



REVERSIBLE ACYLATION OF ELASTASE BY γ -LACTAM ANALOGUES OF β -LACTAM INHIBITORS

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Abstract: The reaction of a monocyclic γ -lactam with the serine protease elastase occurs via reversible formation of a hydrolytically labile acyl-enzyme complex; in contrast analogous β -lactam inhibitors irreversibly react to form a relatively stable acyl-enzyme complex. © 1997 Elsevier Science Ltd.

β -Lactam antibiotics exert their biological activity by acylation of nucleophilic serinyl residues at the active sites of enzymes involved in bacterial cell wall biosynthesis (penicillin binding proteins, PBPs).^{1a} β -Lactams have also been shown to inhibit a range of other enzymes with nucleophilic residues at their active sites, including mammalian serine proteases.² Various structural sub-families of β -lactam antibiotics have been isolated from micro-organisms including the penicillins, cephalosporins, carbapenems, clavams and monocyclic β -lactam antibiotics such as the norcardicins.^{1a} The number of naturally occurring β -lactam antibiotics isolated contrasts sharply with the relative lack of larger ring lactam antibiotics identified. The lacticins are the only isolated naturally occurring γ -lactams which demonstrate their antibacterial activity by interaction with PBPs.³

Many studies have attempted to define the reasons for the apparent general effectiveness of β -lactams as antibacterials or as inhibitors of 'serine' (trans)peptidases/hydrolases.^{1b,c} The subject remains somewhat confused, but it is clear that their activity is not simply related to reactivity nor to lack of resonance in the β -lactam amide.¹ Efforts from several groups have been directed towards synthesising compounds that have the same bacterial PBP targets as the β -lactams, but which do not contain a β -lactam ring. Some of these studies have resulted in the production of new antibacterials, including γ -lactam rings fused to unsaturated ring systems.^{1d,4}

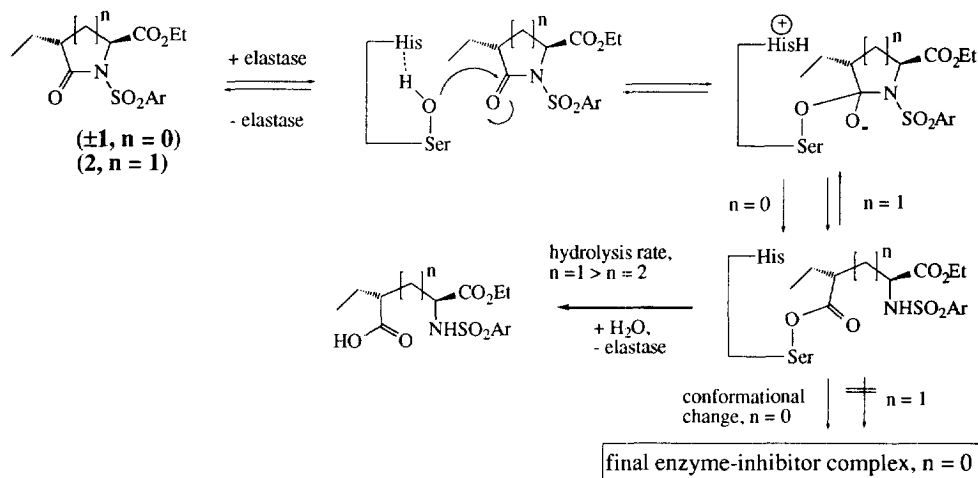
The release of *ca.* 25 kcal of ring strain energy during ring opening of a β -lactam by an alcohol renders reformation of the β -lactam energetically unfavourable with reversal half-lives in the region of tens to thousands of years.⁵ In contrast, the ring strain in most γ -lactams is small. We thus speculated that γ -lactam analogues of β -lactam inhibitors of 'serine' enzymes might (in part) be relatively inactive due to an unfavourable equilibrium position between the free γ -lactam and the acyl-enzyme complex.

We now report studies aimed at exploring the relative efficiency of analogous β - and γ -lactams as inhibitors of 'serine' enzymes, with the long-term objective of pioneering the development of new antibacterials/inhibitors. We chose to work mainly with the serine protease porcine pancreatic elastase (PPE),

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since it is readily available and has been demonstrated to be inhibited by simple monocyclic β -lactams.^{2,6}

The proposed mechanism for the inhibition of elastases by monocyclic β -lactam (**1**) involves reaction to form an initial acyl-enzyme ester complex, which partitions between hydrolysis and a conformational change to form a long lived enzyme-inhibitor complex (Scheme 1, $n = 0$).⁷ The additional methylene group was incorporated into the γ -lactam analogue (**2**) (Scheme 2) between the 3-C and 4-C carbons of (**1**) to give (**2**), since location of the alkyl substituent α to the lactam carbonyl in the S_1 binding pocket of elastase should control the orientation of the lactam within the active site,⁸ enabling nucleophilic attack of Ser-195 (using the chymotrypsin numbering system)⁹ onto the lactam carbonyl.

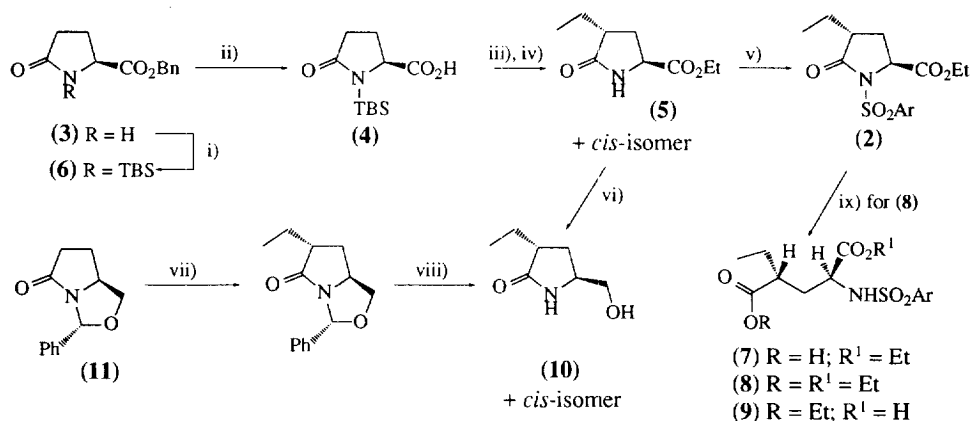


Scheme 1: Ar = $p\text{-NO}_2\text{C}_6\text{H}_4\text{-}$

N-Protected pyrrolidone esters have been used for the synthesis of C-3 alkylated lactams and their derivatives.¹⁰ However, despite precedent for stereoselective alkylation at C-3 in β -lactam chemistry,¹¹ alkylation of *N*-silyl protected pyrrolidone acids has not been reported.^{10d} Pyrrolidone benzyl ester (**3**)¹² was sequentially *N*-silylated and hydrogenolysed to give crude *N*-silylated acid (**4**), which was alkylated by deprotonation with LDA (2 equiv.) and reaction with ethyl iodide (Scheme 2). The crude reaction mixture was esterified and *N*-desilylated to give a mixture of epimeric *trans*- and *cis*-(**5**) esters, *ca.* 10:1 respectively [38% from (**6**)], which were separated by chromatography. *N*-Sulphonation of *trans*-(**5**) was achieved in good yield to give the desired ester (**2**).¹³

Incubation of the γ -lactam (**2**) in phosphate buffer (pD 7.4) in the absence of PPE demonstrated that it was hydrolysed slowly, with <5% reaction being observed after 12 hours (by 500 MHz ^1H n.m.r. analysis). After incubation for 2 weeks a new set of resonances was apparent. Doping experiments showed that they arose from the acid (**7**). A ^1H n.m.r. spectroscopic time course was then used to follow the incubation of (**2**) with PPE.¹⁴ In the presence of PPE the rate of hydrolysis of (**2**) to (**7**) was increased with complete conversion occurring within 3.5 hours (Figure 1). ^1H n.m.r. and mass spectrometric analyses demonstrated that no epimerisation at C-2 and C-4 occurred during the incubation [no incorporation of deuterium into (**2**) was observed]. Catalytic conversion of (**2**) to (**7**) was not observed when using phenylmethylsulfonyl

fluoride (PMSF)-treated PPE¹⁵ (Figure 1.6). Analogous results were obtained when human leucocyte elastase (HLE) was used in place of PPE. Analysis of an incubation of PPE with (2) in water by negative ion electrospray mass spectrometry showed an increase in the ion current corresponding to a peak at 387 (theoretical M_r of (7) = 388.40) with increasing incubation time with this peak being dominant after 16 hours. The negative ion ESI MS spectrum of a fresh solution of (2) did not contain a peak at 387 Da.



Scheme 2: (i) TBSCl, $i\text{Pr}_2\text{NEt}$, CH_2Cl_2 , room temp., 97%; (ii) H_2 , Pd/C, THF, room temp.; (iii) (4) added to LDA (2.1eq), THF, -78°C ; warm to 0°C ; add EtI (2.0eq); iv) EtOH, TsOH. H_2O , Δ ; 38% from (6), *trans:cis* 10:1 based on isolated yields; (v) $\text{NaN}(\text{TMS})_2$, THF, -78°C , $p\text{-NO}_2\text{C}_6\text{H}_4\text{SO}_2\text{Cl}$, 84%; (vi) NaBH_4 , MeOH, 0°C , 90%; (vii) LDA (1.1eq), THF, -78°C ; EtI, 83%, *trans:cis* 2.1:1 with assignments by n.O.e experiments; (viii) MeOH, c.HCl, Δ , 74%; (ix) EtOH, c.HCl, Δ , 87%. Ar = $p\text{-NO}_2\text{C}_6\text{H}_4$.

Kinetic studies¹⁶ demonstrated that (2) was neither an inhibitor of PPE nor HLE up to its solubility limit in the assay system (0.2mM). Thus, (2) presumably acylates the active site serinyl residues of PPE and HLE in an analogous manner to the initial complex formed with (1). However, (2) is a more efficient substrate for PPE or HLE than (1), implying that the acyl-enzyme intermediate formed on reaction of PPE with (2) is more readily hydrolysed to give (7). The lack of inhibition observed for the γ -lactam (2) implies that its initially formed acyl-enzyme complex does not undergo conformational conversion to a hydrolytically stable complex as proposed for the β -lactam inhibitors.⁷

Experiments were conducted to address the possibility of reversible acylation of PPE by (2). It was anticipated that incubation of PPE with the glutamic ester (8) (Scheme 2 and Figure 2.1) would lead to formation of the same acyl-enzyme intermediate formed on incubation of PPE with (2). Thus, if (2) reacts reversibly with PPE, incubation of (8) with PPE could also lead to the formation of (2) itself. ^1H N.m.r. analysis (Figure 2.2) of the incubation of (8) with PPE¹⁴ reproducibly demonstrated that, in addition to the presence of (8), (9) and (7) (the latter presumably formed by hydrolysis of the acyl-enzyme complex), the formation of (2) could be deduced by the presence of the appropriate AA'BB' resonances (*c.f.* Figures 2.3 and 2.2). The assignments were confirmed by doping with authentic (2) (Figure 2.4). A fourth product, resulting from enzymatic hydrolysis of the C_2 -ester functionality of (8), formed in this incubation (see Figure 2.3) was assigned the structure (9).

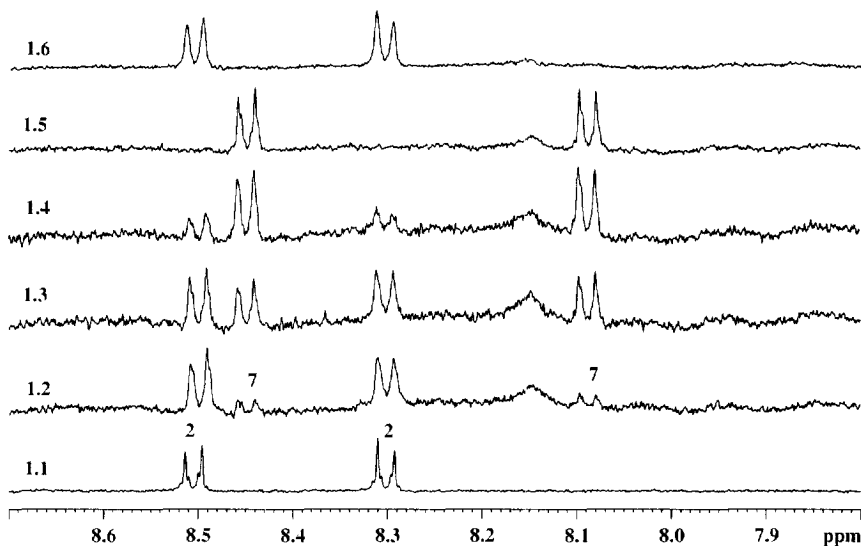


Figure 1: Aromatic region of ^1H n.m.r. (500MHz) spectra obtained on analysis of: **1.1**, Overnight incubation of **(2)** at room temperature in buffer:CD₃CN 10:1 in the absence of PPE; **1.2-1.5**, Incubation of PPE with **(2)** in buffer:CD₃CN 10:1 at incubation times (minutes) of **1.2**; 5; **1.3**; 40; **1.4**; 90; **1.5**; 210; **1.6**, ^1H n.m.r. (500MHz) spectrum of the overnight incubation of PMSF-inhibited PPE with **(2)** in buffer:CD₃CN, 10:1.

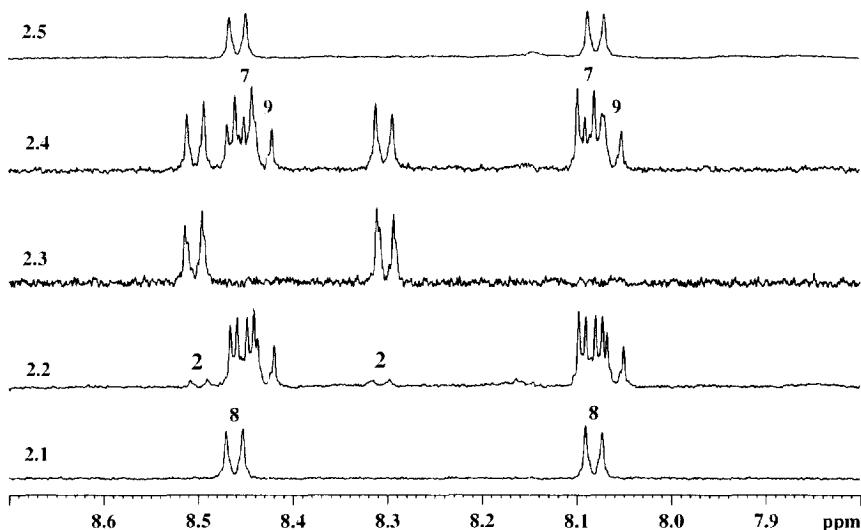


Figure 2: Aromatic region of ^1H n.m.r. (500MHz) spectra obtained on analysis of: **2.1**, Fresh solution of **(8)** in buffer:CD₃CN 10:1 in the absence of PPE; **2.2**, Overnight incubation of PPE with **(8)** at room temperature in buffer:CD₃CN 10:1; **2.3**, Fresh solution of **(2)** in buffer:CD₃CN 10:1; **2.4**, a mixture of the solutions from Figures 2.2 and 2.3. The increase in the conc. of **(7)**, presumably arises by PPE catalysed hydrolysis; **2.5**, Overnight incubation of PMSF-inhibited PPE with **(8)** in buffer:CD₃CN, 10:1.

Control experiments without PPE or using PMSF-inhibited PPE (Figure 2.5) demonstrated that conversion of (8) to (7), (2) and (9) was enzyme-mediated. When the incubation of (8) with PPE was carried out in H_2^{18}O , the resultant (7) and (9) contained only a single ^{18}O atom. Lactam (2) was always observed to be a minor product when (8) was incubated with elastase since hydrolysis of the acyl-enzyme complex dominates cyclisation.

In summary, the results presented demonstrate that γ -lactam (2) can acylate elastases (and potentially other serine proteases) in an analogous manner to β -lactam (1), despite its intrinsically reduced reactivity towards nucleophilic attack at the lactam carbonyl. Two key differences between the reaction of elastases with the β - and γ -lactams were identified (Scheme 1). Firstly, acylation of PPE by (2) is a reversible process while acylation of PPE by β -lactam (1) is effectively irreversible. Secondly, the acyl-enzyme intermediate formed on reaction of β -lactam (1) with PPE is significantly more stable with regard to hydrolysis than is the acyl-enzyme resulting from reaction of (2) with PPE. If these two deficiencies of the γ -lactam (2) can be overcome, then in principle it should be possible to develop efficient acylating γ -lactam inhibitors of serinyl proteases and transpeptidases. The increased stability of γ -lactams relative to analogous β -lactams may be a desirable pharmacokinetic property.

Acknowledgements: We thank J. Bartleet for the X-ray analysis, Dr R.T. Aplin for mass spectrometric analyses, and the M.R.C., the BBSRC, the EPSRC and Zeneca Pharmaceuticals for financial support.

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13. The relative and absolute stereochemistry of (**2**) was confirmed by single crystal X-ray analysis. Crystallographic data will be deposited at the Cambridge Crystallographic Data Centre. Evidence that minimal (<5%) racemisation of the amino acid chiral centre had occurred during the alkylation procedure was obtained by comparison of the optical rotation of *trans*-(**10**) $\{[\alpha]_D^{23} +47.7$ (c 0.6 in CHCl₃) $\}$ prepared from *trans*-(**5**) by NaBH₄ reduction with *trans*-(**10**) prepared by alkylation of the known^{10f} bicycle (**11**) followed by acid mediated hydrolysis $\{[\alpha]_D^{23} +49.0$ (c 0.4 in CHCl₃) $\}$. In an alternative procedure, (2*S*)-ethyl-1-(*tert*-butyloxycarbonyl)-pyroglutamate was treated with lithium *bis*(trimethylsilyl)amide (1.1eq) in THF at -78°C. Subsequent addition of ethyl trifluoromethanesulfonate (2.0eq) and acidic work up gave ethyl (2*S*,4*R*)-1-(*tert*-butyloxycarbonyl)-4-ethylpyroglutamate and ethyl (2*S*,4*S*)-1-(*tert*-butyloxycarbonyl)-4-ethylpyroglutamate the in overall 71% yield in a *trans*:*cis* ratio of 1:1.6, respectively (with assignments by n.O.e. experiments). Chromatographic separation of the diastereomers followed by treatment of the *trans* compound with CF₃CO₂H (anisole in CH₂Cl₂) at room temperature gave *trans*-(**5**) in 77% yield.
14. A solution of (**2**) or (**8**) in CD₃CN (15μl) was added to a solution of PPE in 0.1M NaD₂PO₄/Na₂DPO₄ buffer [prepared by dissolution of Na₂DPO₄ (1.107g) and NaD₂PO₄ (276mg) in D₂O (100ml), pD 7.4, 150μl] and the incubation was centrifuged. N.m.r. experiments were performed at 499.98MHz on a Bruker AMX500 instrument equipped with a 3mm Nalorac MID500-3B microprobe at 303 K with sample volumes of 130-150 μl. All spectra were referenced to internal residual CHD₂CN at 2.05ppm.
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(Received in Belgium 2 September 1997; accepted 23 October 1997)