Pergamon

Bioorganic & Medicinal Chemistry, Vol. 3, No. 8, pp. 1063–1078, 1995

0968-0896/95 $9.50 + .00

0968-0896(95)00105-0

Amide and α-Keto Carbonyl Inhibitors of Thrombin Based on Arginine and Lysine: Synthesis, Stability and Biological Characterization

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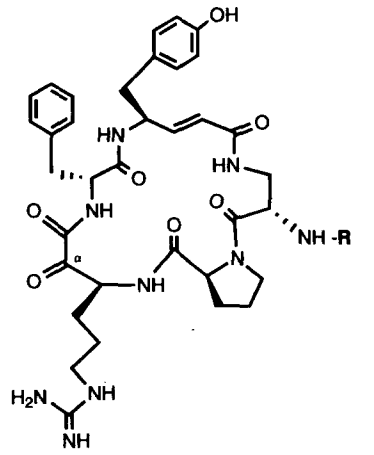
Abstract—We report structure–activity investigations in a series of tripeptide amide inhibitors of thrombin, and the development of a series of highly potent active site directed α-keto carbonyl inhibitors having the side chain of lysine at P_1. Compounds of this class are unstable by virtue of reactivity at the electrophilic carbonyl and racemization at the adjacent carbon (CH). Modifications of prototype α-keto-ester **8a** have afforded analogs retaining nanomolar K_i. Optimal potency and stability have been realized in α-keto-amides **11b** (K_i = 2.8 nM) and **11c** (K_i = 0.25 nM).

Introduction

Inhibitors of the enzyme thrombin, a key serine protease within the blood coagulation cascade,[1] have for some time been considered as potential candidates for anticoagulant prophylaxis and therapy.[2] In particular, the multiple roles played by thrombin in its actions on coagulation factors, circulating blood components, and the cells of the vessel wall make it a particularly attractive target in a variety of pathological states.[3] Moreover, limitations associated with currently employed anticoagulants, in particular the occurrence of hemorrhagic complications, highlight the need for more specifically-acting agents.

Recent studies in these laboratories of the protease-inhibitory properties of the novel natural product cyclotheonamide-A (CyA) (Fig. 1) have shown it to exert its anti-thrombin action via an initial slow-binding process.[4] Early attempts at molecular modeling, subsequently supported by the results of X-ray crystallography,[5a,5b] suggest the formation of a covalent intermediate which mimics the postulated tetrahedral species in the transition state during substrate proteolysis. This mode of action places CyA within the class of direct-acting inhibitors of thrombin considered to be 'reaction intermediate-based'[6a] or 'reversible covalent'.[6b] Structural parallels between CyA and known synthetic thrombin inhibitors, in particular the electrophilic arginyl α-keto-amide residue, prompted us to consider the natural product as a model for possibly novel synthetic inhibitors of thrombin.

CyA **(R = CHO)** ***CyB*** **(R = $COCH_3$)**



Isolation/structure: Fusetani, N.; Matsunaga, S.; Matsumoto, H.; Takebayashi, Y. *J. Am. Chem. Soc.* **1990**, *112*, 7053.

Synthesis: Hagihara, M.; Schreiber, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 6570 (CyB); Maryanoff, B. E.; Qiu, X.; Padmanabhan, K. P.; Tulinsky, A.; Almond, Jr H. R.; Andrade-Gordon, P.; Greco, M. N.; Kauffman, J. A.; Nicolaou, K. C.; Liu, A.; Brungs, P. H.; Fusetani, N. *Proc. Natl Acad. Sci. U.S.A.* **1993**, *90*, 8048 (CyA); Wipf, P.; Kim, H. *J. Org. Chem.* **1993**, *58*, 5592 (CyA).

Figure 1.

A number of examples of electrophilic carbonyl-containing compounds which exhibit high affinity for thrombin are known,[7] which also retain the guanidinopropyl side chain of arginine in the specificity

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pocket (S_1), corresponding to the preference of the enzyme for basic amino acids, arginine in particular, at the P_1 position in natural substrates. Ligands which mimic the P_2 and P_3 positions are moieties that enter into specific non-covalent interactions with the corresponding S_2 and S_3 subsites on the enzyme. We are fully aware that the specificity may differ significantly between the initial recognition and the covalent intermediate states. In our current effort we assessed a number of tripeptide C-terminal amides, chosen to mimic substrate-like binding interactions with the enzyme, as an expedient guide to the incorporation of α-keto-carbonyl residues as mimetics of the P_1–P_1' (Arg-X) scissile bond. We also hoped the amides would comprise a set of SAR affording enhanced selectivity for thrombin over trypsin. Parallel to this effort, we explored routes for the synthesis of precursors of α-oxy derivatives having the side chains of lysine (aminobutyl) and arginine (guanidinopropyl).

Two specific structural types were made (see Fig. 2): a series of substrate-like analogs represented by general structure **I** (X = $-CH_2NH_2$ or X = $-NHC(=NH)NH_2$), and a set of potential transition state analogs of general type **II** (Y = $-OCH_3$ or -N<), capable of presenting the electrophilic carbonyl function to interact with the active-site serine (S-195) of thrombin.

Substrate-like Inhibitors

We chose initially to study a series of readily accessible tripeptide amides in an effort to establish an optimal sequence for a prototype keto-carbonyl thrombin inhibitor. Simple amides were selected because of their relative inertness as thrombin substrates, but nonetheless having the scissile carbonyl appropriately disposed for enzyme interaction. The sequence -D-Phe-Pro-Arg-, common to many examples of potent thrombin inhibitors[6] and exemplified by **1a** (Table 1), was chosen as the basis structure for the present investigation. We were initially encouraged by

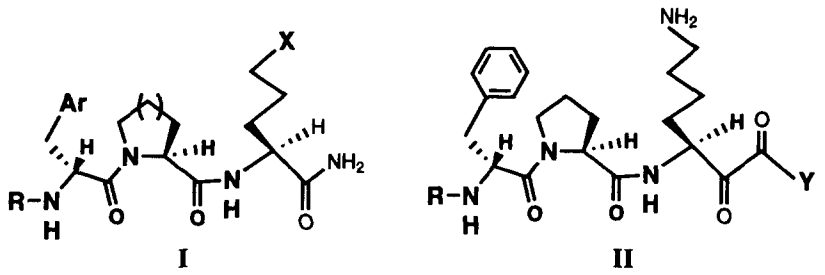


Figure 2.

Table 1. Enzyme-inhibitory activities of substrate-like tripeptide amides varied in the P_3 position[a]

No.	Compound P_3 P_2 P_1	K_i (μm) thrombin	trypsin
1a	(H)D-Phe-Pro-Arg-NH_2	0.77	43
1b	(H) N-Me-D-Phe-Pro-Arg-NH_2	0.63	8
1c	N $(Me)_2$-D-Phe-Pro-Arg-NH_2	31	314
1d	Ac-D-Phe-Pro-Arg-NH_2	189	229
1e	Boc-D-Phe-Pro-Arg-NH_2	5	4.8
1f	$C_6H_5CH_2CH_2CO$-Pro-Arg-NH_2	125	-
1g	(H) L-Phe-Pro-Arg-NH_2	> 1000	-
1h	(H) D-Ph-Gly[b]-Pro-Arg-NH_2	18	30
1i	(H) D-*h*-Phe[b]-Pro-Arg-NH_2	30	12
1j	(H) D-α-Me-Phe-Pro-Arg-NH_2	1.4	2.3
1k	(H) D-3-Ph-Lactyl-Pro-Arg-NH_2	38	54
1l	(H) D-Tyr-Pro-Arg-NH_2	0.60	100
1m	(H) D-Nal(1)[b]-Pro-Arg-NH_2	0.50	19
1n	(H) D-Nal(2)[b]-Pro-Arg-NH_2	0.60	202
1o	(H) D-penta-Me-Phe-Pro-Arg-NH_2	16	165
1p	(H) D-Trp-Pro-Arg-NH_2	11	110
1q	(H) D-β-(3-benzothienyl)-Ala-Pro-Arg-NH_2	1.4	51
1r	(H) D-β-(3-pyridyl)-Ala-Pro-Arg-NH_2	8.2	105
1s	D-*p*-Cl-Phe-Pro-Arg-NH_2	0.11	10.4

[a]Enzyme-inhibitory potencies were determined according to a standardized protocol for assay of fast-binding inhibitors (see Experimental).
[b]Abbreviations: -Ph-Gly- = phenylglycyl, -*h*-Phe- = homophenylalanyl, -Nal(1)- = l-naphthylalanyl, -Nal(2) = 2-naphthylalanyl, -Aze- = azetidine-2-carboxy, -Pip- = piperidine-2-carboxy, -Amf- = *p*-aminomethyl-phenylalanyl.

the finding of sub-micromolar potency (K_i) exhibited by **1a**, which suggested that inhibition differences observed in the amide series (type I) would enable us to apply the SAR to analogs in the α-keto-carbonyl series (type II). Syntheses were accomplished in a straightforward fashion by solid phase methods, followed by cleavage from solid support and purification by preparative HPLC. Variants made at each of the three positions are depicted in Tables 1–3, along with relative potencies for thrombin versus trypsin.

Thus, in accord with a number of published findings,[7b,8] N-methylation (**1b**) has little effect on potency. Acetylation, on the other hand (**1d**), markedly reduces inhibitory activity, whereas the Boc derivative **1e** largely retains activity, suggesting the importance of added hydrophobic interaction. Deletion of the amine (**1f**), N-dimethylation (**1c**), or replacement with a hydroxyl group (**1k**) reduce potency somewhat, which is consistent with the elimination or attenuation of a key specific hydrogen-bond interaction from the P_3 NH to the carbonyl oxygen of Gly(216) on the enzyme.[9] Both chirality (**1g**) and the length of the phenyl-containing side chain is important as demonstrated by comparable losses of potency in the shortened Ph-Gly (**1h**) and *h*-Phe (**1i**) analogs, respectively. On the other hand, retention of potency upon α-methylation (**1j**) is noteworthy, as one might propose a conformation-fixing role for the added methyl. Modified aromatic residues in place of phenyl in **1a** have varying effects, with no clear pattern emerging (**1m–1s**); one residue, D-*p*-chloro-Phe (**1s**) does afford a 7-fold potency enhancement over **1a**. In general, the P_3 position seems to be more sensitive to modification in substrate-like analogs, relative to transition state inhibitors, for instance C-terminal aldehydes.[7b]

Of a group of P_2 substitutions for prolyl in **1a** (Table 2), all are poorly tolerated except for the 4-membered azetidinyl ring (**2b**). Even the minimally altered proline analogs (**2d** and **2e**) result in large potency loss.

Of possible significance in our investigation was the general observation in the ^{1}H NMR spectrum of a characteristic upfield shift of one of the proline δCH by approximately 1 ppm from its usual value of *ca* 3.5 ppm. The shielding effect was observed in all analogs containing the -D-Phe-Pro- segment. This observation, which is ascribable to the proximity of the phenyl ring to the proline, has been reported by other investigators,[10a] and is also consistent with positioning of proline as a hydrophobic ligand within the P_2 pocket at the active site, as evidenced by results of crystallographic studies on related compounds.[10b]

Table 2. Enzyme-inhibitory activity of substrate-like tripeptide amides varied in the P_2 position[a]

No.	Compound	K_i (μm)	
	P_3 P_2 P_1	thrombin	trypsin
1a	(H) D-Phe-Pro-Arg-NH_2	0.77	43
2a	(H) D-Phe-Ala-Arg-NH_2	73	90
2b	(H) D-Phe-Aze[b]-Arg-NH_2	0.87	26
2c	(H) D-Phe-Pip[b]-Arg-NH_2	5.2	213
2d	(H) D-Phe-*t*-4-OH-Pro-Arg-NH_2	429	–
2e	(H) D-Phe-thio-Pro-Arg-NH_2	50	27
2f	(H) D-Phe-N-Me-Phe-Arg-NH_2	> 1000	–
2g	(H) D-Phe-Val-Arg-NH_2	129	595
2h	(H) D-Phe-Phe-Arg-NH_2	459	–
2i	(H) D-Phe-Glu-Arg-NH_2	40	576

[a]Enzyme-inhibitory potencies were determined according to a standardized protocol for assay of fast-binding inhibitors (see Experimental).
[b]Abbreviations: -Ph-Gly- = phenylglycyl, -*h*-Phe- = homophenylalanyl, -Nal(1)- = 1-naphthylalanyl, -Nal(2) = 2-naphthylalanyl, -Aze- = azetidine-2-carboxy, -Pip- = piperidine-2-carboxy, -Amf- = *p*-aminomethyl-phenylalanyl.

Table 3. Enzyme-inhibitory activity of substrate-like tripeptide amides varied in the P_1 position[a]

No.	Compound	K_i (μm)	
	P_3 P_2 P_1	thrombin	trypsin
1a	(H) D-Phe-Pro-Arg-NH_2	0.77	43
3a	(H) D-Phe-Pro-D-Arg-NH_2	21	481
3b	(H) D-Phe-Pro-N-Me-Arg-NH_2	12	> 1000
3c	(H) D-Phe-Pro-Lys-NH_2	368	350
3d	(H) D-Phe-Pro-Amf[b]-NH_2	118	124

[a]Enzyme-inhibitory potencies were determined according to a standardized protocol for assay of fast-binding inhibitors (see Experimental).
[b]Abbreviations: -Ph-Gly- = phenylglycyl, -*h*-Phe- = homophenylalanyl, -Nal(1)- = 1-naphthylalanyl, -Nal(2) = 2-naphthylalanyl, -Aze- = azetidine-2-carboxy, -Pip- = piperidine-2-carboxy, -Amf- = *p*-aminomethyl-phenylalanyl.

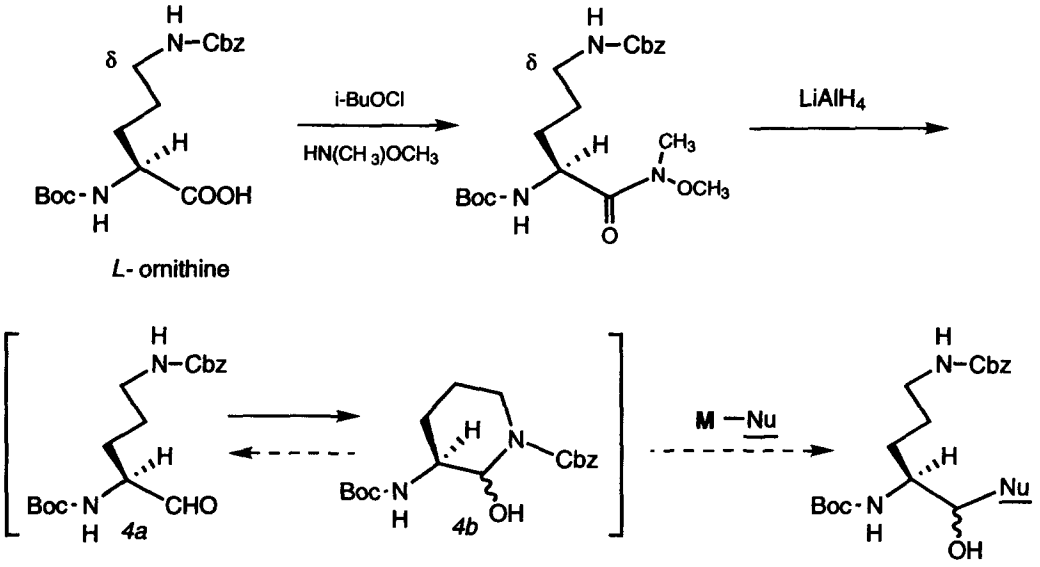
A limited set of P_1 replacements for arginine in **1a** (Table 3) resulted in clear potency loss. As expected, the D-Arg residue (**3a**) reduced potency by over 20-fold, N-methylation (**3b**) by somewhat less. Drastic loss of potency experienced when guanidine (Arg) was replaced by amine (Lys, **3c** or *p*-aminomethyl-Phe, **3d**) led us to consider substitutions at P_1 as less useful than those at P_2/P_3 in guiding extrapolation to keto-carbonyl analogs.

Keto-carbonyl Inhibitors

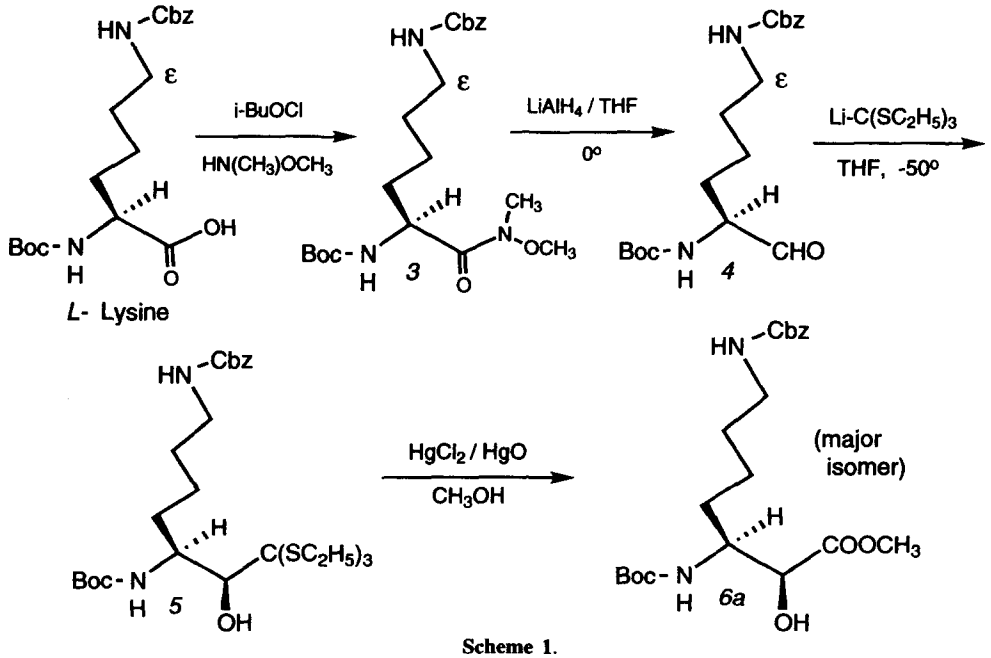
Proceeding from CyA, a stable prototype enzyme inhibitor offering potential binding elements in both P and P' regions vis-à-vis the enzyme, we were prompted to consider design of prototype keto-carbonyl thrombin inhibitors based on the α-keto-arginyl residue found in CyA. The inhibitory action of CyA against thrombin and other serine proteases had previously been characterized as 'time-dependent' (slow-binding), with a measured K_i = 1.0 nM upon full enzyme–inhibitor equilibration prior to assay.[4]

Initially our synthetic plan encompassed an approach to the α-keto-arginyl residue in CyA via its aminopropyl precursor, as depicted in Scheme 1(A). Proceeding from protected L-ornithine, the *N,O*-dimethylhydroxamide could be obtained without difficulty, but treatment under standard reduction conditions failed to

(A) Attempted synthesis of keto-Arg precursor.



(B) Synthesis of keto-lysine precursor.



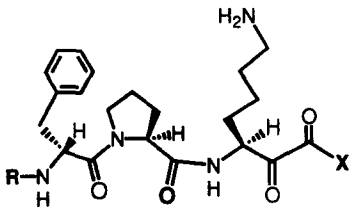
Scheme 1.

yield any usable amount of aldehyde **4a**. Spectral evidence suggested that product was mainly in the form of the 6-membered cyclic aminal **4b**.

Parallel to this effort we approached the homologous aminobutyl precursor by an analogous route, starting from protected L-lysine [Scheme 1(B)]. During the course of our investigation there appeared a report of the synthesis of α-keto-ester inhibitors of thrombin derived from lysine[11] by similar methodology, specifically the *O*-methylhemiketal of **8a** and its precursor α-hydroxy-ester isomers **13a** and **13b**, but lacking a full experimental description. We accomplished the four-step conversion to hydroxy ester **6a** along lines similar to previously reported work.[11,12] Thus, the procedure of Castro and Fehrentz[13] was applied to α-Boc, ε-Cbz-L-lysine via the *N,O*-dimethylhydroxamide amide **3** to afford aldehyde **4** in 89% overall yield, with no evidence for the presence of any of the 7-membered cyclic aminal corresponding to **4b**. The two subsequent steps, coupling with lithio tris-ethylthiomethane followed by mercuric-assisted methanolysis of the mixture of orthothioester diastereoisomers **5**, afforded isomeric hydroxy esters **6a** and **6b** in a roughly 5:1 ratio as determined by NMR. Optimal conversion of **4** to **5** was somewhat sensitive to temperature control during and after addition of the organolithium reagent, the reaction proceeding best under a condition of warming from −50 to −30 °C over a period of 30–60 min.

Intermediate **6a** could be made in quantity and was well disposed for elaboration at the N- or C-terminus, to provide various analogs of interest. In initial studies, the dipeptide (P_2–P_3) units of -D-Phe-Pro- and -*N*-Me-D-Phe-Pro- were individually incorporated to afford, ultimately, the α-keto- and α-hydroxy-carbonyl derivatives shown in Tables 4 and 5, respectively. At an early stage of this work the keto-ester **8a** (see Scheme 2) served as the prototype inhibitor in this series. Although it proved to be both chemically and chirally unstable under conditions of enzyme assay (see below), ester **8a** helped define some of the key chemical issues we had to address in working with the α-keto-carbonyl class of compounds. The final steps in the synthesis of **8a** were

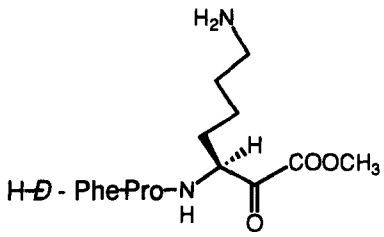
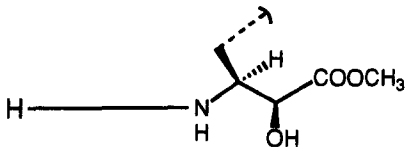
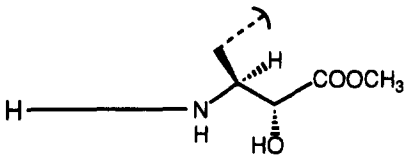
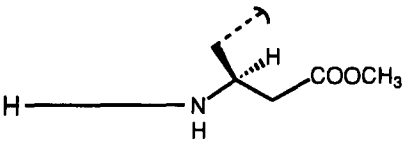
Table 4. Chemical and biological properties of analogs



			K_i (nM)		$t_{1/2}$	
No.	R	X	Thrombin	Trypsin	Chem.[a]	Racem.[b]
8a	H-	$-OCH_3$	0.25	1.8	2 h.	---
11a	H-	$-NHCH_3$	1.6	12	2 d.	*ca.* 6 h.
11b	CH_3-	$-NHCH_3$	2.8	7.8	stable	*ca.* 4 h.
11c	CH_3-	$-NH_2$	0.25	2.6	"	*ca.* 1 d.
11d	CH_3-	$-N(CH_3)_2$	27	1500	"	< 10 m.[c]
11e	CH_3-		4.8	530	"	< 10 m.[c]
11f	CH_3-		0.5	60	"	ND[d]
11g	CH_3-	$-NHCH_2CH_3$	3.1	4.2	"	ND[d]
11h	CH_3-	$-NHCH_2COOH$	15	8.5	"	ND[d]
11i	CH_3-	$-NHCH_2$ 	0.6	0.5	"	ND[d]

[a]Determined by HPLC analysis of solution at pH 7.4.
[b]Determined by ^{1}H NMR of equilibration of $^{\alpha}$CH to $^{\alpha}$CD at pH 7.4.
[c]^{1}H NMR study shows complete equilibration of $^{\alpha}$CH to $^{\alpha}$CD within 15 min at pH 7.4.
[d]Not determined.

Table 5. Potencies of hydroxy-esters

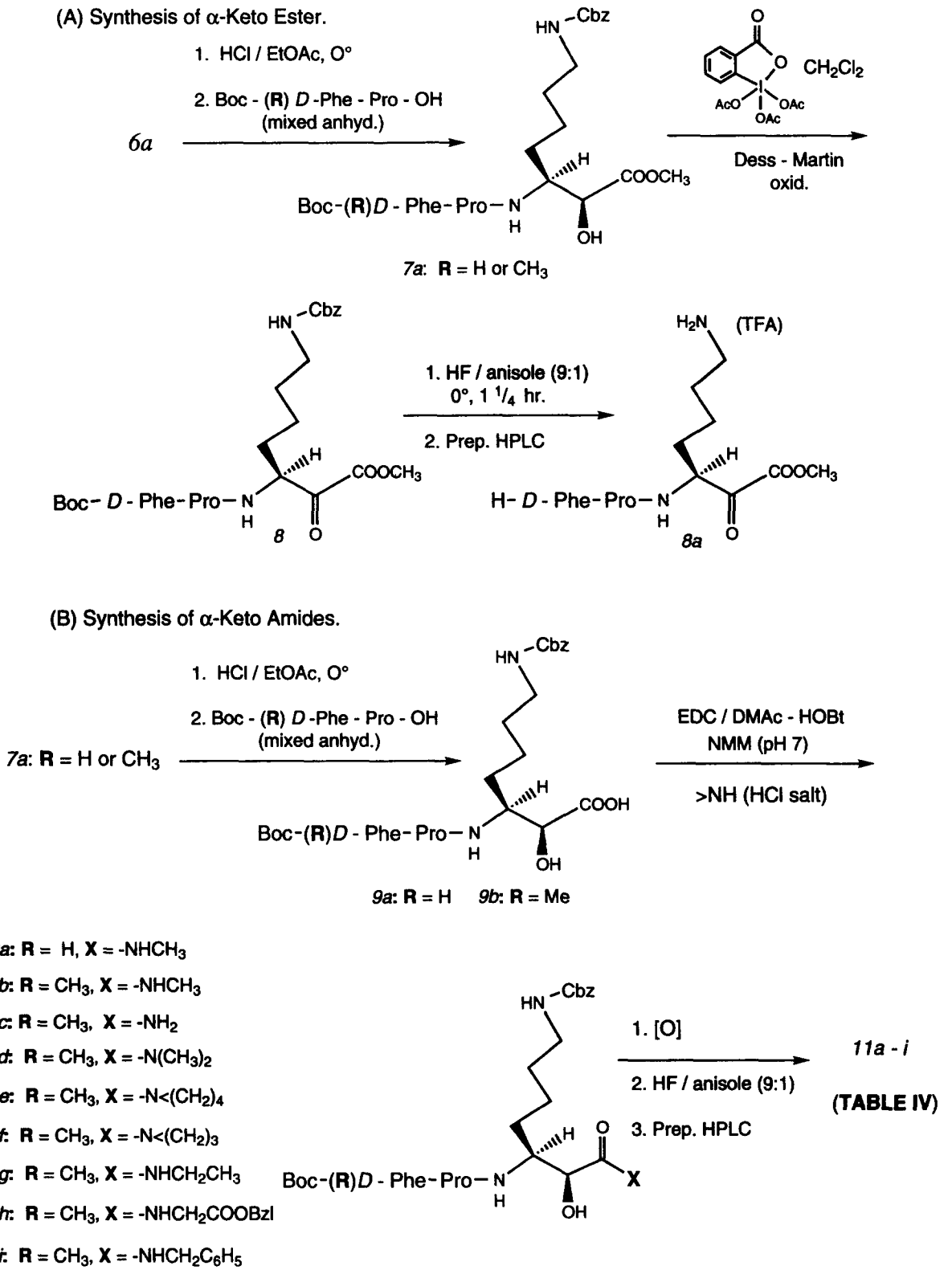
No.	Structure	K_i (nM) Thrombin	Trypsin
8a		0.25	1.8
13a		4100	2000
13b		8100	5600
14		30400	184000

carried out by coupling hydroxy ester **6a** as shown, after removal of the Boc group, under mixed anhydride conditions, to give product **7a** (R = H) in high yield. The mild pentavalent iodine Dess–Martin (periodinane) oxidant was conveniently employed for the conversion of hydroxyl to ketone in **7a** (R = H), as applied previously in a similar system,[14] and with the hoped-for result of preserving the stereo-integrity of the adjacent (*S*) chiral carbon. Efficient conversion was inferred from TLC data, and the crude α-keto ester **8** was treated with anhydrous HF to cleave the protecting groups, followed by preparative HPLC to isolate final product **8a**. In an analogous fashion hydroxy ester **6a** was coupled as above to give product **7a** (R = CH_3), precursor to N-methyl analogs **11b–i**.

Keto-ester **8a** was isolated as a mixture consisting almost entirely of the hydrated form (-$C(OH)_2$-), as clearly evidenced from the presence of a signal for the αCH at 4.13 ppm (hydrated C=O) in the ^{1}H NMR spectrum (D_2O). A weak αCH signal at 4.93 ppm indicated the presence of ≤ 5% of the keto (-C=O) form. Compound **8a** was stable for days in solution at ambient pH (*ca* 3.5), but when stored at pH 7.4 (Tris buffer) underwent rapid degradation to multiple constituents ($t_{1/2}$ ≤ 2 h). ^{1}H NMR studies also established that the (*S*) configuration at the α-carbon of the starting lysine was retained throughout the synthesis and isolation process. Reliable enzyme assays were possible when carried out under strict conditions of protocol and time limitations (see below).

Choice of C-terminal modification (P_1' amides) was implemented, starting from hydroxy esters **7a** (R = H) or **7a** (R = CH_3), after saponification in mixed aqueous/THF medium to afford acids **9a** and **9b** by coupling with appropriate amine hydrochlorides to obtain examples of primary, secondary, and tertiary hydroxy amides **10a–i** in high yield (Scheme 2). The oxidative and protecting group removal steps were then applied as above with the ester **7a** (R = H), to afford final products **11a–i** (see Table 4).

Characteristic physico-chemical distinctions among these compounds were apparent upon comparison of spectral data and the stability (HPLC) profile under



Scheme 2.

conditions of the enzyme assay (0.05 M Tris, 0.15 M NaCl, 0.1% PEG, pH 7.4). By ^{1}H NMR, amides **11a–i** exhibited a measurable proportion of keto form at ambient pH (*ca* 4.5) in D_2O, in contrast to ester **8a**, signifying hydration as a property dependent upon the nature of the C-terminal substitution. Both *N*-methylamides **11a** and **11b** were shown to consist of 80–90% of the hydrated species, and the unsubstituted amide **11c** showed slightly more (> 90%) hydrate. On the other hand, the presence of > 95% of keto species (see Experimental) in the *N,N*-dimethylamide **11d** indicates that the hydrated form is destabilized by substitution at the C-terminal amide nitrogen. Also, the rate of racemization is seen to correlate with the amount of keto

form (electron-withdrawing), since in marked contrast to **11b** and **11c**, the αCH of **11d** is seen to undergo nearly complete exchange ($t_{1/2}$ < 10 min) at pH 7.4, making that position far more labile configurationally by virtue of the disubstitution at the amide. The similarly substituted pyrrolidyl amide **11e**, which also exists essentially entirely in the keto form, likewise undergoes αCH exchange very rapidly at pH 7.4. In contrast, racemization of the unsubstituted α-keto amide **11c** is slow compared with any substituted example, exhibiting a half-life of about 1 day at pH 7.4, establishing the importance of free NH to chiral stability at the αCH.

Unlike ester **8a** and the corresponding amide **11a** (see above), the N-methyl derivatives **11b–i** were essentially completely stable at pH 7.4 over several days, except for the racemization at the methine (α) carbon of the lysine residue. Thus, in the enzyme assay buffer medium, equilibration to two peaks of equal intensity on HPLC was observed within varying time periods, parallel to loss of the αCH in the ^{1}H NMR spectrum (see Table 4). Stabilization of keto-ester **8a** through N-terminal methylation and C-terminal amide formation nonetheless was of value in assessing this series of inhibitors and suggested means by which α-keto-carbonyl compounds may be made more tractable in general. We view the unusual lability of compound **8a** as a combination of several factors, among which is a likely intramolecular association of the N-terminal free amine with the active (electrophilic) α-keto moiety, by analogy with a similar phenomenon detailed in a study of the thrombin inhibitor H-D-Phe-Pro-Arg(H).[8] In that case N-terminal methylation stabilized the arginal and is in agreement with the observed stabilization of **8a** and **11a** to degradation. Thus, in combination with the C-terminal amide, N-terminal methylation suppresses decomposition to allow consistent evaluation of the enzymic properties of K_i and rate of binding.

Further comparisons were made of keto-ester **8a** with the (two) isomeric hydroxy esters **13a** and **13b**, along with the keto-methylene compound **14**. As shown in Scheme 3, the major (*syn*)[11] isomer **13a** was readily obtained from an 8:1 hydroxy ester mixture **6a**, **6b** via coupling with Boc-D-Phe-Pro-OH to give **7a** (R = H), and two deprotection steps, followed by preparative HPLC to remove small amounts of **13b**. The minor (*anti*) isomer was most easily obtained from the same mixture **6a**:**6b**, after oxidation to the corresponding keto-ester and regeneration of hydroxy-ester mixture enriched in isomer **6b**. Coupling with Boc-D-Phe-Pro-OH and deprotection with liquid HF afforded hydroxy ester **13b**, which was readily isolated from the isomeric mixture. The des-oxy ester **14** (see Table 5) was prepared starting from α-Boc, ε-Cbz-L-lysine, which upon conversion to the homologous methyl ester, afforded des-oxy product **14** via an analogous sequence of operations.

*The α-keto acid corresponding to amide **11c** was also prepared and found to be of comparable inhibitory potency (K_i = 0.2 nM); this compound was shown to be moderately labile with respect to several modes of decomposition under various assay conditions similar to ester **8a**.

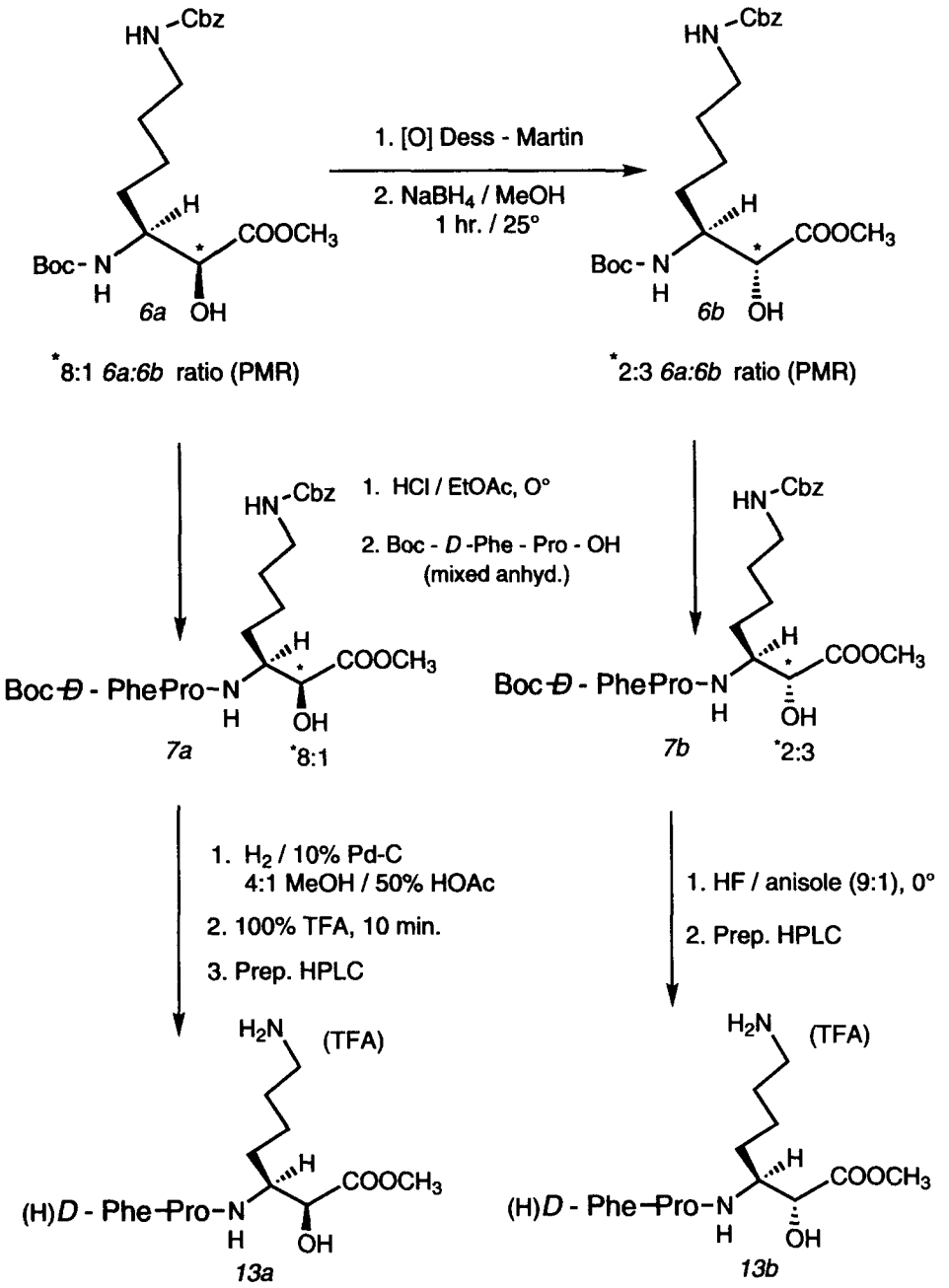
Biological Characterization and Discussion

The salient result in the ester series, as depicted in Table 4, is the finding of high potency for keto-ester **8a** (K_i = 0.25 nM). It is important that a high concentration of inhibitor be used, to quickly attain equilibrium between enzyme and inhibitor, so that degradation is minimal. Previously we had determined[4] that CyA inhibits thrombin by slow-binding kinetics via a single step (mechanism A). Using similar procedures in the present work we determined that **8a** and the other keto-carbonyl inhibitors reported in this paper are likewise slow-binding. Thus, all the α-keto-carbonyl inhibitors reported herein were pre-equilibrated with thrombin for a period sufficient to yield K_i independent of equilibration time (see Experimental). Our results are consistent with the slow-binding kinetics reported for the corresponding *O*-methyl hemiketal (BMS 181,412) reported by Iwanowicz and co-workers.[11] The parallel findings constitute evidence for the rapid equilibration of hydrated and hemi-hydrated forms of the keto-carbonyl moiety in aqueous media, irrespective of the starting form. Comparison of the much less potent hydroxy and des-oxy esters (see Table 5) shows the key contribution of the electrophilic carbonyl to productive interaction at the enzyme active site. Similar results were also observed by Iwanowicz and co-workers for the α-hydroxy-ester BMS 181,316.[11]

Amides are seen to largely retain the high potency associated with the ester, but with some noteworthy distinctions. For example, there is a significant drop in potency with substitution of the amide NH, as depicted in Table 4. Thus, only the primary amide **11c** (K_i = 0.25 nM) retains the full inhibitory activity of ester **8a**;* the *N*-methyl amide 11b (K_i = 2.8 nM) is significantly less potent, and *N*,*N*-dimethyl amide **11d** (K_i = 27 nM) is less potent still. The conclusion that increasing bulk at the P_1' site impacts to reduce inhibitory potency is suggested by this relationship. However, steric factors alone fail to account for the increase in potency seen in going from cyclic amide **11e** to **11f** (see below).

Substitution onto the amide *N*-methyl of analog **11a** is seen to have varying effects. Simple extension to the *N*-ethyl-amide **11g** results in little change in inhibitory potency, but placement of the relatively polar carboxyl function within the same space (analog **11h**) decreases potency by five-fold. By comparison, the nearly 20-fold increase in potency afforded by incorporation of phenyl in the *N*-benzyl amide **11i** strongly implicates a hydrophobic ligand–enzyme binding mode within the P_1' pocket. Such an effect may explain, at least in part, the marked recovery of potency seen in analogs **11e** and **11f**, particularly as compared with the *N*,*N*-dimethyl

Synthesis of isomeric α-Hydroxy Esters.



Scheme 3.

amide **11d**. It should be noted, however, that this reasoning cannot explain the increased potency of the azetidine amide **11f** relative to the pyrrolidine amide **11e**; perhaps stereoelectronic factors associated with strain in the 4-membered heterocycle, which result in greater localization of the amide bond electrons onto the nitrogen atom, are operative here.

As a group, the *N,N*-disubstituted amides, in particular the heterocyclic analogs **11e** and **11f**, exhibit the best selectivity (*ca* 100-fold) for thrombin over trypsin, further evidence that the pyrollidyl and azetidinyl moieties may be involved in unique interactions within the P_1' pocket. On the other hand, the free NH may be more important for interaction (H-bonding?) with trypsin than with thrombin, its absence in analogs **11d**, **11e**, and **11f** attenuating trypsin potency.

Conclusions

We have reported structure–activity investigations in a series of substrate-like inhibitors of thrombin and developed a series of highly potent active site directed α-keto-carbonyl inhibitors. Characterization of this class of compounds has been accomplished both chemically, with respect to features which contribute to lability or enhance stability, and biologically, with respect to

enzyme kinetics and mode of binding. Optimal prototypes with nanomolar-level potencies have been recognized in α-keto-amides **11b** and **11c**. Compounds of this class, however, are shown to exhibit specific lability with respect to racemization at the carbon (CH) adjacent to the electrophilic carbonyl.

Structural and biological studies of compound **11b** have been carried out which establish its mode of binding to thrombin.[15] Likewise, the kinetic and biological properties of these analogs have been further defined, and compound **11b** has been shown to exhibit significant inhibition of thrombogenesis in *in vivo* thrombosis models.[16]

Experimental

Biological assays

Human thrombin was generously supplied by Dr John W. Fenton, II (New York State Department of Health). The materials and methods for the determination of equilibrium constants (K_i) and the mechanism of inhibition for the α-keto-carbonyl inhibitors reported in this paper toward thrombin and trypsin are essentially identical to those previously described.[4] Assays were performed at room temperature in 50 mM Tris buffer pH 7.4, 150 mM NaCl, 0.1% PEG 8000, unless otherwise indicated; the assay for trypsin also included 10 mM $CaCl_2$. To protect against degradation inhibitors were stored and diluted under acidic conditions (pH 2–3) before final addition into assay buffer. Each K_i represents the composite of at least four determinations at different inhibitor concentrations. Additionally, multiple determinations of the K_i values of compounds **8a**, **11b**, and **11c** were found to fall within 20% of each other.

Substrate-like inhibitors. Apparent equilibrium constants ($K_{i,app}$) for dissociation of enzyme–inhibitor complexes were determined from studies of the dependence on inhibitor concentration of the initial rate of release of *p*-nitroaniline (pna) from Sar-PR-pna, as determined from the rate of increase of absorbance at 405 nm. The amides described in Tables 1–3, and the esters **13a**, **13b** and **14**, are fast-binding inhibitors at the concentrations necessary to achieve inhibition; hence, pre-incubation is not relevant. Typically, enzyme (5 nM) was added to a solution containing substrate (25 μM) and inhibitor (varying concentrations). In these studies, the inhibitor (I_t) was in large excess over enzyme (E_t), so the variation of the inhibited velocity (V_i) with I_t is described by equation 1. V_i and V_o are the initial rates of substrate hydrolysis in the presence and absence of I_t, respectively. The apparent inhibition ($K_{i,app}$) is related to K_i* by equation 2.

$$V_o/V_i = 1 + [I_t]/K_i^* \quad (1)$$

$$K_{i,app} = K_i^*/(1 + [S]/K_m) \quad (2)$$

Enzymic turnover of substrate-like amide inhibitors was minimal (e.g., **1a** with thrombin, < 10% hydrolysis) as determined by HPLC (data not shown); hence $K_{i,app}$ is identical to K_i reported in Tables 1–3.

Keto-carbonyl inhibitors. All of the α-keto-carbonyl compounds are slow-binding inhibitors, as evidenced by results of determination of progress curves exhibiting characteristic time-dependence (data not shown). Hence, inhibitor and enzyme were co-incubated for a period of time sufficient to allow the system to reach equilibrium (i.e. no time dependence on inhibitor concentration) before substrate addition. The enzyme-catalyzed hydrolysis of the fluorogenic substrate Z-GPR-afc[4] was monitored (SLM 8000 Spectrofluorometer, SLM Instruments, Urbana, IL) using excitation and emission wavelengths of 400 and 500 nm, respectively. Typical final reagent concentrations were: enzyme, 0.1–0.2 nM; inhibitor, 0.05–20 nM; Z-GPR-afc, 1.25–2.5 μM. When I_t and E_t concentrations were comparable, equation 3[4] was used to calculate the inhibition constant (K_i) from the dependence of substrate hydrolysis on the concentration of I_t at a fixed concentration of E_t.

$$V_i/V_o = \{[E_t] - [I_t] - K_i + \{([I_t] + K_i - [E_t])^2 + 4K_i[E_t]\}^{1/2}\}/2[E_t] \quad (3)$$

When the inhibitor (I_t) was in large excess over enzyme (E_t) equation 1 was used. Since [S] << K_m, there were minimal corrections in K_i* (equation 1) or K_i (equation 3) due to competing substrate.[4]

NMR studies

Reference 1-D proton NMR spectra were recorded for samples on a Varian XL300, a VXR-400S, or a UNITY PLUS 400 instrument with chemical shifts (δ) reported in ppm relative to sodium TMS-propanesulfonate.

Samples for NMR analysis contained approximately 1 mg of compound dissolved in 650 μL of 100% D_2O (99.96 atom% D, Isotec, Inc. or 99.9 atom% D, Aldrich Chem. Co.). ^1H NMR spectra were acquired on a Varian Unity-500 spectrometer at ambient pH, 25 °C. Typical one-dimensional spectra consisted of 64–512 transients acquired with 8K complex points using a 4500 Hz sweep width. The residual HDO signal was suppressed by weak coherent irradiation of the HDO resonance during the 1 second recycle delay. Chemical shifts are referenced internally to sodium 3-(trimethyl)-silyl-propionate-2,2,3,3-d_4 at 0.00 ppm.

^1H chemical shift assignments were made with a two-dimensional TOCSY experiment. TOCSY data sets were acquired with 1K complex points in t_2 and 512 increments in t_1 consisting of 4–32 transients per t_1 increment using a sweep width of 4400 Hz in both dimensions. The spin lock was achieved with a MLEV-17 mixing scheme for a duration of 50–65 ms preceded by a 2.0 ms trim pulse. Typical radio frequency field strengths of the mixing pulse were in the order of 9.6 kHz. Zero-filling the data matrix in t_1 resulted in a final transformed 1K by 1K matrix. A phase-shifted sine bell with Gaussian broadening in t_2 and t_1 was applied prior

to Fourier transformation. Baseline correction was performed by fitting the baseline to a second order polynomial.

Chemistry

Unless otherwise noted, all solvents and reagents were obtained from commercial sources and used without further purification. DMF was degassed before use. HPLC analyses were carried out on a Spectra-Physics SP8000 system having an SP4270 integrator with a Vydac Protein & Peptide C_{18} column, 300 Å, 5 μ, 150 × 4.6 mm, using a 0.1% TFA/H_2O–acetonitrile gradient system over 30 min, with detection by UV at 210 or 280 nm. Preparative HPLC was run on a Separations Technology ST/LAB 800B instrument fitted with a Waters 1000 PrepPak module and Delta-Pak C_{18} radial compression column, 300 Å pore size, 15 μ particle size, 13" × 2" i.d., eluting with a gradient system: 0.1% TFA:99.9% H_2O (solvent A) and 0.1% TFA:0.9% H_2O:99.0% CH_3CN (solvent B) over 30 min. FAB mass spectra were obtained on a Fisons 7070E spectrometer. Thin layer chromatography (TLC) was performed on 250 mm 2.5 × 10 cm silica gel plates (Analtech), using UV light, ceric ammonium molybdate and/or *tert*-butyl hypochlorite/starch-iodine spray for visualization. Solvent systems employed were: EtOAc:hexane (EH); $CHCl_3$:MeOH:H_2O (CMW); EtOAc:pyridine:HOAc:H_2O (EPAW); EtOAc:HOAc:isooctane:H_2O (EAIW, upper layer). Amino acid analyses were performed on a Beckman Instruments 6300 amino acid analyzer.

Abbreviations: DMF, dimethylformamide; DMAc, dimethylacetamide; NMM, *N*-methylmorpholine; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt, *N*-hydroxybenzotriazole; TFA, trifluoroacetic acid.

*Synthesis of peptide amides **1a–s, 2a–i and 3a–d**.* Except as indicated below, all the analogs listed in Tables 1–3 were synthesized by assembly on an Applied Biosystems Model 430A automated peptide synthesis instrument, using manufacturer-supplied reagents and protocols. Boc amino acids, obtained from commercial suppliers, were used throughout, in two-fold excess, with coupling mediated by DCC in either CH_2Cl_2 or DMF with 1-hydroxybenzotriazole (HOBt) catalysis. Products were cleaved from the solid support using liquid HF:anisole (9:1); the crude products contained ≥ 80% one component and were purified by preparative HPLC to a purity of > 98%. Identity was confirmed by FABMS, ^1H NMR and amino acid analysis. A typical preparation was that of peptide **1a**, which afforded 345 mg (36% overall yield) of lyophilized product: HPLC (95→5) 9.17 min; amino acid analysis (6 N HCl, 20 h) Phe 1.50, Pro 1.49, Arg 1.54; mmol mg^{-1}. FABMS m/z 418 (calcd M + H = 418); ^1H NMR (400 MHz, CD_3OD) δ (ppm) 4.38 *dd* 1H, 4.3 *m* 2H (Phe,Pro,Arg αCH), 3.5 *m* 1H, 2.63 *m* 1H (Pro δCH_2), 3.20 *t* 2H (Arg γCH_2), 3.1 *m* 2H (Phe βCH).

Peptide **1c** was prepared by exhaustive reductive methylation ($NaBH_3CN/CH_2O$–CH_3OH) of peptide **1a**. Peptides **1d** and **1f** were prepared by acylation of resin-bound intermediate, respectively, with acetic acid and hydrocinnamic acid prior to cleavage. Analog **1e** was prepared by reaction of **1a** with the reagent BOC-ON® in solution. Analogs **1j** and **1o** incorporated DL-α-Me-Phe and DL-penta-Me-Phe, respectively, via the corresponding Boc derivatives and the diastereoisomeric products were separated on preparative HPLC. For analog **3d** Amf was incorporated as the α-Boc,Cbz derivative.[17]

*Attempted synthesis of aldehyde **4a** derived from L-ornithine.* The procedure was adapted from that of Angelastro and co-workers.[18] To a solution of 11.0 g (30.0 mmol) of α-Boc,δ-Cbz-L-ornithine in 150 mL of EtOAc, under an atmosphere of N_2 and cooled to −15 °C, was added 3.33 mL of NMM, followed by 3.97 mL (4.18 g, 30.0 mmol) of isobutyl chloroformate. After 10 min, 3.09 g (31.0 mmol) of *N,O*-dimethylhydroxylamine hydrochloride was added as solid, followed by 3.3 mL of *N*-methylmorpholine (NMM), then 5 mL of DMF. The reaction mixture was allowed to warm slowly to 20 °C, and after 20 h 50 mL of H_2O was added. The mixture was stirred for 1 h, and the CH_2Cl_2 extract was washed with dilute $KHSO_4$, H_2O, dilute $NaHCO_3$, twice with dilute NaCl, dried over anhydrous Na_2SO_4, and the solvent was removed at reduced pressure to give 12.1 g (98% yield) of crude *N,O*-dimethylhydroxamide: TLC R_f (95:5:0.5, CMW) 0.73; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 5.20 *d*, 4.9 (2 NH), 4.65 *br* (αCH), 3.75 *s* (O-CH_3), 3.20 *s* (N-CH_3).

According to the procedure of Castro and Fehrentz,[13] 1.28 mL (1.28 mmol) of a 1.0 M solution of $LiAlH_4$ in THF was added slowly to an ice-cooled solution of 423 mg (1.03 mmol) of the above amide in 10 mL of ether under N_2. After 30 min a solution of 5% $KHSO_4$ was slowly added, as H_2 gas evolved initially, and a white precipitate appeared which dissolved upon addition of further H_2O. Ether was added, the water layer was drawn off, and the ether solution was washed with dilute $KHSO_4$, H_2O, saturated $NaHCO_3$, H_2O, and saturated NaCl, dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure to give an oily product identified as an equilibrating mixture of **4a** and **4b** by the ^1H NMR spectrum (300 MHz, $CDCl_3$) δ (ppm) 9.50 *s* (-CHO), 5.65 *s*/5.72 *s* [>N-C(H)(OH)-], approx. 1:2 ratio.

*Synthesis of hydroxy-esters **6a** and **6b** via aldehyde **4** derived from L-lysine.*

*Hydroxy ester **6a**.* The procedure was implemented according to Iwanowicz *et al.*,[11] as adapted from Burkhart *et al.*[12] and Angelastro *et al.*[18] To a solution of 11.4 g (30.0 mmol) of α-Boc,ε-Cbz-L-lysine in 150 mL of CH_2Cl_2, under an atmosphere of N_2 and cooled to −15 °C, was added 3.35 mL of NMM, followed by 3.95 mL (4.20 g, 30.1 mmol) of isobutyl chloroformate. After 10 min, 3.10 g (31.0 mmol) of *N,O*-dimethylhydroxylamine hydrochloride was added as solid, followed by 3.0 mL of NMM, then 2.0 mL in increments over 1 h (pH ~7). The reaction mixture was allowed to warm slowly to 20

°C, and after 20 h 50 mL of H_2O was added. The mixture was stirred for 1 h, and the CH_2Cl_2 extract was washed with dilute $KHSO_4$, H_2O, dilute $NaHCO_3$, twice with dilute NaCl, dried over anhydrous Na_2SO_4, and the solvent was removed at reduced pressure to give 13.0 g (99% yield) of crude Weinreb amide (*N,O*-dimethylhydroxamide) **3**: TLC R_f (95:5:0.5, CMW) 0.71, (1:1 EH) 0.30; HPLC 97% (100 → 5) rt 18.6 min; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 5.1, 4.9 (2 NH), 4.55 *br* (αCH), 3.73 *s* (O-CH_3), 3.20 *s* (N-CH_3).

To a solution of 7.9 g (18.6 mmol) of the amide in 230 mL of ether, cooled to –50 °C under N_2 was added 24 mL of a solution of 1.0 M $LiAlH_4$ in THF, not allowing the temperature to rise above –35 °C. After the addition, the reaction mixture was allowed to warm to 0 °C, stirred for 1.5 h, cooled again to –40 °C, and quenched by addition of 80 mL of 20% $KHSO_4$, as the temperature rose to –10 °C. The solution and suspended solids were stirred for another 2 h, followed by decantation of the solution, which was washed three times with ice-cold 1 N HCl,[19] then H_2O, dilute $NaHCO_3$, and dilute NaCl (2 ×). After drying over anhydrous $MgSO_4$ the solvent was removed under reduced pressure to give 6.11 g (90 yield) of aldehyde **4**, as a colorless oil which solidified to a crystalline product: TLC R_f (1:1 EH) 0.57; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 9.57 *s* (1 C<u>H</u>O); 7.35 *s* (5 C_6H_5), 5.18, 4.85 *bs* (2 NH), 5.10 *bs* (2 C<u>H</u>$_2$-O), 4.21 *bq* (1 αCH), 3.20 *m* (2 C<u>H</u>$_2$-N), 1.40 *s* (9 *t*-Bu); (400 MHz, CD_3OD) 4.41 *m* (1 αCH), 3.5 *m* [1 -C<u>H</u>(OD)(OCD_3)-], 3.10 *t* (2 C<u>H</u>$_2$-N). This product was used without further purification.

Lithio tris-ethylthiomethane was generated at –60 °C by the addition of 30 mL (75 mmol) of 2.5 M butyllithium in hexane to a solution of 14.9 mL (75 mmol) of tris-ethylthiomethane, stirring for 30 min. Then a solution of 6.1 g (16.7 mmol) of aldehyde **4** in 50 mL of THF was added over 30 min to the lithio anion, keeping the temperature below –50 °C. The clear yellow solution was allowed to warm to –30 °C and stirred for 2.5 h, and a solution of 8 g of NH_4Cl in 180 mL H_2O was added to quench the excess anion, followed by partition with 200 mL each of ether/H_2O, washing with dilute $KHSO_4$, H_2O, dilute $NaHCO_3$, and H_2O. After drying over anhydrous Na_2SO_4 and solvent removal under reduced pressure, flash chromatography on silica gel (3:7, EtOAc:hexane) yielded 5.37 g (57% yield) of crude product **5** as a colorless oil: HPLC (95→5) hydroxyl isomers 26.6/27.0 min (1:4 ratio).

To a solution of 5.37 g (9.61 mmol) of isomer mixture **5** in 280 mL of 12:1 (v/v) CH_3OH:H_2O was added, with stirring, 12.2 g (45.2 mmol) of $HgCl_2$ and 3.3 g (15.4 mmol) of HgO, and the suspension was stirred for 1.5 h at 25 °C, followed by 1 h at 60 °C. The cooled reaction mixture was filtered through Celite®, evaporated under reduced pressure to a small volume, and partitioned with CH_2Cl_2/H_2O, washing the aqueous with CH_2Cl_2. The organic layer was washed in turn with 200 mL each of 70% NH_4OAc, saturated NH_4Cl, and dilute NaCl, dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to give a colorless oil which solidified on standing. Trituration with hexane: EtOAc (1:4, v/v) afforded a total of 1.85 g (45% yield) of crystalline hydroxy ester **6a**: HPLC > 99% (95 → 5) 18.7 min; TLC R_f (4:6, EH) 0.23; FABMS m/z 425 (M + H = 425); ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 7.30–7.35 *d/m* (5 C_6H_5), 4.15 *d* (1 -C<u>H</u>(OH)-), 3.88 *m* (1 -NH-C<u>H</u>-), 3.70 *s* (3 -O-CH_3), 3.11 *t* (2 -NH-C<u>H</u>$_2$-), 1.39 *s* (9 *t*-butyl). The filtrate was concentrated to an oil, which was subjected to silica gel chroma-tography (2:3, EtOAc:hexane) to yield 1.44 g (35% yield) of product **6a** containing about 30% of its hydroxyl epimer **6b** (see below), which was not resolved on either TLC or HPLC. Either of these products was suitable for subsequent steps.

Hydroxy ester 6b. To a solution of 1.48 g (3.5 mmol) of the Dess–Martin periodinane[14] in 12 mL of CH_2Cl_2 was added a solution of 750 mg (1.77 mmol) of crystalline **6a**:**6b** (8:1 ratio) in 10 mL of CH_2Cl_2. The reaction was monitored by TLC (1:1, EH), and more reagent was required over the course of 2 h (5.1 mmol total). The reaction mixture was diluted with 60 mL of ether and added to a solution of 9.0 g of $Na_2S_2O_3{\cdot}5H_2O$ in 80 mL of saturated $NaHCO_3$, partitioned after 10 min stirring, and the organic phase washed with dilute $NaHCO_3$ and dilute NaCl (2 ×), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure to give solid keto-ester; TLC R_f (1:1, EH) 0.59. A solution of this sample in 25 mL of anhydrous CH_3OH was treated portionwise with 75 mg of $NaBH_4$ and stirred for 1h The reaction was cooled to 0 °C and 0.56 mL of glacial HOAc was added, followed by partition with ether/H_2O and washing with dilute $NaHCO_3$ and dilute NaCl (2 ×), drying over anhydrous Na_2SO_4, and removal of solvent under reduced pressure to give 0.71 g (100% yield) of a mixture containing about 60% of hydroxy ester **6b**, TLC R_f (1:1, EH) 0.40; ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 7.30–7.35 *d/m* (5 C_6H_5), 4.12 *d* (1 -C<u>H</u>(OH)-), 3.80 *m* (1 -NH-C<u>H</u>-), 3.74 *s* (3 -O-CH_3), 3.11 *t* (2 -NH-C<u>H</u>$_2$-), 1.42 *s* (9 *t*-butyl), along with about 40% of epimer **6a** (see above).

Synthesis of keto-ester **8a**.

Coupling to give 7a (R = H). A solution of 256 mg (0.60 mmol) of hydroxy ester **6a** (*ca* 5:1 isomers) in 10 mL of EtOAc, cooled to –25 °C under N_2, was saturated with HCl gas, taking care to keep the temperature below 0 °C, allowed to stir 10 min, then purged with N_2 for 45 min. The solvent was then removed under reduced pressure, and the residual solid was dried *in vacuo* while the following procedure was carried out.

A solution of 219 mg (0.61 mmol) of Boc-D-Phe-Pro-OH[20] in 12 mL of CH_2Cl_2 under N_2 was cooled to –15 °C and treated with 66 μL (62 mg, 0.61 mmol) of NMM, followed by 79 μL (83 mg, 0.61 mmol) of isobutyl chloroformate. After 20 min a solution of the above HCl salt in 5 mL of CH_2Cl_2 was added, followed by 75 μL of NMM. After 1 h H_2O was added, followed by extractive workup to afford, after flash chroma-tography of the crude product on silica gel (95:5:0.5

CMW), 220 mg (55%) of **7a** (R = H) as a white solid; TLC R_f (95:5:0.5, CMW) 0.32.

Oxidation/deblocking to give 8a. Similar to the oxidation of **6b** above, a solution of 218 mg (0.33 mmol) of hydroxy ester **7a** (R = H) in 10 mL of CH_2Cl_2 was treated with a total of 750 mg (1.7 mmol) of the Dess–Martin periodinane over a period of 8 h, followed by workup by adding 30 mL of ether and a solution of 1.98 g of $Na_2S_2O_3 \cdot 5H_2O$ in 20 mL of saturated $NaHCO_3$. The aqueous washes were dried over anhydrous Na_2SO_4, and the solvent was removed to afford 185 mg of a white foam, 50 mg of which was transferred to a Kel-F® reaction vessel and dissolved in 1.0 mL of anisole. Liquid HF was introduced at –70 °C to a volume of 10 mL, followed by stirring at 0 °C for 1.5 h. After removal of HF *in vacuo*, addition of 5 mL of ether and 5 mL of pet. ether afforded a tacky gum, from which the supernatant was decanted, and the solid was washed with 1:1 ether:pet. ether. The crude product was purified on semi-preparative scale HPLC (100 → 60, 60 min) to give, upon lyophilization of pooled fractions, 19 mg (38% yield) of **8a**: HPLC 95% (100 → 60, 30 min) rt 15.5 min; amino acid analysis Phe 1.36, Pro 1.38 μmol mg^{-1}; FABMS m/z 465 (M + H + CH_3OH), 451 (M + H + H_2O), 433 (M + H); ^1H NMR (500 MHz, D_2O) δ(ppm) (see Table 6).

The ^{13}C spectrum indicated three carbonyl carbon resonances at 170–180 ppm. A single resonance at 98 ppm was assigned to the hydrated carbonyl.[11]

Synthesis of keto-amides 11a–i.

Coupling to give 7a (R = CH_3). As in the synthesis of hydroxy ester **7a** (R = H) above, the Boc group was removed from 200 mg of protected intermediate **6b** (*ca* 5:1 isomers), and the crude HCl salt was added to a preformed solution of the mixed anhydride from isobutylchloroformate and 196 mg (10% excess) of Boc-*N*-Me-D-Phe-Pro-OH.[20] Following reaction protocol and workup as described above, 187 mg (58% yield) of product was obtained after chromatography on silica gel 99:1:0.1, CMW; TLC R_f (90:10:1, CMW) 0.58. A scaled-up run provided 4.5 g, of **7a** (R = CH_3) in 93% yield.

Saponification of 7a (R = H) → 9a and 7a (R = CH_3) → 9b. Identical conditions were used for each reaction. Thus, a solution of 150 mg (0.22 mmol) of ester **7a** (R = H) in 8 mL of 1:1 (v/v) THF:H_2O was treated with 0.12 mL of 2.1 N LiOH over 3 h, likewise a solution of 183 mg (0.27 mmol) of ester **7a** (R = CH_3) in 12 mL of 1:1 (v/v) THF:H_2O was treated with 0.17 mL of 2.1 N LiOH over 2 h. Each was worked up by partition with 30 mL of EtOAc and 15 mL of dilute $KHSO_4$, followed by washing with dilute NaCl, drying over anhydrous Na_2SO_4 and solvent removal at reduced pressure to give, respectively, 150 mg **9a**, TLC R_f (12:5:1:3, EPAW) 0.67, and 182 mg **9b**, TLC R_f (10:5:1:1, EPAW) 0.59. A scaled-up run provided 1.72 g of **9b** in 97% yield.

Amide formation from 9a → 10a and 9b → 10b–i. All reactions were carried out under essentially the same conditions. Thus, a solution of 199 mg (0.30 mmol) of hydroxy acid **9a** in 7.5 mL of DMAc was treated, in order, with: 60 mg (0.39 mmol) of HOBt·H_2O, 60 μL of NMM, and 42 mg (0.62 mmol) of $CH_3NH_2 \cdot HCl$, stirring until complete dissolution; then 67 mg (0.42 mmol) of EDC was added. After dissolution the pH (determined on moistened narrow-range paper) was 6.5, and the mixture was stirred for 20 h, then worked up by the addition of 0.5 mL of H_2O, stirring for 1 h, then partitioned with 75 mL EtOAc and 30 mL of H_2O, washing with dilute $KHSO_4$, H_2O, dilute $NaHCO_3$, and dilute NaCl (2 ×), and dried over anhydrous Na_2SO_4. Solvent removal *in vacuo* afforded 188 mg (94% recovery) of hydroxy amide **10a**: TLC R_f (12:2:2:10, EAIW) 0.46, (85:15:1.5, CMW) 0.63.

In a similar manner, 870 mg (1.30 mmol) of hydroxy acid **9b** afforded 810 mg (92% yield) of hydroxy amide **10b**: TLC R_f (85:15:1.5, CMW) 0.59, (12:2:2:10, EAIW) 0.50; 520 mg (0.78 mmol) of hydroxy acid **9b** (reaction solvent DMF) afforded 555 mg (100% recovery) of hydroxy amide **10c**: TLC R_f (12:2:2:10, EAIW) 0.27; and 300 mg (0.45 mmol) of hydroxy acid **9b** afforded 311 mg (99% yield) of hydroxy amide **10d**: TLC R_f (12:2:2:10, EAIW) 0.55; 240 mg (0.36 mmol) of hydroxy acid **9b** afforded 220 mg (83% yield) of hydroxy amide **10e**; 240 mg (0.36 mmol) of hydroxy acid **9b** afforded 270 mg (99% yield) of hydroxy amide **10f**; 232 mg (0.35 mmol) of hydroxy acid **9b** afforded 222 mg (93% yield) of hydroxy amide **10g**; 550 mg (0.83 mmol) of hydroxy acid **9b** afforded 672 mg (99% yield) of hydroxy amide **10h**; and 208 mg (0.31 mmol) of hydroxy acid **9b** afforded 220 mg (93% yield) of hydroxy amide **10i**.

Oxidation/deblocking to give 11a–i. All reactions were carried out under essentially the same conditions. Thus, a solution of 180 mg (0.27 mmol) of hydroxy amide **10a** in 9 mL of CH_2Cl_2 was treated with 0.52 g (1.27 mmol) of the Dess–Martin periodinane. Con-

Table 6.

	αCH	βCH	γCH	other
Phe	4.52 (*dd*)	3.23, 3.11 (*dd*)	——	C_6H_5 7.29,7.40
Pro	4.27 (*dd*)	2.04, 1.71 (*m*)	1.81, 1.52 (*m*)	δCH 3.48,2.71(*m*)
Lys (keto) ≤ 5%	4.93 (*dd*)	nd	nd	nd
Lys (hyd) ~95%	4.13 (*dd*)	1.52	1.64, 1.36	εCH 2.98 O-CH_3 3.75

version to keto-amide was complete within 30 min, as determined by TLC; R_f (12:2:2:10, EAIW) 0.50 (**10a**), 0.71 (**11a**). The reaction mixture was diluted with 30 mL of ether and worked up as described for the keto-ester precursor to **8a** (above) to give 188 mg (100% recovery) of protected keto-amide, which was mixed with 1.0 mL of anisole and treated with liquid HF, to give, after dissolution in H_2O and preparative HPLC (see above), 129 mg (72% yield) of lyophilized final product **11a** as the TFA salt: HPLC 99% (100 → 65) rt 16.6 min; amino acid analysis Phe 1.36, Pro 1.36 μmol mg^{-1}; FABMS m/z 464 (M + H + CH_3OH), 450 (M + H + H_2O), 432 (M + H); ^1H NMR (500 MHz, D_2O) δ (ppm) (see Table 7).

In a similar manner, a sample of 800 mg (1.18 mmol) of hydroxy amide **10b** afforded 475 mg (60% yield) of keto-amide **11b** as lyophilized powder: HPLC 98% (100 → 60, 30 min) rt 16.7 min; FABMS m/z 478 (M + H + CH_3OH), 464 (M + H + H_2O), 446 (M + H); ^1H NMR (500 MHz, D_2O) δ(ppm) (see Table 8).

Likewise, 247 mg (0.37 mmol) of hydroxy amide **10c** afforded 50 mg (20% yield) of keto-amide **11c** as lyophilized powder: HPLC 99% (95 → 5, 45 min) rt 6.9 min; FABMS m/z 464 (M + H + CH_3OH), 450 (M + H + H_2O), 432 (M + H); ^1H NMR (500 MHz, D_2O) δ (ppm) (see Table 9).

Likewise, 310 mg (0.45 mmol) of hydroxy amide **10d** afforded 95 mg (31% yield) of keto-amide **11d** as lyophilized powder: HPLC 99% (100 → 65) rt 20.1 min; FABMS m/z 492 (M + H + CH_3OH), 478 (M + H + H_2O), 460 (M + H); ^1H NMR (500 MHz, D_2O) δ (ppm) (see Table 10).

A ^{13}C NMR spectrum acquired on the same sample showed four carbonyl resonances (165.9, 166.3, 173.4 amide C=O; 199.8 ppm ketone C=O) with no resonance corresponding to hydrated carbonyl (>$C(OH)_2$) (expected *ca* 98 ppm).

Likewise, 220 mg (0.31 mmol) of hydroxy amide **10e** afforded 85 mg (40% yield) of keto-amide **11e** as lyo-

Table 7.

	αCH	βCH	γCH
Phe	4.54 (*dd*)	3.22, 3.12 (*dd*)	——
Pro	(keto) 4.27 (*dd*)	nd	nd
	(hyd) 4.36 (*dd*)	2.04, 1.71 (*m*)	1.81, 1.52 (*m*)
Lys (keto) ~10%	4.93 (*dd*)	1.95 (*m*)	1.5 (*m*)
Lys (hyd) 90%	4.13 (*dd*)	1.76 (*m*)	1.5 (*m*)

Table 8.

	αCH	βCH	γCH	other
Phe	4.55 (*dd*)	3.25, 3.15 (*dd*)	——	C_6H_5 7.30,7.41
Pro	(keto) 4.37 (*dd*)	nd	nd	δCH 3.47,2.65 (*m*)
	(hyd) 4.30 (*dd*)	2.04, 1.76 (*m*)	1.76, 1.54 (*m*)	δCH 3.48,2.71 (*m*)
Lys (keto) ~20%	5.13 (*dd*)	1.97, 1.71	1.64, 1.53	εCH 3.02
Lys (hyd) 80%	4.12 (*dd*)	1.52	1.64, 1.36	εCH 3.00 N-CH_3 2.72, 2.70

Table 9.

	αCH	βCH	γCH	other
Phe	4.50 (*dd*)	3.35, 3.10 (*dd*)	——	C_6H_5 7.30,7.40
Pro	(keto) 4.35 (*dd*)	nd	nd	nd
	(hyd) 4.30 (*dd*)	2.03, 1.76 (*m*)	1.75, 1.55 (*m*)	δCH 3.46,2.63 (*m*)
Lys (keto) ~10%	5.12 (*dd*)	nd	nd	nd
Lys (hyd) ~90%	4.12 (*dd*)	1.50	1.70, 1.40	εCH 3.01 N-CH_3 2.72

Table 10.

	αCH	βCH	γCH	other
Phe	4.52 (*dd*)	3.36, 3.12 (*dd*)	——	C_6H_5 7.29,7.41
Pro	(keto) 4.37 (*dd*)	2.09, 1.75 (*m*)	1.52 (*m*)	δCH 3.50,2.66 (*m*)
Lys (keto) ~100%	4.72 (*dd*)*	2.08,1.82	1.58, 1.51	εCH 3.05 N-CH_3 2.96,2.72

*Signal diminishes by *ca* 40% during the acquisition period (~15 min), indicating rapid racemization at ambient pH (measured pD = 6.3).

philized powder: HPLC 99% (100 → 65) rt 22.2 min; FABMS m/z 516 (M + H + CH_3OH), 502 (M + H + H_2O), 484 (M + H); ^1H NMR (500 MHz, D_2O) δ (ppm): 4.75 *dd* (Lys keto αCH ~100%); Signal diminishes by *ca* 40% during the acquisition period (~15 min), indicating rapid racemization at ambient pH (measured pD = 5.9).

Likewise, 267 mg (0.38 mmol) of hydroxy amide **10f** afforded 52 mg (20% yield) of keto-amide **11f** as lyophilized powder: HPLC 99% (95 → 35) rt ~20 min (broad); FABMS m/z 504 (M + H + CH_3OH), 490 (M + H + H_2O), 472 (M + H); ^1H NMR (400 MHz, D_2O) δ (ppm): 4.7–4.8 (Lys keto αCH ~100%).

Likewise, 213 mg (0.30 mmol) of hydroxy amide **10g** afforded 80 mg (38% yield) of keto-amide **11g** as lyophilized powder: HPLC > 99% (95 → 35) rt 8.5 min; FABMS m/z 492 (M + H + CH_3OH), 478 (M + H + H_2O), 460 (M + H); ^1H NMR (400 MHz, D_2O) δ (ppm): 5.42 *dd* (Lys keto αCH ~10%), 4.10 *dd* (Lys hyd αCH ~90%).

Likewise, 650 mg (0.80 mmol) of hydroxy amide **10h** afforded 409 mg (71% yield) of keto-amide **11h** as lyophilized powder: HPLC > 99% (100 → 65) rt 16.2 min; FABMS m/z 522 (M + H + CH_3OH), 508 (M + H + H_2O), 490 (M + H); ^1H NMR (400 MHz, D_2O) δ (ppm): 5.18 *dd* (Lys keto αCH ~10%), 4.11 *dd* (Lys hyd αCH ~90%).

Likewise, 220 mg (0.31 mmol) of hydroxy amide **10i** afforded 54 mg (23% yield) of keto-amide **11i** as lyophilized powder: HPLC > 99% (95 → 35) rt 12.7 min; FABMS m/z 554 (M + H + CH_3OH), 540 (M + H + H_2O), 522 (M + H); ^1H NMR (400 MHz, D_2O) δ (ppm): 5.08 *dd* (Lys keto αCH ~10%), 4.12 *dd* (Lys hyd αCH ~90%).

*Synthesis of hydroxy-esters **13a** and **13b*** from ***6a*** and ***6b***.

*Compound **13a***. A solution of 150 mg (0.27 mmol) of hydroxy ester **7a** (R = H) in 50 mL of 8:1:1 (v/v) CH_3OH:H_2O:HOAc was hydrogenated at 1 atm with 150 mg of 10% Pd/C for 1 h. The catalyst was filtered off and the filtrate concentrated under reduced pressure to give an oil, which upon lyophilization yielded 130 mg of crude *des*-Cbz intermediate.

A 35 mg sample of this material was treated with 4 mL of 100% TFA, then after 15 min concentrated to dryness under reduced pressure. Lyophilization afforded 35 mg of recovered crude product **13a**, which was purified by semi-preparative HPLC (100 → 60, 60 min) to give, after lyophilization of pooled fractions, 20 mg (55% yield) of white solid **13a**: HPLC 99% (100 → 60) rt 16.3 min; FABMS m/z 435 (M + H); ^1H NMR (500 MHz, D_2O) δ (ppm) 7.48, 7.32 *dd* C_6H_5, 4.55 *dd* (Phe αCH), 4.41 *d* (>CH(OH)), 4.29 *dd* (Pro αCH), 4.25 *dd* (Lys αCH), 3.70 *s* (O-CH_3), 3.50, 2.75 *dd* (Pro δCH_2), 3.25, 3.14 (Phe βCH_2), 3.03 *t* (Lys εCH_2), 2.07 *br* (Pro βCH), 1.6–1.8, 1.4–1.6 env. (Pro β,γCH, Lys β,γCH_2).

*Compound **13b***. A solution of 680 mg (1.60 mmol) of hydroxy ester **6b** (*ca* 2:3 isomers) in 80 mL of EtOAc, cooled to –30 °C under N_2, was saturated with HCl gas, taking care to keep the temperature below 0 °C, allowed to stir for 10 min, then purged with N_2 for 45 min. The solvent was then removed under reduced pressure, and the residual solid, TLC R_f (85:15:1.5, CMW) 3:2 0.39, 0.52, was dried *in vacuo* while the following procedure was carried out.

A solution of 600 mg (1.65 mmol) of Boc-D-Phe-Pro-OH[20] in 50 mL of CH_2Cl_2/10 mL of EtOAc under N_2 was cooled to –15 °C and treated with 0.18 mL (equiv. 0.166 g, 1.6 mmol) of NMM, followed by 0.22 mL (equiv. 0.22 g, 1.6 mmol) of isobutyl chloroformate. After 20 min a solution of the above HCl salt in 10 mL of CH_2Cl_2 was added, followed by 0.15 mL of NMM, then 0.09 mL resulting in pH 8. After 1 h, H_2O was added, followed by extractive workup to afford, after flash chromatography of the crude product on silica gel (98:2:0.2, CMW), 741 mg (69% recovery) of **7b** as a white solid; TLC R_f (95:5:0.5, CMW) 0.25.

A sample of 131 mg of this solid was transferred to a Kel-F® reaction vessel and dissolved in 1.0 mL of anisole. Liquid HF was introduced at –70 °C to a volume of 10 mL, followed by stirring at 0 °C for 1.5 h. After removal of HF *in vacuo*, addition of 5 mL of ether and 5 mL of pet. ether afforded a tacky gum, from which the supernatant was decanted, and the solid washed with 1:1 ether:pet. ether. The crude product was purified on preparative scale HPLC (100 → 60, 60 min) to give, upon lyophilization of pooled fractions, 33 mg of **13b**: HPLC 99% (100 → 60) rt 18.0 min; FABMS m/z 435 (M + H); ^1H NMR (500 MHz, D_2O) δ (ppm): 7.48, 7.30 *dd* (C_6H_5), 4.53 *dd* (Phe αCH), 4.24 *d* (>CH(OH)), 4.29 *dd* (Pro αCH), 4.13 *dd* (Lys αCH), 3.75 *s* (O-CH_3), 3.50, 2.75 *dd* (Pro δCH_2), 3.24, 3.12 (Phe βCH_2), 2.97 *t* (Lys εCH_2), 2.06 *br* (Pro βCH), 1.8, 1.5–1.7, 1.4 env. (Pro β,γCH, Lys β,γCH_2).

*Synthesis of ester **14***.

Mixed anhydride formation. The mixed anhydride was generated from 4.0 g (10.5 mmol) of $^{\alpha}$Boc,$^{\varepsilon}$Cbz-L-lysine in 100 mL of EtOAc, upon cooling to –15 °C under N_2 and addition of 1.27 mL (11.0 mmol, 10% excess) of NMM followed by 1.50 mL (11.5 mmol) of *i*-butyl chloroformate. After 30 min, 100 mL of H_2O was added, stirred for 1 min, the lower layer drawn off and the EtOAc washed with 100 mL saturated NaCl and dried over $MgSO_4$ for 5 min, then filtered into an Erlenmeyer flask.

Diazoketone formation. Diazomethane was generated by addition of 5.41 g (36.8 mmol) of 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) portion-wise with stirring to 125 mL of ice-cold ether over 50 mL of 40% KOH. After 15 min the yellow ether layer was decanted over KOH pellets, rinsing the aqueous layer twice with ether; the solution thus prepared was added to the above mixed anhydride and allowed to stir for 1.5 h at 0 °C. The excess CH_2N_2 was removed by purging with N_2, then addition of 0.5 mL of HOAc, and the solution was concentrated to an oil under reduced pressure.

Rearrangement to methyl ester. To a solution of this oil in 125 mL of dry CH_3OH was added at 0 °C a solution of 0.71 g (3.1 mmol) of silver benzoate in 1.76 mL (12.6 mmol, 20% excess) of triethylamine; the mixture turned black and was allowed to stir for 20 h, whereupon a black precipitate was evident that was filtered through Celite. The filtrate was concentrated *in vacuo* to an oil which was subjected to silica gel chromatography in 3:7 EtOAc:hexane to afford 1.22 g (27% yield) of homologated methyl ester as a colorless oil: FABMS m/z 409 (M + H); ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 7.3 *br* (C_6H_5), 6.52 *d* (NH), 5.05 *s* (-CH_2-Ph), 3.85 *br* (αCH), 3.65 *s* (-OCH_3), 3.10 *t* (-CH_2-N-), 2.44 *d* (-CH_2COOCH_3), 1.45 *s* (Boc).

Boc removal, coupling, deprotection to give ***14.*** Exactly analogous to the preparation of **13a** (Scheme 4), a solution of 0.76 g (1.97 mmol) of the homologated methyl ester in 90 mL of EtOAc was treated with HCl to remove the Boc moiety; then coupling with Boc-D-Phe-Pro-OH was accomplished via the mixed anhydride to give, after purification by silica gel chromatography, 700 mg (53% yield) of white solid: HPLC 99% (95 → 5) rt 22.1 min.

A 600 mg (0.89 mmol) sample of this material was hydrogenated as described for **7a** (Scheme 3), to give 0.53 g of lyophilizate, 140 mg of which was treated with TFA to remove the Boc group. Then preparative HPLC afforded after lyophilization of pooled fractions, 97 mg (56% yield) of white solid **14**: HPLC 97% (95 → 5) rt 10.7 min; FABMS m/z 419 (M + H); ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 7.35, 7.25 *dd* (C_6H_5), 4.38 *m* (D-Phe αCH), 4.2 *m* (Pro αCH, Lys αCH), 3.65 *s* (-OCH_3), 3.45, 2.6 *m* (Pro αCH), 3.10 *m* (Phe βCH_2), 2.90 *t* (Lys εCH_2) 2.4–2.5 *m* (-CH_2COOCH_3), 1.4–2.0 env. (Pro β,γCH_2, Lys β,γCH_2).

Acknowledgements

We extend our thanks to several individuals for their assistance in this work: Mrs Mei-Jy Tang and Mr D. Garrett Kolodin for amino acid analyses; Mr Arthur B. Coddington and Dr Harri G. Ramjit for mass spectral determinations; and Ms Jean F. Kaysen for preparation of the manuscript. We also thank Dr Adel M. Naylor-Olsen for discussion and insight from preliminary molecular modeling studies. Special thanks go to Dr Paul A. Friedman and Dr Paul S. Anderson for their support of this research effort.

References and Notes

1. (a) Davie, E. W.; Fujikawa, K.; Kisiel, W. *Biochemistry* **1991**, *30*, 10363; (b) Maraganore, J. M. *Thromb. Haemostasis* **1993**, *70*, 208.

2. (a) Hijikata-Okunomiya, A.; Okamoto, S. *Seminars in Thromb. Hemostasis* **1992**, *18*, 135; (b) Anderson, H. V.; Willerson, J. T. *New England J. Med.* **1992**, *329*, 703.

3. Maffrand, J. P. *Nouv. Rev. Fr. Hematol.* **1992**, *34*, 405.

4. Lewis, S. D.; Ng, A. S.; Baldwin, J. J.; Fusetani, N.; Naylor, A. M.; Shafer, J. A. *Thrombosis Research* **1993**, *70*, 173.

5. (a) Maryanoff, B. E.; Qiu, X.; Padmanabhan, K. P.; Tulinsky, A.; Almond, Jr H. R.; Andrade-Gordon, P.; Greco, M. N.; Kauffman, J. A.; Nicolaou, K. C.; Liu, A.; Brungs, P. H.; Fusetani, N. *Proc. Natl Acad. Sci. U.S.A.* **1993**, *90*, 8048; (b) Lee, A. Y.; Hagihara, M.; Karmacharya, R.; Albers, M. W.; Schreiber, S. L.; Clardy, J. *J. Am. Chem. Soc.* **1993**, *115*, 12619.

6. (a) Tapparelli, C.; Metternich, R.; Ehrhardt, C.; Cook, N. S. *Trends Pharm. Sci.* **1993**, *14*, 366; (b) Lyle, T. A. *Perspectives in Drug Discovery and Design* **1994**, *1*, 453.

7. (a) Balasubramanian, N.; St. Laurent, D. R.; Federici, M. E.; Meanwell, N. A.; Wright, J. J.; Schumacher, W. A.; Seiler, S. M. *J. Med. Chem.* **1993**, *36*, 300; (b) Shuman, R. T.; Rothenberger, R. B.; Campbell, C. S.; Smith, G. F.; Gifford-Moore, D. S.; Gesellchen, P. D. *J. Med. Chem.* **1993**, *36*, 314.

8. Bajusz, S.; Szell, E.; Bagdy, D.; Barabas, E.; Horvath, G.; Dioszegi, M.; Fittler, Z.; Szabo, G.; Juhasz, A.; Tamori, E.; Szilagyi, G. *J. Med. Chem.* **1990**, *33*, 1729.

9. Stubbs, M. T.; Bode, W. *Thromb. Res.* **1993**, *69*, 1.

10. (a) Lim, M. S. L.; Johnston, E. R.; Kettner, C. A. *J. Med. Chem.* **1993**, *36*, 1831; (b) Banner, D. W.; Hadváry, P. *J. Biol. Chem.* **1991**, *266*, 20085.

11. Iwanowicz, E. J.; Lin, J.; Roberts, D. G. M.; Michel, I. M.; Seiler, S. M. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1607.

12. Buckhart, J. P.; Peet, N. P.; Bey, P. *Tetrahedron Lett.* **1990**, *31*, 1385.

13. Fehrentz, J.-A.; Castro, B. *Synthesis* **1983**, 676; see also Goel, O. P.; Krolls, U.; Stier, M.; Kesten, S. *Org. Syn.* **1988**, *67*, 69.

14. Burkhart, J. P.; Peet, N. P.; Bey, P. *Tetrahedron Lett.* **1988**, *29*, 3433; see also Ireland, R. E.; Liu, L. *J. Org. Chem.* **1993**, *58*, 2899.

15. Chen, Z.-G.; Li, Y.; Mulichak, A. M.; Kuo, L. unpublished results.

16. Lewis, S. D.; Ng, A. S.; Lyle, E. A.; Mellott, M. J.; Appleby, S. D.; Brady, S. F.; Stauffer, K. J.; Sisko, J. T.; Mao, S.-S.; Veber, D. F.; Nutt, R. F.; Lynch, J. J.; Cook, J. J.; Gardell, S. J.; Shafer, J. A. *Thromb. Haemostasis* in press.

17. Nutt, R. F.; Curley, P. E.; Pitzenberger, S. M.; Freidinger, R. M.; Saperstein, R.; Veber, D. F. In: *Peptides, Structure and Function*, pp. 441–444, Deber, C.; Hruby, V.; Kopple, K., Eds; Pierce Chemical Co.; Rockford, IL, 1985.

18. Angelastro, M. R.; Peet, N. P.; Bey, P. *J. Org. Chem.* **1989**, *54*, 3913.

19. This washing was critical to ensure decomposition of the aluminate complex of the aldehyde product.

20. Prepared by synthesis according to conventional methodology: coupling of Boc-protected N-terminal D-amino acid with either L-proline benzyl ester or L-proline methyl ester, followed by hydrogenation or saponification, respectively.

(*Received in U.S.A.* 8 *February* 1995)