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Cyclotheonamide Derivatives: Synthesis and Thrombin Inhibition. Exploration of Specific Structure–Function Issues

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Abstract—Macrocyclic pentapeptide analogues (**5–9**) of the sponge natural product cyclotheonamide A (CtA, **3**) were prepared by means of our convergent [3 + 2] synthetic protocol, in which a late-stage primary amine group is available for substitution (Maryanoff *et al. Proc. Natl Acad. Sci. U.S.A.* **1993**, *90*, 8048). These analogues, as well as CtA and cyclotheonamide B (CtB, **4**), were examined for their ability to inhibit the serine protease α-thrombin, in comparison with suitable reference standards. We characterized Michaelis–Menten and slow-binding kinetics for the cyclotheonamide derivatives. An attempt was made to utilize the unoccupied hydrophobic S_3 subsite of thrombin via analogues **5** and **6**. Also, removal of the hydroxyphenyl group, which is thought to be involved in an aromatic stacking interaction with Trp^{60D} of thrombin, was explored via analogue **9**. The importance of the α-keto and olefin groups was examined via **7** and **8**, respectively. The relationship of structure and function with the analogues proved to be less predictable than anticipated.

Introduction

Thrombin (EC 3.4.21.5) is an enzyme central to the blood coagulation pathway, where it regulates thrombosis and hemostasis.[1–6] Among its other functions, this spherically shaped, 37-kDa, trypsin-like serine protease is responsible for converting fibrinogen into clottable fibrin[7] and for stimulating platelet aggregation, ultimately leading to thrombus formation.[1,4] Thrombin's active site cleft contains a standard His^{57}-Asp^{102}-Ser^{195} catalytic triad and an Asp^{189} in the primary S_1 substrate binding site, the latter of which plays an important role in the molecular recognition of basic functional groups (e.g. see Fig. 1).[8,9] Relative to serine proteases such as trypsin, thrombin is distinguished by two peptide loops located on its surface around the opening to the active site: the Tyr^{60A}–Thr^{60I} insertion loop and the Lys^{145}–Gly^{150} autolysis loop.[9,10] These loops restrict access of macromolecular substrates to the comparatively deep active site cavity in thrombin, thus enhancing substrate selectivity. Also, interactions with the unique insertion loop could control the selectivity of inhibitors for thrombin over other trypsin-like serine proteases.

There has been substantial interest in the search for natural and synthetic thrombin inhibitors as potential therapeutic agents.[1,5,6,10–12] Considering the smaller, active site-directed inhibitors of thrombin, many potent agents possess a guanidine or benzamidine group for strong interaction with the side chain of Asp^{189} in the S_1 subsite, an electrophilic carbonyl or boronate for transition-state adduct formation with the hydroxyl of Ser^{195}, and groups for varied hydrophobic interactions. For example, the archetypical complex between human α-thrombin and D-Phe-Pro-Arg-CH_2Cl (PPACK), an irreversible transition-state analogue, has the guanidine occupying the S_1 subsite, a tetrahedral adduct with Ser^{195}, alkylation of the ε-nitrogen of His^{57}, methylene groups of Pro tucked into a hydrophobic surface partly defined by residues of the insertion loop (proline pocket at the S_2 subsite), and the phenyl group of D-Phe occupying a hydrophobic pocket with an aromatic stacking interaction involving Trp^{215} (Fig. 1).[9] The X-ray crystal structure of this complex also reveals opportunities for specific interactions with the unique 60A–60I insertion loop, particularly with the side chains of Tyr^{60A}, Trp^{60D} and Lys^{60F}. Besides the classical D-Phe-Pro-Arg structural motif, other inhibitor motifs, such as those of argatroban (**1**) and NAPAP (**2**), afford high-affinity complexes with thrombin.[13,14] However, unlike inhibitors in the tripeptide family, compounds in these two classes do not bind in a 'substrate-like' manner (i.e. with a substrate-like alignment of the P_3-P_2-P_1-P_1' positions of the ligand and the S_3-S_2-S_1-S_1' subsites of the enzyme).[10] Furthermore, whereas argatroban is very selective for thrombin relative to other serine proteases,[13] tripeptide-based inhibitors are generally not very selective in particular for trypsin.[10,15]

In the area of molecular recognition between inhibitor ligands and thrombin, we became intrigued with the cyclotheonamides, macrocyclic pentapeptide natural products from the Japanese marine sponge *Theonella* sp. that inhibit various serine proteases, including α-thrombin.[15–17] Cyclotheonamides A (CtA, **3**) and B (CtB, **4**)[16] consist of L-proline, D-phenylalanine, and three uncommon nonproteinogenic amino acids: L-α-keto-homoarginine (h-Arg), L-β-aminoalanine (a-Ala), and vinylogous L-tyrosine (v-Tyr). Significantly, the macrocycle bears an α-keto amide functionality within

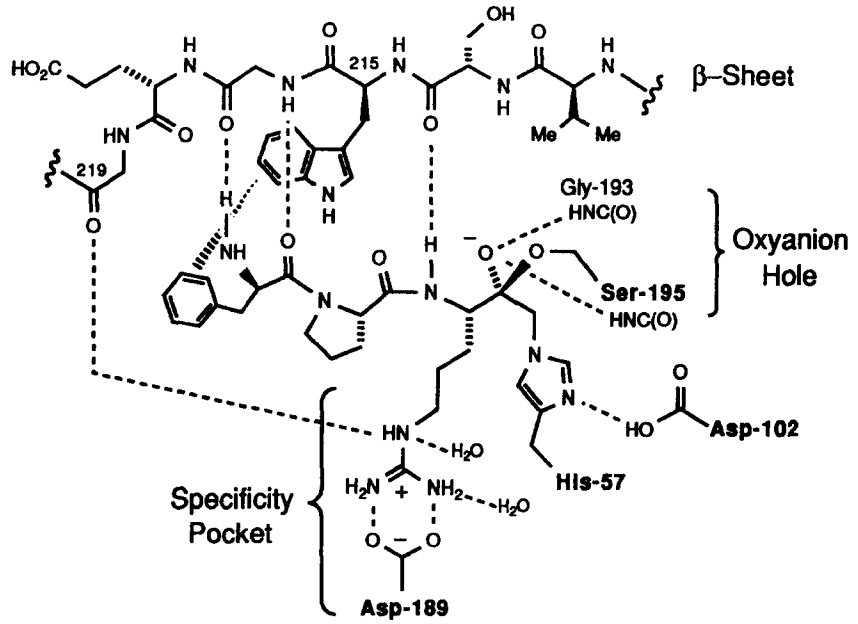
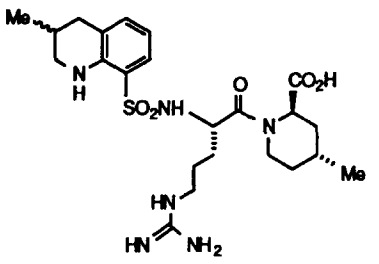


Figure 1. Schematic showing the key interactions of PPACK–thrombin derived from the X-ray crystal structure.[9]



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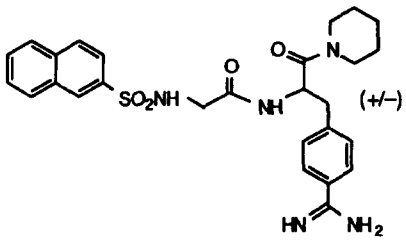

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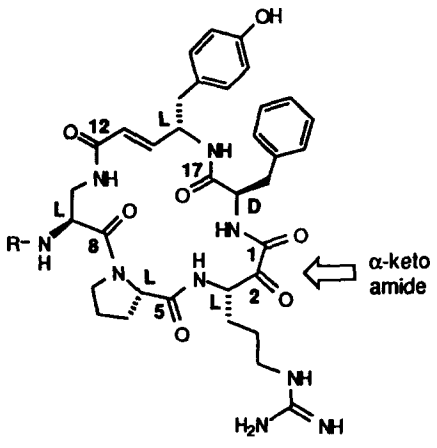
the h-Arg unit, which could impart characteristics of an enzyme transition-state analogue. Although Fusetani *et al.*[16] assigned the D stereochemistry to v-Tyr of the cyclotheonamide backbone, this was revised to L by Hagihara and Schreiber on the basis of the total synthesis of CtB.[18] The stereochemistry for this ring position, as well as the entire structure of CtA, was subsequently corroborated by a single-crystal X-ray diffraction study of the ternary complex formed between CtA, human α-thrombin and hirugen.[15,19]

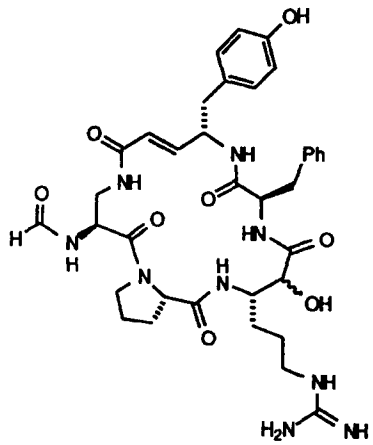


3 R = HC(O) (CtA)

4 R = MeC(O) (CtB)

5 R = $PhCH_2C(O)$

6 R = $PhCH_2CH_2$



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Since CtA is a rare example of a serine protease inhibitor possessing a small cyclopeptide architecture,[20] we had the notion that this agent might reveal new modes of molecular recognition within the active-site cleft of thrombin, particularly in regard to the 60A–60I insertion loop. Although we expected the Pro–Arg motif of CtA to correlate with the P_2–P_1 positions in the tripeptide class of thrombin inhibitors, and the α-keto amide group to serve as a transition-state analogue,[21] we hoped that the cyclic array and aromatic substituents of CtA would offer possibilities for novel interactions. X-Ray crystallography of the ternary complex (1.7-Å resolution) confirmed the expected interactions of the Pro–Arg segment (h-Arg side chain in the S_1 specificity pocket and a hydrogen-bonded, two-strand antiparallel β-sheet with Ser-214 to Gly-216) and the α-keto amide group (tetrahedral adduct with Ser-195 stabilized by an intricate hydrogen-bonding network) (see Fig. 2).[15,22] Of special note here is a novel intermolecular aromatic stacking interaction between Trp^{60D} of the insertion loop and the hydroxyphenyl of CtA, which 'sandwiches' the indole of Trp^{60D} between the Pro and hydroxyphenyl rings.[23] A crystallographic study on the binary complex between CtA and bovine β-trypsin revealed many comparable structural features for the ligand–enzyme interactions, although trypsin lacks the insertion loop residues and the associated special interactions.[24] In the CtA–trypsin complex, the aromatic rings of D-Phe, Tyr^{39} (instead of Glu^{39} in thrombin) and Phe^{41} (instead of Leu^{41} in thrombin) interact favorably, while the hydroxyphenyl of CtA is exposed to the solvent.

As a continuation of our studies in this field, we have prepared additional macrocyclic pentapeptide analogues (**5**–**9**) of CtA by utilizing our convergent synthetic protocol,[15,23] in which a late-stage primary amine is available for substitution. These analogues, as well as CtA and CtB, were examined for their ability to inhibit the serine protease thrombin, under conditions of Michaelis–Menten and slow-binding kinetics.

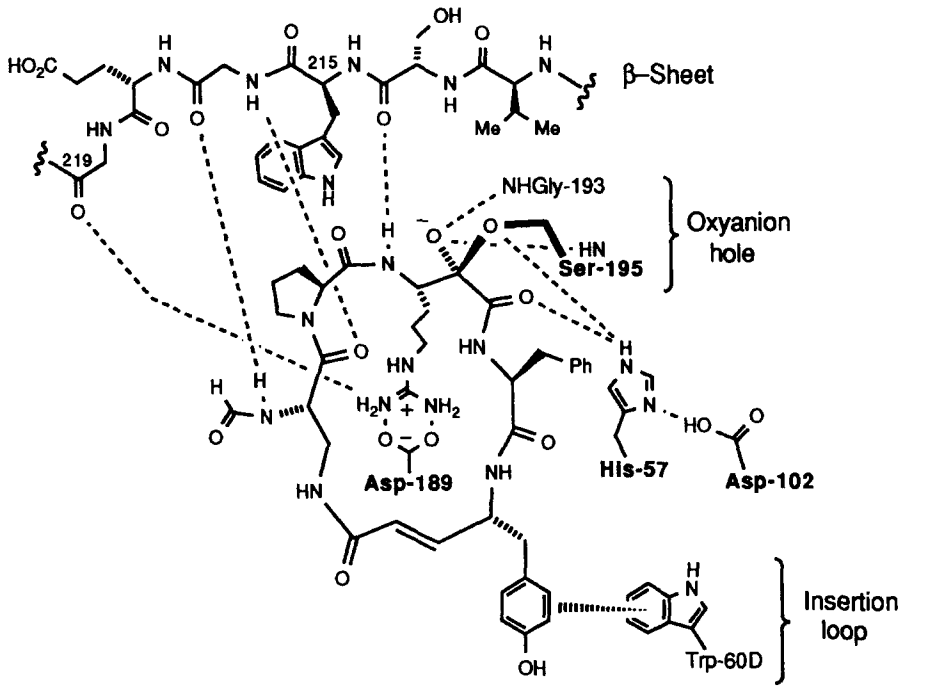
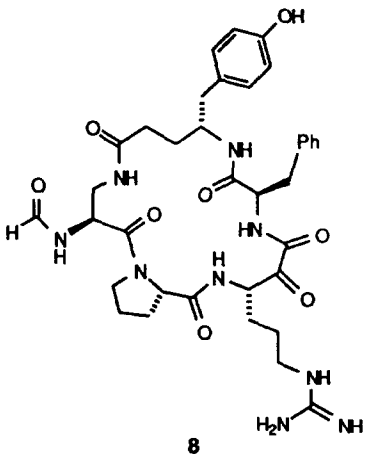


Figure 2. Schematic showing the key interactions of CtA–thrombin derived from the X-ray crystal structure.[15,22]



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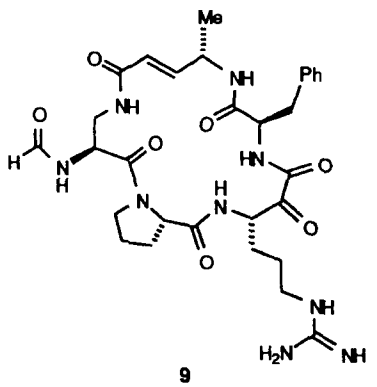

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Results and Discussion

The structural features of the 19-membered ring and the Pro–Arg segment of CtA are virtually the same in its complexes with thrombin[15] and trypsin;[24] the main distinctions relate to the orientations of the v-Tyr and D-Phe aromatic groups. Since thrombin has a more restrictive catalytic cleft, with the presence of a 60A–60I insertion loop that interacts with the v-Tyr side chain, differences with respect to the aromatic side chains were to be expected. In comparing enzyme–inhibitor interactions and steric fit between the two X-ray structures,[15,22,24] there are only limited factors to explain the greater effectiveness of CtA for inhibition of trypsin relative to thrombin. Although the CtA–thrombin complex has the aromatic stacking interaction between v-Tyr of CtA and Trp^{60D},[15] which is absent from the CtA–trypsin complex, the CtA–trypsin complex has the D-Phe side chain ensconced in a hydrophobic area defined by two aromatic residues of the enzyme (Tyr^{39} and Phe^{41}).[24] To examine the importance of the v-Tyr/Trp^{60D} interaction, we decided to synthesize a v-Ala analogue that lacks the hydroxyphenyl group (viz. **9**).

The formamide group of CtA is situated in the thrombin active site such that the hydrophobic S_3 subsite is vacant.[15,23] In the PPACK–thrombin complex, the phenyl group of D-Phe nicely occupies this hydrophobic pocket, while stacking with Trp^{215}.[9] Since trypsin does not have such a pronounced, hydrophobic S_3 region, this factor may partly account for the difference in biological activity. It was our objective to modify the C_9 formamide group to take advantage of this hydrophobic space by incorporating more hydrophobic phenylacetyl (**5**) and phenylethyl (**6**) substituents. This took advantage of our synthetic route to cyclotheon-amide derivatives that involves the late stage, key amine intermediate **10** (Scheme 1).

The ^{13}C NMR spectrum for CtA in D_2O (pH 4.5) indicates a predominance of the *gem*-diol form (covalent hydrate), as opposed to the α-keto amide form.[16,23] Methanol adducts of CtA have also been observed by mass spectrometry, presumably caused by addition of methanol to C_2.[16,23] It is unclear whether the tendency for CtA to exist as a covalent adduct at C_2 has a bearing on its enzyme inhibitory properties, but the X-ray structures of the CtA–thrombin[15] and CtA–trypsin[24] complexes indicate the importance of covalent adduct formation with Ser^{195}. In this connection, we synthesized the keto-reduced (C_2 hydroxy) analogue, as a diastereomeric mixture (viz. **7**), for biological evaluation.

We also synthesized an analogue in which the carbon–carbon double bond of CtA is saturated (viz. **8**). It was thought that this might exert an influence on the conformation of the 19-membered-ring macrocycle and thereby affect biological activity.

Synthetic chemistry

For the preparation of analogues **5** and **6**, we made use of amine **10**, which is a key late-stage intermediate in the synthesis of CtA and CtB[23] (Scheme 1). Treatment of **10** with pentafluorophenyl phenylacetate[25] afforded amide **11**, which was subjected to our sequential two-step protocol of Dess–Martin periodinane oxidation of the α-hydroxy amide at 65 °C in acetonitrile followed by deprotection of the guanidine group with anhydrous HF to provide **5**. Similarly, amine **10** was converted to the BOC-protected phenethyl derivative **12** by standard reductive amination methodology; subsequent oxidation and deprotection afforded analogue **6**. Analogue **7** was prepared by treatment of **13**[23] with excess anhydrous HF and was isolated by HPLC as a single, undefined stereoisomer at C_2 (other diastereomer was not isolated). Double bond reduction of **13** was carried out with excess diimide, generated from hydrazine and hydrogen peroxide, and the resulting saturated derivative was subjected to the oxidation/deprotection protocol to afford analogue **8**. The Dess–Martin oxidation of this saturated analogue proceeded more smoothly (at 23 °C; assisted by *tert*-butanol) than that for the corresponding unsaturated derivatives, e.g. **11**, **12**, and **13**.[23] Perhaps, the less constrained saturated macrocycle may experience improved oxidation because of greater conformational flexibility.

The synthesis of analogue **9** required prior assembly of the (v-Ala)–(a-Ala) segment **14** according to our [3 + 2] segment condensation approach[23] (Scheme 2). Toward that end, the α-amino group of methyl alanine hydrochloride (**15**) was protected as the FMOC derivative by use of standard methodology, and the ester was converted to the corresponding aldehyde with DIBAL-H. The crude aldehyde product was olefinated with (*tert*-butoxycarbonylmethylene)triphenylphosphorane to yield the desired v-Ala derivative **16** with essentially complete stereoselectivity in 35% overall yield from **15**. Cleavage of the *tert*-butyl ester with TFA produced **17**, which was coupled with the a-Ala subunit **18**[23] to give **19**. Removal of the FMOC group of **19** with diethylamine afforded segment **14**, which was employed in the coupling step, mediated by BOP-Cl, with Pro-(h-Arg)-(D-Phe) segment **20**[23] to give **21** in 58% yield. After sequential removal of FMOC and *tert*-butyl protecting groups from **21**, amino acid **22** was treated with BOP-Cl and DMAP to effect macrolactamization in 38% overall yield. At this point in the synthesis, the remaining steps were essentially the same as those in our total synthesis of CtA.[23] Thus, removal of the phthalimide protecting group, formylation, and oxidation/deprotection afforded analogue **9**.

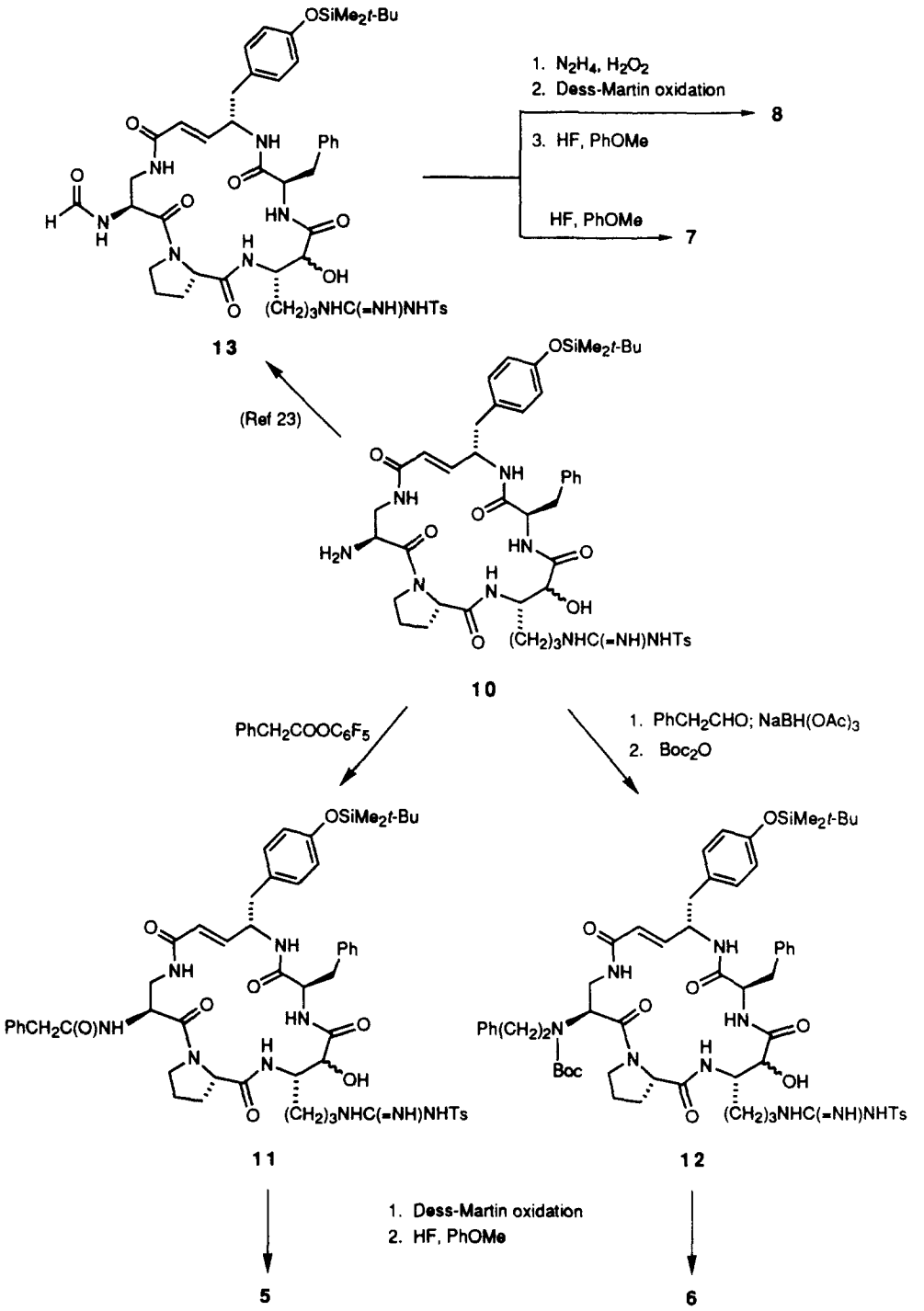
The C_3 stereogenic center in CtA and its α-keto analogues is potentially stereochemically labile in the presence of base. To assess the base stability of CtA in this respect, we treated a solution of CtA (8 mg) with saturated Na_2CO_3 at 23 °C. After stirring for 1.5 h, we obtained a mixture consisting of two new components, along with unchanged CtA, according to HPLC. Separation of the reaction products by HPLC afforded CtA and 3.3 mg of a new substance (**23**). We evaluated this material (**23**) in our thrombin inhibition assay (*vide*

infra) and found relatively weak inhibitory activity (Table 1).

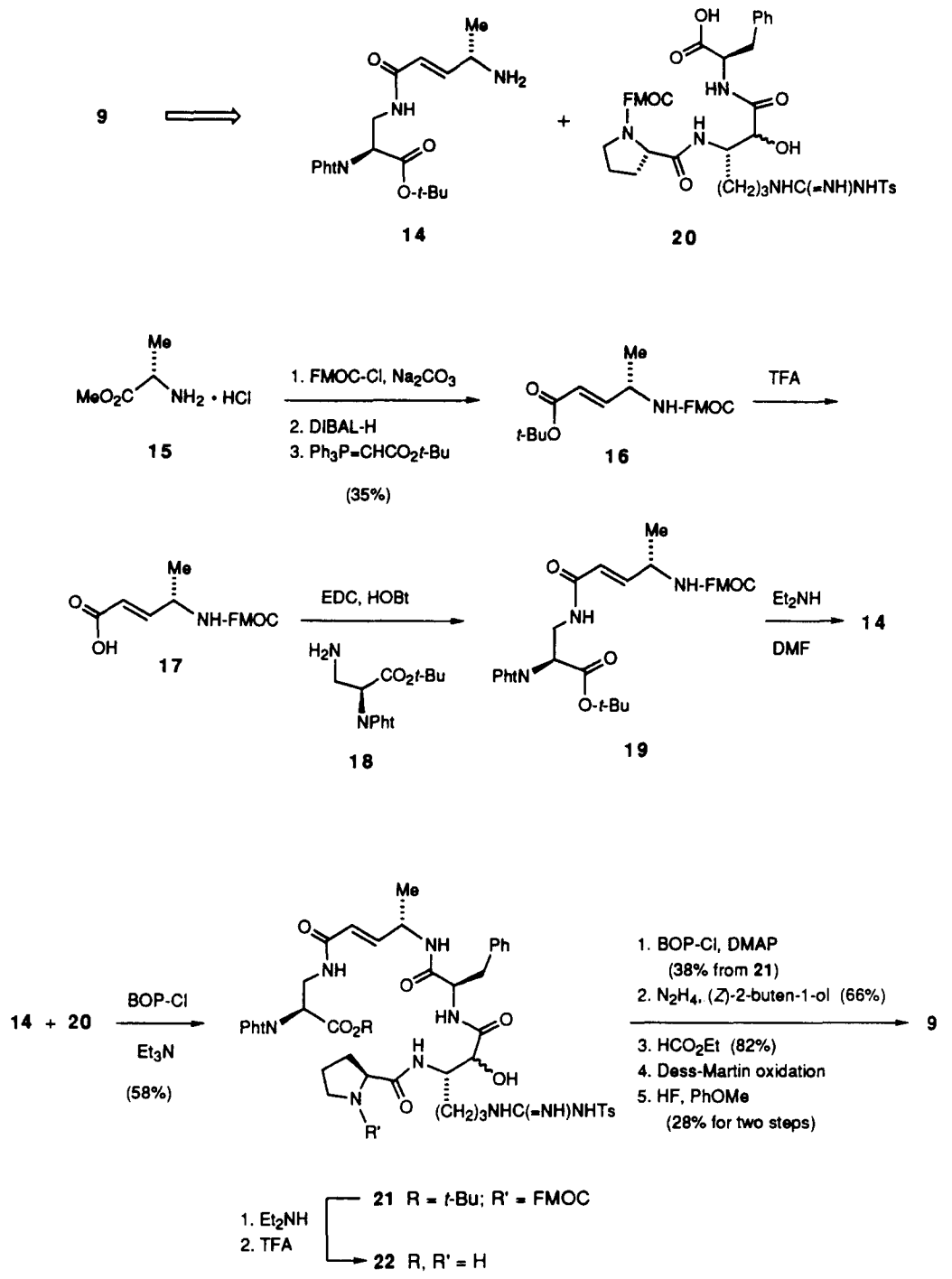
The structure of **23** was difficult to assign unambiguously by ^{1}H NMR or FABMS techniques.[26] Although product **23** had the same mass as CtA (FABMS: m/z 732, MH^+), no water or methanol adduct ions were detected;[16,23] also, a major M-H_2O peak was present. A comparison of the ^{1}H NMR spectrum of **23** with that of CtA showed gross discrepancies. The new material, on exposure to the basic reaction conditions, remained unchanged instead of providing any CtA. Thus, base-induced equilibration at C_3 to give *epi*-C_3 CtA is not likely. The new material may be a diastereomeric mixture of six-membered ring cyclic adducts from addition of the proximal guanidine nitrogen to the ketone.

Enzyme inhibition

Thrombin inhibition data were obtained for the cyclotheonamide derivatives according to two different protocols. Thus, we determined K_i values for the candidate inhibitors under conditions that afford Michaelis–Menten and slow-binding kinetics.



Scheme 1.



Scheme 2.

We had reported a K_i value of 180 ± 40 nM for the inhibition of human α-thrombin by natural CtA.[15] Under the same conditions, synthetic CtA (bis-trifluoroacetate hexahydrate) and synthetic CtB (assumed to be a bis-trifluoroacetate hexahydrate) gave K_i values (n = 2) of 72 ± 22 nM and 84 ± 32 nM, respectively (reversible, competitive behavior). While our paper was in press, a paper by Lewis *et al.*[17] appeared, which observed that CtA is a slow, tight-binding inhibitor with a K_i value of 1.0 nM. The disagreement between our K_i value (at 37 °C) and that of Lewis *et al.* (at 25 °C) is connected with the conditions employed for the enzyme kinetics. Neither the temperature (37 °C versus 25 °C) nor pH differences turned out to account for the difference; rather the factors were concentration of thrombin, substrate employed, and time for pre-equilibration. A comparison of results indicates that CtA can manifest standard competitive inhibition or slow-binding inhibition according to the protocol of the experiment. Under conditions where thrombin was added to a mixture of substrate and inhibitor, the occurrence of slow-binding inhibition was dependent on the substrate and on thrombin concentration (more apparent at 0.1 nM than at 1.0 nM). However, under conditions where

Table 1. Kinetic constants for thrombin inhibition by CtA, CtB and analogues

Cmpd	K_i (nM)[a,b]	K_i (nM)[a,c]
CtA (**3**)[d]	170 ± 80 (11)	4.1 ± 1.9 (4)
CtB (**4**)	200 ± 170 (3)	3.7 ± 2.1 (3)
5	320 ± 180 (3)	3.1 ± 0.8 (3)
6	290 ± 150 (6)	1.5 ± 0.2 (3)
7	11,000 ± 7,000 (4)	100 ± 30 (3)
8	400 ± 230 (3)	6.3 ± 4.3 (3)
9	200 ± 80 (3)	5.3 ± 0.5 (3)
23[e]	47,000 (1)	220 ± 90 (2)
argatroban (**1**)	10 ± 2 (6)	f
DuP-714	0.92 ± 0.20 (4)	0.028 ± 0.014 (4)

[a]The number of experiments (N) is given in parentheses.
[b]Conditions biased to Michaelis–Menten kinetics (see Experimental).
[c]Conditions biased to slow-binding kinetics (see Experimental).
[d]The data reported here are for natural CtA.
[e]Product from base treatment of CtA.
[f]This inhibitor is not reported to show slow-binding behavior; however, we were able to detect a second steady-state slope that corresponds to a K_i value of 0.84 ± 0.30 nM (3).

the substrate was added to an equilibrated mixture of CtA and thrombin, slow-binding inhibition occurred consistently. The K_i values were obtained by fitting the data to the equation $P = v_s t + (v_o - v_s)(1 - e^{-kt})/k$, where P is the *p*-nitroaniline product and, v_o, v_s and k represent the initial velocity, steady-state velocity, and apparent first-order rate constant, respectively.[27,28] Our reported[23] K_i value of 40 ± 20 pM (n = 2) for the reference slow-binding inhibitor DuP-714 (with chromogenic substrate S2238) was close to that reported in the literature (K_i = 44 pM).[29]

We studied CtA (**3**), CtB (**4**), analogues **5**–**9**, **23**, DuP-714, and argatroban under conditions conducive to competitive Michaelis–Menten kinetics and slow-binding kinetics (with chromogenic substrate Spectrozyme® TH; see Experimental) and results for the two protocols are presented in Table 1. Argatroban, a reference thrombin inhibitor which does not operate by a transition-state analogue mode involving Ser^{195},[14] exhibits only competitive Michaelis–Menten kinetics with a K_i of 10 nM. By contrast, DuP-714 exhibits two kinetic behaviors, which give rise to an initial K_i of 0.92 nM and a final K_i of 0.028 nM.

The phenylacetyl analogue (**5**) of CtA is approximately the same potency as CtA (**3**) under the 'Michaelis-Menten' and 'slow-binding' conditions, which contradicts the idea about utilizing the S_3 pocket to amplify the binding of CtA with thrombin. Given this result, we considered the possibility that the amide linkage might not allow the hydrophobic group to access the S_3 pocket properly due to conformational constraints. Thus, it seemed worthwhile to study the phenylethyl analogue **6**. Surprisingly, the K_i values for **6** are nearly the same as those for the parent compounds CtA and CtB (although the slow-binding K_i is twice as good). It is also interesting to note that there is little consequence in having a basic amine substituent (**6**) versus an amide substituent (**5**). In the final analysis, our attempt to utilize the S_3 pocket through analogues **5** and **6** failed to enhance inhibitor potency. This outcome is rather perplexing at the moment.

The C_2 alcohol analogue **7** (one of the two diastereomers), from reduction of the electrophilic keto group, shows 60-fold (Michaelis–Menten) or 25-fold (slow-binding) lower potency than CtA. This loss of potency was expected because of the importance of the keto group for interaction with Ser^{195} of thrombin, although it is somewhat less than the 100-fold loss in potency in going from Me-D-Phe-Pro-Arg-CHO to Me-D-Phe-Pro-Arg-CH_2OH (Michaelis–Menton conditions).[30] The results for **7** may be significant relative to the question of slow-binding behavior of CtA. The ultimate complex formed between CtA and thrombin is presumably the one observed by X-ray crystallography.[15] If one supposes that an initial complex involves a C_2 diol adduct of CtA, which would need to dissociate to the keto form for addition of Ser^{195}, then **7** affords a model, of sorts, for this complex. However, the observation of two kinetic behaviors for **7** is inconsistent with the dissociation hypothesis. On this basis, it might be more appropriate to consider that a series of conformational adjustments may be responsible for the divergent kinetics of CtA derivatives.

Compound **23**, presumably also lacking an α-keto amide pharmacophore, proved to be a relatively weak inhibitor of thrombin.

Analogue **8**, with the double bond of CtA saturated, and analogue **9**, with a vinylogous alanine also afford nearly the same K_i values. It is not unexpected that the vinyl group of CtA has little to do with affinity for thrombin, but replacement of the hydroxyphenyl group of CtA with a methyl group was expected to have a significant impact, given the aforementioned aromatic stacking with Trp^{60D}. The outcome with **9** is certainly contradictory to this view of molecular recognition.

Conclusion

It is important to appreciate the effect of different conditions on the enzyme kinetics for CtA and its congeners. There is an initial loose-binding state that develops into a final tight-binding state, as manifested by divergent kinetic behavior. Since the slow-binding kinetics can be obscured under conditions where the reaction of chromogenic substrate is relatively fast, we used 10-fold less thrombin (0.1 versus 1.0 nM) and a lower temperature (25 versus 37 °C) to emphasize the slow-binding process. The slow-binding inhibition is more representative of the CtA–enzyme complexes studied by X-ray crystallography because of the time

elapsed for growth of the crystals. Interestingly, we observed (Table 1) that there is a parallelism of the relative potencies of thrombin inhibitors between the 'Michaelis–Menten' and 'slow-binding' sets of inhibition data.

Structural alteration of CtA at certain important sites of the molecule did not result in a significant change in thrombin inhibition under either type of kinetic behavior. An attempt to improve potency by using the S_3 pocket, via analogues **5** and **6**, was unsuccessful. Also, replacement of the hydroxyphenyl group, suggested to be involved in a stacking interaction with Trp^{60D}, by a methyl group did not provide the expected 10- to 50-fold loss of inhibition. From these results, it is clear that further studies with other cyclotheonamide analogues should be pursued. Since the Pro-Arg-C(O)NH motif is unlikely to offer enough molecular recognition elements for the potent K_i values that we have observed (Table 1), other interactions between the CtA assembly and thrombin may be critical. In this respect, potential hydrogen-bonding interactions with N_{11} and/or N_{16}, according to the X-ray crystal structure of the CtA–thrombin–hirugen complex (e.g. Glu^{192} proximate to N_{11}, which is hydrogen bonded to O_5), may have some relevance. We intend to explore for additional interactions in future structure–function studies.

Experimental

General procedures

^1H NMR spectra were recorded on a Bruker AC-300 B (300 MHz), AM-360 WB (360 MHz), or AM-400 (400 MHz) spectrometer with Me_4Si as an internal standard, unless otherwise indicated. NMR abbreviations: *s* = singlet; *d* = doublet; *t* = triplet; *q* = quartet; *dd* = doublet of doublets; *m* = multiplet; *br* = broad; *dist* = distorted. ^{13}C NMR spectra were obtained at 100.6 MHz on a Bruker AM-400 with either Me_4Si or sodium 3-trimethylsilylpropanoate-d_4 as an internal standard, unless otherwise indicated. ^{19}F NMR spectra were recorded on a Bruker AM-400 spectrometer at 376.5 MHz with a 5-mm QNP probe: 30° fluorine pulse of 7 μs; acquisition time of 0.4 s; 2-s recycling delay; 55-KHz sweep; chemical shifts referenced to $CFCl_3$ (δ 0.0). The CtA:TFA ratio was analyzed by correlation with a known amount of CF_3CH_2OH, used as an internal or external ^{19}F and ^1H reference. Chemical-ionization mass spectra (CIMS) were recorded on a Finnigan 3300 system with CH_4 or NH_3 as the reagent gas. Fast-atom-bombardment mass spectra (FABMS) were recorded on a VG 7070E high-resolution or Finnigan TSQ-70B triple-quadrupole mass spectrometer by using an argon beam at 7 kV and 2 mA of current in a thioglycerol or nitrobenzyl alcohol (NBA) matrix. Accurate mass measurements were obtained by using a VG ZAB 2-SE or a VG 7070E spectrometer in the FAB mode (thioglycerol, glycerol, and/or NBA matrices) with voltage scanning over a 200-Da mass window, which included three PEG reference peaks as well as the species of interest. Masses were calculated by using a software peak matching algorithm that extrapolates based on the PEG reference masses. Proposed empirical formulae fell within a 95% confidence window based on instrumental performance (± 2σ). TLC separations were conducted on 250-μm silica plates with visualization by UV fluorescence and iodine staining. Preparative TLC was performed with tapered silica gel plates (300–1700 μm) or Analtech 1000-μm silica gel GF plates. Flash chromatography was performed with flash-column silica gel (32–63 μm). Unless otherwise noted, reversed-phase HPLC separations were carried out on three Waters PrepPak® Cartridges (25 × 100 mm, Bondapak® C18, 15-20 mm, pore size = 125 Å) connected in series (Waters 486 UV detector at 254 nm). Melting points were determined on a Thomas–Hoover apparatus calibrated by a set of melting point standards.

Chemical abbreviations not defined in the text: TBDMS, *tert*-butyldimethylsilyl; SEM, = 2-trimethylsilylethoxymethyl; Cbz, carbobenzoxy; FMOC, fluorenylmethoxycarbonyl; Pht, phthalimidoyl; DCC, dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; BOP-Cl, bis(2-oxo-3-oxazolidinyl)phosphinic chloride; BOP reagent, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; THF, tetrahydrofuran; EDTA, ethylenediaminetetraacetic acid; pNA, *p*-nitroanilide.

The numbering system used in the body of the text for CtA and CtB has C_1 as the amide carbonyl of the homoarginine residue; numbering then progresses around the 19-membered-ring up to N_{19} of D-Phe. This is meant for convenience as the macrocycle is the focus of interest. In this Experimental section, we use standard CAS nomenclature with standard numbering. Intermediates containing the h-Arg subunit were isolated as diastereomeric mixtures (*ca* 1:1).

(5S,11S,14R,18S)-N-[3-[14-Benzyl-11-(4-hydroxybenzyl)-5-phenylmethylcarbonylamino-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1H-3a,7,12,15,19-pentaazacyclopentacyclononadecen-18-yl]propylaminoiminomethyl]amine (5)

Pentafluorophenyl phenylacetate (15 mg, 0.050 mmol) was added to a solution of amine **10**[23] (17 mg, 0.017 mmol) in dry DMF (3 mL) and the mixture was stirred at 23 °C under nitrogen for 2 h. The solvent was removed *in vacuo* at ambient temperature and the residue was purified by preparative TLC (1:9, MeOH:CH_2Cl_2) to give 16 mg (86%) of **11** as a colorless solid: FABMS m/z 1092 (MH^+). A solution of **11**, combined with 2 mg of product from a previous preparation, (18 mg, 0.016 mmol) in MeCN (4 mL) was treated with Dess–Martin periodinane (21 mg, 0.049 mmol). The

reaction mixture was stirred at 65 °C for 70 min, then quenched with MeOH (1 mL). The volatiles were removed *in vacuo* at ambient temperature, and the residue was partitioned between CH_2Cl_2 and water. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic solutions were washed with brine, dried (Na_2SO_4), and concentrated to afford the crude α-keto amide as a white solid. The crude material was transferred to a Teflon® vessel and suspended in anhydrous anisole (1.5 mL). The mixture was cooled to –78 °C and treated with anhydrous HF (3 mL). The reaction mixture was then warmed to 0 °C, stirred for 3 h, and HF was removed under reduced pressure at 0 °C. Ether (8 mL) was added to the residue, and the resulting solid was collected by filtration and washed with ether. The crude product was purified by reversed-phase HPLC (MeCN:water:TFA, 35:65:0.2; 15 mL min^{-1}) and lyophilized to give 3.4 mg (19% for two steps) of **5** as an off-white powder: ^1H NMR (400 MHz, D_2O) δ 7.40–7.19 (*m*, 10H, Phe *m*- and *p*-arom., v-Tyr *o*-arom., $PhCH_2CO$ arom.), 6.91 (*d*, *J* = 8.6 Hz, 2H, v-Tyr *m*-arom.), 6.80 (*dd*, *J* = 15.5, 2.6 Hz, 1H, CH=CHCO), 6.61 (*dd*, *J* = 7.7, 1.4 Hz, 2H, Phe *o*-arom.), 5.93 (*dd*, *J* = 15.6, 2.2 Hz, 1H, CH=CHCO), 4.66–4.46 (*m*, 4H, a-Ala α-H, Phe α-H, v-Tyr CHN, Pro α-H), 4.25–4.18 (*m*, 1H, a-Ala β-H), 3.97 (*dd*, *J* = 10.9, 2.4 Hz, 1H, h-Arg α-H), 3.77–3.71 (*m*, 1 H, Pro δ-H), 3.61 (*s*, 2H, $PhCH_2CO$), 3.52–3.46 (*m*, 1H, Pro δ-H), 3.21–3.13 (*m*, 2H, h-Arg δ-H), 3.00 (*dd*, *J* = 14.2, 4.9 Hz, 1H, v-Tyr ArCH), 2.96–2.90 (*m*, 1H, a-Ala β-H), 2.84 (*dd*, *J* = 13.6, 6.0 Hz, 1H, Phe β-H), 2.75 (*dd*, *J* = 13.6, 4.9 Hz, 1H, Phe β-H), 2.54 (*dd*, *J* = 14.1, 10.5 Hz, 1H, v-Tyr Ar CH), 2.31–2.25 (*m*, 1H, Pro β-H), 2.00–1.80 (*m*, 4H, Pro γ-H, h-Arg β-H, Pro β-H, Pro γ-H), 1.69–1.60 (*m*, 1H, h-Arg γ-H), 1.58–1.48 (*m*, 2H, h-Arg β-H and γ-H); FABMS (in MeOH–water) m/z 822 (MH^+), 840 [(M + H_2O)H^+], 854 [(M + MeOH)H^+]. FABHRMS (NBA; in MeOH): calcd for $C_{43}H_{51}N_9O_8$ (MH^+ + MeOH) 854.4201, found 854.4315.

(5S,11S,14R,18S)-N-[3-[14-Benzyl-11-(4-hydroxybenzyl)-5-phenylethylamino-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1H-3a,7,12,15,19-pentaazacyclopentacyclononadecen-18-yl]propylaminoiminomethyl]amine (6)

A solution of amine **10** (80 mg, 0.082 mmol) in 1.5 mL of THF:1,2-dichloroethane (2:1) containing phenylacetaldehyde (16 mg, 0.133 mmol) was treated with $NaBH(OAc)_3$ (26 mg, 0.123 mmol) and stirred for 1.5 h. The reaction was quenched with water (0.25 mL) and the aqueous phase was made basic with solid $NaHCO_3$. The solution was concentrated and the residue was extracted with $CHCl_3$ (5 × 3 mL). The combined organic layers were washed with brine (1 × 2 mL) and the aqueous layer was extracted with $CHCl_3$ (2 × 1 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated to give the crude product as a yellowish solid. Two sequential prep TLC purifications (CH_2Cl_2:MeOH, 95:5, then 9:1) gave **12** (55 mg, 63%) as an off-white solid containing a mixture of diastereomers (*ca* 2:1). TLC for the major and minor diastereomers, respectively: R_f 0.59, 0.57 (CH_2Cl_2:MeOH, 7:1); ^1H NMR (300 MHz, CD_3OD) δ 7.73 (overlapping *d*, 2H, *J* = 8.2 and 7.8 Hz), 7.26–7.07 (*m*, 12H), 6.79–6.72 (*m*, 5H), 6.09 (*dd*, 1H, *J* = 2.1, 15.4 Hz), 6.08 (*d*, 1H, *J* = 15.4 Hz), 4.68–4.60 (*m*, 2H), 4.57–4.49 (*m*, 1H), 4.29–4.11 (*m*, 1H), 4.06 (*d*, 1H, *J* = 0.7 Hz), 3.70 (*dd*, 1H, *J* = 5.7, 10.6 Hz), 3.63–3.40 (*m*, 3H), 3.23–2.99 (*m*, 2H), 2.88 (*dd*, 1H, *J* = 5.7, 10.6 Hz), 3.63–3.40 (*m*, 3H), 3.23–2.99 (*m*, 2H), 2.88 (*dd*, 1H, *J* = 5.8, 14.4 Hz), 2.72–2.56 (*m*, 5H), 2.61–2.42 (*m*, 2H), 2.38 (*s*, 3H), 2.27–2.19 (*m*, 1H), 2.06–1.82 (*m*, 3H), 1.78–1.41 (*m*, 5H), 0.91 (*s*, 9H), 0.10 (*s*, 6H); FABMS m/z 1079 (MH^+). A solution of this material (45 mg, 0.041 mmol) in dry CH_2Cl_2 (0.25 mL) was treated with excess di-*tert*-butyl dicarbonate (36 mg, 0.160 mmol) and stirred at 23 °C for 20 h. The reaction mixture was concentrated *in vacuo* and dried. The resulting solid was washed with petroleum ether (3 × 5 mL) and the residue was purified by preparative TLC (CH_2Cl_2:MeOH, 9:1) to provide the two BOC-protected diastereomers as white solids; 15 mg (30%) of major diastereomer and 10 mg (20%) minor diastereomer. TLC for major and minor diastereomers, respectively: R_f 0.32 and 0.29 (CH_2Cl_2:MeOH, 9:1; eluted twice). Major isomer: ^1H NMR (300 MHz, CD_3OD) δ 7.70 (d, 2H, *J* = 7.7 Hz), 7.27–7.13 (overlapping *m*, 12H), 6.94–6.87 (*m*, 2H), 6.82–6.74 (*m*, 2H), 6.16 (*dd*, 1H, *J* = 1.3, 15.2 Hz), 4.79–4.70 (*m*, 1H), 4.68–4.60 (*m*, 2H), 4.57–4.40 (*m*, 1H), 4.25–4.11 (*m*, 1H), 4.09–4.03 (*m*, 1H), 3.61–3.46 (*m*, 3H), 3.21–3.02 (*m*, 3H), 2.95–2.63 (overlapping *m*, 5H), 2.50 (*dd*, 1H, *J* = 8.4, 13.7 Hz), 2.35 (*s*, 3H), 2.30–2.15 (*m*, 1H), 2.06–1.84 (*m*, 3H), 1.55–1.51 (*m*, 4H), 1.49 (*s*, 9H), 1.31–1.20 (*m*, 2H), 0.90 (*s*, 9H), 0.11 (*s*, 6H). Minor diastereomer: ^1H NMR (300 MHz, CD_3OD) δ 7.69 (*d*, 2H, *J* = 7.9 Hz), 7.30–7.23 (overlapping *m*, 12H), 6.89 (*m*, 2H), 6.81–6.76 (overlapping *m*, 3H), 6.11 (*dd*, 1H, *J* = 2.1, 15.4 Hz), 4.79–4.58 (*m*, 3H), 4.45–4.40 (*m*, 1H), 4.17–4.02 (*m*, 1H), 3.87–3.74 (*m*, 1H), 3.63–3.37 (*m*, 3H), 3.22–3.02 (*m*, 3H), 2.99–2.72 (*m*, 3H), 2.70–2.65 (*m*, 2H), 2.50 (*dd*, 1H, *J* = 8.9, 13.7 Hz), 2.35 (*s*, 3H), 2.31–2.05 (overlapping *m*, 2H), 2.44–1.83 (overlapping *m*, 2H), 1.68–1.08 (overlapping *m*, 6H), 1.46 (*s*, 9H), 0.87 (*s*, 9H), 0.12 (*s*, 6H). FABMS m/z 1179 (MH^+) for each isomer. A solution of the major diastereomer (13 mg, 0.011 mmol) in MeCN (4 mL) was treated with Dess–Martin periodinane (14 mg, 0.033 mmol). The reaction mixture was stirred at 65 °C for 70 min, then quenched with 5% aqueous $Na_2S_2O_3$ solution (0.5 mL). The volatiles were removed *in vacuo*, and the residue was partitioned between $CHCl_3$ and water. The layers were separated, and the aqueous layer was extracted with $CHCl_3$ (4 × 2 mL). The combined organic layers were washed with brine (1 × 2 mL). The aqueous layer was extracted with $CHCl_3$ (2 × 1 mL). The combined organic layers were dried ($MgSO_4$), and concentrated to afford the crude α-keto amide as a white solid (15 mg, 100%): TLC R_f 0.47 (CH_2Cl_2:MeOH, 9:1; eluted twice); FABMS m/z 1077 (MH^+). The crude α-keto amide was transferred to a Teflon® vessel and sus-

pended in anhydrous anisole (1.5 mL). The mixture was cooled to –78 °C and treated with anhydrous HF (3 mL). The reaction mixture was warmed to 0 °C, stirred for 3 h, and HF was removed under reduced pressure at 0 °C. The resulting yellow oil was triturated with ether (10 mL) at –20 °C overnight. After removal of most of the ether, the resulting residue was dried under a stream of nitrogen to provide a crude tan solid. The product was purified by reversed-phase HPLC (MeCN:water:TFA, 30:70:0.2; 30 mL min^{-1}; t_R = 26 min) and lyophilized to give 5.2 mg of **6** (36% for two steps, based on the presence of 3.4 TFA and 6.0 H_2O) as a white fluffy powder: TLC R_f 0.69 (BuOH:water:HOAc, 9:1:1); ^1H NMR (400 MHz, D_2O) δ 7.44–7.40 (*m*, 2H), 7.37–7.31 (*m*, 3H), 7.26–7.20 (*m*, 5H), 6.92 (*d*, 2H, *J* = 8.2 Hz), 6.84 (*dd*, 1H, *J* = 2.2, 15.6 Hz), 6.63 (*d*, 2H, *J* = 6.8 Hz), 5.86 (*dd*, 1H, *J* = 15.5, 1.5 Hz), 4.61–4.58 (*m*, 3H), 4.44–4.36 (*m*, 2H), 3.98 (*d*, 1H, *J* = 8.6 Hz), 3.55–3.51 (*m*, 1H), 3.49–3.40 (*m*, 1H), 3.37–3.25 (*m*, 2H), 3.20–3.17 (*m*, 2H), 3.09–2.98 (*m*, 4H), 2.84 (*dd*, 1H, *J* = 5.7, 13.7 Hz), 2.75 (*dd*, 1H, *J* = 5.1, 13.7 Hz), 2.54 (*dd*, 1H, *J* = 10.7, 14.0 Hz), 2.37–2.25 (*m*, 1H), 2.01–1.90 (*m*, 3H), 1.82–1.75 (*m*, 1H), 1.69–1.60 (*m*, 1H), 1.59–1.51 (*m*, 2H); FABMS (in MeOH–water) m/z 808 (MH^+), 836 [(M + H_2O)H^+], 868 [(M + MeOH)H^+]. FABHRMS (NBA): calcd for $C_{43}H_{54}N_9O_7$ (MH^+) 808.4146, found 808.4189.

(5S,11S,14R,18S)-N-{3-[14-Benzyl-11-(4-hydroxybenzyl)-5-formylamino-17-hydroxy-4,8,13,16,20-pentaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1H-3a,7,12,15,19-pentaazacyclopentacyclononadecen-18-yl]propylaminoiminomethyl}amine (7)

To a Teflon® vessel containing **13** (7.0 mg, 0.007 mmol) was added dry anisole (0.5 mL). The mixture was cooled to –78 °C and treated with anhydrous HF (1 mL). The reaction mixture was then warmed to 0 °C and stirred for 3 h. After removal of HF under reduced pressure at 0 °C, ether (5 mL) was added to the residue and the solid was collected by filtration and washed with ether. The crude product was purified by reversed-phase HPLC (MeCN:water:TFA, 25:75:0.2; 15 mL min^{-1}) and lyophilized to give 2.9 mg (39%) of **7** as an off-white powder: ^1H NMR (400 MHz, D_2O) δ 8.05 (*s*, 1H, CHO), 7.28–7.20 (*m*, 5H, Phe *m*- and *p*-arom., v-Tyr *o*-arom.), 6.90 (*d*, *J* = 8.5 Hz, 2H, v-Tyr *m*-arom.), 6.82 (*dd*, *J* = 15.5, 2.5 Hz, 1H, CH=CHCO), 6.64 (*d*, *J* = 7.6 Hz, 2H, Phe *o*-arom.), 5.91 (*dd*, *J* = 15.6, 2.1 Hz, 1H, CH=CHCO), 4.80 (*m*, 1H, a-Ala α-H), 4.64–4.51 (*m*, 3H, Phe α-H, v-Tyr CHN, Pro α-H), 4.26–4.13 (*m*, 3H, h-Arg CHOH, a-Ala β-H, h-Arg α-H), 3.78–3.72 (*m*, 1H, Pro δ-H), 3.58–3.52 (*m*, 1H, Pro δ-H), 3.21–3.13 (*m*, 2H, h-Arg δ-H), 3.04 (*dd*, *J* = 14.0, 4.5 Hz, 1H, v-Tyr ArCH), 2.92–2.86 (*m*, 1H, a-Ala β-H), 2.77 (*dd*, *J* = 13.8, 5.8 Hz, 1H, Phe β-H), 2.67 (*dd*, *J* = 13.7, 5.9 Hz, 1H, Phe β-H), 2.56 (*dd*, *J* = 14.0, 10.7 Hz, 1H, v-Tyr Ar CH), 2.33–2.24 (*m*, 1H, Pro β-H), 2.01–1.80 (*m*, 4H, Pro γ-H, h-Arg β-H, Pro β-H, Pro γ-H), 1.72–1.53 (*m*, 3H, h-Arg γ-H, h-Arg β-H and γ-H); FABMS in MeOH m/z 734 (MH^+). FABHRMS (NBA; in MeOH): calcd for $C_{36}H_{47}N_9O_8$ (M + H^+) 734.3626, found 734.3541.

(5S,11S,14R,18S)-N-{3-[14-Benzyl-11-(4-hydroxybenzyl)-5-formylamino-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,20a-eicosaahydro-1H-3a,7,12,15,19-pentaazacyclopentacyclononadecen-18-yl]propylaminoiminomethyl}amine (8)

To a solution of **13** (37 mg, 0.038 mmol) in ethanol (4 mL) at 0 °C was added hydrazine (85 μL, 2.7 mmol), followed by 30% aqueous hydrogen peroxide (53 μL, 0.46 mmol). The reaction mixture was warmed to 23 °C and stirred for 20 h or until ^1H NMR (CD_3OD) indicated that complete reduction was achieved (disappearance of the olefinic signal at δ 6.0). The reaction mixture was concentrated *in vacuo* at ambient temperature, treated with MeOH (1 mL), and concentrated again. Purification of the residue by preparative TLC (CH_2Cl_2:MeOH, 8:1) afforded 21 mg (57%) of the reduced material as a white solid: TLC R_f 0.45 (CH_2Cl_2:MeOH, 9:1; eluted twice); ^1H NMR (300 MHz, CD_3OD) δ 8.06 (*s*, 1H), 7.78 (*d*, 1H, *J* = 8.0 Hz), 7.77 (*d*, 1H, *J* = 8.1 Hz), 7.33–7.10 (overlapping *m*, 7H), 7.00–6.95 (*m*, 2H), 6.79 (overlapping *m*, 2H), 4.83 (*m*, 2H), 4.51–4.45 (*m*, 2H), 4.27–4.18 (*m*, 1H), 4.07 (d, 1H, *J* = 1.3 Hz), 4.03–3.89 (*m*, 1H), 3.83–3.76 (*m*, 1H), 3.55–3.40 (*m*, 1H), 3.21–3.00 (*m*, 2H), 2.99–2.87 (*m*, 1H), 2.75–2.64 (overlapping *m*, 3H), 2.54–2.49 (*m*, 2H), 2.42 (*s*, 3H), 2.25–2.18 (*m*, 2H), 2.05–1.93 (*m*, 4H), 1.72–1.59 (*m*, 3H), 1.53–1.32 (overlapping *m*, 2H), 0.97 (*s*, 9H), 0.14 (*s*, 6H); FABMS m/z 1004 (MH^+). A solution of the saturated derivative (19.5 mg, 0.019 mmol) in dry CH_2Cl_2 (3 mL) was treated with *tert*-butanol (8 mg, 0.11 mmol), followed by the Dess–Martin periodinane (28 mg, 0.066 mmol). The reaction mixture was stirred at room temperature for 75 min, then quenched with 5% aqueous $Na_2S_2O_3$ solution (0.5 mL). The organic layer was removed and the aqueous layer was washed with CH_2Cl_2:EtOAc (4:1, 3 × 5 mL). The combined organic solution was washed sequentially with water (1.5 mL) and brine (1.5 mL), then dried (Na_2SO_4), filtered and concentrated (15 °C) to give the keto amide as an off-white residue (33 mg, 100%): TLC R_f 0.46 (CH_2Cl_2:MeOH, 7:1; eluted twice); FABMS m/z 1002 (MH^+). The crude material was transferred to a Teflon® vessel, dried *in vacuo* (1–2 h), then dissolved in anisole (1 mL) and cooled to 0 °C. Anhydrous HF (3 mL) was added, and the reaction mixture was allowed to stir for 3 h at 0 °C. After removal of excess HF *in vacuo*, the residue was triturated at –20 °C with ether. Purification by HPLC (water:MeCN:TFA, 75:25:0.2; 30 mL min^{-1}; t_R = 23 min) provided 3.8 mg (18% for two steps, based on 1.8 TFA and 6 H_2O) of **8** as a flocculent white solid: ^1H NMR (400 MHz, D_2O) δ 8.10 (*s*, 1H), 7.24 (*m*, 7H), 6.89 (*d*, 2H, *J* = 8.4 Hz), 6.64 (*m*, 2H), 4.85 (*m*, 1H), 4.46 (*m*, 2H), 3.99 (*d*, 2H, *J* = 10.8 Hz), 3.89 (*m*, 1H), 3.79 (*dd*, 1H, *J* = 6.9, 17.1 Hz), 3.49 (*m*, 1H), 3.18 (*dd*, 2H, *J* = 6.3, 12.5 Hz), 2.94 (*t*, 1H, *J* = 11.6 Hz), 2.86 (*dd*, 1H, *J* = 4.5, 13.7 Hz), 2.73 (*dd*, 2H, *J* = 5.4, 13.6 Hz), 2.63 (*dd*, 2H, *J* = 5.6, 13.4 Hz), 2.35 (*m*, 4H), 2.04 (*m*, 2H), 1.89 (*m*, 3H), 1.65 (*m*, 1H), 1.50 (*m*, 3H). FABHRMS (NBA; in MeOH): calcd for $C_{36}H_{47}N_9O_8$ (MH^+ + MeOH) 766.3888, found 766.3838.

{2(S)-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)-3-[(4(S)-aminopenten-2-enoyl)amino]}propanoic acid tert-*butyl ester (14)*

Diethylamine (1 mL) was added to a solution of 0.60 g (0.98 mmol) of **19** in DMF (10 mL) and allowed to stir for 1 h. The volatiles were removed under reduced pressure and the remaining solution was diluted with ether:ethyl acetate (5:1, 30 mL) and washed with water (3 × 15 mL). The aqueous layers were extracted with ether:ethyl acetate (5:1, 2 × 20 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated to furnish a crude oil. Column chromatography on silica gel (CH_2Cl_2:MeOH, gradient from 9:1 to 5:1) gave **14** (220 mg, 58%) as an off-white foam: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.87–7.84 (*m*, 2H), 7.75–7.28 (*m*, 2H), 6.81–6.69 (overlapping *m*, 2H), 5.87 (*d*, 1H, *J* = 15.4 Hz), 5.01–4.97 (*m*, 1H), 4.12–4.03 (*m*, 1H), 3.94–3.86 (*m*, 1H), 3.63 (*t*, 1H, *J* = 6.2 Hz), 2.57 (*s*, *br*, 2H), 1.42 (*s*, 9H), 1.20 (*d*, 3H, *J* = 6.6 Hz). FABHRMS (NBA): calcd for $C_{43}H_{54}N_9O_7$ (MH^+) 808.4146, found 808.4189.

4(S)-[(9-Fluorenylmethoxycarbonyl)amino]-2-pentenoic acid tert-*butyl ester (16)*

To a solution of 1.0 g (7.2 mmol) of methyl alanine hydrochloride (**15**) and 1.4 g (14.4 mmol) of Na_2CO_3 in 15 mL of 1,4-dioxane and 15 mL of H_2O at 0 °C was added 1.9 g (7.2 mmol) of FMOC-Cl in 15 mL of 1,4-dioxane. After 4.5 h of stirring at 23 °C, the reaction was diluted with CH_2Cl_2 (100 mL) and water (130 mL). The layers were separated, and the aqueous layer was extracted twice with CH_2Cl_2. The organic layers were combined and washed with H_2O, dried (Na_2SO_4) and concentrated; the residue was recrystallized from ethyl acetate–hexanes to afford 6.0 g (86%) of the N-protected methyl ester as a white solid: mp 115–116 °C; $[\alpha]_D^{25}$ –22.7° (*c* 1.00, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.90 (*d*, 2H, *J* = 7.4 Hz), 7.81 (*d*, 1H, exch. with D_2O, *J* = 7.3 Hz), 7.70–7.74 (*m*, 2H), 7.31–7.45 (*m*, 4H), 4.20–4.36 (*m*, 3H), 4.04–4.14 (*m*, 1H), 3.62 (*s*, 3H), 1.28 (*d*, 3H, *J* = 7.3 Hz); CIMS (NH_3) m/z 326 (MH^+). Anal. calcd for $C_{19}H_{19}NO_4$: C, 70.14; H, 5.89; N, 4.30; found: C, 70.20; H, 5.96; N, 4.25. A solution of 2.0 g (6.2 mmol) of methyl ester in 46 mL of toluene was cooled to –78 °C and treated with a precooled (–78 °C) solution of 15.5 mL (15.5 mmol) of 1 M DIBAL-H in hexanes. After the addition was complete (*ca* 8 min), the reaction was stirred for 7 min, then quenched slowly at –78 °C with 10 mL of methanol followed by *ca* 30 mL of saturated, aqueous solution of Rochelle's salt. After warming to room temperature, the reaction was diluted with ether and water. The layers were separated, and the cloudy aqueous layer was extracted twice with ether. The combined ether extracts were dried (Na_2SO_4), filtered, and concentrated to give 1.4 g of the corresponding aldehyde as an off-white powder which was used without purification in the following step [CIMS (CH_4) m/z 296 (MH^+)]. A solution of 1.4 g (4.7 mmol) of the aldehyde in 16 mL of DMF was treated with 2.3 g (6.1 mmol) of (*tert*-butoxycarbonylmethylene)triphenylphosphorane and the reaction was stirred for 3.0 h. The reaction mixture was diluted with 50 mL ether, washed with brine (4 × 15 mL), dried (Na_2SO_4), and concentrated. The residue was purified by flash column chromatography (silica gel; hexanes:ethyl acetate, 9:1) to afford 1.0 g of **16** as a tacky white solid: $[\alpha]_D^{24}$ –22.3° (*c* 1.00, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79–7.30 (*m*, 8H), 6.76 (*dd*, 1H, *J* = 15.5, 5.7 Hz), 5.82 (*d*, 1H, *J* = 15.5 Hz), 4.71 (*br d*, 1H, *J* = 9.3 Hz), 4.45–4.43 (overlapping *m*, 3H), 4.22 (*t*, 1H, *J* = 6.8 Hz), 1.56 (*s*, 9H), 1.30 (*d*, 3H, *J* = 6.8 Hz); CIMS (CH_4) *m/z* 394 (MH^+). FABHRMS (PEG-400): calcd for $C_{20}H_{20}NO_4$ (MH^{+2} – *t*-Bu) 338.1392, found 338.1402.

4(S)-[(9-Fluorenylmethoxycarbonyl)amino]-2-pentenoic acid (17)

A solution of **16** was dissolved in 25 mL of CH_2Cl_2 and added to 35 mL of a 1:1 solution of CH_2Cl_2:TFA at 0 °C. After stirring at 0 °C for 15 min and 23 °C for 1.5 h, volatiles were removed under reduced pressure at 23 °C. The residue was triturated with ether, collected and rinsed with ether to yield 830 mg of **17** as a white powder which was used in the next step without further purfication: mp 146–148 °C; FABMS m/z 388 (MH^+).

{2(S)-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)-3-[[4(S)-(9-fluorenylmethoxycarbonyl)amino]penten-2-enoyl-amino]}propanoic acid tert-*butyl ester (19)*

A solution containing *ca* 2.4 mmol of **18**[23] in 50 mL of CH_2Cl_2 was combined with **17** (0.49 g, 1.4 mmol) and HOBt (0.49 g, 3.6 mmol), followed by treatment with EDC hydrochloride (0.42 g, 2.2 mmol) at 0 °C. After gradual warming to 23 °C, the reaction was stirred overnight (*ca* 16 h), then concentrated. The residue was taken up in ethyl acetate and washed sequentially with 5% citric acid, satd $NaHCO_3$, and brine. The organic extract was dried (Na_2SO_4), concentrated, and purified by flash column chromatography (silica gel; CH_2Cl_2:MeOH, 9:1) to afford 0.63 g (70%) of **19** as a white solid: mp 108–111 °C (petroleum ether/ethyl acetate); $[\alpha]_D^{23}$ –80.3° (*c* 1.00, $CHCl_3$); TLC R_f 0.68 (CH_2Cl_2:MeOH, 9:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.88–7.83 (*m*, 2H), 7.80–7.75 (*m*, 4H), 7.59–7.53 (*m*, 2H), 7.43–7.39 (*m*, 2H), 7.38–7.30 (*m*, 2H), 6.71 (*dd*, 1H, *J* = 5.7, 15.2 Hz), 6.40 (*s*, *br*, 1H), 5.79 (*d*, 1H, *J* = 15.3 Hz), 4.99 (*t*, 1H, *J* = 6.1 Hz), 4.79–4.68 (*br*, *s*, 1H), 4.52–4.36 (*m*, *br*, 3H), 4.24–4.09 (overlapping *m*, 2H), 3.81–3.93 (*m*, 1H), 1.42 (*s*, 9H), 1.27 (*d*, 3H, *J* = 6.6 Hz); FABMS m/z 388 (MH^+).

3-{4(S)-[2-(3(S)-{[1-(9-Fluorenylmethoxycarbonyl)pyrrolidine-2(S)-carbonyl]amino}-1-oxo-6-{[imino(toluene-4-sulfonylamino)methyl]amino}-2-(hydroxyhexylamino)-3-phenylpropanoyl-2(R)-amino]pent-2-enoylamino}-2(S)-(1,3-dioxo-1,3-dihydroisoindol-2-yl)propionic acid tert-*butyl ester (21)*

A solution of **14** (200 mg, 0.52 mmol) and **20** (489 mg, 0.59 mmol) in CH_2Cl_2 (10 mL) at 0 °C was treated with

BOP-Cl (197 mg, 0.77 mmol) followed by Et_3N (216 mL, 1.5 mmol). The reaction mixture was allowed to warm to room temperature and stirred under nitrogen overnight (*ca* 16 h). Ethyl acetate (100 mL) was added to the mixture and the solution was washed with 5% aqueous citric acid (2 × 20 mL), satd $NaHCO_3$ (2 × 20 mL), and brine (25 mL). The organic phase was dried (Na_2SO_4), the volatiles were removed *in vacuo*, and the residue was separated by flash column chromatography (CH_2Cl_2:MeOH, 97:3 to 95:5) to give 360 mg (58%) of **21** as a white solid:[31] TLC R_f 0.14 and 0.17 (CH_2Cl_2:MeOH, 9:1; eluted twice); FABMS m/z 1194.4 (MH^+).

3-{4(S)-[2-(3S-{[Pyrrolidine-2(S)-carbonyl]amino}-1-oxo-6-{[imino(toluene-4-sulfonylamino)methyl]amino}-2-(hydroxyhexylamino)-3-phenylpropanoyl-2(R)-amino]-pent-2-enoylamino}-2(S)-(1,3-dioxo-1,3-dihydroisoindol-2-yl)propionic acid tert*-butyl ester (22)*

A solution of **21** (320 mg, 0.26 mmol) in MeCN (4 mL) was treated with diethylamine (1 mL) and the mixture was stirred for 1 h, at which time TLC showed that **21** had been consumed. The volatiles were removed *in vacuo* to give a crude product: FABMS m/z 972.4 (MH^+). The crude product was dissolved in CH_2Cl_2 (4 mL), cooled to 0 °C, and treated with cold TFA:CH_2Cl_2 (1:1, 8 mL; 0 °C). The reaction mixture was allowed to warm to 23 °C and then stirred for 3 h. The volatiles were removed under a stream of nitrogen and the residue was triturated with ether, filtered, and washed with ether to yield the crude deprotected product (280 mg, 96% crude yield from **21**): FABMS m/z 916.4 (MH^+).

*(5S,11S,14R,18S)-*N*-{3-[14-Benzyl-11-methyl-5-formyl-amino-4,8,13,16,17,20-hexaoxo-2,3,4,5,6,7,8,11,12,13,14,15,16,17,18,19,20,20a-octadecahydro-1H-3a,7,12,15,19-pentaazacyclopentacyclononadecen-18-yl]propyl-aminoiminomethyl}amine (9)*

A solution of 279 mg of **22** (0.26 mmol) in CH_2Cl_2 (270 mL) was treated with BOP-Cl (170 mg, 0.67 mmol) and DMAP (162 mg, 1.3 mmol). After stirring for 70 h, solvent was removed *in vacuo*, and the residue was purified by flash column chromatography (CH_2Cl_2:MeOH, 95:5) to give 93 mg (38% from **21**) of the macrolactam product as a white solid: TLC R_f 0.27 and 0.30 (CH_2Cl_2:MeOH, 9:1; eluted twice); ^1H NMR (300 MHz, CD_3OD) δ 7.85–7.70 (*m*, 6H), 7.27–7.02 (*m*, 7H), 6.66 (*dd*, 1H, *J* = 3.0, 15.0 Hz), 6.11–6.02 (overlapping *d*, 1H, *J* = 15.1 Hz), 5.10–4.96 (*m*, 1H), 4.73–4.58 (*m*, 1H), 4.56–4.26 (*m*, 3H), 4.22–4.05 (*m*, 1H), 4.02–3.80 (*m*, 2H), 3.13–2.99 (*m*, 2H), 2.93 (*d*, 2H, *J* = 7.1 Hz), 2.34 (*s*, 3H), 2.30–2.08 (*m*, 2H), 2.06–1.82 (*m*, 3H), 1.72–1.44 (*m*, 3H), 1.40–1.32 (*m*, 1H), 1.03–0.98 (overlapping *d*, 3H, *J* = 7.3 Hz); FABMS m/z 898.4 (MH^+). To a solution of the macrolactam (62 mg, 0.069 mmol) and 4-penten-1-ol (107 μL, 1.03 mmol) in MeOH (1.5 mL) was added anhydrous hydrazine (23 μL, 0.69 mmol). The mixture was stirred for 5 h under argon and the volatiles were removed under reduced pressure (below 25 °C). The residue was separated by preparative TLC (CH_2Cl_2:MeOH, 7:1) to afford the free amine (35 mg, 66%) as a white solid: TLC R_f 0.10 (CH_2Cl_2:MeOH, 7:1); ^1H NMR (300 MHz, CD_3OD) δ 7.79–7.72 (*m*, 2H), 7.29–7.19 (*m*, 7H), 6.62 (*dd*, 1H, *J* = 2.2, 15.5 Hz), 6.04–5.95 (overlapping *dd*, 1H, *J* = 2.2, 15.5 Hz), 4.60–4.57 (*m*, 1H), 4.56–4.24 (overlapping *m*, 3H), 4.20–4.07 (*m*, 1H), 3.82–3.69 (*m*, 1H), 3.65–3.50 (*m*, 2H), 3.46–3.34 (*m*, 1H), 3.24–3.02 (*br*, *s*, 2H), 2.92 (*d*, 2H, *J* = 7.7 Hz), 2.60–2.57 (*m*, 1H), 2.37 (*s*, 3H), 2.30–2.09 (*m*, 1H), 2.06–1.79 (*m*, 3H), 1.76–1.41 (*m*, 3H), 1.40–1.23 (*m*, 1H), 1.03–0.96 (overlapping *d*, 3H, *J* = 7.3 Hz); FABMS m/z 768.4 (MH^+). A mixture of the amine (33 mg, 0.043 mmol) in ethyl formate (2.5 mL) was stirred at 55 °C for 24 h. The reaction mixture was concentrated *in vacuo* and purified by preparative TLC (MeOH:CH_2Cl_2, 1:8) to give the corresponding formylated product (28 mg, 82%) as a solid: TLC R_f 0.38 and 0.40 (MeOH:CH_2Cl_2, 1:7; eluted twice); ^1H NMR (300 MHz, CD_3OD) δ 8.03 (*s*, 1H), 7.75–7.71 (*m*, 2H), 7.30–7.19 (*m*, 7H), 6.67–6.61 (overlapping *dd*, 1H, *J* = 15.5, 2.2 Hz), 6.06–5.98 (over-lapping *dd*, 1H, *J* = 15.5, 2.1 Hz), 4.79–4.59 (*m*, 2H), 4.55–4.14 (*m*, 3H), 3.94–3.65 (*m*, 1H), 3.60–3.52 (*m*, 2H), 3.51–3.40 (*m*, 1H), 3.22–3.02 (*m*, 2H), 2.92 (*d*, 2H, *J* = 7.2 Hz), 2.84–2.76 (*m*, 1H), 2.38 (*s*, 3H), 2.32–2.15 (*m*, 1H), 2.03–1.82 (*m*, 3H), 1.75–1.48 (*m*, 3H), 1.41–1.24 (*m*, 1H), 1.02–0.96 (overlapping *d*, 3H, *J* = 7.3 Hz); FABMS m/z 796.4 (MH^+). The formylated product (23 mg, 0.029 mmol) was dissolved in dry MeCN (10 mL) and treated with Dess–Martin periodinane (24 mg, 0.058 mmol). The reaction mixture was stirred at 65 °C for 1 h, then quenched with 5% aqueous $Na_2S_2O_3$ solution (1 mL). The volatiles were removed *in vacuo* at 23 °C, and the residue was partitioned between $CHCl_3$ and water. The layers were separated, and the aqueous layer was extracted with $CHCl_3$. The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated to afford the crude α-keto amide as a white solid: FABMS m/z 794.4 (MH^+), 812.5 [$(M + H_2O)H^+$]. The crude amide was transferred to a Teflon® vessel and suspended in anhydrous anisole (1.5 mL). The mixture was cooled to −78 °C, treated with anhydrous HF (3 mL), warmed to 0 °C, and stirred for 3 h. The HF was removed under reduced pressure at 0 °C. Ether (10 mL) was added to the residue and the resulting white solid was collected by filtration and washed with ether. The crude product was purified by reversed-phase HPLC (MeCN:water:TFA, 75:25:0.2; 30 mL min^{-1}; t_R = 18 min) and lyophilized to give 8.0 mg of **9** (28% for two steps; based on 1.9 TFA and 6.0 H_2O) as a white powder: TLC R_f 0.45 (BuOH:water:HOAc, 4:1:1); ^1H NMR (400 MHz, D_2O) δ 8.09 (*s*, 1H), 7.40 (*m*, 2H), 7.34 (*d*, 1H, *J* = 7.1 Hz), 7.25 (*d*, 2H, *J* = 7.0 Hz), 6.67 (*dd*, 1H, *J* = 2.5, 15.6 Hz), 5.85 (*dd*, 1H, *J* = 2.1, 15.6 Hz), 4.76 (*dd*, 1H, *J* = 5.8, 11.0 Hz), 4.55 (*ddd*, 2H, *J* = 5.5, 8.8, 14.3 Hz), 4.34–4.30 (*m*, 1H), 4.24 (*dd*, 1H, *J* = 6.0, 12.8 Hz), 4.08 (*dd*, 1H, *J* = 2.4, 8.4 Hz), 3.79–3.73 (*m*, 1H), 3.49–3.43 (*m*, 1H), 3.27–3.16 (*m*, 2H), 3.08 (*dd*, 1H, *J* = 6.0, 13.2 Hz), 2.94 (*dd*, 1H, *J* = 9.2, 13.0 Hz), 2.91 (*t*, 1H, *J* = 12.0 Hz), 2.36–2.26 (*m*, 1H), 2.01–1.94 (*m*, 2H), 1.91–1.79 (*m*, 2H), 1.73–1.66 (*m*, 1H),

1.65–1.53 (*m*, 2H), 0.95 (*d*, 3H, *J* = 7.3 Hz); FABMS (in MeOH) m/z 640.3 (MH^+), 672.4 [(M + MeOH)H^+]. FABHRMS (NBA): calcd for $C_{30}H_{42}N_9O_7$ (MH^+) 640.3207, found 640.3160.

Base stability of CtA

CtA (8 mg) was dissolved in MeCN:water (1:3, 4 mL) and the solution was adjusted to pH 11 with saturated aqueous Na_2CO_3. The resulting mixture was stirred at 23 °C for 1.5 h, then purified by reversed-phase HPLC (MeCN:water:TFA, 25:75:0.2). There were two faster-eluting products in a ratio of *ca* 3:1. The major product was collected and lyophilized to afford 3.3 mg of an off-white powder, **23** (t_R = 16.6 min); ^1H NMR (400 MHz, D_2O) δ 8.12, 8.07 (2 *s*, 1H, 4:1 ratio), 7.32 (*m*, 3H), 7.15 (*d*, *J* = 8.5 Hz, 2H), 6.85 (*d*, *J* = 8.2 Hz, 4H), 6.77–6.68 (*m*, 1H), 6.00–5.95 (*m*, 1H), 5.01 (*dd*, *J* = 5.0, 7.6 Hz, 1H), 4.80–4.69 (*m*, 2H), 4.52 (*m*, 1H), 4.22 (*m*, 1H), 4.10 (*dd*, *J* = 4.2, 11.7 Hz, 1H), 3.86–3.70 (*m*, 3H), 3.46 (*dd*, *J* = 8.2, 14.0 Hz, 1H), 3.33 (*m*, 1H), 3.01–2.88 (*m*, 3H), 2.61 (*dd*, *J* = 9.8, 13.8 Hz, 1H), 2.27 (*m*, 1H), 2.04–1.71 (*m*, 7H); FABMS (MeOH or water as solvent) m/z 732 (MH^+), 754 (M + Na)$^+$, 714 [(M − H_2O)H^+]. The CtA (1.5 mg, t_R = 24.9 min) was recovered in similar fashion (FABMS in MeOH m/z 764 [(M + MeOH)H^+]; ^1H NMR of the recovered CtA showed no 3-*epi*-CtA present. A portion of the major product, **23**, was dissolved in MeCN:water (1:3), adjusted to pH 11 with saturated aqueous Na_2CO_3, and stirred at 23 °C for 3 h. HPLC analysis under the conditions described above showed only starting material **23**. We suggest that the new compounds formed on base treatment of CtA may be diastereomeric (at C_3), six-membered cyclic hemiaminal adducts (in a 4:1 ratio), generated by addition on the proximal guanidine nitrogen to the keto group.

Thrombin inhibition studies[23]

DuP-714 was purchased from Sigma Chemical Co., natural CtA was a gift from Professor N. Fusetani (University of Tokyo), and argatroban was a gift from Mitsubishi Kasai Corp. Enzyme-catalyzed hydrolysis rates were measured spectrophotometrically by using commercial human α-thrombin (American Diagnostica) and the chromogenic substrate Spectrozyme® TH (H-D-HHT-Ala-Arg-pNA·2 AcOH; American Diagnostica) in aqueous buffer (10 mM Tris, 10 mM Hepes, 150 mM NaCl, 0.1% PEG; pH 7.4) with a microplate reader (Molecular Devices). Changes in absorbance at 405 nM were monitored on addition of enzyme, with and without the inhibitor present. Inhibition constants (K_i values) were determined under conditions conducive to competitive, Michaelis–Menten kinetics (1 nM thrombin, 50 μM Spectrozyme® TH, 37 °C, monitored for 30 min), analyzed by using the program K·Cat (Bio Metallics Inc.), and conditions conducive to slow, tight-binding kinetics (0.1 nM thrombin, 50 μM Spectrozyme® TH, 25 °C, monitored for 4 h), analyzed according to the method of Williams and Morrison.[27]

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