Pure & Appl. Chem., Vol. 65, No. 6, pp. 1293–1298, 1993.
Printed in Great Britain.

The biosynthesis of triterpenoids of the hopane series in the Eubacteria: A mine of new enzyme reactions

Michel Rohmer

Ecole Nationale Supérieure de Chimie de Mulhouse, Université de Haute Alsace
3 rue Alfred Werner, 68093 Mulhouse Cedex, France

Abstract. Incorporation of ^{13}C labelled acetate or glucose into the hopanoids, a triterpenic series widespread in Eubacteria, threw light on two unique features of terpenoid biochemistry: i) a carbon/carbon linkage between a triterpenic moiety and a D-pentose in the bacteriohopane skeleton and ii) a new biogenetic pathway for the formation of the isoprenic units, fully different from the classical Bloch-Lynen route determined for Eukaryotes and Archaebacteria.

STRUCTURAL DIVERSITY OF BACTERIAL HOPANOIDS

Triterpenoids of the hopane series are widespread in bacteria and now well known. The first and also the most readily detected ones, diploptene **1** and diplopterol **2** (Fig. 1), have been already described in the early 70's (ref. 1-4). These triterpenes with the usual C_{30} skeleton are present, at least in traces amounts, in all hopanoid synthesizing bacteria (ref. 4). The major triterpenoids in these microorganisms are however always the C_{35} bacteriohopanepolyol derivatives which present the unusual feature of an additional C_5 unit linked by a carbon/carbon bound to the isopropyl group of the hopane framework (ref. 4-6). Structural variations in the bacteriohopane series are numerous and affect the pentacyclic ring system (presence of an additional methyl group at C-2α, C-2β or C-3β and introduction of double-bonds at C-6 and/or C-11) (ref. 7-10) as well as the side-chain (Fig. 1). Up to now 23 different bacteriohopanepolyol side-chains have been identified, the most common compounds being derived either from aminobacteriohopanetriol **3** or from bacteriohopanetetrol **4** (Fig 1). Structural modifications of these two basic side-chains include:

- the presence of one or two additional hydroxy groups at C-30 and/or C-31 (hopanoids **18**, **19**, **20** and **21**) (ref. 8, 11, 12),
- carbamoylation of the hydroxy group at C-35 or at C-35 and C-34 (**16** and **17**) (ref. 13),
- methylation of the C-35 hydroxy group (**6**) (ref. 14),
- introduction of polar moieties at C-35, *e.g.* carbohydrates linked to the hydroxy group via glycosidic or ether bonds (hopanoids **9** to **14**, **25**) (ref. 15-19), or amino-acyl residues linked via a peptidic bond to the amino group (hopanoids **7** and **8**) (ref. 20),
- presence of the two diastereoisomers at C-34 in the bacteriohopanetetrols **4** and **15** (only observed in the *Acetobacter* species) (ref. 21) or at C-22 (only known for the saturated bacteriohopanetetrols **4** and **5** and the adenosylhopanes **22** and **23**) (ref. 13, 22).

The combination of all known structural variations would lead to an enormous number of possible compounds. Many have been already isolated and identified, but the list is far from being closed. Indeed, in several hopanoid producers we are looking at, new compounds have been detected and are under investigation.

This diversity of side-chain structures hampered until now the development of a simple and general method for the detection of intact bacteriohopanepolyols. Simultaneous analysis of all intact

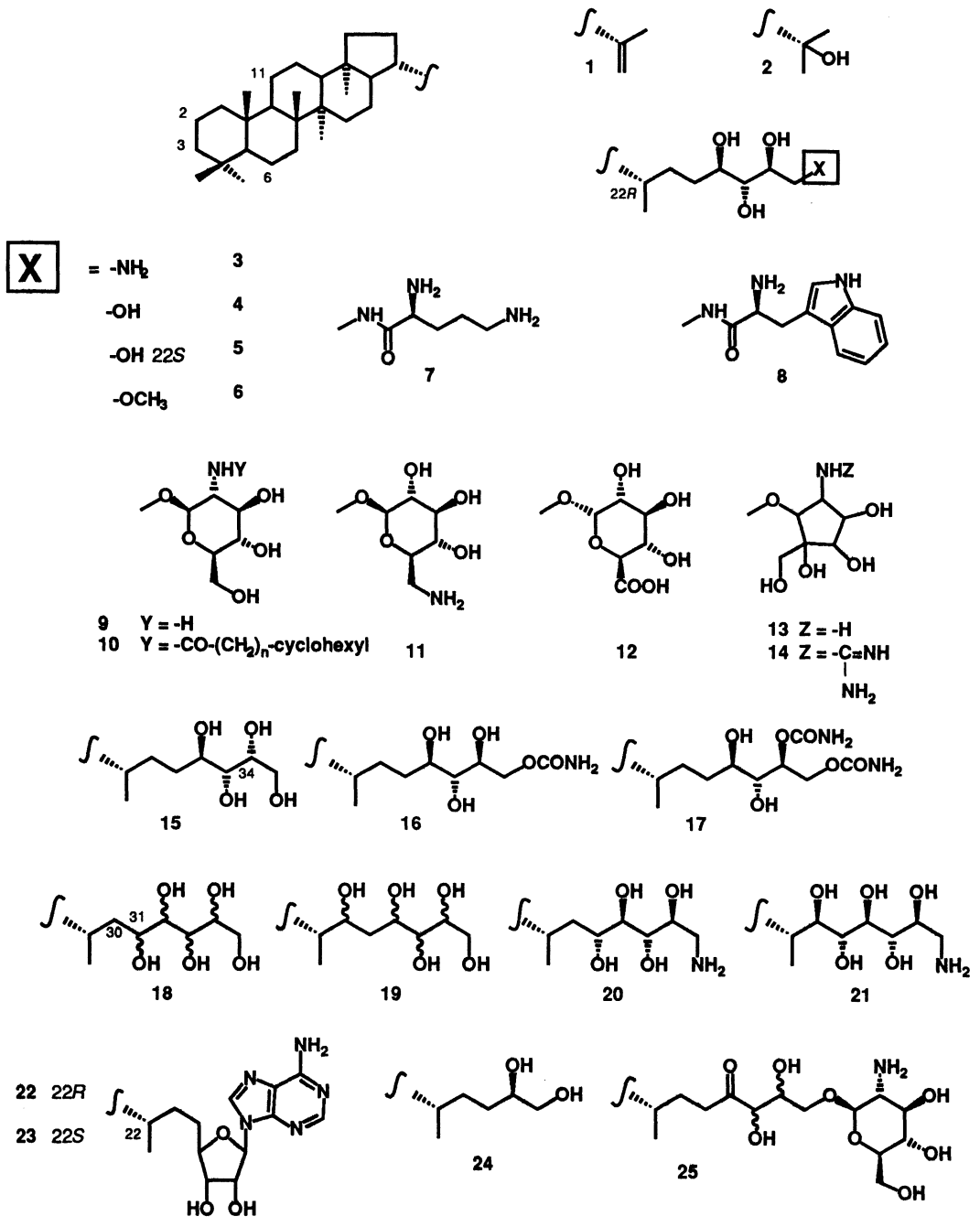

Fig. 1. Bacterial hopanoids.
Structural variations on the pentacyclic skeleton include introduction of an extra methyl group at C-2α, C-2β or C-3β and of double-bonds at C-6 and/or C-11.

acetylated hopanoids from *Zymomonas mobilis* by HPLC has been for instance successfully developped (ref. 23), but, as the hopanoid composition is fully different from one bacterium to another, the method has to be adapted to each case. Detection of hopanoids is until now directly performed on the crude $CHCl_3/CH_3OH$ extract by periodic acid oxidation followed by $NaBH_4$ reduction. This treatment converts bacteriohopanepolyols into primary alcohols which are readily isolated by TLC and analyzed after acetylation by GLC and GLC/MS (ref. 4, 22) or after naphtoylation by HPLC (ref. 24). Although nearly all

information concerning the side-chains is lost by this degradation method, it is until now the most suitable one for the detection of hopanoids in bacteria. It has been widely utilized for the screening of about 200 strains, giving a first glance at the distribution of bacteriohopanepolyols.

The C_{35} bacteriohopane derivatives are characteristic metabolites of the sole Eubacteria (ref. 4). They have never been detected in Archaebacteria or in Eukaryote cells, the traces found for instance in some mosses or higher plants being only the mark of the presence of hopanoid producing bacteria living on the leaf surface (*e.g.* facultative methylotrophs of the genus *Methylobacterium*) (M. Knani and M. Rohmer, unpublished results). Although hopanoids have been found in numerous species, no clear-cut conclusions could be drawn until now on their repartition. Indeed, the negative rsults call for some comments. Hopanoids without suitable 1,2-diol groups (such as **19**, **22**, **23**, **25**) can not be detected by the $H_5IO_6/NaBH_4$ degradation method. Furthermore, those eventually linked to biological macromolecules (polysaccharides, peptides) are most probably not extracted by $CHCl_3/CH_3OH$. Finally, the negative results have been obtained in most cases with cells obtained in single growth conditions. As hopanoid biosynthesis is largely dependent on environmental factors such as pH, temperature or composition of the culture medium (ref. 25, 26), the utilisation of different growth conditions might modify or induce the expression of hopanoid biosynthesis.

RÔLE

Hopanoids are essential metabolites for the bacteria synthesizing them. Indeed, addition of inhibitors of the squalene cyclase to the culture medium, making impossible the formation of the pentacyclic ring system, inhibited selectively the growth of hopanoid producers often at low concentrations (about 1 μM), whereas the growth of other bacteria was not affected even at much higher concentrations (up to 200 μM) (ref. 27, 28).

The intracellular localization of hopanoids could be determined for two Gram-negative bacteria. They have been found in the plasma membrane as well as in the outer membrane of *Zymomonas mobilis* (ref. 29) and in the outer membrane and in the thylakoids of the photosynthetic apparatus of the cyanobacterium *Synechocystis* sp. (ref. 30). Many experiments have been now performed with hopanoids on artificial biological membrane models and on living cells of Eubacteria, mycoplasms and ciliates and confirmed their cholesterol like rôle as bacterial membrane stabilizers (ref. 25).

HOPANOID BIOSYNTHESIS

Only one enzyme of the biosynthetic pathway leading to the hopanoids, the squalene cyclase, is fairly well known. This enzyme has been even isolated (ref. 31-33), cloned and the sequence of its amino-acids determined (ref. 34). Its low specificity towards substrates and its "primitive" features (compared to those of squalene oxide cyclases from Eukaryotes) have been already largely discussed (ref. 25, 35). Only two unusual aspects of bacterial polyterpenoid biosynthesis, brought to light by feeding bacteria with ^{13}C labelled precursors, will be therefore presented here.

The formation of the C_{35} bacteriohopane skeleton

The C_{35} bacteriohopane skeleton shows an unusual carbon/carbon bond between a triterpenic hopane moiety and an additional polyhydroxylated C_5 unit. The origin of this side-chain could be determined by feeding bacteria either with ^{13}C labelled acetate (*Methylobacterium organophilum*, *Rhodopseudomonas palustris*, *Rhodopseudomonas acidophila*) (ref. 36) or with ^{13}C labelled glucose (*Zymomonas mobilis*) (ref. 37). The distribution of the isotopic enrichments showed unambiguously that this C_5 unit is a D-pentose derivative issued from the non-oxidative pentose phosphate pathway and linked by its C-5 carbon atom to the hopane isopropyl group. According to the stereochemistry of the two basic bacteriohopane derivatives found in nearly all hopanoid synthesizing bacteria, aminobacteriohopanetriol **3** and bacteriohopanetetrol **4** (ref. 13, 38-41), a D-ribose derivative could be the precursor. In spite of several attempts to elucidate this new

enzymatic reaction, no data could be collected neither on the triterpene or carbohydrate precursors, nor on the enzymatic reaction itself permitting this coupling. Few enzymatic reactions with probably fairly similar characteristics are known: the alkylation of an aromatic ring by a pentose derivative in the biosynthesis of methanopterin, a cofactor found in methanogenic Archaebacteria (ref. 42, 43), the coupling of arginine and adenosine triphosphate by a pyridoxal phosphate enzyme (ref. 44) and the condensation of isopentenyl pyrophosphate and dimethylallyl pyrophosphate in isoprenoid biosynthesis. A reasonable hypothetical biogenetic pathway could thus involve the addition of a nucleophilic hopanoid on a D-pentose derivative with a good leaving group at C-5 yielding 30-(5'-ribosyl)hopane **27** or **28** via a cationic intermediate such as **26** (Fig. 2) which can explain the simultaneous presence in some cases of the two C-22 diastereoisomers of bacteriohopanetetrol (**4** and **5**) and adenosylhopane (**22** and **23**). Reduction or reductive amination of ribosylhopane **27** can respectively lead to tetrol **4** or aminotriol **3**, which are simultaneously present in some bacteria such as *Beijerinckia indica*, *Microcystis* sp. or *Methylocystis parvus* (M. Knani, P. Simonin, C. Vilchèze and M. Rohmer, unpublished results). Its isomerization of the chiral centre in α position of the carbonyl group of the open form **28** can directly give the 34*S* tetrol **15** series found in the *Acetobacter* species. The biochemistry of the bacteriohopanetetrols rises many other questions that will not be easily answered. Are the adenosylhopanes **22** and **23** the precursors of other bacteriohopane derivatives or do they derive from the adenylation of ribosylhopane **27** (Fig. 2)? Can arabinose derivatives be implied in this biosynthetic pathway to yield directly the tetrols of 34*R* configuration? Do the C_{33} diols **24** (Fig. 1) found in *Acetobacter* species (ref. 45) result from the coupling of a C_3 instead of a C_5 unit, or are they degradation products of the C_{35} bacteriohopane derivatives?

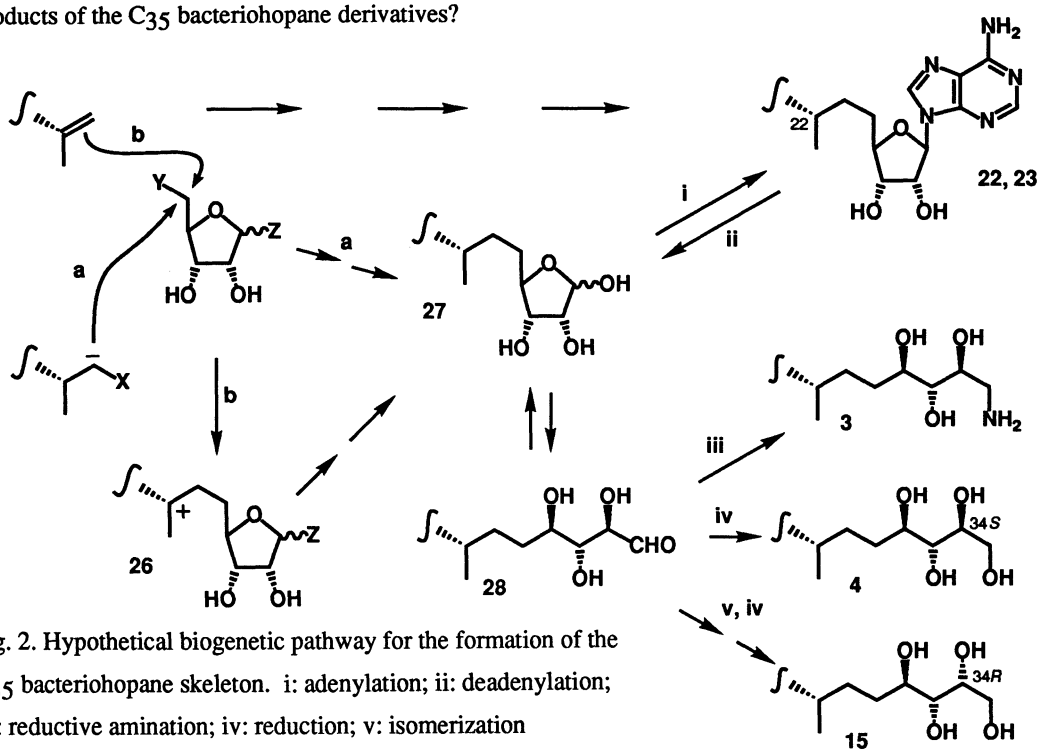

Fig. 2. Hypothetical biogenetic pathway for the formation of the C_{35} bacteriohopane skeleton. i: adenylation; ii: deadenylation; iii: reductive amination; iv: reduction; v: isomerization

The biosynthesis of polyterpenoids in Eubacteria: a new pathway for the early steps

As mentioned above the first incorporation experiments have been performed with ^{13}C labelled precursors essentially in order to determine the origin of the bacteriohopane side-chain. They reserved however an enormous surprise. Indeed, formation of isoprenic units was thought to be a trivial problem, even when working with bacteria. The distribution of the isotopic enrichments on the triterpenic framework was completely different from the labelling pattern normally expected from the classical Bloch-Lynen biosynthetic pathway which has been verified for Eukaryotes (ref. 46) as well as for Archaebacteria (ref. 47-

49). Our results have been obtained with several bacteria using ^{13}C labelled acetate and glucose, usually present as sole carbon sources for the microorganisms and can be summarized as follows.

1) Exogenous acetate is not directly incorporated into isoprenoid biosynthesis.

2) With all tested ^{13}C labelled precursors no scrambling of the isotopic enrichments occurred.

3) If isoprenoid biosynthesis follows in Eubacteria the classical scheme involving in the early steps acetyl coenzyme A, acetoacetyl coenzymeA and hydroxymethylglutaryl coenzyme A for the formation of mevalonate, one has to admit the intervention of at least two different, non interconvertible acetyl coenzyme A pools in the case of the experiments performed with ^{13}C labelled acetate (ref. 36), and three in the case of those performed with glucose (B. Sutter, H. Sahm and M. Rohmer, unpublished results).

4) Such a compartmentation of acetyl coenzyme A metabolism is most unlikely in prokaryotes. The discrepancy observed in the labelling patterns is better explained by different intermediates and different enzymatic reactions involved in the formation of mevalonate-5-phosphate and isopentenyl diphosphate. This novel hypothesis is currently being tested. The results obtained until now tend to confirm it and will be reported elsewhere after completion of all experiments.

5) The conclusions are of general scope. Identical labelling patterns have been obtained with two different isoprenoid series (hopanoids and ubiquinones) from several Gram-negative bacteria (*Methylobacterium* spp., *Rhodopseudomonas* spp., *Zymomonas mobilis* and *Escherichia coli*) and one Gram-positive bacterium (*Bacillus acidoterrestris*).

This breakthrough was only made possible by working on hopanoids. Indeed, these polyterpenoids are present in sufficient amounts (up to 30mg/g, dry weight, in *Zymomonas mobilis*) allowing the use of ^{13}C NMR spectroscopy for biosynthetic studies. Other bacterial isoprenoids (bactoprenols, ubiquinones and menaquinones), although of general occurrence, are found at low or very low concentrations in bacterial cells. Their biosynthesis has been repeteadly studied using mainly radiolabelled precursors, but the radioactivity has not been localized in most experiments. Furthermore free mevalonate failed to be incorporated into bacterial terpenoids (ref. 50-53). Only the biosynthesis of ubiquinone in *Escherichia coli* has been studied by incorporation of ^{13}C and ^{2}H labelled precursors. The labelled positions have been determined by mass spectrometry, and the labelling pattern was again clearly not in accordance with the classical biogenetic pathway (54).

According to Woese and Fox, living organisms are distributed among three kingdoms: Archaebacteria, Eubacteria and Eukaryotes (ref. 55). One of the main argument in favor of this phylogenetic classifying was the comparison of the nucleotide sequences of the 16S ribosomal RNAs. This view is supported by many other biochemical features such as the structure of the cell walls, the lipid composition, the presence of peculiar cofactors, the sensitivity towards antibiotics... The early steps of polyterpenoid biosynthesis and the formation of the C_{35} bacteriohopane skeleton could represent two additional criteria permitting to distinguish the Eubacteria from the two other kingdoms.

Acknowledgements This work forms part of our activities supported by the Centre National de la Recherche Scientifique (Unité de Recherche Associée 135, Laboratoire de Syntèse Organique et de Chimie Microbienne).

REFERENCES

1. E. Gelpi, H. Schneider, J. Mann and J. Oro, *Phytochemistry* **9**, 603-612 (1970).

2. C.W. Bird, J.M. Lynch, S.J.Pirt and W.W. Reid, *Tetrahedron Lett.* 3189-3190 (1971).

3. M. de Rosa, A. Gambacorta, B. Nicolaus and J. D. Bu'lock, *J. Chem. Soc. Chem. Commun.* 619-620 (1971).

4. M. Rohmer, P. Bouvier-Navé and G. Ourisson, *J. Gen. Microbiol.* **130**, &137-1150 (1984), and references cited therein.

5. H. J. Förster, K. Biemann, W.G. Haigh, N.H. Tattrie and J.R. Colvin, *Biochem. J.* **135**, 133-143 (1973).

6. M. Rohmer, P. Bisseret and B. Sutter, *Progr. Drug Res.* **37**, 271-285 (1991).

7. M. Zundel and M. Rohmer, *Eur. J. Biochem.* **150**, 23-27 (1985).
8. P. Bisseret, M. Zundel and M. Rohmer, *Eur. J. Biochem.* **150**, 29-34 (1985).
9. P. Stampf, D. Herrmann, P. Bisseret and M. Rohmer, *Tetrahedron* **47**, 7081-7090 (1991).
10. M. Rohmer and G. Ourisson, *J. Chem. Res.* (S) 356-357, (M) 3037-3059 (1986).
11. S. Neunlist and M. Rohmer, *J. Gen. Microbiol.* **13**, 1363-1367 (1985).
12. S. Neunlist and M. Rohmer, *Biochem. J.* **231**, 635-639 (1985).
13. S. Neunlist, P. Bisseret and M. Rohmer, *Eur. J. Biochem.* **171**, 245-252 (1988).
14. L.M. Kogan, I.V. Kozlova, T.M. Filipova, E.A. Obolnikova, G.T. Orekh, S.V. Skriabina and Y.Y. Kazantzev, *Khim.-Farm. Zh. SSSR* **25**, 83-85 (1991).
15. T.A. Langworthy, W.R. Mayberry and P.F. Smith, *Biochim. Biophys. Acta* **431**, 550-569 (1976).
16. J.-M. Renoux and M. Rohmer, *Eur. J. Biochem.* **151**, 405-410 (1985).
17. G. Flesch and M. Rohmer, *Biochem. J.* **262**, 673-675 (1989).
18. P. Simonin, U.J. Jürgens and M. Rohmer, *Tetrahedron Lett.* **33**, 3629-3632 (1992).
19. P. Llopiz, S. Neunlist and M. Rohmer, *Biochem J.*, in the press.
20. S. Neunlist and M. Rohmer, *Eur. J. Biochem.* **147**, 561-568 (1985).
21. B. Peiseler and M. Rohmer, *J. Chem. Res.*, in the press.
22. M. Rohmer and G. Ourisson, *Tetrahedron Lett.* 3633-3636 (1976).
23. H. Schulenberg-Schell, B. Neuss and H. Sahm, *Anal. Biochem.* **181**, 120-124 (1989).
24. K.D. Barrow and J.-A. Chuck, *Anal. Biochem.*,**184**, 395-399 (1990).
25. G. Ourisson, M. Rohmer and K. Poralla, *Annu. Rev. Microbiol.* **41**, 301-333 (1987), and references cited therein.
26. M.A.F. Hermans, B. Neuss and H. Sahm, *J. Bacteriol.* **173**, 5592-5595 (1991).
27. G. Flesch and M. Rohmer, *Arch. Microbiol.* **147**, 100-104 (1987).
28. S. Horbach, B. Neuss and H. Sahm, *FEMS Microbiol. Lett.* **79**, 347-350 (1991).
29. Y. Tahara, H. Yuhara and Y. Yamada, *Agric. Biol. Chem.* **52**, 607-609 (1988).
30. U. J. Jürgens, P. Simonin and M. Rohmer, *FEMS Microbiol. Lett.* **92**, 285-288 (1992).
31. B. Seckler and K. Poralla, *Biochim. Biophys. Acta* **220**, 356-363 (1986).
32. S. Neumann and H. Simon, *Biol. Chem. Hope-Seyler* **367**, 723-729 (1986).
33. D. Ochs, C.H. Tappe, P. Gärtner, R. Kellner and K. Poralla, *Eur. J. Biochem.* **194**, 75-80 (1990).
34. D. Ochs, C. Kaletta, K.-D. Entian, A. Beck-Sickinger and K. Poralla, *J. Bacteriol.* **174**, 298-302 (1992).
35. I. Abe and M. Rohmer, *J. Chem. Soc. Chem. Commun.* 902-903 (1991).
36. G. Flesch and M. Rohmer, *Eur. J. Biochem.* **175**, 405-411 (1988).
37. M. Rohmer, B. Sutter and H. Sahm, *J. Chem . Soc. Chem Commun.* 1471-1472 (1989).
38. S. Neunlist and M. Rohmer, *J. Chem. Soc. Chem. Commun.* 830-832 (1988).
39. P. Bisseret and M. Rohmer, *J. Org. Chem.* **54**, 2958-2964 (1989).
40. P. Zhou, N. Berova, K. Nakanishi and M. Rohmer, *J. Chem. Soc. Chem. Commun.* 256-258 (1991).
41. P. Zhou, N. Berova, K. Nakanishi, M. Knani and M. Rohmer, *J. Am. Chem. Soc.* **113**, 4040-4042 (1991).
42. R.H. White, *J. Am. Chem. Soc.* **108**, 6434-6435 (1986).
43. P.J. Keller and H.G. Floss, *J. Am. Chem. Soc.* **108**, 344-345 (1986).
44. H. Malina, C. Tempête and M. Robert-Gero, *J. Antibiotics* **40**, 505-511 (1987).
45. B. Peiseler and M. Rohmer, *J. Chem. Res.*, in the press.
46. W.R. Nes and M.L. McKean, *Biochemistry of steroids and other isopentenoids*, pp. 147-169 (1977).
47. T.A. Langworthy, T.G. Tornabene and G. Holzer, *Zentralbl. Bacteriol. Hyg.* **3**, 228-244 (1982).
48. M. de Rosa, A. Gambacorta and B. Nicolaus, *Phytochemistry*, **19**, 791-793 (1990).
49. N. Moldoveanu and M. Kates, *Biochim. Biophys. Acta* **960**, 164-182 (1988).
50. T.S. Raman, B.V.S. Sharma, J. Jayaraman and T. Ramasarma, *Arch. Biochem. Biophys.* **110**, 75-84 (1965).
51. A. Peterkofsky, *Biochemistry* **7**, 472-482 (1968).
52. H. Takatsuji, T. Nishino, I. Miki and H. Katsuki, *Biochem. Biophys. Res. Commun.* **110**, 187-193 (1983).
53. S. Fujisaki, T. Nishino and H. Katsuki, *J. Biochem (Tokyo)* **99**, 1137-1146 (1986).
54. D. Zhou and R.H. White, *Biochem. J.* **273**, 627-634 (1991).
55. C.R. Woese and G.E. Fox, *Proc. Natl. Acad. Sc. USA* **74**, 5088-5090 (1977).