PAPAIN-CATALYZED FRAGMENT SYNTHESIS OF PROTECTED CHOLECYSTOKININ DERIVATIVES*

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Sodium salts of methyl esters of N-tert-butyloxycarbonyl-β-tert-butylaspartyl-O^4-sulfotyrosine (*II*) or N-tert-butyloxycarbonyl-O^4-sulfotyrosine (*III*) were condensed with amino components derived from peptide amides *IVb*—*IVe* and *IVg* (simulating the carboxy-terminal part of cholecystokinin) under catalysis with papain. Rates and yields of conversion of these peptides to the corresponding derivatives *Ib*—*If* were compared with the results reported previously for analogous papain-catalyzed fragment synthesis of the protected carboxy-terminal octapeptide of cholecystokinin *Ia*. In the condensation reactions with the individual amino components quantitative changes were observed in the ability of papain to catalyze the peptide bond synthesis which appeared as differences in the maximum yield (and the time required for achieving it) of the condensation reaction, monitored by HPLC. The observed differences are related not only with the distance of the amino acid substitution from the P_1' subsite in the given amino component but also with the side-chain structure of the substituting amino acid.

Within the framework of our studies concerning the use of enzymes in the peptide synthesis we recently described[1] the papain-catalyzed formation of peptide bond between the protected dipeptide Boc-Asp(OBu^t)-Tyr($SO_3^-.Na^+$)OMe**, containing the sulfated side-chain of the tyrosine moiety, with the cholecystokinin carboxy-terminal hexapeptide Met-Gly-Trp-Met-Asp-Phe-NH_2 under formation of the cholecystokinin derivative *Ia*. After deblocking, the thus-prepared cholecystokinin octapeptide (CCK-8) exhibited the same analgetic, sedative, anorectic and gall bladder contracting activity as commercial samples of this peptide. Papain was also used by Japanese authors[3] in the synthesis of the same peptide by condensation of the fragments Fmoc-Asp-Tyr($SO_3^-.Ba^{2+}$)-Met-Gly-Trp-OMe and Met-Asp-Phe-NH_2.

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** The nomenclature and symbols of amino acids, peptides and protecting groups obey the published recommendations[2]. Cle cycloleucine, denotes 1-aminocyclopentane-1-carboxylic acid. The amino acids used in this work are of the L-configuration.

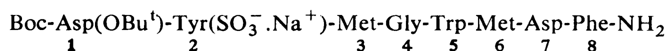
TABLE I
Data on peptide derivatives synthesized in this paper

Compound	M.p., °C Yield, %	Formula M.w./M + H$^+$	$[\alpha]_D^{22}$ a	k b	Amino acid analysis Asp Trp	 Tyr Phe	 Met Cle	 Gly Aib	Calculated/Found % C	 % H	 % N
Ib	179–181[c]	$C_{61}H_{80}N_{10}O_{18}S_3Na_2.2\,H_2O$	−16·6°	6·9	2·16	1·01[d]	1·87	0·94[e]	51·62	5·68	9·89
	45	1 419·5	0·4 DMF	65	0·93	1·00	—	—	51·53	5·79	10·15
Ic	221–224[f]	$C_{52}H_{73}N_{11}O_{18}S_3Na_2$	−7·3°	5·6	2·12	0·97[d]	1·94	1·00	49·20	5·69	11·91
	34	1 294·5	0·3 DMF	62	0·98[g]	0·98	—	—	49·53	5·53	11·64
Id	[h]	$C_{59}H_{76}N_{10}O_{18}S_2Na_2.H_2O$	−12·9°	4·7	2·11	0·97[d]	1·04	0·98	52·83	5·86	10·44
	28	1 341·5/1 324	0·2 DMF	67·5	0·99	1·00	1·04	—	53·09	5·74	10·26
Ie	[h]	$C_{60}H_{79}N_{11}O_{19}S_3Na_2$	−19·2°	2·8	2·10	0·96[d]	1·93	2·17	51·45	5·69	11·00
	19	1 400·6/1 396	0·2 DMF	67·5	0·89	1·00	—	—	51·21	5·61	10·72
If	[h]	$C_{50}H_{63}N_9O_{15}SNa_2$	−12·3°	3·6	1·08	0·96[d]	0·94[i]	1·03	54·89	5·80	10·24
	43	1 094·1	0·1 DMF	65	0·89	1·00	—	0·87	55·16	5·87	9·98
IVb	152–155	$C_{44}H_{60}N_8O_{10}S_2$	+53·5°	3·1	1·10	—	1·89	0·91[e]	57·13	6·54	12·11
	56	925·1	0·1 MeOH	65	1·03	1·02	—	—	57·30	6·48	11·89
IVc	139–141	$C_{36}H_{53}N_9O_{10}S_2.CF_3COOH$	−24·5°	2·6	1·00	—	1·88	1·00	48·04	5·73	13·27
	55	950·0	0·2 MeOH	65	0·98[g]	1·01	—	—	48·87	5·52	12·97
IVd	143–145	$C_{42}H_{56}N_8O_{10}S$	−56·8°	2·8	1·00	—	1·00	1·05	58·32	6·53	12·95
	53	865·0/865	0·1 DMF	70	0·88	0·98	0·99	—	58·09	6·59	13·04
IVe	188–190	$C_{43}H_{59}N_9O_{11}S_2$	−18·5°	2·2	1·12	—	2·05	1·98	54·82	6·31	13·38
	57	942·1/842[j]	0·2 MeOH	70	0·85	1·00	—	—	54·99	6·24	13·51

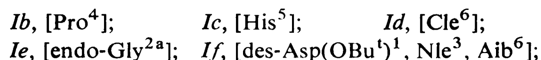
IVf	154–157	$C_{48}H_{62}N_8O_{10}$	−15·6°	3·8	1·06	—	1·01[i]	1·08	63·28	6·86	12·30
	89	911·1/911	0·1 DMF	70	0·94	1·00	—	1·09	63·12	6·97	12·49
IVg	135–137	$C_{41}H_{56}N_8O_{10}$	−11·1°	2·1	1·08	—	1·04[i]	1·06	59·98	6·88	13·65
	92	821·0/821	0·2 MeOH	70	0·89	1·00	—	1·02	60·12	6·82	13·54
Va	210–211	$C_{34}H_{44}N_6O_8S$	−37·2°	7·1	1·10	—	1·02	—	58·61	6·36	12·06
	51	696·8	1·0 DMF[k]	60	0·92	1·00	—	—	58·90	6·29	11·89
Vb	198–199	$C_{29}H_{41}N_7O_8S.CF_3COOH.H_2O$	−34·6°	6·5	1·04	—	1·06	—	47·75	5·69	12·57
	82	779·8	0·2 MeOH	60	0·98[g]	1·00	—	—	47·46	5·44	12·45
Vc	176–178	$C_{35}H_{44}N_6O_8$	−6·32°	8·6	1·07	—	—	—	62·11	6·55	12·42
	45	676·8	0·2 MeOH[l]	60	0·96	1·00	1·02	—	61·89	6·52	12·42
Vd	110–112	$C_{40}H_{48}N_6O_8$	−36·2°	10·9	1·08	—	—	—	64·85	6·53	11·34
	81	740·9/741	0·2 DMF	60	0·91	1·00	—	0·94	65·08	6·44	11·49
VIa	209	$C_{23}H_{34}N_4O_7S$	−39·9°	3·7	1·07	—	1·01	—	54·10	6·71	10·97
	74	510·6	1·0 DMF[m]	60	—	1·00	—	—	53·96	6·84	10·81
VIb	111–113	$C_{24}H_{34}N_4O_7$	−8·42°	4·1	1·06	—	—	—	58·76	6·99	11·42
	47	490·6	0·2 MeOH	60	—	1·00	1·02	—	59·02	7·12	11·34
VIc	77–80	$C_{29}H_{38}N_4O_7$	−38·4°	6·2	1·01	—	—	—	62·80	6·91	10·10
	59	554·7	0·1 DMF	60	—	1·00	—	0·94	63·07	6·84	10·23

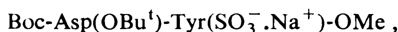
[a] Concentration and solvent; [b] % of methanol; [c] crystallized from ether; [d] on alkaline hydrolysis with $Ba(OH_2)$, only $Tyr(SO_3^-)$ was detected; [e] Pro; [f] crystallized from heptane; [g] His; [h] freeze dried; [i] Nle; [j] $(M\text{-}Boc)^+$; [k] refs[6,7], m.p. 212–213°C, $[\alpha]_D^{20}$ −35·7° (*c* 1·0, DMF); [l] for trifluoroacetate; [m] ref.[7], m.p. 209–210°C, $[\alpha]_D^{20}$ −39·3° (*c* 1·0, DMF).

In our present synthesis of other derivatives of CCK-8 (*Ib*–*If*) we were interested how the catalytic activity of papain is influenced by modification of the amino acid residues at various distance from the reaction site P_1' which in the original sequence in CCK-8 is methionine in position 3, and at which the amino component is acylated with the sulfated dipeptide-ester *II* (for a preliminary communication see ref.[4]). To this end, we first prepared a series of peptides *IVb*–*IVe* and *IVg* (Table I) from

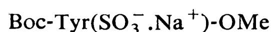


Ia





II



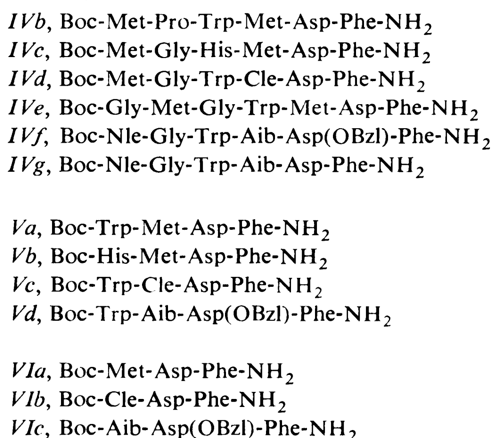
III

which we removed the N^α-tert-butyloxycarbonyl protecting group (Boc) and the corresponding amino components (trifluoroacetates of peptides *IVb*–*IVe* and *IVg*, Table II) were used for the mentioned papain-catalyzed fragment condensation.

In peptides *IVb*–*IVd* the amino acid residues in positions 4,5 or 6 (P_2'–P_4' subsites) were substituted with proline, histidine or cycloleucine, respectively. In the case of the amino component derived from peptide *IVe* the hexapeptide sequence of CCK-8 (3–8) was elongated at the amino terminus by the glycine moiety which replaced the methionine moiety as the P_1' subsite. In the case of the peptide *IVg*, the methionine moieties in position 3 and 6 were replaced by norleucine and 2-amino-isobutyric acid (P_1' or P_4' subsites, respectively).

The peptides *IVb*–*IVe* were prepared in solution by stepwise constructions of the peptide chain starting from the dipeptide amide Asp-Phe-NH_2 (refs[5–7]) which was first acylated with N-hydroxysuccinimide esters of N-tert-butyloxycarbonylmethionine or -cycloleucine. After removal of the amino-protecting tert-butyloxycarbonyl group with trifluoroacetic acid in the presence of ethanedithiol, the obtained tripeptide amides were further acylated with N-hydroxysuccinimide esters of N^α-tert-butyloxycarbonyltryptophane or N^α,N^{im}-di-tert-butyloxycarbonylhistidine. The amino protecting groups were removed by treatment with trifluoroacetic acid containing as scavenger ethanedithiol and indole (which proved to be useful when working with tryptophan unblocked in the side chain) and the corresponding tetrapeptides were subjected to fragment condensation with pentafluorophenyl esters

of N-tert-butyloxycarbonylmethionyl-glycine or N-tert-butyloxycarbonylmethionyl--proline. In the preparation of compound *IVe* the amine protecting group was again removed from the amide of the Boc-hexapeptide and the free hexapeptide amide was acylated with N-hydroxysuccinimide ester of N-tert-butyloxycarbonylglycine. The hexapeptide *IVg* was obtained only using dicyclohexylcarbodiimide with 1--hydroxybenzotriazol as condensation agents and therefore it was necessary to work with β-benzylaspartic acid. The starting β-benzylaspartyl-phenylalanine amide[7] was elongated at the amino terminus by successive treatment with N^{α}-tert-butyloxy-carbonyl derivatives of 2-aminoisobutyric acid, tryptophan and norleucyl-glycine,



IVb, Boc-Met-Pro-Trp-Met-Asp-Phe-NH_2
IVc, Boc-Met-Gly-His-Met-Asp-Phe-NH_2
IVd, Boc-Met-Gly-Trp-Cle-Asp-Phe-NH_2
IVe, Boc-Gly-Met-Gly-Trp-Met-Asp-Phe-NH_2
IVf, Boc-Nle-Gly-Trp-Aib-Asp(OBzl)-Phe-NH_2
IVg, Boc-Nle-Gly-Trp-Aib-Asp-Phe-NH_2

Va, Boc-Trp-Met-Asp-Phe-NH_2
Vb, Boc-His-Met-Asp-Phe-NH_2
Vc, Boc-Trp-Cle-Asp-Phe-NH_2
Vd, Boc-Trp-Aib-Asp(OBzl)-Phe-NH_2

VIa, Boc-Met-Asp-Phe-NH_2
VIb, Boc-Cle-Asp-Phe-NH_2
VIc, Boc-Aib-Asp(OBzl)-Phe-NH_2

TABLE II

Papain-catalyzed reaction of amino components *IVa*–*IVe* and *IVg* with acyl components *II* and *III* leading to peptides *Ia*–*If*

Acyl component	Amino component	Conversion %[a]	Time min[b]	Yield %[c]
II	H-Met-Gly-Trp-Met-Asp-Phe-NH_2	90	20	42
II	H-Met-Pro-Trp-Met-Asp-Phe-NH_2	93	120	45
II	H-Met-Gly-His-Met-Asp-Phe-NH_2	80	15	34
II	H-Met-Gly-Trp-Cle-Asp-Phe-NH_2	80	120	28
II	H-Gly-Met-Gly-Trp-Met-Asp-Phe-NH_2	70	75	19
III	H-Nle-Gly-Trp-Aib-Asp-Phe-NH_2	90	30	43

[a] Conversion of the amino component according to HPLC; [b] optimum time for conversion of the amino component before proteolysis of the products *Ia*–*If* with papain; [c] yield of products *Ia*–*If* after purification with HPLC.

using the already mentioned condensation reagents to give the protected hexapeptide *IVf*. Also during this synthesis the N^{α}-tert-butyloxycarbonyl protecting group was removed in the same manner as described above. The β-benzyl group on the aspartate moiety was finally removed by hydrogenolysis over palladium.

After removal of the Boc protecting group in peptides *IVb*–*IVe* and *IVg* with a mixture of trifluoroacetic acid, ethanedithiol and indole, the corresponding trifluoroacetates were further acylated with the protected dipeptide *II* (trifluoroacetate of *IVg* was reacted with the tyrosine derivative *III*) in the presence of papain under similar conditions as in the synthesis of the parent sequence of CCK-8, i.e. at pH 9 and 30°C. In both the acyl components *II* and *III* the tyrosine hydroxyl had been sulfated prior to the reaction[8].

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. Analytical samples were dried over phosphorus pentoxide at room temperature and 150 Pa. Samples for amino acid analysis were hydrolyzed with 6M-HCl at 110°C for 20 h, tryptophan containing samples were hydrolyzed under the same conditions with 4% thioglycolic acid added. Samples containing O^4-sulfotyrosine were also hydrolyzed with 0·2M-$Ba(OH)_2$ at the same temperaure for 20 h and the barium hydroxide was removed by introduction of gaseous CO_2. The amino acid analyses were performed on an AAA 339 analyzer (Mikrotechna, Czechoslovakia). Mass spectroscopy with FAB technique was used for determination of M^+ of the corresponding peptides (VG Analytical, England). Optical rotations were determined on a Perkin–Elmer 141 MCA polarimeter. For HPLC a Spectra Physics SP 8700 instrument with an SP 8400 UV-detector and an SP 4100 integrator was used. The course of the enzymatically catalyzed reactions was followed by HPLC. The analytical HPLC was carried out on a 15 × 0·4 cm column packed with Separon SIX C-18 (7 μm), flow rate 42 ml/h, detection at 222 nm, mobile phase methanol with 0·05% aqueous trifluoroacetic acid (Table I contains the percentage of methanol and the corresponding values of the capacity factor k'). Preparative HPLC was done on a 25 × 0·8 cm column packed with the same stationary phase, flow rate 180 ml/h, mobile phase a gradient mixture of methanol with 0·05M ammonium acetate buffer pH 6·8. Papain was purchased from Sigma (U.S.A.).

Preparation of Protected Tripeptides *VIa*– *VIb* (Table I)

Aqueous ammonia was added at 40°C to a stirred aqueous suspension of N-benzyloxycarbonyl-aspartyl-phenylalanine amide[5–7] (4·13 g, 10 mmol) until the compound dissolved. The solution was then hydrogenated over freshly prepared mossy palladium (from 8 ml of 10% $PdCl_2$ solution). After removal of the catalyst by filtration, the ammonia was evaporated in vacuo, the solution was freeze-dried and the obtained dipeptide amide was dissolved in dimethylformamide (30 ml). N-Hydroxysuccinimide ester of the N^{α}-tert-butyloxycarbonylamino acid (9·2 mmol) and diisopropylethylamine (1·6 ml, 9·2 mmol) were added and the reaction mixture was stirred at room temperature for 20 h. The dimethylformamide was distilled off, the residue was dissolved in ethyl acetate and this solution was washed three times with saturated solution of potassium hydrogen sulfate and water, dried over sodium sulfate and the solvent was evaporated.

Preparation of Protected Tetrapeptides *Va*— *Vc* (Table I)

The N$^{\alpha}$-tert-butyloxycarbonyl tripeptide amide *VIa* or *VIb* (7 mmol) was dissolved in a mixture of trifluoroacetic acid (5 ml) and ethanedithiol (0·4 ml). After 15 min at room temperature the reaction mixture was evaporated and the residue was triturated with anhydrous ether until it turned into a powder which was then filtered, washed with ether, dried in a desiccator and dissolved in dimethylformamide (30 ml). N-Hydroxysuccinimide ester of the corresponding N$^{\alpha}$-tert-butyloxycarbonylamino acid (6·6 mmol) and diisopropylethylamine (1·2 ml, 7 mmol) were then added at room temperature. After stirring at room temperature for 20 h, the dimethylformamide was evaporated and the obtained oily product solidified on addition of water. The product was washed three times with saturated solution of potassium hydrogen carbonate, water and ether.

Preparation of Protected Hexapeptides *IVb*—*IVd* (Table I)

The N$^{\alpha}$-tert-butyloxycarbonyl tetrapeptide amide (*Va*— *Vc*; 3 mmol) was dissolved in a mixture of trifluoroacetic acid (5 ml), ethanedithiol (0·5 ml) and indole (0·2 g). This reaction mixture was evaporated and the dry corresponding trifluoroacetate was triturated with ether until it turned into a powder, filtered, washed with ether and dried in a desiccator. This product was dissolved in a mixture of dimethylformamide (20 ml) and diisopropylethylamine (0·43 ml, 2·5 mmol), and mixed with a solution of pentafluorophenyl ester of the corresponding tert-butyloxycarbonyl dipeptide (2·5 mmol), prepared by reaction of dicyclohexylcarbodiimide–pentafluorophenol (1 : 3) complex[9,10] (1·9 g, 2·5 mmol) with the corresponding N$^{\alpha}$-tert-butyloxycarbonyl dipeptide (Boc-Met-Gly-OH, Boc-Met-Pro-OH or Boc-Nle-Gly-OH). After stirring at room temperature for 24 h, the protected hexapeptides *IVb*— *IVd* were isolated from the reaction mixture by evaporation of the solvent and threefold washing the residue with saturated solution of potassium hydrogen carbonate, water and ether.

The above-mentioned Boc-dipeptides were prepared by acylation of proline or glycine (3·5 mmol) with N-hydroxysuccinimide esters of N-Boc-derivatives of methionine or norleucine (3 mmol) in dimethylformamide (20 ml) in the presence of diisopropylethylamine (0·51 ml, 3 mmol) for 20 h. The solvent was evaporated, the residue dissolved in ethyl acetate, the solution washed three times with 10% citric acid and water, dried over sodium sulfate and the solvent distilled. The products were used directly for the preparation of the pentafluorophenyl esters.

N-Tert-butyloxycarbonylglycyl-methionyl-glycyl-tryptophyl-
-methionyl-aspartyl-phenylalanine Amide (*IVe*)

N-Hydroxysuccinimide ester of N-tert-butyloxycarbonylglycine (0·11 g, 0·7 mmol) was added to a solution of diisopropylethylamine (0·07 ml) and trifluoroacetate of Met-Gly-Trp-Met-Asp--Phe-NH_2 (ref.[1]) in dimethylformamide (6 ml), prepared by treatment of the corresponding protected peptide[1] (0·36 g, 0·4 mmol) with trifluoroacetic acid (3 ml) in the presence of ethanedithiol (0·3 ml) and indole (0·1 g). The reaction mixture was stirred at room temperature for two days and then worked up using the general procedure described for hexapeptides *IVb*—*Id*. Yields and analytical data are given in Table I.

N-Tert-butyloxycarbonyl-2-aminoisobutyryl-O-benzylaspartyl-phenylalanine Amide (*VIc*)

A mixture of N-tert-butyloxycarbonyl-2-aminoisobutyric acid (0·45 g, 2·2 mmol), dicyclohexylcarbodiimide (0·52 g, 2·5 mmol), diisopropylethylamine (0·39 ml), dimethylaminopyridine (0·27 g, 2·2 mmol), O-benzylaspartyl-phenylalanine amide (prepared from the corresponding

N-benzyloxycarbonyl derivative[7] (1·08 g, 2·3 mmol) by hydrogenation over a palladium catalyst in a mixture of methanol (20 ml) and acetic acid (20 ml) at room temperature) and dimethylformamide (10 ml) was stirred at −20°C for 30 min, at 5°C for 48 h and at room temperature for 48 h. The separated dicyclohexylurea was filtered off, the dimethylformamide was evaporated and the solid residue was washed three times with 5% solution of sodium hydrogen carbonate, 10% citric acid and water. For yield and analytical data see Table I.

N^{α}-Tert-butyloxycarbonyltryptophyl-2-aminoisobutyryl-β-benzylaspartyl-phenylalanine Amide (*Vd*)

N^{α}-Tert-butyloxycarbonyltryptophan (0·25 g, 0·87 mmol), diisopropylethylamine (0·08 ml), 1-hydroxybenzotriazole (0·12 g, 0·89 mmol) and dicyclohexylcarbodiimide (0·18 g, 0·89 mmol) were added to a solution of trifluoroacetate of 2-aminoisobutyryl-β-benzylaspartyl-phenylalanine amide (prepared from the corresponding N-Boc derivative *VIc* (0·28 g, 0·5 mmol) by the general procedure described in the preparation of tetrapeptides *Va*—*Vc*) in dimethylformamide (10 ml). The reaction mixture was stirred at 0°C for 20 h, the separated dicyclohexylurea was filtered and the solvent was evaporated. The dry residue was dissolved in ethyl acetate, the solution washed three times with 5% solution of sodium hydrogen carbonate, 10% solution of citric acid and water, dried over sodium sulfate and the solvent evaporated. For yield and analytical data see Table I.

N-Tert-butyloxycarbonylnorleucyl-glycyl-tryptophyl-2--aminoisobutyryl-β-benzylaspartyl-phenylalanine Amide (*IVf*)

Trifluoroacetate of Trp-Aib-Asp(OBzl)-$PheNH_2$ (prepared from 0·11 g (0·14 mmol) of the corresponding N^{α}-Boc derivative by the procedure described in the preparation of hexapeptides *IVb*—*IVe*) in dimethylformamide (6 ml) was acylated by treatment with N-tert-butyloxycarbonylnorleucyl-glycine (0·12 g, 0·42 mmol), dicyclohexylcarbodiimide (0·09 g, 0·4 mmol), 1-hydroxybenzotriazol (0·06 g, 0·4 mmol) and diisopropylethylamine (0·03 ml) at 0°C for 4 days. The reaction mixture was worked up as described for the tripeptide *VIc* to give the protected hexapeptide *IVf*. For yield and analytical data see Table I.

N-Tert-butyloxycarbonylnorleucyl-glycyl-tryptophyl-2--aminoisobutyryl-aspartyl-phenylalanine Amide (*IVg*)

N-Boc-hexapeptide amide *IVf* (0·2 g, 0·22 mmol) in methanol (15 ml) was hydrogenated in the presence of Pd-black at room temperature. Acetic acid (5 ml) was added after three hours of the hydrogenation. The total hydrogenation time was 4·5 h. The catalyst was filtered through Celite, the solvent evaporated and the crude product recrystallized from aqueous methanol. For yield and analytical data see Table I.

Preparation of Protected Derivatives of CCK-8 *Ib*—*If* (Table I)

Methyl ester of the protected dipeptide *II* or of the tyrosine derivative *III* (0·1 mmol), ethylenediaminetetraacetic acid (0·3 mg) and dithiothreitol (1 mg) were added to a solution of the trifluoroacetate (0·05 mmol, prepared from the corresponding peptide *IVb*—*IVe* or *IVg* by treatment with trifluoroacetic acid (300 μl), ethanedithiol (30 μl) and indole (10 mg) at room temperature for 30 min, evaporation and trituration with ether) in methanol (0·24 ml) and 0·2M carbonate–bicarbonate buffer pH 9·5 (0·96 ml). The solution was kept at pH 9·1 by dropwise addition of 0·5M-NaOH. Papain (0·25 mg) in an aqueous solution (5 μl), containing dithiothreitol, was

added. When the incubation reaction slowed down, another portion of the enzyme was added which increased the reaction rate considerably. The reaction mixture was incubated at 30°C for a time required for maximum conversion of the starting amino component into the desired product *Ib*—*If* before side products began to form (Table II). The reaction was then quenched by addition of methanol (0·3 ml) and rapid cooling, the reaction mixture was filtered through Dowex 50 (1·5 ml) in 50% aqueous methanol, the solution was titrated with 0·1M-NaOH to pH 7, the methanol was evaporated and the aqueous solution was freeze-dried. The peptides *Ib*—*If* were purified by gradient HPLC with the mobile phase 0 to 70% methanol in 0·05M ammonium acetate buffer, pH 6·8. For analytical data see Table I.

RESULTS AND DISCUSSION

From the experimental results obtained in this and previous[1] communication we can conclude that also other peptide sequences with substituted individual amino acid residues in the carboxyterminal hexapeptide of cholecystokinin can be acylated with methyl esters of sulfated tyrosine derivatives in the presence of papain under formation of the peptide bond. In this so-called kinetic approach papain showed only negligible peptidase activity so that practically no peptide bond cleavage was observed. On the other hand, its esterease activity remained fully intact and enabled acylation of the amino components derived from peptides *IVb*—*IVe* and *IVg* with esters *II* and *III* (i.e. with tyrosine derivatives containing a sulfate ester group in the side chain) in relatively high yield (Table II). However, in comparison with the original acylation reaction[1] affording the protected peptide *Ia* we observed quantitative changes in papain capability to catalyze peptide bond formation that were apparent as differences in the total yield of the condensation reactions in question and in the time required for achieving the maximum yield of these reactions (Table II)leading to the protected peptides *Ib*—*If* (Table I). It appeared that the observed differences, monitored by HPLC, are connected not only with the distance of the amino acid substitution from the P'_1 subsite but also with the structure of the side chain of the substituting amino acid in the amino components derived from peptides *IVb*—*IVe* and *IVg*. In accord with our considerations, the fragment condensation was slower when proline was present in the P'_2 subsite in peptide *IVb* whereas a more distant substitution with histidine moiety (P'_3 subsite) in peptide *IVc* lowered the reaction yield only negligibly and the reaction time was the same as in the case of the amino component *IVa*. Surprisingly, the cycloleucine moiety in the still more distant position (P'_4 subsite, peptide *IVd*) from the P'_1 reaction subsite decreased the conversion into the octapeptide similarly as did the substitution in the peptide *IVb*. Although the cycloleucine moiety is considerably far from the synthesized peptide bond, its presence probably leads to such conformation of the peptide fragment *IVd* in which an optimum interaction of the peptide with the enzyme and formation of an enzyme–-substrate complex is unfavourable. On the other hand, in the papain-catalyzed conversion to derivative *If* there was no marked influence on the reaction rate of the

amino component derived from the hexapeptide *IVg* in which the norleucine moiety in the P_1' subsite is highly isosteric with the methionine residue and the further substituent in this peptide – 2-aminoisobutyric acid in the P_4' subsite – does not represent any steric hindrance to the formation of the required interactive conformation of this amino component. The slow conversion of the amino component derived from peptide *IVe* with elongated amino acid sequence into the protected peptide *Ie* can be explained by absence of a hydrophobic side chain in the "new" P_1' subsite which in this peptide is occupied by another glycine moiety. Moreover, prolonged reaction time of this condensation led to digestion of peptide bonds in the arising nonapeptide *Ie* instead of increase of yield.

The results obtained in this study may serve as further example of utilization of enzymes in the peptide synthesis, particularly in special cases where the "chemical" approach is accompanied by difficulties. In the described case of relatively difficult preparation of peptides containing an acid-labile sulfate ester group attached to tyrosine, we have proven on several examples the effective use of papain in an alkaline medium for catalysis of peptide bond formation between various types of amino components on the one hand and tyrosine derivatives with sulfated hydroxyl in the side chain on the other hand. Biological activities of the peptides *Ib*–*If* and their deprotected derivatives will be published in a separate communication.

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