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ISOPRENOIDS OF *BACILLUS ACIDOCALDARIUS*

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Abstract—The isoprenoids of *Bacillus acidocaldarius*, a notably acidophilic thermophilic organism, were investigated. Besides normal bacterial isoprenoids such as menaquinone and polyprenols, which latter were resolved in α-*cis* and α-*trans* fractions, and some minor components (α-*tertiary*-prenols and the corresponding anhydroderivatives), not fully characterized, and probably 'natural' artefacts, we have isolated and characterized squalene and pentacyclic triterpene hydrocarbons belonging to the hopane class, which must be accounted as unusual. Radioactivity from mevalonate-[2-^{14}C] is incorporated into hopene-b (the major triterpene component), thus establishing the origin of cyclized squalene derivatives in *B. acidocaldarius* as a result of *de novo* synthesis.

INTRODUCTION

Bacillus acidocaldarius is a notably acidophilic thermophilic organism[1,2] which is also distinguished by its high content of unusual fatty acids[3] containing shikimate-derived cyclohexyl residues.[4] We have also reported in preliminary form the existence of pentacyclic triterpene hydrocarbons in this bacterium.[5] In this paper we describe a fuller investigation of the isoprenoids of this organism, which comprise normal bacterial isoprenoids (prenols and menaquinone) and some minor components, not fully characterized, in addition to squalene and the triterpenes which must be accounted as unusual. We include data which demonstrate that these triterpenes are genuinely synthesized *de novo* by the bacterium from mevalonic acid.

RESULTS

Chromatography on silica gel of the combined light petroleum and chloroform–methanol extract from 100 g of lyophilized cells in light petroleum with increasing proportions of diethyl ether gave four principal fractions, viz. (a) in light petroleum, a fraction (200 mg) consisting principally of hop-22(29)-ene (hopene fraction); (b) in 99:1 light petroleum–ether, a hydrocarbon fraction (20 mg) (squalene fraction); (c) in 98:2 light petroleum–ether,

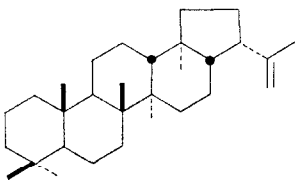
[1] DARLAND, G. and BROCK, T. D. (1971) *J. Gen. Microb.* **67**, 9.
[2] DE ROSA, M., GAMBACORTA A. and BU'LOCK, J. D. (1973) *G. Microbiologia* in press.
[3] DE ROSA, M., GAMBACORTA, A., MINALE, L. and BU'LOCK, J. D. (1971) *Chem. Commun.* 1334.
[4] DE ROSA, M., GAMBACORTA, A., MINALE, L. and BU'LOCK, J. D. (1972) *Biochem. J.* **128**, 751.
[5] DE ROSA, M., GAMBACORTA, A., MINALE, L. and BU'LOCK, J. D. (1971) *Chem. Commun.* 619.

a menaquinone fraction (98 mg), (d) in 80:20 light petroleum–ether, a prenol fraction (42 mg).

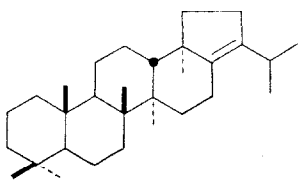
Hopene Fraction

TLC of this fraction on $AgNO_3$-treated silica gel gave as the major component hop-22-(29)-ene [hopene-b, diploptene] (I), (C, 87·2; H, 12·3, $C_{30}H_{50}$ requires: C, 87·7; H, 12·3%), which was identified by comparison with authentic material [GLC data, see below; MS M^+/e 395, 191, 189; NMR as described by Ageta *et al.*;[6] ν_{max} 885 cm^{-1} (=CH_2); m.p. 190° (crystallized from light petroleum), 205° (crystallized from methanol) (lit. values 207–212°)][6] and as a very minor component hop-17(21)-ene (hopene 1) (II)[7] [comparison with material produced from hop-22(29)-ene by treatment with $CHCl_3$–I_2 and with authentic hop-17(21)-ene, having identical TLC behaviour on SiO_2–$AgNO_3$, GLC retention time, NMR and MS]. Direct GLC of this fraction revealed the presence of four more very minor components (see Table 1), of which one was identified as hopane (comparison with authentic hopane obtained by hydrogenation of hopene, giving identical GLC retention time and MS, M^+/e = 412 with major fragmentation at M^+/e = 191 only).

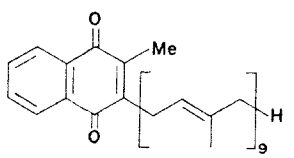
During MS investigations of this fraction, several spectra revealed, in addition to the molecular ions at M^+/e = 410 and 412 ascribed to hopenes and hopane, relatively small molecular ions at M^+/e = 424 and 426 which must be ascribed to C_{31} homologues.



(I)



(II)



(III)

Hydrocarbon Fraction

By carefully graded elution from the silica gel column this fraction was recovered in two portions, of which the larger, less polar, portion was identified as all-*trans*-squalene by comparison of MS, NMR and GLC retention time. The minor, slightly more polar fraction, examined by MS, contained some squalene together with hydrocarbons giving molecular ions at M^+/e = 680 and 748, corresponding to 10 and 11 C_5H_8 units. The NMR spectrum of this fraction showed the presence of both *cis*- and *trans*-C–CMe=CH–C units (CH_3 signals at δ 1·65 and 1·58 respectively),[8] the former predominating. We suppose that these hydrocarbons are a type of anhydro derivative of the corresponding C_{50} and C_{55} prenols (see below and Discussion).

Menaquinone

This fraction crystallized from methanol, m.p. 56° (lit. 57°)[9] gave a MS with substantially

[6] Ageta, H., Iwata, K. and Otaka, Y. (1963) *Chem. Pharm. Bull. (Tokyo)* **11,** 407.

[7] Schaffner, K., Caglioti, L., Arigoni, D., Jeger, O., Fazakerley, H., Halsall, T. G. and Jones, E. R. H. (1957) *Proc. Chem. Soc.* 353; Farakerley, H., Halsall, T. G. and Jones, E. R. H. (1959) *J. Chem. Soc.* 1877.

[8] Bates, R. B. and Gale, D. M. (1960) *J. Am. Chem. Soc.* **82,** 5749.

[9] Pennock, J. F. (1966) *Vitamins Hormones* **24,** 307; Sommer, P. and Kofler, M. (1966) *Vitamins Hormones* **24,** 349.

a single molecular ion, $M^+/e = 648$, corresponding to menaquinone-9 (III). The UV, NMR and MS of this material conform fully with the literature data[10] and confirm this identification. The peak at $M^+/e = 650$ in the mass spectrum corresponds to the natural isotope abundance and provides no evidence for the presence of any dihydromenaquinone; the NMR spectrum shows that the nonaprenyl side-chain is all-*trans*.

Prenol Fraction

Careful elution of this fraction from the silica gel column gave three components which were also resolved by TLC on silica gel in 1:1 light petroleum: Et_2O, $R_f = 0{\cdot}2$, 0·35, 0·6 (approx.) respectively, in approximately equal proportions. In terms of known chromatographic behaviour[11] we suggest that these correspond to *α-trans-*, *α-cis-* and *α-tertiary-*prenols respectively; the most relevant criteria confirming these assignments are the NMR spectra.

The middle component, R_f *ca.* 0·35, showed in the MS a series of molecular ions corresponding to a mixture of prenols with 9–12 C_5 units, with the C_{60} prenol as the main component and the C_{45} and C_{55} prenols as rather minor components. In the NMR (Fig. 1a) this material shows, (a) a small peak at $\delta = 1{\cdot}72$ corresponding to the CH_3 of the *α-cis*-C–CMe=CH.CH_2OH residue (carbinol CH_2 doublet, J 7 Hz, at $\delta = 3{\cdot}98$), (b) a large peak at $\delta = 1{\cdot}66$ corresponding to other *cis*-methyl groups, and (c) a medium-sized peak at $\delta = 1{\cdot}59$ corresponding to *trans*-methyl groups.[12] From the relative intensities of the two main signals, and allowing for the existence of both *α-cis* and a *trans*-methyl group in the *ω*-terminal unit, it is most probable that the prenols in this *α-cis* group contain two internal *trans*-residues.

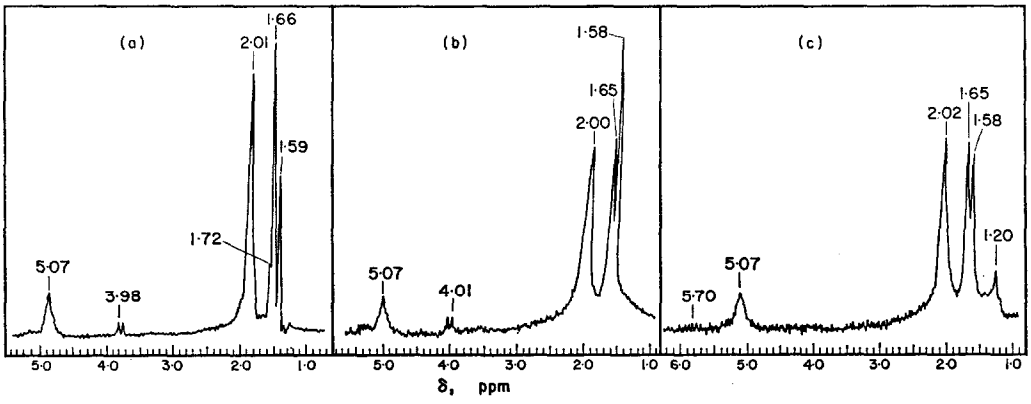


FIG. 1. 100 MHz NMR SPECTRUM (CCl_4) OF *α-cis* (a), *α-trans* (b) AND *α-tert*iary (c) PRENOL FRACTIONS FROM *Bacillus acidocaldarius*.

The most polar prenol fraction (R_f *ca.* 0·2) gave an NMR spectrum (Fig. 1b) in which the carbinol group was apparent (CH_2 doublet, J 7 Hz, at δ 4·01); in the =CMe– region there was, (a) no resolved signal in the region $\delta = 1{\cdot}72$, indicating the absence of an *α-cis*-unit,

[10] LAGEMANN, A. and ISLER, O. (1965) in *Biochemistry of Quinones* (MORTON, R. A., ed.), pp. 128–136, Academic Press, London and New York; DAS, B. C., LORCASMAA, M., TENDILLE, C. and LEDERER, E. (1965) *Biochem. Biophys. Res. Commun.* **21**, 318.

[11] HEMMING, F. W. (1967) in *Terpenoids in Plants* (PRIDHAM, J. B. ed.), p. 226, Academic Press, London.

[12] HEMMING, F. W. (1967) in *Terpenoids in Plants* (PRIDHAM, J. B., ed.), p. 230, Academic Press, London.

(b) a signal at $\delta = 1{\cdot}65$ which is ascribed to the *cis*-methyl of the ω-terminal unit and to an α-*trans*-methyl, (c) a large signal at $\delta = 1{\cdot}58$ due to *trans*-methyl groups. We conclude that this material comprises all-*trans*-prenols of the solanesol type; the mass spectrum of this sample indicated that it is a mixture of prenols predominantly with 10 and 11 C_5 units.

TABLE 1. GAS CHROMATOGRAPHY OF THE HOPENE FRACTION*

Retention time (min)	6·2	7·2	9·7	10·4	11·6	15·6
(%) Total	Trace	3	7	trace	4	86
Identification	—	—	—	Hop-17(21)ene (hopene-1)	Hopane	Hop-22(29)ene (hopene-b)

* 2 m Column (internal dia. 4 mm) packed with SE-30 5% on Chromosorb W 60–80 mesh operating at 290° with N_2 at 60 ml/min.

The identification of the least polar prenol fraction as a mixture of tertiary alcohols (i.e. with the normal α-unit of prenols replaced by the isomeric $-CH_2-CMe(OH)-CH=CH_2$) is confirmed by the presence in the NMR spectrum (Fig. 1c) of a one-proton quartet around $\delta = 5{\cdot}7$, ascribed to the vinyl –CH– proton, the remaining signals of the vinyl *ABM* system being apparent as a broadening of the main olefinic proton signal around $\delta = 5{\cdot}02$, and by the absence of the carbinol CH_2 signal at $\delta = 4{\cdot}0$. The signal at $\delta = 1{\cdot}72$ seen in the α-*cis*-prenol fraction is also absent; the main –CMe= signals at $\delta = 1{\cdot}65$ and $\delta = 1{\cdot}58$ are of nearly equal intensity, and a small sharp signals at $\delta = 1{\cdot}20$ is assigned to the tertiary Me group of the α-terminal unit (This assignment was confirmed by measurements of the NMR spectrum of synthetic faemesylgeranyl-linalool). The mass spectrum of this fraction showed the same preponderance of C_{50} and C_{55} prenols as in the α-*cis* and α-*trans* prenol fractions described above.

TABLE 2. ^{14}C-INCORPORATION INTO FATTY ACIDS AND TRITERPENES*

Fed (dpm)	[1-^{14}C]-acetate $1{\cdot}8 \times 10^8$	[2-^{14}C]-mevalonolactone $5{\cdot}4 \times 10^8$
Recovered		
Bacteria (g)	10·5	12·0
Hop-22(29)-ene (mg)	21	27·4
Fatty acid Me esters (mg)	650	1100
dpm in hop-22(29)-ene	$21{\cdot}4 \times 10^3$	$18{\cdot}8 \times 10^3$
dpm in Me esters	6×10^6	$2{\cdot}6 \times 10^3$
Ratio of specific activities hopene:esters	0·2	50

* Each incubation experiment refers to a 25 l. batch culture.

Incorporation of Mevalonate into Hopene

To verify that the pentacyclic triterpenes of *B. acidocaldarius* are synthesized *de novo* [1-^{14}C]-acetate (0·68 mg, $115{\cdot}10^9$ dpm) were fed to similar 25 l. fermentations at the beginning of the growth phase; after 18 hr the cells were collected, lyophilized, and extracted

(Soxhlet) with light petroleum followed by chloroform–methanol. From the light petroleum extract the hop-22(29)-ene (I) was recovered chromatographically and recrystallized; the chloroform–methanol extract was saponified (10% KOH in methanol, 6 hr reflux) and the resultant fatty acid fraction was methylated giving an ester fraction which was purified chromatographically; final elution with 49:1 light petroleum–Et_2O gave a purified ester fraction.[4] The results of the two incubations are summarized in Table 2. The incorporation of mevalonate was relatively low but this was not unexpected in view of the pH and temperature of the incubation; nevertheless it is very clear, (a) that both the fatty acids and the triterpenes are synthesized *de novo* from acetate, and (b) that the labelling from mevalonate is specifically in the triterpenes, as expected.

DISCUSSION

The isoprenoid metabolites of *B. acidocaldarius* can be divided into those which are normal bacterial constituents and those which are not. As normal constituents we must include the menaquinone, the α-*cis*-prenols, and perhaps the all-*trans*-prenols. Threlfall and Whistance[13] have reviewed the occurrence of menaquinones in bacteria; while menaquinone-9, found here, is relatively common, previous characterizations from *Bacillus* species have given menaquinone-7 as the principal homologue. The absence of analogues with partly hydrogenated chains in *B. acidocaldarius* is to be noted. Though prenols have been shown to have fundamental importance as lipophilic carriers for the synthesis of cell-wall and other extra-membrane polymers in bacteria,[14] they have only been successfully characterized in a few cases. From *Lactobacillus casei*, Thorne and Kodicek obtained a dihydro-undecaprenol of uncertain stereochemistry;[15] Scher *et al.*[16] characterized an undecaprenol from *Micrococcus lysodeikticus* as having two internal *trans*-residues, and eight *cis*-residues (one α-terminal). Subsequently Gough *et al.*[17] obtained the same α-*cis*-undecaprenol from *Lactobacillus plantarum* and also showed that it is accompanied by higher and lower homologues and that there are three biogenetically-*trans* isoprene units which probably correspond to the ω-residue and the two internal *trans*-units. The α-*cis*-prenols of *B. acidocaldarius* apparently belong to the same class and differ only in that the predominant chain length is C_{50} (with a significant proportion of C_{55}). The all-*trans*-prenol which we have also found represents the type of prenol utilized in the formation of ubiquinones and menaquinones, the synthesis of which in bacteria is particularly well attested.[12] The apparent absence of significant amounts of ubiquinones in *B. acidocaldarius* is typical of Gram-positive bacteria.

The α-*tert*-prenol fraction in *B. acidocaldarius* represents a type not previously encountered; it is not likely an artefact of the work-up procedure (in which, for example, saponification steps were excluded), but it may in a sense be a 'natural artefact' of the conditions under which the organism is grown (pH 3, 60°). A similar conclusion applies equally to the very small and less well-characterized hydrocarbon ('anhydro-prenol') fraction.

[13] THRELFALL, D. R. and WHISTANCE, G. R. (1971) in *Aspects of Terpenoid Chemistry and Biochemistry* (GOODWIN, T. W., ed)., Academic Press, London.
[14] HEMMING, F. W. (1970) in *Natural Substances Formed Biologically from Mevalonic Acid* (GOODWIN, T. W., ed.), p. 105, Academic Press, London.
[15] THORNE, K. J. I. and KODICEK, E. (1966) *Biochem. J.* **99,** 123.
[16] SCHER, M., LENNARZ, W. J. and SWEELEY, C. C. (1968) *Proc. Natn. Acad. Sci. Wash.* **59,** 1313.
[17] GOUGH, D. P., KIRBY, A. L., RICHARDS, J. B. and HEMMING, F. W. (1970) *Biochem. J.* **118,** 167.

The triterpenes of *B. acidocaldarius* are very characteristic of this organism. Since our preliminary account of the isolation of hop-22(29)-ene in about 0·3% yield from this organism,[5] this hydrocarbon has also been identified in similar proportion in the lipids of methane-grown *Methylococcus capsulatus*[18] where it is accompanied by squalene (0·5%) and by significant amounts (0·2%) of sterols, 4-methylsterols, and 4,4-dimethylsterols.[19] The present account adds squalene and hopane to the list of triterpenes from *B. acidocaldarius*. In addition, some mass spectra of fractions co-chromatographing with the prenol mixtures showed minor peaks, not readily explained as prenol fragments, which could correspond to oxygenated triterpenes (i.e. at $M^+/e = 424$, 426 and 428); this is, however, a very tentative explanation applied to some very minor components. Similar reservation attaches to the identification of very minor peaks in mass spectra of the hopene fractions, at $M^+/e = 424$ and 426, as homologues of hopene and hopane, and attention is only drawn to these observations in view of the fact that such homologues regularly accompany triterpenes of this type in hydrocarbon fractions of geothermal origin.[20]

In view of the acknowledged rarity of genuine triterpene derivatives in prokaryotic organisms (and the somewhat uncertain status of certain reports of their existence),[21] it seemed particularly desirable to establish their origin in *B. acidocaldarius* as a result of *de novo* syntheses. Circumstantial evidence for this was presented in our preliminary communication[5] but the ^{14}C incorporation studies now described seem to establish the question beyond doubt.

EXPERIMENTAL

Instrumental details. M.ps were determined using a Kofler block and were not corrected. MS were obtained with an AEI MS9 double focus spectrometer at 70 eV; NMR spectra with a Varian HA100 spectrometer in CCl_4 solution with tetramethylsilane as an internal standard. Column chromatography was carried out on silica gel 0·05–0·2 mm (Merck), TLC was carried out on precoated silica gel plates (Merck). GLC was run using a Carlo Erba Fractovap model GV apparatus. Radioactivity measurements were made in Bray's solution (10 ml) on samples weighed on a Cahn electrobalance using a Beckman LS-250 liquid scintillation counter (efficiency 88–91% by internal standardization).

Organism and culture conditions. An isolate of *Bacillus acidocaldarius* from hot springs in the volcanic area of Naples, described by De Rosa *et al*,[2] was used. The medium contained (g/100 ml H_2O): yeast extract (Difco), 0·1; glucose, 0·1; $(NH_4)_2SO_4$, 0·01, $MgSO_4;7H_2O$, 0·01; KH_2PO_4, 0·01; $CaCl_2.2H_2O$, 0·001 and $FeCl_3$, 0·0001; the pH was adjusted to 2·6 with 0·2 M citrate buffer. The organism was grown in 25 l. batches at 60° with aeration (0·5 l. air/min/l. culture vol.) and agitation in a 30 l. fermentor (Terzano). The culture vessel was inoculated by adding 3 l. of a 12 hr broth culture. Cells were harvested in the stationary growth phase (20 hr incubation) by continuous-flow centrifugation, washed with 0·1 M NaCl, and liophilized (8–12 g dried cells from 25 l. cultures).

Extraction and fractionation of isoprenoids. The dried cells (100 g) were extracted continuously (Soxlet) for 6 hr with 40–70° light petrol. followed by $CHCl_3$–MeOH (1:1, for 6 hr). The combined lipid extracts were evaporated and the residue (8·4 g) was chromatographed on a silica gel column (400 g). Elution first with 40–70° light petrol. and then with increasing proportions of Et_2O gave four principal fractions as indicated above (see Results). Hopene and prenol fractions were then subjected to TLC on analytical silica gel plates (20 × 20 cm; 5 mg on each plate) as indicated in the Results.

Labelled experiments. The labelled substrates [1-^{14}C]-CH_3COONa (0·5 mCi; sp.act. 61 mCi/mM; the Radiochemical Centre, Amersham), and DL-mevalonic acid [2-^{14}C]-lactone (0·25 mCi; sp.act. 7·1 mCi/mM; the Radiochemical Centre, Amersham) were added to the cultures (25 l. fermentations) at the beginning of the exponential phase. The freeze-dried cells from each incubation experiment were continuously (Soxlet) for 12 hr extracted with 40–70° light petrol. followed by $CHCl_3$–MeOH (1:1) for 12 hr. From the light petrol.

[18] Bird, C. W., Lynch, J. M., Pirt, S. J. and Reid, W. W. (1971) *Tetrahedron Letters* 3189.
[19] Bird, C. W., Lynch, J. M., Pirt, F. J., Reid, W. W., Brooks, C. J. W. and Middledicth, B. S. (1971) *Nature* **230**, 473.
[20] Ourisson, G. (1972) private communication.
[21] Goldfine, H. (1972) in *Advances in Microbial Physiology* (Rose, A. H. and Tempest, B. W., eds.), pp. 17–19, Academic Press, London.

extract, hopene-b was recovered by SiO_2 column chromatography as above and then, after recrystallization from methanol, assayed for ^{14}C. The $CHCl_3$–MeOH extract was evaporated and treated with KOH in MeOH (10%, w/v; 40 ml per 100 mg lipids) under reflux; after 6 hr the MeOH was removed *in vacuo* and the remaining solution washed with Et_2O, then acidified with 2 M HCl and extracted with Et_2O. The extract was methylated with excess CH_2N_2 to give crude fatty acid methyl esters. Purification was carried out on silica gel column chromatography (10 g SiO_2 per 100 mg esters) eluting first with cyclohexane to remove residue hydrocarbons, etc. and then with 2% Et_2O in cyclohexane to remove the methyl esters, which were assayed for ^{14}C.

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