Biosynthesis of Citreoviridin. A Carbon-13 N.M.R. Study

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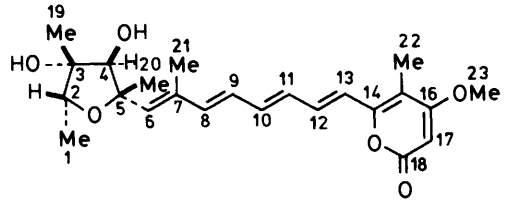
The complete assignment of the natural abundance ^{13}C n.m.r. spectrum of citreoviridin, a mycotoxin produced by *Penicillium pulvillorum* CSIR 1406, allowed a study of its biosynthetic origin using ^{13}C-labelled precursors. The results show that citreoviridin is derived from a C_{18}-polyketide formed from an acetate chain-starter unit and eight malonate units. The five remaining carbon atoms, *i.e.* the five methyl groups, are derived from (2*S*)-methionine.

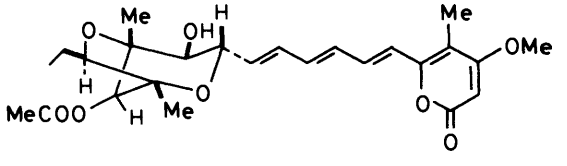
CITREOVIRIDIN (1), a potent neurotoxic mycotoxin produced by *Penicillium citreoviride* and *P. pulvillorum* is reputed to be the causative agent of the epidemic-like occurrence of cardiac beri-beri in man in parts of East-Asia.[1] The compound's acute toxicity, like that of the related fungal metabolites, the aurovertins and asteltoxin is due to its inhibition of ATP-synthesis and ATP-hydrolysis catalysed by mitochrondrial enzyme systems.[2]

The participation of propionate as a starter unit in the biosynthesis of aurovertin B(2) [3] prompted us to investigate the biosynthesis of citreoviridin by ^{13}C n.m.r. spectroscopy using ^{13}C-labelled precursors. In previous studies with ^{14}C-labelled precursors [4] only a few positions of incorporation in citreoviridin could be determined by chemical degradation. The results, however led to the conclusion that the metabolite is derived from a C_{18}-polyketide formed from acetylcoenzyme A as a starter unit and eight malonylcoenzyme A units.

The assignment of the ^1H n.m.r. spectrum of citreoviridin (1) as presented in Table 1 is based on proton-proton



(1)



(2)

coupling patterns, chemical-shift considerations, and the reported chemical-shift values for the corresponding protons in the aurovertins.[3] The signal of the C-2 proton is obscured by the resonance of the methoxy-protons (23-H) at δ 3.83; however, on recording the spectrum of citreoviridin using [$^2H_6$]benzene as solvent, this resonance is shifted upfield and the C-2 proton appears as a quartet (J 6.5 Hz). The assignment of the two three-proton singlets at δ 1.23 and 1.39, attributed to the C-19 and C-20 methyl protons, respectively follows from heteronuclear ^{13}C-{^1H} selective population inversion (SPI) experiments [5] (see below).

The assignment of the natural abundance ^{13}C n.m.r. spectrum of citreoviridin (1) (see Table 1) is based on the results obtained from single frequency n.O.e., proton-noise decoupled (p.n.d.), off-resonance proton-decoupled and selective proton-decoupled ^{13}C n.m.r. spectra, from SPI experiments [6] and the reported chemical-shift values of related compounds.[3,7]

The residual splittings observed in a series of off-resonance proton-decoupled ^{13}C n.m.r. experiments enabled us to correlate all the signals of proton-bearing carbon atoms with specific proton resonances.[8] With this relationship, the resonances due to C-2, C-4, C-6, C-12, C-17, and C-23 could be unambiguously assigned. The magnitude of the observed, directly-bonded (C,H) coupling constants (Table 1) supports these assignments.

The remaining methyl and quaternary carbon resonances were assigned mainly from the results of heteronuclear ^{13}C-{^1H} SPI experiments. The main parameters used in SPI studies are (C,H) couplings and, normally, only one-, two-, and three-bond (C,H) couplings are observed. The reported (C,H) couplings over four bonds are of the order of 1 Hz [9] and need not be considered at the power levels used in the SPI experiments (*ca.* 5 Hz).

Chemical-shift considerations dictate that the resonances at δ 80.5 and 83.9 must be attributed to the two oxygen-bearing aliphatic quaternary carbon atoms, C-3 and C-5. Selective decoupling of 6-H (δ 5.94) leads to significant sharpening of the signal at δ 83.9 thereby assigning it to C-5. The remaining resonance at δ 80.5 is therefore due to C-3. The assignment of the singlet signals at δ 163.7 (C-18), δ 170.4 (C-16), δ 107.5 (C-15), and δ 154.2 (C-14) follows from a comparison with the chemical-shift values for these carbon atoms in the aurovertins [3] and in citreomontanin.[7] As a consequence the remaining quaternary sp^2 carbon resonance at δ 133.3 must be due to C-7.

Application of a selective π-pulse with $\gamma H_2 = 10$ Hz to the highfield ^{13}C transitions of the protons resonating at δ_H 1.20 (1-H), 1.23, and 1.39 confirms the correlation of these proton resonances with the carbon resonances at δ 12.3, 17.6, and 21.2, respectively and assigned the

resonance at δ 12.3 to C-1. The signals at δ 8.6 and 13.1, both of which had been correlated with the proton resonances at δ_H 1.95 (characteristic of the protons of methyl groups on double bonds), were assigned to C-22 and C-21, respectively, on the basis of long-range (C,H) couplings observed for the C-21 resonance. At this stage it is possible to assign unambiguously the ^{13}C and ^{1}H C-12 if the chemical shifts for the comparable carbon atoms in the aurovertins [3] and citreomontanin [7] are taken into account. The remaining signals of citreoviridin at δ 126.9, 138.5 and 130.5 were thus assigned to C-9, C-10, and C-11, respectively. Analysis of the ^{13}C n.m.r. spectra of citreoviridin derived from [1-^{13}C]- and [1,2-^{13}C]acetate enabled us to confirm these assignments.

TABLE 1

N.m.r. data for citreoviridin (1)

Carbon atom	δ_C [a]	^1J(CH)/Hz	$^{>1}J$(CH)/Hz	J(CC)/Hz [b]	δ_H [c]	J(HH)/Hz
1	12.3 Q	126.5		41.1	1.20d	6.5
2	77.3 Dm	142		41.2	3.85q	6.5
3	80.5 Sbr	—		41.1		
4	85.3 Dbr	150.3		40.7	4.02s	
5	83.9 Sbr	—		50.0		
6	141.8 Dm	158		50.2	5.94s	
7	133.3 Sm	—		55.0		
8	140.7 Dm	152.0		55.1	*d*	
9	126.5 Dm	155.6		58.4	*d*	
10	138.5 Dm	155.2		57.7	*d*	
11	130.5 Dm	151		58.1	*d*	
12	135.8 Dm	150		58.6	7.19dd	10.0; 15.0
13	118.1 Dm	154.3		70.6	*d*	
14	154.2 Sm	—		70.5		
15	107.5 Sm	—		62.4		
16	170.4 Sm	—		62.5		
17	88.1 Dbr	168.3		78.1	5.51s	
18	163.7 S	—	—	78.8		
19	17.6 Q	126.7	—	S	1.23s	
20	21.2 Q	128.3	—	S	1.39s	
21	13.1 Qdm	126.5	6.8	S	1.95s	
22	8.6 Q	129.6	—	S	1.95s	
23	56.0 Q	146.4	—	S	3.83s	

[a] Relative to Me_4Si; solvent $CDCl_3$. Capital letters refer to the pattern resulting from directly-bonded (C,H) couplings [^1J(CH)] and small letters to that from (C,H) couplings over more than one bond [$^{>1}J$(CH)]. S = singlet, D = doublet, Q = quartet, m = multiplet and br = broad. [b] Obtained from the spectrum of citreoviridin derived from [1,2-$^{13}C_2$]acetate. [c] Relative to internal Me_4Si; solvent $CDCl_3$. [d] Multiplet at δ_H 6.2—6.6.

resonances of the C-19 and C-20 methyl groups. Application of a π-pulse with $\gamma H_2 = 5$ Hz at a position 5 Hz to highfield of the methyl singlet at δ_H 1.39 influenced the C-5 (δ 83.9) and C-4 (δ 85.3) resonances. The affected proton transition must, therefore, belong to 20-H and proved that the signal at δ 21.2 arises from C-20. The signal at δ 17.6 is therefore assigned to C-19.

The assignment of the signals at δ 77.3 and 85.3 to C-2 and C-4, respectively (see above) was confirmed by a SPI experiment in which a π-pulse ($\gamma H_2 = 5$ Hz) was applied 5 Hz to highfield of the highfield transition of the doublet due to the 1-H protons. This pulse affected only the signals at δ 77.3 (C-2) and 80.5 (C-3).

The five remaining olefinic carbon resonances, at δ 140.7, 126.5, 138.5, 130.5, and 118.1 due to C-8—C-11 and C-13 were assigned as follows. The assignment of the signal at δ 118.1 to C-13 is based on a comparison with the chemical shift value of this carbon atom in both the aurovertins [3] and citreomontanin.[7] When a π-pulse ($\gamma H_2 = 5$ Hz) is applied either 5 Hz to high- or low-field of the proton resonances at δ_H 1.95 (21-H and 22-H) the resonances due to C-6, C-7, C-14, C-15, C-16 and the signal at δ 140.7 are in each case affected. The latter resonance must thus be assigned to C-8. The signals due to C-9, C-11, and C-13 in citreoviridin are expected to appear at higher field than those of C-6, C-8, C-10, and

Our assignments, as presented in Table 1, differ considerably from those reported in the literature.[10]

Biosynthetic Studies.—Cultures of *Penicillium pulvillorum* CSIR 1406 (ATCC 26219) were grown in the dark at 23 °C in a stationary culture on an F14 medium.[11] Studies on the course of fermentation indicated that citreoviridin production commenced on day 2 and that satisfactory yields of the metabolite could be obtained 12 days after the inoculation of the medium. Preliminary feeding experiments with [1-^{14}C]acetate as the precursor established conditions which would give a suitable ^{13}C enrichment at each individual, acetate-derived carbon atom of citreoviridin on feeding [^{13}C]acetate. A satisfactory dilution value [3] for citreoviridin (19.5, assuming 9 labelled positions) and good incorporation (1.5%) was obtained by pulsing cultures of *P. pulvillorum* every 12 h from day 3 to day 11 with sodium acetate to a total amount of 1.0 g l^{-1}.

The p.n.d. ^{13}C n.m.r. spectrum of [1-^{13}C]acetate-derived citreoviridin showed nine enhanced signals, attributed to C-2, C-4, C-6, C-8, C-10, C-12, C-14, C-16, and C-18 whereas the spectrum of citreoviridin derived from [2-^{13}C]acetate showed enhanced signals for C-1, C-3, C-5, C-7, C-9, C-11, C-13, C-15, and C-17. Satisfactory enrichment factors [3] were obtained for both the [1-^{13}C]- and [2-^{13}C]-acetate-derived carbon atoms (average 5.3

and 4.0, respectively). These results are in complete agreement with those reported by Franck and Gehrken.[10]

The arrangement of intact acetate units in citreoviridin was studied by addition of [1,2-^{13}C]acetate to cultures of *P. pulvillorum*. All the signals in the p.n.d. ^{13}C spectrum of citreoviridin derived from [1,2-^{13}C]acetate, with the exception of those for C-19, C-20, C-21, C-22, and C-23, exhibited ^{13}C–^{13}C spin-spin coupling. The measured ^{1}J(CC) values are given in Table 1 and prove the presence of nine intact acetate units, *viz.* C-1–C-2, C-3–C-4, C-5–C-6, C-7–C-8, C-9–C-10, C-11–C-12, C-13–C-14, C-15–C-16, and C-17–C-18.

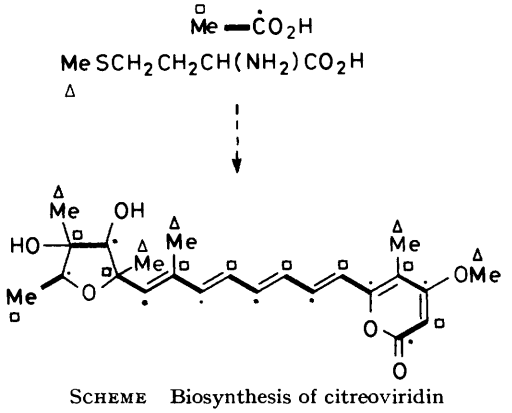
The appearance of a number of AB spin systems in the 25.2 MHz p.n.d. ^{13}C n.m.r. spectrum was actually advantageous since these spin systems are useful for assignment purposes as well as for determining pairs of coupled carbon atoms with similar ^{1}J(CC) values.[3] The general features of the observed AB spin systems for citreoviridin derived from [1,2-^{13}C]acetate were used to confirm the assignment of the olefinic carbon atoms C-9–C-11. The resonance at δ 130.5 exhibits a (C,C) coupling of 58.1 Hz and forms an AB spin system with the signal at δ 135.8 which has been assigned to C-12 (see above). The resonance at δ 130.5 is therefore assigned to C-11. The signals of the AB system at δ 126.5 and 138.5 are due to C-9 and C-10, respectively as the former signal is enhanced in the p.n.d. ^{13}C spectrum of citreoviridin derived from [2-^{13}C]acetate. Conversely, the signal at δ 138.5 is enhanced when [1-^{13}C]acetate is used as the precursor.

The above results, obtained from feeding experiments using ^{13}C-labelled acetate, account for the origin of 18 of the 23 carbon atoms in citreoviridin. The origin of the remaining five carbon atoms was investigated using (2*S*)-methionine, an excellent source of one-carbon units in nature. In preliminary experiments (2*S*)-[*methyl*-^{14}C]methionine was efficiently incorporated into citreoviridin (absolute incorporation 11.5%). Feeding experiments at a level of 390 mg l^{-1} and using 200 ml of medium were chosen in order to obtain sufficient material for ^{13}C n.m.r. spectra, as well as a low dilution value (and thus a satisfactory enrichment). On feeding (2*S*)-[*methyl*-^{13}C]methionine, containing 50 μCi (2*S*)-[*methyl*-^{14}C]methionine as a tracer, citreoviridin with a specific activity of 46.4 μCi mmol^{-1} was obtained. This result indicates a dilution value [3] of 10.4 for each labelled position (assuming the presence of five labels) and thus an enrichment factor [3] of 8.9. The p.n.d. ^{13}C n.m.r. spectrum of the metabolite showed enrichment of the signals attributed to C-19, C-20, C-21, C-22 and C-23 (average enrichment factor 8.8).

The presence of an acetate-starter unit in citreoviridin was demonstrated by feeding experiments with [2-^{13}C, 2-$^{2}H_3$]acetate. Experimental evidence has shown that the methyl hydrogens of acetyl-CoA are incorporated into fatty acids in varying degrees: the predominant species (*ca.* 80%) at the terminal methyl group (*i.e.* the methyl group of the starter unit), using [2-^{13}C, 2-$^{2}H_3$]-acetate as precursor, is $^{13}C^{2}H_3$.[12,13] Little loss of deuterium from the precursor, occurs before the incorporation.[13] This effect has been observed for the starter-acetate unit in griseofulvin.[14]

The presence of carbon atoms bearing deuterium atoms can be investigated with ^{13}C n.m.r. spectroscopy. The ^{13}C signals of ^{13}C–^{2}H species appear as multiplets shifted to higher field (because of isotope effects) compared with the ^{13}C signal due to the corresponding ^{13}C–^{1}H species in p.n.d. ^{13}C n.m.r. spectra; the latter signal exhibits a concomitant decrease in intensity.[14,15] In a parallel experiment [2-^{13}C]- and [2-^{13}C, 2-$^{2}H_3$]-acetate were separately administered to cultures of *P. pulvillorum*. A comparison of the p.n.d. ^{13}C n.m.r. spectra of citreoviridin obtained from each precursor showed no significant difference in the enhancement of the signals of the enriched carbon atoms, except for the intensity of the C-1 methyl signal which was decreased by 70% in the spectrum of the [2-^{13}C, 2-$^{2}H_3$]acetate-derived citreoviridin.

The results obtained from the feeding experiments with ^{13}C-labelled acetate and methionine prove that citreoviridin is derived from a C_{18}-polyketide formed from acetyl-CoA as a starter unit and eight malonyl-CoA units with methionine contributing the remaining five C_1-units.



SCHEME Biosynthesis of citreoviridin

EXPERIMENTAL

^{1}H n.m.r. spectra were recorded on a Varian EM-390 continuous-wave instrument (90 MHz) using Me_4Si as lock signal and internal reference. ^{13}C n.m.r. spectra were recorded on a Varian XL-100-15 F.T. spectrometer equipped with a 16 K Varian 620i computer and a gated gyrocode decoupler.

Isolation of Citreoviridin.—Conical flasks (10 × 500 ml) containing F14 medium [11] (100 ml) were inoculated with a spore suspension of *Penicillium pulvillorum*, CSIR 1406 (ATCC 26219). The mould was grown in stationary culture at 23 °C in the dark. After 12 days the cultures were filtered and the mycelium macerated with acetone in a Waring blender. The acetone solution was evaporated to dryness.

The chloroform extract of the culture filtrates was concentrated and the residue combined with that obtained from the acetone solution. The residues were partitioned between n-hexane and 90% methanol. The 90% methanol solution was concentrated and the residue partitioned

between chloroform and water. The chloroform solution was dried (Na_2SO_4), filtered, and evaporated to dryness. The crude citreoviridin was purified by column chromatography on silica gel using ethyl acetate to give citreoviridin (1) [16] (220 mg).

Incorporation of Labelled Precursors.—To each of two 500-ml flasks containing the 3-day old growth of *P. pulvillorum* was added the requisite, labelled precursor every 12 h from day 3 to day 11. The cultures were harvested on day 12 as described and the labelled citreoviridin separated and purified by column chromatography on silica gel using ethyl acetate. A summary of the yield of citreoviridin for the differently labelled precursors is given in Table 2.

TABLE 2

Feeding experiments with labelled precursors [a]

Precursor	Atom % ^{13}C	Amount (mg)	Yield of (1) (mg)
Sodium [1-^{13}C]acetate [b]	92.6	200	32 [e]
Sodium [2-^{13}C]acetate	90.5	200	22
Sodium [2-^{13}C, 2-$^{2}H_3$]acetate [c]	93.0	200	32
Sodium [1,2-^{13}C]acetate	C-1 90.0; C-2 91.3	200	23
(2*S*)-[*methyl*-^{13}C]Methionine [d]	90.0	78	50 [f]

[a] Values relative to 0.2 l of culture medium. [b] Admixed with 250 μCi sodium [1-^{14}C]acetate. [c] 99 Atom % ^{2}H. [d] Admixed with 50 μCi (2*S*)-[*methyl*-^{14}C]methionine. [e] Specific activity 47.9 μCi mmol^{-1}. [f] Specific activity 46.4 μCi mmol^{-1}.

We thank Dr. A. E. de Jesus for expert microbiological assistance.

[2/247 *Received*, 10*th February*, 1982]

REFERENCES

1 Y. Ueno in 'Mycotoxins,' ed. I. F. H. Purchase, Elsevier, New York, 1974, p. 283.
2 P. E. Linnett, A. D. Mitchell, M. D. Osselton, L. J. Mulheirn, and R. B. Beechey, *Biochem. J.*, 1978, **170**, 503.
3 P. S. Steyn, R. Vleggaar, and P. L. Wessels, *J. Chem. Soc., Chem. Commun.*, 1979, 1041; *ibid., J. Chem. Soc., Perkin Trans. 1*, 1981, 1298.
4 D. W. Nagel, P. S. Steyn, and N. P. Ferreira, *Phytochemistry*, 1972, **11**, 3215.
5 T. G. Dekker, K. G. R. Pachler, and P. L. Wessels, *Org. Magn. Reson.*, 1976, **8**, 530.
6 K. G. R. Pachler and P. L. Wessels, *J. Magn. Reson.*, 1973, **12**, 337; *ibid.*, 1977, **28**, 53.
7 S. Rebuffat, D. Davoust, and D. Molho, *Phytochemistry*, 1981, **20**, 1279.
8 K. G. R. Pachler, P. L. Wessels, J. Dekker, J. J. Dekker, and T. G. Dekker, *Tetrahedron Lett.*, 1976, 3059.
9 V. A. Chertkov and N. M. Sergeyev, *J. Am. Chem. Soc.*, 1977, **99**, 6750; H. Seel, R. Aydin, and H. Günther, *Z. Naturforsch., Teil B*, 1978, **33**, 353.
10 B. Franck and H.-P. Gehrken, *Angew. Chem. Int. Ed. Engl.*, 1980, **19**, 461.
11 C. L. Baldwin, L. C. Weaver, R. N. Brooker, T. N. Jacobsen, C. E. Osborne, and H. A. Nash, *Lloydia*, 1964, **27**, 88.
12 R. H. White, *Biochemistry*, 1980, **19**, 9.
13 A. G. McInnes, J. A. Walter, and J. L. C. Wright, *Tetrahedron Lett.*, 1979, 3245.
14 Y. Sato, T. Oda, and H. Saitô, *Tetrahedron Lett.*, 1976, 2695; Y. Sato, T. Oda, E. Miyata, and H. Saitô, *FEBS Lett.*, 1979, **98**, 271.
15 U. Sankawa, H. Shimada, and K. Yamasaki, *Tetrahedron Lett.*, 1978, 3375.
16 D. W. Nagel, P. S. Steyn, and D. B. Scott, *Phytochemistry*, 1972, **11**, 627; N. Sakabe, T. Goto, and Y. Hirata, *Tetrahedron Lett.*, 1964, 1825.