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Highly conformationally constrained halogenated 6-spiroepoxypenicillins as probes for the bioactive side-chain conformation of benzylpenicillin*

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SUMMARY

The halogenated 6-spiroepoxypenicillins are a series of novel semisynthetic β-lactam compounds with highly conformationally restricted side chains incorporating an epoxide. Their biological activity profiles depend crucially on the configuration at position C-3 of that epoxide. In derivatives with aromatic-containing side chains, e.g., anilide, the 3*R*-compounds possess notable Gram-positive antibacterial activity and potent β-lactamase inhibitory properties. The comparable 3*S*-compounds are antibacterially inactive, but retain β-lactamase inhibitory activity.

Using the molecular simulation programs COSMIC and ASTRAL, we attempted to map a putative, lipophilic accessory binding site on the PBPs that must interact with the side-chain aromatic residue. Comparative computer-assisted modelling of the 3*R*-, and 3*S*-anilides, along with benzylpenicillin, indicated that the available conformational space at room temperature for the side chains of the 3*R*- and the 3*S*-anilides was mutually exclusive. The conformational space for the more flexible benzylpenicillin could accommodate the side chains of *both* the constrained penicillin derivatives. By a combination of van der Waals surface calculations and a pharmacophoric distance approach, closely coincident conformers of the 3*R*-anilide and benzylpenicillin were identified. These conformers must be related to the antibacterial, 'bioactive' conformer for the classical β-lactam antibiotics. From these proposed bioactive conformations, a model for the binding of benzylpenicillin to the PBPs relating the three-dimensional arrangement of a putative lipophilic S_2-subsite, specific for the side-chain aromatic moiety, and the 3α-carboxylate functionality is presented.

INTRODUCTION

A major stumbling-block to the rational design of improved β-lactam antibiotics has been the continued lack of X-ray crystallographic data for their bacterial target enzymes, the so-called peni-

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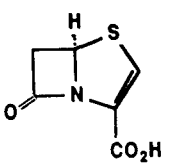
cillin binding proteins, or PBPs. This has placed ever more emphasis on detailed structure-activity studies to elicit information on the likely enzymic binding sites for these important chemotherapeutic agents [1]. Such studies have been greatly assisted by the profusion of compounds synthesised and tested during the last 50 or so years of intensive research in the field of β-lactam antibiotics [2]. These evaluations have revealed a number of the features important for biological activity; for example, it is now accepted that the minimum requirements for antibacterial activity are a β-lactam ring and a suitably positioned adjacent acidic functionality [1, 3]. This particular axiom implies at least two essential enzymic binding sites, one for the β-lactam carbonyl and one for the acid-derived anion functionality, and is best exemplified by the simple antibacterial penem and Δ^2-carbapenem compounds I and II [4]; see Fig. 1.

However, in the case of the 'classical' β-lactam antibiotics, the penicillins and cephalosporins, the simple carboxyl-substituted ring systems, penicillanic acid (III) and Δ^3-cephem-4-carboxylic acid (IV) do not possess notable antibacterial activity [5]. For these compounds, it would appear that a mere two-point association with the PBPs is insufficient for an effective binding that can lead to irreversible inactivation and hence to disruption of bacterial cell-wall biosynthesis. However, attachment of a suitable arylacetamido or aryloxyacetamido side chain to the nucleus, at the 6β-(penams) or 7β-(cephems) position is capable of restoring potent antibacterial activity.

The classical β-lactam antibiotics, with their aromatic-containing side chains, can be looked upon as tri-, or in some cases tetra-, peptide analogues of the enzymes' natural substrate, acyl-D-Ala-D-Ala, with the penam, or cephem nucleus bridging the P_1–P_1' positions (utilising the terminology of Schechter and Berger [6] wherein the β-lactam replaces the hydrolysable D-Ala-D-Ala peptide bond). It is possible then to infer a third binding site, a lipophilic pocket equivalent to an enzymic subsite designated S_2, which is capable of binding the aromatic moiety of the side chain (Fig. 2).

One can thus envisage effective active site docking of the penicillin or cephalosporin by means of a strong electrostatic binding of the essential carboxyl functionality into a suitably positioned,



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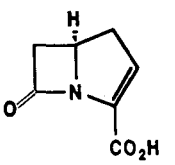

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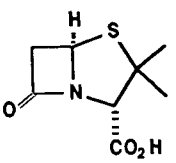
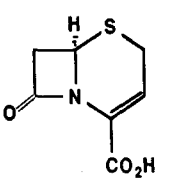


III



IV

Fig. 1. Structures of compounds I – IV.

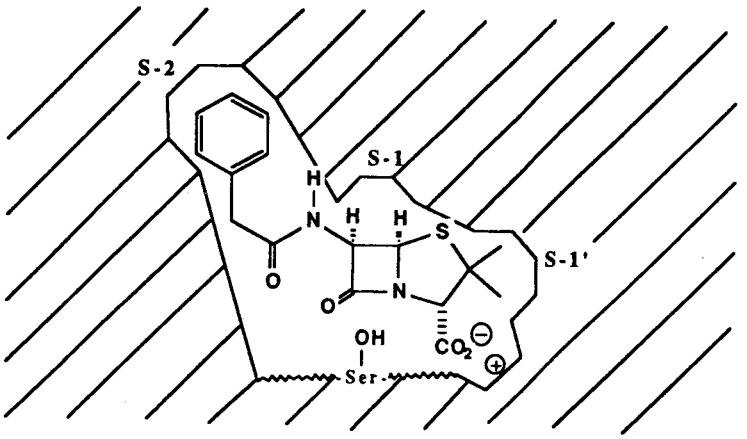


Fig. 2. Diagrammatic representation of the enzymic subsites for binding of benzylpenicillin.

positively charged enzymic pocket, and anchoring of the side chain, through interaction of the aromatic ring with the S_2 subsite. The β-lactam carbonyl is then expediently aligned to allow irreversible inactivation of the PBPs to occur by means of acylation of an active site serine-derived hydroxyl. It should also not be overlooked that the classical β-lactam antibiotic side chains possess a secondary amide bond, which may be important for hydrogen bonding at or near the active site. This would lead to an even more effective enzyme ligand interaction.

To date it has been impossible to obtain information on the position of this putative S_2 subsite, firstly, as has been stated, because of the lack of X-ray crystallographic data on the bacterial PBPs, and secondly because of the considerable conformational flexibility of the arylacetamido side chain preventing identification of a 'bioactive' side-chain conformation.

Restriction of the conformational freedom of flexible, biologically active molecules in order to constrain three-dimensionally their likely binding functionalities, is being increasingly utilised as a means to provide topographical information on receptors whose structures would not otherwise be accessible using alternative, physical methods [7]. In this regard, the conformational restrictions imposed on endogenous mammalian peptides which have been directed towards the dissection of the hormones' often multiple binding conformations have been particularly fruitful [8]. As yet, such approaches have not been applied extensively to the side chains of β-lactam antibiotics. Recently, however, we reported two novel series of highly conformationally constrained, halogenated 6(7)-spiroepoxy penicillins (cephalosporins) [9, 10] whose biological and conformational properties may prove useful for probing PBP active site topography. The two series differ structurally only in their stereochemistry at one crucial position on the side chain, the C-3 position of the epoxide, but this seemingly innocuous difference leads to a marked disparity in their biological properties. In this study we have concentrated on the two representative penicillin analogues (**1** and **2**). These semisynthetic penicillin derivatives can be considered to be 'locked' side-chain analogues of benzylpenicillin (**3**); see Fig. 3.

With respect to the biological activity profiles for the two compounds, the 3*R*-anilide (**1**) possesses notable Gram-positive antibacterial activity, and is also a potent β-lactamase inhibitor, particularly of class A enzymes [11]. Preliminary PBP assay data indicates that compound **1** has affinity

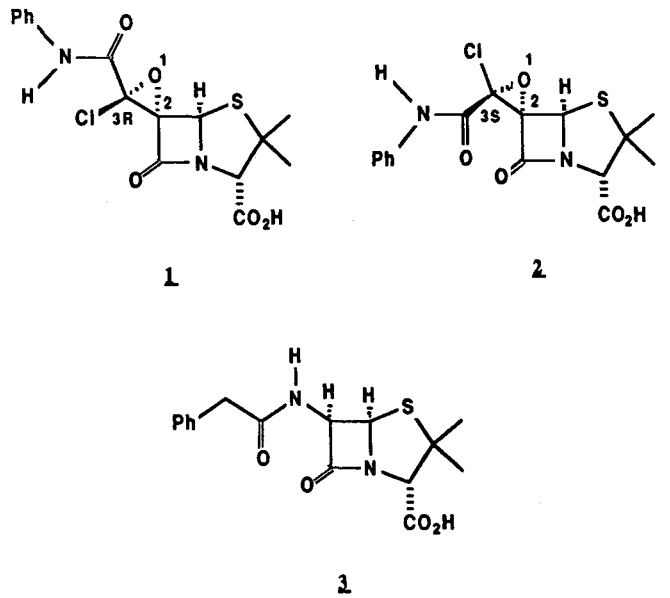


Fig. 3. Structures of compounds **1** – **3**.

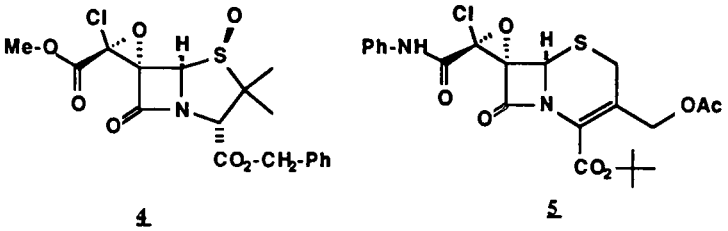
for the PBPs not only of Gram-positive, but also of Gram-negative bacteria [12]. This affinity is highest for the low-molecular-weight PBPs. The 3*S*-anilide (**2**), on the other hand, is antibacterially inactive, but retains β-lactamase inhibitory activity, albeit to a lesser extent than compound **1**.

This marked difference in the antibacterial activity of the two compounds, combined with their structurally similar, but highly constrained side chains, suggested to us that a comparative side-chain conformational analysis of (**1**), (**2**), and benzylpenicillin (**3**), might divulge meaningful information on the position and orientation of the proposed S_2 subsite that is specific for the aromatic moiety of classical penicillin (cephalosporin) side chains. This paper reports the details of our molecular modelling study into this side chain conformational flexibility.

MATERIALS AND METHODS

For this study we utilised the programs COSMIC and ASTRAL [13], mounted on a MicroVAX II with ethernet (DECnet) interface to an Evans & Sutherland PS390, or via a DECserver 200, to Sigma 5684 or Wyse PC+ terminals running Tektronix 4107 emulation software. The Cambridge Crystallographic database was accessed via the joint academic network (JANET) at the Daresbury Laboratory, Warrington, U.K.

X-ray data for compounds **1** and **2** are not available as no derivative of either has been found suitably crystalline. However, coordinates for a carboxyl-protected, methyl ester sulphoxide (**4**) from the 3*S*-series [9], i.e., analogous to (**2**), and the *tert*-butyl-ester-protected cephalosporin anilide (**5**) [10] are accessible (see Fig. 4). These yield sufficient information, particularly on the dimensions of the extremely unusual, and highly functionalised spiro-3,4-fused-4,5 ring system, to allow modelling of the desired compounds.



4 5

Fig. 4. Structures of compounds **4** and **5**.

Although this study was to be directed towards analysis of the side chain, an important consideration in any structure-activity study of penicillins is the conformation of the penam nucleus. It is well established that the 5-membered thiazolidine ring can exist in two possible ring-flip conformations, or puckers [1, 14]. One, where both the 2β-methyl and the 3α-carboxyl are pseudo-axial (henceforth termed 'penam-diaxial'); and one, where the 2β-methyl and the 3α-carboxyl are both pseudo-equatorial (henceforth termed 'penam-diequatorial'). These two puckered conformations have also been termed 'C-3 type' and 'S-1 type' respectively, referring to the atom of the 5-membered ring which is displaced from the plane through the other 4 atoms [15]; alternatively, they have been termed 'closed' and 'open' [16]. We consider the 'axial-equatorial' terminology to be more three-dimensionally informative and consequently have adopted it throughout.

Various studies have indicated that, in solution at room temperature, the two penam conformers are rapidly interconverting [17, 18]. Evidence is strongest for the penam-diequatorial conformation being not only the more energetically stable (by about 0.5 kcal mol^{-1}), but also being most closely related to the 'bioactive' penicillin nucleus conformation [19]. For these reasons, we chose to model the penam-diequatorial ring conformations of (**1**), (**2**) and benzylpenicillin (**3**). For (**1**) and (**2**) this entailed no modification of the penicillin nucleus coordinates from the 3*S*-methyl ester sulphoxide (**4**) (penam-diequatorial conformation) crystal data. However, the coordinates for (**3**) logged in the CSSR database reveal benzylpenicillin, potassium salt, possessing the penam-diaxial conformation [20]. We therefore modelled (**3**) from the sulphoxide (**4**) by removal of the epoxide and sulphoxide functionalities, reassignment of the various atoms, and addition of the phenylacetyl side chain. This afforded benzylpenicillin with not only the diequatorial conformation which we desired, but also with penam geometry identical to compounds (**1**) and (**2**), so any influence on the side-chain orientation caused by the nucleus during the conformational analysis would be identical for all three compounds.

Replacement of the 3α-carboxylate benzyl ester of (**4**) with a proton in the modelling of (**1**), (**2**) and (**3**) yielded free carboxylic acids as opposed to carboxylate anions. This precluded the modelling of fully charged compounds. Partial charges were added to (**1**), (**2**) and (**3**) using the CNDO facility in the MO menu of COSMIC.

Once the charges had been assigned to the atom types, all the substituents attached to the penam nucleus of (**3**), and the spiro-3,4-fused-4,5 ring systems of (**1**) and (**2**) were minimised using the COSMIC molecular mechanics potentials and their current force fields [13]. The penam nucleus and the spiro-epoxide were left unminimised due to the lack of suitable parameters for these systems in the program. The final structures became the starting conformations for the conformational analysis reported below.

CONFORMATIONAL ANALYSIS

Within the MULTIMOL menu in COSMIC, the program MIN01 samples the conformational space of a molecule by cycling through, in defined rotational increments, all the specified bonds in a molecule after 'randomisation' of the starting conformation a set number of times [13]. The local minima for rotation about the single bonds are identified, then all the local minima over all the selected rotatable bonds are permuted, and the conformations (up to 100) giving rise to the lowest energies, up to a specified upper energy limit, are stored. The parameters for our MIN01 calculations are presented in Table 1. The atom numbering scheme used throughout (Fig. 5) has been assigned to identify analogous atoms between the three structures; it is not systematic.

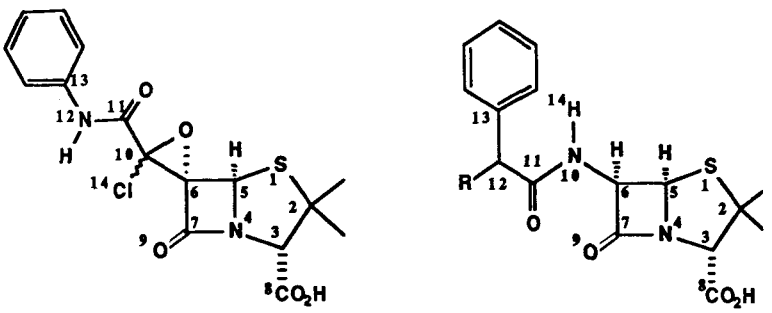
It should be noted that for (**1**) and (**2**), using the minimum rotational increment allowable (5°) and an upper energy limit of 7 kcal mol^{-1} (i.e., any conformations with an energy > 7 kcal mol^{-1} above the lowest energy conformer are discarded), only 32–33 conformations were identified. This gives an indication of the conformational inflexibility of the two compounds. Using the same parameters for (**3**), the maximum number of conformers (100) was calculated, all of which fell within 3 kcal mol^{-1} of the lowest energy conformer. In order to sample the whole conformational space accessible to benzylpenicillin up to 7 kcal mol^{-1}, it was necessary to relax the rotational increment parameter to 20°. This yielded a suitable range of conformers (58) with energies $\leqslant 7$ kcal mol^{-1}.

RESULTS

The data files generated by the MIN01 calculations were analysed and processed using ASTRAL, a suite of programs designed to display and manipulate output files from COSMIC on the Evans & Sutherland PS390.

TABLE 1
MIN01 PARAMETERS

	Compound		
	1	**2**	**3**
Specified rotatable bonds	(a) C(13) – N(12) (b) C(11) – C(10) (c) —	C(13) – N(12) C(11) – C(10) —	C(13) – C(12) C(12) – C(11) N(10) – C(6)
Rotational increment (deg.)	5	5	20
Upper energy limit (kcal mol^{-1})	7	7	7
Number of starting conformation randomisations	15	15	15
Number of local minima/ conformations generated	32	33	58



(1) and (2) (3)

Fig. 5. Numbering scheme for compounds (1), (2) and (3).

In the main, analysis of the MIN01 files for (**1**), (**2**) and (**3**) was undertaken by three methods:

(i) Manual docking of the three 'supermolecules' (ASTRAL terminology for MIN01 data incorporating up to 100 different structures) and visual assessment of the degree of overlap of conformational space.

(ii) Use of a pharmacophoric distance approach to define the position of the side-chain phenyl ring relative to the β-lactam carbonyl. This uses DISTSEARCH within the MULTIMOL menu of COSMIC to select conformers from the MIN01 files that fit the specified distance parameters. In a sense, this approach is analogous to that employed by Cohen [19] to identify the 'active' position of the 3α-carboxyl functionality.

(iii) Calculation of a van der Waals (VDW) surface map for each supermolecule, and manipulation of those maps to identify common conformational volume.

(i) Superimposition of (1), (2) and (3)

By displaying both MIN01 supermolecule files for (**1**) and (**2**) and manually superimposing the penam nuclei and the epoxide (Fig. 6), it can be seen that the conformational space accessible to each molecule is wholly independent of that space available to the other. The side chains of (**1**) and (**2**) do not overlap in any orientation. This observation, combined with the known disparity in the antibacterial activity of the two compounds, confirmed that the bioactive conformation for benzylpenicillin could only lie within the conformational space of (**1**).

When the same supermolecules were compared to the MIN01 file for (**3**) (Fig. 7), it was apparent that the conformational space accessible to the more flexible side chain of benzylpenicillin (green) could overlap, though not entirely, *both* the 3*R*-anilide, antibacterially active (yellow), *and* the 3*S*-anilide, antibacterially inactive (magenta). The bioactive conformer must, therefore, be contained only in the overlapping conformational space of (**1**) and (**3**).

It also bears mention that the overlapping conformational space for (**1**) and (**3**) tended to coincide with the lower energy (0–4 kcal mol^{-1}) conformers of (**3**), whereas the overlapping space for (**2**) and (**3**) coincided with the higher energy (4–7 kcal mol^{-1}) benzylpenicillin conformers.

(ii) Pharmacophoric distance approach

From further visual inspection of the MIN01 files for (**1**) and (**2**), particularly with respect to

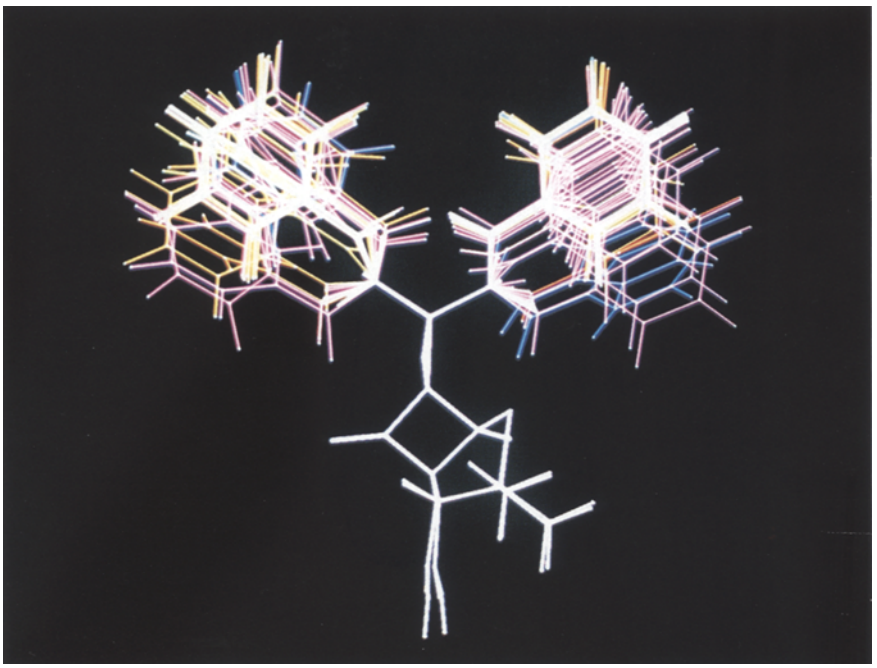


Fig. 6. MIN01 data for 3*R*-anilide (**1**), and 3*S*-anilide (**2**) superimposed to show non-overlap of side-chain conformational space. Relative energy coded above minimum energy conformer in the range: 0 (yellow) to 7 (blue) kcal mol^{-1}.

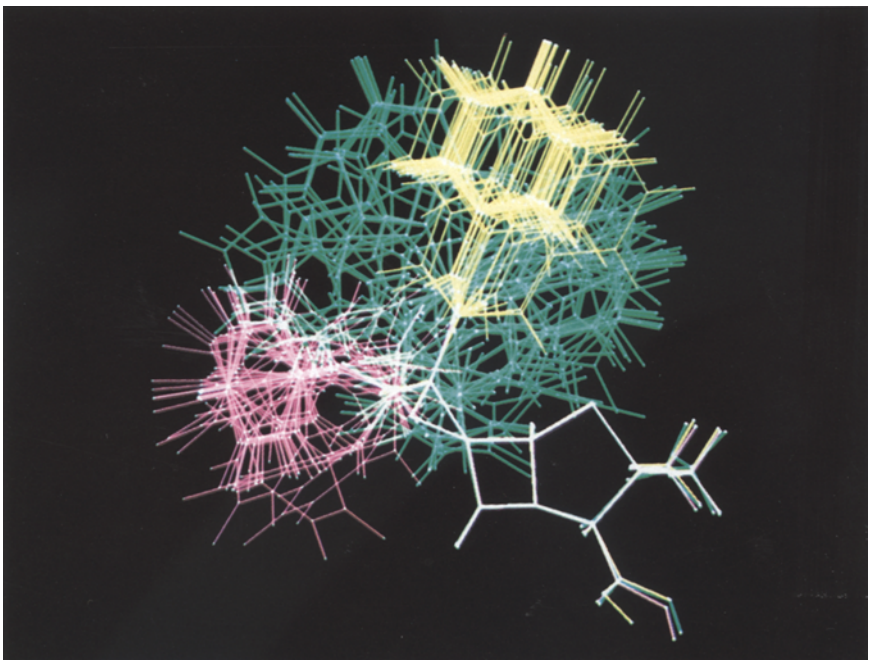


Fig. 7. MIN01 data for benzylpenicillin (**3**), green; 3*R*-anilide (**1**), yellow; and 3*S*-anilide (**2**), magenta, superimposed to show overlap of conformational space of (**3**) with both (**1**) and (**2**).

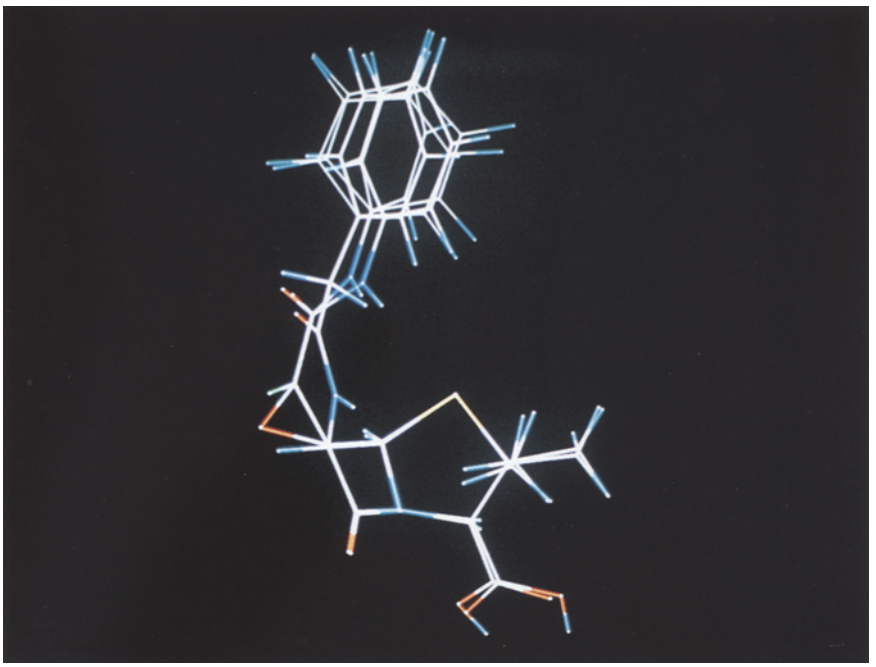


Fig. 8. Cluster of five closely coincident conformers, three from the MIN01 data for (**1**) and two from (**3**), chosen via the pharmacophoric distance approach.

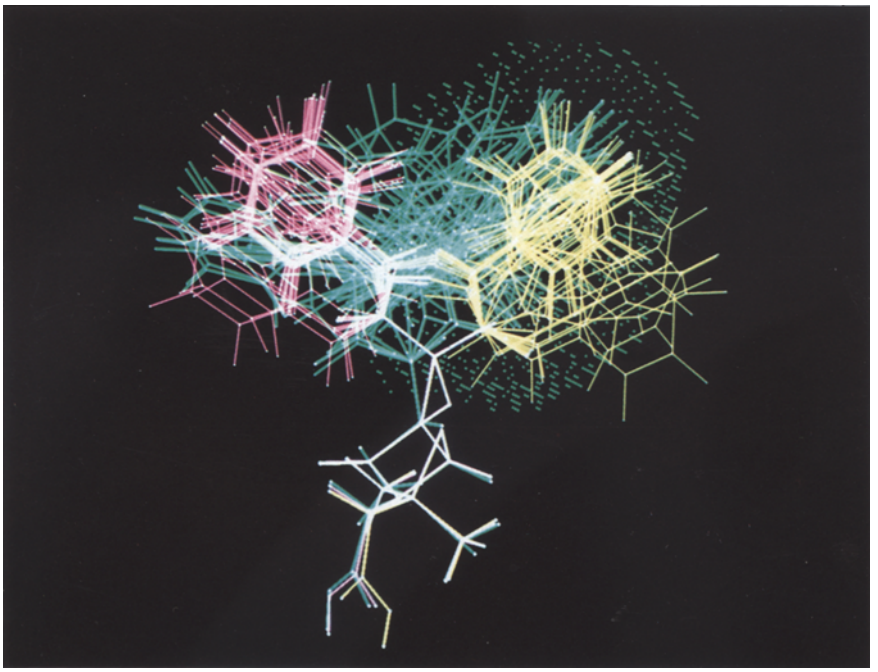


Fig. 9. Result of overlapping the VDW surfaces of (**1**) and (**3**) at 1.5 Å, and subtraction of the VDW Surface of (**2**) at 1.7 Å. Surface displayed in green (hatched) is that conformational volume accessible to the side chains of both (**1**) and (**3**).

the position of the aromatic ring, it became apparent that the phenyl group, as defined by position C-1′ [C(13) in Fig. 5], was situated further from the β-lactam carbonyl in the 3*R*-anilide supermolecule, (**1**), than it was in the 3*S*-anilide supermolecule, (**2**). Closer examination revealed that all 32 structures for the antibacterially active isomer, (**1**), could be accommodated when the distance from C-1′ to the β-lactam carbonyl oxygen (i.e., C(13) - 0(9)] was in the range 6.4 – 7.4 Å, whereas for the antibacterially inactive isomer, (**2**), C(13) – O(9) = 3.6 – 5.6 Å. Similarly, the distance between the β-lactam carbonyl carbon and C-1′ [i.e., C(13) – C(7)] was in the range 5.5 – 6.5 Å for all conformers of (**1**), but for the supermolecule (**2**), C(13) – C(7) = 4.1 – 5.7 Å. It seemed to us that these distance constraints, particularly those derived from the active isomer (**1**), might be useful for selecting potential 'bioactive' conformations from the MIN01 data for (**3**). Moreover, active benzylpenicillin conformers revealed by such a 'pharmacophoric distance' approach might divulge information not only on the optimal geometry of the side chain, but also on the topography of the putative PBP S_2-subsite.

A more critical evaluation of the two pharmacophoric distances, C(13) – O(9) and C(13) – C(7), revealed, however, an insufficiency in the latter constraint. The C(13) – C(7) distance ranges derived from the conformational analyses of (**1**), 'active' (5.5 – 6.5 Å), and (**2**), 'inactive' (4.1 – 5.7 Å), were not completely exclusive; they overlapped between 5.5 Å and 5.7 Å. A hypothetical benzylpenicillin conformer with C(13) – C(7) = 5.6 Å would thus appear, nonsensically, to be both active and inactive. This distance constraint was therefore considered too lax for the selection of active benzylpenicillin conformers. On the other hand, the C(13) – O(9) pharmacophore appeared much more stringent, in that its active (6.4 – 7.4 Å) and inactive (3.6 – 5.6 Å) distance ranges were clearly non-overlapping. C(13) – O(9) hence became our chosen distance criterion for the selection of benzylpenicillin conformers.

Using MULTIMOL:DISTSEARCH within COSMIC, it is possible to analyse a MIN01 file and select all conformers which satisfy a defined distance constraint. Analysis of the MIN01 data for (**3**) by setting C(13) – O(9) = 6.4 – 7.4 Å, the active pharmacophoric distance, caused 13 benzylpenicillin conformers to be selected out of the 58 possible. These 13 were analysed as before, using ASTRAL.

When the chosen conformers of (**3**) were compared with the MIN01 data for (**1**), again by manual superimposition of their penam nuclei, a cluster of five structures could be identified, three from (**1**) and two from (**3**), whose aromatic rings were particularly closely coincident. These five, extracted and examined separately (Fig. 8), will be discussed below.

(iii) VDW surface map analysis

In order to analyse more closely the overlapping and non-overlapping conformational space of the molecules, van der Waals surface maps for all three supermolecules were calculated (at 1.5 Å) using the VDW MAP facility in the SURFACES menu of ASTRAL. It was again possible to demonstrate, by superimposition of the maps (not shown), that the conformational volume for benzylpenicillin was able to accommodate, though not entirely, the side chains of both (**1**) and (**2**).

When, however, the VDW surface for (**2**) (at 1.7 Å, to allow for slight geometrical differences and inexactitudes in the superimposition of the ring systems) was *subtracted* from the overlapping VDW surface map of (**1**) and (**3**) only that conformational volume accessible to the side chains of *both* (**1**) and (**3**) was left. This volume must contain the bioactive side chain conformation of (**3**) (Fig. 9; hatched green).

Analysis of the structures whose side chains fell within this 'bioactive conformational volume' revealed the same cluster of five conformers (Fig. 8) selected from the pharmacophore approach. No other such closely fitting conformers could be identified within the overlapping conformational volumes of (**1**) and (**3**).

EXAMINATION OF SELECTED CONFORMERS

The two benzylpenicillin conformers chosen from both the VDW and pharmacophore approaches differ only in their orientation of the phenyl ring [i.e., by rotation around the C(13) – C(12) bond]; their main side chain atoms are completely superimposable. Similarly, two of the selected conformers of (**1**) differ only in their phenyl-ring orientation. The third conformer differs from the first two by 15° in its C(6) – C(10) – C(11) – N(12) torsional angle (−93.4° as opposed to −108.4°). This slightly alters the position of the phenyl ring, but when all five conformers are superimposed (Fig. 8), it is possible, nevertheless, to see their close coincidence particularly with respect to the aromatic rings. By visual inspection again, it is possible to select the two most closely fitting conformers of (**1**) and (**3**); these are shown in Fig. 10.

The close coincidence of the aromatic rings of the two conformers is again re-emphasised. Not unexpectedly however, owing to the geometrical distortions produced by the epoxide, the side chain backbone atoms coincide rather less well, though it should be pointed out that the equivalent carbonyls in the side chains of both (**1**) and (**3**) occupy similar space; this may be important if a specific hydrogen bond to this group is required for optimal receptor binding. Furthermore, the presence in (**1**) of a C–Cl bond instead of the N–H of (**3**) suggests that H-bonding of this group to the enzyme surface cannot be a prerequisite for optimal side-chain interaction. This observation is borne out by the presence of Gram-positive antibacterial activity, albeit low, in the penicil-

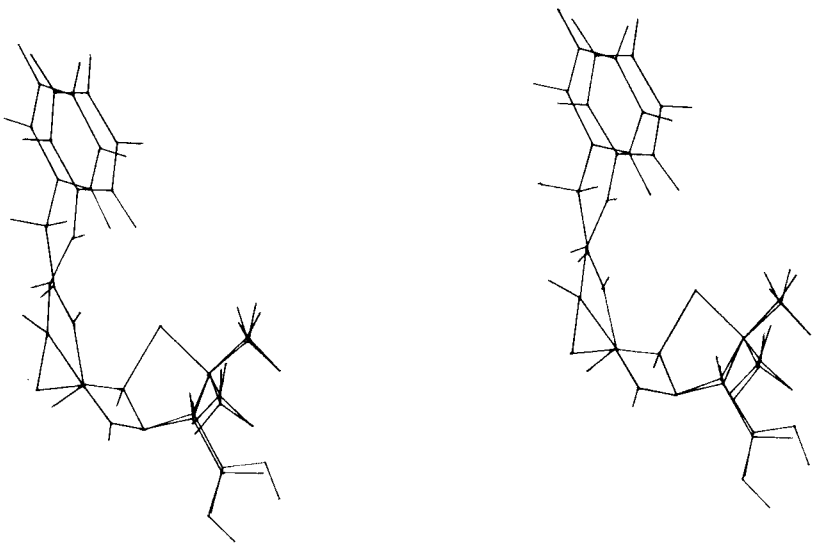


Fig. 10. Stereoview of the two most closely coincident, 'bioactive' conformers of (**1**) and (**3**). Geometrical data for these two conformers are reported in Table 2.

lin-V analogues (**6**) and (**7**) as seen in Fig. 11, which also lack a secondary amide N–H [21, 22]. Moreover, there can be no particular steric constraint in this region of the enzyme if one considers the size difference between C–Cl and N–H [typical bond lengths: C–Cl = 1.77 Å; N–H = 1.00 Å; VDW radius: Cl = 1.8 Å, H = 1.2 Å].

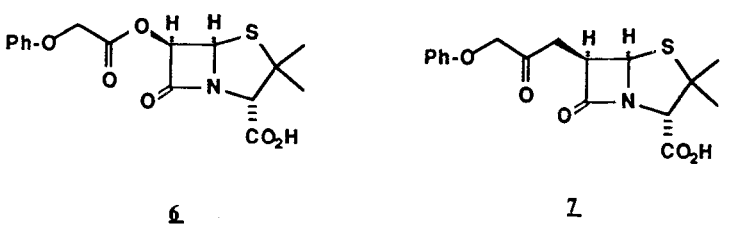
Geometrical data for the selected conformers of (**1**) and (**3**), particularly with respect to the side chain, are reported in Table 2. Also contained in Table 2 are the analogous data for benzylpenicillin (data taken from the crystal structure of the potassium salt [20]) and ampicillin [15], as revealed by the CSSR database.

DISCUSSION

Comparison of the conformational analysis data for (**1**) and (**3**) with the X-ray crystal data for benzylpenicillin, potassium salt, and ampicillin (Table 2) highlights the differences between X-ray crystallography and conformational analysis for the identification of likely biologically active conformations. All three compounds, (**1**), (**3**) and ampicillin, are antibacterially active, yet according to the CSSR data the side-chain conformations of benzylpenicillin and ampicillin possess quite different geometry from our proposed bioactive conformations (Fig. 10). With respect to the pharmacophoric distances for their aromatic residues, ampicillin almost fulfils the active requirements [that C(13) – O(9) should be in the range 6.4 – 7.4 Å, and C(13) – C(7) in the range 5.5 – 6.5 Å)]. The comparable distances from the crystal structure of benzylpenicillin, on the other hand, are more indicative of an inactive conformation [C(13) – O(9) to be in the range 3.6 – 5.6 Å, and C(13) – C(7) in the range 4.1 – 5.7 Å)]. Furthermore, when the side chain torsional angles are compared, large discrepancies stand out that must preclude any close overlap of the backbone atoms. These observations re-emphasise the importance of gaining an appreciation of conformational space available to flexible molecules when searching for biologically active conformations, they further highlight the dangers of using X-ray crystallographic data alone in the simulation of biological processes.

From the C(13) – O(9) and C(13) – C(7) distances defined above, combined with the geometry constraints proposed by Cohen [19], a total of five distances in all (see Table 2), it is now possible to construct a putative model (see Fig. 12) for the binding of benzylpenicillin to the PBPs.

The model attempts to relate spatially the 3α-carboxyl binding site, the β-lactam carbonyl binding site and the proposed lipophilic S_2-subsite, as defined by the geometry of the chosen, bioactive conformer of (**3**). The importance of these three binding sites for the effective interaction of classical β-lactam antibiotics with the PBPs has been noted previously [1], but until now no attempts to define their relative three-dimensional arrangement have been reported.



6 **7**

Fig. 11. Structures of compounds **6** and **7**.

TABLE 2
SELECTED GEOMETRICAL DATA FOR MOST CLOSELY FITTING CONFORMERS OF (**1**) AND (**3**) (SEE FIG. 10), ALONGSIDE COMPARABLE CSSR DATA FOR AMPICILLIN AND BENZYLPENICILLIN, POTASSIUM SALT (**3**, K^+)

	Compound			
	1	**3** (R = –H)[a]	Ampicillin (R = $-NH_2$)[a]	**3** K^+ (R = –H)[a]
Distances (Å)[a] (No.[b])				
C(8) – O(9)[c] (No. 1)	3.89	3.78	3.90	4.35
C(8) – C(7) (No. 2)	3.52	3.46	3.58	3.76
C(13) – O(9) (No. 3)	6.77	6.75	6.36	5.37
C(13) – C(7) (No. 4)	5.90	6.02	5.80	5.03
C(13) – C(8) (No. 5)	7.81	8.20	8.84	7.79
Torsional Angles (deg)[a]				
N(4) – C(7) – C(6) – At.(10)	–152.5	–118.5	–126.3	–123.8
C(5) – C(6) – At.(10) – C(11)	5.0	74.7	141.9	168.2
C(7) – C(6) – At.(10) – C(11)	141.3	171.8	–117.3	–94.1
C(6) – At.(10) – C(11) – At.(12)	–93.4	–177.8	–177.8	176.3
At.(10) – C(11) – At.(12) – C(13)	179.5	138.2	98.8	3.4
Angles (deg)[a]				
O(9) – C(7) – C(13)	132.5	123.7	113.5	100.1
C(7) – O(9) – C(13)	40.0	47.8	56.7	67.1
O(9) – C(7) – C(8)	99.0	96.1	96.8	111.3
C(7) – O(9) – C(13)	63.3	65.5	65.7	53.6
C(8) – O(9) – C(13)	90.1	98.3	117.0	106.0
O(9) – C(8) – C(13)	60.1	54.6	39.9	41.5

[a]See Fig. 5.
[b]Distance number, see Fig. 12.
[c]Active position within the range 3.0 Å – 3.9 Å[19]; At. = Atom.

Finally, it should be noted that some antibacterials, such as penicillin V (**8**), have one more atom in their side chain than the typical arylacetamido-substituted β-lactam antibiotics, and some, e.g., methicillin (**9**), one less (see Fig. 13). This is likely to alter markedly the binding orientation of their aromatic residues compared to (**1**) and (**3**) (Fig. 3). Thus, to accommodate such differences, the S_2-subsite must be quite large and/or extended. However, both penicillin V and methicillin are antibacterially less active than benzylpenicillin [23]. This indicates that optimal S_2-binding is provided by the benzylpenicillin topology, i.e., an aryl group separated by 3 atoms, as opposed to 2 or 4, from the β-lactam ring. It also explains the predominance of substituted arylacetamido side chains in the most potent β-lactam antibacterials [23] and provides a further justification for the distance constraints proposed in this modelling study. Other exceptions to these observations are those β-lactam antibiotics which do not possess an aromatic moiety in their side chain, the best example (Fig. 13) being the antibacterial mecillinam (**10**). This compound not only has a highly unusual structure, incorporating an azepine/amidine side chain, but also unusual biochemistry, in

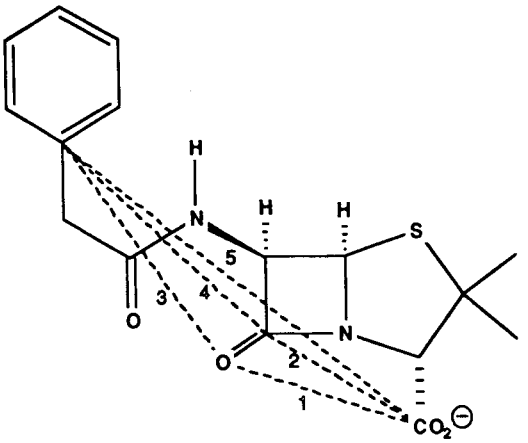


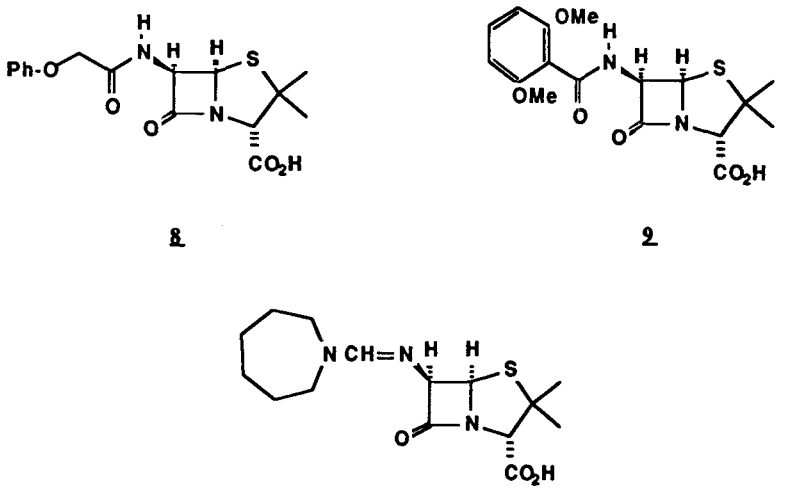
Fig. 12. Defined distances (see Table 2) between essential functional groups, indicating proposed S_2 subsite and 3α-carboxyl topography in benzylpenicillin-binding model.

that it binds specifically to *E. coli* PBP 2[24]. Very few β-lactam antibiotics with aromatic-containing side chains bind effectively to this protein, indicating that PBP 2, at least, is unlikely to possess an S_2-subsite specific for an aromatic residue. Despite these exceptions, the distance constraints and the model reported herein should assist in the future prediction of likely bioactive conformations of, for example, the highly potent third-generation cephalosporins and the expanded-spectrum penicillins. Work to this end is currently being instigated in this laboratory.

CONCLUSIONS

This study has utilised two similarly conformationally constrained penicillin derivatives, which possess differing biological activity profiles, to probe likely bioactive side chain conformations of benzylpenicillin. This has been undertaken in an attempt to map, for the first time, a putative S_2 enzymic subsite on the PBPs that is apparently specific for the side chain aromatic moiety of the classical β-lactam antibiotics. The conformational analysis yielded a proposed bioactive conformation of benzylpenicillin which was then used to construct a model for the three-dimensional arrangement of three enzymic binding sites on the PBPs; those for the 3α-carboxyl, the β-lactam carbonyl and the side chain aromatic residue.

It should be stressed that this study has been concerned primarily with *antibacterial activity*, i.e., with binding to penicillin-binding proteins; in this respect the differential activities of (**1**), antibacterially active, and (**2**), inactive, are crucial. However, the PBPs are a diverse *family* of proteins, differing between bacterial species and genera, and no one protein of the group has been implicated solely in the killing action of β-lactam antibiotics. One is thus dealing with multiple-target enzymes that may well possess quite considerable differences in their active-site topographies, but which co-operatively must be inactivated in order to produce cell lysis. We are suggesting that at least some of the essential PBPs, those enzymes whose inhibition by β-lactam antibiotics leads to fatal disruption of cell-wall biosynthesis, possess an accessory binding site that is capable of interacting with the side chain aromatic moiety of the classical β-lactam antibiotics, hence enhancing binding efficiency. The putative S_2-site is likely to be positioned somewhat differently in the va-



8 9 10

Fig. 13. Structures of compounds **8** – **10**.

rious PBPs however, which may explain the subtle differences in PBP-binding preferences which we have seen for (**1**) and (**3**) [12] and which have been reported for other antibacterials [24]. In this respect, the more flexible side chains of the classical β-lactam antibiotics may, in fact, be advantageous for optimal binding to the various PBPs, resulting in a more effective and broad spectrum antibacterial activity.

Finally, it should not be overlooked that the differential antibacterial activities of (**1**) and (**2**) are not entirely mirrored in their β-lactamase-inhibitory activity; both are, to a greater or lesser extent, inhibitors of β-lactamases. This is contrary to the PBP situation, where they must *both* be capable of binding to these proteins. This observation has important general implications with respect to the binding of ligands to β-lactamases as opposed to the PBPs, and immediately raises the question: how are the β-lactamases able to accommodate both compounds (**1**) and (**2**), that have been revealed by this particular study to be so conformationally different, whereas the PBPs are apparently only able effectively to bind (**1**)?

An earlier but not directly related study analysed the side-chain conformations of a range of β-lactamase-resistant penicillins including methicillin (**9**) which bind effectively to, but are not turned over by, β-lactamases. It was concluded that the lack of flexibility of these side chains results in a distorting effect within the enzyme active site, and is the major factor which determines the resistance to hydrolysis [25]. At this time, the molecular basis for these and the above observations remains elusive. However, with the expectation of even more highly refined X-ray crystallographic data on the various β-lactamases [26] and the penicillin-binding proteins [27] becoming available, the possibilities for future meaningful computer-modelling studies based on genuine enzyme ligand docking, rather than mere simulation, appear promising. These will, hopefully, lead to better-designed, improved β-lactam antibacterials and β-lactamase inhibitors for the treatment of bacterial infection well into the 21st century.

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