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Dehydroalanine-Based Inhibition of a Peptide Epimerase from Spider Venom

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Ribosomally produced peptides that contain D-amino acids have been isolated from a number of vertebrate and invertebrate sources. In each case, the D-amino acids are introduced by a posttranslational modification of a parent peptide containing only amino acids of the L-configuration. The only known enzyme to catalyze such a reaction is the peptide epimerase (also known as peptide isomerase) from the venom of the funnel web spider, *Agelenopsis aperta*. This enzyme interconverts two 48-amino-acid-long peptide toxins that differ only by the stereochemistry at a single serine residue. In this paper we report the synthesis and testing of two pentapeptide analogues that contain modified amino acids at the site normally occupied by the substrate serine residue. When the L-chloroalanine-containing peptide **3** was incubated with the epimerase it was converted into the dehydroalanine-containing peptide **4** via an elimination of HCl. The dehydroalanine peptide **4** was independently synthesized and found to act as a potent inhibitor of the epimerase ($IC_{50} = 0.5\ \mu M$). These results support a direct deprotonation/reprotonation mechanism in which a carbanionic intermediate is formed. The observed inhibition by **4** can be attributed to the sp^2-hybridization of the α-carbon in the dehydroalanine unit that mimics the planar geometry of the anionic intermediate.

Introduction

A central tenet of biochemistry is that all proteins are derived from the 20 common amino acids, all of which are of the L-configuration. This is largely true, since in most organisms the ribosomal machinery responsible for the translation of RNA can only accept these L-amino acids as substrates. The occurrence of D-amino acids is restricted mainly to nonribosomally produced peptides such as the cyclic peptide antibiotics and peptidoglycan.[1−3] A notable exception is found in the bacterial lantibiotics, which are complex polycyclic peptides containing extensive posttranslational modifications.[4,5] One such modification is the amino acid lanthionine that is formed by the dehydration of a serine residue to give a dehydroalanine residue, followed by the addition of a thiol from a cysteine residue in the same peptide. The resulting cross-linked amino acid is formed with the D-configuration at its α-carbon. In the 1980s, several ribosomally derived peptides, the dermorphins and the deltorphins, were isolated from the skin of the South American tree frog, *Phyllomedusa sauvagei*, and were found to contain D-amino acids.[6] These potent analgesics bind to the μ-type and δ-type opiate receptors, respectively, and were found to contain D-alanine, D-leucine, or D-methionine. In all cases, the D-amino acids were introduced from the corresponding genetically encoded L-amino acids via a posttranslational process. More recently, D-amino acids have been found in peptides isolated from snails, bivalves, and spiders, indicating that this is a widespread phenomenon in nature.[6]

A great deal of information is available concerning the enzymes responsible for the racemization of both free amino acids[7−12] and amino acids bound to nonribosomal peptide synthases.[13,14] However, only a single example of an enzyme capable of catalyzing the posttranslational epimerization of an intact polypeptide has been reported to date. In 1994, the first peptide epimerase (also known as peptide isomerase) was isolated from the venom of the funnel web spider, *Agelenopsis aperta*.[15] This enzyme was found to interconvert two 48-amino-acid-long peptide toxins (IVC and IVB) that differed only by the stereochemistry at serine 46 (L-Ser and D-Ser, respectively)

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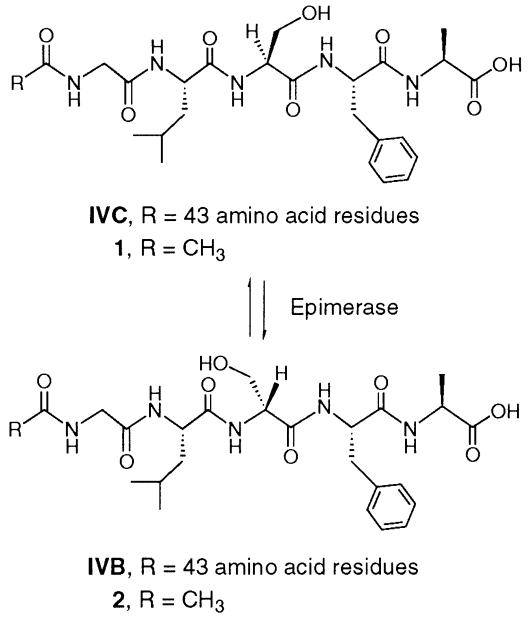
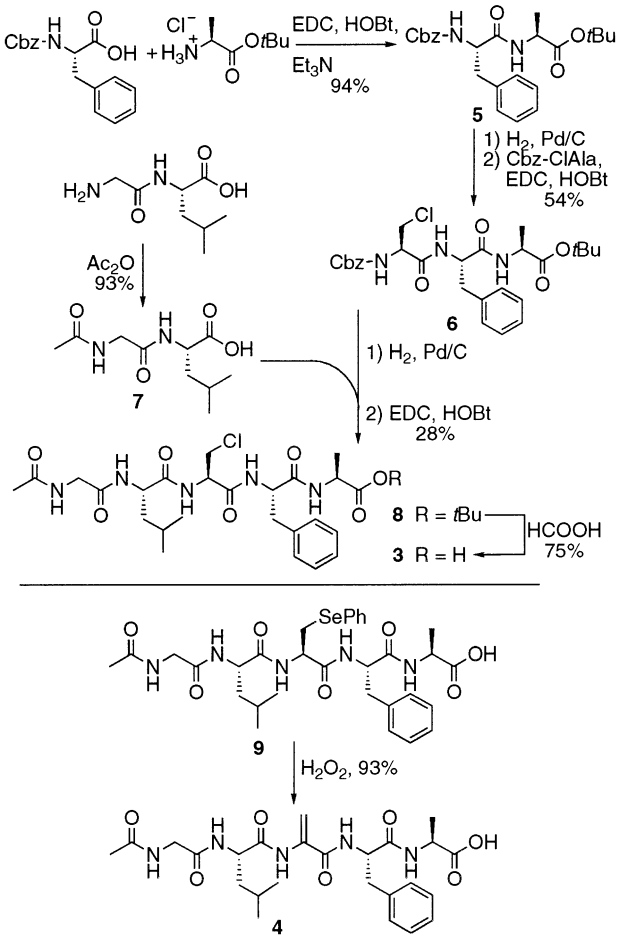


FIGURE 1. The interconversion of *R*-Gly-Ile-LSer-Phe-Ala and *R*-Gly-Ile-DSer-Phe-Ala catalyzed by the peptide epimerase from spider venom.

(Figure 1). The D-serine-containing toxin IVB was found to be a more potent blocker of the P-type voltage-sensitive calcium channel in rats than its L-serine-containing counterpart. Subsequently, the epimerase was sequenced and reported to consist of an 18-residue light chain and a 243-residue heavy chain interlinked by disulfide bonds.[16] Surprisingly, the heavy chain bears remarkable sequence identity to serine proteases such as thrombin and kallikrein (26% and 35%, respectively), particularly in the region of the conserved catalytic triad.

Initial mechanistic studies on the peptide epimerase have been carried out using the truncated pentapeptides **1** and **2** as alternate substrates (Figure 1).[17] It has been shown that the epimerization is accompanied by the incorporation of solvent-derived deuterium into the C-2 position of serine in the product epimer. Furthermore, the reaction of a substrate bearing deuterium at the C-2 position of serine is slowed by a primary kinetic isotope effect. These observations support a mechanism involving an initial deprotonation at the α-carbon to form a carbanionic intermediate, followed by reprotonation on the opposite face to generate the epimeric product (Figure 2a). A related mechanism could be invoked to explain the strong sequence similarity, and presumed common ancestry, that this epimerase shares with the serine proteases. The enzyme could first form an acyl−enzyme intermediate using the serine of its conserved catalytic triad in a step that is analogous to the first step of amide hydrolysis (Figure 2b). Epimerization via a nonstereo-

SCHEME 1. Syntheses of Pentapeptides 3 and 4



specific deprotonation/reprotonation event would generate a serine residue of the opposite configuration, and reformation of the peptide bond would produce the epimeric product. A final mechanism is similar to that involved in lanthionine formation and involves the elimination of water from serine to generate a dehydroalanine-containing peptide (Figure 2c). The readdition of water with protonation on the opposite face would give the epimeric product. Evidence arguing against this mechanism includes the observations that a pentapeptide containing alanine in the place of serine can still serve as a substrate and that no ^{18}O isotope is incorporated during epimerization in $H_2{}^{18}O$.[17]

In this paper, we report the syntheses of both the chloroalanine-containing pentapeptide **3** and the dehydroalanine-containing pentapeptide **4** and studies of their interactions with the spider venom peptide epimerase. We have found that compound **4** acts as a potent inhibitor of the epimerization process and that the enzyme catalyzes the conversion of **3** into **4** by the elimination of HCl (Figure 3).

Results

Compound **3** was prepared from Cbz-L-chloroalanine[18] using solution-phase peptide chemistry (Scheme 1). An

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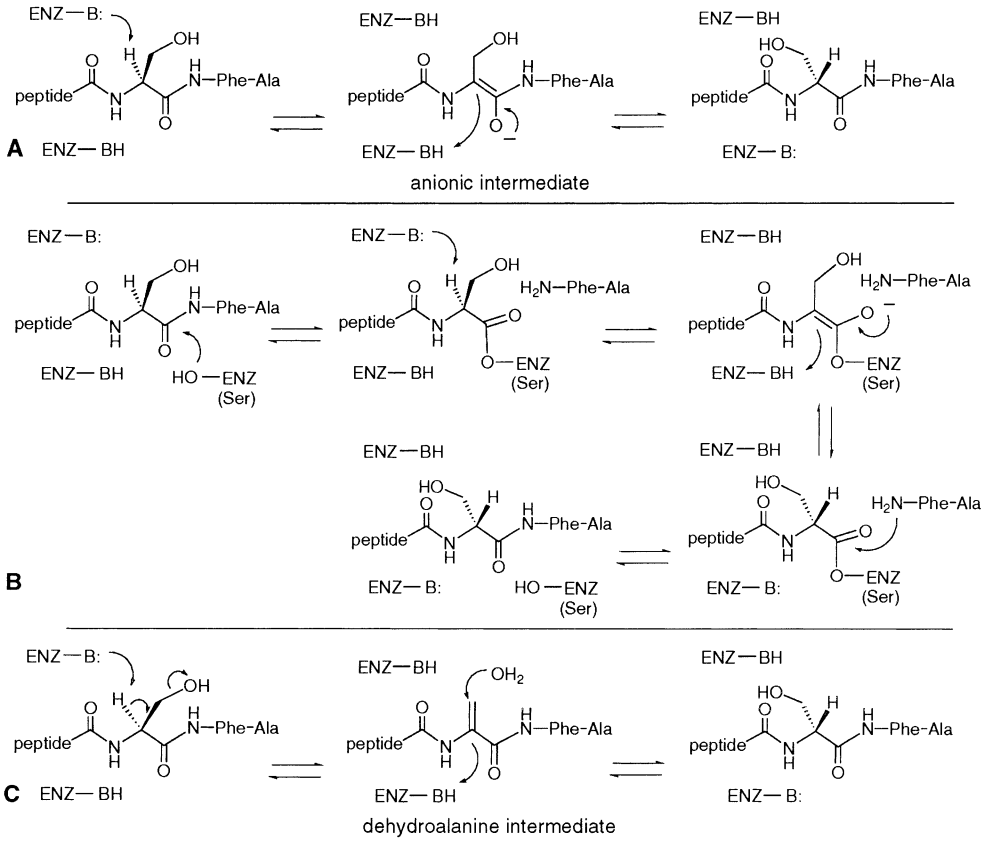

FIGURE 2. Three possible mechanisms for the reaction catalyzed by the peptide epimerase: (A) the direct deprotonation/reprotonation mechanism, (B) the acyl−enzyme mechanism, (C) the dehydration/rehydration mechanism.

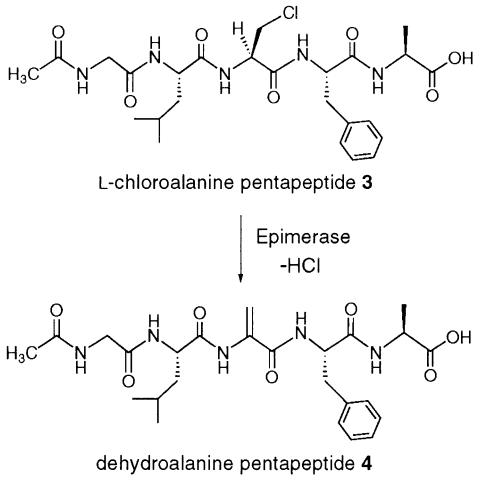

FIGURE 3. The conversion of the L-chloroalanine pentapeptide **3** into the dehydroalanine pentapeptide **4** catalyzed by the peptide epimerase.

initial condensation between Cbz-L-phenylalanine and the *tert*-butyl ester of L-alanine gave the dipeptide **5**. Removal of the carboxybenzyl protecting group was accomplished by hydrogenolysis, and coupling with Cbz-L-chloroalanine afforded the tripeptide **6**. Hydrogenolysis of **6** and coupling with *N*-acetylglycyl-L-leucine **7** gave the protected pentapeptide **8**. A final deprotection with formic acid gave the target pentapeptide **3**. Compound **3** was found to be reasonably stable to incubation under the conditions employed for the enzymatic assays; however, even in the absence of enzyme it was gradually converted ($t_{1/2}$ = 48 h) to a compound with a retention time identical to that of **1**. Attempts to prepare the D-chloroalanine-containing pentapeptide were hampered by the production of inseparable mixtures of diastereomers during the final coupling step.

Compound **4** was prepared from Fmoc-phenylselenocysteine as outlined in the methodology described by van der Donk and co-workers (Scheme 1).[19] Solid-phase peptide synthesis followed by HPLC purification was used to prepare the pentapeptide **9**. The conversion of **9** into the dehydroalanine target **4** was achieved by treatment with hydrogen peroxide in H_2O/MeOH followed by HPLC purification. Compound **4** was found to be indefinitely stable under the conditions employed for the enzymatic assays.

The epimerase was isolated from the venom of *A. aperta* using size exclusion chromatography as described previously.[17] Analysis by gel electrophoresis (SDS−PAGE) showed a single band, and mass spectral analysis gave signals at 29 473 Da (major) and 29 314 Da (minor). These masses are consistent with the reported values for the glycosylated protein with and without a terminal sugar residue (either mannose or fucose), respectively.[16,20] The previously reported background metalloprotease activity that acts on the pentapeptide substrate **1** was also found to be present in the sample when analyzed

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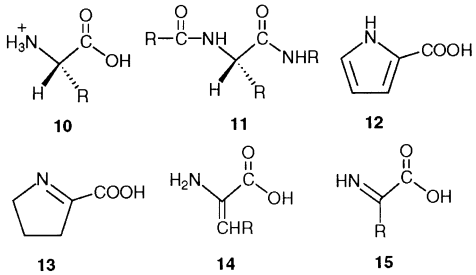
by HPLC.[17] This protease activity did not affect the D-serine-containing substrate **2**, suggesting it was coming from an impurity, as opposed to the epimerase itself. In an attempt to further purify the epimerase, an additional cation exchange chromatography step was employed (HiTrap SP HP). This gave active enzyme; however, the metalloprotease activity was still present, suggesting that the two proteins coelute under orthogonal chromatographic conditions. To suppress the background metalloprotease activity, all incubations were carried out in the presence of 5 mM EDTA, as described previously.[17] The epimerase activity was monitored using HPLC analysis and a specific activity of 1×10^{-4} μmol/min/mg was determined for the epimerization of **1** under subsaturating conditions (100 μM **1**). The low activity was consistent with reported values[17] and made it necessary to employ prolonged incubations during the analyses (typically 5−20 h).

When the L-chloroalanine peptide **3** was incubated with the epimerase and the reaction was monitored by HPLC, a new peak appeared that had a retention time identical to that of the dehydroalanine analogue **4**. Mass spectral analysis (HPLC/ESI-MS) confirmed that this product also had the same mass as compound **4**. No HPLC peaks attributable to the D-chloroalanine epimer of **3** were detected. When **3** was tested as an inhibitor of the epimerase, it was found to inhibit the epimerization of **1** at submicromolar concentrations (presumably due to the formation of **4**, vide infra). When compound **4** was incubated with the epimerase alone, no reaction was observed. It was, however, found to strongly inhibit the epimerization of **1**, and the IC_{50} value for **4** in the presence of 0.1 mM **1** was 0.5 μM. This value is considerably lower than the reported K_M value of 8 mM for the epimerization of **1**.[17] These results indicate that the epimerase catalyzes the elimination of HCl from peptide **3** to generate the dehydroalanine peptide **4** and that the latter acts as a potent inhibitor of the enzyme. It should be noted that the Bradford assay indicated that the protein concentration was 9.5 μM, suggesting that only a fraction of the added protein was in an active form. No evidence for the formation of a covalent adduct was obtained when samples of the epimerase were incubated with either **3** or **4** (100 μM) and analyzed by ESI-MS.

Discussion

Racemases that act on free amino acids all employ a mechanism involving an initial deprotonation at the α-carbon to form a carbanionic intermediate, followed by reprotonation on the opposite face to generate the enantiomeric product.[8] These enzymes are therefore faced with the problem of overcoming the barrier toward the deprotonation of a relatively nonacidic proton. In some cases, such as alanine racemase, a pyridoxal phosphate cofactor forms an imine linkage with the amino functionality and serves to stabilize the intermediate by delocalization of the negative charge.[10] Other amino acid racemases, such as proline racemase and glutamate racemase, operate in a cofactor independent fashion and must deprotonate the amino acid directly.[7,11,12,21−31] In these cases, it is likely that the amino acid is bound in its fully protonated form, **10**, in which both the ammonium group and the neutralized carboxylate are in a protonation state best suited to stabilize the incipient negative charge. In this situation, the pK_a of the α-proton has been estimated to be 21.[32,33] The problem faced by a peptide epimerase is somewhat greater, since the pK_a of the α-proton in a peptide (**11**) is expected to be much higher. The absence of an ammonium functionality could raise the pK_a value by as much as 4 units, similar to the comparison between ethyl acetate (pK_a = 25.6) and the methylene in the protonated form of the glycine methyl ester (pK_a = 21).[32,33] Furthermore, the electron-withdrawing ability of an amide carbonyl is much lower than that of a carboxylic acid or ester, and this difference could raise the pK_a value by an additional 5 units.[34] Therefore, the pK_a of the α-proton in a peptide could be expected to be as high as 30, and this value is approaching the upper limit at which enzymes are known to catalyze proton-transfer reactions.[35] The direct deprotonation/reprotonation mechanism (Figure 2a) and the elimination/readdition mechanism (Figure 2c) must face this barrier directly, and this may explain why the activity of this enzyme is so low. In the acyl−enzyme mechanism (Figure 2b), however, the second factor is eliminated, since the deprotonation occurs at a carbon adjacent to an ester functionality and the pK_a may be as low as 26.



The observations that the chloroalanine pentapeptide **3** is converted to the dehydroalanine pentapeptide **4** (Figure 3) and that this compound acts as a potent inhibitor are consistent with the direct deprotonation/reprotonation mechanism (Figure 2a). The introduction of a good leaving group in place of the serine hydroxyl

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promotes an elimination reaction that generates the dehydroalanine-containing species. The observed inhibition by **4** can be attributed to the sp^2-hybridization of the α-carbon in the dehydroalanine unit that mimics the planar geometry of the anionic intermediate shown in Figure 2a. These results argue strongly against the dehydration/rehydration mechanism (Figure 2c), since **4** is the putative intermediate in that mechanism, yet no signs of the hydration of **4** (to give a mixture of **1** and **2**) were observed. No evidence for the formation of covalent adducts was observed, as might be expected if the unnatural elimination step occurred at the covalent ester adduct stage (Figure 2b). Furthermore, if covalent catalysis were employed, one might not expect compound **4** to be a potent inhibitor.

Elimination reactions of *β*-halides and inhibition by planar intermediate analogues have long been hallmarks of the reactions catalyzed by cofactor-independent amino acid racemases, all of which utlilize deprotonation/reprotonation mechanisms.[9,11,12,21,26,36−40] Notable examples include the inhibition of proline racemase by the planar compounds **12** and **13**.[21,36] In addition, the enzyme-catalyzed elimination of HCl or HF from *β*-halo amino acids, as well as the elimination of water from *N*-hydroxy amino acids, has led to the transient formation of the planar enamine and imine species **14** and **15** (they ultimately hydrolyze when free in solution). These species have been shown to serve as potent inhibitors of the enzymes diaminopimelate epimerase[38,39] and glutamate racemase.[9,40] This work strongly suggests that a similar mechanistic strategy is at play in this newly discovered class of peptide epimerases.

Experimental Section

General. Et_3N, CH_2Cl_2, and EtOAc were distilled under N_2 from CaH_2. MeOH was distilled under N_2 from magnesium methoxide. Anhydrous DMF was dried over 4 Å molecular sieves and stored under argon. The pentapeptide substrates **1** and **2** were purchased from the Nucleic Acids Protein Services Unit at the University of British Columbia (UBC). Flash chromatography was performed on 230−400 mesh silica gel. ^{1}H NMR data were obtained on a 200, 300, or 400 MHz spectrometer. Mass spectrometry was performed by the Mass Spectrometry Centre at UBC by liquid secondary ionization mass spectrometry (LSIMS) or chemical ionization (CI). Elemental analyses were performed by Mr. Peter Borda in the Microanalytical Laboratory at UBC and by Canadian Microanalytical Service, Ltd. Deprotected peptides were purified by reversed-phase HPLC on a C_{18} column (19 × 300 mm, 15-*μ*m particle size, 100-Å pore) with detection at 220 nm. Elution was achieved in mixtures of 0.1% TFA in water and 0.05% TFA in CH_3CN using linear gradients optimized for each peptide with a flow rate of 14 mL/min.

***N*-(Benzyloxycarbonyl)-L-phenylalanyl-L-alanine *tert*-Butyl Ester (5).** A solution of Cbz-Phe (6.60 g, 22.0 mmol), 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide (EDC, 4.42 g, 23.1 mmol), and 1-hydroxybenzotriazole hydrate (HOBt, 3.54 g, 23.1 mmol) in 60 mL of dry CH_2Cl_2 was stirred for 10 min under argon. Ala-O*t*Bu·HCl (4.00 g, 22.0 mmol) and Et_3N (3.05 mL, 22.0 mmol) were added, and the solution was stirred at room temperature for 20 h. The mixture was diluted to 200 mL with CH_2Cl_2 and washed with 200 mL of water, resulting in the precipitation of triethylamine hydrochloride salt. Following filtration, the organic layer was separated and washed with 200 mL portions of 5% $KHSO_4$, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$, filtered, and rotary evaporated. Recrystallization from EtOH/H_2O gave 8.4 g (90%) of **5** as white crystals. Spectral properties were in agreement with those in the literature.[41]

***N*-(Benzyloxycarbonyl)-3-chloro-L-alanyl-L-phenylalanyl-L-alanine *tert*-Butyl Ester (6).** A solution of **5** (2.50 g, 5.86 mmol) in 15 mL of CH_3OH containing 5% Pd/C (250 mg) was stirred under 1 atm of hydrogen at room temperature for 20 h. The mixture was filtered through Celite, and the filtrate was rotary evaporated to give 1.71 g (100%) of the amine as a colorless oil. In a separate flask, a solution of *Z*-3-chloro-L-alanine[18] (1.17 g, 4.55 mmol), EDC (0.916 g, 4.78 mmol), and HOBt (0.766 g, 5.00 mmol) in 20 mL of dry CH_2Cl_2 was stirred for 10 min under argon. A solution of the amine (1.33 g, 4.55 mmol) in 5 mL of dry CH_2Cl_2 was then added, and the mixture was stirred at room temperature for 22 h. The mixture was diluted to 50 mL with CH_2Cl_2 and washed with 50 mL portions of water, 5% $KHSO_4$, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$, filtered, and rotary evaporated. The residue was purified by silica gel chromatography (40:40:1 petroleum ether/EtOAc/MeOH) to give 1.31 g (54%) of **6** as a colorless glass: ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.35 (s, 5H), 7.28−7.13 (m, 5H), 6.76 (d, 1H, J = 7.8 Hz), 6.25 (d, 1H, J = 7.1 Hz), 5.45 (d, 1H, J = 7.3 Hz), 5.11 (s, 2H), 4.63 (dd, 1H, J = 7.1 Hz, J = 7.1 Hz), 4.51 (m, 1H), 4.32 (m, 1H, J = 7.1 Hz), 3.96 (dd, 1H, J = 3.9 Hz, J = 11.2 Hz), 3.68 (dd, 1H, J = 5.1 Hz, J = 11.2 Hz), 3.08 (m, 2H), 1.43 (s, 9H), 1.29 (d, 3H, J = 7.1 Hz); +LSIMS (thioglycerol matrix) 532 ($M + H^+$). Anal. Calcd for $C_{27}H_{34}ClN_3O_6$: C, 60.95; H, 6.44; N, 7.90. Found: C, 61.29; H, 6.57; N, 8.20.

***N*-Acetylglycyl-L-leucine (7).** To a stirred solution of glycyl-L-leucine (5.42 g, 28.8 mmol) in 20 mL of 0.1 M AcOH was added Et_3N to pH 6. Acetic anhydride (15.9 mL, 144 mmol) was added in small portions, while pH 6 was maintained by addition of Et_3N. Amberlite IR-120 (H) resin was added to pH < 2 and then filtered off. The filtrate was rotary evaporated under reduced pressure to give 6.19 g (93%) of **7** as a colorless glass: ^{1}H NMR (200 MHz, D_2O) δ 4.37 (dd, 1H, J = 5.7 Hz, J = 8.9 Hz), 3.88 (s, 2H), 2.00 (s, 3H), 1.73−1.50 (m, 3H), 0.89 (d, 3H, J = 6.1 Hz), 0.84 (d, 3H, J = 6.1 Hz); +DCI-MS (NH_3) 231 ($M + H^+$). Anal. Calcd for $C_{10}H_{18}N_2O_4$: C, 52.16; H, 7.88; N, 12.17. Found: C, 51.99; H, 7.81; N, 12.01.

***N*-Acetylglycyl-L-leucyl-3-chloro-L-alanyl-L-phenylalanyl-L-alanine *tert*-Butyl Ester (8).** A solution of **6** (3.01 g, 5.66 mmol) in 30 mL of distilled CH_3OH containing 5% Pd/C (300 mg) was stirred under 1 atm of hydrogen at room temperature for 24 h. The mixture was filtered through Celite, and the filtrate was rotary evaporated to give 2.10 g (93%) of the amine as a pale yellow foam. In a separate flask, a solution of **7** (1.22 g, 5.28 mmol), EDC (1.06 g, 5.54 mmol), and HOBt (0.848 g, 5.54 mmol) in 20 mL of DMF was stirred for 10 min under argon. A solution of the amine in 20 mL of DMF was then added, and the solution was stirred at room temperature for 58 h. The solvent was removed by rotary evaporation under reduced pressure, and the resulting amber residue was triturated in 50 mL of CH_2Cl_2, leaving **8** as a white solid that was collected by filtration. The filtrate was concentrated and partitioned between 50 mL of water and 50 mL of EtOAc. The organic layer was washed with 50 mL portions of 5% $KHSO_4$, saturated $NaHCO_3$, and brine and then dried over $MgSO_4$. The mixture was filtered and rotary evaporated, and the resulting

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residue was triturated with ether to give additional **8** (total yield: 0.866 g, 27%): ^{1}H NMR (400 MHz, CD_3OD) δ 7.27−7.16 (m, 5H), 4.63 (dd, 1H, J = 5.2 Hz, J = 9.0 Hz), 4.54 (dd, 1H, J = 5.2 Hz, J = 7.3 Hz), 4.38 (dd, 1H, J = 5.8 Hz, J = 9.4 Hz), 4.26 (q, 1H, J = 7.3 Hz), 3.86 (s, 2H), 3.80 (dd, 1H, J = 4.9 Hz, J = 11.3 Hz), 3.74 (dd, 1H, J = 7.3 Hz, J = 11.3 Hz), 3.20 (dd, 1H, J = 5.2 Hz, J = 14.0 Hz), 2.93 (dd, 1H, J = 9.1 Hz, J = 14.0 Hz), 1.98 (s, 3H), 1.74−1.61 (m, 1H), 1.61−1.54 (m, 2H), 1.45 (s, 9H), 1.35 (d, 3H, J = 7.3 Hz), 0.96 (d, 3H, J = 6.7 Hz), 0.92 (d, 3H, J = 6.4 Hz); +LSIMS (thioglycerol matrix) 610 (M + H$^+$). Anal. Calcd for $C_{29}H_{44}ClN_5O_7$: C, 57.09; H, 7.27; N, 11.48. Found: C, 56.95; H, 7.36; N, 11.51.

***N*-Acetylglycyl-L-leucyl-3-chloro-L-alanyl-L-phenylalanyl-L-alanine (3).** A solution of **8** (20 mg, 0.033 mmol) in 3 mL of 88% formic acid was stirred at room temperature for 48 h. After removal of solvent by rotary evaporation under reduced pressure at <35 °C, the residue was dissolved in HPLC-grade MeOH and evaporated to dryness. The resulting colorless foam was dissolved in 9 mL of HPLC-grade MeOH and purified in ∼2-mL portions by preparative reversed-phase HPLC, followed by concentration and lyophilization to give 14 mg (75%) of **3** as a white solid: ^{1}H NMR (400 MHz, CD_3OD) δ 7.24−7.13 (m, 5H), 4.60 (dd, 1H, J = 5.2 Hz, J = 8.8 Hz), 4.52 (dd, 1H, J = 5.2 Hz, J = 7.3 Hz), 4.39−4.29 (m, 2H), 3.84 (s, 2H), 3.78 (dd, 1H, J = 5.2 Hz, J = 11.3 Hz), 3.72 (dd, 1H, J = 7.6 Hz, J = 11.3 Hz), 3.18 (dd, 1H, J = 5.2 Hz, J = 14.0 Hz), 2.91 (dd, 1H, J = 8.8 Hz, J = 14.0 Hz), 1.96 (s, 3H), 1.70−1.59 (m, 1H), 1.59−1.50 (m, 2H), 1.37 (d, 3H, J = 7.0 Hz), 0.93 (d, 3H, J = 6.4 Hz), 0.89 (d, 3H, J = 6.4 Hz); +LSIMS (thioglycerol matrix) 554 (M + H$^+$).

***N*-Acetylglycyl-L-leucyl-Se-phenyl-L-selenocysteinyl-L-phenylalanyl-L-alanine (9).** The title peptide was synthesized on a 0.25-mmol scale by standard solid-phase peptide synthesis using Fmoc-phenylselenocysteine.[19] After cleavage from the resin, the peptide was purified by reversed-phase HPLC to give 94 mg (56%) of **9** as a white solid: ^{1}H NMR (300 MHz, CD_3OD) δ 7.53−7.46 (m, 2H), 7.29−7.14 (m, 8H), 4.58 (dd, 1H, J = 5.0 Hz, J = 8.9 Hz), 4.41−4.27 (m, 3H), 3.86 (s, 2H), 3.25−3.14 (m, 2H), 3.05 (dd, 1H, J = 9.2 Hz, J = 12.3 Hz), 2.90 (dd, 1H, J = 9.2 Hz, J = 13.9 Hz), 1.97 (s, 3H), 1.71−1.60 (m, 1H), 1.60−1.50 (m, 2H), 1.39 (d, 3H, J = 7.3 Hz), 0.95 (d, 3H, J = 6.2 Hz), 0.91 (d, 3H, J = 6.2 Hz); +LSIMS (thioglycerol matrix) 676 (M + H$^+$).

***N*-Acetylglycyl-L-leucyl-2,3-didehydroalanyl-L-phenylalanyl-L-alanine (4).** To a solution of **9** (40 mg, 0.059 mmol) in 10 mL of HPLC-grade MeOH was added 30% aqueous H_2O_2 (27 μL, 0.24 mmol), and the mixture was stirred at room temperature for 30 min. Reaction completion was confirmed by reversed-phase HPLC on an analytical column, and the mixture was purified by HPLC on a preparative column to give 28 mg (93%) of **4** as a white solid: ^{1}H NMR (400 MHz, D_2O) δ 7.31−7.17 (m, 5H), 5.51 (s, 1H), 5.46 (s, 1H), 4.59 (dd, 1H, J = 6.4 Hz, J = 8.5 Hz), 4.30−4.23 (m, 2H), 3.84 (s, 2H), 3.14 (dd, 1H, J = 6.4 Hz, J = 14.0 Hz), 2.97 (dd, 1H, J = 8.5 Hz, J = 14.0 Hz), 1.93 (s, 3H), 1.61−1.48 (m, 3H), 1.31 (d, 3H, J = 7.3 Hz), 0.85 (d, 3H, J = 5.8 Hz), 0.80 (d, 3H, J = 5.8 Hz); +LSIMS (3-nitrobenzyl alcohol matrix) 518 (M + H$^+$), 540 (M + Na$^+$).

Enzyme Purification. For routine assays the epimerase was purified according to a literature[17] method with the following change: in lieu of lyophilization, the isolated enzyme was concentrated by centrifugation through a membrane (10 000 MWCO) to a volume of 1.0−1.5 mL, divided into 50 μL aliquots, and stored at −80 °C. To obtain orthogonally purified enzyme, cation exchange chromatography followed by size exclusion chromatography was performed as follows. A 200-μL sample of lyophilized venom was dissolved in 1 mL of distilled H_2O and loaded onto a 1-mL HiTrap SP HP column (Amersham Pharmacia Biotech) equilibrated with 20 mM potassium phosphate buffer, pH 6.8. Elution was achieved by a linear gradient of phosphate buffer containing 0.4 M NaCl. The relevant fractions were pooled and concentrated by centrifugation to 400 μL, which was further purified by size exclusion chromatography as described.[17]

Enzyme Assay. Typically, a 50 μL (0.018 mg by Bradford assay[42]) solution of enzyme in phosphate-buffered saline, pH 7.4, was equilibrated with 6 μL of 50 mM EDTA at 37 °C for 30 min. To this was added 4 μL of inhibitor **3** (in DMSO) or **4** (in H_2O) of varying concentration (0.1−100 μM), followed by 4 μL of 100 μM substrate **1**. Aliquots were analyzed after 5−20 h by reversed-phase HPLC on a C_{18} column (3.9 × 150 mm) using 0.1% TFA in H_2O with a linear gradient of 0.05% TFA in CH_3CN with detection at 220 nm. Relative retention times were as follows: **1**, 13.8 min; **2**, 11.1 min; **3**, 15.9 min; **4**, 16.5 min.

To obtain a mass of spectrum of **4** generated enzymatically from **3**, a solution of enzyme (50 μL, 0.018 mg) in phosphate-buffered saline, pH 7.4, was equilibrated with EDTA (6 μL of a 50 mM solution) at 37 °C for 30 min. Inhibitor **3** (4 μL of a 100 μM stock solution in DMSO) was added and the reaction was incubated for 20 h. The sample was subjected to liquid chromatography−mass spectrometry (HPLC−ESI-MS) and the peak with a retention time corresponding to **4** was found to have masses 518 (M + H$^+$) and 540 (M + Na$^+$).

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Supporting Information Available: ^{1}H NMR spectra for compounds **3**, **4**, and **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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