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Nodule-Inducing Activity of Synthetic *Sinorhizobium meliloti* Nodulation Factors and Related Lipo-Chitooligosaccharides on Alfalfa. Importance of the Acyl Chain Structure[1]

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***Sinorhizobium meliloti* nodulation factors (NFs) elicit a number of symbiotic responses in alfalfa (*Medicago sativa*) roots. Using a semiquantitative nodulation assay, we have shown that chemically synthesized NFs trigger nodule formation in the same range of concentrations (down to 10^{-10} M) as natural NFs. The absence of *O*-sulfate or *O*-acetate substitutions resulted in a decrease in morphogenic activity of more than 100-fold and approximately 10-fold, respectively. To address the question of the influence of the structure of the *N*-acyl chain, we synthesized a series of sulfated tetrameric lipo-chitooligosaccharides (LCOs) having fatty acids of different lengths and with unsaturations either conjugated to the carbonyl group (2E) or located in the middle of the chain (9Z). A nonacylated, sulfated chitin tetramer was unable to elicit nodule formation. Acylation with short (C8) chains rendered the LCO active at 10^{-7} M. The optimal chain length was C16, with the C16-LCO being more than 10-fold more active than the C12- and C18-LCOs. Unsaturations were important, and the diunsaturated 2E,9Z LCO was more active than the monounsaturated LCOs. We discuss different hypotheses for the role of the acyl chain in NF perception.**

Rhizobia are symbiotic bacteria that elicit on the roots of specific leguminous hosts the formation of new organs (nodules) in which they multiply and fix nitrogen. Both loss- and gain-of-function genetic experiments have shown that host recognition, the initiation of infection thread formation, and the induction of nodules require rhizobial nodulation (*nod*) genes. Most products of *nod* genes are involved in the synthesis and transport of bacterial symbiotic signals, the NFs (Dénarié et al., 1996; Long, 1996). NFs synthesized by all rhizobial species are LCOs. They consist of a chitin oligomer backbone that is made up of four or five GlcNAc residues, mono-*N*-acylated at the nonreducing end and carry diverse substitutions at both ends. Each rhizobial species (or biovar) has a defined host range and produces a set of NFs with specific substitutions (Dénarié et al., 1996). NF structure does not correlate with rhizobial taxonomy and phylogeny as derived from molecular studies of rRNA, but it correlates instead with the rhizobial host range, indicating that a given plant has defined NF structural requirements for the triggering of symbiotic responses (Dénarié et al., 1996).

Rhizobia can be schematically grouped into two classes according to the type of *N*-acylation of their NFs. Rhizobia that nodulate tropical and temperate legumes of the Genisteae and Loteae tribes (*Azorhizobium caulinodans*, *Bradyrhizobium japonicum*, *Rhizobium* sp. *NGR234*, *Rhizobium fredii*, and *Rhizobium loti*) produce NFs that are *N*-acylated with fatty acids of general lipid metabolism such as C18:1Δ11Z (vaccenic acid) (Cohn et al., 1998). In contrast, rhizobia that nodulate legumes of the related Galegeae, Trifolieae, and Vicieae tribes produce NFs that are *N*-acylated by polyunsaturated fatty acids, whose major species are C16:2Δ2E,9Z in *Sinorhizobium meliloti*; C18:4Δ2E,4E,6E,11Z in *Rhizobium leguminosarum* bv *viciae* and *Mesorhizobium huakuii*; C20:3 and C20:4 in *R. leguminosarum* bv *trifolii*; and C18:2 and C18:3 in *Rhizobium galegae* (Lerouge et al., 1990; Spaink et al., 1991, 1995; van der Drift et al., 1996; G.P. Yang, F. Debellé, A. Savagnac, M. Ferro, O. Schiltz, F. Maillet, D. Promé, M. Treilhou, C. Vialas, K. Lindstrom, and others, unpublished data). The *nodFE* genes, which determine the synthesis of these polyunsatu-

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Abbreviations: LCOs, lipo-chitooligosaccharides; NF, nodulation (Nod) factor; X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds.

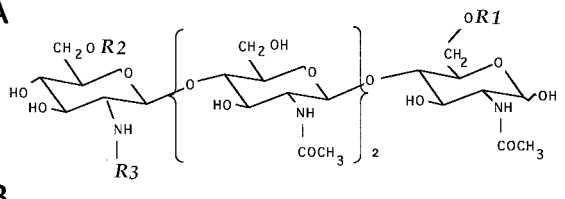
rated fatty acids, are major host-range determinants, indicating that the *N*-acyl-specific structure (carbon-chain length and degree of unsaturation) is involved in host specificity (Spaink et al., 1989; Ardourel et al., 1994; Bloemberg et al., 1995). The use of purified NFs has shown that acylation by polyunsaturated fatty acids is important for biological activity in vetch, clover, and alfalfa (Spaink et al., 1991, 1995; Ardourel et al., 1994).

Strains of *S. meliloti*, an alfalfa symbiont, produce NFs that have three specific substitutions (Fig. 1): *O*-sulfation at the C6 position of the reducing GlcNAc residue, *O*-acetylation at the C6 position, and *N*-acylation by an unsaturated C16 fatty acid (C16:1Δ9Z, C16:2Δ2E,9Z, and C16:3Δ2E,4E, 9Z, respectively) of the terminal nonreducing glucosamine residue (Lerouge et al., 1990; Roche et al., 1991b; Schultze et al., 1992; Demont et al., 1993). Purified NFs of *S. meliloti* in the nanomolar to picomolar range elicit various responses on alfalfa roots: electrophysiological changes in root hairs (Ehrhardt et al., 1992, 1996; Felle et al., 1995, 1996, 1998; Kurkdjian, 1995), root hair deformations (Lerouge et al., 1990), activation of cortical cells (Ardourel et al., 1994), activation of early nodulin genes (Journet et al., 1994; Fang and Hirsch, 1998), induction of cell divisions in the inner cortex, and formation of empty nodules (Truchet et al., 1991). Two approaches have been used to study the NF structural requirements for the elicitation of alfalfa responses: treatment of plants with *S. meliloti nod* mutants that produce modified NFs and treatment with purified NFs prepared from these mutants. The requirement for the three NF substitutions seems to vary with the plant response, suggesting the involvement of different mechanisms of NF perception and transduction in alfalfa (Ardourel et al., 1994; Felle et al., 1995) and underlining the importance of structure-activity studies.

An important limitation when using natural NFs to study structure-activity relationships is that rhizobia produce a mixture of NFs having different substitutions and it is presently not possible to separate all of the molecular species. The chemical synthesis of NFs of *S. meliloti* (Nicolaou et al., 1992; Wang et al., 1993; Ikeshita et al., 1994; Tailler et al., 1994) and *B. japonicum* (Ikeshita et al., 1995) has now been achieved, making possible the synthesis of a variety of chemically pure authentic NFs, the introduction of single, defined, structural modifications, and the synthesis of analogs that may not occur naturally. Stokkermans et al. (1995) used synthetic NFs and related LCOs to study NF structural requirements to elicit symbiotic responses in soybean, a plant nodulated by rhizobia that produce fucosylated NFs and do not possess active *nodFE* genes. These studies have shown the importance of the length of the chitin oligomer backbone, the requirement for the fucosyl group at the reducing end, and the relative unimportance of the structure of the acyl chain on soybean symbiotic responses.

In the present study we used chemically synthesized *S. meliloti* NFs and a semiquantitative nodulation bioassay to show that synthetic NFs have nodule-inducing activities similar to those of their natural analogs. We also address the role of the *O*-sulfate and *O*-acetate substitutions and use a set of new synthetic LCOs to address the role of the structure of the *N*-acyl moiety in this morphogenic activity.

A



B

	R1	R2	R3
LCO-IV (S, Ac, C16:2Δ2E,9Z)	SO_3H	$COCH_3$	C16:2(Δ2E,9Z)
LCO-IV (S, C16:2Δ2E,9Z)	SO_3H	-	C16:2 (Δ2E,9Z)
LCO-IV (Ac, C16:2Δ2E,9Z)	-	$COCH_3$	C16:2(Δ2E,9Z)
LCO-IV (C16:2Δ2E,9Z)	-	-	C16:2 (Δ2E,9Z)
CO-IV (S, NH_2)	SO_3H	-	H
LCO-IV (S, C8:1 Δ2E)	SO_3H	-	C8:1 (Δ2E)
LCO-IV (S, C12:1Δ2E)	SO_3H	-	C12:1 (Δ2E)
LCO-IV (S, C16:1Δ2E)	SO_3H	-	C16:1 (Δ2 E)
LCO-IV (S, C16:1Δ9Z)	SO_3H	-	C16:1 (Δ9Z)
LCO-IV (S, C16:2Δ2E,9Z)	SO_3H	-	C16:2 (Δ2E,9Z)
LCO-IV(S, C18:1Δ2E)	SO_3H	-	C18:1 (Δ2E)
LCO-IV (S, C18:2Δ2E,9Z)	SO_3H	-	C18:2 (Δ2E,9Z)

Figure 1. A, Structure of the synthetic *S. meliloti* NFs. B, Related LCOs with different *N*-acyl moieties used in this study.

MATERIALS AND METHODS

Preparation and Purification of NFs and Related LCOs

Natural NFs were isolated from the overproducing *Sinorhizobium meliloti* 2011 (pMH682) strain as previously described (Roche et al., 1991a). The following LCOs, analogs to natural NFs, were chemically synthesized by Nicolaou et al. (1992): LCO-IV(Ac,S,C16:2Δ2,9), LCO-IV(S,C16:2Δ2,9), LCO-IV(Ac,C16:2Δ2,9), and LCO-IV(C16:2Δ2, 9; see Fig. 1). Sulfated LCO-IV with modified aliphatic chains (described in Fig. 1B) were synthesized according to a modification of the procedure described by Tailler et al. (1994). The synthetic intermediate consisting of a chitotetramer with a free amino group at the nonreducing end and an *O*-sulfate group located on C6 of the GlcNAc reducing residue, was acylated by a variety of fatty acid chlorides as described below.

To a solution of the sulfated tri-*N*-acetyl chitotetraose (20–40 μmol), either in a 5:2 (v/v) dimethylformamide: water mixture (1 μmol 20 μL^{-1}) for acylation with the C8:1Δ2, C12:1Δ2, C16:1Δ2, and C18:1Δ2 acyl chlorides or in a 1:1:1 (v/v) ethyl acetate:methanol:water (25 μL μmol^{-1}

oligosaccharide) for acylation with the C16:1Δ9, C16:2Δ2,9, and C18:2Δ2,9 acyl chlorides, we added 3 equivalents of sodium hydrogenocarbonate and 2 equivalents of the acyl chloride, either as a 0.7 M solution in tetrahydrofuran for the former solvent system or as a 0.7 M solution in ethyl acetate for the latter system. The reaction mixture was stirred at room temperature; we then added 2 equivalents of hydrogenocarbonate and acyl chloride after 4 and 24 h, respectively. The reaction was monitored on silica gel 60 F_{254} TLC plates (Merck, Darmstadt, Germany) using ethyl acetate:methanol:water (2:1:1, v/v) as an eluant, with detection by charring with H_2SO_4:ethanol (10:1).

After 48 h the reaction mixture was concentrated in vacuo and excess acylating agent was removed with ethyl ether. The acylated products were purified by column chromatography on silica gel (Merck, 40–63 μm, 1.5 g 10 μmol^{-1} starting oligosaccharide) with ethyl acetate: methanol:water (5:2:1, v/v) as an eluant, followed by chromatography on a Sephadex G-25 column (1.4 × 28 cm) with water as an eluant. The unreacted amine (20%–30%) was recovered from the silica gel column. LCOs were isolated as white, amorphous powders after lyophilization, and we obtained the following yields: LCO-IV (C8:1,Δ2E), 68%; LCO-IV (C12:1,Δ2E), 55%; LCO-IV (C16:1,Δ2E), 61%; LCO-IV (C18:1,Δ2E), 65%; LCO-IV (C16:1,Δ9Z), 60%; LCO-IV (C16:2,Δ2E,9Z), 60%; and LCO-IV (C18:2,Δ2E,9Z), 56%. The structure and purity of the LCOs was determined by proton-NMR and MS. NMR spectra were recorded at 25°C for samples in ^2H_2O (LCO-IV [C8:1,Δ2E]) or methanol-d*4* (other LCOs) on a spectrometer (model AM-300WB, Bruker Instruments, Billerica, MA); for details, see Tailler et al. (1994) and D. Tailler, J.C. Jacquinet, and J.M. Beau (unpublished data). Mass spectra of LCOs were recorded on a mass spectrometer (Autospec VG-Analytical, Fisons, Manchester, UK), equipped with liquid secondary ion MS, as previously described (Demont et al., 1993).

Plant Assays

Seeds of alfalfa (*Medicago sativa* cv *gemini*) were provided by V. Gensollen (Groupe d'Etude et de Contrôle des Variétés et des Semences, Montpellier, France). Seeds were sterilized and germinated, and seedlings were transferred to test tubes as previously described by Truchet et al. (1985). Plants were grown in a growth chamber (20°C, 80% RH, 16 h under 300 mE m^{-2} s^{-1} for 24 h). For each treatment three series of dilutions of LCOs were tested, ranging from 10^{-8} to 10^{-10} M for the LCOs described in Figure 1B, top, and from 10^{-7} to 10^{-9} M for the LCOs described in Figure 1B, bottom. LCOs were applied in melted Fahraeus agar medium immediately after it was poured into test tubes. To minimize experimental errors in the preparation of these highly diluted solutions, we prepared three independent LCO solutions for each dilution; each of the three served to inoculate 10 test tubes. We used 30 tubes, each containing two seedlings, for each treatment. To limit subjective bias in the estimation of nodulation, two persons counted the nodules independently and the results were averaged.

Seeds of common vetch (*Vicia sativa* subsp. *nigra*) were kindly provided by G. Genier (Station d'Amelioration des Plantes, Institut National de la Recherche Agronomique, Lusignan, France). The vetch root-hair initiation and thick and short root assays were performed as previously described by Roche et al. (1991a), except that Jensen medium was replaced with Fahraeus medium. We used the Fisher's test, with Statview SE+ software (Abacus Koncept, Alfa System Diffusion, Meylan, France), to perform the variance analysis. We estimated the activity of root-hair initiation with the limit-dilution method, as previously described (Roche et al., 1991a).

Microscopic Methods

Light microscopy of root deformations took place as follows. Roots were fixed in 2.5% glutaraldehyde buffered at pH 7.2 with sodium cacodylate (30 min under vacuum and 1 h at room temperature), stained with methylene blue (0.005% in distilled water), cleared 3 min with sodium hypochlorite (Truchet et al., 1989b), and finally, stained with potassium iodide (Vasse et al., 1990). Root sections (80 μm thick) were made after the fixation procedure (Micro-Cut H 1200, Bio-Rad). We viewed the entire plants or the root sections by bright- or dark-field microscopy with a light microscope (Vanux, Olympus).

RESULTS

Morphogenic Activity of Synthetic NFs on Alfalfa and the Importance of *O*-Sulfate and *O*-Acetate Groups

Our first goal was to determine whether chemically synthesized NFs showed the activities and limitations that are characteristic of biological material. The two major natural NFs produced by *S. meliloti* are β-1,4-linked GlcNAc tetramers, *O*-6-sulfated on the reducing sugar, *N*-acylated by 2E,9Z hexadecadienoic acid on the nonreducing sugar, and either *O*-6-acetylated or not on C6 of this sugar; these NFs were termed NodRm-IV(Ac,S,C16:2) and NodRm-IV(S,C16:2), respectively (Roche et al., 1991a). These two compounds were chemically synthesized by Nicolaou et al. (1992) (Fig. 1). *S. meliloti* NFs can induce the formation on alfalfa seedlings of root-derived structures that share common features with the nodules induced by bacteria (Truchet et al., 1991). Nodule formation is NF dose dependent and can be used as a semiquantitative bioassay. Synthetic LCO-IV(Ac,S,C16:2) and LCO-IV(S,C16:2) and the mixture of natural NFs were tested at concentrations varying from 10^{-8} to 10^{-10} M. Root-derived structures having the appearance of small nodules appeared rapidly and could be observed within 2 weeks at concentrations of 10^{-8} and 10^{-9} M (Fig. 2A).

Cytological studies revealed that the root-derived structures induced by LCO-IV(Ac,S,C16:2), LCO-IV(S,C16:2), and by natural NFs were indistinguishable and shared a number of features with *S. meliloti*-induced nodules: cortical origin, peripheral vascular bundles, and endodermis. In contrast to rhizobium-induced nodules, those induced by both synthetic and natural NFs exhibited the following

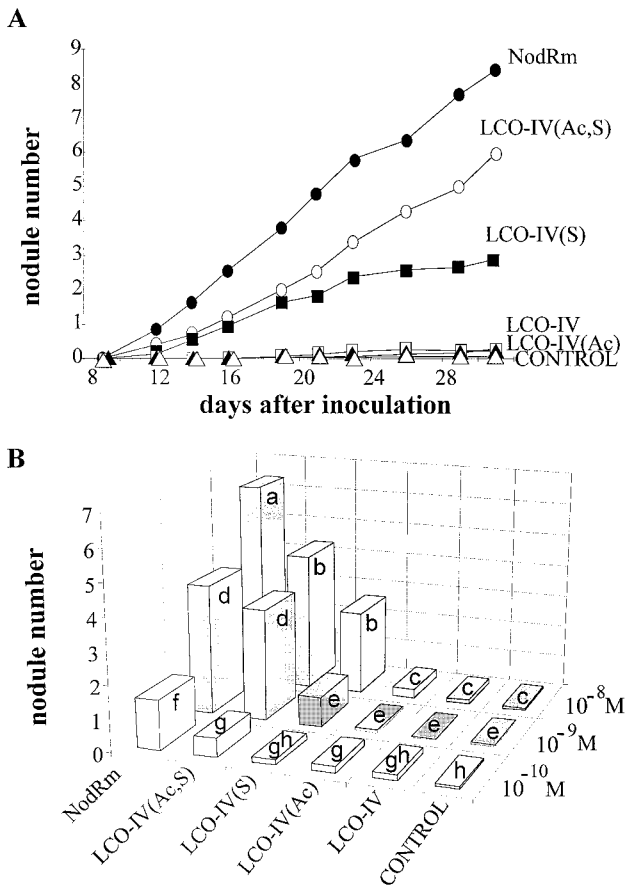


Figure 2. Nodule induction on alfalfa by natural and synthetic *S. meliloti* NFs. A, Nodulation kinetics at 10^{-8} M. B, Nodule number 26 d after addition of NFs. NodRm, Natural *S. meliloti* NFs; LCO-IV, synthetic *S. meliloti* NFs. All LCOs tested had a C16:2Δ2E,9Z acyl chain (see Fig. 1B). Nodule numbers represent the average of 30 tubes, each containing two seedlings. Statistical analysis of nodule numbers was performed separately for each concentration by analysis of variance with Fisher's test: treatments with letters in common do not differ significantly at the P = 0.05 level.

features: they generally had transient meristems and thus remained small and nonelongated (Fig. 3, A and C), and root hairs located at the distal ends of emerging nodules (Fig. 3, A and C) and distally to the meristematic areas elicited in the plant cortex (Fig. 3, B and D) were extremely long and deformed. These characteristics allowed us to easily distinguish between NF-induced nodules and the rare, spontaneous nodules that developed in the absence of rhizobia (Truchet et al., 1989a). In the control seedlings without NFs, the formation of non-rhizobium-induced nodules was a rare event: two or three nodule-like structures could be observed among 60 seedlings approximately 3 weeks after germination. Morphologically, such spontaneous nodules are glabrous, and because they have a persistent meristem, they are elongated (Pichon et al., 1994). These differences suggest that NFs induce a nodule-organogenic program that does not correspond to the activation of non-rhizobium-induced nodule formation. These results show that synthetic NFs are responsible for the induction of nodule morphogenesis and that the alfalfa nodulation assay can be used to study the structure-activity relationship with synthetic NFs and related LCOs.

At the three concentrations used, the natural NFs elicited the formation of slightly more nodules than the synthetic ones (Fig. 2B), suggesting that a mixture of various NFs may be more efficient in triggering nodule formation than a single molecular species. The nonacetylated LCO-IV(S,C16:2) was less efficient than the acetylated form in eliciting nodule formation, confirming the importance of the *O*-acetyl substitution for morphogenic activity. Root treatment with chemically synthesized nonsulfated LCO-IV(Ac,C16:2) and LCO-IV(C16:2) did not result in a detectable induction of nodule formation, confirming that *O*-sulfation of NFs is required for nodule induction in alfalfa.

Activity of Synthetic NFs on Vetch

The original definition of NFs as specific signals was strengthened by observing the positive and negative relationships between the NF structures and the host range. *S. meliloti nodH* mutants have acquired the ability to infect and nodulate vetch, a nonhost (Faucher et al., 1988). An *S. meliloti nodH*::Tn5 mutant produces nonsulfated NFs that elicit root-hair initiation and thick and short root formation on vetch (Faucher et al., 1989; Roche et al., 1991a). Using these two vetch bioassays we compared the biological activity of synthetic sulfated and nonsulfated NFs using natural nonsulfated NFs purified from a *nodH* mutant as a control. Synthetic, nonsulfated LCO-IV(C16:2,Ac) and LCO-IV(C16:2) factors induced significant root-hair initiation (P = 0.01) at concentrations as small as 10^{-11} M, as did the natural, nonsulfated NFs (Table I). In contrast, the sulfated NFs had no detectable effect, even at 10^{-9} M, showing that the nonsulfated factors are at least 1000-fold more active than the sulfated factors in eliciting biological responses on this nonhost.

Because natural NFs do not elicit nodule formation on vetch (Spaink et al., 1991), we used the thick and short root assay for an assessment of NF activity on vetch root morphology. At 10^{-9} M, nonsulfated synthetic factors induced a significant (P = 0.01) thick and short root effect, whereas the sulfated factors had no detectable effect (Fig. 4). Thus, as is the case with natural NFs, nonsulfated synthetic NFs are able to elicit morphological changes on the roots of vetch. The natural, nonsulfated NFs had a stronger thick and short root effect than the synthetic NFs (significant at the P = 0.01 level), suggesting that in this assay a mixture of NFs, including tetramers and pentamers and *N* substitutions by various unsaturated fatty acids, might be more active than single molecular species.

Synthesis of LCOs Differing in Their Acyl Moiety

The above results confirm the general usefulness of synthetic molecules for studies of symbiotic signals (Stokkermans et al., 1995). Therefore, we chose to use this approach to examine the effects of *N*-acyl chain structure in isolation from other changes in the LCO molecule. *S. meliloti nodFE*

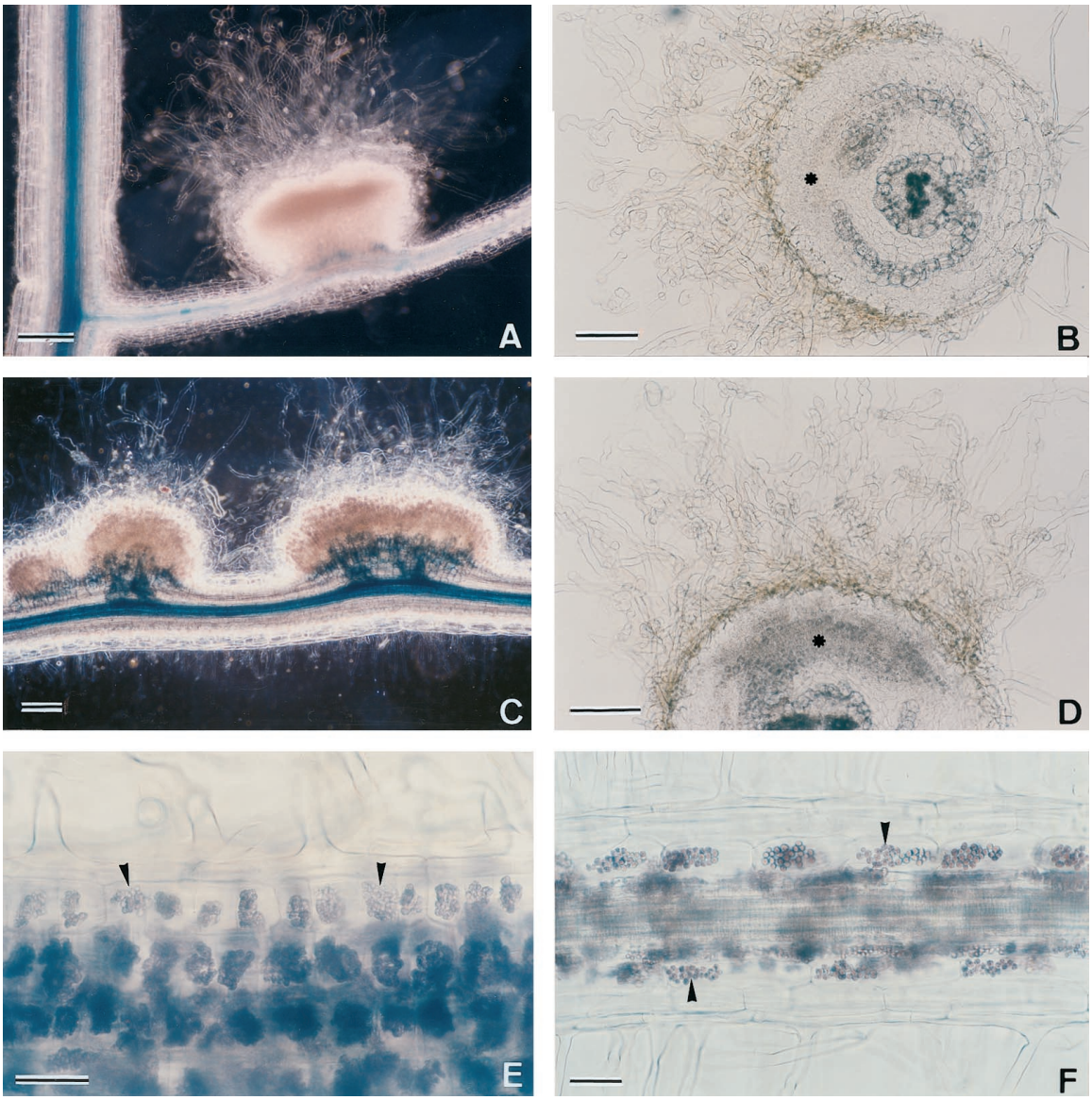


Figure 3. Comparison between biological activities of natural *S. meliloti* NFs (A and B) and synthetic NFs and related LCOs (C–F). After fixation, entire plants (A, C, E, and F) or root sections (B and D) were cleared with sodium hypochlorite (A–F), stained with methylene blue (A and C), stained with potassium iodide (C–F), and finally viewed by dark-field microscopy (A and C) or bright-field microscopy (B, D, E, and F). A to D, Abundant development of root hairs at the distal part of nodules (A and C) or in the vicinity of the root area where cortical cell divisions (asterisks) are observed. A and B, Plants treated with natural NFs. C and D, Plants treated with synthetic NFs. E and F, Amyloplasts (arrowheads) in dividing cortical cells of a plant treated with LCO-IV(S,C16:1Δ2E) (E) and in nondividing inner cortical cells of a plant treated by LCO-IV(S,C8:1Δ2E) (F). In A and C, bars = 250 μm; in B and D, bars = 100 μm; in E and F, bars = 50 μm.

mutants produce NFs that are *N*-acylated by 18:1 (vaccenic acid) instead of unsaturated C16 fatty acids (Demont et al., 1993). These mutants exhibit a strong decrease in the ability to infect alfalfa root hairs, indicating that the structure of the *N*-acyl chain is important for bacterial entry into root hairs (Ardourel et al., 1994). NFs from *nodFE* mutants elicit nodule formation with a reduced efficiency, suggesting that the structure of the *N*-acyl chain is also important at the step of nodule organogenesis induction (Ardourel et al., 1994).

To obtain reagents to study acyl chain effects, we synthesized LCOs differing in the length and in the presence of double bonds in the acyl moiety. The synthesis of the two *O*-acetylated LCOs described in the previous section required six chemical steps after the introduction of the acyl chain (Nicolaou et al., 1992). To provide direct and easy access to a collection of LCOs with different *N*-acyl chains in a single operation with a single precursor, we used another synthetic route in which the last step was the introduction of the acyl chain. This approach was not com-

Table I. *Root-hair initiation assays on vetch*

All LCOs tested had a C16:2 (Δ2E,9Z) acyl chain. Ten plants were used for each treatment and each dilution. Forty untreated control plants were used to estimate the intrinsic plant variability for the root-hair initiation character. The responses were classified "+" when the proportion of root hair initiation was significantly higher (at P = 0.05) among the treated plants compared with the untreated control. Data were analyzed using Fisher's exact test.

	10^{-9} M	10^{-10} M	10^{-11} M	10^{-12} M
NodRm *nodH* Nat	+[a]	+	+	−[b]
LCO-IV(Ac)	+	+	+	−
LCO-IV	+	+	+	−
LCO-IV(S,Ac)	−	−	−	−
LCO-IV(S)	−	−	−	−

[a]+, Strong induction of root-hair formation and growth. [b]−, No strong induction of root-hair formation and growth.

patible with the presence of an *O*-acetyl group on the same GlcNAc unit because in the basic conditions used the acetyl group either migrated to the amino group or was partially removed. Thus, the synthetic LCOs described below did not contain an acetyl group at the C6 position of the nonreducing GlcNAc residue. We synthesized the LCOs by treating sulfated tri-*N*-acetyl-chitotetraose with the appropriate acid chlorides following a published procedure (Tailler et al., 1994). The synthesized compounds are described in Figure 1.

The structure of the purified compounds was confirmed by the ^{1}H-NMR spectra in MeOH-*d4* showing the expected signals for the carbohydrate domain and the olefinic protons of the acyl chain at 6.83 to 6.80 ppm (H-3, dt, *J* 15.5, and 7 Hz) and 5.99 to 5.95 ppm (H-2, dt, *J* 15.5, and 1.5 Hz) for the E-conjugated double bond (when present) and at 5.35 ppm (H-9 and H-10, m) for the Z internal double bond (when present). The presence of the sulfate group was readily ascertained by the downfield shift of the H-6 protons of the sulfated reducing sugar unit at 4.21 to 4.25 ppm (H-6a of the major α anomer, dd, *J* 3.0, and 10.5 Hz) and 4.06 to 4.09 ppm (H-6b of the major α anomer, dd, *J* 2, and 10.5 Hz) (Tailler et al., 1994; D. Tailler, J.C. Jacquinet, and J.M. Beau, unpublished data). The structure and the purity of each compound have also been tested by MS in the positive ion mode after alkali cationization with alkali salts. The specific fragmentations of the molecules resulted in the loss of the sulfate group and the cleavage of the interglycosidic residues corresponding to a tetramer of GlcNAc and revealed an acyl moiety having the expected masses for the various derivatives (data not shown).

Acyl Chain Structural Requirements for Alfalfa Nodulation

An exogenous supply of unsubstituted chitin oligomers does not elicit symbiotic responses in vetch (Spaink et al., 1991) or alfalfa (Journet et al., 1994). Given the importance of the sulfate group for inducing symbiotic responses in alfalfa, we first checked whether the *o*-sulfation on the C6 of the reducing GlcNAc residue of chitotetraose having no *N*-acyl substitution (with a free NH_2 group at the nonreducing end) would be sufficient to generate activity on this plant. To increase the chance of detecting a response, a high concentration (10^{-7} M) of CO-IV(S,NH_2) was used and a large number (n = 100) of alfalfa seedlings were treated. No significant difference could be detected (P = 0.05) with the untreated control. This result indicates that CO-IV(S,NH_2) neither induced nodule organogenesis nor increased the frequency of appearance of spontaneous nonrhizobium-induced nodules. Thus, the presence of an acyl group seems to be an essential requirement for nodule formation in alfalfa.

The most abundant *N*-acyl chain found in natural *S. meliloti* NFs is C16:2Δ2,9. To determine the role of the double bond number and position in the acyl chain, synthetic LCOs containing C16 acyl chains with different types

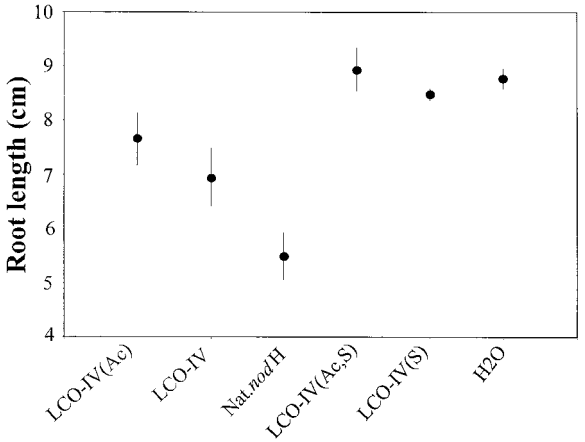


Figure 4. Induction of root shortening on vetch by sulfated and nonsulfated synthetic *S. meliloti* NFs. Nat.*nod*H, Natural nonsulfated NFs from a *nodH* mutant of *S. meliloti*; LCO-IV, synthetic NFs (see Fig. 2 legend). Vetch roots were treated with 10^{-9} M NFs and LCOs. All LCOs tested had a C16:2Δ2E,9Z acyl chain (see Fig. 1A). The vertical bars correspond to confidence intervals at the 5% probability level.

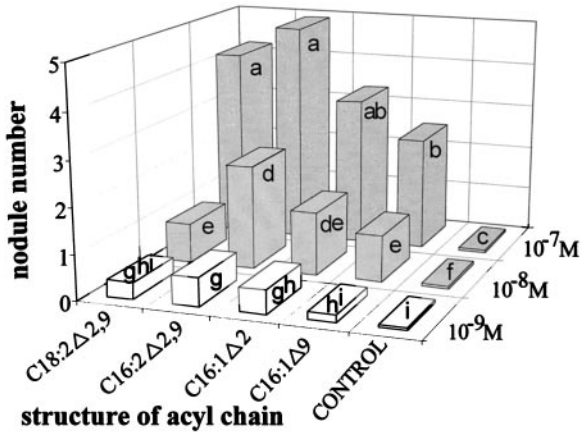


Figure 5. Influence of the number and location of unsaturations on the *N*-acyl chain on the morphogenic activity of synthetic LCOs. LCOs are tetrameric and sulfated (see Fig. 1B) and only the structure of the *N*-acyl chain is represented. Nodule number 23 d after addition of LCOs (see Fig. 2B for comparison with NF activity) is shown. Experimental treatment and statistical analysis are the same as in the legend of Figure 2.

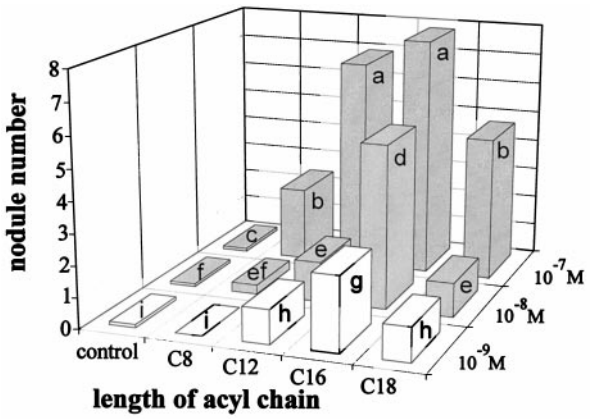


Figure 6. Influence of the length of the *N*-acyl chain on the morphogenic activity of synthetic LCOs. LCOs are tetrameric and sulfated (see Fig. 1B), and only the structure of the *N*-acyl chain is represented. Nodule number 26 d after addition of LCOs is shown (see Fig. 2B for comparison with NF activity). Experimental treatment and statistical analysis are the same as in the legend of Figure 2.

of unsaturation were tested: LCO-IV(S,C16:2Δ2E,9Z), LCO-IV(S,C16:1Δ2E), and LCO-IV(S,C16:1Δ9Z) at 10^{-7} to 10^{-9} M. Results presented in Figure 5 clearly show that at all concentrations tested LCO-IV(S,C16:2Δ2E,9Z) had a better nodulation efficiency than the monounsaturated LCO-IV(S,C16:1Δ9Z), with the unsaturation located in the middle of the chain at position C9. It can be concluded that the presence of the two double bonds in the acyl moiety of LCO results in an enhanced efficiency of nodulation. LCO-IV(S,C16:1Δ2E), with a double bond conjugated to the carbonyl group, elicited the formation of slightly more nodules than NodRmIV(S,C16:1Δ9Z) at all concentrations, with the unsaturation located in the middle of the chain at position C9, but the differences were not significant at $P = 0.05$.

The next series of experiments addressed the question of the influence of the carbon chain length. We first compared LCOs having C16 or C18 acyl chains unsaturated at positions C2 and C9. LCO-IV(S,C16:2Δ2E,9Z) and LCO-IV(S,C18:2Δ2E,9Z) were applied to alfalfa seedlings at 10^{-7} to 10^{-9} M. NodRm-IV(S,C16:2Δ2E,9Z) was more active than LCO-IV(S,C18:2Δ2E,9Z) at all concentrations, but the difference was only significant at 10^{-8} M (Fig. 5).

We further analyzed the influence of the carbon chain length by testing a series of LCOs with a single Δ2 unsaturation and varied carbon chain lengths: C8, C12, C16, and C18. Figure 6 shows that synthetic C16-LCO was clearly the most active compound. Comparison of data obtained at 10^{-8} to 10^{-9} M indicated that the C16-LCO was more than 10 times as active as the other LCOs. C12-LCO and C18-LCO had similar activities at 10^{-8} and 10^{-9} M. The activity of the C8-LCO was very weak: nodule formation could be observed only at 10^{-7} M. We can conclude that, of LCOs having one conjugated double bond and different carbon chain lengths, the LCO with a C16 chain was the most efficient in triggering nodule formation in alfalfa.

Histological studies have determined the ability of LCOs with a single Δ2E substitution and varied carbon lengths to elicit another cortical response, the accumulation of starch in plastids (Ardourel et al., 1994). A correlation was found between the ability of LCOs to trigger nodule primordium formation and their ability to provoke amyloplast formation. Strong starch accumulation was observed in plastids of dividing cortical cells in plants treated with the most morphogenic compound, LCO-IV(S,C16:1Δ2E) (Fig. 3E), whereas the lowest (but still significant) accumulation was seen in cortical cells of plants treated with the less morphogenic LCO-IV(S,C8:1Δ2E) (Fig. 3F).

DISCUSSION

Chemically synthesized *S. meliloti* NFs have been reported to elicit alfalfa root-hair deformation (Bono et al., 1995). We have shown here that they elicit nodule organogenesis in alfalfa roots in the same range of concentrations (down to 10^{-9} to 10^{-10} M) as their natural analogs. The biological activity of the synthetic products is definitive evidence that the chemical structures initially proposed for the natural *S. meliloti* NFs were correct (Lerouge et al., 1990; Roche et al., 1991b; Schultze et al., 1992) and that NFs alone act as potent morphogenic compounds and elicit alfalfa nodule organogenesis (Truchet et al., 1991).

Natural NFs are made up of a mixture of related LCOs. For example, *S. meliloti* NFs are all sulfated but comprise tetra- and pentamers (Roche et al., 1991b; Schultze et al., 1992) with or without an *O*-acetyl group (Roche et al., 1991b; Truchet et al., 1991) and are *N*-acylated by a variety of C16 unsaturated fatty acids (Lerouge et al., 1990; Schultze et al., 1992; Demont et al., 1993) or a series of (ω-1)-hydroxylated fatty acids (Demont et al., 1994). We found that such a mixture of NFs was slightly more active than the synthetic analog of the major natural product, NodRm-IV(Ac,S,C16:2Δ2,9), a single molecular species. Similar results have already been described with different legume hosts. Mixtures of NFs from *B. japonicum* elicited the transcription of early nodulin genes in soybean more efficiently than did single molecular species (Minami et al., 1996a), which could explain the diversity observed in NFs synthesized by a single rhizobial strain: different NFs could differentially activate plant genes involved in the symbiotic process.

The NF structural requirements to trigger responses in alfalfa are varied. Most responses, e.g. root-hair membrane depolarization, root-hair deformation, induction of *ENOD12* transcription, and nodule formation, require the presence of the *O*-sulfate substitution. In contrast, with regard to the structure of the acyl chain, two types of responses have been identified. Nonstringent responses that seem not to require the presence of the specific unsaturated C16 acyl moiety include root-hair deformation (Ardourel et al., 1994), induction of *ENOD12* transcription in epidermal cells (Journet et al., 1994), and starch accumulation in cortical cells (Ardourel et al., 1994). Stringent responses such as root-hair plasma membrane potential changes (Felle et al., 1995), bacterial entry into root hairs, and initiation of infection thread formation and nodule formation (Ardourel et al., 1994) require the presence of the polyunsaturated C16:2 chain. These differences are not tissue specific, because both stringent and nonstringent

requirements have been found in both epidermal and cortical cells. The use of synthetic LCOs has confirmed that induction of nodule formation is also a stringent response and we now can address the question of the respective roles of carbon chain length and the degree and position of unsaturations.

Requirement for the Presence of an *N*-Acyl Group

All NFs identified so far from various rhizobial strains are *N*-acylated. This observation is not due simply to a bias in the NF-extraction procedures that eliminated the nonacylated chitin oligosaccharides. A major nodulation gene, *nodA*, was present in all rhizobial strains studied so far and this gene specifies the transfer of an acyl moiety on the amino group of the terminal, nonreducing GlcNAc residue of the oligochitin backbone (Atkinson et al., 1994; Röhrig et al., 1994; Debellé et al., 1996; Ritsema et al., 1996; Roche et al., 1996). When supplied exogenously to legume roots, unsubstituted chitin oligomers were unable to elicit plant responses such as root-hair deformations, cortical cell divisions, and the formation of a nodule primordium (Lerouge et al., 1990; Spaink et al., 1991; van Brussel et al., 1992; Relic et al., 1993). Similarly, *O*-acetylated chitin oligomers were unable to trigger cortical cell divisions when added exogenously (Schlaman et al., 1997). Given the importance of the sulfate moiety in triggering responses in alfalfa, we have tested the morphogenic activity of a chitin tetramer *O*-sulfated at C6 of the reducing GlcNAc residue and no nodule or bump could be detected. The addition of a short (C8) acyl chain was sufficient to provide some morphogenic activity at a high concentration (10^{-7} M). Thus, the presence of an acyl moiety seems essential for triggering symbiotic responses when LCOs are added exogenously. It is worth noting that a simple, nonacylated chitin pentamer was found to transiently elicit *ENOD40* transcription in soybean roots (Minami et al., 1996b). However, sustained expression of *ENOD40* required an acylated Nod signal (Minami et al., 1996b), and *ENOD40* could also be expressed in nonsymbiotic conditions (Asad et al., 1994; Papadopoulou et al., 1996).

In rhizobia that produce NFs acylated by fatty acids of the general lipid metabolism, such as 18:1 (vaccenic acid), 16:0 (palmitic acid), and 18:0 (stearic acid) , it is unlikely that the acyl moiety plays an important role in specific recognition by the host plant. The same fatty acid substitutions are present in NFs of diverse rhizobial strains irrespective of their host range, and the nature of the fatty acid chain does not seem to play a significant role in biological activity (Stokkermans et al., 1995; Cohn et al., 1998). However, the presence of an acyl group is nonetheless essential for NF activity. What is the role of the acyl chain? We hypothesize that one of the functions of the *N*-acyl chain is to provide a hydrophobic tail, making possible the insertion of NF into the lipid bilayer of the plant plasma membrane (lipid trapping of the ligand). Such an insertion would facilitate two-dimensional lateral diffusion of NFs toward a (putative) membrane receptor and would also give an orientation to NFs that might facilitate ligand-receptor interactions. *N*-acylation could also influence the stability of the signal against degradation by chitinases (Staehelin et al., 1994).

Recently, it was shown that, whereas *O*-acetylated (and nonacylated) chitin oligomers do not induce cortical cell division in vetch when applied exogenously, they do act as mitogens when delivered inside the roots by ballistic microtargeting (together with uridine, a putative stele factor required for cortical cell division; Schlaman et al., 1997). This result suggests that the acyl group of NFs is involved in the transport (internalization) of the signal and that substituted oligosaccharides could activate an intracellular receptor(s). A transport role of the NF hydrophobic tail is also supported by the recent observation, using fluorescent probes, that the acyl chain was required for uptake of LCOs by root cells (Philip-Hollingsworth et al., 1997).

Influence of the Structure of the *N*-Acyl Group

In rhizobia that nodulate legumes of the tribes Galegeae, Trifolieae, and Vicieae, NFs are acylated by specific polyunsaturated fatty acids. In these symbiotic associations the structure of the acyl chain appears to be important for host specificity and biological activity. We have observed that the presence of two double bonds, one conjugated to the carbonyl group (Δ2E) and the other located in the middle of the acyl chain (Δ9Z), makes the LCO more active than an LCO with a single double bond. The Δ2E-conjugated unsaturation might influence the rotation of the acyl chain around the chitin backbone.

The length of the acyl chain is very important for morphogenic activity. If a short C8 chain was sufficient to provide some activity, increasing the chain length to C12 and C16 resulted in a dramatic increase. C16 was clearly the optimal length, about 10-fold more active than C18 and C12. It is unlikely that the NF acyl chain length corresponds to an adaptation to the fatty acid composition of the host plasma membrane. The genera *Medicago* and *Trifolium* are closely related within the Trifolieae tribe, and fatty acid composition studies of plants do not suggest drastic differences between related plants of similar habits, with a majority of C16 in alfalfa and C20 in clover. This suggests that a specific recognition mechanism of subtle structural differences in the structure of the NF acyl chain exists in the roots of alfalfa and of other legumes of the Galegeae, Trifolieae, and Vicieae tribes. We are currently using a genetic approach to identify *Medicago truncatula* genes involved in NF perception and transduction and have found *M. truncatula* ecotypes exhibiting different requirements for the *O*-acetyl and *N*-acyl substitutions. Their genetic analysis should allow the identification of a gene(s) involved in *N*-acyl chain recognition and open the way to positional cloning and molecular characterization of these genes.

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