B. With Aqueous Dimethyl Sulfoxide.—A solution of 16.5 mg of IIm in 4 ml of DMSO was diluted with 6 ml of H_2O and then kept at 25° for 2 days. The resulting mixture was diluted with 60 ml of H_2O and extracted with CH_2Cl_2. This extract was washed twice with H_2O, dried, and concentrated. The residue was resolved into two components by adsorption chromatography on a magnesia–silica gel column. The component eluted by CH_2Cl_2 was identical in ir spectrum with starting material, and the component eluted by CH_2Cl_2 containing 25% of Me_2CO was identical in ir spectrum with IIi.

Acknowledgment.—We wish to thank Mr. L. M. Brancone and staff for microanalyses, Mr. W. Fulmor and staff for spectral data, Mr. A. C. Dornbush and staff for *in vitro* antibacterial assays, and Dr. G. R. Allen, Jr., for generously furnishing all starting materials. We also thank Miss R. H. Roth and Mr. J. F. Poletto for permission to publish the indicated experiments.

The Mitomycin Antibiotics. Synthetic Studies. XXII.[1] Antibacterial Structure–Activity Relationships in the Indoloquinone Series

MARTIN J. WEISS, GUNNAR S. REDIN, GEORGE R. ALLEN, JR., ALBERT C. DORNBUSH, HARRY L. LINDSAY, JOHN F. POLETTO, WILLIAM A. REMERS, RETA H. ROTH, AND ADOLF E. SLOBODA

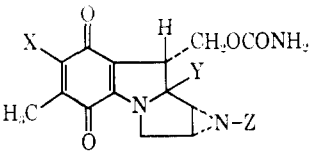
Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York

Received March 13, 1968

The *in vivo* antibacterial structure–activity relationships of a considerable number of indoloquinone analogs of the mitomycin antibiotics are reviewed. The most active members of the series were certain 5-ethyleneimino derivatives, of which 1-ethyl-2,6-dimethyl-5-ethyleneiminoindoloquinone N-(β-hydroxyethyl)carbamate (IV) was the most extensively investigated. This compound shows potent oral activity in mice infected with a spectrum of representative gram-positive organisms, but apparently still retains some of the cytotoxicity manifested by the parent antibiotics and therefore is not of clinical interest.

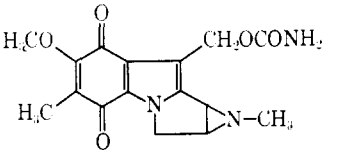
In previous papers of this series we have described the synthesis and *in vitro* antibacterial activity of a series of indoloquinone analogs of the mitomycin antibiotics. Our interest in these antibiotics derives from their very potent broad-spectrum oral antibacterial activity in mice, an activity which extends to tetracycline- and penicillin-resistant strains.[2] Moreover, the mitomycins show an important antitumor effect, and, in fact, mitomycin C has found clinical use in this connection, particularly in Japan.[3] On the other hand, these substances are powerful general cytotoxic agents, which precludes them from consideration as clinical antibacterial agents and presumably limits their utility as antitumor agents. Thus, the mitomycins represent a challenging structure-activity problem, and in this paper we review the relationships which we have found for the indoloquinone series with regard to *in vivo* antibacterial activity.

The structures of the four mitomycin antibiotics were determined by Webb and coworkers[4] and are as shown below.

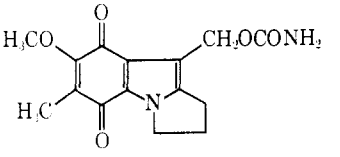


	X	Y	Z
Mitomycin A	OCH_3	OCH_3	H
Mitomycin B	OCH_3	OH	CH_3
Porfiromycin	NH_2	OCH_3	CH_3
Mitomycin C	NH_2	OCH_3	H

The conversion of mitomycin B to the equally potent pyrroloindoloquinone I was achieved by Patrick and coworkers.[5] And in a further simplification the desaziridino analog of I, the pyrroloindoloquinone 7-methoxymitosene (II), was synthesized in our laboratory.[6] Although this latter substance was considerably less potent than the parent antibiotics or I, it retained enough antibacterial effect to be of significant interest. *In vitro* it was markedly active against a variety of gram-positive organisms, including strains resistant to tetracycline and penicillin, but it was relatively ineffective *vs.* gram-negative organisms. In the *Staphylococcus aureus*, strain Smith, infection in mice, II was about one-third as active orally as tetracycline hydro-



I



II

(1) Paper XXI: W. A. Remers and M. J. Weiss, *J. Med. Chem.*, **11**, 737 (1968).

(2) (a) T. Hata, Y. Sano, R. Sugawara, A. Matsumae, K. Kanamori, T. Shima, and T. Hoshi, *J. Antibiotics* (Tokyo), **A9**, 141 (1956); (b) 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); (c) A. C. Dornbush and G. S. Redin, unpublished data.

(3) R. Jones, Jr., U. Jonsson, J. Colsky, H. E. Lessner, and A. Franzino in "Fourth National Cancer Conference Proceedings, 1960," J. B. Lippincott, Philadelphia, Pa., 1961, p 175; B. Sokoloff, *et al.*, *Growth*, **23**, 109 (1959); L. H. Manheimer and J. Vital, *Cancer*, **19**, 207 (1966); M. Kutsumi, *Wakayama Med. Rept.*, **9**, 153 (1965).

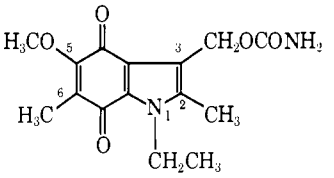
(4) J. S. Webb, D. B. Cosulich, J. H. Mowat, J. B. Patrick, R. W. Broschard, W. E. Meyer, R. P. Williams, C. F. Wolf, W. Fulmor, C. Pidacks, and J. E. Lancaster, *J. Am. Chem. Soc.*, **84**, 3185, 3187 (1962); see also A. Tulinsky, *ibid.*, **84**, 3188 (1962).

(5) J. B. Patrick, R. P. Williams, W. E. Meyer, W. Fulmor, D. B. Cosulich, R. W. Broschard, and J. S. Webb, *ibid.*, **86**, 1889 (1964).

(6) G. R. Allen, Jr., J. F. Poletto, and M. J. Weiss, *ibid.*, **86**, 3877 (1964); *J. Org. Chem.*, **30**, 2897 (1965).

chloride. However, despite its marked *in vitro* activity against the tetracycline-resistant *S. aureus*, strain Rose, and *Streptococcus pyogenes*, β-hemolytic strain C203, 7-methoxymitosene (II) was not effective *in vivo* against these organisms.[6]

The discovery of significant antibacterial activity for II was then rationally followed by the synthesis of the indoloquinone analog III, which had a spectrum and level of activity about the same as that observed for II.[7]



III

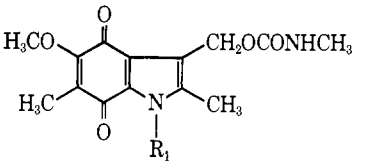
Encouraged by these results,[8] we undertook an extensive analog program in the indoloquinone series, which was chosen in preference to the pyrroloindole series (see II) for convenience of synthesis. A considerable number of pertinent analogs were prepared and the structure–activity relationships which can be drawn from this study are discussed below. In this discussion, activities, unless otherwise noted, were obtained from an *S. aureus*, strain Smith, assay[9] in mice (subcutaneous treatment). Those compounds active by the subcutaneous route, which were also submitted to the corresponding oral assay, were invariably active in the latter assay as well.

Variations at N_1.—Optimum activity was recognized at this position with either the methyl or ethyl substituent, the activity decreasing when these substituents were replaced with propyl, isopropyl, or butyl groups (see Table I).[10] Substitution at this site with a series of β-substituted ethyl groups ($-CH_2CH_2X$: X = F, Cl, N_3, OH, SCH_3)[11] proved unrewarding, although the members of this series were marked by *in vitro* activities nearly as high as that of the corresponding ethyl compound.[11]

Variations at C_2.—Only the original methyl group at C_2 appeared compatible with high potency. Modest activity was noted for the $-CH{=}NNHCONH_2$ group[12] (estd ED_{50} = 128 mg/kg), but the replacement of the methyl group by hydrogen,[10] ethyl,[10] or certain substituted methyl groups ($-CH_2X$: X = OH, OAc, $OCONH_2$, OCH_3, NH_2, SAc, Cl, F)[12] did not give compounds of interest. The poor *in vivo* showing noted for the substituted-methyl derivatives was disappointing because *a priori* these analogs appeared to be especially pertinent since a benzylic-type function at C_2 conceivably affords an additional locus for biological alkylation; furthermore, this site corresponds to that in the parent antibiotics which is most prone to chemical solvolysis[4] of the fused aziridine ring. This possibility is of interest since biochemical evidence indicates that biological alkylation may play an important role in the mechanism by which the mitomycins exert their effect.[13] However, these compounds were among the more active analogs according to the *in vitro* assay.[12]

TABLE I

ACTIVITY OF N_1 VARIANTS IN MICE INFECTED WITH *Staphylococcus aureus*, SMITH



N_1 substituent (R_1)	Median effective dose range (ED_{50}), mg/kg sc
Methyl	16–32
Ethyl[a]	16–32 (16–32 *po*)
Ethyl	16–32
n-Propyl	32–128
Isopropyl	>128
n-Butyl	>128

[a] NH_2 carbamate derivative.

Variations at C_3.—Here, exchange of the carbamoyloxymethyl side chain for hydrogen, methyl, hydroxymethyl,[7b] chloromethyl, methoxymethyl, acetylthiomethyl, formyl,[7b] or acetyl groups gave compounds[14] inactive at the 128-mg/kg dose level (subcutaneous), nor were a variety of carboxylic acid esters[15] of the hydroxymethyl group of any particular interest. Moreover, in contrast to the C_2 series, these analogs, with the exception of the carboxylates, were essentially inactive in the *in vitro* as well as in the *in vivo* assays, although in several instances the new functions also provided good leaving groups, so that in principle a site for biological alkylation was maintained.

Variations of the substituents on the carbamate nitrogen proved more fruitful and it was possible to obtain analogs[15] which showed a retention and even an enhancement of activity. In the N-monoalkyl series, the methyl and propyl members were about as active as the original unsubstituted carbamate III. Of the N,N-dialkyl derivatives, the dimethyl analog was the most interesting, affording substantial activity, which was also observed for the piperazine derivative. Introduction of the phenyl or allyl group gave compounds of relatively modest activity. Of considerable interest was the activity observed following monosubstitution of the carbamate nitrogen by various substituted alkyl groups, particularly by the β-hydroxyethyl group which afforded the most active member of the 5-methoxy and possibly also of the 5-ethylenimino series (see below). Results with the more interesting of the carbamate nitrogen-substituted derivatives are given in Table II.

Variations at C_6.—Peak activity at this position in the quinone ring apparently was obtained with the

(7) (a) G. R. Allen, Jr., J. F. Poletto, and M. J. Weiss, *J. Am. Chem. Soc.*, **86**, 3878 (1964); (b) G. R. Allen, Jr., and M. J. Weiss, *J. Med. Chem.*, **10**, 1 (1967).

(8) Also noteworthy was the observation that, in a bone-marrow depressant assay in mice (see ref. 18) in which the parent antibiotics and I were virulently active, II and III appeared to show, at most, border-line depressant action.

(9) (a) *In vivo* antibacterial assays were carried out according to the procedure of G. S. Redin and M. E. McCoy, "Antibiotics Annual, 1959–1960," Antibiotica Inc., New York, N. Y., 1960, p 213. (b) *In vitro* antibacterial assay data are included in the various synthetic papers of this series.

(10) G. R. Allen, Jr., L. J. Binovi, and M. J. Weiss, *J. Med. Chem.*, **10**, 7 (1967).

(11) G. R. Allen, Jr., and M. J. Weiss, *ibid.*, **10**, 23 (1967).

(12) G. R. Allen, Jr., J. F. Poletto, and M. J. Weiss, *ibid.*, **10**, 14 (1967).

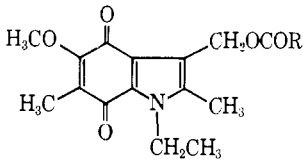
(13) (a) V. N. Iyer and W. Szybalski, *Science*, **145**, 55 (1964); (b) A. Weissbach and A. Lisio, *Biochemistry*, **4**, 196 (1965); (c) for a recent review see W. Szybalski and V. N. Iyer in "Antibiotics," Vol. I, D. Gottlieb and P. D. Shaw, Ed., Springer Verlag, New York, N. Y., 1967, p 211.

(14) Except as noted, for these compounds see J. F. Poletto, G. R. Allen, Jr., and M. J. Weiss, *J. Med. Chem.*, **10**, 95 (1967).

(15) J. F. Poletto, G. R. Allen, Jr., and M. J. Weiss, *ibid.*, **11**, 000 (1968).

TABLE II

ACTIVITY OF CARBAMATE DERIVATIVES IN MICE INFECTED WITH *Staphylococcus aureus*, SMITH



R	ED_{50} range, mg/kg—Subcutaneous	ED_{50} range, mg/kg—Oral
NH_2	16–32	16–32
$NHCH_3$	8–32	64–128
$NHCH_2CH_2CH_3$	32–64	32–64
$NH(CH_2)_3CH_3$	>32	*Ca.* 128
$N(CH_3)_2$	16–32	16–64
$N(CH_2CH_3)_2$	>128	
	8–32	32–128
	>32	
	>128	
$NHCH_2CH{=}CH_2$	*Ca.* 128	64–128
$NHCH_2CH_2OH$	4–8	8–32
$NHCH_2CH_2CH_2OH$	>128	
$NHCH_2CH_2OCH_2CH_3$	32–128	
$NHCH_2CH_2CN$	32–128	

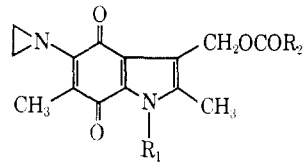
original methyl group, since potency was diminished when this group was replaced by hydrogen[16] (N-propylcarbamate, estd ED_{50} = 32–128 mg/kg sc) or ethyl[10] (N-methylcarbamate, estd ED_{50} > 128 mg/kg sc).

Variations at C_5.—Homologation of the 5-methoxy function to ethoxy[1] in one instance (N-unsubstituted carbamate) resulted in at least a lowering of activity, but in another instance (N-methylcarbamate) equivalent activity was noted. The reverse of this situation obtained in the 5-amino series[1] wherein substitution of methoxy by the primary amino group afforded a compound of equivalent activity for the N-unsubstituted carbamate and a compound of little or no activity for the N-methylcarbamate. Several substituted amino derivatives were prepared,[1] but none of these, with the exception of the benzylamino analog (N-methylcarbamate, estd ED_{50} = 32–128 mg/kg sc) was of interest. Substitution of the 5-methoxy group by hydrogen[17] gave a compound of lessened potency (N-methylcarbamate, estd ED_{50} ~ 128 mg/kg sc), and substitution by methyl[17] or chlorine[1] (good leaving group) gave compounds of no particular importance.

The 5-Ethylenimino Series.—Maximum activity was observed when the methoxy group was replaced by the ethylenimino group.[1] This exchange afforded several compounds with oral activity *vs.* the tetracycline-resistant *S. aureus*, strain Rose, and also *S. pyogenes* C203 (see Table III),[9a] a significant forward step since even the most potent members of the 5-methoxy series (also 5-amino), according to the *Staphylococcus* Smith assay, were ineffective *in vivo* against these two organisms. Introduction of the 2-methylethylenimino group (N-β-hydroxyethylcarbamate) gave a compound[1] which was about equipotent against *Staphylococcus* Rose when administered subcutaneously (estd ED_{50} = 4–16 mg/kg), but which suffered a much greater loss of its effectiveness than did the corresponding ethylenimino derivative IV when administered orally (estd ED_{50} > 128 mg/kg).

TABLE III

ACTIVITY OF 5-ETHYLENIMINO DERIVATIVES AGAINST BACTERIAL INFECTIONS IN MICE



R_1	R_2	ED_{50} range, mg/kg—Subcutaneous—*Staph.* Rose	*Staph.* Smith	*Strep.* C203	Oral *Staph.* Rose
Ethyl	$NHCH_2CH_2OH$	5	10	20	42
Ethyl	$NHCH_3$	4–8	9	20	32–64
Ethyl	$NHCH_2CH_3$	4–16			32–64
Methyl	$NHCH_3$	16–64			>64
Propyl	$NHCH_3$	32–64			>64
Isopropyl	$NHCH_3$	Inact. 64			
Ethyl	NH_2	Inact. 64	>32	>64	
Ethyl	$NHCH_2CH_2CH_3$	>64			
Ethyl		16–64			>64

Of the 5-ethylenimino series, the N-methyl-, N-ethyl-, and N-hydroxyethylcarbamate derivatives were the most potent members, and of these, the last compound (IV) was chosen, more or less arbitrarily, for further study. It was found to have substantial oral activity in mice when tested against the representative gram-positive organisms, *Diplococcus* SVI, *Streptococcus* C203, *Staphylococcus* Smith, as well as the aforementioned *Staphylococcus* Rose, but was essentially inactive by the subcutaneous route *vs.* the gram-negative *Escherichia coli* and *Proteus mirabilis* (see Table IV). This activity is comparable to that found in our laboratory for the clinically useful antibiotic novobiocin, a comparison with which is provided in Table IV.

TABLE IV

COMPARISON OF 5-ETHYLENIMINO N-β-HYDROXYETHYLCARBAMATE IV WITH NOVOBIOCIN IN BACTERIAL INFECTIONS IN MICE

Organism	Estd ED_{50}, mg/kg—Subcutaneous—IV	Subcutaneous—Novobiocin	Oral—IV	Oral—Novobiocin
S. aureus, strain Smith	10	10	40	20
S. aureus, strain Rose	5	15	40	40
S. pyogenes, β-hemolytic, strain C203	20	320	50	<320
D. pneumoniae, type 1, strain SVI	20	200	60	...
E. coli 311	Inact. 128	...	...	...
P. mirabilis	Inact. 128	60	...	300

Further studies with IV indicate a bactericidal action when tested *in vitro* against *S. aureus* ATCC 6538P. After a 16-hr incubation period, half-maximum inhibition was achieved at a concentration of 0.65 μg/ml. At the lowest concentration at which no growth

(16) W. A. Remers and M. J. Weiss, *J. Am. Chem. Soc.*, **88**, 804 (1966).
(17) R. H. Roth, W. A. Remers, and M. J. Weiss, *J. Org. Chem.*, **31**, 1012 (1966).

TABLE V

INCREASE IN RESISTANCE TO IV AND MITOMYCIN C

Organisms	Initial MIC, μg/ml	Fold increase in resistance — No. of transfers: 1	2	3	4	5	7	9	11	13	15	17	19
Compound IV													
S. aureus Smith	1.25	8	16	64	125	125	125	125	125	125	250	125	
S. aureus Rose	2.5–5.0	2	16	64	64	32	64	64	64	64	125	125	125
S. pyogenes NY5	1.25–2.5	4	16	32	64								
S. pyogenes C203	0.62–1.25	8	8	16	16	32	32	250	125	250	500	250	
Mitomycin C													
S. aureus Smith	0.16–0.31	2	2	4	4	4	4	16	32	250	125	500	
S. aureus Rose	0.16–0.31	2	2	4	4	4	4	4	8	8	16	16	16
S. pyogenes NY5	0.04	0	0	2	2	16	32	32	64	125	250	500	500
S. pyogenes C203	0.02	2	2	0	2	2	4	8	4	32	32	500	2000

TABLE VI

TOXICITY OF COMPOUND IV IN MICE

Dose, mg/kg	Acute toxicity, dead/total[a] — Subcutaneous	Oral	Marrow nucleated cell count, controls/treated[b] — Subcutaneous	Oral
512	2/2	2/2	9.2	2.2
256	1/2	1/2	6.3	2.0
128	0/2	0/2	2.1	1.0
64	0/2	0/2	1.3	1.1

[a] Fourteen days after administration of IV. [b] The count was made on the third day after administration of IV; two mice per group. The femoral marrow was used.

was apparent, 2.5 μg/ml, more than 97% of the microorganisms introduced with the inoculum were no longer viable. At higher concentrations the bactericidal activity was even more pronounced.

A study[18] of resistance development by selected organisms to compound IV and mitomycin C was also carried out (see Table V). Toward IV, resistance developed more rapidly than toward mitomycin C, and a plateau was reached at about the fourth or fifth transfer for the staphylococci and at about the ninth transfer for *S. pyogenes* C203. In contrast, the resistance developed to mitomycin C began to increase in a stepwise pattern for three of the organisms ranging from the sixth to thirteenth transfer, whereas for *S. aureus* Rose there was only a 16-fold increase at the end of the 19th transfer. Note, too, that the initial minimal inhibitory concentrations for IV were about ten to fifty times as great as that observed for mitomycin C.

A preliminary acute toxicity determination with IV and a bone-marrow depression study[19] gave the results shown in Table VI.

Gross examination of the contents of the abdominal cavity revealed gelatinous, toneless intestines in the dead mice. However, all survivors appeared normal and, upon sacrifice, gross examination of the abdominal cavity contents revealed nothing unusual. According to the data of Table VI, bone-marrow depression would appear to be definitely established by the subcutaneous route. However, the oral data, in view of the limited number of animals, is of questionable significance. In any case, these toxicity manifestations are sufficient to preclude any interest in IV as a possible clinical antibacterial agent. Thus, the development of a useful antibacterial agent based upon the mitomycin class of antibiotics, one of the most potent groups of orally effective antibacterial substances known, remains an achievement yet to be accomplished.

The antibacterial activity manifested by the various indoloquinone carbamates leads to an observation of some interest to the question of the biochemical mechanism by which the mitomycins exert their biological effect. There is accumulated evidence that these antibiotics can cross-link DNA and that this ability to cross-link depends upon an initial biological reduction.[13] In the mitomycins the two alkylating sites required for cross-linking can be conceived as provided by the aziridine and the carbamoyloxymethyl functions; a third possibility, the methoxy- or amino-substituted carbon in the quinone ring, is inactivated by reduction to the hydroquinone state. It is, of course, quite possible that the antibacterial activity, as well as the toxicity manifestations, of these synthetic analogs and the mitomycins does not result from a common mechanism, or, if it does, this mechanism does not involve a cross-linking process. However, in the event of a common cross-linking mechanism, the observed activity of the desaziridino synthetic analogs requires that, in the mitomycins, the aziridino function, usually considered the biological alkylating group *par excellence*, in fact does not participate in the cross-linking process. Cross-linking still could be achieved by use of the carbamate and quinone sites. However, inasmuch as an initial biological reduction is required,[13,20] a subsequent biological reoxidation, presumably after alkylation by the carbamate function, would then be necessary in order to reestablish the quinone site. Unfortunately, we can offer no biochemical evidence concerning these speculations.

Finally, we would note that a broad selection of the indoloquinone carbamates prepared in the course of this study was submitted to an antitumor assay[21] using the 72j mammary adenocarcinoma in C_3H mice. In view of the important antitumor properties manifested by mitomycin C, it was disappointing that none of the indoloquinones could be found active at a nontoxic dose.

Acknowledgment.—We wish to acknowledge the support and encouragement of Drs. J. J. Denton and J. M. Smith, Jr., throughout the course of this investigation.

(18) For the assay procedure see E. J. Kirsch, A. C. Dornbush, and E. J. Backus, ref 9a, p 205.

(19) For the assay procedure see A. W. Vogel, *Cancer Res.*, **21**, 636 (1961).

(20) H. S. Schwartz, J. E. Sodergren, and F. S. Philips, *Science*, **142**, 1181 (1963).

(21) A. W. Vogel and J. D. Haynes, *Cancer Chemotherapy Rept.*, **No. 22**, 23 (1962).