Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 503–510

BIOORGANIC & MEDICINAL CHEMISTRY

Cysteinyl Peptide Inhibitors of *Bacillus cereus* Zinc β-Lactamase

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Received 17 July 2000; accepted 4 October 2000

Abstract—Several cysteinyl peptides have been synthesised and shown to be reversible competitive inhibitors of the *Bacillus cereus* metallo-β-lactamase. The pH dependence of pK_i indicates that the thiol anion displaces hydroxide ion from the active site zinc(II). D,D-Peptides bind to the enzyme better than other diastereoisomers, which is compatible with the predicted stereochemistry of the active site.

Introduction

The major mechanism of bacterial resistance to the normally lethal action of β-lactam antibiotics is the microorganism's production of β-lactamase enzymes which catalyse the hydrolysis of the β-lactam. There are two major types of β-lactamase — serine enzymes and those requiring zinc(II) ions for their activity.[1] In 1985 there were just two microorganisms which had been identified as producing metallo-β-lactamases but to date there are over 20 bacterial sources of the metallo-enzyme, including those found in *Bacillus cereus*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Aeromonas hydrophila* and *Bacteroides fragilis*,[2] all of which are classified as class B metallo-β-lactamases.

Although these metallo-enzymes were initially thought to be clinically unimportant, some pernicious strains have been shown to owe their antibiotic resistance to their ability to produce zinc-β-lactamases.[3] Of particular concern is the ability of these metallo-β-lactamases to catalyse the hydrolysis of some of the newer β-lactam antibiotics such as the carbapenems. The mechanism-based inactivators which have been used against the serine enzymes are generally ineffective against the Zn(II)-dependent enzymes, and, at present, there are no clinically useful inhibitors known of the metallo-β-lactamases.

The metallo-β-lactamases are extremely efficient at catalysing the hydrolysis of imipenem with values of k_{cat}/K_m of ca. $10^6\,M^{-1}\,s^{-1}$, which is at least 1000-fold greater than that shown by "classical" class A β-lactamases such as TEM-1. A review of the catalytic properties of the well-characterised class B β-lactamases showed that the *A. hydrophila* enzyme clearly exhibits the most specific substrate profile, while the other enzymes are rather broad-spectrum.[4]

The sequences of the metallo-β-lactamases have been established and they all contain a single peptide chain, composed of 220–230 residues.[5] A comparison of these amino acid sequences indicates that they appear to constitute a fairly homogeneous group and exhibit up to 37% sequence identity. The enzymes from *B. cereus*, *Aeromonas hydrophila*, *B. fragilis* and L1 from *S. maltophilia* have the capacity to bind up to 2 mole equivalents of metal ion per mole of protein.[6–10] However, despite these structural similarities the functional role of the second metal ion is controversial[11–17] and the different metallo-enzymes may have different metal ion requirements. Although the *B. cereus* enzyme can bind two metal ions,[7–9] it appears that only one is required for maximum catalytic activity.[18] For the other enzymes the effect of binding the second metal ion is not clear and it may either increase or even decrease activity.[11–14] The role of the first zinc ion appears to be to provide a metal bound, but nucleophilic, hydroxide ion to attack the β-lactam carbonyl carbon, which generates a tetrahedral intermediate with the oxyanion stabilised by the metal ion.[13,18]

There have been several recent reports of inhibitors which inhibit the metallo-β-lactamases.[18–22] A series of thioester substrates of IMP-1 metallo-β-lactamases are reversible competitive inhibitors of the enzyme, probably by producing thiols as hydrolytic products.[22] In contrast

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0968-0896/01/$ - see front matter
PII: S0968-0896(00)00257-1

thioesters were thought to irreversibly inhibit *B. cereus* metallo-β-lactamases by covalent modification of the active site by mercaptoacetic acid,[20] but in a separate study,[21] thioesters of mercaptophenylacetic acid were shown to be weak competitive inhibitors of the *B. cereus* enzyme as the unreacted thioesters.

Whatever the detailed mechanism of action of the metallo-β-lactamases,[13,18] it is likely that zinc(II) stabilises the tetrahedral intermediate presumed to be formed during the catalytic process. Thiols are well known inhibitors of metallo-proteases because of their ability to coordinate to the active site zinc. We have reported earlier[1,18] the use of thiol derivatives, with structures containing suitable sites for molecular recognition, to inhibit the class B metallo-β-lactamases from *Bacillus cereus* 5/B/6 and herein we develop that theme, including the importance of stereochemistry.

Results and Discussion

A series of substituted thiols and cysteinyl dipeptides were synthesised as outlined in Scheme 1 and then tested as inhibitors of the *B. cereus* 569/H/9 class B metallo-β-lactamase. Even the simple thiol, *N*(2-mercaptoethyl)-phenylacetamide (**1**), was found to be a competitive inhibitor of the enzyme and the effects of substituents and their stereochemistry in various mercapto dipeptides on K_i were determined (Table 1).

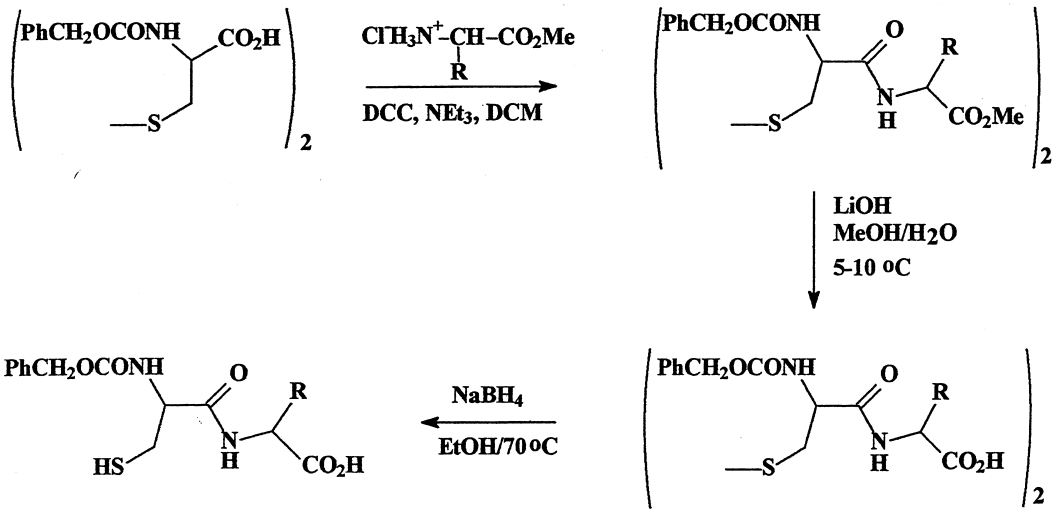
A plot of the logarithm of the inhibition constant, pK_i, for *N*-carboxy-L-cysteinyl-glycine (**6**) against pH is pH independent between pH 6 and 9 but decreases in both acidic and basic solution (Fig. 1). The inflections are very similar to those observed for enzyme catalysed hydrolysis of penicillins and cephalosporins, with calculated pK_as of 5.66±0.4 and 9.93±0.1, which indicates that the ionisation states of the enzyme required for catalysed hydrolysis are also those required for binding the inhibitor.

The pK_a of the thiol **6** is 9.3 so the predominant species at pHs below this is the undissociated neutral form. The zinc-bound water is thought to have a pK_a of 5.7[18] and so it will be fully deprotonated above pH 6. If the thiol binds to the metal ion as the thiolate anion then the measured binding constant of the neutral undissociated thiol would be pH independent between pH 6 and 9, as seen in Figure 1.

Table 1. Inhibitor dissociation constants for the β-lactamase II catalysed hydrolysis of β-lactams, at 30 °C, pH 7.0 and ionic strength 1.0 M (NaCl). Enzyme concentration 0.1 μM

Inhibitor	K_i μM ($ZnSO_4$ 2×10^{-6} M)
N(2-Mercaptoethyl)phenylacetamide (**1**)	56.2±11
N-Phenylacetylglycine (**2**)	1000±150
N-Phenylacetyl-L-cysteine (**3**)	108±3
N-Phenylacetyl-DL-penicillamine (**4**)	515±38
N-Carbobenzoxy-L-cysteine (**5**)	97±5
N-Carbobenzoxy-D-cysteine (**5**)	20.1±1.6
N-Carbobenzoxy-L-cysteinyl-glycine (**6**)	42.2±10
N-Carbobenzoxy-DL-cysteinyl-glycine (**6**)	46.3±0.1
N-Carbobenzoxy-L-cysteinyl-DL-alanine (**7**)	62.0±9.3
N-Carbobenzoxy-L-cysteinyl-DL-valine (**8**)	48.3±2.3
N-Carbobenzoxy-L-cysteinyl-L-leucine (**9**)	135.5±17
N-Carbobenzoxy-L-cysteinyl-DL-serine (**10**)	72.5±0.5
N-Carbobenzoxy-DL-cysteinyl-L-serine (**10**)	84±4
N-Carbobenzoxy-D-cysteinyl-D-penicillamine (**11**)	Chelates Zn^{++}
N-Carbobenzoxy-L-cysteinyl-DL-phenylalanine (**12**)	17.3±0.2
N-Carbobenzoxy-L-cysteinyl-L-phenylalanine (**12**)	26.9±0.5
N-Carbobenzoxy-L-cysteinyl-D-phenylalanine (**12**)	9.3±0.4
N-Carbobenzoxy-D-cysteinyl-L-phenylalanine (**12**)	11.0±1
N-Carbobenzoxy-D-cysteinyl-D-phenylalanine (**12**)	3.0±0.1
N-Carbobenzoxy-L-cysteinyl-DL-proline (**13**)	95.0±1.5
Captopril	41.6±9

The thiol group is necessary for inhibition and, for example, *N*-phenylacetylglycine (**2**) is a very poor inhibitor. Conversely, the addition of a carboxylate residue to the simple thiol, **1**, as in *N*-phenylacetyl-L-cysteine **3**, slightly decreases potency, probably simply as a result of increasing the pK_a of the thiol group and indicating that a negatively charged carboxylate anion in this position does not facilitate binding. There is an order of magnitude decrease in binding when two gem-dimethyl groups are α-substituted to the thiol, as in *N*-phenylacetyl-DL-



Scheme 1.

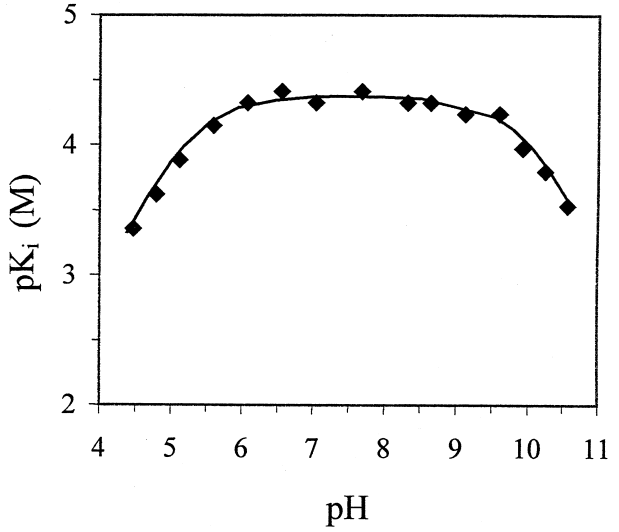


Figure 1. The pH dependence of pK_i for *B. cereus* β-lactamase class B inhibited by *N*-carboxy-L-cysteinyl-glycine at 30 °C and 1.0 M (NaCl). The values of pK_i were determined using cephaloridine as the substrate.

penicillamine (**4**). This is indicative of some selectivity in binding and suggests that thiol co-ordination is important and can be sterically inhibited.

The effect of substituents on the inhibition was investigated in various *N*-carbobenzoxy-cysteinyl amino acids, although the dipeptide cbzCysGly (**6**) shows no significant increased binding compared with the simple thiol **1**, despite potential increased recognition. However, hydrophobic substituents at the α-carbon of the C-terminal amino acid do increase binding, and K_i decreases as the size of the R group increases from CH_3 in *N*-carbobenzoxy-L-cysteinyl-DL-alanine (**7**) to $CH(CH_3)_2$ in *N*-carbobenzoxy-L-cysteinyl-DL-valine (**8**), but increases again if the alkyl substituent becomes too large (**9**). When the α-substituent is CH_2-Ph as in *N*-carbobenzoxy-L-cysteinyl-DL-phenylanine (**12**), the K_i of 17 μM indicates a more potent inhibitor, presumably due to the hydrophobic effect of the aromatic group. An α-hydroxymethyl substituent as in *N*-carbobenzoxy-L-cysteinyl-serine (**10**) has little effect upon binding and a proline residue (**13**) does not improve the inhibition.

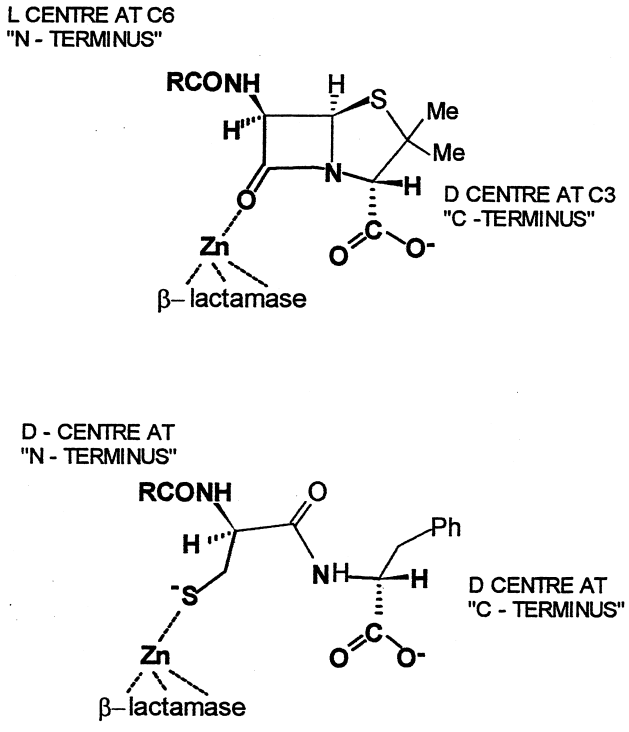
The thiol inhibitors do not act as chelating agents. The measured inhibition constants were independent of zinc ion concentration and the enzyme activity was not restored by the addition of zinc ions. However, the enzyme was completely inactivated by the dithiol, *N*-carbobenzoxy-D-cysteinyl-D-penicillamine (**11**), but the activity was, in this case, mostly recovered upon addition of excess zinc, indicating that the dithiol was acting as a chelating agent and removed the zinc ion from the protein. As expected, other dithiol compounds, such as 2,3-dimercaptosuccinic acid and 2,3-dimercapto-1-propane sulfonic acid, exhibit a similar phenomenon.

Captopril and enalapril are potent inhibitors of the zinc-dependent angiotensin converting enzyme (ACE), and with the *B. cereus* zinc β-lactamase captopril was found to inhibit the enzyme with a K_i of 41 μM, whereas enalapril, which does not contain a thiol group, is practically ineffective.

There are two asymmetric centres in the cysteinyl dipeptides and the effect of the stereochemistry at each centre on inhibition was determined. For example, the D-enantiomer of the *N*-carbobenzoxy-cysteine (**5**) is five times more potent than the L-enantiomer. The four stereoisomers of *N*-carbobenzoxy-cysteinyl-phenylalanine (**12**) display stereoselectivity in their inhibition of β-lactamase II. As with other cysteinyl derivatives, the D-centre is preferred, and this configuration is also favoured at the carboxy terminal residue. Thus the D,D-enantiomer is an order of magnitude better than the L,L-one.

It is concluded that the best interactions with the enzyme are obtained with compounds with a thiol residue and a hydrophobic side chain α to carboxyl terminus. The D,D isomers exhibit the highest potency, *N*-carbobenzoxy-D-cysteinyl-D-phenylalanine (**12**) with a K_i of 3.0 μM being the most efficient in vitro inhibitor of the *B. cereus* β-lactamase.

The most effective thiol peptide inhibitors have the D configuration at the cysteinyl chiral centre and the D configuration at the carboxyl terminus. By contrast, the stereochemistry of penicillin is L at C6 and D at C3. However, this is compatible with similar binding interactions at the active site with the carboxylate anion of penicillin and of the thiol inhibitor binding to His 210 or Lys 171 and the β-lactam carbonyl oxygen or the thiolate anion of the inhibitor binding to the active-site zinc (Scheme 2).



Scheme 2.

Summary

Several cysteinyl peptides have been synthesised and shown to be reversible competitive inhibitors of the *B. cereus* metallo-β-lactamase. The pH dependence of pK_i indicates that the thiol anion probably displaces hydroxide ion from the active site zinc(II) and that the terminal carboxylate binds to His 210 or Lys 171. D,D-Peptides bind to the enzyme better than other diastereoisomers, which is compatible with the predicted stereochemistry from the binding of penicillin substrates to the active site.

Experimental

Synthesis

^{1}H and ^{13}C NMR spectra were recorded at 270 MHz on a Bruker AC-270 spectrometer. The chemical shifts (δ_H) are quoted in p.p.m. and referenced to TMS. Infrared spectra were obtained on a Perkin Elmer 1600 Series FTIR, as nujol mulls or chloroform solutions. The mass spectra were recorded on a VG (Fisons Instruments) Quattro (II) S Q mass spectrometer.

***N*(2-Mercaptoethyl)phenylacetamide (1).** Phenylacetyl chloride (5.5 mmoles) was added dropwise to a solution of cystamine (2.5 mmoles) in water (40 mL) containing sodium hydroxide (11.25 mmoles) and was cooled to 0 °C. At the end of the reaction, the white precipitate formed was filtered and washed with water. The solid was recrystallised from hot methanol. The disulfide thus obtained was reduced by adding sodium borohydride (88 mmoles) to a solution of the disulfide (4.4 mmoles) in ethanol:water (5:3, v/v), which was heated to 90 °C for 1 h, cooled, then acidified to pH 2 with dilute hydrochloric acid. The solvent was evaporated, filtered and the filtrate extracted with chloroform, washed with water and dried over anhydrous sodium sulfate. The solvent was evaporated and the solid recrystallised. Yield 87%. Mp 48–50 °C. ^{1}H NMR δ_H ($CDCl_3$) 7.30 (5H, m), 5.75 (1H, m), 3.55 (2H, s), 3.35 (2H, m), 2.52 (2H, dt, $J=6.6$ and 8.5, CH_2-S), 1.18 (1H, t, $J=8.5$, SH). IR (nujol) ν 3253, 2544, 1637 cm^{-1}. A pK_a of 9.5±0.10 of the thiol was determined from the dependence of the absorbance of an aqueous 2.52×10^{-4} M solution at 238 nm as a function of pH.

***N*-Phenylacetyl-L-cysteine (3).** The disulfide was obtained from phenylacetyl chloride (13 mmoles) and cystine (5.9 mmoles). Yield 80%. ^{1}H NMR δ_H (DMSO) 8.54 (1H, d, $J=7.9$), 7.26 (5H, m), 4.50 (1H, m), 3.50 (2H, s), 3.16 and 2.95 (2H, m, $J=4.6$, $J=9.5$, $J=13.6$). ^{13}C NMR δ_C (DMSO) 171.87, 170.25, 136.0, 129.0, 128.1, 126.3, 51.3, 41.9, 40.3. IR (nujol) ν 3319, 1720, 1660 cm^{-1}. ES MS $[M-H]^-$ m/z 475.

The disulfide (5 mmoles) was then reduced with sodium borohydride (100 mmoles). Yield 90%. Mp 85–88°C. ^{1}H NMR δ_H (DMSO) 8.47 (1H, d, $J=7.8$), 7.25 (5H, m), 4.40 (1H, m), 3.53 (2H, s), 2.82 (2H, m), 2.45 (1H, m). ^{13}C NMR δ_C (DMSO) 171.6, 170.4, 136.3, 129.1, 128.2, 126.4, 54.5, 41.9, 25.7. IR (nujol) ν 3342, 2550, 1731, 1623 cm^{-1}. ES MS $[M-H]^-$ m/z 238.

***N*-Phenylacetyl-DL-penicillamine (4).** Obtained from phenylacetyl chloride (0.98 mmoles) and penicillamine (6.7 mmoles). Yield 42%. Mp 118–120 °C. ^{1}H NMR δ_H (DMSO) 8.34 (1H, d, $J=9.2$), 7.32 (5H, m), 4.44 (1H, d), 3.58 (2H, dd, $J_{AB}=13.8$), 2.86 (1H, s) and 1.38 and 1.36 (6H, 2 s). ^{13}C NMR δ_C (DMSO) 171.2, 170.3, 136.3, 129.0, 128.1, 126.3, 61.3, 45.5, 41.8, 29.7, 29.6. IR (nujol) ν 3365, 2571, 1728, 1635 cm^{-1}. ES MS $[M-H]^-$ m/z 266.

***N*-Carbobenzoxy-L- and -D-cysteine (5).** Obtained from cystine (0.0125 moles) and carbobenzoxy chloride (0.0375 moles). Yield 50%. ^{1}H NMR δ_H (DMSO) 7.77 (1H, d, $J=8.4$), 7.35 (5H, m), 5.07 (2H, s), 4.36 (1H, m), 3.20 and 2.97 (2H, m, $J=4.1$, $J=10.1$, $J=13.7$). ^{13}C NMR δ_C (DMSO) 172.3, 156.2, 136.9, 128.4, 128.1.1, 127.9, 65.7, 53.1, 39.6. IR (nujol) ν 3329, 1730, 1655 cm^{-1}. ES MS $[M-H]^-$ m/z 509. The disulfide (2.18 mmoles) was then reduced with sodium borohydride (8.74 mmoles). Yield 90%. ^{1}H NMR δ_H (DMSO) 7.64 (1H, d, J 7.9), 7.36 (5H, m), 5.08 (2H, s), 4.14 (1H, m), 2.91 and 2.75 (2H, m), 2.51 (1H, m). ^{13}C NMR δ_C (DMSO) 172.3, 156.2, 136.9, 128.5, 127.9.1, 127.8, 65.7, 56.6, 25.6. IR (nujol) ν 3327, 2571, 1719, 1640 cm^{-1}. ES MS $[M-H]^-$ m/z 254.

***N*-Carbobenzoxy-L-cysteinyl amino acids.** Synthesised using the following general procedure. A solution of *N*,*N*′-dicarbobenzoxycystine (6 mmoles) in dichloromethane (50 mL), cooled to 0 °C, was treated with dicyclohexylcarbodiimide (12 mmoles), a solution of triethylamine (12 mmoles) and amino acid methylester hydrochloride (12.6 mmoles) in dichloromethane (50 mL). The mixture was stirred at 0 °C for 1 h and then for 24 h at room temperature. The precipitated dicyclohexylurea was filtered, the filtrate washed twice with 1 M sulfuric acid (30 mL) and twice with 5% sodium bicarbonate (30 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated. The residue was dissolved in acetone, the insoluble part was removed by filtration and the solvent evaporated to give a white product. Yield 90%. To a solution of (Z-Cys-AaOMe)$_2$ (5 mmoles) in 50 mL of methanol, cooled to 5 °C, was added dropwise lithium hydroxide (10.2 mmoles) in water (17 mL). After 18 h, 50 mL of water were added, the methanol evaporated and the solution washed with diethyl ether (2×25 mL). The aqueous phase was acidified to pH 2 with hydrochloric acid and the product filtered, washed with water, and dried. Yield 70%.

To a solution of (Z-Cys-AaOH)$_2$ (2 mmoles) in ethanol (20 mL) at 68–70 °C was added dropwise a solution containing 4 equivalents of sodium borohydride (8.74 mmoles) in ethanol (10 mL) and the mixture heated at 75–80 °C for 1 h. After cooling to 25 °C, the solution was added to 50 mL iced water, to which was added 6 M hydrochloric acid to give pH 3. After evaporating the ethanol, the solution was extracted three times with ethyl acetate, washed three times with distilled water and dried over anhydrous sodium sulfate. Removal of

the solvent gave an oil which was purified by chromatography. Yield 97%.

(Z-Cys-GlyOMe)$_2$. ^{1}H NMR δ_H (DMSO) 8.50 (1H, t, $J=5.6$), 7.66 (1H, d, $J=8.5$), 7.36 (5H, m), 5.05 (2H, s), 4.36 (1H, m), 3.85 (2H, d), 3.58 (3H, s), 3.14 and 2.85 (2H, m, $J=4.0$, $J=10.1$, $J=13.3$). ^{13}C NMR δ_C (DMSO) 170.8, 170.1, 156.1, 136.9, 128.4, 127.8, 127.7, 65.6, 53.6, 51.7, 40.8, 38.6. IR (nujol) ν 3303, 1740, 1687 and 1659 cm^{-1}. ES MS $[M+Cl]^-$ m/z 685.

(Z-Cys-GlyOH)$_2$. ^{1}H NMR δ_H (DMSO) 8.31 (1H, t, $J=5.9$), 7.63 (1H, d, $J=8.5$), 7.36 (5H, m), 5.05 (2H, s), 4.36 (1H, m), 3.76 (2H, d), 3.16 and 2.85 (2H, m). ^{13}C NMR δ_C (DMSO) 170.8, 170.6, 156.1, 136.9, 128.4, 127.7, 127.2, 65.6, 53.6, 40.9, 40.0. IR (nujol) ν 3309, 1729, 1689, 1653 cm^{-1}. ES MS $[M-H]^-$ m/z 621, $[M-312]^-$ m/z 310.

Z-Cys-GlyOH (6). ^{1}H NMR δ_H (DMSO) 12.63 (1H, s), 8.34 (1H, t), 7.51 (1H, d), 7.36 (5H, m), 5.05 (2H, s), 4.18 (1H, m), 3.77 (2H, m), 2.83 and 2.67 (2H, m), 2.37 (1H, t, $J=8.5$). ^{13}C NMR δ_C (DMSO) 171.3, 170.7, 156.2, 137.1, 128.5, 127.9, 127.2, 65.8, 57.4, 41.0, 26.6. IR (nujol) ν 3285, 3215, 2500, 1728, 1686, 1635 cm^{-1}. ES MS $[M-H]^-$ m/z 311.

(Z-L-Cys-DL-AlaOMe)$_2$. ^{1}H NMR δ_H (DMSO) 8.49 (1H, m), 7.60 (1H, m), 7.36 (5H, m), 5.05 (2H, s), 4.29 (2H, m), 3.62 (3H, s), 3.11 and 2.88 (2H, m), 1.27 (3H, d, $J=7.3$). ^{13}C NMR δ_C (DMSO) 172.7, 170.2, 156.1, 136.9, 128.5, 128.4, 128.1, 65.9, 53.6, 51.9, 47.8, 40.1, 17.0, 16.8. IR (nujol) ν 3420, 3333, 1743, 1690, 1651 cm^{-1}. ES MS $[M-H]^-$ m/z 677.

(Z-L-Cys-DL-AlaOH)$_2$. ^{1}H NMR δ_H (DMSO) 8.32 (1H, m), 7.59 (1H, m), 7.35 (5H, m), 5.05 (2H, s), 4.38 (1H, m), 4.23 (1H, m), 3.15 and 2.87 (2H, m), 1.29 (3H, m). ^{13}C NMR δ_C (DMSO) 174.1, 170.2, 156.2, 137.0, 128.5, 128.0, 128., 65.8, 53.8, 47.9, 40.7, 17.5. IR (nujol) ν 3326, 1718, 1669, 1659 cm^{-1}. ES MS $[M-H]^-$ m/z 649.

Z-L-Cys-DL-AlaOH (7). mp 40–44 °C ^{1}H NMR δ_H (DMSO) 8.33 (1H, m), 7.44 (1H, m), 7.34 (5H, m), 5.06 (2H, s), 4.26 (2H, m), 2.73 (2H, m), 2.25 (1H, m), 1.29 (3H, m). ^{13}C NMR δ_C (DMSO) 174.4, 170.3, 156.2, 137.2, 128.7, 128.4, 128.0, 66.0, 57.3, 48.1, 27.0, 17.7. IR (nujol) ν 3413, 3322, 2571, 1719, 1671, 1659 cm^{-1}. ES MS $[M-H]^-$ m/z 325.

(Z-L-Cys-DL-ValOMe)$_2$. ^{1}H NMR δ_H (DMSO) 8.30 (1H, m), 7.60 (1H, m), 7.35 (5H, m), 5.05 (2H, s), 4.40 (1H, m), 4.21 (1H, m), 3.64 (3H, s), 3.09 and 2.90 (2H, m), 2.05 (1H, m), 0.86, 0.85 (6H, d, $J=4.6$). ^{13}C NMR δ_C (DMSO) 171.8, 170.5, 155.9, 136.9, 128.4, 127.8, 127.6, 65.6, 57.5, 53.7, 51.8, 40.5, 30.1, 29.9, 19.0, 18.1. IR (nujol) ν 3303, 1739, 1693, 1656 cm^{-1}. ES MS $[M-H]^-$ m/z 733.

(Z-L-Cys-DL-ValOH)$_2$. ^{1}H NMR δ_H (DMSO) 8.17 (1H, m), 7.65 (1H, m), 7.35 (5H, m), 5.05 (2H, s), 4.43 (1H, m), 4.17 (1H, m), 3.13, 2.90 (2H, m), 2.06 (1H, m), 0.86 (6H, d). ^{13}C NMR δ_C (DMSO) 172.7, 170.3, 155.9, 136.9, 128.4, 127.7, 127.1, 65.6, 57.2, 53.9, 40.6, 30.1, 19.2, 17.9. IR (nujol) ν 3309, 1715, 1661 cm^{-1}.

Z-L-Cys-DL-ValOH (8). ^{1}H NMR δ_H (DMSO) 8.15 (1H, m), 7.60 (1H, m), 7.35 (5H, m), 5.05 (2H, s), 4.19 (2H, m), 2.80 (2H, m), 2.05 (1H, m), 0.86 (6H, d). ^{13}C NMR δ_C (DMSO) 172.9, 170.5, 156.1, 137.1, 128.5, 128.2, 127.9, 65.7, 57.2, 30.2, 26.9, 19.2 18.0. IR (nujol) ν 3317, 2625, 1718, 1668 cm^{-1}. ES MS $[M-H]^-$ m/z 353. Mp 37–40 °C.

(Z-L-Cys-L-LeuOMe)$_2$. ^{1}H NMR δ_H (DMSO) 8.39 (1H, m), 7.62 (1H, m), 7.35 (5H, m), 5.05 (2H, s), 4.33 (2H, m), 3.61 (3H, s), 3.13 and 2.89 (2H, m), 1.55 (3H, m), 0.85 (6H, m). ^{13}C NMR δ_C (DMSO) 172.7, 170.4, 156.0, 136.9, 128.3, 127.7, 127.2, 65.6, 53.7, 51.9, 50.4, 40.4, 39.5, 24.2, 22.8, 21.3. IR (nujol) ν 3266, 1746, 1703, 1655 cm^{-1}.

(Z-L-Cys-L-LeuOH)$_2$. ^{1}H NMR δ_H (DMSO) 8.20 (1H, d, $J=7.9$), 7.60 (1H, d, $J=8.1$), 7.35 (5H, m), 5.05 (2H, s), 4.34 (1H, m), 4.23 (1H, m), 3.13 and 2.87 (2H, m), 1.59 (3H, m), 0.86 (6H, m); ^{13}C NMR δ_C (DMSO) 173.7, 170.2, 156.0, 136.9, 128.4, 128.0, 127.8, 65.6, 53.8, 50.5, 40.1, 39.8, 24.3, 22.9 and 21.4; IR (nujol) ν 3312, 1693, 1655 cm^{-1}.

Z-L-Cys-L-LeuOH (9). ^{1}H NMR δ_H (DMSO) 8.22 (1H, m), 7.53 (1H, m), 7.35 (5H, m), 5.05 (2H, s), 4.25 (2H, m), 2.85 (2H, m), 1.58 (3H, m), 0.88 (6H, m); ^{13}C NMR δ_C (DMSO) 174.9, 170.2, 156.0, 136.9, 128.4, 127.8, 127.7, 65.6, 57.18, 50.5, 40.1, 26.45, 24.5, 22.9 and 21.4; IR (nujol) ν 3308, 2503, 1714, 1661 cm^{-1}; ES MS $[M-H]^-$ m/z 361.

(Z-L-Cys-DL-SerOMe)$_2$, (Z-DL-Cys-L-SerOMe)$_2$. ^{1}H NMR δ_H (DMSO) 8.30 (1H, m), 7.65 (1H, m), 7.36 (5H, m), 5.06 (2H, s), 4.42 (2H, m), 3.72 (2H, m), 3.63 (3H, s), 3.15 and 2.88 (2H,m); ^{13}C NMR δ_C (DMSO) 170.8, 170.5, 156.1, 136.9, 128.4, 128.1, 127.8, 65.6, 61.2, 54.9, 53.7, 52.0, 40.5; IR (nujol) ν 3505, 1738, 1700, 1651 cm^{-1}; ES MS $[M-H]^-$ m/z 709.

(Z-L-Cys-DL-SerOH)$_2$, (Z-DL-Cys-L-SerOH)$_2$. ^{1}H NMR δ_H (DMSO) 8.10 (1H, m), 7.65 (1H, m), 7.35 (5H, m), 5.05 (2H, s), 4.42 (1H, m), 4.28 (1H, m), 3.67 (2H, m), 3.16 and 2.86 (2H, m); ^{13}C NMR δ_C (DMSO) 171.8, 170.3, 156.2, 136.9, 128.5, 127.9, 127), 65.7, 61.4, 54.9, 53.8, 40.5; IR (nujol) ν 3298, 1718, 1703 and 1650 cm^{-1}; ES MS $[M-H]^-$ m/z 681.

Z-L-Cys-DL-SerOH, Z-DL-Cys-L-SerOH (10). ^{1}H NMR δ_H (DMSO) 8.10 (1H, m), 7.53 (1H, m), 7.37 (5H, m), 5.06 (2H), 4.30 (2H, m), 3.68 (2H, m), 3.15 and 2.87 (2H, m), 2.29 (1H, m); ^{13}C NMR δ_C (DMSO) 171.8, 171.7, 170.2, 170.1, 156.0, 136.9, 128.4, 127.8, 127.7, 65.6, 61.4, 61.2, 57.5, 55.2, 55.1, 26.6; IR (nujol) ν 3301, 2609, 1718, 1663; ES MS $[M-H]^-$ m/z 341.

(Z-D-Cys-D-PenicillamineOMe)$_2$. ^{1}H NMR δ_H (DMSO) 8.25 (1H, m), 7.60 (1H, m), 7.35 (5H, m), 5.04 (2H, s), 4.50 (2H, m), 3.63 (3H, s), 3.04 and 2.73 (2H, m), 1.33 (6H, m); ^{13}C NMR δ_C (DMSO) 170.5, 169.9, 156.0,

136.9, 128.4, 127.9, 127.7, 65.6, 61.5, 54.9, 51.9, 45.21, 40.4, 29.5 and 24.19; IR (nujol) ν 3324, 1734, 1675 cm^{-1}; ES MS $[M+Cl]^-$ m/z 835.

(Z-D-Cys-D-PenicillamineOH)$_2$. ^{1}H NMR δ_H (DMSO) 8.20 (1H, m), 7.57 (1H, m), 7.35 (5H, m), 5.04 (2H, s), 4.67 (1H, m), 4.34 (1H, m), 3.63 (3H, s), 2.89 (2H, m), 1.35 (6H, m); ^{13}C NMR δ_C (DMSO) 171.4, 170.2, 155., 136.9, 128.4, 128.1, 127.9, 65.6, 59.4, 53.0, 45.21, 40.2, 26.8 and 19.7; IR (nujol) ν 3416, 2500, 1719, 1671 cm^{-1}.

Z-D-Cys-D-PenicillamineOH (11). ^{1}H NMR δ_H ($CDCl_3$) 8.31 (1H, d), 7.35 (6H, m), 5.11 (2H, s), 4.82 (1H, m), 4.68 (1H, m), 2.96 (2H, m), 1.41 (6H, m); ^{13}C NMR δ_C ($CDCl_3$) 173.1, 171.8, 155.2, 135.9, 128.5, 128.3, 128.1, 67.2, 59.5, 53.6, 57.0, 41.6, 26.7 and 19.8; IR (nujol) ν 3303, 2570, 1719, 1669 cm^{-1}; ES MS $[M-3H]^-$ m/z 383.

(Z-L-Cys-DL-PheOMe)$_2$. ^{1}H NMR δ_H (DMSO) 8.50 (1H, m), 7.60 (1H, m), 7.35 (5H, m), 7.24 (5H, m), 5.05 (2H, s), 4.52 (1H, m), 4.34 (1H, m), 3.61 (3H, m), 2.87 (4H, m); ^{13}C NMR δ_C (DMSO) 171.6, 170.2, 155.9, 137.0, 136.9, 129.2, 129.1, 127.8, 127.0, 126.6, 65.6, 53.7 and 53.5, 52.0 and 51.9, 40.4, 40.0, 36.8 and 36.5; IR (nujol) ν 3321, 1743, 1717 and 1669 cm^{-1}.

(Z-L-Cys-L-PheOMe)$_2$, (Z-D-Cys-D-PheOMe)$_2$. ^{1}H NMR δ_H (DMSO) 8.45 (1H, d, $J=7.9$), 7.62 (1H, d, $J=8.5$), 7.34 (5H, m), 7.21 (5H, m), 5.06 (2H, s), 4.53 (1H, m), 4.39 (1H, m), 3.59 (3H, s), 3.05 and 2.85 (4H, m); ^{13}C NMR δ_C (DMSO) 171.7, 170.4, 156.0, 137.0, 136.9, 129.2, 128.4, 128.3, 127.9, 127.8, 127.7, 65.8, 53.8, 52.0, 40.4, 39.5.

(Z-L-Cys-D-PheOMe)$_2$, (Z-D-Cys-L-PheOMe)$_2$. ^{1}H NMR δ_H (DMSO) 8.51 (1H, d, $J=7.9$), 7.56 (1H, d, $J=8.5$), 7.34 (5H, m), 7.21 (5H, m), 5.04 (2H, s), 4.52 (1H, m), 4.33 (1H, m), 3.62 (3H, s), 3.00 and 2.75 (4H, m); C NMR δ_C (DMSO) 171.7, 170.3, 156.0, 137.0, 136.9, 129.3, 128.4, 128.3, 127.9, 127.8, 127.7, 65.7, 53.6, 52.0, 39.9, 36.8.

(Z-L-Cys-DL-PheOH)$_2$. ^{1}H NMR δ_H (DMSO) 8.28 (1H, m), 7.53 (1H, m), 7.35 (5H, m), 7.21 (5H, m), 5.03 (2H, s), 4.50 (1H, m), 4.32 (1H, m), 2.90 (4H, m); ^{13}C NMR δ_C (DMSO) 172.6, 170.1, 170.0, 156.0, 137.3, 136.9, 129.3, 129.2, 128.4, 128.2, 127.9, 127.7, 127.1, 126.5, 65.7, 53.8, 53.6, 53.4, 40.0, 36.9 and 36.6; IR (nujol) ν 3308, 1712, 1651 cm^{-1}; ES MS $[M-H]^-$ m/z 801.

(Z-L-Cys-L-PheOH)$_2$, (Z-D-Cys-D-PheOH)$_2$. ^{1}H NMR δ_H (DMSO) 8.13 (1H, d, $J=7.8$), 7.54 (1H, d, $J=8.7$), 7.32 (5H, m,), 7.22 (5H, m), 5.04 (2H, s), 4.44 (1H, m), 4.31 (1H, m), 3.06 and 2.88 (4H, m); ^{13}C NMR δ_C (DMSO) 172.4, 169.9, 155.9, 137.2, 136.8, 129.1, 128.3, 128.1, 127.7, 127.6, 126.4, 65.6, 53.8, 53.4, 40.4, 36.5.

(Z-L-Cys-D-PheOH)$_2$, (Z-D-Cys-L-PheOH)$_2$. ^{1}H NMR δ_H (DMSO) 8.21 (1H, d, $J=8.2$), 7.48 (1H, d, $J=8.7$), 7.32 (5H, m), 7.20 (5H, m), 5.04 (2H, s), 4.48 (1H, m), 4.32 (1H, m), 3.00 and 2.74 (4H, m); ^{13}C NMR δ_C (DMSO) 172.5, 169.9, 155.9, 137.2, 136.8, 129.2, 128.3, 128.1, 127.8, 127.6, 126.4, 65.6, 53.7, 53.3, 39.9, 36.8

Z-L-Cys-DL-PheOH (12). ^{1}H NMR δ_H (DMSO) 8.28 (1H, m), 7.53 (1H, m), 7.35 (5H, m), 7.21 (5H, m,), 5.04 (2H, s), 4.50 (1H, m), 4.19 (1H, m), 2.90 (4H, m); ^{13}C NMR δ_C (DMSO) 173.0, 170.4 and 170.1, 156.3, 137.7, 137.3, 129.6, 128.7, 128.2, 128.0, 126.8, 126, 66.0, 57.5, 57.4, 53.9, 53.0, 37.3, 37.0, 26.8 and 26.7; IR (nujol) ν 3314, 2570, 1719, 1669 cm^{-1}; ES MS $[M-H]^-$ m/z 401

Z-L-Cys-L-PheOH, Z-D-Cys-D-PheOH. ^{1}H NMR δ_H (DMSO) 8.21 (1H, d, $J=7.7$), 7.36 (6H, m), 7.23 (5H, m), 5.04 (2H, s), 4.44 (1H, m), 4.14 (1H, m), 3.00, 2.68 (4H, m), 2.25 (1H, m); ^{13}C NMR δ_C (DMSO) 172.6, 170.0, 155.9, 137.3, 136.9, 129.1, 128.3, 128.1, 127.8, 127.7, 126.4, 65.5, 57.1, 53.5, 36.5, 26.3; IR (Z-L-Cys-L-Phe-OH) (nujol) ν 3312, 2569, 1717, 1692 and 1658 cm^{-1}; IR (Z-D-Cys-D-Phe-OH) (nujol) ν 3314, 2567, 1715, 1693 and 1658 cm^{-1}; ES MS $[M-H]^-$ m/z 401; mp 143–146 °C.

Z-L-Cys-D-PheOH, Z-D-Cys-L-PheOH. ^{1}H NMR δ_H (DMSO) 8.28 (1H, d, $J=8.2$), 7.34 (6H, m), 7.23 (5H, m), 5.04 (2H, s), 4.49 (1H, m), 4.15 (1H, m), 2.99 and 2.52 (4H, m), 2.03 (1H, m); ^{13}C NMR δ_C (DMSO) 172.3, 169.7, 155.9, 137.3, 136.9, 129.2, 128.4, 128.2, 127.9, 127.8, 127.7, 65.76, 57.0, 53.3, 36.9, 26.5; IR (nujol) ν 3308, 2568, 1718, 1669 cm^{-1}; ES MS $[M-H]^-$ m/z 401; mp 120–122 °C.

Z-L-Cys-DL-ProOH (13). ^{1}H NMR δ_H (DMSO) 7.64 (1H, m), 7.34 (5H, m), 7.21 (5H, m), 5.05 (2H, m), 4.22 and 3.66 (2H, m), 2.88, 2.70, 2.12 and 1.88 (8H, m); ^{13}C NMR δ_C (DMSO) 173.5, 173.4, 169.1, 156.2, 137.3, 127.7, 128.4, 128.1, 127.9, 65.9, 60.7, 57.2, 56.9, 46.9, 29.1, 25.7, 24.4, 24.7; IR (nujol) ν 3295, 2606, 1716, 1630 cm^{-1}; ES MS $[M-H]^-$ m/z 351; mp 60–65 °C.

Kinetic studies

Benzylpenicillin, cefuroxime and cephaloridine were gifts from GlaxoWellcome. Deionised water was used for preparation of buffers and other aqueous solutions. The buffers were acetate (pK_a 4.75), MES (pK_a 6.15), MOPS (pK_a 7.20), TAPS (pK_a 8.40), CAPSO (pK_a 9.60), hexafluoroisopropanol (pK_a 9.30) and CAPS (pK_a 10.4). The ionic strength was maintained at a constant level with sodium chloride or potassium chloride (AnalaR grade). Kinetic studies were carried out using the class B β-lactamase enzyme from *Bacillus cereus* 569/H which was isolated using the previously described procedure.[18] UV–Vis wavelength scans were performed using a Cary 1/3 (Varian) spectrophotometer. The temperature was kept constant at 30 °C with a water circulator and a peltier system. The pHs of solutions were measured using either a JENWAY 3020 or a Beckman Φ40 pH meter fitted with Ag/AgCl, orHg/Hg_2Cl_2 Russell semi-micro electrodes, which were calibrated prior to use.

Generally, 25 μl of a stock solution of substrate was injected into 2.5 mL of buffer solution (0.05 M, I = 1.0 M, 30 °C) and stirred. 25 μl of a stock solution of enzyme was then added to the system. Usually, the substrate and enzyme concentrations in the cell were 0.12 to 1.1 mM and 0.04–2.0 μM respectively. The experiment was repe-

ated by adding more substrate at the end of the reaction. Hydrolysis of the substrate was followed by measuring the decrease in absorbance, at 260 nm for cephaloridine and cefuroxime, and at 230 nm for benzylpenicillin, as a function of time. Enzyme catalysed reactions followed Michaelis–Menten kinetics, but the kinetic constants were generally measured by following the entire course of the reaction. Complete time curves were studied because of increased accuracy and there was no evidence of either product inhibition or enzyme denaturation. Repeat kinetics runs, performed by injecting more substrate into the cell after the first reaction was complete, gave identical rate constants. Furthermore, rate constants obtained from initial rate studies also gave identical rate constants. The rate constants have an estimated error of ±3%. Curve fittings were achieved using ENZFITTER (Elsevier Biosoft, Cambridge, UK) or SCIENTIST (MicroMath Scientific Software, Utah, USA) software.

The zinc ion content of the β-lactamase II stock solution was determined by atomic absorption spectrometry on a Perkin Elmer AAnalyst 100 Atomic Absorption Spectrometer. The hollow cathode lamp wavelength was set at 213.9 nm, with a current of 7 mA, and a slit width of 0.7 nm. Zinc nitrate solutions of different concentrations were used as standards.

Inhibition studies

Values of K_i were determined by the effect of varying concentrations of the thiol on the value of the second-order rate constant k_{cat}/K_m for the hydrolysis of two substrates — benzylpenicillin and cephaloridine — using Eq. (1).

$$\left(\frac{k_{cat}}{K_m}\right)_I = \left(\frac{k_{cat}}{K_m}\right)_O \cdot \left(\frac{K_i}{[I] + K_i}\right) \quad (1)$$

where $(k_{cat}/K_m)_I$ and $(k_{cat}/K_m)_O$ are the observed second-order rate constants for hydrolysis of cephaloridine or benzylpenicillin in the presence or absence, respectively, of a given concentration of the inhibitor, I, and K_i is the dissociation constant of the enzyme–inhibitor complex. The steady-state kinetics were obtained using concentrations of the thiol inhibitors ranging from 1 to 10^3 μM and similar values of K_i were obtained whether cephaloridine or benzylpenicillin was used as a substrate to monitor enzyme activity.

The stock solutions of the inhibitors were prepared in ethanol. Unless otherwise stated, 25 μl of solution of inhibitor at a given concentration were added to 2.5 mL of buffer containing the substrate. The substrate was shown to be stable in the presence of inhibitor. The hydrolysis of the substrate was initiated by the addition of 25 μl of enzyme solution and enzyme activity was measured as described in the previous section by following the complete time course of the reaction. The time dependence of the inhibition was studied either by incubating the enzyme with inhibitor for different times before adding the substrate and by adding more substrate at the end of the hydrolysis reaction. Zinc sulfate was also added to the mixture in order to investigate metal chelating reactions, and restoration of the enzyme activity.

The kinetic parameters were determined from the second-order rate constant k_{cat}/K_m. Inhibition constants K_i were calculated using the equation for competitive inhibition:

$$\text{Rate} = \frac{k_{cat} \cdot [E] \cdot [S]}{[S] + K_m\left(\frac{K_i + [I]}{K_i}\right)}$$

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