Mechanistic Insights into the Inhibition of Serine Proteases by Monocyclic Lactams[†,‡]

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ABSTRACT: Although originally discovered as inhibitors of pencillin-binding proteins, β-lactams have more recently found utility as serine protease inhibitors. Indeed through their ability to react irreversibly with nucleophilic serine residues they have proved extraordinarily successful as enzyme inhibitors. Consequently there has been much speculation as to the reason for the general effectiveness of β-lactams as antibacterials or inhibitors of hydrolytic enzymes. The interaction of analogous β- and γ-lactams with a serine protease was investigated. Three series of γ-lactams based upon monocyclic β-lactam inhibitors of elastase [Firestone, R. A. et al. (1990) *Tetrahedron 46*, 2255−2262.] but with an extra methylene group inserted between three of the bonds in the ring were synthesized. Their interaction with porcine pancreatic elastase and their efficacy as inhibitors were evaluated through the use of kinetic, NMR, mass spectrometric, and X-ray crystallographic analyses. The first series, with the methylene group inserted between C-3 and C-4 of the β-lactam template, were readily hydrolyzed but were inactive or very weakly active as inhibitors. The second series, with the methylene group between C-4 and the nitrogen of the β-lactam template, were inhibitory and reacted reversibly with PPE to form acyl-enzyme complexes, which were stable with respect to hydrolysis. The third series, with the methylene group inserted between C-2 and C-3, were not hydrolyzed and were not inhibitors consistent with lack of binding to PPE. Comparison of the crystal structure of the acyl-enzyme complex formed between PPE and a second series γ-lactam and that formed between PPE and a peptide [Wilmouth, R. C., et al. (1997) *Nat. Struct. Biol. 4*, 456−462.] reveals why the complexes formed with this series were resistant to hydrolysis and suggests ways in which stable acyl-enzyme complexes might be obtained from monocyclic γ-lactam-based inhibitors.

The β-lactam antibiotics are among the most successful therapeutic agents yet discovered. They exert their biological activity by acylation of the nucleophilic serinyl residue at the active site of enzymes involved in bacterial cell wall biosynthesis (penicillin-binding proteins, PBPs[1]) (*1*). Following from pioneering studies by the Merck group, they have also shown utility as inhibitors of serine proteases, most notably human elastase (*2*, *3*). Like the penicillins, which were the first β-lactam antibiotics to be discovered, almost all medicinally used β-lactams are based upon naturally occurring core structures, the subfamilies of which exhibit significant structural diversity and include the bicyclic β-lactams, such as the penicillins, cephalosporins, carbapenems, clavams, and monocyclic β-lactams such as the norcardicins (Figure 1). The first β-lactam inhibitors of elastase were also based upon natural compounds, such as the clavams and cephalosporins (*4*). More recently, synthetic monocyclic β-lactams have been developed as inhibitors of other proteases.

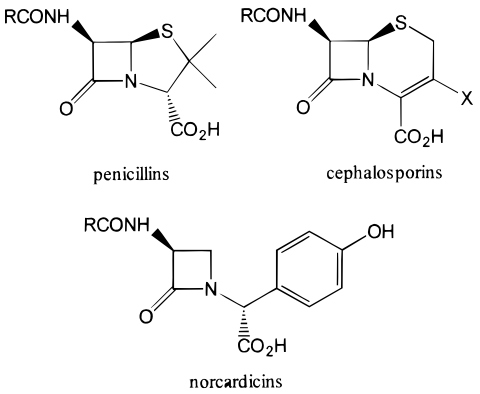

FIGURE 1

There has been much speculation and some experimentation aimed at defining the reasons for the apparent general effectiveness of β-lactams as antibacterials or as inhibitors of "serine" (trans)peptidases/hydrolases [for a review, see ref *5*]. The subject remains somewhat unclear, but it is clear that any "special" properties of β-lactams are not directly

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[‡] Coordinates for the structure described in this paper have been deposited with the Brookhaven Protein Data Bank (accession number 1QGF).

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[1] Abbreviations: BCM7, β-casomorphin-7; ESIMS, electrospray ionization mass spectrometry; HLE, human leukocyte elastase; PBP, penicillin-binding protein; PNBS, *para*-nitrobenzenesulphonyl; PPE, porcine pancreatic elastase; PTFMBS, *para*-trifluoromethylbenzenesulphonyl; Wat, water.

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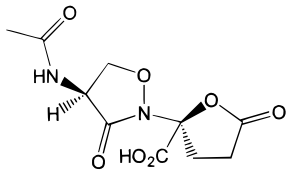
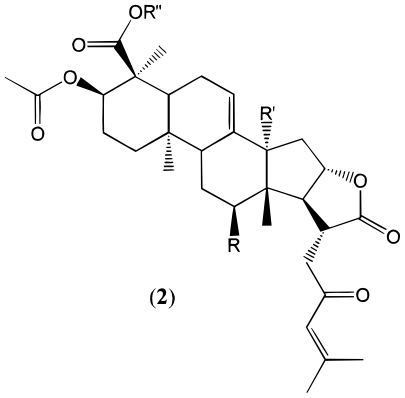
related to hydrolytic stability or to lack of resonance in the β-lactam amide (*5*, *6*).

In contrast to the range of naturally occurring β-lactam antibiotics, relatively few larger ring lactam antibiotics binding to PBPs have been identified, from either naturally occurring or synthetic sources. While it may be argued that the latter reflects a prejudice of the medicinal chemist in favor of the four-membered ring, the lack of naturally occurring γ-, δ-, etc. antibiotics isolated is notable. The lactivicins (**1**), which are based upon a cycloserine nucleus,



(**1**)

are the only isolated naturally occurring γ-lactams which demonstrate their antibacterial activity by interaction with PBPs (*7*). It is also noteworthy that, a *trans*-5,5-substituted γ-lactone (**2**) has been isolated recently from the plant



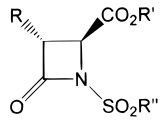
(**2**)

Lantana camara which inhibits human α-thrombin via acylation of the active site serine (*8*).

Stimulated by the problem of β-lactamase-mediated bacterial resistance to β-lactams, efforts have been directed toward the preparation of compounds that inhibit PBPs, but which do not contain a β-lactam ring [for a review, see ref *9*]. Although no clinically used compound has resulted from this work, new antibacterials have been synthesized, including γ-lactam rings fused to unsaturated ring systems (*10*−*13*).

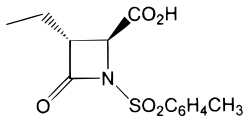
The release of ~25 kcal of ring strain energy during ring opening of a β-lactam by an alcohol renders reformation of the β-lactam energetically unfavorable (*6*), but in the case of most γ-lactams the ring strain is small. Consequently, we speculated that the relative lack of activity of γ-lactams as inhibitors of "serine" enzymes might be due partly to an unfavorable equilibrium position between the free γ-lactam and the acyl-enzyme (E/I) complex (*16*). We are interested in exploring the relative efficiency of analogous β- and larger ring lactams as inhibitors of "serine" enzymes, with the objectives of defining the special nature, if it exists, of the β-lactam and developing new antibacterials/inhibitors. We have chosen to work with the serine protease porcine pancreatic elastase (PPE), since it has been well studied from a structural perspective, is readily available, and has been demonstrated to be inhibited by simple monocyclic β-lactams, in which the only covalent modification is acylation of the active site serine[2] (*2*, *3*, *14*).

The inhibition of elastases by β-lactams has been studied in detail. In the case of the monocyclic β-lactam (**3**), an initial



(**3**)

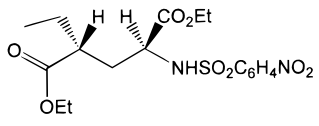
reaction occurs to form an acyl-enzyme ester complex, which then partitions between a conformational change to form a long-lived enzyme/inhibitor (E/I) complex or hydrolysis to give a β-amino acid (*15*). Comparison of X-ray crystallographic studies on the E/I complex formed from reaction of **4** with the productive conformation adopted in peptidic



(**4**)

acyl-enzyme complex (albeit at pH 5) has revealed the nature of the conformational change (*14*). It was proposed that the β-lactam initially reacts to form a hydrolytically labile acyl-enzyme complex mimicking that formed with a peptide, in which the ester carbonyl is located within the oxyanion hole. However, in the case of the β-lactam-derived complex, rotation of the ester carbonyl out of the oxyanion hole and displacement of the hydrolytic water gives rise to a hydrolytically stable complex (Scheme 1).

To test the reversibility proposal we have synthesized γ-lactam analogues based upon the β-lactam inhibitor (**3**). In a preliminary report we described the interaction between PPE and the γ-lactam series in which a methylene was 'inserted' between the C-2 and C-3 carbons of **3**, to give, for example, **6** (*16*). In this series the positioning of the additional methylene ensures the C-3 alkyl substituent was adjacent to the lactam carbonyl, allowing interaction with the S_1 subsite in the PPE active site. Incubation in phosphate buffer (pD 7.4) demonstrated that **6** was hydrolyzed very slowly in the absence of PPE, with <5% reaction being observed after 12 h (by 500 MHz ^{1}H NMR analysis). In the presence of PPE, the rate of hydrolysis was increased with complete conversion to the γ-amino acid occurring within 3.5 h. PPE was also shown to catalyze the cyclization of the γ-amino ester (**5**) to **6**, thereby indicating that **6** reacts

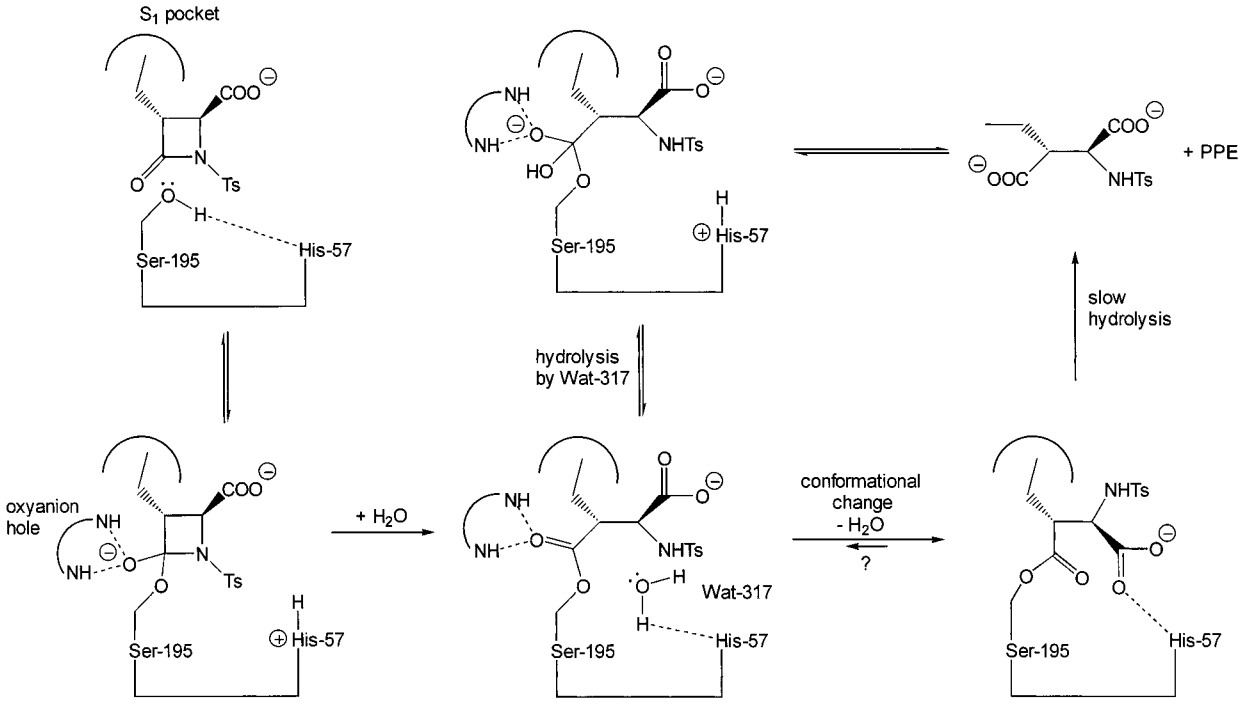


(**5**)

reversibly with PPE. Further NMR experiments showed that this reaction was mediated via an acyl-enzyme complex. Kinetic studies demonstrated that **6** was an inhibitor of

[2] The inhibition of elastase by some other β-lactams involves a "double-hit" mechanism (*3*, *28*).

Scheme 1



neither PPE nor HLE (human leukocyte elastase) up to its solubility limit in the assay system (0.2 mM). This lack of inhibition implied that the initially formed acyl-enzyme complex does not undergo conformational conversion to a hydrolytically stable complex as proposed for the β-lactam inhibitors.

To investigate the interaction of analogous β- and γ-lactams with elastases, we synthesized two further series of γ-lactam analogues in which a methylene was inserted between C-2 and the lactam nitrogen (series 2), and between C-1 and C-2 (series 3) of **1** (Figure 2). Herein, we report NMR, ESIMS, kinetic, and crystallographic studies on the interaction with PPE of these compounds and further compounds in the first series. The results demonstrate why some γ-lactams react reversibly with PPE, but also suggest ways in which the efficient inhibition of serine enzymes by monocyclic γ-lactams might be achieved.

EXPERIMENTAL PROCEDURES

Electrospray Ionization Mass Spectrometry. Electrospray ionization mass spectra were recorded on a VG Bio-Q triple quadrupole atmospheric pressure mass spectrometer equipped with an electrospray interface. Samples (10 μL) were introduced into the electrospray source via a loop injector as a solution with a final protein concentration of typically 20 pmol μL^{-1} in water/acetonitrile (1:1 v/v) containing 1% (v/v) formic acid. Mass spectra typically consisted of 15 10-second scans over the range 700−1900 M_r. Mass spectra were acquired using a cone voltage of 50 V and a source temperature of 50 °C. The instrument was calibrated with horse heart myoglobin or bovine ubiquitin.

NMR Experimental Procedures. NMR experiments were performed at 499.98 MHz on a Bruker AMX500 instrument equipped with a 3 mm microprobe. Sample volumes were 130−150 μL, and the sample temperature was regulated at 303 K. All spectra were referenced to internal MeCN at 2.05 ppm. The phosphate buffer was prepared by dissolving Na_2DPO_4 (1.107 g) and NaD_2PO_4 (276 mg) in D_2O (100 mL).

Enzyme Assays. Enzyme assays were performed using a Shimadzu 1601PC spectrophotometer equipped with a thermostated multicell transport system. The hydrolysis of the *para*-nitroanilide substrate (Suc-AlaAlaProAla-pNa) was measured at 405 nm and at a constant temperature of 25 °C in 0.1 M Tris-HCl buffer (pH 7.5) (*17*). Duplicate to quadruplicate measurements of the initial rate were taken at five or six substrate concentrations between approximately a third and six times K_m. Two or three inhibitor concentrations were used. Where solubility allowed, inhibitor concentrations below, at, and above the K_i were used. Data were analyzed using standard kinetic equations programmed into Excel (Microsoft Corp.) and Grafit (Erithacus Inc.).

Synthesis of γ-Lactams. Synthesis of the first series of γ-lactams was carried using previously reported protocols (*16*). Details of the synthesis of the second and third series of γ-lactams will be reported elsewhere (*16*, *18*, *19*). The first series of γ-lactams were synthesized as optically active enantiomers, and the second and third series were synthesized as racemates.

Crystal Preparation and Soaking. The PPE crystals were prepared as previously described (*20*). The γ-lactam (**21**) (20 mg mL^{-1}) was dissolved in a solution containing sodium acetate buffer (pH 5.0, 50 mM), sodium sulfate (25 mM), PPE (25 mg mL^{-1}), and DMSO (10% v/v). The mother liquor containing the crystals was exchanged with this solution in a stepwise manner over a 2 h period. After 24 h of soaking in the inhibitor solution, the crystals were placed in a cryoprotectant solution containing 20% (v/v) glycerol and flash frozen in liquid nitrogen.

Data Collection and Processing. Data were collected at 100 K using a Rigaku rotating anode generator (Cu Kα radiation) and 30 cm MAR Research imaging plate to a resolution limit of 1.7 Å. The crystal belonged to the space

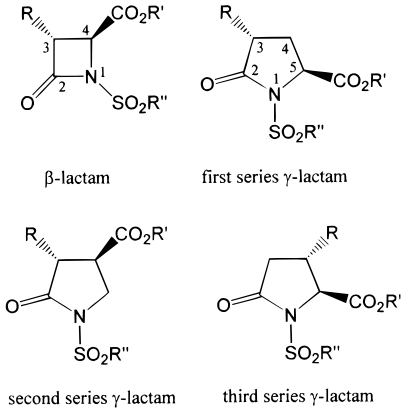


FIGURE 2

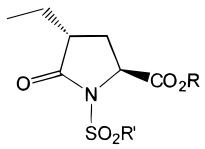
group $P2_12_12_1$ with $a = 50.22$ Å, $b = 57.63$ Å, and $c = 74.48$ Å. Raw data were processed with the programs DENZO and SCALEPACK (*21*). R_{merge} was 3.6% for all data. Data were 97.6% complete [95.2% complete for the highest resolution shell (1.70−1.75 Å)] with 153 319 reflections of which 23 759 were unique after reduction.

Structure Refinement. The 1.65 Å native PPE structure obtained by Meyer et al. (*20*) was used as the starting model. The structure was refined using a combination of XPLOR 3.1 (*22*) and REFMAC [CCP4 suite (*23*)]. A total of 4% of the reflections in the entire dataset were randomly selected in order to provide a test set for the R_{free} calculations (*24*). These reflections were not used during refinement, but were included in the electron density map calculations. Models and electron density maps were displayed with the program O (*25*). At the stage in refinement when the $F_o - F_c$ map showed clear and unambiguous density for the γ-lactam, it was included in the model and appropriate topology files for REFMAC and XPLOR created. The ester bond was simulated solely with bond length restraints. During the latter stages of refinement, 159 water molecules were added along with the bound calcium and sulfate ions (*20*), giving a final *R*-factor of 18.4% and a free *R*-factor of 21.3%. The average *B*-factor for the atoms in the γ-lactam inhibitor molecule was 18.7 Å^2. The rms deviations of the bond lengths and angles in the final structure were 0.015 Å and 1.3° respectively. There were no unusual outliers in the Ramachandran plot, and the final $F_o - F_c$ difference map did not demonstrate any large areas of unexplained electron density.

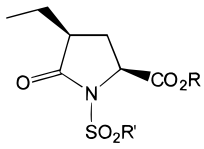
RESULTS AND DISCUSSION

Solution ESIMS and NMR Studies. It was reported that incubation of the γ-lactam (**6**) in phosphate buffer (pD 7.4) in the presence of PPE resulted in complete hydrolysis within 3.5 h (*16*). ^{1}H NMR analysis of the overnight incubation of PPE with **7**, **8**, **9**, and **10** demonstrated that resonances relating to the corresponding ring-opened γ-lactams (**11**, **12**, **13**, **14**) appeared. These increased in intensity with incubation time, at the expense of those corresponding to the intact monocyclic γ-lactams. In addition, a dominant peak at 356 Da was observed upon negative ion ESIMS analysis of the incubation of PPE with **7** in water, corresponding to the formation of **11** (theoretical $M_r = 357.43$ Da). These results implied that the *p*-nitrobenzenesulfonyl activating group in **6** may be replaced by a *p*-toluenesulfonyl group with little

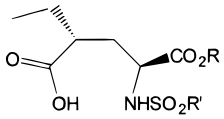
First series:



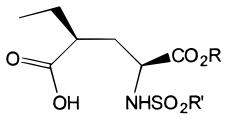
(**6**) R = Et, R' = *p*-$NO_2C_6H_4$
(**7**) R =Et, R' = *p*-$CH_3C_6H_4$
(**8**) R = Bn, R' = *p*-$NO_2C_6H_4$
(**9**) R = Bn, R' = *p*-$CH_3C_6H_4$
(**17**) R = H, R' = *p*-$CH_3C_6H_4$
(**18**) R = H, R' = *p*-$NO_2C_6H_4$



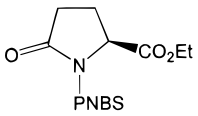
(**10**) R = Et, R' = *p*-$NO_2C_6H_4$



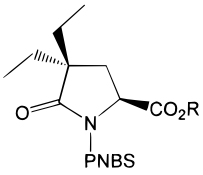
(**11**) R =Et, R' = *p*-$CH_3C_6H_4$
(**12**) R = Bn, R' = *p*-$NO_2C_6H_4$
(**13**) R = Bn, R' = *p*-$CH_3C_6H_4$
(**19**) R = H, R' = *p*-$CH_3C_6H_4$
(**20**) R = H, R' = *p*-$NO_2C_6H_4$



(**14**) R = Et, R' = *p*-$NO_2C_6H_4$



(**15**)



(**16**) R = Et; (**26**) R = H

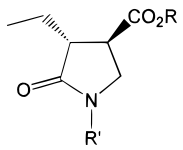
effect on the ability of the nucleophilic serine residue of PPE to react with the monocyclic γ-lactam ring. They also indicated that PPE is able to react with and hydrolyze γ-lactams of this series in which the relative stereochemistry of the C-3 and C-5 substituents was either cis, as in **6**, or trans, as in **10**. The hydrolysis of the benzyl esters (**8**, **9**) to the corresponding ring-opened compounds (**12**, **13**) was confirmed by ESIMS analysis. This demonstrated that the C-5 ester functionality can also be modified and still allow PPE acylation by the γ-lactam ring to occur. Further, the observed (partial) hydrolysis of **15** demonstrated that a C-3 substituent is not essential for PPE-catalyzed hydrolysis of the γ-lactam ring. However, when the C-3 diethyl-substituted γ-lactam (**16**) was incubated with PPE, no hydrolysis was evident, suggesting that the increased steric interactions decreased the stability of the acyl-enzyme complex.

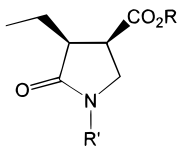
^{1}H NMR analysis of the overnight incubations of the C-5 carboxylic acid containing γ-lactams (**17**, **18**) with PPE in buffer demonstrated that hydrolysis to give **19** and **20**, respectively, in the ratio of 1:10 (ring-opened/intact γ-lactam) had occurred. These results contrasted with those for the incubation of PPE with the C-5 ester containing compounds (**6**, **7**, **10**, and **15**) in which the hydrolyzed products were observed in a much higher ratio (if not exclusively) compared with the starting γ-lactam under the same conditions. Negative ion ESIMS analysis of the incubation of the toluenesulfonyl compound (**17**) with PPE in water failed to

provide evidence in support of the hydrolysis of **17** by PPE to give **19**. This presumably resulted from the lowering in pH of the incubation on addition of the carboxylic acid (**17**), rendering the enzyme inactive. Repetition of the overnight incubation in phosphate buffer in $H_2{}^{18}O$ instead of $D_2{}^{16}O$, acidification of the incubation mixture with 1N HCl, and extraction of the aqueous solution with ethyl acetate gave a mixture of compounds which on negative ion ESIMS analysis demonstrated the formation of singly ^{18}O exchanged **19** (observed peak at 330 Da; theoretical $M_r = 331.37$ Da). This indicates that the hydrolysis of **17** was effectively irreversible under the reaction conditions.

All of the compounds of the second series

Second series (±):





(**21**) R = H, R' = Ts
(**23**) R = Et, R' = Ts
(**24**) R = Me, R' = Ts
(**28**) R = Me, R' = H
(**29**) R = H, R' = PNBS
(**30**) R = H, R' = PTFMBS
(**31**) R = H, R' = SO_2Me

(**22**) R = H, R' = Ts
(**25**) R = Me, R' = Ts
(**27**) R = Me, R' = H

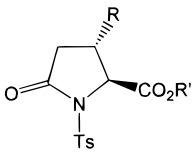
had an ethyl side chain at the C-3 position. ^1H NMR analysis of the C-4 carboxylic acid containing γ-lactams [**21**, trans and **22**, cis] and the C-4 ethyl ester γ-lactam (**23**) demonstrated that they were stable (<5% hydrolyzed) in phosphate buffer in the absence of PPE over a period of a week. Although similar experiments performed on the C-4 methyl ester γ-lactams [trans, **24**, and cis, **25**] provided no evidence for ring opening, some hydrolysis of the methyl ester was observed. About 10% hydrolysis of the *trans*-methyl ester (**24**) was observed overnight with almost complete hydrolysis observed after two weeks. The *cis*-methyl ester (**25**) was hydrolyzed at a slower rate with a trace of methanol being produced over a period of one month.

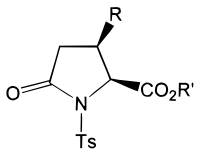
In contrast to the results obtained for the compounds of the first series, there was no change in the NMR spectrum of the racemic trans and cis acid compounds (**21**, **22**) after an overnight incubation with PPE, implying that hydrolysis of the γ-lactam ring had not occurred. Even after a 100 h incubation, no lactam-derived peaks were visible in the ESIMS spectrum apart from the 310 Da signal, corresponding to the MH^- parent ion. NMR analyses of the *trans*- and *cis*-methyl ester compounds (**24**, **25**) after an overnight incubation demonstrated the production of methanol from hydrolysis of the ester functionality. The methanol signal was significantly higher than that observed for the control experiments, implying the involvement of enzyme-mediated catalysis. Doping of the incubation mixture with methanol and the respective carboxylic acid compounds (**21**, **22**) confirmed that all of the observable peaks arose from ester hydrolysis and that no ring opening of the γ-lactam had occurred. Negative ion ESIMS analyses on the incubation mixture at various time points (1, 2, 24, 48, and 96 h) showed a gradual increase in the amount of the ester hydrolyzed product. Complete hydrolysis was observable after 24 h, which was consistent with the ^1H NMR results. Analogous results were obtained for the *trans*-ethyl ester compound (**23**), which demonstrated that the ester functionality was completely hydrolyzed within 1 h. Again, no evidence for ring opening was observed. The trans acid (**21**) MH^- peak at 310 Da remained at 100% for the 24, 48, and 96 h intervals.

In contrast to the monocyclic β-lactam (**3**), where HPLC-ESIMS analysis of tryptic digests of the enzyme/inhibitor complex demonstrated acylation at Ser-195 of a peptide fragement (*14*), no evidence for acylation was observable in a similar experiment conducted with the γ-lactams (**9**) (unpublished data). Although suggestive of it, by itself this observation does not mean that the γ-lactam **9**/enzyme complex is less stable than that of the β-lactam, since it would be anticipated that any simple ester formed from the γ-lactam might be less stable due to the possibility of more favorable intermolecular nucleophilic attack. Thus, deacylation may have occurred during peptide digestion or purification.

Analogous NMR experiments on the third series compounds (**32** and **34**) demonstrated that, like the second series, no hydrolysis was observable after an overnight incubation with PPE. In addition, in the case of **34**, no ethanol production was demonstrated, indicating that the C-5 ester functionality was not hydrolyzed.

Third series (±):





(**32**) R = Et, R' = H
(**34**) R = Et, R' = Et
(**35**) R = H, R' = H
(**36**) R = H, R' = Bn

(**33**) R = Et, R' = H

Kinetic Studies. Kinetic studies on the first series of γ-lactams showed that neither of the compounds with an ethyl ester at the C-5 position (**6**, **7**) were inhibitors of PPE up to their solubility limits. However, the carboxylic acid toluenesulfonyl γ-lactam (**17**) was a very weak inhibitor (the K_i was too high to be accurately measured). The diethyl-substituted PNBS γ-lactam (**26**) was found to be inactive which further demonstrated that these γ-lactams, like their monocyclic β-lactam analogues, are not inhibitors of PPE. Kinetic analysis of the second series of γ-lactams demonstrated that the *trans*- and *cis*-methyl esters of the second series of γ-lactams both with (**24**, **25**) and without (**27**, **28**) a toluenesulfonyl group on the nitrogen atom were inactive. However, the *trans*-carboxylic acid toluenesulfonyl γ-lactam (**21**) was found to be a weak inhibitor ($K_i = 1.32 \pm 0.13$ mM), which indicated that hydrolysis of the ester side chain was insignificant for the duration of the kinetic assay (~5 min.). The *trans*-carboxylic acid compounds with PNBS and PTFMBS N-substituents (**29**, **30**) were more potent inhibitors ($K_i = 0.20 \pm 0.09$ and 0.60 ± 0.03 mM, respectively), but the *N*-methanesulfonyl compound (**31**) was inactive.

All of the third series γ-lactams synthesized had a toluenesulfonyl substituent on the amide nitrogen. For the

Table 1: ESIMS Studies on the Interaction of γ-Lactams with PPE[a]

compound	calcd. mass	PPE	E/I	relative int. (%)[b]	diff.[c]
6	370.1	25898.7	26266.5	4	367.8
7	339.1	25898.1	26239.3	19	341.2
9	401.1	25897.6	26299.8	31	402.2
21	311.1	25898.8	26210.2	13	311.4
22	311.1	25898.1	26209.3	18	311.2
23	339.1	25898.0	26236.6	18	338.5
24	325.1	25898.1	26224.6	43	326.5
25	325.1	25897.7	26223.7	18	326.0

[a] The incubations contained PPE/γ-lactam in a 1:2 ratio for the first series (except for compound **9** which was in a 1:10 ratio) and in a 1:3 ratio for the second series. The incubation times were 5 min except for compounds **7**, **9**, **22**, and **24** where it was 15 min, 17 h, 3 min, and 24 h, respectively. [b] Relative intensity refers to the intensity of the adduct peak normalized to the unmodified enzyme. [c] Mass shift relative to unmodified PPE observed in the same analysis.

compounds with an ethyl group on the C-4 carbon, kinetic analyses showed that, although the *trans*-carboxylic acid compound (**32**) was very weakly active (K_i was too high to be accurately measured), the *cis*-compound (**33**) was inactive (the ethyl ester (**34**) was insoluble in the assay conditions). Both of the compounds without a C-4 substituent (**35**, **36**) were also found to be inactive.

Protein ESIMS Studies. Positive ion ESIMS analyses of incubations of the first series γ-lactams (**6**, **7**, **9**) with PPE gave rise to mass shifts corresponding to the formation of 1:1 complexes between PPE and the respective γ-lactams (Table 1). The intensities of the signals for the complex were low, typically less than 20% of the peak intensity observed for native PPE. It was observed that the intensities of the observed PPE/γ-lactam complexes could be increased by increasing the ratio of γ-lactam used in the incubations, consistent with the presence of an equilibrium between PPE and the γ-lactam. In one case, incubation of PPE with **7** in a ratio of 1:10 (PPE/γ-lactam), the relative intensity of the observed PPE/**7** complex compared to native PPE was 65−70%. A low-intensity peak (of less than 10%) corresponding to formation of a PPE/**7** 1:2 complex was also observed. Increasing the cone voltage from 50 to 110 V resulted in the observation of mass series for native PPE only. At a cone voltage of 90 V, only the E/I complex was observed (not the E/I_2 complex) but at a significantly reduced intensity compared with the studies at a cone voltage of 50 V.

Positive ion electrospray studies on incubations of the second series γ-lactams (**21**, **22**, **23**, **24**, **25**) gave rise to mass shifts corresponding to the formation of both 1:1 and 1:2 complexes. E/I_2 complexes were observed sometimes at low levels (<5% relative intensity) after prolonged incubation (24−100 h). In the case of compound (**22**), when the cone voltage was raised to 70 V, the second adduct was no longer observed, suggesting that it was due to nonspecific (i.e., not active site directed), weak, and probably noncovalent binding. In addition, the 96 h incubation of **24** and the 24 h incubation of **23** give rise to signals that were consistent with adducts between PPE and their respective C4-ester hydrolyzed products. This correlated with the NMR experiments, which demonstrated the production of methanol and ethanol due to hydrolysis of the C4 ester functionality.

The ESIMS studies were consistent with tighter binding of the second series compounds, compared to the first series, in support of the kinetic and crystallographic (vide infra) results. The observation of low levels of adducts consistent with E/I_2 complexes in both series can probably be attributed to relatively weak nonspecific binding in the ESIMS experiments. In light of the crystallographic studies it is tempting to ascribe the differences in the ESIMS experiments between the first and second series as arising from noncovalent and covalent binding, respectively. However, it is possible that a weak covalent interaction (e.g., a tetrahedral complex or readily reversible acyl-enzyme complex) is partly responsible for the E/I adducts observed in the analyses of the first series.

Crystallographic Studies. As the C-4 carboxylic acid compound of the second series (**21**) was one of the monocyclic γ-lactams to produce significant inhibition of PPE, it was chosen for crystallographic study. Native PPE crystals were soaked for 24 h in a high concentration (64 mM) of **21** since the ESIMS studies had suggested that this compound gave rise to the highest concentration of E/I adduct. The soaked crystals were isomorphous to native PPE (*20*), and there was no significant change in unit cell parameters. The resultant 1.7 Å resolution structure (Figures 3 and 4) shows well-defined electron density for all of the atoms in the γ-lactam inhibitor except for the aromatic ring of the *N*-toluenesulfonyl group. The position of the toluenesulfonyl ring was modeled into the structure using the location of the oxygen atoms of the sulfonamide and the electron density visible for the carbon atom in the phenyl ring nearest the sulfur atom as a guide, but the occupancy of the atoms was set to zero. The absolute stereochemistry of the ring-opened γ-lactam bound to PPE is 3*R*,4*S*. This does not prove that only this enantiomer is active, but it is suggestive that the 3*R*,4*S* isomer is more more potent than the 3*S*,4*R* γ-lactam. Thus, the K_i values for the *3R*,4*S* enantiomer are likely to be lower than those reported above for the racemate.

Recently, using a mass spectrometric screening approach, we discovered that a natural heptapeptide, human β-casomorphin-7 (BCM7), forms an unusually stable acyl-enzyme complex with PPE. Comparison of the PPE/BCM7 structure (*26*) with the PPE/γ-lactam complex is interesting. The ring-opened γ-lactam is located within the active site of PPE, and the electron density is consistent with the presence of an ester linkage between O_γ of Ser-195 and the C-2 atom of the γ-lactam. One of the three residues forming the catalytic triad, His-57, is partially disordered in this structure. Although a small amount of discontinuous electron density is visible in the native location, the side chain of His-57 is best fit by a conformation rotated about 90° away from its position in the PPE/BCM7 structure. In this new position, the imidazole ring is positioned between Trp-45 and the phenyl ring of the toluenesulfonyl group of the γ-lactam, with which it may be in position to form a $\pi-\pi$ interaction. However, this is dependent on the rotational angle of the phenyl ring which is unascertainable due to the absence of electron density for this group.

Unlike the structure for the β-lactam compound (**4**) (Figure 5a), the serine-ester carbonyl of the PPE/**21** complex is located in the oxyanion hole in a conformation similar to that observed for it in the PPE/BCM7 structure and is in position to form hydrogen bonds with the amido-nitrogens of Gly-193 (3.22 Å) and Ser-195 (2.76 Å). The ethyl group on C-3 is located in the S_1 subsite. The C-4 carboxylate forms

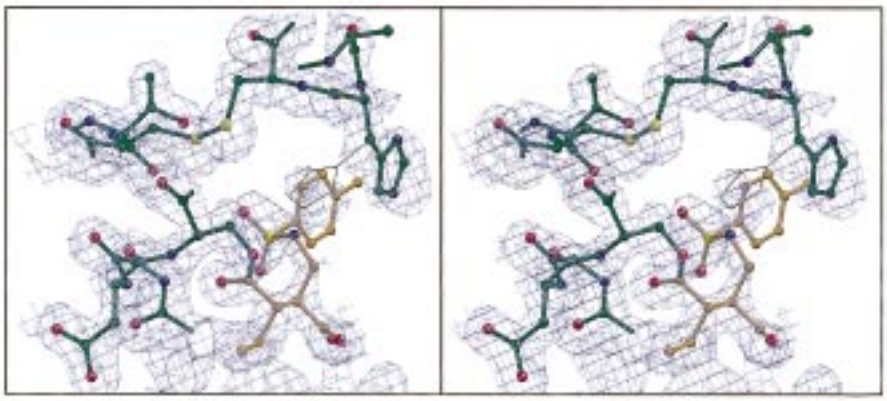


FIGURE 3: A stereoview of the active site of PPE (in green) showing compound **21** (in beige) covalently linked via an ester bond to Ser-195. The phenyl ring of the *p*-toluenesulfonyl group was not included in the refinement. The conformation of His-57 in native PPE is shown in thin lines. The $2F_o - F_c$ electron density map was contoured at 1σ.

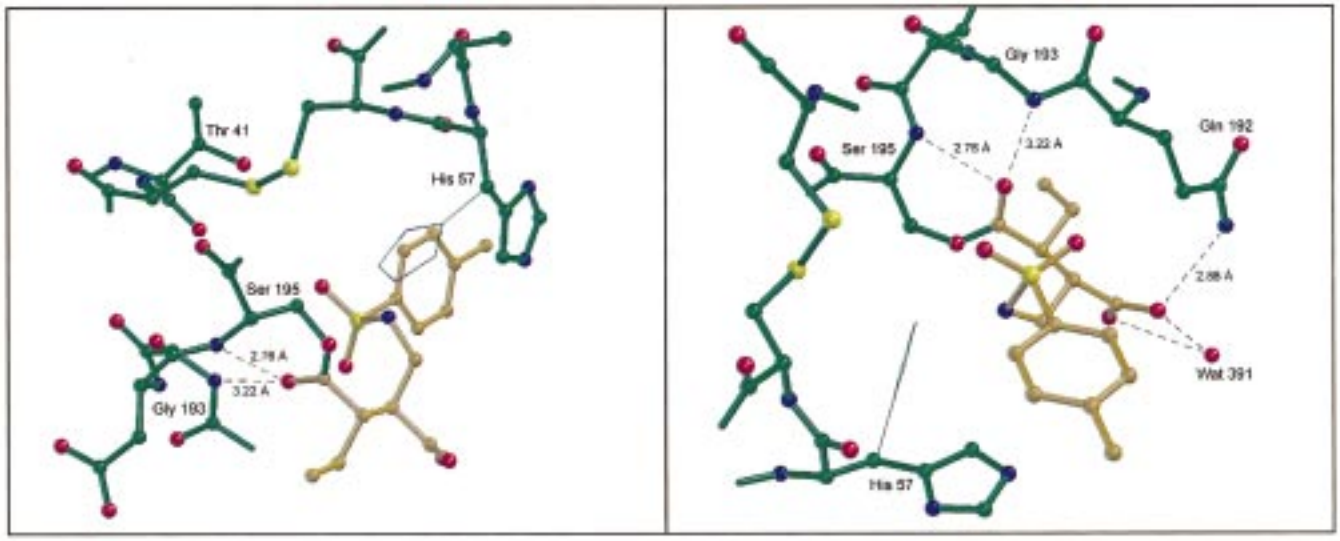


FIGURE 4: Two annotated views of the active site of PPE (in green) with the γ-lactam inhibitor shown in beige. As before, the conformation of His-57 in native PPE is shown in thin lines.

hydrogen bonds with the side chain of Gln-192 (2.88 Å) and the amido-nitrogen of Val-216 (3.23 Å). Both of the oxygen atoms of the C-4 carboxylate also appear to form hydrogen bonds with Wat-391 (2.93 and 2.71 Å), which is within hydrogen-bonding distance of the amide oxygen of Val-216 (2.91 Å). The γ-lactam adopts a similar conformation in the active site as BCM7 in the PPE/BCM7 structure up to the point of the C-5 methylene group, which lies close to the carbonyl of the proline amide in the PPE/BCM7 structure.

The nitrogen atom of the sulfonamide group lies close to (1.01 Å away) the position occupied by Wat-317 in the PPE/BCM7 structure (Figure 5b) and is also positioned relatively close to the carbon atom of the ester carbonyl (2.99 Å). It may be readily envisaged that if His-57 rotates into its "productive" conformation, then the $N_{\epsilon 2}$ atom would be very close ($\sim$2.4 Å) to the sulfonamide NH. Further, the location of the latter nitrogen atom, almost overlapping with the location of Wat-317 in the PPE/BCM7 structure, suggests that it is in a good position to effect deacylation and recyclize the free γ-lactam (Scheme 2). Although the sulfonamide nitrogen is in the correct plane for nucleophilic attack onto the ester and the angle between the nitrogen atom and the ester carbonyl is 105° which is close to the optimum ($\sim$100−110°) for nucleophilic attack upon a carbonyl group (*27*), in the crystal structures its lone pairs of electrons are probably not correctly orientated for nucleophilic attack. It appears that the bond angle between C-5 and the nitrogen atom needs to rotate $\sim$50−60° to allow the toluenesulfonyl group to be correctly positioned for deacylation to occur.

Since the sulfonamide displaces Wat-317 (the hydrolytic water in the PPE/BCM7 structure), it may be considered the only functional group capable of effecting nucleophilic attack on the ester linkage between the ring-opened γ-lactam and Ser-195. This would lead to ring closure and reformation of the original γ-lactam structure, thus rationalizing the NMR experiments on the second series of γ-lactams in which no PPE-catalyzed ring opening was observed even under extended incubation conditions.

Conclusions. The NMR experiments on the first series of monocyclic γ-lactams demonstrated that they were readily ring-opened by PPE. Despite the evidence for reversible binding of this series to PPE, they were shown not to be inhibitors by the kinetic analyses. Thus the acyl-enzyme complex formed must be unstable, and these compounds appear to act as substrates. These results contrast with those for the respective monocyclic β-lactams which partition between hydrolysis and a conformational change resulting in a hydrolytically stable acyl-enzyme intermediate (*14*). The

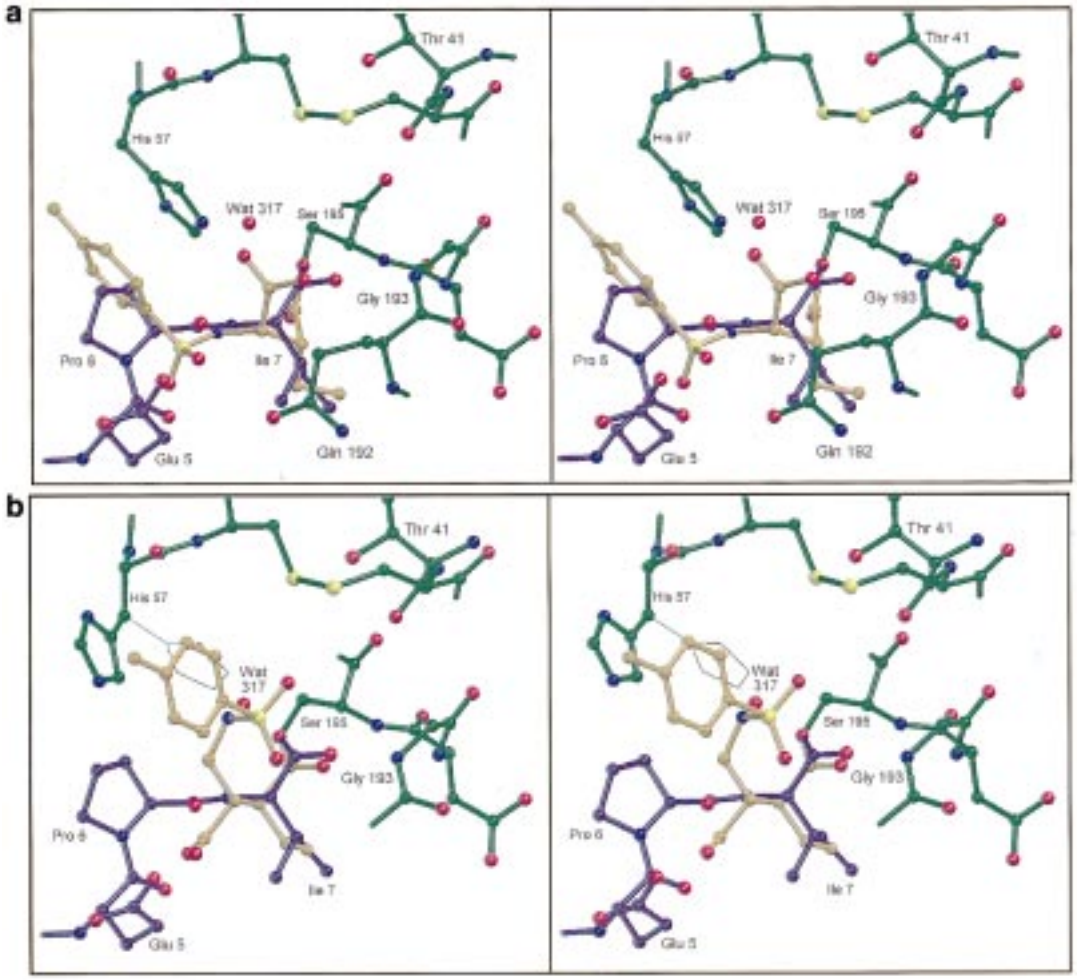
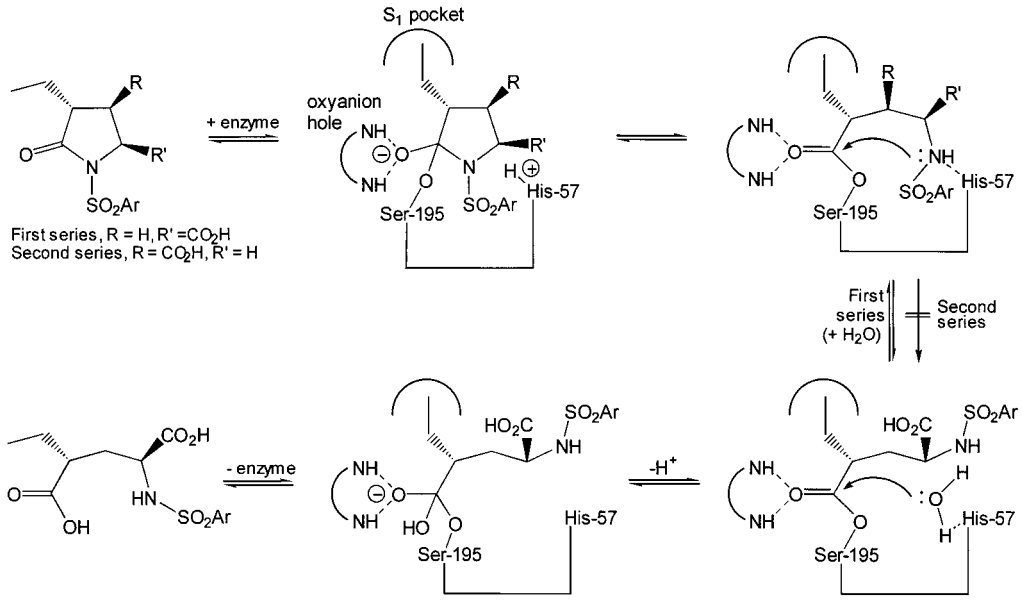


FIGURE 5: A comparison of the location of the acyl enzyme complex for (a) the β-lactam (**4**) and (b) the monocyclic γ-lactam (**21**) in the active site of PPE compared to that of the natural peptide, β-casomorphin-7 (shown in purple). Only the three C-terminal residues of β-casomorphin (Glu-Pro-Ile) are shown. In both cases the inhibitor molecule is shown in beige and the PPE active site is shown in green. Figures 3, 4, and 5 were produced using the programs Bobscript (*29*), Raster3D (*30*), ImageMagick, Illustrator 7.0 (Adobe Inc.), and Photoshop 4.0 (Adobe Inc.).

Scheme 2



lack of inhibitory activity of these γ-lactam compounds is therefore probably due, in part, to the presence of the extra methylene group which prevents a conformational change rather than any lack of reactivity toward acylation by PPE. Crystallographic analysis of the binding of compound **17** with PPE was attempted, but the resultant electron density map

only revealed a very low-occupancy intact γ-lactam molecule apparently noncovalently bound within the active site.

γ-Lactam compounds of the second series that demonstrated significant inhibitory activity toward PPE were those with a carboxylic acid functionality at the C-4 position (**21**, **29**, **30**). The stabilizing role of the C-4 group was revealed by crystallographic analysis on **21**, which demonstrated that it formed good hydrogen bonds with the side chain of Gln-192 and the amide nitrogen of Val-216. It may be relevant that a hydrogen bond from a glutamate residue in the P_3 position of the peptide inhibitor with Gln-192 may be partially responsible for the unusual stability of the acyl-enzyme complex formed between BCM7 and PPE. A similar interaction could be envisaged for **17** which exhibited weak inhibitory properties.

The third series of γ-lactams were devoid of inhibitory activity, and the NMR results demonstrated that, like the second series, no lactam hydrolysis was observable. These observations most probably reflect the relative positioning of the ethyl substituent and the γ-lactam carbonyl in this series; that is, the ethyl group cannot productively orientate the lactam carbonyl in the active site for nucleophilic attack by the serine.

The PPE-catalyzed hydrolysis of the side chain esters was observed only in the second and not in the first or third series. This observation probably reflects the relative positions of the ester and ethyl substituents. In the second series, the ester and ethyl groups are adjacent, allowing positioning of the ethyl group in the S_1 pocket and the ester carbonyl in a "productive" conformation for attack by the nucleophilic serine. Although this is also the case in the third series, steric hindrance by the aryl-sulfonyl group may prevent the reaction of the serine with the ester.

In conclusion, it seems that despite the inherently lower reactivity of the monocyclic γ-lactams compared to their β-lactam counterparts, their ability to acylate PPE is not significantly impaired. The relative stability of the γ-lactam may indeed be an advantage in any potential therapeutic application. However, the introduction of an extra methylene group appears to prevent the analyzed γ-lactams from undergoing a conformational change in the active site as observed for the β-lactams. In addition, the facile reversibility of the lactam hydrolysis allows the acyl-enzyme complex of the first and second series of γ-lactams to readily reform the γ-lactam. However, the results demonstrate that alteration of the substitution pattern on the γ-lactam can significantly alter the reactivity within the PPE active site; for example, the acyl-enzyme complexes formed for the first series compounds are hydrolytically labile, while those for the second series are not. This result indicates that, with appropriate substitution, it should be possible to form an acyl-enzyme complex for a simple monocyclic γ-lactam which is stable enough for the γ-lactam to be a useful inhibitor. Stabilization of the acyl-enzyme might be achieved by some or all of the following: (a) introducing an electron-withdrawing group onto the lactam nitrogen [note that **29** and **30** are more active than **21**]; (b) mesomerically removing the nitrogen lone pairs of the ring-opened lactam (as proposed in the mechanism for PBP inhibition by lactivicin (*7*)); (c) hindering recyclization by steric buttressing; (d) displacing the histidine from its productive conformation (modeling studies indicate that this might be achieved by substitution of the second series at the 5-position or by modification of the N-substituent); or (e) displacing the hydrolytic water.

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