Macrocyclic Peptide Inhibitors of Serine Proteases. Convergent Total Synthesis of Cyclotheonamides A and B via a Late-Stage Primary Amine Intermediate. Study of Thrombin Inhibition under Diverse Conditions[1]

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Received June 28, 1994[⊗]

Abstract: Cyclotheonamide A (CtA, **1**), a cyclic pentapeptide isolated from the marine sponge *Theonella* sp., is an inhibitor of serine proteases such as α-thrombin and trypsin. We describe, in detail, our total synthesis of CtA by a convergent [3 + 2] fragment-condensation route. The requisite protected amino acid starting materials were processed and converted into two segments, A (**13**) and B (**21**), which were coupled with BOP reagent in 75% yield (65% with BOP-Cl) to give pentapeptide intermediate **23**. After selective removal of the terminal protecting groups on **23**, the critical macrocyclization was effected with BOP-Cl in 65% yield under high-dilution conditions to provide **25** (25% yield for **23** → **25**). Macrocycle **25** was then processed in four steps to CtA (**1**), which was isolated and purified by HPLC (trifluoroacetate salt). The synthetic CtA was identical to the natural product by 500-MHz ^{1}H NMR, 100-MHz ^{13}C NMR, HPLC, TLC, FAB-MS, optical rotation, and bioassay. The ^{13}C NMR spectrum of CtA in D_2O shows virtually exclusive population by the hydrated form of the α-keto amide (*gem*-diol structure). We also synthesized cyclotheonamide B (CtB, **2**) by utilizing key amine intermediate **28**, through an analogous three-step "end game". Our chemical protocol offers a useful vehicle for the systematic preparation of cyclotheonamide analogues, and because of the late-stage primary amine intermediate, analogues with a modified *N*-acyl or *N*-alkyl substituent should be conveniently accessible. This could be important for satisfying the hydrophobic S_3 binding pocket of thrombin (viz. Figure 3), which is vacant for the CtA−thrombin complex but effectively utilized by the standard D-Phe-Pro-Arg tripeptide inhibitors. Other chemical highlights of the synthesis include (1) homologation of a protected arginal via a cyanohydrin to obtain the h-Arg subunit, (2) use throughout of a monoprotected guanidine, and (3) macrocyclic lactam formation with an unprotected hydroxyl substituent. The characteristics of CtA as a thrombin inhibitor were also examined. Either competitive, Michaelis−Menten kinetics or slow, tight-binding kinetics were observed, depending on the substrate, the thrombin concentration, and the order of addition of components. Given sufficient time for equilibration of CtA and thrombin, slow-binding inhibition is generally displayed.

Marine sponges have proven to be a bountiful source of complex natural products, encompassing a wide range of molecular diversity, such as peptides, macrolides, saccharides, polycyclic aromatics, lipids, and steroids. Representative of the more intriguing structures identified over the past few years are the swinholides,[2] norhalichondrin A,[3] calyculins A−D,[4] erylusamine B,[5] theonellamide F,[6] and kiheisterones A−E.[7,8] From sponges of the genus *Theonella*, in particular, researchers have isolated remarkable macrocyclic peptides that possess significant biological activities.[6,9] Indeed, cyclotheonamide A

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⊗ Abstract published in *Advance ACS Abstracts*, December 15, 1994.

(1) (a) Our total synthesis of cyclotheonamide A (CtA, **1**), and the X-ray crystallographic results for the CtA−thrombin−hirugen complex, were first reported at the 205th National Meeting of the American Chemical Society, Denver, CO, March 28−April 1, 1993; ORGN-311. (b) Chemical abbreviations not defined in the text: PPACK = D-Phe-Pro-Arg-CH_2Cl, TBDMS = *tert*-butyldimethylsilyl, SEM = [2-(trimethylsilyl)ethoxy]methyl, Cbz = carbobenzoxy, FMOC = fluorenylmethoxycarbonyl, TROC = 2,2,2-trichloroethoxycarbonyl, Pht = phthalimido, DCC = dicyclohexylcarbodiimide, EDC = 1-ethyl-3-[3′-(dimethylamino)propyl]carbodiimide, HOBt = 1-hydroxybenzotriazole, BOP-Cl = bis(2-oxo-3-oxazolidinyl)phosphinic chloride, BOP reagent = [(benzotriazol-1-yl)oxy]tris(dimethylamino)-phosphonium hexafluorophosphate, DMAP = 4-(dimethylamino)pyridine, DPPA = diphenylphosphoryl azide, BBC = *O*-(benzotriazol-1-yl)-*N*,*N*:*N*′,*N*′-bis(tetramethylene)uronium hexafluorophosphate, DMF = dimethylformamide, THF = tetrahydrofuran, EDTA = ethylenediaminetetraacetic acid, pNA = *p*-nitroanilide. (c) Ring numbering: The numbering system used in the body of the text for CtA and CtB has C1 as the amide carbonyl of the homoarginine residue; numbering then progresses around the 19-membered-ring up to N19 of D-Phe. This is meant for convenience as the macrocycle is the focus of interest. In the Experimental Section, we use standard CAS nomenclature with standard numbering.

(2) Kitagawa, I.; Kobayashi, M.; Katori, T.; Yamashita, M.; Tanaka, J.; Doi, M.; Ishida, T. *J. Am. Chem. Soc.* **1990**, *112*, 3710−3712. Doi, M.; Ishida, T.; Kobayashi, M.; Kitagawa, I. *J. Org. Chem.* **1991**, *56*, 3629−3632.

(3) Uemura, D.; Takahashi, K.; Yamamoto, T. *J. Am. Chem. Soc.* **1985**, *107*, 4796−4798.

(4) Kato, Y.; Fusetani, N.; Matsunaga, S.; Hashimoto, K.; Fujita, S.; Furuya, T. *J. Am. Chem. Soc.* **1986**, *108*, 2780−2781. Kato, Y.; Fusetani, N.; Matsunaga, S.; Hashimoto, K.; Koseki, K. *J. Org. Chem.* **1988**, *53*, 3930−3932.

(5) Fusetani, N.; Sata, N.; Asai, N.; Matsunaga, S. *Tetrahedron Lett.* **1993**, *34*, 4067−4070.

(6) Matsunaga, S.; Fusetani, N.; Hashimoto, K.; Wälchli, M. *J. Am. Chem. Soc.* **1989**, *111*, 2582−2588.

(7) Carney, J. R.; Scheurer, P. J.; Kelly-Borges, M. *J. Org. Chem.* **1993**, *58*, 3460−3462. Carney, J. R.; Yoshida, W. Y.; Scheurer, P. J. *Ibid.* **1992**, *57*, 6637−6640. For other unusual steroids, see: Kerr, R. G.; Baker, B. J. *Nat. Prod. Rep.* **1991**, *8*, 465−497.

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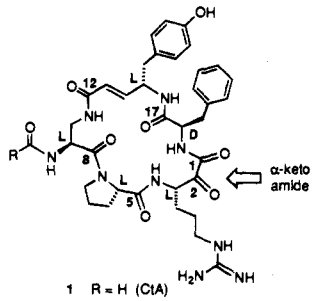
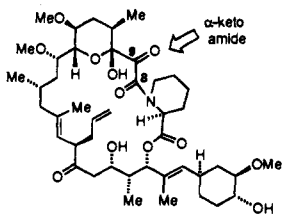


1 R = H (CtA)
2 R = Me (CtB)



3 (FK-506)

(CtA, **1**),[1c] isolated in low yield from the Japanese sponge *Theonella* sp. and found to inhibit certain serine proteases,[9a] is a rare example of a naturally occurring protease inhibitor based on a small cyclopeptide architecture.[10]

Cyclotheonamides A and B (CtB, **2**)[1c] are macrocyclic pentapeptides consisting of one standard proteinogenic amino acid, L-proline, one enantiomer of a proteinogenic amino acid, D-phenylalanine, and three uncommon nonproteinogenic amino acids, L-α-ketohomoarginine (h-Arg), L-β-aminoalanine (a-Ala), and vinylogous L-tyrosine (v-Tyr). The latter amino acid, v-Tyr, is especially noteworthy as a naturally occurring member of a recently described class of vinylogous amino acids which can form polymeric structures with a regular secondary structure.[11] The cyclotheonamide macrocycle also bears an α-keto amide functionality, harbored within the h-Arg unit, which could impart characteristics of an enzyme transition-state analogue. In this respect, one can compare CtA with macrocyclic α-keto amide immunosuppressants, such as FK-506 (**3**), which inhibit peptidyl−prolyl cis−trans isomerases.[12]

Originally, Fusetani et al. assigned the D stereochemistry to v-Tyr of the cyclotheonamide backbone;[9a] however, Hagihara and Schreiber put forth a revision to L on the basis of their total synthesis of CtB.[13] This stereochemistry for ring position 15, as well as the entire structure of CtA, was corroborated unambiguously by a single-crystal X-ray diffraction study of the ternary complex formed from CtA, human α-thrombin, and hirugen.[1a,14] Furthermore, the X-ray data reveal that CtA occupies the active site of thrombin with h-Arg and Pro positioned in the conventional S_1 specificity and S_2 apolar binding sites,[15] respectively, and with the α-keto group of CtA involved in an intricate transition-state array (hemiketal structure). Notably, the Ser-195 of the catalytic triad (Ser-195, His-57, Asp-102)[18] has its γ-oxygen positioned well within bonding distance (C−O distance = ca. 1.6 Å) from, and orthogonal to, the *re* face of the α-keto carbon (C2) of CtA; also, the α-keto oxygen (O2) makes a bifurcated hydrogen bond with thrombin.[1c,14a] There is a direct interaction between the insertion loop of thrombin, Tyr-60A to Thr-60I, and CtA via an intriguing aromatic stacking chain comprised of Tyr-60A, Trp-60D, v-Tyr, and D-Phe, respectively.[14a] A subsequent crystallographic study on a complex between bovine β-trypsin and CtA shows comparable structural features for the ligand interactions, although trypsin lacks the insertion loop and the special interactions relating to it.[19]

Enzyme inhibition data (K_i values) for CtA indicate that it is substantially more potent in inhibiting trypsin and streptokinase than in inhibiting α-thrombin.[14a,20] Since we are primarily interested in the discovery and understanding of novel thrombin inhibitors, because of their potential as antithrombotic drugs,[22] we sought to conduct a structure−function study with cyclotheonamide analogues to explore issues of potency and selectivity. As a prerequisite, we have developed a synthetic route to CtA, based on a convergent [3 + 2] fragment-condensation approach.[23] We now report the evolution and full details of

(8) For a recent review on sponge peptides, see: Fusetani, N.; Matsunaga, S. *Chem. Rev.* **1993**, *93*, 1793−1806.

(9) (a) Cyclotheonamides A and B: Fusetani, N.; Matsunaga, S.; Matsumoto, H.; Takebayashi, Y. *J. Am. Chem. Soc.* **1990**, *112*, 7053−7054. (b) Orbiculamide A: Fusetani, N.; Sugawara, T.; Matsunaga, S.; Hirota, H. *J. Am. Chem. Soc.* **1991**, *113*, 7811−7812. (c) Keramamides B−D: Kobayashi, J.; Itagaki, F.; Shigemori, H.; Ishibashi, M.; Takahashi, K.; Ogura, M.; Nagasawa, S.; Nakamura, T.; Hirota, H.; Ohta, T.; Nozoe, S. *Ibid.* **1991**, *113*, 7812−7813. (d) Theonella-peptolides: Kitagawa, I.; Lee, N. K.; Kobayashi, M.; Shibuya, H. *Chem. Pharm. Bull.* **1987**, *35*, 2119−2132 and references cited therein.

(10) The term "small cyclopeptide" is meant to distinguish the compounds of interest from naturally occurring protein inhibitors of proteases. There are several families of proteins that inhibit proteases with very potent K_i values, and a common structural hallmark of these is a binding domain for the protease active site that is contained within a macrocyclic array. Reviews: Laskowski, M., Jr. *Adv. Exp. Med. Biol.* **1986**, *199*, 1−17. Laskowski, M., Jr.; Kato, I. *Annu. Rev. Biochem.* **1980**, *49*, 593−626. Carrell, R.; Travis, J. *Trends Biochem. Sci.* **1985**, *10*, 20−24. Barrett, A. J., Salvensen, G., Eds. *Proteinase Inhibitors*; Elsevier Publishing Co.: Amsterdam, 1986.

(11) (a) Hagihara, M.; Anthony, N. J.; Stout, T. J.; Clardy, J.; Schreiber, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 6568−6570. (b) For a naturally occurring antibiotic containing vinylogous alanine, see: Oka, M.; Yaginuma, K.; Numata, K.; Konishi, M.; Oki, T.; Kawaguchi, H. *J. Antibiot.* **1988**, *41*, 1338−1350.

(12) For reviews, see: Sigal, N. H.; Dumont, F. J. *Annu. Rev. Immunol.* **1992**, *10*, 519−560. Shinkai, I.; Sigal, N. H. In *Advances in Medicinal Chemistry*; Maryanoff, B. E., Maryanoff, C. A., Eds.; JAI Press, Inc.: Greenwich, CT, 1992; Vol. 1, pp 55−108. Schreiber, S. L. *Science* **1991**, *251*, 283−287. Rosen, M. K.; Schreiber, S. L. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 384−400. Schreiber, S. L.; Liu, J.; Albers, M. W.; Rosen, M. W.; Standaert, R. F.; Wandless, T. J.; Somers, P. K. *Tetrahedron* **1992**, *48*, 2545−2558.

(13) Hagihara, M.; Schreiber, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 6570−6571.

(14) (a) Maryanoff, B. E.; Qiu, X.; Padmanabhan, K. P.; Tulinsky, A.; Almond, H. R., Jr.; 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*, 8048−8052. (Also see: Borman, S. *Chem. Eng. News* **1993**, *71* (Sept 20), 34−35.) (b) The X-ray crystallographic structure for this ternary complex is now refined to 1.7-Å resolution (A. Tulinsky, unpublished results, manuscript in preparation).

(15) The Pro-Arg unit is a P_2−P_1 structural motif generally present in the tripeptide class of thrombin inhibitors[16] and in several thrombin substrates (human platelet receptor, protein C, factors V, VIII, XI, and XIII, and prothrombin).[17]

(16) E.g.: (a) Kettner, C.; Shaw, E. *Thromb. Res.* **1979**, *14*, 969−973. Kettner, C.; Shaw, E. *Methods Enzymol.* **1981**, *80*, 826−848. Hussain, M. A.; Knabb, R.; Aungst, B. J.; Kettner, C. *Peptides* **1991**, *12*, 1153−1154. Shuman, R. T.; Rothenberger, R. B.; Campbell, C. S.; Smith, G. F.; Gifford-Moore, D. S.; Gesellchen, P. D. *J. Med. Chem.* **1993**, *36*, 314−319. (b) Bajusz, S.; Szell, E.; Bagdy, D.; Barabas, E.; Horvath, G.; Dioszegi, M.; Fittler, Z.; Szabo, G.; Juhasz, A.; Tomori, E.; Szilagyi, G. *J. Med. Chem.* **1990**, *33*, 1729−1735. (c) Kaiser, B.; Hauptmann, J. *Cardiovasc. Drug Rev.* **1992**, *10*, 71−87.

(17) Qui, X.; Padmanabhan, K. P.; Carperos, V. E.; Tulinsky, A.; Kline, T.; Maraganore, J. M.; Fenton, J. W., II. *Biochemistry* **1992**, *31*, 11689−11697. Ni, F.; Ripoll, D. R.; Martin, P. D.; Edwards, B. F. P. *Ibid.* **1992**, *31*, 11551−11557.

(18) (a) The chymotrypsinogen numbering system is employed herein.[18b] (b) Bode, W.; Turk, D.; Karshikov, A. *Protein Sci.* **1992**, *1*, 426−471. Bode, W.; Mayr, I.; Baumann, U.; Huber, R.; Stone, S. R.; Hofsteenge, J. *EMBO J.* **1990**, *8*, 3467−3475.

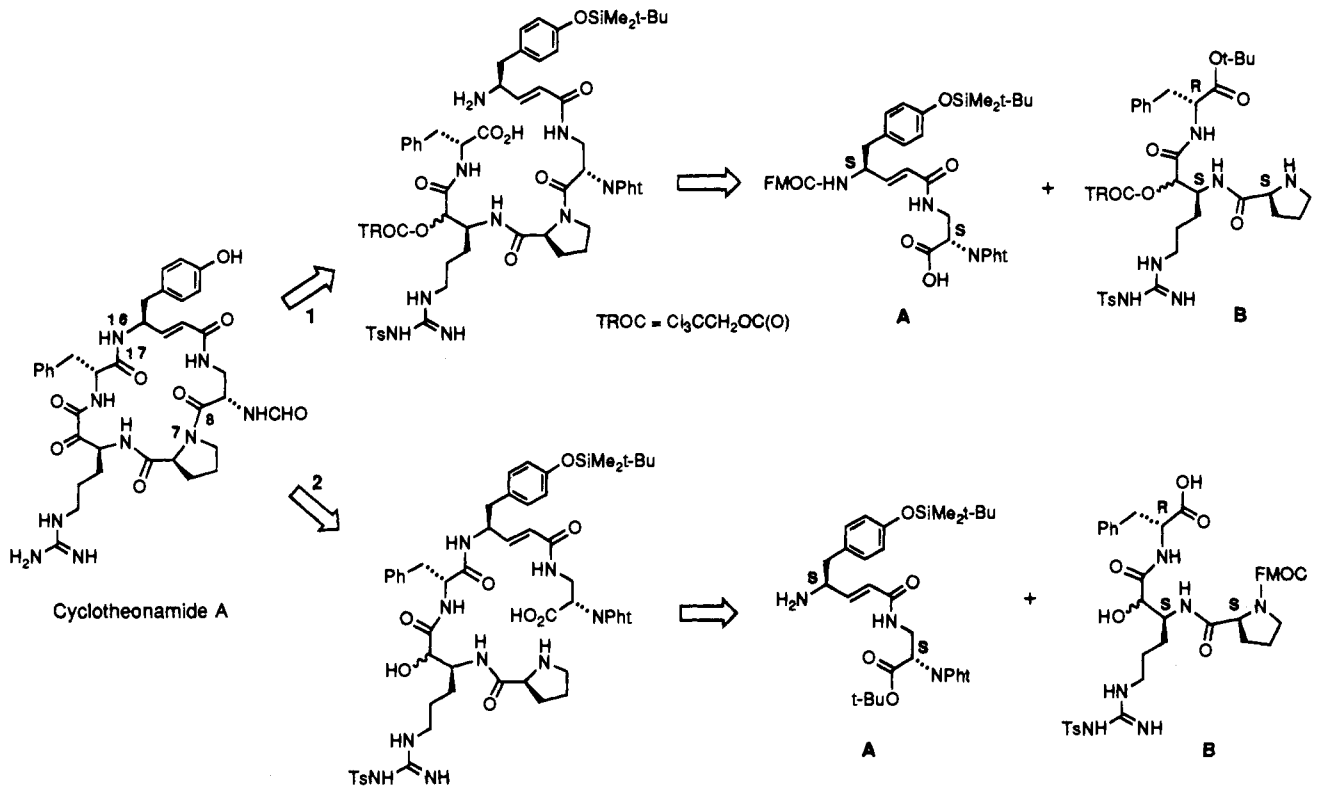
(19) (a) Lee, A. Y.; Hagihara, M.; Karmacharya, R.; Albers, M. W.; Schreiber, S. L.; Clardy, J. *J. Am. Chem. Soc.* **1993**, *115*, 12619−12620. (b) In the CtA−trypsin complex, the aromatic groups of D-Phe and Phe-41 are involved in a stacking interaction; the hydroxyphenyl of CtA is exposed to the solvent.

(20) Different enzyme inhibition data for CtA have been reported by us,[14a] Fusetani et al.,[9a] and Lewis et al.[21] This is a consequence of CtA behaving as a slow-binding inhibitor,[21] which causes the observed K_i to depend on the precise conditions of the enzyme inhibition experiment. This issue is discussed in some detail later (see Enzyme Inhibition).

(21) Lewis, S. D.; Ng, A. S.; Baldwin, J. J.; Fusetani, N.; Naylor, A. M.; Shafer, J. A. *Thromb. Res.* **1993**, *70*, 173−190.

(22) Thrombin is a serine protease central to the bioregulation of thrombosis and hemostasis, see: Berliner, L. J., Ed. *Thrombin: Structure and Function*; Plenum Press: New York, 1992. Mann, K. G. *Trends Biochem. Sci.* **1987**, *12*, 229−233.

Scheme 1



our synthetic work on CtA (**1**), the total synthesis of CtB (**2**), and thrombin inhibition results with CtA.

Synthetic Chemistry

For the synthesis of CtA and CtB, we intended to employ suitably protected, naturally occurring or commercially available, amino acids. Consequently, each subunit would be imported into the target structure in enantiomerically pure form (relative to the α-carbon stereocenters) and with some of the desired protecting groups already in place on the commercial starting materials. The retrosynthetic analysis that we explored first involves formation of the N16−C17 bond by macrocyclization (Scheme 1, pathway 1), whereas the retrosynthetic analysis that we ultimately applied with success involves formation of the the N7−C8 bond by macrocyclization (Scheme 1, pathway 2).[1c] A key requirement of our plan is unveiling the amino group of the a-Ala subunit at a very late stage in the synthesis. Thus, we would be able to assemble both CtA and CtB via an advanced, common intermediate and conveniently introduce other, more hydrophobic acyl groups as well.[24] Additionally, the macrocycle construction entails a convergent [3 + 2] fragment-condensation process, rather than a linear sequence, which was conducive to a division of labor between the R. W. Johnson and Scripps laboratories. After the initial total synthesis of CtA,[14a] we repeated the entire sequence with an eye toward optimizing certain steps, particularly the macrocyclization and Dess−Martin oxidation. We also investigated side reaction issues and effected the synthesis of CtB.

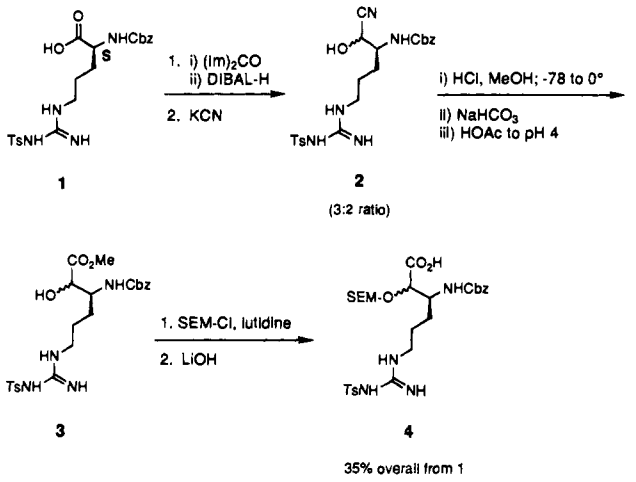
In planning the synthetic chemistry, the choice of protecting groups was viewed as very critical to our ultimate success. It was necessary to orchestrate this aspect of the project such that (1) the alkene group would not be reduced or substituted, (2) the amino group of a-Ala could be unblocked selectively, (3) the hydroxyphenyl group would not be oxidized, (4) the h-Arg side chain would be kept inert, and (5) the electrophilic α-keto group would not pose a problem. We decided to generate the α-keto group near the end of the synthesis rather than having to protect it and then facing a late-stage deprotection (Scheme 1). The *p*-toluenesulfonyl (Ts) group was chosen to protect the guanidine of the Arg/h-Arg entity (although nitro was also a candidate) because of its effectiveness for monoprotection, as long as we did not have to perform carbanion addition chemistry, and because of commercial availability. The phthalimide (Pht) group was chosen to block N9 because it would permit clean differentiation of this primary 9-amino substituent and be removable with hydrazine under relatively mild conditions. Protection of the h-Arg subunit by Ts, and protection of the v-Tyr subunit by TBDMS, set the stage for using anhydrous HF to deblock in the final step with subsequent conversion of CtA·HF to CtA·CF_3CO_2H during isolation/purification by reverse-phase HPLC.

We began the synthesis of CtA according to pathway 1 in Scheme 1 because macrocyclization in this case would involve a reactive primary amine. For the alternative approach in Scheme 1 (pathway 2), we were originally concerned about performing efficient macrocyclization onto the proline nitrogen. We also considered keeping the hydroxyl group protected until

(23) (a) We reported the first total synthesis of CtA in a preliminary context.[1a,14a] Hagihara and Schreiber reported the first total synthesis of a cyclotheonamide, namely CtB.[13] (b) For a subsequent total synthesis of CtA, see: Wipf, P.; Kim, H. *J. Org. Chem.* **1993**, *58*, 5592−5594. We thank Prof. Wipf for communicating results to us prior to publication [Addition and Correction: Wipf, P.; Kim, H. *J. Org. Chem.* **1994**, *59*, 2914]. (c) There have been preliminary synthetic reports on the D-v-Tyr subunit of 15-epi-CtA,[23d] the tripeptide (a-Ala)-Pro-(h-Arg),[23e] and a tetrapeptide segment of 15-epi-CtA.[23f] (d) Roth, P.; Metternich, R. *Tetrahedron Lett.* **1992**, *33*, 3993−3996. (e) Wipf, P.; Kim, H. *Ibid.* **1992**, *33*, 4275−4278. (f) Peterli-Roth, P.; Metternich, R.; Dalvit, C.; Breckenridge, R.; Ehrhardt, C.; Tapparelli, C. *Abstracts of the XIIth International Symposium on Medicinal Chemistry*, Basel, Switzerland, Sept 13−17, 1992.

(24) The formamide group of the CtA ligand is situated in a highly hydrophobic region of the thrombin active site (S_3 subsite),[14a] which is usually satisfied in the tripeptide class of thrombin inhibitors by a D-Phe (or analogous) group.[16] Thus, it might be advantageous to install a more hydrophobic group at this position of CtA.

Scheme 2

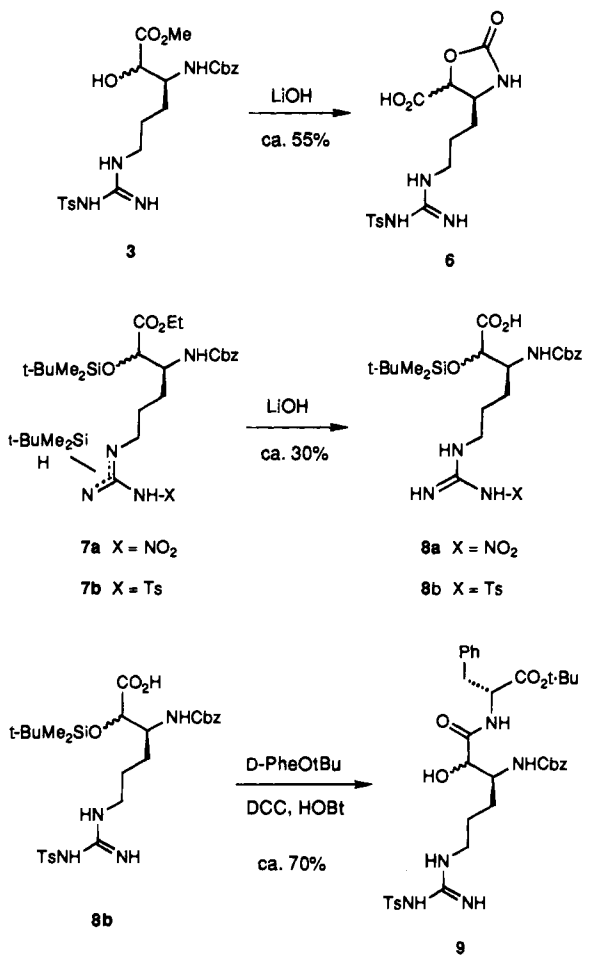


oxidation to the ketone was necessary. Hydroxyl protection, such as by a TROC group, was deemed important for pathway 1 in Scheme 1 because formation of a six-membered-ring lactone could compete with macrocyclization. Our [3 + 2] strategy benefits from flexibility in that the termini of segments A and B are orthogonally protected, allowing either approach in Scheme 1 to be tried in the event that one became unwieldy or nonviable. CtA was successfully synthesized by pathway 2 in Scheme 1.

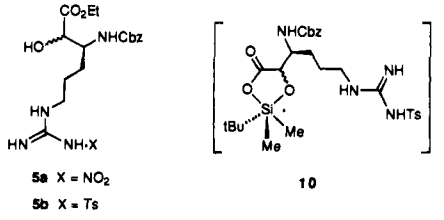
Segment A. By a reported method,[25] **1** was converted via its imidazolide derivative to the corresponding aldehyde (Scheme 2). The crude white solid was treated with excess KCN for 24 h to furnish cyanohydrin **2**. This white solid was a 3:2 mixture of diastereomers, which were not separated. Since the new stereochemically mixed center was to be eradicated by oxidation in the penultimate step of the synthesis, we decided to carry a diastereomeric mixture throughout the synthetic scheme. It turned out that the two diastereomers for each step were usually detectable by TLC as neighboring spots, and this was often of diagnostic value in judging formation of the correct products. A solution of **2** in absolute MeOH was treated with an excess of gaseous HCl at −78 °C, let stand at 0 °C, neutralized, and adjusted to pH 4 to give methyl ester **3**, which was reacted with 2,6-lutidine and SEM-Cl.[26] The crude SEM-protected methyl ester was saponified with LiOH to give SEM-acid **4**, isolated by chromatography as a white solid in 35% overall yield from **1**.

In early work, we had difficulty with direct saponification of ethyl ester **5a** in that no desired acid was obtained. Further investigation of the saponification of **3** revealed the major product to be a diastereomeric mixture of oxazolidinones **6**, isolated in 55% yield (Scheme 3).[27] Since the hydroxyl was apparently attacking the Cbz group, displacing benzyl alcohol, we opted to introduce a silyl protecting group.[28] Reaction of either **5a** or **5b** with *t*-BuMe$_2$SiCl and imidazole afforded the corresponding bis(silyl) derivative, **7a** or **7b**, treatment of which with LiOH gave acid **8a** or **8b** in ca. 30% yield.[27] Unfortunately, the silyl ether protecting group was being lost in the saponification, even when a *t*-BuPh$_2$Si protecting group was

Scheme 3



used. We were also surprised to find facile desilylation in the subsequent, mild peptide coupling step: when silyloxy acid **8b** was combined with the *tert*-butyl ester of D-Phe (i.e., D-PheO-*t*-Bu)[29] in the presence of DCC and HOBt, desilylated dipeptide **9** (19% overall from **5b**) was produced.[27] We considered that the carboxylate moiety may be exerting a neighboring group effect by attacking the silicon center to give a pentacoordinate siliconate species, such as **10**, which could lose the silyl group to form oxazolidinones in the presence of LiOH. However, in a control experiment with the TBDMS ether of methyl mandelate, saponification went cleanly to give the TBDMS ether of mandelic acid.[30] Alternatively, the urethane group or guanidine side chain may be the source of this problem, possibly due to catalysis by an NH group.



Envisioning later reprotection of the hydroxyl group, if necessary, we used routine protecting group manipulations and

(25) Austen, B. M.; Cliffe, S.; Grant, D.; Hermon-Taylor, J. U.S. Patent 4,593,018, 1986.

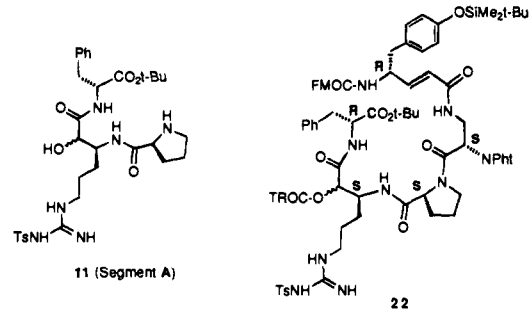
(26) Lipshutz, B. H.; Tegram, J. J. *Tetrahedron Lett.* **1980**, *21*, 3343–3346.

(27) See the paragraph at the end of this article regarding supplementary material.

(28) For a related observation on the stability of the TBDMS ether of mandelic acid to base, see: Burnell-Curty, C.; Roskamp, E. J. *Tetrahedron Lett.* **1993**, *33*, 5193–5196.

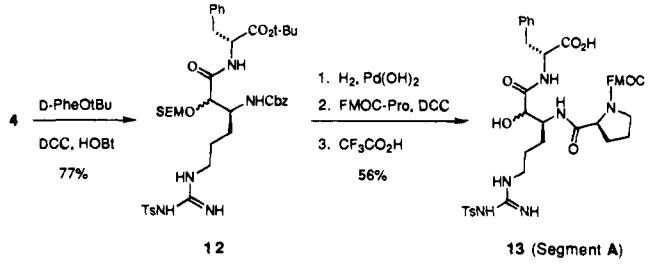
(29) Anderson, G. W.; Callahan, F. M. *J. Am. Chem. Soc.* **1960**, *82*, 3359–3363.

(30) (a) In the synthesis of CtB,[13] oxazolidinone formation was not observed with a Boc group on the α-amino group of the h-Arg subunit. (b) For a report of oxazolidinone formation on base (NaH) treatment of a β-hydroxy *tert*-butylurethane, see: Dondoni, A.; Franco, S.; Merchán, F. L.; Merino, P.; Tejero, T. *Tetrahedron Lett.* **1993**, *33*, 5475–5478.



coupling procedures to transform dipeptide **9** into tripeptide **11** (segment A): (1) the Cbz group was removed by hydrogenolysis in 89% yield, (2) Cbz-Pro was coupled by using DCC and HOBt in 76% yield, and (3) the Cbz group was removed by hydrogenolysis with $Pd(OH)_2$ in 83% yield.[31] Alternatively, SEM-acid **4** was coupled with D-PheO-*t*-Bu[29] by using DCC and HOBt to furnish **12**, isolated by chromatography in 77% yield, and we converted **12** into **13** (segment A) in 56% yield (Scheme 4): (1) the Cbz group was removed by hydrogenolysis, (2) FMOC-Pro was coupled by using DCC, and (3) the *tert*-butyl group was removed by trifluoroacetic acid (TFA).[31] Unfortunately, the SEM group was eliminated by the TFA treatment, but we hoped that the free hydroxyl would not pose a problem later on. Since abandoned pathway 1 in Scheme 1 eventually included a step involving *tert*-butyl ester deprotection with TFA, just prior to macrocyclization, the SEM ether would not have survived in this case either, exposing us to the threat of δ-lactone formation. Consequently, pathway 1 suffers from a touch of inelegance: the SEM group was needed early on to avoid oxazolidinone formation, but it would have to be exchanged for another protecting group (e.g., TROC) prior to macrocyclization.

Scheme 4



Segment B. Methyl tyrosine (**14**) was protected on the hydroxyl and α-amino groups by standard procedures[32,33] and reduced to the corresponding aldehyde with *i*-Bu_2AlH (5 min at −78 °C, MeOH quench) in good yield (Scheme 5). For olefination[34] of the intermediate aldehyde, a Wittig reagent bearing a bulky *tert*-butyl ester group was chosen to maximize *E* stereoselectivity; this was also conducive to mild deprotection in the subsequent step. We obtained the protected v-Tyr compound, **15**, with essentially 100% *E* stereoselectively in 73% isolated yield from **14**. Treatment of **15** with TFA removed the *tert*-butyl ester without disturbing the silyl ether (*vide infra*) to give **16** in 81% yield. L-2,3-Diaminopropanoic acid (**17**) was readily converted to the 3-*N*-Cbz derivative **18** (41%),[35] which was treated with *N*-carbethoxyphthalimide, followed by aqueous K_2CO_3, to generate an oily imide (80% yield). Removal of the Cbz group by hydrogenolysis in the presence of HOAc yielded a solution of thermally labile **19** (present as an acetate salt).[36] The organic solution of **19**·HOAc was concentrated to dryness carefully, diluted, washed with base at 0 °C, and coupled with **16** (1 mol equiv) by using EDC and HOBt in 72% yield. Recrystallization afforded FMOC-dipeptide **20**, as a white solid, which was deprotected[33] with Et_2NH to afford pure **21** (segment B) in 95% yield.

In early work, we performed this reaction sequence with D-Tyr, because the v-Tyr subunit of CtA was originally assigned the D (*R*) configuration,[9a] and proceeded to acyclic pentapeptide **22** en route to 15-epi-CtA.[27,37] When the correct stereochemistry of CtB (and thus CtA) was disclosed, we immediately revised our synthesis. In conjunction with the changeover, we decided to pursue pathway 2 in Scheme 1, which worked out nicely despite a macrocyclization involving acylation of a secondary amine.[39]

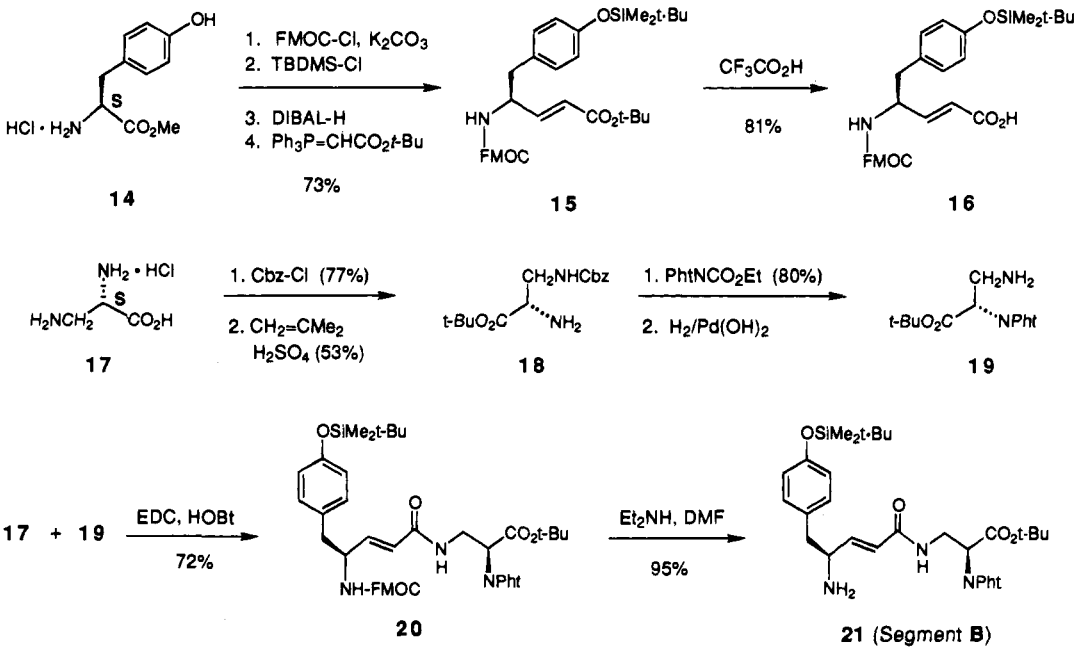
Segment Coupling and Macrocyclization. Equimolar amounts of **13** (segment A) and **21** (segment B), in a 0.1 M solution (CH_2Cl_2) containing Et_3N (3 mol equiv), were coupled by using BOP-Cl[40] (1 mol equiv) at 0−5 °C to give **23** in 65% yield (Scheme 6). The yield of this coupling was elevated to 75% when BOP reagent and DMAP were used.[41] We were concerned about the possible intramolecular reaction of **13** to form **24** in the coupling process (Scheme 7). Thus, **13** was independently treated with BOP reagent and DMAP to generate **24**, as confirmed by FAB-MS. The TLC of authentic **24** and the crude product from coupling **13** and **21** were compared, indicating that no more than a trace of **24** could have been produced during coupling. As far as we could ascertain, there was little epimerization during production of **23** because of stereomutation at the α-carbon of the D-Phe subunit.[42]

In the synthesis of cyclic peptides, the degree of success for macrocyclic coupling depends on the ring size, the type of amino acid residues, the carboxyl-activating reagent, and the concentration.[43] Such coupling of a linear pentapeptide would be hampered in the absence of amino acids that stabilize turn structures. Fortunately, the case at hand would be facilitated by the Pro and D-Phe subunits. If the macrocyclization process were too sluggish because of unfavorable factors, significant side reactions, such as epimerization (from racemization of the C-terminal α stereocenter) or cyclodimerization, would ensue.[43] We did not detect any products resulting from these side reactions; however, we did find one resulting from participation of the free hydroxyl group (vide infra).

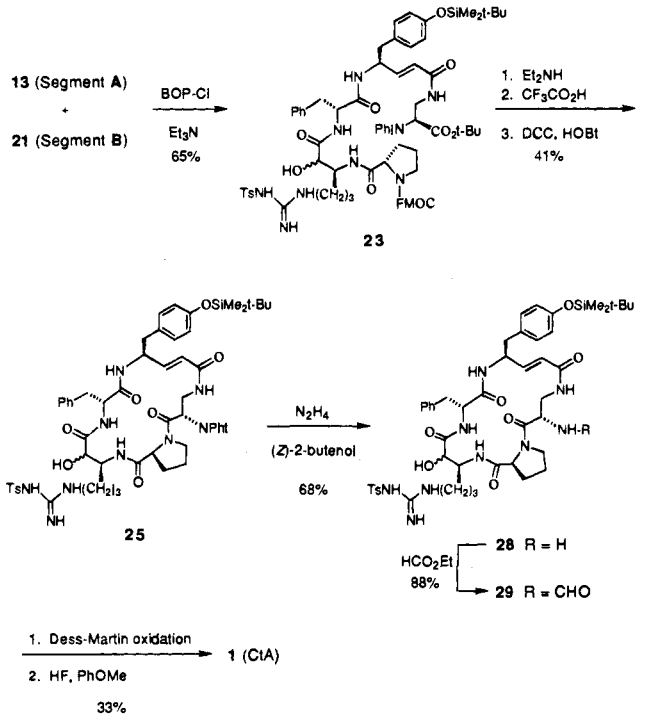
After removal of the FMOC group with diethylamine in MeCN (1.5 h) and the *tert*-butyl group with excess TFA−CH_2

(31) Bodanszky, M.; Klausner, Y. S.; Ondetti, M. A. *Peptide Synthesis*; John Wiley & Sons: New York, 1976.
(32) Corey, E. J.; Venkateswarlu, A. *J. Am. Chem. Soc.* **1972**, *94*, 6190−6191.
(33) Carpino, L. A.; Han, G. Y. *J. Org. Chem.* **1972**, *37*, 3404−3409.
(34) Maryanoff, B. E.; Reitz, A. B. *Chem. Rev.* **1989**, *89*, 863−927.
(35) Mokotoff, M.; Logue, L. W. *J. Med. Chem.* **1981**, *24*, 554−559.
(36) Attempts to isolate **19** resulted in the rapid formation of intractable white material; the acetic acid was an essential stabilizer. We presume that the instability of free base **19** is due to attack on the phthalimide by the reactive β-amino group.
(37) Coupling of the a-Ala and D-v-Tyr synthons with triphenylphosphine and 2,2′-dipyridyl disulfide (Mukaiyama, T.; Matsueda, R.; Suzuki, M. *Tetrahedron Lett.* **1970**, 1901−1904) was not effective (24% yield).
(38) Hagihara, M.; Schreiber, S. L. Presentation at the 203rd National Meeting of the American Chemical Society, San Francisco, CA, April 5−10, 1992; ORGN-508.
(39) Thaisrivongs, S.; Blinn, J. R.; Pals, D. T.; Turner, S. R. *J. Med. Chem.* **1991**, *34*, 1276−1282.
(40) Diago-Meseguer, J.; Palomo-Coll, A. L.; Fernandez-Lizarbe, J. R.; Zugaza-Bilbao, A. *Synthesis* **1980**, 547−551.
(41) Castro, B.; Dormay, J. R.; Evin, G.; Selve, C. *Tetrahedron Lett.* **1975**, 1219−1222.
(42) Dudash, J., Jr.; Jiang, J.; Mayer, S. C.; Joullié, M. M. *Synth. Commun.* **1993**, *23*, 349−356.
(43) Ehrlich, A.; Rothemund, S.; Brudel, M.; Beyermann, M.; Carpino, L. A.; Bienert, M. *Tetrahedron Lett.* **1993**, *34*, 4781−4784 and references cited therein. A comparison of different peptide coupling reagents in macrocyclization is reported.

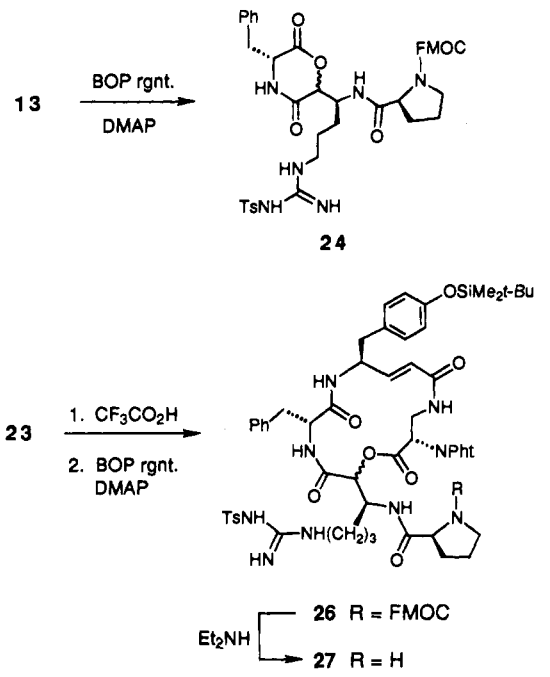
Scheme 5



Scheme 6



Scheme 7



Cl_2 (0−25 °C) from **23**, macrocyclic lactam formation[39] was effected under high-dilution conditions (0.001 M in CH_2Cl_2) to give **25** (Scheme 6). Our first macrocyclization involved BOP-Cl (2 mol equiv) with DMAP (5.05 mol equiv) and afforded a 25% isolated yield (flash column chromatography on silica gel; Table 1). The isolated yield of **25** from the three-step procedure was improved to 38% by using BOP reagent with DMAP[39,43] and to 41% by using DCC−HOBt (Table 1).[43] Again, we had a concern about the potential for side products due to the unprotected hydroxyl substituent. Consequently, a control experiment was conducted by reacting **23** sequentially with TFA, BOP reagent and DMAP (giving **26**), and diethylamine to generate 15−membered lactone **27** (FAB-MS), which might have been formed in the macrocyclization to lactam **25** (Scheme 7). In fact, TLC comparison of independently prepared **27** with the crude macrocyclization product indicated the presence of a small amount (ca. 10%) of this material, in a spot previously identified as being isomeric with the diastereomers of **25**. There was no evidence of epimerization during production of **25** because of stereomutation at the α-carbon of the a-Ala subunit.

In the deprotection of **23** for macrocyclization (Scheme 6), unblocking of the *tert*-butyl ester with TFA resulted in a byproduct due to removal of the TBDMS group. For a small test-scale reaction, this occurred to a very modest extent; however, on a larger scale, it became a problem (ca. 50% removal). The difference was connected to the longer reaction and the workup procedure: on the test scale, the TFA was removed by a stream of nitrogen, whereas on a larger scale, the TFA was removed by an evaporator at 23 °C. The phenol

Table 1. Macrocyclization of **23** to **25**

reagent[a]	yield,[b] %
BOP-Cl, DMAP	25
BOP reagent, DMAP	38
EDC, HOBt	24
DCC, HOBt	41
DPPA, $NaHCO_3$	25
BBC, *i*-Pr_2NEt	36

[a] For chemical abbreviations see ref 1b. [b] Isolated yield for three-step process.

byproduct could be effectively recycled to produce CtA or CtB by selective resilylation.[44]

Prior to macrocyclization, high-field NMR spectra of intermediates were readily interpretable in terms of the anticipated structures, despite the presence of two diastereomers at each step. However, after macrocyclization, NMR spectra of the large-ring intermediates were often poorly resolved and difficult to interpret.[27] As a consequence, for the chemistry from **25** to CtA, only FAB-MS and TLC were helpful to insure that we were proceeding correctly. Our discomfort persisted until verification was at hand by completion of the synthesis and comparison of synthetic CtA with authentic material.

Conversion of 25 to CtA and CtB. Removal of the phthalimide group from **25** with hydrazine was expected to be routine; however, this proved not to be the case (Scheme 6). Under various conditions, the desired amine **28** was contaminated, sometimes to the extent of 50%, with material that was two mass units higher (FAB-MS). MS and ^1H NMR data suggested that the carbon–carbon double bond was being reduced to the corresponding alkane, presumably by diimide arising from the hydrazine. Although we endeavored to eschew possible oxidation of the hydrazine by operating under argon in degassed solvent in EDTA-rinsed glassware, the side reaction persisted. Consequently, we were compelled to add an olefin as a diimide scavenger. Cyclohexene turned out to be only partially effective in this role, probably because it was being lost before complete hydrazine removal during evaporative workup. Indeed, the side reaction was successfully suppressed by use of less volatile olefin scavengers, such as 4-pentenol and (*Z*)-2-butenol. Thus, a 0.02 M solution of **27** in MeOH, containing hydrazine hydrate and (*Z*)-2-butenol, was stirred at 25 °C for 24 h to furnish amine **30** without any saturated byproduct (68% yield).

Crude amine **28** was heated at reflux in ethyl formate for 3 h to smoothly provide formamide **29**, which was oxidized with excess Dess–Martin periodinane[45] in CH_2Cl_2 at 25 °C to give the macrocyclic α-keto amide. Although this oxidation was successful, the reaction did not proceed to completion in spite of addition of excess periodinane; there was usually about 40–50% of unreacted starting material, most of which was recoverable.[46] Final deprotection was effected with excess anhydrous HF at 0 °C for 90 min, in the presence of anisole as an electrophile scavenger (Scheme 6). HPLC purification and lyophilization provided CtA (**1**), as a white, powdery trifluoroacetate salt in 16% yield from **29**.[47] The percent conversion of the Dess–Martin oxidation was improved to some extent by addition of *tert*-butyl alcohol to the medium,[45] but the reaction was still incomplete. Now, after HF deprotection, we obtained a 23% yield for the final two steps (**29** → **1**). Further improvement was realized by performing the oxidation at 65 °C in acetonitrile,[23b] thereby elevating the yield for the last two steps of our CtA synthesis to 33%. The synthetic CtA was identical to the natural product by 500-MHz ^1H NMR, 100-MHz ^{13}C NMR, HPLC, TLC, FAB-MS, optical rotation, and bioassay;[48] comparative ^1H NMR spectra are depicted in Figure 1.[49] It should be noted that the ^{13}C NMR spectrum of CtA in D_2O (pH 4.5) shows only the hydrated (*gem*-diol) form (δ C2, 97.2 ppm), rather than the α-keto amide form. Such adduct formation between CtA and hydroxylic solvents is often detected by FAB-MS.[9a] A combination of ^{19}F and ^1H NMR analysis afforded a CtA:TFA ratio of 1:1.9 for the synthetic substance and 1:1 for the natural product. The elemental analysis of synthetic CtA was consistent with this result and with the molecular formula CtA·1.9TFA·$6H_2O$, which was used in the bioassay to calculate K_i values.

At this point, the synthesis of CtB was very straightforward. Key amine intermediate **28** was selectively acetylated with pentafluorophenyl acetate (DMF, 90% yield),[50] then oxidized with the periodinane reagent in CH_2Cl_2/*t*-BuOH and deprotected with HF and anisole (33% yield for two steps). The Dess–Martin oxidation was effected under much less drastic conditions in this instance. Isolation in analogy with CtA afforded CtB in 30% overall yield from **30**. The TLC homogeneous product, a white, powdery trifluoroacetate salt, was characterized by 400-

(44) (a) The acyclic phenol byproduct was not isolated, rather the mixture was macrocyclized with DCC/HOBt in CH_2Cl_2–THF (1:1). Macrolactam **25** and its phenol analogue were separated by flash chromatography (silica gel, CH_2Cl_2–MeOH), whence ^1H NMR and FAB-MS confirmed the byproduct to be a desilylated version of **25**. The phenol macrocycle was also independently generated from **25** by desilylation with anhydrous CsF in THF or TFA–CH_2Cl_2–water (20:20:1). To obtain CtA, the phenol analogue of **25** was treated with hydrazine ((*Z*)-2-butenol, MeOH) and formylated (excess ethyl formate, DMF), affording the phenol analogue of **29** (44% yield), which was selectively silylated to give **29** (TBDMS-Cl, DMAP, Et_3N, CH_2Cl_2; 71% yield). Resilylation is necessary because the phenol analogue of **29** does not survive Dess–Martin oxidation. (b) For a comparison of different conditions for removal of a TBDMS ether from Tyr, see: Fischer, P. M. *Tetrahedron Lett.* **1992**, *33*, 7605–7608. (c) For desilylation of a TBDMS ether with TFA–water (9:1), see: Baker, R.; Cummings, W. J.; Hayes, J. F.; Kumar, A. *J. Chem. Soc., Chem. Commun.* **1986**, 1237–1239.

(45) Dess, D. B.; Martin, J. C. *J. Am. Chem. Soc.* **1991**, *113*, 7277–7287.

(46) The difficulty with attaining high-percent conversion in the Dess–Martin oxidation may be caused by conformational features of the macrocycle. A constrained ring geometry may subject the target hydroxyl to a sterically or electronically unfavorable environment. Indeed, although analogous acyclic peptide alcohols **13** and **23** were completely oxidized within 45 min at 23 °C in CH_2Cl_2, **25** remained largely unchanged under the same conditions.

(47) Although the C3 stereocenter of CtA, and of its immediate precursor, is potentially labile to base, no epimerization occurred under the conditions for transforming **29**. For the purpose of our bioassays, the stability of CtA under mildly basic conditions, pH 7.85 and 8.40 buffers, was investigated. After 35 min at 37 °C, only 0–1% degradation of CtA was apparent, as determined by HPLC (25 cm × 4.6 mm Supelcosil LC 18-DB column; water–MeCN, 3:1, containing 0.2% TFA; 1.5 mL/min). However, given much longer reaction times (2 days at 23 °C), we observed 5% and 45% degradation of CtA at pH 7.85 and 8.40, respectively. HPLC analysis showed the formation of two major, faster-eluting products (in nearly equal amounts) that had the same mass spectra (HPLC thermospray CI-MS; Supelcosil column as above) and UV spectra (diode-array detector): the molecular ions had the same mass as CtA, but the spectra were distinguishable. As a followup, exposure of CtA to triethylamine in MeCN–water at 23 °C showed a total loss of CtA within 1.5 h and the formation of the same new products, albeit in a different ratio. On the basis of FAB-MS and ^1H NMR data for the isolated mixture, we suggest that the new compounds may be diastereomeric, six-membered cyclic hemi-aminal adducts generated by addition of the proximal guanidine nitrogen to the keto group. After partial conversion of CtA with aqueous Na_2CO_3, there was no evidence for the presence of 3-epi-CtA by ^1H NMR analysis of the recovered CtA.

(48) Natural and synthetic CtA were comparable in competitively inhibiting human α-thrombin (see Enzyme Inhibition).

(49) The high-field NMR characterization of CtA included complete TOCSY (50 ms) and ROESY (225 ms) analysis in H_2O (G. Leo and P. MacDonnell, unpublished results). Our observations were generally consistent with the NMR data disclosed by Fusetani et al. (supplementary material to ref 9a).

(50) Kisfaludy, L.; Mohacsi, T.; Low, M.; Drexler, F. *J. Org. Chem.* **1979**, *44*, 654–656.

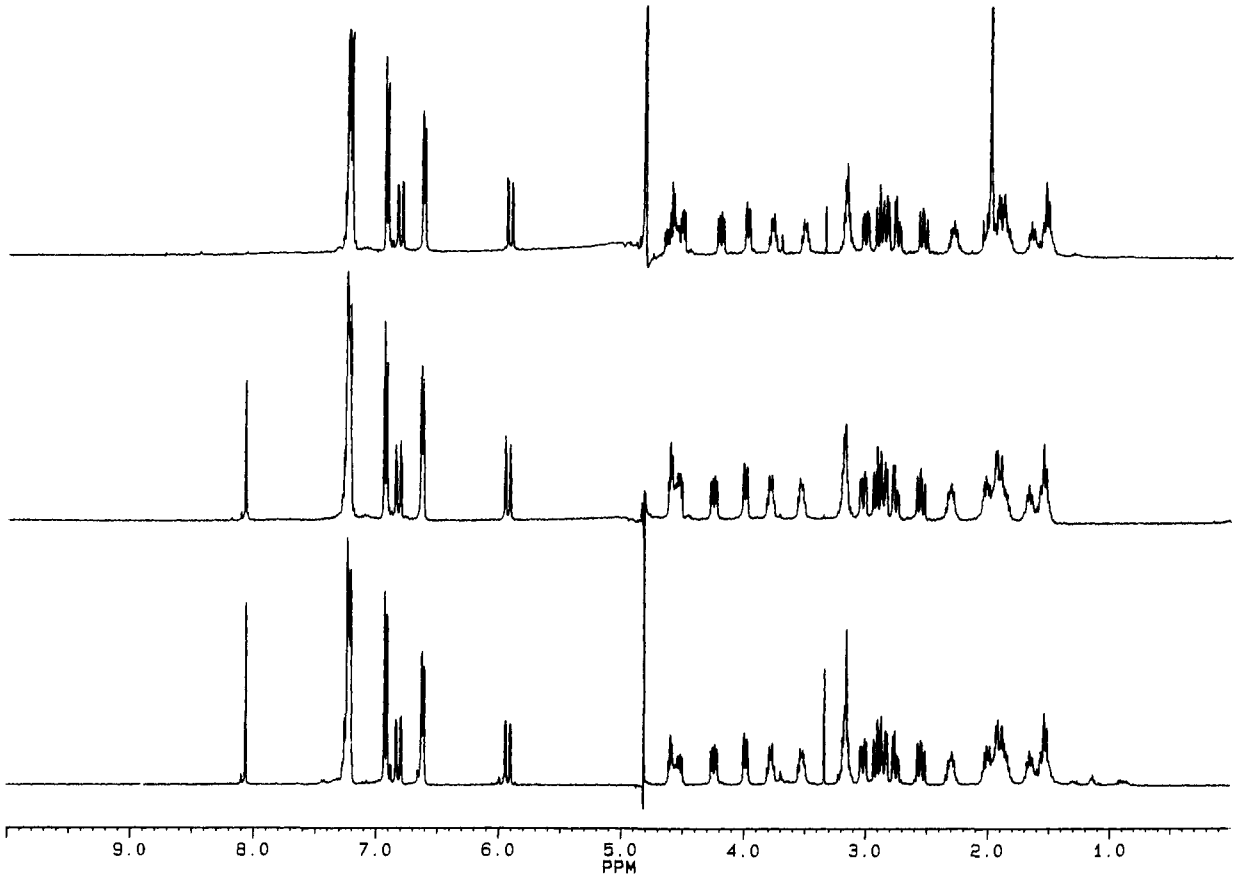


Figure 1. Comparison of 400-MHz NMR spectra in D_2O for authentic CtA (bottom panel), synthetic CtA (middle), and synthetic CtB (top).

MHz ^1H NMR (Figure 1), FAB-MS, ^{13}C NMR, IR, optical rotation, and bioassay.[51]

Comparison of Cyclotheonamide Syntheses. The synthesis of CtB reported by Hagihara and Schreiber[13] employs a linear approach, whereas our synthesis and that of Wipf and Kim[23b] employ a convergent [3 + 2] approach. All three routes are reasonably applicable to the synthesis of cyclotheonamide analogues in which the amino acid subunits are systematically modified. There is no significant difference between the three routes in the way that the unusual amino acids v-Tyr and a-Ala are obtained. However, relative to the a-Ala residue, our synthesis is uniquely well-suited for the convenient synthesis of analogues with modified acylamido and alkylamino substituents because of the late-stage unveiling of the primary amino group (viz. **28**).

Our route also differs by the use throughout of Arg/h-Arg with a monoprotected guanidine moiety, while the other routes use double protection. Thus, by starting with commercially available N^{α}-Cbz-N^{ω}-Ts-arginine (**1**), we obtain h-Arg synthon **4** via a five-step process in good overall yield and with just one purification. We elected to homologate the arginal intermediate generated from **1** via cyanohydrin formation (viz. **2**) because the process is amenable to guanidine monoprotection and directly supplies the alcohol oxidation state. In the synthesis of CtB,[13] homologation of the *N,O*-dimethylhydroxamate of arginine by [tris(methylthio)methyl]lithium is effected with double protection of the guanidine.[52] Although Wipf and Kim also apply the cyanohydrin reaction to preparation of h-Arg, they use double protection of the guanidine, which is restored once during the sequence because of loss of one of the groups.[23b] The Ts and TBDMS protecting groups are efficiently removed in the final step of our synthetic protocol.

For cyclotheonamide synthesis, macrocyclic lactamization can be effected by ring closure of a primary or secondary amine. The Hagihara–Schreiber route closes the Pro–(h-Arg) N4–C5 bond and the Wipf–Kim route closes the (D-Phe)–(v-Tyr) N16–C17 bond, both of which involve primary amines. By contrast, we close the (a-Ala)–Pro N7–C8 bond, involving a secondary amine, in a relatively standard manner.[39] It is worth noting that in the Wipf–Kim route the tetrahydropyranyl (THP) protecting group on the hydroxyl is lost prior to macrolactamization at the (D-Phe)–(v-Tyr) N16–C17 bond; however, this macrocyclization does not appear to suffer seriously from competitive δ-lactone formation,[23b] as we had feared in our early synthetic approach (Scheme 1, pathway 1).

Enzyme Inhibition

A major reason for our interest in CtA and CtB has stemmed from their ability to inhibit thrombin,[9a] an important enzyme in the regulation of hemostasis and thrombosis. We reported a K_i value of 180 ± 40 nM for the inhibition of thrombin by natural CtA.[14a] In the context of our total syntheses, we assessed the synthetic products CtA and CtB for inhibition of human α-thrombin (substrate = Spectrozyme TH or H-D-HHT-Ala-Arg-pNA, $K_m = 6\ \mu M$, $T = 37$ °C, pH = 7.4). Under the same conditions,[14a] synthetic CtA [bis(trifluoroacetate) hexahydrate] and synthetic CtB [assumed to be bis(trifluoroacetate) hexahydrate] provided K_i values ($n = 2$) of 72 ± 22 nM and 84 ± 32 nM, respectively, with reversible, competitive behavior.

(51) Synthetic CtB competitively inhibited human α-thrombin (see Enzyme Inhibition).

(52) Organometallic addition to arginine via the *N,O*-dimethylhydroxamate can be performed without guanidine double protection, by using N^{α}-Boc-N^{ω}-Ts-arginine (DiMaio, J.; Gibbs, B.; Lefebvre, J.; Konishi, Y.; Munn, D.; Yue, S. Y.; Hornberger, W. *J. Med. Chem.* **1992**, *35*, 3331–3341).

Some enzyme inhibition data for CtA were first reported by Fusetani et al.,[9a] as IC_{50} values. While our paper on CtA[14a] was in press, an interesting paper by Lewis et al.[21] appeared with data for the inhibition of thrombin by CtA. They observed that CtA is a slow, tight-binding thrombin inhibitor with a K_i value of 1.0 nM. There is disagreement between our K_i values for thrombin inhibition (at 37 °C) and those of Lewis et al. (at 25 °C), although the relative rankings are similar for various serine proteases. Besides the different temperatures, enzyme concentrations, pH, and buffers used in these two studies, different enzyme substrates were also used. The slow-binding inhibition for CtA found by Lewis et al., through a full kinetics treatment, was divergent from our linear (below saturation), competitive results. Thus, we have probed this issue further.

Since neither the temperature (37 °C vs 25 °C) nor pH (7.85[14a] vs 7.4) difference turned out to have serious consequences, we turned attention to the different chromogenic substrates and different enzyme concentrations ranging from 0.1 to 1.0 nM. Initially, we elected to examine four commercially available thrombin substrates using 0.1 nM thrombin at 25 °C and pH 7.4: Spectrozyme TH ($K_m = 1.6 \pm 0.2$ μM), Sar-Pro-Arg-pNA ($K_m = 101 \pm 6$ μM), D-Phe-Pip-Arg-pNA (S2238, $K_m = 2.0 \pm 0.1$ μM), and Ts-Gly-Pro-Arg-pNA (T1637, $K_m = 9.0 \pm 2.2$ μM). Reaction conditions are described in the Experimental Section, and data were analyzed according to Williams and Morrison.[53] At first, the reactions were started by the addition of enzyme to a mixture of CtA or CtB and substrate ("enzyme last"). The well-known thrombin inhibitor DuP-714 was also studied as a reference slow, tight-binding inhibitor.[54] Two sets of curves, reflecting time-dependent inhibition of thrombin with increasing concentrations of synthetic CtA or CtB in the presence of S2238, are depicted in Figure 2. The curves in Figure 2 display a short-lived pre-steady-state phase and a second steady-state that fits the integrated rate equation for slow-binding inhibitors.[53,55] The observation of slow-binding kinetics under these conditions for CtA with S2238 (Figure 2A) agrees with the characterization made by Lewis et al.[21] The K_i values were obtained by fitting the data to the equation $P = v_{st} + (v_o - v_s)(1 - e^{-kt})/kt$, 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. The K_i values ($n = 2$, S2238) determined for natural CtA, synthetic CtA, synthetic CtB, and Dup-714 were 0.5 ± 0.4 nM, 3.5 ± 0.8 nM, 6.3 ± 4.7 nM, and 40 ± 20 pM (lit.[54] $K_i = 44$ pM), respectively. Subsequently, we tested the four substrates under 4-h preincubation conditions, similar to those described by Lewis et al. ("substrate last").[21] Consistent with their observations, CtA exhibited slow-binding inhibition with Sar-Pro-Arg-pNA; the three other substrates exhibited similar behavior after a 4-h CtA–thrombin equilibration, as well.

A comparison of our previous results[14a] with those shown above indicates that CtA can manifest standard competitive inhibition (Michaelis–Menten kinetics) or slow-binding inhibition according to the protocol of the enzymology experiment. Under conditions where thrombin was added to a mixture of substrate and inhibitor ("enzyme last"), the occurrence of slow-binding inhibition was dependent on the substrate and on thrombin concentration (more apparent at 0.1 than at 1.0 nM).[56] However, under conditions where the substrate was added to an equilibrated mixture of CtA and thrombin ("substrate last"), slow-binding inhibition occurred consistently.

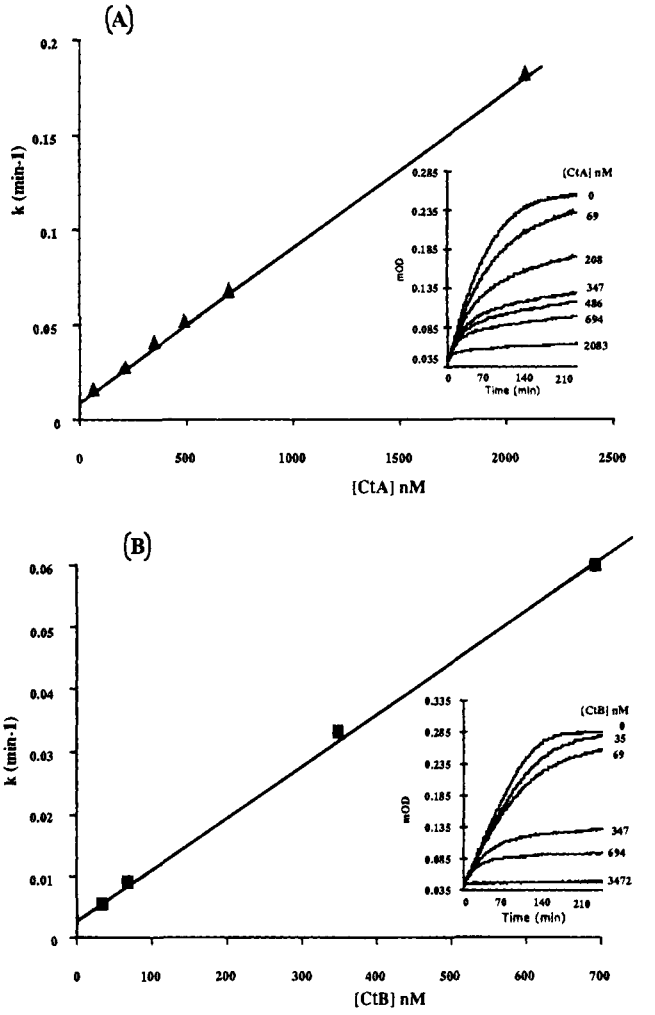


Figure 2. Representative progress curves showing time-dependent inhibition of human α-thrombin by (A) synthetic CtA and (B) synthetic CtB. The reactions were started by addition of human α-thrombin (0.1 nM) in the presence of the chromogenic substrate S2238 (50 μM) at 25 °C.

In considering CtA as a slow, tight-binding inhibitor,[19,23f,57] it is important to appreciate the effect of different conditions on the enzyme kinetics. Specifically, a confluence of various factors can give rise to dichotomous observations, but a common thread is the reaction rate. Given the competition between the rate of substrate turnover and the rate of formation of the final CtA–thrombin complex ("tight-binding state") from the initial CtA–thrombin complex ("loose-binding state"), the slow-binding behavior can be completely obscured. The slow-binding inhibition for CtA, although not particularly representative of physiological conditions, is representative of the CtA–enzyme complexes studied by X-ray crystallography[14a,19] because of the time elapsed for growth of the crystals.

Discussion

Small cyclic peptides, such as CtA, are attractive structural formats for the design of ligands for enzymes or receptors because of certain specific features. First, cyclization of a linear peptide reduces the number of available low-energy conformations and alters the distribution of conformations. Thus, molecular recognition involving the modified, macrocyclic ligand may show distinct differences from the original ligand.

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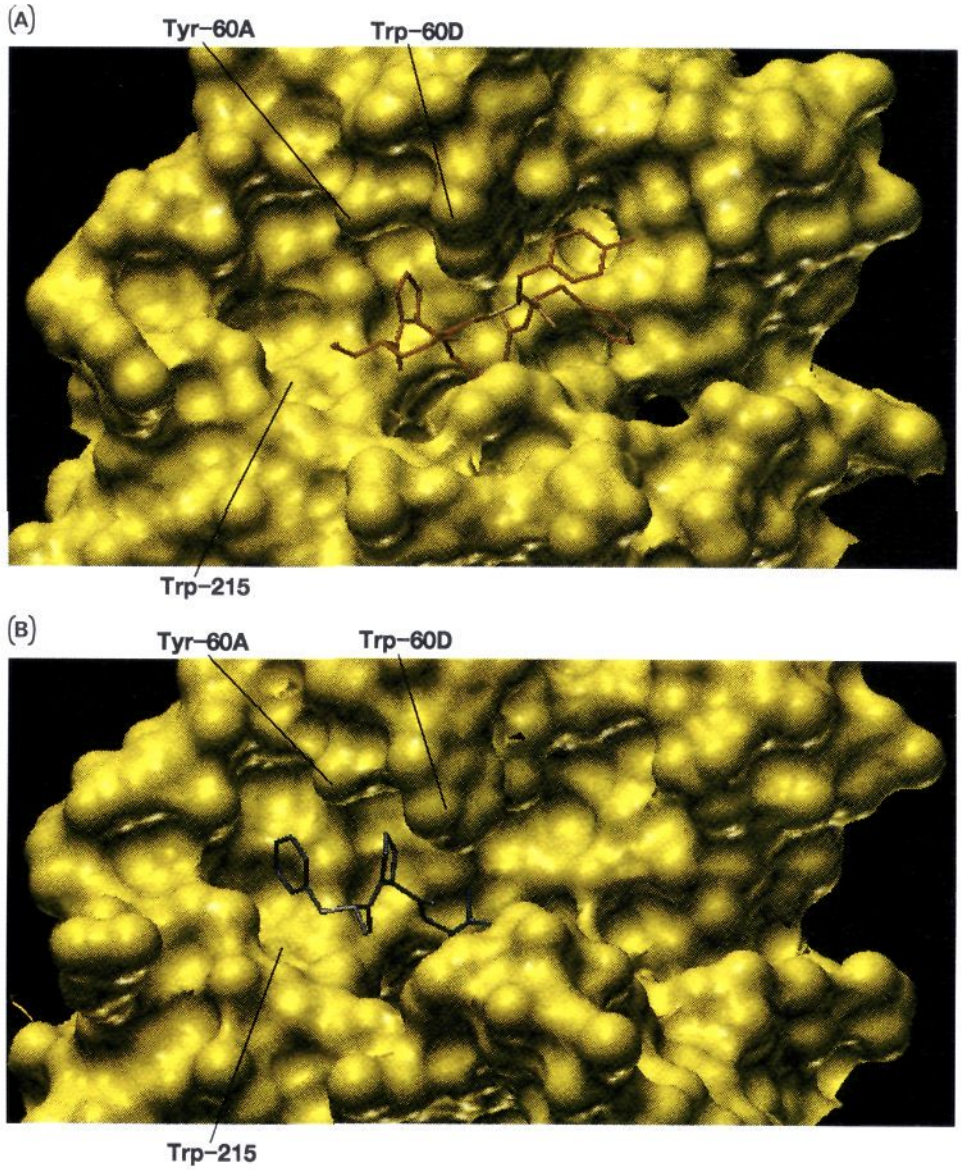

Figure 3. Comparison of the binding modes for CtA–thrombin and PPACK–thrombin. (A) Representation of the CtA–thrombin complex from X-ray crystallography[14a] displaying the active-site region. Thrombin (yellow) is shown with a Connolly surface; CtA (orange) is shown as a stick model of its non-hydrogen atoms. The S3 hydrophobic pocket, located between residues Tyr-60A and Trp-215, is vacant. (B) Representation of the PPACK–thrombin complex from X-ray crystallography[18b] displaying the active-site region. Thrombin (yellow) is shown with a Connolly surface; PPACK (blue) is shown as a stick model of its non-hydrogen atoms. The S_3 hydrophobic pocket, located between residues Tyr-60A and Trp-215, is occupied by the phenyl ring of PPACK.

This could, for example, turn an enzyme substrate into an inhibitor or a receptor agonist into an antagonist. Second, cyclic peptides gain advantage for possible in vivo activity, which is important for therapeutic applications, because they lack polar end groups and are more sterically hindered. Relative to acyclic peptides, this can confer greater resistance to degradation by peptidases and enhanced ability to traverse membrane barriers.

The conformations of CtA in its complexes with thrombin[14a] and trypsin[19] are known from X-ray crystallography. The structural features of the 19-membered ring and the Pro-Arg segment are virtually the same in these two situations; the main distinctions relate to the orientations of the D-Phe and v-Tyr aromatic groups. Since thrombin has a more restrictive catalytic cleft, with the presence of the 60A–60I insertion loop that appears to interact 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,[14a,19a] only limited factors can be pinpointed to explain the greater effectiveness of CtA for inhibition of trypsin relative to thrombin. The CtA–thrombin complex shows an aromatic stacking interaction between v-Tyr of CtA and Trp-60D of the thrombin insertion loop (Figure 3A),[14a] which is absent from the CtA–trypsin complex. However, in the CtA–trypsin complex, the D-Phe side chain is ensconced within a hydrophobic area defined by two aromatic residues of the enzyme (Tyr-39 and Phe-41).[19a]

One thing that is clear is the formamide group of CtA is situated in the thrombin active site such that the hydrophobic S_3 subsite is left vacant (cf. Figure 3A,B).[14a] In the X-ray crystal structure of the complex between PPACK and thrombin, the phenyl group of D-Phe nicely occupies this hydrophobic pocket, while stacking with Trp-215 (Figure 3B).[18b] Since trypsin does not have such a pronounced, hydrophobic S_3 area, this factor may partly account for the difference in biological activity.

It is interesting to note that the ^{13}C NMR spectrum for synthetic 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 (signal for C2 at 97.2 ppm; there was no signal for a C2 keto carbon). This coincides with ^{13}C NMR data reported for the natural product (C2 at 97.4 ppm).[9a] We have also observed methanol adducts of CtA by mass spectrometry, as reported by Fusetani and co-workers,[9a] presumably caused by addition of methanol to C2. It is unknown at this time whether the propensity of CtA to exist as a covalent adduct at C2 has any bearing on its enzyme inhibitory properties. The rate of dissociation of the covalent adduct to the keto form might play a role in enzyme inhibition under certain circumstances and may be associated with the observed slow-binding inhibition.[21]

The slow-binding inhibition of thrombin by CtA, although not particularly relevant to physiological conditions, is relevant to the CtA−thrombin complex studied by X-ray crystallography because of the time elapsed for growth of the crystals.[14a,58] Hence, the earlier viewpoint that CtA has a deficiency in binding interactions within thrombin is inaccurate.[14a] In the face of the low nanomolar K_i value under conditions that evince the slow-binding component, CtA probably benefits from supportive interactions outside of the Pro−(h-Arg) domain, such as the aromatic stacking between v-Tyr and Trp-60D (Figure 3A). Clearly, CtA must be considered as an effective ligand for the thrombin active site. Despite this revised interpretation, the issue surrounding the S_3 region still exists and presents a curious course for structural modification. Specific cyclotheonamide analogues with a more hydrophobic acyl (or alkyl) substituent on the amino group at C9 might show enhanced thrombin inhibition and provide insight into molecular recognition. Our synthetic route to cyclotheonamide derivatives is especially well-suited for these modifications at a late stage, via key intermediate **28**, and we are currently pursuing this direction.

Experimental Section

General Procedures. Proton NMR spectra were recorded on a Bruker AC 300 B (300 MHz), Bruker AM-360 WB (360 MHz), Bruker AM-400 (400 MHz), or Bruker AMX 500 (500 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, and dist = distorted. Carbon-13 NMR spectra were obtained at 100.6 MHz on the Bruker AM-400 or at 125 MHz on the Bruker AMX 500 with either Me_4Si or sodium 3-(trimethylsilyl)propanoate-d_4 as an internal standard, unless otherwise indicated. Fluorine-19 NMR spectra were recorded on the 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, and chemical shifts referenced to $CFCl_3$ (δ 0.0). The CtA:TFA ratio was analyzed by correlation with a known amount of trifluoroethanol, used as an internal or external ^{19}F and ^1H reference. Infrared spectra were recorded on a Nicolet SX 60 FT spectrometer or a PE 1600 Series FT-IR (relative intensities: s = strong, m = medium). Chemical-ionization mass spectra (CI-MS) were recorded on a Finnigan 3300 system with CH_4 or NH_3 as the reagent gas. Fast-atom-bombardment mass spectra (FAB-MS) 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. FAB-MS data were also obtained on a VG ZAB 2-SE double-focusing high-resolution mass spectrometer equipped with a cesium ion gun. A 35-kV cesium ion beam was used as the primary ion beam in a glycerol or 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 on the basis of the PEG reference masses. Proposed empirical formulas 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 done with flash-column silica gel (32−63 μm). HPLC separations were carried out on three Waters PrepPak cartridges (25 × 100 mm, Bondapak C18, 15−20 μm, pore size = 125 Å) connected in series, 254 nm on a Waters 486 UV detector. Melting points were determined on a Thomas−Hoover apparatus calibrated by a set of melting point standards. Microanalysis was performed by Robertson Microlit Laboratories, Inc., or by Galbraith Laboratories, Inc.; water was determined by the Karl Fischer method.

Materials. Natural CtA was obtained from Prof. N. Fusetani,[9a] as a monotrifluoroacetate monohydrate. N^{α}-Cbz-N^{ω}-tosylarginine and N^{α}-Cbz-D-phenylalanine were purchased from Bachem; tyrosine methyl ester hydrochloride, *N*-FMOC-proline, and DuP-714 were purchased from Sigma Chemical Co.; L-α,β-diaminopropanoic acid (a-Ala) hydrochloride was purchased from Schweizerhall, Inc.; (*Z*)-2-butenol was purchased from Wiley Organics.

6-[[Imino[(4-methylbenzenesulfonyl)amino]methyl]amino]-2(*R*,*S*)-[[2-(trimethylsilyl)ethoxy]methoxy]-3(*S*)-[(phenylmethoxycarbonyl)-amino]hexanoic Acid (4). To a solution of 25.2 g of Cbz-Arg(Ts)-OH (**1**) in 160 mL of THF at 0−10 °C was added carbonyldiimidazole (9.89 g, 0.053 mmol) in one portion.[24] After the solution was stirred for 1 h, it was cooled to −40 °C and 1 M *i*-Bu_2AlH in hexanes (130 mL) was added over 15 min. After 30 min of stirring, the reaction mixture was quenched with 1.2 N HCl (365 mL) and warmed to 23 °C. The mixture was treated with 360 mL of 0.6 N HCl and 400 mL of $CHCl_3$, then stirred for 2.5 h. The organic phase was separated off, and the aqueous phase was extracted twice with $CHCl_3$. The combined extracts were washed three times with water, dried (Na_2SO_4), and concentrated in vacuo at 40 °C to give 22.3 g of the desired aldehyde as a white solid [FAB-MS *m/z* 447 (MH^+)]. A solution of the aldehyde (22.0 g, 49 mmol) in 120 mL of MeOH−water (1:1) and 110 mL of ethyl acetate was treated with KCN (6.0 g, 92 mmol). After 16 h of stirring, the layers were separated and the aqueous phase was extracted twice with ethyl acetate. The combined organic extracts were washed with water, dried (Na_2SO_4), and concentrated to afford 20.5 g of cyanohydrin **2** as a white foam [FAB-MS *m/z* 474 (MH^+)]. A solution of **2** (20.0 g) in 220 mL of absolute MeOH at −78 °C was treated with 90 g of gaseous HCl at such a rate as to maintain the temperature below −40 °C. After 30 h at 0 °C, the mixture was added slowly to an aqueous $NaHCO_3$ (273 g) with vigorous stirring, maintaining pH > 5−6. Following neutralization, we added glacial acetic acid to attain pH 4.0, then 400 mL of ethyl acetate was added. The two-phase mixture was stirred for 4 h, the layers were separated, and the aqueous phase was extracted three times with ethyl acetate. The combined organic solution was washed sequentially with water, saturated $NaHCO_3$, and water, dried (Na_2SO_4), and concentrated to give 18.2 g of **3** as a white foam [FAB-MS *m/z* 507 (MH^+)]. To a solution of **3** (18.0 g, 36 mmol) and 2,6-lutidine (17.0 g, 160 mmol) in 150 mL of CH_2Cl_2 was added dropwise [2-(trimethylsilyl)ethoxy]methyl chloride (SEM-Cl; 16 g, 96 mmol). After 3 days of stirring, solvent was removed in vacuo; the residue was treated with excess toluene and concentrated in vacuo. The process was repeated, then the residue was taken up in ethyl acetate and washed sequentially with water, 10% aqueous citric acid, and water. Drying (Na_2SO_4) and concentration afforded 27 g of the SEM-protected ester as a tan oil [FAB-MS *m/z* 637 (MH^+)]. A solution of the SEM-protected ester (25 g) in 9:1 dioxane−water (168 mL) was treated with 7.0 g of pulverized LiOH. After stirring for 4 h, the reaction was adjusted to pH 4 with HOAc, then diluted with ethyl acetate. The mixture was washed twice with brine, dried (Na_2SO_4), and concentrated. The residue was purified by flash column chromatography (gradient from 92:8 to 85:15 CH_2Cl_2−MeOH) to give 11.0 g of **4** as a white solid (two diastereomers, 35% yield from **1**): TLC R_f 0.26, 0.16 (85:14.5:0.5 CH_2Cl_2−MeOH−HOAc); ^1H NMR δ 7.68 (d, *J* = 7.7 Hz, 2 H), 7.36 (s, 5 H), 7.29 (d, *J* = 7.7 Hz, 2 H), 7.17−6.78 (br m, 2 H, exch), 5.11−4.96 (m, 2 H, OCH_2Ph), 4.79−4.58 (overlapping m, 2 H, OCH_2O), 3.99−3.84 (m, 1 H,

(58) This applies to the CtA−trypsin X-ray situation, as well.[19]

SEMOC*H*), 3.83−3.61 (m, 2 H, OCH_2CH$_2$), 3.57−3.46 (m, 1 H, OCHC*H*N), 3.12−2.94 (m, 2 H), 2.35 (s, 3 H), 1.64−1.25 (m, 4 H), 0.94−0.77 (m, 2 H, SiCH_2CH$_2$), 0.04 (s, 9 H, Me_3Si); IR (KBr) ν_{max} 3434, 3337, 3165, 2953, 2894, 1702 (s), 1622 (s), 1591 (s), 1550 (s), 1412, 1250 (s) cm^{-1}; FAB-MS *m/z* 623 (MH$^+$); FAB-HRMS exact mass calcd for $C_{28}H_{41}N_4O_8SSi$ (M + Na$^+$) 645.2390, found 645.2437.

2(*R*)-[[3(*S*)-[(Benzyloxycarbonyl)amino]-6-[[imino[(4-methylbenzenesulfonyl)amino]methyl]amino]-2(*R*,*S*)-[[2-(trimethylsilyl)ethoxy]methoxy]hexanoyl]amino]-3-phenylpropanoic Acid *tert*-Butyl Ester (12). To a solution of 0.70 g of **4** (1.1 mmol), 0.27 g of D-PheO-*t*-Bu (1.2 mmol), 0.20 g of HOBt, and 10 mL of MeCN was added a solution of DCC (0.25 g, 1.2 mmol) in 1 mL of MeCN, dropwise. The reaction was stirred overnight, then filtered and concentrated. The residue was taken up in ethyl acetate, washed with saturated $NaHCO_3$, dried (Na_2SO_4), and concentrated in vacuo. Flash column chromatography (95:5 CH_2Cl_2−MeOH) afforded 0.70 g of **12** as an off-white foam (77%): ^{1}H NMR ($CDCl_3$);[27] FAB-HRMS exact mass calcd for $C_{41}H_{59}N_5O_9SSi$ 826.3881, found 826.3942.

2(*R*)-[[6-[[Imino[(4-methylbenzenesulfonyl)amino]methyl]amino]-3(*S*)-[[[1-(9*H*-9-fluorenylmethylcarbonyl)pyrrolidin-2(*S*)-yl]carbonyl]amino]-2(*R*,*S*)-hydroxyhexanoyl]amino]-3-phenylpropionic Acid (13, Segment A). A solution of **12** (3.2 g, 3.9 mmol) in 50 mL of MeOH was treated with 20% $Pd(OH)_2$ (1.5 g) and shaken with hydrogen (10 psig) for 3 h. The slurry was filtered, and the filtrate was concentrated to give 2.6 g (100%) of deprotected intermediate as a foam. The crude product was dissolved in 60 mL of MeCN containing FMOC-Pro (1.4 g, 4.1 mmol) and HOBt (1.1 g, 8.1 mmol). The mixture was treated with a solution of DCC (0.84 g, 4.1 mmol) in 10 mL of MeCN and stirred overnight. The reaction was filtered and concentrated in vacuo; the residue was taken up in CH_2Cl_2, washed with saturated $NaHCO_3$, dried (Na_2SO_4), concentrated, and purified by flash column chromatography (95:5 CH_2Cl_2−MeOH) to give 2.8 g of coupled product as a foam: ^{1}H NMR (CD_3OD) δ 7.82−7.55 (m, 6 H, arom.), 7.42−7.16 (m, 11 H, arom.), 4.70−3.94 (m, 9 H), 3.70−3.39 (m, 4 H), 3.23−2.88 (m, 4 H), 2.36 (s, 3 H, Ts*Me*), 2.23−2.07 (m, 1 H), 2.02−1.82 (m, 3 H), 1.42−1.35 (m, 13 H), 0.89 (m, 2 H, SiCH_2-CH_2), 0.00 (m, 9H, Me_3Si); IR (KBr) ν_{max} 3416, 3330, 1681 (s), 1626, 1550 (s), 1451, 1416, 1359, 1250, 1151, 1132 (s), 1083 cm^{-1}; FAB-MS *m/z* 1012 (MH$^+$). Anal. Calcd for $C_{53}H_{70}N_6O_{10}SSi$: C, 62.95; H, 6.98; N, 8.31. Found: C, 62.58; H, 7.05; N, 8.43.

A solution of this material in 10 mL of CH_2Cl_2 was added to 1:1 CH_2Cl_2−TFA (30 mL) at 0 °C. The reaction was gradually warmed to 23 °C and stirred for a total of ca. 4 h, and the volatiles were removed by a stream of argon. The residue was triturated twice with ether to afford 2.3 g of **13** as a white powder: FAB-MS *m/z* 825 (MH$^+$).

5-[4-[(*tert*-Butyldimethylsilyl)oxy]phenyl]-4(*S*)-[(9-fluorenylmethoxycarbonyl)amino]pent-2-enoic Acid *tert*-Butyl Ester (15). A solution of tyrosine ester **14** (14.48 g, 62.5 mmol) in water (60 mL) was treated with K_2CO_3 (17.97 g, 130 mmol) in water (30 mL) at 0 °C. The resulting precipitate was dissolved with additional dioxane (120 mL), and FMOC-Cl (14.23 g, 55.0 mmol) was then added in small portions. The milky mixture was stirred at 0 °C for 4 h, then extracted with ethyl acetate (2 × 70 mL). The combined extracts were dried ($MgSO_4$) and concentrated to yield the FMOC-protected material (29.1 g) as an oil. A solution of this material (15.88 g, ca. 38 mmol) and imidazole (7.76 g, 114 mmol) in dry CH_2Cl_2 (150 mL) at 0 °C was treated with TBDMS-Cl (14.33 g, 95 mmol) in CH_2Cl_2 (50 mL). The mixture was the stirred at 23 °C for 4 h, diluted with CH_2Cl_2 (150 mL), and quenched with water (300 mL). The aqueous phase was extracted with ether (2 × 80 mL), and the combined organic extracts were dried ($MgSO_4$) and concentrated. The residue was purified by flash column chromatography (15:85 EtOAc−petroleum ether) to give a silylated product (15.54 g, 86% for two steps) as a glass: mp 47−49 °C (from EtOAc−petroleum ether); TLC R_f 0.19 (1:9 EtOAc−petroleum ether); $[\alpha]_D^{23}$ +31.0° (*c* 1.0, $CHCl_3$); IR (thin film);[27] ^{1}H NMR (300 MHz, $CDCl_3$);[27] ^{13}C NMR (125 MHz, $CDCl_3$);[27] FAB-HRMS (NBA/CsI) exact mass calcd for $C_{31}H_{37}NO_5Si$ (M + Cs$^+$) 664.1495, found 664.1499. This compound (15.0 g, 28.2 mmol) in toluene (250 mL) was stirred at −78 °C and *i*-Bu_2AlH (71 mL, 1.0 M in hexane, 71 mmol, cooled to −78 °C) was added over 45 min via cannula under argon. The reaction mixture was stirred for 5 min and quenched with MeOH (50 mL). After the mixture was removed from the cooling bath, saturated aqueous Rochelle's salt (150 mL) was added and the mixture was stirred vigorously for 1.5 h until the phases became clear. The mixture was extracted with ether (2 × 200 mL); the combined extracts were washed with brine (150 mL), dried ($MgSO_4$), and concentrated in vacuo to afford the corresponding aldehyde (14.7 g) as a glassy solid, which was not purified because of partial racemization during chromatography with silica gel. A solution of crude aldehyde (13.78 g, 27 mmol) in DMF (95 mL) was treated with [(*tert*-butoxycarbonyl)methylene]triphenylphosphorane (11.38 g, 30 mmol) and stirred at 25 °C for 2 h. The reaction mixture was diluted with ether (200 mL) and washed with brine (4 × 80 mL), and the combined aqueous layers were extracted with ether (100 mL). The combined extracts were dried ($MgSO_4$), concentrated in vacuo, and purified by flash column chromatography (11:89 EtOAc−petroleum ether) to give **15** (13.54 g, 85% for two steps; 73% overall yield from **14**) as a solid: mp 70−72 °C (from EtOAc−petroleum ether); TLC R_f 0.32 (1:9 EtOAc−petroleum ether); $[\alpha]_D^{23}$ −5.9° (*c* 1.0, $CHCl_3$); IR (KBr);[27] ^{1}H NMR (500 MHz, $CDCl_3$);[27] ^{13}C NMR (125 MHz, $CDCl_3$);[27] FAB-HRMS (NBA/CsI) exact mass calcd for $C_{36}H_{45}NO_5Si$ (M + Cs$^+$) 732.2121, found 732.2121.

5-[4-[(*tert*-Butyldimethylsilyl)oxy]phenyl]-4(*S*)-[(9-fluorenylmethoxycarbonyl)amino]pent-2-enoic Acid (16). A solution of **15** (13.5 g, 22.6 mmol) in CH_2Cl_2 (75 mL) at 0 °C was treated with TFA (60 mL), then stirred at 0 °C for 5 min and 25 °C for 1 h. The reaction mixture was diluted with ether (120 mL), then washed with cold aqueous $NaHSO_4$ (50 mL, saturated) and cold brine (50 mL). The organic phase was dried ($MgSO_4$), concentrated in vacuo (25 °C), and purified by flash column chromatography (gradient from 1:4 EtOAc−petroleum ether to 100% EtOAc) to give **16** (9.94 g, 81%) as a solid: mp 79−81 °C (from EtOAc−petroleum ether); TLC R_f 0.32 (1:1 EtOAc−petroleum ether); $[\alpha]_D^{23}$ −14.5° (*c* 1.1, EtOH); IR (KBr) ν_{max} 3424 (br), 2954 (m), 1702 (s), 1512 (s), 1259 (s), 915 (m), 839 (m), 740 (m) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2 H, FMOC arom.), 7.55−7.51 (m, 2 H, FMOC arom.), 7.40 (t, *J* = 7.4 Hz, 2 H, FMOC arom.), 7.31 (t, *J* = 7.4 Hz, 2 H, FMOC arom.), 7.06−6.98 (br, 1 H, C*H*=CHCO), 6.99 (d, *J* = 7.7 Hz, 2 H, v-Tyr arom.), 6.77 (d, *J* = 8.2 Hz, 2 H, v-Tyr arom.), 5.82 (d, *J* = 15.6 Hz, 1 H, CH=C*H*CO), 4.74 (d, *J* = 8.6 Hz, 1 H, NH), 4.69−4.60 (m, 1 H, C*H*CH=CHCO), 4.48−4.37 (m, 2 H, OCH$_2$), 4.19 (t, *J* = 6.6 Hz, 1 H, C*H*-Ar), 2.85 (br, 2 H, TyrCH_2), 0.94 (s, 9 H, Si-*t*-Bu), 0.18 (s, 6 H, SiMe_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8 (CO), 155.5 ($NHCO_2$), 154.6 (arom.), 149.5 (*C*H=CHCO); 143.6, 141.2, 130.2, 128.5, 127.7, 127.0, 124.9 (7 arom.); 120.6 (CH=*C*HCO), 120.12 (arom.), 119.9 (arom.), 66.67 (OCH$_2$), 52.9 (C*H*CH=CH), 47.1 (CH-Ar), 39.6 (Tyr-*C*H$_2$), 25.6 (SiC*Me*$_3$), 18.1 (Si*C*Me$_3$), −4.5 ($SiMe_2$); FAB-HRMS (NBA/CsI) exact mass calcd for $C_{32}H_{37}NO_5Si$ (M + Cs$^+$) 676.1495, found 676.1470.

(*S*)-3-Amino-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)]propanoic Acid *tert*-Butyl Ester (19). A solution of **18**[35] (2.38 g, 8.1 mmol) in THF (50 mL) was treated with *N*-carbethoxyphthalimide (1.77 g, 8.1 mmol) and stirred at 25 °C for 18 h. The solvent was evaporated, and the residue was dissolved in 35 mL of CH_2Cl_2−ethyl acetate−petroleum ether (1:3:3). After storage at 5 °C for 36 h, the precipitate was filtered and washed with cold ether (15 mL) to give 2.8 g of pure intermediate. The filtrate was concentrated and the residue was purified by flash column chromatography (1:1 EtOAc−petroleum ether) to give 0.51 g more. A solution of the combined intermediate (3.31 g, 6.5 mmol) in THF (20 mL) was treated with K_2CO_3 (12 mL, 2.5% aqueous solution, 2.2 mmol) and stirred for 5 min. The reaction mixture was diluted with ether (60 mL), then washed with water (30 mL) and brine (30 mL). The extract was dried ($MgSO_4$) and concentrated in vacuo to give the phthalimido derivative (2.77 g, 80%) as an oil: TLC R_f 0.50 (1:2 EtOAc−petroleum ether); $[\alpha]_D^{22}$ −22.0° (*c* 2.0, $CHCl_3$); IR (thin film) ν_{max} 3388 (br, s), 1775 (m), 1714 (s), 1637 (s), 1526 (m), 1390 (s), 1256 (s), 1157 (s) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.87−7.83 (m, 2 H, Pht arom.), 7.76−7.71 (m, 2 H, Pht arom.), 7.31−7.28 (m, 5 H, Cbz arom.), 5.44 (t, *J* = 5.9 Hz, 1 H, NH), 5.04 (s, 2 H, OCH$_2$), 4.98 (dd, *J* = 7.3, 5.3 Hz, 1 H, α-CH), 3.97−3.82 (m, 2 H, β-CH$_2$), 1.42 (s, 9 H, *t*-Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.6 (CO), 166.6 (CO), 156.2 ($NHCO_2$); 136.3, 134.2, 131.6, 128.34, 128.0, 123.5 (6 arom.), 83.2 (O*C*Me$_3$), 66.6 (OCH$_2$), 52.4 (α-CH), 40.3 (β-

CH_2), 27.7 (CMe_3); FAB-HRMS (NBA/CsI) exact mass calcd for $C_{23}H_{24}N_2O_6$ (M + Cs^+) 557.0689, found 557.0689.

A solution of the phthalimide derivative (460 mg, 1.1 mmol) and $Pd(OH)_2$ (250 mg, moist, 20% on carbon) in a mixture of ethanol (8.75 mL), water (2.5 mL), and acetic acid (1.25 mL) was stirred at 25 °C for 4 h under 1 atm of hydrogen. The mixture was diluted with cold ethyl acetate (20 mL), filtered (Celite), and rinsed with cold ethyl acetate (20 mL). Unstable **20** was retained in solution at −20 °C.

(2*S*,4*S*)-2-[(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)]-3-[[4-[(9-fluorenylmethoxycarbonyl)amino]-5-[4-[(*tert*-butyldimethylsilyl)oxy]-phenyl]pent-2-enoyl]amino]propanoic Acid *tert*-Butyl Ester (20). The solution of **19** (ca. 0.87 mmol) was concentrated in vacuo (0 °C) and rediluted with CH_2Cl_2 (0 °C, 30 mL). The solution was washed with 2 N NaOH (25 mL, 0 °C), the aqueous layer was reextracted with CH_2Cl_2 (0 °C, 2 × 15 mL), and the combined organic extracts were dried ($MgSO_4$). This solution was treated with acid **16** (470 mg, 0.87 mmol), HOBt (130 mg, 0.96 mmol), and EDC hydrochloride (325 mg, 1.70 mmol), then stirred at 0 °C for 50 min and at 25 °C for 16 h. The solvent was evaporated and the residue dissolved in ethyl acetate (50 mL). The organic solution was washed with 5% aqueous citric acid (25 mL), saturated $NaHCO_3$ (25 mL), and brine (25 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue was crystallized from EtOAc−petroleum ether to give dipeptide **20** (638 mg, 72%) as a white solid: mp 166−167 °C (from EtOAc−petroleum ether); TLC R_f 0.21 (1:2 EtOAc−petroleum ether); $[\alpha]_D^{22}$ −7.5° (*c* 1.0, $CHCl_3$); IR (KBr) ν_{max} 3398 (br), 1714 (s), 1512 (m), 1391 (m), 1260 (s), 1159 (m), 917 (m), 841 (m), 595 (m) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.87−7.83 (m, 2 H, Pht arom.), 7.77−7.73 (m, 4 H, Pht and FMOC arom.), 7.53−7.50 (m, 2 H, FMOC arom.), 7.40 (t, J = 7.5 Hz, 2 H, FMOC arom.), 7.32−7.29 (m, 2 H, FMOC arom.), 6.98 (d, J = 7.5 Hz, 2 H, v-Tyr arom.), 6.88−6.73 (m br, 1 H, C*H*=CHCO), 6.74 (d, J = 7.5 Hz, 2 H, v-Tyr arom.), 6.37 (br, 1 H, NH), 5.71 (d, J = 15.5 Hz, 1 H, CH=C*H*CO), 5.00−4.96 (m, 1 H, NPhtC*H*), 4.73 (br, 1 H, NH), 4.58 (br, 1 H, C*H*CH=CH), 4.44−4.33 (m, 2 H, OCH_2), 4.17 (t, J = 6.5 Hz, 1 H, C*H*-Ar), 4.14−4.08 (m, 1 H, $NHCH_2$), 3.87−3.84 (m, 1 H, $NHCH_2$), 2.85−2.75 (m, 2 H, TyrCH_2), 1.42 (s, 9 H, O-*t*-Bu), 0.97 (s, 9 H, Si-*t*-Bu), 0.16 (s, 6 H, $SiMe_2$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.6 (CO), 166.8 (CO), 165.2 (CO), 155.6 ($NHCO_2$), 154.3 (arom.), 143.7 (arom.), 143.3 (*C*H=CH), 141.2, 134.1, 131.6, 130.3, 129.0, 127.5, 127.0, 124.9, 123.5 (9 arom.), 123.1 (CH=*C*H), 120.0 (arom.), 119.8 (arom.), 83.2 ($OCMe_3$), 66.4 (OCH_2), 52.9 (*C*HC=CH), 52.1 (NPht*C*H), 47.0 (*C*H-Ar), 39.9 (TyrCH_2), 38.7 ($NHCH_2$), 27.7 ($OCMe_3$), 25.5 ($SiCMe_3$), 18.0 ($SiCMe_3$), −4.6 ($SiMe_2$); FAB-HRMS (NBA/CsI) exact mass calcd for $C_{47}H_{53}N_3O_8Si$ (M + Cs^+) 948.2656, found 948.2618.

(4*S*,2*S*)-3-[[4-Amino-5-[4-[(*tert*-butyldimethylsilyl)oxy]phenyl]-pent-2-enoyl]amino]-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)]-propanoic Acid *tert*-Butyl Ester (21, Segment B). A solution of **20** (1.69 g, 2.07 mmol) in DMF (23 mL) was treated with diethylamine (1.11 mL, 10.73 mmol) and stirred at 25 °C for 15 min. The diethylamine was evaporated at 25 °C, and the residue was diluted with ether (75 mL) and ethyl acetate (15 mL) and washed with water (3 × 30 mL). The combined aqueous layers were extracted with a mixture of ether−EtOAc (5:1, 30 mL); the combined organic extracts were dried ($MgSO_4$), concentrated in vacuo, and purified by flash column chromatography (12:88 MeOH−EtOAc) to give **21** (1.17 g, 95%) as a solid: mp 144−147 °C (from EtOAc−petroleum ether); TLC R_f 0.23 (1:9 MeOH−EtOAc); $[\alpha]_D^{21}$ +8.9° (*c* 1.0, $CHCl_3$); IR (KBr);[27] ^1H NMR (500 MHz, $CDCl_3$);[27] ^{13}C NMR (125 MHz, $CDCl_3$);[27] FAB-HRMS (NBA/CsI) exact mass calcd for $C_{32}H_{43}N_3O_6Si$ (M + Cs^+) 726.1975, found 726.2004. Anal. Calcd for $C_{32}H_{43}N_3O_6Si$: C, 64.73; H, 7.30; N, 7.08. Found: C, 64.43; H, 7.32; N, 7.04.

Pentapeptide 23. Segment B (**21**) (469 mg, 0.79 mmol) and crude segment A (**13**) (890 mg, 0.95 mmol) were dissolved in CH_2Cl_2 (25 mL), and the solution was treated with DMAP (578 mg, 4.74 mmol) followed by BOP reagent (667 mg, 1.58 mmol). The reaction mixture was stirred at 23 °C under nitrogen for 2 days. Ethyl acetate (120 mL) was added to the mixture, and the solution was washed with 5% aqueous citric acid (2 × 20 mL), saturated $NaHCO_3$ (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 (1:15 MeOH−EtOAc) to give 840 mg (75%) of **24** as a colorless solid: ^1H NMR (400 MHz, CD_3OD);[27] FAB-MS *m/z* 1401 (MH^+). Anal. Calcd for $C_{75}H_{89}N_9O_{14}SSi{\cdot}0.75H_2O$: C, 63.73; H, 6.40; N, 8.92; H_2O, 0.96. Found: C, 63.36; H, 6.39; N, 8.67; H_2O, 0.83.

Coupling Control Experiment. Formation of 24. A solution of segment A (**13**) (9.4 mg, 0.010 mmol) in CH_2Cl_2 (3 mL) was treated with DMAP (6.0 mg, 0.049 mmol) followed by BOP reagent (7.6 mg, 0.018 mmol). The reaction mixture was stirred at 23 °C under nitrogen for 2 days. The solution was diluted with ethyl acetate and washed with 5% aqueous citric acid, saturated $NaHCO_3$, and brine. The organic solution was dried (Na_2SO_4), the volatiles were removed in vacuo, and the residue was analyzed by TLC/FAB-MS. The major product produced a molecular ion corresponding to lactone **24**: FAB-MS *m/z* 807 (MH^+).

Macrocyclic Lactam Formation. Compound 25. A solution of **23** (830 mg, 0.593 mmol) in MeCN (20 mL) was treated with diethylamine (2 mL, 19.3 mmol), and the mixture was stirred for 2 h, at which time TLC showed that **23** had been consumed. The volatiles were removed in vacuo to give a crude product [FAB-MS *m/z* 1178.5 (MH^+)]. The crude product was dissolved in CH_2Cl_2 (10 mL), cooled to 0 °C, and treated with cold TFA−CH_2Cl_2 (1:1, 24 mL; 0 °C). The reaction mixture was allowed to warm to 23 °C and then stirred for 3.5 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 (690 mg, 87% crude yield from **23**, based on a bis(TFA) salt) as a white solid: FAB-MS *m/z* 1122 (MH^+). A solution of 200 mg of the deprotected material (0.148 mmol) in MeCN (160 mL) was treated with HOBt (88 mg, 0.65 mmol) and DCC (84 mg, 0.41 mmol). After 60 h of stirring, the solvent was removed in vacuo and the residue was purified by flash column chromatography (1:20 MeOH−CH_2Cl_2) to give 78 mg (41% from **23**) of macrolactam **25** as a white solid: ^1H NMR (400 MHz, CD_3OD);[27] FAB-MS *m/z* 1104.5 (MH^+). The other macrocyclization reactions in Table 1 were conducted in a similar fashion, with DCC and HOBt being replaced by the other coupling reagents.

Macrolactonization Control Experiment. Formation of 27. A solution of **23** (4.0 mg, 0.003 mmol) in CH_2Cl_2 (1 mL) was cooled to 0 °C and then treated with 1 mL of TFA−CH_2Cl_2 (1:1). The mixture was stirred at 23 °C for 2 h. The volatiles were removed under a stream of nitrogen, and the residue was triturated with ether to give a colorless solid. The crude product was dissolved in CH_2Cl_2 (2 mL) and treated with DMAP (2 mg, 0.016 mmol) followed by BOP reagent (2.4 mg, 0.0057 mmol). The reaction mixture was stirred for 4 days and then worked up as for **24** to afford the crude macrolactone **26**, which was confirmed by FAB-MS [*m/z* 1326 (MH^+)]. To a solution of **26** in MeCN (2 mL) was added diethylamine (0.2 mL). After the mixture was stirred for 1.5 h, the volatiles were evaporated to give crude **27**. The TLC behavior of **27** displayed characteristics of a free amine (lower R_f with streaking relative to **25**): FAB-MS *m/z* 1104 (MH^+).

(5*S*,11*S*,14*R*,18*S*)-*N*-[[[3-[5-Amino-14-benzyl-17-hydroxy-11-[4-[(*tert*-butyldimethylsilyl)oxy]benzyl]-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-1*H*-3a,7,12,15,19-pentaazacyclopentacyclononadecen-18-yl]propyl]amino]iminomethyl]-4-methylbenzenesulfonamide (28). To a solution of **25** (8 mg, 0.0072 mmol) and (*Z*)-2-buten-1-ol (9 μL, 0.11 mmol) in MeOH (2 mL) was added hydrazine monohydrate (3.5 μL, 0.072 mmol). The mixture was stirred for 24 h under nitrogen, and the volatiles were removed in vacuo (below 25 °C). The residue was separated by preparative TLC with CH_2Cl_2−MeOH (5:1) to afford **28** (4.8 mg, 68%) as a white solid: IR (KBr) ν_{max} 3426 (s), 1654 (s), 1635 (s), 1544 (m), 1260 (s), 1132 (m), 912 (m) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 8.38 (br, 1 H, NH), 8.20 (d, J = 8.2 Hz, 1 H, NH), 8.05 (d, J = 8.0 Hz, 1 H, NH), 7.85−7.81 (br, 1 H, NH), 7.62 (d, J = 8.1 Hz, 2 H, Ts arom.), 7.50 (br, 1 H, NH), 7.26 (d, J = 8.1 Hz, 2 H, Ts arom.), 7.23 (d, J = 8.5 Hz, 2 H, arom.), 7.13−7.08 (m, 3 H, arom.), 6.80−6.76 (m, 4 H, arom.), 6.59 (dd, J = 15.3, 1.8 Hz, 1 H, C*H*=CHCO), 5.90 (dd, J = 15.3, 2.0 Hz, 1 H, CH=C*H*CO), 4.62−4.55 (m, 1 H), 4.47−4.39 (m, 2 H), 4.03−3.93 (m, 4 H), 3.64−3.58 (m, 1 H), 3.40−3.30 (overlapping m, 1 H), 3.08−2.92 (m, 2 H), 2.90 (dd, J = 13.6, 4.6 Hz, 1 H), 2.60−2.43 (m, 3 H), 2.31 (s, 3 H, Ts Me), 2.08−2.00 (m, 1 H), 1.97−1.90 (m, 1 H), 1.83−1.75 (m, 2 H), 1.45−

1.20 (m, 4 H), 0.87 (s, 9 H, Si-*t*-Bu), 0.08 (s, 3 H, SiMe), 0.07 (s, 3 H, SiMe); ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 171.2, 169.8, 164.2, 156.6, 153.6, 141.8, 137.0, 131.5, 130.4, 129.4, 129.1, 127.7, 126.0, 125.6, 124.0, 119.5, 70.9, 59.2, 53.2, 51.5, 50.9, 47.4, 43.7, 38.1, 30.1, 29.0, 25.5, 24.5, 20.9, 17.9, −4.60, −4.65; FAB-HRMS (NBA/CsI) exact mass calcd for $C_{48}H_{67}N_9O_9SSi$ (M + Cs^+) 1106.3606, found 1106.3606.

(5*S*,11*S*,14*R*,18*S*)-*N*-[[[3-[14-Benzyl-11-[4-[(*tert*-butyldimethylsilyl)oxy]benzyl]-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-1*H*-3a,7,12,15,19-pentaazacyclopentacyclononadecen-18-yl]propyl]amino]iminomethyl]-4-methylbenzenesulfonamide (29). A mixture of the amine **28** (10 mg, 0.010 mmol) in ethyl formate (1 mL) was stirred at 55 °C for 3 h until dissolution occurred. The reaction mixture was concentrated in vacuo and purified by preparative TLC (9:91 MeOH−CH_2Cl_2) to give **29** (9.0 mg, 86%) as a solid: TLC R_f 0.30 (11:89 MeOH−CH_2Cl_2); IR (KBr);[27] ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.95 (s, 1 H, CHO);[27] ^{13}C NMR (125 MHz, DMSO-d_6);[27] FAB-HRMS (NBA/CsI) exact mass calcd for $C_{49}H_{67}N_9O_{10}SSi$ (M + Cs^+) 1134.3555, found 1134.3498.

Cyclotheonamide A (1, CtA). The formylated product (**29**) (21 mg, 0.021 mmol) was dissolved in MeCN (4 mL) and treated with Dess−Martin periodinane[43] (23 mg, 0.054 mmol). The reaction mixture was stirred at 65 °C for 1 h, then quenched with MeOH (1 mL). The volatiles were removed in vacuo at room 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 layers were washed with brine, dried (Na_2SO_4), and concentrated to afford the crude α-keto amide as a white solid; FAB-MS *m/z* 1000 (MH^+), 1032 [(M + MeOH)H^+]. The crude α-keto amide 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 and stirred for 3 h. 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 reverse-phase HPLC (MeCN−water−TFA, 25:75:0.2; 15 mL/min) and lyophilized to give 7.2 mg (33% for two steps) of CtA (**1**) as an off-white fluffy powder which was identical in all respects (R_f, HPLC, ^{1}H NMR, FAB-HRMS) to authentic CtA:[9a] TLC R_f 0.47 (silica, BuOH−water−HOAc, 4:1:1); $[\alpha]^{24}_D$ −12.8° (*c* 0.13, MeOH) (lit.[9] $[\alpha]^{23}_D$ −12.8°, *c* 0.2, MeOH); ^{1}H NMR (500 MHz, D_2O) δ 8.08 (s, 1 H, CHO), 7.28−7.22 (m, 5 H, Phe *m*- and *p*-arom., v-Tyr *o*-arom.), 6.93 (d, *J* = 6.8 Hz, 2 H, v-Tyr *m*-arom.), 6.83 (dd, *J* = 15.6, 2.6 Hz, 1 H, CH=C*H*CO), 6.64 (d, *J* = 7.5 Hz, 2 H, Phe *o*-arom.), 5.94 (dd, *J* = 15.6, 2.3 Hz, 1 H, C*H*=CHCO), 4.87−4.83 (m, 1 H, a-Ala α-H), 4.61 (dd, *J* = 5.5, 5.5 Hz, Phe α-H), 4.60−4.55 (m, 1 H, v-Tyr C*H*N), 4.56−4.52 (m, 1 H, Pro α-H), 4.26 (dd, *J* = 12.9, 6.0 Hz, 1 H, a-Ala β-H), 4.00 (dd, *J* = 10.3, 1.2 Hz, 1 H, h-Arg α-H), 3.83−3.77 (m, 1 H, Pro δ-H), 3.57−3.52 (m, 1 H, Pro δ-H), 3.25−3.13 (m, 2 H, h-Arg δ-H), 3.04 (dd, *J* = 14.1, 4.8 Hz, 1 H, v-Tyr ArC*H*), 2.96−2.89 (m, 1 H, a-Ala β-H), 2.86 (dd, *J* = 13.6, 6.1 Hz, 1 H, Phe β-H), 2.77 (dd, *J* = 13.6, 4.9 Hz, 1 H, Phe β-H), 2.57 (dd, *J* = 14.0, 10.1 Hz, 1 H, v-Tyr Ar C*H*), 2.36−2.27 (m, 1 H, Pro β-H), 2.07−2.00 (m, 1 H, Pro γ-H), 2.00−1.84 (m, 3 H, h-Arg β-H, Pro β-H, Pro γ-H), 1.71−1.64 (m, 1 H, h-Arg γ-H), 1.61−1.51 (m, 2 H, h-Arg β-H and γ-H); ^{13}C NMR (100.5 MHz, D_2O, pH 4.5) δ 176.1 (Pro C=O), 174.5 (Arg C=O), 173.6 (Phe C=O), 173.0 (a-Ala C=O), 170.4 (v-Tyr C=O), 166.5 (CHO), 159.5 (C=NH), 157.2 (v-Tyr C4), 146.1 (*C*H=CHCO), 137.9 (Phe C1), 133.2 (v-Tyr C2,6), 132.2 (v-Tyr C1), 132.0 (Phe C2,6), 131.1 (Phe C3,5), 129.9 (Phe C4), 125.2 (CH=*C*HCO), 118.4 (v-Tyr C3,5), 97.2 (h-Arg *C*(OH)$_2$), 63.3 (Pro Cα), 57.3 (h-Arg Cα), 56.8 (Phe Cα), 55.4 (v-Tyr *C*N), 51.6 (a-Ala Cα), 51.4 (Pro Cδ), 43.4 (h-Arg Cδ), 42.1 (a-Ala Cβ), 41.6 (Phe Cβ), 40.3 (v-Tyr *C*H$_2$), 32.9 (Pro Cβ), 27.3 (Pro Cγ), 27.1 (Arg Cγ), 25.9 (Arg Cβ); FAB-MS (NBA) in MeOH *m/z* 764 (MH^+ + MeOH); FAB-HRMS (NBA) in H_2O calcd for $C_{36}H_{45}N_9O_8$ (MH^+ + H_2O) 750.3575, found 750.3606. A combination of ^{1}H and ^{19}F NMR furnished a CtA:TFA ratio of 1:1.9. Anal. Calcd for $C_{36}H_{45}N_9O_8 \cdot 1.9CF_3CO_2H \cdot 6H_2O$: C, 45.25; H, 5.62; N, 11.93; F, 10.25; H_2O, 10.22. Found: C, 44.83; H, 5.03; N, 11.37; F, 9.78; H_2O, 8.31.[59]

Cyclotheonamide B (2, CtB). Pentafluorophenyl acetate (5.0 mg, 0.021 mmol) was added to a solution of amine **28** (4.0 mg, 0.004 mmol) in dry DMF (1.5 mL), and the mixture was stirred for 3 h. The solvent was removed in vacuo at 23 °C, and the residue was purified by preparative TLC (1:8 MeOH−CH_2Cl_2) to give 3.7 mg (90%) of acetylated product as a colorless solid: ^{1}H NMR (CD_3OD) δ 7.61 (d, *J* = 8.2 Hz, 2 H, Ts arom.), 7.16 (d, *J* = 8.2 Hz, 2 H, Ts arom.), 7.04 (m, 5 H, arom.), 6.71 (m, 4 H, arom.), 6.65 (dd, *J* = 15.4, 2.2 Hz, 1 H, C*H*=CHCO), 6.02 (dd, *J* = 15.4, 2.3 Hz, 1 H, CH=C*H*CO), 4.52 (m, 3 H), 4.37 (m, 1 H), 4.08 (m, 2 H), 3.72 (m, 1 H), 3.38 (m, 1 H), 3.02 (m, 2 H), 2.80−2.53 (m, 4 H), 2.38 (dd, *J* = 13.7, 9.7 Hz, 1 H), 2.26 (s, 3 H, Ts*Me*), 2.13 (m, 1 H), 1.92−1.38 (m, 10 H), 0.82 (m, 9 H, Si-*t*-Bu), 0.02 (m, 6 H, $SiMe_2$); FAB-MS *m/z* 1017 (MH^+). The acetylated product (2.8 mg, 0.003 mmol) was dissolved in CH_2Cl_2 (1.5 mL) and treated with *tert*-butyl alcohol (0.8 mg, 0.011 mmol) followed by the Dess−Martin periodinane[45] (6.0 mg, 0.014 mmol). The reaction mixture was stirred for 2 h, then quenched with aqueous sodium thiosulfate. The layers were separated, and the aqueous phase was extracted with CH_2Cl_2. 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: FAB-MS *m/z* 1014 (MH^+), 1046 [(M + MeOH)H^+]. The α-keto amide was transferred to a Teflon vessel containing anhydrous anisole (1 mL). The mixture was cooled to −78 °C and treated with anhydrous HF (2 mL). The reaction mixture was then warmed to 0 °C and stirred for 3 h. The HF was removed 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 reverse-phase HPLC (MeCN−water−TFA, 25:75:0.2; 15 mL/min) and lyophilized to give 1.0 mg (33% for two steps) of CtB (**2**) as a white solid: $[\alpha]^{24}_D$ −13.6° (*c* 0.13, MeOH) (lit.[13] $[\alpha]^{23}_D$ −13.5°, *c* 0.2, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 7.26−7.19 (m, 5 H, Phe *m*- and *p*-arom. and v-Tyr *o*-arom.), 6.91 (d, *J* = 8.4 Hz, 2 H, v-Tyr *m*-arom.), 6.80 (dd, *J* = 15.5, 2.5 Hz, 1 H, CH=C*H*CO), 6.60 (d, *J* = 6.4 Hz, 2 H, Phe *o*-arom.), 5.91 (dd, *J* = 15.6, 2.1 Hz, 1 H, C*H*=CHCO), 4.64−4.48 (m, 4 H, a-Ala α-H, Phe α-H, v-Tyr C*H*N, Pro α-H), 4.19 (dd, *J* = 12.8, 5.9 Hz, 1 H, a-Ala β-H), 3.97 (dd, *J* = 10.8, 2.3 Hz, 1 H, h-Arg α-H), 3.79−3.73 (m, 1 H, Pro δ-H), 3.53−3.47 (m, 1 H, Pro δ-H), 3.21−3.10 (m, 2 H, h-Arg δ-H), 3.00 (dd, *J* = 14.1, 4.8 Hz, 1 H, v-Tyr ArC*H*), 2.92−2.81 (m, 2 H, a-Ala β-H, Phe β-H), 2.74 (dd, *J* = 13.7, 4.9 Hz, 1 H, Phe β-H), 2.53 (dd, *J* = 14.0, 10.6 Hz, 1 H, v-Tyr Ar C*H*), 2.33−2.23 (m, 1 H, Pro β-H), 2.04−2.01 (m, 1 H, Pro γ-H), 1.98 (s, 3 H, MeCO), 1.97−1.81 (m, 3 H, h-Arg β-H, Pro β-H, Pro γ-H), 1.69−1.60 (m, 1 H, h-Arg γ-H), 1.58−1.49 (m, 2 H, h-Arg β-H and γ-H); ^{13}C NMR (D_2O) δ 176.8, 176.2, 174.5, 173.8, 173.6, 170.4, 159.4, 157.3, 146.2, 138.0, 133.4, 132.4, 132.1, 131.2, 130.0, 125.3, 118.4, 97.2, 63.3, 57.3, 56.8, 55.4, 53.1, 51.5, 43.4, 41.8, 41.7, 40.4, 33.0, 27.5, 27.2, 25.9, 24.0; FAB-MS in MeOH−water *m/z* 746 (MH^+), 764 [(M + H_2O)H^+], 778 [(M + MeOH)H^+]; FAB-HRMS (NBA) in H_2O calcd for $C_{37}H_{47}N_9O_8$ (MH^+ + H_2O) 764.3731, found 764.3818. Since insufficient CtB was available for combustion or ^{1}H/^{19}F NMR analysis, we assumed a molecular formula similar to that of CtA.

Thrombin Inhibition Studies. Enzyme-catalyzed hydrolysis rates were measured spectrophotometrically at 25 °C or 37 °C for 4 h by using commercial human α-thrombin (American Diagnostica) at 0.1 or 1.0 nM and chromogenic substrates at 50 μM (Molecular Devices microplate reader). Reactions were conducted in aqueous buffer [10 mM Tris, 10 mM Hepes, 150 mM NaCl, 0.1% PEG (pH 7.4)] and started by addition of enzyme ("enzyme last") or by addition of substrates after a 4-h preincubation of enzyme and inhibitor ("substrate last"). We employed four commercial substrates: Spectrozyme TH (H-D-HHT-Ala-Arg-pNA·2AcOH, American Diagnostica), Sar-Pro-Arg-pNA (Sigma Chemical Co.), S2238 (D-Phe-Pip-Arg-pNA, Kabi Pharmacia Hepar, Inc.), and Ts-Gly-Pro-Arg-pNA (Sigma). Changes in absorbance at 405 nm were monitored with and without inhibitor to

(59) The microanalytical data are reasonably consistent with the molecular formula CtA·1.9TFA·6H_2O. Insufficient material was available for repeated attempts at elemental analysis.

furnish kinetic curves from which K_i values were determined. The values for K_m were obtained by extrapolation from Lineweaver–Burk plots by using the commercial software package K-Cat (Biometallics, Inc.). Slow-binding kinetics were analyzed according to the method described by Williams and Morrison.[53]

Notes Added in Proof: (1) Deng et al. recently reported an additional total synthesis of CtB.[60] (2) We have now prepared suitable analogues to probe the S_3 pocket and have not observed any significant improvement in potency.[61]

Acknowledgment. We are grateful to Prof. Nobuhiro Fusetani (University of Tokyo) for generous samples of natural CtA (**1**). We thank Prof. Alexander Tulinsky (Michigan State University) for X-ray crystallography of CtA–thrombin–hirugen and helpful discussions, Dr. Harold Almond for molecular modeling studies, Eugene Powell for technical assistance, Dr. Michael Costanzo for the degradation studies on CtA (**1**), Dr. Gregory Leo, Dr. Patricia McDonnell, and Diane Gauthier for NMR data, and John Masucci and William Jones for MS data.

Supplementary Material Available: Text describing experimental procedures for **6**, **7a,b**, **8a,b**, **9**, and **11**, listings of IR, ^{1}H NMR, and ^{13}C NMR data for **15**, **21**, **29**, and an intermediate en route to **15**, and ^{1}H NMR spectra for **2**, **3**, **6**, **7a,b**, **8a,b**, **9**, **11**, **12**, **20**, **22**, **23**, **25**, the SEM derivative of **3**, and intermediates in the conversion of **9** to **11** (28 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JA942080A

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