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Peptidyl allyl sulfones: a new class of inhibitors for clan CA cysteine proteases

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Abstract—A new series of peptidyl allyl sulfone inhibitors was discovered while trying to synthesize epoxy sulfone inhibitors from vinyl sulfones using basic oxidizing conditions. The various dipeptidyl allyl sulfones were evaluated with calpain I, papain, cathepsins B and L, cruzain and rhodesain and found to be potent inhibitors. In comparison to the previously developed class of vinyl sulfone inhibitors, the novel dipeptidyl allyl sulfones were more potent inhibitors than the corresponding dipeptidyl vinyl sulfones. It was observed that the stereochemistry of the vinyl sulfone precursor played a role in the potency of the dipeptidyl allyl sulfone inhibitor.

1. Introduction

Proteases or proteolytic enzymes catalyze the hydrolysis of a wide variety of peptides and proteins. Proteases can be divided into classes of cysteine, serine, aspartate, threonine, and metallopeptidases depending on the catalytic nucleophile.[1,2] Cysteine proteases employ a thiolate residue, which performs a nucleophilic attack on the amide bond of the peptide backbone to form a tetrahedral intermediate. The intermediate collapses to release the first product and the resulting acyl enzyme then undergoes hydrolysis. Based on their sequence homology, cysteine proteases are divided into several clans and families. Clan CA and clan CD contain the majority of cysteine proteases. Clan CD contains several important enzymes including caspases, legumains, gingipain and clostripain. The papain family of cysteine proteases belongs to the clan CA and includes cathepsins, calpains, and the parasite cysteine proteases cruzain and rhodesain.

Calpains are a family of cysteine proteases in clan CA, which are activated by calcium. So far, eight homologs of human calpain have been identified, two of which have received most attention: calpain I (or μ-calpain) and calpain II (or m-calpain). Their activity in vitro is dependent on a calcium concentration in micromolar amounts for calpain I and millimolar amounts for calpain II.[3–6] Both forms are present in the cytosol of mammalian cells and are involved in physiological processes that control the degradation of the cytoskeleton as well as hormone receptors.[7,8] Enhanced calpain activity has been associated with cell injury due to ischemic stroke,[9] physical damage,[10] and hypoxia.[11] Thus, calpains are attractive targets for the development of inhibitors, which should be useful for treatment of a variety of diseases.

The clan CA proteases, cruzain and rhodesain, are essential for the development and survival of the protozoan parasites *Trypansoma cruzi* and *T. brucei*, respectively. *T. cruzi* causes Chagas' disease in humans in South and Central America, whereas *T. brucei* causes sleeping sickness in humans in large areas of central and southern Africa. Current drug therapies are accompanied by serious side effects and widespread resistance. Thus, the need of new medicinal agents is urgent.[12,13]

Keywords: Cysteine protease inhibitor; Vinyl sulfone; Cathepsins; Cruzain.
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Cathepsins comprise a large family of lysosomal cysteine proteases and are involved in the degradation of host connective tissues, the generation of bioactive proteins and antigen processing.[14,15] They have been implicated in a variety of disease states such as rheumatoid arthritis,[16] muscular dystrophy,[17,18] and tumor metastasis.[19] The high similarity in the substrate specificity among the individual cathepsins presents a challenge in finding selective and thus effective cathepsin inhibitors.

Clan CA cysteine proteases are effectively inhibited by several classes of peptidyl inhibitors including transition state, reversible and irreversible inhibitors.[20] Examples of reversible transition state inhibitors are peptide aldehydes,[21–24] α-diketones,[25–28] α-ketoesters,[29,30] α-ketoamides,[29,31] and α-ketoacids.[29] Clan CA proteases are also irreversibly inhibited by peptidyl diazomethyl ketones and fluoromethyl ketones,[32,33] peptidyl epoxides (E-64, E-64-c, E-64-d),[34] and vinyl sulfones.[35] However, the potency and specificity of many of the irreversible inhibitors could be improved.

In order to develop new functional groups suitable for irreversible inhibition of cysteine proteases, we attempted the synthesis of peptidyl epoxy sulfones. The design of the peptidyl epoxy sulfone structure was based on vinyl sulfones, which are a class of promising irreversible peptidyl inhibitors for clan CA cysteine proteases.[35] Vinyl sulfones contain a Michael acceptor functional group and react irreversibly with the active site cysteine. Another widely used warhead in cysteine protease inhibitors is the epoxide moiety of the natural product inhibitor E-64. We recently reported aza-peptide epoxides as potent and selective inhibitors for clan CD cysteine proteases including caspases and legumains.[36,37] Here, we propose that by combining the reactivity of the E-64 epoxide electrophile with the selectivity of the vinyl sulfone moiety will result in both a potent and specific new class of inhibitors for cysteine proteases. We initially focused on the clan CA cysteine proteases calpain and papain, and thus decided to attach this novel warhead to an optimal dipeptide sequence for effective calpain and papain inhibition. We chose the Leu-Phe, Ala-Phe, and Val-Phe sequences with the N-terminal Cbz-protecting group (Cbz = Ph–CH_2–OCO–, benzyloxycarbonyl).[25]

2. Results and discussion

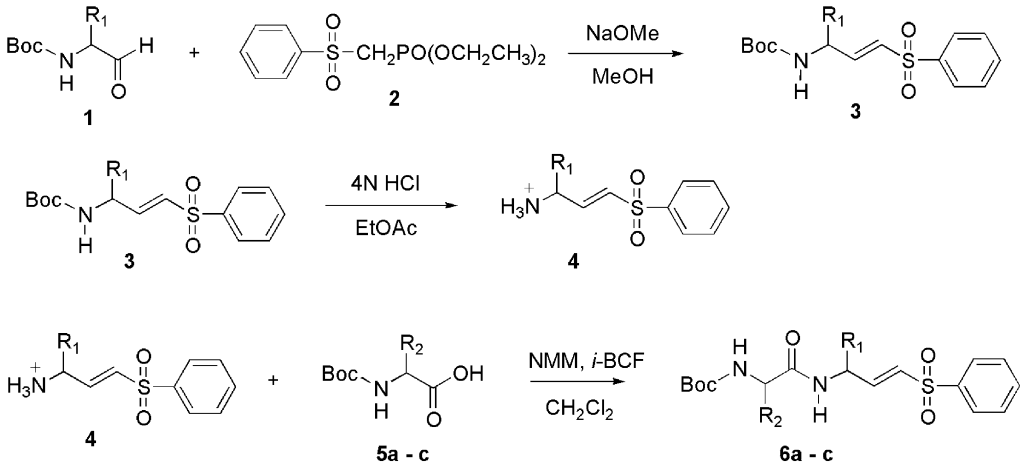
2.1. Synthesis

Our strategy for the synthesis of epoxy sulfones began with the synthesis of the appropriate peptidyl vinyl sulfone (Scheme 1). Vinyl sulfones have been previously synthesized and prepared by coupling the chiral amino acid aldehydes **1** and the corresponding sulfonyl phosphonate **2** using Wadsworth–Emmons chemistry.[35] The stereochemistry of the vinyl sulfone is primarily E, but the presence of small amounts of the Z isomer cannot be excluded.[38] The Boc-group (Boc = butyloxycarbonyl) was then removed using 4 N HCl in ethyl acetate. Coupling of the salt **4** to a Cbz-protected amino acid **5** using standard mixed anhydride conditions furnished the dipeptidyl vinyl sulfone precursors **6**.

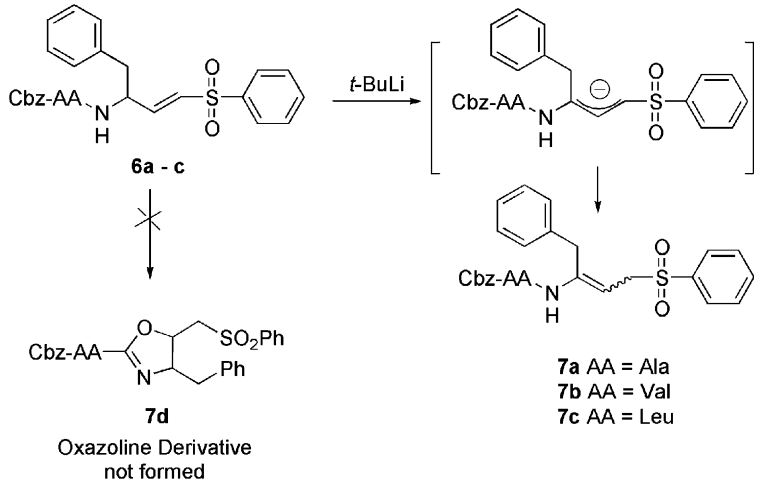
In order to synthesize the epoxy sulfone, we decided to epoxidize the double bond moiety in the peptidyl vinyl sulfone precursor using basic peroxidizing conditions. When we tried to epoxidize the vinyl sulfone using *t*BuLi and *n*BuOOH in freshly distilled THF at −78 °C, the formation of a new compound was observed by TLC. The vinyl sulfone starting material was consumed within 20 min. The new compound was purified by column chromatography and carefully characterized using several spectroscopic methods. We found that the conjugated double bond of the vinyl sulfone moiety was no longer present in the ^{1}H NMR. However, according to the mass spectrum (MS), the molecular weight of the newly formed product was unchanged. Elemental analysis also confirmed that the new compound was a constitutional isomer of the vinyl sulfone starting material. The characteristic sulfone stretch was still present in the FT-IR spectrum. Since the new compound was formed by isomerization and not oxidation, the critical reagent was the *t*BuLi base and not the oxidizing *n*BuOOH. Treatment of the vinyl sulfone with *n*BuLi without the peroxide *t*BuOOH gave the same product. However, the reaction was much more sluggish, and required several hours at room temperature.

We considered several possible isomeric structures for the *n*BuLi reaction product (Scheme 2). Two likely possibilities were a substituted oxazoline (**7d**) formed by cyclization or an allyl sulfone (AS), with the double bond isomerized toward the adjacent α-carbon. Oxazolines have characteristic ^{13}C NMR signals at 170 ppm for the C-2 carbon atom.[39] Oxazolines have previously been synthesized, and we prepared the parent compound **11** following the synthetic method described by Wipf and Miller.[39] This oxazoline could potentially acylate the active site cysteine of a cysteine protease, but the ^{1}H NMR spectrum was not identical to our isomerization product. The ^{13}C NMR spectrum of our product has a new peak at 106 ppm and not one at 170 ppm for the oxazoline. In addition, we observed the presence of an NH in the ^{1}H NMR. This ruled out the formation of the oxazoline in the isomerization reaction.

The allyl sulfone structure (**7a**–**c**) was supported by all the spectroscopic data. The methylene of the phenylalanine side chain shifts substantially from 2.9 to 3.8 ppm in the ^{1}H NMR spectrum, and the shift is explained by the close proximity to the double bond in the newly formed isomerization product. The distinct triplet at 4.9 ppm is consistent with a vinylogous proton. Upon decoupling the vinyl proton triplet at 4.9 ppm, the methylene group next to the sulfone moiety at 3.83 ppm appears as a singlet, indicating that the protons involved are located on adjacent carbons. Supporting information was also provided by a series of COSY (correlation spectroscopy) and NOESY (nuclear Overhauser effect spectroscopy) experiments for Cbz-Val-Phe-AS-Ph (**7b**, Fig. 1). We have determined NOE (nuclear Overhauser effect) correlations between the vinyl proton and the *ortho* ring protons of the phenylalanine side chain. These results



Scheme 1. Synthesis of dipeptidyl vinyl sulfones.



Scheme 2. Isomerization of dipeptidyl vinyl sulfones to allyl sulfones.

indicate that the allyl sulfones derived from the L-Phe vinyl sulfone precursor assume Z stereochemistry after isomerization. It has previously been reported that treatment of vinyl sulfones with strong base results in the formation of allyl sulfones (Scheme 2), as allyl sulfones are thermodynamically more stable than vinyl sulfones.[40,41] The stereochemistry of the isomerized double bond was not determined, however, according to the ^{1}H NMR, we are certain that only one isomer is present. Both E and Z or an E/Z mixture have been obtained upon base catalyzed isomerization of vinyl sulfones.[42,43]

2.2. Inhibition of clan CA cysteine proteases

The novel, newly synthesized peptidyl allyl sulfones had the potential of being cysteine protease inhibitors and were tested along with the oxazoline **11** with papain, calpain I, cathepsin B and L, cruzain and rhodesain (Table 1). This new class of compounds shows moderate inactivation rates with calpain and papain, and faster rates of inactivation with rhodesain and cruzain. The oxazoline was also a slow inhibitor of calpain I. Kinetic inhibition assays were performed using calpain I from bovine erythrocytes and Suc-Leu-Tyr-AMC (AMC = 7-amino-4-methylcoumarin) as the fluorogenic substrate.[44] Cathepsin B was tested using commercially available enzyme and Cbz-Arg-Arg-AMC as the substrate.[45] The substrate Cbz-Phe-Arg-AMC was used with the cathepsin L inhibition assays.[46] Papain inhibition was measured using commercially available papain and Cbz-Arg-Phe-*p*NA (*p*NA = *para*-nitroaniline) as the substrate.[47] The dipeptidyl allyl sulfones do not inhibit cathepsin B and show limited rates with both calpain I and papain. In contrast are the striking second-order inhibition rate constant ($k_{obs}/[I]$) values of $564\,M^{-1}s^{-1}$ and $1062\,M^{-1}s^{-1}$ for Cbz-Leu-Phe-AS-Ph (isomer B) with calpain I and cathepsin L, respectively. This dipeptidyl allyl sulfone was synthesized from the dipeptidyl vinyl sulfone precursor Cbz-Leu-D-Phe-VS-Ph. When the double bond isomerizes, the original Z stereochemistry of the vinyl sulfone is lost and the product has an unknown ratio of E to Z isomers at the new allyl double bond. There are several factors, which can contribute to a mixed ratio of isomers, such as the so-called '*syn*-effect'[42,43] and the chelating lithium metal. This explains the difference in inhibitor potency between Cbz-Leu-Phe-AS-Ph (isomer A), which is derived from the vinyl sulfone precursor Cbz-Leu-L-Phe-VS-Ph, and

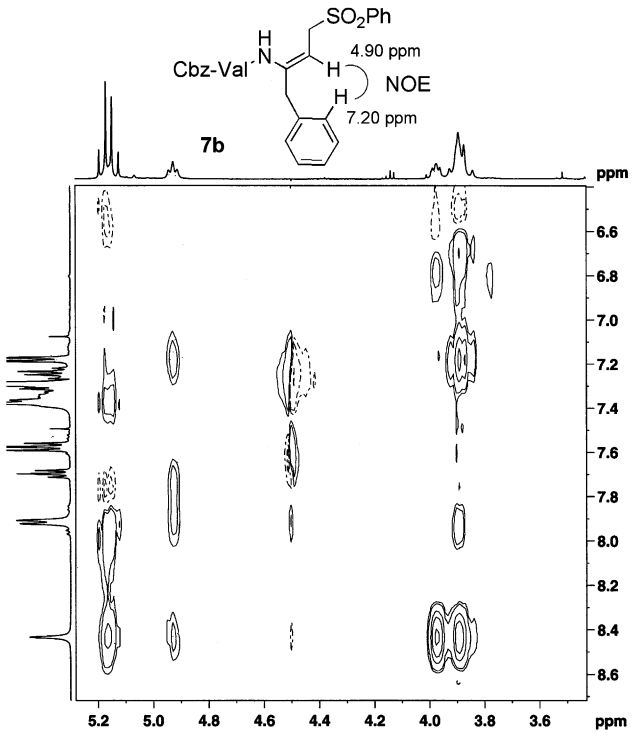


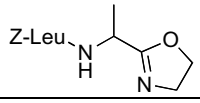
Figure 1. NOESY spectrum of Cbz-Val-Phe-AS-Ph (**7b**) at 500 MHz in $CDCl_3$ showing the correlation of the vinyl proton at 4.90 ppm with the *ortho* ring proton of the Phe side chain at 7.20 ppm. We have designated the allyl sulfone 'AS'. Thus, an allyl sulfone derivative will be abbreviated AA-AS-R, where R is the phenyl substituent on the sulfone functional group; Cbz = Ph–CH_2–OCO–.

Cbz-Leu-Phe-AS-Ph (isomer B). The initial stereochemistry of the phenylalanine side chain probably plays a significant role in the final ratio of Z to E isomers in the allyl sulfone inhibitor. The difference in potency of the two isomers is also observed with inhibition of cruzain and rhodesain. However, in this case the ranking is reversed. The Cbz-Leu-Phe-AS-Ph (isomer A), which is derived from the vinyl sulfone precursor Cbz-Leu-L-Phe-VS-Ph, is more potent (IC_{50} = 0.06 μM for cruzain and 0.04 μM for rhodesain) than isomer B (IC_{50} = 0.3 μM for cruzain and 0.18 μM for rhodesain).

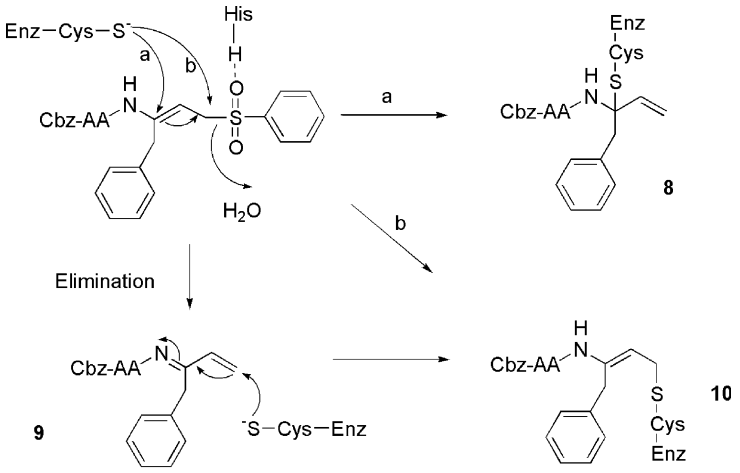
We were surprised to find the novel allyl sulfone inhibitors Cbz-Leu-Phe-AS-Ph (both isomers) to be more potent than the corresponding vinyl sulfone Cbz-Leu-Phe-VS-Ph. Vinyl sulfones have so far been the most effective inhibitors for cruzain and rhodesain.[48] It has been shown that the peptide sequence and the nature of the substituent on the prime side of the vinyl sulfone functionality plays an important role in the potency of the vinyl sulfone inhibitor towards the target enzyme.[35,49] Using extensive SAR studies, vinyl sulfones have been improved in potency by factors of 10^3. Therefore, it is likely that with further modifications of the peptide sequence and the P′ sulfonyl substituent, this new class of dipeptidyl allyl sulfone inhibitors has the potential of becoming as effective a class of inhibitors as the vinyl sulfones.

One mechanism for the inhibition reaction could involve re-isomerization of the peptidyl allyl sulfone to the vinyl sulfone. This isomerization could be catalyzed by the active site histidine and would be reflected by a higher inhibition rate ($k_{obs}/[I]$) for the parent vinyl sulfone inhibitor. However, as the parent vinyl sulfone Cbz-Leu-Phe-VS-Ph was less potent with papain ($k_{obs}/[I] = 10\ M^{-1}s^{-1}$) than the corresponding allyl sulfone Cbz-Leu-Phe-AS-Ph (isomer A) ($k_{obs}/[I] = 49\ M^{-1}s^{-1}$), we conclude that the mechanism of inhibition does not

Table 1. Inhibition of various cysteine proteases by dipeptidyl allyl sulfones

	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)[a]				IC_{50} (μM)[b]	
		Calpain I[c]	Papain[d]	Cathepsin B[e]	Cathepsin L[e]	Cruzain	Rhodesain
7a	Cbz-Ala-Phe-AS-Ph	3 ± 0	9 ± 0	N.I.[f]	183 ± 10[g]	>10	>10
7b	Cbz-Val-Phe-AS-Ph	3 ± 0	6 ± 1	N.I.	310 ± 52[g]	6	5
7c	Cbz-Leu-Phe-AS-Ph (isomer A)[h]	23 ± 4	49 ± 6	N.I.	700 ± 43[g]	0.06	0.04
8	Cbz-Leu-Phe-AS-Ph (isomer B)[i]	564 ± 32[g]	15 ± 1	N.I.	1060 ± 121[g]	0.3	0.18
6c	Cbz-Leu-Phe-VS-Ph	550 ± 2[g]	10 ± 0	N.I.	219 ± 96[g]	2	0.5
11		13 ± 1	N.I.	N.I.	N.I.	>10	>10

[a] k_{obs} is the pseudo-first-order rate constant obtained from plots of ln v_t/v_0 versus time unless indicated otherwise.
[b] Cruzain (2 nM) or rhodesain (3 nM) was incubated with 0.0001 to 1 μM inhibitor in 100 mM sodium acetate buffer, pH 5.5 and 5 mM DTT (buffer A), for 5 min at room temperature prior to substrate addition.
[c] Calpain I assay conditions: Irreversible kinetic assays were performed by the incubation method with calpain I from porcine erythrocytes. Enzymatic activities of calpain I were measured at 23 °C in 50 mM Hepes buffer (pH 7.5) containing 10 mM cysteine and 5 mM $CaCl_2$.
[d] Papain assay conditions: Incubation kinetics were measured using an enzyme stock solution for the papain assays, which was freshly prepared from 330 μL of enzyme storage solution (1.19 mg/mL) diluted with 645 μL papain buffer (50 mM Hepes, and 2.5 mM EDTA at pH 7.5) and 25 μL of DTT (0.1 M).
[e] Cathepsin B and L assay conditions: Enzymatic activities of cathepsin B and L were measured in 0.1 M $KHPO_4$, 1.25 mM EDTA, 0.01% Brij, pH 6.0 buffer and at 23 °C.
[f] N.I.=no inhibition after 20 min of incubation.
[g] k_{obs} is obtained by the progress curve method and corrected for substrate.
[h] Allyl sulfone is derived from the L-isomer of phenylalanine.
[i] Allyl sulfone is derived from the D-isomer of phenylalanine.



Scheme 3. Proposed mechanisms of inhibition of cysteine proteases by allyl sulfones.

involve re-isomerization of the allyl sulfone to the vinyl sulfone with a subsequent attack of the cysteine thiol on the Michael acceptor double bond. This was even more pronounced with cruzain and rhodesain, where the same vinyl sulfone was over 30- and 12-fold less potent with cruzain ($IC_{50} = 2\,\mu M$) and rhodesain ($IC_{50} = 0.5\,\mu M$), respectively, than the corresponding allyl sulfone Cbz-Leu-Phe-AS-Ph. With calpain I it is possible that the isomerization mechanism is occurring, since the vinyl sulfone is a much more effective inhibitor of calpain.

Two other mechanisms can be envisaged for enzyme inhibition by allyl sulfones (Scheme 3). The active site cysteine could directly displace phenyl sulfinic acid in an S_N2 reaction (pathway b) or could attack the allylic double bond with loss of phenyl sulfinic acid (pathway a). Both pathways result in alkylation of the active site cysteine residue. It has previously been reported that allyl sulfones when reacted with nucleophiles undergo a tosyl elimination or displacement process, in which the allyl sulfone moiety undergoes alkylation at either the α- or γ-carbon.[41,50,51] An alternative possibility for enzyme inactivation follows a mechanism-based pathway, which is initiated by removal of the amide nitrogen proton by a base or the active site histidine, followed by the elimination of phenyl sulfinic acid and the formation of the imine **9**. The active site cysteine then reacts with **9** in a Michael addition, which also irreversibly alkylates the enzyme. Several PLP-dependent enzymes (PLP = pyridoxal phosphate) are irreversibly inhibited by mechanism-based inactivators, where the pathway also follows displacement and β-elimination reactions. A vinylic imine forms as an intermediate before undergoing attack by a nucleophile in the active site of the enzyme.[52]

3. Conclusions

In summary, we report the synthesis of a new class of inhibitors, peptidyl allyl sulfones. In an effort to synthesize epoxy sulfone inhibitors, allyl sulfones were unexpectedly produced from vinyl sulfones by isomerization of the double bond. This new class of inhibitors contains a novel cysteine protease warhead and shows moderate rates of inactivation of calpain, papain and cathepsin L. The inhibitors are potent inactivators of the parasitic proteases cruzain and rhodesain.

We observed that the allyl sulfones were generally more effective inhibitors in comparison to the vinyl sulfone precursor. We have not yet determined the optimum sequences for the peptidyl allyl sulfones. Therefore, we would expect substantial improvement in the potency of allyl sulfones using an SAR study in order to determine the optimum peptide sequence and the nature of the prime side substituent. However, the two classes of inhibitors have different inhibition mechanisms and different points of attack. Therefore, it remains to be seen whether the peptidyl allyl sulfones can be improved as much as peptidyl vinyl sulfones, which have been highly optimized by SAR studies.

We envision achievement of even greater potency with both cruzain and rhodesain by attaching the allyl sulfone warhead to a cruzain and rhodesain specific peptide sequence in combination with the optimization of the P′ sulfonyl substituent. It would be of interest to extend this warhead to other cysteine proteases and investigate the effect of the stereochemistry of the double bond on the potency of the inhibitor. It would also be of interest to determine the mechanism of inhibition since allyl sulfones are likely mechanism-based inhibitors.

4. Experimental

4.1. Materials and methods

Materials were obtained from Acros, Bachem Bioscience Inc., or Sigma Aldrich and used without further purification. The purity of each compound was confirmed by TLC, ^1H NMR, MS, and elemental analysis. Chemical shifts are reported in ppm relative to an internal standard (trimethylsilane). Thin layer chromatography (TLC) was performed on Sorbent Technologies

(250 μm) silica gel plates. The ^{1}H NMR spectra were obtained on a Varian Mercury 400 MHz spectrometer. The NOESY and COSY spectra were obtained on a Bruker DRX 500 MHz spectrometer. Electrospray ionization (ESI), fast-atom-bombardment (FAB) and high-resolution mass spectrometry were obtained using Micromass Quattro LC and VG Analytical 70-SE instruments. Elemental analysis was carried out by Atlantic Microlab Inc., Norcross, GA.

4.1.1. Papain and cathepsin B assays. The incubation method was used to measure the irreversible inhibition of papain and cathepsin B. With cathepsin B, 30 μL of a stock inhibitor solution was added to 300 μL of a 0.1 M potassium phosphate buffer containing 1.25 mM EDTA, 0.01% Brij 35 at pH 6.0, followed by the addition of 30 μL of a freshly prepared cathepsin B solution (approximate concentration 6.98×10^{-3} μg/μL) in the same potassium phosphate buffer containing 1 mM DTT (freshly prepared). Aliquots (50 μL) from the inhibition mixture were withdrawn at various time intervals and added to 200 μL of a 0.1 M potassium phosphate buffer containing 1.25 mM EDTA, 0.01% Brij 35 at pH 6.0, and the substrate Cbz-Arg-Arg-AMC (499 μM). The release of 7-amino-4-methylcoumarin was monitored (λ_{ex} = 360 nm, λ_{em} = 465 nm) using a Tecan Spectra Fluor microplate reader. Pseudo first-order inactivation rate constants were obtained from plots of ln v_t/v_o versus time.

The incubation method was also used for commercially available papain. The inhibition incubation buffer for papain was 50 mM Hepes buffer at pH 7.5, containing 2.5 mM DTT (dithiothreitol) and 2.5 mM EDTA. The assay used the substrate Cbz-Phe-Arg-*p*NA (53.7 μM) in the same buffer. The approximate concentration of papain added to the incubation buffer was 0.29 mg/mL. The release of *p*-nitroanilide was monitored at 405 nm with a Molecular Devices Thermomax microplate reader. All assays were run in duplicate.

4.1.2. Calpain assays. Calpain I was purchased from Calbiochem (La Jolla, CA) in a solution of 30% glycerol at a concentration of 6.96 μM and stored at −20 °C prior to use. The calpain I assay was conducted with 235 μL of a solution of 50 mM Hepes, 0.5 M $CaCl_2$, 0.5 M cysteine, at pH 7.5 (calpain I buffer), 6.5 μL of Suc-Leu-Tyr-AMC substrate solution in DMSO and 4.2 μL of the enzyme solution (6.96 μM) at 23 °C. The enzymatic activity was monitored by following the change in fluorescence for 10 min at 465 nm. The k_2 values were obtained by nonlinear regression analysis and corrected for substrate ($1 + [S]/K_M = 1.274$). All assays were run in duplicate and the standard deviation was determined.

4.1.3. Cathepsin L assays. Cathepsin L was purchased from Athens Research Technology (Athens GA) in a 20 mM malonate buffer solution at pH 5.5 with 1 mM EDTA and 400 mM NaCl with a specific activity of 4.133 U/mg. The progress of cathepsin L inhibition was conducted with 243 μL of a solution of 0.25 M NaAc, 2 mM EDTA, 0.015% Brij, 5 mM DTT (dithiothreitol) at pH 5.5, 4 μL of Cbz-Phe-Arg-AMC substrate solution (16 mM) in DMSO (2% final concentration). Activity was monitored by following the change in fluorescence for 20 min at 465 nm. The k_{obs} values were obtained by nonlinear regression analysis and corrected for substrate ($1 + [S]/K_M = 1.274$). All assays were run in duplicate and the standard deviation was determined.

4.1.4. Cruzain and rhodesain IC_{50} determinations. Inhibitors were screened for effectiveness against purified recombinant cruzain[53] and rhodesain.[48] Cruzain (2 nM) or rhodesain (3 nM) was incubated with 0.0001 to 1 μM inhibitor in 100 mM sodium acetate buffer, pH 5.5 and 5 mM DTT (buffer A), for 5 min at room temperature. Buffer A containing Cbz-Phe-Arg-AMC (Bachem, K_M = 1 μM, AMC = 7-amino-4-methylcoumarin) was added to the enzyme and inhibitor to give 20 or 10 μM substrate concentration for cruzain and rhodesain, respectively, in 200 μL. The increase in fluorescence (excitation at 355 nm and emission at 460 nm) was followed with an automated microtiter plate spectrofluorimeter (Molecular Devices, Flex Station). Inhibitor stock solutions were prepared at 20 mM in DMSO and serial dilutions were made in DMSO (0.7% DMSO in assay). Controls were performed using enzyme alone, and enzyme with DMSO. IC_{50} values were determined graphically using inhibitor concentrations in the linear portion of a plot of enzyme activity versus log[*I*] (7 concentrations tested with at least 2 in the linear range).

4.1.5. Diethyl phenylsulfonylmethanephosphonate (2, $PhSO_2CH_2PO(OEt)_2$). A mixture of chloromethyl phenyl sulfide (3.2 g, 20 mmol) and triethyl phosphite (3.3 g, 20 mmol) was heated at 130–140 °C for 5 h. The resultant mixture was distilled under reduced pressure, and the starting materials were distilled out. The oily residue was diethyl phenylmercaptomethanephosphonate, yield 3.4 g (65%). ^{1}H NMR ($CDCl_3$) δ 1.2–1.3 (t, 6H, $2 \times CH_3$), 3.1–3.2 (d, 2H, S–CH_2), 4.0–4.2 (m, 4H, $2 \times CH_2$), 7.2–7.4 (m, 5H, Ph). Diethyl phenylsulfonylmethanephosphonate was prepared by oxidation of diethyl phenylmercaptomethanephosphonate with potassium permanganate, yield 55%. ^{1}H NMR ($CDCl_3$) δ 1.2–1.3 (t, 6H, $2 \times CH_3$), 3.7–3.8 (d, 2H, SO_2–CH_2), 4.1–4.2 (m, 4H, $2 \times CH_2$), 7.5–7.7 (m, 3H, Ph), 7.9–8.0 (d, 2H, Ph). MS *m*/*z* 293 (M+1).

4.1.6. General procedure for mixed anhydride coupling. 2-(*tert*-butoxycarbonylamino)-3-phenylpropionaldehyde (1, Boc-Phe-H). *N*-Methylmorpholine (2 mmol) was added to Boc-Phe-OH in CH_2Cl_2 at −15 °C followed by isobutyl chloroformate. *N*-Methylmorpholine (2 mmol) was added to a cooled solution (−15 °C) of *N*,*O*-dimethylhydroxylamine hydrochloride (2 mmol) in CH_2Cl_2. This solution was added to the Boc-Phe-OH mixture, which had been stirring at −15 °C. The mixture was continued to stir at −15 °C for 30 min, then warmed to room temperature and continued to stir over night. The amount of solvent was doubled, then washed with citric acid (10%, 3×50 mL), saturated $NaHCO_3$ (3×50 mL), and brine (3×50 mL), and finally dried ($MgSO_4$). The solvent was evaporated to give Boc-Phe-N(OCH_3)CH_3 as a pure white solid in good yield (93%). ^{1}H NMR ($CDCl_3$) δ 1.4 (s, 9H, Boc), 2.8–2.9 (m, 1H, CH_2–Phe),

3.0–3.1 (m, 1H, CH_2–Phe), 3.2 (s, 3H, N-CH_3), 3.6 (s, 3H, O–CH_3), 4.9 (m, 1H, α-H), 5.2 (b, 1H, NH), 7.1–7.3 (m, 5H, Ph). MS (FAB^+) *m/z* 309 (M+1, 30%), 253 (M−*t*Bu+1, 100%). Reduction of Boc-Phe-$N(OCH_3)CH_3$ with lithium aluminum hydride according to a previously described method[54] gave Boc-Phe-H, yield 88%. ^1H NMR ($CDCl_3$) δ 1.4 (s, 9H, Boc), 3.1 (d, 2H, CH_2–Phe), 4.4 (m, 1H, α-H), 5.0 (b, 1H, NH), 7.1–7.3 (m, 5H, Ph), 9.6 (s, 1H, CHO). MS (FAB^+) *m/z* 250 (M+1, 15%), 150 (M−Boc+1, 100%).

4.1.7. Phenyl (3*S*)-3-amino-4-phenylbut-1-enyl sulfone hydrochloride (4, Phe-VS-Ph · HCl). Boc-Phe-VS-Ph was prepared by reaction of Boc-Phe-H with diethyl phenylsulfonylmethanephosphonate in the presence of 2 N sodium methoxide, yield 85%. ^1H NMR ($CDCl_3$) 1.3–1.4 (s, 9H, Boc), 2.9 (d, 2H, CH_2–Phe), 4.4–4.5 (b, 1H, α-H), 4.6–4.7 (b, 1H, NH), 6.3 (d, 1H, CH=), 6.9–7.0 (dd, 1H, CH=), 7.1–7.3 (m, 5H, Ph), 7.5–7.8 (m, 5H, SO_2–Ph). MS (FAB^+) *m/z* 388 (M+1, 15%), 288 (M−Boc+1, 100%). Boc-Phe-VS-Ph was deblocked with 6.7 N HCl in EtOAc to give Phe-VS-Ph · HCl, yield 88%. ^1H NMR (DMSO-d_6) δ 2.9–3.0 (m, 1H, CH_2–Phe), 3.1–3.2 (m, 1H, CH_2–Phe), 4.2 (b, 1H, α-H), 6.7–6.8 (m, 2H, CH=), 7.1–7.3 (m, 6H, CH= and Ph), 7.6–7.8 (m, 5H, SO_2–Ph), 8.6–8.8 (b, 2H, NH_2). MS (FAB^+) *m/z* 288 (M−Cl, 100%).

4.1.8. Phenyl (3*R*)-3-amino-4-phenylbut-1-enyl sulfone hydrochloride (D-Phe-VS-Ph · HCl). Boc-D-Phe-VS-Ph was prepared by reaction of Boc-D-Phe-H with diethyl phenylsulfonylmethanephosphonate in the presence of 2 N sodium methoxide, yield 85%. ^1H NMR ($CDCl_3$) 1.3–1.4 (s, 9H, Boc), 2.9 (d, 2H, CH_2–Phe), 4.4–4.5 (b, 1H, α-H), 4.6–4.7 (b, 1H, NH), 6.3 (d, 1H, CH=), 6.9–7.0 (dd, 1H, CH=), 7.1–7.3 (m, 5H, Ph), 7.5–7.8 (m, 5H, SO_2–Ph). MS (FAB^+) *m/z* 388 (M+1, 15%), 288 (M−Boc+1, 100%). Boc-D-Phe-VS-Ph was deblocked with 6.7 N HCl in EtOAc to give D-Phe-VS-Ph · HCl, yield 88%. ^1H NMR (DMSO-d_6) δ 2.9–3.0 (m, 1H, CH_2–Phe), 3.1–3.2 (m, 1H), 4.2 (b, 1H, α-H), 6.7–6.8 (m, 2H, CH=), 7.1–7.3 (m, 6H, CH= and Ph), 7.6–7.8 (m, 5H, SO_2–Ph), 8.6–8.8 (b, 2H, NH_2). MS (FAB^+) *m/z* 288 (M−Cl, 100%).

4.1.9. Phenyl (3*S*)-3-(*N*-carbobenzyloxyleucyl)amino-4-phenylbut-1-enyl sulfone (6c, Cbz-Leu-Phe-VS-Ph). Cbz-Leu-Phe-VS-Ph was prepared from Cbz-Leu-OH and Phe-VS-Ph · HCl using standard mixed anhydride coupling method, yield 82%. ^1H NMR ($CDCl_3$) δ 0.8–0.9 (2d, 6H, 2 × Leu–CH_3), 1.4–1.6 (m, 2H, Leu–CH_2), 2.06 (m, 1H, Leu–CH), 2.9–3.0 (m, 2H, CH_2–Phe), 3.9–4.0 (m, 1H, α-H), 4.8–4.9 (b, 1H, NH), 4.9–5.0 (m, 1H, α-H), 5.1 (m, 2H, Cbz) 6.3–6.4 (d and b, 2H, NH and CH=), 6.9–7.0 (dd, 1H, CH=), 7.1–7.4 (m, 10H, 2 × Ph), 7.5–7.7 (m, 5H, SO_2–Ph). MS (ESI) *m/z* 535.

4.1.10. Phenyl 3-(*N*-carbobenzyloxyleucyl)amino-4-phenylbut-2-enyl sulfone (7c, Cbz-Leu-Phe-AS-Ph). Butyllithium (3.63 mL, 6.17 mmol, 1.7 M in pentane) was added dropwise to a solution of *tert*-butylhydroperoxide (2.55 mL, 8.42 mmol, 3.3 M in toluene) in freshly distilled THF (80 mL) at −78 °C under argon. A solution of Cbz-Leu-Phe-VS-Ph (3.00 g, 5.61 mmol) in dry THF (30 mL) was added dropwise. The reaction was continued to stir at −20 °C for 45 min (TLC hexane/EtOAc 1:1). The reaction mixture was quenched with saturated aqueous ammonium chloride (50 mL) and allowed to warm to room temperature. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (3 × 30 mL). The extracts were then washed with aqueous sodium sulfite (10%, 3 × 20 mL). The combined organic layers were dried ($MgSO_4$) and evaporated to give Cbz-Leu-Phe-AS-Ph as a white powder, yield 63%. ^1H NMR ($CDCl_3$) δ 0.8–0.9 (2d, 6H, 2 × Leu–CH_3), 1.4–1.6 (m, 2H, Leu–CH_2), 3.8 (s, 2H, CH_2–Phe), 3.9 (d, 2H, CH_2–SO_2), 4.1 (m, 1H, α-H), 4.8 (t, 1H, CH=), 4.9–5.0 (b, 1H, NH), 5.1 (m, 2H, Cbz), 7.1–7.4 (m, 10H, 2 × Ph), 7.5–7.7 (m, 5H, SO_2–Ph), 8.4 (b, 1H, NH). ^{13}C NMR (400 MHz, $CDCl_3$) δ 172.1, 156.3, 144.4, 140.8, 135.7, 134.2, 133.6, 131.2, 129.5, 129.4 x 2, 129.3, 128.8 × 2, 128.7, 128.6, 128.4, 128.3, 127.8, 127.3, 126.9, 106.3, 67.6, 56.0, 55.6, 41.7, 40.4, 22.1, 21.0, 18.4. MS (FAB^+) *m/z* 535 (M+1, 100%). Anal. Calcd for $C_{30}H_{34}N_2O_5S$: C, 67.39; H, 6.56; N, 5.43. Found: C, 67.41; H, 6.56; N, 5.43.

4.1.11. Phenyl (3*R*)-3-(*N*-carbobenzyloxyleucyl)amino-4-phenylbut-1-enyl sulfone (6c, Cbz-Leu-D-Phe-VS-Ph). Cbz-Leu-D-Phe-VS-Ph was prepared from Cbz-Leu-OH and D-Phe-VS-Ph · HCl using standard mixed anhydride coupling method, yield 81%. ^1H NMR ($CDCl_3$) δ 0.8–0.9 (2d, 6H, 2 × Leu–CH_3), 1.4–1.6 (m, 2H, Leu–CH_2), 2.06 (m, 1H, Leu–CH), 2.9–3.0 (m, 2H, CH_2–Phe), 3.9–4.0 (m, 1H, α-H), 4.8–4.9 (b, 1H, α-H), 4.9–5.0 (m, 1H, NH), 5.1 (m, 2H, Cbz), 6.3–6.4 (d and b, 2H, NH and CH=), 6.9–7.0 (dd, 1H, CH=), 7.1–7.4 (m, 10H, 2 × Ph), 7.5–7.7 (m, 5H, SO_2–Ph). MS (ESI) *m/z* 535.

4.1.12. Phenyl 3-(*N*-carbobenzyloxyleucyl)amino-4-phenylbut-2-enyl sulfone (7c, Cbz-Leu-Phe-AS-Ph) (isomer B). Cbz-Leu-D-Phe-VS-Ph was treated with butyllithium and *tert*-butylhydroperoxide in freshly distilled THF as described with Cbz-Leu-Phe-VS-Ph above to give Cbz-Leu-Phe-AS-Ph (isomer B) as a white powder, yield 15%. ^1H NMR ($CDCl_3$) δ 0.8–0.9 (2d, 6H, 2 × Leu–CH_3), 1.4–1.6 (m, 2H, Leu–CH_2), 2.06 (m, 1H, Leu–CH), 3.8 (s, 2H, CH_2–Phe), 3.9 (d, 2H, CH_2–SO_2), 4.1 (m, 1H, α-H), 4.8 (t, 1H, CH=), 4.9–5.0 (b, 1H, NH), 5.1 (m, 2H, Cbz), 7.1–7.4 (m, 10H, 2 × Ph), 7.5–7.7 (m, 5H, SO_2–Ph); 8.4 (b, 1H, NH). MS (ESI) *m/z* 535 (M+1, 100%). Anal. Calcd for $C_{30}H_{34}N_2O_5S$: C, 67.39; H, 6.56; N, 5.43. Found: C, 67.12; H, 6.61; N, 5.33.

4.1.13. Phenyl (3*S*)-3-(*N*-carbobenzyloxyvalyl)amino-4-phenylbut-1-enyl sulfone (6b, Cbz-Val-Phe-VS-Ph). Cbz-Val-Phe-VS-Ph was prepared from Cbz-Val-OH and Phe-VS-Ph · HCl using standard mixed anhydride coupling method, yield 98%. ^1H NMR ($CDCl_3$) δ 0.70–0.84 (2d, 6H, Val-CH_3), 2.06 (m, 1H, Val-CH), 2.91 (m, 2H, CH_2–Phe), 3.82 (m, 1H, α-H), 4.80–4.90 (m, 1H, α-H), 5.10 (s, 2H, Cbz), 5.96 (d, 1H, NH) 6.34 (d, 1H, CH=), 6.9–7.0 (dd, 1H, CH=), 7.1–7.4 (m, 10H, 2 × Ph), 7.5–7.7 (m, 5H, SO_2–Ph). MS (ESI) *m/z* 521 (M+1, 100%).

4.1.14. Phenyl 3-(*N*-carbobenzyloxyvalyl)amino-4-phenylbut-2-enyl sulfone (7b, Cbz-Val-Phe-AS-Ph). Cbz-Val-Phe-VS-Ph was treated with butyllithium and *tert*-butylhydroperoxide in freshly distilled THF as described with Cbz-Leu-Phe-VS-Ph above to give Cbz-Val-Phe-AS-Ph as a white powder, yield 21%. ^{1}H NMR ($CDCl_3$) δ 0.70–0.85 (2d, 6H, Val–CH_3), 2.1 (m, 1H, Val–CH), 3.80 (d, 2H, CH_2–Phe), 3.95 (m, 3H, CH_2–SO_2 and α-H), 4.90 (t, 1H, CH=), 5.12 (m, 3H, NH and Cbz), 7.1–7.4 (m, 10H, 2 × Ph), 7.55 (t, 2H, SO_2–Ph), 7.65 (t, 1H, SO_2–Ph), 7.91 (d, 2H, SO_2–Ph), 8.42 (b, 1H, NH). ^{13}C NMR (300 MHz, $CDCl_3$) δ 172.1, 156.3, 144.4, 140.8, 135.7, 134.2, 133.6, 131.2, 129.5, 129.4 × 2, 129.3, 128.8 × 2, 128.7, 128.6, 128.4, 128.3, 127.8, 127.3, 126.9, 106.3, 67.6, 56.0, 55.6, 41.7, 22.0, 21.0, 18.4. MS (FAB^+) *m/z* 521 (M+1, 100%). Anal. Calcd for $C_{29}H_{32}N_2O_5S \cdot 1/10\ H_2O$: C, 66.67; H, 6.21; N, 5.36. Found: C, 66.31; H, 6.23; N, 5.26.

4.1.15. Phenyl (3*S*)-3-(*N*-carbobenzyloxyalanyl)amino-4-phenylbut-1-enyl sulfone (6a, Cbz-Ala-Phe-VS-Ph). Cbz-Ala-Phe-VS-Ph was prepared from Cbz-Ala-OH and Phe-VS-Ph · HCl using standard mixed anhydride coupling method, yield 88%. ^{1}H NMR ($CDCl_3$) δ 1.25 (d, 3H, Ala-CH_3), 2.91 (dq, 2H, CH_2–Phe), 4.10 (m, 1H, α-H), 4.80–4.90 (m, 1H, α-H), 5.10 (s, 2H, Cbz), 6.34 (b, 1H, NH), 6.39 (d, 1H, CH=), 6.90–6.98 (dd, 1H, CH=), 7.10–7.39 (m, 10H, 2 × Ph), 7.55 (t, 2H, SO_2–Ph), 7.65 (t, 1H, SO_2–Ph), 7.91 (d, 2H, SO_2–Ph). ^{13}C NMR (400 MHz, $CDCl_3$) δ 172.1, 156.3, 144.4, 140.8, 135.7, 134.2, 133.6, 131.2, 129.5, 129.4 × 3, 129.3, 128.8 × 2, 128.7, 128.6, 128.4, 128.3, 127.8, 127.3, 126.9, 67.6, 51.7, 51.6, 40.2, 19.4.

4.1.16. Phenyl 3-(*N*-carbobenzyloxyalanyl)amino-4-phenylbut-2-enyl sulfone (7a, Cbz-Ala-Phe-AS-Ph). Cbz-Ala-Phe-VS-Ph was treated with butyllithium and *tert*-butylhydroperoxide in freshly distilled THF as described with Cbz-Leu-Phe-VS-Ph above to give Cbz-Ala-Phe-AS-Ph as a white powder, yield 93%. ^{1}H NMR ($CDCl_3$) δ 1.21 (d, 3H, Ala–CH_3), 3.80 (m, 4H, CH_2–Phe and CH_2–SO_2), 4.12 (q, 1H, α-H), 4.88 (t, 1H, CH=), 5.11 (m, 3H, NH and Cbz), 7.30 (m, 10H, 2 × Ph), 7.50 (t, 2H, SO_2–Ph), 7.64 (t, 1H, SO_2–Ph), 7.83 (d, 2H, SO_2–Ph), 8.40 (b, 1H, NH). ^{13}C NMR (400 MHz, $CDCl_3$) δ 172.1, 156.3, 144.4, 140.8, 135.7, 134.2, 133.6, 131.2, 129.5, 129.4 × 2, 129.3, 128.8 × 2, 128.7, 128.6, 128.4, 128.3, 127.8, 127.3, 126.9, 107.2, 67.6, 51.7, 51.6, 40.2, 19.4. MS (FAB^+) *m/z* 493 (M+1, 100%). HRMS calcd for $C_{27}H_{29}N_2O_5S$: 493.17972. Obsd. 493.17663. Anal. Calcd for $C_{27}H_{28}N_2O_5S$: C, 65.83; H, 5.73; N, 5.69. Found: C, 65.73; H, 5.82; N, 5.68.

4.1.17. *N*-Benzyloxycarbonylleucylalanine methyl ester (Cbz-Leu-Ala-OMe). Cbz-Leu-Ala-OMe was prepared from Cbz-Leu-OH and Ala-OMe using standard mixed anhydride coupling, yield 93%. ^{1}H NMR ($CDCl_3$) δ 0.90 (d, 6H, Leu–CH_3), 1.41 (d, 3H, Ala–CH_3), 1.58 (m, 1H, Leu–CH), 1.62 (m, 2H, Leu–CH_2), 3.88 (s, 3H, OCH_3), 4.21 (m, 1H, α-H), 4.59 (m, 1H, α-H), 5.11 (s, 2H, Cbz), 5.23 (b, 1H, NH), 6.45 (b, 1H, NH), 7.31 (m, 5H, Ph).

4.1.18. *N*-Benzyloxycarbonylleucylalanine (Cbz-Leu-Ala-OH). Cbz-Leu-Ala-OMe (4.23 g, 12.2 mmol) was suspended in methanol at room temperature. Aqueous sodium hydroxide (14.69 mL, 1 N, 14.69 mmol) was added. The reaction was stirred for 1 h (TLC Hex/EtOAc 1:1). The reaction mixture was cooled to 0 °C and acidified with 1 N HCl (pH 2). The mixture was extracted with ethyl acetate (3 × 50 mL). The organic layer was washed with water (3 × 20 mL) and brine (3 × 20 mL) and dried ($MgSO_4$). The solvent was evaporated to give Cbz-Leu-Ala-OH as a white powder, yield 85%. ^{1}H NMR ($CDCl_3$) δ 0.90 (d, 6H, Leu–CH_3), 1.41 (d, 3H, Ala–CH_3), 1.59 (m, 1H, Leu–CH), 1.63 (m, 2H, Leu–CH_2), 4.30 (m, 1H, α-H), 4.59 (m, 1H, α-H), 5.11 (s, 2H, Cbz), 5.60 (b, 1H, NH), 6.97 (b, 1H, NH), 7.31 (m, 5H, Ph).

4.1.19. *N*-(*N*-Benzyloxycarbonylleucylalanyl)-*N*-2-ethanolamine (Cbz-Leu-Ala-NH(CH$_2$)$_2$OH). Cbz-Leu-Ala-OH was coupled to ethyl hydroxyl amine using standard mixed anhydride coupling to give crude Cbz-Leu-Ala-$NH(CH_2)_2OH$, which was recrystallized from cold ethyl acetate to give a white powder, yield 63%. ^{1}H NMR ($CDCl_3$) δ 0.90 (d, 6H, Leu–CH_3), 1.41 (d, 3H, Ala–CH_3), 1.58 (m, 1H, Leu–CH), 1.62 (m, 2H, Leu–CH_2), 2.92 (b, 1H, OH), 3.38 (m, 1H, NHCH_2), 3.41 (m, 1H, NHCH_2), 3.69 (d, 2H, CH_2OH), 4.13 (m, 1H, α-H), 4.43 (m, 1H, α-H), 5.11 (s, 2H, Cbz), 5.19 (b, 1H, NH), 6.45 (b, 1H, NH), 6.79 (b, 1H, NH), 7.31 (m, 5H, Ph).

4.1.20. 2-(1-Benzyloxycarbonylleucylaminoethyl)oxazoline (11, Cbz-Leu-Ala-oxazoline). Cbz-Leu-Ala-$NH(CH_2)_2OH$ (1.15 g, 3.02 mmol) was dissolved in THF (20 mL). Triphenyl phosphine (1.19 g, 4.54 mmol) was added followed by dropwise addition of diisopropylazadicarboxylate (0.92 g, 4.54 mmol). The reaction mixture was stirred for 2 h at room temperature. The solvent was evaporated, and the crude oil was subjected to column chromatography (silica, MeOH/CH_2Cl_2 7%) to give Cbz-Leu-Ala-oxazoline as a white powder, yield 75%. ^{1}H NMR ($CDCl_3$) δ 0.90 (d, 6H, Leu–CH_3), 1.41 (d, 3H, Ala-CH_3), 1.58 (m, 1H, Leu–CH), 1.69 (m, 2H, Leu–CH_2), 3.83 (t, 2H, NCH_2), 4.19 (m, 1H, α-H), 4.36 (t, 2H, OCH_2), 4.62 (m, 1H, α-H), 5.11 (s, 2H, Cbz), 5.19 (b, 1H, NH), 6.62 (b, 1H, NH), 7.36 (m, 5H, Ph). MS (FAB^+) *m/z* 362 (M+1, 100%). HRMS calcd for $C_{19}H_{28}N_3O_4$: 362.20813. Obsd. 362.20798. Anal. Calcd for $C_{19}H_{27}N_3O_4 \cdot 1/4\ H_2O$: C, 62.36; H, 7.57; N, 11.48. Found: C, 62.22; H, 7.55; N, 11.64.

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