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Research Note

Biological Activities and Structures of *Bradyrhizobium elkanii* Low Abundance Lipo Chitin-Oligosaccharides

Thomas J. W. Stokkermans[1], Ron Orlando[3], V. S. Kumar Kolli[3], Russell W. Carlson[3], and N. Kent Peters[1,2]

[1]Ohio State Biotechnology Center, Ohio State University, Columbus 43210-1002, U.S.A.; [2]Department for Plant Physiology and Microbiology, University of Tromsø, N-9037 Tromsø, Norway; [3]Complex Carbohydrate Research Center, University of Georgia, Athens 30602-4712, U.S.A.

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We report the structures and biological activity of low abundance lipo chitin-oligosaccharides (LCOs) or Nod factors isolated from *Bradyrhizobium elkanii* USDA 61. These new LCOs have structures similar to those previously isolated from *B. elkanii*, but with unique combinations of substituents. Each LCO-containing peak has nodule initiation activity on *Glycine soja* and *Vigna umbellata*. As a result of this further analysis, it is likely that all major and most minor biologically active LCOs from *Bradyrhizobium elkanii* have been characterized.

Rhizobia synthesize a class of lipo chitin-oligosaccharide (LCO) molecules that induce nodule structures on legume roots. Rhizobia are induced to synthesize these LCOs upon exposure to flavonoids exuded from the legume root and seed coat (Fisher and Long 1992). These LCO molecules are tetra- and penta-oligosaccharides of *N*-acetylglucosamine (GlcNAc) residues with an acyl substitution on the nonreducing end. The structures of 10 different LCO molecular species isolated from major high-pressure liquid chromatography (HPLC) peaks from *Bradyrhizobium elkanii* USDA 61 have been reported (Carlson et al. 1993). The previously characterized LCO molecules were purified from peaks 1, 2, 3, and 4c (Carlson et al. 1993) as depicted in a representative HPLC elution profile shown in Figure 1. However, biological activities have been demonstrated only for peaks 1, 2, and 3 on *Glycine soja* Siebold et Zucc. (Stokkermans and Peters 1994).

In an attempt to complete the biological and chemical characterization of LCO molecules synthesized by strain USDA 61, minor absorbance peaks X, 1′, 2′, 3′, 4a, 4b, and Y (Fig. 1) were purified as described by Carlson et al. (1993). To de-

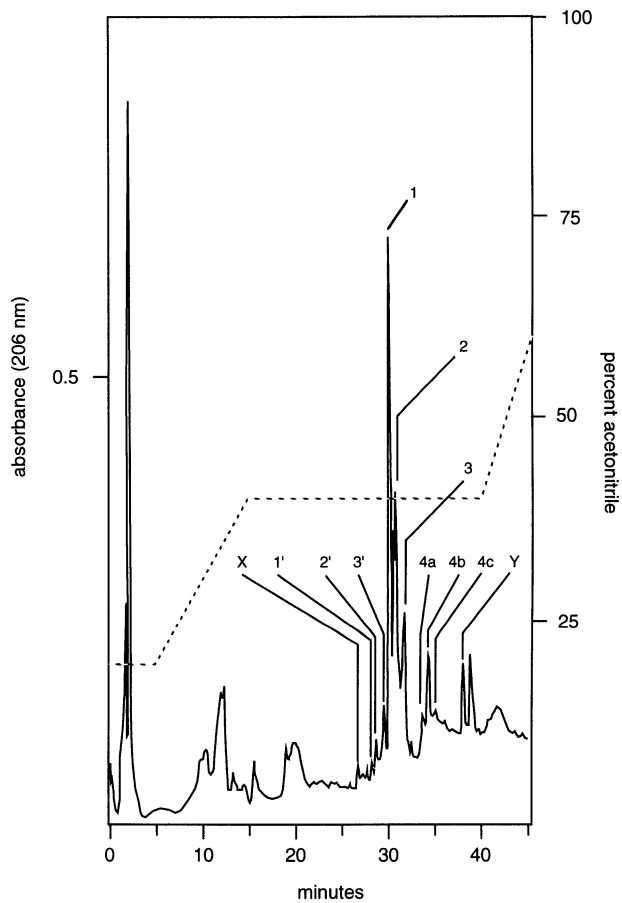

Fig. 1. High-pressure liquid chromatography trace of lipo chitin-oligosaccharide (LCO) peaks from *B. elkanii*. LCOs were monitored at 206 nm (—) and eluted from a C_{18}-reverse phase column on a 20 to 82% acetonitrile/water gradient (– –). Individual peaks were assigned numbers or letters arbitrarily. Peaks were collected and further purified using different step gradients according to their hydrophobicity as determined by the time of elution from the gradient described above.

Corresponding author: N. Kent Peters, Institute of Biology and Geology, University of Tromsø, N-9037 Tromsø, Norway, Telephone 47 77 64 63 24; FAX 47 77 64 56 00; E-mail kent@ibg.uit.no

Present address of N. Kent Peters: Institute of Biology and Geology, University of Tromsø, N-9037 Tromsø, Norway

Present address of Thomas J. W. Stokkermans: 91 Westland Ave. Box 31, Boston, MA 022115 USA

termine the biological activity of the uncharacterized peaks, samples from peaks 1′, 2′, 3′, 4a, 4b, 4c, X, and Y were spot inoculated onto roots of *G. soja* and *Vigna umbellata* (Thunb.) Ohwi & Ohashi as previously described (Stokkermans and Peters 1994). Briefly, roots were spot inoculated with single drops of LCO containing 100 ng of LCO per spot. After 12 days incubation, roots were cleared and examined for evidence of nodule initiation (NOI). Each spot inoculation was scored positive for NOI if cortical cell divisions, nodule primordia, or emerging nodules were observed on cleared roots. Each uncharacterized peak was assumed to contain LCO-like molecules and amounts of LCO for spot inoculation assays were estimated from absorbance measurements using an extinction coefficient of $1{,}400 \cdot cm^{-1} \cdot M^{-1}$ at 210 nm as estimated by J. Sanjuan (Department of Microbiology, University of Tennessee, Knoxville, TN, personal communication). Because the structures were not known at the time the biological assays were done, a molecular weight of 1,400 was assumed. Potential errors introduced by this assumption are not more than 10% and do not alter the conclusions about biological activity of the low abundance LCO.

NOI was found on both *Glycine soja* and *Vigna umbellata* in peaks 1′, 2′, 3′, 4a, 4b, and 4c, but not in peaks X and Y (Table 1). These results suggest that peaks 1′, 2′, 3′, 4a, 4b, and 4c contain LCO molecules while peaks X and Y either do not contain LCO molecules or contain LCO molecules inactive on these two legume species.

Structural characterization of the molecular species contained within the peaks demonstrated that all biologically active peaks contained LCO molecules. From absorbance measurements, the estimated yields for peaks 1′, 2′, 3′, 4a, 4b, and 4c were 8, 4, 12, 5, 18 and 8 $\mu g \cdot liters^{-1}$ culture, respectively. Therefore, insufficient amounts of these relatively minor LCO components were available for linkage or nuclear magnetic resonance analyses. Therefore, tandem mass spectrum (MS) and compositional analyses were used to obtain structural information. Thus, structures described in this section were hypothesized from mass spectrometric data, compositional data, and knowledge of the linkage and common substituent groups reported previously for LCO from *Bradyrhizobium* species including *B. elkanii* USDA 61 (Sanjuan et al. 1992; Carlson et al. 1993).

All MS were obtained using a JEOL (Tokyo, Japan) SX/SX 102A tandem four-sector mass spectrometer, which was operated at 10 kV accelerating potential. Ions were produced by fast atom bombardment (FAB) with xenon using a JEOL FAB gun operated at 6 kV in a conventional FAB ion source. Spectra acquired for the first MS are averaged profile data of 3 scans as recorded by a JEOL complement data system. These spectra were acquired from 200 to 2,000 *m/z* at a rate that would scan from *m/z* 0 to *m/z* 2,500 in 1 min. A filtering rate of 100 Hz and an approximate resolution of 1,000 (a 10% valley) were used in acquiring these spectra. This resolution was sufficient to resolve the isotopic masses for all of the samples and, therefore, the monoisotopic mass values are reported. The samples were dissolved in dimethyl sulfoxide and 1-µl aliquots were mixed with an equal volume of the FAB matrix, thioglycerol (TG), on the probe tip. The tandem MS analyses were performed on the monoisotopic mass ions. Collisional activation was performed in the third field free region, using helium as the collision gas. The helium pressure was sufficient to attenuate the primary ion beam by 75%. The

Table 1. Summary of observed lipo chitin-oligosaccharide (LCO) structures and their biological activity[a]

HPLC peak[b]	Observed mass values[c]	Calculated mass values[d]	Proposed LCO	*Glycine* NOI/total[e]	*Vigna* NOI/total[e]
1′	1,391.1	1,390.7	NodBe-V($C_{16:0}$,MeFuc)	9/11	3/6
2′	1,433.7 (major)	1,433.7	NodBe-V(Cb,$C_{16:0}$,MeFuc)	10/12	3/4
	1,417.7[f]	1,416.7 (1,417.6)[f]	NodBe-V($C_{18:1}$,MeFuc)		
	1,391.7[f]	1,390.7 (1,391.6)[f]	NodBe-V($C_{16:0}$,MeFuc)		
	1,261.7	1,261.7	NodBe-IV($C_{16:0}$,NMe,Fuc,Gro)		
	1,247.7	1,247.6	NodBe-IV($C_{16:0}$,Fuc,Gro)		
3′	1,477.1	1,476.7	NodBe-V(2Cb,$C_{16:0}$,MeFuc)	7/11	5/6
	1,477.1	1,447.1	NodBe-V($C_{18:1}$,Fuc,Gro)		
	1,448.1	1,476.7	NodBe-V(Cb,$C_{16:0}$,NMe,MeFuc)		
	1,290.6[f]	1,290.7 (1290.4)[f]	NodBe-IV(Ac,$C_{16:0}$,Fuc,Gro)[g]		
4a	1,611.2[h]	1,610.8[h]	NodBe-V(2Cb,$C_{18:1}$,MeFuc)	5/10	4/4
	1,545.3	1,544.8	NodBe-V(Ac,2Cb,$C_{18:1}$,MeFuc)		
	1,476.4	1,475.7	NodBe-V(Ac,Cb,$C_{16:0}$,MeFuc)		
4b	1,459.1	1,458.7	NodBe-V(Ac,$C_{18:1}$,MeFuc)	8/12	3/5
4c	1,502.1	1,501.7	NodBe-V(Ac,Cb, $C_{18:1}$,MeFuc)	7/11	2/4
X	1,986			0/12	
	1,970				
Y	2,639			0/12	
	2,655				

[a] See text for explanation of abbreviations and nomenclature for proposed LCO structures.
[b] High-pressure liquid chromatography.
[c] These are the observed monoisotopic $[M+H]^+$ values unless otherwise indicated.
[d] These are the calculated monoisotopic $[M+H]^+$ values of the proposed structures; those in parentheses are the calculated average chemical $[M+H]^+$ values.
[e] Each spot inoculation was scored for nodule structure induction (NOI) if cortical cell divisions, nodule primordia, or emerging nodules were observed on cleared roots.
[f] These masses are of minor intensity and due to the contribution of baseline noise there was insufficient resolution to distinguish the monoisotopic mass peak. Therefore, these values probably represent the average chemical $[M+H]^+$ mass.
[g] Due to the baseline noise it was not possible to determine if the observed mass was the average or monoisotopic value and therefore this ion could also be assigned to a carbamyl- rather than an acetyl-containing structure.
[h] These values are the thioglycerol adducts of the observed calculated $[M+H]^+$ masses for this proposed structure.

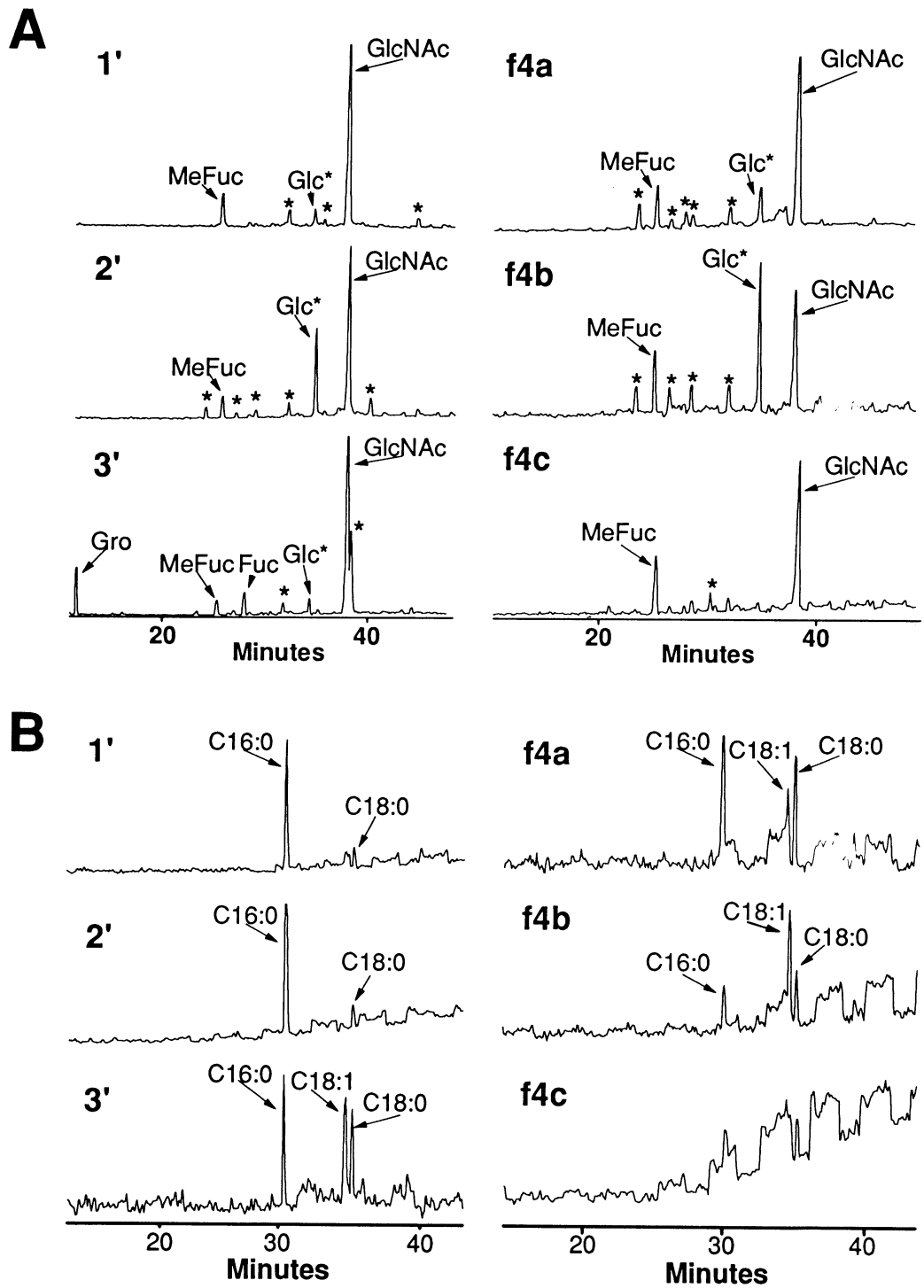

Fig. 2. A, Gas chromatographic profile of the alditol acetates derived from the minor *Bradyrhizobium elkanii* lipo chitin-oligosaccharide (LCO) peaks. Carbohydrate constituents are identified as 2-*O*-methylfucose (MeFuc), fucose (Fuc), *N*-acetylglucosamine GlcNAc, glucose (Glc), noncarbohydrate contaminants (*). Due to the very small sample amounts analyzed (between 5 to 20 μg), a number of unidentified noncarbohydrate contaminants are present and may be derived from the solvents used in the preparation of the alditol acetates or in the purification of the various LCO peaks. The glucose contaminate is also common when very small amounts of sample are present. Additionally, some of the samples still contained some carboxymethylcellulose that was used in preparing samples for the biological assays. **B,** Gas chromatographic profile of the fatty acid methyl esters derived from the minor *B. elkanii* LCO peaks. Small amounts of $C_{18:0}$ are observed in all samples indicating some contamination by this fatty acyl residue. A detectable level of fatty acid was not recovered from peak 4c.

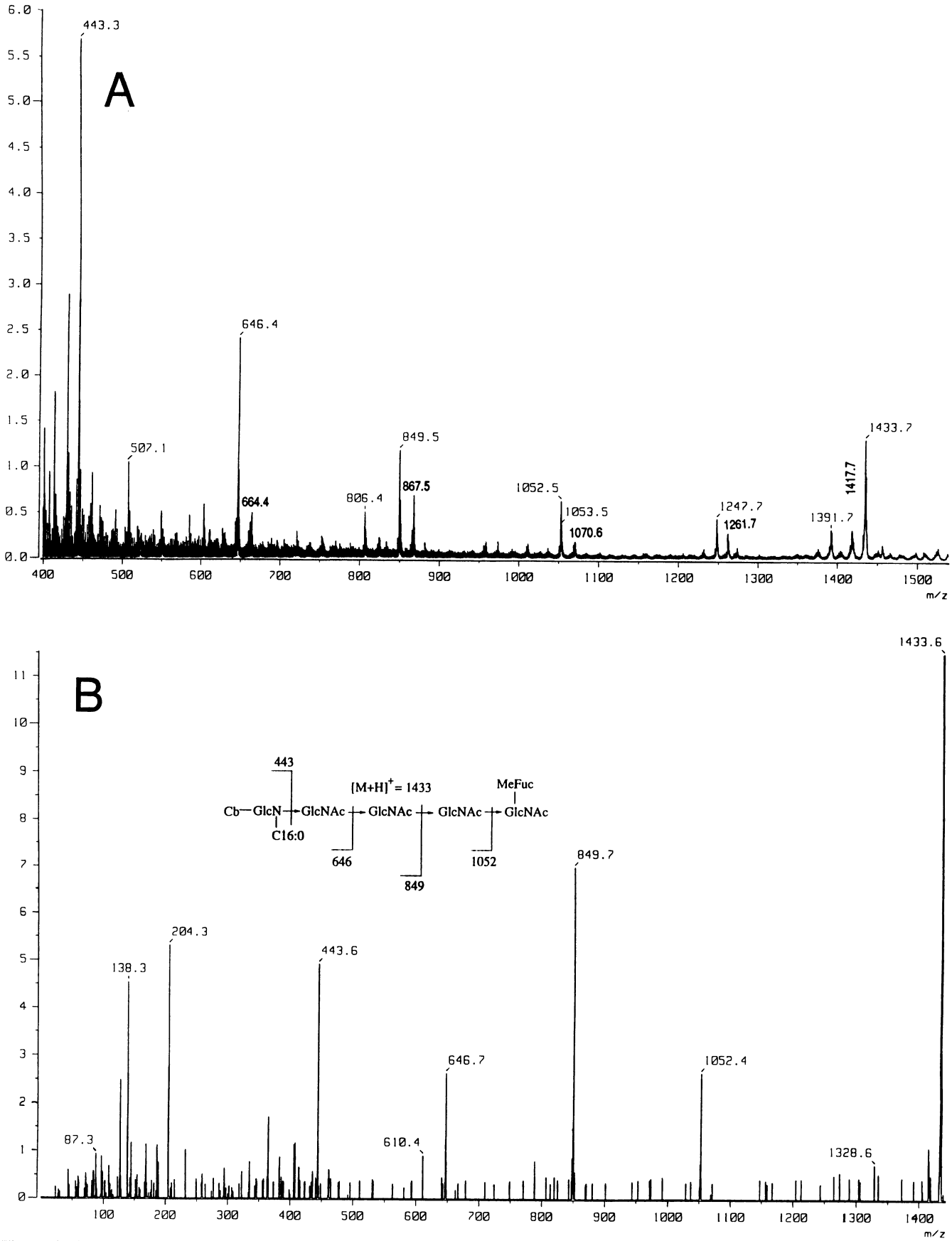

Fig. 3. The fast atom bombardment mass spectrum (FAB-MS) and tandem MS of peak 2′. **A,** the FAB-MS; **B,** the tandem MS of the *m/z* 1,433.6 ion and proposed lipo chitin-oligosaccharide structure of BeNod-V(Cb,$C_{16:0}$,MeFuc) (for explanation of nomenclature, see text).

collision cell was floated at 3 kV, providing a collision energy of 7 kV. Acquired tandem MS are averaged profiles of 4 scans as recorded by the JEOL complement data system. The following discussion of the MS data uses the observed values for the molecular ions. Due to the natural distribution of isotopes, these values are usually between 1 and 2 mass units larger than the calculated nominal mass values.

For compositional analysis, samples were hydrolyzed in 2 M trifluoroacetic acid at 121°C for 2 h. Alditol acetates of the glycosyl residues in the aqueous layer were prepared as previously described (York et al. 1985). The released fatty acids were extracted into hexane. Hexane in the organic fraction was evaporated under a stream of dry air and the fatty acids were converted into their methyl esters by methanolysis in 1 M HCl at 80°C for 2 h. The solvent was evaporated at room temperature under a stream of dry air. The alditol acetates were dissolved in acetone and the fatty acid methyl esters in hexane and the samples were analyzed by GC (gas chromatography)–MS.

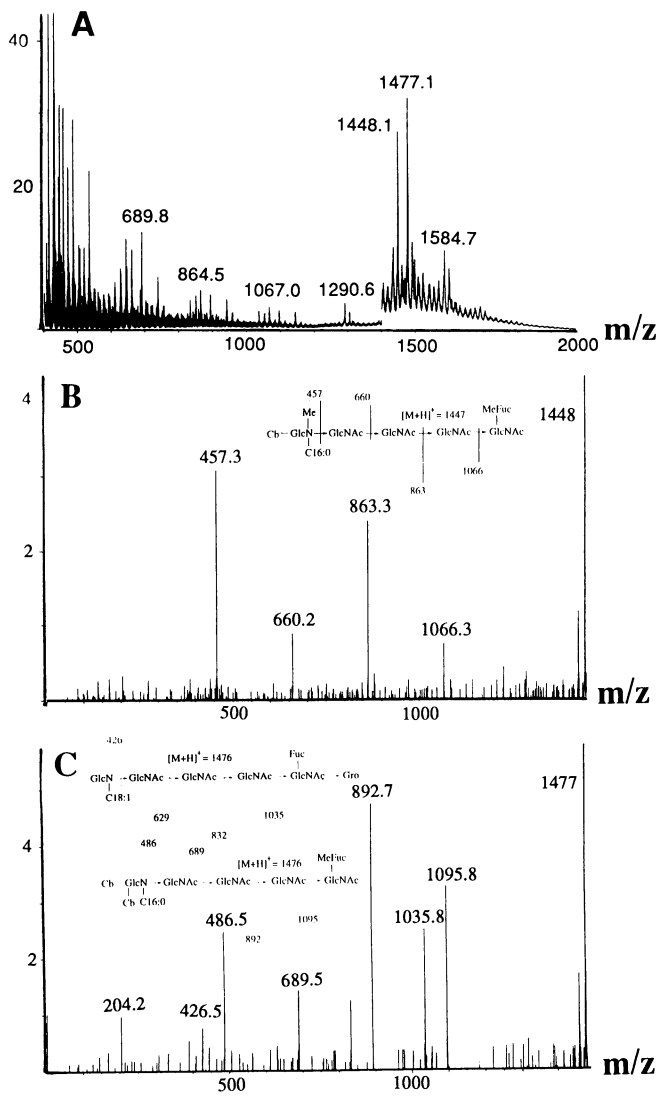

Fig. 4. The fast atom bombardment mass spectrum (FAB-MS) and tandem MS of peak 3′. **A,** the FAB-MS; **B,** the tandem MS of the *m/z* 1,448 ion and proposed lipo chitin-oligosaccharide (LCO) structure BeNod-V(Cb,$C_{16:0}$,NMe,MeFuc); **C,** the tandem MS of the *m/z* 1,477 ion and the proposed LCO structures BeNod-V(2Cb,$C_{16:0}$,MeFuc) and BeNod-V($C_{18:1}$,Fuc,Gro) (for explanation of nomenclature, see text).

The accepted nomenclature for Nod factors includes an abbreviation for the species from which the factor is isolated and structural information. For example, NodBe-V(Ac [Acetyl],$C_{18:1}$,MeFuc[2-*O*-methylfucose]) describes a molecule isolated from *Bradyrhizobium elkanii*. The Roman numeral (e.g., "V") preceding the parentheses indicates the number of glucosamine residues and modifications of this oligo are listed within the parentheses. Abbreviations for modifications appearing before the fatty acyl designation indicate additional substituents on the nonreducing end of the glycosyl chain. Abbreviations for modifications appearing after the acyl substituent designation indicate substitutions of the reducing-end residue.

Analysis of peak 1′ by FAB-MS and tandem MS (data not shown) gives a molecular species of $[M+H]^+$ *m/z* 1,391.1. Tandem MS gave a fragmentation series of 1,009.7, 806.7, 603.6, and 400.4. Glycosyl and fatty acid composition analyses demonstrate the presence of 2-*O*-methylfucose, *N*-acetylglucosamine and $C_{16:0}$ as the *N*-acetyl substituent (Fig. 2). These results are consistent with a structure previously reported for *Bradyrhizobium japonicum*, NodBj-V($C_{16:0}$, MeFuc) (Carlson et al. 1993).

Glycosyl composition analysis of peak 2′ demonstrates the presence of 2-*O*-methylfucose and *N*-acetylglucosamine (Fig. 2A). The fatty acid profile shown in Figure 2B demonstrates the presence of $C_{16:0}$ as the *N*-acetyl substituent. The FAB-MS of peak 2′ is shown in Figure 3A. This spectrum shows a major molecular species of $[M+H]^+$ *m/z* 1,433.7, with minor species of *m/z* 1,417.7, 1,391.7, 1,261.7, and 1,247.7. Figure 3B shows the tandem MS of the major molecular species of *m/z* 1,433.7. These results are consistent with the structure shown in Figure 3B: NodBe-V(Cb,$C_{16:0}$,MeFuc). The fragment ion 443.6 clearly indicates the presence of a carbamyl rather than an acetyl group on the terminal *N*-acyl GlcN residue. The *m/z* 1,417.7 and 1,391.7 ions are consistent with the average $[M+H^+]$ values for the previously reported structures of NodBj (or Be)-V($C_{18:1}$,MeFuc) and NodBj (or Be)-V($C_{16:0}$,MeFuc), respectively (San Juan et al. 1992; Carlson et al. 1993). The low intensity together with increased baseline noise in this region of this spectrum did not allow resolution of the isotopic masses for these two molecules. The 1,247.7 and 1,261.7 ions were not sufficiently intense to obtain tandem MS data; however, they are consistent with the monoisotopic $[M+H^+]$ values for NodBe-IV($C_{16:0}$,Fuc,Gro[Glycerol]) and NodBe-IV($C_{16:0}$,NMe,Fuc,Gro), respectively. Baseline noise in this region of the spectrum was minimal and allowed resolution of the isotopic masses. While other structures for these ions are possible, analogous compounds containing $C_{18:1}$ as the *N*-acyl component have been reported for *B. elkanii* (Carlson et al. 1993).

The FAB-MS and tandem MS of peak 3′ are shown in Figure 4. The FAB-MS in Figure 4A shows two major molecular species of $[M+H]^+$ *m/z* 1,448.1 and 1,477.1, with a TG adduct of the 1,477.1 ion at *m/z* 1,584.7 of minor intensity, indicating that the latter species contains an unsaturated fatty acyl group. The tandem MS of the 1,448 ion shows fragment ions that are consistent with the structure NodBe-V(Cb,$C_{16:0}$,NMe,MeFuc) as shown in Figure 4B. The tandem MS of the 1,477 ion indi-

cates the possibility of two species with identical molecular weights. The major fragment ions observed were those of *m/z* 1,095.8, 892.7, 689.5, and 486.5, which are consistent with the structure NodBe-V(2Cb,$C_{16:0}$,MeFuc) as shown in Figure 4C. The minor fragmentation series of *m/z* 1,035.8, 832.7, 629.5, and 426.5 is consistent with the structure NodBe-V($C_{18:1}$,Fuc,Gro), which would give the TG adduct at *m/z* 1,584.7. The FAB-MS also shows a minor species of $[M+H]^+$ *m/z* 1,290.6. The low abundance of this ion made it impossible to obtain a tandem MS. Also, due to baseline noise, it is likely that this ion represents an average $[M+H]^+$ value. A structure consistent with this ion would be NodBe-IV(Ac, $C_{16:0}$,Fuc,Gro); however, the structure NodBe-IV(Cb,$C_{16:0}$, Fuc,Gro) cannot be excluded. The MS data and the proposed structures for peak 3′ would dictate that this peak contains glycerol, fucose, 2-*O*-methylfucose, and *N*-acetylglucosamine, and all these components are present as determined by the composition analysis as shown in Figure 2A. Fatty acid analysis also demonstrates the presence of the expected $C_{16:0}$ and $C_{18:1}$ molecular species.

The FAB-MS of peak 4a is shown in Figure 5. Several molecular ions, $[M+H]^+$, are observed, namely, *m/z* = 1,476.4, 1,545.3, and 1,611.2. Of these, the major ions are those at 1,476.4 and 1,545.3. In addition, the $[M+Na]^+$, $[M+H+TG]^+$, and $[M+Na+TG]^+$ adducts of the 1,545.3 ion are observed: 1,567.9, 1,653.6, and 1,675.3, respectively. The TG adducts (+108 a.m.u.) are common with molecules containing an unsaturated fatty acyl substituent. Fragment ions from the following molecular ions were also observed: 1,476.4 (1,094, 891, 688, and 485); 1,545.3 (1,163, 960, 757, and 554); 1,567.9 (1,185, 982, 779, and 576); and 1,653.6 (1,271, 1,068, 865, and 662). These data for the molecules of *m/z* 1,476.4 and 1,545.3 are consistent with the structures shown in Figure 5: NodBe-V(Ac,Cb,$C_{16:0}$,MeFuc) and NodBe-V(Ac,2Cb, $C_{18:1}$,MeFuc), respectively. The exact positions of the acetyl and carbamyl groups on the non-reducing terminal GlcNAc (*N*-acetylglucosamine) residue cannot be determined, other than that they must be at C3, C4, and/or C6. Fragment ions for the *m/z* 1,611.2 ion were not observed; however, they are consistent with the $[M+H+TG]^+$ ion of NodBe-V(2Cb,$C_{18:1}$, MeFuc). An ion of low intensity at *m/z* 1,503.2 was observed and may represent the $[M+H]^+$ ion for this structure. The compositional analysis demonstrated the presence of 2-*O*-methylfucose and *N*-acetylglucosamine along with both $C_{16:0}$ and $C_{18:1}$ fatty acid molecular species (Fig. 2).

The FAB-MS and tandem mass of peak 4b (data not shown) give a molecular species of $[M+H]^+$ *m/z* 1,459.1 with a fragmentation series of 1,078.0, 874.8, 671.7, and 468.5, which is consistent with the structure NodBe-V(Ac,$C_{18:1}$, MeFuc). Interestingly, this structure has been previously identified in peak 2 (Carlson et al. 1993). The position of the acetyl group of NodBe-V(Ac, $C_{18:1\Delta}11$, MeFuc) from peak 2 was determined to be located on the C-6 of the nonreducing end of the LCO (Carlson et al. 1993). Although a position was not assigned to the acetyl group on this molecule from peak 4b, because of the different elution time it is possible that the acetyl is not on the C-6 but on either the C-3 or C-4 at the nonreducing end of the LCO. From the MS data, peak 4b

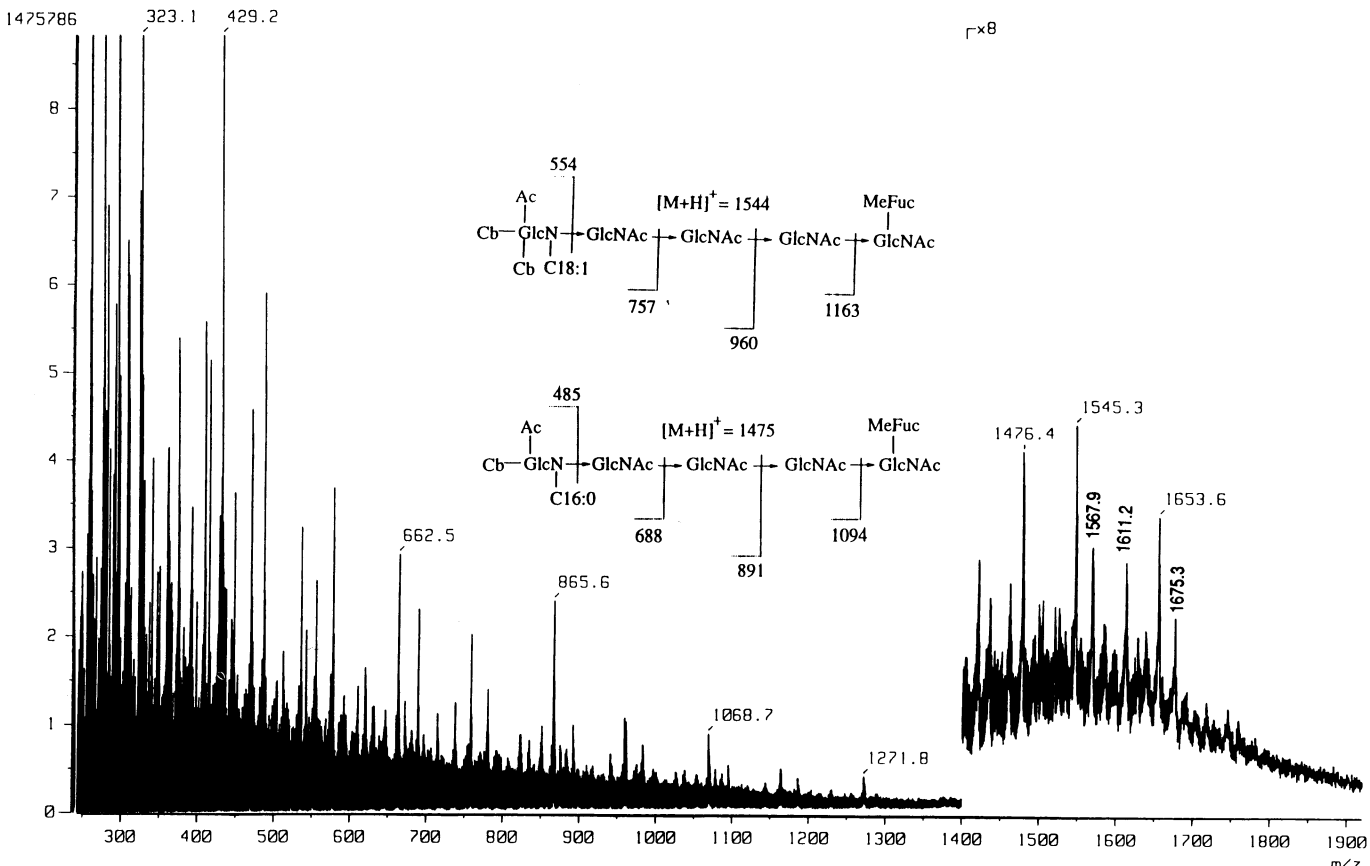

Fig. 5. The fast atom bombardment mass spectrum (FAB-MS) of peak 4a with proposed lipo chitin-oligosaccharide structures BeNod-V(Ac,Cb, $C_{16:0}$,MeFuc) and BeNod-V(Ac,2Cb,$C_{18:1}$,MeFuc) (for explanation of nomenclature, see text).

should contain only $C_{18:1}$. Small amounts of $C_{16:0}$ and $C_{18:0}$ in this sample may be due to slight contamination by other lipids containing these fatty acyl substituents.

Analysis of peak 4c gave a molecular ion and fragmentation series of *m/z* 1,502.1 (1,120.8, 917.8, 714.6, and 511.5), which is consistent with the data and structure NodBe-V(Ac,Cb,$C_{18:1}$,MeFuc), which was previously reported for that peak (Carlson et al. 1993). A detectable level of fatty acid was not recovered from peak 4c.

The two biologically inactive peaks, X and Y, were analyzed (see Figure 1), but these peaks did not give fragmentation patterns characteristic of rhizobial LCO (Table 1).

Three of the peaks analyzed for biological activity, 1′, 4b, and 4c, each contain a single LCO molecular species. Common to each of these active LCOs is a pentameric backbone structure with a 2-*O*-methylfucose substitution. Each of the peaks containing mixtures of LCO also contains molecules with these features. These findings are consistent with the structure activity relationships described for *G. soja* elsewhere (Stokkermans et al. 1995).

LCOs have been isolated and characterized for a number of rhizobia (Lerouge et al. 1990; Spaink et al. 1991; Price et al. 1992; Sanjuan et al. 1992; Schultze et al. 1992; Carlson et al. 1993; Mergaert et al. 1993). The collection of LCO characterized from *B. elkanii* varies in backbone length, type of fatty acid, and the presence of acetyl and carbamyl moieties or an *N*-methyl moiety on the nonreducing end and the presence of 2-*O*-methylfucose or fucose and glycerol on the reducing end of the LCO. This structural and positional variability allows for the potential formation of at least 96 different LCO structures, of which about 20 have now been identified, demonstrating the metabolic diversity of this species. The variety of LCO synthesized by *B. elkanii* rivals that of the broad host range *Rhizobium* NGR234 (Price et al. 1992), which is consistent with *B. elkanii* having a broad host range. It is of interest to note that on *G. soja* and *V. umbellata* a successful NOI response is obtained with any one of the LCO peaks isolated. The biological significance of the production of so many LCOs by *B. elkanii* and the ability of host legumes to respond to all these LCOs are still to be determined.

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