Symbiotic host-specificity of Rhizobium meliloti is determined by a sulphated and acylated glucosamine oligosaccharide signal

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RHIZOBIA are symbiotic bacteria that elicit the formation on leguminous plants of specialized organs, root nodules, in which they fix nitrogen[1]. In various *Rhizobium* species, such as *R. leguminosarum* and *R. meliloti*, common and host-specific nodulation (*nod*) genes have been identified which determine infection and nodulation of specific hosts[1]. Common *nodABC* genes[2–5] as well as host-specific *nodH* and *nodQ* genes[4,6–8] were shown recently, using bioassays, to be involved in the production of extracellular Nod signals. Using *R. meliloti* strains overproducing symbiotic Nod factors, we have purified the major alfalfa-specific signal, NodRm-1, by gel permeation, ion exchange and C_{18} reverse-phase high performance liquid chromatography. From mass spectrometry, nuclear magnetic resonance, ^{35}S-labelling and chemical modification studies, NodRm-1 was shown to be a sulphated β-1,4-tetrasaccharide of D-glucosamine (M_r 1,102) in which three amino groups were acetylated and one was acylated with a C_{16} bis-unsaturated fatty acid. This purified Nod signal specifically elicited root hair deformation on the homologous host when added in nanomolar concentration.

Cytological studies of nodules induced by *R. meliloti* mutants unable to form infection threads or to be released into host cells suggested that the bacteria are able to elicit, at a distance, cortical cell dedifferentiation and initiation of nodule organogenesis by means of a 'nodule inducing principle' (refs 9, 10). Recent studies showing that alfalfa clones may develop nodules of normal anatomy in the absence of *Rhizobium* indicated that the host plant possesses the entire genetic programme for nodule organogenesis and that the role of the microsymbiont *R. meliloti* is to switch on this programme[11]. The common *nodABC* and host specific *nodH* and *nodQ* genes of *R. meliloti*, which are required for specific infection and for triggering nodule formation, determine the production of extracellular symbiotic signals[1,4,6–8,12,13].

The transcription of *R. meliloti nod* genes is under the control of *syrM* and three *nodD* regulatory genes, and requires the presence of flavonoids from plant exudates[1,14,15]. After induction of *nod* gene transcription by the flavonoid luteolin, the sterile filtrate of a *R. meliloti* wild type strain culture exhibits specific root hair deformation (Had) activity on alfalfa, the homologous host, but not on vetch, a heterologous host[4]. Both the common *nodABC* genes and host range genes *nodH* and *nodQ* are required for the production of the alfalfa specific extracellular Nod signals[4,6–8]. In an attempt to purify these active signal molecules, the filtrate of a luteolin induced culture of *Rhizobium meliloti* 2011 was extracted by organic solvents (see legend to Fig. 1). Most of the Had activity assayed on alfalfa seedlings was detected in the butanol soluble and ethyl acetate insoluble fraction. This extract was fractionated by high performance liquid chromatography (HPLC) on a C_{18} reverse phase column. However, the amount of compounds present in the fraction showing the highest Had activity was too small for structural analysis. To improve the bacterial production of these compounds we introduced the plasmid pGMI149 into *R. meliloti* to provide extra copies of the nodulation region. The plasmid pGMI149 carries the whole set of common and specific *nod* genes as well as three regulatory genes, *nodD1*, *nodD3* and *syrM*, cloned in an Inc P1 vector present at 5 10 copies per cell[1,12,15] (Fig. 1*a*). The introduction of pGMI149 into *R. meliloti* 2011 resulted in both an increase of more than a 100 fold of the Had activity on alfalfa, as estimated by assaying serial dilutions of extract, and the appearance of two far UV (220 nm) absorbing peaks on HPLC chromatograms (Fig. 1*b*).

To ascertain the role of the *nodABC* genes in the production of these compounds, *nodA*::Tn5 or *nodC*::Tn5 insertions were introduced into pGMI149. To avoid interference between the *nod* genes present on the pSym megaplasmid and those present on pGMI149, we introduced pGMI149 and its mutant derivatives either into *R. meliloti* strain GMI766, which carries a deletion of the whole pSym *nod* region, or into GMI6059, a strain carrying a deletion of the *nodD1ABC* genes. The inactivation of the *nodABC* operon by a *nodA*::Tn5 insertion resulted simultaneously in the disappearance of the two major UV-absorbing peaks in the HPLC profiles and in the loss of Had activity on alfalfa (Fig. 1*b*). Inactivation of *nod C* also resulted in no accumulation of these factors. Insertion of Tn5 downstream of *nodC* in the putative *nodIJ* region[1] did not suppress NodRm production, showing that *nodC*::Tn5 insertion was not acting by a polar effect on downstream genes (data not shown). Thus the ultraviolet detected compounds are symbiotically active molecules and the *nodC* gene is required for their production.

To facilitate large scale culture filtration, the pGMI149 plasmid was introduced into the *R. meliloti exoB* mutant EJ355 which is deficient for exopolysaccharide production[10]. The Exo EJ355 (pGMI149) strain exhibited the same Nod factor peaks in HPLC profile as the Exo^+ strains 2011 (pGMI149) and GMI766 (pGMI149). The filtrates of fermenter cultures of the non mucoid EJ355 (pGMI149) strain were extracted and successively purified by preparative reverse phase C_{18} HPLC, by gel permeation on a Sephadex LH20 column and by ion exchange chromatography on a DEAE column as described in Fig. 1. Only one peak absorbing at 220 nm could be observed which showed Had activity on alfalfa. Analytical HPLC on a C_{18} reverse phase column resolved the active fraction into two close UV absorbing peaks which showed Had activities on alfalfa but not on vetch (Fig. 1*b*). Ten litres of induced rhizobial culture yielded around 4 mg of purified Nod factors.

Chemical analysis of the two Nod factors was carried out using either each peak or a mixture of both, and this revealed that the two compounds correspond to the α and β anomers of the same molecule, at the C 1 position of the reducing end sugar. We propose calling this molecule NodRm 1. A clear separation of α and β anomers of *N* acetyl D glucosamine oligomers by reverse phase HPLC has already been reported[16]. The structure of NodRm 1 was determined by mass spectrometry (Fig. 2*a*, *b*), chemical modification (Fig. 2) and NMR spectroscopy (Fig. 3). The presence of a sulphate group was confirmed by ^{35}S-labelling (Fig. 1*c*). These data, together with methylation analysis, led to the structure proposed in Fig. 4. NodRm 1 is an *N* acyl tri *N* acetyl β 1,4 D glucosamine tetrasaccharide bearing a sulphate group on carbon 6 of the reducing sugar moiety. The aliphatic chain is carried by the non reducing terminal sugar and is a 2,9 hexadecadienoic *N*-acyl group. The rationale for the structural assignments is given in the legends of Figs. 2 and 3. Minor compounds were detected which differed slightly from NodRm 1 in the length of the aliphatic chain (C_{16}, C_{18}) and number and location of double bonds. These molecules were designated NodRm factors since they could not be detected in the extracts of *nodA* or *nodC* mutants. Chemical

and biological studies of the various NodRm factors produced by mutants altered in different specific *nod* genes should indicate whether these minor molecules are intermediates in the biosyn thetic pathway of the NodRm 1 symbiotic signal. NodRm-1 was also clearly the major Nod factor in filtrates of Exo^+ strains 2011 (pGMI149) and 766 (pGMI149), as determined by mass and NMR spectrometry and alfalfa Had activity. Breaking gly cosidic bonds by methanolysis resulted in a complete loss of Had activity, as assayed on alfalfa seedlings at concentrations equivalent to 10^{-8} 10^{-11} M NodRm-1.

The following evidence shows that the NodRm factor described is a plant specific symbiotic signal: (1) After three types of purification based on different physicochemical proper ties, ion exchange, gel filtration and partition chromatography, we always observed an absolute correlation between the pres ence of this molecule (characterized by HPLC profile, mass spectrometry and ^{1}H NMR spectroscopy) and specific Had activity on alfalfa. (2) An increase in *nod* gene activity, either by induction of *nod* gene transcription or by gene dosage, was correlated with an increased production of this molecule, whereas a Tn5 mutation in *nodA* or *nodC* genes resulted in no production (see legend to Fig. 1). (3) The biological activity of NodRm, as detected by the hair branching bioassay, was very high and specific. A NodRm solution in the 10^{-8} 10^{-11} M range elicited Had reactions on the homologous host alfalfa but not on vetch, a heterologous host (data not shown). The NodRm factor was still clearly active on alfalfa at a 10^{-11} M concentra tion. NMR studies indicated that NodRm 1 was at least 95% pure. Molecules having aromatic rings, like the plant hormones auxin and cytokinin, could not be detected. The study of ultra violet absorbance of NodRm 1 at 260 and 280 nm showed that if such hormone like compounds were present in the active fraction, they should represent less than 1% of the purified product. Therefore any possible hormone like contaminants would be below 10^{-13} M in our assays, that is, at a concentration around a million fold below the level (10^{-7} M) at which these phytohormones act when added exogenously[17]. Indole acetic acid, isopentyl adenine and zeatin did not comigrate with NodRm 1 and did not exhibit any Had activity on alfalfa in the range 10^{-6} 10^{-13} M. We thus conclude that such contaminants are very unlikely to account for the observed effects.

In Gram negative bacteria *N* acetyl D glucosamine is in

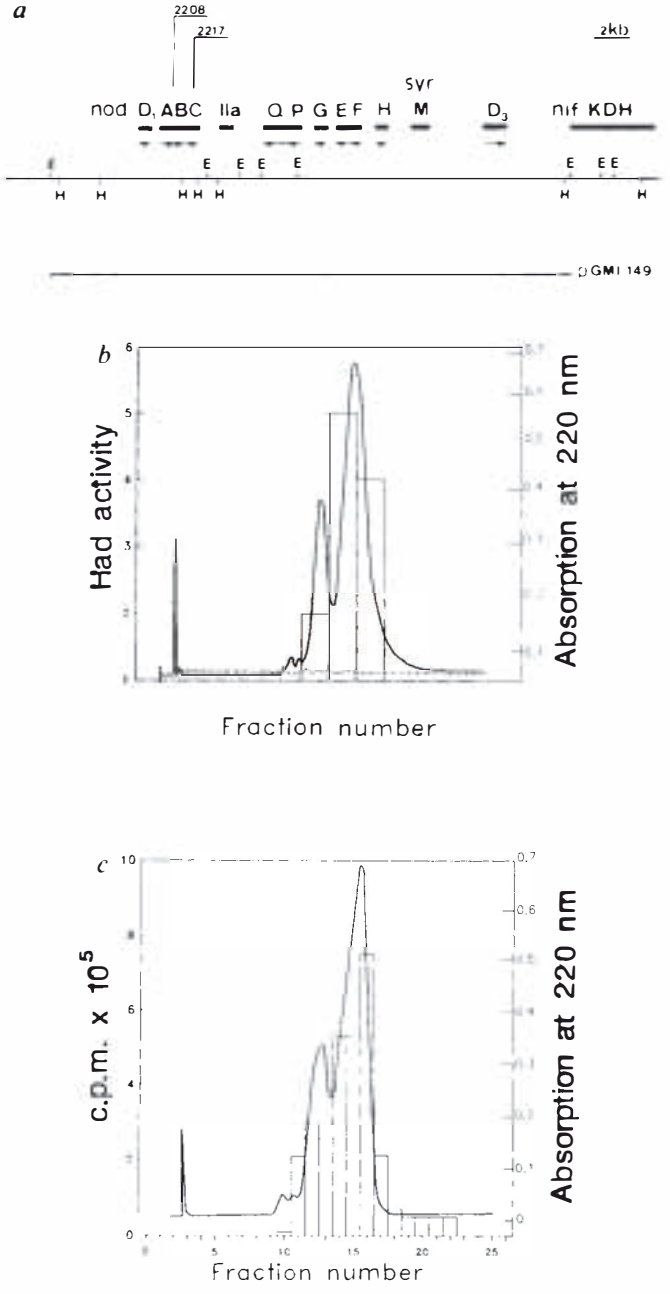

FIG. 1 *a*, Physical and genetic map of the *nod* region of *R. meliloti* 2011 (refs 1, 4, 13, 15). The restriction map (E, *Eco*RI; H, *Hin*dIII) is shown with the pGMI149 plasmid represented below[12]. Arrows indicate the direction of transcription of the *nod* genes. The position of *nodA*::Tn5 (2,208) and *nodC*::Tn5 (2,217) mutations is given above the map[12]. The putative *nodIJ* genes are located downstream of *nodABC* (ref. 1). *b*, Analytical HPLC chromatogram (isocratic conditions) of butanol-extracted compounds from a filtrate of *R. meliloti* EJ355 (pGMI149), a NodRm factor-overproducing strain. The elution profile of a sample of 300 μg of NodRm-1, as monitored by absorption at 220 nm, is represented by a solid line. The fraction size was 3 ml. The hair-branching activity on alfalfa is shown as a bar graph. Similar results were obtained with two other overproducing strains 2011 (pGMI149) and GMI766 (pGMI149). In contrast, for strains having a *nod* deletion in the pSym (GMI766 and GMI6059) and a Tn5 insertion in the *nodA* or *nodC* genes located on pGMI149, no peaks of far ultraviolet-absorption (dotted line) and no Had biological activity could be detected. *c*, Demonstration of the presence of a sulphate group in NodRm factors by labelling with [^{35}S]sodium sulphate. Analytical HPLC chromatogram run in the same conditions as for *b*. The bar graph represents ^{35}S radioactivity. The radioactive peak co-migrated with the UV-absorbing peak. In GMI6059 (pGMI149*nodA*::Tn*5*), a NodA derivative, no peak of ^{35}S radioactivity could be detected.

METHODS. *R. meliloti* EJ355 is an Exo derivative of strain 2011 (ref. 10). GMI766 carries a deletion of the whole *nod* region[12]. GMI6059 was construc ted by introducing a *nodD1ABC* deletion[12] into EJ355 by marker exchange. pGMI149, pGMI149*nodA*::Tn5 or pGMI149*nodC*::Tn5 were introduced into GMI766 and GMI6059 as already described[12]. Bacteria were grown in a liquid minimal medium[23] containing Na glutamate (1 g l^{-1}) and Na succinate (2 g l^{-1}) as nitrogen and carbon sources, and luteolin (10 μM) as a *nod* gene inducer[14]. Log phase cultures ($\sim 5 \times 10^8$ colony-forming units per ml) were centrifuged, sterilized through filter membranes (0.45 μm) and extracted by butanol. The residue was dissolved in water and extracted with ethyl acetate. The water fraction was lyophilized and chromatographed successively as follows: (1) on a preparative reverse-phase C_{18} column (10 μm, 7.5 × 250 mm) in a linear water-acetonitrile gradient (solvent x 80/20, solvent y 50/50) at a flow rate of 2 ml min^{-1}; (2) on a Sephadex LH20 column (99% ethanol); (3) on a DEAE trisacryl column equilibrated with Tris-HCl (10^{-3} M, pH8) and eluted with a linear gradient of sodium chloride (up to 0.1 M); (4) the final purification of the active fraction was achieved with an analytical reverse-phase C_{18} column (5 μm, 4.6 × 250 mm) in isocratic conditions (water-acetonitrile 80/20) at a flow rate of 1 ml min^{-1}. NodRm was labelled by growing bacteria overnight with 80 μCi of ^{35}S-sodium sulphate. Incorporation was assayed by liquid scintillation counting. The biological Had activity was assayed microscopically by observing root hair deformation on alfalfa[7] (*Medicago sativa*) and on common vetch[3] (*Vicia sativa* subsp. *nigra*). Fractions were serially diluted and a minimum of 10 plants were observed for each dilution. Intensity of the Had reaction was scored from 0 to 6 as a function of both the proportion of branched root hairs and the extent of the root region affected. To avoid subjective bias during visual rating, samples were scored without previous knowledge as to the specific treat-ment used. Had results given in this figure were obtained with dilutions corresponding to around 10^{-10} M NodRm-1 factor.

volved in the biosynthesis of two major components of the cell wall, peptidoglycan in the periplasmic space and lipidA in the outer membrane[18]. Future study of the biosynthesis of Nod signals will address the question of whether the *nod* gene products mediate a new synthetic pathway from *N*-acetyl-D glucosamine or, rather, if they are involved in the degradation of pre-existing cell wall macromolecules. Some fungal oligosaccharide elicitors, which trigger host defence mechanisms such as phytoalexin production, have been shown to be derived from fungal cell wall degradation and are active in the nanomolar range[19]. It is worth noting that NodRm is a plant-specific signal, in contrast to the purified fungal oligosaccharide elicitors so far described which are not host species-specific[19].

In addition to the common *nodABC* genes, the host-range

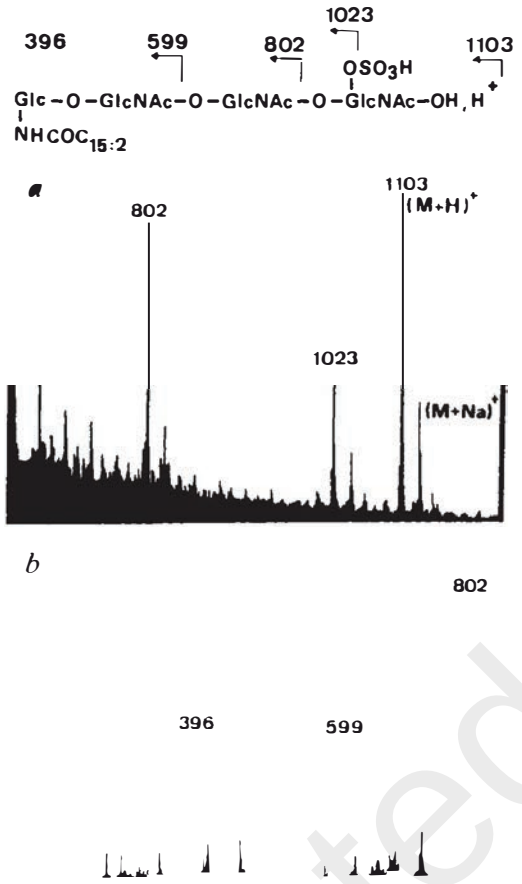

FIG. 2 Mass spectrometry (VG Analytical, ZAB 2E) and analysis by chemical degradation of NodRm-1. *a*, Fast atom bombardment (FAB-MS) spectra were obtained through a magnetic scan from 4,000 to 100 mass units. NodRm-1 exhibited a pseudomolecular ion $(M+H)^+$ at $m/z=1,103$ by positive ion MS and a $(M-H)^-$ ion at $m/z=1,101$ in the negative mode. A fragment was observed at m/z 1,023 in the positive spectrum. It arose from the decomposition of the $(M+H)^+$ ion as confirmed by the metastable fragmentation (B/E linked scan) of the latter. The 80 mass unit loss suggested either a phosphate or a sulphate group[24]. The latter was confirmed by ^{35}S-labelling. Furthermore, no signal could be detected in ^{31}P NMR spectrometry. Sodium borodeuteride-reduced NodRm-1 ($NaBD_4/H_2O$; 40 °C; 2 h) showed a shift of three mass units on both $(M+H)^+$ and $(M+H)^+$ minus 80. However the fragment ion at $m/z=802$, due to the loss of sulphated N-acetyl glucosamine, was not shifted. This indicated that the sulphate group was carried by the sugar moiety at the reducing end. *b*, Collision-induced decomposition of $m/z=802$ (B/E linked scan) showing the successive elimination of two *N*-acetyl glucosaminyl units by glycosidic cleavages[25] (losses of 203 mass units).

METHODS. Chemical analysis of NodRm-1: Acidic hydrolysis (3N HCl; 100 °C; 3 h) released glucosamine which was of the D series as determined by gas chromatography (GC) analysis of its glycoside with (−)-2-butanol (peracetylated derivative). A milder acidic cleavage using 1 N HCl in methanol (80 °C; 1 h) afforded both *N* acetyl glucosamine and a long chain *N*-acylated glucosamine, the structure of which was confirmed by electron impact-mass spectrometry (EI-MS) of its trimethylsilyl derivative[26]. The main characteristic fragments were at $m/z=365$ (ion containing the C2 C3 part of the sugar ring) and at $m/z=452$ (C2–C3–C4 part), indicating a bis-unsaturated C_{16} *N*-linked fatty acid. The *N*-linked fatty acid was liberated from NodRm-1 by saponification and analysed by gas chromatography coupled to tandem mass spectrometry (negative ion GC/MS/MS) of its perfluoro-benzyl-ester derivative, inducing remote charge fragmentation processes[27]. This characterized the major $C_{16:2}$ fatty acid as bis-unsaturated on both positions 2 and 9 with no chain branching. Other minor C_{16} and C_{18} fatty acids were also detected (less than 5%). The interglycosidic linkage mode of the different glucosamine moieties was determined by methylation analysis of reduced NodRm-1 (ref. 28). The partially methylated alditol acetates, obtained after subsequent chemical reactions, were characterized by EI/GC/MS and this indicated (1 → 4) linkages between all monosaccharidic moieties.

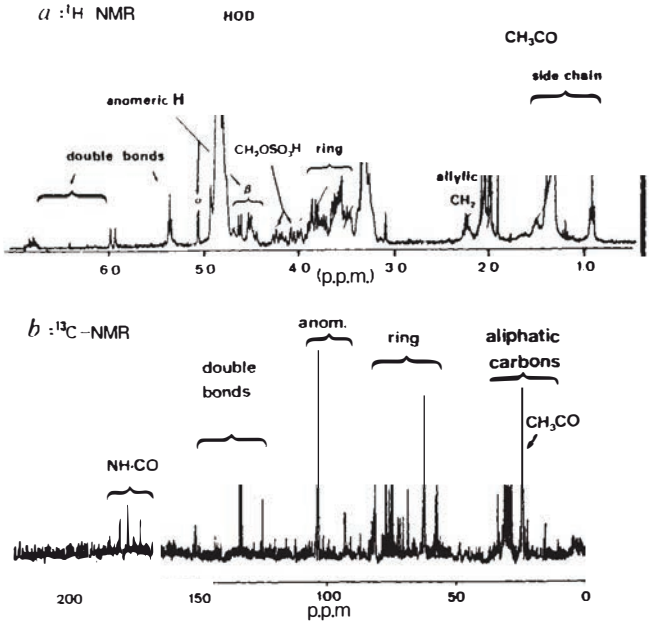

FIG. 3 NMR spectrometry of NodRm-1. *a*, The ^1H NMR spectrum (CD_3OD; Bruker AM 300 MHz) showed four olefinic protons which were assigned to the internal double bond of the acyl chain ($\delta=5.34$ p.p.m.; 2H) and to the E conjugated double bond ($\delta=5.95$ p.p.m.; d; J 15 Hz and $\delta=6.83$ p.p.m.; dt; $J=7$ and 15 Hz). The structure of the acyl chain was confirmed by ^1H NMR-COSY experiments (data not shown). The proton anomeric region revealed three β-interglycosidic linkages ($J=8.5$ Hz). The α ($J=3$ Hz) and β ($J=8.5$ Hz) anomeric protons of the terminal reducing *N*-acetyl glucosamine were also observed, and they disappeared after NodRm-1 reduction ($NaBD_4/H_2O$; 40 °C; 2 h). The 3.4 to 3.9 p.p.m. region was assigned to the sugar ring protons[29]. Two downfield signals ($\delta=4.07$ and 4.26 p.p.m.; dd) were attributed to two H-6 protons allowing the location of the sulphate group at C-6 (see ref. 30). Six methyl signals (6 × 1.5 H) were assigned to the three *N*-acetyl groups of each α and β anomer of native NodRm-1. *b*, All the signals observed in the ^{13}C NMR spectrum (D_2O; ref: TMSPS; Bruker AC 200 MHz) have been assigned to the different carbons of NodRm-1, in agreement with previous reports[29,30]. This spectrum confirmed the $\beta(1\rightarrow4)$ *N*-acetyl-glucosamine tetrasaccharide structure (δ C1 103.9 p.p.m. and δ C4 = 81.8 82.0 p.p.m.), the presence of a bis-unsaturated chain and a sulphate substitution characterized by the downfield shift of one C-6 of a glucosamine residue ($\delta=68.8$ p.p.m.).

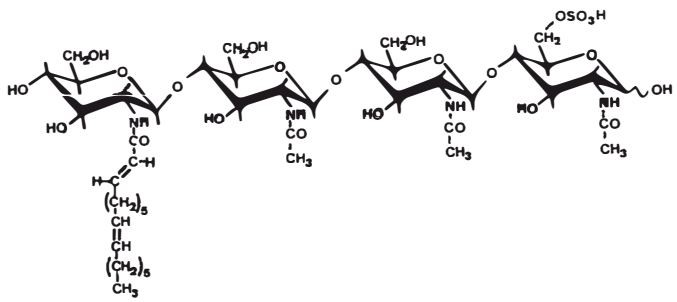

FIG. 4 The proposed NodRm-1 structure is consistent with NMR spectroscopy, mass spectrometry, ^{35}S-labelling and methylation analysis data.

nodH and *nodQ* genes are required for the production of alfalfa specific signals[4,6,8]. These *nod* genes are involved in the control of host specific steps such as recognition, infection and nodulation[1,12,13]. Studies of host range *nod* mutants of *R. meliloti* revealed an absolute correlation between the specificity of the symbiotic behaviour of living rhizobial cells and the Had bio assay specificity of their sterile supernatants, which indicates that the Nod signals are involved in determining specific infec tion and nodulation[4,7]. We have established here that NodRm is a symbiotic signal which is highly plant-specific. The transfer of a pea lectin gene into white clover seedlings recently achieved resulted in the transfer of the ability to be infected and nodulated by a pea specific *Rhizobium* (ref. 20). This finding supports the hypothesis that plant lectins are involved in the *Rhizobium* legume specific recognition. The alfalfa specific NodRm 1 signal has structural properties which are characteristic of effective lectin ligands such as hexosamine oligosaccharides[21]. A plant root lectin located at the surface of the cytoplasmic membrane of the susceptible root hairs might be part of the Nod signal plant receptor complex[22]. Purification and characterization of *Rhizobium meliloti* symbiotic signals open up new ways for studying the molecular basis of plant microbe specific recognition and the mechanisms by which symbiotic microbes signal to plants to elicit various host reactions, including the induction of organogenesis, without triggering plant defence reactions. □

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