A Search for Peptide Ligase: Cosolvent-Mediated Conversion of Proteases to Esterases for Irreversible Synthesis of Peptides[1]

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Abstract: Serine and cysteine proteases (trypsin, chymotrypsin, papain, subtilisin) in the presence of certain concentrations of water-miscible organic solvents express no amidase activities. The esterase activities, however, remain significant. These modified enzymes can be used as peptide ligases in a kinetically controlled approach (aminolysis) for the stepwise synthesis and fragment coupling of peptides, the products of which are free from secondary hydrolysis. Preparative syntheses of peptides containing both D- and L-amino acids and penicillin precursors containing *O*-methyl-D-allothreonine and α-aminoadipic acid under such conditions are demonstrated. A comparative study of the enzymatic synthesis of Z-Phe-Leu-NH_2 in anhydrous DMF and in aqueous DMF (50% water, v/v) indicates that the rate in aqueous DMF is >10 000 times faster. Chymotrypsin, subtilisin, and *Streptomyces griseus* protease are inactive toward Z-Phe-OMe + Leu-NH_2 in anhydrous DMF.

The utilization of proteases as catalysts in peptide synthesis began early this century[2] and is still an area of active research.[3] Some of the processes have been commercialized; of particular importance is the production of aspartame[4] and human insulin.[5] The advantages of enzymatic peptide synthesis are freedom from racemization, minimal activation and side-chain protection, mild reaction conditions, high regio- and stereoselectivity, and enzyme immobilization allowing for catalyst recovery in large-scale processes. Further, the reactions can be carried out in a mixture of organic solvent and water, devoid of the problem of low solubility of protected peptides in the organic solvents employed in chemical synthesis. With these advantages, however, come the disadvantages that the amidase activity of proteases causes a secondary hydrolysis of the growing peptides and that the substrates of proteases are generally limited to natural L-amino acid derivatives.[2,3]

Two strategies are generally used in enzymatic peptide synthesis: One is a thermodynamically controlled synthesis (i.e., a direct reversal of the catalytic hydrolysis of peptides; eq 1), and the other

$$RCO_2H + H_2NR' \overset{K}{\rightleftharpoons} RCONHR' + H_2O \qquad (1)$$

$$RCO_2R' + E \overset{k_1}{\rightleftharpoons} RCO_2R'\cdot E \overset{k_2}{\rightleftharpoons} RCOE \xrightarrow{k_4\ H_2NR} RCONHR + E \qquad (2)$$

$$\downarrow k_3\ H_2O$$

$$RCO_2H + E$$

is a kinetically controlled synthesis (i.e., aminolysis of N-protected amino acid or peptide esters; eq 2). The former is an endergonic process; manipulation of reaction conditions is thus required to increase the product yield.[2,3] The addition of water-miscible organic solvents to increase the pK value of the carboxyl component[6] (thus increasing the concentration of the uncharged, reactive substrate), the use of a bisphasic system,[7] reverse micelles,[8] or anhydrous media saturated with water,[9] and the selection of appropriate N- or C-protecting groups to reduce the solubility of products[2,3] are very often employed. The latter is faster, and the product yield can be improved by manipulating the reaction conditions as that used in the thermodynamic approach.[2,3] The kinetic approach, however, requires the use of an ester as an acyl donor and is limited to those enzymes forming an acyl-enzyme intermediate. The hydrolyses of esters and amides catalyzed by such proteases are similar in mechanism but different in rate-determining steps.[10] Formation of acyl-intermediate in amide hydrolysis is rate-determining and pH independent (at pH higher than the pK of active site His-imidazole), while deacylation in ester hydrolysis is rate-determining, general-base catalyzed, and pH dependent. This difference allows us to find working conditions to control the esterase vs amidase activity for use in the kinetic approach in which amidase activity is not desirable. For example, a marked increase of esterase vs amidase activity of papain at pH 9 was observed,[11,12] and the alkaline condition was used in papain-catalyzed stepwise and fragment coupling of peptides, where a secondary hydrolysis of peptide products was not observed.[12] An ideal case would be the use of esterases without amidase activity as catalysts if the enzyme would accept amino acid or peptide derivatives as substrates. Lipases[13,14] and pig liver esterase[13] with no amidase activity have been used in the synthesis of peptides in biphasic systems[13] or in anhydrous media.[14] Certain microbial lipases also accept D-amino acid derivatives as acyl donor[13] and acceptor,[13] allowing for synthesis of peptides con-

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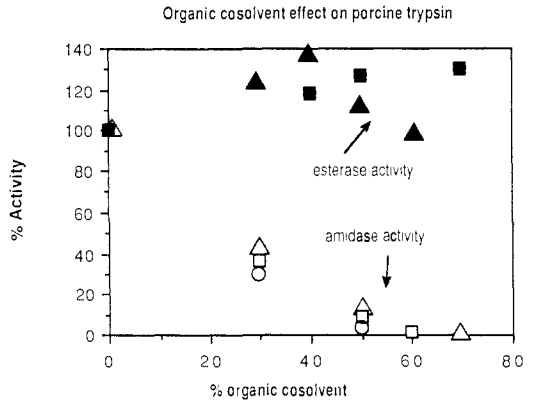

Figure 1. Effect of water-miscible organic solvents on the activities of porcine trypsin. The esterase activity toward *N*-benzoylarginine methyl ester (40 mM) was determined in 0.02 M $CaCl_2$ (2 mL) containing organic solvent. A 50-μL enzyme solution (4.17×10^{-5} M) in 0.004 M Tris, pH 7, was added to the substrate solution, and the solution was held at pH 8 by automatic addition of 0.025 M NaOH through a Radiometer pH-stat. The activity was determined on the basis of consumption of base. (□) dioxane; (▲) DMF. To determine the amidase activity, the enzyme was added to a solution (1 mL) containing *N*-benzoyl-DL-arginine *p*-nitroanilide, Tris (0.1 M), $CaCl_3$ (0.02 M), pH 8.0. The absorbance change at 410 nm was monitored. (○) acetonitrile; (△) DMF; (□) dioxane.

taining D and other unusual amino acids. Some proteases have been found to behave similarly when used in high concentrations of organic solvents.[12,15] This provides a new route to a number of unnatural peptides generally not accessible by recombinant DNA technology. More recently, subtilisin was reported to accept both D- and L-amino acids as acyl donors in anhydrous media.[16]

In the kinetic approach, the undesirable amidase activity of proteases can also be selectively destroyed by chemical modification. Thiosubtilisin[17] and $N^{\epsilon 2}$-methyl-His_{57}-α-chymotrypsin[18] both express only esterase activity and have been used as catalysts in peptide-fragment coupling. The catalytic profile of methyl-chymotrypsin was characterized by the accumulation of an acyl intermediate at room temperature detected by ^{13}C NMR spectroscopy.[18] These interesting results led us to reinvestigate the effects of water-miscible organic solvents on the esterase and amidase activities of proteases. The difference of such effects on the two activities has been known for some time.[19] The applicability of amidase-damaged, solvent-modified proteases toward synthesis, however, has not been examined, although cosolvents have been used for other reasons. We have found the cosolvent effect is quite general for several serine and thiol proteases and report here the preparation of such amidase-damaged proteases for use in the synthesis of peptides containing both D and L and other unusual amino acids. We also report here a comparative study of protease-catalyzed peptide synthesis in anhydrous DMF (a solvent used previously in subtilisin reactions)[16] and aqueous DMF solution.

Results and Discussion

As illustrated in Figure 1, addition of an appropriate cosolvent (acetonitrile, DMF, dioxane) up to 50–60% can selectively eliminate undesirable amidase activity for the porcine protease trypsin while the esterase activity remains significant. A similar phenomenon was observed in other serine and cysteine proteases including bovine trypsin,[19] papain, subtilisin (*Bacillus subtilis* protease), and α-chymotrypsin. Table I indicates the esterase activities of several proteases at complete elimination of the amidase activity. The remaining esterase activities are high enough (5–270 units/mg) to be effective catalysts in peptide synthesis. $Met(O)_{192}$-α-chymotrypsin, in which Met_{192} is oxidized to methionine sulfoxide, is of interest since it is more stable to the synthetic conditions of high pH than the native enzyme.[15] The modification of activity is reversible with amidase activity being restored upon return of the enzyme to water.

Table I. Esterase Activities of Proteases at Elimination of the Amidase Activity[a]

enzyme	org solv concn	apparent pH	V_{max}, units/mg
papain[b]	40% dioxane	8	5
		9	16
	50% DMF	9	8
porcine trypsin[c]	70% dioxane	8	38
	50% DMF	9	31
	50% DMSO	9	30
	50% CH_3CN	9	35
bovine trypsin[c]	60% dioxane	8	30
	45% DMF	9	20
α-chymotrypsin[d]	60% CH_3CN	9	200
	65% CH_3CN	8	155
Met $(O)_{192}$-chymotrypsin[d]	60% CH_3CN	8	60
	50% CH_3CN	9	100
Subtilisin[e]	40% Dioxane	8	270

[a] All enzymes are from Sigma; all reactions are performed at 25 °C. [b] The esterase and amidase activities were determined similarly to the procedure used for trypsin in Figure 1. In addition to all the components, mercaptoethanol was added (10 mM). [c] The activities were determined according to the procedures described in Figure 1. [d] *N*-benzoyltyrosine ester and *N*-benzoyltyrosine-*p*-nitroanilide were used to determine the activities. [e] The activities were determined in the same manner as that for chymotrypsin.

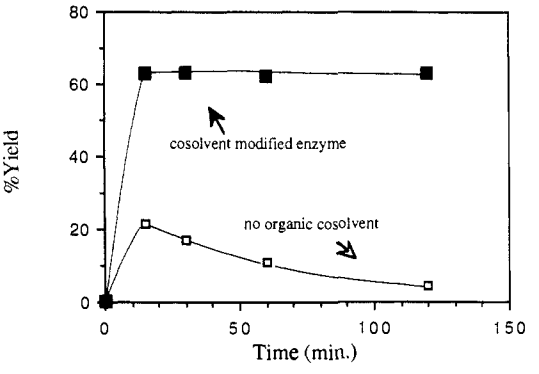

Figure 2. Comparison of cosolvent-modified enzyme and all-aqueous system for the synthesis of Bzl-Arg-Gly-NH_2, as monitored by HPLC with a C-18 column. Conditions: 0.2 M Bzl-Arg-OEt, 0.2 M Gly-NH_2, 0.25 M Tris, 0.02 M $CaCl_2$, 0.1 mM TPCK-treated bovine trypsin, pH 10; cosolvent-modified system is 60% by volume dioxane, and approximately 30% of the initial esterase activity is retained after 2.5 h. No peptide formation was observed in the absence of enzymes. The product is soluble in both systems.

The advantage of such amidase-damaged enzymes is clearly demonstrated in the kinetically controlled peptide synthesis (Figure 2, Table II). No secondary hydrolysis of the peptide bond was observed during the course of reactions. Hydrolysis of the product peptide in solution over time in the all-aqueous solution was observed by HPLC. This behavior becomes even more problematic in fragment coupling when the specificity of the enzyme allows for reaction at more than one position within the fragment, resulting in fragment scrambling.[18] The acyl donors used are conveniently prepared and form stable methyl and ethyl esters (either is applicable for all enzymes presented here; deacylation is rate-determining in each case), whereas reports utilizing other

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Table II. Synthesis of Peptides Catalyzed by Amidase-Deficient Esterases[a]

no.	acyl donor	acyl acceptor	product	enzyme	yield, %
1	Pha-Cys(SAcm)-OMe	D-Val-OMe	Pha-Cys(SAcm)-D-Val-OMe	CT	72
2	Pha-Cys(SSBu^t)-Glc	D-Val-OBzl	Pha-Cys(SSBu-*t*)-D-Val-OBzl	CCL	45[b]
3	BOC-Cys(SBzl)-OMe	D-Val-OBzl	Boc-Cys(SBzl)-D-Val-OBZl	CT	72
4	Boc-Cys(SAcm)-OMe	D-alloThr(OMe)-OMe	Boc-Cys(Acm)-D-alloThr(OMe)-OMe	CT	30
				subtilisin	40
5	Z-Adp(OMe)OH	Cys(SAcm)OMe	Z-Adp-δ-Cys(SAcm)-OMe	GT	40
6	Z-Adp(OMe)OH	Cys(SBzl)-D-Val-OBzl	Z-Adp-δ-Cys(SBzl)-D-Val-OBzl	papain	42
		Cys(SAcm)-D-alloThr(OMe)OMe	Z-Adp-δ-Cys(SAcm)-D-alloThr(OMe)-OMe	GT	26
7	Z-Tyr-Gly-Gly-Phe-OCH_2CN	Leu-NH_2	Z-Tyr-Gly-Gly-Phe-Leu-NH_2	Me-CT	99[c]
8	Z-Phe-Gly-OEt	D-Leu-OMe	Z-Phe-Gly-D-Leu-OMe	papain	87
9	Z-Phe-OMe	Gly-OPr-*i* + D-Leu-OMe	Z-Phe-Gly-D-Leu-OMe	papain	70
10	Bz-Arg-OEt	Gly-NH_2	Bz-Arg-Gly-NH_2	trypsin	94[d]
11	Z-D-Phe-OGlc	glycidal dimethyl acetal	Z-D-Phe-glycidal dimethyl acetal	CCL	45[b]
12	Z-Tyr-OMe	L-Arg-OMe	Z-Tyr-Arg-OMe	CT	71
13	Z-Tyr-OMe	D-Arg-OMe	Z-Tyr-D-Arg-OMe	CT	72

[a] Abbreviations: Pha, phenylacetyl; Acm, acetamidomethyl; Bzl, benzyl; SBu-*t*, *tert*-butylmercapto; Adp, α-aminoadipic acid; Glc, glyceryl; Bz, benzoyl; Z, benzyloxycarbonyl; CT, α-chymotrypsin; CCl, *Candida cylindraceae* lipase; GT, γ-glutamyl transpeptidase; MeCT, α-chymotrypsin methylated at $N^{2\epsilon}$ of His-57. All amino acids used are of the L configuration unless otherwise indicated. [b] Reference 13. [c] Reference 18. [d] Determined by HPLC. See Experimental Section for conditions used to selectively destroy the amidase activity. The low yields in some reactions are due to the hydrolysis of the acyl donor. Isolated yields are reported unless otherwise indicated. The thiol groups are protected to avoid oxidation. Reaction yields are not optimized.

amidase-deficient catalysts rely for the most part on active[17,18] or unusual esters[13] and, in the case of lipases, require extensive reaction time. The peptides prepared here are precursors to enkephalin and its releasing factors, lactam antibiotics and derivatives.[20] It is worth noting that *O*-methyl-D-allothreonine can be used as a nucleophile and α-aminoadipic acid δ-monoester can be used as an acyl donor forming a δ-peptide bond catalyzed by papain or γ-glutamyl transpeptidase. Z-Aminoadipic acid δ-monoester instead of diester was used as a substrate to avoid the formation of undesired α-peptide, as both papain and γ-glutamyltranspeptidase catalyze the hydrolysis of both α- and δ-esters. The δ-monoester was prepared by porcine pancreatic lipase catalyzed selective hydrolysis of Z-aminoadipic acid diester. The regioselectivity was >95%. We have also found that by taking advantage of the subsite primary specificity of proteases such as papain,[21] a one-pot synthesis of tripeptide from three distinct amino acid derivatives can be achieved with 70% overall yield (entry 9 of Table II).

The interesting results reported by Klibanov and co-workers[16] that subtilisin is active in anhydrous DMF encouraged us to test if this and other proteases could be used as effective catalysts in peptide synthesis in this solvent. The results of a comparative study on protease-catalyzed synthesis of Z-Phe-Leu-NH_2 from Z-Phe-OMe or the activated ester Z-Phe-OCH_2CN and Leu-NH_2 are shown in Table III. All the proteases tested showed no activity toward the methyl ester and very low activity toward the activated ester (<0.1% of the activity toward the methyl ester). The cyanomethyl ester used here is about 10 times more active than the methyl ester in the enzymatic reactions[18] yet stable in aqueous solution, and no nonenzymatic peptide coupling was observed under the reaction conditions in the reaction time frame. In the case of chymotrypsin, it was reported[22] that a suspension of the crystalline enzyme in dichloromethane containing 0.25% water was stable and catalytically active toward the hydrolysis of acetyl tyrosine ethyl ester. In water-miscible organic solvents including DMF, DMSO, formamide, and methylacetamide, the enzyme undergoes conformational change (based on ORD study) to a random coil and expresses no hydrolytic activity toward the active ester *p*-nitrophenyl acetate,[23] except in DMSO where the enzyme loses substrate specificity and catalyzes the hydrolyses of several *p*-nitrophenyl esters. The rate for *p*-nitrophenyl acetate hydrolysis is approximately equal to that of chymotrypsinogen, trypsin, trypsinogen, lysozyme, and serum albumin, suggesting that the proteins in DMSO all behave similarly as general bases. In fact, organic solvent induced structural changes of proteins has been studied since 1960.[23] When chymotrypsin was dissolved in an

Table III. Synthesis of Z-L-Phe-L-Leu-NH_2 in High DMF Systems[a]

acyl donor	enzyme	aq, %	init velocity, nmol/min per mg of enz	yield,[b] % (peptide/acid)
Z-L-Phe-OMe	CT	0	0	0/0
	subtilisin	0	0	0/0
	subtilisin[c]	0	0	0/0
	S. Griseus[d]	0	0	0/0
	papain	0	0	0/0
	CT	50	>10000	87/13
	subtilisin	50	12000	30/70
	S. Griseus	50	5000	45/55
Z-L-Phe-OCH_2CN	CT	0	0[e]	0/0
	subtilisin	0	3.5[e]	5/0
	subtilisin[c]	0	5.1[e]	7/0
	S. Griseus	0	15.2[e]	44/0
	papain	0	5.3[e]	15/0

[a] Reaction conditions: 20 mM acyl donor, 50 mM L-Leu-NH_2, aqueous component of system was 0.2 M K_2HPO_4, pH 9.0, 2 mg of enzyme; all reaction volumes, 1 mL. Dimethylformamide was fractionally distilled from BaO under reduced pressure and stored over 4-Å molecular sieves under dry argon in the dark. Extent of reaction was monitored by HPLC (gradients of MeOH in 0.1 M NH_4OAc, pH 5.6, Beckman C-18 column). [b] After 48 h. Remainder in reactions not summing to 100% is unreacted acyl donor. [c] Subtilisn dissolved in 50 mM phosphate buffer, pH 7.6, (20 mg/mL) and lyophilized. [d] Protease type XXI (Sigma) from *Streptomyces griseus*. [e] Corrected for background, nonenzymatic peptide synthesis, as determined by blanks containing no enzyme or 2 mg of bovine serum albumin, the velocity of which did not exceed 0.4 nmol/min or 2% peptide produced in 48 h.

(20) Both Tyr-L-Arg and Tyr-D-Arg are enkephalin-releasing peptides. The D peptide is about 5 times as active as the L peptide: Takagi, H.; Shiomi, H.; Ueda, H.; Amano, H. *Eur. J. Pharmac.* **1979**, *55*, 109–111; *Nature (London)* **1979**, *282*, 410–412. Pha-Cys-Val can be converted to penicillin G catalyzed by isopenicillin N synthetase: Luengo, J. M.; Alemany, M. T. S.; Salto, F.; Ramos, F.; Lopez-Nieto, M. J.; Martin, J. F. *Bio/Technology* **1986**, *4*, 44. Adp-δ-Cys-D-Val (the natural substrate) can be converted to isopenicillin N: Wolfe, S.; Demain, A. L.; Jensen, S. E.; Westlake, D. W. S. *Science (Washington, D.C.)* **1984**, *226*, 1386. Adp-δ-Cys-D-alloThr-(OMe)-OH can be converted to the isopenicillin N analogue catalyzed by the enzyme: Baldwin, J. E.; Adlington, R. M.; Basak, A.; Flitsch, S. L.; Petursson, S.; Turner, N. J.; Ting H. H. *J. Chem. Soc., Chem. Commun.* **1986**, 975. All of the substrates were prepared chemically. R = δ-(L-α-aminoadipyl) or *m*-carboxylphenylacetyl. R_1 = Me, R_2 = Me or OMe.

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aqueous solution, pH 7.8, containing acetylphenylalanine (0.25%) and lyophilized to reduce the water content to 2.5% (w/w), the enzyme was active in octane (a water-immiscible solvent) with <0.02% water and a change of substrate specificity was observed.[24] The report on *Bacillus* subtilisin[16] indicates that the enzyme is active in a number of anhydrous organic solvents, including *tert*-amyl alcohol, THF, acetone, acetonitrile, ethyl acetate, and dioxane, and catalyzes the formation of a peptide bond between N-protected amino acid chloroethyl ester and amino acid amides. No coupling was observed in aqueous solution containing 40% DMF at pH 7 or 10! All these interesting results generate new questions regarding the origin of catalytic power: Are the catalytic mechanisms of these enzyme reactions in anhydrous, water-miscible organic solvents such as DMF the same as that in aqueous solution, or do the enzymes simply act as general bases with low selectivity? In any event, our initial conclusion based on the comparative study of enzymatic coupling of Z-Phe-OMe or Z-Phe-OCH_2CN and Leu-NH_2 in aqueous DMF and anhydrous DMF is that in anhydrous DMF the enzymes tested are either inactive or too slow to be useful for preparative synthesis. The reactions, however, can be done in aqueous DMF to achieve high-yield synthesis.

The role of organic cosolvent interactions in modifying the behavior of proteases in such a dramatic way is unclear. It could be due to a conformational change, thus decreasing the efficiency in the higher energy pathway, namely amide hydrolysis, and to a lesser extent in the lower energy pathway, ester hydrolysis. It could also be due to a local pH increase, since the rate-determining deacylation in ester hydrolysis is a general-base catalysis and is thus pH dependent. This effect appears to be general, and experiments are ongoing to determine its true origin. It is clear that this approach represents a simple solution to the problems associated with protease-catalyzed peptide synthesis and a convenient alternative to site-directed mutagenesis or chemical modification in producing peptide-forming catalysts without undesirable amidase activity.

Experimental Section

All enzymes were obtained from Sigma without further treatment or purification unless otherwise indicated. The following grades were used: papain (type III, crystallized), α-chymotrypsin (type II, crystallized), substilisin (protease from *Bacillus* subtilisin, type VIII, crystallized), γ-glutamyltranspeptidase from bovine kidney (type II). Assays were carried out according to the protocols described in Figure 1 and Tables I–III. Melting points were taken on a Thomas-Hoover micro melting point apparatus and are uncorrected. HPLC analyses were performed on a Gilson Model 43 Gradient LC, equipped with a Vydac C-18 column and a variable wavelength UV detector set at 270 nm. A linear gradient of MeOH (60–80%, 15 min) in 0.1 M NaOAc, pH 5.6, was employed. Measurement of UV or visible absorbance was performed on a Beckman Model-7 UV–vis spectrophotometer. Amino acid analyses were carried out with a Beckman amino acid analyzer. Sample was hydrolyzed at 110 °C in constant boiling HCl (6 N) for 22 h in a sealed test tube, evaporated under vacuum in the presence of P_2O_5 and KOH, and dissolved in a buffer before analysis. Optical rotations were measured at room temperature with a Perkin-Elmer polarimeter, Model 241. Thin-layer chromatography (TLC) was developed with Merck Silica gel plates coated on plastics. NMR spectra were recorded with a 200-MHz Varian instrument. All protected amino acid derivatives and authentic peptides were prepared according to the standard procedures.[21] α-L-Aminoadipic acid was prepared from α-ketoadipic acid catalyzed by glutamic dehydrogenase according to the procedure developed previously.[22] The COSY 2D-NMR technique was employed to help assign the chemical shifts. All compounds prepared are pure by TLC and NMR. No racemization was observed in peptide synthesis according to NMR and HPLC analyses.[15]

Selective Enzymatic Hydrolysis of α-*N*-(Benzyloxycarbonyl)adipic Acid Dimethyl Ester [Z-Adp(OMe)OMe] To δ-Monomethyl Ester [Z-Adp(OMe)OH]. To a stirred suspension of Z-Adp(OMe)OMe (0.4 g, 1.2 mmol) in a potassium phosphate buffer (0.05 M, pH 7.5, 15 mL) was added porcine panceatic lipase (100 mg). The suspension was maintained at pH 7.5 with addition of 0.05 N NaOH until 1 equiv of base was consumed. The mixture was then acidified to pH 2 with 1 N HCl and extracted with diethyl ether (3 × 20 mL). The extracts were combined, dried over Na_2SO_4, and evaporated to give Z-Adp(OMe)OH (0.33 g, 92%) as a single oily product: $[\alpha]_D^{25}$ −5.50° (*c* 0.03, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.73 (m, 2 H, γ-CH_2), 1.74, 1.93 (dm, 2 H, β-CH_2), 2.36 (t, 2 H, δ-CH_2), 3.67 (s, 3 H, δ-OCH_3) 4.42 (m, 1 H, α-H), 5.13 (s, 2 H, Z-CH_2), 5.44 (d, 1 H, NH), 7.35 (s, 5 H, PhH), 7.99 (br, OH). Anal. Calcd for $C_{14}H_{17}O_6N$: C, 56.95; H, 5.76; N, 4.75. Found: C, 56.88; H, 5.80; 4.77.

Synthesis of *O*-Methyl-D-allothreonine [D-AlloThr(OMe)]. Z-Allothreonine methyl ester (3.56 g, 13.3 mmol) was dissolved in a mixture of THF (40 mL) and HMPA (4 mL). The mixture was deoxygenated with Ar and cooled with an ice bath. Potassium hydride (1.68 g of 35% w/w in mineral oil, 14.6 mmol) was added and the solution stirred for 1 min. Methyl iodide (4 mL) was added and the salt precipitated out immediately. The reaction was allowed to proceed for $^1/_2$ h, and then the mixture filtered. The mother liquid was evaporated, taken up in diethyl ether, and washed with saturated NaCl (3 × 10 mL). The ether layer was dried over Na_2SO_4 and evaporated to dryness to give Z-D-alloThr(OMe)OH in quantitative yield, pure by TLC (CH_2Cl_2:MeOH = 9:1, R_f 0.8): ^{1}H NMR ($CDCl_3$) δ 1.74 (d, 3 H, γ-CH_3), 3.04 (s, 3 H, OCH_3), 3.75 (s, 3 H, ester CH_3), 4.68 (s, 2 H, Z-CH_2), 5.12 (m, 2 H, α- and β-H), 6.83 (d, 1 H, NH), 7.35 (s, 5 H, PhH); $[\alpha]_D$ +2.0 (*c* 0.06, MeOH). Anal. Calcd for $C_{14}H_{19}NO_5$: C, 59.79, H, 6.76; N, 4.98. Found: C, 59.88; H, 6.77; N, 4.90. This compound (1.31 g) was dissolved in anhydrous MeOH (50 mL), and the flask was purged with Ar. Pd (10% on carbon, 100 Mg) was added, the reaction vessel (pressure bottle) was purged with Ar again. The bottom was then loaded with H_2 (10 psi) and the reaction allowed to proceed overnight to deprotect the Z group. The product is taken up in 1 N HCl in MeOH and evaporated to a thick oil, which is stored in the freezer for next use.

Enzymatic Synthesis of *N*-(Phenylacetyl)-*S*-(acetamidomethyl)-L-cysteinyl-D-valine Methyl Ester [Pha-Cys(SAcm)-D-Val-OMe]. Pha-Cys(SAcm)-OMe (0.3 g, 0.9 mmol) and D-val-OMe·HCl (1.31 g, 6.71 mmol) were added to a solution (20 mL) containing DMSO (50% v/v) and $NaHCO/Na_2CO_3$ (0.1 M). The mixture was adjusted to pH 9.5, and α-chymotrypsin (100 mg) was added. The mixture was slowly stirred for 15 h until the ester was consumed. Cold water (50 mL) was added to the mixture, and the precipitate obtained was dissolved in $CHCl_3$ (200 mL). The organic phase was washed with 1 N $NaHCO_3$, 1 N HCl, and water, dried over Na_2SO_4, and evaporated to give 0.29 g of product. After purification with a silica gel column with CH_2Cl_2:MeOH = 9:1, v/v, as elutent (R_f 0.75), the product was obtained in 72% yield (0.27 g): $[\alpha]_D$ −42.0° (*c* 0.03, DMSO); ^{1}H NMR ($CDCl_3$) δ 0.73 (d, 3 H, *J* = 6 Hz, Val δ-H), 0.81 (d, 3 H, *J* = 6.3 Hz, Val δ-H), 1.85 (s, 3 H, Acm-CH_3), 2.02 (m, 1 H, val β-H), 2.56 (dd, 1 H, J_1 = 5.2 Hz, J_2 = 14 Hz, Cys β-H), 2.82 (dd, 1 H, J_1 = 5.2 Hz, J_2 = 14 Hz, Cys β-H), 3.50 (s, 2 H, Pha-CH_2), 3.58 (s, 3 H, Val-OMe), 4.13–4.41 (m, 3 H, Val α-H + Cys SCH_2), 4.75 (m, 1 H, Cys α-H), 7.18 (s, 5 H, PhH), 7.55 (d, 1H, *J* = 8.3 Hz, Cys-Val-NH), 7.77 (m, 1 H, AcmNH), 7.12 (d, 1 H, CysNH). Anal. Calcd for $C_{20}H_{29}N_3O_5S$: C, 56.74; H, 6.86; N, 9.93. Found: C, 56.80; H, 6.88; N, 9.88.

Enzymatic Synthesis of *N*-(*tert*-Butyloxycarbonyl)-*S*-benzyl-L-cysteinyl-D-valine Benzyl Ester [Boc-Cys(SBzl)-D-Val-OBzl]. The same procedure as above was used. The product was crystallized from MeOH/H_2O (1:2) to give an overall yield of 72%: $[\alpha]_D$ −40.1° (*c* 0.1, DMF); ^{1}H NMR ($CDCl_3$) δ 0.89 (d, 3 H, Val C-4), 0.99 (d, 3 H, *J* = 6.2 Hz, Val 4-H), 1.48 (s, 9 H, *t*-Bu), 2.25 (m, 1 H, Val β-H), 2.85 (dd, 2H, Cys β-H), 3.80 (s, 2 H, SCH_2Ph), 4.33 (m, 1 H, Val α-H), 4.62 (dd, 1 H, J_1 = 6.4 Hz, J_2 = 3.2 Hz, Cys α-H), 5.20 (dd, 2 H, *J* = 3.4 Hz, OCH_2Ph), 5.38 (d, 1 H, *J* = 6.0 Hz, Val NH), 6.86 (d, 1 H, *J* = 7.6 Hz, Cys NH), 7.3–4 (br, 10 H, Ph). Anal. Calcd for $C_{27}H_{36}N_2O_5S$: C, 64.80; H, 7.20; N, 5.60. Found: C, 64.88; H, 7.30; N, 5.58.

Enzymatic Synthesis of *N*-(*tert*-Butyloxycarbonyl)-*S*-(acetamidomethyl)-L-cysteinyl-*o*-methyl-D-allothreonine Methyl Ester [Boc-Cys(SAcm)-D-alloThr(OMe)OMe]. The same procedure as above was used: $[\alpha]_D$ −39.8° (*c* 0.05, DMF); ^{1}H NMR ($CDCl_3$) δ 1.45 (s, 9 H, Boc), 1.80 (d, 3 H, Thr γ-H), 2.05 (s, 3 H, Acm CH_3), 2.9, 3.1 (ddd, 2 H, Cys β-H), 3.14 (s, 3 H, OMe), 3.84 (s, 3 H, ester OMe), 4.39 (d, 2 H, Acm, CH_2), 4.50 (m, 1 H, Cys α-H), 5.18 (m, 1 H, Thr β-H), 5.25 (m, 1 H, Thr α-H), 5.60 (d, 1 H, Cys NH), 6.0 (d, 1 H, Thr NH), 7.25 (t, 1 H, Acm NH). Anal. Calcd for $C_{17}H_{31}N_3O_7S$: C, 48.46; H, 7.36; N, 9.98. Found: C, 48.80; H, 7.44; N, 9.88.

Enzymatic Synthesis of δ-[*N*-(Benzyloxycarbonyl)-L-α-(aminoadipyl)]-*S*-benzyl-L-cysteinyl-D-valine Benzyl Ester [Z-Adp-δ-Cys(SBZl)-D-Val-OBZl]. To Z-Adp(OMe)OH (1 mmol) and Cys(SBzl)-D-Val-OBzl–TFA (4 mmol, prepared by deprotection of the Boc derivative with TFA) in sodium phosphate buffer (0.1 M)/DMF (1:1 v/v, pH 9, 15 mL) were added papain (100 mg) and 50 μL of 2 M mercaptoethanol. The

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mixture was slowly stirred at room temperature until the ester was consumed (~15 h). The product was isolated as above and purified by silica gel column chromatography (CH_2Cl_2:MeOH = 95:5, R_f 0.25): $[\alpha]_D$ = -11.92° (c 0.02, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.85 (d, 3 H, J = 6.8 Hz, Val γ-H), 0.92 (d, 3 H, J = 6.8 Hz, Val γ-H), 1.71 (m, 4 H, Adp β- and γ-H), 2.19 (m, 3 H, val β-H + Adp δ-H), 2.70 (dd, 1 H, J_1 = 5.6 Hz, J_2 = 14 Hz, Cys β-H), 2.89 (dd, 2 H, J_1 = 5.6 Hz, J_2 = 14 Hz, Cys β-H), 3.63 (s, 3 H, OMe), 3.78 (s, 2 H, SCH_2Ph), 4.38 (m, 1 H, Val α-H), 4.56 (m, 2 H, Cys α-H + Adp α-H), 5.11 (s, 2 H, Z-CH_2), 5.15 (m, 2 H, Val-OCH_2Ph), 5.62 (d, 1 H, J = 8.8 Hz), 6.33 (d, 1 H, J = 8.0 Hz, Adp NH), 6.82 (d, 1 H, J = 9.2 Hz, Cys NH), 7.33 (s, 15 H, PhH). Anal. Calcd for $C_{36}H_{43}N_3O_8S$: C, 63.81; H, 6.35; N, 6.20. Found: C, 63.90; H, 6.50; N, 6.18.

Bovine kidney γ-glutamyl transpeptidase (60 mg) also catalyzed the same reaction to give the product in 20% yield. When the nucleophile was replaced with Cys(SAcm)OMe-TFA, the yield of Z-Adp-Cys(SAcm)OMe was 45%: ^1H NMR ($CDCl_3$) δ 1.55-1.85 (m, 4 H, Adp β, γ-H), 2.05 (s, 3 H, Acm CH_3), 2.20 (m, 2 H, Adp γ-H), 3.15, 2.95 (ddd, 2 H, Cys β-H), 3.70 (s, 3 H, Adp OCH_3), 3.80 (s, 3 H, Cys OCH_3), 4.30 (m, 1 H, Adp α-H), 4.38 (d, 2 H, Acm CH_2), 4.5 (m, 1 H, Cys α-H), 5.05 (s, 2 H, Z-CH_2), 6.0 (d, 1 H, Adp NH), 6.8 (d, 1 H, Cys NH), 6.9 (m, 1 H, Acm NH), 7.3 (s, 5 H, Ph). Anal. Calcd for $C_{21}H_{29}O_8N_3S$: C, 52.17; H, 6.00; N, 8.70. Found: C, 52.30; H, 5.98; N, 8.80.

Enzymatic Synthesis of δ-[*N*-(Benzyloxycarbonyl)-L-α-(aminoadipyl)]-*S*-(acetamidomethyl)-L-cysteinyl-*o*-methyl-D-allothreonine Methyl Ester [Z-Adp-δ-Cys(SAcm)-D-alloThr(OMe)OMe]. This was prepared with the same procedure as above. The yield obtained from the papain-catalyzed reaction was 46% and that from the glutamyl transpeptidase reaction was 26%: ^1H NMR ($CDCl_3$) δ 1.80 (d, 3 H, Thr γ-H), 1.55-1.85 (m, 4 H, Adp, β, γ-H), 2.05 (s, 3 H, Acm CH_3), 2.10 (m, 2 H, Adp δ-H) 3.15, 2.95 (ddd, 2 H, Cys β-H), 3.10 (s, 3 H, OCH_3), 3.8 (s, 3 H, ester CH_3), 4.40 (d, 2 H, Acm CH_2), 4.45 (m, 1 H, Adp α-H), 4.6 (m, 1 H, Cys α-H), 5.15 (m, 4 H, Thr α, β-H, and Z-CH_2), 6.1 (d, 1 H, Thr NH), 6.35 (d, 1 H, Adp NH), 6.5 (d, 1 H, Cys NH), 7.25 (m, 1 H, Acm NH), 7.35 (s, 5 H, Ph). Anal. Calcd for $C_{26}H_{38}N_4O_{10}S$: C, 52.17; H, 6.35; N, 9.37. Found: C, 52.11; H, 6.33; N, 9.40.

Enzymatic Synthesis of *N*-(Benzyloxycarbonyl)-L-phenylalanyl-L-glycyl-D-leucine Ethyl Ester (Z-Phe-Gly-D-Leu-OEt). Z-Phe-Gly-OEt) (2 mmol) and D-Leu-OMe·HCl (4 mmol) were added to 10 mL of Tris (0.2 M) containing 0.02 M $CaCl_2$, 10 mM meraptoethanol, 40% dioxane, and 10% methyl isobutyl ketone by volume, pH 9. Papain (0.8 g) was added, and the mixture was stirred for 2.5 h. The product was extracted with $CHCl_3$, (3 × 50 mL) and washed with 0.1 N $NaHCO_3$ (2 × 20 mL), 0.1 N HCl (2 × 20 mL), and water, dried over Na_2SO_4, and evaporated to give a crystalline solid (0.87 g, 87% yield), which is pure by TLC and NMR: mp 115-116 °C; $[\alpha]_D$ = +9.6° (c 2.4, MeOH); ^1H NMR ($CDCl_3$) δ 3.40 (t, 1 H, Gly NH), 8.20 (d, 1 H, Leu NH), 7.25 (br, 8 H, Z and Phe), 4.91 (s, 2 H, Z-CH_2), 4.30 (br, α-CH of Phe + Leu), 3.90 (br, 2 H, Gly-CH_2), 3.61 (s, 3 H, OCH_3), 3.01 (dd, 2 H, Phe-CH_2), 1.55 (br, 2 H, Leu-CH_2), 0.85 (q, 6 H, Leu-CH_3). Anal. Calcd for $C_{27}H_{35}N_3O_6$: C, 64.80; H, 7.00; N, 8.40. Found: C, 65.18; H, 7.10; N, 7.78. Amino acid analysis: Phe (1) 1.01, Gly (1) 1.0, Leu (1) 1.0. In another synthesis, Z-Phe-OMe (1 mmol), Gly-OPr*i* (2 mmol), and D-Leu-OMe (2 mmol) were used and other conditions were the same. The same product was isolated in 70% yield.

Enzymatic Syntheses of *N*-(Benzyloxycarbonyl)-L-tyrosyl-L(or D)-arginine Methyl Ester (Z-Tyr-L(or D)-Arg-OMe). To 50 mL of sodium carbonate buffer (0.2 M) containing CH_3CN (50%, v/v) was added Z-Tyr-OMe (0.5 M), L- or D-Arg-OMe·HCl (1.5 M), and chymotrypsin (0.2 mM). The reaction was monitored by HPLC and stopped after 10 min when it reached the maximal yield. The mixture was then evaporated to an oily residue under vacuum and 1-butanol (400 mL) was added. The solution was washed with water, 5% $NaHCO_3$, and water and then concentrated. The solid was recrystallized from MeOH/ether to give the product in 75-80% yield. Z-Tyr-L-Arg-OMe: TLC (CH_3Cl_3:MeOH = 9:1 v/v) R_f 0.14; mp 118-121 °C. Z-Tyr-D-Arg-OMe: TLC (CH_3Cl_3:MeOH = 9:1 v/v), R_f 0.14; mp 120-124 °C. Both compounds were identical with those prepared previously.[15]

A New Class of Phospholipase A_2 Substrates: Kinetics of the Phospholipase A_2 Catalyzed Hydrolysis of 3-(Acyloxy)-4-nitrobenzoic Acids

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Abstract: 3-(Acyloxy)-4-nitrobenzoic acids were synthesized with acyl groups ranging from butyryl to dodecanoyl. All these compounds yielded monomeric solutions in water with 1.6% (v/v) acetonitrile in the neutral pH range, and they were hydrolyzed by catalytic amounts of phospholipases A_2 from a variety of sources as shown by the spectral change at 425 nm due to the appearance of nitrophenolate ion. Most of the kinetic studies were performed using *Agkistrodon piscivorus piscivorus* phospholipase A_2, but similar results were obtained with porcine pancreatic and *Crotalus atrox* phospholipase A_2. The catalytic reaction requires the presence of Ca^{2+}, but unlike the hydrolysis of lecithins, the hydrolysis of these substrates also occurs in the presence of Ba^{2+} and Sr^{2+}, while Mg^{2+} and Zn^{2+} are not catalytically competent. Increasing the acyl chain length increases the enzymatic rate mainly by enhancing the hydrophobic interaction in the E-Ca^{2+}-S complex. Among structural isomers of the octanoyl compound, 3-nitro-4-(octanoyloxy)benzoic acid shows the highest specificity toward the enzyme, suggesting that it is in this compound that the distance between the negatively charged carboxylate and the reactive ester approximates best that found in the lecithin-enzyme complex. All kinetic characteristics of the enzymatic hydrolysis indicate that the reaction occurs by the same mechanism as that of the hydrolysis of lecithins. The highest catalytic efficiency observed with this series of substrates occurs with 3-dodecanoyl-4-nitrobenzoic acid, and the second-order rate constant of this reaction (k_{cat}/K_m = 9.1 × 10^4 M^{-1} s^{-1}) is only 1 order of magnitude lower than that of the hydrolysis of egg phosphatidylcholine in unilamellar vesicles. The reactivity of all isomers, especially that of the *p*-carboxy ester, shows that Ca^{2+} does not act as a catalyst in the phospholipase A_2 catalyzed hydrolysis but rather serves to bind and orient the substrate at the active site of the enzyme. The octanoyl compounds, **1** and **2**, are ideally suited for a rapid and sensitive spectrophotometric assay of phospholipases A_2, and the conditions for the assay are described.

Phospholipases A_2 (PLA_2, EC 3.1.1.4) are a class of calcium-dependent enzymes that catalyze the hydrolysis of the fatty acid ester in the 2-position of 3-*sn*-phospholipids (for a review, see ref 1). The essential structural elements of the substrate that appear to be required for recognition by PLA_2 are the glycerol backbone, the ester function in position 2, and the negative charge of the phosphate in position 3 (see Figure 1). While the enzyme has a pronounced preference for substrates aggregated into micelles,

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