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MOLECULAR RECOGNITION BY SECONDARY METABOLITES

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Abstract—Aspects of molecular recognition based on the interaction between the vancomycin group of antibiotics and bacterial cell wall precursor analogues are discussed. The energetically unfavourable folding-in of the residue 1 sidechain in vancomycin and ristocetin A is discussed in terms of the favourable entropy associated with simultaneous release of solvent molecules. The effect of the sugar amino substituent on the strength of an adjacent hydrophobic interaction in the vancomycin/acetyl-D-Ala-D-Ala complex is rationalised as an intramolecular "salting-out" of hydrocarbon entities. The slow on-rate for dimerisation of the ristocetin A/*N,N*-diacetyl-L-Lys-D-Ala-D-Ala complex is attributed to the need for the relatively rigid peptide backbone of the antibiotic to be extensively desolvated before dimerisation can occur. Some of these concepts are then applied to understanding the interactions between antibiotics and the minor groove of double-helical DNA, the receptor site with which they have probably evolved to interact. Two structural motifs (π-polarised aromatic rings and deoxy sugars) are postulated to be important in this recognition process. The possible roles of these structural features are discussed.

We have discussed previously the molecular basis of the activity of antibiotics of the vancomycin group [1]. More recently, we have presented the case that this class of antibiotics, which acts by binding to cell wall mucopeptide precursors terminating in D-Ala-D-Ala, has specifically evolved to perform this physiological function [2]. Strong support for this view derives not only from the beautiful complementarity between the antibiotics and the cell wall mucopeptide precursors, but also from the large amount of DNA which must be required to code for their biosynthesis. In the present paper, we point out some sophistications of the antibiotic/cell-wall molecular recognition processes which support further the Darwinian arguments, and also note that an extension of the arguments leads to the view that all secondary metabolites should have evolved to fit specific receptors (see also [2]). In particular, we argue that in the case where the receptor has a known structure, specifically in the case of the DNA double helix, a perusal of natural product structures that bind to DNA can lead to predictions as to how the molecular recognition may be effected.

Dynamics and specificity of vancomycin group antibiotics when recognising peptides terminating in D-Ala-D-Ala

The structure of vancomycin, and an exploded view of the complex that it forms with acetyl-D-Ala-D-Ala, are given in Fig. 1. In referring to this diagram, we will number the amino acid residues from the N-terminus to the C-terminus of the antibiotic as 1–7, and the NH protons associated with each amino acid will be coded as w_1–w_7. We have reported previously [1] that when vancomycin is present as its free form in d_6-DMSO† solution, the amide bond formed between residues 2 and 3 exhibits dynamic behaviour. In particular, the NH w_3 shows NOEs to both w_2 and w_4, which are on the front face of the molecule as presented in Fig. 1, and also to x_2 which is on the back face of the molecule. This observation necessitates a rotation of w_3 from the front to the back face of the molecule which we were able to calculate involves a barrier of about 13 kcal/mol [1]. However, when the antibiotic is bound to the cell wall analogue *N*-acetyl-D-Ala-D-Ala in the same solvent, we find evidence for only that conformation of the antibiotic in which w_2, w_3, and w_4 are all at the front of the molecule, and therefore orientated in a direction suitable to bind the two oxygen atoms of the carboxylate anion of the cell wall analogue. Thus, the negative charge associated with the carboxylate anion of the terminal D-Ala, through its interaction with the fractional positive charge on the w_3 amide NH, is able to induce the formation of the carboxylate anion binding pocket in a geometry which is appropriate for a strong interaction.

In the case of ristocetin A (Fig. 2), another member of the vancomycin group, the above flexibility of the carboxylate anion binding pocket is not observed because the sidechain of residue 1 in ristocetin A is covalently linked to the sidechain of residue 3. However, in this case we have been able to show [3] that there is dynamic behaviour of the aromatic side chain of residue 1. Before binding of the cell wall analogue, the aromatic ring of residue 1 lies so that the vector which joins the α-carbon of this residue to its attached proton is approximately orthogonal to the plane of the benzene ring. However, when the cell wall analogue binds, this aromatic ring undergoes a rotational motion such that proton 1f moves into

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† Abbreviations: DMSO, dimethyl sulfoxide; and NOE, nuclear Overhauser effect.

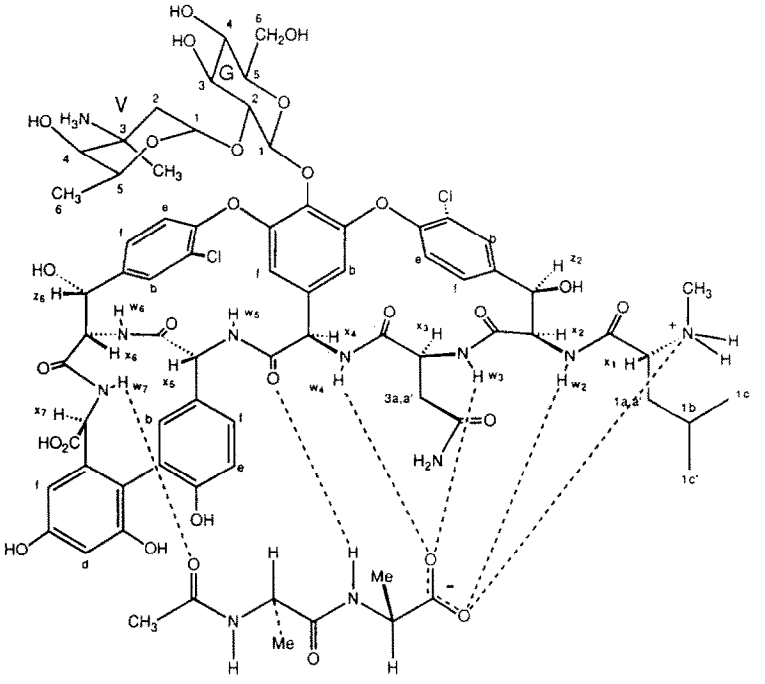


Fig. 1. An exploded view of the vancomycin/*N*-acetyl-D-Ala-D-Ala complex.

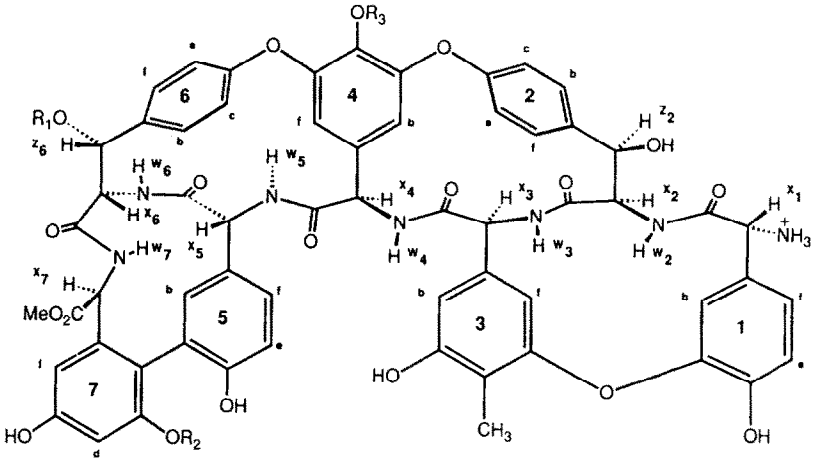


Fig. 2. Ristocetin A. R_1, R_2 and R_3 are sugar g ups.

a position in which it is close in space to x_1 (see Fig. 3). This conclusion is based on approximately equal and weak NOEs which are observed from both 1f and 1b to x_1 before the addition of the cell wall analogue, but a strong NOE from x_1 to 1f after the addition of the cell wall analogue. That this change in magnitude of the NOE is due to a change in relative distance rather than a change in correlation time associated with the sidechain of residue 1 is indicated by the fact that the x_1 to 1b NOE remains weak.

The spontaneous folding-in of the substituted benzene ring, over the carboxylate anion as it binds to the three adjacent NH protons of amino acids 2, 3 and 4, is not a process that could have been predicted. On the contrary, it is normally held that the energy of such systems would be lower if the polar groups involved in the interactions could be exposed simultaneously to polar solvent molecules. Since the above data were obtained in a polar medium (5:2 D_2O/CD_3CN), similar behaviour might have been expected in the present case. The experimental observation may be rationalised by noting that the benzene ring which moves over the polar interaction is intramolecularly bound as part of the antibiotic structure. Therefore, the unfavourable entropic change associated with the folding-in of the benzene ring is probably more than offset by the favourable

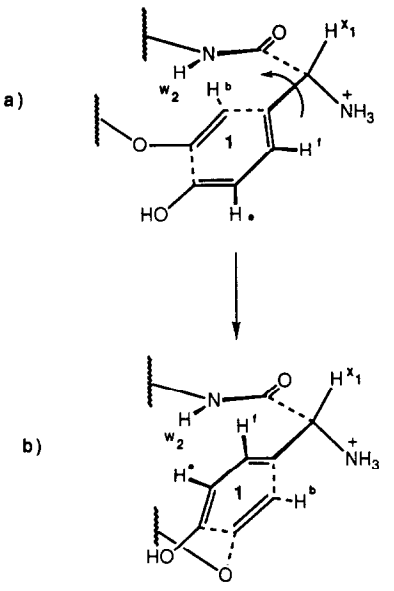


Fig. 3. Two orientations of ring 1 of ristocetin A; before (a) and after (b) binding to the cell wall analogue.

entropic change associated with the release of water molecules—which otherwise would be associated with the polar groups involved in the interaction between the carboxylate anion and the three adjacent NH groups. Additionally, we note that if the benzene ring of residue 1 lay in the open position which is characteristic of the free antibiotic, then a limited space would remain between this ring and the carboxylate anion and the amide NH groups with which the anion interacts. The presence of such a limited space is likely to be very unfavourable in free energy terms, since (i) it will either be unable to accommodate solvent molecules because of its limited size, and thereby represent a loss in dispersion energy, or alternatively (ii) it will accommodate solvent molecules in a high energy configuration, since in a limited space they are unlikely to be able to make favourable interactions with neighbouring solvent molecules. These points emphasise the importance of solvent structure in determining the free energy of molecular recognition processes in polar media.

We have also noted a similar "folding-in" of the sidechain of the N-terminal *N*-methyl-leucine sidechain in vancomycin itself [4]. For this dynamic behaviour, we offer a similar rationalisation of a favourable net entropy change associated with release of polar solvent molecules from the vicinity of the binding pocket.

In earlier work [5], we had proposed an additional factor involved in the folding of the hydrocarbon entities over the polar interaction—that the electrostatic intermolecular interactions (hydrogen bonds) would thereby become enthalpically more favourable. This argument relies on the assumption that the removal of polar solvent would decrease the local effective dielectric, and thus strengthen the hydrogen bonds. However, recently we were able to measure the local effective dielectric in this pocket and found it to be *ca.* 70.* Therefore, since the dielectric constant of water is 80, any local dielectric effect would at best be small. That a pocket with hydrocarbon walls should have a relatively high local dielectric is perhaps not too surprising when it is recognised that the effective local dielectric for stabilisation of the carboxylate anion will be high when numerous polar groups are orientated in a manner which stabilises the negative charge of the anion. This is the case in both vancomycin and ristocetin A, since no less than 3 amide NH groups are held in favourable orientations.

Further sophistication in the interaction between vancomycin and cell wall peptides is evident from the fact that the 6-methyl group of the amino sugar vancosamine (V, Fig. 1) forms a hydrophobic interaction with the methyl group of the C-terminal alanine of the cell wall analogue [6]. Thus, the glucose (G) of vancomycin can be regarded as a "molecular jig" which suitably orientates the amino sugar vancosamine to aid the binding process (Fig. 1). Remarkably, acetylation of the amino group of vancosamine almost completely removes the favourable binding energy (*ca.* 0.7 kcal/mol) associated with the above hydrophobic interaction. Nevertheless, the NOE between the 6-methyl group of the sugar and the methyl group of the C-terminal alanine of the cell wall analogue remains, indicating no significant change in geometry associated with the N-acetylation of the amino sugar. We conclude that the hydrophobic interaction is only significant in the presence of the polar amino group. When the polar amino group is present, the water around it will be highly ordered, and the cost of disturbing this highly ordered structure by inserting hydrocarbon moieties into it would be high. Therefore, the negative free energy change associated with the hydrocarbon–hydrocarbon interaction is relatively high. The situation is analogous to the use of brine to isolate organic compounds, of limited solubility in non-polar solvents, in the workup of organic reactions. In these cases, high concentrations of ions in the aqueous solution render the free energy change of the organic molecule on passing from the water to the non-polar environment more negative. Thus, we propose that in the case of the amino group of the amino sugar, the positive charge causes an intramolecular "salting out" of neighbouring hydrocarbon entities—that is, the hydrophobic interaction of these entities is promoted.

Dimerisation of the antibiotics

We have reported recently that, in a 5:2 D_2O/CD_3CN solvent, the ristocetin A/*N,N*-diacetyl-L-Lys-D-Ala-D-Ala complex forms a dimer [3]. The structure which we have elucidated for this dimer is shown in Fig. 4. From a study of the concentration dependence of the dimer signals, we have calculated that the binding constant for dimer formation is about 2×10^3 L/mol. Additionally, from saturation transfer experiments, we have shown that the off-rate for the dimer is about 10 sec^{-1}. Hence, dimerisation has an on-rate of *ca.* 2×10^4 $L \cdot mol^{-1} \cdot sec^{-1}$.

* Williams DH, Stone MJ and Booth P, unpublished results.

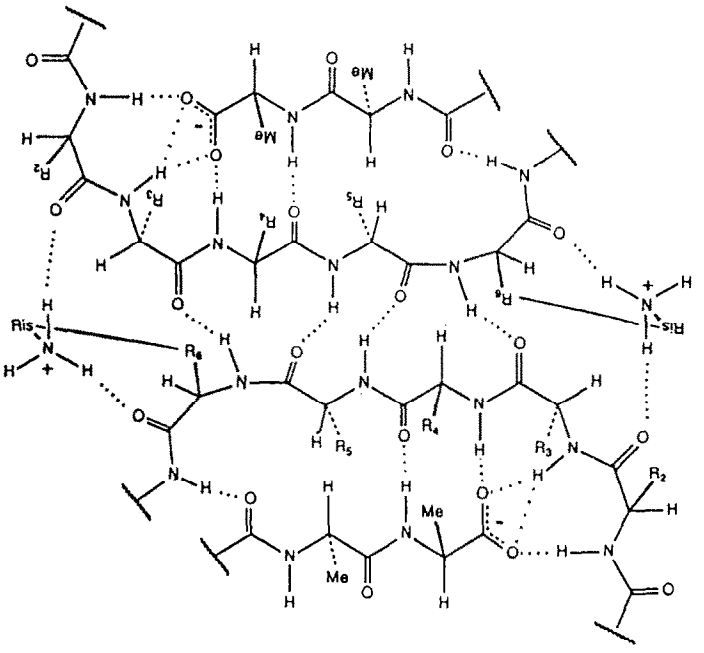


Fig. 4. Ristocetin A/*N*,*N*-diacetyl-L-Lys-D-Ala-D-Ala dimer. Ris = ristosamine sugar on residue 6.

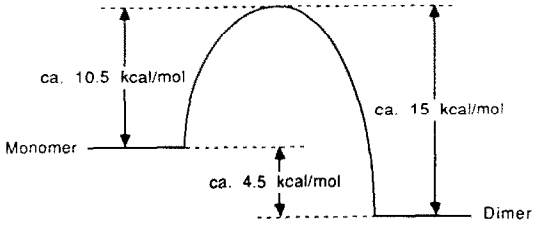


Fig. 5. Free energy profile for the dimerisation process of the ristocetin A/*N*,*N*-diacetyl-L-Lys-D-Ala-D-Ala complex.

The free energy profile for this dimerisation process of the ristocetin A/tripeptide complex therefore appears as in Fig. 5. The slow on-rate for, and hence the large barrier to, dimerisation is noteworthy. We conclude that it arises because of the relative rigidity on the amide bonds and associated sidechains involved in the dimerisation. This leads to a slow on-rate because the relative rigidity of the two components necessitates that the two halves of the dimer be desolvated extensively before they can come together. This desolvation is costly in energy and gives rise to the large barrier which is observed. The result is significant in terms of antigen recognition by antibodies. In such processes, it is observed that it is the flexible regions of the antibodies rather than their rigid regions which are involved in antigen recognition. This is because flexibility permits fast on-rates. It is possible that the dimerisation of the antibiotics, which is a phenomenon of some generality [3], may be of physiological relevance.

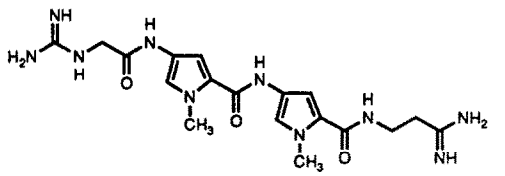
Some comments on double-stranded DNA as a receptor for secondary metabolite structures

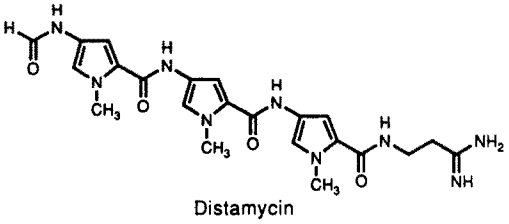
On the basis of the above work on molecular recognition by vancomycin group antibiotics, and on the comments made in the introduction section, it might be argued that secondary metabolites will, in general, have specific receptors and recognise these receptors with a sophistication comparable to that involved in enzyme–substrate recognition [2]. The main limitation in testing this hypothesis lies in the general lack of availability of the receptor structures which may be recognised by natural products. However, double helical DNA as a receptor is an exception to this generalisation, and additionally the structure of the double helix is reasonably well defined.

We now compare a series of compounds that are known to interact with the minor groove of double helical DNA, although in many cases the details of the interactions are unknown. Our aim is to identify common structural features of these molecules and to use our understanding of molecular recognition, gained from studies such as those described above, to draw some conclusions about the likely roles of these structural moieties.

Netropsin, distamycin and CC-1065 (Fig. 6) share structural similarities in that they are built up from aromatic residues and that each molecule contains an inherent right-handed twist analogous to that of DNA itself. NMR [7] and X-ray crystallographic [8] studies have revealed the structure of the netropsin–DNA complex in which the drug lies completely within the DNA minor groove; distamycin is expected to have a similar mode of interaction [8]. CC-1065 has been shown to act within the minor groove by two separate experiments: netropsin, known to bind there, is displaced by CC-1065 [9]; and CC-1065 binds to forms of DNA which are glucosylated in the major groove [10].

We note that all these compounds contain π-polarised aromatic systems. That is, the inner edge is





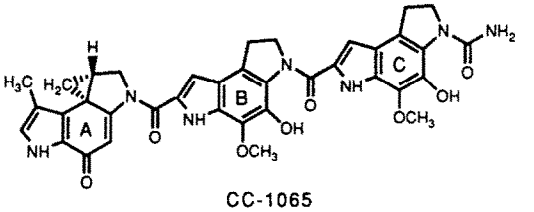


Fig. 6. Minor groove binders containing π-polarised aromatic moieties.

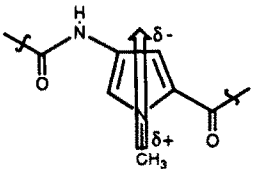


Fig. 7. Orientation of the aromatic ring dipole in the pyrrole systems of netropsin and distamycin.

unsubstituted (and hence relatively hydrophobic), while the outer edge bears methoxyl or hydroxyl functionalities, or in the case of the netropsin series of compounds, is N-methylated. Other workers speculate that a layer of water molecules may bridge the hydrophilic substituents of the outer edge and the phosphate backbone of DNA [11]. We agree with this hypothesis, but add that the combination of an unsubstituted inner edge and a hydrophilic outer edge *in a polarisable π system* confers a net positive charge on the outer edge substituents. This may play an important role in stabilising the complex by interacting with the negatively charged phosphates.

CC-1065 [12] contains an oxygenated outer edge and a hydrophobic inner edge. The outer edge substituents of the benzenoid rings can act in a mesomeric manner so as to confer δ-positive charges on the outside edge. The drug has a twist which promotes its fit into the minor groove, and it can become irreversibly bound by covalent modification of adenine residues via the reactive cyclopropyl ring [9]. CC-1065 analogues which do not have the oxygenated substituents in the pyrroloindole rings ("B" and "C") bind less avidly than the native compound [13]. We note that the sequence specificity is contained in the "A" unit of CC-1065 and that the "B" and "C" units mainly affect the strength of binding of the drug [14]. It may then be that the lower binding constant observed in the tetradeoxy analogues is derived from a lower complex stability induced by a less favourable interaction between the now hydrophobic outer edge, lacking a δ-positive charge, and the phosphate backbone.

Netropsin and distamycin do not have specifically hydrophilic outer edges—indeed, at first sight, the outer edge may seem hydrophobic. They are, however, composed of π-polarised pyrrole units joined by amide linkages. This means that the N-methyl moieties of these units carry a δ-positive charge (Fig. 7). The sequence specificity of these minor groove binders is derived from the pattern of hydrophobic contacts made between the inner edge and the DNA core [11]. Complexes to GC base pairs are disfavoured because of a repulsive steric interaction which would be made between the C2 amino group of guanine and the inner pyrrole protons of the drug molecule. Exchange of a pyrrole unit for imidazole should mean that a GC base pair will be tolerated at that position and this has been confirmed [15, 16]. Thus, the role of the inner edge is well understood. We believe that the manner in which the outer edge is constructed must also have an effect on complex stability and kinetics, and suggest that this may be rationalised in terms of π-polarisation.

Another structural feature common to many DNA-binding antibiotics is deoxy sugar substituents. Calichemycin [17, 18], neocarzinostatin chromophore (NCS-C) [19], chromomycin [20] and the anthracyclines [21–24] (Fig. 8) interact with DNA by various modes, but in every case the deoxy sugar moieties lie in or near to the minor groove. Calichemycin and NCS-C are converted to radical species which cleave double-stranded DNA [17, 25]. In the case of NCS-C, the napthoate group is thought to intercalate between DNA base pairs, which positions the rest of the molecule in the minor groove [26, 27]. Conflicting models of the calichemycin–DNA complex have been proposed [28, 29]. They agree, however, that the molecule lies in the minor groove, as evidenced by the asymmetry observed in affinity cleavage experiments [29], and that the sugar chain stretches along the direction of the groove. Chromomycin, an example of the aureolic acid class of antitumour antibiotics, has been shown to bind as a dimer to specific sites in the minor groove of A-DNA [30]. While the origin of the high site specificity is unknown, it is clear that at least some of the five deoxy sugars bind into the minor groove. Finally, the anthracyclines, examples of which are daunomycin [21, 22], aclacinomycin A [23] and ditrisarubicin B [24], interact with DNA by intercalation of their planar chromophores between DNA base pairs. X-ray structures of daunomycin/DNA complexes [31, 32] show that this interaction positions the deoxy sugar in the minor groove. While the anthracyclines containing more sugars have not been studied so closely, we note that the stereochemistry of attachment of the sugar chains to the chromophore allows

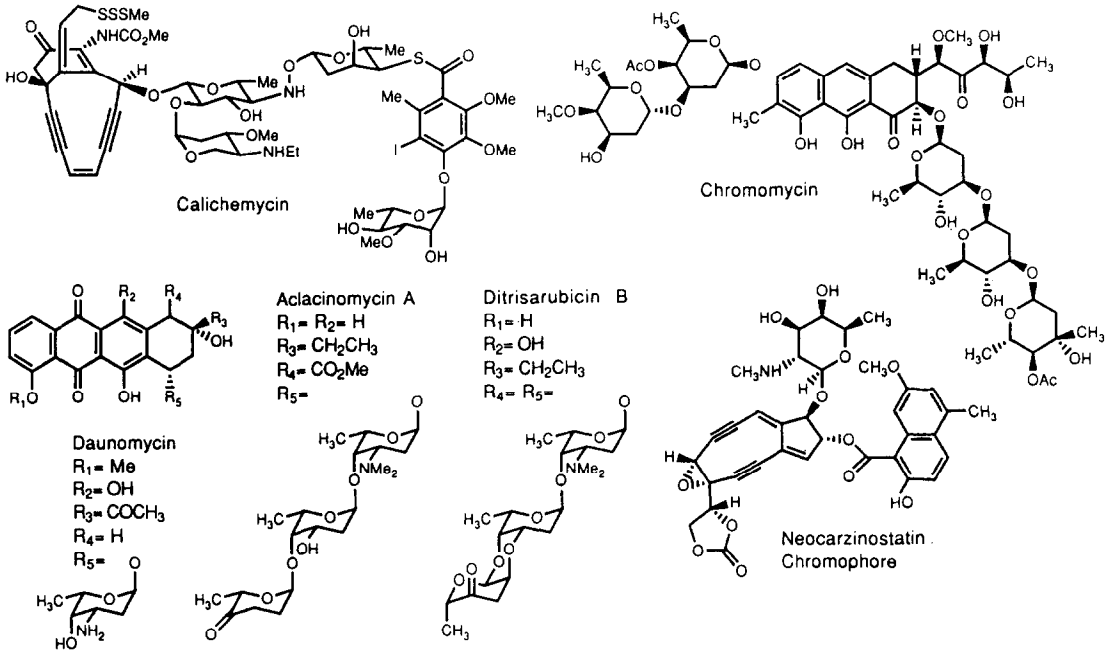


Fig. 8. Minor groove binders containing deoxy sugars.

these chains to extend along the minor groove away from the intercalation site [31]. In the case of ditrisarubicin B, the two sugar chains are related by a pseudo 2-fold axis coincidental with the 2-fold axis of the DNA duplex.

It is clear from the above examples that the deoxy sugars have some role in or adjacent to the minor groove. Such a role might be: (i) to strengthen the drug/DNA complex by direct binding into the minor groove; (ii) to regulate the kinetics of drug/DNA binding by a sugar-minor groove interaction; (iii) to contribute to site specificity by shape recognition; or (iv) to interact with other compounds that normally recognise DNA. In assessing each of these roles we must consider the structural details of the deoxy sugars and the types of interactions in which they can participate.

Several of these sugars carry amino substituents which would be protonated at physiological pH. The presence of this positive charge may be important in stabilising an interaction close to the negatively charged DNA phosphate groups [33]. This interaction could contribute to binding of the sugars into the minor groove or could help regulate the kinetics of the drug–DNA interaction. The fact that all of these sugars are deoxygenated is particularly striking; deoxygenation at positions 2 and 6 is most common. The removal of hydrophilic substituents, which would tend to decrease the solubility of the compounds, must be necessary to participate in some hydrophobic interaction(s). Theoretically, such interactions could give rise to high site specificity by shape recognition. This effect has been observed for some synthetic DNA binders [34]. However, available evidence does not support this role for the given examples. Anthracyclines show only mild selectivity, while Patel's structure of the chromomycin/$[d(TTGGCCAA)]_2$ complex [30] cannot explain the selectivity of the drug in this way. It is more likely that the deoxy sugars are able to stabilise the complex by making non-specific hydrophobic contacts with the inner edge of the minor groove. Site specificity could then be provided by hydrogen bonds involving other parts of the molecules. An alternative role of the deoxy sugars could be to make hydrophobic contacts with a compound that normally recognises DNA and to inhibit the usual interaction. In this regard, it has been suggested [32] that the sugar group of daunomycin interacts with a topoisomerase giving rise to the observed inhibition of topoisomerase activity [35]. A further possibility is that deoxy sugars are advantageous for trans-membrane transport of these compounds, thus enabling them to reach their target receptor. It is not possible to comment further on the latter two hypotheses without knowing the structures of the potential receptors involved.

We observed recently* that aclacinomycin A stabilised the helix to coil transition of a $[d(CGAATTCG)]_2$ double helix by $12 \pm 4°$ in high salt solution, compared with a stabilisation of *ca.* $10 \pm 4°$ by daunomycin. These results imply that the extra duplex stabilisation provided by the second and third sugars is not significant compared with the stabilisation provided by the intercalating chromophore. However, DuVernay and coworkers [36] have demonstrated that the second and third sugars of aclacinomycin and related anthracyclines do contribute to the binding of the drugs to various types of DNA. Removal of both sugars results in a 5- to 12-fold decrease in binding constant. Despite this decrease, the anthracycline bearing only one sugar

* Stone MJ and Williams DH, unpublished results.

still binds with an affinity of *ca.* $10^6\,M^{-1}$. If this binding constant gives rise to a 10° stabilisation of the helix, then it is quite reasonable to conclude that a binding constant of 5×10^6 will only stabilise the helix by a further 1–2°. Thus, our results are consistent with those of DuVernay. We conclude that the sugars play only a minor role in stabilising the duplex, probably by binding into the minor groove. NMR experiments aimed at establishing the structure of the duplex have been complicated by broad spectral lines resulting from exchange processes occurring at comparable rates to the chemical shift differences between resonances of bound and free species.

Kinetic studies of daunomycin–DNA binding [37–39] have revealed that binding occurs in three steps, viz.: (i) formation of an "outside" (non-intercalated) complex of unspecified structure; (ii) intercalation of the chromophore; and (iii) conformational rearrangement or repositioning of the chromophore in the final complex. On the basis of the above observations, it is now proposed that the "outside" complex involves binding of the anthracycline sugars to the DNA minor groove. This interaction is then sufficiently long lived to allow the kinetically slow yet energetically favourable chromophore intercalation. It is further suggested that the occurrence of such deoxy sugars in the other antitumour antibiotics of Fig. 8 may be due to their abilities to form initial non-specific interactions with the DNA minor groove and hence to position the rest of the molecule in a suitable position to form stronger and more specific interactions.

Conclusion

Work on the mode of action of the vancomycin group of antibiotics has shown that sophistication and subtlety are involved in the molecular recognition processes. This, along with the sophisticated mechanisms of other natural products, has led us to propose [2] that secondary metabolites will, in general, have receptors in other organisms against which they have evolved to act. As a consequence, hypotheses can be put forward as to how certain natural products may recognise double-stranded DNA, not only in terms of intercalation, but also by π-polarised aromatic groups and deoxy sugars that may bind into the minor groove of DNA. Current work is providing elegant support for these ideas.

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