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Carbohydrate Research 303 (1997) 435–443

CARBOHYDRATE
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Structural determination of the lipo-chitin oligosaccharide nodulation signals produced by *Rhizobium fredii* HH103

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Received 4 February 1997; accepted 20 June 1997

Abstract

Rhizobium fredii HH103 produces extracellular signal molecules that are able to induce deformation of root hairs and nodule organogenesis of soybean. This strain produces a large variety of nodulation factors, consisting of a linear backbone of GlcNAc with different degrees of polymerization, bearing on the non-reducing residue various different *N*-acyl residues. The reducing terminal residue is 2-*O*-methylfucosylated at position 6. Several analogous molecules substituted with fucose were also detected. © 1997 Elsevier Science Ltd.

Keywords: *Rhizobium fredii* HH103; Nod factors; Lipo-chitin oligosaccharides

1. Introduction

Rhizobia are nitrogen-fixing bacteria that are able to invade the roots of leguminous plants and trigger the formation of the nodule that contains the nitrogen-fixing microsymbiont [1,2]. Infection and nodule development are highly specific and depend on both partners, the bacterium and the plant, to be effective. During the symbiotic process, the host plant roots secrete flavonoids that induce the rhizobial nodulation genes (*nod* and *nol* genes) [3]. These genes have been shown to be involved in the synthesis and excretion of bacterial nodulation signals called nodulation factors or lipo-chitin oligosaccharides (LCOs). These molecules have been characterized from, amongst others, *Rhizobium meliloti* [4,5], *R. legumi-*

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PII S0008-6215(97)00189-4

nosarum bv. *viciae* [6,7], *R.* NGR234 [1,8], *Bradyrhizobium japonicum* [9,10], *Azorhizobium caulinodans* [11], *R. fredii* [12,13], and *R. tropici* [14,15].

The nodulation factor LCOs consist of a backbone of three to five GlcNAc residues, bearing an amide-bound fatty acyl residue (saturated or unsaturated) on the non-reducing terminal GlcNAc residue. This basic structure has structural variations that determine the host-specificity. Thus, the host-specificity of *R. leguminosarum* bv. *viciae* and *trifolii* are determined by the lipid moiety [16]. The sulfate group on O-6 of the reducing GlcNAc residue is necessary for the nodulation by *R. meliloti* of alfalfa plants [17]. Finally, bacteria that are symbionts of soybean (*B. japonicun* and *R. fredii)* and the broad host-range *R.* sp. NGR234, produce nodulation factors that are 6-*O*-substituted on the reducing GlcNAc residue with 2-*O*-methylfucose [1,8,10,12].

The structures of some of the Nod factors produced by *R. fredii* have already been reported. However, these studies have been carried out using *R. fredii* USDA257, a strain that fails to nodulate with a large number of American soybean cultivars [18]. This fact set forth the notion that the value of *R. fredii* strains as commercial soybean inoculates is very limited. Dowdle and Bohlool [19] reported the isolation of new wild type *R. fredii* strains (as HH103) that in greenhouse experiments are highly effective on American soybean cultivars. Moreover, field experiments have shown that *R. fredii* strain HH103 is a good inoculant of commercial soybean cultivars [20,21].

This paper describes the isolation and the structural analysis of the Nod factors from *R. fredii* HH103.

2. Experimental

Bacterial strains and cell cultures.—Rhizobium fredii strain HH103 was grown at 28 °C in a minimal B^- medium [22] until the cultures reached an A_{660} of 0.6–0.8. Induction of the bacterial nodulation genes was achieved by adding 3.8 μM genistein aq soln to the bacterial cultures.

Thin-layer chromatography analysis of Nod factors.—Nodulation factors were labelled in vivo and analyzed by TLC using the procedure described by Spaink et al. [22]. Briefly, *R. fredii* HH103 was grown in 1 mL of minimal B^- medium supplemented with genistein and the appropriate antibiotics and was radioactively labelled by the addition of 0.5 μCi of [^{14}C]-glucosamine hydrochloride (52 mCi/mmol) from Amersham, Int. and 1 μCi of L-(methyl-[^{14}C])-methionine (55 mCi/mmol) from Amersham, Int. Cultures were grown to the stationary phase and the supernatant was extracted with 0.5 mL of water-saturated *n*-butanol. The solvent was evaporated to dryness and the residue was resuspended in 40 μL of *n*-butanol. Five microliters of this solution was applied to the TLC plate (ODS, 100% octadecyl silanization, from Sigma Co.) where the Nod factors were separated using 1:1 acetonitrile–water as the mobile phase. TLC plates were dried and exposed to Kodak X-Omat R film for 15 days at room temperature.

Nod factor purification.—Ten liters of culture medium were extracted with 3 L of *n*-butanol. The butanol extract was evaporated to dryness under vacuum and the residue was suspended in 50 mL of 3:2 acetonitrile–water which was then brought to 1:4 acetonitrile–water. A pre-purification step was performed by passing the crude extract through a Super Clean LC-18, Supelco column from which the Nod factors were eluted using different acetonitrile–water ratios (20, 45, and 60%). These fractions were purified by HPLC on a semi-preparative C_{18} reversed phase column (250 × 7.5 mm, Spherisorb ODS2, 5 μm, Tracer) using isocratic elutions of acetonitrile–water (20%, 5 min; 30%, 30 min; 40%, 30 min; 60%, 15 min), and then a linear gradient in 10 min from 60 to 100% of acetonitrile. The eluent from the HPLC was monitored at 206 nm.

Carbohydrate composition and methylation analysis.—Glycosyl composition analysis was carried out after methanolysis using anhydrous methanolic 0.625 M HCl (16 h, 80 °C). The samples were reacetylated using 1:1 Ac_2O–pyridine, then trimethylsilylated with 1:1 pyridine–BSTFA and analyzed by GLC–MS [23].

Glucosamine was assigned as D-$GlcNH_2$ following GLC–MS analysis of its trimethylsilylated 2-butyl glycosides prepared by using (+)-2-butanol and (±)-2-butanol [24] as above. 2-*O*-Methyl-fucose was assigned as L-Fuc as follows: authentic samples of L-Fuc and the Nod factors were derivatized with (+)-2-octanol and (±)-2-octanol and then permethylated by the method of Ciucanu and Kerek [21] and the permethylated 2-octyl glycosides were analyzed by GC–MS.

The LCOs were permethylated [25] and the samples were hydrolyzed with 2 M CF_3COOH (120°, 1 h), reduced, and acetylated by the method of Blakeney [26]. The permethylated alditol acetates were

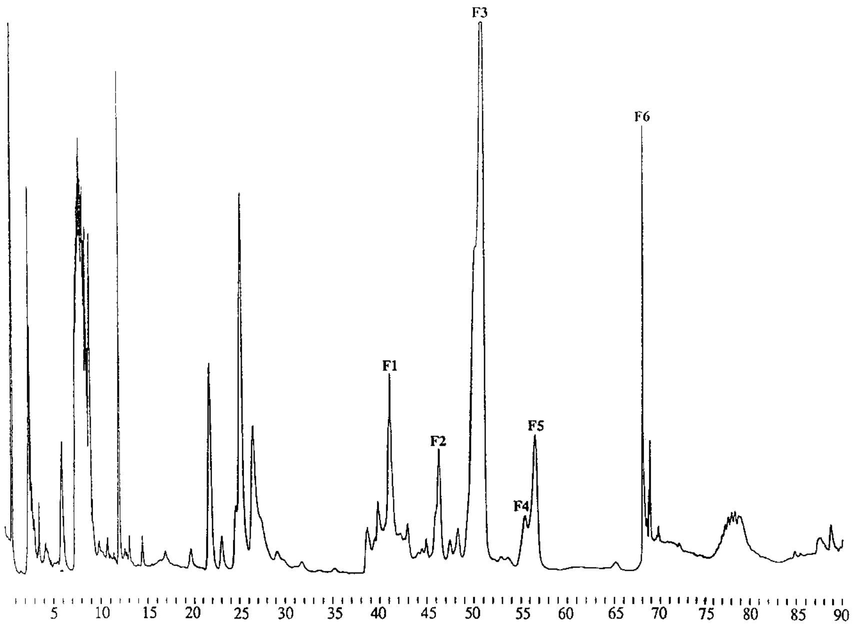


Fig. 1. HPLC profiles of *n*-butanol extract from *R. fredii* HH103, eluted from Super Clean LC-18 with 45% acetonitrile–water (see text for details).

analyzed by GLC–MS. The GLC–MS instrument, column, and conditions for the different derivatizations have been described previously [27].

Fatty acid analysis.—The fatty acids were identified as their methyl esters. The methyl esters were prepared by methanolysis in methanolic 0.625 M HCl

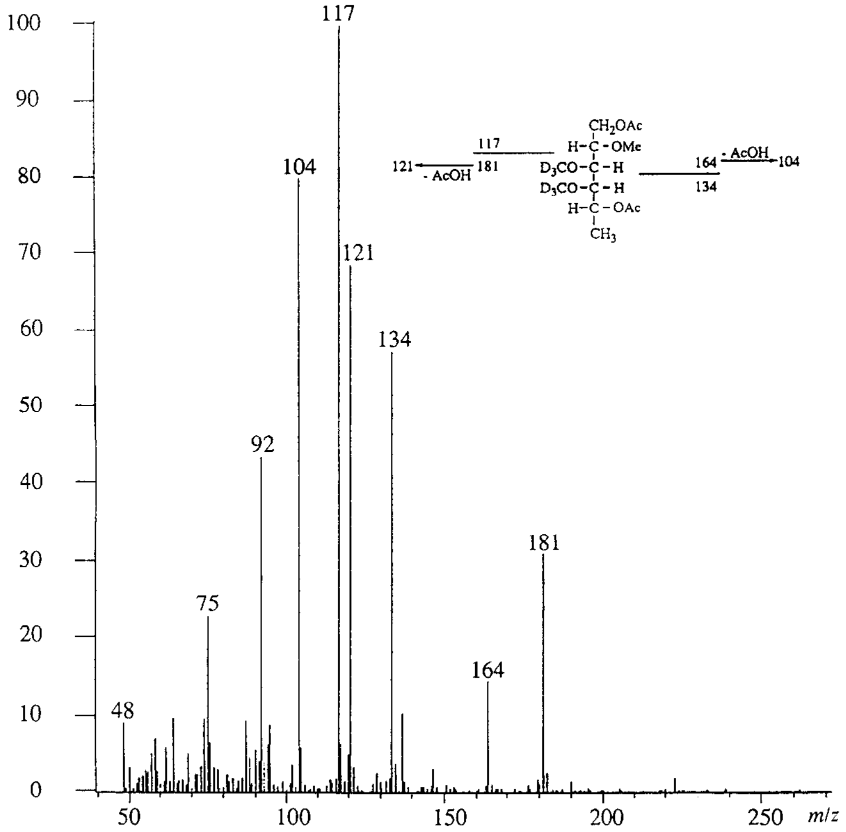


Fig. 2. EI mass spectrum of 1,5-di-*O*-acetyl-3,4-di-*O*-trideuteriomethyl-2-*O*-methylfucitol showing the presence of a methyl group at O-2.

at 80° for 16 h. The methyl esters of fatty acids were identified by GLC–MS. The location of the double bond in the unsaturated fatty acyl residues was determined by the preparation of dimethyl disulfide ethers of the methyl esters [28] followed by GLC–MS.

FABMS analysis.—Positive FABMS was performed on a Kratos MS80-RFA instrument with a xenon atom beam at an accelerating voltage of 4 kV. Thioglycerol was used as the matrix and NaI as the cationizing agent. The samples were dissolved in Me_2SO. Tandem MS–MS analysis was performed using a Jeol JMS-SX/SX102A tandem mass spectrometer at 10 kV accelerating voltage, using xenon as the bombarding gas. Helium was used as the collision gas (in the third field free region). The samples dissolved in Me_2SO were loaded into a matrix of thioglycerol.

NMR analysis.—The sample was deuterium exchanged several times by freeze-drying from D_2O and then examined in solution in deuterated Me_2SO. The spectrum was recorded at 45° with a Bruker AMX 500 at 500 MHz using the standard pulse sequence supplied by Bruker. Chemical shifts are reported in ppm using the Me_2SO signal (2.49 ppm) as reference.

3. Results

Preliminary characterization and purification of the Nod metabolites.—*Rhizobium fredii* HH103 was grown either in the presence or absence of genistein as inducer, with the addition of L-(methyl-[^{14}C])-methionine and [^{14}C]-glucosamine hydrochloride. TLC analysis showed one major Nod metabolite and four additional spots. For preparative purposes, 10 L of medium from the genistein-induced culture were extracted with *n*-butanol. The *n*-butanol extract was pre-purified using Super Clean LC-18 columns and eluting with different acetonitrile–water compositions (20, 45, and 60%, v/v). Each fraction was subjected to HPLC analysis using a semi-preparative C_{18} col-

Table 1
FABMS and CID-MS–MS analysis of LCOs from *R. fredii* HH103

Fraction	$[M+H]^+$	$[M+Na]^+$	$[M+H+TG]^+$	$[M+Na+TG]^+$	Oxonium-type ions	Ion from β-cleavage	Designation
F1	1171 $^{\mathrm{m}}$		1279				Rf-IV($C_{16:1}$, Fuc)
	1185		1293				Rf-IV($C_{16:1}$, MeFuc)
	1374		1482				Rf-V($C_{16:1}$, Fuc)
	1388 $^{\mathrm{M}}$	1410	1496	1518	398, 601, 804, 1007	1228	Rf-V($C_{16:1}$, MeFuc)
F2	982 $^{\mathrm{m}}$		1090				Rf-III($C_{16:1}$, MeFuc)
	1376						Rf-V($C_{16:0}$, Fuc)
	1390 $^{\mathrm{M}}$	1412			400, 603, 806, 1009	1230	Rf-V($C_{16:0}$, MeFuc)
F3	1187	1209					Rf-IV($C_{16:0}$, MeFuc)
	1402 $^{\mathrm{m}}$		1510				Rf-V($C_{18:1}$, Fuc)
	1416 $^{\mathrm{M}}$	1438	1524	1546	426, 629, 832, 1035	1256	Rf-V($C_{18:1}$, MeFuc)
F4	1199 $^{\mathrm{m}}$		1307				Rf-IV($C_{18:1}$, Fuc)
	1213 $^{\mathrm{M}}$	1235	1321	1343	426, 629, 832	1053	Rf-IV($C_{18:1}$, MeFuc)
	1416		1524				Rf-V($C_{18:1}$, MeFuc)
F5	1010	1032	1118	1140	426, 629	850	Rf-III($C_{18:1}$, MeFuc)
F6	1012 $^{\mathrm{m}}$	1034			428, 631	852	Rf-III($C_{18:0}$, MeFuc)
	1215	1237			428, 631, 834	1055	Rf-IV($C_{18:0}$, MeFuc)
	1418 $^{\mathrm{M}}$	1440			428, 631, 834, 1037	1258	Rf-V($C_{18:0}$, MeFuc)

$^{\mathrm{M}}$ Major component in each fraction.
$^{\mathrm{m}}$ Minor component in each fraction.

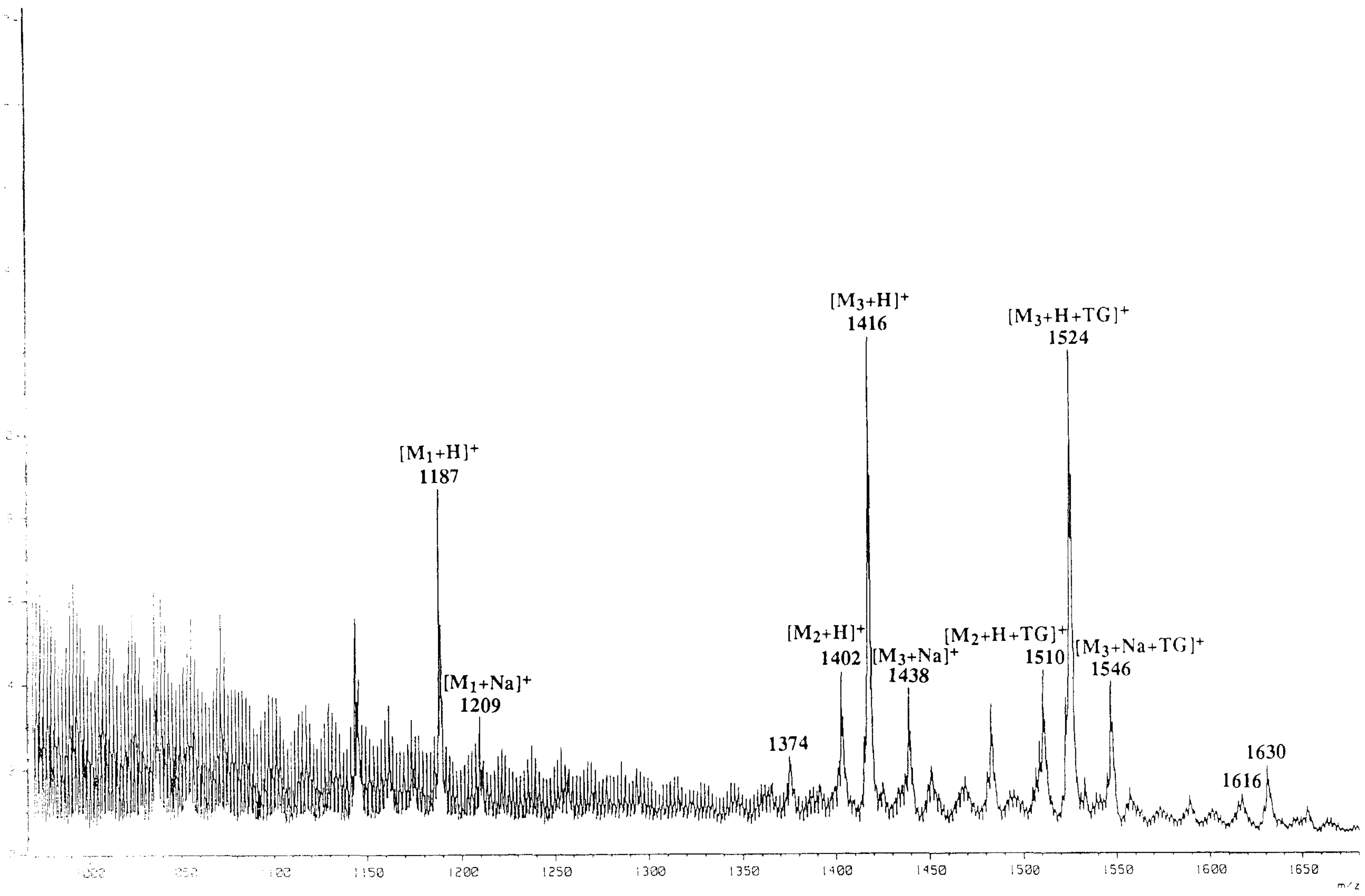


Fig. 3. Positive FAB mass spectrum of fraction F3 from *R. fredii* HH103 showing three different $[M+H]^+$ pseudomolecular ions (M_1, M_2, and M_3).

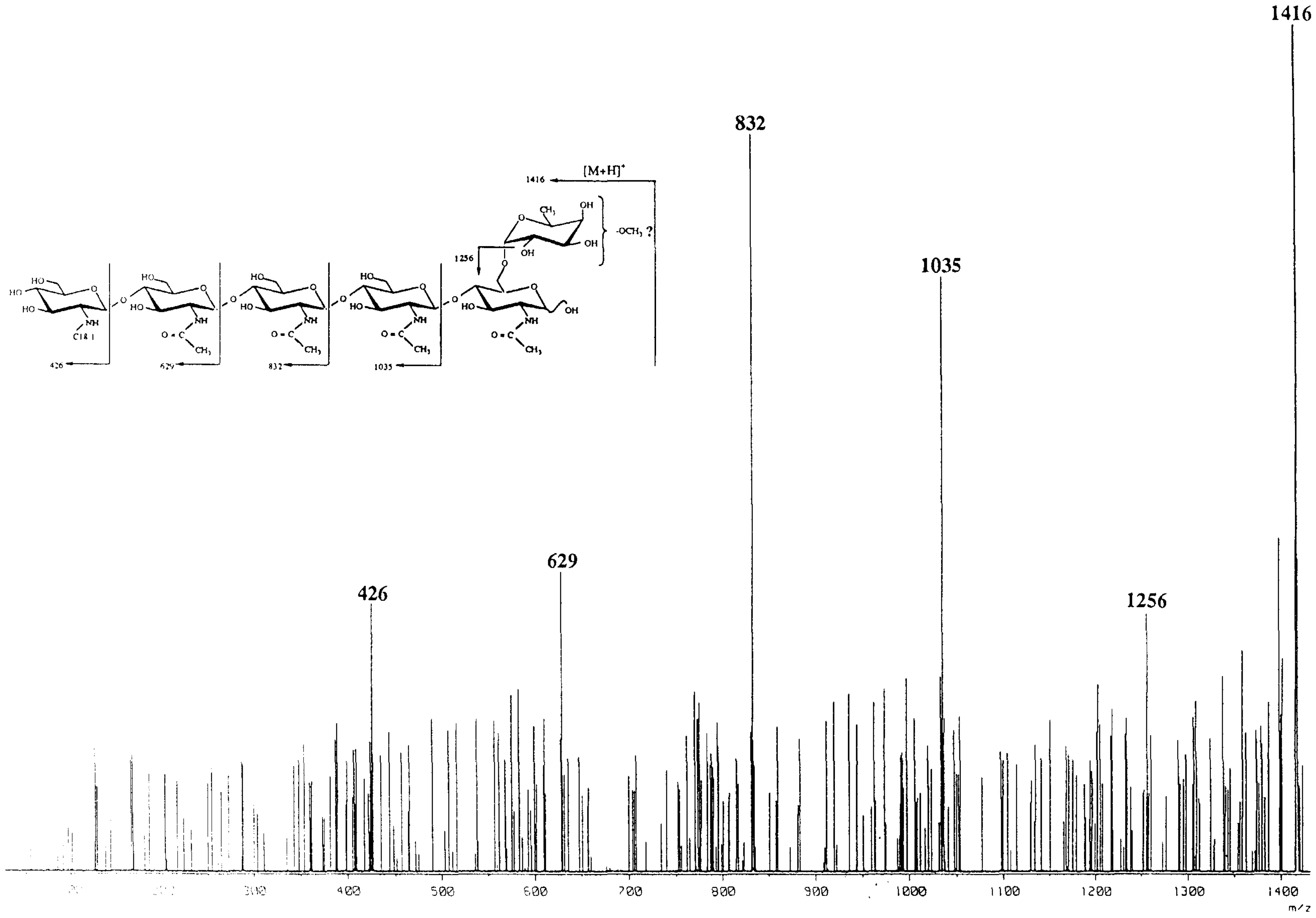


Fig. 4. Collision-induced dissociation mass spectrum of the $[M+H]^+$ at m/z 1416 in fraction F3, and fragmentation scheme for NodRf-V($C_{18:1}$, MeFuc).

umn, and the HPLC profile of the 45% fraction is shown (Fig. 1). Six fractions (F1–F6) were isolated.

Composition and methylation analysis.—GLC–MS of the TMS ethers of methyl glycosides showed 2-*O*-methylfucose and glucosamine. Glucosamine was assigned as D-GlcNH$_2$ following GLC–MS analysis of its trimethylsilylated (+)-2-butyl glycoside and (±)-2-butyl glycosides, and L-Fuc was assigned by comparing the GLC results for the permethylated (+)-2-octyl glycosides and (±)-2-octyl glycosides of standard L-fucose and the Nod factors. Methylation analysis of all factors showed the presence of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol derived from terminal 2-*O*-methylfucose, 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-*N*-acetyl-*N*-methyl-glucosaminitol from the non-reducing terminal glucosamine residue, 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-*N*-acetyl-*N*-methyl-glucosaminitol from the internal glucosamine residues, and 1,4,5,6-tetra-*O*-acetyl-3-*O*-methyl-*N*-acetyl-*N*-methyl-glucosaminitol from the reducing terminal glucosamine residue substituted at O-6. To demonstrate and locate the substitution of the methyl group on the fucose, methylation analysis was performed using trideuteriomethyl iodide. The fragments in the mass spectra (Fig. 2) allowed the identification of 1,5-di-*O*-acetyl-3,4-di-*O*-trideuteriomethyl-2-*O*-methyl-fucitol and showed the substitution of a methyl group at the O-2 of the fucose. The fatty acid compo-

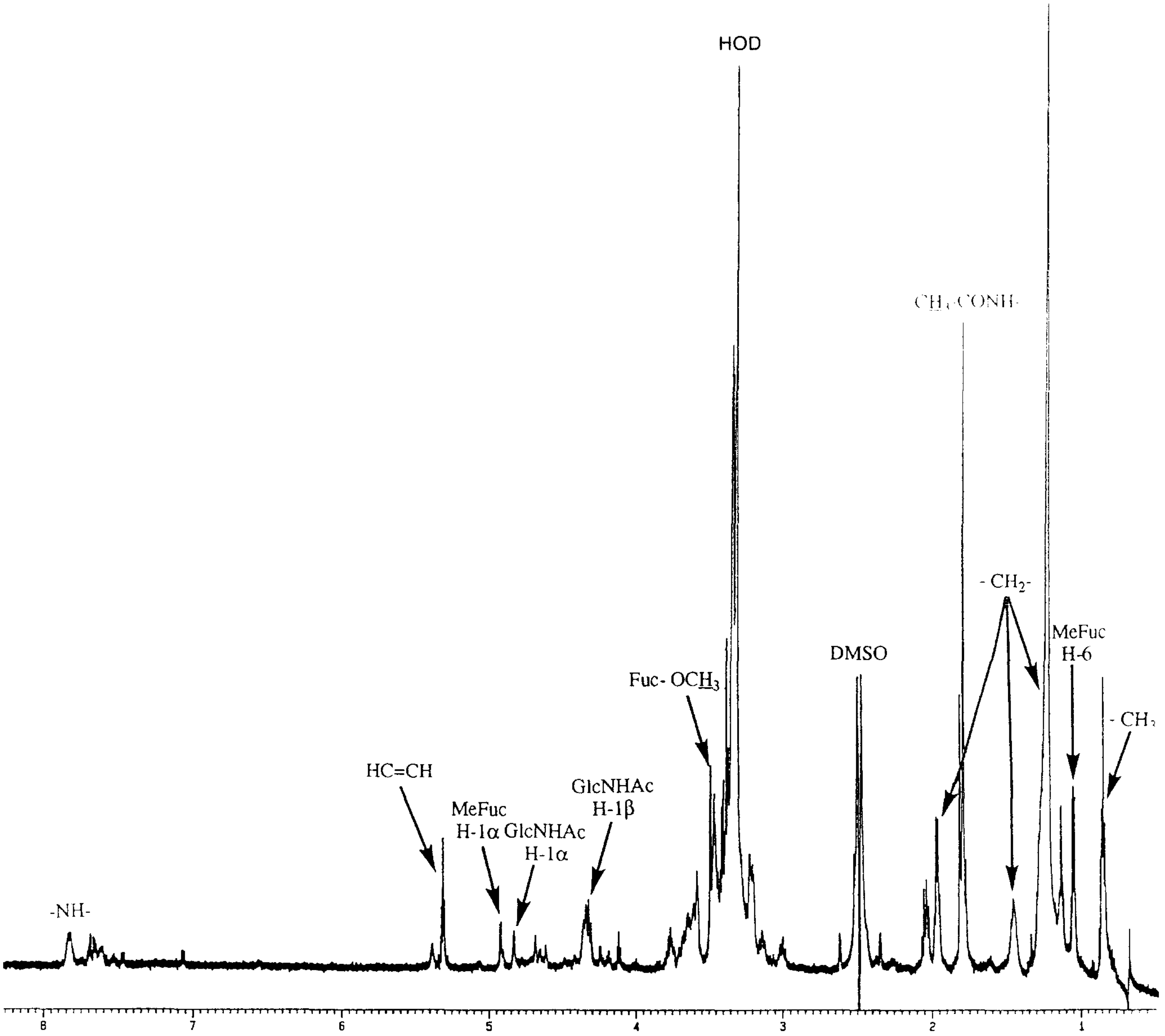
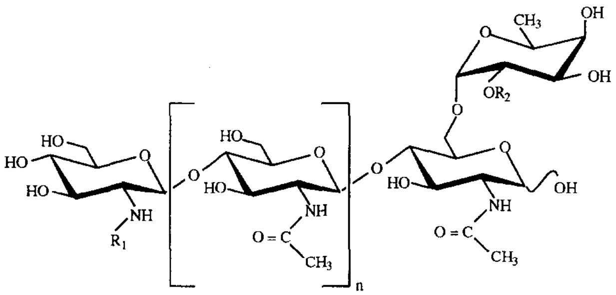


Fig. 5. ^{1}H NMR spectrum of fraction F3 [mainly NodRf-V(C$_{18:1}$, MeFuc)].

sition was hexadecanoic acid ($C_{16:0}$), octadecanoic acid ($C_{18:0}$), hexadecenoic acid ($C_{16:1}$), and octadecenoic acid ($C_{18:1}$). The mass spectra of the dimethyl disulfide derivatives of the unsaturated fatty acid methyl esters (data not shown) contain $C_{18:1}$ mass fragments at *m/z* 390 (molecular ion), *m/z* 145 and 245 (from the fragmentation between the carbons that carry the dimethyl disulfide groups) which demonstrates that the double bond is at the carbon number 11. For $C_{16:1}$ the mass fragments are at *m/z* 362, 145, and 217, and have the same origin as above. The presence of the fragments at *m/z* 145 and 217 demonstrate that the double bond is located at carbon 9. Fraction F1 contains only $C_{16:1}$, F2 contains both $C_{16:1}$ and $C_{16:0}$, F3 contains both $C_{18:1}$ and $C_{16:0}$, F4 contains only $C_{18:1}$, F5 contains only $C_{18:1}$, and the fraction F6 contains only $C_{18:0}$.

FABMS analysis of fractions F1–F6.—The results of the FABMS analysis of fractions F1–F6 are summarized in Table 1. The FAB mass spectra revealed the presence of $[M + H]^+$ and $[M + Na]^+$ pseudomolecular ions. Thioglycerol adduct ions $[M + H + TG]^+$ and $[M + Na + TG]^+$ were also observed when unsaturated fatty acids were present. The positive-ion collision-induced dissociation tandem mass spectrum (CID-MS–MS) contained oxonium-type fragment ions formed by sequential cleavage of each glycosidic linkage with charge retention on the non-reducing portion of the molecule. The *m/z* of the smallest fragment of the oxonium-type fragment ion series allowed the identification of the fatty acyl group on the non-reducing terminal GlcN residue. In addition, the CID-tandem mass spectra contained an ion at *m/z* $[M + H - 160]^+$ arising by β-cleavage of 2-*O*-methyl fucose. The FAB mass spectrum and the collision-induced dissociation mass spectrum of the fraction F3 are shown in Figs. 3 and 4.

NMR analysis.—The ^1H NMR spectrum obtained for fraction F3 [mainly NodRf-V($C_{18:1}$, MeFuc)] is shown (Fig. 5). The signal at 4.93 ppm (*J* 3.3 Hz) was assigned to the anomeric proton of an α-linked 2-*O*-methylfucosyl residue. A second minor doublet at 4.92 ppm was assigned to the 2-*O*-methylfucosyl residue, linked to the β-anomeric lipo-chitin oligosaccharide [10]. The signal at 3.49 ppm was assigned to the methoxy protons of the 2-*O*-methylfucosyl residue and the doublet at 1.05 ppm (*J* 6.2 Hz) was assigned to the methyl group of the 2-*O*-methylfucosyl residue. The signal at 4.84 ppm (*J* ≈ 2 Hz) was assigned to the anomeric proton of the reducing α-*N*-acetylglucosamine residue of the lipo-chitin oligosaccharide. The resonances at 4.32–4.40 ppm (*J* 7.9 Hz) are due to the anomeric protons of the β-linked *N*-acyl and *N*-acetylglucosaminosyl residues. The signal at 5.32 (*J* 4.8 Hz) was assigned to the olefinic protons of the unsaturated acyl residue. The small coupling constant value allowed the identification of the *cis*-anomer. The signals from methyl and methylene groups of the fatty acid residue, the methyl group of the acetamido substituent, and the NH protons, are assigned in the ^1H NMR spectrum.



Fraction	$[M+H]^+$	n	R_1 (fatty acid)	R_2
F1	1171	2	$C_{16:1\Delta 9}$	H
	1185	2	$C_{16:1\Delta 9}$	CH_3
	1374	3	$C_{16:1\Delta 9}$	H
	1388	3	$C_{16:1\Delta 9}$	CH_3
F2	982	1	$C_{16:1\Delta 9}$	CH_3
	1376	3	$C_{16:0}$	H
	1390	3	$C_{16:0}$	CH_3
F3	1187	2	$C_{16:0}$	CH_3
	1402	3	$C_{18:1\Delta 11}$	H
	1416	3	$C_{18:1\Delta 11}$	CH_3
F4	1199	2	$C_{18:1\Delta 11}$	H
	1213	2	$C_{18:1\Delta 11}$	CH_3
	1416	3	$C_{18:1\Delta 11}$	CH_3
F5	1010	1	$C_{18:1\Delta 11}$	CH_3
F6	1012	1	$C_{18:0}$	CH_3
	1215	2	$C_{18:0}$	CH_3
	1418	3	$C_{18:0}$	CH_3

Fig. 6. Summary of structures of Nod factors from *R. fredii* HH103.

4. Discussion

The structures of sixteen Nod factors isolated from genistein-induced cultures of *Rhizobium fredii* HH103 have been determined. These LCOs were present in six C_{18}-HPLC fractions and were studied using methylation analysis, FABMS, NMR, and CID-MS–MS. From the results of this analysis we conclude that *R. fredii* HH103 produces four different series of Nod factors having a backbone of chitin oligomers ranging from trimer to pentamers, substituted by 2-*O*-methyl-fucose at O-6 of the reducing terminal *N*-acetylglucosamine residue. Each series is *N*-acylated at the non-reducing terminal residue with a different fatty acid residue: octadecanoic acid (stearic acid), cis-octadec-11-enoic acid (vaccenic acid), cis-hexadec-9-enoic acid (palmitoleic acid), or hexadecanoic acid (palmitic acid). In the last series, the trimer was not found. In five different cases, in addition to the Nod factors described above, a series of structures corresponding to 6-*O*-fucosylated species was identified (Fig. 6). Together, these results show that the array of Nod factors produced by HH103 is even wider than that produced by *R. fredii* USDA259. This difference might be responsible for the broader effective host-range exhibited by strain HH103.

Acknowledgements

We thank the Comision Interministerial de Ciencia y Tecnología (grants Nos BIO093-0716-C04 and BIO096-1469-C03) and the Netherlands Organisation for Scientific Research, NWO, (J.T.-O. and H.P.S.) for financial support.

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