

111. Enzyme-Catalyzed Peptide-Bond Formation: Elastase- and δ -Chymotrypsin-Assisted Synthesis of Oligopeptides

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A series of penta- and hexapeptides of the structure $\text{Ac-L}_{xm}\text{-}\dots\text{-L}_{x1}\text{-L}_{y1}\text{-}\dots\text{-L}_{ym}\text{-NH}_2$ with phenylalanine or tyrosine in L_{x1} and glycine or alanine in the other positions were synthesized using elastase and δ -chymotrypsin to catalyse the formation of the peptide bonds adjacent to the aromatic residue. Such oligopeptides are useful substrates for studying the secondary specificity of chymotrypsin-like proteases.

Introduction²⁾. – Growing interest in protease-catalyzed synthesis of peptides is documented by a large number of model studies showing the mode of application of various proteases and by several reports on the successful use of this approach in the synthesis of biologically active peptides. Comprehensive reviews of these investigations have been published [3] [4]. Further examples of preparative peptide synthesis for which the enzymatic approach presents clear advantages over the conventional methods will help recognising cases in which the enzymatic method may open valuable alternative synthetic routes.

In our laboratory, enzyme-catalyzed formation of peptide bonds has become a key step in the synthesis of numerous peptide substrates we need for our investigation of the specificity of serine proteases. The systematical variation of the peptide structure required by these studies is achieved by synthesizing various small peptides as building blocks and then assembling them in different combinations. According to a general strategy, the small fragments are prepared stepwise by conventional methods and then assembled using proteases as catalysts, thus assuring racemization-free fragment condensation. In relation to our study of the interactions of the extended active site of α -chymotrypsin, we were let to design a series of peptide substrates containing an internal aromatic residue to specify the scissible peptide bond and several adjacent alanine or glycine residues. The δ -chymotrypsin-assisted synthesis of a number of such peptides has been described in a previous communication [2]. In the present paper we report on the synthesis of further members of this series, the pentapeptides $\text{Ac-Ala-Ala-Ala-Tyr-Gly-NH}_2$ (**1x-1y**) and $\text{Ac-Ala-Ala-Ala-Tyr-Ala-NH}_2$ (**1x-2y**) and the hexapeptides $\text{Ac-Ala-Ala-Ala-Tyr-Ala-Ala-NH}_2$ (**1x-3y**), $\text{Ac-Ala-Ala-Ala-Phe-Ala-Ala-NH}_2$ (**1x-4y**), and $\text{Ac-Gly-Ala-Ala-Tyr-Ala-Ala-NH}_2$ (**2x-3y**). In the synthesis of these peptides, the formation of the bonds preceding and following the amino acid containing the aromatic residue was carried out enzymatically using porcine elastase and bovine δ -chymotrypsin, respectively. These

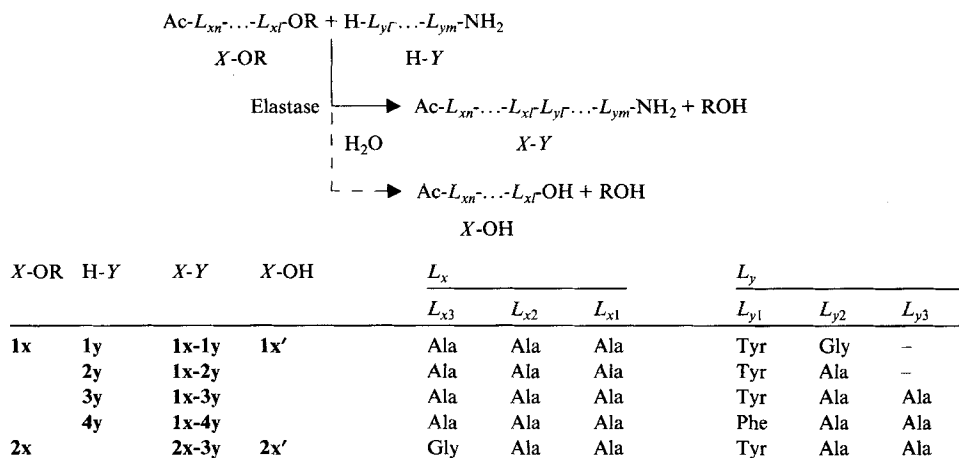
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²⁾ Nomenclature and abbreviations see [1] [2]. All amino acids are of the L-configuration.

examples serve to illustrate the enzymatic synthesis of different peptide bonds by the combined use of proteases of different specificities.

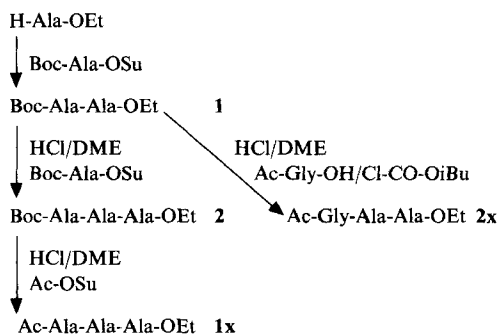
Results and Discussion. – The main step of the syntheses was the elastase-catalyzed condensation of acetyl-tripeptide ethyl esters with dipeptide and tripeptide amides according to *Scheme 1*. The former fragments were prepared by conventional stepwise synthesis (*Scheme 2*), whereas the synthesis of the latter involved δ -chymotrypsin-catalyzed coupling of Z-Tyr-OH or Z-Phe-OH with the required amino-acid or dipeptide amides (*Scheme 3*).

Scheme 1. Structure of the Carboxyl Components X-OR, the Amino Components H-Y, the Peptide Products X-Y and the Acidic By-products X-OH^{a)}

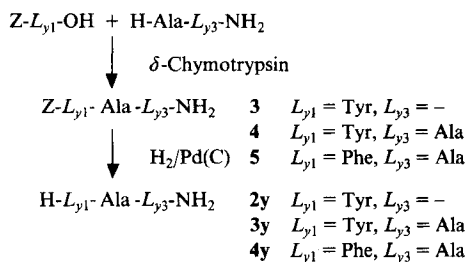


^{a)} The symbols L_{x1}, L_{x2}, etc. representing the amino-acid residues are used to specify their position relative to the peptide bond between L_{x1} and L_{y1}, formed by elastase.

Scheme 2. Synthesis of the Carboxyl Components X-OR



Scheme 3. Synthesis of the Amino Components H-Y



Elastase-Catalyzed Fragment Condensation. The reaction conditions and the yields obtained are summarized in *Table 1*, and the analytical data of the products are presented in *Table 2*. These reactions are examples of protease-catalyzed fragment condensations in

Table 1. *Elastase-Catalyzed Fragment Condensation: Reaction Conditions and Yields*

Peptide	<i>X</i> -OR conc. [mM]	H-Y conc. [mM]	H-Y molar excess [%]	Cosolvent	pH	Yield [%]
1x-1y	0.10	0.15	50	31% MeOH	8.9	12
1x-2y	0.15	0.22	52	45% MeOH	8.5	68
1x-3y	0.12	0.18	50	42% MeOH	8.5	70
1x-4y	0.13	0.22	50	39% MeOH	8.5	75
2x-3y	0.076	0.11	49	11% DMF	8.4	52

Table 2. *Analytical Data of the Products of the Elastase-Catalyzed Syntheses*

Compound	m.p. [°C]	[α] _D ²⁵	Composition	Mol. wt. calc.	C H N		
					[%] calc. [%] found		
1x-1y	272–275 (dec.)	– 34.3° (<i>c</i> = 0.7, DMSO)	C ₂₂ H ₃₂ N ₆ O ₇ · 2H ₂ O	528.55	49.99	6.87	15.90
					50.29	6.77	15.60
1x-2y	> 300	– 25.1° (<i>c</i> = 1.0, DMSO)	C ₂₃ H ₃₄ N ₆ O ₇ · HCOOH	552.57	52.17	6.57	15.21
					52.50	6.86	15.43
1x-3y	273–276 (dec.)	– 13.4° (<i>c</i> = 1.0, DMSO)	C ₂₅ H ₃₇ N ₇ O ₈ · H ₂ O	581.62	51.62	6.76	18.86
					51.52	6.75	16.45
1x-4y	266–269	– 78.0° (<i>c</i> = 1.0, HCOOH)	C ₂₆ H ₃₉ N ₇ O ₈ · 3/4 HCOOH	612.16	52.48	6.67	16.02
					52.51	6.77	16.09
2x-3y	> 300	– 82.1° (<i>c</i> = 1.0, HCOOH)	C ₂₆ H ₃₉ N ₇ O ₇ · 3/4 HCOOH	596.14	53.89	6.85	16.45
					53.20	6.90	16.80

which alkyl esters *X*-OR of the carbonyl components serve as acyl-enzyme precursors according to a mechanism considered in detail previously [2]. Concurrent nucleophilic attack of the acyl-enzyme *X*-E by the amino component H-Y, water, and alcohol (if present as cosolvent) lead to the formation of peptide product *X*-Y, carboxylic acid *X*-OH as a by-product, and regeneration of the starting ester *X*-OR, respectively. Necessary condition for successful application of the method is rapid formation of the acyl-enzyme from the starting ester. This condition is satisfied if the ester *X*-OR is a specific substrate of the protease to be used. Knowledge of proteases specificity is, therefore, important for determining which enzyme is suitable for a particular sequence of the ester fragment. Chymotrypsin is an example of a protease for which consideration of the primary specificity is sufficient. Accordingly, any ester fragment of the type *E*_{*x*}-*L*_{*xn*}-...-*L*_{*x1*}-OR is adequate, provided that *L*_{*x1*} is a residue with an aromatic or another large hydrophobic side chain. The rate constant of acylation measured for a number of such esters was found in the range 10³–10⁴ s^{–1} [5]. Use of elastase is possible if *L*_{*x1*} is a residue with less extended side chain like alanine or valine. Specificity studies [6] indicate that for this enzyme secondary specificity becomes more important so that the acylation rate is expected to increase considerably with the length of the fragment. The present experiments show that fragments with three or more residues are suitable for use with elastase.

In the subsequent step, kinetically controlled partitioning of the acyl-enzyme *X*-E between the amino component H-Y and H₂O occur according to the ratio of reaction rates $v_{(H-Y)}/v_{(H_2O)} = k_{34}[H-Y]/k_{31}[H_2O]$, where *k*₃₄ and *k*₃₁ are the rate constants of acyl-enzyme attack by the amino component and water, respectively. The parameters appearing

in this ratio deserve particular consideration since they directly determine the yield of peptide product. Low values for k_{31} are desirable. However, values of the order of 10^2 s^{-1} are inherent to carboxyl components satisfying the specificity requirements of chymotrypsin and elastase. Among such carboxyl components, k_{31} is little dependent on their structure. In contrast, k_{34} is strongly dependent on the structure of the amino component, as is known from specificity studies with α -chymotrypsin [7] [8]. Accordingly, it is not surprising that H-Tyr-Gly-NH₂, which has few L_{y2} interactions and therefore a low k_{34} , leads to a considerably lower yield (12%) compared with H-Tyr-Ala-NH₂ which leads to a yield of 68% under similar conditions (see Table 1).

The concentration of the amino component H-Y is critical for high yield. Typically, a 50% molar excess of H-Y relative to X-OR is used to avoid that the concentration of H-Y reaches too low a value at the end of the reaction. High concentration of the carboxyl component is often impeded by its low solubility. Improvement can be obtained by addition of cosolvents such as MeOH or DMF to an extent not to preclude enzyme activity, usually up to 50% (v/v). This has the additional advantage of reducing H₂O concentration. Alternatively, high molar excesses of the well soluble amino component H-Y can be used which will be recovered and recycled to a successive run.

Since the reaction mixture at the end of synthesis only consists of three well-defined components, peptide product X-Y, acidic by-product X-OH, and excess of amino component H-Y, separation is generally easily achieved by gel permeation or ion-exchange chromatography. From the examples presented here, it can be seen that with 50% molar excess of amino component at 0.2M concentration, in the presence of 40–50% MeOH, satisfactory yields of 70% or more can be obtained (Table 1). A lower amino-component concentration (ca. 0.1M) leads to a considerable yield reduction, as shown by the 52% yield in the case of Ac-Gly-Ala-Ala-Tyr-Ala-Ala-NH₂ (2x-3y).

δ -Chymotrypsin-Catalyzed Fragment Condensation. The preparation of the fragments used for the above syntheses was carried out according to Scheme 2 and 3. The synthesis of the amino components included a further enzymatic step in which δ -chymotrypsin was used for introducing *N*-benzyloxycarbonyl-tyrosine or -phenylalanine. This reaction is essentially different from those described above, in that the carboxyl component is not activated. Condensation simply occurs as the reverse reaction of peptide hydrolysis and is *per se* thermodynamically controlled. Since equilibrium strongly favours the starting components relative to the peptide product at usual conditions, appreciable yields of peptide product can only be obtained when its solubility is lower than its equilibrium concentration. This is the case for the Z-protected peptides shown in Scheme 3 which can therefore be obtained in high yield as crystalline precipitates by simply incubating concentrated mixtures of the well soluble starting materials with the enzyme. This mode of synthesis has often been reported to give good results [3] but is obviously applicable to a limited number of cases.

The work presented in this paper shows that the use of proteases as catalysts of peptide-bond formation provides a valuable method for carrying out fragment condensation on a preparative scale. The enzymatic fragment condensation does not require side-chain protection and is free of racemization. Owing to the virtual absence of side reactions, the reaction mixtures can easily be analyzed by HPLC. This allows monitoring of the reaction progress and consequently optimal choice of the reaction time. A limitation in the application of the method lies in the fact that not every peptide bond of a given

target peptide is suitable for enzymatic synthesis. However, this limitation can be partially overcome by employing various proteases of different specificity. Examples of this possibility are shown in this paper by the combined use of δ -chymotrypsin and elastase.

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Experimental Part

General. The composition of solv. mixtures is indicated in volume parts. M.p.'s were measured in open capillaries and are uncorrected. $[\alpha]_D^{25}$'s were measured with a *Perkin-Elmer-141* polarimeter and $^1\text{H-NMR}$ spectra recorded with a *Varian-HA-100* spectrometer. The chemical shifts are given in ppm relative to TMS and the coupling constants in Hz. Elemental analyses were performed in the microanalytical laboratory of this institute. Amino-acid derivatives and the dipeptide $\text{H-Tyr-Gly-NH}_2 \cdot \text{Cl}$ were purchased from *Bachem AG*, Bubendorf, porcine pancreatic elastase and bovine pancreatic δ -chymotrypsin from *Sigma*. Solutions of these enzymes were prepared by dissolving the required amounts in 1 mM HCl. DME = dimethoxyethane.

Methods: Procedures I–IV, Isolation of Peptides, HPLC. Standard procedures for coupling with *N*-hydroxy-succinimide esters of *N*-(*t*-butoxycarbonyl)amino acids (*Procedure I*), removal of the amino-protecting *t*-butoxycarbonyl group (*Procedure II*), acylation with *N*-acetoxysuccinimide (*Procedure III*) have been described previously [2].

Removal of the amino-protecting benzyloxycarbonyl group (*Procedure IV*) was carried out as follows. The benzyloxycarbonyl-peptide amide (1 mol-equiv.) was dissolved in dry DMF (0.1–0.2M) containing 4–5 mol-equiv. of HCl and hydrogenated at normal pressure over 10% Pd/C (250 mg/mmol). The mixture was stirred for 3–5 h until evolution of CO_2 (detected by sat. $\text{Ba}(\text{OH})_2$) subsided. After removal of the catalyst by filtration through *Celite*, the soln. was evaporated to dryness. The residue was freed of HCl by repeated addition and evaporation of anhyd. EtOH and by drying at 0.001 Torr over KOH.

The peptide products were isolated by gel chromatography on *Bio-Gel P-2* (200–400 mesh of *Bio-Rad*) in the following way. The reaction mixtures were concentrated under reduced pressure, applied to the column, and eluted with $\text{HCOOH}/\text{H}_2\text{O}$ 1:1 at a flow rate of 27 ml/h. The fractions were collected at 15 min intervals. HPLC and TLC analysis were performed as described previously [2]. The solv. systems used for TLC were $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1 (A), 4:1 (B), and 2:1 (C).

The progress of the elastase-catalyzed condensations was followed by measuring the concentrations of the reaction partners at regular intervals with HPLC. The retention-time data are given in *Table 3*.

Table 3. HPLC-Data^{a)}

Synthesized peptides	Solvent system ^{b)}	Flow rate component [ml/min]	Amino component H-Y	Carboxyl component X-OR	Peptide product X-Y	Acidic byproduct X-OH
1x-1y	a	1.0	1.84–1.87	3.34–3.37	2.47–2.51	1.93–1.95
1x-2y	a	1.4	1.82–1.85	2.76–2.80	2.38–2.41	1.64–1.66
1x-3y	b	1.0	2.29–2.31	4.83–4.90	5.72–5.86	3.02–3.10
1x-4y	a	1.0	2.55–2.57	3.34–3.37	7.59–7.63	1.93–1.95
2x-3y	a	1.4	1.67–1.69	2.40–2.42	2.05–2.08	1.47–1.49

^{a)} The retention times (min) of the fragments, the product, and the by-product are given for each elastase-catalyzed condensation.

^{b)} The samples were injected onto a *LiChrosorb RP-18* column and eluted with $\text{MeOH}/\text{H}_2\text{O}/\text{H}_3\text{PO}_4$ 50:100:1 (a) or 40:100:1 (b).

Boc-Ala-Ala-OEt (1). Boc-Ala-OSu (20.0 g, 70 mmol) in DME (300 ml) were coupled in the presence of 1 mol-equiv. of *N*-methylmorpholine with $\text{H-Ala-OEt} \cdot \text{HCl}$ (10.7 g, 70 mmol) in H_2O (15 ml), according to *Procedure I*. Crystallisation of the crude product from $\text{MeOH}/\text{H}_2\text{O}$ gave 16.42 g (82%) of 1 as large colourless crystals. R_f 0.60 (A), m.p. 97–98°, $[\alpha]_D^{25} = -51.5^\circ$ ($c = 1.0$, EtON). Anal. calc. for $\text{C}_{13}\text{H}_{24}\text{N}_2\text{O}_5$ (288.34): C 54.15, H 8.39, N 9.72; found: C 53.98, H 8.24, N 9.66.

Boc-Ala-Ala-Ala-OEt (2). Compound 1 (15.0 g, 52 mmol) was deprotected with HCl-sat. DME according to *Procedure II*. The homogeneous dipeptide hydrochloride obtained (R_f 0.30 (B)) was dissolved in 25 ml of H_2O , neutralized with 5.3 g (52 mmol) of *N*-methylmorpholine and coupled with 14.9 g (52 mmol) of Boc-Ala-OSu dissolved in 230 ml of DME. Crystallisation of the crude product from EtOH/ H_2O gave 17.0 g (91%) of 2 as white needles. R_f 0.51 (A), m.p. 172–173°, $[\alpha]_D^{25} = -67.1^\circ$ ($c = 1.0$, EtOH). Anal. calc. for $C_{16}H_{29}N_3O_6$ (359.41): C 53.46, H 8.19, N 11.65; found: C 53.49, H 8.14, N 11.83.

Ac-Ala-Ala-Ala-OEt (1x). Compound 2 (16.0 g, 44.5 mmol) was treated with HCl-sat. DME to remove the protecting group. After evaporation of the solv., the tripeptide hydrochloride was dissolved in 25 ml of H_2O and acetylated in the presence of 1 mol-equiv. of *N*-methylmorpholine with 7.0 g (44.5 mmol) of acetoxy succinimide in 220 ml of DME. The crude product was crystallized from ethyl methyl ketone: 10.7 g (80%) of 1x as white needles. R_f 0.50 (B), m.p. 242–243° ([9]: 246–247°), $[\alpha]_D^{25} = -96.8^\circ$ ($c = 1.0$, DMSO). Anal. calc. for $C_{13}H_{23}N_3O_5$ (301.34): C 51.82, H 7.69, N 13.94; found: C 51.62, H 7.67, N 13.52.

Ac-Gly-Ala-Ala-OEt (2x). A soln. of 1.17 g (10 mmol) of Ac-Gly-OH and 1.01 g (10 mmol) of *N*-methylmorpholine in 50 ml of abs. DME/abs. DMF 1:1 was cooled to -15° and treated under stirring with 1.50 g (11 mmol) of isobutyl chloroformate. After 7 min, a cool soln. of 2.25 g (10 mmol) of H-Ala-Ala-OEt · HCl in 50 ml of DME/ H_2O 9:1, neutralized with 1 mol-equiv. of *N*-methylmorpholine, was added. The mixture was stirred for 2 h, meanwhile being allowed to reach r.t. Part of the crude product precipitated during reaction and was collected by filtration, part was extracted from the filtrate with AcOEt. Both portions were combined and crystallized from EtOH: 2.0 g (70%) of 2x. R_f 0.46 (B), m.p. 231–233°, $[\alpha]_D^{25} = -74.3^\circ$. Anal. calc. for $C_{12}H_{21}N_3O_5$ (287.31): C 50.16, H 7.37, N 14.63; found: C 50.13, H 7.43, N 14.62.

Z-Tyr-Ala-NH₂ (3). A soln. of 1.25 g (10 mmol) of H-Ala-NH₂ · HCl in 3 ml of H_2O was adjusted to pH 6.5 with ca. 0.5 ml of 1M NaOH. This was added to a soln. of 3.52 g (10 mmol) of Z-Tyr-OH · 2H₂O in 15 ml of 0.67M NaOH. The mixture was incubated at r.t. with a soln. of 200 mg of δ -chymotrypsin in 2 ml of 1mM HCl. The product began to precipitate after 12 h, and sedimentation was favoured by repeated centrifugations. After a week, the supernatant soln. was discarded and the precipitate suspended in cold H_2O , collected by filtration, washed carefully with H_2O , and dried *in vacuo*. The product obtained (3.9 g) was dissolved in MeOH and the residual chymotrypsin which remained in suspension removed by filtration. The clear soln. was concentrated and allowed to crystallize: 3.52 g (91%) of 3. R_f 0.70 (B), m.p. 199–200°, $[\alpha]_D^{25} = +17.0^\circ$ ($c = 1.0$, DMSO). Anal. calc. for $C_{20}H_{23}N_3O_5$ · 1MeOH (417.46): C 60.42, H 6.52, N 10.07; found: C 60.33, H 6.33, N 10.26.

Z-Tyr-Ala-Ala-NH₂ (4). Synthesis was carried out as described for 3 with 1.45 (7.4 mmol) of H-Ala-Ala-NH₂ · HCl [2] in 4 ml of H_2O and adjusted to pH 6.5 with ca. 0.4 ml of 1M NaOH, 2.61 g (7.4 mmol) of Z-Tyr-OH · 2H₂O in 11 ml of 0.67M NaOH, and 200 mg of δ -chymotrypsin in 2 ml of 1mM HCl. The precipitate was collected as described for 3 and crystallized from acetone/MeOH 1:1 to give 3.21 g (95%) of pure 4. R_f 0.63 (B), m.p. 250–251°, $[\alpha]_D^{25} = +13.2^\circ$ ($c = 1.0$, DMSO). Anal. calc. for $C_{23}H_{28}N_4O_6$ (456.50): C 60.51, H 6.18, N 12.27; found: C 60.34, H 6.22, N 12.31.

Z-Phe-Ala-Ala-NH₂ (5). The reaction mixture consisted of 1.67 g (8.54 mmol) of H-Ala-Ala-NH₂ · HCl [2] in 5 ml of H_2O adjusted to pH 6.5 with ca. 0.45 ml of 1M NaOH, 3.0 g (8.54 mmol) of Z-Phe-OH in 13 ml of 0.67M NaOH, and 200 mg of δ -chymotrypsin in 2 ml of 1mM HCl. After 5 days, the product precipitated was collected by filtration and crystallized from MeOH to give 3.54 g (94%) of 5 as colourless needles. R_f 0.63 (B), m.p. 241–242°, $[\alpha]_D^{25} = +11.3^\circ$ ($c = 1.0$, DMSO). Anal. calc. for $C_{23}H_{28}N_4O_5$ (440.50): C 62.71, H 6.41, N 12.72; found: C 62.60, H 6.54, N 12.61.

Ac-Ala-Ala-Ala-Tyr-Ala-Ala-NH₂ (1x–3y). To 685 mg (1.50 mmol) of 4 in 20 ml of dry DMF, a suspension of 350 mg of 10% Pd/C in abs. MeOH was added followed by 1 ml of 7M HCl in abs. DME. The mixture was treated 5 h with H_2 and worked up according to *Procedure IV* to give 546 mg of homogenous 3y which was used without further purification as the amine component for the following enzymatic coupling reaction. To the soln. of 3y in 3 ml of H_2O adjusted to pH 8.5 with 1.5 ml of 1M NaOH were added 200 μ l of 70 μ M elastase. To this mixture, which was kept under stirring at 25° in a thermostat, a warm soln. of 300 mg (0.99 mmol) of 1x in 3.5 ml of MeOH was added dropwise. The progress of the reaction was monitored by HPLC as previously described [2] and the slowly decreasing pH kept at 8.5 by the addition of small amounts of 1M NaOH. After 30 min, the product began to precipitate. At this time, the reaction rate had decreased considerably and was increased again with additional 100 μ l of elastase soln. After 90 min, as 1x completely disappeared, the enzyme was deactivated by lowering the pH with 1 ml of HCOOH. The mixture was evaporated and the residue dissolved in 3.5 ml of HCOOH, diluted with 2.5 ml of H_2O , applied to a *Bio-Gel-P-2* column (2.6 $\times$ 70 cm), and eluted with HCOOH/ H_2O 1:1 (flow rate 27 ml/h). The fractions were collected at 15 min interval and analyzed by HPLC. The product from *Fractions* 26–30 was dried over KOH at 0.001 Torr: 406 mg (70%) of pure 1x–3y as a light yellow crystalline powder. ¹H-NMR (TFA):

1.4–1.7 (*m*, 5 CH₃ (Ala)); 2.36 (*s*, CH₃CO); 3.0–3.2 (br. 2-line system, 2 H–C(β) (Tyr)); 4.5–5.0 (*m*, 6 H–C(α) (Ala, Tyr)); 6.8–7.25 (4 lines, AA'BB', 4 arom. H (Tyr)).

From *Fractions 21–24*, 21 mg (2.5%) of a by-product, the nonapeptide Ac-(Ala)₃-Tyr-(Ala)₂-Tyr-(Ala)₂-NH₂ was isolated.

Ac-Gly-Ala-Ala-Tyr-Ala-Ala-NH₂ (2x–3y). A soln. of 1.04 mmol of **3y** (obtained from 475 mg (1.04 mmol) of **4** by removing the protecting group as described in the above exper.) in 1.5 ml H₂O was adjusted to pH 8.4 with 0.5 ml of 2N NaOH. Then, 100 μl of 100 μM elastase were added, followed by 200 mg (0.7 mmol) of **2x** in 7 ml of H₂O/DMF 6:1. The slowly sinking pH was maintained at its initial value by additions of NaOH and the progress of the reaction monitored by HPLC. After 30 min, 100 μl of elastase soln. were added again, and after 60 min, the reaction was stopped with 1 ml of HCOOH. The mixture was evaporated and the residue dissolved in 3 ml of HCOOH/H₂O 1:1, applied to a *Bio-Gel-P-2* column (2.6 × 70 cm), and eluted with HCOOH/H₂O 1:1. The residue of *Fractions 28–31* (15 ml each) were dried *in vacuo* over KOH: 205 mg (52%) of pure **2x–3y**. ¹H-NMR ((D₆)DMSO): 1.1–1.3 (*m*, 4CH₃ (Ala)); 1.82 (*s*, CH₃CO) 2.5–3.1 (*m*, 2 H–C(β) (Tyr)); 3.6–3.8 (*d*, *J* = 3.0, 2 H–C(α) (Gly)); 4.0–4.5 (*m*, 5 H–C(α) (Ala, Tyr)); 6.5–7.15 (*m*, AA'BB', 4 arom. H, NH); 7.15 (br.*s*, NH); 7.6–8.2 (*m*, 6 NH); 8.1 (*s*, OH (Tyr)).

Ac-Ala-Ala-Ala-Tyr-Ala-NH₂ (1x–2y). A soln. of 1.49 mmol of crude **2y** (obtained from 623 mg (1.49 mmol) of **3** by removing the benzyloxycarbonyl protecting group according to *Procedure IV*) in 2 ml of H₂O was adjusted to pH 8.5 with ca. 1.5 ml of 1M NaOH, treated with 100 μl of 70 μM elastase, and placed in a thermostat at 25°. Then, 294 mg (0.98 mmol) of **1x** in 3 ml of warm MeOH were added dropwise under stirring, followed by additional 100 μl of elastase soln. After ca. 1 h (HPLC: complete consumption of **1x**), the enzyme was inactivated with 1 ml of HCOOH. The mixture was evaporated, dissolved in 4 ml of HCOOH/H₂O 1:1, and chromatographed on *Bio-Gel P-2* (2.6 × 70 cm column) with HCOOH/H₂O 1:1. The product was eluted with *Fractions 34–36* which gave 337 mg (68%) of **1x–2y** as a white crystalline powder. ¹H-NMR (TFA): 1.4–1.7 (*m*, 4CH₃ (Ala)); 2.34 (*s*, CH₃CO); 3.1–3.3 (*m*, 2H–C(β) (Tyr)); 4.5–5.0 (*m*, 5H–C(α) (Ala, Tyr)); 6.85–7.3 (4 lines, AA'BB', 4 arom. H (Tyr)).

Ac-Ala-Ala-Ala-Tyr-Gly-NH₂ (1x–1y). A soln. of 206 mg (0.75 mmol) of H-Tyr-Gly-NH₂·HCl in 1.2 ml of H₂O, adjusted to pH 8.9 with 0.4 ml of 2N NaOH, and a soln. of 151 mg (0.50 mmol) of **1x** in 2.5 ml of MeOH/H₂O 3:2 were mixed, and the reaction started with 200 μl of 70 μM elastase. Since the reaction was slow, further amounts of elastase soln. (200 μl each) were added after 25, 45, and 75 min. After 100 min, the mixture was treated with 1 ml of HCOOH and chromatographed in the usual manner on *Bio-Gel P-2*. *Fractions 27* and *28* gave 29 mg (12%) of pure **1x–1y**. ¹H-NMR: identical to that described in [2].

Ac-Ala-Ala-Ala-Phe-Ala-Ala-NH₂ (1x–4x). According to *Procedure IV*, 662 mg (1.5 mmol) of **5** were hydrogenated. The resulting crude **4y** was dissolved in 2 ml of H₂O, and after adjusting to pH 8.5, the soln. was combined with a soln. of 201 mg (1.0 mmol) of **1x** in 4 ml of MeOH/H₂O 3:1. The condensation was started with 100 μl of 70 μM elastase, and the product began to precipitate few min later. After 30 min, further 100 μl of elastase were added, and after 65 min, the reaction was stopped with HCOOH. The mixture was evaporated under reduced pressure, redissolved in HCOOH/H₂O 1:1, and chromatographed on *Bio-Gel P2* with HCOOH/H₂O 1:1. From *Fractions 25–28*, 419 mg (75%) of pure **1x–4y** were obtained. ¹H-NMR (TFA): 1.4–1.7 (*m*, 5 CH₃ (Ala)); 3.36 (*s*, CH₃CO); 3.05–3.25 (br. 2-line system, 2H–C(β) (Phe)); 4.5–5.1 (*m*, 6H–C(α) (Ala, Phe)); 7.1–7.4 (*m*, 5 arom. H (Phe)).

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