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Analysis of the Lipid Moiety of Lipopolysaccharide from *Rhizobium tropici* CIAT899: Identification of 29-Hydroxytriacontanoic Acid

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The lipid moieties of two lipid A's isolated from the phenolic and aqueous fractions of lipopolysaccharide from *Rhizobium tropici* CIAT899 have been studied. Several 3-hydroxy fatty acids and two long-chain hydroxy fatty acids, 27-hydroxyoctacosanoic acid, and 29-hydroxytriacontanoic acid were identified; the ratios of these acids are the same in both lipid A's. These results can be used for chemotaxonomic purposes.

Rhizobia are gram-negative bacteria and are able to form nitrogen-fixing symbiotic relationships with leguminous plants. As gram-negative bacteria, they have the usual surface polysaccharides, and these molecules have been hypothesized to be involved in the molecular mechanism of symbiotic infections. The lipopolysaccharide (LPS) is essential for later symbiotic steps (1, 3, 4, 6, 13, 14).

The LPS are anchored in the outer membrane by their lipid A component, which exhibits a very low structural diversity and is the main endotoxic component. Lipid A generally contains a (1-6)-linked glucosamine disaccharide and different fatty acids. The lipid moiety of lipid A has been analyzed in relation to chemotaxonomic aspects, and 3-hydroxy fatty acids, *cis*-11, 12-methyleneoctadecanoic acids, and a series of ring-opened products have been identified (7, 8, 12, 16, 17, 20). Recently, the identification of 27-hydroxyoctacosanoic acid from members of the family *Rhizobiaceae* (9, 10, 15) and other bacteria (11, 18) was described.

In this communication, we report the structures of the fatty acids found in the lipid A from two LPS (phenolic and aqueous fractions) isolated from *Rhizobium tropici* CIAT899 and the identification of 29-hydroxytriacontanoic acid.

Bacteria were grown for 3 days at 28°C in shaken TY medium containing (per liter) 5 g of tryptone, 3 g of yeast extract, and 0.83 g of $CaCl_2$. The culture was centrifuged, and the pellet was washed three times with 0.5 M NaCl to remove the exopolysaccharide. The LPS was extracted from the bacterial cells by the hot phenol-water method (19), and two fractions (phenolic and aqueous phases) were isolated. Both phases were dialyzed against water, concentrated, and passed through an anionic exchange resin, Amberlite IRA 400 (acetate form) with water as eluent. The eluates were lyophilized and then dissolved in 10 mM $MgSO_4$ and 50 mM Tris-HCl solution (100 ml, pH 7.0). DNase I (1 mg) and RNase A (1 mg) were added, and the solution was stirred overnight at 5°C and then lyophilized (5). LPS fractions were hydrolyzed with 1% acetic acid at 100°C for 90 min, and the lipid A's formed precipitates. The supernatant was extracted with dichloromethane, and the combined organic layers were added to the former precipitate and evaporated. Two lipid A's were obtained, one from LPS in the aqueous phase (LAW) and the other from LPS in the phenolic phase (LAP).

Fatty acids were determined as methyl esters by gas chromatography-mass spectrometry after release by methanolysis with 0.625 M methanolic hydrogen chloride at 80°C for 16 h. The hydroxy fatty acid methyl esters were silylated [pyridine-bis(trimethylsilyl)trifluoroacetamide (1:1), 2 h, at room temperature] and also acetylated (1-methylimidazole–acetic anhydride [1:10]). Unsaturated fatty acids were converted into the dimethyldithio derivatives (2). The methyl esters were treated with iodine, diethylether, and dimethyldisulfide for 24 h at 58°C. $Na_2S_2O_3$ (5% aqueous) was then added, and the derivatives were extracted four times with hexane. Gas chromatography-mass spectrometry was performed with a Carlo Erba model MFC 500 capillary gas chromatograph fitted with a CP-Sil 5 CB WCOT column (25 m by 0.32 mm) attached to a Kratos MS-80RFA. For the methyl esters, methyl ester acetates, and methyl ester dithio derivatives, the temperature program was isothermal at 100°C for 2 min, followed by a 10°C/min gradient up to 280°C, followed by holding at 280°C for 30 min. For the trimethylsilyl derivatives of methyl esters, the isothermal step was at 140°C for 2 min followed by an 8°C/min gradient up to 280°C. The ionization potential was 70 eV.

In the chromatogram of LAW, the following saturated fatty acids, as their methyl esters, were identified: 14-methylpentadecanoic acid (i-16:0), *cis*-11,12-methyleneoctadecanoic acid (19:0 cyc, lactobacillic acid) and 11-methoxynonadecanoic acid (11-OMe-19:0). Three 3-hydroxy fatty acids and two long-chain hydroxy fatty acids were also detected. To identify the hydroxy fatty acids, the corresponding methyl esters were trimethylsilylated and acetylated. 3-Hydroxytetradecanoic acid (3-OH-14:0), 3-hydroxypentadecanoic acid (3-OH-15:0), and 3-hydroxyhexadecanoic acid (3-OH-16:0) were identified. The electron impact mass spectrum of the 27-O-acetyl derivative of the methyl ester of the 27-hydroxyoctacosanoic acid (27-OH-28:0) (Fig. 1) was identical to that previously reported (10). The EI mass spectrum of the 29-O-acetyl derivative of the methyl ester of 29-hydroxytriacontanoic acid (29-OH-30:0) (Fig. 1) showed the expected fragments: *m/z* 464 (M-60, loss of acetic acid), *m/z* 432 (M-92, loss of acetic acid and methanol), and *m/z* 390 (M-134, loss of acetic acid, methanol, and ketene). The spectrum of the trimethylsilyl derivative of the methyl ester of 27-OH-28:0 is shown in Fig. 2. The main fragments

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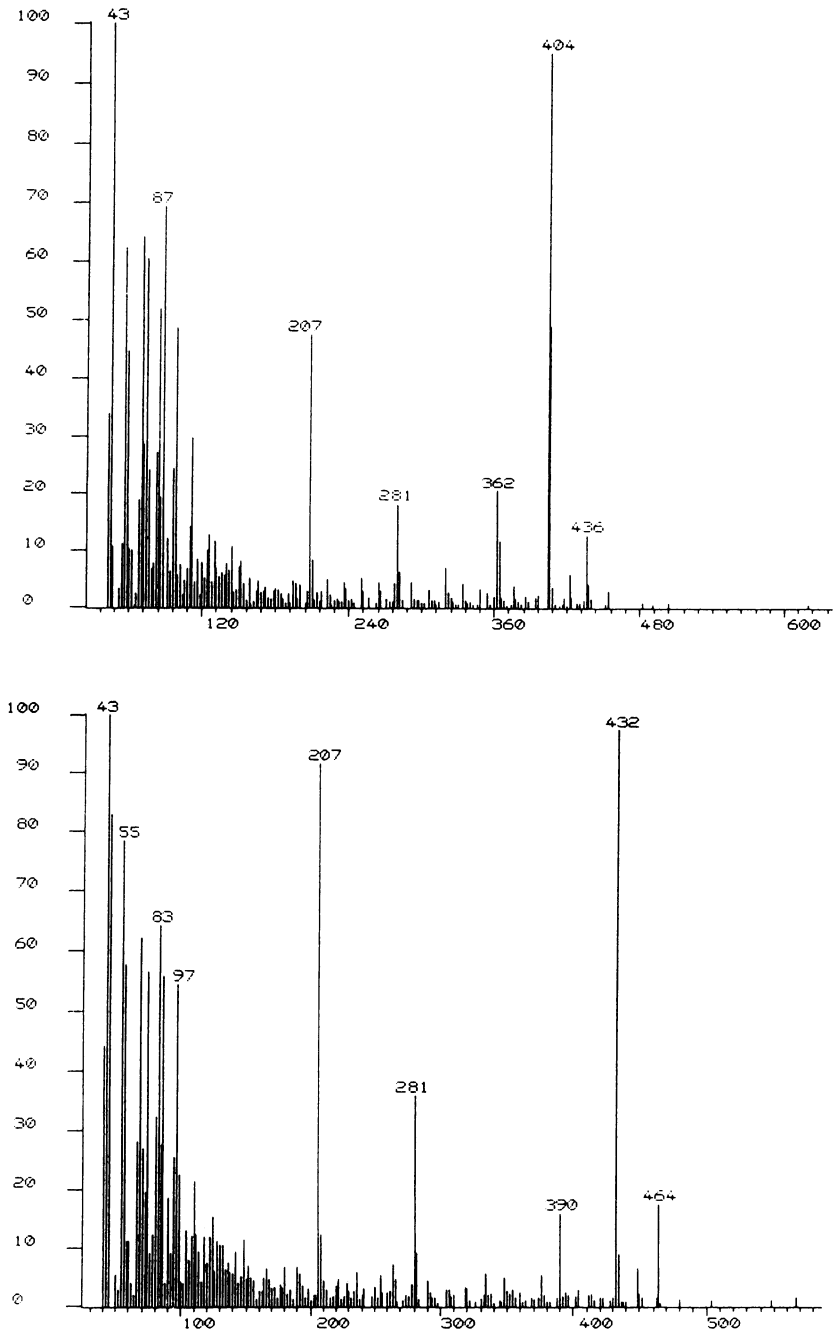

FIG. 1. Mass spectra of the acetylated methyl esters of 27-hydroxyoctacosanoic acid (top) and 29-hydroxytriacontanoic acid (bottom).

were *m/z* 117 ($C_5H_{13}OSi$, from the cleavage between C26 and C27, base peak), *m/z* 511 (M-15, loss of methyl), *m/z* 482 (M-44, loss of carbon dioxide) and *m/z* 479 (M-47, loss of methyl and methanol). The mass spectrum (Fig. 2) of 29-OH-30:0 showed the expected fragments, *m/z* 117, 539, 510, and 508, which correspond to the same losses mentioned above.

For the lipid A isolated from aqueous LPS (LAW), the major components were hydroxy fatty acids, and approximately 54% of the total fatty acid content corresponds to 3-hydroxy fatty acids, accordingly to the data reported (12, 16). A 40% component of the total fatty acids corresponds to the long-chain hydroxy fatty acids. 27-OH-28:0 is the major component, in agreement with the results of Ramadas Bhat et al. (15). 29-OH-30:0, which has been identified in *Agrobacterium* spp. and *Bradyrhizobium* spp. (15), was identified and estimated. These two long-chain hydroxy fatty acids are in a 3:2 ratio. Vaccenic acid was not found, but the related lactobacillic acid and the ring-opened product 11-OMe-19:0 (7, 8) have been identified.

The gas chromatogram of the methyl esters of the fatty acids from LAP allowed the identification of the methyl esters of the following saturated fatty acids: i-16:0, 19:0 cyc, 11-OMe-19:0, 13-methoxynonadecanoic acid (13-OMe-19:0), and 11-methoxy-12-methyloctadecanoic acid (11-OMe-12-Me-18:0). The

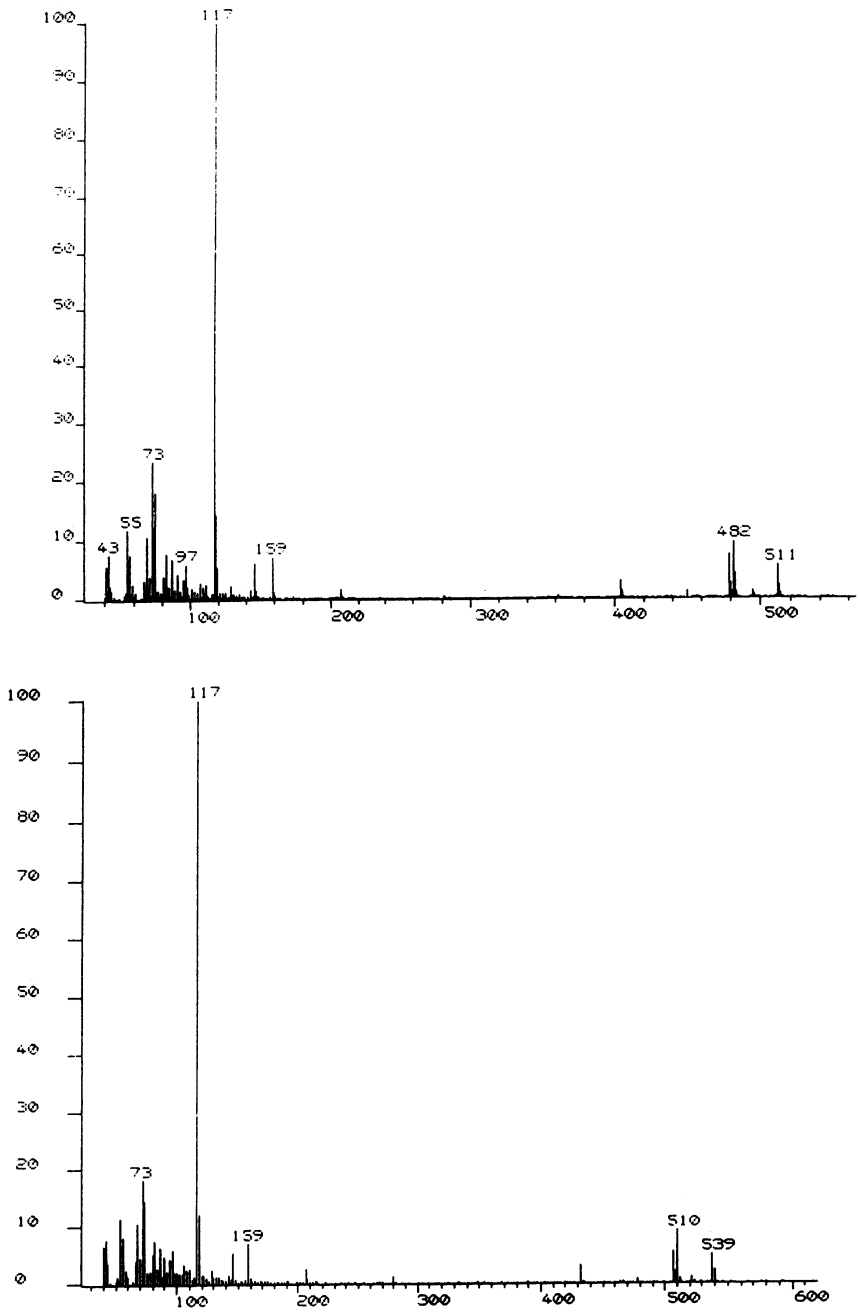

FIG. 2. Mass spectra of the silylated methyl esters of 27-hydroxyoctacosanoic acid (top) and 29-hydroxytriacontanoic acid (bottom).

following hydroxy fatty acids were identified as the O-trimethylsilyl and O-acetyl derivatives of their methyl esters: 3-OH-14:0, 3-OH-15:0, 3-OH-16:0, 3-hydroxyheptadecanoic acid (3-OH-17:0), 3-hydroxyoctadecanoic acid (3-OH-18:0), 27-OH-28:0, and 29-OH-30:0. Two unsaturated fatty acids were also detected. To identify them and to locate the double bond positions, the methyl esters of the corresponding fatty acids were treated with dimethyldisulfide. Octadec-11-enoic acid [vaccenic acid, 18:1(11)] and 11-methyl-octadec-11-enoic acid [11-Me-18:1(11)] were thus identified as dimethyldithio derivatives of their methyl esters.

The analysis of LAP showed an increase of the relative proportion of i-16:0 and a decrease of the relative proportions of 3-hydroxy fatty acids (38%) and long-chain hydroxy fatty acids (18%) compared with those for LAW. In LAP, the 27-OH-28:0 is not the major component, but the ratio of 27-OH-28:0 to 29-OH-30:0 is the same as for the aqueous fraction. Additionally, two 3-hydroxy fatty acids (3-OH-17:0 and 3-OH-18:0) were identified. Vaccenic acid, the biosynthetically related lactobacillic acid, and four ring-opened products, 11-OMe-19:0, 13-OMe-19:0, 11-OMe-12-Me-18:0, and 11-Me-18:1 (11) have been also identified.

Gas chromatography-mass spectrometry results are summarized in Table 1.

TABLE 1. Fatty acid composition of lipid A's from *R. tropici* CIAT899

Fatty acid	% Fatty acid composition of[a]:	
	LAW	LAP
i-16:0	4	10
19:0 cyc	1	10
3-OH-14:0	18	13
3-OH-15:0	19	14
3-OH-16:0	17	8
3-OH-17:0		1
3-OH-18:0		2
11-OMe-19:0	1	11
13-OMe-19:0		1
11-OMe-12-Me-18:0		3
18:1(11)		6
11-Me-18:1(11)		5
27-OH-28:0	24	11
29-OH-30:0	16	7

[a] Calculated as relative percentages of the total peak areas in the methyl ester chromatograms.

In conclusion, the analysis of the lipid moiety of the lipid A obtained from *R. tropici* CIAT899 showed the presence of 29-hydroxytriacontanoic acid, which may be used as chemotaxonomic marker.

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