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Structural identification of the lipo-chitin oligosaccharide nodulation signals of *Rhizobium loti*

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Summary

***Rhizobium loti* is a fast-growing *Rhizobium* species that has been described as a microsymbiont of plants of the genus *Lotus*. Nodulation studies show that *Lotus* plants are nodulated by *R. loti*, but not by most other *Rhizobium* strains, indicating that *R. loti* produces specific lipo-chitin oligosaccharides (LCOs) which are necessary for the nodulation of *Lotus* plants. The LCOs produced by five different *Rhizobium loti* strains have been purified and were shown to be *N*-acetylglucosamine pentasaccharides of which the non-reducing residue is *N*-methylated and *N*-acylated with *cis*-vaccenic acid (C18:1) or stearic acid (C18:O) and carries a carbamoyl group. In one *R. loti* strain, NZP2037, an additional carbamoyl group is present on the non-reducing terminal residue. The major class of LCO molecules is substituted on the reducing terminal residue with 4-*O*-acetylfucose. Addition of LCOs to the roots of *Lotus* plants results in abundant distortion, swelling and branching of the root hairs, whereas spot inoculation leads to the formation of nodule primordia.**

Introduction

Bacteria belonging to the genera *Rhizobium, Bradyrhizobium* and *Azorhizobium*, collectively called rhizobia, are able to invade the roots of their legume hosts and trigger the formation of a new organ, namely the nodule. In these root nodules a differentiated form of the rhizobium, the bacteroid, is able to fix nitrogen into ammonia which can then be utilized by the plant. During the onset of this symbiosis, flavonoids, secreted by the host plant roots, cause the induction of rhizobial nodulation genes (called *nod* and *nol* genes). Several of these genes have been shown to be involved in the synthesis and excretion of bacterial nodulation signals. These molecules have been characterized from *Rhizobium meliloti* (Lerouge *et al.*, 1990; Schultze *et al.*, 1992), *Rhizobium leguminosarum* bv. viciae (Spaink *et al.*, 1991; Firmin *et al.*, 1993), *Rhizobium* NGR234 (Price *et al.*, 1992), *Bradyrhizobium japonicum* (Sanjuan *et al.*, 1992; Carlson *et al.*, 1993), *Azorhizobium caulinodans* (Mergaert *et al.*, 1993), *Rhizobium fredii* (Bec-Ferté *et al.*, 1993) and *Rhizobium tropici* (Poupot *et al.*, 1993). They are all β-1,4-linked oligomers of three to five *N*-acetylglucosamine residues with an amide-linked fatty acyl moiety on the non-reducing terminal residue. In view of their resemblance to chitin, these molecules are referred to as lipo-chitin oligosaccharides (LCOs). Specific modifications of the LCOs produced by different rhizobia appear to determine host-specificity. Structural variations influencing the biological activity of the LCOs are sulphation of the reducing terminal residue (Roche *et al.*,1991), variations in the degree of unsaturation of the fatty acid (Spaink *et al.*, 1991; Truchet *et al.*, 1991), *O*-acetylation of the non-reducing terminal residue (Spaink *et al.*, 1991), and variations in the length of the oligosaccharide 'backbone' (Schultze *et al.*, 1992).

Purified LCOs are able to elicit various responses from the host plant root. For instance, they can induce the deformation of root hairs, the formation of pre-infection threads, and the division of root cortical cells (Carlson *et al.*, 1993; Lerouge *et al.*, 1990; Relic *et al.*, 1993; Sanjuan *et al.*, 1992; Schultze *et al.*, 1992; Spaink *et al.*, 1991; Truchet *et al.*, 1991; van Brussel *et al.*, 1992). On *Medicago*, *Sesbania* and *Glycine soja* the host-specific Nod factors trigger the formation of nodule-like structures (Truchet *et al.*, 1991; Mergaert *et al.*, 1993; Stokkermans and Peters, 1994).

Lotus japonicus has been proposed as a model leguminous plant for classical and molecular genetic studies. For this purpose it has several advantages, including its small genome size and the existance of systems for high-frequency transformation with *Agrobacterium*, and for regeneration from protoplasts and cell culture (Handberg and Stougaard, 1992; Jiang and Gresshoff, 1993). *Lotus* plants can be nodulated by *Rhizobium loti*, but not by

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most other rhizobial species. This suggests that *R. loti* produces specific LCO signals.

In this paper we elaborate on the bacterial requirements for nodulation of *Lotus* plants. We describe the structure of the LCOs produced by *R. loti* and their biological activity on *Lotus* and *Vicia* plants.

Results

Host specificity of R. loti *for nodulation on* Lotus *plants*

R. loti strain E1R, which was originally isolated from nodules of *Lotus corniculatus*, appears to have a very narrow host range for nodulation being limited mainly to plants of the genus *Lotus* (data not shown). *L. corniculatus* plants were inoculated with several broad-host-range strains to establish the general bacterial requirements for nodulation of *Lotus* plants. *L. corniculatus* plants are not nodulated by any of the strains tested (*Azorhizobium* ORS571, *R. fredii* USDA191, *Rhizobium* NGR234 and *R. tropici* CIAT 899), whereas normal nodulation is seen with *R. loti* E1R. Using the test system in Magenta jars, described by Lewin *et al.* (1990), we observed delayed induction of pseudonodules by *Rhizobium* strain NGR234. To test whether the lack of nodulation in our system by broad-host-range strains is caused by the absence of *nod* gene-inducing flavonoids, we introduced plasmid pMP604 into each of the strains listed above. Plasmid pMP604 contains a *nodD* FITA (flavonoid-independent transcription activation) gene, which confers the ability to produce LCOs in the absence of inducer. The ability of these strains to produce LCOs in the absence of inducer was shown by radiolabelling of cells with [1-^{14}C]-D-glucosamine and subsequent TLC analysis of *n*-butanol extracts (Fig. 1A). The nodulation results show that only strain *R. loti* E1R.pMP604 is able to induce nodulation. In conclusion, in our test system, *L. corniculatus* is only nodulated by *R. loti* and not by any of the broad-host-range strains tested.

Production of lipo-chitin oligosaccharides

Using thin-layer chromatography (TLC) we have demonstrated the production of root exudate-dependent [1-^{14}C]-D-glucosamine-labelled metabolites by *R. loti* strain E1R. The results (Fig. 1B) show two inducible radiolabelled spots on reversed-phase TLC which, on the basis of their migration behaviour, we assume to represent the LCOs of *R. loti*. Various flavonoids and one betaine that was reported to be a *nod* gene inducer (Phillips *et al.*, 1992) were tested for their effect on the production of inducible metabolites in strain *R. loti* E1R. However, none of these compounds is able to induce the production of LCOs.

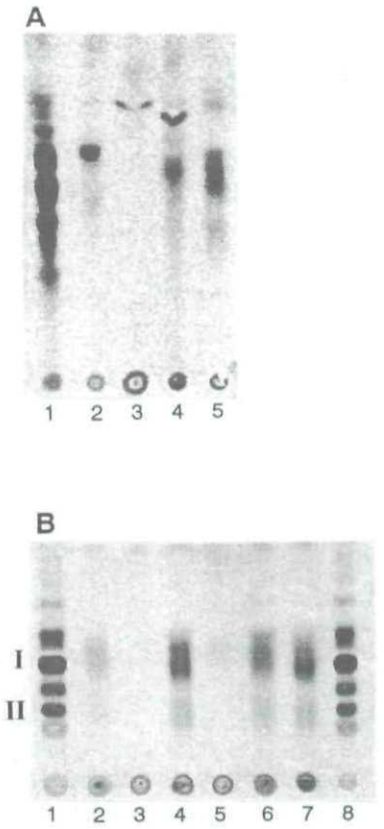

Fig. 1. Detection of ^{14}C-labelled LCOs produced by various strains. A. *A. caulinodans* ORS571.pMP604 (lane 1); *R. fredii* USDA 191.pMP604 (lane 2); *Rhizobium* sp. NGR234.pMP604 (lane 3); *R. tropici* CIAT 899.pMP604 (lane 4); and *R. loti* E1R.pMP604 (lane 5). B. *R. loti* E1R induced with 2 ml of *L. corniculatus* root exudate (lane 2); *R. loti* E1R.pMP280 induced with naringenin (lane 3); *R. loti* E1R.pMP283 induced with naringenin (lane 4); *R. loti* E1R.pMP284 induced with luteoline (lane 5); *R. loti* E1R.pMP604 without inducer (lane 6); and *R. loti* E1R.pMP2112 induced with naringenin (lane 7). Lanes 1 and 8 contain reference compounds which consist of ^{14}C-labelled LCOs isolated from *Rhizobium* RBL5560 (Spaink *et al.*, 1991).

The TLC experiments in Fig. 1A show that *R. loti* strain E1R.pMP604 produces ^{14}C-labelled metabolites having TLC retention times similar to those of the wild-type metabolites produced after induction with exudates (Fig. 1B). The following additional plasmids containing *nodD* genes were transferred to strain E1R: pMP280 (*nodD* of *R. leguminosarum* bv. viciae); pMP283 (*nodD* of *R. leguminosarum* bv. trifolii); and pMP284 (*nodD1* of *R. meliloti*). The results (Fig. 1B) show that the best production of radiolabelled metabolites is achieved with *nodD* from bv. trifolii, while there is hardly any production with *nodD* from *R. meliloti* or with *nodD* from *R. leguminosarum* bv. viciae (Fig. 1B). However, a major problem with the use of all of the IncP-class plasmids is the very poor bacterial growth in liquid cultures (generation times longer than 24 h). For this reason, we constructed plasmid pMP2112 of the IncW class, containing the *R. leguminosarum* bv. trifolii *nodD*

Table 1. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Relevant characteristics	Source/Reference
Strain		
E1R	Rif^R derivative of wild-type *R. loti* isolate E1	This work
CIAM 1801	Wild-type *R. loti*	Novikova, St. Petersburg
NZP2037	Wild-type *R. loti*	Scott, *et al.* (1985)
NZP2235	Wild-type *R. loti*	DSIR culture collection
NZP2238	Wild-type *R. loti*	DSIR culture collection
ORS571	Wild-type *A. caulinodans*	Mergaert *et al.* (1993)
USDA191	Wild-type *R. fredii*	Bec-Ferté *et al.* (1993)
NGR234	Wild-type *Rhizobium* sp.	Price *et al.* (1992)
CIAT899	Wild-type *R. tropici*	Gil-Serrano *et al.* (1990)
RBL5560	LPR5045 harbouring Sym plasmid pJB5JI (=pRL1JI *mep*::Tn*5*)	Spaink *et al.* (1987a)
Plasmid		
pMP280	IncP, contains *nodD* from *R. leguminosarum* bv. viciae, Tc^R	Spaink *et al.* (1987b)
pMP283	IncP, contains *nodD* from *R. leguminosarum* bv. trifolii, Tc^R	Spaink *et al.* (1987b)
pPM284	IncP, contains *nodD1* from *R. meliloti*, Tc^R	Spaink *et al.* (1987b)
pMP604	IncP, contains *nodD* FITA, Tc^R	Spaink *et al.* (1989)
pMP2112	IncW, contains *nodD* from *R. leguminosarum* bv. trifolii, $Spec^R$	This work

Tc^R, tetracycline resistance; $Spec^R$, spectinomycin resistance; Rif^R, rifampicin resistance.

gene. *R. loti* E1R harbouring the plasmid pMP2112 nodulates *Lotus* plants as efficiently as the parental wild-type strain, has a growth rate in liquid culture which is similar to that of the wild type, and produces LCOs as abundantly as *R. loti* E1R.pMP283 (Fig. 1B). Therefore, strain *R. loti* E1R.pMP2112 was used for the purification of LCOs. In order to study the LCOs produced by different *R. loti* strains the plasmid pMP2112 was introduced into four additional *R. loti* strains (see Table 1, *Experimental procedures*). The ^{14}C-labelled spots observed in the TLC analysis of the butanol extract of the four *R. loti* strains harbouring plasmid pMP2112 have similar mobilities to those spots observed from strain *R. loti* E1R.pMP2112. The patterns of these spots are indistinguishable from those observed after induction with root exudates of the corresponding wild-type strain (data not shown).

Purification of LCOs

LCOs from strain *R. loti* E1R.pMP2112 were obtained from an 8 l culture grown in the presence of naringenin. The entire culture was extracted with *n*-butanol and the LCOs in this extract were subsequently purified using the methods described in the *Experimental procedures* section. The final high-performance liquid chromatography (HPLC) profiles of the samples derived from uninduced cultures and from cultures induced with naringenin are shown in Fig. 2. Two inducible peaks are present, which are numbered I and II. These peaks correspond to the inducible radiolabelled spots I and II observed in TLC analysis (Fig. 1B). By following an identical method, the LCOs were obtained from 2 l cultures of each of the other *R. loti* strains harbouring plasmid pMP2112. The HPLC profiles of the *n*-butanol extracts from the different *R. loti* strains induced with naringenin are almost identical to the HPLC profile shown in Fig. 2A. Peaks I and II have identical retention times in all strains, except for strain *R. loti* NZP2037.pMP2112 (in which the peaks elute slightly later). The fractions containing these two peaks were collected from each strain for further analysis.

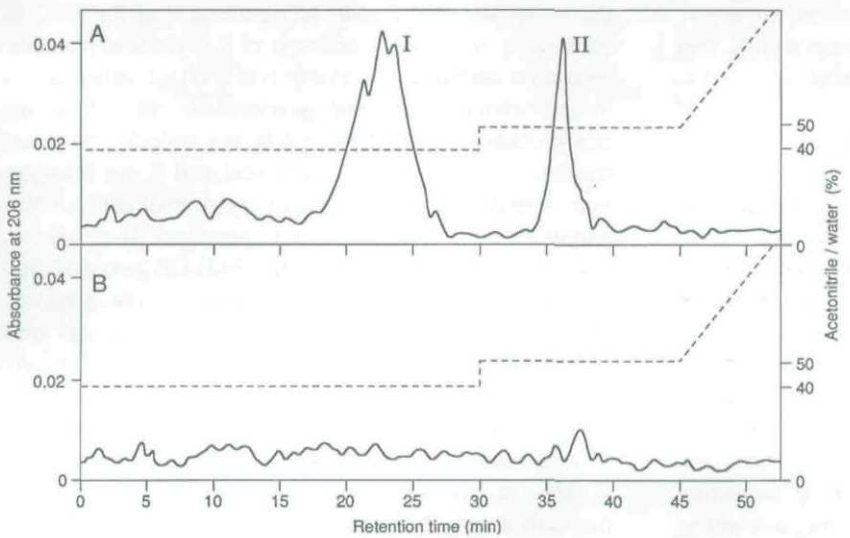

Fig. 2. HPLC profiles of *n*-butanol extracts from a *R. loti* E1R.pMP2112 culture induced with naringenin (A) or uninduced (B). The amount of sample loaded was equivalent to that isolated from 20 ml of culture (see the text for details).

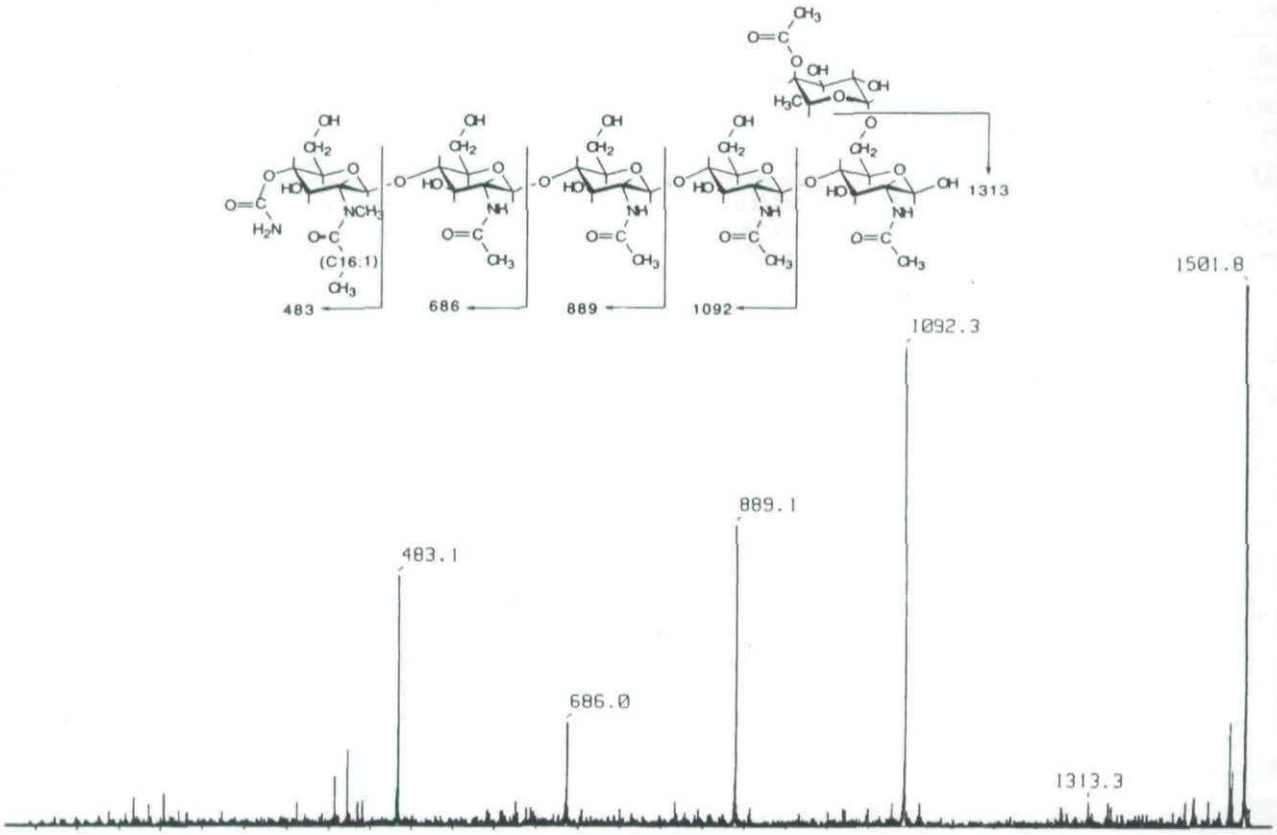

Fig. 3. CID mass spectrum and fragmentation scheme of parent ion m/z 1501 from peak I.

Mass-spectrometric analysis

The fractions corresponding to peak I from *R. loti* E1R.pMP2112 (Fig. 2) were analysed using fast atom bombardment mass spectra (FAB-MS) in the positive-ion mode. The spectra (data not shown) contain $[M+H]^+$ pseudomolecular ions at m/z 1501, 1459, and 1458, consistent with LCO species composed of five *N*-acetylglucosamine (GlcNAc) residues and one deoxyhexose, an acetyl, a carbamoyl, a methyl and a C18:1 fatty acid moiety, and related components lacking an acetyl group, or a carbamoyl group, respectively. Thioglycerol adduct ions of these species are observed at m/z 1609, 1567, and 1566, consistent with the presence of a double bond in the fatty acid chain (Fukuda *et al.*, 1985). Tetramethylsilane (TMS)-monosaccharides obtained after acid hydrolysis of peak I were analysed using gas chromatography–mass spectrometry (GC-MS), and their retention times and mass spectra were compared with those of authentic fucose and rhamnose standards. The results demostrate that the only deoxyhexose present is fucose. The collision induced dissociation (CID) mass spectrum (Fig. 3), obtained on collision of the ion at m/z 1501 $[M+H]^+$ with helium, contains oxonium-type fragment ions formed by sequential cleavage of each glycosidic linkage, with charge retention on the non-reducing portion of the molecule. These fragment ions, having m/z values of 1092, 889, 686, and 483, arise as shown in the fragmentation scheme (Fig. 3). The mass intervals between them allow the glycan sequence and sites of fatty acid, methyl, carbamoyl and acetyl substitution to be determined. The minor ion at m/z 1313 arises by β-cleavage of acetylfucose from the molecular ion. These data indicate that the LCO is composed of a linear backbone of five GlcNAc residues bearing a carbamoyl, a methyl and a C18:1 fatty acid on the non-reducing terminal glucosamine, as well as an acetylfucose residue attached to the reducing terminal residue. The nature of the fatty acid and acetyl linkages was established following mild base treatment of the pentasaccharide species under conditions which hydrolyse ester but not amide bonds. FAB-MS and CID-MS analysis of the product (data not shown) reveal a reduction of 42 amu in the mass of the pseudomolecular ions at m/z 1458 and 1501, indicating removal of an ester-linked acetyl moiety from the fucose residue. The LCO species with $[M+H]^+$ at m/z 1459 fails to show this 42 amu mass shift, as expected, since it lacks the acetyl substituent. When an aliquot of peak I was subjected to permethylation, the tandem (MS/MS) mass spectrum of

the resulting $[M+H]^+$ pseudomolecular parent ion at m/z 1668 contains fragment ions at m/z 1218, 972 and 727 (data not shown), indicating removal of the acetyl and carbamoyl moieties under the basic conditions used, and incorporation of methyl groups in accordance with the proposed structure, and allowing the formal possibility of the presence of an unsaturated C19 fatty acid in place of the C18 chain and a methyl group to be excluded. If the permethylation conditions are changed slighty, so that methyl iodide is added immediately following the addition of the NaOH, an $[M+H]^+$ pseudomolecular ion is observed at m/z 1725 corresponding to the fully methylated species, which has retained its carbamoyl group (which gains two methyl groups). A very small amount of the species lacking the carbamoyl group is also observed.

The location of the double bond in the fatty acyl chain was determined following mild alkaline methanolysis of an aliquot of peak I, which releases the fatty acid moiety from the LCO backbone, followed by methyl esterification and dimethyl disulphide derivatization (Scribe *et al.*, 1988). GC-MS analysis of the product yields a mass spectrum (data not shown) characteristic of a vaccenic acid (18:1 Δ11) derivative.

The positive-ion FAB mass spectrum of the fractions corresponding to peak II from strain E1R.pMP2112 contains $[M+H]^+$ pseudomolecular ions at m/z 1503, 1461, 1460 (data not shown), which correspond to closely related LCO species differing from those in peak I only with regard to the extent of unsaturation of their fatty acid moieties. The CID mass spectrum of the parent ion m/z 1503 contains oxonium-type fragment ions at m/z 1094, 891, 688 and 485. These data correspond to a mass shift of +2 amu of all ions compared to those in peak I, indicating the presence of a fully saturated C18 fatty acyl moiety. The absence of thioglycerol adducts of the pseudomolecular ions is in accordance with this conclusion.

In order to determine the position of the methyl group on the non-reducing terminal $GlcNH_2$, an aliquot of peak II was *O*-acetylated; the incorporation of only one *O*-acetyl group into the first oxonium ion would be consistent with the presence of an *O*-substituted methyl group, whereas the incorporation of two *O*-acetyl groups would indicate that the methyl group is substituted on the amino nitrogen. The positive-ion FAB spectrum of the *O*-acetylated derivative of peak II (not shown) contains an $[M+H]^+$ pseudomolecular ion at m/z 2007, which corresponds to the incorporation of a total of 12 acetyl groups. The first oxonium ion in this spectrum is observed at m/z 569, corresponding to the incorporation of two acetyl groups into the non-reducing terminal $GlcNH_2$ residue. From these data it is concluded that the methyl group is located on the nitrogen of the non-reducing terminal $GlcNH_2$.

Linkage analysis was performed on peak I from *R. loti* E1R.pMP2112. After permethylation, hydrolysis, reduction and acetylation of an aliquot of peak I, the resulting partially methylated alditol acetates were analysed using GC-MS (data not shown). Derivatives corresponding to non-reducing terminal HexNAc, 4-substituted HexNAc, and 4,6-substituted HexNAc residues are observed. Together with the FAB data these results demonstrate that the fucose residue is linked to C-6 of the reducing terminal GlcNAc. Linkage analysis carried out on the permethylated species still bearing its carbamoyl group gives similar results, although the amount of the derivative corresponding to non-reducing terminal HexNAc is much reduced. Derivatives corresponding to 3- or 6-substituted HexNAc are not observed, and the relative intensity of the peak corresponding to H-substituted HexNAc increases, consistent with the carbamoyl group being substituted on C-4 of the non-reducing terminal GlcNAc residue.

For *R. loti* strains NZP2235, NZP2238 and CIAM1801 the FAB mass spectra corresponding to peaks I and II contain pseudomolecular ions having m/z values identical to those for peaks I and II from strain E1R, indicating that these four strains all produce the same LCOs. The FAB mass spectra of peaks I and II from strain *R. loti* NZP2037 contain major pseudomolecular ions at m/z 1544 (peak I) and 1546 (peak II). This mass increase of 43 amu compared to the m/z values of the pseudomolecular ions obtained from strain E1R indicates the presence of a second carbamoyl substituent on these LCOs. CID-MS analysis of the parent ion at m/z 1544 produces oxonium-type fragment ions at m/z 1135, 932, 729, and 526, indicating substitution of the additional carbamoyl group on the non-reducing terminal residue.

NMR analysis

Nuclear magnetic resonance (NMR) analysis was performed on peak I from strain E1R.pMP2112. The data indicate a mixture of oligomers of β-linked *N*-acetylglucosamine, containing a fucosyl residue and a fatty acyl chain. There are two sharp peaks around δ2.1 from distinct *O*-acetyl methyl groups (data not shown). The peaks labelled F1–F4 in the COSY spectrum of Fig. 4 indicate that the 4-*O*-acetylfucosyl residue is a major component. A similar set of peaks can be attributed to 3-*O*-acetylfucose (data not shown). Other downfield signals in the region δ4.6–5.0 cannot be traced clearly to the signals of the β-anomeric protons of the glucosamine residues. Therefore, the position of the carbamoyl group cannot be confirmed by the NMR analysis.

Biological activity of the LCOs

The activity of LCOs was tested in a root-hair-deformation assay. Microscopic studies were carried out on the root

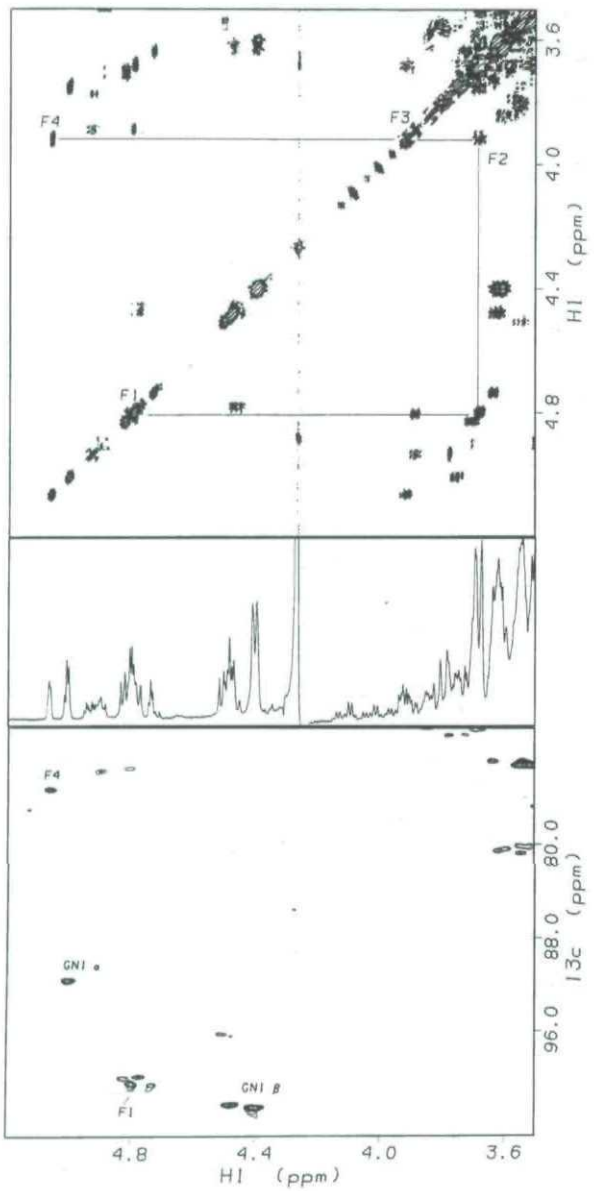

Fig. 4. Regions of a two-dimensional DQF-COSY (top), a one-dimensional proton (middle) and a two-dimensional HSQC carbon proton correlated spectrum (bottom) showing the presence of a 4-*O*-acetyl fucosyl residue. The downfield signal at δ5.06 (F4), attributed to H4 of fucose, has a corresponding carbon signal at δ75.4, consistent with a ring carbon. It is connected to crosspeak F1, which has a carbon chemical shift of δ100.5, characteristic of an α-fucosyl anomeric carbon.

systems of *L. corniculatus*, *L. japonicus* and *Vicia sativa* subsp. nigra seedlings grown for 1, 2 or 4 d in the presence of purified LCOs from *R. loti* E1R (10^{-7} to 10^{-9} M). As a control, NodRlv-V (C18:4, Ac) from *R. leguminosarum* bv. viciae was applied in the same concentration to plants of all three species. The results showed that *R. loti* and *R. leguminosarum* bv. viciae LCO elicit abundant root-hair deformation in plants of both genera. The deformations present in the root hairs of the plants treated with LCOs are distortions, branching and swellings, while these phenotypes are not found in untreated roots (data not shown). In comparison with the LCOs from *R. leguminosarum* bv. viciae applied at the same concentration, significantly more branching and swelling of root hairs is observed on *V. sativa* when these plants are treated with *R. loti* factors. In conclusion, the *R. loti* LCOs are active on the host plant and also on a non-host plant.

The observation of nodule primordia on cleared roots of *L. corniculatus* and *L. japonicus* plants inoculated with *R. loti* is very difficult, probably because of the low number of nodules developed by these plants. In a screening among 20 different *Lotus* plant species, *Lotus preslii* is the fastest- and best-nodulating plant, and nodule primordia are easily identifiable 5–6 d after inoculation with *R. loti*. Even at this early stage they are macroscopically visible because of the red coloration which appears at the surface of these structures (Fig. 5A). NodRlv-V(C18:4, Ac) and NodRlo-V (C18:1, NMe, Carb, AcFuc) (peak I from *R. loti*) were spot-inoculated on the roots of the plants using a newly developed technique as described in the *Experimental procedures*. After 6 d, all of the plants inoculated with purified *R. loti* LCOs develop a nodule primordium at the site of inoculation (Fig. 5, B and D). The LCO-induced nodule primordia are indistinguishable from *R. loti*-induced nodule primordia and are clearly different from lateral root initiations, since the focus of cell division is broad and originates in the outer cortex, while lateral initiations contain a dense conical primordium originating in the pericycle (Fig. 5, B and D). However, although the LCO-induced primordia continue growing, they never develop into the nodule structures induced by *R. loti* (Fig. 5, E and F). The root hairs at the sites of LCO-induced nodule primordia have the characteristic morphology caused by the addition of purified LCOs (i.e. distortions and branching) (Fig. 5D). In contrast, the root hairs at the sites of *R. loti*-induced nodule primordia show marked curling (Fig. 5C). Nodule primordia never formed in uninoculated roots or in roots spot-inoculated with *R. leguminosarum* bv. viciae LCOs, indicating that these structures are specifically determined by the purified *R. loti* LCOs.

Discussion

As a commercially available inducer of the *nod* genes was not found for strain *R. loti* E1R, we have introduced different *nodD* genes which are inducible with naringenin or luteolin. The results show that the strategy of introducing a foreign *nodD* gene leads to good production of LCOs. The best production of LCOs was achieved by the introduction of *nodD* from *R. leguminosarum* bv. trifolii cloned in an IncW plasmid. Furthermore, the TLC profile

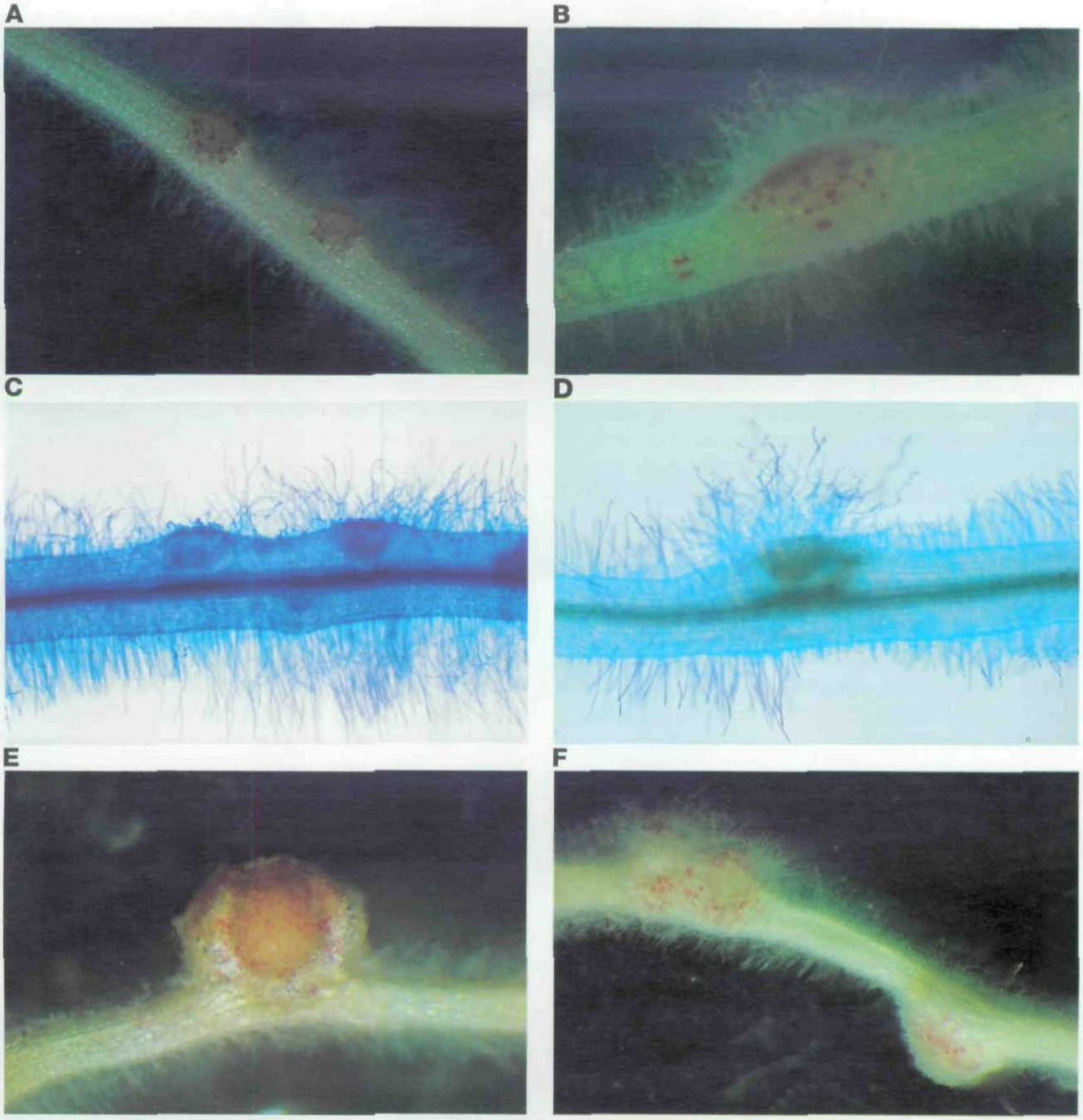

Fig. 5. Nodule primordia induced on *L. preslii*.
A, C and E. Nodule primordia induced by spot-inoculation with *R. loti* E1R.pMP2112.
B, D and F. Nodule primordia induced by spot-inoculation with purified *R. loti* LCOs.
C and D. The roots were cleared with hypochloride and stained with methylene blue.
E. This photograph was taken 15 d after inoculation, whereas the photograph of the LCO-induced structure (in F) was taken one month after the spot application.

produced was the same as that observed with the strain *R. loti* E1R induced with *Lotus* root exudates (Fig. 1B).

Chemical and structural analysis of the *nodD*-inducible metabolites produced by the strain *R. loti* E1R revealed them to be β-1,4 linked pentasaccharides consisting of one *N*-acyl-D-glucosamine and four *N*-acetyl-D-glucosamines. The major compounds are substituted with a carbamoyl group and a methyl group on the non-reducing terminal residue, and a 4-acetylfucose on the reducing terminal residue. The fatty acyl chain carried by the non-

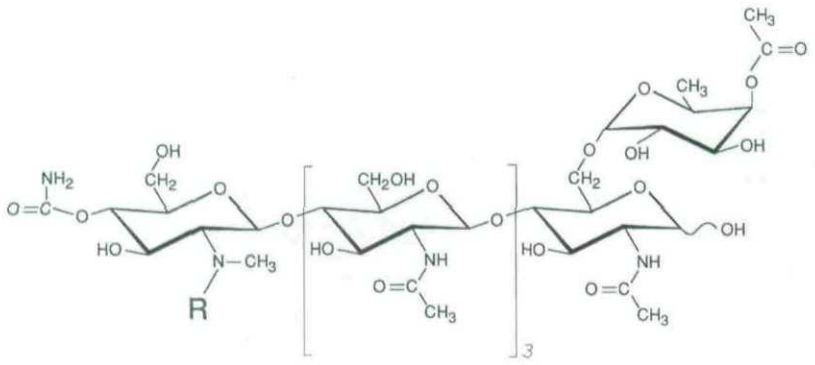

Fig. 6. Structure of the major LCOs produced by *R. loti*. Strains E1R, CIAM 1801, NZP2235 and NZP2238 produce the same type of LCOs. In the case of NZP2037 an additional carbamoyl is present on the non-reducing terminal saccharide. R = vaccenoyl or stearyl.

reducing terminal glucosamine is either vaccenic acid (C18:1) or stearic acid (C18:0). The structures of these LCO molecules are represented in Fig. 6. *R. loti* strains CIAM 1801, NZP2235 and NZP2238 produce LCOs which appear to have the same structures as strain E1R, while the LCOs produced by strain NZP2037 bear an additional carbamoyl group on the non-reducing terminal residue. Interestingly, it has been shown that *R. loti* NZP2037 has a broader host range than most of the other *R. loti* strains (Scott *et al.*, 1985). This suggests that the second carbamoyl group in the LCOs of strain NZP2037 might be involved in determining the broad host range. Knowledge of the structures of the *R. loti* LCOs, as well as an effective method for their isolation, is an important basis for further molecular studies on nodulation by the proposed model legume *L. japonicus*.

LCO spot I in the TLC system (Fig. 1B) and the corresponding HPLC peak I (Fig. 2) are broader than expected for a single compound. This is consistent with the results of the NMR and mass-spectrometric studies, which show that peak I is a mixture of compounds. Some minor compounds, such as the de-*O*-acetylated compounds or those lacking the carbamoyl group, could also have been generated during purification as a result of partial degradation.

The bacterial requirements of *L. corniculatus* plants for nodulation suggest that specific LCOs must be produced by *R. loti*. Indeed, the 4-*O*-acetylfucose residue has not been reported for LCOs produced by other rhizobial species. Other LCOs with a specific sugar attached to the reducing terminal residue are found in: (i) *B. japonicum*, where a 2-*O*-methylfucose (Sanjuan *et al.*, 1992; Carlson *et al.*, 1993) or fucose (Carlson *et al.*, 1993) is found; (ii) *R. fredii*, where fucose or 2-*O*-methylfucose is observed (Bec-Ferté *et al.*, 1993); (iii) *Rhizobium* sp NGR234, having 2-*O*-methylfucose which can be either sulphated (in position 3) or acetylated (in position 4) (Price *et al.*, 1992); and (iv) *A. caulinodans*, where arabinose is present (Mergaert *et al.*, 1993).

A carbamoyl substituent has also been found in the LCOs of *A. caulinodans*, *Rhizobium* sp. NGR234 and *B. japonicum*. Although this substitution is always found on the non-reducing terminal glucosamine, its position on this residue may differ. In the *R. loti* LCOs the carbamoyl group is substituted on C-4, while in *A. caulinodans* LCO it is on C-6 (Mergaert *et al.*, 1993). In *Bradyrhizobium elkanii* LCOs the carbamoyl group may be present on C-3, C-4 or C-6 (Carlson *et al.*, 1993). LCOs of the broad-host-range *Rhizobium* NGR234 may or may not be substituted by one or two carbamoyl groups on C-3 or C-4 positions in the non-reducing saccharide (Price *et al.*, 1992).

In the *R. loti* LCOs the *N* atom that carries the acyl chain is also methylated. The methyl group in this position has also been found in *Rhizobium* NGR234 (Price *et al.*, 1992), *R. tropici* (Poupot *et al.*, 1993), and *A. caulinodans* (Mergaert *et al.*, 1993), and it can be present in the LCOs produced by *B. elkanii* (Carlson *et al.*, 1993).

The presence in LCOs of a common fatty acid, such a vaccenic or stearic acid, seems to be a general characteristic of bacteria that are associated with plant hosts which form determinate nodules. This is in contrast with the host-specific LCOs from *R. meliloti* and *R. leguminosarum* bv. viciae in which the fatty acids have conjugated double bonds (Lerouge *et al.*, 1990; Spaink *et al.*, 1991).

Little is known about the genes of *R. loti* involved in the synthesis of LCOs. *nodA*, *nodB* and *nodC* genes have been described in *R. loti* strain NZP2037 (Collins-Emerson *et al.*, 1990; Scott *et al.*, 1988). *nodI* has been described in *R. loti* strain NZP2213 (Young *et al.*, 1990). The predicted NodS product of *A. caulinodans* has been implicated in the addition of the methyl group to the LCO. The NodS amino acid sequences of *A. caulinodans* ORS571, *B. japonicum* and *Rhizobium* sp. strain NGR234 display similarity with *S*-adenosylmethionine-utilizing methyl transferases (Krishnan *et al.*, 1992; Geelen *et al.*, 1993). Geelen *et al.* (1993) have provided evidence that *nodS* encodes a methyltransferase involved in Nod-factor modification. As the *R. loti* LCOs carry a methyl group, it can be speculated that *R. loti* contains a homologue of the *nodS* gene. Indeed, DNA fragments homologous to *nodSU* have been found in three strains

of *R. loti* (Krishnan *et al.*, 1992). In *B. japonicum*, *nodZ* is involved in the addition of the 2-*O*-methylfucose residue, and weak DNA hybridization with *nodZ* has been found for *Rhizobium* sp. NGR234 and for *R. tropici* (Stacey *et al.*, 1994). It is not known whether a gene homologous to *nodZ* is responsible for the addition of the 4-acetylfucose in *R. loti*.

Jiang and Gresshoff (1993) pointed out the slow nodulation behaviour of the proposed model legume *L. japonicus*. In this study we have found that the species *L. preslii* shows a fast and efficient nodulation. Using a newly developed spot-inoculation assay we have been able to show that purified *R. loti* LCOs induce nodule primordia on *L. preslii* plants. We are presently testing whether *L. preslii* can be used as an alternative for *L. japonicus* as a model legume.

Experimental procedures

Strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pMP2112 was derived by cloning the 2 kb *Hin*dIII fragment of pMP283 (Spaink *et al.*, 1987b) into plasmid pMP2733. pPM2733 is an IncW cloning vector (Spaink *et al.*, 1994). Broad-host-range plasmids were mobilized from *E. coli* to rhizobia using pRK2013 as a helper plasmid (Ditta *et al.*, 1980). *Rhizobium* was grown in B^- medium (Spaink *et al.*, 1989). Antibiotics were added, when required, at the following concentrations ($\mu g\ ml^{-1}$): tetracyline, 2; spectinomycin, 400; rifampicin, 20. All of the inducers were added to the medium at a final concentration of 1 μM, except for luteolin (3 μM) and trigonelline (20 μM). The compounds tested as inducers of the production of LCOs in *R. loti* E1R were naringenin, luteolin, eriodictyol, hesperitin, apigenin, 7,8-dihydroxyflavone, genistein, daidzein, 7-hydroxiflavone, umbelliferone, trigonelline, dihydrorobinetin, dihydroquercetin, dihydrofisetin, kaempferid, pinocembrin, morin, taxifolin, scutellarein, kaempferol-7-neohesperosid, prunetin, chrysin, 5-hydroxyflavone, 6-hydroxyflavanone, kaempferol, 3′-*O*-methyldihydroquercitin, malvin, fisetin, geraldol, quercetin and isosakuretin (suppliers: Aldrich, Merck and Sigma).

Detection of LCOs by TLC

In vivo radiolabelling of Nod metabolites was achieved using [1-^{14}C]-D-glucosamine (Amersham, specific activity 54 mCi $mmol^{-1}$) as precursor. To 1 ml of the appropriate cells (OD = 0.1) 0.1 μCi [1-^{14}C]-D-glucosamine was added, together with a suitable inducer, and the cultures were grown overnight at 28°C. A volume of 2 ml of *Lotus corniculatus* root exudates, prepared as described previously (van Brussel *et al.*, 1986), was added to 1 ml of culture (OD = 0.1) for induction with root exudates. LCOs were isolated from the cultures using *n*-butanol extraction. Samples were concentrated by evaporation and chromatographed on reversed-phase C18-coated silica plates (Sigma) using a mobile phase of acetonitrile:water (1:1, v:v). Radiolabelled components were detected using a Molecular Dynamics PhosphoImager using the Image Quant™ software.

Purification of LCOs

LCOs were extracted from the culture with a 0.4 volume of *n*-butanol. The butanol layer was collected, and the butanol was removed by rotatory evaporation. The residue was resuspended in 60% acetonitrile in water. This mixture was then pre-purified on an octadecyl extraction column (J. T. Baker, Phillipsburg, USA) and eluted with a volume of 45% acetonitrile corresponding to 0.1% of the volume of the culture. This sample was further purified using HPLC on a Pharmacia SuperPac Pep-S column (5 μm, 4 × 250 mm) and the following protocol: (i) 5 min of isocratic elution with 30% acetonitrile; (ii) 30 min of isocratic elution with 40% acetonitrile; (iii) 15 min of isocratic elution with 50% acetonitrile; and (iv) a linear gradient in 10 min from 50–100% acetonitrile. The HLPC elution was performed at a flow rate of 0.7 ml min^{-1} and the eluent was monitored at 206 nm.

NMR analysis of the LCO

A sample, corresponding to peak I extracted from a 4 l culture of strain *R. loti* pMP2112 was dissolved in a 7:5 mixture of D_2O and acetonitrile-D_3. All spectra were recorded on a Bruker AMX 600 MHz specrometer at 25°C. Two-dimensional DQF-COSY (Piantini *et al.*, 1982), TOCSY (Braunschweiler and Ernst, 1983; Bax and Davis, 1985), and HSQC (Bodenhausen and Ruben, 1980) experiments were run in a phase-sensitive mode using the TPPI method (Marion and Wuthrich, 1983). In all experiments, low-power pre-saturation was applied to the residual HDO signal. The TOCSY pulse program used a 112 ms MLEV-17 mixing sequence flanked by 2 ms trim pulses, and the HSQC experiment used a GARP (Shaka *et al.*, 1985) sequence for ^{13}C decoupling.

For homonuclear experiments, 1024 FIDS of 2048 complex points were collected and for the HSQC, 512 FIDS of 2048 complex points were acquired. Data were processed on an SGI workstation using FELIX software (Byosym). Typically, Lorentzian-to-Gaussian weighting functions were applied in the t_2 domain, and shifted squared sinebell functions with zero-filling applied in the t_1 domain. Proton chemical shifts were referenced to internal acetonitrile-D_3 at 1.93 p.p.m., relative to TMS.

Chemical modification of the LCOs

For acetylation, samples were dried and 500 μl of trifluoroacetic anhydride/acetic acid (2:1 v/v) was added and after 30 min the sample was dried under reduced pressure.

For permethylation, the dried sample was redissolved in 300 μl of DMSO in a screw-capped tube and a few freshly ground NaOH pellets were added. After 10 min (or immediately, if the carbamoyl group was to be retained) 500 μl of iodomethane was added and the mixture was stirred gently. After 10 min this was repeated, and after another 10 min 1 ml was added. The reaction was stopped after 30 min by the addition of 1 ml of sodium thiosulphate solution (100 mg ml^{-1}), immediately followed by the addition of 1 ml of chloroform. After

shaking and centrifugation, the aqueous layer was removed and the chloroform was washed three times with 1 ml of water and dried under reduced pressure.

The dried sample was hydrolysed in 500 µl of 2 M trifluoroacetic acid (TFA) at 120°C for 1 h. The TFA was then evaporated, after the addition of 250 µl toluene.

For reduction, 250 µl of 10 mg ml^{-1} $NaBD_4$ in 0.5 M NH_4OH was added to the dried sample and this mixture was left at room temperature for 18 h. The reaction was quenched with 1 ml of glacial acetic acid and dried. Hereafter (3×) 1 ml of 10% acetic acid in methanol was added, mixed, and dried under a stream of nitrogen. This procedure was repeated with (3×) 1 ml of methanol.

For acetylation, 500 µl acetic anhydride was added to the dried sample and heated for 3 h at 120°C. A volume of 0.5 ml of distilled water was added and the sample was neutralized by the addition of solid Na_2CO_3. After extraction into 0.75 ml of dichloromethane, the organic phase was concentrated under a stream of N_2.

For de-*O*-acetylation, the sample was dried and 50 µl of methanol and 50 µl of an ammonium hydroxide solution (25% NH_3 in H_2O) were added. The mixture was allowed to react overnight at room temperature and thereupon dried under a stream of N_2.

Composition analysis

To a third of the dried de-*O*-acetylated sample, 200 µl of 1 M HCl in dry methanol was added and incubated at 80°C for 4 h. After cooling, 50 µl pyridine was added, the mixture was stirred, and 50 µl of acetic anhydride was added. After 30 min the mixture was dried under reduced pressure. The residue was redissolved in 250 µl of methanol and the solvent was evaporated. Hereafter the residue was redissolved in 40 µl of TMS reagent (pyridine/BSTFA/TMCS; 10/5/1; v/v/v) and allowed to react for 10–15 min at room temperature. Standards containing approximately 50 µg of fucose and rhamnose were prepared in the same way.

For alkaline methanolysis, 100 µl of 5% KOH/MeOH was added to the dried sample and allowed to react at 70°C overnight. The reaction was quenched by the addition of 20 µl of acetic acid (3.5 M). The product was extracted into 200 µl of diethylether which was washed with 200 µl of water. The aqueous layer was removed and the organic layer was dried under a stream of N_2.

For methyl esterification, 0.5 ml of diazomethane solution in diethylether was added to the residue and the mixture was allowed to react for 20 min at room temperature. Hereafter the solvent was evaporated under nitrogen.

For dimethyl disulphide (DMDS) derivatization, the dried residue was dissolved in 200 µl of hexane, 200 µl of DMDS; 20 µl of 6% (w/w) I_2/diethylether was added and the mixture was incubated at 50°C for 48 h. The sample was diluted with 200 µl of hexane and the solution was decolorized by treatment with 200 µl of 5% $Na_2S_2O_3$ solution. The organic phase was removed and the aqueous phase was extracted a second time with 200 µl under a stream of N_2.

FAB-MS(MS) analysis

FAB mass spectra were recorded on a JEOL JMS-SX/SX 102A tandem mass spectrometer, operated in the positive-ion mode, using a 10 kV accelerating voltage. The FAB-gun was operated at 6 kV with a 10 mA emission current, using xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used and recorded and processed on a Hewlett Packard HP900 series data system using the JEOL Complement software. Tandem (MS/MS) mass spectra were obtained on the same instrument, using helium as collision gas in the third field free-region collision-cell, at a pressure sufficient to reduce the parent ion to one-third of its original intensity. The probe was loaded with 1 µl of sample solution in DMSO using a matrix of thioglycerol.

GC-MS analysis

GC-MS was performed on a Jeol JMS-AX505 W mass spectrometer fitted with a Hewlett Packard 5890 gas chromatograph using an on-column injector and helium as the carrier gas. Monosaccharide derivatives were separated on an SE-54 column (0.25 mm × 30 m; Alltech), using the following temperature programme: 60°C for 2 min, a gradient of 40°C/min to 130°C, holding for 2 min at 130°C, then a gradient of 4°C/min to 230°C, and finally at 230°C for 10 min. Mass spectra were obtained under conditions of electron impact in the positive-ion mode and were recorded using linear scanning from m/z 50–450 at an accelerating voltage of 3 kV.

Analysis of the products of the DMDS derivatization was performed on a Fisons MD800 mass spectrometer fitted with a Carlo Erba GC8060 gas chromatograph using an on-column injector and helium as the carrier gas. The fatty acid derivative was separated on a BPX-5 column (0.25 mm × 25 m; SGE), using the following temperature programme: 80°C for 1 min, a gradient of 4°C/min to 100°C, then a gradient of 8°C/min to 240°C, and finally at 240°C for 10 min. Mass spectra were obtained under conditions of electron impact in the positive-ion mode with an electron energy of 70 eV and were recorded using linear scanning from m/z 50–500.

Biological tests

The nodulation test on *L. corniculatus* was performed as described by van Brussel *et al.* (1982). To test for root-hair deformation activity of the LCOs, seeds were germinated and the plants grown as described previously (van Brussel *et al.*, 1986), and LCOs were added to the plants in solution in 2 µl of dimethyl sulphoxide.

For spot-inoculation, we made use of the fact that LCOs can be coated onto quartz sand. Small quantities of the LCO-coated sand were added to the plants for the induction of nodule primordia. The sand (0.1–0.3 mm) was washed, dried, and sterilized. To 100 mg of sterile sand, 100 µl of a 10^{-4} M solution of purified LCOs in 50% acetonitrile was added. This mixture was vortexed for a few seconds and dried under vacuum. Before spot-inoculation, plants were grown in agar tubes (van Brussel *et al.*, 1982) for 2 d and then the LCO-coated sand was carefully placed with a spatula close to the root tip. This part of the root has been shown to be responsive to infection by rhizobia (Bhuvaneswari *et al.*, 1981). As a negative control, sterile sand was

added to the plants. For spot-infection with *R. loti,* 100 mg of sterile sand was mixed with 50 µl of a culture at OD = 1, and the plants were inoculated in the same way as with LCO-coated sand. After the desired time the sand was removed from the roots using a stereomicroscope. Roots were cleared with hypochlorite and stained with methylene blue using the method of Truchet *et al.* (1989).

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