REVIEW

Functional evolution of the serine β-lactamase active site

R. F. Pratt

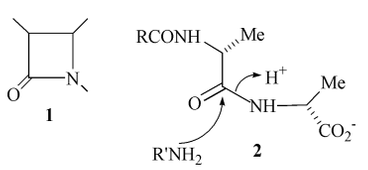
Department of Chemistry, Wesleyan University, Middletown, CT 06459, USA

Received (in Cambridge, UK) 12th December 2001
First published as an Advance Article on the web 22nd March 2002

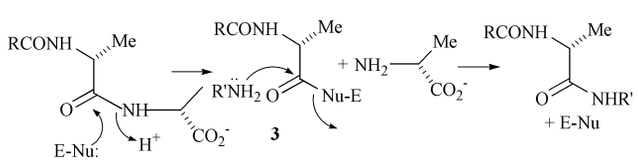
Covering: 2001

1 Introduction

Despite the challenge of ever-increasing resistance by bacteria, β-lactams **1** are still our main general defense against infection by these organisms. These antibiotics inhibit the final step of bacterial cell wall biosynthesis, the peptide-crosslinking of peptidoglycan strands. The enzymes that catalyze this reaction are termed transpeptidases, DD-peptidases, or penicillin-binding proteins (PBPs), from the result of their covalent inhibition by β-lactams. They catalyze the aminolysis of an *N*-acyl-D-alanyl-D-alanine peptide, **2**, in order to achieve the crosslink. The



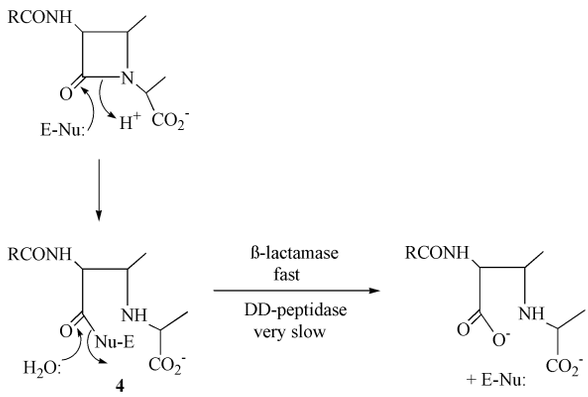
substituents R and R′ are contributed by the acyl donor and acyl acceptor peptidoglycan strands, respectively. DD-Peptidase catalysis involves a double displacement mechanism (Scheme 1) with an acyl-enzyme intermediate, **3**. β-Lactams



Scheme 1

also react with the enzyme (Scheme 2) but yield an inert acyl-enzyme **4** which cannot be either aminolyzed or hydrolyzed in a facile manner. The active site nucleophile, Nu, in these enzymes is a serine hydroxy group.

Serine β-lactamases also catalyse cleavage of β-lactams in double displacement mechanisms (Scheme 2) but differ from DD-peptidases in that hydrolytic deacylation is fast.



Scheme 2

β-Lactamases thus, in general, catalyze rapid β-lactam hydrolysis and represent the major bacterial defense system against β-lactam antibiotics. One important aim of the pharmaceutical industry over the last 50 years has been to find β-lactams that inhibit DD-peptidases but avoid (do not interact strongly with), or inhibit, β-lactamases. For reasons that become clear below, this goal remains unrealized, on a long term basis at least.

It is not possible to usefully discuss the enzymes of β-lactam activity except in an evolutionary framework.[1] Many years ago,[2] the cell wall of bacteria evolved, and, in concert, the enzymes of its biosynthesis. Somewhat more recently, presumably, β-lactams evolved, most likely as chemical warfare agents among bacteria, targeted at each others' DD-peptidases. Subsequently, it seems,[3] the biosynthetic pathways generating β-lactams (two separate routes are now known[4]) were taken up by fungi (where, eventually, they came to our attention[5]).

Next in this sequence of events, but still long ago, β-lactamases arose in bacteria to combat the chemical menace of β-lactams. Finally, β-lactamase inhibitors[6] were selected for and optimized among certain bacteria. It seems likely at this stage that a kind of evolutionary stasis may have been established and maintained for many millions of years. This steady state was interrupted dramatically however in 1945 when the first β-lactam was introduced into medical practice and, shortly after this, when β-lactams became available to millions of people. This event, felicitous from our point of view, in the short term at least, set in motion among bacteria an evolutionary race whose end is not yet in sight. Since that time, an intense selection process has ensued in the direction of DD-peptidases more resistant to β-lactams, and β-lactamases more effective in hydrolyzing them. We have, unwittingly, at the beginning at least, aided in this process by excessive use of these drugs[7] and by providing bacteria with greatly enhanced travel options. The future of β-lactams in human medicine is thus under serious threat.

As indicated above, an important evolutionary development in the saga of β-lactams was the appearance of β-lactamases (Fig. 1). It is now clear that the majority of these enzymes

DOI: 10.1039/b107097p

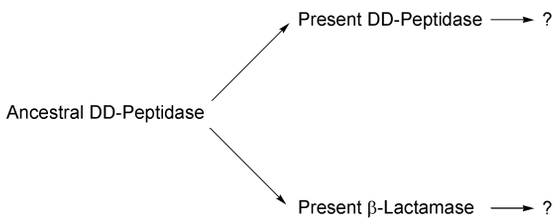


Fig. 1 β-Lactamase evolution.

evolved directly from parent DD-peptidases. This was first suggested by Tipper and Strominger[8] on the basis of the similarity of substrate structures between these two types of enzyme, an *N*-acyl-D-Ala-D-Ala peptide on one hand and penicillin on the other (see Schemes 1 and 2). Subsequently, it has become clear from direct X-ray structural studies that these enzymes are closely related.[9,10] Although there is, in general, little amino acid sequence homology between them, the overall folds of these proteins are very similar, with the active site lodged between two domains, one a collection of helices, the other based on a β-sheet platform (Fig. 2). Further, the functional groups of the active site are very similar, as described below.

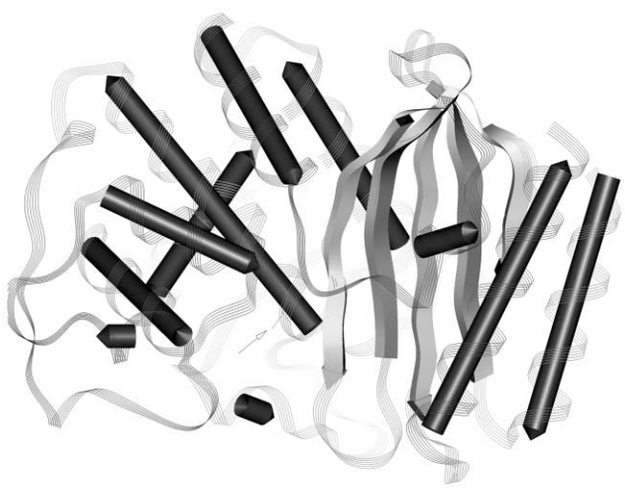


Fig. 2 General fold of a β-lactam-recognizing enzyme. Shown is the structure of the class A TEM β-lactamase.[67]

Although many reviews of β-lactamases have been written, most are from a structural or clinical perspective. This review will describe issues relating to the evolution of chemical reactivity of these active sites, from DD-peptidase to β-lactamase.

2 Functional differences between β-lactamases and DD-peptidases

On the basis of amino acid homology and active site structure, serine β-lactamases fall into three classes, A, C, and D[11] (class B β-lactamases are zinc metalloenzymes and do not fall within the scope of this review). In all three cases, the double displacement mechanism of Scheme 2 holds, with a specific serine hydroxy group as the active site nucleophile. The three differ however in the auxiliary functionality involved in proton transfer and electrostatic stabilization of the transition states. A schematic diagram of β-lactamase active sites is shown as Fig. 3. At the time of writing, the situation with respect to the class D enzymes is unresolved. These β-lactamases have the lysines and serines (including the nucleophilic Ser_1) of class A β-lactamases but not the glutamate. It is possible however that Lys_1 is carboxylated and that the carbamate anion acts as a general acid/base catalyst.[12] In general, the details of the roles of Ser_2(Tyr), Lys_1, and the Glu in catalysis are not universally agreed upon although all are considered general acid/base

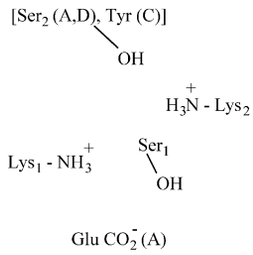


Fig. 3 Generic β-lactamase active site functional groups.

catalysts. Lys_2 appears to act as an electrostatic catalyst. From the point of view of active site structure and mechanistic homology, it seems that the class A and D β-lactamases most resemble and may therefore derive from low molecular weight class A DD-peptidases (using the DD-peptidase/penicillin-binding protein classification of Ghuysen[13]), and class C β-lactamases from low molecular weight class B DD-peptidases (of which only one appears known, the water-soluble DD-peptidase of *Streptomyces* R61).

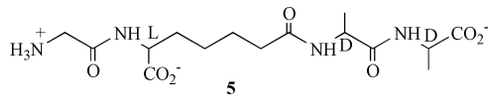
Although, as noted above, the reactions catalyzed by DD-peptidases and β-lactamases are very similar, and the catalytic apparatus available to them is very similar, there are clear and important differences in substrate specificity between them that relate directly to biological function. These differences are seen in both the acylation and deacylation steps and are treated separately under these categories below.

2.1 Enzyme acylation

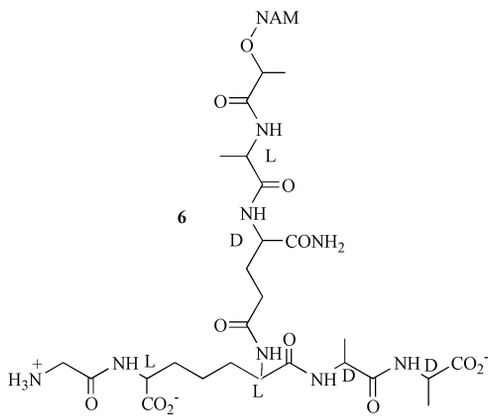
2.1.1 DD-Peptidases are acylated by (specific!) peptides

One would expect that the natural substrates of a DD-peptidase would be peptidoglycan precursors and that, when isolated and purified, these enzymes would catalyze the reaction of Scheme 1 as they appear to do *in vivo*. *In vitro*, however, employing purified enzymes, it has been difficult to demonstrate peptide hydrolase or transpeptidase activity at the level required for bacterial growth even with peptidoglycan fragments.[14,15] Although small peptides such as *N*,*N*′-diacetyllysyl-D-alanyl-D-alanine (DALAA) have been successfully used to demonstrate DD-peptidase activity with low MW enzymes, the high MW DD-peptidases, which represent the critical β-lactam targets, do not appear to catalyze any reaction of these molecules. Even in the former case, turnover numbers and specificity constants are low. Although somewhat more of these enzymes are found to catalyse the hydrolysis and aminolysis of ester analogs of these peptides,[16] turnover rates are commonly still small. There is also evidence[16,17] that some details of the reaction pathway of amides during turnover by these enzymes, involving particularly the side chain, differ from that of esters; this difference must occur during the acylation step of course.[18] In view of the problems described above, there has been speculation that the optimal activity of these enzymes may only be obtained in the specific membranous environment or multienzyme complex specific to their *in vivo* activity.[19,20] This activity in such an environment has not yet been reproduced *in vitro* with purified enzymes however.

Some progress in this puzzle concerning peptide specificity was recently achieved. Although the DD-peptidase of *Streptomyces* R61 (a class B low MW PBP) hydrolyzes and aminolyzes small, non-specific peptides, such as DALAA, only inefficiently, a tetrapeptide glycyl-L-α-amino-ε-pimelyl-D-alanyl-D-alanine, **5**, was found to be an excellent substrate for hydrolysis by this enzyme.[21] This peptide is a direct mimic of the amine and carboxy termini of the pentapeptide precursor of the peptidoglycan of *Streptomyces* sp., **6**. It is turned over quite rapidly by the R61 DD-peptidase (k_{cat} = 69 s^{-1}), binds tightly (K_m = 7.9 μM), and is very specific to this enzyme ($k_{cat}/K_m = 8.7 \times 10^6$ s^{-1} M^{-1}); this situation contrasts sharply, particularly with



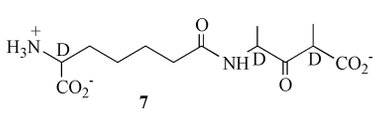
5



6

respect to binding and specificity, with DALAA where k_{cat} = 34.5 s^{-1}, K_m = 9.8 mM, and $k_{cat}/K_m = 3.5 \times 10^3$ s^{-1} M^{-1} under the same conditions. Crystal structures of this peptide and its hydrolysis products (glycyl-L-α-amino-ε-pimelyl-D-alanine and D-alanine), bound to the R61 DD-peptidase, support the specificity of the interaction, with appropriate binding sites for the penultimate methyl group (see below), the hydrophobic tetramethylene chain, the L-carboxylate, and the terminal ammonium ion.[22]

In contrast to the above result, the pentapeptide Ac-Ala-D-isoGln-Ala-D-Ala-D-Ala was not a good substrate of the R61 enzyme[23]—k_{cat}/K_m = 270 s^{-1} M^{-1}. Thus, the specificity of the R61 DD-peptidase towards its peptide substrates is centered in the N-terminal glycyl-L-α-aminopimelyl moiety. This theme does not appear, however, to extend generally to other DD-peptidases. The *Streptomyces* K15 DD-peptidase (a class A, low MW PBP), which, in principle, should also recognize **5** as a good substrate, did little or nothing with it;[24] nor, apparently, was Ac-Ala-D-isoGln-Ala-D-Ala-D-Ala hydrolyzed at any significant rate.[23] Further, although, by the rationale employed above, **7** should be a good substrate of *E. coli* DD-peptidases, this compound was not significantly turned over by PBP2 (class B, high MW) or PBP5 (class A, low MW) from that organism. On the other hand, the DD-peptidase of *Actinomadura* R39 (class C, low MW), which has a peptidoglycan structure the same as that of *E. coli*, finds **7** to be a very good hydrolysis substrate.[24]

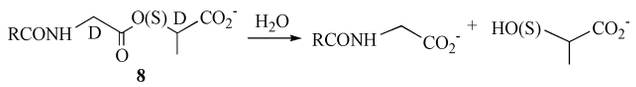


7

Thus, although it has now been shown that certain DD-peptidases are able to impressively catalyze the hydrolysis of specific peptides bearing elements of the expected peptidoglycan structure, it is clear that a general method of "activating" these enzymes has not yet been found, particularly those in the important high molecular weight classes. The crystal structure[25] of the R61 DD-peptidase shows that this enzyme has an active site homologous to a class C β-lactamase and thus contains a tyrosine functional group (Fig. 3) which may act as a general acid/base catalyst in acylation and deacylation. On the other hand, the crystal structures of the *Streptomyces* K15 DD-peptidase,[26] PBP5 of *E. coli*,[27] and of a class B high molecular weight PBP (PBP2x of *Streptococcus pneumoniae*,[28] which is probably closely similar to *E. coli* PBP2) show their active sites to resemble class A β-lactamases, although lacking an analog of Glu 166 to aid catalysis, particularly deacylation. Presumably these enzymes employ the conserved lysine homologous to Lys 73 of a class A β-lactamase (Lys_1, Fig. 3) as the general acid/base catalyst. The absence of the Glu 166 homolog may account for the slower turnover of non-specific substrates by these enzymes and their slower (than class A β-lactamases) acylation by β-lactams. Although the *Actinomadura* R39 DD-peptidase also has the imprint of a class A β-lactamase, it seems unclear at this time whether a homolog of Glu 166 is present.[29] It is rapidly acylated by β-lactams and, like the R61 DD-peptidase, appears to be a secreted soluble enzyme and thus may well function *in vivo* without assistance from other proteins. Its biological role *in vivo*, however, like that of the R61 enzyme, appears to be unknown.

2.1.2 β-Lactamases are not acylated by peptides

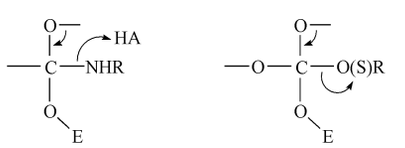
It has long been known[30] that β-lactamases do not catalyze the hydrolysis or aminolysis of *N*-acyl-D-alanyl-D-alanine peptides at any significant absolute rate. This illustrates what was probably an important early event in β-lactamase evolution, since it would seem counterproductive for bacteria to produce large amounts of a β-lactam-resistance enzyme that also hydrolyzed cell wall precursors. The very slow rates of peptide turnover by β-lactamases have however been quantitatively measured ($k_{cat}/K_m \leqslant 10^{-2}$ s^{-1} M^{-1})[31] and it is clear that the acylation step is much slower than deacylation and that non-covalent binding of *N*-acyl-D-Ala-D-Ala peptides to β-lactamases is very weak. On the other hand, analogous depsipeptides or esters and their thio analogs, **8**, are modest to good substrates ($k_{cat}/K_m = 10^2$–10^6 s^{-1} M^{-1}), not only of DD-peptidases but also of β-lactamases (Scheme 3).[32–35] There are two likely reasons for the reactivity of



8

Scheme 3

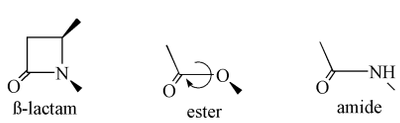
β-lactamases with these depsipeptides. First, these substrates have better leaving groups than amides, and ones that may not (esters) or do not (thioesters[36]) require protonation prior to departure during enzyme acylation (Scheme 4). Thus, precise



amide substrate (thio) ester substrate

Scheme 4

positioning of the leaving group with respect to the general acid of the active site (HA) is not needed. (Note that such a general acid should be a necessary part of the β-lactamase active site since a β-lactam nitrogen would require protonation concomitant with ring opening.[37]) Second, the greater ease of rotation around the C–(S)O bond of a (thio)ester than the C–N bond of an amide would allow these depsipeptides to bind at the active site in a conformation closer to that of a bicyclic β-lactam, the optimal substrate (Scheme 5).

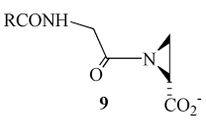


ß-lactam ester amide

Scheme 5

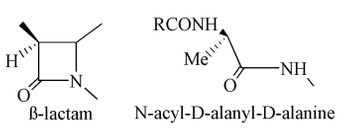
These ideas have been supported by experiments with an acyclic acylaziridine substrate **9**.[38] This molecule has the quasi-tetrahedral nitrogen of a bicyclic β-lactam, but one which also would require protonation in the acylation step. It was found that the class C *Enterobacter cloacae* P99 β-lactamase had

greater specificity ($k_{cat}/K_m = 3.9 \times 10^4$ s^{-1} M^{-1}) for **9** than did the structurally very similar DD-peptidase of *Streptomyces* R61 ($k_{cat}/K_m = 600$ s^{-1} M^{-1}) with respect to depsipeptide or peptide substrates. Representative class A and B β-lactamases also preferred **9**. Thus the nitrogen-distorted peptide **9**, like bicyclic β-lactams, has greater relative affinity for the β-lactamase active site than its planar (in the ground state) analogs. This finding supports the notion that β-lactamase evolution involved exclusion of D-Ala-D-Ala peptides containing the rigid planar amide group from productive binding at the active site by subtle active site geometry changes. The precise nature of these changes in any particular β-lactamase, however, has not yet been clearly identified.



It was also found that the P99 β-lactamase efficiently catalyzed aminolysis of **9** by D-amino acids. This reaction is of course a direct analog of the transpeptidase reaction catalyzed by DD-peptidases (Scheme 1) and emphasizes again the similarity of the active sites of these two groups of enzymes. The P99 β-lactamase also catalyzes the aminolysis of acyclic depsipeptides[35] but not of bicyclic β-lactams, the latter presumably because of steric interactions between the incoming amine nucleophile and the pendant leaving group of the β-lactam at the acyl-enzyme stage (see below).

Another clear structural difference between β-lactams and *N*-acyl-D-Ala-D-Ala peptides is the presence in the latter of the D-methyl substituent on the penultimate amino acid of the substrate (Scheme 6). A study of the effect of this methyl group on



Scheme 6

the access of peptides to the active site of a structurally similar β-lactamase (*E. cloacae* P99) and DD-peptidase (*Streptomyces* R61) pair showed a clear preference for the methyl group by the peptidase and against the methyl group by the β-lactamase.[39] Analysis of free energy–reaction coordinate diagrams showed that the DD-peptidase favored the methyl group at all accessible points along the reaction coordinate, whereas the β-lactamase disfavored the methyl group only at the peptide acylation transition state. This seems reasonable since the DD-peptidase has presumably evolved to turn over *N*-acyl-D-Ala-D-Ala peptides while the β-lactamase needs only to prevent acylation by such a peptide; selective pressure would not be applied further along the reaction coordinate in the latter case. Thus, it seems likely that the penultimate D-methyl group of an *N*-acyl-D-Ala-D-Ala peptide provided a handle for an evolving β-lactamase to select against.

Molecular modeling of acylation tetrahedral intermediates into the active sites of the above enzymes revealed the structural basis for rejection of the D-methyl substituent by the P99 β-lactamase. In the *Streptomyces* R61 DD-peptidase, a hydrophobic methyl binding site exists snugly adjacent to the benzenoid ring of the side chain of Trp 233 (Figs. 4A and 5). The same conformation of the peptide at the β-lactamase active site brought the methyl group into van der Waals contact with the more rigidly placed β-methylene of Tyr 221 at the C-terminus of the Ω loop (Fig. 4B); this conformation was, unlike in the DD-peptidase case, less stable than one where the penultimate D-methyl group was directed out into solvent. A similar study of the structures of *E. coli* PBP5, *S. pneumoniae* PBP2x, and the

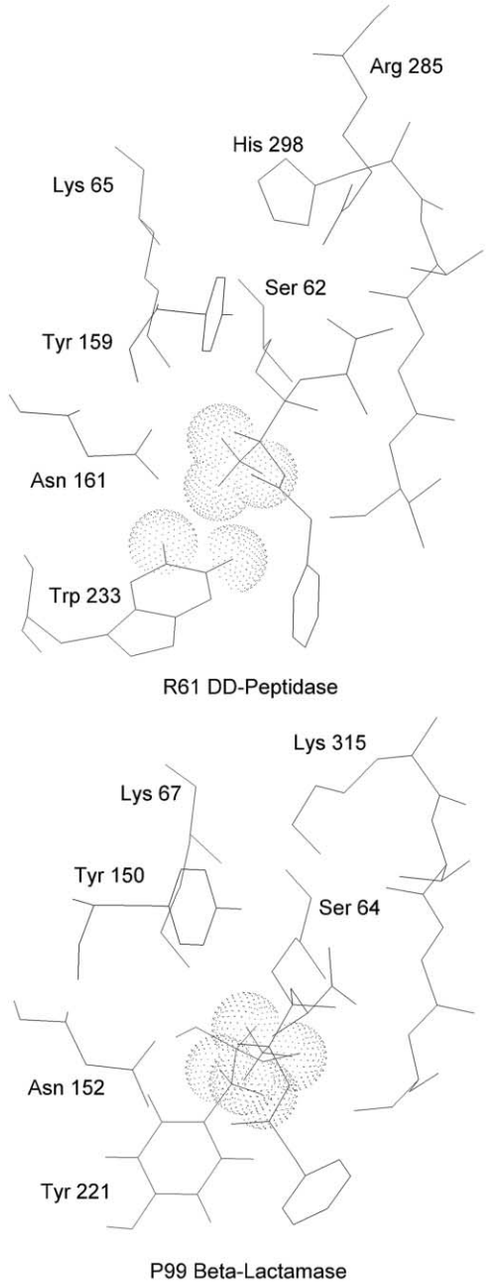


Fig. 4 Energy-optimized tetrahedral intermediate structures formed on reaction of *N*-(phenylacetyl)-D-alanyl-D-alanine with the *Streptomyces* R61 DD-peptidase (A, upper) and the *Enterobacter cloacae* P99 β-lactamase (B, lower).[39] Interactions between the methyl group of the penultimate D-alanine residue of the substrate and the enzyme are emphasized by van der Waals surfaces of hydrogen atoms on the methyl groups and the adjacent protein residues.

Streptomyces K15 DD-peptidase in comparison with a class A β-lactamase would be of considerable interest. Certainly, inspection of the former three crystal structures suggests the presence of a methyl group pocket in a position comparable to that in the R61 peptidase (Fig. 5); no such site seems present on the β-lactamase.

A comparative study[36] of the β-secondary and solvent kinetic isotope effects engendered on turnover of peptide and depsipeptide substrates by the P99 β-lactamase and the R61 DD-peptidase provides further insight into the similarities and differences between the transition states stabilized by these enzymes. The β-secondary kinetic isotope effects (k_{cat}/K_m) indicate a similar conformation of bound acyclic substrates on the two enzymes during acylation. This similarity encompasses the conformational relationship between the carbonyl group of the scissile bond and substituents on the carbon atom α to it.

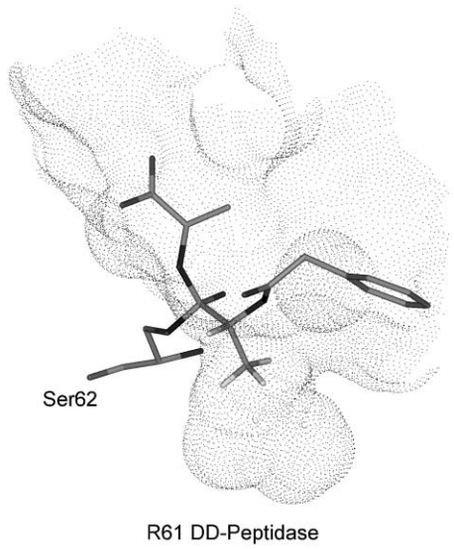
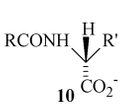


Fig. 5 Connolly surface of the *Streptomyces* R61 DD-peptidase adjacent to the active site showing the D-alanyl methyl group pocket. The ligand shown is the tetrahedral intermediate of Fig. 4.[39]

Differences may be present however (see below) between the conformation of the amido side chain with respect to the above-mentioned elements. Solvent kinetic isotope effects, close to unity, on the acylation step (k_{cat}/K_m) were found to be similar for all substrates, including β-lactams, examined with these enzymes; similar values were also found for turnover of a depsipeptide and a β-lactam by representative class A β-lactamase.[40] This result, interpreted superficially, suggests little proton transfer in the acylation transition state, a counterintuitive result since general acid/base catalysis would be expected. More detailed analysis showed however that this result probably arises from compensation between the effects of loss of a tightly bound proton during substrate binding and of proton(s) in flight during general acid/base catalysis. It was suggested that the tightly bound proton in the free enzyme could be that in the hydrogen bond between Lys_1 and Ser_2 (Tyr) (Fig. 3).[36,41]

Not only does it seem important that an optimally evolved β-lactamase should not catalyze D-Ala peptide hydrolysis, but it would also be prudent for such an enzyme not to strongly and non-covalently bind to an extended peptidoglycan structure, which would then act as a competitive inhibitor of the β-lactamase. It seems likely that DD-peptidases must have an extended binding site to specifically accommodate both acyl donor and acceptor peptides.[22,42] Remnants of such extended binding sites may be detectable on present-day β-lactamases. For example, kinetic[43,44] and physical data,[45] indicate that the P99 β-lactamase has a binding site distinct from the current β-lactamase active site that is specific for structures containing the motif **10** which can be found in the peptidoglycan



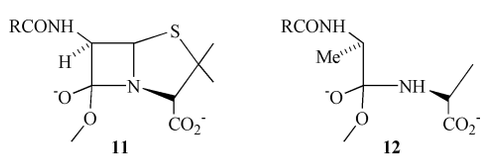
10

of gram negative bacteria. Interactions between this site and the β-lactamase site affect substrate turnover at the latter. To date, however, the position of this binding site on the P99 β-lactamase has not been determined.[45] Analogous secondary binding sites could not be detected on the class A TEM β-lactamase by similar methods.[24] Although such fragments of an ancestral DD-peptidase binding site may still survive in β-lactamases, it is likely that selection of mutants where the extended binding was blocked would also be a significant part of β-lactamase evolution. Suggestions on how this has been achieved in specific β-lactamases have been made.[10,46]

2.1.3 Both β-lactamases and DD-peptidases are acylated by β-lactams

Since β-lactamases most likely evolved to hydrolyse β-lactams, it is not surprising that these enzymes are rapidly acylated by most β-lactams and that the ensuing acyl-enzyme rapidly hydrolyzes. The broad side-chain specificity of β-lactamases, an important attribute of a resistance enzyme, has made the search for a general inhibitor difficult since tight binding is generally only achieved by formation of the acyl-enzyme. Potent inhibition is therefore only generally achieved by disruption of, or interaction with, the active site in very close proximity to the chemical reaction. The most effective small molecule β-lactamase inhibitors known are therefore covalent inhibitors of the mechanism-based variety[47] or transition state analogue species (see below). The reactivity and specificity of serine β-lactamases is significantly influenced, in both class A[48] and class C[49] enzymes, by the structure and mobility of the Ω loop which forms a substantial part of the floor of the binding site for substrate side chains. In both classes of enzyme, extension of the loop can lead to broader specificity.[49,50]

A less understood and perhaps more interesting issue is why DD-peptidases are acylated by β-lactams and why it seems very difficult for a DD-peptidase that efficiently catalyzes the transpeptidase reaction (Scheme 1), but is inert to β-lactams, to evolve. Certainly, the evidence suggests that as DD-peptidases evolve to better resist β-lactams, they also become less efficient at handling substrate analogs,[51] although the issue of the appropriate substrate to use in these comparisons is still a real one. The effectiveness of β-lactams as DD-peptidase inhibitors arises, not from strong non-covalent interaction with the active site prior to reaction, but from the covalent acylation step which, in some cases, can be very facile.[13] This reactivity most likely stems from the ability of the DD-peptidase to stabilize, *i.e.* strongly bind to, the tetrahedral intermediate–transition state of the acylation reaction with β-lactams, which closely resembles that of acylation by peptide substrates (Scheme 7).



11 **12**

Scheme 7

The similarity of these structures was first pointed out by Lee.[52] The enzyme is apparently unable to evolve a means of distinction between **11** and **12**; if the ability to bind to **11** and thus to react with β-lactams is lost, so too, apparently, is the ability to bind **12** and catalyze transpeptidation.

A striking feature of the acylation of high molecular weight DD-peptidases by β-lactams is that the rates appear to be very similar in water-solubilized constructs (where, typically, an N-terminal membrane-anchor peptide has been removed) as in the holo-enzyme *in vivo* (*i.e.* in the milieu of the bacterial membrane with all of the accessory proteins that may be needed[53]). This contrasts sharply with the peptidase activity which must occur *in vivo* but, as noted above, is difficult to demonstrate *in vitro*. Despite the structural similarity between **11** and **12**, the side chain (R) specificity is different for acylation by a β-lactam and by a peptide.[17,54] This suggests a different application of the side-chain "handle" to correctly align the active site in the two cases, where that for the peptide must be more specifically exerted.

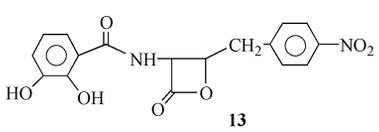
β-Lactams appear to be extraordinarily specific as DD-peptidase inhibitors. Of the myriad of other cyclic inhibitors tested by pharmaceutical chemists, and those naturally occurring compounds presumably tested under evolutionary pressure in the past, very few classes of compound other than β-lactams

Table 1 Acyl-enzyme conformations in β-lactamases and DD-peptidases

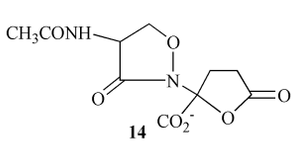
Enzyme[a]	Ligand	Dihedral angle/°[b] *abcd*	Dihedral angle/°[b] *bcde*	Sum/° *abcd* + *bcde*	Deacylation rate constant /s^{-1}	PDB[k]
Amp C	Loracarbef	64.8	−58.4	6.4	500[d]	1FCN
Amp C	Ceftazidime	101.2	−49.4	51.8	7×10^{-3}[e]	1IEL
Amp C	Cloxacillin	102.7	−12.7	90.0	6×10^{-3}[f]	1FCM
Amp C	Moxalactam[f]	69.2	74.6	143.8	9×10^{-3}[g]	1FCO
Citrobacter	Aztreonam	91.3	14.4	115.7	1×10^{-4}[h]	1FR6
R61	Cephalothin	99.3	−43.8	55.5	5×10^{-6}[i]	1CEG
R61	Cefotaxime	127.4	−45.2	82.2	5×10^{-6}[i]	1CEF
PBP2x	Cefuroxime	79.2	1.6	80.2	1×10^{-6}[j]	1QMF

[a] The enzymes are the class C amp C β-lactamase from *E. coli*, the class C β-lactamase from *Citrobacter freundii*, the DD-peptidase from *Streptomyces* R61, and penicillin-binding protein 2x of *Streptococcus pneumoniae*. [b] Defined in structure **18** (see text). [c] The angles here are to the methoxy oxygen rather than the heterocycle nitrogen. [d] A typical number for a good cephalosporin substrate of a class C β-lactamase. [e] A value for the P99 β-lactamase; ref. 49. [f] A value for the P99 β-lactamase; ref. 92. [g] A value for the P99 β-lactamase; ref. 93. [h] Ref. 72. [i] Ref. 61. [j] A value for cefotaxime; ref. 94. [k] Protein Data Bank ID; ref. 96.

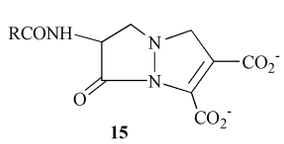
have been selected as DD-peptidase inhibitors, for use either in medicine or in inter-bacterial warfare. Notable among these few are β-lactones such as obafluorin, **13**[55] and γ-lactams such as lactivicin, **14**,[56] and the pyrazolidinones, **15**.[57] Recently, a series of bicyclic δ and γ-lactones *e.g.* **16**, **17**[58] were examined for reactivity with β-lactam-recognizing enzymes. They were found to be β-lactamase substrates, particularly reactive with class C enzymes, but had little reactivity with DD-peptidases. The rigid planarity of the ring systems of **16** and **17** is probably the main cause of the latter result. The difficulty of finding effective alternatives continues to extend the search for new β-lactams as antibiotics and as β-lactamase inhibitors.[59,60]



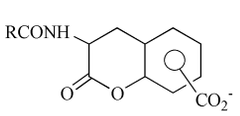
13



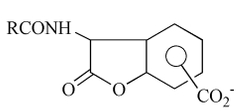
14



15



16

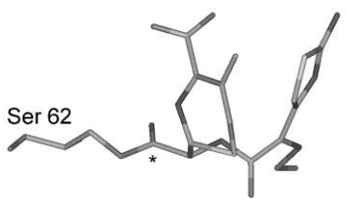


17

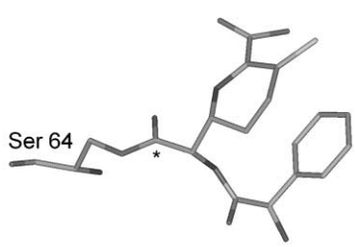
2.2 Enzyme deacylation

2.2.1 DD-Peptidases cannot catalyze the hydrolysis of acyl-enzymes derived from β-lactams

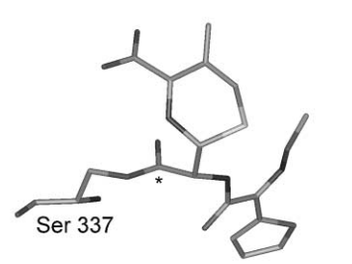
Following acylation by a β-lactam, a DD-peptidase then finds itself unable to catalyze hydrolysis of the ensuing acyl-enzyme **18**, and is thus inactivated. Herein lies the antibiotic activity of β-lactams. Traditionally, it has been believed that the continued presence of the pendant leaving group after β-lactam ring opening blocked the nucleophilic attack at the acyl-enzyme carbonyl group required for deacylation. Certainly, acyl groups such as **19**, with the same side chains as β-lactams but lacking the attached leaving group, appear to deacylate readily, as do those, **20**, with the D-methyl group of the natural peptide substrates.[39] Recent crystal structures support this rationale. The structures of complexes of cefotaxime and cephalothin with the *Streptomyces* R61 DD-peptidase,[61] and of cefuroxime with PBP2x of *Streptococcus pneumoniae*,[28] clearly show that attack of water with its presumed attendant general base would be hindered by the pendant heterocycle (Fig. 6). This point is reinforced by some interesting structures of complexes between the amp C β-lactamase and loracarbef (a good substrate; the structure is actually of a weakly active mutant enzyme)[62] and ceftazidime (an inhibitory substrate that forms a stable acyl-enzyme).[63] The class C amp C β-lactamase is very similar in structure to the R61 DD-peptidase.[10] The relevant detail of the loracarbef structure is also shown in Fig. 6. In this case, the incoming nucleophile is able to avoid the heterocycle, but access is blocked in the ceftazidime complex.



R61 DD-peptidase/cefotaxime

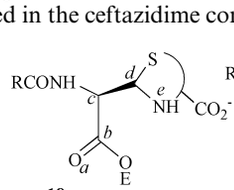


class C beta-lactamase/loracarbef

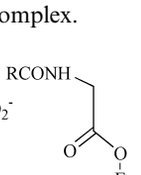


S. pneumoniae PBP2x/cefuroxime

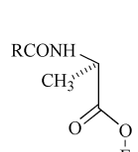
Fig. 6 Nucleophile access to the acyl-enzyme carbonyl group (carbon indicated by an asterisk) of β-lactam adducts of two peptidases and a β-lactamase. The sources of these structures are given in Table 1.



18



19



20

The conformation of the bound ligand found in loracarbef is not possible with ceftazidime since, in the case of the latter, the side chain would repulsively interact with the side chain of Tyr 221.[63] Interestingly, as mentioned above, it is just this residue that prevents productive binding of *N*-acyl-D-Ala-D-Ala peptides and thus, probably, DD-peptidase activity in the class C β-lactamases.[39] It is clear that the dihedral angles *abcd* and *bcde* (see structure **18**) are primarily involved in situating the leaving group nitrogen in a position in the acyl-enzyme to retard approach of a nucleophile (*e.g.* water) along the usual trajectory towards a carbonyl group.[64] Table 1 shows values for these angles, taken from the available crystal structures. Their sum seems a useful estimate of the degree of hindrance. This sum appears to be >50° for the DD-peptidases and for the class C β-lactamase with inhibitory substrates, whereas it is much smaller for the good substrate loracarbef. The van der Waals surface of the heterocyclic nitrogen in each case is shown in Fig. 7. This visually reinforces the message implied by the numbers in Table 1.

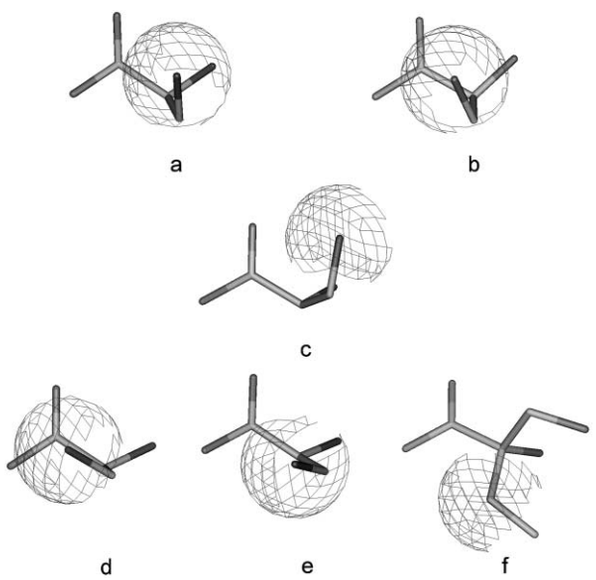


Fig. 7 Nucleophile access to the acyl-enzyme carbonyl group of β-lactam adducts with a variety of enzymes: a, ceftazidime with the amp C β-lactamase; b, cephalothin with the R61 DD-peptidase; c, loracarbef with the amp C β-lactamase; d, cefuroxime with *S. pneumoniae* PBP2x; e, aztreonam with the *C. freundii* β-lactamase; f, moxalactam with the amp C β-lactamase. In each case the van der Waals surface of the nitrogen or oxygen atom most directly hindering attack at the carbonyl is shown; in each case this atom is above the plane of the carbonyl (paper). The sources of these structures are given in Table 1.

It should be noted that in the DD-peptidases and class C β-lactamases depicted in Fig. 7, the nucleophile and the presumed general base (tyrosine in the case of class C β-lactamases and the R61 DD-peptidase and lysine in the case of PBP2x) attack the carbonyl from the (outer) solvent side of the carbonyl (*Re* face).[65] The above analysis does not apply directly to class A β-lactamases where the nucleophile (water) and general base (glutamate) are thought to attack from the (inner) protein side (*Si* face);[66] DD-peptidases, however, do not have this glutamate or another base in that position, presumably because their primary biological role is transpeptidation where the nucleophile (a peptide amine) is much larger than water and therefore cannot approach the acyl-enzyme from the inner side; the amino group of the general base (Lys_1) is positioned on the outer, solvent side of a plane comprising the β3-strand and the Ser_1 O_γ oxygen atom in the available low MW class A[26] and high MW class B[28] DD-peptidase structures. Class A β-lactamases do not effectively catalyze the aminolysis of acyclic depsipeptide substrates, presumably because of the position of their catalytic general base (the Glu 166 carboxylate group lies below the above-mentioned plane in class A β-lactamase crystal structures[67]).

An important remaining question then is why it is not possible for a DD-peptidase to evolve an active site that can catalyze hydrolysis of these acyl-enzymes and still retain DD-peptidase activity. In order to do so, it seems that rotation about the dihedral angle *abcd* would be needed to give a ligand conformation closer to that in the amp C–loracarbef complex. Such a rotation in a D-Ala-D-Ala substrate however would bring the D-Ala methyl group out of its pocket into a position eclipsing the active site Ser O_γ oxygen, hindering nucleophilic attack on the acyl-enzyme (Fig. 8), and change the orientation

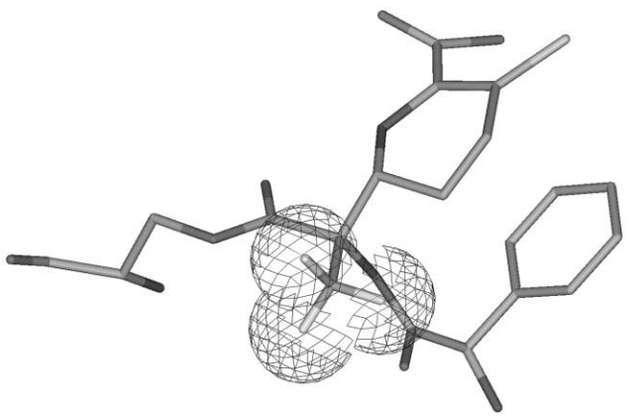


Fig. 8 The acyl-enzyme of the adduct of loracarbef with the amp C β-lactamase[62] to which a D-methyl group has been added. The methyl group (van der Waals surface shown) will clearly hinder nucleophilic attack on the acyl-enzyme carbonyl group.

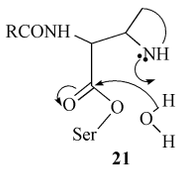
of the acyl side chain with respect to the enzyme. As noted above, the positioning of both of these elements seems critical to DD-peptidase activity and it is probably not possible to change these significantly without disrupting the entire active site and its adaptation to substrate.

2.2.2 β-Lactamases catalyze rapid deacylation of adducts with β-lactams

β-Lactamases have evolved mechanisms for catalyzing rapid deacylation of the acyl-enzymes formed on their reaction with β-lactams. In the class A enzymes, this involved the revolutionary step of incorporating a new general base catalyst, the carboxylate of Glu 166 and thus a mechanism asymmetric with respect to the general acid/base catalysts of acylation and deacylation.[66] In the catalytically more conservative class C enzymes that still have difficulty with deacylation (k_{cat} is the deacylation rate constant for class C β-lactamases with most substrates), deacylation has apparently been optimized by a more subtle rearrangement of the contours of the active site such that, as described above, nucleophiles have access to the carbonyl group of the acyl-enzyme. Kinetic isotope effects indicate that acyl-enzymes have different substrate conformations in the P99 β-lactamase and R61 DD-peptidase.[36] Bulky side chains and 6(7)α-substituents on the β-lactam ring are able to block access to the carbonyl group by restricting the accessible space available to the heterocyclic ring after acylation and thus produce inhibitory substrates, for example, of class C β-lactamases.[63] The naturally occurring class C β-lactamase mutant, GCl, is able to reestablish access to the acyl-enzyme carbonyl group derived from third generation cephalosporins, probably because of the greater flexibility of the extended Ω loop.[60] 6(7)α-Substituents also effectively incapacitate class A β-lactamases, but in this case largely by perturbation/displacement of the occluded nucleophilic water molecule.[68] With various mechanism-based inhibitors, interactions with the enzyme at the acyl-enzyme stage, often after rearrangement of the

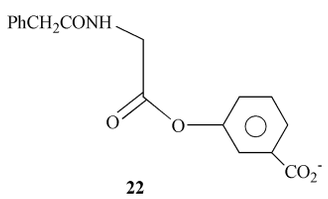
inhibitor, force the carbonyl group of the acyl-enzyme into a non-optimal orientation with respect to the oxyanion hole, thus deactivating it with respect to deacylation.[60,69] In solution, a number of conformations of the bound inhibitor may be present.[70]

It has been suggested[63,71] that deacylation of β-lactams from class C β-lactamases is catalyzed by the heterocyclic nitrogen of the erstwhile β-lactam, *i.e.* substrate-assisted catalysis, **21**. The



21

loracarbef/amp C β-lactamase acyl-enzyme structure has been interpreted in this way, for example.[63] The evidence from the latter source is certainly not conclusive however. The proposed deacylating water molecule (wat 402) is not close to an optimal position for attack on the acyl-enzyme carbonyl and the lone pair on the β-lactam-derived nitrogen is neither hydrogen-bonded to this water molecule nor directed towards a position on the carbonyl attack trajectory. Actually, the evidence *against* significant participation by the substrate seems stronger. It is known that class C β-lactamases efficiently catalyze hydrolysis of acyclic depsipeptides such as **22**,[33,35] with turnover numbers,

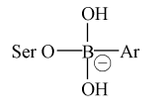


22

representing deacylation rate constants, in excess of 100 s^{-1}, which may be compared with the k_{cat} of *ca.* 500 s^{-1} (Table 1) for loracarbef/amp C. The leaving group in these cases is lost from the enzyme active site (it can be replaced by alternative nucleophiles) and cannot assist deacylation. Thus, an efficient catalyst of deacylation other than the β-lactam nitrogen must exist at the active site. Much evidence suggests that this is the dissociated hydroxy group of Tyr 150.[65,72] It is possible that substrate-assisted catalysis may play a role as a slow default mechanism with mutant enzymes or with poor/inhibitory substrates of various kinds.[63,71] If substrate-assisted catalysis by β-lactam nitrogens were such an efficient mechanism of deacylation, the extended lifetimes of acyl-enzymes derived from reactions of β-lactams with DD-peptidases, *i.e.* the whole basis of the antibiotic action of β-lactams, would be rather surprising. The latter enzymes would still, of course, need an efficient general acid/base catalyst to catalyze the transpeptidase reaction where, again, substrate-assisted catalysis of the kind proposed is impossible.

3 Transition state analogue inhibitors

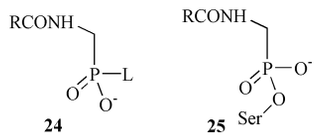
One would expect that β-lactamases and DD-peptidases, like other acyl transferases and especially serine proteases,[73] would be inhibited by transition state analogues that contain a tetrahedral atom in place of the acyl carbon and thus mimic the tetrahedral intermediates/transition states of acyl transfer reactions. Indeed, inhibition of β-lactamases by borate and boronates has been known and studied for many years. Both class A[74] and class C β-lactamases can be strongly inhibited by suitably designed boronates. In recent years, the affinity of class C β-lactamases for arylboronates has been examined in detail, and crystal structures showing tetrahedral boronate adducts, **23**, have been obtained.[75] In these adducts, one boronate hydroxy is found in the oxyanion hole and the other is generally hydrogen-bonded to the putative general acid/base catalyst of



23

the deacylation step. This, of course, is the disposition expected of a transition state analogue.

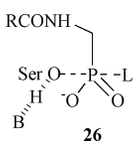
Suitably structured phosphonate monoesters (**24**; L is a leaving group) also inhibit β-lactamases.[76] These form covalent anionic tetrahedral adducts **25** at the active site and



24 **25**

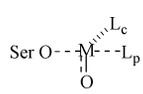
several crystal structures of these are available.[65,77] These also show the expected interactions with the active site that were mentioned above, and also demonstrate the positioning of the amido side chain in the fashion expected of a β-lactam substrate. By the appropriate thermodynamic criterion these adducts behave as complexes of the enzyme with transition state analogues.[78]

The ability of phosphonyl derivatives such as **24** to efficiently inhibit a β-lactamase requires that the active site of the enzyme must have substantial affinity for the pentacoordinated transition state **26**, where B represents the general base catalyst



26

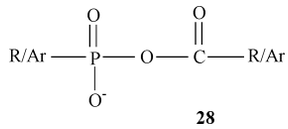
of the active site.[79] Class C β-lactamases, in particular, seem to have such an affinity, although with careful structural design, class A affinity can also be achieved.[80] The properties of the leaving group are important. Since the position of a leaving group L_p in a trigonal bipyramidal phosphoryl transfer transition state, **27**, will be different from that, L_c, in a tetrahedral



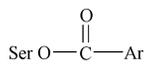
27

acyl transfer transition state, general acid assistance to the leaving group, essential for β-lactamase catalysis, will not be optimally placed for a phosphoryl transfer reaction. Thus, the leaving group in a phosphonate inhibitor must be a good one, not requiring protonation to leave. For this reason, phosphonamidates (L_p = NHR or Ar) are, at best, modest β-lactamase inhibitors.[81]

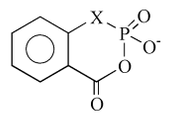
Exploration of good leaving groups (**24**, L) led to the acyl phosph(on)ates **28**.[82] These compounds have proved very interesting, not so much perhaps as phosphylating inhibitors, but rather as acylating agents. The class C β-lactamase active site apparently has considerable affinity for the phosph(on)ate leaving group in the acyl-transfer transition state, which allows acylation of the active site by non-specific acyl groups. A number of such acyl-enzymes, the aroyl derivatives **29**, for example, are quite inert and thus **28** represents a new class of inhibitory substrate for these enzymes. Notably, the arylboronate adducts **23** are transition state analogues for hydrolysis of **29**.[83] Cyclic variants of **28** are also effective inactivators of class C β-lactamases. The phosph(on)ates **30**, for example, appear to phosphylate the enzyme rather than acylate it, *cf.* the acyclic derivatives **28**. Enzyme activity is restored by recyclization of the phosphyl-enzyme to **30** rather than by hydrolysis.[84] This reversal is fast in the case of the phosphonate **30**b but slow for the phosphate **30**a.



28

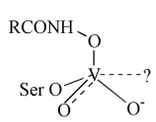


29



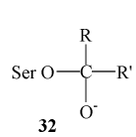
30a X = O
30b X = CH_2

The affinity of the β-lactamase active site for the pentacoordinated transition state **26** suggests that stable pentacoordinated species should also be β-lactamase inhibitors. The most commonly used source of such species is vanadium. Vanadium complexes have been much used as inhibitors of phosphoryl transfer enzymes and crystal structures of inhibitory complexes reveal pentacoordinated vanadium.[85] In principle, readily accessible vanadium isosteres of **26** would be complexes of vanadate with hydroxamic acids **31**. Indeed 1 : 1 complexes of these components do inhibit class C β-lactamases very effectively, with dissociation constants ranging down to less than micromolar.[86] Spectroscopic studies indicate penta- or hexa-coordinated vanadium; X-ray crystal structures will be needed, however, to completely decide this structural issue. The arrangement of ligands in these complexes should inform further inhibitor design. These vanadium complexes also inhibit the *Streptomyces* R61 DD-peptidase (see below) and other serine amidohydrolases.[86]

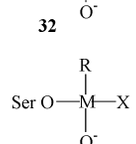


31

In contrast to the boronates and phosph(on)ates, electrophilic carbonyl species, potentially also leading to tetrahedral adducts **32**, have proved disappointing as β-lactamase inhibitors. Aldehydes,[87] α-keto acids and esters[87] and α-ketoheterocycles[88] have not appeared to be effective. A computational analysis[87] of the relative effectiveness of tetrahedral adducts **33** concluded that in those that interacted strongly with the active site, *viz.* those derived from boronates (M = B, X = OH), phosphonates (M = P, X = O), and substrates (M = C, X = O, N), X is a heteroatom. This of course excludes the carbonyl adducts. Further, strong interaction of MXO^- with the active site lysines of a class C β-lactamase was required for effective inhibition; this excluded other interesting possibilities such as M = Si, X = O. Carbonyl compounds will therefore require X groups more strongly interacting with the enzyme to yield good inhibitors.



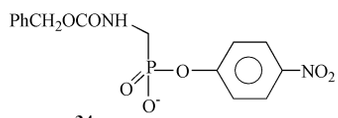
32



33

In contrast to the situation with β-lactamases, there appear to be no specific and effective transition state analogue inhibitors of DD-peptidases known at present. The situation here is comparable to that with DD-peptidase substrates, and probably arises for the same reasons. This is unfortunate since, in principle, such molecules would represent new antibiotics. Simple boronates do not appear to have antibiotic activity and therefore are presumably not strong general inhibitors of DD-peptidases; they do however potentiate the effect of β-lactams, presumably because of their effect on β-lactamases.[89]

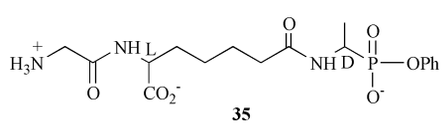
Phosphonates, even with β-lactam-like side chains and good leaving groups, **34** for example, have little or no inhibitory activity against DD-peptidases. The compound **34** and the analogous fluoride do not even inhibit the *Streptomyces* R61 DD-peptidase at any significant rate despite the latter enzyme's close structural similarity to class C β-lactamases which are very susceptible to **34**.



34

The negative results with phosphonates may reflect, in part, the fact that the DD-peptidase active site does not stabilize the pentacoordinate transition state **26** to the same degree that β-lactamases do. Computational studies in fact indicate that although the R61 DD-peptidase active site interacts with tetrahedral species derived from substrates and phosphonates as strongly as does the class C P99 β-lactamase active site, the latter site interacts considerably more strongly with **26** than does the former.[90] Further, the phosphonate analog, **35**, of **5**, the specific peptide substrate of the R61 DD-peptidase, is a poor inhibitor of the peptidase, *i.e.* it phosphonylates the enzyme only very slowly.[91]

Despite the fact that these phosphonates do not inhibit the R61 DD-peptidase, the vanadate complex **31** is a very effective inhibitor.[86] Thus, the DD-peptidase active site may be very sensitive to the specific arrangement of oxygen atoms in "transition state analogues". A crystal structure of the complex of vanadate with the R61 DD-peptidase would be of considerable interest in this regard. The question of whether the combination of a specific side chain with a source of tetrahedral adduct other than a phosphonate, the boronic acid analogue of **35**, for example, would yield a strong DD-peptidase inhibitor is yet to be answered.



35

4 Concluding summary

The β-lactamases have evolved from bacterial DD-peptidases along a path where changes in both acylation and deacylation steps have occurred. β-Lactamases have lost the ability to be acylated by acyl-D-alanyl-D-alanine peptides; the means by which this has been accomplished includes removal of the penultimate D-methyl binding site and changes in the active site topography to prevent binding of a substrate rigidly planar at the nitrogen of the scissile peptide bond in favor of substrates with the tetrahedral nitrogen of bicyclic β-lactams. The results of a hypothetical intermediate stage, involving monocyclic β-lactams, may not be visible today because the latter compounds do not appear, in general, to have strong antibiotic properties. The side chain specificity of DD-peptidases, presumably targeted to the peptidoglycan structure, has also been largely lost in present-day β-lactamases. The susceptibility of present-day DD-peptidases to β-lactams has presumably been minimized by evolution, but apparently cannot be lost entirely while maintaining DD-peptidase activity; the reason for this is likely to be the close resemblance between the transition states of acylation by β-lactamases and DD-peptidases.

β-Lactamases have acquired the ability to catalyze hydrolysis of the acyl-enzyme derived from their acylation by β-lactams. This has been achieved either by incorporation of a new general acid/base catalyst (class A, class D (?)) or by rearrangement of the active site to permit access of water and an accompanying general base to the acyl group. The latter cannot apparently be achieved in a DD-peptidase because of the adverse effect of such a change in the disposition of the penultimate D-methyl group and the acyl side chain of the acyl-D-alanyl-D-alanine substrate. It seems unlikely that good, present-day β-lactamases and DD-peptidases can be functionally interchanged by simple mutations.[95]

The broader specificity of β-lactamases than DD-peptidases, both with respect to the environment of the scissile bond and to the side chain, is probably responsible for the greater success achieved to date in the development of transition state analogue inhibitors of the former enzymes. Development of such inhibitors in the latter case is still slow, reflecting the related unsolved problems of the substrate side chain specificity and active site potentiation of the high MW DD-peptidases. The challenge in that regard is very nicely illustrated by the awe-inspiring active site cleft of *Streptococcus pneumoniae* PBP2x (Fig. 9).[28]

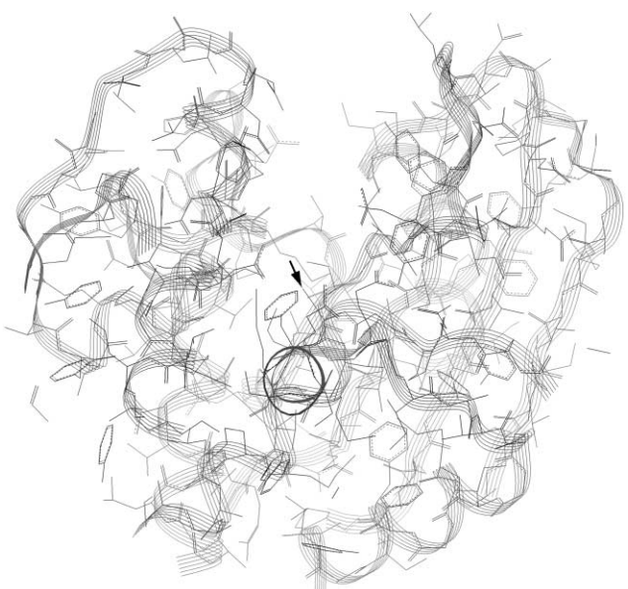


Fig. 9 The active site cleft of *Streptococcus pneumoniae* PBP2x.[28] Shown is a 10 Å sphere centered at the active site serine hydroxy group which is indicated by an arrow.

The author hopes that the train of thought followed in this review will guide further rational design of inhibitors of both β-lactamases and DD-peptidases; the need for such inhibitors to combat the challenges that bacteria present to human health in the twenty-first century is certainly very great.

5 Acknowledgements

The author is grateful to Wesleyan University and the National Institutes of Health for support of his research from which the general themes of this review grew.

6 References

1 A. A. Medeiros, *Clin. Infect. Dis.*, 1997, **24**(Suppl. 1), 519.
2 J. L. Siefert and G. E. Fox, *Microbiology*, 1998, **144**, 2803; A. L. Koch, *CRC Crit. Rev. Microbiol.*, 2000, **26**, 1.
3 Y. Aharonowitz, G. Cohen and J. F. Martin, *Annu. Rev. Microbiol.*, 1992, **46**, 461.
4 S. J. Mcgowan, M. Sebaihia, L. E. Porter, G. S. A. B. Stewart, P. Williams, B. W. Bycroft and G. P. C. Salmond, *Mol. Microbiol.*, 1996, **22**, 415; R. Li, A. Stapon, J. T. Blanchfield and C. A. Townsend, *J. Am. Chem. Soc.*, 2000, **122**, 9296.
5 A. Fleming, *Br. J. Exp. Pathol.*, 1929, **10**, 226.
6 A. G. Brown, D. Butterworth, M. Cole, G. Handscomb, J. D. Hood, C. Reading and G. N. Rolinson, *J. Antibiot.*, 1976, **29**, 668; N. C. J. Strynadka, S. E. Jensen, K. Johns, H. Blanchard, M. Page, A. Matagne, J.-M. Frère and M. N. G. James, *Nature*, 1994, **368**, 657.
7 S. B. Levy, *Sci. Am.*, 1998, **278**, 46.
8 D. J. Tipper and J. L. Strominger, *Proc. Natl. Acad. Sci. USA*, 1965, **54**, 1133.
9 J. A. Kelly, O. Dideberg, P. Charlier, J. P. Wery, M. Libert, P. C. Moews, J. R. Knox, C. Duez, C. Fraipont, B. Joris, J. Dusart, J.-M. Frère and J.-M. Ghuysen, *Science*, 1986, **231**, 1429.
10 J. R. Knox, P. C. Moews and J.-M. Frère, *Chem. Biol.*, 1996, **3**, 937.
11 A. Matagne, A. Dubus, M. Galleni and J.-M. Frère, *Nat. Prod. Rep.*, 1999, **16**, 1.
12 L. Maveyraud, D. Golemi, L. P. Kotra, S. Tranier, S. Vakulenko, S. Mobashery and J.-P. Samama, *Structure*, 2000, **8**, 1289; D. Golemi, L. Maveyraud, S. Vakulenko, J.-P. Samama and S. Mobashery, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 14280.
13 J.-M. Ghuysen, *Annu. Rev. Microbiol.*, 1991, **45**, 37.
14 J.-M. Frère and B. Joris, *CRC Crit. Rev. Microbiol.*, 1985, **11**, 299.
15 J.-M. Ghuysen and G. Dive, in *Bacterial Cell Wall*, ed. J.-M. Ghuysen and R. Hakenbeck, Elsevier Science B.V., Amsterdam, 1994, p. 103; M. Terrak, T. K. Ghosh, J. van Heijenoort, J. Van Beeumen, M. Lampilas, J. Aszodi, J. A. Ayala, J.-M. Ghuysen and M. Nguyen-Distèche, *Mol. Microbiol.*, 1999, **34**, 350; B. A. Schwartz, J. A. Markwalder and Y. Wang, *J. Am. Chem. Soc.*, 2001, **123**, 11638.
16 M. Adam, C. Damblon, M. Jamin, W. Zorzi, V. Dusart, M. Galleni, A. El Kharroubi, G. Piras, B. G. Spratt, W. Keck, J. Coyette, J.-M. Ghuysen, M. Nguyen-Distèche and J.-M. Frére, *Biochem. J.*, 1991, **279**, 601.
17 Y. Xu, G. Soto, H. Adachi, M. P. G. van der Linden, W. Keck and R. F. Pratt, *Biochem. J.*, 1994, **302**, 851.
18 Y. Xu and R. F. Pratt, *Bioorg. Med. Chem. Lett.*, 1994, **4**, 2291.
19 M. Matsuhashi, in *Bacterial Cell Wall*, ed. J.-M. Ghuysen and R. Hakenbeck, Elsevier Science B.V., Amsterdam, 1994, p. 55.
20 J.-V. Höltje, *Microbiol. Mol. Biol. Rev.*, 1998, **62**, 181.
21 J. W. Anderson and R. F. Pratt, *Biochemistry*, 2000, **39**, 12200.
22 M. A. McDonough, J. W. Anderson, N. R. Silvaggi, R. F. Pratt, J. R. Knox and J. A. Kelly, submitted for publication.
23 S. A. Adediran and R. F. Pratt, unpublished work.
24 J. W. Anderson and R. F. Pratt, unpublished work.
25 J. A. Kelly and A. P. Kuzin, *J. Mol. Biol.*, 1995, **254**, 223.
26 E. Fonzé, M. Vermeire, M. Nguyen-Distèche, R. Brasseur and P. Charlier, *J. Biol. Chem.*, 1999, **274**, 21853.
27 C. Davies, S. W. White and R. A. Nicholas, *J. Biol. Chem.*, 2001, **276**, 616.
28 E. Gordon, N. Mouz, E. Duée and O. Dideberg, *J. Mol. Biol.*, 2000, **299**, 477.
29 B. Granier, C. Duez, S. LePage, S. Englebert, J. Dusart, O. Dideberg, J. Van Beeumen, J.-M. Frère and J.-M. Ghuysen, *Biochem. J.*, 1992, **282**, 781.
30 A. K. Saz, D. L. Lowery and L. L. Jackson, *J. Bacteriol.*, 1961, **82**, 298.
31 N. Rhazi, M. Galleni, M. I. Page and J.-M. Frère, *Biochem. J.*, 1999, **341**, 409.
32 R. F. Pratt and C. P. Govardhan, *Proc. Natl. Acad. Sci. USA*, 1984, **81**, 1302.
33 C. P. Govardhan and R. F. Pratt, *Biochemistry*, 1987, **26**, 3385.
34 M. Adam, C. Damblon, B. Platin, L. Christiaens and J.-M. Frère, *Biochem. J.*, 1990, **270**, 525.
35 Y. Xu, G. Soto, K. R. Hirsch and R. F. Pratt, *Biochemistry*, 1996, **35**, 3595.
36 S. A. Adediran and R. F. Pratt, *Biochemistry*, 1999, **38**, 1469.
37 M. I. Page, *Adv. Phys. Org. Chem.*, 1987, **23**, 165.
38 B. P. Murphy and R. F. Pratt, *Biochemistry*, 1991, **30**, 3640.
39 N. J. Bernstein and R. F. Pratt, *Biochemistry*, 1999, **38**, 10499.
40 S. A. Adediran, S. A. Deraniyagala, Y. Xu and R. F. Pratt, *Biochemistry*, 1996, **35**, 3604.
41 M. I. Page, B. Vilanova and N. J. Layland, *J. Am. Chem. Soc.*, 1995, **117**, 12092.
42 W. Lee, M. A. McDonough, L. P. Kotra, Z.-H. Li, N. R. Silvaggi, Y. Takeda, J. A. Kelly and S. Mobashery, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 1427.
43 S. Pazhanisamy and R. F. Pratt, *Biochemistry*, 1989, **28**, 6875.
44 M. Dryjanski and R. F. Pratt, *Biochemistry*, 1995, **34**, 3561.
45 M. Dryjanski and R. F. Pratt, *Biochemistry*, 1995, **34**, 3569.
46 I. Massova and S. Mobashery, *Antimicrob. Agents Chemother.*, 1998, **42**, 1.
47 R. F. Pratt, in *The Chemistry of β-Lactams*, ed. M. J. Page, Chapman & Hall, London, 1992, ch. 7; M. G. P. Page, *Drug Resist. Updates*, 2000, **3**, 109.

48 O. Herzberg, G. Kapadia, B. Blanco, T. S. Smith and A. Coulson, *Biochemistry*, 1991, **30**, 9503; S. Banerji, U. Pieper, G. Kapadia, L. K. Pannell and O. Herzberg, *Biochemistry*, 1998, **37**, 3286.
49 M. Nukaga, S. Haruta, K. Tanimoto, K. Kogure, K. Taniguchi, M. Tomaka and T. Sawai, *J. Biol. Chem.*, 1995, **270**, 5729.
50 C. Arpin, R. Labia, C. Andre, C. Frigo, Z. el Harif and C. Quentin, *Antimicrob. Agents Chemother.*, 2001, **45**, 2480.
51 G. Zhao, W.-K. Yeh, R. H. Carnaton, J. Flokowitsch, T. I. Meier, W. E. Alborn, Jr., G. W. Becker and S. R. Jaskunas, *J. Bacteriol.*, 1997, **179**, 4901; N. Mouz, E. Gordon, A.-M. DiGuilmi, I. Petit, Y. Petillot, Y. Dupont, R. Hakenbeck, T. Vernet and O. Dideberg, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 13403.
52 B. Lee, *J. Mol. Biol.*, 1971, **61**, 463.
53 H. Adachi, T. Ohta and H. Matsuzawa, *FEBS Lett.*, 1987, **226**, 150; H. Ellerbrak and R. Hakenbeck, *Eur. J. Biochem.*, 1988, **171**, 219; C. Y. E. Wu, J. Hoskins, L. C. Blaszczak, D. A. Preston and P. L. Skatrud, *Antimicrob. Agents Chemother.*, 1992, **36**, 533; M. P. G. van der Linden, L. de Haan and W. Keck, *Biochem. J.*, 1993, **289**, 593.
54 J.-M. Ghuysen, J.-M. Frère, M. Leyh-Bouille, J. Coyette, J. Dusart and M. Nguyen-Distèche, *Annu. Rev. Biochem.*, 1979, **48**, 73.
55 A. A. Tymiak, C. A. Culver, M. F. Malley and J. Z. Gougoutas, *J. Org. Chem.*, 1985, **50**, 5491.
56 S. Harada, S. Tsubotani, T. Hida, K. Koyama, M. Kondo and H. Ono, *Tetrahedron*, 1988, **21**, 6589.
57 L. N. Jungheim, S. K. Sigmund and J. W. Fisher, *Tetrahedron Lett.*, 1987, **28**, 285.
58 D. Cabaret, S. A. Adediran, M. J. Garcia Gonzalez, R. F. Pratt and M. Wakselman, *J. Org. Chem.*, 1999, **64**, 713; S. A. Adediran, D. Cabaret, R. F. Pratt and M. Wakselman, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 341; S. A. Adediran, D. Cabaret, B. Drouillat, R. F. Pratt and M. Wakselman, *Bioorg. Med. Chem.*, 2001, **9**, 1175.
59 I. Heinze-Krauss, P. Angehrn, R. L. Charnas, K. Gubernator, E.-M. Gutkneckt, C. Hubschwerlen, M. Kania, C. Oefner, M. G. P. Page, S. Sogabe, J.-L. Specklin and F. K. Winkler, *J. Med. Chem.*, 1998, **41**, 3961; M. Vilar, M. Galleni, T. Solmajer, B. Turk, J.-M. Frère and A. Matagne, *Antimicrob. Agents Chemother.*, 2001, **45**, 2215.
60 G. Crichlow, M. Nukaga, V. Doppalapudi, J. D. Buynak and J. R. Knox, *Biochemistry*, 2001, **40**, 6233.
61 A. Kuzin, H. Liu, J. A. Kelly and J. R. Knox, *Biochemistry*, 1995, **34**, 9532.
62 A. Patera, L. C. Blaszczak and B. K. Shoichet, *J. Am. Chem. Soc.*, 2000, **43**, 10504.
63 R. A. Powers, E. Casseli, P. J. Focia, F. Prati and B. K. Shoichet, *Biochemistry*, 2001, **40**, 9207.
64 H. B. Bürgi, J. D. Dunitz and E. Shefter, *J. Am. Chem. Soc.*, 1973, **45**, 5065; H. B. Bürgi, J.-M. Lehn and G. Wipff, *J. Am. Chem. Soc.*, 1974, **96**, 1956.
65 E. Lobkovsky, E. M. Billings, P. C. Moews, J. Rahil, R. F. Pratt and J. R. Knox, *Biochemistry*, 1994, **33**, 6762.
66 O. Herzberg and J. Moult, *Curr. Opin. Struct. Biol.*, 1991, **1**, 946.
67 O. Herzberg, *J. Mol. Biol.*, 1991, **217**, 701; J. R. Knox and P. C. Moews, *J. Mol. Biol.*, 1991, **220**, 435; C. Jelsch, L. Mourey, J.-M. Masson and J.-P. Samama, *Proteins: Struct. Funct. Genet.*, 1993, **16**, 364.
68 A. Matagne, J. Lamotte-Brasseur, G. Dive, J. R. Knox and J.-M. Frère, *Biochem. J.*, 1993, **293**, 607; L. Maveyraud, I. Massova, C. Brick, J. P. Miyashita, J.-P. Samama and S. Mobashery, *J. Am. Chem. Soc.*, 1996, **118**, 7435.
69 L. Maveyraud, L. Mourey, L. P. Kotra, J.-D. Pedelacq, V. Guillet, S. Mobashery and J.-P. Samama, *J. Am. Chem. Soc.*, 1998, **120**, 9748; P. Swarén, J. Massova, J. R. Bellettini, A. Bulychev, L. Maveyraud, L. P. Kotra, M. J. Miller, S. Mobashery and J.-P. Samama, *J. Am. Chem. Soc.*, 1999, **121**, 5353.
70 A.-S. Wilkinson, S. Ward, M. Kania, M. G. P. Page and C. W. Wharton, *Biochemistry*, 1999, **38**, 3851.
71 A. Bulychev, I. Massova, K. Miyashita and S. Mobashery, *J. Am. Chem. Soc.*, 1997, **119**, 7619.
72 C. Oefner, A. D'Arcy, J. J. Daly, K. Gubernator, R. L. Charnas, I. Heinze, C. Hubschwerlen and F. K. Winkler, *Nature*, 1990, **343**, 284.
73 J. Kraut, *Annu. Rev. Biochem.*, 1977, **46**, 331.
74 S. Ness, R. Martin, A. M. Kindler, M. Paetzel, M. Gold, S. E. Jensen, J. B. Jones and N. C. J. Strynadka, *Biochemistry*, 2000, **39**, 5312.
75 R. A. Powers, J. Blazquez, G. S. Weston, M.-I. Morosini, F. Baquero and B. K. Shoichet, *Protein Sci.*, 1999, **8**, 2330.
76 R. F. Pratt, *Science*, 1989, **246**, 917.
77 C. C. H. Chen. J. Rahil, R. F. Pratt and O. Herzberg, *J. Mol. Biol.*, 1993, **234**, 165; L. Maveyraud, R. F. Pratt and J.-P. Samama, *Biochemistry*, 1998, **37**, 2622.
78 J. Rahil and R. F. Pratt, *Biochemistry*, 1994, **33**, 116.
79 J. Rahil and R. F. Pratt, *Biochemistry*, 1992, **31**, 5869; M. J. Slater, A. P. Laws and M. I. Page, *Bioorg. Chem.*, 2001, **29**, 77.
80 J. M. Besterman, D. Delorme and J. Rahil, *PCT Int. Appl. WO 2000-US18344* (*Chem. Abstr.*, 2001, **134**, 95480).
81 J. Rahil and R. F. Pratt, *Biochemistry*, 1993, **32**, 10763; A. P. Laws, M. I. Page and M. J. Slater, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 2317; J. H. Bateson, B. C. Gasson, T. Khushi, J. E. Neale, D. J. Payne, D. A. Tolson and G. Walker, *Bioorg. Med. Chem. Lett.*, 1994, **4**, 1667.
82 N. Li and R. F. Pratt, *J. Am. Chem. Soc.*, 1998, **120**, 4264.
83 K. Kaur and R. F. Pratt, *Biochemistry*, 2001, **40**, 4610.
84 R. F. Pratt and N. J. Hammar, *J. Am. Chem. Soc.*, 1998, **120**, 3004; K. Kaur and R. F. Pratt, *J. Am. Chem. Soc.*, 2001, **123**, 10436.
85 P. J. Stankiewicz, A. S. Tracey and D. C. Crans, in *Metal Ions in Biological Systems, Vanadium and its Role in Life*, ed. H. Sigel and A. Sigel, Marcel Dekker, New York, 1995, ch. 9, vol. 31.
86 J. H. Bell, K. Curley and R. F. Pratt, *Biochem. Biophys. Res. Commun.*, 2000, **274**, 732; J. H. Bell and R. F. Pratt, *Biochemistry*, in the press.
87 K. Curley and R. F. Pratt, *J. Am. Chem. Soc.*, 1997, **119**, 1529.
88 S. Kumar, A. L. Pearson and R. F. Pratt, *Bioorg. Med. Chem.*, 2001, **9**, 2035.
89 G. Amicosante, A. Felici, B. Segatore, L. DiMarzio, N. Franceschini and M. D. Girolamo, *J. Chemother.*, 1989, **1**, 394; R. L. Then, C. Hubschwerlen and R. Charnas, in *50 Years of Penicillin Application*, ed. H. Kleinkauf and H. von Dôhren, Public Ltd., Czech Republic, 1991, p. 383; G. S. Weston, J. Bláquez, F. Baquero and B. K. Shoichet, *J. Med. Chem.*, 1998, **41**, 4577.
90 R. F. Pratt, unpublished work.
91 J. W. Anderson and R. F. Pratt, unpublished work.
92 M. Galleni and J.-M. Frère, *Biochem. J.*, 1988, **255**, 119.
93 M. Galleni, G. Amicosante and J.-M. Frère, *Biochem. J.*, 1988, **255**, 123.
94 M. Jamin, C. Damblon, S. Millier, R. Hakenbeck and J.-M. Frère, *Biochem. J.*, 1993, **292**, 735.
95 Y.-H. Chang, M. R. Labgold and J. H. Richards, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 2823; C. Bourguignon-Bellefroid, J.-M. Wilkin, B. Joris, R. T. Aplin, C. Houssier, F. G. Prendergast, J. Van Beeumen, J.-M. Ghuysen and J.-M. Frère, *Biochem. J.*, 1992, **282**, 361; C. R. Lewis, K. M. Winderberg and A. L. Fink, *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 443.
96 H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, J. N. Shindyalov and P. E. Bourne, *Nucleic Acids Res.*, 2000, **28**, 235.