Analysis of intact bacteriohopanepolyols from methanotrophic bacteria by reversed-phase high-performance liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry

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Abstract

Direct detection of most intact biohopanoids is not possible using conventional GC–MS techniques due to their highly functionalised and amphiphilic nature. Here we report the application of a new reversed-phase high-performance liquid chromatography method for the direct analysis of acetylated, intact bacteriohopanepolyols in solvent extracts of methanotrophic bacteria. Atmospheric pressure chemical ionisation mass spectrometric detection provides structural information relating to the number and types of functional groups present in the four biohopanoids detected: bacteriohopanetetrol, aminobacteriohopanetriol, -tetrol and -pentol. The method should facilitate the assessment of hopanoid composition of both bacteria and environmental samples. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Bacteria; Bacteriohopanepolyols; Hopanoids; Triterpenoids; Terpenoids

1. Introduction

The hopanoids (see Fig. 1 for examples) are triterpenoids which are biosynthesised by many bacteria (e.g. [1,2]) as cell membrane stabilising components [3]. Their pentacyclic carbon skeleton is relatively resistant to degradation thus allowing their preservation in soils and sediments and ultimately (as hopanes and other stable degradation products) in the geological record. Accordingly they have been described as "the most abundant natural products on Earth" [4]. Despite the ubiquity of these "chemical fossils" in the geosphere, relatively little is known about the structures and composition of their precursors (the biohopanoids) in the original source biota, and particularly their composition and preservation in modern sedimentary environments. The processes involved in their diagenesis are also poorly understood and consequently the full potential for

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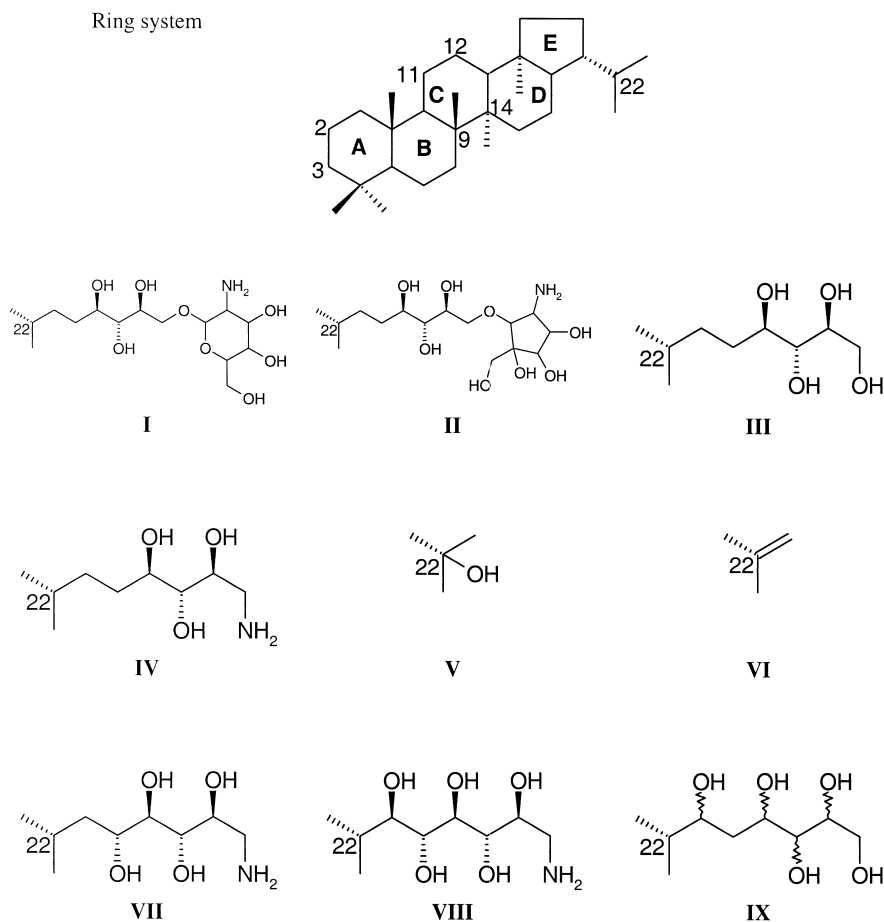*E-mail address:* h.m.talbot@ncl.ac.uk (H.M. Talbot).

Ring system

Fig. 1. Hopanoid structures.

use of hopanoids as biomarkers of bacterial activity and population structure has not yet been recognised.

Characterisation of individual biohopanoids is problematical as they are highly functionalised and amphiphilic, and are not readily amenable to analysis by conventional GC–MS methods (e.g. [5]). The most important biohopanoids, the bacteriohopanepolyols, have four, five or six functional groups on their side chains, and include "composite" hopanoids wherein the terminal functional group is a more complex biochemical moiety (e.g. Fig. 1, **I** and **II**). Of the bacteriohopanepolyols, only the tetra-acetates of bacteriohopanetetrol (BHT from herein; **III**) and aminobacteriohopanetriol (aminotriol from herein; **IV**) are known to be amenable to analysis by GC–MS [5,6]. To circumvent this problem periodic acid has been employed to oxidise the

1,2-diols yielding aldehyde products which are then reduced to terminal alcohols using sodium borohydride [1]. This procedure converts the polyhydroxylated hopanols to simple primary alcohols with shortened side chains (Fig. 2), the structures of the products being related to the number and configuration of the hydroxyl groups in the precursor. The hopanol product can then be quantified by GC–MS after acetylation. Although this technique can provide valuable information regarding the abundance of tetra-, penta- and hexafunctionalised components it is of limited use in the determination of the exact nature of the functionalities as the majority are lost during the side-chain cleavage. More specific structural information is typically obtained by isolation of the product from an acetylated total extract of a bacterium using methods such as TLC and prepara-
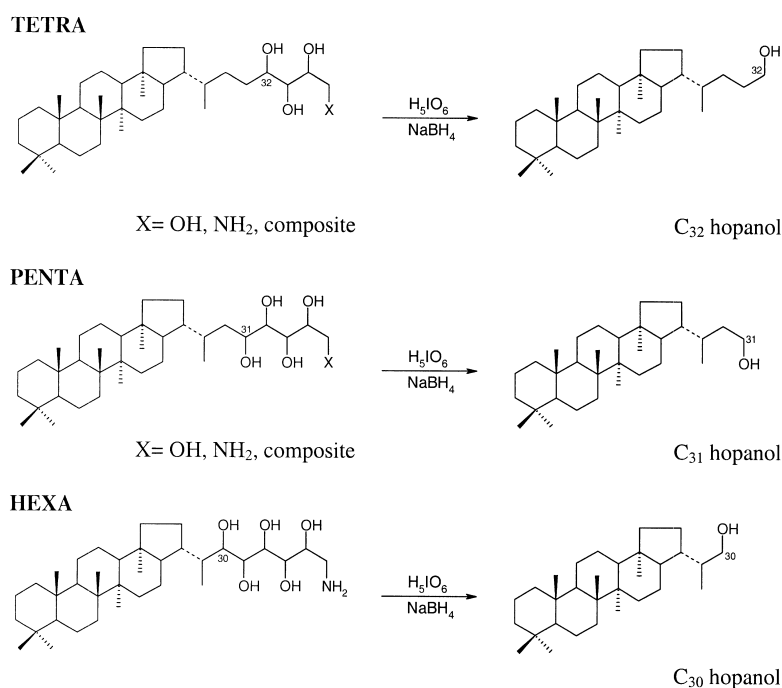
Fig. 2. Periodic acid/sodium borohydride reactants and products.

tive HPLC followed by NMR and probe MS (e.g. [7,8]). The development of an analytical scheme capable of characterising intact biohopanoids in complex mixtures is therefore required to allow the biohopanoid compositions in both bacterial extracts and recent sedimentary environments to be assessed.

The use of HPLC for the direct separation and determination of intact biohopanoids has been described previously in a small number of studies [9–11]. Each of these methods was based on the analysis of the composite biohopanoids of the gram-negative bacterium *Zymomonas mobilis*, previously reported to contain high amounts of BHT (**III**), BHT-glycoside (**I**) and BHT-cyclitol ether (**II**) along with minor amounts of diplopterol (**V**) and diploptene (**VI**) [12]. The earliest of these studies [9] described a reversed-phase HPLC method utilising a C_{18} bonded phase and a tertiary solvent system in which acetonitrile was gradually added to a water–methanol mixture. After acetylation of the total extract all five components were separated and detected using absorbance detection at 206 nm. The second study [10] employed normal-phase HPLC with a silica column and a solvent system comprising

hexane, propan-2-ol and water (+0.004% triethylamine) with flame ionisation detection. This method, itself adapted from an earlier method [13] which was devised to examine different lipid classes in nitrogen-fixing root nodules and *Frankia* cells, proved successful in detecting the three composite hopanoids but not the two C_{30} components diplopterol and diploptene. The most recent study [11] utilised the same normal-phase HPLC conditions but employed negative ion chlorine addition atmospheric pressure chemical ionisation (APCI) for detection. The chlorinated species produced from a polyhydroxylated species are very stable and thus readily detectable even at low concentrations [14]. Furthermore, comparison of the results of the bacterial extract with those obtained for a recent sediment extract (Priest Pot, UK) revealed the presence of two of the bacterial components, BHT and BHT-cyclitol ether, in the sediment and it was postulated that these compounds may account for the high abundance of tetrafunctionalised components known to occur in sediments of this lake [15].

Following the pilot study we have further developed an HPLC-based method for the characterisa-

tion of hopanoids in both bacterial and sedimentary extracts. Initial results from methanotrophic bacteria, known to contain abundant aminobacterio-hopanepolyols, using the normal-phase HPLC method [11] showed that the polyhydroxylated components containing a single amino group were strongly retained resulting in extended analysis times and poor peak shapes (Talbot, unpublished results).

As the negative ion chlorine addition method used in the pilot study provided molecular mass information only, we have now investigated the use of positive ion APCI to provide structural information on individual hopanoids. Unequivocal identification of hopanoid structure is necessary for the analysis of more complex environments in which previously unknown hopanoids are expected to occur. For example, in a recent review [15] regarding the hopanoid composition of bacteria, it was shown that out of a total of 136 strains and species from a total of 10 different bacterial phyla ca. 50% contained hopanoids. The number of bacteria studied from each of the 10 classes varied considerably and at least 11 other bacterial phyla are yet to have any species analysed for hopanoid content. Of those species that have been analysed for the presence of hopanoids, only a fraction have had their intact structures fully elucidated.

This paper describes recent work using a new RP-HPLC method adapted from that of Schullenberg-Schell et al. [9] with positive ion APCI-MS detection allowing the direct detection of intact BHT and three aminobacteriohopanepolyols in six methanotrophs. These results are compared with those obtained by the existing GC–MS-based method [5]. It is hoped that the method described will facilitate the rapid assessment of hopanoid composition in a large number of bacterial species and in recent sediments thus addressing the current shortfall in the understanding of hopanoid chemistry.

2. Experimental

2.1. Samples

Samples of the Type I methanotrophic bacteria *Methylomicrobium album* (BG8), *Methylococcus capsulatus* (Bath), *Methylomonas methanica* (S1)

and *Methylomonas rubrum*, and the Type II methanotrophs *Methylocystis parvus* (OBBP) and *Methylosinus trichosporium* (OB3b) were from The University of Warwick culture collection and were grown on a nitrate mineral salts medium with methane as the carbon source as described by Murrell and Dalton [16]. Samples were provided as cell suspensions in 20 mM Tris–HCl buffer, pH 6.8. The buffer was removed by repeated dilution and centrifugation in Milli-Q grade water. Cells were then freeze-dried prior to extraction.

2.2. Extraction

Freeze-dried cells (30–60 mg) were extracted using a Gerhardt Soxtherm apparatus with chloroform–MeOH (180 ml; 2:1, v/v). Extracts were evaporated to near dryness, transferred to a pre-weighed vial and evaporated under a stream of nitrogen. Internal standards (5α-androstane and 5α-androstan-3β-ol) were added for quantitation during GC–MS analysis. After redissolving in warmed chloroform–methanol (2:1, v/v) the total extract was split into aliquots (2–5 mg) for derivatisation by methods targeted at specific groups [5].

2.3. Periodic acid and sodium borohydride treatment

The full procedure has been described elsewhere [5]. Briefly, the extract was stirred at room temperature for 1 h with periodic acid (H_5IO_6; 300 mg) in tetrahydrofuran–water (3 ml; 8:1, v/v). Water (10 ml) was added and the mixture extracted with light petroleum, boiling point 40–60°C (15 ml; $\times$3). The combined extracts were rotary evaporated to dryness then the resulting extract was stirred for 1 h at room temperature with sodium borohydride ($NaBH_4$; 100 mg) in ethanol (3 ml) to produce terminal alcohols (Fig. 2). After careful addition of potassium dihydrogenphosphate (KH_2PO_4; 15 ml; 100 mM) the mixture was extracted with petroleum ether (15 ml; $\times$3). The extract was then rotary evaporated to near dryness and transferred to large vials for acetylation of the hopanols by the procedure described below. The acetylated hopanols were analysed by GC–MS only.

2.4. Acetylation

Aliquots of the total extract and the products of the periodic acid treatment (hopanols) were analysed as their acetate derivatives, formed by heating with acetic anhydride–pyridine (4 ml; 1:1, v/v) at 50°C for 1 h and leaving at room temperature overnight. Each derivatised extract was rotary evaporated to dryness and treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; heated at 50°C for 1 h) prior to GC–MS analysis.

2.5. High-performance liquid chromatography

Initial method development was carried out using a Gilson ternary solvent delivery system fitted with a Jones Chromatography (Hengoed, UK) Genesis C_{18} (150×4.6 mm, 4 µm) column and C_{18} (10×4.6 mm) guard column. Chromatography was achieved at ambient temperature with a flow-rate of 1 ml/min and the following gradient profile: A–B (90:10) (0–5 min); A–B–C (40:1:59) (at 45 min) then isocratic to 70 min [where A=MeOH, B=water and C=propan-2-ol (IPA); A and C are Fisher HPLC grade; B is Milli-Q grade]. Samples (acetylated total extracts) were injected in MeOH–IPA (60:40) via a Rheodyne 7125 injector fitted with a 20 µl loop. Detection was achieved with a Sedex 55 evaporative light scattering detection (ELSD) system, with the nebulizer gas (air) at 2.5 bar and evaporator temperature of 85°C.

2.6. Liquid chromatography–mass spectrometry

The HPLC system comprised a Waters (Watford, UK) MS 600 silk quaternary LC system. Column and gradient composition were as described above for use with ELSD. Solvents were Rathburn HPLC grade. The LC system was linked to a Finnigan MAT TSQ 700 triple quadrupole mass spectrometer via a Finnigan MAT APCI interface operated in positive ion mode. Interface and mass spectrometer instrument parameters were optimised during repeated infusion into the source of a solution of BHT standard in MeOH–IPA (60:40, v/v) at 1 ml/min. Final optimised conditions were: vaporiser 400°C; capillary 150°C; corona 8 µA; capillary and octapole offset voltages of 50 and −2.1 V, respectively;

nitrogen sheath gas pressure 80 p.s.i. and auxiliary gas pressure 2 p.s.i. (1 p.s.i.=6894.76 Pa). Data were recorded by scanning from *m/z* 150 to 900 or 400 to 1200 in 2 s.

The HPLC-based results are non-quantitative as standards were available for only two of the compounds of interest and we were unable to calculate response factors for each component.

2.7. Gas chromatography–mass spectrometry

GC–MS analysis of the acetylated total extracts and periodic acid treatment products was conducted on a Hewlett-Packard 5890 II GC system (split/splitless injector; 350°C) linked to a Hewlett-Packard 5972 mass-selective detector (electron energy 70 eV; filament current 220 µA; source temperature 270°C; multiplier voltage 2000 V; interface temperature 350°C). A DB5-HT column (15 m×0.25 mm I.D.; 0.1 µm film thickness) was used with helium as the carrier gas. The oven temperature was programmed from 50 to 200°C at 15°C/min (held for 1 min), from 200 to 250°C at 10°C/min (held for 1 min) and from 250 to 350°C at 5°C/min (held for 8 min). The mass spectrometer was operated in full scan mode (*m/z* 50–700; 1.1 s).

3. Results and discussion

3.1. Optimisation of chromatography

Authentic standards were only available of BHT and aminotriol. Initially we examined the two underivatised standards using the NP-HPLC method described previously [11]. Although BHT showed good chromatographic performance the aminotriol was strongly retained, eluting significantly later than BHT as a broad, tailing peak. Examination of several of the bacterial extracts using this method revealed that all three amino derivatives demonstrate similar chromatographic behaviour (Talbot, unpublished results). The addition of acetic acid (from 0.05 to 1%) to the aqueous phase improved peak shape and reduced retention times but caused all three amino species to co-elute even with the lowest amount of acid. This also increased the overall analysis time as additional equilibration time was required to ensure

complete removal of water and acid prior to beginning a subsequent run.

We then examined the two underivatised standards using an adaptation of the method of Schullenberg-Schell et al. [9]. Although BHT eluted after about 20 min as a sharp peak the aminotriol standard was unretained and eluted with the solvent peak. Modifying the polarity of the starting solvent composition by the addition or removal of water failed to result in this standard being retained.

In contrast, the acetylated standards were retained by the column and demonstrated similar retention characteristics. Initial trials with bacterial samples, however, revealed that a number of the more strongly retained components eluted as broad or tailing peaks. Replacing acetonitrile with propan-2-ol was found to significantly improve the chromatographic performance. Typical chromatograms for the acetylated standards and an example bacterium, *Methylosinus trichosporium*, are shown in Fig. 3. This species has been reported to contain the tetra-functionalised aminotriol and pentafunctionalised

aminobacteriohopanetetrol (aminotetrol from herein; **VII**) in significant abundance [17].

3.2. Optimisation of mass spectrometry

Whilst the negative ion chlorine addition technique used during the pilot study is sensitive when examining known polyhydroxylated species it provides little structural information with the spectra being dominated by a single chlorinated molecular ion $[M+Cl]^-$. We have previously shown the negative ion APCI-MS spectrum of BHT with a chlorinated molecular ion at m/z 581 [11] and similar studies have shown that the spectrum of the aminotriol standard is also dominated by the chlorinated molecular ion of m/z 580 (not shown).

Positive ion APCI mass spectral analysis of an authentic standard of underivatised BHT (Fig. 4a) produced some characteristic ions including the base
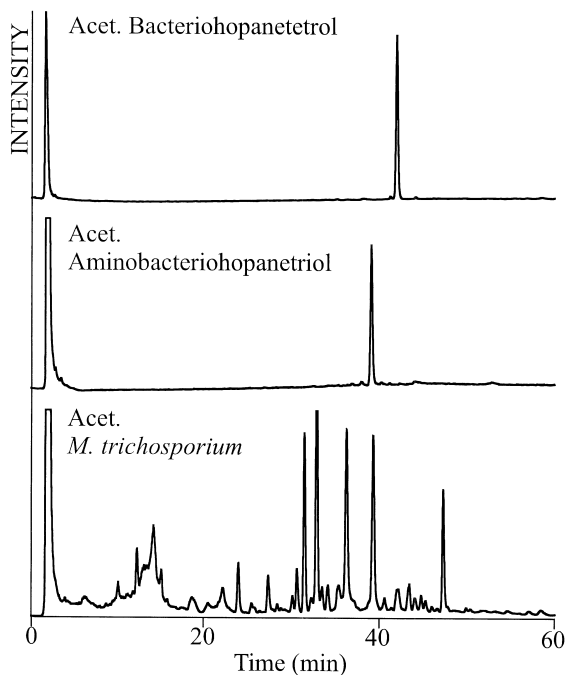
Fig. 3. RP-HPLC–ELSD chromatograms of acetylated standards of BHT (ca. 4 μg) and aminotriol (ca. 2 μg), and of acetylated total extract of *Methylosinus trichosporium* (ca. 400 μg).
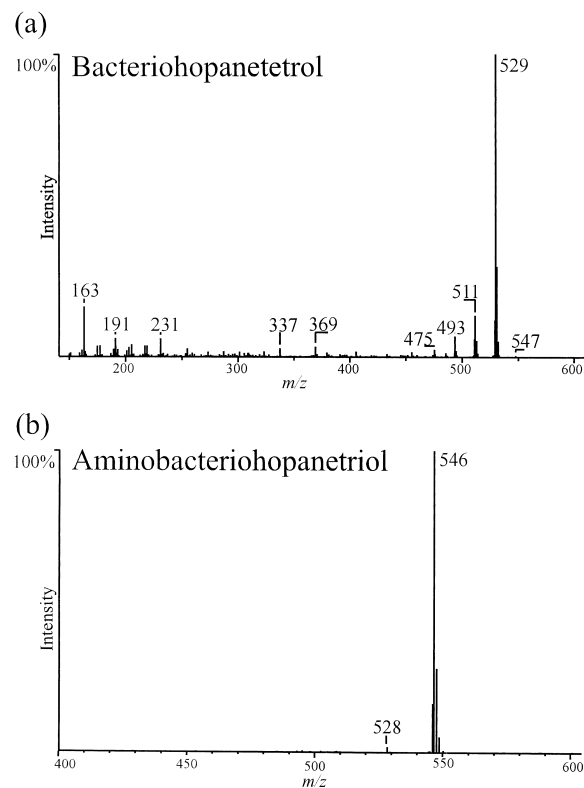
Fig. 4. Positive ion APCI mass spectra of (a) BHT and (b) aminotriol.

peak at m/z 529 showing loss of H_2O from the protonated molecule $[M+H]^+$ at m/z 547. Loss of the neutral species (H_2O) to produce a stable, even electron cation $[M+H-H_2O]^+$ is energetically more favorable than the equivalent cation ($[M+H]^+$). Three further losses of m/z 18 consistent with consecutive losses of the three remaining hydroxy functionalities (m/z 511, 493 and 475) are also observed. The ion at m/z 369 represents the hopanoid ring system after loss of the entire side chain. The ions between m/z 150 and 250 indicate cleavage in ring C of the hopanoid pentacyclic system. Under electron ionisation conditions cleavage in ring C occurs between carbons 10 and 11 and 9 and 14 producing the highly abundant fragment at m/z 191. Under the APCI conditions employed here the most abundant ring cleavage fragment is seen at m/z 163 presumably resulting from cleavage between carbons 11 and 12 as well as 9 and 14. Other ring cleavage fragments are present at m/z 177, 191 and 205.

Under identical conditions the mass spectrum observed for a standard of aminotriol (Fig. 4b) showed considerably less fragmentation and no ions indicative of the ring system. The protonated molecule (m/z 546) is also the base peak and the only observable fragmentation is m/z 529 indicating loss of a single OH group demonstrating that the basic nature of the amino functionality leads to ready formation of an ammonium ion and this protonated species is more stable under these conditions than the protonated BHT ion. Therefore, due to the differing chemistries of the molecules involved it was not possible to produce conditions in the interface which would favour production of $[M+H]^+$ as the base peak in the spectra of both molecules.

Analysis of tetraacetylated BHT (Fig. 5a), under identical positive ion APCI conditions to those used for the unacetylated species, produces similar fragmentation in the side chain with the base peak (m/z 655) resulting from loss of one functional group as acetic acid (i.e. loss of m/z 60). This was again due to the energetically more favorable production of an even electron cation (see above). The protonated molecule (m/z 715) is present in very low abundance. An ion at m/z 775 $[M+H+60]^+$ is thought to be an association ion formed between the protonated molecule and a free acetic acid in the interface.
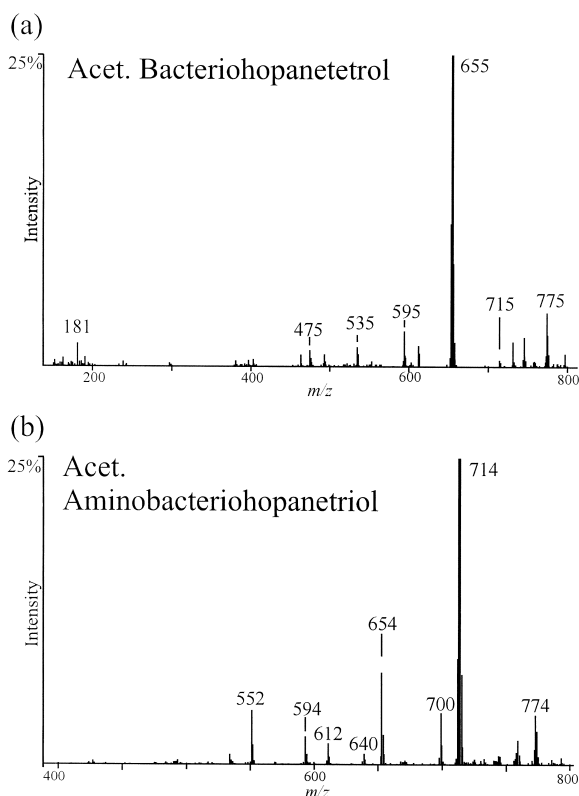

Fig. 5. Positive ion APCI mass spectra of tetraacetylated (a) BHT and (b) aminotriol.

Increasing the temperature of the ion transfer capillary was found to reduce the abundance of this ion due to desolvation and breakage of cluster ions. Further fragmentations of the side chain reveal the presence of the remaining three functional groups, each lost as acetic acid, producing ions of m/z 595, 535 and 475. Losses of m/z 42 were also observed and assigned as loss of a partial fragment of the acetyl group $[CH_3CO]$, with the addition of a hydrogen ion. Full assignments of observed ions are given in Table 1.

The spectrum of the tetraacetylated aminotriol (Fig. 5b) was also similar to that of its unacetylated form with the base peak again being the protonated molecule (m/z 714). The association ion at $[M+H+60]^+$ observed in the BHT spectrum was again present (m/z 774). Loss of functional groups was evident as three sequential losses of acetic acid with the terminal acetamido (CH_3CONH_2, m/z 59) group

Table 1
Assignments of mass spectral fragment ions of peracetylated BHT (**III**), aminotriol (**IV**), aminotetrol (**VII**) and aminopentol (**VIII**)

Fragment	**III**	**IV**	**VII**	**VIII**
$[M+H+AcOH]^+$	775	774	–	–
$[M+H]^+$	715	<u>714</u>	<u>772</u>	<u>830</u>
$[M+2H-Me]^+$	–	700	758	816
$[M+2H-Ac]^+$	–	–	730	787
$[M+H-AcOH]^+$	<u>655</u>	654	712	770
$[M+2H-AcOH-e]^+$	–	640	–	–
$[M+2H-AcOH-Ac]^+$	613	612	670	728
$[M+H-2(AcOH)]^+$	595	594	652	710
$[M+2H-2(AcOH)-Ac]^+$	553	552	610	668
$[M+H-3(AcOH)]^+$	535	534	592	650
$[M+2H-3(AcOH)-Ac]^+$	493	–	550	608
$[M+H-4(AcOH)]^+$	475		532	590
$[M+H-3(AcOH)-AcNH_2]^+$		475	–	–
$[M+2H-4(AcOH)-Ac]^+$			490	548
$[M+H-5(AcOH)]^+$				530
$[M+H-4(AcOH)-AcNH_2]^+$			473	–
$[M+H-5(AcOH)-Ac]^+$				489
$[M+H-5(AcOH)-AcNH_2]^+$				471

Underlined, base peak; –, not observed.

lost last. There were again no fragmentations indicative of the ring system. Full assignment of observed ions is given in Table 1.

3.3. Bacteria

Methanotrophic bacteria can be divided into two groups: Type I and Type II. The hopanoid compositions of these two phylogenetically different groups have been shown to be distinguished by particularly abundant hexafunctionalised components in Type I methanotrophs with Type II methanotrophs being dominated by tetra- and pentafunctionalised compounds [1].

Acetylated total extracts from the six methanotrophic bacteria were analysed using the RP-HPLC method with positive ion APCI-MS detection. Based on the mass spectrum for the aminotriol standard we expected to observe similar spectra for aminotetrol and aminobacteriohopanepentol (aminopentol from herein; **VIII**), i.e. both being dominated by the protonated molecular ion. Data for each extract were examined using mass chromatography to detect the presence of m/z 655 to indicate the base peak $[M+H-AcOH]^+$ for tetraacetylated BHT. Chromato-

grams at m/z 714, 772 and 830 were examined to indicate $[M+H]^+$ for aminotriol, -tetrol and -pentol, respectively. An example of these mass chromatograms are shown for *M. trichosporium* in Fig. 6. This Type II methanotroph has previously been reported to contain aminotriol and aminotetrol [17] but is here also shown to contain BHT and a trace of aminopentol. Although standards were not available for the aminotetrol and -pentol, interpretation of the mass spectra by comparison to that of the aminotriol revealed high similarity with the protonated molecule as the base peak and minor ions indicating the sequential loss of each of the acetyl groups followed
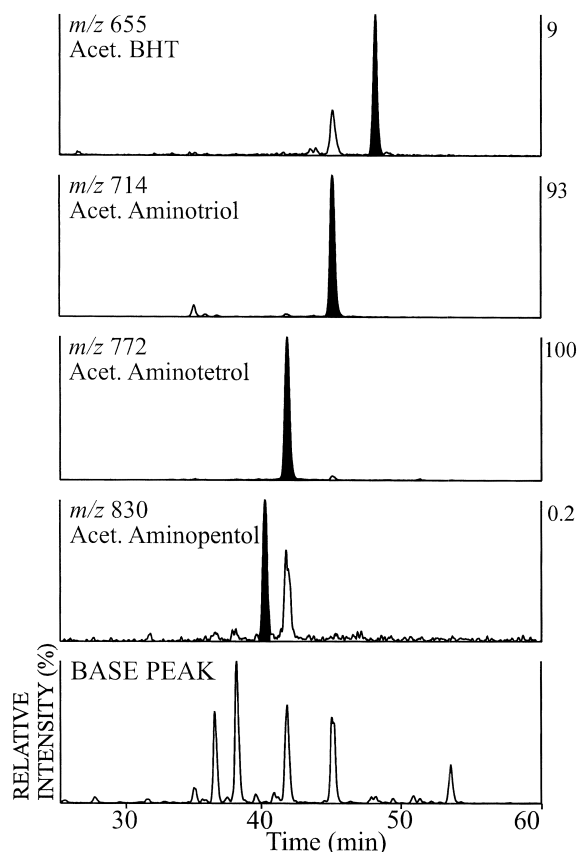

Fig. 6. Partial base peak (most abundant ion in each 2 s scan) and mass chromatograms indicating bacteriohopanepolyols in the acetylated total extract of *Methylosinus trichosporium*. (Key: m/z 655 = BHT $[M+H-AcOH]^+$; m/z 714 = aminotriol $[M+H]^+$; m/z 772 = aminotetrol $[M+H]^+$; m/z 830 = aminopentol $[M+H]^+$.)

by final loss of the terminal acetamido group. Full assignment of the observed fragment ions is given in Table 1.

Fig. 7 shows combined mass chromatograms (m/z $655 + 714 + 772 + 830$) which clearly indicate the presence of some or all of these four bacteriohopanepolyols in each of the six methanotrophs. *Methylococcus capsulatus* (Bath), whilst being phylogenetically related to Type I methanotrophs, does appear to have some properties of both Type I and Type II methanotrophs and is sometimes referred to as Type X. Here we observed high abundance of both the hexa- (**VIII**; Fig. 7a) and penta- (**VII**) functionalised components in cells of *M. capsulatus* (Bath) in agreement with its assignment as a Type I
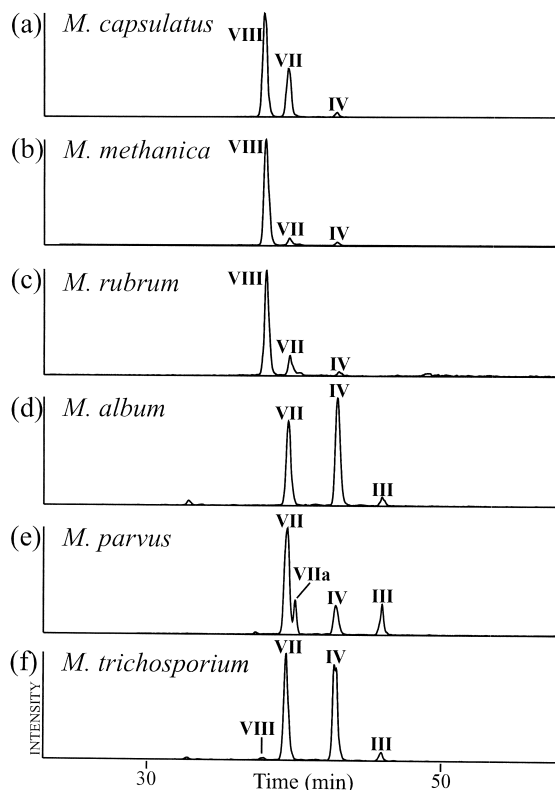

Fig. 7. Combined mass chromatograms (m/z $655 + 714 + 772 + 830$) indicating bacteriohopanepolyols in the acetylated total extract of methanotrophic bacteria. (Key: **VIII** = aminopentol; **VII** = aminotetrol; **VIIa** = possible second isomer of aminotetrol; **IV** = aminotriol; **III** = BHT.) *M. album*, identity uncertain (see text).

methanotroph, as well as a minor contribution from aminotriol (**IV**). Although this species has been shown to contain aminobacteriohopanepolyols substituted with a methyl group at position 3 [17] we found no evidence of 3-Me hopanoids in this or any of the other species examined. This may be due to differences in the growth conditions used to culture the bacterium, an effect known to alter the hopanoid composition in other bacteria [18,19].

Methylomonas methanica is also a Type I methanotroph previously reported to contain all three desmethyl aminobacteriohopanepolyols in addition to the 3-Me counterparts of aminotetrol and aminopentol [7]. Our results show the distribution to be clearly dominated by the hexafunctionalised component (Fig. 7b) with very minor contributions from aminotriol and aminotetrol. *Methylomonas rubrum* (Fig. 7c) has not previously been examined for hopanoids but like all *Methylomonas* species is a Type I methanotroph (e.g. [20]) and our results correspond to this assignment with aminopentol (**VIII**) being significantly more abundant than aminotetrol (**VII**) or -triol (**IV**).

Methylomicrobium album (previously *Methylomonas albus*) has been screened for the presence of hopanoids by the periodic acid method but not with full, unambiguous characterisation of the individual components [1]. This species has been assigned as a Type I methanotroph, however the results shown here are indicative of a Type II species. The reason for this apparent discrepancy is unclear but may result from a contaminated culture; i.e. this sample was not *M. album* (Murrell, pers. comm.). The distribution was dominated by aminotriol and -tetrol along with only a minor contribution from BHT (**III**; Fig. 7d) and with the hexafunctionalised component being absent. A hexafunctionalised component was indicated by the GC–MS results and may not have been detected due to lower sensitivity of the LC–MS system (see Section 3.4).

Methylocystis parvus, a Type II methanotroph, has previously been reported to contain BHT and aminotriol [21] as well as a pentafunctionalised component [1]. Here we found evidence of the pentafunctionalised aminotetrol together with a possible second isomer (**VIIa**; Fig. 7e) and the two tetrafunctionalised components reported previously. The nature of the second isomer of aminotetrol is unknown but

could be the C-35 aminofunctionalised counterpart of the unusual bacteriohopane-30,32,33,34,35-pentol (**IX**) reported in Ref. [22]. As for *M. album* there was no indication of the hexafunctionalised component in the LC–MS results, although it was indicated by GC–MS (see Section 3.4). The hopanoid distribution in *Methylosinus trichosporium*, another Type II methanotroph, was also dominated by aminotriol and aminotetrol, with apparently only minor contributions from BHT (**III**) and aminopentol (**VIII**) (Fig. 7f).

3.4. Comparison with GC–MS results

Quantification of the hopanoids was achieved using GC–MS analysis of the terminal alcohols produced by the periodic acid and sodium borohydride treatment (Fig. 2). The relative abundances
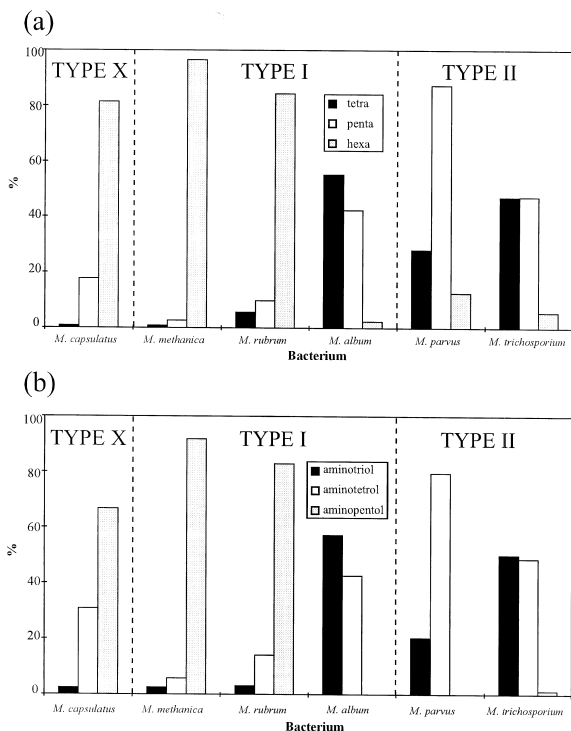
(a)

(b)

Fig. 8. (a) Relative abundance of total tetra-, penta- and hexa-functionalised hopanoids in methanotrophic bacteria calculated based on peak areas of m/z 191 GC–MS mass chromatograms. (b) Relative abundance of aminotriol, -tetrol and -pentol based on area of base peak ions in APCI mass chromatograms.

of the total tetra-, penta- and hexa-functionalised components are shown in Fig. 8a.

Although the LC–MS technique was not quantitative it was still possible to approximate the relative abundances of the three amino species by integrating the areas of peaks in the APCI mass chromatograms. BHT is excluded from the calculations as response factors are not yet known for the different components. Response factors for the three amino compounds are assumed to be the same for this first estimate using LC–MS measurements (Fig. 8b). By comparing the results shown in Fig. 8a and b it is apparent that the LC–MS data correlate extremely well with the GC–MS results, and all six species can be clearly distinguished from each other using the LC–MS method. Therefore, with the application of an appropriate internal standard and calculation of response factors this method should provide accurate quantitation as well as qualitative identification. The absence of the aminopentol from the LC–MS results for *M. album* and *M. parvus* indicates that in this preliminary study the LC–MS method may not be as sensitive as the GC–MS method. However, sensitivity would be improved by the use of selected ion monitoring (SIM) and this technique will be applied in future studies of both bacteria and environmental samples.

4. Conclusions

A new RP-HPLC–APCI-MS technique provides an effective method for the separation and analysis of certain polyfunctionalised hopanoids in bacterial extracts. Analyses are performed on acetylated total extracts without the need for further cleanup or fractionation. The use of mass chromatograms of the protonated molecular ions of peracetylated aminobacteriohopanetriol, -tetrol and -pentol (m/z 714, 772 and 830, respectively) is highly effective in the identification of these compounds along with BHT (m/z $655 = [M+H-AcOH]^+$) in six different species of methanotrophic bacteria.

Relative abundance measurements made using the HPLC–MS-based method are in good agreement with those from the established GC–MS method with all six species being clearly distinguishable, indicating that with the application of appropriate

standards this method should prove highly quantitative as well as qualitative.

Acknowledgements

References

[1] M. Rohmer, P. Bouvier-Nave, G. Ourisson, J. Gen. Microbiol. 130 (1984) 1137.

[2] R.E. Summons, L.L. Jahnke, J.M. Hope, G.A. Logan, Nature 400 (1999) 554.

[3] G. Ourisson, M. Rohmer, Curr. Top. Membr. Transp. 17 (1982) 153.

[4] G. Ourisson, P. Albrecht, Acc. Chem. Res. 25 (1992) 398.

[5] H.E. Innes, A.N. Bishop, I.M. Head, P. Farrimond, Org. Geochem. 26 (1997) 565.

[6] H.E. Innes, Ph.D. Thesis, University of Newcastle, Newcastle upon Tyne, 1998.

[7] S. Neunlist, M. Rohmer, Biochem. J. 231 (1985) 635.

[8] P. Simonin, J. Jürgens, M. Rohmer, Eur. J. Biochem. 241 (1996) 865.

[9] H. Schulenberg-Schell, B. Neuss, H. Sahm, Anal. Biochem. 181 (1989) 120.

[10] R.R. Moreau, M.J. Powell, S.F. Osman, B.D. Whitaker, W.F. Fett, L. Roth, D.J. O'Brien, Anal. Biochem. 224 (1995) 293.

[11] P.A. Fox, J.F. Carter, P. Farrimond, Rapid Commun. Mass Spectrom. 12 (1998) 609.

[12] S. Bringer, T. Härtner, K. Poralla, H. Sahm, Arch. Microbiol. 140 (1985) 312.

[13] A.M. Berry, R.A. Moreau, A.D. Jones, Plant Physiol. 95 (1991) 111.

[14] A.K. Ganguly, N.F. Cappuccino, Fujiwara, A.K. Bose, J. Chem. Soc., Chem. Commun. (1979) 148.

[15] P. Farrimond, P.A. Fox, H.E. Innes, I.P. Miskin, I.M. Head, Ancient Biomolecules 2 (1998) 147.

[16] J.C. Murrell, H. Dalton, Microbiology 129 (1983) 1187.

[17] S. Neunlist, M. Rohmer, J. Gen. Microbiol. 131 (1985) 1363.

[18] K. Poralla, T. Härtner, E. Kannenberg, FEMS Microbiol. Lett. 23 (1984) 253.

[19] A. Schmidt, B. Bringer-Meyer, K. Poralla, H. Sham, Appl. Microbiol. Biotechnol. 25 (1986) 32.

[20] J.P. Bowman, L.I. Sly, A.C. Hayward, Int. J. Syst. Bacteriol. 41 (1991) 301.

[21] M. Rohmer, Pure Appl. Chem. 65 (1993) 1293.

[22] P. Bisseret, M. Zundel, M. Rohmer, Eur. J. Biochem. 150 (1985) 29.