of substituted rifamycin S but not SV derivatives suggests that the observed conformational changes are linked to enzymatic activity, perhaps in the sense of exposing the ansa hydroxyls as alluded to above. On the other hand, the conformational effects seem to parallel size of substituent while inhibitory effects parallel electronegativity. We are thus led to one of two possible conclusions.

(1) The observed conformational changes are unrelated to the question of enzymatic activity. This seems unlikely on the face of it but it could be that the only role of the ansa bridge is to afford a hydrophobic cross-ring bridge. Alternatively, the enzyme "fit" and conformational mobility of the ansa bridge could be such that minor changes in the latters' solution conformation can be tolerated without appreciable modification of the interaction between the enzyme and the ansa functionality.

(2) The observed conformational changes are indeed of prime importance in defining inhibitory activity, and the basic Karplus equation approach is too crude to pick out the rather subtle interplay between the steric and electronic effects of the substituents. In this respect we find it quite frustrating to be unable to translate the observed chemical shift changes into meaningful conformational changes. One could do this if one could assume a fixed geometry for all derivatives but this is demonstrably not the case. One can only note with a sense of wonderment that changing a chlorine to an iodine gives rise to, as its major effect, a twisting about a bond some ten atoms away! The present data are just too inexact to decide between these two alternatives.[9]

References and Notes

(1) H. W. Whitlock and M. F. Dampier, *J. Am. Chem. Soc.*, **97**, 6254 (1975).
(2) M. L. Casey and H. W. Whitlock, *J. Am. Chem. Soc.*, **97**, 6231 (1975).
(3) S. G. Gallo, E. Martinelli, V. Pagani, and P. Sensi, *Tetrahedron*, **30**, 3093 (1974).
(4) W. Kump and H. Bickel, *Helv. Chim. Acta*, **56**, 2323 (1973).
(5) M. Brufani, W. Fedeli, G. Giacomello, and A. Vaciago, *Experientia*, **20**, 339 (1964).
(6) J. Leitich, V. Prelog, and P. Sensi, *Experientia*, **23**, 505 (1967).
(7) R. C. Fort, Jr., and P. v. R. Schleyer, *J. Org. Chem.*, **30**, 789 (1965); F. W. van Deursen and P. K. Korver, *Tetrahedron Lett.*, **40**, 3923 (1967).
(8) "Handbook of Chemistry and Physics", 55th ed, CRC Press, Cleveland, Ohio, 1975.

(9) Support of this work by NIH is acknowledged.

Studies on the Mode of Action of the Mitomycin Antibiotics. Reversible Conversion of Mitomycin C into Sodium 7-Aminomitosane-9a-sulfonate

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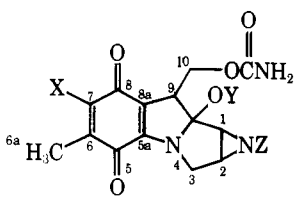
Abstract: Reduction of mitomycin C with sodium dithionite in Tris buffer at 0 °C for a short period of time and subsequent reoxidation with oxygen gas yields 7-aminomitosane-9a-sodium sulfonate and red compounds of unknown structures. 7-Aminomitosane-9a-sodium sulfonate is also obtained at low temperature by catalytic reduction of mitomycin C over a palladium/charcoal catalyst in aqueous solution containing sodium sulfite and subsequent reoxidation. It can be converted back into the starting material by catalytic reduction at 0 °C in methanolic solution in the presence of sodium methoxide followed by reoxidation. These reactions constitute novel interconversions of mitosanes which may have a bearing on the formation of the naturally occurring mitomycin relative, mitiromycin. In addition, the formation of 7-aminomitosane-9a-sodium sulfonate suggests the possibility that C-9a of the mitomycins may be involved to some extent in the alkylation of biological macromolecules. 7-Aminomitosane-9a-sodium sulfonate is the first compound ever to be derived from any member of the mitomycins in a reduction–reoxidation sequence in aqueous solution. It is more stable in acidic medium than mitomycin C and like the parent antibiotic it shows antibacterial activity, albeit at a reduced level. The red compounds obtained show uv spectra similar to those of 7-aminoindoloquinones and they lack the carbamoyl group present in their precursor. The results of this investigation are considered to support, in part, proposals made by others on the mechanism of alkylation of biological macromolecules by mitomycin C.

It is well established that the mitomycin antibiotics[1,2] (I), which are elaborated by *Streptomyces verticillatus*[3] and by other strains of *Streptomyces*,[4] require reductive activation to become alkylating agents of biological macromolecules including DNA.[5] Bifunctional alkylation of the latter leading to the cross-linking of the two strands is considered to be an important feature in the mode of action of mitomycin C[6] and this bifunctional alkylation is presumably at least in part responsible for its effectiveness as an anticancer agent.[7,8]



I

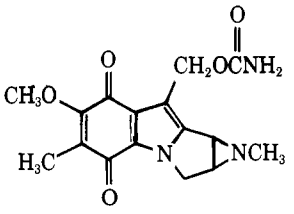
Ia, X = OCH_3; Y = CH_3; Z = H
b, X = OCH_3; Y = H; Z = CH_3
c, X = NH_2; Y = CH_3; Z = H

Studies have been undertaken in several laboratories to gain information on the mutual binding sites between mitomycin C and DNA but these binding sites have not been unambiguously identified. Szybalski and Iyer[9] introduced sodium dithionite as an efficient reducing reagent for in vitro studies and showed that reduced mitomycin C can react with DNA by monofunctional and bifunctional alkylation. These authors proposed that alkylation involved C-1 after opening of the aziridine ring and/or C-10 after displacement of the carbamoyl

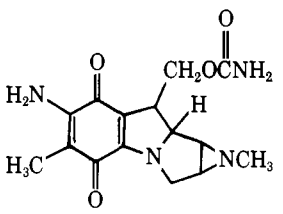
group, and they suggested that cross-linked DNA could arise by interaction of these centers with nucleophilic groups on DNA most likely with O-6 of guanine residues on opposite strands. Support for these suggestions was provided by Mercado and Tomasz[10] and by Otsuji and Murayama[11] who showed that mitomycin derivatives lacking the aziridine ring or the carbamoyl group were inferior antibacterial agents and were considerably less active than mitomycin C in the in vitro cross-linking of DNA, respectively. Tomasz[12] provided evidence that N-7 of guanine is not involved in alkylation and Tomasz and co-workers[13] reported that the amount of mitomycin C that could be bound to DNA could be increased 10- to 20-fold simply by adding sodium dithionite in small portions over a longer period of time rather than at once as done by Szybalski and Iyer. Unfortunately, the alkylated DNA proved to be resistant to attack by *Escherichia coli* K_{12} DNAse and snake venom diesterase precluding the isolation of alkylated nucleotides for detailed structural study. Tomasz and co-workers[13] and our laboratory,[14] in contrast to results reported by Lipsett and Weissbach,[15] were unable to obtain alkylation products by the interaction of reduced mitomycin C with mono- and dinucleotides. Thus it has been impossible to gain knowledge on the binding sites by direct investigation of low molecular weight mitomycin-DNA alkylation products.

In view of these difficulties we decided to undertake a detailed study of the chemistry of reduced mitomycin C in aqueous solution with special emphasis on its interaction with nucleophiles, hoping to gain information on the nature of the activation reaction and on the binding sites of this antibiotic.

Previous attempts elsewhere to generate and to characterize products arising by reduction of mitomycins in aqueous solution have not led to identifiable products;[16] however, reduction-reoxidation reactions carried out in nonaqueous media afforded derivatives in some instances. Thus catalytic reductions of *N*-methylmitomycin A (Ia, Z = CH_3) and of mitomycin B (Ib) over a palladium catalyst using dimethylformamide as solvent and subsequent air oxydation yielded the aziridinomitosene II,[17] a compound which is biologically almost as



II



III

active as the parent antibiotics. The discovery of II led Patrick et al. to suggest that loss of the 9a-substituent together with the 9-proton is an early stage in the activation of the mitomycins. Attempts to obtain a related aziridinomitosene from mitomycin C were futile. A second example for the generation of a product arising from a reduction-reoxidation experiment concerns the formation of demethoxyporfiromycin (III)[8] by reduction of 1a-*N*-methylmitomycin C (porfiromycin; Ic, Z = CH_3) with sodium borohydride in methanol and reoxidation with Fremy's salt. Compound III, a biologically inactive acid-stable material, was not only obtained by this route but also from mitomycin B in a number of steps. Details of the NMR spectrum of III which are crucial for an evaluation of this structure were not given; it was only stated that the 9a-OCH_3 signal was absent. Mitomycins A and C were reported to afford the respective 9a-demethoxy derivatives only in very poor yield.

During the course of our attempts to isolate and to characterize compounds arising from the reduction of mitomycin C in aqueous solution, we discovered the blue title compound and red conversion products thereof. We report here our structural studies on these compounds and discuss their potential significance in terms of the mode of action of the mitomycins.

Results

Initial experiments were carried out to explore the reactivity of C-10 of mitomycin C toward nucleophiles by investigating the stability of the carbamoyl group of the reduced antibiotic in aqueous medium. [$OCONH_2$-^{14}C]Mitomycin C obtained biosynthetically by feeding L-[*guanidino*-^{14}C]arginine to *S. verticillatus*[18] was mixed with carrier material and dissolved in Tris-HCl buffer, pH 7.4, and the violet solution was reduced with sodium dithionite ($Na_2S_2O_4$) for different periods of time. The resulting colorless solution was reoxidized by bubbling oxygen gas to give a blue-violet or violet-red solution depending on reaction time and temperature. Aliquot fractions were subjected to paper chromatography and to radioactivity analysis. Rapid disappearance of the antibiotic and its associated radioactivity and a short-term labeling of a blue compound were noted when the reduction was carried out at room temperature, but the rate of disappearance of both the antibiotic and the radioactivity were decreased and the amount of the blue compound formed was considerably increased when the reaction was carried out at 0 °C. The results of these studies are shown in Figure 1. At both temperatures mitomycin C and the blue compound formed from it appeared to be converted into the red compounds which were devoid of radioactivity and thus were lacking the carbamoyl group. The precursor product relationships were clarified by showing that the isolated and purified blue compound is converted into the red compounds by reduction with sodium dithionite and subsequent reoxidation.

The fact that the blue compound retained the radioactivity of mitomycin C and, hence, the carbamoyl group suggested the possibility that compounds representing early intermediates of the mitomycin C activation reaction could be isolated from low-temperature experiments and could be stabilized by reoxidation. On the other hand, the formation of the red compounds suggested the possibility that compounds arising from the interaction of reduced mitomycin, presumably with nucleophiles, could also be trapped and that the elucidation of their structures might reveal the binding sites of the antibiotic. In order to isolate these materials in quantities sufficient for structure elucidation, the reduction reaction was carried out repeatedly on a 0.1-mmol scale, which appeared to be optimal. Reaction products were purified by preparative paper chromatography, column chromatography, and, for the preparation of analytical samples, by high-pressure liquid chromatography. The blue compound was isolated in up to 32% yield as a green solid while the red compound was isolated in 10-20% yield as a brown powder. Both compounds are highly polar and they are soluble with blue and with red color, respectively, only in water, methanol, dimethyl sulfoxide, and dimethylformamide. They both move to the anode on electrophoresis while mitomycin C moves slightly toward the cathode. The data collected on the blue compound identify it as 7-aminomitosane-9a-sodium sulfonate (IV).[19]

Besides preparing IV by reduction of mitomycin C with sodium dithionite (reaction condition a, Scheme I), it was possible to obtain it by catalytic reduction of the antibiotic in Tris-HCl buffer at 0 °C over palladium on charcoal in the presence of sodium sulfite and subsequent reoxidation with oxygen gas (reaction condition b, Scheme I). The product obtained showed the same R_f value in systems A and D and the same electrophoretic mobility in systems G and H as the material obtained in the first reaction. The facile formation of IV by the second route suggested the possibility of converting it back into mitomycin C by a related catalytic reduction

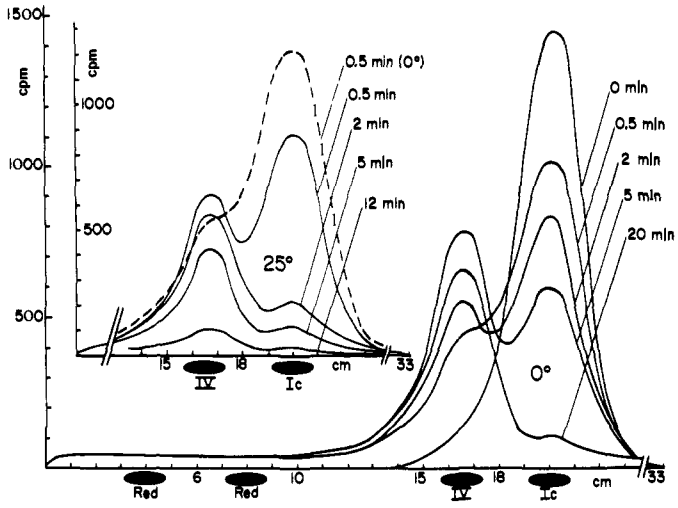


Figure 1. Radiochromatographic analysis of the conversion of Ic into IV and red compounds at different temperatures.

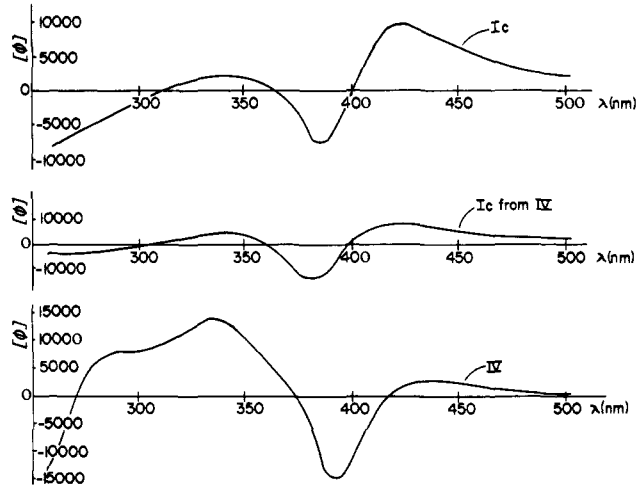
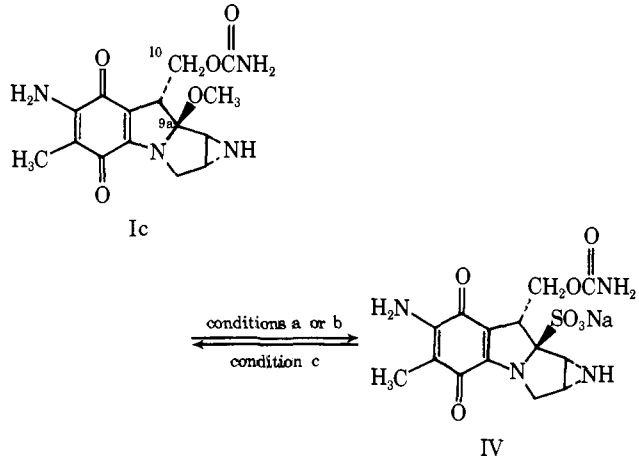


Figure 2. ORD spectra of Ic, of Ic obtained from IV, and of IV.

Scheme I

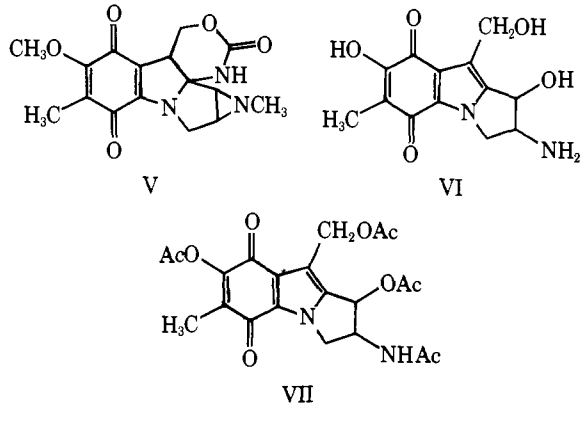


a, (1) $Na_2S_2O_4$, Tris-HCl buffer, pH 7.4, 0 °C, 3 min; (2) O_2
b, (1) H_2 (Pd/C), Na_2SO_3, 0 °C, 3 min; (2) O_2
c, (1) H_2 (Pd/C), $CH_3ONa(CH_3OH)$; (2) O_2

procedure. This was indeed possible by reduction over a palladium/charcoal catalyst with hydrogen gas in methanolic solution at 0 °C in the presence of sodium methoxide and subsequent oxidation by bubbling oxygen (reaction condition c, Scheme I). The reaction product showed the same chromatographic mobility in three TLC systems and in one paper chromatography system as well as the same mass and ORD spectra as mitomycin C. Mitomycin C could not be obtained from IV when the reduction step was omitted.

Electron-impact mass spectroscopy of IV gave no useful ions at higher masses; however, ions at *m/e* 48 and 64 and corresponding satellite peaks at *m/e* 50 and 66 indicated the presence of sulfur. Field-desorption mass spectroscopy (FD MS) afforded an ion at *m/e* 302 which is also seen in the electron-impact mass spectrum (EI MS) of mitomycin C.[20] This ion arises by the loss of methanol from C-9 and C-9a of mitomycin C and it could arise by loss of sodium bisulfite (HSO_3Na, mol wt = 104) from the same carbons of IV, suggesting that the molecular weight of IV is 406. Additional peaks seen in the FD MS of IV at *m/e* 241 and 259 also occur in the EI MS of mitomycin C, but a peak at *m/e* 267 has no parallel in the mitomycin C spectrum. None of these peaks have been assigned. The ir spectrum is very similar to that of mitomycin C[21] but shows three additional strong bands at 1195, 1030, and 630 cm^{-1} which are diagnostic for sulfonates.[22] The 1195-cm^{-1} absorption band partially overlapped the 1200 region where the aziridine ring absorption occurs. The uv spectrum of IV is almost superimposable with that of the parent antibiotic indicating that IV is a mitosane.[4] The ^1H NMR spectrum lacked the $-OCH_3$ signal found in the spectrum of mitomycin C but otherwise it is very similar.[23] The signals for H_1 and H_2 moved slightly downfield, 3.23 vs. 3.07 and 3.04 vs. 2.20, those for H_3 and $H_{3'}$ moved closer together, 4.14 vs. 4.50 and 3.76 vs. 3.56, while H_{10} and $H_{10'}$ showed upfield shifts, 4.31 vs. 5.00 and 4.58 vs. 5.36, reminiscent of similar shift changes of the corresponding protons in mitiromycin (V),[24] a compound isolated together with the mitomycins from the fermentation broth of *S. verticillatus*.[3] The ^{13}C NMR spectrum of IV is also very similar to that of Ic for which assignments have been published.[23] The only major differences are seen in the area around 32–37 and 93–112 ppm. The ORD spectra of Ic and IV shown together with the ORD spectrum of Ic obtained from IV (Figure 2) were also very similar, suggesting that both compounds possess the same absolute configuration.[2]

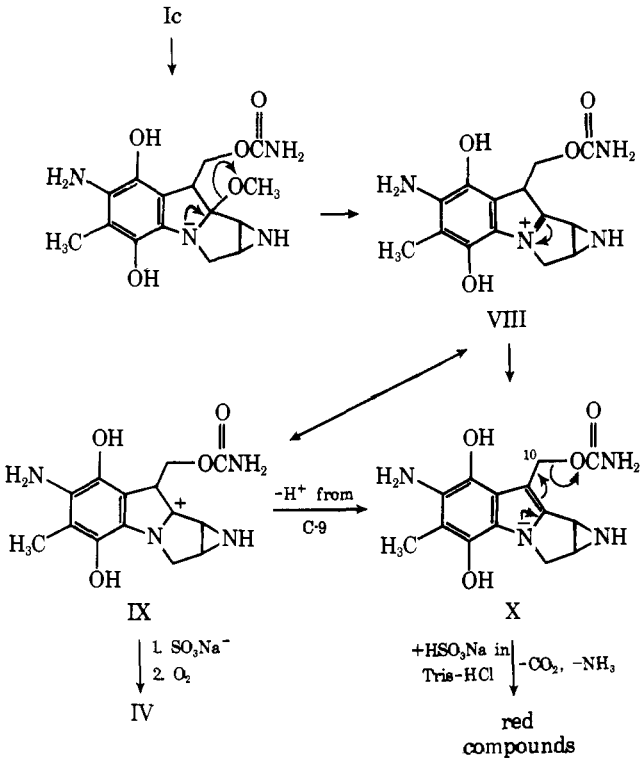
Compound IV in concert with mitiromycin (V)[24] shows a



substantially increased acid stability in comparison with mitomycin C and it is also quite stable in basic solution. Concentrated hydrochloric acid treatment and subsequent acetylation with acetic anhydride in pyridine afford a tetraacetate VII by way of the intermediate VI, giving identical ^1H NMR and mass spectra as the same compound obtained from mitomycin C by the same sequence of reactions.[25] This observation provides additional proof that the basic skeleton is identical in both Ic and IV, thus supporting the formulation of IV as a bisulfite adduct. IV showed weaker antibacterial activity in agar diffusion assays than Ic; its possible cross-linking ability of DNA has not been investigated.

The structures of the red compounds have not been eluci-

Scheme II



dated. The compound moving the greater distance in system A was prepared in larger quantity. As mentioned above, this compound lacks the $-OCONH_2$ group and it is assumed that this group may have been replaced by a nucleophilic group such as an $-SO_3Na$ group. In line with this assumption is the observation that this compound is highly polar and moves toward the anode in electrophoresis systems G and H. The fact that it moves less far than IV suggests that an amino function is also present which is more basic than an aziridine nitrogen. The ir spectrum of the red compound showed the presence of a sulfonyl group, thus supporting the above assumptions, and the uv spectrum showed a chromophore similar to that of apomitomycin C (VI), suggesting that is a mitosene.[4] The ^1H NMR spectrum measured on a very small sample showed the presence of the C-7 amino group and an unassigned signal also containing two protons close by, which may be assumed to belong to an amino group as well as the C-6 methyl group, but other resonances could not be assigned. Californium-252 plasma desorption mass spectroscopy, a novel method for the mass spectroscopic analysis of nonvolatile compounds,[26] permitted the assignment of a molecular weight of 437. The red compound showed no antibacterial activity except for very weak activity against *Bacillus subtilis*.

Discussion

Two main conclusions can be drawn from the present investigation. First, it is possible to isolate and to characterize compounds arising from the interaction of reduced and reoxidized mitomycin C with nucleophiles in aqueous medium and, second, the proposed activation mechanism[6,17] for the mitomycins receives support through the observation that 7-aminomitosane-9a-sodium sulfonate (IV) is an early reaction product. While a full evaluation of the importance of the isolation of reaction products from long-term reactions will only be possible after their structures have been elucidated, it is of interest to examine the importance of the discovery of IV in terms of the proposed activation mechanism for the mitomycins and the general understanding of the chemistry of 9a-substituted mitosanes.

Szybalski and Iyer[6] proposed that generation of the cation VIII (Scheme II) by expulsion of the OY substituent is the first event after reduction of the mitomycins and that this cation, for which the resonance contributor IX can be written, loses a proton from C-9 to afford a reduced aziridinomitosene (X) which can undergo a number of transformations. One possible transformation is loss of the C-10 carbamoyl group and interaction of the cation thus generated at C-10 with nucleophiles to give rise to compounds having structures presumably similar to decarbamoylapomitomycins such as VI.[4] The formation of IV affords a strong indication for the existence in aqueous solution of the cation IX or the reduced aziridinomitosene X. It is possible that IV arises by trapping of cation IX through its interaction with the SO_3Na^- anion provided directly in reaction condition b (Scheme I) and generated from $Na_2S_2O_4$ by oxidation in reaction condition a (Scheme I), followed by stabilization of the resulting reduced mitosane by oxidation. Alternatively, it is possible that IV arises via X by addition of bisulfite across the 9-9a double bond and subsequent oxidation. No experimental evidence is presently available that would allow us to choose between these possibilities; however, we favor IX as an intermediate. We consider the conversion of the blue compound into the red compounds to be in accord with the generation of a C-10 cation as proposed by Szybalski and Iyer, but we cannot exclude other reactions which might effect the loss of the $-OCONH_2$ group.

Besides providing substantiation for the Szybalski-Iyer activation mechanism, the formation of the blue compound suggests a possible mechanism for the formation of mitiromycin (V) via a similar redox sequence and reaction of C-9a with the amino group of the carbamoyl group possibly of mitomycin B. In addition, the formation of the blue compound suggests that at least in part interaction of reduced mitomycin with nucleophilic groups on biological macromolecules via C-9a may be possible and may be contributing to monofunctional alkylation which, as estimated by Szybalski and Iyer, is ten times more prevalent than bifunctional alkylation by this antibiotic.

The synthesis of the blue compound and its conversion back into mitomycin C by the reactions outlined in Scheme I constitute novel interconversions of mitosanes. The stereochemical course of these conversions is not presently completely known, although retention of configuration is considered to be highly likely. This assumption is based on the great similarity of the ORD spectrum of 7-aminomitosane-9a-sodium sulfonate with that of mitomycin C and the identity of the ORD spectrum of mitomycin C generated from IV with that of the authentic antibiotic. It is of interest to note that IV is derived from Ic, a member of the mitomycin family which lacks an NCH_3 group, while the two previously reported reduction-reoxidation products II and III, generated in nonaqueous medium, were both obtained from starting mitomycins containing NCH_3 groups. The presence of the NCH_3 group appeared to be a requirement for the successful isolation of products since mitomycins lacking this group gave only very poor yields.[11,17]

Future studies will be directed toward the complete structure elucidation of the red compound(s) and the generation and structure elucidation of additional related compounds arising in the interaction of reduced mitomycin C with nucleophiles in anticipation that this will permit us to gain knowledge on the binding sites of this antibiotic. It will also be of interest in future studies to investigate the structure and the possible role in alkylation of a semiquinone radical which has been shown to form in nonaqueous[17] and in aqueous[27] solution upon reduction of mitomycin B and mitomycin C, respectively, and which has been postulated[13] to be responsible for the increased amount of mitomycin that can be bound to DNA. In addition, future reactions patterned on those outlined in Scheme I may be of interest to prepare novel mitomycin analogues, especially

since it can be expected that some of them like IV may show an increased acid stability and thus may be good oral drugs.

Experimental Section

Materials. Nonlabeled mitomycin C was obtained from the Kyowa Hakko Kogyo Co., Tokyo, Japan, and from Bristol Laboratories, Syracuse, N.Y., and was used without purification. [$OCONH_2$-^{14}C]Mitomycin C was prepared by feeding 100 μCi of L-[*guanidino*-^{14}C]arginine, specific radioactivity 5.9 mCi/mM, to a replacement culture of *S. verticillatus* and isolating labeled mitomycin A as previously described.[18] The red antibiotic was spotted on three TLC plates (5 × 20 cm), and the plates were exposed to vapors of concentrated ammonia for 15 min and the now violet zone was developed in system C. The zone containing mitomycin C was scraped off, extracted twice with ligroine to remove 1-octanol, and then extracted ten times with 2 ml of ethyl acetate. The solvent was removed and the radioactive antibiotic was dissolved in water. An aliquot developed in systems D and E was shown by analysis with a radiochromatogram scanner to be radiochemically pure mitomycin C. All other materials were commercially available and were used without purification except for solvents which were redistilled.

Analytical Methods. Melting points were determined on a Thomas-Hoover apparatus. Elemental analyses were obtained from Midwest Microlab, Indianapolis, Ind. Optical rotations were recorded on a Perkin-Elmer 141 polarimeter. Electron-impact mass spectra were obtained on a CEC 21 110B instrument (direct inlet probe, 70 eV, temperature between 150 and 200 °C). Field-desorption mass spectra were obtained on a Varian MAT CH5 DF mass spectrometer equipped with a combination field desorption–field ionization–electron impact ion source.[28] Ir spectra were recorded on Perkin-Elmer IR 237 and Beckman IR 33 instruments and only the major peaks are reported. Uv and visible spectra were recorded on a Cary Model 17 recording spectrophotometer. The ^1H NMR spectra were obtained on Jeol PFT 100 and on Varian XL 100 instruments using approximately 10–15% (w/v) solutions. The proton-decoupled ^{13}C NMR spectrum was recorded on the Jeol instrument operating in the Fourier transform mode under conditions similar to those described elsewhere.[29] The ORD and CD spectra were recorded on a Cary Model 60 spectropolarimeter, using 10^{-4} M solutions in methanol–water (1:9).

Chromatography and Electrophoresis. Spots were identified by their own color: mitomycin C (Ic), violet; 7-aminomitosane-9a-sodium sulfonate (IV), blue; red compounds, bordeaux red. Ascending and descending paper chromatography was performed on sheets of Whatman No. 1 paper for analytical separations and for preparative isolations on sheets (46 × 57 cm) of Whatman 3MM paper in system A [methanol–water (9:1); Ic R_f 0.60; IV, R_f 0.49; red compound (major), R_f 0.24; red compound, R_f 0.12] and system B [2-propanol–ethyl acetate–water (6:4:1); 36-h descending (front ran through) relative distances, Ic 0.90, IV 0.65, red compound (major) 0.20, red compound 0.10]. Thin-layer chromatography on 5 × 20 cm plates precoated with silica gel G (Merck) was used for analytical and for some preparative separations: systems C, acetone–ligroine (boiling range 100–115 °C)–1-octanol (5:5:2), run twice, Ic R_f 0.14, IV R_f 0.01, red compound R_f 0.01; system D, 2-propanol–ethyl acetate–water (6:4:1), Ic R_f 0.47, IV R_f 0.13, red compound R_f 0.52; system E, diglyme [bis(2-methoxyethyl) ether]–2-propanol (3:2), Ic R_f 0.60, IV R_f 0.14, red compound R_f 0.23; system F, 2-propanol–ethyl acetate (2:1), Ic R_f 0.29, IV R_f 0.04. Electrophoretic separations were carried out with a Savant FP-30A high-voltage electrophoresis apparatus using Whatman 3MM paper in system G [0.05 M sodium phosphate buffer, pH 7.3; 1 h; 2 kV; 10–15 °C; distance traveled (cm) Ic, −2.4; IV, +11.1; red compound (major), +6.2; red compound, +3.4] and system H [0.05 M sodium borate buffer, pH 10.2; 1 h; 2 kV; 10–15 °C; Ic, −7, IV, +6.3, red compound, +5.3].

Isotope Analysis. Radioactivity measurements were carried out in Beckman LS 100 and LS 250 liquid scintillation counters. A solution (10 ml) containing 7 g of PPO and 0.3 g of Me_2-POPOP in 1 l. of toluene was used as the scintillator fluid for counting mitomycin C samples. Radioactivity on paper chromatograms was determined by cutting 1 cm wide zones, agitation of the paper with 1 ml of water in a counting vial, and subsequent addition of Bray's solution (10 ml).[30] Samples were counted to at least 2% statistical error.

Time Course Experiments. Two solutions, each containing 2.6 mg (7.8 μmol) of [$OCONH_2$-^{14}C]mitomycin C (5.1 × 10^4 dpm) and 1.25 ml of 0.1 M Tris–HCl, pH 7.4, were purged with oxygen-free nitrogen gas for 1 h. One solution was then cooled to 0 °C while the other was left at room temperature. Freshly prepared solutions (50 μl) of sodium dithionite (3.0 μmol), one at 0 °C and the other at room temperature, were added to the respective mitomycin solutions under a stream of nitrogen to initiate the reduction. Aliquots (0.25 ml) were withdrawn with a syringe from the reaction mixture at room temperature after 0.5, 2.0, 5.0, and 12.0 min and from the reaction mixture at 0 °C after 0.5, 2.0, 5.0, and 20 min, respectively, diluted with the buffer solution (0.25 ml), and purged for 5 min with oxygen gas to terminate each reaction. The total volume was then applied to a 3 cm wide zone on Whatman 3MM paper for chromatography in system A and subsequent radioactivity analysis.

Synthesis of 7-Aminomitosane-9a-sodium Sulfonate (IV). Method a. Mitomycin C (35 mg) was dissolved in 0.05 M Tris–HCl (22 ml), pH 7.4, and the resulting blue solution was cooled to 0 °C and purged with oxygen-free nitrogen gas for 45 min. A freshly prepared cooled solution (0.2 ml) of sodium dithionite (35 mg) was added at once, which rendered the solution colorless, and the purging with nitrogen gas was continued for 3 min at 0 °C. The reaction was stopped by passing oxygen through the reaction mixture for 5 min. The resulting blue-violet solution was applied to two full-size Whatman 3MM sheets, the solvent was removed with a stream of cold air (duration of the application, 2 h), and the chromatograms were developed in solvent B. A blue-green zone representing IV at R_f 0.65 and a red zone at R_f 0.24 were cut separately, the blue-green zone was eluted with distilled methanol, and the red zone was eluted with water. The methanol was removed and IV was added to a silica gel column which was developed with chloroform–methanol (8:2). The greenish-bluish fraction was collected and the solvent was removed by evaporation. The solid residue was dissolved in water and added to a Sephadex G-200 column which was eluted with water. Sephadex G-200 clearly afforded a purer product than Sephadex G-25 which was also tried. An analytical sample of IV (approximately 3 mg) was prepared by high-pressure liquid chromatography using a Porasil II column and methanol–water (9:1) as eluent on a Waters liquid chromatograph. The purified material was dissolved in methanol and crystallization was induced by the addition of ethyl acetate. The resulting material was homogeneous on TLC in systems C and D and in electrophoresis in systems G and H. It melted above 350 °C and showed $[\alpha]^{25}D$ −11 ± 1° (*c* 0.018 in water).

Method b. Mitomycin C (5 mg) was dissolved in 0.1 M Tris–HCl buffer (2.0 ml), pH 7.4, and the solution was cooled to 0 °C and flushed with nitrogen gas for 20 min. 5% palladium on charcoal (1 mg suspended in 1 ml of water) and sodium sulfite (30 mg) dissolved in 0.25 ml of water were added and the mixture was reduced by bubbling hydrogen gas for 20 min. The reaction was terminated by bubbling nitrogen gas for 5 min and then oxygen gas for 10 min. The color of the solution remained violet during the course of the reaction. Aliquots of the reaction mixture were analyzed in systems A, D, G, and H and showed in each case one major spot matching exactly with reference IV besides a smaller amount of unreacted Ic and unidentified red compounds. The yield of IV is estimated to be 50%. Compound IV, prepared by method a, was used in all subsequent investigations. The field-desorption mass spectrum run on 1 mg of material at approximately 120 °C effective wire temperature showed peaks (relative intensity) at *m/e* 241 (0.4), 259 (1.0), 267 (0.2), and 302 (0.3) besides peaks of much lower intensity at lower masses; uv λ_{max} (MeOH) 212, 248, (s), 367 (ϵ 14 350), and 555; uv λ_{max} (H_2O) 372 (ϵ 14 280); ir ν_{max} (KBr) 3300 (br, s), 2900 (m), 1720 (s), 1580 (s), 1520 (s), 1430 (s), 1330 (s), 1240 (s), 1195 (s), 1030 (s), 850 (m), 700 (m), 630 cm^{-1} (s); ^1H NMR δ (in H_2O, DDS) 1.68 (3 H, s, CH_3), 3.04 (1 H, dd, $J_{2,3'}$ = 2 Hz, $J_{1,2}$ = 5 Hz, H_2), 3.23 (1 H, d, H_1), 3.76 (1 H, dd, $J_{3,3'}$ = 13 Hz, $H_{3'}$), 4.00 (1 H, dd, $J_{9,10}$ = 5 Hz, $J_{9,10'}$ = 11 Hz, H_9), 4.14 (1 H, d, H_3), 4.31 (1 H, t, $J_{10,10'}$ = 11 Hz, H_{10}), 4.58 (1 H, dd, $H_{10'}$); ^1H NMR δ (in Me_2SO-d_6, Me_4Si) 6.2 (2 H, s, NH_2), 6.9 (2 H, s, -$OCONH_2$); ^{13}C NMR δ (in D_2O) 178.6, 176.9, 160.6, 159.3, 153.4, 111.1, 105.9, 93.4, 63.2, 52.8, 44.3, 36.2, 36.0, 9.1.

Conversion of 7-Aminomitosane-9a-sodium Sulfonate into Mitomycin C. Compound IV (5 mg) was dissolved in anhydrous methanol (1 ml), 5% palladium on charcoal (1 mg) was added, the mixture was cooled to 0 °C, and oxygen-free nitrogen gas was bubbled for 10 min. Sodium (approximately 2 mg) was added and hydrogen gas was bubbled through the mixture for 5 min, followed by nitrogen gas (2 min) and oxygen gas (5 min). Aliquots were analyzed in systems A, D, E, and F and in every system appeared a spot exactly matching the R_f value of reference Ic besides red spots of unknown structure of

identical R_f with those formed from Ic in a control sodium methoxide–methanol solution. Omission of palladium on charcoal and hydrogen gas from the reaction mixture afforded no mitomycin C as shown by analysis in system D. A sample for mass spectral analysis and for determination of the ORD spectrum of Ic formed from IV was obtained by diluting 4.5 ml of the reaction mixture with water (5 ml), extracting with ethyl acetate (3 × 5 ml), washing the extract with water (2 × 10 ml), drying with Na_2SO_4, concentrating, and purifying in system A. The band traveling the same distance as reference Ic was eluted with methanol, the solvent was removed, and the sample was analyzed. Ic was formed in approximately 40% yield.

Acid Stability of Ic and IV. Hydrochloric acid (0.2 N, 1 ml) was added separately to 10^{-4} M aqueous solutions (1 ml) of Ic and IV and each resulting mixture was quickly transferred into a cuvette for measurement over a time period of 2–4 h of the decrease of the absorption maximum at 363 and 372 nm, respectively, using a Perkin-Elmer Coleman 124 spectrophotometer. The pseudo-first-order rate constants and the half-lives were calculated. The latter were found to be 0.16 and 26.5 h, respectively, indicating that IV is 166 times more stable than Ic in 0.1 N HCl at 25 °C.

Conversion of IV into VII. A solution of IV (30 mg) in 6 N HCl (3 ml) was kept at 55–60 °C for 2 h. It turned red after 2 min and orange after 15 min. The reaction mixture was worked up and the resulting apomitomycin C (VI) was converted into the tetraacetate derivative VII as described.[25] The reaction product (10 mg) showed ^{1}H NMR and mass spectra indistinguishable from those of VII obtained directly from Ic.

Antibacterial Activity. *Klebsiella pneumoniae* (ATCC 10031), *Staphylococcus aureus* (ATCC 13709), *Escherichia coli* (ATCC 14948), and *Bacillus subtilis* (ATCC 6633) were maintained on nutrient agar slants. Organisms used for testing were incubated at 37 °C overnight in nutrient broth or in the case of *B. subtilis* in a tryptone-glucose medium,[31] collected by centrifugation, suspended in 0.5 M potassium phosphate buffer (pH 7.5), mixed with "soft" agar (0.7 g of nutrient agar/100 ml of H_2O) at 39–40 °C, and then poured onto solid agar plates. Compound IV (100 μg) and, for comparison, Ic (100 μg) were dissolved in water and placed on paper disks. The disks were dried and placed on the prepared agar plate which was then incubated at 37 °C for 24–48 h and the inhibition zones were measured (results for Ic are given in brackets): *K. pneumoniae*, 5 mm (cloudy) [4 mm]; *S. aureus*, 0.5 mm [5 mm]; *E. coli*, 1 mm (cloudy) [5 mm]; and *B. subtilis*, 3 mm (only 50 μg was used) [15 mm, 21 mm halo].

Conversion of Ic and IV into Red Compounds. A solution of Ic or IV (5 mg) in 0.01 M Tris–HCl buffer (2 ml), pH 7.4, was purged with nitrogen for 30 min and cooled to 0 °C. A freshly prepared solution of $Na_2S_2O_4$ (5 mg/50 μl of water) was added and aliquot samples were withdrawn from the colorless reaction mixture after 2, 5, 15, and 30 min. The samples were immediately purged with oxygen gas to stop the reaction and then spotted for chromatographic analysis in system A. Red compounds obtained from mitomycin C in a reaction mixture similar to the one described for the preparation of IV were also spotted on the chromatograms. All chromatograms showed red spots at R_f 0.24 and R_f 0.12 and a spot for IV at R_f 0.65. The intensity of the spot of IV was decreasing while the intensities of the spots at R_f 0.24 and R_f 0.12 were increasing initially and then decreasing to give rise to a red spot of unknown nature remaining at the origin. Material moving at R_f 0.24 in system A obtained from several runs of the Ic → IV conversion reactions as a side product in approximately 10–20% yield was eluted with water from the chromatography paper and purified by column chromatography on silica gel using chloroform–methanol (8:2) as eluent and by high-pressure liquid chromatography on a Porasil II column using methanol–water (9:1) as solvent. Electron-impact mass spectroscopy showed no ions above *m/e* 100 but clearly indicated the presence of sulfur through ions at *m/e* 48 and 64 and respective satellite peaks. Plasma-desorption mass spectroscopy[26] showed *m/e* 460 in the positive ion spectrum and *m/e* 414 in the negative ion spectrum presumably resulting from sodium addition or abstraction, respectively, from the compound having mol wt 437. Additional peaks at *m/e* 265 and 241 were observed in the positive ion spectrum as well as an additional peak at *m/e* 195 in the negative ion spectrum: uv λ_{max} (MeOH) 205, 254, 308 (s), 350 (s), 515; ir ν_{max} (KBr) 3400 (br, s), 2900 (m), 1600 (s), 1380 (m), 1200 (w), 1110 (m), 1030 (s), 620 cm^{-1} (w); ^{1}H NMR δ (D_2O, DSS) 1.68 (3 H, s, CH_3), 6.28 (2 H, s), 6.94 (2 H, s).

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References and Notes

(1) J. S. Webb, D. B. Cosulich, J. H. Mowat, J. B. Patrick, R. W. Broschard, W. E. Meyer, R. P. Williams, C. F. Wolf, W. Fulmor, C. Pidacks, and J. E. Lancaster, *J. Am. Chem. Soc.*, **84**, 3185, 3186 (1962).
(2) A. Tulinski and J. H. Van den Hende, *J. Am. Chem. Soc.*, **89**, 2905 (1967).
(3) D. V. Lefemine, M. Dann, F. Barbatschi, W. K. Hausmann, V. Zbinovsky, P. Monnikendam, J. Adam, and N. Bohonos, *J. Am. Chem. Soc.*, **84**, 3184 (1962).
(4) E. J. Kirsch in "Antibiotics. Volume II. Biosynthesis", D. Gottlieb and P. D. Shaw, Ed., Springer, New York, N.Y., 1967, pp 66–76.
(5) I. H. Goldberg and P. A. Friedman, *Annu. Rev. Biochem.*, **40**, 775 (1971).
(6) W. Szybalski and V. N. Iyer in "Antibiotics. Volume I. Mechanism of Action", D. Gottlieb and P. D. Shaw, Ed., Springer, New York, N.Y., 1967, pp 211–245.
(7) R. L. Comis and S. K. Carter, *Cancer*, **34**, 1576 (1974).
(8) S. Kinoshita, K. Uzu, K. Nakano, and T. Takahashi, *J. Med. Chem.*, **14**, 103, 109 (1971), and references cited therein.
(9) W. Szybalski and V. N. Iyer, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **23**, 946 (1964).
(10) C. M. Mercado and M. Tomasz, *Antimicrob. Agents Chemother.*, **1**, 73 (1972).
(11) N. Otsuji and I. Murayama, *J. Bacteriol.*, **109**, 475 (1972).
(12) M. Tomasz, *Biochim. Biophys. Acta*, **213**, 288 (1970).
(13) M. Tomasz, C. M. Mercado, J. Olson, and N. Chatterjie, *Biochemistry*, **13**, 4878 (1974).
(14) Unpublished observations.
(15) M. N. Lipsett and A. Weissbach, *Biochemistry*, **4**, 206 (1965).
(16) J. B. Patrick et al., unpublished results, personal communication.
(17) J. B. Patrick, R. P. Williams, W. E. Meyer, W. Fulmor, D. B. Cosulich, R. W. Broschard, and J. S. Webb, *J. Am. Chem. Soc.*, **86**, 1889 (1964).
(18) U. Hornemann, J. P. Kehrer, C. S. Nunez, and R. L. Ranieri, *J. Am. Chem. Soc.*, **96**, 320 (1974).
(19) Systematic name: sodium 6-amino-1,1a,2,4,8,8b-hexahydro-8-(hydroxymethyl)-5-methyl-4,7-dioxoazirino[2′,3′:3,4]pyrrolo[1,2-*a*]indole-8a(7*H*)-sulfonate carbamate (ester).
(20) G. E. Van Lear, *Tetrahedron*, **26**, 2587 (1970).
(21) K. Uzo, Y. Harada, and S. Wakaki, *Agric. Biol. Chem.*, **28**, 388 (1964).
(22) D. J. Pasto and C. R. Johnson, "Organic Structure Determination", Prentice-Hall, Englewood Cliffs, N.J., 1969, p 132.
(23) J. W. Lown and A. Begleiter, *Can. J. Chem.*, **52**, 2331 (1974).
(24) G. O. Morton, G. E. Van Lear, and W. Fulmor, *J. Am. Chem. Soc.*, **92**, 2588 (1970).
(25) C. L. Stevens, K. G. Taylor, M. E. Munk, W. S. Marshall, K. Noll, G. D. Shah, L. G. Shah, and K. Uzu, *J. Med. Chem.*, **8**, 1 (1965).
(26) D. F. Torgerson, R. P. Skowronski, and R. D. Macfarlane, *Biochem. Biophys. Res. Commun.*, **60**, 616 (1974), and personal communication.
(27) C. Nagata and A. Matsujama, *Prog. Antimicrob. Anticancer Chemother., Proc. Int. Congr. Chemother, 6th, 1969*, **2**, 423–427 (1970); *Chem. Abstr.*, **74**, 41 068 (1971).
(28) K. L. Rinehart, J. C. Cook, K. H. Maurer, and U. Rapp, *J. Antibiot.*, **27**, 1 (1974).
(29) C. J. Chang, H. G. Floss, and G. E. Peck, *J. Med. Chem.*, **18**, 506 (1975).
(30) A. Bray, *Anal. Biochem.*, 1, 279 (1960).
(31) F. Reusser, *J. Bacteriol.*, **96**, 1285 (1968).