Human Leukocyte Cathepsin G. Subsite Mapping with 4-Nitroanilides, Chemical Modification, and Effect of Possible Cofactors†

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ABSTRACT: The extended substrate binding site of cathepsin G from human leukocytes has been mapped by using a series of peptide 4-nitroanilide substrates. The enzyme has a significant preference for substrates with a P_1 Phe over those with the other aromatic amino acids Tyr and Trp. The S_2 subsite was mapped with the substrates Suc-Phe-AA-Phe-NA where AA was 13 of the 20 amino acid residues commonly found in proteins. The best residues were Pro and Met. The S_3 subsite was mapped with the sequence Suc-AA-Pro-Phe-NA by using 14 different amino acid residues for AA. The two best residues were the isosteric Val and Thr. No significant improvement in reactivity was obtained by extending the substrate to include seven different P_4 residues. The kinetic parameters for cathepsin G are significantly slower than those for many other serine proteases. Changes in the reaction conditions and addition of possible cofactors or ligands were in general found to have little effect on the enzymatic activity, while chemical modifications and proteolysis destroyed the activity of cathepsin G. Cathepsin G hydrolyzed peptides containing model desmosine residues and prefers the hydrophobic picolinoyllysine derivative over lysine by substantial margins at both the S_4 and S_2 subsites but will not tolerate it at S_3. Substrates with sequences related to the cathepsin G cleavage site in angiotensin I and angiotensinogen, and the reactive site of α_1-antichymotrypsin, were hydrolyzed effectively by enzyme, but with unexceptional rates. Our results indicate that the natural substrate(s) and function(s) of cathepsin G still remain to be discovered.

The granule fraction of human polymorphonuclear leukocytes contains almost equal amounts of two serine proteases, cathepsin G and human leukocyte (HL)[1] elastase. These proteases have given rise to increasing interest in recent years due to their possible involvement in connective tissue turnover and in diseases such as inflammation, rheumatoid arthritis, and emphysema in which connective tissue is destroyed. Both cathepsin G and HL elastase have major plasma inhibitors, α_1-antichymotrypsin and α_1-protease inhibitor (α_1-antitrypsin), respectively (Travis & Salvensen, 1983). Although the involvement of HL elastase in lung elastin turnover and emphysema is now quite clear, the exact function of cathepsin G is not yet known.

Cathepsin G has been implicated in the proteolysis of lung elastin (Reilly & Travis, 1980), collagen (Starkey et al., 1977), cartilage proteoglycan (Feinstein et al., 1976; Malemud & Janoff, 1975; Roughley & Barrett, 1977), and other connective tissue proteins and has been identified in rheumatoid synovium (Saklatvala & Barrett, 1980). It will cleave four human IgG subclasses (Baici et al., 1982a), human IgM (Baici et al., 1982b), and fibrinogen (Wintroub et al., 1980; Gramse et al., 1980). Fibronectin either in solution or in matrix form is a rather selective substrate for cathepsin G (Vartio et al., 1981), and the enzyme appears to slightly stimulate the elastolytic activity of HL elastase (Boudier et al., 1981a; Reilly et al., 1984).

The substrate specificity of HL cathepsin G has been studied with the oxidized insulin B chain (Blow & Barrett, 1977; Levy & Feinstein, 1979), extended peptide substrates (McRae et al., 1980), peptide 4-nitroanilide substrates (Yoshida et al., 1980; Nakajima et al., 1979; Zimmerman & Ashe, 1977; Boudier et al., 1981b), peptide thio esters (Harper et al., 1981, 1984), and with peptide chloromethyl ketone inhibitors (Powers et al., 1977). The enzyme is inactivated by various classes of inhibitors including heterocyclic transition-state analogues and acylating agents (Teshima et al., 1982; Zimmerman et al., 1980), sulfonyl fluorides (Yoshimura et al., 1982), and azapeptides (Gupton et al., 1984). Azapeptides are also useful for active-site titration of the enzyme.

In this paper, we report a systematic study of the subsite preference of cathepsin G at the S_4 through S_1' subsites.[2] We have investigated substrates containing residues similar to those found in cross-linking regions of elastin and containing sequences related to those of the α_1-antichymotrypsin reactive site and to the cathepsin G cleavage site in angiotensinogen. We have investigated the effect of proteolysis and possible cofactors on the enzymatic activity of HL cathepsin G. In addition, we have studied the pH optimum, the effect of organic solvents, and the effect of chemical modification. These studies should be useful in future efforts to define the exact physiological role of this abundant mammalian tissue serine protease.

Materials and Methods

Cathepsin G was prepared by previously described methods (Travis et al., 1978). Histamine, cortisone, hydrocortisone,

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[1] Abbreviations: NA, 4-nitroanilide; NA(2), 2-nitroanilide; NA(3), 3-nitroanilide; Boc, *tert*-butyloxycarbonyl; HL, human leukocyte; Me_2SO, dimethyl sulfoxide; Lys(Pic), ϵ-(2-picolinoyl)lysine; Suc, succinyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate; MeO-Suc, methoxysuccinyl; Ac, acetyl; Z, benzyloxycarbonyl; SBzl, $-SCH_2C_6H_5$; SBzl(Cl), $-SCH_2C_6H_4$-4-Cl.

[2] The nomenclature used for the individual amino acid residues (P_1, P_2, etc.) of a substrate and the subsites (S_1, S_2, etc.) of the enzyme is that of Schechter & Berger (1967).

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N-acetylneuraminic acid, *N*-acetylgluosamine, bovine brain lipid extract, calf skin collagen (type III), salmon sperm protamine, whale and shark cartilage chondroitin sulfate, ATP, and ADP were obtained from Sigma Chemical Co., St. Louis, MO. Mannose, glucose, and galacturonic acid were obtained from Eastman Organic Chemicals, Rochester, NY. Ascorbic acid was obtained from Aldrich Chemical Co., Milwaukee, WI.

The reagents used for chemical modifications were 1,2-cyclohexanedione, tetranitromethane, dithiothreitol, and *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K), which were obtained from Aldrich Chemical Co., and *N*-hydroxysuccinimide acetate, which was synthesized by the method of Lindsay & Shall (1971).

Boc amino acids were purchased from Bachem, Torrance, CA. All other chemicals were reagent grade. The syntheses of all new compounds are described in the supplementary material (see paragraph at end of paper regarding supplementary material).

Kinetic Measurements. The nitroanilide substrates were dissolved in 0.1 M Hepes buffer, pH 7.5, containing 0.5 M NaCl and 10% Me_2SO. The enzymatic reactions were initiated by addition of a 50-μL aliquot of the appropriate enzyme solution to 2.0 mL of a substrate (with or without added cofactor) solution contained in a spectrophotometer cuvette. The rates were measured at 25 °C with a Beckman Model 25 spectrophotometer. The wavelengths were 410 nm for the 4- and 3-nitroanilides and 450 nm for the 2-nitroanilide, respectively. The following ϵ values were used: 8800 M^{-1} cm^{-1} for the 4-nitroanilides (Erlanger et al., 1961); 2700 and 1300 M^{-1} cm^{-1} for the 2- and 3-nitroanilides, which were determined by the method of Erlanger et al. (1961) using Suc-Val-Pro-Phe-NA(2) [or -NA(3)]. Kinetic constants were determined from the initial rates of hydrolysis by using Lineweaver–Burk plots and five separate substrate concentrations. Correlation constants were greater than 0.99 in all cases. The concentration of cathepsin G was determined with Suc-Ala-Ala-Pro-PhehNA using kinetic constants that were based on active-site titrated enzyme (Nakajima et al., 1979).

Chemical Modifications. Arginine residues were modified with 1,2-cyclohexanedione by the method of Patthy & Smith (1975). The reaction mixture contained 0.2 M sodium borate buffer, pH 8.5, 0.5 M NaCl, 0.5 μM cathepsin G, and 1 mM reagent in a final volume of 2.0 mL. The mixture was incubated at 25 °C, and 50-μL aliquots were removed for assay of the enzymatic activity.

Carboxyl groups were modified with Woodward's reagent K (Petra, 1971). The reaction mixture contained 0.1 M Hepes buffer, pH 6.5, 0.5 M NaCl, 0.5 μM cathepsin G, and 0.1 mM reagent in 1 mM HCl, in a final volume of 2.0 mL.

Tyrosine residues were modified with tetranitromethane by the method of Sokolovsky et al. (1966), and amino groups were modified with *N*-hydroxysuccinimide acetate by the method of Lindsay & Shall (1971). Reduction of the enzyme in the presence of dithiothreitol was carried out by the method of Acharya & Taniuchi (1974).

Proteolytic Reactions. Reaction mixtures containing 0.5 μM cathepsin G and either HL elastase (5 nM) or trypsin (5 nM) in a final volume of 8.0 mL were incubated. Aliquots were removed at specified time intervals and measured for cathepsin G hydrolytic activity with Suc-Val-Pro-Phe-NA as a substrate.

RESULTS

Kinetic Studies. The kinetic parameters for the reaction of cathepsin G with a series of 4-nitroanilide substrates are reported in Table I. The effects of amino acid substitutions at the P_1, P_2, P_3, and P_4 subsites of the substrate were each investigated in turn.

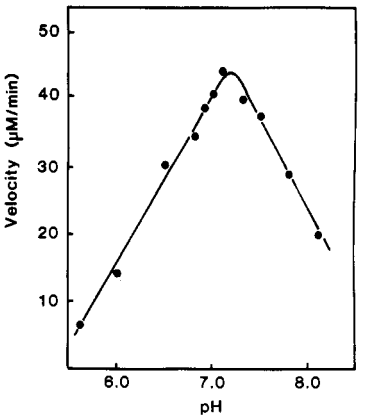

FIGURE 1: Plot of velocity (micromolar per minute) as a function of pH for the hydrolysis of Suc-Val-Pro-Phe-NA (1.46 mM) by cathepsin G (0.12 μM). A 0.1 M Hepes buffer containing 0.5 M NaCl and 10% Me_2SO at 25 °C was used.

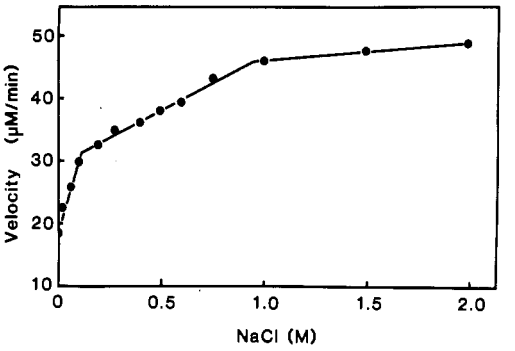

FIGURE 2: Plot of velocity (micromolar per minute) as a function of NaCl concentration for the hydrolysis of Suc-Val-Pro-Phe-NA (1.46 mM) by cathepsin G (0.12 μM). A 0.1 M Hepes buffer containing 10% Me_2SO at 25 °C was used.

The 2- and 3-nitroanilide substrates corresponding to the 4-nitroanilide Suc-Val-Pro-Phe-NA were synthesized and studied to learn if the P_1 leaving group was significantly affecting hydrolysis (Table II). In each case, the extent of hydrolysis of substrate was determined by measurement of 2-nitroaniline at 450 nm or 3-nitroaniline at 410 nm, wavelengths at which the extinction coefficients were 2700 or 1340 cm^{-1} M^{-1}, respectively. The starting anilides made no contribution to the absorbance at these wavelengths. The hydrolysis rates of these chromogenic substrates by cathepsin G were 53-fold (2-nitro) and 18-fold (3-nitro) slower than that of the corresponding 4-nitroanilide.

Effect of Kinetic Conditions and Metal Ions. We next investigated various reaction conditions in order to improve hydrolysis rates. The pH optimum for the hydrolysis of Suc-Val-Pro-Phe-NA was found to be 7.1–7.2 (Figure 1). Under our standard conditions at pH 7.5, the enzymatic activity of cathepsin G was ca. 80% of the optimal value.

The hydrolytic activity of the enzyme was 2- or 2.7-fold higher in the presence of 0.5 or 2.0 M NaCl, respectively, than that in the absence of NaCl (Figure 2). There was no effect on the hydrolysis rate of the substrate Suc-Val-Pro-Phe-NA by the enzyme in buffers containing from 2.5 up to 18% (v/v) Me_2SO (Figure A, supplementary material). Furthermore, we tested the effect of other cations on the activity of cathepsin G (Figure 3). The activity was strongly stimulated by 1.6 M KCl (ca. 70% increase). $MgCl_2$ was almost as effective as NaCl, whereas $CaCl_2$ depressed activity at 1.2 M by 50%.

Table I: Kinetic Constants for Hydrolysis of 4-Nitroanilide Substrates by Human Cathepsin G[a]

substrate P_5 P_4 P_3 P_2 P_1	substrate concn range (mM)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
		Effect of P_1 Residue		
Suc-Val-Pro-Phe-NA	2.0–0.25	1.4	9.6	6900
Suc-Val-Pro-Leu-NA	1.4–0.17	2.6	4.5	1700
Suc-Val-Pro-Met-NA	2.1–0.26	3.4	5.5	1600
Suc-Val-Pro-Tyr-NA	0.20–0.025	0.26	0.29	1100
Suc-Val-Pro-Trp-NA	1.0–0.13	0.22	0.15	680
		Effect of P_2 Residue		
Suc-Phe-Pro-Phe-NA[b]	2.0–0.25	1.5	5.3	3500
Suc-Phe-Met-Phe-NA	0.8–0.10	0.52	1.8	3500
Suc-Phe-Ala-Phe-NA	0.8–0.10	1.0	2.2	2200
Suc-Phe-Val-Phe-NA	0.4–0.05	1.4	2.3	1600
Suc-Phe-Leu-Phe-NA[b]	0.2–0.05	0.62	0.95	1500
Suc-Phe-Thr-Phe-NA	0.4–0.05	1.2	1.4	1200
Suc-Phe-Ser-Phe-NA	1.0–0.13	1.7	1.2	710
Suc-Phe-Phe-Phe-NA	0.05–0.006	0.060	0.023	380
Suc-Phe-Glu-Phe-NA	2.0–0.25	3.4	1.0	290
Suc-Phe-Gln-Phe-NA	0.4–0.05	0.53	0.14	260
Suc-Phe-Lys-Phe-NA	0.4–0.05	2.6	0.8	260
Suc-Phe-Trp-Phe-NA	0.05–0.006	0.11	0.0036	32
Suc-Phe-Gly-Phe-NA	2.0			NR[c]
		Effect of P_3 Residue		
Suc-Thr-Pro-Phe-NA	2.0–0.25	3.4	18	5300
Suc-Met-Pro-Phe-NA	2.0–0.25	2.5	9.0	3600
Suc-Glu-Pro-Phe-NA	2.0–0.25	2.6	7.2	2800
Suc-Lys(Z)-Pro-Phe-NA	0.012–0.25			220
Suc-Gln-Pro-Phe-NA	2.0–0.25	8.9	9.5	1100
Suc-Leu-Pro-Phe-NA	2.0–0.25	4.7	5.1	1100
Suc-Ser-Pro-Phe-NA	2.0–0.25	1.9	1.9	1000
Suc-Ala-Pro-Phe-NA	1.0–0.13	3.3	2.0	610
Suc-Lys-Pro-Phe-NA	0.93–0.12	4.0	0.90	230
Suc-Gly-Pro-Phe-NA	2.0–0.33	16	0.67	42
Suc-Pro-Pro-Phe-NA	2.0–0.25	29	0.22	7.6
		Effect of P_4 residue		
Suc-Met-Val-Pro-Phe-NA	2.0–0.25	2.0	14	7000
Suc-Leu-Val-Pro-Phe-NA	2.1–0.26	2.5	13	5200
Suc-Phe-Val-Pro-Phe-NA	0.32–0.040	1.0	5.1	5100
Suc-Ala-Val-Pro-Phe-NA	0.23–0.029	1.2	6.0	5000
Suc-Lys-Val-Pro-Phe-NA	3.3–0.41	8.5	24	2800
Suc-Glu-Val-Pro-Phe-NA	2.3–0.29	8.3	18	2200

[a] Conditions: pH 7.5, 0.1 M Hepes, 0.5 M NaCl, and 10% Me_2SO at 25 °C. [b] Data of Yoshida et al. (1980). [c] NR, no reaction.

Table II: Effect of the Leaving Group on Hydrolysis of Suc-Val-Pro-Phe Nitroanilides[a]

nitroanilide	wavelength (nm)[b]	cleavage (1%/min)	rel act.
4-nitro (para)	410 (8800)	4.3	1.0
2-nitro (ortho)	450 (2730)	0.083	0.019
3-nitro (meta)	410 (1340)	0.24	0.056

[a] Conditions: pH 7.5, 0.1 M Hepes, 0.5 M NaCl, and 10% Me_2SO at 25 °C, Substrate concentration was 1.1 mM for the para isomer, 0.99 mM for the ortho isomer, and 0.98 mM for the meta isomer. [b] Values in parentheses in this column are ϵ values (M^{-1} cm^{-1}).

Effect of Possible Cofactors or Ligands. The effects of several low molecular weight ligands [histamine, saccarides (glucose, mannose, *N*-acetylneuraminic acid, galacturonic acid, *N*-acetylglucosamine, and ascorbic acid), steroids (cortisone and hydrocortisone), ATP, ADP, H_2O_2, and SDS] and a few high molecular weight substances (collagen, protamine, bovine brain lipid, and chondroitin sulfate) on the hydrolysis of Suc-Val-Pro-Phe-NA by cathepsin G were studied at a variety of concentrations (Figures 4 and 5).

Galacturonic acid, *N*-acetylglucosamine, and ascorbic acid slightly inhibited the enzyme. However, the other small molecules generally had no effect except for SDS which inhibited the enzyme. On the other hand, protamine and collagen stimulated the rate up to 35% at 1.2 mg/mL and 50% at 1.9 mg/mL, respectively. The activity was slightly increased by chondroitin sulfate or bovine brain lipid.

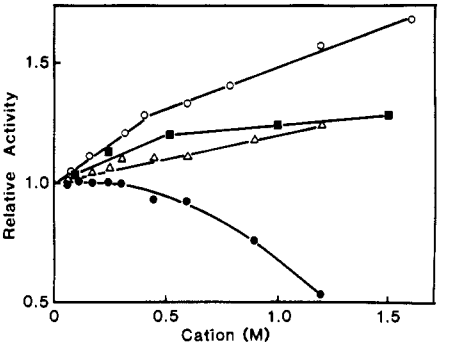

FIGURE 3: Effect of cation concentration on the activity of cathepsin G. Assays were performed at pH 7.5 in 0.1 M Hepes buffer, 0.5 M NaCl, and 10% Me_2SO, 25 °C, and with Suc-Val-Pro-Phe-NA (1.46 mM) as a substrate. The results are expressed as the relative activity. The concentration of sodium (squares) is given in term of the concentration greater than 0.5 M NaCl which was contained in all the assays. Other cations used were potassium (open circles), magnesium (triangles), and calcium (closed circles).

Effect of Proteolysis. To evaluate the possibility of increased enzymatic activity upon proteolysis, cathepsin G was incubated with HL elastase or trypsin at 25 °C (cathepsin G/protease ratio = 100). As shown in Figure B in the supplementary material, the activity of cathepsin G was stable for ca. 30 min before it began to decrease. After 1200 min,

Table III: Kinetic Constants for Cathepsin G Hydrolysis of Peptide 4-Nitroanilide Substrates Containing Model Desmosine Residues[a]

substrate P_4 P_3 P_2 P_1	substrate concn range (mM)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat}/K_M, Lys derivative (M^{-1} s^{-1})	k_{cat}/K_M ratio, Lys(Pic)/Lys
Suc-Phe-Lys(Pic)-Phe-NA	0.04–0.21			3400	260	13
Suc-Lys(Pic)-Pro-Phe-NA	0.28			NH[b]	230	0
Suc-Lys(Pic)-Val-Pro-Phe-NA	0.40–0.79	0.63	5.1	8100	2800	2.9

[a] Conditions: 0.1 M Hepes, 0.5 M NaCl, and 10% Me_2SO, pH 7.5, 25 °C. [b] No hydrolysis.

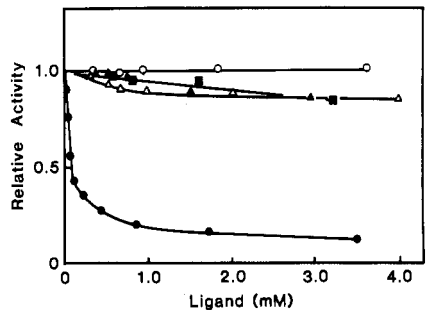

FIGURE 4: Effect of ligand concentration on the activity of cathepsin G. Assays were performed at pH 7.5 in 0.1 M Hepes buffer, 0.5 M NaCl, and 10% Me_2SO, 25 °C, and with Suc-Val-Pro-Phe-NA (1.46 mM) as a substrate. The results are expressed as relative activity. The compounds tested were SDS (closed circles), ascorbic acid (open triangles), galacturonic acids (closed triangles), *N*-acetylglucosamine (squares), and histamine (open circles). The following compounds had no effect at the same concentration range employed with histamine: cortisone, hydrocortisone, sialic acid, glucose, mannose, ATP, ADP, and H_2O_2.

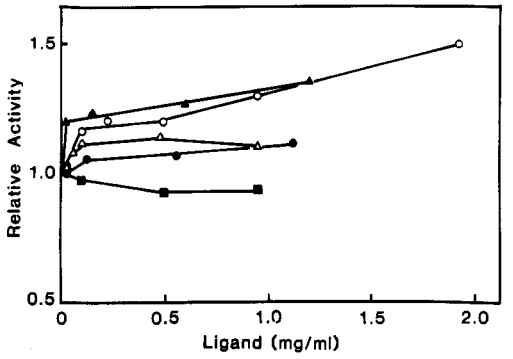

FIGURE 5: Effect of ligand concentration on the activity of cathepsin G. Assays were performed at pH 7.5 in 0.1 M Hepes buffer, 0.5 M NaCl, and 10% Me_2SO, 25 °C, and with Suc-Val-Pro-Phe-NA (1.45 mM) as a substrate. The results are expressed as relative activity. The compounds tested were protamine (closed triangles), type III collagen (open circles), bovine brain lipid extract (open triangles), chondroitin sulfate (closed circles), and heparin (squares).

the enzyme had only 40% of the control activity. Both HL elastase and trypsin inactivated cathepsin G at roughly the same rates.

Chemical Modifications. We next evaluated the effect of chemical modification on the enzymatic activity. All of the modifications were carried out in the presence of NaCl to stabilize the enzyme. With 1 mM 1,2-dicyclohexanedione, the enzymatic activity of cathepsin G decreased to 10% of the control value after 60 min (Figure 6). Woodward's reagent K at a concentration of 0.1 mM caused an 80% loss of activity within 30 min (Figure C of the supplementary material). Modification of the tyrosine residues with tetranitromethane resulted in a slight loss of the activity. Acylation of the amino groups with *N*-hydroxysuccinimide acetate and reaction with dithiothreitol had no effect on the enzyme activity.

Substrates with Model Desmosine Residues. We have previously shown that HL elastase preferentially hydrolyzes peptide substrates containing Lys residues which have been

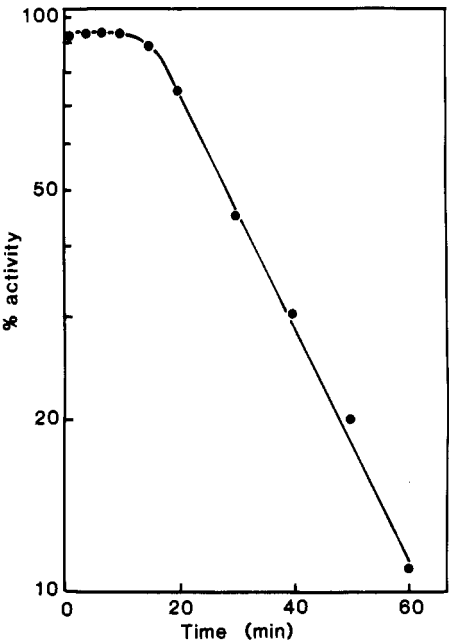

FIGURE 6: Time course for the inactivation of cathepsin G by 1,2-cyclohexanedione. The reaction mixture contained 0.48 μM cathepsin G and 1.0 mM 1,2-cyclohexanedione in a final volume of 2.0 mL. Incubation was carried out at 25 °C, pH 8.5, in 0.2 M sodium borate buffer and 0.5 M NaCl. At the time intervals shown, a 50-μL aliquot of the mixture was removed and added to a cuvette containing 2.0 mL of Suc-Val-Pro-Phe-NA (1.46 mM) solution (pH 7.5, 0.1 M Hepes buffer, 0.5 M NaCl, and 10% Me_2SO) and assayed for activity. The remaining enzyme activity was plotted vs. time.

Table IV: Kinetic Constants for Hydrolysis of Peptide 4-Nitroanilide Substrates Containing Model Desmosine Residues by Human Leukocyte Elastase[a]

substrate P_4 P_3 P_2 P_1	substrate concn range (mM)	k_{cat}/K_M (M^{-1} s^{-1})
Suc-Phe-Lys(Pic)-Phe-NA	0.38–0.08	21
Suc-Lys(Pic)-Pro-Phe-NA	0.29–0.09	54
Suc-Lys(Pic)-Val-Pro-Phe-NA	0.13–0.08	26

[a] Human leukocyte elastase was pretreated with 100 equiv of the cathepsin G inhibitor Z-Gly-Leu-Phe-CH_2Cl (Powers et al., 1977) to remove any possible contaminating cathepsin G in the HL elastase sample. Conditions were pH 7.50, 1 M Hepes buffer, 0.5 M NaCl, and 10% Me_2SO at 25 °C.

modified to resemble the desmosine cross-links in elastin (Yasutake & Powers, 1981). We decided to investigate the effect of one of these amino acid residues, the picolinoyl derivative of lysine, at the P_2, P_3, and P_4 subsites of a good cathepsin G peptide substrate. The results are listed in Table III. As can be seen, cathepsin G prefers the hydrophobic lysine derivatives over lysine by substantial margins at both the S_4 and S_2 subsites but will not tolerate it at the S_3 subsite. In order to see if the desmosine-like residues would overcome the S_1 specificity of HL elastase, we also measured hydrolysis rates with that enzyme (Table IV). In each case, the substrates were hydrolyzed with small but measurable rates.

Table V: Kinetic Constants for Hydrolysis by Human Cathepsin G of 4-Nitroanilide Substrates with Sequences of Physiologically Important Substrates and Inhibitors[a]

substrate P_4 P_3 P_2 P_1 P_1'	substrate concn range (mM)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Human Angiotensinogen and Angiotensin I				
-Ile-His-Pro-Phe-His-Leu		0.29	0.6	2070[b]
		0.22	3.4	15000[c]
Suc-His-Pro-Phe-NA	0.11–0.014	0.36	0.17	470
Suc-Ile-His-Pro-Phe-NA	0.10–0.017	0.47	0.18	380
Human α_1-Antichymotrypsin				
Ile-Thr-Leu-Leu-Ser-				
Suc-Ile-Thr-Leu-Leu-Ser-NA	0.11–0.03			24[d]
Suc-Ile-Thr-Leu-Leu-Phe-NA	0.017–0.008	0.052	0.087	1700
Suc-Ile-Thr-Leu-Leu-NA	1.03–0.16	0.36	0.94	2600

[a] Conditions: pH 7.5, 0.1 M Hepes, 0.5 M NaCl, and 10% Me_2SO at 25 °C. [b] Cleavage of angiotensin I to angiotensin II at pH 7.5 and 37 °C (Reilly et al., 1982). [c] Cleavage of angiotensin I to angiotensin II at pH 7.4 in 0.01 M Tris, pH 7.4, and 1.0 M NaCl at 37 °C (Klickstein et al., 1982). [d] The Lineweaver–Burk plot went through the origin, and only k_{cat}/K_M could be determined.

Substrates with Sequences Related to Physiologically Important Substrates and Inhibitors. Cathepsin G has recently been shown to be capable of cleaving angiotensinogen or angiotensin I with the release of angiotensin II (Tonnesen et al., 1982; Klickstein et al., 1982; Reilly et al., 1982 Weintroub et al., 1984). Since we had previously shown that the Pro-Phe sequence was quite favorable for cathepsin G [Suc-Phe-Pro-Phe-NA was the best substrate reported by Yoshida et al. (1980)], we decided to investigate the cathepsin G hydrolysis of 4-nitroanilide substrates with the angiotensin I sequence. The data are reported in Table V and are compared with the data reported for the cleavage of angiotensin I itself. The K_M values for both the tri- and the tetrapeptide are quite similar to those reported for angiotensin I itself. The k_{cat} values for the two 4-nitroanilides are at least 4-fold lower than that for angiotensin I. There is a discrepancy between the k_{cat}/K_M values reported for angiotensin I cleavage by the two separate research groups. The first value in the table was measured with titrated cathepsin G, while the second group did not appear to standardize the enzyme and this may explain the different k_{cat} values. If we compare the lower value of 2070 s^{-1} with the values for the synthetic peptide and take into account the temperature difference, we conclude that the k_{cat} values for the synthetic peptides are within a factor of 2 of the angiotensin I value.

The reactive site sequence of α_1-antichymotrypsin has recently been determined (Morii & Travis, 1983). We synthesized three sequences related to this sequence, and kinetic results with these peptides are reported in Table V. The most reactive peptide was the tetrapeptide Suc-Ile-Thr-Leu-Leu-NA with the P_4–P_1 sequence of the reactive site. The other two peptides contain the P_4–P_1' sequence of α_1-antichymotrypsin or the P_4–P_1' sequence in which the P_1' residue was replaced by Phe. These peptides were synthesized at a time when it was uncertain whether cathepsin G was cleaving the Leu-Ser bond or the bond following Ser. Suc-Ile-Thr-Leu-Leu-Ser-NA is a very poor substrate, which is consistent with the observed reactive site sequence of α_1-antichymotrypsin.

Since it was possible that Suc-Ile-Thr-Leu-Leu-Ser-NA was being cleaved at the Leu-Ser bond in addition to the Ser-NA bond, we examined the cathepsin G reaction mixture using thin-layer chromatography. The reaction mixture (pH 7.5, 0.1 M Hepes, 0.5 M NaCl, and 10% Me_2SO at 25 °C) of Suc-Ile-Thr-Leu-Leu-Ser-NA (0.11 mM) and cathepsin G (9.7×10^{-8} M) was spotted on a silica gel plate (Merck; 5 × 20 cm) at several reaction times and developed by using a $CHCl_3$–MeOH (5:1 v/v) system with Suc-Ile-Thr-Leu-Leu-Ser-NA, Ser-NA, and 4-nitroaniline as standards. Ninhydrin-positive products were identified by spraying with 0.3% ninhydrin–acetone and heating on a hot plate. Another TLC plate was sprayed with concentrated sulfuric acid and then heated on a hot plate to identify all products. After 24 h, a faint spot with R_f of 4-nitroaniline was detected. No Ser-NA or any other spots were detected.

We then evaluated the two pentapeptides as competitive inhibitors. Using Suc-Val-Pro-Phe-NA as a substrate, we found Suc-Ile-Thr-Leu-Leu-Ser-NA to be a moderate competitive inhibitor with a K_I value of 0.11 mM using a Dixon plot. Suc-Ile-Thr-Leu-Leu-Phe-NA showed no inhibition at 0.016 mM which was the maximum that its solubility allowed us to measure.

Discussion

Cathepsin G is still something of an enigma. It is present in large amounts in the granule fraction of leukocytes and has a specific plasma inhibitor, α_1-antichymotrypsin. Yet there is still no prescribed function for the enzyme, and no disease state has yet been correlated with an excess of cathepsin G or with the absence of α_1-antichymotrypsin. In contrast, the companion protease of cathepsin G (human leukocyte elastase) and its inhibitor [α_1-protease inhibitor (α_1-antitrypsin)] are intimately involved in the pathogenesis of emphysema.

Another enigma concerns the kinetic behavior of cathepsin G. Thus far, it has exhibited quite low reactivity toward many types of peptide substrates and inhibitors. It is much more difficult to inhibit than enzymes such as HL elastase, and cathepsin G hydrolyzes synthetic substrates much more slowly. We began this study with systematic subsite mapping of cathepsin G to learn whether the low kinetic reactivity of cathepsin G was simply due to an inappropriate choice of substrate sequences.

Subsite Mapping. The extended substrate binding site of HL cathepsin G has previously been investigated with 4-nitroanilide substrates (Yoshida et al., 1980; Nakajima et al., 1979; Zimmerman & Ashe, 1977), peptide substrates (McRae et al., 1980), peptide chloromethyl ketone inhibitors (Powers et al., 1977), and peptide thio ester substrates (Harper et al., 1981, 1984). None of these studies evaluated systematically the subsite preferences of HL cathepsin G using a large number of amino acid residues chosen to be representative of those found in proteins. In particular, most previous studies only dealt with amino acid residues that offered no particular problems in synthesis. In this study, we have deliberately chosen at least one amino acid residue to represent each class of amino acid residue normally found in proteins.

Since cathepsin G had previously been shown to prefer aromatic amino acid residues at P_1 (Zimmerman & Ashe, 1977; Harper et al., 1984; Blow & Barrett, 1977; Levy &

Feinstein, 1979), we only investigated substrates containing P_1 aromatic residues (Phe, Tyr, and Trp) and those with large hydrophobic side chains (Met and Leu). The most effective 4-nitroanilide substrate previously reported is Suc-Phe-Pro-Phe-NA which has a k_{cat}/K_M value of 3500 M^{-1} s^{-1} (Yoshida et al., 1980).

The S_1 subsite of cathepsin G exhibited a substantial preference for Phe over the other two aromatic amino acids, Tyr and Trp. Indeed, the P_1 Met and Leu substrates were much better than either Tyr or Trp. The effect was in both k_{cat} and K_M; the Tyr (Suc-Val-Pro-Tyr-NA) and Trp (Suc-Val-Pro-Trp-NA) peptides bound more tightly to the enzyme than Suc-Val-Pro-Phe-NA but were hydrolyzed less effectively. This specificity should be contrasted with chymotrypsin A_α, where the preference of bond cleavage in peptide substrates is Trp > Tyr > Phe > Leu > Met (A. D. Harley, D. V. Myers, and J. C. Powers, unpublished results).

In order to further evaluate the nature of the S_1 binding pocket of cathepsin G, we measured K_I values for eight aromatic compounds, 6 derivatives of phenylethylamine, and 19 indoles (Tables A, B, and C of the supplementary material). The best inhibitors of the group of aromatic compounds were phenothiazine (K_I = 0.16 mM) and chalcone (K_I = 0.2 mM), both compounds with several aromatic rings. All of the phenylethylamine derivatives (phenylethylamine amides contain the equivalent of the side chain of Phe) had K_I values >5 mM. The most potent inhibitors were the indoles, where K_I values as low as 0.01 mM were observed. All the good indolic inhibitors such as 2-methyl-3-(4-nitrophenyl)-5-nitroindole and 2-methyl-3-phenylindole had several aromatic rings, and the simple indoles which resembled the Trp side chain were much poorer (K_I > 2 mM). This indicates that the good indolic inhibitors are probably binding to additional hydrophobic regions in the vicinity of the primary specificity site of cathepsin G.

We mapped the S_2 subsite of cathepsin G using 4-nitroanilides with Suc-Phe-AA-Phe-NA where AA was 13 of the 20 amino acid residues commonly found in proteins. The best amino acid residue at P_2 was Pro or Met. It is interesting to note that the substrates Suc-Phe-Phe-Phe-NA and Suc-Phe-Trp-Phe-NA, which have the lowest K_M values, also have several adjacent aromatic rings similar to the best indolic inhibitors of cathepsin G. This would indicate that the extended substrate binding site of cathepsin G is quite hydrophobic. The high K_M values for the substrates with a P_2 Lys or Glu are consistent with this view.

Since a P_2 Pro residue restricts multiple binding modes in most serine proteases, we decided to use the Suc-AA-Pro-Phe-NA sequence to map the S_3 subsite of cathepsin G using 14 different amino acid residues. the two best substrates were Suc-Val-Pro-Phe-NA and Suc-Thr-Pro-Phe-NA. Both of these substrates have side chains which are isosteric with each other. The two substrates with a P_3 Met or Phe were also quite good. All of the best substrates had good k_{cat} values, with their K_M being no better than those of the other substrates.

Extension of the sequence to evaluate the importance of P_4 was accomplished with Suc-AA-Val-Pro-Phe-NA, where AA was seven different amino acid residues. Again the peptides with hydrophobic amino acid residues in P_4 were much better than those with polar residues (Lys or Glu). However, no significant improvement was obtained by extending Suc-Val-Pro-Phe-NA (k_{cat}/K_M = 6900 M^{-1} s^{-1}) to Suc-Met-Val-Pro-Phe-NA (k_{cat}/K_M = 7000 M^{-1} s^{-1}).

Effect of Possible Cofactors or Ligands. One disappointment in this study was the limited improvement that we were able to obtain by subsite mapping of cathepsin G. The best substrate Suc-Val-Pro-Phe-NA discovered is only 4.6-fold better than our previous best substrate, Suc-Phe-Pro-Phe-NA (Yoshida et al., 1980). Therefore, we decided to optimize the assay conditions, and we measured the pH dependence and the effect of cations and Me_2SO. In agreement with previous reports, we found that cathepsin G activity was stimulated by NaCl, KCl, and $MgCl_2$ but was inhibited by $CaCl_2$ (Starkey & Barrett, 1976). This is in contrast to many other serine proteases which are stabilized by calcium. Cathepsin G requires high ionic strength (Schmidt & Havemann, 1977) and was not significantly affected by Me_2SO. However, all of the observed effects were quite small and still did not explain the low kinetic parameters obtained with peptide 4-nitroanilide substrates.

A number of serine proteases have been shown to be influenced by external cofactors or ligands. Examples include the ATP-dependent protease from *Escherichia coli* and the vitamin K dependent blood coagulation proteases which require cofactors such as phospholipids and calcium. Therefore, we next decided to search for such a cofactor for cathepsin G. Although we investigated a larger number of possible cofactors or ligands which could conceivably be present in cathepsin G's environment, none of the low molecular weight substances significantly affected the enzyme's activity. Several carbohydrates slightly inhibited the enzyme which is consistent with the recent observation that glycosaminoglycans are moderate inhibitors of cathepsin G (Marossy, 1981).

Two high molecular weight ligands, collagen and protamine, stimulated the activity of cathepsin G. The effect of protamine is surprising since cathepsin G is cationic and would not be expected to interact with basic materials. Collagen is slowly hydrolyzed by cathepsin G, and in addition, it slightly stimulates the activity of the enzyme toward synthetic peptide 4-nitroanilide substrates. Cathepsin G is tightly bound to the neutrophil granule and requires high salt concentration for its solubilization. Once released, it is possible that macromolecules such as collagen are acting as a reaction matrix on which cathepsin G carries out its function.

The ionic properties of cathepsin G also seem to play an important role in its enzymatic activity. Chemical modification of the arginine residues with 1,2-dicyclohexanedione and of the carboxyl groups with Woodward's reagent K resulted in a loss of activity.

Conclusion. The primary function of the neutrophilic granulocyte is phagocytosis, i.e., the killing of microorganisms. Two major proteases are present, HL elastase and cathepsin G in roughly equal amounts, and the plasma contains two protease inhibitors (α_1-protease inhibitor and α_1-antichymotrypsin) to protect against either of these enzymes which may escape. If both enzymes are involved in the same physiological function, why do they have such different catalytic properties? Comparison of the rates of hydrolysis of the best 4-nitroanilide substrates (HL elastase, MeO-Suc-Ala-Ala-Pro-Val-NA, k_{cat}/K_M = 330000 M^{-1} s^{-1}; cathepsin G, Suc-Val-Pro-Phe-NA, 6900 M^{-1} s^{-1}), best extended peptide substrate (HL elastase, Ac-Ala-Ala-Pro-Val*Thr-Ala-NH_2, where the asterisk indicates the bond cleaved, k_{cat}/K_M = 71000 M^{-1} s^{-1}; cathepsin G, Ac-Ala-Ala-Pro-Phe*Ala-Ala-NH_2, 1000 M^{-1} s^{-1}), best peptide chloromethyl ketone inhibitor (HL elastase, MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl, $k_{obsd}/[I]$ = 1560 M^{-1} s^{-1}; cathepsin G, Z-Gly-Leu-Phe-CH_2Cl, 51 M^{-1} s^{-1}), best sulfonyl fluoride irreversible inhibitor [HL elastase, 2-$(CF_3CF_2CONH)C_6H_4S_2F$, $k_{obsd}[I]$ = 1700 M^{-1} s^{-1}; cathepsin G, 2-(Z-Gly-NH)$C_6H_4SO_2F$, 19 M^{-1} s^{-1}], and the best peptide

thio ester substrate [HL elastase, Boc-Ala-Pro-Nva-SBzl(Cl), k_{cat}/K_M = 130 000 000 M^{-1} s^{-1}; cathepsin G, Boc-Ala-Ala-Phe-SBzl, 1 000 000 M^{-1} s^{-1}] shows that cathepsin G is in each case much less enzymatically active than its leukocyte partner HL elastase. Usually, cathepsin G is ca. 2 orders of magnitude less reactive. Many other serine proteases such as bovine pancreatic trypsin, chymotrypsin, and the rat mast cell protease I among others have kinetic properties with their best substrates and inhibitors of the same magnitude as those of HL elastase. Therefore, one must conclude that cathepsin G is exceptionally unreactive. Since this behavior is exhibited by such a variety of substrates and inhibitors, and since our efforts to improve the reactivity of the enzyme have not yet met with significant success, we conclude that the low intrinsic kinetic reactivity of cathepsin G is an inherent property of the enzyme and is related to the function of this enzyme.

Cathepsin G appears to be extremely effective at cleaving cartilage proteoglycans (Roughley & Barrett, 1977), and fibronectin acts as a specific substrate (Vartio et al., 1981). Is it possible that there is some sequence or structural element in these macromolecules that increases the kinetic reactivity of the enzyme? This would appear to be unlikely since there is a large amount of enzyme present in leukocytes. A highly specific enzyme would probably only be needed in small amounts, while a general degradative enzyme would been needed in more substantial amounts. Cathepsin G will cleave peptide sequences related to those in cross-linking regions of elastin, but with unexceptional rates. It will cleave angiotensinogen, angiotensin I (Tonnesen et al., 1982; Klickstein et al., 1982; Reilly et al., 1982; Wintroub et al., 1984) and related peptide sequences. It will bind to and cleave sequences related to the reactive site of α_1-antichymotrypsin, but again with unexceptional rates. Thus, although there are intriguing suggestions and possibilities, the natural substrate(s) and function(s) of cathepsin G still remain to be elucidated.

Acknowledgments

The studies on the inhibition of cathepsin G by aromatic compounds, amides of phenylethylamine, and indoles, which are reported in the supplementary material, were carried out by Larry Barker.

Supplementary Material Available

Experimental details describing the synthesis of all new compounds (24 pages). Ordering information is given on any current masthead page.

Registry No. Suc-Val-Pro-Phe-NA, 95192-11-3; Suc-Val-Pro-Leu-NA, 95363-62-5; Suc-Val-Pro-Met-NA, 95192-15-7; Suc-Val-Pro-Tyr-NA, 95192-12-4; Suc-Val-Pro-Trp-NA, 95192-13-5; Suc-Phe-Pro-Phe-NA, 75651-68-2; Suc-Phe-Met-Phe-NA, 95192-22-6; Suc-Phe-Ala-Phe-NA, 95192-21-5; Suc-Phe-Val-Phe-NA, 95192-18-0; Suc-Phe-Leu-Phe-NA, 75651-69-3; Suc-Phe-Thr-Phe-NA, 95192-20-4; Suc-Phe-Ser-Phe-NA, 95192-24-8; Suc-Phe-Phe-Phe-NA, 95363-63-6; Suc-Phe-Glu-Phe-NA, 95192-25-9; Suc-Phe-Gln-Phe-NA, 95192-23-7; Suc-Phe-Lys-Phe-NA, 95192-19-1; Suc-Phe-Trp-Phe-NA, 95363-64-7; Suc-Thr-Pro-Phe-NA, 95192-27-1; Suc-Met-Pro-Phe-NA, 95192-26-0; Suc-Glu-Pro-Phe-NA, 95192-31-7; Suc-Lys(Z)-Pro-Phe-NA, 95363-65-8; Suc-Gln-Pro-Phe-NA, 95192-29-3; Suc-Leu-Pro-Phe-NA, 95192-28-2; Suc-Ser-Pro-Phe-NA, 95192-30-6; Suc-Ala-Pro-Phe-NA, 83329-41-3; Suc-Lys-Pro-Phe-NA, 95192-33-9; Suc-Gly-Pro-Phe-NA, 95363-66-9; Suc-Pro-Pro-Phe-NA, 75651-79-5; Suc-Met-Val-Pro-Phe-NA, 95192-37-3; Suc-Leu-Val-Pro-Phe-NA, 95192-40-8; Suc-Phe-Val-Pro-Phe-NA, 95192-39-5; Suc-Ala-Val-Pro-Phe-NA, 95192-38-4; Suc-Lys-Val-Pro-Phe-NA, 95192-42-0; Suc-Glu-Val-Pro-Phe-NA, 95192-41-9; Suc-Val-Pro-Phe-NA(2), 95363-67-0; Suc-Val-Pro-Phe-NA(3), 95363-68-1; Suc-Phe-Lys(Pic)-Phe-NA, 95363-69-2; Suc-Lys(Pic)-Pro-Phe-NA, 95363-70-5; Suc-Lys(Pic)-Val-Pro-Phe-NA, 95363-71-6; Suc-Ile-His-Pro-Phe-NA, 95192-47-5; Suc-Ile-Thr-Leu-Leu-Ser-NA, 95192-48-6; Suc-Ile-Thr-Leu-Leu-Phe-NA, 95192-49-7; Suc-Ile-Thr-Leu-Leu-NA, 95192-50-0; Suc-His-Pro-Phe-NA, 95192-46-4; BOC-Gly-Phe-NA, 95363-72-7; BOC-Ala-Phe-NA, 70968-19-3; BOC-Val-Phe-NA, 95363-73-8; BOC-Phe-Phe-NA, 95363-74-9; BOC-Trp-Phe-NA, 95363-75-0; BOC-Met-Phe-NA, 95363-76-1; BOC-Gln-Phe-NA, 95363-77-2; BOC-Glu(OtBu)-Phe-NA, 95363-78-3; BOC-Lys(Z)-Phe-NA, 95363-79-4; BOC-Ser-Phe-NA, 95363-80-7; BOC-Thr-Phe-NA, 75651-87-5; BOC-Phe-Gly-Phe-NA, 95363-81-8; BOC-Phe-Ala-Phe-NA, 95363-82-9; BOC-Phe-Val-Phe-NA, 95363-83-0; BOC-Phe-Phe-Phe-NA, 95363-84-1; BOC-Phe-Trp-Phe-NA, 95363-85-2; BOC-Phe-Gln-Phe-NA, 95363-86-3; BOC-Phe-Lys-(Z)-Phe-NA, 95363-87-4; 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BOC-Leu-Leu-NA, 85697-78-5; BOC-Ile-Thr-Leu-Leu-NA, 95364-13-9; BOC-Phe-SBzl, 95364-14-0; BOC-Pro-Phe-SBzl, 95364-15-1; BOC-Val-Pro-Phe-SBzl, 95364-16-2; BOC-D-MeVal-OH, 89536-85-6; BOC-L-MeVal-OH, 45170-31-8; BOC-Gly-OH, 4530-20-5; BOC-Ala-OH, 15761-38-3; BOC-Val-OH, 13734-41-3; BOC-Phe-OH, 13734-34-4; BOC-Trp-OH, 13139-14-5; BOC-Met-OH, 2488-15-5; BOC-Gln-OH, 13726-85-7; BOC-Glu-(OtBu)-OH, 13726-84-6; BOC-Lys(Z)-OH, 2389-45-9; BOC-Ser-OH, 3262-72-4; BOC-Leu-OH, 13139-15-6; BOC-Pro-OH, 15761-39-4; BOC-Thr-OH, 2592-18-9; BOC-His(Tos)-OH, 35899-43-5; BOC-Ile-OH, 13139-16-7; BOC-Val-Pro-OH, 23361-28-6; BOC-Val-Pro-Trp-OH, 95364-17-3; BOC-D-Val-OH, 22838-58-0; H-Phe-NA, 2360-97-6; H-Gly-Phe-NA, 21027-72-5; H-Ala-Phe-NA, 95364-18-4; H-Val-Phe-NA, 89499-05-8; H-Phe-Phe-NA, 95364-19-5; H-Trp-Phe-NA, 95364-20-8; H-Gln-Phe-NA, 95364-21-9; H-Lys(Z)-Phe-NA, 95364-22-0; H-Met-Phe-NA, 95364-23-1; H-Glu-Phe-NA·CF_3CO_2H, 95364-24-2; H-Ser-Phe-NA, 95364-25-3; H-Thr-Phe-NA, 95364-26-4; H-Pro-Phe-NA, 95364-27-5; H-Val-Pro-Phe-NA, 95364-28-6; H-Tyr-NA, 52551-07-2; H-Met-NA, 6042-04-2; H-Ser(OBzl)-NA, 95364-29-7; H-Ser(OBzl)-OH, 4726-96-9; H-Ser-NH, 95364-30-0; H-Thr-OMe, 3373-59-9; H-Leu-OBzl·TsOH, 1738-77-8; H-Leu-Leu-OBzl, 77167-53-4; H-Leu-NA, 4178-93-2; H-Leu-Leu-NA, 81928-67-8; H-His(Tos)-Pro-Phe-NA·TFA, 95364-32-2; Z-Phe-OH, 1161-13-3; Ac-PEA, 877-95-2; Ac-Leu-PEA, 52201-09-9; Ac-Gly-Gly-PEA, 52201-10-2; Ac-Gly-Leu-PEA, 52201-11-3; Ac-Gly-PEA, 52201-07-7; Ac-Ala-PEA, 52201-08-8; CF_3CO_2H, 76-05-1; benzyl mercaptan, 100-53-8; *p*-nitroaniline, 100-01-6; angiotensin I, 484-42-4; α_1-antichymotrypsin, 9004-07-3; angiotensinogen, 20845-02-7; benzothiazole, 95-16-9; chalcone, 94-41-7; naphthalene, 91-20-3; phenothiazine, 92-84-2; phenol, 108-95-2; 4-methylphenol, 106-44-5; benzaldehyde, 100-52-7; indole, 120-72-9; 2-methylindole, 95-20-5; 5-methylindole, 614-96-0; 7-methylindole, 933-67-5; 5-cyanoindole, 15861-24-2; indole-5-carboxamide, 1670-87-7; 5-hydroxyindole, 1953-54-4; 5-methylindole-3-carboxaldehyde, 52562-50-2; 3-indolylacetic acid, 87-51-4; 5-methylindole-2-carboxylic acid, 10241-97-1; 3-phenylindole, 1504-16-1; 2,3-diphenylindole, 3469-20-3; 2-phenyl-3-(4-nitrophenyl)indole, 1741-96-4; 2-methyl-3-phenylindole, 4757-69-1; 2-phenyl-3-methyl-5-nitroindole, 2047-87-2; 2-phenyl-3-(4-nitrophenyl)-5-nitroindole, 1741-97-5; 2-methyl-3-(4-nitrophenyl)-5-nitroindole, 1678-07-5; 2-phenyl-5-methylindole, 13228-36-9; cathepsin G, 56645-49-9.

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