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^{13}C-NMR ASSIGNMENTS AND BIOSYNTHETIC DATA FOR THE ETHER LIPIDS OF *CALDARIELLA*

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Key Word Index—*Caldariella*; thermophile bacteria; biphytanyl; ether lipids; biosynthesis; ^{13}C-NMR.

Abstract—Fully assigned ^{13}C-NMR spectra confirm the C_{40} (16,16-biphytanyl) structures of the alkyl chains in the ether lipids of extreme thermoacidophile bacteria of the *Caldariella* group. The incorporations of ^{13}C- and ^{14}C-labelled acetate and mevalonate provide further structural confirmation and define their biosynthetic origin.

INTRODUCTION

The membrane lipids of extreme thermoacidophile bacteria of the *Caldariella* series are based on macrocyclic tetraethers containing pairs of bifunctional C_{40} alkyl chains, and evidence for the structures of three of these C_{40} components, obtained from the diglycerol tetraethers, has been presented in the accompanying paper [1]. They are represented by structures (1)–(3), which are made up of two phytanyl (perhydrogeranylgeranyl) units joined 'head-to-head' [i.e. at the 16,16'-positions, cf. (1)] and differ in the additional feature of up to two cyclopentane rings. These structures have been deduced from MS, chemical, and ^{1}H-NMR data. In this paper we report ^{13}C-NMR data which fully confirm these structures. The same data provide a useful basis for biosynthetic studies, and results which establish the regular isoprenoid origins of (1)–(3) are described.

RESULTS

C_{40} *Hydrocarbons*

Table 1 lists the chemical shifts, multiplicities (from off-resonance decoupling) and assignments of the natural-abundance ^{13}C-NMR spectra of the hydrocarbons (1)–(3), obtained by cleavage of the tetraethers with HI and reduction with $LiAlH_4$ [1] and separated by prep GLC. The assignments are based on chemical shift rules [2, 3], comparisons with appropriate model compounds [4–6] and observed multiplicities, combined with additional spectral data obtained for the corresponding diols (4)–(6), for which shift reagent data [7–9] and acetylation shifts [10] were also measured (see below). Spectra for samples of (1)–(3) labelled with ^{13}C from both [1-^{13}C]- and [2-^{13}C]-acetate were also measured (see below) and provided additional discriminatory data.

The ^{13}C spectrum of the acyclic hydrocarbon (1) shows only 13 resolved signals, because so many of the 40 carbon atoms are either strictly, or effectively, equivalent. The assignments are fully consistent with the 16,16'-biphytanyl structure and in particular they confirm the central head-to-head linkage. Thus the chemical shifts of the pairs 16,16' and 14,14' differ from those of the biogenetically-related pairs 6,6'; 8,8'; 10,10' and 12,12' by -3.11 ($-\gamma_2 + \delta_2$ effects [2, 3] and $+0.14$ ($+\varepsilon_2$ effect) [2, 3] respectively; that of the pair 15,15' differs from that of the biogenetically-related pairs 7,7' and 11,11' by $+0.27$ ($+\delta_3 + \varepsilon_3$ effects). This establishes the structure of the head-to-head region of the molecule of (1). In addition, the spectrum shows three resolved methyl signals, two at δ 11.32 and 19.41 (1,1' and 17,17' respectively) confirming the existence of two free 'tails' and one at 19.80 (18,18',19,19',20,20').

The ^{13}C-NMR spectrum of the bicyclic hydrocarbon (3) shows just 20 resolved signals, as its symmetrical C_{40} structure requires. Of the four methyl signals, three are also present in the spectrum of (1) and are ascribed to carbons 1,1',17,17', and 20,20', while the fourth at δ 17.75 is assigned to the carbons 19,19' which are β to the cyclopentane rings. Chemical shifts of carbons distant from the cyclopentane rings are the same as in (1); assignments for those near or in the rings have been calculated, from data for model compounds [6], for both 1,3-*cis* and 1,3-*trans* stereochemistry, and the calculations confirm the

Table 1. ^{13}C Chemical shifts (from TMS, ± 0.02) and multiplicities for the C_{40} hydrocarbons (1)–(3)

Carbon no.	(1)	(2)	(3)
1,1'	11.32 (*q*)	11.32 (*q*)	11.32 (*q*)
2,2'	29.71 (*t*)	29.71 (*t*)	29.71 (*t*)
3,3'	34.73 (*d*)	34.73 (*d*)	34.73 (*d*)
4,4'	37.02 (*t*)	36.91 (*t*); 37.02 (*t*)	36.91 (*t*)
5,5'	24.35 (*t*)	25.87 (*t*); 24.35 (*t*)	25.87 (*t*)
6,6'	37.46 (*t*)	37.19 (*t*); 37.46 (*t*)	37.19 (*t*)
7,7'	32.85 (*d*)	39.13 (*d*); 32.85 (*d*)	39.13 (*d*)
8,8'	37.40 (*t*)	33.37 (*t*); 37.46 (*t*)	33.37 (*t*)
9,9'	24.53 (*t*)	31.28 (*t*); 24.53 (*t*)	31.28 (*t*)
10,10'	37.46 (*t*)	44.89 (*d*); 37.46 (*t*)	44.89 (*d*)
11,11'	32.85 (*d*)	38.29 (*d*); 32.85 (*d*)	38.29 (*d*)
12,12'	37.46 (*t*)	35.77 (*t*); 37.46 (*t*)	35.77 (*t*)
13,13'	24.53 (*t*)	24.53 (*t*)	24.53 (*t*)
14,14'	37.60 (*t*)	37.60 (*t*)	37.60 (*t*)
15,15'	33.12 (*d*)	33.12 (*d*)	33.12 (*d*)
16,16'	34.35 (*t*)	34.35 (*t*)	34.35 (*t*)
17,17'	19.41 (*q*)	19.41 (*q*)	19.41 (*q*)
18,18'	19.80 (*q*)	36.00 (*t*); 19.80 (*q*)	36.00 (*t*)
19,19'	19.80 (*q*)	17.75 (*q*); 19.80 (*q*)	17.75 (*q*)
20,20'	19.80 (*q*)	19.80 (*q*)	19.80 (*q*)

Table 2. Chemical shifts and lanthanide-induced shifts for monocyclic diol (5), with Eu(fod-d_9)$_3$ at 1:1 and 2:1 reagnet:diol

Carbon no.		1:1	2:1
1,1′	61.22	5.43	22.28
2,2′	40.10	0.76	3.07
3,3′	29.67	0.74	2.75
17,17′	19.70	0.57	1.83
4,4′	37.52, 37.42	0.52	2.10
5,5′	25.87, 24.35	0.33	1.23
6,6′	37.19, 37.46	0.22	0.71
7,7′	39.13, 32.85	0.12	0.34
18,18′	36.00, 19.80	<0.02	0.17
8,8′	33.37, 37.46	<0.02	0.17
9,9′	31.28, 24.53	<0.02	0.14
10,10′	44.49, 37.46	<0.02	0.13, 0.06
rest	—	<0.02	<0.02

location of the rings and unambiguously indicate *trans* stereochemistry in each. The mutual stereochemistry of the two rings, of course, remains unknown.

In the ^{13}C-NMR spectrum of the hydrocarbon (2) the two sets of signals given by (1) and (3) are both observed, confirming the monocyclic structure assigned to it.

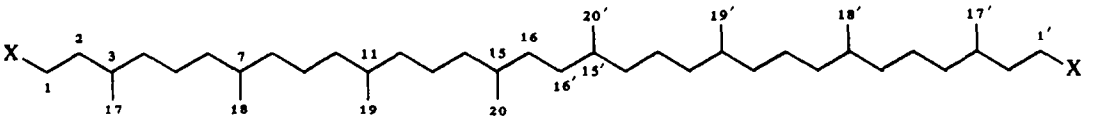
C_{40} *Diols and diacetates*

Confirmatory data were obtained from the ^{13}C-NMR spectra of the diols (4)–(6) (cf. [1]), for which the effects of acetylation and of the europium shift reagent Eu(fod-d_9)$_3$ could be observed. For the case of the monocyclic diol (5), in which both the open-chain and the cyclized structures are present, the Eu shift data are reported in Table 2. The shifts are linear up to a molar ratio of 1:1; at a molar ratio of 2:1 reagent: diol, effects can be seen up to the ninth carbon from each end of the chain. The effects of acetylation are more localised (δ + 1.88 at 1,1′, −4.54 at 2,2′, +0.25 at 3,3′, −0.33 at 4,4′, −0.13 at 17,17′). Results with the acyclic and bicyclic diols, (4) and (6) respectively, and their acetates, proved to be identical with those for the two 'halves' of (5) and are consequently not presented.

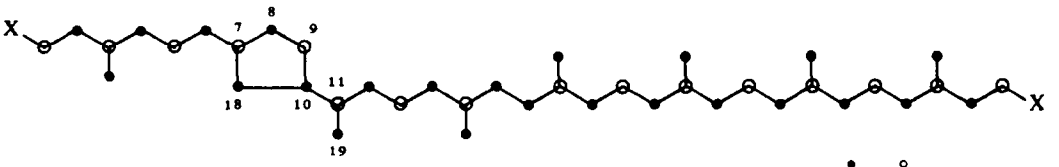
Biosynthesis

In preliminary experiments the incorporations of [1-^{14}C] acetate and of [2-^{14}C] mevalonate into the lipids was studied. The purified diglycerol tetraether fraction was cleaved with BCl_3 and the products (C_{40} dihalides and glycerol) separated chromatographically [1]. From the acetate, the dilution value (specific activity of precursor: product) was 7000 for the mixed tetraethers, 7200 for the C_{40} dichlorides, and 250000 for the glycerol. From the mevalonate, corresponding values were 1200, 1200, and 560000. Clearly the incorporation of mevalonate is particularly selective, as expected.

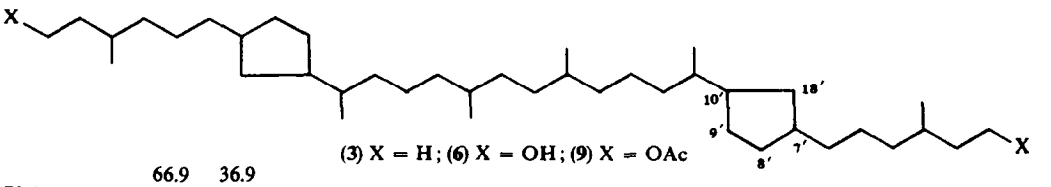
As a preliminary to ^{13}C-labelling, further experiments with ^{14}C-acetate were carried out to optimize conditions; maximum incorporation was obtained when the precursor was added at the beginning of the logarithmic phase of bacterial growth, and to reduce losses (from volatilization of acetic acid in the hot acidic aerated cultures), and also to minimize the disturbance of normal metabolism, the precursor was added slowly, using a peristaltic pump



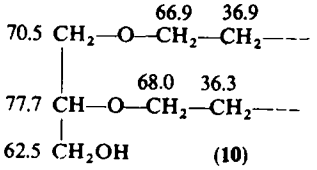
(1) X = H
(4) X = OH
(7) X = OAc



(2) X = H; (5) X = OH; (8) X = OAc [label from $\overset{\bullet}{C}H_3$–$\overset{\bullet}{C}O_2H$]



(3) X = H; (6) X = OH; (9) X = OAc



(10)

Table 3. Incorporation of [1-^{13}C]- and [2-^{13}C]-acetate into the acyclic C_{40} diacetate (7)

Carbon no.	Enriched peak height from [1-^{13}C]acetate	natural abundance peak height* from [2-^{13}C]acetate
1,1′	3.30	1.02
2,2′	1.07	3.50
3,3′	3.40	1.06
17,17′	0.98	3.60
4,4′	0.98	3.40
5,5′	3.40	0.94
6,6′(with 8,8′,10,10′, 12,12′)	0.99	3.10
7,7′(with 11,11′)	3.50	1.00
18,18′(with 19,19′,20,20′)	1.02	3.38
9,9′(with 13,13′)	3.20	0.94
14,14′	0.92	3.50
15,15′	3.50	0.90
16,16′	1.06	3.40

*Normalized with respect to the natural-abundance acetoxy (methyl carbon) signal.

over a 10 hr period. The cultures were harvested at the beginning of stationary phase. In the experiments with added [1-^{13}C]- and [2-^{13}C]-acetate, the three C_{40} components were recovered as the acetates and separated by prep GLC [1].

The biosynthetic origins of the individual carbon atoms of the C_{40} chain were deduced from the line intensities in the spectra of the biosynthetically-enriched diacetates, compared with the corresponding natural-abundance line intensities, the two sets of spectra being independently normalised with respect to the line intensities of the methyl carbons of the acetoxy groups (at natural abundance in both sets of spectra). Results for the acyclic diacetate (7) and the bicyclic diacetate (9) are presented in Tables 3 and 4; entirely consistent results were also

Table 4. Incorporation of [1-^{13}C]- and [2-^{13}C]-acetate into the bicyclic C_{40} diacetate (9)

Carbon no.	Enriched peak height from [1-^{13}C]acetate	natural abundance peak height* from [2-^{13}C]acetate
1,1′	3.60	0.97
2,2′	1.03	3.70
3,3′	3.50	1.05
17,17′	0.97	3.50
4,4′ and 6,6′	0.93	3.20
5,5′	3.35	1.02
7,7′	3.70	1.04
18,18′	0.89	3.70
8,8′	1.00	3.20
9,9′	3.60	0.94
10,10′	0.95	3.20
11,11′	3.40	1.01
19,19′	0.98	3.60
12,12′	1.04	3.30
13,13′	3.40	1.04
14,14′	1.06	3.50
15,15′	3.30	1.00
20,20′	1.06	3.70
16,16′	0.98	3.60

*Normalized with respect to the natural-abundance acetoxy (methyl carbon) signal.

obtained for the monocyclic diacetate (8). The results are unambiguous and establish the labelling pattern indicated in the formulae. In particular, whereas in (7) [2-^{13}C]acetate selectively enriches four pairs of methyls and eight pairs of methylenes, in (9) it enriches three pairs of methyls, seven of methylenes, and one of methines, just as the location and origin of the five-membered rings require. In (7) all the methine carbons derive from acetate C-1, as expected.

In none of the samples was there sufficient enrichment to allow useful observations of ^{13}C–^{13}C splittings. Spectra of the intact diglycerol tetraethers confirmed that, as indicated by the ^{14}C results, acetate is not a significant precursor of the glycerol units.

Glycerol unit in tetraethers

Assignments for the ^{13}C spectrum of the terminal region of the intact diglycerol tetraether, based on chemical shifts and multiplicities, were as shown in (10), thus confirming the 2,3-dialkoxypropan-1-ol structure assigned on other grounds [1].

EXPERIMENTAL

The organism used was the MT-3 strain of *Caldariella* for which culture conditions are fully described [11]. Growth was in 90l. batch cultures at 75°, pH controlled to 3.5. Labelled substrates, Na[1-^{13}C]acetate (1 g; 90 atom %), Na[2-^{13}C]acetate. (1 g; 90 atom %), Na[1-^{14}C]acetate (0.3 mCi) or DL-[2-^{14}C]mevalonolactone (0.75 mCi), were dissolved in water (1l.) and added over the first 10 hr of the exponential phase by means of a peristaltic pump. Cells were harvested at *ca* 36 hr (yield *ca* 0.2 g lyophilized cells/l. Extraction of lipids, cleavage of the tetraethers, separation of the diol acetates and preparation of the diols were as previously described [1, 12]. The hydrocarbons were separated by GLC (column 2m × 10 mm packed with 10% SE-30 on 80–100 mesh Chromosorb W, run at 290° with 180 ml N_2/min. Radioactive samples, weighted on a Cahn electrobalance, were counted at 88–91% efficiency in 10 ml aliquots of Bray's solution [13]. ^{13}C-NMR spectra were run on the Varian XL-100-15 equipped with a Digi-Lab Fourier transform accessory; multiplicity was determined by off-resonance decoupling. The samples (10–100 mg) were spun in 5 mm tubes using C^2HCl_3 (0.5 ml) as solvent. The solvent deuterium provided the lock signal, and TMS the int. stand. Chemical shifts are accurate to within ± 0.02 ppm. The shift reagent experiments were performed with commercial Eu(fod-d_9)$_3$, used without further purification. The spectra were first recorded in the proton noise decoupling mode to measure chemical shifts and the degree of substitution of each carbon atom then determined by obtaining a second series of spectra in the single frequency off-centre decoupling mode. Subsequently shift reagent was added in increments up to a final molar ratio (reagent : C_{40} diol) 2:1. The effect of the additions was linear in the range 0 to 1:1.

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