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Structure identification of natural rhamnolipid mixtures by fast atom bombardment tandem mass spectrometry

A. MANSO PAJARRON[1], C. G. DE KOSTER[1]*, W. HEERMA[1], M. SCHMIDT[2] and J. HAVERKAMP[1,2]

[1] *Bijvoet Center for Biomolecular Research, Department of Mass Spectrometry, University of Utrecht, P.O. Box 80083, 35008 TB Utrecht, The Netherlands*

[2] *Uniliver Research Laboratories, P.O. Box 114, 3130 AC Vlaardingen, The Netherlands*

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Two rhamnobiose-lipid preparations have been studied by fast atom bombardment (FAB) tandem mass spectrometry. The principal rhamnobiose-lipids contain the β-hydroxydecanoyl-β-hydroxydecanoate Rha-Rha-C_{10}-C_{10} and the β-hydroxytetradecanoyl-β-hydroxytetradecanoate Rha-Rha-C_{14}-C_{14}. Both preparations contain minor components which are heterogenous in β-hydroxy fatty acid composition. FAB ionization of rhamnobiose-lipids in the presence of Na^+ shows the formation of both $[M + Na]^+$, $[M + 2Na - H]^+$, $[M + 3Na - 2H]^+$ and $[M - H]^-$ ions. Tandem mass spectrometry of the $[M + 2Na - H]^+$ and $[M - H]^-$ ions give information about the sequence of the building blocks. Particularly, heterogeneity in β-hydroxy fatty acid composition is determined for the principal components and all the minor components present in the preparations.

Keywords: fast atom bombardment, tandem mass spectrometry, rhamnolipids, *Pseudomonas* sp.

Introduction

A wide variety of biosurfactants occur in nature [1, 2]. In particular, biosurfactants from microbial sources (bacteria, yeasts and filamentous fungi) receive a great deal of attention as they may find technical application potential in a wide range of industrial areas including natural oil recovery, cosmetics and detergents. Commercial interests in biosurfactants as alternatives to the generally used petrochemical surfactants is stimulated by environmental concerns with the latter. Although application of biosurfactants is still restricted because of the relatively high cost of production, the interesting functional properties, good biodegradability and producibility from renewable raw materials are the major driving forces for the development of improved production systems and new industrial applications.

An interesting class of low molecular weight microbial biosurfactants is the glycolipids. These contain a hydrophobic part composed usually of one or more fatty acid residues ester-bound to a hydrophilic part being a mono- or oligosaccharide unit. Typical representatives of this type of glycolipid are the anionic rhamnolipids secreted by *Pseudomonas* sp. during the stationary phase of growth of these bacteria. Although their exact biological function is not yet clear, it is postulated that they may support microbial adhesion and growth on hydrophobic substrates [3] and that they may serve as a microbial virulence factor [4].

The *Pseudomonas* rhamnolipids are composed of rhamnose or rhamnobiose linked to a β-hydroxy fatty acid or a dimer thereof, according to the general formula as given in Fig. 1. The structure of the fatty acid part may vary depending on the strain, culture medium and growth conditions [5]. These structure variations can influence strongly physical-chemical properties of the (mixtures of) rhamnolipid produced.

The present study towards the detailed structural analysis of (natural mixtures of) rhamnolipids was carried out in a larger framework, directed at achieving insight into relations between chemical structure and surface active properties of these substances. In the context of research directed towards applications of rhamnolipids, rapid and sensitive methods for detection and structure analysis are required. The recent availability of four sector mass spectrometers has added a new dimension in the analysis of biomolecules [6]. In tandem mass spectrometry (MS/MS) the ion of interest is selected in the first mass spectrometer at unit resolution. This resolution for the selection of the precursor ion in collision experiments is important, because components that vary in degree of unsaturation or other modifications resulting in mass shifts of only a few daltons are often present

* To whom correspondence should be addressed.

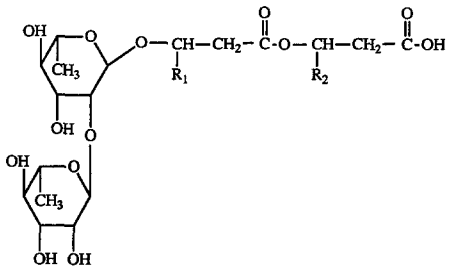


Figure 1. Structure of 2-*O*-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (R_1 and R_2 = C_7H_{15}) and 2-*O*-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxytetradecanoyl-β-hydroxytetradecanoate (R_1 and R_2 is $C_{11}H_{23}$).

in mixtures of glycoconjugates even in extensively purified extracts. The mass selected ions collide with an inert gas such as helium in a collision cell located in the field-free region between MS-1 and MS-2. The product ions are analysed at unit resolution in the second mass spectrometer MS-2. Domon and Costello [7] described the application of this approach for the characterization of glycosphingolipids and ganliosides. Their investigation has shown that much information regarding both the lipophilic part and the glycosidic part can be gained. The combination of FAB and tandem mass spectrometry has been applied successfully to the structure determination of several classes of glycolipids [8–10]. The major application of MS/MS lay in structure elucidation and mixture analysis with low chemical noise. For the analysis of glycolipid mixtures, particularly when the sample size is very limited, it is especially desirable to eliminate background interferences by using MS/MS.

Two rhamnolipid preparations (RL I and II) from *Pseudomonas* are studied by FAB-MS and FAB-MS/MS. The principal rhamnobiose-lipid in RL I (I_p) is 2-*O*-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}). The major compound in RL II (II_p) is 2-*O*-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxytetradecanoyl-β-hydroxytetradecanoate (Rha-Rha-C_{14}-C_{14}). The positive and negative ion FAB-MS spectra of Rha-C_{10} and Rha-Rha-C_{10}-C_{10} have been published by Bosch *et al.* [11]. Rendell *et al.* [12] identified several rhamnolipids in cultures of clinical isolates of *Pseudomonas* sp. by FAB and electron impact (EI) mass spectrometry of native and derivatized compounds.

Materials and methods

Materials

The rhamnolipids with a chain length of C_{14} were recovered from the acidified culture broth of the strain *Pseudomonas glumae* by exhaustive extraction with ethyl acetate. From the crude extract the lipids were isolated by means of reversed-phase column chromatography, using Merck LiChrospher RP-8 silica gel and a solvent gradient CH_3OH:H_2O from 80:20 to 100:0. The lipids were then applied to preparative thin layer chromatography on Merck Silica gel F60 TLC plates using $CHCl_3$:MeOH:HOAc(20%), 65:15:2 by vol, as solvent prior to mass spectrometry. The exact procedure of isolation, structure elucidation with NMR methods and investigation of the properties of these new type of rhamnolipids will be described elsewhere. The well known C_{10}-rhamnolipids which served here as reference compounds were isolated from a crude rhamnolipid mixture by recrystallization from hexane. The rhamnolipid samples were a gift from Petrogen Inc., IL, USA.

Mass spectrometry

Positive and negative ion FAB mass spectra and collisional activation (CA) spectra were obtained with a Jeol JMS-SX102/102A four sector instrument of a B_1E_1-B_2E_2 geometry. FAB mass spectra were obtained with MS-1. Tandem mass spectra were acquired by selecting the desired precursor ion with MS-1, and colliding the selected ion at 10 keV translational energy in a collision cell at ground potential located in the third field free region of the instrument. The resulting fragment ions were determined by scanning MS-2. The CA spectra were recorded at a main beam attenuation of 50% with helium as collision gas. The resolution was 1000 for MS-1 as well as for MS-1. Xenon was used as the FAB gas; the gun was operated at 6 kV and a 5 mA discharge current.

Samples were dissolved in methanol at a concentration of 1 μg μl^{-1}. Aliquots (1 μl) were transferred by syringe and mixed with 1 μl glycerol on the stainless steel FAB probe tip. In the positive ion mode, 1 μl of a saturated NaCl solution was added to the matrix, because the resulting $[M + Na]^+$ signal is much more abundant than the $[M + H]^+$ ion intensity from a sodium-free or acidified matrix.

Results and discussion

The positive ion FAB mass spectrum of RL I is presented in Fig. 2. The mass region of the pseudo-molecular ion is extended in Fig. 3. Sodium cationization of Rha-Rha-C_{10}-C_{10} (I_p) gives $[M + Na]^+$ ions at *m/z* 673, $[M + 2Na - H]^+$ ions at *m/z* 695 and $[M + 3Na - 2H]^+$ at *m/z* 717 of which the $[M + 2Na - H]^+$ cation is the most abundant. Additional molecular weight information is obtained from the $[I_p + 2Na + NaCl - H]^+$ and $[I_p + 2Na + 2NaCl - H]^+$ adduct ions at *m/z* 753 and *m/z* 811, respectively. This cluster formation is indicative of a high sodium affinity of the rhamnolipid. The mass differences between the Na and Na/NaCl adduct ions enables a tentative assignment of the molecular weight of the compound.

An interesting feature is the presence of *m/z* 667 and *m/z* 723 ions in the spectrum of RL I (Fig. 2). These ions have a nominal mass difference of −28 and +28, respectively, with respect to *m/z* 695 $[I_p + 2Na - H]^+$, pointing to the

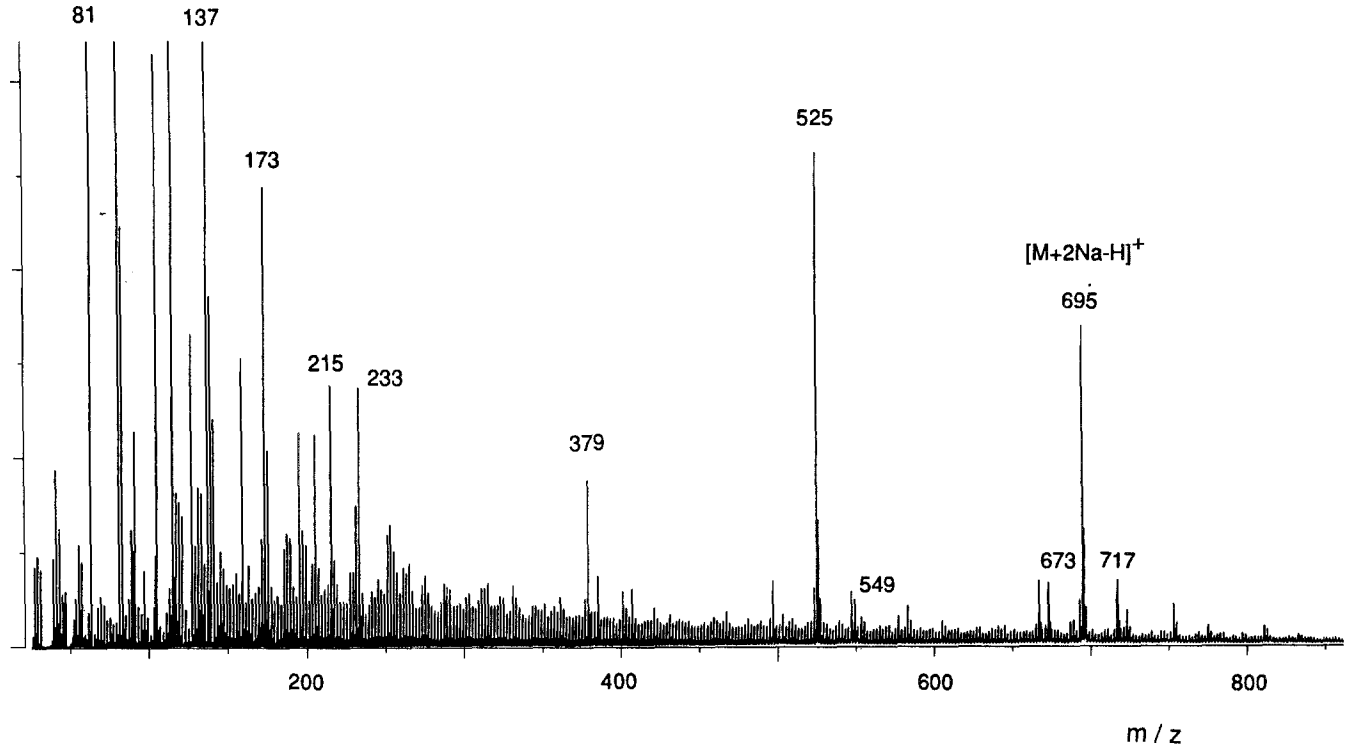


Figure 2. Positive ion FAB mass spectrum RL I.

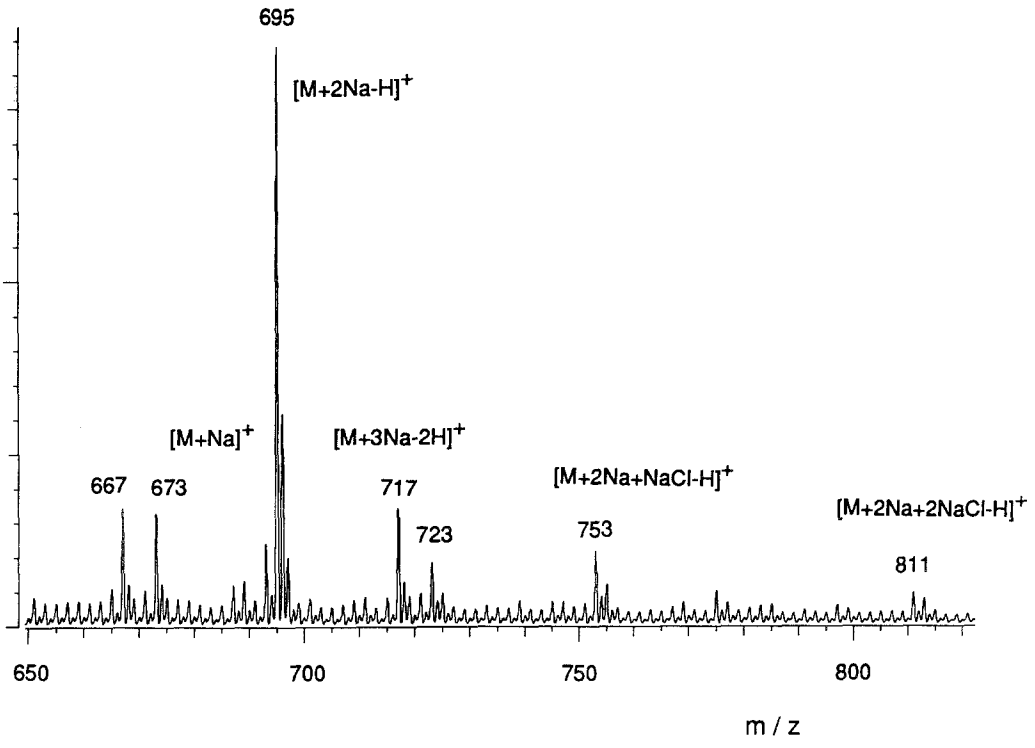


Figure 3. Partial positive ion FAB spectrum RL I.

presence of minor quantities of rhamnolipid homologues of I_p containing β-hydroxy fatty acid groups with other alkyl chain lengths. The *m/z* 667 ion can be ascribed to the $[M + 2Na - H]^+$ adduct ion of Rha-Rha-C_{10}-C_8 and/or Rha-Rha-C_8C_{10} (further denoted as I_a) and *m/z* 723 to disodium ions of Rha-Rha-C_{10}-C_{12} and/or Rha-Rha-C_{10}-C_{12} (I_b).

The base peak at *m/z* 525 orginates from $[I_p + 2Na - H]^+$

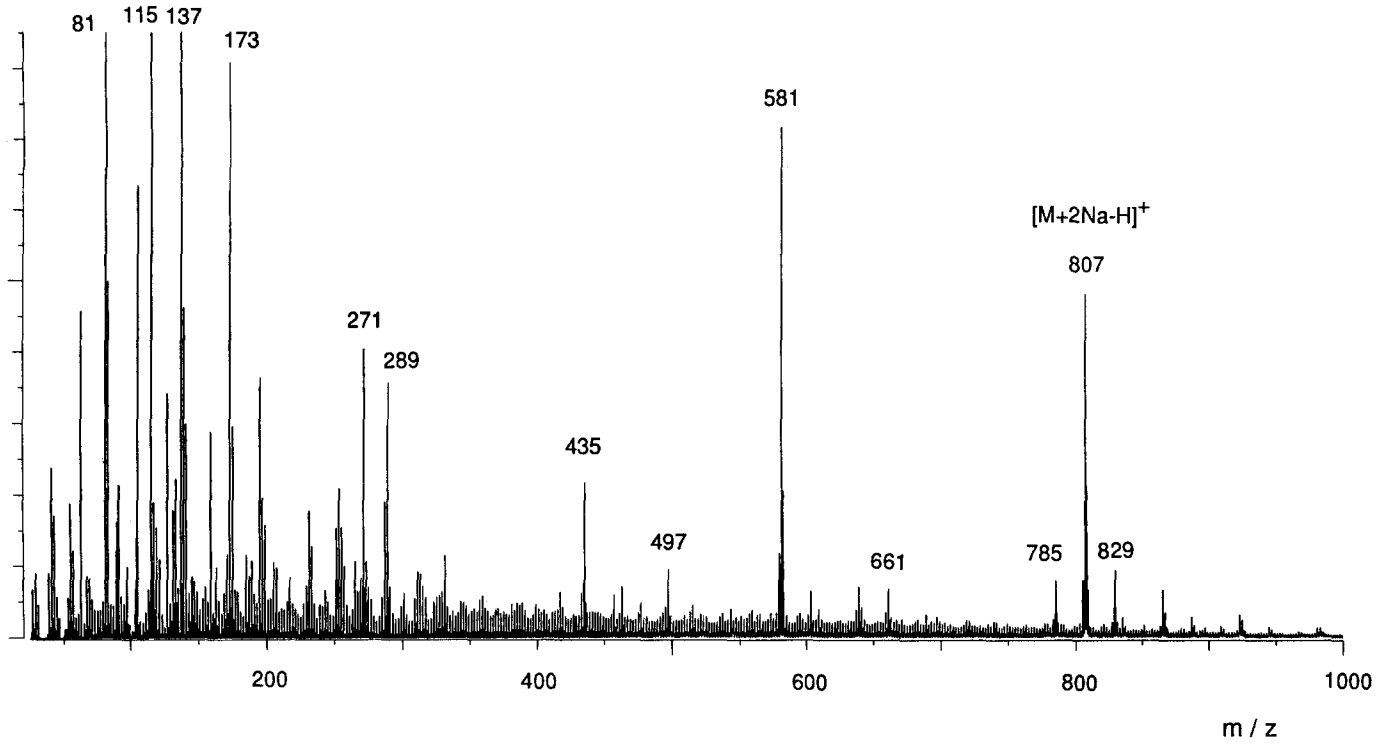


Figure 4. Positive ion FAB mass spectrum of RL II.

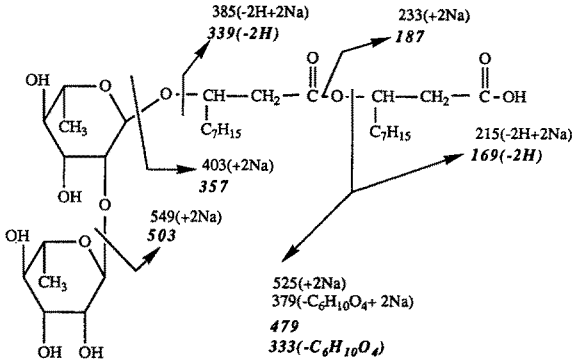
by loss of the terminal alkenoic acid $C_9H_{17}COOH$. Small peaks at *m/z* 497 and *m/z* 553, 28 atomic mass units equidistant from *m/z* 525, indicate the presence of I_a (Rha-Rha-C_8-H_{10}) and I_b (Rha-Rha-C_{12}-C_{10}) as minor components of the sample. The possible presence of the analogues Rha-Rha-C_{10}-C_8 and Rha-Rha-C_{10}-C_{12} cannot be concluded from these fragments. The *m/z* 549 fragment ion is formed by loss of $C_6H_{10}O_4$ from *m/z* 695 $[I_p + 2Na - H]^+$. Other indicative fragment ions are found at *m/z* 379 $[I_p + 2Na - H - C_6H_{12}O_5 - C_9H_{17}COOH]^+$, *m/z* 233, $[C_{10}H_{20}O_3 + 2Na - H]^+$ and *m/z* 215 $[C_9H_{17}COOH + 2Na - H]^+$. The *m/z* 525, *m/z* 549 and *m/z* 215 fragment ions were also found by Bosch *et al.* [11]. In addition, the authors observed ions at *m/z* 172 and *m/z* 194 which were assigned to $[C_{10}H_{19}O_2 + H]^+$ and $[C_{10}H_{19}O_2 + Na]^+$ cations, respectively. At first we should like to state that the *m/z* value of these ions is even. Consequently, they are radical cations at *m/z* 172 $[C_{10}H_{19}O_2 + H]^{+\cdot}$ and *m/z* 194 $[C_{10}H_{19}O_2 + Na]^{+\cdot}$. Generation of these radical cations under FAB ionization is rather unlikely, as their formation is expected to be thermochemically unfavourable and their formation may be considered as a violation of the even-electron rule. A more plausible explanation is that the *m/z* 172 and *m/z* 194 ions arise from protonated and sodiated triethanolamine (MW 171), respectively, used as FAB matrix.

Similar results were obtained for RL II (Fig. 4). The major component in this isolate is Rha-Rha-C_{14}-C_{14} (II_p). The most abundant quasi-molecular ion is $[M + 2Na - H]^+$ at *m/z* 807. The ions at *m/z* 785 and at *m/z* 829 can be assigned as the $[M + 2Na - H]^+$ adducts of Rha-Rha-C_{14}-C_{12} and/or Rha-Rha-C_{12}-C_{14} (II_a) and Rha-Rha-C_{14}-C_{16} and/or Rha-Rha-C_{16}-C_{14} (II_b), respectively. The base peak *m/z* 581 is formed by elimination of $C_{13}H_{25}COOH$ from II_p. Fragment ions 28 mass units equidistant from *m/z* 581 are the result of losses of the terminal C_{14} alkenoic acids from II_a and II_b. Other characteristic fragment ions are found at *m/z* 661, $[II_p + 2Na - H - C_6H_{10}O_4]^+$, *m/z* 497, $[II_p + 2Na - H - C_6H_{10}O_4 - C_6H_{12}O_5]^+$, *m/z* 435, $[II_p + 2Na - H - C_6H_{10}O_4 - C_{13}H_{25}COOH]^+$, *m/z* 289, $[C_{14}H_{28}O_3 + 2Na - H]^+$ and *m/z* 271 $[C_{13}H_{25}COOH + 2Na - H]^+$.

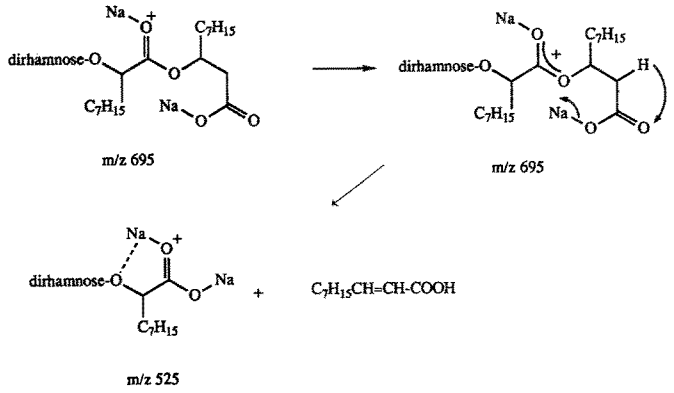
The disodium ions of the individual compounds in the rhamnolipid isolates I and II have been studied by collisional activation mass spectroscopy. The CA spectra of the $[M + 2Na - H]^+$ ions of I_a, I_p, I_b, II_a, II_p and II_b are listed in Table 1. The spectra show common fragmentation reactions. These fragmentations are rationalized for CA of I_p in Scheme 1. As is seen in Scheme 1 and Table 1, CA of the rhamnolipid $[M + 2Na - H]^+$ cations gives sequence information about the building blocks. The most abundant collisionally induced fragmentation is the elimination of the terminal alkenoic acid. For I_p and II_p this reaction leads to the formation of *m/z* 525 and *m/z* 581 fragment ions, respectively. In the CA spectra of I_a, I_b, II_a and II_b the ion flux for alkenoic acid elimination is divided into two reaction channels. Collisional activation of $[I_a + 2Na - H]^+$ shows the loss of $C_7H_{13}COOH$ leading to *m/z* 525 and the loss

Table 1. CA spectra of the $[M + 2Na - H]^+$ cations of I_a, I_p, I_b, II_a, II_p, II_b. Relative abundances of the fragment ions are given in parenthesis. RHA corresponds to (rhamnose $- H_2O$]. FA is $C_nH_{2n-1}COOH$, $n = 7, 9, 11, 13$ or 15.

Cation	I_a	I_p	I_b	II_a	II_p	II_b
M + 2Na − H	667	695	723	779	807	835
M + 2Na − H − RHA	521 (36)	549 (25)	577 (45)	633 (6)	661 (23)	689 (7)
M + 2Na − H − FA	525 (655)	525 (1000)	553 (596)	581 (1000)	581 (1000)	609 (1000)
	497 (1000)		525 (1000)	553 (928)		581 (421)
M + 2Na − H − 2RHA	375 (15)	403 (11)	431 (10)	487 (24)	515 (2)	543 (2)
M + 2Na − H − 2RHA − H_2O	357 (48)	385 (32)	413 (24)	469 (49)	497 (34)	525 (8)
M + 2Na − H − RHA − FA	379(50)	379 (23)	407 (63)	435 (98)	435 (26)	463 (4)
	351 (60)		379 (70)	407 (98)		435 (2)
M + 2Na − H − 2RHA − FA	233 (27)	233 (18)	261 (31)	289 (49)	289 (23)	317 (2)
	205 (13)		233 (37)	261 (32)		289 (1)
M + 2Na − H − 2RHA − FA − H_2O	215 (39)	215 (36)	243 (46)	271 (42)	271 (40)	299 (5)
	187 (40)		215 (46)	243 (42)		271 (7)



Scheme 1. Collisionally induced fragmentation reactions of (a) $[I_p + 2Na - H]^+$ cations and (b) $[I_p - H]^-$ anions. The *m/z* values and losses of the neutrals for the negative ion fragmentations are depicted in bold and italics.



Scheme 2.

of $C_7H_{13}COOH$ leading to *m/z* 525 and the loss of $C_9H_{17}COOH$ leading to the formation of *m/z* 497. Thus, it can be concluded thus I_a is a mixture of two isomeric rhamnolipids differing in the sequence of the β-hydroxy alkanoic acid Rha-Rha-C_{10}-C_8 and Rha-Rha-C_8-H_{10}, respectively. Similarly, the results in Table 1 show that I_b is a mixture of Rha-Rha-C_{10}-C_{12} (formation of *m/z* 525 ions) and Rha-Rha-C_{12}-C_{10} (formation of *m/z* 553 ions). Similar phenomena are observed in the CA spectra of II_a and II_b. Activation of II_a leads to the formation of *m/z* 581 and *m/z* 553 fragment ions by loss of C_{12} and C_{14} alkenoic acid neutrals, respectively, indicative of the presence of Rha-Rha-C_{14}-C_{12} and Rha-Rha-C_{12}-C_{14}. The spectrum of II_b reveals the presence of both Rha-Rha-C_{14}-C_{16} and Rha-Rha-C_{16}-C_{14} in RL II. The heterogeneity in β-hydroxy fatty acid sequence is also observed for other collisional induced fragmentations in which loss of the terminal fatty acid or formation of ions containing one of the β-hydroxy alkanoic acid are involved.

A mechanism for the loss of the alkenoic acid is proposed in Scheme 2. In the disodium compound the acid H-atom of the terminal β-hydroxy alkanoic acid is replaced by a sodium atom, a common reaction in the FAB ionization of fatty acids in the presence of sodium ions [13]. From proton affinity data it can be extrapolated that the second sodium cation is bound to one of the carbonyl O-atoms [14]. So an Na shift of the distal Na atom is required for elimination of the terminal fatty acid. The β-position of the hydroxyl group allows a sterically favourable six-membered ring transition state for a γ-sodium rearrangement. This γ-sodium rearrangement reaction is initiated by the localized positive charge on the acceptor O-atom and facilitated by the fact that in the course of the reaction the electrons remain paired. Generally, decomposition of even-electron ions, involving rearrangement reactions in which the charge site retains its location and the electrons have not to be unpaired, are favoured from an energetic point of view [15].

Another interesting fragmentation reaction which is abundant in the positive ion FAB-MS spectra and is present in the CA spectra of the $[M + 2Na - H]^+$ ions is the loss of $C_6H_{10}O_4$. In this reaction the glycosidic bond between the two rhamnose moieties is cleaved, accompanied by

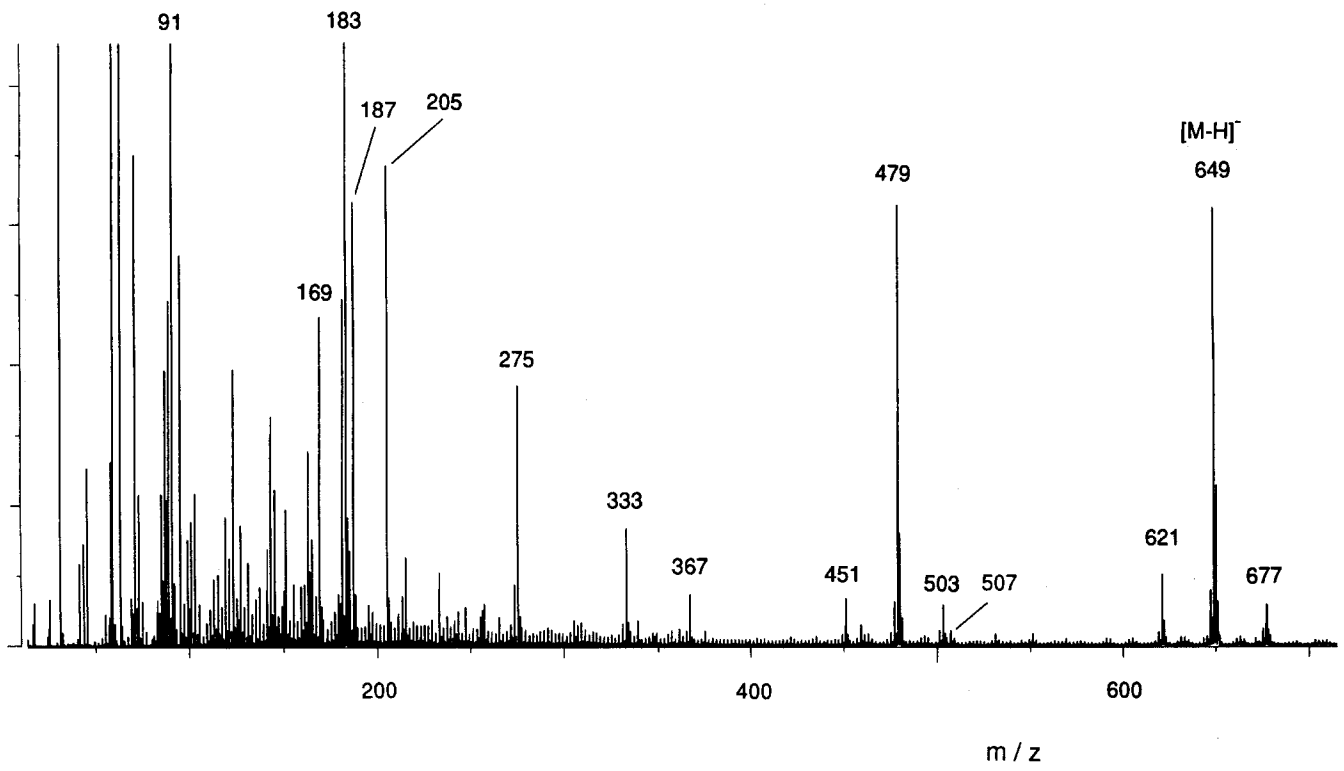


Figure 5. Negative ion FAB mass spectrum of RL I.

hydrogen transfer, with retention of the glycosidic O-atom in ions containing the aglycon of the rhamnolipid. This process is a common fragmentation reaction occurring in the mass and CA spectra of glycoconjugates [16]. In the nomenclature of Domon and Costello, these ions are assigned as Y fragment ions. Formally, the Y fragment ions result from protonation of the glycosidic bond, which is subsequently broken accompanied by a proton transfer yielding the Y ions. Assuming that the preferred site for cationization is the carbonyl O-atom of the rhamnolipid, cleavage of the glycosidic bond is not driven by cationization of the glycosidic O-atom and must be the result of a charge-remote fragmentation [13]. A similar glycosidic cleavage is observed upon collisional activation of sodium cationized sucrose esters [17]. Other examples of CID of carbohydrates [18] and glycosides [19, 20] in favour of a charge-remote fragmentation of the glycosidic bonds are known. The $[M + H]^+$ ions of many flavonoid and steroidal glycosides undergo cleavage of the glycosidic bond, where the site of protonation is the carbonyl oxygen of the flavonoid or steroidal glycoside [19, 20].

As can be seen in Fig. 3, the intensity of *m/z* 723, the $[I_b + 2Na - H]^+$ is only about 2–3 times the S/N ratio. Nevertheless, it appears possible to obtain a collisional spectrum containing structurally diagnostic ions not present in the normal FAB-MS spectrum by tandem mass spectrometry with a four sector instrument. Four sector MS/MS offers good possibilities for the structure analysis of even minor components in mixtures of closely related rhamnolipids, because it reduces background interference and allows the selective analysis of individual components.

The negative ion FAB-MS spectrum of RL I is presented in Fig. 5. Proton abstraction of I_p yields abundant $[M - H]^-$ anions at *m/z* 649. It is obvious that $[M - H]^-$ ions of I_a and I_b are also present at *m/z* 621 and *m/z* 677, respectively. Loss of the $C_9H_{17}COOH$ from the principal component I_p of the rhamnolipid isolate gives an abundant fragment ion at *m/z* 479. Similarly as in the positive ion spectra homologous fragment ions from the minor components I_a and I_b are found at *m/z* 451 and *m/z* 507. Other characteristic ions are *m/z* 503, $[I_p - C_6H_{10}O_4]^-$, *m/z* 333, $[I_p - C_6H_{10}O_4 - C_9H_{17}COOH]^-$, *m/z* 187 the β-hydroxy-tetradecane carboxyl anion and *m/z* 169 with the elemental composition of a tetradecane alkanoic acid. Similar results can be observed in the negative ion FAB-MS spectrum of RL II (Fig. 6). In both the negative ion FAB-MS spectra (Figs 5 and 6) an ion is present at *m/z* 205. The generation of this *m/z* 205 anion, which yields information about the linkage of the dirhamnose, will be discussed below.

The four sector MS/MS collision induced dissociation spectra of the $[M - H]^-$ anions of I_a, I_p, I_b, II_a and II_b are presented in Table 2. For all homologues the same fragmentations are observed. The fragmentation reactions of I_p $[M - H]^-$ anions are visualized in Scheme 1. As is seen in Scheme 1, CA of the rhamnolipid anions gives sequence information of the building blocks. The most abundant collisional induced dissociation fragment results from elimination of the distal β-hydroxy alkenoic acid with

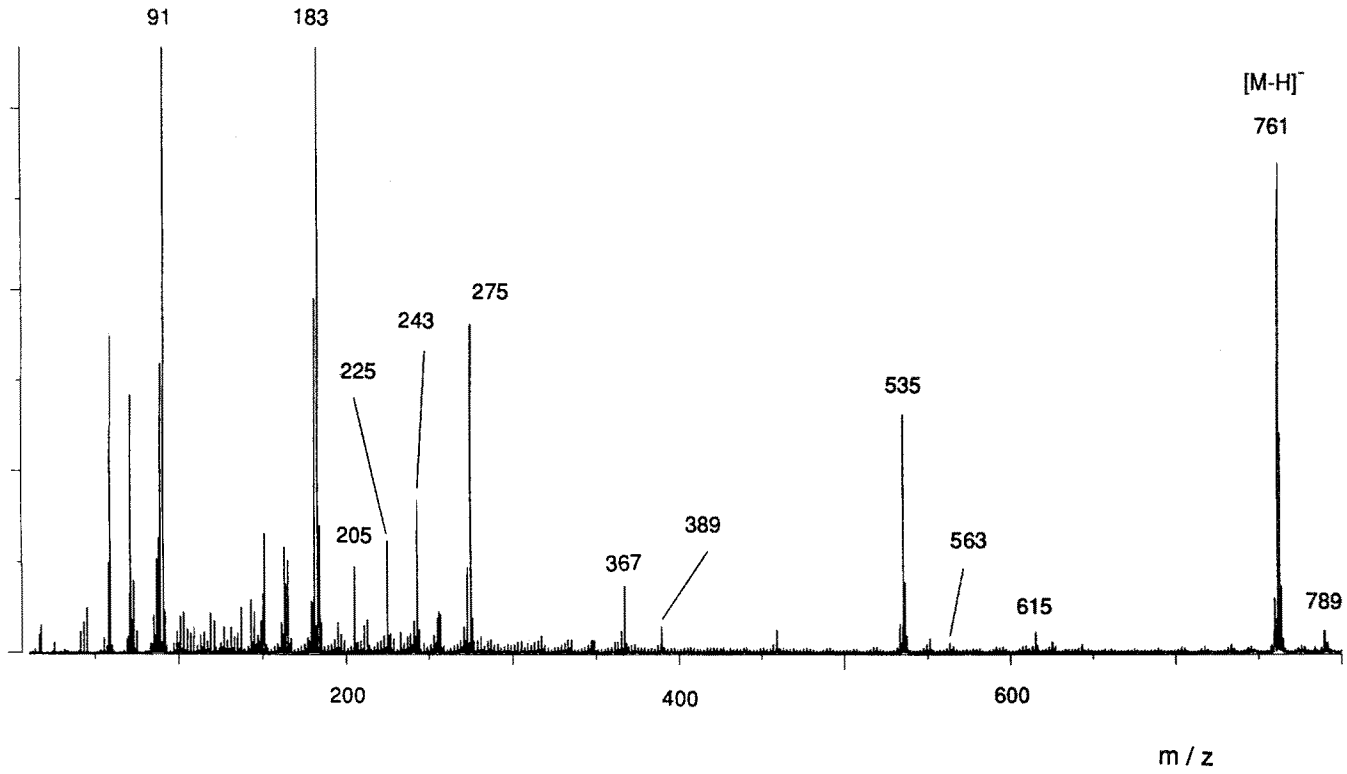


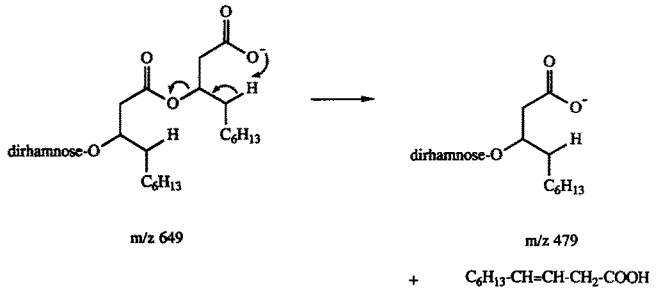
Figure 6. Negative ion FAB mass spectrum of RL II.

Table 2. CA spectra of the $[M - H]^-$ anions of I_a, I_p, I_b, II_a, II_p, II_b. Relative abundances of the fragment ions are given in parentheses. RHA corresponds to [rhamnose $- H_2O$]. FA is $C_nH_{2n-1}COOH$, $n = 7, 9, 11, 13$ or 15.

Anion	I_a	I_p	I_b	II_a	II_p	II_b
M – H	621	649	677	733	761	789
M – H – RHA	475 (58)	503 (21)	531 (35)	587 (47)	615 (26)	643 (32)
M – H – FA	479 (753)	479 (1000)	507 (529)	535 (1000)	535 (1000)	563 (1000)
	451 (1000)		479 (1000)	507 (624)		535 (458)
M – H – 2RA	329 (20)	357 (7)	385 (3)	441 (5)	469 (12)	497 (10)
M – H – 2RA – H_2O	311 (88)	339 (73)	367 (109)	423 (157)	451 (40)	479 (126)
M – H – RHA – FA	333 (11)	333 (16)	361 (9)	389 (11)	389 (15)	417 (27)
	305 (14)		333 (20)	361 (5)		389 (2)
M – H – 2RHA – FA	187 (74)	187 (62)	215 (65)	215 (13)	243 (82)	271 (61)
	159 (31)		187 (41)	243 (117)		243 (58)
M – H – 2RHA – FA – H_2O	169 (96)	169 (110)	197 (113)	225 (222)	225 (176)	253 (132)
	141 (53)		169 (128)	197 (117)		225 (81)
	205 (228)	205 (109)	205 (165)	205 (173)	205 (89)	205 (72)

retention of the O-atom in the remaining glycolipid anion. A mechanism for this elimination reaction is proposed in Scheme 3. Again, from this type of fragmentation the presence of the fatty acid sequence isomers of I_a, I_b, II_a and II_b can be concluded. These negative ion experiments corroborate successfully the conclusions drawn from the collision experiments of the disodium rhamnolipids.

An interesting peak in all the negative ion spectra of the molecular anions $[M - H]^-$ is *m/z* 205 (see Table 2). This fragment ion is indicative of the 1-2 linkage of the rhamnoses in the glycolipids. It has been found by Ballistreri *et al.* [21] and Garozzo *et al.* [22] that linkage positions in small linear oligosaccharides can be determined by negative ion FAB of the native compounds. The authors stated that the negative ionization is believed to occur by selective deprotonation of the free (reducing) anomeric hydroxyl group, which is more acidic than to the other OH groups. Once the negative charge is localized at the oligosaccharide reducing end, fragmentation of this ring occurs and the mass losses are found to be diagnostic of the glycoside linkage with the



Scheme 3.

adjacent sugar unit. The presence of *m/z* 221 $[M - H - 120]^-$ in the spectrum of sophorose Glcα1-2Glc) allowed the discrimination between this glucobiose and glucobioses with other glycosidic linkages. By means of labelling experiments, precursor ion scans and CA mass spectroscopy, Dallinga *et al.* [23] were able to elucidate some of the reactions and mechanisms involved in the FAB fragmentation of $[M - H]^-$ ions of several dialdohexopyranoses. However, it appeared that *m/z* 221 is also found in the CA spectra of 1,4-linked glucose dimers. This fragment ion is absent in spectra of 1,3-linked dimers. A plausible route of formation of *m/z* 205 fragment ions in the negative ion spectra (illustrated for Rha-Rha-C_{10}-C_{10} anions) is given in Eqn (1). Generation of *m/z* 205 anions by mechanisms are depicted by several authors [21–23] requires first the formation of *m/z* 309 rhamnobiose anions. The abundance of these ions is small (Figs 5 and 6). The relatively low abundance of these *m/z* 309 ions in the spectra (Figs 5 and 6) can be rationalized by assuming that the final reaction step in Eqn (1), the combined losses of $C_2H_4O_2$ and C_2H_4O [23], is fast with respect to the formation of rhamnobiose anions. The rhamnobiose anions are most likely the result from consecutive eliminations (Scheme 3) of alkenoic acid molecules.

$$[M - H]^- \xrightarrow[-C_{10}H_{18}O_2]{} m/z\ 479 \xrightarrow[-C_{10}H_{18}O_2]{} m/z\ 309 \xrightarrow[-C_2H_4O_2\ -C_2H_4O]{\text{Fast}} m/z\ 205 \quad (1)$$

So, reasoned by analogy, the fragment ion at *m/z* 205 in the spectra of our rhamnolipids may be indicative of a 1,2- or 1,4-linkage between the rhamnose molecules.

In conclusion, FAB in combination with tandem mass spectrometry provides detailed information on the structure of natural rhamnolipids. Addition of NaCl to the glycerol matrix gives rise to formation of abundant $[M + 2Na - H]^+$ cations and, to a lesser extent, $[M + Na]^+$ and $[M + 3Na - 2H]^+$ cations, from which information about the molecular mass of the rhamnolipids can be derived. Tandem mass spectrometry of the $[M + 2Na - H]^+$ cations gives information about the sequence of the building blocks (Scheme 1). Particularly, the β-hydroxy fatty acid sequence can be determined for rhamnolipids containing dimers of β-hydroxy fatty acids with unequal alkyl chain lengths. Similar information can be derived from the negative ion spectra and more in particular from the collisional induced fragmentation behaviour of the $[M - H]^-$ molecular anions (Scheme 1). As rhamnolipids from biological sources often contain mixtures of structural homologues resulting from heterogeneity in the lipophilic part of the molecules, CA of the $[M + 2Na - H]^+$ cations or $[M - H]^-$ anions is of direct interest for the structural analysis of natural rhamnolipid isolates.

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