USE OF CARBON-13 IN BIOSYNTHETIC STUDIES: ORIGIN OF THE MALONYL COENZYME A INCORPORATED INTO TETRACYCLINE BY *STREPTOMYCES AUREOFACIENS*†

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The proton noise decoupled ^{13}C nuclear magnetic resonance spectrum of tetracycline hydrochloride prepared from *Streptomyces aureofaciens* cultures supplemented with [1-^{13}C]-acetate and [2-^{13}C]acetate showed enrichment of nine alternating ring carbons. In addition, a small enrichment of the carboxamide carbon by [1-^{13}C]acetate was observed. The labelling patterns clearly demonstrated the polyketide origin of the tetracyclic nucleus. The ^{13}C nuclear magnetic resonance spectrum of tetracycline hydrochloride derived from [1,2-^{13}C]acetate showed all 18 ring carbons as doublets with coupling constants appropriate for the incorporation of nine intact two-carbon precursors, confirming that head-to-tail condensation of C_2 units had occurred. Absence of bond scission within the C_2 units and a low level of uncoupled ^{13}C in the carboxamide substituent indicated that when the organism is supplemented with acetate, malonyl coenzyme A used for tetracycline biosynthesis is formed by direct carboxylation of acetyl coenzyme A.

Radioisotope studies seeking to determine the primary precursors of the tetracyclines have indicated a predominantly polyketide origin for these antibiotics[1,2]. The two-carbon units are probably derived from malonyl coenzyme A, and the chain-initiating unit is postulated to be malonamyl coenzyme A[1]. In organisms producing oligoketide-derived metabolites such as the tetracyclines, malonyl coenzyme A (malonyl CoA) is generally assumed to be formed by carboxylation of acetyl coenzyme A (acetyl CoA) and to form a common pool with the malonyl CoA used in fatty acid biosynthesis. However, based mainly on enzyme analysis, HOSTALEK *et al.*[3] have reported that decarboxylation of oxaloacetate provides the main supply of malonyl CoA for chlortetracycline formation in idiophase cultures of the producer, *Streptomyces aureofaciens*.

The present study examines the route by which the malonyl CoA used in tetracycline biosynthesis is formed. It is apparent that [1,2-^{13}C]acetate supplied to *S. aureofaciens* during tetracycline production would, if incorporated *via* malonyl CoA formed by direct carboxylation of acetyl CoA, suffer no bond scission within the two-carbon acetate units. On the other hand, if the acetate were incorporated *via* malonate formed from oxaloacetate, the bond between C-1 and C-2 of acetate would be broken (Fig. 1).

By obtaining ^{13}C-enriched samples of the antibiotic from cultures supplemented with singly and doubly labelled acetate, and by locating ^{13}C-enriched and ^{13}C-^{13}C bonded atoms in these molecules, the labelling patterns can be established and the route used to form malonyl CoA during tetracycline bio-

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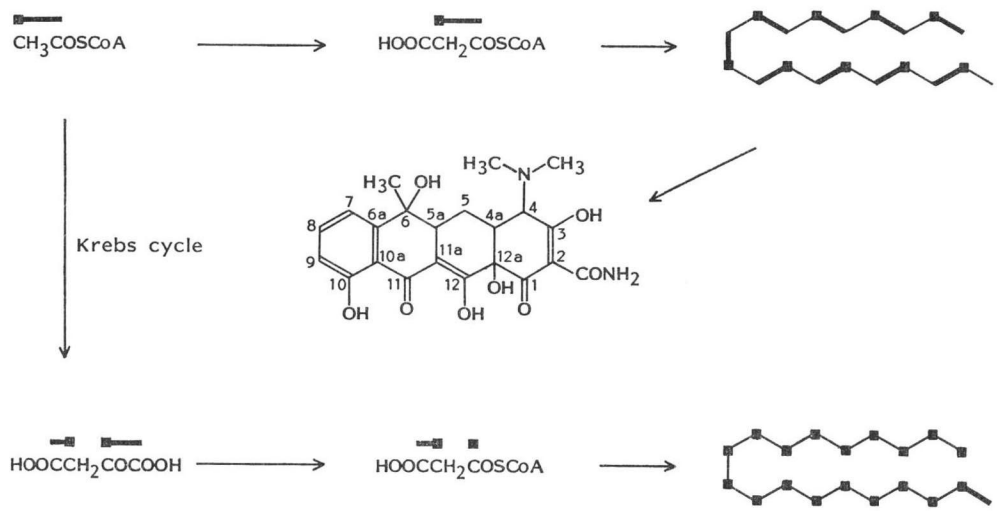
Fig. 1. Postulated pathways for the incorporation of [1,2-^{13}C]acetate into tetracycline.



synthesis thereby deduced.

Materials and Methods

Culture

Streptomyces aureofaciens XT99-IL was obtained from Bristol-Myers Company, Syracuse, N.Y. Variations in colony morphology were observed when the organism was plated on agar medium, and single colony isolates of the parent strain varied over an 8-fold range in their ability to produce tetracycline; isolate 113-1 was selected for this study. Because storage as frozen spore suspensions in 20% glycerol led to a marked decrease in tetracycline production, stock cultures were lyophilized and stored at −20°C; for short-term maintenance the cultures were grown to sporulation at 27°C on 0.4% maltose, 0.4% yeast extract, 1% malt extract (MYM) agar slants and then kept at −20°C. Spores and mycelia from a slant were transferred to a 250-ml Erlenmeyer flask containing 50 ml of MYM liquid medium and incubated for 24 hours at 27°C on a rotary shaker (220 rpm, 3.8 cm eccentricity). The vegetative mycelium was washed aseptically with H_2O, then dispersed in H_2O in a 250-ml Ryan flask (side arm path length, 1.2 cm) to give an optical density at 640 nm of between 0.80 and 0.85.

Cultures for producing tetracycline were grown in 250-ml Erlenmeyer flasks containing 50 ml of production medium consisting of (g/liter): sucrose (110), $(NH_4)_2SO_4$ (6), $CaCO_3$ (8), K_2HPO_4 (0.3), $MgSO_4 \cdot 7H_2O$ (1.0), NaBr (0.2), $ZnSO_4 \cdot 7H_2O$ (0.3), $MnSO_4 \cdot 4H_2O$ (0.03), $FeSO_4 \cdot 7H_2O$ (0.0012), $CoSO_4 \cdot 6H_2O$ (0.035). The medium was prepared by combining separately autoclaved solutions of sucrose and ammonium sulfate with finely divided calcium carbonate dispersed in a solution containing the remaining ingredients; the latter was brought to pH 6.5 with sodium hydroxide before autoclaving.

Labelled Supplements

Sodium [2-^{14}C]acetate was obtained from New England Nuclear Corporation, Boston, Massachusetts. Singly and doubly ^{13}C-labelled sodium acetate, all 90% enriched, and [2-^{13}C, 2-$^{2}H_3$]acetate (90%^{13}C, 98%^{2}H), were obtained from Merck, Sharp and Dohme Canada, Ltd., Montreal, Quebec. Supplements were prepared as aqueous solutions and sterilized by autoclaving.

Based upon preliminary optimization studies using [2-^{14}C]acetate, carbon-13 labelled supplements were added to cultures in cumulative amounts totalling 10 mmol per liter of broth. Cultures were harvested on the 9th day, and tetracycline was extracted and purified as the crystalline hydrochloride for nuclear magnetic resonance (NMR) spectroscopic examination. The yield of enriched metabolites in all cases was about 100 mg from 400 ml of culture fluid.

Analyses

Tetracycline in cultures and extracts was assayed spectrophotometrically at 445 nm after acid-catalyzed conversion to anhydrotetracycline. The conditions were modified from those described by LEVINE *et al.*[4] by using a less concd hydrochloric acid solution (1.7 M) and shorter reaction time (3 minutes) to avoid interference from sucrose.

In radioisotope incorporation studies, tetracycline was extracted into butanol and the concd extract applied to a 20×20 cm thin-layer plate of silica gel GHLF (Analtech Inc., Newark, DEL) which had been predeveloped with satd aq disodium ethylenediamine tetraacetate. The chromatogram was developed with 2-propanol - 0.1 M phosphate buffer, pH 7.0 (5: 2). Tetracycline was detected by viewing the chromatogram under UV light. The zone was excised and eluted with 0.01 M HCl. The concentration of tetracycline in the eluate was determined by measuring its absorbance at 356 nm; radioactivity was measured with a liquid scintillation spectrometer.

Isolation of Tetracycline

Cultures were acidified with H_2SO_4 to pH 2, and the solids were separated by centrifugation. The supernatant solution after concentration *in vacuo* at 32°C to about one-third volume and filtration was brought to pH 8.5 with NaOH and extracted three times with equal volumes of butanol. The organic phase was washed twice with one-tenth of its volume of distilled water at pH 8.5, then concd at 32°C. Tetracycline was recovered from the butanolic solution with 0.01 M HCl and the aq extracts were concd at 32°C to a volume that contained at least 7 mg of the antibiotic per ml. The free base was precipitated by carefully raising the pH of the concentrate to 5.0 in an ice bath, then reconverted to the hydrochloride by crystallization from ethanolic HCl. The product was recovered as yellow needles.

NMR Spectroscopy

^{13}C NMR spectra were recorded at 29°C with a Varian model XL-100/15 Fourier transform spectrometer with a Nicolet 1280 computer and 293B pulse programmer under the following conditions: frequency, 25.16 MHz; spectral width, 6,000 Hz; acquisition time, 0.68 s; delay between acquisitions, 1.0 s; flip angle, 40°; sample concentration *ca.* 190 mg/ml; solvent, dimethyl sulfoxide-d_6 (DMSO-d_6), solution volume, 0.4 to 0.5 ml; chromium acetylacetonate ($Cr(acac)_3$) concentration (when used), 10 mg/ml; sample tubes, 5-mm diameter; internal lock to ^2H in solvent; trimethylsilane (TMS) as internal standard. Free induction decays recorded over 8K data points were "zero-filled" to 16K before Fourier transformation, yielding 0.74 Hz digital resolution. When $Cr(acac)_3$ was used as relaxation reagent, ^1H decoupling was applied during the acquisition time (0.68 s) only, and switched off for 2.0 s following acquisition for suppression of nuclear Overhauser enhancement (NOE). ^{13}C NMR spectra of tetracycline hydrochloride labelled with [2-^{13}C, 2-$^2H_3$]acetate were obtained with simultaneous broadband decoupling of ^1H and ^2H. The 15.4 MHz signal for ^2H-irradiation (2W, 0 to 180 degree phase-modulated at 40 Hz) was provided from a General Radio 1061 synthesizer and ENI 320L RF amplifier *via* a double tuned (100 MHz, 15.4 MHz) matching network on the decoupler coil of the NMR probe.

Results

Selection of Conditions for Isotopic Enrichment

Under the culture conditions used, tetracycline was first detected in the culture broth on the third day and the titre increased at a linear rate until the seventh day. Initiation of antibiotic production correlated with exhaustion of phosphate from the medium. Ammonium was depleted by the fifth day but sucrose remained in excess throughout the fermentation. Cell proliferation and production of antibiotic were distinctly biphasic, with no further accumulation of biomass occurring after the fifth day. Thus most of the tetracycline was biosynthesized during late idiophase, after active gowth had ended.

In preliminary experiments it was observed that supplementation of cultures on or before day 5 with

sodium acetate markedly depressed production of the antibiotic. At the same time, early supplementation enhanced the specific incorporation of [2-^{14}C]acetate. To reduce the severity of the inhibition, the supplement was added in small doses at daily intervals, with the size of the dose being increased in the later stage of production. With the feeding regimen adopted, a total of 10 mmol/liter supplied between days 3 and 7 reduced the yield of tetracycline by less that 20% from that obtained in unsupplemented cultures. The specific incorporation of radioactivity from [2-^{14}C]acetate was approximately 2.2% at each labelled position. This compromise between yield and isotopic enrichment was considered satisfactory for analyses of a ^{13}C-labelled product by NMR spectrometry. A radiochromatogram scan of the ^{14}C-labelled product extracted from these cultures and examined by TLC showed it to contain 63% tetracycline, 25% chlortetracycline and 12% of other radioactive products.

Incorporation of ^{13}C-Labelled Acetate

The proton noise decoupled ^{13}C NMR spectrum of tetracycline hydrochloride derived from either [1-^{13}C] or [2-^{13}C]acetate showed enrichment of nine ring carbons (Table 1). Small peaks due to the presence of chlortetracycline hydrochloride in the samples could also be detected and some of these showed enrichment in the products derived from ^{13}C-labelled acetate.

The sample enriched from [1,2-^{13}C]acetate yielded a spectrum in which all 18 ring carbons of tetracycline appeared as doublets with coupling constants that corresponded to the incorporation of nine intact C_2 units (Table 1). The tetracyclic nucleus of the compound is, therefore, assembled from intact pairs of carbon atoms derived from acetate. The ^{13}C enrichments indicated a uniform incorporation of the precursor throughout the metabolite.

Assignment of ^{13}C Resonances

Multiplicities in the high resolution spectrum defining the number of hydrogens bonded to carbon, and ^{13}C-^{13}C couplings introduced by incorporation of [1,2-^{13}C]acetate, were used to assign resonances to the individual carbon atoms of tetracycline hydrochloride. Assignments have been reported earlier by ASLESON and FRANK[5] and most of these were confirmed by the present study. However, a rational allocation of resonances consistent with coupling information is possible only when the previous assignments for C-1 and C-11 and also C-7 and C-9 are interchanged.

Labelling Patterns

Taking into account the revised assignment of resonances, the incorporation of [1-^{13}C]acetate into tetracycline enriched C-1, C-3 C-4a, C-5a, C-6a, C-8, C-10, C-11 and C-12, whereas [2-^{13}C]acetate enriched C-2, C-4, C-5, C-6, C-7, C-9, C-10a, C-11a and C-12a (Table 1). The following pairs of coupled carbons were detected in tetracycline enriched from [1,2-^{13}C]acetate: C-1/C-2; C-3/C-4; C-4a/C-5; C-5a/C-6; C-6a/C-7; C-8/C-9; C-10/C-10a; C-11/C-11a; C-12/C-12a (Table 1). These results allow the unambiguous linking of precursor acetate units to establish the polyketide origin of the tetracycline nucleus. Isotopically-shifted resonances for C-5, C-7 and C-9 were absent from the spectrum of the metabolite labelled with [2-^{13}C, 2-$^{2}H_3$]acetate, the anticipated isotope shift (>5 Hz) being large compared to the digital resolution (0.74 Hz). This establishes that hydrogens bonded to these carbons had exchanged completely with the hydrogens of water in the cytosol at some stage in the biosynthetic pathway. It is noteworthy that the carboxamide carbon was enriched from [1-^{13}C]acetate, although to only about one-third the extent of the labelled ring carbons. Moreover, with [1,2-^{13}C]acetate as the precursor, no coupling was observed between C-2 and the carboxamide carbon. The similarity of the calculated

Table 1. ^{13}C NMR data for tetracycline hydrochloride enriched from ^{13}C-labelled acetate samples[a].

Carbon	δ_C[b] (ppm)	[1-^{13}C]	[2-^{13}C]	[1,2-^{13}C]	
		E[c] (%)	E[c,d] (%)	$^1J_{CC}$[e] (Hz)	E[f] (%)
C-1	193.3	1.9		61.2	1.5
C-2	95.7		2.3	61.3	1.6
C-3	187.2	2.3		42.3	1.5
C-4	68.1		1.8±0.6	42.9±0.8	1.3
C-4a	35.1	2.1		33.2±2	1.8
C-5	27.0		2.5	33.2±2	1.3
C-5a	42.1	2.3±0.6		36.4±2	1.6
C-6	67.9		1.8±0.6	37.3	[g]
C-6a	147.8	1.7		60.8	1.5
C-7	115.2		2.4±0.6	60.4	1.4
C-8	136.5	2.4		57.0±1	1.6±0.5
C-9	117.0		2.5	57.0±1	1.6
C-10	161.3	2.1		62.8	1.3
C-10a	114.3		2.0	62.9	1.9
C-11	192.8	1.9		57.0	1.3
C-11a	106.8		1.9	55.9	1.4
C-12	174.9	2.4		52.4	1.5
C-12a	73.1		1.9	52.4	1.4
$CONH_2$	172.0	0.7±0.3	<0.3	—	0.5±0.2
$N(CH_3)_2$	41.5			—	—
CH_3	22.5			—	—
Error (except where shown)		±0.4	±0.4	*ca.* ±0.5	*ca.* ±0.3

[a] The samples contained 23~35% chlortetracycline. As far as could be determined, this was enriched in the same manner and to the same extent as tetracycline. Overlap of resonances from the two compounds has been taken into account, as has the slow conversion to anhydrotetracycline which occurred after the samples were dissolved.

[b] Relative to the internal TMS standard; the solvent was DMSO-d_6.

[c] Calculated from integrated resonance intensities according to the procedure described by WRIGHT *et al.*[12] for incorporation of single labells. Values shown are averages from experiments with and without addition of $Cr(acac)_3$ and NOE suppression. Corresponding spectra of natural abundance material were obtained under identical conditions.

[d] Tetracycline hydrochloride enriched from [2-^{13}C, 2-$^2H_3$]acetate showed ^{13}C enrichments as for [2-^{13}C]-acetate labelling, with no detectable isotopically shifted ^{13}C resonances indicating ^2H-retention at C-5, C-7 or C-9.

[e] Couplings are for ^{13}C-^{13}C within doubly labelled units incorporated intact. Free induction decays (8K data points) were "zero-filled" to 16K before Fourier transformation to improve digital resolution. Lorentzian interpolations were used to locate peak maxima.

[f] Calculated from $E(\%)=1.1(Id_1+Im)/[Ic-f(Id_1+Im)]$ (See ref 12 for derivation and definitions; $Im=0$ and $f=1/9$ in this case) except for the carboxamide peak for which the enrichment was measured from absolute resonance intensities.

[g] Could not be estimated accurately due to overlap of the C-5a resonance with the solvent signal.

enrichments from each labelled carbon indicates that a uniform precursor pool was maintained in the mycelium during the biosynthetic phase.

Discussion

There have been reports[1,6] that although [1-^{14}C]acetate supplied to *Streptomyces rimosus* labelled oxytetracycline in alternate positions (C-1, 3, 4a, 5a, 6a, 8, 10, 11 and 12), [2-^{14}C]acetate labelled the

molecule somewhat randomly. When the organism was exposed to the precursor during a 72 hour period, CATLIN *et al.*[7] found that about 14% of the radioactivity entering oxytetracycline from [2-^{14}C]-acetate was in the position normally labelled by [1-^{14}C]acetate. Since results recently reported by THOMAS and WILLIAMS[8] for oxytetracycline biosynthesis from [1-^{13}C]acetate in *S. rimosus* show clearly the polyketide derivation of the antibiotic, it is apparent that some of the [2-^{14}C]acetate used in the earlier studies had participated in other metabolic reactions before its incorporation into the antibiotic. The results are most easily accounted for by assuming passage through the Krebs cycle. In the present experiments the ^{13}C labelling pattern of tetracycline hydrochloride derived from either [1-^{13}C]acetate or [2-^{13}C]acetate demonstrated clearly that this kind of metabolic processing of the precursor had not occurred. The distribution of the label conformed strictly to the polyketide hypothesis of tetracycline biogenesis originally proposed by ROBINSON[9].

Incorporation of [1,2-^{13}C]acetate into tetracycline generated satellites at every ring carbon resonance, with coupling constants showing that nine intact C_2 units were present. This result confirmed that the tetracycline nucleus is formed exclusively from acetate-derived two-carbon units. THOMAS and WILLIAMS[8] reported a similar result for oxytetracycline enriched from [1,2-^{13}C]acetate in cultures of *S. rimosus*. They have also shown[10] that [1,2,3-^{13}C]malonate is incorporated intact into the carboxamide substituent of oxytetracycline and thus probably serves as the chain-initiating unit of the polyketide intermediate. Assuming that malonate has a similar role in *S. aureofaciens*, the relatively low ^{13}C enrichment of the carboxamide carbon atom by [1-^{13}C] and [1,2-^{13}C]acetate, and the negligible labelling of this carbon by [2-^{13}C]acetate in comparison with ring carbon atoms, indicates that the carboxamide carbon was derived from a different biogenetic unit. Its likely origin is from an endogenous pool of carbon dioxide which had become partially enriched with ^{13}C by processing of labelled acetate through the Krebs cycle. This implicates a route to malonyl CoA involving acetyl CoA carboxylase.

The presence of ^{13}C-^{13}C coupling between C-2 and the carboxamide carbon, predicted if the malonyl CoA starter unit had been generated from oxaloacetic acid after passage of [1,2-^{13}C]acetate through the Krebs cycle, was not observed. This and the failure to detect scission of the bond between the two acetate carbons gives no indication that malonyl coenzyme A can be formed by oxidative decarboxylation of oxaloacetic acid during idiophase production of tetracycline or oxytetracycline. However, participation of a pathway *via* oxaloacetate, as postulated by HOSTALEK *et al.*[3], may be difficult to detect when large amounts of labelled acetate are supplied as the precursor. It is apparent from the labelling results with [2-^{13}C]acetate that negligible amounts of the precursor had been passed through the Krebs cycle where they would have undergone conversion to oxaloacetic acid. Under conditions where disproportionate concentrations of acetyl CoA and oxaloacetic acid are present, even low levels of acetyl coenzyme A carboxylase in the cells may yield malonyl coenzyme A derived directly from the acetate pool.

In addition to providing information on the way intact two-carbon units derived from acetate are inserted into a polyketide chain, the ^{13}C NMR spectrum of carbocyclic metabolites constructed from [1,2-^{13}C]acetate contains information on the mode of cyclization[11], provided the rings are assumed to be formed from a single polyketide chain which is folded and cross-linked. Thus, the ^{13}C-^{13}C coupling pattern reported in Table 1 requires that the polyketide chain leading to tetracycline be folded as shown in Fig. 1. The revised assignment of resonances and the results from ^{13}C-enrichment of tetracycline by singly and doubly labelled acetate have led to a consistent interpretation of all NMR spectra and it can be concluded that the biosynthetic process for constructing the tetracycline nucleus under the conditions used in this study is by condensation of malonyl intermediates generated by the carboxylation of acetyl CoA with a malonyl or malonamyl starter unit drawn from the same pool.

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