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BIOSYNTHESIS OF CITREOMONTANIN IN *PENICILLIUM PEDEMONTANUM*

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Key Word Index—*Penicillium pedemontanum*; Aspergillaceae; biosynthesis; citreomontanin; ^{13}C NMR; shift reagents.

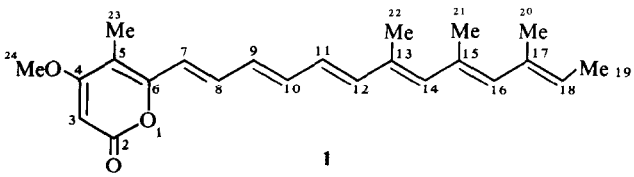
Abstract—The ^{13}C NMR spectrum of citreomontanin has been fully assigned by analysis in the presence of a shift reagent. The intramolecular distribution patterns of ^{13}C were determined for citreomontanin labelled from singly or doubly labelled [^{13}C]acetate and 2*S*-[Me^{13}C]methionine. The mechanism of the *C*-methylation reactions was investigated by MS analysis of citreomontanin labelled from 2*S*-[Me-$^{2}H_3$]methionine. The results show that the carbon skeleton of citreomontanin is biosynthesized by the polyketide pathway and that the four C—Me groups and the O—Me group are derived by transmethylation from *S*-methionine.

INTRODUCTION

We reported recently on the isolation and characterization of citreomontanin (**1**), a polyene pyrone produced by *Penicillium pedemontanum* [1]. This metabolite has a close structural similarity to citreoviridin [2] and the aurovertins [3], compounds which have been shown to be potent inhibitors of oxidative phosphorylation [4]. It was thus of interest to compare the biosynthetic origin of the citreomontanin molecule to those previously established for citreoviridin [5] and aurovertin B [6], especially as propionic acid was found to be a possible starter for aurovertin biogenesis [6]. In this paper, we report on the patterns of incorporation of ^{13}C-enriched precursors into citreomontanin.

RESULTS AND DISCUSSION

A prerequisite for ^{13}C biosynthetic studies is an unambiguous assignment of the ^{13}C NMR spectrum of the metabolite. Some of the previous assignments [1], based on literature and standard chemical shift data only, were rather ambiguous, especially in the cases of the ethylenic carbons (C-7 to C-14) which exhibit similar shifts. As a result of the use of selective irradiation in lanthanide-shifted spectra, it has been necessary to reassign some of the resonances (Table 1). The ^{1}H NMR resonances were previously assigned in the presence of the same chelate [1], hence the ^{1}H and ^{13}C NMR spectra can be correlated. It should be noted that the shifts induced at C-8, C-14, C-16 and C-18 respectively were greater than those observed for C-7, C-13, C-15 and C-17. This result can be used to distinguish between carbons C-9 and C-10, and C-11 and C-12 by comparing the intensities of the shifts induced at these carbons.



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In a preliminary study, the production of citreomontanin by *P. pedemontanum* was monitored by UV-visible spectrophotometry. The metabolite appeared after

Table 1. ^{13}C NMR data (20.115 MHz, $CDCl_3$, TMS as internal standard) for citreomontanin

Signal δ (ppm)	Signal + Eu (fod)$_3$ / [Eu(fod)$_3$] / [citreomontanin] = 0.6		Carbon atom
	δ (ppm)	Δ δ (ppm)	
170.6	178.4	7.8	4
163.7	156.3	−7.4	2
154.8	158.8	4.0	6
142.2	142.9	0.7	12
139.7	140.1	0.4	14
139.1	140.5	1.4	10
136.5	136.7	0.2	16
136.2	139.5	3.3	8
133.6	133.7	0.1	15
133.4	133.5	0.1	13
132.1	132.1	0	17
130.6	131.2	0.6	9
127.1	127.2	0.1	11
125.9	126.0	0.1	18
118.3	119.6	1.3	7
107.5	111.7	4.2	5
88.5	98.0	9.5	3
56.0	57.3	1.3	24
18.9	18.9	0	21
16.6	16.6	0	20
14.0	14.1	0.1	22
13.8	13.8	0	19
8.8	10.3	1.5	23

Table 2. ^{13}C NMR enrichment data

Carbon atom	Enrichment ratios*		$^1J^{13}C-^{13}C$ (Hz)
	[1-^{13}C]Acetate†	[2-^{13}C]Acetate‡	[1,2-^{13}C]Acetate†
2	2.24	1.32	80
3	1.03	5.74	80
4	1.92	1.32	62.5
5	0.50	3.40	62.5
6	1.56	1.37	71
7	0.77	5.20	71
8	2.18	1.91	60
9	1.17	5.18	60
10	2.08	1.45	58.5
11	0.80	5.40	58.5
12	2.32	1.43	57
13	0.60	3.96	57
14	2.11	1.57	56
15	0.68	4.63	56
16	2.55	1.95	50
17	0.82	6.02	50
18	2.38	0.98	45
19	0.79	4.61	45
20	0.71	1.71	—
21	0.86	1.95	—
22	0.91	1.69	—
23	0.91	1.69	—
24	—	—	—

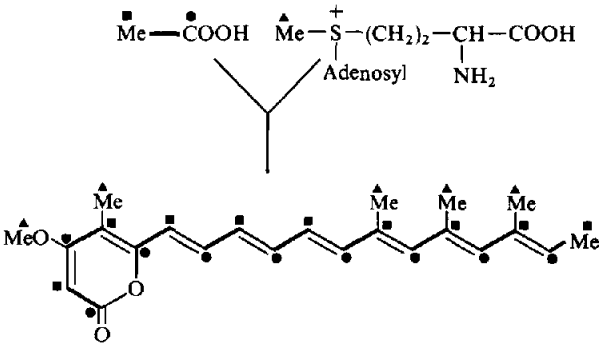
* Obtained from the ratios of the intensities in the enriched and non-enriched ^{13}C NMR spectra, whose values were normalized using OMe as a standard.

† Precursor concentration, 4.8 mM.

‡ Precursor concentration, 8.5 mM.

5 days' growth and reached its highest level 10 days thereafter. From these results, we decided that three separate additions of the enriched precursors on the 5th, 8th and 11th day should lead to a good incorporation of ^{13}C (or ^2H) into citreomontanin. To determine if citreomontanin is biosynthetized by the polyketide pathway, *P. pedemontanum* was grown in the presence of [1-^{13}C]-, [2-^{13}C]- or [1,2-^{13}C]-labelled acetate and the patterns of incorporation of ^{13}C determined (see Experimental for enrichment values). The natural abundance ^{13}C NMR spectrum of citreomontanin determined under usual conditions displays a wide range of resonance intensities which makes the identification and comparison of enriched resonances difficult. On the other hand, if the spectrum is determined under inverse gated decoupling conditions, the differences in intensity are removed. This procedure was used for all of the spectra from which we calculated the enrichment ratios at each labelled position (Table 2). The ^{13}C NMR spectrum of citreomontanin derived from [1-^{13}C]acetate showed nine enhanced signals due to C-18, C-16, C-14, C-12, C-10, C-8, C-6, C-4 and C-2 whereas the spectrum of citreomontanin derived from [2-^{13}C]acetate showed nine enhanced signals corresponding to C-19, C-17, C-15, C-13, C-11, C-9, C-7, C-5 and C-3. These labelling patterns established that the citreomontanin skeleton is formed by the polyketide pathway and that acetate (acetyl CoA) is the starter molecule. This result was confirmed by the analysis of the ^{13}C NMR spectrum of the citreomontanin labelled from [1,2-^{13}C]acetate (average % enrichment for each C, calculated according to ref. [7] $\simeq$ 2.5). It should be noted that the detection of the $^{13}C-^{13}C$ coupling satellites is extremely difficult due to the severe overlap of the AB spin systems owing to the similarity in the chemical shift of some of the olefinic carbon signals. However, we could measure the directly bonded $^{13}C-^{13}C$ coupling constants. They are compatible with the formation of citreomontanin from nine intact acetate units (Table 2).

The above data accounted for the origin of 18 of the 23 carbon atoms in citreomontanin. As the five remaining carbons are C_1 units (four Me groups and one OMe group), the possibility that they are derived from methionine was tested. We found that when cultures were supplemented with 2*S*-[Me-^{13}C]methionine a high level of incorporation of ^{13}C was obtained (MS and ^{13}C NMR, average enrichment ratio at each carbon $\simeq$ 25) at C-20, C-21, C-22 and C-24 of citreomontanin. The mechanism of the *C*-methylation and *O*-methylation reactions was investigated by feeding 2*S*-[Me-$^2H_3$]methionine. The MS of the ^2H-labelled citreomontanin exhibited intense ions at $[M+3]^+$, $[M+6]^+$, $[M+9]^+$, $[M+12]^+$ and $[M+15]^+$, consistent with the incorporation of five intact C^2H_3 groups (see Experimental). These results show unambiguously that the four C–Me groups are



Scheme 1. Derivation of carbon skeleton of citreomontanin.

formed by transmethylation of the S–Me group of 2*S*-methionine without the participation of methylene intermediates. The MS-measured incorporation of ^{13}C and ^{2}H into citreomontanin from 2*S*-methionine ([Me-^{13}C] and [Me-$^{2}H_3$]) showed that the probability of detecting five labelled Me groups is approximately the same as that of detecting one labelled group (see Experimental). This can be explained by the fact that the addition of a large amount of labelled methionine to the culture probably inhibits the formation of endogenous methionine. Thus for a brief period, the C–Me and O–Me groups of citreomontanin are formed entirely from added methionine, and the five carbons are highly enriched. When the exogenous methionine pool falls, the production of endogenous methionine and hence of unlabelled citreomontanin increases again. The results of this study show that the carbon skeleton of citreomontanin is biosynthetized by the polyketide pathway (using acetate as the starter molecule) and that the four C–Me groups and the O–Me group are derived by transmethylation of the S–Me group of *S*-methionine (Scheme 1).

EXPERIMENTAL

^{13}C NMR (20.115 MHz, $CDCl_3$, TMS as internal standard) were recorded in the Fourier transform mode on a Brücker WP 80 DS spectrometer. The typical conditions for obtaining the FT data were as follows: spectral width 5000 Hz; data points 8 K; pulse angle 60°; acquisition time 0.8 sec; pulse delay without decoupling 4 sec (for the spectra recorded with ^{1}H inverse gated decoupling). MS: direct inlet; 10 eV. ^{13}C-enriched precursors were purchased from CEA, France.

Incorporation of ^{13}C-labelled precursors. P. pedemontanum was grown in Roux flasks containing 200 ml Czapek Dox medium in stationary culture at 25°. The production of citreomontanin was monitored by measuring the UV–visible spectrum of the culture, the increase in A_{410nm} indicating the appearance of citreomontanin. In the labelling expts, aq. solns of the precursors ([1-^{13}C]NaOAc, 90% enriched 500 mg/l. of culture; [2-^{13}C]NaOAc, 90% enriched, 900 mg/l. of culture; [1,2-^{13}C]NaOAc, 87% enriched, 500 mg/l. of culture; 2*S*-[Me-^{13}C]- or 2*S*-[Me-$^{2}H_3$]methionine, 90% enriched, 250 mg/l. of culture) were added to the flasks after 5,8 and 11 days of growth. After a further 4 days, the mycelium was harvested by filtration, lyophilized, extracted with EtOH and the citreomontanin isolated as described previously [1].

MS data. Ion intensities in the M^+ region of the MS of natural, ^{13}C-labelled and ^{2}H-labelled citreomontanin ($[M]^+ = 100$): natural, $[M+1]^+ = 1.0$, $[M+2]^+ = 2.2$; [1-^{13}C]acetate (final conc in culture medium 4.8 mM) $[M+1]^+ = 13.1$; [2-^{13}C] acetate 8.5 mM, $[M+1]^+ = 80.1$; [1,2-^{13}C] acetate 4.8 mM, $[M+1]^+ = 8.4$, $[M+2]^+ = 23.4$; 2*S*-[Me-^{13}C] methionine 1.3 mM, $[M+1]^+ = 16.3$, $[M+2]^+ = 9.3$, $[M+3]^+ = 11.8$, $[M+4]^+ = 20.0$, $[M+5]^+ = 23.7$; 2*S*-[Me-$^{2}H_3$]-methionine 1.3 mM, $[M+3]^+ = 20.5$, $[M+6]^+ = 10.5$, $[M+9]^+ = 12.1$, $[M+12]^+ = 17.1$, $[M+15]^+ = 14.0$.

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