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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 lactivicins 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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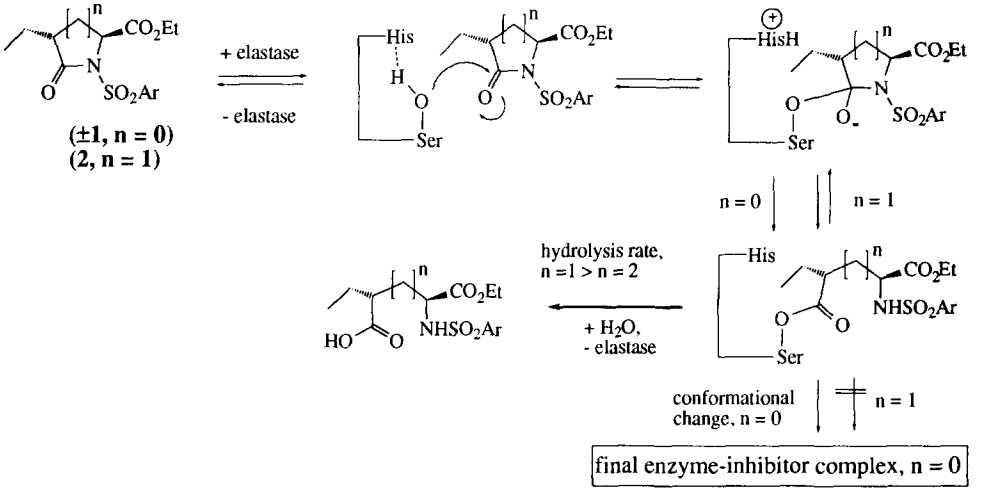
since it is readily available and has been demonstrated to be inhibited by simple monocyclic β-lactams.[2,6]

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.

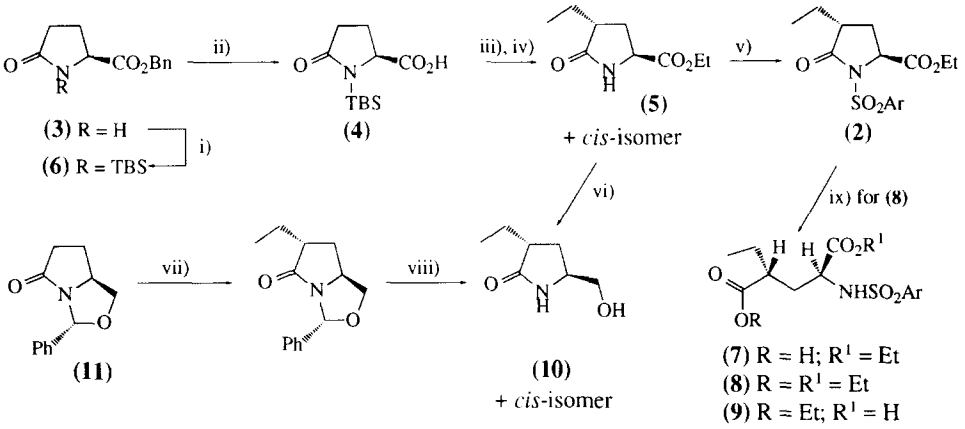


Scheme 1: Ar = *p*-$NO_2C_6H_4$-

N-Protected pyroglutamate 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 pyroglutamate acids has not been reported.[10d] Pyroglutamate 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 ^{1}H 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 ^{1}H 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). ^{1}H n.m.r. and mass spectrometric analyses demonstrated that no epimeristaion 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 PPE[15] (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, $^{i}Pr_2NEt$, 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) $NaN(TMS)_2$, THF, -78°C, *p*-$NO_2C_6H_4SO_2Cl$, 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*-$NO_2C_6H_4$-.

Kinetic studies[16] 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 PPE[14] 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**).

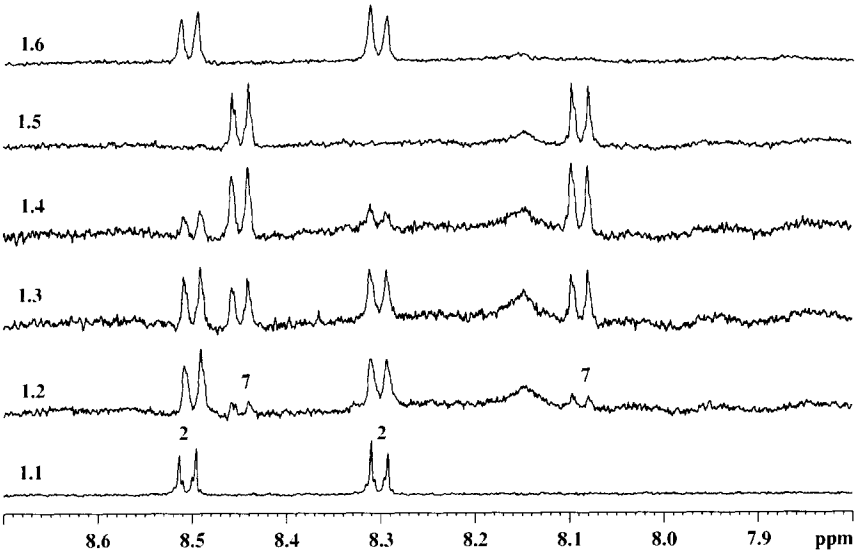


Figure 1: Aromatic region of ^{1}H n.m.r. (500MHz) spectra obtained on analysis of: **1.1**, Overnight incubation of (**2**) at room temperature in buffer:CD3CN 10:1 in the absence of PPE; **1.2**-**1.5**, Incubation of PPE with (**2**) in buffer:CD3CN 10:1 at incubation times (minutes) of **1.2**; 5;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

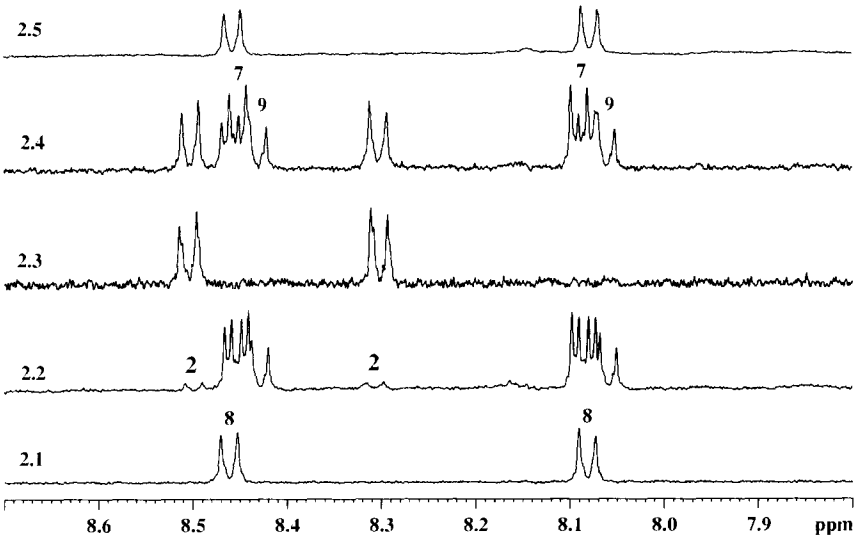


Figure 2: Aromatic region of ^{1}H n.m.r. (500MHz) spectra obtained on analysis of: **2.1**, Fresh solution of (**8**) in buffer:CD3CN 10:1 in the absence of PPE; **2.2**, Overnight incubation of PPE with (**8**) at room temperature in buffer:CD3CN 10:1; **2.3**, Fresh solution of (**2**) in buffer:CD3CN 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 pharmokinetic property.

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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 $CHCl_3$)} prepared from *trans*-(**5**) by $NaBH_4$ 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 $CHCl_3$)}. 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 CF_3CO_2H (anisole in CH_2Cl_2) at room temperature gave *trans*-(**5**) in 77% yield.

14. A solution of (**2**) or (**8**) in CD_3CN (15μl) was added to a solution of PPE in 0.1M NaD_2PO_4/Na_2DPO_4 buffer [prepared by dissolution of Na_2DPO_4 (1.107g) and NaD_2PO_4 (276mg) in D_2O (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 CHD_2CN at 2.05ppm.

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