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5,5-*TRANS* LACTONE-CONTAINING INHIBITORS OF SERINE PROTEASES: IDENTIFICATION OF A NOVEL, ACYLATING THROMBIN INHIBITOR

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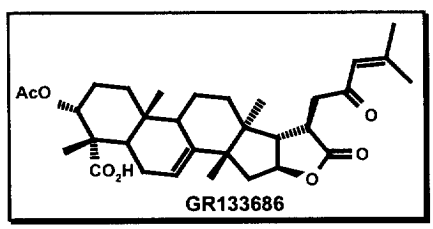
Abstract: Synthesis of a variety of 5,5-*trans* fused lactones, related to compounds found in extracts of *Lantana camara*, has provided a series of novel acylating inhibitors of human thrombin, trypsin, chymotrypsin and human leucocyte elastase. The most effective thrombin inhibitor is **7** with an IC_{50} of 130nM and a $K_{obs}/[I]$ of 4,000 M^{-1} s^{-1}.

There is currently great interest in identifying compounds that can selectively inhibit serine protease enzymes for the potential therapeutic benefits they are likely to provide.[1] For example, whilst inhibitors of thrombin[2] should be beneficial for a variety of thrombotic disorders, human leucocyte elastase (HLE) inhibitors[3] are being developed for the treatment of emphysema and chronic bronchitis. Our interest in serine proteases was initially focussed on thrombin and a desire to discover an orally active inhibitor for the treatment of venous thrombosis. This paper describes our initial studies.

At the outset of our work most of the previously described thrombin inhibitors were peptidic in nature and suffered from lack of oral bioavailability and short duration of action.[1] More recently, there has been an explosion of reports on non-peptidic thrombin inhibitors with major advances being made.[4] Our strategy to discover a non-peptidic lead molecule was to use high throughput screening of natural products and compounds from our file.[5] This strategy has allowed us to identify extracts of *Lantana camara* that potently inhibit a variety of serine proteases, including thrombin and chymotrypsin.[6] The substances responsible for this have been separated and characterised and shown to belong to the euphane triterpene class of natural products,[7] the presence of the rare 5,5-*trans* lactone moiety[8] being necessary for potent inhibitory activity (Figure 1).[6] X-ray crystallographic and electrospray ionisation-mass spectrometric[9] studies have demonstrated that human α-thrombin is acylated[10] on Ser^{195} by the strained lactone system present in **GR133686** and related molecules (**Figure 1**).[6,11]

Figure 1: Serine Protease Inhibitory Activity of a Representative Euphane Triterpene Lactone



GR133686

	IC_{50} nM
Thrombin	4[6]
Trypsin	70
Chymotrypsin	70
Factor Xa	>20,000
HLE	>10,000

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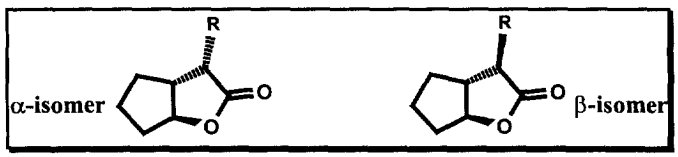
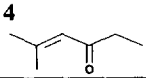
We have begun to elucidate the structural features necessary for selective inhibitory activity within this series of novel compounds and have previously reported the synthesis of the parent 5,5-*trans* lactone and a number of analogues.[12] To aid the speed of these initial investigations we chose to generate only racemic compounds and the enzyme inhibitory activities of representative examples are shown in **Table 1**. Although the *Lantana camara* extracts showed no inhibitory activity against HLE it was anticipated that 5,5-*trans* lactone analogues containing small lipophilic sidechains proximate to the lactone moiety would demonstrate some activity. In practice, the β-allyl compound **1** showed reasonable activity (IC_{50} 7μM) against HLE.[13] In addition, and as expected from the known peptide substrate preferences for serine proteases,[1] those compounds containing an aromatic group were inactive against HLE but were potent inhibitors of chymotrypsin (e.g. **3**, β-SPh, IC_{50} 20nM). In most instances the β-substituted isomer is more active than its α-substituted analogue.

Several of the compounds described showed time-dependent inhibition of the target enzymes which strongly suggests that they are acting by acylation of the relevant Ser^{195} in a similar manner to that proven for the euphane lactones.[6] This was confirmed for the β-allyl lactone **1** following an X-ray crystal structural analysis of the complex with pancreatic porcine elastase.[14] Although it is recognised that the use of IC_{50} values alone is of limited use as a measure of potency for time dependent enzyme inhibitors,[15] these results were only used to identify quickly the compounds requiring further study.

Table 1: Enzyme Inhibitory Activity of Substituted 5,5-*Trans* Lactones [a]



N° R	Thrombin IC_{50} μM [b]		Chymotrypsin IC_{50} μM [b]		HLE IC_{50} μM [b]	
	α–	β-	α–	β-	α–	β-
1 Allyl	>50	98	41	26	37	7[c]
2 Benzyl	92	73	2[c]	0.6[c]	>100	>100
3 Phenylthio	6	10	0.12[c]	0.02[c]	>100	>100
4 	19	21	33	0.5	-	>100

a. All compounds are racemic. b. IC_{50}'s after a 15 min. preincubation. c. Time-dependent inhibition was observed.

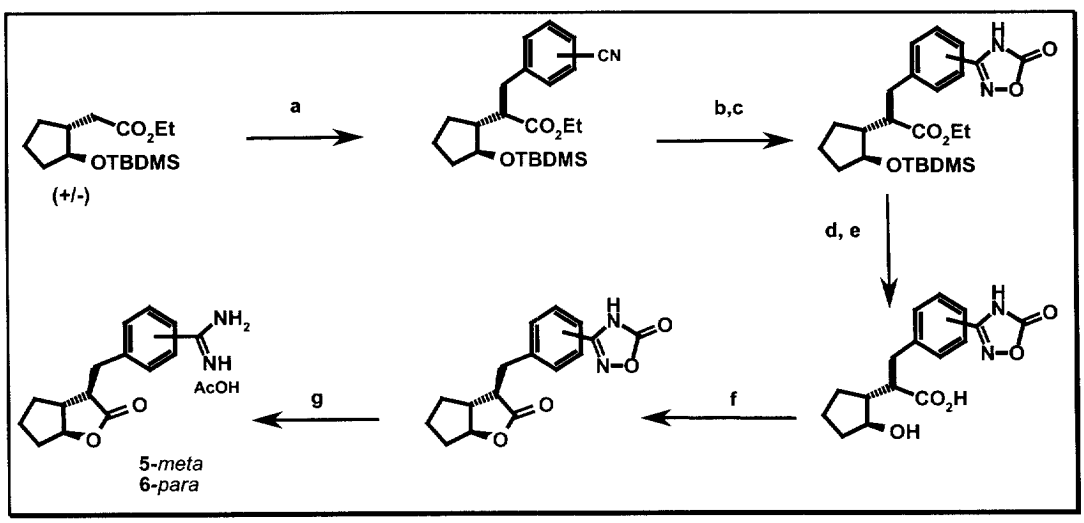
Inspection of **Table 1** reveals that only weak activity was evident against thrombin with lactones **1-3**. Since the natural products showed some selectivity of action towards thrombin, the parent lactone, substituted with the enone sidechain **4**, was prepared.[16] The weak activity observed with this derivative indicated that there was no special property associated with the unsaturated sidechain, but that the potency observed with the natural product, **GR133686** is a consequence of complementary shape and multiple van der Waals interactions between the euphane hydrophobic skeleton and the enzyme active site. This is supported by the X-ray crystal

structure[6] of the **thrombin:GR133686** complex which shows the enone sidechain bound in the S-1 pocket but with no obvious, specific interactions.[17]

The data shown above indicates that with appropriate modification, synthetic 5,5-*trans* lactones can have good enzyme inhibitory activity against a range of serine proteases. Since our initial goal was to discover novel thrombin inhibitors, we needed a strategy that would significantly enhance thrombin inhibitory activity. Realising that the potency of enzyme inhibitors that act by an acylation mechanism is dependent on two properties of the enzyme-inhibitor interaction; a) the initial, reversible association of the inhibitor molecule, which is controlled by the affinity for the enzyme and, b) the acylation event, which is dependent upon the reactivity of the acylating moiety present, we focussed our approach on enhancing affinity. Since the majority of thrombin inhibitors contain a strongly basic sidechain that forms a coulombic interaction with Asp^{189} in the enzyme S-1 specificity pocket,[1,2] we chose to introduce the benzamidine function, to fulfil this purpose.

The synthesis of the desired benzamidine-containing 5,5-*trans* lactones is described in **Scheme 1**. Notable is the use of the oxadiazolidinone group as a protected amidine.[18] This moiety survives the deprotection and lactonisation sequences and is then liberated *via* a hydrogenation reaction in the presence of acetic acid. This process allowed preparation of both the *meta* and *para* benzamidine analogues **5** and **6**.

Scheme 1: Synthesis of Benzamidine 5,5-*Trans* Lactones 5 and 6



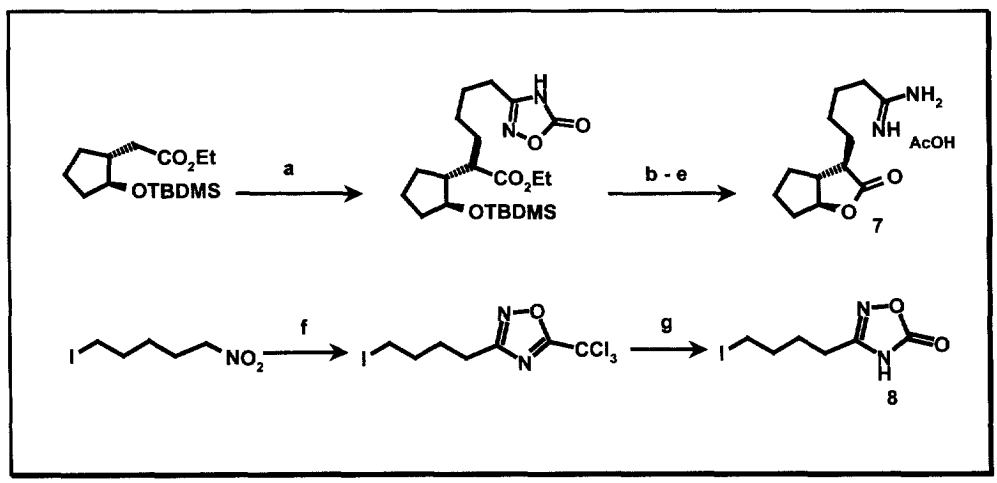
a. LDA, CN-$C_6H_4CH_2Br$, THF; 68, 76% (*meta*- and *para*- respectively). **b**. $NH_2OH.HCl$, EtOH; 100, 96%. **c**. $ClCO_2Et$, Pyridine; 59, 67%. **d**. HF/MeCN; 71, 91%. **e**. KOH, EtOH, H_2O; 92, 96%. Steps a-e provided mixtures of diastereomers that were not separated. **f**. Trichlorobenzoyl chloride, Et_3N, DMAP, THF, Toluene; 82, 64%. **g**. H_2, EtOAc, AcOH, 10% Pd/C; 100, 99%: (ratio β:α–isomers, *meta*. 4:1, *para* 3:1)

Although both compounds were reasonably good inhibitors of trypsin (IC_{50}'s of 3.3 and 0.22μM respectively), they were unexpectedly weak inhibitors of thrombin (IC_{50}'s of >100 and 16.8μM) (**Table 2**). The observation that the *para*-benzamidine **6** was of very similar potency to the enone lactone **4** strongly suggested that the coulombic interactions expected with Asp^{189} of thrombin were not being realised. Subsequent molecular modelling using the X-ray data from the **thrombin:GR133686**[6] complex suggested that Ser^{195}

attack on the lactone carbonyl could not be accomplished whilst the *meta*- or *para*-benzamidine moiety was engaged in a salt-bridge interaction with Asp^{189} in the S-1 specificity pocket. This non-optimal situation is presumably responsible for the observed weak inhibitory effect.

Further modelling suggested that a more flexible, substrate-like sidechain would overcome the above problem. After several failures to introduce a guanidine function we chose to incorporate the corresponding alkyl amidine sidechain. We were able to utilise a similar synthetic strategy to that described above, the alkylamidine moiety being protected as an alkyloxadiazolidinone (**Scheme 2**). The resultant amidine lactone **7** showed a 100 fold enhancement in inhibitory activity against thrombin and potent activity versus trypsin (**Table 2**). Our modelling studies were unable to provide an explanation why the lactones **5-7** possess enhanced inhibitory activity against trypsin relative to thrombin. The kinetics of inactivation of human α-thrombin by the amidine-lactone **7** have been examined and compared with the euphane lactone **GR133686.** The lactone **7** acylates thrombin with an apparent second order rate constant ($k_{obs}/[I]$) of $4x10^3$ $M^{-1}s^{-1}$, demonstrating it is approximately one thousand times less effective than **GR133686.**[6] However, the deacylation rate constant for **thrombin:7** is $2.1x10^{-5}s^{-1}$ ($t_{1/2}$ 550min) compared to $1.7x10^{-4}s^{-1}$ ($t_{1/2}$ 50min) for **thrombin:GR133686** indicating the generation of a more stable enzyme complex.

Scheme 2: Synthesis of Alkylamidine Lactone 7



a. LHMDS, HMPA, THF; **8**, 76%. **b**. Bu_4NF, THF; 70%. **c**. KOH, EtOH, H_2O; 63%. Steps a-d provided mixtures of diastereomers that were not separated. **d**. Trichlorobenzoyl chloride, Et_3N, DMAP, THF, Toluene; 43%. **e**. H_2, EtOAc, AcOH, 10% Pd/C; 72%; (>99:1 ratio β:α–isomers). **f**. Cl_3CN, PhNCO, toluene, Et_3N; 72%. **g**. KOH , EtOH; 77% .

Acylation of human α-thrombin by lactone **7** was proven initially by electrospray-ionisation mass spectrometry.[9] The mass spectrum of thrombin inhibited by **7** shows an increase of 222.5 mass units compared to the native enzyme (MW of **7** is 224.3). Absolute confirmation was obtained from an X-ray crystal structure of the alkylamidine **7** complexed with human α-thrombin, this showing the expected binding of the amidine moiety in the S-1 specificity pocket and the presence of an acylated Ser^{195} residue (**Scheme 3**).[11,19]

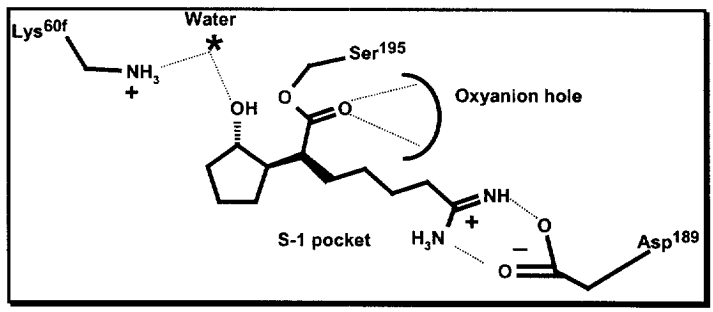
Table 2: Enzyme Inhibitory Activity of Amidine-Containing 5,5-*Trans* Lactones

N°	Thrombin IC_{50} μM	Trypsin IC_{50} μM	Factor Xa IC_{50} μM
5	>100	3.3	-
6	16.8[a]	0.22[a]	70.3[a]
7	0.13[a]	0.04[a]	10.3[a]

a. Time-dependent inhibition was observed

The amidine containing lactones weakly inhibited Factor Xa, another important trypsin-like enzyme involved in the coagulation cascade, but as anticipated none of them exhibited inhibitory activity towards HLE or chymotrypsin (IC_{50}'s >100μM) (**Table 2**).

Scheme 3: Schematic Diagram Showing the Binding Mode of 7 in the Thrombin Active Site[19]



The potent thrombin inhibitory activity demonstrated with the alkylamidine **7** prompted us to test for it's ability to prolong the coagulation of human plasma; the concentration required to double the activated partial thromboplastin time (APTT) being used as a standard measure of anticoagulant potency. Although the alkylamidine **7** is able to double the APTT at 130μM, preincubation in plasma for only 10 minutes virtually abolishes this activity (1.1x APTT). This is consistent with the measured (by h.p.l.c.) $t_{1/2}$ values in plasma for these synthetic lactones which are in the region of 2-5 minutes (rat, marmoset and human). Presumably this instability is due to plasma esterase-mediated lactone cleavage. These results are in contrast to the *Lantana camara* extract **GR133686** which possessed substantially greater stability in rat and human plasma ($t_{1/2}$ of 1 and 6 hours, respectively).

Conclusions: Substituted 5,5-*trans* lactones have been synthesised that respectively can inhibit HLE (e.g. **1**; β-isomer, IC_{50} 7μM); chymotrypsin (e.g. **3**, β-isomer, IC_{50} 20nM) and human α-thrombin (e.g. **7**, β-isomer, IC_{50} 130nM). Unfortunately, these compounds, unlike their natural product analogues, are highly unstable in plasma and are therefore not suitable candidates for extensive evaluation. Strategies which have resulted in improved enzyme specificity, potency, and plasma stability of these novel agents will be the subject of future publications.

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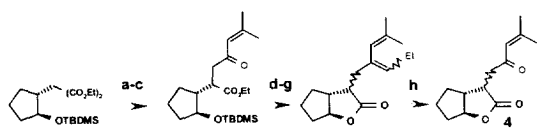
References and notes

1. Barrett, A.J.; Salvesen, G. *Proteinase Inhibitors*, Elsevier, **1986**.
2. Tapperelli, C.; Metternich, R.; Ehrhardt, C.; Cook, N.S. *Trends Pharmacol. Sci.*, **1993**, *14*, 366; Hauptmann, J.; Markwardt, F. *Seminars in Thrombosis and Haemostasis*, **1992**, *18*, 200.
3. Finke, P.E.; Shah, S.K.; Fletcher, D.S.; Ashe, B.M.; Brause, K.A.; Chandler, G.O.; Dellea, P.S.; Hand, K.M.; Maycock, A.L.; Osinga, D.G.; Underwood, D.J.; Weston, H.; Davies, P.; Doherty, J.B. *J. Med. Chem.*, **1995**, *38*, 2449 and references therein.
4. For some recent references see; Oh, Y.S.; Yun, M.; Hwang, S.Y.; Hong, S.; Shin, Y.; Lee, K.; Yoon, K.H.; Yoo, Y.J.; Kim, D.S.; Lee, S.H.; Lee, Y.H.; Park, H.D.; Lee, C.H.; Lee, S.K.; Kim, S. *Bioorg. Med. Chem. Lett.*, **1998**, *8*, 631; Fevig, J.M.; Buriak, J.,Jr.; Cacciola, J.; Alexander, R.S.; Kettner, C.A.; Knabb, R.M.; Pruitt, J.R.; Weber, P.C.; Wexler, R.R. *Bioorg. Med. Chem. Lett.*, **1998**, *8*, 301; Brady, S.F.; Stauffer, K.J.; Lumma, W.C.; Smith, G.M.; Ramjit, H.G.; Lewis, S.D.; Lucas, B.J.; Gardell, S.J.; Lyle, E.A.; Appleby, S.D.; Cook, J.J.; Holahan, M.A.; Stranieri, M.T.; Lynch, J.J., Jr.; Lin, J.H.; Chen, I.W.; Vastag, K.; Naylor-Olsen, A.M.; Vacca, J.P. *J. Med. Chem.*, **1998**, *41*, 401; Feng, D-M.; Gardell, S.J.; Lewis, S.D.; Bock, M.G.; Chen, Z.; Freidinger, R.M.; Naylor-Olsen, A.M.; Ramjit, H.G.; Woltmann, R.; Baskin, E.P.; Lynch, J.J.; Lucas, R.; Shafer, J.A.; Dancheck, K.B.; Chen, I-W.; Mao, S-S.; Krueger, J.A.; Hare, T.R.; Mulichak, A.M.; Vacca, J.P. *J. Med. Chem.*, **1997**, *40*, 3726; Sall, D.J.; Bastian, J.A.; Briggs, S.L.; Buben, J.A.; Chirgadze, N.Y.; Clawson, D.K.; Denney, M.L.; Giera, D.D.; Gifford-Moore, D.S.; Harper, R.W.; Hauser, K.L.; Klimkowski, V.J.; Kohn, T.J.; Lin, H-S.; McCowan, J.R.; Palkowitz, A.D.; Smith, G.F.; Takeuchi, K.; Thrasher, K.J.; Tinsley, J.M.; Utterback, B.G.; Yan, S-C.B.; Zhang, M. *J. Med. Chem.*, **1997**, *40*, 3489; Tucker, T.J.; Lumma, W.C.; Lewis, S.D.; Gardell, S.J.; Lucas, B.J.; Sisko, J.T.; Lynch, J.J.; Lyle, E.A.; Baskin, E.P.; Woltmann, R.F.; Appleby, S.D.; Chen, I-W.; Dancheck, K.B.; Naylor-Olsen, A.M.; Krueger, J.A.; Cooper, C.M.; Vacca, J.P. *J. Med. Chem.*, **1997**, *40*, 3687.
5. For a reference debating this strategy for discovering non-peptide leads see: Rees, C.D. *Ann. Rep. Med. Chem.*, **1993**, 59.
6. Weir, M.P.; Bethell, S.S.; Cleasby, A.; Campbell, C.J.; Dennis, R.J.; Dix, C.J.; Finch, H.; Jhoti, H.; Mooney, C.J.; Patel, S.; Tang, C-M.; Ward, M.; Wonacott, A.J.; Wharton, C.W. *Biochemistry*, **1998**, *37*, 6645. A full kinetic analysis for **GR133686** gave the following data: Ki 60nM, Ki* 0.045nM, apparent 2nd order rate constant, k_3/Ki (or Kobs/[Ki]) $2.7x10^6$ $M^{-1}s^{-1}$, k_3 $0.22s^{-1}$, k_4 $1.7x10^{-4}s^{-1}$ for E + I k_1→ ← k_2 EI k_3→ EI* (acyl enzyme) k_4→E + I*.
7. Ourisson, G.; Crabbe, P.; Rodig, O. *Tetracyclic Triterpenes*, Holden-Day, San Francisco, **1964**; for a synthetic approach to euphane triterpenes see Kolaczkowski,W.; Reusch, W. *J. Org. Chem.*, **1985**, *50*, 4766.
8. For example see Chiang, C.; Chiang, F.C. *Tetrahedron*, **1973**, *29*, 1911; Chiang, C.; Chiang, F.C. *Tetrahedron Letters*, **1969**, *11*, 891; Ma, K.W.; Chiang, F.C.; Clardy, J.C. *J. Chem. Soc. Chem. Commun.*, **1971**, 424; Ochi, M.; Kotsuki, H.; Tokoroyama, T.; Kubota,T. *Bull. Chem. Soc. Jpn.*, **1977**, *50*, 2499; Nakai, H.; Shiro, M. *Acta. Cryst.*, **1978**, *B34*, 2063.
9. For some recent uses of electrospray ionisation mass spectrometry (EI-MS) with serine proteases see; Knight, W.B.; Swidereck, K.M.; Sakuma,T.; Calaycay, J.; Shively, J.E.; Lee, T.D.; Covey, T.R.; Shushman, B.; Green, B.G.; Chabin, R.; Shah, S.; Mumford, R.; Dickenson, T.A.; Griffen, P.R. *Biochemistry*, **1993**, *32*, 2031; Sall, J. S.; Kaiser Jnr., R. E. *J. Med. Chem.*, **1993**, *36*, 2350; Mini review on EI-MS, Ashton, D. S.; Beddell, C. R.; Green, B. N.; Oliver, R. W. A. *FEBS Letters*, **1994**, *342*, 1.
10. For other acylating inhibitors see reference 1, Powers, J.C.; Harper, J.W. *Inhibitors of serine proteinases*, pg. 55-152.
11. A detailed analysis of the X-ray structures of thrombin inhibited with **GR133686** and **7** will be published in due course.
12. In press; Kelly, H.A.; Bolton, R.; Brown, S.A.; Coote, S.J.; Dowle, M.; Dyer, U.; Finch, H.; Golding, D.; Lowdon, A.; McLaren, J.; Montana, J.G.; Owen, M.R.; Pegg, N.A.; Ross, B.C.; Thomas, R.; Walker, D.A. *Tetrahedron Letters*,; n.b. all the synthetic compounds described are racemic and possess satisfactory spectroscopic and / or analytical data.
13. IC_{50} is the concentration of compound required to inhibit 50% of the enzyme-catalysed hydrolysis of the chromogenic substrate, the compounds being incubated with the enzyme for 15 mins. prior to substrate addition. Chymotrypsin substrate: Suc.Ala.Ala.Pro.Phe-pNA. Human leucocyte elastase substrate: MeOSuc.Ala.Ala.Pro.Val.-pNA, Thrombin substrate: Tos.Gly.Pro.Lys.pNA. Trypsin and FXa substrate: S2222. Reported values are the mean of triplicate determinations.
14. Singh, O.; Wonacott, A.; Jhoti, H. Unpublished work.
15. For a recent commentary on factors that affect the potency of suicide enzyme inhibitors see; Ternansky, R.J.; Draheim, S.E. *J. Med. Chem.*, **1993**, *36*, 3219.
16. 

a. NaH, DMF, 'enonebromide'[20]; 52%. **b**. KOH, EtOH; **c**. Cu_2O, MeCN; 50% overall for **b** and **c**. **d**. $EtPh_3^+Br^-$, $KOBu^t$, THF; 52%. **e**. EtOH, KOH. **f**. Bu_4NF, THF; 57% overall for **e** and **f**. **g**. Mukaiyama lactonisation, 55%. **h**. O_3, CH_2Cl_2, -78°; α-isomer 0.5%; β-isomer 2%
17. A recent paper from the Merck laboratories describes a potent thrombin inhibitor with a highly optimised, non-basic P-1 sidechain; Lumma Jr., W.C.; Witherup, K.M.; Tucker, T.J.; Brady, S.F.; Sisko, J.T.; Naylor-Olsen, A.M.; Lewis, S.D.; Lucas, B.J.; Vacca, J.P. *J. Med. Chem.*, **1998**, *41*, 1011.
18. Bolton, R.E.; Coote, S.J.; Finch, H.; Lowdon, A.; Pegg, N.; Vinader, M.V. *Tetrahedron Letters*, **1995**, *16*, 4471.
19. **Scheme 3**: Hydrogen bonds (broken lines) are observed between the former lactone carbonyl and backbone amides from the 'oxyanion hole' residues **Gly**[193] and **Ser**[195]. The alkyl amidine sidechain interacts with **Asp**[189] in the **S1** pocket and a water-mediated interaction is observed between the inhibitor hydroxyl and **Lys**[60f] from the thrombin 'sixties loop'. It was not possible to establish the absolute configuration of the bound inhibitor from the X-ray data.
20. Grundke, G.; Keese, W.; Rimpler, M.; *Chem. Ber.*, **1985**, *118*, 4288.