167. Serine-Protease-Assisted Synthesis of Peptide Substrates for α-Chymotrypsin

by **Spartaco A. Bizzozero, Bruno A. Rovagnati** and **Hans Dutler**

Laboratorium für Organische Chemie, ETH-Hönggerberg, CH-8093 Zürich

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Summary

δ-Chymotrypsin catalyzes peptide-bond formation between acylated amino-acid and peptide esters as the carboxyl component and amino-acid and peptide amides as the amino component. The conditions under which enzyme-catalyzed coupling can be used for fragment condensation in peptide synthesis is investigated. To illustrate the method the synthesis of tetra-, penta- and hexapeptides of the structure $Ac\text{-}L_{xn}-\cdots-L_{xl}-L_{yl}-\cdots-L_{ym}-NH_2$ with L_{xl}=Tyr, designed as substrates for α-chymotrypsin, is described.

Introduction[1]. – Kinetic investigations of the interactions occurring between serine proteases and peptide substrates as carried out in our laboratory for several years [1] require a large number of peptide substrates with systematically varied structure. Often the desired structural variations involve substitution of amino-acid residues at a single position or at a limited number of adjacent positions, the rest of the peptide chain remaining unchanged. Similar synthetic problems are also met in other fields, *e.g.* in the study of structure-activity relationships with peptide hormones. In all these cases assembly of preformed building blocks by fragment condensation represents the most appropriate synthetic pathway. Conventional fragment condensation involves considerable hazards of azlactone-induced racemization at the C-terminal residue of the carboxyl component, which is activated and not urethan-protected. Thus for studies, like those mentioned, where high optical purity is essential, safe application of conventional methods is restricted to post-glycine and post-proline condensations. In contrast, enzyme-catalyzed fragment condensation using a variety of tissue and bacterial proteases with different specificities is entirely free of racemization and likely to meet the requirement for structural flexibility. Application of proteases for catalyzing peptide-bond formation was first reported in the late thirties [2] [3]. The many examples which recently appeared in the literature document the renewed interest in this method [4–9].

[1]) Nomenclature and abbreviations see [22]. In addition: DME = 1,2-dimethoxyethane, DMF = dimethylformamide, DMSO = dimethylsulfoxide, MeOH = methanol, HPLC. = high-performance liquid chromatography, TLC. = thin-layer chromatography, r.t. = room temperature.

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Two modes of protease-catalyzed peptide synthesis can be distinguished according to the type of carboxyl component used. In the first mode this component is free carboxylic acid and peptide synthesis occurs as the reverse reaction of hydrolysis. Since in aqueous solutions the equilibrium constant favours hydrolysis, appreciable yields of condensation product can only be obtained when its solubility is lower than equilibrium concentration [10]. Alternatively, yields can be improved by shifting equilibrium with organic co-solvents [11] or by performing the condensation in the presence of non-miscible organic solvents [12] [13]. In the second mode the carboxyl component is employed in an activated form, usually as an alkyl ester. Peptide synthesis then proceeds *via* a double acyl transfer, first from the alcohol moiety of the ester to the hydroxyl group of the active serine and then to the amino group of the amino component. In this second mode reaction conditions must be carefully chosen in order to minimize concurrent ester hydrolysis and secondary hydrolysis of the peptide product.

In the course of our studies on serine-protease specificity we designed a number of peptide substrates for α-chymotrypsin having the general formula

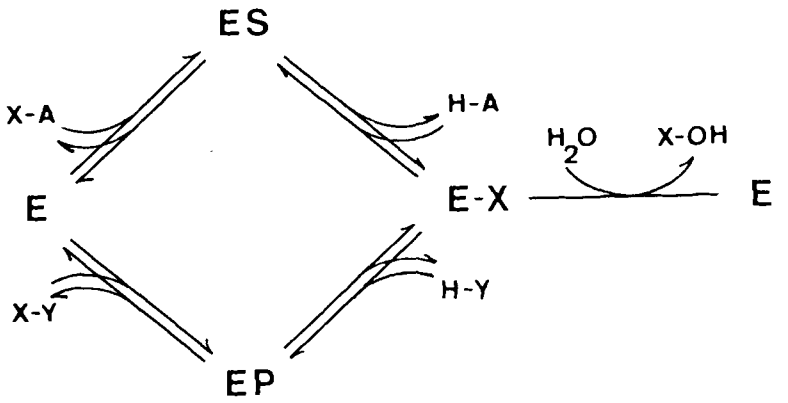
$$\text{Ac-L}_{xn}-\cdots-\text{L}_{x2}-\text{L}_{x1}\overset{\downarrow}{-}\text{L}_{y1}-\text{L}_{y2}-\cdots-\text{L}_{ym}-\text{NH}_2$$

with L_{x1} = tyrosine. In this paper we describe the synthesis of such peptides to illustrate applicability of the second mode of enzyme-assisted fragment condensation using δ-chymotrypsin as coupling enzyme.

Theoretical considerations on serine-protease-catalyzed peptide synthesis using alkyl esters as the carboxyl component. – Chymotrypsin is known to catalyze acyl transfer from specific substrates to various O- and N-nucleophiles *via* an acyl enzyme intermediate arising from nucleophilic attack of Ser-195 on the susceptible carbonyl group of the substrate. Transfer of the acyl moiety X of an amino-acid or peptide ester X-A to the amino group of an amino acid or peptide H-Y leads to formation of the peptide product X-Y. This reaction is described by *Scheme 1;* it is evident that the distribution of the acyl moiety of the starting ester between peptide product X-Y and undesired by-product X-OH is primarily the result of the competition between the amine component H-Y and water for the acyl enzyme. With increasing reaction time the formation of by-product increases as a consequence of secondary hydrolysis of the peptide product and since acyl enzyme hydrolysis is the only irreversible step in the *Scheme*, all peptide will ultimately be degraded to X-OH. Therefore the reaction parameters must be carefully considered in order to obtain optimal product yield.

The criteria underlaying the choice of the reaction conditions can be established by two complementary approaches: 1) empirically with the aid of model reactions, and 2) theoretically on the basis of kinetic considerations. Most of the research activity in this field is concerned with the first approach and produced an experimental background which demonstrates the application range of protease-catalyzed peptide synthesis [14] [15]. The second, theoretical approach involves kinetic analysis of the reaction steps appearing in *Scheme 1* and reveals which reaction parameters are to be considered, why they are important, and in what range they need to be chosen; this approach is currently under investigation in our laboratory.

Scheme 1



E = Enzyme
ES = Enzym complex with the ester X – A
X – A = Ester
E – X = Acyl enzyme
EP = Enzyme complex with the peptide product X – Y
X – Y = Peptide product
H – A = Alcohol released from the ester upon acyl enzyme formation
H – Y = Amine component
X – OH = Carboxylic acid resulting from (E – X)-hydrolysis

The information resulting from the two approaches is used as a guide line for the synthetic work presented in this paper and can be summarized as follows.

Influence of concentration of the amino component H-Y. Amino acid and peptide amides are much better nucleophiles than H_2O for acylchymotrypsin attack. For example 1 M H-Ala-NH_2 and H-Ala-Ala-NH_2 are, respectively, 90 and 240 times more reactive than 55 M H_2O in attacking the acyl enzyme formed from Ac-Tyr-OMe. From such reactivity difference preferential formation of peptide product X-Y over formation of by-product X-OH can be expected. Nevertheless 50% molar excess of H-Y with respect to the starting ester X-A should be used to ensure sufficiently high concentration at the end of the reaction. In the case of syntheses with H-Gly-NH_2, which is the least reactive [16] of the H-Y used, a larger excess is necessary. At the end of reaction the remaining H-Y can easily be separated by gel chromatography and, if desired, recovered.

Influence of pH. Since only the unprotonated form of the amino component H-Y reacts with the acyl enzyme and the pK of this component is about 8 the pH should preferably be higher than this value. The increase of pH, however, is limited by the pH-dependence of enzyme activity. α-Chymotrypsin activity decreases rapidly at pH higher than 8 as a consequence of displacement of a conformational equilibrium to the side of an inactive conformer [17]; for this enzyme the upper pH limit is at about 8.5. For δ-chymotrypsin, whose reactivity decrease at high pH is less pronounced, the upper pH limit is as high as 10.

Influence of addition of alcohol product H-A. With the aim of minimizing acyl enzyme hydrolysis alcohol product H-A was added to the reaction mixture. Kinetic

experiments with methanol showed that the enzyme remains active, though at a considerably lower level, up to concentration of 50% (*v/v*). It should be noted that nucleophilic attack of the acyl enzyme by methanol is not detrimental for the yield because it regenerates the starting ester. In addition, methanol may often be necessary to bring the starting ester into solution.

Influence of enzyme concentration. Correct choice of the enzyme concentration is critical in connection with minimalization of secondary hydrolysis of the peptide product. If the reaction is stopped as soon as the starting ester is consumed, secondary hydrolysis is negligible and maximum amount of peptide product is formed. This end point of the reaction is conveniently found by analyzing the reaction mixture at regular intervals by HPLC. Reaction rate and hence enzyme concentration must be chosen such that analysis can keep pace with reaction. Reaction times of 30–60 min with a sampling frequence of 5–10 min has been found appropriate.

δ-Chymotrypsin-catalyzed fragment condensation. – To illustrate the method we describe the synthesis of tetra-, penta-, and hexapeptides which will be used as substrates for kinetic studies with α-chymotrypsin. The structure of these peptides and that of their parent fragments is given in *Scheme 2*. Some of these fragments were commercial products, others were synthesized by conventional methods in homogenous solution according to *Scheme 3*. Data about physical properties and elemental analysis for the fragments are given in *Table 1*.

The fragment-condensation experiments were carried out as follows. The carboxyl component X-A, dissolved in the smallest possible volume of CH_3OH, was coupled in the presence of 0.1–2 μM δ-chymotrypsin with 1.5- to 30-fold molar excess of amino component, dissolved in H_2O and brought to pH 8.5–8.6 with NaOH. Whenever possible the starting X-A concentrations were kept higher than

Scheme 2. *Structure of the peptide products X – Y, the components X – A and H – Y and the by-product X – OH*

Carboxyl components X – A: Ac-L_{xn} – ··· – L_{x1} – OMe

Amino component H – Y: H – L_{y1} – ··· – L_{ym} – NH_2

Peptide product X – Y: Ac-L_{xn} – ··· – L_{x1} – L_{y1} – ··· – L_{ym} – NH_2

Acidic by-product X – OH: Ac-L_{xn} – ··· – L_{x1} – OH

X – A	H – Y	X – Y	X – OH	L_x				L_y		
				L_{x4}	L_{x3}	L_{x2}	L_{x1}	L_{y1}	L_{y2}	L_{y3}
1x	3y	1x – 3y	1x′	–	–	–	Tyr	Gly	Gly	Ala
	4y	1x – 4y		–	–	–	Tyr	Ala	Ala	Ala
2x	2y	2x – 2y	2x′	–	–	Gly	Tyr	Ala	Ala	–
3x	1y	3x – 1y	3x′	–	Ala	Pro	Tyr	Gly	–	–
		3x – 2y		–	Ala	Pro	Tyr	Ala	Ala	–
4x		4x – 1y	4x′	Ala	Ala	Ala	Tyr	Gly	–	–
5x		5x – 1y	5x′	Pro	Ala	Pro	Tyr	Gly	–	–
		5x – 2y		Pro	Ala	Pro	Tyr	Ala	Ala	–

Scheme 3. *Synthesis of the carboxyl components X – A*

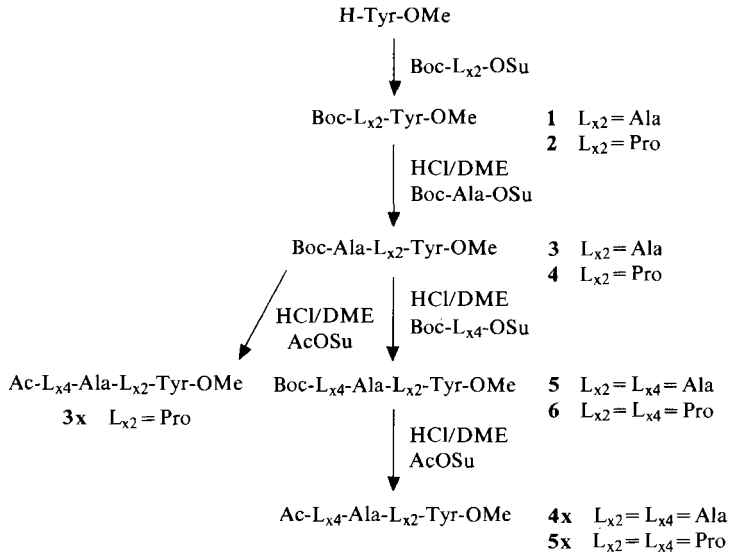


Table 1. *Physical and analytical data of the fragments*

Compound	M.p. °C	$[\alpha]_D^{25}$	pK^a)	Composition	Mol-Wt. Calc.	% Calc. / % Found C	H	Cl	N
3x		−78.5° (c = 1.5, C_2H_5OH)	-	$C_{20}H_{27}N_3O_6 \cdot \frac{1}{3}\,CHCl_3$	445.19	54.84	6.19		9.44
						54.72	6.32		9.24
4x	252–253	−18.7° (c = 1, DMSO)	-	$C_{21}H_{30}N_4O_7$	450.50	55.99	6.71		12.44
						55.53	6.67		12.23
5x	-	−54.2° (c = 1, DMSO)	-	$C_{25}H_{34}N_4O_7 \cdot H_2O$	520.57	57.68	6.97		10.76
						57.75	6.81		10.39
2y	220–221	−17.0° (c = 1, H_2O)	8.05 ± 0.03	$C_6H_{14}ClN_3O_2$	195.65	36.83	7.21	18.12	21.48
						36.78	7.21	17.95	21.31
3y	-	−31.0° (c = 1, H_2O)	7.99 ± 0.03	$C_7H_{15}ClN_4O_3 \cdot \frac{1}{2}\,H_2O$	247.68	33.94	6.51		22.62
						33.70	6.64		22.73
4y	-	−4.3° (c = 1, DMSO)	8.09 ± 0.03	$C_9H_{19}ClN_4O_3$	266.72	40.53	7.18	13.29	21.01
						40.44	7.07	13.25	20.91

a) pK_a of α-amino groups determined in 0.2 M NaCl, 25°.

0.1 M and the methanol concentration did not exceed 50%. The reaction mixture was stirred at room temperature and the pH kept constant by repeated additions of small amounts of NaOH. Progress of the reaction was followed by analyzing the composition of the reaction mixture at regular time intervals by HPLC. on reverse-phase columns. 5-µl-Samples were withdrawn from the reaction mixture at 5–15 min intervals, diluted 10 times with 1 M H_3PO_4, injected into the HPLC.-apparatus and eluted with MeOH/H_2O/H_3PO_4 (composition is given in *Table 2*). The four components of the reaction mixture, the amino component H-Y, the peptide product

X-Y, the carboxylic acid by-product X-OH and the carboxyl component X-A, generally appeared as well-resolved peaks *(Fig. 1);* retention times are given in *Table 2.* When analysis indicated complete consumption of the starting ester the enzyme was inactivated by lowering the pH below 3 with formic acid.

Product isolation consisted mainly in separation based on the differences of molecular size using gel-chromatography with *Bio-Gel P-2* or *Sephadex G 10.* Since the peptide products were sparingly soluble in water, $HCOOH/H_2O$ mixtures were used as the eluant. A typical example is given in *Figure 2.* Failure of complete resolution was only observed between **5x-1y** and **5x'**[2]) for which the difference in

Table 2. *HPLC.-Data of the four components in the reaction mixture.* Retention times (min) of the four components of the reaction mixtures for each fragment-condensation experiment. The samples were injected onto a *LiChrosorb RP-18* column and eluted with $MeOH/H_2O/H_3PO_4$ 50:100:1.

Synthesized peptides	Flow rate ml/min	Amino component H–Y	Product X–Y	Acidic by-product X–OH	Carboxyl component X–A
1x–3x	1.4	1.10–1.15	2.39–2.43	3.43–3.46	6.70–6.75
2x–2y	1.5	0.98–1.00	1.34–1.36	1.71–1.74	2.65–2.68
3x–1y	1.5	1.02–1.03	1.64–1.66	2.35–2.38	4.00–4.03
3x–2y	1.4	1.15–1.18	2.14–2.18	2.64–2.66	4.50–4.54
4x–1y	1.4	1.15–1.18	1.67–1.70	2.28–2.32	3.73–3.80
5x–1y	1.0	1.57–1.59	2.72–2.74	4.09–4.12	6.71–6.90
5x–2y	1.4	1.15–1.20	1.62–1.70	1.96–2.05	2.83–2.94

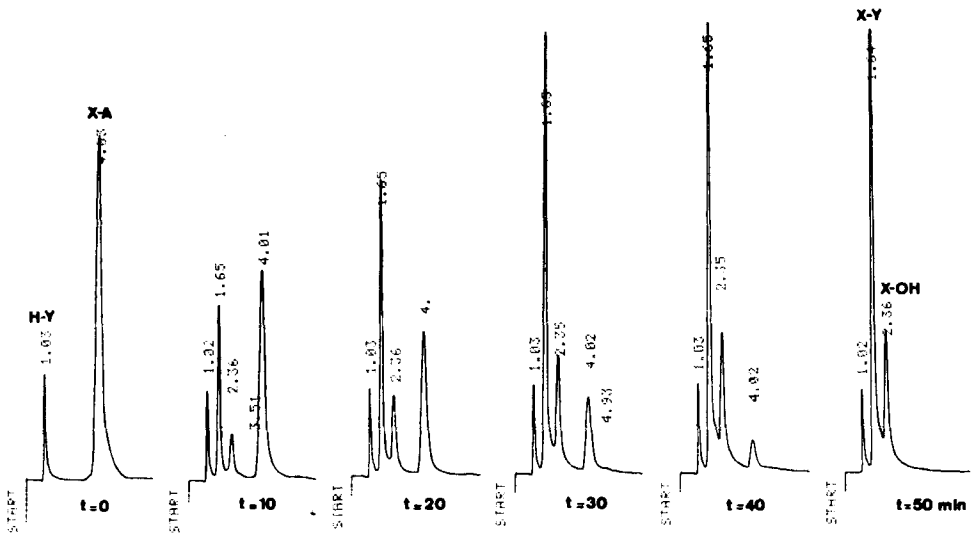


Fig. 1. *Progress of δ-chymotrypsin-catalyzed synthesis of* **3x–1y** *monitored by HPLC.* (X–A = **3x**, H–Y = **1y** and X–OH = **3x'**)

[2]) For the meaning of these symbols see *Scheme 2.*

molecular weight was the smallest. It is interesting to note that when the relative difference in molecular weight is only a little increased as in the case of **4x-1y** and **4x'**, complete separation is again obtained. The analytical data and the yields of the final products are given in *Tables 3* and *4*.

As can be seen from *Table 4* yields were particularly good when a single gel-chromatography operation was sufficient to achieve full purification as for **1x-3y**, **2x-2y**, **3x-1y**, **3x-2y** and **5x-2y**. In the case of **1x-3y** and **3x-1y** the by-product

Table 3. *Analytical data of the enzymatically synthesized peptides*

Compound	M.p. °C	$[\alpha]_D^{25}$	Composition	Mol-Wt.	% Calc. / % Found C	H	N
1x–3y	209–210	+ 12.0° (c = 0.5 DMF/H_2O 1:1)	$C_{18}H_{25}N_5O_6 \cdot \frac{1}{2} H_2O$	416.42	51.91	6.29	16.91
					52.08	6.33	16.84
1x–4y	282–284	– 24.2° (c = 0.5 DMF/H_2O 1:1)	$C_{20}H_{29}N_5O_6$	435.47	55.16	6.71	16.08
					55.04	6.85	16.03
2x–2y	238–239	– 15.4° (c = 1, DMSO)	$C_{19}H_{27}N_5O_6 \cdot \frac{1}{2} H_2O$	430.45	53.01	6.56	16.26
					52.95	6.40	16.28
3x–1y	254–255	– 103.8° (c = 1, HCOOH)	$C_{21}H_{29}N_5O_6 \cdot \frac{1}{4} CH_3OH$	455.50	56.03	6.64	15.37
					56.02	6.58	15.15
3x–2y	–	– 44.9° (c = 1, DMSO)	$C_{25}H_{36}N_6O_7 \cdot H_2O$	550.60	54.53	6.96	15.26
					54.38	6.87	15.14
4x–1y	273–277	– 34.2° (c = 1, DMSO)	$C_{22}H_{32}N_6O_7 \cdot H_2O$	510.55	51.76	6.71	16.46
					51.95	6.55	16.33
5x–1y	239–241	– 96.5° (c = 1, DMSO)	$C_{26}H_{36}N_6O_7 \cdot 3\ CH_3COOH$	724.77	53.03	6.68	11.60
					52.80	6.63	11.66
5x–2y	138–140	– 127.5° (c = 1, HCOOH)	$C_{30}H_{43}N_7O_8 \cdot 2\ H_2O$	665.72	54.12	7.12	14.73
					54.09	6.92	14.56

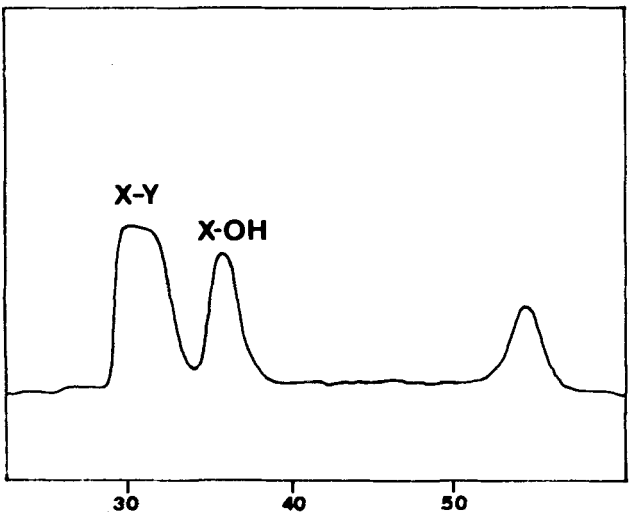


Fig. 2. *Purification of* **3x – 1y** *by* Bio-Gel P-2 *chromatography* (X – OH = **3x'**)

Table 4. *Yields of the enzymatically synthesized peptides*

Peptide	Separation method[a])	Yield of product[b])	Yield of acidic by-product[b])
1x–3y	B	85	15
1x–4y	P, K	70	
2x–2y	S	90	
3x–1y	B	80	22
3x–2y	B	82	
4x–1y	P, B	59	
5x–1y	B, F, B	43	
5x–2y	B	82	

[a]) P = precipitation, K = crystallization, B = gel-chromatography with *Bio-Gel P-2*, S = gel-chromatography with *Sephadex G10*, F = flash-chromatography with silicagel.

[b]) Calculated on the basis of the carboxyl component.

X-OH was also isolated and it was obtained in 15% and 22% yield, respectively. Since the product yield was 85% and 80%, respectively, this indicates that recovery of product and by-product from gel-chromatography is virtually quantitative. Thus, for the experiments in which product isolation was carried out by gel-chromatography alone, the yield is solely determined by the distribution of the starting ester between product and by-product. From these experiments it can be concluded that under the condition used the loss due to ester hydrolysis is between 10 and 20%.

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Experimental Part

General remarks. – The composition of solvent mixtures is indicated in volume parts. Melting points were measured in open capillaries and are uncorrected. Optical rotations were measured with a *Perkin-Elmer* 141 polarimeter. ^{1}H-NMR. spectra (100 MHz) were recorded with a *Varian HA-100* spectrometer using D_6-DMSO as solvent and TMS as internal standard. They showed the expected signals for all intermediates and final products. Elemental analyses were performed in the microanalytical laboratory of this institute *(D. Manser).*

Materials and methods. – TLC. was run on *Merck F 254* silica gel plates with CH_2Cl_2/MeOH 9:1 (A), 4:1 (B), 2:1 (C) and with 1-butanol/pyridine/AcOH/H_2O 50:12:12:25 (D). The compound spots were detected with iodine vapour or ninhydrin. The HPLC.-apparatus consisted of an *Altex* pump model *110A*, a *Waters* injector model *U6K* and a *Kontron* UV.-detector *Uvikon 725* connected with a *Shimadzu RPR-G1* processor. Analyses were carried out by injecting 10 µl of test solution onto a column (3.2×250 mm) filled with *LiChrosorb RP-18*, 10 µm mean particle size, and eluting with MeOH/H_2O/H_3PO_4. For purifications over silica gel the rapid chromatographic technique ('flash'-chromatography) described by *Still et al.* [18] was adopted throughout. Columns of 2–5 cm diameter were chosen according to sample size and filled with *ca.* 20 cm silica gel of 40–63 µm particle size *(Merck).* After application of the sample, eluent was forced through the column at a constant flow rate of 4 cm/min with pressure varying from 0.3 bar for the 5-cm-column to 0.5 bar for the 2-cm-column.

The peptides synthesized by enzymatic fragment condensation were isolated by gel-chromatography on *Bio-Gel P-2* (200–400 mesh of Bio-Rad) or *Sephadex G 10 (Pharmacia)* in the following way. The

reaction mixture was concentrated under reduced pressure, applied to the column and eluted with HCOOH- or CH_3COOH-solutions at a flow rate of 27 ml/min, unless otherwise indicated. The fractions were collected at 15 min intervals. UV. absorbance of the eluate at 280 nm was continuously monitored by an *Uvicord 4701 A (LKB)*. The composition of the relevant fractions was determined by HPLC.

Most solvents and reagents were purchased from *Fluka AG*, Buchs, and amino acid derivatives from *Bachem AG*, Bubendorf. Solutions of bovine pancreatic δ-chymotrypsin *(Sigma)* were prepared by dissolving the required amounts of enzyme in 10^{-3}M HCl. DME was dried by distillation from NaH. HCl-saturated DME and NH_3-saturated MeOH were prepared by bubbling dry gas through stirring anhydrous solvent at 0°. *N*-Acetoxysuccinimide, m.p. 129–130°, was prepared by reacting *N*-hydroxysuccinimide with acetic anhydride in acetone and crystallizing from ethanol. 1-Acetoxybenzotriazole, m.p. 94–95°, was prepared by acetylating 1-hydroxybenzotriazole with acetic anhydride in ethyl methyl ketone and crystallizing from benzene/cyclohexane.

Standard preparative procedures. – *Coupling with* N*-hydroxysuccinimide esters of* t*-butoxycarbonyl amino acids (Procedure I).* The hydrochloride of the amino-acid or peptide ethyl ester and *N*-methylmorpholine were dissolved in DME/H_2O 10:1 (0.2M each). The *N*-hydroxysuccinimide ester of *t*-butoxycarbonyl amino acid (1 mol-equiv.) was then added and the solution stirred at r.t. for 4–5 h. The reaction mixture was reduced to a small volume, diluted with water, and extracted several times with AcOEt. The combined AcOEt-phases were washed with 1N HCl, 1M $NaHCO_3$, and water, dried over Na_2SO_4 and evaporated to dryness under reduced pressure at a temp. not exceeding 40°.

Removal of the t*-butoxycarbonyl amino protecting group (Procedure II).* The solution or suspension of *t*-butoxycarbonyl peptide ester (1 mol-equiv.) in anhydrous DME (0.3–0.5M) was treated with HCl-saturated DME (15–25 mol-equiv.) at 0° and stirred at r.t. for 1 h. The solvent was evaporated under reduced pressure, and the residue freed of HCl by several cycles of addition/evaporation of abs. DME/abs. MeOH 1:1 and drying at 0.001 Torr over KOH.

N*-Acetylation with* N*-acetoxysuccinimide (Procedure III).* The hydrochloride of the peptide ester (1 mol-equiv.) was dissolved in water (1M) and treated with *N*-methylmorpholine. After dilution with 5–10 volumes of DME, *N*-acetoxysuccinimide (1 mol-equiv.) was added. The reaction mixture was stirred at r.t. for 48 h and worked up as described in *Procedure I.*

Ammonolysis of the C-terminal ester group (Procedure IV). A solution of the acetyl peptide ester (1 mol-equiv.) in a small volume of anhydrous MeOH was treated with NH_3-saturated MeOH (40–50 mol-equiv.) at 0° and set aside at r.t. for 1–2 days. The solvent was evaporated and the residue dried at 0.001 Torr.

Synthesis of the fragments X-A and H-Y. – *Boc-Ala-Tyr-OMe* (**1**). H-Tyr-OMe (5.0 g, 21.6 mmol) was coupled with Boc-Ala-OSu (6.18 g, 21.6 mmol) in the presence of *N*-methylmorpholine (2.19 g, 21.6 mmol) according to *Procedure I.* The chromatographically homogeneous crude product, Rf 0.65 (B), yield 6.89 g (87%), was used without further purification for the next reaction. A sample was recrystallized from MeOH/H_2O to give colorless needles, m.p. 115–116°, $[\alpha]_D^{25} = +5.0°$ ($c = 1.0$, DMSO).

$C_{18}H_{26}N_2O_6$ (366.40) Calc. C 59.00 H 7.15 N 7.65% Found C 58.90 H 7.15 N 7.67%

Boc-Ala-Ala-Tyr-OMe (**3**). The protected dipeptide **1** (6.34 g, 17.3 mmol) was treated with HCl-saturated DME according to *Procedure II* to give the corresponding dipeptide methyl ester hydrochloride. This was coupled with Boc-Ala-OSu (4.95 g, 17.3 mmol) in the presence of *N*-methylmorpholine (1.75 g, 17.3 mmol) to give 7.10 g (94%) of crude product which was chromatographed over silica gel with CH_2Cl_2/MeOH 9:1. The oily residue obtained by evaporating the combined pure fractions under reduced pressure was repeatedly dissolved in $CHCl_3$ and evaporated, until it could be collected as an amorphous white powder which was dried *in vacuo*. Rf 0.49 (B), $[\alpha]_D^{25} = -24.4°$ ($c = 0.5$, C_2H_5OH).

$C_{21}H_{31}N_3O_7 \cdot 1/2\ CHCl_3$	Calc.	C 51.94	H 6.39	Cl 10.71	N 8.45%
(497.17)	Found	„ 51.31	„ 6.39	„ 10.92	„ 8.32%

Ac-Ala-Ala-Ala-Tyr-OMe (**4x**)[2]. The tripeptide methyl ester hydrochloride obtained from **3** (6.45 g, 13.0 mmol) according to *Procedure II* was treated with 1 mol-equiv. *N*-methylmorpholine and coupled with Boc-Ala-OSu (3.72 g, 13.0 mmol). The homogeneous protected tetrapeptide **5**,

Rf 0.54 (B), was isolated by extraction with AcOEt (yield 5.79 g, 11.4 mmol, 88%) and converted to the corresponding tetrapeptide methylester hydrochloride *(Procedure II)* without further purification. Acetylation of the latter with *N*-acetoxysuccinimide (1.79 g, 11.4 mmol) in the presence of *N*-methylmorpholine *(Procedure III)* gave crude **4x** which was repeatedly crystallized from MeOH. Yield 2.11 g (41%).

Boc-Pro-Tyr-OMe (**2**). H-Tyr-OMe · HCl (7.41 g, 32.0 mmol) was coupled with Boc-Pro-OSu (10.0 g, 32.0 mmol) in the presence of 1 mol-equiv. *N*-methylmorpholine. The crude product was chromatographed on a silica gel column (5 × 21 cm) by eluting with CH_2Cl_2/MeOH 9:1. Compound **2** was obtained as a chromatographically homogeneous material (10.81 g, 86%) which could not be crystallized: Rf 0.53 (A), $[\alpha]_D^{25} = -37.6°$ ($c = 1.0$, C_2H_5OH).

$C_{20}H_{28}N_2O_6$ (392.43) Calc. C 61.21 H 7.19 N 7.14% Found C 61.08 H 7.35 N 7.09%

Boc-Ala-Pro-Tyr-OMe (**4**). Compound **2** (5.0 g, 12.7 mmol) was treated with HCl-saturated DME *(Procedure II)*. The dipeptide methyl ester hydrochloride obtained was coupled with Boc-Ala-OSu (3.65 g, 12.7 mmol) in the presence of 1 mol-equiv. *N*-methylmorpholine. Purification of the crude product by flash chromatography (5 × 21 cm column) with CH_2Cl_2/MeOH 9:1 gave 3.07 g (52%) of pure **4**: Rf 0.43 (A), $[\alpha]_D^{25} = -33.0°$ ($c = 1.0$, DMSO).

$C_{23}H_{33}N_3O_7$ (463.51) Calc. C 59.59 H 7.18 N 9.07% Found C 59.64 H 7.30 N 8.78%

Ac-Ala-Pro-Tyr-OMe (**3x**). Compound **4** (1.80 g, 3.9 mmol) was converted to the hydrochloride *(Procedure II)*. This was treated with 1 mol-equiv. *N*-methylmorpholine and acetylated according to *Procedure III* but using acetoxybenzotriazole (700 mg, 3.9 mmol) instead of acetoxysuccinimide. The crude product was transferred to a silica gel column (4 × 21 cm) and eluted with $CHCl_3$/MeOH 4:1. The combined pure fractions gave 0.75 g (50%) acetylated tripeptide **3x** as an homogeneous amorphous powder: Rf 0.48 (B). NMR. and elemental analysis (see *Table 1*) indicated the presence of 1/3 mol-equiv. $CHCl_3$.

Ac-Pro-Ala-Pro-Tyr-OMe (**5x**). Compound **4** (4.45 g, 9.6 mmol) was deprotected by treatment with HCl-saturated DME. The tripeptide methyl ester hydrochloride obtained was treated with 1 mol-equiv. *N*-methylmorpholine and coupled with Boc-Pro-OSu (2.98 g, 9.6 mmol). Purification of the crude product over silica gel (5 × 21 cm column) with CH_2Cl_2/MeOH 9:1 gave 2.47 g (4.4 mmol, 64%) homogeneous protected tetrapeptide **6**, Rf 0.32 (A), which was converted to the corresponding hydrochloride without further purification. This was dissolved in 20 ml abs. DMF/abs. pyridine 1:1 and treated with *N*-methylmorpholine (0.45 g, 4.4 mmol); 4-nitrophenyl acetate (1.20 g, 6.6 mmol) was added and the reaction mixture stirred for 18 h at r.t. After evaporation of the solvent under reduced pressure, the oily residue was applied to a silica gel column (5 × 21 cm) and eluted with CH_2Cl_2/MeOH 1:1. In the fractions containing the product a considerable amount of 4-nitrophenol was still present. They were combined and evaporated; the oily residue was dissolved in 10 ml MeOH/0.1M AcOH 1:1 and applied to a *Bio-Gel P-2* column (3 × 100 cm). Elution with 0.1M acetic acid gave 2.36 g (95%) pure **5x**[2]), Rf 0.59 (B), as a resinous colorless material.

H-Gly-Gly-Ala-OEt · HCl (**7**). H-Gly-Ala-OEt · HCl [19] (4.52 g, 17.7 mmol) was coupled with Boc-Gly-OSu (4.81 g, 17.7 mmol) in the presence of 1 mol-equiv. *N*-methylmorpholine. The protected tripeptide obtained after extraction from AcOEt (5.95 g, 100%) showed a single spot in TLC., Rf 0.67 (A) and was converted to the corresponding hydrochloride by treatment with HCl-saturated DME. The crude product was crystallized from ethanol to give 3.41 g (72%) pure **7**, Rf 0.44 (D), m.p. 194–195°.

$C_9H_{18}ClN_3O_4$	Calc.	C 40.38	H 6.78	Cl 13.24	N 15.70%
(267.71)	Found	„ 40.49	„ 6.77	„ 13.33	„ 15.60%

H-Gly-Gly-Ala-NH_2 · HCl (**3y**). Compound **7** (1.0 g, 3.7 mmol) was dissolved in 20 ml abs. MeOH and treated with 40 ml NH_3-saturated MeOH at r.t. for 48 h. After evaporation of the solvent the solid residue was dried at 0.001 Torr. Yield 0.92 g (100%), Rf 0.13 (D). Elemental analysis indicates the presence of 1/2 mol-equiv. H_2O. Titration: theoretical 0.106 mmol, found 0.102 mmol (96%).

H-Ala-Ala-Ala-NH_2 · HCl (**4y**). H-Ala-Ala-Ala-OMe · AcOH (0.50 g, 1.6 mmol, commercial) was treated with NH_3-saturated MeOH according to *Procedure IV*. The residue obtained after evaporation of the solvent was dissolved in 1.64 ml 1N HCl and lyophilized to give 0.39 g (91%) of **4y** as a homogeneous white powder, Rf 0.20 (B). Titration: theoretical 92.4 µmol, found 87.2 µmol (95%).

Boc-Ala-Ala-NH_2 (**8**). H-Ala-OMe (4.88 g, 35 mmol) was coupled with Boc-Ala-OSu (10.02 g, 35 mmol) in the presence of 1 mol-equiv. *N*-methylmorpholine. The crude protected dipeptide methyl ester was crystallized from MeOH/H_2O, yield 7.92 g (28.0 mmol, 83%), Rf 0.63 (B). This was converted to the corresponding dipeptide amide according to *Procedure IV*. After evaporation of the solvent 7.49 g (100%) of **8** were recovered as an homogeneous product, Rf 0.42 (B). A sample was recrystallized for analysis. M.p. 162–163°, $[\alpha]_D^{25} = +56.3°$ ($c = 1.0$, C_2H_5OH).

$C_{11}H_{21}N_3O_4$ (259.30) Calc. C 50.95 H 8.16 N 16.21% Found C 50.83 H 8.11 N 16.06%

H-Ala-Ala-$NH_2 \cdot HCl$ (**2y**). Compound **8** (4.97 g, 19.2 mmol) was treated according to *Procedure II* to remove the *N*-protecting group. After evaporation of the solvent under reduced pressure the residue was dried at 0.001 Torr over KOH, dissolved in abs. MeOH and precipitated with abs. ether. Yield 3.74 g (100%), Rf 0.41 (D). Titration: theoretical 0.103 mmol, found 0.101 mmol (98%).

δ-Chymotrypsin-catalyzed fragment condensation. – *Preparation of Ac-Tyr-Gly-Gly-Ala-NH_2* (**1x-3y**). A stirred solution of **3y**[2]) (351 mg, 1.42 mmol) in 1.5 ml H_2O, adjusted to pH 8.5 with 0.8 ml 1N NaOH, was mixed with 100 µl of a 65 µM δ-chymotrypsin. Condensation was initiated by dropwise addition of a solution of **1x** (240 mg, 1.01 mmol) dissolved in 1.5 ml MeOH. *Ca.* 20 min after start the product **1x-3y** began to precipitate and HPLC.-analysis showed that the peak of **1x** had completely disappeared. The reaction was stopped and the mixture evaporated under reduced pressure. The residue, dissolved in 4 ml HCOOH/H_2O 1:1, was applied to a *Bio-Gel P-2* column (26×75 cm) and eluted with HCOOH/H_2O 1:1. All product was eluted in Fractions 33–37 which were pure as judged by HPLC. The by-product **1x'** was contained in Fractions 49–53. Evaporation of the solvent from the combined fractions gave 356 mg (85%) **1x-3y** and 34 mg (15%) **1x'**. – ^{1}H-NMR.-Data of **1x-3y** (D_6-DMSO): 1.22 (*d*, $J = 3.5$, 3 H, CH_3 (Ala)); 2.75 (*s*, 3 H, CH_3CO); 2.50–3.04 (*m*, 2 H, 2 H–C(β) (Tyr)); 3.58–3.82 (br. 2-lines system, 4 H, 2×2 H–C(α) (Gly)); 3.94–4.52 (*m*, 2 H, 2 H–C(α) (Ala, Tyr)); 6.52–7.12 (*m*, *AA'BB'*-system, 5 H, 4 arom. H and 1 *H*–NH); 7.2 (br. *s*, 1 H, *H*–NH); 7.80–8.10 (*m*, 3 H, 3 H–N); 8.14–8.36 (*m*, 1 H, H–NH); 9.10 (br. *s*, 1 H, OH (Tyr)).

Ac-Tyr-Ala-Ala-Ala-NH_2 (**1x-4y**). To a solution of 590 mg (2.0 mmol) **4y**[2]) in 2 ml water adjusted to pH 8.6 with 0.8 ml 2M NaOH, 0.2 ml of 40 µm δ-chymotrypsin solution was added. The reaction was started by the addition of 300 mg (1.26 mmol) **1x** dissolved in 2 ml MeOH. Few minutes after reaction start product began to precipitate. After 20 min the precipitate was collected by centrifugation, washed by three cycles of suspension/centrifugation in 3 ml cold water and dried *in vacuo* over P_2O_5. A white powder (503 mg, 92%), homogeneous in TLC. (Rf 0.20 (B)), was obtained. Crystallization from MeOH/H_2O gave 385 mg (70%) crystalline tetrapeptide. – ^{1}H-NMR. (D_6-DMSO): 1.08–1.38 (2-lines system, 9 H, 3 CH_3 (Ala)); 1.81 (*s*, 3 H, CH_3CO); 2.50–3.04 (*m*, 2 H, 2 H–C(β) (Tyr)); 3.96–4.56 (*m*, 4 H, 4×1 H–C(α)); 6.54–7.12 (*m*, 5 H, 4 arom. H and 1 *H*–NH); 7.22 (br. *s*, 1 H, *H*–NH); 7.72 (*d*, $J = 3.5$, H–N); 7.81–8.15 (*m*, 3 H, 3 H–N); 9.05 (*s*, 1 H, OH (Tyr)).

Ac-Gly-Tyr-Ala-Ala-NH_2 (**2x-2y**). To a solution of 315 mg (1.61 mmol) **2y**[2]) in 2.5 ml H_2O, adjusted to pH 8.6 with 0.4 ml of 2N NaOH, 307 mg (1.04 mmol) **2x** [20] dissolved in 3 ml MeOH were added. The reaction was started by the addition of 100 µl of a 15 µM δ-chymotrypsin. Since the progress of the reaction was rather slow a second portion of 100 µl enzyme solution was added after 15 min. After 2 h the **2x**-peak had disappeared and the reaction was stopped. The amount of by-product **2x'** formed was estimated by HPLC. to be between 5 and 10%. After most of the methanol was removed from the reaction mixture in the rotatory evaporator, the concentrated solution was applied to a *Sephadex G-10* column (3.5×60 cm) and elution was performed with water at a flow rate of 36 ml/h. Fractions were collected at 15 min intervals and analyzed by HPLC. The product was eluted in Fractions 63–78. Yield 394 mg (90%). Crystallization from MeOH gave 319 mg (71%) colorless needles. – ^{1}H-NMR. (D_6-DMSO): 1.00–1.36 (4-lines system, 6 H, 2 CH_3 (Ala)); 1.79 (*s*, 3 H, CH_3CO); 2.56–3.06 (*m*, 2 H, 2 H–C(β) (Tyr)); 3.48–3.86 (*m*, 2 H, 2 H–C(α) (Gly)); 3.92–4.50 (*m*, 3 H, 3×1 H–C(α)); 6.48–7.10 (*m*, 5 H, 4 arom. H and 1 *H*–NH); 7.18 (br. *s*, 1 H, *H*–NH); 7.67 (*d*, 1 H, H–N); 7.82–8.22 (*m*, 3 H, 3 H–N); 9.12 (br. *s*, 1 H, OH (Tyr)).

Ac-Ala-Pro-Tyr-Gly-NH_2 (**3x-1y**). To a solution of 1.5 g (13.6 mmol) **1y**[2]) in 3.0 ml H_2O, adjusted to pH 8.5 with 3.5 ml 2N NaOH, a solution of 296 mg (0.66 mmol) **3x** in 2.5 ml MeOH was added. The synthesis was started with 50 µl of a 11 µM δ-chymotrypsin and followed with HPLC. *(Fig. 1)*. After 75 min **3x** was totally consumed and the reaction was stopped with formic acid. The reaction mixture

was concentrated to half its volume, applied to a *Bio-Gel P-2* column (2.6×70 cm) and eluted with $HCOOH/H_2O$ 1:3 *(Fig. 2)*. The product was contained in Fractions 29–32 and crystallized upon evaporation of the solvent. Yield 238 mg (80%). A sample was recrystallized for elemental analysis. The by-product **3x′** (54 mg, 22%) appeared in Fractions 34–37. In consideration of the small difference in the molecular weights, this by-product was unexpectedly well resolved from the product. Excess of H-Gly-NH_2 came with fractions 52–56. – *^{1}H-NMR. Data of* **3x-1y** (D_6-DMSO): 1.04–1.22 (*d*, 3 H, CH_3 (Ala)); 1.80 (*s*, 3 H, CH_3CO); 1.62–2.00 (*m*, 4 H, 4 H–C(β,γ) (Pro)); 2.70–2.96 (*m*, 2 H, 2 H–C(β) (Tyr)); 3.40–3.68 (*m*, 4 H, 2 H–C(δ) (Pro) and 2 H–C(α) (Gly)); 4.14–4.60 (*m*, 3 H, 3×1 H–C(α)); 6.50–7.15 (*m*, 6 H, 4 arom. H and NH_2); 7.80–8.18 (*m*, 3 H, 3 H–N); 9.10 (*s*, 1 H, OH (Tyr)).

Ac-Ala-Pro-Tyr-Ala-Ala-NH_2 (**3x-2y**). The solution of 125 mg (0.64 mmol) **2y**[2]) in 1.5 ml H_2O was adjusted to pH 8.5 with 0.4 ml 1N NaOH. After addition of 100 µl 22 µM δ-chymotrypsin the reaction was started by the addition of 130 mg (0.29 mmol) **3x** dissolved in 0.5 ml MeOH. HPLC. indicated depletion of **3x** after *ca.* 30 min. The enzyme was then inactivated and the reaction mixture concentrated under reduced pressure. Chromatography on a *Bio-Gel P-2* column (2.6×75 cm) with $HCOOH/H_2O$ 1:1 gave the product in Fractions 29–31, well separated from a small amount of **3x′** which was contained in Fractions 37–40. Fractions 29–31 were combined, evaporated and dried several days at 0.001 Torr. Yield 130 mg (82%) pure **3x-2y**. In a similar experiment a 89% yield was obtained [21]. – ^{1}H-NMR. (D_6-DMSO): 1.04–1.42 (3-line system, 9 H, 3 CH_3 (Ala)); 1.60–2.10 (*m*, 4 H, 4 H–C(β,γ) (Pro)); 1.80 (*s*, 3 H, CH_3CO); 2.64–3.10 (*m*, 2 H, 2 H–C(β) (Tyr)); 3.15–3.68 (*m*, 2 H, 2 H–C(δ) (Pro)); 4.00–4.62 (*m*, 5 H, 5×1 H–C(α)); 6.50–7.08 (*m*, 5 H, 4 arom. H and 1 *H*–NH); 7.15 (br. *s*, 1 H, *H*–NH); 7.56–7.94 (*m*, 3 H, 3 H–N); 8.10 (br. *d*, 1 H, H–N); 9.08 (*s*, 1 H, OH (Tyr)).

Ac-Ala-Ala-Ala-Tyr-Gly-NH_2 (**4x-1y**). To a suspension of 402 mg (0.89 mmol) **4x**[2]) in 15 ml H_2O/MeOH 1:1 10 ml of 3M **1y** of pH 8.5 was added. Condensation was started with 0.3 ml 20 µM δ-chymotrypsin. After 30 and 60 min 0.2 ml of this enzyme solution were added again. HPLC. indicated complete consumption of **4x** after 90 min. Part of the product, which precipitated during reaction, was collected by centrifugation and washed once with water. The supernatant was concentrated under reduced pressure, treated with the same volume of HCOOH and applied to a *Bio-Gel P-2* column (2.6×75 cm). Elution was achieved with $HCOOH/H_2O$ 1:1. The Fractions 28–31 containing the product, were combined and evaporated to give 69 mg pure **4x-1y** as a crystalline powder. HPLC. indicated that the precipitate consisted of 90% **4x-1y** and 10% by-product **4x′**. The latter was removed by gel chromatography over *Bio-Gel P-2* in the same way as described for the supernatant. From Fractions 26–31 197 mg pure **4x-1y** were recovered. Total yield 266 mg (59%). – ^{1}H-NMR. (D_6-DMSO): 1.02–1.30 (4-lines system, 9 H, 3 CH_3 (Ala)); 1.81 (*s*, 3 H, CH_3CO); 2.56–3.08 (*m*, 2 H, 2 H–C(β) (Tyr)); 3.50–3.70 (*m*, 2 H, 2 H–C(α) (Gly)); 4.00–4.46 (*m*, 4 H, 4×1 H–C(α)); 6.52–7.10 (*m*, 6 H, 4 arom. H and NH_2); 7.72–8.12 (*m*, 5 H, 5 H–N).

Ac-Pro-Ala-Pro-Tyr-Gly-NH_2 (**5x-1y**). The solution of 193 mg (0.37 mmol) **5x** in 2 ml MeOH/H_2O 1:1 was added to 3.8 ml of 1.5M **1y**[2]) of pH 8.5. Condensation was started with 50 µl 11 µM δ-chymotrypsin and stopped after 30 min by addition of HCOOH. The concentrated reaction mixture was chromatographed on a *Bio-Gel P-2* column (2.6×61 cm) using 0.1M CH_3COOH at a flow rate of 36 ml/h. Poor resolution of product **5x-1y** from by-product **5x′** was observed and Fractions 24–26, though mainly containing product, were contaminated with a considerable amount of by-product. The latter was removed by flash chromatography on silica gel (3×21 cm column) using CH_2Cl_2/MeOH 2:1 as eluent. Rechromatography on *Bio-Gel P-2* under the same conditions as described above gave 115 mg (43%) pure **5x-1y** as a crystalline powder, – ^{1}H-NMR. (D_6-DMSO): 1.02–1.26 (*m*, 3 H, CH_3 (Ala)); 1.60–2.10 (*m*, 20 H, CH_3CO, 2×4 H–C(β,γ) (Pro) and 3 CH_3COOH (solv.)); 2.64–3.00 (*m*, 2 H, 2 H–C(β) (Tyr)); 3.20–3.70 (*m*, 6 H, 2×2 H–C(δ) (Pro) and 2 H–C(α) (Gly)); 4.10–4.58 (*m*, 4 H, 4×1 H–C(α)); 6.50–7.14 (*m*, 6 H, 4 arom. H and NH_2); 7.80–8.36 (*m*, 3 H, 3 H–N).

Ac-Pro-Ala-Pro-Tyr-Ala-Ala-NH_2 (**5x-2y**). A solution of 200 mg (1.02 mmol) **2y**[2]) in 1 ml H_2O, adjusted to pH 8.5 with 0.7 ml 1N NaOH, was mixed with 290 mg (0.56 mmol) **5x** dissolved in 3.5 ml MeOH/H_2O 1:2.5. The condensation was started by the addition of 100 µl 10 µM δ-chymotrypsin. After *ca.* 60 min the reaction was stopped and the concentrated reaction mixture chromatographed on a *Bio-Gel P-2* column (2.6×66 cm) and eluted with $HCOOH/H_2O$ 1:3. The product **5x-2y** was eluted in Fractions 27–31 which, after evaporation to dryness, gave 305 mg (82%) product **5x-2y**, as a white crystalline powder, pure by HPLC. A small amount of by-product **5x′** was eluted in

Fractions 33–37 and the unreacted **2y** in Fractions 41–44. – *^{1}H-NMR.-Data of* **5x-2y** (D_6-DMSO): 1.04–1.32 (*m*, 9 H, 3 CH_3 (Ala)); 1.60–2.10 (*m*, 8 H, 2×4 H–C(β,γ) (Pro)); 1.93 (*s*, 3 H, CH_3CO); 2.60–2.96 (*m*, 2 H, 2 H–C(β) (Tyr)); 3.20–3.60 (*m*, 4 H, 2×2 H–C(δ) (Pro)); 4.00–4.50 (*m*, 6 H, 6×1 H–C(α)); 6.50–7.05 (*m*, 5 H, 4 arom. H and 1 *H*–NH); 7.06–7.20 (*m*, 1 H, *H*–NH); 7.50–8.04 (br. *m*, 3 H, 3 H–N); 8.06–8.40 (br. *s*, 1 H, H–N).

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