48, 364.
Vandlen, R. L., and Tulinsky, A. (1973), *Biochemistry 12*, 4193.
Weiner, H., Batt, C. W., and Koshland, Jr., D. E. (1966), *J. Biol. Chem. 241*, 2687.
Wilcox, P. E. (1967), *Methods Enzymol. 11*, 605.
Yapel, A., Han, M., Lumry, R., Rosenberg, A., and Shiao, D. F. (1966), *J. Am. Chem. Soc. 88*, 2573.

Pyrrolo[1,4]benzodiazepine Antibiotics. Biosynthesis of the Antitumor Antibiotic 11-Demethyltomaymycin and Its Biologically Inactive Metabolite Oxotomaymycin by *Streptomyces achromogenes*†

Laurence H. Hurley,* Chandrachuranand Gairola, and Nobel V. Das

ABSTRACT: 11-Demethyltomaymycin, an antitumor antibiotic produced by *Streptomyces achromogenes*, and its biologically inactive metabolite oxotomaymycin are biosynthesized from L-tyrosine, DL-tryptophan, and L-methionine. The anthranilate part of 11-demethyltomaymycin is derived from tryptophan probably via the kynurenine pathway. The predominant loss of tritium from DL-[5-^{3}H]tryptophan, during its conversion to 11-demethyltomaymycin and oxotomaymycin is interpreted to mean by NIH shift rules, that the main pathway to the 5-methoxy-4-hydroxy anthranilate moiety is through hydroxylation at C-8 prior to hydroxylation at C-7. The methoxy carbon is derived from the *S*-methyl group of methionine by transfer of an intact methyl group. The ethylideneproline moiety of 11-demethyltomaymycin is biosynthesized from tyrosine, without a 1-carbon unit from methionine. The results of biosynthetic feeding experiments with L-[1-^{14}C, 3- or 5-^{3}H]tyrosine are consistent with a "meta" or extradiol cleavage of 6,7-dihydroxycyclodopa as has also been demonstrated previously for anthramycin and lincomycin A. An experiment in which L-[1-^{14}C, Ala-2,3-^{3}H]tyrosine was fed showed that both of the β hydrogens of this amino acid are retained in 11-demethyltomaymycin. It has been demonstrated in cultures and washed cell preparations that 11-demethyltomaymycin is enzymatically converted to oxotomaymycin by an intracellular constitutive enzyme. Conversion of oxotomaymycin to 11-demethyltomaymycin by these same preparations could not be demonstrated. The enzymatic activity associated with the conversion of 11-demethyltomaymycin to oxotomaymycin is not limited to the 11-demethyltomaymycin production phase, since trophophase cells and even cells from 11-demethyltomaymycin nonproducing cultures of *S. achromogenes* were equally active in converting 11-demethyltomaymycin to oxotomaymycin.

11-Demethyltomaymycin (DMT)[1] is one of three pyrrolo[1,4]benzodiazepine antitumor antibiotics isolated from culture filtrates of actinomycetes. The other antibiotics within this class are anthramycin and sibiromycin. The structures of these compounds and the DMT related compound oxotomaymycin (OT) are shown in Scheme I. DMT was originally isolated from the culture filtrates of *Streptomyces achromogenes* by Professor K. Arima and co-workers at the University of Tokyo and the Fujisawa Pharmaceutical Company (Arima et al., 1972). The structures of DMT and the closely related compound, OT, which is also produced by cultures of *S. achromogenes*, were established by Kariyone et al. (1971). DMT and its methylated product tomaymycin have been shown to possess antiviral activity against *Escherichia coli* T_1 and T_3 phages, antibacterial activity against gram-positive bacteria (Arima et al., 1972), and cytotoxic activity towards Leukemia L1210 cells (Nishioka et al., 1972). All of the biological properties of these pyrrolo[1,4]benzodiazepine antibiotics can be attributed to the effects of these compounds on nucleic acid biosynthesis. DMT and tomaymycin appear to act by virtue of their ability to bind to DNA and thereby interfere with the function of DNA (Nishioka et al., 1972).

As part of a continuing effort to discover more about the biosynthesis of these pyrrolo[1,4]benzodiazepine antibiotics, we are currently studying in our laboratory the biosynthesis of anthramycin, DMT, OT, and sibiromycin. Our work on anthramycin (Hurley et al., 1975b) and preliminary data on DMT (Hurley et al., 1975a) have already appeared. For both of these antibiotics and sibiromycin (Hurley et al., unpublished data) we have shown that the anthranilate moiety of these antibiotics is derived from tryptophan probably via the kynurenine pathway. Our studies on anthramycin (Hurley et al., 1975b) show conclusively that the acrylamide proline moiety of this antibiotic is derived from tyrosine (7 carbons) and methionine (1 carbon) to give rise to the demonstrated labeling pattern shown in Scheme II. Dopa, or more likely its condensation product with the anthranilate part of the molecule, is considered to be the intermediate that undergoes "meta" or extradiol cleavage.

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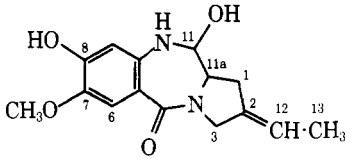
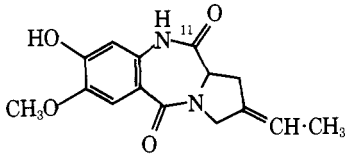
[1] Abbreviations used are: DMT, 11-demethyltomaymycin; OT, oxotomaymycin; DMAA, methyl-4,5-dimethoxyanthranilate; TLC, thin-layer chromatography; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

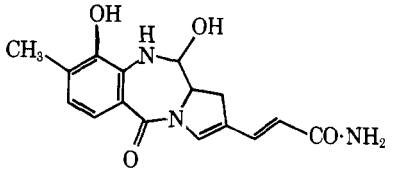
SCHEME I: Comparison of the Structures of DMT and Related Compounds.



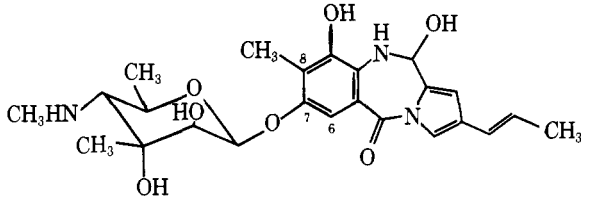
11-demethytomaymycin
(DMT)



oxotomaymycin
(OT)



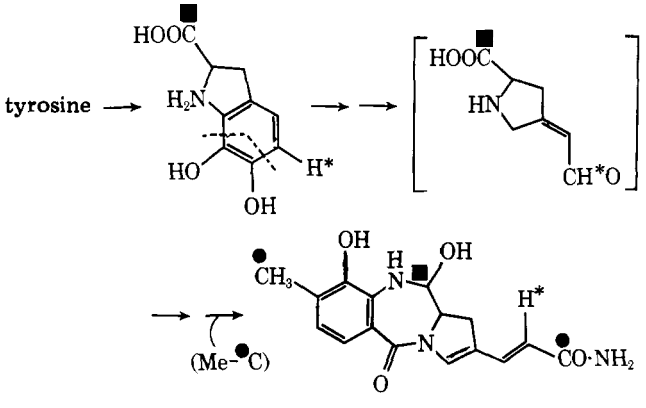
anthramycin



sibiromycin

This report described the results of radioactive tracer studies using both single- and double-labeling techniques and the subsequent degradations of the purified crystalline radiolabeled DMT. These results are interpreted in terms of possible biogenetic pathways. The biogenetic relationship between DMT and OT is also described.

SCHEME II: Established Labeling Pattern of Tyrosine and Methionine in Anthramycin.



Experimental Section

Fermentations. S. achromogenes var. *tomaymyceticus* ATCC 21353 was maintained on Bennett's agar slants. Frequent transfer was required in order to maintain a viable antibiotic-producing strain. To prepare seed cultures mycelial fragments were transferred aseptically to 500-ml erlenmeyer flasks containing 100 ml of a medium consisting of (g/l.) phytone BBL-lot 305617(5), yeast extract BBL-lot A6DATJ(5), glucose Baker-lot 38515(10), Bacto-casitone Difco-lot 588134(1), and NaCl(5) adjusted to pH 7. The seed cultures were incubated for 48 h at 29 °C in a New Brunswick Model G-25 gyrotary shaker at 250 rpm. Samples (5 ml) of the seed culture were used to inoculate 500-ml erlenmeyer flasks containing 100 ml of a medium consisting of (g/l.) Bacto-lactose Difco-lot 608376(30), polypepto BBL-lot G8DDTl(10), beef extract BBL-lot B7DDHU(10), yeast extract BBL-lot AGDATJ(10), NaCl(2.5), KH_2PO_4(15), Na_2HPO_4(4), adjusted to pH 6.2, if necessary, in distilled water. After 72 h, the radioactive precursors, usually dissolved in 0.5 ml of distilled water, were added to the cultures and incubated for a further 12 h. For short-term experiments aliquots of 5 ml of the culture medium were removed at appropriate times. Washed cell preparations were prepared by collecting the mycelium by filtration, washing twice with saline, and once with 0.2 M phosphate buffer (pH 7.1). The washed cells were finally suspended in the same buffer so as to attain a four-times-concentrated cell suspension. This concentrated suspension (100 or 25 ml) was then transferred to 500- or 250-ml erlenmeyer flasks that were incubated for usually 6 or 12 h under the same conditions as described for routine growth of the *S. achromogenes*.

Chromatography. Thin-layer chromatography on either 5 × 20 cm precoated silica gel F-254 (Merck) or 20 × 20 cm on preparative thick-layer plates (0.5 mm) was used throughout this study. Visualization of DMT and OT on TLC plates was carried out by observing the developed plates under short uv light with authentic reference materials. Chromatography system A consisted of ethyl acetate. DMT R_f 0.32, OT R_f 0.46. Chromatography system B consisted of hexane, 2-propanol, ethanol 6:3:1. DMT R_f 0.65. Chromatography system C consisted of benzene, ethyl acetate 9:1, DMAA R_f 0.37.

Radioactively Labeled Compound. L-[1-^{14}C]Tyrosine and L-[3,5-^{3}H]tyrosine were purchased from New England Nuclear, DL-5-hydroxy[G-^{3}H]tryptophan, L-[Ala-2,3-^{3}H]tyrosine, L-[U-^{14}C]tyrosine, L-[Me-^{14}C]methionine, L-[1-^{14}C]-dihydroxyphenylalanine, and L-[Me-$^{3}H_3$]methionine were from Amersham Searle, DL-[1-^{14}C]tyrosine and DL-[7a-^{14}C]tryptophan were from I.C.N., and DL-[5-^{3}H]tryptophan was from Research Products International. [11-^{14}C]DMT (1.04 μCi/μmol) and [11-^{14}C]OT (1.2 μCi/μmol) were prepared biosynthetically by adding 50 μCi of DL-[1-^{14}C]tyrosine to a 72-h *S. achromogenes* culture. After a 12-h incubation period, the DMT and OT were removed from the culture by repeated extractions with ethyl acetate and purified by preparative TLC in system A. Radiochemical purity was determined in solvent system B. The [11-^{14}C]DMT was converted to [11-^{14}C]tomaymycin by heating in methanol for 2–3 min and then recrystallized with carrier tomaymycin. This procedure showed the antibiotic to be >95% radiochemically pure.

Nonlabeled Compounds. Tomaymycin and OT were a kind gift from Dr. Kohsaka of Fujisawa Pharmaceuticals. Other chemicals were obtained from commercial sources.

Isotope Analysis. Radioactivity measurements were carried out on a Packard Model 2425 liquid scintillation counter. A solution (15 ml) containing 6 g of PPO, 1.2 g of Me_2POPOP, and 500 ml of Triton-X in 1 l. of toluene was used as the scintillator solution, except for radioactivity determinations of DMT–DNA complexes. In this latter case the buffer solution containing the DMT–DNA complex (0.5 ml) was treated with 0.5 ml of 1 N HCl for 30 min at 70 °C before adding 10 ml of Aquasol. Samples were counted to 1% standard deviation and counting efficiencies were determined using an external standardization technique. Where double-label samples were

counted, appropriate adjustments were made for spillover of carbon-14 into the tritium channel. Radioactivity on chromatograms was detected using a Packard Model 7201 radiochromatograph scanner.

General Techniques

(*a*) *Determination of Mycelial Dry Weight.* Cultures were filtered on a Büchner funnel by suction on tared Whatman No. 1 paper (7 cm) disks. After washing with cold distilled water, the filter paper and mycelium were dried at 80 °C to constant weight.

(*b*) *Quantitative Determination of DMT.* The concentration of DMT in the culture broth was determined by measurement of the absorbance at 320 nm after purifying the antibiotic by preparative TLC. Since the recovery of DMT from different plates, as determined by radioactivity measurement, varied between 65 and 80%, an adjustment for the percent recovery from each isolation was made. This latter figure was determined by following the amount of recovered radioactive DMT through to the end of the isolation procedure.

(*c*) *Uptake of DMT into Cells.* 4.5×10^4 dpm of DMT (1.09 μCi/μmol) was added to 20 ml of a washed cell preparation, and 0.2-ml aliquots were taken at various time intervals up to 6 h. The washed cell suspension was prepared from a 74-h culture as described under fermentations. The samples were filtered through a 0.45-nm Millipore filter. The mycelium and filter were washed twice with about 2-ml of cold saline solution. The filter and washed mycelium were transferred to a scintillation vial, 0.5 ml of 1 N HCl was added, and the contents were heated at 80 °C for 30 min, cooled, and 1 ml of ethyl acetate was added to dissolve the filter. After incubating at room temperature for 30 min ten milliliters of Aquasol was added and the sample was counted for radioactivity.

(*d*) *Other Analytical Methods.* Ultraviolet spectra were obtained on a Cary 15. Mass spectral studies were carried out on a Hitachi RMU-7. Melting points were obtained with a Thomas–Hoover apparatus and were uncorrected.

Isolation of DMT and OT from Cultures and Determination of Percent Incorporation of Precursors. At the end of the fermentation period the combined beer and mycelium were extracted with three 100-ml portions of ethyl acetate. The organic phases were combined, dried over anhydrous sodium sulfate, and then concentrated under vacuum at below 36 °C using a rotary evaporator. The residue was redissolved in methanol up to about 3 ml to convert the DMT to tomaymycin. An aliquot of this solution, usually 10 μl, was counted to determine the total radioactivity in this phase. Another aliquot equivalent to 20 000 dpm was chromatographed on a 5×20 cm TLC plate in chromatography system A together with reference tomaymycin and OT. The plate was then scanned for radioactivity and the tomaymycin and OT zones were located by inspection under short uv light. To determine the percentage of radioactivity of the ethyl acetate extract residing in tomaymycin and OT, the area under the recorder tracing from the radiochromatogram scanner was integrated by cutting out and weighing the paper. These percentage figures multiplied by the total radioactivity of ethyl acetate extract gave the total radioactivity incorporated into tomaymycin and OT, which was divided by the total radioactivity fed and multiplied by 100 to give percentage incorporation. If no radioactivity was detected at the position of tomaymycin or OT, it was assumed on the basis of the limits of detection by the scanner that less than 2×10^3 dpm ^{14}C or 2×10^4 dpm ^3H had been incorporated and these figures were used to calculate upper limits of the percentage of incorporation. Tomaymycin and OT samples that were radioactively labeled were then isolated by preparative TLC and the zones corresponding to these compounds eluted with methanol. Aliquots of these isolated components were rechromatographed on precoated glass plates in the same system to ensure degradation had not taken place, before they were subjected to further purification procedures. Those samples of DMT shown to be significantly labeled from added precursors were further purified by cocrystallization with carrier antibiotic to constant specific activity to substantiate the combined counting and scanning result (see below).

Further Purification of DMT Isolation from Culture Filtrates

Samples of tomaymycin isolated by preparative TLC were further purified by either recrystallization or covalent complexation with DNA and in some cases by both procedures.

(*a*) *Recrystallization.* Tomaymycin was recrystallized to constant specific activity by adding exactly 25 mg of cold carrier material and recrystallizing repeatedly from methanol–H_2O. After each crystallization, about 2–3 mg of tomaymycin was weighed out accurately and used to measure the specific radioactivity. The recovery in each crystallization was about 70%.

(*b*) *Covalent Complexation with DNA.* Tomaymycin dissolved in methanol was transferred to a test tube and the methanol was removed by evaporation over a stream of nitrogen. One milliliter of SSC (sodium chloride 0.015 M, sodium citrate 0.0015 M) was added to the residue, mixed, and allowed to stand for 1 h. This procedure reconverts tomaymycin to DMT. To the DMT dissolved in SCC, 1 ml of a 1.0 mM calf thymus DNA (Sigma Chemical Co.) solution was added and allowed to incubate at room temperature for 3–4 h. The final concentration of the antibiotic was generally about 0.4 mM, which represents an excess of antibiotic. The noncovalently DNA-bound material (unreacted DMT and other impurities) was removed from the incubation mixture by dialysis against 50 volumes of SSC buffer, changed twice over a 48-h period. An aliquot, usually 0.5 ml, of the purified DMT–DNA complex from the dialysis bag was counted to determine tritium to carbon-14 ratio. A similar experiment in which the biologically inactive OT (this compound does not react with DNA to form a covalent complex) was carried through this procedure resulted in an almost negligible amount of DNA-bound radioactive material (<0.1%). This was in contrast to the reaction with DMT in which about 40–50% of the activity remains bound to the complex. The less than 100% reaction of DMT with DNA is due to the excess of DMT present for maximum binding to DNA.

Degradation of Tomaymycin. A known amount of radiochemically pure tomaymycin and an amount of nonlabeled carrier tomaymycin to make a total of 50 mg were dissolved in about 5 ml of methanol. A freshly prepared solution of diazomethane in ether was added and allowed to stand overnight to form the 8-methoxy-8-deoxytomaymycin (Scheme III). Excess diazomethane was removed by evaporation at room temperature under vacuum and the volume was reduced to about 1 ml. Four milliliters of 6 N NaOH was added to this solution and the mixture was stirred at room temperature for 1 h. At the end of the reaction, the alkaline reaction mixture was acidified to pH 2 with concentrated HCl and extracted three times with 10 ml of ethyl acetate to remove the 4,5-dimethoxyanthranilate. The anthranilate derivative was removed from the organic phase by extraction three times with about 5 ml of 10% sodium carbonate solution. The sodium

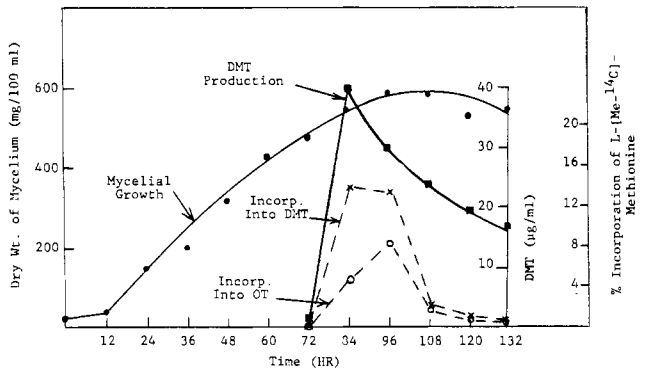
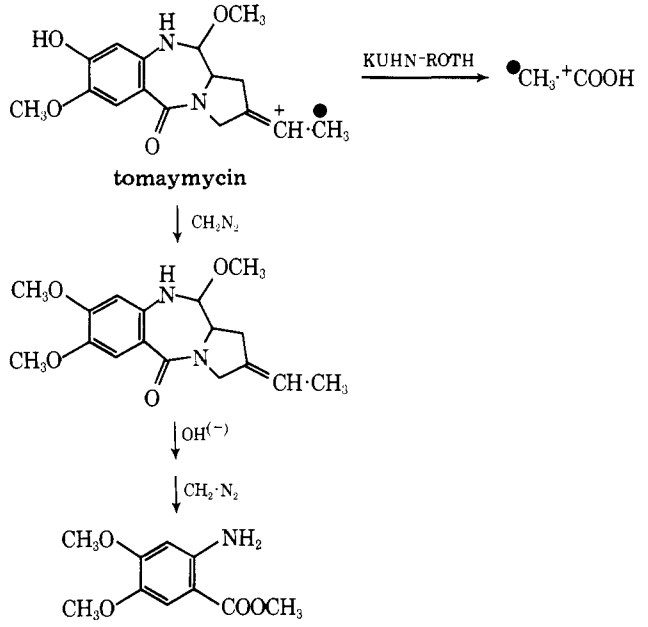


FIGURE 1: Time-course production of DMT and incorporation of L-[*methyl*-^{14}C]methionine into DMT and OT by *S. achromogenes*. At the times indicated flasks were harvested and L-[*methyl*-^{14}C]methionine was added 12 h prior to harvesting. (●—●) mg/100-ml dry weight of mycelium; (■—■) μg/ml of DMT; (x--x) % incorporation in DMT; (O- -O) % incorporation into OT.

SCHEME III: Degradation of Tomaymycin.



carbonate phases were combined, acidified to pH 2 with 2 N HCl, and the anthranilate finally extracted into ethyl acetate. This organic phase was dried over sodium sulfate, concentrated under vacuum, and redissolved in about 2 ml of ether. Excess diazomethane in ether was added and allowed to stand for a few minutes at room temperature to form the methyl ester of 4,5-dimethoxyanthranilate. The reaction mixture was taken to dryness by evaporation at 35 °C under vacuum and redissolved in a small amount of ethyl acetate. Since the DMAA could not be induced to crystallize at this state, preparative TLC was carried out in system C. The DMAA zone was located by inspection under short uv light, and eluted from the silica gel with methanol. After removing the solvent, the yellowish residue was redissolved in hot benzene and allowed to crystallize at room temperature. The crystals that formed were collected and an aliquot was weighed out accurately and counted for radioactivity. A further aliquot was chromatographed and scanned to determine radiochemical purity. The melting point of the DMAA (133–134 °C) was in close agreement to the literature value (133 °C) (Heidelberger and Jacobs, 1919). Mass spectral data was also consistent with that

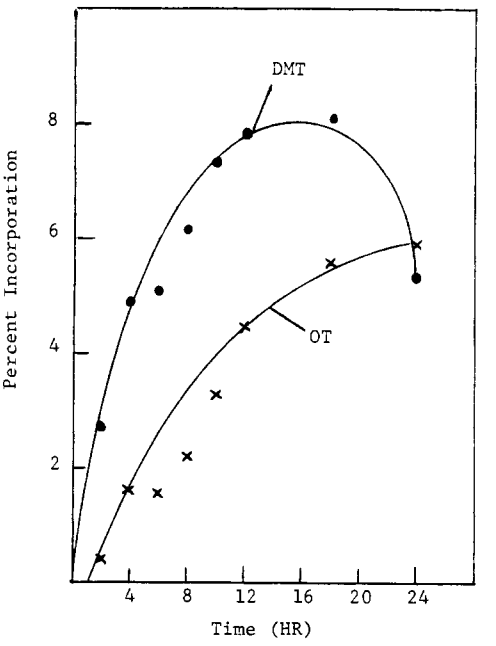


FIGURE 2: Short-term time-course incorporation of DL-[1-^{14}C]tyrosine into DMT and OT by an 80-h culture of *S. achromogenes*. 2.14 × 10^7 dpm of tyrosine was added to 95 ml of medium and 5-ml aliquots were taken at the times indicated.

expected (mol wt = 211).

Carbon atoms 12 and 13 of tomaymycin were isolated from the antibiotic by Kuhn–Roth oxidation. A known amount of radiochemically pure tomaymycin was added to an amount of carrier tomaymycin to make a total of 50 mg and subjected to Kuhn–Roth oxidation (Simon and Floss, 1967).

Results

The biosynthetic experiments were carried out with shake cultures of the DMT and OT producing *S. achromogenes* ATCC 21353 grown in a complex medium. DMT and OT were isolated from the culture medium by repeated extractions with ethyl acetate. Purification was carried out by preparative TLC and in the case of DMT followed by recrystallization or covalent complexation with DNA.

Representative data on mycelial growth and production of DMT and OT are shown in Figure 1. Onset of DMT production and also OT biosynthesis, as judged by incorporation of precursors, takes place between 72 and 84 h. Typically, like most other microbial secondary metabolites, production of DMT and OT is delayed until almost the end of the growth phase. The maximum production of DMT occurred at 84 h and titers reached about 40 μg/ml. The optimum time for incorporation of labeled precursors into these compounds occurred when the precursor was added at 72 h. The short-term time course incorporation of L-[1-^{14}C]tyrosine into DMT and OT is shown in Figure 2. Maximum incorporation of this amino acid into DMT occurred between 12 and 16 h and into OT at 24 h. Partially for convenience and also because DMT was of prime interest in this investigation, cultures were harvested 12 h after addition of the labeled precursors to 72-h-old cultures.

In order to establish the precursors of DMT and OT, various carbon-14 and tritium labeled compounds were fed to the *S. achromogenes* under optimum conditions for incorporation into DMT and OT. The choice of possible precursors was based upon our previous studies on anthramycin in which we had demonstrated that tyrosine, dopa, tryptophan, and methionine

TABLE I: Incorporation of Labeled Substrates into DMT and OT.

	Quantity Fed (μmol)	Radioactivity Fed (dpm)	Radioactivity in EtOAc extract (% of Total Fed)	% Radioactivity of EtOAc Extract in		% Incorporation into	
				DMT	OT	DMT	OT
L-[1-^{14}C]Dihydroxyphenylalanine	0.1	3.59×10^7	8.3	83.9	16.1	6.9	1.4
5-Hydroxy-DL-[G-^{3}H]tryptophan	0.1	2.21×10^8	0.4	*a*	*a*	<0.01	<0.01
L-[*methyl*-^{14}C]Methionine	0.2	4.45×10^6	23.4	76	24	17.8	5.6
L-[7a-^{14}C]Tryptophan	1.3	9.29×10^6	11.6	54	25	6.3	2.8
DL-[1-^{14}C]Tyrosine	0.3	2.14×10^7	17.4	47	32	8.1	5.6
L-[1-^{14}C]Tyrosine	0.5	8.66×10^7	11.9	85	10	10.1	1.2
L-[U-^{14}C]Tyrosine	0.01	1.82×10^6	10.7	70	30	7.6	3.1

[a] Below limit of detection.

contributed to the antibiotic molecule (Hurley et al, 1975b). As anticipated, these amino acids were all well incorporated into DMT and OT (Table I).

The role of methionine, tryptophan, and tyrosine as DMT precursors was substantiated by degradation of the biosynthetically labeled samples of the antibiotic. Prior to chemical degradation as outlined in Scheme III, DMT was converted to tomaymycin by recrystallization from methanol, and then methylated with diazomethane to produce 8-methoxy-8-deoxytomaymycin. Alkaline hydrolysis of this compound with 6 N NaOH produced the 4,5-dimethoxyanthranilate that was converted to its methyl ester (DMAA) after extraction from the reaction mixture with ether. The radiochemical purity of DMAA was checked by TLC. Carbon atoms 12 and 13 of DMT were isolated from tomaymycin by Kuhn–Roth oxidation (Simon and Floss, 1967). The data obtained from these degradations are shown in Table II. These results show that whereas L-[CH_3-^{14}C]methionine and DL-[7a-^{14}C]tryptophan label DMAA exclusively, virtually none of the activity from L-[U-^{14}C]tyrosine was found in this degradation product. Kuhn–Roth oxidation on tomaymycin shows that, whereas all the radioactivity in DMT labeled from L-[3- or 5-^{3}H]tyrosine resides at carbon atom 13 of the antibiotic, the acetic acid representing C-12 and -13 of DMT from the sample labeled from L-[CH_3-^{14}C]methionine is almost devoid of radioactivity. Kuhn–Roth oxidation on a sample of tomaymycin labeled from L-[U-^{14}C]tyrosine shows that 30% of the activity resides with the acetic acid. The theoretical figure should be about 29% (i.e., two of the seven carbons of the ethylideneproline moiety isolated as acetic acid).

Based on the known biosynthesis of anthramycin and reported work on the biosynthesis of the ethyl- and propylproline moieties of lincomycins A and B, respectively (Witz et al., 1971; Argoudelis et al., 1973) the three alternative pathways shown in Scheme IV for the biosynthesis of the "ethylidene proline" grouping of DMT can be postulated. Pathways "a" and "b" each require that 5,6-dihydroxycyclodopa be the substrate that undergoes ring cleavage.[2] However, these pathways differ in that b involves ortho cleavage and gives rise directly to a seven-carbon unit, whereas a involves meta cleavage and would require an extra carbon from the C-1 pool

TABLE II: Degradation of Tomaymycin.

Precursor	% Radioactivity of Tomaymycin Recovered in	
	DMAA	CH_3·COOH
L-[*methyl*-^{14}C]Methionine	107	2
L-[U-^{14}C]Tyrosine	10	30
L-[3- or 5-^{3}H]Tyrosine	NE[a]	102
DL-[7a-^{14}C]Tryptophan	108	NE[a]

[a] NE, not examined.

TABLE III: Incorporation of Double-Labeled Precursors into DMT and OT by *S. achromogenes*.

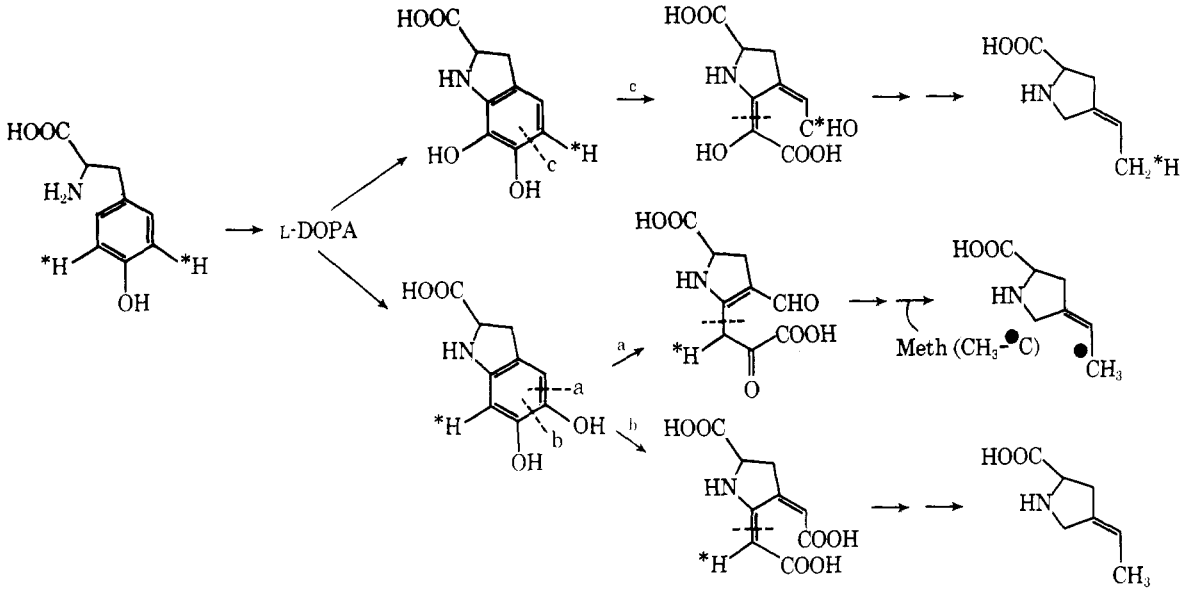
Expt	Precursor	% Retention of ^{3}H	
		DMT	OT
1	L-[1-^{14}C, 3- or 5-^{3}H]Tyrosine	50,[a] 49,[b] 44[c]	–
2	L-[U-^{14}C, 3- or 5-^{3}H]Tyrosine	64,[a] 62[b]	–
3	L-[1-^{14}C, *Ala*-2, -3-^{3}H]Tyrosine	49,[a] 51[c]	48[a]
4	L-[U-^{14}C, *Ala*-2, -3-^{3}H]Tyrosine	63,[a] 62,[b] 63[c]	64[a]
5	DL-[7a-^{14}C, 5-^{3}H]Tryptophan	17,[a] 16,[b] 14[c]	17[a]
6	L-[*methyl*-^{14}C, *methyl*-$^{3}H_3$]-Methionine	96,[a] 87[c]	91[a]

[a] Purification by preparative TLC. [b] Recrystallization to constant specific activity. [c] Complexation with DNA and dialysis against two changes of buffer.

to produce a total of seven carbons. Pathway "a" would be analogous to that suggested for lincomycin B biosynthesis (Witz et al., 1971). The third possibility, pathway "c" utilizes 6,7-dihydoxycyclodopa as the substrate for ring cleavage, and would be analogous to the anthramycin and lincomycin A biosynthetic pathways, except that an extra carbon from methionine would not be required for DMT biosynthesis. Differentiation of pathways b and c from a can be made on the basis of the origin of the terminal carbon of the ethylideneproline moiety of DMT and the number of carbon atoms tyrosine contributes to DMT. Furthermore, it is possible to differentiate between pathways b and c on the basis of the complete loss or 50% retention of tritium from L-[3- or 5-^{3}H]tyrosine in DMT. In the case of pathway "c" meta cleavage leads

[2] It is equally likely that the anthranilate condensation product with dopa or cyclodopa is the real substrate that undergoes ring cleavage; however, for convenience sake we will consider the uncondensed cyclodopa as the example for our argument, while maintaining an open mind as to the true identity for this intermediate.

SCHEME IV: Alternative Pathways for the Conversion of Tyrosine into the Ethylideneproline Moiety of DMT.



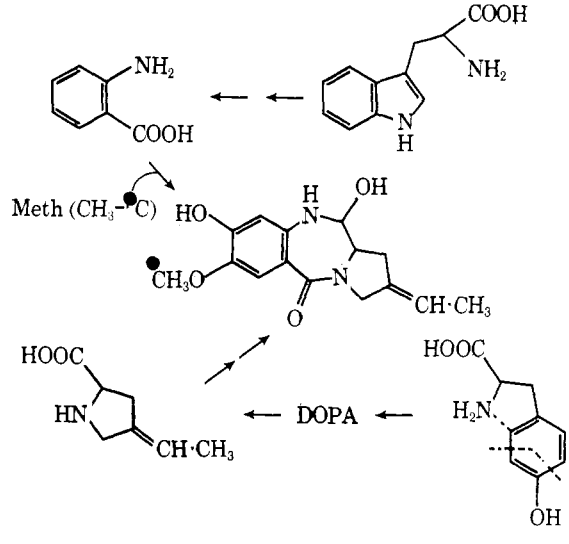
to retention of half of the tritium from L-[3- or 5-^{3}H]tyrosine in DMT, whereas in pathway b ortho cleavage will lead to complete loss of tritium during the conversion of tyrosine to DMT. The appropriate experiments were carried out to determine the correct pathway.

The results demonstrate that pathway a can be eliminated on the basis of both chemical degradation of tomaymycin and the number of carbon atoms from tyrosine that appear in DMT and OT. First, the results in Table II on the chemical degradation of tomaymycin, biosynthetically labeled from L-[Me-^{14}C]methionine, show that virtually all of the radioactivity was located in the DMAA moiety, presumably in the methoxy group, while the acetic acid derived from carbon atoms 12 and 13 was almost devoid of radioactivity. Second, our results show that tyrosine contributes seven of its nine carbon atoms to DMT, again clearly not in accord with pathway a which demands only six carbons in DMT should arise from tyrosine. In order to determine how many carbon atoms of L-tyrosine were actually incorporated into DMT and OT, we compared the incorporation of L-[U-^{14}C]tyrosine with that of L-[1-^{14}C]tyrosine using L-[Ala-2,3-^{3}H]tyrosine and L-[3- or 5-^{3}H]tyrosine as reference labels. The data (experiments 1, 2, 3, and 4) obtained are presented in Table III. The 62–64% retention of tritium is indicative of a transfer of seven of the nine carbon atoms of tyrosine to the ethylideneproline moiety of DMT and OT. Incorporation of six or seven carbons of tyrosine into DMT and OT would be represented by tritium retentions of 75 and 64%, respectively.[3]

Elimination of pathway b as a viable alternative is based upon the results of the double-labeling experiment with L-[1-^{14}C, 3- or 5-^{3}H]tyrosine in which almost exactly half of the tritium was retained in DMT, a result which is not compatible with this pathway (Table III). Pathway c is in agreement with the retention of seven of the nine carbon atoms of tyrosine in DMT and OT and should also lead to acetic acid, derived from Kuhn–Roth oxidation of tomaymycin, biosynthetically labeled from L-[3- or 5-^{3}H]tyrosine, which has the same specific activity as the antibiotic. This was found in practice (Table II). Taken together these results lead to the inescapable conclusion that only pathway c is compatible with these results and pathway a and b cannot be operative. This, therefore, established that the ring cleavage of cyclodopa occurs extradiol (meta), as is also the case with anthramycin, lincomycin A, and probably sibiromycin.

Chemical degradation of DMT labeled from DL-[7a-^{14}C]tryptophan revealed that all of the activity from this amino acid resides with the DMAA moiety. The origin of the 5-methoxy-4-hydroxyanthranilic acid from tryptophan probably involved a diversion of the well-known metabolic pathway leading to the formation of anthranilic acid from L-tryptophan (Greenberg, 1969). The double-labeling experiment with L-[Me-^{14}C, Me-^{3}H$_3$]methionine and subsequent degradation indicates that the methyl group is transferred intact, barring an unexpected tritium isotope effect. (Chemical degradation of tomaymycin biosynthetically labeled from L-[*methyl*-^{14}C, *methyl*-^{3}H$_3$]methionine gave DMAA which had an 88% tritium retention.) Our results are compatible with the proposed building blocks shown in Scheme V.

SCHEME V: Building Blocks for DMT.



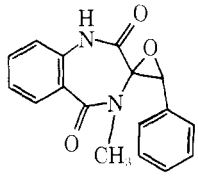
In an effort to learn more about the structures of the possible intermediates in DMT biosynthesis before we embarked upon lengthy synthetic procedures designed to ultimately test the intermediacy of these compounds in DMT biosynthesis, we

[3] Taking into account the 50% loss of tritium from L-[*Ala*-2,3-^{3}H]tyrosine during its conversion to DMT and OT then retentions of 7 or 6 carbons of L-tyrosine in DMT or OT will be $\frac{7}{9} \times \frac{1}{2} \times 100 = 64.3\%$, $\frac{6}{9} \times \frac{1}{2} \times 100 = 75\%$, respectively.

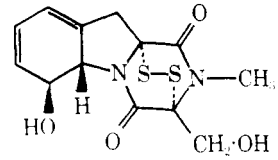
carried out a number of double-labeling experiments with tyrosine and tryptophan.

The existence of the fungal metabolites cyclopenin (Mohammed, 1963) and cyclic dipeptides, such as gliotoxin (Brannon et al., 1971) and brevianamide A (Baldas et al., 1974) (Scheme VI), led us to suspect that a cyclopenin-like

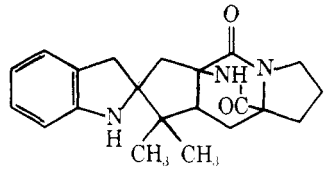
SCHEME VI: Structure of Cyclopenin, Gliotoxin, and Brevianamide A.



cyclopenin



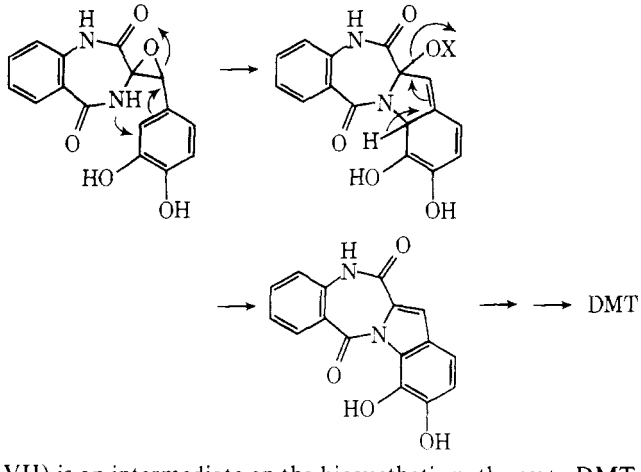
gliotoxin



brevianamide A

compound might be an intermediate in DMT, OT, anthramycin, and sibiromycin biosynthesis. An epoxide similar in structure to cyclopenin could be looked upon as the reactive intermediate that undergoes cyclization to form cycloanthranilylcyclodopa (Scheme VII). If such an epoxide (Scheme

SCHEME VII: Postulated Mechanism for the Formation of the Pyrrolo Ring of DMT.



VII) is an intermediate on the biosynthetic pathway to DMT, then the α-hydrogen and one of the two β hydrogens of tyrosine must be lost during conversion of this amino acid to DMT. In order to test this hypothesis, L-[1-^{14}C,Ala-2,3-^{3}H]tyrosine was added to a culture of *S. achromogenes* and the DMT was isolated in radiochemically pure form. This sample of DMT showed a tritium retention of 50% (Table III). The relative distribution of tritium between the α hydrogen and the two β hydrogens in L-[Ala-2,3-^{3}H]tyrosine has recently been published (Kirby et al., 1975). Results of the study show that as expected 50% of the total activity resides in the α carbon and the remaining 50% of activity is distributed unequally between the *pro-R* (8.5%) and *pro-S* (41.5%) hydrogens at the β carbon.[4] Therefore, our 50% retention of tritium in DMT and OT is most likely related to the loss of just the α hydrogen of tyrosine.[5] Therefore, our conclusion from this experiment is that the epoxide shown in Scheme VII is not a feasible intermediate in DMT biosynthesis.

Since DMT and OT are hydroxylated in the aromatic ring, it was of interest to determine whether these hydroxylation reactions involved an intramolecular migration of aromatic ring substituents (the NIH shift). Such migrations have been demonstrated in a variety of biological systems including plant, animal, and bacterial tissues (Daly et al., 1972). The wealth of knowledge on the NIH shift has led to the formulation of a number of basic rules concerning the migration or loss of the hydrogen atom from the carbon atom at which hydroxylation has occurred and, consequently, the order in which substituents are inserted into the aromatic ring (Daly et al., 1972).

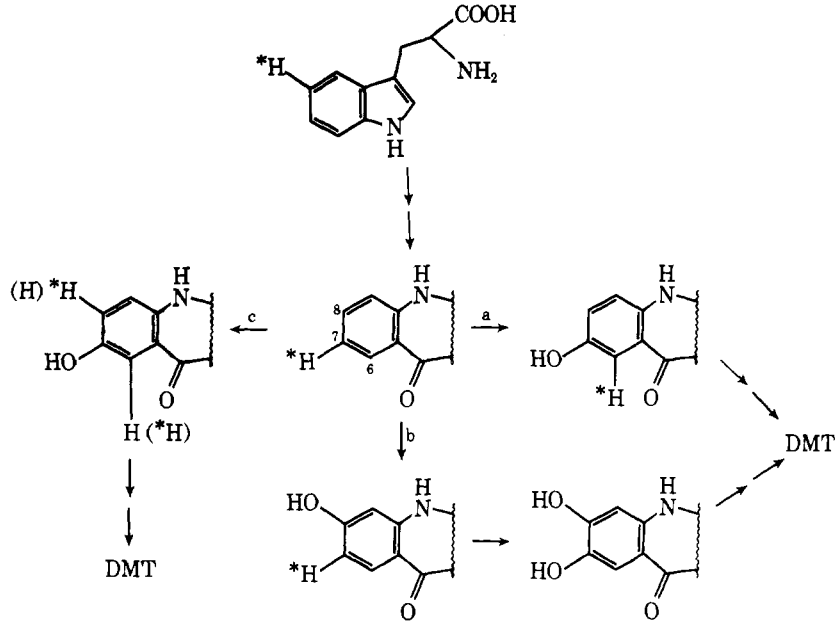
The possible pathways for conversion of DL-[5-^{3}H]tryptophan into DMT are as shown in Scheme VIII. Selective migration of displaced tritium to C-6 during hydroxylation at C-7 but prior to hydroxylation at C-8 would result in 85% or greater retention of tritium in DMT (pathway a). Nonselective migration of tritium to C-6 and -8, during hydroxylation at C-7 but again prior to hydroxylation at C-8 would result in about 42–47% retention in DMT (pathway c). Hydroxylation at C-8 prior to hydroxylation at C-7 would result in complete loss of tritium during the conversion of DL-[5-^{3}H]tryptophan to DMT (pathway b). The experimentally determined retention of 16% (Table III) can be explained in two ways. First, it is suggested that b is the major pathway, with a or c being minor alternate pathways, or second, that a modification of pathway c is operative such that an 84% migration to C-8 and a 16% migration to C-6 occurs during the first hydroxylation reaction. The DL-[5-^{3}H]tryptophan used in this experiment was prepared by catalytic dehalogenation of 5-bromo-DL-tryptophan with tritium gas (Research Products International). This procedure has been demonstrated to produce tryptophan with greater than 90% of the tritium in the 5 position (Renson et al., 1966), and, therefore, our retention cannot adequately be explained by nonspecific labeling. Finally, the possibility that 5-hydroxytryptophan is an intermediate on the pathway to DMT has been excluded, since this substrate, fed in labeled form (see Table I) with tritium predominately in the aromatic ring, was not detectably incorporated (i.e., less than 0.1%).

The next step in our study of the biosynthesis of DMT and OT was to determine the biogenetic relationship between these two compounds. Upon feeding in parallel flasks DMT and OT (both substrates were prepared in radiolabeled form in a previous biosynthetic experiment in which DL-[1-^{14}C]tyrosine had been fed) to 72-h cultures of *S. achromogenes*, the results shown in Table IV were obtained. This data reveals that, while DMT was converted into OT, the reverse does not occur. It is also interesting to note that while virtual complete recovery of radioactivity from the flask-fed OT was found both at 24 and 48 h, in contrast, after addition of DMT only about two-thirds of the total radioactivity could be recovered. The explanation for this observation is probably that, while some of the biologically active DMT becomes bound to the *S. achro-*

[4] The relative distribution of radioactivity between carbon atoms 2 and 3 was also checked by us on our sample of L-[*Ala*-2,3-^{3}H]tyrosine using the racemization procedure described by Kirby et al. (1975). Approximately 50% of the activity was lost during this procedure.

[5] The incorporation of DL-[1-^{14}C,3*RS*-^{3}H)-tyrosine and the two stereospecifically labeled substrates, DL-[1-^{14}C,(3*S*)3-^{3}H]tyrosine, DL-[1-^{14}C,(3*R*)3-^{3}H]tyrosine, together with the equivalent deuterated substrates in DMT, anthramycin and sibiromycin will be the subject of another publication (L. Hurley, N. Das, and R. Parry, manuscript in preparation). However, our results with these radioactively labeled substrates into DMT do show complete retention of tritium in this antibiotic substantiating our conclusion that both β hydrogens of tyrosine are retained in DMT.

SCHEME VIII: Alternative Pathways for the Conversion of DL-[5-^{3}H] Tryptophan into DMT.



mogenes DNA and therefore becomes unavailable for reextraction into ethyl acetate, the biologically inactive OT does not bind to DNA and is completely available for reextraction into ethyl acetate.

The bioconversion or inactivation of DMT through its conversion into OT was further examined in washed cell preparations. The kinetics of conversion of DMT to OT in a 72-h-washed cell preparation is shown in Figure 3. In this experiment, DMT biosynthetically radiolabeled from DL-[1-^{14}C]tyrosine was added to a 100-ml concentrated washed cell preparation. Aliquots of 5 ml were taken at appropriate times and worked up in the usual manner to determine the amount of conversion of DMT to OT. Uptake of DMT from the medium into the cells was also measured and found to occur initially in a linear manner, then leveling out by about 60 min at which time 42.6% of the added [11-^{14}C]DMT had been taken up from the medium. Control experiments in which a boiled washed cell preparation or buffer was used did not show appreciable conversion of DMT to OT (Table V). An incubation with broth from which the mycelium has been removed by filtration did show a small amount of conversion of DMT into OT, but this was probably due to traces of cells which had passed through the filter.

The ability of different aged mycelium to carry out this conversion was also tested. Washed cell preparation from the trophophase (44 and 60 h) and idiophase (78 and 102 h) of equal density were prepared, radiolabeled DMT was added, and the preparations were incubated for 12 h. The results in Figure 4 show that comparable conversion of DMT into OT and DMT recovery are found at 44, 60, and 78 h, thus indicating that this enzymatic activity is not restricted to the idiophase. The markedly lower conversion of DMT to OT at 102 h is probably due to a larger competitive DNA binding reaction, which occurs due to release of DNA into the medium after lysis of the cells and possibly enzyme inactivation in these advanced cultures.

TABLE IV: Conversion of DMT to OT by *S. achromogenes* Culture.

Species Fed	Incubation Time (h)	% of Added Total Act. Recovered in DMT	% of Added Total Act. Recovered in OT	% Not Extractable into Ethyl acetate
OT	24	0	98	2
OT	48	0	92	8
DMT	24	34	30	36
DMT	48	24	36	40

Discussion

By a combination of feeding experiments with specifically labeled amino acids and chemical degradation of the resulting biosynthetically labeled DMT molecues, the building blocks for DMT have been established as tryptophan, tyrosine, and methionine. The manner in which these amino acids are incorporated into DMT is as shown in Scheme V.

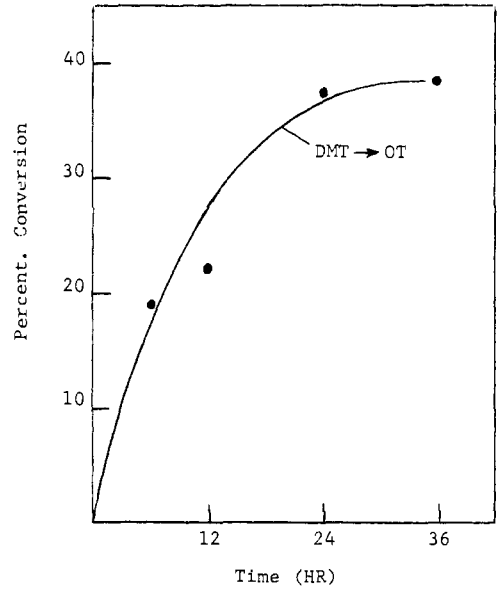


FIGURE 3: Time-course conversion of [11-^{14}C]DM into OT by a 78-h washed cell preparation. 1.05 × 10^5 dpm of DTM was added to 100 ml of the washed cell preparation and 20-ml aliquots were taken at the times indicated.

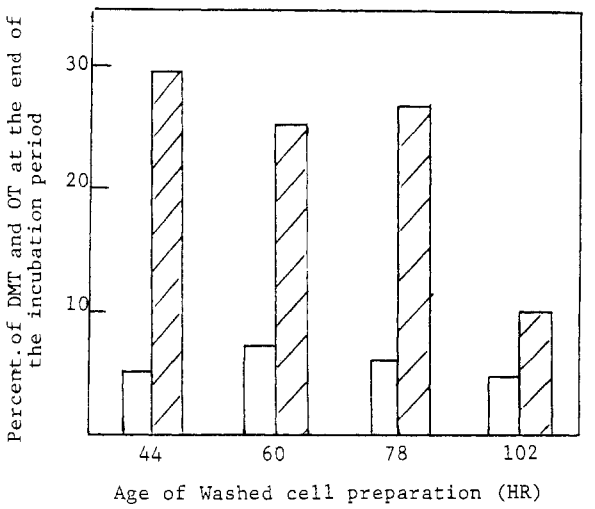


FIGURE 4: Conversion of DMT into OT by different aged washed cell suspensions of *S. achromogenes*. At the times indicated DMT (5.25×10^4 dpm/25 ml of suspension) was added to the washed cell preparation and incubated for 12 h. (□) Recovered DMT; (▨) OT produced.

TABLE V: Conversion of DMT to OT by 78-h Washed Cell Preparation and Various Controls. DMT (6.0×10^4 dpm) Was Added to 25 ml of the Incubation System.

Incubation Conditions	Incubation Time (h)	DMT Recovered (%)	OT Converted (%)
Washed cells	6	6.7	22.3
Washed cells	12	5.8	27.0
Boiled cells[a]	6	50.0	3.05
Boiled cells	12	26.3	1.37
Broth[b]	6	64.4	7.14
Broth	12	63.4	6.97
Buffer[c]	6	93.0	<0.1
Buffer	12	95.0	<0.1

[a] A 25-ml suspension of the washed cells was heated in a water bath at 100 °C for 30 min. [b] The broth was obtained by filtering the culture under sterile conditions through Whatman No. 1 filter paper to remove mycelium. [c] Phosphate buffer, 0.02 M, pH 7.1.

The incorporation of tryptophan, labeled in the aromatic ring, exclusively in the MHAA moiety of DMT and presumably OT is analogous to the biosynthesis of anthramycin, although the aromatic substitution pattern differs in each antibiotic. The predominant loss of tritium from DL-[5-^{3}H]tryptophan during its conversion to DMT and OT is interpreted to mean either that the major pathway for DMT biosynthesis requires hydroxylation at C-8 prior to hydroxylation at C-7, or that only partially selective migration of tritium occurs during the first hydroxylation at C-7. In sibiromycin a 91% retention of tritium from DL-[5-^{3}H]tryptophan is found (Hurley et al., 1976), indicating that in this case migration of tritium from C-7 occurs either selectively to C-6 in a substrate where C-6 and -8 are unsubstituted or that hydroxylation at C-7 occurs after methylation at C-8. In any event, this latter example does indicate that NIH shifts do occur in actinomycetes and, therefore, our interpretation of data leading to the prediction of the order in which the hydroxylation reactions take place in DMT and OT is not unreasonable. This report apparently provides the first example of an antibiotic containing a C_2-proline moiety in which the carbon skeleton is derived in its entirety from tyrosine. Argoudelis et al. (1973) have reported that the C_2-proline moiety of lincomycin B is derived from tyrosine (6 carbons) and methionine (1 carbon). On the other hand, our results do fall in line with anthramycin, lincomycin A, and sibiromycin[6] in that "meta cleavage" of 6,7-dihydroxycyclodopa is implicated. In this respect, the proposed "meta cleavage" of 5,6-dihydroxycyclodopa to give rise ultimately to the C_2-proline moiety of lincomycin B seems unlikely considering the generality of the pathway to the other antibiotics.[7] Additional evidence that the "meta cleavage" pathway is operative in the biosynthesis of these antibiotics is that we are able to isolate from the acid phases of all our pyrrolo[1,4]benzodiazepine antibiotics-producing strains a very unstable compound that has all the characteristics of a typical α-hydroxymuconic semialdehyde, which results from meta cleavage of a *o*-diphenol.

The results of our feeding experiment with L-[1-^{14}C, *Ala*-2,3-^{3}H]tyrosine indicate that the α hydrogen of tyrosine is lost prior to conversion into DMT and OT, while both β-hydrogens are retained in these compounds. In an experiment with L-[1-^{14}C, *Ala*-2,3^{3}H]tyrosine in which pool and protein tyrosine were examined, it was found that a tritium loss in excess of 50% was found in both cases. This indicates that the loss of the α hydrogen of tyrosine in DMT and OT may not necessarily be directly related to the biosynthesis of these compounds. While it is highly likely that the two β hydrogens are located at C-1 of DMT, this conclusion should be treated with some caution at this time in the absence of stable isotope (deuterium labeled) experiments or exhaustive chemical degradation studies. Further studies in our laboratory utilizing specifically deuterated molecules are in progress to substantiate this result. The biogenetic relationship between DMT and OT was particularly interesting, since OT is biologically inactive and its production from DMT might represent a means to protect the cells against intracellular antibiotic that fails to be excreted or DMT retaken up by the cells. Our studies show that the conversion of DMT to OT is due to an intracellular enzyme that is active during both trophophase and idiophase. It was also found that cultures of *S. achromogenes* that have lost their ability to produce DMT were capable of converting the antibiotic to OT. This is suggestive that the DMT metabolizing enzyme is constitutive. The enzyme that inactivates chloramphenicol, chloramphenicol hydrase (Malik and Vining 1971), has similar characteristics in that the enzyme is also found in chloramphenicol nonproducing cultures, and is intracellular.

Acknowledgments

We are indebted to the Fujisawa Pharmaceutical Co. for providing us with samples of tomaymycin and OT through Dr. Kohsaka. We also acknowledge the technical assistance of M. Zmijewski.

References

Argoudelis, A. D., Eble, T. E., Fox, J. A., and Mason, D. J. (1973), *J. Antibiot. 26*, 142.

[6] Our preliminary results on sibiromycin indicate that some retention of tritium from L-[1-^{14}C,3- or 5-^{3}H]tyrosine occurs in sibiromycin compatible with a "meta cleavage" pathway.

[7] After this manuscript was submitted for publication Rolls et al. (1976) reported experiments using stable isotopes demonstrating that the C_2-proline unit of lincomycin B is derived in an analogous fashion to the C_2-proline unit of DMT. This, therefore, refutes the previous finding by Argoudelis et al. (1973) and confirms the generality of the "meta cleavage" pathway to these antibiotics.

Arima, K., Kohsaka, M., Imanaka, H., and Sakai, H. (1972), *J. Antibiot. 25,* 437.
Baldas, J., Birch, A. J., and Russell, R. A. (1974), *J. Chem. Soc., Perkin Trans. 1* 50.
Brannon, D. R., Mabe, J. A., Molloy, B. B., and Daly, W. A., (1971), *Biochem. Biophys. Res. Commun. 43,* 588.
Daly, J. W., Jerina, D. M., and Witkop, B. (1972), *Experientia 28,* 1129.
Greenberg, D. M. (1969), *Metab. Pathways 3rd Ed.* 153.
Heidelberger, M., and Jacobs, W. A. (1919), *J. Am. Chem. Soc. 41,* 2142.
Hurley,, L. H., Das, N., Gairola, C., and Zmijewski, M. (1976), *Tetrahedron Lett.* 1419.
Hurley, L. H., Gairola, C., and Zmijewski, M. (1975a), *J. Chem. Soc., Chem. Commun.* 120.
Hurley, L. H., Zmijewski, M., and Chang, C-J. (1975b), *J. Am. Chem. Soc. 97,* 4372.
Kariyone, K., Yazawa, H., and Kohsaka, M. (1971), *Chem. Pharm. Bull. 19,* 2289.
Kirby, G. W., Narayanaswami, S., and Rao, P. S. (1975), *J. Chem. Soc., Perkin Trans. 1* 645.
Malik, V. S., and Vining, L. C. (1971), *Can. J. Microbiol. 17,* 1287.
Mohammed, Y. (1963), *Tetrahedron Lett.* 1953.
Nishioka, Y., Beppu, T., Kohsaka, M., and Arima, K. (1972), *J. Antibiot. 25,* 660.
Renson, J., Daly, J., Weissbach, H., Witkop, B., and Udenfriend, S. (1966), *Biochem. Biophys. Res. Commun. 25,* 504.
Rolls, J. P., Ruff, B. D., Haak, W. J., and Hessler, E. J. (1976), *Abstr. Annu. Meet. A.S.M. 76th* 027.
Simon, H., and Floss, H. G. (1967), Bestimmung der Isotopenverteilung in markierten verbendungen, West Berlin and Heidelberg, Springer-Verlag, p 12.
Witz, D. F., Hessler, E. Z., and Miller, T. L. (1971), *Biochemistry 10,* 1128.

Coumermycin A_1: A Preferential Inhibitor of Replicative DNA Synthesis in *Escherichia coli.* I. In Vivo Characterization†

Michael J. Ryan‡

ABSTRACT: Coumermycin A_1, an antibiotic related to novobiocin, inhibited nucleic acid synthesis in intact *Escherichia coli* with replication being slightly more sensitive to this drug than transcription. The ultraviolet-induced repair synthesis of DNA was only partially inhibited under conditions where replication was eliminated by coumermycin A_1. Inhibition of protein synthesis was a secondary effect. Coumermycin A_1-resistant *E. coli* were isolated and the mutation was mapped near *dna*A. Chromatography of crude protein extracts of sensitive and resistant bacteria on drug affinity columns implicated a soluble protein of approximately 37,000 molecular weight as the target site for coumermycin A_1. Depending on the medium used, this antibiotic had either a bacteriocidal or a bacteriostatic effect on *E. coli.* Results showed that the effect of coumermycin A_1 cannot be explained by the degradation of DNA under bacteriocidal growth conditions.

Although a relatively large number of antibiotics inhibit chromosomal replication by interacting with the DNA template or by inhibiting the synthesis of precursors (Corcoran and Hahn, 1975; Kersten and Kersten, 1974; Gale et al., 1972), few interfere with the actual polymerization reactions. Only one compound, the chemically synthesized 6-(*p*-hydroxyphenylazo)uracil, specifically inhibits a DNA polymerase from *Bacillus subtilis* (Mackenzie et al., 1973; Gass et al., 1973).

This is surprising in view of the fact that a substantial segment of contemporary cancer research is aimed at the biochemistry and pharmacology of antitumor agents, many of which interfere with some aspect of nucleic acid metabolism. One reason for our meager understanding lies in the nature of the mechanism of DNA replication, which is still unclear relative to those for transcription and translation. However, this position has improved considerably with the recent classification of thermosensitive mutants in DNA replication (Wechsler and Gross, 1971) and the reconstitution of an in vitro system for DNA replication (Wickner and Hurwitz, 1974; Schekman et al., 1975).

Coumermycin A_1 and novobiocin (Kawaguchi et al., 1965a; Berger et al., 1966) are related coumarin and carbohydrate-containing antibiotics (Figure 1) produced by *Streptomyces.* Coumermycin A_1 is particularly active against *Staphylococcus aureus,* inhibiting the growth of many strains at a very low concentration (0.004 μg/ml) (Kawaguchi et al., 1965b; Grunberg and Bennett, 1966; Fedorko et al., 1969). The antimicrobial spectrum of both drugs was qualitatively very similar, but coumermycin A_1 was up to 50-fold more active than novobiocin (on a weight basis). The mode of action of novobiocin has been widely studied in a variety of systems but its elucidation has been complicated by a number of pleiotropic, secondary effects (Brock, 1967). However, one study with *E. coli* indicated that this antibiotic inhibited the in vivo synthesis of DNA (Smith and Davis, 1967). Recent in vitro studies

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