Multiplatform Mass Spectrometry-Based Approach Identifies Extracellular Glycolipids of the Yeast *Rhodotorula babjevae* UCDFST 04-877

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Supporting Information

**ABSTRACT:** A multiplatform mass spectrometry-based approach was used for elucidating extracellular lipids with biosurfactant properties produced by the oleaginous yeast *Rhodotorula babjevae* UCDFST 04-877. This strain secreted 8.6 ± 0.1 g/L of extracellular lipids when grown in a benchtop bioreactor fed with 100 g/L glucose in medium without addition of hydrophobic substrate, such as oleic acid. Untargeted reversed-phase liquid chromatography–quadrupole/time-of-flight mass spectrometry (QTOFMS) detected native glycolipid molecules with masses of 574–716 Da. After hydrolysis into the fatty acid and sugar components and hydrophilic interaction chromatography–QTOFMS analysis, the extracellular lipids were found to consist of hydroxy fatty acids and sugar alcohols. Derivatization and chiral separation gas chromatography–mass spectrometry (GC-MS) identified these components as D-arabitol, D-mannitol, (R)-3-hydroxymyristate, (R)-3-hydroxypalmitate, and (R)-3-hydroxysearate. In order to assemble these substructures back into intact glycolipids that were detected in the initial screen, potential structures were *in-silico* acetylated to match the observed molar masses and subsequently characterized by matching predicted and observed MS/MS fragmentation using the Mass Frontier software program. Eleven species of acetylated sugar alcohol esters of hydroxy fatty acids were characterized for this yeast strain.

**SECRETION:** Extracellular lipids such as glycolipids have been reported in some yeasts, bacteria, and mycelial fungi. Specifically, rhamnolipids, mannosylerythritol lipids, sophorolipids, cellobiose lipids, and trehalose lipids are the best known extracellular glycolipids. Because of their physicochemical properties, they can be used as biosurfactants. In addition, multiple biological activities and potential applications of these compounds were reported in the food industry, bioremediation, pharmaceutics, and medicine.

Over the past decades, several approaches have been used for chemical analysis of glycolipids. Previous studies focused mainly on the determination of primary constituents of these compounds. To this end, isolated lipids were hydrolyzed under acidic or alkaline conditions releasing the fatty acid, sugar, and/or further constituents (e.g., acetic acid originating from acetylated structures) followed by analysis using gas chromatography with flame ionization detection (GC-FID) or more recently mass spectrometry (MS). Because polar analytes were formed during the hydrolysis, derivatization steps were needed to increase their volatility.

Recent advances in liquid chromatography–mass spectrometry (LC-MS) instrumentation and computation methods have permitted characterization of small molecules (e.g., lipids) by analyzing the intact molecules without a need for hydrolysis and/or derivatization. Using tandem mass spectrometry (MS/MS), fragment ions originating from intact molecule precursors can be used for structure elucidation. For instance, Ongena et al. focused on characterization of mannosylerythritol lipid biosurfactants. Fractions of mannosylerythritol lipids from preparative column chromatography were used for subsequent LC-MS analysis. MS/MS data allowed the unambiguous identification of the fatty acids present in the mannosylerythritol lipids. These data were also supported by GC-MS analysis of fatty acids after the previous hydrolysis of mannosylerythritol lipids.

For unknown identification, several *in-silico* fragmentation software programs such as Mass Frontier, CSI:FingerID,
CFM-ID, MS-FINDER, or MetFrag are currently available in order to confirm or reject the hypothetical structures based on fragment ion pattern. While some of these programs utilize compound libraries such as PubChem or KEGG (e.g., MetFrag), for truly unknown identification it is important to have the option to submit one’s own structures for structure elucidation (e.g., Mass Frontier, CFM-ID). Here, a workflow is presented to identify a series of novel glycolipids, polyol esters of fatty acids (PEFA), produced by the yeast Rhodotorula babjevae UCDFST 04-877 (formerly known as Rhodosporidium babjevae UCDFST 04-877 before recent taxonomic revision of this clade of yeasts).19 By combining multiplatform analyses with in-silico software for interpreting the mass spectral data, this group of extracellular lipids could be characterized.

RESULTS AND DISCUSSION

R. babjevae UCDFST 04-877 was isolated from a female olive fly trapped in an olive tree at UC Davis Campus, California, USA, in 2004. This yeast is of commercial interest since it has been described to produce high amounts of intracellular lipid mainly in the form of triacylglycerols as well as being able to grow in carbon sources different from glucose (e.g., glycerol) and the presence of inhibitors.20 R. babjevae is a close relative of R. glutinis and R. graminis, which were identified in the 1960s to produce a heavy oil.9 Given the analytic capabilities of the time, researchers were able to identify only broadly the constituents of the oil, but failed to provide a detailed profile of the molecules and the exact structures as the present study is providing. These glycolipids are observed as droplets that sink when grown in a nutrient-limited media under aeration conditions like the one used in the present study.

A multiplatform mass spectrometry-based approach was used for identification and characterization of extracellular lipids produced by yeast R. babjevae UCDFST 04-877. Figure 1 summarizes the workflow integrating the analysis of native molecules and the detailed characterization of hydrolyzed glycolipid precursors with the interpretation of mass spectra by in-silico software for structure elucidation. As discussed in subsequent sections, combining information obtained from both liquid chromatography–mass spectrometry (LC-MS) and gas chromatography–mass spectrometry (GC-MS) was essential for this task.

Structural Elucidation of Glycolipids by Chemical Analysis. The isolated extracts of glycolipids were separated using reversed-phase liquid chromatography (RPLC) on a C18 column followed by mass spectrometric (MS) detection in positive electrospray ionization (ESI) mode. Several features in the mass range of m/z 592–734 (as ammonium adducts) were found in the chromatogram (Figure 2). For some of them, calculated elemental compositions matched those of published sophorolipid species.21 However, follow-up analysis of a commercially available standard of sophorolipids containing dominantly 17-1′-[(2′-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-cis-9-octadecenoic acid 1′,4″-lactone 6′,6″-diacetate (>70% purity) showed that this glycolipid

Figure 1. General workflow of glycolipid analysis. (FA, fatty acid; FAME, fatty acid methyl ester; GC-EIMS, gas chromatography–electron ionization mass spectrometry; HILIC-ESI(−)MS, hydrophilic interaction chromatography–negative electrospray ionization mass spectrometry; HMDB, Human Metabolome Database; LLE, liquid–liquid extraction; MTPA, (R)-(-)-α-methoxy-α-trifluoromethylphenylacetyl derivatives; RPLC-ESI(+)MS/MS, reversed-phase liquid chromatography–positive electrospray ionization–tandem mass spectrometry; TFA, trifluoroacetyl derivatives; TMS, trimethylsilyl derivatives).

Figure 2. Overlay of RPLC-ESI(+)MS extracted ion chromatograms of extracellular lipids isolated from Rhodotorula babjevae UCDFST 04-877. For masses and peak annotations see Table 1.
detected as [M + NH₄]⁺ and [M + Na]⁺ molecular species had a retention time of 1.48 min, while the unknown glycolipid with the same elemental composition (C₃₄H₅₆O₁₄) and detected as the same molecular species eluted later at 3.16 min (Supplemental Figure S1). Additional MS/MS analysis confirmed also differences in MS/MS spectra of these compounds (Supplemental Figure S2). Because most of the elemental compositions obtained by the analysis of these native glycolipids did not provide satisfactory results in databases such as PubChem,²² SciFinder,²³ or ChemSpider,²⁴ further experiments were conducted in order to determine the structure of these extracellular lipids.

Typical glycolipids consist of both lipophilic (e.g., fatty acid) and hydrophilic (e.g., sugar) constituents.²⁵ Individual substructural components of glycolipids must be characterized by hydrolysis, under either acidic, alkaline, or enzymatic conditions.²⁶ Hydrolysis using HCl and NaOH was used, both in methanol. The hydrolysis of sophorolipids consisting mainly of 17-L-[(2′-O-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy]-cis-9-octadecenoic acid 1′,4″-lactone 6′,6″-diacetate was used as a positive control, which correctly provides 17-hydroxyoctadecenoic acid and glucose after hydrolysis treatment. This standard was used for quality control to ensure that the hydrolysis and derivatization reactions were working properly.

The hydrolysis was conducted at 55 °C for 2 h using (i) 1 M HCl in methanol and (ii) 1 M NaOH in methanol. The hydrolysates were neutralized for initial untargeted screening. A small volume of the extract was further diluted with acetonitrile–water (80:20, v/v) and separated using hydrophilic...
interaction chromatography (HILIC). Accurate mass detection in negative ESI was used because it permits detection of both fatty acids and sugars, typical constituents of glycolipids, including the determination of sum formulas, which is very hard to do in GC-MS. Using this method, both free fatty acids and polar constituents of these glycolipids were annotated (Figure 3). Querying the Metlin27 and HMDB28 databases for the detected accurate masses, the fatty acid constituents were annotated as saturated hydroxy fatty acids with elemental compositions of C_{14}H_{28}O_{3}, C_{16}H_{32}O_{3}, and C_{18}H_{36}O_{3}. The polar constituents were found to be C_{6}H_{12}O_{3} and C_{6}H_{14}O_{3}, indicating sugar alcohols (polyols), rather than the hexoses (C_{6}H_{12}O_{3}) observed in the sophorolipid control.

The position of the hydroxy group in the hydroxy fatty acids could not be identified by LC-MS/MS because current databases, including Metlin, lack MS/MS spectra for these compounds. In addition, the fragment ions of fatty acids are in general less abundant compared to precursor ions, making the identification even more difficult. Similarly, common sugar alcohols provide almost identical MS/MS spectra in LC-MS analyses. When retention time was used for reliable identification of different sugar alcohol standards under HILIC conditions, most sugar alcohols did not separate chromatographically under the conditions used in the present study (Supplemental Figure S3). Gas chromatography–mass spectrometry (GC-MS) was therefore used, permitting improved peak capacity and availability of more comprehensive electron ionization (EI) libraries (e.g., NIST 14) for compounds such as fatty acids.

The hydrolyzed extracts were submitted to liquid–liquid extraction using hexane in order to isolate hydroxy fatty acid methyl esters. Because the hydrolysis was conducted in methanol solution, it was possible to inject the hexane extracts directly and detect the hydroxy fatty acids as methyl esters. However, for confirmation purposes, follow-up derivatization using N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was conducted for derivatization of the free hydroxy group of particular hydroxy fatty acids. Separation of both types of derivatized extracts was conducted on a GC column with a DB-225ms polar stationary phase. The lipids were unambiguously assigned as hydroxy fatty acids with the hydroxy group in the β-position (C3), interpreting the characteristic fragments m/z 103 and m/z 175 for 3-hydroxy fatty acids detected as methyl esters or 3-trimethylsiloxy methyl esters, respectively (Figure 4). These assignments were confirmed by analyzing authentic standards of 3-hydroxypalmitic and 3-hydroxystearic acids with matching electron ionization spectra and retention times (Supplemental Figure S4).

For analysis of polar constituents, the reaction mixture was neutralized, followed by evaporation of small aliquots, conducting a two-step derivatization (methoximation and silylation) and separation of derivatives on a GC column with a nonpolar stationary phase (Rtx-5Sil MS). A series of authentic standards of sugar alcohols (CS-polyols: xylitol, D- /L-arabitol, ribitol; C6-polyols: D- /L-mannitol, dulcitol, glucitol) were derivatized and analyzed in the same way. Although only silylation is needed for derivatization of sugar alcohols, methoximation was also used because the hydrolysates might contain less volatile matrix components containing a carbonyl group. Indeed, methoximation was needed for derivatization of glucose during analysis of a sophorolipid standard (quality control). Matching retention time and mass spectra confirmed that the glycolipids contain arabitol and mannitol in their structures (Figure 5).

Finally, an enantioselective analysis was performed using GC-MS in order to determine the D- or L-configuration of arabitol and mannitol and the R- or S-configuration of 3-hydroxy fatty acids. For enantioselective analysis of polyols, the chiral GC Chirasil-Dex CB phase was used, which permitted excellent separation of both D- /L-arabitol and D- /L-mannitol as trifluoroacetyl (TFA) derivatives. Analysis of the extracellular lipid hydrolysates confirmed that polyols are D-arabitol and D-mannitol (Figure 6).

Under these conditions, the R- or S-configuration of 3-hydroxy fatty acids did not separate, mainly because they eluted at the upper temperature limit of the GC column (200 °C), where the effect of the chiral phase for separation is negligible. In order to verify the stereoconfiguration, 3-hydroxy fatty acids were converted into methyl esters followed by derivatization of the free 3-hydroxy group using (R)-(−)-α-methoxy-α-trifluoro-
omethylphenylacetyl chloride [(R)-(−)-MTPA-Cl, Mosher’s reagent]. Corresponding MTPA-O-fatty acid methyl esters (Mosher’s esters) were then separated on a nonpolar GC column.29 As Figure 7 shows, extracellular lipid hydrolysates contained all detectable 3-hydroxy fatty acids in the R-configuration. When running R- and S-standards of 3-hydroxy C14:0, the S-diastereomer eluted earlier compared to its R-counterpart, as reported earlier.29,30

Structural Elucidation of Glycolipids by in-Silico Assembly. Similar components of extracellular glycolipids were reported for R. glutinis more than 50 years ago,9 albeit without proving absolute configurations and without detailing the masses of the intact lipids. As stated before, the masses of the intact glycolipids were observed in the initial untargeted RPLC-QTOFMS screen in a mass range of m/z 592–734 (as ammonium adducts, Table 1). Condensing 3-hydroxystearate (or -palmitate) to mannitol or arabitol resulted in masses ranging between 406 and 464 Da. The difference from the observed masses indicated that these glycolipids were acetylated by 4–6 acetyl groups. In all cases, the hydroxy groups of the fatty acids were acetylated, as evidenced by treating 3-acetoxy fatty acid polyol esters with alkaline sodium methoxide, which led to the formation of αβ-unsaturated palmitic and stearic esters via β-elimination of acetic acid (data not shown).

In order to confirm the complete, intact structures of the observed glycolipids, Mass Frontier software was used to evaluate and interpret mass spectral data based on acquired MS/MS spectra and proposed structures.31 Structures were first submitted consisting of acetylated (R)-3-hydroxypalmitic acid and acetylated (R)-3-hydroxypalmitic stearic acid condensed to completely acetylated D-arabitol (Table 1 and Figure 2; peaks 5 and 10). Predicted fragment structures matched accurate masses (<5 ppm) of fragments. This was further confirmed by comparing fragment structures differing by 28 Da (corresponding to C2H4 increase in the fatty acyl chain), containing fatty acid moieties such as m/z 497 (peak 5 in Figure 2) vs m/z 525 (peak 10 in Figure 2) or m/z 237 (peak 5, Figure 2) vs m/z 265 (peak 10, Figure 2), and fragments originating from acetylated D-arabitol, which were identical for both glycolipids (e.g., m/z 141, m/z 201, and m/z 303); see Figure 8.

Less abundant glycolipids (Table 1 and Figure 2; peaks 4 and 9) consisted of acetylated (R)-3-hydroxypalmitic acid or acetylated (R)-3-hydroxystearic acid, each condensed to partially acetylated D-mannitol with one nonacetylated hydroxy group in the sugar alcohol. All possible structures containing one free hydroxy group were probed; however, MS/MS prediction results using Mass Frontier were ambiguous with respect to the exact location of the nonacetylated hydroxy group. In fact, the observed peaks 4 and 9 in Figure 2 might be a mixture of isomers. Fragment structures were observed differing in a 28 Da fatty acid moiety (e.g., m/z 237 vs m/z 265; m/z 467 vs m/z 495; m/z 527 vs m/z 555; m/z 569 vs m/z 597) and fragments originating from partially acetylated mannitol, which were identical for both glycolipids (e.g., m/z 153, m/z 231, m/z 273, m/z 333) (Figure 9).
Combining all information collected, the full structures (3-hydroxy fatty acid, polyol, and degree of acetylation) of all detected glycolipids were completed (Figure 10 and Table 1). Polyol lipids containing 3-hydroxypalmitic acid were the most abundant, followed by (R)-3-hydroxystearic acid and (R)-3-hydroxymyristic acid (detectable as trace amount, see peak 1 in Figure 2). D-Arabitol was the dominating polyol unit in these extracellular lipids (Table 1 and Figure 2).

Summary of the Approaches Used. This study demonstrated the combined use of several chromatography–mass spectrometry-based methods together enabled the complete identification and characterization of 11 extracellular lipids produced by the yeast R. babjevae UCDFST 04-877, including absolute configurations. Use of LC–accurate mass fragmentation analysis was indispensable to obtain knowledge of the intact glycolipids, including final validation of complete structures by software-guided interpretation of the MS/MS spectra. However, LC-MS analysis alone was incapable of providing sufficient information for full elucidation, either with or without hydrolysis of the glycolipids into substructure components. GC-MS with various derivatization methods showed superior ability to distinguish sugar alcohol isomers, readily identified the hydroxy fatty acids, and proved to be required for defining absolute configuration analysis. This workflow shows the importance of combining different mass spectrometry-based methods together with structure elucidation using in-silico fragmentation software programs in order to determine structures of intact glycolipids.

### Experimental Section

**General Experimental Procedures.** LC-MS-grade solvents, mobile-phase modifiers, derivatives, and other reagents were obtained from Fisher Scientific, Hampton, NH, USA (water, acetonitrile, methanol), and Sigma-Aldrich/Fluka, St. Louis, MO, USA (2-propanol, hexane, dichloromethane, methyl tert-butyl ether, formic acid, ammonium formate, N-methyl-N-(trimethylsilyl)-trifluoroaceticamide, methoxyamine hydrochloride, trifluoroacetic anhydride, (R)-(-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride, K₂CO₃, pyridine, hydrochloric acid, sodium hydroxide). Standards of d-mannitol, xylitol, D-(+)-arabitol, L-(−)-ribitol, α-mannitol, and glycine were from Sigma-Aldrich, while 3-hydroxytetradecanoic acid was from Matreya LLC, State College, PA, USA. (S)-3-Hydroxymyristic acid and (R)-3-hydroxymyristic acid were purchased from Carbosynth (Compton, UK).

**Extracellular Lipids.** The oleaginous yeast *Rhodotorula babjevae* UCDFST 04-877 was obtained from the Phaff Yeast Culture Collection (UCDFST), University of California Davis, and its identity was confirmed by ITS and 26S ribosomal sequencing as described previously. The GenBank accession numbers are KR149271 and KU609429, respectively.

### Table 1. Identified Extracellular Lipids (Polyol Esters of Fatty Acids) Secreted by Rhodotorula babjevae UCDFST 04-877

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<th>Peak number</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; (min)</th>
<th>m/z [M + NH₄]&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Elemental composition</th>
<th>Acetylated (R)-3-hydroxy fatty acid</th>
<th>α-polyl</th>
<th>Degree of acetylation of polyol</th>
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*Peak numbers refer to the numbering in Figure 2.*
temperature, 200 °C; drying gas (nitrogen), 14 L/min; nebulizer gas (nitrogen), 35 psi; sheath gas temperature, 350 °C; sheath gas flow (nitrogen), 11 L/min; acquisition rate MS+, 10 spectra/s; acquisition rate MS/MS, 13 spectra/s; total cycle time, 0.508 s; number of precursor ions per cycle, 4; mass range for selection of precursor ions, m/z 500–1200. The instrument was tuned using an Agilent tune mix (mass resolving power ~20 000 fwhm). A reference solution (m/z 121.0509, m/z 922.0098) was used to correct small mass drifts during the acquisition. For the data processing, MassHunter Qualitative B.05.00 and Quantitative B.05.01 Analysis (Agilent) software programs were used.

Untargeted Analysis of Hydrolyzed Samples Using HILIC-MS. (1) Sample preparation: Samples (1 mg) were hydrolyzed in a 1.5 mL Eppendorf tube with (i) 0.3 mL of 1 M HCl in methanol at 55 °C for 2 h and (ii) 0.3 mL of 1 M NaOH in methanol at 55 °C for 2 h. After cooling to room temperature, both extracts were neutralized to pH 7. An aliquot of 10 μL was added to 1990 μL of an acetonitrile–water (80:20, v/v) mixture. After brief vortexing and centrifugation.

Figure 8. Predicted fragment structures of polyol esters of fatty acids based on MS/MS spectra acquired in ESI(+): (A) acetylated 3-hydroxypalmitic acid condensed with completely acetylated arabitol (C_{31}H_{52}O_{12}; peak 5 in Figure 2); (B) acetylated 3-hydroxystearic acid condensed with completely acetylated arabitol (C_{33}H_{56}O_{12}; peak 10 in Figure 2).
(13 400 rcf for 2 min), a 100 μL aliquot was transferred to a glass vial and submitted to HILIC-MS analysis. The reagent blanks were run in the same manner. (2) HILIC-MS analysis: The system consisted of an Agilent 1290 Infinity LC system (Agilent Technologies) with a pump (G4220A), a column oven (G1316C), an autosampler (G4226A), and an Agilent 6550 QTOFMS. Extracts were separated on an Acquity UPLC BEH Amide column (150 × 2.1 mm; 1.7 μm) coupled to an Acquity UPLC BEH Amide VanGuard precolumn (5 × 2.1 mm; 1.7 μm) (Waters). The column was maintained at 45 °C at a flow rate of 0.4 mL/min. The mobile phases consisted of (A) water with ammonium formate (10 mM) and formic acid (0.125%) and (B) 95:5 (v/v) acetonitrile—water with ammonium formate (10 mM) and formic acid (0.125%). The separation was conducted under the following gradient: 0 min 100% B; 0−2 min 100% B; 2−7.7 min 70% B; 7.7−9.5 min 40% B; 9.5−10.25 min 30% B; 10.25−12.75 min 100% B; 12.75−17.75 min 100% B. A sample volume of 1 μL was used for Figure 9. Predicted fragment structures of polyol esters of fatty acids based on MS/MS spectra acquired in ESI(+): (A) acetylated 3-hydroxypalmitic acid condensed with partially acetylated mannitol (C32H54O13; peak 4 in Figure 2); (B) acetylated 3-hydroxystearic acid condensed with partially acetylated mannitol (C34H58O13; peak 9 in Figure 2).
the injection. Sample temperature was maintained at 4 °C. The QTOFMS instrument was operated in electrospray ionization in negative mode with the following parameters: mass range, m/z 50–1700; capillary voltage, −3 kV; nozzle voltage, −1 kV; gas temperature, 200 °C; drying gas (nitrogen), 14 L/min; nebulizer gas (nitrogen), 35 psi; sheath gas temperature, 350 °C; sheath gas flow (nitrogen), 11 L/min; acquisition rate, 2 spectra/s. For the data processing, the MassHunter Qualitative B.05.00 (Agilent) software program was used.

**GC-MS Analysis of Free Fatty Acids.** (1) Sample preparation: Samples were prepared similarly to those in "Untargeted Analysis of Hydrolyzed Samples Using HILIC-MS". After cooling to room temperature, 800 μL of hexane was added to each tube. The tube was vortexed for 10 s followed by centrifugation at 13 400 rcf for 2 min. An aliquot of 20 μL was evaporated and submitted to derivatization. Pyrline (100 μL) and (R)-(−)-MTPA-CI (10 μL) were added to dry extracts. The mixture was allowed to react at room temperature for 2 h. Then, water (700 μL) and solid K2CO3 (one spatula tip) were added, and the mixture was vortexed for 20 s followed by addition of MTBE (700 μL). The tube was vortexed for 20 s followed by centrifugation at 13 400 rcf for 2 min. An aliquot of 100 μL of the organic phase with methylated hydroxy fatty acids as their MTPA derivatives was evaporated to dryness. The residues were dissolved in 200 μL of MTBE and subjected to GC analysis. The reagent blanks were run in the same manner. Stock solutions of (R)-3-hydroxymyristic acid, (S)-3-hydroxymyristic acid, (R,S)-3-hydroxyoctadecanoic acid (racemate), and (R,S)-3-hydroxyoctadecanoic acid (racemate) were prepared in methanol (1 mg/mL). An aliquot of 10 μL was treated in the same way as yeast glycolipid samples. (2) GC-MS analysis: The system consisted of an Agilent GC-MS system (Agilent Technologies), a 7693 Series autosampler, a split/splitless injector, a 7890A GC system, and a quadrupole mass spectrometer 5977A. Injection parameters were as follows: injection volume, 1 μL; injector temperature, 250 °C; helium carrier gas flow, 1 mL/min; splitless period, 1.5 min. For GC separation a 30 m × 0.25 mm, 0.25 μm DB-225ms (Agilent) capillary column was used with an oven temperature program: 60 °C (0.5 min), 15 °C/min to 135 °C (hold 5 min), 15 °C/min to 235 °C (hold 10 min). MS detection parameters were as follows: acquisition rate, 3.2 scans/s; mass range, m/z 50–500; MS ion source temperature, 230 °C; MS quadrupole temperature, 150 °C; electron multiplier voltage, 7000 V. For the data processing, the MSD ChemStation E.02.00 (Agilent) software program was used.

**GC-MS Analysis of Free Polyols.** (1) Sample preparation: Samples were prepared similarly to those in "Untargeted Analysis of Hydrolyzed Samples Using HILIC-MS". An aliquot of 20 μL of neutralized extracts was transferred to a two-step derivatization. First, 10 μL of 40 mg/mL methoxyamine hydrochloride in pyridine was added to dry extracts followed by shaking at 30 °C for 90 min. Second, 90 μL of MSTFA was added to the extracts followed by shaking at 37 °C for 30 min. The contents were diluted 100-fold with MSTFA, transferred to a glass vial, and submitted to gas chromatography—mass spectrometry (GC-MS) analysis. The reagent blanks were run in the same manner. (2) GC-MS analysis: The system consisted of an Agilent GC-MS system (Agilent Technologies), a 7890A Series autosampler, a split/splitless injector, an 8900N GC system, and a quadrupole mass spectrometer 5977C. Injection parameters were as follows: injection volume, 0.2 μL; injector temperature, 225 °C; helium carrier gas flow, 1 mL/min for 28 min, 4 mL/min for 2 mL/min (hold for 8.5 min); splitless period, 0.5 min. For GC separation, a 30 m × 0.25 mm, 0.25 μm DB-225ms (Agilent) capillary column was used with an oven temperature program: 60 °C (0.5 min), 15 °C/min to 135 °C (hold 5 min), 15 °C/min to 235 °C (hold 10 min). MS detection parameters were as follows: acquisition rate, 3.2 scans/s; mass range, m/z 50–500; MS ion source temperature, 230 °C; MS quadrupole temperature, 150 °C; electron multiplier voltage, 2060 V. For the data processing, the MSD ChemStation E.02.00 (Agilent) software program was used.

**GC-MS Enantioselective Analysis of 3-Hydroxy Fatty Acids.** (1) Sample preparation: Samples were prepared similarly to those in "Untargeted Analysis of Hydrolyzed Samples Using HILIC-MS". After cooling to room temperature, 800 μL of hexane was added to each tube. The tube was vortexed for 10 s followed by centrifugation at 13 400 rcf for 2 min. An aliquot of 20 μL was evaporated and submitted to derivatization. Pyrline (100 μL) and (R)-(−)-MTPA-CI (10 μL) were added to dry extracts. The mixture was allowed to react at room temperature for 2 h. Then, water (700 μL) and solid K2CO3 (one spatula tip) were added, and the mixture was vortexed for 20 s followed by addition of MTBE (700 μL). The tube was vortexed for 20 s followed by centrifugation at 13 400 rcf for 2 min. An aliquot of 100 μL of the organic phase with methylated hydroxy fatty acids as their MTPA derivatives was evaporated to dryness. The residues were dissolved in 200 μL of MTBE and subjected to GC analysis. The reagent blanks were run in the same manner. Stock solutions of (R)-3-hydroxymyristic acid, (S)-3-hydroxymyristic acid, (R,S)-3-hydroxyoctadecanoic acid (racemate), and (R,S)-3-hydroxyoctadecanoic acid (racemate) were prepared in methanol (1 mg/mL). An aliquot of 10 μL was treated in the same way as yeast glycolipid samples. (2) GC-MS analysis: The system consisted of an Agilent GC-MS system (Agilent Technologies), a 7693 Series autosampler, a split/splitless injector, a 7890A GC system, and a quadrupole mass spectrometer 5977A. Injection parameters were as follows: injection volume, 1 μL; injector temperature, 250 °C; helium carrier gas flow, 1 mL/min; splitless period, 1.5 min. For GC separation a 30 m × 0.25 mm, 0.25 μm SLB-5ms (Supelco) capillary column was used with an oven temperature program: 60 °C (1.5 min), 40 °C/min to 180 °C (hold 2 min), 2 °C/min to 220 °C (hold 35 min), 10 °C/min to 250 °C (hold 15 min), 20 °C/min to 300 °C (hold 10 min). MS detection parameters were as follows: acquisition rate, 2.3 scans/s; mass range, m/z 50–600; MS ion source temperature, 230 °C; MS quadrupole temperature, 150 °C; electron multiplier voltage, 1700 V. For the data processing, the MSD ChemStation E.02.00 (Agilent) software program was used.
structure elucidation. MS/MS spectra were submitted in MSP format (text files containing spectra in the NIST MS Search format) and structures in MOL format prepared in ACD/ChemSketch software (Advanced Chemistry Development, Toronto, Ontario, Canada).

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00497.

Supplemental Figures S1–S4: GC-MS and LC-MS chromatograms and mass spectra (PDF)

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**Notes**
The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Multiplatform Mass Spectrometry-Based Approach Identifies Extracellular Glycolipids of the Yeast *Rhodotorula babjevae* UCDFST 04-877

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Supplemental Figure S1. RPLC separation of (A) the sophorolipid 17-L-[(2′-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-cis-9-octadecenoic acid 1’,4″-lactone 6′,6″-diacetate and (B) an unknown glycolipid with the same accurate mass.
Supplemental Figure S2. MS/MS spectra of (A) 17-L-[\(2\prime-O-\beta-D\)-glucopyranosyl-\(\beta-D\)-glucopyranosyl]oxy]-\(cis\)-9-octadecenoic acid 1\(^\prime\),4\(^\prime\)-lactone 6\(^\prime\),6\(^\prime\)-diacetate and (B) an unknown glycolipid. Both compounds had the same precursor ion (\(m/z\) 706.4). Spectra acquired at a collision energy of 20 eV in ESI(+).
Supplemental Figure S3. Separation of common sugar alcohols under HILIC-ESI(−)MS conditions: (A) C5-polyols (m/z 151.0607); (B) C6-polyols (m/z 181.0712). Polyols were detected as deprotonated molecules [M−H]−.
Supplemental Figure S4. GC-MS chromatogram ($m/z$ 175) of 3-hydroxypalmitic acid (16.81 min) and 3-hydroxystearic acid (17.99 min): (A) glycolipid extract after acidic hydrolysis; (B) standard analysis. Derivatized 3-hydroxy fatty acids were detected as 3-trimethylsiloxy methyl esters.