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The synthesis of inhibitors for processing proteinases and their action on the Kex2 proteinase of yeast

Herbert ANGLIKER,* Peter WIKSTROM,*‡ Elliott SHAW,* Charles BRENNER,† and Robert S. FULLER†

*Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland,
and †Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305, U.S.A.

Peptidyl chloromethane and sulphonium salts containing multiple Arg and Lys residues were synthesized as potential inhibitors of prohormone and pro-protein processing proteinases. The potencies of these compounds were assayed by measuring the kinetics of inactivation of the yeast Kex2 proteinase, the prototype of a growing family of eukaroytic precursor processing proteinases. The most potent inhibitor, Pro-Nvl-Tyr-Lys-Arg-chloromethane, was based on cleavage sites in the natural Kex2 substrate pro-α-factor. This inhibitor exhibited a K_i of 3.7 nM and a second-order inactivation rate constant (k_2/K_i) of 1.3×10^7 $M^{-1} \cdot s^{-1}$ comparable with the value of $k_{cat.}/K_m$ obtained with Kex2 for the corresponding peptidyl methylcoumarinylamide substrate. The enzyme exhibited sensitivity to the other peptidyl chloromethanes over a range of concentrations, depending on peptide sequence and α-amino decanoylation, but was completely resistant to peptidyl sulphonium salts. Kinetics of inactivation by these new inhibitors of a set of 'control' proteinases, including members of both the trypsin and subtilisin families, underscored the apparent specificity of the compounds most active against Kex2 proteinase.

INTRODUCTION

Active-site-directed irreversible inhibitors have been useful reagents in proteinase studies. This approach takes advantage of a synthetic peptidyl portion, which directs the inhibitor to a target enzyme, attached to a mechanism-specific reactive group. Thus peptides having a C-terminal arginine chloromethane (-Arg-CH_2Cl) have provided specific reagents for quantifying the extent of zymogen activation of trypsin-related coagulation factors such as prothrombin and Factors VII and X (Kettner and Shaw, 1981). Highly specific inhibitors can be used in biochemical and cellular studies to elucidate the physiological role of a proteinase. A functional change in cellular properties can be related to modification of a particular protein because the peptidyl-CH_2Cl forms a covalent adduct with the target enzyme.

Prohormone processing and the analogous proteolytic activation of inactive pro-proteins represent areas in which specific inhibitors and affinity labelling reagents should be valuable. Conversion of proinsulin into insulin by cleavage after -Lys-Arg and -Arg-Arg sites, followed by trimming of exposed basic residues (Steiner et al., 1969), has been the paradigm for maturation of numerous polypeptide precursors in the secretory pathway. Insight into the nature of the processing enzymes emerged from studies of the biosynthesis of the yeast α-mating pheromone (reviewed by Fuller et al., 1988). The yeast *KEX2* gene encodes a membrane-bound proteinase responsible for processing pro-α-factor at -Lys-Arg- sites *in vivo* (Julius et al., 1984). Kex2 protein, which contains a domain showing sequence similarity to the bacterial serine proteinase subtilisin (Fuller et al., 1988, 1989b; Mizuno et al., 1988) is a Ca^{2+}-dependent serine proteinase that can be inhibited by Ala-Lys-Arg-CH_2Cl and affinity-labelled by ^{125}I-Tyr-Ala-Lys-Arg-CH_2Cl (Fuller et al., 1989a; Mizuno et al., 1989). A secreted, soluble (ss) form of Kex2 proteinase, ss-Kex2, was created by deleting C-terminal sequences, retaining only the subtilisin domain and an additional essential region, the 'P-domain' (P. Gluschankof and R. S. Fuller, unpublished work). Highly purified ss-Kex2 exhibited a high degree of specificity for cleaving at -Lys-Arg- and -Arg-Arg- sites (Brenner and Fuller, 1992).

The recent characterization of Kex2 homologues in metazoans suggests that Kex2 proteinase is the prototype of a family of eukaryotic processing enzymes that cleave a variety of secretory precursors on the carboxy side of pairs of basic residues and other related sites [reviewed by Hutton (1990), Steiner (1991) and Barr (1991)]. Information about the possible functions of these Kex2-related enzymes is accumulating. The first identified mammalian Kex2 homologue, 'furin' (Fuller et al., 1989a, b; van den Ouweland et al., 1990), exhibits a wide tissue distribution (Schalken et al., 1987) and may process diverse pro-proteins in the constitutive secretory pathway, including the precursors of the fusion proteins of lipid-enveloped viruses (Stieneke-Gröber et al., 1992; Hallenberger et al., 1992). Two other enzymes, PC2 and PC3, have been implicated in prohormone and neuropeptide processing in mammals (Benjannet et al., 1991; Thomas et al., 1991; Bennett et al., 1992).

In addition to a pair of basic residues, usually -Lys-Arg- or -Arg-Arg-, at the P_2 and P_1 positions in the cleavage site, other residues may also be important. Furin appears to discriminate at the P_4 position as well, cleaving on the carboxy side of the consensus sequence -Arg-Xaa-Lys/Arg-Arg- ['Xaa' indicates that multiple substitutions are possible (Hosaka et al., 1991; Stieneke-Gröber et al., 1992)].

The present paper describes the synthesis of various peptidyl chloromethanes that mimic processing sites in prohormones and pro-proteins, incorporating substitutions at the P_1, P_2, and P_4 positions. Because the new compounds were also intended for inhibiting processing in living cells, certain species incorporate an N-terminal lipophilic group similar to palmitoyl, which has

Abbreviations used: Boc, t-butoxycarbonyl; Dec, decanoyl; DMF, dimethylformamide; f.a.b., fast atom bombardment; MA, mixed anhydride; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulphonyl; NHMec, 4-methyl-7-coumarylamide; NMM, *N*-methylmorpholine; Nvl, norvaline; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Su, succinimide; Z, benzyloxycarbonyl; OBu^t, *O*-t-butyl ester.

‡ Present address: Pentapharm A.G., P.O. Box, CH-4002 Basel, Switzerland.

been shown previously to improve inhibitor potency *in vivo* (Garten et al., 1989). The effectiveness of this new series of inhibitors in blocking the cellular processing of the influenza-virus haemagglutinin and the HIV-1 (human-immunodeficiency-virus-1) glycoprotein has been established (Hallenberger et al., 1992; Stieneke-Gröber et al., 1992).

In the present work, ss-Kex2, as the first member of the family of processing enzymes available in homogeneous form, has been used as a model target for the new peptidyl chloromethanes. High specificity constants obtained for inhibitors based on the physiological Kex2 cleavage site in pro-α-factor suggest that the design of high-affinity inhibitors selective for members of the Kex2 family may be feasible, which would facilitate the design of inhibitors of possible therapeutic importance.

MATERIALS AND METHODS

Materials

ss-Kex2 was purified to homogeneity and determined to be fully active by initial-burst titration as described by Brenner and Fuller (1992). Tosylphenylalanylchloromethane ('TPCK')-treated bovine trypsin was from Worthington, and human plasmin was from Boehringer-Mannheim. Human thrombin (Stone et al., 1989) was kindly provided by Dr. S. R. Stone, Department of Haematology, University of Cambridge, Cambridge, U.K. Subtilisin Carlsberg and Proteinase K were obtained from Sigma.

Fluorogenic substrates and peptide intermediates were purchased from Bachem, Bubendorf, Switzerland. Other reagents were obtained from Fluka, Switzerland. Silica 60 was from Merck, Darmstadt, Germany. Ala-Lys-Arg-$CH_2S^+(CH_3)_2$ (Ala-Lys-Arg methyl dimethylsulphonium salt) was obtained as described by Zumbrunn et al. (1988).

H.p.l.c. was carried out on a C_{18} reversed-phase column (5 μm particle size; 125 mm × 4.6 mm); solvent A was 0.1% trifluoroacetic acid (TFA) in water, and solvent B was acetonitrile; program 1 was a linear gradient of solvent A from 60 to 0% within 7 min, then isocratic, the flow rate being 1.5 ml/min; program 2 was a linear gradient of solvent A from 90 to 0% within 7 min, then isocratic, the flow rate being 1.5 ml/min.

Preparative h.p.l.c. was carried out with a C_{18} reversed-phase Vydac column 218TP1022 (10 μM particle size; diameter 2.2 cm; length 25 cm). The solvents were the same as those used above; a linear gradient of solvent A from 90 to 50% within 30 min, then isocratic was used; eluates were monitored at 230 nm.

Synthesis of blocked inhibitors

Some syntheses were carried out by coupling the blocked peptide lacking the C-terminal arginine residue to a chloromethyl ketone of arginine. To gain experience with more labile guanidino protecting groups, we have synthesized *H*-Arg(Mtr)-CH_2Cl for incorporation into peptides since the 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr) group can be removed with TFA (Fujino et al., 1981). Previous work has used nitroarginine (Kettner and Shaw, 1981) or $Arg(Z)_2$ (Aplin et al., 1983; Rauber et al., 1988). The chloromethane group has also been introduced as the final step on the completed peptide sequence.

H-Arg(Mtr)-CH₂Cl·HCl

Boc-Arg(Mtr)-OH (4.87 g, 10 mmol) was converted into the diazomethane via the mixed anhydride (MA) with isobutyl chloroformate (1.37 ml, 10.5 mmol) and N-methylmorpholine (NMM) (1.21 ml, 11 mmol) in tetrahydrofuran (THF) (100 ml) at −20 °C. After 10 min, 30 mmol of diazomethane in diethyl ether (84 ml) was added. After stirring for 1 h at −20 °C and for 1 h at room temperature, ethyl acetate (300 ml) was added. The organic phase was washed twice with satd. $NaHCO_3$ and satd. NaCl, dried over anhydrous $MgSO_4$, filtered and evaporated. The resulting oil was purified on silica-gel 60 column with chloroform containing increasing ethanol, yielding a yellow oil [3.8 g, 75% pure (h.p.l.c.), 74% yield]. A solution of the diazomethane (2.5 g, 4.9 mmol) in 1 M HCl in ethyl acetate (65 ml) gradually precipitated the *H*-Arg(Mtr)-CH_2Cl·HCl obtained by decantation of the supernatant solution after 1 h and washing with ethyl acetate and drying (2.15 g, 96% yield, 98% pure by h.p.l.c. program 2, 254 nm); f.a.b.m.s.: m/z $(M+H)^+$ 419 ($C_{17}H_{27}N_4O_4SCl$ formula 418, $C_{17}H_{27}N_4O_4SCl \cdot HCl$ formula M_r 455.405).

Z-Pro-Nvl-Tyr-Lys(Z)-Arg(Mtr)-CH₂Cl

The compound was obtained by a 4+1 coupling, as follows.

Z-Pro-Nvl-Tyr-Lys(Z)-OH. The N-terminal tetrapeptide was synthesized as the *O*-t-butyl ester (OBu^t). For this, Boc-Tyr-OH, as the MA, was coupled to *H*-Lys(Z)-OBu^t. The Boc group was removed in 1.5 M gaseous HCl in ethyl acetate for 1 h. The resultant *H*-Tyr-Lys(Z)-OBu^t was treated with the MA from Z-Pro-Nvl-OH (15% excess) to give the tetrapeptide t-butyl ester, which was converted into Z-Pro-Nvl-Tyr-Lys(Z)-OH in ethyl acetate saturated with HCl.

Z-Pro-Nvl-Try-Lys(Z)-Arg(Mtr)-CH₂Cl. Z-Pro-Nvl-Tyr-Lys(Z)-OH (484 mg, 0.625 mmol) in THF (20 ml) was converted into the MA at −20 °C with isobutyl chloroformate (86 μl, 0.656 mmol) and NMM (76 μl, 0.688 mmol). After 15 min, *H*-Arg(Mtr)-CH_2Cl·HCl (569 mg, 1.25 mmol, 100% excess) and NMM (137 μl, 1.25 mmol) were added. After stirring for 2 h at 0 °C, then overnight at room temperature, the solvent was evaporated. The residue was taken up in ethyl acetate, washed twice with 1 M HCl, twice with satd. $NaHCO_3$ and satd. NaCl, dried over $MgSO_4$, filtered and evaporated. The dried residue was washed with small volumes of THF; the insoluble portion (370 mg, 50% yield) was 75% pure pentapeptide derivative (h.p.l.c. program 2, 254 nm).

Z-Pro-Met-Tyr-Lys(Boc)-Arg(Mtr)-CH₂Cl

Z-Pro-Met-OH was coupled with *H*-Tyr-OEt via the MA. The tripeptide ethyl ester in aqueous THF was saponified below pH 12.4. The tripeptide, as the MA, was coupled to *H*-Lys(Boc)-OMe. Saponification, as described above, provided the acid, Z-Pro-Met-Tyr-Lys(Boc)-OH. Activation as the MA and treatment with *H*-Arg(Mtr)-CH_2Cl (1.5 equiv.) gave Z-Pro-Met-Tyr-Lys(Boc)-Arg(Mtr)-CH_2Cl (34% yield for the 4+1 coupling); f.a.b.m.s.: m/z $(M+H)^+$ 1172 ($C_{55}H_{78}N_9O_{13}S_2Cl$ formula 1171, M_r 1172.9).

Z-D-Tyr(Bzl)-Ala-Lys(Z)-Arg(Z)₂-CH₂Cl

Z-D-Tyr(Bzl)-Ala-Lys(Z)-OH was coupled via the MA to *H*-$Arg(Z)_2$-CH_2Cl (Found: C 56.78; H, 6.69; N, 10.83; $C_{48}H_{67}N_8O_{12}SCl$ requires C, 56.77; H, 6.65; N, 11.03).

Decanoyl(Dec)-Phe-Ala-Lys(Z)-Arg(Z)₂-CH₂Cl

Dec-Phe-OH was synthesized from H-Phe-OBu^t and decanoyl chloride for hydrolysis with HCl in ethyl acetate. The acid was

converted via the MA into Dec-Phe-Ala-OBu^t. The dipeptide ester was hydrolysed as described above. Elongation as above with *H*-Lys(Z)-OBu^t gave, after hydrolysis, Dec-Phe-Ala-Lys(Z)-OH. This was coupled via the MA with *H*-Arg(Z_2)-CH_2Cl to give a colourless solid, m.p. 154–155 °C (10% yield for the 3+1 coupling) [Found: C, 63.59; H, 7.08; N, 10.17; Cl, 2.94. $C_{59}H_{77}N_8O_{11}Cl$ (1109.763) requires C, 63.91; H, 6.91; N, 10.11; Cl, 3.2].

Boc-D-Tyr(Bzl)-Pro-Gly-Lys(2ClZ)-Arg(Z)$_2$-CH$_2$Cl

Boc-D-Tyr(Bzl)-Pro-Gly-Lys(2ClZ)-OH was prepared by coupling the dipeptides via the succinimide ester (OSu) derivative. The tetrapeptide was coupled with *H*-Arg(Z_2)-CH_2Cl·HCl (1 equiv.) via the MA (Found: C, 60.52; H, 6.03; N, 9.64; Cl 6.04; $C_{65}H_{77}N_9O_{14}Cl_2$ requires C, 60.03; H, 6.07; N, 9.85; Cl, 5.54).

Boc-Phe-Ala-Lys(Boc)-Arg(Mtr)-CH$_2$Cl

Boc-Phe-Ala-Lys(Boc)-OH was obtained by coupling Boc-Phe-Ala-OSu with H-Lys(Boc)-OH. The blocked tripeptide was converted into an OSu derivative and coupled with H-Arg(Mtr)OH. The tetrapeptide was converted into the diazomethane via the MA. The blocked chloromethane was obtained by a rapid reaction (1 min) of the diazomethane at 0 °C with 1.5 M gaseous HCl in ethyl acetate.

Boc-Ala-Lys(Z)-Arg(Mtr)-CH$_2$Cl

Boc-Lys(Z)-OSu was coupled to *H*-Arg(Mtr)-OH. The Boc group of the product was cleaved off with TFA at 0 °C for 10 min. The free dipeptide was converted with Boc-Ala-OSu into Boc-Ala-Lys(Z)-Arg(Mtr)-OH. Treatment of the MA with diazomethane led to the diazomethane, and subsequent rapid treatment with gaseous HCl in ethyl acetate at 0 °C yielded Boc-Ala-Lys(Z)-Arg(Mtr)-CH_2Cl.

Dec-Arg(Mtr)-Ala-Lys(Z)-Arg(Mtr)-CH$_2$Cl

Dec-Arg(Mtr)-OH [obtained from Dec-OSu and H-Arg(Mtr)-OH] was coupled via the MA with *H*-Ala-Lys(Z)-Arg(Mtr)-CH_2Cl which had been obtained by treating the Boc derivative with TFA at 0 °C for 15 min; f.a.b.m.s.: *m/z* $(M+H)^+$ 1274 ($C_{60}H_{92}N_{11}O_{13}S_2Cl$ formula 1273, M_r 1275.041).

Boc-Tyr-Ala-Arg(Mtr)-Ala-Lys(Z)-Arg(Mtr)-CH$_2$Cl

Boc-Tyr-OSu was coupled with Ala. The resulting Boc-Tyr-Ala-OH was coupled via the MA with *H*-Arg(Mtr)-Ala-Lys(Z)-Arg(Mtr)-CH_2Cl obtained by treating Boc-Arg(Mtr)-Ala-Lys(Z)-Arg(Mtr)-CH_2Cl with TFA at 0 °C for 10 min; f.a.b.m.s.: *m/z* $(M+H)^+$ 1454 ($C_{67}H_{96}N_{13}O_{17}S_2Cl$ formula 1453, M_r 1455.16).

Dec-Arg(Mtr)-Glu(OBzl)-Lys(Z)-Arg(Z)$_2$-CH$_2$Cl

Boc-Glu(OBzl)-Lys(Z)-Arg($Z)_2$-CH_2Cl was synthesized with the same procedure as described for Boc-Ala-Lys(Z)-Arg(Mtr)-CH_2Cl. Boc-Arg($Z)_2$-OH was coupled via the MA with H-Glu(OBzl)-Lys(Z)-Arg(Z,Z)-CH_2Cl (Boc group removed with TFA). The Boc group of the product was removed with TFA and the decanoyl group introduced by the use of Dec-OSu. The protected tetrapeptide was obtained as a colourless solid; f.a.b.m.s.: *m/z* $(M+H)^+$ 1534 ($C_{81}H_{100}N_{11}O_{17}Cl$ formula 1533, M_r 1535.204).

Dec-Arg(Mtr)-Val-Lys(Z)-Arg(Mtr)-CH$_2$Cl

Boc-Val-OSu was coupled with *H*-Lys(Z)-OMe and the Boc group cleaved off with gaseous HCl in ethyl acetate. Boc-Arg(Mtr)-OH was coupled with the free dipeptide via the MA to provide Boc-Arg(Mtr)-Val-Lys(Z)-OMe, which was hydrolysed with 1 M-NaOH. The tripeptide was converted into the OSu ester and coupled with *H*-Arg(Mtr)-OH. The resulting tetrapeptide was converted into the diazomethane. Treatment with gaseous HCl in ethyl acetate gave *H*-Arg(Mtr)-Val-Lys(Z)-Arg(Mtr)-CH_2Cl which was converted into Dec-Arg(Mtr)-Val-Lys(Z)-Arg(Mtr)-CH_2Cl with Dec-OSu; f.a.b.m.s.: *m/z* $(M+H)^+$ 1302 ($C_{62}H_{96}N_{11}O_{13}S_2Cl$ formula 1301, M_r 1303.095).

Dec-Arg(Mtr)-Ala-Ile-Arg(Mtr)-CH$_2$Cl

Boc-Ala-Ile-Arg(Mtr)-CH_2Cl was synthesized as described for Boc-Ala-Lys(Z)-Arg(Mtr)-CH_2Cl. Dec-Arg(Mtr)-OH was coupled via the MA with H-Ala-Ile-Arg(Mtr)-CH_2Cl obtained from the Boc derivative with gaseous HCl in ethyl acetate. Dec-Arg(Mtr)-Ala-Ile-Arg(Mtr)-CH_2Cl was obtained as a colourless solid; f.a.b.m.s.: *m/z* $(M+H)^+$ 1125 ($C_{52}H_{85}N_{10}O_{11}S_2Cl$ formula 1124, M_r 1125.892).

Boc-Tyr-Ala-Arg(Mtr)-Ala-Lys(Z)-Arg(Mtr)-CH$_2$Cl

Boc-Tyr-OSu was coupled with Ala. The resulting Boc-Tyr-Ala-OH was coupled via the MA with H-Arg(Mtr)-Ala-Lys(Z)-Arg(Mtr)-CH_2Cl obtained by treating Boc-Arg(Mtr)-Ala-Lys(Z)-Arg(Mtr)-CH_2Cl with TFA at 0 °C for 10 min. The product was obtained as a colourless solid; f.a.b.m.s.: *m/z* $(M+H)^+$ 796 ($C_{34}H_{58}N_{13}O_7Cl$ formula 795, M_r 796.375.

Deblocking of inhibitors

The blocked peptides described above were deblocked either by HF or by TFA (Table 1). The blocked peptide was left at room temperature in TFA (about 0.5 ml/100 mg) for 17–20 h. If Lys(Z) was present, about 40 h was allowed. In the case of peptides containing tyrosine, 10% thioanisole was included. The solvent was removed in a stream of nitrogen and finally by high vacuum; thioanisole, if used, was removed by trituration with diethyl ether. The washed residue was taken up in water (about 10 ml/100 mg of blocked peptide starting material), with addition of methanol if needed to achieve complete solution. This was poured over a small column of SP (Sulphopropyl)-Sephadex C-25 (H^+ form) (7–10 ml), which was washed with water (50 ml) before elution of the peptide with 0.4 M HCl (50 ml). At this stage fractionation was possible with careful monitoring (h.p.l.c. program 2, 230 nm) and, in some cases (cf. Table 1), provided adequate purification. If not, this procedure removed u.v.-absorbing by-products and thus simplified subsequent preparative h.p.l.c. runs analysed at 230 nm. On some occasions identification of inhibitory activity by enzymic assay (trypsin) was useful for identifying the product before isolation in bulk. The 0.4 M HCl eluate containing the purified inhibitor was evaporated without heating at water-pump pressure.

Enzymic studies

Survey of inactivation of ss-Kex2 by peptidyl chloromethanes

Experiments reported in Table 2 were carried out as follows. A quantity of proteinase sufficient for at least five assays was incubated at room temperature in 200 mM Bistris buffer, pH 7.2, plus 1 mM Ca^{2+}; inhibitor was added and timed aliquots were

Table 1 Formation of deblocked inhibitors

Inhibitor	Method	Isolation	Formula M_r	Formula m/z	F.a.b.m.s. m/z $(M+H)^+$
H-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl $C_{32}H_{52}N_9O_6Cl \cdot 3$ TFA	TFA	H.p.l.c.	694.3 1036.4	693	694
H-Phe-Ala-Lys-Arg-CH_2Cl $C_{25}H_{41}N_8O_4Cl \cdot 3$ TFA	TFA	H.p.l.c.	553.1 895.2	552	553
H-Tyr-Ala-Arg-Ala-Lys-Arg-CH_2Cl $C_{34}H_{58}N_{13}O_7Cl \cdot 4$ HCl	TFA	SP-S*	796.4 942.2	795	796
Dec-Phe-Ala-Lys-Arg-CH_2Cl $C_{35}H_{59}N_8O_5Cl \cdot 2$ HCl	HF	SP-S	707.4 780.3	706	707
D-Tyr-Pro-Gly-Lys-Arg-CH_2Cl $C_{29}H_{46}N_9O_6Cl \cdot 3$ TFA	HF	H.p.l.c.	652.2 994.3	651	652
Dec-Arg-Glu(OCH_3)-Lys-Arg-CH_2Cl† $C_{35}H_{66}N_{11}O_7Cl \cdot 3$ HCl	HF	SP-S	788.4 897.8	787	788
Dec-Arg-Val-Lys-Arg-CH_2Cl $C_{34}H_{66}N_{11}O_5Cl \cdot 3$ HCl	TFA	SP-S	744.4 853.8	743	744
Dec-Arg-Ala-Lys-Arg-CH_2Cl $C_{32}H_{62}N_{11}O_5Cl \cdot 3$ HCl	TFA‡	SP-S	716.4 825.8	715	716
Dec-Arg-Ala-Lys-D-Arg-CH_2Cl $C_{32}H_{62}N_{11}O_5Cl \cdot 3$ HCl	TFA‡	SP-S	716.4 825.8	715	716
D-Tyr-Ala-Lys-Arg-CH_2Cl $C_{25}H_{41}N_8O_5Cl \cdot 3$ HCl	HF	SP-S	569.1 678.5	568	569
Arg-Ala-Ala-Arg-CH_2Cl $C_{19}H_{37}N_{10}O_4Cl \cdot 3$ HCl	TFA	SP-S	505.0 614.4	504	505
Dec-Arg-Ala-Ile-Arg-CH_2Cl $C_{32}H_{61}N_{10}O_5Cl \cdot 2$ HCl	TFA	SP-S	701.4 774.8	700	701

* SP-S, SP-Sephadex.
† Contains 30% of Dec-Arg-Glu-Lys-Arg-CH_2Cl as determined on the basis of the proton signals of CH_2Cl and OCH_3 in the n.m.r. and of the M^+ in the mass spectra.
‡ 3 days.

Table 2 Inactivation of Kex2 by peptidyl chloromethanes

Inhibitor structure	Concn. (μM)	$t_{\frac{1}{2}}$ (min)	$k_{app.}/[I]$* ($M^{-1} \cdot s^{-1}$)
H-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl	0.005	3.5	$> 6.6 \times 10^5$
H-Phe-Ala-Lys-Arg-CH_2Cl	0.005	3.2	7.2×10^5
H-Tyr-Ala-Arg-Ala-Lys-Arg-CH_2Cl	0.010	4.5	2.6×10^5
Dec-Phe-Ala-Lys-Arg-CH_2Cl	0.050	2.6	8.9×10^4
Dec-Arg-Glu(OMe)-Lys-Arg-CH_2Cl	0.050	3.5	6.6×10^4
Dec-Arg-Val-Lys-Arg-CH_2Cl	0.050	4.1	5.6×10^4
Dec-Arg-Ala-Lys-Arg-CH_2Cl	0.100	5.4	3.1×10^4
H-D-Tyr-Ala-Lys-Arg-CH_2Cl	0.050	12.1	1.9×10^4
H-D-Tyr-Pro-Gly-Lys-Arg-CH_2Cl	0.50	5.2	4.5×10^3
Dec-Arg-Ala-Lys-D-Arg-CH_2Cl	1.0	3.3	3.5×10^3
Dec-Arg-Ala-Ile-Arg-CH_2Cl	5.0	5.1	4.5×10^2
H-Ala-Lys-Arg-$CH_2S^+(Me)_2$	50	No effect (46 min)	
Dec-Arg-Ala-Lys-Arg-$CH_2S^+(Me)_2$	30	No effect (28 min)	
Dec-Phe-Ala-Lys-Arg-$CH_2S^+(Me)_2$	100	No effect (20 min)	

* When $[I] \ll K_i$, $K_{app.}/[I]$ approaches k_2/K_i (Kettner and Shaw, 1979, 1981).

removed for assay at 37 °C with 0.1 mM Z-Ala-Arg-Arg-aminomethyl coumarylamide in the same buffer. A recording fluorimeter was used. Replicate inactivations were carried out, and the results reported were typically obtained with an agreement of ±20 %, generally less. In addition, variations in inhibitor concentration were examined to ensure that measurements were being made below saturating levels of inhibitor but at concentrations high enough to produce an apparent-first-order loss of activity (Kitz and Wilson, 1962) as used earlier with arginylchloromethanes (Kettner and Shaw, 1981).

Table 3 Observations on other selected serine proteinases

Inhibitor structure and proteinase used	Concn. (μM)	$t_{\frac{1}{2}}$ (min)	$k_{app.}/[I]$ ($M^{-1} \cdot s^{-1}$)
Phe-Ala-Lys-Arg-CH_2Cl			
Trypsin	2	6.05	9.1×10^2
Plasmin	50	3.5	67
Thrombin	100	8.3	14
Subtilisin Carlsberg	200	No effect (60 min)	
Proteinase K	200	No effect (60 min)	
Dec-Phe-Ala-Lys-Arg-CH_2Cl			
Trypsin	0.2	2.45	2.35×10^4
Plasmin	5	4.4	5.25×10^2
Thrombin	40	14.75	20
Subtilisin Carlsberg	200	2.1	27.5
Proteinase K	200	12	4.9
Dec-Arg-Ala-Ile-Arg-CH_2Cl			
Trypsin	0.4	2.2	1.3×10^4
Plasmin	10	16.1	72
Thrombin	1	3.4	3.4×10^3
Subtilisin Carlsberg	200	2.8	21
Proteinase K	200	18.5	3.1

The same considerations apply to the determinations made with the remaining serine proteinases (Table 3), which were assayed in the same buffer. However, for trypsin (10 μM) and for plasmin (30 μM), Boc-Val-Leu-Lys-NHmec was used. For thrombin, 20 μM Boc-Val-Pro-Arg-NHmec was the substrate. These have been shown to be appropriate by Lottenberg et al. (1981). These proteinases were titrated with nitrophenyl *p*-guanidinobenzoate (Chase and Shaw, 1970) to guide the selection of a low enzyme concentration when examining the more effective inhibitors. Subtilisin Carlsberg and proteinase K concentrations were based on weighed samples and assay with 0.1 mM Ala-Ala-Phe-NHmec.

Inhibitor activity titration with ss-Kex2

The experiment reported in Figure 1 was carried out as follows. In duplicate, ss-Kex2 [50 fmol by initial-burst titration (Brenner and Fuller, 1992)] was mixed with 0, 10, 20, 30, 40, 50, 60, 70 and 80 fmol (by weight) of H-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl or H-Phe-Ala-Lys-Arg-CH_2Cl in 40 μl of 200 mM Bistris/HCl (pH 7.0)/0.01 % Triton X-100/1 mM $CaCl_2$ and incubated for 2 h at room temperature. Activity remaining was assayed by adding 10 μl of 100 μM Ac-Pro-Met-Tyr-Lys-Arg-4-methyl-7-coumarylamide (-NHmec) (9-fold > K_m at final concentration). Following incubation for 3 min at 36 °C, reactions were terminated by adding 950 μl of 0.125 M $ZnSO_4$, and aminomethylcoumarin release was determined fluorimetrically. Fractional activity remaining was plotted against the ratio of the nominal concentration of inhibitor to the enzyme concentration.

Determination of the kinetic constants for *H*-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl and H-Phe-Ala-Lys-Arg-CH_2Cl with ss-Kex2

The experiments reported in Figure 2 were carried out as follows.

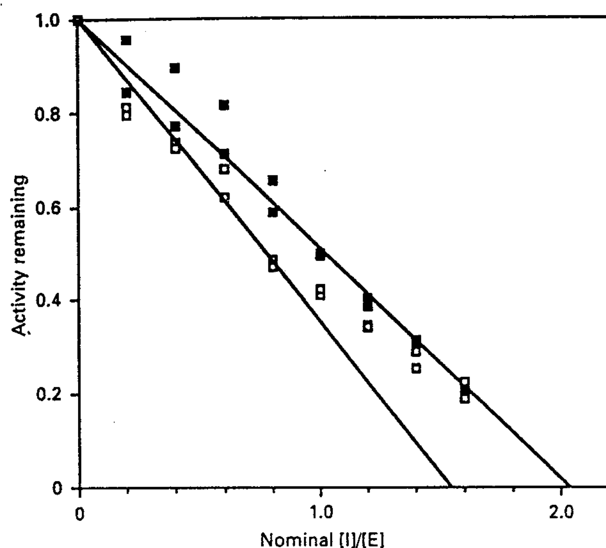

Figure 1 Inhibitor activity titration

H-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl (■) and *H*-Phe-Ala-Lys-Arg-CH_2Cl (□) were examined as described in the Methods section. In the latter case, for reasons that were not apparent, activity remaining did not decrease linearly with increasing inhibitor concentration when the inhibitor concentration exceeded 80% of the enzyme concentration. Those data were not used for linear regression. Lower-than-nominal inhibitor activities may be due to several factors, including hydration and instability.

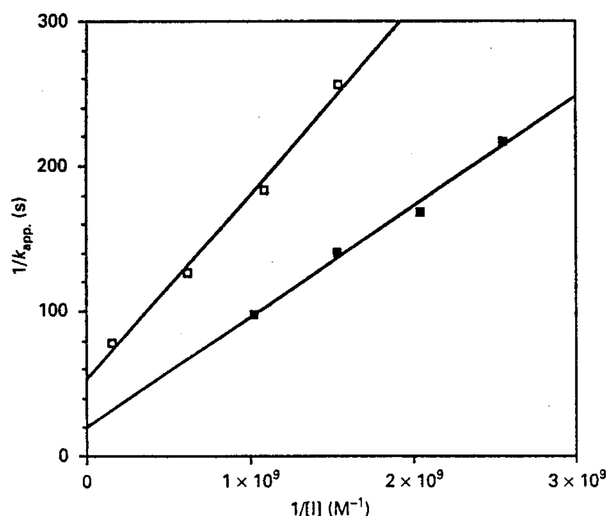

Figure 2 Determination of kinetic constants

H-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl (■) and *H*-Phe-Ala-Lys-Arg-CH_2Cl (□) were examined as described in the Methods section. Values of $1/k_{app.}$ were plotted against 1/[I] according to the equation: $1/k_{app.} = (K_i/k_2)(1/[I]) + 1/k_2$ (Kitz and Wilson, 1962).

Inhibitor concentrations were adjusted to reflect the inhibitor activity titrations (Figure 1). *H*-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl was assayed at concentrations of 980, 650, 490 and 390 pM. *H*-Phe-Ala-Lys-Arg-CH_2Cl was assayed at concentrations of 6.5 nM, 1.6 nM, 930 pM and 650 pM. All incubations were at 37 °C. For each determination of $k_{app.}$ (the apparent first-order rate of enzyme inactivation), 2.6 ml of 200 mM Bistris/HCl (pH 7.0)/0.01 % Triton X-100/1 mM $CaCl_2$, containing ss-Kex2 at one-tenth the concentration of inhibitor to be added, was prepared; 0.5 ml of this enzyme solution was added to 2.0 ml of 200 mM Bistris/HCl (pH 7.0)/0.01 % Triton X-100/1 mM $CaCl_2$ containing 250 μM Ac-Pro-Met-Tyr-Lys-Arg-NHmec to establish the uninhibited activity of the enzyme. Inhibitor was added to the remaining enzyme solution and, at time points ranging from 15 s to 6 min, 0.5 ml of the inhibition reaction mixture was removed and added to 2.0 ml of assay mix with 250 μM Ac-Pro-Met-Tyr-Lys-Arg-NHmec to determine residual activity. The high concentration of substrate (90-fold > K_m at final concentration) was used to minimize competitive inhibition. Reciprocal values of $k_{app.}$ were determined with ENZFITTER software, version 1.05 (Biosoft), and plotted against the reciprocal of the inhibitor concentration (Kettner and Shaw, 1981).

RESULTS AND DISCUSSION

The usefulness of coupling *H*-Arg(Mtr)-CH_2Cl to small blocked tri- and tetra-peptides in solution was demonstrated in several cases. This intermediate is analogous to *H*-Arg$(Z)_2$-CH_2Cl (Aplin et al., 1983), which was also used but which requires HF deblocking. In other syntheses the blocked peptide was completed before converting the C-terminal Arg into the chloromethane. Several of the peptides were converted into the *N*-decanoyl derivative to improve their effectiveness as inhibitors of cellular processing instead of palmitoyl (Garten et al., 1989) to reduce membrane-lytic activity. The structures of the final products (Table 1) were confirmed by f.a.b.m.s.

In view of the high degree of specificity of Kex2 proteinase for cleaving at the carboxy end of the sequence Ac-Pro-Met-Tyr-Lys-Arg (Brenner and Fuller, 1992) the synthesis of the corresponding chloromethane was undertaken and allowed to proceed to the protected state, as described above. This material was somewhat unstable, slowly converting at room temperature into a form migrating more slowly on t.l.c. with a half-life of about 2 weeks. This was evidently due to self-alkylation at the thioether group of methionine by either an intra- or an inter-molecular process. Evidence for this was also found in the u.v. spectrum by the appearance of a pH-sensitive band at 266 nm, a property of sulphonium salts (Shaw, 1988). To avoid this complication, the synthesis of the norvaline-containing analogous sequence was carried out.

The sequence of *H*-D-Tyr-Pro-Gly-Lys-Arg-CH_2Cl is based on a site (-Pro-Gly-Lys-Arg-) in the thyrotropin-releasing hormone precursor that is cleaved by an as-yet-unidentified processing proteinase (Richter et al., 1984). Several of the remaining peptides incorporate an Arg residue at the P_4 subsite, a feature of numerous pro-proteins that are cleaved in the constitutive secretory pathway. Such sites are of particular interest in the activation of precursors of fusion glycoproteins of lipid-enveloped viruses, a step required for re-infection of host cells. The activation sites in influenza-virus haemagglutinins are important examples of this type, often containing not only Arg at P_4 but also additional Arg residues at P_3, P_5, and P_6.

The peptidyl chloromethanes synthesized in the present study permitted the *in vitro* evaluation of the effect of sequence variation on the inactivation of an authentic prohormone-processing enzyme. In an initial survey of the newly synthesized inhibitors (Table 2) it was of interest that the range of inhibitory effects on ss-Kex2 was quite wide. The sensitivity of the proteinase to some members of the group is remarkable. With the most effective inhibitors, nanomolar concentrations resulted in rapid inactivation. Initially, in the case of *H*-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl, the rate of inactivation observed was not clearly sensitive

to dilution at the inhibitor concentration used, and observations were limited by the need to approach the enzyme concentration itself. In all other cases the observations were clearly below saturation. To obtain more quantitative information on the interaction of ss-Kex2 with the two best inhibitors, *H*-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl and H-Phe-Ala-Lys-Arg-CH_2Cl were examined further. To ascertain the effective concentration of the inhibitors, inhibitor activity titrations were performed (see Figure 1 and the Methods section). The concentration of ss-Kex2 active sites measured by initial-burst titration (Brenner and Fuller, 1992) was taken as a constant. The effective concentration of *H*-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl and H-Phe-Ala-Lys-Arg-CH_2Cl were calculated to be 0.49- and 0.65-fold their nominal concentrations, respectively (Figure 1).

The rates of enzyme inactivation as a function of inhibitor concentration were measured (Figure 2). Reciprocal inactivation rates were plotted against reciprocal inhibitor concentrations (Kitz and Wilson, 1962; Kettner and Shaw, 1981) to obtain values of K_i (equilibrium constant for reversible binding of inhibitor to enzyme), k_2 (rate of covalent reaction of enzyme–inhibitor complex) and k_2/K_i (second-order rate constant for the overall reaction) according to the reaction scheme:

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} EI \xrightarrow{k_2} \text{alkylated } E \qquad (1)$$

H-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl had a K_i of 3.7 nM, a k_2 of 0.049 s^{-1} and a k_2/K_i value of $1.3 \times 10^7\ s^{-1} \cdot M^{-1}$. Binding as a transition-state analogue was indicated by the fact that the K_m for the corresponding synthetic substrate (Ac-Pro-Met-Tyr-Lys-Arg-NHMec, $K_m = 2.2\ \mu M$; Brenner and Fuller, 1992) exceeded K_i by a factor of 590. On the other hand, the overall rate of acylation and deacylation of the Michaelis complex of good substrates ($k_{cat.} = 25\ s^{-1}$) exceeded k_2 by 510-fold. k_2 for the peptide chloromethanes presumably reflects the rate at which the catalytic histidine attacks the chloromethyl group of the inhibitor, a reaction the enzyme has not evolved to perform. Nevertheless, the second-order rate for the reaction of ss-Kex2 with *H*-Pro-Nvl-Tyr-Lys-Arg-CH_2Cl slightly exceeded the $k_{cat.}/K_m$ reported ($1.1 \times 10^7\ s^{-1} \cdot M^{-1}$; Brenner and Fuller, 1992) with the best known ss-Kex2 substrate. This value also exceeded the k_2/K_i ($1.15 \times 10^7\ s^{-1} \cdot M^{-1}$; Kettner and Shaw, 1979) estimated for the reaction of D-Phe-Pro-Arg-CH_2Cl against thrombin, perhaps the most effective irreversible inhibitor of a serine proteinase previously created. *H*-Phe-Ala-Lys-Arg-CH_2Cl reacted with ss-Kex2 nearly as effectively as did the pentapeptide inhibitor, with a K_i of 2.4 nM, a k_2 of 0.019 s^{-1} and k_2/K_i value of $7.9 \times 10^6\ s^{-1} \cdot M^{-1}$. Measurement of inactivation rates as a function of inhibitor concentration is limited by the detectability of enzyme (Kettner and Shaw, 1979). Enzyme concentration was kept at 10% of inhibitor concentration so that the concentration of free inhibitor would not change much over the course of enzyme inactivation. Measurement of the kinetics of inactivation in this study were made possible by fluorescent detection of ss-Kex2 activity at levels below 100 pM.

The spread of values in Table 2 is considerable and reflects the fact that some of these sequences are less appropriate for Kex2. The presence of arginine at P_4 is relevant to certain processing events in mammalian cells, and concurrent studies are in progress on the inhibition of viral glycoprotein processing by host cells (Stieneke-Gröber et al., 1992). The importance of the Lys-Arg sequence for effectiveness against Kex2 is indicated by the low activity of Dec-Arg-Ala-Ile-Arg-CH_2Cl (cf. Table 2). One inhibitor was synthesized incorporating D-Arg-CH_2Cl, and the inactivation of ss-Kex2 by this compound was unexpected. The high reactivity encountered with certain members of this series of inhibitors is remarkable considering the resistance of subtilisin itself as discussed below.

In contrast with the chloromethanes, the peptidyl dimethylsulphonium salts containing two basic residues had no effect on Kex2 when examined at relatively high concentrations (Table 2). At higher concentrations some inhibition was observable, but was not progressive with time in a manner characteristic of affinity-labelling reagents. This behaviour was in contrast with the susceptibility of the processing activities acting on rat proinsulin (Rhodes et al., 1989). In extracts from that tissue, two distinguishable activities cleaving after -Lys-Arg- and -Arg-Arg- were inactivated readily by sulphonium salts corresponding to the specificity of each proteinase.

To assess the relative specificity of these inhibitors for Kex2, assays were performed with other serine proteinases.

We selected trypsin, plasmin, and thrombin which represent a subgroup of chymotrypsin enzymes family with specificity for cleavage carboxyl to lysine or arginine. Trypsin, a digestive protease, is the least specific of this group, whereas plasmin, a fibrinolytic proteinase, and thrombin, a procoagulant proteinase, are dependent on additional substrate sequence features that limit their physiological action. Among the inhibitors, we chose Phe-Ala-Lys-Arg-CH_2Cl, one of the most effective ss-Kex2 inactivators, and its decanoyl derivative. Finally we also included Dec-Arg-Ala-Ile-Arg-CH_2Cl which lacks a basic residue at P_2.

As shown in Table 3, Phe-Ala-Lys-Arg-CH_2Cl inactivated trypsin with a second-order rate constant of about $10^3\ M^{-1} \cdot s^{-1}$, about 1/800 the rate against ss-Kex2. Its effectiveness against plasmin and thrombin was even less. That this specificity for ss-Kex2 is due to the sequence of the inhibitor was shown by the superior rates of inactivation of plasmin and thrombin when sequences more appropriate for these specialized proteinases are contained in the reagent (Kettner and Shaw, 1981). The decanoyl derivative, Dec-Phe-Ala-Lys-Arg-CH_2Cl, was decisively more effective in inactivating thrombin and plasmin than the non-decanoylated derivative (Table 3). The result was quite different from the effect of this change on the response of ss-Kex2 (Table 2), where a loss was observed. In spite of these opposing results, the decanoylated derivative is still about 100-fold more effective in inactivating ss-Kex2 than plasmin and thrombin. The final inhibitor compared, Dec-Arg-Ala-Ile-Arg-CH_2Cl, is a relatively poor inhibitor of ss-Kex2, lacking the essential basic residue in P_2 (Table 2) and, in fact, at this point the inhibitor is more effective in inactivating trypsin and thrombin. The increase in the latter case is remarkable (Table 3).

The second major family of serine proteinases is the subtilisin family, to which Kex2 belongs. We therefore also examined two members of this family of microbial origin in our survey, subtilisin Carlsberg (Ottesen and Svendsen, 1970) and Proteinase K (Ebeling et al., 1974). These exhibit a conserved structural difference. Unlike subtilisin, proteinase K has disulphide bonds and a free -SH. However, these two have in common a specificity for binding hydrophobic amino acids in their P_1–P_4 subsites (Morihara et al., 1970; Ermer et al., 1990). It was, therefore, expected that the ss-Kex2 inhibitors described in the current work would not be effective against these proteinases. In fact, as shown in Table 3, at the highest concentration tested, 2×10^{-4} M, Phe-Ala-Lys-Arg-CH_2Cl, the chloromethane with unblocked amino group as well as the charged side chains in P_1 and P_2, showed no effect on either enzyme during a 1 h incubation. With decanoylation, an inhibitory activity was demonstrated, which was somewhat more pronounced with subtilisin Carlsberg (Table 3). The third inhibitor behaved similarly. The rates achieved in the inactivation of subtilisin Carlsberg with Dec-Phe-Ala-Lys-

Arg-CH_2Cl, (27.5 $M^{-1} \cdot s^{-1}$) and with Dec-Arg-Ala-Ile-Arg-CH_2Cl (21 $M^{-1} \cdot s^{-1}$) can be compared with the rate reported for the action of Z-Ala-Ala-Phe-CH_2Cl (2560 $M^{-1} \cdot s^{-1}$; Ermer et al., 1990). In connection with inactivation by peptidyl chloromethanes, the possible involvement of the -SH group near the active-centre histidine became of concern. However, the product formed on the inactivation of proteinase K by Z-Ala-Ala-CH_2Cl was examined and shown by Betzel et al. (1986) to be alkylated on the ring of His-68, leaving the side chain of -Cys free. A similar observation was made with thermitase by Ermer et al. (1990). These results are analogous to the site of chloromethane inactivation of subtilisin BPN′, which has no -SH group (Wright et al., 1969). In view of this common modification pattern, it is our expectation that the inhibitors described in our study with ss-Kex2 are also acting on the active-centre histidine and not on the conserved -SH group. The increase in reactivity towards ss-Kex2 compared with subtilisin Carlsberg and proteinase K can be attributed to great specificity of this processing proteinase compared with the microbial subtilisins and the fact that the superior reagents in Table 2 satisfy this specificity.

Although the decanoyl group has had a useful effect in promoting cellular permeability in these small inhibitors, the favourable or adverse effect of a decanoyl group in P_5 on the isolated proteinases may be due to local interactions in an extended substrate binding region. It is advisable to examine this detail of inhibitor structure on furin itself in the hope of optimizing its location.

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