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Biosynthesis of Anthramycin. Determination of the Labeling Pattern by the Use of Radioactive and Stable Isotope Techniques

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Abstract: The building blocks for anthramycin, an antitumor antibiotic produced by a strain of *Streptomyces refuineus*, have been shown to be L-tryptophan, probably via 3-hydroxyanthranilic acid, L-tyrosine which loses two of its aromatic carbons, and L-methionine which contributes two methyl groups. While one of the two methyl groups is transferred intact, the other loses all of its hydrogens and becomes the carbonyl of an amide group. A mechanism involving extradiol cleavage of Dopa is proposed on the basis of double labeling and stable isotope experiments. A general scheme for the biosynthetic origin of the C_3-proline moieties of anthramycin, lincomycin A, and sibiromycin and the C_2-proline moieties of tomaymycin and lincomycin B is proposed.

Anthramycin (Ia) is an antitumor antibiotic produced by *Streptomyces refuineus* var *thermotolerans*.[2] The structure and stereochemistry of anthramycin have been elucidated[3,4] and the total synthesis of anthramycin has been reported.[5] Anthramycin has been shown to possess antitumor,[6] antibiotic,[6] amebicidal,[6] and chemosterilant properties.[7] All the biological properties of the antitumor agent can be attributed to the effect of anthramycin on nucleic acid biosynthesis. Anthramycin appears to act by virtue of its ability to bind to DNA and therefore interfere with the function of DNA.[8-10] Anthramycin causes inhibition of DNA and RNA synthesis in both bacterial and mammalian cells. However, the synthesis of RNA is most powerfully affected.[11]

The biosynthetic origin of the non-4-methyl-3-hydroxyanthranilic acid part of anthramycin was of prime interest in this investigation. A similarly structured element can be found in tomaymycin (II),[12] sibiromycin (III),[13] and the lincomycin group of antibiotics (IVa,b).[14] (See Chart I.) This structural similarity might suggest a common biogenetic origin for all of these antibiotics. The biogenetic origin of the propylproline group of lincomycin A has been shown to be tyrosine and two one-carbon units via methionine.[15] In this case the *N*-methyl and terminal methyl groups were those derived from methionine. In the case of tomaymycin, we have established that this antibiotic is derived in an analogous way to anthramycin.[16]

In this paper we wish to report on the determination of the biogenetic origin of anthramycin. Some of these results have been communicated in preliminary form.[17]
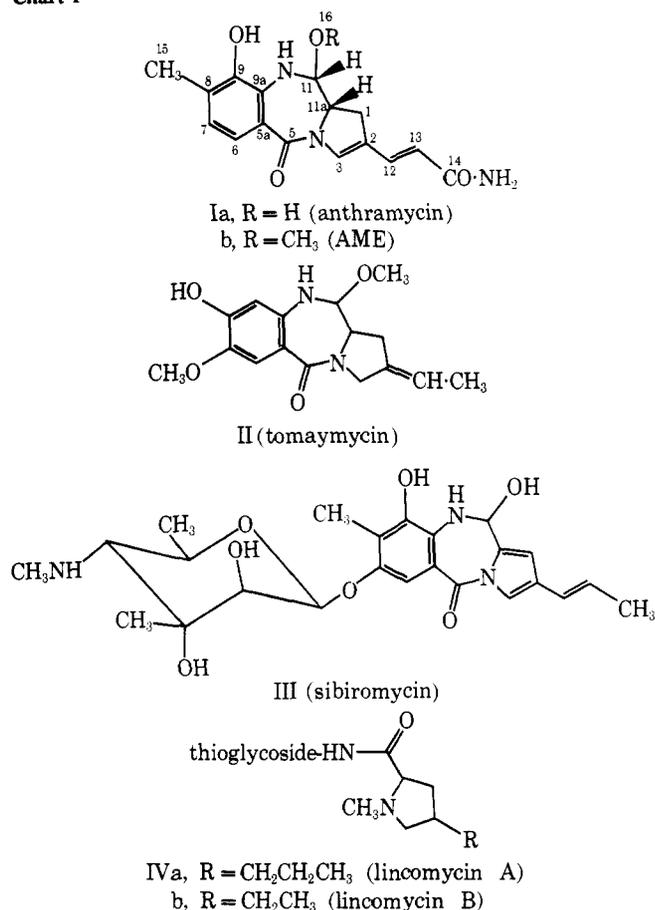
Results

Biosynthetic experiments were carried out with shake cultures of the anthramycin-producing *S. refuineus* strain NRRL 3143 grown in a complex medium. The amount of anthramycin produced at the time of harvest was about 80 μg/ml, as determined by a spectroscopic method.[18] The antibiotic was isolated from the culture medium by extraction with butanol and further purified if necessary by countercurrent distribution.

The time course of anthramycin production was followed by measuring spectroscopically the amount of anthramycin produced and the incorporation of radioactive methionine into the antibiotic at different times during the culture period. As shown in Figure 1, maximum production of anthramycin occurred at 15 hr and maximum incorporation of methionine into anthramycin occurred when the precursor was added at 12 hr.

The efficiency of incorporation of potential radioactive precursors into anthramycin was evaluated as follows. The thin-layer chromatograms of the methanol solubilized extracts were scanned for radioactivity and the areas under the peaks were integrated to obtain the percentage of the radioactivity of the extract residing in anthramycin. From this value and from the total radioactivity of the methanol extract, the total radioactivity incorporated into anthramycin was calculated, which was related to the total radioactivity of the precursor fed to give the percentage incorporation. This latter figure was also obtained independently by recrystallizing to constant specific activity those samples of

Chart I

Ia, R = H (anthramycin)
b, R = CH₃ (AME)

II (tomaymycin)

III (sibiromycin)

thioglycoside-HN

IVa, R = CH₂CH₂CH₃ (lincomycin A)
b, R = CH₂CH₃ (lincomycin B)

anthramycin which appeared to be appreciably labeled from fed compounds.

Using these conditions a number of possible precursors were fed to *S. refuineus* cultures and the incorporations given in Table I were obtained. It is apparent that the aromatic ring of tryptophan but not its side chain, L-tyrosine, L-Dopa, and the methyl group of L-methionine are utilized efficiently for anthramycin synthesis. A number of other compounds were fed to the organism (Table I, experiments 1, 2, 4, 5, 7, and 8) but were not detectably incorporated.

The role of tryptophan, methionine, and tyrosine as anthramycin precursors was substantiated by degradation of the labeled samples of the antibiotic. As outlined in Scheme I, acid hydrolysis yields 4-methyl-3-hydroxyanthranilic acid
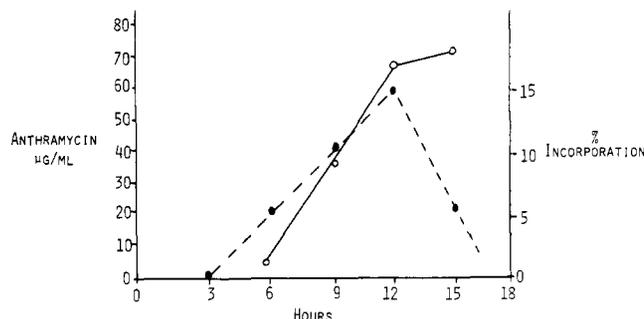
Figure 1. Time course of the production of anthramycin and incorporation of L-[Me-¹⁴C]methionine into anthramycin by *S. refuineus*. At the times indicated the cultures were incubated with 2.2 × 10⁷ dpm of L-[Me-¹⁴C]methionine for a period of 3 hr: O—O, µg/ml of anthramycin; ●- - -●, % incorporation.

Scheme I. Degradation of Anthramycin
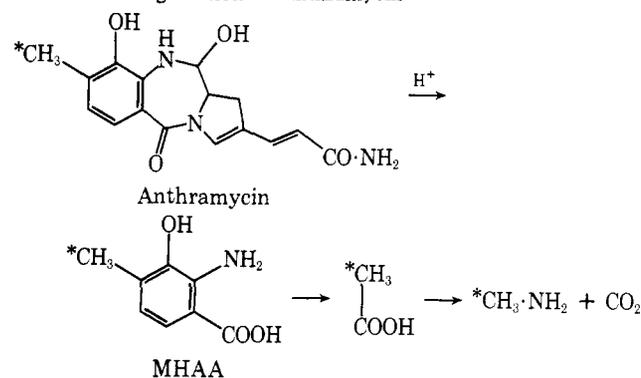
(MHAA) which was extracted from the reaction mixture and crystallized. The purity of MHAA was checked by TLC. After dilution with carrier material it was degraded further by Kuhn Roth oxidation,[19] and the resulting acetic acid was subjected to Schmidt degradation.[20] The data obtained are presented in Table II. The results of these degradations show that whereas all the radioactivity from tryptophan was found in MHAA, only 42% of that from methionine and none of that from the tyrosine were found in this compound. Subsequent degradation of the MHAA labeled from methionine showed that all the radioactivity in MHAA was located in the methyl group. The origin of the MHAA from tryptophan and methionine is analogous to that in actinomycin biosynthesis[21,22] and probably involved a diversion of the well-known metabolic pathway leading to

Table I. Incorporation of Labeled Substrates into Anthramycin by *S. refuineus*

Expt		Quantity fed, µmol	Radioactivity fed, dpm	Radioactivity in butanol extract, % of total fed	% radioactivity of butanol extract in anthramycin as determined by scanning	Incorporation into anthramycin, %
1	[1-¹⁴C] Acetic acid	3.2	2.80 × 10⁷	5.8	a	<0.1
2	[4-¹⁴C]-δ-Aminolevulinic acid	0.7	2.14 × 10⁷	7.3	a	<0.1
3	L-[1-¹⁴C] Dihydroxyphenylalanine	0.1	3.2 × 10⁶	35.6	62.5	22.1
4	D-[1-¹⁴C] Glucose	0.1	4.46 × 10⁶	2.3	a	<0.3
5	D-[6-¹⁴C] Glucose	0.1	7.37 × 10⁶	3.7	a	<0.2
6	L-[Me-¹⁴C] Methionine	0.3	7.8 × 10⁶	26.6	62.0	16.4
7	L-[U-¹⁴C] Phenylalanine	0.1	2.2 × 10⁷	2.3	a	<0.1
8	L-[U-¹⁴C] Proline	1.0	1.7 × 10⁷	0.8	a	<0.1
9	DL-[7a-¹⁴C] Tryptophan	13.4	2.2 × 10⁷	42.8	31.8	13.7
10	L-[G-³H] Tryptophan	10.0	5.7 × 10⁷	7.6	14.4	1.1
11	L-[Ala-3-¹⁴C] Tryptophan	14.9	5.24 × 10⁶	15.2	a	<0.3
12	L-[U-¹⁴C] Tyrosine	0.1	2.2 × 10⁷	13.2	85.5	11.7
13	L-[1-¹⁴C] Tyrosine	5.7	2.06 × 10⁷	20.0	51.8	10.4

a Below limit of detection.

Table II. Degradation of Anthramycin

	% radioactivity of anthramycin recovered in			
Precursor	MHAA[a]	CH₃COOH	CH₃NH₂	CO₂
L-[7a-¹⁴C] Tryptophan	104	NE[b]	NE[b]	NE[b]
L-[U-¹⁴C] Tyrosine	0.3	NE[b]	NE[b]	NE[b]
L-[Me-¹⁴C] Methionine	42.4	42.2	40.8	0.1

[a] MHAA = 4-methyl-3-hydroxyanthranilic acid. [b] NE, not examined.

Table III. Cocrystallization of AME[a] from Feeding Experiments with L-[1-¹⁴C,3- or 5-³H] Tyrosine and L-[U-¹⁴C,3- or 5-³H]-Tyrosine with Unlabeled AME

| Expt	Precursor	³H/¹⁴C ratio of tyrosine	AME[a] ³H/¹⁴C ratio, cocrystallization 1st	2nd	3rd	Final ³H retention[b]
1	L-[1-¹⁴C,3- or 5-³H]Tyrosine	9.17	4.74	4.68	4.68	51
2	L-[1-¹⁴C,3- or 5-³H]Tyrosine	3.73	1.85	1.81	1.82	49
3	L-[U-¹⁴C,3- or 5-³H]Tyrosine	8.96	6.08	6.02	5.73	64
4	L-[U-¹⁴C,3- or 5-³H]Tyrosine	10.1	6.84	6.92	6.86	68

[a] Anthramycin methyl ether. [b] Tritium retention = (³H/¹⁴C of product)/(³H/¹⁴C of substrate) × 100%.

the formation of 3-hydroxyanthranilic acid from L-tryptophan.[23]

By analogy with the known biosynthesis of the propylproline moiety of lincomycin A[15] the building blocks for anthramycin were proposed as shown in Scheme II.

Scheme II. Building Blocks for Anthramycin
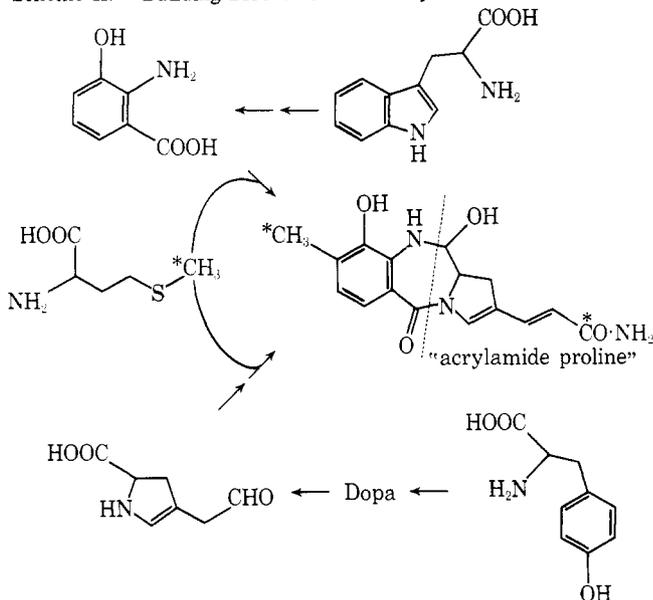
In this scheme the MHAA moiety would be derived from tryptophan via 3-hydroxyanthranilic acid and the methyl group of methionine. In addition, 7 of the 8 carbons of the acrylamide proline moiety would be derived from L-tyrosine, the eighth being a further C-1 unit from methionine. In order to determine how many carbon atoms of L-tyrosine were actually incorporated into anthramycin, we compared the incorporation of L-[U-¹⁴C]tyrosine with that of L-[1-¹⁴C]tyrosine using L-[3- or 5-³H]tyrosine as a reference label. The data obtained are presented in Table III. The 64-68% retention of tritium is indicative of a transfer of 7 of the 9 carbon atoms of tyrosine to the acrylamide proline

Table IV. Feeding Experiment with Doubly Labeled Methionine

	Rel specific radioactivities of anthramycin and its degradation products	³H/¹⁴C ratio	Tritium retention, %	No. of H retained on transferred C-1 unit
[Me-¹⁴C,Me-³H₃]-Methionine		2.92	100	
Anthramycin	100	1.41	48	
Acetic acid[a]	44	2.90	99	2.98
Values calcd from above data for the acrylamide proline moiety	56	0.24	8	0.25

[a] Derived from Kuhn Roth oxidation of anthramycin.

moiety of anthramycin. Incorporation of 6, 7, or 8 carbons of tyrosine into anthramycin would be represented by tritium retentions of 75, 64, and 56%, respectively.[24] By analogy with the biosynthesis of the propylproline group of lincomycin A from tyrosine and methionine, the amide carbon of the acrylamide side chain should be that which is derived from methionine. If this were true, then a feeding experiment with doubly labeled [Me-¹⁴C,Me-³H₃]methionine should show transfer of a one carbon unit to the acrylamide proline group with complete loss of tritium. The data from this experiment (Table IV) substantiate our postulation but do not supply unequivocal proof of this. The data also are indicative of a transfer of an intact methyl group to the 3-hydroxyanthranilic acid group.

During the conversion of tyrosine to anthramycin, ring cleavage of the aromatic ring must take place. Two types of ring cleavage are possible, either intradiol (ortho) or extradiol (meta) cleavage. These two alternative pathways are shown in Scheme III. It is possible to differentiate between

Scheme III. Alternative Pathways for the Conversion of [1-¹⁴C,3- or 5-³H]Tyrosine into the "Acrylamide Proline" Group of Anthramycin Involving either Ortho or Meta Cleavage
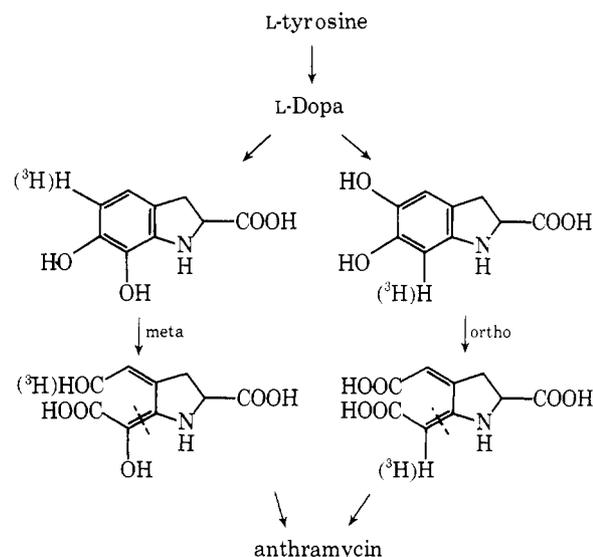
these types of ring cleavage because "meta" cleavage will lead to retention of half of the tritium from [3- or 5-³H]tyrosine in anthramycin, whereas "ortho" cleavage will lead to complete loss of tritium during the conversion of tyrosine to anthramycin. The results in Table III (experiments 1 and 2) are in agreement with a "meta" cleavage pathway and rule out the alternative "ortho" cleavage.

One ambiguity exists, in that a mechanism involving an

Table V. Dilution of L-Tyrosine in Anthramycin, Obtained by Feeding Varying Amounts of the Amino Acid to the *S. refuineus*

L-[1-^{14}C]-Tyrosine, μM, fed at		Specific act. of tyrosine, dpm/μmol	Anthramycin		
6 hr	12 hr		μM	dpm/μmol	Dilution[a]
27.6	27.6	3.98 × 10^5	27.7	5.23 × 10^4	7.6
82.8	82.8	1.33 × 10^5	26.6	2.91 × 10^4	4.6
138.0	138.0	7.97 × 10^4	21.1	2.96 × 10^4	2.7
276.0	276.0	3.98 × 10^4	20.5	1.73 × 10^4	2.3

[a] Specific activity of L-tyrosine/specific activity of anthramycin.

NIH shift with 100% retention of tritium, followed by "ortho" cleavage, could conceivably lead to the same result, that is, retention of 50% of the label in anthramycin. This is illustrated in Scheme IV. It is possible to differentiate between these pathways (A and B in Scheme IV) providing

Scheme IV. Alternative Mechanisms Leading to the Retention of One-Half of the Tritium from [1-^{14}C,3- or 5-^{3}H]Tyrosine in Anthramycin
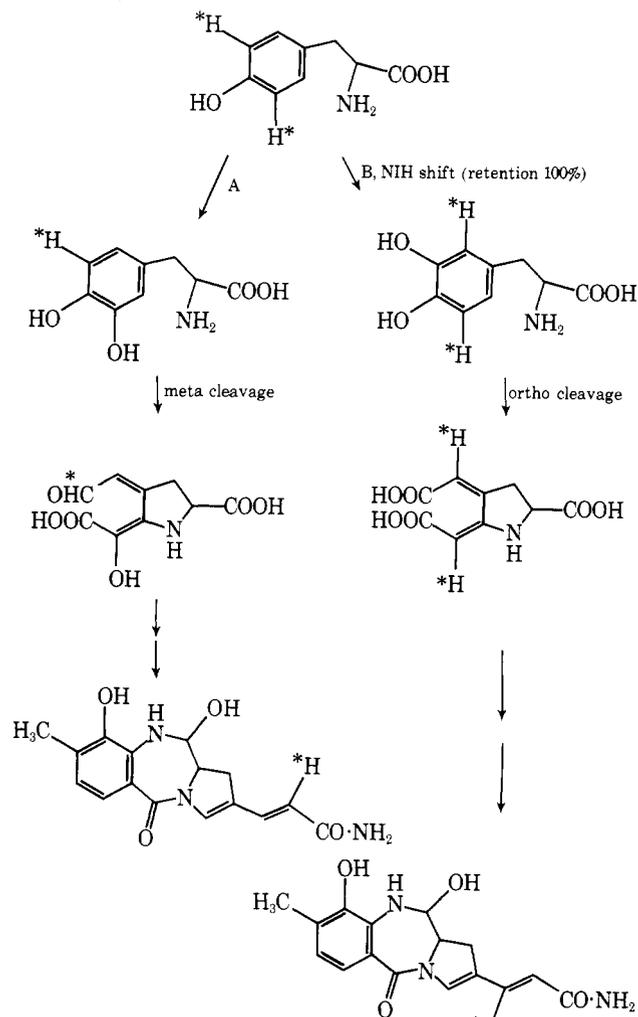
one can determine the position of the isotopically labeled hydrogen in anthramycin. Pathway A leads to isotopically labeled hydrogen at position 13 whereas pathway B results in the label at position 12. In order to differentiate between these two pathways we prepared L-[3,5-^{2}H]tyrosine which was fed to the anthramycin producing organism under conditions for least dilution of the label (see Table V). The anthramycin from this feeding experiment was isolated by

**Table VI.** Enrichments of Carbons in AME from Feeding Experiments with L-[Me-^{13}C]Methionine and L-[1-^{13}C]Tyrosine

Carbon atom[a]	Chemical shift[b]	Multiplicity[c]	Rel enrichment[d] from L-[Me-^{13}C]-Methionine	Rel enrichment[d] from L-[1-^{13}C]-Tyrosine
1	34.7	t	1.2	1.2
2	121.1	s	1.0	1.3
3	135.1	d	1.1	0.8
5	163.4	s	1.1	0.9
5a	114.5	s	1.4	0.7
6	123.7	d	1.3	0.8
7	119.0	d	1.2	0.9
8	127.5	s	1.2	1.5
9	141.7	s	0.7	0.6
9a	135.1	s	1.1	0.8
11	86.7	d	1.1	27.5
11a	59.1	d	1.1	0.9
12	120.2	d	1.2	0.9
13	134.1	d	1.3	1.0
14	167.7	s	3.6	0.7
15	17.1	q	7.1	0.8
16	54.2	q	1.0	1.0

[a] Numbering for AME is shown in Chart I (Ib). [b] Chemical shifts are given relative to TMS. [c] This multiplicity arises from one-bond ^{13}C proton coupling. [d] Calculated by measuring peak heights in the spectrum of enriched AME relative to the height of the methoxy group of AME, then dividing these relative heights by the relative heights of the same peaks (calculated in the same way) in the natural abundance spectrum.

countercurrent distribution and recrystallized twice from methanol to produce anthramycin methyl ether (AME, Ib). The mass spectrum of AME showed only species of D_0, D_1, and D_2 molecules in the ratio of 53.2, 46.0, and 0.9. This therefore confirmed our result with the double labeled tyrosine in which we had shown that only one of the two tritium atoms from [3- or 5-^{3}H]tyrosine was retained. The ^{1}H NMR on the same sample shows clearly a reduction of approximately 0.5 H for the doublet due to substitution of deuterium for the proton at position 13 of AME (Figure 2).[25] This therefore is in accord with pathway A and rules out the mechanism involving an NIH shift.

Subsequent experiments to confirm the labeling pattern of methionine and tyrosine in the acrylamide proline moiety of anthramycin were carried out using carbon-13 NMR. The carbon NMR assignments for AME are shown in Table VI. Assignments for carbon atoms 1, 11, 11a, 15, and 16 were made on the basis of multiplicity and specific neighboring groups.[26] Resonance signals for quaternary aromatic carbons were assigned on the basis of chemical shift calculations of model compounds such as methyl anthranilate[27] and the shielding constants of the methyl and hydroxyl substituents of other aromatic compounds.[28] Since all these calculated values differ by at least 5 ppm, none of these assignments are in doubt. The remaining quaternary carbon signal which occurs at 121.2 ppm can therefore be assigned to C-2 of AME. On the basis of the calculated values for the methyl ester of MHAA and other model compounds of unsaturated carboxylic compounds,[29,30] the 123.7-ppm signal can be designated to C-6 of AME. The two pairs of signals at 119.0 and 120.2 ppm and 134.1 and 135.1 ppm cannot be so readily distinguished by simple chemical shift theory. However, based on the known proton

calculated values
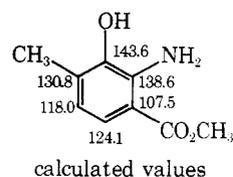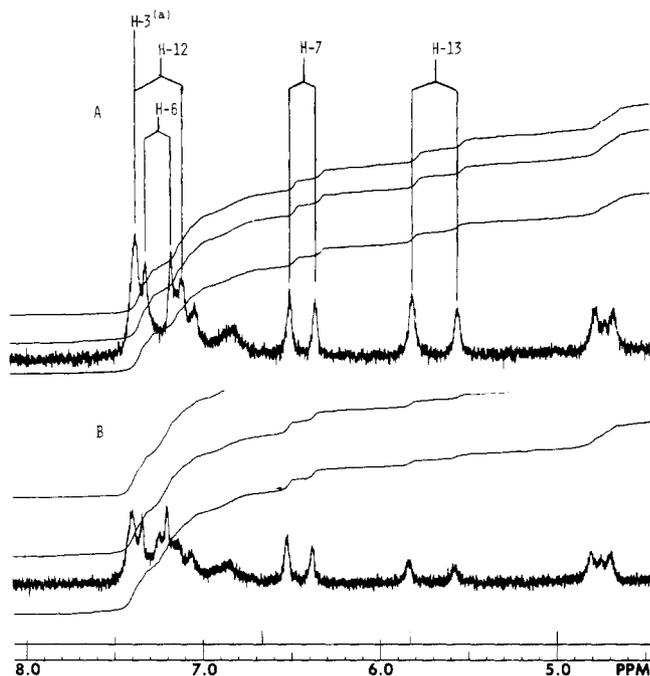
Figure 2. ¹H NMR spectra of nonisotopically labeled AME (A) and AME isotopically labeled from L-[3,5-²H]tyrosine (B) in [6-²H]DMSO (60 MHz). (a) The numbering for Ib is shown in Chart I.

spectral analysis of the nonisotopically labeled AME,[4] in conjunction with samples of AME biosynthetically specifically enriched with deuterium at C-12[31] and C-13, we have been able to unambiguously differentiate C-7 from C-12 and C-3 from C-13 in the ¹³C NMR spectra. The assignment of C-3 and C-13 to the signals at 135.1 and 134.1 ppm, respectively, is substantiated by the large difference in one-bond ¹³C-¹H coupling constants [$^1J_{CH}$ = 194 Hz (C-3) and 157.6 Hz (C-13)].[32-34] In order to differentiate between the two amide carbonyls which give rise to signals at 163.4 and 167.7 ppm, we used tomaymycin (II) as a model compound. This antibiotic lacks the terminal amide carbonyl group found in AME and consequently only one signal at 165.5 ppm is found in the 160–170-ppm range. This signal can therefore be assigned to C-5 of tomaymycin. A small upfield shift of the carbonyl carbon resonance from an unconjugated to a conjugated amide can be anticipated.[35] Thus the 163.4-ppm signal in the ¹³C NMR of AME can be assigned to C-5, and furthermore the remaining signal at 167.7 ppm must be that due to C-14.[36]

The [Me-¹³C]methionine (90% ¹³C) was fed to 12 flasks of the anthramycin producing *S. refuineus* just prior to anthramycin production. After 9 hr the anthramycin was purified by countercurrent distribution, 30 mg of unlabeled AME added to the 7 mg of enriched anthramycin and recrystallized from methanol.[37] Analysis of the ¹³C NMR spectra of AME (Table VI) showed that [Me-¹³C]methionine enriched the aromatic methyl group and the terminal amide carbonyl of the antibiotic. The enrichment of the amide carbonyl at C-14 of AME from [Me-¹³C]methionine confirms that the biogenetic origin of the acrylamide proline moiety of anthramycin is the same as that of the propylproline moiety of lincomycin A.[15]

L-[1-¹³C]tyrosine (95% ¹³C) prepared by the method of Loftfield[38] was fed to *S refuineus* as described for the specifically deuterated tyrosine molecules. Isolation and recrystallization of anthramycin in the usual manner gave a sample of antibiotic in which the ¹³C NMR spectra only showed enrichment of the expected C-11 of anthramycin

(Table VI). This result in conjunction with that of the specific incorporation of L-[3,5-²H]tyrosine into anthramycin firmly establishes the manner in which tyrosine is incorporated into anthramycin.

Discussion

The results of this study firmly establish the origin of the carbon skeleton of anthramycin from tryptophan, tyrosine, and methionine. The manner in which these three amino acids are incorporated into anthramycin is as shown in Scheme II. The incorporation of tryptophan, labeled in the aromatic ring, exclusively in the MHAA moiety of anthramycin is probably analogous to the biosynthesis of MHAA in actinomycin biosynthesis.[21,22]

This report provides the second example of an antibiotic containing a C₃-proline moiety which is derived from tyrosine (7C) and methionine (1C), the first report being the propylproline moiety of lincomycin A (IVa).[15] In both cases comparison of the incorporation of L-[U-¹⁴C]tyrosine with L-[1-¹⁴C]tyrosine leads to the conclusion that tyrosine contributes seven of its nine carbons to the C₃-proline moiety. Also, in both cases the terminal carbon atom of the C₃ side chain is derived from methionine. However, anthramycin differs from lincomycin A in that the terminal carbon atom is an amide carbonyl, whereas in lincomycin A it is a methyl group. To our knowledge this is the first example of an amide carbonyl biogenetically derived from the S-methyl group of methionine, although it has been previously reported that the carboxyl group in leucensomycin[39] and the carbomethoxylcarbonyl carbon in streptovaricin D[40] are biogentically derived from the methyl group of propionate. Tomaymycin (II) and lincomycin B (IVb) are antibiotics containing proline moieties with 2-carbon side chains attached at equivalent positions to the 3-carbon side chains of anthramycin and lincomycin A. The biogenetic origin of the C₂-proline unit of lincomycin B has been previously shown to be tyrosine (6C) and the terminal methyl group from methionine. We have recently shown[16] that the entire C₂-proline moiety of tomaymycin is derived from tyrosine, without an additional C-1 unit from methionine. Our results from double labeling experiments using [1-¹⁴C,3- or 5-³H]tyrosine with both anthramycin and tomaymycin are in accord with an extradiol ring cleavage reaction. Similar experiments carried out at Upjohn on lincomycin A are also in accord with extradiol cleavage of the aromatic ring.[41] It is therefore tempting to predict that extradiol cleavage of 5,6-dihydroxycyclo-Dopa and 6,7-dihydroxycyclo-Dopa would lead to lincomycin B and sibiromycin, respectively. Scheme V represents an attempt to generalize the biogenetic origin of C₂- and C₃-proline moieties of antibiotics, which are known or expected to be derived from tyrosine.

The intermediates in the pathway to anthramycin are as yet largely unresolved. The most urgent question is whether tyrosine or Dopa condenses with the anthranilic acid moiety before or after conversion to the C₃-proline moiety. Preliminary results based on the incorporation of double labeled methionine and tyrosine into an intermediate in anthramycin biosynthesis suggest that tyrosine condensed with MHAA prior to subsequent modification. Further evidence for the existence of this type of compound can be found in the known structure of the fungal metabolite cyclopenin (V).[42] (See Chart II.)
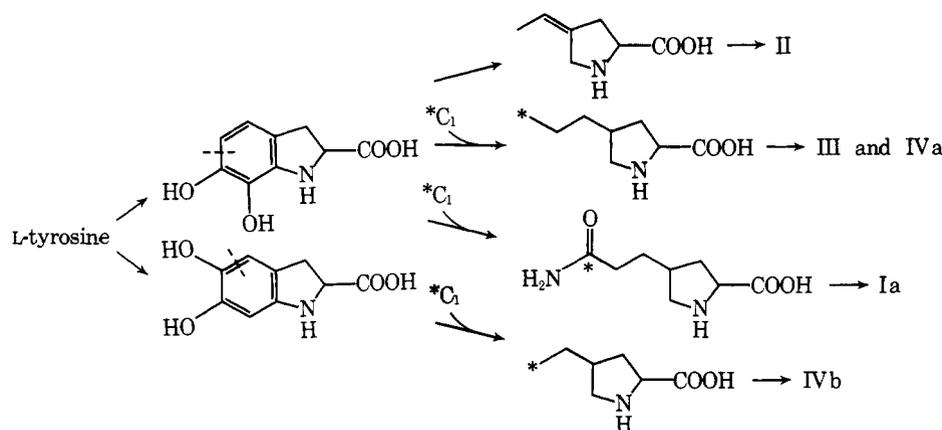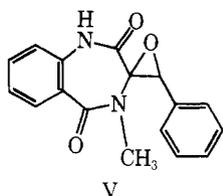
Experimental Section

Scheme V. Proposed General Scheme for the Biogenetic Origin of C_2- and C_3-Proline Moieties of Antibiotics Derived from L-Tyrosine

Chart II

V

distilled water. To prepare seed cultures spores were transferred aseptically to 500-ml baffled, erlenmeyer flasks containing 50 ml of a medium consisting of (grams per liter) peptonized milk (20), dried yeast (3), and cornstarch (10) adjusted to pH 7 in distilled water. The seed cultures were incubated for 24 hr at 47° in a New Brunswick Model G-25 gyrotory shaker. Samples (2 ml) of the seed culture were used to inoculate 500-ml baffled erlenmeyer flasks containing 50 ml of the same medium and these were incubated with shaking as before. After 12 hr the radioactive precursors dissolved in 1 ml of water were added to the cultures and these were incubated for another 3 hr. Where stable isotope experiments were carried out, the precursors (138 μM) were added at 6 and 12 hr and the cultures harvested at 15 hr.

Chromatography. Thin-layer chromatography on 5 × 20 cm precoated silica gel F-254 (Merck) was used throughout this study. Visualization of anthramycin occurred spontaneously if the plates were exposed to air for a few hours, when a brownish yellow coloration formed. The chromatography system consisted of hexane-2-propanol-ethanol (6:3:1): anthramycin R_f 0.25, MHAA R_f 0.80. The development time was about 2.5 hr.

Radioactively Labeled Compounds. [1-^{14}C]Acetic acid and L-[1-^{14}C]tyrosine were purchased from New England Nuclear, [4-^{14}C]-δ-aminolevulinic acid, D-[1-^{14}C]glucose, D-[6-^{14}C]glucose, L-[Me-^{14}C]methionine, L-[1-^{14}C]dihydroxyphenylalanine, L-[G-^{3}H]tryptophan, L-[U-^{14}C]tyrosine, L-[U-^{14}C]proline, and L-[U-^{14}C]phenylalanine from Amersham/Searle, and DL-[7a-^{14}C]tryptophan and L-[Ala-3-^{14}C]tryptophan from ICN.

Stable Isotope Labeled Compounds. L-[Me-^{13}C]Methionine and K^{13}CN were purchased from Merck (Canada); L-[1-^{13}C]tyrosine was synthesized by the method of Loftfield.[38] L-[3,5-^{2}H]Tyrosine was prepared by refluxing unlabeled L-tyrosine in 5N ^{2}HCl for 1 hr. After lyophilizing off the ^{2}HCl the exchangeable deuterium was removed by repeated freeze drying, first from dilute HCl and then from water. Complete exchange of the (3-5) hydrogens was confirmed by mass spectrometry and ^{1}H NMR.

Nonlabeled Compounds. 4-Methyl-3-hydroxyanthranilic acid was a gift from Dr. U. Hornemann, Purdue University, and Dr. D. Perlman of Wisconsin University. Anthramycin methyl ether was a kind gift of Dr. Julius Berger of the Hoffmann-La Roche Company.

Isotope Analysis. All radioactivity measurements were carried out on a Packard Model 3375 liquid scintillation counter. A solution (15 ml) containing 6 g of PPO and 1.2 g of Me$_2$POPOP and 500 ml of Triton-X in 1 l. of toluene was used as the scintillator solution. Samples were counted to at least 2% statistical error and

counting efficiencies were determined using an external standardization technique. Radioactivity on chromatograms was detected using a Packard Model 7201 radiochromatogram scanner.

General Techniques. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian A-60 and Jeol PFT-100 NMR spectrometers, respectively. The NMR spectra in deuteriodimethyl sulfoxide ([6-^{2}H]Me$_2$SO) were obtained in 10 (^{13}C NMR) and 5 mm (^{1}H NMR) spinning tubes. Tetramethylsilane (Me$_4$Si) was used as internal reference for (^{1}H NMR) spectra. Integration of signals in the proton NMR was obtained by averaging five successive scans. The ^{13}C resonance of [6-^{2}H]Me$_2$SO served as internal reference for ^{13}C NMR spectra and conversion to the Me$_4$Si scale involved the following correction: δ(Me$_4$Si) = δ[6-^{2}H]Me$_2$SO + 39.6 ppm. The instrument employed was a Jeol PFT-100 spectrometer operating at 23.5 kG, interfaced with a Jeol EC-100 Fourier transform computer with 20K memory. The spectra were recorded at ambient temperature using a deuterium lock. All proton lines were decoupled by a broad-band (2.5 kHz) irradiation from an incoherent 99.99-MHz source. The chemical shifts were measured for 5000-Hz sweep width. The proton-coupled ^{13}C spectrum was obtained using gated, noise decoupling in order to achieve greater sensitivity. The typical pulse width was 13 μsec, and the repetition time between pulses was 3.0 sec. Uv spectra were recorded on a Cary and mass spectra on a Hitachi RMU-7. For quantitative isotope analysis, several slow scans of the ion region were recorded at 12 mV. AME was assayed spectrophotometrically as described by Stefanovich.[18]

Calculation of Isotope Composition from Mass Spectral Data. The actual isotope composition of deuterium labeled compounds was calculated from the intensity of the ion at 296, and its satellites of the labeled compound and the corresponding unlabeled compound, using Biemann's formula[43] for the correction for natural isotope abundances. Anthramycin methyl ether (molecular weight 329) was used for the mass spectral analysis. This compound loses methanol (M − 32) and one more mass unit to give rise to a high mass ion of 296. Leimgruber et al.[4] report only an ion at 297 (M − 32) but in our hands we always recorded a high mass of 296 for unlabeled compounds.

Isolation of Anthramycin from Cultures and Determination of Percentage Incorporation of Precursors. At the end of the fermentation period the combined beer and mycelium were extracted with two 50-ml portions of water-saturated butanol. The phases were separated by centrifugation and concentrated under vacuum at below 36° by distilling off the butanol with water. The residue was redissolved in methanol up to about 5 ml to produce the methyl ether of anthramycin. An aliquot of this solution, usually 0.1 ml, was counted to determine the total radioactivity in this phase. Another aliquot equivalent to 10,000 dpm was chromatographed on a 5 × 20 cm TLC plate in the chromatography system together with reference spots of authentic anthramycin methyl ether. The plate was then scanned for radioactivity and the anthramycin zone was located by inspection under ordinary light after a few hours. To determine the percentage of the radioactivity of the neutral fraction residing in anthramycin, the area under the recorder tracing from the radiochromatogram scanner was integrated by cutting out and weighing the paper. This percentage figure multiplied by the total

radioactivity of the methanol extract gives the total radioactivity incorporated into anthramycin, which was divided by the total radioactivity fed and multiplied by 100 to give the percentage incorporation. If no radioactivity was detected at the position of anthramycin, it was assumed on the basis of the limits of detection by the scanner that less than 10^4 dpm ^{14}C or 5×10^4 dpm ^{3}H had been incorporated and these figures were used to calculate upper limits of the percentage of incorporation. Since all attempts to elute anthramycin methyl ether from the plates without causing extensive breakdown failed, we were unable to rechromatograph in alternative solvents to confirm the radiochemical purity. Therefore, those samples of anthramycin shown to be significantly labeled from added precursors were cocrystallized with carrier AME to constant specific activity to substantiate the combined counting and scanning result. The radioactive AME samples were diluted with exactly 25 mg of cold carrier material and recrystallized, firstly from methanol–H$_2$O, then acetone–H$_2$O, and finally methanol–H$_2$O again. After each crystallization about 2–3 mg of AME was weighed out accurately and used to measure the specific radioactivity. The recovery in each crystallization was about 70%. The radiochemical purity of the AME obtained this way was checked by TLC.

Purification of Anthramycin Labeled with Stable Isotopes from Culture Broths. The concentrated butanol extracts of the culture media were taken to dryness and subjected to countercurrent distribution in a solvent system consisting of chloroform–2-propanol–ethanol–water (3:1:1:5), 99 transfers; $K = 0.69$ for anthramycin methyl ether.[4] The anthramycin was located in the collection tubes by uv absorption at 335 nm. Those tubes containing anthramycin were pooled, concentrated under reduced pressure, and crystallized twice from methanol–H$_2$O.

Degradation of Anthramycin. A known amount of radiochemically pure AME and an amount of nonlabeled carrier AME to make a total weight of 50 mg were placed in a round-bottomed flask equipped with a gas inlet and a reflux condenser. Five milliliters of 5 N HCl was added to the flask and the reaction mixture was refluxed for 2 hr in a sand bath. At the end of the reaction the acid solution was diluted to 25 ml with water and extracted with three 25-ml portions of ethyl acetate. This extract was dried over sodium sulfate, taken to dryness, and redissolved in about 2 ml of methanol; a few drops of water were added and the solution was allowed to stand overnight. The brownish crystals which formed were weighed out accurately and redissolved in methanol and an aliquot was counted for radioactivity. A further aliquot was chromatographed to determine radiochemical purity. The MHAA obtained in the degradation of AME labeled from L-[Me-^{14}C]methionine was mixed with a known amount of synthetic MHAA to give a total weight of 50 mg and subjected to Kuhn Roth oxidation.[19] The acetic acid obtained from the Kuhn Roth oxidation on MHAA labeled from L-[Me-^{14}C]methionine was further degraded by the Schmidt procedure.[20]

AME labeled from L-[Me-^{14}C,Me-^{3}H$_3$]methionine was added to a known amount of nonlabeled carrier material to make a total of 50 mg and subjected to Kuhn Roth oxidation.

References and Notes

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(25) The proton NMR has been previously assigned by Leimgruber et al.[4] The change in complexity of signals in the region 7.2–7.5 ppm is due to the coupling of ^{2}H at C-13 to the ^{1}H at C-12 of AME. Although there is an apparent reduction in the intensity of the doublet for the proton at C-12, this is compensated for by an appearance of a signal at 7.32 ppm, which can be attributed to those protons which are adjacent to deuterium atoms. There was no decrease in the overall integration of the region between 7.1 and 7.5 ppm.
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