Nodulation Factors from *Rhizobium tropici* Are Sulfated or Nonsulfated Chitopentasaccharides Containing an *N*-Methyl-*N*-acylglucosaminyl Terminus†

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ABSTRACT: *Phaseolus vulgaris* (common bean) can be nodulated by several *Rhizobium* species. Among them, *Rhizobium tropici* has a relatively broad host range, as it is able to infect beans, *Leucaena* trees, and several other legumes. This work describes the isolation and the characterization of extracellular factors (Nod factors) whose production from *R. tropici* was triggered by the transcriptional activation of its *nod* genes. These factors consist of a chitopentaose backbone in which the *N*-acetyl group of the nonreducing end glucosaminyl residue is replaced by an *N*-methyl-*N*-vaccenoyl one. Some of these molecules are sulfated on position 6 of the terminal reducing glucosamine.

Gram-negative soil bacteria of the three genera *Azorhizobium*, *Bradyrhizobium*, and *Rhizobium* establish symbiotic relationships with leguminous plants through the elicitation of root–nodule organogenesis. In these new vegetative organs, molecular atmospheric nitrogen (N_2) is reduced to ammonia (NH_3), which is then used as the nitrogen source in the plant's metabolism. One of the most important properties of this symbiotic interaction is its specificity: Each rhizobial species has a characteristic host range which can be narrow or wide depending on whether the bacteria can infect and nodulate either a few plant varieties or a large number of them.

The infection and nodulation processes are controlled by both bacterial and plant genes (Long, 1989; Nap & Bisseling, 1990). The expression of the bacterial nodulation genes (so-called *nod* and *nol* genes) is activated by flavonoids secreted by the plant roots (Peters et al., 1986). This activation leads to the synthesis of extracellular signals (Faucher et al., 1988), the so-called Nod factors. These factors were isolated for the first time from a modified strain of *Rhizobium meliloti*, possessing multicopies of the *nod* genes, and were characterized as lipooligosaccharides consisting of a chitotetrameric backbone, sulfated on the reducing end residue and N-acylated on the nonreducing glucosaminyl end by replacement of the *N*-acetyl group by an *N*-(2,9)-hexadecadienoyl one (Lerouge et al., 1990). Some of these molecules were also O-acetylated on the nonreducing glucosaminyl end (Roche et al., 1991a). These factors induce root hair deformation on alfalfa (a normal host plant) at a concentration as low as 10^{-12} M and organogenesis of root nodules at 10^{-9} M (Truchet et al., 1991).

Other Nod factors from different rhizobial species have been isolated such as nonsulfated factors from *Rhizobium leguminosarum* bv. *viciae* (Spaink et al., 1991), a methylfucosylated factor from *Bradyrhizobium japonicum* (Sanjuan et al., 1992), and a complex mixture of Nod factors from the tropical strain *Rhizobium* sp NGR 234 which bear methylfucosyl, carbamoyl, *N*-methyl, acetyl, and sulfate substituents (Price et al., 1992). It is now very likely that the common *nodABC* genes are involved in the biosynthesis of the lipooligochitin core, whereas other *nod* genes determine the host specificity by substitutions or modifications of this backbone.

Because of the importance of cultivating beans in some countries, we focused our attention on bacteria that nodulate the legume *Phaseolus vulgaris*, i.e., *Rhizobium tropici* and *Rhizobium etli*, the latter taxon being a new name proposed for *R. leguminosarum* bv. *phaseoli* (Segovia et al., 1993). These two species have a different host range: relatively broad for *R. tropici*, more specific for *R. etli*.

Herein we report the isolation and the structural elucidation of the Nod factors produced by *R. tropici* (strain CFN 299) which infects *P. vulgaris* L. beans, *Leucaena* sp. trees, and other legumes (Martinez-Romero et al., 1991). These factors were designated NodRt factors (for nodulation factors of *R. tropici*) according to the proposed nomenclature (Roche et al., 1991b). They belong to the same lipooligosaccharide family as described for Nod factors from other rhizobial species. However, NodRt factors exhibit characteristic structural features: (i) both sulfated and nonsulfated molecules are present; (ii) these molecules are all pentameric oligomers; (iii) an *N*-methyl-*N*-acyl-D-glucosaminyl residue is present at the nonreducing end of the oligosaccharide backbone.

MATERIALS AND METHODS

Cell Cultures. R. tropici strains CFN 299 and CFN 299-10 (Nod⁻ ΔpSym) were grown on liquid minimal medium containing sodium glutamate (1 g/L), sucrose (2 g/L), magnesium sulfate ($MgSO_4 \cdot 7H_2O$; 0.1 g/L), calcium chloride ($CaCl_2 \cdot 2H_2O$; 0.15 g/L), iron(III) chloride ($FeCl_3$; 10 mg/L), and biotin (0.5 mg/L) in a potassium dihydrogen phosphate/dipotassium hydrogen phosphate (KH_2PO_4, 9.5

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[1] Abbreviations: EI, electron impact; FAB-MS, fast atom bombardment mass spectrometry; GC–MS, gas chromatography coupled to mass spectrometry; GC–MS–MS, gas chromatography coupled to tandem mass spectrometry; HPLC, high-pressure liquid chromatography; MIKE, mass-analyzed ion kinetic energy; TLC, thin-layer chromatography.

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g/L; K_2HPO_4, 0.5 g/L) buffer (pH 5.5). Naringenin (4',5,7-trihydroxyflavanone) was used to induce *nod* genes (1.5 μM). Growth temperature was regulated at 32 °C.

Log-phase cultures were centrifuged, and the supernatant was filtered through a 0.45-μm filter membrane. The sterile growth medium obtained was then neutralized with ammonia.

TLC and Radioactivity Assays. For labeling experiments, culture medium (100 mL) was supplemented either with 200 μCi of sodium (^{35}S) sulfate (1000 Ci/mmol) or 100 μCi of sodium (^{14}C) acetate (56 mCi/mmol) (Amersham).

After centrifugation and membrane sterilization, the growth medium was applied to a C_{18} reversed-phase packed Sep-Pak cartridge (Waters Associates). After washing with water (5 mL), the Nod factors were eluted with 5 mL of methanol.

Methanol eluates obtained from either induced or noninduced wild-type strain cultures or from pSym⁻ strain cultures were loaded on a C_{18} reversed-phase coated TLC plate. The plate was developed twice with methanol/ammonia 5.5 N (9:1 v/v) (solvent 1). Radiolabeled components were detected by autoradiography on a Kodak X-Omat film.

In parallel experiments, methanol eluates were dried and redissolved in a citrate (130 mM)/phosphate (65 mM) buffer at pH 6.8. Five units of chitinase from *Streptomyces* sp. (Boehringer, Mannheim) were then added, and the mixture was incubated overnight at 37 °C. After freeze-drying, the residue was dissolved in methanol and loaded on TLC plates developed with solvent 1.

Purification of Nod Factors. Nod factors were removed from the culture medium by butanol extraction and ethyl acetate washing, as previously described (Roche et al., 1991a). The crude extract was first purified by HPLC on a semi-preparative C_{18} reversed-phase column (250 × 7.5 mm, Spherisorb ODS2, 5 μm, ColoChrom). A linear gradient from water/acetonitrile (80:20 v/v) (solvent A) to pure acetonitrile (solvent B), at a flow rate of 2 mL/min, was used to elute the factors. The UV absorbance of the eluate was monitored at 220 nm.

A sulfated fraction eluted first, followed by a nonsulfated one. These two UV-absorbing fractions were purified again by HPLC on an analytical C_{18} reversed-phase column (250 × 4.6 mm, Spherisorb ODS1, 5 μm, ColoChrom) using the following protochols: (i) for sulfated factors—5 min isochratic with solvent A, then a linear gradient in 20 min from solvent A to solvent B; (ii) for nonsulfated factors—a linear gradient in 25 min from solvent A to solvent B. Both were performed at a flow rate of 1 mL/min; UV absorbance was monitored at either 220 or 206 nm, depending on the required sensitivity.

Analytical Methods. Gas chromatography analyses were achieved on a Girdel 30 gas chromatograph equipped with an OV1 bound capillary column (0.32 mm × 30 m, Spiral France). Temperature gradient was 2 °C/min from 100 to 280 °C.

Mass spectra were recorded on a ZAB-HS instrument (VG Analytical, Manchester, U.K.). In the positive ion mode, the matrix was a (1:1 v/v) mixture of *m*-nitrobenzyl alcohol/glycerol, spiked either with 1% trichloroacetic acid (TCA) in water or with a solution of sodium iodide (1 mg/mL). In the negative ion mode, a glycerol matrix was used. The location of the fatty acid double bond by remote charge fragmentation was carried out as described (Promé et al., 1987).

GC–MS experiments were done on a Hewlett-Packard 5989A mass spectrometer working in EI ionization mode.

^1H NMR spectra were obtained on a Brüker AC-200 spectrometer (Karlsruhe, Germany) using 1 mg of sample dissolved in 0.5 mL of methanol (CD_3OD 99.95% from Euriso Top); 320 scans were accumulated at 303 K.

Carbohydrate Determination and Methylation Studies. Carbohydrate determination was carried out in two consecutive steps. Limited hydrolysis using 1 N aqueous HCl (3 h, 65 °C) was followed by methylene chloride extraction. After solvent evaporation under nitrogen, an aliquot of the organic layer was hydrolyzed again using 3 N HCl (3 h, 80 °C) and extracted with diethyl ether. Sugars from the aqueous phases were separately derivatized as alditol acetates and analyzed by capillary GC–MS. 2-Butyl glycosides of individual sugars were prepared by reaction with 1 N HCl in (±)- or (–)-2-butanol (3 h, 60 °C) obtained by reaction of acetyl chloride with the suitable anhydrous alcohol. These derivatives were further acetylated [acetic anhydride/pyridine (1:1 v/v) mixture; 3 h, 40 °C] and analyzed by capillary GC.

Pertrimethylsilyl 1-*O*-methyl derivatives of N-acylated glucosamines were obtained by reaction of the methylene chloride extract from mild hydrolysis with 1 N HCl in anhydrous methanol for 2 h at 60 °C, followed by solvent evaporation and solubilization in bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane.

Permethylation analysis was done according to the method of Ciucanu and Kerek (1984). One milligram of $NaBD_4$-reduced Nod factors in 300 μL of anhydrous dimethyl sulfoxide was mixed with 3 pellets of dry sodium hydroxide. After 15 min in an ultrasonic bath, 300 μL of methyl iodide was added and the solution sonicated for an additional 15 min. After 1.5 h at room temperature, the reaction was stopped by the addition of a few drops of a saturated sodium thiosulfate solution. The methylated molecules were extracted with methylene chloride. This procedure was repeated twice. Hydrolysis was achieved using a 4 N trifluoroacetic acid (TFA) solution (4 h, 110 °C). Partially methylated sugars were then converted into alditol acetates and studied by GC–MS. A slight modification of this procedure was used for sulfate location. After the permethylation steps, the sulfate group was removed by mild acid hydrolysis (0.1 N HCl; overnight, 30 °C), and the desulfated compound was trideuteromethylated using ICD_3 instead of ICH_3. Hydrolysis and derivatization were done as above.

Synthesis of the 1,3,4,5,6-Penta-O-acetyl-N-acetyl-N-methyl-D-glucosaminitol. Fifty milligrams of commercial *N*-acetyl-D-glucosamine (Sigma) was dissolved in 2 mL of anhydrous diethyl ether and 0.5 mL of tetrahydropyran with 5 mg of *p*-toluenesulfonic acid. The mixture was stirred for 3 h at room temperature. The amide group of hydroxyl-protected *N*-acetyl-D-glucosamine was then methylated according to Ciucanu and Kerek's procedure, as above. The tetrahydropyranyl ether derivatives and the acetyl group were removed by strong acidic hydrolysis (2 N HCl, 5 h at 110 °C). The resulting *N*-methyl-D-glucosamine was borohydride-reduced and acetylated according to the standard procedure. GC–MS analysis showed a major peak corresponding to the expected product, characterized by ions at *m/z* 158 and 116 (C_1–C_2 fragment, the latter arising from additional loss of acetic acid) and *m/z* 374 (C_2–C_3–C_4–C_5–C_6 fragment).

Fatty Acid Analysis. Fatty acids were liberated from Nod factors either by aqueous acidic hydrolysis (3 N HCl; 3 h, 80 °C) or by saponification (KOH 5%, 20 h, 70 °C). Methyl esters were prepared by reaction with diazomethane. Pentafluorobenzyl derivatives were synthesized by dissolving fatty acids (about 100μg) in 60 μL of a (1:5 v/v) mixture of dry methanol and acetonitrile followed by addition of 2 μL of pentafluorobenzyl bromide and 2 μL of diisopropylethylamine. After 1 h at room temperature, reagents were removed by evaporation. The pentafluorobenzyl esters were dissolved in

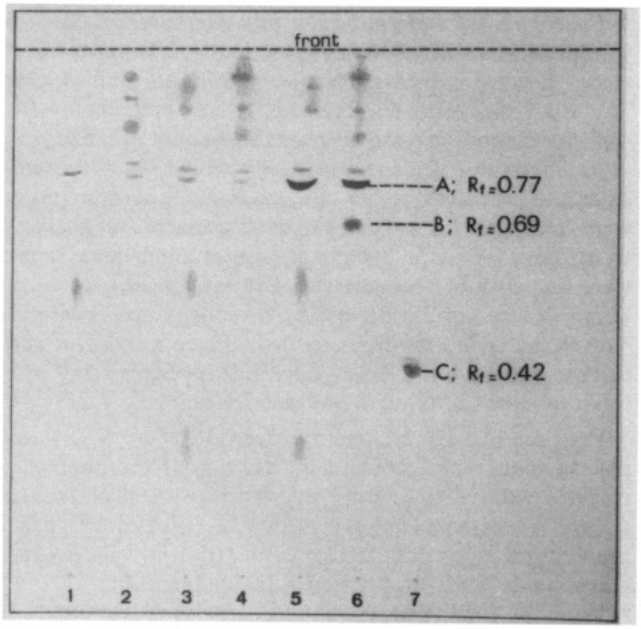

FIGURE 1: TLC (C_{18} reversed-phase) autoradiogram from the Sym plasmid-cured strain induced with naringenin, labeled with ^{35}S (lane 1) or ^{14}C (lane 2), from the noninduced wild-type strain labeled with ^{35}S (lane 3) or ^{14}C (lane 4), and from the naringenin-induced wild-type strain labeled with ^{35}S (lane 5) or ^{14}C (lane 6). Lane 7 represents the latter experiment after chitinase treatment.

cyclohexane and analyzed by negative ion GC–MS–MS (Promé et al., 1987).

RESULTS

Detection of Extracellular Nod Metabolites after nod Gene Induction. R. tropici (wild-type strain CFN 299) was grown in the presence or in the absence of naringenin as inducer, using either sodium (^{14}C)acetate or sodium (^{35}S)sulfate labeled precursors.

Lipophilic compounds from the growth medium were reversed-phase extracted on Sep-Pak and eluted with methanol. Reversed-phase TLC of this eluate indicated the presence of both sulfated (compound **A**; R_f = 0.77) and nonsulfated (compound **B**; R_f = 0.69) metabolites (Figure 1, lanes 5 and 6) from the naringenin-induced culture. These compounds were not detected from a noninduced culture (Figure 1, lanes 3 and 4) or from an induced culture of the same strain cured of the symbiotic plasmid (CFN 299–10) (Figure 1, lanes 1 and 2), and hence they are presumed to be Nod metabolites.

The methanol eluate from a ^{14}C-labeled and naringenin-induced culture was separately treated with chitinase. After this treatment, both labeled spots corresponding to compounds **A** and **B** disappeared and an additional spot, with a R_f of 0.42 (compound **C**) (Figure 1, lane 7), was observed. This was consistent with an enzymatic hydrolysis of both compounds **A** and **B** leading to the formation of a more hydrophobic compound as already described, suggesting an chitooligomeric backbone (Roche et al., 1991a).

Purification of Nod Factors. Four liters of sterile medium from a *nod* gene induced culture was extracted with butanol, and the evaporated butanol extract was partitioned between ethyl acetate and water to remove highly hydrophobic compounds (Roche et al., 1991a). Nod factors remained in the water phase. They were purified by two consecutive separations on C_{18} reversed-phase HPLC. The location of Nod-factor-containing fractions was done by comparison of the HPLC profiles from the wild-type strain and the Sym-plasmid-cured strain which could not produce Nod factors. A crude separation between sulfated and nonsulfated molecules was achieved in the first semipreparative HPLC run. Each fraction was purified further by an analytical HPLC step. About 1.5–2.0 mg of each species was isolated from a 4-L culture.

Carbohydrate Determination and Fatty Acid Analysis. After mild acidic hydrolysis (1 N HCl in water) of both the sulfated and nonsulfated molecules, *N*-acetylglucosamine was the only water-soluble product that could be detected, whereas the acyl-containing truncated fractions remained in the organic phase. A stronger hydrolysis of the latter (3 N HCl in water) liberated *N*-methylglucosamine. Both *N*-acetyl- and *N*-methylglucosamine were assigned to the D series from the GC analysis of their peracetylated (–)-2-butyl glycosides as described (Gerwig et al., 1979).

The liberated fatty acid was identified as *cis*-vaccenic acid by looking for the decomposition of its carboxylate anion by tandem mass spectrometry (Promé et al., 1987) which localized the double-bond on position 11 of octadecenoic acid. Confirmation was obtained by co-injection on capillary GC of an authentic standard (methyl ester derivatives).

Mass Spectrometry Analysis. In the positive ion mode, both fractions exhibited $(M + H)^+$ ions when studied by FAB-MS with acidified matrix. This ion was at *m/z* 1270 for the nonsulfated fraction and at *m/z* 1350 for the sulfated one. These assignments were confirmed by cationization with the sodium ion, giving $(M + Na)^+$ at *m/z* 1292 for the former and $(M - H + 2Na)^+$ at *m/z* 1394 for the latter. The presence of an additional sodium atom was due to the strongly acidic sulfate group. In the negative ion mode, both compounds showed $(M - H)^-$ ions at *m/z* 1268 and 1348, respectively, thus confirming the above results.

Clear fragmentations of the backbone were seen in the positive ion mass spectra (acid-doped matrix), assigning an *N*-acetylglucosamine oligomer core for both compounds (series of peaks separated by 203 mass units). The highest mass peak of this series was 221 mass units below the $(M + H)^+$ ion of the nonsulfated molecule. This indicated a nonsubstituted *N*-acetylglucosamine at the reducing end for the latter. This sequence was confirmed by the metastable ion spectrum from the $(M + H)^+$ ion at *m/z* 1270 (Figure 2b). The $(M + H)^+$ ion from the sulfated molecule at *m/z* 1350 showed a loss of 80 mass units (SO_3 loss), giving *m/z* 1270. Below this mass, all fragments were identical to those from the nonsulfated fraction (Figure 2a). This located the sulfate group on the reducing glucosaminyl end (Lerouge et al., 1990).

These fragmentation series ended at *m/z* 440, which should correspond to the acylated glucosaminyl end as its oxonium ion. To examine the nature of this part of the molecules, the organic phase from a mild acidic hydrolysis (see above) was treated with HCl containing anhydrous methanol to obtain *O*-methyl glycosides of the acylated sugar which were then converted to trimethylsilyl derivatives. The EI mass spectrum showed the expected fragment ions containing the C_2–C_3 (*m/z* 409), C_3–C_4 (*m/z* 204), and C_2–C_3–C_4 (*m/z* 422) part of the molecule. The difference of 14 additional mass units between the *m/z* values of the ions containing C_2 and their calculated value, assuming that the terminal glucosamine bore an *N*-vaccenoyl group, indicated the presence of an *N*-methyl group (Figure 3). Thus, the glucosamine residue at the terminal nonreducing end was both N-methylated and N-acylated.

Permethylation Studies. The (1 → 4) linkage between saccharidic residues was confirmed by methylation analysis of both compounds **A** and **B**. The permethylated $NaBD_4$-

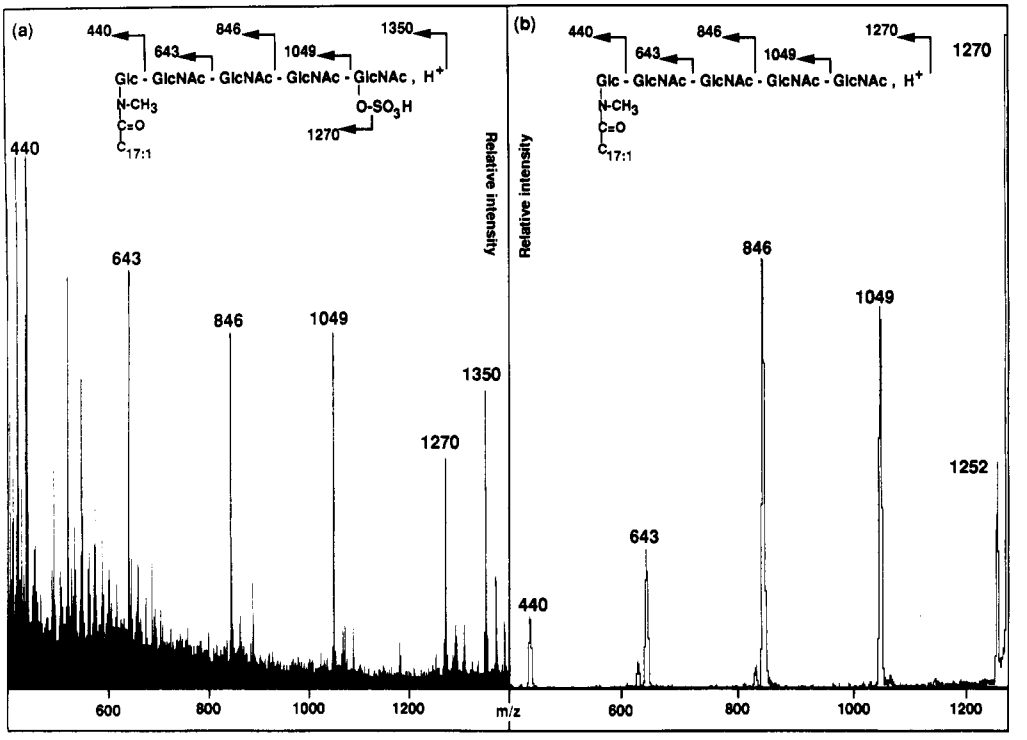

FIGURE 2: (a) FAB mass spectrum in the positive ion mode of the sulfated NodRt-V (Me,S) factor. (b) FAB MIKE spectrum in the positive ion mode of the protonated ion at *m/z* 1270 of the nonsulfated NodRt-V (Me) factor with the corresponding fragmentations.

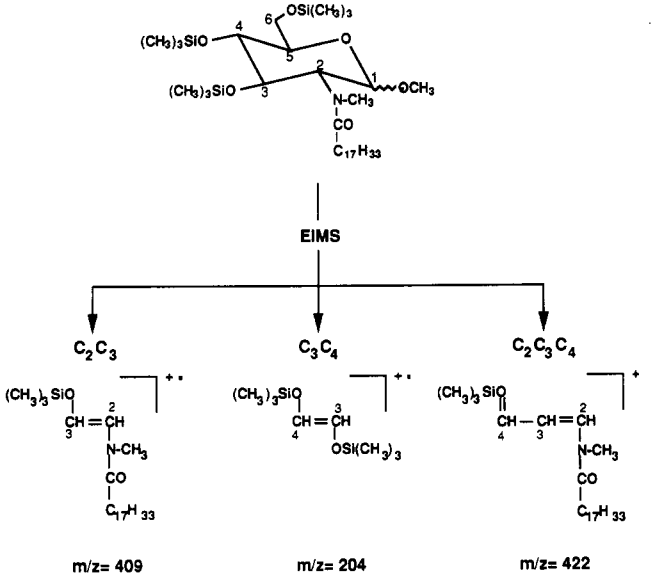

FIGURE 3: Characteristic fragments in the EI mass spectrum from the pertrimethylsilyl derivative of the 1-*O*-methyl-*N*-methyl-*N*-acylglucosamine obtained after methanolysis of NodRt factors.

reduced compound **B**, when analyzed by FAB-MS in the positive ion mode, showed a $(M + Na)^+$ ion at *m/z* 1533. Thus, as expected, 17 methyl groups have been introduced into the molecule. Total hydrolysis of this permethylated compound, followed by $NaBH_4$ reduction and peracetylation, afforded three partially methylated N-acetylated glucosaminitol acetates that were identified by GC–EIMS. Glucosamine at the nonreducing end afforded alditols methylated on positions 2, 3, 4, and 6. The three internal residues gave 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-*N*-acetyl-*N*-methylglucosaminitol, whereas glucosamine at the reducing end produced the corresponding 4-*O*-acetyl-1,3,5,6-tetra-*O*-methyl-*N*-acetyl-*N*-methylglucosaminitol.

$NaBD_4$-reduced compound **A** was permethylated by the same procedure. Then, the sulfate group was selectively removed by mild acid hydrolysis, and the resulting free hydroxyl group was trideuteromethylated. Hydrolysis afforded a mixture of partially methylated glucosamines and glucosaminitol, the latter coming from the reducing part of the original molecule. GC–MS analysis of the acetyl derivatives led to the identification of 4-*O*-acetyl-1,3,5-tri-*O*-methyl-6-*O*-trideuteromethyl-*N*-acetyl-*N*-methylglucosaminitol. The overall study was consistent with (1 → 4) linkage between glucosamine residues in both compounds and location of the sulfate group on O-6 from the reducing end residue in compound **A**.

^1H NMR Analysis. The one-dimensional spectra of sulfated and nonsulfated molecules were relatively similar to those already described for NodRm and NodRlv factors (Nod factors from *R.meliloti* and from *R. leguminosarum* bv. *viciae*, respectively) (Roche et al., 1991a; Spaink et al., 1991). The four doublets corresponding to anomeric protons at the interglycosidic linkages (δ 4.55–4.70) showed a 8.5-Hz value for the coupling constant $^3J_{H1-H2}$: This was characteristic of β linkages. The doublet ($^3J_{H1-H2}$= 3.5 Hz) at δ 5.20 ppm was attributed to H1α of the reducing sugar. The *N*-methyl protons clearly appeared as a singlet at δ 3.00 ppm. The protons of the internal double bond of the fatty acid gave a triplet at δ 5.45 (Figure 4). Comparison of the spectra from compounds **A** and **B** indicated in the former a downfield shift for two protons (δ 4.35 and δ' 4.30). These were attributed to the deshielding effect of the sulfate group on H-6 protons at the reducing end (Figure 4, inset). A similar effect was observed in the sulfated Nod factor from *R. meliloti*.

DISCUSSION

Nodulation Factors Produced by R. tropici. We have reported herein the structural characterization of the Nod factors produced and excreted by the recently described *R. tropici* species (Martinez-Romero et al., 1991). The wild-

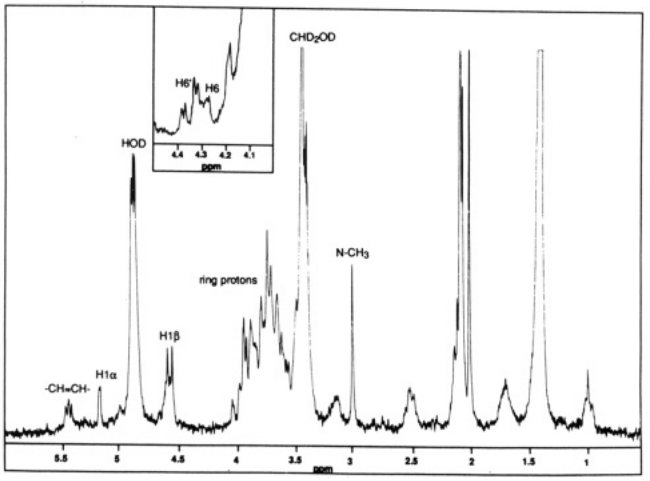

FIGURE 4: ^{1}H NMR spectrum of the nonsulfated NodRt-V (Me) factor in CD_3OD. (Inset) ^{1}H NMR spectrum of the sulfated NodRt-V (Me,S) factor for the region from 4.0 to 4.5 ppm.

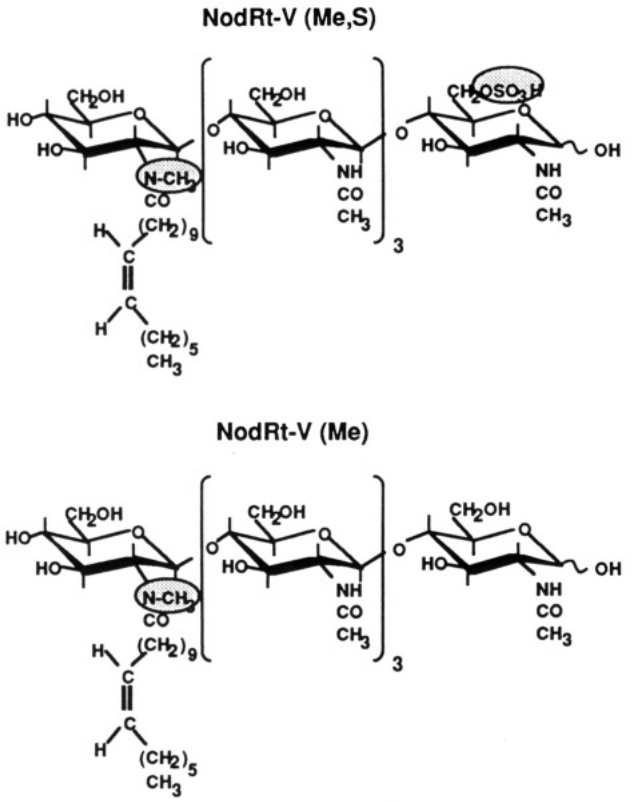

FIGURE 5: Structural features of the NodRt-V (Me,S) and NodRt-V (Me) factors.

type strain (CFN 299) naturally produced relatively large amounts of Nod factors in contrast to *R. meliloti* and *R. leguminosarum* bv. *viciae*, where genetic engineering was required to increase production of Nod factors.

Two types of Nod factors are produced: sulfated (compound **A**) and nonsulfated (compound **B**) molecules. The production yield is about 0.3–0.5 mg/L of *nod* gene induced culture medium for each factor. According to the nomenclature proposed by Roche and co-workers (Roche et al., 1991b), we propose to name these compounds NodRt-V (Me, S) and NodRt-V (Me), respectively (Figure 5).

The structural characteristics of these new Nod factors are the following: (i) they all possess a pentameric chitooligomeric backbone; (ii) the glucosaminyl residue at the nonreducing end is both N-methylated and N-acylated by vaccenic acid; (iii) some of these molecules are sulfated on carbon 6 of the reducing glucosamine; and (iv) all of the glucosaminyl residues, except the nonreducing terminal one, are N-acetylated.

The fatty acid which amidifies the terminal nonreducing glucosamine (vaccenic acid) is a major fatty acid found in the total lipids of several *Rhizobium* species (MacKenzie et al., 1979). An *N*-methyl group located on the same nitrogen atom as the vaccenic acid has previously been described both in Nod factors from *R.* sp. NGR 234 (Price et al., 1992) and from *Azorhizobium caulinodans* (Mergaert et al., 1993).

Only pentameric glucosamine oligomers were isolated from *R. tropici* Nod factors. This contrasts with the NodRm factors which have been described mainly as tetramers (Roche et al., 1991b), although trimers and pentamers occur as minor compounds (Schultze et al., 1992). *R. leguminosarum* bv. *viciae* produces roughly similar amounts of tetra- and pentameric Nod factors (Spaink et al., 1991), whereas *B. japonicum* produces only pentameric ones (Sanjuan et al., 1992).

Sulfated Nod factors have been isolated so far from *R. meliloti* and *R.* sp. NGR 234, the latter species being similar to *R. tropici* insofar as it produces both sulfated and nonsulfated molecules. This peculiarity has been proposed to partially explain the extended host range of the tropical NGR 234 strain (Price et al., 1992).

Anyway, biological activities of the NodRt factors are now tested on different legumes (hosts and nonhosts). First results show that the sulfated NodRt factors have a true morphogenic activity on bean at 10^{-6}–10^{-8} M (E. Martinez, 1993). Extended data on biological activities and relationships between NodRt factor structure and host range will be published later.

Nodulation Genes and Biosynthesis of Nod Factors. It is now clearly established for several rhizobial species that specific bacterial *nod* genes modify the lipooligosaccharide core structure of the Nod factors (Fisher & Long, 1992).

In *R. meliloti*, the 6-O-sulfation of NodRm factors is under the control of two *nod* operons: *NodH* and *nodPQ*. Mutations in these genes modify (*nodH*$^-$) or extend (*nodPQ*$^-$) the host range of the bacterium by producing exclusively (*nodH*$^-$) or partly (*nodPQ*$^-$) nonsulfated factors (Roche et al., 1991b).

In *A. caulinodans*, *nodS*, which shares sequence homology with *N*-methylases, is likely to encode the N-methylation of nodulation factors (M. Holsters, personal communication).

Since NodRt factors are both sulfated (in part) and N-methylated, sequence homologies with *nodH* and *nodS* were searched successfully within the *R. tropici* symbiotic plasmid (E. Martinez-Romero, personal communication).

The simultaneous production of sulfated and nonsulfated NodRt factors may be explained by a partially efficient sulfation process which cannot transform all of the precursors produced by action of other *nod*-gene-dependent enzymes. The same idea has been proposed to explain the partial sulfation and the extended host range exhibited by the *R. meliloti* *nodPQ*$^-$ strain (Roche et al., 1991b).

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