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Structural characterization of a novel diglycosyl diacylglyceride glycolipid from *Rhizobium trifolii* ANU843

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ABSTRACT

A novel glycolipid was isolated by chloroform–methanol extraction of *Rhizobium trifolii* ANU843 cells. Compositional analysis, methylation studies, ^{1}H NMR and spectroscopies led to the identification of a diglycosyl diacylglyceride: 1,2-di-*O*-acyl-3-*O*-[α-D-glucopyranosyl-(1 → 3)-*O*-α-D-mannopyranosyl]glycerol. Iso-hexadecanoic and anteiso-heptadecanoic acids were the predominant fatty acids esterifying the glyceryl moiety, but a microheterogeneity in fatty acid composition was found, resulting in at least five distinct molecular species of the glycolipid. Although widespread in plants, animals and Gram-positive bacteria, glycosyl glycerides have been seldom reported in Gram-negative bacteria and this work is the first evidence of their occurrence in the bacterial family *Rhizobiaceae*.

INTRODUCTION

Rhizobium leguminosarum biovar *trifolii* (hereafter called *R. trifolii*) is a Gram-negative bacterium that specifically infects root hairs of its white clover host and induces cortical cell-divisions leading to the development of nitrogen-fixing root nodules. The bacterial lipopolysaccharide and a family of acylated chitooligosaccharides have been recently found to modulate critical stages of host-specific interactions between rhizobia and their symbiotic plant hosts in the process of the establishment of the symbiosis[1–3]. Therefore, the glycolipid structures of *Rhizobium* species become a focus of major interest and the present work is the first report of a glycosyl diacylglyceride membrane-constituent in *Rhizobium*. Glycosyl diacylglycerides are widespread components of cell membranes of plants[4–6], animals[7,8], and Gram-positive eubacteria, and archaebacteria[5,6,9], but their occurrence in Gram-negative microorganisms appears rare, since it has been reported

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only for a few species of *Pseudomonads, Bacteroides, Spirochaetes, Mycoplasma* and photosynthetic bacteria[5,9].

MATERIALS AND METHODS

Bacterial culture, cell extraction and purification of BF-7.—Rhizobium trifolii ANU843 cells[10] were grown at 30° in 8 L of BIII broth medium[11] in the presence of 4 μM 4,7′-dihydroxyflavone to induce the pSym nodulation genes[12]. The culture was harvested at a cell density of 8×10^8 cells/mL (late exponential phase) by centrifugation. The cell pellet was washed twice with 10 mM phosphate-buffered saline (pH 7.2) containing 0.5 M NaCl, then with distilled water, and lyophilized. The dry cell residue (1.6 g) was extracted with 1:1 $CHCl_3$–MeOH (125 mL) for 7 h at 60°. The mixture was filtered through a Millipore GVWP04700 (0.22 μm) membrane, the cell-free filtrate (290 mg, dry weight) was redissolved in 19:1 $CHCl_3$–MeOH (5 mL) and applied to a column of silica gel (Silicar, Mallinckrodt, gel bed: 1×30 cm). The column was developed by stepwise elutions with 5, 10, 20, 30, and 50% MeOH in $CHCl_3$ (2×25 mL each), followed by a terminal elution with 100% MeOH (50 mL). Fractions (5 mL) were collected and individually analyzed by TLC on silica gel (solvent *A*, 8:4:1 $CHCl_3$–MeOH–NH_4OH, detection with an orcinol spray). The fractions containing the compound of interest (hereafter called BF-7) were pooled (8.5 mg, dry weight) and the final purification of BF-7 was achieved by successive chromatography on reversed-phase C-18 Sep-Pak (using 3×2 mL stepwise elutions with increasing concentrations of $CHCl_3$ in 3:1 MeOH–H_2O) and silica Sep-Pak (using 3×2 mL stepwise elutions with increasing concentrations of MeOH in $CHCl_3$), followed by reversed-phase C-18 preparative TLC (solvent *B*, 4:3:1 MeOH–$CHCl_3$–H_2O), and silica Sep-Pak chromatography as already described. The compound was eluted from silica Sep-Pak with 8–12% MeOH in $CHCl_3$ and from reversed-phase Sep-Pak with 15–20% $CHCl_3$ in 3:1 MeOH–H_2O. Its R_F value was 0.54 on silica TLC (solvent *A*) and 0.43 on reversed-phase TLC (solvent *B*). A final yield of 1.3 mg of pure compound was obtained.

Compositional analysis.—A sample of BF-7 (200 μg) was treated with $NaBD_4$ (10 mg/mL, 7 h, 25°), methanolysed (1 M MeOH–HCl, 5 h, 80°) and peracetylated (1:1 Ac_2O–pyridine, 15 h, 25°). Preparation of alditol acetates from BF-7 with special attention devoted to the recovery of the glycerol derivative was performed as follows. The compound (200 μg) was hydrolyzed (2 M CF_3CO_2H, 3 h, 110°), reduced with $NaBD_4$, and peracetylated. The acetylation mixture was mixed with 2 mL of 2 M NaCl, the solution acidified to pH 3.0 with 3 M HCl, and extracted with 2 mL EtOAc. The organic phase was washed twice with 2 M NaCl and dried by evaporation under a gentle stream of N_2 at room temperature. The preparation of partially methylated alditol acetates was performed on 200 μg of BF-7 according to a standard procedure[13]; however, the prereduction of the glycoconjugate and reduction of glycosyluronic carboxyl groups involved in this procedure were irrele-

vant in this case, and were thus omitted. The fatty acids from BF-7 were obtained as methyl esters by $CHCl_3$ extraction of a methanolysis (1 M MeOH–HCl, 2 h, 80°) mixture of the sample (100 μg).

The carbohydrate and fatty acid derivatives obtained in the foregoing experiments were identified by GLC–MS analysis by matching both their retention times and mass spectra to those of authentic standards, and were quantified by FID–GLC. The GLC–MS analyses were carried out on a Hewlett–Packard 5995C instrument equipped with a Supelco SP-2330 column for the methyl glycosides, alditol acetates, and partially methylated alditol acetates, or a JNW DB-1 column for fatty acid methyl esters. The FID–GLC analyses were performed on a Varian model 3740 gas chromatograph, with the same columns.

Spectroscopic methods.—^{1}H NMR analyses were performed on a Varian VXR500 spectrometer (500 MHz for protons) at 25°, in CD_3OD for the native compound and in $CDCl_3$ for its peracetylated derivative (obtained by treatment of BF-7 with 1:1 Ac_2O–pyridine for 18 h at 25°). The chemical shifts are expressed in ppm downfield from an external standard of Me_4Si and actually measured by reference to internal $CDCl_3$ (δ 7.24 ppm). FABMS was conducted in a Jeol HX-110 instrument with 1-thioglycerol as the matrix and an accelerating voltage of 10 kV. The FTIR spectrum of the native product was obtained on a Nicolet 710 spectrophotometer, using $CHCl_3$ as solvent. The digalactosyl diacylglyceride standard was obtained from Serdary Research Laboratories Inc. (London, ON, Canada) and peracetylated before ^{1}H NMR analysis as for BF-7.

RESULTS AND DISCUSSION

The ^{1}H NMR spectrum of the purified BF-7 compound (Fig. 1A) exhibited characteristic resonances of both fatty acid chains (δ 0.6–2.3 ppm) and a simple pattern of sharp-lined carbohydrate signals (δ 3.1–5.1 ppm), and was compatible with a lipooligosaccharide-type structure. After successive treatment with sodium borodeuteride, methanolysis and peracetylation, BF-7 yielded two methyl glycoside peracetate derivatives, corresponding to mannose and glucose in a 1:1 ratio, but no alditol acetate, indicating glycosylation of the reducing sugar of the oligosaccharide moiety. The native compound was hydrolyzed, reduced with sodium borodeuteride and peracetylated; careful processing of the peracetylation mixture was designed to optimize the recovery of the most volatile compounds (see Material and Methods section). GLC–MS analysis of the derivatives produced in this experiment confirmed the occurrence of mannose and glucose residues, detected here as their (C-1 monodeuterated) alditol acetate derivatives, and revealed the additional presence of glycerol triacetate. The identity of this latter component was ascertained by matching both its GLC retention time and mass spectrum with those of an authentic standard. The FID–GLC analysis of the same mixture established the relative ratios of carbohydrate components Man–Glc–Glycerol as 1:1:0.7.

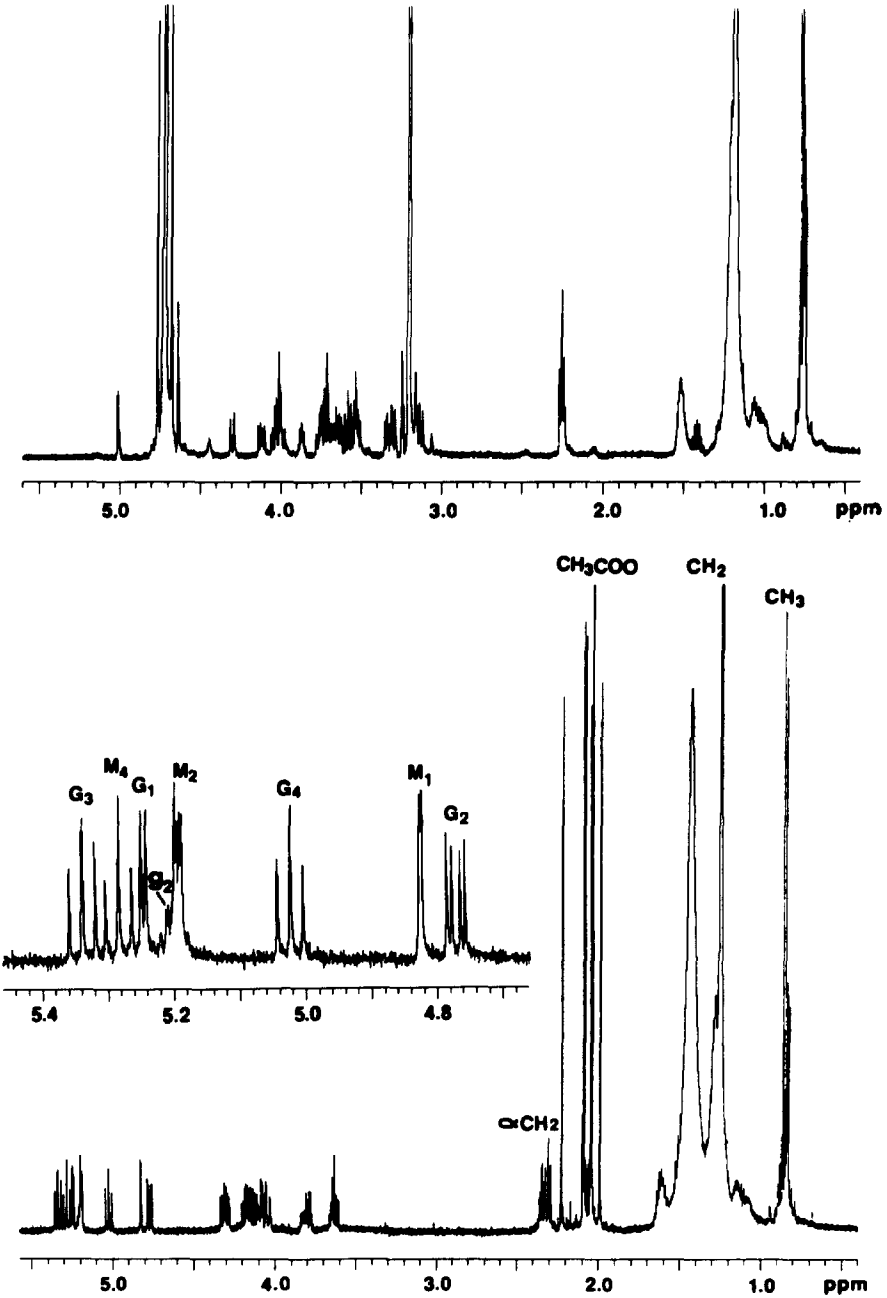

Fig. 1. ^{1}H NMR spectrum of the *R. trifolii* diglycosyl diacylglyceride (BF-7): A, native compound; B, peracetylated derivative.

A microheterogeneity in the composition of BF-7 was revealed by FID–GLC and GLC–MS analyses of the fatty acid methyl esters released by methanolysis of the pure compound. A large predominance of branched fatty acids was observed:

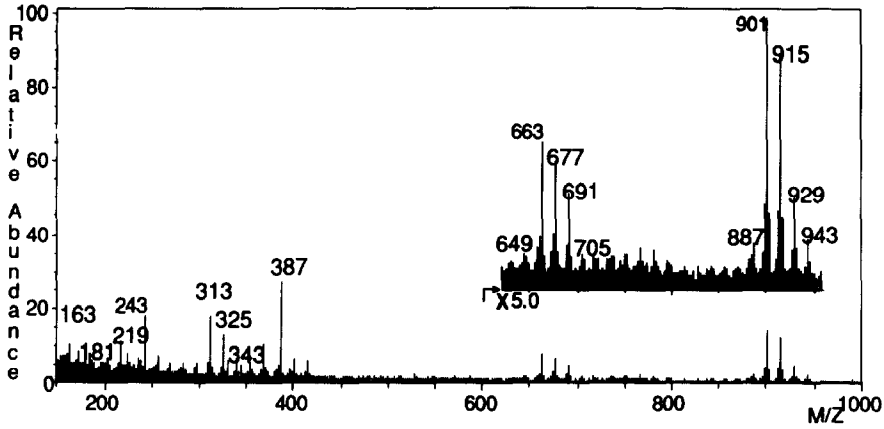

Fig. 2. FABMS of the *R. trifolii* diglycosyl diacylglyceride (BF-7, native form).

i-15:0 (14%), i-16:0 (30%), a-17:0 (42%), together with low levels of 16:0 (8%) and 18:0 (6%) chains. Several lines of evidence indicated that the fatty acids were ester-linked to the glycerol moiety. First, the FTIR spectrum of BF-7 exhibited strong carboxyl ester bands at 1719 and 1733 cm^{-1} and no amide band. Second, the ^1H NMR spectrum of BF-7 did not show any downfield resonance assignable to protons carried by *O*-acylated carbons of sugar residues[14]. Third, the positive mode FABMS spectrum of the native BF-7 (Fig. 2) showed B1- and B2-type fragments[15] at *m/z* 163 and 325, corresponding to unsubstituted monoglycosyloxonium and diglycosyloxonium ions from the reducing end of the oligomer, respectively. The corresponding C1- and C2-type fragments were also found with lower intensities at *m/z* 181 and 343, respectively. This FABMS spectrum of the native compound confirmed the structural microheterogeneity resulting from the occurrence of variable chain-length fatty acids on the molecule. At least five molecular species were revealed through their molecular ions detected as sodium adducts $(M + Na)^+$ at *m/z* 887, 901, 915, 929, and 943. These masses were fully consistent with a diglycosyl diacylglyceride structure existing with at least five distinct fatty acid combinations, described together with their respective frequencies in Table I. A family of ions at *m/z* 649, 663, 677, 691, and 705 (Fig. 2) could be explained by

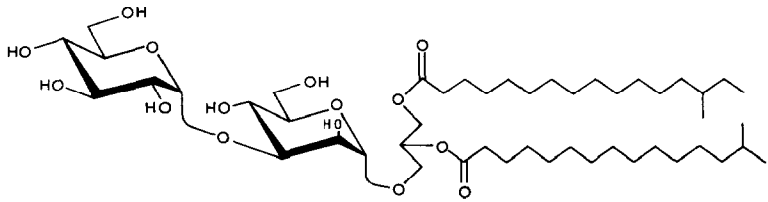

Fig. 3. Structure of BF-7, a diglycosyl diacylglyceride from *R. trifolii*. This structure features arbitrarily the two major fatty acids occurring as esters of the glyceryl moiety (i-16:0, a-17:0).

TABLE I

Frequency of the fatty acid combinations occurring in the BF-7 diglycosyl diacylglyceride structure [a]

Molecular ion $(M+Na)^+$	Combination of fatty acids [b]	Frequency (%)
887	$C_{15}+C_{15}$	6
901	$C_{15}+C_{16}$	39
915	$C_{16}+C_{16}$ or $C_{15}+C_{17}$	34
929	$C_{16}+C_{17}$ or $C_{15}+C_{18}$	14
943	$C_{17}+C_{17}$ or $C_{16}+C_{18}$	7

[a] Based on the relative intensity of the molecular ion of each molecular species in the FABMS spectrum of the native compound. Values are the mean of two independent analyses. [b] Only the chain length is reported here; see text for the actual structures of these fatty acids.

the loss from the molecular-ion adducts of a C_{15}, C_{16}, C_{17}, or C_{18} fatty acid as ketene species by elimination from the carboxyl end. This scheme of fragmentation appears typical of diglycosyl diacyglyceride structures, since an identical pattern was observed on the positive FABMS spectrum of a similar glycoglycerolipid isolated from *Mycobacterium tuberculosis*[16].

GLC–MS analysis of the partially methylated alditol acetates obtained from BF-7 yielded a 2,3,4,6-tetra-*O*-methylhexitol acetate, identified by characteristic primary fragments at *m/z* 118 (57%), 161 (37%), 162 (38%), 205 (21%), and 206 (2%), and a 2,4,6-tri-*O*-methylhexitol acetate defined by primary fragments at *m/z* 118 (100%), 161 (44%), 234 (24%), and 277 (1%)[17,18]. The tetra-*O*-methylated alditol was identified as 2,3,4,6-tetra-*O*-methylglucitol acetate by comparison to the GLC retention time and mass spectrum of an authentic standard (the equivalent derivative of mannose exhibited a clearly different retention time). The tri-*O*-methylated alditol was consequently assigned to 2,4,6-tri-*O*-methylmannitol acetate. These conclusions were further confirmed by the ^{1}H NMR analysis of peracetylated BF-7, which showed a strong downfield shift of the H-2, H-3, and H-4 resonances of the glucose residue (Fig. 1B, Table II) as compared to their chemical shifts in the native molecule. This effect, resulting from the *O*-acetylation of the carbons bearing those protons[14], was also observed for the H-2 and H-4 of the mannose moiety but not for its H-3 proton (Table II), whose corresponding carbon was thus glycosylated since, as shown earlier by FABMS, no substituents were detected on the disaccharide. Based on both methylation analysis and ^{1}H NMR data, a sequence was thus established with the glucosyl residue at the non-reducing end of the disaccharidic unit and glycosylating the mannosyl residue on its C-3 position.

The ^{1}H NMR spectrum of peracylated BF-7 (Fig. 1B) displayed a complex pattern of aliphatic methyl resonances at 0.8–0.9 ppm reflecting the diversity of anteiso-, iso-, and linear fatty acids involved in the glycolipid structure. The integration of the methyl signals from the *O*-acetyl moieties (δ 1.95–2.25 ppm) indicated that seven acetate groups had been added by acetylation of the native

TABLE II

Assignments of ^{1}H NMR resonances of peracetylated BF-7 diglycosyl diacylglyceride

Residue		Chemical shift (ppm)	Coupling constant (Hz)
Glucose	H-1	5.24 (5.01) [a]	$J_{1,2}$ 3.8
	H-2	4.77 (3.29)	$J_{2,3}$ 9.7
	H-3	5.33 (3.57)	$J_{3,4}$ 10.1
	H-4	5.03 (3.13)	$J_{4,5}$ 9.8
	H-5	4.11	
	H-6	nd	
Mannose	H-1	4.82	$J_{1,2}$ 1.8
	H-2	5.19	$J_{2,3}$ 3.7
	H-3	4.17	$J_{3,4}$ 9.9
	H-4	5.28	$J_{4,5}$ 9.7
	H-5	3.83	
	H-6	nd	
Glycerol [b]	H-1a	4.31 (4.30) [c]	$J_{1a,1b}$ 12.2 (11.9) [c] $J_{1a,2}$ 3.8 (3.3)
	H-1b	4.13 (4.13)	$J_{1b,2}$ 6.4 (6.3)
	H-2	5.21 (5.18)	
	H-3a	3.79 (3.78)	$J_{3a,3b}$ 11.4 (10.7)
	H-3b	3.62 (3.64)	$J_{3a,2}$ 4.6 (4.9) $J_{3b,2}$ 5.9 (5.5)
Fatty acids	CH_3	0.8–0.9	
	CH_2	1.1–1.6	
	αCH_2	2.3–2.4	

[a] Values in parentheses for the glucose residue correspond to assignments from the ^{1}H NMR spectrum of the native BF-7 (due to overlapping of signals, the resonances of the mannose protons could not be unambigously ascribed in the spectrum of the native compound). [b] The glycerol protons are not stereospecifically numbered. [c] Values in parentheses for the glycerol residue correspond to homologous assignments obtained from the ^{1}H NMR spectrum of a peracetylated 1,2-di-*O*-acyl-3-*O*-[α-D-galactopyranosyl-(1 → 6)-*O*-β-D-galactopyranosyl]-*sn*-glycerol standard. nd: not determined.

compound. Two sequences of resonances, corresponding to protons of the glucose and the mannose residues, respectively, were identified based on selective homonuclear decoupling experiments and measurement of the coupling constants between adjacent protons (Table II). The two sets of the $J_{H\text{-}2,H\text{-}3}$, $J_{H\text{-}3,H\text{-}4}$, and $J_{H\text{-}4,H\text{-}5}$ coupling constants: 9.7, 10.1, 9.8 Hz (H-2*a*, H-3*a*, H-4*a*) and 3.7, 9.9, 9.7 Hz (H-2*e*, H-3*a*, H-4*a*) were characteristic of pyranosidic rings in their $^4C_1$

conformation of glucose and mannose, respectively[19]. Both the downfield chemical shift of the glucose anomeric doublet (5.01 ppm for the native compound, 5.24 ppm for the peracetylated derivative), and the low $J_{H\text{-}1,H\text{-}2}$ coupling constant (3.8 Hz) indicated the α-anomeric configuration of this sugar residue[19]. The relatively large $J_{H\text{-}1,H\text{-}2}$ coupling constant measured from the H-1 doublet (1.8 Hz) was indicative of an α-anomeric configuration (H-1*e*–H-2*e* interaction) for the mannose residue[20]. The position of glycosylation of the glycerol was deduced from the ^{1}H NMR pattern of resonances observed for this moiety. Based on this pattern, an unambiguous distinction can be made between a 1,2-di-*O*-acylglyceryl group, for which the C-1 methylene protons will be deshielded due to the *O*-acylation of this position and resonate clearly downfield as compared to the methylene protons at the C-3 glycosylated position[21,22], and a 1,3-di-*O*-acylglyceryl group for which the two sets of methylene protons, both carried by *O*-acylated carbons, will be close to magnetic equivalency and will yield a broad band of overlapping ^{1}H NMR resonances[21]. The spectral dispersion of the glycerol H-1a, H-1b versus H-3a, H-3b methylene resonances (Table II) was in full agreement with a 1,2-di-*O*-acyl-3-*O*-glycosyl-glycerol structure[21–23]. To further substantiate this conclusion, a comparative ^{1}H NMR study was performed between the peracetylated forms of BF-7 and of an authentic 1,2-di-*O*-acyl-3-*O*-[α-D-galactopyranosyl-(1 → 6)-*O*-β-D-galactopyranosyl]-*sn*-glycerol[24]. The observed identity in both chemical shifts and coupling constants of the glycerol resonances for the two compounds (Table II) allowed the final establishment of BF-7 as a 1,2-di-*O*-acyl-3-*O*-[α-D-glucopyranosyl-(1 → 3)-*O*-α-D-mannopyranosyl]glycerol (the stereochemistry of the glycerol moiety was not verified).

Since glycosyl glyceride structures are widespread in Gram-positive bacteria and up to now scarcely reported in Gram-negative organisms, the issue of culture purity was thoroughly examined. The purity of the *R. trifolii* culture that provided the initial BF-7 preparation was established based on: (*a*) uniform morphology of isolated colonies on BIII streak plates and cellular morphology examined by phase contrast microscopy, (*b*) no trace of bacterial growth when inoculated on trypticase soy agar (*R. trifolii* does not grown on this medium), and (*c*) strong positive reactivity of cells to both polyclonal and monoclonal anti-*R. trifolii* ANU843 antibodies when examined by immunofluorescence microscopy. In addition, BF-7 could be reproducibly isolated from independent culture batches of *R. trifolii* ANU 843 grown as described. This isolated glycolipid features a large predominance of branched fatty acids, which is unusual for a Gram-negative bacterial membrane lipid. However, *R. trifolii* has been previously reported to produce minor levels of branched fatty acids present as ester substituents of the 3-hydroxy-fatty acids in the lipopolysaccharide structures. Preliminary studies indicate that the purified BF-7 glycolipid alters the development of root hairs and cortical cells of the white clover host for *R. trifolii* in a way that resembles the thickening and shortening of roots (Tsr effect) observed at the early stages of the interaction between the bacterial and the plant symbionts.

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