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Transformation of the Marine Natural Product Cyclotheonamide A by Aqueous Base. X-Ray Analysis of a Novel Ligand Complexed with Human α-Thrombin

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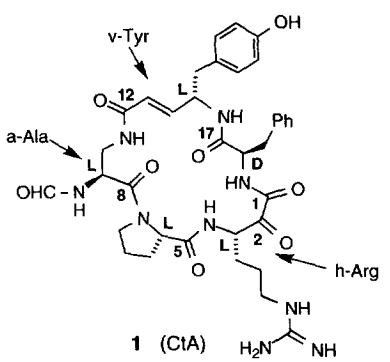
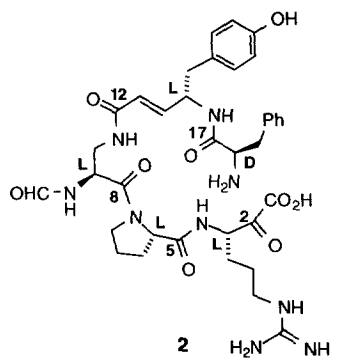
Summary: Treatment of the macrocyclic pentapeptide cyclotheonamide A (**1**) with aqueous sodium carbonate or triethylamine at 23°C generated two isomeric products. X-ray analysis of a complex with α-thrombin indicates a ring-opened pentapeptide, **2**, from cleavage at the α-keto amide bond. However, given the MS data for **3** and a model study in which **4** provides **5**, structure **3** is suggested for the product from base treatment of **1**.

Cyclotheonamide A (**1**, CtA) is a cyclic pentapeptide natural product from the Japanese marine sponge *Theonella* sp. that inhibits certain serine proteases, such as α-thrombin and trypsin.[1] Its chemical structure has been confirmed by total synthesis[1b,2] and X-ray crystallography of enzyme-CtA complexes.[1c,3] In our continuing studies of CtA and its analogues,[1b,2a,4] we became concerned about the potential stereochemical lability of the C_3 stereogenic center adjacent to the α-keto amide group, and wondered if it would be possible to obtain the *epi*-C_3 isomer of CtA for biological testing by treating CtA with base. However, the chemistry took a different course from that originally envisioned.



1 (CtA)

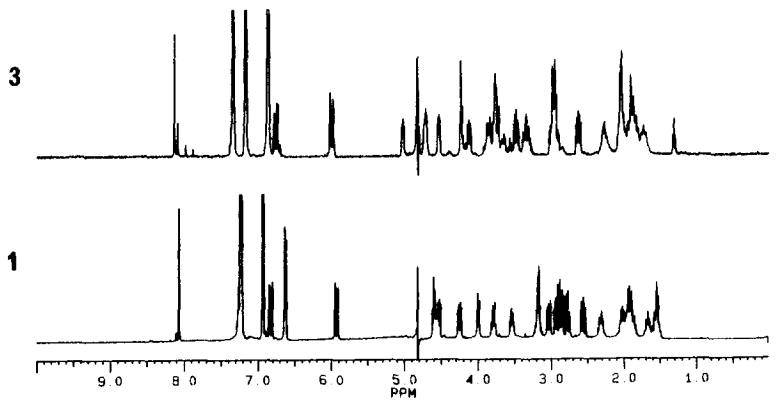


2

To assess the base stability of CtA, we treated a solution of synthetic CtA (8 mg)[2a] in MeCN-water (1:3) with saturated Na_2CO_3 at 23 °C. After stirring for 1.5 h, we obtained a mixture consisting of a new component (ca. 65%), along with unchanged CtA (ca. 35%), according to HPLC.[5] Triethylamine as base (MeCN-water) resulted in a rapid, complete conversion of CtA to the new material. Separation of the products from

the Na_2CO_3 reaction by HPLC afforded 3.3 mg of an amorphous substance, whose structure was difficult to assign unambiguously by ^1H NMR or FAB-MS data. Although the product had the same mass as CtA (FAB-MS: *m/z* 732, MH^+), no molecular ions for water or MeOH covalent adducts were detected, and a major [M - OH] ion was present, in contrast to what is observed with CtA. Furthermore, comparison of the ^1H NMR spectrum with that of CtA showed gross discrepancies (D_2O, 400 MHz), as one can appreciate from Fig. 1. The new material, on re-exposure to the basic reaction conditions, remained unchanged instead of providing any CtA, thereby ruling out the *epi*-C_3 isomer of CtA from base-induced equilibration at C_3.

Figure 1. Comparison of ^1H NMR spectra.

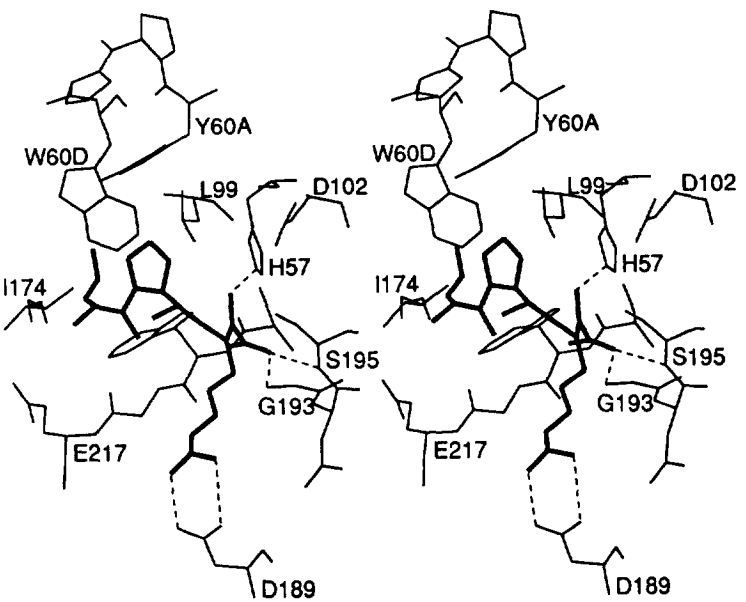


The new material was first surmised to be a diastereomeric mixture (4:1 by ^1H NMR) of six-membered ring adducts from addition of the proximal guanidine nitrogen to the ketone (viz. **3**), which were not readily reconvertible to CtA via ring opening [FAB-MS: 714.5 (M - OH), 754.5 (M + Na^+)]. The relatively weak thrombin inhibitory activity (slow-binding K_i = 220 nM vs. 4.1 nM for CtA) was consistent with this view, but we sought more convincing structural information. Given this inhibition constant, we attempted to form a complex of the noncrystalline material with human α-thrombin, inhibited by hirugen, for an X-ray study to probe the molecular structure of the ligand.

A crystal of the thrombin-hirugen complex, grown by the hanging-drop method,[6] was transferred to a 4-μL drop containing a storage solution [0.1 M phosphate buffer (pH 7.3), 0.188 M NaCl, 26% PEG-8000, 1 mM NaN_3; reservoir had 1 mL of this solution]. We added 1 μL of a solution of the CtA-derived compound in the same medium (final concentration = 15 mM) slowly to the hanging drop and let the crystal soak overnight; we then removed 1 μL of solution from the drop and added 1 μL of ligand solution (final ligand concentration of 5.4 mM). The crystal was kept for 10 days before being mounted for X-ray diffraction. Two intensity data sets were collected by using a Rigaku RU200 fine-focus rotating-anode generator operating at 50 kV and 100 mA. Both crystals diffracted to 2.1 Å resolution and are isomorphous with crystals of thrombin-hirugen[6] (space group *C*2: a = 71.22 Å, b = 72.47 Å, c = 73.16 Å, β = 101.0°). The merged and averaged sets gave 47,279 measured observations with 17,946 independent reflections (R-merge = 7.5%; 84.4% completeness at

2.25 Å resolution). The structure of thrombin-hirugen without water molecules was energy minimized with X-PLOR (R = 20%; resolution, 10.0-2.5 Å), and the difference electron-density map between thrombin-hirugen-ligand and thrombin-hirugen was calculated, phased with this model. The map showed the h-Arg (α-keto-homoarginine) and Pro groups in the active site region. The structure was refined by restrained least-squares methods (PROLSQ) with data selected from 7.0-2.0 Å (3.0σ cut-off on $|F|$*2) to afford a final model with 194 water molecules (R = 16.4%). Although no density was found for v-Tyr and D-Phe, some density developed linked to Pro (fitted with a-Ala) and density for a carboxylate attached to h-Arg became clear.

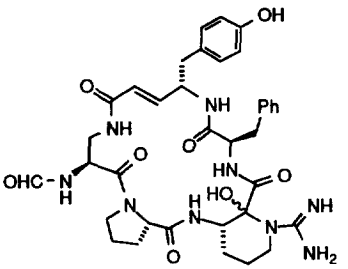
Figure 2. Stereoview of **2** in the active site of thrombin (**2** in bold; broken lines for hydrogen bonds).



The X-ray result (Fig. 2) shows Pro occupying the standard S_2 pocket and h-Arg occupying the standard S_1 specificity pocket with the guanidine bound to Asp-189. There is a C-terminal carboxylate (C_1) instead of a carboxamide and the C_3-C_2-O_2 bonds appear as an enol grouping instead of a C_2 hemiketal adduct with the γ-oxygen of Ser-195. A carboxylate oxygen occupies the oxy-anion hole and makes hydrogen bonds with the Gly193N and Ser195N, in analogy with the thrombin complex of CVS-1347.[7] The D-Phe and v-Tyr residues are not visible presumably because they are exposed to the solvent, and are highly flexible and disordered. Part of the a-Ala residue, -C(O)C-(NCO)-C-, is present, while the segment from N_{11} to the amino terminus is not. This indicates that hydrolysis, probably enzymic, occurred at the C_1-N_{19} amide bond of **3** to give acyclic pentapeptide **2**. Thus, we presume that the two components seen for **3** by ^{1}H NMR reflect the C_2 epimers.[8]

As a further probe, we experimented with simplified CtA analogue **4**. Treatment of a solution of **4** with triethylamine in MeCN-water produced a mixture of a new compound (62%; t_R = 15.9 min) and unchanged **4** (27%; t_R = 20.0 min), as detected by HPLC. Unambiguous structural assignment of the new product by

spectral means was not straightforward. However, inspection of ^{1}H NMR and FAB-MS data indicated an analogous material to the CtA conversion (*m/z* 689.5, MH^+);[9] in particular, there were gross discrepancies between the ^{1}H NMR spectra of **4** and the new product, similar to the CtA case. This suggests cyclic hemiaminal **5** as the product from base treatment of **4**, with **5** presumably existing with mixed C_2 stereochemistry. Both **5** and **3** have MS molecular ions identical to the corresponding starting materials and each can generate a stabilized ion (M - OH) under fragmentation. Altogether, these observations support the formation of **3** from CtA under mild treatment with aqueous base.



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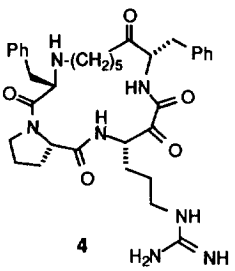

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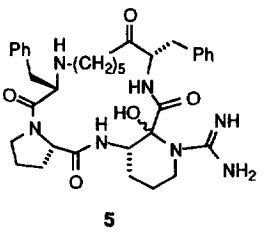

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5. HPLC conditions: Bondapak® C18, 15-20 μm, 125 Å, 3 x 40 x 100 mm; MeCN-water-TFA, 25:75:0.2 (pH ca. 3); 30 mL/min; t_R = 16.6 min for the new product (isolated by lyophilization), 24.9 min for CtA.
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8. On exposure of **3** to human α-thrombin at 23 °C [**3** (1.5 mg; 2 μmol) in phosphate buffer (0.67 mL, pH 7.3) was added to a 1:1 glycerol-water solution of human α-thrombin (114 μL, 0.04 μmol; final concentration of 2.6 mM)], there was no loss of **3** after 5 days (HPLC). Although this indicates that **3** is not a substrate for thrombin under these conditions, the possibility of enzymic hydrolysis during cocrystallization remains since a much higher effective concentration of thrombin exists within the crystal lattice.
9. A signal for [M - OH] (*m/z* 671.5) was observed by FAB-MS; there were no H_2O or MeOH adduct ions.

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