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Structural characterisation of lipo-chitin oligosaccharides isolated from *Bradyrhizobium aspalati*, microsymbionts of commercially important South African legumes

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Abstract

The shoots of the South African legume *Aspalathus linearis* spp. *linearis* (*A. linearis*) are used in the manufacture of an increasingly popular beverage that has acclaimed beneficial effects on health; this important export product is known as Rooibos (or Redbush) tea. Three strains of *Bradyrhizobium aspalati*, which are the nitrogen-fixing symbionts of *Aspalathus carnosa*, *A. hispida* and *A. linearis*, were tested for the production of lipo-chitin oligosaccharide signal molecules using thin-layer chromatographic analysis after induction with different inducers, including Rooibos tea extract, and radioactive labelling. Large-scale separation, using high-performance liquid chromatography, of lipo-chitin oligosaccharides from *B. aspalati* isolated from *A. carnosa* was performed for structural characterisation using fast-atom bombardment mass spectrometry and chemical modifications followed by gas chromatography–mass spectrometric analysis. The strain was shown to secrete a family of unusual lipo-chitin oligosaccharides that are highly substituted on the nonreducing-terminal residue but unsubstituted on the reducing-terminal residue. They have a backbone of three to five β-(1→4)-linked *N*-acetyl-D-glucosamine residues substituted on the nonreducing terminus with a C16:0, C16:1, C18:0, C18:1, C19:1cy, or C20:1 fatty acyl chain, and are both N-methylated and 4,6-dicarbamoylated.

Keywords: *Bradyrhizobium aspalati*; Lipo-chitin oligosaccharides; Rooibos tea; Rhizobia; Legumes; Mass spectrometry

1. Introduction

Aspalathus linearis spp. *linearis* (*A. linearis*) is a leguminous shrub indigenous to the unique biome of the mountain areas of the north-western Cape in South Africa [1]. Its leaves and stems are used for the production of Rooibos tea, a beverage with acclaimed beneficial health effects, which has become an important export product. Rooibos, or Redbush, tea is caffeine-free, rich in ascorbic acid and minerals [1], and contains large quantities of the flavonoids quercetin, luteolin, aspalathin, orientin, iso-orientin, isoquercetin, and rutin [2–5], which are believed to be antioxidants.

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Leguminous plants are characterised by their ability to establish a symbiotic relationship with bacteria, collectively known as rhizobia. When these soil-living bacteria invade the roots of their host plants, they trigger the formation of a new organ called the root nodule. In the nodule a differentiated form of rhizobium, the bacteroid, reduces nitrogen from the air to produce ammonia, which the plant can incorporate into organic compounds. The symbiosis is species-specific and this specificity is mediated by signal molecules from both the plant and the bacterium. A cocktail of flavonoids secreted by the host plant induces the production of species-specific lipo-chitin oligosaccharides (LCOs) by the bacterium. LCOs consist of an oligosaccharide backbone of β-(1→4)-linked *N*-acetyl-D-glucosamine (GlcNAc), varying in length between two and six residues and which are decorated with a combination of chemical moieties that helps encode species-specificity. Specificity may be further determined by the structure of the fatty acyl chain that is attached in amide linkage to the nonreducing-terminal residue. Substitutions occurring on the terminal residues of the chitin backbone include O-linked acetyl, carbamoyl, glycerol, sulfate, and additional monosaccharide moieties, (e.g., fucose), and N-linked methyl groups [6,7].

Aspalathus species are nodulated by typically slow-growing rhizobial strains belonging to the *Bradyrhizobium* group [8]. A recent report [9] has shown strong nodulation specificity within the genus *Aspalathus* (which consists of 245 species) as well as between *Aspalathus* bradyrhizobia and other agricultural legumes, and vice versa. Consequently, the symbioses formed between these bacteria and their *Aspalathus* hosts have relevance to our understanding of their co-evolutionary relationships as well as to their role as the major nitrogen-fixers in the fynbos biome. Another important biological feature of these bacterial strains is their ability to nodulate *Aspalathus* species and fix nitrogen in soils at pH 3 or 4 [10]. This makes them among the most acid-tolerant rhizobial strains ever reported, with potential for use as inoculants in the many acidic soils of the world. This, coupled with the fact that Rooibos tea is a major commercial product in South Africa and that increasing its production and export is of key commercial relevance, requires a deeper understanding of the *Aspalathus*–bradyrhizobium symbiosis, which begins with determining the structures of the bacterial signals that control nodule formation.

In this study, we have examined bradyrhizobia isolated from root nodules of *A. carnosa*, *A. hispida* and *A. linearis* and authenticated to be the nodule-forming organisms of their respective host plants [11]. For the first time, LCOs from *Aspalathus* strains have been isolated and examined using radiolabelling and thin-layer chromatography (TLC). Various flavonoids and Rooibos tea, root and shoot extracts were tested for their inducing effect on the rhizobia. By means of high-performance liquid chromatography (HPLC), LCOs from *Bradyrhizobium aspalati* from *A. carnosa* were successfully isolated on a large scale using Rooibos tea extract as an inducer, and structurally characterised using fast-atom bombardment mass spectrometry (FABMS) and chemical modifications followed by gas chromatography–mass spectrometry (GC–MS).

The LCOs from *B. aspalati* were established to have a backbone consisting of three to five GlcNAc residues substituted on the nonreducing terminus with a C16:0, C16:1, C18:0, C18:1, C19:1cy, or C20:1 fatty acyl chain, additionally being N-methylated and 4,6-dicarbamoylated. The structures of the LCOs from *B. aspalati* are novel, being highly substituted on the nonreducing-terminal residue, while being unsubstituted on the reducing-terminal residue.

2. Experimental

Bacteria.—Bacteria were isolated from the root nodules of *A. carnosa*, *A. hispida* and *A. linearis* [12] growing in the Western Cape, and authenticated to be the nodule-forming organisms of their respective host plants [11].

Inducers.—Flavonoids and tea, shoot and root extracts were prepared as follows and tested for their LCO-inducing effect. Genistein

and naringenin were added to the cultures to a concentration of 1 μg/mL. Commercial Rooibos tea was extracted with water (100 g/L) by shaking for several hours, and autoclaved. The Rooibos tea extract was tested at the following induction temperatures: 18, 23, and 28 °C, while all other inducers were tested only at 28 °C. Shoot and root extracts from *A. linearis* were prepared by extracting 311 g shoots and 240 g roots with 80% (v/v) MeOH in water, drying down the extracts, redissolving 5 g in 50 mL water and autoclaving. HPLC analysis demonstrated that autoclaving has no influence on the R_f values of the peaks observed from Rooibos tea, shoot and root extracts (data not shown).

TLC analysis.—Bacteria were grown in B^- medium [13], including 10 mL/L potassium phosphate at 28 °C and 200 rpm, and diluted to an optical density at 620 nm (OD_{620}) of 0.05–0.1. To 3 mL of the culture, 0.6 μCi [1-^{14}C]-D-glucosamine (specific activity 50 mCi/mmol, Amersham) was added together with 50 μL of inducer solution, and the cultures were incubated at 28 °C (unless otherwise indicated) for at least 16 h. The LCOs were then extracted from the cultures by adding 2 mL water-saturated *n*-butanol, boiling for 10 min, and evaporating the butanol layer. The residue was redissolved in 30 μL water-saturated *n*-butanol after which 2 μL was used for TLC analysis. Reversed-phase C_{18}-coated silica plates (E. Merck) were developed with 1:1 MeCN–water, and normal-phase silica plates (Aldrich) were developed with 5:3:2 butanol–EtOH–water. Radioactive compounds were detected using a phosphorimaging system (Molecular Dynamics, Co.) and ImageQuant software.

Isolation of LCOs using HPLC.—Bacteria were cultured in 25 mL B^- medium including 2.5 mL potassium phosphate at 28 °C and 200 rpm to an OD_{620} of 0.5–1.0. The cultures were then diluted to 3 L, and 50 mL inducer was added. Inducers were chosen on the basis of TLC results, and included commercial Rooibos tea extract for *B. aspalati* strains from *A. carnosa* and *A. linearis*, and root extract for *B. aspalati* from *A. hispida*. LCO production was induced for at least 16 h at 28 °C for all strains. However, as an additional treatment, *B. aspalati* from *A. carnosa* was also induced at 12 °C. LCOs were extracted by shaking for 1 h with 900 mL *n*-butanol, after which the butanol layer was removed and evaporated to dryness. The residue was resuspended in 15 mL 60% (v/v) MeCN in water, shaken overnight, and prepurified using octadecyl silica columns (Baker, 1 mg of adsorbent). Samples were submitted to HPLC using a reversed-phase Pharmacia SuperPac Pep-S column (5 μm, 4 × 250 mm). Elution was with a stepwise gradient of 30% MeCN (30 min), 40% MeCN (30 min), 42, 45, 50, 60 and 80% MeCN in water (highest grade, Baker) for 10 min each, using a flow rate of 1 mL/min. The eluate was monitored at 206, 260, 303 and 330 nm with an RSD 2140 photodiode array detector (Pharmacia LKB Biotechn. Inc.). Fractions corresponding to peaks of UV absorption were collected, evaporated to dryness, and subjected to structural analysis.

Chemical modifications of LCOs

Peracetylation. A 500 μL sample of trifluoroacetic anhydride (Aldrich)–AcOH (E. Merck) (2:1, v/v) was added to 8% of Fraction F5 from the culture filtrate produced at 12 °C. After 20 min at room temperature, the samples were evaporated to dryness and redissolved in 15 μL Me_2SO (Aldrich).

Linkage analysis. Partially methylated alditol acetates (PMAAs) were prepared from 15% of Fraction F5 from the culture filtrate produced at 12 °C, with and without retention of the carbamoyl group [14]. Briefly, the sample was dissolved in 300 μL Me_2SO and a few freshly ground NaOH pellets (Lamers &Pleuger) were added. After 10 min (or immediately, if the carbamoyl group was to be retained) five drops of iodomethane (Aldrich) were added, and repeated after another 10 min. After 10 min, 20 drops were added and the reaction stopped after 30 min by the addition of 1 mL aq sodium thiosulfate solution (E. Merck, 100 mg/mL), immediately followed by the addition of 1 mL chloroform (E. Merck). Following shaking and centrifugation, the aqueous layer was removed and the chloroform washed three times with 1 mL water and dried under reduced pressure. The dried permethylated sample was hydrolysed in 500 μL 2 M trifluoroacetic acid (Aldrich) at

120 °C for 1 h, and evaporated to dryness. The sample was reduced on addition of 250 μL 10 mg/mL $NaBD_4$ (Aldrich, 98% D) in 0.5 M NH_4OH (E. Merck) to the dried sample. This mixture was left at room temperature for 1 h and the reaction quenched with 1 mL glacial AcOH (E. Merck) followed by drying. A total of 1 mL of 10% AcOH in MeOH (Biosolve Ltd) was added, mixed and evaporated to dryness, and the procedure repeated twice. The same procedure was repeated three more times using 1 mL MeOH. Acetylation was achieved on addition of 500 μL acetic anhydride (Aldrich) to the dried sample followed by heating for 3 h at 120 °C. A volume of 0.5 mL water was added and the sample neutralised by the addition of solid Na_2CO_3 (E. Merck) until evolution of gas stopped. After extraction with 0.75 mL CH_2Cl_2 (E. Merck), the organic phase was concentrated under a stream of nitrogen gas and 1 μL used for GC–MS analysis.

FABMS analysis.—Positive-ion mode FAB mass spectra were obtained using a Jeol JMS-SX/SX 102A tandem mass spectrometer at +10 kV accelerating voltage. The FAB gun was operated at an accelerating voltage of 4 kV and an emission current of 10 mA using xenon as the bombarding gas. Collision-induced dissociation (CID) mass spectra of selected ions were recorded using air as the collision gas at a pressure sufficient to reduce the parent ion to one third of its original intensity. HPLC fractions were redissolved in 15 μL Me_2SO and 1–2 μL of sample loaded into a matrix of 2–3 μL thioglycerol.

GC–MS analysis.—PMAAs were analysed using a Fisons MD800 mass spectrometer fitted with a Fisons GC8060 gas chromatograph, an on-column injector and helium as the carrier gas. A DB-5MS column (0.32 mm × 30 m; J&W Scientific) was used. The GC temperature program was: 50 °C for 2 min, a gradient of 40 °C/min to 130 °C holding for 2 min at 130 °C, a gradient of 4 °C/min to 230 °C and holding for 20 min at 230 °C. Mass spectra were recorded under conditions of electron impact in the positive-ion mode and were recorded using linear scanning of the range m/z 50–350 with an electron energy of 70 eV.

3. Results

TLC analysis of LCOs.—The flavonoids genistein and naringenin, and Rooibos tea, shoot and root extracts were tested for their effect on the production of radiolabelled LCOs in *B. aspalati* from *A. carnosa*, *A. hispida* and *A. linearis*. The behaviour of the radiolabelled LCOs was compared on both normal- and reversed-phase TLC plates.

Genistein was able to induce in *B. aspalati* from *A. carnosa* a weakly radiolabelled spot on normal- and reversed-phase TLC, which, on the basis of its migration behaviour, corresponds to LCOs. On induction with naringenin, spots for LCOs were only observed with *B. aspalati* from *A. carnosa* and *A. linearis* (data not shown).

Induction with Rooibos tea, shoot and root extracts provided various radiolabelled spots for LCOs from all three strains. Induction with water extract of commercial Rooibos tea yielded intense radioactive spots for LCOs from *B. aspalati* strains isolated from *A. carnosa* and *A. linearis*, while induction with root extract resulted in the most intense LCO spots for *B. aspalati* from *A. hispida* (data not shown). Induction with Rooibos tea extract resulted in LCOs giving reversed-phase TLC profiles (Fig. 1) with an intense spot from the *B. aspalati* strain from *A. carnosa* (Lane 2) and an intense and a very much weaker spot, both with higher R_f values from *B. aspalati* from *A. linearis* (Lane 1), corresponding to metabolites more hydrophilic than those produced by *A. carnosa*. A very weak spot is observed from *B. aspalati* from *A. hispida* (Lane 3), having an R_f value comparable to that of the intense spot observed from *B. aspalati* from *A. linearis*.

Induction with Rooibos tea extract resulted in LCOs giving reversed-phase TLC profiles with an intense spot from the *B. aspalati* strain from *A. carnosa*, and less intense spots with higher R_f values from the other two strains, indicating less efficient radioactive incorporation into metabolites that are more hydrophilic (see Fig. 1). The normal-phase TLC profile obtained on induction with Rooibos tea extract yielded no LCO spots from the *B. aspalati* strains from *A. hispida* and *A.*

linearis, while two LCO spots were observed from *B. aspalati* from *A. carnosa*.

The influence of induction temperature on LCO production was studied by incubating *B. aspalati* from *A. carnosa* with Rooibos tea extract at 18, 23 and 28 °C. The normal-phase TLC profile of the LCOs produced (Fig. 2) showed that the intensity of the radioactive spots increases with increasing induction temperature, with the most efficient labelling occurring at 28 °C.

Isolation of LCOs.—Large-scale preparation of LCOs for structural characterisation was therefore performed using *B. aspalati* from *A. carnosa* at 28 °C. However, cultures were also grown at 12 °C to determine whether the apparent temperature-dependent trend in radioactive incorporation into LCOs observed on TLC plates was an accurate reflection of a difference in the level of LCO production. The inducer chosen was Rooibos tea extract because it resulted in the most intense spots on TLC plates.

Several peaks with UV absorbance at 206 nm are present in the HPLC profile of LCOs isolated from *B. aspalati* (*A. carnosa*) at both 12 and 28 °C. In spite of the marked temperature dependency of radioactive incorporation into LCOs, apparently comparable amounts of LCOs are produced at both 12 and 28 °C. This is perhaps not surprising considering the fact that the members of the genus *Aspalathus* (which includes *A. linearis*, *A. carnosa* and *A. hispida*) germinate and nodulate with the winter rains when temperatures are around 12–15 °C. The only difference between the profiles obtained from the LCOs produced at the two temperatures was in the relative intensities of some of the peaks. Fig. 3 shows the HPLC profile of the LCOs produced at 28 °C. Since no absorbance was observed at 260, 303 or 330 nm, it was concluded that polyunsaturated fatty acyl components are not produced. An intense peak corresponding to compounds present in Rooibos tea extract eluted at ca. 40 min. Fractions F1–F9 were collected from the HPLC separation of the LCOs produced at both 12 and 28 °C, and the LCOs were subjected to structural analysis.

Structural identification of LCOs from B. aspalati.—The results of the FABMS analysis of the LCOs isolated on HPLC are summarised in Table 1. Similar LCOs were produced at both 12 and 28 °C.

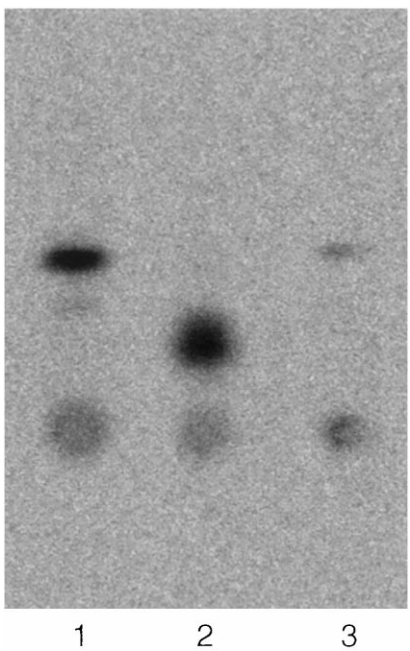

Fig. 1. Reversed-phase TLC profile of radiolabelled LCOs produced by *B. aspalati* from *A. linearis*, *A. carnosa* and *A. hispida* (Lanes 1, 2 and 3, respectively), after induction of strains with Rooibos tea extract at 28 °C.

Fig. 2. Normal-phase TLC profile of radiolabelled LCOs produced by *B. aspalati* from *A. carnosa* after induction with Rooibos tea extract at 18, 23 and 28 °C (Lanes 1, 2 and 3, respectively).

The FAB mass spectra revealed the presence of $[M+H]^+$ and $[M+Na]^+$ pseudomolecular ions. The major component (Fraction F5) contained an $[M+H]^+$ pseudomolecular ion at m/z 1153, consistent with an LCO having four GlcNAc residues, substituted with two carbamoyl groups, one methyl group, and a C18:1 fatty acyl moiety (IV(C18:1,Me,Cb,Cb)). The thioglycerol adduct ion present at m/z 1261 is consistent with the presence of a component bearing an unsaturated fatty acyl moiety (glycolipids having unsaturated fatty acids readily form thioglycerol adducts, while saturated fatty acid-containing glycolipids do not [15]).

The CID mass spectrum of the ion at m/z 1153 (Fig. 4 and Table 1) contains signals corresponding to oxonium ions formed by cleavage of each successive glycosidic bond, at m/z 932, 729 and 526. This fragmentation pattern demonstrates that all substituents are present on the nonreducing-terminal residue of the chitin backbone. The signals at m/z 1153 and 526 are accompanied by weaker satellite ions 61 mass units lower, consistent with the elimination of one carbamoyl group as carbamoic acid (NH_2COOH).

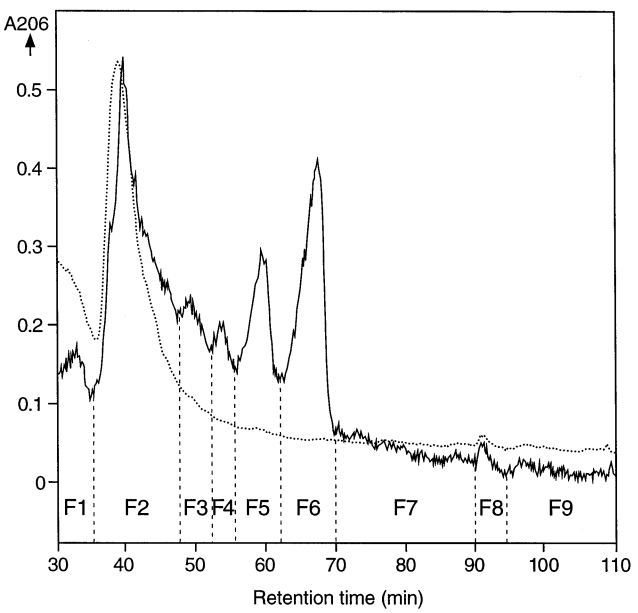

Fig. 3. HPLC profile of *n*-butanol extract from *B. aspalati* from *A. carnosa*, after induction with Rooibos tea extract at 28 °C. The absorbance was recorded at 206 nm. Fractions were pooled as indicated. Dotted line shows HPLC profile of Rooibos tea extract alone (206 nm).

Table 1
Summary of FABMS data and structural assignments for LCOs produced by *B. aspalati* from *A. carnosa*

Fraction	$[M+H]^+$	B_i ions	Assignment
F1	1125	904, 701, 498 [a]	IV(C16:1,Me,Cb,Cb)
F2	1330		V(C16:0,Me,Cb,Cb)
F3	1330	1109, 906, 703, 500 [b]	V(C16:0,Me,Cb,Cb)
F4	1096	875, 672, 469 [b]	IV(C18:1,Cb)
	1127	906, 703, 500	IV(C16:0,Me,Cb,Cb)
	1356	1135, 932, 729, 526	V(C18:1,Me,Cb,Cb)
F5	1153	932, 729, 526	IV(C18:1,Me,Cb,Cb)
F6	950	729, 526	III(C18:1,Me,Cb,Cb)
	1153		IV(C18:1,Me,Cb,Cb)
F7	1155	934, 731, 528	IV(C18:0,Me,Cb,Cb)
	1167	946, 743, 540 [b]	IV(C19:1cy,Me,Cb,Cb)
F8	1155		IV(C18:0,Me,Cb,Cb)
	1167		IV(C19:1cy,Me,Cb,Cb)
	1181	960, 757, 554 [a]	IV(C20:1,Me,Cb,Cb)
F9	1155		IV(C18:0,Me,Cb,Cb)
	1181		IV(C20:1,Me,Cb,Cb)

[a] CID spectrum recorded only from the fraction produced at 12 °C.
[b] CID spectrum recorded only from the fraction produced at 28 °C.

Structurally related LCOs varying only in the number of GlcNAc residues (three to five) and the composition of their fatty acyl chains (C16:0, C16:1, C18:0, C18:1, C19:1cy, and C20:1) were identified similarly in the other fractions (see Table 1). Thioglycerol adducts were observed for all LCOs containing an unsaturated fatty acyl group. Where sufficient material was available, FAB-CID-MS/MS was performed. The second most abundant LCO, V(C18:1,Me,Cb,Cb), was in Fraction F4, and also bears all substituents on the nonreducing-terminal residue. Fig. 5 shows the CID spectrum of V(C18:1,Me,Cb,Cb). We assume that the two spots observed from this strain on normal-phase TLC analysis (e.g., Lane 3, Fig. 2) correspond to the major LCOs. A minor LCO (IV(C18:1,Cb)) containing a single carbamoyl group and no methyl

group, probably as the result of incomplete biosynthesis, was also identified.

In order to determine whether the methyl group is attached to the nitrogen of the nonreducing-terminal GlcNAc or to an oxygen, 8% of Fraction F5 was peracetylated. FAB mass spectrometric analysis produced a pseudomolecular ion at m/z 1573, which corresponds to the incorporation of ten acetyl groups, consistent with the presence of eight hydroxyl groups and two carbamoyl groups in the native material, indicating that the methyl group is located on the nitrogen atom of the nonreducing-terminal GlcNAc.

In order to preclude the theoretical possibility of the presence of a C19:1 fatty acyl group in place of a methyl group and a C18:1 fatty acyl chain, an aliquot of Fraction F6 was permethylated. The FAB mass spectrum of the product contains a signal at m/z 1249 (from the m/z 1153 component). This mass corresponds to the removal of two carbamoyl groups and the incorporation of 13 (rather than 14) methyl groups, indicating that the nitrogen on the nonreducing-terminal GlcNAc bears a C18:1 fatty acyl and a separate methyl group.

To determine the linkage position of the carbamoyl moieties, linkage analysis was performed on two aliquots of the LCOs in Fraction F5, one with and the other without retention of the carbamoyl groups (see Section 2).

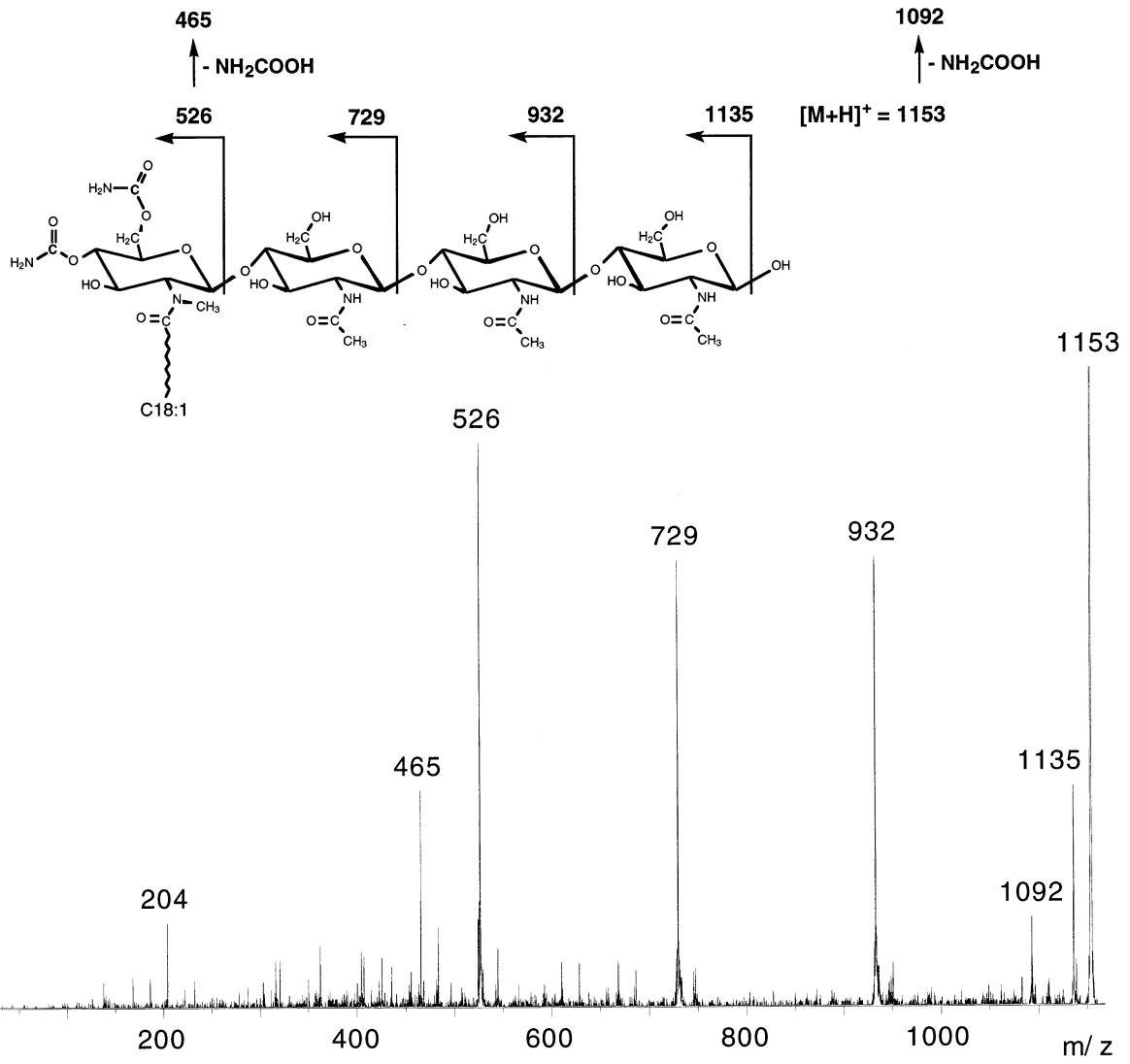

Fig. 4. Collision-induced dissociation mass spectrum of the $[M + H]^+$ at m/z 1153 in Fraction F5, and fragmentation scheme for IV(C18:1,Me,Cb,Cb). m/z 204: double cleave ion.

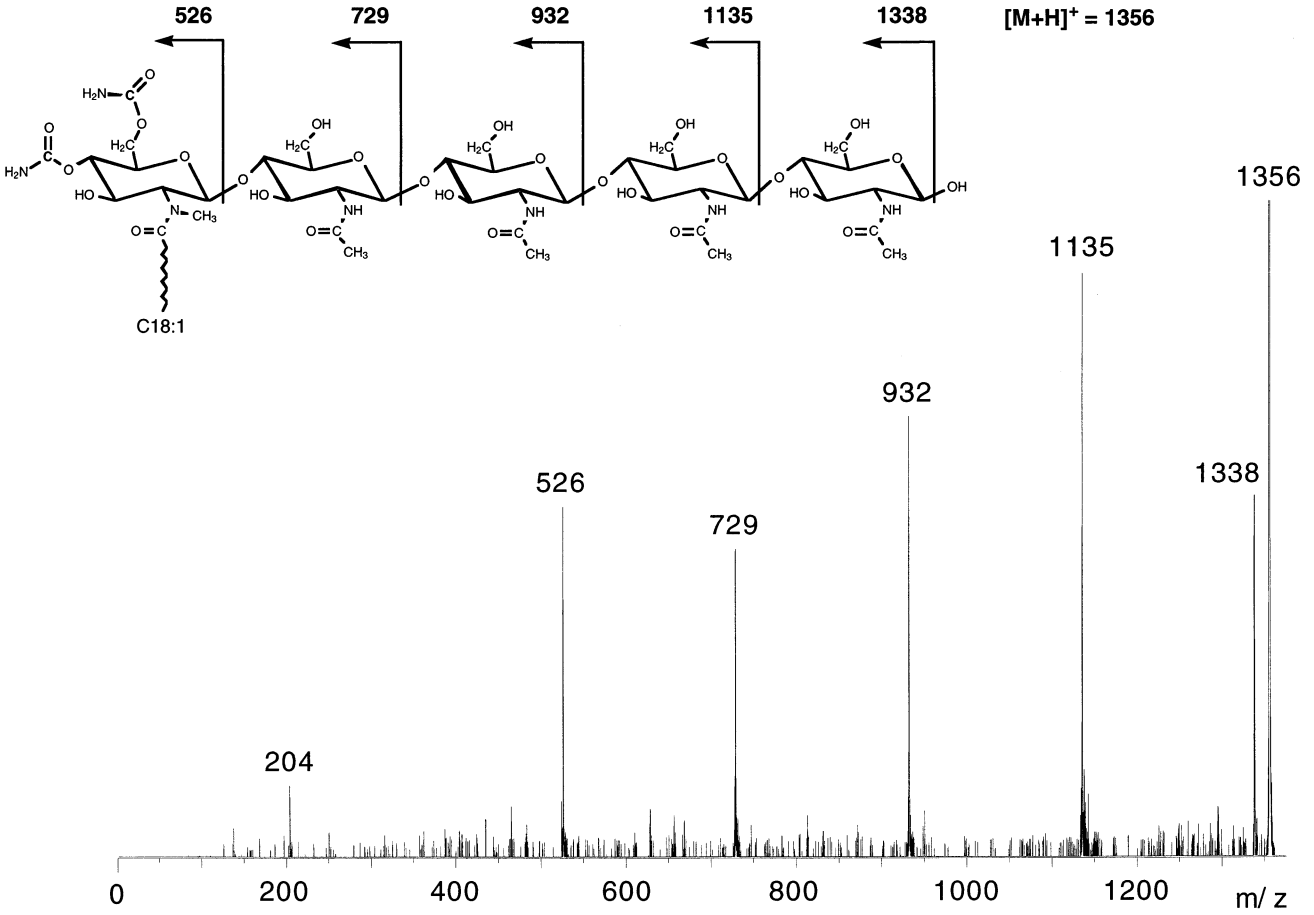

Fig. 5. Collision-induced dissociation mass spectrum of the $[M + H]^+$ at m/z 1356 in Fraction F4, and fragmentation scheme for V(C18:1,Me,Cb,Cb). m/z 204: double cleave ion.

FAB mass spectra of the aliquot without retention of the carbamoyl groups obtained after permethylation showed pseudomolecular ions at m/z 1249 and 1306. The first is consistent with the presence of an LCO having lost both carbamoyl groups and having incorporated 13 methyl groups, and the second with a species partially retaining one carbamoyl group and having incorporated 14 methyl groups. Ions indicating the presence of an LCO retaining both carbamoyl groups were absent. In contrast, FAB mass spectra obtained from the aliquot prepared with retention of the carbamoyl groups on permethylation showed a weak additional pseudomolecular ion at m/z 1363, which is consistent with the presence of an LCO having incorporated 15 methyl groups and retaining both carbamoyl groups.

GC–MS analysis of the PMAAs from Fraction F5 prepared without retention of carbamoyl groups showed the presence of derivatives corresponding to terminal HexNAc and 4-substituted HexNAc. Spectra obtained from the PMAAs produced from Fraction F5 prepared with retention of carbamoyl groups showed the presence of terminal HexNAc, 4-substituted HexNAc, and 4,6-disubstituted HexNAc. It was therefore concluded that the LCOs in Fraction F5 consist of a $(1 \rightarrow 4)$-linked GlcNAc backbone with carbamoyl moieties present on carbons 4 and 6 of the nonreducing-terminal residue.

4. Conclusions

The legume *A. linearis* is used for manufacturing Rooibos tea, a beverage with beneficial effects on health, and which has become an important South African export product. To gain a better understanding of the symbiotic system of this commercially important species, we have studied LCO production in three

strains of *B. aspalati*, which are the microsymbionts of *A. carnosa*, *A. hispida* and *A. linearis*. TLC experiments showed that Rooibos tea extract, which is rich in flavonoids, is a suitable inducer for LCO production in the three strains. Large-scale isolation of LCOs followed by structural identification showed that *B. aspalati* from *A. carnosa* secretes a family of O-carbamoylated and N-methylated LCOs. LCOs were established to have a backbone of between three and five GlcNAc residues substituted on the nonreducing terminus with a C16:0, C16:1, C18:0, C18:1, C19:1cy, or a C20:1 fatty acyl chain, an N-methyl group, and two carbamoyl groups on carbons 4 and 6. The two most abundant LCOs were determined to be IV(C18:1, Me,Cb,Cb) and V(C18:1,Me,Cb,Cb). The species-specific decorations, while in themselves not unusual, are remarkable in yielding LCOs that are so highly decorated on the nonreducing-terminal residue while being unsubstituted on the reducing-terminal residue.

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