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THE X-RAY CRYSTAL STRUCTURE DETERMINATION, AND BIOSYNTHETIC STUDIES OF THE ANTIBIOTIC, HEPTELIDIC ACID

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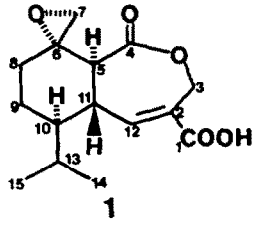
Abstract—A sesquiterpene lactone isolated from *Gliocladium virens*, was identical to the antibiotic, heptelidic acid. The complete structure of heptelidic acid, including the orientation of the epoxide ring was determined by single crystal X-ray defraction analysis. Feeding experiments with carbon-13 labeled acetate allowed complete assignment of the ^{13}C-NMR spectrum and showed that *trans*, *trans*- or *cis*, *trans*-farnesyl pyrophosphate is the probable biosynthetic precursor to heptelidic acid. These experiments also showed that during the biosynthesis of cadalene type sesquiterpenes, the isopropylidene group is held rigid during ring closure and protonation. Thus, in the feeding experiment with [1,2-^{13}C] acetate the methine carbon of the isopropyl group in heptelidic acid is asymmetric since the carbon of one methyl group is ^{13}C and the other is ^{12}C. An enzymatic Baeyer–Villiger type oxygen insertion is proposed for the formation of the lactone ring.

During our screening of antibiotic producing soil microbes, we found that *Gliocladium virens* produced a sesquiterpenoid ($C_{15}H_{20}O_5$) antibiotic that was active against *Rhizoctonia solani*. We identified this compound as 1,5a,6,7,8,9a-hexahydro-6-(1-methylethyl)-1-oxo-spiro[2-benzoxepin-9(3H),2′oxirane]-4-carboxylic acid (**1**). This compound has previously been identified in extracts from *Chaetomium globosum*, *Trichoderma viride*, and *G. virens* by skillful chemical and spectral analyses by Y. Itoh *et al.*,[1,2] who named compound **1**, heptelidic acid. This compound also has been identified in extracts from *Anthostoma avocetta* by Arigoni *et al.*,[3,4] and they named it avocettin. We call the compound heptelidic acid in this manuscript because of the extensive structure identification work and antibiotic activity reported by Itoh *et al.*,[1,2] and their recent patent.[5] Neither group of investigators were able to assign the stereochemistry of the epoxide ring.

We report here the single-crystal X-ray defraction analysis of heptelidic acid which establishes the orientation of the epoxide ring. We also report our biosynthetic studies of heptelidic acid using [^{13}C] acetate.

Unequivocal proof for the structure and stereochemistry of heptelidic acid (**1**) was obtained by X-ray analysis. A view of the molecular conformation is shown in Fig. 1. The ring junction of the molecule is *trans* with an equatorial isopropyl group as determined by Itoh *et al.* using proton–proton coupling constants.[2] The oxygen of the epoxide ring is equatorial. The X-ray crystallographic study also shows, as expected, that the preferred rotamer of the isopropyl group has the methine proton facing the lactone ring with the Me groups directed away from the lactone ring. Thus, C-14 and its protons encounter proton–proton interactions with the axial protons on C-9 and C-11. This is an important factor in the discussion of the biosynthetic study which follows.

The biosynthetic studies with [^{13}C] acetate were undertaken to aid the structure determination and the assignment of the ^{13}C-NMR spectrum. Using the information from the [^{13}C] acetate feeding experiments, chemical shift values, and carbon–proton coupling data it was possible to assign all C resonances in the ^{13}C-NMR spectrum (Fig. 2). The CO carbons are at 170.2 and 169.2δ. The olefinic carbons at 128.3 and 147.1 δ are assigned to C-2 and C-12, respectively, since in the proton coupled spectra, the peak at 128.3δ is a singlet and the peak at 147.1δ is a doublet. C-7 is assigned to the peak at 51.9δ because its multiplicity changes from a triplet in the off resonance decoupling mode to a doublet or to a doublet of doublets, depending on the setting of the heteronuclear decoupler frequency in the selective proton decoupling mode. This indicates that the



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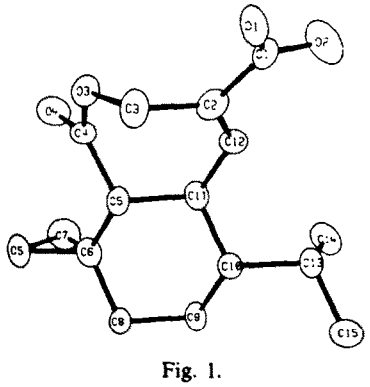

Fig. 1.

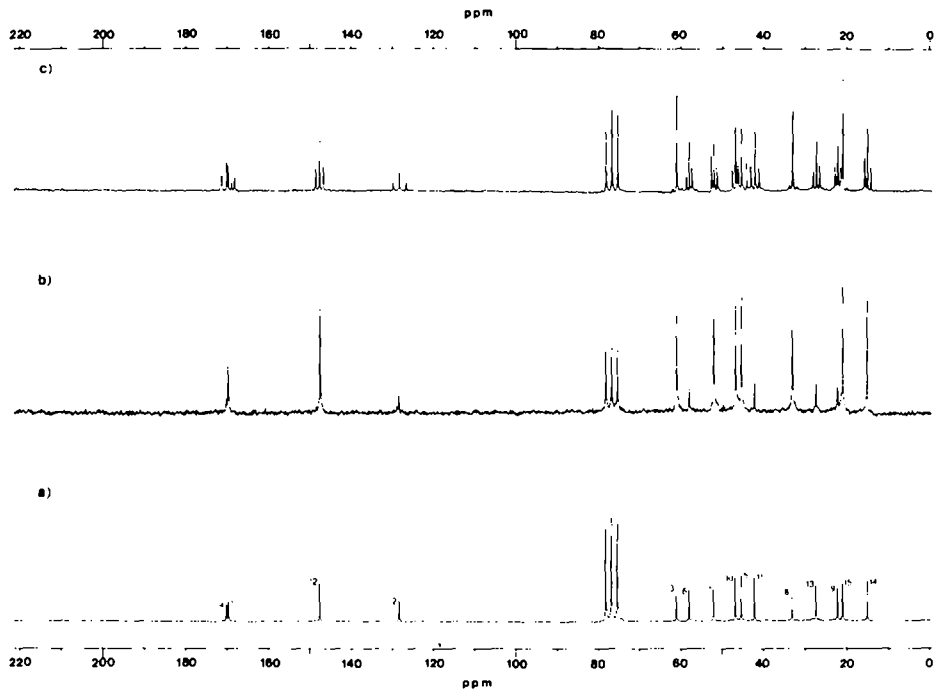

Fig. 2. 22.5 MHz proton noise-decoupled ^{13}C spectra of heptelidic acid (1): (a) natural abundance; (b) ^{13}C-enriched from [2-^{13}C] acetate; (c) ^{13}C-enriched from [1,2-^{13}C] acetate.

protons coupled to this C resonate at significantly different frequencies. This is consistent with the epoxide protons which appear as doublets (J = 5 Hz) at 3.83 and 2.59δ. The peak at 3.83δ is assigned to the proton closest to lactone CO. The other C's to which O is attached appear as a 61.0δ triplet assigned to C-3, and a singlet at 58.2δ assigned to C-6. C-13 is assigned to the doublet at 27.2δ because it is selectively decoupled on irradiation of the doublet of septets at 1.95δ. The other C atoms could be assigned with certainty by referring to the ^{13}C-enriched spectra.

Sodium [1-^{13}C] acetate, sodium [2-^{13}C] acetate, and sodium [1,2-^{13}C] acetate were fed in parallel to cultures of *G. virens*. The proton decoupled ^{13}C-NMR spectrum of ^{13}C-enriched heptelidic acid derived from [1-^{13}C] acetate showed peak enhancement for the carbons at 170.2, 128.3, 58.2, 42.0, 27.2 and 22.2δ (Table 1). Thus, these C's originate from the CO group of acetate. The ^{13}C-enriched heptelidic acid

Table 1. ^{13}C chemical shifts, C–C coupling constants, and [1-^{13}C] acetate, [2-^{13}C] acetate incorporation

Carbon No.	δ (ppm)	Mult.[a]	$^1J_{C-C}$ (Hz)	[1-^{13}C][b] Acetate	[2-^{13}C][b] Acetate
1	169.6	s	C_1-C_2=70.8		+
2	128.3	s		+	
3	61.0	t			+
4	170.2	s	C_4-C_5=54.8	+	
5	45.0	d			+
6	58.2	s	C_6-C_7=31.0	+	
7	51.9	t			+
8	33.1	t			+
9	22.2	t	C_9-C_{10}=34.0	+	
10	46.7	d			+
11	42.0	d	C_{11}-C_{12}=41.6	+	
12	147.1	d			+
13	27.2	d	C_{13}-C_{14}=35.2	+	
14	14.9	q			+
15	20.9	q			+

[a] Multiplicity caused by one bond C-H coupling

[b] + Indicates ^{13}C incorporation greater than 1.1%

from [2-^{13}C] acetate showed enhanced peaks at 169.6, 147.1, 61.0, 51.9, 46.7, 45.0, 33.1, 20.9 and 14.9δ (Fig. 2, Table 1), and thus are due to the Me group of acetate. With the doubly labeled [1,2-^{13}C] acetate (Fig. 2) only C's at 61.0, 33.1, and 20.9δ do not have satellite signals resulting from ^{13}C at an adjacent position. This indicates that these resonances are due to C's from which the CO group of acetate is lost during biosynthesis (e.g. C-2 of mevalonic acid, Fig. 4). The ^{13}C–^{13}C coupling constants showed that the C's at the following resonances were coupled: 169.6–128.3δ; 170.2–45.0δ; 58.2–51.9δ; 46.7–22.2δ; 147.1–42.0δ; and 27.2–14.9δ. These ^{13}C–^{13}C coupling constants are listed in Table 1. Since the peak at 128.3δ has already been assigned to C-2, the acid CO, C-1, must occur at 169.6δ. The lactone CO, C-4, must therefore absorb at 170.2δ, and since it can be coupled only to C-5, the resonance at 45.0δ is assigned to C-5. By similar reasoning, C-11 can be assigned to the resonance at 42.0δ. The resonances at 46.7 and 22.2δ are assigned to C-10 and C-9, respectively. The only remaining methylene group in the molecule, C-8, is assigned to the 33.1δ triplet.

C-14 and C-15 are assigned by consideration of the following: the biosynthesis of the cadalene type terpenoids; the C–C coupling observed in the [1,2-^{13}C] acetate feeding experiment; and the difference in chemical shifts. The biosynthetic route to heptelidic acid as indicated by our [^{13}C] acetate feeding experiment is outlined in Fig. 3. The results from the [1,2-^{13}C] acetate feeding experiment show that C-3, C-8 and C-15, originate from the acetate that loses CO during the synthesis of isopentenyl pyrophosphate (Fig. 4). The [1,2-^{13}C] acetate feeding experiment shows that C-14 and C-15 are not scrambled during heptelidic acid synthesis and that C-13 becomes a chiral center since C-14 is ^{12}C and C-15 is ^{13}C. Thus, the ring closure step in the synthesis of γ-cadinene[5] must proceed on the enzyme surface through a carbonium ion (**4**) with a very short life, which does not allow rotation around the C_{10}–C_{13} bond. As indicated above, the X-ray defraction study shows the isopropyl group is in the most stable rotamer with C-14 pointing down and interacting with the axial protons on C-9 and C-11. If, as expected, this is also the favored rotamer in solution, then C-14 should experience an upfield shift in the ^{13}C-NMR spectrum.[6] C-14 is thus assigned to the quartet at 14.9δ. C-15 occurs slightly downfield (20.9δ) from a freely rotating isopropyl group such as found in menthane (19.0δ).[7] Proton–proton coupling constants further substantiate that the rotamer shown for the isopropyl group in Fig. 1 from the X-ray defraction data is also the predominant rotamer in solution. Thus $J_{H\text{-}10}$–$J_{H\text{-}13}$ is found to be 2.8 Hz, which from the Karplus eqns (8, 9) indicates a torsional bond angle of 52° for H_{10}–C_{10}–C_{13}–H_{13}. Using the torsional bond angles derived from the X-ray

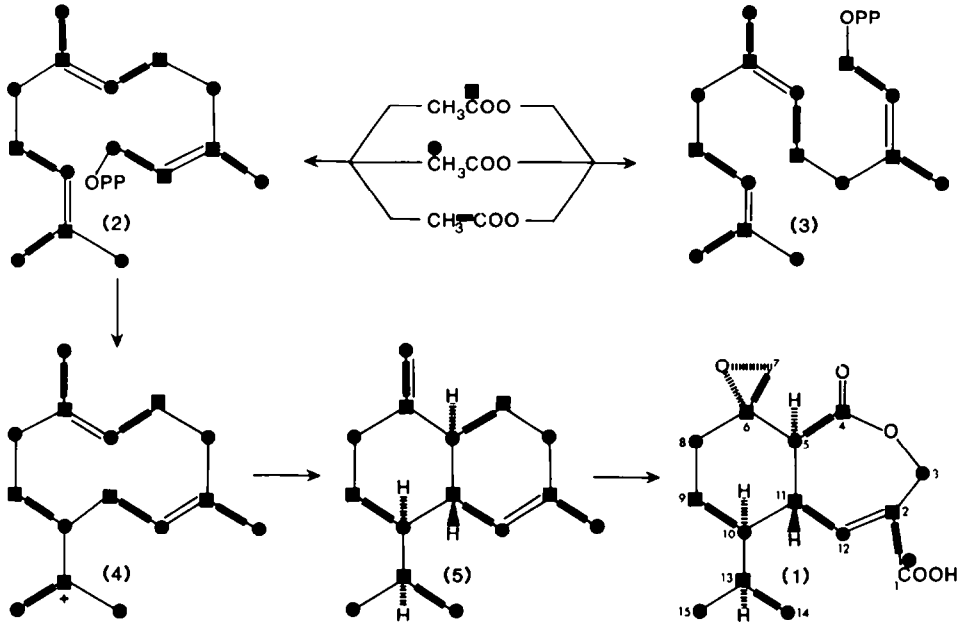

Fig. 3.

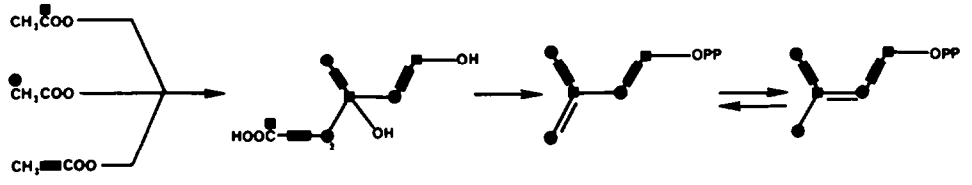

Fig. 4.

defraction study and assuming that H-10 and H-13 are equidistant from C-9 and C-11, and from C-14 and C-15, respectively, it is possible to calculate the torsional angle H_{10}–C_{10}–C_{13}–H_{13}. This angle is found to be 59.7°, which is in good agreement with that obtained from the proton–proton coupling constant. Furthermore, the observed stereochemistry in **1** requires that the carbonium ion in **4** must be protonated from the back side of the molecule. The X-ray defraction data, the proposed biosynthetic pathway, the results of the [1,2-^{13}C] acetate feeding experiment, the ^{13}C chemical shifts, and the proton–proton coupling constant agree with the ring closure and protonation as outlined above.

The observed ^{13}C–^{13}C coupling between C-4 and C-5 show that these C's are derived from the same acetate unit, and thus **3** (Fig. 3) cannot be the precursor to heptelidic acid. The O insertion between C-4 and C-3 apparently proceeds by an enzymatic Baeyer–Villiger type O insertion known to occur in microorganisms.[10] At the conclusion of this work, during a literature search for other biosynthetic studies on cadalene type sesquiterpenes, we found Arigoni's report on the biosynthesis of this same compound in which he named **1**, avocettin.[4] Arigoni's work was apparently omitted by Itoh *et al.* since the structure elucidation research was published only in Hagenbach's dissertation.[3] Arigoni's elegant ^{3}H and ^{14}C tracer work showed that *cis, trans*-farnesyl pyrophosphate can act as the biosynthetic precursor to heptelidic acid without intervention of *trans, trans*-farnesyl pyrophosphate. Ring closure occurs through a stereospecific 1,3-hydride shift in which the Me's of the isopropyl group retain their biogenetic identity (Fig. 5). The results of our [^{13}C] acetate experiments agree in all respects with Arigoni's results.

EXPERIMENTAL

M.ps were determined using a Kofler hot-stage microscope and are uncorrected. The optical rotation was determined on a Perkin–Elmer Model 241 polarimeter. Mass spectra were measured at low resolution on a Varian-MAT-CH7 spectrometer, and at high resolution on a CEC 21-110 spectrometer by direct probe insertion (probe temp 200° for high resolution mass spectrum). The ^{1}H-NMR and ^{13}C-NMR were recorded on either a JEOL FX-90Q or a Varian XL-200. Elemental analyses were performed by Galbraith Laboratory. The single crystal X-ray structure determination was performed by Molecular Structure Corporation.

Potato dextrose broth (12 × 1 l. of Difco PDA with agar removed) was inoculated with a conidial suspension of strain GV-P of *G. virens* and incubated at 25° in shake culture for 5 days. The cultures were filtered through cheese cloth and extracted with $CHCl_3$ (1 ml $CHCl_3$/10 ml of filtrate). The aqueous filtrate was adjusted to pH 3.5 with 6 N HCl and extracted again with $CHCl_3$ (1 ml $CHCl_3$/6 ml filtrate), and the $CHCl_3$ extract was dried over anhyd Na_2SO_4 and evaporated to dryness. The residue was chromatographed over silica gel (75 g of Mallinckrodt CC-4) eluting successively with $CHCl_3$ (100 ml); $CHCl_3$:$(CH_3)_2CO$ (250 ml, 98:2); $CHCl_3$:$(CH_3)_2CO$ (250 ml, 95:5). Fractions (11 ml) number 15 to 22 contained the heptelidic acid. The crude heptelidic acid was chromatographed over silica gel (65 g) eluting successively with $CHCl_3$:cyclohexane (C_6H_{12}) (100 ml, 1:1); $CHCl_3$:C_6H_{12} (100 ml, 3:1); $CHCl_3$ (300 ml). Fractions (11 ml) number 11 to 14 contained heptelidic acid. The crude product was crystallized from CH_2Cl_2:C_6H_{12}.

The compound gave white needles from CH_2Cl_2:C_6H_{12} containing one half mole of C_6H_{12} (m.p. 62–65°); $[\alpha]_D^{20}$ + 7.4° (*c* 1.0, $CHCl_3$); MS *m/e* (% base peak): 280 (1.6), 262 (8), 244 (15), 234 (13), 219 (43), 217 (18), 191 (23), 180.117165 (85, Calc for $C_{11}H_{16}O_2$: 180.1150), 175 (26), 174 (20), 173 (24), 165 (21), 164 (27), 163 (26), 148 (27), 147 (25), 145 (30), 138 (20), 137 (50), 136 (20), 135 (32), 133 (22), 131 (27), 129 (30), 124 (22), 123 (21), 121 (19), 119 (34), 117 (41), 115 (33), 107 (35), 105 (40), 93 (47), 91 (100). Calc for $C_{15}H_{20}O_5$ 1/2 C_6H_{12}: C, 67.06; H, 8.13; O, 24.81. Found: C, 66.64; H, 8.23; O, 24.90%. IR, UV and ^{1}H-NMR are reported in Ref. 2.

G. virens was grown as described above in 500 ml of PDA in 3 l. Fernbach flask. On the second and fourth day after inoculation, 250 mg of sodium [1-^{13}C] acetate (90% isotopic purity) or sodium [2-^{13}C] acetate (90% isotopic purity) were added to parallel flasks. Sodium [1,2-^{13}C] acetate (60% isotopic purity) (250 mg) was added to a third flask on the

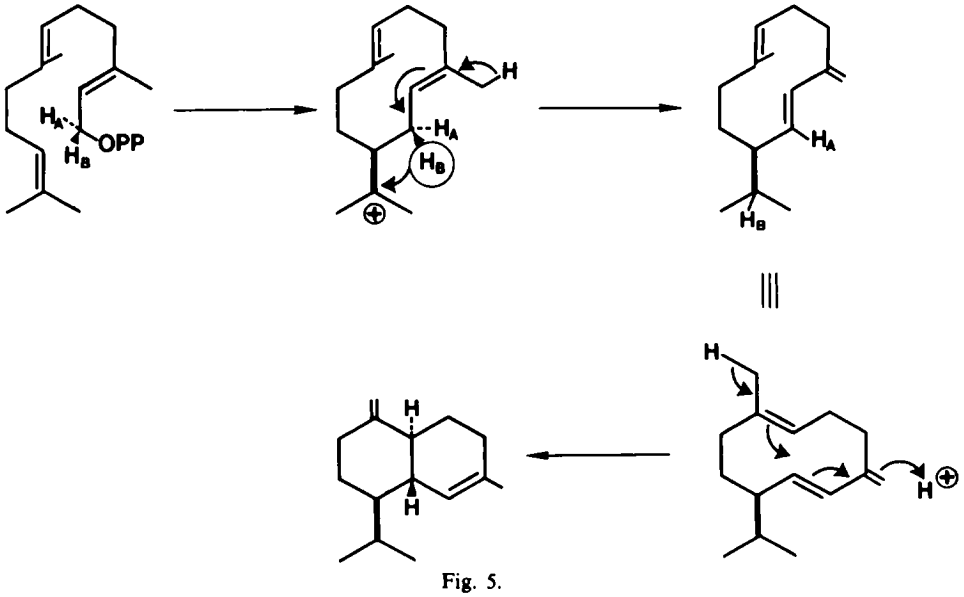

Fig. 5.

third day. Controls for each treatment were also run. The controls appeared to be completely normal. Per cent enrichment due to incorporation of [1-^{13}C] acetate (a), and [2-^{13}C] acetate (b) as calculated by peak intensity is as follows: (a) 1.1 (C-2), 1.4 (C-4), 2.9 (C-6), 2.7 (C-9), 2.6 (C-11), 2.9 (C-13); (b) 1.1 (C-1), 2.8 (C-3), 2.9 (C-5), 3.0 (C-7), 2.3 (C-8), 3.0 (C-10), 3.1 (C-12), 4.4 (C-14), 4.1 (C-15).

A colorless prismatic crystal of heptelidic acid obtained from benzene ($C_{15}H_{20}O_5$ plus 1:1 benzene–water solvate) having approximate dimensions of 0.20 × 0.25 × 0.30 mm was mounted in a glass capillary with its long axis roughly parallel to the ϕ axis of the goniometer. Preliminary examination and data collection were performed with CuK radiation (λ = 1.54184A) on an Enraf–Nonius CAD4 computer controlled kappa axis diffractometer equipped with a graphite crystal incident beam monochromator. The orthorhombic cell parameters and calculated volume were: a = 15.682; b = 15.497; c = 16.799 A; V = 4082.5 A^3, for z = 8 calc d = 1.22 g/cm^3, space group $P2_12_12_1$. The data were collected at −112 + 1°. A total of 4878 reflections were collected, of which 4660 were unique and not systemically absent. The scan rate varied from 2° to 20°/min (in omega) using the w–θ scan technique. Data were collected to a maximum 2θ of 150.0°. The structure was solved by direct methods. Using 308 reflections (minimum E of 1.70) and 3679 relationships, a total of 80 phase sets were produced. A total of 43 atoms were located from an E-map prepared from the phase set with probability statistics: absolute figure of merit = 1.13, residual = 0.19, and psi zero = 1.110. The remaining atoms were located in succeeding difference Fourier synthesis. Hydrogen atoms were not included in the calculations. Non-H atoms were refined anisotropically. Tables of bond distances, bond angles, torsional angles, and of observed and calculated structure factors have been deposited in the Cambridge Crystallographic Data Center, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.

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