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The Application of N^{α}-Formyl Amino Acid Esters in the Enzyme-Catalyzed Peptide Synthesis**

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The synthesis of N^{α}-formyl protected amino acid esters and their use in kinetically controlled α-chymotrypsin-, trypsin- and carboxypeptidase *Y*-catalyzed peptide synthesis is described. Using the synthesis of H-Tyr-Ala-OH as example it is demonstrated that optically uniform products are obtained. Reaction yields are discussed with regard to the ester component employed. In comparison to commonly used α-amino protecting groups in chemical peptide synthesis, N^{α}-formyl amino acid derivatives show improved solubilities in aqueous-alcoholic media.

(Keywords: Enzyme catalyzed peptide synthesis; Formyl protecting group; Optical purity)

Die Anwendung von N^{α}-Formyl-Aminosäureester in der enzymkatalysierten Peptidsynthese

Die Synthese N^{α}-Formyl-geschützter Aminosäureester und deren Anwendung in der kinetisch kontrollierten α-Chymotrypsin-, Trypsin- und Carboxypeptidase *Y*-katalysierten Peptidsynthese wird beschrieben. Man erhält hierbei optisch einheitliche Produkte, was anhand der Synthese von H-Tyr-Ala-OH gezeigt wurde. Reaktionsausbeuten bei Verwendung verschiedener Ester werden diskutiert. Im Verhältnis zu gängigen α-Aminoschutzgruppen der chemischen Peptidsynthese weisen N^{α}-Formyl-geschützte Derivate in wäßrig-alkoholischen Medien verbesserte Löslichkeiten auf.

** *Abbreviations:* IUPAC-IUB rules for peptides are followed, see Pure Appl. Chem. (1984) 56:595. *Boc* = *tert.* butyloxycarbonyl, *CT* = α-chymotrypsin, *DCC* = N,N'-dicyclohexylcarbodiimide, *DIPEA* = diisopropylethylamine, *Fmoc* = 9-fluorenylmethyloxycarbonyl, *For* = formyl, GC = gaschromatography, HO*Su* = N-hydroxysuccinimide, HPLC = high pressure liquid chromatography, *Me*CN = acetonitrile, —O*Me* = methyl ester, —O*Prop* = *n*-propyl ester, *PFPA* = decafluoropropionic anhydride, *T* = trypsin, *TBA* = tetrabutylammonium hydrogen sulfate, TLC = thin layer chromatography, *TMS* = tetramethylsilane, *Y* = carboxypeptidase *Y*, *Z* = benzyloxycarbonyl.

Introduction

Esters of amino acid and peptide derivatives are utilized as carboxyl components in the kinetically controlled approach to enzymatic peptide synthesis, which enables in general peptide bond formation in short reaction times with low enzyme concentrations [1–3]. For operating enzymatic synthesis commercially, concentrations of the reaction components as high as possible are desired [4]. Commonly used N^{α}-protecting groups of chemical peptide synthesis, especially the *Fmoc*-group besides the *Z*- and *Boc*-group [5, 6], lead to a decreased solubility of amino acid derivatives in aqueous media, the preferred reaction media employing proteolytic enzymes as catalysts [1]. Therefore our interest has been to look for more suitable N^{α}-protecting groups for application in enzymatic peptide synthesis. Refering to this, the *For*-group reveals some attractive features, which will be discussed in the following report.

Results and Discussion

For use in aqueous media the formyl group shows favourable solubilization properties [7]. An additional advantage over conventional N^{α}-protecting groups is the extremely simple, low cost preparation and introduction of this protecting group [8–11]. We examined the usefulness of the formyl-group in the enzymatic peptide synthesis applying N^{α}-formyl esters of amino acids with hydrophobic side chains, such as Tyr, Phe, and Trp. In aqueous-alcoholic solutions, concentrations up to some mmol per litre were reached with these derivatives. Table 1 illustrates the peptide synthesis carried out. As amino components amino acid amides (H-Leu-NH_2, H-Met-NH_2), a free amino acid (H-Ala-OH), and amino acid esters [H-Lys-O*Prop*, H-Arg-O*Prop*, H-Arg-O*Me*, H-Glu(O*Me*)$_2$, and H-Ala-O*Prop*] have been used. The yield depends in a high degree on the affinities of the derivatives towards the corresponding S_1- and S_1'-subsites of the enzymes employed (binding site notation according to *Berger* and *Schlechter* [12]). The CT-catalyzed synthesis of *For*-Tyr-Lys-O*Prop* (**1.**) reached a selectivity of 85% using 14% excess of the amino component only. In the corresponding synthesis of *For*-Trp-Lys-O*Prop* (**2.2.**), 650% excess of the same amino component was necessary to give a comparable selectivity of 78%. This discrepancy can be explained by the different substrate specificity of CT towards Tyr- and Trp-derivatives [13]. The following examples (**3**–**5**), synthesis of *For*-Tyr-Glu(O*Me*)$_2$, *For*-Tyr-Ala-O*Prop*, and *For*-Tyr-Ala-OH, show, that under comparable conditions higher yields result using the methyl ester of the carboxyl component instead of the propyl ester. This may be explained, presuming that bulkier ester residues of the carboxyl component are more overlap-

ping with the S_1'-binding site of the enzyme [14]. To certain extent, this might render it more difficult to arrange the amino-component and therefore the attack of water molecules on the acyl enzyme might be favoured. The selectivities of example **6**, synthesis of *For*-Tyr-Arg-O*R* (*R*=*Me*, *Prop*), indicate that the arginine propyl ester (selectivity: 73–75%) has distinctly a better affinity towards the S_1'-subsite of *CT* than the methyl ester (33–40%).

For checking the optical purity of the peptides synthesized, as representative example, *For*-Tyr-Ala-O*Prop* was examined in detail. N- and C-terminal protecting groups were removed from the enzymatically synthesized product. Chromatographic data were then compared with those of chemically synthesized stereoisomers by reversed phase HPLC and capillar GC (equipped with a chiral column) (Table 2 summarizes analytical data). By HPLC 0.5% and by GC 0.1% racemization are well detectable. Using these techniques no stereoisomers could be detected contaminating the enzymatically synthesized product.

In conclusion, it may be said, that formyl amino acid esters are suitable substrates for kinetically controlled enzymatic peptide synthesis. The synthesis, including preparation of formylated substrates, enzymatic reaction, and removal of protecting groups, proceeds without racemization as shown with the example of the synthesis of H-Tyr-Ala-OH via *For*-Tyr-Ala-O*Prop*. The formyl group improves the solubility of hydrophobic amino acid derivatives in aqueous media considerably and is, compared to conventional N^{α}-protecting groups, much cheaper.

Experimental

^{1}H-NMR spectra were recorded on a Varian EM 360 spectrometer using *TMS* as internal standard. Optical rotation measurements were carried out with a Perkin-Elmer 241 MC polarimeter. Melting points were taken on a SG 01 melting point apparatus (HWS, Mainz, FRG). UV spectra were obtained using a Shimadzu UV-vis Recording Spectrophotometer UV-160. HPLC analysis were performed on a Gynkotek (Germering, FRG) HPLC equipped with an ODS-Hypersil(5 μ) 4.6 × 250 mm HPLC-column, a Shimadzu C-R3A data processor, and a variable wavelength detector (Shimadzu) (solvent systems: 10 m*M* *TBA*/*Me*CN; 0.5–2.0 ml/min, 40 °C). The racemization analysis was performed using 10 m*M* *TBA* (1 ml/min). For GC analysis a Shimadzu GC-9A equipped with a chiral column [FS-L-Chirasil-Val, 25 m × 0.32 mm; Macherey & Nagel (Düren, FRG)] was used (mobile phase: He; 60 ml/min, split 1 : 50, 190 °C). For GC analysis, peptide derivatization was performed with 2 *N* HCl in 2-*Prop*OH during 1 h and *PFPA*/CH_2Cl_2 = 1 : 1 for 15 min at room temperature. TLC was performed with Merck TLC-plates silica gel 60 F_{254} [solvent systems: (A) Cyclohexane/ethyl acetate/acetic acid (25 : 70 : 5); (B) $CHCl_3$/*Me*OH/acetic acid (35 : 60 : 5); (C) *Me*CN/*Me*OH/H_2O (20 : 5 : 5); (D) pyridine/acetic acid/*n*-butanol/H_2O (24 : 6 : 30 : 20)]. Amino acid analysis were determined with a Biotronik Amino Acid Analyzer LC 5001. *Y* was obtained from Carlsberg

Table 1. *Enzyme catalyzed peptide synthesis*

Reaction components	Concentration (M)	Conditions	Selectivity (%)[a]	Example	R_F of product
For-Tyr-O*Prop*/ H-Lys-O*Prop*	0.20 0.23	*pH* 9.0; 5.1 ml 37% *Prop*OH; 15 μM *CT*	85	**1.**	0.70 (B)[b]
For-Trp-O*Prop*/ H-Lys-O*Prop*	0.12 0.26	*pH* 9.2; 1.3 ml 31% *Prop*OH; 50 μM *CT*	55	**2.1.**	
For-Trp-O*Prop*/ H-Lys-O*Prop*	0.12 0.90	*pH* 9.4; 0.85 ml 47% *Prop*OH; 120 μM *CT*	78	**2.2.**	0.82 (B)
For-Tyr-O*Prop*/ H-Glu(O*Me*)$_2$	0.08 0.30	*pH* 9.2; 1.2 ml 25% *Prop*OH; 29 μM *CT*	30	**3.1.**	
For-Tyr-O*Me*/ H-Glu(O*Me*)$_2$	0.08 0.30	*pH* 9.2; 0.7 ml 25% *Me*OH; 65 μM *CT*	55	**3.2.**	0.20 (A)
For-Tyr-O*Prop*/ H-Ala-O*Prop*	0.14 0.16	*pH* 9.2; 0.7 ml 33% *Prop*OH; 34 μM *CT*	55	**4.1.**	
For-Tyr-O*Me*/ H-Ala-O*Prop*	0.10 0.16	*pH* 9.2; 14 ml 33% *Me*OH; 40 μM *CT*	90	**4.2.**	0.39 (A)[c]
For-Tyr-O*Prop*/ H-Ala-OH	0.02 1.00	*pH* 8.6; 2 ml 2.2 μM *Y*	45	**5.1.**	

For-Tyr-O*Me*/ H-Ala-OH	0.02 1.00	*pH* 8.6; 1 ml 3 μM *Y*	55	**5.2.**	0.77 (B)
For-Tyr-O*Me*/ H-Arg-O*Me*	0.10 0.17	*pH* 9.0; 5 ml 33% *Me*OH; 28 μM *CT*	40	**6.1.**	0.35 (D)
For-Tyr-O*Me*/ H-Arg-O*Prop*	0.10 0.17	*pH* 9.0; 1.5 ml 33% *Me*OH; 20 μM *CT*	73	**6.2.**	
For-Tyr-O*Prop*/ H-Arg-O*Me*	0.15 0.26	*pH* 9.0; 1 ml 33% *Prop*OH; 40 μM *CT*	33	**6.3.**	
For-Tyr-O*Prop* H-Arg-O*Prop*	0.15 0.26	*pH* 9.0; 1 ml 33% *Prop*OH; 40 μM *CT*	75	**6.4.**	0.75 (D)[d]
For-Tyr-Lys-O*Prop*/ H-Met-NH_2	0.10 0.18	*pH* 9.2; 0.6 ml 17% *Prop*OH; 30 μM *T*	86	**7.**	0.77 (D)[e]
For-Phe-O*Me*/ H-Leu-NH_2	0.04 0.08	*pH* 9.0; 1 ml 38 μM *CT*	90	**8.**	0.82 (D)

[a] Selectivity: % peptide formation/turnover of carboxyl component
[b] Yield 67%; $[\alpha]_D^{25} = +6.4$ (1, *Me*OH)
[c] Yield 66%; $[\alpha]_D^{25} = +3.24$ (1, *Me*OH)
[d] Yield 64%; $[\alpha]_D^{25} = -2.69$ (0.7, 50% *Me*OH)
[e] Yield 59%; $[\alpha]_D^{25} = +3.6$ (0.2, 85% *Me*OH)

Table 2. *Analytical and physical data of* H-*Tyr-Ala*-OH *stereoisomers*

Compound	Amino acid analysis Tyr : Ala	$[\alpha]_D^{25}$ (c = 0.2, 1 N HCl)	R_F (C)[a]	Retention times (min)	
				HPLC[d]	GC[e]
H-L-Tyr-L-Ala-OH[b]		+ 9.8	0.73	6.13	78.0
H-D-Tyr-D-Ala-OH	1.04 : 1.00	− 13.0	0.84	6.13	37.4
H-L-Tyr-D-Ala-OH	1.14 : 1.00	− 74.8	0.78	10.13	37.7
H-D-Tyr-L-Ala-OH	1.08 : 1.00	+ 69.7	0.84	10.13	37.1
H-L-Tyr-L-Ala-OH[c]	1.03 : 1.00	+ 9.9	0.73	6.13	78.0

[a] Chiral plates (Macherey & Nagel, Düren, FRG)
[b] Purchased from Serva (Heidelberg, FRG)
[c] Enzymatically synthesized
[d] 1 ml/min; 10 m*M TBA*
[e] 60 ml/min; He; 190 °C; N,O-pentafluoropropinyl-iso-propyl ester derivatives

Biotechnology (Copenhagen, DK), and *CT* and *T* (*TPCK*-treated) from E. Merck (Darmstadt, FRG). Amino acid propyl ester hydrochlorides were synthesized by the method of *Brenner* et al. [15], and identified by ^{1}NMR-spectroscopy and elemental analysis. Analytical data are given in Table 3. H-Tyr-Ala-OH-stereoisomers were synthesized with the HO*Su*/*DCC*-method [16] using the *Boc*-protection group. Analytical data are summarized in Table 2.

Table 3. *Yields and physical properties of amino acid propyl esters*

Compound	Yield (%)	m.p. (°C)	$[\alpha]_D^{25}$ ($c = 2$, *Me*OH)	R_F	
				(B)	(D)
HCl · H-Tyr-O*Prop*	71	160	+ 9.4	0.86	0.90
HCl · H-Trp-O*Prop*	78	225 (dec.)	+ 17.4	0.88	0.89
2 HCl · H-Arg-O*Prop*	90	oil	+ 10.1	0.57	0.67
2 HCl · H-Lys-O*Prop*	59	143	+ 12.2	0.50	0.60
HCl · H-Ala-O*Prop*	94	107	+ 1.62	0.75	0.81

Synthesis of N$^{\alpha}$-Formyl Amino Acid Esters

60 mmol amino acid ester hydrochloride was dissolved in a mixture of 80 ml 98% formic acid and 20 ml acetic anhydride, which was precooled on an ice-bath. Subsequently 66 mmol *DIPEA* (as 1 *N* solution in methylene chloride) was added. In the case of the tryptophan ester, 121 mmol *DIPEA* was used. The reaction mixtere was stirred at room temperature for 15 min. Thereupon, 80 ml ice-cold, saturated NaCl-solution was added, and the reaction mixture was adjusted to *pH* 2.0 with 1 *N* hydrochloric acid. The products were extracted with methylene chloride (4 × 70 ml), the combined organic phases were washed with 50 ml acidic NaCl-solution (*pH* 2), concentrated under vacuum, crystallized from *Me*OH/water, dried over P_2O_5, and identified by ^{1}H-NMR spectroscopy [$CDCl_3$/$\delta = 8.1$ ppm (s, 1 H, H—CO—)] and elemental analysis. Data are given in Table 4.

Table 4. *Yields and physical properties of N$^{\alpha}$-formyl amino acid esters*

Compound	Yield (%)	m.p. (°C)	R_F (A)	$[\alpha]_D^{25}$ ($c = 2$, *Me*OH)
For-Phe-O*Me*	74	43 (43–44) [18]	0.68	+ 24.9
For-Tyr-O*Me*	70	140 (141.5) [19]	0.50	+ 20.7
For-Tyr-O*Prop*	60	113.5	0.84	+ 23.5
For-Trp-O*Prop*[a]	74	oil	0.53	+ 4.86

[a] UV (50% ethanol): λ_{max} (lg ε) = 280 nm (3.490), 220 nm (4.114); ^{1}H-NMR ($CDCl_3$): $\delta = 9.8$ ppm [s (broad), 1 H, N^{In}—H], 8.1 (s, 1 H, H—CO—), 6.8 [d (broad), 1 H, N$^{\alpha}$—H]

Enzymatic Peptide Synthesis

Amino acids were dissolved in mixtures of alcohol and 0.1 *M* *Tris* buffer (*pH* 9.5) as indicated in Table 1. The *pH* was adjusted to the desired value with 2 *N* NaOH. Enzymes were dissolved in 0.2 ml 0.1 *N* Ca^{2+}/0.5 *N* Na^{+}-solution and subsequently added to the reaction mixtures. During the reactions, *pH*-values were held constant using a Radiometer (Copenhagen, DK) Titralab TM. The reactions were stopped by addition of acetic acid. The selectivity of peptide bond formation was assayed by HPLC analysis. Preparative recovery of peptides (*For*-Tyr-Lys-O*Prop*, *For*-Tyr-Arg-O*Prop*, and *For*-Tyr-Lys-Met-NH_2) followed by cation exchange chromatography using a Pharmacia FPLC equipment (CM-Sepharose FF, 40 × 2.6 cm, 20 m*M* citrate buffer), and gel filtration (Sephadex G 10, 2.6 × 80 cm, 20% *Prop*OH). *For*-Tyr-Ala-O*Prop* was isolated by extraction procedures and crystallized from methylene chloride (m.p. 174–175.5 °C). The other peptides were isolated on analytical scale, carried out by one- and two-dimensional TLC. The peptides were identified by amino acid analysis after hydrolysis in 6 *N* HCl at 106 °C for 16 h; *For*-Trp-Lys-O*Prop* however was cleaved by *CT* after removal of protecting groups. Analytical amounts of peptides have been deprotected. In all cases, HPLC data were identical with commercial available products. Hydrolysis of methyl esters were performed in dilute NaOH (*pH* 10.0; 30 min; 20 m*M* solution). Hydrolysis of C-terminal Arg- and Lys-containing peptides was carried out with *T* (*pH* 9.5; 1 min; 40 m*M* solutions; 20 μ*M* *T*). *For*-Tyr-Ala-O*Prop* was suspended in 50% aqueous dioxane at *pH* 10.0 and deesterified under vigorous stirring for 10 h. The deformylation was performed in 0.3–0.5 *N* HCl solution (20 m*M*) at 56 °C during 2–6 h [17].

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