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INTERACTION BETWEEN SYNTHETIC ANALOGUES OF QUINOXALINE ANTIBIOTICS AND NUCLEIC ACIDS: ROLE OF THE DISULPHIDE CROSS-BRIDGE AND D-AMINO ACID CENTRES IN DES-*N*-TETRAMETHYL-TRIOSTIN A

K.R. FOX, R. K. OLSEN* & M.J. WARING
With an appendix by P.K. CHAKRAVARTY* & R.K. OLSEN*

University of Cambridge. Department of Pharmacology, Medical School, Hills Road, Cambridge CB2 2QD and *Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322, U.S.A.

1 [Ala^3, Ala^7] TANDEM is an analogue of des-*N*-tetramethyl-triostin A (TANDEM) in which both L-Cys residues of the octapeptide ring are replaced by L-Ala; accordingly it lacks the disulphide cross-bridge which limits the conformational flexibility of TANDEM.

2 In [L-Ser^1] TANDEM the configuration of one of the serine residues is inverted, altering the disposition of one of the quinoxaline chromophores with respect to the peptide ring.

3 Both compounds interact weakly but detectably with natural DNAs as judged by spectral shifts and increases in the thermal denaturation ('melting') temperature T_m. They also raise the T_m of poly rA.poly rU.

4 Binding isotherms determined by solvent partition analysis with [Ala^3, Ala^7] TANDEM yield association constants of about 10^3 M^{-1} for its interaction with natural DNAs. A Scatchard plot for binding to poly(dA-dT) determined by solvent partition and spectrophotometric methods shows marked evidence of cooperativity with an intrinsic association constant 1.9×10^4 M^{-1}, 8.7 nucleotides per binding site, and cooperativity parameter 15.

5 Binding of [Ala^3, Ala^7] TANDEM to short rod-like fragments of poly(dA-dT) increases their contour length by almost the theoretical amount expected for an ideal process of bifunctional intercalation.

6 No effect of either compound on the winding of the DNA helix could be detected in sedimentation experiments with closed circular duplex PM2 DNA.

7 It is concluded that the cross-bridge of TANDEM greatly stabilizes its binding to DNA, most probably via entropic factors, but is not the only structural feature that influences its AT sequence-selectivity. The consequences of epimerising one of the D-Ser residues appear as disastrous as epimerising both.

8 The experimental details for the synthesis of [Ala^3, Ala^7] TANDEM and [L-Ser^1] TANDEM are given in an appendix to this paper.

Introduction

Quinoxaline antibiotics are secondary metabolites produced by several species of streptomycetes. They are highly active against Gram positive bacteria, viruses, and a variety of experimental tumours (reviewed by Katagiri, Yoshida & Sato, 1975). There is general agreement that the biological effects of these antibiotics are directly attributable to their binding to DNA (Ward, Reich & Goldberg, 1965; Sato, Shiratori & Katagiri, 1967; Waring & Makoff, 1974; Katagiri *et al.*, 1975). The best-known member of the group is echinomycin (quinomycin A), whose structure has recently been revised from that originally proposed (Dell, Williams, Morris, Smith, Feeney & Roberts, 1975; Martin, Mizsak, Biles, Stewart, Baczynskyj & Meulman, 1975), and which was the first reported example of a bifunctional DNA-intercalating agent (Waring & Wakelin, 1974).

Thanks to the availability of numerous naturally-occurring as well as synthetic analogues of echinomycin, it has been possible to probe the nature of structure-activity relations for DNA binding by quinoxaline antibiotics in some detail, and to attempt to relate their important sequence-selectivity to aspects of their molecular structure (Wakelin & Waring, 1976; Lee & Waring, 1978a, b). In particular, it has been surmised that the disposition of the quinoxaline chromophores with respect to the octapeptide ring, and the nature and conformation of the sulphur-containing linkage which bridges across that ring, are crucial determinants of the strength, intercalative

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character and specificity of the antibiotic-DNA interaction. In echinomycin the cross-bridge is a thioacetal; in triostin A it is a disulphide; and in the synthetic quinoxaline TANDEM (des-*N*-tetramethyltriostin A; Ciardelli, Chakravarty & Olsen, 1978) the disulphide bridge is retained but there are no methylated peptide bonds (see Figure 1). These three closely related compounds share the same bifunctional intercalative mode of binding to DNA but their patterns of nucleotide sequence-selectivity are radically different (Wakelin & Waring, 1976; Lee & Waring, 1978a, b; Waring, 1979). The evidence of n.m.r. investigations and energy calculations suggests that in all three compounds the peptide ring tends to adopt much the same rigid conformation but that important differences occur in the positioning of the cross-bridge (Ughetto & Waring, 1977; Cheung, Feeney, Roberts, Williams, Ughetto & Waring, 1978; Kalman, Blake, Williams, Feeney & Roberts, 1979; J. R. Kalman, personal communication).

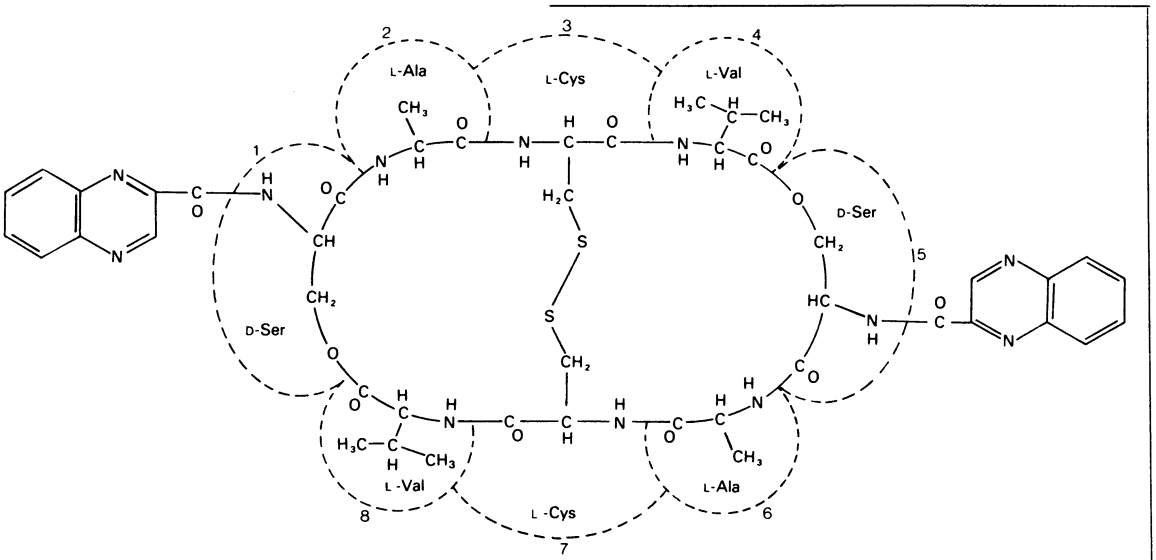


Figure 1 Structural formula of des-*N*-tetramethyltriostin A (TANDEM).

In this paper we examine the role of the cross-bridge directly, using an analogue of TANDEM in which the two Cys residues are replaced by Ala so that the cross-bridge is totally lacking. Contrary to what was found previously with a product derived from echinomycin in which the thioacetal cross-bridge had been cleaved (Lee & Waring, 1978b), this new cross-bridgeless analogue is not devoid of interaction with DNA, and its binding to poly (dA-dT) reveals novel features of interest in their own right. We also examine the effect of epimerising one of the D-serine centres in TANDEM to L-, thus altering the disposition of one of the chromophores with respect to the peptide ring, and compare the results with those found previously for ELSERTA, an analogue having both centres L- (Lee & Waring, 1978b). Experimental details for the preparation of the above two analogues are given in the appendix.

Methods

All DNA-binding experiments were conducted in HEPES–NaOH buffer pH 7.0, ionic strength 0.01, designated 0.01 SHE. General reagents and solutions of naturally occurring and synthetic nucleic acids were obtained as previously described (Lee & Waring, 1978b).

Extinction coefficients

Measurements of ultraviolet absorption were made with a Unicam SP500 Series II spectrophotometer. Molar extinction coefficients were determined according to the procedure of Lee & Waring (1978b). Although the aqueous solubility of [Ala^3, Ala^7] TANDEM is much greater than that of naturally-occurring quinoxaline antibiotics (such that its aqueous extinction coefficient could be checked by direct weighing), the slow rate of dissolution together with the tendency to lose material by adhesion to glass surfaces from aqueous solutions posed certain problems in handling this analogue. They could be largely, but not entirely, circumvented by scrupulous cleaning of glassware in dichromate-H_2SO_4 cleaning mixture. Relevant extinction coefficients of the compounds studied are listed in Table 1.

Solvent partition analysis for [Ala³, Ala⁷] TANDEM

The partition coefficient of [Ala³, Ala⁷] TANDEM between iso-amyl acetate and 0.01 SHE buffer is lower than that recommended by Waring *et al.* (1975) for solvent-partition work, attributable no doubt to its unusually high aqueous solubility. However, initial experiments indicated that its binding to nucleic acids was weaker than for other quinoxaline antibiotics, so that the concentration of free drug in equilibrium with the DNA would be likely to be greater and thus require a smaller amplification factor for accurate measurement. The partition coefficient was measured by shaking 1.5 ml of [Ala³, Ala⁷] TANDEM dissolved in iso-amyl acetate with 4 ml of 0.01 SHE buffer for two hours at 20°C. The antibiotic concentration in the organic phase was measured from the absorption at 315 nm in 10 mm and 2 mm light path quartz cuvettes, while the concentration in the aqueous phase was measured from the absorbance at 322 nm in 40 mm or 10 mm cuvettes. The relationship between the concentrations in the two phases was found to be linear over the working range and yielded a partition coefficient of 4.95 ± 0.07, as shown in Figure 2. The high precision of this partition coefficient compared with that determined for other quinoxaline antibiotics (cf. Table 1 of Waring, 1979) is again due to the much larger aqueous solubility of the drug, which enables the longer wavelength maximum to be used where interference from small quantities of iso-amyl acetate present is much reduced.

Binding isotherms were measured by an identical procedure except that the aqueous phase now contained 150 μM nucleic acid. Absorption measurements on the organic phase, together with the partition coefficient, yielded an estimate of c, the free drug concentration in the aqueous phase. The amount of drug bound was determined by treating the aqueous phase with an equal volume of dimethyl sulphoxide (DMSO) to dissociate the complex and measuring the absorption at 325 nm to obtain the total drug concentration in the aqueous phase; from this was subtracted the measured free drug concentration. The resulting value divided by the nucleotide concentration gives r (mol of drug bound/mol of nucleotides). Values of r and c were analyzed in terms of equations (10) or (15) of McGhee & Von Hippel (1974) to yield estimates of the intrinsic binding constant $K(0)$, the number of nucleotides occluded by the binding of one ligand molecule n, and, where relevant, the cooperativity parameter ω.

Viscometry

Measurements were made according to the method of Cohen & Eisenberg (1966; 1969) with a viscometer having a 10 cm capillary of 0.4 mm bore and a bulb of volume 2 ml. It was clamped in a water bath containing 53 litres of deionized water and maintained at $20° \pm 0.2°$ by the use of an Accurostat bath heater opposing the action of a cooling coil. The DNA used in these experiments was poly(dA-dT) which had been sonicated for 5 min at 0°C by use of the exponential probe of an MSE 150 W ultrasonic disintegrator at the highest power level. To prevent excessive heating, sonication was performed in bursts of 30 s with a delay of 30 s between bursts. This gave poly(dA-dT) with a reduced viscosity of 2.0 dl/g from which the molecular weight was estimated to be approximately 3.7×10^5 (Crothers & Zimm, 1965). This sonicated DNA was extensively dialysed against 0.01 SHE and filtered through two Whatman GF/C glass fibre filters before use. The viscometer was filled by weighing in 1.8 ml of the DNA solution at a concentration of 600 μM. Flow times were measured in triplicate with a stopwatch accurate to 0.1 s. The viscometer had a flow time for buffer of 186.5 s and an estimated shear gradient of 1000 s^{-1}. Complexes of known drug to nucleotide ratio (D/P) were generated by adding 10 μl aliquots of antibiotic (110 μM in 0.01 SHE) delivered from a Burkard syringe. It was assumed that in the calculation of L/L_o, the relative contour length of

Table 1 Molar extinction coefficients of synthetic quinoxalines

Compound	*Solvent*	*ε at λmax*
[Ala³· Ala⁷] TANDEM	Iso-amyl acetate	12190 (315 nm)
	Iso-amyl acetate-saturated 0.01 SHE buffer	11840 (322 nm) 53900 (241 nm)
	50% (v/v) Dimethyl sulphoxide/iso-amyl acetate-saturated 0.01 SHE buffer	13800 (323 nm)
[L-Ser¹] TANDEM	0.01 SHE buffer	14130 (322 nm) 68700 (244 nm)
	50% (v/v) Dimethyl sulphoxide/0.01 SHE buffer	13670 (325 nm)

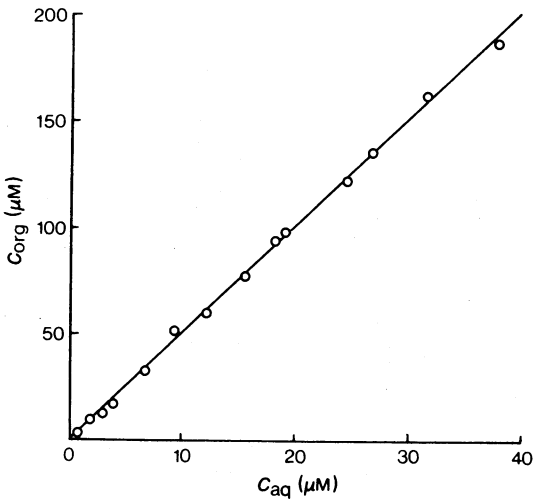


Figure 2 Partition of [Ala^3, Ala^7] TANDEM between iso-amyl acetate and 0.01 SHE buffer. The ordinate and abscissa scales represent the concentrations of [Ala^3, Ala^7] TANDEM in the organic and aqueous phases (corg, caq respectively) after equilibration by shaking for 2 h at 20°C. The line drawn was fitted to the points by the method of least-squares and constrained to pass through the origin; its slope is 4.95 ± 0.07.

DNA molecules with bound drug (L) compared to control drug-free DNA (L_o), the intrinsic viscosity can be approximated by the measured reduced viscosity, as discussed previously (Wakelin & Waring, 1976; Lee & Waring, 1978a). Since the antibiotics of interest are peptides the possibility was considered that [Ala^3, Ala^7] TANDEM might be surface-active. This was eliminated by comparing the effect of adding antibiotic solution, instead of solvent, to 1.8 g of buffer in the viscometer. There was negligible difference between flow time-dilution calibration curves determined by addition of either solution.

Spectrophotometric titrations

For [L-Ser^1] TANDEM a near-saturated solution of the drug in buffer was first prepared by vigorous shaking for 2 h at room temperature followed by clarification by filtration and/or centrifugation. The spectrum was then measured in 40 mm cells, after a small dilution with buffer. The procedure was repeated using the same initial drug solution but diluting with a small amount of concentrated DNA solution to yield the same final total drug concentration and give a known drug/nucleotide ratio (D/P), measuring the spectrum against a blank containing the same DNA concentration. For [Ala^3, Ala^7] TANDEM the procedure was similar, except that 1 cm cells could be used because of the higher solubility.

The binding of [Ala^3, Ala^7] TANDEM to poly(dA-dT) was determined spectrophotometrically by a method similar to that described by Waring (1970). To measure low levels of binding a Beer's law plot at 322 nm was first determined for the free drug by addition of 50 μl aliquots of a concentrated drug solution in 0.01 SHE to 100 mm light path quartz cuvettes containing 30 ml of buffer. The procedure was repeated with both cuvettes containing 10 μM poly(dA-dT). To measure higher binding levels, aliquots of concentrated poly(dA-dT) were added to a drug solution in 10 mm cells and the change in absorbance at 322 nm was measured. After correcting the values for dilution caused by adding the polynucleotide the decrease in absorption was recorded. The extinction coefficient of the drug when bound to poly(dA-dT) was determined as follows. An equilibrium dialysis cell of the type described by Müller, Crothers & Waring (1973) was set up with one side containing a solution of poly(dA-dT) and the other side containing a solution of the drug in 0.01 SHE. The solutions were stirred to equilibrium at room temperature for several days. It had previously been shown that this was sufficient for equilibration of the drug across the membrane. The spectrum of the bound drug was determined by measuring the absorbance of the two sides of the cell against each other, and is shown in Figure 4. In this way the true absorption spectrum of the ligand in the DNA-bound state was determined directly. The extinction coefficient could then be calculated having determined the concentration of bound drug by dissociating the complex with DMSO, measuring the absorbance at 322 nm, and subtracting the known concentration of free drug. By this means the molar extinction coefficient at 323 nm of the drug when bound to poly(dA-dT) was calculated to be 7470.

Analytical ultracentrifugation

Sedimentation coefficients were determined by boundary sedimentation in a Beckman model E analytical ultracentrifuge using mixtures of bacteriophage PM2 DNA with the compound of interest prepared as previously described (Lee & Waring, 1978a). For [L-Ser^1] TANDEM the initial complex was formed by adding a small volume of concentrated DNA solution to a saturated solution of the drug to yield a final DNA concentration of 50 μM. An 0.6 ml portion of this solution was weighed into one of the ultracentrifuge cells; the other contained 0.6 ml of 50 μM DNA in buffer alone. After the run the cells were vigorously shaken and reweighed. Further complexes were generated by mixing the two starting solutions in appropriate proportions as described by Lee & Waring (1978a).

Initial experiments with [L-Ser^1] TANDEM showed that the presence of the drug seemed to cause nicking of the closed circular DNA so that only one boundary was observed. This was presumed to be due to the presence of a trace impurity in the drug solution. Accordingly, a simple repurification procedure was adopted as follows: 2 mg of the drug was dissolved in a small volume of chloroform and chromatographed on Silica gel plates with ethyl methyl ketone as the liquid phase. The spot corresponding to the drug was scraped off the plate and extracted from the gel with a mixture of chloroform and methanol (50/50, v/v) several times. The washings were pooled and the organic solvents removed by vacuum distillation. The solid was redissolved in chloroform and passed over a column of Sephadex LH20 in chloroform. The solvent was again removed by vacuum distillation and the resulting drug used in the experiment. After this procedure the nicking of PM2 DNA to the open circular form II was no longer observed.

In experiments with [Ala^3, Ala^7] TANDEM the complexes were formed by method 2 of Waring (1970). As before, 0.6 ml of PM2 DNA (91 μM in 0.01 SHE) was introduced into each cell and the cell was weighed. A small amount of drug solution at 110 μM in 0.01 SHE was introduced by means of a Hamilton syringe and the cell was reweighed and shaken. After each run the cells were shaken and reweighed to check for any leakage that could have occurred. The procedure was then repeated by adding a further quantity of the antibiotic solution to prepare a complex having a higher input ratio of added drug to DNA nucleotides.

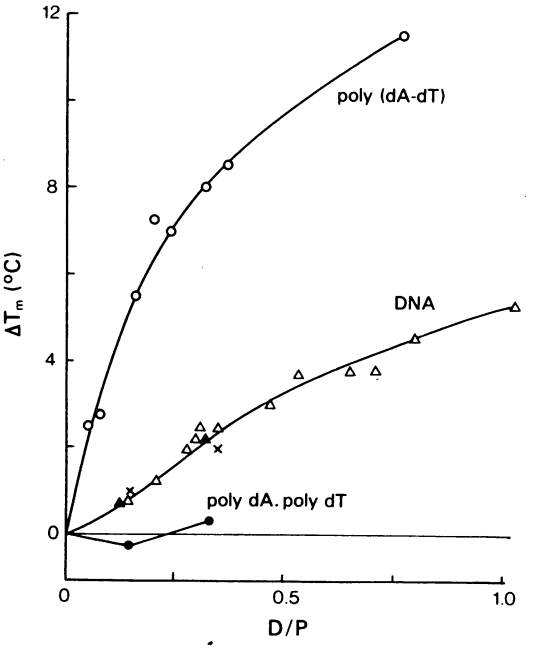


Figure 3 Effect of [Ala^3, Ala^7] TANDEM on the thermal-denaturation transition midpoint (T_m) of poly(dA-dT) (○), *Clostridium perfringens* DNA (△), calf thymus DNA (▲), polyrA.polyrU (×), and polydA.polydT (●). The change in 'melting' temperature (ΔT_m) is shown as a function of the molar ratio of ligand to total nucleotides (D/P).

Thermal denaturation profiles

'Melting' curves were measured as described by Lee & Waring (1978b). Complexes of [Ala^3, Ala^7] TANDEM were prepared by adding small quantities of concentrated DNA solution to the drug solution to give a final DNA concentration of 90 μM and known D/P value. For [L-Ser^1] TANDEM complexes were prepared by shaking a solution of DNA (90 μM in buffer) with a few mg of solid drug for about 30 min. The excess antibiotic was removed by centrifugation and filtration, after which the D/P of the complex was measured by dissociating a portion with DMSO and measuring the absorbance at 325 nm against an optical reference containing no drug which had been prepared in an analogous manner. The reproducibility of melting temperatures (T_m) was shown to be better than ±0.5°C (Lee & Waring, 1978b). It is possible to estimate approximate binding constants from values of ΔT_m (McGhee, 1976) using the equation $K_1c_1 \approx K_2c_2$ where K_1 and K_2 are the binding constants for two analogous compounds and c_1 and c_2 are the concentrations of free drug in equilibrium with the DNA giving this ΔT_m value (details in Lee & Waring, 1978b).

Results

[Ala^3, Ala^7] TANDEM

The effect of this quinoxaline on the 'melting temperature' (T_m) of several different DNAs is shown in Figure 3 as a function of the input ratio of added drug molecules to DNA nucleotides. Because of the relatively high solubility of this compound it was possible to attain quite high values of D/P. It can be seen that there is weak but easily detectable interaction with both natural DNAs and polyrA.polyrU. However, there is no detectable effect on the melting of polydA.polydT, a result typical of other quinoxaline antibiotics (Lee & Waring, 1978a, b). In contrast, the interaction with poly(dA-dT) is relatively strong, giving a maximum observed increase in melting temperature of 11°C at D/P 0.8. This could be explained by preferential binding of the drug to (A + T) sequences,

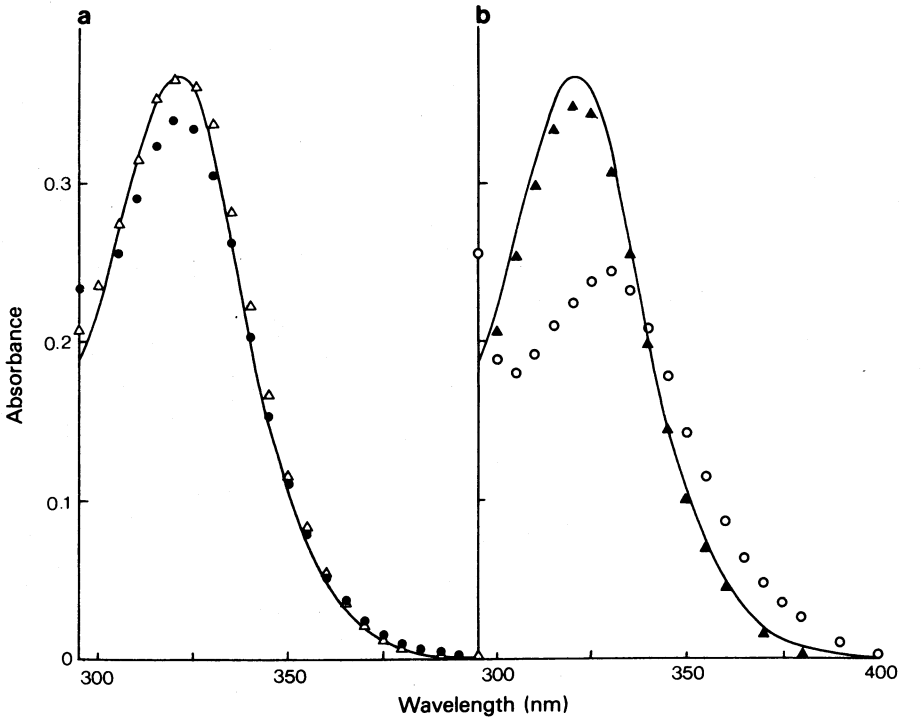


Figure 4 Effect of various DNAs on the absorption spectrum of 7.4 μM [Ala^3, Ala^7] TANDEM measured in 40 mm light path quartz cuvettes. The spectrum of the drug alone in buffer is represented in (a) by a continuous line, in the presence of 600 μM calf thymus DNA by (●), in the presence of 150 μM *Cl. perfringens* DNA by (△), and in (b) in the presence of 100 μM poly(dG-dC) by (▲). The spectrum when bound to poly(dA-dT), determined as described under Methods, is represented in (b) by (○).

or perhaps because this synthetic DNA is more susceptible to changes in T_m than other DNAs. It is possible to calculate approximate binding constants from these data by comparison with known values of ΔT_m and binding constants for a related compound. In this case TANDEM is the closest related compound which has been investigated in detail. The following will serve as an example of the method by which binding constants can be estimated. The complex of [Ala^3, Ala^7] TANDEM with poly(dA-dT) at D/P 0.32 displayed a ΔT_m of 8.0°C (Figure 3). This same change in T_m is produced by TANDEM at an r value of 0.035, where it is in equilibrium with a free TANDEM concentration c_T of 1.88 nM (calculated from the binding curve determined previously by Lee & Waring, 1978b). It is then assumed that in order to produce the same change in T_m, the value of r for the complex with [Ala^3, Ala^7] TANDEM must also have been 0.035. From the difference the free drug concentration in equilibrium with the DNA must have been 25.7 μM. Since $K_T c_T \approx K_A c_A$ where the subscripts refer to TANDEM and its alanyl analogue respectively, the binding constant of [Ala^3, Ala^7] TANDEM to poly(dA-dT) is estimated to be $1.5 \times 10^7 \times 1.88 \times 10^{-9}/25.7 \times 10^{-6} = 1100\ M^{-1}$. The same procedure was repeated for the other values of ΔT_m with poly(dA-dT) and with *Cl. perfringens* DNA. The resulting values of K_A averaged for each DNA were 1390 M^{-1} for poly(dA-dT) and 216 M^{-1} for *Cl. perfringens* DNA.

The effect of calf thymus and *Cl. perfringens* DNAs on the u.v. absorption spectrum of [Ala^3, Ala^7] TANDEM is shown in Figure 4. Although the observed changes are small they are not negligible and appear as hypochromic and bathochromic shifts like those seen with other quinoxalines (Waring *et al.*, 1975; Lee & Waring, 1978b). In the right-hand panel the spectrum of the free drug is compared with its spectrum when bound to poly(dA-dT), and in the presence of an excess of poly(dG-dC). The spectrum of the poly(dA-dT)-bound form shows large hypochromic and bathochromic shifts with a cross-over point at 337 nm, close to the isosbestic point seen with echinomycin at

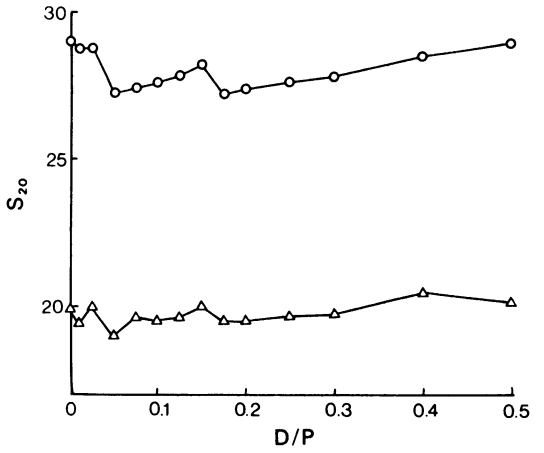


Figure 5 Effect of [Ala^3, Ala^7] TANDEM on the sedimentation coefficient of bacteriophage PM2 DNA. The DNA preparation contained 60–80% closed circular duplex molecules whose S_{20} is represented by (○). The S_{20} of the nicked circles is represented by (△). The abscissa scale shows the molar ratio of [Ala^3, Ala^7] TANDEM to DNA nucleotides.

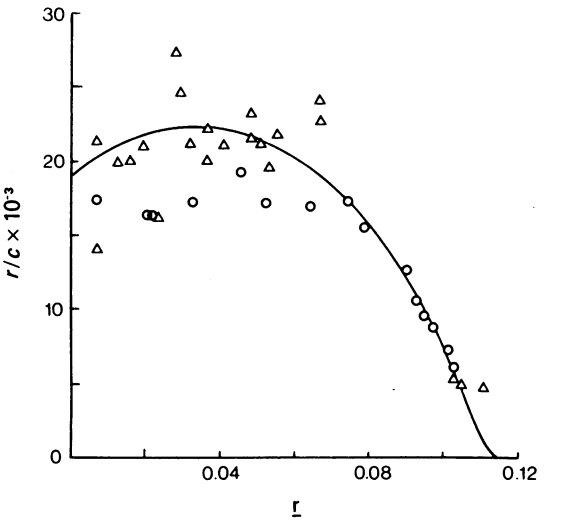


Figure 6 Interaction between [Ala^3, Ala^7] TANDEM and poly(dA-dT). The data are presented in the form of a Scatchard plot where r represents [Ala^3, Ala^7] TANDEM molecules bound per nucleotide and c is the free ligand concentration. Data determined by solvent partition analysis are shown by (○); results from spectrophotometric titration are shown by (△). The curve drawn corresponds to equation (15) of McGhee & Von Hippel (1974) for a cooperative binding process.

337 nm (Waring *et al.*, 1975). Thus in respect of its interaction with poly(dA-dT), [Ala^3, Ala^7] TANDEM clearly behaves like a typical quinoxaline antibiotic. This is in marked contrast to the small changes that are seen with poly(dG-dC).

The effect of [Ala^3, Ala^7] TANDEM on the sedimentation coefficient of closed circular duplex PM2 DNA is shown in Figure 5. It produces no significant change up to an input ratio of 0.5 molecules/nucleotide. This does not prove a total lack of interaction with PM2 DNA but the removal and reversal of supercoiling at low D/P values characteristic of the natural quinoxaline antibiotics and TANDEM is certainly not observed. In this respect the behaviour of [Ala^3, Ala^7] TANDEM appears similar to the non-binding quinoxaline analogue, echinomycin split product 1 (Lee & Waring, 1978b).

A Scatchard plot for the interaction of [Ala^3, Ala^7] TANDEM with poly(dA-dT) is shown in Figure 6. Results from both solvent partition analysis and spectrophotometric titration are included. The data show the clearest evidence to date of the cooperativity previously noticed for binding of quinoxalines to poly(dA-dT) (Lee & Waring, 1978a, b) and accordingly have been fitted to equation (15) of McGhee & Von Hippel (1974). This involves the introduction of a cooperativity parameter, ω, such that the binding constants to singly and doubly contiguous sites are ωK and $\omega^2 K$ respectively. Trial curves were calculated by a PDP/8E computer, the parameter $K(0)$, n, and ω being varied by trial and error until the best fit (chosen by eye) was obtained. The parameters $K(0)$ and n, defined by the intercepts, are relatively easy to fix and the value of ω is then altered by trial and error until the best 'hump' is produced. The curve drawn in Figure 6 corresponds to $K(0) = 1.9 \times 10^4$ M^{-1}, $n = 8.7$, $\omega = 15$. The points for the spectrophotometric titration are more scattered due to the inaccuracies involved in measuring the small absorbance changes between the free drug and the drug with DNA present. Nevertheless the reasonable agreement between data points obtained by the two methods is important because it provides the first independent check on the validity of the solvent partition method for measuring binding constants of quinoxalines to nucleic acids.

Figure 7 shows binding curves for the interaction with two natural DNAs compared with the curve for binding to poly(dA-dT). As anticipated from the thermal denaturation results, they reveal a much reduced interaction. Due to the weakness of the binding, Scatchard plots of the data were too scattered to be analysed rigorously in terms of equation (10) of McGhee & Von Hippel (1974). However, an approximate estimate of the binding constant could be obtained from

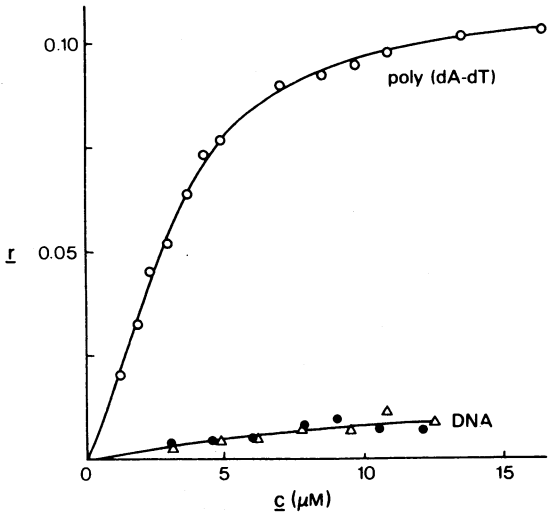


Figure 7 Binding of [Ala^3, Ala^7] TANDEM to poly-(dA-dT) (○), *Cl. perfringens* DNA (●), and calf thymus DNA (△) determined by solvent-partition analysis.

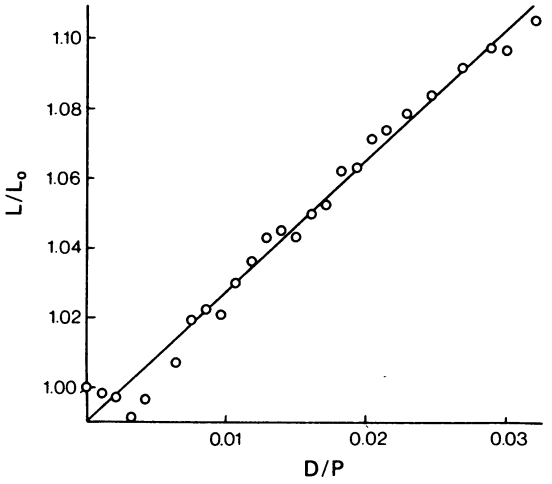


Figure 8 Effect of [Ala^3, Ala^7] TANDEM on the contour length of sonicated poly(dA-dT) fragments. The ordinate scale represents the calculated contour length in the presence of antibiotic (L) relative to the length in its absence (L$_o$). The abscissa scale shows the molar ratio of ligand to DNA nucleotides (D/P). The line drawn represents a least-squares fit to all the data points and has slope 3.74 ± 0.11.

the probable intercept on the *r/c* axis. In this way the binding constants for the natural DNAs were estimated to be approximately 10^3 M^{-1}.

Despite the failure to detect appreciable unwinding of the helix in the sedimentation experiments with PM2 DNA (Figure 5) a viscometric titration with poly(dA-dT) was attempted in the hope that it might reveal evidence of *bis*-intercalative interaction with this polydeoxynucleotide. The results are shown in Figure 8 as the fractional helix extension (L/L$_o$) against the input ratio of drug to nucleotides. The poly(dA-dT) was first mildly sonicated in order to reduce its molecular weight to the range within which it may be expected to behave as a rod-like fragment. Small variations occurred in the flow time for the control DNA between experiments, no doubt due to 'slippage' of the strands relative to each other (a problem which is likely to be encountered with any alternating, self complementary synthetic DNA), and the data have been corrected for this effect. The straight line plotted represents a least-squares fit to all the data points; its slope is 3.74, which is typical of the values reported for bifunctional intercalators (Waring, 1979). It falls slightly below the theoretical line for idealized bifunctional intercalation, which predicts the relationship $L/L_o = 1 + 4r$, but its gradient is well over twice the value seen for ethidium (Reinert, 1973). However, the line does not pass through 1.00 and there is an apparent initial decrease in L/L$_o$. This decrease was seen in more than one set of observations and appears to be real.

These results establish that [Ala^3, Ala^7] TANDEM does bind to nucleic acids and that its interaction is most evident with poly(dA-dT). Its apparent preference for (A + T)-rich sequences is shared with the parent compound TANDEM, although the binding constants are three orders of magnitude lower than those of TANDEM.

[*L-Ser^1*] *TANDEM*

The absorption spectrum of this derivative in the presence of various DNAs is shown in Figure 9. Only small changes from the spectrum of the free drug occur, which mostly appear as small hypochromic shifts. The largest effect is again seen with poly-(dA-dT). Figure 10 shows that [L-Ser^1] TANDEM has no detectable effect on the sedimentation of covalently closed circular duplex bacteriophage PM2 DNA up to the highest accessible concentration, corresponding to a D/P value of 0.06. These two experiments established that any interaction between [L-Ser^1] TANDEM and DNA was likely to be weak and would not prove amenable to investigation by the standard solvent partition analysis because of the low binding constant and the high value anticipated for the partition coefficient.

Results of thermal denaturation experiments performed with various DNAs are shown in Figure 11,

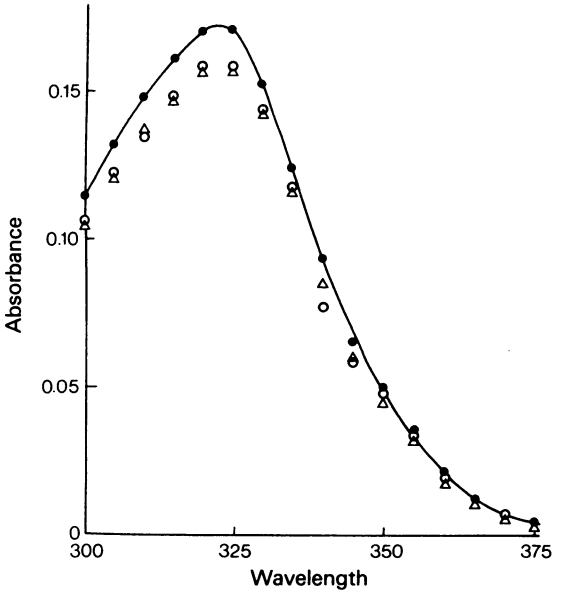


Figure 9 Effect of various DNAs on the absorption spectrum of 3.0 μM [L-Ser[1]] TANDEM measured in 40 mm light path quartz cuvettes. The spectrum of the drug alone in buffer is represented by a continuous line, in the presence of 300 μM calf thymus DNA by (○), in the presence of 60 μM poly(dG-dC) by (●), and in the presence of 30 μM poly(dA-dT) by (△).

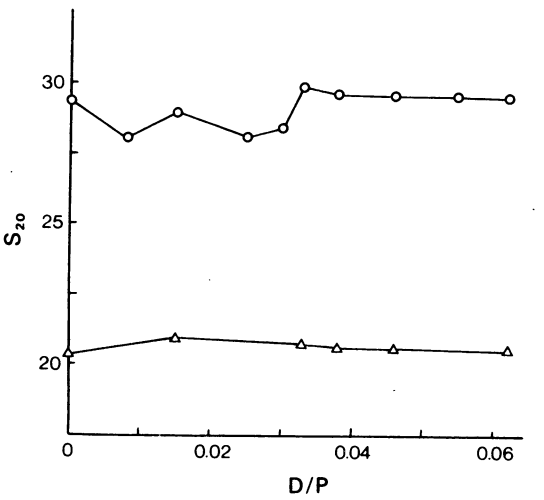


Figure 10 Effect of [L-Ser[1]] TANDEM on the sedimentation coefficient of bacteriophage PM2 DNA. The DNA preparation contained 60–80% closed circular duplex molecules whose S_{20} is represented by (○). The S_{20} of the nicked circles is represented by (△). The abscissa scale shows the molar ratio of [L-Ser[1]] TANDEM to DNA nucleotides. At the low DNA concentration employed the minor sedimenting boundary corresponds to nicked circles was insufficiently stable to resist convection due to small temperature changes, so in a few runs no S_{20} for this component could be calculated.

together with the changes produced by TANDEM and its *bis*-L-Ser analogue (from Lee & Waring, 1978b) for comparison. With both poly(dA-dT) and *Cl. perfringens* DNA [L-Ser[1]] TANDEM produces effects similar to those seen with the *bis*-L-Ser analogue. These suggest binding constants of 3×10^3 M^{-1} for poly(dA-dT) and less than 10^3 M^{-1} for *Cl. perfringens* DNA, calculated as above. With polyrA.polyrU, [L-Ser[1]] TANDEM produces much the same ΔT_m as TANDEM itself, suggesting a binding constant of 5×10^3 M^{-1} for this polymer.

[L-Ser[1]] TANDEM thus appears to interact only weakly with DNA, certainly several orders of magnitude less tightly than does TANDEM. In fact there is little to distinguish its behaviour from that of the doubly L-Ser-substituted analogue, despite its intermediate position between that substance and TANDEM.

Discussion

The first broad conclusion to be drawn from this work is that both derivatives of TANDEM display much weaker interaction with DNA than do the naturally-occurring quinoxaline antibiotics (Waring, 1979). Binding is most evident with the synthetic poly(dA-dT) in both cases. However, the fact that [Ala[3], Ala[7]] TANDEM binds to DNA at all is important because it establishes that the bridge across the peptide ring in the quinoxaline group of antibiotics cannot be absolutely essential for binding to DNA, though evidently it markedly increases the strength of binding. Why, then, was no interaction detectable with echinomycin split product 1, a derivative in which the cross-bridge was split by attack with methyl iodide (Lee & Waring, 1978b). The lower aqueous solubility of the latter compound may be partly responsible, though at the highest D/P value attained in thermal denaturation studies with poly(dA-dT) a ΔT_m of approx. 2.7°C would have been expected if it produced the same stabilization as [Ala[3], Ala[7]] TANDEM. More plausibly, the discrepancy may be related to the other structural differences between these compounds, i.e. the retention of the four peptide *N*-methyl groups and the likely presence of residual substituents replacing the cross-bridge in echinomycin split product 1 (Lee & Waring, 1978b).

Presumably the effect of the cross-bridge is to hold the peptide ring in a rigid conformation, thereby

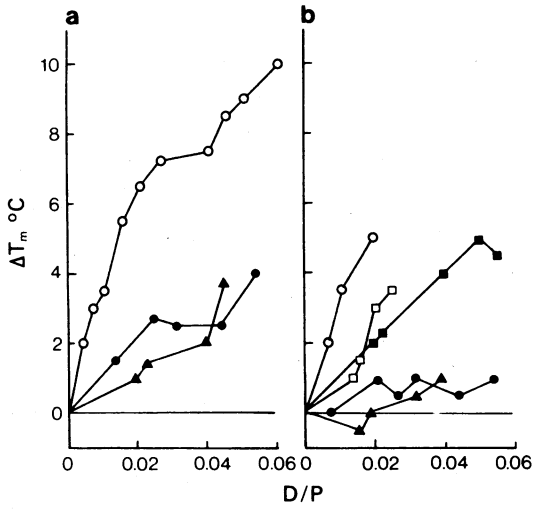


Figure 11 Effect of TANDEM (○), [L-Ser^1] TANDEM (●) and [L-Ser^1, L-Ser^5] TANDEM (▲) on the T_m of poly(dA-dT) (a) and *Cl. perfringens* DNA (b). Included in (b) are values for polyrA.polyrU in the presence of TANDEM and [L-Ser^1] TANDEM (□ and ■ respectively). The abscissa scale represents the molar ratio of added ligand to total nucleotides. Data for [L-Ser^1, L-Ser^5] TANDEM (designated 'ELSERTA') are taken from Lee and Waring (1978b).

reducing an unfavourable entropy term in the binding equilibrium by restricting the number of conformations that the peptide can adopt and holding the chromophores in a position favourable for intercalation. Because [Ala^3, Ala^7] TANDEM shares with the parent compound a strong preference for (A + T) nucleotide sequences we may further conclude that the presence and conformation of the disulphide cross-bridge is not the only factor influencing the sequence-selectivity of these antibiotics. If the (A + T) preference of TANDEM were due to steric hindrance between the 2-amino group of guanine in the narrow groove of DNA and the disulphide bridge, then removing the cross-bridge should remove this negative influence and return the base preference to that of echinomycin for which it is proposed that the cross-bridge is positioned so that there is no such steric hindrance (Lee & Waring, 1978b). This leaves open the question of the origin of the sequence-selectivity of these compounds. Indeed, in view of the evidence that poly(dA-dT) may adopt an unusual conformation under certain circumstances (Klug, Jack, Viswamitra, Kennard, Shakked & Steitz, 1979) it remains entirely possible that selective binding to this polymer may not accurately reflect preferences for binding to specific sequences in natural DNAs.

The slope of the line in Figure 8 provides compelling evidence that [Ala^3, Ala^7] TANDEM intercalates into the poly(dA-dT) helix in a bifunctional manner. However, the initial decrease in the L/L_o plot is rather puzzling. In principle it could be due to the polynucleotide undergoing certain structural changes, possibly related to the conformation hypothesized by Klug *et al.* (1979), on binding the drug at low ratios. The idea of such changes occurring is attractive because they might also account for the observed co-operativity in the binding of this compound and other quinoxaline antibiotics to poly(dA-dT). Alternatively, the initial decrease might reflect nothing more than a rearrangement of the chains of the alternating polynucleotide by a 'slippage' mechanism in which the gross helical structure of the DNA would remain unchanged but the average fragment length might decrease, promoted in some unknown fashion by the binding of a small amount of ligand. At all events, it seems clear that over practically the whole range studied the drug appears to be intercalating bifunctionally, notwithstanding the anticipated flexibility of its peptide ring. These observations contrast with its lack of detectable effect on the supercoiling of circular PM2 DNA. Reference to the calculated binding constant for calf thymus DNA suggests that, if the same constant were applicable to PM2 DNA, the level of binding achieved in the experiment of Figure 5 should have been sufficient to produce a measurable decrease in the S_{20} of the circular DNA if the drug were binding purely *bis*-intercalatively. The fact that no such decrease was seen probably means that one of the assumptions was not entirely justified: it does not rule out the possibility of limited intercalative binding of [Ala^3, Ala^7] TANDEM to natural DNA, bifunctional or otherwise.

The behaviour of [L-Ser^1] TANDEM contrasts sharply with that of TANDEM itself and is quite similar to that of the *bis*-L-Ser analogue ELSERTA (Lee & Waring, 1978b). It seems reasonable to suppose that the basic conformation of the peptide ring in these three compounds is much the same, so that if, for TANDEM, the chromophores are positioned parallel to allow for bifunctional intercalation into DNA, the chromophores of ELSERTA should lie in approximately the same plane as the peptide ring while with [L-Ser^1] TANDEM one chromophore should lie in the plane of the ring and one perpendicular to it. Bifunctional intercalation of ELSERTA would require a radically altered peptide conformation in order to bring the chromophores parallel. However, for [L-Ser^1] TANDEM it would seem possible for the chromophore attached to the D-Ser to become intercalated in the normal way while the other would be left dangling free in solution or stacked against the outside of the helix. The results presented here suggest that this is not the case. Any

interaction between [L-Ser^1] TANDEM and DNA is very weak, being three or four orders of magnitude weaker than that of TANDEM. This result is not entirely surprising since the quinoxaline-2 carboxamide chromophores themselves have been shown not to interact detectably with DNA (Wakelin & Waring, 1976). The binding of [L-Ser^1] TANDEM would be expected to be stronger than that of the chromophores alone because of interaction between the peptide portion and the DNA. However, this could be countered by sterically unfavourable interactions of the second chromophore with the DNA and it is possible that a molecule having only one chromophore attached to the intact peptide would have a higher affinity for DNA than does [L-Ser^1] TANDEM. According to this view the binding energy of the quinoxaline antibiotics does not derive from any single identifiable structural feature but depends upon the disposition of substituents throughout the whole molecule, involving both chromophores as well as the peptide portion. Additional support for this concept is provided by the observation that the peptide portion of TANDEM alone causes an elevation of the T_m of DNA (unpublished observations).

It is interesting that [L-Ser^1] TANDEM has the same effect on the T_m of polyrA.polyrU as TANDEM itself. As a group, quinoxaline antibiotics show no detectable interaction with natural RNAs but they do interact weakly with this synthetic ribopolymer helix (Wakelin & Waring, 1976; Lee & Waring, 1978b). Since [L-Ser^1] TANDEM elicits the same response as TANDEM it can be concluded that this mode of interaction is insensitive to the disposition of the chromophores with respect to the peptide ring and thus is highly unlikely to involve intercalative binding. While it cannot be dismissed as entirely 'non-specific' (witness the fact that it does not occur with RNA) it clearly differs from the interaction with DNA and may proceed via peculiar contacts between the peptide ring and an ordered 11 or 12 fold ribopolymer helix (Arnott, Fuller, Hodgson & Prutton, 1968).

The poly(dA-dT)-binding parameters measured for [Ala^3, Ala^7] TANDEM by solvent partition and spectrophotometric titration are in reasonably good agreement. They differ by less than a factor of 2 and this difference can easily be accounted for by the inaccuracies inherent in the techniques. The consonance between these determinations constitutes the first evidence which directly supports the validity of the solvent partition method (Waring *et al.*, 1975). However, they are higher by factors of 5 to 15 than the values estimated from the changes in melting temperatures. This is almost certainly attributable to incorrect assumptions in calculating binding constants from ΔT_ms. The treatment employed assumed that TANDEM and [Ala^3, Ala^7] TANDEM share the same mode of binding so that for similar values of r the two compounds should cause the same change in melting temperature. This assumption may be valid for compounds having similar values of $K(0)$ but could be seriously in error for compounds whose binding constants differ by as much as three orders of magnitude. The approach in this case is also complicated by the cooperative nature of the interaction of both ligands with poly(dA-dT). There is, in fact, some evidence for different modes of binding of these two compounds from the shapes of the thermal denaturation profiles. At intermediate r values the profiles seen with TANDEM were distinctly biphasic, as predicted by the theory of helix-coil transitions (McGhee, 1976). This is attributable to redistribution of ligand molecules released from parts of the helix which melt early rebinding to the remaining helical portions causing their further stabilization. With [Ala^3, Ala^7] TANDEM no such biphasic melting curves were seen, suggesting that, at elevated temperatures, once the drug has come off the helix, it does not associate with remaining helical regions within the experimental time course.

We thank Drs L.P.G. Wakelin, J.S. Lee and D.C. Goodwin for helpful criticism and stimulating discussion. This work was aided by grants from the Medical Research Council, the Royal Society, and the Cancer Research Campaign. K.R.F. acknowledges the support of an M.R.C. Research Studentship.

APPENDIX

CHEMICAL SYNTHESIS OF TRIOSTIN ANALOGUES

P.K. CHAKRAVARTY & R.K. OLSEN

Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra (NMR) were obtained with a Varian EM-360, EM-390 or XL-100-12 spectrometer; data are reported for selected intermediates. Optical rotations were recorded with a Rudolf and Sons Model 80 polarimeter. Thin layer chromatography (TLC) was performed on commercial silica gel on glass plates using the following solvent systems; A, chloroform–absolute ethanol (80:20); B, chloroform–absolute ethanol (90:10); C, chloroform–acetone (80:20); D, *n*-BuOH–acetic acid–water (10:2:3). Elemental analyses were performed by H-M-W Laboratories, Phoenix, Arizona. D-Serine and 2-quinoxalinecarbonyl chloride were purchased from Sigma and from Aldrich, respectively. All coupling reagents and L-amino acid derivatives were obtained from commercial sources. E. Merck silica gel 60 (230–400 mesh) was used for column chromatography.

Figure 1 Scheme for synthesis of [L-Ser[1]]-des-*N*-tetramethyltriostin A (3).

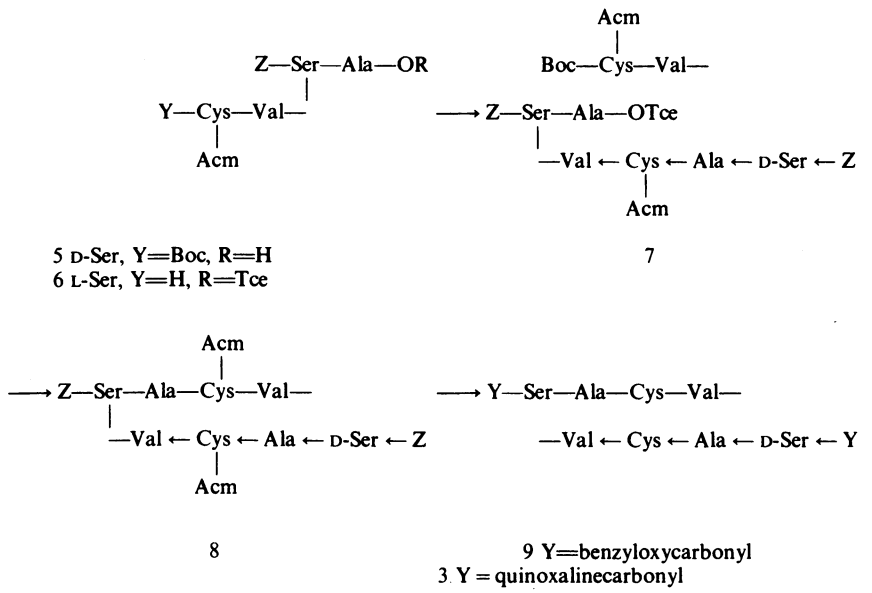
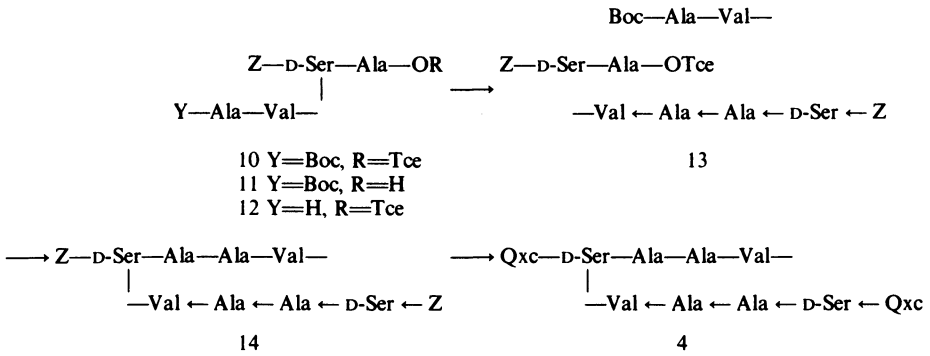


Figure 2 Scheme for synthesis of [Ala[3], Ala[7]]-des-*N*-tetramethyltriostin A (4)



The general procedure developed for synthesis of triostin A (1) and TANDEM (2) (Ciardelli *et al.*, 1978) was employed for the preparation of [L-Ser[1]] TANDEM (3) and [Ala[3], Ala[7]] TANDEM (4) as summarized in Figures 1 and 2 respectively. This procedure involves fragment coupling of appropriate tetradepsipeptides to give a linear octadepsipeptide which, after deprotection, is caused to undergo cyclization. The final steps in the syntheses involve disulphide bond formation, as required for analogue 3, and introduction of the quinoxalinecarbonyl moiety.

Preparation of N-*benzyloxycarbonyl*-O-[N-*benzyloxycarbonyl*-O-(N-tert-*butyloxycarbonyl*-S-*acetamidomethyl*-L-*cysteinyl*-L-*valyl*)-D-*seryl*-L-*alanyl*-S-*acetamido*-L-*cysteinyl*-L-*valyl*]-L-*seryl*-L-*alanine* β,β,β-*trichloroethyl ester* (7)

A stirred solution of *N*-benzyloxycarbonyl-*o*-(*N*-*tert*-butyl-oxycarbonyl-*S*-acetamidomethyl-L-cysteinyl-L-valyl)-D-seryl-L-alanine (5) (2.7 g, 3.92 mmol) (Ciardelli *et al.*, 1978) in dry tetrahydrofuran (30 ml) cooled to −15°C in a dry ice–acetone bath was treated with

N-methylmorpholine (0.44 ml, 4.0 mmol) and isobutylchloroformate (0.54 ml, 4.0 mmol). After 15 min, a solution of *N*-benzyloxycarbonyl-*O*-(*S*-acetamidomethyl-L-cysteinyl-L-valyl)-L-seryl-L-alanine β,β,β-trichloroethyl ester (4.0 mmol) (Ciardelli *et al.*, 1978) in 20 ml of tetrahydrofuran was added and the reaction mixture stirred at −15°C for an additional 30 min and then at room temperature overnight. The reaction mixture was concentrated *in vacuo* and the residue triturated with 10% sodium bicarbonate and filtered. The solid residue was washed successively with 10% sodium bicarbonate, water, 1 N hydrochloric acid, water, and dried. The product was recrystallized from methanol to give 4.6 g (80%) of white crystals, m.p. 164–166°C; $[\alpha]_D^{25} - 87 \pm 2°C$ (c 1.7, MeOH); R_F (solvent A) 0.80; (Found: C, 49.4; H, 6.20; N, 10.1; $C_{57}H_{81}N_{10}O_{19}S_2Cl_3$ requires C, 49.6; H, 5.91; N, 10.1).

(N-benzyloxycarbonyl-L-seryl-L-alanyl-S-acetamidomethyl-L-cysteinyl-L-valyl-N-benzyloxycarbonyl-D-seryl-L-alanyl-S-acetamidomethyl-L-cysteinyl-L-valine)(serine hydroxyl)dilactone (8)

Octadepsipeptide 7 (2.0 g, 1.45 mmol) in 90% aqueous acetic acid (40 ml) was stirred with 4.8 g of zinc powder at 0°C for 1.5 h. The mixture was filtered, washed with 90% aqueous acetic acid, and the combined filtrate and washings were concentrated to dryness *in vacuo*. The residue was shaken with a mixture of ethyl acetate (100 ml) and 1 N hydrochloric acid (40 ml) until the solid dissolved. The organic phase was washed with water and extracted with saturated sodium bicarbonate (4 × 70 ml) and water (50 ml). The combined bicarbonate extracts and water wash were washed once with ethyl acetate and then acidified with solid citric acid with cooling in an ice bath. The aqueous phase was saturated with sodium chloride and extracted with ethyl acetate (3 × 70 ml). The combined ethyl acetate extracts were washed with water and dried over magnesium sulphate. Evaporation of the solvent *in vacuo* gave 1.67 g of a solid material; m.p. 157–162°C.

The above material was dissolved in anhydrous trifluoroacetic acid (7 ml) at room temperature and allowed to stir for 30 min. The solvent was removed *in vacuo* and the residue triturated with dry ether, filtered, washed with ether and dried over phosphorous pentoxide and sodium hydroxide. The solid material was dissolved in dimethylformamide (38 ml). The resulting solution was cooled to 0°C and *N*-methylmorpholine (0.14 ml, 1.29 mmol) and 1-hydroxybenzotriazole monohydrate (0.75 g, 4.9 mmol) were added, followed by dilution of the reaction mixture with 480 ml of tetrahydrofuran. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.60 g, 3.13 mmol)) was added and the reaction mixture was stirred at 0°C for 30 min and at room temperature for 4 days. The solvent was removed *in vacuo* and the residue was triturated with water, filtered, and washed successively with 10% sodium bicarbonate, 1 N-hydrochloric acid, and water. After drying, the crude product was purified on a column of silica gel 60 by elution with chloroform–ethanol (9:1). The fractions containing 8, having R_F 0.35 in solvent B, were pooled and concentrated *in vacuo*. Recrystallization from ethyl acetate-light petroleum (b.p. 30–60°C) gave 420 mg (30% yield) of product: m.p. 138–140°C; $[\alpha]_D^{25} - 52 \pm 2°C$ (c 1, MeOH); NMR ($CDCl_3$–DMSO-d_6, 1:1) δ 1.0 (d, 12 H, valyl methyls), 1.4 (q, 6 H, alanyl methyls), 1.8–2.2 (m, 8 H, valyl methynes and Acm methyls), 2.9 (m, 4 H, cysteinyl methylenes), 4.0–5.0 (m, 16 H, α-hydrogens, Acm and seryl methylenes), 5.2 (d, 4 H, benzylic), 7.2–8.9 (m, 20 H, phenyl and NH); (Found: C, 52.9; H, 6.40; N, 12.2; $C_{50}H_{70}N_{10}O_{16}S_2$ requires C, 53.1; H, 6.24; N, 12.4).

(N-benzyloxycarbonyl-L-seryl-L-alanyl-L-cysteinyl-L-valyl-N-benzyloxycarbonyl-D-seryl-L-alanyl-L-cysteinyl-L-valine)(serine hydroxyl)dilactone disulphide (9)

To a solution of 8 (150 mg, 0.13 mmol) in 24 ml of methanol was added dropwise a solution of iodine (170 mg, 0.67 mmol) in methanol (40 ml) over 1.5 h at room temperature. The reaction mixture was stirred for 3 h, then cooled to 0°C and decolourized with 1 N sodium thiosulphate. The solvent was removed *in vacuo* and the residue was triturated with water, filtered, washed with water, and dried. The product was purified on a column of silica gel 60 by elution with chloroform–ethanol (9:1). The fractions containing product (R_F 0.62 in solvent B) were pooled and evaporated *in vacuo*. Recrystallization of this material from chloroform-diethyl ether gave 120 mg (92%) of 9: m.p. 163–166°C, $[\alpha]_D^{25} - 78 \pm 2°C$ (c 1, MeOH); (Found: C, 53.4; H, 5.86; N, 11.3; $C_{44}H_{58}N_8O_{14}S_2$ requires C, 53.5; H, 5.92; N, 11.4).

[L-Ser^1]-des-N-tetramethyltriostin A (3)

The benzyloxycarbonyl groups were removed by treatment of a solution of 9 (160 mg, 0.16 mmol) in 4.3 ml of acetic acid with 4 N hydrogen bromide in acetic acid (9 ml) for 1 h at room temperature. The dihydrobromide salt was precipitated by the addition of anhydrous diethyl ether, filtered, washed with ether, and dried over P_2O_5 and NaOH.

The above salt was dissolved in dimethylformamide (7 ml) and neutralized with triethylamine (0.045 ml, 0.32 mmol) at 0°C. Quinoxaloyl chloride (63 mg, 0.32 mmol, obtained from Aldrich Chemical Co.) and triethylamine (0.045 ml, 0.32 mmol) were added simultaneously in small portions to the stirred reaction mixture, which was then stirred at 0°C for 30 min and

at room temperature for 24 h. The solvent was removed *in vacuo* and the residue obtained was triturated with water, filtered, washed with 10% sodium bicarbonate, water, ether, and dried. Chromatography of this material on a column of silica gel 60 with elution by chloroform–ethanol (9:1) gave product (R_F 0.8, solvent A). Recrystallization from chloroform–ether gave 70 mg (43%) of 3; m.p. 230–243°C; $[\alpha]_D^{25} - 36 \pm 2°C$ (c 1, $CHCl_3$); NMR ($CDCl_3$) δ 0.9–1.5 (m, 18 H, valyl and alanyl methyls), 2.1 (m, 2 H, valyl methynes), 2.9 (m, 4 H, cysteinyl methylenes), 4.3–5.3 (m, 10 H, α-hydrogens and seryl methylenes), 5.6 (m, 2 H, α-hydrogens), 6.6 (m, 2 H, NH), 7.0 (m, 2 H, NH), 7.6–8.3 (m, 8 H, quinoxaline 5, 6, 7, 8 hydrogens), 8.4–8.9 (m, 4 H, NH), 9.6 (pair of S, 2 H, quinoxaline 3 hydrogens); (Found: C, 53.5; H, 5.34; N, 16.2; $C_{46}H_{54}N_{12}O_{12}S_2$ requires C, 53.6; H, 5.24; N, 16.3).

N-*benzyloxycarbonyl*-O-(N-tert-*butyloxycarbonyl*-L-*alanyl*-L-*valyl*)-D-*seryl*-L-*alanine*,β,β,β-*trichloroethyl ester* (*10*)

The free amine obtained from *N*-benzyloxycarbonyl-*O*-(*N*-*tert*-butyloxycarbonyl-L-valyl)-D-seryl-L-alanine β,β,β-trichloroethyl ester (3.2 g, 5.0 mmol), prepared as previously described (Ciardelli, Chakravarty & Olsen, 1978), was treated with *N*-*tert*-butyloxycarbonyl-L-alanine (0.97 g, 5.13 mmol) and 1-hydroxybenzotriazole monohydrate (1.54 g, 10.0 mmol) in 30 ml of tetrahydrofuran at 0°C. A solution of *N*,*N*'-dicyclohexylcarbodimide (1.1 g, 5.4 mmol) in 15 ml tetrahydrofuran was added dropwise to the stirred reaction mixture. After stirring an additional 2 h at 0°C, the mixture was allowed to stand overnight at 0°C. The solid material was removed by filtration and the filtrate was concentrated *in vacuo*. The oil obtained was taken up in ethyl acetate (100 ml), washed with 10% sodium bicarbonate, water, 10% citric acid, water and dried over magnesium sulphate. Filtration and removal of solvent *in vacuo* gave a solid, which upon recrystallization from diethyl ether-light petroleum (b.p. 30–60°C) yielded 3.40 g (96%) of product: m.p. 88–89°C; $[\alpha]_D^{25} - 39 \pm 2°C$ (c 1, MeOH); R_F 0.40 (Solvent C); (Found: C, 48.7; H, 5.72; N, 8.03; $C_{29}H_{41}N_4O_{10}Cl_3$ requires C, 48.9; H, 5.76; N, 7.87).

N-*benzyloxycarbonyl*-O-(N-tert-*butyloxycarbonyl*-L-*alanyl*-L-*valyl*)-D-*seryl*-L-*alanine* (*11*)

A solution of 10 (2.50 g, 3.51 mmol) in 80% aqueous acetic acid (80 ml) was stirred at 0°C and zinc powder (6.6 g) was added in portions during a period of 30 min. The reaction mixture was stirred at 0°C for 1 h. filtered, and the solvent was removed *in vacuo*. The residue obtained was shaken with ethyl acetate (60 ml) 1 N hydrochloric acid (30 ml) to dissolve all solid material. The organic phase was washed with water and extracted with 10% sodium bicarbonate (3 × 40 ml) and water. The bicarbonate extract and washings were combined, washed once with ethyl acetate, and acidified at 0°C with solid citric acid. The product was extracted with ethyl acetate (3 × 30 ml) and the combined organic extracts were washed with water and dried over magnesium sulphate. The solvent was removed *in vacuo* and the solid obtained was recrystallized from ethyl acetate-diethyl ether-light petroleum (b.p. 30–60°C): 1.85 g of 11 (91%); m.p. 156–157°; $[\alpha]_D^{25} - 84 \pm 2°C$ (c 1.6, MeOH); R_F 0.70 (Solvent D); NMR ($CDCl_3$) δ 0.9 (d, 6 H, valyl methyls), 1.2–1.8 (m, 15 H, t-butyl and alanyl methyls), 2.0 (m, 1 H, valyl methyne), 4.0–5.0 (m, 6 H, α-hydrogens and seryl methylenes), 5.1–5.8 (m, 3 H, benzylic and NH), 6.6 (m, 1 H, NH), 7.0–8.5 (m, 8 H, aromatic, NH, and COOH); (Found: C, 55.9; H, 6.81; N, 9.52; $C_{27}H_{40}N_4O_{10}$ requires C, 55.9; H, 6.90; N, 9.66).

N-*benzyloxycarbonyl*-O-[N-*benzyloxycarbonyl*-O-(N-tert-*butyloxycarbonyl*-L-*alanyl*-L-*valyl*)-D-*seryl*-L-*alanyl*-L-*alanyl*-L-*valyl*]-D-*seryl*-L-*alanine* β,β,β-*trichloroethyl ester* (*13*)

Tetradepsipeptide 10 (1.85 g, 2.60 mmol) was stirred with anhydrous trifluoroacetic acid (3 ml) for 30 min at room temperature. The solvent was removed *in vacuo* and the residue was taken up in ethyl acetate. The organic phase was washed twice with 10% sodium bicarbonate, with water, and dried over magnesium sulphate. Filtration and removal of the solvent *in vacuo* gave the amine 12 as a solid having R_f 0.51 in solvent E.

The amine 12 was combined with 11 (1.50 g, 2.60 mmol) and 1-hydroxybenzo-triazole monohydrate (0.79 g, 5.16 mmol) in tetrahydrofuran (30 ml) at 0°C. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.55 g, 2.86 mmol) was added and the reaction mixture was stirred for 4 h at 0°C; during which time some product separated, and overnight at room temperature. The solvent was removed *in vacuo* and the residue obtained was triturated with water and filtered. The solid product was washed with 10% sodium bicarbonate, water, cold 1 N hydrochloric acid, and water. After drying, the solid material was recrystallized from methanol-diethyl ether to yield 2.7 g (89%) of product: m.p. 170–172°C; $[\alpha]_D^{25} - 58 \pm 2°C$ (c 1, MeOH); R_F 0.52 (Solvent A); NMR ($CDCl_3$–DMSO-d_6, 1:1) δ 0.95 (d, 12 H, valyl methyls), 1.45 (m, 21 H, t-butyl and alanyl methyls, 2.1 (m, 2 H, valyl methynes), 4.0–5.0 (m, 14 H, seryl and Tce methylenes, α-hydrogens), 5.1 (s, 4 H, benzylic), 6.2 (m, 1 H, NH). 7.0–8.3 (m, 17 H, aromatic and

NH); Found: C, 52.4; H, 6.21; N, 9.78; $C_{51}H_{71}N_8O_{17}Cl_3$ requires C, 52.2; H, 6.05; N, 9.54).

(N-benzyloxycarbonyl-D-seryl-L-alanyl-L-alanyl-L-valine)$_2$(serine hydroxyl) dilactone (14)

A solution of 13 (920 mg, 0.78 mmol) in 25 ml of 90% aqueous acetic acid was treated with zinc powder (2 g) in a similar manner as described above for 8 to yield solid material (770 mg, 94%), m.p. 198–200°C.

The above material was treated with trifluoroacetic acid (2.5 ml) at room temperature for 30 min. The solvent was removed *in vacuo* and the residue was triturated with diethyl ether, filtered, and dried over phosphorous pentoxide and sodium hydroxide. This product, having a melting point of 205–208°C dec, was dissolved in dry dimethylformamide (21 ml) and the solution was cooled in an ice bath. *N*-methylmorpholine (0.083 ml, 0.74 mmol) was added followed by dilution with 265 ml of dry tetrahydrofuran. *N*-hydroxysuccinimide (350 mg, 3.0 mmol) was added followed by the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (420 mg, 2.18 mmol). The reaction mixture was stirred at 0°C for 1 h and at room temperature for 4 days. The reaction mixture was worked up as described above for compound 8. The crude product was purified on a column of silica gel 60 by elution with chloroform–ethanol (9:1). The fractions containing product (R_f 0.37, Solvent B) were combined and the solvent removed *in vacuo*. Recrystallization from chloroform-diethyl ether gave 230 mg (34%) of 14: m.p. 265–267°C, $[\alpha]_D^{25}$ − 75 ± 2°C (c 1, MeOH); (Found: C, 57.3; H, 6.45; N, 12.0; $C_{44}H_{60}N_8O_{14}$ requires C, 57.2; H, 6.39; N, 12.1).

[Ala^3, Ala^7]-des-N-tetramethyltriostin A (4)

Cyclic depsipeptide 14 (350 mg, 0.38 mmol) was treated with 15 ml of 4 N hydrogen bromide in acetic acid at room temperature for 1 h. The hydrobromide salt was precipitated by the addition of anhydrous diethyl ether, filtered, and dried *in vacuo* over phosphorous pentoxide and sodium hydroxide for 1 h. The hydrobromide salt was dissolved in 10 ml of anhydrous dimethylformamide and neutralized with triethylamine (0.11 ml, 0.78 mmol). Quinoxaloyl chloride (160 mg, 0.85 mmol) and triethylamine (0.12 ml, 0.85 mmol) were added simultaneously in small portions at room temperature. The reaction mixture was stirred for 24 h and then worked up as described above for analogue 3. Following chromatography on a column of silica gel 60 and elution with chloroform–ethanol (8:2), the fractions containing product (R_f 0.20, solvent B) were combined, and the solvent evaporated *in vacuo*. The product was recrystallized from chloroform–diethyl ether to give 190 mg (51%) of 4: m.p. 188–190°C; $[\alpha]_D^{25}$ − 67 ± 2°C (c 1, MeOH); NMR ($CDCl_3$–DMSO-d_6, 1:1) δ 0.85 (dd, 12 H, valyl methyls), 1.35 (dd, 12 H, alanyl methyls), 2.12 (m, 2 H, valyl methynes), 4.2–5.1 (m, 12 H, seryl methylenes and α-hydrogens), 7.53 (d, 2 H, NH), 7.7–8.2 (m, 12 H, quinoxaline 5, 6, 7, 8 hydrogens, NH), 8.80 (d, 2 H, NH), 9.47 (s, 2 H, quinoxaline 3 hydrogens); (Found: C, 56.9; H, 5.79; N, 17.2; $C_{46}H_{56}N_{12}O_{12}$ requires C, 57.1; H, 5.69; N, 17.4).

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