Review Article

PYRROLO(1,4)BENZODIAZEPINE ANTITUMOR ANTIBIOTICS. COMPARATIVE ASPECTS OF ANTHRAMYCIN, TOMAYMYCIN AND SIBIROMYCIN

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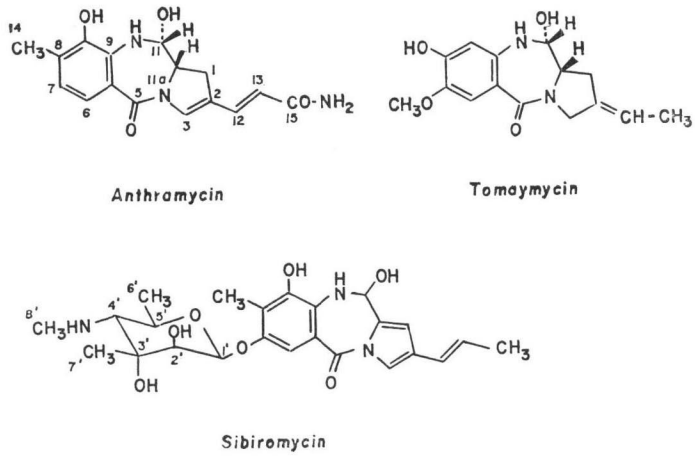
Anthramycin, sibiromycin and tomaymycin are structurally related antibiotics produced by various actinomycetes. Anthramycin was originally isolated from the fermentation broth of a thermophilic actinomycete, *Streptomyces refuineus* var. *thermotolerans* found in a compost heap in the 1950's by M. D. TENDLER[1]. The active compound, originally called "refuin" (from the Hebrew "refuah" meaning a medicine), was isolated as a pure crystalline antibiotic by LEIMGRUBER[2,3] in 1965. This antibiotic was subsequently shown to have antibiotic, antitumor, antiprotozoal and chemosterilant activity against houseflies. Tomaymycin, a Japanese antibiotic, produced by *Streptomyces achromogenes* var. *tomaymyceticus,* was isolated from a soil sample collected in Musashikoganei-city. The isolation and properties of this antibiotic were first reported by ARIMA and coworkers[4] in 1972. The structure of tomaymycin and the structurally related but biologically inactive compound, oxotomaymycin, were reported by KARIYONE[5] in 1971. Tomaymycin has been shown to have antitumor, antiviral and antibiotic activities. Sibiromycin, the most recent of the three antibiotics to be fully characterized, is produced by the actinomycete, *Streptosporangium sibiricum* and was first reported by GAUSE and coworkers[6] at the Moscow Institute for New Antibiotics. The isolation and partial characterization of this antibiotic was published in 1972.[7] However, its full structure was not published until 1974 by MESENTEV and coworkers.[8] Sibiromycin has been shown to have antitumor as well as antibiotic activity. In addition to anthramycin, sibiromycin and tomaymycin, three further structurally related antibiotics have appeared in the literature, although full structural information has not been published. The first of these compounds, dextrochrysin which is produced by *Streptomyces calvus* var. *dextrochrysus*[9], has been demonstrated to have antiviral as well as antibiotic activity. Most recently two isomeric anthramycin-related compounds, neothramycins A and B produced by *Streptomyces* No. MC916-C4[10] have been reported. These compounds have been shown to have weak antibiotic and antifungal activity as well as antitumor activity. Reviews on the mechanism of action of anthramycin[11,12] and sibiromycin have appeared.[13~15]

Chemistry

The structures of anthramycin, tomaymycin and sibiromycin are shown in Fig. 1. A structural feature common to all three antibiotics is the pyrrolo(1,4)benzodiazepine nucleus. The antibiotics differ in the following respects: (1) The side chain at C-2 may either be an acrylamide (anthramycin), ethylidene (tomaymycin) or a propylidene (sibiromycin) group, (2) the aromatic substitution pattern may

Fig. 1. Structures of the pyrrolo(1,4)benzodiazepine antitumor antibiotics.



either be 8-methyl-9-hydroxy (anthramycin), 7-methoxy-8-hydroxy (tomaymycin), or 7-sibirosaminide-8-methyl-9-hydroxy (sibiromycin), and (3) the pyrrolo ring has either two degrees of unsaturation (sibiromycin), one degree of unsaturation (anthramycin) or is completely saturated (tomaymycin).

The absolute configuration of anthramycin[16] and tomaymycin[5] at C–11a has been demonstrated to be "S", by chemical synthesis starting from L-hydroxyproline. The relative configuration of the two asymmetric centers (11 and 11a) of anthramycin[3] and tomaymycin[17] has been assigned from their N.M.R. spectra, leading to the conclusion that the absolute configuration at C–11 of both antibiotics is "R". Information on the configuration of sibiromycin at C–11 is not published. The trans stereochemistry for both anthramycin[3] and sibiromycin[8] at C–12 and 13 has been deduced from the coupling constants for the olefinic protons.

The structure of anthramycin has been determined by complete chemical synthesis[16] and a simplified synthetic procedure for the synthesis of the anthramycin skeleton described by STEVANS[18]. The structure of tomaymycin[5] and the aglycone moiety of sibiromycin[8] have been determined by a combination of chemical degradation and partial synthesis. The amino sugar moiety of sibiromycin, sibirosaminide, has been shown, utilizing a combination of chemical degradation and spectroscopic evidence, to be 4-methylamino-4,6-dideoxy-3-C-methyl-β-D-altropyranoside[19].

The literature on tomaymycin is confusing, since although the parent antibiotic is the 11-hydroxy derivative, the original workers have used the name tomaymycin to refer to the 11-methoxy compound. This latter compound is only formed upon heating the 11-hydroxy derivative in methanol, whereupon the 11-methyl ether is formed. The names for the other two known antibiotics in this series, anthramycin and sibiromycin, are reserved for the parent 11-hydroxy antibiotics. In order to avoid unnecessary confusion I will use the name tomaymycin to refer to the 11-hydroxy compound. The 11-methyl ethers will then be designated, anthramycin methyl ether (AME), tomaymycin methyl ether (TME) and sibiromycin methyl ether.

All three antibiotics are usually isolated as their more chemically stable 11-methyl ether derivatives, which are formed upon recrystallization from boiling methanol. AME and TME are rapidly hydrolyzed *via* the anhydro-(mycin) to yield anthramycin (tomaymycin) and the 11-epi compounds[2] (see Fig. 2). Sibiromycin, on the other hand, when converted into anhydrosibiromycin is not readily

Fig. 2. Interconversion of AME and TME *via* the anhydro compounds to anthramycin (tomaymycin) or epianthramycin (epitomaymycin).

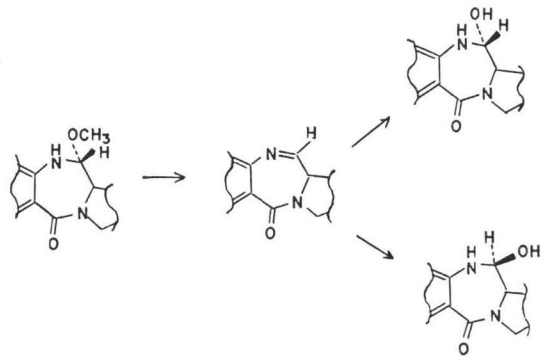


Table 1. Table of comparative physical and chemical properties of the pyrrolo(1,4)benzodiazepine antibiotics.

	Anthramycin[2]	Sibiromycin[7]	Tomaymycin methyl ether[4]	Dextro-chrysin[9]	Neothramycin A[10]	Neothramycin B[10]
Melting point	120 (dec.)	120 (dec.)	145~146 (dec.)	250~255 (dec.)	132~147 (dec.)	144~151 (dec.)
Molecular formula	$C_{16}H_{17}N_3O_4$	$C_{24}H_{31}N_3O_7$	$C_{16}H_{20}N_2O_4$	not recorded	$C_{13}H_{14}N_2O_4 \cdot 1/2H_2O$	$C_{13}H_{14}N_2O_4 \cdot 1/2H_2O$
Elemental analysis	C 61.17 H 5.56 N 13.26	C 61.50 H 7.00 N 9.20	C 62.95 H 6.66 N 9.05	C 61.47 H 6.82 N 10.25	C 57.46 H 5.76 N 9.84	C 57.00 H 5.58 N 9.75
UV Spectrum	CH_3CN λ_{max} nm (E) 235 (18,200) 333 (31,800)	CH_3OH λ_{max} nm (E) 230 (25,950) 310 (21,800)	CH_3OH λ_{max} nm (E) 224 (36,000) 235s(30,000) 260s(9,000) 320 (3,600)	CH_3OH λ_{max} nm ($E^{1\%}_{1cm}$) 240 (410) 335 (850)	CH_3OH λ_{max} nm (E) 223 (22,400) 240 (sh) 265 (7,600) 318 (4,100)	CH_3OH λ_{max} nm (E) 224 (24,200) 240 (sh) 265 (sh) 318 (4,380)
$[\alpha]_D^{20}$	+930° (*c* 1.00, DMF)	+525° (DMF)	+423° (*c* 0.5, pyridine)	+837.9° (*c* 1, DMF)	+272° (*c* 0.52, dioxane)	+314° (*c* 0.48, dioxane)
TLC on silica gel (A) EtOAc - MeOH, 4:1 (B) $CHCl_3$ - MeOH, 10:1	Rf 0.5 (A)	Rf 0.25 (A)	Rf 0.62 (A)	Rf 0.46(A)	Rf 0.57 (B)	Rf 0.50 (B)

reconverted to the parent compound[8]. This is probably because the 10,11 double bond is less reactive due to its conjugation in the sibiromycin molecule.

A comparison of some of the physical and chemical properties of these three antibiotics and dextrochrysin and neothramycins A and B is shown in Table 1.

Biosynthesis

Studies in my laboratory have clearly demonstrated that anthramycin, sibiromycin and tomaymycin are biogenetically as well as structurally closely related. Radioisotope labelling experiments have shown that anthramycin, tomaymycin and the aglycone moiety of sibiromycin are biogenetically derived from tryptophan, tyrosine and methionine (see Table 2). The distribution of the radioactivity in the antibiotics derived from feeding experiments with DL-[7a-^{14}C] tryptophan, L-[U-^{14}C] tyrosine and L-[CH_3-^{14}C] methionine, as determined by chemical degradation is shown in Fig. 3.

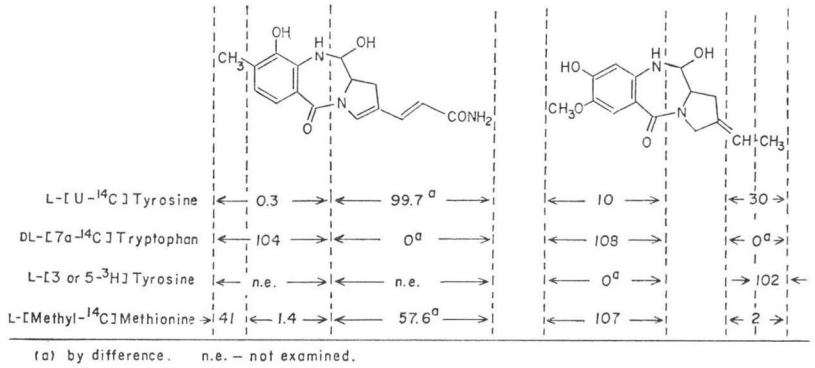
These results led us to speculate that the biogenetic building blocks for anthramycin, sibiromycin

Table 2. Incorporation of labelled substrates into anthramycin, tomaymycin and sibiromycin[24~26].

Precursor Fed	% Incorporation into		
	Anthramycin	Tomaymycin	Sibiromycin
L-[1-^{14}C]Dihydroxyphenylalanine	22.1	6.9	18.4
L-[Methyl-^{14}C]methionine	16.4	17.8	35.2
DL-[7a-^{14}C]Tryptophan	13.7	6.1	8.1
L-[1-^{14}C]Tyrosine	10.4	10.1	15.4
L-[U-^{14}C]Tyrosine	11.7	7.6	9.9
D-[6-^{14}C]Glucose	<0.2	n.e.	2.1
[1-^{14}C]Acetic acid	<0.1	n.e.	n.e.
L-[U-^{14}C]Phenylalanine	<0.1	n.e.	n.e.
L-[U-^{14}C]Proline	<0.1	n.e.	n.e.
L-[Ala-3-^{14}C]Tryptophan	<0.3	n.e.	n.e.
[4-^{14}C]-δ-Aminolevulinic acid	<0.1	n.e.	n.e.

n.e. — not examined.

Fig. 3. Relative distribution of radiolabel from fed precusors in anthramycin and tomaymycin.



and tomaymycin were as shown in Figs. 4 and 5.

Substantiation of the intermediacy of kynurenine and its derivatives in the biosynthesis of the anthranilic acid moiety of sibiromycin was established by competition experiments[20]. In addition, 4-methyl-3-hydroxy-(2-^{14}C) anthranilic acid was efficiently incorporated into this antibiotic. Since (2-^{14}C) anthranilic acid was not incorporated directly into tomaymycin, this led us to suspect derivatization of the aromatic acid ring of tomaymycin also takes place at the kynurenine stage. Corresponding results with anthramycin were inconclusive due to the impermeability of the cells that produced this antibiotic. Some information on the order in which the substituents are introduced into the aromatic ring of tomaymycin was obtained by feeding DL-[5-^{3}H] [7a-^{14}C] tryptophan to the producing organism and determining the tritium retention in the antibiotic[21]. The low tritium retention in tomaymycin (17%) by consideration of NIH shift rules[22] was indicative that the most likely pathway involved insertion of the 8 hydroxy substituent prior to the 7 hydroxy group. When the same species of tryptophan was fed to the sibiromycin-producing culture an 85% retention of tritium was found in the antibiotic indicative of an NIH shift. These results led us to suggest the general scheme shown in Fig. 6 for the biosynthesis of the anthranilic acid moieties of anthramycin, tomaymycin and sibiromycin.

Fig. 4. Building blocks for the anthranilate moieties of anthramycin, tomaymycin and sibiromycin.

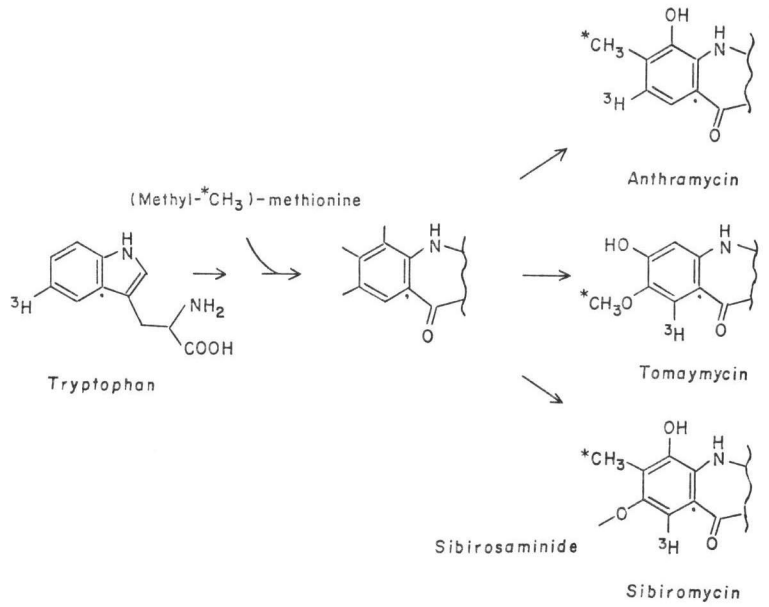
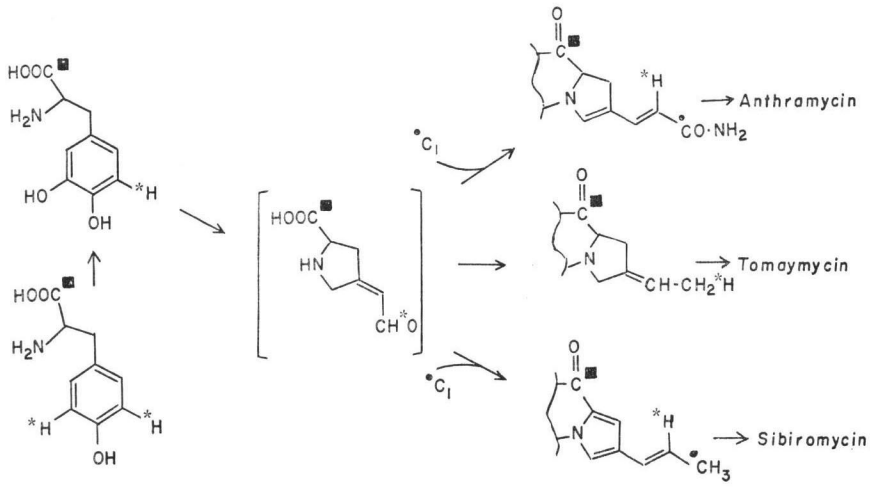


Fig. 5. Building blocks for the C_2 and C_3 proline moieties of anthramycin, tomaymycin and sibiromycin.



Of prime interest in our biosynthetic studies was the origin of the C_2 and C_3-proline moieties of anthramycin, sibiromycin and tomaymycin (see Fig. 7). The first step was to determine the biogenetic building blocks for each of these moieties. This was accomplished by a combination of radio and stable isotope techniques. Tyrosine and dihydroxyphenylalanine (dopa) were both found to serve as excellent precursors for all three antibiotics (Table 2). In addition a comparison of incorporation values for L [U-^{14}C] tyrosine and L-(1-^{14}C) tyrosine, using L-(3 or 5)^{3}H-tyrosine or L-(2 or 6)^{3}H-tyrosine internal reference labels, demonstrated that this amino acid contributed 7 of its 9 carbon atoms to each of these antibiotics[23~25]. This then accounted for 7 of the 8 carbon atoms of the C_3-proline moieties of anthramycin and sibiromycin and all 7 carbon atoms of the C_2-proline moiety of tomaymycin.

The manner in which tyrosine was incorporated into these units was elucidated using L-(1-^{13}C) tyrosine[25,26] and L-(3-5)$^{2}H_2$ tyrosine[26] or L-(3 or 5)^{3}H tyrosine[24] in combination with carbon-13 NMR, proton-NMR or chemical degradation respectively. The labelling pattern established is shown in Fig. 5. The origin of the eighth carbon atom of the C_3-proline unit of anthramycin and sibiromycin was found to be the S-methyl group of methionine. The biogenetic fate of the carbon atom derived from methionine should then be the amide carbonyl (C–15) or the propylidene methyl (C–15) of anthramycin and sibiromycin respectively. This anticipated labelling pattern was confirmed using L-(CH_3–^{13}C) methionine in conjunction with carbon-13 NMR[25,26]. Since some possible ambiguity in the carbon-13 NMR assignment between C–5 and C–15 existed in anthramycin, confirmation for the carbon-13 enrichment specifically at C–15 was obtained by examination of the proton-NMR for long-range ^{1}H–^{13}C spin-spin coupling[27].

Fig. 6. Proposed general pathway for the conversion of tryptophan to the anthranilate moieties of anthramycin, tomaymycin and sibiromycin.

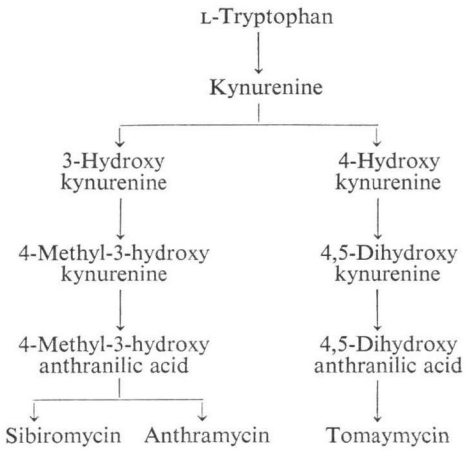


Fig. 7. C_2 and C_3-proline moieties of the pyrrolo(1,4)benzodiazepine antibiotics.

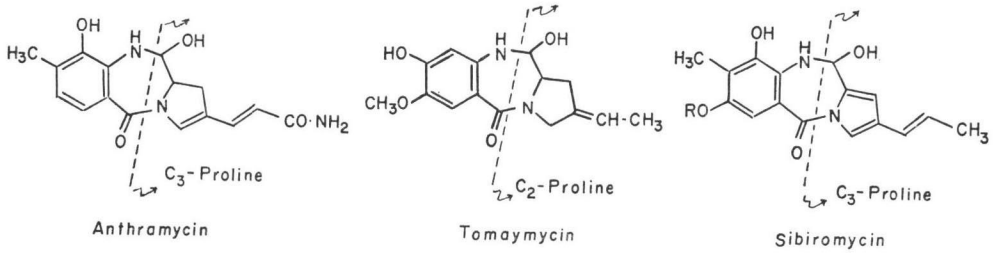
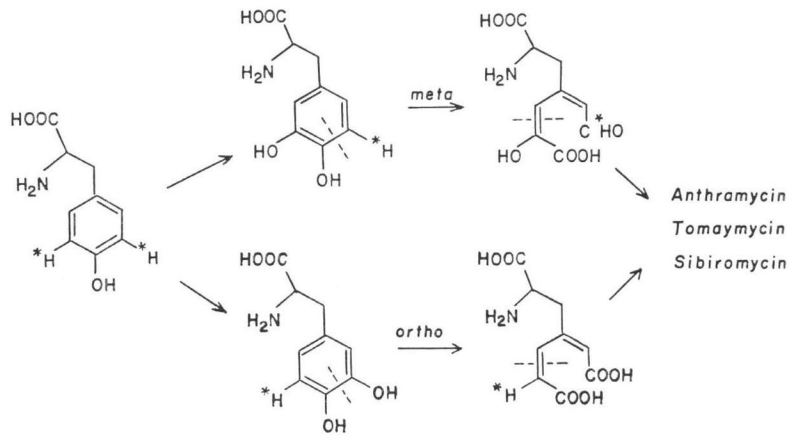


Fig. 8. Alternative pathways for the conversion of L-[3 or 5-^{3}H]tyrosine into the C_2 or C_3-proline moieties involving either *ortho* or *meta* cleavage.



During the conversion of tyrosine through dopa to the C_2 and C_3-proline moieties of the pyrrolo-(1,4)benzodiazepine antibiotics ring cleavage of the aromatic ring must take place. At least two types of ring cleavage are possible, either intradiol (*ortho*) or extradiol (*meta*) cleavage. These two alternative pathways are shown in Fig. 8. It is possible to differentiate between these types of ring cleavage because *meta* cleavage will lead to retention of half of the tritium from L-[3 or 5-^{3}H] tyrosine in the antibiotics, whereas *ortho* cleavage will lead to complete loss of tritium during the conversion of tyrosine to anthra-

Table 3. Incorporation of double-labeled precursors into anthramycin, tomaymycin and sibiromycin[24~26].

Precursor	% Retention of ^{3}H in		
	Anthramycin	Tomaymycin	Sibiromycin
L-[1-^{14}C,3 – or 5-^{3}H]Tyrosine	51	48	17
L-[1-^{14}C,2 – or 6-^{3}H]Tyrosine	52	86	72

Fig. 9. Alternative pathways leading to the C_2 and C_3-proline moieties of the pyrrolo(1,4)benzodiazepine antibiotics involving ring cleavage either before or after cyclization of the proline ring.

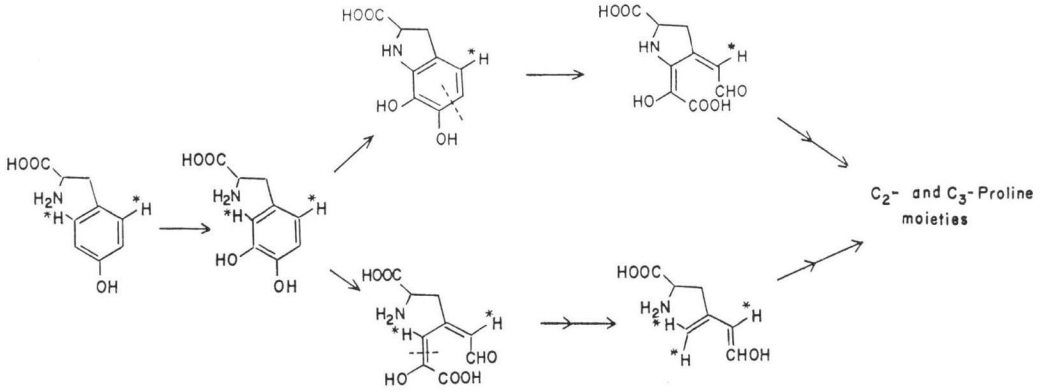
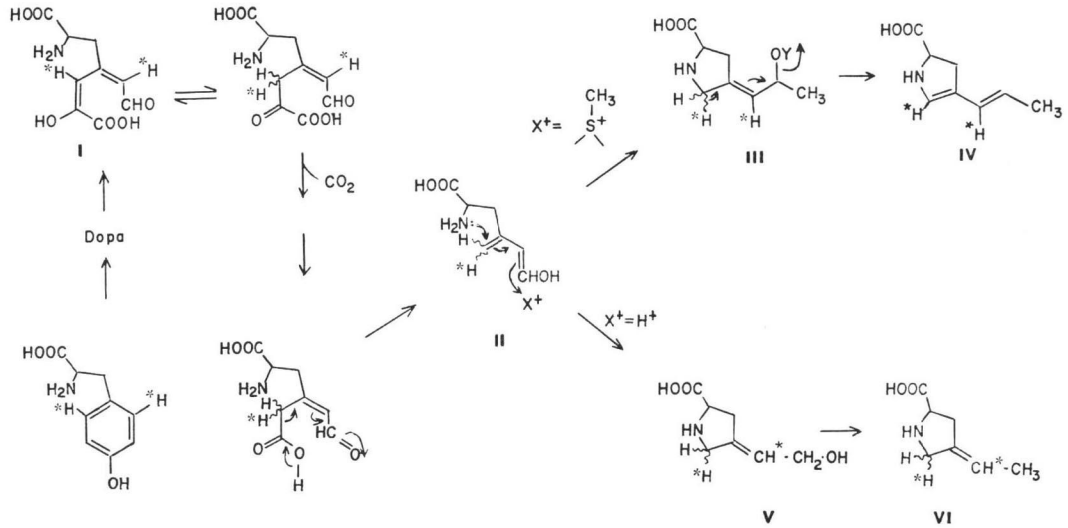


Fig. 10. Proposed parallel pathways for conversion of tyrosine into the C_2 and C_3-proline moieties of anthramycin, tomaymycin and sibiromycin.



mycin. The results in Table 3 are in excellent agreement with a *meta* cleavage pathway for anthramycin and tomaymycin and are in accord with this same pathway for sibiromycin, although the retention in this case is considerably less than expected. In the case of tomaymycin and anthramycin the biogenetic fate of the isotopically labelled hydrogen (either tritium or deuterium) from L-(3 or 5)^{3}H tyrosine or L-(3,5)$^{2}H_2$ tyrosine in the antibiotics was found as anticipated at carbon atom 13[24,26].

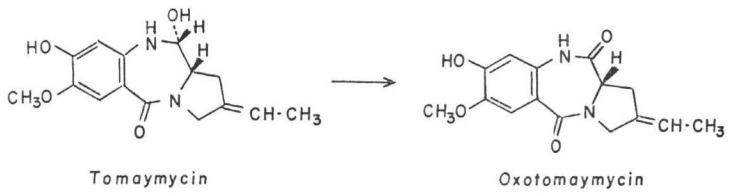
In order to answer the question as to whether ring cleavage of dopa takes place before or after cyclization to form the "proline ring" (see Fig. 9), the fate of the aromatic hydrogens at carbon atoms 2 and 6 of tyrosine were examined in each antibiotic. Experiments utilizing L-(2 or 6)^{3}H/(1-^{14}C) tyrosine showed retentions significantly greater than 50% for tomaymycin and sibiromycin (Table 3) therefore ruling out a cyclodopa intermediate for these antibiotics, since this would require at least a 50% loss of tritium.

A postulated mechanism for the conversion of tyrosine, through dopa and its ring cleavage product to a key seven carbon intermediate (**II**) is shown in Fig. 10. The principle features of this hypothesis and how they accomodate data are: (1) Enolization of **I** leads to either extensive (anthramycin) or smaller (tomaymycin or sibiromycin) losses of tritium from C-3, (2) a concomitant scrambling of the label at C-3 of **I** takes place such that the 1,4 loss of HOY from **III** during its conversion to **IV**, occurs in an apparently non-stereospecific manner, which rationalizes the loss of less than 50% of the tritium in sibiromycin, (3) the nature of X (H^+ or the S-methyl group of methionine) dictates the product of the parallel pathways leading to the C_2 or C_3-proline moieties, (4) relatively minor modifications lead to the ethylidene proline unit of tomaymycin (**V**→**VI**) and the appropriate unsaturation pattern in anthramycin and sibiromycin (**III**→**IV**).

An interesting bioinactivation of tomaymycin occurs in the culture medium of *S. achromogenes*, whereby a constitutive "tomaymycin dehydrogenase" converts this antibiotic into the biologically inactive oxotomaymycin[24] (see Fig. 11). The enzymatic activity associated with the conversion of tomaymycin to oxotomaymycin is not limited to the production phase, since trophophase cells and even cells from tomaymycin-nonproducing cultures of *S. achromogenes* were equally active in converting tomaymycin to oxotomaymycin.

Cultures of *S. refuineus* that are supplemented with methionine produce significantly lower yields of anthramycin. The mechanism for this reduction in antibiotic titre has been demonstrated to be due to interaction of a metabolic product derived from methionine with anthramycin[28]. It seems likely that the identity of the metabolic product from methionine may be SO_2 since this compound has been demonstrated to react directly with anthramycin[29].

Fig. 11. Mechanism for enzymatic inactivation of tomaymycin through conversion into oxotomaymycin[24].



Tomaymycin Oxotomaymycin

Mechanism of Action

The pyrrolo(1,4)benzodiazepine antitumor antibiotics do not share any of the structural features that are normally associated with compounds that form covalent adducts with DNA. In spite of

the lack of obvious alkylating groups on these antibiotics, they react specifically with DNA and other guanine containing polydeoxynucleotides to form nearly irreversibly polymer-bound drugs. The comparative kinetics of reaction of anthramycin, tomaymycin and sibiromycin with DNA are shown in Fig. 12[30]. A peculiarity of the reaction of these compounds with DNA relative to other DNA-binding drugs such as the mitomycins, adriamycin or actinomycin is that the reaction is comparatively slow. In the case of anthramycin and tomaymycin the time required to saturate DNA is about 60 minutes, whereas sibiromycin reacts somewhat faster reaching saturation in about 15 minutes; however, even this is much slower than other DNA-binding drugs. Upon formation of the adduct with DNA, bathochromic and hypochromic changes in the absorption spectra of the antibiotics appear (see Table 4). The final saturation binding capacity of calf thymus DNA for these antibiotics is: 1 anthramycin molecule per 13 nucleotides, 1 tomaymycin molecule per 16 nucleotides and 1 sibiromycin molecule per 8.8 nucleotides (Table 5).

Anthramycin, sibiromycin and tomaymycin have been demonstrated to have a high specificity for reaction with DNA. Little if any reaction occurs with RNA or protein and heat-denaturated DNA binds less antibiotic than native DNA (Table 5). Anthramycin has been demonstrated to react specifically with DNA and polydeoxynucleotides containing guanine[31]. Significantly anthramycin was un-

Fig. 12. Relative kinetics of the reaction of the pyrrolo(1,4)benzodiazepine antibiotics with calf thymus DNA at pH 7.4 in SSC buffer at 23°C[30].

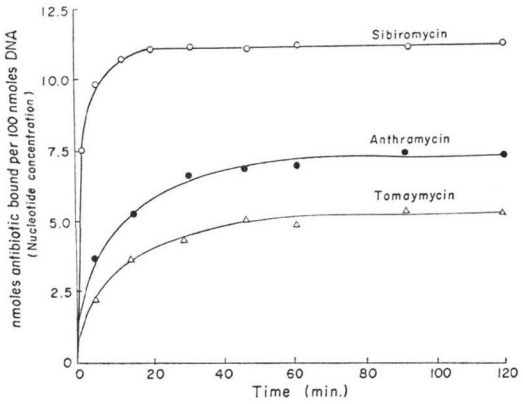


Table 4. Bathochromic and hypochromic changes in the UV absorption spectra of the antibiotics upon reaction with DNA[29].

Antibiotic	Δ max[a]	Δ max (%)[b]
Anthramycin	+11	−15
Sibiromycin	+18	−13
Tomaymycin	+15	−12

a) Difference in γ max (in nm) between a solution containing antibiotic and DNA and a solution containing only antibiotic.

b) Difference in molar absorptivity at γ max (expressed in per cent) between a solution containing antibiotic and a solution containing a mixture of antibiotic and DNA.

Table 5. Relative specificity of binding of the pyrrolo(1,4)benzodiazepine antibiotics for different macromolecules[30].

Macromolecule	Antibiotic to base ratio[a]		
	Anthramycin	Sibiromycin	Tomaymycin
Calf thymus DNA (native)	1: 12.9	1: 8.8	1: 18.2
Calf thymus DNA (denatured)	1: 33	1: 17.8	1: 72
RNA	1: 306	1: 250	1: 2476
Protein[b]	1: 1666	1: 555	1: 1428

a) 1×10^{-3} M polydeoxynucleotide or 0.5 mg/ml human serum albumin was reacted with a 4×10^{-4} M antibiotic in SSC buffer (pH=7.2) at 23°C for 3 hours. The antibiotic base ratio was determined after repeated dialysis to remove unbound antibiotic.

b) Antibiotic (μmole): protein (300 μg).

reactive towards poly (dI) poly (dC) suggesting the importance of the 2-amino group of guanine in the reactivity of the base with anthramycin. Both anthramycin[32,33] and sibiromycin[15] have been shown to be unreactive towards bases of nucleic acids, ribonucleosides and ribonucleotides and deoxyribonucleotides. DNA denatured by formaldehyde does not bind either anthramycin[32] or sibiromycin[34]. DNA with a high GC content binds more sibiromycin than DNA with a low GC content[15]. Conditions of high salt concentration inhibit the rate of binding of sibiromycin[14] and anthramycin[35] to DNA. Sodium dodecylsulfate in concentrations of 5~10% completely inhibits the binding of sibiromycin to DNA[14]. These results indicate that the reaction of these antibiotics with DNA is dependent upon the proper conformation in DNA.

A comparison of the reactivity of the three antibiotics towards DNA provides some information of the importance of various groups on the antibiotic molecules in their reaction with DNA. Since anthramycin and sibiromycin are relatively similar in structure except for the amino sugar at C–7 of sibiromycin, but possess such markedly different rates of reaction with DNA, this is very suggestive that the amino sugar of sibiromycin plays a significant role in the interaction with DNA. It can be assumed that the interaction of the pyrrolo (1,4) benzodiazepine antibiotics with DNA occurs in at least two discrete steps: the first being a non-covalent type of interaction in which the reactive group on the antibiotic is brought into juxtaposition to the reactive species on DNA, and the second step resulting in covalent attachment of the antibiotic to DNA. The initial (non-covalent) binding is more likely to be a rapidly reversible reaction, with an equilibrium constant favoring dissociation in the case of anthramycin and tomaymycin, so that at any instant only a small fraction of the anthramycin or tomaymycin is bound. The amino sugar of sibiromycin would increase the fraction of this antibiotic bound reversibly and thereby enhance the rate of the second (covalent) step. This could be represented by $A+DNA \underset{}{\overset{K}{\rightleftharpoons}} A\cdot DNA \xrightarrow{k} A\text{-}DNA$ where A·DNA is the reversible complex, A-DNA is the covalent complex, K is an equilibrium constant, and k is a first-order rate constant. It is pertinent to note that antitumor compounds such as adriamycin and daunomycin[36], which are believed to intercalate with DNA, require the amino sugar portion for biological activity, indicative of the importance of this part of the molecule for non-covalent interactions with DNA. Since tomaymycin, the smallest least polar molecule, binds to DNA with a lower frequency than either anthramycin or sibiromycin, it would appear steric interactions are probably less significant in binding than electronic attractive interactions.

The properties of the complex (antibiotic-DNA adduct) have been examined for all three antibiotics. The stability of antibiotic and the DNA are both increased in the complex. The ΔTm for the complexes with anthramycin[35], tomaymycin[37] and sibiromycin[14] are 7°, 13° and 20°C respectively. In the case of anthramycin[35] it has been shown that after strand separation by alkali the antibiotic remains bound to the separate strands. No inhibition of strand separation was evident ruling out inter-strand cross linking[35] as is common with bifunctional alkylating agents. All three complexes are stable to alkali conditions but dissociate at acid pH's of 5 or below (see Table 6). The sibiromycin-DNA complex is most stable to acid conditions dissociating only at pH of 2 or lower. The anthramycin-DNA complex shows an intermediate stability at acid pH's between sibiromycin and tomaymycin, whereas the tomaymycin-DNA complex is the least stable of the three complexes to acidic conditions. The dialyzed antibiotic DNA complexes are not disrupted by solvent extraction with *n*-butanol or DMSO, solvents which readily dissolve the uncomplexed antibiotics.

Physical measurements on the sibiromycin and anthramycin-DNA complexes have further characterized the properties of the adducts. Electric dichroism measurements have clearly shown that the anthramycin chromophore is not held by intercalation since the plane of the chromophore is at least 40° to the plane of the DNA bases[38]. Viscosity and sedimentation data indicate that anthramycin causes stiffening without lengthening of the DNA helix[38]. Sibiromycin has no action upon the viscosity of DNA in solution[14]. The binding of sibiromycin causes a drastic change in the circular dichroism spectra of DNA suggesting alterations in the DNA conformation (increase in winding angle of the base pairs)[39].

The anthramycin and tomaymycin-DNA complexes have been demonstrated to show resistance to nuclease treatment[12,37]. In addition anthramycin and sibiromycin-DNA complexes are inactive as templates for RNA and DNA synthesis[12,14].

Experiments in which competition between the three pyrrolo(1,4)benzodiazepine for the same binding sites on DNA or between these antibiotics and other classes of antibiotics have been carried out. Studies in my laboratory have demonstrated that there is complete competition between sibiromycin and anthramycin or tomaymycin for binding to DNA, whereas only partial competition occurs between anthramycin and tomaymycin[30]. Studies have demonstrated that sibiromycin competes with actinomycin D and olivomycin A for the binding sites on DNA[40]. Likewise anthramycin competes with actinomycin D[31] and tomaymycin with methyl green[37]. In cases where the second antibiotic was specific for GC pairs (actinomycin D, olivomycin A) complete displacement by anthramycin or sibiromycin was found; however, in the case of tomaymycin and methyl green only 50% of the latter compound was released. None of these compounds, whether a pyrrolo(1,4)benzodiazepine antibiotic or actinomycin D, *etc.* was able to displace an already bound pyrrolo(1,4)benzodiazepine antibiotic from DNA[30,31].

Of the three pyrrolo(1,4)benzodiazepine antibiotics the reaction of anthramycin with DNA has been studied most thoroughly, chiefly by KOHN and coworkers[31,35,38]. In a series of systematic studies, they have shown that the rate of reaction of anthramycin with DNA has the following characteristics.

(1) The reaction rate of anthramycin with DNA is proportional to hydrogen ion concentration between pH 4 and 7[35].

(2) The rate of reaction with native DNA decreases with increasing salt concentration[35].

Table 6. Stability of antibiotic·DNA complexes at different pH values[30].

pH	Amount of antibiotic released from complex (%)[a]		
	Anthramycin-DNA	Sibiromycin-DNA	Tomaymycin-DNA
2.0	98.0	89.0	100.0
3.0	75.0	3.0	100.0
4.0	15.5	3.0	91.0
5.0	10.0	4.0	81.0
6.0	6.0	3.0	64.5
7.2	0.0	0.0	37.0
10.0	0.0	2.0	13.0
11.0	2.0	3.0	24.0

[a] Antibiotic-DNA complexes were dialyzed for 2 successive 24 hours periods each time against 50 vols of buffer at the pH shown in the table.

(3) Both anthramycin and anthramycin methyl ether are able to react directly with DNA; however, the reaction of the methyl ether is slower[31].

(4) The reaction rates are much higher with helical DNA than with single-stranded DNA.[35]

These characteristics lead to the conclusion that the reaction rate is dependent upon a protonated intermediate, conformational aspects of DNA and the nature of the substituent at C–11 of anthramycin. Information on the reactive site on the pyrrolo(1,4)benzodiazepine antibiotics is available from a comparison of the structure activity relationship of these antibiotics and their derivatives. These are shown in Table 7. The structure activity relationships show that: (1) while an unsaturated side chain at C–2 of the pyrrolo ring may be a requirement for activity, a variety of C_2 and C_3 unsaturated side chains will suffice, (2) substitution of the aromatic ring may vary except that a methoxy group at C–9 destroys activity and (3) the carbinolamine at C–10, 11 is essential for activity. With respect to this latter point, oxidation to the 10, 11 amide or dehydration to the 10, 11 enamine in the case of sibiromycin results in complete loss of activity[8].

These structure activity relationships lead us to believe that it is the 10, 11 positions, that is, the carbinolamine, which is involved in the reaction of these compounds with a guanine residue in DNA. Further support for this view can be found in that rapid and complete exchange of the 11-methoxy group for a hydroxy group occurs when the pyrrolo(1,4)benzodiazepine-11-methyl ethers are dissolved in aqueous buffer at neutral pH. This reaction is catalyzed by acid conditions (between pH 7 and 4), as is also the reaction of these antibiotics with DNA[31].

Table 7. Structure activity relationships of anthramycin, tomaymycin and sibiromycin and their derivatives.

Antibiotic or derivative	Biological activity	Systems and references
Anthramycin	+	a, b, c
-11 epimer	+	b
-11 methyl ethers	+	a
-11 ethyl ether	+	a
-11 benzyl ether	+	a
-10, 11 anhydro	+	a, b
terminal CO NH_2 replaced by CN	+	b
-9 methyl ether	−	a, b
-11 keto	−	b
-10 acetyl	−	b
-9 desoxy-2, 3, 12, 13 tetrahydro	−	a
2, 3 dihydro- and side chain at position 2 replaced by -OH	−	b
Tomaymycin	+	c, d
-11 keto	−	c d
Sibiromycin	+	c. e
-10, 11 anhydro	−	f
-SO_2 complex	+	g

(a) Spectral change with DNA, antibacterial activity, antitumor activity[32].
(b) Inhibition of RNA polymerase reaction and chemosterilizant action of houseflies[51].
(c) Covalent complexation with DNA[30].
(d) Inactivating effect against various bacteriophages and animal viruses[5].
(e) Spectral change with DNA[14], antibacterial activity, antitumor activity[6].
(f) Biological screen not specified[5].
(g) Antitumor activity[53].

Studies in my laboratories using dual tagged radiolabelled molecules (^{3}H and ^{14}C) on the interaction of anthramycin, tomaymycin and sibiromycin with DNA have allowed us to rule out loss of any major portion of the antibiotic molecule upon formation of the covalently DNA bound antibiotic.[30]

On the basis of the fact that the acid released compound from the anthramycin-DNA complex was able to react with DNA at neutral pH at the normal rate, KOHN and SPEARS[35] had concluded that the released compound was unchanged antibiotic. We have confirmed this conclusion by isolating the released radio-labelled antibiotic from the same antibiotic-DNA complex and comparing its Rf in known chromatography systems together with authentic reference anthramycin[30]. Similar results were obtained with the tomaymycin-DNA complex[30].

The inability of these antibiotics to react with RNA, mononucleotides, or polydeoxynucleotides lacking guanine residues indicates a high degree of selectivity for structural requirements in the polymer before the interaction can take place. This can best be interpreted to mean that a special orientation or juxtaposition must be present before formation of the covalent linkage between C–11 of the antibiotic and probably guanine occurs.

We have suggested a number of possibilities for the reactivity of the carbinolamine with a nucleophilic center on DNA[30]. For example, one can suggest that the reactive species might be the 10, 11 anhydro-compound (enamine), which in the case of anthramycin and tomaymycin is in equilibrium, in aqueous solution, with the parent antibiotics and their epimers. However, since the enamine of sibiromycin, which is less reactive than the corresponding enamines of anthramycin and tomaymycin (presumably due to its conjugation in the sibiromycin molecule) is biologically unreactive[8] this effectively rules out this mechanism.

A second mechanism that we had considered (Fig. 13) involved the formation of a SCHIFF base linkage between the aldehydic carbonyl of the previously cleaved carbinolamine group and the 2-amino group of guanine. This we were able to rule out since reduction of the antibiotic-DNA complexes did not result in any increase in the amount of acid-stable complex[30].

A third possibility, which we favor at this time, involves the nucleophilic attack originating from a basic group on DNA (see Fig. 14), at C–11 of the antibiotics in an analogous manner to the demonstrated hydrolytic changes which occur at C–11 upon conversion of the anthramycin methyl ether to

Fig. 13. Proposed mechanism for the formation and acid cleavage of the antibiotic-DNA complex involving a SCHIFF base linkage between the antibiotic and DNA[30].

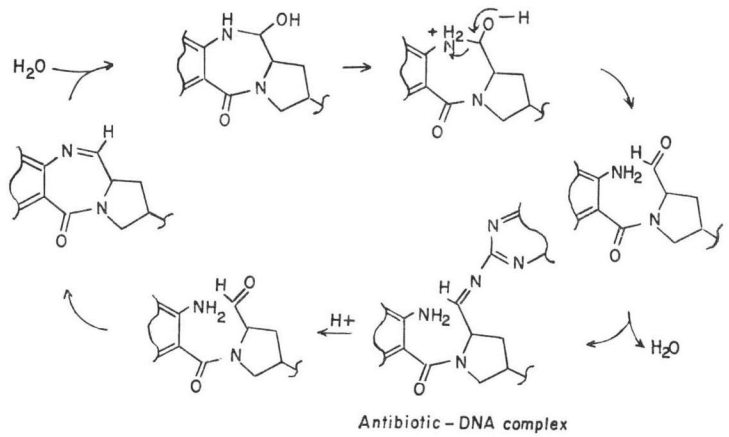
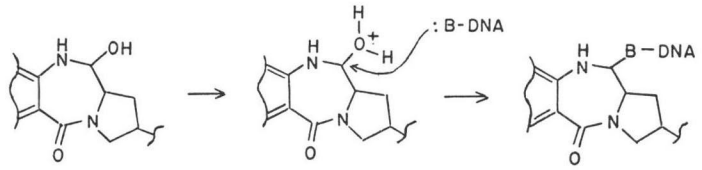


Fig. 14. Proposed mechanism for the formation of the antibiotic-DNA complex involving an Sn_1c mechanism[30].



anthramycin[31]. Since both the hydrolysis of the 11-methoxy group and the reaction with DNA were found to be acid catalyzed, between pH 4 and 7, this supports a relationship between the two processes. Evidence which would unequivocally confirm the mechanism is as yet unavailable.

A sulfur derivative of sibiromycin has been prepared by BRAZHNIKOVA[41] by bubbling SO_2 through a solution of sibiromycin. Significantly this SO_2-sibiromycin adduct retains biological activity. Upon reaction of the labeled (^{35}S) derivative with DNA the ^{35}S is lost from the complex and the kinetics of the reaction of this derivative with DNA are markedly slower[42]. The loss of sulfur from the sibiromycin molecule is a prerequisite for reaction with DNA. Studies from my laboratory have demonstrated the same is true for the anthramycin and tomaymycin molecules[29]. The identification of the site on the pyrrolo(1,4)benzodiazepine with which SO_2 reacts will most likely lead to a determination of the DNA reactive group on these antibiotics.

The effects of anthramycin, tomaymycin and sibiromycin on macromolecular synthesis have been studied, but most thoroughly for anthramycin and sibiromycin. As would be expected for drugs which form covalently bound adducts with DNA, these drugs selectively inhibit RNA and DNA synthesis without adversely affecting protein synthesis. Whereas anthramycin appears to produce a slightly more marked inhibition of RNA than DNA synthesis in mammalian cells[43,44], sibiromycin inhibits DNA and RNA synthesis to approximately the same degree in mammalian cells[34]. On the other hand in bacterial cells tomaymycin[37] and sibiromycin[15] were found to preferentially inhibit DNA synthesis while having a lesser effect on RNA synthesis. Incorporation of amino acids into protein was not adversely affected at these same concentrations of antibiotics in any of these systems. KOHN[11] has summarized the comparative effects of anthramycin on RNA and DNA synthesis.

KANN and KOHN[44] have examined the effect of anthramycin on RNA synthesis in mouse leukemia L1210 cells and compared these results to those obtained with actinomycin. The inhibition of RNA synthesis in L1210 cell cultures brought about by anthramycin was gradual in onset and increased progressively with time. This differed markedly from actinomycin where prompt inhibition was noted. This difference is most likely related to the comparative rates of reaction of anthramycin and actinomycin with DNA, the latter antibiotic reacting within a few seconds with DNA *in vitro*.

Since many DNA reactive drugs such as actinomycin and daunomycin selectively inhibit the synthesis of ribosomal precursor RNA in nucleoli while having little effect on nucleoplasmic RNA synthesis, the effect of anthramycin was evaluated on these processes in L1210 cells[45]. Anthramycin was found to inhibit nucleolar RNA synthesis to the same extent as nucleoplasmic RNA synthesis and also cause a reduction in chain lengths of both types of RNA. Since nitrogen mustard mimicked the effects of anthramycin on these processes, it was suggested that these types of effects on RNA synthesis may be characteristic of drugs that form covalent adducts with DNA.

Sibiromycin has been shown to selectively inhibit the elongation of RNA while having a lesser

effect on initiation[34]. GAUSE and DOLGILEVICH[46] have reported that sibiromycin selectively inhibits the incorporation of [^{3}H] thymidine into the H-strand of mitochondrial DNA, using an *in vitro* isolated rat liver mitochondria system. These workers have speculated that this selectivity results from the fact that sibiromycin binds only to double stranded DNA and since L-strand synthesis proceeds on a single-stranded template, which has been despiralized earlier in replication, it is resistant to the action of sibiromycin. Since H-strand synthesis requires opening of the double helix, this process is inhibited by the binding of sibiromycin to the double-stranded DNA.

Indirect evidence for the ability of the DNA repair mechanism to repair sibiromycin damaged DNA is evident from the fact that mutants (uvr$^-$) of *E. coli* AB–1157 with impaired ability to repair DNA lesions induced by ultraviolet radiation had increased sensitivity to the bactericidal effects of sibiromycin.[47]

Biological Effects

All of the drugs belonging to the pyrrolo(1,4)benzodiazepine group of antibiotics are extremely potent compounds. They have all been demonstrated to have pronounced antibiotic, antitumor and antiviral activities.

In vitro, anthramycin has a wide antibacterial spectrum at a dose range of 0.1 to 5 μg/ml against a variety of organisms including *Staphylococcus aureus, Bacillus subtilis, Sarcina lutea, Mycobacterium smegmatis, Candida albicans* and *Cryptococcus neoformans*[48]. However, *in vivo* anthramycin was found to be without chemotherapeutic activity in experimental infections with bacteria, fungi, viruses and other protozoan and helminthic infections[49], except experimental infections with the protozoans *Trichomonas vaginalis* and *Endamoeba histolytica* and the helminth *Syphacia obvelata.*

Tomaymycin showed MIC's of 25 μg/ml or less when tested against *Staphylococcus aureus*, *Bacillus subtilis, Corynebacterium xerosis, Sarcina lutea,* and *Penicillium chrysogenum*[4]. In general this drug was primarily active against gram-positive bacteria and weakly active against yeasts and fungi[4].

Sibiromycin inhibits the growth of *Bacillus mycoides* and *Bacillus subtilis* (0.3 μg/ml), *Staphylococcus aureus* (1 μg/ml) and *Escherichia coli* (20 μg/ml)[41].

The antiphage activity of anthramycin and tomaymycin has been demonstrated to be selective for certain, closely related phage systems of *E. coli*[4,48]. For example, when 0.1 μg/ml of anthramycin is incorporated into the media in the T_1 or T_5 system, the phage production is inhibited by more than 99%, whereas T_2 and T_3 were inhibited far less severely.

Dextrochrysin has potent antibiotic-activity against gram-positive and gram-negative bacteria (except *Pseudomonas*) and *Mycobacterium* sp 607[9]. Dextrochrysin is also active against some bacteriophages[9].

The neothramycins A and B have been demonstrated to have MIC's of 100 μg/ml against *Staphylococcus aureus, Bacillus subtilis* PCI 219, *Klebsiella pneumoniae* PCI 602, various strains of *Escherichia coli, Aeromonas salmonecida* ATCC 14174, *Vibrio anguillarum* NCBM6 and a number of fungi including *Aspergillus niger* and *Saccharomyces cerevisiae*[10].

The potent antitumor activity of all of the members of the pyrrolo(1,4)benzodiazepine antibiotic series is probably of most potential clinical significance.

Anthramycin has been shown to have broad antitumor activity against a variety of transplanted tumors[49]. This antibiotic was found to be active parenterally and p.o. against EHRLICH solid carcinoma, sarcoma 180, WALKER carcinosarcoma 256 and the ascitic form of the EHRLICH carcinoma. When

anthramycin was administered s.c. against human heterotransplants, Human epidermoid carcinoma No. 3 and Human adenoma No. 1, an appreciable antitumor effect was obtained with fully tolerated doses.

Sibiromycin has pronounced antitumor activity against 6 transplanted tumors in mice[50]. While it was most effective against squamous praegastric cancer cells (strain OG-5), sibiromycin also inhibited ascitic forms of tumors and sarcoma 180.

Tomaymycin has been reported to have a marked inhibitory effect on leukemia L1210 cells[37].

The neothramycins A and B produced up to a 200% prolongation in the survival period in mice implanted with mouse leukemia L-1210 or EHRLICH ascites carcinoma cells when the drugs were given intraperitoneally[10]. In addition the neothramycins inhibited multiplication of YOSHIDA rat sarcoma cells and C3H cells transformed by SV40 in tissue culture.

Anthramycin has a chemosterilizant action on male houseflies[51,52].

The antitumor activity of a sibiromycin-SO_2 adduct has been compared to the parent antibiotic[53]. Although it was found the sibiromycin-SO_2 adduct had a lower toxicity than sibiromycin in healthy mice, this was not evident when administered to mice with lymphosarcoma, strain L10-1.

Dextrochrysin lacked antitumor activity against the two tumors it was tested against, EHRLICH ascites carcinoma and YOSHIDA sarcoma[9].

Both anthramycin and sibiromycin have been used clinically. Approximately 300 terminal cancer patients with solid tumors, lymphomas or HODGKIN's disease have been treated either with pure anthramycin or fractions obtained from the fermentation broth of *S. refuineus*[48,54]. The dose of the pure crystalline anthramycin was 0.02 mg per kg per day for two weeks administered intravenously in one to two hours with a maximum of 1 mg per day. Thereafter this dose of anthramycin was administered only one to three times weekly and the patients were placed on an oral form of anthramycin. Of a total of 146 patients treated with the crude unpurified preparation, 70 responded favorably, whereas of a total of 73 patients treated with pure anthramycin 40 responded favorably.

The tumors most responsive to either the crude preparation or pure anthramycin included gastrointestinal and breast neoplasms as well as lymphomas and sarcomas. Conversely ovarian, pancreatic carcinomas and malignant melanomas were the least responsive[48]. Significantly no patient who has been treated with anthramycin or the crude preparation developed any depression of the bone marrow, nor hepatic, renal or gastrointestinal toxicity[48,55].

Preliminary clinical studies with sibiromycin indicate that intravenous injection of this drug was effective in the treatment of some forms of squamous cell carcinoma[15].

Mammalian Toxicity and Pharmacology

Toxicity in both animals and patients has been noted with anthramycin. In man the most common side effect was due to its severe irritating properties which frequently led to phlebitis, painful infiltration and necrosis at the site of injection[48]. These reactions usually occurred 24 hours after administration of the drug. This reaction was prevented in some patients by the local injection of hydrocortisone and albumin within eight hours of the infiltration[48]. Evidence of some neurotoxicity was apparent since some patients developed lethargy and somnolence and two became disorientated in the study reported by KORMAN and TENDLER[48]. Following rapid intravenous of anthramycin to seventy three patients irreversible shock occurred in seven patients, of which four registered low voltage on the electrocardiogram[48]. This latter reaction only occurred at doses of greater than 1 mg given by slow intravenous infusion.

In rats repeated intraperitoneal injections of anthramycin causes changes in the electrocardiogram which were related to dose and duration of treatment[56]. The principal effect was an alteration of the T wave.

Acute intestinal necrosis has been noted at 0.5 mg/kg and higher of anthramycin given subcutaneously to mice[57]. However this dose is higher than that normally given and the gut was able to return to normal within 36 hours after injection of the drug.

Dogs and rats treated with various fractions of the culture broth of *S. refuineus* at tolerated doses up to 30 days did not reveal any leukopenia or bone marrow damage[55]. In a comparative study of selective toxicity in mice with and without EHRLICH solid tumor it was found that whereas FUDR inhibited slightly to markedly spermatogenesis no such inhibition was noted with the crude anthramycin preparation. Likewise, whereas, in FUDR-treated mice the tumor cells showed various degrees of degeneration with polymorphism of the cells and some necroses, there was no morphological changes in the tumor cells except for large areas of necrosis when treated with the anthramycin preparation.

Anthramycin has been shown to have a marked effect on early chick embryos, particularly stages 4~5 embryos, during the 19~21 hours of incubation *in vitro*[58].

ADAMSON *et al.*[59] have studied the effect of anthramycin on various other pharmacological parameters. Hexobarbital sleeping times were doubled in mice receiving daily injections of anthramycin for 4 days, but single injections at times varying from 30 minutes to 4 hours prior to hexobarbital had no effect. Anthramycin in doses of up to 1 mg/kg did not effect blood pressure, respiration or ECG in anesthetized dogs.

The toxicity of sibiromycin has been reported in animals. At maximum tolerated doses in dogs (0.37 μg/kg given intravenous for 30 days) no impairment of the heart, kidney or liver function was noted[60]. With animals treated with lethal doses of sibiromycin, injuries to the myocardium and kidneys were found to be the cause of death. No changes in the blood characteristics were noted when dogs were given the maximum tolerated dose[61]. Following intravenous administration sibiromycin could be detected in the blood for 15~30 minutes. Almost half of the injected dose was found in the urine and small amounts were detected in all organs[61].

Relationship of the Pyrrolo(1,4)benzodiazepine Antibiotics to other Antibiotics

Strong evidence points to the direct involvement of a carbinolamine, a common feature of all the pyrrolo(1,4)benzodiazepine antibiotics, in the reaction with DNA. Significantly, a number of other antibiotics which interact with DNA also contain carbinolamine functionalities. The structures of these antibiotics are shown in Fig. 15. Of these antibiotics the interaction of the mitomycins with DNA has been most thoroughly studied. Recently HORNEMANN[62] has suggested that this functionality may possibly be involved in the interaction of the mitomycins with nucleophilic groups on biological molecules, and as such may contribute to the monofunctional alkylation of DNA by these antibiotics. Mitomycin C has been demonstrated to react with sodium sulfite at C-9a (the position bearing the carbinolamine function) to form 7-aminomitosane-9a-sodium sulfonate. This reaction is of significance since the pyrrolo(1,4)benzodiazepine antibiotics undergo a reaction with sulfur dioxide to form a sulfur-containing derivative[41] which upon reaction with DNA loses the sulfur atom[42].

Biosynthetically the pyrrolo(1,4)benzodiazepine antibiotics are related to the lincomycin antibiotics (see Fig. 16), in that the lincomycins also contain C_2 and C_3-proline moieties which are derived from

Fig. 15. Structures of other antitumor agents containing a carbinolamine moiety.

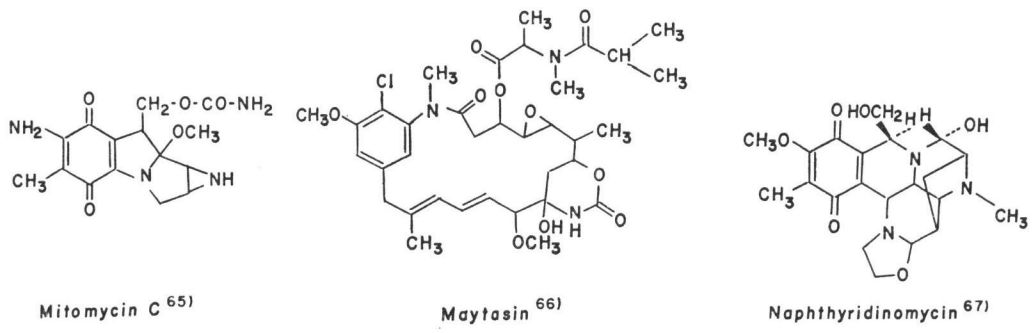


Fig. 16. Structures of lincomycins A and B.

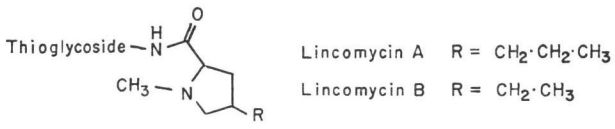
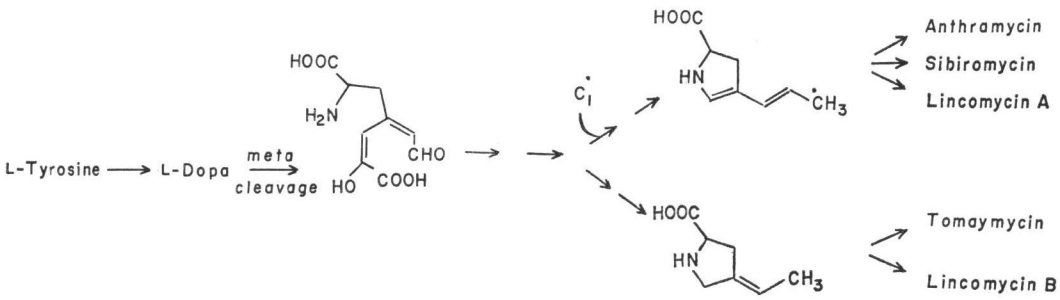


Fig. 17. Parallel pathways leading to antibiotics containing C_2 or C_3-proline moieties.



tyrosine[63,64]. The biosynthesis of the C_2-proline moieties of lincomycin B is formed in an analogous manner to that of a similar unit in tomaymycin[24] whereas the C_3-proline moiety of lincomycin A is derived in an analogous manner to similar units in anthramycin and sibiromycin. A general pathway for the formation of C_2 and C_3-proline units in these antibiotics is shown in Fig. 17.

Summary and Conclusions

1. The pyrrolo(1,4)benzodiazepine antibiotics comprise the antibiotics, anthramycin, tomaymycin, sibiromycin, dextrochrysin and the neothramycins A and B. Structure activity relationships indicate that in addition to a pyrrolo(1,4)benzodiazepine nucleus, these compounds also require a 10,11-carbinolamine and an unsaturated side chain at C–2 to retain their potent biological activities.

2. The antibiotics within this group show antiviral and most significantly antitumor activity. Anthramycin and sibiromycin have been shown to have a wide range of antitumor activity in animal models, and in the case of anthramycin, activity against both solid and liquid tumors in man.

3. Tryptophan, methionine and tyrosine supply the biosynthetic building blocks for anthramycin, tomaymycin and the aglycone moiety of sibiromycin. Parallel pathways from tryptophan, *via* kynure-

nine lead to the anthranilate moieties of these three antibiotics. Conversion of tyrosine, *via* dopa, to the C_2 and C_3-proline moieties of anthramycin, tomaymycin and sibiromycin involves meta cleavage of the aromatic ring of a tyrosine metabolite and loss of two aromatic carbon atoms.

4. Addition of the pyrrolo(1,4)benzodiazepine antibiotics to bacterial or mammalian cells leads to a potent inhibition of RNA and DNA synthesis, while protein synthesis is virtually unaffected at these same antibiotic concentrations.

5. Overwhelming evidence suggests that the pyrrolo(1,4)benzodiazepine antibiotics form a covalent linkage with DNA. No major modification of the pyrrolo(1,4)benzodiazepine antibiotics occurs upon reaction with DNA, and intact antibiotic can be released from their respective DNA adducts under certain acidic conditions.

6. Structure activity relationships lead to the conclusion that the DNA reactive site on the pyrrolo (1,4)benzodiazepine antibiotics is the 10,11-carbinolamine. Most likely the covalent linkage with DNA involves a nucleophilic attack originating from a basic group on DNA at C–11 of the antibiotics resulting in loss of the conjugate acid.

7. The pyrrolo(1,4)benzodiazepine antibiotics have a high specificity for reaction with double-stranded polydeoxynucleotides containing guanine moieties. No reaction occurs with RNA, protein or polydeoxynucleotides lacking guanine moieties.

8. The pyrrolo(1,4)benzodiazepine antibiotics represent a unique group of DNA reactive drugs. Their reaction with DNA is characterized by a relatively slow rate of reaction, a high degree of selectivity for the polydeoxynucleotide, and formation of a covalent adduct that is reversible at low pH's.

9. The pyrrolo(1,4)benzodiazepine antibiotics have a unique biosynthetic origin. The biosynthetic transformations of tryptophan and tyrosine which lead to the building blocks for these antibiotics are more generally associated with catabolic enzymes leading to the biodegradation of aromatic compounds. Why a microorganism chooses to synthesize a proline unit by condensation of the α-amino group of tyrosine with one of the *ortho* aromatic carbon atoms of tyrosine and partially degrade the aromatic ring is an interesting but unfortunately unanswered question. It is difficult to believe that this necessary genetic information was not retained to serve an important role for the producing organism.

10. Anthramycin is remarkable in that in spite of being a potent cytotoxic drug to rapidly proliferating tissues, such as tumor cells, no bone marrow depression has been detected, either in animal or human studies. If the reported cardiotoxicity and tissue necrosis problems could be solved or at least controlled, the application of such a drug with lack of bone marrow depression in combination chemotherapy or even alone would be a major achievement in cancer chemotherapy. In view of the advances made in the design of dosage scheduling for antitumor agents either given singly or in combination since 1965 when anthramycin was first evaluated in the clinic, it is perhaps time for a reevaluation of this drug.

11. The high specificity of the pyrrolo(1,4)benzodiazepine antibiotics for double-stranded polydeoxynucleotides containing guanine residues suggests that these antibiotics may be useful agents to probe DNA. Since not every guanine residue in DNA is reactive towards the pyrrolo(1,4)benzodiazepine antibiotics, this is suggestive that there may be a regional specificity for guanine residues extending to the neighboring bases before reaction will take place. The selective inhibition of H-strand synthesis in isolated mitochondria by sibiromycin suggests strongly that these compounds may be useful tools for examining DNA replication in various biological systems.

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