Phosphoramidate analogues of dipeptides with carboxypeptidase A and β -lactamase-inhibitory activity: elucidation of the mechanism of β -lactamase inhibition by electrospray mass spectrometry

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A series of phosphoramidate compounds with different P_1' amino acid residues have been shown to be irreversible inactivators of the serine β -lactamase from *Enterobacter cloacae* P99. The efficiency of inhibition (based on k_3/K values) of P99 by these derivatives, ordered in decreasing potency, is: β -phenyl- β -Ala > L-Phe > β -Ala > Gly > D-Phe > D-Pro > D-thiazolidine. The D- and L-Phe compounds also inhibit carboxypeptidase A. The proline and thiazolidine derivatives were phosphoramidate methyl esters, whereas the others were salts of diacids. Electrospray mass spectrometry showed that equimolar mixtures of the P99 enzyme with each of the following derivatives, Gly, D-Phe, L-Phe, β -Ala and β -phenyl- β -Ala, effected efficient adduct formation (70–95 % of enzyme modified), illustrating the particularly active nature of some of these compounds. All the primary

amino acid derivatives gave a similar mass increment, which suggests the displacement of the variable P_1' part of the molecule. This observation provides evidence that the compounds phosphorylate the active-site serine, with the phosphoramidate bond as the scissile bond and the amino acid as the leaving group. The thiazolidine derivative (phosphoramidate methyl ester) also appeared to work by the same mechanism. The comparable proline derivatives caused lower than expected mass shifts of 227–229, and therefore it is proposed that with these compounds both the amino acid and the phosphoramidate ester methoxy group were displaced at the phosphorus atom during the inhibition process. Therefore, electrospray mass spectrometry has provided both a measure of potency and a rationale for the mechanism of inhibition of P99 by these compounds.

INTRODUCTION

Serine active-site β -lactamases hydrolyse the β -lactam ring of β -lactam antibiotics and therefore pose one of the greatest threats to the successful clinical use of β -lactam antimicrobials. Consequently, the use of a β -lactamase inhibitor in combination with a β -lactam has become one of the most successful strategies used to overcome the resistance problems caused by this group of microbial enzymes. All the commercially available β -lactamase inhibitors contain a β -lactam ring and these compounds are active against the vast majority of Bush Group 2 [1] enzymes, which are the most common β -lactamases produced by clinical pathogens [2].

Other compound classes have been identified that have β -lactamase-inhibitory activity. One such group is the phosphonate series of depsipeptide analogues. Pratt [3] has illustrated that phosphonate monoesters inhibit the *Enterobacter cloacae* P99 Group 1 serine β -lactamase by phosphorylation of the active-site serine. In addition, phosphoramidates have been shown to inhibit P99 *via* the same mechanism [4,5]. We were also intrigued by the structural similarity (Scheme 1) in their peripheral topology of linear phosphoramidate analogues of dipeptides (Scheme 1, compound 1) to the bicyclic β -lactam substrates of β -lactamases (generalized structure 2 in Scheme 1). In a previous publication [6] we reported the synthesis of a variety of phosphoramidates (Figure 1), incorporating increasing substrate-recognition features (1; R^1 , R^2) into the P_1' amino acid residue. These had inhibitory activity against a broad range of serine β -lactamases [6]. The compounds showed greatest potency against the P99 enzyme but one compound (9), containing a β -alanyl residue at

P_1' , had IC_{50} values of less than or equal to 50 μ M for β -lactamases from each of the following Bush Groups: 1, 2a, 2b, 2b' and 2d [1]. In addition, synergy minimum inhibitory concentrations illustrated that some of these compounds potentiated the antibacterial activity of amoxicillin against bacteria producing the P99 enzyme. One of the phosphoramidates containing L-Phe at P_1' (5) is a known inhibitor of the zinc metalloprotease carboxypeptidase A, for which the mechanism of inhibition occurs as expected by transition-state mimicry, rather than by any phosphorylation process [7].

In this study we report on the use of electrospray mass spectrometry (ESMS) to investigate both the potency and

Scheme 1 Peripheral relationship between linear phosphoramidates (1) and generalized bicyclic β -lactam structures (2) of β -lactamases

Abbreviation used: ESMS, electrospray mass spectrometry.

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Figure 1 Structures of phosphoramidate compounds

mechanism of inhibition of the P99 β -lactamase by eight-phosphoramidate compounds (3–10). In addition, we have examined the activity of each of the compounds against the mammalian carboxypeptidase A metalloprotease and a series of bacterial metallo- β -lactamases to determine whether any of the inhibitors are able to inhibit enzymes operating by this very different mechanism.

MATERIALS AND METHODS

Materials

P99 β -lactamase was purchased from Porton Products. Hippuryl-L-phenylalanine (Sigma) and carboxypeptidase A (Sigma) were used as purchased. The phosphoramidate compounds are shown in Figure 1 and were prepared in our laboratories [6] using methods similar to or derived from known procedures [7]. The dilithium salts (3–5 and 9) are single diastereoisomers; the β -phenyl- β -alanine compound (10) is a 1:1 mixture of enantiomers, epimeric at the phenyl substituent. The lithium salt phosphoramidate esters (6–8) were each 1:1 mixtures of diastereoisomers, epimeric at the phosphorus. The methyl ester function present in compounds 6–8 could not be cleaved hydrolytically without concomitant hydrolysis of the phosphoramidate linkage.

ESMS

Each compound was mixed with P99 (1 mg/ml final concentration) to achieve the desired ratio of enzyme/inhibitor. All assays were performed in 5 mM Tris/HCl, pH 7.0, buffer. The mixture was then incubated for 5 min at 37 °C and a 20 μ l aliquot examined by ESMS using a gradient system in order to prevent possible interference from excess inhibitor. Chromatography was performed on a Hewlett-Packard 1090 liquid chromatograph using an Applied Biosystems Aquapore RP-300

column (100 mm $\times$ 2.1 mm) with the following gradient at a flow rate of 100 μ l/min:

The eluate from the column was fed directly into the electrospray interface of a Finnigan MAT TSQ 700 mass spectrometer. Spectra were averaged over the peak of interest and deconvoluted using ICIS V1.00 software. In spectra that show both unbound P99 and the adduct peak, mass-shift calculations were internally calibrated. Where no free P99 was observed, the mass of P99 was taken as 39195 ± 1.0 which was the average of five mass determinations (Figure 2, top, shows an example of one of the mass determinations). This is lower than the mass of P99 determined from the amino acid sequence (39206). This anomaly has been observed in our previous studies with this enzyme [8].

Enzyme assays and kinetic analysis

All β -lactamase assays were performed at 37 °C in 25 mM sodium phosphate buffer (pH 7.0). The assays described below were initiated by the addition of enzyme and utilized nitrocefin as the reporter substrate (final concentration 100 μ M). The enzyme concentration was chosen so that only 10% of the reporter substrate ($\sim 10 \mu$ M) was hydrolysed at the end of the inactivation. A pseudo-first-order rate constant k_i characterizing the rate of inactivation was determined at different inhibitor concentrations. Table 1 shows the range of concentrations used for each compound. For all inhibitors, k_i increased linearly with the concentration of inhibitor (I). Under these conditions, only the k_2/K ratio can be computed. Limit values of k_2 and K were given.

$$k_i = k_3 + \frac{k_2 I}{\{K[(K_m^{SR} + S)/K_m^{SR}]\} + I}$$

where S is the concentration of the reporter substrate and K_m^{SR} is the K_m of the reporter substrate.

Here, $K[(K_m^{SR} + S)/K_m^{SR}] \gg I$ and $k_3 = 0$, so

$$k_i = \left\{ \frac{k_2}{K[(K_m^{SR} + S)/K_m^{SR}]} \right\} I$$

IC₅₀ assays for the carboxypeptidase A enzyme (0.54 unit/ml) were carried out after a 5 min incubation of inhibitor and enzyme. Assays were performed at 37 °C in 25 mM Tris/HCl/500 mM NaCl, pH 7.5, with hippuryl-L-phenylalanine (0.39 mM) as the reporter substrate. Hydrolysis of the reporter substrate was monitored at 254 nm.

RESULTS

Kinetic analysis showed that these compounds were inactivators of the P99 enzyme, and the k_2/K ratio for inhibition of P99 was determined for each of the compounds (Table 1). On the basis of the k_2/K data the activities of the compounds were ranked as follows: β -phenyl- β -Ala (10) > L-Phe (5) > β -Ala (9) > Gly

Figure 2 ESMS of P99 β -lactamase (top) and P99 β -lactamase and compound 9 mixed in a 1:1 molar ratio (bottom)

The mass of P99 increased by m/z 229 when mixed with compound 9.

Table 1 Kinetic constants for the inhibition of P99 by phosphoramidate compounds

Results for k_2/K are means $\pm$ S.E.M. from three determinations.

Compound	Phosphoramidate side chain	Concentrations of inhibitor used to determine k_2/K (μ M)	k_2/K ($M^{-1} \cdot s^{-1}$)	$10^3 \times k_2$ (s^{-1})	K (μ M)
3	Gly	200–1500	94 ± 3	> 141	> 1500
4	D-Phe	200–4000	60 ± 2	> 240	> 4000
5	L-Phe	100–1000	226 ± 7	> 226	> 1000
6	D-Pro	500–5000	5.5 ± 0.1	> 28	> 5000
8	D-Thiazolidine	5000–10 000	0.20 ± 0.02	> 2	$> 10 000$
9	β -Ala	100–1000	157 ± 6	> 157	> 1000
10	β -Phenyl- β -Ala	100–1000	250 ± 5	> 250	> 1000

(**3**) $>$ D-Phe (**4**) $>$ D-Pro (**6**) $>$ D-thiazolidine (**8**). Interestingly, the L-Phe (**5**) derivative was more than 3-fold more effective at inactivating P99 than the D-isomer (**4**). Compounds **3–10** at a

concentration of 1 mM had no inhibitory activity against the *Bacteroides fragilis* CfiA, *Stenotrophomonas maltophilia* L-1 and *Aeromonas hydrophila* CphA metallo- β -lactamases [9].

Table 2 Summary of ESMS data

–, No adduct observed.

Compound	Ratio of enzyme/inhibitor	$\Delta\text{Mass } (m/z)$ (± 2)	Percentage of enzyme modified
3	1:1	225	70
4	1:1	228	80
5	1:1	226*	> 95
6	1:50	229	40
7	1:10	–	–
	1:50	227	50
	1:50	228	40
8	1:10	–	–
	1:50	244	20
	1:50	246	15
9	1:1	229*	> 95
10	1:1	228*	> 95

* No unbound P99 observed, therefore mass of P99 taken as 39195.

ESMS studies on the inhibition of P99 by phosphoramidate compounds

ESMS analysis, in turn, of a 1:1 molar mixture of each of the most potent phosphoramidates, (**10**, **5** and **9**) with P99 showed a mass increment in the range m/z 226–229 (Figure 2, bottom, illustrates the mass shift observed with compound **9**), suggesting that a covalent enzyme–inhibitor adduct had formed. This adduct did not correspond to an increment representing the masses of any of the intact compounds but indicated incorporation of a common fragment. Under these conditions, no free P99 was observed on ESMS. An adduct of similar mass was also observed when 1:1 mixtures of P99 and compounds **3** and **4** were examined by ESMS (Table 2). However, spectra from these investigations revealed both unbound P99 together with an enzyme–inhibitor adduct, illustrating that the P99 was incompletely inhibited. Moreover, spectra obtained for P99 mixed with a 10-fold molar excess of compound **7** did not reveal any P99 adduct. However, on increasing the concentration of inhibitor (50:1 molar excess), the spectra for compounds **6** and **7** showed both free P99 and a peak corresponding to a similar mass increment of m/z 227–229 (Table 2). At this ratio, approximately 40–50% of the P99 remained unbound. Finally, P99 mixed with a 50:1 molar excess of the least active compound, the D-thiazolidine derivative (**8**), gave rise to only a minor peak which corresponded to a fragment m/z 246 binding to the P99 β -lactamase (Table 2).

Inhibition of carboxypeptidase A by phosphoramidate compounds

The D-Phe (**4**) and L-Phe (**5**) derivatives inhibited the carboxypeptidase A metalloprotease with IC_{50} values of 8.95 and 0.32 μM respectively. Interestingly, the L-isomer was more than 25 times more active than the compound with the D-Phe substituent. The latter feature corresponds to the β -lactam P_1' -recognition stereochemistry: see Scheme 1, structure **2**. All the other compounds showed no significant inhibition of carboxypeptidase A at 1 mM.

DISCUSSION

The kinetic analysis described in this report has illustrated that these phosphoramidate compound irreversibly inhibit the P99 β -lactamase. The ESMS data have further substantiated the nature

of this irreversible inhibition by illustrating the covalent binding of fragments of the compounds to the P99 enzyme.

The efficiency of inhibition of P99 by each of these compounds compared well with the ESMS data. For example, the spectra of a 1:1 mixture of the three compounds with $k_2/K = 157\text{--}250 \text{ M}^{-1}\cdot\text{s}^{-1}$ showed that P99 was completely inhibited, but under the same conditions only a small proportion of P99 had formed an adduct in the presence of a 50:1 molar excess of the least active phosphoramidate (**8**) ($k_2/K = 0.2 \pm 0.02 \text{ M}^{-1}\cdot\text{s}^{-1}$). A compound similar to **7** has been synthesized by Laws et al. [4] and was the phosphoramidate ethyl ester rather than the methyl ester; these two esters would be expected to have similar potencies. On the basis of k_2/K comparisons, compounds **10**, **9** and **5** were between 50 and 30 times more effective inactivators of P99 than the compound described by Laws et al. [4].

The masses of the five most active compounds (**3–5**, **9**, **10**) vary from 404 to 314. However, each compound gave an ESMS adduct of similar mass increment (225–229) which suggests elimination of the variable P_1' part of the molecule. Therefore, to explain these mass shifts, we propose that the compounds phosphorylate the active-site serine of the β -lactamase (Scheme 2) with the phosphoramidate bond as the scissile bond and the amino acid as the leaving group. The predicted mass shift for this mechanism depends (Scheme 2) on whether the inhibitor was the phosphoramidate ($\text{X} = \text{H}$) or its methyl ester ($\text{X} = \text{Me}$). The predicted mass shift for the phosphoramidate form (227) was close to the observed values for compounds **3–5**, **9** and **10** (Table 2), supporting the proposed mechanism. The thiazolidine analogue (**8**) is a phosphoramidate methyl ester and, according to Scheme 2, should give rise to an m/z increment of 241. A mass shift of $+(244\text{--}246)$ mass units was observed, suggesting that compound **8** inhibits P99 by the mechanism illustrated in Scheme 2, with its phosphoramidate methyl ester bond retained intact.

Both the proline derivatives (**6** and **7**) are also phosphoramidate methyl esters. Consequently, a mass shift of approx. 241 should also have been measured according to the mechanism in Scheme 2. However, mass shifts of 227–229 were observed. It is possible that with these compounds both the amino acid and the phosphoramidate methoxy group were displaced sequentially at the phosphorus atom during the enzyme inhibition. The observed

Scheme 2 Proposed mechanism of inhibition of P99 by phosphoramidates

mass changes would not permit a subsequent nucleophilic displacement of methanol at phosphorus by a proximate enzyme lysine residue [requiring a nett loss of CH_3OH , $\Delta\text{mass} -32$ mass units], but necessitate rather a hydrolytic displacement by water [nett loss of CH_2 , $\Delta\text{mass} -14$ mass units]. This would result in a mass shift of 227 which is close to the observed change.

Clearly, the second hydrolytic event cannot be fully enzyme-mediated since the serine residue is now blocked. It may, however, be accelerated by the presence of proximate amino acid residues. Our data do not rule out the alternative possibility that, for the proline compounds, displacement of the methanol precedes that of the P_1' amino acid residue. As both compounds **6** and **7** provided the expected $[M+H]^+$ ions (m/z 347) in their spectra, it is extremely unlikely that the observed methyl ester loss from the enzyme adduct is an artifact of electrospray-induced fragmentation. We are unable to account for the difference in behaviour between thiazolidine (**8**) and prolines (**6** and **7**) other than it may be a consequence of a much slower methyl ester hydrolysis reaction for the former. It is noteworthy that Laws et al. [4] report that, in the enzyme reaction of one diastereoisomer of their ethyl ester analogue of compound **7**, ethanol loss was detected.

The L-Phe derivative (**5**) is a potent inhibitor of carboxypeptidase A by transition-state mimicry [7]. This reflects both the known preference for a Phe substituent in the P_1' residue of carboxypeptidase A inhibitors (see for example ref. [10]) and a preference for the natural (L-) amino acid stereochemistry at this position. As discussed above, our work suggests that this compound inhibits the P99 serine β -lactamase by a phosphorylation mechanism. It is quite remarkable that this compound can inhibit both a serine and an unrelated metallo active-site enzyme by two such different mechanisms. Both enzymes also have preference for the L-isomer over the D-isomer (**4**). In the case of the proline derivatives (**6** and **7**), the amino acid stereochemistry was a less important criterion for inhibition of P99.

The parallel study by Laws et al. [4] on a single phosphoramidate structural type (the ethyl phosphoramidate esters analogous to **7**) also supports the hypothesis that this type of compound inhibits P99 by phosphorylation at the active-site serine with elimination of the inhibitor amino acid residue. Phosphonate monoesters have also been shown to inhibit Class C β -lactamases by phosphorylation and the validity of this concept has been demonstrated by an extensive X-ray study [11].

In the present work we have demonstrated how ESMS can be successfully used to deduce the mechanism of inhibition of a Class C β -lactamase by eight phosphoramidate compounds possessing six different P_1' amino acid residues. Only two of the compounds were potent inhibitors of both carboxypeptidase A

and P99. It is also worthy of note that the activity of some of these compounds against Class C β -lactamases exceeds the activity of some of the commercially available β -lactamase inhibitors [12]. In fact, ESMS studies have shown that the L-Phe, β -Ala and β -phenyl- β -Ala derivatives bind to P99 at a 1:1 molar ratio of inhibitor/enzyme. BRL 42715 (a 6-alkylidene penem, prepared in these laboratories) and β -halogenopenicillanates were also reported to bind and inhibit P99 at this ratio [13,14], but the former was much more efficient ($k_2/K = 1000\text{--}2000 \text{ mM}^{-1}\cdot\text{s}^{-1}$) than the latter ($k_2/K = 0.11 \text{ mM}^{-1}\cdot\text{s}^{-1}$). Therefore this series of linear phosphoramidate analogues of dipeptides represents an interesting alternative to bicyclic β -lactam structures in its efficacy against a clinically important serine β -lactamase, while utilizing a drastically different (phosphonylative) inhibition mechanism. It is equally remarkable that representatives (**4** and **5**) of the series are able to inhibit a mammalian metalloprotease (carboxypeptidase A) in transition-state inhibitory mechanistic mode and yet all the compounds remain ineffective against a range of bacterial metalloenzymes, the zinc metallo- β -lactamases. They are therefore unable to inhibit this bacterial series of metalloenzymes by functioning as transition-state isosteres of the bicyclic β -lactam substrates (see Scheme 1).

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