Structural Determination of Bacterial Nodulation Factors Involved in the Rhizobium meliloti-Alfalfa Symbiosis*

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Extracellular signals produced by Rhizobium meliloti are able to induce root hair deformations and nodule organogenesis on alfalfa. The production of these signals is controlled by bacterial nod genes. To enable their isolation in significant amounts, an overproducing strain was constructed. These Nod factors were first extracted by butanol from the culture medium and further purified by reverse-phase high performance liquid chromatography, ion-exchange, and Sephadex LH-20 chromatographies. The structure of the major signal, called NodRm-1, was determined by mass spectrometry, nuclear magnetic resonance, 35S labeling, chemical analysis, and enzymatic degradation, and was shown to be a sulfated and acetylated tetramer of glucosamine namely, \( \beta-D\text{-GlcP}(2,9\text{-hexadecadienoyl}) \cdot (1 \rightarrow 4) \cdot \beta-D\text{-GlcPa}c \cdot (1 \rightarrow 4) \cdot \beta-D\text{-GlcP}a c \cdot (1 \rightarrow 4) \cdot \beta-D\text{-GlcP}a c \cdot (1 \rightarrow 4) \cdot D\text{-GlcP}a c\text{-SO}_4\text{-H}. \)

Another Nod factor (called Ac-NodRm-1) was co-purified and identified as NodRm-1 acetylated on the C-6 of the nonreducing end sugar. NodRm-1 elicits root hair deformation specifically on alfalfa at a concentration less than 10^{-10} M but has no effect on vetch (a heterologous host plant).

Some microorganisms establish parasitic or symbiotic relationships with plants. Gram-negative soil bacteria of the genus Rhizobium are able to form nitrogen-fixing nodules on the roots of leguminous plants (see Ref. 1 for recent review). This partnership is specific: a particular bacterium will invade and form nodules only on some host plants and not others. Thus Rhizobium meliloti can only nodulate Medicago, Melilotus, and Trigonella species. The development of nitrogen-fixing nodules is a complex process including root hair deformation, infection thread formation within root hairs, and nodule organogenesis. Relatively few bacterial-encoded genes are required for the nodule initiation and development. Among these genes, the nodulation (nod) genes (2, 3) are involved in the earliest stages of the infection process. Nod genes are classified into three groups: the common nod genes which are conserved in most Rhizobium species, the specific nod genes that determine the host specificity of nodulation, and the regulatory nod genes nodD1, nodD2, nodD3, and SyrM. Expression of common and specific nod genes is strongly enhanced in the presence of flavonoid compounds that are exuded from the roots of the host plants (4).

Even though the Rhizobium-legumes symbiosis has received a great deal of attention, relatively little is known about the molecular mechanisms of this interaction. Previous studies have shown that common and specific nod genes determine the production of extracellular symbiotic signals involved in the root hair deformation (Had1 phenotype) and exhibiting the same host specificity as the bacterium (5–7).

This paper describes the first isolation of symbiotic signals from the cell-free filtrate of R. meliloti and the determination of their structure. A preliminary report concerning mainly the biological aspects of this work has been published (8).

EXPERIMENTAL PROCEDURES

Analytical Methods

HPLC was carried out on Waters model 510 pumps, a Waters model 600 solvent programmer, and a Shimatzu model SPD-6A variable wavelength detector.

Gas chromatography was performed on a Girdel series 30 (He; 0.7 atm) equipped with a OV1 capillary column (0.32 mm \times 30 m, Spiral France), a Ross injector, and a flame ionization detector.

**Mass Spectrometry**—Mass spectra were recorded on a ZAB-2E reverse geometry instrument by cesium ion-induced desorption (liquid secondary ion mass spectrometry). In positive mode of ionization, the matrix was a 1:1 mixture of m-nitrobenzylalcohol and glycerol, containing 1% of trifluoroacetic acid. Thioglycerol was the matrix for negative chemical ionization. Collision activation dissociation spectra (CAD) were recorded using the B/E-linked scan method. The location of double bonds by remote charge fragmentation was carried out on a ZAB-HS instrument, coupled with capillary GC. The dissociative electron capture of pentafluorobenzyl esters followed by CAD/MIKE spectrometry of the carboxylate ions was performed as described (10).

The resistive gas for negative chemical ionization was methane and the collision gas was helium.

**NMR**—1H NMR was obtained on a Bruker AM-300WB spectrometer (reference: tetramethyl silane; 293 K). A one-dimensional spectrum was recorded by using 35 pulses and a repetition rate of 3 s. Sample was dissolved in CD,OD at a concentration of 4 mg/0.3 ml, and 128 scans were accumulated. Two-dimensional correlation spectroscopy (COSY) was performed using 256\( \times (90^\circ\text{t1})\cdot 90^\circ\text{t2})\)-acquisition(t2) sequences of 128 scans. The spectrum was recorded using a 256\( \times 1024 \) points time domain matrix over 1874 Hz along the t1 and \( t2 \) directions. 13C NMR (1H decoupled) was obtained on a Bruker AC-200 spectrometer using 50 pulses and a repetition rate of 1 s. The sample was dissolved in D,OD at a concentration of 10 mg/3 ml and 14,000 scans were accumulated.

1 The abbreviations used are: Had, hair deformation; NodRm-1, nodulation factor of R. meliloti; Ac-NodRm-1, acetylated NodRm-1; HPLC, high performance liquid chromatography; GC, gas chromatography; GC-MS, gas chromatography combined to mass spectrometry; GC-MS-MS, gas chromatography combined to random wall spectrometry; CAD/MIKE, collision-activated dissociation-mass analyzed ion kinetic energy; LSIMS, liquid secondary ion mass spectrometry; COSY, correlation spectroscopy; GlepNAc, 2-deoxy-2-acetamido glucopyranose.

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Cell Cultures
Both wild type and overproducing Exo EJ355(pGMI149) R. meliloti strains were grown on a liquid minimal medium (11) containing sodium glutamate (1 g/liter), sodium succinate (2 g/liter), a vitamin mixture containing biotin (0.5 mg/liter), inositol (0.5 mg/liter), nicotinic acid (1 mg/liter), calcium pantothenate (1 mg/liter), pyridoxine (1 mg/liter), thiamine (1 mg/liter) and a nod gene inducer (luteolin, 10 μM). Log phase cultures (5 × 10⁶ colony-forming units/ml) were centrifuged and resuspended in a 0.45-μm filter membrane. For the [3S]-labeling experiment, the culture medium (250 ml) was supplemented with 25 μCi of [3S]sodium sulfate.

Nod Factors Purification
10 liters of culture medium were extracted twice with 2 liters of butanol. The butanol phase was concentrated to dryness, and the residue was treated with 0.3 ml of water and heated at 80 °C for an additional 5 h. The reaction mixture was then applied to a 1 × 20 cm Sephadex LH 20 column (Pharmacia, Sweden) eluted with benzene and previously calibrated with polyethylene glycols. The flow rate was 4 ml/h (Fig. 1b). Further purification was done by HPLC on a DEAE column (4.6 × 150 mm; Spherisorb 5 SAX; Biochrom) with linear gradient (100 mM ammonium acetate in ethanol, at a flow-rate of 1 ml/min (Fig 1c).

Carbohydrate Determination
The active fraction was hydrolyzed by 3 n aqueous hydrochloric acid at 100 °C. After drying under nitrogen, the residue was treated with 1 N hydrogen chloride in anhydrous methanol for 1 h at 80 °C. The methyl glycosides thus obtained were acetylated by an acetic anhydride/pyridine (1:1) mixture for 2 h at 50 °C. The resulting 1-O-Me glycose peracetates were analyzed by GC on an OV1 column. The α- and β-anomers were identified both by coinjection with standards chromatographed from the electrophoretic map spectra obtained by combined GC-MS. GLC/GC-MS was assigned to the D-series by GC analysis of its peracetylated 2-butyl glycosides prepared by using (−)-2-butanol or (+)-2-butanol instead of methanol in the procedure described above. On nonchiral stationary phases, the enantiomeric (+)-2-butyl D-glycosides and (−)-2-butyl L-glycosides are eluted from the peaks derived from the (+)-2-butyl glycosides of D-glucosamine could be assigned as (−)-2-butyl glycosides of L- and D-glucosamine (9). Hydrolysat of NodRm-1 reacted with (−)-2-butanol to afford only (−)-2-butyl D-glycoside as shown by coinjection with authentic compound.

Hydrolysis by Chitinase
Chitinase from Streptomyces griseus(2 units, Sigma, 10 units/mg) was added to a solution of methyl diethylphosphonoacetate (250 mg) in 5 ml of dry tetrahydrofuran was added dropwise to a suspension of sodium hydride (30 mg) in the same solvent (20 ml) under nitrogen at room temperature. After 20 min, a solution of 7Z-tetradecenal (210 mg, Sigma) in 2 ml of tetrahydrofuran was added, and the reaction mixture was stirred for 2 h. After addition of 100 ml of water, the ester was extracted twice with 100 ml of ethyl ether. The organic phase was concentrated to dryness and the residue obtained chromatographed on a silica gel plate. Methyl 2E,9Z-hexadecadienoate was eluted with a 9:1 hexane-ethyl acetate mixture.

Methylation Analysis of NodRm-1
Reduced NodRm-1 was desulfated by mild methanolsysis using 0.05 N HCl in methanol, overnight at room temperature (12). About 1 mg of desulfated and reduced NodRm-1 was permethylated according to the Hakomori procedure (13). Hydrolysis, reduction, and acetylation were performed according to literature (14). The permethylated oligosaccharide was hydrolyzed in 0.5 ml of 5 M sulfuric acid at 80 °C overnight. The reaction mixture was then mixed with 0.3 ml of water and heated at 80 °C for an additional 5 h. The hydrolysate was passed through a AG3X4A column, followed by NaBH₄ reduction and acetylation. Partially methylated alditol acetates were analyzed by GC-electromass impact mass spectrometry.

Decaytation of Ac-NodRm-1
1 mg of the biologically active fraction, containing both NodRm-1 and Ac-NodRm-1, was treated with 1 ml of 0.5 N NaOH in methanol at 40 °C for 1 h. The mixture was acidified by acetic acid and evaporated to dryness. The residue was dissolved in 1 ml of water and applied to a C₁₈ Sep Pnak cartridge. After washing with water, NodRm-1 was eluted with 3 ml of methanol.

Hydrolysis by Carbohydrate
Specific root hair deformation (Had activity) was tested on alfalfa (Medicago sativa) (6) and on vetch (Vicia sativa subsp. nigra) (16). Fractions were serially diluted and a minimum of 10 plants were observed for each dilution.

Results
Isolation of Nod Factors—Purification of the extracellular symbiotic signals NodRm-1 and Ac-NodRm-1 from R. meliloti was followed by the specific root hair deformation on alfalfa (Had activity) (6). Our preliminary attempts to isolate Nod factors from the wild type bacterial strain were unsuccessful. Although Had activity was detected in several chromatographc fractions no significant UV or light scattering detector

Fatty Acid Analysis
Fatty acids were released from nod factors by saponification (KOH 5%, 18 h, 80 °C), acidification, and extraction into diethyl ether. Methyl esters were prepared by reaction with diazomethane. The perfluorobenzyl derivatives were synthesized by dissolving fatty acids (0.1–0.5 mg) in a mixture of dry methanol (20 μl) and acetonitrile (50 μl) followed by the addition of pentafluorobenzyl bromide (2 μl) and diisopropylethylamine (2 μl). After 1 h at room temperature, the reagents were removed by evaporation. The perfluorobenzyl esters were dissolved in hexane (100 μl) and analyzed by negative ion GC-MS as previously described (10).

Chemical Synthesis of 2E,9Z-Hexadecadienoic Acid
A solution of methyl diethylphosphonoacetate (250 mg) in 5 ml of dry tetrahydrofuran was added dropwise to a suspension of sodium hydride (30 mg) in the same solvent (20 ml) under nitrogen at room temperature. After 20 min, a solution of 7Z-tetradecenal (210 mg, Sigma) in 2 ml of tetrahydrofuran was added, and the reaction mixture was stirred for 2 h. After addition of 100 ml of water, the ester was extracted twice with 100 ml of diethyl ether. The organic phase was concentrated to dryness and the residue obtained chromatographed on a silica gel plate. Methyl 2E,9Z-hexadecadienoate was eluted with a 9:1 hexane-ethyl acetate mixture.

1H NMR (CDCl₃) −0.87 ppm (t, 3H); 1.2–1.5 ppm (m, 14H); 2.00 ppm (m, 4H); 2.18 ppm (m, 2H); 3.71 ppm (m, 3H); 5.33 ppm (m, 2H); 5.80 ppm (d, 1H, J = 15.6 Hz) and 6.96 ppm (dd, 1H, J = 7.0 and 15.6 Hz).

13C NMR (CDCl₃) − δ ppm (14.1) (C6); 22.7 (C15); 27.1 (C5); 27.3 (C6); 28.0 (C7); 28.8 (C12); 29.1 (C13); 29.5 (C8); 29.8 (C7); 31.8 (C14); 32.3 (C4); 51.4 (OHCl); 120.9 (C9); 129.5 (C9); 130.3 (C10); 149.8 (C3); 167.0 (C1).

Accurate mass measurement of the methyl ester by electron impact mass spectrometry, M+ at m/z = 266 226.4. Calculated for C₁₇H₃₀O₂ 266.2244. infrared spectrum, νC=O = 1720 cm⁻¹.
responses could be observed. Thus, an overproducing strain was constructed by genetic engineering (8). This Exo-EJ355(pGMI149) strain did not produce acidic exopolysaccharides which can interfere with the isolation of Nod factors. The pGMI149 plasmid carries the common and specific nod genes, as well as three regulatory genes, nodD1, nodD3, and syrM cloned in an Inc-P1 vector present at 5-10 copies/cell (1, 17, 18).

The filtered culture medium from this strain exhibited a strong HAD activity which was fully recovered in a butanol extract but remained water-soluble on ethyl acetate extraction. The butanol-soluble and ethyl acetate-insoluble extract from a 1-liter culture was first fractionated on a preparative C18 reverse-phase column using a water-ethanol gradient (Fig. 1a). The activity corresponded to a wide UV-absorbing peak (220 nm). The pooled active fractions were submitted to gel permeation on a Sephadex LH-20 column in ethanol. Activity was recovered in a broad peak, eluting with a molecular weight of 1100-1500 relatively to polyethylene glycol calibration (Fig. 1b). This fraction was further purified on a DEAE-trisacryl column, using an ammonium acetate gradient in ethanol (Fig. 1c). Final purification on an analytical C18 reverse-phase HPLC column resolved Nod factors in two close peaks, both exhibiting HAD activity (Fig. 1d). Chemical and spectroscopic analyses of these two peaks are described below. The direct evidence that UV-absorbing peaks are related to Nod factors was produced by a comparative purification from a culture medium of a R. meliloti mutant where the nodABC operon was inactivated by a nodA::Tn5 insertion. This mutant was unable to synthesize Nod factors. Neither of the two UV-absorbing peaks were detected, nor did the corresponding HPLC fractions exhibited any HAD activity.

**Constituent Analysis of Nod Factors**—Complete acidic hydrolysis (3 N HCl, 3 h, 100 °C), followed by treatment with acidic methanol and acetylation (see “Experimental Procedures”), afforded an anomeric mixture of methyl N-acetylglucosamine (GlcNAc) peracetate as determined by capillary GC. Glucosamine was assigned to the D-series from the GC analysis of its peracetylated (–)-2-butyl glycoside (9) (see “Experimental Procedures”). Sodium borohydride reduction of the Nod factors (mixture of the two HPLC peaks) gave a product which behaved as a single peak on an analytical C18 reverse-phase HPLC. Water-soluble compounds from a mild acidic methanolysis of this reduced compound, followed by a water-methanol chloride partition, were identified as a mixture of 1-O methyl N-acetylglucosamine and N-acetylgalactosaminol. The methyl chloride-soluble compounds were permethylylated and analyzed by electron impact-mass spectrometry. The main characteristic fragments at m/z = 204 and m/z = 365 were attributed respectively to the C3C4 and C2C3 parts of an hexosamine moiety which was N-acetylated by a bis-unsaturated C16 fatty acid (19) (Fig. 2). The structure of this N-acetylated hexosamine was confirmed by chemical derivations. After catalytic hydrogenation followed by complete methanolysis and acetylation both 1-O methyl glucosamine peracetate and methyl palmitate were identified (a small amount of methyl stearate was also detected).

These chemical modifications showed that the two close peaks observed in the final HPLC purification (Fig. 1d) corresponded to α-and β anomers at the reducing end of the oligosaccharide. A similar separation of anomers of GlcNAc oligomers has been previously observed (20). The monosaccharide analysis also clearly established the presence of reducing and nonreducing glucosamines, and a nonreducing glucosamine which was N-acetylated by a bis-unsaturated C16 fatty acid.

In order to avoid any acid-catalyzed isomerization, fatty acids were released from NodRm-1 by alkaline hydrolysis. Their carbon chain length and the number of double bonds were deduced from a GC-electron impact mass spectrometry analysis of their methyl esters (data not shown). A major doubly unsaturated C16:2 fatty acid was identified. However, other saturated and monounsaturated C16:0 and C16:1 fatty acids were also detected as minor compounds. The location of the double bonds in the C16:2 fatty acid was deduced from the CAD/MIKE spectrum (Fig. 3) of its carboxylate anion. This ion was generated from dissociative electron capture ionization of its perfluorobenzylester, after capillary gas chromatography (negative ion GC-MS-MS) as previously described (10). The fragmentation pattern allowed the location of the two double bonds on positions 2 and 9, respectively (Fig. 3). From
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FIG. 2. Characteristic fragments in the electron impact mass spectrum from the pertrimethylsilyl derivative of the 1-O methyl N-acetylated glucosamine obtained by methanolysis of NodRm-1.

right to left are five regularly spaced signals corresponding to the group of five methylenes between the terminal methyl group and the first double bond. The five signals, after the set of three weak ones, indicated the presence of five successive methylene groups between the two double bonds. The intense ion at \( m/z = 83 \) was attributed to the allylic cleavage at the conjugated double bond. To confirm this structure, the 2E,9Z-hexadecadienoic acid was synthesized. The methyl ester of this synthetic compound behaved similarly on capillary GC. Moreover, its carboxylate ion exhibited the same fragmentation pattern in negative CAD/MIKE spectrometry.

Mass Spectrometry—Ionization of the Nod factors by cesium ion-induced desorption (LSIMS) produced a pseudomolecular ion \( \text{M}^+ \text{H}^+ \) at \( m/z = 1103 \) in the positive ion spectrum (Fig. 4a) and a \( \text{M}^+ \text{H}^+ \) ion at \( m/z = 1101 \) in the negative ion spectrum. Another ion of lower intensity was also detected at 42 mass units higher using both modes of ionization. After a mild basic treatment (NaOMe/MeOH 0.5 N, 40 °C, 1 h) of Nod factors, only \( m/z = 1103 \) was observed in the positive mode. Therefore, the ion at 42 mass units higher was attributed to an acetylated form. Because of the large variability

FIG. 3. CAD/MIKE spectrum of the deprotonated molecular anion from the bis-unsaturated C_{16:2} fatty acid isolated by alkaline hydrolysis of Nod factors. The deprotonated molecular ion was generated from electronic capture of pentafluorobenzyl esters in a GC-MS-MS experiment.

FIG. 4. a, positive ions cesium-induced desorption mass spectrum (LSIMS) of purified Nod factors. b, metastable fragmentation (B/E linked scan) of protonated NodRm-1 \( (m/z = 1103) \). c, metastable fragmentation of protonated Ac-NodRm-1 \( (m/z = 1145) \).
between the relative abundance of the acetylated and unacetylated molecular ions according to the origin of the sample, it seems likely that these two ions correspond to two different products and not to a ketene loss (42 mass units) in the gas phase. Moreover, no ketene loss was observed in the positive ion spectrum arising from the decomposition of the protonated molecular ions, respectively, of NodRm-1 and its acetylated form Ac-NodRm-1. These 80 mass unit losses were confirmed by metastable fragmentation (B/E linked scan; Fig. 4b). This suggested a loss of a SO₃ (21). The presence of a sulfate group was further demonstrated by growing the bacteria in a medium containing [³⁵S]sodium sulfate which led to a high incorporation of ³⁵S into Nod factors (8). Other fragment ions were also observed both in the positive ion spectrum (Fig. 4a) and in the collision-induced dissociation of the protonated molecular ions (Fig. 4b). The ions at m/z = 396, 599, and 802, arising from the decomposition of NodRm-1, were attributed to the cleavages of glycosidic bonds from a N-acetylglicosamine sequence: the difference of 203 mass units between these fragments corresponded to GlcNAc residues (22). The fragment ion at m/z = 396 was assigned to the oxonium ion from a glucosamine residue N-acetylated by a C₂₀₂ fatty acid. A similar pattern, shifted up by 42 mass units was observed in the collision-induced dissociation spectrum of the MH⁺ from Ac-NodRm-1 (Fig. 4c). This result allowed the location of the acetyl group on the glucosamine at the nonreducing end. These fragmentation patterns are consistent with a linear glucosaminyl sequence possessing a sulfate function on the reducing sugar, a N-acetylglicosamine at the nonreducing end and three consecutive N-acetylglicosaminie moieties. This structure was confirmed by the negative ion spectra. This spectrum exhibited cleavages at each glycosidic linkage leading to fragment ions at m/z = 300, 503, and 706 for both products. Because of the acidity of the sulfate group, charge retention occurs at the reducing end residue. Ion at m/z = 300 corresponded to a deprotonated and sulfated N-acetylglicosamine and the other ions to successive additions of anhydro N-acetylglicosaminie moieties.

The reducing character of Nod factors was confirmed by positive ion LSIMS of their sodium borohydride reduction products. Thus, NaBD₄ reaction led to both complete reduction of the anomeric carbons and O-deacetylation of Ac-NodRm-1. The mass spectrum of reduced Nod factors showed a shift of three mass units for both the pseudomolecular ion of NodRm-1 and for the fragment corresponding to the loss of SO₃ from this ion, whereas all other fragment ions remained at the same m/z values. Acetylated analogues were no longer detected.

Location of the linkages sites between the glucosamine residues was determined by methylation analysis. The permethylation reaction was carried out on the desulfated and reduced NodRm-1 (see “Experimental Procedure”) (13). The permethylated oligosaccharide analyzed by Fab-MS spectrometry, showed a pseudomolecular ion M⁺Na⁺ at m/z = 1257 (Fig. 5) corresponding to the introduction of 15 methyl groups. Fragment ions corresponding to glycosidic cleavages at m/z = 942, 697, and 452 were also detected. Hydrolysis of the permethylated product, followed by the formation of the alditol acetates (14) afforded three partially methylated N-acetylated glucosaminol acetates which were identified by GC-electron impact mass spectrometry, as O-methylated on positions 1, 2, 3, 6; 2, 3, 6; and 2, 3, 4, 6. These partially methylated and acetylated sugars indicated respectively a 4-

substituted glucosamine at the reducing end, (1, 4) linked glucosamines as intermediary sequence, and a glucosamine at the nonreducing end.

**NMR Analysis—**The one-dimensional ¹H NMR spectrum was realized on the mixture of NodRm-1 and Ac-NodRm-1 (Fig. 6). Sugar ring protons appeared between 3.4–3.9 ppm. Five downfield signals were assigned to protons deshielded by either the acetate or the sulfate function. The three signals at δ = 3.90, 4.07, and 4.26 ppm which remained unchanged after O-deacetylation were assigned respectively to H5, H6a, and H6b of the sugar bearing the sulfate function as determined by the connectivity pattern of the two-dimensional-COSY spectrum (24, 25) (Fig. 7). The two signals at δ = 4.16 and
Fig. 7. One-dimensional and two-dimensional 1H NMR of the 3.30–5.30 ppm region.

4.46 ppm disappeared completely after mild basic treatment. From the connections observed in the two-dimensional-COSY spectrum, they were assigned to the H6α' and H6β' of the acetylated nonreducing end sugar of Ac-NodRm-1. After O-deacetylation, no change was observed in the signals assigned to aliphatic and anomeric protons. Thus, all others assignments are common to NodRm-1 and Ac-NodRm-1. Four olefinic protons were assigned to the two double bonds of the aliphatic chain. Two signals were attributed to an E double bond conjugated with the carbonyl function of the fatty acid amide (δH2' = 5.95 ppm, 3J = 15 Hz, and δH3' = 6.83 ppm, dt, 3J = 7 and 15 Hz). The two magnetically equivalent protons of the internal double bond appeared as a single signal (δH9'- H10' = 5.32 ppm, 2H, t). The fatty acid chain proton assignments were achieved by two-dimensional 1H NMR COSY (Fig. 6). From olefinic protons, successive correlations were observed with allylic methylene protons (δH8',H11' = 2.05 ppm and δH4' = 2.30 ppm), other aliphatic protons (δt = 1.30–1.60 ppm) and terminal methyl protons (δt = 0.90 ppm). Such a connectivity pattern was in agreement with the bis-unsaturated C16 structure of the side chain. The resonances from anomeric protons showed three doublets (δt = 4.50–4.65 ppm, 3J = 8.5 Hz) assigned to three H1 of β-interglycosidic linkages (Fig. 7) and a doublet at δt = 5.05 ppm (3J = 3.4 Hz) attributed to H-1α of the reducing sugar, according to data previously reported on 1H NMR assignment of GlcNAc oligomers (23).

The structure of NodRm-1 was confirmed by the analysis of the fully decoupled 13C NMR spectrum after O-deacetylation (Fig. 8). Chemical shifts of all carbons of the aliphatic side chain were in agreement with the proposed structureconjugated double bond (δC2' = 125 and δC3' = 155 ppm) and internal double bond (δC9' and C10' = 133 ppm). Assignments for the oligosaccharidic carbons were proposed according to a previous report on 13C NMR studies of β(1→4)GlcNAc oligomers (23). Nonreducing sugars were characterized by a single signal due to C1 (δC1β = 103.9 ppm), whereas reducing GlcNAc appeared as a mixture of α- and β-anomers as indicated by two C1 signals (δC1(1α) = 93.1 ppm and δC1(1β) = 97.8 ppm). The linkage modes between the glucosamine residues were deduced from the down-field chemical shift of linked C4 (δC4 = 81.8–82.0 ppm). All the other ring-carbon resonances were in agreement with the literature data except C6 of the sugar bearing the sulfate group which appeared 5 ppm downfield (δC6(1) = 68.7 ppm) as already reported (26).

Enzymatic Hydrolysis by Chitinase—From permethylation analysis and NMR data, NodRm-1 and Ac-NodRm-1 were shown to possess β(1→4) N-acetylglucosamine linkages as chitin. Exochitinase from Streptomyces griseus cleaved sequentially β(1→4)GlcNAc bonds. However, it was known that the replacement of a N-acetyl group by a N-fatty acyl one precludes the corresponding hydrolysis of the glycosidic bond (15). In order to present an additional evidence for the proposed structure, NodRm-1 was submitted to chitinase hydrolysis. The degradation was monitored by reverse-phase HPLC (see "Experimental Procedures"). After 18 h of reaction time, NodRm-1 disappeared completely and two more hydrophobic and UV-absorbing compounds were detected. The positive ion mass spectrum (LISIMS) of these two products showed pseudomolecular ions at m/z = 617 and 820, corresponding respectively to the di- and trisaccharide bearing the nonreducing end of NodRm-1. After 2 days of enzymatic digestion, only the disaccharide could be detected. This result confirmed both the presence of β(1→4)GlcNAc linkages in NodRm-1, the location of the sulfate function on the reducing GlcNAc, and the presence of a N-acyl group on the glucosamine at the nonreducing end.

Biological Activity—Both the purified NodRm-1 and the mixture NodRm-1/Ac-NodRm-1 exhibited specific root hair deformation on alfalfa from 10^{-6} M to 10^{-11} M concentrations. However, all attempts to get pure Ac-NodRm-1 were unsuccessful; thus, we cannot define the contribution of this signal in the effects observed on plants.

DISCUSSION

NodRm-1 and Ac-NodRm-1 are the first structurally identified Rhizobium nodulation factors. Their production is controlled by the nod genes of R. meliloti. A nod A::Tn5 insertion completely suppressed both biological activity of the culture supernatant and Nod factor production. An enzymatic hydrolysis by chitinase also destroyed both the Nod factors and biological effects. We conclude that contaminants are unlikely to account for the observed effects on plants.

From chemical analysis, 35S-labeling, mass spectrometry,
to be a new-found active lipo-oligosaccharide inducing specific bacterial or plant cell walls. These oligosaccharides are isolated by Albersheim and co-workers by degradation of unlikely that the tetraglucosaminyl backbone of Nod factors resistance to infection (32-37). In this way, NodRm-1 seems involved in plant signaling, including growth, development, and root nodule organogenesis even in the absence of bacteria (28). The sulfate group controls the host specificity on alfalfa. Its removal by chemical modification or by inactivation of nodH completely supresses activity on alfalfa. However, the desulfated compound (NodRm-2) is able to induce root hair deformation on vetch (28). Relationships between structure and biological effects are now under consideration and will be published soon. It is also interesting to point out that plants possess a chitinase activity which is elicited as a defense reaction (38). Since NodRm-1 and Ac-NodRm-1 are hydrolyzed by chitinase, a control phenomenon of the nodulation process may act by this way.

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REFERENCES
1. Long, S. R. (1989) Cell 56, 203-214
2. Long, S. R., Buikema, W. J., and Ausubel, F. M. (1982) Nature 298, 485-488
3. Truchet, G., Debélè, F., Vasse, J., Terzaghi, B., Garnerone, A. M., Rosenberg, C., Batut, J., Maillet, F., and Dénarié, J. (1985) J. Bacteriol. 164, 1200-1210
4. Peters, N. K., Frost, J. W., and Long, S. R. (1986) Science 233, 977-980
5. Faucher, C., Maillet, F., Vasse, J., Rosenberg, C., Van Brussel, A. A. N., Truchet, G., and Dénarié, J. (1988) J. Bacteriol. 170, 5489-5499
6. Faucher, C., Camut, S., Dénarié, J., and Truchet, G. (1989) Mol. Plant-Microbe Interact. 2, 291-300
7. Faucher, C., Lerouge, P., Roche, P., Maillet, F., Dénarié, J., Promé, J. C., and Truchet, G. (1989) in Signal Molecules in Plant-Microbe Interactions (Lugtenberg, B. J. J., edl) pp. 379-386, Springer-Verlag, Berlin
8. Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C., and Dénarié, J. (1990) Nature 344, 781-784
9. Gerwig, G. J., Kamerling, J. P., and Vliegenthart, J. F. G. (1979) Carbohydr. Res. 77, 1-7
10. Promé, J. C., Aurelle, H., Couderc, F., and Savagnac, A. (1987) Rapid Commun. Mass. Spectrom. 1, 50-52
11. Vincent, J. M. (1970) A Manual for the Practical Study of Root-Nodule Bacteria, IBP Handbook 15, Blackwell Scientific Publications, Oxford
12. Karlson, K. A., Samuelson, B. E., and Steen, G. O. (1971) J. Membr. Biol. 5, 169-184
13. Hakomori, S. (1964) J. Biochem. (Tokyo) 55, 205-208
14. Stellner, K., Saito, H., and Hakomori, S. (1973) Arch. Biochem. Biophys. 155, 464-472
15. Hirano, S., Saito, N., Yoshida, S., and Kitagawa, S. (1987) Prog. Biotech. 3, 163-176
16. Zaat, S., Van Brussel, A., Tak, T., Fees, E., and Lugtenberg, B. J. J. (1987) J. Bacteriol. 169, 3388-3391
17. Debélè, F., Rosenberg, C., Vasse, J., Maillet, F., Martinez, E., Dénarié, J., and Truchet, G. (1986) J. Bacteriol. 168, 1075-1086
18. Mulligan, J. T., and Long, S. R. (1989) Genetics 122, 7-18
19. Denary, M., Puzo, G., and Asselinenu, J. (1977) Nouv. J. Chimie 2, 373-379
20. Mulligan, K., Linerie, F., Pustilnik, L., and Bush, C. A. (1982) Anal. Biochem. 119, 407-412
21. Dell, A., Rogers, E. M., Thomas-Oates, J., Huckerby, E. T. N., Sanderson, P. N., and Niedzysnki, I. A. (1988) Carbohydr. Res. 179, 7-19
22. Dell, A. (1987) Adv. Carbohydr. Chem. Biochem. 45, 19-72
23. Boyd, J., Porieux, R., and Soffe, N. (1985) Carbohydr. Res. 139, 35-46

Fig. 9. Structures of NodRm-1 and Ac-NodRm-1. The oligosaccharide chain is numbered from the reducing to the nonreducing end. Thus, Cx′ refers to the Cx carbon of the ith saccharide unit. The side chain is numbered from the carbonyl to the terminal methyl group. Thus, Cx′′ refers to the xth carbon of the fatty acyl chain.

NMR, and enzymatic hydrolysis, NodRm-1 and Ac-NodRm-1 were determined to be sulfated and N-acylated glucosamine tetramers, as shown in Fig. 9. The O-acylated derivative Ac-NodRm-1 was copurified with NodRm-1. A nodL gene has been found in R. meliloti and its putative product exhibits homologies both with the Rhizobium leguminosarum bv viciea NodL (27) and with bacterial acetyl transferases. A R. meliloti mutant having a deletion removing the nodL region exhibited a slightly delayed nodulation and produced NodRm-1 but not Ac-NodRm-1. These results indicate that Ac-NodRm-1 is also an active symbiotic signal.

What are the different functions of the other nod genes in the biosynthesis of these nodulation factors? We have recently ascertainment that nodH and nodPOQ are involved in the sulfation of the signals produced by R. meliloti (28). Other specific nod genes, nodFEG, are known to have high homology with genes encoding for acyl carrier protein (29), β-ketoacylase (30), and alcohol dehydrogenase (29). Therefore, these genes could be involved in the fatty acid chain biosynthesis. NodABC are common genes present in all the Rhizobium species and may specify the production of a common structural backbone which is further modified by the products of the specific nod genes to give a host-specific Nod signal. The role of nodFEG genes and the structure of the precursor synthesized through the activity of the nodABC genes are under investigation.

The complete structural analysis of the signals produced by R. meliloti allowed the detection of some minor nod factors possessing acyl side chains differing both in length and in the number of double bonds. This heterogeneity could be explained by a low specificity of a fatty acyl transferase but also by the presence of biosynthetic intermediates of the lipid chain.

In Gram-negative bacteria N-acetylglucosamine is an important constituent of two major components of the cell wall. β-(1→6) linked glucosaminyl moieties are found in the lipid A of lipopolysaccharides (31). N-acetylglucosamine β-(1→4) linked to a N-acetyl muramic acid constitutes the repeated unit of the peptidoglycan. But neither chitin nor oligomers of chitin have been described yet in bacteria. Thus, it seems unlikely that the tetraglucosaminyl backbone of Nod factors comes from the partial degradation of a polymeric precursor. On the contrary, other active oligosaccharides have been isolated by Albersheim and co-workers by degradation of bacterial or plant cell walls. These oligosaccharides are involved in plant signaling, including growth, development, and resistance to infection (32-37). In this way, NodRm-1 seems to be a new-found active lipo-oligosaccharide inducing specific nodule organogenesis on plants.

What is the role of the peculiar structural details in the activity of these Nod factors? NodRm-1 exhibits a specific activity on alfalfa (homologous host) and not on vetch (heterologous host). It elicits root hair deformation at concentrations from 10⁻⁴ to 10⁻¹ M (8) and induces cortical cell divisions and root nodule organogenesis even in the absence of bacteria (28). The sulfate group controls the host specificity on alfalfa. Its removal by chemical modification or by inactivation of nodH completely supresses activity on alfalfa. However, the desulfated compound (NodRm-2) is able to induce root hair deformation on vetch (28). Relationships between structure and biological effects are now under consideration and will be published soon. It is also interesting to point out that plants possess a chitinase activity which is elicited as a defense reaction (38). Since NodRm-1 and Ac-NodRm-1 are hydrolyzed by chitinase, a control phenomenon of the nodulation process may act by this way.

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24. Hounsell, E. F., Feeney, J., Scudder, P., Tang, W. P., and Feizi, T. (1986) *Eur. J. Biochem.* **157**, 375-384
25. Torri, G., Casu, B., Gatti, G., Petitou, M., Choay, J., Jacquinet, J. C., and Sinao, P. (1986) *Biochem. Biophys. Res. Commun.* **128**, 134-140
26. Strecker, G., Wierszczak, J. M., Martel, C., and Montreuil, J. (1989) *Carbohydr. Res.* **185**, 1-13
27. Downie, J. A. (1989) *Mol. Microbiol.* **3**, 1649-1652
28. Roche, P., Lerouge, P., Promé, J. C., Faucher, C., Vasse, J., Maillet, F., De Billy, F., Dénarié, J., and Truchet, G. (1990) in *Advances in Molecular Genetics of Plant-Microbe Interactions* (Hennecke, H., and D. P. S. Verma, D. P. S., eds) Vol. 1, pp. 119-126, Kluwer Academic Publishers, Dordrecht
29. Debelle, P., and Sharma, S. B. (1986) *Nucleic Acids Res.* **14**, 7453-7472
30. Bibb, M. J., Biro, S., Motamedi, H., Collins, J. F., and Hutchinson, C. R. (1989) *EMBO J.* **8**, 2727-2736
31. Urbanik-Szpiewak, T., Seydel, U., Greck, M., Weckesser, J., and Mayer, H. (1989) *Arch. Microbiol.* **152**, 527-532
32. Sharp, J. K., Valent, B., and Albersheim, P. (1984) *J. Biol. Chem.* **259**, 11312-11320
33. Gollin, D. J., Darvill, A. G., and Albersheim, P. (1984) *Biol. Cell* **51**, 275-280
34. Darvill, G., and Albersheim, P. (1984) *Annu Rev. Plant Physiol.* **35**, 243-275
35. York, W. S., Darvill, A. G., and Albersheim, P. (1984) *Plant Physiol.* **75**, 295-297
36. Tran Thanh Van, K., Toubart, P., Cousson, A., Darvill, A. G., Gollin, D. J., Chelf, P., and Albersheim, P. (1985) *Nature* **314**, 615-617
37. McDougall, G. J., and Fry, S. C. (1988) *Planta* **175**, 412-416
38. LeGrand, M., Kauffmann, S., Geoffroy, P., and Fritig, B. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6750-6754