We and others[24] have examined complex **1** in order to try to mimic the reactions controlled by P-450; yet, in the attempted epoxidation of styrene, complex **1** exhibited no reactivity. We suspect that the axial thiolate ligand which is absent from our model systems may be critical and oxidations may not be observed until we can incorporate into complex **1** a good π-donor electron rich ligand.

The closely related monooxygenase, heme oxidase, which participates in the conversion of heme to bile pigments has also recently been shown to be activated by the reduction of an oxygenated ferrous heme.[25] At this time the number of electrons involved is not apparent, but since the reductant is NADPH-cytochrome *c* reductase one can assume that the electrons are added one at a time. This suggests that an intermediate such as **1** may also be involved in this system.

Acknowledgment. This work is a contribution from the Bioinorganic Chemistry Group and was supported by grants from the N.S.E.R.C. of Canada and the United States NIH (AM 17989).

(24) Groves, J. T., personal communication.
(25) Yoshida, T.; Noyuchi, M.; Mikuchi, G. *J. Biol. Chem.* **1980**, *255*, 4418-4420.

Biosynthesis of Streptothricin F. 1. Observing the Interaction of Primary and Secondary Metabolism with [1,2-$^{13}C_2$]Acetate

Steven J. Gould,*[1] Kazys J. Martinkus, and Chou-Hong Tann

School of Pharmacy
Section of Medicinal Chemistry and Pharmacognosy
University of Connecticut, Storrs, Connecticut 06268

Received January 20, 1981

Streptothricin F (**1**)[2,3] is representative of a ubiquitous family of broad spectrum antibiotics[4] produced by *Streptomyces* species. All have the same unusual heterocyclic moiety, streptolidine (**2**), for which King et. al.[5] proposed the biogenesis from arginine **3** shown in Scheme I.

Subsequent biosynthetic studies by two different groups[6-9] produced conflicting results and led one group to claim[7,8] that there are two fundamentally different pathways leading to **2**: one derived from arginine and the other from acetate. [U-^{14}C]Arginine was used in all cases, making it impossible to determine the specificity of incorporation, much less the pathway involved. In one organism sodium [1-^{13}C]- and [2-^{13}C]acetates were specifically incorporated, but the authors were unable to propose a pathway and concluded that "Streptolidine... seems to be formed by at least two different pathways in the streptothricins".[8] We now present evidence that there is only one pathway to **2**, with **3** the direct primary precursor.

Scheme I. Biogenesis of the Streptolidine Portion of Streptothricin F from Arginine

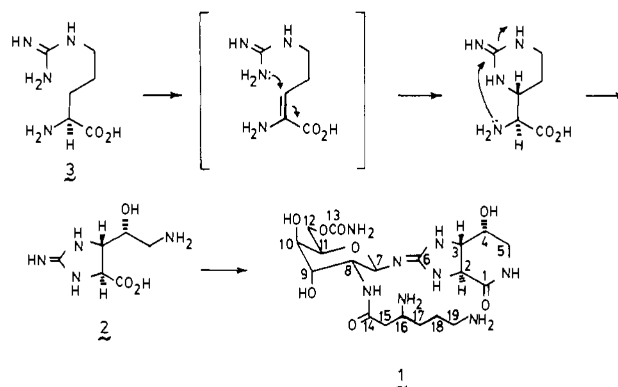


Table I. ^{13}C NMR of Streptothricin F·3HCl[a]

assignment	chemical shift, δ[b]	J_{CC}, Hz	assignment	chemical shift, δ[b]	J_{CC}, Hz
14	169.7 s[c]	48.1	4	58.5 d	37.0
1	167.7 s	55.5	12	57.9 t	
6, 13	{160.4 s		2	52.1 d	55.5
	{155.4 s		5	46.8 t	37.0
7	76.5 d		8	46.6 d	
9, 10, 11	{71.1 d		16	45.9 d	37.0
	{67.6 d		19	36.6 t	
	{64.1 d		15	34.2 t	48.1
3	58.9 d		17	26.9 t	37.0, 35.1
			18	20.5 t	35.1

[a] Bruker WH-270, 67.88 MHz; spectral width 15 000 Hz; 30° pulse angle; 0.54-s aquisition time; 66 500 transients; 48 mg in 0.5-mL D_2O containing 2% pyridine. The observed signal enhancements were calculated by comparing the normalized area of each set of signals with that of the natural abundance antibiotic. [b] Middle pyridine signal = 135.5 ppm. [c] Multiplicity in gated decoupled spectrum: s = singlet, d = doublet, t = triplet.

In their [^{13}C]acetate feedings the Japanese group fed half the labeled material at the beginning of the fermentation and the second half at the onset of antibiotic production. Acetate, as its coenzyme A thioester, is the most central of all primary metabolites and the feeding protocol used was likely to label a large array of intermediates, interconnected by primary metabolic grids, before streptothricin biosynthesis began. These specific labelings unrelated to secondary metabolism could obscure the branch point from primary metabolism into the streptothricin pathway.

Using *Streptomyces* L-1689-23 and the same feeding protocol as the previous workers,[8] we have obtained specific incorporations of sodium [1,2-$^{13}C_2$]acetate into the streptolidine and β-lysine portions of **1**. Sodium [1,2-$^{13}C_2$]acetate (281 mg, 3.35 mmol, 90 atom %/^{13}C), sodium acetate trihydrate (456 mg, 3.35 mmol), and sodium [2-^{14}C]acetate (112 μCi) were dissolved in 20 mL of water. To each of 4 250-mL fermentations,[10] 2.5 mL of this solution was added aseptically immediately after innoculation with a seed culture.[11] The fermentations were then shaken on a New Brunswick Rotary Shaker at 200 rpm and 29 °C. At the onset of streptothricin F production, 12 h later, the second half of the acetate mixture was divided aseptically among the four flasks, and the fermentations were continued for an additional 36 h.

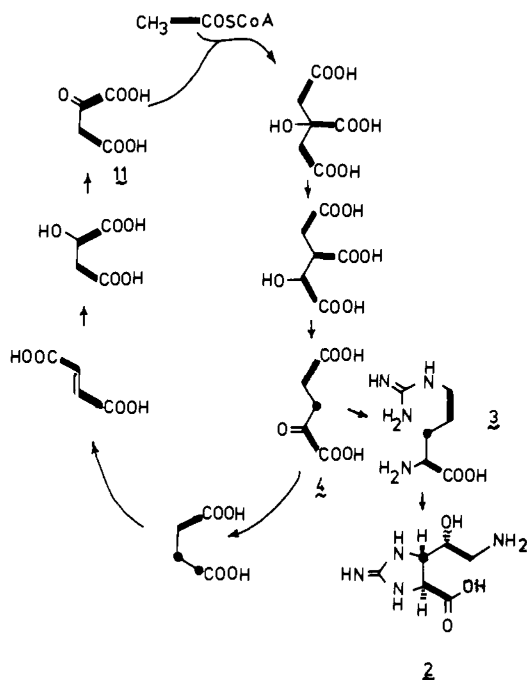
Workup of the fermentations[12] eventually afforded, after re-

(1) Career Development Awardee of the National Cancer Institute (CA00627), 1979-1984.
(2) Waksman, S. A. *J. Bacteriol.* **1943**, *46*, 299-310.
(3) van Tamelen, E. E.; Dyer, J. R.; Whaley, H. A.; Carter, H. E.; Whitfield, G. B., Jr., *J. Am. Chem. Soc.* **1961**, *83*, 4295-4298.
(4) Khokhlov, A. S. *J. Chromatogr. Libr.* **1978**, *15*, 617-713.
(5) Bycroft, B. W.; King, T. J. *J. Chem. Soc. Commun.* **1972**, 652-654.
(6) Gräfe, U.; Reinhardt, G.; Bocker, H.; Thrum, H. *J. Antibiot.* **1977**, *30*, 106-110.
(7) Sawada, Y.; Kubo, T.; Taniyama, H. *Chem. Pharm. Bull.* **1976**, *24*, 2163-2167.
(8) Sawada, Y.; Kawakami, S.; Taniyama, H.; Inamori, Y. *J. Antibiot.* **1977**, *30*, 632-640.
(9) Sawada, Y.; Nakashima, S.; Taniyama, H.; Inamori, Y.; Sunagawa, S.; Tsuruga, M. *Chem. Pharm. Bull.* **1977**, *25*, 1161-1163.

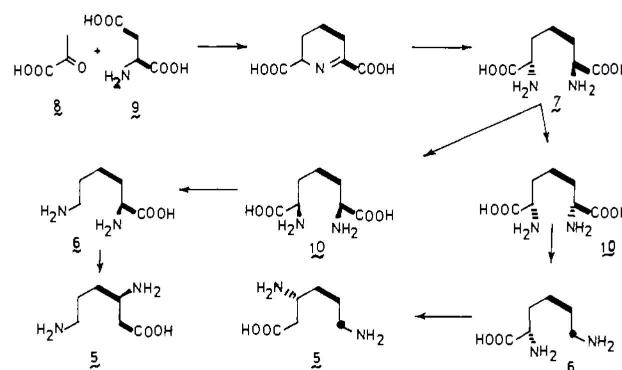
(10) The medium for production fermentations consisted of corn starch, 30%; molasses, 2%; soy flour, 1.5%; and calcium carbonate, 1%. In 1-L Erlenmeyer flasks, 250-mL fermentations were run.
(11) The medium for the seed culture consisted of beef extract, 0.3%; yeast extract, 0.5%; tryptone, 0.5%; dextrose, 0.1%; and corn starch, 2.4%. In baffled 250-mL Erlenmeyer flasks, 50-mL fermentations were run.

0002-7863/81/1503-2871$01.25/0 © 1981 American Chemical Society

Scheme II. Labeling of Streptolidine by [1,2-$^{13}C_2$]Acetyl-CoA via α-ketoglutarate and the TCA Cycle



Scheme III. Labeling of β-Lysine by [1,2-$^{13}C_2$]Acetyl-CoA via the Diaminopimelic Acid Pathway and the TCA Cycle



peated recrystallization, 314 mg[13] of radiochemically pure streptothricin F helianthate with a specific radioactivity of 5.7 × 10^6 dpm/mmol,[14] indicating a 1.3% incorporation of acetate. A portion of the salt (120 mg) was converted to the amorphous trihydrochloride (48 mg),[15] and the 67.88-MHz proton-noise-decoupled ^{13}C NMR spectrum of the sample in 2% pyridine/D_2O was obtained. The spectroscopic data are presented in Table I. All 19 singlets of **1** were observable, and 9 of these were flanked by doublets due to the $^{13}C-^{13}C$ splitting of labeled acetate units incorporated intact. ^{13}C enrichments measured for seven of these doublets were in the range of 0.2–0.5%, while the doublets for C-4 and C-5 indicated approximately 1.3% enrichment for each. At these levels, only enrichment of the latter two carbons would have been detectable with singly ^{13}C-labeled acetate. Using the signal for the anomeric carbon, C-7, as a standard, and comparing this spectrum with one of unlabeled antibiotic, none of the sugar carbons were found to be enriched with ^{13}C but all the other singlets showed some enhancement due to extensive metabolism of the labeled acetate. Excluding the sp^2 carbonyls, for which C-7 is a poor reference, by far the most enhanced singlets were those due to C-3 and C-19 (0.8 and 0.9% enrichments, respectively).

As seen in Table I, the doublets could easily be paired on the basis of the coupling constants, revealing the locations of acetate units that had been incorporated intact. The pattern of couplings and the enhancement of the C-3 singlet observed for the streptolidine moiety is exactly that which is predictable for the incorporation of acetate into **3** via α-ketoglutarate **4** (Scheme II) and then of **3** into **2** according to Scheme I. The first dose of [1,2-$^{13}C_2$]acetate would have eventually led to low—but specific—labeling of all the intermediates of the tricarboxylic acid cycle. The second dose of labeled acetate—added at the start of antibiotic production—would be expected to have been metabolized with considerably less dilution and recycling before entering the streptolidine pathway and would label C-4 and C-5 most heavily. Thus, acetate does label **2** specifically but only indirectly via **3**.

All but the terminal carbon of the β-lysine portion exhibited small doublets. Of these, the C-17 satellites were actually an unresolved pair of doublets; in two different spectra obtained with the same sample the C-17 splittings were 37.0 and 35.1 Hz, respectively (the spectrum was obtained with 1.8 Hz/data point). Thus, the antibiotic sample is made up of some molecules in which C-16 and C-17 are derived from an acetate unit and some molecules in which C-17 and C-18 are so derived. This pattern is consistent with β-lysine (**5**) being derived from α-lysine (**6**) which was produced *via* the diaminopimelic acid (DAP) pathway shown in Scheme III. In this pathway (2*S*,6*S*)-DAP (**7**), derived from pyruvic acid (**8**) and aspartic acid (**9**) is epimerized to *meso*-DAP (**10**) and then decarboxylated. Aspartic acid is obtained by transamination of oxaloacetic acid (**11**) (see Scheme II), thus revealing the specific but indirect labeling by acetate of this portion of **1** as well.[16]

Two distinct pathways from acetate to lysine are known to occur in microorganisms.[17] While there has been strong circumstantial evidence for the DAP pathway in *Streptomyces*, obtained through the use of blocked mutants and cell-free enzymes,[18] the work presented here clearly establishes the involvement of this pathway. This is consistent with the accepted designation of *Streptomyces* as funguslike bacteria.[19]

The results so far obtained are most easily explained by the incorporation of acetate into **2** and **5** via **3** and **6**, respectively. Experiments using arginine and lysine multiply labeled with $^{15}N/^{13}C$ and $^{2}H/^{13}C$ to confirm this and elucidate the mechanistic details of these secondary pathways are in progress.[20]

Acknowledgment. This work was supported by Public Health Service Grant GM 25996 from the National Institute of General Medical Sciences. We are grateful to Dr. Donald Borders, Lederle Laboratories, Pearl River, NY, for generous gifts of streptothricin F and *Streptomyces* L-1689-23 and BL-287. Peter Demou of the Chemistry Department, Yale University, and Dr. Thomas Leipert of the Chemistry Department, University of Connecticut, are thanked for obtaining the ^{13}C NMR spectra.

(12) Workup of the fermentations involved centrifugation to remove the mycelium, adsorption of the supernatant on Amberlite IRC-50(K^+), washed with water, and then eluted with 0.3 N HCl, neutralization of the ninhydrin-positive fractions with 1 N KOH, and lyophilization. The residue was then extracted with methanol, and the extracts concentrated to dryness, taken up in water, and chromatographed on Sephadex LH-20 eluted with water. Only those fractions not containing low R_f impurities (circular chromatography on silica gel) were used further. These were combined, lyophilized, and the antibiotic converted to its crystalline methyl orange salt.

(13) This corresponds to a 38% recovery of antibiotic originally in the fermentation broth as determined by bioassay with *B. subtilis* ATCC 6633 grown on brain-heart infusion agar.

(14) Weighed samples of the highly colored salt were combusted to CO_2 in a Packard Model 306 sample oxidizer and counted by liquid scintillation in a Beckman Model 8000 counter.

(15) This was effected by acidifying, removing the precipitate by centrifugation, passing the supernatant through a column of Dowex AG-3(OH^-) to remove the last traces of methyl orange, and lyophilization of the effluent.

(16) Since only the C-19 singlet showed excessive enhancement, and not the C-16 and C-18 singlets as well, it would appear that under the experimental conditions **11** was not also being converted to pyruvic acid.

(17) Rodwell, V. W. *Metab. Pathways, 3rd Ed.* **1969**, *3*, 335–349.

(18) Kirkpatrick, J. R.; Doolin, L. E.; Godfrey, O. W. *Antimicrob. Agents Chemother.* **1973**, 542–550.

(19) Bainbridge, B. W. "Genetics of Microbes"; Wiley: New York, 1980; p 165.

(20) Intact incorporation of arginine has now been demonstrated with the incorporation of [*guanido*-^{13}C,$^{15}N_2$,1-^{14}C]arginine. These and other results will be reported in the future.