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Use of ^{13}C in biosynthetic studies. Incorporation of ^{13}C-labeled acetate into chartreusin by *Streptomyces chartreusis*[1]

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Examination by ^{13}C nuclear magnetic resonance spectroscopy of chartreusin produced in cultures of *Streptomyces chartreusis* supplemented with [1-^{13}C] and [2-^{13}C]acetate showed that the 19-carbon aglycone component was derived entirely from acetate. In the spectrum of chartreusin enriched from [1,2-^{13}C]acetate the signals for 16 of the carbon atoms were accompanied by satellites due to spin–spin coupling of intact ^{13}C—^{13}C units. The coupled pairs were matched with the aid of homonuclear single ^{13}C-frequency decoupling. Of the uncoupled carbon atoms, two were derived from methyl groups of acetate and the third came from an acetate carboxyl group. The arrangement of paired and unpaired ^{13}C atoms in chartreusin suggests that the aglycone is derived from a single 22-carbon polyketide chain. Cyclization to a benzpyrene-like intermediate followed by ring cleavage and loss of three carbon atoms provides a plausible route from the polyketide to the substituted isocoumarin structure of the aglycone.

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L'examen des spectres rmn du ^{13}C de la chartreusine produite dans les cultures de *Streptomyces chartreusis* additionnées de [1-^{13}C] et [2-^{13}C]acétate montre que le composant aglycone contenant 19 atomes de carbone provient entièrement de l'acétate. Dans le spectre de la chartreusine enrichie à partir de [1,2-^{13}C]acétate, les signaux de 16 des atomes de carbone sont accompagnés par des satellites dus à un couplage spin–spin des unités ^{13}C–^{13}C intactes. On a pu comparer les paires couplées à l'aide d'un découplage de fréquence ^{13}C homonucléaire et simple. Des atomes de carbone non-couplés, deux proviennent des groupes méthyles de l'acétate et le troisième d'un groupe carboxyle de l'acétate. L'arrangement des atomes de carbone ^{13}C pairés et non-pairés dans la chartreusine, suggère que l'aglycone dérive d'une seule chaîne polykétide contenant 22 atomes de carbone. Une cyclisation conduisant à un intermédiaire ressemblant à du benzpyrène suivie par une fermeture de cycle et la perte de trois atomes de carbone fournit une route possible menant d'un polykétide à la structure isocoumarine substituée de l'aglycone.

[Traduit par le journal]

Introduction

Streptomyces chartreusis produces a glycosidic antibiotic, chartreusin, the aromatic dilactone component of which has been reported to originate from acetate (1, 2). Schmid located the carbon atoms derived from the methyl and carboxyl groups of acetate as shown in Fig. 1, but no experimental evidence was provided (1). Brown *et al.* (2) examined the distribution of ^{14}C-labeled carbon atoms in chartreusin enriched by growing cultures of *S. chartreusis* on diethyl [1-^{14}C] or [2-^{14}C]malonate. By chemical degradation of the aglycone they were able to confirm that C-3 and C-9 originated with the carboxyl and methylene carbons of malonate, and that the relative specific activities of substituted phthalic acids containing the carbon atoms of rings A and C were consistent with the distribution of label reported by Schmid. However, no complete chemical dissection of the labeled molecule has been described. Even if the proposed labeling pattern is assumed to be correct, the manner in which acetate and malonate units are assembled to generate chartreusin aglycone is not obvious. As observed by Brown and his colleagues, the carbon skeleton cannot be formed directly by the folding of a single polyketide chain; either an intermediate

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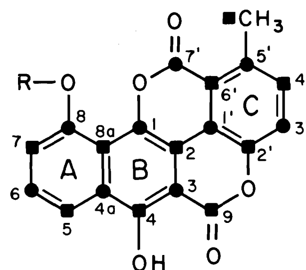
of single-chain origin undergoes rearrangement or more than one polyketide chain is involved.

To obtain information on the assembly of two-carbon units in chartreusin we have prepared the antibiotic from cultures supplemented with ^{13}C-enriched acetate and measured the incorporation of isotopic label by ^{13}C nuclear magnetic resonance (^{13}C nmr) spectroscopy. A preliminary account of these experiments has already been reported (3). We present here the evidence supporting ^{13}C nmr assignments, biosynthetic labeling patterns, and our conclusion that chartreusin aglycone is derived from a single polyketide chain of 22 carbon atoms.

Biosynthetic Labeling

Of three isolates of *S. chartreusis* (strains K-180, ATCC 14922, and X-465) compared on defined (2) and complex (4) media for chartreusin production, strain X-465 gave the highest titre. Yields of 80–100 mg/ℓ were obtained at first, but deteriorated rapidly when the stock culture was transferred. To maintain good production we found it necessary to select a high-producing variant and prepare inocula for biosynthetic labeling experiments directly from stock cultures preserved by lyophilization. The culture medium favouring chartreusin formation was adapted from that of Berger *et al.* (4). An appropriate concentration of sodium acetate supplement, and suitable times for adding the supplement and harvesting the cultures to obtain optimum isotopic enrichment of the antibiotic, were selected in pilot experiments. Incorporation of radioactivity from sodium [2-^{14}C]acetate was used as a measure of isotopic enrichment.

The ^{13}C nmr spectra of chartreusin from cultures supplemented with sodium [1-^{13}C] acetate,



R= D-DIGITALOSE -D-FUCOSE

FIG. 1. Structure of chartreusin and labeling pattern proposed by Schmid (1) and Brown *et al.* (2).

[2-^{13}C]acetate, and [1,2-^{13}C]acetate are compared in Fig. 2 with the spectrum of the antibiotic at natural abundance. Signals at δ 15 (CH_3), 100 (anomeric carbon), and in the range 55–80 ppm (hydroxymethine carbons) could be assigned to the glycosidic moiety. They were enhanced to only a small extent in the spectra of samples enriched from ^{13}C-labeled acetate. Chartreusin derived from singly labeled acetate showed marked enhancement of a reciprocal series of signals, *i.e.* all of the non-glycosidic signals not intensified in the sample labeled from [1-^{13}C]-acetate were enhanced in the sample from [2-^{13}C]acetate. This result verifies the earlier deductions based on radiotracer studies that all of the aglycone carbon atoms are derived from acetate (1, 2). In the spectrum of the sample labeled from [1,2-^{13}C]acetate, signals for 16 of the 19 aglycone carbons were accompanied by satellites due to ^{13}C–^{13}C coupling between covalently bonded ^{13}C carbon atoms.

Assignment of ^{13}C Resonances

Aglycone resonances in the ^1H broad-band decoupled pulse Fourier transform ^{13}C nmr spectrum of chartreusin were assigned from chemical shift trends (5), direct and long-range spin–spin couplings to hydrogen ($^nJ_{CH}$, which were more easily measured in the high resolution (HR) spectra of samples labeled with [1-^{13}C] and [2-^{13}C]acetate), and unambiguous pairing of carbons that were spin–spin coupled ($^1J_{cc}$) in the spectrum of the [1,2-^{13}C]acetate-labeled metabolite. Values for chemical shifts and spin–spin coupling constants are presented in Table 1.

Chemical shift values and a characteristic one-bond ^{13}C–^{13}C coupling of 43.5 Hz identified the aglycone methyl carbon (δ_c 21.6 ppm) and the adjacent C-5′ quaternary aromatic carbon atom (δ_c 138.5 ppm). The HR signal for C-5′ was probably a doublet of quartets due to coupling with H-3′ and the methyl hydrogens ($^2J_{CH}$) but spectral resolution was insufficient to permit the small couplings to be measured. Signals for the five aromatic methine carbon atoms were easily recognized in the HR spectrum from the splitting due to one-bond ^{13}C–H couplings (5). Those for C-5 and C-7 could be distinguished by the additional splitting caused by three-bond ^{13}C–H coupling to H-7 and H-5. The carbon atom resonating at δ_c 116.2 ppm was assigned to C-5 because it was ^{13}C–^{13}C coupled ($^1J_{cc}$ 56.2 Hz) to a quaternary aromatic carbon (C-4a) at δ_c 126.0

FIG. 2. ^{13}C nmr spectra (broadband (^{1}H)-decoupled) of chartreusin at natural abundance ^{13}C concentration (A), and from cultures supplemented with [1-^{13}C]acetate (B), [2-^{13}C]acetate (C), and [1,2-^{13}C]acetate (D). In D regions containing resonances of the aglycone carbon atoms are expanded and show satellites due to ^{13}C–^{13}C spin–spin coupling ($^{1}J_{cc}$). C^{s} denotes the glycosidic resonances.

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TABLE 1. Assignments of resonances and ^{13}C–^{1}H spin–spin couplings in ^{13}C nmr spectra of ^{13}C-enriched chartreusin

δ (ppm)	Assignment	Multiplicity*	$^{1}J_{CH}$† (Hz)	$^{3}J_{CH}$† (Hz)	$^{n}J_{C(OH)}$† (Hz)
163.6	C-9	s	—	—	—
158.3	C-7′	s	—	—	—
155.4	C-4	dd→d‡	—	3.1	4.1
154.0	C-8	bd	—	6.3	—
145.8	C-2′	d	—	7.5	—
138.5	C-5′	dq	—	¶	—
138.4	C-1	s	—	—	—
133.1	C-4′	bdq	164.4	¶	—
128.3	C-6	d	165.0	—	—
126.0	C-4a	dd→d‡	—	8.7	6.9
120.6	C-3′	d	166.3	—	—
119.2	C-1′	bd	—	2.5	—
117.8	C-8a	dd	—	¶	—
116.7	C-6′	dq	—	¶	—
116.2	C-5	bdd	167.8	6.2	—
114.4	C-7	bdd	162±2	6.9	—
108.1	C-2	s	—	—	—
96.5	C-3	d→s‡	—	—	3.5
21.6	CH_3	q	129.7§	—	—

*s = singlet, d = doublet, q = quartet, b = broad.
†Error *ca.* ±0.5 Hz except where indicated; n = 2, 3, 3 for C-4, C-4a, C-3, respectively.
‡Multiplicity change caused by deuteration.
¶Accurate measurements of long-range coupling constants precluded by broadening or overlap of multiplets.

ppm which in turn was long-range coupled to an aromatic hydrogen (H-6, $^{3}J_{CH}$ 8.7 Hz) as well as to a hydroxyl hydrogen ($^{3}J_{C(OH)}$ 6.9 Hz). Replacing the hydroxyl hydrogen with deuterium removed the $^{13}C(OH)$ coupling. The signal at δ_c 114.4 ppm was allocated to C-7, since this carbon atom was ^{13}C–^{13}C coupled ($^{1}J_{cc}$ 53.9 Hz) to an aromatic methine carbon atom at δ_c 128.3 ppm which could only be C-6. The remaining carbons bearing hydrogen, namely C-3′ (δ_c 120.6 ppm) and C-4′ (δ_c 133.1 ppm), were also derived biosynthetically from an intact [1,2-^{13}C]acetate unit ($^{1}J_{cc}$ 54.5 Hz). The higher field resonance was assigned to C-3′ because the oxygen substituent at C-2′ would be expected to shield it, but not C-4′ (5). Consistent with those assignments the HR resonance attributed to C-4′ was a doublet of quartets due to coupling with H-4′ and the methyl hydrogens, whereas that for C-3′ was a sharp doublet due to coupling with H-3′ only. There was not sufficient spectral resolution to measure the small $^{3}J_{CH}$ values.

Resonances could be assigned to C-4 (δ_c 155.4 ppm, $^{2}J_{C(OH)}$ 4.1 Hz) and C-3 (δ_c 96.5 ppm, $^{3}J_{C(OH)}$ 3.5 Hz) because both carbon atoms (like C-4a) were spin–spin coupled to hydroxyl hydrogens, belonged to a ^{13}C–^{13}C unit ($^{1}J_{cc}$ 70.6 Hz), and possessed chemical shifts consistent with one (C-4) being deshielded and the other (C-3) being shielded by a hydroxyl substituent (5). Supporting these assignments, the resonance attributed to C-3 was enhanced in the spectrum of the metabolite enriched with [1-^{13}C]acetate, as predicted from the radiotracer study (2) which established that C-3 was derived from the carbonyl carbon of acetate. The long-range coupling ($^{3}J_{CH}$ 3.1 Hz) at C-4 must involve H-5.

Of the nine ^{13}C resonances still unassigned the five at lowest field were allocated to the two carbonyl carbon atoms (C-7′ and C-9) and the three aromatic carbon atoms bonded to oxygen (C-1, C-8, and C-2′). In HR spectra the signals at δ_c 154.0 and 145.8 ppm could be distinguished within this group as belonging to C-8 and C-2′ since they were present as doublets due to coupling with H-6 and H-4′, respectively. Of the three other resonances, those at δ_c 138.4 and δ_c 158.3 ppm were enhanced in the spectrum of chartreusin biosynthesized from [1-^{13}C]acetate whereas the carbon atom with a chemical shift of δ_c 163.6 ppm was derived from the methyl group of acetate. Since the earlier radiotracer study (2) established that C-9 was labeled by

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[2-^{14}C]malonate the δ_c 163.6 ppm resonance was assigned to this carbon atom. Such an assignment is consistent with chemical shift expectations (5) and also with the absence of ^{13}C–^{13}C satellites at this resonance in the spectrum of chartreusin labeled by [1,2-^{13}C]acetate. Since C-3 and C-4 were incorporated as an intact two-carbon unit, C-9 is necessarily a product of acetate C—C bond fission. The signal at δ_c 158.3 ppm was attributed to C-7′, which should have a chemical shift similar to that of C-9. Moreover, it was ^{13}C–^{13}C coupled ($^1J_{cc}$ 74.1 Hz) to an aromatic quaternary carbon atom with δ_c 116.7 ppm, which should be C-6′ as it was also ^{13}C–H coupled to an aromatic hydrogen (H-4′) and to the hydrogens of the methyl group bonded to C-5′. The signal at δ_c 138.4 ppm was assigned to C-1, the only remaining carbon atom bonded to oxygen but not ^{13}C–H coupled; its chemical shift value is primarily accounted for by the shielding effect of substituents at C-4 and C-8 (6, 7).

The ^{13}C nmr spectrum of chartreusin labeled from [1,2-^{13}C]acetate showed that C-1, like C-9, was one of the three carbons in the aglycone not derived from an intact ^{13}C—^{13}C unit. The third such carbon atom resonated at δ_c 145.8 ppm and was one of the two low-field carbon atoms (C-2′ and C-8) that appeared as a doublet in HR spectra. The signal was assigned to C-2′, because C-8, with C-8a, comprised a ^{13}C—^{13}C pair ($^1J_{cc}$ 68.4 Hz) incorporated intact from [1,2-^{13}C]acetate. C-8 was assigned the resonance at δ_c 154.0 ppm and C-8a the one at δ_c 117.8 ppm. Confirming these assignments, HR spectra showed that the latter signal was coupled to two aromatic hydrogens (H-5 and H-7) as expected for C-8a, whereas signals attributed to C-8 and C-2′ were each coupled to one hydrogen (H-6 and H-4′, respectively). The carbon atoms at C-2 and C-1′, each shielded by lactone oxygens on adjacent carbons, were expected to resonate at high field. Of the two unassigned signals given by chartreusin, that at δ_c 119.2 ppm was a doublet ($^3J_{CH}$ 2.5 Hz) in HR spectra. It was assigned to C-1′ because this carbon should be spin–spin coupled with H-3′, whereas the other resonance, at δ_c 108.1 ppm, was a singlet and was more appropriately assigned to C-2. In samples labeled from [1,2-^{13}C]acetate the signals were coupled ($^1J_{cc}$ 53.9 Hz), confirming that the two carbon atoms to which they were assigned were adjacent.

Labeling Pattern

Inspection of the ^{13}C nmr spectra of chartreusin enriched from [1-^{13}C] and [2-^{13}C]acetate in the light of ^{13}C resonance assignments established the origin of the aglycone carbons (Table 2). Enrichments were approximately 6 and 3%, respectively, and were thus large enough to show the labeling pattern unequivocally. The distribution of carbon atoms derived from the carboxyl and methyl groups of the precursor was identical with that proposed earlier by Schmid (1) and partially confirmed by Brown and colleagues (2). However, evidence from ^{13}C–^{13}C coupling between carbons of chartreusin enriched from [1,2-^{13}C]acetate (Table 1) revealed that only eight two-carbon precursor units had been incorporated intact into the antibiotic. This result, together with the information on origin and location of the remaining three carbon atoms of chartreusin aglycone that were not coupled, exclude all previous biogenetic schemes based on a 20-carbon polyketide chain intermediate.

A plausible alternative, consistent with all of the ^{13}C nmr evidence for the labeling pattern as well as the arrangement of bonded and single carbon units, is shown in Fig. 3. In this a polyketide chain (**1**) with 22 carbon atoms derived by head-to-tail condensation of acetate and malonate units is condensed to a polycyclic aromatic intermediate (**2**) of the benzpyrene type. Oxidative cleavage of two rings, with elimination of three carbon atoms by decarboxylation, would generate the phenylnaphthalene dicarboxylic acid derivative (**3**). Rotation of the phenyl ring would orient the substituents to facilitate formation of the dilactone (**4**) with the labeling pattern observed in chartreusin aglycone. Attachment of sugars at the C-8 hydroxyl could take place at any stage after formation of the polyketide, but by analogy with the sequence of biosynthetic steps shown for other glycosidic antibiotics of *Streptomyces* species (8–10), it is probably a terminal step.

If chartreusin is derived from a single-chain 22-carbon polyketide as suggested, it is the first example of a metabolite in this biogenetic subgroup (11). Hitherto, the 24-carbon metabolite, siphulin (12), was the only compound of apparent polyketide derivation formed by participation of more than ten acetate units. Although the measurement of ^{13}C enrichment is subject to appreciable error the results obtained suggest that each precursor enriched uniformly those

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TABLE 2. Isotopic enrichment (%) in chartreusin from cultures containing singly and doubly ^{13}C-labeled acetate

Aglycone carbon	$^{1}J_{cc}$ (Hz)	Position of label in precursor		
		1-^{13}C	2-^{13}C	1,2-^{13}C
CH_3	43.4		3.3	0.5†
C-5′	43.5	6.4‡		0.70
C-7	54.5		4.0	0.67
C-6	55±1	7.5		0.73
C-2	53.9		1.8	0.63
C-1′	54±2	6.8		0.54
C-4′	54.5		4.0	0.55
C-3	56±2	7.4		0.51
C-5	56.2		3.8	0.49
C-4a	56.7	4.4		0.7±0.1
C-8a	68±1		1.5	0.59
C-8	68.4	5.5		0.55
C-4	70.6		2.4	0.44±0.12
C-3	70±1	4.9		0.55±0.08
C-6′	74.0		2.0	0.45
C-7′	74.1	4.2		0.55
C-1	—	6.4‡		—
C-2′	—		2.2	—
C-9	—		1.6	—
Average ±SD		5.9±1.3	2.7±1.0	0.58±0.09
Measuring error	*ca.* ±0.5*	*ca.* ±1.3	*ca.* ±0.8	*ca.* ±0.07§

*Except those indicated in the Table.
†Inexact value due to peak overlap.
‡Average value for C-5′ and C-1, due to peak overlap in spectrum of sample from [1-^{13}C]acetate.
§Weighted average error. Individual errors were *ca.* ±0.05 where not shown.

chartreusin aglycone carbon atoms that it labeled. This is consistent with biosynthesis via a single-polyketide-chain intermediate, but it should be noted that the experimental conditions were not designed to test multichain alternatives. Precursors were added intermittently during growth of the cultures to produce a sustained isotopic enrichment of the acetate pool, the objective being to obtain maximum ^{13}C incorporation. To examine the possibility that chartreusin aglycone is formed from more than one polyketide chain a single-pulse addition of precursor would have been more informative, since it might have made it possible, from differences in enrichment at different positions in the molecule, to distinguish between chain-initiating units derived directly from acetate and chain-extending units derived via malonate. The limiting factor in such experiments is likely to be the accuracy with which small differences in enrichment can be detected and our results indicate that the use of doubly labeled acetate would be advantageous.

If chartreusin aglycone is derived from a single-chain-polyketide intermediate we see no reasonable alternative to the folding pattern and processing steps proposed in Fig. 3. In speculating about a multichain origin one might include a variation of this pathway in which the initial polyketide folding pattern is retained but the single-chain intermediate (**1**) is replaced by two or more shorter chains, assembled and condensed head-to-tail to form the same polycyclic aromatic intermediate (**2**). Clearly a large number of such short-chain variations can be entertained. Other routes, beginning with the condensation of two or more polyketide chains folded in different patterns, are conceivable. Since the presence in chartreusin aglycone of acetate-derived carbon atoms which are no longer part of bonded two-carbon units implies that processing reactions occur after the initial polyketide condensation, and since the nature of these reactions are not known at present, it is possible to construct and modify polyketide precursors with a variety of chain lengths and

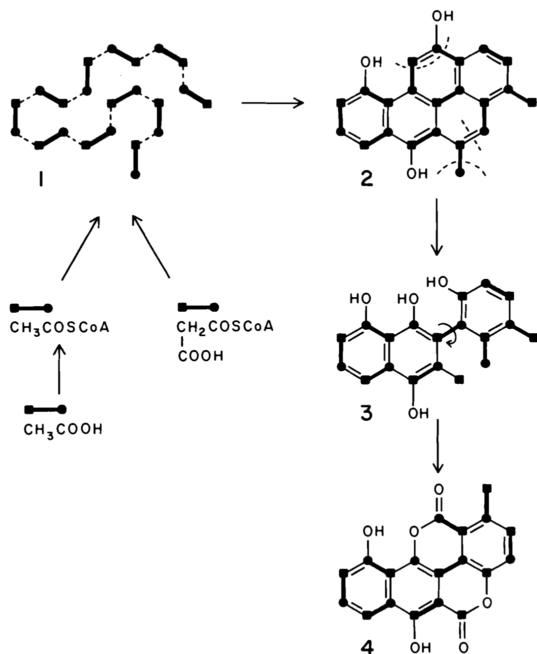

FIG. 3. Suggested biogenesis of chartreusin aglycone.

folding patterns to fit the labelling results. However, none of these alternatives appear to be more plausible than the route we have proposed.

Experimental

Labeled Compounds

Sodium [1-^{13}C]acetate, [2-^{13}C]acetate, and [1,2-^{13}C]acetate, all 90% enriched, were obtained from Merck, Sharp and Dohme, Pointe Claire, Quebec. An authentic sample of chartreusin was obtained from Dr. G. Whitfeld, the Upjohn Co., Kalamazoo, Mich.

Production, Extraction, and Purification of Chartreusin

In early experiments a sporulated slant of *S. chartreusis* X-465-4222 from Hoffmann-LaRoche, Nutley, NJ, maintained on a medium of Heinz Tomato Paste (2%), Heinz Baby Oatmeal (2%), and agar (1.5%), was used to seed 50-ml portions of a medium containing glucose (6%), soybean meal (2%), dipotassium hydrogen phosphate (0.1%), calcium carbonate (0.3%), zinc sulphate heptahydrate (0.001%), cupric sulphate pentahydrate (0.0002%), manganese sulphate tetrahydrate (0.0002%), and ferrous sulphate heptahydrate (0.001%) in 250-ml Erlenmeyer flasks. For later experiments it was necessary to plate spores of strain X-465-4222 and select a chartreusin-producing variant from the population. The variant was propagated on tomato paste – oatmeal agar and a spore suspension, subdivided into ampoules, was lyophilized and stored at −20°C. One of the lyophilized spore preparations was used to seed the liquid medium. Cultures seeded by either method were incubated in the dark for 2 days at 26°C on a shaker platform rotating at 220 rpm with an eccentricity of 3.8 cm, and used in 2-ml portions as a vegetative inoculum for chartreusin production medium. Cultures for producing chartreusin were grown under the same conditions as the vegetative inoculum.

In separate experiments, sterile solutions containing 10 mmol of sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate or sodium [1,2-^{13}C]acetate were added in aliquots to each of 20 flask cultures. The supplement was given in two portions, one on the fourth and one on the fifth day after inoculation. Cultures were harvested on the sixth day and extracted using the following adaption of previously described methods (4, 13). The broth was acidified to p*H* 5.5, centrifuged, and finally filtered through Whatman No. 5 paper to remove fine particles. Culture solids removed in this process were heated to boiling successively with three 100-ml portions of 80% aqueous acetone. The filtered extracts were combined with the culture filtrate and the mixture was extracted with an equal volume of acetone – methylene chloride (1:1) to completion. The combined extracts, evaporated and dried *in vacuo*, were redissolved in chloroform and applied to a column (2.6 × 25 cm) of silicic acid. The column was washed with acetone–chloroform (1:9) and chartreusin was eluted with acetone–chloroform (2:3). It was crystallized from methylene chloride and ethanol in yields ranging from 53 to 199 mg per litre of culture; the identity and purity of the samples was established by thin-layer chromatography (silica gel treated with citric acid, irrigated with chloroform–methanol, 9:1), ultraviolet absorption spectroscopy, and the absence of extraneous peaks in its ^{13}C nmr spectrum. Samples were compared with a reference specimen of chartreusin.

Spectra of chartreusin (50 to 120 mg samples dissolved in dimethyl sulfoxide-d_6) were obtained with a Varian XL-100/15 Fourier transform spectrometer under the following conditions: frequency 25.16 MHz, acquisition time 0.8 or 1.6 s (data accuracy ±0.6 or ±0.3 Hz), spectral width 5120 Hz, flip angle 40°, internal ^{2}H lock to solvent, temperature *ca.* 30°C, internal reference to tetramethylsilane. Broadband (^{1}H)-decoupling was achieved by phase modulation of the decoupling field ($\gamma H_2/2\pi$ 3800 Hz) from 0° to 180° at 150 Hz (14). During the acquisition of high-resolution spectra the nuclear Overhauser enhancement was maintained by applying this field for *ca.* 1.7 s between acquisition periods. For homonuclear (^{13}C) decoupling, a 25.12 MHz signal from a General Radio model 1061 frequency synthesizer was fed continuously via a tuned RF amplifier and BNC T connector to the transmitter coil of the probe. The higher frequency sideband of this signal, generated by the 40.96 KHz modulation field, was used to irradiate the resonance to be decoupled, the effective $\gamma H_2/2\pi$ being *ca.* 40 to 70 Hz. Coupled ^{13}C–^{13}C pairs of resonances were located from either direct irradiation of resonances or from the residual couplings caused by off-resonance decoupling effects.

The distribution of isotopic label in chartreusin from cultures supplemented with [1-^{13}C] or [2-^{13}C]acetate was determined by comparing ^{13}C nmr spectra of the enriched and natural abundance compounds recorded under identical experimental conditions. The ratio, *R*, of the intensity of each resonance to that of a reference peak known not to be enhanced was calculated for each spectrum. Spectra were compared via the ratio, *r*, for each peak, where $r = R$ (labeled compound)/*R* (natural

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abundance compound). Thus $r \gg 1.0$ for peaks corresponding to enriched carbons; otherwise $r \simeq 1.0$. The percentage ^{13}C at each site was obtained by multiplying r for that carbon by the factor required to scale the average r for unenriched carbons to 1.108%. This procedure clearly distinguished enhanced peaks at the levels of enrichment encountered, and should detect enrichments $\geq 0.4\%$ above natural abundance. Errors stem mainly from the variation in r for unenhanced peaks, which places a limit on the accuracy of 'scaling' to natural abundance ^{13}C concentration. The measuring errors shown in the footnote to Table 2 are related to the standard deviation Δr in r for unenhanced peaks: error = $\Delta r/r \times$ average enrichment. In both cases the standard deviation (SD) of the enrichments is very similar to this error, showing that the relative variation in resonance intensity, due to factors other than enrichment, is almost the same for unenriched and enriched carbons. The method is independent of the choice of a reference peak for calculating R: if a peak enhanced by enrichment were chosen $r \ll 1$ for those peaks not enhanced. The percentage enrichment at labeled positions in chartreusin produced from doubly labeled acetate was calculated from the equation $1.1I_s/(I_c\text{-}fI_s)$, where I_s is the sum of the intensities of the two satellite resonances, I_c the intensity of the central peak, and f is the ratio of the concentrations of appropriate singly and doubly labeled precursors present in the doubly enriched acetate (15).

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