

Rhizobium leguminosarum bv. *trifolii* produces Lipo-chitin Oligosaccharides with *nodE*-dependent Highly Unsaturated Fatty Acyl Moieties

AN ELECTROSPRAY IONIZATION AND COLLISION-INDUCED DISSOCIATION TANDEM MASS SPECTROMETRIC STUDY*

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Koen M. G. M. van der Drift[‡], Herman P. Spaijk[§], Guido V. Bloemberg[§],
Anton A. N. van Brussel[§], Ben J. J. Lugtenberg[§], Johan Haverkamp[‡],
and Jane E. Thomas-Oates[‡]

From the [‡]Department of Mass Spectrometry, Utrecht University, F.A.F.C. Went Gebouw, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands and the [§]Institute of Molecular Plant Sciences, Clusius Laboratory, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

The lipo-chitin oligosaccharides (LCO) or nodulation factors synthesized by *Rhizobium leguminosarum* bv. *trifolii* were analyzed using positive mode fast atom bombardment and positive and negative mode electrospray ionization mass spectrometry. From their mass spectrometric behavior it is clearly possible to distinguish between the $[M + Na]^+$ pseudomolecular ion of the *nodE*-independent molecule IV(C18:1,Ac) and the $[M + H]^+$ pseudomolecular ion of the *nodE*-dependent molecule IV(C20:4,Ac), although they both have the same mass value. The results unequivocally show that the bacterial strain investigated produces *nodE*-dependent LCOs with highly unsaturated fatty acyl moieties. We further demonstrate that the interpretation of the mass spectrometric data by Philip-Hollingsworth *et al.* (Philip-Hollingsworth, S., Orgambide, G. G., Bradford, J. J., Smith, D. K., Hollingsworth, R. I., and Dazzo, F. B. (1995) *J. Biol. Chem.* 270, 20968) is incorrect and that their data do not contradict our hypothesis that the *nodE* gene determines the host specificity of *R. leguminosarum* bv. *trifolii*.

The symbiosis between bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* and their leguminous host plants results in the formation of nitrogen-fixing root nodules. This symbiosis is regarded as being highly host-specific. Some bacterial strains, for example *Rhizobium* sp. NGR234 (1), are able to nodulate a broad range of host plants, while other strains are able to nodulate only a few plant species. Examples of rhizobia with a narrow host range are *Rhizobium leguminosarum* (*R. leguminosarum*) bv. *trifolii*, which nodulates plants of the genus *Trifolium* and the closely related *R. leguminosarum* bv. *viciae* which nodulates plants of the genera *Lathyrus*, *Lens*, *Pisum*, and *Vicia*.

It is generally recognized that the host specificity is mediated by signal molecules from both the plant and the bacterium. Flavonoids secreted by the plant are recognized by the guest bacteria resulting in the transcription of the nodulation genes (2, 3). These genes encode the nodulation enzymes involved in the biosynthesis of lipo-chitin oligosaccharide (LCO)¹ signal

molecules, also called Nod metabolites or Nod factors. In general, LCOs consist of a chitin backbone of three to six β 1 \rightarrow 4-linked *N*-acetylglucosamine (GlcNAc) residues, *N*-acylated on the nonreducing terminal residue and bearing various biovar-specific modifications on both terminal residues (see *e.g.* Refs. 4–6). Although the mechanism is not yet clear, the LCOs are involved in mediating the host-specific biological activity on the plant (4, 6).

In a previous article (7) we described the analysis of LCOs produced by *R. leguminosarum* bv. *trifolii*. Several analytical techniques, such as reversed phase high performance liquid chromatography (reversed phase-HPLC) with photodiode array detection, fast atom bombardment mass spectrometry (FAB-MS), collision-induced dissociation tandem mass spectrometry (CID-MS-MS), and chemical modifications prior to mass spectrometry were applied. It was demonstrated that a mixture of LCOs is produced, with a chitin-type backbone of three to five GlcNAc residues, and bearing an *O*-acetyl group on the nonreducing terminal residue. The fatty acyl moieties vary from the common C16:0, C16:1, C18:0, C18:1, and C20:1 to the specific *nodE*-dependent highly unsaturated C18:3, C20:2, C20:3, and C20:4. It was postulated that these highly unsaturated fatty acyl moieties, although produced in a minor quantity compared to the *nodE*-independent common LCOs, determine the host specificity of *R. leguminosarum* bv. *trifolii*.

In a recent study, Philip-Hollingsworth *et al.* (8) rejected our assignment of highly unsaturated LCOs and suggested that they have “critically evaluated the proposed function of the *nodE* gene by performing detailed structural analysis” of LCOs produced by *R. leguminosarum* bv. *trifolii*. Based on data obtained from techniques such as electrospray ionization (ESI) mass spectrometry and gas chromatography coupled with mass spectrometry, no LCOs with highly unsaturated fatty acyl moieties could be detected in the cell membrane extracts. Furthermore, no differences were found in the spectra of LCOs produced by a wild-type strain, a strain lacking the *nodE* gene and a strain overexpressing the *nodE* gene. Since the two conflicting studies were carried out using different strains of *R. leguminosarum* bv. *trifolii*, and employing different methods of culturing and extracting the LCOs from the cell cultures, the fact that the authors of Ref. 8 were unable to detect LCOs with highly unsaturated fatty acyl moieties certainly does not dem-

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¹ The abbreviations used are: LCO, lipo-chitin oligosaccharides;

HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; CID-MS-MS, collision-induced dissociation tandem mass spectrometry; ESI, electrospray ionization.

FIG. 1. Photodiode array UV spectrogram of reversed phase HPLC eluent of LCOs derived from strain LPR5045.pRtRF101. The absorbance of the late eluting fractions is not shown, since it is obscured by the peaks around fraction 35. For this HPLC analysis an extract (obtained after prepurification as described previously (7)) derived from 200 ml of culture was used.

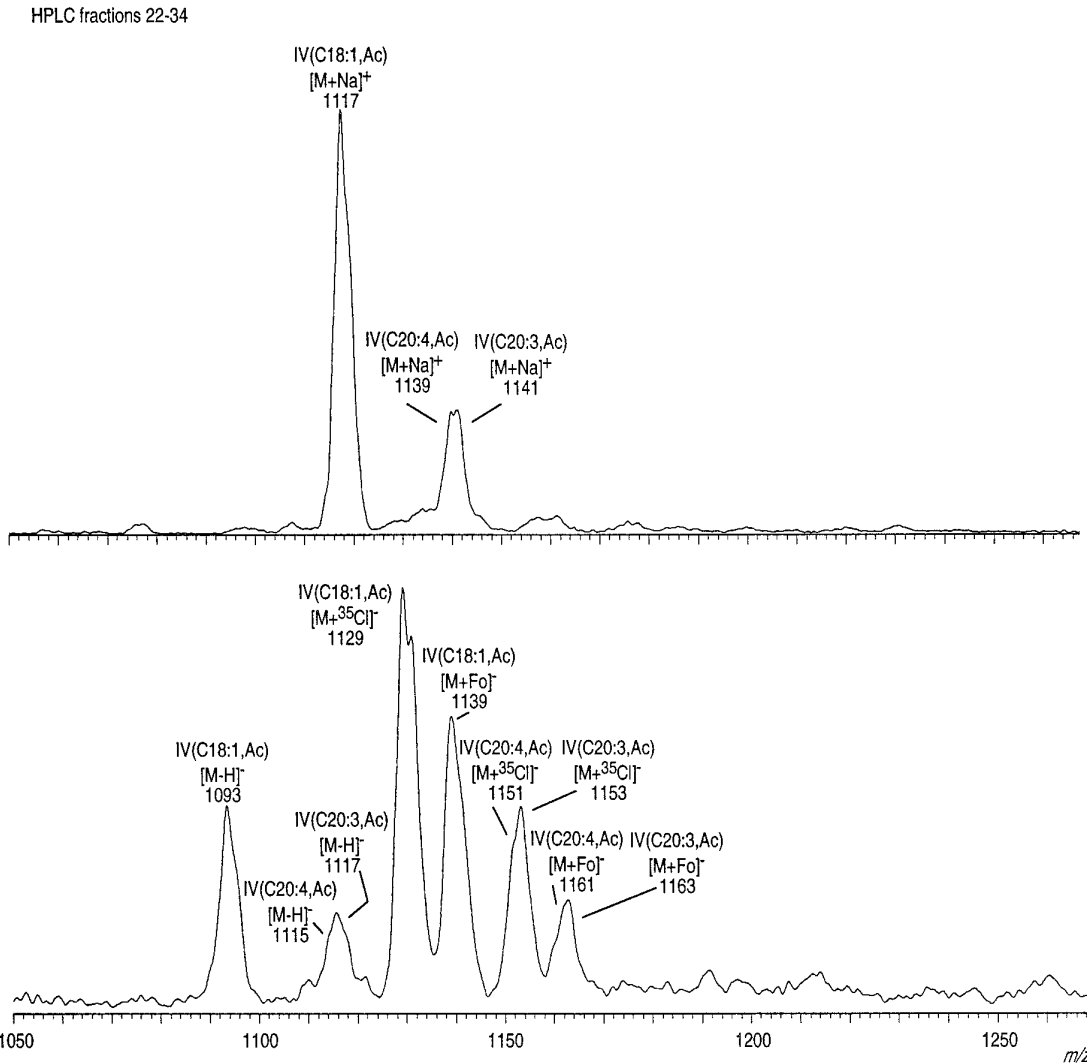
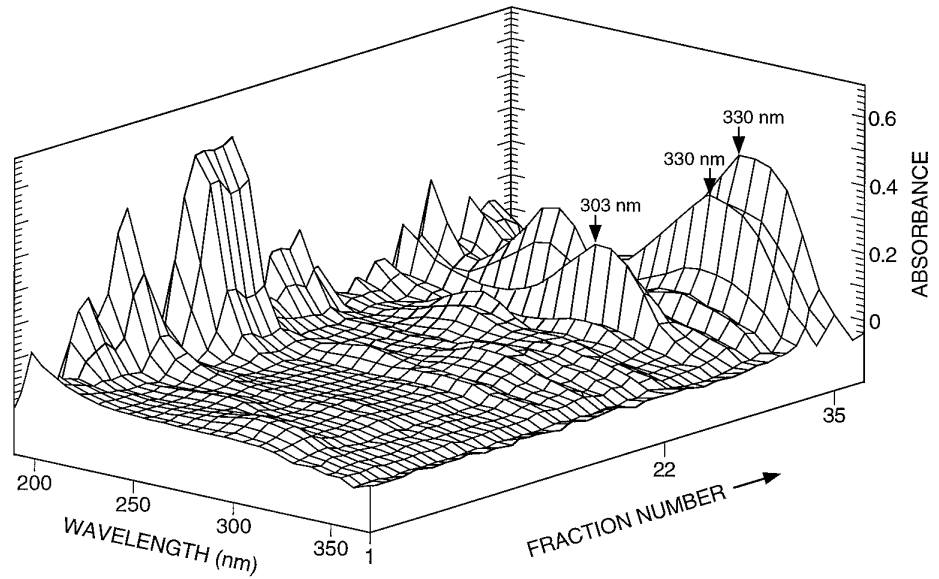


FIG. 2. Electrospray ionization mass spectra of HPLC pool A (fractions 22-34), positive mode (*top*), negative mode (*bottom*). In the positive mode spectrum $[M + Na]^+$ pseudomolecular ions are observed at m/z 1117, 1139, and 1141. The corresponding pseudomolecular ions in the negative mode are $[M - H]^-$, $[M + ^{35}Cl]^-$ and $[M + Fo]^-$.

onstrate that these molecules cannot be produced by the bacteria under any conditions.

To account for the absence of LCOs with highly unsaturated

fatty acyl moieties, Philip-Hollingsworth *et al.* (8) suggest that the ions in the mass spectra assigned as *nodE*-dependent LCOs by Spaink *et al.* (7) were actually due to sodium adducts of the

TABLE I

Summary of lipo-chitin oligosaccharides found in *R. leguminosarum* *bv. trifolii* wild-type strain LPR5045.pRTRF101 obtained on positive and negative mode electrospray mass spectrometry

Fraction pool	<i>m/z</i>	Pseudomolecular ion	Assignment
A (22–34)	1093	[M – H] [–]	IV(C18:1,Ac)
	1117	[M + Na] ⁺	
	1129	[M + ³⁵ Cl] [–]	IV(C20:3,Ac)
	1139	[M + Fo] [–]	
	1117	[M – H] [–]	
	1141	[M + Na] ⁺	
	1153	[M + ³⁵ Cl] [–]	IV(C20:4,Ac)
	1163	[M + Fo] [–]	
	1115	[M – H] [–]	
	1139	[M + Na] ⁺	
1151	[M + ³⁵ Cl] [–]		
1161	[M + Fo] [–]		
B (35–45)	1095	[M – H] [–]	IV(C18:0,Ac)
	1119	[M + Na] ⁺	IV(C20:3,Ac)
	1131	[M + ³⁵ Cl] [–]	
	1141	[M + Fo] [–]	
	1117	[M – H] [–]	
	1141	[M + Na] ⁺	IV(C20:4,Ac)
	1153	[M + ³⁵ Cl] [–]	
	1163	[M + Fo] [–]	
	1115	[M – H] [–]	
	1139	[M + Na] ⁺	
1151	[M + ³⁵ Cl] [–]		
1161	[M + Fo] [–]		

major *nodE*-independent LCOs. This reassignment was based on the fact that “no corresponding molecular ions were found in the negative mode electrospray ionization mass spectra of the same samples” (8). We present data that prove that it is not possible to confuse an [M + H]⁺ with an [M + Na]⁺ pseudomolecular ion.

In this paper we describe additional experiments we have carried out to reinforce our structural assignment of the structures of the *nodE*-dependent LCOs produced by *R. leguminosarum* *bv. trifolii*. For this purpose the LCOs were submitted to both positive and negative mode ESI-MS and to FAB-MS and CID-MS-MS experiments. The results clearly support our earlier findings (7) that *R. leguminosarum* *bv. trifolii* indeed produces LCOs with highly unsaturated fatty acyl moieties.

EXPERIMENTAL PROCEDURES

Isolation and Separation of Lipo-chitin Oligosaccharides—The LCOs were isolated from wild-type *R. leguminosarum* *bv. trifolii* strain LPR5045.pRtRF101 and separated using HPLC as described previously (7), and 1-min fractions were collected. To elute all LCOs from the HPLC column, a gradient of acetonitrile and water was used: 30% acetonitrile (20 min), 40% (20 min), 42% (20 min), and 50% (20 min).

Electrospray Mass Spectrometry—Positive and negative ion mode electrospray mass spectra were obtained using a VG Platform II single quadrupole mass spectrometer. Aliquots of 10 μl of the pooled fractions were infused into a mobile phase of acetonitrile/water (50: 50, v/v) and introduced into the electrospray source at a flow rate of

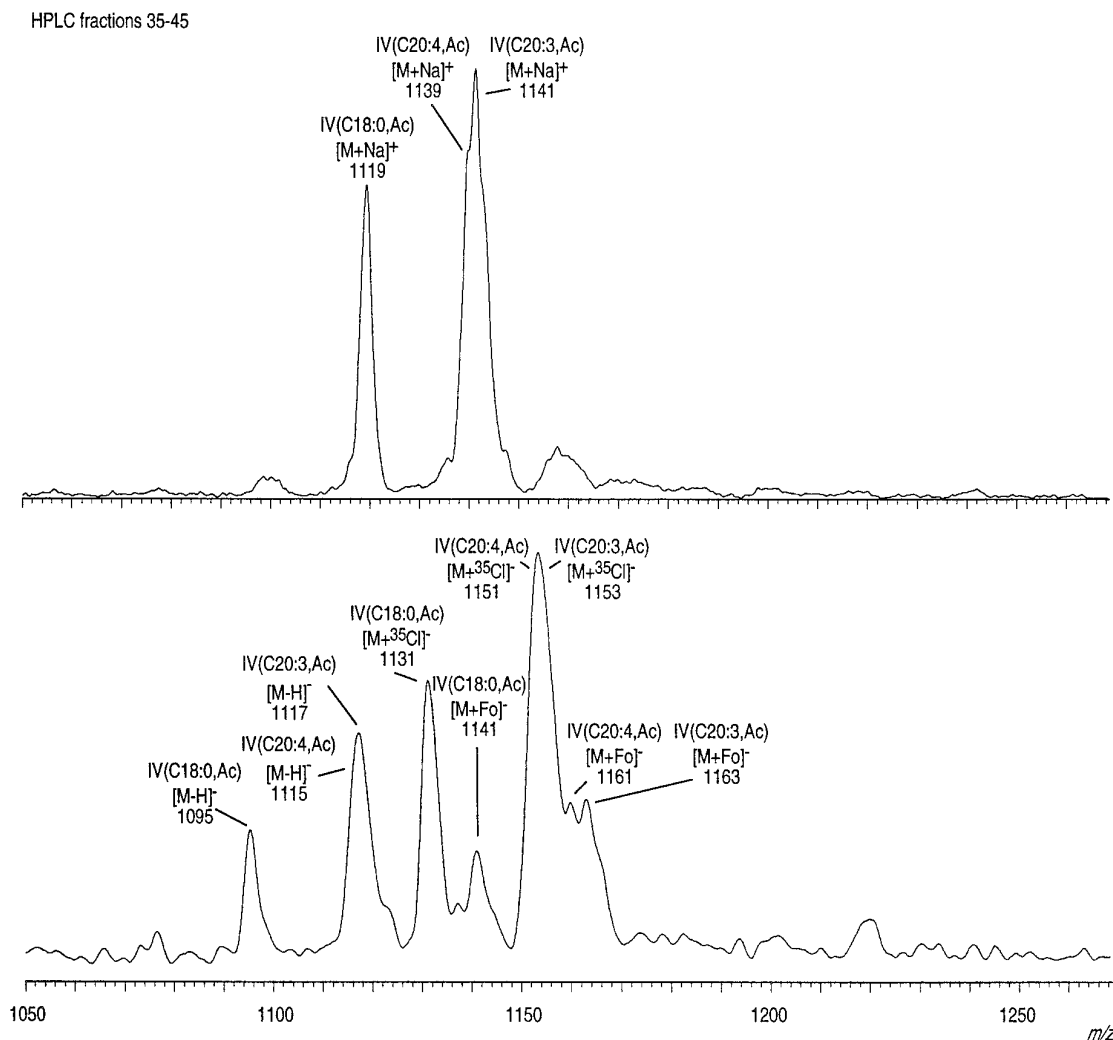


FIG. 3. Electrospray ionization mass spectra of HPLC pool B (fractions 35–45), positive mode (top), negative mode (bottom). In the positive mode spectrum [M + Na]⁺ pseudomolecular ions are observed at *m/z* 1119, 1139, and 1141. The corresponding pseudomolecular ions in the negative mode are [M – H][–], [M + ³⁵Cl][–] and [M + Fo][–].

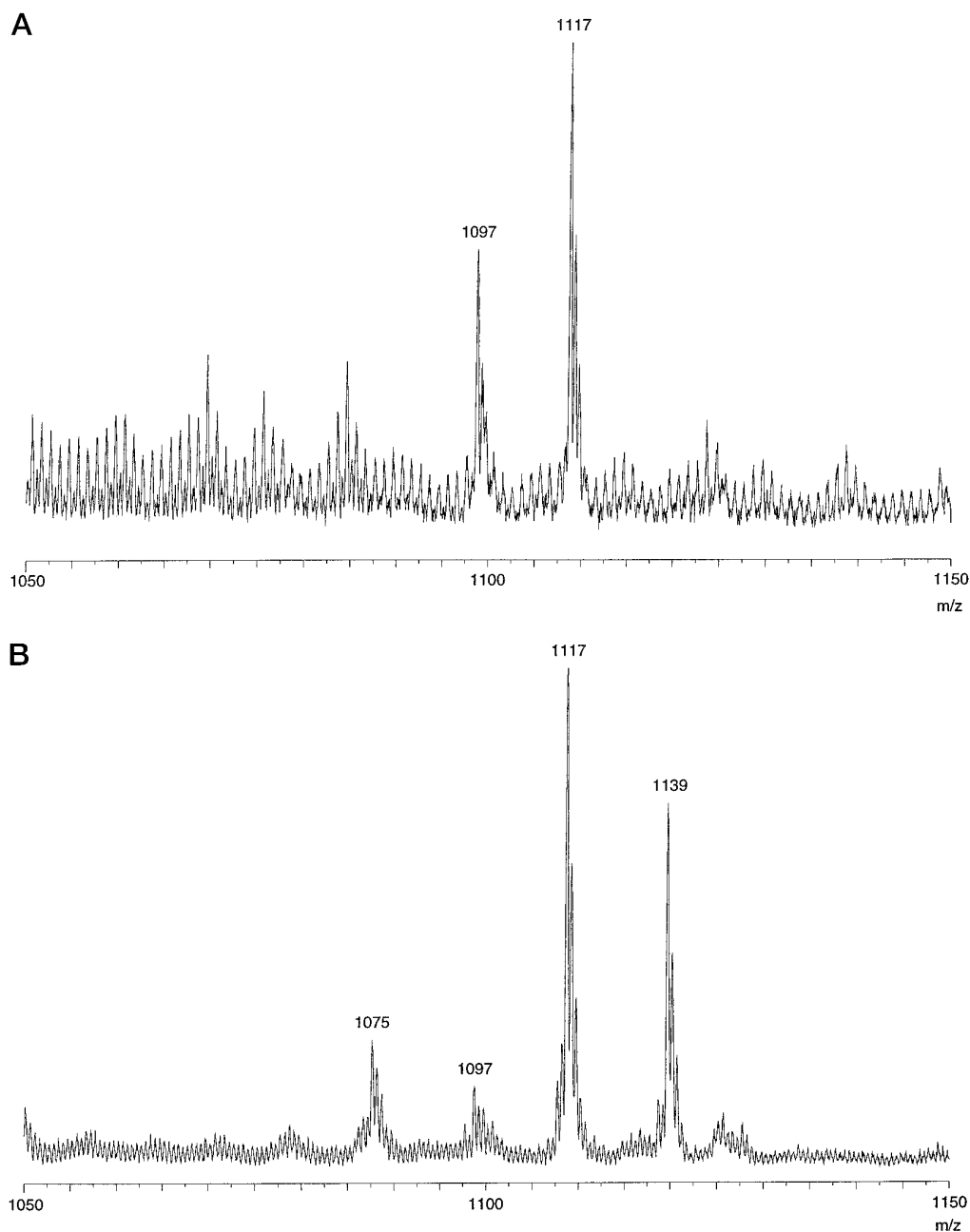


FIG. 4. Partial positive mode fast atom bombardment spectra of an early eluting HPLC fraction in a thioglycerol matrix (A) and *m*-nitrobenzyl alcohol matrix (B). The ions at m/z 1077 and 1095 are derived from LCO molecules lacking the acetyl group on the nonreducing terminal GlcNAc residue (7).

5 μ l/min. In the positive mode spectra were scanned at a speed of 10 s for m/z 800-1400, with a cone voltage of 90 V. In the negative mode spectra were scanned at a speed of 10 s for m/z 100-1500, using a cone voltage of -40 V. All spectra were recorded and processed using the MassLynx 2.0 software.

Fast Atom Bombardment and Collision-induced Dissociation Tandem Mass Spectrometry—FAB-mass spectra were obtained in the positive ion mode using a JEOL JMS-SX/SX102A tandem mass spectrometer using 10 kV accelerating voltage. The FAB gun was operated at 6 kV with an emission current of 10 mA using xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for m/z 100-2000 and recorded and processed on a Hewlett-Packard HP9000 series data system using the JEOL Complement software. Collision-induced dissociation tandem mass spectra were obtained on the same instrument, using helium as the collision gas (in the third field free region) at a pressure sufficient to reduce the parent ion to one third of its original intensity. Aliquots of 2 μ l of the pooled fractions were loaded into a matrix of either thioglycerol or *m*-nitrobenzyl alcohol dosed with sodium.

RESULTS AND DISCUSSION

Isolation and Separation of Lipo-chitin Oligosaccharides—The HPLC diode-array profile (Fig. 1) clearly shows the presence of compounds having UV absorption maxima at 260, 303, or 330 nm, which we have previously assigned as corresponding to LCOs containing highly unsaturated fatty acyl moieties. These absorption maxima are indicative of a conjugated π -system with two, three, or four double bonds, respectively, in conjugation with a carbonyl group (9, 10). The UV absorption spectra of synthetically produced fatty acyl moieties, which contain three or four such double bonds, support this assignment (11).² It should be noted that the species absorbing with

² S. van Leeuwen, H. P. Spaink, G. A. van der Marel, and J. H. van Boom, manuscript in preparation.

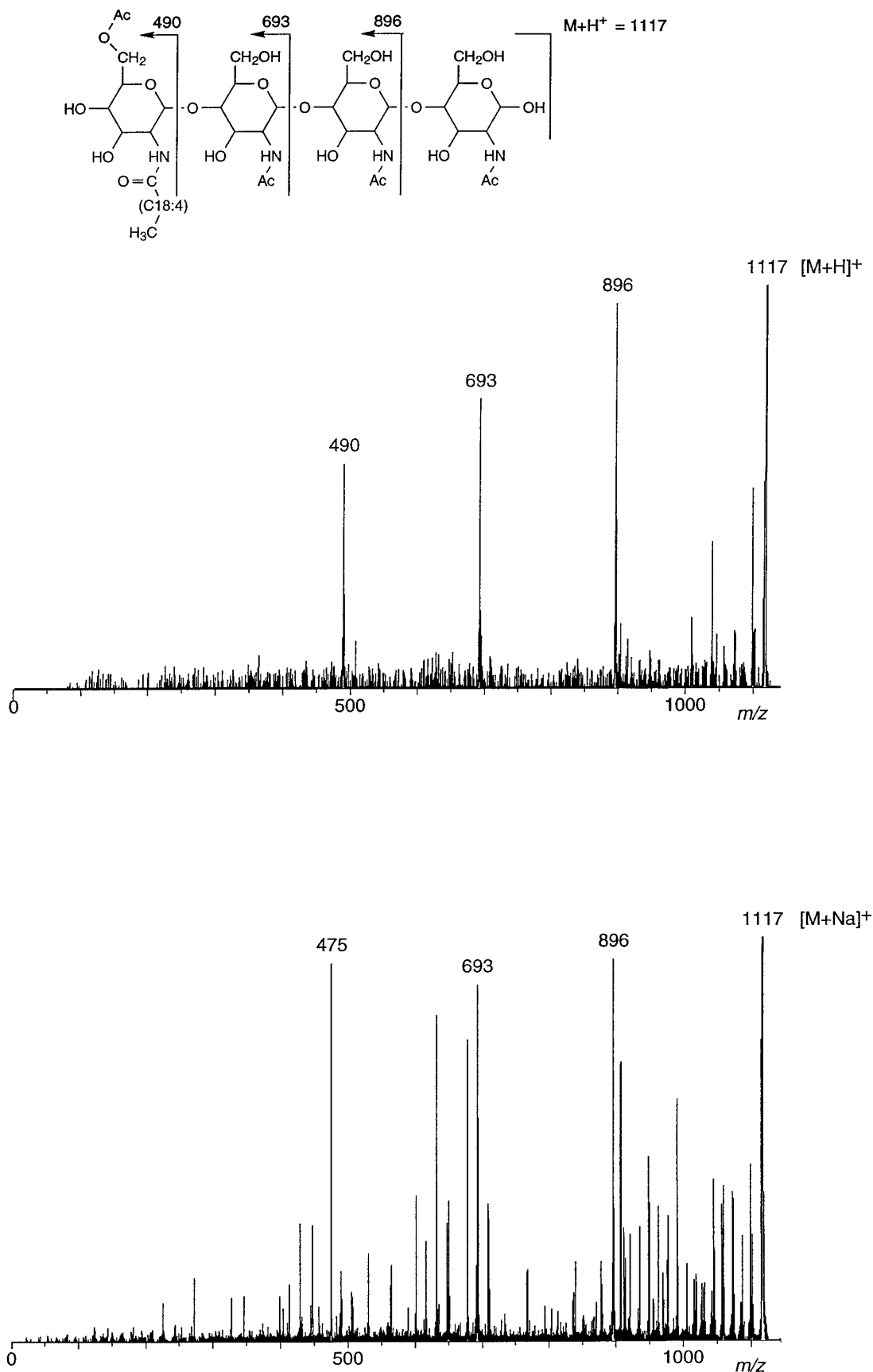


FIG. 5. Collision-induced dissociation mass spectra of two LCO molecules, both having pseudomolecular ions at m/z 1117. *Top panel*, spectrum of IV(C20:4,Ac), with $[M + H]^+$ pseudomolecular ion and its fragmentation scheme. *Bottom panel*, the $[M + Na]^+$ pseudomolecular ion of IV(C18:1,Ac).

a maximum of 260, 303, and 330 nm give UV absorbances of sufficient intensity to be detectable with any commercially available HPLC diode array detection system. The failure of Philip-Hollingsworth *et al.* (8) to observe such UV-absorbing peaks in their extracts can be explained in several ways. First, it has been observed that different growth conditions can change the relative quantities of the different types of LCOs produced by one bacterial strain (10, 13, 14). It is therefore quite possible that the strain studied by Philip-Hollingsworth *et al.* (8) does not produce LCOs with UV absorption maxima at 303 or 330 nm, due to their employing growth conditions that are unfavorable for the production of highly unsaturated fatty acids by Node. Second, the solvent conditions chosen by Philip-Hollingsworth *et al.* (8) might not be suitable for the extraction of the C20 acyl containing LCOs; in our hands their conditions are not suitable for dissolving the classes of LCOs eluting later than 22 min in our HPLC chromatogram. Third, since the wild-type strain ANU843 has a rather unstable symbiotic phenotype,³ the strain used by Philip-Hollingsworth *et al.* (8) might have suffered from mutations affecting *nodE* expression. However, regardless of the cause for the failure of Philip-Hollingsworth *et al.* (8) to detect specific LCOs, it should be noted that their results do not imply that the production and detection of highly unsaturated LCOs is not possible using a different strain, different growth conditions, and/or different purification methods.

ESI-MS—The so-called soft ionization mass spectrometric techniques, such as electrospray or fast atom bombardment ionization, generate ions in the positive mode by the addition of a cation to the sample molecule. In most cases the molecules are protonated, yielding an $[M + H]^+$ pseudomolecular ion, which is detected at an m/z value 1 mass unit higher than the molecular mass of the sample molecule. Because of the use of an aqueous mobile phase to transport the sample molecules to the electrospray interface, the sample solution is exposed to sodium ions, which leads to the formation of an $[M + Na]^+$ pseudomolecular ion. In the negative mode, pseudomolecular ions are generated either by the loss of a proton or by the addition of an anion. Common negative mode pseudomolecular ions are $[M - H]^-$ or, in an aqueous solution $[M + ^{35}Cl]^-$. These are detected at m/z values of 1 mass unit lower or 35 and 37 mass units higher than the molecular mass of the sample molecule, respectively.

In the ESI mass spectrum of the pool corresponding to fractions 22–34 abundant $[M + Na]^+$ pseudomolecular ions were observed in the positive mode at m/z 1117, 1139, and 1141 (Fig. 2, *top panel* and Table I). In the negative mode, abundant clusters of three pseudomolecular ions were observed in the mass spectrum (Fig. 2, *lower panel*, and Table I). These can be assigned as the $[M - H]^-$ (at m/z 1093, 1115, and 1117), $[M + ^{35}Cl]^-$ (at m/z 1129, 1151, and 1153) and $[M + Fo]^-$ (at m/z 1139, 1161, and 1163) pseudomolecular ions corresponding in each case to the same species observed as $[M + Na]^+$ ions in the positive mode. $[M + Fo]^-$ is the adduct with formate ($HCOO^-$), which arises from formic acid used to rinse the syringe, injection loop, etc. The ions undoubtedly correspond to the LCOs abbreviated as IV(C18:1,Ac),⁴ IV(C20:4,Ac), and IV(C20:3,Ac), respectively, confirming the data from Ref. 7. In fractions 35–45 ions were observed in the positive mode (Fig. 3, *top panel*, and Table I) at m/z 1119, 1139, and 1141 corresponding

to $[M + Na]^+$ pseudomolecular ions for IV(C18:0,Ac), IV(C20:4,Ac), and IV(C20:3,Ac). The corresponding pseudomolecular ions in the negative mode (Fig. 3, *lower panel*, and Table I) were observed at m/z 1095, 1115, and 1117 (all $[M - H]^-$), 1131, 1151, and 1153 (all $[M + ^{35}Cl]^-$) and 1141, 1161, and 1163 (all $[M + Fo]^-$). It is clear from these ESI data that both the *nodE*-independent IV(C18:0,Ac) and IV(C18:1,Ac) and the *nodE*-dependent polyunsaturated fatty acid-containing IV(C20:3,Ac) and IV(C20:4,Ac) LCOs are present in our extracts.

Philip-Hollingsworth *et al.* (8) summarize the results of their mass spectrometric analysis of LCOs in their Table I. Remarkably, ions recorded in negative mode ESI are labeled as M^- ions; e.g. the ion detected at m/z 1094 is assigned as the M^- molecular ion of IV(C18:1,Ac). Unfortunately, this conclusion is based on a critical flaw, since in negative mode ESI, oligosaccharides, and glycoconjugates do not form molecular ions (such as M^-), but pseudomolecular ions (such as $[M - H]^-$). So, the ion detected at m/z 1094 by the Michigan group, must actually correspond to the $[M - H]^-$ pseudomolecular ion of IV(C18:1,Ac), which should give an ion at m/z 1093. The small discrepancy in mass is presumably due to a slight deterioration in the calibration of the mass analyzer.

FAB-MS and CID-MS-MS—Although the $[M + H]^+$ pseudomolecular ion of IV(C20:4,Ac) and the $[M + Na]^+$ pseudomolecular ion of IV(C18:1,Ac) are isobaric (both having m/z values of 1117), these two species may be very readily distinguished from each other on the basis of their tandem mass spectrometric behavior. In order to demonstrate this, IV(C18:1,Ac) ($[M + H]^+$ at m/z 1095) was loaded into a sodium-dosed *m*-nitrobenzyl alcohol matrix, forcing the LCOs to form sodiated instead of protonated pseudomolecular ions. It should be noted that the nonpooled HPLC fractions described in Ref. 7 were used, in order to be quite certain that only the IV(C18:1,Ac) species was present. In the pseudomolecular ion region of the FAB mass spectrum (Fig. 4B), all $[M + H]^+$ pseudomolecular ions are indeed shifted by 22 amu to higher mass, compared with the FAB mass spectrum obtained in the non-sodiated thioglycerol matrix (Fig. 4A), due to the replacement of the proton by a sodium ion. The CID mass spectrum of the $[M + H]^+$ ion from IV(C20:4,Ac) (Fig. 5, *top panel*; already published in Ref. 7, but added here for comparison) shows typical B ions at m/z 490, 693, and 896, well known to be formed from $[M + H]^+$ precursor ions (9, 15). The CID mass spectrum of the $[M + Na]^+$ compound (Fig. 5, *bottom panel*) is very clearly different and is a classic example of the fragmentation occurring mainly via cross-ring cleavages, characteristic of alkali-cationized oligosaccharide-containing species (12, 16–18). Most evidently, the suggestion in Ref. 8 that the fragmentation spectrum in Ref. 7 is that of an $[M + Na]^+$ pseudomolecular ion can therefore be excluded.

Taken together with the UV absorption data and the previously published mass spectrometric analysis obtained on palladium-catalyzed reduction of the LCOs (7), we feel our findings to be unambiguous proof that *R. leguminosarum* bv. *trifolii* does indeed produce *nodE*-dependent nodulation factors with highly unsaturated fatty acyl moieties.

REFERENCES

- Relic, B., Talmont, F., Kopcinska, J., Golinowski, W., Promé, J. C., and Broughton, W. J. (1993) *Mol. Plant-Microbe Interact.* **6**, 764–774
- Schlaman, H. R. M., Okker, R. J. H., and Lugtenberg, B. J. J. (1992) *J. Bacteriol.* **174**, 5177–5182
- Fisher, R. F., and Long, S. R. (1993) *J. Mol. Biol.* **233**, 336–348
- Spaink, H. P., and Lugtenberg, B. J. J. (1994) *Plant Mol. Biol.* **26**, 1413–1422
- Carlson, R. W., Price, N. P. J., and Stacey, G. (1994) *Mol. Plant-Microbe Interact.* **7**, 684–695
- Schultz, M., Kondorosi, É., Ratet, P., Buiré, M., and Kondorosi, Á. (1995) *Int. Rev. Cytol.* **156**, 1–75
- Spaink, H. P., Bloembergen, G. V., Van Brussel, A. A. N., Lugtenberg, B. J. J., van der Drift, K. M. G. M., Haverkamp, J., and Thomas-Oates, J. E. (1995)

³ H. P. Spaink, unpublished results.

⁴ Nomenclature of the lipo-chitin oligosaccharides: a Roman numeral indicates the number of *N*-acetylglucosamine units, and the term in parenthesis refers to the nature of the fatty acyl moiety and substituents; e.g. IV(C18:1,Ac): LCO consisting of four GlcNAc residues, containing an *O*-acetyl group and a C18:1 acyl chain.

- Mol. Plant-Microbe Interact.* **8**, 155–164
8. Philip-Hollingsworth, S., Orgambide, G. G., Bradford, J. J., Smith, D. K., Hollingsworth, R. I., and Dazzo, F. B. (1995) *J. Biol. Chem.* **270**, 20968–20977
 9. Spaink, H. P., Sheeley, D. M., van Brussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenburg, B. J. J. (1991) *Nature* **354**, 125–130
 10. Demont, N., Debelle, F., Aurelle, H., Dénarié, J., and Promé, J. C. (1993) *J. Biol. Chem.* **268**, 20134–20142
 11. Verduyn, R., Lagas, R. M., Dreef, C. E., van der Marel, G. A., and van Boom, J. H. (1992) *Recl. Trav. Chim. Pays-Bas* **111**, 367–368
 12. Carroll, J. A., Willard, D., and Lebrilla, C. B. (1995) *Anal. Chim. Acta* **307**, 431–447
 13. Demont, N., Ardourel, M., Maillet, F., Promé, D., Ferro, M., Promé, J. C., and Dénarié, J. (1994) *EMBO J.* **13**, 2139–2149
 14. Ritsema, T., Wijfjes, A. H. M., Lugtenberg, B. J. J., and Spaink, H. P. (1996) *Mol. & Gen. Genet.* **251**, 44–51
 15. Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C., and Dénarié, J. (1990) *Nature* **344**, 781–784
 16. Cerny, R. L., Tomer, K. B., and Gross, M. L. (1986) *Org. Mass. Spectrom.* **21**, 655–660
 17. Zhou, Z., Ogden, S., and Leary, J. A. (1990) *J. Org. Chem.* **55**, 5444–5446
 18. Orlando, R., Bush, C. A., and Fenselau, C. (1990) *Biomed. Environ. Mass Spectrom.* **19**, 747–754

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