_2CH_2–	24.8–25.3		1.54–1.72 b
$-(CH_2)_n-$	22.7–32.1		1.20–1.40 b
–CH=CH–	128.3–131.5		5.26–5.46 b
–CH=CH–CH_2–	26.6–27.4		1.96–2.10 b
$-CH_3$	14.2		0.85–0.93 b

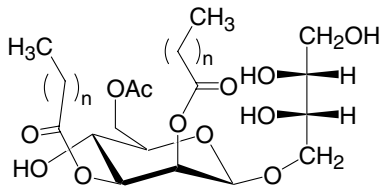

Figure 3. Chemical structure of the mannosylerythritol lipid produced by *P. tsukubaensis*.

of the *meso*-erythritol moiety. Further studies on the difference in the interfacial and biochemical properties between the two diastereomers are underway.

1. Experimental

1.1. Materials and microorganisms

All reagents and solvents were commercially available and were used as received.

P. tsukubaensis JCM10324^T was obtained from the Japan Collection of Microorganisms. *P. antarctica* T-34 was isolated according to methods previously described.[24] Stock cultures were cultivated for three days at 25 °C on an agar medium containing 4% glucose, 0.3% $NaNO_3$, 0.03% $MgSO_4$, 0.03% KH_2PO_4, and 0.1% yeast extract. They were stored at 4 °C and renewed every two weeks.

1.2. General methods of the production and isolation of MELs

The following standard procedure for the production of MEL by fermentation was adopted. Seed cultures were prepared by inoculating cells grown on slants into test tubes containing a growth medium [4% glucose, 0.3% $NaNO_3$, 0.03% $MgSO_4$, 0.03% KH_2PO_4, 0.1% yeast extract (pH 6.0)] at 25 °C on a reciprocal shaker (150 rpm) for two days.

Seed cultures (1 mL) were transferred to 300 mL Erlenmeyer flasks containing 30 mL of a basal medium [4% olive oil, 0.3% $NaNO_3$, 0.03% $MgSO_4$, 0.03% KH_2PO_4, 0.1% yeast extract (pH 6.0)], and then incubated on a rotary shaker (220 rpm) at 28 °C for 7 days, unless otherwise indicated.

The produced glycolipids were extracted from the culture medium with an equal volume of ethyl acetate. The extracts were analyzed by thin-layer chromatography (TLC) on silica plates (Silica gel 60F; Wako) with a solvent system consisting of chloroform/methanol/7 M ammonia solution (65:15:2, by vol). The compounds on the plates were located by charring at 110 °C for 5 min after spraying an anthrone/sulfuric acid reagent as previously reported.[24] The purified MEL fraction including MEL-A, -B, and -C prepared as reported previously[29] was used as a standard.

The above organic layer was separated and evaporated. The concentrated glycolipids were dissolved in

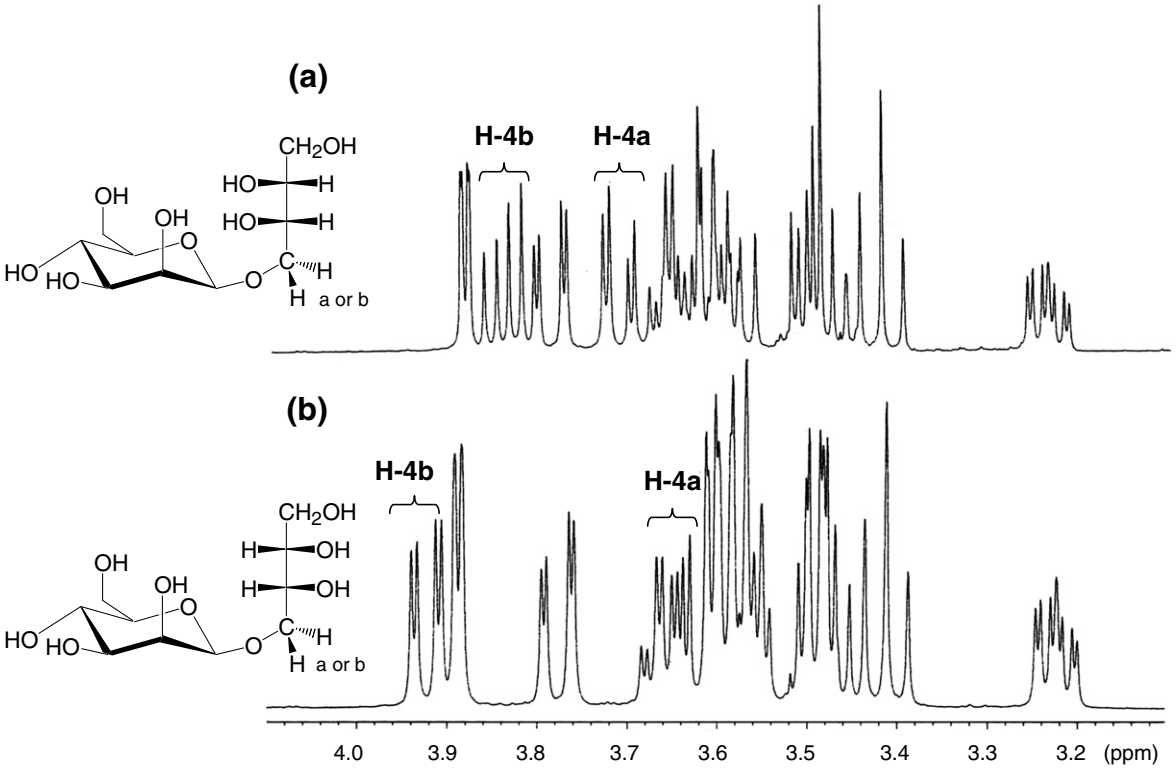

Figure 4. Partial ^{1}H NMR spectra of mannosylerythritol derived from: (a) *P. tsukubaensis*, and (b) *P. antarctica*.

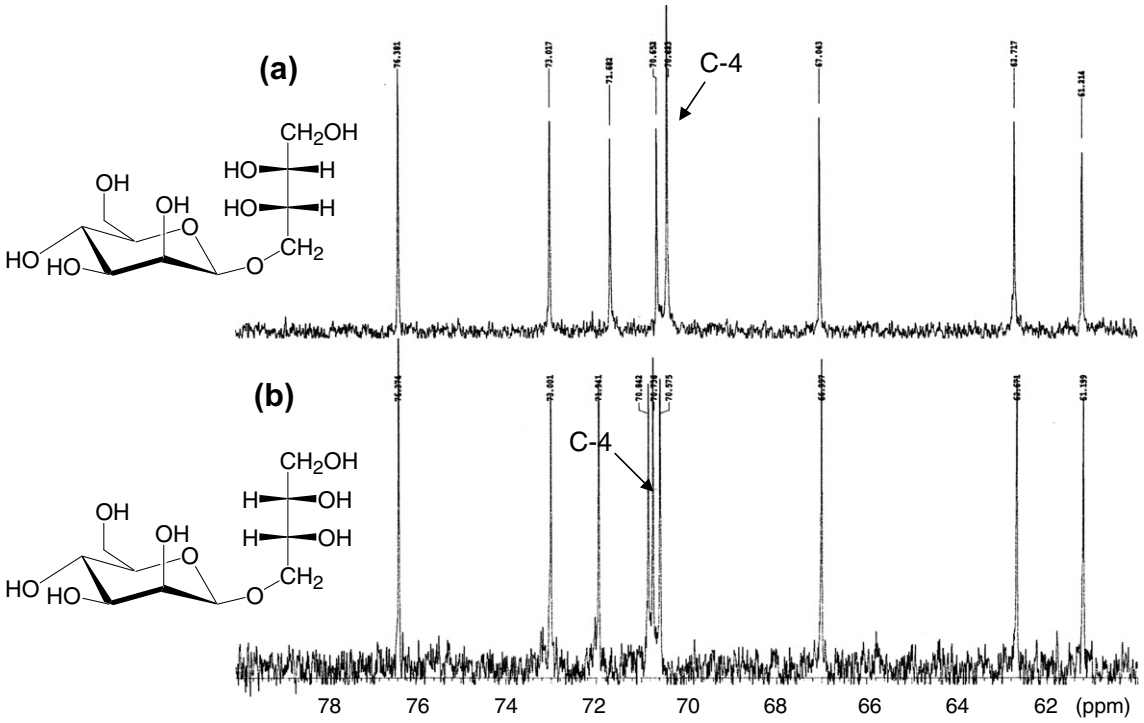

Figure 5. Partial ^{13}C NMR spectra of mannosylerythritol derived from: (a) *P. tsukubaensis*, and (b) *P. antarctica*.

Table 2. NMR data for mannosylerythritol (D_2O, 400 MHz)

	^{13}C NMR δ (ppm)			^{1}H NMR δ (ppm)	
	tsukubaensis	*antarctica*		*tsukubaensis*	*antarctica*
Mannose					
C-1′	100.1	100.6	H-1′	4.55 d	4.54 d
C-2′	70.4	70.5	H-2′	3.89 dd	3.89 dd
C-3′	73.0	73.0	H-3′	3.50–3.52 dd	3.50–3.52 dd
C-4′	67.0	67.0	H-4′	3.42 t	3.42 t
C-5′	76.4	76.4	H-5′	3.21–3.26 m	3.21–3.26 m
C-6′	61.2	61.2	H-6′a	3.56–3.60 dd	3.54–3.58 dd
			H-6′b	3.77–3.81 dd	3.76–3.80 dd
Erythritol					
C-1	62.7	62.7	H-1a	3.46–3.50 m	3.46–3.50 m
			H-1b	3.64–3.68 m	3.64–3.68 m
C-2	70.7	70.7	H-2	3.62–3.64 m	3.59–3.61 m
C-3	71.9	71.9	H-3	3.59–3.62 m	3.56–3.59 m
C-4	70.4	70.8	H-4a	3.69–3.73 dd	3.63–3.67 dd
			H-4b	3.82–3.86 dd	3.90–3.94 dd

chloroform and then purified by silica gel (Wako-gel C-200) column chromatography using a gradient elution of chloroform–acetone (10:0–0:10, vol/vol) mixtures as solvent systems.[24,29] The purified glycolipids were used in the following experiments.

1.3. Synthesis of ME by the alkaline hydrolysis of MELs

To a solution of the mixture of MELs (680 mg, 1 mmol) in dry MeOH (5 mL) was added NaOMe (20 mg, 370 μmol) in MeOH (5 mL). After stirring at room temperature for 1 h, cation exchange resin (DOWEX H+ form, >1 g) was added and the mixture was stirred further. After stirring for 15 min, the resin was removed by filtration, and the filtrate was evaporated. To this residue was added a small amount of water (1 mL) and ethyl acetate (10 mL), then the fatty acids were extracted with ethyl acetate. The resulting aqueous layer containing the sugar moiety was concentrated to a syrup. Purification of the glycoside (ME) was performed by recrystallization from 90% ethanol. ME from *P. antarctica* was obtained (224 mg, 0.79 mmol) as a white needle crystal, and that from *P. tsukubaensis* was obtained (257 mg, 0.90 mmol) as a colorless syrup. Their NMR data were described above.

1.4. Structure determination of the purified MEL and ME

Structure determination of the purified MEL and ME dissolved in $CDCl_3$ or D_2O was performed by ^1H, ^{13}C nuclear magnetic resonance (NMR) and two-dimensional NMR analysis, such as ^1H–^1H correlation spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC) using a Varian INOVA 400 (400 MHz).

Specific rotations of the purified MEL and ME were measured by JASCO Digital Polarimeter DIP-370 for $CHCl_3$ or aqueous solution.

The molecular weight of the purified MEL was measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (Voyager-DE PRO) with an α-cyano-4-hydroxycinnamic acid matrix.

The fatty acid profiles of the purified MEL were examined by gas chromatography–mass spectrometry (GC–MS) as previously reported.[6] The methyl ester derivatives of fatty acids were prepared by mixing the above purified glycolipids (10 mg) with 5% HCl–methanol reagent (1 mL) (Tokyo Kasei Kogyo, Tokyo, Japan) at 80 °C for 20 min. After the reaction was quenched with water (1 mL), the methyl ester derivatives were extracted with *n*-hexane and then analyzed by GC–MS (Hewlett Packard 6890 and 5973N) with a TC-WAX (GL-science, Tokyo) with the temperature programmed from 90 °C (held for 3 min) to 240 °C at 5 °C/min.

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