Synthesis of Tight Binding Inhibitors and Their Action on the Proprotein-Processing Enzyme Furin[1]

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Furin is a subtilisin-like eukaryotic serine endoprotease which processes proproteins to biologically active proteins and peptides. Also, the envelope proteins of viruses, such as influenza and HIV viruses, need to be processed by furin for infectivity. This enzyme has a consensus substrate specificity for Arg-Xxx-Lys/Arg-Arg at the cleavage site. Two kinds of transition state analog peptides were designed and tested *in vitro* with furin. The ketomethylene series, $\Psi(COCH_2)$, have K_i's in the submicromolar range; the aminomethyl ketone series, $\Psi(COCH_2NH)$, have K_i's in the nanomolar range. The best inhibitor is Dec-Arg-Val-Lys-Arg-CH_2-Ala-Val-Gly-NH_2 (**2c**) with a K_i of 3.4 nM.

Introduction

Endoproteolysis of precursor proteins is an obligatory step common to the synthesis of many biologically active proteins and peptides in yeast, invertebrate, and mammalian cells. Typically, cleavage occurs after paired basic amino acid residues (especially Lys-Arg and Arg-Arg).

Many viruses have membrane glycoproteins that are also activated at cleavage sites containing multiple arginine and lysine residues. The cleavage of hemagglutinin (HA) of the influenza, fowl plague virus, which is essential for the ability to enter the cell, proved to be an important determinant for the spread of infection through the organism and for virus pathogenicity. The HA protein is activated in a broad range of host cells. The ubiquitous protease responsible for the activation of HA is furin,[2,3] a subtilisin-like eukaryotic serine endoprotease, which has a consensus substrate specificity Arg-Xxx-Lys/Arg-Arg at the cleavage site.[4] Similarly, the envelope protein of human immunodeficiency virus (HIV) initiates infection by mediating fusion of the viral envelope with cell membranes. Proteolytic cleavage of the gp160 protein into gp120 and gp41 by furin is required for activity.[5,6]

For the investigation of the role of furin in these processes, active site-directed irreversible inhibitors have been used. Such inhibitors consist of a peptidyl portion which directs the inhibitor to the target enzyme and a reactive part which irreversibly labels the enzyme by forming a covalent bond. Inhibitors with the sequence Arg-Xxx-Lys-Arg (such as Dec-Arg-Val-Lys-Arg-CH_2Cl) efficiently prevent cleaving of the envelope proteins resulting in reduced infectivity of the released viruses.[1,4]

Furin has been expressed as a soluble secreted form of the endoprotease lacking the putative transmembrane and cytoplasmic domains. This form of furin still retains full protease activity[7] and can be used for analysis of its enzymatic properties.

Fluorogenic substrates, such as Boc-Arg-Val-Arg-Arg-AMC, are cleaved only slowly by furin. Internally quenched fluorogenic substrates,[8] such as Abz-Arg-Val-Lys-Arg-Gly-Leu-Ala-Tyr(NO_2)-Asp-OH, are cleaved faster which allows shorter assay time and measurement at lower concentrations of furin.[9] These substrates contain, in addition to the fluorescent group (anthranilic acid), a quenching chromophore (nitrotyrosine) such that cleavage of a peptide bond (Arg–Gly) situated in between leads to an increase in fluorescence.[10] With the availability of soluble furin and internally quenched fluorogenic substrates, structure–activity studies can be carried out efficiently.

As the fluorogenic substrate extends to the P′ region, the higher efficiency might be due to additional binding to the enzyme. Therefore, reversible inhibitors extending to the P′ region may offer the chance to examine a possible increase of inhibition. Also, for therapeutic use, reversible inhibitors are generally preferred over irreversible inhibitors.

Here, we synthesize two kinds of reversible inhibitors and measure their inhibitory effect on furin. One type is a ketomethylene pseudopeptide analog, $\Psi(COCH_2)$, in which the -NH- group of the scissile P–P′ bond has been replaced by a methylene group. The second type is an aminomethyl ketone, $\Psi(COCH_2NH)$, in which a methylene group has been inserted between -CO- and -NH- of the scissile peptide bond. Those analogs have several advantages. Firstly, the bonds are resistant to enzymatic degradation. Secondly, the keto group can form a tetrahedral semiketal with the active site serine hydroxyl. Thirdly, the amino acid sequence on the carbonyl side of those bonds can add binding affinity to the inhibitor.

Results and Discussion

The sequences of the peptides are based on the sequence at the cleavage site of the envelope protein gp160, ArgGluLysArg-AlaValGlyIle.[5] Cleavage by furin occurs after Arg-Glu-Lys-Arg. As in the series of irreversible chloromethane inhibitors of furin, Glu has been replaced by Val. It has been shown that the inhibitor Dec-Arg-Val-Lys-Arg-CH_2Cl shows the same efficient inhibitory activity as the one with Glu.[2,11]

In the first series of reversible inhibitors, the amide moiety -CONH- at the cleavage site was replaced by an uncleavable ketomethylene moiety, $-COCH_2$ (Scheme 1). The ketomethylene pseudopeptide bond was introduced

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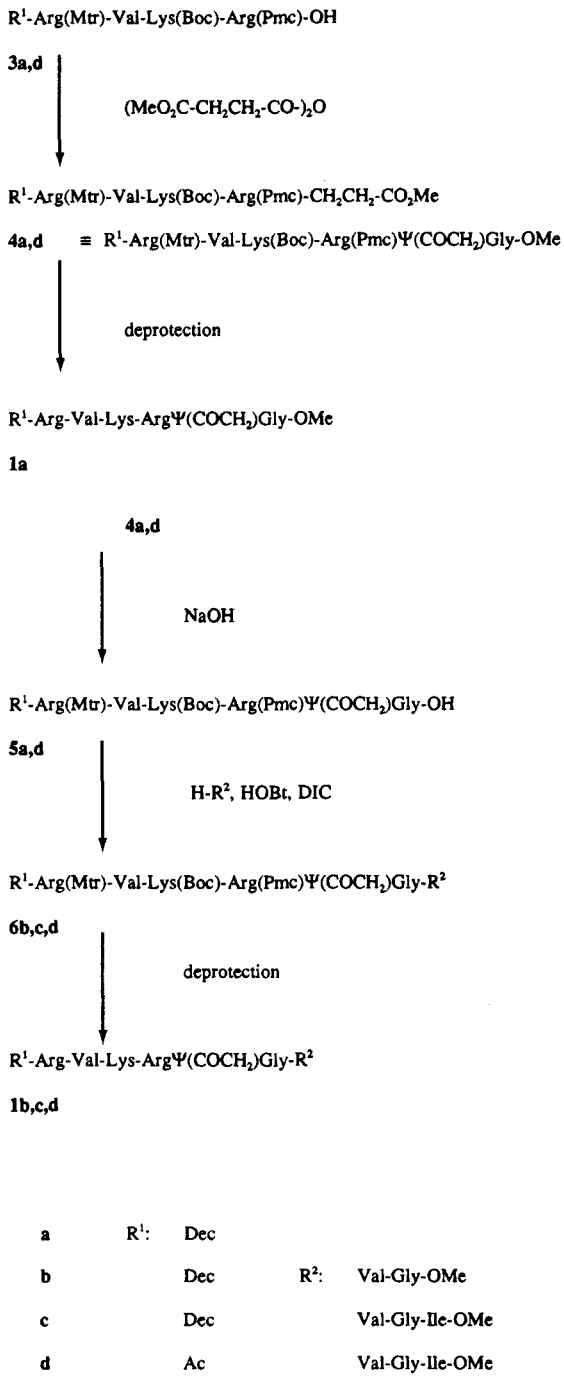
Table 1. Inhibition Constants (K_i) for Furin[a]

inhibitor	K_i	K_i, relative
1a, Dec-ArgValLysArgΨ(COCH$_2$)Gly-OMe[b]	0.24 μM	71
1b, Dec-ArgValLysArgΨ(COCH$_2$)GlyValGly-OMe[b]	0.37 μM	109
1c, Dec-ArgValLysArgΨ(COCH$_2$)GlyValGlyIle-OMe[b]	0.33 μM	97
1d, Ac-ArgValLysArgΨ(COCH$_2$)GlyValGlyIle-OMe[b]	0.35 μM	103
2a, Dec-ArgValLysArg-CH$_2$-Ala-OMe[c]	7.5 nM	2.2
2b, Dec-ArgValLysArg-CH$_2$-AlaValGly-OMe[c]	5.1 nM	1.5
2c, Dec-ArgValLysArg-CH$_2$-AlaValGly-NH$_2$	3.4 nM	1
2d, Dec-ArgValLys-D-Arg-CH$_2$-AlaValGly-NH$_2$	26.0 nM	7.6

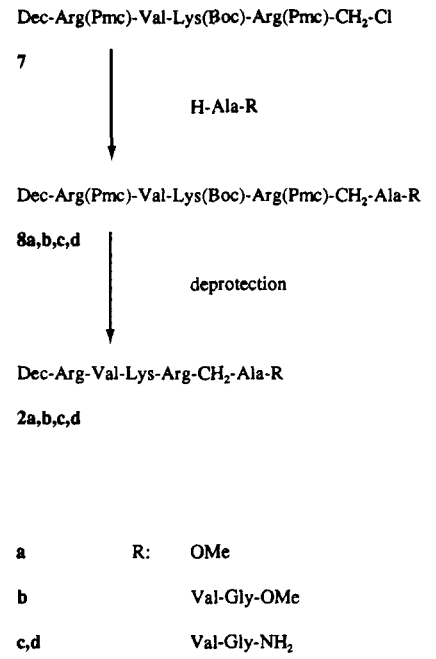
[a] K_i values determined in 100 mM HEPES (pH 7.5), 0.5% Triton X-100, 1 mM $CaCl_2$, and 1 mM 2-mercaptoethanol at 30 °C. [b] L/D ratio 1:1. [c] L/D ratio 6:4.

Scheme 1



by the Dakin–West reaction of the peptide with the anhydride of monomethyl succinate.[12] By the nature of this reaction, the ketomethylene peptide was obtained as a racemate. After hydrolysis of the resulting methyl ester, the peptide was elongated with the appropriate di- or tripeptide methyl ester. In the second series of

Scheme 2



reversible inhibitors, the aminomethyl ketones, the amide moiety -CONH- at the cleavage site was replaced by -COCH$_2$NH- (Scheme 2). Dec-Arg(Pmc)-Val-Lys(Boc)-Arg(Pmc)-CH$_2$Cl (**7**) was synthesized as a mixture of epimers containing 34% epimer with D-arginine in the P_1 position. **7** was combined with Ala-OMe, Ala-Val-Gly-OMe, or Ala-Val-Gly-NH$_2$. The epimers **2c,d** could be separated on a silica gel column. Deprotection with TFA gave the final peptides.

The decanoyl group, introduced at the N-terminal end of the peptide, improves the effectiveness as inhibitors of cellular processing by the ability to penetrate the cell membrane.[13] In one case, inhibitor **1d**, it was replaced by the acetyl group to test whether the nonpolar peptide sequence of the P′ region is able to take over the function of the decanoyl group. Comparison with inhibitor **1c** (Table 1) shows that this alteration does not influence the K_i value.

The K_i values of the ketomethylene compounds **1** are in the submicromolar range, the ones of the aminomethyl ketones **2** in the nanomolar range (Table 1). The K_i of the aminomethyl ketones **2** is up to 109 times lower than that of the ketomethylene compounds **1**. It was expected that the insertion of a methylene group into the scissile peptide bond would disturb the optimal alignment of the inhibitor to the enzyme. The tighter binding, however, suggests that additional interactions must occur. Most probably, the amino nitrogen must be involved, either by interaction of the free electron pair with the enzyme or by forming hydrogen bridges between the nitrogen hydrogen and the enzyme.

Table 2. Comparison of Two Inhibitors with Furin, Subtilisin Carlsberg, and Trypsin

inhibitor	K_i[a]
1c, Dec-ArgValLysArgΨ($COCH_2$)GlyValGlyIle-OMe	
furin	0.33 μM
subtilisin Carlsberg	no inhibition[b]
trypsin	20.9 μM
2b, Dec-ArgValLysArg-CH_2-AlaValGly-OMe	
furin	5.1 nM
subtilisin Carlsberg	no inhibition[b]
trypsin	1.9 μM

[a] K_i values determined in 100 mM HEPES (pH 7.5), 0.5% Triton X-100, 1 mM $CaCl_2$, and 1 mM 2-mercaptoethanol at 30 °C. [b] No inhibition at 50 μM inhibitor concentration.

It is remarkable that the D-amino methyl ketone **2d** inhibits furin and that the K_i value is only 7.6 times higher than that of the L-isomer **2c**. A similar small difference, but between the second-order rate constants of inactivation, k_{obs}/K_i, of the D- and L-isomers was also observed in the case of the inactivation of Kex2 by irreversible inhibitors.[9]

Decroly et al.[14] made inhibitors in which the cleavable peptide bond has been replaced by Ψ(CH_2NH). The K_i was 0.6 μmol for Dec-Arg-Lys-Arg-Arg-Ψ(CH_2NH)-Phe-Leu-Gly-Phe-NH_2 and is thus in the range of our inhibitors with Ψ($COCH_2$) as the pseudopeptide bond. These results show that the carbonyl moiety alone, or nitrogen moiety alone, is not sufficient for tight binding. Only when the two moieties are combined, as in Ψ(CO-CH_2-NH), a very efficient inhibitor results.

The length of the inhibitors was varied to see whether the length in the P′ region influences the K_i. As depicted in Table 1, the length of the P′ region in both series Ψ-($COCH_2$) and Ψ($COCH_2NH$) has no effect on the K_i. While the length of the P′ region might not control the binding to furin, the amino acid in P_1' does influence the cleavage of substrates. Although furin can tolerate many different amino acids in P_1',[15] some have a big effect: (i) the variant proalbumin Benheim in which Asp in the P_1' position has been replaced by Val is not cleaved at all by furin;[16] (ii) a proalbumin-derived 15-residue peptide with Lys in P_1' is also not cleaved;[15] (iii) with a pro-PC1 (the proprotein of the prohormone convertase-1)-derived 10-residue peptide, γ-Hyp (γ-hydroxyproline) in P_1' is not cleaved.[17]

To assess the relative specificity of these inhibitors for furin, assays were performed with two other serine proteases: (i) trypsin belonging to the chymotrypsin family and cleaving peptides at the carboxyl side of lysine or arginine and (ii) subtilisin Carlsberg belonging, as furin, to the subtilisin family but cleaving peptides after hydrophobic residues. Among the inhibitors, one of each category was chosen, Dec-ArgValLysArgΨ-($COCH_2$)GlyValGlyIle-OMe (**1c**) and Dec-ArgValLysArg-CH_2-AlaValGly-OMe (**2b**) (Table 2). With trypsin, compound **1c** is 63 times less effective than with furin, and compound **2b** is 373 times less effective. The aminomethyl ketone **2b** remains 11 times more effective than the ketomethylene **1c**. As expected subtilisin Carlsberg is not inhibited by both inhibitors which lack a hydrophobic residue in P_1.

It would be advisable to investigate the tight binding of the amino ketones **2** to furin by X-ray studies. Replacing the nitrogen hydrogen of Ψ($COCH_2NH$) by alkyl groups or exchanging the nitrogen by other heteroatoms should lead to an explanation of the tight binding. Furthermore, cellular studies should clarify

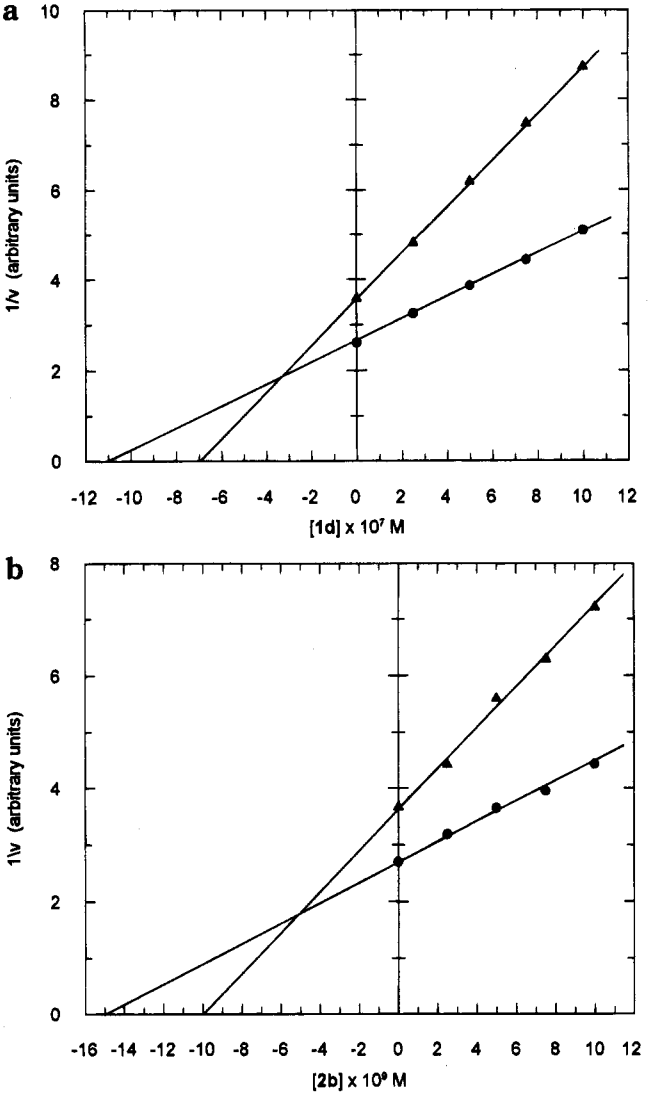


Figure 1. Dixon plot of Ac-ArgValLysArgΨ($COCH_2$)GlyVal-GlyIle-OMe (**1d**) (a) and Dec-ArgValLysArg-CH_2-AlaValGly-OMe (**2b**) (b) using 5 μM (▲) and 10 μM (●) internally quenched fluorogenic substrate Abz-Arg-Val-Lys-Arg-Gly-Leu-Ala-Tyr(NO_2)-Asp-OH at pH 7.5 and 30 °C.

if those inhibitors are as effective in blocking furin as in inhibitor/enzyme assays and thus establish if those kinds of inhibitors are potential agents for antiviral therapeutic treatment.

Experimental Section

Amino acids and their derivatives were purchased from Bachem Feinchemikalien AG, 4416 Bubendorf, Switzerland. Furin was obtained from Sean S. Molloy and Gary Thomas, Vollum Institute, Oregon Health Sciences University, Portland, OR 97201-3098. Proton nuclear magnetic resonance spectra were recorded on a Bruker (400 MHz) spectrometer. HPLC was performed on a Nucleosil C18, 5 μm, 4.6 × 125 mm column; flow rate 1.5 mL/min; solvent A, 0.1% TFA in water; solvent B, acetonitrile; solvent C, 0.1% TFA in acetonitrile; program 1, solvent A and B, gradient 60% solvent A to 0% within 7 min, monitoring wavelength 254 nm; program 2, solvent A and B, gradient 90% solvent A to 0% within 7 min, 220 nm; program 3, solvent A and C, gradient 90% solvent A to 50% within 10 min, 220 nm.

Synthesis of Ketomethylene Compounds. H-Val-Gly-OMe (9). Boc-Val-OH was coupled via the mixed anhydride with H-Gly-OMe. Boc-Val-Gly-OMe (**10**) was then deprotected with 2 N gaseous HCl in ethyl acetate.

H-Val-Gly-Ile-OMe (11). **10** was hydrolyzed with NaOH and coupled via the mixed anhydride with H-Ile-OMe. The Boc group was removed with 2 N gaseous HCl in ethyl acetate.

Dec-Arg(Mtr)-Val-OH (12). Boc-Arg(Mtr)-OH was coupled via the mixed anhydride with H-Val-OMe to give Boc-Arg-(Mtr)-Val-OMe (**13**). Hydrolysis of **13** with NaOH, removal of the Boc group with 2 N gaseous HCl in ethyl acetate, and treating with DecOSu gave **12**.

Ac-Arg(Mtr)-Val-OH (14). The Boc group of **13** was removed with 2 N gaseous HCl in ethyl acetate. Acetylation with acetic anhydride and triethylamine and hydrolysis of the methyl ester with NaOH gave **14**.

Dec-Arg(Mtr)-Val-Lys(Boc)-Arg(Pmc)-OH (3a). The OSu ester of Fmoc-Lys(Boc)-OH was coupled with H-Arg(Pmc)-OH. After removal of the Fmoc group, the dipeptide was coupled with the OSu ester of **12**.

Ac-Arg(Mtr)-Val-Lys(Boc)-Arg(Pmc)-OH (3d). Coupling as above with the OSu ester of **14** provided **3d**.

Dec-Arg(Mtr)-Val-Lys(Boc)-Arg(Pmc)Ψ($COCH_2$)Gly-OMe (4a). *N,N'*-Dicyclohexylcarbodiimide (1.25 g, 6.07 mmol) was added to MMS (1.6 g, 12.15 mmol) in CH_2Cl_2 (40 mL). After 30 min, the reaction mixture was cooled with ice/water, filtered, and evaporated. The anhydride of MMS was dissolved in CH_2Cl_2 (4 mL). Triethylamine (266 μL, 1.91 mmol), 4-(dimethylamino)pyridine (11 mg, 0.09 mmol), and **3a** (1.12 g, 0.87 mmol) were added. The reaction mixture was heated at 40 °C for 1.25 h. After evaporation the residue was taken up in ethyl acetate (100 mL) and washed with 1 N HCl (2 × 20 mL), saturated $NaHCO_3$ (3 × 20 mL), and brine (20 mL). The extracts were dried over $MgSO_4$, filtered, and evaporated. After chromatography over silica gel with $CHCl_3$ containing 3% MeOH, a colorless solid was obtained, 618 mg (52.4%): pure and single peak on HPLC (program 1); TLC [silica gel, chloroform–methanol (19:1)] R_f 0.40, 0.42; FAB/MS *m/z* 1360 $(M + H)^+$.

Ac-Arg(Mtr)-Val-Lys(Boc)-Arg(Pmc)Ψ($COCH_2$)Gly-OMe (4d). **4d** was obtained from **3d** as above.

Dec-Arg(Mtr)-Val-Lys(Boc)-Arg(Pmc)Ψ($COCH_2$)-Gly-OH (5a). NaOH (1 N, 468 μL, 0.468 mmol) was added to **4a** (424 mg, 0.312 mmol) in acetonitrile (2.5 mL) and water (156 mL). After 1 h additional 1 N NaOH (312 μL, 0.312 mmol) was added and after another 2 h 1 N NaOH (156 μL, 0.156 mmol). After 5 h (pH 7.5) acetonitrile was evaporated. The aqueous reaction mixture was diluted with 1 N HCl (20 mL) and extracted with ethyl acetate (50 mL). The organic phase was washed with 1 N HCl (20 mL), water (2 × 20 mL), and brine (20 mL). The extract was dried over $MgSO_4$ and filtered. Evaporation yielded the acid as a colorless solid, 330 mg (78.6%).

Ac-Arg(Mtr)-Val-Lys(Boc)-Arg(Pmc)Ψ($COCH_2$)-Gly-OH (5d). **4a** was hydrolyzed as above.

Dec-Arg(Mtr)-Val-Lys(Boc)-Arg(Pmc)Ψ($COCH_2$)Gly-Val-Gly-OMe (6b). To **9** [from **10** (46 mg, 0.16 mmol)] and NMM (36 μL, 0.32 mmol) in 1.6 mL of THF were added **5a** (165 mg, 0.123 mmol), HOBt·13% H_2O (18 mg, 0.129 mmol), and CME-CDI (114 mg, 0.17 mmol). After stirring for 24 h at room temperature, the reaction mixture was evaporated, taken up in ethyl acetate (100 mL), and washed with 1 N HCl (3 × 20 mL), saturated $NaHCO_3$ (3 × 20 mL), and brine (20 mL). The extracts were dried over $MgSO_4$, filtered, and evaporated. After chromatography over silica gel with $CHCl_3$ containing 5% MeOH, colorless plates were obtained, 92 mg (46.0%): pure and single peak on HPLC (program 1); TLC [silica gel, chloroform–methanol (19:1)] R_f 0.12, 0.14; FAB/MS *m/z* 1516 $(M + H)^+$.

Dec-Arg(Mtr)-Val-Lys(Boc)-Arg(Pmc)Ψ($COCH_2$)Gly-Val-Gly-Ile-Ome (6c). **5a** was coupled as above to **11**.

Ac-Arg(Mtr)-Val-Lys(Boc)-Arg(Pmc)Ψ($COCH_2$)Gly-Val-Gly-Ile-OMe (6d). **5d** was coupled as above to **11**.

Synthesis of Aminomethyl Ketones. Boc-Ala-Val-Gly-OMe (15). **9** was coupled to the mixed anhydride of Boc-Ala-OH.

H-Ala-Val-Gly-NH_2 (16). **15** was hydrolized with NaOH, and the mixed anhydride was treated with aqueous ammonia. The Boc group was removed with TFA.

Dec-Arg(Pmc)-Val-Lys(Boc)-Arg(Pmc)-CH_2Cl (7). Fmoc-Lys(Boc)-OH was converted to the succinimide ester and coupled to H-Arg(Pmc)-OH. The dipeptide was converted via the mixed anhydride to the diazomethyl ketone with diazomethane and purified on a silica gel column with ethyl acetate/hexane (9:1). After removing the Fmoc group with piperidine/DMF (1:4), the dipeptidyl diazomethyl ketone was coupled to Fmoc-Val-OH with HOBt and DCC. After removing the Fmoc group with piperidine/DMF (1:4), the tripeptidyl diazomethyl ketone was coupled to Fmoc-Arg(Pmc)-OH and HOBt and DCC yielding the tetrapeptide diazomethyl ketone which was epimerized containing L- and D-arginine in the P_1 position [HPLC Spherisorb Si 80, 5 μm, 4.6 × 125 mm column, chloroform–methanol (97:3), 254 nm, flow rate 1.5 mL/min, t_R 3.27 min (27% D-epimer) and 4.79 min (73% L-epimer)]. After removing the Fmoc group with piperidine/DMF (1:4), the tetrapeptidyl diazomethyl ketone was treated with DecOSu and then converted to the chloromethyl ketone with 2.8 N gaseous HCl in ethyl acetate. The product which could not be separated on silica gel contained 34% D-epimer [HPLC Spherisorb Si 80 column, t_R 2.55 min (34% D-epimer) and 3.25 min (66% L-epimer)]: TLC [silica gel, chloroform–methanol (19:1)] R_f 0.23 (L-epimer), 0.27 (D-epimer); FAB/MS *m/z* 1376 $(M + H)^+$; NMR (DMSO) δ 0.75–0.88 [9H, m, CH_3, (Dec), 2CH_3 (Val)], 1.21 [12H, s, 6CH_2 (Dec)], 1.25 [6H, s, C(CH_3)$_2$ (Pmc)], 1.35 [9H, s, C(CH_3)$_3$ (Boc)], 1.30–1.80 [12H, m, 5 CH_2 (Lys, Arg), CH_2 (Dec)], 1.76 [2H, t, J = 7 Hz, $CH_2CH_2C(CH)_3$ (Pmc)], 1.87–1.98 [1H, m, $CH(CH_3)_2$ (Val)], 2.01 [3H, s, arom CH_3 (Pmc)], 2.09 [2H, t, J = 7 Hz, CH_2CO (Dec)], 2.46 [6H, s, 2 arom CH_3 (Pmc)], 2.57 [2H, t, J = 7 Hz, $CH_2CH_2C(CH)_3$ (Pmc)], 2.86 [q, 2H, J = 7 Hz, CH_2NH (Lys)], 3.01 [q, 4H, J = 6 Hz, 2 CH_2NH (Arg)], 4.09–4.20 [2H, m, 2 α-CH (2 Arg)], 4.20–4.28 [2H, m, 2 α-CH (Val, Lys)], 4.49 (2H, s, CH_2Cl), 6.30–6.54, 6.54–6.90 [4H, 2 br s, 4 NH (2 guanidino)], 6.72 [1H, t, J = 7 Hz, ε-NH (Lys)], 7.62 [1H, d, J = 7 Hz, CONH (Dec-Arg)], 7.97, 7.99, 8.06 (D) [2H, 3d, J = 7 Hz, 1 CONH not epimer resolved, CONH L- and D-epimers, 2:1 (Arg-Val, Val-Lys)], 8.37 (L), 8.41 (D) (1H, 2d, J = 7 Hz, CONH (Lys-Arg) L- and D-epimers, 2:1].

Dec-Arg(Pmc)-Val-Lys(Boc)-Arg(Pmc)-CH_2-Ala-OMe (8a). H-Ala-OMe·HCl (67 mg, 0.48 mmol) was added to **7** (551 mg, 0.4 mmol), $NaHCO_3$ (101 mg, 1.2 mmol), and NaI (15 mg, 0.1 mmol) in DMF (4 mL). The reaction mixture was heated at 60 °C for 4 h. DMF was evaporated and the residue distributed between ethyl acetate (50 mL) and 1 N HCl (10 mL). The organic phase was separated, washed with 1 N HCl (2 × 10 mL), saturated $NaHCO_3$ (3 × 10 mL), and brine (10 mL), dried over $MgSO_4$, and filtered. Purification on a silica gel column with ethyl acetate containing 5% methanol (no separation of the epimers) and trituration in ethyl acetate/hexane gave a yellow solid (147 mg, 25% yield): TLC [silica gel, chloroform–methanol (19:1)] R_f 0.22 (D-epimer), 0.25 (L-epimer).

Dec-Arg(Pmc)-Val-Lys(Boc)-Arg(Pmc)-CH_2-Ala-Val-Gly-OMe (8b). The Boc group of **15** was removed with TFA and then coupled to **7** as above: TLC [silica gel, chloroform–methanol (19:1)] R_f 0.20 (D-epimer), 0.23 (L-epimer).

Dec-Arg(Pmc)-Val-Lys(Boc)-Arg(Pmc)-CH_2-Ala-Val-Gly-NH_2 (8c) and Dec-Arg(Pmc)-Val-Lys(Boc)-D-Arg(Pmc)-CH_2-Ala-Val-Gly-NH_2 (8d). **16** was coupled to **7**. The epimers were separated on a silica gel column with $CHCl_3$/5% methanol: HPLC (program 1) t_R 9.22 min (D-epimer), 9.35 min (L-epimer); TLC [silica gel, chloroform–methanol (9:1)] R_f 0.13 (D-epimer), 0.21 (L-epimer).

General Procedure for Deprotection. Dec-ArgVal-LysArgΨ($COCH_2$)Gly-OMe·3HCl (1a). **4a** (129 mg) was deprotected in a mixture (1 mL) of TFA, anisole, and thioanisole (18:1:1). After 3 h TFA was evaporated and gaseous HCl (2.8 M) in ethyl acetate (2 mL) and dry ether (10 mL) was added. Trituration, centrifugation, and decantation gave a colorless solid. This was triturated three times with 10 mL of ether, centrifuged, and decanted. It was purified on a SP-Sephadex (1 g, H^+-form) column (1 cm i.d., 7 cm length) by eluting with 0.5 N HCl. The fractions were evaporated without heating on a rotavapor and examined on HPLC (program 2). The desired fractions were pooled, evaporated, taken up in 2 mL of water, and lyophilized, yielding a colorless solid (55 mg, 65 % yield): FAB/MS *m/z* 782 $(M + H)^+$; NMR (DMSO and D_2O) δ 0.76–0.87 [9H, m, CH_3 (Dec), 2CH_3 (Val)], 1.21 [12H, s, 6CH_2 (Dec)], 1.30–1.80 [16H, m, 7 CH_2 (Lys, Arg), CH_2 (Dec)], 1.92–2.01 [1H, m, $CH(CH_3)_2$ (Val)], 2.11 [2H, t, J = 7 Hz, CH_2CO (Dec)], 2.32–2.39, 2.39–2.48 [2H, 2m, $COCH_2CH_2$-CO_2CH_3, D/L (1:1)], 2.62–2.80 (2H, m, $COCH_2CH_2CO_2CH_3$, D/L),

2.73, 2.74 [2t, 2H, J = 7 Hz, CH_2NH (Lys), D/L (1:1)], 3.06 [4H, br s, 2 CH_2NH (Arg)], 3.56 (3H, s, OCH_3), 4.11–4.19 [2H, m, 2 α-CH (2 Arg)], 4.19–4.29 [2H, m, 2 α-CH (Val, Lys)], 7.66, 7.68 [1H, 2d, J = 7 Hz, CONH (Dec-Arg) D/L (1:1)], 8.11, 8.13 [2H, 2d, J = 7 Hz, 2 CONH (Arg-Val, Val-Lys)], 8.18, 8.19 [1H, 2d, J = 7 Hz, CONH (Lys-Arg) D/L (1:1)]; HPLC (program 3) t_R 10.7 min (L), 11.2 min (D); L/D ratio 1:1.

Dec-ArgValLysArgΨ(COCH$_2$)GlyValGly-OMe·3HCl (1b): 54% yield; FAB/MS m/z 938 (M + H)$^+$; t_p 11.4 min (L), 11.6 min (D); 1:1.

Dec-ArgValLysArgΨ(COCH$_2$)GlyValGlyIle-OMe·3HCl (1c): 36% yield; FAB/MS m/z 1051 (M + H)$^+$; t_R 11.7 min (L), 12.1 min (D); 1:1.

Ac-ArgValLysArgΨ(COCH$_2$)GlyValGlyIle-OMe·3TFA (1d): 30% yield after additional preparative HPLC purification (Vydac C18, 10 μm, 22 × 250 mm column, solvent A and B, gradient 90% solvent A to 50% within 15 min, 220 nm, flow rate 8 mL/min); FAB/MS m/z 939 (M + H)$^+$; t_R 8.4 min (L), 8.6 min (D); 1:1.

Dec-ArgValLysArg-CH$_2$-Ala-OMe·4HCl (2a): 50% yield; FAB/MS m/z 811 (M + H)$^+$; NMR (CD_3OD) δ 0.86–1.00 [12H, m, CH_3 (Dec), 2 CH_3 (Val), CH_3 (Ala)], 1.30 [12H, s, 6 CH_2 (Dec)], 1.40–2.04 [16H, m, 7 CH_2 (Lys, Arg), CH_2 (Dec)], 2.04–2.12 [1H, m, CH(CH$_3$)$_2$ (Val)], 2.265, 2.270 [2H, 2t, J = 7 Hz, CH_2CO (Dec)], 2.94 [br t, 2H, J = 7 Hz, CH_2NH (Lys)], 3.20 [4H, br s, 2 CH_2NH (Arg)], 3.84 (3H, s, OCH_3), 4.06–4.45 [7H, m, 5 α-CH (2 Arg, Val, Lys, Ala), COCH_2NH], 7.26–7.37 [1H, 2d, J = 7 Hz, CONH (Dec-Arg)], 7.87, 7.89, 7.95 (D) [2H, 3d, J = 7 Hz, 2 CONH (Arg-Val, Val-Lys)], 8.25 (L), 8.29 (D) [1H, 2d, J = 7 Hz, CONH (Lys-Arg) L/D (6:4)]; HPLC (isochratic, 67% solvent A, 33% solvent C, 220 nm, 1.5 mL/min) 4.74 min (L), 5.17 min (D); 6:4.

Dec-ArgValLysArg-CH$_2$-AlaValGly-OMe·4HCl (2b): 63% yield; FAB/MS m/z 967 (M + H)$^+$; 3.05 min (L), 4.43 min (D); 6:4.

Dec-ArgValLysArg-CH$_2$-AlaValGly-NH$_2$·4HCl (2c): 63% yield; FAB/MS m/z 952 (M + H)$^+$; 3.90 min (L).

Dec-ArgValLys-D-Arg-CH$_2$-AlaValGly-NH$_2$·4HCl (2d): 69% yield; FAB/MS m/z 952 (M + H)$^+$; 3.90 min (D); mixing **2c,d** gave only one peak on HPLC.

Enzymic Studies. Fluorometric assays were performed in a 2 mL reaction volume containing 100 mM HEPES (pH 7.5), 0.5% Triton X-100, 1 mM $CaCl_2$, 1 mM 2-mercaptoethanol, and 10 μM internally quenched fluorogenic substrate Abz-Arg-Val-Lys-Arg-Gly-Leu-Ala-Tyr(NO_2)-Asp-OH[7] at 30 °C. Inhibitors (5 μL) in water (**1b,c** and **2b** dissolved in DMSO to 2 × 10^{-3} M and then further diluted with water) and 0.1 μM furin[6] (5 μL) in 100 mM HEPES (pH 7.5) and 0.5% Triton X-100 were added (final enzyme concentration 0.25 nM). The fluorescence emission of the cleaved substrate was measured with a Perkin Elmer luminescence spectrometer (LS 50B; 320 nm excitation, 425 nm emission). The K_i values of the ketomethylene inhibitor Ac-Arg-Val-Lys-ArgΨ(COCH$_2$)Gly-Val-Gly-Ile-OMe (**1d**) and Dec-Arg-Val-Lys-Arg-CH$_2$-Ala-Val-Gly-OMe (**2b**) were determined by a Dixon plot with two substrate concentrations, [S], 5 and 10 μM. The apparent inhibition constant, K_i', of the other inhibitors was determined with 10 μM substrate concentration at four inhibitor concentrations. The K_i was calculated using a K_m of 5.0 μM and the relationship $K_i = K_i'/(1 + [S]/K_m)$. For trypsin, the K_i values of **1c** and **2b** were determined in the same buffer by a Dixon plot with two substrate concentrations, 5 and 10 μM, of Boc-Val-Leu-Lys-AMC. Subtilisin Carlsberg was assayed with H-Ala-Ala-Phe-AMC.

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References

(1) Abbreviations: Abz, 2-aminobenzoyl (anthranilyl); AMC, 7-amido-4-methylcoumarin; Dec, decanoyl; Tyr(NO_2), 3-nitrotyrosine; MMS, monomethyl succinate; HOBt, 1-hydroxybenzotriazole; CME-CDI, *N*-cyclohexyl-*N'*-(morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Ψ(COCH$_2$), ketomethylene pseudopeptide bond; Ψ(COCH$_2$NH), Aaa-CH$_2$-Bbb, aminomethyl ketone; FAB/MS, fast atom bombardment mass spectroscopy. The binding site notation is that of Schechter and Berger (see ref 18), i.e., P_n denotes a substrate position, and S_n denotes an enzyme-binding subsite.

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