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Liquid chromatographic investigation of quinoxaline antibiotics and their analogues by means of ultraviolet diode-array detection

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SUMMARY

Numerical formats for evaluation of spectral purity and for spectral comparison of ultraviolet diode-array detector data, together with library search routines, were applied to the liquid chromatographic analysis of echinomycin, triostin A and their synthetic and biosynthetic analogues. Samples of monoquinoline and bisquinoline analogues of echinomycin were found to contain echinomycin and the other respective analogue. Triostin A and its undermethylated synthetic analogues, des-N-tetramethyltrioestin A (TANDEM) and [MeCys³,MeCys⁷]-TANDEM, were each composed of two or more components. Triostin A primarily consisted of a major chromatographic component and a minor component with very similar ultraviolet spectral features. TANDEM exhibited three chromatographic components with nearly identical ultraviolet spectral characteristics. Apparent conformational interconversion of at least two forms of the [MeCys³,MeCys⁷]-TANDEM analogue was observed by reversed-phase liquid chromatography. An activation energy of 15 kcal/mol was estimated for the interconversion based upon an Arrhenius plot of the data.

INTRODUCTION

Quinoxaline antitumor antibiotics are produced by several species of *Streptomyces* and are characterized by two planar quinoxaline rings linked by a cross-bridged, cyclic octadepsipeptide. Two families of quinoxaline antibiotics are known, the triostins and the quinomycins, which differ in their respective cross-bridge structure. The quinomycins, of which echinomycin is the most prominent member, contain a thioacetal cross-bridge while the triostins, of which triostin A has been most widely studied, contain a disulfide cross-bridge (Fig. 1). Olsen¹ has reviewed the quinoxaline depsipeptide antibiotics. Their biological activity has been extensively documented by

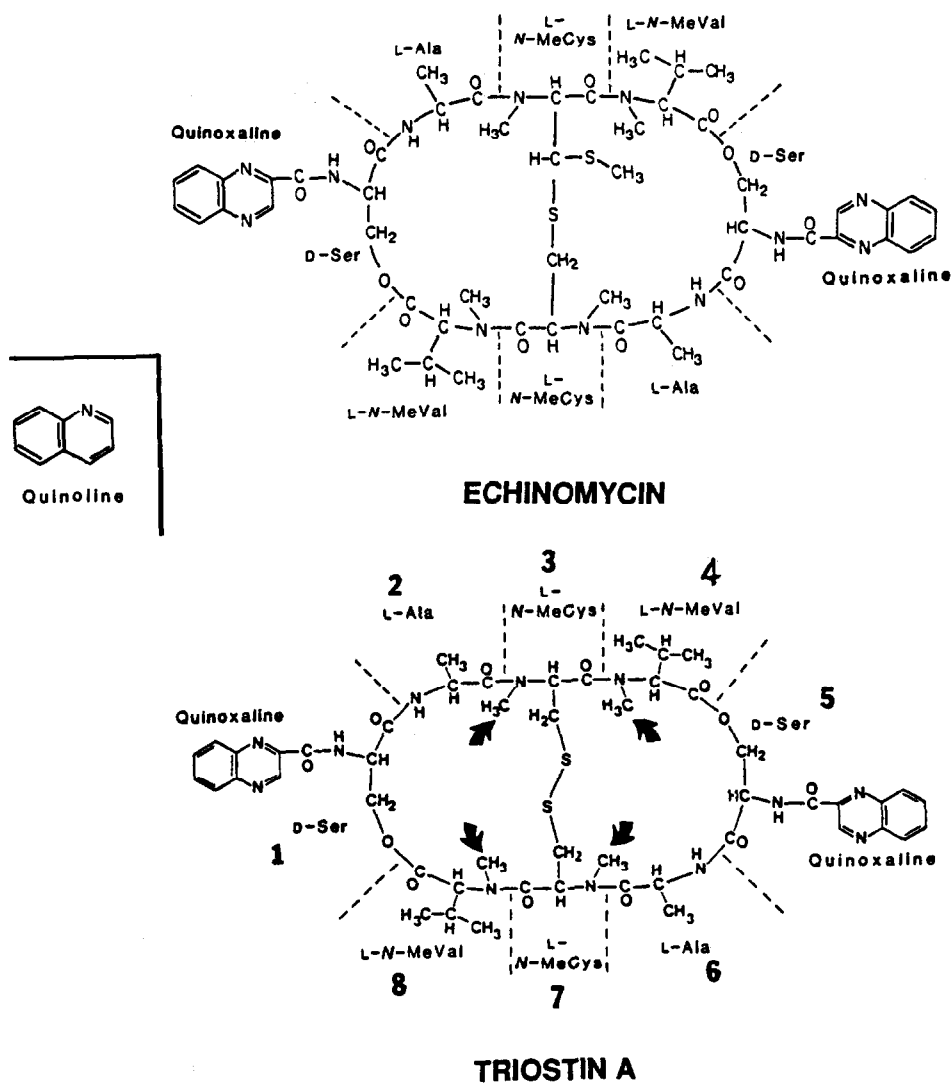


Fig. 1. Structures of echinomycin and triostin A. In the quinoline analogue of echinomycin (1QN) one quinoxaline ring is replaced by a quinoline ring. In the bisquinoline analogue of echinomycin (2QN) both quinoxaline rings are replaced by quinoline rings. The arrows in the triostin A structure represent locations at which the N-methyl groups are replaced by H atoms in the des-N-tetramethyltriostin A (TANDEM) analogue. In the [MeCys³,MeCys⁷]-TANDEM analogue of triostin A, only the N-methyl groups at the 4-MeVal and 8-MeVal positions are replaced by H atoms (see peptide ring numbering scheme for residue positions 1–8).

Katagiri *et al.*² and recent developments with respect to their mode of action have been reviewed by Waring³.

Waring and Wakelin^{4,5} proposed over ten years ago that echinomycin interaction with DNA occurs via a bifunctional intercalation (bisintercalation) mode of binding. Recent crystallographic investigations by Wang and co-workers^{6,7} and

Quigley *et al.*⁸ of the structure of echinomycin and triostin A, complexed with duplex oligonucleotides, have corroborated the earlier findings. These quinoxaline antibiotics were found to bind to the minor groove of the double helix where the quinoxaline rings sandwich a CpG base sequence. Several studies have demonstrated that changes in the peptide ring structure^{9,10} and in the aromatic ring moiety^{11,12} of these antibiotics affect DNA binding specificity. Directed biosynthesis efforts by Fox *et al.*¹³ and Gauvreau and Waring¹⁴ along with synthetic work by Ciardelli *et al.*¹⁵, Chakravarty and Olsen¹⁶, Shin *et al.*¹⁷ and Helbecque *et al.*¹⁸ have yielded a wide variety of depsipeptide drug analogues. Echinomycin and its monoquinoline (1QN) and bisquinoline (2QN) biosynthetic analogues, along with triostin A and its synthetic analogues TANDEM (des-N-tetramethyltriostrin A) and [MeCys³,MeCys⁷]-TANDEM, were investigated in this liquid chromatographic study. The quinoxaline and quinoline rings of the drugs were utilized as intrinsic probes for UV spectroscopic diode-array detection.

UV spectral features of a compound have been exploited by liquid chromatography photodiode-array detectors to confirm peak identification and to determine peak homogeneity. The high spectral acquisition rates of diode-array detectors (up to *ca.* 25 Hz) have been a driving force in the development of computer-aided strategies for signal processing, data reduction and data manipulation. Developments in photodiode-array detection for liquid chromatography and spectral data interpretation techniques have been recently reviewed¹⁹.

Conceptually, the numerical formats for spectral data interpretation employed in this study treat spectra as vectors in order to utilize scalar functions for evaluation and comparison of data sets. The numerical technique employed for evaluation of spectral purity, labeled the purity parameter format, has been described previously^{20,21}. White²² has recently reported a detailed comparison of the purity parameter format with the absorbance ratio technique for spectral discrimination between compounds with similar UV spectra. The purity parameter format makes use of an absorbance-weighted mean wavelength to describe a spectrum by a single number. Absorbance-weighting minimizes the effect of noise in the calculation. The purity parameter value ($\bar{\lambda}_w$) of a spectrum is mathematically defined by the following algorithm:

$$\bar{\lambda}_w(\vec{A})_{(\lambda_i, \lambda_f)} = \frac{\sum_{n=i}^f a_n^2 \lambda_n}{\sum_{n=i}^f a_n^2}$$

where \vec{A} is a spectrum that has absorbance values a_0 at λ_0 , a_1 at λ_1, \dots, a_n at λ_n, \dots , etc. and (λ_i, λ_f) is the wavelength interval included in the calculation. The wavelength range over which $\bar{\lambda}_w$ is calculated can be selected to focus on characteristic spectral features of a chromophore or to enhance discrimination between the spectrum of a compound of interest and that of an impurity.

Spectral comparison was accomplished in this study by treating two spectra as vectors and measuring the differences by means of correlation coefficients [similarity (SIM) and dissimilarity (DISSIM) coefficients] which, together with library search

routines, were used to confirm peak identification. A similar approach was taken by Hill *et al.*²³ who utilized a goodness of fit value in a computerized library search routine for matching UV spectra of unknown drugs to UV spectra of reference drugs.

The SIM and DISSIM coefficients employed in this work for comparison of two spectra can be mathematically defined as follows:

$$\text{SIM}(\vec{A}, \vec{B})_{(\lambda_i, \lambda_j)} = \frac{\sum_{n=i}^j a_n b_n}{\sqrt{\sum_{n=i}^j a_n^2 \sum_{n=i}^j b_n^2}} = \cos \theta$$

$$\text{DISSIM}(\vec{A}, \vec{B})_{(\lambda_i, \lambda_j)} = \sin \theta = \sqrt{1 - \cos^2 \theta}$$

where \vec{A} is a spectrum as defined previously, \vec{B} is a spectrum that has absorbance values b_0 at λ_0 , b_1 at λ_1, \dots , b_n at λ_n, \dots , etc. and θ is the angle between the vectors \vec{A} and \vec{B} .

The SIM coefficient (correlation coefficient) is simply the dot product between two normalized vectors. Excoffier *et al.*²⁴ have demonstrated that for purity determination the DISSIM coefficient is more linearly related to the amount of impurity present, and thus offers a greater advantage than the SIM coefficient.

The purpose of this study is to apply library search routines that utilize SIM and DISSIM coefficients and the purity parameter numerical format to the confirmation of peak identity and peak homogeneity in the liquid chromatographic analysis of quinoxaline antibiotics and their analogues. The apparent interconversion between two forms of [MeCys³, MeCys⁷]-TANDEM was also investigated using reversed-phase column temperature studies in conjunction with UV photodiode-array detection.

EXPERIMENTAL

Materials

A Varian Series 9010 solvent delivery system, a Polychrom 9065 diode-array detector, with the LC Star 9021 workstation and Polyview spectral processing software, and a 9095 autosampler (Varian, Walnut Creek, CA, U.S.A.) were employed for all chromatographic separations and data collection. The autosampler was equipped with a Rheodyne (Cotati, CA, U.S.A.) 7125 automated loop-valve injector and 20- μ l sample loop. A Spark-Holland (Amsterdam, The Netherlands) high-performance liquid chromatography (HPLC) column heater was used for temperature studies. Collected fractions were concentrated or evaporated to dryness in a Pierce (Rockford, IL, U.S.A.) Model 18780 Reacti-Vac evaporator using high-purity nitrogen gas. All measurements of drug concentration were made using a Hewlett-Packard 8450 UV spectrophotometer.

Acetonitrile, methanol and HPLC-grade water were B&J Brand high-purity solvents obtained from Baxter Healthcare, Burdick & Jackson Division (Muskegon, MI, U.S.A.). Buffers were prepared from HPLC-grade potassium dihydrogen-phosphate (Fisher, Pittsburgh, PA, U.S.A.).

Echinomycin was a gift from the National Cancer Institute (Bethesda, MD,

U.S.A.). A sample of echinomycin was also kindly supplied by Drs. H. H. Peter and K. Scheibli of Ciba-Geigy (Basle, Switzerland). IQN and 2QN samples were products of directed biosynthesis studies¹⁴. Triostin A samples were generously provided by Drs. J. Shoji and K. Sato of Shionogi Research Laboratories (Osaka, Japan). Samples of TANDEM and [MeCys³,MeCys⁷]-TANDEM were a gift from Dr. R. K. Olsen, Department of Chemistry and Biochemistry, Utah State University (Logan, UT, U.S.A.). All quinoxaline drugs were stored in a dessicator in the dark at -20°C . Quinoxaline and quinoline were obtained from Aldrich (Milwaukee, WI, U.S.A.).

Sample preparation and chromatography

All quinoxaline antibiotic and analogue samples were dissolved in methanol at 100–800 μM concentrations and centrifuged prior to injection. Solutions were freshly prepared whenever possible. No precipitates were observed in the sample solutions. Quinoxaline drug concentrations were determined spectrophotometrically (echinomycin, $\epsilon_{315} = 11\,500$; IQN, $\epsilon_{315} = 8700$; 2QN, $\epsilon_{315} = 6000$; triostin A, $\epsilon_{316} = 12\,100$; TANDEM, $\epsilon_{316} = 12\,400$; [MeCys³,MeCys⁷]-TANDEM, $\epsilon_{316} = 12\,400$) from standards obtained by collection of the major chromatographic band of each sample and verified by NMR and other spectroscopic techniques. Extinction coefficients of the standards were calculated from methanol solutions using a 1-cm light path quartz cuvette.

A MicroPak SP C-8 IP-5 (Varian) column (150 mm \times 4 mm I.D.) with a 0.05 M phosphate buffer (pH 7.0)–acetonitrile (1:1) mobile phase at a flow-rate of 1.2 ml/min was employed for all chromatographic separations. Detection was performed at 239 nm and the raw data were collected for subsequent chromatographic and spectral processing. Fraction collection of [MeCys³,MeCys⁷]-TANDEM components was performed manually using a short piece (4 in.) of 0.009 in. I.D. stainless-steel tubing connected to the detector outlet to minimize band-broadening.

Data reduction

Purity parameter, SIM and DISSIM calculations were performed by using 38 fixed-wavelength values across the UV region (190–367 nm) from which a subset could be defined. The library search routines and attendant spectral processing software employed in this report have been described recently by Sheehan *et al.*²⁵. Basically, the library search utilizes a settable wavelength range, purity parameter ($\bar{\lambda}_w$) window, and retention time window as primary search criteria. An activated search routine calculates $\bar{\lambda}_w$ values for all library spectra and compares all $\bar{\lambda}_w$ values and retention data to the search windows. SIM and DISSIM values are then calculated for the unknown and for each selected library spectrum that falls within the search parameter windows. The top five library matches are listed in a search report.

RESULTS AND DISCUSSION

The low solubility of the quinoxaline antibiotics in aqueous solutions (*ca.* 1–6 μM) precluded direct dissolution of the drug samples in the mobile phase solvent at the concentrations desired for analysis²⁶. Methanol, in which the antibiotics are freely soluble, was employed for sample dissolution in order to ensure that the drugs were in true solution prior to chromatographic separation. Column plugging or back-pressure

problems were not experienced using this methodology. Studies with quinoxaline and echinomycin dissolved in the mobile phase solvent and in methanol revealed no differences in peak shape or position upon chromatographic analysis.

Echinomycin and its biosynthetic quinoline analogues

Reversed-phase liquid chromatographic separation of echinomycin and its 1QN and 2QN analogues is displayed in Fig. 2. Substitution of the quinoxaline ring with quinoline enhances retention. Consistent with these results, it was observed that quinoline is more highly retained than quinoxaline. Although the echinomycin sample displays only one chromatographic band, the samples of 1QN and 2QN analogues exhibit two and three principal bands, respectively, as well as some evidence of other minor constituents.

Fig. 3 displays the UV spectra and purity parameter values ($\bar{\lambda}_w$) for echinomycin,

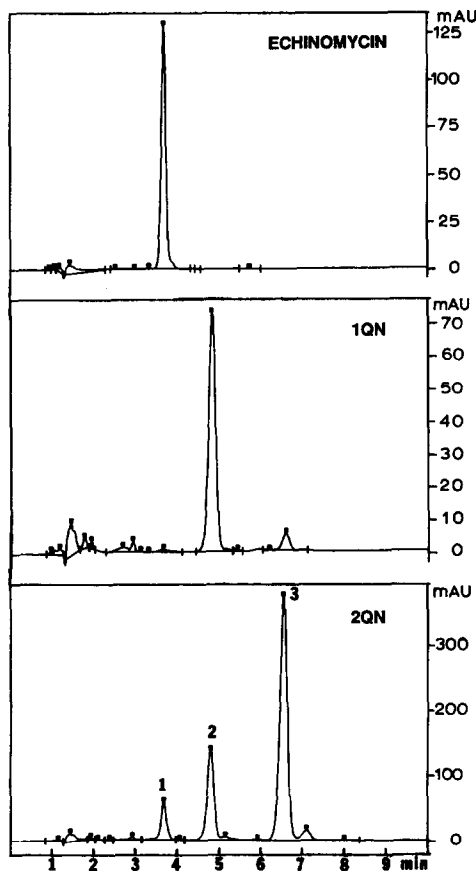


Fig. 2. Reversed-phase HPLC analysis of echinomycin and its 1QN and 2QN analogues. Chromatographic conditions are described in the text. The 1QN sample contains 5% of the 2QN analogue. The 2QN sample contains 8.1% echinomycin (component 1) and 21% of the 1QN analogue (component 2) in addition to 59.7% (by area percent at 239 nm) of 2QN (component 3).

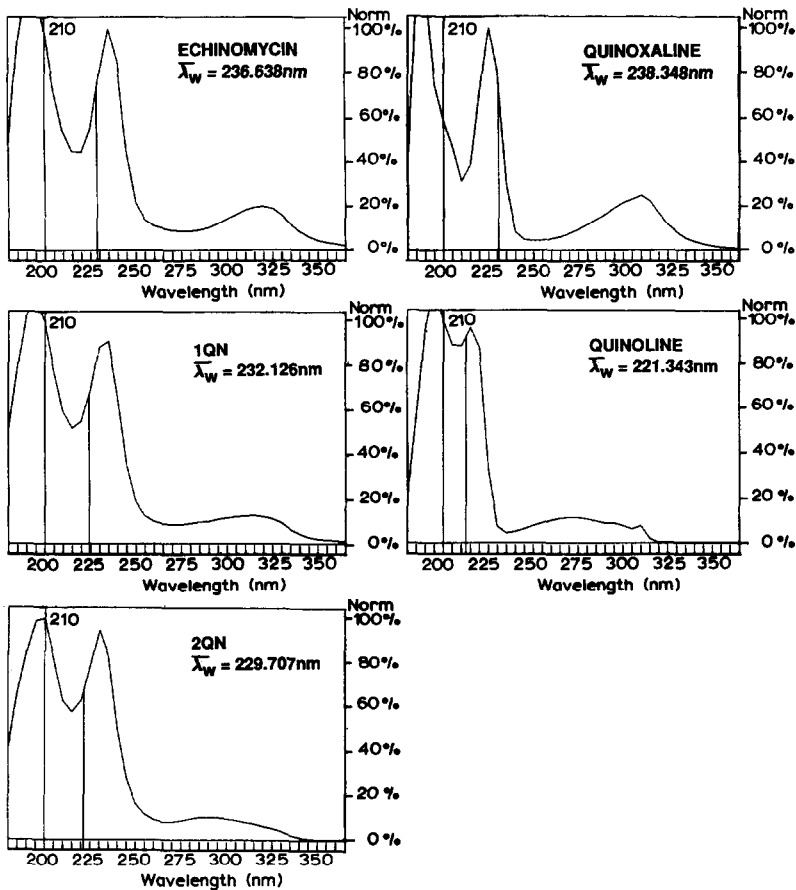


Fig. 3. UV spectra of echinomycin, 1QN, 2QN, quinoxaline and quinoline standards. λ_i values listed in the spectra (starting wavelength for calculation) and $\bar{\lambda}_w$ (purity parameter value) are denoted by solid vertical lines in each spectrum. See text for chromatographic conditions.

1QN and 2QN standards as well as for quinoxaline and quinoline. Table I lists the $\bar{\lambda}_w$ values and their standard deviations calculated over a broad wavelength range (210–367 nm) and over an optimized range (263–344 nm) for enhanced spectral discrimination of echinomycin and its analogues. As can be seen, the $\bar{\lambda}_w$ values calculated over the broad wavelength range (210–367 nm) for echinomycin, 1QN and 2QN differ by *ca.* 7 nm and reflect the spectral differences of the chromophores. However, an optimized spectral range (263–344 nm) yields $\bar{\lambda}_w$ values for echinomycin and its analogues that differ by over 17.5 nm, again reflecting chromophore spectral features. Based upon statistical comparison (*t*-test) of $\bar{\lambda}_w$ values acquired at several points (upslope inflection point, apex, downslope inflection point) across the peak elution profile, the major chromatographic components of each sample were found to exhibit a high degree of homogeneity.

A spectral reference library was constructed by acquiring spectra at several

TABLE I

ECHINOMYCIN, IQN, 2QN, QUINOXALINE AND QUINOLINE PURITY PARAMETER VALUES (λ)

Standard deviations (σ) were calculated on the basis of spectra acquired from different chromatographic experiments over a six-month period. See text for chromatographic conditions.

Compound	Wavelength range 210–367 nm		Optimized range 263–344 nm	
	λ_w (nm)	σ (n = 5)	λ_w (nm)	σ (n = 5)
Echinomycin	236.638	0.052	310.788	0.024
IQN	232.126	0.197	302.656	0.469
2QN	229.707	0.016	293.285	0.019
Quinoxaline	238.348	0.094	309.395	0.151
Quinoline	221.343	0.084	286.206	0.055

points across the elution profile of each quinoxaline depsipeptide in addition to quinoxaline and quinoline (over 100 spectra in all). A library search was then conducted in order to confirm identification of the major chromatographic components of the IQN and 2QN analogues. Results from a library search report for the 2QN analogue of echinomycin are summarized in Table II. Component 1 of the 2QN sample ($t_R = 3.6$ min) was matched to echinomycin (DISSIM = 0.01523); component 2 ($t_R = 4.7$ min) was matched to IQN (DISSIM = 0.00324); and component 3 (major component with $t_R = 6.4$ min) was matched to 2QN (DISSIM = 0.00091). About 2% of an unidentified component ($t_R = 6.9$ min) was present, having spectral features nearly identical with those of 2QN. In an analogous manner, the IQN sample was found to consist of about 5% 2QN plus a few other components (including 12% of a poorly retained material) in addition to the IQN analogue (67% by area percent at 239 nm).

TABLE II

LIBRARY SEARCH REPORT SUMMARY FOR 2QN

See Fig. 2 for a representative chromatographic separation from which spectra were acquired. Library search parameters: λ_w value interval, ± 2.00 nm; wavelength range, 210–367 nm; retention time range, 1.00–9.99 min; retention time interval, $\pm 5.00\%$; minimum peak height, 5 mA.U.

	Peak No.		
	1	2	3
Peak retention time (min)	3.613	4.706	6.396
λ_w value (nm)	236.007	232.188	229.678
Library match identification	Echinomycin	IQN	2QN
Library match retention time (min)	3.679	4.835	6.562
Library match λ_w value (nm)	236.565	232.245	229.695
SIM coefficient	0.99988	0.99999	1.00000
DISSIM coefficient	0.01523	0.00324	0.00091

Due to the asymmetric nature of the quinomycin cross-bridge structure, the IQN analogue of echinomycin is composed of two positional isomers in which one quinoline ring is substituted for a quinoxaline ring at each respective quinoxaline moiety position. Based upon a lack of any observed resolution, the two IQN isomers were not apparently separable under the chromatographic conditions used in this study. Williamson *et al.*²⁷ have reported that although not separable by the liquid chromatographic procedures employed in their study, the IQN isomers were clearly distinguishable by NMR techniques.

Triostin A and its undermethylated synthetic analogues

Fig. 4 displays the chromatograms of triostin A and its undermethylated analogues TANDEM and [MeCys³,MeCys⁷]-TANDEM (TANDEM with additional N-methyl groups present at both cysteine residues at positions 3 and 7 in the peptide ring). Two chromatographic components are evident for the triostin A sample in addition to a few minor peaks. The sample of TANDEM displays three major chromatographic components as well as a poorly retained minor component. The [MeCys³, MeCys⁷]-TANDEM analogue exhibits an unusual chromatographic profile which is characterized by a broad, flat absorbance region linking two well resolved peaks.

Table III lists the purity parameter values at peak apex calculated over a broad wavelength range (210–367 nm) and their standard deviations for each major chromatographic component of triostin A, TANDEM and [MeCys³,MeCys⁷]-TANDEM. The $\bar{\lambda}_w$ values for the components of all three samples are similar, reflecting the fact that the only structural differences among them are the presence or absence of N-methyl groups at the cysteine and valine residues in the peptide ring. However, the major component of triostin A and TANDEM can be spectrally discriminated ($\bar{\lambda}_w$ values of 237.072 and 240.794 nm, respectively). On the other hand, no significant spectral difference seems to exist between the three apparent components of TANDEM or between the two apparent components of triostin A. In contrast, however, the two major components of [MeCys³,MeCys⁷]-TANDEM have statistically different spectral features. Component 1 of this analogue has a $\bar{\lambda}_w$ value of 235.346 and component 2 has a $\bar{\lambda}_w$ value of 238.908 nm.

Several NMR studies^{28–30} have determined that triostin A exists in deuteriochloroform solutions as two symmetrical conformers separated by an energy barrier of 20–22 kcal/mol. Based upon solvent effects on conformer equilibrium, one conformer is known to be favored in non-polar solvents while the other is favored in polar solvents. Blake *et al.*²⁸ designated the conformer predominant in [²H₆]dimethylsulfoxide([²H₆]DMSO) and other polar solvents as the *p* conformer (*p*-triostin A) and the conformer predominant in non-polar solvents as the *n* conformer (*n*-triostin A). They observed that in [²H]chloroform, [²H₆]benzene–[²H]chloroform and [²H]chloroform–carbon tetrachloride solutions *n*-triostin A predominates although both forms of the drug are present. In polar solvents such as [²H₆]DMSO only the *p*-triostin A conformer was apparent. Kyogoku *et al.*³⁰ have postulated that based upon ¹H and ¹³C NMR studies of the drug and its S-benzyl triostin A analogue, the triostin A conformers result from *cis*–*trans* isomerism at either the Ala–MeCys or MeCys–MeVal peptide bonds. Since both the mobile phase and sample dissolution solvent employed in this chromatographic study are polar solvents, *p*-triostin A would be

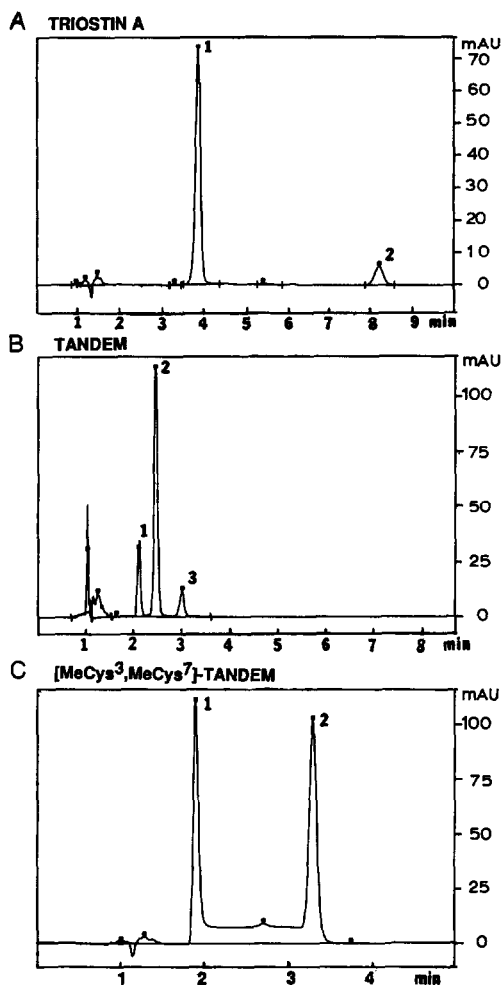


Fig. 4. Reversed-phase HPLC analysis of triostin A, TANDEM and [MeCys³,MeCys⁷]-TANDEM. Chromatographic conditions are described in the text. (A) Triostin A: component 1, $t_R = 3.9$ min (88.3%); component 2, $t_R = 8.25$ min (11.7% by area percent at 239 nm). (B) TANDEM: component 1, $t_R = 2.1$ min (18.7%); component 2, $t_R = 2.5$ min (71%); component 3, $t_R = 3.0$ min (10.3%). (C) [MeCys³, MeCys⁷]-TANDEM: component 1, $t_R = 2.05$ min; component 2, $t_R = 3.5$ min.

expected to predominate in solution. Triostin A dissolved in DMSO and chromatographed by our reversed-phase methods gave nearly identical results to those shown in Fig. 4A. Although no unequivocal determination of triostin A conformers can be obtained from this work, it is speculated that the major chromatographic band of triostin A (component 1) may be *p*-trioestin A. Since both chromatographic components of triostin A have nearly identical UV spectra (λ_w values of 237.072 and 237.530 nm, respectively), it is likely that both have very similar chromophore environments.

NMR investigations by Hyde *et al.*³¹ of the conformation of TANDEM in [²H]chloroform, [²H₅]pyridine and [²H₆]DMSO have shown that, unlike triostin A,

TABLE III

TRIOSTIN A, TANDEM AND [MeCys³,MeCys⁷]-TANDEM PURITY PARAMETER VALUES (λ_w)

Standard deviations were calculated on the basis of spectra acquired from different chromatographic experiments over a six-month period. See text for chromatographic conditions.

Compound	Wavelength range 210–367 nm	
	λ_w (nm)	σ (n = 5)
Triostin A		
Component 1 ^a	237.072	0.069
Component 2	237.530	0.343
TANDEM		
Component 1	240.877	0.375
Component 2 ^a	240.794	0.168
Component 3	239.450	1.711
[MeCys ³ ,MeCys ⁷]-TANDEM		
Component 1	235.346	0.570
Component 2	238.908	0.409

^a Major component.

the TANDEM analogue seems to adopt a single conformation in solution. The reversed-phase chromatographic separation of TANDEM (Fig. 4B) reveals three apparent components with very similar UV spectral characteristics. Further work is currently in progress to elucidate the nature of these components.

Of particular interest is the unusual chromatographic profile obtained for the [MeCys³,MeCys⁷]-TANDEM analogue in our reversed-phase study (Fig. 4C). The profile was noted to be similar to the chromatographic profile of the cyclic undecapeptide cyclosporine, reported by Bowers and Mathews³² and to the profile of the dipeptide L-alanyl-L-proline investigated by Melander *et al.*³³. Investigation of peak broadening in the reversed-phase chromatography of cyclosporine led to the conclusion that the mechanism involved interconversion of several conformers of the drug. The postulate of solution conformers was supported by NMR spectroscopic results. The L-alanyl-L-proline dipeptide chromatographic study consisted of a detailed analysis of the effects of molecular structure and conformational changes of proline-containing dipeptides. The results were interpreted as evidence for the slow kinetics of *cis-trans* isomerism about the proline amide bond. Due to its smaller hydrophobic surface area, the *trans* conformer of the proline dipeptide eluted faster than the corresponding *cis* conformer. Proline *cis-trans* isomerism in proteins has recently been examined by two-dimensional NMR in the work of Chazin *et al.*³⁴.

In an attempt to elucidate the nature of our [MeCys³,MeCys⁷]-TANDEM results, the analogue was chromatographed at column temperatures from ambient to 90°C (Fig. 5; chromatogram at 90°C is not shown as it was very similar to that at 75°C). As can be seen, the two sharp major components of the drug vanish and a broad central peak grows as the temperature is increased. A third, sharp component, eluted at a point intermediate to the two major chromatographic bands, is evident in several of these

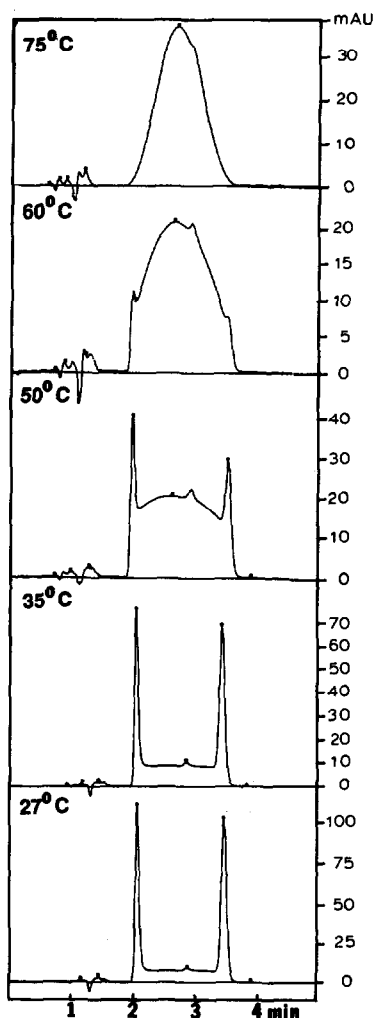


Fig. 5. Reversed-phase HPLC analysis of [MeCys³,MeCys⁷]-TANDEM as a function of column temperature. See text for chromatographic conditions.

chromatograms. Analogy to similar results with the proline dipeptides and cyclosporine seems to indicate that the [MeCys³,MeCys⁷]-TANDEM analogue consists of at least two conformers in polar solutions. Their kinetics are such that interconversion takes place on the chromatographic time scale (a few minutes). Using the height of the broad central peak as a measure of the extent of interconversion, an Arrhenius plot of the column temperature study data was made (Fig. 6). A linear plot is obtained up to about 60°C at which point interconversion may be so rapid that the height of the central peak is no longer a good measure of rate. An activation energy of about 15 kcal/mol was estimated from the slope of a least-squares fit to the linear portion of the data. The activation energy estimated for [MeCys³,MeCys⁷]-TANDEM is lower than that calculated for interconversion of triostin A conformers from NMR results²⁸, but

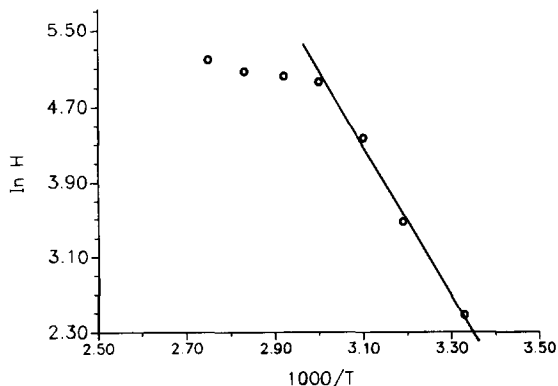


Fig. 6. Arrhenius plot of [MeCys³,MeCys⁷]-TANDEM conformer interconversion data. H is the height of the broad central peak, measured as a function of column temperature (see Fig. 5).

it is similar to the energy barrier calculated for the conformer interconversion of the cyclic tetrapeptide tentoxin³⁵. A detailed investigation of conformer content versus time in methanol prior to chromatography is currently in progress to further define the solution kinetics of [MeCys³,MeCys⁷]-TANDEM conformer interconversion.

Fig. 7A shows the chromatogram of the [MeCys³,MeCys⁷]-TANDEM analogue which contains two major components (termed conformer 1 and conformer 2). As previously demonstrated, the two conformers exhibit statistically different spectra indicating a slight difference in chromophore environments. Collection of the conformer 1 fraction followed by concentration in aqueous mobile phase and reversed-phase chromatography gave the results displayed in Fig. 7B. Similar procedures with conformer 2 yielded nearly identical results. Library search results indicated that the major component in the chromatogram matched conformer 1 and the minor peak matched conformer 2. If the conformer 1 fraction of the [MeCys³,MeCys⁷]-TANDEM is collected, evaporated to dryness under a stream of nitrogen and redissolved in methanol for chromatographic reanalysis, the results as shown in Fig. 7C are obtained. In addition to the two conformers observed in the parent compound, a third sharp component eluted between conformers 1 and 2 is obvious in the chromatogram. Spectral characteristics of this component ($\lambda_w = 239.280$ nm) are nearly identical with those of conformer 2 ($\lambda_w = 238.908$ nm), but differ considerably from conformer 1 ($\lambda_w = 235.346$ nm). Apart from the prominence of this intermediate component, the chromatogram is very similar to that obtained for the parent compound (Fig. 7A). Similar procedures carried out with conformer 2 of [MeCys³,MeCys⁷]-TANDEM yielded results comparable to those obtained with conformer 1 but with a slight enhancement of the intermediate component. Assignment of conformers 1 and 2 were again verified by library search methods. The results of this fraction collection study seem to indicate that the ratio of conformers 1 and 2 obtained from reversed-phase chromatography is a function of the sample dissolution solvent. If [MeCys³,MeCys⁷]-TANDEM or its individual conformers are dissolved in methanol, nearly equal amounts of each conformer are obtained upon reversed-phase chromatography with an aqueous buffer-acetonitrile mobile phase

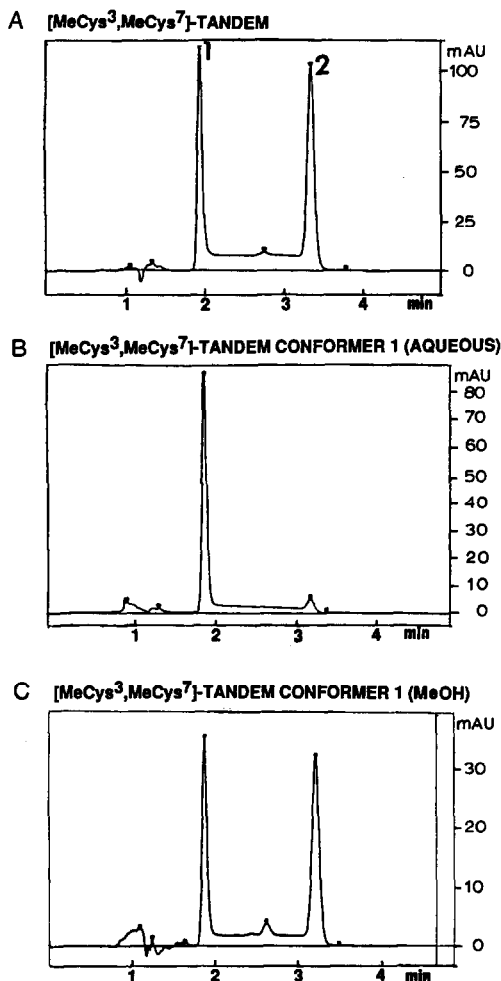


Fig. 7. Reversed-phase analysis of [MeCys³,MeCys⁷]-TANDEM and individual conformer fractions. Chromatographic conditions are described in the text. (A) [MeCys³,MeCys⁷]-TANDEM reversed-phase analysis; (B) HPLC analysis of conformer 1 fraction from (A), concentrated in aqueous mobile phase; (C) HPLC analysis of conformer 1 fraction from (A) evaporated to dryness and redissolved in methanol prior to chromatography.

(see Fig. 7A and C). If conformer 1 or 2 is dissolved in aqueous solvent prior to chromatographic analysis, conformer 1 predominates although both forms of the drug are present (see Fig. 7B). Additionally, if [MeCys³,MeCys⁷]-TANDEM itself is dissolved in the aqueous mobile phase and then chromatographed, conformer 1 is found to predominate over conformer 2. Apparently conformer 1 is favored in aqueous solutions.

Preliminary ¹H NMR investigation of [MeCys³,MeCys⁷]-TANDEM reveals, like the TANDEM analogue, one set of resonances for each set of symmetry-related pair of protons in [²H]chloroform. Additional NMR studies of [MeCys³,MeCys⁷]-

TANDEM in methanol and other polar solvents should help elucidate the nature of the conformers and their interconversion in solution.

CONCLUSIONS

Estimation of purity and UV spectral matching of quinoxaline antibiotics and their analogues was greatly aided by numerical formats for evaluation of spectral purity and spectral comparison in conjunction with library search routines. Echinomycin consisted of one major chromatographic component which exhibited a high degree of peak homogeneity based upon UV spectral data. Samples of IQN and 2QN analogues of echinomycin contained 5 and 21% (by area percent at 239 nm) of the other analogue, respectively. In addition, the 2QN sample contained 8% echinomycin based upon library search results. Analysis of the triostin A sample revealed two apparent chromatographic components (minor component 12% by peak area) with similar spectral characteristics. The TANDEM sample exhibited three apparent components with nearly identical spectral features. The purity parameter format displayed the ability to spectrally discriminate the major component of each decapeptide drug investigated in this study.

[MeCys³,MeCys⁷]-TANDEM analogue of triostin A was found to consist of about equal amounts of two distinguishable conformers (conformer 1 and conformer 2) when dissolved in methanol prior to chromatography. Conformer 1 appears to be favored in aqueous solutions. Since neither triostin A nor TANDEM display evidence of any interconversion taking place on a chromatographic time scale, it is postulated that the [MeCys³,MeCys⁷]-TANDEM conformers may be the result of *cis-trans* isomerism involving the MeCys peptide bond. UV spectral data suggest that the quinoxaline ring environment is slightly different in the two conformers. An activation energy of about 15 kcal/mol was estimated for the interconversion process.

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