PERKIN 1

Enthalpic (electrostatic) contribution to the chelate effect: a correlation between ligand binding constant and a specific hydrogen bond strength in complexes of glycopeptide antibiotics with cell wall analogues

Mark S. Searle,† Gary J. Sharman, Patrick Groves, Bellinda Benhamu,‡ Daniel A. Beauregard, Martin S. Westwell, Robert J. Dancer, Alison J. Maguire, Andrew C. Try and Dudley H. Williams *

Cambridge Centre for Molecular Recognition, University Chemical Laboratories, Lensfield Road, Cambridge CB2 1EW, UK

The ^{1}H NMR chemical shift of amide protons in the binding pocket of glycopeptide antibiotics has been used to monitor the interaction of these amide protons with the carboxylate group of cell wall analogues and related ligands. A good correlation is observed between overall ligand binding energy ($\Delta G°$) and amide NH chemical shift. We conclude that the strength of the electrostatic interaction of the carboxylate group, which is crucial to recognition and binding by the antibiotics, is cooperatively enhanced by adjacent functional groups on the same ligand template. Hydrogen bonding and burial of hydrocarbon in adjacent sites produce an enhancement of electrostatic binding of the carboxylate group. The data provide experimental evidence for an enthalpic contribution to the chelate effect that is distinct from, and works in addition to, the classic entropic chelate effect. The correlation between amide NH chemical shift and overall binding energy has been used to show binding affinity for eremomycin and chloroeremomycin by di-*N*-Ac-Lys-D-Ala-D-Lac (Lac = lactate), which is a cell wall analogue of bacteria which exhibit vancomycin resistance. Binding constants for this ligand have also been determined by UV difference spectrophotometry (70 dm^3 mol^{-1} and 240 dm^3 mol^{-1} respectively).

Introduction

The extent to which the binding of a molecule X–Y to a receptor is enhanced over the sum of the binding of the separate components X and Y (Fig. 1) is related, in part, to the classic entropic chelate effect described by Jencks and Page.[1–3] In a recent analysis[4,5] we have shown that this expression of cooperativity is greatest when each of the associations of X and Y are sufficiently exothermic to approach the limiting cost in entropy on binding. Conversely, when X and Y bind with very small exothermicities, the entropic chelate effect should be less favourable.

An additional feature of this model for the mutual aiding of two binding interactions is that cooperativity can also be expressed as an *enthalpic benefit* when X and Y are tethered together in X–Y. If the bonding of X alone to a receptor (Fig. 1) corresponds to binding in a relatively shallow electrostatic well, then the binding of X will normally be associated with considerable residual motion. However, if the restriction of motion of X is aided by the interactions of Y when X and Y are tethered together, then the average position of X in its electrostatic (enthalpic) well will correspond to some greater binding exothermicity. Thus, the electrostatic binding of the X part of X–Y will be aided by Y, and *vice versa*. Therefore, in the general case, the exothermicity of binding X–Y is expected to be greater than the sum of the exothermicities of binding X and Y alone. This enthalpic cooperative effect is distinct from, and operates in addition to, the entropic chelate effect.

In this paper we illustrate, in an experimental system, this mutual aiding of binding interactions through a study of the complexation of cell wall peptide analogues with a variety of glycopeptide antibiotics. ^{1}H NMR Chemical shift changes have enabled us to monitor a key binding interaction, namely a carboxylate group hydrogen bonding to a pocket of three amide NHs (Fig. 2). We show that by introducing other functional groups (giving rise to either polar or hydrophobic interactions) within the same ligand template, the electrostatic binding

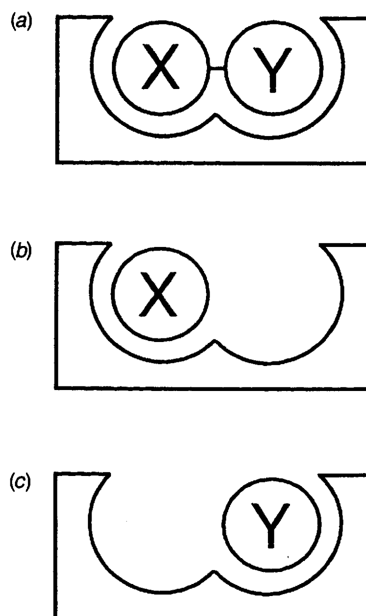


Fig. 1 Schematic representation of ligands X and Y binding to a receptor site separately and when covalently linked as X–Y

† Current address: Department of Chemistry, University of Nottingham, University Park, Nottingham NG7 2RD, UK.
‡ Current address: Departo de Quimica Organica, Facultad de Ciencias Quimicas, Universidad Complutense de Madrid, 28040 Madrid, Spain.

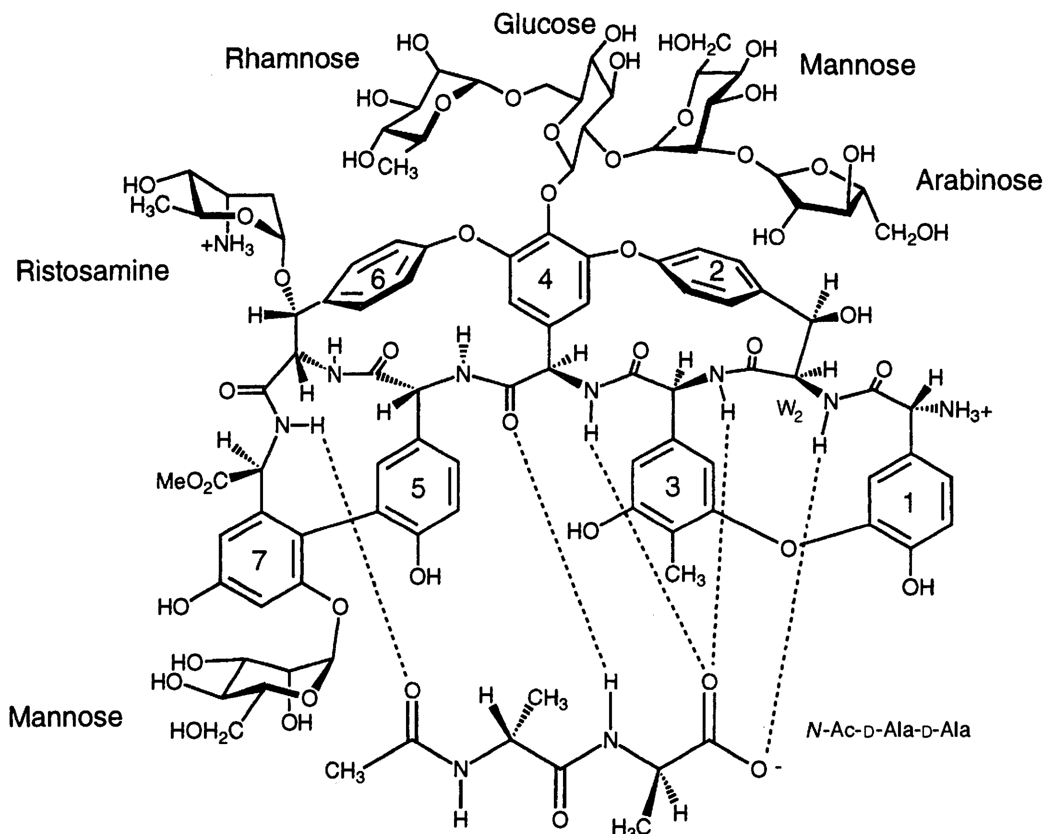


Fig. 2 Exploded view of the ristocetin A complex with *N*-Ac-D-Ala-D-Ala. Dotted lines represent intermolecular hydrogen bonds.

interaction of the ligand carboxylate group can be enhanced. The implications for understanding drug receptor interactions are discussed.

Results and discussion

Probably the most convincing method of demonstrating the enthalpic contribution of the chelate effect described above is by comparing the exothermicities of binding of **X**, **Y** and **X–Y** derived from isothermal calorimetry experiments. Unfortunately, cases where the binding of **X**, **Y** and **X–Y** have been measured with the necessary precision are not readily available. The strength of electrostatic interactions can, however, be implied from the measurement of ^{1}H NMR chemical shift values.[6,7] When a proton is involved in a hydrogen bond, its chemical shift typically moves downfield. The bigger this shift, the stronger the hydrogen bond. This effect is responsible for the well known temperature dependence of the H_2O signal, which shifts downfield when the sample is cooled and hydrogen bonding interactions become stronger.

The glycopeptide group of antibiotics provide an excellent system in which we can use this method to study one interaction when others are present on the same template.[8,9] Members of this clinically important group of compounds block the biosynthesis of the peptidoglycan cell wall of Gram-positive bacteria by binding to the terminal –Lys-D-Ala-D-Ala sequence present in the growing cell wall.[10] The interaction of the antibiotic amide protons w_2, w_3 and w_4 with the C-terminal carboxylate group of the ligand represents a key element of cell wall recognition by these antibiotics (Fig. 2). These amide protons form hydrogen bonds to the carboxylate anion in a shallow binding pocket on the surface of the antibiotic. The strong interaction of the tripeptide cell wall analogue di-*N*-Ac-Lys-D-Ala-D-Ala with ristocetin A ($\Delta G° = -35$ kJ mol^{-1}),[11] is thus reflected in large changes in the ^{1}H chemical shifts for these amide NHs. The largest effect is observed for w_2 which is displaced ≈ 3.6 ppm ($\Delta\delta$) downfield of its shift in the ligand-free antibiotic.

The natural tripeptide ligand makes a number of other interactions with the antibiotic in addition to that of the carboxylate group described above. These include the two additional hydrogen bonds shown in Fig. 2, and hydrophobic interactions through burial of Ala methyl groups against hydrophobic surfaces on the antibiotic. A series of ligands were synthesised in which these interactions were systematically deleted. The limiting chemical shift of w_2 (*i.e.* at ligand concentrations where the antibiotic is more than 95% bound) for the complexes of these ligands then provided a measure of the strength of this particular interaction when neighbouring interactions were varied.[8,9] Overall binding energies, $\Delta G°$, were also measured for the same complexes by UV difference spectrophotometry. In Fig. 3(*a*)–(*d*) we have plotted the chemical shift of w_2 against the overall ligand binding energy $\Delta G°$ for a variety of glycopeptide antibiotics which have now been studied (Fig. 4). The excellent correlations clearly indicate that the strength of the carboxylate binding interaction increases as other interactions are introduced on the same template. This correlation is even more striking when the data from these different plots are combined on one plot (Fig. 5).

All of the ligands studied share a common carboxylate anchoring group, but differ from the natural tripeptide by having different numbers of hydrogen bonds or different hydrophobic interactions. For example, one series of ligands consisted of truncated natural peptides, from tripeptide to *N*-Ac-D-Ala-D-Ala to *N*-Ac-D-Ala to acetate (**1–4**). Another series of ligands (**5–7**) had the same dipeptide template, with identical hydrogen bonding sites, but differed in the number of hydrophobic methyl groups, with alanine being replaced by glycine. Unnatural ligands which had similar arrangements of hydrogen bond donors and acceptors, but which contained non-peptidic backbones, were also used (**8–12**). To emphasise the generality of the results, this correlation was examined for a

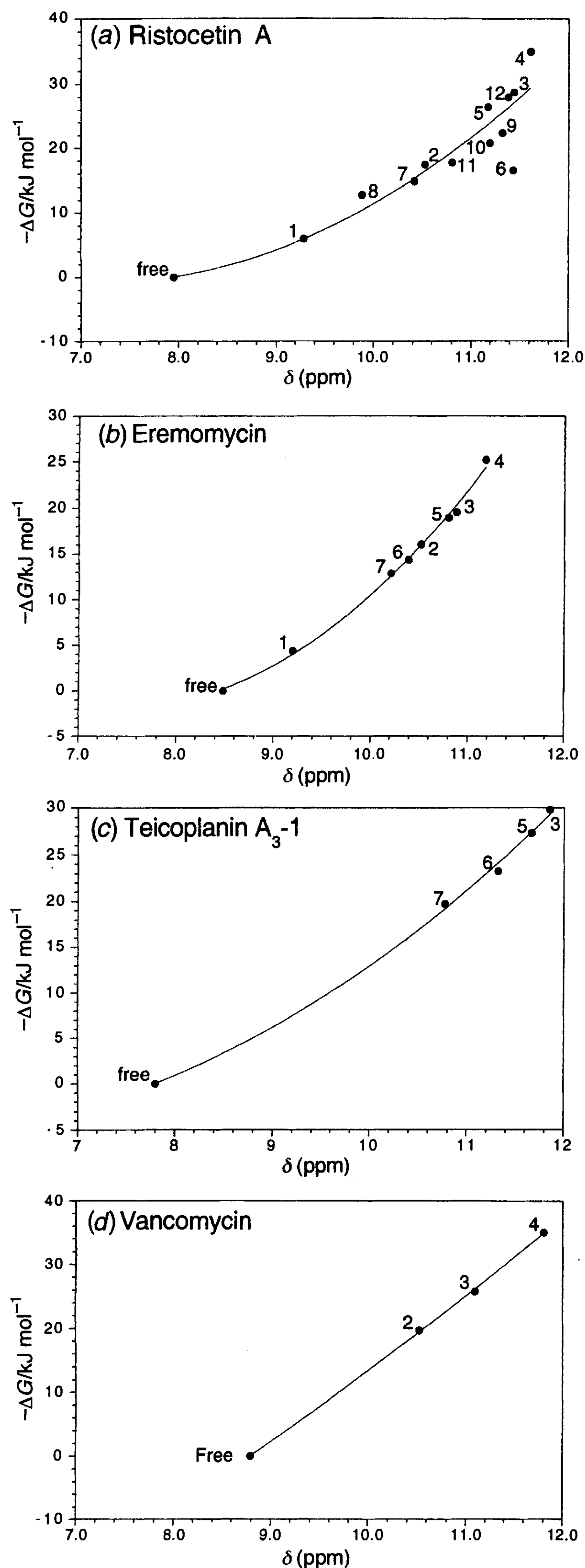
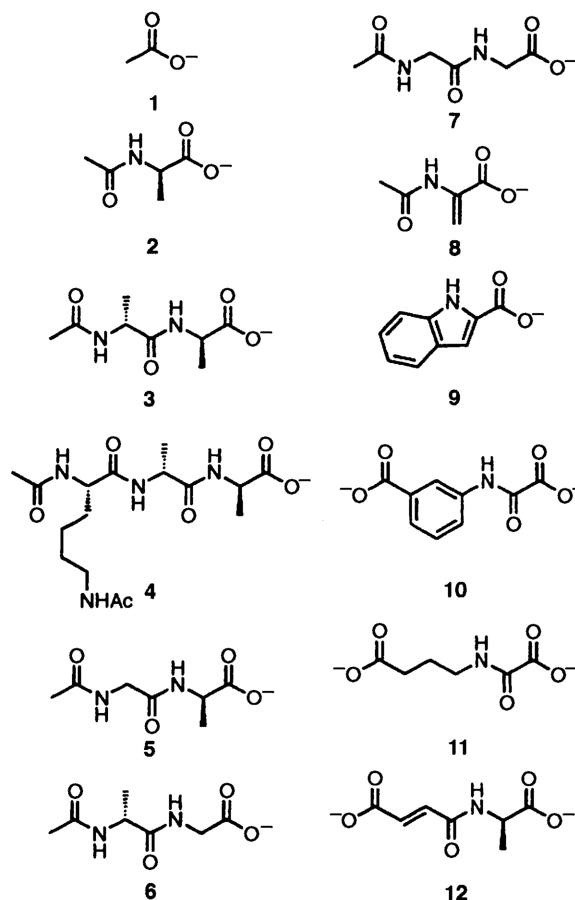


Fig. 3 Plot of ligand binding energy *versus* limiting chemical shift of antibiotic amide NH w_2 for (*a*) ristocetin A, (*b*) eremomycin, (*c*) teicoplanin A_3-1 and (*d*) vancomycin with the various cell wall analogues **1–12** used in this study. ^{1}H Chemical shifts were determined at 500 MHz at 298 K and are referenced to trimethylsilylpropionic acid. In all cases the signals were assigned on the basis of 2D NOE experiments. Ligand binding energies are from UV titration measurements and from the calorimetry data of Rodriguez-Tebar *et al.*[31]



range of glycopeptide antibiotics. Members of the group have been shown to dimerise in aqueous solution. To show this was not an important factor, experiments were performed on antibiotics with quite different dimerisation constants. Teicoplanin A_3-1 does not measurably dimerise, vancomycin and ristocetin A dimerise relatively weakly ($K_{dim} \approx 700$ and 500 $dm^3 mol^{-1}$ respectively), whereas eremomycin dimerises strongly ($K_{dim} \approx 3 \times 10^6$ $dm^3 mol^{-1}$).[12] Moreover, ristocetin A and vancomycin show important structural differences in the peptide backbone. Residues 1 and 3 of ristocetin A have cross-linked aromatic side chains, whereas the corresponding residues in vancomycin are aliphatic (*N*-methylleucine and asparagine respectively). Despite these differences in ligand and antibiotic structure, a very good correlation is still observed.

Other factors could affect the limiting chemical shift of w_2, such as conformational changes of the antibiotic associated with ligand binding or proximity of antibiotic and ligand aromatic groups. However, we conclude that the interaction of w_2 with the ligand carboxylate anion is by far the most significant effect. This is for two reasons. First, molecular modelling studies, guided by NOE data, establish that the hydrogen bond geometries of the bound ligands are relatively invariant, as are the structures of the antibiotics (which are relatively inflexible on account of extensive cross-linking of the aromatic side chains). Second, contributions from other factors would be variable, contributing only to the observed scatter of the data. The major effect on the chemical shift of w_2 (of up to $\approx$ 3.6 ppm) is therefore concluded to arise from the electrostatic interaction with the carboxylate group of the ligand concerned.

The above results show that the binding energy of the carboxylate–amide interaction is not a fixed quantity, but is cooperatively enhanced by neighbouring interactions. This would at first seem to deal a fatal blow to attempts to quantify binding interactions in molecular recognition phenomena.

Fig. 4 Structures of the antibiotics used in this study

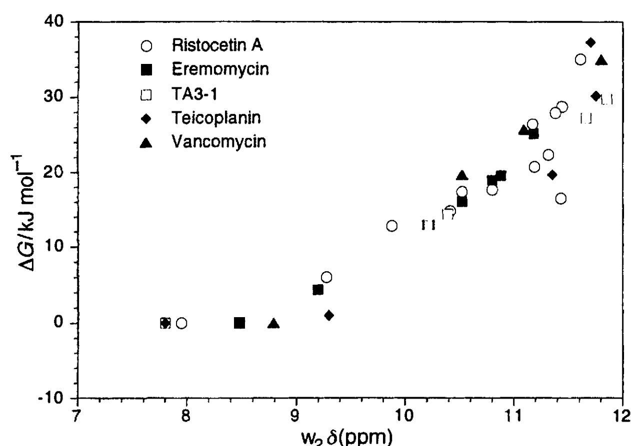


Fig. 5 Combined plot of ligand binding energy *versus* limiting chemical shift of antibiotic amide NH w_2 for ristocetin, eremomycin, teicoplanin A_3-1 and vancomycin

However, the present results show that in this system at least, the degree of cooperative enhancement is predictable, since it is strongly related to the overall binding energy. That is, carboxylate binding energy correlates well with the magnitude of $\Delta G°$, regardless of the origin of the binding energy, either through hydrogen bonds or the burial of hydrocarbon (we use the term 'burial of hydrocarbon' to include contributions from van der Waals contacts and any conformational bias towards that required for binding caused by ligand methyl groups, as well as the hydrophobic effect). This is striking when it is considered that the former represents an enthalpic component to binding, but the latter is largely entropically driven, at room temperature.

In all the ligand complexes studied, the carboxylate group provides the key binding interaction. We have illustrated this cooperativity for this one specific interaction, but this effect must operate for other interactions on the same ligand template. In the present case, the effects on w_2 chemical shift are the most convenient to monitor since the shift changes are very large and consequently very sensitive to differences in binding interactions.

Use of the correlation to demonstrate the binding of di-*N*-Ac-Lys-D-Ala-D-Lac (Lac = lactate) to eremomycin and chloroeremomycin

The good correlation observed for a large number of data points, representing a varied group of ligands and antibiotics, suggested that it could be used as a rapid way to investigate ligand binding. We were particularly interested in the ligand di-*N*-Ac-Lys-D-Ala-D-Lac (Lac = lactate), which is a cell wall analogue of bacteria which exhibit vancomycin resistance. The substitution of lactate for alanine results in the hydrogen bond between the terminal alanine amide proton and the antibiotic backbone being replaced by a repulsive interaction between oxygen atoms (Fig. 6). This repulsion dramatically

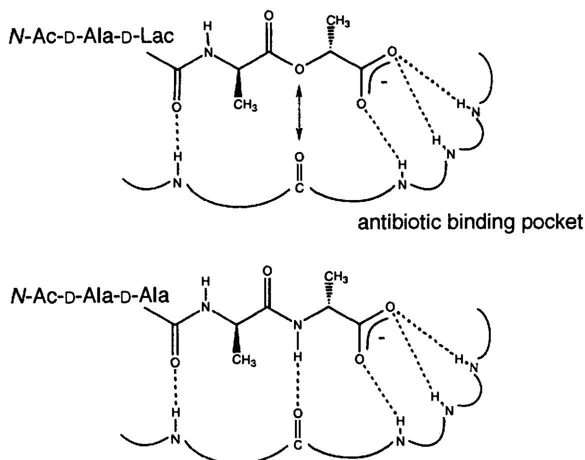


Fig. 6 Many bacteria which exhibit resistance to glycopeptide antibiotics do so by producing cell wall peptides which terminate in D-lactate instead of D-alanine. This substitution leads to a repulsive interaction between the ester oxygen of the lactate and the carbonyl of residue 4 of the antibiotic.

reduces ligand binding and so confers resistance to the bacteria.[13,14]

In order to determine whether the antibiotics were still able to bind di-*N*-Ac-Lys-D-Ala-D-Lac in the same way as di-*N*-Ac-Lys-D-Ala-D-Ala, the w_2 probe was used. If the carboxylate of the ligand were to associate with the antibiotic binding pocket, a large change in the chemical shift of w_2 would be observed. The w_2 chemical shift was therefore measured for eremomycin with a tenfold excess of di-*N*-Ac-Lys-D-Ala-D-Lac (to ensure a high fraction of bound antibiotic). It was found to be 10.50 ppm, 2 ppm downfield of the free w_2 shift. This showed that the carboxylate, at least, was binding into the binding pocket. The w_2 shift was also measured for chloroeremomycin (eremomycin with a chlorine on ring 6, which is directed toward the binding pocket), and was found to be 10.98 ppm. The larger downfield shift indicated stronger binding, thus suggesting the chlorine fulfils a similar role in the binding of di-*N*-Ac-Lys-D-Ala-D-Lac as for di-*N*-Ac-Lys-D-Ala-D-Ala, where its presence gives rise to a similar enhancement.[12] Together, this suggests that di-*N*-Ac-Lys-D-Ala-D-Lac binds in a similar manner to di-*N*-Ac-Lys-D-Ala-D-Ala, albeit with much reduced binding affinity. Indeed, this conclusion is supported by analysis of crosspeaks from NOESY spectra.[15] For the complex of di-*N*-Ac-Lys-D-Lac with chloroeremomycin, the methyl group of the lactate is located adjacent to ring 2, and the methyl group of alanine is close to rings 5 and 7, as for the complex of di-*N*-Ac-Lys-D-Ala-D-Ala.

Given these indications, binding constants were determined using UV difference spectrophotometry. These were found to be 70 $dm^3\ mol^{-1}$ and 240 $dm^3\ mol^{-1}$ for eremomycin and chloroeremomycin respectively, similar to those found by other workers of Eli Lilly.[16] When these values are plotted against the respective w_2 shifts on the curve in Fig. 3, the points do not fit the correlations as well as the data for **1–12**. The binding constants are almost an order of magnitude less than would be predicted from the w_2 shifts used in conjunction with the correlation shown in Fig. 3. However, all of the points in these figures are for ligands with a carboxylate and neighbouring amide. It would seem likely that the repulsion between the two oxygens subtly alters the conformation, such that the correlation between ΔG and w_2 is different. Thus, although glycopeptides appear to bind ligands terminating in lactate in an essentially similar manner as those terminating in alanine, the subtle conformational differences caused by the O–O repulsion give rise to the somewhat anomalous w_2 shift.

Conclusions

A desire to rationalise the forces involved in molecular complexation[17–19] and the stabilisation of highly ordered molecular assemblies[20–24] has lead to useful numerical estimates for assessing apparent binding contributions in molecular recognition studies.[19,24–26] Of necessity, these approaches use the effects of changes to ligand or protein structure at specific sites, but must measure effects on overall stability constants. The present analysis illustrates the complexity of the problem because extended arrays of weak interactions can cooperatively interact such that specific changes at one site can affect interactions elsewhere in the structure. The results presented show that specific interactions can be aided both enthalpically as well as entropically (classic chelate effect) by adjacent binding interactions. In the present context, this cooperative enhancement depends on the overall ligand binding energy in a predictable way, regardless of the nature of the interactions that stabilise the complex.

The correlation between chemical shift and binding has also allowed us to demonstrate that di-*N*-Ac-Lys-D-Ala-D-Lac does bind to the glycopeptide antibiotics in a similar manner to di-*N*-Ac-Lys-D-Ala-D-Ala, albeit with greater reduced affinity.

Experimental

Preparation of ligands

Benzyl D-lactate, which was used to prepare depsipeptide ligands, was prepared from lithium D-lactate according to the method of Losse and Bachmann.[27] Peptides and depsipeptides were prepared using Boc and benzyl ester protecting groups and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate couplings, as per standard literature methods.[28] Unnatural ligands were prepared as previously described.[29,30]

Measurement of equilibrium constants

Equilibrium constants were measured by UV difference spectrophotometry using a dual beam Uvikon 960 spectrophotometer equipped with a temperature control bath to maintain a constant temperature of 298 K. Typically, 50 $\mu mol\ dm^{-3}$ antibiotic, buffered at pH 4.5 with 0.1 $mol\ dm^{-3}$ sodium phosphate, was added to both spectrophotometer cells and ligand was titrated into one cell. For eremomycin, 1 $mol\ dm^{-3}$ NaCl was included in the buffer to avoid changes in ionic strength caused by differing ligand concentrations. The wavelengths used to follow change in absorbance were determined by adding an excess of ligand and taking a difference spectrum. Data were collected at four wavelengths; subtraction was used to minimise errors due to baseline drift. Binding constants were determined by a least squares fitting method using Kaleidagraph (v. 2.1.3, Abelbeck software). Determinations were performed in triplicate and usually gave results within 10%. Binding energies were calculated from $\Delta G^\circ = -RT \ln K$.

NMR Spectroscopy

All NMR experiments were performed on a Bruker DRX500 or Varian Unity-plus 500 spectrometer. Samples were prepared in 9:1 H_2O–D_2O buffered at pH 4.5 with 50 $mmol\ dm^{-3}$ sodium phosphate. 1–10 $mmol\ dm^{-3}$ antibiotic was used; ligand concentrations were calculated from K values so as to give >95% bound antibiotic. For eremomycin, 1 $mol\ dm^{-3}$ NaCl was also included in the buffer to show that changes in ionic strength caused by differing ligand concentrations were not responsible for any observed changes in w_2 chemical shift. One dimensional experiments were performed using WATERGATE or presaturation to suppress the intense H_2O peak. 32K Points were collected and the spectrum was referenced to trimethylsilylpropionic acid (TSP). For two dimensional NOESY experiments, typically 2K points were collected in $f2$, whereas 512 points were collected in $f1$ using TPPI (time proportional phase increment).

In the investigation of the binding of di-*N*-Ac-Lys-D-Ala-D-Lac, 10 mmol dm^{-3} antibiotic and 100 mmol dm^{-3} ligand was used, with the same buffer as used in other experiments.

Acknowledgements

We are grateful to Glaxo (M. S. S.), EPSRC (G. J. S. and M. S. W.), BBSRC (P. G.), Zenova (A. C. T.), the British Council and Association of Commonwealth Universities (D. A. B.), Smithkline Beecham (A. J. M.) and the Wellcome Trust (R. J. D.) for financial support. We thank the NIMR, Mill Hill, UK, for access to high resolution NMR facilities. We also thank Eli Lilly (Indianapolis), SKB (Brockham Park, UK), Abbott Laboratories (Chicago) and Lepetit (Geranzano, Italy) for samples of chloroeremomycin (LY264826B), eremomycin, ristocetin A and teicoplanin respectively.

References

1 W. P. Jencks, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1975, **43**, 219.
2 W. P. Jencks, *Proc. Natl. Acad. Sci. USA*, 1981, **78**, 4046.
3 M. I. Page, *Chem. Soc. Rev.*, 1973, **2**, 295.
4 M. S. Searle, M. S. Westwell and D. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1995, 141.
5 D. H. Williams, M. S. Searle, M. S. Westwell, J. P. Mackay, P. Groves and D. A. Beauregard, *Chemtracts Org. Chem.*, 1994, **7**, 133.
6 J. A. Pople, W. G. Schneider and H. J. Bernstein, in *High Resolution Nuclear Magnetic Spectroscopy*, McGraw-Hill, New York, 1959, vol. 1, ch. 15.
7 U. Sternberg and E. Brunner, *J. Magn. Reson. Ser. A*, 1994, **108**, 142.
8 P. Groves, M. S. Searle, M. S. Westwell and D. H. Williams, *J. Chem. Soc., Chem. Commun.*, 1994, 1519.
9 G. J. Sharman, M. S. Searle, B. Benhamu, P. Groves and D. H. Williams, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 1483.
10 J. C. J. Barna and D. H. Williams, *Ann. Rev. Microbiol.*, 1984, **38**, 339.
11 M. Nieto and H. R. Perkins, *Biochem. J.*, 1971, **124**, 845.
12 J. P. Mackay, U. Gerhard, D. A. Beauregard, R. A. Maplestone and D. H. Williams, *J. Am. Chem. Soc.*, 1994, **116**, 4573.
13 G. D. Wright and C. T. Walsh, *Acc. Chem. Res.*, 1992, **25**, 468.
14 C. T. Walsh, S. L. Fisher, I.-S. Park, M. Prahalad and Z. Wu, *Chem. Biol.*, 1996, **3**, 21.
15 R. J. Dancer, A. C. Try, G. J. Sharman and D. H. Williams, *Chem. Commun.*, 1996, 1445.
16 D. L. LeTourneau, J. N. Hobbs and N. E. Allen, Abstract F250, p. 157, in Program and abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy, American Society for Microbiology, Washington, DC, 1995.
17 P. R. Andrews, D. J. Craik and J. L. Martin, *J. Med. Chem.*, 1984, **27**, 1648.
18 A. R. Fersht, *Trends Biochem. Sci.*, 1987, **12**, 301.
19 M. S. Searle, D. H. Williams and U. Gerhard, *J. Am. Chem. Soc.*, 1992, **114**, 10697.
20 K. A. Dill, *Biochemistry*, 1990, **29**, 7133.
21 A. Fersht, in *Enzyme structure and mechanism*, W. H. Freeman and Company, New York, 1985.
22 L. Serrano, A. Horovitz, B. Avron, M. Bycroft and A. R. Fersht, *Biochemistry*, 1990, **29**, 9343.
23 B. A. Shirley, P. Stanssens, U. Hahn and C. N. Pace, *Biochemisry*, 1992, **31**, 725.
24 L. Serrano, J.-L. Neira, J. Sancho and A. R. Fersht, *Nature (London)*, 1992, **356**, 453.
25 J. T. Kellis, K. Nyberg and A. R. Fersht, *Biochemistry*, 1989, **28**, 4914.
26 D. Shortle, W. E. Stites and A. K. Meeker, *Biochemistry*, 1990, **29**, 8033.
27 G. Losse and G. Bachmann, *Chem. Ber.*, 1964, **97**, 2671.
28 M. Bodanszky, and A. Bodanszky in *The Practice of Peptide Synthesis*, Springer-Verlag, Berlin, 1994.
29 P. Groves, M. S. Searle, I. Chicarelli-Robinson and D. H. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1994, 659.
30 S. E. Holroyd, P. Groves, M. S. Searle, U. Gerhard and D. H. Williams, *Tetrahedron*, 1993, **49**, 9171.
31 A. Rodriguez-Tebar, D. Vazquez, J. L. Perez Velazquez, J. Laynez and I. Wadso, *J. Antibiot.*, 1986, **39**, 1578.

Paper 6/05297E
Received 29th *July* 1996
Accepted 2nd *September* 1996