CHANGES IN CONTOUR LENGTH OF POLYDEOXYNUCLEOTIDE FRAGMENTS

Direct evidence for bifunctional intercalative binding of antibiotic ligands

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1. Introduction

Bifunctional intercalating agents, such as the quinoxaline antibiotic echinomycin (fig.1), are of major importance as potential chemotherapeutic drugs [1,2]. They are expected to bind to DNA more tightly, and with greater selectivity, than comparable simple (monofunctional) intercalators. Echinomycin was the first bifunctional intercalator to be identified [3] and consequently its DNA-binding properties have been extensively characterised: it does indeed display considerable sequence-selectivity, which is evident in its interaction with natural DNAs as well as with synthetic polynucleotides [4]. The same is true of other quinoxaline antibiotics but their patterns of sequence-selectivity differ radically from that seen with echinomycin, especially as regards their binding to poly(dA–dT) and poly(dG–dC) [5–7]. To date, however, there has been no direct evidence that the nature of the complex formed with a synthetic polynucleotide resembles that formed with natural DNA, though it has generally been assumed that all such

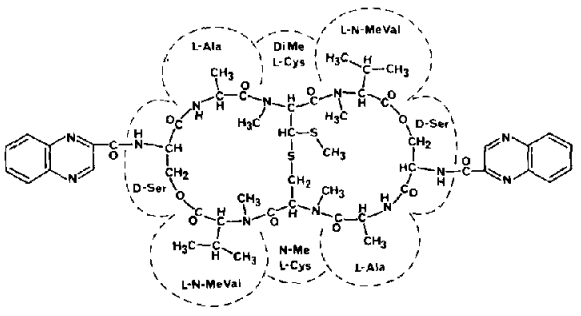

Fig.1. Structure of echinomycin.

complexes involve bifunctional intercalation. It therefore seemed important to investigate whether differences in apparent sequence selectivity could be the result of altered modes of binding to different DNAs, especially with respect to their mono- or bifunctional character.

Intercalative binding to poly(dA–dT) can be evidenced by simple viscometry [8]. Here, this technique has been extended to permit direct investigation of the intercalative binding of various quinoxaline antibiotics to poly(dA–dT), and for the first time to poly(dG–dC). By way of comparison similar studies were also performed with the monofunctional intercalators ethidium and actinomycin D.

2. Materials and methods

All experiments were conducted at 20°C in a Hepes [4(2-hydroxyethyl)-1-piperazine–ethane sulphonic acid]/NaOH buffer at pH 7.0 and ionic strength 0.01, designated 0.01 SHE [4]. Reagent grade water from a Millipore Milli Q2 system was used throughout. Echinomycin was a gift from Drs H. Bickel and K. Scheibli, Ciba-Geigy Ltd (Basel). Triostin A, a product of Shionogi and Co. (Osaka) was a gift of Drs H. Otsuka and T. Yoshida. The *bis*-quinoline analogue of echinomycin, referred to as 2QN, was produced by supplementing cultures of *Streptomyces echinatus* with quinoline-2-carboxylic acid as in [9]. Actinomycin D was a product of Merck, Sharp and Dohme; ethidium bromide was a gift from Dr S. S. Berg, May and Baker Ltd (Dagenham). Poly(dG–dC) was a product of P. L. Biochemicals. Poly(dA–dT) was obtained from Boehringer Corp (London) Ltd. These

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synthetic DNAs were found to be readily soluble in 0.01 SHE buffer; concentrated solutions were dialysed extensively against 0.01 SHE, filtered through 2 Whatman GF/C glass-fibre filters, and stored frozen at −22°C. Nucleic acid concentrations are expressed with respect to nucleotides and are based on the following values of $\epsilon(P)_{260}$: poly(dA–dT), 6700 [10]; poly(dG–dC), 7100 [11]. Molar extinction coefficients of the quinoxaline antibiotics were taken from [7,12]. Concentrations of other drugs were based on the following extinction coefficients: actinomycin D, 24 450 (440 nm) [13]; ethidium bromide, 5900 (480 nm) [14].

The viscometric technique is well established as a method for investigating the extension of the DNA helix associated with intercalation [15–18]. Under conditions where the DNA molecules behave as stiff rods:

$$L/L_0 = (\eta/\eta_0)^{1/3} \quad [16]$$

where L, L_0 are the contour lengths of the DNA in the presence and absence of the drug respectively; η and η_0 are the corresponding values of the intrinsic viscosity (approximated by the reduced viscosity) of the solution. The 'base-pair sandwich' model for intercalation requires that the helix be extended by an amount equal to the thickness of a base-pair (3.4 Å) for each intercalation event. This gives the helix extension ratio $L/L_0 = 1 + 2r$ for monofunctional intercalation and $L/L_0 = 1 + 4r$ for bifunctional intercalation [18], where r represents the binding ratio

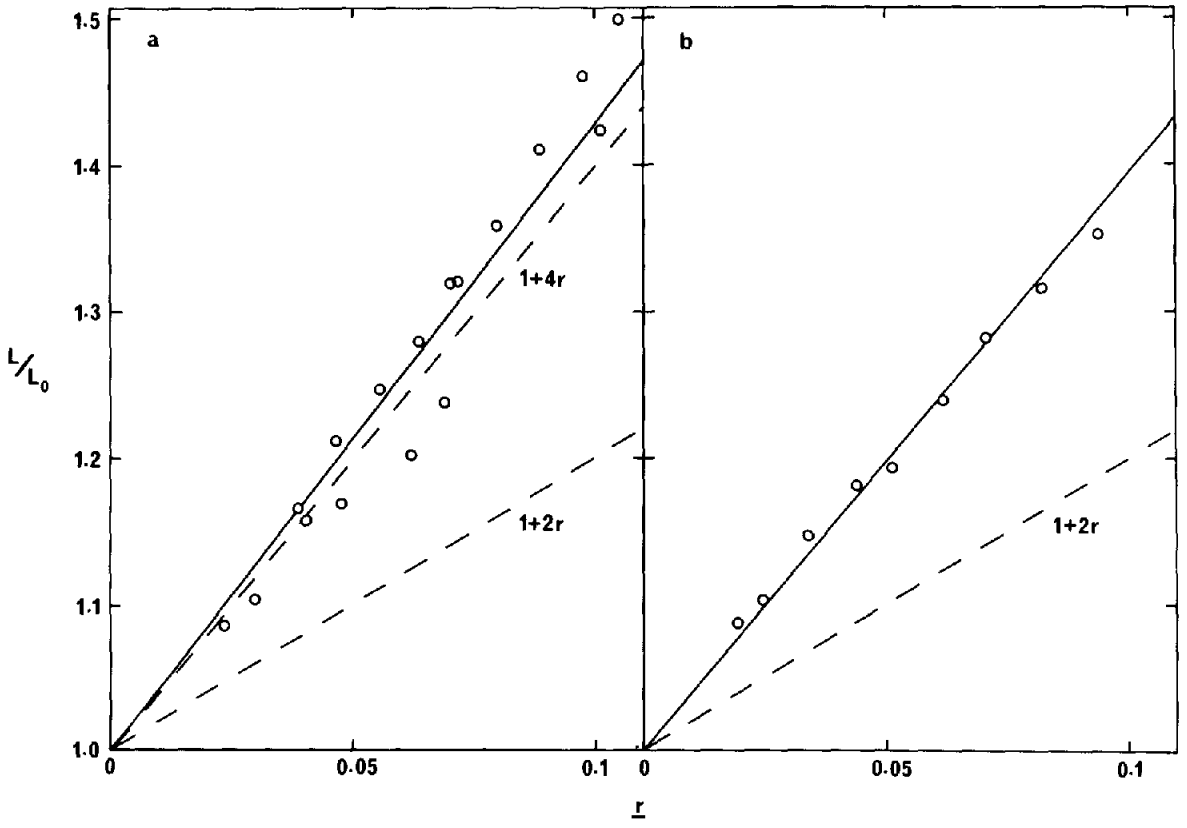

Fig.2. Effect of echinomycin on the relative contour length of (a) poly(dA–dT) and (b) poly(dG–dC). The ordinate represents the fractional increase in helix length; the abscissa shows the binding ratio (r), drug molecules bound/nucleotide, calculated from the input ratio (D/P) using the binding parameters in [4]. The lines passing through the experimental points were fitted by the method of least squares and constrained to pass through the origin (0,1). Their slopes are 4.29 ± 0.33 for poly(dA–dT) and 3.95 ± 0.16 for poly(dG–dC). Also shown are theoretical lines (broken) representing slopes of 2 and 4 corresponding to idealised monofunctional and bifunctional intercalation, respectively.

expressed as mol ligand bound/mol. nucleotides.

All the poly(dG–dC) and some of the poly(dA–dT) samples used in these experiments were sonicated to reduce their M_r-value using the exponential probe of an MSE 150W ultrasonic disintegrator at the highest power level, as in [8].

Experiments with quinoxaline antibiotics were performed using apparatus and procedures as in [5]. Complexes with a high binding ratio (r) were prepared by 'solid shake' procedure. Mixtures having lower r were generated from these by diluting with DNA in the viscometer. Complexes of ethidium and actinomycin D with the polynucleotides were prepared by direct addition of small amounts of a concentrated drug solution to the DNA in the viscometer as in [18]. Estimates of the binding ratio, r, were based on published values of association constants and site-sizes in buffer of 10 mM ionic strength [7,12, 19].

3. Results

Figure 2 shows helix extension plots for echinomycin binding to poly(dA–dT) and poly(dG–dC). The slopes of the least-squares lines fitted to the experimental points are, in each case, close to the theoretical value of 4 for ideal bifunctional intercalation. Two samples of poly(dA–dT) were employed to establish that the result was not dependent on the length of the DNA. One had reduced viscosity 5.08 dl/g, corresponding to an av.M_r of 6.5×10^5, just above the value of 5×10^5 recommended to

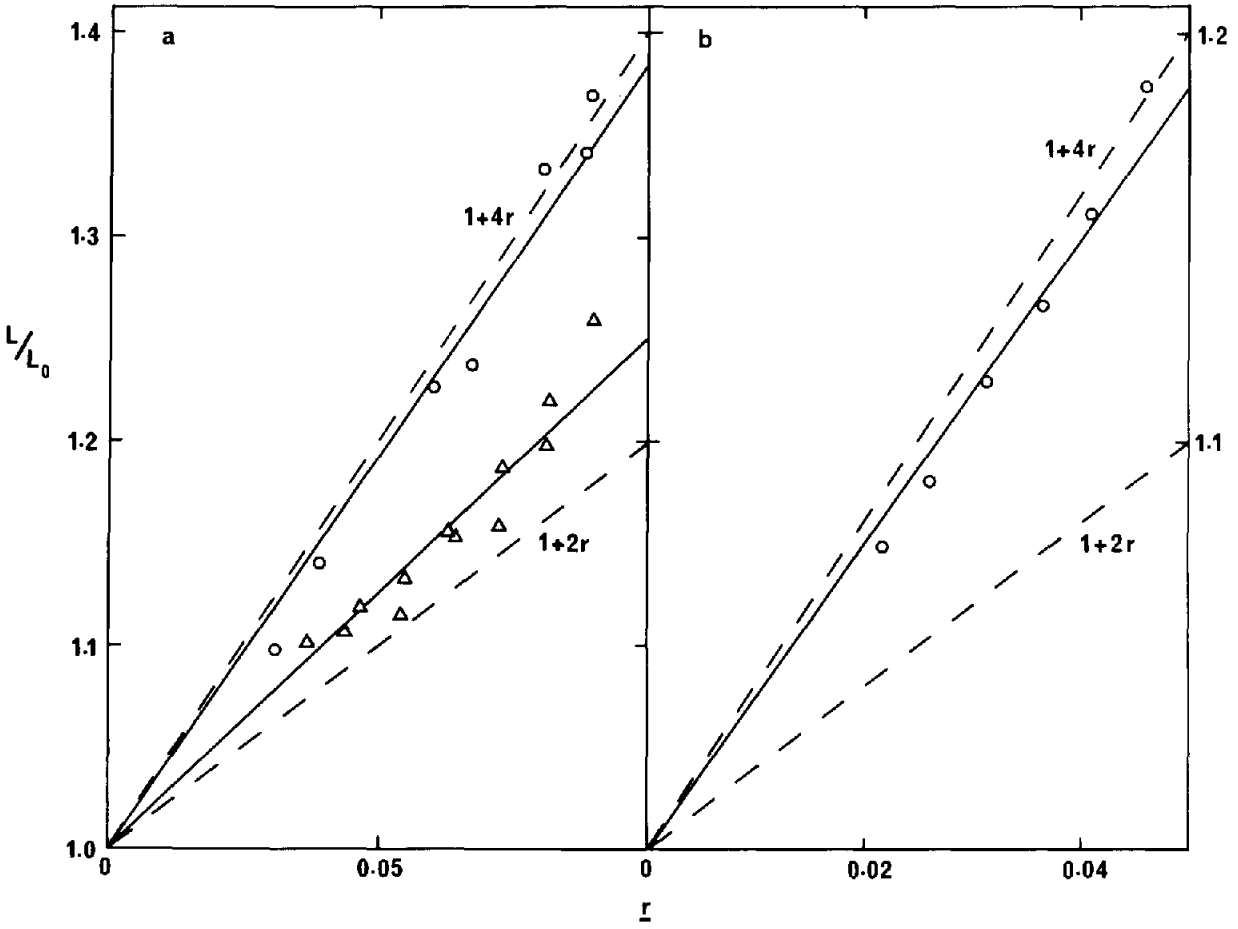

Fig.3.(a) Effect of triostin A on the relative contour length of poly(dA–dT) (○) and poly(dG–dC) (△). The slopes of the lines fitted to the experimental points are 3.85 ± 0.36 for poly(dA–dT) and 2.51 ± 0.29 for poly(dG–dC). (b) Effect of 2QN on the relative contour length of poly(dA–dT). The least-squares line fitted to the experimental points has slope 3.72 ± 0.46.

ensure that the DNA behaves as a rigid rod [15,16]. The other sample had been sonicated for 5 min so as to lower its reduced viscosity to 1.05 dl/g, corresponding to an av. M_r of 3×10^5. There was no significant difference between the two sets of data.

In fig.3a the corresponding plots for interaction with triostin A are presented. Here the least-squares slopes are rather lower, though that for poly(dA–dT) again lies close to the theoretical value of 4. However, the slope measured for poly(dG–dC), while greater than that predicted for simple (monofunctional) intercalation, is clearly much less than 4. The helix extension with poly(dA–dT) produced by 2QN is shown in fig.3b. As with the two natural antibiotics the slope approximates to 4, indicative of bifunctional intercalation.

To assist the interpretation of these results in terms of mono- or bifunctional interaction we sought to compare them with the effects of a 'classical' monofunctional intercalator: ethidium (fig.4). With this drug the changes in contour length for both polynucleotides were much smaller, yielding least-squares lines having slopes well below the predicted value of 2 or the experimental value of 1.79 reported for calf thymus DNA [17].

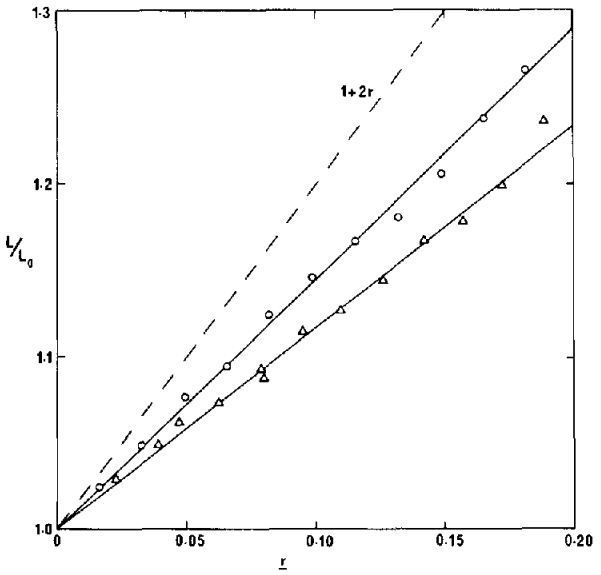

Fig.4. Effect of ethidium on the relative contour length of poly(dA–dT) (○) and poly(dG–dC) (△). The abscissa shows the binding ratio calculated using the parameters given in [19]. The least-squares lines drawn through the experimental points are characterised by slopes of 1.44 ± 0.03 for poly(dA–dT) and 1.17 ± 0.04 for poly(dG–dC).

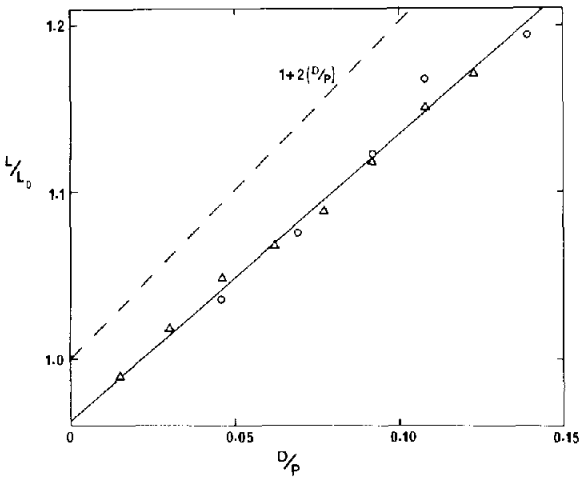

Fig.5. Effect of actinomycin D on the relative contour length of poly(dG–dC). The abscissa shows the input ratio of drug molecules to nucleotides. The line fitted to the experimental points by the method of least squares has slope 1.72 ± 0.05 and intercept 0.9378.

Perhaps a better control against which to compare the actions of quinoxaline antibiotics is the effect of actinomycin D, an intercalating peptide antibiotic of comparable M_r [1,2]. Binding of this ligand is restricted to poly(dG–dC) by virtue of its requirement for at least 1 GC base-pair; however, the resulting plot quite clearly does not pass through the origin (fig.5). This curious effect might be due to some sort of rearrangement of the DNA strands, possibly by a slippage mechanism, at low levels of drug binding. At all events, the slope of the best straight line shown in fig.5 approximates to 2, though it is presented in terms of ratio of total added antibiotic molecules to DNA nucleotides (D/P) rather than the actual binding ratio (r). Unfortunately, reliable binding parameters for the interaction of actinomycin with poly(dG–dC) at this ionic strength are not available. The correction from D/P to r should be small at low values of D/P but could become significant at higher values, which would result in an upward curvature of the line.

4. Discussion

The results of all the experiments with quinoxalines and poly(dA–dT) are clear-cut: each antibiotic produces a good straight line through the origin having slope close to 4, exactly as expected for ideal bifunc-

tional reaction. A similar experiment using TANDEM, the synthetic *des*-tetramethyl analogue of triostin A, has also yielded the same result [20]. We can therefore state with confidence that the binding of all these ligands to poly(dA–dT) displays an essential characteristic of *bis*-intercalation.

The results with poly(dG–dC) are less easily interpreted. Although the helix extension measured for echinomycin lies close to the expected bifunctional value, that seen with triostin A seems closer to the value expected for a monofunctional intercalator. However, the extension produced by both antibiotics is well over twice the value obtained with ethidium. On that basis we might reasonably conclude that triostin A does indeed interact with poly(dG–dC) in *bis*-intercalative fashion, but, if so, with echinomycin certain additional interactions must occur to produce an even greater effect on the apparent contour length of this polynucleotide. In actual fact, although the extension produced by ethidium falls short of the 'ideal' value of 3.4 Å it does lie within the range of values determined for the interaction between other intercalators and natural DNAs [21]. This could mean either that a 3.4 Å increase is only an approximation or that the structure of our synthetic DNAs is significantly different from B-DNA. What is more surprising is the observation that ethidium produces a different helix extension with the two DNAs (fig.4). This could reflect minor differences in the mechanism of intercalation into various sequences, and/or differences in the structure of the two polynucleotides. An alternating B-structure has been proposed for poly(dA–dT) [22] whereas at low ionic strengths poly(dG–dC) appears to adopt a more classical B-form [23]. Thus even a simple monofunctional intercalator, displaying no pronounced sequence preferences, can produce different helix extensions with different sequences.

There could be several reasons why echinomycin produces a greater helix extension with poly(dG–dC) than does triostin A, and why both effects are more than double that of ethidium. The simple *bis*-intercalation model assumes that the two base-pairs sandwiched between the chromophores of the antibiotic are unperturbed. It seems quite likely that some distortion of their structure may occur, optimizing any hydrogen bonding contacts with the peptide backbone of the antibiotic. This extra distortion could easily be less marked with triostins than quinomycins, for the longer cross-bridge of the triostins can adopt more than one stable conformation in solution, and is generally more flexible [24]. However, it is not obvious why this could only result in different helix extension with poly(dG–dC).

The extension of poly(dG–dC) seen with actinomycin D is entirely consistent with its intercalative mode of binding and is larger than found with ethidium, as was also observed with calf thymus DNA [21]. The unusual feature of its effect is the apparent decrease in contour length at low binding ratios. It is possible that this effect may be general but was not observed with quinoxaline antibiotics because of the different method used to prepare the complexes. With actinomycin the drug was titrated into the DNA, whereas with the quinoxalines a high *r* complex was gradually 'diluted' with fresh DNA. A similar decrease was also observed for the binding of [Ala^3, Ala^7]-TANDEM to poly(dA–dT) where the complexes were again prepared by direct addition of drug to the DNA [8]. We are inclined to the view that polynucleotides may undergo certain structural changes at low binding ratios, possibly related to those suggested in [25] for poly(dG–dC) and in [20] for poly(dA–dT) from kinetic investigations. They may reflect little more than rearrangement of the chains of alternating polynucleotides by a slippage mechanism, in which the gross helical structure of the DNA remains unchanged but the average fragment length might decrease.

Acknowledgements

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References

[1] Waring, M. J. (1981) Annu. Rev. Biochem. 50, 159–192.
[2] Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H. and Waring, M. J. (1981) The Molecular Basis of Antibiotic Action, 2nd edn, Wiley, London.
[3] Waring, M. J. and Wakelin, L. P. G. (1974) Nature 252, 653–657.
[4] Wakelin, L. P. G. and Waring, M. J. (1976) Biochem. J. 157, 721–740.
[5] Lee, J. S. and Waring, M. J. (1978) Biochem. J. 173, 115–128.
[6] Lee, J. S. and Waring, M. J. (1978) Biochem. J. 173, 129–144.

[7] Fox, K. R., Gauvreau, D., Goodwin, D. C. and Waring, M. J. (1980) Biochem. J. 191, 729–742.
[8] Fox, K. R., Olsen, R. K. and Waring, M. J. (1980) Brit. J. Pharmacol. 70, 25–40.
[9] Bradley, S. G., Gauvreau, D. and Waring, M. J. (1980) Dev. Industr. Microbiol. 21, 245–253.
[10] Wells, R. D. and Wartell, R. M. (1974) in: Biochemistry of Nucleic Acids (Kornberg, H. L. et al. eds) vol. 6, pp. 41–64, University Park Press, Baltimore MD.
[11] Winkle, S. A. and Krugh, T. R. (1981) Nucleic Acids Res. 9, 3175–3186.
[12] Waring, M. J. (1979) in: Antibiotics, vol. 5/pt 2: Mechanism of Action of Antieukaryotic and Antiviral Compounds (Hahn, F. E. ed) pp. 173–194, Springer-Verlag, Berlin, New York.
[13] Bittman, R. and Blau, L. (1975) Biochemistry 14, 2138–2145.
[14] Wakelin, L. P. G. and Waring, M. J. (1974) Mol. Pharmacol. 10, 544–561.
[15] Cohen, G. and Eisenberg, H. (1966) Biopolymers 4, 429–440.
[16] Cohen, G. and Eisenberg, H. (1969) Biopolymers 8, 45-55.
[17] Reinert, K. E. (1973) Biochim. Biophys. Acta 319, 135–139.
[18] Wakelin, L. P. G., Romanos, M., Chen, T. K., Glaubiger, D., Canellakis, E. S. and Waring, M. J. (1978) Biochemistry 17, 5057–5063.
[19] Baguley, B. C. and Falkenhaug, E. M. (1978) Nucleic Acids Res. 5, 161–171.
[20] Fox, K. R., Olsen, R. K. and Waring, M. J. (1981) in preparation.
[21] Hogan, M., Dattagupta, N. and Crothers, D. M. (1979) Biochemistry 18, 280–288.
[22] Klug, A., Jack, A., Viswamitra, M. A., Kennard, O., Shakked, Z. and Steitz, T. A. (1979) J. Mol. Biol. 131, 669–680.
[23] Cohen, J. S., Wooten, J. B. and Chatterjee, C. L. (1981) Biochemistry 20, 3049–3055.
[24] Kalman, J. R., Blake, T. J., Williams, D. H., Feeney, J. and Roberts, G. C. K. (1979) J. Chem. Soc. Perkin I, 1313–1321.
[25] Krugh, T. R., Hook, J. W., Lin, S. and Chen, F.-M. (1979) Stereodynamics of Molecular Systems (Sarma, R. H. ed) pp. 423–434, Pergamon Press, New York.