Enzyme-Catalyzed Irreversible Formation of Peptides Containing D-Amino Acids

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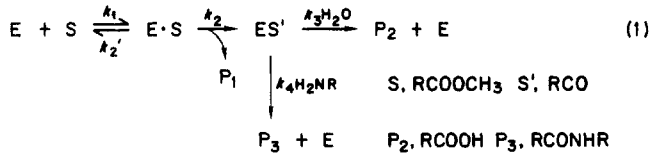
Received August 27, 1985

Procedures have been developed for the preparation of dipeptides Z-L-Tyr-D-X and Z-L-Phe-D-X using Z-L-Tyr-OMe (or Z-L-Phe-OMe) and D-amino acid esters or amides (D-X) as substrates and soluble or immobilized α-chymotrypsin as a catalyst. The formation of each of these peptides in miscible or immiscible organic solvent–water systems in a kinetically controlled approach is virtually irreversible with no side reactions or racemization. Kinetic studies indicate that D-amino acid esters are about 100 times that of water and 10% that of L-amino acid esters as a nucleophile in deacylation reactions. The effects of pH, organic solvents, temperature, and substrate and enzyme concentrations on the yield and the stability of the enzyme in syntheses have been studied and the results compared with those in the enzyme-catalyzed formation of L-L-dipeptides.

Great interest has been shown in the use of proteases to catalyze peptide bond formation, and many studies to this end have been reported.[1] The advantages of these techniques include (a) general freedom from racemization, (b) less need for side chain protection, and (c) enzyme immobilization allows for the recovery of catalytic activity, giving these techniques considerable economic advantage over chemical, stoichiometric procedures. The methodology, however, has not been fully exploited for possible synthesis of a number of biologically important peptides containing D-amino acids or other unusual amino acid derivatives. Antibiotic peptides,[2] synthetic peptides of enhanced hormonal or neural activity,[3] and many prodrugs used in chemotherapy[4] often contain D-amino acid residues. Recombinant DNA technology is limited to the production of peptides containing only L-amino acids[5] as, in general, have been approaches based on protease catalysis.[6]

Since α-chymotrypsin was reported in 1977 to be a poor catalyst in the synthesis of dipeptides using D-Leu-NH_2 as nucleophile,[7] it was not used for preparative synthesis of peptides containing D-amino acids until 1984 when Petkov and others illustrated the practicality of the system in a kinetically controlled approach. The reaction, however, was slow, and the enzyme was deactivated quickly at room temperature under the alkaline conditions used. We have attempted to investigate the generality of this type of unusual catalysis and to optimize the reactions.

In order to improve the reaction rate by using a D-amino acid ester as a nucleophile, we have chosen to utilize the kinetically controlled approach to peptide synthesis.[7,8] In this procedure, the acyl donor to the peptide bond is an ester, here Z-L-Tyr-OMe. Such an ester facilitates the formation of an acylenzyme, which is then subsequently deacylated slowly by a nucleophile. If the nucleophile is water, the acid of the acyl donor is formed (eq 1).[9] In a



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kinetically controlled synthesis, the reaction must generally be quenched rapidly; continued exposure to the enzyme will result in secondary hydrolysis of the newly formed peptide. This is not necessary when D-amino acids (or arginine) are the C-terminal components of the dipeptides;

Table I. Physical Data of the Dipeptides Prepared in Chymotrypsin-Catalyzed Syntheses[a]

product	yield, %[b]	mp, °C	$[\alpha]^{20}_D$, deg (c 1, $CHCl_3$)	R_f[c]
Z-L-Tyr-D-Met-OMe	80	143–144	+18.1	0.28 (I)
Z-L-Tyr-D-Ser-OMe	72	164–165	−9.2	0.37 (I)
Z-L-Tyr-D-Phe-OMe	75	170–173	+8.1	0.48 (I)
Z-L-Tyr-D-Arg-OMe	70	120–124	+10.1	0.14 (II)
Z-L-Tyr-L-Arg-OMe	71	118–121	−7.3	0.14 (II)
Z-L-Tyr-D-Leu-OMe	74	114–116	+12.1	0.48 (I)
Z-L-Tyr-L-Val-OMe	20	143–145[d]	−11.1[d]	0.53 (I)
Z-L-Tyr-D-Val-OMe	20	118–119	+5.2	0.53 (I)
Z-L-Phe-D-Met-OMe	76	191–193	+10.0	0.50 (I)
Z-L-Phe-D-Ala-OMe	57	125–126	+11.3	0.60 (II)
Z-L-Phe-D-Val-NH_2	20	125–127	+17.6	0.58 (I)

[a] Conditions: 50 mM acyl donor, 800 mM acyl acceptor, 0.2 mM α-chymotrypsin, 0.2 M carbonate (pH 9.1, before addition of organic solvent)/50% DMF, 25 °C; total volume, 50 mL. Reaction was stopped when no donor ester was detected (5–10 min). The physical constants of the products (mp, specific rotations, C and N analyses) are in agreement with those prepared chemically. [b] Determined by HPLC before isolation. [c] Solvent systems for TLC on silica gel plates: (I) $CHCl_3$/EtOAc (7:3, v/v); (II) $CHCl_3$/MeOH (9:1, v/v). [d] The literature values are 144–147 °C and −11.7°, respectively (Schroder, E. *Justus Liebigs Ann. Chem.* **1964**, *680*, 142.)

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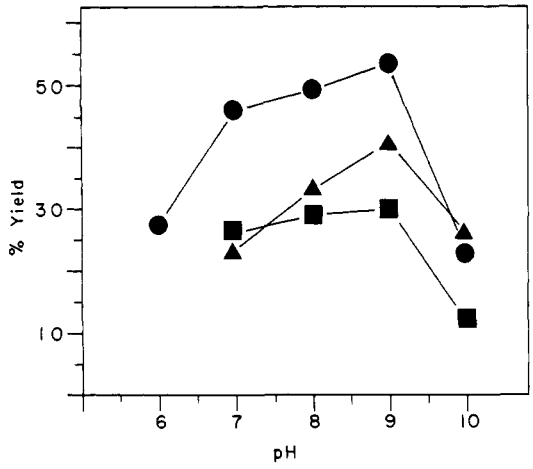

Figure 1. pH profile for the synthesis of Z-L-Tyr-D-Met-OMe. The concentrations of Z-Tyr-OMe and D-Met-OMe·HCl were 50 and 200 mM, respectively. Reactions were done in 50% organic solvent. The pH reported was of components in buffer before addition of organic solvent. The concentration of enzyme was 0.2 mM. Organic solvents: (▲) DMF; (●) Me_2SO; (■) MeOH.

chymotrypsin can only hydrolyze the peptide bond slowly, if at all,[10] thus making the peptide synthesis virtually irreversible.

Results and Discussion

Synthesis. Kinetically controlled enzymatic peptide synthesis is but a special case of deacylation of acyl-α-chymotrypsin by a nucleophile other than water (hydrolysis). Indeed, Fersht and co-workers have shown 1 M L-Ala-NH_2 to be 44 times as effective as 55 M water in deacylating Ac-Phe-chymotrypsin[11] and 100 times as effective in deacylating Ac-Tyr-chymotrypsin.[12] D-Amino acids (an arginine by virtue of tis charged side chain) can be expected to be less efficient nucleophiles than L-amino acid esters. In a carbonate buffer (0.2 M, pH 9)–DMF (1:1 v/v) solution containing 50 mM Z-L-Tyr-OMe, 800 mM D-amino acid methyl ester, and 0.2 mM α-chymotrypsin, the yield of the peptides produced reached 70–80% within 10 min when all Z-amino acid ester was consumed.

Table II. Effect of Cosolvent Concentration on Peptide Yield[a,b]

% solvent	solvent: MeOH	DMF	Me_2SO	formamide	1,4-butanediol
		Z-L-Tyr-D-Met-OMe			
0			29 [80.4][b]		
10	8 [76]	15 [78.2]	10 [79.4]	37	
25	16 [71]	28 [75.0]	12 [78.7]	27	
40		33 [70.4]	48 [77.2]	23	
50	19 [60.3]	38 [66.5]	55 [76.0]	21	31
60			61 [73.6]		
80					49
		Z-L-Tyr-L-Arg-OMe			
0			16		
10	11	14	22		
25	21	17	22		
50	26	37	42		
60			53		

[a] In %. Same reaction conditions as in Table I except that the concentration of D-Met-OMe was 200 mM. [b] The values in brackets are dielectric constants of the solvent mixture (ref 19).

Table I presents yield data and physical constants for several peptides of the form Z-L-Tyr-X-OMe or Z-L-Phe-X-OMe. In all cases, α-chymotrypsin was seen to be at least a moderately effective catalyst for synthesis of the peptides. We undertook, therefore, a closer examination of reaction conditions, utilizing Z-L-Tyr-D-Met-OMe and Z-L-Tyr-L-Arg-OMe as models.

Effects of pH, Organic Solvents, Temperature, and Concentrations. Figure 1 presents pH profiles for the synthesis of Z-L-Tyr-D-Met-OMe in 50% organic solvent. The optimal pH is 9 which is roughly comparable to the results found by other authors.[7,13] The pH values given are of the buffer solution containing the reaction components before the addition of organic solvent. The apparent pH of the medium can be expected to be 1–2 units higher in the presence of organic cosolvent.[14] At high pH, the predominant conformation of chymotrypsin is inactive,[15] accounting for the fall-off of activity above pH 9.

The effect of added cosolvent on peptide synthesis is summarized in Table II. With the exception of formamide, peptide yield increases with added solvent up to 50% (60% for Me_2SO), beyond which no reaction occurs, due to enzyme denaturation. This denaturation is not completely irreversible, as hydrolytic activity can be restored by adding buffer to the reaction. Suitability for peptide synthesis generally increases with increasing polarity[16] or dielectric constant of the solution. Values in brackets next to yield values are the dielectric constants for the respective cosolvent mixtures;[17] peptide yield can be seen to correlate quite well with these values. Formamide is atypical in that, although its dielectric constant is greater than water (ca. 100), peptide synthesis drops with increasing solvent concentration. Since formamide is in effect a "bare" peptide bond, it may bind weakly in active site near where the nucleophile amino acid binds, while at the same time being less effective in excluding water. The unexpected high yield of Z-L-Tyr-D-Met-OMe in the absence of organic solvent is surprising. In this system the peptide rapidly precipitates; removal of the product from

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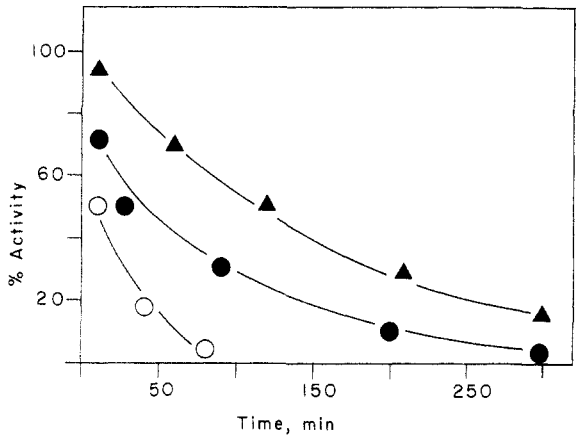

Figure 2. Stability of soluble α-chymotrypsin in different solvent systems. See Experimental Section for details of assay. 100% activity was defined as the activity of a 20-μL aliquot 10 s after addition of enzyme. The enzyme concentration was 0.2 mM: (●) 0.2 M phosphate, pH 7/50% DMF; (○), 0.2 M carbonate, pH 9.1/50% DMF; (▲) 0.2 M carbonate, pH 9.1/80% butane-1,4-diol.

the reaction medium may serve to shift the partitioning of the acyl enzyme toward ammonolysis. The lower concentrations of organic solvent (25% and 10%) may lower the yield of peptide by increasing its solubility while not greatly altering the nucleophile to water ratio. This is supported by the fact that no large increase in yield is seen for Z-L-Tyr-D-Arg-OMe in buffer alone, as this peptide is considerably more water soluble. Also atypical is the relatively low yield of Z-L-Tyr-D-Met-OMe in 80% 1,4-butanediol. Chymotrypsin is known to be stable in high concentrations of such polyols,[18] yet the yield is low for a system containing only 11 M water. Similar results were reported by Nilsson and Mosbach,[13] and were attributed to the observation that polyols are selectively excluded from the protein domain (at moderate concentrations), thus raising the water concentration at the active site.[19]

Since optimal synthesis seems to occur under conditions where enzyme activity is not likely to be long-lived, we examined whether this denaturation might interfere with synthesis. At room temperature in most solvents, the acyl donor ester was consumed within 2 min, stopping the reaction (the acid once formed can not react any further due to being charged at the pH of the reaction). Figure 2 is the time course of enzyme inactivation under typical reaction condition (pH 9, 50% cosolvent, 0.2 mM enzyme). The 100% activity was defined as the hydrolytic activity of an aliquot of the mixture toward Z-L-Tyr-*p*-nitrophenyl ester (Z-L-Tyr-ONp), 10 s after addition of the enzyme to the reaction mixture. In all solvents except 1,4-butanediol, nearly 50% of the original activity has been lost within 5 min; however, since typical reaction times are less than 2 min, this denaturation should not be a problem. Activity curves for the enzyme in the presence of the other synthesis components (acyl donor and acceptor) were found to exactly parallel these results. In a companion experiment, the effect of enzyme concentration of synthetic yield was examined (Figure 3). The yield of Z-L-Tyr-D-OMe was invariant in the enzyme concentration range of 0.01–1 mM, falling off rapidly at 4 μM, indicating that above this latter concentration, enough active enzyme remains to catalyze the reaction.

The effect of nucleophile concentration is summarized in Figure 4. The yield of both peptides can be seen to be nonlinear in nucleophile concentration. This phenomenon,

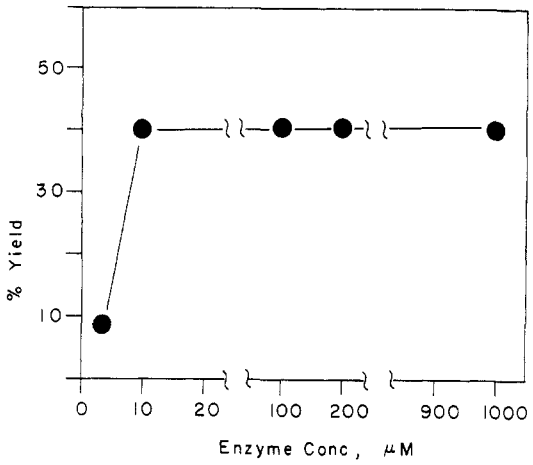

Figure 3. Effect of enzyme concentration on the synthesis of Z-L-Tyr-D-Met-OMe. The reaction mixture contained 50 mM Z-L-Tyr-OMe and 200 mM D-Met-OMe in 0.2 M carbonate, pH 9.1/50% DMF.

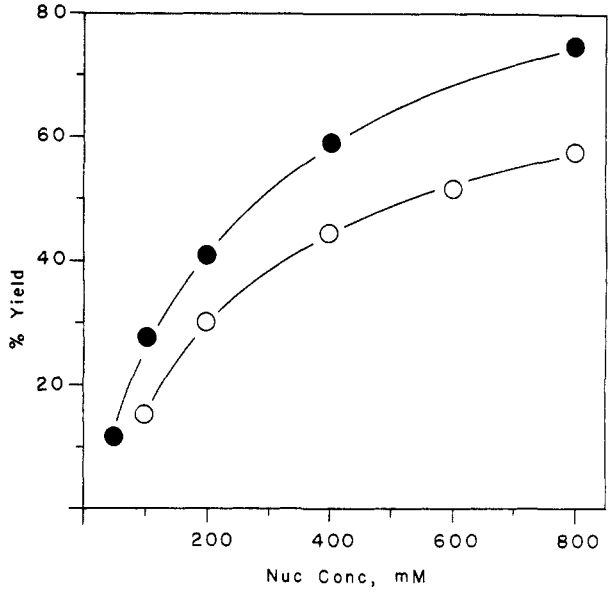

Figure 4. Effect of nucleophile concentration on peptide yield. Conditions are the same as that in Figure 3: (●) Z-L-Tyr-D-Met-OMe; (○) Z-L-Tyr-L-Arg-OMe (the concentration of enzyme was 0.2 mM, and the concentration of Z-L-Tyr-OMe was 200 mM).

which has also been observed for "better" nucleophiles, has been interpreted as indicative of a nucleophile binding site, the nonlinear response being due to the saturation of the site.[20] Simpler nucleophiles (methanol,[21] simple amines[22]) do not exhibit such saturation effects. Interestingly, in one study L-Val-NH_2 was found not to competitively inhibit hydrolysis of Ac-Tyr-*p*-nitroanilide, suggesting that the nucleophile binding site may be distinct from the site for the hydrolytic leaving group (S'_1).[20] Different nucleophiles can be viewed, then, as occupying a broad continuum of deacylating efficiency. Nonspecific nucleophiles, such as water or methanol require high concentration to be effective, while a much better nucleophile, L-Ala-NH_2, is about 5500 times as reactive as an equimolar amount of water in deacylating Ac-Tyr-chymotrypsin. From our product ratios, D-Met-OMe and L-Arg-OMe can be seen to be 250 and 170 times as reactive, respectively, as an

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Table III. Effect of Component Concentration on Peptide Yield[a]

[acyl donor],[b] mM	[acyl acceptor],[c] mM	yield, %
	Z-L-Tyr-D-Met-OMe	
250	250	41[d]
250	500	52[d]
100	400	54[d]
200	800	61[d]
400	1600	76[d]
500	2000	79[d]
	Z-L-Tyr-L-Arg-OMe	
100	400	31
200	800	44
400	1600	67[e]

[a] Reaction conditions: 0.2 M carbonate (pH 9.1, before addition of solvent)/50% DMF, 0.2 mM chymotrypsin. [b] A-L-Tyr-OMe. [c] D-Met-OMe·HCl or L-Arg-OMe·2HCl. [d] Determined on the basis of the product precipitated within 2 min. [e] Reaction stopped with 30% donor ester unreacted.

equimolar amount of water (i.e., $k_4/k_3 = 250$ and 170, respectively). To estimate the ratio of k_4/k_3, we use eq 2–4.

$$V_{H_2NR}/V_{H_2O} = k_4[H_2NR]/k_3[H_2O] = n \quad (2)$$

$$\text{yield} = n/(n+1) \quad (3)$$

$$[H_2NR] = [\text{total amine}]/(1 + 10^{pK-pH}) \quad (4)$$

The n values calculated from the observed yields were used to estimate the rate of aminolysis and hydrolysis and their rate constants. The k_{cat}, $k_3[H_2O]$, value for α-chymotrypsin in hydrolysis is about 100 s^{-1} ($k_3 \sim 2$ s^{-1} M^{-1}),[9] and k_4 with L-amino acid ester as a nucleophile is about 5×10^3 s^{-1} M^{-1}. The D-amino acid ester is thus about 10% as effective as the L-form as a nucleophile.

Practically, however, one wishes to avoid large excesses of one component over another, and Figure 4 indicates acyl acceptor:acyl donor ratio of 20 or greater is necessary for high yields at this concentration (50 mM donor). However, if the critical determinant for high synthetic yield is the ratio of nucleophile concentrations ([acceptor]/[water]), as indicated by the cosolvent concentration data, then increasing the concentrations of both components should increase yield without requiring large excesses of one component over the other. This is borne out by the data in Table III. In the case of Z-L-Tyr-D-Met-OMe, the product precipitated in all reactions. Yield can be seen to depend on acceptor concentration more strongly than on donor concentration. The situation for Z-L-Tyr-L-Arg-OMe is slightly different. No precipitation was seen in any experiment, and in the experiments where acceptor concentration exceeded 800 mM, the reaction stopped with approximately 30% of the donor ester unreacted. Addition of more enzyme reduced this to approximately 22% but no further. It seems likely that the high concentration of the peptide in solution (ca. 200 mM) is inhibitng the enzyme. This inhibition is not evident in the synthesis of Z-L-Tyr-D-Met-OMe, since the product rapidly separates from the medium.

At least two authors[13,20] have reported increased synthetic yields using chymotrypsin at lower temperatures (−15 to 20 °C), both with immobilized and soluble enzyme. This yield increase has been suggested to be due to tighter binding of the nucleophiles at lower temperatures. It has also been suggested that cooling can offset the deleterious effect the organic cosolvent has on the enzyme by increasing the dielectric constant of the medium.[23] Figure 5 presents time course for the synthesis of Z-L-Tyr-D-Met-OMe at various temperatures, with both soluble and immobilized enzyme. No significant increase of yield is seen, perhaps indicating that D-Met-OMe cannot bind well enough to take advantage of the better contacts available to its enantiomer.

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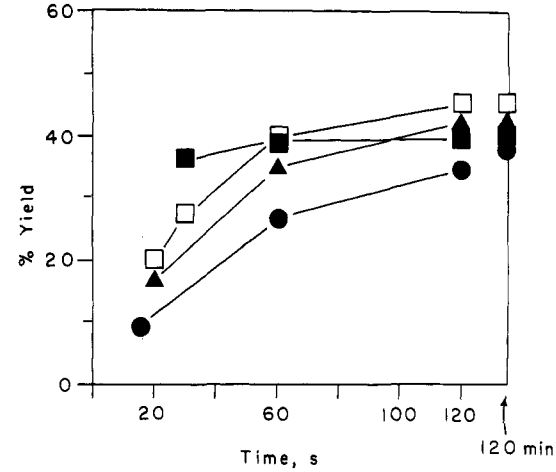

Figure 5. Temperature effect on peptide yield. Conditions are the same as that in Figure 3: (●) −15 °C; (▲) 0 °C; (□) 12 °C; (■) 20 °C. Enzyme concentration was 0.2 mM.

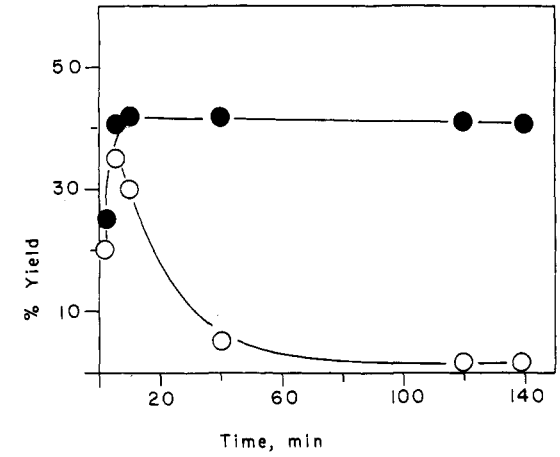

Figure 6. Time course for the enzyme-catalyzed synthesis of Z-L-Tyr-D-Met-OMe (●) and Z-L-Tyr-L-Met-OMe (○). Conditions are the same as that in Figure 3. After 2 h, 5 mg of enzyme was added. The original enzyme concentration was 0.2 mM.

Irreversibility of Peptide Bond Formation. To demonstrate the irreversibility of the synthesis when D-amino acids are used, Figure 6 presents the time course for the synthesis of Z-L-Tyr-D-Met-OMe and Z-L-Tyr-L-Met-OMe. The amount of the L-L-peptide can be seen to pass through a maximum, then decrease, while the L-D-peptide reaches an amount which remains the same level even after addition of additional enzyme after 2 h. Surprisingly, the apparent rate of the L-L-peptide synthesis is slower than that of the L-D-peptide. This is attributable to two processes. First, the L-L-peptide is a good substrate for the enzyme; that any peptide is produced at all is due to the inherent rapidity of ester hydrolysis vs. amide hydrolysis. Secondly, L-Met-OMe itself is a substrate. If the reaction is followed on TLC, the free acid can be seen to be rapidly generated.

Optical Purity. The specific rotations and the melting points of the peptides prepared enzymatically are in agreement with those prepared chemically, indicating that no significant racemization was occuring during the synthesis. To further verify that the products prepared enzymatically were free from racemization, two approaches were employed. Reverse-phase HPLC was used to detect

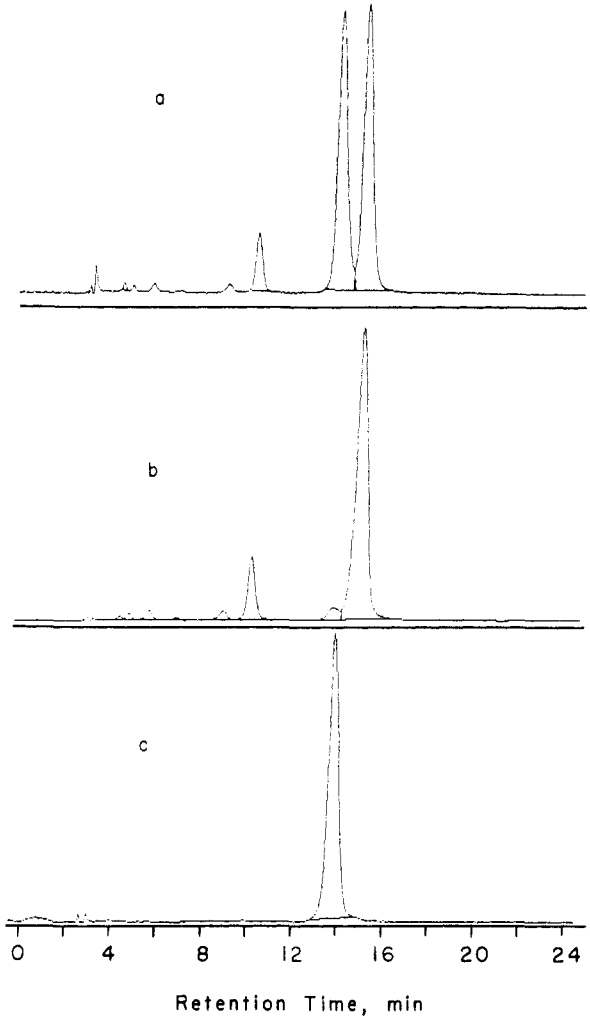

Figure 7. HPLC Analyses of synthetic peptides: (a) coinjection of Z-D-Tyr-D-Met-OMe (chemically synthesized; retention time, 15.41 min) and Z-L-Tyr-D-Met-OMe (enzymatically synthesized; retention time, 14.28 min); (b), Z-D-Tyr-D-Met-OMe (chemically synthesized) showing 3% racemization; (c) Z-L-Tyr-D-Met-OMe (enzymatically synthesized) with no detectable racemization. The flow rate was 1 mL/min. Samples were analyzed before purification by recrystallization. See Experimental Section for details. This technique will detect 0.1% racemization.

the presence of Z-D-Tyr-D-Met-OMe, the racemization product. Figure 7 shows that in the chemical synthesis of Z-D-Tyr-D-Met-OMe, 3% racemization was seen, whereas none is evident in the enzymatic reaction. The product was also examined by proton and ^{13}C NMR. As shown in the product prepared chemically (Figure 8b), a shift of 0.1–0.2 ppm in the α, β, or OCH_3 protons can be seen due to the presence of the other diastereomers generated by racemization. In no instance was any racemization detected in the enzymatic reaction (Figure 8). This is consistent with the results obtained in kinetic resolution of racemic *N*-acyl amino acid esters with chymotrypsin in which an optically pure *N*-acyl L-amino acid was obtained.[9] Since deacylation of the acyl enzyme intermediate (ES′, the activated intermediate which may racemize before reaction with nucleophiles) with water is the rate-determining step in hydrolysis and no racemization was observed, it is conceivable that deacylation with a more effective nucleophile such as D-amino acid ester would not cause racemization because the lifetime of the acylenzyme intermediate is shorter than that in hydrolysis.

Preparative Synthesis. We also investigated preparative-scale synthesis using the optimal conditions we have

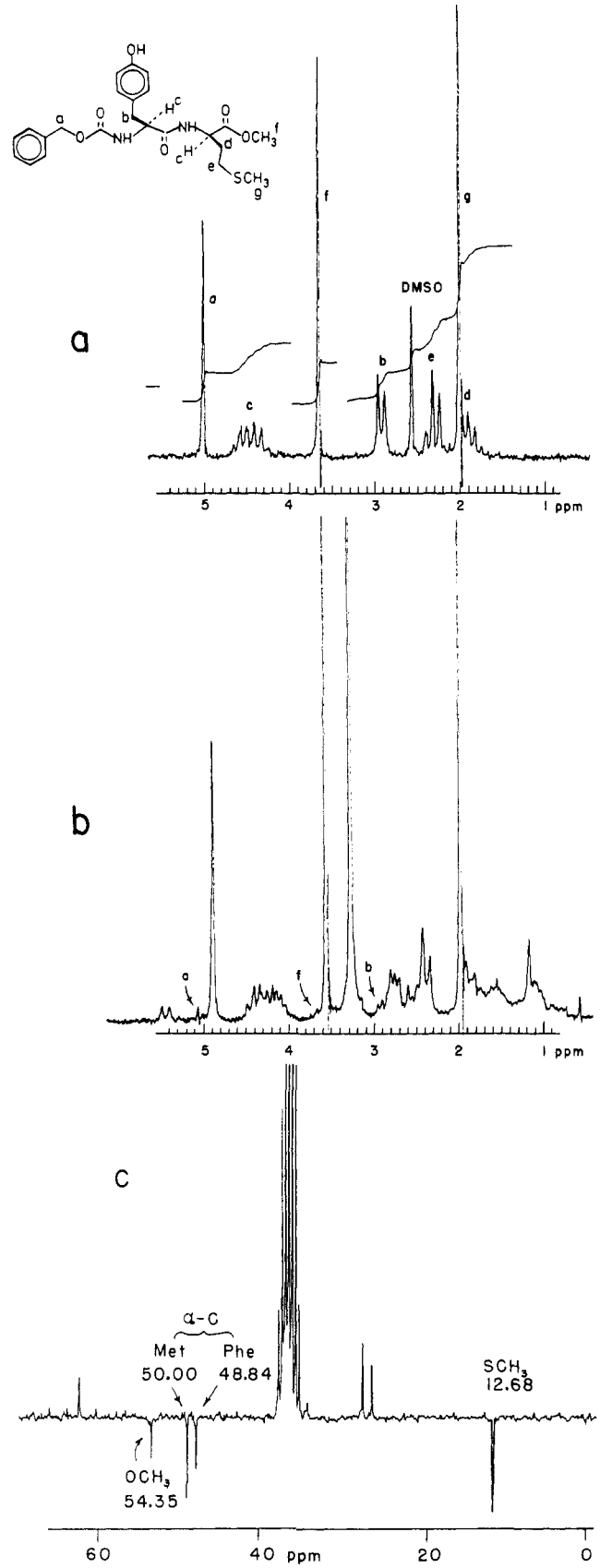

Figure 8. ^1H NMR (90MHz) spectra of Z-L-Tyr-D-Met-OMe (a) prepared enzymatically and Z-D-Tyr-D-Met-OMe (b) prepared chemically. Samples were analyzed before purification by recrystallization. (c) The ^{13}C NMR (50 MHz) spectrum with attached proton test (APT) of Z-L-Tyr-D-Met-OMe in Me_2SO-d_6, obtained by using a 2-s repetition rate and 45° observe pulses with single spin echo, τ 7.4 ms. Assignments of some nuclei are indicated. All samples were analyzed before purification by recrystallization. Both ^1H and ^{13}C NMR techniques will detect 3% racemization.

Table IV. Peptide Synthesis Using Chymotrypsin Immobilized on Eupergit C. Synthesis of Z-L-Tyr-D-Met-OMe

	EC_{NH}[a]		EC_{SH}[b]	
% DMF	reacn time, min	yield, %	reacn time, (min)	yield, %
50	40	34	40	36
70	60	42	60	39
80	240	46	240	42

[a] Conditions: 50 mM Z-Tyr-OMe, 200 mM D-Met-OMe·HCl in 0.2 M carbonate, pH 9.1; total volume, 2 mL, 300 mg of immobilized enzyme containing 15 U/g of wet gel. [b] Same conditions as in *a*, 320 mg of immobilized enzyme containing 10 U/g of wet gel.

developed. Two systems were developed: one containing 50 mM Z-L-Tyr-OMe and 800 mM D-Met-OMe or L-Arg-OMe in 50 mL of 60% Me_2SO/0.2 M carbonate buffer (pH 9) and 0.2 mM chymotrypsin. The isolated yield was 70%. In the second system, 0.5 M Z-L-Tyr-OMe and 1.5 M D-Met-OMe in 10 mL of 60% Me_2SO/carbonate buffer (pH 9), 0.2 mM chymotrypsin was used. The isolated yield was 78%.

Use of Immobilized Enzyme. Elimination of water should in general improve peptide yield, and several approaches to achieve this while retaining enzyme activity have appeared in the literature. These include protein immobilization,[13,24] the use of biphasic reaction systems involving water–immiscible cosolvents[25] or reverse micelles, or the derivatization of the enzyme with an amphoteric polymer, making it soluble in organic solvents and active with no added water.[27] We have begun to investigate immobilization. In the present work, α-chymotrypsin was immobilized in two different media, Eupergit C, a methacrylamide polymer containing oxirane groups,[28] and polyacrylamide (PAN, poly[acrylamide-*co*-*N*-(acryloxy)-succinimide]),[29] cross-linked with triethylenetetramine (TET). Such immobilization should stop the aggregation of the enzyme in high concentration of organic solvents, the predominant mode of denaturation of chymotrypsin in such systems.[24]

The immobilization yield on Eupergit C was generally low (about 10% soluble activity), but this was deemed sufficient for our purposes. Table IV presents representative yield data for the synthesis of Z-L-Tyr-D-Met-OMe using two forms of this immobilized catalyst: one (EC_{SH}) that has had excess oxirane groups digested by treatment with mercaptoethanol, the other (EC_{NH}) has had these groups digested by treatment with 2-aminoethanol.[30] These catalysts allow reaction to continue up to 80% DMF (although it is slower), but the yield is not significantly different from that of soluble enzyme in 50% DMF. Of course, immobilization does allow the enzyme activity to be recovered (see Figure 2), and we have observed less than 30% loss of activity over several syntheses. Further improvement of the enzyme stability at pH 9 can be achieved by modifying the Met-192 residue to sulfoxide (see West and Wong in ref 1). The methacrylamide polymer backbone is fairly hydrophilic; it may be that the microenvironment of the enzyme attached to the polymer has a

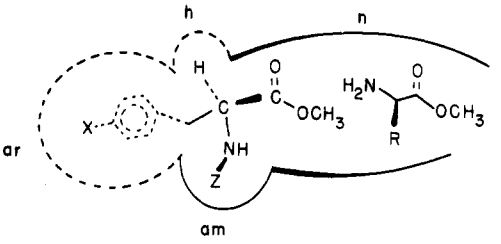

Figure 9.

higher water concentration than that of the bulk solvent. This situation is even more severe in the case of PAN-immobilized enzyme. This procedure produces a hydrated gel, which is then crushed. The gel is not amenable to filtration but can be isolated by gentle centrifugation. The gel is approximately 90% water by weight; in the presence of water–miscible organic solvents it tends to dehydrate, and the gel structure collapses, leaving a hard material which is catalytically inactive. Activity can be restored by soaking the hardened gel in water. It was found that moderate concentrations of glycerol (20–40%) stabilized the gel structure when DMF was used as cosolvent. Also the gel can be pretreated with mixtures of glycerol and buffer, thus further lowering the amount of water. Tables V and VI present data from the systems and show some yield enhancement. Again, the microenvironment of the enzymes may be more aqueous than that of the bulk solvent due to the hydrophilic nature of the polymer.

We have found, however, that water–immiscible solvents do not dehydrate the gel. Use of ethyl acetate, for example, produces a biphasic system, which has been shown to be an effective synthetic system by several authors.[31,32] The amount of water in the system can be varied by pretreatment as before, or by increasing the reaction volume relative to the gel volume. Reaction time for this system is slow, on the order of days rather than minutes, but quite high yields of peptides were achieved (greater than 90%). Tables V and VI summarize these results.

The Active Site Model. Examination of the active site model developed on the basis of X-ray structure provides the rationale for the observed substrate specificity of chymotrypsin (Figure 9).[33] As shown, the *ar*, *h*, and *am* regions can only accept groups with specific configuration such as *N*-acyl-L-Tyr, which must be bound in such a way that the phenolic group is oriented in the hydrophobic pocket (the *ar* region) and the acyl amino group in the *am* region. The *n* region (P′ site, the charge-delay system is located here),[34] however, is quite flexible and will accommodate different groups. It is not surprising that D-amino acid derivatives could enter this region and act as a slow substrate.

In summary, the procedures illustrated here describe the practical application of chymotrypsin in peptide synthesis, particularly in the synthesis of peptides containing D-amino acids. The irreversibility of the reactions makes isolation of the products very simple. Further, the starting materials used in the syntheses are readily available and need not be pure; contamination of free amino acids or inorganic salts would not cause problems. The compounds prepared

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Table V. Peptide Synthesis Using PAN–Chymotrypsin in Water-Miscible Solvents. Synthesis of Z-L-Tyr-D-Met-OMe

% DMF	% glycerol	% buffer	PAN-A[a] reacn time, h	PAN-A[a] yield, %	PAN-B[b] reacn time, h	PAN-B[b] yield, %
50		50	1	43	1	44
60	20	20	1	44	1	39
70	25	5	3	48	3	45

[a] PAN–α-chymotrypsin pretreated with 0.2 M carbonate buffer, pH 9.1, followed by centrifugation to obtain the wet gel; 400 mg of wet gel was used. [b] PAN–α-chymotrypsin pretreated with 0.2 M carbonate buffer/50% glycerol; 400 mg of gel was used. The concentrations of substrates are the same as in Table IV.

Table VI. Peptide Synthesis Using PAN–Chymotrypsin in Water–Immiscible Solvents. Synthesis of Z-L-Tyr-D-Met-OMe

ethyl acetate, mL	mg of PAN[a]	reacn time, day	yield, %
4	(A) 200	5	82
4	(B) 210	5	86
8	(A) 200	4	88
8	(B) 190	5	91

[a] PAN-A and PAN-B were prepared as described in Table V. The wet gels were mixed with substrate and ethyl acetate as described in Table V.

in this study are useful intermediates for a variety of peptides with neural activities.

Experimental Section

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). Measurement of UV absorbance was performed on a Beckman Model 6 UV–vis spectrophotometer. Amino acid analyses were carried out at the University of Texas in Austin using a Beckman amino acid analyzer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Optical rotations were measured at room temperature with Perkin-Elmer polarimeter, Model 241. Thin-layer chromatography was developed with Merck silica gel plates coated on plastics.

Amino acids were purchased from Sigma, as was chymotrypsin (Type III, 3× crystallized). Z-Tyr-ONp, benzyl chloroformate, dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, and thionyl chloride were from Aldrich.

Amino acid methyl esters were synthesized by suspending the amino acid in cold, dry methanol and slowly adding 1 equiv of thionyl chloride. The solution was then stirred at room temperature for 1 h and then allowed to stand overnight. The solvent was removed under vacuum and the resulting oil taken up in 5 mL of methanol, to which 40 mL of ether was added. The resulting crystals were collected, and recrystallized from methanol/ether.

Carbobenzoxy-L-tyrosine Methyl Ester (*Z*-L-Tyr-OMe). The procedure is similar to that reported previously.[35] L-Tyrosine methyl ester hydrochloride (20 g, 43 mmol) was suspended in 400 mL of chloroform and 50 mL of water containing 9.1 g (86 mmol) of sodium carbonate. The solution was cooled in an ice bath, and 7.33 g (43 mmol) of benzyl chloroformate and 40 mL of 2 Na_2CO_3 were added simultaneously wtih vigorous stirring. The solution was stirred for 3 h at room temperature. Water (200 mL) was added and the solution stirred for 10 min more, and the chloroform was separated and dried over $MgSO_4$. The solvent was removed under vacuum, and the resulting oil was rubbed with petroleum ether, affording a solid, which was recrystallized from $CHCl_3$/petroleum ether; yield, 20.1 g (71%), mp 91–92 °C.

Z-Tyrosine (Z-Tyr-OH) was prepared according to the literature procedure.[36] The compound Z-Tyr-OMe (4 g, 12 mmol) was dissolved in 10 mL 4 N NaOH/dioxane (1:1 v/v) and allowed to stand for 2 h. Water (20 mL) was added, the solution slowly acidified with 4 N HCl, and the resulting precipitate collected and washed with cold water. This solid was dissolved in hot sodium acetate solution (0.5 M), filtered, then allowed to cool, and then slowly acidified with 1 N HCl. The resulting white needles were collected and washed with cold water; yield, 3.3 g (89%); mp 92–94 °C.

Chemical Synthesis of Peptides. All peptides except those containing Arg were synthesized by the DCC-HONSu method.[37] In a typical synthesis, 470 mg (1.5 mmol) of Z-Tyr-OH, 300 mg (1.5 mmol) of D-Met-OMe·HCl, and 0.21 mL of triethylamine were dissolved in dry THF and cooled to 0 °C. *N*-Hydroxysuccinimide (172 mg) and DCC (310 mg) were added, and the solution was stirred for 2 h and then stored at 4 °C overnight. A few drops of acetic acid were added, and the solution was allowed to stand at room temperature for 2 h. The dicyclohexylurea and triethylamine hydrochloride were filtered off, and the solution was concentrated under vacuum. The resulting oil was taken up in 5 mL of ethanol and poured into cold water. The peptide, which quickly crystallized, was collected and washed successively with 1 N HCl, water, 5% $NaHCO_3$, and water. The product was recrystallized from ethanol/water; yield 0.52 g (67%).

Arginine-containing peptides were synthesized by the carbonic anhydride method.[38] A mixture of Z-Tyr-OH (470 mg) and *N*-methylmorpholine (150 mg) were dissolved in dry THF (10 mL), and the solution was cooled to −15 °C. To this mixture was added 204 mg of isobutyl chloroformate, and the solution was stirred for 1 min. A solution of 390 mg of L-Arg-OMe·2HCl (1.5 mmol) and 0.21 mL of triethylamine in 3 mL dry DMF was added and the solution stirred for 5 min. The reaction was allowed to come to room temperature, the hydrochloride was filtered off, and the solution was concentrated under vacuum. The residue was taken up in 1-butanol, washed with water, 5% $NaHCO_3$, and water, and then concentrated. The resulting oil was rubbed with diethyl ether, which afforded a solid, which was recrystallized from MeOH/ether; yield, 0.42 g (56%).

Enzyme Assays. Chymotrypsin activity was assayed photometrically by measuring the absorbance increase at 405 nm due to the release of *p*-nitrophenol (ϵ 18.5 mM^{-1} cm^{-1}) from Z-Tyr *p*-nitrophenyl ester. The assay buffer contained 0.2 M tris-maleate (pH 6.8), 0.1 M $CaCl_2$, and 10% dioxane. Substrate solution was 2.4 mM Z-Tyr-ONp in dioxane. Typical assay contained 2.8 mL of buffer, 0.1 mL of substrate, and 0.02 mL of solution being assayed.

HPLC Analysis. All yields were determined by HPLC on a Gilson Model 43 Chromatograph equipped with a Data Master gradient programmer/data manipulation package. Peaks were detected at 270 nm. Peak identification was made by comparing retention times with those of authentic compounds prepared chemically. Peak intensities were used to calculate relative concentrations. With a given buffer preparation, retention times varied by no more than 3%. For peptide yield analysis, linear gradients of methanol in 0.1 M ammonium acetate (pH 3.1, methanol was increased linearly from 30% to 60% in 20 min) were run with a flow rate of 1 mL/min. Aliquots of enzymatic reactions (20 μL) were diluted with methanol (at least 20-fold), heated at 60 °C for 2 min to coagulate the enzyme, and centrifuged (13 000 rpm) for one min. The supernatant (15 μL) was then injected on HPLC. The racemization analysis was performed by using an isocratic system. The mobile phase was 0.1 M ammonium acetate (pH 4.6)/methanol (56/44, v/v).

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Enzyme Immobilization. Chymotrypsin (146 mg) was dissolved in 4 mL of 1 M phosphate buffer (pH 7.1) containing 0.1% butyl acetate. The mixture was added to 510 mg of Eupergit C. The suspension was stirred to mix and then allowed to stand for 3 days. The resin was then filtered and washed with 20 mL of 10 mM phosphate buffer (pH 7.5). Residual oxirane groups (which might react with the amino acid nucleophiles during synthesis) were digested by treatment of the resin with 5% aqueous mercaptoethanol, pH 7.7, or 10% aqueous 2-aminoethanol, pH 8.1, as suggested by the manufacturer. No significant difference in immobilized activity or synthetic activity was detected in samples treated by either method.

Chymotrypsin was also immobilized on cross-linked polyacrylamide gel.[39] The active polymer PAN 800 (poly[acrylamide-*co*-*N*-(acryloxy)succinimide]) containing 800 mequiv of succinimide ester per gram of polymer was prepared in our laboratory. The cross-linking reagent was triethylenetetramine (TET). In a typical procedure, 2 g of PAN 800 was dissolved rapidly in 7.2 mL of 0.3 M Hepes buffer, pH 7.5, to which was added 24 mg of Z-Phe in 0.8 mL of DMF (Z-Phe acts to protect the active site during the immobilization process). As soon as complete dissolution was achieved, 0.85 mL of 0.5 M TET in water and 0.01 mL of 0.5 M dithiothreitol were added, immediately followed by a solution of 100 mg of chymotrypsin in 2 mL of 0.3 M Hepes buffer, pH 7.5. The mixture was stirred for 2 min, by the end of which the gel had solidified. The gel was allowed to stand for 1 h and was then crushed in a mortar, to which was added 5 mL of 50 mM Hepes/50 m*M* ammonium sulfate buffer (pH 7.5). The gel was crushed for another minute, then transferred to a centrifuge tube with another 10 mL of Hepes/ammonium sulfate buffer, and stirred for 15 min. The gel was separated by gentle centrifugation (2000 rpm). The immobilized yield based on activity was 25% of the soluble enzyme.

Enzymatic Peptide Synthesis. Phosphate buffers were used for reactions at pH 7 and 8, while carbonate buffers were used for pH 9 and 10. The pH reported is of the aqueous solution before addition of organic cosolvent. Acyl donor (Z-Tyr-OMe) and the given acyl acceptor hydrochloride were added to the buffer (Z-Tyr-OMe is only slightly soluble in aqueous media), and the pH was adjusted to the desired value with 20% NaOH. Cosolvent was added, and the solution was allowed to cool to room temperature. Solid enzyme was then added. Under normal conditions containing 0.2 mM enzyme the reaction is over within 1 min. Longer time in the presence of the enzyme was deleterious only in the cases where the nucleophile was an L-amino acid ester, in which case the reaction was quenched by the addition of large amount of methanol. If peptide was to be isolated, the reaction was heated to 60 °C for 5 min after the addition of methanol, and the coagulated enzyme was filtered off. The solution was concentrated and worked up in the same manner as were the chemically synthesized peptides.

Preparative Enzymatic Peptide Synthesis. A carbonate buffer (0.2 M, pH 9)–Me_2SO (4:6, v/v), 50 mL, containing Z-Tyr-OMe (50 m*M*), D-amino acid methyl ester (800 mM), and 0.2 mM α-chymotrypsin was stirred at room temperature. The reaction was monitored by HPLC and stopped when the yield reached the highest (about 5 min). The mixture was added to an equal volume of cold water. After filtration, the precipitate was washed with 1 N HCl (2 × 20 mL), water (2 × 20 mL), 5% $NaHCO_3$ (2 × 20 mL), and water, then recrystallized from water/ethanol. Similar procedures were employed for the synthesis of other peptides. Typical yields are in the range of 70–80%.

In the second experiment, 10 mL of the carbonate/Me_2SO solution containing 0.5 M Z-Tyr-OMe and 1.5 M D-amino acid ester and 0.2 mM chymotrypsin was stirred at room temperature. Within 10 min the precipitate formed was dissolved in a minimum amount of methanol and heated to 60 °C and the enzyme filtered off. The methanol solution was added to an equal volume of cold water, and the precipitate was treated as above.

NMR Analyses. Proton NMR spectra were obtained on a Varian EMS-390 (90 MHz) instrument. Samples were dissolved in Me_2SO-d_6 containing Me_4Si as the internal standard. ^{13}C NMR spectra were taken on a Varian XL-200 instrument (50 MHz). Peak identification was aided by an attached proton test (APT) spectrum.[40] Methyls and methines have opposite sign from methylenes and quaternary carbons. The α-carbons of the dipeptide Z-L-Tyr-D-Met-OMe were detected at 50.00 and 48.84 ppm. The chemical shifts are typical for dipeptides and in agreement with those reported.[41] A shift of the α-C (0.2–0.4 ppm) can be seen if racemization occurs.

Acknowledgment. Support of this research by the National Science Foundation (CHE 8318217) and by the Searle Scholars Program/the Chicago Community Trust is gratefully acknowledged.

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