Solution structures of thiopeptide antibiotics†

Richard J. Lewis,[a] Rachael A. Hughes,[b] Lilian Alcaraz,[a] Stewart P. Thompson[b] and Christopher J. Moody*[bc]

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A detailed NMR study of the thiopeptide amythiamicin D establishes its solution conformation and the presence of a single intramolecular hydrogen bond involving NH13 and O28, and also provides the first evidence for self-association of thiopeptides in solution.

The thiopeptide family of antibiotics is a class of sulfur-containing highly modified cyclic peptides, characterized by several common structural features such as the presence of thiazole, and in some cases, oxazole rings, unusual and dehydro amino acids, and a heterocyclic core of tri- or tetra-substituted pyridine moieties all in a macrocyclic array.[1] Since the isolation of the first member of the class, micrococcin, in 1948, the thiopeptides have been the subject of a number of biological studies. Many of them inhibit protein synthesis in bacteria and share common modes of action, acting directly on the ribosome or its associated elongation factor proteins.[1] More recently the thiopeptides have attracted the attention of synthetic chemists and this has led to Ciufolini and Shen's synthesis of the reported structure for micrococcin P1,[2] and the landmark synthesis of thiostrepton **1** (Fig. 1) by Nicolaou and co-workers.[3,4] Our own efforts in this area have resulted in the total synthesis of promothiocin A[5,6] and amythiamicin D **2**.[7,8]

With molecular weights of over 1000 Da, and arrays of rings linked by peptide bonds, the thiopeptides can be regarded as exhibiting protein-like structural features. Thus, in a detailed X-ray crystallographic analysis, building on Hodgkin and co-workers' original structural work,[9] Hunter and colleagues described thiostrepton **1** as a microcosm of the protein world,[10] containing bound water molecules, a hydrophobic core and a conformation held together by five intramolecular hydrogen bonds. The solution conformation of thiostrepton **1** has also been studied using ^{1}H NMR spectroscopy.[11] We now report a detailed NMR study of the thiopeptide amythiamicin D **2**. This not only establishes the presence of a single intramolecular hydrogen bond involving NH13 and O28, suggesting a solution conformation closely related to that exhibited in the crystal structure of protein-bound GE2270A **3**,[12] but also provides the first evidence for the self-association of thiopeptides in solution.

Our interest in the solution conformation of amythiamicin D **2** was triggered by two observations. Firstly, the ROESY spectrum of our synthetic material showed transannular cross peaks that would indicate the presence of a restricted conformation. Secondly, we noted that whilst our synthetic material had an identical ^{13}C NMR spectrum to that of the natural product,[13] the chemical shift of one signal in the ^{1}H NMR spectrum, assigned to NH29, showed significant concentration dependence.[7,8] This prompted a much more detailed NMR study in which we have established the solution conformation using quantitative ROE measurements,

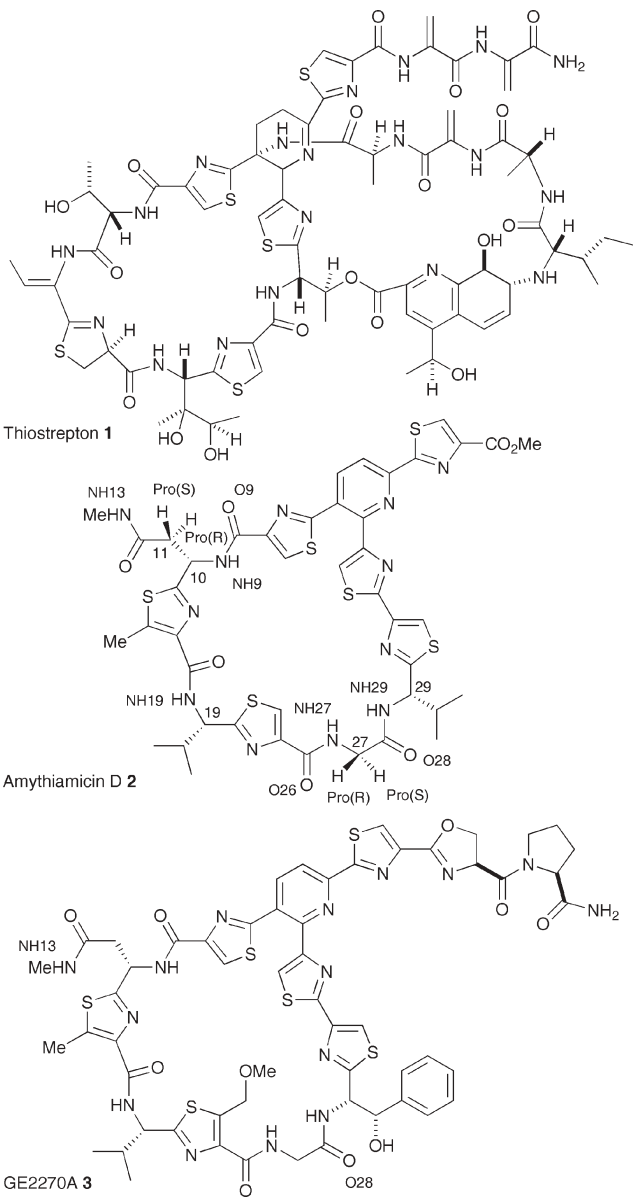

Fig. 1 Structures of the thiopeptide antibiotics thiostrepton **1**, amythiamicin D **2** and GE2270A **3**.

[a] *AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leicestershire, UK LE11 5RH*
[b] *Department of Chemistry, University of Exeter, Stocker Road, Exeter, UK EX4 4QD*
[c] *School of Chemistry, University of Nottingham, University Park, Nottingham, UK NG7 2RD. E-mail: c.j.moody@nottingham.ac.uk*
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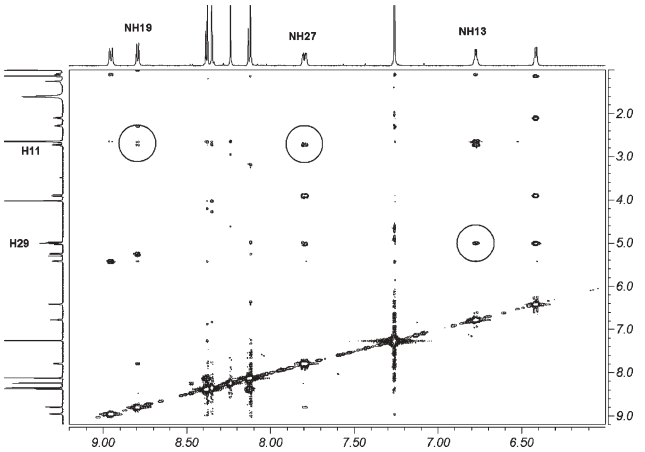

Fig. 2 ROESY spectrum of amythiamicin D **2** in $CDCl_3$ solution (4.4 mM); unexpected cross peaks between transannular protons are circled.

used temperature coefficients of the NH protons to establish hydrogen bonding patterns and investigated the self-association of amythiamicin D **2**.

A ROESY spectrum of amythiamicin D **2** (Fig. 2) in $CDCl_3$ suggested the proximity of certain key pairs of transannular protons (NH13 and H29, H11 and NH19, and H11 and NH27).‡

The structure of the related thiopeptide antibiotic GE2270A **3** (Fig. 1) has been determined to 2.35 Å resolution when bound to the elongation factor EF-Tu.[12,14] This protein-bound conformation is stabilized by an intramolecular hydrogen bond between NH13 and O28, and the presence of such a hydrogen bond in amythiamicin D **2** could explain these unexpected ROE cross peaks. However, the authors suggested that the bound conformation differed considerably from that determined in dimethyl sulfoxide solution. In order to investigate whether amythiamicin D **2** was actually forming the NH13–O28 hydrogen bond spontaneously *in solution*, a series of double-offset ROESY experiments[15–17] were performed at mixing times ranging from 50 to 150 ms at a concentration of 30 mM. Using a reference distance of 1.78 Å for the H27a–H27b geminal proton pair, the initial slope of the ROE build up was used to derive distances for the other proton pairs, and the derived distance information was compared to that derived from the X-ray structure of bound GE2270A **3** (Table 1).

As can be seen in Table 1, the derived distances for **2** in solution are in agreement with those in the crystal structure of protein-bound GE2270A **3**. The slightly shorter NH13–H29 distance observed in solution suggests a small change in the torsion angle about the C10–C11 bond for amythiamicin D **2**.

Table 2 Temperature coefficients for the amide protons in amythiamicin D **2**

Concentration/mM	0.09	3.2	20	44
Proton	Temperature coefficient (ppb K^{-1})			
NH9	-1.8 ± 0.5	-2.7 ± 0.6	-2.6 ± 0.7	-1.9 ± 0.4
NH13	-5.2 ± 0.1	-6.8 ± 0.2	-7.2 ± 0.6	-5.4 ± 0.1
NH19	-1.3 ± 0.2	-1.9 ± 0.3	-2.0 ± 0.4	-1.4 ± 0.2
NH27	-2.1 ± 0.4	-2.8 ± 0.5	-3.6 ± 0.5	-3.1 ± 0.4
NH29	-2.0 ± 0.4	-6.6 ± 1.3	-12.8 ± 1.7	-10.8 ± 0.9

Coupling constants can also be used to derive conformational information, and we therefore measured the values of the three bond proton–proton coupling constants from the NH protons as these can be related to torsion angles using the expression derived by Ludvigsen *et al.*[18] We found that there is generally good agreement between the values of the derived torsion angles and those observed in the X-ray structure of GE2270A **3** (data given in ESI†). The match of distance and coupling constant information between the solution conformation of amythiamicin D **2** and the bound conformation of GE2270A **3** is good evidence that the gross conformations are similar and, in particular, is strongly supportive of a hydrogen bond between NH13 and O28.

In order to obtain further evidence for the presence of hydrogen bonds, we investigated the amide proton temperature coefficients. In peptides and other small molecules, the amide proton chemical shifts are usually sensitive to temperature. The temperature coefficients ($\Delta\delta/\Delta T$) of these protons can be used as a measure of the extent of involvement of individual NH protons in hydrogen bonds. In non-polar solvents, an NH proton which is not hydrogen bonded is expected to show a small temperature coefficient *ca.* -2 ppb K^{-1}.[19–21] Protons that are hydrogen bonded tend to have higher temperature coefficients of around -5 ppb K^{-1} due to a lengthening of the hydrogen bond and consequent reduction in the deshielding effect of the carbonyl moiety on the NH proton as the temperature increases. The values of the temperature coefficients measured for the five amide protons in amythiamicin D **2** at four concentrations between 0.09 and 44 mM are shown in Table 2.

The temperature coefficients clearly show that NH13 is involved in a hydrogen bond at all concentrations, thus confirming the interpretation of the ROE distance measurements above. The coefficients also show that amide protons NH9, NH19 and NH27 are not involved in any interactions over the concentration range measured. Amide proton NH29 shows different behaviour however. At low concentration, the temperature coefficient suggests no involvement in any hydrogen bonding interaction. However, as the concentration increases, the coefficient increases

Table 1 ROE results and derived distances for key proton pairs in amythiamicin D **2** at a concentration of 30 mM

Proton pair	NH27 Pro(*S*) H11	NH19 NH27	NH19 H10	NH19 Pro(*S*) H11	NH13 H29	H27a H27b
Initial slope /% s^{-1}	5.16	4.00	0.72	1.86	4.81	112
R^2	0.997	0.996	0.966	0.980	0.997	0.995
Derived distance/Å (95% confidence limits)	2.97 ($\pm$ 0.46)	3.10 ($\pm$ 0.46)	4.13 ($\pm$ 0.46)	3.52 ($\pm$ 0.46)	3.01 ($\pm$ 0.46)	1.78[a]
X-Ray distance/Å of equivalent protons in GE2270A **3**	3.06	3.35	4.49	3.45	3.70	

[a] Reference distance.

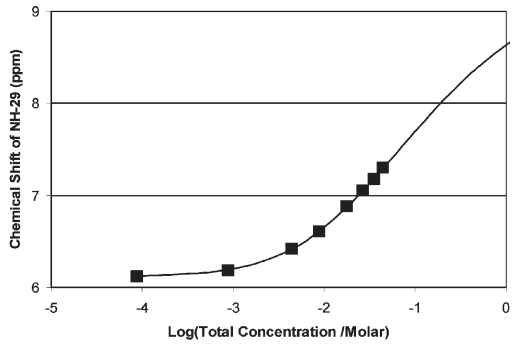

Fig. 3 Observed (■) *vs.* predicted chemical shift of NH29 for the dimerization model (solid line) for amythiamicin D **2**.

in magnitude, before falling off again slightly, indicating that NH29 is involved in a hydrogen bonding interaction at high, but not at low, concentrations. The behaviour of the magnitude of the coefficients for NH29 may be indicative of oligomer formation (see below).

Initial observations had suggested that the chemical shift of NH29 was concentration dependent, and the amide temperature coefficients implied that this proton is involved in a hydrogen bond at higher concentrations. These observations are indicative of self-association of **2**, and therefore the concentration dependence of the chemical shift of NH29 was investigated. We prepared samples of **2** in chloroform-*d* at concentrations ranging from 90 μM to 44 mM. The concentration of amythiamicin D **2** was assayed using NMR methods,[22,23] giving confidence as to the accuracy of the concentrations reported. Over this concentration range, the chemical shift of the NH29 proton exhibits by far the greatest change (1.18 ppm), though other protons (notably NH13, NH27 and Pro(*S*) H27) exhibit shift changes greater than 0.1 ppm. The profile of the shift change gives information about the nature of the interaction, and, in order to test whether the shift variation could be explained by dimerization, the data for NH29 were fitted to a dimerization model (see ESI†). Fig. 3 shows these data and the calculated fit for the dimerization model. Although ideally it would be preferable to measure to higher concentrations, this was not possible for experimental reasons. However, the excellent fit to the model ($R^2 = 0.9997$) is still strong evidence for dimerization. In the light of this, the concentration behaviour of the temperature coefficient for NH29 (see above) can thus be understood as the result of interplay between the effect of temperature on the hydrogen bonding of the individual monomer and dimer, and the effect on the equilibrium itself.

The NH proton shift data implicate NH29 in an intermolecular hydrogen bond at higher concentrations; however, we could find no evidence to suggest the identity of the hydrogen bond acceptor. ROESY spectra do not show correlations that we could conclusively assign as being due to intermolecular interactions. Similarly, carbon shift changes were inconclusive, showing only a small shielding (*ca.* 0.2 ppm) for carbonyl-28 on a change in concentration from 1.9 to 15.7 mM and a slightly smaller change (0.1 ppm) for carbonyl-26 (data not shown). The former change would support a slight shortening of the intramolecular hydrogen bond on dimerization. The latter shift however, is too small to draw any firm conclusions as to the identity of the hydrogen bond acceptor.

In conclusion, the NH proton temperature coefficients, ROE distance data and ^3J proton–proton coupling constants together convincingly show that, in dilute chloroform solution, the conformation of amythiamicin D **2** is very similar to that seen in the crystal structure of protein-bound GE2270A **3**,[12] being constrained by a single hydrogen bond between NH13 and O28. Our data also suggest that amythiamicin D **2** aggregates at higher concentrations by formation of a hydrogen bond from NH29, and the form of the shift change of this proton with concentration suggests that this aggregation is by formation of a dimer.

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Notes and references

‡ The spectrum was initially recorded in DMSO-*d* and showed the same cross peaks, but the data was of poorer quality and therefore subsequent work was done in $CDCl_3$ solution. The use of $CDCl_3$ is justified as being biologically relevant in that the protein binding site for the closely related antibiotic GE2270A **3** is completely non-polar.[12]

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