Inhibitors of the Isoprenylated Protein Endoprotease†

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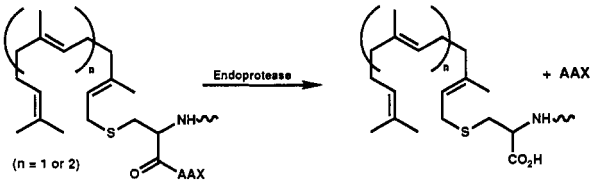
ABSTRACT: The isoprenylation pathway requires an endoprotease that cleaves the modified protein at the isoprenylated cysteine residue. This endoprotease was readily assayed with simple tetrapeptide substrates of the type *N*-acetyl-*S*-farnesyl-L-Cys-(AFC)-Val-Ile-Met, where AFC and the tripeptide are the products of the hydrolysis. The endoprotease proved to be unaffected by (1) serine protease inhibitors, including (4-amidinophenyl)methanesulfonyl fluoride, aprotinin, and leupeptin, by (2) cysteine protease inhibitors, including E-64 and leupeptin [the enzyme is, however, inhibited by *p*-(hydroxymercuri)benzoate], by (3) metalloprotease inhibitors, including phosphoramidon, EDTA, and 1,10-phenanthroline, or by (4) the aspartyl protease inhibitor pepstatin. The conclusion from these data is that the enzyme is probably not a metalloenzyme. *N*-Boc-*S-all-trans*-farnesyl-L-cysteine (BFC) derivatives containing a statine moiety are also not inhibitory, strongly suggesting that the enzyme is not an aspartyl protease. However, the enzyme is potently inhibited by the aldehyde derivative of BFC (K_I = 1.9 μM), which is consistent with the idea that the enzyme is a serine or cysteine protease. Potent tetrapeptide-based competitive inhibitors were prepared. Analogs with the scissile bond modified so that hydrolysis could not occur were excellent inhibitors. An analog containing BFC-statine-Val-Ile-Met inhibited the endoprotease with a K_I = 64 nM. The equivalent pseudopeptide $\psi(CH_2\text{-}NH)$ analog was almost as potent, indicating that the statine moiety simply represents a nonhydrolyzable linker.

Hydrophobic posttranslational modifications are important in the activation of a diverse group of proteins (James & Olson, 1990). The isoprenylation pathway is an example of this type of hydrophobic modification (Casey et al., 1989; Farnsworth et al., 1990; Hancock et al., 1989; Lai et al., 1990; Maltese, 1990; Mumby et al., 1990; Schafer et al., 1989). Proteins with a carboxyl-terminal CAAX (Casey et al., 1989; Hancock et al., 1989; Schafer et al., 1989; Lowy & Willumsen, 1990) or, less frequently, a CXC sequence (where C = cysteine, A = aliphatic amino acid, and X is an undefined amino acid) (Horiuchi et al., 1991; Farnsworth et al., 1991; Khosravi-Far et al., 1991) or a CXXX sequence (Glenn et al., 1992) are first isoprenylated at the cysteine residue(s) with either *all-trans*-farnesyl (C15) or *all-trans*-geranylgeranyl (C20) pyrophosphate (Manne et al., 1990; Reiss et al., 1990; Schaber et al., 1990). In the case of modifications at a CAAX motif, proteolysis follows, to generate the isoprenylated cysteine residue as the new carboxyl terminus (Hancock et al., 1989). This set of modifications is completed by the biologically reversible carboxymethylation of the isoprenylated cysteine residue (Seabra et al., 1991; Hancock et al., 1991; Clarke et al., 1988; Fukada et al., 1990; Gutierrez et al., 1989; Kawata et al., 1990; Perez-Sala et al., 1991; Stephenson & Clarke, 1990; Yamane et al., 1991; Yamane et al., 1990).

The various *ras* oncogene products are all farnesylated and methylated at their carboxyl-terminal cysteine residues (Hancock et al., 1991). Farnesylation is essential for the functioning of these oncogene products (Hancock et al., 1991). Proteolysis and methylation appear also to be important for efficient membrane binding of the *ras* products (Hancock et al., 1991). Thus, potent inhibitors of the enzymes in the isoprenylation pathway are of some interest.

Scheme I: Endoproteolysis in the Isoprenylation Pathway



Proteolysis occurs in mammals primarily by endoproteolytic cleavage between the modified cysteine residue and the adjacent aliphatic amino acid to liberate the intact AAX tripeptide as shown in Scheme I (Ashby et al., 1992; Ma & Rando, 1992). A liver and pancreatic microsomal endoproteolytic activity was identified that produces a single cut between the modified cysteine residue and the AAX tripeptide, using a synthetic radiolabeled tetrapeptide substrate L-AFC-Val-Ile-Ser[1] (Ma & Rando, 1992). The protease also specifically cleaves the tripeptide L-AFC-Val-Ile and the dipeptide L-AFC-Val, but not L-AFC-amide (Ma & Rando, 1992). Therefore, minimally, a dipeptide is required for substrate activity. The enzyme does not cleave substrates containing D-AFC as the first amino acid, demonstrating that the cleavage reaction is stereospecific at the scissle bond (Ma & Rando, 1992). Moreover, the isoprenyl group is essential for substrate activity, and either stereospecificity or stereoselectivity is observed at AAX (Ma et al., 1992). Thus far, no information is available on the mechanistic class that the enzyme belongs to, nor have inhibitors been prepared for it. Standard commercially available protease inhibitors designed

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[1] Abbreviations: DMSO, dimethyl sulfoxide; Boc, *tert*-butyloxycarbonyl; AFC, *N*-acetyl-*S-all-trans*-farnesyl-L-cysteine; BFC, *N*-Boc-*S-all-trans*-farnesyl-L-cysteine; DCM, dichloromethane; THF, tetrahydrofuran; Dibal, diisobutylaluminum hydride; IPCF, isopropenyl chloroformate; DMF, dimethylformamide; EDC, 1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride; HOBT, 1-hydroxybenzotriazole hydrate; NMM; *N*-methylmorpholine; DMAP, 4,4-(dimethylamino)pyridine; Val, valine; Ile, isoleucine; Met, methionine; Ser, serine.

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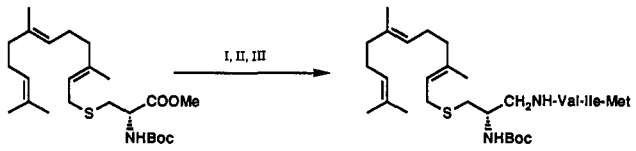
Scheme II[a]



[a] Reagents: (I) Dibal, toluene; (II) $NaBH_3CN$, Val-Ile-Met-OMe, MeOH; (III) 0.25N $Ba(OH)_2$, MeOH-H_2O (1:1).

to inhibit either serine proteases, cysteine proteases, aspartyl proteases, or metalloproteases have thus far not been shown capable of inhibiting the endoprotease. Here we present the syntheses and properties of several potent inhibitors of the endoprotease.

MATERIALS AND METHODS

Materials

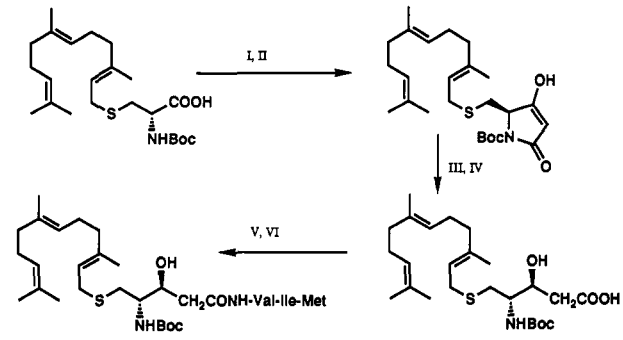
Fresh bovine calf liver was obtained from a local slaughterhouse. Amino acids and dipeptides were purchased from Bachem Bioscience Inc. Di-*tert*-butyl dicarbonate and sodium cyanoborohydride were purchased from Fluka Chemical. Ethyl bromodifluoroacetate was purchased from PCR Inc. Other reagents, unless otherwise stated, were acquired from Aldrich Chemical. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was recorded on a Varian VRX 500S spectrometer operating at a proton frequency of 499.843 MHz. Chloroform ($CDCl_3$), or dimethyl sulfoxide (DMSO-d_6) was used as the ^{1}H NMR solvent. The residual proton absorption of the deuterated solvent was used as the internal standard. HPLC solvents were from J. T. Baker, Inc. All chemicals and solvents purchased were of the highest purity available.

Methods

Syntheses. The synthesis of *N*-[^{3}H]acetyl-*S-all-trans*-farnesyl-L-Cys-L-Val-L-Ile-L-Met has been previously reported (Ma et al., 1992). *N*-Boc-*S-all-trans*-farnesyl-L-cysteine and *N*-Boc-*S-all-trans*-farnesyl-L-cysteine methyl ester were prepared according to the procedure of Brown (Brown et al., 1992). *N*-Boc-*S-all-trans*-farnesyl-L-cysteine alcohol and *N*-Boc-*S-all-trans*-farnesyl-L-cysteine aldehyde were prepared from *N*-Boc-*S-all-trans*-farnesyl-L-cysteine methyl ester by the method of McNulty and Still (1992). *N*-Boc-*S-all-trans*-farnesyl-L-cysteine methyl ketone was generated from *N*-acetyl-*S-all-trans*-farnesyl-L-cysteine (Tan et al., 1991) or from *S-all-trans*-farnesyl-L-cysteine and acetic anhydride via a Dakin–West reaction (Dakin & West, 1928). The $\psi(CH_2$-NH) pseudopeptides were prepared from *N*-Boc-*S-all-trans*-farnesyl-L-cysteine aldehyde and the corresponding amino acid/peptide by the procedure of Rodriguez et al. (1986) (Scheme II). The peptides were prepared by the procedure of Bodanszky and Bodanszky (1984). (3*S*,4*S*)-*N*-Boc-4-amino-3-hydroxy-5-(*S-all-trans*-farnesyl)pentanoic acid was synthesized from *N*-Boc-*S-all-trans*-farnesyl cysteine and Meldrum's acid by the procedure of Jouin et al. (1987) (Scheme III). (4*S*)-*N*-Boc-4-amino-2,2-difluoro-3-hydroxy-5-(*S-all-trans*-farnesyl)pentanoic acid was prepared from *N*-Boc-*S-all-trans*-farnesyl-L-cysteine aldehyde and ethyl bromodifluoroacetate by the procedure of Doherty et al. (1992) (Scheme IV). Complete experimental details of the synthetic protocols are provided as supplementary material. The ^{1}H NMR data and the chromatographic behavior of the inhibitors are described below.

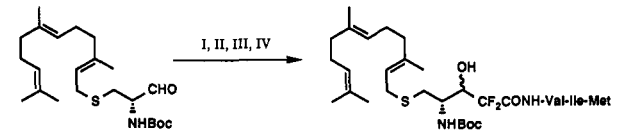
N-Boc-S-all-trans-Farnesyl-L-cysteine Alcohol (**1**). The compound was purified by silica gel chromatography (hexane/EtOAc 80:20): ^{1}H NMR ($CDCl_3$, 500 MHz) 5.24 (1 H, t, J = 8 Hz), 5.09 (2 H, t, J = 7 Hz), 4.99 (1 H, br s), 3.74 (3 H, m), 3.18 (2 H, dd, J = 2, 8 Hz), 2.68 (1 H, dd, J = 6, 13 Hz), 2.63 (1 H, dd, J = 7, 13 Hz), 2.07 (6 H, m), 1.97 (2 H, t, J = 8 Hz), 1.68 (3 H, s), 1.67 (3 H, s), 1.60 (6 H, s), 1.45 (9 H, s).

Scheme III[a]



[a] Reagents: (I) IPCF, DMAP, Meldrum's acid, DCM; (II) heat, EtOAc; (III) $NaBH_4$, DCM; (IV) 1N NaOH; (V) Val-Ile-Met-OMe, EDC, HOBT, NMM DMF; (VI) 10% Na_2CO_3, CH_3CN.

Scheme IV[a]



[a] Reagents: (I) Zn, I_2, $BrF_2CCOOEt$, THF; (II) 5% KOH, MeOH; (III) Val-Ile-Met-OMe, EDC, HOBT, NMM, DMF; (VI) 10% Na_2CO_3, CH_3CN.

N-Boc-S-all-trans-Farnesyl-L-cysteine Aldehyde (**2**). The compound was purified by silica gel chromatography (hexane/EtOAc 80:20): ^{1}H NMR ($CDCl_3$, 500 MHz) 9.67 (1 H, d, J = 8 Hz), 5.34 (1 H, br s), 5.22 (1 H, t, J = 8 Hz), 5.09 (2 H, t, J = 6 Hz), 4.49 (1 H, brs), 3.20 (2 H, m), 2.96 (1H, dd, J = 4, 13.5 Hz), 2.88 (1 H, dd, J = 5, 13.5 Hz), 2.12–2.02 (6 H, m), 1.98–1.90 (2 H, m), 1.67 (3 H, s), 1.66 (3 H, s), 1.59 (6 H, s), 1.45 (9 H, s).

N-Boc-S-all-trans-Farnesyl-L-cysteine Methyl Ketone (**3**). The compound was purified by silica gel chromatography (hexane/acetone 80:20): ^{1}H NMR ($CDCl_3$, 500 MHz) 5.34 (1 H, br s), 5.22 (1 H, t, J = 8 Hz), 5.09 (2 H, t, J = 6 Hz), 4.49 (1 H, br s), 3.20 (2 H, m), 2.96 (1 H, dd, J = 4, 13.5 Hz), 2.88 (1 H, dd, J = 5, 13.5 Hz), 2.12–2.02 (6 H, m), 1.96 (2 H, t, J = 9 Hz), 1.67 (3 H, s), 1.66 (3 H, s), 1.59 (6 H, s), 1.45 (9 H, s).

Synthesis of (3S,4S)-N-Boc-4-Amino-3-hydroxy-5-(S-all-trans-farnesyl)pentanoic Acid (**4**). The peptide was purified by silica gel chromatography (hexane/acetone 80:20): ^{1}H NMR ($CDCl_3$, 500 MHz) 5.23 (1 H, t, J = 8 Hz), 5.09 (1 H, t, J = 7 Hz), 5.01 (1 H, d, J = 9.5 Hz), 4.35 (1 H, d, J = 10 Hz), 3.66 (1 H, dd, J = 7, 15 Hz), 3.19 (2 H, d, J = 8 Hz), 2.71 (1 H, dd, J = 8, 13.5 Hz), 2.64 (2 H, m), 2.53 (1 H, dd, 3, 16.5 Hz), 2.12–1.95 (8 H, m), 1.67 (3 H, s), 1.66 (3 H, s), 1.59 (6 H, s), 1.55 (9 H, s).

Synthesis of (4S)-N-Boc-4-Amino-2,2-difluoro-3-hydroxy-5-(S-all-trans-farnesyl)pentanoic acid (**5**). The compound was purified by silica gel chroamtography (hexane/EtOAc 80:20): ^{1}H NMR (DMSO-d_6, 500 MHz) 5.17 (1 H, t, J = 7 Hz), 5.05 (2 H, t, J = 5 Hz), 4.18 (1 H, dd, J = 7, 14 Hz), 3.87 (1 H, t, J = 5 Hz), 3.14 (2 H, d, J = 6 Hz), 2.55 (1 H, dd, J = 6, 13 Hz), 2.46 (1 H, dd, J = 8, 13 Hz), 2.04 (6 H, m), 1.92 (2 H, t, J = 7 Hz), 1.68 (3 H, s), 1.67 (3 H, s), 1.57 (6 H, s), 1.38 (9 H, s).

Synthesis of N-Boc-S-all-trans-Farnesyl-L-cysteine (**6**). The peptide was purified by silica gel chromatography (hexane/acetone 80:20, 70:30): ^{1}H NMR ($CDCl_3$, 500 MHz) 5.34 (1 H, br s), 5.22 (1 H, t, J = 8 Hz), 5.09 (2 H, t, J = 6 Hz), 4.49 (1 H, br s), 3.20 (2 H, m), 2.96 (1 H, dd, J = 4, 13.5 Hz), 2.88 (1 H, dd, J = 5, 13.5 Hz), 2.12–2.02 (6 H, m), 1.96 (2 H, t, J = 9 Hz), 1.67 (3 H, s), 1.66 (3 H, s), 1.59 (6 H, s), 1.45 (9 H, s).

N-Boc-S-all-trans-Farnesyl-L-Cys-ψ(CH_2-NH)-L-Val (**7**). The peptide was purified by HPLC (Rainin silica, 250 × 4.6 mm, hexane/2-propanol/TFA (95:5:0.01) as eluant at a flow rate of 2.5 mL/min with UV detection at 210 nm). The retention time was 4.80 min: ^{1}H NMR (DMSO-d_6, 500 MHz) 8.80 (1 H, br s), 8.53 (1 H, br s), 6.71 (1 H, d, J = 8.9 Hz), 5.14 (1 H, t, J = 7.9 Hz), 5.04 (2 H, t, J = 5.9 Hz), 3.55 (1 H, br s), 3.28 (2 H, d, J = 11.5 Hz), 3.09 (1 H, d, J = 7.9), 2.83 (1 H, d, J = 5.5 Hz), 2.62–2.52 (2 H, m), 2.42–2.32 (2 H, m), 2.06–1.92 (6 H, m), 1.94–1.86 (2 H, m), 1.61 (3 H, s), 1.59 (3 H, s), 1.53 (6 H, s), 1.35 (9 H, s), 0.83 (6 H, t, J = 7 Hz).

N-Boc-S-all-trans-Farnesyl-L-Cys-ψ(CH_2-NH)-L-Val-L-Ile (**8**). The peptide was purified by HPLC (Rainin Silica, 250 × 4.6 mm, hexane/2-propanol/TFA (95:5:0.01) as eluant at a flow rate of 2.5 mL/min with UV detection at 210 nm). The retention time was 3.50 min: ^{1}H NMR (DMSO-d_6, 500 MHz) 8.84 (1 H, br s), 8.64 (1 H, d, J = 7.9 Hz). 8.57 (1 H, br s), 6.94 (1 H, d, J = 8.9 Hz), 5.16 (1 H, t, J = 7.9 Hz), 5.05 (2 H, t, J = 5.9 Hz), 4.25 (1 H, dd, J = 8, 6 Hz), 3.88–3.76 (2 H, m), 3.18 (1 H, dd, J = 13, 8 Hz), 3.11 (1 H, dd, J = 13, 8 Hz), 2.97 (1 H, br s), 2.89 (1 H, br s), 2.52 (2 H, d, J = 7 Hz), 2.28–2.16 (1 H, m), 2.06–1.92 (6 H, m), 1.94–1.88 (2 H, m), 1.86–1.80 (1 H, m), 1.61 (3 H, s), 1.59 (3 H, s), 1.53 (6 H, s), 1.38 (9 H, s), 1.22–1.19 (1 H, m), 1.15 (3 H, t, J = 7 Hz), 1.01 (3 H, d, J = 7 Hz), 0.92 (3 H, d, J = 7 Hz), 0.88–0.83 (5 H, m).

N-Boc-S-all-trans-Farnesyl-L-Cys-ψ(CH_2-NH)-L-Val-L-Ile-L-Ser (**9**). The peptide was purified by HPLC (Rainin silica, 250 × 4.6 mm, hexane/2-propanol/TFA (90:10:0.01) as eluant at a flow rate of 2.5 mL/min with UV detection at 210 nm). The retention time was 10.10 min: ^{1}H NMR (DMSO-d_6, 500 MHz) 8.86 (1 H, br s), 8.62 (1 H, br s), 8.14 (1 H, d, J = 8 Hz), 8.12 (1 H, d, J = 8 Hz), 7.85 (1 H, d, J = 8.5 Hz), 7.81 (1 H, d, J = 9.5 Hz), 5.15 (1 H, t, J = 8 Hz), 5.05 (2 H, m), 4.46 (1 H, ddd, J = 2, 6, 14.5 Hz), 4.39 (1 H, dd, J = 5.5, 9.5 Hz), 4.33 (1 H, ddd, J = 5, 10, 14 Hz), 4.23 (1 H, dd, J = 7, 8.5 Hz), 3.17 (1 H, dd, J = 8, 14 Hz), 3.12 (1 H, dd, J = 7.5, 14 Hz), 2.74 (1 H, dd, J = 6, 13.5 Hz), 2.52 (1 H, dd, J = 7.5, 13.5 Hz), 2.46–2.35 (3 H, m), 2.00 (3 H, s), 2.04–1.90 (10 H, m), 1.83 (3 H, s), 1.61 (3 H, s), 1.60 (3 H, s), 1.53 (6 H, s), 1.24 (2 H, m), 1.08 (1 H, m), 0.80 (12 H, m).

N-Boc-S-all-trans-Farnesyl-L-Cys-ψ(CH_2-NH)-L-Val-L-Ile-L-Met (**10**). The peptide was purified by HPLC (Rainin silica, 250 × 4.6 mm, hexane/2-d/propanol/TFA (97:3:0.01) as eluant at a flow rate of 1.5 mL/min with UV detection at 210 nm). The retention time was 21.20 min: ^{1}H NMR (DMSO-d_6, 500 MHz) 8.94 (1 H, br s), 8.57 (1 H, br s), 8.37 (1 H, d, J = 7 Hz), 7.86 (1 H, d, J = 8 Hz), 6.68 (1 H, d, J = 9 Hz), 5.15 (1 H, t, J = 7.9 Hz), 5.05 (2 H, t, J = 5.9 Hz), 4.39–4.34 (1 H, m), 4.22 (1 H, t, J = 8 Hz), 3.88–3.76 (2 H, m), 3.18 (1 H, dd, J = 13, 8 Hz), 3.11 (1 H, dd, J = 13, 8 Hz), 2.97 (1 H, br s), 2.89 (1 H, br s), 2.52 (2 H, d, J = 7 Hz), 2.28–2.16 (1 H, m), 2.06–1.92 (6 H, m), 1.94–1.88 (2 H, m), 1.86–1.80 (1 H, m), 1.61 (3 H, s), 1.59 (3 H, s), 1.53 (6 H, s), 1.44–1.40 (1 H, m), 1.35 (9 H, s), 1.12–1.04 (1 H, m), 1.15 (3 H, t, J = 7 Hz), 0.84–0.77 (12 H, m).

(3S,4S)-N-Boc-4-Amino-3-hydroxy-5-(S-all-trans-farnesyl)-1-(L-Ile-L-Met)pentanoic Acid (**11**). The peptide was purified by silica gel chromatography (hexane/acetone 70:30, hexane/acetone/methanol 70:30:10): ^{1}H NMR (DMSO-d_6, 500 MHz) 7.50 (1 H, br s), 6.34 (1 H, br s), 5.16 (1 H, t, J = 8 Hz), 5.04 (2 H, dd, J = 6, 11.5 Hz), 4.10 (1 H, dd, J = 6.5, 8.5 Hz), 3.92 (1 H, s), 3.49 (1 H, dd, J = 7, 15 Hz), 3.15 (2 H, dd, J = 4.5, 6.5 Hz), 2.60 (1 H, dd, J = 6, 14 Hz), 2.37 (3 H, m), 1.96 (3 H, s), 2.04–1.89 (10 H, m), 1.81 (1 H, m), 1.61 (3 H, s), 1.60 (3 H, s), 1.54 (6 H, s), 1.41 (1 H, m), 1.32 (9 H, br s), 1.08 (1 H, m), 0.80 (3 H, d, J = 6.5 Hz). 0.77 (3 H, t, J = 7.5 Hz).

(4S)-N-Boc-4-Amino-2,2-difluoro-3-hydroxy-5-(S-all-trans-farnesyl)-1-(L-Ile-L-Met)pentanoic Acid (**12**). The peptide was purified by silica gel chromatography (hexane/acetone 70:30, hexane/acetone 50:50, acetone, methanol): ^{1}H NMR (DMSO-d_6, 500 MHz) 6.26 (1 H, d, J = 9 Hz), 5.15 (1 H, t, J = 8 Hz), 5.04 (2 H, br d, J = 6 Hz), 4.13 (3 H, m), 3.92 (1 H, dd, J = 8, 16 Hz), 3.14 (2 H, m), 2.54 (1 H, dd, J = 8, 13.5 Hz), 2.44 (1 H, dd, J = 7, 13.5 Hz), 2.41 (2 H, t, J = 8 Hz), 1.98 (3 H, s), 2.05–1.78 (11 H, m), 1.61 (6 H, s), 1.53 (6 H, s), 1.46 (1 H, m), 1.34 (9 H, s), 1.06 (1 H, m), 0.84 (3 H, d, J = 7 Hz), 0.79 (3 H, t, J = 7 Hz).

(3S,4S)-N-Boc-4-Amino-3-hydroxy-5-(S-all-trans-farnesyl)-1-(L-Val-L-Ile-L-Met)pentanoic Acid (**13**). The peptide was purified by silica gel chromatography (hexane/acetone 70:30, hexane/acetone/methanol, 70:30:10): ^{1}H NMR (DMSO-d_6, 500 MHz) 7.92 (1 H, d, J = 8 Hz), 7.82 (1 H, d, J = 7 Hz), 7.40 (1 H, br s), 6.40 (1 H, d, J = 9 Hz), 5.15 (1 H, t, J = 8 Hz), 5.04 (1 H, dd, J = 6.5, 12.5 Hz), 4.91 (1 H, br s), 4.22 (1 H, t, J = 8 Hz), 4.10 (1 H, t, J = 8 Hz), 3.94 (1 H, br s), 3.88 (1 H, s), 3.51 (1 H, dd, J = 9, 16 Hz), 3.10 (2 H, d, J = 6.5 Hz), 2.59 (1 H, dd, J = 5, 13 Hz), 2.32 (3 H, m), 1.95 (3 H, s), 2.12–1.88 (10 H, m), 1.74 (3 H, m), 1.61 (3 H, s). 1.59 (3 H, s), 1.53 (6 H, s), 1.40 (1 H, m), 1.36 (9 H, s), 1.04 (1 H, m), 0.79 (12 H, m).

(4S)-N-Boc-4-Amino-2,2-difluoro-3-hydroxy-5-(S-all-trans-farnesyl)-1-(L-Val-L-Ile-L-Met)pentanoic Acid (**14**). The peptide was purified by silica gel chromatography (hexane/acetone 70:30, hexane/acetone 50:50, acetone, methanol): ^{1}H NMR (DMSO-d_6, 500 MHz) 7.25 (2 H, br s), 6.65 (2 H, br s), 5.15 (1 H, t, J = 7 Hz), 5.04 (2 H, t, J = 5 Hz), 4.22 (1 H, d, J = 8 Hz), 4.16 (1 H, dd, J = 8, 21.5 Hz), 4.12 (1 H, d, J = 6.5 Hz), 3.91 (1 H, t, J = 9 Hz), 3.89 (1 H, br s), 3.13 (2 H, m), 2.54 (1 H, dd, J = 9, 13.5 Hz), 2.43 (1 H, dd, J = 7, 13.5 Hz), 2.35 (3 H, m), 1.95 (3 H, s), 2.07–1.88 (10 H, m), 1.76 (1 H, m), 1.61 (6 H, s), 1.53 (6 H, s), 1.41 (1 H, m), 1.35 (9 H, s), 1.08 (1 H, m), 0.84 (3 H, d, J = 6.5 Hz), 0.80 (9 H, m).

Protease Assays. The substrates and inhibitors were dissolved in DMSO and incubated with calf liver microsomal membranes (0.2 mg of protein/mL) (Walter & Blobel, 1983) according to the detailed previously published procedure (Ma & Rando, 1992). The amount of radioactive product was also determined as before (Ma & Rando, 1992). The reaction was quenched with 500 μL of $CHCl_3$/MeOH (1:1 v/v), and the radioactive N-terminal amino acid, i.e., *N*-[^{3}H]AFC, was extracted after this mixture was thoroughly agitated. Phase separation was achieved by adding 500 μL of 1 M citric acid. After the chloroform layer was evaporated under nitrogen, the residue was resuspended in *n*-hexane/2-propanol/TFA (90:10:0.01 v/v/v), and nonradioactive AFC was added as a standard for UV detection (210 nm). The sample was injected

Table I: Inhibition of the Isoprenyl Endoprotease with Commercial Protease Inhibitors[a]

inhibitor	specificity	concn	inhibition (%)
antipain-2HCl	papain, trypsin, cathepsin A and B	74 μM	0
APMSF	serine protease	20 μM	0
aprotinin	serine protease	0.6 μM	0
bestatin	amino peptidase	0.13 mM	0
chymostatin	α-, β-, γ-, δ-chymotrypsin	0.16 mM	0
E-64	cysteine protease	2.8 μM	0
EDTA-Na_2	metalloprotease	0.1 mM	0
leupeptin	serine and cysteine protease	1.0 μM	0
pepstatin	aspartyl protease	1.0 μM	0
phosphoramidon	metallo endopeptidases	0.5 mM	0
DFP	serine protease	1.0 mM	0
ebelactone		0.2 mM	0
1,10-phenanthroline		0.6 mM	0
p-(hydroxymercuri)-benzoate		0.5 mM	41
N-ethylmaleimide		1.0 mM	0
zinc chloride		0.5 mM	35

[a] The inhibitors were preincubated with the calf liver microsomal membrane (0.2 mg of protein/mL) in 100 mM phosphate buffer (pH = 7.0) for 10 min. N-[^{3}H]AFCVIM (10 μM) was added and the incubation was continued for an additional 30 min. The work-up and enzymatic assays were performed as indicated in Materials and Methods. The % inhibition values are average values of two determinations.

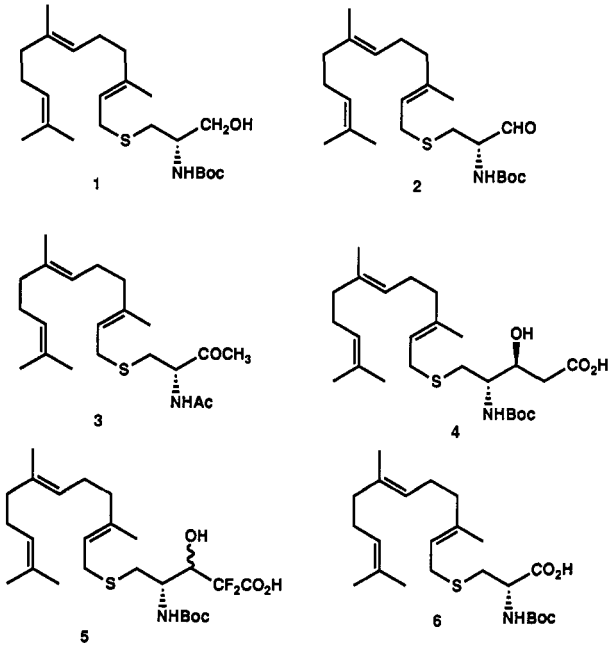
on a normal-phase HPLC column (Dynamax 60A, Rainin), and elution was performed with the same solvent at a flow rate of 1.5 mL/min. Radioactivity was counted with an on-line Berthold (Nashua, NH) LB 506-C HPLC radioactivity monitor. The limit of efficiency of this assay was 3X the background cpm (~150 cpm). This would readily allow us to assay molecules as substrates whose activities were 0.9% of the V_{max} of AFC-Val-Ile-Met (1.13 pmol/(min·mg)).

RESULTS

As a starting point for inhibitor design, a series of commercial protease inhibitors was tested as putative inhibitors of the endoprotease (Table I). It was found that the endoprotease was not affect by (1) serine protease inhibitors, including (4-amidinophenyl)methanesulfonyl fluoride, aprotinin, and leupeptin or by (2) cysteine protease inhibitors, including E-64 and leupeptin (the enzyme was, however inhibited by p-(hydroxymercuri)benzoate (0.5 mM, 41%)) or by (3) metalloprotease inhibitors, including phosphoramidon, EDTA, and 1,10-phenanthroline or by (4) the aspartyl protease inhibitor pepstatin. Given the protease is designed to recognize modified amino acids, these results are not necessarily surprising. The fact that high concentrations of p-(hydroxymercuri)benzoate inhibit the enzyme cannot, of course, be taken as evidence that the endoprotease is a thiol protease. Peptide-based inhibitors were then studied for their abilities to inhibit the endoprotease.

Previously, we have shown that tetrapeptides containing an L-AFC moiety are readily processed by the endoprotease, which specifically cleaves after the L-AFC moiety (Ma & Rando, 1992). Tripeptides and dipeptides were also processed, although much less effectively than tetrapeptides. An isoprenoid moiety was essential for substrate activity. In order to begin to develop specific inhibitors of the endoprotease, AFC and BFC analogs were synthesized and tested as putative inhibitors of the enzyme. The simple farnesyl-L-cysteine derivatives shown in Scheme V were synthesized and tested as inhibitors of the enzyme. Interestingly, only the aldehyde

Scheme V: Farnesyl-L-Cysteine-Based Inhibitors



analog (**2**) proved to be a potent competitive inhibitor (K_I = 1.9 μM) of the enzyme (Figure 1 and Table II). The remaining inhibitors proved to be inactive in our assay system. The fact that the aldehyde **2** is a potent inhibitor suggests the possibility that the protease is a serine- or cysteine-based enzyme (Vinitsky et al., 1992). It is noteworthy that neither the statine nor difluorostatine analog (**4** and **5**) was active as an inhibitor, suggesting that the enzyme is not an aspartyl protease (Doherty et al., 1992).

In order to prepare more potent inhibitors, peptide-based analogs were sought. Simple ψ(CH_2-NH) pseudopeptides and statine analogs in the di(pseudopeptide only), tri-, and tetrapeptide series (Table II) were synthesized and studied as inhibitors of the endoprotease. The K_Is are also recorded in Table II, and the kinetic data for analogs **10** and **13** are shown in Figures 2 and 3, respectively. Clearly, increasingly potent analogs are generated as the peptide is increased in length. Both pseudopeptide **10** and statine **13** analogs are of approximately the same potency. The best inhibitor **13**, which is a statine analog, is exceedingly potent, and has a K_I = 64 nM.

DISCUSSION

Earlier we had demonstrated that bovine liver and dog pancreatic microsomes contain an endoproteolytic activity capable of processing L-AFC-L-Val-L-Ile-L-Ser to L-AFC plus the intact tripeptide Val-Ile-Ser, and L-AFC-L-Val-L-Ile to L-AFC plus the dipeptide Val-Ile (Ma & Rando, 1992). The sequence of the tetrapeptide is taken from the carboxyl terminus of retinal transducin (Hurley et al., 1984). K_M and V_{max} values for the tetrapeptide were measured to be 5.8 μM and 251 pmol/(min·mg of protein), respectively (Ma & Rando, 1992). Rat liver microsomes have also been shown to process *ras* in a way consistent with the presence of an endoproteolytic activity being present which cleaves after the farnesylated cysteine residue (Ashby et al., 1992). Yeast extracts apparently contain several proteolytic activities capable of hydrolyzing farnesylated peptides (Hrycyna & Clark, 1992). The activities uncovered thus far apparently function in a nonspecific exoproteolytic manner (Hrycyna & Clark, 1992). Further studies will be required to determine whether yeast

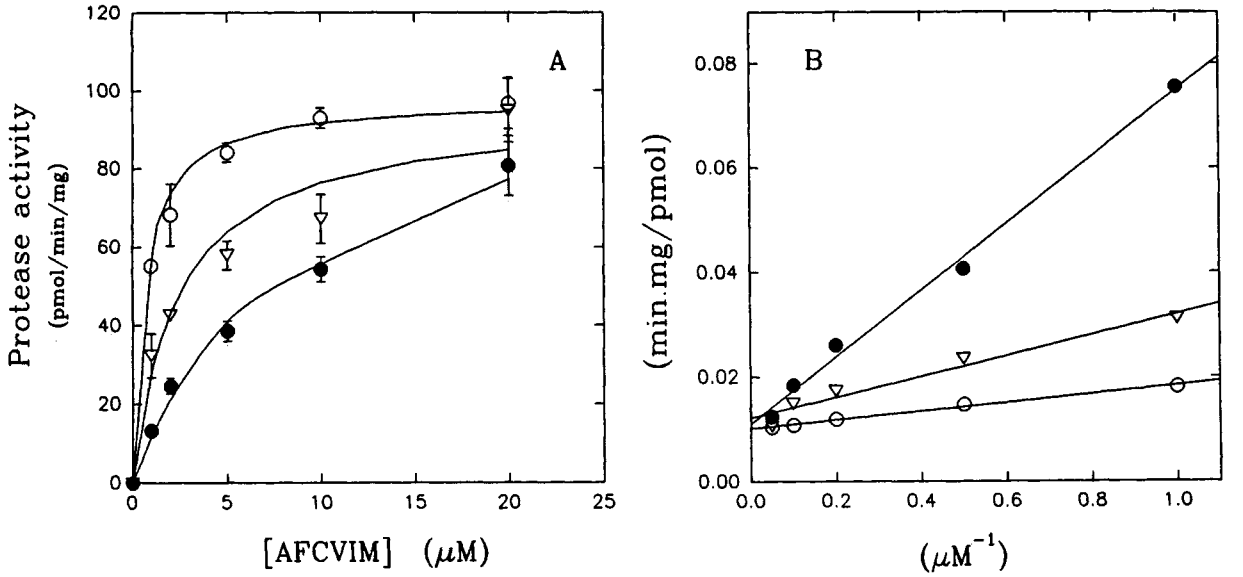


FIGURE 1: *N*-Boc-*S-all-trans*-farnesyl-L-cysteine aldehyde (**2**) inhibition of proteolysis of *N*-[^{3}H]AFCVIM. Michaelis–Menten (A) and Lineweaver–Burk (B) plots for the formation of L-[^{3}H]AFC as a function of *N*-[^{3}H]AFCVIM in the presence of increasing concentrations of **2**: 0 (○), 5 μM (▽), and 25 μM (●). Symbols represent average values of two determinations, and error bars represent the standard deviation from the mean.

Table II: Inhibitors and Kinetic Constants for the Isoprenyl Endoprotease[a]

entry	compound	K_I (μM)
2	Farn-S-CHO, NHBoc	1.95±0.15
7	Farn-S-CH_2NH-Val, NHBoc	0.0
8	Farn-S-CH_2NH-Val-Ile, NHBoc	20.95±2.62
9	Farn-S-CH_2NH-Val-Ile-Ser, NHBoc	0.31±0.07
10	Farn-S-CH_2NH-Val-Ile-Met, NHBoc	0.086±0.017
11	Farn-S-OH-CONH-Ile-Met, NHBoc	28.7±3.00
12	Farn-S-OH-CONH-Ile-Met, NHBoc	36.4±6.8
13	Farn-S-OH-CONH-Val-Ile-Met, NHBoc	0.064±0.011
14	Farn-S-OH-CF_2CONH-Val-Ile-Met, NHBoc	3.60±0.70

[a] Determined as described in Materials and Methods with *N*-[^{3}H]AFCVIM as substrate.

contains a specific endoproteolytic activity for isoprenylated proteins, as mammalian cells do.

Structure–activity studies have been useful in demonstrating the specificity of the isoprenylated protein endoprotease (Ma & Rando, 1992; Ma et al., 1992). The enzyme cleaves AFC containing tetra-, tri-, and dipeptide substrates after the AFC moiety (Ma & Rando, 1992). Proteolytic cleavage of the substrates is stereospecific, because the substitution of a farnesylated D-cysteine residue for the L-amino acid leads to the abolition of substrate activity. In fact, stereospecificity or stereoselectivity is found at all amino acids of a tetrapeptide substrate, demonstrating binding interactions between the enzyme and the individual amino acids (Ma et al., 1992). As might be expected, in a particular series the order of substrate activity was tetrapeptide > tripeptide ≫ dipeptide. This strongly suggests that the endoprotease has subsites at its active site which recognize the individual amino acids of the tetrapeptide, as diagrammed below in Scheme VI.

A free terminal carboxyl group is also required for substrate activity, because methyl esterification renders the substrates inert. In addition, an isoprenoid moiety is essential for activity (Ma et al., 1992). The work described here had as its goal the design of specific inhibitors of the enzyme, for three reasons. First, specific inhibitors of the enzyme would help enable us to determine the mechanistic class that the enzyme belongs to. Second, inhibitors will be useful in determining the physiological role of the enzyme. Hancock et al. have shown that dog pancreas microsomes can specifically proteolyze P21K-*ras*(B) to generate mature *ras* protein (Hancock et al., 1991). These authors demonstrated that proteolysis of pro-*ras* was essential for the efficient binding of *ras* protein to membranes (Hancock et al., 1991). Finally, specific inhibitors of the enzyme will also be useful as ligands for affinity chromatography.

Initial studies on various group specific protease inhibitors did not yield useful information (Table I). The difficulty here may be related to the fact that the endoprotease is designed to proteolyze isoprenylated amino acid containing proteins, and the group specific inhibitors are designed to inhibit proteases which process unmodified amino acid containing proteins. The results using *p*-(hydroxymercuri)benzoate are not viewed as necessarily supporting the idea that the endoprotease is a thiol protease, because many enzymes contain noncatalytic sulfhydryl groups which if blocked lead to the

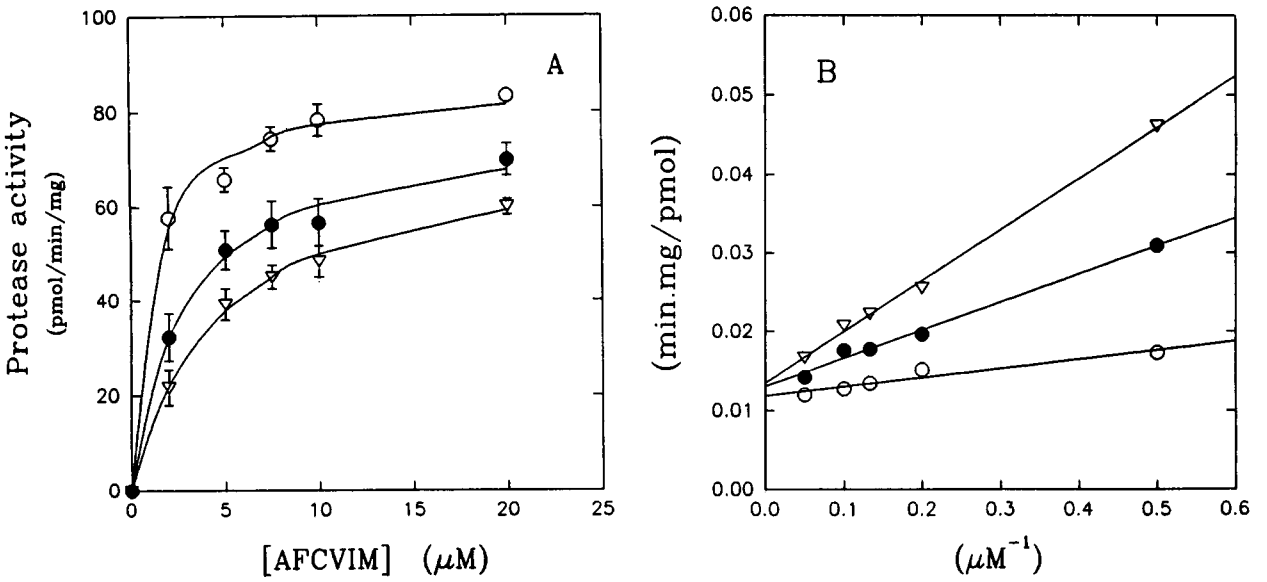


FIGURE 2: Statine analog **13** inhibition of proteolysis of *N*-[^{3}H]AFCVIM. Michaelis–Menten (A) and Lineweaver (B) plots for the formation of L-[^{3}H]AFC as a function of *N*-[^{3}H]AFCVIM in the presence of increasing concentrations of **13**: 0 (○), 0.1 μM (●), and 0.5 μM (▽). Symbols represent average values of two determinations, and error bars represent the standard deviation from the mean.

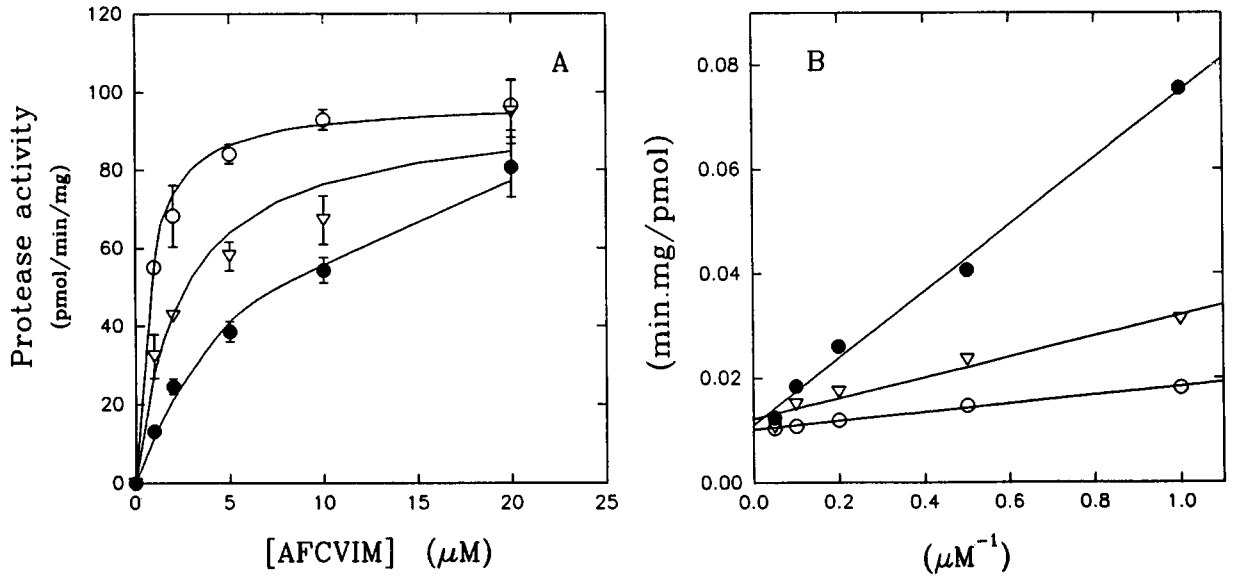
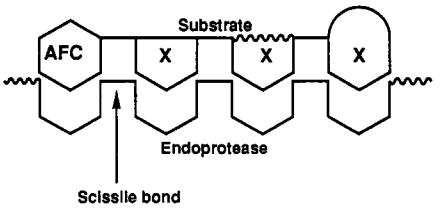


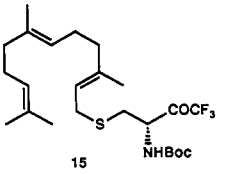
FIGURE 3: Pseudopeptide analog **10** inhibition of proteolysis of *N*-[^{3}H]AFCVIM. Michaelis–Menten (A) and Lineweaver (B) plots for the formation of L-[^{3}H]AFC as a function of *N*-[^{3}H]AFCVIM in the presence of increasing concentrations of **10**: 0 (○), 0.05 μM (●), and 0.2 μM (▽). Symbols represent average values of two determinations, and error bars represent the standard deviation from the mean.

Scheme VI: Recognition of AFC-X-X-X by the Endoprotease



inactivation of the enzyme. The results with chelating agents are probably the most telling here and strongly suggest that the enzyme is not a metalloprotease.

Of the AFC or BFC derivatives studied, only the aldehyde analog (**2**) proved to be inhibitory toward the endoprotease. It is well-known that aldehyde analogs can be potent transition-state derivatives of serine- and cysteine-dependent enzymes by mimicking tetrahedral intermediates which are normally part of the catalytic mechanism (Vinitsky et al., 1992). This can occur either via the hydrated form of the aldehyde or through the complexation of the active-site serine/cysteine by the aldehyde (Westerik & Wolfenden, 1972; Thompson, 1973). Because of chemical complexities inherent in the BFC moiety, we were unable to prepare either fluoroketone (**15**) or phosphoramidate (**16**) analogs as possible transition-state inhibitors of the putative serine/cysteine dependent enzyme.



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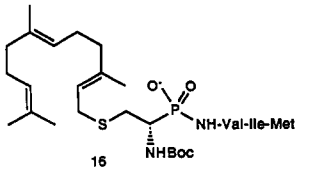

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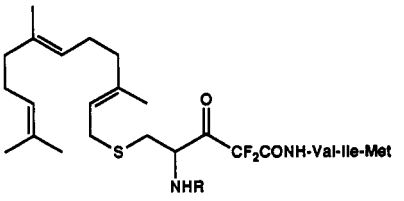
For example, fluoroketone analogs are normally prepared from the alcohol by oxidation (Imperiali & Abeles, 1986). Oxidizing conditions used to carry out this transformation did not produce the desired product (Linderman & Graves, 1987). It is interesting to note that the statine derivative of AFC (**4**)

is also inactive as an inhibitor. This result strongly suggests that the enzyme is not an aspartyl protease (Doherty et al., 1992).

Although we were not able to synthesize AFC or BFC analogs mimicking tetrahedral transition states, other than the aldehyde **2**, we were nevertheless able to prepare several potent peptide-based inhibitors. As is apparent from the pseudopeptide analogs **7**, **8**, and **9**, the potency of the inhibitors is simply a function of the number of amino acid residues in the inhibitor. This is completely consistent with previous studies on peptide-based substrates of the enzyme, where the best substrates proved to be tetrapeptides (Ma & Rando, 1992). Dipeptides were almost inactive as substrates (Ma & Rando, 1992). A subsite mechanism of substrate (or inhibitor) binding is shown in Scheme VI. Interestingly, the most potent inhibitor (**13**), which has a K_I = 64 nM, is a statine analog. From the results with simple AFC and BFC analogs, it is highly unlikely that the enzyme is an aspartyl protease. Given that the pseudopeptide analog **10** is almost as active as **13**, it appears that many modifications in the tetrapeptide in which cleavage at the scissile bond is prevented will generate active inhibitors. Nevertheless, even without transition-state peptide analogs, potent inhibitors are already on hand which will allow for a variety of studies on the endoprotease. For example, the potent inhibitors already prepared will be useful as affinity ligands for the purification of the endoprotease which has already been solubilized in a stable form (Chaudhuri and Rando, unpublished experiments).

Given the results with the BFC-based aldehyde inhibitor (**2**), it is likely that exceedingly potent transition-state inhibitors of the endoprotease, along the lines of the difluoroketone derivative (**17**), could be prepared. Molecules of this type



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combine the transition-state stabilization found in **2** with the substrate binding affinities found in tetrapeptide substrates and inhibitors. Because of synthetic problems alluded to earlier, a C for S substitution analog might be a more accessible target. Nevertheless, the results reported here clearly indicate what direction the design of inhibitors of the endoprotease should proceed in.

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SUPPLEMENTARY MATERIAL AVAILABLE

Details of the syntheses of the modified peptides (19 pages). Ordering information is given on any current masthead page.

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