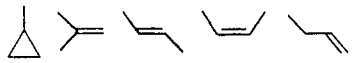
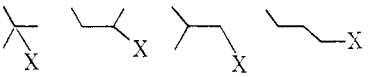
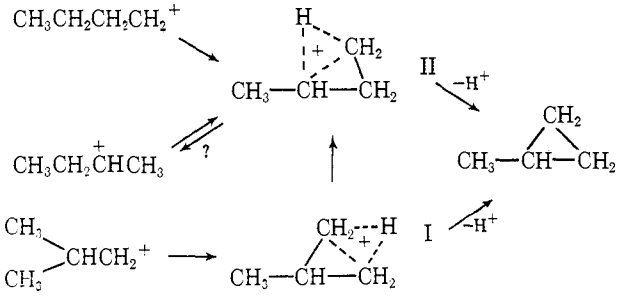
Table I. Diazotization of Isobutyl- and *n*-Butylamine[a]

Acid	Solvent	Hydrocarbon products[b] % yield[c]	Composition					Butyl derivatives[b] % yield	Product composition[d]			
												
Isobutylamine												
HOAc	$CHCl_3$	33	14	73	4.2	2.1	6.9	54	5	9	86	
HCl	$CHCl_3$	8	15	57	5.0	3.7	19					
HOAc	Glyme[g]	35	10	76	4.1	2.2	7.7	44	4	11	85	
HCl	Glyme[g]	20	9.7	68	5.6	3.2	13	54[e,f]	5	9	86	
HOAc	HOAc	20	4.5	62	14	7.1	12	51	47	28	25	
HOAc	50% aq. HOAc	12	2.5	40	25	11	21	21	54	20	26	
***n*-Butylamine**												
HOAc	$CHCl_3$	8	4.3		4.5	2	89			4		96
HCl	$CHCl_3$	9	3.1		3.1	2	92					
HOAc	Glyme[g]	18	2.9		6.6	3	88	75[e]		4		96
HCl	Glyme[g]	12	3.5		6.6	3	87	72[e]		2		98
HOAc	HOAc–$NaNO_2$[h]	5	0.7		25	11	63	30		30		70
HOAc	50% aq. HOAc–$NaNO_2$	11	0.3		29	14	56	48		32		68

[a] Amine (0.005 mole), acid (0.005 mole), and alkyl nitrite (0.0055 mole) in 10 ml. of solvent, at reflux (*t*-$C_4H_9NH_2$ data not included here, since only isobutene and *t*-butyl products are formed). [b] Hydrocarbons analyzed on Dowtherm-A (20%), Chromosorb P.; and $AgNO_3$–benzyl cyanide, Chromosorb P (room temperature). Acetates, alcohols, and halides analyzed on diisodecyl phthalate (5%), Bentone (5%), Chromosorb P (Hy-Fi), at 75°. [c] Yields determined *via* quantitative g.l.p.c. [d] Alcohols and acetates (or halides) combined. [e] Ether cleavage products not included here (see A. T. Jurewicz, J. H. Bayless, and L. Friedman, *J. Am. Chem. Soc.*, **87**, 5788 (1965)). [f] N.m.r. analysis of unresolved g.l.p.c. trapped sample. [g] Hydrocarbon compositions from thermal decomposition of nitrosoamides, nitroamides, and nitrourethans are essentially identical. Slightly more rearrangement is observed in butyl derivatives. [h] *Cf.* ref. 16c.

In addition to other routes,[2] poorly solvated cations may yield by "neighboring group participation" (*internal solvation*) the more stable protonated cyclopropane intermediate.[14] Cyclopropanes could then result by simple loss of proton.[15] Some of the rearranged solvent- and counterion-derived products could result from nucleophilic attack at the quaternary carbon with concomitant ring cleavage.



Since *n*- and *sec*-butyl cations do not give any iso- and *t*-butyl derivatives it may be concluded that II best describes the intermediate. It is significant that *t*-butyl derivatives cannot be derived from either I or II.

The results obtained are essentially independent of the cation precursor[11] (amine diazotization, rearrangement of nitroso- and nitrourethans and ureas)[16] and reflect mainly the environment of the cation, thus suggesting that all of these precursors generate the same *primary reactive intermediate(s)* in a given solvent system. Product fallout can then occur as described or *via* the other accepted pathways.[2]

(14) G. J. Karabatsos, C. E. Orzech, Jr., and S. Meyerson, *J. Am. Chem. Soc.*, **87**, 4394 (1965), and references contained therein.

(15) (a) C. C. Lee and J. E. Kruger, *ibid.*, **87**, 3986 (1965). (b) Dimethylcyclopropane (1%) was formed by aprotic diazotization of neopentyl amine. *Cf.* ref. 5b. (c) Protic diazotization of *trans*-8-hydrindanylcarbinylamine (a neopentyl system) gave tricyclo[4.3.1.0^{1,6}]-decane in 74% yield: W. G. Dauben and P. Laug, *Tetrahedron*, **20**, 1259 (1964).

(16) (a) Table I; (b) E. H. White and D. W. Grisley, Jr., *J. Am. Chem. Soc.*, **83**, 1191 (1961), and references contained therein; (c) A. Streitwieser and W. D. Schaffer, *ibid.*, **79**, 2888 (1957).

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Received October 29, 1965

Isolation and Characterization of Anthramycin, a New Antitumor Antibiotic

Sir:

It has been observed by Tendler and Korman[1] that a thermophilic actinomycete (*Streptomyces refuineus* var. *thermotolerans*, NRRL 3143) produces a fermentation broth possessing antibiotic properties and exhibiting *in vivo* antitumor activity. We wish to report the isolation of a pure, crystalline antibiotic which is responsible for the observed antitumor activity.

The isolation of this antibiotic, which we have named anthramycin,[2] was followed by the use of a quantitative cup-plate assay method employing *Sarcina lutea* (PCI-1001) and *Bacillus sp. TA* (NRRL B-3167) as test organisms. Anthramycin was removed from the fermentation broth filtrate[3] by countercurrent column extraction with 1-butanol, and the resulting extract was subjected to a fractional liquid extraction employing 1-butanol

(1) M. D. Tendler and S. Korman, *Nature*, **199**, 501 (1963).

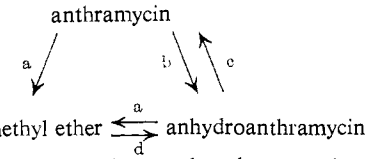
(2) The name "refuin" has been used by Tendler and Korman for the initial designation of the antitumor active principle, which they assumed to be a protein; *cf.* S. Korman and M. D. Tendler, Abstracts, Meeting of the American Association for Cancer Research, Toronto, Canada, May 1963. Their crude preparation contained approximately 0.5% of anthramycin. We have subsequently designated the anticancer active principle as Roche 5-9000. The generic name, anthramycin, has been derived from the structure of this antibiotic, an integral part of which is an anthranilic acid moiety.

(3) The fermentation procedure will be published elsewhere.

and water as the solvents. Fractional precipitation of the butanol extract with petroleum ether, followed by a Craig countercurrent distribution (solvent system: chloroform–2-propanol–ethanol–water, 3:1:1:5; 196 transfers; $K = 0.69$), yielded anthramycin of high purity.

The antibiotic afforded crystals either from hot methanol–water *or* from boiling acetone; repeated recrystallization from these solvents gave analytically pure samples which were suitable for characterization. Spectroscopic data indicated that the two crystalline samples were chemically different and that neither represented unaltered anthramycin. Thus, the n.m.r. spectrum (in DMF-d_7) of methanol–water crystallized antibiotic revealed the presence of a methoxyl group (3 H singlet at δ 3.35, 0.04 p.p.m. downfield from added methanol) which was absent in anthramycin prior to its crystallization. The possibility that this product represented a methanol solvate of anthramycin was ruled out by the fact that it remained unchanged (as revealed by n.m.r.) upon recrystallization from boiling deuterated methanol–water (CD_3OD–D_2O). The crystallization of the antibiotic from boiling acetone had also altered the chemical structure of anthramycin, since the ultraviolet spectrum of the resulting product, when measured in nonhydroxylic solvents (*e.g.*, acetonitrile), was significantly different from that of anthramycin prior to its crystallization. In addition, it was found that this compound reacted with water which converted it in high yield to a new crystalline product. Subsequent structure work[4] established that this latter substance is pure anthramycin, whereas methanol–water and acetone crystallized antibiotics are anthramycin derivatives, designated as anthramycin methyl ether and anhydroanthramycin, respectively. Our findings were substantiated by the fact that crystalline anthramycin could be converted to anthramycin methyl ether and to anhydroanthramycin by crystallization under conditions which were identical with those employed in their original preparation. These transformations, which also include the interconversion of anthramycin methyl ether and anhydroanthramycin, are summarized in Scheme I.

Scheme I[a]



[a] Path a, crystallization from hot methanol–water; b, crystallization from boiling acetone; c, crystallization from acetone–water at room temperature; d, refluxing acetonitrile (plus catalytic amount of Amberlite IRC-50) or refluxing isopropenyl acetate.

Anthramycin and its derivatives are very labile substances which are difficult to characterize. They are extremely sensitive to heat and are stable in solution only under essentially neutral conditions. Anthramycin methyl ether, which possesses comparatively the highest stability, could be fully characterized by spectroscopic methods and was therefore selected for subsequent structure work.

Anthramycin methyl ether crystallized from methanol–water as a hydrate, pale yellow needles, which decomposed above 120° *in vacuo*. *Anal.* Calcd. for $C_{17}H_{19}N_3O_4 \cdot H_2O$: C, 58.78; H, 6.09; N, 12.10; O, 23.03; OCH_3, 8.94; mol. wt., 347. Found: C, 58.99; H, 6.27; N, 12.10; O, 22.96; OCH_3, 9.03; mol. wt. (isothermal distillation), 329. The high-resolution mass spectrum[5] indicated a molecular weight of 297 (instead of 329) and an empirical formula of $C_{16}H_{15}N_3O_3$ (Calcd.: 297.1113. Found, 297.1117), which corresponded to that of anhydroanthramycin; $\lambda_{max}^{CH_3CN}$ mμ (ϵ): 231 (22,100), 334 (36,800), and 360 s (22,500); ν_{max}^{KBr} (cm.$^{-1}$): 3400, 3340, 3220, 1665, 1615, 1600, 1590, 1560, and 1520; $[\alpha]^{25}D$ +1002° (*c* 1.01, DMSO).

Anhydroanthramycin crystallized from acetone in yellow plates, m.p. 200–201°, dec. *in vacuo*, which tenaciously retained solvent. Recrystallization from acetonitrile gave yellow prisms, m.p. 205–206°, dec. *in vacuo*, which were solvent free after drying under high vacuum at 80°. *Anal.* Calcd. for $C_{16}H_{15}N_3O_3$: C, 64.63; H, 5.09; N, 14.14. Found: C, 64.98; H, 5.15; N, 14.39. The low-resolution mass spectrum confirmed the calculated molecular weight of 297; $\lambda_{max}^{CH_3CN}$ mμ (ϵ): 228 (16,200), 236 s (15,800), 300 s (19,400), 313 (21,400), and 352 (22,800); ν_{max}^{KBr} (cm.$^{-1}$): 3480, 3420, 3320, 1680, 1630, 1610, 1600, 1565, and 1495; $[\alpha]^{25}D$ +1796° (*c* 1.00, N,N-dimethylacetamide).

Anthramycin was obtained from an acetone–water solution of anhydroanthramycin in small yellow prisms, m.p. 188–194°, dec. *in vacuo*. *Anal.* Calcd. for $C_{16}H_{17}N_3O_4$: C, 61.00; H, 5.43; N, 13.31. Found: C, 61.17; H, 5.56; N, 13.26; $\lambda_{max}^{CH_3CN}$ mμ (ϵ): 235 (18,200) and 333 (31,800); ν_{max}^{KBr} (cm.$^{-1}$): 3370, 1665, 1660, 1615, 1595, 1555, 1535, 1480, 1420, 1250, 1220, 1150, 1085, 970, 935, 840, and 760; $[\alpha]^{25}D$ +930° (*c* 1.00, DMF).

We also wish to report the isolation of a minor fermentation product which was of importance in connection with the structure elucidation of anthramycin. This compound, referred to as "yellow pigment,"[4,6] was extracted from the fermentation broth along with anthramycin and was separated from the antibiotic in the countercurrent distribution ($K = 0.25$) mentioned above. Subsequent column chromatography (Florisil) afforded material of high purity which could be crystallized from methanol.

The "yellow pigment" was obtained as a hydrate in bright yellow prisms, m.p. 280–282°, dec. *in vacuo*. The high-resolution mass spectrum[5] indicated a molecular weight of 269 and an empirical formula $C_{15}H_{15}N_3O_2$ (Calcd.: 269.1164. Found: 269.1147). Recrystallization from methanol–benzene afforded the compound in anhydrous form as yellow needles, m.p. 280–282°, dec. *in vacuo*. (*Anal.* Calcd. for $C_{15}H_{15}N_3O_2$: C, 66.90; H, 5.61; N, 15.61. Found: C, 66.66; H, 5.80; N, 15.56); $\lambda_{max}^{2\text{-propanol}}$ mμ (ϵ): 238 (18,100), 253 s (15,200), 324 (33,100), 335 (32,950), and 361 s (16,000); $\lambda_{max}^{0.1N HCl}$ mμ (ϵ): 230 (11,250) and 328 (32,200); ν_{max}^{KBr} (cm.$^{-1}$): 3395, 3335, 3210, 1680, 1655, 1615, 1605, 1585, and 1575; $[\alpha]^{25}D$ +883° (*c* 1.03, DMSO).

(4) W. Leimgruber, A. D. Batcho, and F. Schenker, *J. Am. Chem. Soc.*, 87, 5793 (1965).

(5) High-resolution mass spectra were determined with a CEC 21-110 spectrometer using photographic recording. For techniques of mass measurement and data processing see K. Biemann, *et al.*, Abstracts, 12th Annual Conference on Mass Spectrometers, Montreal, Canada, June 1964, pp. 428, 433, and 442.

(6) This compound showed no antibiotic properties.

Anthramycin exhibited *in vitro* cytotoxic activity and *in vivo* antitumor activity against transplantable tumors in mice.[7] It also showed substantial *in vitro* antimicrobial activity, particularly against Gram-positive bacteria and, to a lesser extent, against Gram-negative organisms and fungi. The direct comparison of anthramycin and anthramycin methyl ether showed no significant differences in biological activity. Preliminary clinical trials indicate that the antibiotic possesses appreciable carcinostatic activity in a variety of malignant human tumors.[8,9]

(7) (a) G. Zbinden in "Proceedings of the International Symposium on the Chemotherapy of Cancer," Pl. A. Plattner, Ed., Elsevier Publishing Co., Amsterdam 1964, pp. 303–310; (b) E. Grunberg, H. N. Prince, E. Titsworth, G. Beskid, and M. D. Tendler, to be published.

(8) Initial clinical trials were conducted with various preparations of the crude antibiotic; when crystalline anthramycin methyl ether, also designated as Roche 5-9000/15, became available, it was used exclusively.

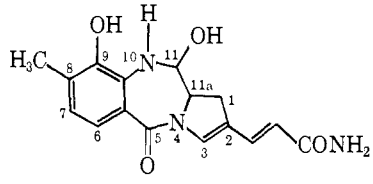
(9) S. Korman and M. D. Tendler, to be published.

W. Leimgruber, V. Stefanović, F. Schenker, A. Karr, J. Berger
Hoffmann-La Roche, Research Division
Nutley, New Jersey
Received October 27, 1965

The Structure of Anthramycin

Sir:

We wish to present evidence which permits the assignment of structure **1** (5,10,11,11a-tetrahydro-9,11-dihydroxy-8-methyl-5-oxo-1H-pyrrolo[2,1-*c*][1,4]benzodiazepine-2-acrylamide) to anthramycin.[1]



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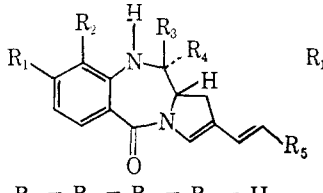
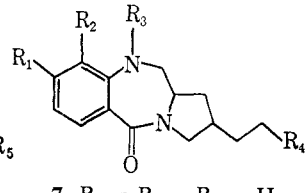
The structure elucidation of this antibiotic was facilitated by the investigation of another fermentation product, designated as "yellow pigment,"[1] which possesses the closely related, but less complex structure **2**.[2]





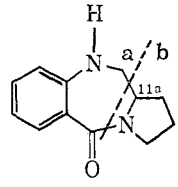
2, $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = CONH_2$
3, $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = CN$
4, $R_1 = CH_3$, $R_2 = OH$, $R_3 = H$, $R_4 = OCH_3$, $R_5 = CONH_2$
5, $R_1 = CH_3$, $R_2 = OH$, $R_3 = OCH_3$, $R_4 = H$, $R_5 = CONH_2$
6, $R_1 = CH_3$, $R_2 = OCH_3$, $R_3 = H$, $R_4 = OCH_3$, $R_5 = CONH_2$
7, $R_1 = R_2 = R_3 = H$, $R_4 = CONH_2$
8, $R_1 = R_2 = R_3 = H$, $R_4 = COOCH_3$
9, $R_1 = CH_3$, $R_2 = OH$, $R_3 = H$, $R_4 = CONH_2$
10, $R_1 = CH_3$, $R_2 = OCOCH_3$, $R_3 = COCH_3$, $R_4 = CONH_2$
11, $R_1 = CH_3$, $R_2 = OCH_3$, $R_3 = H$, $R_4 = CONH_2$

(1) W. Leimgruber, V. Stefanović, F. Schenker, A. Karr, and J. Berger, *J. Am. Chem. Soc.*, **87**, 5731 (1965).

(2) We have not assigned a generic name to the "yellow pigment" since it may be regarded as an anthramycin derivative, namely, desdihydroxydesmethylanthramycin.

The distinctive features of the n.m.r. spectrum[3] of the "yellow pigment" could be interpreted in terms of structure **2** as follows: two doublets at δ 5.81 and 7.37 (J = 15 c.p.s.) for the *trans*-olefinic protons, a singlet at δ 7.47 for C-3–H, a doublet of doublets at δ 7.80 for C-6–H, and a broad multiplet at $\delta \sim 4.2$ for C-11a–H. The presence of a conjugated primary amide was established by the conversion of **2** to the conjugated nitrile **3**[4] [m.p. 233–234°; ν_{max}^{KBr} 2210 cm.$^{-1}$ (intense); δ 5.36 and 7.52 (two 1 H doublets, J = 16 c.p.s.)], effected by the action of phosphorus pentoxide in boiling quinoline. Hydrolysis of **3** with concentrated hydrochloric acid reconverted it to **2**. Catalytic reduction (Pd–C) of **2** resulted in the formation of a mixture of two epimeric (at C-2) tetrahydro derivatives of structure **7**. The presence of a nonconjugated, primary amide was substantiated by the conversion of **7** (ν_{max}^{KBr} 1670 cm.$^{-1}$) to the corresponding saturated ester **8**[5] ($\nu_{max}^{CHCl_3}$ 1735 cm.$^{-1}$), obtained by hydrolysis (6 *N* HCl) and subsequent esterification with diazomethane. The presence of an anthranilamide moiety in compounds **7** and **8** was deduced from their n.m.r. (aromatic region) and ultraviolet spectra (*cf.* Table I) which were very similar to those of anthranilamide.

The evidence presented above accounts for all the hetero atoms and functional groups of the "yellow pigment" and demands a tricyclic expression for its structure. The n.m.r. and ultraviolet data particularly favored two formulas: One is represented by expression **2** which contains the pyrrolobenzodiazepine nucleus **12**; the corresponding alternative possesses the pyridoquinazoline skeleton **13**. The spectroscopic properties of the parent tricyclic compounds **12** and **13**[6] revealed the striking similarity between **12** and **8** (Table I). The presence of a pyrrolobenzodiazepine skeleton in the "yellow pigment" and its transformation products was further supported by their mass spectra, all of which exhibited two intense peaks (**3**, *m/e* 133, 119; **8**, *m/e* 133, 156; **12**, *m/e* 133, 70) which could be attributed to fragments of the type a and b (always observed in



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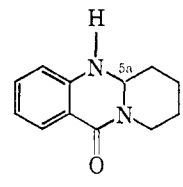

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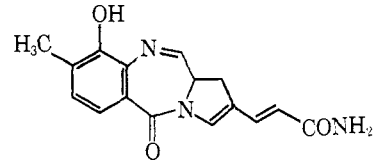

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(3) The n.m.r. spectra were recorded in DMSO-d_6 solution (unless otherwise stated), and the chemical shifts are reported in p.p.m. (δ) downfield from an internal tetramethylsilane reference.

(4) Satisfactory elemental analyses were obtained for all new compounds reported here.

(5) Mixture of epimers.

(6) Compound **12**, m.p. 183–184°, was prepared by lithium aluminum hydride reduction of the dilactam, m.p. 231–232°, obtained from the fusion of proline and isatoic anhydride. Compound **13**, m.p. 135–136°, was prepared from the quinazolinone [E. Späth and N. Platzer, *Ber.*, **68**, 2225 (1935)] by reduction with sodium borohydride in the presence of aluminum chloride.