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THE BIOSYNTHESIS OF FULVIC ACID, A FUNGAL METABOLITE OF HEPTAKETIDE ORIGIN

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Summary: Analyses of the regiochemistry of [^{13}C]acetate incorporation into fulvic acid (2) by *Penicillium brefeldianum* indicates that the metabolite is biosynthesized via a heptaketide intermediate assembled as a single chain of seven C_2 units rather than from two smaller polyketide chains. This information favours a route leading through common C_{14} intermediates to the three fungal metabolites, citromycetin, fulvic acid and fusarubin.

Within the large class of fungal metabolites of polyketide origin, those formed from seven C_2 units have been subdivided by Turner,[1] on the basis of structure and available biosynthetic information, into eight groups. Compounds in the first seven are logically derived from a common linear heptaketide intermediate which is made from one acetyl and six malonyl units. The different groups of end-products are postulated to arise from folding pattern variations during subsequent cyclizing reactions. Citromycetin (1) and fulvic acid (2), the two compounds in Turner's eighth group, were distinguished from the heptaketide-based metabolites in that their structures could not be derived by simple linear chain folding.

Birch and co-workers[2] showed that seven C_2 units originating from [^{14}C]acetate were incorporated into 1 by *Penicillium frequentans*. Later, Gatenbeck and Mosbach[3] found evidence for two starter units by chemical degradation of 1 labeled biosynthetically from diethyl [2-^{14}C]-malonate and concluded that two polyketide chains contributed to the structure. A ^{13}C NMR study of 1 labeled from [1-^{13}C]-,[2-^{13}C]- and [1,2-^{13}C]acetate showed a labelling pattern consistent with a two-chain origin[4] but also did not exclude pathways in which a single chain is cyclized and then ring-opened to a branched intermediate such as has been postulated on several occasions[5,6] to be the progenitor of 1, 2 and fusarubin (3). Two such pathways have been noted, one involving a palitantin-type[1] and the other a fusarubin-type[7] folding of the heptaketide.

We are persuaded by evidence presented here for the biosynthesis of 2 via a single-chain heptaketide that 1, 2 and 3 arise from common C_{14} intermediates as shown in Fig. 1. The heptaketide folding pattern (*A*) that gives *B* upon intramolecular aldol cyclization provides a direct route to the 4a,10a-dihydrofusarubins, from which 3 is formed by non-enzymic oxidation.[8] This idea has recently been validated by labeling experiments with [^{13}C]- and [^{2}H]-labeled acetate.[9] Oxidative fission of the indicated bond in *B*, followed by appropriate bond reconnections (shown later in Scheme 1) would give rise to 1 and 2. The hypothesis is attractive because it accounts for the biogenesis of all fungal metabolites containing seven C_2 units as derivatives of a single linear heptaketide which is modified by organism-specific cyclizing and processing reactions.

Penicillium brefeldianum Dodge (NRRL 2083) produces 2 and palitantin (4) when grown in Raulin-Thom medium.[10,11] Both products reach their maximum yield on day 5 of the fermentation.

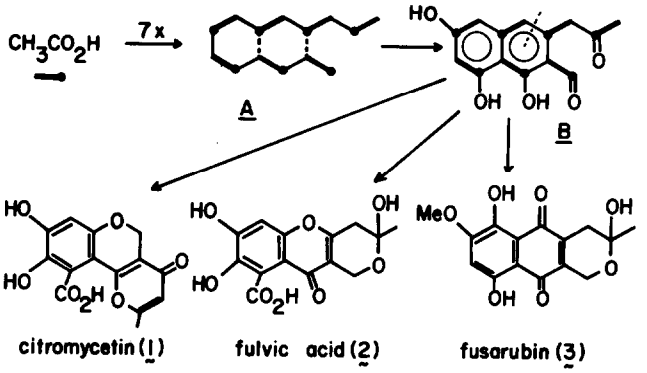


Figure 1. Probable biogenetic origin of citromycetin, fulvic acid and fusarubin.

Experiments with [1-^{14}C]acetate showed that ^{14}C enrichment of 2 was highest (18-19%) if the precursor was fed on days 2 and 3 whereas 4 was maximally enriched by precursor feeding on days 3 and 4. To achieve high ^{13}C incorporation, therefore, we administered [1-^{13}C]acetate and [1,2-$^{13}C_2$]acetate (each containing 90 atom % ^{13}C) to give 5 m*M* broth concentrations twice during the two-day periods of maximum metabolite production. The labeled products were isolated on day 5.

The δ_C relative to internal Me_4Si of all signals in the ^{13}C NMR spectrum of fulvic acid were assigned on the basis of: comparison of δ_C values for 2 and anhydrofulvic acid (6)[10] in pyridine and their respective trimethyl derivatives,[10] 5 and 7, in chloroform; the J_{CH} coupling relationships in these four compounds observed by gated proton irradiation (NOE) experiments; and $^1J_{CC}$ coupling data obtained from the ^{13}C NMR spectra of ^{13}C-enriched 5 and 7. THE NOE spectrum of 2 had two triplet signals in the upfield region; that of 6 had only one. Similarly, a high-field quartet and a mid-field doublet appeared in the NOE spectrum of 2 and 5. From this evidence we could assign the δ_C of C-2, C-7, C-9 and C-11 securely. The resulting assignments enabled us to use the unique $^1J_{CC}$ relationships visible for all seven intact C_2 units in the spectrum of ^{13}C-enriched 7 to assign the chemical shifts of C-2a, C-7a, C-8a and C-10. The δ_C of C-3a and C-4 were distinguished by the value of $^1J_{C_4C_{12}}$ and by the presence of a $^3J_{CH}$ coupling for C-3a but the absence of any long range C,H coupling for C-4. From these data we could assign the δ_C of C-3 and C-12 by noting the value of their respective carbon coupling constants with C-3a and with C-4. The δ_C for C-12 is also defined by its characteristic upfield shift on methylation. Finally assignment of C-5 and of C-6 was based on the relative magnitudes of long-range J_{CH} patterns and δ_C values in 2 vs. 5 and 6 vs. 7. ([1-^{13}C]Acetate also labeled C-5 and not C-6.) The pertinent spectral data are shown in Figure 2.

We then determined the complete regiochemistry of the incorporation of [1-^{13}C]acetate and of [1,2-$^{13}C_2$]acetate into fulvic acid by analyzing the ^{13}C NMR spectra of ^{13}C enriched 2, 5, 6 and 7. Fulvic acid was labeled only at the (●) carbons (Fig. 2) by [1-^{13}C]acetate with a ^{13}C enrichment of 2.6±0.2% per site. Its (—●) carbon pairs were labeled by [1,2-^{13}C]acetate

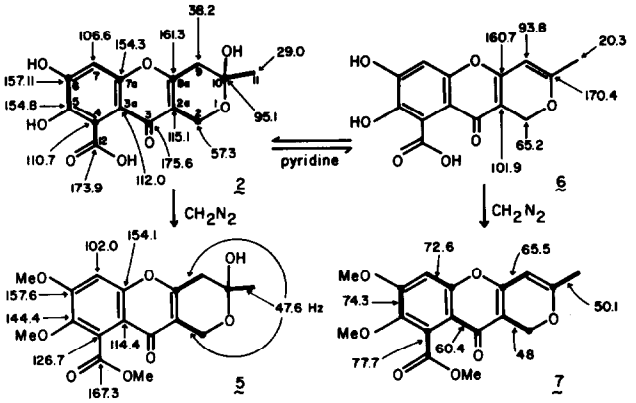
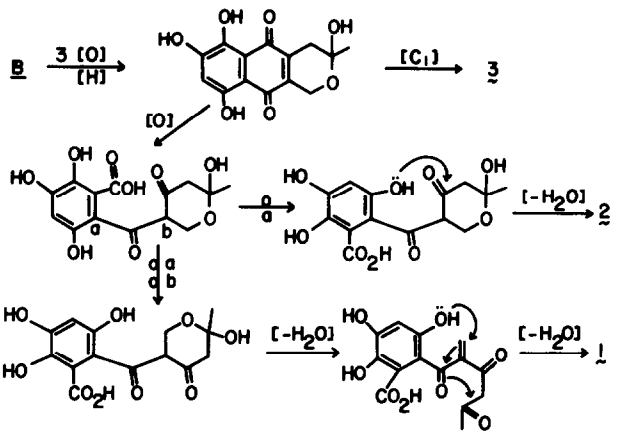


Figure 2. ^{13}C NMR data for fulvic acid and its derivatives. J_{CC} values shown for 5 and 7 are ±1.2 Hz and all spectra were determined at 22.5 MHz at 27°C.

(Fig. 2) with a ^{13}C enrichment of 1.8±0.2% per site. Since we could clearly see the small quartet resonances due to $^{1}J_{CC}$ coupling between carbons in neighboring $^{13}C_2$ sub-sets for at least one carbon of each C_2 unit in the ^{13}C NMR spectrum of 5, we could calculate that the ^{13}C enrichment of the individual acetyl/malonyl units was 45±2% at the time of polyketide chain assembly.[9]

The manner in which fulvic acid is labelled by [^{13}C]acetate is compatible with its formation from a single heptaketide chain folded as in *A* (Fig. 1), which is then cyclized and ring-opened without C_2-unit bond fission as in the biosynthesis of citromycetin. The nearly equal enrichment by [^{13}C]acetate of all one- and two-carbon subsets and the consistent high ^{13}C enrichment of all seven C_2 subsets at the time of chain assembly are more likely for a single-chain origin than for one in which separately formed chains come together. In particular, the intermolecular enrichment of two polyketide chains would probably have fallen below the observed 45% due to dilution with unlabeled material before fusion occurred; we should not then have observed coupling between the neighboring carbons of C_2 units joined in the fusion process. Finally, we can discount palitantin as a precursor of fulvic acid in *P. brefeldianum* because 2 was enriched by acetate significantly earlier than 4.[12]

The weight of evidence from this study and that on fusarubin[9] thus favours the conclusion that 1, 2 and 3 have a common biosynthetic parentage.[13] Scheme 1 shows a reasonable sequence of biochemical transformations by which these metabolites could be formed from intermediate *B* of Fig. 1.[14]



Scheme 1. Biogenetic hypothesis for the formation of citromycetin (1), fulvic acid (2) and fusarubin (3) from a common intermediate (B).

References and Notes

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(10) Dean, F.M., Eade, R.A., Moubasher, R.A. and Robertson, A., J. Chem. Soc., 1957, 3497.

(11) On Czapek-Dox medium the fungus is variously reported to produce only brefeldin A (Harri, E., Loeffler, W., Sigg, H.P., Stahelin, H. and Tamm, Ch., Helv. Chim. Acta, 1963, 46, 1235) or chiefly griseofulvin (ref. 6 above).

(12) We determined the regiochemistry of 4 labeled from [^{13}C]acetate and found it to be as previously reported for [^{14}C]acetate (Birch, A.J. and Cocor, M., J. Chem. Soc., 1960, 866; Chaplen, P. and Thomas, R., Biochem. J., 1960, 77, 91).

(13) Although the results cited in ref. 3 do not support this conclusion we do not wish to comment until the biosynthesis of 1 is reexamined with appropriate ^{13}C-labeled precursors.

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