Biochem. J. (1988) 256, 271–278 (Printed in Great Britain)

^{1}H- and ^{13}C-n.m.r. studies of the antitumour antibiotic luzopeptin

Resonance assignments, conformation and flexibility in solution

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The depsipeptide DNA-intercalating antibiotic luzopeptin was studied in solution by n.m.r. methods. Two-dimensional ^{1}H double-quantum-filtered correlation spectroscopy (DQF-COSY) and nuclear-Overhauser-effect spectroscopy (NOESY) confirm the primary structure and twofold symmetry of luzopeptin and provide details of its three-dimensional conformation in solution. Trans-annular hydrogen bonds between the glycine NH groups and carbonyl oxygen atoms have been identified in the crystalline state [Arnold & Clardy (1981) J. Am. Chem. Soc. **103**, 1243–1244], and are important in maintaining an antiparallel β-sheet conformation. The n.m.r. data indicate that the glycine NH protons are appreciably shielded from the solvent molecules, which suggests that these hydrogen bonds are maintained in solution. The orientation of the quinoline chromophores is defined by two-dimensional NOE cross-peaks that position the *N*-methyl group of the L-β-hydroxyvaline residue close in space to both the quinoline H-8 and serine NH proton. This pattern of NOEs is in accord both with the chromophore configuration found in the crystal and one where the quinoline rings are aligned in a parallel manner at right-angles to the depsipeptide ring. The n.m.r. data are consistent with a hydrogen bond between the quinoline hydroxy groups and the quinoline carbonyl oxygen atoms. The pyridazine acetylmethyl groups give NOEs to the $C_{(\alpha)}H$ groups of the β-hydroxy-*N*-methylvaline residues, showing that the acetyl groups, for at least some of the time, stretch over the depsipeptide ring, occluding one face of the molecule. Both of the latter features are also found in the crystal structure. Resonances in the ^{13}C-n.m.r. spectrum of luzopeptin have been assigned by transferring ^{1}H assignments to their covalently bonded carbon atoms via a heteronuclear shift-correlation experiment (HETCOR). The measurement of spin–lattice relaxation times and ^{1}H–^{13}C NOEs at specific sites in the molecule has led us to conclude that segmental motions within the depsipeptide ring are restricted and that the ^{13}C relaxation data for luzopeptin's protonated carbon atoms are adequately described by isotropic tumbling in solution. Furthermore, relaxation data for the carbon atoms of the quinoline chromophores show that these rings exhibit similar motion to the depsipeptide ring and are not rotating rapidly with respect to it. Taken together all the data imply that luzopeptin is fairly rigid in solution, on the time scale of molecular tumbling, and has, or can readily attain, a staple-like structure suitable for bisintercalation. An important conclusion from the n.m.r. and X-ray studies is that the interchromophore separation in the (putative) DNA-binding configuration is large enough to encompass three base-pairs of a B-type helix. The trans-annular hydrogen bonds are very stable under our conditions, implying that changes to the conformation of the depsipeptide ring will be necessary if the glycine NH and carbonyl groups are to be involved in intermolecular hydrogen-bonding interactions with the DNA bases. In the unperturbed structure only the carbonyl oxygen atoms of the D-serine residues are appropriately positioned for hydrogen-bonding to DNA.

INTRODUCTION

Luzopeptin (synonym BBM-928A) is the principal component of a complex of antibiotics isolated from the organism *Actinomadura luzonensis* [1,2]. These drugs resemble the quinoxaline family of antibiotics, the parent of which is echinomycin [3]. Luzopeptin (Fig. 1) is a cyclic depsipeptide containing ten amino acid residues and two 3-hydroxy-6-methoxyquinaldic acid chromophores per molecule. The antibiotic comprises 2 molar equivalents each of the amino acids D-serine, sarcosine and glycine and the unusual residues L-β-hydroxy-*N*-methylvaline and *trans*-(3*S*,4*S*)-4-acetoxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid [4,5]. Preliminary ^{13}C- and ^{1}H-n.m.r. studies of luzopeptin [4,5] indicate that it consists of two equivalent halves related by a twofold axis of symmetry. X-ray-crystallographic studies have provided details of the three-dimensional structure of the antibiotic [6], showing that it retains a twofold symmetry axis in the crystal, and that it has a right-handed twisted rectangular conformation reminiscent of that of echinomycin [7], triostin A [7,8] and TANDEM [9]. Luzopeptin shows activity against a wide range of animal tumours, being significantly active

Abbreviations used: NOE, nuclear Overhauser effect; DEPT, distortionless enhancement by polarization transfer; NOESY, two-dimensional nuclear-Overhauser-effect spectroscopy; DQF-COSY, two-dimensional double-quantum-filtered correlation spectroscopy; HETCOR, two-dimensional heteronuclear correlation spectroscopy; T_1, spin–lattice relaxation time.

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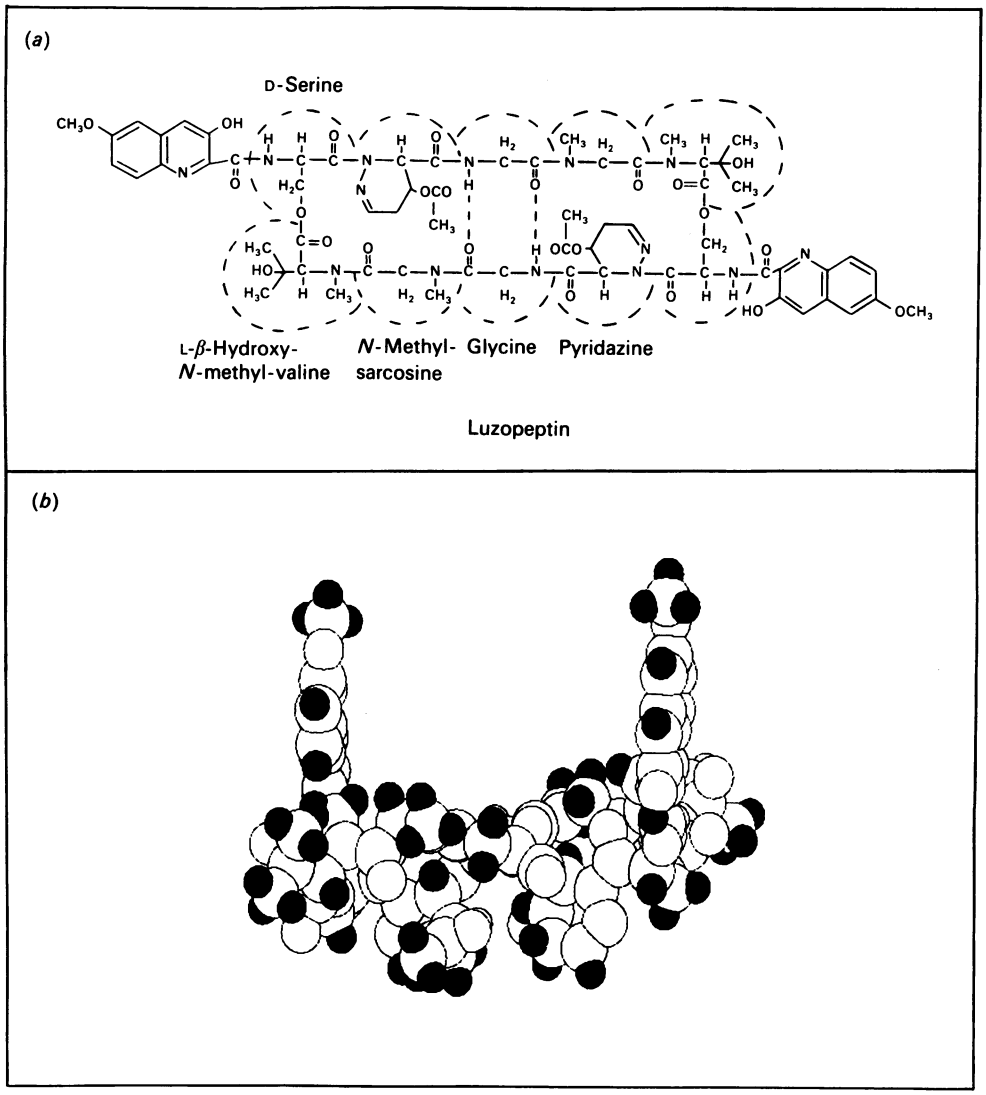

Fig. 1. (*a*) Chemical structure of luzopeptin and (*b*) space-filling model representing the appropriate conformation for bifunctional intercalation between the base-pairs of DNA

The chromophores are rotated compared with the crystal conformation to bring the quinoline rings into parallel alignment.

in L1210 and P388 leukaemia screens, as well as in Lewis lung carcinoma, B16 melanoma and sarcoma 180 models [1]. Measurements with covalently closed circular and rodlike fragments of linear DNA reveal that luzopeptin, like echinomycin [3], is a bifunctional DNA-intercalating agent [10,11], with an affinity constant of 3×10^6 M^{-1} and a binding-site size of five to six nucleotide pairs per bound ligand molecule [10]. The DNA-binding properties of luzopeptin differ from those of echinomycin, however, in the ability of the former, but not the latter, to cross-link two DNA molecules as well as to bind to RNA and heat-denatured DNA [3,10–12]. Given the larger size of the peptide ring of luzopeptin, these differences may arise as a consequence of greater conformational flexibility within the luzopeptin structure. To evaluate this possibility we have studied luzopeptin by using n.m.r. spectroscopy, which is recognized as a powerful method for characterizing the conformation and dynamics of molecules in solution.

A prerequisite for investigating solution conformation and dynamics is the assignment of resonances in the n.m.r. spectrum to specific atoms in the antibiotic molecule. Methodologies are now well established enabling ^{1}H resonances to be assigned on the basis of two-dimensional ^{1}H-homonuclear-chemical-shift correlation experiments in the form of DQF-COSY and NOESY. Similarly, ^{13}C resonances can be identified initially as belonging to methine, methylene or methyl carbon atoms by using DEPT spectral editing techniques [13,14], then by a two-dimensional heteronuclear ^{1}H–^{13}C shift-correlation experiment, HETCOR, to transfer ^{1}H assignments to their covalently bonded carbon atoms. Specific assignment of protonated carbon atoms, together with measurements of ^{13}C spin–lattice relaxation times, T_1, and nuclear Overhauser effects, NOE, for both the backbone and side-chain carbon atoms at specific sites, also yield information about the overall rate of molecular tumbling and the mobility of individual structure elements within the molecule [15,16]. This approach enables the solution dynamics and molecular

flexibility to be characterized. Assignment of the amide and hydroxy resonances in the ^{1}H-n.m.r. spectrum to specific residues in the molecule also provides a starting point for probing intramolecular hydrogen-bonding interactions in solution, which may be important in defining molecular conformation. Such experiments are described here for luzopeptin, and the implications for its DNA-binding properties are discussed.

MATERIALS AND METHODS

Samples for n.m.r. studies

Luzopeptin was a gift from Bristol Myers and was used without further purification. Two-dimensional ^{1}H-n.m.r. and one-dimensional and two-dimensional ^{13}C-n.m.r. data were collected from samples of luzopeptin dissolved in [^{2}H]chloroform: for ^{1}H-n.m.r. studies 0.8 ml of a 7 mM solution was used, and for ^{13}C experiments 3.0 ml of a 5.4 mM solution.

^{1}H-n.m.r. experiments

All ^{1}H-n.m.r. spectra were obtained at 400 MHz on a Varian VXR 400/WB spectrometer. Homonuclear ^{1}H double-quantum-filtered COSY and NOESY experiments were performed in the phase-sensitive mode with the hypercomplex method of data collection [17,18]. Data were collected with the carrier frequency placed at the centre of the spectrum with quadrature detection in both dimensions: data were recorded as 1024 to 2048 points in t_2 for each of 512 t_1 values. A relaxation delay of 3.0–3.5 s was incorporated into each sequence. The total accumulation time in each case was approx. 16 h. Data sets were zero-filled to 1024 points in t_1 before Fourier transformation, and an apodization function was used to weight the data to ensure that the interferogram decayed to zero by the end of accumulation. All ^{1}H chemical shifts are referenced to internal tetramethylsilane at 20 °C.

^{13}C-n.m.r. experiments

All ^{13}C-n.m.r. data were collected at 100.58 MHz on the Varian spectrometer. DEPT subspectra were obtained by using the methods outlined in ref. [13]. Coherence transfer was optimized for a $^1J_{(CH)}$ value of 150 Hz and a series of spectra were obtained with θ pulses of 38, 90 and 142°. A sweepwidth of 15 kHz was used for which 16 k data points were collected with a relaxation delay of 2 s between scans. ^{1}H decoupling was achieved by using the Waltz method [19] during the acquisition period only. The two-dimensional heteronuclear ^{1}H–^{13}C correlation spectrum (see refs. [20] and [21]) was obtained by using a standard pulse sequence from the Varian library. Experimental conditions were again optimized for a $^1J_{(CH)}$ value of 150 Hz. In all 2 k data points were collected in the ^{13}C dimension (t_2) with the use of a spectral width of 15 kHz for 64 t_1 increments over a ^{1}H spectral width of 4 kHz. Waltz decoupling during acquisition only was used to collapse ^{13}C multiplets. The data were zero-filled to 512 points in t_1 before Fourier transformation, and a pseudo-echo function was used to weight the data. The total data accumulation time was 48 h. Spin–lattice relaxation times were obtained by using the fast-inversion recovery method [22] with a composite 180° pulse. T_1 values were determined by non-linear regression analysis from a total of 12 time points. Nuclear Overhauser enhancements were measured by comparison of spectra obtained with normal Waltz decoupling and with gated Waltz decoupling. For T_1 and NOE measurements relaxation delays of 5 and 20 s were used respectively. All ^{13}C chemical shifts are referenced to internal [^{2}H]-chloroform at 77.00 p.p.m.

RESULTS AND DISCUSSION

^{1}H-resonance assignments

Resonances were assigned in the ^{1}H-n.m.r. spectrum of luzopeptin by using a two-step procedure. Firstly, phase-sensitive double-quantum-filtered COSY experiments enabled us to identify scalar coupling interactions between protons separated by two or three chemical bonds. Several spin systems are unique, and a number of protons were assigned specifically on the basis of scalar coupling interactions alone. Secondly, protons that are correlated by dipolar through-space interactions were identified in phase-sensitive NOESY spectra. Fig. 2 illustrates the DQF-COSY spectrum of luzopeptin at 20 °C. Horizontal and vertical lines delineate correlations between scalar-coupled protons for all spin systems in the antibiotic. Correlations involving the tetrahydropyridazine ring, glycine and serine residues are readily identified. The sarcosine $C_{(\alpha)}H_2$ protons are revealed by elimination of the other geminally coupled hydrogen atoms. Scalar-coupling interactions between H-8 and H-7 and a meta coupling between H-7 and

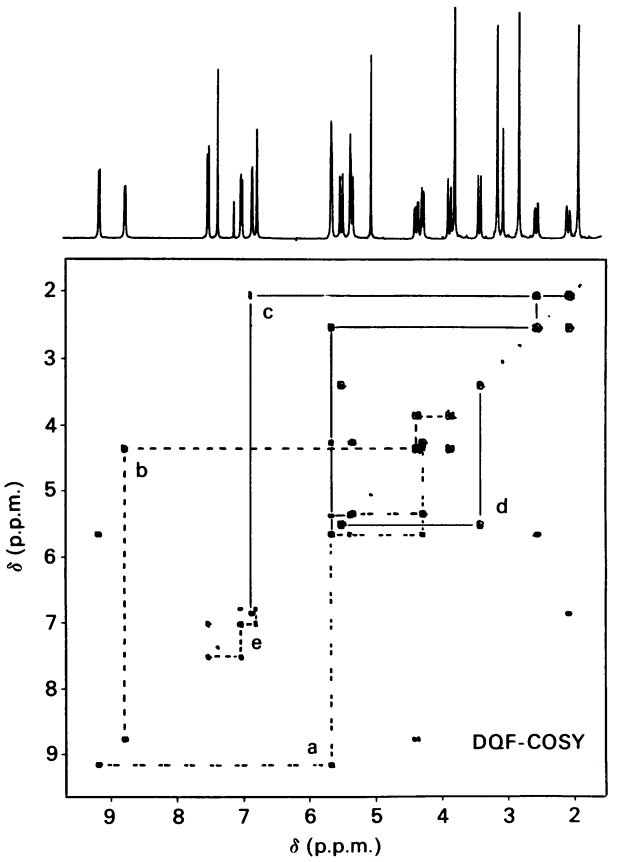

Fig. 2. DQF-COSY spectrum of luzopeptin in [^{2}H]chloroform at 20 °C

Scalar couplings within the spin systems of serine (a), glycine (b), tetrahydropyridazine (c), sarcosine (d) and quinoline (e) are identified.

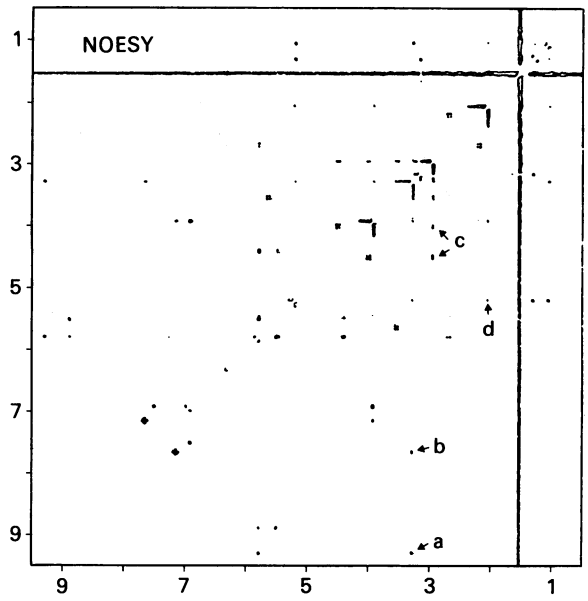

Fig. 3. NOESY spectrum of luzopeptin in [^{2}H]chloroform at 30 °C (1.0 s mixing time)

A number of inter-residue NOEs are highlighted that identify pairs of protons that are less than 0.4 nm apart: β-hydroxy-*N*-methylvaline *N*-methyl group to serine NH group (a), β-hydroxy-*N*-methylvaline *N*-methyl group to quinoline H-8 (b), sarcosine *N*-methyl group to glycine $C_{(\alpha)}H$ groups (c) and pyridazine acetylmethyl group to β-hydroxyvaline $C_{(\alpha)}H$ group (d).

H-5 are also readily apparent within the quinoline chromophore. Fig. 3 illustrates a portion of the NOESY spectrum of luzopeptin recorded at 30 °C with a mixing time of 1 s. In the phase-sensitive experiment cross-peaks appear with opposite phase to the diagonal as a consequence of fast molecular motion on the n.m.r. time scale ($\omega\tau \ll 1$, where ω is the spectrometer frequency and τ is the correlation time of an interproton vector), and thus only the cross-peaks are plotted in Fig. 3. The observation of NOEs between the glycine NH group and pyridazine $C_{(\alpha)}H$ and $C_{(\beta)}H$ groups confirms the sequential positioning of these two residues within the depsipeptide. The NOESY data enable the singlet resonances to be assigned, particularly those of the six methyl groups that are filtered out of the DQF-COSY spectrum. The two highest-field singlet resonances between 1.0 and 1.3 p.p.m. are assigned to the side-chain methyl groups of β-hydroxy-*N*-methylvaline, these groups giving NOESY cross-peaks to the singlet at 5.17 p.p.m., which we can assign to the $C_{(\alpha)}H$ group of this same residue. The $C_{(\alpha)}H$ group in turn is close to its *N*-CH_3 group at 3.26 p.p.m. The 6-OCH_3 group of the quinoline chromophore is identified at 3.91 p.p.m., giving NOEs to both the H-5 and H-7 on the quinoline ring. The *N*-CH_3 group of sarcosine is assigned on the basis of NOEs to the $C_{(\alpha)}H$ groups of both sarcosine and the adjacent glycine residue. The remaining 4-acetoxy methyl group of the tetrahydropyridazine ring is identified by the process of elimination, all other methyl resonances having been accounted for. The H-4 resonance from the quinoline is assigned by a NOESY cross-peak from the H-5; the 3-hydroxy proton, on the same ring, appears at very low field (11.75 p.p.m.) in [^{2}H]chloroform and is assigned on the basis of its correlation to the adjacent H-4. The hydroxy proton of β-hydroxy-*N*-methylvaline, by comparison, resonates at much higher field (3.18 p.p.m.) and is assigned by a cross-peak to one of the CH_3 groups of that residue. This completes the assignment of the ^{1}H-n.m.r. spectrum. All ^{1}H chemical-shift values are listed in Table 1.

Hydrogen-bonding interactions and solvent accessibility

Four resonances have been identified and assigned in the ^{1}H-n.m.r. spectrum to the amino and hydroxy protons of luzopeptin. Three of these resonances, namely those of the glycine NH group, the serine NH group and most notably that of the quinoline 3-hydroxy group, have particularly low-field chemical shifts (> 8.5 p.p.m.). In contrast, the hydroxy proton of the β-hydroxyvaline residue appears at much higher field, 3.18 p.p.m. Several methods are available for probing the solvent accessibility and hydrogen-bonding characteristics of these protons in solution, including solvent and temperature effects on chemical-shift values [23,24]. In hydrogen-bonding solvents such as acetone and dimethyl sulphoxide solvent-exposed NH and OH protons generally have large temperature coefficients ($d\delta/dT > 0.003$ p.p.m./K) whereas those that are solvent-shielded generally have much smaller coefficients, typically < 0.003 p.p.m./K [23,24]. Plots of δ versus T (not shown) yield linear profiles for the low-field resonances in [^{2}H]chloroform, [$^2H_6$]acetone, [$^2H_6$]acetone/water (9:1, v/v) and [$^2H_6$]-acetone/water (4:1, v/v) solutions over the temperature range from 20 to 60 °C. The $d\delta/dT$ values for the glycine NH and quinoline 3-hydroxy protons are 0.0022 ($\pm$0.0002) p.p.m./K in all of the solvent mixtures studied. The serine NH proton has a smaller coefficient of 0.0009 ($\pm$0.0001) p.p.m./K which is also invariant to changes in solvent composition. The β-hydroxyvaline hydroxy group can only be assigned in [^{2}H]chloroform, for which $d\delta/dT = 0.0034$ p.p.m./K. At a given temperature the dependence of chemical-shift values on solvent composition also provides useful information about the degree of solvation. Solvent-exposed NH or OH protons are expected to show large downfield changes in chemical shifts (> 0.5 p.p.m.) on changing from a non-hydrogen-bonding ([^{2}H]chloroform) to a hydrogen-bonding solvent ([$^2H_6$]acetone/water); however, differences of < 0.15 p.p.m. are observed when comparing the three low-field resonances in [^{2}H]chloroform and a [$^2H_6$]-acetone/water (4:1, v/v) solvent mixture. We conclude that the glycine NH, serine NH and quinoline 3-hydroxy protons are shielded from the bulk solvent. The small temperature-dependence of the chemical shifts of the glycine NH and quinoline 3-hydroxy protons can be accounted for in terms of intramolecular hydrogen-bonding interactions that have been seen in the crystal structure of luzopeptin [6]. Arnold & Clardy [6] have shown that the glycine NH groups and carbonyl oxygen atoms participate in trans-annular hydrogen bonds (see Fig. 1). Similarly, the crystal structure revealed a hydrogen bond between the quinoline 3-hydroxy group and its carbonyl group [6], and the n.m.r. data are also consistent with the presence of this interaction in solution. The serine NH group, however, also appears to be solvent-shielded, and yet no specific hydrogen bond is identified in the crystal that could account for this observation. Its particularly low-field chemical shift may

Table 1. ^{1}H and ^{13}C chemical shifts and ^{13}C relaxation data for luzopeptin

^{1}H chemical shifts were measured at 20 °C and are ±0.005 p.p.m. ^{13}C chemical shifts were measured at 23 °C and are ±0.01 p.p.m. T_1 and NOE values are estimated to be accurate to within ±15%. The values were obtained at 23 °C; N is the number of attached protons. Terms in parentheses are the NOE values calculated from the observed NT_1 values.

	^{1}H	^{13}C			
Atom	δ (p.p.m.)	δ (p.p.m.)	NT_1 (s)	NOE	$10^{10} \times \tau_{eff.}$ (s)
Pyridazine					
$C_{(\alpha)}H$	5.49	52.03	0.24	2.3 (2.5)	2.7
$C_{(\beta)}H$	5.76	60.97	0.22	2.7 (2.7)	3.2
$C_{(\gamma)}H_2$	2.19/2.67	26.21	0.28	2.3 (2.2)	2.1
$C_{(\delta)}H$	6.97	141.16	0.20	2.3 (2.2)	4.1
$COCH_3$	2.04	20.84	2.37	2.0 –	–
Serine					
NH	9.28	–	–	– –	–
$C_{(\alpha)}H$	5.77	56.98	0.22	2.3 (2.4)	3.2
$C_{(\beta)}H_2$	5.46/4.39	63.59	0.22	2.1 (2.4)	3.2
β-Hydroxy-*N*-methylvaline					
$C_{(\alpha)}H$	5.17	60.82	0.27	2.4 (2.6)	2.2
$C_{(\beta)}OH$	3.18	72.12	–	– –	–
$C_{(\gamma)}H_3$	1.04/1.29	25.53/28.86	0.90/1.35	2.6/2.6	–
N-CH_3	3.26	34.84	2.46	2.1 –	–
Sarcosine					
$C_{(\alpha)}H_2$	5.62/3.53	48.90	0.22	2.3 (2.4)	3.2
N-CH_3	2.93	32.67	2.55	2.2 –	–
Glycine					
NH	8.88	–	–	– –	–
$C_{(\alpha)}H_2$	4.48/3.98	42.00	0.24	2.6 (2.5)	2.7
Quinoline					
3-OH	11.75	–	–	– –	–
4-H	7.48	118.98	0.29	2.0 (2.7)	2.0
5-H	6.89	103.03	0.27	2.7 (2.6)	2.2
6-OCH_3	3.91	55.61	2.40	2.9 –	–
7-H	7.13	121.14	0.27	2.3 (2.6)	2.2
8-H	7.63	130.82	0.28	2.0 (2.7)	2.1

arise as a result of a ring-current contribution from the quinoline chromophore. For example, a configuration about the D-serine peptide bond that places the serine NH group in the plane of the quinoline ring system will result in it experiencing a ring-current-induced downfield shift. Analogously, the 3-hydroxy group of the quinoline also lies in the plane of the aromatic ring in forming the hydrogen bond to its own carbonyl oxygen atom, and its low field chemical shift is also likely to have a downfield ring-current contribution. In contrast, the hydroxy proton of the β-hydroxyvaline residue has a larger temperature coefficient, suggesting limited hydrogen-bonding involvement and greater solvent accessibility.

Conformational constraints

A number of inter-residue NOEs observed in the NOESY spectrum of Fig. 3 place considerable constraints on the conformation of some portions of luzopeptin, in particular the orientation of the quinoline chromophores. In the following discussion the NOEs are interpreted in terms of a single dominant conformation. NOESY cross-peaks are observed from the *N*-methyl group of the β-hydroxyvaline to the H-8 of the quinoline ring and to the serine NH group. It is apparent that this configuration of the chromophore and serine residue effectively occludes the solvent from access to the serine NH group, which accords with our findings of the temperature- and solvent-dependence of its chemical shift. The pattern of NOESY cross-peaks from the side-chain methyl groups of the β-hydroxyvaline residue suggest that the conformation about the valine $C_{(\beta)}$–$C_{(\alpha)}$ bond is well defined in solution. The hydroxy proton gives an NOE to only one of the $C_{(\alpha)}H_3$ groups, while the *N*-methyl group of the valine residue appears to be close in space to the other $C_{(\gamma)}H_3$. These conformational features are illustrated in Fig. 4. NOESY cross-peaks of equal intensity from the sarcosine *N*-methyl group to both glycine $C_{(\alpha)}$ protons indicate a *trans* configuration for the glycine–sarcosine peptide bond. The pyridazine acetylmethyl group gives a weak NOE to the $C_{(\alpha)}H$ group of the β-hydroxy-*N*-methylvaline residue, showing that the acetyl group stretches across the depsipeptide ring, in an analogous manner to the conformation identified in the crystal, and occludes one face of the molecule. This, of course, may not be the sole orientation of the acetyl group, given that the observed NOE is weak. NOEs from the glycine NH group to pyridazine $C_{(\alpha)}H$ and $C_{(\beta)}H$ groups identifies a *trans* configuration about this peptide bond.

Several three-bond coupling constants are resolved in the ^{1}H-n.m.r. spectrum of luzopeptin and have been used to determine possible dihedral angles for various

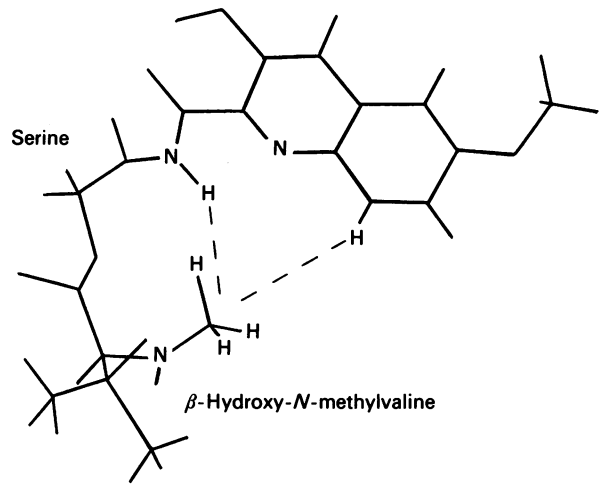

Fig. 4. Proximity of the β-hydroxy-*N*-methylvaline *N*-methyl group to the serine NH group and quinoline H-8 in the crystal structure of luzopeptin

fragments of the molecule. This analysis, together with proximity relationships derived from NOESY data, enables the conformation of the serine and glycine fragments to be determined. The observed $^3J_{(NH-CH)}$ coupling constants for conformationally constrained fragments can be related to the dihedral angle ϕ for the NH–CH bond of L-amino acids by the Bystrov relationship [25,26]. The glycine $^3J_{(NH-CH)}$ coupling constants have values of 5.7 and < 1 Hz, which yield two possible combinations of dihedral angles of −90 and 150° and of 90 and −30° (±5°) for this residue. The more likely configuration is the former, in which the glycine NH and δ carbonyl groups are eclipsed, and are thus fixed in a suitable orientation to hydrogen-bond to the corresponding residues on the opposite side of the depsipeptide ring. This configuration is of course consistent with the pattern of NOEs identified above involving the sarcosine *N*-methyl group and also with the absence of NOEs from the glycine NH group to its own $C_{(\alpha)}H$ groups.

The NH–CH portion of the D-serine fragment, which is not part of the depsipeptide ring structure but which connects the quinoline chromophore to the peptide, has a $^3J_{(NH-CH)}$ value of 6.6 Hz, suggesting four possible values for ϕ of 30, 90, −160 and −80°. It is likely, however, given the exocyclic position of the serine NH group, that the observed value of $^3J_{(NH-CH)}$ represents an average of a number of interconverting rotamers. By contrast, the $C_{(\alpha)}$ and $C_{(\beta)}$ atoms of the D-serine residue do form part of the depsipeptide ring and have ^1H coupling constants that are indicative of a rigidly defined conformation. Small values of $^3J_{C_{(\alpha)}H-C_{(\beta)}H)}$ of 2.5 and < 1 Hz are observed, which we reconcile with a conformation in which the $C_{(\alpha)}$ proton is *gauche* to both $C_{(\beta)}$ protons, although the measured coupling constants for this residue are unusually small. This configuration is consistent with the pattern of NOEs from the serine $C_{(\alpha)}H$ group to the two serine $C_{(\beta)}H$ groups.

^{13}C-resonance assignments

The ^{13}C-n.m.r. spectrum of luzopeptin has also been analysed using a two-step procedure to assign resonances in the spectrum to specific carbon atoms in the molecule. DEPT spectral editing methods [13,14] provide us with an initial analysis of the ^{13}C-n.m.r. spectrum by generating subspectra containing carbon resonances of only a single multiplicity. Thus the subspectra contain CH, CH_2 and CH_3 carbon resonances only. The serine $C_{(\beta)}$, glycine and sarcosine $C_{(\alpha)}$ and tetrahydropyridazine $C_{(\gamma)}$ carbon atoms are clearly resolved in the CH_2 subspectrum (not shown). Specific ^{13}C-resonance assignments have been obtained by using a two-dimensional heteronuclear ^1H–^{13}C shift-correlation experiment [21,22] to enable the transfer of ^1H-resonance assignments to their covalently bonded carbon atoms (results not shown). All protonated carbon atoms were assigned in this manner, and their ^{13}C chemical-shift values are listed in Table 1.

^{13}C-n.m.r. relaxation measurements

Spin-relaxation measurements on ^{13}C nuclei have proved to be a useful method for obtaining information concerning dynamic processes in complex biological molecules [27–31]. With luzopeptin we were particularly interested in evaluating the effects of the glycine-NH-to-δ-carbonyl hydrogen bonds on the structural rigidity of the cyclic depsipeptide ring. By the same token, the hydrogen bond identified between the aromatic 3-hydroxy group of the quinoline and its carbonyl group suggests that these chromophores may also be motionally restricted. We have investigated the conformational flexibility of luzopeptin by measurement of spin-relaxation parameters: the spin–lattice relaxation time, T_1, and the nuclear Overhauser effect, NOE. Under conditions of continuous decoupling, the dominant relaxation mechanism for protonated carbon atoms is through dipole–dipole interactions with directly bonded hydrogen atoms. The dipolar mechanism arises when the magnetic dipole of a proton produces a fluctuating magnetic field at a nearby carbon nucleus as a result of molecular motion. The relaxation process produced by this fluctuating field is represented quantitively by the value of NT_1 (where N is the number of attached protons) [9]. Measured NOEs of between 2.0 and 2.9 (±0.3) for luzopeptin's protonated carbon atoms confirm that the dipole–dipole mechanism is the dominant relaxation process and that overall molecular motion is fast on the ^{13}C-n.m.r. time scale ($\omega\tau < 1$). However, the observation of non-maximal NOEs (< 2.98) for the majority of carbon atoms in luzopeptin may indicate either the presence of other relaxation mechanisms in addition to the dipole–dipole contribution, or, more probably at this field strength (100 MHz), molecular motions that lie just outside the extreme narrowing limit. In the latter case the use of simple equations to describe molecular motions is not applicable. We have therefore used the molecular dynamics program MOLDYN [32] to describe motions in terms of an effective correlation time, $\tau_{eff.}$, for individual carbon atoms. This parameter was optimized from both the NT_1 and NOE data assuming that the molecule is undergoing isotropic tumbling in solution; the results are given in Table 1.

The effective correlation times for the backbone methine and methylene carbon atoms lie in the range 2.2×10^{-10}–4.1×10^{-10} s, there being good agreement between the experimental and calculated values for the NOEs (see Table 1). Furthermore the presence of only small variations in the atomic motions along the backbone of the depsipeptide ring indicates a uniformity

of motion throughout the structure on the time scale of molecular tumbling. Using the Stokes–Einstein equation we have calculated the rate of isotropic tumbling for a rigid structure (approximated by a solid sphere of diameter 1.5 nm) in solution of similar viscosity and obtained a value for $\tau_{eff.}$ of 2.3×10^{-10} s. This value agrees closely with the experimental values for $\tau_{eff.}$. Thus we are led to conclude that isotropic reorientation of luzopeptin in solution is an acceptable approximation to its overall molecular motion and that segmental motions within the ring are restricted. It seems likely that the trans-annular hydrogen-bonding interaction between the glycine residues contributes significantly to this molecular rigidity. Furthermore $\tau_{eff.}$ values for the aromatic carbon atoms and those in the pyridazine ring fall in the same range as those in the backbone of the depsipeptide. This indicates that the quinoline rings do not rotate rapidly relative to the peptide backbone and that the pyridazine ring shows similar restricted motion to the rest of the depsipeptide.

A more complex model than simple isotropic molecular reorientation is required to describe adequately methyl group motions, and therefore here we interpret their NT_1 values only semi-quantitatively. Restricted motion of the β-hydroxyvaline methyl groups is evident from their NT_1 values, which are substantially smaller than those of any of the other methyl groups in the molecule. This is consistent with restricted rotation about the $C_{(\beta)}$–$C_{(\gamma)}$ bond arising, for example, from a concerted rotation of the two methyl groups i.e. 'geared motion' [33].

Implications for DNA-binding properties of luzopeptin

Together, the results of the crystallographic and n.m.r. studies provide a comprehensive picture of the shape and dynamic properties of luzopeptin in solution. The n.m.r. data suggest that all of the principal structural features of the antibiotic revealed by the crystallographic analysis are retained in solution, even in solvents containing 20% water, where competing solute–solvent hydrogen-bonding schemes are possible. Thus in solution, as in the solid phase, the peptide portion of luzopeptin comprises a symmetrical right-handed rectangular β-sheet, the antiparallel strands held firmly together by a pair of trans-annular hydrogen bonds between the glycine residues. The n.m.r. data imply that these hydrogen-bonding interactions are strong, whereas in the crystal the bond lengths appear long, which indicates a weak interaction. Perhaps there is some relaxation of the structure in solution that permits closer approach of the glycine groups. It is clear that the relative position of the tetrahydropyridazine rings and their substituents is largely unperturbed in solution compared with the crystal. The n.m.r. and crystallographic data show that the acetyl groups stretch across the peptide, lying parallel to its minor axis, and occlude one face of the molecule. However, the hydrogen bond between the hydroxy group of *N*-methyl-β-hydroxyvaline and its own carbonyl oxygen atom, which is identified in the crystal configuration, appears to be weak if not absent in solution.

To enable bisintercalation into DNA the quinoline chromophores must be on the same side of the molecule, be aligned approximately parallel to one another and lie at right-angles to the peptide ring. However, in the crystal structure, although the chromophores are both on the opposite face of the peptide to the tetrahydropyridazine rings as a consequence of the stereochemistry about the D-serine residue, they are inappropriately oriented for intercalation. They are arranged edge-on to one another, lying along a diagonal axis bisecting the serine $C_{(\alpha)}$ atom at either end of the molecule. We have found from molecular-modelling studies that rotations about the serine $C_{(\alpha)}$–N and serine *N*-quinoline carbonyl bonds of -70 and $+30°$ respectively bring the quinoline rings into a parallel alignment perpendicular to the long axis of the depsipeptide. The orientation of the chromophores with respect to the peptide ring is defined in solution by the NOE from the *N*-CH_3 group of the β-hydroxyvaline residue to the quinoline H-8. Since the relevant inter-proton distance in both the crystal structure and modified configuration is less than 0.4 nm, the n.m.r. data are consistent with either conformation and cannot be used to distinguish between them. Molecular-mechanics calculations indicate that the energy difference between the two conformers is small, implying that they may readily interconvert. The hydrogen bond between the quinoline hydroxy group and the quinoline carbonyl oxygen atom was maintained in the molecular-mechanics simulation.

The ^{13}C measurements of the molecular dynamics of luzopeptin point to a relatively inflexible structure in solution that behaves essentially as a rigid body on the time scale of molecular tumbling. Under our experimental conditions, even up to 70 °C in solvents comprising 20% water, there is no evidence for breathing motions within the trans-annular hydrogen bonds, nor does it appear that the quinoline chromophores spin rapidly with respect to the depsipeptide. The tetrahydropyridazine rings also have internal motions that are typical of the molecule as a whole. Thus all the data taken together suggest that luzopeptin is fairly rigid in solution and has, or can readily attain at little energy cost, the correct staple-shaped 'C-like' structure appropriate for bisintercalation. An important conclusion from the structural data is that the interchromophore separation in the (putative) DNA-binding configuration is large enough to encompass three base-pairs of a B-type Watson–Crick helix. A second conclusion is that, if bisintercalation of luzopeptin were to proceed via a mechanism that involves hydrogen-bonding between its glycine residues and the DNA bases, then there would have to be changes in the solution conformation of the depsipeptide to disrupt the trans-annular hydrogen-bonding scheme. Indeed, with regard to potential intermolecular hydrogen bonds, we note that in the configurations found in the crystal and in solution the only hydrogen-bonding group of luzopeptin appropriately positioned for directly interacting with the DNA bases is the D-serine carbonyl oxygen atom. We are somewhat sceptical of the capacity of luzopeptin to cross-link two DNA molecules by binding in a Z-like manner as suggested by Huang *et al.* [34], since the n.m.r. and crystallographic data exclude the possibility that one of the chromophores and its neighbouring tetrahydropyridazine ring ever find themselves on the opposite face of the peptide ring to the other pair. It remains to be seen whether this conformation can be induced by DNA on binding.

We thank Dr. A. Clardy and Dr. E. Arnold for providing the crystal co-ordinates of luzopeptin and Dr. R. T. C. Brownlee

for allowing us access to the molecular-graphics facilities in the Department of Chemistry at La Trobe University. This work was supported by the Australian Research Grants Scheme.

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Recieved 2 February 1988/17 May 1988; accepted 23 May 1988