FREE CHYMOTRYPSIN-CATALYZED SYNTHESIS OF PEPTIDE BOND IN ALIPHATIC ALCOHOLS WITH LOW WATER CONTENT*

Václav ČEŘOVSKÝ and Karel MARTINEK

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received March 15, 1988
Accepted April 7, 1988

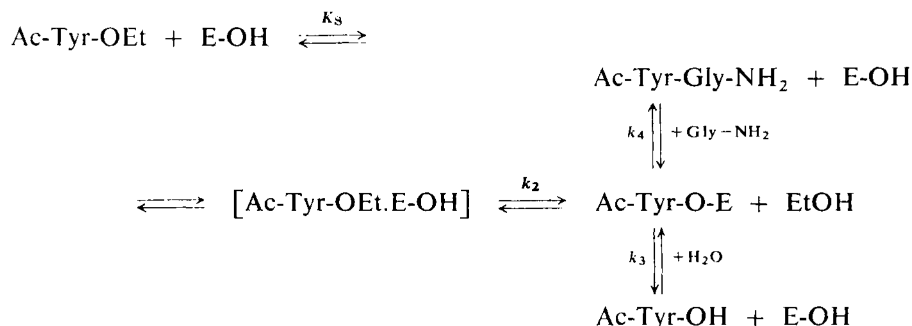
The effect of water content on free chymotrypsin-catalyzed reaction of Ac-Tyr-OEt with HBr. .Gly-NH_2 in triethylamine-containing 2-propanol was studied. Maximum yield of dipeptide Ac-Tyr-Gly-NH_2 was obtained in 2-propanol with 2% of water. Lower water content retards the reaction. Although higher water content accellerates the process, the yield of the dipeptide is reduced by enzymatically catalyzed hydrolysis of Ac-Tyr-OEt. The studied reaction proceeds analogously also in other aliphatic alcohols with low content of water except in methanol; it does not take place in dimethylformamide or dimethyl sulfoxide containing 2% or 20% of water. In 2-propanol with 2% or 5% of water, syntheses of the protected amino-terminal oxytocine and vasopressin tripeptide, as well as other model peptides, were studied. In all the described experiments, α-chymotrypsin without any stabilization or immobilization was employed.

Peptide syntheses, catalyzed by proteolytic enzymes, are usually performed in an aqueous medium containing a water-miscible organic solvent such as dimethylformamide or methanol to dissolve the starting components. As it is commonly assumed that higher concentrations of water-miscible solvents rapidly reduce the enzymatic activity[6,7], the maximum employed concentration of these solvents is usually about 50% (refs[1-5]). Another approach utilizes biphasic systems where some starting components are distributed in a water-immiscible organic solvent and the enzyme remains during the reaction in the aqueous phase, being protected from the attack by the organic solvent[8,9]. The use of enzymes in synthetic organic chemistry has required to perform the enzyme-catalyzed reactions in all types of organic solvents[10-13]. Water-miscible organic solvents as a medium for enzymatically catalyzed synthesis of peptides or amino acid derivatives have become popular only recently. Under conditions of thermodynamic equilibrium the synthesis of the peptide bond using proteolytic enzymes in various organic solvents with varying water content was studied[14]. There were described esterifications of N-acetyl-L-tyrosine and N-acetyl-L-tryptophan with free α-chymotrypsin in ethanol and other alcohols, containing

* Part CCXI in the series Amino Acids and Peptides; Part CCX: Int. J. Pept. Protein Res. in press.

less than 10% of water[15], syntheses of Ac-Tyr-Gly-NH_2* from Ac-Tyr and Gly-NH_2 in acetonitrile or ethanol with 5% of water, using free[16] or various types of immobilized α-chymotrypsin[16,17]. Model dipeptide Z-Ala-Val-OBu^t was synthesized in acetonitrile containing 0·3–0·5% of water in the presence of immobilized papain[18]. Subtilisin, suspended in anhydrous organic solvents, catalyzed the synthesis of model peptides containing D-amino acids[19]; in anhydrous dimethylformamide this enzyme catalyzed the synthesis of sugar esters of protected amino acids[20]. 4-Methylpentan-2-one was employed as a medium for the immobilized papain-catalyzed synthesis of a model dipeptide[21].

Within the framework of our methodical studies on enzymatically catalyzed syntheses of peptides and amino acids performed during the last several years (see e.g. ref.[22]) we have studied free α-chymotrypsin-catalyzed peptide bond syntheses in water-miscible organic solvents with low content of water. For this purpose aliphatic alcohols seem to be very advantageous. We used the so-called kinetic approach[23–25] which for the studied model reaction of Ac-Tyr-OEt with Gly-NH_2, catalyzed with α-chymotrypsin (E-OH) in a basic medium can be described by the following very simplified scheme[23–25].



The reaction was performed with 0·1M Ac-Tyr-OEt and 0·2M HBr.Gly-NH_2 in the presence of triethylamine (0·22 mol l^{-1}), the concentration of α-chymotrypsin being 0·02 mmol l^{-1}. The composition of the reaction mixture was analyzed at appropriate time intervals by HPLC.

Table I shows the dependence of the reaction course on the ratio of the solvents used as the reaction medium (2-propanol : water). It is evident that high water content enhances the enzymatically catalyzed hydrolysis of the ester (Ac-Tyr-OEt) at the expenses of the synthetic reaction. The 1 : 1 2-propanol–water mixture suits least to synthetic purposes because the enzymatic activity is destroyed and thus no complete conversion of the starting Ac-Tyr-OEt is achieved. In more concentrated

* The abbreviations used obey the published recommendations (Eur. J. Biochem. *134*, 9 (1984)).

2-propanol (80%, 90%) the dipeptide synthesis is already preferred to the ester hydrolysis and the starting compound is completely converted during several minutes. Optimum conditions for the dipeptide synthesis were achieved in 2-propanol with low percentage of water. With water concentration 2% (1·11 mol l^{-1}), Ac-Tyr-OEt reacted completely during relatively short time (2 h) and the dipeptide was obtained

TABLE I

Composition of mixtures from α-chymotrypsin-catalyzed reaction between Ac-Tyr-OEt and Gly-NH_2 in 2-propanol with various water concentration (vol. %)

Ac-Tyr-Gly-NH_2, % k'_{40} = 0·16	Ac-Tyr-OH, % k'_{40} = 0·43	Ac-Tyr-OEt, % k'_{40} = 2·01	Ac-Tyr-OPr^i, % k'_{40} = 3·87	H_2O[a]	Time min
49	50	1	0	80	2
49·5	50·5	0	0		5
36	37	16	11		2
37	38	14	11	60	5
37	39	14	10		15
48	29	13	10		2
48	30	13	9	40	5
48	30	13	9		15
73	22	3·5	1·5	20	2
76	24	0	0		5
62	11	15	12		2
80	13·5	3	3·5	10	5
85·5	14·5	0	0		15
63	16	10	11		2
73	20	1	6	5	5
79	21	0	0		15
51	6	36·5	6·5		5
70·5	9	14	6·5	2	15
86	9·5	1·5	3		60
89	9·5	0	1·5		120
47	3	45	5		4 h
74	4·5	16·5	5	1	28 h
81·5	5	9	4·5		72 h
40·5	3	51	5·5		4 h
65	3	26	6	0·5	28 h
76	4	13·5	6·5		72 h

[a] Reaction media with 80% to 10% of water contain 0·2M carbonate–bicarbonate buffer, pH 9·5.

in almost 90% yield. In a preparative experiment, the desired product was isolated in 83% yield which is substantially higher than that achieved[16,17] using the so-called thermodynamic approach[23-25]. Although further decrease of the water content to 1% or 0·5% still more preferred the synthesis, the reaction was many times slower. In anhydrous 2-propanol no reaction of Ac-Tyr-OEt was observed 24 hours after addition of the solid enzyme (performed as the blank experiment). Upon addition of water (2%) only 10% of Ac-Tyr-OEt was converted into the dipeptide and Ac-Tyr-

TABLE II

Composition of mixtures from α-chymotrypsin-catalyzed reaction between Ac-Tyr-OEt and Gly-NH_2 in aliphatic alcohols containing 2 vol. % of water

Ac-Tyr-Gly-NH_2 %	Ac-Tyr-OH %	Ac-Tyr-OEt %	Ac-Tyr-OR %	Alcohol (R–OH)	Time min
0	0	100	0	methanol	2 to 180
41·5	1	57·5	—		5
55	1	44	—	ethanol	15
68·5	1·5	30	—		60
71·5	1·5	27	—		120
58	8	18	16		5
76	10·5	5	8·5	1-propanol	15
88	12	0	0		60
9	0·5	89·5	1		15
27	1	69	3	1-butanol	60
41·5	1·5	51·5	5·5		120
93	7	0	0		28 h
6	0·5	93·5	0		15
24	1·5	74·5	0	2-butanol	60
35	1·5	63·5	0		120
83	5	10·5	1·5		28 h
39	11	50	0		5
56	16	28	0	tert-butyl	15
68	24	8	0	alcohol	60
73	26·5	0·5	0		120
5·5	0·5	94	0		15
23	1	76	0	1-pentanol	60
43	2	55	0		120
93	5·5	1·5	0		24 h
59·5	12·5	9·5	18·5		2
85·5	12·5	0	2	1,4-butanediol	5
87	13	0	0	(8% H_2O)	15

TABLE III

Composition of reaction mixture after reaction of Z-Cys(Bzl)-Tyr-OEt with derivatives of amino acids (X), catalyzed with α-chymotrypsin in 2-propanol, containing 5% or 2% of water

Z-Cys(Bzl)-Tyr-X %	Z-Cys(Bzl)-Tyr-OH %	Z-Cys(Bzl)-Tyr-OEt %	Z-Cys(Bzl)-Tyr-OPr[i] %	X (% H_2O)	Time min
28·5	4·5	65	2	HBr.Gly-NH_2	5
44·5	6	47	2·5	(5)	15
66	8·5	23	2·5		60
72	9	16	3		120
77	9·5	11·5	2		25 h
16	1·5	82·5	0	HBr.Gly-NH_2	5
25	2	72·5	0·5	(2)	15
37	3	59	1		60
55	4	39	2		8 h
67	5	26	2		25 h
8	26	54	12	HCl.Ile-OMe	5
14	44	23	19	(5)	15
23	60	2	15		60
26	65	0	9		120

11	9	70	10	HCl.Ile-OMe (2)	60
24	19	40	17		4 h
47	31	5	17		24 h
51·5	35	2·5	11		48 h
53[a]	—[b]	0	6[c]	TFA.Ile-N_2H_2Ph (5)	120
46[a]	—[b]	27[c]	11[c]	TFA.Ile-N_2H_2Ph (2)	48 h
53[a,d]	20[c]	20[c]	0	HCl.Phe-NH_2 (5)	18 h
54[a,e]	—[b]	13[c]	15[c]	TFA.Phe-N_2H_2Ph (5)	24 h

[a] The product precipitates from the reaction mixture after several minutes of reaction; this value is determined after isolation of the product; [b] not evaluated, retention time very similar to that of X; [c] relative to the peak of Z-Cys(Bzl)-Tyr-OEt in time 0; [d] homogeneous according to TLC and HPLC, m.p. 231—232°C, amino acid analysis: Tyr 0·94, Phe 1·08, Cys(Bzl) 0·98; [e] homogeneous according to TLC and HPLC, m.p. 243—245°C, amino acid analysis: Tyr 0·97, Phe 1·05, Cys(Bzl) 0·98 (k' and m.p. agree with those in ref.[28]).

TABLE IV

Composition of reaction mixture after reaction of Boc-Met-Gly-Trp-OMe with derivatives of amino acids (X), catalyzed with α-chymotrypsin in 2-propanol containing 5% or 2% of water

Boc-Met-Gly-Trp-X %	Boc-Met-Gly-Trp-OH %	Boc-Met-Gly-Trp-OMe %	Boc-Met-Gly-Trp-OPr^i %	X (% H_2O)	Time min
46	14·5	33·5	6	HBr.Gly-NH_2	5
63·5	17·5	13	6	(5)	15
75	19	2	4		60
77·5	19	0·5	3		120
21	3	75	1	HBr.Gly-NH_2	60
29·5	3	66	1·5	(2)	4 h
42	3·5	52·5	2		24 h
49	3·5	45·5	2		48 h
27	9	59	5	TFA.Met-N_2H_2Ph	5
47	16	32	5	(5)	15
68	24	5	3		60
71	26	1	2		120
19	3·5	77·5	0	TFA.Met-N_2H_2Ph	60
33	5·5	60	1·5	(2)	4 h
54	11	32·5	2·5		24 h
61	14·5	22·5	2		48 h

-OH after further 24 hours. One has to keep in mind that in a kinetically controlled enzymatically catalyzed peptide synthesis in water-miscible organic solvents, even reducing the water content to very low values cannot completely suppress the concurrent hydrolysis of the acylating ester. Even a concentration of 0·5% (0·28 mol l^{-1}) is still comparable with that of the nucleophile (Gly-NH_2). In all experiments we detected (HPLC) at appropriate time intervals also the presence of acetyltyrosine 2-propyl ester, arising by enzymatically catalyzed transesterification, similarly as described[26] for an α-chymotrypsin-catalyzed reaction. The ester was further converted into the corresponding products.

Table II shows the composition of the reaction mixture at various time intervals for the same α-chymotrypsin-catalyzed reaction in other aliphatic alcohols containing 2% of water. Similarly as in 2-propanol, the compounds reacted in 1-propanol, tert-butyl alcohol and 1,4-butanediol. In 1-butanol, 2-butanol and 1-pentanol the reaction was slower. In 1,2-ethanediol the ester hydrolysis prevailed over the dipeptide synthesis (the reaction rate was very slow). Also here the ester of acetyltyrosine and the corresponding alcohol was detected in the course of some reactions. In methanol there was no reaction, in ethanol the reaction slowed down after several minutes and ceased entirely after 2–4 hours, probably due to loss of enzymatic activity. Similar results were obtained with free α-chymotrypsin-catalyzed esterification of acetyl-L-tryptophan in various alcohols with low content of water, the synthesis of Ac-Trp-OEt did not proceed in methanol and tert-butyl alcohol[15]. No reaction was also observed in dimethylformamide and dimethyl sulfoxide with 2% or 20% of water. This is at variance with a study[27] which measured the cleavage of *p*-nitrophenyl acetate and *p*-nitrophenyl ester of benzyloxycarbonyl-DL-phenylalanine with α-chymotrypsin in anhydrous dimethyl sulfoxide. In a 3 : 2 mixture of dimethylformamide–carbonate-bicarbonate buffer, pH 9, we detected 5% of Ac-Tyr-Gly-NH_2, 1% of Ac-Tyr-OH and 94% of Ac-Tyr-OEt after 5 min as well as 60 min; using a reversed solvent ratio (2 : 3) the respective percentages were 49%, 24% and 27% after 15 as well as 60 min. Just the last-mentioned solvent composition has been very often used in α-chymotrypsin-catalyzed syntheses of peptides[2–4,28].

We aplied the obtained results to enzymatically catalyzed synthesis of protected tripeptides of the N-terminal oxytocin and vasopressin sequence and to the preparation of other peptides. Tables III and IV present the composition of reaction mixtures at various time intervals for α-chymotrypsin-catalyzed reactions of Z-Cys(Bzl)-Tyr-OEt and Boc-Met-Gly-Trp-OMe with various amino acid derivatives in 2-propanol with 2% or 5% of water. As compared with the data in Table I, the dependence of the reaction course on the water content in 2-propanol is here much more pronounced. It seems that the increase in the size of the acylating molecule generally retards the reaction and that in preparative experiments a slightly higher water content (5%) would be advantageous. The syntheses of Z-Cys(Bzl)-Tyr-Ile-N_2H_2Ph, Z-Cys(Bzl)-Tyr-Phe-N_2H_2Ph and Z-Cys(Bzl)-Tyr-Phe-NH_2 were accompanied by complete precipitation

of the desired products from 2-propanol, enabling their easy isolation. In this manner we synthesized Z-Cys(Bzl)-Tyr-Ile-N_2H_2Ph in 53% yield on a preparative scale in 95% 2-propanol.

Aliphatic alcohols (excepting methanol and ethanol) with low water content thus prove to be very suitable media for free α-chymotrypsin-catalyzed peptide syntheses using the kinetic approach. In aliphatic alcohols the activity of α-chymotrypsin is retained for several days[15] and therefore the syntheses require no stabilization or immobilization.

EXPERIMENTAL

α-Chymotrypsin was a Serva product of activity 45 U/mg, Ac-L-Tyr-OEt.H_2O was purchased from Koch-Light Laboratories, other amino acid and peptide derivatives were synthesized in our Laboratory by standard procedures and according to ref.[29]. Z-Cys(Bzl)-Tyr-OEt and Boc-Met-Gly-Trp-OMe were prepared according to ref.[30] and ref.[22], respectively. Amino acid analyses were performed on a Durrum D-500 analyzer, optical rotations were measured on a Perkin-Elmer 141 MCA polarimeter. Melting points were determined on a Kofler block and are uncorrected. High-performance liquid chromatography (HPLC) was done on a Spectra Physics SP 8700 instrument equipped with an SP 8400 UV detector and an SP 4100 integrator, using a 15 × 0·4 cm column packed with Separon SIX C-18 (7 μm); flow rate 42 ml/h, detection at 280 nm, mobile phase a mixture of methanol with 0·05% aqueous trifluoroacetic acid (the amount of methanol in vol. % is given as a subscript at the k'values of the corresponding compounds). k'_{40} of Ac-Tyr-Gly-NH_2 = 0·16, Ac-Tyr-OH = 0·43, Ac-Tyr-OEt = 2·01, Ac-Tyr-OPr^i = 3·87. k'_{73} of Z-Cys(Bzl)-Tyr-Gly-NH_2 = 0·88, Z-Cys(Bzl)-Tyr-OH[30,31] = 1·23, Z-Cys(Bzl)-Tyr-OEt = 2·39, Z-Cys(Bzl)-Tyr-OPr^i = 3·20. k'_{78} of Z-Cys(Bzl)-Tyr-OH = 0·64, Z-Cys(Bzl)-Tyr-OEt = 1·18, Z-Cys(Bzl)-Tyr-OPr^i = 1·55, Z-Cys(Bzl)-Tyr-Ile-OMe[31] = 1·86, Z-Cys(Bzl)-Tyr-Ile-N_2H_2Ph = 1·85, Z-Cys(Bzl)-Tyr-Phe-NH_2 = 1·12, Z-Cys(Bzl)-Tyr-Phe-N_2H_2Ph[28] = 2·18. k'_{70} of Boc-Met-Gly-Trp-Gly-NH_2 = 0·72, Boc-Met-Gly-Trp-OH[22] = 1·09, Boc-Met-Gly-Trp-OMe = 1·65, Boc-Met-Gly-Trp-OPr^i = 3·14, Boc-Met-Gly-Trp-Met-N_2H_2Ph = 3·00.

Reaction of Ac-Tyr-OEt with HBr. Gly-NH_2

Triethylamine (30 μl, 0·22 mmol), followed by Ac-Tyr-OEt.H_2O (27 mg; 0·1 mmol), was added to a solution of HBr.Gly-NH_2 (31 mg; 0·2 mmol) in a given organic solvent (200, 400, 600, 800 or 900 μl). The mixture was mixed with 0·2M carbonate-bicarbonate buffer pH 9·5 (750, 550, 350, 150 or 50 μl). Mixtures with low content of water were prepared similarly by dissolving the above-mentioned components in an anhydrous organic solvent (950, 980, 990 or 995 μl). After addition of α-chymotrypsin (0·5 mg) solution in water (50, 20, 10 or 5 μl), the mixtures were incubated at 30°C. At regular time intervals aliquots (1 μl) for HPLC analysis were withdrawn which were diluted with 50% aqueous methanol, containing 0·1% trifluoroacetic acid (100 μl).

Enzymatic Synthesis of Ac-Tyr-Gly-NH_2

Triethylamine (300 μl) and Ac-Tyr-OEt.H_2O (270 mg) were added to a solution of HBr.Gly-NH_2 (310 mg) in 2-propanol (9·8 ml). After addition of α-chymotrypsin (5 mg) in water (200 μl) the mixture was incubated at 30°C for 4 h, cooled, diluted with water (10 ml) and filtered through a column of Dowex-50 (20 ml) in 50% aqueous methanol. The eluate (about 80 ml) was con-

centrated in vacuo and filtered through an Amberlite IR-4B column (20 ml) in the same solvent. After evaporation to dryness, the residue was crystallized from methanol–ether, affording 240 mg (83%) of the product, m.p. 99–101°C, homogeneous according to TLC and HPLC. Amino acid analysis: Gly 1·00, Tyr 0·96. An analytical sample was recrystallized from the same solvent mixture, m.p. 108–110°C, $[\alpha]_D$ +42·6° (*c* 0·3, water). For $C_{13}H_{17}N_3O_4.0{\cdot}5\ H_2O$ (288·3) calculated: 54·16% C, 6·29% H, 14·58% N; found: 53·75% C, 6·04% H, 14·30% N.

Reaction of Z-Cys(Bzl)-Tyr-OEt and Boc-Met-Gly-Trp-OMe with Derivatives of Amino Acids

Triethylamine (3 μl; 0·022 mmol) and Z-Cys(Bzl)-Tyr-OEt (5·5 mg; 0·01 mmol) or Boc-Met-Gly--Trp-OMe (5·1 mg; 0·01 mmol) were added to a solution of salt of the given amino acid derivative (0·02 mmol) in 2-propanol (95 or 98 μl). After addition of α-chymotrypsin (0·05 mg) in water (5 or 2 μl) the mixtures were incubated at 30°C for various times. The reaction was monitored by HPLC as described above.

Enzymatic Synthesis of Z-Cys(Bzl)-Tyr-Ile-N_2H_2Ph

Triethylamine (30 μl) and Z-Cys(Bzl)-Tyr-OEt (55 mg; 0·1 mmol) were added to a solution of isoleucine phenylhydrazide trifluoroacetate (67 mg; 0·2 mmol) in 2-propanol (950 μl). After addition of α-chymotrypsin (0·5 mg) in water (50 μl) the mixture was incubated at 30°C for 2 h. The precipitate was filtered, washed with water and 2-propanol and dried; yield 38 mg (53%) of product, m.p. 258–260°C, TLC and HPLC homogeneous. Amino acid analysis: Ile 1·04, Tyr 0·96, Cys(Bzl) 1·01. $[\alpha]_D$ −33·8° (*c* 0·2, dimethylformamide). For $C_{39}H_{44}N_5O_6S.0{\cdot}5\ H_2O$ (720·9) calculated: 64·98% C, 6·29% H, 9·71% N; found: 65·13% C, 6·18% H, 9·81% N.

REFERENCES

1. Tsuzuki H., Oka T., Morihara K.: J. Biochem. *88*, 669 (1980).
2. Kullmann W.: J. Biol. Chem. *255*, 8234 (1980).
3. Kullmann W.: Proc. Natl. Acad. Sci. U.S.A. *79*, 2840 (1982).
4. Sakina K., Ueno Y., Kawazura K., Morihara K.: *Peptide Chemistry 1986. Proc. 24th Symp. Pept. Chem.* (T. Miyazawa, Ed.), p. 305. Protein Research Foundation, Osaka 1987.
5. Mitin Yu. V., Zapevalova N. P., Gorbunova E. Yu.: Int. J. Pept. Protein Res. *23*, 528 (1984).
6. Pliura D. H., Jones J. B.: Can. J. Chem. *58*, 2633 (1980).
7. Reslow M., Adlercreutz P., Mattiasson B.: Appl. Microbiol. Biotechnol. *26*, 1 (1987).
8. Martinek K., Semenov A. N., Berezin I. V.: Biochem. Biophys. Acta *658*, 76 (1981).
9. Kuhl P., Walpuski J., Jakubke H.-D.: Pharmazie *37*, 766 (1982).
10. Fukui S., Tanaka A.: Endeavour *9*, 10 (1985).
11. Klibanov A. M.: CHEMTECH *16*, 354 (1986).
12. Laane C., Boeren S., Vos K., Veeger C.: Biotechnol. Bioeng. *30*, 81 (1987).
13. West J. B., Wong C.-H.: Tetrahedron Lett. *28*, 1629 (1987).
14. Isowa Y., Kakutani M., Yaguchi M.: *Peptide Chemistry 1981. Proc. 19th Symp. Pept. Chem.* (T. Shioiri, Ed.), p. 25. Protein Research Foundation, Osaka 1982.
15. Kise H., Shirato H., Noritomi H.: Bull. Chem. Soc. Jpn. *60*, 3613 (1987).
16. Noritomi H., Kise H.: Biotechnol. Lett. *9*, 383 (1987).
17. Kise H., Hayakawa A., Noritomi H.: Biotechnol. Lett. *9*, 543 (1987).
18. Mitin Yu. V., Schellenberger V., Jakubke H.-D.: Bioorg. Khim. *14*, 5 (1988).
19. Margolin A. L., Tai D.-F., Klibanov A. M.: J. Am. Chem. Soc. *109*, 7885 (1987).

20. Riva S., Chopineau J., Kieboom A. P. G., Klibanov A. M.: J. Am. Chem. Soc. *110*, 584 (1988).
21. Barbas C. F., Wong C.-H.: J. Chem. Soc., Chem. Commun. *1987*, 533.
22. Čeřovský V., Hlaváček J., Slaninová J., Jošt K.: Collect. Czech. Chem. Commun. *53*, 1234 (1988).
23. Jakubke H.-D., Kuhl P., Könnecke A.: Angew. Chem., Int. Ed. *24*, 85 (1985).
24. Kullmann W.: J. Protein Chem. *4*, 1 (1985).
25. Morihara K.: Trends Biotechnol. *5*, 164 (1987).
26. Zaks A., Klibanov A. M.: J. Am. Chem. Soc. *108*, 2767 (1986).
27. Klyosov A. A., Van Viet N., Berezin I. V.: Eur. J. Biochem. *59*, 3 (1975).
28. Čeřovský V.: Collect. Czech. Chem. Commun. *51*, 1352 (1986).
29. Čeřovský V., Jošt K.: Collect. Czech. Chem. Commun. *49*, 2557 (1984).
30. Čeřovský V., Jošt K.: Collect. Czech. Chem. Commun. *50*, 2775 (1985).
31. Boissonnas R. A., Guttmann S., Jaquenoud P. A., Waller J. P.: Helv. Chim. Acta *38*, 1491 (1955).

Translated by M. Tichý.