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Rhamnolipids as Platform Molecules for Production of Potential Anti-zoospore Agrochemicals

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Supporting Information

ABSTRACT: Rhamnolipid biosurfactants have potential applications in the control of zoosporic plant pathogens. However, rhamnolipids have not been closely investigated for the anti-zoospore mechanism or for developing new anti-zoospore chemicals. In this study, RhL-1 and RhL-3 groups of rhamnolipids were used to generate the corresponding RhL-2 and RhL-4 groups and the free diacids. Conversion of RhL-3 to RhL-1 was also accomplished *in vitro* with cellobiase as the catalyst. The anti-zoospore effects of RhL-1−RhL-4 and the diacids were investigated with zoospores of *Phytophthora sojae*. For RhL-1−RhL-4, approximately 20, 30, 40, and 40 mg/L, respectively, were found to be the lowest concentrations required to stop movement of all zoospores, which indicates that the anti-zoospore effect remains strong even after RhL-1 and RhL-3 are hydrolyzed into RhL-2 and RhL-4. The free diacids required a significantly higher critical concentration of about 125 mg/L. Rhamnose can be obtained as a co-product.

KEYWORDS: *rhamnolipid, zoospore, HPLC−MS, biosurfactant, fungi*

INTRODUCTION

Soybean is an important agricultural product. In 2010, 222 million bushels of soybeans were harvested in Ohio, which ranked the sixth state in the U.S. for soybean production.[1] In Ohio, the number one soybean disease is arguably the root disease caused by *Phytophthora sojae*, which is also among the most serious soybean diseases worldwide and causes an estimated loss of $1−2 billion each year globally.[2−4]

P. sojae survives in soil and plant debris as oospores, which germinate to form mycelia under suitable conditions.[5,6] The mycelia can produce sporangia and zoospores.[5,6] Zoospores can swim in water to reach the soybean roots, then germinate, and infect the root tissues. Zoospores are generally considered to be responsible for the spreading of this pathogen.[5,7] Killing the zoospores is an efficient way to control this pathogen because zoospores are cell-wall-less, enclosed only by a cell membrane, while the fungus has a protective cell wall during other life stages.[5,6]

Rhamnolipids are a class of sugar fatty acid biosurfactants that are capable of damaging fungal zoospores.[7,8] In comparison to usual chemical surfactants, rhamnolipids have several advantages. First, rhamnolipids can be produced from renewable resources by bacterial fermentation.[9−12] Second, rhamnolipids are degradable in the soil environment.[13,14] Third, rhamnolipids are less toxic; their use lowers the health threat of remaining pesticides on food.[7,9] Rhamnolipids are not commercially available as anti-zoospore chemicals because the high price to manufacture is cost-prohibitive. Production of high-value co-products might improve the overall economics. It should be mentioned that other biosurfactants, such as sophorose lipid, trehalose lipid, and surfactin, were found to have no anti-zoospore effects at concentrations lower than 1000 mg/L, while rhamnolipids could lyse zoospores at 30 mg/L.[7] Understanding the relationship between the molecular structures of rhamnolipids and their anti-zoospore effects might lead to the development of potent rhamnolipid-based anti-zoospore chemicals and the production of high-value co-products.

Rhamnolipids are produced in microbial fermentations as a mixture of many congeners, which have one or two rhamnose sugar residues combined with the hydrophobic moiety composed of one or two β-hydroxyalkanoic acids.[15,16] Structures of major rhamnolipid types are shown in Figure 1, hereinafter referred to as rhamnolipid 1 (RhL-1; compound **1**), rhamnolipid 2 (RhL-2; compound **2**), rhamnolipid 3 (RhL-3; compound **3**), and rhamnolipid 4 (RhL-4; compound **4**).[17,18] The fatty acid chains shown in Figure 1 all have 10 carbons in length, but they can vary from 8 to 12 carbons and can contain double bonds in the typical mixtures produced by *Pseudomonas aeruginosa*. In the most common rhamnolipids, the rhamnose residue is linked to a chain of two β-hydroxydecanoic acid residues, referred to as R−C_{10}−C_{10} and R−R−C_{10}−C_{10}, respectively, which belong to the RhL-1 and RhL-3 groups (Figure 1).[15] The rhamnolipids that have been studied for anti-zoospore effects are mostly RhL-1 and RhL-3.[7,8]

In the present study, free diacids and RhL-2 and RhL-4 were generated from RhL-1 and RhL-3. Enzymatic conversion of RhL-3 to RhL-1 was also studied to provide a way to adjust the composition of rhamnolipid mixtures. High-performance liquid chromatography−mass spectrometry (HPLC−MS) was the main method used to monitor these processes. The anti-zoospore effects of every rhamnolipid group (RhL-1−RhL-4) and the free diacids were then individually determined. In an attempt to quantitate the potency of rhamnolipids against zoospores at low concentration levels, the results help advance

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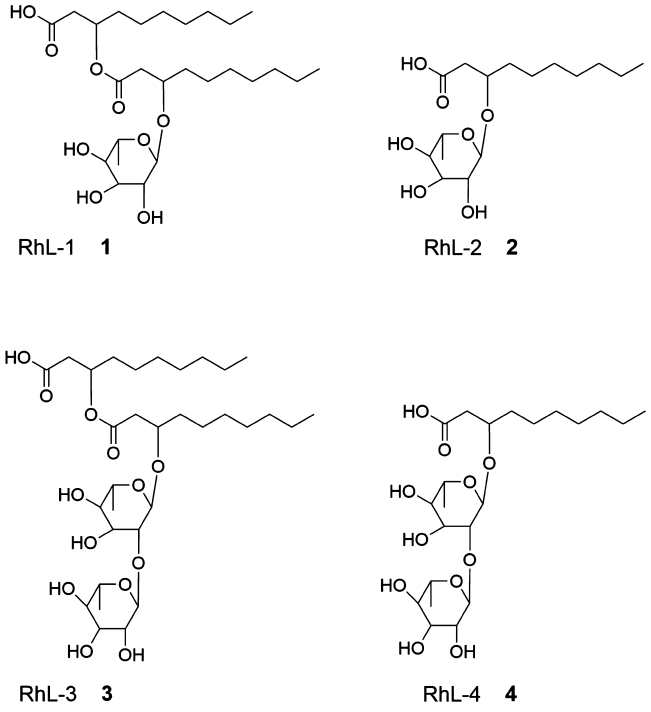

Figure 1. Representative molecular structures of four main groups of rhamnolipids, RhL-1−RhL-4.

the economic production of anti-zoospore chemicals and potentially valuable co-products from rhamnolipids.

MATERIALS AND METHODS

Materials. Ethanol, ethyl acetate, citric acid, sodium citrate, acetic acid, sodium bicarbonate, sodium hydroxide, sulfuric acid, sodium methoxide methanol solution (25%, w/w), cellulase (EC 3.2.1.4) from *Trichoderma reesei* [American Type Culture Collection (ATCC) 26921], and cellobiase from *Aspergillus niger* were purchased from Sigma-Aldrich (St. Louis, MO). Milli-Q water (Millipore, Billerica, MA) was used. V8 high-fiber 100% vegetable juice from Campbell Soup Company (Camden, NJ) was purchased from a local store. All other reagents were of analytical grade.

HPLC−MS Analysis. HPLC analysis was performed with the Hewlett-Packard LC1100 series equipment (Agilent Technologies, Santa Clara, CA). The column used was a 250 × 4.6 mm inner diameter, 5 μm, Supelcosil LC-18 (Supelco, Bellefonte, PA). The column temperature was controlled at 24 °C. Several mobile-phase solvents were used in this study for different sample groups. For clarity, they are described with the specific samples in later sections. The mobile phase flow rate used was always the same at 0.4 mL/min. All chromatographic peaks were confirmed to appear during the retention time period of 5−45 min. MS analysis was made with a Bruker Esquire-LC system. Compounds were ionized by electrospray ionization (ESI). The detection was made in the "smart mode" with the following conditions: gas flow pressure, 40 psi; gas flow rate, 8 mL/min; drying gas temperature, 365 °C; target m/z, 750; trap drive, 60%; compound stability, 75%; and under the wide-mode optimization.

Production and Purification of Rhamnolipids. Rhamnolipids were produced by *P. aeruginosa* fermentation. Inoculum was grown for 8 h in a 30 g/L tryptic soy broth (TSB) medium in a shaker operating at 34 °C and 250 rpm. A total of 10 mL inoculum was added to 90 mL of TSB solution and similarly grown for 18 h to prepare the preculture. The entire (100 mL) preculture was added to a 3 L fermentor (BIOFLO 110, New Brunswick Scientific, Edison, NJ) with 1 L of medium and agitated at 600 rpm with two six-blade turbines. The pH was maintained at 6.2 ± 0.1 by automatic addition of 1 N H_2SO_4 or NaOH. The dissolved oxygen concentration was kept at 10% (±3%) by controlled bubbling of pure oxygen; the temperature was controlled at 32.0 ± 0.1 °C. The fermentation medium contained 100 g/L glycerol, 6 g/L KH_2PO_4, 5.73 g/L NH_4Cl, 5 g/L yeast extract, 5 g/L peptone, 1.5 g/L NaCl, 0.9 g/L $MgSO_4·7H_2O$, 0.1 g/L $FeSO_4·7H_2O$, 0.03 g/L $CaCl_2·2H_2O$, 0.03 g/L $MnCl_2·4H_2O$, and 2 mL/L of a trace element solution. The trace element solution had 0.75 g/L $MnSO_4·H_2O$, 0.75 g/L $ZnSO_4·7H_2O$, 0.15 g/L H_3BO_3, 0.08 g/L $FeCl_3·6H_2O$, 0.08 g/L $CoCl_2·6H_2O$, 0.075 g/L $CuSO_4·5H_2O$, and 0.05 g/L Na_2MoO_4.

The rhamnolipids produced in the fermentation broth were mainly RhL-1 and RhL-3. A glass chromatography column (600 × 50 mm inner diameter) was used for separation of RhL-1 and RhL-3. The height of silica gel in the column was about 500 mm. The mobile phase was a chloroform/methanol/water (100:23:3, v/v/v) mixture. The column was flushed with about 1.4 L mobile phase. The eluate was collected in 40 consecutive fractions of 25 mL. The 40 fractions were air-dried separately. RhL-1 was in the fractions 3−15, and RhL-3 was in the fractions 17−35, which was confirmed with HPLC−MS analysis.

Generation of Free Diacids from Rhamnolipids. Free diacids were generated in two steps: ethyl diacids were first formed and then hydrolyzed to free diacids, according to the route shown in Figure 2.

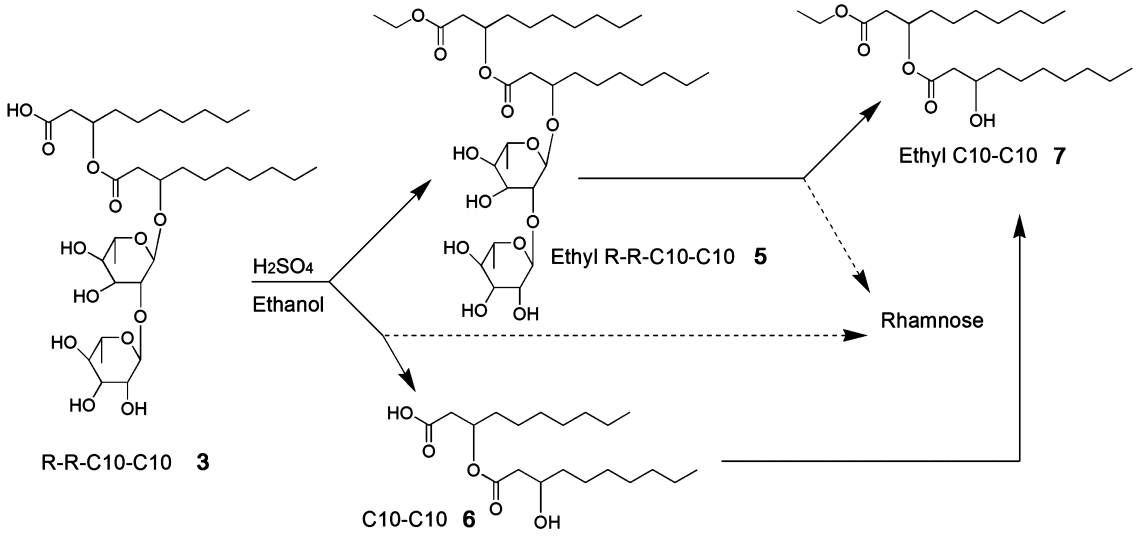

Figure 2. Reaction scheme for generating ethyl diacids from RhL-3, illustrated with R−R−C_{10}−C_{10} as the structural example.

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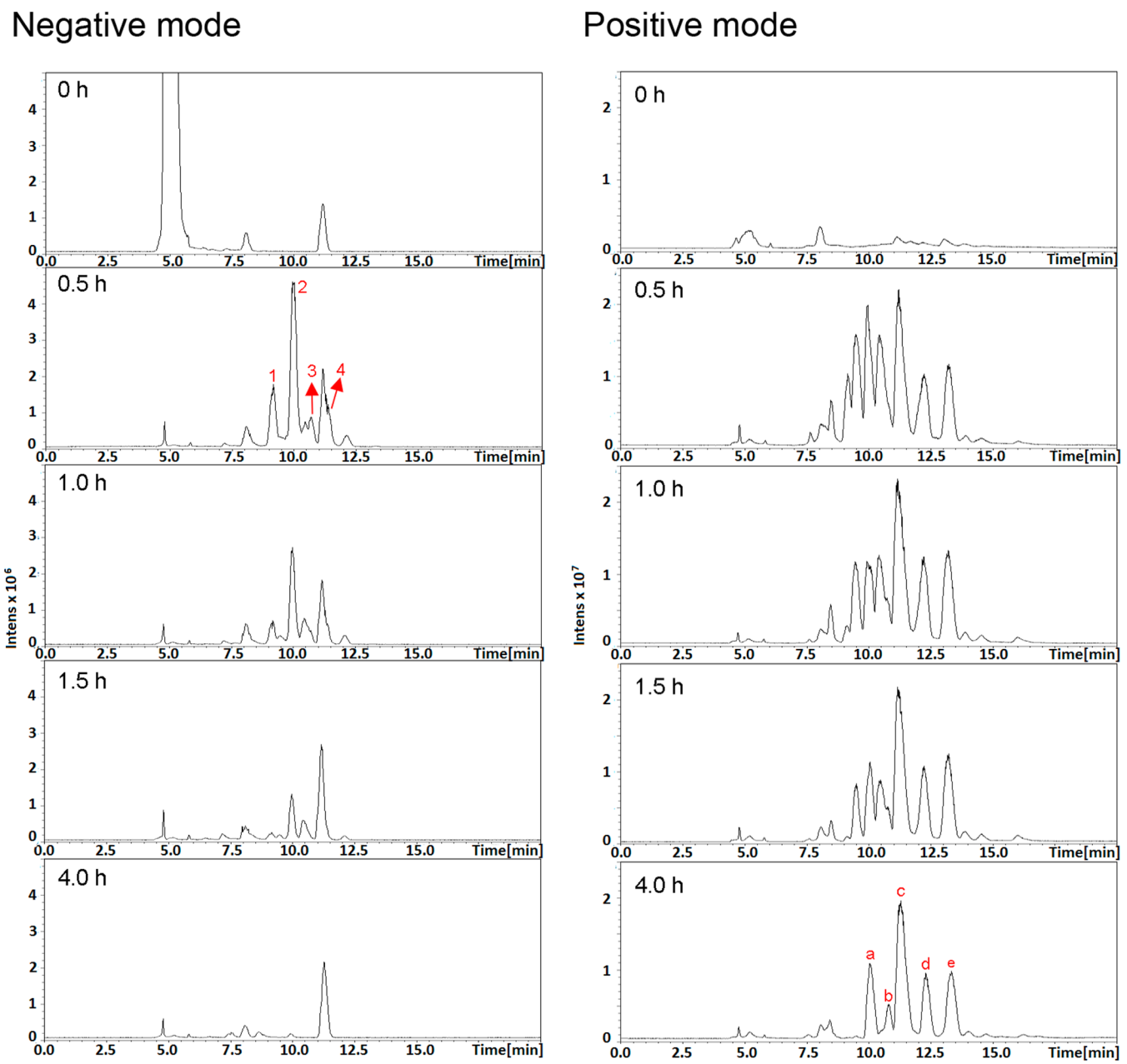

Figure 3. Total ion chromatograms from HPLC−MS analysis of samples during the generation of ethyl diacids from RhL-3.

For the production of ethyl diacids, 100 mg of RhL-3 and 5 mL of ethanol were charged into a 20 mL glass vial. Then, 0.2 mL of sulfuric acid was added dropwise. The mixture was reacted for 4 h in a 70 °C shaking water bath operating at 150 rpm. The reaction progress was followed by taking samples (typically 50 μL) at 0.5, 1.0, 1.5, 2.0, and 4.0 h. The sulfuric acid in these samples was neutralized by the addition of 100 μL of saturated aqueous solution of sodium bicarbonate. The neutralized samples were dried and extracted with ethyl acetate. The ethyl acetate phase was collected and dried, and the residue was redissolved in 95% methanol in water and then subjected to HPLC−MS analysis, which was performed with an isocratic flow (0.4 mL/min) of the following mobile phase: methanol/4 mM aqueous ammonium acetate (95:5).

Free diacids were then obtained in the second reaction step, by hydrolysis of the ethyl diacids with an aqueous sodium hydroxide solution. Typically, 2.5 mL of ethyl diacid solution (10 mg/mL in ethanol) was mixed with 1.5 mL of water. Then, 1 mL of sodium hydroxide stock solution (10 mg/mL in water) was added. The reaction mixture was shaken at 150 rpm and 25 °C for 2 h. The mixture was then neutralized with acetic acid, dried, and extracted with ethyl acetate to remove the remaining salt. The synthesized free diacids in the ethyl acetate extract were characterized by MS analysis with the following conditions: positive mode; gas flow pressure, 10 psi; gas flow rate, 8 mL/min; drying gas temperature, 310 °C; target m/z, 381; trap drive, 100%; compound stability, 100%; and under the normal-mode optimization.

Conversion of RhL-1 and RhL-3 to RhL-2 and RhL-4. A total of 100 mg of RhL-3 (or RhL-1) and 5 mL of aqueous NaOH solution (40 g/L) were reacted in a 20 mL glass vial for 4 h at 25 °C in a shaker operating at 150 rpm. Samples (50 μL each) were taken at 0.5, 1.0, 1.5, 2.0, and 4.0 h and neutralized with 50 μL of anhydrous acetic acid. The samples were dried and then dissolved with 40% methanol in water for HPLC−MS analysis. The mobile phase used for these samples was a gradient of (A) methanol and (B) 4 mM aqueous ammonium acetate according to the following: a 5 min isocratic hold at 40% A, a 20 min gradient to increase A from 40 to 95%, a 10 min isocratic hold at 95% A, a 15 min gradient to decrease A from 95 to 40%, and a 5 min isocratic hold at 40% A. The total run time was 55 min for each

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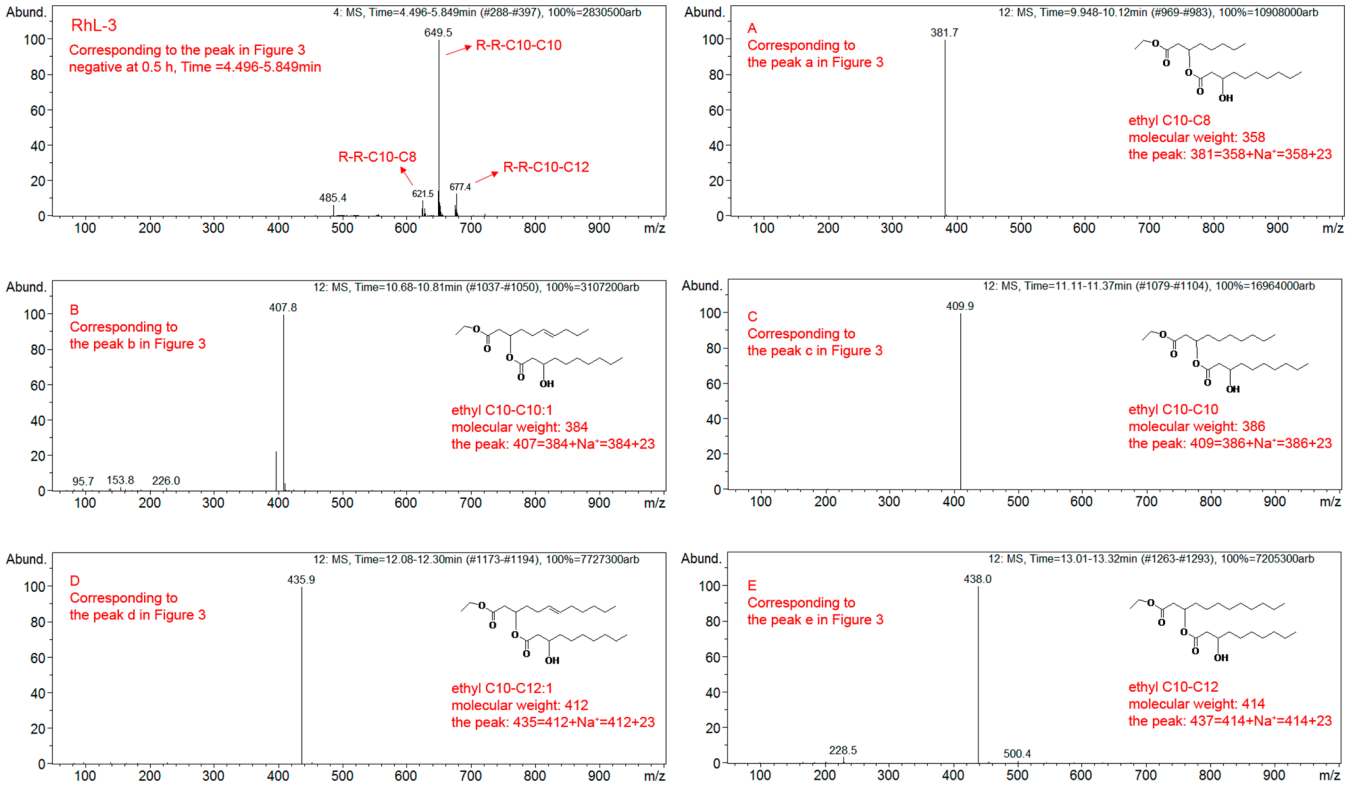

Figure 4. MS spectra for some isolated peaks shown in Figure 3, confirming the structures of the RhL-3 reactants and the five ethyl diacid products.

sample. MS analysis was in the negative mode, while other operating parameters were as described previously.

Enzymatic Conversion of RhL-3 to RhL-1. Into a 20 mL glass vial, 5 mL of RhL-3 solution (0.5 mg/mL in water) and 5 mL of enzyme solution (cellulase or cellobiase in 0.05 M citrate buffer at pH 4.8) were added. For cellulase, the concentration was 1 mg/mL (8.9 units/mL), assayed with cellulose as the substrate at pH 5.0 and 37 °C for 2 h of incubation time; 1.0 unit liberates 1.0 μmol/min glucose from cellulose. For cellobiase, the enzyme solution was prepared by adding 50 μL of cellobiase to 5 mL of citrate buffer. The reaction mixture was shaken at 150 rpm and 37 °C for 24 h. Samples (1 mL) were taken at 6 and 24 h. The samples were dried and redissolved in acetonitrile/water (40:60) for HPLC−MS analysis. The mobile phase used for these samples was a gradient of (A) acetonitrile and (B) 4 mM aqueous ammonium acetate according to the following: a 3 min isocratic hold at 40% A, a 17 min gradient to increase A from 40 to 95%, and a 10 min isocratic hold at 95% A. MS analysis was conducted in the negative mode as described previously.

Anti-zoospore Test. *P. sojae* was cultured on V8 juice agar. Zoospores were obtained by washing a 7-day-old plate culture with tap water, once every 30 min for a total of 6 h. Multiple 2 mL plastic tubes were used for the anti-zoospore test. Each tube was filled with 100 μL of rhamnolipid solution that had a concentration varying among 20, 40, 60, 80, 100, 120, 160, and 250 mg/L. A total of 50 μL of water and 50 μL of zoospore suspension were added to the tubes. The zoospore suspension had about 1×10^5 zoospores/mL as measured by counting with a hemocytometer under a light microscope coupled with a DP71 digital camera (Olympus America, Inc., Center Valley, PA). Immediately after the addition of zoospores, the tube was gently swirled for mixing for 5 s, followed by extraction of one drop of the mixture and placement on a glass slide for observation of zoospore motility under the microscope. The critical anti-zoospore concentration was defined in this study as the lowest rhamnolipid concentration at which the motility of all observed zoospores was stopped.

RESULTS AND DISCUSSION

Generation of Free Diacids from Rhamnolipids. There are several hypotheses as to why bacteria produce rhamnolipids.[19−21] None assumes that rhamnolipids are specifically produced to kill fungal zoospores.[19−21] The mechanism of the anti-zoospore effects of rhamnolipids is poorly understood. There have been no studies on the correlation of rhamnolipid molecular structures with their anti-zoospore effects. It was recognized at an early stage of this study that the structures of the diacid residue of RhL-1 and RhL-3 are somewhat similar to another class of biosurfactants, corynomycolic acids, which also have branched β-hydroxyl fatty acid structures.[22−24] On the other hand, other well-known biosurfactants, such as sophorolipids and surfactin, which have no strong anti-zoospore properties,[7] do not have the diacid structure. It was therefore hypothesized that, upon initial breakdown of rhamnolipids in natural environments, the free diacids generated may play a role in lysing zoospores (and/or other biological activities). If so, the anti-zoospore and/or other biological activities of the diacids may offer some longer term benefits to the rhamnolipid-producing bacteria, such as *P. aeruginosa*. Free diacids were therefore synthesized from rhamnolipids for the later anti-zoospore evaluation.

The reaction scheme for production of ethyl diacids from RhL3 precursors is shown in Figure 2. The total ion spectra from HPLC−MS analysis of the samples taken along the reaction of ethyl diacid production are shown in Figure 3. In the negative detection mode, RhL-3, with the free carboxyl group, is expected to give a strong signal. This is seen with the 0 h sample, as the large peak at the retention time of 4.5−5.5 min. The isolated mass spectrum for this large RhL-3 peak is given in Figure 4, showing three major structures: R−R−C_{10}−C_8, R−R−C_{10}−C_{10}, and R−R−C_{10}−C_{12}. In fact, five groups of

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Table 1. Components of RhL-1−RhL-4 and the Free Diacids Detected in this Study

RhL-1 from fermentation	RhL-2 (produced from RhL-1)
$R-C_{10}-C_8$ or $R-C_8-C_{10}$	$R-C_8$
$R-C_{10}-C_{10:1}$ or $R-C_{10:1}-C_{10}$	$R-C_{10}$
$R-C_{10}-C_{10}$	$R-C_{12}$
$R-C_{10}-C_{12:1}$ or $R-C_{12:1}-C_{10}$ or $R-C_{10:1}-C_{12}$ or $R-C_{12}-C_{10:1}$	
$R-C_{10}-C_{12}$ or $R-C_{12}-C_{10}$	

RhL-3 from fermentation	RhL-4 (produced from RhL-3)	diacids (produced from RhL-3 and RhL-1/RhL-3 mixture)
$R-R-C_{10}-C_8$ or $R-R-C_8-C_{10}$	$R-R-C_8$	$C_{10}-C_8$ or C_8-C_{10}
$R-R-C_{10}-C_{10:1}$ or $R-R-C_{10:1}-C_{10}$	$R-R-C_{10}$	$C_{10}-C_{10:1}$ or $C_{10:1}-C_{10}$
$R-R-C_{10}-C_{10}$	$R-R-C_{12}$	$C_{10}-C_{10}$
$R-R-C_{10}-C_{12:1}$ or $R-R-C_{12:1}-C_{10}$ or $R-R-C_{10:1}-C_{12}$ or $R-R-C_{12}-C_{10:1}$		$C_{10}-C_{12:1}$ or $C_{12:1}-C_{10}$ or $C_{10:1}-C_{12}$ or $C_{12}-C_{10:1}$
$R-R-C_{10}-C_{12}$ or $R-R-C_{12}-C_{10}$		$C_{10}-C_{12}$ or $C_{12}-C_{10}$

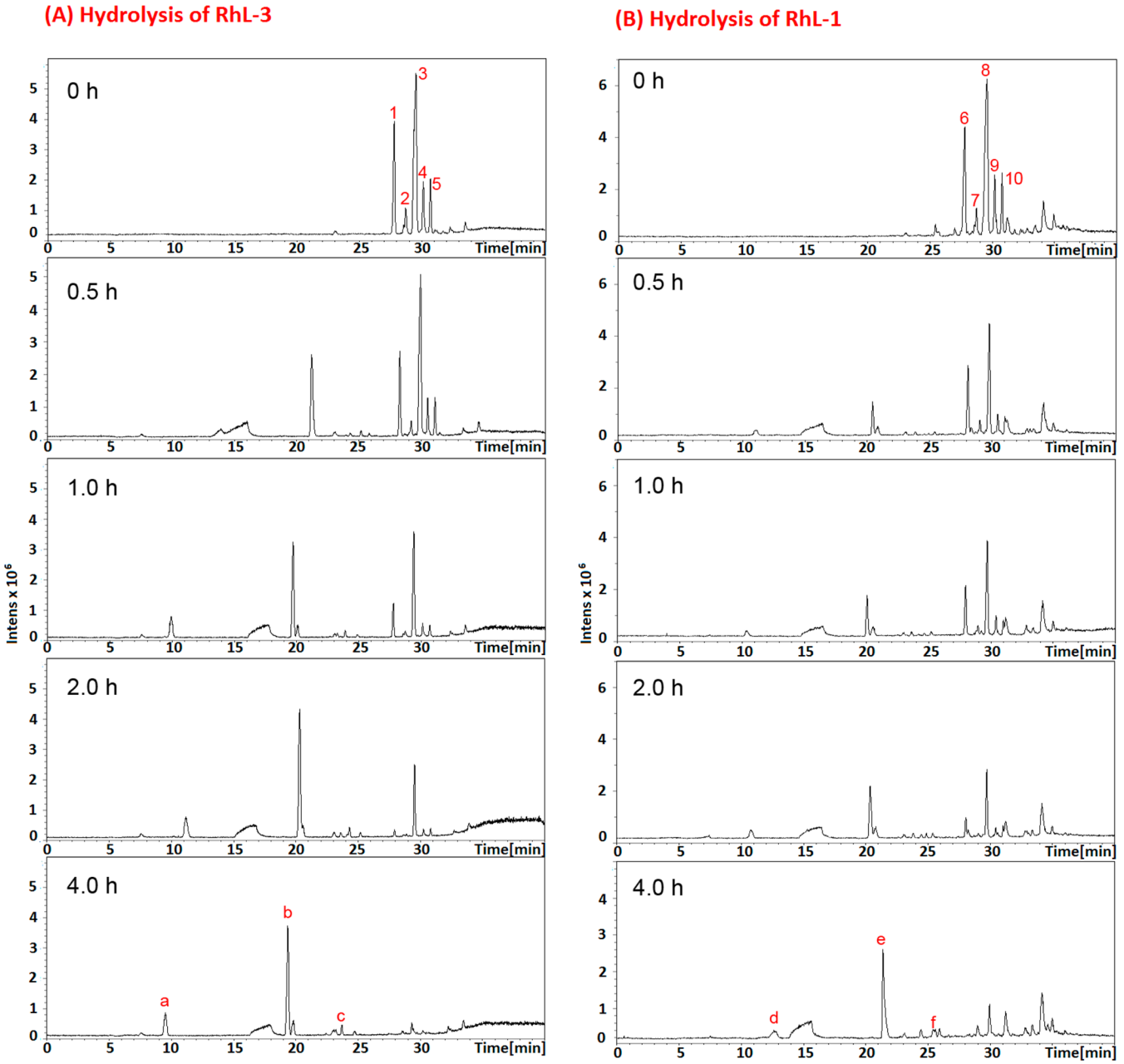

Figure 5. Total ion chromatograms from HPLC−MS analysis of samples from the hydrolysis reaction of (A) RhL-3 to RhL-4 and (B) RhL-1 to RhL-2.

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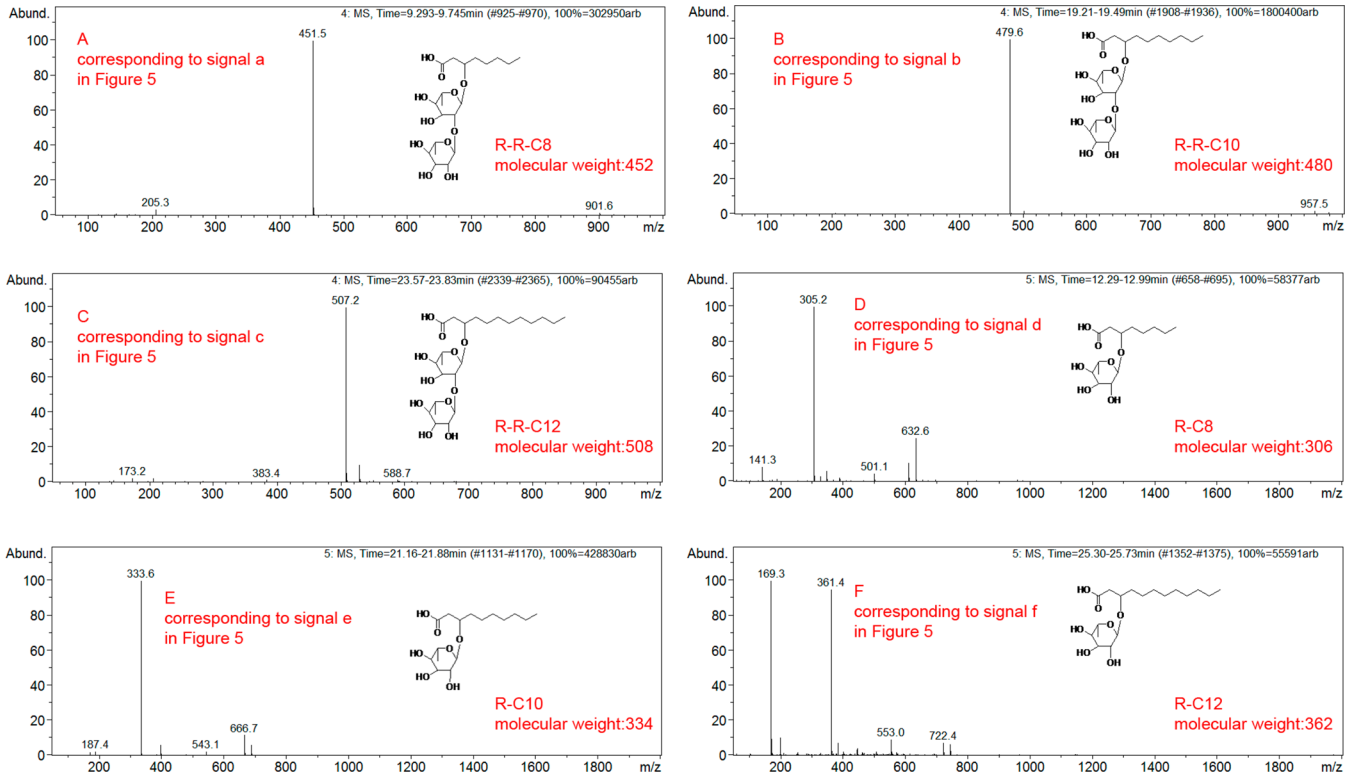

Figure 6. (A–F) MS spectra for the isolated product peaks, shown in Figure 5.

structures have been detected in RhL-3 produced in our laboratory, as given in Table 1. They are more clearly shown in Figures 5 and 7 in later sections. The two smaller groups of congeners are not apparent in this spectrum for the isolated peak.

In the negative-mode spectrum for the sample taken after 0.5 h of reaction, a group of new signals, labeled as 1–4 in Figure 3, appears in the retention time range of 8.5–11.5 min. According to their mass spectra, the new signals correspond to the ethylated rhamnolipids: (1) ethyl R–R–C_{10}–C_8, (2) ethyl R–R–C_{10}–C_{10}, (3) ethyl R–R–C_{10}–$C_{12:1}$, and (4) ethyl R–R–C_{10}–C_{12}. Among the five detectable structures in RhL-3, the group of R–R–C_{10}–$C_{10:1}$ and R–R–$C_{10:1}$–C_{10} is present in the smallest quantity; the ethylated rhamnolipid of this group is not seen in the negative-mode spectra in Figure 3.

The above four new peaks are seen to decrease in intensity with a longer reaction time and nearly disappear after 4.0 h. The results indicate that rhamnolipid ethylation is a fast reaction and the ethyl rhamnolipids formed are further converted to other products. These final products are more clearly seen in the positive-mode spectra in Figure 3. In the positive-mode spectrum for the 0 h sample, the signal of RhL-3 is much weaker than the signal in the negative-mode spectrum. However, more new peaks appear in the positive-mode spectrum for the 0.5 h sample. These peaks correspond to not only the ethyl rhamnolipids, which give strong signals at both negative and positive modes, but also the ethyl diacids formed by the reaction. For the 4.0 h sample, five peaks, labeled as a–e, remain in the positive-mode spectrum. These peaks are from the five synthesized ethyl diacids, as confirmed by the corresponding mass spectra shown in Figure 4 for the five isolated peaks.

Structures of the free diacids subsequently obtained by hydrolysis of the ethyl diacids with sodium hydroxide are summarized in Table 1. The synthesized free diacids were used in the later anti-zoospore study.

Besides ethyl diacids, rhamnose can be obtained as a byproduct. The production of rhamnose by sulfuric acid hydrolysis of rhamnolipids has been reported[25,26] and is not investigated in detail here. Currently, the commercial rhamnose is obtained through an extraction and hydrolysis process.[25,26] Rhamnose-containing materials are first extracted from plant materials, such as oak bark and citrus peels. Rhamnose is then produced by hydrolyzing these extracts. This labor-intensive process needs toxic, corrosive chemicals and produces undesirable waste products. The co-production of rhamnose from rhamnolipids represents an alternative route.

RhL-3 was used as the starting material to generate free diacid for easier tracking of the progress of reactions involving more manageable numbers of rhamnolipids and ethyl rhamnolipids. However, RhL-1 and RhL-3 have the same fatty acid residue (Table 1), and the diacids can be obtained from the mixture of RhL-1 and RhL-3. This process of generating diacids directly from the rhamnolipid mixture collected from fermentation, without the preceding chromatographic separation into RhL-1 and RhL-3, was also carried out in this study. The ethyl diacids obtained have the same final HPLC–MS positive-mode spectrum as that in Figure 3 (data not shown).

Generation of RhL-2 and RhL-4 from RhL-1 and RhL-3. RhL-2 and RhL-4, with one β-hydroxyalkanoic acid as the hydrophobic moiety, have been reported to be produced in the bacterial fermentation but typically make up very small fractions of the rhamnolipid mixtures.[17,18] In the rhamnolipids produced in our laboratory, the contents of RhL-2 and RhL-4 are very low, not observed in our MS analysis. RhL-1 and RhL-3, with two ester-linked β-hydroxyalkanoic acids as the hydrophobic moiety, are the most common groups of rhamnolipids. These

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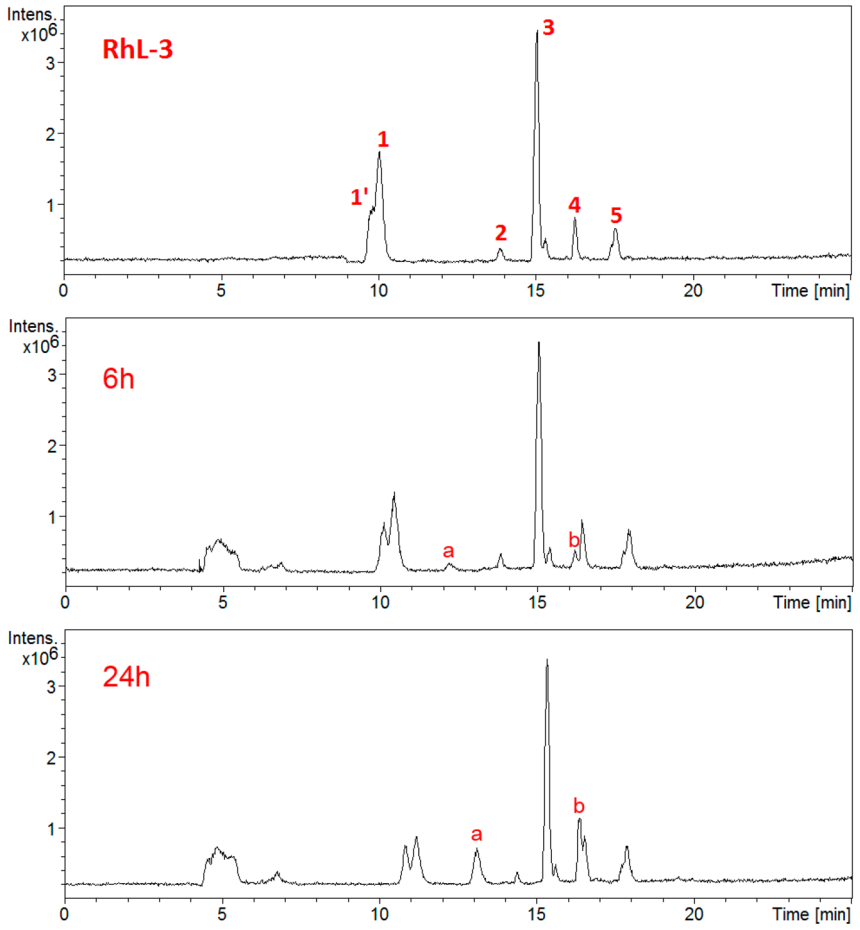

Figure 7. Total ion chromatograms from HPLC−MS analysis of samples from the cellobiase-catalyzed conversion of RhL-3 to RhL-1.

were the groups studied for rhamnose production and anti-zoospore effects in previous literature reports.[7,25] It was desirable to evaluate the anti-zoospore effect of RhL-2 and RhL-4, as compared to that of RhL-1 and RhL-3. The results might provide some insights into the potential importance of the unique diacid structure in rhamnolipids to the biological activities of the biosurfactants. In addition, if RhL-2 and RhL-4 would be found to have superior anti-zoospore property, there might be advantages to tailor the bacterial strain and fermentation conditions to produce RhL-2 and RhL-4, instead of RhL-1 and RhL-3. For example, RhL-2 and RhL-4 have smaller molecular weights than RhL-1 and RhL-3. The molar yield of RhL-2 and RhL-4 can be higher from the same amount of substrate.

RhL-1 and RhL-3 were hydrolyzed with sodium hydroxide to produce RhL-2 and RhL-4. The progress of the hydrolysis reaction is illustrated by the HPLC−MS results shown in Figure 5 for the samples taken at 0, 0.5, 1.0, 2.0, and 4.0 h. For the 0 h samples, the peaks numbered as 1−5 correspond to the five major structures in RhL-3 and those numbered as 6−10 correspond to the five structures in RhL-1. These structures are summarized in Table 1. As shown in Figure 5A, with increasing reaction time, peaks 1−5 of RhL-3 all decrease in intensity simultaneously and three new peaks, labeled as a−c, appear in the spectrum for the 4.0 h sample. The mass spectra corresponding to peaks a−c are shown in panels A−C of Figure 6, confirming that these new peaks formed are dirhamnolipids with the RhL-4 structure, i.e., R−R−C_8, R−R−C_{10}, and R−R−C_{12}. Similarly, three new peaks d−f are formed from hydrolysis of RhL-1, as shown in Figure 5B, and their corresponding mass spectra are shown in panels D−F of Figure 6, confirming that the new compounds produced are monorhamnolipids with the RhL-2 structure, i.e., R−C_8, R−C_{10}, and R−C_{12}. There are other possible RhL-2 and RhL-4 structures (i.e., R−$C_{10:1}$, R−$C_{12:1}$, R−R−$C_{10:1}$, and R−R−$C_{12:1}$) in addition to the three RhL-2 rhamnolipids formed from hydrolysis of RhL-1 and the three RhL-4 rhamnolipids from RhL-3. However, these structures are not seen in the product spectra. It should be noted that the broad signal appearing around 16−18 min of retention time in both panels A and B of Figure 5 (except for the 0 h starting reactants of RhL-3 and RhL-1) is from the sodium acetate present in the reaction mixture samples. The final reaction mixture required further purification techniques to collect only the RhL-2 and RhL-4 produced. Purification was performed by silica column chromatography, to remove the salt and hydroxyl fatty acids (see part 3 of the Supporting Information). The hydroxyl fatty acids collected were analyzed, and their structures were confirmed by MS (see part 3 of the Supporting Information). RhL-2 and RhL-4 thus produced were used in the later anti-zoospore tests. Industrial uses of the hydroxyl fatty acids obtainable from this simple base-catalyzed hydrolysis potentially improve the overall process economics of rhamnolipid production and applications.

Enzymatic Conversion of RhL-3 to RhL-1. Rhamnolipids mostly include RhL-1 and RhL-3, and the ratio of RhL-1/RhL-3 affects the rhamnolipid mixture physical properties. The monorhamnose RhL-1 is less hydrophilic than its dirhamnose

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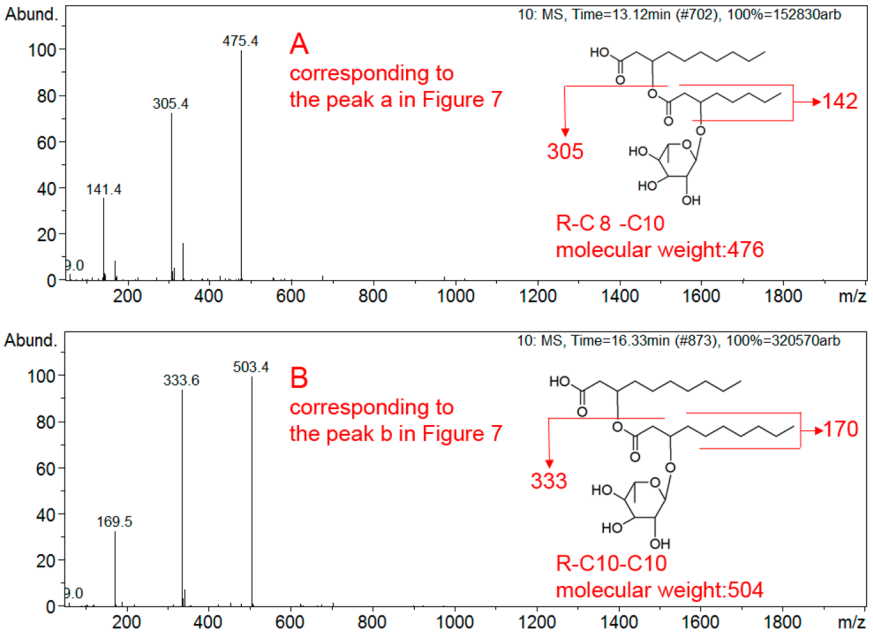

Figure 8. MS spectra for the isolated product peaks a and b in Figure 7.

RhL-3 counterpart with the same fatty acid residue. Accordingly, the critical micelle concentration (cmc) of RhL-1 is usually lower than that of RhL-3. For example, at pH 7, the cmc of RhL-1 is about 0.035 mM, while that of RhL-3 is about 0.110 mM.[23,27−29] These different properties might make different rhamnolipid mixtures more suitable for different applications. There are several ways to alter the ratio of RhL-1/RhL-3. This ratio varies with the fermentation conditions.[30,31] For example, mixtures of higher RhL-1 contents can be produced using vegetable oil as the carbon source in the fermentation.[31] The disadvantage is that the total yield of rhamnolipids also changes significantly with different fermentation substrates and/or conditions. A second approach is to first separate RhL-1 and RhL-3 and then blend them at different ratios, but the initial separation cost is high, usually relying on silica column chromatography. In current work, an enzymatic method was studied to convert RhL-3 to RhL-1. The enzymatic method not only suggests a way to tailor this ratio but also provides valuable information on the breakage of the glycosidic bond in RhL-3 *in vitro* with an enzyme as the catalyst. The latter is helpful to the understanding of the degradation of rhamnolipids in nature.

Both cellulase and cellobiase (β-glucosidase, EC 3.2.1.21) were tested for converting RhL-3 to RhL-1. No RhL-3 conversion could be detected with the cellulase alone. It is possible that the β-glucosidase activity was too low in the cellulase preparation used for this study. Note that β-glucosidase activity is mainly responsible for the intended hydrolysis of R−R disaccharide in RhL-3. On the other hand, RhL-3 hydrolysis was observed to take place with the cellobiase as the catalyst, as shown in Figure 7, in which RhL-3 rhamnolipids appear as a group of peaks 1 (and 1′)−5, similar to those in earlier figures. However, here, peak 1, which in earlier figures was described as the structure R−R−C_{10}−C_8 without differentiation of the order of the two fatty acid residues C_{10} and C_8, can clearly be seen to consist of two overlapping peaks. Peak 1′, which appears slightly earlier, has the structure of R−R−C_{10}−C_8, and the following peak 1 is R−R−C_8−C_{10}, as confirmed by MS analysis. With increasing reaction time, up to 24 h, the intensities of peaks 1 and 1′ are seen to clearly decrease but not the intensities of other peaks 2−5. Furthermore, two new peaks a and b appear and increase in intensity with the progress of reaction. The mass spectra for these new peaks a and b are shown in Figure 8, indicating that peak a is a combination of R−C_{10}−C_8 and R−C_8−C_{10} and peak b is R−C_{10}−C_{10}. According to the product peaks formed, R−R−C_{10}−C_8, R−R−C_8−C_{10}, and R−R−C_{10}−C_{10} were hydrolyzed by cellobiase to their corresponding monorhamnolipid counterparts, while no conversion was detected for R−R−C_{10}−$C_{12:1}$ and R−R−C_{10}−C_{12}. The conversions of R−R−C_8−C_{10} and R−R−C_{10}−C_8 appear to be the most significant, with the R−R−C_8−C_{10} conversion likely being faster. This is supported by two observations: the intensity of fragment R−C_8−C_{10} (m/z 305) is stronger than that of fragment R−C_{10}−C_8 (m/z 329) in Figure 8A, and peak 1 intensity decreases faster than peak 1′ intensity in Figure 7. It is possible that the enzymatic conversion is related to the fatty acid chain length. R−R−C_8−C_{10} and R−R−C_{10}−C_8 have the shortest chain length and are hydrolyzed the most. R−R−C_{10}−C_{10} is also hydrolyzed. The longest fatty acid chains in R−R−C_{10}−$C_{12:1}$ and R−R−C_{10}−C_{12} might have affected the affinity of these rhamnolipids for the active site of the enzyme. Cellobiase is supposed to act on the cellobiose released during enzymatic breakdown of cellulose. Rhamnose is not present in usual cellulose. The observed results of RhL-3 conversion with cellobiase suggest that the enzyme can also hydrolyze the dirhamnose residue of RhL-3 but not those with fatty acid residues longer than C_{10}−C_{10}.

Anti-zoospore Tests. RhL-1−RhL-4 and the synthesized diacids were investigated for their anti-zoospore effects. The results are shown in Table 2. RhL-1−RhL-4 have similar anti-zoospore effects, although RhL-1 appears to be most potent. This is inconsistent with the previous report that RhL-3 has better anti-zoospore property than RhL-1.[7,8] The discrepancies might arise from different choices for zoospores and different rhamnolipid compositions used in the various studies. More importantly, RhL-2 and RhL-4 are found to have anti-zoospore effects similar to RhL-1 and RhL-3. This finding sheds some

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Table 2. Anti-zoospore Effects of RhL-1−RhL-4 and the Diacids Evaluated at Different Concentrations[a]

concentration (mg/L)	10	20	30	40	50	60	80	125
RhL-1	−	*	*	*	*	*	*	*
RhL-2	−	−	*	*	*	*	*	*
RhL-3	−	−	−	*	*	*	*	*
RhL-4	−	−	−	*	*	*	*	*
diacids (e.g., C_{10}−C_{10})	−	−	−	−	−	−	−	*

[a]"−", moving zoospores were observable; "*", all zoospores ceased moving; most were lysed.

light on the anti-zoospore mechanism of rhamnolipids. It does not support the hypothesis that the unique diacid structure of rhamnolipids plays a critical role in the anti-zoospore effect.

A wide range of cmc has been reported for rhamnolipid mixtures, because of the varying structures, e.g., 5−40 mg/L for RhL-1 and RhL-3 with two hydrophobic tails and around 200 mg/L for RhL-2 and RhL-4 with only one hydrophobic tail.[32] In this study, the critical anti-zoospore concentrations to stop movement of all zoospores for RhL-1−RhL-4 are found to be in the range of 20−40 mg/L (Table 2), which are much lower than the cmc of about 200 mg/L for RhL-2 and RhL-4. Therefore, while micelles might be present in the test solutions of RhL-1 and RhL-3 at their critical anti-zoospore concentrations, micelles were unlikely to be formed in the test solutions of RhL-2 and RhL-4 at those concentrations (30 and 40 mg/L). The finding implies that micelle formation and the associated solubilization of membrane lipids or other hydrophobic cell components are not an essential mechanism responsible for the anti-zoospore effect.

The diacids also exhibit the anti-zoospore effect (Table 2). The diacids are more effective against zoospores than most of the other biosurfactants tested in the literature;[7] more than 1000 mg/L of those biosurfactants (sophorolipids, trehalose lipid, and surfactin) are required to totally cease zoospore movement,[7] while approximately 125 mg/L diacids are required. Diacids are more hydrophobic and less soluble than RhL-1−RhL-4. The diacids make a clear solution in water at 100 mg/L but form a slightly white, turbid suspension at 1000 mg/L. The hydrophobicity of the diacids might affect their interactions with the surface of zoospores; the rhamnose residue in rhamnolipids lowers this hydrophobicity and significantly enhances the anti-zoospore effect of the biosurfactants. Overall, the results indicate that rhamnolipids are very effective against the zoospores of *P. sojae* and that, when applied to natural environments, the degradation intermediates, including RhL-2 and RhL-4 (which have lost one fatty acid residue) and the free diacids separated from the rhamnose residue, can continue to exert the anti-zoospore effect.

In summary, although RhL-1 and RhL-3 are produced in large quantities in *P. aeruginosa* fermentation, RhL-2 and RhL-4 and the free diacids can be synthesized from RhL-1 and RhL-3 using the methods developed in this study. Rhamnose, a valuable co-product, can be obtained from some of the conversion reactions developed. β-Hydroxy fatty acids can also be produced when generating RhL-2 and RhL-4 from RhL-1 and RhL-3. The possibility of adjusting the ratio of RhL-1/RhL-3 in the rhamnolipid mixture to better suit the specific application is further demonstrated using the cellobiase enzyme as a catalyst, and the enzyme is shown to have higher selectivity toward dirhamnolipids with shorter fatty acid chains. RhL-1−RhL-4 and the free diacids are all found to be very effective in damaging the zoospores of *P. sojae*, with RhL-1 being the most effective and the diacids the least effective among them. The results indicate that the combined structure of β-hydroxy fatty acid and sugar significantly enhances the anti-zoospore effect. This finding may be helpful to the further development of anti-zoospore chemicals. These results also indicate that, when applied to natural environments, the degradation intermediates, including the RhL-2 and RhL-4 that have lost one fatty acid residue (from RhL-1 and RhL-3) and the free diacids separated from the rhamnose residue, can all continue to exert the anti-zoospore effect.

■ ASSOCIATED CONTENT

Supporting Information

Mass spectra of ethyl rhamnolipids (part 1), proton nuclear magnetic resonance (^{1}H NMR) of ethyl diacids and free diacids (part 2), and mass spectra of hydroxyl fatty acids (part 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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