NOVEL METHYL RHAMNOLIPIDS FROM *PSEUDOMONAS AERUGINOSA*

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1. Introduction

A group of rhamnolipids produced by *Pseudomonas aeruginosa* was described in [1–5] to act as biologically active compounds with antibacterial, mycoplasmacidal, and antiviral activities. The lipid moiety of these molecules is characterized by β-hydroxydecanoyl-β-hydroxydecanoic acid bound to L-rhamnopyranose or 2-*O*-α-L-rhamnopyranosyl-L-rhamnopyranose with α-configuration.

We have confirmed and extended the lipid analyses of the cytotoxic rhamnolipids isolated from *Ps. aeruginosa* 158 culture medium. This paper presents evidence for the existence of a substance on their partial acid hydrolysates that migrates faster than β-hydroxydecanoic acid on thin-layer chromatography (TLC) and could be identified as methyl β-hydroxydecanoate. We show that the cytotoxic rhamnolipids are L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid methyl ester and L-rhamnopyranosyl-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid methyl ester.

2. Materials and methods

2.1. *Preparation of rhamnolipids*

Ps. aeruginosa 158 was kindly supplied by Dr F. Lutz, Institute of Pharmacology and Toxicology, Justus-Liebig-University. The organism was grown on Difco trypticase soy broth [6]. $ZnCl_2$ (3.7 M) was added to the culture supernatant fluid up to 75 mM. After the precipitate was collected and dissolved in 0.4 M sodium phosphate buffer (pH 6.5) this solution was dialyzed against Ca^{2+} + Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS, 0.01 M sodium phosphate, 0.15 M NaCl, and 2.7 mM KCl). Solid NaCl was added to the dialysate to 1.5 M final conc. and the mixture was allowed to stand at 4°C. Precipitated material was collected, dissolved in PBS, and dialyzed against the same buffered saline. After addition of 5 vol. chloroform to the dialysate, the chloroform phase was separated and evaporated. The residual material was dissolved in PBS and applied to a Sephadex G-200 column (2.5 × 85 cm) equilibrated with the same buffered saline. The pooled fractions with orcinol- [7] and iodine- [8]positive material eluted from the column were concentrated, and then extracted repeatedly with 5 vol. chloroform as above. Since this chloroform extract contained 2 glycolipids, provisionally named GL-I and GL-II, differing in R_F on TLC, final separation of these glycolipids was done by preparative TLC.

2.2. *Thin-layer chromatography*

Preparative TLC was carried out on precoated silica gel 60 F_{254} plates using solvent system (A), chloroform:methanol:water (60:30:5, by vol.). To establish the purity of each glycolipid, TLC plates of silica gel 60 were used with the following solvent systems: (A); (B), chloroform:methanol:acetic acid:water (60:50:10:4, by vol.); and (C), chloroform:methanol:7 N NH_4OH (60:30:5, by vol.). The products of partial acid hydrolysis were examined by TLC on silica gel 60 and silica gel 60 F_{254} plates in solvent system (D), benzene:acetone:acetic acid (90:50:1, by vol.).

2.3. *Methanolysis and partial acid hydrolysis experiments*

After the glycolipids (100 μg) were methanolyzed by treatment with 5% (w/v) methanol–HCl at 100°C for 3 h, the hydrolysates were quantitatively analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) as their trimethylsilyl (TMS) ether derivatives. A glycolipid sample (250 μg)

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was suspended in 1 ml 2 N HCl and heated at 100°C. An aliquot of the hydrolysate was withdrawn at the time indicated. Fatty acids were extracted with hexane and analyzed by TLC and GC–MS.

2.4. *GC and GC–MS analysis*

GC was performed on a 30 m capillary column coated with OV-1, programmed at 2°C/min from 130–220°C for analyses of TMS–ethers. Mass spectra of TMS-derivatives were obtained on Shimadzu Auto GC–MS 6020 interfaced with 0.3% OV-1 column (0.5 m × 3 mm) or 1.0% OV-1 column (2 m × 3 mm), operated at ion source temperature of 270°C, ionizing potential of 70 eV and accelating voltage of 3.5 kV for electron impact (EI) analysis and at an ion source temperature of 290°C, ionizing potential of 100 eV and accelating voltage of 3.5 kV using NH_3 gas for chemical ionization (CI) analysis.

3. Results and discussion

The complete separation of cytolytic glycolipids, GL-I and GL-II, was performed by TLC (fig.1). With solvent system (A), when both glycolipids GL-I and GL-II were examined by TLC with 3 different pH solvent systems, (A), (B) and (C), individual glycolipids appeared as one discrete spot after the visualization for carbohydrate (fig.1(2,3)). The yields of the highly purified GL-I and GL-II were ~88 mg/l and ~70 mg/l culture medium, respectively. These glycolipids produced crystals in the form of rectangular plates by acidifying the 0.01 M sodium phosphate solution of the glycolipids with 0.05 N HCl. They did not show appreciable differences in their infrared spectra with absorptions assigned to aliphatic hydrocarbon at 2950–2850 cm^{-1} and hydroxy groups at ~3400 cm^{-1}. Both glycolipids had the band characteristic of the ester bond with absorption at 1730 cm^{-1}. The presence of amide and phosphate groups was excluded by the absence of absorption at 1650 cm^{-1} and 1550 cm^{-1}, and 1240 cm^{-1}, respectively. The signal at 840 cm^{-1} showed that the anomeric configuration of the oligosaccharide moiety may be α. Gas chromatograms (fig.2) of TMS-derivatives of methanol–HCl hydrolysates from GL-I and GL-II revealed 5 peaks (I–V) having retention times of 9.2 (I), 10.6 (II), 10.7 (III), 12.1 (IV), and 12.5 (V) min. The retention time of peak I was equal to that of the TMS-derivative of erythritol or methyl β-hydroxydecanoate. Accordingly, further analysis of this peak I by GC–MS was done. Peak I was shown to correspond to the TMS-derivative of methyl β-hydroxydecanoate by comparison of mass spectrum with that of authentic compound, containing prominent mass ions at *m/e* 259 (M-15), 201 and 175. When authentic rhamnose derivatized in an identical fashion was subjected to GC, 4 peaks from II–V were obtained as in [9]. In addition, inspection of the mass spectrum of peak II suggested that this peak corresponded to the TMS-derivative of methanolyzed rhamnose, with prominent mass ions at *m/e* 394 (M), 363 (M-31) and 204. Thus, we concluded that both glycolipids consisted of rhamnose and β-hydroxydecanoic acid and/or methyl β-hydroxydecanoate. To examine the lipid sequence of these glycolipids, partial hydrolysis with 2 N HCl was done as above. The hydrolysates were monitored by TLC and a chromatogram of GL-I hydrolysates is shown in

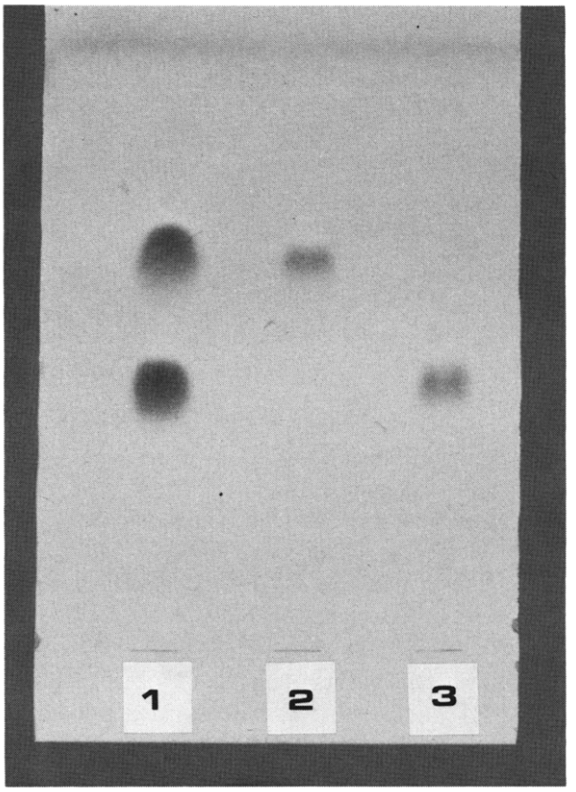

Fig.1. Separation of GL-I and GL-II by TLC. Precoated silica gel 60 plates were developed with solvent system A and visualized with orcinol–H_2SO_4 reagent [7]: (1) chloroform extract after Sephadex G-200 column chromatography; (2) purified GL-I (5 μg); (3) purified GL-II (5 μg).

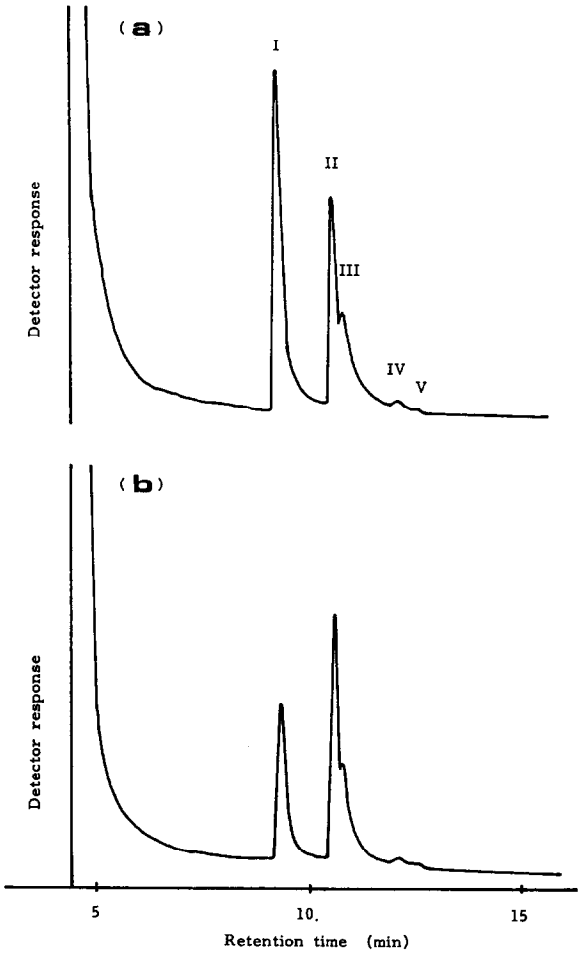

Fig.2. Gas chromatogram of the TMS–methanolysates from GL-I (a) and GL-II (b). Conditions for GC are described in the text.

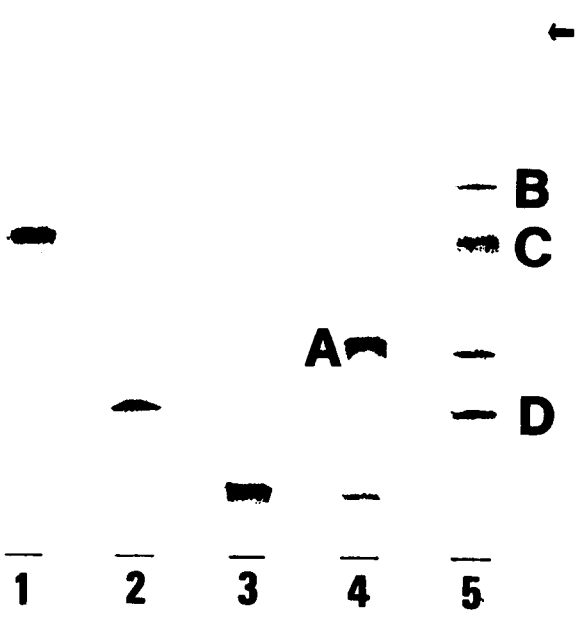

Fig.3. Acid hydrolysis of GL-I with 2 N HCl. A 100 μl aliquot of the reaction mixture was removed at the indicated time, and fatty acids were extracted with hexane. All hexane extracts were spotted on a silica gel plate which was developed with solvent system D. (1) Methyl β-hydroxydecanoate; (2) β-hydroxydecanoic acid; (3) products of 0 min hydrolysis; (4) products of 30 min hydrolysis; (5) products of 2 h hydrolysis. Bands were revealed by spraying with H_2SO_4:ethanol (1:1, v/v). The front (arrow) migrated at a position of 7.2 cm.

fig.3. One major spot (A) migrating faster than intact GL-I appeared at 30 min hydrolysis and became a minor spot after 2 h hydrolysis, at which time another minor spot (B) and 2 major spots (C,D) having mobilities similar to authentic methyl β-hydroxydecanoate and β-hydroxydecanoic acid, respectively, were detectable on TLC. The resulting fraction of the spot A isolated by preparative TLC was converted to the TMS-derivative and was subjected to GC–MS. The gas chromatogram of this TMS–ether revealed one peak with prominent mass ions at *m/e* 429 (M-15), 243, 227, 201 and 143 analyzed by GC–EIMS (fig.4) and a molecular ion (M + 1) gaining a hydrogen ion with an *m/e* of 445 by GC–CIMS. The fatty acid moiety of GL-I is β-hydroxydecanoyl-β-hydroxydecanoic acid methyl ester, but not β-hydroxydecanoyl-β-hydroxydecanoic acid. Furthermore, the hydrolysate of GL-II also revealed the same products as that of GL-I on TLC. These results strongly suggested that the fatty acid moieties of these glycolipids were composed of the same sequence. In addition, quantitative analysis of the constituents of GL-I and GL-II by GC of their TMS–methanolysates revealed that GL-I and GL-II contained 28.8% and 45.9% of rhamnose and 61.2% and 47.4% of the combined amount of β-hydroxydecanoic acid and methyl β-hydroxydecanoate, respectively. Based on these data, GL-I and GL-II were assigned to be L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid methyl ester and L-rhamnopyranosyl-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid methyl ester, respectively (fig.5).

These glycolipids exhibit cytotoxicity to rabbit leukocytes, erythrocytes and Chinese hamster lung cells with detergent-like effects. Work is in progress to elucidate the cytotoxic action of the rhamnolipids on some cell membranes.

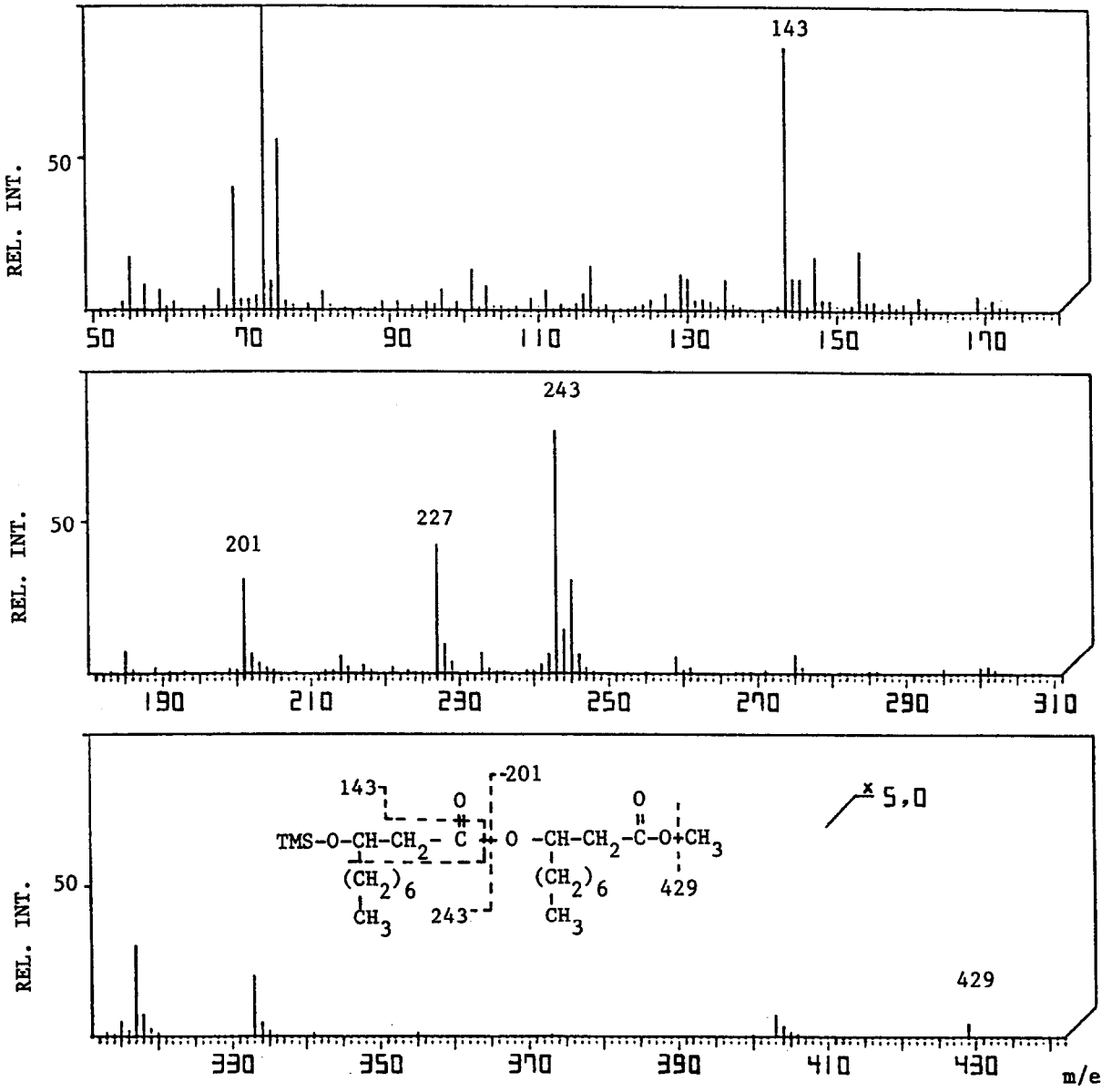

Fig.4. Electron impact/desorption mass spectrum of the TMS ether derivative of the spot A material.

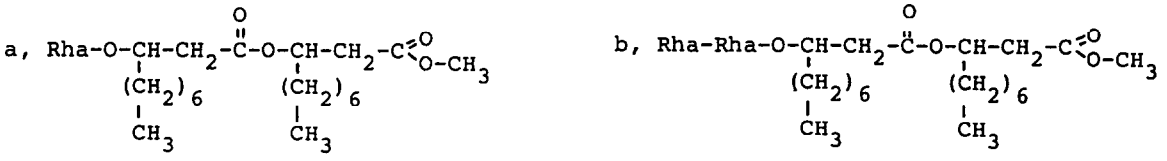

Fig.5. Proposed chemical structures of rhamnolipids from *Ps. aeruginosa*: (a) GL-I; (b) GL-II.

References

[1] Jarvis, F. C. and Johnson, M. J. (1949) J. Am. Chem. Soc. 71, 4124–4126.
[2] Edward, J. R. and Hayashi, J. A. (1965) Arch. Biochem. Biophys. 111, 415–421.
[3] Hisatsuka, K., Nakahara, T., Sano, N. and Yamada, K. (1971) Agric. Biol. Chem. 35, 686–692.
[4] Itoh, S., Honda, H., Tomita, F. and Suzuki, T. (1971) J. Antibiot. 24, 855–859.
[5] Yamaguchi, M., Sato, A. and Yukuyama, A. (1976) Chem. Indust. 4, 741–742.
[6] Scharmann, W. (1976) J. Gen. Microbiol. 93, 283–291.
[7] François, C., Marshall, R. D. and Neuberger, A. (1962) Biochem. J. 83, 335–341.
[8] Skidmore, W. D. and Entenmann, C. (1962) J. Lipid Res. 3, 471–475.
[9] Yamakawa, T., Ueta, N. and Ishizuka, I. (1964) Jap. J. Exptl. Med. 34, 231–240.