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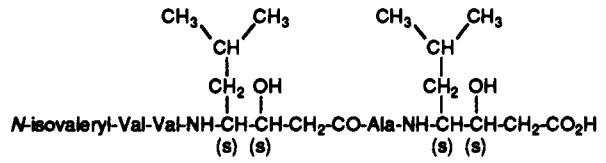
New Pepstatin Analogues: Synthesis and Pepsin Inhibition

Since the isolation of pepstatin, isovaleryl-L-valyl-L-valyl-(3*S*,4*S*)-statyl-L-alanyl-(3*S*,4*S*)-statine (1), by Umezawa,[1,2] investigators have pursued the development and biochemical study of proteinase inhibitors similar in structure to pepstatin.[3-11] The recent discovery of the aspartyl proteinase of the human immunodeficiency virus 1, (HIV-1), and its inhibition by pepstatin, has increased the search for synthetic analogues of pepstatin with increased substrate specificities.[12-16] However, the therapeutic value of pepstatin is limited.



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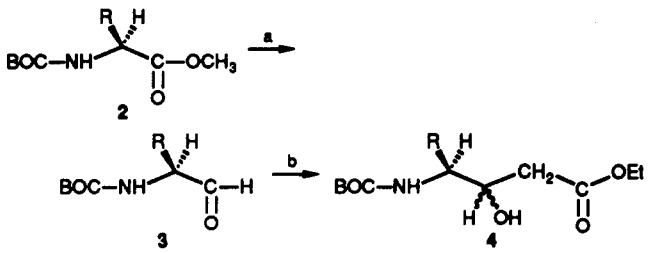
Scheme I[a]



R = $CH_2CH(CH_3)_2$, $CH_2C_6H_5$, $CH_2C_6H_4OH$, $CH_2CH_2CH_2CH_3$

[a] (a) Diisobutylaluminum hydride, toluene, −78 °C; (b) ethyl acetate is pretreated with lithium diisopropylamide in THF at −78 °C for 30 min followed by addition of aldehyde in THF, 2 h at −78 °C.

For a proteinase inhibitor to have therapeutic value, it should be chemically stable, active as low concentrations, selective for a particular proteinase in the presence of other proteinases with similar substrate specificities, and should readily penetrate cell membranes. Pepstatin satisfies the first two of these criteria. The third criterion, specificity, is satisfied only at the level of the major mechanistic class. Pepstatin, while a very effective inhibitor of aspartyl proteinases, is not very selective within this class of enzymes. Pepstatin is also known not to penetrate well cell membranes due to its size and lipophilicity.

The exact mode of inhibition of many aspartyl proteinases by pepstatin has not been well documented. However, crystal structure studies of the HIV-1 aspartyl proteinase inhibited by pepstatin show that the statine residue occupies both the P_1 and P_1' sites, acting as a dipeptide in the bound enzyme.[17] Several studies have also been conducted concerning the nature of inhibition of other

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Table I. Physical Constants for Tripeptide Analogues

no.	compound	mp, °C	$[\alpha]^{25}_D$, deg (c 1.0, CH_3OH)	molecular formula	anal.
5a	Cbz-Val-Val-(*S*,*S*)-AHMHA[a]	201–203	−70.3	$C_{26}H_{41}N_3O_7$	C,H,N
5b	Cbz-Val-Val-(*R*,*S*)-AHMHA	216–218	−49.5	$C_{26}H_{41}N_3O_7$	C,H,N
6a	Cbz-Val-Val-(*S*,*S*)-AHPPA[b]	168–170	−37.9	$C_{29}H_{39}N_3O_7$	C,H,N
6b	Cbz-Val-Val-(*R*,*S*)-AHPPA	189–191	−27.5	$C_{29}H_{39}N_3O_7$	C,H,N
7a	Cbz-Val-Val-(*S*,*S*)-AHHPPA[c]	197–200	−73.5	$C_{29}H_{39}N_3O_8$	C,H,N
7b	Cbz-Val-Val-(*R*,*S*)-AHHPPA	217–219	−52.3	$C_{29}H_{39}N_3O_8$	C,H,N
8a	Cbz-Val-Val-(*S*,*S*)-AHOA[d]	218–220	−36.2	$C_{26}H_{41}N_3O_7$	C,H,N
8b	Cbz-Val-Val-(*R*,*S*)-AHOA	232–235	−30.3	$C_{26}H_{41}N_3O_7$	C,H,N

[a] AHMHA = 4-amino-3-hydroxy-6-methylheptanoic acid. [b] AHPPA = 4-amino-3-hydroxy-5-phenylpentanoic acid. [c] AHHPPA = 4-amino-3-hydroxy-5-*p*-hydroxyphenylpentanoic acid. [d] AHOA = 4-amino-3-hydroxyoctanoic acid.

aspartyl proteinases by pepstatin and its fragments. The novel amino acid statine, (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA), appears to be the major structural component responsible for the inhibitory activity of pepstatin.[18] It has also been suggested that the shape of the side chain of the statine unit greatly influences the inhibitory activity. For example, Gunn et al. found peptide analogues containing a statine unit derived from L-valine to be much less active pepsin inhibitors than similar analogues containing statine units derived from L-leucine.[10]

In this communication we now report the preparation and determination of pepsin inhibition of eight tripeptide analogues of pepstatin. Since pepstatin is known not to penetrate cell membranes as readily as small peptides, such as leupeptin, we have synthesized tripeptide analogues of pepstatin. This was done in an effort to find a small peptide analogue with inhibitory activity comparable to that of pepstatin.

Synthesis. The preparation of the statine derivatives **4** is outlined in Scheme I. The *tert*-butyloxycarbonyl (BOC) protected amino acid methyl esters **2** were reduced directly to the aldehydes **3** with diisobutylaluminum hydride in toluene for 10 min at −78 °C. The reactions were then worked up with methylene chloride and Rochelle salt to isolate the aldehydes **3** in 65 to 90% yield. The aldehydes were then condensed with the lithium enolate of ethyl acetate at −78 °C to give the ethyl esters as mixtures of diastereomers in 70–85% yields. The lithium enolate of ethyl acetate was generated from lithium diisopropylamide (Aldrich) and ethyl acetate as a modification of Steulmann and Klostermeyer's method.[19] The diastereomers were separated via high-performance liquid chromatography. Normal-phase silica gel columns were used in conjunction with SSI Model 232D gradient liquid chromatograph utilizing both UV and refractive index detection. The stereochemistry was assigned by ^1H NMR with use of the chemical shifts and coupling constants of the third and fourth carbons of the 2-oxazolidinone derivatives in comparison to similar known data.[13,20–22]

Table II. Inhibition of Pepsin Hydrolysis of Hemoglobin by Pepstatin and Analogues

no.	compound	IC_{50}, μM	K_i(app), μM
1	pepstatin	0.007	4.60×10^{-11}
5a	Cbz-Val-Val-(3*S*,4*S*)-AHMHA[a]	0.20	1.90×10^{-7}
5b	Cbz-Val-Val-(3*R*,4*S*)-AHMHA	39.4	3.94×10^{-5}
6a	Cbz-Val-Val-(3*S*,4*S*)-AHPPA[b]	0.42	4.10×10^{-7}
6b	Cbz-Val-Val-(3*R*,4*S*)-AHPPA	346	3.46×10^{-4}
7a	Cbz-Val-Val-(3*S*,4*S*)-AHHPPA[c]	1130	1.13×10^{-3}
7b	Cbz-Val-Val-(3*R*,4*S*)-AHHPPA	1540	1.54×10^{-3}
8a	Cbz-Val-Val-(3*S*,4*S*)-AHOA[d]	0.010	3.10×10^{-9}
8b	Cbz-Val-Val-(3*R*,4*S*)-AHOA	17.7	1.77×10^{-5}

[a] AHMHA = 4-amino-3-hydroxy-6-methylheptanoic acid. [b] AHPPA = 4-amino-3-hydroxy-5-phenylpentanoic acid. [c] AHHPPA = 4-amino-3-hydroxy-5-(*p*-hydroxyphenyl)pentanoic acid. [d] AHOA = 4-amino-3-hydroxyoctanoic acid.

Removal of the *tert*-butoxycarbonyl protecting groups was achieved with 30% trifluoroacetic acid in chloroform. The trifluoroacetate salts of the statine ethyl ester derivatives were then condensed with the mixed anhydride of carbobenzoxy-L-valyl-L-valine to give the Cbz-protected tripeptide ethyl esters in 40–70% yield. Saponification of the ethyl esters then provided the Cbz-protected tripeptide analogues (**5**–**8**) of pepstatin. The *N*-carbobenzoxy protecting group was used on the pepstatin analogues rather than the *N*-isovaleryl protecting group because Cbz-L-valyl-L-valyl-(3*S*,4*S*)-statine has been reported to be a slightly better inhibitor of pepsin than *N*-isovaleryl-L-valyl-L-valyl-(3*S*,4*S*)-statine.[23] Physical constants for **5**–**8** are found in Table I.

Pepsin Assay. The potency of the synthetic pepstatin analogues was measured as inhibitors of the peptic hydrolysis of hemoglobin (Sigma), and the results are presented in Table II. Inhibition of porcine pepsin (Sigma) was measured by the following method: 0.9 mL of the inhibitor of appropriate concentration in 0.02 M KCl–HCl buffer (pH 2.0) and 1.0 mL of 0.5% hemoglobin solution were mixed and incubated for 3 min at 37 °C. Porcine pepsin, 100 μL of a 10 μg/mL solution, was added and mixed. The incubator was continued for 27 min at 37 °C. After this, 2.0 mL of 1.5 M perchloric acid was added to quench the reaction. The mixtures were allowed to warm to room temperature for 1 h. The precipitated protein was centrifuged and the absorbance measured at 280 nm. The absorbance of a blank containing no enzyme was subtracted from each reading. The inhibition of the enzyme activity was measured four times at five or more inhibitor concentrations. The average absorbance at each inhibitor concentration was utilized in calculations of the IC_{50} values. All absorbance values were ≤0.002 standard deviations from the mean for a given inhibitor concentration; thus the relative error is minimal. The standard error for the linear regression plots was calculated and is in each case

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less than 5%. A plot of percent inhibition versus the log of the inhibitor concentration provided a value for 50% inhibition concentration (IC_{50}). All plots are linear through the 50% inhibition value and have slopes ranging from 15 to 41. The apparent inhibition constants (K_i (app) were calculated as K_i (app) = $IC_{50} - 0.5[E]_0$, where $[E]_0$ is the enzyme concentration.

Results and Conclusions

The eight Cbz-protected tripeptide analogues were compared to pepstatin as inhibitors of the pepsin hydrolysis of hemoglobin (Table II). On comparing the activities of Cbz-Val-Val-AHMHA with that of pepstatin, it was found that the length of the peptide chain alters the inhibitor activity. This agrees with data reported in the literature.[10,22,24] Also, as expected, the stereochemistry of carbon-3, bearing the hydroxyl group, is very important to the activity of the inhibitors. The 3*S*,4*S* diastereomers was in each case shown to be a better inhibitor of the peptic hydrolysis of hemoglobin than the 3*R*,4*S* diastereomer.

Modifications made in the side chain of the novel amino acid statine, however, had the greatest effect on the pepsin inhibition. Analogues containing a statine unit derived from L-phenylalanine (**6a,b**) were significantly less active as pepsin inhibitors, and very little inhibition of pepsin activity is shown by the analogues containing a statine unit derived from L-tyrosine (**7a,b**). This is indicative of steric restrictions within the enzyme pepsin. Interestingly, it was also found that the analogues containing a statine unit derived from L-norleucine (**8a,b**) displayed a better inhibition of pepsin activity than those analogues containing statine units derived from L-leucine.

In view of the preliminary success achieved in finding a small peptide with potency similar to pepstatin for pepsin inhibition, further modifications to improve hydrophilicity of aspartyl proteinase inhibitors are warranted. Further synthesis and testing of tripeptide analogues of pepstatin is currently underway.

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Registry No. **2** (R = *i*-Bu), 63096-02-6; **2** (R = CH_2Ph), 51987-73-6; **2** (R = $CH_2C_6H_4$-*p*-OH), 4326-36-7; **2** (R = Bu), 87974-77-4; **3** (R = *i*-Bu), 58521-45-2; **3** (R = CH_2Ph), 72155-45-4; **3** (R = $CH_2C_6H_4$-*p*-OH), 134081-15-5; **3** (R = Bu), 104062-70-6; (*S*,*S*)-**4** (R = *i*-Bu), 67010-43-9; (*R*,*S*)-**4** (R = *i*-Bu), 67010-44-0; (*S*,*S*)-**4** (R = CH_2Ph), 72155-46-5; (*R*,*S*)-**4** (R = CH_2Ph), 72155-47-6; (*S*,*S*)-**4** (R = $CH_2C_6H_4$-*p*-OH), 134081-16-6; (*R*,*S*)-**4** (R = $CH_2C_6H_4$-*p*-OH), 134081-17-7; (*S*,*S*)-**4** (R = Bu), 134081-18-8; (*R*,*S*)-**4** (R = Bu), 134081-19-9; **5a**, 69980-30-9; **5b**, 69980-31-0; **6a**, 134081-20-2; **6b**, 134175-09-0; **7a**, 134081-21-3; **7b**, 134175-10-3; **8a**, 134081-22-4; **8b**, 134175-11-4; AcOEt, 141-28-6; Cbz-Val-Val-OH, 19542-54-2; pepsin, 9001-75-6.

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