CHEMICAL CONVERSION OF ANTHRAMYCIN 11-METHYL ETHER TO DIDEHYDROANHYDROANTHRAMYCIN AND ITS UTILIZATION IN STUDIES OF THE BIOSYNTHESIS AND MECHANISM OF ACTION OF ANTHRAMYCIN[1a, b]

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ABSTRACT.—Reaction of anthramycin 11-methyl ether (AME) with trifluoroacetic acid results in formation of (1,11a)-didehydroanhydroanthramycin (DAA). Anthramycin biosynthetically labelled from DL-[3'RS(3'-^{3}H)]; DL-[3'S(3'-^{3}H)] and DL-[3'R(3'-^{3}H)]tyrosine each lose approximately 50% of their tritium during this conversion to DAA confirming the labelling pattern of 3'-tritiated species of tyrosine in AME. As expected negligible losses of tritium occurred from AME biosynthetically labelled from L-[2- or 6-^{3}H] or L-[3- or 5-^{3}H]tyrosine. DAA did not form a stable adduct with DNA in accord with the postulated mechanism of action of anthramycin.

Anthramycin is produced by *Streptomyces refuineus* (1) and belongs to the pyrrolo(1,4)benzodiazepine class of antitumor agents (2). It is of interest because of its unique biosynthesis and reaction with DNA. Biosynthetically, this group of metabolites is derived from tyrosine, tryptophan and methionine (3–6). Although anthramycin and related antibiotics lack any of the structural features commonly associated with drugs that bind tightly to DNA, they react specifically with it to form a labile covalent adduct (7–10). During attempts to find a more suitable nmr solvent for stable isotope biosynthetic work, we discovered that the addition of trifluoroacetic acid (TFA) to anthramycin 11-methyl ether (AME) produces a yellow didehydro derivative (DAA) that crystallizes upon the addition of water. The results reported in this study establish the structure of DAA, its use in determining the labelling pattern of 3'-tritiated tyrosine in anthramycin, and further evidence on the mechanism for reaction of anthramycin with DNA.

RESULTS AND DISCUSSION

Reaction of AME with TFA results in the formation of DAA by facile elimination of methanol from the carbinolamine and a novel dehydrogenation to create a pyrrolo ring system (scheme 1).[2] Dehydrogenation reactions with TFA resulting in aromatization have been previously reported (11). Structural characterization of DAA was based upon high resolution mass spectral analysis and comparison of the ^{1}H(100 MHz)- and ^{13}C(25.16 MHz)-nmr data (tables 1 and 2) for DAA and AME.

When Leimgruber *et al.* published the structure of anthramycin, they included an assigned 60 MHz proton spectrum of AME in DMSO-d_6 (12). Thus if DAA has the structure shown in scheme I the following spectral changes (table 1) would

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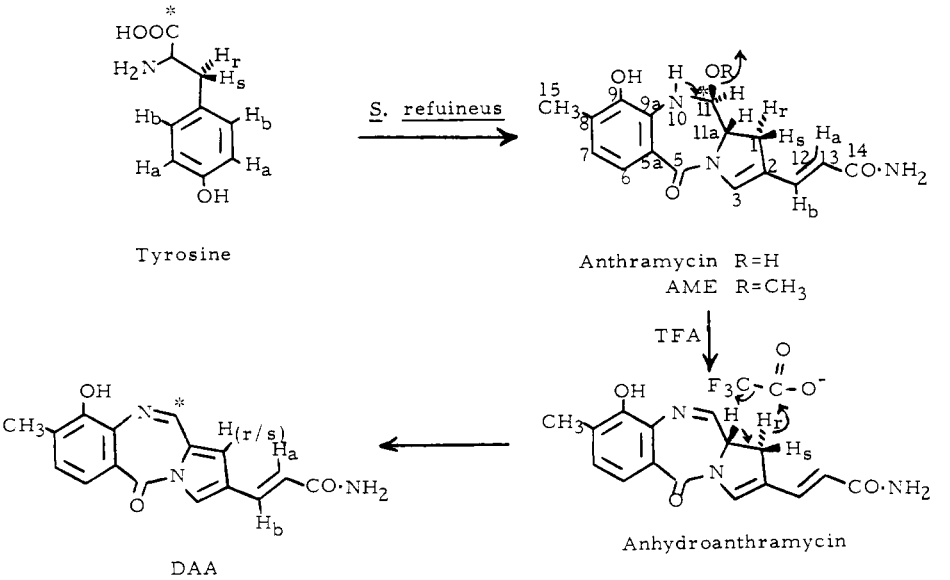
[2]A referee points out that our 50% yield is compatible with a TFA catalyzed disproportionation reaction. Although we lack evidence of this we feel it is not an unreasonable postulate.



SCHEME 1. Biosynthetic conversion of tyrosine to AME and suggested chemical conversion of AME to DAA.

be expected. Conversion of the methylated carbinolamine residue of AME to the aldimine grouping in DAA would result in the disappearance of the H-10 and methoxyl signals and would deshield H-6, H-7 and H-11 (13). Dehydrogenation of AME to give the pyrrolo ring in DAA would, according to our postulate, remove the signal for H-11a, deshield H-13 but not H-12, and result in H-11 being observed as a singlet resonance and H-1 and H-3 as an AB system ($^{1,3}J_{AB}$ 1.9 Hz)

TABLE 1. ^{1}H NMR (100 MHz) data for anthramycin methyl ether (AME) and didehydroanhydroanthramycin (DAA).[a]

Compound	H-1	H-3	H-6	H-7	H-11	H-11a	H-12	H-13	H-15
DAA (in TFA)	8.32 $J_{1,3}$ 1.8	9.22	8.48 $J_{6,7}$ 8.6	7.73	8.89		8.05 $J_{12,13}$ 15.9	6.94	2.60
DAA (in DMSO-d_6)	7.78 $J_{1,3}$ 1.9	8.62	7.95 $J_{6,7}$ 8.3	7.42	8.64		7.49 $J_{12,13}$ 15.8	6.60	2.35 $J_{7,15}\sim$ 0.6
AME (in DMSO-d_6)	2.71 (β) 3.11 (α) $J\alpha,\beta$ 16.0	7.46 $J_{1\alpha\ 3}\sim$ $J_{1\beta,\ 3}\sim$1	7.32 $J_{6,7}$ 8.3	6.50	4.80 $J_{11,10}$ 6.2 $J_{11,11a}$ $\leq$0.6	4.24 $J_{11a,1\beta}$ 6.0 $J_{11a,1\alpha}$ 11.0	7.31 $J_{12,13}$ 15.3	5.77	2.21
	OCH_3	OH	H-10	NH_2					
	3.28	6.90[b]	7.16	9.6[b]					

[a]Chemical shifts relative to internal TMS. J_{pq} in Hz. Spectra were recorded on a Varian HA-100D or XL-100/15 NMR spectrometer. The six spin sub-system formed by H-1α, H-1β, H-3, H-11, H-11a, H-10 in AME was analyzed with the simulation program SIMEQ on the Varian FT-80 spectrometer.

[b]These assignments may be interchanged. The OH and NH_2 signals could not be identified in the spectra of DAA because of superposition and broadening of peaks.

TABLE 2. ^{13}C NMR data (25.16 MHz) for anthramycin methyl ether (AME) and didehydroanhydroanthramycin (DAA).[a]

Compound	C-1	C-2	C-3	C-5	C-5a	C-6	C-7	C-8	C-9
DAA (in TFA)	137.25(dm) $^1J_{174.5}$	130.19(m)	139.56(ddd) $^1J_{197.6}$; $^3J_{4.8,6.7}$	160.81(d) $^3J_{4.4}$	119.48(d) $^3J_{8.7}$	129.57(d) $^1J_{172.0}$	135.98(dq) $^1J\sim163$; $^3J\sim6$	125.78(m)	148.92(dq) $^3J_d6.7$; $^3J_q4.8$
AME (in DMSO-d_6)	34.38(tm) $^1J\sim134$	120.52(m)	134.64(dm) $^1J_{183.8}$	163.01(d) $^3J_{4.1}$	114.74(dd) $^3J_{6.3,8.7}$	123.51(dq) $^1J_{163.8}$	118.64(dq) $^1J_{161.4}$;$^3J_{5.8}$	127.23(m)	141.47(m)
	C-9a	C-11	C-11a	C-12	C-13	C-14	C-15	OCH_3	
DAA (in TFA)	126.03(d) $^3J_{6.4}$	145.89(dd) $^1J_{188.2}$; $^3J_{1.8}$	137.34(dd) $^2J_{8.4}$; $^3J_{6.0}$	138.76(d) $^1J_{161.3}$	121.37(d) $^1J_{163.7}$	174.40(dd) $^2J_{4.8}$; $^3J_{7.2}$	17.14(dq) $^1J_{129.2}$; $^3J_{4.3}$		
AME (in DMSO-d_6)	134.83(dd) $^3J_{5.6,8.1}$	86.53(dm) $^1J_{158.4}$	58.82(dm) $^1J_{146.0}$	133.52(dm) $^1J_{164.4}$	120.10(d) $^1J_{164.4}$	167.11(d) $^2J_{3.8}$, $^3J_{5.9}$	16.67(dq) $^1J_{126.9}$, $^3J_{4.4}$	53.94(qm) $^1J_{141.9}$	

[a]Spectra were recorded with a Varian XL-100/15 Fourier transform spectrometer under the following conditions: acquisition time 1.6s (data accuracy ±0.3 Hz), spectral width 5120 Hz, flip angle ca. 40°, internal lock to ^{19}F in trifluoroacetic acid (TFA) or ^2H in DMSO-d_6, temperature 30°, concentration 30 mg/ml. For off-resonance decoupling protons were irradiated with $\gamma H_2/2\pi$ ca. 460 Hz at δ_H6.94 and 9.22. Broadband ^1H decoupling was by phase modulation of the decoupling field from 0 to 180° at 150 Hz, with $\gamma H_2/2\pi$ ca. 3800 Hz.

(14). Comparison of the ^{1}H spectra of DAA and AME shows these predicted changes do indeed occur, providing strong evidence for the structure of DAA given in scheme 1.

The ^{13}C nmr data for AME (table 2) is more detailed than that previously given (3), and assignments for C–12 and C–13 have been reversed to take into account known substituent effects (9). Resonances of carbons bonded to hydrogen in DAA were correlated with ^{1}H signals by plotting residual ^{13}C-^{1}H couplings in the off-resonance ^{1}H-decoupled spectrum as a function of the proton irradiation frequency (15, 16). Despite changes in chemical shifts, ^{13}C–H couplings and signal multiplicities due to structural changes and recording the ^{13}C spectra in different solvents, it was evident that the substituted benzene ring and unsaturated sidechain attached to C–2 of AME had been retained intact in DAA. Off-resonance decoupling experiments identified the signals arising from the carbons bonded to the vicinally coupled pairs of hydrogens, H–6, H–7 and H–12, H–13 and the high resolution ^{13}C spectrum showed that C–7 was further coupled to methyl hydrogens. Apart from chemical shift considerations, the characteristic multiplicities of the signals for the quaternary carbons in the benzene ring of DAA simplified assignments: C–5a was coupled to H–7, C–8 to H–6 and the methyl hydrogens, C–9 to H–7 and the methyl hydrogens, and C–9a to H–6. Assignment of the methyl carbon signal was trivial, and the spectrum of cinnamide in DMSO-d_6 (C=0, δ_c 166.64, dd, $^{2}J_{CH}$ 3.5, $^{3}J_{CH}$ 6.3 Hz) and in TFA (C=0, δ_c 175.29) was used to assign C–14 in both AME and DAA. The nmr parameters for C–5 of AME and DAA were not significantly different.

C–3 of DAA was identified by its chemical shift and characteristically large one-bond ^{13}C–H coupling (14). Additional coupling of C–3 with two other hydrogens, which can only be H–1 and H–12, establishes the position of the unsaturated sidechain and points of attachment to the substituted benzene ring. The remaining pyrrolo ring carbons were readily assigned: C–1 was coupled to the attached hydrogen and to H–3, H–12 and another hydrogen which must be H–11. C–2 was a multiplet as a result of coupling to H–1, H–3, H–12 and H–13, and C–11a was coupled to H–1 and H–3. The magnitude of the one-bond ^{13}C–H coupling between the remaining olefinic carbon (C–11) and the attached hydrogen requires that this carbon be bonded to nitrogen also, and the additional small coupling to a hydrogen (H–1) establishes the location of this methine group. The combined ^{1}H and ^{13}C nmr evidence is consistent with the structure shown for DAA, hence conversion of AME to DAA involves dehydration and dehydrogenation without skeletal rearrangement. Anhydrosibiromycinone (the aglycone and dehydration product of sibiromycin) contains the same pyrrolo(1,4)benzodiazepine nucleus and has, as expected, similar spectral characteristics (17, 18).

According to our proposed biosynthetic scheme, C–1 of AME is derived from C–3' of tyrosine (see scheme 1); we have previously noted that both diastereotopic hydrogens at C–3' of tyrosine are retained in anthramycin (4), presumably at C–1. If the anticipated labelling pattern is correct, there should be a 50% loss of tritium on the conversion of AME, biosynthetically labelled with DL-3'RS[3'-^{3}H]tyrosine, to DAA. The results of experiments in which ^{14}C, ^{3}H-labelled tyrosine molecules were fed to *S. refuineus* and the anthramycin isolated, recrystallized as AME and chemically converted to DAA are shown in table 3. AME from feeding experiments with 3'-tritiated tyrosine molecules showed approximately 50% loss of tritium during conversion to DAA, indicative of a non-stereospecific loss of hydrogen from C–1 of AME during its conversion to DAA. These results

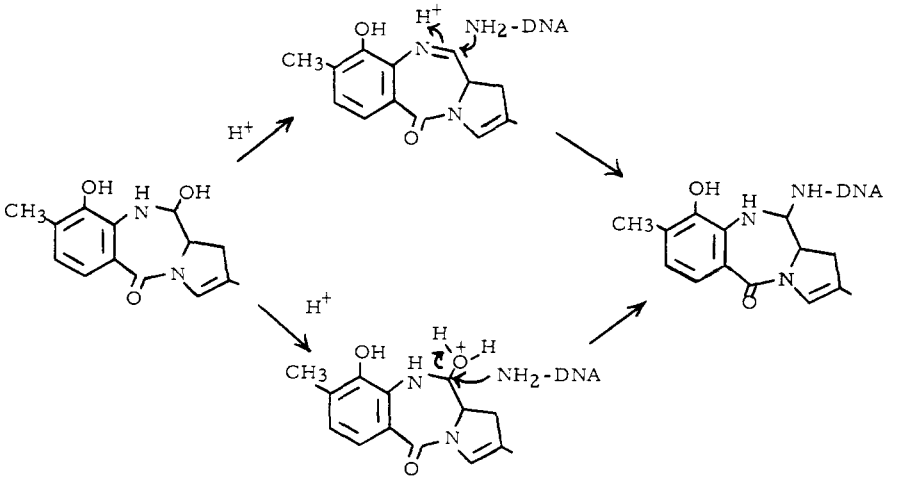
TABLE 3. Relative tritium retention in DAA obtained from reaction of AME with TFA.

Species of tryosine fed	$^3H/^{14}C$ Ratio of tyrosine	AME $^3H/^{14}C$ Ratio recrystallization[a]		DDA $^3H/^{14}C$ Ratio recrystallization[b]			% Final tritium in DAA retention relative to AME[c]
		1st	2nd	1st	2nd	3rd	
DL-[1'-^{14}C, 3'RS (3'-^{3}H)]	12.70	10.09	9.97	4.76	4.92	5.44	54.5
DL-[1'-^{14}C, 3'R (3'-^{3}H)]	11.04	9.03	9.12	6.51	5.10	4.63	50.6
DL-[1'-^{14}C, 3'S (3'-^{3}H)]	9.85	8.12	7.81	4.04	3.48	3.43	44.2
L-[1'-^{14}C, 2- or 6-^{3}H]	18.38	9.55	8.86	8.05	8.45	——	95.4
L-[1'-^{14}C, 3- or 5-^{3}H]	11.20	5.37	5.79	5.53	5.69	——	98.4

[a]Recrystallized from $MeOH/H_2O$.
[b]Recrystallized from TFA/H_2O.
[c]Tritium retention = $\frac{^3H/^{14}C \text{ ratio of DAA}}{^3H/^{14}C \text{ ratio of AME}} \times 100$

therefore confirm beyond doubt the origin of C–1 of anthramycin as being 3' of tyrosine. Species of AME biosynthetically labelled from tyrosine ring tritiated molecules did not show loss of tritium during conversion to DAA (table 3). These latter results were anticipated from our knowledge of the labelling patterns at C–12 and C–13 of anthramycin following incorporation of L–[2– or 6–^{3}H] and L–[3– or 5–^{3}H]tyrosine, respectively.

The interaction of anthramycin with DNA has been studied by both of us at Kentucky (7–9) and by other groups (10, 19). We have proposed that anthramycin forms a covalent adduct through carbon 11 of the drug to the 2–NH_2 group of guanine and lies hidden in the narrow groove of DNA (7). Furthermore, we have suggested that a protonated carbinolamine is the reactive intermediate that undergoes nucleophilic attack by the 2-amino group of guanine (9), while Lown and Joshua consider that the imine is the reactive form, since, when the azomethine function is conjugated to the carbonyl through the benzene ring, it can act as a powerful alkylating agent (10) (see scheme 2). We have shown DDA does not



SCHEME 2. Alternative mechanisms for the formation of the pyrrolo(1,4)-benzodiazepine antibiotic-DNA adducts.

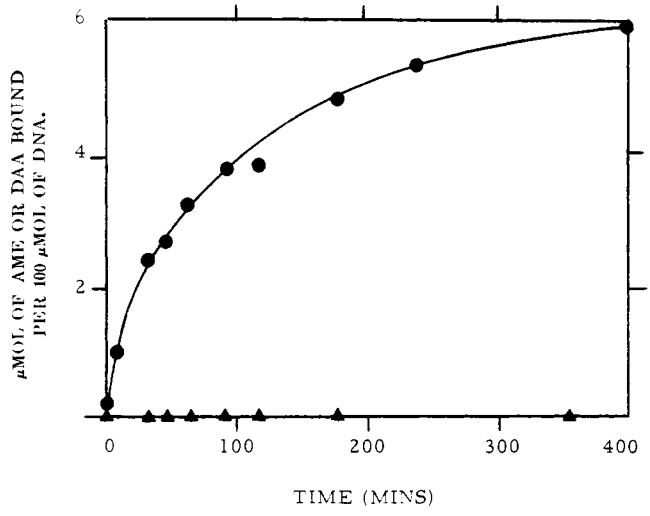


FIG. 1. Time course binding of (15-^{3}H)anthramycin (-●-) and (15-^{3}H)DAA (-▲-) to calf thymus DNA. Solutions of anthramycin, 0.90 μmoles/ml (Sp.Act.=2.93 μCi/-μmole) or DAA, 0.89 μmoles/ml (Sp.Act.=1.70 μCi/-μmole) dissolved in DMF (0.1 mls) were added to 2.1 mls of a DNA solution (0.15 μmoles/ml). At various times 0.1 ml aliquots were withdrawn from each reaction mixture diluted to 1.0 ml with 0.1 SSC and extracted immediately with 2 x 2 ml of water saturated n-butanol. A 0.5 ml aliquot of the remaining aqueous phase was then prepared for liquid scintillation counting as described previously (9).

form a covalent adduct with DNA (see figure 1), although it possesses the azomethine function. This we believe may be due to the lower reactivity of the azomethine group in DAA, compared to anhydroanthramycin, because of its conjugation into the pyrrolo ring. Since sibiromycin has an unsaturated pyrrolo ring similar to that introduced into AME by reaction with TFA but is reactive towards DNA, we feel it is unlikely that the azomethine function is the reactive intermediate in this case. In accord with this idea, anhydrosibiromycin is biologically unreactive (18), although it is able to form an unstable adduct with DNA (20). Lown and Joshua (10) have suggested that the Sn_1ca (21) mechanism proposed for the protonated carbinolamine is unlikely since the *N*-acetyl derivative is unreactive towards DNA. However, studies using our CPK space filling model of this anthramycin derivative and DNA show that steric hindrance prevents the *N*-acetyl anthramycin from approaching the reactive group on DNA, and therefore the Sn_1ca mechanism cannot be eliminated on these grounds. We therefore conclude that the Sn_1ca mechanism or some transient intermediate not involving an azomethine function is the likely reactive compound which forms an adduct with DNA.

EXPERIMENTAL SECTION

CHEMICALS AND RADIOISOTOPES.—AME was kindly supplied to us by Hoffmann-LaRoche Inc., Nutley, N.J. Calf thymus DNA and TFA were purchased from Sigma. L-[CH_3-^{3}H]-methionine, L-[2- or 6-^{3}H]tyrosine, L-[3- or 5-^{3}H]tyrosine and L-[1'-^{14}C]tyrosine were obtained from New England Nuclear. DL-[1'-^{14}C]tyrosine was purchased from ICN. DL-[3'RS-

(3'-^{3}H)], DL-[3'S(3'-^{3}H)] and DL-[3'R(3'-^{3}H)]tyrosine were kindly provided by Dr. Ronald Parry at Rice University (Texas). [15-^{3}H]Anthramycin was biosynthetically prepared and purified as previously described (9).

CHEMICAL CONVERSION OF AME TO DDA.—To 100 mg of crystalline AME, 2 ml of TFA was added and stirred at room temperature for 30 mins. The addition of water until the solution became cloudy and partial evaporation overnight resulted in a yellow crystalline product (about 50% yield), mp 283-285°. The high resolution mass spectrum indicated a molecular weight of 295 and an empirical formula of $C_{16}H_{13}N_3O_3$. (Calculated 295.09569, Found 295.0966± 0.0009). $[\alpha]^{20}D$ 0° (c 1.00 DMF); λ max (DMF) nm (E) 280 (24,000); 384s (4,550); 402 (6,270); 426 (5,180). λ max (KBr) (cm^{-1}) 3350, 3290, 3160, 1675, 1645, 1630, 1610 and 1560.

BIOSYNTHETIC EXPERIMENTS.—Optimum conditions for production, isolation of anthramycin, and the incorporation of precursors into anthramycin have been described previously (3).

REACTION OF DAA WITH DNA.—Conditions for the time course reaction of [15-^{3}H]DAA and [15-^{3}H]AME with calf thymus DNA have been described previously (9).

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