Hopanoids. 2. Biohopanoids: A Novel Class of Bacterial Lipids

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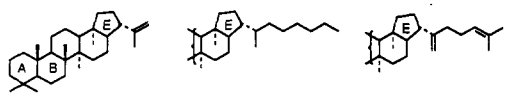
Introduction

This Account describes a novel family of bacterial lipids, the "biohopanoids", whose existence was unsuspected until their molecular fossils (the "geohopanoids", varied and widespread, described in Part 1 of this series) happened to be found in sediments and identified. These biohopanoids are abundant and diverse, and in the bacteria containing them, they are essential for life, at least as membrane constituents, surrogates of the cholesterol of the membranes of higher organisms. Their study, begun by Rohmer in Strasbourg, has so far been nearly entirely pursued in his laboratory in Mulhouse and in Karl Poralla's in Tübingen, while the Strasbourg group has pursued biophysical studies on these substances. The study of biohopanoids has led to new hypotheses on the biochemical evolution of lipidic membrane constituents on the way to cholesterol and to a new dogma: terpenoids are fundamentally membrane constituents and only accessorily anything else, e.g., scents, hormones, vitamins, etc. It has led to the discovery of a novel general biosynthetic pathway for the first stages of the biosynthesis of terpenoids in bacteria. It has also led Karl Poralla to the first isolation and sequence determination of a squalene cyclase and still raises many exciting biochemical and biomedical questions.

Discovery of "Extended Hopanoids"

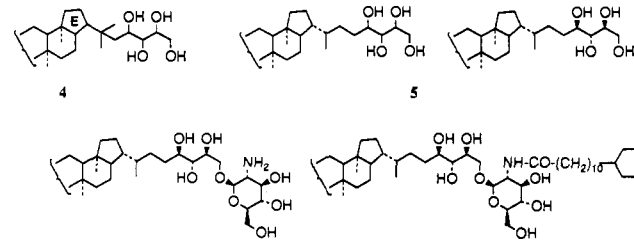
As we have noted in the preceding Account in this issue, a most embarrassing problem was posed by the discovery of geohopanoids in sediments: their ubiquitous presence called for a general interpretation, and this had to "explain" not only the presence of substances derived from the known hopane skeleton, such as diploptene (hop-22(29)-ene) **1**, but also that of the "extended hopanes" based on skeleton **2**. Diploptene



1 **2** **3**

was found in several unicellular organisms, and this was proposed by Bird et al. to explain the presence of some of the C_{30} or smaller geohopanoids then known.[1] However, aging might have explained all the degradative changes observed, but not the acquisition of additional carbon atoms in the side chain, and it had been proposed by Whitehead that this could have arisen from the cyclization of a C_{35} isoprenolog of squalene to an homolog of hopene bearing an additional isopentenyl unit, as in **3**.[2] This in turn became untenable once we had shown by synthesis that the additional carbon atoms attached to hopane were in the form of a straight chain. In another context, Ch. de Gaulle said: "La vieillesse, quelle déchéance!" This rule of entropy increase linked with senescence must apply to sedimentary molecular fossils as well as to statesmen and excludes the idea that standing in a sediment could provide a molecule with such an improbable appendix as an *n*-pentyl chain. The C_{35} geohopanoids, and by extension all geohopanoids, were therefore "orphan" fossils: they had to have had parents, but these were still unknown.

The riddle began to clear when Förster et al. isolated, from a cellulose-producing bacterium *Acetobacter xylinum*, bacteriohopanetetrol (BHT), a hybrid of hopane and a C_5 sugar, for which they proposed structures **4** and **5**, favoring the first.[3] They noted the highly amphiphilic nature of BHT and thought this justified their study of this particular strain: it could explain, by the surfactant properties of BHT, the orientation into fibrils of cellulose micelles synthesized by the bacterium. We repeated their work and proved that **5**, the structure "needed" if BHT were to serve as a precursor of geohopanoids, was indeed correct with the stereochemistry indicated.[4] Independently and simultaneously, Langworthy et al. also isolated the same substance and its glucosamine derivatives **6** and **7** in the thermo-acidophilic *Bacillus acidocaldarius* and gave them their correct structures.[5] They, too, noted the amphiphilic nature of these substances, from which they deduced that the compounds could be membrane constituents, and they "explained" their unusual structures as needed



4 **5**

6 **7**

Guy Ourisson, born in 1926, was educated in Paris (Ecole Normale Supérieure; Dr.Sc., G. Dupont, 1954) and at Harvard University (Ph.D., L. F. Fieser, 1952). In 1955, he was appointed to the University of Strasbourg, where he has since been a Professor. From 1971 to 1975, he was Founding President of the new Université Louis Pasteur. From 1985 to 1989, he was Director of the CNRS Institut de Chimie des Substances Naturelles in Gif-sur-Yvette, France.

Michel Rohmer, born in 1948, was educated in Strasbourg, where he obtained his Doctorate in 1975 (G.O., P. Benveniste), and at Stanford University, where he completed postdoctoral work with C. Djerassi. He was Instructor in Pharmacognosy in the Pharmaceutical School of Université Louis Pasteur and in 1979 was appointed Professor of Organic Chemistry at the Université de Haute Alsace in Mulhouse, France.

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(2) Whitehead, E. V. *Adv. Org. Geochem. 1972* **1973**, 187.
(3) Förster, H. J.; Biemann, K.; Haig, W. G.; Tattrie, N. H.; Colvin, J. R. *Biochem. J.* **1973**, *135*, 133–143.
(4) Rohmer, M.; Ourisson, G. *Tetrahedron Lett.* **1976**, 3633–3636.
(5) Langworthy, T. A.; Mayberry, W. R. *Biochim. Biophys. Acta* **1976**, *431*, 570–577. Langworthy, T. A.; Mayberry, W. R.; Smith, P. F. *Ibid.* **1976**, *431*, 550–569.

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to ensure the extreme resistance of *B. acidocaldarius* to hot acid. In agreement with this hypothesis, eight years later Poralla et al. confirmed that the content of BHT increases markedly in *B. acidocaldarius* when the growth temperature is increased from 50 to 65 °C and the pH decreased from 5 to 2.5.[6]

Some potential precursors of the C_{35} geohopanoids had thus now been identified, but others had to be found besides those isolated from strains of bacteria studied from their particular properties. We thus embarked on a systematic search of biohopanoids in microorganisms.[7] The finding that they were indeed often present, in many groups of bacteria, solved the initial riddle, but only to pose other questions: we now had to understand which biological role(s) these novel microbial constituents could play, and how they were biosynthesized. This Account summarizes this work.

Biohopanoids of Bacteria

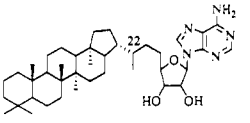
The discovery of diploptene in some ferns, bacteria, and cyanobacteria had been noted by Bird et al.[1] as potentially relevant for the interpretation of origin of the few C_{27}, C_{29}, and C_{30} hopane derivatives isolated a short time earlier in one crude oil. The systematic search we then undertook was influenced by the recognition that bacterial hopanoids might well be related to BHT and thus be highly polar. We therefore treated the crude extracts with periodate to cleave any polyol, then with borohydride to reduce the resulting aldehydes to primary alcohols, and finally with acetic anhydride to obtain the corresponding acetates, which could be separated by chromatography and characterized structurally.[7] Of course, this can only be a preliminary step: it leaves unattacked any derivative presenting no 1,2-diols, does not define the exact nature of any polyhydroxylated side chain clipped off, etc. Thus, isolation and identification of each *intact bacteriohopane derivative* had to be developed. For instance, after peracetylation, separation can be achieved by HPLC, and NMR/MS characterization of the pure acetates can lead to the proposal of hypothetical structures, authenticated definitely (or corrected) by comparison with synthetic samples or by strict chemical correlations.[8]

The solubility of many biohopanoids, linked with their amphiphilic character, is so low that failure to find any of them in a given strain may, in some cases, only reflect the presence of novel structures and/or the inefficiency of our isolation methods. Some "hopanoid-free" strains may therefore later turn out to be untapped sources of novel hopanoid structures.

How could chemists run a reliable survey of the distribution of biohopanoids in the microbial world?[7] We were in danger of running a very biased survey by selecting, for their easy availability, microbial strains too closely related taxonomically to be significantly different. We were fortunately guided very efficiently by the late Roger Stanier, of the Institut Pasteur, who selected for us strains covering some of the major groups of bacteria and cyanobacteria (excluding, for reasons obvious in a chemical laboratory, pathogenic strains).

In fact, the results of this study were still more exciting than we could have anticipated:[7] biohopanoids were found to be very widespread and inordinately varied. Some taxonomic families of microorganisms, for instance the Archaeales (Archaebacteria), the Chromatiaceae, or the Enterobacteria, appeared to be free of biohopanoids (with the important caveat noted above), but biohopanoids were encountered in numerous strains of Gram-positive and Gram-negative eubacteria (and cyanobacteria). However, in tighter taxa, e.g., in *Pseudomonas*, some strains did and some did not give evidence of the presence of biohopanoids. Surprisingly, although the biosynthesis of hopanoids is independent of dioxygen, these triterpenoids have not been found in the few strict anaerobes examined until now. They were however present in most Rhodospirillaceae grown either under photosynthetic and anaerobic conditions or under heterotrophic and microaerophilic conditions in the dark. As it is highly probable that the distribution of biohopanoids does follow some taxonomic rules, the matter should be reinvestigated when a less time-consuming and less leaky isolation method becomes available; for instance, direct laser desorption mass spectrometry on the intact bacterial cells might prove workable.

From a structural point of view, the results have been just as surprising. The structures defined so far, nearly exclusively in Rohmer's laboratory, are represented in Chart I. They form a novel and colorful family of bacterial lipids, combining the triterpene skeleton of hopane C–C linked to polyols, as in **5**,[3,5,8] or their glucosamine conjugates such as **6**, **7**,[5] ketopolyols,[9] aminopolyols,[10] carbamic acid derivatives,[11] aminocyclitols,[12] peptides,[13] and even the very remarkable C–C linked adenosine **8** (Chart I).[14] The pentacyclic system itself can bear an additional methyl group at C-2 or C-3[15] (more on this point later) and additional double bonds at 6(7) and/or 11(12).[8,16] The side chain is usually the *n*-pentyl one of BHT, but it can also be shorter, with only 3 carbon atoms,[8] or probably longer, as judged by the occurrence of the corresponding C_{36} molecular fossils in sediments. The bacteriohopane derivative most frequently found so far is not BHT **5**, but the corresponding 35-amino triol, BHAT.



Adenosylhopanes (22R and 22S)

8

The structures indicated have been mostly derived from an analysis of the NMR and MS data and from

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(8) Peiseler, B.; Rohmer, M. *J. Chem. Soc., Perkin Trans.* **1991**, 2449–2453. Peiseler, B.; Rohmer, M. *Eur. J. Biochem.*, submitted for publication.

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Scheme I
Lack of Substrate Specificity in the Biosynthesis of Biohopanoids

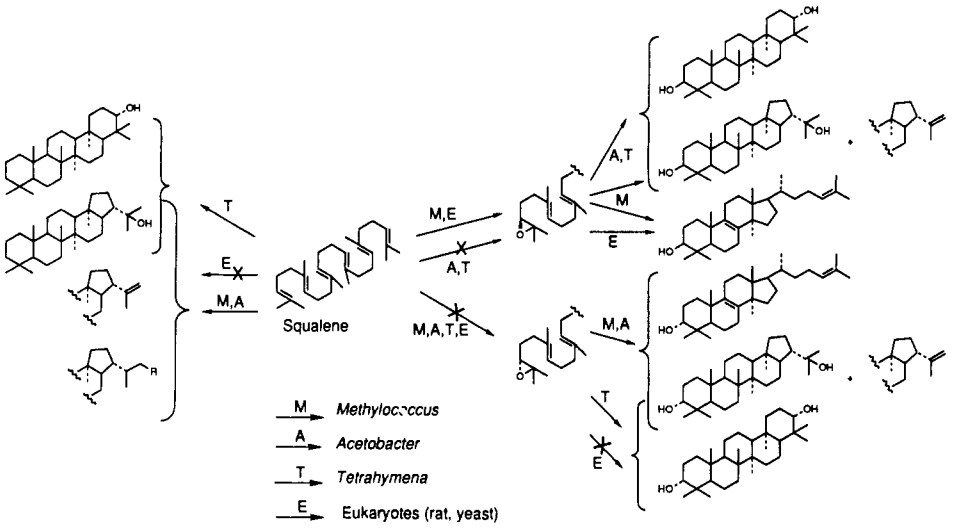
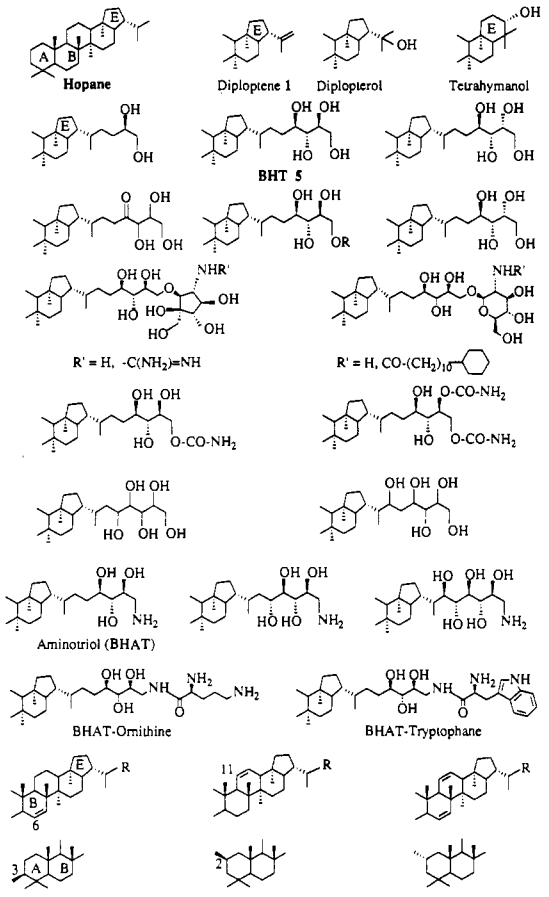


Chart I
Biohopanoids; Structural Variants



the correlation by degradation and partial synthesis with diploptene. The synthesis of all eight diastereomers of BHT[17] and that of the two diastereomers of the C_{33} diols of *Acetobacter aceti ssp. xylinum*,[8] the use of CD exciton chirality measurements with BHAT and other aminopolyols,[18] as well as the recent isolation and full structure determination, in two strains of *Acetobacter*, of 13 different, individually characterized C_{35} and C_{33} polyols, with or without ring double bonds and/or 3β-methyl groups, some of which represent less than 1% of the BHT fraction,[8] are some of the major developments in this field. The finding of these (obviously optically active) precursors of the geohopanoids provides the answer to a question often asked: are the geohopanoids optically pure? They are, in particular, because any optical impurity would lead to diastereomers. The same question cannot be answered as firmly in the case of the tricyclopolyprenanes mentioned in the preceding Account: we do not know their bio-precursors, and they might even have been formed abiotically from acyclic precursors.

Biosynthesis of Biohopanoids

The structure of bacterial hopanoids suggests a number of questions regarding their biosynthesis. The pentacyclic skeleton of hopane must of course be derived from squalene by cyclization, and the squalene-hopene cyclase of *B. acidocaldarius* is the first cyclase to have been purified, partially sequenced by degradation, cloned in *Escherichia coli*, expressed, and sequenced through its gene.[19]

The squalene-hopene cyclases of microorganisms have a very low substrate specificity. As cell-free systems, *Acetobacter* (giving hopanoids), *Tetrahymena* (giving hopan-22-ol besides much tetrahymanol), and *Methylococcus* (giving hopanoids and, through an accompanying epoxysqualene cyclase, lanosterol) cyclize not only the natural substrate, squalene, but also both enantiomeric squalene epoxides to give mixtures of 3α- and 3β-hydroxyhopanes, while the epoxysqualene cyclase of *Methylococcus* transforms both its natural substrate (3*S*)-epoxysqualene and its (3*R*) enantiomer to give a mixture of lanosterol and its unnatural 3α epimer (Scheme I).[20] This is in sharp contrast with the strict substrate specificity of the epoxysqualene cyclases of eukaryotes (yeast and mammals), which accept only (3*S*)-epoxysqualene. The cyclases of microorganisms are in fact still less choosy, as they also cyclize more

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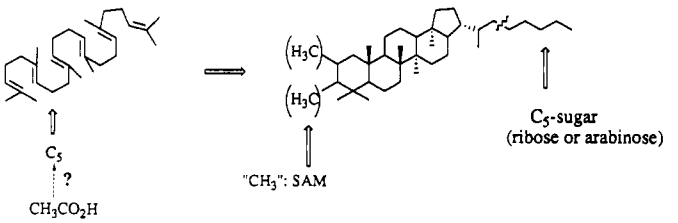
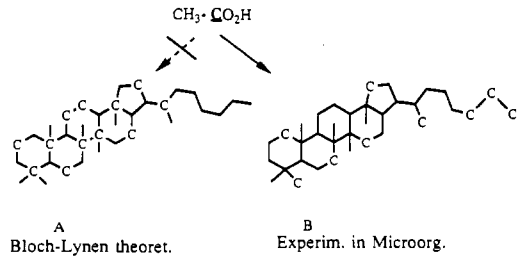
Scheme II
Origin of the Various Parts of the Bacteriophane Skeleton



foreign substrates: smaller or larger polyterpenes[21] or dihydrosqualene (giving unnatural triterpenes).[22]

The reactions by which the C_5 side chain is attached to the triterpene skeleton are not known. The labeling pattern observed in the side chains of the hopanoids from *Rhodopseudomonas palustris*, *Rh. acidophila*, and *Methylobacterium organophilum* fed [1- and [2-^{13}C]-labeled acetate and of BHT from *Zymomonas* fed with ^{13}C-labeled glucose[23] is in agreement with their derivation from a pentose intermediate linked via its C-5 carbon atom to the triterpenic framework. The configurations demonstrated for the BHT isomers and analogs would then suggest ribose and arabinose as the C_5 unit (Scheme II). One can of course surmise from the nature of adenosylhopane 8[14] that such a molecule or the still unknown ribosylhopane might play an intermediate role, as it is a structural hybrid of BHT and the corresponding amino triol. But this is pure speculation at present, and the passage from C_{30} to C_{35} remains an interesting problem. Similarly, the origin of the C_3 side chain of the C_{33} trishomohopanediols is not clear: they could be either products of degradation of bacteriohopane derivatives or native metabolites arising from hopene and a C_3 unit such as glycerol or glyceraldehyde.[8]

In the ^{13}C-labeled acetate and glucose incorporations mentioned,[23] the hopane skeleton itself was found by NMR studies *not* to bear the ^{13}C labels at the positions expected from the classical Bloch–Lynen route from acetate to the prenyl units; this implies a different pathway, yet unknown, to mevalonate. The details and the consequences of such a remarkable dichotomy in biosynthetic pathways remain to be explored; they obviously offer some promise for the discovery of selective inhibitors of polyterpene biosynthesis in bacteria and not in their hosts, a potentially useful perspective in therapeutics or agriculture.[24]



Incorporation of labelled acetate into bacteriohopane:
A: expected; B: Found

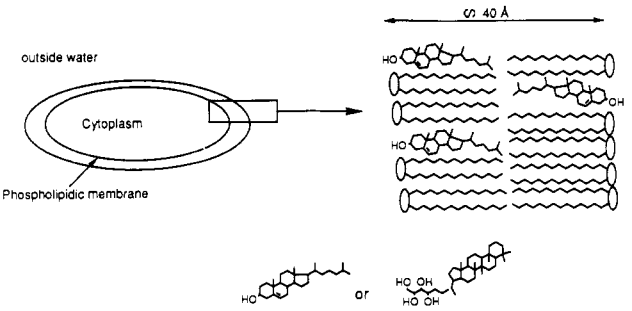


Figure 1. Reinforcement of phospholipid vesicles by cholesterol or BHT.

Another problem was raised by the 2α- and 3β-methylated hopanoids initially found as hydrocarbons in sediments and later identified with the appropriate polar side chains in several bacteria.[15] By analogy with known methylations, we postulated that the methyl group of these novel triterpenes might be provided by the methyl group of *S*-adenosylmethionine; this was proved by the incorporation of ^{14}C-, ^{3}H-, or ^{2}H-labeled *S*-adenosylmethionines, with full retention of the three hydrogen atoms, into 2α- and 2β-methyldiplopterol in *M. organophilum* and 3β-methyl-BHT in *A. pasteurianus*.[15] The concentration of methionine in the growth medium is the limiting factor: adding this amino acid in large amounts dramatically enhances the 3-methylation of the hopanoids (up to 60% of the total content, in *A. pasteurianus*).

Role of Hopanoids in Membranes

We mentioned above the hypothesis linking the resistance of *B. acidocaldarius* to hot acids to the presence of BHT in its membranes. It appears to be true that high concentrations of BHT in bacteria can be correlated with stress conditions: high temperature and acidity[6,7] or high ethanol concentration.[25] However, their distribution extends, as we have seen, to strains living in more normal niches.

We have linked this observation with the classical dispute about the presence or absence of sterols in bacteria. While it is established that some particular strains do contain amounts of sterols comparable with those of eukaryotic cells, such is usually not the case, and the traces found (which in many cases are apparently contaminations) are not compatible with a structural role comparable to that of cholesterol or phytosterols in higher organisms. This role implies

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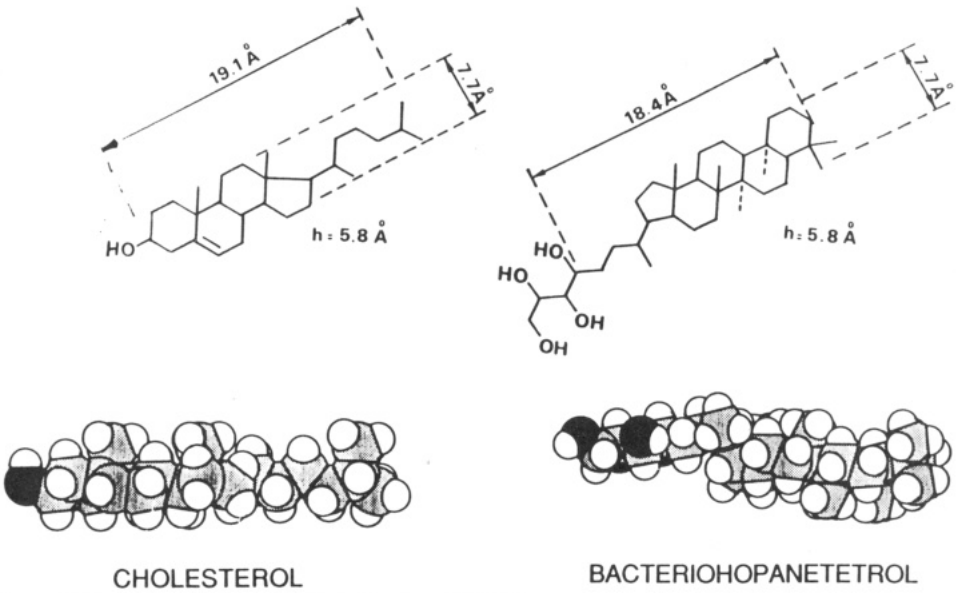

Figure 2. Comparison of the size and shape of cholesterol and BHT.

relatively high concentrations in membranes, where the sterols play the role of rigid, oriented inserts, exerting a condensing effect on the phospholipid partners to improve the mechanical strength and the impermeability to water (Figure 1).

We thus proposed that biohopanoids, or at least BHT, could be cholesterol surrogates in bacteria.[26] This is in agreement with their general dimensions and amphiphilic nature (Figure 2) and is now rather firmly established by the following methods: (1) experiments using Langmuir's classical film balance, showing that BHT and its complex glycosides exert a condensing effect similar to that of cholesterol on phospholipid monolayers;[27] (2) the diminution of permeability of phospholipid vesicles[28] and the enhancement of stability of liposomes[29] containing amphiphilic hopanoids; (3) solid-state NMR spectroscopy of stacked bilayers, using a deuterated phospholipid probe to measure the order of the chains in bilayers;[28] (4) molecular modeling, showing their close analogy with cholesterol (Figure 2, bottom); (5) in vivo, in the absence of sterols in the growth medium, the quasihopanoid tetrahymanol is biosynthesized by the protozoan *Tetrahymena*, which stops producing it if it can import sterols from its environment.[30]

Other Biological Roles of Biohopanoids

While it can be considered established that BHT and its near analogs are indeed cholesterol surrogates in bacterial membranes, it looks rather improbable that the most elaborate biohopanoids, for instance the aminocyclitol, the peptides, or mostly the adenosylhopanes, are limited to such a passive role. However, so far no other function is known for these metabolites. More work will be required to show whether these complex biohopanoids, instead of being sterol surrogates, are not rather steroid surrogates, playing more specific roles. What has been shown, which may be of potential practical application in devising new types of antibiotics, is that specific squalene cyclase inhibitors prevent the growth of hopanoid-synthesizing bacteria.[31] Also, 30-hopanylethanol (incorrectly named bacteriohopan-32-ol) has been found to be cytotoxic against two strains of leukemia cells.[32]

It will also remain to be seen whether the very preliminary indications obtained by some medical investigators converge to show that biohopanoids could play a role in certain pathological conditions.

Conclusion

Once again, it seems appropriate to call attention to the very particular circumstances which have led us stepwise, from answer to question, from a study of petroleums to that of these novel polyterpenes. It appears to be the first time that a whole family of natural products has been discovered through their molecular fossils.

The function assigned to hopanoids as membrane reinforcers has also led us to postulate a similar role, in hopane-free bacteria, for the α,ω-dipolar carotenoids so often present in bacteria and to show that this is indeed the case.[33] It has also led us further astray, in postulating a phylogenetic derivation of terpenes involved in membrane stabilization and in assigning to polyterpenes the general role of membrane reinforcers.[34]

This whole story is an excellent example of the difficulty of planning research when it is not limited to obtaining answers already expected, but also of the fruitfulness of an "open chase", not limited by overspecialization.

We thank our many partners and students on this long trail; the names of some of them are mentioned in the references. However, G.O. owes a special gratitude to Pierre Albrecht and Michel Rohmer, the principal actors in the story told in this and

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the preceding Account; they have let me use freely their work, and let me enjoy the illusion that what they do on their own is still partly mine, an illusion I also enjoy with my children and grandchildren; to Yoichi Nakatani, who has made it possible to engage in the biophysical developments only superficially mentioned here but essential; and to Marie-Claire Dillenseger, who has often been more ambitious than myself for what we were doing and who has constantly challenged me to do better.

Mapping the Genesis of Helical Structure in Polymers of the Trihaloacetaldehydes

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Macromolecules are capable of occupying a host of different conformational forms. These range from randomly coiled wormlike chains to more spatially ordered structures. Helical geometries are encountered when internal rotational angles along the polymer backbone can repeat in a regular, sequential fashion. Widely recognized native helical structures include the right-handed α-helix[1] found in many proteins and poly(α-amino acids) and single-, double-, and even triple-stranded helical arrangements common to the nucleic acid family of biopolymers.[2] Synthetically derived macromolecules can also adopt helical architectures. In contrast to their naturally occurring cousins, however, many of these polymers lack secondary bonding interactions which help to stabilize helical order in solution. Here, helicity is often confined to the solid state where crystalline packing forces encourage the formation of compact helical coils. Over the past five decades, a variety of helical motifs have been identified through X-ray diffraction studies.[3] For the stereoregular polyolefins and polyaldehydes, these include both right- and left-handed helices with 3_1, 4_1, and nonintegral 7_2 arrangements[4] of the polymer backbone.

The loss or absence of helical structure can have a profound impact on the behavior associated with a macromolecule. The physiochemical and biological consequences of helix to random coil transitions in nucleic acids and proteins have been a subject of intense scrutiny.[5] For synthetic polymers, including many of

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