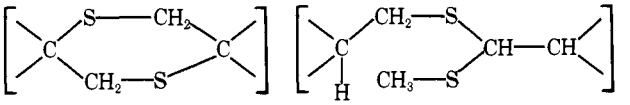
Structure Revision of the Antibiotic Echinomycin

Anne Dell, Dudley H. Williams,* Howard R. Morris, Gerald A. Smith, James Feeney, and Gordon C. K. Roberts

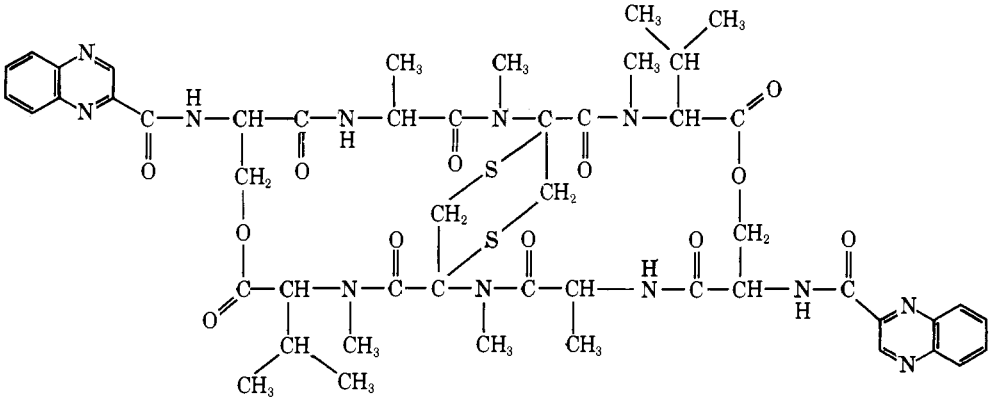
Contribution from the University Chemical Laboratory, Cambridge CB2 1EW, England, and the National Institute for Medical Research, London N.W.7, England. *Received August 6, 1974*

Abstract: In the light of evidence based on ^1H and ^{13}C nuclear magnetic resonance experiments, and on electron impact and field desorption mass spectrometry, the structure of the antibiotic echinomycin has been revised. The hitherto accepted structure incorporated a dithian cross-link (i) which is now modified to a thioacetal cross-link (ii). Since the structures of quinomycins B_0, C, D, B, and E are based on analogy to the behavior of echinomycin (quinomycin A), the nature of the cross-link in these antibiotics should presumably be modified in the same way.



i ii

In 1959, structure **1** was proposed for the antibiotic echinomycin, largely on the basis of chemical degradation experiments, and on the basis of a molecular formula derived from microanalysis.[1] Structure **1** has been accepted since the original proposal, and indeed, five related antibiotics (quinomycins B_0, C, D, B, and E) have been assumed[2] to contain the dithian ring proposed for echinomycin.



1

We were first led to question the proposed structure for echinomycin upon examination of its NMR spectrum (Figure 1). A feature which is immediately striking is the presence of two nonequivalent protons (due to the 3 H's of the quinoxaline rings), indicating that the antibiotic does not have a symmetrical structure. In addition a strongly coupled doublet (J = 15 Hz) is present at δ 3.40 ppm; this proton is coupled (J = 15 Hz) to a proton centered at δ 2.86 ppm and, upon irradiation of the latter proton, the doublet centered at 6.1 ppm is collapsed to a broad singlet (Figure 1, superimposed trace A). In the light of the coupling constant and chemical-shift data, we conclude that there is present in echinomycin a -CHH'-C'HX system (J_{gem} = 15 Hz) other than those associated with the serine residues (since all the CH's of the serine residues would be anticipated to resonate in the δ 4.5–5.2 region).[3]

We therefore decided to undertake a detailed analysis of the echinomycin structure, using spin decoupling, shift reagents, deuterium-exchange experiments, and mass spectrometry. Although most of the structural units in the proposed structure **1** for echinomycin are based on sound chemical evidence, the NMR experiments on the intact molecule should be able to confirm (or otherwise) that no unusual chemical changes occurred in the degradation experiments.

The results of some spin-decoupling experiments on the unshifted spectrum (Figure 1) are summarized in Table I. Some additional assignments that could have been made at this stage are reserved for the discussion of shifted spectra. The assignments given in Table I merit brief comment. First, the valine α-CH's are clearly evident in Figure 1 [δ 5.08 (J = 10 Hz), 5.11 (J = 10 Hz)], and the sharpness of the doublets confirms N-methylation of the valine residues as in **1**. Second, the assignment of the overlapping doublets at δ 7.99 and 8.08 to benzene ring protons is confirmed by additional evidence to be given subsequently. Third, the collapse of the doublet centered at δ 6.46 to a singlet upon irradiation at δ 4.96 (see Figure 1, superimposed trace B) indicates the presence of a W-CH-CH-Z unit (where W and Z are electronegative groups) in echinomycin, and this unit is not accounted for in structure **1**.

The spectrum of echinomycin was now spread out by the sequential addition of Eu(fod)$_3$. After the addition of approximately 0.25 mol equiv of Eu(fod)$_3$, the δ 8.2–11.0 region of the spectrum appeared as shown in Figure 2a; the two low-field singlets are the signals associated with the 3 H's of the quinoxaline ring. The six remaining doublet signals were assigned as follows. The doublets centered at δ 8.6 and 8.84 ppm could be assigned to the benzene ring protons identified in Table I by following their shifts during stepwise addition of Eu(fod)$_3$. The doublet centered at δ 10.08 is the signal shifted from δ 8.58 in the normal spectrum, from which it can be removed upon addition of CF_3COOD.

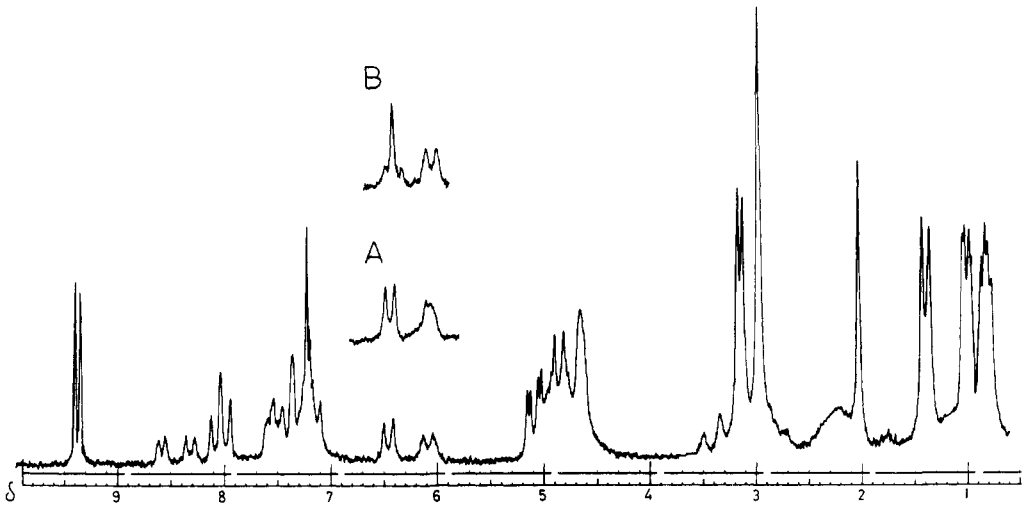


Figure 1. ^{1}H NMR spectrum of echinomycin (~0.1 *M*) in $CDCl_3$ solution. Superimposed trace A recorded with double irradiation at δ 2.86 ppm. Superimposed trace B recorded with double irradiation at δ 4.96 ppm.

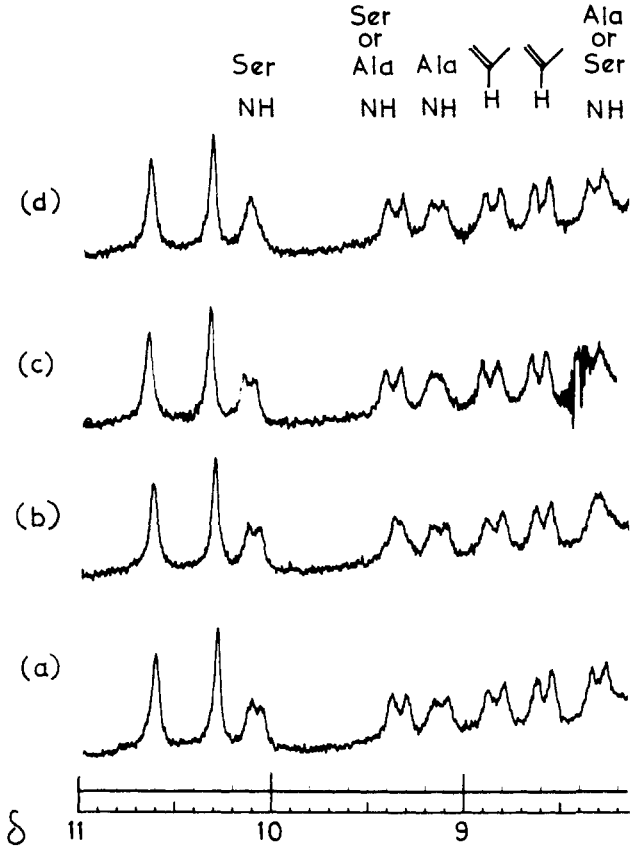


Figure 2. ^{1}H NMR spectrum (δ 8.2–11.0 ppm region) of echinomycin in $CDCl_3$ after addition of approximately 0.25 mol equiv of $Eu(fod)_3$: (a) normal spectrum; (b) with double irradiation at δ 6.23 ppm (Ala NH and Ser NH decoupled); (c) with double irradiation at δ 7.51 ppm (Ala NH decoupled); (d) with double irradiation at δ 7.10 ppm (Ser NH decoupled).

In the shifted spectrum, double irradiation at δ 7.10 (of a one proton signal originally occurring in the δ 4.6–5.2 region of Figure 1) collapses the δ 10.08 doublet to a singlet (Figure 2d) without collapsing either of the now nonequivalent alanine CH_3 doublet resonances; this signal is therefore associated with a serine NH. Double irradiation at δ 7.51 collapses the δ 9.12 doublet to a singlet (Figure 2c) while simultaneously collapsing an alanine CH_3 doublet (at δ 2.6 ppm in the shifted spectrum) to a singlet. Finally, double irradiation of 2 protons centered at δ 6.23 in the shifted spectrum simultaneously collapses the doublets centered at δ 9.44 and 8.30 to singlets (Figure 2b) and, in the same experiment, the remaining alanine CH_3 doublet (at δ 2.10) is collapsed to a singlet. This experiment, coupled with deuterium exchange experiments, allows the two collapsed dou-

Table I. Structural Deductions from Spin-Decoupling Experiments on the NMR Spectrum of Echinomycin

Irrad freq, Hz	Protons irrad (number)	Resulting assignments[a]
230	Isopropylmethines (2)	4 nonequivalent valine CH_3 groups in δ 0.7–1.1 region 2 nonequivalent valine α-CH's at δ 5.08 and 5.11
756	Benzene ring (2)	2 benzene ring protons (peri to nitrogen) at δ 7.99 (d, J = 8 Hz) and 8.08 (d, J = 8 Hz)
496	>CH–X (1)	W–CH–CH–Z (see text; unit not evident in structure 1)

[a] d = doublet.

Table II. Assignment of the Spectrum (Figure 1) of Echinomycin[a]

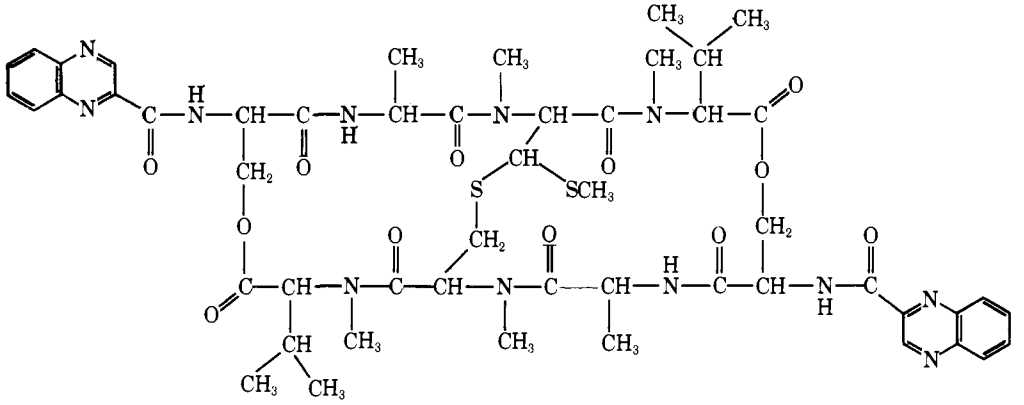
Signal, δ	No. of protons	Assignment
0.8–1.1	12	4 nonequiv Val CH_3
1.3–1.5	6	2 nonequiv Ala CH_3
2.04	3	$-X-CH_3$[b]
2.24	2	2 Val $-CH(CH_3)_2$
2.86	1 (J = 9 and 15 Hz)	$-CHH'-CH-Y$[b]
3.00	6	$2NCH_3$
3.14	3	$N-CH_3$
3.18	3	$N-CH_3$
3.40	1 (J = 15 and ⩽ 4 Hz)	$-CHH'-CHY$[b]
4.6–5.0	6	2 Ser $>CHCH_2O$
4.7–4.9	2	2 Ala–NH–CHCO
4.96	1 (J = 9 Hz)	$W-CH-CHZ$[b]
5.08	1 (J = 10 Hz)	Val CH_3NCHCO
5.11	1 (J = 10 Hz)	Val CH_3NCHCO
6.10	1 (J = 9 and ⩽4 Hz)	$-CHH'-CH-Y$[b]
6.44	1 (J = 9 Hz)	$W-CH-CH-Z$[b]
	6	Arom ⩾CH
7.1–7.6	1	Ala NH
	1	Ser or Ala NH
7.24		$CHCl_3$ (solvent)
7.99	1 (J = 8 Hz)	2 arom ⩾CH
8.08	1 (J = 8 Hz)	
8.32	1 (J = 8 Hz)	Ser or Ala NH
8.58	1 (J = 6 Hz)	Ser NH
9.36	1	2 nonequiv
9.41	1	

[a] W, X, Y, and Z represent electronegative groups which do not carry directly attached protons. [b] Unit not present in structure 1.

blets of Figure 2b to be assigned to serine and alanine NH's (when taken in conjunction with the chemical evidence).[1]

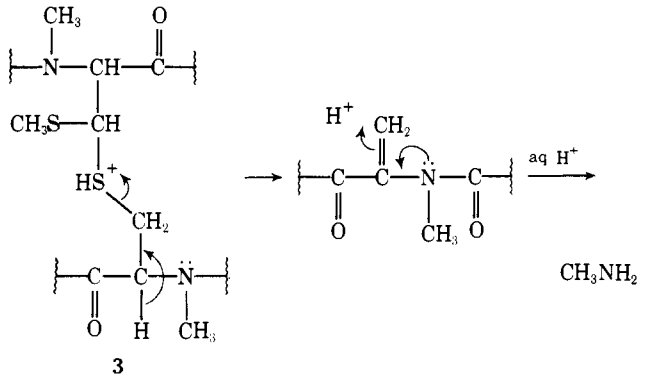
As a result of further related decoupling and shift experiments and integration of spectra (both in $CDCl_3$ and CF_3COOD solutions), the NMR data and chemical evidence allow complete assignment of the spectrum (Figure 1) as indicated in Table II.

On the basis of the NMR evidence, the modified structure **2** can be proposed for echinomycin. First, it is clear



2

that echinomycin must have a structure which can account for the nonequivalence of the two halves of the molecule in the NMR spectrum. Second, structure **2** incorporates the new units -CHH'-CH-Y, W-CH-CH-Z, and X-CH_3 (Table II) which bridge the two peptide chains in place of the dithian ring of **1**. The NMR spectrum establishes the absence of the two -C-CH_2-S units of structure **1**. The chemical evidence which led to the proposal of two -CH_2-S-C-NCH_3 units (see **1**) in echinomycin was the isolation of 1.54 mol of volatile bases per mole of echinomycin; these bases were identified as methylamine plus a trace of ammonia.[1] However, from echinomycin and five closely related antibiotics, Otsuka and Shoji[2] isolated as volatile bases, ammonia (0.33-0.47 mol) and methylamine (0.93-1.25 mol; mean value 1.12 mol). Thus the data seem to be at least as well satisfied by a structure **2** which can afford only 1 mol of methylamine upon acid hydrolysis (see **3**).



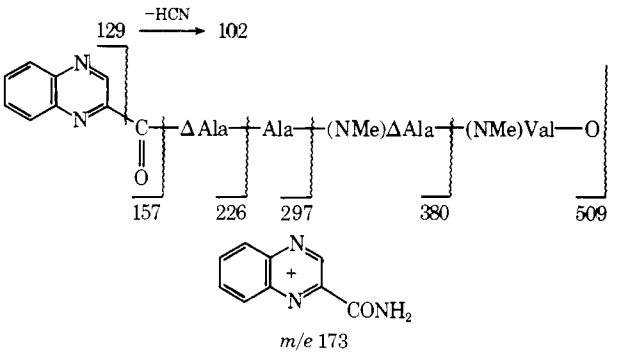
3

In addition, it is noteworthy that acid hydrolysis of the product of desulfurization of echinomycin with Raney nickel (desthioechinomycin) gives L-*N*-methylalanine.[1] The production of an optically active amino acid suggests that the asymmetric CH_3N-CH-C(=O)- group is present in echinomycin since Raney nickel desulfurization is generally accepted to proceed through radical intermediates. Hence the production of racemic *N*-methylalanine would seem more likely from **1**.

The data from microanalysis[1] of echinomycin are closely satisfied by structure **2** (cf. Anal. Calcd for **1**: C, 55.34; H, 5.57; N, 15.49; S, 5.91. Calcd for **2**: C, 55.62; H, 5.86; N, 15.27; S, 5.82. Found: C, 55.45; H, 5.88; N, 15.33; S, 5.27), whose molecular formula ($C_{51}H_{64}O_{12}N_{12}S_2$) is greater by CH_4 than that originally proposed. A critical test of our proposed structure would therefore be the determination of the molecular weight of echinomycin. Conventional electron impact methods of ionization of echinomycin failed to give a molecular ion; neither were useful spectra obtained following derivatization of echinomycin using dimethyl sulfoxide anion-methyl iodide in a 1 min reaction (despite the fact that this derivatization technique is frequently successful for peptides incorporating sulfur-containing amino acids).[4] However, when echinomycin was introduced in chloroform solution onto a 10 μm diameter tungsten wire (previously conditioned with benzonitrile), and a field desorption mass spectrum[5] obtained after introducing the wire into the spectrometer via a vacuum lock, only a molecular ion peak was obtained in the high-mass region. The *m/e* value of the molecular ion was determined as 1100.421 by peak matching against 2,4,6-triperfluoroheptyltriazine, in agreement with a molecular formula $C_{51}H_{64}O_{12}N_{12}S_2$.

Following this determination, a complete spectrum of echinomycin was obtained using a modified electron impact technique[6] in which the sample is evaporated close to the electron beam. The spectrum produced (Figure 3) is discussed below using the abbreviation Quin for the quinoxaloyl moiety, the normal amino acid codes, and ΔAla to indicate dehydroalanine. The low-mass region of the spectrum is dominated by fragments attributable to the quinoxaline moiety (*m/e* 102, 129, 157, and 173). The proposed sequence of amino acids between the aromatic residue and the cross-link is confirmed (Scheme I) by signals (due to

Scheme I



m/e 173

cleavage of peptide bonds, with or without hydrogen rearrangement) at *m/e* 226 (Quin-ΔAla), *m/e* 269, 268 (Quin-ΔAla-Ala minus CO), and *m/e* 297, 296 (Quin-ΔAla-Ala). The signal at *m/e* 310 is unassigned. Beyond this point, the signal at *m/e* 509, assigned to Quin-ΔAla-Ala-(NMe)ΔAla-(NMe)Val-O, corresponds to the bottom part of structure **2** derived by cleavage at the lactone linkages and the

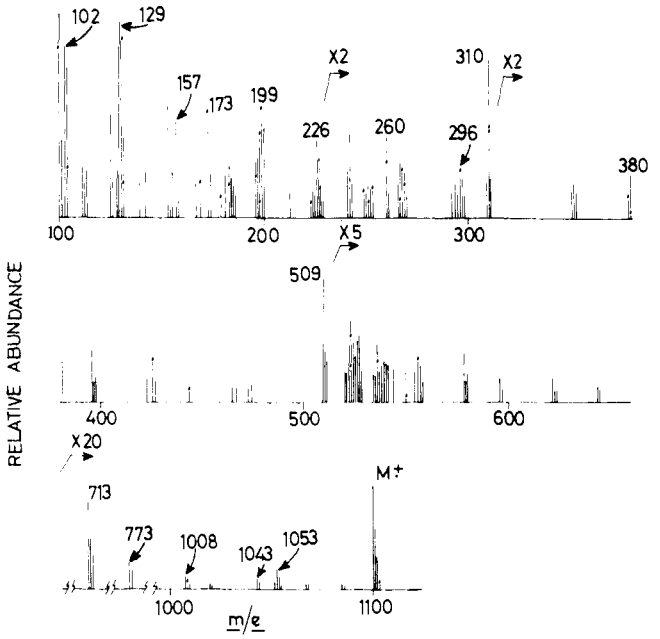


Figure 3. Electron impact mass spectrum (70 eV) of echinomycin (evaporation near the electron beam).

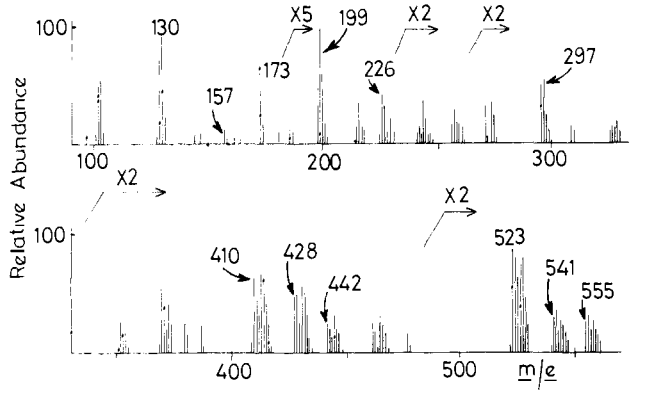


Figure 4. Electron impact mass spectrum (70 eV) of the product obtained by successive treatment of echinomycin with: (a) 0.04 *M* NaOH in methanol;[1] and (b) CH_3OH-CD_3OH-HCl.

cross-link. The less abundant signal at *m/e* 380 is consistent with further breakdown of this fragment at the (NMe)ΔAla-(NMe)Val amide bond. Finally, the molecular weight of intact echinomycin is reaffirmed by the presence of a molecular ion at *m/e* 1100; *m/e* 1053 is significant since it most probably arises by expulsion of $CH_3S\cdot$ from the molecular ion.

Further confirmation of structure **2** was sought by cleaving the lactone ring with 0.04 *M* NaOH in methanol.[1] The free acid was then esterified with methanolic hydrogen chloride, using equal quantities of methanol and methanol-d_4 in order to facilitate identification of fragments containing the C-terminal amino acid residue.[7] The mass spectrum of the product is shown in Figure 4, the most notable feature being the groups of signals between *m/e* 410 and 445 and *m/e* 523 and 558, all of which contain doublet patterns indicative of d_3-labeled fragments. It is also noted that the cross-link has not remained intact since the presence of two carboxyl groups would be indicated by a triplet signal.[7]

The signals at *m/e* 524, 527 correspond to the signal at *m/e* 509 in the echinomycin spectrum (Figure 3), i.e., Quin-ΔAla-Ala-(NMe)ΔAla-(NMe)Val-OMe. The signals at *m/e* 542 and 556 are respectively 18 and 32 mass units higher than *m/e* 524 and indicate Michael addition of water and methanol to the molecule, probably during the basic

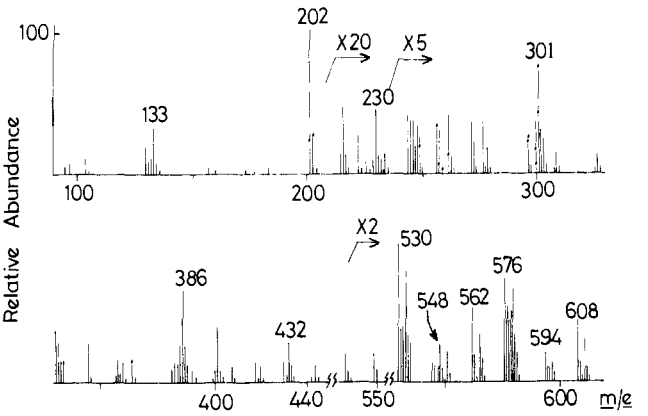
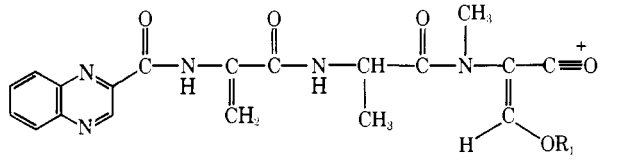


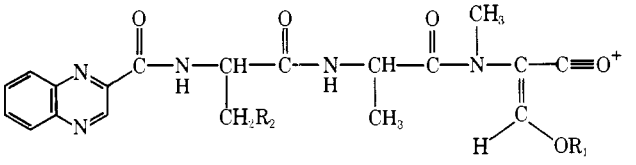
Figure 5. Electron impact mass spectrum (70 eV) of the product obtained by successive treatment of echinomycin with: (a) 0.04 *M* NaOH in mehanol;[1] (b) CH_3OH-CD_3OH-HCl; (c) Ni_2B-MeOH.

hydrolysis, after generation of the α,β-unsaturated amide. This was confirmed by hydrolysis with 0.04 *M* NaOH in a 1:1 mixture of methanol-methanol-d_4. The mass spectrum of the ester showed signals at *m/e* 524, 542, 556, and 559 confirming the above conclusion. The signals at *m/e* 410, 413, 428, 431, 442, and 445 are related in an analogous manner but do not appear in the mass spectrum of a product in which the carboxylic acid group of the valine residue is esterified with diazomethane. We conclude therefore that these ions originate from the product of an acid-catalyzed modification of the cross-link and do not contain the C-terminal methoxyl group of the valine residue. The peaks in fact appear to be due to the fragments iii and iv. Formation



iii

($R_1 = OCH_3, OCD_3$; *m/e* 410, 413)



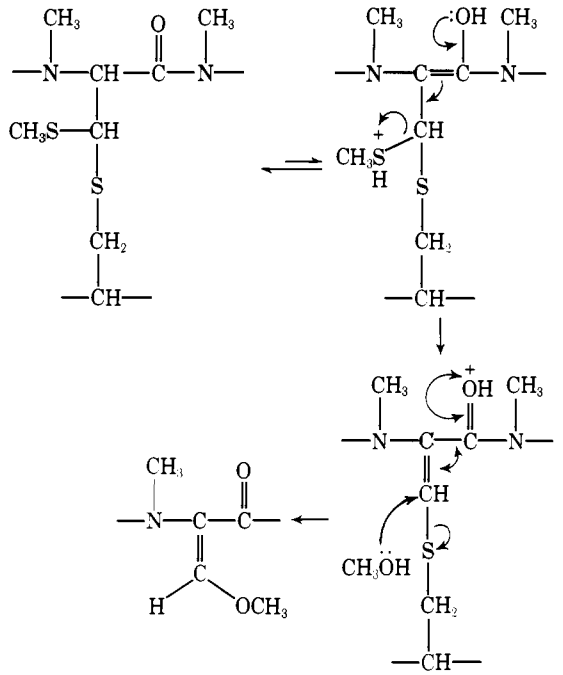
iv

$R_1 = OCH_3, OCD_3$
$R_2 = OH$; *m/e* 428, 431
$R_2 = OCH_3$; *m/e* 442, 445

of these products can be simply rationalized from our proposed structure **2** as shown in Scheme II.

Further structurally informative spectra were obtained following reaction of the diester with nickel boride[8] in methanol which reductively cleaves the cross-link and reduces the aromatic nuclei to tetrahydroquinoxalines. Fragments containing methoxy residues are immediately obvious in the spectrum (Figure 5), and again the products before and after Michael addition can be recognized via signals at *m/e* 530, 533; 548, 551; 562, 565; and *m/e* 576, 579; 594, 597; 608, 611. The presence of two distinct sets of Michael addition products due to addition of water and methanol to the products associated with *m/e* 530, 533 and *m/e* 576, 579 strongly suggests an unsymmetrical cross-link which has cleaved to give two separate chains differing by 46 mass units (RH vs. $RSCH_3$) upon reduction. The major signals in the spectrum are assigned as shown in Scheme III. These

Scheme II



experiments give independent support to the nature of the cross-link and to the sequence of amino acids in the antibiotic.

Lastly, further evidence in support of the revised structure **2** is provided by ^{13}C magnetic resonance spectroscopy. Figure 6 shows the high-field region of the proton noise decoupled ^{13}C spectrum of echinomycin at 25.2 MHz. In the high-field region, there are signals corresponding to 25 aliphatic carbon atoms (the original structure of echinomycin would only give rise to 24 such carbons). Off-resonance proton-decoupling experiments reveal that none of the aliphatic carbons are quaternary, and that there are three methylene carbons; this is consistent with the proposed new structure for echinomycin but inconsistent with the original structure. The magnetic nonequivalence of the amino acid residues of the same type noted in the ^1H spectrum is even more pronounced in the ^{13}C spectrum. This is seen in Figure 6 and also in the low-field part of the spectrum (not shown) where ten distinct carbonyl signals are observed.

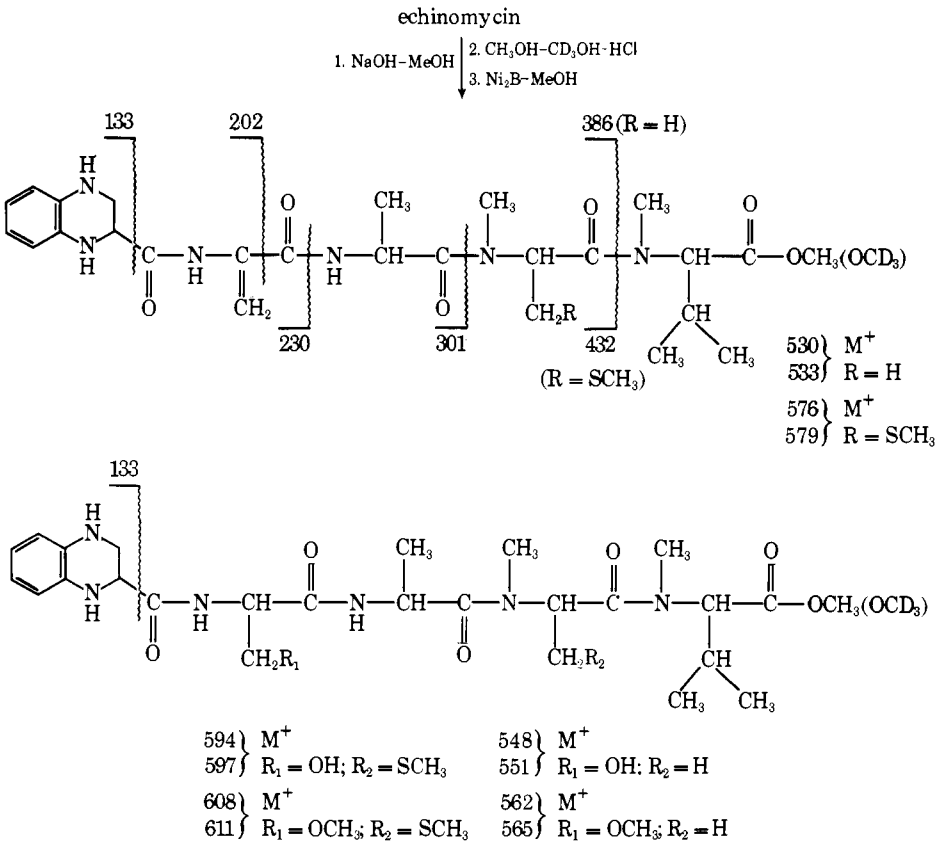
In the CH_3 region of the spectrum (15–21 ppm), there are seven carbons, and the highest field one is fully consistent with a S-CH_3 group (observed shift 15.24 ppm; shift of S-Me in methionine and S-methylcysteine 15.00 ppm). Likewise the methylene signal at 27.16 ppm can be assigned to a S-CH_2 group and the methine carbon signal at 59.93 ppm to the S-CH-S carbon. No ideal model compounds were available to confirm these assignments directly, but empirical ^{13}C chemical-shift calculations based on related compounds and assuming that ^{13}C substituent shielding effects act in an additive manner predict shifts of ~28 and 60 ppm, respectively.

The revised structure **2** seems biogenetically more plausible than **1**, bearing in mind that both echinomycin and the triostin antibiotics are produced from strains related to *Streptomyces aureus*. The nature of the cross-link in the triostin antibiotics appears to be on secure grounds in the light of the isolation of *N,N'*-dimethylcystine from antibiotics of this group (see **4**). Monomethylation of the cross-link, followed by rearrangement (see **5**), therefore provides a facile route from triostin antibiotics to the echinomycin type. Since the structures of quinomycins B_0, C, D, B, and E are based on analogy to the behavior of echinomycin (quinomycin A),[2] the nature of the cross-link in these antibiotics should presumably be modified in the same way.

Experimental Section

^1H NMR spectra were recorded on a Varian HA-100 spectrometer, in deuteriochloroform solution at normal probe temperature

Scheme III



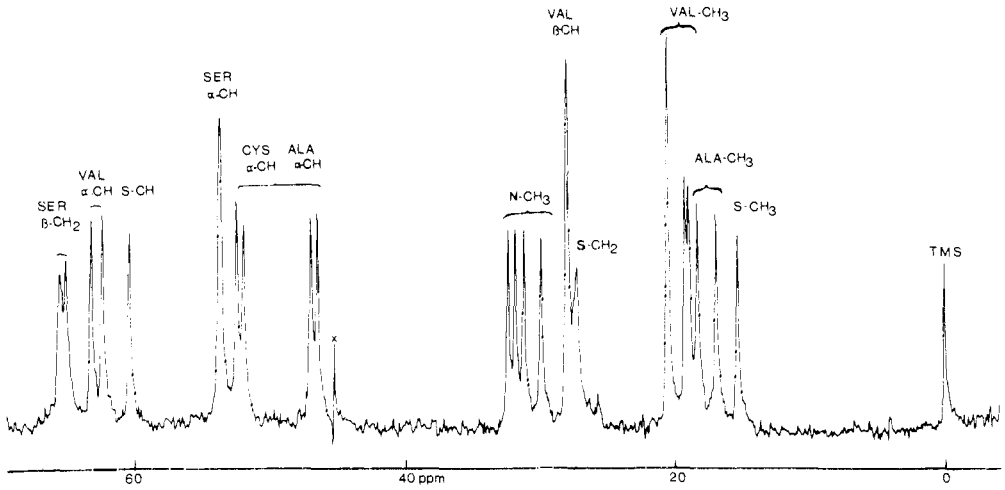
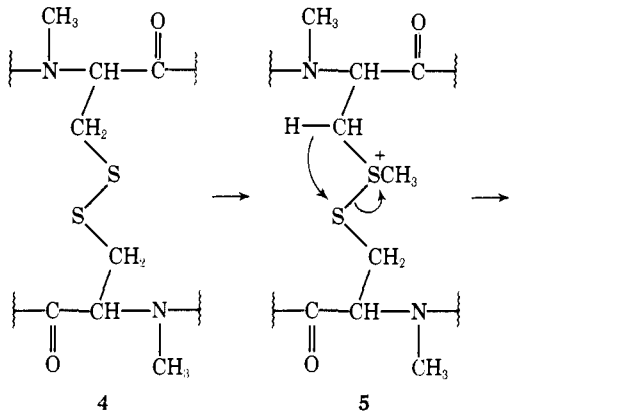
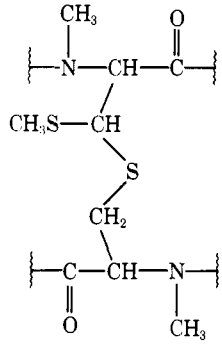


Figure 6. The aliphatic carbon region of the proton noise decoupled ^{13}C spectrum at 25.2 MHz of echinomycin in chloroform solution. The spectrum is the result of 62,500 pulses using an aquisition time of 0.8 sec without a pulse delay.



4 5



unless otherwise stated; spin-decoupling experiments were carried out in the frequency sweep mode. The ^{13}C spectrum was recorded at 25.2 MHz using a Varian XL100 spectrometer equipped with Fourier transform facilities. A solution containing 140 mg/ml of echinomycin in $CDCl_3$ (~1 mg of $Cr(acac)_3$ relaxation reagent was present) was examined. Tetramethylsilane was used as an internal reference.

All mass spectra were determined using an AEI MS 902 double focusing mass spectrometer. Samples for conventional electron impact mass spectrometry were loaded into the cup of the quartz tip of the direct insertion probe and introduced into the mass spectrometer ion source via the standard vacuum lock. Source temperatures of 200–235° were used. To obtain the "in beam" electron impact mass spectrum, the sample was loaded onto the outside of the quartz tip of the direct insertion probe. The probe length had been preadjusted so that the end of the quartz tip just interrupted the electron beam when fully inserted into the ion source. After introduction of the probe into the ion source via the vacuum lock, the tip was gently moved toward the electron beam until interference with the latter was observed. Spectra were then recorded immediately. A source temperature of 250° was used.

Field desorption spectra were obtained at a resolving power of 1200 using a modified AEI EI–FI source equipped with a vacuum lock and probe. The sample was loaded by dipping the emitter into a few μl. of a solution of echinomycin in $CHCl_3$. The echinomycin was found to desorb at an emitter heating current value of 17 mA (the wire being 1 mm from the cathode plate) and an anode–cathode potential difference of 10^4 V. The unit mass of the molecular ion was located by comparison on the oscillograph recorder paper with the fragment ions of trisperfluoroheptyltriazine introduced via the heated inlet system. Following this, the molecular ion was peak matched against *m/e* 1116 in the reference spectrum ($C_{23}N_3F_{42}$) to yield an observed molecular weight of 1100.421 (calcd for $C_{51}H_{64}O_{12}N_{12}S_2$, 1100.420774).

Preparation of Echinomycin Ester. The lactone linkages of echinomycin were cleaved with mild base according to the procedure of Keller-Schierlein et al.[1] The product was taken up in methanolic HCl (pH 1) prepared by bubbling dry HCl into an equivalent volume mixture of methanol and methanol-d_4. After standing at 18° overnight, the volatile components were evaporated to afford the esterified product as a yellow solid.

Ni_2B Reduction of Echinomycin Ester. Ni_2B catalyst (4 g; freshly prepared according to the method of Paz et al.)[8] was added to a methanolic solution (10 ml) of the esterified material (prepared above; 10 mg), and the mixture was shaken at 18° overnight. The catalyst was filtered off and washed (3 × 3 ml) with warm methanol. The combined filtrates were centrifuged to remove a fine suspension of catalyst, and the methanol was evaporated to afford a light-yellow solid which was taken up in the two-phase system ethyl acetate (10 ml)–aqueous EDTA (1g/100 ml) (10 ml). The organic layer was separated and washed with aqueous EDTA (1g/100 ml) (2 × 2 ml), and the solvent was evaporated to afford a dark-yellow oil (7 mg). This was examined by mass spectrometry without further purification.

Acknowledgment. We thank CIBA for a generous gift of echinomycin, the Science Research Council and the Medical Research Council for financial support, and Dr. M. Waring for useful discussions. A.D. thanks the Commissioners for the Exhibition of 1851 for a Research Scholarship.

References and Notes

(1) W. Keller-Schierlein, M. Lj. Mihailovic, and V. Prelog, *Helv. Chim. Acta*, **42**, 305 (1959).
(2) H. Otsuka and J. Shoji, *Tetrahedron*, **23**, 1535 (1967).
(3) The serine β-CH_2 resonances normally occur at δ 3.7–4.1, but the presence of the carbonyl group will cause a downfield shift into the range δ 4.4–4.8 ppm.
(4) H. R. Morris, R. J. Dickinson, and D. H. Williams, *Biochem. Biophys. Res. Commun.*, **51**, 247 (1973).
(5) For an account of this technique, see H. D. Beckey, *Int. J. Mass Spectrom. Ion Phys.*, **2**, 500 (1969); H. D. Beckey, A. Heindricks, and H. U. Winkler, *ibid.*, **3**, app 9 (1970).
(6) H. R. Morris, A. Dell, and M. R. Thompson, unpublished results on peptides and carbohydrates.
(7) E. Hunt and H. R. Morris, *Biochem. J.*, **135**, 833 (1973).
(8) M. A. Paz, A. Bernath, E. Henson, O. O. Blumenfield, and P. M. Gallop, *Anal. Biochem.*, **36**, 527 (1970).