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Carbohydrate Research 339 (2004) 1061–1067
Carbohydrate RESEARCH

Acacia senegal and *Prosopis chilensis*-nodulating rhizobia *Sinorhizobium arboris* HAMBI 2361 and *S. kostiense* HAMBI 2362 produce tetra- and pentameric LCOs that are N-methylated, O-6-carbamoylated and partially sulfated

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Received 28 November 2003; received in revised form 6 February 2004; accepted 16 February 2004

Abstract—*Sinorhizobium arboris* and *S. kostiense* are rhizobia that nodulate the tropical leguminous trees *Acacia senegal* and *Prosopis chilensis*. The lipochito-oligosaccharidic signalling molecules (LCOs) of *S. arboris* HAMBI 2361 and *S. kostiense* HAMBI 2362 were analyzed by mass spectrometry. The major LCOs produced by the strains were shown to be pentameric, acylated with common fatty acids, N-methylated, O-6-carbamoylated and partially sulfated, as are the LCOs characterized to date for other *Acacia*-nodulating rhizobia. Besides the major LCOs the two strains produced (i) tetrameric LCOs, (ii) LCOs acylated with fatty acids other than those commonly found, (iii) LCOs with only an acyl substituent and (iv) noncarbamoylated LCOs. Production of LCOs (i) to (iii) are novel among *Acacia*-nodulating rhizobia. The roles of the different structural characteristics of LCOs in the rhizobium–*A. senegal* symbiosis are discussed. Specific structural features of the LCOs are proposed to be important in the selection of effective nitrogen-fixing rhizobia by *A. senegal*.

Keywords: Lipochitin oligosaccharides (LCOs); Nodulation; *Sinorhizobium arboris*; *Sinorhizobium kostiense*; *Acacia senegal*; *Prosopis chilensis*

1. Introduction

Sinorhizobium arboris and *S. kostiense* are rhizobia that nodulate the tropical leguminous trees *Acacia senegal* and *Prosopis chilensis*.[1] The type strains of both species originate from the Kosti area in Sudan.[2] Of their hosts, *A. senegal* is one of the dominant trees of the Sahelian proper biogeographical zone, while *P. chilensis* is originally from South America and has been introduced into Sudan.[3] The arid lands of the tropics are commonly poor in nutrients, especially nitrogen. The nitrogen-fixing symbiosis of rhizobia and the leguminous trees has been utilized in reforestation and agroforestry. In addition to fodder and fuel, the trees provide shelter and nutrients, and the cultivability of the lands may be restored.

A successful symbiosis requires signal exchange between the rhizobium and plant partners. Rhizobial signalling molecules are N-acylated glucosamine oligosaccharides, called lipochitin oligosaccharides (LCOs).[4] They are also known as nodulation factors, in short Nod factors. The chitin oligosaccharide backbone consists of two to six *N*-acetylglucosamine (GlcNAc) residues.[5,6] Attached to the GlcNAc residue at the nonreducing end of the oligosaccharide, an acyl group is present on all biologically active LCOs. In addition to the acyl group, LCOs may carry other substitutions, for example, acetyl and carbamoyl groups. The characteristic structure of LCOs serves two purposes: specific host recognition and protection against degradation by plant enzymes.[7,8] Each rhizobial strain produces a wide range of

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doi:10.1016/j.carres.2004.02.013

structurally related LCOs that may be needed at different stages of the symbiosis and to complement one another.[9–11] To circumvent the problem caused by the small amount of LCOs produced, genetically modified LCO over-producing strains have been used since the first study of LCO structures.[4] The wild-type strains produce similar LCOs to the over-producing strains.[12]

Mass spectrometry (MS) has proven to be an excellent method for studying heterogenous biological samples, such as LCOs.[13] Analytes in solution are delivered to the mass spectrometer either by direct infusion or on interfacing liquid chromatography (LC) on-line. Closely related structural variants may be detected and identified according to their masses. In MS/MS, protonated or cationized precursor molecules may be fragmented using collision induced dissociation (CID), and the structure of the precursor ion may be deduced from the masses of the fragments generated from it.[13] The nature of the charge-bearing species has impact on the fragmentation of oligosaccharides. Protonated oligosaccharides may lose internal fragments and appear as a set of isomeric species, while sodiated oligosaccharides do not.[14,15]

In a specific extension of the MS/MS approach, Treilhou et al. studied the fragmentation patterns of fragment ions generated from *N*-acetylglucosamine esters and from LCOs, concentrating on ions containing the nonreducing terminal residues of LCOs.[16] They observed characteristic fragmentation patterns and product ion intensity ratios depending on the location of substituents on the nonreducing GlcNAc residue. They also noticed a characteristic ratio of product ions depending on the presence and absence of an *N*-methyl group.

However, determining the full structure of an LCO requires the use of chemical methods in combination with MS.[13] Composition of the oligosaccharide backbone and the types of linkages between backbone monosaccharide residues need to be determined by gas chromatography–MS of chemically derivatized samples.

In this work, the structures of *S. arboris* strain HAMBI 2361 and *S. kostiense* strain HAMBI 2362 LCOs were determined by electrospray-quadrupole orthogonal time-of-flight mass spectrometry (ES-Q-o-TOF-MS) and LC-ES-ion trap-MS. Comparison of results from this work and from earlier studies on *Acacia*-nodulating rhizobia have led us to a proposal of roles of the LCO structural characteristics in the rhizobium-*A. senegal* symbiosis.

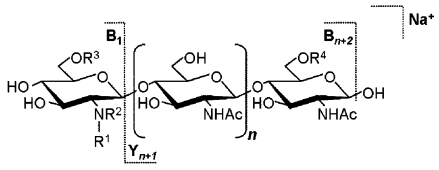
2. Results

Lipochito-oligosaccharidic signalling molecules (LCOs) of *S. arboris* HAMBI 2361 and *S. kostiense* HAMBI 2362 were analyzed using two different approaches. In an off-line LC approach, extracted LCOs were fractionated by HPLC. Pooled HPLC fractions were then analyzed by direct infusion ES-Q-o-TOF-MS. In a parallel on-line LC approach, LCO extracts from separate fermentations were analyzed using a reversed phase microbore HPLC system coupled on-line with an ES-ion trap mass spectrometer. Data from the two approaches is summarized in Table 1.

The fragmentation patterns of LCO compounds as analyzed using CID-MS/MS in the Q-TOF and resonance excitation-MS/MS in the ion trap showed that *S. arboris* HAMBI 2361 and *S. kostiense* HAMBI 2362 produces tetra- and pentameric, N-methylated and partly carbamoylated LCOs, in which the fatty acyl groups are saturated or monounsaturated (Table 1). Besides the fragment ions of types B and Y listed in Table 1, complete B-ion series were present in all the other MS/MS spectra but in the spectra of m/z 1396, m/z 1278, m/z 1351. Also, other Y-ions and fragment ions of types C and Z, double cleavage fragment ions of type B_mY_n and cross-ring cleavage fragment ions of type ${}^{l,m}A$ and ${}^{l,m}X$ were detected in most of the spectra (data not shown) (nomenclature for fragmentation after Domon and Costello).[17] The major LCO from both strains is an N-methylated and carbamoylated pentamer acylated with a C18:1 fatty acyl chain (Fig. 1). A sulfate group was detected on some LCO variants. The fragmentation data indicated that the methyl and carbamoyl groups were located on the nonreducing terminal and the sulfate group on the reducing terminal residues. Comparison of the extracted ion chromatograms (EICs) showed that all the LCOs listed in Table 1 eluted separately, indicating that they represent distinct LCO structures (data not shown). The EIC comparison also showed that in-source fragments were formed, but they were easily identified on the basis of their retention times and spectra, and therefore not misassigned (data not shown).

The results of the analyses of the LCOs produced in the two separate fermentations and analyzed in the first instance using off-line LC and the Q-TOF, and in the second instance using on-line LC-ion trap-MS were very similar. In general, the on-line approach offered better limits of detection, resulting in the identification of more structural variants than the off-line approach (Table 1). In the *S. kostiense* samples, the sodiated molecules at m/z 1307 and m/z 1351 were detected only only using the Q-Tof. Q-TOF CID-MS/MS analysis of the sodiated molecule at m/z 1351 indicated that *S. kostiense* HAMBI 2362 produces an LCO with a C18:1-OH or C19:0 fatty acyl group (Fig. 1). In the on-line LC-ES-ion trap experiment m/z 1351 was observed only as an isotope of m/z 1349. LC-ion trap MS/MS analysis of the sodiated molecule at m/z 1349 indicated an LCO with a C18:1-methyl, C18:2-OH, C19-cyclopropyl or C19:1 fatty acyl group.

Table 1. The m/z values of sodiated ions and characteristic sodiated fragment ions, and the structures of *Sinorhizobium arboris* HAMBI 2361 and *S. kostiense* HAMBI 2362 LCOs as determined by LC-ES-ion trap-MS and ES-Q-o-TOF-CID-MS/MS



$[M+Na]^{+a}$ (ion trap)	$[M+Na]^{+a}$ (Q-Tof)	B_1	B_{n+2}	Y_{n+1}	n	R^1	R^2	R^3	R^4	LCO structure[b]	*S. arboris* HAMBI 2361[c]	*S. kostiense* HAMBI 2362[c]
1437		505	1419	955	3	C18:1	Me	Cb	SO_3H	V(C18:1, *N*-Me, Cb, S)	+	+
1234	1234	505	1216	752	2	C18:1	Me	Cb	SO_3H	IV(C18:1, *N*-Me, Cb, S)	+	+
1396		667[d]	1378	955	3	C18:0	Me	H	SO_3H	V(C18:0, *N*-Me, S)	+	–
1439	1439	507	1421	955	3	C18:0	Me	Cb	SO_3H	V(C18:0, *N*-Me, Cb, S)	+	+
1266		436	1248	853	3	C16:0	Me	H	H	V(C16:0, *N*-Me)	+	–
	1307	477	1289	853	3	C16:1	Me	Cb	H	V(C16:1, *N*-Me, Cb)	–	+
1309	1309	479	1291	853	3	C16:0	Me	Cb	H	V(C16:0, *N*-Me, Cb)	+	+
1278		651[d]	1260	853	3	C18:1	H	H	H	V(C18:1)	+	+
1292		462	1274	853	3	C18:1	Me	H	H	V(C18:1, *N*-Me)	+	+
<u>1335</u>	1335	505	1317	853	3	C18:1	Me	Cb	H	V(C18:1, *N*-Me, Cb)	+	+
1132	1132	505	1114	650	2	C18:1	Me	Cb	H	IV(C18:1, *N*-Me, Cb)	+	+
1349		519	1331	853	3	Acyl[e]	Me	Cb	H	V(Acyl, *N*-Me, Cb)	+	+
	1351	521	1130[f]	853	3	Acyl[g]	Me	Cb	H	V(Acyl, *N*-Me, Cb)	–	+
1294		464	1276	853	3	C18:0	Me	H	H	V(C18:0, *N*-Me)	+	–
<u>1337</u>	1337	507	1319	853	3	C18:0	Me	Cb	H	V(C18:0, *N*-Me, Cb)	+	+
1363	1363	533	1345	853	3	C18:0	Me	Cb	H	V(C20:1, *N*-Me, Cb)	+	+
1134		507	1116	650	2	C18:0	Me	Cb	H	IV(C18:0, *N*-Me, Cb)	+	–

[a] Sulfated LCOs observed as $[M-H+2Na]^+$ ions. The major ions are underlined.
[b] LCO nomenclature: roman numeral = no of GlcNAcs in the oligosaccharide backbone, other substituents clockwise starting from the fatty acyl. Abbreviations: C16:0 = the length and the degree of unsaturation of the acyl group; Cb = carbamoyl group; *N*-Me = *N*-methyl group. S = sulfate group.
[c] + = pseudomolecular ion observed; – = pseudomolecular ion not observed.
[d] m/z of the B_2 ion. B_1 ion was not observed in MS/MS of m/z 1396 and m/z 1278.
[e] C18:1-methyl, C18:2-OH, C19-cyclopropyl or C19:1.
[f] m/z of the B_4 ion. B_{n+2} ion was not observed in MS/MS of m/z 1351.
[g] C18:0-methyl, C18:1-OH or C19:0.

In the CID-MS/MS spectrum of the protonated B_1-ion (m/z 483) generated in-source from LCO SkV(C18:1, *N*-Me, Cb), domination of the loss of carbamic acid and water (B_1-79) over the loss of carbamic acid (B_1-61), and the low abundance ratios of fragment ions at m/z 158 and m/z 152 to m/z 140 suggest that the carbamoyl group is located on O-6 (Fig. 2). The low abundance ratio of m/z 84 to m/z 98 shows that the LCO SkV(C18:1, *N*-Me, Cb) is N-methylated and does not have a methyl branched or a cyclopropyl-bearing acyl group (Fig. 2).

GC–MS analyses of TMS methyl glycosides and partially methylated alditol acetates derived from purified LCO fractions showed that that *S. arboris* and *S. kostiense* LCOs are (1→4)-linked *N*-acetylglucosamine oligosaccharides (data not shown). Substitutions are

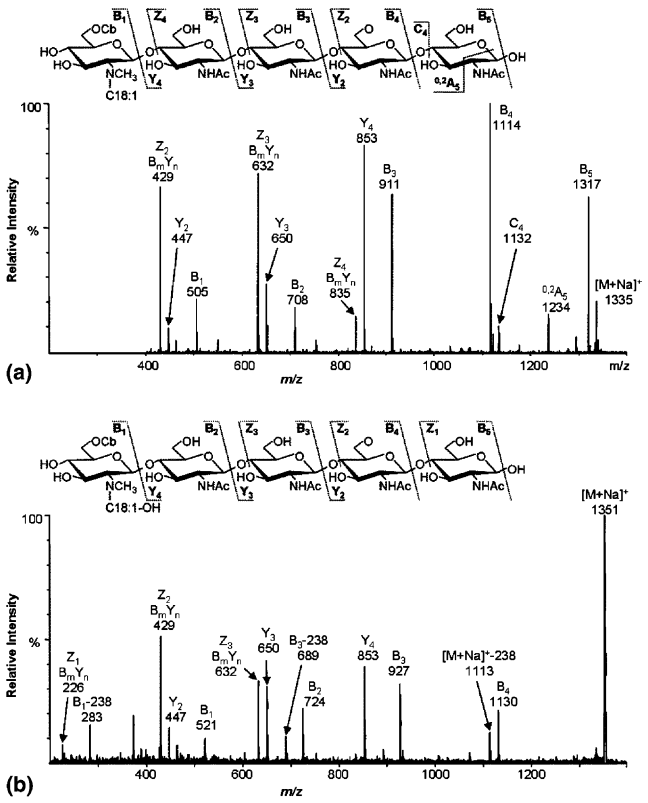


Figure 1. Schematic representations and MS/MS spectra of two LCOs. (a) The major *Sinorhizobium arboris* HAMBI 2361 LCO at m/z 1335, determined by LC-ES-ion trap-MS at scan range from 400 to 1600 Th. The LCO V(C18:1, *N*-Me, Cb) was also the major *S. kostiense* HAMBI 2362 LCO. (b) A minor *S. kostiense* HAMBI 2362 LCO at m/z 1351. For an LCO without a reducing terminal substitution, the m/z values of Z ions and double cleavage ions (B_m/Y_n) with equal number of residues are the same (nomenclature for fragmentation after Domon and Costello).

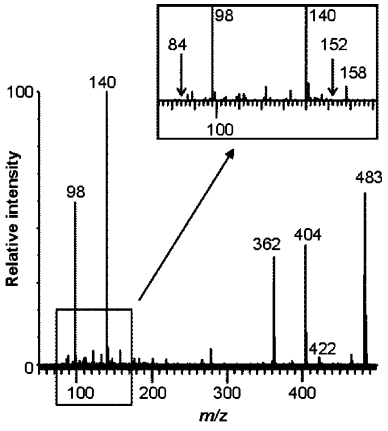


Figure 2. The MS/MS spectrum of the protonated B_1-ion (m/z 483) of an in-source fragmented LCO SkV(C18:1, *N*-Me, Cb). The inset shows an enlargement of the m/z range 80–160 Th.

located on O-6 on both the reducing and nonreducing terminal residues.

3. Discussion

The major LCO of both *S. arboris* HAMBI 2361 and *S. kostiense* HAMBI 2362 carries a C18:1 fatty acyl group. This is in accordance with the results of fatty acid analyses by Tighe et al. who show that over two-thirds of cellular fatty acids in these strains are C18:1.[18] Our identification of LCOs carrying C18:0, C16:0 and C16:1 is also in accordance with the cellular fatty acid group analyses of Tighe et al.[18] The fatty acyl on the sodiated molecules at m/z 1349 could have been C18:1-methyl, C18:2-OH, C19-cyclopropyl or C19:1, and at m/z 1351 C18:0-methyl, C18:1-OH or C19:0. From the CID-MS/MS-data, it is clear that the 2 Th difference between the pair of ions at m/z 1307/1309 arises from different degrees of saturation in the fatty acyl group. Similarly, the 2 Th difference between the pair of ions at m/z 1349/1351 could also be due to such a difference in the degree of saturation. However, in our data there are indications that the fatty acyl on the species ionizing to generate the ion at m/z 1351 could be hydroxylated. Alternatively, a 16 Th increment on the ion at m/z 1335, the major sodiated molecule, could arise by cationization with potassium instead of sodium. This possibility was ruled out, since in the Q-TOF-CID tandem-mass spectrum of m/z 1351 Y_n ions and ions assigned as Z_n ions or double cleavage ions (B_mY_n), that is, ions not carrying the nonreducing terminal residue, had similar m/z values to the corresponding product ions of m/z 1335. In support of the hydroxylation assignment, peaks at m/z 283 (B_1-238), m/z 689 (B_3-238) and m/z 1113 ($[M+Na]^+-238$) are in accordance with the proposed fragmentation mechanism observed for 3-hydroxy C18 fatty acyl groups of LCOs.[19,20]

Pacios-Bras et al. demonstrated that a reversed phase nano-HPLC coupled directly to an ion trap mass spectrometer allows fast, precise and sensitive analysis of LCOs.[21] In this study, we applied reversed phase microbore HPLC coupled to an ion trap and noticed that more LCO structural variants were detected with the on-line LC-ion trap-MS than with the off-line Q-TOF-MS. Use of on-line LC–MS gave us the opportunity to apply EIC comparisons of different sodiated molecules for securing our interpretations, excluding the possibility of the species we assign arising by in-source fragments or corresponding to isotopes of lower mass species. We were able to confirm that the ions assigned as arising from LCOs that are noncarbamoylated, tetrameric or carrying a saturated fatty acyl group correspond to genuine molecular species. Nevertheless, the datasets of the two MS techniques we applied were not absolutely identical, as might be expected when analyzing the extracts of two different fermentations. In our opinion, this is because of slight variations in the LCOs produced by the strains, and not due to any major variation in the analytical techniques employed. One

cannot conclude that a certain rhizobium produces a certain number of LCO variants, as some variants may be missed due to being produced at quantities below the detection limit of the analytical techniques used.[22] The fatty acyl composition of LCOs acylated with common fatty acids reflect the fatty acid pool, which is known to vary in response to growth conditions and growth phase.[23] It is also probable that there are biosynthetically incomplete LCOs in the culture flask at the time of extraction.

Rhizobial strains that nodulate tropical leguminous trees have been shown to produce similar LCOs even if they are derived from geographically distant sources or represent different rhizobial species, as is the case for *Sesbania*-nodulating *Rhizobium* sp. *mus10* from India and *Azorhizobium caulinodans* and *Sinorhizobium terangae* biovar *sesbaniae* from Senegal.[12,24,25] In this study, we again see that rhizobia sharing the same tropical trees as hosts produce similar LCOs. *S. arboris* HAMBI 2361 and *S. kostiense* HAMBI 2362 produce N-methylated, partially carbamoylated and partially sulfated LCOs, acylated with common fatty acids, as do the other *Acacia*-nodulating rhizobia analyzed to date.[5,26–30] The LCOs currently characterized from *Acacia*-nodulating strains have all been pentamers, whereas *S. arboris* and *S. kostiense* have been shown to produce tetramers as well. In addition, LCOs acylated with fatty acyl chains other than C16 and C18 fatty acyls have not been reported before for *Acacia*-nodulating rhizobia.[5,26–30] Production of an LCO without any substitution other than the acyl group is also a novel finding for *Acacia*-nodulating rhizobia.

Tropical leguminous trees are nodulated by rhizobia that produce LCOs that are partially sulfated or glycosylated or both on the reducing terminal residue. *Leucaena leucocephala* is nodulated by strains producing sulfated, glycosylated or glycosylated and sulfated LCOs.[26,31–36] *Sesbania* species are nodulated by rhizobia producing glycosylated LCOs.[12,25] *A. senegal* is nodulated by strains producing pentameric, mainly carbamoylated and partially sulfated LCOs.[28,30] As seen in this study, *A. senegal*-nodulating strains may also produce tetrameric LCOs. *Bradyrhizobial* strains and *Rhizobium* sp. strain NGR234 that nodulate some *Acacia* species but not *A. senegal* produce LCOs with (i) methyl, (ii) acetyl and methyl and (iii) sulfate and methyl fucosyl groups.[26,29] Two closely related strains, *Acacia*-nodulating *S. terangae* biovar *acaciae* and *Acacia*-nonnodulating *S. terangae* biovar *sesbaniae*, produce LCOs that differ in their reducing terminal residue decoration: the LCOs of biovar *sesbaniae* are glycosylated whereas those of biovar *acaciae* are not.[25,28] *A. senegal* seems to have strict requirements in regard to the LCOs the rhizobia that nodulate it produce and, moreover, those requirements effectively narrow the host range of the rhizobia nodulating *A. senegal*: rhizobia that produce LCOs suitable for *A. senegal* do not nodulate *Sesbania*.[30] Knowing the structures of LCOs of *Acacia*-nodulating rhizobia allows interpretations of the roles of different structural characteristics of LCOs. In the formation of the rhizobium–*A. senegal* symbiosis, a mixture of partially sulfated, N-methylated pentameric LCOs acylated with common fatty acids are needed. In the *R. etli–Phaseolus vulgaris* symbiosis, the plant response to both carbamoylated and noncarbamoylated LCOs was similar.[37] Taking this together with the findings that *A. senegal* can be nodulated by strains producing noncarbamoylated LCOs[30] and that *S. arboris* and *S. kostiense* also produce noncarbamoylated LCOs, the role of the carbamoyl group is probably not in defining the host specificity of nodulation, but to protect the LCO against degradation or to increase nodulation efficiency, as proposed for acetyl groups in *S. meliloti* and *R. etli* LCOs.[8,38] Further insight into this could be gained by conducting nodulation tests with *S. arboris* and *S. kostiense* wild-type strains and mutants not expressing *nodU*, a 6-O-carbamoyl transferase gene.[39] Additional decorations, such as reducing terminal glycosylations, might result in unsuccessful nodulation. The complexity limits set by the plant partner would select for effective nitrogen-fixing rhizobia.

4. Experimental

4.1. Microbiological techniques

Plasmid pA28, carrying a *nodD1* containing fragment from *Sinorhizobium* sp. strain NGR234 pNGR234a[26] was conjugated to spontaneous streptomycin resistant mutants of *S. arboris* HAMBI 1552 and *S. kostiense* HAMBI 1489 as described.[40] The LCO over-producing strains were included in the HAMBI culture collection (Department of Applied Chemistry and Microbiology, University of Helsinki, Finland) as *S. arboris* HAMBI 2361 and *S. kostiense* HAMBI 2362. The over-producing strains were maintained on YEM agar with Congo red[41] and tetracycline.

4.2. LCO production, extraction and purification

For the production of LCOs, the over-producing strains were inoculated in 2.5 mL TY-broth,[42] incubated overnight, and diluted in 250 mL of TY-broth. Apigenin (4′,5,7-trihydroxyflavone, Sigma Chemicals Co., St Louis, USA) was added to a final concentration of 1 μM for the induced cultures. The cultures were incubated at 28 °C for 24 h in a rotary shaker at 150 rpm.

The culture supernatants were extracted with 1-butanol (1-butanol, Analytical Reagent, Riedel-deHaën GmbH & Co. KG, Seelze, Germany). The extracts were dried under diminished pressure

(Rotavapor, Glasapparatefabrik Flawil, Switzerland) and resuspended in 50% (v%) aq MeCN (HPLC Grade, Rathburn Chemicals Ltd, Walkerburn, Scotland). The extracts were sonicated (Sonorex Super 10P, Bandelin Electronic, Berlin, Germany) and filtered through a 0.2 μm filter (PVDF Filter Media, Whatman Inc., Clifton, USA). The extracts were diluted with water to lower the MeCN concentration below 20% and purified over octadecyl columns (Sep-Pak tC18, Waters Co., Milford, USA) according to the manufacturer's instructions. LCOs were eluted with MeOH, dried under vacuum (Speedvac SVC-100II, Savant Instruments, Inc., Hicksville, USA) and resuspended in 50% (v%) aq MeCN. For the ES-Q-TOF-MS, LCOs were separated by HPLC using a SMART^R System (Pharmacia Biotech, Sweden) with a reverse phase column (Sephasil™ C18 SC 2.1/10, Pharmacia Biotech, Sweden) in a gradient from 30% to 100% (v%) aq MeCN. The effluent was monitored at 206 nm. Fractions were collected and pooled, and the pooled fractions were dried under vacuum. Dried fractions were resuspended in 50% (v%) aq MeCN; compared to the initial culture, the fractions were 1000 or 2000 times concentrated.

4.3. Mass spectrometric analyses

4.3.1. Structural analysis using ES-MS. LCO structures were determined in positive mode with electrospray mass spectrometry (ES-MS). ES-Q-o-TOF-MS was carried out on a Q-TOF mass spectrometer (Micromass, Manchester, UK). Pooled HPLC fractions were directly infused through a Z-spray sample introduction system at 1 μL min^{-1}. A capillary voltage of 3000 or 3500 V and a cone voltage of 75 V were applied. CID-MS/MS was performed with the MS1 quadrupole set to select the precursor ion for fragmentation in the hexapole collision cell. Argon was used as the collision gas and the collision energy varied between collision energy offset setting of 40 and 90 V. Product ions were analyzed with the orthogonal TOF analyzer. LC-ES-ion trap-MS was carried out on an Agilent 1100 system (Agilent Technologies, Palo Alto, USA) connected to a Esquire LC ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany). Amounts of 10–20 μL of 1000× concentrated LCO extract were separated on-line in a microbore (1 × 150 mm) Luna C18(2) column with particle size of 5 μm and pore size of 100 Å (Phenomenex, Torrance, USA) at 50 μL min^{-1} over a 44–95% (v%) aq MeCN gradient in 20.5 min followed by isocratic elution at 95% (v%) aq MeCN for 10 min. Formation of sodium adducts was promoted by making both eluents 1 mM in NaOAc. ES-parameters were set as follows: capillary voltage 4500 V, nebulizer pressure 20 psi, drying temperature 300 °C and drying gas flow 5 L min^{-1}. The ion trap was set such that one full MS scan from m/z 400 to m/z 1600 was followed by auto-MS/MS scans of the most the four most intense ions from m/z 800 to m/z 1600 in the MS spectrum.

The location of the carbamoyl substituent on the nonreducing terminal HexNAc was determined by CID-MS/MS with ES-Q-o-TOF-MS of the protonated B_1-ion (m/z 483) of an in-source fragmented LCO SkV(C18:1, *N*-Me, Cb). A capillary voltage of 3500 V and a cone voltage of 46 V were applied.

4.3.2. Composition analysis. The monosaccharide composition of the LCOs was determined by GC–MS of trimethylsilylglycoside derivatives of LCO samples.[43] The retention times and fragmentation patterns of the resulting Me_3Si methyl glycosides were compared with those of authentic monosaccharide standards following the same derivatization protocol.

4.3.3. Linkage analysis. Substitution patterns of the monosaccharides in the LCOs were determined following identification of the partially methylated alditol acetate derivatives (PMAAs) of LCO samples following GC–MS analysis,[43] with the exception that the partially hydrolytically released monosaccharides were reduced with $NaBH_4$ instead of $NaBD_4$.

Acknowledgements

This work was supported with a grant from the University of Helsinki, Finland. P. Nowak acknowledges the Federation of European Microbiological Societies for the 2001 FEMS Fellowship grant.

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