Hopanoids in Bacteria and Cyanobacteria – Their Role in Cellular Biochemistry and Physiology, Analysis and Occurrence

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Abstract: Hopanoids, widespread compounds of triterpene nature, are found mainly in different bacterial types (G+ and G- bacteria, aerobs, facultative anaerobs, strict anaerobs), in methanotrophs, acetic acid bacteria, nitrogen fixers, purple non-sulphur bacteria, cyanobacteria, and also in sediments. They occur in a variety of structures from simple hopanoids to elongated compounds with polyfunctional side chains such as bacteriohopanepolyols (BH-polyols). This review summarizes current knowledge of the occurrence of these compounds in different bacterial types, genera and species, their biosynthesis and functions in cellular membranes and in important biochemical and physiological processes, and surveys advances and trends in the field of hopanoid analysis, especially as concerns LC-MS with soft ionization techniques such as APCI and/or ESI.

Keywords: Bacteriohopanol, APCI, HPLC, bacteria, cyanobacteria.

1. INTRODUCTION

Compounds with hopane skeleton, i.e. bacteriohopanepolyols (BH-polyols), are an important subgroup of naturally occurring pentacyclic triterpenoids which are present as membrane constituents in certain bacteria [1,2]. They have been found in many bacterial groups including gram-negative and gram-positive bacteria, methanotrophs, cyanobacteria, acetic acid bacteria, nitrogen fixers and purple non-sulfur bacteria [3-6]. In an extensive literature survey, Talbot *et al.* [7] reported that over 280 pure cultures of bacteria representing at least 206 different species, 117 genera and 10 major groups or phyla have been tested for BH-polyol production.

Pearson *et al.* [8] have brought other interesting findings concerning the genetics of BH-polyol producers. Over 600 bacterial cultures have had their genomes sequenced, but only about 10 % were found to contain squalene-hopene cyclase genes that encode the enzyme squalene-hopene cyclase that catalyses cyclization of BH-polyols from their acyclic precursor, squalene. This suggests a lower frequency of BH-polyol biosynthesis among bacteria than originally expected [8].

Traditionally, BH-polyols have been regarded as being restricted to aerobic organisms [2,9], although the biosynthesis of BH-polyols in general does not require the presence of free oxygen [9,10]. Observations of BH-polyol biosynthesis in a range of facultative anaerobic bacteria, including photosynthetic purple non- sulfur bacteria or the fermentative *Zymomonas mobilis* [2,11,12] have brought a new view of its distribution in bacteria. Moreover, recent studies revealed the capability of BH-polyol synthesis in strictly anaerobic bacteria such as *Planctomycetes* performing the anaerobic oxidation of ammonium [12,13], iron-oxidizing *Geobacter* species [14,15] as well as sulfate-reducing *Desulfovibrio* species, which can tolerate some level of oxygen [16]. Elevated occurrence of BH-polyols in recent and fossil anoxic deposits indicates that additional groups of anaerobic bacteria are probably able to biosynthesize BH-polyols [16].

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No hopanoids have been found in *Archaea* [14,17,18]. This fact was confirmed by genetic analysis that showed that squalene-hopene cyclase gene is universally absent from the *Archea* and from known species of purple and green sulfur bacteria [8].

The occurrence of hopanoids in eukaryotic organisms is rather rare [5,16]. Simple hopanoids with a normal C_{30} triterpenic framework have been found in several scattered eukaryotic organisms (e.g. higher plants, ferns, mosses, lichens and fungi) [19].

BH-polyols have been isolated also from several marine sponges [20-22]. However, sponges are well-known for their symbiont microorganisms, which, in many cases, are the source of secondary metabolites. Thus, the real sources of the BH-polyols from sponges are very likely bacteria or cyanobacteria living in the sponge [21,22].

1.1. Biohopanol Structures

Hopanoid structures can be divided into two classes. The first class is represented by simple hopanoids, amphipathic molecules possessing a C_{30} quasi-planar polycyclic hopane skeleton (e.g. diploptene (**1a**)) [14,23]. The second class includes elongated hopane moiety resulting from the carbon-carbon linkage of the C_{30} hopane skeleton to a polyfunctionalized side chain derived from a carbohydrate [14]. The polar side chains modify the charge and function of the molecule [23]. The second class includes the most important BH-polyols, which have four, five or six functional groups on their side chains [19,24,25]. The terminal functional group can be a more complex moiety. These structures are called composite BH-polyols [19], in which the hydroxyl or amino group at C_{35} may be linked to diverse complex moieties such as an amino sugar (glucosamine or *N*-acylglucosamine), amino acid (tryptophan and ornithine) or other polar entities the structures of which are still unidentified [2,20,26-29].

Amongst BH-polyols have been found many other structural variations including double bonds in the ring system, methylations of ring A [2,26,27] and one or two additional OH group(s) at position(s) C_{30} and/or C_{31} [25,26]. These structures have been found in a limited number of organisms or in a specific group of organisms and can serve for specific bacterial marker information [2,26].

The most commonly occurring BH-polyols contain four functional groups, typically three hydroxyl groups at C_{32}, C_{33} and C_{34},

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with the C_{35} position occupied by either another hydroxyl (**2**) or an amino group (**3**), or a more complex group [2].

To date there has been only one hexafunctionalised side chain structure reported from a bacterium. This has five hydroxyl groups and a NH_2 functionality at C_{35} (**4a**), and has only been observed in Type I methanotrophic bacteria [19,26,30,31]. Although no other hexafunctionalised side chain structures have been reported from cultured organisms, Talbot and Farrimond [2] reported the occurrence of a BH-hexol (**5a**) in a Loch Ness sediment and a composite hexafunctionalised structure also in a sediment from Lake Druzhby (Antarctica).

BH-polyols are a complex group of biomarkers which occur ubiquitously in sediments [6]. Their pentacyclic carbon skeleton is relatively resistant to degradation, thus allowing their preservation in soils and sediments and ultimately in the geological record [19]. After the death of the organism, diagenetic processes modify the initial side chain composition of the biohopanoids, leading to the formation of geohopanoids including hopanoic acids, hopanols, hopenes and hopanoidal aldehydes and ketones. Compounds such as these represent intermediate stages in the diagenesis of the polyfunctionalised biohopanoid precursors to the hopanes, which are routinely analysed in ancient sedimentary rocks and petroleums [6].

These important biological markers (BH-polyols and their wholly or partially defunctionalised diagenetic products) record inputs of bacterial biomass to sediments and can therefore provide a record of palaeoenvironmental conditions at the time of deposition [1,4]. These compounds are among the oldest molecular biomarkers, dating back to late Archean times 2.77 billion years ago [16]. Bacterial triterpenoids of the hopane series are typical biomarkers for eubacteria [32].

In some cases, where no BH-polyols could be detected by chemical analyses of the cells, genome studies revealed nevertheless the presence of squalene cyclase, a key enzyme of the BH-polyol biosynthesis. At least two explanations can be found for this apparent contradiction. First, BH-polyols can be tightly bound to macromolecules escaping in this way chemical extraction. Second, the expression of the squalene cyclase can be activated or deactivated so that the cells only produce BH-polyols under peculiar conditions. Abundant literature suggests relationships between the growth phase, chemistry of the growth medium and membrane composition. Differences in BH-polyol distribution in bacteria depending on the growth carbon sources were observed for *Methylobacterium* species [32].

1.2. Biohopanol Biosynthesis

The biosynthesis of the hopane skeleton involves an acid-catalysed cyclization of the acyclic triterpene squalene *via* carbocationic intermediates, yielding diploptene (**1a**) and diplopterol (**6a**) as main products [18]. The key enzyme in the BH-polyol biosynthesis is squalene-hopene cyclase, which catalyzes the cyclization of the linear triterpenoid squalene to hopene [8,41]. The supplementary C_5 polyhydroxylated side chain arises from a D-pentose unit *via* the non-oxidative pentose phosphate pathway [42-44]. The absolute configuration of the C_5 polyhydroxylated unit is 32*R*,33*R*,34*S* as in D-ribose. Thus, D-ribose is assumed to be the precursor of these additional five C atoms of the BH-polyol side chain [21].

Combination of all the known structural variations leads to an enormous number of different compounds. The explanation for this large structural variability, which is unusual for compounds involved in primary metabolism, is still missing [5].

1.3. Biohopanoid Functions in the Cell

BH-polyols are known to exert a strong influence on the properties of membranes. They maintain membrane stability by increasing the rigidity of the lipid matrix in a manner similar to some sterols in eukaryotes [10,33]. Experiments with model membranes have shown that BH-polyols efficiently condense membranes by increasing Van der Waals forces between lipid molecules thus diminishing the penetration of small molecules [34].

Besides the above role, it has been proposed that BH-polyols may serve to enhance the stability or barrier function of membranes in a number of bacteria, including *Alicyclobacillus acidocaldarius*, *Z. mobilis*, *Frankia* sp., *Methylococcus capsulatus*, and *Streptomyces coelicolor* [18,35]. In *Z. mobilis*, BH-polyols were supposed to be implicated in the high degree of ethanol tolerance of this bacterium. In *A. acidocaldarius*, an acidothermophilic bacterium, BH-polyols were proposed to stabilize the membrane under high temperature and low pH conditions. Under such extreme conditions, the adaptive response of the bacteria leads to an increase in the total BH-polyol content [36].

Analysis of the importance of hopanoids in *Rhodopseudomonas palustris* performed by Welander *et al.* [37] was the first to properly demonstrate the physiological importance of hopanoids in any bacterium and provided evidence that hopanoids can be essential in some bacteria.

The association between BH-polyols and phospholipids is influenced by the acyl chain of the phospholipids. The BH-polyol ring system should extend to the acyl chain ends of phospholipids, with the hydroxyl group at the side chain forming a hydrogen bond with the phospholipid polar head group [33,38]. Thus, BH-polyols have an inverted orientation in the phospholipid bilayers compared to cholesterol [33].

The function of BH-polyols is also influenced by hydrophobic interactions with the surrounding molecules as well as by the ratio of methylated to non-methylated BH-polyols, which is probably important for the regulation of membrane fluidity [39].

High temperature has been observed to give rise to very high global BH-polyol concentrations (up to 14 mg g^{-1} dry cells). This is not really surprising, as the regulation of membrane fluidity needs more reinforcers at higher temperatures [40].

The cultivation experiments with *Frauteria aurantia* suggest that no significant BH-polyol synthesis occurs after the cells have reached the stationary growth phase, even if the growth conditions are changed. In addition, the C_{31} hydroxylated BH-polyols synthesised during the exponential growth phase seemed not to be further modified during the stationary growth phase [40]. The growth conditions under which a bacterium produces extractable BH-polyols are as yet unidentified [40].

The non-uniform and rare distribution of BH-polyol biosynthesis [8] as well as a number of structural variations [41,42] hint at a richer biological function of these compounds than currently acknowledged [8,41,42].

The recently demonstrated relatively low distribution of BH-polyols in bacteria [8] mentioned above clearly argues against their recently proposed fundamental membrane-structural role in bacteria [8,9].

2. BIOHOPANOL ANALYSIS

2.1. GC-MS Analysis

Characterization of individual hopanoids, especially those with extended side chains, is difficult as they are highly functionalized, polar or amphiphilic, low soluble in organic solvents and have high

molecular weight [5,19,45,46]. These structures are not readily amenable to analysis by conventional GC–MS methods [19].

The simplest hopanoids such as diplopterol (**6a**) and diploptene (**1a**) are readily extractable from freeze-dried cells by organic solvents and can be characterized by the GC-MS method. However, it has been observed that diplopterol (**6a**) sometimes undergoes partial decomposition on the GC column, which results in the formation of a mixture of diplopterol (**6a**), diploptene (**1a**) and hop-21-ene (**7a**). This can be avoided by silylation of the hydroxyl prior to GC analysis [5,45].

Of the BH-polyols, only the tetra-acetates of BH-tetrol (**2a**) and aminoBH-triol (**3a**) are known to be amenable to analysis by GC–MS [19].

Rohmer *et al.* [45] developed a standard procedure (Fig. **1**) for the analysis of complex BH-polyols. The method partially overcomes the mentioned analytical problems. This procedure converts the BH-polyols to simple primary alcohols with shortened side chains. The lipid extract is oxidized with periodic acid (H_5IO_6), leading to aldehydes which are reduced by $NaBH_4$ to primary alcohols or eventually to secondary alcohols. Then the hopanol products can be analyzed by GC–MS after acetylation [1,4-6,19,45,47-49]. This degradation technique cleaves off the very polar side chain [12] and thus allows the quantification of total tetra-, penta- and hexa-functionalized BH-polyols in bacterial and sediment samples [19] and the detection of nearly all known composite BH-polyols [46].

The method is widespread in the literature but it has several limitations. First, adenosylhopanes (**8**) cannot be detected by GC-MS analysis after $H_5IO_6/NaBH_4$ treatment [18,50], because this procedure requires two vicinal hydroxyl groups permitting the cleavage of the side chain, which is not met in adenosylhopanes (**8**) [19]. Second, each of the BH-polyol classes is split to the same fragment so the specific structural information in the side chain is lost. This is a major problem as it is the side chain functionality which may be diagnostic of certain bacterial species or environmental conditions [1,4,51].

More specific structural information is typically obtained by isolating the product from an acetylated total extract of a bacterium using methods such as TLC and/or preparative HPLC followed by NMR [46] and probe MS.

2.2. HPLC Analysis

The limitations of the GC-MS method promoted a considerable interest in finding a reliable qualitative and quantitative method capable of characterizing intact hopanoids in complex mixtures. The scientific world thus opened the way to HPLC methods. Table **1** summarizes HPLC and MS conditions for separation and identification of hopanoids.

One of the earlier methods was developed for the extended BH-polyols occurring in *Z. mobilis*. Total bacterial extract was acetylated and separated on a reversed-phase HPLC column with a gradient of acetonitrile in methanol-water mixture using UV detection at 206 nm [5,19,52]. The same method was used for analysis of acetylated lipid extract from *Frankia* sp. and from *Z. mobilis* but with a flame ionisation detector [51,53].

Other studies employed normal-phase HPLC and the flame ionisation detection (FID) [51,53,54]. In the case of lipid extract of *Frankia* sp., lipids were eluted with a gradient of isooctane-isopropanol-water. Most of the peaks were identified by comparison of retention times with known standards [53]. The method was further modified and the solvent system comprised hexane, propan-2-ol and 0.04 % triethyl amine in water in the case of *Z. mobilis*. The total lipid extract of *Z. mobilis* was injected directly into HPLC without derivatization and UV detector was placed in front of FID [51,54]. Moreau *et al.* [51] described in detail a comparison of normal-phase and reversed-phase HPLC as well as FID and UV detection.

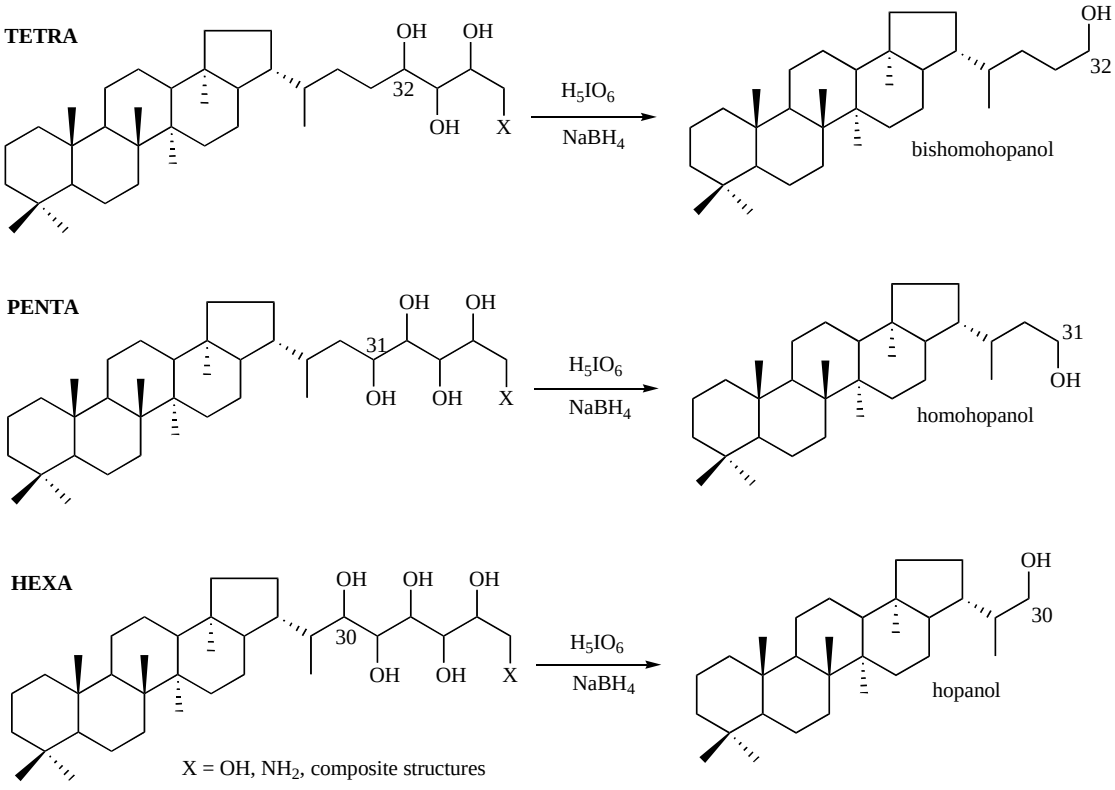


Fig. (1). Periodic acid/sodium borohydride cleavage products [45].

Table 1. Sources of Hopanoids, HPLC Techniques and Analytical Conditions Used for their Separation and Identification

Ref.	Column Type	Column Characteristic[1]	Mobile Phase	Flow (mL/min)	Corona/ Electrospray Voltage[5]	Vap. Temp (°C)	Scan Type	Mass Range[2] (*m/z*)	Analyzed Samples
[1]	Hichrom	(100 × 3.2); 5; RT[3]	gradient (min/%A/%B%C): 0/100/0/0; 5/95/5/0; 10/85/15/0;15/40/60/0; 53/40/51/9; 68/40/51/9; 73/40/60/0;78/100/0/0 to 100 min with (A) hexane, (B) propan-2-ol, (C) 0.04% triethylamine in water	0.5	7 μA	350	-	400 - 800	*Z. mobilis*; a sediment from the deepest part of the lake Priest Pot, Great Britain
[4]	Sperisorb ODS 2	(150 × 4.6); 3; RT	gradient (min/%A/%B%C): 0/90/10/0; 6/59/1/40 to 70 min (A) MeOH, (B) water, (C) propan-2-ol	1.0	8 μA	400	-	-	acetylated bacterial extracts (*Z. mobilis*, methanotrophic bacteria; a sediment from the lake Druzhby, Antarctica)
[19]	Genesis C_{18}	(150 × 4.6); 4; RT	gradient (min/%A/%B%C): 0/90/10/0; 6/40/1/59 to 70 min (A) MeOH, (B) water, (C) propan-2-ol	1.0	8 μA	400	-	150 - 900 or 400 - 1200	several strains of methanotrophic bacteria
[55]	Genesis C_{18}	(150 × 4.6); 4; RT	gradient (min/%A/%B%C): 3/90/10/0; 25/59/1/40 to 60 min (A) MeOH, (B) water, (C) propan-2-ol	0.2	8 μA	400	-	-	surface sediment from Laguna Grande (a saline lake in Spain); methanotroph fermenter sample; *Prochlorothrix hollandica* culture; *Chlorogloeopsis*
[16]	Phenomenex Gemini C_{18}	(150 × 3); 5; 30	gradient (min/%A/%B%C): 0/90/10/0; 25/59/1/40 to 45 min (A) MeOH, (B) water, (C) propan-2-ol	0.5	8 μA	490	-	-	bacteria isolated from a Black Sea microbial mat (genus *Desulfovibrio*) and deep-sea sediment
[35, 44]	Nova-Pak C_{18}	(150 × 3.9)	gradient (min/%A/%B): 1/100/0; 8/0/100 to 12 min (A) 90% MeOH + 10% water, (B) 59% MeOH + 1% water + 40% propan-2-ol	-	3 μA	300	-	-	dry cells from postexponential acetate-grown cultures (cyanobacterial strains)

Table 1. contd…..

Ref.	Column Type	Column Characteristic[1]	Mobile Phase	Flow (mL/min)	Corona/ Electrospray Voltage[5]	Vap. Temp (°C)	Scan Type	Mass Range[2] (*m/z*)	Analyzed Samples
[2,7]	Genesis C_{18}	(150 × 4.6); 4; RT	gradient (min/%A/%B%C): 3/90/10/0; 25/59/1/40 to 60 min (A) MeOH, (B) water, (C) propan-2-ol	0.2; 1.0	8 μA	490	full SCAN1	*m/z* 400-1300	Cyanobacteria, cyanobacterial mat, sediments
							SCAN2	depend on MS^2 spectrum of most intense ion from SCAN1	
							SCAN3	depend on MS^3 spectrum of most intense ion from SCAN2	
[7]	Phenomenex Gemini C_{18}	(150 × 3); 5; 30	gradient (min/%A/%B%C): 0/90/10/0; 25/59/1/40 to 45 min[4] (A) MeOH, (B) water, (C) propan-2-ol	0.5	8 μA	490	full SCAN1	*m/z* 400-1300	Cyanobacteria, cyanobacterial mat, sediments
							SCAN2	depend on MS^2 spectrum of most intense ion from SCAN1	
							SCAN3	depend on MS^3 spectrum of most intense ion from SCAN2	

[1]length (mm); particle size (μm); temperature (°C).
[2]in case of SIM single mass.
[3]room temperature.
[4]returning to the starting conditions in 5 min and stabilizing for 10 min.
[5]ion source was APCI.

Fox *et al.* [1] applied the same normal-phase HPLC conditions as Moreau *et al.* [51] but employed negative ion in LC/MS with APCI to detect BH-polyols. A highly characteristic isotopic distribution was produced by addition of chlorine (from the solvent). Using this method the authors identified BH-tetrol (**2a**) and the two composite hopanoids, BH-tetrol cyclitol ether (**9a**) and BH-tetrol glycoside (**10a**) in a complex mixture of lipids extracted from a culture of *Z.mobilis*. The same technique allowed the identification of BH-tetrol (**2a**) and BH-tetrol cyclitol ether (**9a**) in an extract from a modern lake sediment. A composite BH-polyol has been identified directly in a sediment sample [1,9].

The advantage of the method is that chlorinated species produced from a polyhydroxylated species are very stable and thus readily detectable even at low concentrations. On the other hand, this method has limitations since it provides little structural information, with the spectra being dominated by a single chlorinated pseudomolecular ion $[M+Cl]^-$ [19].

The LC/MS^n APCI recognize, and at least partially characterize, unknown biohopanoid structures, and this method of detection appears to be ideally suited to the study of intact BH-polyols and composite hopanoids in bacterial and sedimentary extracts [4].

2.3. Fragmentation of Different Hopanol Compounds

Underivatised BH-tetrol (**2a**) - some characteristic ions are produced including the base peak at *m/z* 529 showing the loss of H_2O from the protonated molecule $[M+H]^+$ at *m/z* 547 [19]. 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 BH-polyol ring system after the loss of the entire side chain. The ions between *m/z* 150 and 250 indicate cleavage in ring C of the BH-polyol pentacyclic system. 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.

Underivatised aminoBH-triol (**3a**) - gives considerably less fragmentation and no ions indicative of the ring system [19]. The protonated molecule at *m/z* 546 is the base peak and the only observable fragmentation is *m/z* 528 indicating again loss of a water and demonstrating that the basic nature of the amino functionality leads to ready formation of an ammonium ion and this protonated BH-tetrol ion is more stable under these conditions than the protonated ($[M+H]^+$ at *m/z* 547) of underivatised BH-tetrol (**2a**).

Tetraacetylated BH-tetrol (**2a**) - readily loses an acetate group (i.e. loss of 60 Da) from the protonated molecule ($[M+H]^+$ at *m/z* 715), producing a base peak ion at *m/z* 655 ($[M+H–CH_3COOH]^+$) [4,19]. The protonated molecule 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 [19]. Loss of a second acetate group is indicated by the ions at *m/z* 613 and 595 (loss of CH_2=C=O and CH_3COOH, respectively) [4].

The MS^2 spectrum from *m/z* 655 ($[M+H–CH_3COOH]^+$) shows a number of ions corresponding to full (*m/z* 595, 535 and 475) losses of the three remaining acetate groups [4,55]. Ions at *m/z* 463, 403, 343 and 283 represent cleavage in ring-C between C_9 and C_{11}, and C_8 and C_{14} with charge retention on the side-chain-containing fragment after the loss of a 192 Da neutral fragment [4,55].

Tetraacetylated aminoBH-triol (**3a**) **-** also showed a spectrum similar to that of its unacetylated form, with the base peak again being the protonated molecule ($[M+H]^+$ at *m/z* 714). The adduct ion at *m/z* 774 $[M+H+60]^+$ was again present. Loss of functional groups was evident as three sequential losses of acetic acid with the terminal acetamido (CH_3CONH_2, 59 Da) group lost last [4].

MS^2 spectrum of *m/z* 714 ($[M+H]^+$ resulted in the formation of just three major ions from the loss of the three acetylated hydroxyls as neutral species (i.e. multiple losses of CH_3COOH or 60 Da) to give *m/z* 654, 594 and 534. However, a number of key ions become apparent upon close scrutiny of the spectrum between *m/z* 190 and 500. The ion at *m/z* 475 indicates loss of the final functional group (i.e. neutral loss of CH_3CONH_2). As for BH-tetrol (**2a**), *m/z* 191 represents the A and B rings after cleavage through ring-C. The ions at *m/z* 462, 402, and 342 represent charge retention by the side-chain-containing fragment following ring-C cleavage and loss of one, two or three acetylated hydroxyls, respectively. The ion at *m/z* 283 represents charge retention on the side-chain-containing fragment subsequent to neutral loss of all four acetylated functional groups with ring-C cleavage [55].

Pentaacetylated aminoBH-tetrol (**11a**) produced four successive losses of 60 Da (peaks at *m/z* 712, 652, 592 and 532) indicating the presence of four acetate groups. A single loss of 59 Da (CH_3CONH_2) to give ion at *m/z* 473 indicates the presence of the terminal acetamido group, which appeared to be removed last, in agreement with the study [19]. Loss of a single acetate group produced by far the most abundant ion at *m/z* 712. No ions indicative of the ring system were observed in MS^2 of ion at *m/z* 772 [4]. MS^3 of *m/z* 712 produced ions indicating loss of the remaining three acetates and the one acetamido group. Ring C cleavage in this component is apparently a minor process compared with that occurring in BH-tetrol (**2**). Possible losses of 192 Da from *m/z* 652, 592, 532 and 473 produced very low abundance ions at *m/z* 460, 400, 340 and 281 [4].

Hexaacetylated aminoBH-pentol (**4a**) - MS^2 of the protonated molecule indicative of aminopentol (**4a**) ($[M+H]^+$ at *m/z* 830) demonstrated very similar fragmentations to those of aminotetrol (**11a)**, with ions indicative of loss of the five acetate groups (*m/z* 770, 710, 650, 590 and 530). In this case, however, loss of the terminal acetamido group was not observed in the MS^2 spectrum. MS^3 of *m/z* 770 ($[M+H-CH_3COOH]^+$) also shows multiple losses of 60 Da and one loss of 59 Da at *m/z* 471, again suggesting that the terminal group is removed last. In MS^3 loss of 192 Da from each of the ions arising from successive losses of acetate groups is suggested with ions at *m/z* 518, 458, 398 and 338; however, these ions are again very weak. The loss of 192 Da from the fragment containing the fully defunctionalised side chain was not observed [4].

BH-polyols containing an amino functionality at C_{35} produce a strong $[M+H]^+$ ion, and fragmentation in both the side chain and ring system is delayed relative to that observed in similar fully hydroxylated compounds [55].

Peracetylated 2β-methylBH-tetrol (**2b**) - the MS^2 of 2-Me-BH-tetrol (**2b**) would result in the loss of 206 Da (cleavage in ring-C) from the side-chain-containing fragment. This assignment is further confirmed by the observation of an ion at *m/z* 205 indicating the presence of an additional methyl group on the A or B rings [4].

Peracetylated 3β-methylated aminoBH-polyols - (3β-methyl aminoBH-tetrol (**11c**), and 3β-methylaminoBH-pentol (**4c**)) - MS^2 of the protonated molecule produces similar results to those for aminoBH-pentol (**4a**) with full loss of acetate groups but no indication of the loss of the acetamido group [4]. MS^3 of *m/z* 726 ($[M+H-CH_3COOH]^+$ for 3β-Me aminoBH-tetrol (**11c**)) shows the loss of three acetate groups (*m/z* 666, 606 and 546) and the terminal acetamido group (ion at *m/z* 487). Also present are ions at *m/z* 340 and 281. The authors have tentatively assigned these ions as representing loss of 206 Da from the ions at *m/z* 546 and 487, resulting from cleavage of ring C with loss of rings A and B. MS^3 of *m/z* 784 in the spectrum of 3β-Me-aminoBH-pentol (**4c**) indicates only one apparent loss of 206 Da following the loss of all six functional groups (*m/z* 279).

Compounds with the same molecular weight can be distinguished and isolated, e.g., by RP-HPLC, in which **BH-tetrol cyclitol ether** (**9a**) is eluted earlier than **BH-tetrol glycoside** (**10a**) [4]. Both have a molecular mass of 1001 Da and they are therefore indicated by a value at *m/z* 1002 ($[M+H]^+$ by SIM). MS^2 of *m/z* 1002 shows three successive losses of acetate (to *m/z* 942, 882 and 822). The ion at *m/z* 655 results from loss of the terminal group due to cleavage between C_{35} and the oxygen atom attached to C_{35}, indicating the tetrafunctionalised nature of the component. The ion at *m/z* 984 is of particular importance; it indicates the presence of the nonacetylated hydroxyl at $C_{5'}$ (tertiary OH) on the composite group of BH-tetrol cyclitol ether (**9a**) and this functionality is not present in the structure of BH-tetrol glycoside (**10a**). The mild acetylation procedure does not usually acetylate tertiary alcohols. The minor ion at *m/z* 348 corresponds to fragmentation with charge retention on the terminal group, *via* cleavage of the bond between C_{35} and the oxygen atom at C_{35}. The ion at *m/z* 330 corresponds to the terminal group following cleavage between the oxygen at C_{35} and C_{10} on the composite moiety. MS^3 of *m/z* 942 shows similar ions as MS^2 but with differences in their relative abundance. The ions at *m/z* 595 and 475 indicate losses of the terminal group and either one or all three remaining acetates from the side chain, and *m/z* 348 is confirmed as a fragment ion.

BH-tetrol glycoside (**10a**) with the same base peak is indicating the protonated molecule also at *m/z* 1002. The MS^2 spectrum is significantly different from BH-tetrol cyclitol ether (**9a**). In this case, the most abundant ion is *m/z* 330 arising from the cleavage between the oxygen atom at C_{35} and $C_{1'}$ of the terminal group with charge retention on the terminal group. This ion was so abundant that it was possible to perform MS^3 on *m/z* 330. In the MS^3 spectrum the ion at *m/z* 210 suggests loss of two acetate groups from *m/z* 330, and *m/z* 168 and 150 indicate loss of a third acetate and/or ketene (i.e. $CH_2=C=O$) respectively [4].

3. STEREOCHEMISTRY OF HOPANOIDS

In most triterpenoids the ring A has a chair conformation. In 2β-methylBH-polyols a chair conformation of cycle A would lead to an axial position for the three C-2β, C-4β and C-10β methyl groups, resulting in unfavorable steric interactions. These interactions are minimized in a boat conformation where the two C-2β and C-4β methyl groups are equatorial [39, 56, 57].

The absolute stereochemistry of the side chain of BHtetrols was established by Bisseret [58] who synthesized the eight possible diastereomers which could be distinguished well by their ^1H NMR spectra. Unfortunately, the 32 diastereomers for BHhexols have not yet been synthesized and hence a different approach was used. The CD exciton chirality method [59] has been applied to numerous different acyclic polyols such as BHpolyols [24] with up to five contiguous OH groups, or aminoBHpentols [60,61].

These CD studies clearly showed that with flexible compounds it is preferable to explore the exciton coupling between two different chromophores such as anthroyl and cinnamoyl. Based on the similarity of CD spectra of model compounds, predominantly different sugar alcohols [61] and BH-polyols, it was possible to deter-

mine the absolute configuration of the side chain, as shown in Fig. (**2**).

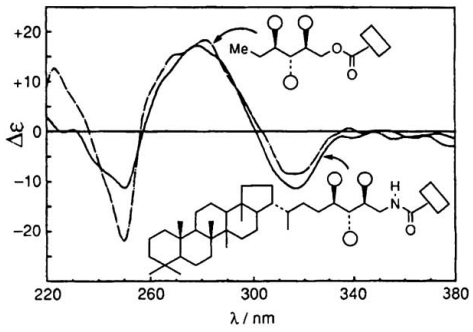


Fig. (2). CD spectra of derivatized aminohopanetriol and L-ribose derivative [57]. Reproduced by permission of the author and The Royal Society of Chemistry.

4. OCCURRENCE OF BH-POLYOLS IN BACTERIA

4.1. Methanotrophs

Methanotrophs belong to the best-known BH-polyol-producing bacteria. They are divided into three groups on the basis of intracellular membrane type and pathways of carbon assimilation [4,62]: type I (genera *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylococcus*,) [31,62,63], type II (genera *Methylosinus*, *Methylocystis*, *Methylobacterium* and *Methylocella*) [62,63] and type X (genus *Methylocaldum*) [4]. Each group has been shown to produce a distinctive distribution of BH-polyols [4].

An analysis of acetylated total extract of several methanotrophic bacterial strains (*Methylomicrobium album*, *Methylococcus capsulatus*, *Methylomonas methanica*, *Methylomonas rubrum*, *Methylocystis parvus* and *Methylosinus trichosporium*) revealed BH-tetrol (**2a**), aminotriol (**3a**), aminoBH-tetrol (**11a**) and aminoBH-pentol (**4a**) [19].

Type I methanotrophs typically possess a high abundance of hexafunctionalised components, although to-date only one hexafunctionalised side-chain structure has been identified (aminoBH-pentol (**4a**)). This component has been isolated in a variety of methanotrophs [4], e.g. *M. methanica* and *M. capsulatus* [30] and *Methylococcus luteus* [31]. *Methylocaldum szegediense* contained aminoBH-pentol (**4a**) in 24 % of the total BH-polyol content; 68 % represented its saturated 3β-methyl homologue (**4c**) and 8 % its unsaturated 3β-methyl homologue with a Δ^{11} double bond (**4h**) [31]. In *Methylocaldum tepidum*, aminoBH-tetrol (**2a**) (32 % of the total BH-polyol content) and its 3β-methyl homologue (**2c**) (7 %) represented major BH-polyols and were accompanied by the 40 % of aminoBH-pentol (**4a**) and 21 % of its 3β-methyl homologue (**4c**) [31].

M. methanica and *M. trichosporium*, which are species more distant from the genus *Methylocaldum*, do not synthesise 3β-methylBH-polyols. The BH-polyol fingerprint is thus consistent with the presumed phylogeny of these bacteria [31].

Type II methanotrophs are characterised by abundant tetra- and pentafunctionalised structures including aminoBH-triol (**3a**) and aminoBH-tetrol (**11a**). An aminoBH-triol (**3a**) was the major BH-polyol for instance in *M. trichosporium* [31].

The following compounds were found in *Methylobacterium organophilum*: BH-tetrol (**2a**), BH-tetrol glycoside (**10a**), BH-tetrol ethers (**9a, 12a**), and also 2β-methyl-BH-tetrol (**2b**) though in a very low amount. The total isolated amount of BH-polyols (about 4-13 mg/g dry weight), as well as the relative amounts of all these compounds varied considerably from one culture to another, depending probably on the culture conditions (fermentor or flasks) and the nature of the culture medium. Usually BH-tetrol (**2a**) and the glycoside (**10a**) were minor compounds, and one of the two ethers was the major compound (**9a**) or (**12a**) [28].

Type X methanotrophs, whilst being phylogenetically related to type I, also contain some properties of type II. They have also been shown to produce a high abundance of hexafunctionalised BH-polyols together with BH-polyols containing a modified ring system with a methyl group at position C-3 in ring A [4].

4.2. Purple Non-Sulphur Bacteria

The purple non-sulphur bacterium *Rhodomicrobium vannielii* is a good source of BH-polyols, the essential was derived from aminoBH-triol (**3a**). Three bacteriohopane derivatives aminoBH-triol (**3a**), *N*-tryptophanyl-aminoBH-triol (**13a**) and *N*-ornithinyl-aminoBH-triol (**14a**) are the major BH-polyols of this bacterium. They represent the quasi-totality of the BH-polyol content of the cells (nearly 6 mg/g dry weight). The simple C_{30} hopane derivatives diploptene (**1a**) and diplopterol (**6a**), and some other minor compounds, were found in trace amounts [29].

Members of genera *Rhodopseudomonas* and *Rhodoblastus* are also good BH-polyol producers belonging to photosynthetic purple non-sulfur bacteria. Besides diploptene (**1a**) (50 μg/g dry weight) and diplopterol (**6a**) (250 μg/g), a C_{32} alcohol of 22*R* configuration (2.7 mg/g, 95% of the bacteriohopane derivatives) accompanied by small amounts of its 22*S* epimer (5%) were found in the crude extract of *Rhodopseudomonas palustris*. The tetraacetate of aminotriol (**3a**) (3 mg/g) was the only bacteriohopane derivative detected by Neunlist *et al.* [50]. However, several minor BH-polyols could be detected, but were not properly isolated and identified because of their low concentration in the cells (< 20 μg/g). *Rhodoblastus acidophilus* (formerly *Rhodopseudomonas acidophila*) was found to contain diploptene (**1a**) (200 μg/g, dry mass), a C_{32} BH-polyol primary alcohol of 22*R* configuration (2 mg/g, 96% of the bacteriohopane derivative fraction) as major compound, accompanied by its 22*S* epimer as minor companion (4%). Further five complex BH-polyols, i.e. carbamoylBH-triol (**15a**), dicarbamoylBH-diol (**16a**) and two nucleoside analogues, (22*R*)-30-(5'-adenosyl)hopane (**8a**) and (22*S*)-30-(5'-adenosyl)hopane (**8a**) were also detected [50].

Besides diploptene (**1a**) and diplopterol (**6a**), a glucuronopyranosyl residue linked *via* an α-glycosidic bond to the hydroxyl group of C-35 in BH-tetrol was isolated from the bacterium *Rhodospirillum rubrum*. The absolute configuration of BH-tetrol chain was common 32*R*, 33*R* and 34*S* [41]. Unusual is an α-glycosidic bond between a bacteriohopanetetrol and a carbohydrate [41]. Complex tetrol glycosides isolated for example from *A. acidocaldarius* [64,65], *M. organophilum* [28] or *Z. mobilis* [66] are β-glycosides.

4.3. Acetic Acid Bacteria

The acetic acid bacteria have been found to produce the most diverse range of BH-polyol structures. Great variability was found in the ring system, e.g. double-bond at C_6 and/or C_{11}, or additional methyl group at C-3β. Besides commonly occurring 32*R*, 33*R*, 34*S* configuration, the unusual 32*R*, 33*R*, 34*R* configuration and rare 22*S* epimer were found [26,43,67].

A typical member of acetic acid bacteria in terms of occurrence of BH-polyols is *Gluconobacter europaeus* (previously *Acetobacter europaeus*), which contains a very complex mixture of unsaturated and methylated BH-polyols. Furthermore, *G. europaeus*, like *A. pasteurianus* and *A. aceti*, possesses two bacteriohopane side chains differing one from another by the stereochemistry at C-34. This feature has been only found in *Acetobacter* species. However,

BH-polyols isolated from *G. europaeus* are the only ones possessing an extra methyl group at C-31 [67].

Peiseler and Rohmer [43] found a new series of C_{33}-diols with a shortened side chain with the absolute configuration 32*R* in cells of *Gluconobacter xylinus* (formerly *Acetobacter aceti* ssp. *xylinum*).

Frateuria aurantia, formerly classified as *Acetobacter aurantius* according to its phenotypic and physiological properties, was reclassified in a new *Frateuria* genus after rRNA analyses. This bacterium was recently isolated from acidic soils and shows exponential growth under very diverse growth conditions including wide temperature (from 10 to 36°C) and pH ranges (from 3.5 to 8). It is therefore suitable for a study in which the impacts of changing growth conditions are considered. The total hydrocarbon fraction of *F. aurantia* (1‰ of total BH-polyols) showed the presence of diploptene (**1a**) (10 μg g^{-1} dry cells), hop-17(21)-ene (**7a**) (3 μg g^{-1} dry cells) and fern-7-ene (**17a**) (1.5 μg g^{-1} dry cells). Contrary to many other bacteria, no detectable amount of diplopterol (**6a**) was found in the analysed *Frateuria* strain. Among the intact BH-polyols, five compounds could be identified after acetylation and purification: the tetraacetate of the (22*R*)-BH-tetrol (**2a**) (1.1 mg g^{-1} dry cells), heptaacetate and octaacetate of BH-tetrol cyclitol ether (**12a**) (total amount of 4.3 mg g^{-1} dry cells), octaacetate and nonaacetate of BH-pentol cyclitol ether (**18a**) (total amount of 10 mg g^{-1} dry cells) [40].

4.4. Nitrogen Fixers

Frankia is a nitrogen-fixing actinomycete that produces very high levels of BH-polyols. The large amount of BH-polyols (20-87 % of total lipids) is a general feature of the *Frankia* genus and could be used as a phenotypic characteristic of this genus among actinomycetes [36].

In *Frankia*, nitrogenase activity is expressed only within specialized vesicular structures [23] that differentiate mainly under nitrogen-limiting culture conditions [36]. Vesicle envelopes provide an oxygen diffusion barrier for nitrogenase activity [53]. BH-polyols in vesicles represent up to 90% of their total lipids [48]. They are very likely involved in the protection of the oxygen sensitive nitrogenase from oxidation [36,48]. It is expected that they participate in altering permeability of the vesicle to oxygen [53].

The vesicular structures are surrounded by multilayered envelopes that consist almost entirely of four BH-polyols. Two BH-tetrol (**2a**) isomers and two phenyl acetic acid monoesters of BH-tetrol (**19a**) [23,36] represented 84% of the dry weight of purified vesicle envelope preparations and 80% of the total lipids of vesicle clusters [36]. However, while the first tetrol was found at high levels in vegetative (non-envelope-forming) *Frankia* cells, the second tetrol (C-34 epimer) was specifically found in the vesicle envelope, suggesting that the properties of this molecule determine some critical aspect of monolayer (or lamina) formation [23]. Thus the BH-tetrols (**2a**) together with their phenylacetic acid esters (**19a**) were proposed to form the major physical barrier to protect nitrogenase against oxygen. It is not yet known whether these lipids might play an additional role in other stages of the *Frankia* life cycle [36]. Besides the above BH-polyol structures, two other BH-polyols were identified: a BH-tetrol propionate (**20a**) as a minor compound and moretan-29-ol (**21a**), which was one of the major BH-polyols. The moretan-29-ol (**21a**) and the BH-tetrols (**2a**), the most abundant BH-polyols in *Frankia* cells, have been found in different ratios among the *Frankia* strains tested. Quantitative variations under the alternative nitrogen conditions were much less important in experiments, since such variation represented only up to 20%. A similar result was found in another nitrogen-fixing bacterium, *Azotobacter vinelandii*, where production of BH-polyols was not stimulated under nitrogen-fixing conditions [36]. The structural diversity of BH-polyols and their high proportion in all *Frankia* populations throughout different infectivity groups suggest that these compounds play important roles in *Frankia* cells. Their roles could be expressed in symbiotic interactions, i.e. in root nodules, where BH-polyols were first reported [36].

GC-MS analysis after $H_5IO_6/NaBH_4$ treatment revealed in several strains of *Bradyrhizobium* and related bacteria (e.g. *B. japonicum*, *B. elkanii*, *Bradyrhizobium* sp. and the closely related, photosynthetic, nitrogen-fixing *Photorhizobium* sp.) the occurrence of diploptene (**1a**) and diplopterol (**6a**), which are both nearly ubiquitous in BH-polyol producers, as well as dihomohopan-32-ol, which represented the signature of the BH-polyol derivatives. In addition, unidentified alcohols, most probably corresponding to methylated pentacyclic triterpenic alcohols, accompanied in significant amounts the above mentioned triterpenoids. The widespread aminoBH-triol (**3a**), which affords dihomohopan-32-ol after $H_5IO_6/NaBH_4$ treatment, was the main BH derivative. It was accompanied by significant amounts of the rather rare adenosylhopane (**8a**), which cannot be detected by the former derivatization method. So far, adenosylhopane (**8a**) has been only found as minor BH-polyol in *R. acidophila* and *Nitrosomonas europaea*. The unknown triterpenic alcohols were identified as novel methylated gammacerane derivatives. The simultaneous occurrence of the hopane and the gammacerane series seems to be a common feature of *Bradyrhizobium* bacteria, which is also shared with *R. palustris*. Indeed, the latter bacterium contained aminoBH-triol (**3a**) as the only BH-polyol, which was nearly always accompanied by significant amounts of tetrahymanol. According to 16S rRNA analysis, *Bradyrhizobium* bacteria and the purple nonsulfur bacterium *R. palustris* are closely related and cluster together in the phylogenetic tree of the a-subgroup of proteobacteria [18].

4.5. Cyanobacteria

Cyanobacteria are ubiquitous phylogenetically diverse components of the phytoplankton of marine and freshwater environments as well as some extreme settings such as hot springs, and highly saline and ice covered lakes. They have also been shown to be amongst the most prolific sources of BH-polyols and are considered to be the most environmentally significant source of C-2 methylated BH-polyols [7,24]. The accumulation of substantial quantities of 2-methylhopane derivatives in sediments has thus been proposed as a marker for cyanobacteria [7,35,38,44,68]. However, they are not produced by all cyanobacteria [7]. Moreover, methylation at C-2 was found also in *Methylobacterium* spp. [26,28,39], *Bradyrhizobium* spp. [18] and *Beijerinckia* spp. [5].

This indicates that the source of 2-methyl-BH-polyols is more diverse than simply cyanobacteria. However, currently, the vast majority of BH-polyols with a methylated C-2 originate from cyanobacteria and the distribution of these structures within soils decreases with depth, as would be expected for an origin in photosynthetic cyanobacteria. It is therefore reasonable to assume that the major contribution of C-2 methylated structures in soils is from cyanobacteria [69].

A survey of the literature (1984 to date) on BH-polyol occurrence in cyanobacteria reveals that a total of 46 species or strains from five different orders (Chroococcales, Nostocales, Oscillatoriales, Prochlorales and Stigonematales) have been tested and that 41 strains were found to produce BH-polyols in culture [7].

Recently, Talbot *et al.* [7] carried out an extensive study of marine and non-marine cyanobacteria using LC-MS^n method. They analysed 26 strains of cyanobacteria. The study showed that two strains of nitrogen fixing marine cyanobacteria, *Trichodesmium erythraeum* and *Crocosphaera watsonii*, produce tetrafunctionalised BH-polyols, and could contribute significantly to the sources of these dominant BH-polyol compounds in marine environments while three strains of *Prochlorococcus* and one strain of *Synechococcus* were found not to produce BH-polyols. These findings agreed with the results of genetic studies; the presence or ab-

sence of BH-polyols corresponded to the presence or absence of a gene encoding a homologue for the squalene-hopene cyclase protein [7].

In the case of non-marine cyanobacteria many of tested strains were investigated previously, e.g. *Anacystys montana* [70], *Microcystis aeruginosa* [5], *Synechocystis* sp. [27], *Nostoc muscorum* [39], and *Prochlorothrix hollandica* [71]. In *Anabena cylindrica*, BH-tetrol (**2a**) was the only BH-polyol, whereas *Chlorogloeopsis fritschii* has been shown to produce both methylated (**2b**) and non-methylated BH-tetrol (**2a**), the non-methylated component being approximately twice as abundant as the methylated compound [7].

The previously identified methylated (**9b**) and unmethylated BH-tetrol cyclitol ethers (**9a**) [70] were the two major BH-polyols in *A. montana* and were supplemented by a small amount of BH-tetrol (**2a**). *Microcystis* sp. and *M. aeruginosa* contained abundant aminoBH-triol (**3a**) [7].

Nostoc muscorum was found to contain both BH-tetrol (**2a**) and 2-methyl-BH-tetrol (**2b**) [7,39]. Bisseret *et al.* [39] reported further afield detection of methylated and non-methylated BH-pentols with hydroxyl groups in positions 31, 32, 33, 34, 35 (**22a,b**) and 30, 32, 33, 34, 35 (**23a,b**), while Talbot *et al.* [7] detected only non-methylated BH-31, 32, 33, 34, 35-pentol (**22a**).

Simonin *et al.* [71] described eight different BH-polyol structures, including methylated and non-methylated homologues of BH-tetrol (**2a,b**), 35-*O*-β-3,5-anhydrogalacturonopyranosyl-BH-tetrol (**24a**), 35-*O*-β-galacturonopyranosyl-BH-tetrol (**25a**) and 35-*O*-α-altruronopyranosyl-BH-tetrol (**25a**) isolated from *Prochlorothrix hollandica*. The 35-*O*-β-3,5-anhydrogalacturono- pyranosyl-BH-tetrol (**24a**) was further identified in samples of *Cyanothece* strain [7].

Several cyanobacteria such as *A. cylindrica*, *Crocosphaera* sp., *Mycrocystis* sp., *Synechocystis* sp. and *Trichodesmium* sp. have been shown to produce only non-methylated BH-polyols. A double bond in the pentacyclic ring system was revealed in one case, namely the marine cyanobacterium *Trichodesmium* sp. While tetrafunctionalized BH-polyols predominate in cyanobacteria, pentafunctionalized BH-polyols were found in the *Nostoc* species. Hexafunctionalized BH-polyols have so far not been identified in cyanobacteria.

4.6. Sulfur Reducers

Sulfur-reducing bacteria of genus *Desulfovibrio* contribute to the recent and also to the fossil sedimentary pool of hopane derivatives. Three tested strains of *Desulfovibrio* (BSS2, BSS3 and BSS6) contain considerable amounts of diploptene (**1a**) and diplopterol (**6a**), which are both common hopane derivatives in phylogenetically diverse bacteria. In addition, they contain intact BH-polyols, where the most dominant are BH-tetrol (**2a**) and amino-BH-triol (**3a**). BH-polyols are ubiquitous in recent sediments and are putative precursors of hopane derivatives such as hydrocarbons, alcohols, ketones, and acids which are found in sediments. BH-tetrol (**2a**) and the amino-BH-triol (**3a**) accounted for 89–96% of the total BH-polyols of three *Desulfovibrio* strains. LC-MS analyses showed minor amounts of other extended BH-polyols such as amino-BH-tetrol (**11a**) and an unknown structure with mass-spectral characteristics indicative of BH-polyol. Blumenberg *et al.* [9] studied BH-polyol production by sulfate-reducing bacteria and demonstrated that three species of the genus *Desulfovibrio* (*D. halophilus*, *D. vulgaris* Hildenborough, and *D. africanus*) are devoid of BH-polyols. The lack of BH-polyols in *D. vulgaris* Hildenborough was in accordance with the absence of the squalene-hopene-cyclase in this fully sequenced member of the genus *Desulfovibrio*. In contrast, high amounts of BH-polyols accompanied by diploptene (**1a**) and diplopterol (**6a**) were found in *Desulfovibrio bastinii* grown under strictly anoxic conditions. Major BH-polyols were again BH-tetrol (**2a**) and amino-BH-triol (**3a**). Pentafunctionalized amino-BH-tetrol (**11a**) was found in a trace amount [9].

Sulfur-reducing bacteria commonly thrive in anoxic environments. Due to this fact *Desulfovibrio* was long considered to be obligately anaerobic. However, at present many members of the *Desulfovibrio* are known to cope with low amounts of oxygen while some are even able to grow in the presence of nearly atmospheric oxygen levels. It has been hypothesised that the capability of some *Desulfovibrio* strains to survive in suboxygenated settings is because of the BH-polyol content. BH-polyols might provide a protection against oxygen. Nevertheless, this theory needs further investigation [9].

BH-polyols with a hexafunctionalized side-chain and methylated A-ring BH-polyols were not found in sulfur-reducing bacteria [16].

4.7. Thermophiles

Unusually high content of BH-polyols was found in *A. acidocaldarius* (up to 16 % of the total lipid content) [5,72]. Several analyses revealed the presence of diploptene (**1a**), BH-tetrol (**2a**) and 35-*O*-β-(*N*-acylglucosaminyl)-BH-tetrol (**26a**) [64,65,72]. In *A. acidocaldarius*, growth temperature strongly influences the BH-polyol level of the cells. BH-polyol content increased strongly with increasing temperature and moderately with decreasing pH, whereas hopene showed no significant alteration with temperature and pH [5,73].

A. acidocaldarius is an acidophilic thermophile and it was one of the first organisms in which side chain extended hopanoids were identified [65]. As well as BH-tetrol and BH-tetrol glycoside (**26a**), more complex structures were found incorporating an ω -cyclohexane fatty acid attached to the amino group of the glucosamine *via* an amide bond [72]. Mass chromatograms show the presence of peracetylated acylated BHT glycosides (**27a-30a**). The analysis using the mass range up to *m/z* 2000 showed a series of peaks which were interpreted as N-acylglucosaminyl bacteriohopanetetrols with C15, C17, C19, C21 ω-cyclohexyl fatty acid derivatives attached *via* the amide bond. In all cases the mass spectra were very similar and showed two or three minor ions indicating loss of acetic acid from the $[M+H]^+$ ion and a base peak indicating cleavage of the glycosidic bond between the oxygen atom at C-35 and C-10 of the glucosamine.

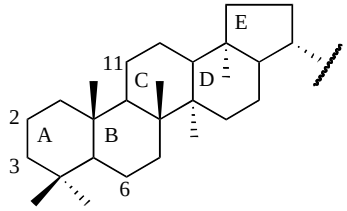
4.8. Anammox Bacteria

Anammox (anaerobic ammonium oxidizing) bacteria form a distinct phylogenetic group within the *Planctomycetes*. The group of anammox bacteria is currently made up of four genera, "*Candidatus* Brocadia", "*Candidatus* Kuenenia", "*Candidatus* Anammoxoglobus" and "*Candidatus* Scalindua" [13]. The recent assembly of the genome of "*Candidatus* K. stuttgartiensis" [74] showed that gene sequences encoding putative squalene hopane cyclases are present. Moreover, study [13] reported that BH-polyols have been identified in all genera of anammox bacteria.

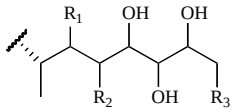
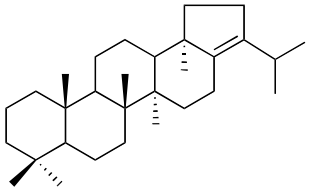
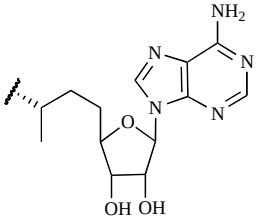
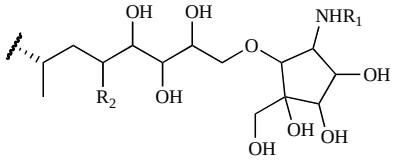
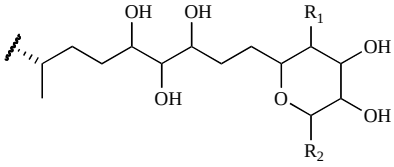
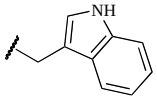
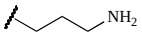
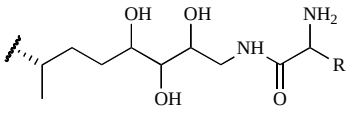
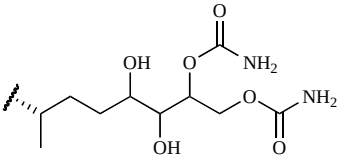
4.9. Strictly Anaerobic Bacteria

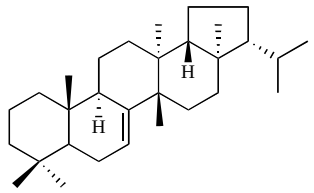
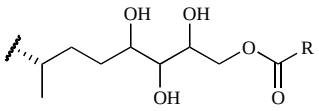
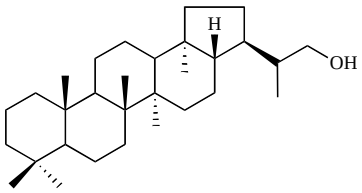
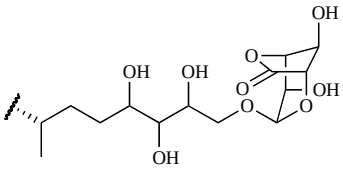
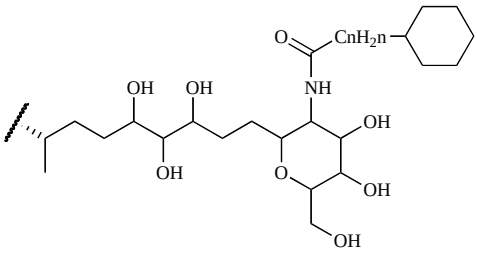
Hartner *et al.* [14] and Fischer *et al.* [15] found BH-polyols in strictly anaerobic species belonging to genus *Geobacter*. In addition, available genome sequencing data of these strains reveal genes that could be involved in BH-polyol formation, e.g. two open reading frames for putative squalene–hopene cyclases [14]. These findings corroborate the assumption that BH-polyol formation does exist in strict anaerobes.

The results of Hartner *et al.* [14] showed that *G. metallireducens* and *G. sulfurreducens* contain squalene and a range of



a – saturated skeleton; **b** - 2β-CH_3; **c** - 3β-CH_3; **d** - Δ^6; **e** - Δ^{11}; **f** - $\Delta^{6,11}$; **g** - 3β-CH_3-Δ^6; **h** - 3β-CH_3-Δ^{11}; **i** - 3β-CH_3-$\Delta^{6,11}$; **j** - 2β-CH_3-Δ^{11}

1 diploptene	
2 bacteriohopane-32,33,34,35-tetrol R_1=H; R_2=H; R_3=OH **3** 35-aminobacteriohopane-32,33,34-triol R_1=H; R_2=H; R_3=NH_2 **4** 35-aminoBH-30,31,32,33,34-pentol R_1=OH; R_2=OH; R_3=NH_2 **5** bacteriohopane-30,31,32,33,34,35-hexol R_1=OH; R_2=OH; R_3=OH **11** 35-aminoBH-31,32,33,34-tetrol R_1=H; R_2=OH; R_3=NH_2 **22** BH-31,32,33,34,35-pentol R_1=H; R_2=OH; R_3=OH **23** BH-30,32,33,34,35-pentol R_1=OH; R_2=H; R_3=OH	
6 diplopterol	
7 hop-17(21)-ene	
8 adenosylhopane	
9 BH-tetrol cyclitol ether R_1=H; R_2=H **12** BH-tetrol cyclitol ether R_1=  ; R_2=H **18** BH-31,32,33,34-pentol-35-cyclitol ether R_1=H; R_2=OH	
10 BH-tetrol glycoside R_1=NH_2; R_2=CH_2OH **25** BH-tetrol glycoside R_1=OH; R_2=COOH **26** BH-tetrol glycoside R_1=NHCO-$(CH_2)_n$-cyclohexyl; R_2=CH_2OH	
13 N-tryptophanyl-35-aminoBH-32,33,34-triol R=  **14** N-ornithinyl-35-aminoBH-32,33,34-triol R= 	
16 34,35-dicarbamoyl-BH-32,33-diol	

17 fern-7-en	
15 35-carbamoyl-BH-32,33,34-triol R=NH_2 **19** phenyl acetic acid monoester of BH-tetrol R=Bz **20** BH-tetrol propionate R=Et	
21 moretan-29-ol	
24 35-*O*-β-3,5-anhydrogalacturonopyranosyl-BH-tetrol	
27 *N*-acetylated BH-tetrol glycoside n = 8 **28** *N*-acetylated BH-tetrol glycoside n = 10 **29** *N*-acetylated BH-tetrol glycoside n = 12 **30** *N*-acetylated BH-tetrol glycoside n = 14	

BH-polyols, i.e. diploptene (**1a**), hop-21-ene (**7a**), and derivatives of BH-polyol. All were identified as bishomohopan-32-ol acetate [14]. Detailed structures have so far not been determined.

Fischer *et al.* [15] found in *G. sulfurreducens* a variety of BH-polyols, which were present in a quantity of approximately 1 $mg.g^{-1}$ of culture wet weight. The major product after H_5IO_6/$NaBH_4$ treatment was a C_{32} derivative obtained from BH-tetrols. This was accompanied by another C_{32} homologue with a double bond. The mass spectral fragmentation pattern indicated that the unsaturation occurred in the side chain. Saturated and unsaturated C_{32} homologues comprised 90% of the total BH-polyols. Further structures were the homohopan-31-ol (9 %) and hopan-30-ol (traces) derived from pentafunctionalized and hexafunctionalized precursors, respectively [15].

Genomic search data indicate that also *Magnetospirillum magnetotacticum* contains a protein homologous to the squalene-hopene cyclase of other known BH-polyol producers. This species commonly grows anaerobically, in or near aerobic-anaerobic transition zones [15].

4.10. Other Bacteria

Z. mobilis is a BH-polyol-producing gram-negative bacterium. It contains high amounts of BH-tetrol derivatives, i.e. BH-tetrol (**2a**), BH-tetrol-glycoside (**10a**) and BH-tetrol cyclitol ether (**9a**) along with minor amounts of diplopterol (**6a**) and diploptene (**1a**) [4].

Z. mobilis produces ethanol and tolerates the presence of high levels of ethanol in the medium. Two possible mechanisms have been proposed to explain the high degree of ethanol tolerance exhibited by this bacterium. In the first mechanism, it is postulated that the high levels of BH-polyols in the cell membranes protect the bacteria from the toxic effects of ethanol [34,54]. In the second mechanism, it is postulated that the bacterium is protected from ethanol toxicity by the high levels of cis-vaccenic acid in the phospholipids of the bacterial membrane. In the absence of ethanol, BH-polyols comprised about 30% (by mass) of the total cellular lipids. The addition of ethanol to culture media caused complex changes in the levels of BH-polyols and other lipids. When the cells were grown either aerobically or anaerobically, the addition of ethanol (3% or 6%) reduced the levels of total BH-polyols [54].

Recent taxonomic changes modified the classification of the bacteria formerly related to the genus *Pseudomonas*, and separated the new genera *Burkholderia* and *Ralstonia* from the genus *Pseudomonas*. Revision of the BH-polyol distribution in these species was therefore done in order to check whether these triterpenoids are useful markers for a chemotaxonomic approach. Whereas no BH-polyols were found in any strain belonging to *Pseudomonas* and *Ralstonia* spp., they were present in all strains related to *Burkholderia*. Bis-homohopan-32-ol was the major hopane derivative in all strains. It was accompanied by significant amounts of its unsaturated analogue with a double bond at C-6. Homohopan-31-ol and homohop-6-en-31-ol were identified in much lower concentrations than those of their C_{32} homologues in nearly all strains. Diploptene (**1a**) and diplopterol (**6a**) were both present in small amounts in most BH-polyol producing strains. The BH-tetrol carbapseudopentose ether (**9a**) was isolated from almost all tested

strains. The saturated BH-polyol were accompanied by significant amounts of the unsaturated BH-6-enetetrol carbapseudopentose ether (**9d**). The presence of unsaturated BH-polyols is an extremely rare feature outside the group of the acetic acid bacteria. The BH-polyol fingerprints in the *Burkholderia* species were quite homogeneous.

The absence of any detectable BH-polyols in *Pseudomonas* and *Ralstonia* species might just be the consequence of a lack of expression of this biosynthetic pathway in the culture conditions used [32].

In the case of the actinomycete *S. coelicolor* BH-polyols were either not found or present only in traces in cells grown in liquid culture while some were found in cells grown on solid media whilst sporulating. Besides minor amounts of hopene, the only detected BH-polyol was amino-BH-triol (**3a**) [17]. Also *Streptomyces griseus* has been found to produce BH-polyols. The BH-polyol content was measured after growth in liquid medium in a similar amount as in a diferentiated culture of *S. coelicolor*. These results are an indication that BH-polyol synthesis is coupled to differentiation [17].

The research performed by Poralla *et al.* [17] showed that the expression of BH-polyol synthesis genes in *Streptomyces* is connected with aerial mycelium formation, which is accompanied by osmotic stress. The membrane condensing effect of BH-polyols very likely helps to minimize the diffusion of water out of the cytoplasm, thereby protecting cells against desiccation [17]. This hypothesis was challenged by Seipke and Loria [75], who reported the first analysis of a hopanoid mutant in any genera of bacteria. The results obtained with *Streptomyces scabies* implied that Poralla's hypothesis is may not be extended beyond *Streptomyces coelicolor*, documented that hopanoids are not required for normal growth or for tolerance of ethanol, osmotic and oxidative stress, high temperature, or low pH, and suggested that hopanoids are not essential for normal streptomycete physiology.

4.11. Sediments

Triterpenoids of the hopane series are widespread in the organic matter of sediments [48]. Their distributions within recent sediments represent an important class of biological marker compounds, preserving information regarding the inputs of bacterial biomass to sediments, and the palaeoenvironmental conditions prevailing at the time of deposition [3]. The presence of BH-polyols in recent and ancient sedimentary organic matter reflects the resistance of the polycylic hydrocarbon skeleton to both biotic and abiotic degradation [8,15]. Nevertheless, the BH-polyols found in geological samples are mostly diagenetic products, i.e. compounds predominantly biosynthesised by certain bacteria. Although specific functional groups within the precursor BH-polyols are lost or modified during diagenesis, the relatively stable carbon skeleton enables the resulting hopanoids (e.g. hopanoic acids, hopanols, hopenes, BH-polyols and their aldehydes or ketones) to be used as biomarkers even in ancient sediments which have experienced long-term burial and heating, and in oils [3].

An analysis of BH-polyols or their degradation products from sediment extracts is widely mentioned in the literature. The results revealed a range of structures including tetra-, penta- and hexafunctionalised methylated and non-methylated components [4]. Talbot *et al.* [4] even reported the observation of an unsaturated composite BH-polyol in the sedimentary environment. However, the most commonly reported BH-polyol in sediments and soils is BH-tetrol, which is found in a diverse suite of organisms and so is not diagnostic for any particular group of organisms or processes [26].

However, many BH-polyols are restricted to a limited number of organisms or a group of organisms, offering much more specific bacterial marker information [26]. As an indicator of aerobic methane oxidizing bacteria in the sample can serve BH-polyols with a NH_2 group at C_{35} [26,49]. The hexafunctionalised side chain structure, amino-BH-pentol (**4a**), is one of the most diagnostic, having been found only in Type I methane oxidizing bacteria [26,49]. Apparent association of 2-methyl-BH-polyols with cyanobacteria was described above, and hence may serve as a biomarker proxy for oxygenic photosynthesis [8]. Composite structures are abundant in acetic acid bacteria [40,67] or cyanobacteria [4].

For a long time, BH-polyols in sedimentary environments were considered to be potential indicators of solely aerobic organisms and processes [16]. This is now known to be false. BH-polyol production occurs in anaerobic sediments and in common environmental groups such as the *Planctomycetales*, *Geobacteraceae* and *Desulfovibrio*s [8,12,14-16].

Belin [48] investigated BH-polyol distribution in oxic and anoxic sediment samples from mountain lakes. He found the distributions of hopanol and homohopanol quite similar in the sediment samples obtained from oxic and anoxic zone. The content of bishomohopanol was higher in the samples from oxic zone [48].

On the other hand, Talbot *et al.* [7] investigated the diversity of the distributions of BH-polyol structures in samples from polar and hot environments and found that the distributions in natural settings are highly variable. The polar samples (sediment and microbial mat) showed the greatest diversity, including a wide range of structures, including a group of tetra and pentafunctionalised methylated, non-methylated and unsaturated compounds, ascribed to cyanobacterial sources. Less variation was observed in the samples from hot environments (deserts and hot springs) although it is clear from other studies that this is not necessarily always the case [7].

CONCLUSION

Hopanoids are attracting an ever increasing attention as important, though as yet not thoroughly explored, natural compounds with essential functions in some microorganisms and plants. The future of hopanoid chemistry and analysis can be seen in two areas. The first is the use of new ionization techniques such as, e.g., atmospheric pressure photoionization mass spectrometry, MS/MS combination, and also the use of ultra performance liquid chromatography. The other area offers the possibility of analyzing a much larger spectrum of samples. To our knowledge, LC-MS/APCI has been used for analyzing only hopanoids from only a few species of bacteria or cyanobacteria, especially those that are found in collections and do not give any problems in cultivation. Virtually completely unexplored are organisms from unusual areas – see the finding of BH-hexols from Antarctica. We foresee an explosive development in this area in the next decade.

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