STUDIES ON THE BIOSYNTHESIS OF ANTIMYCIN A
I. INCORPORATION OF ^{14}C-LABELED METABOLITES INTO THE 3-FORMAMIDOSALICYL MOIETY

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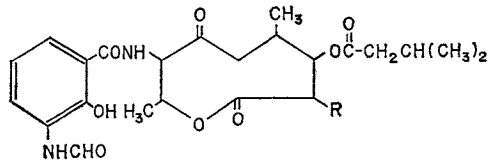
The incorporation of ^{14}C-labeled glucose, shikimate, benzoate, L-phenylalanine, and DL-tryptophan into antimycin A by *Streptomyces* AY-B-265 was examined. The most efficient incorporation was observed with L-phenylalanine-^{14}C (U) (0.18 %) and variously ^{14}C-labeled DL-tryptophan (up to 2.0 %). Analysis of degradation products from ^{14}C-labeled antimycin produced from benzene-ring-^{14}C-DL-tryptophan and L-phenylalanine-^{14}C (U) showed a high proportion of the isotope present in the 3-aminosalicylic acid residue. Incorporation of 2-ring-^{14}C-DL-tryptophan followed by degradation of the antimycin demonstrated that carbon-2 of the indole ring was incorporated into the 3-formamido carbonyl of antimycin in high yield. Tryptophan dilution studies suggested a fairly large tryptophan pool in cells transferred to resting medium for incorporation studies.

Antimycin A (I), an antibiotic complex, first isolated by LEBEN and KEITT[1] in 1948 from a soil organism of the genus *Streptomyces* consists of at least seven components, the most abundant being antimycin A_1 and A_3[2,3]. CHANCE and WILLIAMS[4] in 1955 demonstrated that the inhibition of electron transfer by antimycin occurred specifically between cytochromes b and c. However, neither a majority of bacteria[5] nor antimycin-producing *Streptomyces* sp.[6] containing these cytochromes are sensitive to the antibiotic. Antimycin A has been found to be toxic toward a number of pathologic fungi,[1,7] however, its principle commercial application is as a fish control vehicle[8,9,10].



Antimycin A_1 R = *n*-Hexyl
Antimycin A_3 R = *n*-Butyl

Studies on the biosynthesis of the antimycin A complex have been very limited. Although the relationship of acetate and pyruvate to the biosynthetic routes to the branched aliphatic portion of the dilactone have been reported by BIRCH *et al.*[11], the biosynthetic pathway for the aromatic moiety has not been illucidated. The incorporation of several potential ^{14}C-labeled aromatic precursors into antimycin are reported in this paper.

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Experimental

Materials

Glucose-^{14}C (U) (209 mc/mmole), shikimic-^{14}C (U) acid (1.86 mc/mmole), benzoic-1-^{14}C-acid (2.33 mc/mmole), and L-phenylalanine-^{14}C (U) (369 mc/mmole) were purchased from New England Nuclear, Boston, Massachusetts. DL-Tryptophan [benzene ring-^{14}C (U)] (100 mc/mmole) was purchased from Amersham-Searle (Arlington Heights, Illinois) and DL-tryptophan-2-ring-^{14}C (29.1 mc/mmole) from CalAtomic (Los Angeles, California). Dr. LaVel HENDERSON, of the University of Minnesota, Minneapolis, Minnesota, kindly supplied DL-tryptophan-7^{a}-^{14}C (153 μc/mmole).

Ninhydrin spray used to locate amino acid spots on TLC plates was a 0.2% solution of ninhydrin in a mixture of ethanol, acetic acid and 2,4,6-collidine (50 : 10 : 2). Immediately before use, 25 parts of the stain were mixed with 1.5 parts of a 1% solution of Cu $(NO_3)_2 \cdot 3\,H_2O$ in absolute ethanol.

Streptomyces strain AY-B-265[8] used in these studies was kindly provided by Dr. CLAUDE VEZINA, Department of Microbiology, Ayerst Research Laboratories, Montreal, Canada.

Methods

For liquid scintillation studies, a Beckman CPM-100 Liquid Scintillation System (Beckman Instruments, Palo Alto, California) was used. The liquid scintillation fluid was prepared by adding 42 ml Spectrafluor PPO-POPOP concentrate (Nuclear Chicago, Des Plaines, Illinois) to 1 liter toluene yielding a final concentration of 4 g PPO and 50 mg POPOP per liter. Samples to be counted were dissolved in 10 ml of this fluid.

Preparation, inoculation and incubation of liquid cultures.

Liquid medium[6] was prepared by adding 890 ml distilled water to 7.5 g glycine, 1.0 g DL-tryptophan, 1.0 g DL-alanine, 5.0 g $K_2HPO_4 \cdot 3\,H_2O$, 5.0 g NaCl and 0.24 g $MgSO_4$. The mixture was heated to bring the tryptophan into solution, cooled and 10 ml of a solution which contained 10% $ZnSO_4 \cdot 7\,H_2O$, 0.8% $MnCl_2 \cdot 4\,H_2O$ and 0.1% $CoCl_2 \cdot 6\,H_2O$ added. The pH was adjusted to 7.2 with 1 N HCl. Glucose (20 g/100 ml) was dissolved in water; the solutions autoclaved separately and combined at the time of inoculation.

An aqueous suspension of organisms which had been grown on agar slants was used to inoculate broth cultures. The suspension was prepared by layering 1~2 ml sterile distilled water over the surface of each slant and scraping the white growth with the aid of an inoculating needle. Suspensions were pooled in a sterile flask. To insure a uniform inoculum to each culture, the turbidity of the suspension was determined and the amount of suspension to be introduced into each culture was measured accordingly. For example, when an aliquot of the suspension diluted 1 : 10 gave an absorbance reading of 0.15~0.16 at 600 mμ (13 mm round cuvette, Bausch and Lomb Spectronic 20), 3.5 ml of the undiluted suspension was introduced into 100 ml of the 20% sterile glucose solution; the mixture was then dispensed in 2.5 ml aliquots into each 22.5 ml of liquid medium.

Liquid cultures were shaken at 25°C in 2-liter shake flasks, 250-ml shake flasks or 25 $\times$200 mm culture tubes, on an oscillating shaker (120 oscillations/minute, 1$^1/_2$ inch (3.8 cm) stroke). Tube cultures were placed at a 15~20° angle.

Growth in resting cell medium.

Glucose, tryptophan, and $CaCO_3$ were omitted from the medium used for resting cell cultures. Cells from 72-hour cultures were separated by centrifugation and washed twice with distilled water before being transferred to the resting medium. Each 25 ml of medium was then supplemented with a ^{14}C compound in such quantity as to introduce 1×10^6 dpm. The inoculated cultures were then incubated at 25°C and extracted after 2~3 days shaking.

Extraction of Antimycin

Antimycin was quantitatively extracted by a modification of the method of NAKAYAMA, *et al.*[7] The entire culture was adjusted to pH 1.0 and extracted three times with dichloro-

methane. The extracts were pooled and evaporated to dryness. The residue was dissolved in absolute ethanol and applied to a 20×20 cm silica gel preparative plate (2 mm thickness), which was developed in 25% methanol in $CHCl_3$ against an antimycin A reference. The desired band was located by means of its fluorescence under uv light, scraped and eluted with ethanol. The yield of antimycin was calculated from absorbance at 320 mμ ($\varepsilon=4.8\times10^3$)[12].

Purified antimycin samples were degraded to deformylantimycin by the method described by LIU, *et al.*[12] Complete degradation and isolation of the ^{14}C-labeled 3-aminosalicylic acid was carried out using the methods described by TENER, *et al.*[13]

The antimycin to be degraded for analysis of ^{14}C-formyl activity was placed in 3 ml ethanol-HCl (2 : 1) and boiled 5 minutes under a reflux condenser connected by tubing to a flask of 0.5 N NaOH. A weighed amount of unlabeled sodium formate was added prior to refluxing for the degradation of antimycin A_1.

After cooling, the tubing and condenser were rinsed with water back into the reaction flask, the contents of which were then extracted with $CHCl_3$. The chloroform layer was washed 5 times with water, dried and saved for liquid scintillation studies. The washings were recombined with the aqueous layer, adjusted to pH 7.5 with 5% NaOH and evaporated to dryness. In the degradation of antimycin A_3, unlabeled sodium formate was added at this point.

The residue was then dissolved in 5 ml of concentrated HCl and reduced with magnesium powder[14]. When the reaction mixture no longer showed evoltion of bubbles, water was added to dilute the HCl to around 2 N. Excess 2,4-dinitrophenylhydrazine was added and the mixture allowed to stand overnight at 4°C. The mixture was extracted repeatedly with $CHCl_3$ and the combined extracts evaporated. The residue, dissolved in $CHCl_3$ was applied to a TLC plate and chromatographed in $CHCl_3$ against formyl-2,4-dinitrophenylhydrazone. The desired band was scraped and eluted with $CHCl_3$. The yield of formyl-2,4-dinitrophenylhydrazone was determined spectrophotometrically at 405 mμ, using weighed formyl-2,4-dinitrophenylhydrazone standards. The eluate was dried and ^{14}C-activity determined in a scintillation counter.

Results

^{14}C-Precursor Studies

Actively growing *Streptomyces* cultures were transferred to resting cell medium which was supplemented with a ^{14}C-labeled compound, shaken three days and the antimycin isolated, purified and ^{14}C activity determined. The antimycin A_1 and A_3 yields and specific activities are summarized in Table 1. Variously ^{14}C-labeled DL-tryptophan showed the highest incorporation levels followed by L-phenylalanine-^{14}C (U). Glucose-^{14}C (U), shikimic acid-^{14}C (U) and benzoic acid-1-^{14}C were incorporated to a much lower extent.

^{14}C-Incorporation of Potential Precursors of the Aromatic Portion of Antimycin A

^{14}C-Antimycin A was isolated from cultures containing either L-phenylalanine-^{14}C (U) or DL-trytophan-[benzene ring-^{14}C (U)] and degraded[13]. The threonine, lactone and 3-aminosalicylic acid were separated and ^{14}C-activity determined. The results shown in Table 2 indicated that the benzene ring of tryptophan was very efficiently incorporated into the aromatic portion of antimycin. L-Phenylalanine-^{14}C (U) was also incorporated into the aromatic fraction, however, a low recovery yield precluded a clear interpretation of the results.

Incorporation of DL-Tryptophan-2-ring-^{14}C into the 3-Formamido Position of Antimycin A

Table 1. Efficiency of incorporation of ^{14}C-metabolites into antimycin

Source of label	Supplied per culture			Yield per culture				% Incorporation[b]
				A_1		A_3		
	dpm	mmole	sp. act.[a]	mmoles	sp. act.[a]	mmoles	sp. act.[a]	
Glucose-^{14}C (U)	4.2×10^{6}	9.0×10^{-6}	4.6×10^{11}	5.5×10^{-5}	1.2×10^{7}			0.017[c]
Benzoic-1-^{14}C	2.2×10^{6}	4.3×10^{-3}	5.1×10^{9}	2.9×10^{-5}	3.0×10^{6}			0.004[c]
Shikimic-^{14}C(U)	4.1×10^{5}	1.0×10^{-4}	4.0×10^{9}	1.2×10^{-5}	1.1×10^{6}	2.3×10^{-5}	1.1×10^{6}	0.011
L-Phenylalanine-^{14}C (U)	4.1×10^{5}	5.0×10^{-7}	8.2×10^{11}	3.1×10^{-5}	1.6×10^{7}	1.5×10^{-5}	1.7×10^{6}	0.126
DL-Tryptophan-7[a]-^{14}C	4.1×10^{5}	1.2×10^{-3}	3.4×10^{8}	1.9×10^{-5}	2.2×10^{7}	1.8×10^{-5}	1.8×10^{7}	0.18
DL-Tryptophan (benzene ring)-^{14}C (U)	1.0×10^{6}	4.5×10^{-6}	2.2×10^{11}	2.3×10^{-5}	5.0×10^{8}	1.9×10^{-5}	4.9×10^{8}	1.8
DL-Tryptophan 2-ring-^{14}C	1.0×10^{6}	1.6×10^{-5}	6.4×10^{10}	2.6×10^{-5}	2.1×10^{8}	2.6×10^{-5}	2.6×10^{8}	1.2

a. Specific activity expressed as dpm/mmole.
b. Calculated by dividing total dpm supplied into total dpm in extracted antimycin A_1 and A_3.
c. % Incorporation into A_1.

Table 2. Antimycin labeling pattern from DL-tryptophan and L-phenylalanine incorporation

Precursor	Antimycin	Degraded (dpm)	Recovered (dpm)		
			Lactone	3-Aminosalicylic acid	Threonine
DL-Tryptophan (benzene ring)-^{14}C(U)	A_3	4.45×10^{4}	152	2.1×10^{4}	381
	A_1	2.7×10^{4}	97	4.6×10^{3}[a]	103
L-Phenyl alanine-^{14}C(U)	A_1	4.1×10^{4}	110	3.3×10^{3}[a]	167
	A_1	2.5×10^{8}	0[b]	185	0[b]

a. Some material lost during autoclaving as shown by blackened residue.
b. Insignificantly above background.

Table 3. Incorporation of 2-^{14}C-tryptophan into 3-formamido position of antimycin

	Antimycin degraded		% Original activity $CHCl_3$ soluble[a]	2,4-Dinitrophenylhydrazone	3-Formamido-carbonyl[b]
	mmoles	dpm/mole		dpm/mmole	dpm/mmole
A_1[c]	1.75×10^{-5}	2.6×10^{8}	10.4	8.2×10^{4}	5.3×10^{7}
A_3[d]	1.8×10^{-4}	2.8×10^{8}	2.1	9.5×10^{4}	4.7×10^{7}

a. Unreacted antimycin plus deformylantimycin.
b. Represents minimum specific activity calculated on the basis of 100% recovery of formyl group as formic acid.
c. Unlabeled sodium formate (0.103 mmole) added prior to hydrolysis.
d. Unlabeled sodium formate (0.085 mmole) added to hydrolysate.

The antimycin A_1 and A_3 isolated after DL-tryptophan-2-ring-^{14}C incorporation (Table 1) was deformylated[13] and the deformyl antimycin separated from the aqueous formic acid containing phase. After reduction[14] and dinitrophenylhydrazine treatment of the aqueous phase, the formyl-2,4-dinitrophenylhydrazone was counted and a minimum specific activity for the 3-formamidocarbonyl calculated. The results shown in Table 3 indicated that the labeling of antimycin was limited almost exclusively to the 3-formamido carbonyl. TENER, *et al.*[14] reported a yield of 90% for this deformylation method. This value is in good agreement with our results for antimycin A_1 and we approached 98% deformylation in the case of antimycin A_3 degradation (Table 3).

Table 4. Effect of unlabeled DL-tryptophan on ^{14}C-tryptophan incorporation in antimycin[a]

DL-Tryptophan	Antimycin[b]			
	A_1		A_3	
mmoles/culture	mmoles	dpm/mmole	mmoles	dpm/mmole
None	2.2×10^{-5}	4.8×10^{8}	2.24×10^{-5}	4.8×10^{8}
1.56×10^{-4}	3.0×10^{-5}	3.1×10^{8}	2.3×10^{-5}	2.7×10^{8}
1.56×10^{-3}	2.2×10^{-5}	1.5×10^{8}	2.5×10^{-5}	1.1×10^{8}
1.56×10^{-2}	2.2×10^{-5}	4.3×10^{7}	1.9×10^{-5}	4.1×10^{7}

a. Each culture contained 1.56×10^{-5} (1×10^{6} dpm) DL-tyrptophan-2-^{14}C.
b. Represents amount extracted per culture.

Tryptophan Dilution Studies

DL-Tryptophan-2-^{14}C (1.56×10^{-5} mmoles, 1×10^{6} dpm) was added to each of 16 culture tubes and unlabeled DL-tryptophan was added to 3 sets of 4 tubes in increasing amounts so as to dilute the labeled tryptophan 10-, 100-, and 1,000-fold. Inoculated resting cell medium was then added to each of the tubes. After two days incubation, antimycin having specific activities indicated in Table 4 was isolated.

The results showed that, although the specific activity of the antimycin decreased with increasing amounts of unlabeled tryptophan, a 1,000-fold dilution of the labeled amino acid brought about only a 10-fold decrease in the specific activity of the antibiotic. It was also seen that the amount of tryptophan added did not substantially increase the amount of antimycin produced.

Discussion

A preliminary screening of possible precursors to the aromatic moiety of antimycin suggested that the shikimate pathway for aromatic synthesis was functional. The aromatic amino acids phenylalanine and tryptophan were found to be more direct precursors than shikimate as seen from enhanced incorporation, particularly in the case of tryptophan (Table 1). Degradation of ^{14}C-antimycin followed by ^{14}C-analysis of the 3-amino salicylic acid residue clearly showed that the benzene ring portions of phenylalanine and tryptophan were the main source of incorporation. The fact that phenylalanine incorportion was only 10 % of that observed for benzene ring-labeled tryptophan does not necessarily rule out phenylalanine as a direct precursor to the aromatic portion of antimycin, however, it is possible that phenylalanine may have served as a precursor of tryptophan through a chorismic acid intermediate[15].

With tryptophan serving as an antimycin precursor, it seemed possible that the C-2 of the indole ring might serve as a precursor of the 3-formamido carbonyl group of antimycin in some manner analogous to the formation of the N-formyl group of N^1-formyl kynurenine derived by oxidative cleavage of tryptophan by the enzyme tryptophan pyrrolase. The efficient incorporation of 2-ring-^{14}C-tryptophan into the 3-formamido carbonyl of antimycin (Table 3) confirmed the above hypothesis. However, preliminary attempts to demonstrate tryptophan pyrrolase activity in this *Streptomyces* sp. have not as yet proven successful. Further investigation of the exact nature of the indole ring cleavage is clearly required.

If tryptophan was a direct precursor to the 3-formamidosalicyl portion of antimycin, the specific activity of labeled tryptophan should have been readily diluted out by addition of unlabeled tryptophan. The effect of tryptophan dilution was not dramatic. However, the results obtained in these dilution experiments (Table 4) may be explained if one considers that the cells, which had been grown in a medium containing 1 mg/ml of tryptophan,

may have had a fairly large intracellular tryptophan pool at the time of transfer to resting medium. The addition of 1.56×10^{-2} mmoles of tryptophan to the medium caused a dilution of the specific activity of the antimycin by a factor of 10. If this indicated a total pool of 1.56×10^{-3} mmoles of tryptophan in the transferred cells, the dilution pattern becomes more intelligible. The total radioactivity added per culture was 1×10^{6} dpm. With the estimated pool size taken into consideration, this would bring the specific activity of the total tryptophan to 6×10^{8} dpm/mmole in the undiluted tubes, since the quantity of added labeled material was insignificant (1.56×10^{-5} mmoles). Assuming 100 % assimilation of the added material, or at least free mixing with intracellular pools, the tryptophan pool in the cells to which the 1,000-fold dilution of label had been added would now have a specific activity of 6.0×10^{7} dpm/mmole.

Both figures agree well with the specific activity of the antimycin extracted from the undiluted and diluted cultures, respectively 4.8×10^{8} and 4.1×10^{7} dpm/mmole. The argument explains the slow falling off of specific activity in the intermediate dilutions. The large pool of tryptophan would also explain why there was no increase in amount of antimycin with increasing amounts of tryptophan.

Acknowledgements

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References

1) LEBEN, C. & G. W. KEITT: An antibiotic substance active against certain phytopathogens. Phytopath. 38: 399~906, 1948
2) KLUEPFEL, D.; S. SEGHAL & C. VEZINA: Antimycin A components. I. Isolation and biological activity. J. Antibiotics 23: 75~80, 1969
3) VAN TAMELEN, E. E.; J. P. DICKIE, M. E. LOOMANS, R. S. DEWEY & F. M. STRONG: The chemistry of antimycin A. X. Structure of the antimycins. J. Am. Chem. Soc. 83: 1639~1646, 1961
4) CHANCE, B. & G. R. WILLIAMS: Respiratory enzymes in oxidative phosphorylation. IV. The respiratory chain. J. Biol. Chem. 217: 429~438, 1955
5) SMITH, L.: Bacterial cytochromes. Difference spectra. Arch. Biochem. Biophys. 50: 299~314, 1954
6) REHACEK, Z.; M. RAMANKUTTY & J. KOZOVA: Respiratory chain of antimycin A producing *Streptomyces antibioticus*. Appl. Microbiol. 16: 23~32, 1968
7) NAKAYAMA, K.; F. OKAMOTO & Y. HARADA: Antimycin A. Isolation from *Streptomyces kitazawaensis* and its activity against rice plant fungi. J. Antibiotics, Ser. A 9: 63~66, 1956
8) VEZINA, C.: Antimycin A, a teleocidal antibiotic. Antimicr. Agents & Chemoth. -1966: 757~766, 1967
9) DERSE, P. H. & F. M. STRONG: Toxicity of antimycin A to fish. Nature 200: 600~601, 1963
10) WALKER, C. R.; R. E. LENNON & B. L. BERGER: Investigation of fish control. Preliminary observations on toxicity of antimycin A to fish and other aquatic animals. U. S. Wildlife Service Circular No. 186, 1964
11) BIRCH, A. J.; D. W. CAMERON, Y. HARADA & R. W. RICKARDS: Studies in relation to biosynthesis. XXV. A preliminary study of the antimycin A complex. J. Chem. Soc. 1962: 303~305, 1962
12) LIU, W.; E. E. VAN TEMELEN & F. M. STRONG: The chemistry of antimycin A. VIII. Degradation of antimycin A. J. Am. Chem. Soc. 82: 1652~1654, 1960
13) TENER, G. M.; F. M. BUMPUS, B. R. DUNSHEE & F. M. STRONG: The chemistry of antimycin A. II. Degradation studies. J. Am. Chem. Soc. 75: 1100~1104, 1953
14) STRONGR, F. M.: Structure and properties of antimycin A, from "Topics in Microbial Chemistry", p. 12. John Wiley and Sons, Inc., New York, N. Y. 1958
15) WHITE, A.; P. HANDLER & E.L. SMITH: "Principles of Biochemistry", p. 574. McGraw Hill Book Co., New York, N.Y., 1968