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Crystal Structures of Cephaibols‡

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Abstract: The crystal structures of the peptaibol antibiotics cephaibol A, cephaibol B and cephaibol C have been determined at ca. 0.9 Å resolution. All three adopt a helical conformation with a sharp bend (of about 55°) at the central hydroxyproline. All isovalines were found to possess the D configuration, superposition of all four models (there are two independent molecules in the cephaibol B structure) shows that the *N*-terminal helix is rigid and the *C*-terminus is flexible. There are differences in the hydrogen bonding patterns for the three structures that crystallize in different space groups despite relatively similar unit cell dimensions, but only in the case of cephaibol C does the packing emulate the formation of a membrane channel believed to be important for their biological function. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cephaibol; peptaibol; antibiotic; crystal structure; ion channel; membrane channel

INTRODUCTION

Cephaibols comprise a group of peptaibol antibiotics and have been isolated from the soil fungus *Acremonium tubakii*, DSM 12774 [1]. Apart from their considerable antibacterial potency, it has been established that cephaibols possess pronounced anthelmintic action and activity against ectoparasites. Other surprising biological properties, such as the induction of pigment formation in *Phoma destructiva* and potential neuroleptic effects have recently been reported [2,3].

The amino acid sequence of cephaibols shows marginal variation among the group members (Figure 1), the main difference being some methyl groups at positions 5, 6, 8 and 12. However, as there is an at least 10-fold variation in the antibacterial potency among the members of the group, the presence or absence of these methyl groups seems to have a substantial effect upon antimicrobial action. Unlike other types of antibiotics, peptaibols frequently occur naturally as microheterogeneous mixtures; this peculiarity has long been established and attributed to their non-ribosomal peptide synthesis, in which Aib is frequently replaced by other α,α-dialkylated amino acids [4].

Three-dimensional structures of various peptaibols have been determined by X-ray diffraction for alamethicin [5], [Leu1]zervamicin [6], antiamoebin [7,8] and trichotoxin [9]. The NMR solution structures of chrysospermin C [10], antiamoebin [11] and zervamicin IIb [12,13] have also been reported. These molecules seem to adopt a common structural motif that consists of a long helical section that has a variable bend at a proline or a hydroxyproline residue near to the middle of the helix. Since the action of peptaibols is believed to derive from their interaction with biological phospholipid membranes, in which the formation of ion channels results in increased ion permeability [14], the length seems to be an important feature of the

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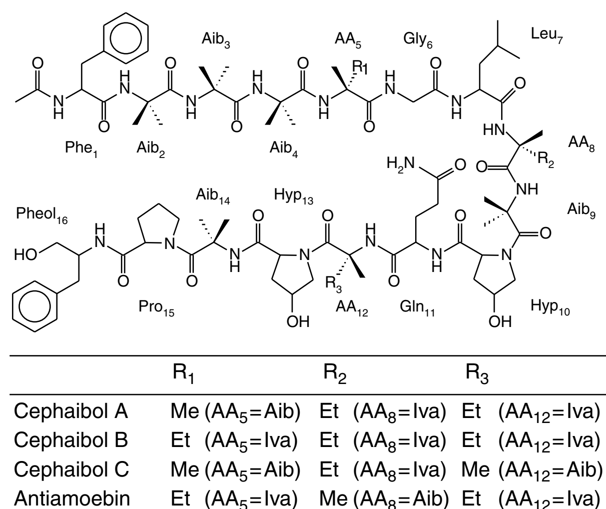

	R_1	R_2	R_3
Cephaibol A	Me (AA_5=Aib)	Et (AA_8=Iva)	Et (AA_{12}=Iva)
Cephaibol B	Et (AA_5=Iva)	Et (AA_8=Iva)	Et (AA_{12}=Iva)
Cephaibol C	Me (AA_5=Aib)	Et (AA_8=Iva)	Me (AA_{12}=Aib)
Antiamoebin	Et (AA_5=Iva)	Me (AA_8=Aib)	Et (AA_{12}=Iva)

Figure 1 Chemical structure of selected cephaibols and comparison with antiamoebin.

molecule. Alamethicin with its 20 residues and moderate bending angle (about 30°) is more than 33 Å long and therefore able to span biological membranes. Conductivity measurements with several artificial and naturally occurring membrane systems did show the formation of highly voltage sensitive and weakly cation selective membrane channels [15–17]. The 18-residue trichotoxin and the 19-residue chrysospermin C possess similar structures, although they are shorter and their bending angles are markedly different (10° and 38°, respectively). [Leu1]zervamicin consists of only 16 residues and its bending angle varies among the different crystal forms in the range 30°–45°. Consequently, it is significantly shorter than alamethicin, only 29 Å long, but conductivity measurements still prove the formation of membrane channels [18]. Moreover, it was found that in all crystal forms [Leu1]zervamicin molecules aggregated in a similar fashion to form water channels and suggested a gating mechanism for cation transport. The structure of antiamoebin has been determined independently in methanol [8] and in a partial membrane-mimetic environment [7]. Although different crystal forms were obtained, the peptide conformation and even the molecular packing show strong similarity between the two structures. The 16-residue antiamoebin, in comparison with [Leu1]zervamicin, possesses a much sharper bend near 55°, but only marginally shorter than [Leu1]zervamicin. Initial membrane conductivity measurement failed to give evidence of membrane channel formation [8], and although later largely voltage-insensitive channel formation has been observed in specific membrane media [19], a hypothesis for an ion carrier mechanism was put forward.

Cephaibols, although similar to antiamoebin, were available to us as pure separate compounds that have marginal structural difference but wide variation in antibacterial potency and provided an opportunity to investigate the effect of small structural changes on backbone conformation and association properties.

MATERIALS AND METHODS

Crystallization and Data Collection

The isolation and purification of cephaibols have already been described in detail [1]. Pure cephaibol A, B and C were dissolved in ethanol : water 1 : 1 mixture (concentration: cephaibol A and B, 5 mg/ml, cephaibol C, 40 mg/ml). Aliquots of these (cephaibol A and B, 4 µl, cephaibol C, 2 µl) were mixed with 2 µl of the reservoir solution (0.1 M NaAc/HAc pH = 4.2–4.8 and 30%–38% ethanol) and crystallized using the hanging drop technique. Before data collection, a suitable crystal was soaked in cryoprotectant solution consisting of 15% ethylene glycol in the crystallization condition and shock frozen in a cold nitrogen stream operating at 100 K. Data sets were collected on a Bruker rotating anode, Osmic focusing mirrors and a Bruker SMART6000 CCD detector using Cu Kα radiation.

Data Processing and Structure Solution

Images were collected and indexed with the program system Proteum [20]. After orientation matrix refinement, the images were integrated with the program SAINT [20] using a three-dimensional profile-fitting algorithm and scaled with the program SADABS [20]. The program XPREP [20] was employed for space group determination, inspection and merging. Data collection statistics have been summarized in Table 1. All three structures were solved using the dual space recycling technique implemented in SHELXD [21] employing default parameters with a reasonable success rate.

Model Building and Refinement

After successful solution, several cycles of temperature factor refinement were performed using the program SHELXL [22] and peaks in the electron density were assigned to appropriate atoms with the

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Table 1 Data Collection Statistics

	Cephaibol A	Cephaibol B	Cephaibol C
Space group	$P2_12_12$	$P2_1$	$P2_12_12_1$
Cell parameters	$a = 30.534$ Å	$a = 32.146$ Å	$a = 9.002$ Å
	$b = 37.787$ Å	$b = 9.126$ Å	$b = 28.619$ Å
	$c = 9.115$ Å	$c = 37.982$ Å	$c = 41.100$ Å
		$\beta = 111.36°$	
No. of reflections	67303	73436	76788
No. of unique reflections	7025	16045	8776
Resolution (last shell) (Å)	0.95 (1.05-0.95)	0.89 (1.00-0.89)	0.89 (1.00-0.89)
Completeness	98.6% (96.6%)	97.0% (90.0%)	99.3% (97.6%)
Redundancy	9.45 (4.66)	4.44 (2.16)	8.69 (4.36)
I/σ	29.21 (10.67)	23.29 (10.37)	50.44 (26.51)
R_{int}	0.0431 (0.1156)	0.0437 (0.0786)	0.0306 (0.0349)

Table 2 Refinement Details

	Cephaibol A	Cephaibol B	Cephaibol C
Resolution range (Å)	37.79–0.95	35.37–0.89	28.62–0.89
R-factor [$F > 4\sigma(F)$/all data]	0.0835/0.0872	0.0709/0.0746	0.0712/0.0719
R-free [$F > 4\sigma(F)$/all data]	0.1082/0.1120	0.0756/0.0793	0.0758/0.0770
No. of non-hydrogen atoms	133	268	135
No. of solvent atoms	17	27	18
R.m.s.d. from ideal geometry			
Bond length (Å)	0.017	0.021	0.018
Angle distances (Å)	0.035	0.040	0.028
Ramachandran plot			
Residues in			
Allowed region (%)	100%	100%	100%
Not allowed region (%)	0%	0%	0%

program XP [20]. When virtually all peptide atoms had been found, the refinement was carried on with SHELXL using suitable bond length, bond angle, chiral volume and planarity restraints. Throughout the refinement, $2mF_o$-DF_c and F_o-F_c type maps were displayed with the program XtalView [23], which was also used for manual editing of the structure and identifying disorder components. Water molecules were added with the program SHELXWAT [22] and by hand. All non-hydrogen atoms were refined anisotropically with rigid bond restraints, similarity restraints and for solvent molecules, approximately isotropic restraints; hydrogen atoms were included in later stages of refinement. Refinement details are shown in Table 2.

Accession Numbers

The coordinates and structure factors have been deposited within the Protein Data Bank under accession numbers 1OB4, 1OB6 and 1OB7 for cephaibol A, B and C, respectively.

RESULTS AND DISCUSSION

Despite their high homology and similar crystallization conditions, different crystal forms were obtained. The overall structures of the molecules, however, were found to be very similar. All three cephaibols assume a helical conformation that is

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sharply bent at Hyp10. The bending angle is near 55° and seems not to vary much. Before the structure determination it was unclear whether the peptides contained D- or L-isovaline, as the chemical structures had been established with NMR spectroscopy and mass spectrometry. The maps unambiguously showed that all isovalines are present as D-isovaline (assuming that the common amino acids are present as L-enantiomers).

Structure Description

Cephaibol A crystallizes in the space group $P2_12_12$ with one molecule in the asymmetric unit. The whole molecule is well defined apart from two residues (Phl16 and Pro15) that each show two distinct conformations. This 1 : 1 disorder implies the flipping of the proline ring into the other envelope conformation and the consequent motion of Phe16. In spite of this fact, the structure shows considerable homology to the previously determined antiamoebin and [Leu1]zervamicin structure in terms of backbone conformation, secondary structure and intramolecular hydrogen bonding pattern.

Cephaibol B crystallizes in the monoclinic space group $P2_1$, but with a very similar cell and therefore has two molecules in the asymmetric unit. Although the overall structures of the two molecules are similar, different regions have been found to be discretely disordered. In the first molecule (molecule I), Hyp10, where the molecule is bent, shows a 9 : 1 disorder for the OH group being in axial and equatorial position, respectively. The backbone of molecule I from Iva12 to Phl16 inclusive also adopts two different conformations. This disorder is thought to be caused by the interaction with the second molecule (molecule II), in which the interacting region (from Ac0 to Aib3) is also disordered, allowing different hydrogen bonds between the two conformations (see later). It is also noteworthy that in molecule II the OH group of Phl16 adopts two conformations, but the sum of occupancies turns out to be slightly higher than one. This fact seems to be accounted for by mass spectrometric evidence that, together with cephaibol B, there is another compound present that has a sequence identical to that of the principal constituent, but with an aldehyde function instead of the alcohol group at Phl16 [1]. As a geminal diol, an aldehyde group would convert into two OH groups, and increase the occupancy for both disorder components. It should be emphasized that according to the electron density map, a maximum of 10% of the molecules end in a phenylalaninal terminus and the rest have phenylalaninol with a disordered OH as the *C*-terminus. However, the presence of the aldehyde seems to be significant.

Cephaibol C crystallizes in the orthorhombic system with cell edges very similar to those of cephaibol A, but in the space group $P2_12_12_1$. This difference in symmetry involves different interactions amongst the molecules and consequently different packing. The structure of cephaibol C was also found to be very similar to that of antiamoebin and [Leu1]zervamicin and contained no disorder. The electron density map showed the presence of some cephaibol E contamination, which manifested itself as Aib12 being partially Iva. This contamination originates from the retention times of cephaibol C and E being nearly identical, making these two components very difficult to separate [1].

Comparison of the Structures

A common way of comparing structures is via a least-squares fitting procedure and giving average r.m.s. distances and the atom pair where the maximum deviation occurs. Although this method works well when considering the molecule as a whole, it distributes the effect of a single conformational movement among several atom pairs and superposition only shows statistical scatter in the atom positions. A better way of identifying any apparent motion would be to find rigid parts of the molecules, which can be considered identical in the two molecules with respect to the preciseness of the analysis [24]. Fitting these two regions onto each other can highlight conformational motion.

To search for rigid fragments, the program ESCET [25] was employed with coordinate esds generated from temperature factors using Cruickshank's DPI equation [26] plus linear B-factor scaling [24]. In total four different molecules were compared simultaneously and in a pairwise manner (side chain atoms were not included). On average, the molecules are about 20% identical within the experimental error (this number is a slight underestimation as the precision of the structure is relatively high), but in special cases the similarity reaches higher levels. Cephaibol A and C are found to share about 60% of identical structure, while cephaibol A also has about 60% in common with one of the cephaibol B molecules. Interestingly, the two cephaibol B molecules were found to be less similar, showing only 30% identity.

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To identify any apparent motion, rigid atoms found by ESCET were fitted together with the program LSQKAB [27], and the whole molecules were transformed using the matrix resulting from this superposition. The two molecules were then compared using computer graphics. In the case of cephaibol A and cephaibol C, the two structures fit very well to each other from their *N*-terminus up to position 12, where the sequence differs (Figure 2a). For cephaibol A and cephaibol B, the centres fit well on each other, only the termini seem to be flexible, and the sequence difference at position 5 seems not to be responsible for the conformational change (Figure 2b). Simultaneous superposition of all models reveals that despite the low structure identity nearly the whole *N*-terminal helix is rigid, while the *C*-terminal is very flexible (Figure 2c). This finding can be confirmed by the analysis of the difference-distance matrices that give no indication of a rigid, but smaller sized *C*-terminal helix and a flexible hinge region. The observed extensive disorder of *C*-termini also suggests that this region can assume multiple conformations.

Packing in the Crystals

In the case of cephaibol C, nearly the same arrangement was found as for antiamoebin and [Leu1]zervamicin, i.e. two V-shaped molecules that are related by a translation along the crystallographic **a** axis face a third one and form a channel that looks like an X when viewed from the side (Figure 3a). Strong intermolecular hydrogen bonds connect the three molecules, especially between O(Gly6)–N^{ε}(Gln11)', O^{δ}(Hyp10)–N^{ε}(Gln11)', O^{δ}(Hyp10)–O(Leu7)', O(Leu7)–O^{δ}(Hyp10)", N^{ε}(Gln-11)–O^{δ}(Hyp10)" and N^{ε}(Gln11)–O(Gly6)" (the apostrophes ' and " denote different symmetry equivalent molecules). This arrangement is believed to be representative of the functional membrane channel [6]. Hydrogen bonds between N(Phe1)–O(Pro15)'",

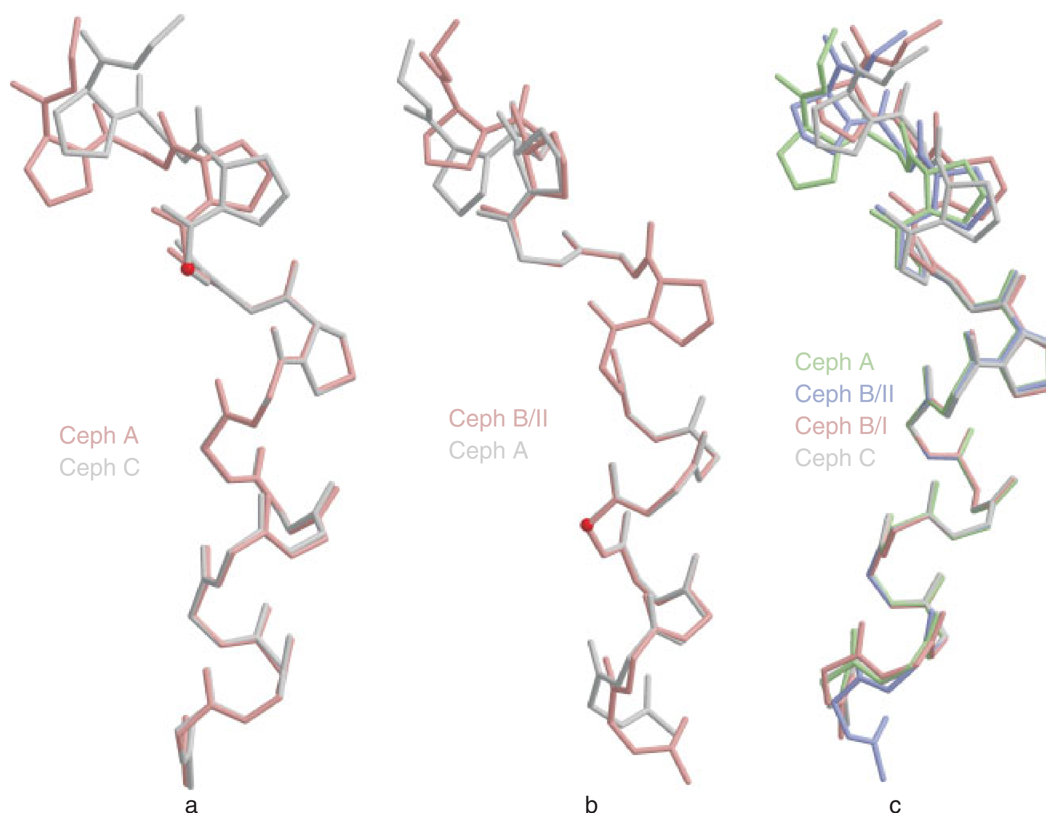

Figure 2 Backbone differences. (a) More than half of the backbone for cephaibol A is virtually identical with that of cephaibol C and the deviation starts at position 12 (marked with the ball), where the sequence differs. (b) Cephaibol B fits very well on cephaibol A and position 5 (where the sequence differs) seems not to be responsible for the different conformation. (c) Simultaneous superposition of cephaibols reveals a rigid *N*-terminal helix, which includes three of four positions where the sequence may differ. These figures were generated with Molscript [28]/Raster3D [29].

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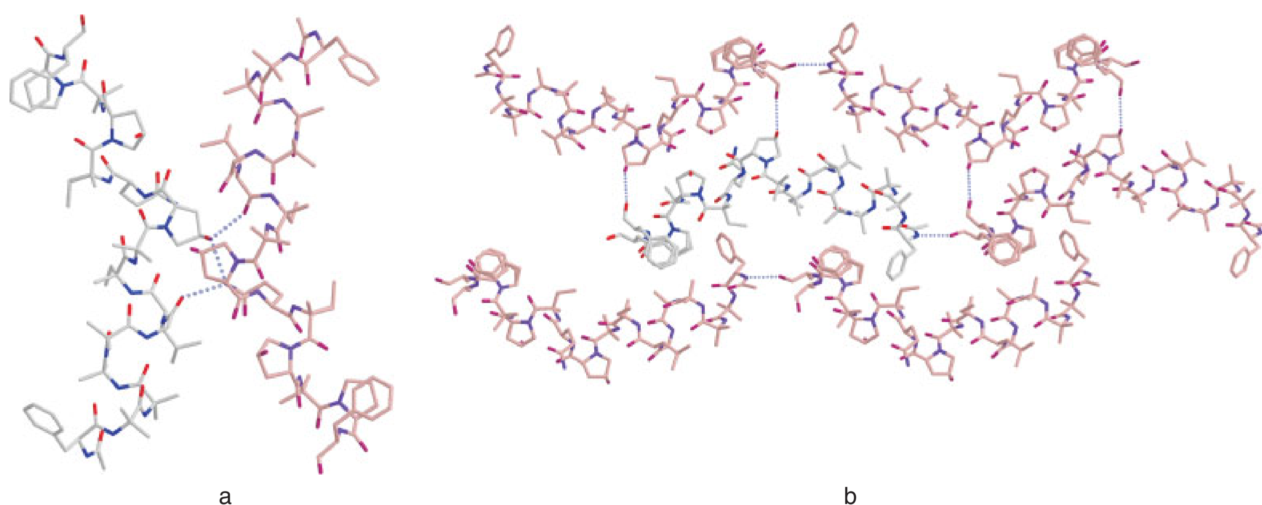

Figure 3 Crystal packing. (a) Three cephaibol C molecules adopt a channel like arrangement (the left molecule represents the asymmetric unit, the right molecule is a symmetry equivalent that is designated by one apostrophe in the text and the third molecule, which is directly behind the right molecule, is designated by two apostrophes; hydrogen bonds that involve the third molecule are not shown), while (b) cephaibol A and B (cephaibol A is shown) form a zigzag structure. Hydrogen bonds of Gln11 are not indicated, because they would involve molecules that are behind the layer that is shown. These figures were generated with Molscript [28]/Raster3D [29].

N(Aib2)–OH(Pheol16)''' and O$^{\delta}$(Hyp13)–O$^{\varepsilon}$(Gln11)'''' connect to other symmetry related molecules and seem to be important in stabilizing the crystal lattice.

Surprisingly, cephaibol A and cephaibol B adopt different packing motifs to cephaibol C. The main hydrogen bonds connect two termini and the centre of one molecule to the terminus of another. The molecules form a layer-like zigzag structure (Figure 3b). There is no sign of any channel formation similar to that observed in the case of cephaibol C, and Hyp10, which was involved in most of the intermolecular interactions, is now pointing towards a water-filled cavity. In the case of cephaibol A the following hydrogen bonds are observed between the molecules: N(Phe1)–OH(Pheol16A)' is the head-to-tail interaction, where A refers to one of two distinct conformations with higher occupancy, while the O$^{\delta}$(Hyp10)–OH(Pheol16B)'', N$^{\varepsilon}$(Gln11)–O$^{\delta}$(Hyp13)''' and O$^{\varepsilon}$(Gln11)–O$^{\delta}$(Hyp13)'''' interactions connect the centre of the molecule with the *C*-termini of three molecules. This pattern also accounts for the disorder observed at the *C*-terminus, since OH(Pheol16) is able to participate in hydrogen bonds in both conformations, although the first is marginally more favourable. The two molecules in the cephaibol B crystal, although having the same packing arrangement as cephaibol A, interact at the termini by means of more hydrogen bonds: N(Phe1/I)–O(Pro15/II)', N(Phe1/I)–OH(Pheol16B/II)', N(Aib2/I)–OH(Pheol-16B/II)' and N(Aib2/I)–OH(Pheol16A/II)' at the *N*-terminus and O(Pro15A/I)–N(Phe1A/II), OH(Pheol-16A/I)–N(Phe1A/II), OH(Pheol16A/I)–N(Aib2A/II), OH(Pheol16B/I)–N(Phe1B/II) and OH(Pheol16B/I)–N(Aib2B/II) at the *C*-terminus (/I and/II refers to molecule I and II, respectively). This higher number of hydrogen bonds seems to be a consequence of the increased flexibility at the termini that enables them to interact in various ways. Both disorder components take part in roughly the same number of hydrogen bonds and therefore have nearly equal occupancies. However, there is a reduction in the number of intermolecular hydrogen bonds involving the centre; only the N$^{\varepsilon}$(Gln11/I)–O$^{\delta}$(Hyp13B/II)'' interaction can be detected.

Solvent Channels

Several water and other solvent (mostly ethanol) molecules have been located during the refinement process. For cephaibol A and B, the solvent molecules seem to be equally distributed along the molecule, although some concentration is observed near the bend in the helix. On the contrary, for cephaibol C the solvent molecules seem to occupy exclusively the cavity between the two legs of the X. This is in accordance with the [Leu1]zervamicin structure with the difference that the cephaibol C

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helix is not amphipathic, and suggests that even this channel would allow the transport of water molecules and ions when immersed in a biological membrane [7].

CONCLUSIONS

Although closely related in chemical structure, the potency of cephaibol antibiotics shows considerable variation among the family members. Nevertheless, the assumption that variations in antibacterial action arise from significant differences in the three-dimensional structure can be rejected since their structures are very similar even at high resolution. However, comparison of intermolecular interactions reveals large differences in association properties. It seems that as the molecules become more flexible, i.e. the amount of disorder in the peptide backbone increases, the number of interactions involving the bend region decreases, so that a new packing arrangement appears when changing from cephaibol C to cephaibol A. For cephaibol B, the centre takes part in only a handful of interactions, while the interactions involving the termini get stronger. It can be assumed that membrane-channel-forming potency decrease in this order. It is interesting to note that the activity of cephaibols increases in the same order. Since cephaibols (as all peptaibol antibiotics) occur naturally as microheterogeneous mixtures, intermolecular interactions involving different members could lead to a large number of complexes with different properties and account for their wide variety of action. It is also suggested that small structural differences should not be disregarded as they might have important effects on the structure and/or on the association properties.

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