Journal of Medicinal Chemistry

VOLUME 8, NUMBER 1 DECEMBER 28, 1964

Chemistry and Structure of Mitomycin C[1]

CALVIN L. STEVENS, K. GRANT TAYLOR, MORTON E. MUNK, W. S. MARSHALL, KLAUS NOLL, G. D. SHAH, L. G. SHAH, AND K. UZU

Department of Chemistry, Wayne State University, Detroit, Michigan

Received July 29, 1963

Mitomycin C (Ia) was converted by acid hydrolysis to a series of degradation products (IIa–f, IIIa and b). Ozonization, reduction, and oxidation reactions were carried out with selected acid hydrolysis products to establish the carbon skeleton. Comparative acid hydrolysis studies of Ia and N-methylmitomycin (porfiromycin) (Ib) together with pK_a' and n.m.r. data, indicated the presence and location of the aziridine ring. Deuterium incorporation studies showed that the methoxyl group in mitomycin C was located at position 9a. The structure for mitomycin C deduced in this investigation is in agreement with that reported in the recent communication of the Lederle workers[2a] and the X-ray analysis.[2b]

Mitomycin C was first isolated from *Streptomyces caesipitosus* by Wakaki and co-workers[3] and preliminary structural investigations were performed by Sato and co-workers.[4] Closely related antibiotics designated as mitomycins A and B[5] and porfiromycin[6] have also been isolated. Porfiromycin has been shown to be an N-methyl derivative of mitomycin C.[7] The promising antitumor and antibiotic activity of these compounds led us to initiate a structure determination program in mid 1960. As this work neared completion, Webb and co-workers[2a] reported their proof of the structure of mitomycin A,[5] confirmed by Tulinsky *via* an X-ray diffraction analysis of the *p*-bromobenzenesulfonyl derivative.[2b] The Lederle workers also established the relationship of mitomycin A to mitomycins B and C, and to porfiromycin by direct chemical interconversion. In this paper we wish to report an independent structure determination of mitomycin C as well as our fairly extensive exploration of the essential chemistry of the molecule through a series of hydrolytic degradation products.

Hydrolysis of mitomycin C at 60° in 2 *N* hydrochloric acid for 2 hr. proceeded with evolution of carbon dioxide and produced an amphoteric ($pK_a' = 5.4$ and 7.3), optically active hydrolysate IIIa,[2a] $[\alpha]^{23}D$ 110 ± 10°. The indicator properties (see Experimental) of IIIa were similar to those reported for α-tocopurple (VIa) and a homolog (VIb).[8]

Since IIIa could not be purified satisfactorily, its empirical formula, $C_{13}H_{14}N_2O_5$, was deduced from that of its yellow tetraacetyl derivative, $C_{21}H_{22}N_2O_9$ (IIIb).[2a] Tetraacetate IIIb exhibited a sharp band at 5.65 μ in its infrared spectrum, which is characteristic of acetates of enolic hydroxyl groups.[9] Also, one acetyl group could be cleanly and selectively removed from IIIb merely on dissolution in cold, 1% methanolic ammonia, and removal of this acetyl function gave IIIc with regeneration of the indicator properties which IIIa had lost on acetylation.

Catalytic hydrogenation of tetraacetate IIIb resulted in the absorption of 1 mole equiv. of hydrogen. The colorless reduction product was isolated as its hexaacetyl derivative (VII) by treating the reduction solution with acetic anhydride prior to exposure to air. When the reduction solution was exposed to air, the reduction product was readily oxidized and tetraacetate IIIb could be recovered from the reaction. Such facile reductions are well documented for both 1,2- and 1,4-quinones[10] and reoxidation to the quinone system would be facilitated by the presence of electron-donating substituents such as oxygen and/or nitrogen.[11]

Treatment of hydrolysate IIIa with acetic anhydride in methanol resulted in formation of an acidic ($pK_a' = 6.50$) N-acetyl derivative (IIId) whose indicator and

(1) (a) This investigation was supported by Research Grant CY 3772 from the National Institutes of Health, Public Health Service. (b) Abstracted in part from the doctoral theses of K. G. Taylor and W. S. Marshall, Wayne State University, 1963.

(2) (a) J. S. Webb, D. Cosulich, J. Mowat, J. Patrick, R. Broschard, W. Meyer, R. Williams, C. Wolf, W. Fulmor, C. Pidacks, and J. Lancaster, *J. Am. Chem. Soc.*, **84**, 3185 (1962); (b) A. Tulinsky, *ibid.*, **84**, 3188 (1962).

(3) S. Wakaki, H. Marumo, K. Tomoika, G. Shimizu, E. Kato, H. Kamada, S. Kudo, and Y. Fujimoto, *Antibiot. Chemotherapy*, **8**, 228 (1958).

(4) S. Wakaki [*Cancer Chemotherapy Rept.*, **13**, 79 (1961)] summarizes work by N. Sato, Y. Harada, K. Kumabe, and K. Uzu which was reported to the Japan Antibiotics Research Association, Tokyo, July 1960.

(5) T. Hata, S. Yoshimoto, R. Sugawara, A. Matsumae, K. Kanamori, T. Shima, and T. Hoshi, *J. Antibiotics* (Tokyo), *Ser. A*, **9**, 141 (1956).

(6) C. DeBoer, P. Gray, B. Tabenkin, and S. Bradley, "Antimicrobial Agents Annual 1960," Plenum Press, New York, N. Y., 1961, pp. 17–40.

(7) S. Wakaki, Y. Harada, K. Uzu, G. B. Whitfield, A. N. Wilson, A. Kalowsky, E. O. Stapley, F. J. Wolf, and D. E. Williams, *Antibiot. Chemotherapy*, **12**, 469 (1962).

(8) V. Frampton, W. Skinner, P. Cambour, and P. Bailey, *J. Am. Chem. Soc.*, **82**, 4632 (1960).

(9) L. J. Bellamy, "The Infrared Spectra of Complex Molecules," John Wiley and Sons, New York, N. Y., 1958, p. 182.

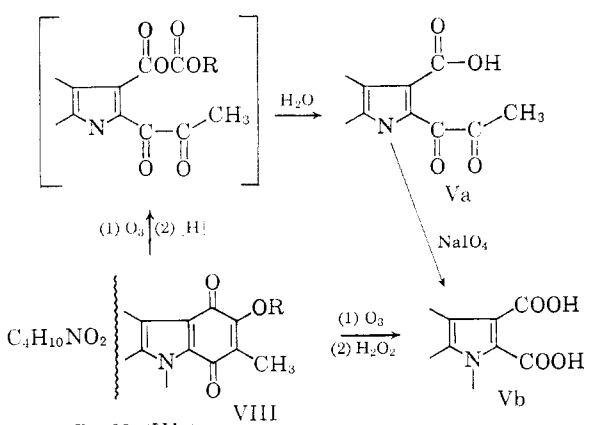
(10) R. B. Wagner and H. D. Zook, "Synthetic Organic Chemistry," John Wiley and Sons, New York, N. Y., 1953, p. 154.

(11) L. Fieser and M. Fieser, "Organic Chemistry," Heath and Co., Boston, Mass., 1956, p. 712.

spectral properties were similar to IIIa. Esterification of the acidic function with diazomethane produced a neutral, nonindicating methoxyacetamide, $C_{16}H_{18}N_2O_6$ (IIIe), which incorporated two more acetyl groups on further treatment with acetic anhydride in pyridine to give IIIf. The infrared spectrum of methoxytriacetate IIIf exhibited a sharp band at 5.75 μ indicative of O-acetate carbonyl absorption, but no band at 5.65 μ for an enolic acetate.

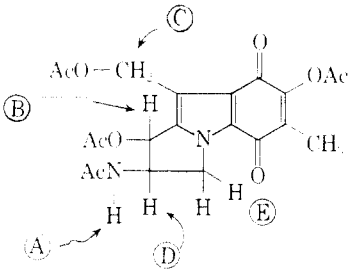
Ozonization of tetraacetate IIIb with a reductive work-up afforded a crystalline optically active ($[\alpha]^{25}D$ $-151°$) triacetate (Va) (Chart I) that contained both acid ($pK_a' = 4.83$) and carbonyl functions (positive dinitrophenylhydrazine test). Compound Va was no longer an indicator, but gave a positive iodoform test.[12] Since the dinitrophenylhydrazone derivative gave a negative iodoform test, the presence of a methyl ketone, rather than a methyl carbinol moiety, was indicated. Accordingly, keto acid Va on treatment with sodium metaperiodate consumed 1 mole equiv. and produced diacid Vb (negative iodoform and dinitrophenylhydrazone tests) identical with that obtained from an ozonization of IIIb followed by hydrogen peroxide oxidative work-up. Treatment of diacid Vb with diazomethane afforded a dimethyl ester (Vc), thus confirming the diacid formulation. Pine-splinter tests on the ozonolysis products indicated the possible existence of a pyrrole- or indole-type nucleus in mitomycin C, and this was confirmed when diacid Vb gave a positive Ehrlich test only when warmed with the reagent. This was indicative of a tetrasubstituted pyrrole nucleus with a carboxyl group as an α-substituent.[13] Since keto acid Va gave negative Ehrlich tests, even on prolonged heating, it appeared that the diketone of Va was located in the α-position of a pyrrole nucleus.

Consideration of these data permitted a partial structure VIII to be drawn for hydrolysate IIIa. The ozonization and periodate reactions are summarized in the following reaction scheme.



The structure of the remaining four-carbon fragment was deduced in the following manner. Methoxyacetamide IIIe (2 hydroxyls free) was found to consume no periodate. However, when diacid Vb (which was optically active: $[\alpha]^{25}D$ $-149°$) was treated with periodate, following a basic cleavage of acetyl protection, 1 mole equiv. was consumed and optical activity was lost. These data indicated that the C_4 fragment should contain an isolated hydroxyl function attached to a nonasymmetric environment, and vicinal hydroxyl and amino groups in an asymmetric environment.

Thus the n.m.r. spectrum ($CDCl_3$) of tetraacetate IIIb supported the assignment of structure IIIb to that compound. In addition to five singlets attributable to

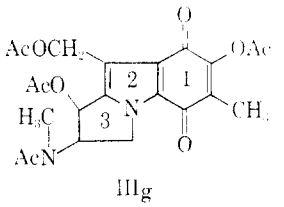


IIIb

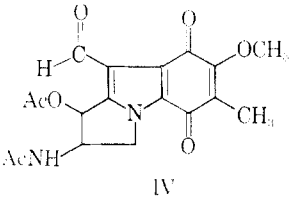
C-methyl and acetyl methyl groups, there was a sixth singlet, equivalent to two protons, located at δ_{TMS} 5.20.[14] This signal was assigned to the protons of a benzylic-like methylene group attached to a β-position on the pyrrole nucleus and labeled C in formula IIIb. A broadened doublet (one hydrogen) centered at δ_{TMS} 6.48 was assigned as the NH signal (A). The NH proton of N-acetylphenethylamine is seen at δ_{TMS} 6.50 and is analogous.[15] A sharp doublet (one hydrogen) centered at δ_{TMS} 6.04 was assigned as the signal of hydrogen B. The signal for hydrogen D was an unsymmetrical quartet centered at about δ_{TMS} 4.7. The remaining two protons (E) were nonequivalent exhibiting a complex multiplet centered approximately at δ_{TMS} 4.0. The two E′ protons of methoxyindole IX[16] arise at δ_{TMS} 4.28 and are analogous.

Oxidation of methoxyacetamide IIIe with Jones' reagent[17] produced an aldehyde, isolated as the diacetyl derivative IV and identified by the aldehyde hydrogen n.m.r. (DMSO-d_3) signal at δ_{TMS} 10.4, thereby confirming the presence of a primary hydroxyl function in tetraacetate IIIb.

The existence of a primary amine function in mitomycin C degradation products was confirmed by hydrolysis experiments performed on N-methylmitomycin C (Ib). Thus, hydrolysis of Ib under conditions



IIIg



IV

(12) Functional group analyses of mitomycin C had indicated the presence of one C-methyl and one methoxyl group.

(13) N. V. Sidgwick, "The Organic Chemistry of Nitrogen," Oxford University Press, Oxford, 1937, p. 484; *cf.* H. Fischer and H. Orth, "Die Chemie des Pyrrols," Edwards Bros., Inc., Ann Arbor, Mich., 1943, p. 66.

(14) The methylene protons of phthalide and furfuryl acetate show singlets at δ_{TMS} 5.26 and 5.03, respectively. *Cf.* G. V. D. Tiers, "Tiers Tables," Minnesota Mining and Manufacturing Co., Minneapolis, Minn., 1958, Table II, p. 8; and N. S. Bhacca, L. F. Johnson, and J. N. Schoolery, "NMR Spectra Catalog," Varian Associates, Palo Alto, Calif., 1962, Spectrum 167.

(15) N. S. Bhacca, L. F. Johnson, and J. N. Schoolery, *ibid.*, Spectrum 650.

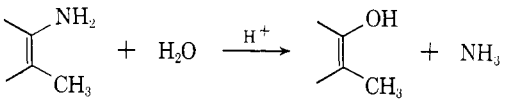
(16) N. S. Bhacca, L. F. Johnson, and J. N. Schoolery, *ibid.*, Spectrum 299.

(17) K. Bowden, I. Heilbron, E. R. H. Jones, and B. Weedon, *J. Chem. Soc.*, 39 (1946); *cf.* also C. Djerassi, R. Engle, and A. Bowers, *J. Org. Chem.*, **21**, 1547 (1956).

used to prepare IIIa from mitomycin C produced, after acetylation, a C_{22} tetraacetate IIIg which, unlike IIIb, showed no –NH band in its infrared spectrum. The n.m.r. spectrum ($CDCl_3$) of IIIg confirmed a tetraacetate formulation and also showed that the δ_{TMS} 6.48 signal, assigned to the NH proton in IIIb, had been replaced by a new signal at δ_{TMS} 3.04, attributable to the N-methyl protons of an acylated methylamine.[18,19] Thus, since the acetylated nitrogen was secondary in IIIg, it must be primary in IIIb.

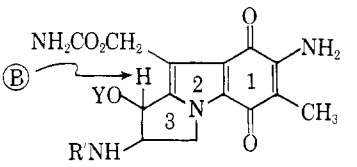
The structures of hydrolysis products intermediate between mitomycin C and hydrolysate IIIa were then determined. An unusual feature of mitomycin C was that although the antibiotic was essentially neutral ($pK_a' = 3.2$), chromatography and pK_a' measurements[20] indicated the generation of one basic group ($pK_a' = 6.5$) soon after dissolution in 0.1 *N* HCl at room temperature. After 24 hr. at room temperature, one acidic function and two basic functions had appeared with pK_a' values of 5.4, 7.3, and 9.3, respectively.[21] Measurements at intermediate times indicated that the acidic and the second basic functions were generated simultaneously. A 15-hr. preparative-scale hydrolysis resulted in the isolation of hydrolysate IIf[2a] as its acetate derivative (IIe).[2a] The spectral properties of IIe indicated that it was closely related to tetraacetate IIIb, and further acid hydrolysis followed by reacetylation converted IIe to IIIb. The concomitant changes in empirical formulas suggested the hydrolysis of a carbamoyl group and explained the origin of the carbon dioxide involved in the more vigorous acid hydrolysis of mitomycin C, and also the infrared band at 5.80 μ seen in mitomycin C and its derivatives of mild acid treatment.

Brief exposure of mitomycin C to dilute, aqueous HCl resulted in the isolation of the initial hydrolysis product of $pK_a' = 6.5$ (IId). Its empirical formula, $C_{14}H_{16}N_4O_5$, indicated it to be a precursor of hydrolysate IIf, $C_{14}H_{15}N_3O_6$, and the resulting empirical formula change was accommodated by the hydrolysis of an amide to an acid. Since the acidic function of these derivatives was an enolic hydroxyl group, the structural change involved in this hydrolysis would be



Acid-catalyzed methanolysis of mitomycin C produced a basic product (IIa), $pK_a' = 6.57$, isomeric with mitomycin C. Aqueous acid hydrolysis of IIa gave, after acetylation, a mixture of triacetate IIe and tetraacetate IIIb.

The action of hot acetic anhydride converted mitomycin C to a diacetate (IIc), which could be converted to a mono-N-acetyl derivative (IIb) with methanolic ammonia. Treatment of mitomycin C with glacial acetic acid resulted in its smooth conversion to the acetamide IIb, identical in all respects with that obtained from the ammonolysis of diacetate IIc. Finally, the aqueous acid hydrolysis product, IId, was converted to diacetate IIc on acetylation in pyridine, thus relating it to both IIb and IIc. Comparison of the n.m.r. spectrum (DMSO-d_6) of diacetate IIc with that of tetraacetate IIIb confirmed their structural similarities; however, IIc showed two broadened peaks (two hydrogens each) in the δ_{TMS} 6.25–6.46 region which replaced two acetyl CH_3 peaks of tetraacetate IIIb.



IIa, Y = CH_3; R′ = H
b, Y = H; R′ = CH_3CO
c, Y = R′ = CH_3CO
d, Y = R′ = H

In the n.m.r. spectrum (DMSO-d_6) of methoxyamine IIa, the doublet usually seen for hydrogen B was moved upfield, as might be expected, and was now part of a complex pattern (between δ_{TMS} 3.87–4.83) equivalent to four hydrogens, representing the hydrogens attached to ring 3.[22]

These data served to establish the position of the carbamoyl group. Since the carbamoyl moiety remained intact during the early stages of hydrolysis, it could not have been located at a site undergoing hydrolytic change, and position 10 (rather than position 1, or possibly 7) fits this requirement. Further, the unusual features of the acid sensitivity of mitomycin C became apparent. First, it could be seen that the methoxyl group originally present in mitomycin C[12] was an extremely labile one being quickly cleaved under the mildly acidic conditions used to prepare IIa, b, and d.[23] Second, while the ultraviolet spectra of all the acid hydrolysis derivatives were markedly similar,[24] they bore little resemblance to that of mitomycin C. Thus, the ultraviolet spectrum of mitomycin C greatly resembled that of 2,5-bis(dimethylamino)benzoquinone,[25] but underwent rapid, irreversible change, in the presence of acid, to that of the hydrolysis derivatives. Third, the nitrogen in mitomycin C which could be methylated to give N-methylmitomycin C (Ib) could not be further acetylated,[26] thereby indicating that it had been converted from a secondary nitrogen to a tertiary nitrogen on methylation. Finally, mitomycin C and hydrolysate IId both contained nine sites of unsaturation. The dissimilarity of their ultraviolet spectra, however, required that the pyrrole nucleus *per se* be absent in mitomycin C and that the ninth site of unsaturation be elsewhere in the molecule. All of these

(18) N. S. Bhacca, L. F. Johnson, and J. N. Schoolery, ref. 15, Spectrum 39.

(19) With splitting caused by the NH function of IIIb eliminated in IIIg, the signals from the ring 3 hydrogens were resolved into a doublet(1H)–quartet(1H)–doublet(2H) pattern with δ-values similar to their IIIb counterparts.

(20) A. Wilson and M. Munk [*Anal. Chem.*, **34**, 443 (1962)] describe the automatic, semimicro titrator used in the pK_a' measurements. The procedure used is described in the Experimental section.

(21) The odor of a volatile amine was easily detected in the basic solutions of the pK_a' measurements. Comparison of the pK_a' of the "second basic function" (9.3) with that of ammonium chloride (9.2) indicated that the volatile amine was ammonia.

(22) Mono-N-acetate IIb could be converted to the N-acetate of IIa by treatment with methyl iodide, thus relating IIa to IIb–d and confirming the n.m.r. interpretation.

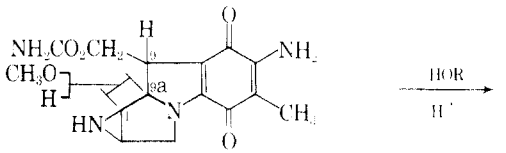
(23) Since the methoxyl was absent in derivatives IIb and d, the methoxyl of IIa must have resulted from external incorporation of methanol, the solvent.

(24) The spectra of those derivatives bearing the 7-amino group were unaltered by pH changes and resembled the pH >7 spectra of the 7-hydroxy derivatives; see Experimental.

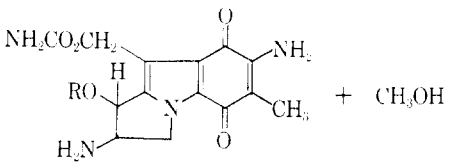
(25) C. Martini and F. Nachod, *J. Am. Chem. Soc.*, **73**, 2953 (1951).

(26) Conversely, acetylmitomycin C (Ic) could be prepared using acetic anhydride in methanol and could not be methylated under conditions used to prepare Ib.

features could be accommodated by structure I and the following reaction sequence.



I



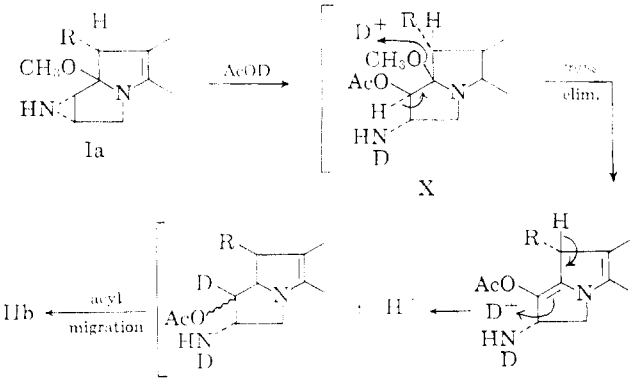
II

The conversion of mitomycin C to the acetamide IIb with glacial acetic acid (R = Ac in the last reaction sequence) was accommodated by an opening of an aziridine ring. Thus, the initial product of opening would be II (R = Ac), after which an O to N acyl migration would yield acetamide IIb. The n.m.r. spectrum of mitomycin C (DMSO-d_6) was in accord with a dihydropyrrole formulation since the singlet usually seen for the methylene group in the acid-reaction derivatives was absent in mitomycin C.[27]

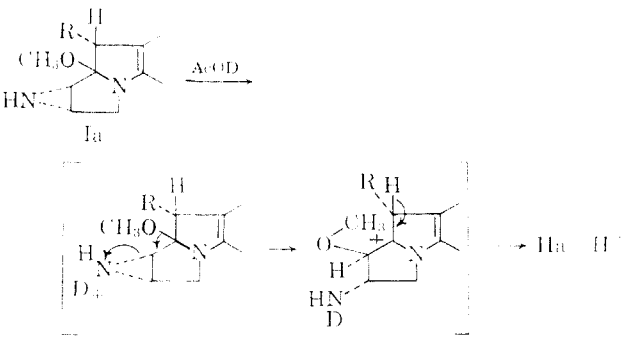
Differentiation between positions 1 and 9a for the labile methoxyl was accomplished by deuterium incorporation studies.[28] Closer examination of the reaction of mitomycin C with acetic acid revealed that, in addition to the acetamide IIb, the methoxyamine IIa was formed as a minor product. Since the solvent, acetic acid, was present in great excess, it was considered unlikely that the methoxy group in the methoxyamine product resulted from elimination of alcohol from mitomycin C followed by opening of the aziridine ring with this alcohol in competition with the acetic acid solvent. Thus, the methoxyamine represents the only product isolated to date which has the aziridine ring opened and still retained the original methoxy group.

When the reaction was repeated using acetic acid-*d*, n.m.r. analysis (DMSO-d_6) of IIb (as diacetate IIc) revealed that the only carbon-bound deuterium present was located at position 1.[29] This would be expected if the methoxyl were originally at position 1, but can also be readily explained with the methoxyl at 9a in its relative stereochemical position as determined by Tulinsky[2b] (see Scheme I). While more than one hydrolytic mechanism is operating, a more favorable *trans* (*vs.* a possible *cis*) elimination of methanol from possible intermediate X would result in deuterium incorporation at position 1 in acetamide IIb. If IIa were to arise *via* an isomerization of I (methoxyl at position 1), deuterium incorporation at position 1 would again be expected. However, if IIa were to arise *via* methoxyl migration[30] from position 9a to position 1, the IIa isolated from the acetic acid-*d* reaction should contain no deuterium at position 1 (see Scheme II). An n.m.r.

SCHEME I



SCHEME II



(DMSO-d_6) comparison with 1-deuterio IIa prepared by methanolysis employing methanol-*d* revealed that the IIa isolated from the acetic acid-*d* reaction contained, cleanly, no deuterium at position 1 and thus provided chemical evidence for the methoxy at position 9a in mitomycin C.

Biological Data.—In a microbiological assay using *Bacillus subtilis*, Ia and Ic, with the aziridine ring intact, were active but the IIa and N-acetyl IIa were inactive. Using 10 other organisms against which mitomycin C was active, IIa was inactive (Table I).

TABLE I

COMPARATIVE MICROBIOLOGICAL ACTIVITY OF MITOMYCIN C AND IIa[a]

Organism	M.I.C.,[b] γ/ml. Mitomycin C	IIa
Staphylococcus aureus Smith	0.16	>20
Staphylococcus aureus 209P	0.16	>20
Sarcina lutea	0.08	>20
Streptococcus faecalis	0.6	>20
Klebsiella pneumoniae	0.16	>20
B. subtilis	0.04	>20
Proteus 28017	2.5	>20
Escherichia coli	5.0	>20
Pseudomonas aeruginosa	2.5	>20
Salmonella typhosa	0.6	>20

[a] We wish to thank Dr. I. R. Hooper of Bristol Laboratories, Inc., for these data. [b] Minimum inhibitory concentration.

Experimental

All melting points are uncorrected and were taken on a Thomas–Hoover capillary melting point apparatus. Ultraviolet spectra were obtained on a Cary-14 spectrophotometer, infrared spectra on a Beckman-IR-4 and Beckman-IR-7; pK_a' determinations were performed on an automatic titrator,[17] and n.m.r. spectra on a Varian DP-60 spectrometer. Analyses were

(27) This fact also served to eliminate position 9 as the site of attachment of the labile methoxyl group.

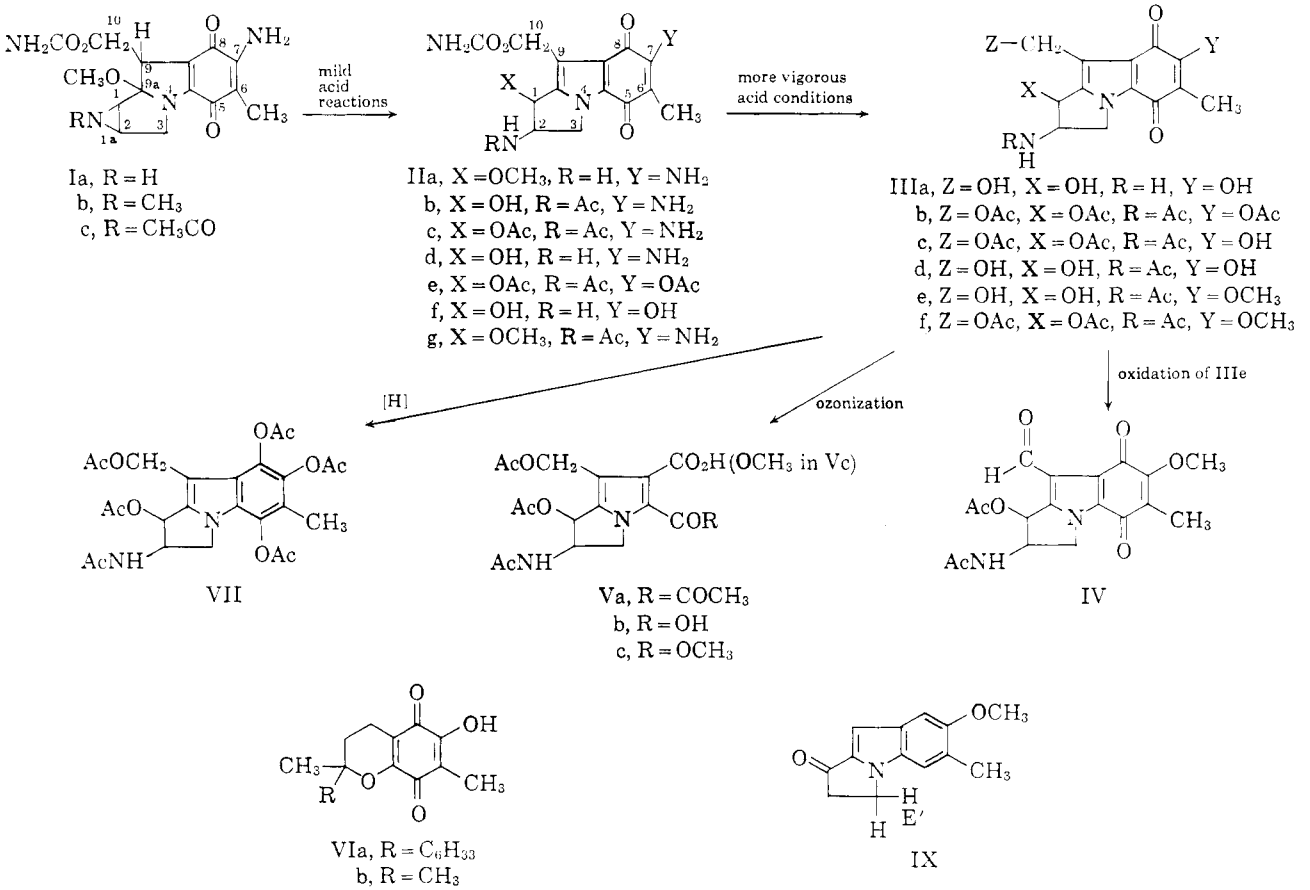
(28) At this stage the Lederle communication[2a] appeared and the deuterium incorporation studies were devised as a chemical means to confirm their X-ray data.

(29) Nitrogen-bound deuterium *via* exchange was present to the extent of about 50% and carbon-bound deuterium to the extent of about 40%.

(30) See, for example, D. Noyce, and B. Bastian, *J. Am. Chem. Soc.*, **82**, 1246 (1960).

CHART I

STRUCTURES OF MITOMYCIN C AND DERIVATIVES



Ia, R = H
b, R = CH_3
c, R = CH_3CO

IIa, X = OCH_3, R = H, Y = NH_2
b, X = OH, R = Ac, Y = NH_2
c, X = OAc, R = Ac, Y = NH_2
d, X = OH, R = H, Y = NH_2
e, X = OAc, R = Ac, Y = OAc
f, X = OH, R = H, Y = OH
g, X = OCH_3, R = Ac, Y = NH_2

IIIa, Z = OH, X = OH, R = H, Y = OH
b, Z = OAc, X = OAc, R = Ac, Y = OAc
c, Z = OAc, X = OAc, R = Ac, Y = OH
d, Z = OH, X = OH, R = Ac, Y = OH
e, Z = OH, X = OH, R = Ac, Y = OCH_3
f, Z = OAc, X = OAc, R = Ac, Y = OCH_3

VII

Va, R = $COCH_3$
b, R = OH
c, R = OCH_3

IV

VIa, R = C_6H_{33}
b, R = CH_3

IX

done by Midwest Microlab, Inc. Paper chromatography employed Whatman Grade No. 1 paper in the following systems: A, 1-propanol–1% ammonium hydroxide, 2:1 (by vol.); B, 2-propanol–1% ammonium hydroxide, 2:1 (by vol.); C, 1-butanol–acetic acid–water, 4:2:1 (by vol.); D, 1-butanol–morpholine–water, 3:1:3 (by vol.); E, acetone–water, 3:1 (by vol.); F, methanol–water, 1:1 (by vol.). Thin layer chromatography was done using Merck silica gel G distributed by Brinkmann Instruments, Inc.; the solvent system used was ethyl acetate–2-propanol, 1:2 (by vol.).

The first sample of mitomycin C was received from the Cancer Chemotherapy National Service Center, and we wish to thank Drs. H. W. Bond and R. Coghill for their cooperation. Later we received generous samples from the Kyowa Hakko Kogyo Co., Ltd., and we are pleased to acknowledge the cooperation and assistance of Drs. N. Sato, S. Wakaki, and Y. Harada of that company.

Purification of Mitomycin C.—A solution of 0.29 g. of crude mitomycin C in a small amount of hot methanol was pipetted onto the top of an alumina column (Woelm, neutral, Brockman activity grade I). Elution with methanol brought the mitromycin C off the column as a purple band. A small pink band trailed the mitomycin down the column, and a brown residue remained at the top. The methanol eluate containing the mitomycin was evaporated to dryness *in vacuo* leaving a purple residue which was then triturated three times with 10-ml. portions of hot distilled water. The combined triturates yielded 0.180 g. of mitomycin C as deep purple needles, m.p. >300°. Mitomycin C thus purified showed a clean single spot on paper chromatography in systems A (R_f 0.78), D (R_f 0.83), and E (R_f 0.85); ultraviolet: $\lambda_{max}^{H_2O}$ 367 mμ (ε 21,840); $\lambda_{max}^{CH_3OH}$ 357 mμ (ε 22,780), 550 (232); ultraviolet of 2,5-bis(dimethylamino)benzoquinone: λ_{max} 377 mμ (ε ~20,000)[25]; infrared (μ, KBr): 2.90, 3.0, 3.04, 5.80, 6.05, 6.25, 6.45, 7.23; basic pK_a' = 3.20 (50% methanol). Elemental and group analyses were determined after drying to constant weight at 100° *in vacuo*. Mitomycin C and certain derivatives consistently gave a high C-methyl value.

Anal. Calcd. for $C_{15}H_{18}N_4O_5$: C, 53.83; H, 5.43; N, 16.76; O, 23.98, mol. wt., 334; C-CH_3, 4.5(1); OCH_3, 9.0(1). Found: C, 53.55; H, 5.57; N, 16.86; O, 24.13; mol. wt., 311 (isothermal distillation, MeOH); C-CH_3, 5.45; OCH_3, 8.73; NCH_3, 0.00.

pK_a' Study on Mitomycin C.—Mitomycin (172 mg.) was triturated twice with 3 ml. of methanol. The undissolved mitomycin weighed 78 mg. leaving 94 mg. of mitomycin C in solution. This was diluted to 10.0 ml. and used as a stock solution for subsequent pK_a' determinations. For titration, 2.0 ml. of the mitomycin stock solution (19 mg. of mitomycin C) was pipetted into the titration cell. This was diluted with 2.0 ml. of CO_2-free distilled water. Then 1.0 ml. of 0.05 *N* HCl in 50% methanol–water was added and the sample was titrated with 0.165 *N* KOH in 50% methanol–water.

A.—When the titration was performed 15–20 sec. after addition of the 0.05 *N* HCl, only a very weakly basic species was present, pK_a' = 3.2.

B.—When the titration was performed 15–20 min. after addition of the HCl, a basic group with pK_a' of 6.5 had appeared.

Hydrolysis Study of Mitomycin C *via* pK_a'.—A solution of 68.6 mg. of mitomycin C in 6.00 ml. of 0.10 *N* HCl was left to stand at room temperature. Periodically 1.00-ml. aliquots were removed, diluted with 1.0 ml. of methanol and 3 ml. of 50% methanol–water, and the pK_a' values were determined by titration with 0.165 *N* KOH. The titration curves were compared with that of a 1.00-ml. blank of 0.10 *N* HCl.

A.—At 15 min., the titration curve showed the presence of a basic function, pK_a' = 6.51. The equivalent weight of this basic species was calculated from the curve (364), and it compared favorably with the molecular weight of mitomycin C (334). Therefore, treatment of mitomycin C with 0.1 *N* acid for 15 min. had liberated one basic function.

B.—At 6 hr. and 40 min., the titration curve showed the presence of 1.7 equiv. of basic functions plus 0.5 equiv. of an acidic function.

C.—At 19 hr. and 40 min., 2.0 equiv. of basic functions and 0.8 equiv. of an acid function were present.

D.—At 24 hr. and 25 min., 2.1 equiv. of basic functions and 0.9 equiv. of an acid function were present, with pK_a' values of 5.4, 7.3, 9.3. At this point a sample was removed from the reaction flask, evaporated to dryness, and acetylated with acetic

anhydride in pyridine. The presence of carbamoyl triacetate IIe was demonstrated by paper chromatography in system B.

Acid Hydrolysis of Mitomycin C.—A solution of 0.500 g. (0.0015 mole) of mitomycin C in 50 ml. of 1 *N* HCl was stirred at room temperature. The color of the solution gradually turned from its original purple to red (1 hr.) and then to orange (8 hr.). After 24 hr., the resulting orange solution was diluted to 150 ml. with distilled water and passed over a Dowex-50-x2 column, the hydrolysate being absorbed at the top as an orange-brown band. The column was washed with distilled water until the eluates gave a negative Cl^- test. The hydrolysate was then eluted as a purple band with 3% ammonium hydroxide. Evaporation of the water solution by freeze drying left 0.440 g. of an amorphous purple powder. Investigation of all colorless fractions yielded nothing.

The hydrolysate was an indicator, purple in base, orange in acid; $[\alpha]^{23}D$ −120° (*c* 0.05, 0.001 *N* HCl), $[\alpha]^{23}D$ −100° (*c* 0.025, 0.0005 *N* HCl). (The observed rotation was doubled when the concentration was doubled.)

Acetylation of Hydrolysate.—A slurry of 0.500 g. of hydrolysate, prepared as above, in 25 ml. of pyridine was treated with 1.0 ml. of acetic anhydride, and the reaction was left to stand at room temperature for 1 hr., with occasional swirling. Solution became complete in approximately 15 min. The reaction was quenched by the addition of ethanol and then evaporated to dryness *in vacuo*, the last traces of pyridine being removed by azeotroping with toluene. The resulting brown residue was taken up in a small volume of chloroform, passed over an alumina column (Woelm neutral, activity grade III), and eluted with chloroform. The chloroform eluate yielded a yellow solid (IIIb) after evaporation which crystallized from absolute ethanol (60 mg.), m.p. 226–228° dec. Three recrystallizations from absolute EtOH yielded 15 mg. of yellow needles of IIIb, m.p. 229–230° dec. Paper chromatography showed a clean single spot in systems A (R_f 0.68), B (R_f 0.68), and D (R_f 0.80), the spot turning purple in the basic systems[31]; ultraviolet: λ_{max}^{EtOH} 231 mμ (ε 18,700), 269 (10,930), 275 (s) (10,700), 342 (1500); infrared: (μ, $CHCl_3$) 2.90, 5.64, 5.71, 5.93 (sh), 5.97, 6.03, 6.13, 6.66, 7.27, 8.05.

Anal. Calcd. for $C_{21}H_{22}N_2O_9$: C, 56.51; H, 4.97; N, 6.28; O, 32.26; C-CH_3, 16.85 (5); CH_3CO, 38.6 (4). Found: C, 56.48, 56.36; H, 4.82, 5.43; N, 6.35, 6.30; O, 32.22; CH_3C, 20.4; CH_3CO, 36.2.

Further elution of the column with 99:1 chloroform–methanol yielded a second yellow compound (IIe) which also crystallized from absolute EtOH. Further purification of this compound was difficult since it contained an impurity (evidenced by paper chromatography) which had similar physical properties. Purification was effected, however, by crystallization from dilute solution in absolute methanol, yielding yellow needles of IIe (25 mg.), m.p. 238–240° dec. Paper chromatography in systems A (R_f 0.50), B (R_f 0.60), and D (R_f 0.70) showed a clean single spot, the spot again being purple in the basic systems; ultraviolet: λ_{max}^{EtOH} 231 mμ (ε 20,350), 268 (12,320) 276 (12,000), 342 (1500); infrared: (μ, null): 2.90, 3.02, 5.65, 5.70, 6.03, 6.15, 6.45, 6.62, 8.10.

Anal. Calcd. for $C_{20}H_{23}N_3O_9$: C, 53.69; H, 4.69; N, 9.39; O, 32.21; CH_3CO, 28.83 (3); CCH_3, 13.44 (4). Found: C, 53.78; H, 5.03; N, 9.35; O, 31.80; CH_3CO, 29.02; CCH_3, 13.65.

Considerable residue remained on the alumina column as a deep purple band and could be eluted only with methanol. The residue appeared to consist of decomposition products and deacetylation products, since it could be reacetylated and rechromatographed to yield more tetraacetate IIIb but never in good yield.

Preparation of Triacetate IIe.—A solution of 0.500 g. (0.0015 mole) of mitomycin C in 100 ml. of 0.1 *N* HCl was allowed to stand at room temperature for 15 hr. A pK_a' curve of the solution showed the presence of three titratable groups, two basic, one acidic. The solution was evaporated to dryness *in vacuo* and the resulting solid (IIf) was treated with 10 ml. of acetic anhydride in 10 ml. of pyridine for 8 hr. at room temperature. Pyridine and acetic anhydride were evaporated *in vacuo*, the residue dissolved in a small volume of methanol, and passed onto a cellulose column (packed in 2-propanol). Elution with 2-propanol and evaporation of solvent produced a brown solid upon scratching with methanol.

(31) The color of the spots indicated that the basic systems had cleaved the enolic acetyl function. Thus, the R_f values of IIIb and IIIc are identical.

Two recrystallizations from methanol yielded 0.10 g. (15%) of yellow needles with decomposition point identical with that of the previous preparation, 238–240°. After three more recrystallizations, the decomposition point was raised by 9° (247–247.5°); however, the analysis for carbon was high (54.06; calcd., 53.69) and for oxygen was low (31.44; calcd., 32.21). R_f values of samples of IIe from the two preparations were identical.

Preparation of Hydrolysate IIIa.—A solution of 1.0 g. (0.003 mole) of mitomycin C in 100 ml. of 2 *N* HCl was warmed at 55–60° for 2 hr. The resulting orange solution was diluted with 150 ml. of distilled water and passed over a previously prepared Dowex-50W-x2 column (containing 130 ml. of wet resin). The hydrolysate was absorbed as an orange band at the top of the column. The column was washed with distilled water until a Cl^- test was negative, and the hydrolysate was then eluted as a purple band with 3% ammonium hydroxide. Excess ammonia was evaporated *in vacuo* with warming, and the remaining water was removed by freeze drying. The resulting fluffy, purple powder weighed 0.820 g. The crude hydrolysate could not be purified satisfactorily for analysis.

Hydrolysate IIIa was an indicator, orange in acid, purple in base; ultraviolet (0.1 *N* NaOH): λ_{max} 312 and 253 mμ; (0.1 *N* HCl): λ_{max} 294 and 236 mμ. It was also amphoteric, pK_a' = 5.37, 7.30 (50% methanol).

Preparation of Tetraacetate IIIb.—A slurry of 750 mg. of crude hydrolysate IIIa (prepared as above) in 10 ml. of dry pyridine and 5 ml. of acetic anhydride was allowed to stand (with occasional swirling) at room temperature for 20 min. The excess pyridine and acetic anhydride were then evaporated *in vacuo*. The yellow oily residue was then taken up in chloroform and the last traces of pyridine and acetic acid were removed by evaporating again *in vacuo* using toluene as a chaser. A small amount of ethanol was then added, whereupon tetraacetate IIIb crystallized. The supernatant liquid was pipetted off and the crude tetraacetate was redissolved in hot ethanol, filtered, and left to crystallize. The recrystallized tetraacetate was collected by filtration, 400 mg., m.p. 226–228° dec. The mother liquor was combined with the above supernatant liquid, evaporated to dryness, taken up in chloroform, and passed over a short alumina column (Woelm, neutral, Brockman activity III), and the chloroform eluate was collected. Crystallization and recrystallization of this eluate yielded an additional 140 mg. of tetraacetate, m.p. 226–228° dec. Further reworking of the mother liquors yielded no more crystalline product. Tetraacetate IIIb of this melting point was of sufficient purity for further reactions; total yield, 540 mg. (44%). Yields in this preparation generally ranged from 20–45%.

Triacetate IIIc.—Dissolution of 0.100 g. of tetraacetate IIIb in 30 ml. of 1% methanolic ammonia at room temperature produced a purple solution which was immediately evaporated to dryness *in vacuo* at room temperature. The resulting red-purple residue was taken up in a small volume of methanol and several drops of 2 *N* HCl were added, whereupon the solution turned yellow. Yellow crystals were deposited on cooling. Recrystallization from methanol–water afforded 0.061 g. (68%) of IIIc as yellow needles, m.p. 199–200° dec. On paper chromatography in systems A and B, IIIc showed a purple spot with R_f values identical with those of tetraacetate IIIb; ultraviolet: λ_{max}^{MeOH} 261 mμ (ε 22,100), 285 (14,000); acid pK_a' = 5.90 (50% methanol). The unusual spectrum may reflect the possibility of tautomerism to the *o*-quinone. The infrared spectrum showed loss of the enolic acetate band at 5.65 μ and presence of a hydroxyl band.

Anal. Calcd. for $C_{19}H_{20}N_2O_8$: C, 56.43; H, 4.98; N, 6.93. Found: C, 56.32; H, 4.85; N, 6.72.

Hydrolysis of Triacetate IIe to Tetraacetate IIIb.—Carbamoyl triacetate (IIe) (2 mg.) was heated at 50° for 1 hr. in 3 ml. of 1 *N* HCl and left to stand at room temperature for 5 hr. Evaporation to dryness *in vacuo* yielded a yellow-orange residue which was dissolved in pyridine (0.5 ml.) and acetic anhydride (few drops) and left to stand at room temperature for 0.5 hr. After removal of solvents *in vacuo*, the resulting yellow residue was examined by paper chromatography and the major component had an R_f value identical with that of tetraacetate IIIb (system D, R_f 0.80). Tetraacetate IIIb was shown to be re-formed after similar hydrolysis and reacetylation.

Dihydrohexaacetate VII.—A suspension of 50 mg. of platinum oxide in 10 ml. of acetic anhydride was reduced in an atmospheric pressure catalytic hydrogenation apparatus until the catalyst was completely reduced and hydrogen uptake had stopped.

Then a solution of 50 mg. of tetraacetate IIIb in 10 ml. of acetic anhydride was added and hydrogenation continued. There was a 2.5-ml. uptake of hydrogen (equivalent to 1 mole of hydrogen) within 10 min., after which no further hydrogen was absorbed. After stirring 0.5 hr., 3 ml. of pyridine was added and the solution was kept at room temperature under the hydrogen atmosphere for 3 hr. The catalyst was then filtered and the filtrate was evaporated to dryness *in vacuo*. An ether–petroleum ether (b.p. 30–60°) mixture (1:1) (100 ml.) was added to the residue and, after standing overnight and scratching, a yellow solid was obtained. This was filtered, dissolved in chloroform, and introduced onto an alumina column (Brockman grade III, packed under benzene). Elution with chloroform gave a compound which was crystallized from benzene–petroleum ether mixture to yield 25 mg. (42%) of colorless cubes (VII), m.p. 223.5°; λ_{max}^{EtOH} 227 mμ (ε 61,700), 278 (11,200).

Anal. Calcd. for $C_{25}H_{28}N_2O_{11}$: C, 56.40; H, 5.26; N, 5.26; O, 33.08; C-CH_3(7), 19.8. Found: C, 56.55; H, 5.45; N, 5.24; O, 32.95; C-CH_3, 25.55.

Acetamide IIId.—A slurry of 360 mg. of crude IIIa in 30 ml. of absolute methanol was treated with 1.5 ml. of acetic anhydride. The mixture was stirred at room temperature for 6 hr. (solution was complete after 30 min.). After filtration, toluene was added and the solution was evaporated to dryness *in vacuo*. The evaporation was repeated to remove traces of acetic acid. The resulting orange powder was then taken up in a small amount of 5% acetic acid–2-propanol, 1:2 (by volume), and passed onto a cellulose column packed in the same solvent. The acetate was eluted as an orange band with the same solvent. A main fraction was collected, which, after evaporation *in vacuo* and washing with ether, yielded 345 mg. of orange powder. Paper chromatography showed a single spot in systems A (R_f 0.41), C (R_f 0.54), D (R_f 0.54), E (R_f 0.72), but the compound could never be crystallized. The N-acetyl derivative was an indicator, purple in base, orange in acid; ultraviolet: λ_{max}^{MeOH} (pH 7–8) 257 mμ ($E_{1\,cm}^{1\%}$ 489), 308 (300); acid pK_a' = 6.50 (50% methanol).

Methoxyacetamide IIIe.—A solution of 290 mg. of acetamide IIId in 50 ml. of absolute ethanol was cooled to 0°. Ethereal alcoholic diazomethane, prepared by the method of DeBoer[32] was then distilled into the IIId solution until a small aliquot did not turn purple on basification, indicating complete reaction of the enolic hydroxyl group. Excess diazomethane was then destroyed by addition of ethanol–acetic acid solution, and the reaction was evaporated to dryness *in vacuo*. Re-evaporation employing toluene as a chaser removed the last traces of acetic acid. The resulting brown residue was dissolved in acetone and chromatographed over alumina (Woelm neutral, activity grade III). An acetone fraction was obtained but not investigated further. Methanol (30%) in acetone yielded two fractions, the second of which gave crystalline methoxyacetamide IIIe on standing in a small volume of chloroform, 50 mg., m.p. 212° dec. Recrystallization from chloroform raised the m.p. to 216° dec.; ultraviolet: λ_{max}^{EtOH} 235 mμ (ε 17,650), 288 (11,250), 353 (2000); infrared (μ, KBr): 2.90–3.10 (broad), 6.10 (broad), 6.45, 6.68, 7.30, 9.03.

Anal. Calcd. for $C_{16}H_{18}N_2O_6$: C, 57.49; H, 5.43; N, 8.38. Found: C, 57.54; H, 5.34; N, 8.27.

The mother liquors from the crystallization and recrystallization were evaporated to dryness *in vacuo* yielding an orange foam, which yielded only a small amount of crystalline material. An infrared spectrum of this foam was almost identical with that of the pure product and paper chromatography in system D gave one spot which had the same R_f (0.83) as the crystalline product. Consequently, these foamy residues were often used for subsequent reactions, provided infrared and paper chromatography indicated sufficient purity.

Acetylation of Methoxyacetamide IIIe.—A crude preparation of IIIe was treated with 1.0 ml. of acetic anhydride in 1.0 ml. of pyridine and left to stand at room temperature for 1 hr. After thorough evaporation of the pyridine and acetic anhydride, the residue was dissolved in a small volume of chloroform and passed onto an alumina column (Woelm, neutral, Brockman activity III). The chloroform fraction was collected and evaporated to dryness *in vacuo* to yield 0.075 g. of yellow needles (IIIf), crystallized from ethanol, m.p. 213° dec. Recrystallization from ethanol afforded an analytical sample, m.p. 217° dec.; ultraviolet: λ_{max}^{EtOH} 236 mμ (ε 23,000), 286 (13,550), 340 (2850); infrared (μ, $CHCl_3$): 2.91, 5.73, 5.91 (sh), 6.00, 6.07, 6.65, 7.28, 8.05, 9.01.

Oxidation of Methoxyacetamide IIIe.—A solution of 0.230 g. (0.69 mmole) of methoxyacetamide IIIe in 50 ml. of acetone (distilled from $KMnO_4$ and K_2CO_3) was cooled to 15° with an ice–water bath. Jones' reagent[17] (0.18 ml., 1 equiv.) was added dropwise from a buret while the acetone solution was swirled. After the addition was completed, the reaction mixture was filtered over Celite to remove the precipitated chromic salts and evaporated to dryness to yield an orange foam. This was slurried in 5 ml. of pyridine, 0.5 ml. of acetic anhydride was added and was left to stand at room temperature for 1.5 hr. The solvents were evaporated *in vacuo*, the residue was redissolved in ethanol, toluene was added as a chaser, and the solution was re-evaporated to dryness. The resulting brown residue was taken up in chloroform and passed over an alumina column (Woelm, neutral; Brockman activity III). Elution with chloroform yielded an orange glass, after evaporation, which could not be crystallized and was not investigated further. A second fraction could be eluted with chloroform, or with 2% acetone–chloroform, which crystallized after evaporation and addition of absolute ethanol. Recrystallization from absolute ethanol yielded 50 mg. (22%) of yellow plates (IV), m.p. 225° dec. The oxidation product IV could be recrystallized to yellow needles with slightly higher decomposition point (230°). The two crystalline forms could be interconverted by dissolution in hot ethanol and seeding with either the plates or needles. The crystals gave a positive Tollens test when warmed with the reagent; ultraviolet: λ_{max}^{EtOH} 214 mμ (ε 20,500), 243 (14,500), 274 (15,400), 329 (4300); infrared (μ, KBr): 3.08, 3.50, 5.70, 5.93, 6.05, 6.40, 7.25, 8.20, 9.05.

Anal. Calcd. for $C_{18}H_{18}N_2O$: C, 57.74; H, 4.84; N, 7.48; CH_3CO, 23.0(2); residual CCH_3, 4.30(1). Found: C, 57.79; H, 5.10; N, 7.30; CH_3CO, 23.99; residual CCH_3, 4.23.

The aldehyde function generated by oxidation was quite resistant to further oxidation and was recovered (in low yield) as the only crystalline product when it was retreated with a tenfold excess of Jones' reagent[17] for 30 sec.

N-Methylmitomycin C (Ib).—A solution of 0.500 g. (0.0015 mole) of mitomycin C in 100 ml. of acetone and 2.5 ml. of methyl iodide was refluxed gently in the presence of 2.5 g. of anhydrous potassium carbonate for 6 hr. After cooling and filtration, the solvent was evaporated *in vacuo* (room temperature) leaving a purple residue. The residue was dissolved in a small volume of methanol and passed over a cellulose column packed in 1-propanol. Elution with 1-propanol yielded a purple solid after solvent evaporation and washing with isopropyl ether. This solid could be crystallized and recrystallized from absolute ethanol to yield dark purple cubes, 0.38 g. (73%) of methylmitomycin C (Ib), m.p. 198° dec. Methylmitomycin thus prepared showed a single spot on paper chromatography in systems A (R_f 0.78) and G (R_f 0.85); ultraviolet: λ_{max}^{MeOH} 215 mμ (ε 27,840), 363 (23,100).

Anal. Calcd. for $C_{16}H_{20}N_4O_5$: C, 55.17; H, 5.74; N, 16.09; O, 22.98; C-CH_3(1), 4.31; OCH_3(1), 8.9; N-CH_3(1), 4.3. Found: C, 55.13; H, 5.90; N, 15.95; O, 23.13; C-CH_3, 4.36; OCH_3, 9.15; NCH_3, 4.7.

Methylmitomycin could be recovered unchanged on attempted acetylation under a variety of conditions.

Acid Hydrolysis and Acetylation of N-Methylmitomycin C (Ib).—A solution of 0.27 g. (0.78 mmole) of methylmitomycin C (Ib) in 120 ml. of 2 *N* HCl was warmed at 60° for 3 hr. The resulting orange solution was then diluted with 30 ml. of distilled water and passed onto a column of Dowex-50W-x2, the hydrolysate being absorbed at the top. When the water washings became chloride free, the hydrolysate was eluted with 2% ammonium hydroxide. Lyophilization of the water yielded an amorphous purple solid. Paper chromatography in systems B (R_f 0.50) and D (R_f 0.54) indicated that the product was fairly pure. Treatment of the amorphous solid with 2.0 ml. of acetic anhydride in 5.0 ml. of pyridine for 1 hr. at room temperature produced a yellow solid after thorough solvent evaporation and washing with ether. This was dissolved in chloroform and passed over a short alumina column (Woelm, neutral; Brockman activity III), eluting with chloroform. Evaporation of the chloroform eluate produced a yellow solid which was crystallized from ethanol–ether to give 0.12 g. (33%) of yellow needles (IIIc), m.p. 172–173° dec. Further recrystallizations provided an analytical sample, m.p. 175–176° dec. Paper chromatography showed one spot in systems B (R_f 0.73) and D (R_f

(32) T. J. DeBoer, *Rec. trav. chim.*, **73**, 229 (1954).

0.78); ultraviolet: λ_{max}^{EtOH} 232 mμ (ε 26,460), 268 (17,830), 275 (17,830); infrared (μ $CHCl_3$): 5.65, 5.73, 6.05, 7.10, 7.30. The n.m.r. ($CDCl_3$) spectrum substantiated the formulation of a tetraacetate.

Anal. Calcd. for $C_{22}H_{24}N_2O_9$: C, 57.30; H, 5.22; N, 6.10; O, 31.27. Found: C, 56.50; H, 5.38; N, 6.47; O, 31.55. Calcd. for a triacetate $C_{20}H_{22}N_2O_8$: C, 64.85; H, 5.95; N, 7.56; O, 21.62.

Ozonization of Tetraacetate IIIb.—A solution of 480 mg. (1.08 mmoles) of tetraacetate IIIb in 100 ml. of chloroform was cooled to about −60°. Ozone from a Wellsbach T-3 ozonizer was then passed through the solution for 2.5 min. at which time the solution had turned from yellow to green. Excess ozone was swept from the reaction with oxygen, and the chloroform was evaporated *in vacuo* at room temperature. The resulting pale yellow foam was dissolved in acetone (10 ml.), diluted with water (15 ml.), and stirred at room temperature with 200 mg. of 5% palladium on carbon overnight. At the end of this time, a starch–iodide test was negative. Filtration over Celite gave a yellow solution, which, after *in vacuo* evaporation of acetone, yielded a pale yellow solid Va. It was collected by filtration and air-dried several hours. It weighed 280 mg. (64%). A portion was recrystallized twice from distilled water yielding fine pale yellow needles, m.p. 160–162°. Paper chromatography showed a clean single spot in systems A (R_f 0.69), B (R_f 0.73), and D (R_f 0.78); $[\alpha]^{25}D$ −151° (*c* 0.8, ethanol). The ozonolysis product gave a positive iodoform test and formed a dinitrophenylhydrazone. An iodoform test on the crude dinitrophenylhydrazone was negative. The ozonolysis product gave a positive pine-splinter test but a negative Ehrlich test, even when heated; acid pK_a' = 4.83; ultraviolet: λ_{max}^{EtOH} 314 mμ (ε 11,670); infrared (μ, mull): 3.02, 5.73, 5.83, 5.90 (sh), 6.00 (sh), 6.36, 8.05.

Anal. Calcd. for $C_{18}H_{20}N_2O_8$: C, 52.95; H, 4.94; N, 6.86; CH_3, 14.72(4); CH_3CO, 31.62(3). Found: C, 53.12; H, 4.84; N, 7.11; CH_3, 18.22; CH_3CO, 33.76.

Periodate Cleavage of Methyl Ketone Va.—To a solution of 12.0 mg. (0.0294 mmole) of methyl ketone Va in a few milliliters of distilled water was added 1.00 ml. of 0.45 *M* sodium metaperiodate. The solution was then diluted to 10.0 ml. and left to stand at room temperature while 1.0-ml. aliquots were removed periodically and titrated using the arsenite method to determine periodate consumption. After 30 min., periodate uptake was essentially complete, 1 mole equiv. being consumed.

In another experiment, 5 mg. of the ozonolysis product was dissolved in 1 ml. of warm water. One drop of this solution gave a positive iodoform test. To this pale yellow solution, 2 ml. of 0.02 *M* sodium metaperiodate was added. After addition of the periodate, 3 drops of the reaction solution was subjected to the iodoform test (after addition of excess 0.01 *N* sodium arsenite solution) and gave a positive test. After 1 hr., the reaction solution had turned colorless and gave a negative iodoform test. At this time, the pH was adjusted to 3 with dilute sulfuric acid and the solution was left to stand in the refrigerator. After 3 days, fine, silky needles had crystallized. These were collected by filtration and dried. The entire amount was used for an infrared spectrum (KBr disk) which was identical with that of the ozonolysis diacid Vb (see below). The KBr disk was then dissolved in water, and a qualitative ultraviolet spectrum was obtained, λ_{max} 260 and 279 mμ, which was qualitatively identical with that of the ozonolysis diacid Vb.

Ozonolysis of Tetraacetate IIIb with Oxidative Work-Up.—Tetraacetate IIIb (200 mg., 0.42 mmole) was dissolved in 75 ml. of chloroform and the solution was cooled to −60° by means of a Dry Ice–acetone bath. An ozone-enriched (*ca.* 1.5%) stream of oxygen was passed through the solution slowly until decolorization was observed. Evaporation of the chloroform solution gave a yellow foamy residue. This was treated with 15 ml. of 30% hydrogen peroxide and the solution was stirred at room temperature for 90 min. At this time a white crystalline precipitate had occurred. This was filtered off and dried to yield 72 mg. (42%) of crude diacid Vb. The diacid was crystallized from absolute acetone to yield 58 mg. (33.9%) of analytical material, only gradual darkening up to 300° without melting: λ_{max}^{EtOH} 260 mμ (ε 7080), 278 (7950); $[\alpha]^{25}D$ −149° (*c* 0.25, EtOH); acid pK_a' values in 50% methanol were 2.55 and 10.15. It gave a positive Ehrlich test on heating, negative iodoform test, and negative reaction with 2,4-dinitrophenylhydrazine reagent.

Anal. Calcd. for $C_{36}H_{38}N_2O_{18}$: C, 50.26; H, 4.74; N, 7.33; three acetyls, 33.8. Found: C, 50.04; H, 5.00; N, 7.35; acetyl, 27.98.

In another run, 250 mg. (0.52 mmole) of tetraacetate IIIb dissolved in 100 ml. of chloroform was ozonized in the same manner. Upon removal of solvents, a light yellow foamy residue remained. This was oxidized by shaking for 2.5 hr. with 10 ml. of 30% hydrogen peroxide. The crystalline precipitate that resulted was filtered and dried in a desiccator overnight; yield, 187 mg. (87.5%) of diacid Vb.

Hydrolysis and Sodium Periodate Cleavage of Ozonolysis Diacid Vb.—The ozonolysis diacid triacetate Vb (16.5 mg., 0.04 mmole) was heated with 20 mg. (0.5 mmole) of NaOH dissolved in 2 ml. of water on the steam bath for 4.5 hr. The solution was cooled, neutralized to pH 8.5 by dropwise addition of 0.1 *N* HCl, 28 mg. (0.13 mmole) of sodium metaperiodate was added, and the solution volume was adjusted to 4.0 ml. by addition of water. Samples were removed at various time intervals, excess standard sodium arsenite solution was added to decompose unreacted periodate, and the arsenite was back-titrated with standard iodine solution (see Table II).

TABLE II

Time elapsed, min.	Uptake of IO_4^-, moles/moles of substrate	Optical rotation
0	0	(−)
10	0.59	...
100	0.89	None observed
170	0.82	...
190	0.89	...
46.5 hr.	1.14	...

Dimethyl Ester of Ozonolysis Diacid Vb.—Recrystallized diacid Vb (100 mg., 0.26 mmole) was dissolved in 100 ml. of absolute methanol and the solution was treated dropwise with an ethereal solution of diazomethane until the yellow color of diazomethane persisted in solution. The excess diazomethane was decomposed by addition of a few drops of glacial acetic acid, and the solution was evaporated to dryness. The residue crystallized from hot carbon tetrachloride to give 79 mg. (74%) of analytically pure Vc, m.p. 161.5–163°, λ_{max}^{EtOH} 275 mμ (ε 11,000), Ehrlich test positive with heating.

Anal. Calcd. for $C_{18}H_{22}N_2O_9$: C, 52.67; H, 5.41; N, 6.83. Found: C, 52.86, 52.62; H, 5.55, 5.61; N, 6.70.

Hydrolysate IId.—Mitomycin C (200 mg., 0.60 mmole) was dissolved in 50 ml. of 0.05 *N* HCl and stirred at room temperature for 25 min. The solution was then neutralized (to pH 8) with solid, anhydrous potassium carbonate. The hydrolysate precipitated on standing for 1 hr. in the refrigerator. It was collected by filtration, dissolved in acetone with warming, absolute ethanol was added, and the solution was evaporated to dryness to remove traces of water. Paper chromatography indicated one main spot (R_f 0.58) in system B. The residue was then dissolved in a small volume of 2-propanol–1% ammonium hydroxide (2:1 by volume) and passed over a tightly packed cellulose column and eluted with the same solvent system. The main band was the first off, and the hydrolysate IId crystallized out as it came off the column. (Crystallization also took place on the column and reduced the yield.) It was collected by filtration and recrystallized twice from hot dimethylformamide–water to give red-purple needles: yield, 41 mg. (22%); ultraviolet: λ_{max}^{MeOH} 249 mμ (ε 17,600), 308 (11,400), 345 (s) (3700); infrared (μ, KBr): 3.00 (broad), 5.85, 6.00, 6.20, 6.65, 7.20.

Anal. Calcd. for $C_{14}H_{16}N_4O_5$: C, 52.50; H, 5.04; N, 17.50. Found: C, 52.30; H, 5.31; N, 17.80.

Treatment of 5 mg. of crystalline IId with acetic anhydride in pyridine with warming yielded 2 mg. of diacetate IIe (see below) as identified by paper chromatography.

Methoxyamine IIa.—One gram of mitomycin C (3.0 mmoles) was dissolved in 250 ml. of anhydrous methanol by warming. The solution was filtered and added dropwise to a stirred slurry of 300 ml. of Dowex 50-x2 resin which had previously been washed thoroughly with methanol. After addition was complete (*ca.* 20 min.), the resin was stirred an additional 30 min., then the reaction flask was immersed in an ice–salt bath and cooled to −5°. The product was desorbed by addition of 750 ml. of 3% ammonia (by weight) in methanol, the temperature during addition never being allowed to rise above 0°. The resin was filtered and the filtrate was concentrated to dryness on a rotary evapo-

rator at aspirator pressure. The residue was dissolved in hot water and crystalline IIa resulted on cooling overnight. Three crops were collected amounting to 0.7203 g. (72%). Two additional crystallizations gave analytically pure material, m.p. 204–205.5° (dec. with bubbling); λ_{max}^{MeOH} 249 mμ (ε 21,400), 308 (14,500), 525 (1290); $\lambda_{max}^{0.1\ N\ HCl}$ 251 mμ (ε 19,100), 309 (12,600), 540 (1450); $\lambda_{max}^{0.1\ N\ NaOH}$ 251 mμ (ε 21,400), 314 (14,100), 543 (1290); basic pK_a' (as the hydrochloride) = 6.76 (50% methanol), 6.6 (water).

Anal. Calcd. for $C_{15}H_{18}N_4O_5$: C, 53.03; H, 5.43; N, 16.76; O, 23.98; one C-methyl, 4.50; one N-methyl, 4.50; one O-methyl, 9.29, two NH_2, 9.60; mol. wt., 334. Found: C, 53.57; H, 5.61; N, 16.47; O, 23.87; C-methyl, 4.12; N-methyl, 0.0; O-methyl, 9.38, 9.09, 9.15; NH_2 (van Slyke nitrogen), 5.84, 6.01; mol. wt., 436, 415 (isothermal distillation in methanol), 345 (titration).

Acetylation of IIa.—A solution of 0.30 g. (0.90 mmole) of IIa in 100 ml. of methanol was stirred with 5 ml. of acetic anhydride for 2 hr. at room temperature. Evaporation of the solvents *in vacuo* left a red residue which crystallized from water. Recrystallization from hot water afforded 0.15 g. (42%) of deep red crystals (IIg), m.p. 230–231° dec. with previous softening at 126°.

Anal. Calcd. for $C_{17}H_{20}N_4O_6$: C, 54.25; H, 5.32; N, 14.89; O, 25.54; C–CH_3, 8.00(2). Found: C, 54.00; H, 5.54; N, 14.54; O, 25.54; C–CH_3, 7.72.

Paper chromatography showed one spot in systems A (R_f 0.81), C (R_f 0.67), and F (R_f 0.64).

Acid Hydrolysis of IIa.—IIa (110 mg., 0.33 mmole) was hydrolyzed by heating with 30 ml. of 1 *N* HCl at 50–60° for 2 hr. The solution was then put onto a Dowex 50-x2 column (1 × 16 cm.) and the column was washed free of Cl^- with distilled water. The hydrolysate was eluted from the column with 3% ammonium hydroxide. The eluate was lyophilized to dryness and the purple, powdery residue was acetylated with 0.5 ml. of acetic anhydride and 10 ml. of pyridine for 2 hr. at room temperature. Volatiles were then removed *in vacuo* and the yellow solid remaining was taken up in absolute ethanol and spotted on paper. Paper strip analysis indicated that the products were mainly tetraacetate IIIb with a small amount of triacetate IIe. The ethanolic solution failed to crystallize so solvent was evaporated and the residue was dissolved in a small amount of chloroform and passed over a neutral alumina column (Brockman grade III, 1 × 10 cm.). Elution with chloroform gave a yellow eluate; the residue after evaporation of the chloroform crystallized from absolute ethanol to yield 83 mg. (45%) of tetraacetate IIIb, m.p. 228–229° dec. A mixture melting point with IIIb prepared directly from mitomycin C was undepressed.

Reaction of Mitomycin with Acetic Anhydride.—Mitomycin (200 mg.) and 1.5 ml. of acetic anhydride were placed in a 5 ml. round-bottomed flask fitted with a condenser and calcium chloride drying tube. The mixture was heated to 125–130° in an oil bath. After 5 min. the reaction mixture had become a thick, crystalline slurry and heating was stopped. The red crystalline product was filtered, washed well with ethyl acetate, and dried to yield 120 mg. (50%) of diacetate IIc. An analytical sample was prepared by crystallization from a large amount of boiling methanol; λ_{max}^{MeOH} 249 mμ (ε 24,550), 308 (15,900).

Anal. Calcd. for $C_{18}H_{20}N_2O_7$: C, 53.47; H, 4.98; N, 13.82; O, 27.73; $(CH_3C)_3$, 11.16; $(CH_3CO)_2$, 21.3. Found: C, 53.40; H, 5.14; N, 13.90; O, 27.30; CH_3C, 10.73; CH_3CO, 20.05.

Acetamide IIb.—The diacetate IIc (100 mg., 0.25 mmole) was dissolved in 120 ml. of absolute methanol to which 1.2 ml. of concentrated ammonium hydroxide was added. The solution was boiled on the steam bath for 15 min., cooled, and evaporated to dryness on the rotary evaporator. The residue was crystallized from absolute methanol to yield 60.7 mg. (67.5%) of pure acetamide IIb having no melting point below 300°; λ_{max}^{MeOH} 248 mμ (ε 24,000), 308 (15,900); pK_a' in 50% methanol, no titratable group; R_f 0.66 in system B and 0.78 in system C.

Anal. Calcd. for $C_{16}H_{18}N_4O_6$: C, 53.04; H, 5.00; N, 15.46. Found: C, 53.06; H, 5.08; N, 15.58.

Reaction of Mitomycin C with Glacial Acetic Acid.—A solution of 30 mg. (0.09 mmole) of mitomycin C in 1.5 ml. of glacial acetic acid was left at room temperature for 2 hr. The acetic acid was evaporated thoroughly and the resulting red residue (IIb) was crystallized from acetone. Infrared and paper chromatographic analysis demonstrated that IIb prepared by deacetylation of diacetate IIc (see above) was identical with the IIb of this preparation.

Methylation of IIb.—Diacetate IIc (50 mg., 0.12 mmole) was monodeacetylated as previously described by the action of 0.6 ml. of concentrated ammonium hydroxide in 60 ml. of absolute methanol (boiling 15 min.). The residue left after evaporation of the ammonia and methanol was dissolved in 100 ml. of acetone. Methyl iodide (2 ml.) and 50 mg. of potassium carbonate were added to this solution and the whole was heated under reflux for 15 hr. Filtration of potassium salts, followed by evaporation of volatiles, left a residue which was crystallized from distilled water to yield 27.8 mg. (60%) of methoxyacetamide IIg.

Mitomycin C Acetate (Ic).—Mitomycin acetate was prepared on microscale for paper chromatography experiments by acetylating 1 or 2 mg. of mitomycin C. The acetylation was run for 30–60 min., volatiles were removed *in vacuo*, and the residue was found to run cleanly on paper giving one nicely formed spot.

In a preparatory experiment 500 mg. (1.5 mmoles) of mitomycin C was acetylated by the action of 2 ml. of pyridine and 10 ml. of acetic anhydride at room temperature for 1 hr., at which time volatiles were evaporated. Last traces of volatiles were removed by adding a few milliliters of toluene and again evaporating to dryness. The residue was dissolved in hot distilled water and filtered, and, on cooling, acetyl mitomycin C (Ic, 160 mg., 28%) was obtained as a fine crystalline deposit. Additional crystalline product, 185 mg. (33%), was obtained by evaporation of the filtrate to dryness, dissolving the residue in warm isopropyl alcohol, and cooling the solution in the refrigerator overnight. The melting point of the product was difficult to determine due to its intense violet color, m.p. 165–169° dec. with prior softening at 150°; λ^{MeOH} 218 mμ (ε 21,900), 358 mμ (ε 21,900).

Anal. Calcd. for $C_{17}H_{20}N_4O_6$: C, 54.25; H, 5.32; N, 14.89; two C-methyls, 8.00; one acetyl, 11.43. Found: C, 54.16; H, 5.53; N, 14.81 (after drying *in vacuo* at 100° for 2 days); C-methyl, 7.74; acetyl, 10.81.

Reaction of Mitomycin C with Acetic Deuterioacid.—Thirty grams of 99.5% deuterium oxide was stirred overnight at room temperature with 95 ml. of acetic anhydride and the product was distilled through a short Vigreux column. The constant boiling (114–115.5°) middle fraction was redistilled through a 30-cm. column packed with 1/8-in. i.d. glass helices with a 10:1 reflux ratio being maintained. The fraction boiling at 114–115° was reserved. This fraction when subjected to n.m.r. spectral analysis showed only a minute amount of proton on oxygen compared to deuterium on oxygen (estimated 99% CH_3COOD or better).

Mitomycin C (300 mg., 0.90 mmole) was stirred with 15 ml. of acetic deuterioacid for 1 hr. at room temperature. The acetic deuterioacid was then removed *in vacuo*, and the residue was acetylated by the action of 12 ml. of pyridine and 12 ml. of acetic anhydride for 1 hr. at room temperature. During this time, 205.8 mg. (57%) of diacetate IIc crystallized from the acetylation mixture. Filtration of this followed by evaporation *in vacuo* of the filtrate left a residue which yielded 34.7 mg. (10%) of methoxyacetamide IIg upon crystallization from absolute methanol. Both products ran with identical rates as previously prepared samples on paper. Diacetate IIc was subjected to n.m.r. spectral analysis to determine amount of deuterium bound to C-1 in formula IIc. This was determined by integration of the area of the signal (doublet) at δ 5.95 and comparison of that area with either the area of the 2 proton singlet at δ 4.94 or the areas of the three singlets (3 protons each) at δ 1.90 and 2.06. Areas under peaks were determined by tracing the spectrum onto graph paper and counting squares. (Note: Previous integration of nondeuterated diacetate spectra has shown this to be a reasonably accurate method.) This method showed at least 40.5% deuterium bound to C-1 (*i.e.*, area corresponded to only 59.5% of that expected for 1 hydrogen). Also 50% deuterium was shown by this spectrum to have exchanged with the hydrogens on the 3 nitrogen atoms.

Test of Deuterium Incorporation in Preformed IIb.—Mitomycin C (250 mg., 0.75 mmole) was stirred for 2 hr. with 20 ml. of glacial acetic acid. The acid was then evaporated *in vacuo*, the residue was dissolved in 20 ml. of acetic deuterioacid, and the solution was stirred an additional 2 hr. Then 10 ml. of acetic anhydride was added to the solution and stirring was continued for an additional hour. The solution was evaporated to dryness *in vacuo*, the residue was triturated with warm ethyl acetate, and the insoluble material was filtered. The ethyl acetate solution upon cooling yielded 65 mg. (23%) of crystalline methoxy-

acetamide IIg which was identical with that previously prepared by acetylation of IIa as indicated by infrared spectra and paper chromatographic analysis. The ethyl acetate insoluble material was crystallized from absolute methanol giving 130 mg. (43%) of diacetate IIc (identical with previously prepared material by infrared spectra and paper chromatographic analysis). This was subjected to n.m.r. spectral analysis to determine whether deuterium had become incorporated at position 1 by exchange. By the methods previously described, it was found that no deuterium exchange had taken place.

Reaction of Mitomycin C with Acetic Deuterioacid without Subsequent Acetylation (Test of Deuterium Incorporation into Methoxyamine IIa).—Mitomycin C (750 mg., 2.2 mmoles) was stirred in a sealed flask with 50 ml. of acetic deuterioacid for 5 hr. at room temperature. The acid was then removed *in vacuo* and a small sample of the residue was spotted on a thin layer plate. Analysis of this plate after development showed the residue to consist primarily of the acetamide IIb with smaller amounts of methoxyamine IIa and an unidentified substance (trace) which ran fast in the system used. The residue was dissolved in the minimum volume of hot acetone and 410 mg. (50.5%) of the acetamide IIb crystallized in three crops on cooling the solution. The filtrate was evaporated to dryness, and the residue was dissolved in the minimum amount of methanol and introduced onto a neutral alumina column (Brockman grade III). Elution with ethyl acetate and then 10% methanol in ethyl acetate removed the fast-running unidentified substance. Then elution with 30% methanol in ethyl acetate gave a red band which yielded an additional 30.5 mg., 5% of the acetamide IIb upon evaporation of the solvent. Finally elution with absolute methanol followed by 50% methanol–water gave an intense purple-red band which was shown to be methoxyamine IIa by thin layer chromatography. This was rechromatographed in a similar manner to yield 106.8 mg., 14.2%, of pure IIa as a residue. An n.m.r. spectrum of this compound showed all of the features of the previously obtained spectrum of IIa and integration of the δ 3.87–4.83 multiplet in the usual manner showed the presence of 4 protons (*i.e.*, no deuterium bound to carbon) as in the previous spectrum.

Preparation of Deuterated Methoxy Derivative IIa.—A solution 0.10 g. (0.3 mmole) of mitomycin C in 20 ml. of absolute methanol and 0.20 ml. of glacial acetic acid was refluxed gently for 5 hr. and left at room temperature for an additional 8 hr. Solvents were then evaporated *in vacuo*, toluene being added to help remove traces of acetic acid. The resulting red residue was dissolved in a small amount of methanol and passed onto an alumina column (Woelm, neutral, Brockman activity III). Elution with methanol produced two fractions, the first being purple and containing mostly unreacted mitomycin C. A second red fraction followed closely and yielded crystals after evaporation and addition of water. Recrystallization from water yielded 30–35 mg. (30–35%) of IIa, homogeneous on thin layer chromatography, R_f 0.08–0.10.

In a similar manner methanol-*d* and acetic acid-*d* were employed in converting mitomycin C to a deuterated methyl ether. All nitrogen-bound deuterium was re-exchanged in the work-up and recrystallization procedure, leaving only carbon-bound deuterium in the product. Analogy with diacetate IIc dictated that deuterium incorporation would be exclusively at position 1, and integration of the δ_{TMS} 3.87–4.83 multiplet in the n.m.r. spectrum showed it to be incorporated there to the extent of approximately 70%.

Potential Anticancer Agents. II. The Synthesis of Some Nitrogen Mustard Containing Sulfones and Thiosulfinates[1a]

ALLEN F. HIRSCH,[1b] CLAUDE PIANTADOSI,[1c] AND J. LOGAN IRVIN

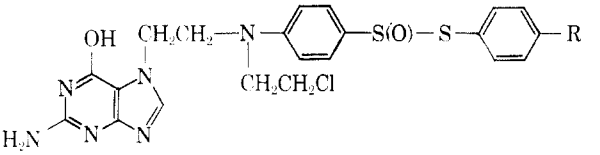
Departments of Medicinal Chemistry and Biochemistry, University of North Carolina, Chapel Hill, North Carolina

Received June 19, 1964

Three new thiosulfinates containing a nitrogen mustard moiety were prepared by treating *p*-[bis(2-chloroethyl)amino]benzenesulfinyl chloride with substituted thiophenols in the presence of pyridine. The sulfinyl chloride was synthesized from the corresponding sulfonyl chloride by reduction with lithium aluminum hydride followed by treatment with oxalyl chloride. In addition, several new sulfones containing a nitrogen mustard moiety were prepared by treating an alkyl halide with sodium *p*-[bis(2-chloroethyl)amino]benzenesulfinate. The antitumor activity of these compounds was studied in mice against the Ehrlich ascites carcinoma.

Weisberger and Pensky[2a,b] observed antitumor activity in several symmetrical thiosulfinates [–S(O)–S–], a moiety present in such naturally occurring plants as garlic.[3a,b] Kametani, *et al.*,[4a,b] synthesized additional symmetrical thiosulfinates which were effective against the Ehrlich ascites carcinoma.

The primary objective of the present research was to synthesize new thiosulfinates which contained a nitrogen mustard moiety. It was considered possible that these compounds might have antitumor activity for several reasons. (1) From the standpoint of reaction with essential sulfhydryl groups, both thiosulfinates[5a,b] and nitrogen mustards[6] could exhibit such reactivities; consequently, a combination of the two moieties in one compound could yield a type of dual antagonist.[7] (2) An interaction between one arm of the nitrogen mustard and the N-7 of guanine nucleotides could yield an unstable quaternary ammonium compound which upon hydrolysis would give com-



I, R = H, CH_3, $N(CH_2CH_2Cl)_2$

(1) (a) This work was supported by Public Health Service Research Grants C-6364, CA-06364-02, CA-06364-03, and CA-02756-08 from the National Cancer Institute, and in a small part from the American Cancer Society Institutional Grant to the University of North Carolina. (b) Fellow of the American Foundation for Pharmaceutical Education (1959–1963). Abstracted in part from the dissertation of Allen F. Hirsch submitted to the Graduate School of the University of North Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1963. (c) To whom inquiries should be sent.

(2) (a) A. S. Weisberger and J. Pensky, *Science*, **126**, 1112 (1957); (b) *Cancer Res.*, **18**, 1301 (1958).

(3) (a) A. Stoll and E. Seebeck, *Helv. Chim. Acta*, **32**, 197 (1949); (b) C. J. Cavallito and J. H. Bailey, *J. Am. Chem. Soc.*, **66**, 1950 (1944).

(4) (a) T. Kametani, K. Fukumoto, and O. Umezawa, *Yankugaku Kenkyu*, **33**, 60, (1959); (b) *ibid.*, **33**, 125 (1959).

(5) (a) C. J. Cavallito, J. S. Buck, and C. M. Suter, *J. Am. Chem. Soc.*, **66**, 1952 (1944); (b) E. D. Wills, *Biochem. J.*, **63**, 514 (1956).

(6) W. C. J. Ross, *Ann. N. Y. Acad. Sci.*, **68**, 669 (1958).

(7) T. J. Bardos, Z. B. Papanastassiou, A. Segaloff, and J. L. Ambrus, *Nature*, **183**, 399 (1959).