Nucleoside Antibiotics. Biosynthesis of the Maleimide Nucleoside Antibiotic, Showdomycin, by *Streptomyces showdoensis**

E. F. Elstner and R. J. Suhadolnik†

ABSTRACT: The biosynthesis of showdomycin, the maleimide nucleoside antibiotic, elaborated by *Streptomyces showdoensis* has been studied. Of the twelve radioactive compounds studied, α-ketoglutarate-*5*-^{14}C is incorporated into the maleimide ring of showdomycin with the highest efficiency. The data strongly suggest that carbons-2, -3, -4, and -5 of α-keto glutarate contribute to the biosynthesis of the four carbons of the maleimide ring of showdomycin. Carbon-14 from succinate-*2,3*-^{14}C, fumarate-*2,3*-^{14}C, and malate-*U*-^{14}C, but not succinate-*1,4*-^{14}C or fumarate-*1,4*-^{14}C, is uniformly incorporated into the maleimide ring. Similarly, ^{14}C from acetate-*1*-^{14}C is only incorporated into the carbonyl carbons of the maleimide ring while ^{14}C from acetate-*2*-^{14}C is incorporated uniformly into all four carbons. The labeling patterns from these ^{14}C acids are in agreement with the notion that carbons-2 and -3 of succinate, fumarate, and malate and both carbons of acetate are incorporated exclusively into the maleimide ring of showdomycin, but only after these mono- and dicarboxylic acids enter the Krebs cycle. Carbons-2, -3, -4, and -5 of glutamate or α-ketoglutarate appear to be direct precursors for the biosynthesis of four carbons of the maleimide ring of showdomycin for the following reasons: (1) all of the ^{14}C in the maleimide ring from the glutamate-*2*-^{14}C or glutamate-*5*-^{14}C experiments were shown to reside in the carboxyl carbons; (2) ^{14}C from α-ketoglutarate-*1*-^{14}C is not incorporated into showdomycin; (3) if glutamate-*2*-^{14}C or α-ketoglutarate-*5*-^{14}C entered the Krebs cycle prior to their incorporation into the maleimide ring, all of the ^{14}C would be lost upon completion of one passage of the Krebs cycle. Carbon-14 from maleic acid-*2,3*-^{14}C, phenylalanine-*U*-^{14}C, and fluoroacetate-*2*-^{14}C is not incorporated into showdomycin. The possibility that glutamate could be decarboxylated to form γ-aminobutyric acid, which then serves as the four-carbon–nitrogen precursor for the maleimide ring has been eliminated. Ribose appears to be the five-carbon pentose that forms the C–C riboside bond in showdomycin biosynthesis. The intermediate compounds between α-ketoglutarate and showdomycin are not yet known. The findings reported here are the first example of the biosynthesis of a nucleoside antibiotic in which purine or pyrimidine nucleosides and/or nucleotides are not the precursors. These studies also add another role of α-ketoglutaric acid in the biosynthesis of heterocyclic rings and the formation of a carbon–carbon bond with ribose.

Showdomycin is one of six C-substituted nucleosides and/or nucleoside antibiotics isolated from natural sources. The other C-substituted nucleosides are formycin, formycin B, oxoformycin B, pyrazomycin, and pseudouridine (Suhadolnik, 1970). A comparison of the structures of uridine, pseudouridine, and showdomycin is shown in Figure 1. Showdomycin was isolated from the culture filtrates of *Streptomyces showdoensis* by the research group at the Shionogi Research Laboratory (Nishimura *et al.*, 1964). The structure of showdomycin has been established as 3-(β-D-ribofuranosyl)maleimide (Darnall *et al.*, 1967; Nakagawa *et al.*, 1967; Kano *et al.*, 1967). Kalvoda *et al.* (1970) utilized several elegant procedures for the total chemical synthesis of showdomycin starting from 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-2,4,6-trimethoxybenzene. This nucleoside antibiotic is moderately active against gram-positive and gram-negative bacteria. It is cytotoxic to tumor cells (Matsuura *et al.*, 1964). Showdomycin inhibits the RNA and DNA polymerases isolated from *Escherichia coli* (Roy-Burman, 1970; Maryanka and Johnston, 1970). Y. Komatsu (private communication) states that showdomycin is a strong inhibitor of DNA synthesis but does not inhibit RNA synthesis in *Escherichia coli*. Komatsu and Tanaka (1970) reported that showdomycin inhibited the incorporation of amino acids and purine and pyrimidine bases into their corresponding macromolecules in *E. coli* K-12 cells. The selective inhibition of showdomycin appears to be related to the chemical reactivity of the maleimide structure with the sulfhydryl groups of proteins (Roy-Burman, 1970; Tsai *et al.*, 1970; Bermek *et al.*, 1970).

As a complement to the biosynthesis of purine and pyrimidine nucleosides, this laboratory has been involved in studying the biosynthesis of the nautrally occurring nucleoside antibiotics. The biosynthesis of ten nucleoside antibiotics has been studied and reported (Suhadolnik, 1970; Seto *et al.*, 1968). In all studies, except showdomycin, either the purine or pyrimidine nucleosides and/or nucleotides or the aglycone have been shown to serve as precursors for the biosynthesis of these nucleoside analogs elaborated by the *Streptomyces* and fungi.

Since acetate-*1*-^{14}C and acetate-*2*-^{14}C are incorporated into the maleimide ring of showdomycin (Suhadolnik and Ramer, 1968), it was of interest to determine if the Krebs cycle or compounds closely related to the Krebs cycle play an important role in the biosynthesis of the C_4-maleimide ring of showdomycin. This report describes the results of radioactive tracer studies and the subsequent degradations of the purified, crystalline ^{14}C-labeled showdomycin. The data strongly suggest that carbons-2, -3, -4, and -5 of α-ketoglutarate contribute to the biosynthesis of the four carbons of the maleimide ring of showdomycin.

* From the Research Laboratories, Department of Bioorganic Chemistry, Albert Einstein Medical Center, Philadelphia, Pennsylvania 19141. *Received March 30, 1971.* This research was supported by Research Grant GB 7434 from the National Science Foundation and Research Grant AI-GM 08932-10. This is the eighth part of a series. The previous paper is Suhadolnik and Uematsu (1970).

† Research Career Development awardee of U. S. Public Health Service (5-K3-GM-7100-10); to whom to address correspondence.

Experimental Section

Cultures of *S. showdoensis*, obtained from Merck and Co., Rahway, N. J., were maintained on nutrient agar slants. The composition of the medium for the production of showdomycin is as follows: 1% glucose, 0.1% asparagine, 0.01% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, and 0.05% yeast extract in distilled water. The medium was adjusted to pH 7.2 and autoclaved for 20 min at 15 lb. Each 2-l. baffled flask contained 300 ml of medium. Inoculations were made as described (Uematsu and Suhadolnik, 1971). Cells were grown at 28° on a New Brunswick Gyrotort (Model G-25) at a speed setting of 6. After 18-hr growth, the ^{14}C-labeled compounds were added with a sterile microfilter syringe. Showdomycin was isolated 14–16 hr later. Showdomycin was isolated by a modification of the method of Nishimura *et al.* (1964) as follows. The culture medium from ten flasks (3 l.) was filtered through Celite; the filtrate was then passed through a column of 70 g of Dowex 50 H^+ (75 ml/min). The effluent was adjusted to pH 5.5 with concentrated ammonium hydroxide. Showdomycin was then adsorbed on partially deactivated Norit A (10 g). After stirring for 30 min, filtering, and washing the carbon with 1 l. of water, showdomycin was eluted from the Norit A by the addition of 500 ml of acetone–water (4:1, v/v). This procedure was repeated three times. The combined acetone filtrates were concentrated under vacuum at 55° to 1 ml, spotted on paper chromatograms (Whatman No. 3MM), and developed in water-saturated 1-butanol for 18 hr. The areas on the chromatograms equivalent to showdomycin (R_F 0.35) were cut out, eluted with water, spotted on Whatman No. 3MM paper chromatograms, and developed in 1-propanol–water (7:3, v/v) for 12 hr. The showdomycin (R_F 0.80) was identified on the chromatograms by ultraviolet absorption. The showdomycin areas on the chromatograms were cut out, eluted, rechromatographed on Whatman No. 3MM paper chromatograms, and developed in water for 5 hr (R_F 0.85). The eluted showdomycin was crystallized to constant specific activity from the following three solvents: ethyl acetate–benzene, acetone–benzene, and absolute alcohol–benzene. Yields were about 6 mg of pure, crystalline showdomycin/flask. Melting points were 141–149° (literature 153-163°; Nakagawa *et al.*, 1967; Nishimura *et al.*, 1964; Kalvoda *et al.*, 1970). The ultraviolet, infrared, and mass spectral properties of the showdomycin were the same as those reported earlier (Nishimura *et al.*, 1964; Nakagawa *et al.*, 1967; Townsend and Robins, 1969).

Degradation of Showdomycin. Ribose was isolated by treatment of showdomycin with aqueous hydrazine (Darnell *et al.*, 1967). The D-ribose was isolated by paper chromatography (Whatman No. 1) in the following systems: (A) pyridine–1-butanol–water (4:6:3, v/v) and (B) 1-butanol–acetic acid–water (5:1:4, v/v). The area corresponding to ribose on the chromatograms was determined by spraying with 2-aminobiphenyl reagent (Gordon *et al.*, 1956). This area was cut out and eluted with water. The amount of ribose was determined quantitatively by the method of Fernell and King (1953). The yields of ribose were 5–10%.

Isolation of CO_2 from the Carbonyl Groups of the Maleimide Ring of Showdomycin. Showdomycin was reduced catalytically to dihydroshowdomycin and isolated by the method of Darnall *et al.* (1967) except that tetrahydrofuran and 10% Pd/C were used. Hydrogenation of showdomycin resulted in the loss of the ultraviolet absorption maxium at 218 mμ. The nmr spectrum of the dihydroshowdomycin showed a loss of the vinylic proton and the appearance of new multiplets

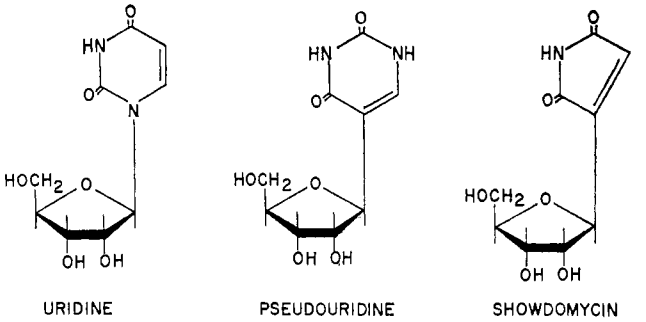
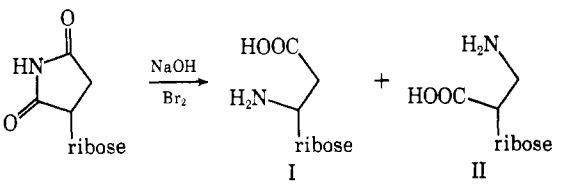


FIGURE 1: Comparison of the structures of uridine, pseudouridine, and showdomycin.

(three protons) in the region δ 2.7–3.58 (Darnall *et al.*, 1967). The yield of dihydroshowdomycin as determined by radiochemical measurements was 60%. Decarboxylation of dihydroshowdomycin was done by the Hofmann degradation method as previously described for the degradation of succinimide (Clarke and Behr, 1943). The decarboxylation of dihydroshowdomycin should proceed as shown in the following scheme. Theoretically, there should be a 50% yield of I and II. Since the succinimide is unsymmetrical, it is unlikely



that equal amounts of these two compounds are produced. This possibility does not invalidate the results on the per cent distribution of ^{14}C in the carboxyl groups. Dihydroshowdomycin (5–10 μmoles) was added to 0.5 ml of water containing 0.08 ml of 19.3 N sodium hydroxide and 50 μmoles of freshly prepared aqueous bromine. The reaction mixture was maintained at 58° for 1 hr in a closed microdistillation apparatus. After 1 hr, 1 ml of 2.7 M sulfuric acid was injected into the reaction mixture. Nitrogen gas was bubbled through the reaction mixture and through 1 ml of 1 N Hyamine to trap the carbon dioxide formed.

The recovery of the $^{14}CO_2$ by the microdistillation method described here was calculated to be greater than 95%. This recovery of CO_2 was determined by two methods: (1) decarboxylation of succinic acid-*1,4*-^{14}C by the Schmidt method (Greenberg and Rothstein, 1957) and (2) oxidation of formic acid to carbon dioxide by mercuric sulfate–sulfuric acid (Friedeman, 1938).

Antimicrobial Activity of Showdomycin. Showdomycin potency was assayed by the pulp disk method (microbiological assay). *E. coli* B was the test organism.

Determination of Dry Weight of S. showdoensis. The cells from 300 ml of culture medium were centrifuged, washed with water, recentrifuged, lyophilized, and weighed.

Radioactive Compounds and Analytical Measurements. Acetate-*1*-^{14}C, acetate-*2*-^{14}C, succinate-*1,4*-^{14}C, succinate-*2,3*-^{14}C, γ-aminobutyric acid-*1*-^{14}C, and phenylalanine-*U*-^{14}C were obtained from Schwarz–Mann; malic acid-*U*-^{14}C, fumaric acid-*2,3*-^{14}C, maleic acid-*2,3*-^{14}C, ribose-*1*-^{14}C, and fluoroacetate-*2*-^{14}C were obtained from International Chemical and Nuclear Corp.; fumaric acid-*1,4*-^{14}C was obtained

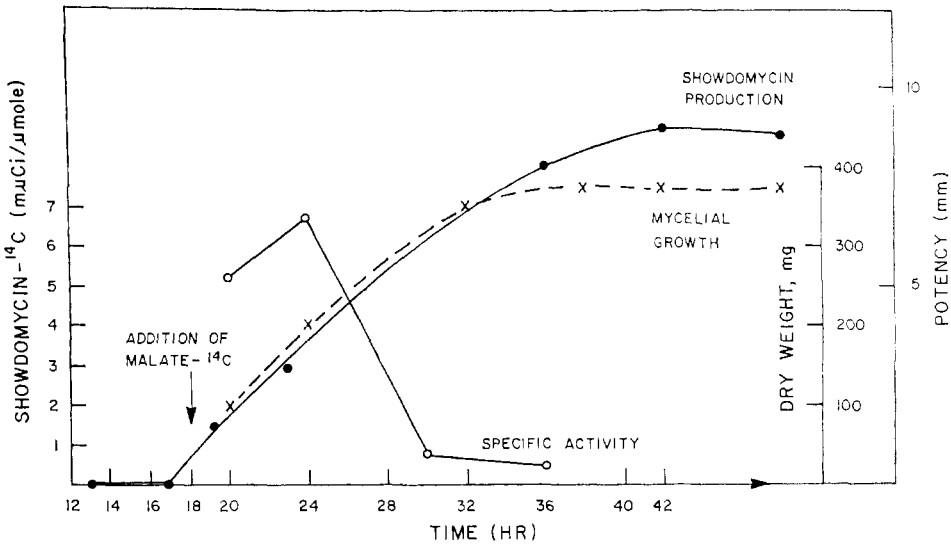


FIGURE 2: Growth of *S. showdoensis*, production of showdomycin, and specific activities of ^{14}C-labeled showdomycin. (×) Mycelial growth, (●) showdomycin potency, and (○) specific activity.

from Amersham–Searle; α-ketoglutarate-*1*-^{14}*C* and α-ketoglutarate-*5*-^{14}*C* were obtained from Calbiochem; glutamate-*2*-^{14}*C*, glutamate-*5*-^{14}*C*, and aspartate-*4*-^{14}*C* were obtained from New England Nuclear Corp. Palladium (10%) on powdered charcoal was obtained from Matheson Coleman & Bell. Radiochemical measurements were made on a Packard liquid scintillation spectrometer, Model 314E, using Bray's solution (1960). Ultraviolet spectra were obtained with a Beckman DB and Gilford Model 2400 spectrophotometers. Mass spectral studies were done with a CEC Model 21492 mass spectrometer by using the direct inlet probe at about 40°. Nmr spectra were determined on a Varian A-60 spectrometer. All samples were run in D_2O with Me_4Si as an internal standard. Melting points were obtained with a Thomas-Hoover apparatus and were uncorrected. Infrared spectra were obtained by a Perkin-Elmer Model 21 spectrophotometer with KBr pellets.

Results

Growth of S. showdoensis, Production of Showdomycin, adn Specific Activities of ^{14}*C-Labeled Showdomycin.* Typical data on mycelial growth and production of showdomycin are shown in Figure 2. Mycelium and showdomycin production begins 18 hr after inoculation. The simultaneous production of showdomycin and mycelial growth is unlike other nucleoside antibiotics where the production of the nucleoside antibiotic begins when the cells go into the stationary phase of growth (Suhadolnik, 1970). Kimura *et al.* (1968) reported similar findings with their mutant strain (N_2-209-56) of *S. showdoensis*. Figure 2 also shows the marked decrease in the specific activity of the ^{14}C-labeled showdomycin following the uptake and incorporation of malate-*U*-^{14}*C*. When malate-*U*-^{14}*C* is added to the culture flasks 18 hr after inoculation, the specific activity of the showdomycin increased up to 24 hr and then decreased. This decrease in specific activity after 24 hr would be expected since showdomycin biosynthesis is maximal at this time. All of the ^{14}C incorporation experiments reporter here were done by adding the ^{14}C-labeled compounds to the culture flasks 18 hr after inoculation. Showdomycin was isolated from the culture medium between 32 and 34 hr after inoculation.

The specific activities of the showdomycin are lower and almost constant at 32–34 hr, but the yields of pure, crystalline compound are much higher. By using this procedure, a more accurate comparison can be made of the specific activities of the ^{14}C compounds incorporated into showdomycin. These interpretations would be more difficult if isolations were done between 22 and 30 hr when the production and specific activities of showdomycin are changing rapidly (Figure 2). In addition, the specific activities of the showdomycin, following the isolation at 32–34 hr, are sufficiently high to perform the necessary degradations to determine the per cent distribution of the ^{14}C-labeled carbons in the maleimide ring.

Incorporation of ^{14}*C-Labeled Compounds into the Maleimide Ring and Ribose Moieties of Showdomycin by S. showdoensis.* In order to elucidate the pathway involved in the biosynthesis of showdomycin, separate experiments were performed in which 17 ^{14}C-labeled compounds were added to the culture filtrates of *S. showdoensis*. While ^{14}C from α-ketoglutarate-*5*-^{14}*C* was incorporated into the maleimide ring most efficiently (Table I), carbon-14 from α-ketoglutarate-*1*-^{14}*C* was not incorporated. Likewise, the carbon-14 from the succinate-*1,4*-^{14}*C*, fumarate-*1,4*-^{14}*C*, and aspartate-*4*-^{14}*C* was not incorporated into showdomycin. Nevertheless, ^{14}C from succinate-*2,3*-^{14}*C*, fumarate-*2,3*-^{14}*C*, malate-*U*-^{14}*C*, glutamate-*2*-^{14}*C*, and glutamate-*5*-^{14}*C* is incorporated into showdomycin.

Although the incorporation of fumarate-*2,3*-^{14}*C* is similar to that of α-ketoglutarate-*5*-^{14}*C* (Table I), fumarate cannot be considered as a direct precursor for the biosynthesis of the maleimide ring of showdomycin for two reasons: first, ^{14}C from fumarate-*1,4*-^{14}*C* is not incorporated into showdomycin; second, fumarate-*2,3*-^{14}*C* is incorporated uniformly into the four carbons of the maleimide ring of showdomycin. These findings are consistent with the idea that the uniform incorporation of carbons-2 and -3 of fumarate into the maleimide ring must take place by passage of fumarate through the tricarboxylic acid cycle (Krebs cycle) (Table II).

Acetate-*1*-^{14}*C* and acetate-*2*-^{14}*C* were incorporated into the maleimide ring of showdomycin but the incorporations were

TABLE I: Incorporation of ^{14}C-Labeled Compounds into Showdomycin by *S. showdoensis*.

Compound Added	Amount/Flask[a]		Sp Act. (mμCi/μmole × 10^{-3})	Showdomycin Isolated	
	mμCi × 10^{3}	μmoles		Sp Act. (mμCi/μmole)	Dilution (× 10^{-3})
α-Ketoglutarate-*5*-^{14}C	1.5	15	0.1	0.20	0.5
Glutamate-*2*-^{14}C	4.2	1.3	3.2	0.90	3.5
Glutamate-*5*-^{14}C	4.7	1.2	3.9	0.90	4.3
Succinate-*2,3*-^{14}C	3.3	0.8	4.2	0.60	7.0
Fumarate-*2,3*-^{14}C	2.1	2.0	1.1	0.75	1.5
Malate-*U*-^{14}C	5.8	0.12	47	0.63	74.0
Acetate-*1*-^{14}C	3.6	0.07	56	0.60	93.0
Acetate-*2*-^{14}C	4.4	0.09	51	1.4	36.0
Ribose-*1*-^{14}C	2.7	0.30	8.9	0.1	89.0
Maleic-*2,3*-^{14}C	1.0	0.61	1.63	0.01	
Aspartate-*4*-^{14}C	7.4	4.5	1.65	0.01	
Succinate-*1,4*-^{14}C	3.1	0.52	6.0	0.01	
Fumarate-*1,4*-^{14}C	3.0	0.25	12.0	0.01	
α-Ketoglutarate-*1*-^{14}C	7.5	0.65	11.0	0.001	
Phenylalanine-*U*-^{14}C	1.3	0.0003	360.0	0.02	
Fluoroacetate-*2*-^{14}C	2.5	0.05	5.0	0.01	
γ-Aminobutyric-*1*-^{14}C	3.2	0.15	21.5	0.01	

[a] The ^{14}C-labeled compounds were added to ten flasks 18 hr after inoculation. All compounds were taken up from the medium by *S. showdoensis* within 10 hr. Showdomycin was isolated 32–34 hr after inoculation. The yield of showdomycin was about 6 mg/flask. All incorporation studies were repeated up to five times.

low as judged by the dilutions shown in Table I. In an attempt to determine if fluoroacetate could also be incorporated into the maleimide ring of showdomycin, fluoroacetate-*2*-^{14}C was added to the culture medium. Fluoroacetate-*2*-^{14}C was not incorporated into showdomycin. Since the *cis*-maleyl isomer is part of the metabolic product in the metabolism of phenylalanine, this aromatic amino acid was considered as the *cis*-dicarboxylic acid, four-carbon donor for the biosynthesis of the maleimide ring of showdomycin. Carbon-14 from phenylalanine-*U*-^{14}C was not incorporated into showdomycin. Maleate-*2,3*-^{14}C, the cis geometric four-carbon dicarboxylic acid isomer, was rapidly taken up by *S. showdoensis*, but ^{14}C was not incorporated into showdomycin. The incorporation of ^{14}C into the ribose moiety of showdomycin provides experimental evidence that ribose serves as the pentose for the biosynthesis of the C–C ribosyl bond of showdomycin.

Distribution of Radioactivity from the ^{14}C-Labeled Compounds Incorporated into Showdomycin. The Hofmann degradation procedure was used to isolate the carboxyl carbons of dihydroshowdomycin as CO_2. One mole of carbon dioxide is released per mole of succinimide by this degradation procedure (Clarke and Behr, 1943). Therefore, if all of the ^{14}C is located in the two carboxyl carbons of the dihydromaleimide ring of dihydroshowdomycin, only 50% of the radioactivity would be isolated as CO_2 and 50% of the radioactivity would remain in the second carboxyl group as part of the nonvolatile residue. If the ^{14}C incorporated into showdomycin is distributed uniformly in all four carbons of the dihydromaleimide ring of dihydroshowdomycin, only 25% of the ^{14}C would be isolated as CO_2 and 75% would remain in the nonvolatile residue. The role of the Krebs cycle in the biosynthesis of the maleimide ring of showdomycin will be discussed in this section. These results are based on the distribution of ^{14}C resulting from the incorporation of radioactive mono- and dicarboxylic acids into showdomycin after passage through the Krebs cycle (Table II). The data on the incorporation of glutamate-*2*-^{14}C and glutamate-*5*-^{14}C into showdomycin show that all of the ^{14}C resides in either or both of the carbonyl carbons. These findings also suggest that carbons-2 and -5 of glutamic acid must become either carbons-1 and/or -4 of the maleimide ring of showdomycin. The incorporation of glutamic acid-*2*-^{14}C or glutamic acid-*5*-^{14}C into showdomycin is proof that this amino acid cannot make a complete passage

TABLE II: Distribution of Radioactivity in Showdomycin Following the Incorporation of ^{14}C-Labeled Compounds.

Compound Incorpd	Showdomycin-^{14}C Degraded[a]	
	Distribution of ^{14}C in	
	CO_2 (%)	Nonvolatile Residue (%)
Glutamate-*2*-^{14}C	44.0	52.0
Glutamate-*5*-^{14}C	49.0	50.0
Succinate-*2,3*-^{14}C	26.0	75.0
Fumarate-*2,3*-^{14}C	24.0	76.0
Malate-*U*-^{14}C	21.0	76.0
Acetate-*1*-^{14}C	53.0	47.0
Acetate-*2*-^{14}C	25.0	75.0
Ribose-*1*-^{14}C	0	94.0

[a] Showdomycin from all incorporation studies was degraded by the Hofmann degradation procedure.

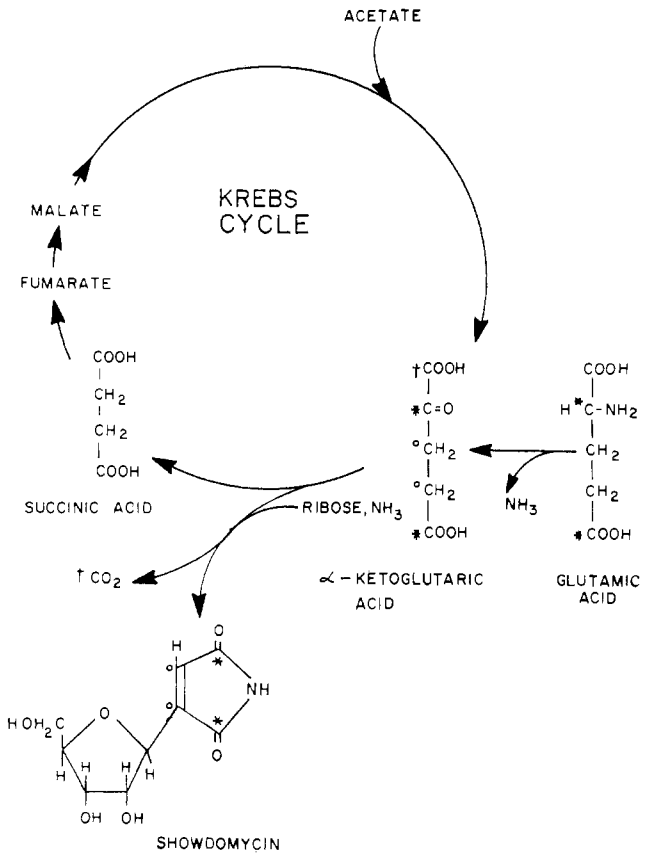


FIGURE 3: Proposed scheme for the biosynthesis of the maleimide ring of showdomycin.

through the Krebs cycle prior to its incorporation into showdomycin. The finding that 25% of ^{14}C from the succinate-*2,3*-^{14}C, fumarate-*2,3*-^{14}C, and malate-*U*-^{14}C experiments resides in each carbonyl carbon of the maleimide ring of showdomycin is in agreement with the labeling patterns for these ^{14}C-labeled acids once they enter the Krebs cycle. The distribution of ^{14}C in the maleimide ring of showdomycin from the succinate-*2,3*-^{14}C, fumarate-*2,3*-^{14}C, and malate-*U*-^{14}C experiments (Table II) and the lack of incorporation of ^{14}C from carboxyl-labeled succinate and fumarate (Table I) show that these four-carbon dicarboxylic acids are incorporated into showdomycin only after they enter the Krebs cycle. They cannot be direct precursors for the biosynthesis of the maleimide ring of showdomycin. In order to explain the labeling pattern in the maleimide ring of showdomycin, succinate and fumarate must enter the Krebs cycle and be converted into α-ketoglutarate. The α-ketoglutarate is then decarboxylated to give rise to a four-carbon dicarboxylic acid. Therefore, carbons-2, -3, -4, and -5 of a α-ketoglutarate are involved in the biosynthesis of the maleimide ring of showdomycin. It is not yet clear if carbon-2 of α-ketoglutarate becomes either carbon-1 or -4 or both of these carbons of the maleimide ring of showdomycin. Since succinate and glutamate are known precursors for the biosynthesis of γ-aminobutyric acid, the possibility that γ-aminobutyrate is the immediate precursor of the maleimide ring was studied by the addition of γ-aminobutyric acid-*1*-^{14}C to the cultures of *S. showdoensis*. The lack of incorporation of ^{14}C into showdomycin is taken as proof that γ-aminobutyric acid is not the carbon–nitrogen precursor arising from succinate or glutamate for maleimide ring formation by *S. showdoensis*.

The ^{14}C in the maleimide ring from the malate-*U*-^{14}C was distributed uniformly in all four carbons. However, malate-*U*-^{14}C cannot be considered a direct four-carbon precursor for several reasons. First, the metabolism of fumarate *via* the Krebs cycle requires its conversion to malate. The data in Table I show that the showdomycin from the fumarate-*1,4*-^{14}C experiments was not radioactive. Therefore, the ^{14}C in the carboxyl carbons of malate-*U*-^{14}C must be lost before incorporation of carbons-2 and -3 of malate into showdomycin. Second, the dilution of malate-*U*-^{14}C is twice that of acetate-*2*-^{14}C (74×10^{-3} and 36×10^{-3}, Table I). These would be the expected orders of dilution of ^{14}C in showdomycin if malate were to lose its two carboxyl carbons during its passage through the Krebs cycle.

The finding that 53% and 25% of the ^{14}C from acetate-*1*-^{14}C and acetate-*2*-^{14}C, respectively, resides in the carboxyl carbons of showdomycin is in excellent agreement with the labeling patterns expected if acetate were to contribute to the biosynthesis of the maleimide ring *via* the Krebs cycle. The known labeling patterns in citric acid from acetate-*1*-^{14}C or acetate-*2*-^{14}C explain the better incorporation of acetate-*2*-^{14}C into the maleimide ring of showdomycin.

All of the data presented in Tables I and II on the incorporation and distribution of ^{14}C from the mono- and dicarboxylic acids strongly support the notion that succinate, fumarate, malate, and acetate are converted into α-ketoglutarate which is then decarboxylated to give rise to a C_4 compound which subsequently becomes the maleimide ring of the nucleoside, showdomycin. All of the ^{14}C from ribose-*1*-^{14}C was found in the ribose moiety of showdomycin as determined by the Hofmann degradation (Table I). In addition, the specific activity of the ribose isolated when showdomycin was treated with hydrazine was the same as the showdomycin degraded.

Discussion

By selecting suitable ^{14}C-labeled mono- and dicarboxylic acids and amino acids and determining their incorporation and distribution of ^{14}C in showdomycin, the biosynthesis of the maleimide ring has been established (Tables I and II). In addition, it appears that ribose is required for the formation of the C–C riboside bond.

This is the first report on the biosynthesis of a nucleoside antibiotic in which the aglycone moiety has its biosynthetic origin with carbons other than purines or pyrimidines already present in the cell. The data presented in Table I clearly show that while carbons-2 and -3 of succinate and fumarate are incorporated uniformly into all four carbons of the maleimide ring of showdomycin, carbons-1 and -4 of succinic and fumaric acid are not incorporated. This type of labeling pattern is consistent with the known distribution of ^{14}C once these acids enter the Krebs cycle. Similarly, the incorporation and distribution of ^{14}C in the maleimide ring of showdomycin from the acetate-*1*-^{14}C and acetate-*2*-^{14}C experiments can be best explained by implementation of the Krebs cycle. If acetate-*2*-^{14}C is incorprated into the maleimide ring of showdomycin *via* the Krebs cycle, the theoretical distribution of ^{14}C should reside in each carbon of the maelimide ring. Experimentally, it was observed that 25% of the ^{14}C resided in one of the carbonyl carbons (Table II). In addition, one would expect a lower incorporation of the ^{14}C from acetate-*1*-^{14}C into the maleimide ring of showdomycin when compared to the incorporation from acetate-*2*-^{14}C if the acetate had to enter the Krebs cycle before incorporation occurred. Experimentally,

the dilutions of the showdomycin from the acetate-*1*-^{14}C and acetate-*2*-^{14}C were 93 $\times$ 10^{-3} and 36 $\times$ 10^{-3}, respectively (Table I).

Carbons-2, -3, -4, and -5 of α-ketoglutarate and/or glutamate must be directly converted to the C_4 precursor for maleimide ring formation. This conclusion is based on the observation that 100% of the ^{14}C from the glutamate-*2*-^{14}C and glutamate-*5*-^{14}C experiments resides in the carbonyl carbons of the maleimide ring of showdomycin. If the glutamate, labeled in the 2 or 5 position, were to enter the Krebs cycle all of the ^{14}C would be lost as $^{14}CO_2$ and the showdomycin would not be labeled with carbon-14.

All of the ^{14}C incorporation data presented in Tables I and II strongly support the idea that acetate, succinate, fumarate, and malate are converted to α-ketoglutarate *via* the Krebs cycle (Figure 3). Several unknown, important biochemical reactions, involving a decarboxylation of α-ketoglutarate, an unsaturation, an addition of ammonia and ribose, and maleimide ring formation, are necessary to complete the biosynthesis of showdomycin (Figure 3). The fact that all of the ^{14}C from glutamic acid-*2*-^{14}C and glutamic acid-*5*-^{14}C is located in the carbonyl carbons of the maleimide ring of showdomycin is taken as evidence that glutamate must be deaminated to form α-ketoglutarate. The latter compound is then decarboxylated and converted into the maleimide ring without any participation in the Krebs cycle. It is not known if the ^{14}C from glutamate-*2*-^{14}C or glutamate -*5*-^{14}C is located in carbon-1 or carbon-4 or both of these carbonyl carbons of the maleimide ring. Experiments are currently under way to determine if the C_4 acid formed following the decarboxylation of α-ketoglutarate is a symmetrical or asymmetrical compound. If there is an addition of the ribose moiety to α-ketoglutarate prior to decarboxylation, then all of the ^{14}C from glutamate-*2*-^{14}C or glutamate-*5*-^{14}C would reside in either carbon-1 or carbon-4 of the maleimide ring of showdomycin. The idea that α-ketoglutarate would be decarboxylated and converted into the four-carbon *cis*-dicarboxylic acid (maleic acid) appears unlikely since maleic acid-*2,3*-^{14}C is not a precursor in the biosynthesis of showdomycin. Likewise, the ^{14}C from phenylalanine-*U*-^{14}C, which is known to be metabolized to maleylacetoacetate, is not incorporated into showdomycin. During the isolation of showdomycin an additional compound was observed on the paper chromatograms. This compound inhibited the growth of *E. coli* at concentrations equivalent to showdomycin. The infrared and ultraviolet spectral properties of this antibiotic are similar to showdomycin; the R_F values on paper chromatograms in several solvents are entirely different. The structure of this new inhibitor is under investigation.

It is now possible to compare the precursors involved in the biosynthesis of the maleimide ring, the pyrrole ring, the pyrrolidone ring, the oxazolinone ring, and the glutarimide ring. The biosynthesis of the maleimide ring of showdomycin requires the decarboxylation of carbon-1 of α-ketoglutarate. This differs from the biosynthesis of the pyrrolidone ring of variotin in which glutamate is the precursor (Tanaka *et al.*, 1962). Hornemann *et al.* (1971) have reported that carbon-2 of the oxazolinone ring of indolmycin was dependent on the guanidino carbon of arginine while carbon-4 and -5 required the side chain of tryptophan. Two carbons of the pyrrole ring of tryptophan and the pyrrolopyrimidine nucleoside antibiotics have been shown to utilize ribose (Yanofsky, 1956; Suhadolnik and Uematsu, 1970). The propylhygric acid moiety of lincomycin appears to be formed from tyrosine, while the pyrrole rings of pyrrolonitrin and protoporphyrin require the alanyl side chain of tryptophan and δ-aminolevulinic acid (Witz *et al.*, 1970; Gorman and Lively, 1967; Shemin, 1970). Finally, the biosynthesis of the six-membered glutarimide ring is formed from malonyl-CoA (Vaněk *et al.*, 1967) and not α-ketoglutarate, as reported for the biosynthesis of the maleimide ring of showdomycin.

In addition to the isolation of showdomycin, two other substituted imides have been isolated as metabolic products of *Penicilium multicolor* and *Aspergillus versicolor*. They are: (1) pencolide, the substituted maleimide which has the structure *cis*-α-citraconimidocrotonic acid (Birkinshaw *et al.*, 1963; Brown and Smale, 1969) and (2) versimide, which has the structure methyl (+)-(*R*)-α-(methylsuccimido)acrylate (Brown, 1970). The biosynthesis of these compounds will be studied to determine if α-ketoglutarate is a general precursor for maleimide and succinimide ring formation.

The biosynthesis of two of the six known C-ribosyl nucleosides has now been reported. The formation of the C–C ribosyl bond in pseudouridine has been reported to proceed *via* two mechanisms: one mechanism involves the condensation of uracil with ribose 5-phosphate (Heinrikson and Goldwasser, 1964; Suzuki and Hochster, 1966); a second mechanism involves an intramolecular rearrangement of uridine residues in a polynucleotide (Johnson and Söll, 1970). From the data presented here on the biosynthesis of showdomycin, it appears that ribose forms a carbon–carbon bond with either α-ketoglutarate or a four-carbon acid arising from α-ketoglutarate.

References

Bermek, E., Krämer, W., Moenkemeyer, H., and Matthäei, H. (1970), *Biochem. Biophys. Res. Commun. 40*, 1311.

Birkinshaw, J. H., Kalyanpur, M. G., and Stickings, C. E. (1963), *Biochem. J. 86*, 237.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Brown, A. G. (1970), *J. Chem. Soc.*, 2572.

Brown, A. G., and Smale, T. C. (1969), *Chem. Commun.*, 1489.

Clarke, H. T., and Behr, H. (1943), Organic Syntheses, Collect. Vol. 2, New York, N. Y., Wiley, p 19.

Darnall, K. R., Townsend, L. B., and Robins, R. K. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 548.

Fernell, W. R., and King, H. K. (1953), *Analyst 78*, 80.

Friedemann, T. E. (1938), *J. Biol. Chem. 123*, 161.

Gordon, H. T., Thornberg, W., and Werum, L. N. (1956), *Anal. Chem. 28*, 849.

Gorman, M., and Lively, D. H. (1967), *in* Gottlieb, D., and Shaw, P. D., Ed., Antibiotics, Vol. II, New York, N. Y., Springer-Verlag, p 433.

Greenberg, D. M., and Rothstein, M. (1957), *Methods Enzymol. 4*, 652.

Heinrikson, R. L., and Goldwasser, E. (1964), *J. Biol. Chem. 239*, 1177.

Hornemann, U., Hurley, L. H., Speedie, M. K., and Floss, H. G. (1971), *J. Amer. Chem. Soc. 93*, 3028.

Johnson, L., and Söll, D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 943.

Kalvoda, L., Farkaš, J., and Šorm, F. (1970), *Tetrahedron Lett.*, 2297.

Kano, H., Nakagawa, Y., Koyama, H., and Tsukuda, Y. (1967), *Int. Congr. Heterocycl. Chem. Proc., 1st.*

Kimura, T., Kyotani, H., and Ozaki, M. (1968), Annual Report of Shionogi Research Laboratory, No. 18, 23.

Komatsu, Y., and Tanaka, K. (1970), *Agr. Biol. Chem. (Tokyo) 34*, 891.

Maryanka, D., and Johnston, I. R. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 125.
Matsuura, S., Shiratori, O., and Katagiri, K. (1964), *J. Antibiot. (Tokyo) 17A*, 234.
Nakagawa, Y., Kano, H., Tsukuda, Y., and Koyama, H. (1967), *Tetrahedron Lett.*, 4105.
Nishimura, H., Mayama, M., Komatsu, Y., Katō, H., Shimaoka, N., and Tanaka, Y. (1964), *J. Antibiot. (Tokyo) 17A*, 148.
Roy-Burman, P. (1970), *Recent Results Cancer Res. 25*, 80.
Seto, H., Otake, N., and Yonehara, H. (1968), *Agr. Biol. Chem. (Tokyo) 32*, 1299.
Shemin, D. (1970), *Naturwissenschaften 57*, 185.
Suhadolnik, R. J. (1970), Nucleoside Antibiotics, New York, N. Y., Wiley.
Suhadolnik, R. J., and Ramer, R. M. (1968), 156th National Meeting of the American Chemical Society, Atlantic City, N. J.
Suhadolnik, R. J., and Uematsu, T. (1970), *J. Biol. Chem. 245*, 4365.
Suzuki, T., and Hochster, R. M. (1964), *Can. J. Biochem. 44*, 259.
Tanaka, N., Sashikata, K., and Umezawa, H. (1962), *J. Gen. Appl. Microbiol. 8*, 192.
Townsend, L. B., and Robins, R. K. (1969), *J. Heterocycl. Chem. 6*, 459.
Tsai, C. M., Holmberg, N., and Ebner, K. E. (1970), *Arch. Biochem. Biophys. 136*, 233.
Uematsu, T., and Suhadolnik, R. J. (1971), *Biochemistry*, manuscript in preparation.
Vaněk, Z., Cudlin, J., and Vondracek, M. (1967), *in* Gottlieb, D., and Shaw, P. D., Ed., Antibiotics, Vol. II, New York, N. Y., Springer-Verlag, p 223.
Witz, D. F., Hessler, E. J., and Miller, T. L. (1970), 160th National Meeting of American Chemical Society, Atlantic City, N. J.
Yanofsky, C. (1956), *Biochim. Biophys. Acta 20*, 438.

Multiple Forms of Hepatic Adenosine 3′:5′-Monophosphate Dependent Protein Kinase*

Lee-Jing Chen and Donal A. Walsh†

ABSTRACT: Cytoplasmic hepatic protein kinase is resolved into three fractions by chromatography on DEAE-Sephadex. Two of these fractions exhibit a sedimentation constant = 6.8 S, whereas the third component is 4.0 S. The activity of each 6.8S fraction is stimulated by adenosine 3′:5′-monophosphate (cAMP) whereas that of the smallest protein kinase (4.0 S) is independent of this nucleotide. The 6.8S protein kinase that is present in the highest proportion can be dissociated to yield two protein kinase fractions, each of 4.0 S, whose activity is independent of cAMP, and neither of which is identical with the 4.0S protein kinase isolated by DEAE-Sephadex chromatography. On the basis of reconstitution experiments and on work recently presented by several laboratories it is interpreted that each 4.0S component represents a species of catalytic subunit of cAMP-dependent protein kinase, whereas the 6.8S protein is a holoenzyme(s) composed of catalytic and nucleotide-binding regulatory subunits.

Adenosine 3′:5′-cyclic monophosphate has been shown to be the intracellular second messenger of a number of hormones that control hepatic metabolism (Sutherland and Rall, 1960; Robison *et al.*, 1968). The stimulation of hepatic glycogenolysis, gluconeogenesis, and lipolysis by glucagon and epinephrine can be mimicked by the action of cAMP.[1] In addition, the elevation of tissue levels of cAMP promoted by the action of either of these hormones precedes all other established metabolic events. Insulin, which promotes the reversal of each of these metabolic processes, has been shown to decrease the level of cAMP in liver (Exton *et al.*, 1966).

With the discovery of the cAMP-dependent protein kinase from skeletal muscle (Walsh *et al.*, 1968), it has been suggested that the initial step of many of the actions of this nucleotide is the activation of this enzyme. This enzyme has been found in a wide variety of tissues (Kuo and Greengard, 1969) and utilizes several different proteins as substrates. The enzyme catalyzes the phosphorylation of phosphorylase *b* kinase (Walsh *et al.*, 1971a) and glycogen synthetase (Schlender *et al.*, 1969) from skeletal muscle, hormone-sensitive lipase from adipose tissue (Corbin *et al.*, 1970; Huttunen *et al.*, 1970), histone from liver (Langan, 1968), RNA polymerase from *Escherichia coli* (Martelo *et al.*, 1970), and microtubules from brain (Goodman *et al.*, 1970).

The question then arises whether a single protein kinase initiates the various hepatic metabolic functions that are mediated by cAMP, or alternatively whether a number of isozymes are involved in its diversity of action. It was originally proposed by Brostrom *et al.* (1970) on the basis of studies of reversible inactivation and has since been demonstrated by several laboratories (Gill and Garren, 1970; Tao *et al.*, 1970; Kumon *et al.*, 1970; Reimann *et al.*, 1971a) that cAMP-de-

* From the Department of Biological Chemistry, University of California, Medical School, Davis, California 95616. *Received May 26, 1971.* Supported by National Institutes of Health Grant AM13613. A preliminary report has been given at the Federation of American Societies of Experimental Biology Meeting, San Francisco, Calif., June 1971.

† Established Investigator of the American Heart Association.

[1] Abbreviations used are: cAMP, adenosine 3′:5′-cyclic monophosphate; *pI*, isoelectric point.