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THROMBIN INHIBITORS BASED ON KETONE DERIVATIVES OF ARGININE AND LYSINE

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Much attention is currently focused on inhibitors of thrombin as potential anticoagulants. We have previously reported thrombin inhibitors based on fragments of fibrinogen containing a ketomethylene isostere at P_1 - P'_1. We now expand on these early findings by reporting on tripeptide based inhibitors of thrombin containing arginine or lysine ketones at the C-terminus. A large variety of such ketones have been studied and compared in their ability to increase the thrombin time in human plasma. In the case of arginine or lysine ketones the order of activity (i.e. decreasing IC_{50} TT) was: alkyl ketones $<\beta$-ketoesters $<$ difluoro β-ketoamides $<$ alkyloxymethyl ketones $<$ fluoroketones. Lysine analogues were generally found to be ca. ten-fold less active than their arginine counterparts. However, in the case of α-ketoesters the lysine derivatives were superior to all the types of arginine ketones studied (including the arginine α-keto ester derived thrombin inhibitor). A mechanistic explanation of the relative inactivity of the arginine α-keto ester derivative is proposed. All the highly electrophilic ketones were found to be slow-binding wih thrombin.

KEY WORDS: Thrombin, enzyme inhibitors, anticoagulants, peptide ketones

INTRODUCTION

In recent years much attention has been focused on inhibitors of thrombin as potential anticoagulants.[1,2] As part of our programme aimed at the development of enzyme inhibitors based on transition-state mimics,[3,4] we discovered in the early nineteen eighties that fragments of human fibrinogen Aα containing a ketomethylene isostere[5] at P_1–P'_1 (nomenclature of Schechter and Berger[6]) were potent inhibitors of thrombin.[7,8] More recently, other reports have also appeared on incorporation of ketomethylene isosteres into thrombin inhibitors.[9,10]

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Abbreviations: Pfp, pentafluorophenyl; WSCDI, water-soluble carbodiimide; Tce, 2,2,2-trichloroethyl; NMM, N-methylmorpholine; IBC, isobutyl chloroformate; Ch, cyclohexyl; 4-DMAP, 4-dimethylamino-pyridine; An, p-anisyl (p-methoxyphenyl); CAN, ceric ammonium nitrate; Tcboc, 2′,2′,2′,-trichloro-1′,1′-dimethylethoxycarbonyl; TBS, tert-butyl dimethyl silyl.

		IC_{50} TT (μM)
1	H-DPhe-Pro-Arg-CH_2CH_2CO-Pro-NHEt	1.0
2	H-DCha-Pro-Arg-CH_2CH_2CO-Phe-NHEt	0.024

FIGURE 1 Thrombin inhibitors containing ketomethylene isostere of Arg-Gly.

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Our initial lead compound **1** (Figure 1) could be markedly improved[8] (as shown by an *in vitro* thrombin time assay and K_i determinations[11,12]) by replacement of the DPhe at P_3 by DCha and Pro at P'_2 by Phe. Compound **1** exhibited significant beneficial effects *in vivo* in animal models of venous thrombosis and was metabolically stable (unpublished data). However, **2** was found to be shorter acting *in vivo* and was metabolically labile at the $P'_1 - P'_2$ amide bond (*in vivo* results to be published separately).

The ketone carbonyl group of compound **1** and its congeners was expected to form a hemi-ketal with the active site serine hydroxyl of thrombin giving a tight-binding tetrahedral transition state mimic in much the same way as peptide aldehyde inhibitors of thrombin[13] form hemi-acetals. We embarked on a programme of synthesis of potential inhibitors of thrombin containing different arginine or lysine ketone derivatives, in the hope of finding compounds that are more stable *in vivo* than **2** with an equal or superior *in vitro* thrombin inhibitory activity and a corresponding *in vivo* anti-coagulant effect.

MATERIALS AND METHODS

Thrombin Time[11,12]

Results from this assay are expressed as IC_{50} TT values i.e. the concentration of inhibitor that doubles the thrombin clotting time in human plasma.

Method Pooled normal human plasma (100 μl) is mixed with a range of concentrations of the test compound dissolved in saline containing 10 g/l of bovine albumin (100 μl). Human thrombin T6759 (Sigma) in 0.05 M tris buffer, pH 7.4 containing 10 g/l of bovine albumin (100 μl, 0.2–0.3 NIH units of thrombin) is added and the clotting time measured at 37°C in an automatic coagulometer (Amelung KC 10). The clotting time in seconds is plotted against the inhibitor concentration and the IC_{50} TT determined by interpolation.

In the case of slow-binding inhibitors, a pre-incubation of inhibitor with thrombin for 10–60 min was used before the addition of plasma.

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TABLE 1
Thrombin inhibitors: analytical data.

No.	Structure	P.C.(%)	Mol. Wt.	Found [M+H] FAB-MS Major peak (†M+H_2O+H)	Hplc system & t_R (min) (L/D at Arg/Lys)
3	H-DPhe-Pro-Arg-CH_3	71	416.57	417	A, 9.0, 9.6
4	H-DCha-Pro-Arg-CH_3	36	422.58	423	B, 8.4, 9.4
5	H-DPhe-Pro-Arg-Ch	73	484.6	485	A, 13, 14
6	H-DPhe-Pro-Arg-CH_2SCh	75	530.7	532	A, 14.8, 15.6
7	H-DPhe-Pro-Arg-CH_2CO_2Et	63	488.6	489	A, 9.8‡
8	H-DPhe-Pro-Arg-CF_2CO_2Et	73	524.57	525	A, 11.6‡
9	H-DCha-Pro-Arg-$CH_2OCH_2CF_3$	71	520.6	521	D, 13.4,14
10	H-DPhe-Pro-Arg-CF_3	48	470.5	489,† 471	A, 9.8, 10.6
11	H-DPhe-Pro-Arg-$CF_2CF_2CF_3$	71	570.6	589,† 571	A, 13.6, 14.4
11a	H-DPhe-Pro-Lys-$CF_2CF_2CF_3$	57	542.5	543, 561†	B, 8.6, 9.6
12	H-DCha-Pro-Lys-$CF_2CONHCH_2CH_2$Ph	41	577.7	578*	B, 9.4, 10.4
13	H-DCha-Pro-Lys-CF_2CONHEt	58	501.65	503*	B, 4.8, 5.2
14	Me-DCha-Pro-Lys-CO_2Me	76	452.6	471,† 453	C, 13.4
15	Me-DCha-Pro-Lys-CO_2H	51	438.5	377 (M+H-H_2O-CO_2)	C, 12.8, 13.2
16	Me-DCha-Pro-Lys-CH_2OH	75	424.59	425	D, 10
17	$PhSO_2$-DCha-Pro-Lys-CO_2Me	70	578.7	597,† 579	C, 22.5
18	$MeSO_2$-DCha-Pro-Lys-CO_2Me	69	516.66	535,† 517	C, 18, 20
19	$ChCH_2$-DCha-Pro-Lys-CO_2Me	58	534.75	553,† 535	C, 19.4
20	HO_2C. CH_2-DCha-Pro-Lys-CO_2Me	65	496.6	515,† 497	C, 13, 13.5
21	$(HO_2C.\ CH_2)_2$-DCha-Pro-Lys-CO_2Me	65	554.65	573,† 555	C, 14.5
22	H-DCha-Pro-Arg-CO_2Me	66	466.59	485,† 467	D, 11.2, 11.4
23	H-DCha-Pro-Arg-CH_2OH	56	438.57	439	D, 11.3, 11.6

‡Evidence of slight splitting of peak due to epimerisation at Arg α carbon; †(M+H + H_2O); *Peaks due to loss of 2 × F also present.

Hplc: Novapak C_{18}(Waters) 100×8 mm cartridge. Flow 1.5 ml/min. Detection uv 210 nm.
Solvent A: 0.1% TFA-H_2O; Solvent B: 0.1% TFA-MeCN.
System A: 10–70% B into A over 20'
System B: 20–80% B into A over 30'
System C: 10–60% B into A over 30'
System D: 10–90% B into A over 30'

Chemistry

All the compounds synthesised are shown in Table 1 together with results of their analysis by Fab mass spectrometry and their retention times found by reverse-phase chromatography. "Peptide Content" (P.C.) expresses % by weight of base, the rest being water and anion. It is calculated from quantitative amino acid analysis of Pro and/or DPhe or DCha after hydrolysis with HCl (Beckman Gold System).

Boc-AA-OH $\xrightarrow{(a), (b)}$ Boc-AA-Pro-OH

AA = DPhe or DCha

$\xrightarrow{(a)}$ Boc-AA-Pro-OPfp

Boc-Arg(Z)$_2$OH $\xrightarrow{(c), (d)}$ HCl. H-Arg(Z)$_2$OTce

$\xrightarrow{(e)}$ Boc-AA-Pro-Arg(Z)$_2$OTce

$\xrightarrow{(f)}$ Boc-AA-Pro-Arg(Z)$_2$OH

$\xrightarrow{(g)}$ BocD-AA-Pro-D,L-Arg(Z)$_2$-R $\xrightarrow{(d), (h)}$ H-AA-Pro-Arg-R

3 AA = DPhe, R = CH_3
4 AA = DCha, R = CH_3
5 AA = DPhe, R = Ch

(a) Pentafluorophenol, WSCDI, CH_2Cl_2; (b) 2 equiv. sodium prolinate, DMF-H_2O;
(c) Trichloroethanol, WSCDI, 4-DMAP, CH_2Cl_2; (d) 4M HCl-dioxan; (e) DMF-CH_2Cl_2, $^iPr_2NEt$;
(f) Zn-HOAc; (g) WSCDI, CH_2Cl_2 then Ac_2O or ChCOCl, Et_3N, 4-DMAP, Pyridine, HOAC
(h) H_2, 5% Pd/C, MeOH-H_2O-HCl

SCHEME 1 Preparation of alkyl ketones **3–5**.

Alkyl ketones **3–5** were synthesised using a modified Dakin-West reaction[14,15] as depicted in Scheme 1. Bis-Z protection was used for the side-chain of arginine and standard coupling methods[16] were used for peptide bond formation.

Excellent yields were obtained in the Dakin-West reaction using acetic anhydride (75–89%) however, with cyclohexane carboxylic acid chloride yields were more modest (ca. 25%). In our methodology the intermediate oxazolones in this reaction were preformed using Benoiton's method[17] prior to acylation. Products **3–5** from this synthesis were completely epimerised at the Arg α-carbon. Compound **6** was produced as shown in Scheme 2.

Boc-Arg(Z)$_2$OH $\xrightarrow{(a), (b), (c)}$ Boc-Arg(Z)$_2$CH$_2$Br

$\xrightarrow{(d)}$ Boc-Arg(Z)$_2$CH$_2$SCh $\xrightarrow{(e), (f)}$ Boc-DPhe-Pro-Arg(Z)$_2$CH$_2$SCh

$\xrightarrow{(e), (g)}$ H-DPhe-Pro-Arg-CH$_2$SCh

6

(a) NMM, IBC, THF; (b) CH_2N_2-ether; (c) HBr, -20°C;
(d) ChSH, NaH, DMF, -20°C; (e) 4M HCl-dioxan;
(f) Boc-DPhe-Pro-OPfp, DMF, $^iPr_2NEt$; (g) H_2, 5% Pd/C, MeOH-H_2O-HCl

SCHEME 2 Preparation of **6**.

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Careful monitoring of the final hydrogenation to produce **6** was necessary as extended reaction times or using acetic acid as a solvent caused hydrogenolysis of the CH_2-S bond giving rise to **6** and ChSH. Partial epimerisation at arginine was observed after step (d) with complete epimerisation occurring during the remaining steps of Scheme 2.

The β-keto ester derivative of arginine required for the preparation of **7** (Scheme 3) was synthesised via a route analogous to that described by Rich[18] for Phe, Leu and Orn (Z).

Boc-Arg(Z)$_2$OH —(a), (b)→ Boc-Arg(Z)$_2$CH$_2$CO$_2$Et

→ standard coupling and deprotection steps as described in Schemes I and II

→ H-DPhe-Pro-Arg-CH$_2$CO$_2$Et **7**

(a) Carbonyldiimidazole, THF, RT; (b) EtOAc, LDA, -78°, THF, 10'.

SCHEME 3 Preparation of β-ketoester **7**.

The difluoro analogue of **7**, compound **8** was obtained via an ultrasound catalysed Reformatsky reaction of Boc-Arg(Z)$_2$H with BrZnCF$_2$CO$_2$Et as described by Thaisrivongs *et al.*[19] for Boc-Leu-H (Scheme 4). Boc-Arg(Z)$_2$H was synthesised by first reducing a mixed anhydride of Boc-Arg(Z)$_2$OH to the alcohol with NaBH$_4$ using the method of Hopkins[20] followed by oxidation with Dess-Martin periodinane.[21] This reagent was also used to oxidise the β-hydroxydifluoro ester to the β-keto ester.

Boc-Arg(Z)$_2$OH —(a), (b)→ [structure: Boc-NH–CH(CH$_2$OH)–(CH$_2$)$_3$–N(Z)–C(=NH)–NH–Z]

—(c)→ Boc-Arg(Z$_2$)H —(d)→ Boc-NH–CH(R)–CH(OH)–CF$_2$CO$_2$Et

—(e)→ Boc-DPhe-Pro-NH–CH(R)–CH(OH)–CF$_2$CO$_2$Et

—(c)→ Boc-DPhe-Pro-Arg(Z$_2$)CF$_2$CO$_2$Et

—(f)→ H-DPhe-Pro-Arg-CF$_2$CO$_2$Et **8**

(a) NMM, IBC, THF, -10°C; (b) NaBH$_4$-H$_2$O, 0°C; (c) Dess-Martin periodinane, CH$_2$Cl$_2$; (d) Zn, BrCF$_2$CO$_2$Et, DMF, ultrasound, 30'; (e) Standard coupling of Boc-DPhe-Pro-OPfp; (f) Standard acidolytic deprotection followed by hydrogenation in EtOH-H$_2$O-HCl.

SCHEME 4 Preparation of the difluoro β-ketoesters **8**.

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The trifluoroethoxymethyl ketone **9** was synthesised essentially as shown for **6** in Scheme 2 but using $Na^{+-}OCH_2CF_3$ rather than $Na^{+-}SCh$. Fluorinated ketones **10** and **11** were synhesised as depicted for **8** in Scheme 4 except that the zinc reagents $IZnCF_3$ and $IZnCF_2CF_2CF_3$ were used rather than $BrZnCF_2CO_2Et$. The zinc reagents were generated from the corresponding halides and activated zinc in DMF using ultrasound catalysis as described by Kitazume and Ishikawa.[22,23] Rather poor yields were obtained in the reaction of the zinc reagents with Boc-Arg$(Z)_2$H ($\leq 20\%$). Edwards has recently described[24] an improved general method of carrying out this reaction with peptidic aldehydes and CF_3ZnI. The lysine analogue **11a** was prepared in the same way from Boc-Lys(Z)-H. The lysine-based difluoro β-keto amides **12** and **13** were prepared likewise from the reaction product of $BrZnCF_2CO_2Et$ and Boc-Lys(Z)-H as shown in Scheme 5.

Boc-Lys(Z)-OH —cf. Scheme IV→ Boc-Lys(Z)-H

—cf. Scheme IV→ Boc-DCha-Pro-NH-CH[(CH₂)₄NHZ]-CH(OH)-CF_2CO_2Et

—(a), (b)→ Boc-DCha-Pro-NH-CH(R)-CH(OH)-CF_2CO_2Pfp

(c) → Boc-DCha-Pro-NH-CH(R)-CH(OH)-$CF_2CONHCH_2CH_2Ph$ —(e)→ Boc-DCha-Pro-Lys(Z)$CF_2CONHCH_2CH_2Ph$ → H-DCha-Pro-Lys-$CF_2CONHCH_2CH_2Ph$ **12**

(d) → Boc-DCha-Pro-NH-CH(R)-CH(OH)-$CF_2CONHEt$ —(e)→ Boc-DCha-Pro-Lys(Z)$CF_2CONHEt$ → H-DCha-Pro-Lys-$CF_2CONHEt$ **13**

Standard deprotection (see Scheme I, d,h)

(a) KOH, H_2O, MeOH; (b) PfpOH, WSCDI, CH_2Cl_2; (c) $PhCH_2CH_2NH_2$, CH_2Cl_2; (d) $EtNH_2$, CH_2Cl_2; (e) Dess-Martin periodinane, CH_2Cl_2.

SCHEME 5 Preparation of difluoro β-ketoamides **12** and **13**.

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Tcboc-Lys(Z)-OH —(see Scheme II a, b, c)→ Tcboc-Lys(Z)CH_2Br —(a)→ Tcboc-Lys(Z)CH_2OAn —(b), (c)→ Tcboc-NH-CH(side chain $(CH_2)_4$NHZ)-CH(OTBS)-CH_2OAn

—(d)→ Tcboc-NH-CH(R)-CH(OTBS)-CH_2OH —(e)→ Tcboc-NH-CH(R)-CH(OTBS)-CHO

—(f)→ Tcboc-NH-CH(R)-CH(OTBS)-CO_2Me

13a

(a) p-Methoxyphenol, NaH, DMF, -20°C; (b) $NaBH_4$, MeOH, 0°C;
(c) TBS triflate, 2-6-Lutidine, -10°C, CH_2Cl_2; (d) Ceric Ammonium Nitrate, MeCN-H_2O, 0°C;
(e) Dess-Martin periodinane, CH_2Cl_2. (f) Br_2, MeOH, H_2O, $NaHCO_3$.

SCHEME 6 Preparation of protected lysine α-hydroxy ester **13a**.

Compounds **14**, **15** and **17–21** were all prepared from the same precursor α-hydroxy ester derivative **13a** of lysine, the synthesis of which is shown in Scheme 6. The 2′,2′,2′-trichloro 1′,1′-dimethylethoxycarbonyl (Tcboc) group[25] was used for N^{α}-protection as it could be removed in the presence of -OTBS with Zn in acetic acid.[25] During reduction of the carbonyl group, the primary alcohol function was protected with *p*-anisyl which was subsequently removed with ceric ammonium nitrate.[26]

A one-step oxidation of alcohol (Scheme 6 from step d) to acid was found impossible to achieve in good yield using a variety of oxidants. However, a two-step method gave very high yields of the methyl ester. First the alcohol was oxidised with Dess-Martin periodinane[21] the aldehyde and then the latter was converted directly to the methyl ester (step f) using Br_2-MeOH-H_2O buffered with $NaHCO_3$.[27]

The Tcboc N-protecting group of ester **13a** was removed (Scheme 7) and either Boc-DCha-Pro-OH or Boc-Me-DCha-Pro-OH (derived from the former by alkylation with MeI-NaH[28]) was coupled to it by means of pentafluorophenyl or hydroxysuccinimide esters. Removal of the Boc group and reaction with sulphonyl halides, ^{t}butyl bromoacetate or reductive alkylation with ChCHO/$NaCNBH_3$ provided the precursors to compounds **17–21**. In all cases the Dess-Martin penodinane reagent was used for oxidation of the α-hydroxy ester to α-keto ester[29] followed by final deprotection.

NHZ

Tcboc-NH ... CO_2Me, OTBS **13a** —(a)→ H_2N ... CO_2Me, OTBS

(b) → Boc-DCha-Pro-NH ... CO_2Me, OTBS

(c) → Boc-Me-DCha-Pro-NH ... CO_2Me, OTBS

(g) → H-DCha-Pro-NH ... CO_2Me, OH

(d), (e) → Boc-Me-DCha-Pro-Lys(Z)CO_2Me

(f) → Me-DCha-Pro-Lys-CO_2Me **14**

(h) → R-DCha-Pro-NH ... CO_2Me, OH

(e), (f) → R-DCha-Pro-Lys-CO_2Me

17 R = $PhSO_2$-
18 R = $MeSO_2$-
19 R = $ChCH_2$-
20 R = HO_2C-CH_2-
21 R = $(HO_2C$-$CH_2)_2$-

(a) Zn, HOAc; (b) Boc-DCha-Pro-OPfp; (c) Boc-Me-DCha-Pro-OPfp; (d) 1M Bu_4NF in THF; (e) Dess-Martin periodinane; (f) Standard deprotection (see Scheme I); (g) 4M HCl-dioxan; (h) $PhSO_2Cl$, Et_3N, CH_2Cl_2 or $MeSO_2Cl$, Et_3N, CH_2Cl_2 or ChCHO, $NaCNBH_4$, MeOH, HOAC or $^tBu_2O_2CCH_2Br$ (1.2 equiv.), K_2CO_3, DMF or $^tBuO_2CCH_2Br$ (excess), K_2CO_3, DMF.

SCHEME 7 Preparation of lysine α-ketoester derivatives **14**, and **17–21**.

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It was subsequently found that partial reduction of α-keto ester to α-hydroxy ester could sometimes occur during the final hydrogenolysis. This was obviated by the use of Boc rather than Z for the protection of the Lys side-chain (Scheme 8).

NHBoc
Tcboc-N(H)–CH–CH(OTBS)–CO_2Me —Scheme VII→ Z-DCha-Pro-N–CH–CH(OTBS)–CO_2Me
—(a)→ H-DCha-Pro-N(H)–CH–CH(OTBS)–CO_2Me
—Scheme VIII→ R-DCha-Pro-Lys(Boc)CO_2Me
↓ (b)
R-DCha-Pro-Lys-CO_2Me
17 - 21

(a) H_2, 5% Pd-C, MeOH; (b) 4M HCl-dioxan

SCHEME 8 Alternative synthesis of lysine α-ketoester derivative avoiding final hydrogenation.

α-Keto acid **15** was produced either by hydrolysis of the protected precursor to methyl ester **14** using 1 M KOH-MeOH followed by deprotection, or by leaving peptide **14** in 0.5 M $KHCO_3$ for several hours followed by hplc purification.

α-Ketol **16** was easily accessible *via* a variation of Scheme 6 using ϵ-Boc protection for lysine (Scheme 9).

NHBoc
—cf. Scheme VI→ Tcboc-N(H)–CH–CH(OH)–CH_2–OAn —(a), (b)→ Boc-Me-DCha-Pro-N(H)–CH–CH(OH)–CH_2–OAn
—(c), (d)→ Boc-Me-DCha-Pro-Lys(Boc)CH_2OH
↓ (e)
Me-DCha-Pro-Lys-CH_2OH
16

(a) Zn-HOAc; (b) Boc-Me-DCha-Pro-OPfp; (c) Dess-Martin periodinane; (d) CAN, MeCN-H_2O, 0°C; (e) 4M HCl-dioxan.

SCHEME 9 Preparation of α-ketol **16**.

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Boc-DCha-Pro-Arg(Z)$_2$OH (see Scheme I)

↓ Scheme II (steps a, b, c)

Boc-DCha-Pro-Arg(Z)$_2$CH$_2$Br

↓ (a)

Boc-DCha-Pro-Arg(Z)$_2$CH$_2$OAn

↓ (b), (c)

H-DCha-Pro-Arg-CH$_2$OH

23

(a) p-Methoxyphenol, NaH, DMF, -20°C; (b) CAN, MeCN-H$_2$O, 0°C;
(c) Standard deprotection

SCHEME 10 Preparation of arginine α-ketol **23**.

Compound **23**, the arginine analogue of **16** lacking the N-Me group was produced using similar methodology (Scheme 10).

Finally, the arginine α-keto ester **22** was produced via a cyanohydrin intermediate as shown in Scheme 11. The acid stable Tcboc group[25] was used for N^{α}-protection as hydrolysis of the cyanohydrin via an imidate required low pH. Facile generation of the cyanohydin was achieved in 65% yield by treating Tcboc-Arg(Z)$_2$-H with KCN (5 equiv.) and 1 M HCl (5 equiv.) for 16 h in a two-phase system[30] containing CHCl$_3$ or EtOAc. Formation of the imidate using MeOH-HCl was followed by *in-situ* hydrolysis at pH 1 and < 10°C (to prevent carboxamide formation[31]) to give good yields of the desired α-hydroxy ester derivative of arginine. Subsequent removal of Tcboc and coupling of Boc-DCha-Pro-OH was uneventful as was the Dess-Martin oxidation to α-keto ester. Hydrogenolysis of the two Z-groups was complete in 12 min during which only minimal reduction of the ketone occurred.

Tcboc-Arg(Z)$_2$OH —see Scheme IV→ Tcboc-Arg(Z)$_2$-H

—(a)→ Tcboc-NH-CH(side chain)-CH(OH)-CN —(b)→ Tcboc-NH-CH(side chain)-CH(OH)-C(=NH.HCl)OMe

—(c)→ Tcboc-NH-CH(side chain)-CH(OH)-CO$_2$Me —cf. Scheme VII→ → → H-DCha-Pro-Arg-CO$_2$Me

22

(a) EtOAc, KCN, 1M HCl; (b) MeOH, 4M HCl-dioxan, 4°C, 16h
(c) H$_2$O, in situ, pH 1, 5°C, 24 h.

SCHEME 11 Preparation of the arginine α-ketoester derivative **22**.

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TABLE 2
The IC_{50} TT values for compounds containing arginine ketones.

No.	R	R′	IC_{50} TT (μM)
1	Ph	$-CH_2CH_2CO$-Pro-NNHEt	1.0
3	Ph	-Ch_3	4.2
4	Ch	$-CH_3$	0.43
5	Ph		2.2
6	Ph	$-CH_2S-$	1.4
7	Ph	$-CH_2CO_2CH_2CH_3$	0.5
9	Ch	$-CH_2O-CH_2CF_3$	0.012

RESULTS AND DISCUSSION

Table 2 shows the effect of replacing the C-terminal portion $-CH_2-CH_2$-CO-Pro-NHEt of compound **1** with Me, Ch- or $ChSCH_2$-. Methyl ketones have been reported as serine protease inhibitors.[32,33] The peptide methyl ketone **3** shows a four-fold loss in activity compared to **1** demonstrating that the $-CH_2$-CO-Pro-NHEt grouping is moderately effective in binding to thrombin. As expected, the DCha analogue **4** is 10 times more active than **3**. Increasing the bulk of the group attached to the carbonyl function by changing methyl to cyclohexyl (compound **5**) only gives a modest increase in activity and moving it further away from the carbonyl function (compound **6**) results in a further small improvement. In **6** the effect of the sulphur could increase the electrophilicity of the ketone, making attack on it by the active site hydroxyl more favourable. This, rather than the presence of the cyclohexane ring, could explain the relatively low IC_{50} of **6**. (Oxidation of S to SO_2 led to a small loss of activity, data not shown). In fact, replacing the sulphur of **6** with oxygen, substituting $-CH_2CF_3$ for -Ch and DCha for DPhe provided **9** which is 80 times more potent than **1**. The β-keto ester **7** displayed only a modest two-fold improvement over **1**. An arginine β-keto amide derivative of hirudin has been reported.[34] It is 10 times less active as a thrombin inhibitor than the corresponding Arg-$\Psi[COCH_2]$Gly derivative.

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TABLE 3
The IC_{50} TT values for compounds containing α-fluoroketones.

No.	R	R′	R″	IC_{50} TT (μM)[a]
8	Ph	$-CF_2CO_2CH_2CH_3$	$-\underset{H}{N}-C(:NH)NH_2$	0.007
10	Ph	$-CF_3$	$-\underset{H}{N}-C(:NH)NH_2$	0.01
11	Ph	$-CF_2CF_2CF_3$	$-\underset{H}{N}-C(:NH)NH_2$	0.009
11a	Ph	$-CF_2CF_2CF_3$	$-CH_2NH_2$	0.045
12	Ch	$-CF_2CO\underset{H}{N}CH_2CH_2-C_6H_5$	$-CH_2NH_2$	0.021
13	Ch	$-CF_2CO\underset{H}{N}CH_2CH_3$	$-CH_2NH_2$	0.12

[a]All IC_{50} TT values were obtained after a 60 min pre-incubation of inhibitor with thrombin.

In an attempt to improve the potency of our compounds beyond that of **9** we next studied more electrophilic ketones. Abeles reported[35] in 1985 on fluoroketones as general inhibitors of various proteolytic enzymes. Since then this area of research has mushroomed with numerous reports of peptidic fluorinated ketones, α-keto esters and α-keto amides appearing in the literature (for an excellent review see Reference 36).

Table 3 shows examples of the fluoroketone derivatives which we have synthesised. The difluoro-β-keto ester[19] type **8** is an excellent inhibitor having an IC_{50} TT of 7 nM. However, this, and the other compounds in Table 3 suffer from a slow-binding behaviour[36,37,38] towards thrombin which necessitates a pre-incubation of inhibitor with thrombin before the thrombin time is measured. The slow binding can be attributed to low concentration of free ketone in solution, the major part being hydrated and thus unavailable for attack by the active site serine. Such hydration of activated peptidyl ketones[36] is well known for difluoro-β-ketoamides,[19] perfluoroalkyl ketones[39] and trifluoromethyl ketones.[40] In the case of inhibitors of aspartic proteinases[19,39] this is

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beneficial since the hydrate is the active tetrahedral transition-state mimic. For serine proteases however, the ketone/hydrate ratio is a major determinant of slow-binding kinetics.[40] Compounds **8, 10** and **11** all display similar IC_{50} TT values. Subsequent to our synthesis of **10** and **11** an independent report of **10** (MDL 73756) and its pentafluoroethyl homologue (MDL 74063) as thrombin inhibitors was published.[41] MDL 74063 was found to have a final $K_i = 0.074\ \mu M$ towards thrombin and was very slow-binding, K_{on} $9.8 \times 10^3\ M^{-1}s^{-1}$.

A further complication with arginine-based fluoroketones is that the δ-N of the arginine side-chain can attack the ketone carbonyl forming a six-membered carbinolamine as observed with arginine aldehydes.[13,42] This is another equilibrium process, like the competing hydrate formation already mentioned, that limits the concentration of free ketone available for attack by serine at the active site of thrombin.

One way to circumvent this last problem would be to use lysine at P_1 as seven-membered ring formation is not favoured. Therefore, the lysine analogue of **11**, compound **11a** was synthesised. **11a** is also a good inhibitor (5 times less active than **11**) but still exhibits slow-binding and thus demonstrates the overriding importance of hydration over carbinolamine formation.

Compound **12** containing a lysine difluoro β-keto-amide is a moderately good inhibitor, the DCha present in **12** obviously enhances binding to thrombin (cf **3** and **4**, Table 2) as does the phenyl group at the C-terminus which was incorporated to mimic the highly beneficial phenylalanine of **2**. Compound **13** demonstrates the deleterious effect of removing this phenyl group.

We next turned our attention towards α-keto esters[43,44,45] (Table 4). Initially we decided to synthesise lysine-based compounds as the chemistry was simpler and we had already shown good prolongation of thrombin time with the lysine containing compounds **11a** and **12**. We also decided to N-methylate the N-terminus as Bajusz had shown[13] that N-alkylation of tripeptide aldehyde did not significantly alter their *in vitro* activity but dramatically improved their chemical stability and thus made them more attractive as drug candidates. Our activated ketones could be subject to an inactivation mechanism similar to that described[13] for H-DPhe-Pro-Arg-H. The H-DPhe analogue of **14** (BMS 181412) was recently reported by Iwanowicz and coworkers.[46]

The α-keto ester exhibited unusual behaviour in the thrombin time assay: it was found to be (not unexpectedly) a slow-binding inhibitor but also to become more active with time after pre-incubation in pH = 7.4 buffer alone. BMS 181412 was found[46] to have an IC_{50} TT value of 0.032 μM after a 3 min pre-incubation with thrombin, a value which agrees very well with that of **14** (10 min pre-incubation). However, on longer incubation (10–120 min) **14** became even more active, its potency approaching the nanomolar range. A pure diastereomer of **14** was shown to be hydrolysed completely to the acid **15** at pH = 7.4 in 24 h with simultaneous racemisation at the Lys α-carbon. At pH = 8.5 and 20°C, **14** decomposed to give Me-DCha-Pro diketopiperazine. Since formation of this compound requires attack by the MeNH-group of DCha on the -CO- of Pro we studied protection of the amine function in **14**. We expected a loss in activity by removing basicity at the N-terminus therefore we made two compounds (**19, 20**) that retained the basic

TABLE 4
The IC_{50} TT values for compounds containing lysine α-keto derivatives.

No.	R	R′	R″	IC_{50} TT (μM)[a]
14	CH_3–	H	$-CO_2CH_3$	0.031
15	CH_3–	H	$-CO_2H$	0.007
17	C_6H_5–SO_2–	H	$-CO_2CH_3$	0.009
18	CH_3-SO_2	H	$-CO_2CH_3$	0.007
19	C_6H_{11}–CH_2–	H	$-CO_2CH_3$	0.011
20	HO_2C–CH_2–	H	$-CO_2CH_3$	0.009
21	HO_2C–CH_2–	HO_2C–CH_2	$-CO_2CH_3$	0.009

[a]All IC_{50} TT values were obtained after a 10 min pre-incubation of inhibitor with thrombin prior to adding human plasma.

NH but had larger substituents in place of the N-methyl group of **14**. Surprisingly, all the N-derivatised compounds **17–21** proved more potent thrombin inhibitors than **14**. The methanesulphonyl compound **18** having an acidic NH at the N-terminus was the most potent and even the more sterically demanding benzene sulphonamide **17** was almost as active. The mono- and di-carboxymethyl compounds **20** and **21** proved equipotent with **17**. These modifications did serve to stabilise these molecules against fragmentation giving diketopiperazine but the methyl ester functions present in **17–21**, were as labile as that of **14**. This variety of permissible N-terminal groups offers a potentially attractive way of modifying the *in vivo* pharmacological properties of these molecules.

In view of the excellent IC_{50} TT values displayed by the lysine α-keto esters it was logical to synthesise the arginine analogues which we expected to be much more potent. To our disappointment the arginine α-keto ester **22** (Table 5) was considerably less active than **14** in the TT assay. Compound **22** is lacking the N-CH_3 of **14** but we did not envisage this as being the cause of reduced potency. This was confirmed

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TABLE 5
Comparison of IC_{50} TT values for compounds containing Arg and Lys α-keto esters and α-ketols.

No.	R	R′	R″	IC_{50} TT (μM)
14	CH_3	$-CH_2NH_2$	$-CO_2CH_3$	0.031
16	CH_3	$-CH_2NH_2$	$-CH_2OH$	0.1
22	H	–N–C(:NH)NH_2 H	$-CO_2Me$	0.49
23	H	–N–C(:NH)NH_2 H	$-CH_2OH$	0.02

by synthesis of the α-ketol analogues **16** and **23**. The latter was more active than its lysine analogue **16** and also more active than the arginine α-keto ester **22** (although the lysine ketol **16** was less active than **14**). Comparison of **14** and **16** illustrates the dramatic effect of the carboxylate group α to the carbonyl of **14**. The inactivity of **22**, was all the more surprising in view of the recently discovered cyclotheonamides.[47] These cyclic pentapeptide analogues contain an arginine α-keto amide residue and are moderate inhibitors of thrombin but potent inhibitors of trypsin and streptokinase. The total syntheses of cyclothenoamides B[48] and A[49,50] have been described. The latter uses methodology similar to that described in Scheme 11 for producing a precursor arginine α-keto acid derivative. An X-ray study of cyclotheonamide A complexed with thrombin[50] at 2.3 Å resolution has confirmed that the ketone carbonyl of the arginine α-keto amide forms a hemi-ketal transition-state mimic with the active site serine hydroxyl. Therefore, why should our α-keto esters of arginine be relatively inactive? The facile hydrolysis of the methyl-ester of **14** (pH 7.4) already described, coupled with the known[13,42] propensity of the δ-N of the arginine side-chain to attack an activated α-carbonyl forming a 6-membered carbinolamine leads us to propose the explanation shown in Figure 2 for the low activity of **22**. Hydrolysis of the ester and formation of a carbinolamine gives a thermodynamically favoured structure which is highly stable by virtue of a salt-bridged six-membered ring and a hydrogen-bonded five-membered ring (Figure 2).

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ASSAY CONDITIONS

pH 7.4

22 (H_2O, pH 4)

STABLE INACTIVE FORM

FIGURE 2 Proposed mechanism to explain the inactivity of arginine α-keto ester **22** as a thrombin inhibitor.

A time-course study of the pre-incuation of **22** with thrombin, followed by measurement of the TT, has not been carried out. However, it would be very interesting to find out whether **22** will achieve better inhibition of thrombin than **14** does, given a sufficiently long incubation time. It is possible that the inactive carbinolamine shown in Figure 2 is so stable that, for all practical purposes, no equilibrium exists between it and the free ketone.

The use of slow-binding inhibitors of thrombin as anti-coagulant drugs is open to criticism. *In vivo*, thrombin is constantly generated in plasma and thrombin inhibitors primarily function by slowing thrombin formation through inhibiting thrombin-mediated amplification steps.[51] To slow such amplification cascades, a fast-binding inhibitor would be preferable. A larger dose of a slow-binding inhibitor would be needed to achieve the same effect,[2] with a correspondingly increased risk of side-effects.

Thrombin is trapped within fibrin clots[51] and a burst of thrombin is released when clots are lysed. This increases the danger of re-occlusion.[52] Ideally, a thrombin inhibitor should be able to quickly neutralise this burst of thrombin activity if it was to be used successfully as an adjunct to thrombolytic therapy.

These considerations led us to rule out further study of highly electrophilic ketones of arginine or lysine. Compound **9** IC_{50} TT = 0.012 μM (Table 2) (no pre-incubation) was therefore chosen for further investigation in view of its low molecular weight and freedom from slow-binding behaviour.

A future publication will describe optimisation of **9** leading to highly potent and selective, low molecular weight inhibitors of thrombin.

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