
SYNTHESIS OF THE CARBOXY-TERMINAL OCTAPEPTIDE OF CHOLECYSTOKININ (CCK-8) BASED ON INCORPORATION OF O⁴-SULFOTYROSINE BY ENZYMATICALLY CATALYZED FORMATION OF PEPTIDE BONDS*

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Papain-catalyzed condensation of sodium salt of tert-butyloxycarbonyl- β -tert-butyloxyaspartyl-O⁴-sulfotyrosine (fragment 1-2) with methionyl-glycyl-tryptophyl-methionyl-aspartyl-phenylalanine amide (fragment 3-8) has been elaborated. Deprotection of the thus-obtained octapeptide afforded CCK-8 which exhibited full biological activities. Benzyloxycarbonylaspartyl-phenylalanine amide (fragment 7-8) was prepared using thermolysin without protecting the aspartic acid side chain. Attempted condensation of tert-butyloxycarbonylmethionyl-glycyl-tryptophan (fragment 3-5) with methionyl-aspartyl-phenylalanine amide (fragment 6-8), catalyzed by α -chymotrypsin, subtilisin or proteinase K, afforded the product (fragment 3-8) in only low yields. Further use of proteolytic enzymes for preparing other peptide fragments of the CCK-8 molecule without side chain protection is investigated.

Recently, the properties and synthesis of the C-terminal cholecystokinin octapeptide** CCK-8 (I) and its analogues have attracted the interest of peptide chemists. These compounds are prepared by the classical solution synthesis (for a review see ref.³) as well as by the solid phase method⁴. In most cases, the last or the last but one synthetic step involves sulfation of the tyrosine hydroxyl with various reagents³. This step is the most critical one because of possible destruction or undesired sulfation and sulfonation of the side chains of some amino acids. An alternative method based on 2 + 6 fragment condensation in which the protected tyrosine-containing dipeptide fragment is sulfated prior to the reaction³ has found a less frequent use. Another synthesis of CCK-8 consists in preparation of the protected peptide fragments (1-4) and (5-8) using proteolytic enzymes (with both side chains of aspartic acid protected), their chemical coupling and sulfation of the protected octapeptide in the usual manner⁵.

* Part CCIV in the series Amino Acids and Peptides; Part CCIII: Collect. Czech. Chem. Commun. 53, 145 (1988).

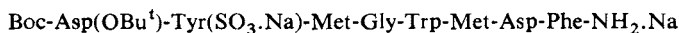
** The nomenclature and symbols of amino acids, peptides and protecting groups obey the published recommendations^{1,2}. The amino acids used in this work are of the L-configuration.

In our previous communication⁶ we have found that papain or thermolysin are capable of synthesizing the peptide bond between O⁴-sulfotyrosine and another amino acid of sufficient affinity to the S₁' subsite of these enzymes. In the present study we investigate the enzymatically catalyzed synthesis of protected sulfated octapeptide *II* by 2 + 6 fragment condensation starting from sulfated dipeptide *III* and hexapeptide *IVb* in order to avoid sulfation of the octapeptide in the last step. As shown by a blank experiment, thermolysin cleaves some peptide bonds in the hexapeptide *IVb* under conditions used for the synthesis of the octapeptide *II*. For this reason, we have chosen papain which is known to possess only negligible peptidase activity in the alkaline pH area whereas its esterase activity remains intact. Papain-catalyzed syntheses of peptide bonds in alkaline media via esters (the so-called kinetic approach) have been used in the synthesis of enkefalin⁷ and shorter peptides⁸. No undesired cleavage of peptide bonds have been reported. Previously⁶, we used this method in the synthesis of potassium salt of a model dipeptide tert-butylloxycarbonyl-O⁴-sulfotyrosyl-leucine α -phenylhydrazide starting from sodium salt of tert-butylloxycarbonyl-O⁴-sulfotyrosine α -methyl ester. Now, we have also proven (using HPLC) that at pH 9 the papain-catalyzed hydrolysis of sodium salt of tert-butylloxycarbonyl-O⁴-sulfotyrosine α -methyl ester is approximately equal to that of tert-butylloxycarbonyltyrosine α -methyl ester under the same reaction conditions (about 50% hydrolysis/30 min): thus the enzyme shows an approximately equal specificity for O⁴-sulfotyrosine as for tyrosine. We have also verified that at pH 9 papain cleaves neither any peptide bond in the hexapeptide *IVb* nor its C-terminal amide group. However, it is very surprising that, under the same conditions, this enzyme cleaved almost completely the glycine-tryptophan peptide bond in the protected hexapeptide *IVa* within 15 minutes (as found by HPLC; the cleavage product had the same retention time as the tetrapeptide *IXb*). The dipeptide *III* was easily prepared by sulfation of the dipeptide *V* with pyridinium acetylsulfate⁹. The octapeptide *II* was synthesized by 2 + 6 fragment condensation using a two-fold molar amount of dipeptide *III* relative to the hexapeptide *IVb* and 20 times lower concentration of papain than in the previous experiments^{7,8}. Under these conditions, HPLC has shown a 90% conversion of the hexapeptide *IVb* into the octapeptide *II* during 10 to 20 minutes. Simultaneously, the octapeptide *II* was transformed to the extent of 15% into an unidentified side product. Isolation of *II* by preparative HPLC was accompanied by some loss of the material. The protecting groups in the octapeptide *II* were removed with trifluoroacetic acid according to the literature³. The thus-obtained CCK-8 (*I*) was tested for its ability to contract guinea-pig gall-bladder in vitro and for its sedative, analgetic and anorectic activity. In all these tests the found activities were identical with those obtained with a standard sample of CCK-8.

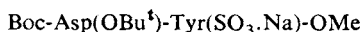
It was intended to base the synthesis of the hexapeptide *IVa* on the enzymatically catalyzed condensation of the tripeptide *VIa* and tripeptide *VIIb* (3 + 3). However,



I



II

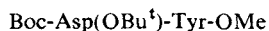


III

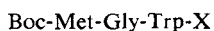


IVa, X = Boc

IVb, X = H

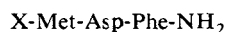


V



VIa, X = OMe

VIb, X = OH



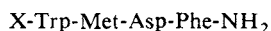
VIIa, X = Boc

VIIb, X = H



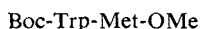
VIIIa, X = OMe

VIIIb, X = OH

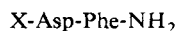


IXa, X = Boc

IXb, X = H



X



XIa, X = Z

XIb, X = H

the synthetic reaction with α -chymotrypsin (at pH 10), subtilisin (at pH 9) or proteinase K (at pH 10) proceeded maximally only to the extent of 15% (the synthesis with papain at pH 9 failed completely because of surprisingly fast enzymatic cleavage of the glycine-tryptophan peptide bond in the tripeptide *VIa*). These reactions were studied in an aqueous buffer, containing dimethylformamide, or in two-phase systems (aqueous buffer – ethyl acetate) or only in a mixture of organic solvents (1,4-butanediol–ethyl acetate 1 : 1). In all these cases the very rapid enzymatically catalyzed hydrolysis of the α -methyl ester *VIa* invariably predominated and the formed tripeptide *VIb* did not react further. In the 1,4-butanediol – ethyl acetate mixture we also observed transesterification of the tripeptide *VIa* leading to 4-hydroxybutyl ester of tripeptide *VIb*, catalyzed by α -chymotrypsin in the presence of 0.5% of water. Similar observations have been already published¹⁰. The mentioned ester was further very rapidly transformed into the tripeptide *VIb*. The failure of this attempted enzymatically catalyzed condensation is obviously due to the presence of a negatively charged side chain of aspartic acid, corresponding to the P_2' subsite of the nucleophile *VIIIb*. The enzymatically catalyzed hydrolysis of the methyl ester *VIa* with α -chymotrypsin was utilized for the preparation of tripeptide *VIb* under mild reaction conditions. The peptide *VIb* was then condensed chemically with the tripeptide *VIIIb* using pentafluorophenol–dicyclohexylcarbodiimide “complex”. The synthesis of the hexapeptide *IVa* by other methods is described elsewhere^{11,12}. An attempted papain-catalyzed condensation (2 + 4) of dipeptide *VIIIa*

(ref.¹¹) with tetrapeptide *IXb* (refs^{13,14}) in an alkaline medium was also unsuccessful. As shown by HPLC monitoring, the formed hexapeptide *IVa*, soluble in the reaction medium, underwent rapid enzymatic cleavage.

The tripeptide *VIa* was prepared from dipeptide *VIIIb* (ref.¹¹) in the chemical way. Since attempts to acylate the dipeptide *XIb* with tert-butyloxycarbonylmethionine in the presence of papain failed, the tripeptide *VIIa* was prepared from the dipeptide *XIb* using a described chemical procedure^{13,14}.

Dipeptide *XIa* was synthesized in high yield by thermolysin-catalysed condensation of benzyloxycarbonylaspartic acid and phenylalanine amide hydrochloride, similarly as in the synthesis of protected aspartam¹⁵. The product *XIa* precipitated from the reaction mixture (containing a twofold molar amount of phenylalanine amide hydrochloride) as the salt *Z-Asp-Phe-NH₂.Phe-NH₂* the formation of which enabled a rapid shift of the chemical equilibrium in the direction of synthesis. On washing, this salt afforded directly the dipeptide *XIa*.

The attempted preparation of tetrapeptide *IXa*, based on papain-catalysed acylation of dipeptide *XIb* (ref.¹³) with dipeptide *X* (ref.¹³) in an alkaline medium, was unsuccessful. HPLC analysis revealed only a rapid enzymatic cleavage of the dipeptide methyl ester *X* (about 50%/5 min) instead of synthesis of the tetrapeptide *IXa*. This result is at variance with the report⁸ according to which in alkaline pH area papain acylates also such nucleophiles that have acidic amino acids in the P₁' position. The tetrapeptide *IXa* was prepared by a described^{13,14} chemical procedure. We have shown that at pH 9 papain cleaves the tryptophan-methionine peptide bond in this compound.

Our study has shown that in alkaline medium papain can be used for the synthesis of peptide bond between the O⁴-sulfotyrosine containing protected dipeptide fragment (1-2) and the remaining part of the CCK-8 molecule (fragment 3-8). However, several examples have proven that even in the alkaline pH area papain retains a considerable ability to cleave peptide bonds in some peptide substrates (peptides *IVa*, *VIa* and *IXa*), probably as a result of presence of other proteases in the commercial samples of papain. Therefore, the principle of papain-catalyzed peptide synthesis in the alkaline area via the esters^{7,8} cannot be taken as general. The use of other proteolytic enzymes in the synthesis of CCK-8 and its analogues may facilitate some steps.

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. Thin-layer chromatography (TLC) was performed on silica gel-coated plates (Silufol, Kavalier, Czechoslovakia) in the following systems: 2-butanol-98% formic acid-water (75 : 13.5 : 11.5) (S1), 2-butanol-25% aqueous ammonia-water (85 : 7.5 : 7.5) (S2), 1-butanol-acetic acid-water (4 : 1 : 1) (S3), 1-butanol-pyridine-acetic acid-water (15 : 10 : 3 : 6) (S4). Electrophoreses were carried out in moist

chamber on a Whatman 3MM paper (20 V/cm) in 1M acetic acid (pH 2.4) and in a pyridine-acetate buffer (pH 5.7). Detection was performed with ninhydrine or by the chlorination method. Samples for amino acid analysis were hydrolyzed by 6M-HCl at 110°C for 20 h, tryptophan-containing samples were hydrolyzed under the same conditions with 4% thioglycolic acid added. Samples containing O⁴-sulfotyrosine were also hydrolyzed with 0.2M-Ba(OH)₂ at the same temperature for 20 h and the barium hydroxide was removed by introduction of gaseous CO₂. The amino acid analyses were performed on an AAA 339 analyzer (Mikrotechna, Czechoslovakia). Optical rotations were determined on a Perkin-Elmer 141 MCA polarimeter. For HPLC a Spectra Physics SP 8 700 instrument with an SP 8 400 UV-detector and an SP 4 100 integrator was used. 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. Values of *k'* were determined with a mobile phase containing 65% of methanol. 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 mixture of methanol with 0.05M ammonium acetate buffer pH 6.5. The tert-butyloxycarbonyl group was removed with a mixture of trifluoroacetic acid (90%), water (9%), thioglycolic acid (0.5%), and dithiothreitol (0.5%) as described in ref.³ The course of the enzymatically catalyzed reactions was followed by HPLC. Papain was purchased from Sigma (U.S.A.), thermolysin, α-chymotrypsin and proteinase K from Serva (F.R.G.) and subtilisin from Novo (DK). The CCK-8 sample was a Cambridge Research Biochemicals (Great Britain) product.

Tert-butyloxycarbonyl-β-tert-butyloxyaspartyl-tyrosine α-Methyl Ester (V)

Dicyclohexylcarbodiimide (230 mg; 1.1 mmol) was added to a solution of β-tert-butyl tert-butyloxycarbonylaspartate (290 mg; 1 mmol) and hydroxybenzotriazole (150 mg; 1.1 mmol) in dimethylformamide (2 ml), pre-cooled below -10°C. The mixture was stirred at -10°C to -5°C for 1 h. After removal of dicyclohexylurea the solution was added to a solution of tyrosine methyl ester hydrochloride (260 mg; 1.1 mmol) in dimethylformamide (4 ml) which had been made alkaline with N-ethylpiperidine. The mixture was stirred at -10°C to -5°C for 1 h and then at room temperature overnight. Dimethylformamide was evaporated and the residue dissolved in ethyl acetate. After filtration, the ethyl acetate solution was washed successively with an HSO₄⁻ buffer (pH 2), water, 5% NaHCO₃, water, dried over sodium sulfate and taken down. Crystallization of the residue from ether-light petroleum afforded 395 mg (85%) of the product, m.p. 55–57°C. *R_F* 0.82 (S1), 0.75 (S2), 0.78 (S3), 0.80 (S4); *k'* = 2.56. [α]_D -6.8° (c 0.3, methanol). Amino acid analysis: Asp 1.00, Tyr 0.97. For C₂₃H₃₄N₂O₈ (466.5) calculated: 59.21% C, 7.35% H, 6.01% N; found: 59.64% C, 7.54% H, 5.93% N.

Sodium Salt of Tert-butyloxycarbonyl-β-tert-butyloxyaspartyl-O⁴-sulfotyrosine α-Methyl Ester (III)

Pyridinium acetyl sulfate (440 mg; 2 mmol) was added to a solution of tert-butyloxycarbonyl-β-tert-butyloxyaspartyl-tyrosine α-methyl ester (240 mg; 0.5 mmol) in a mixture of dimethylformamide (2.5 ml) and pyridine (2.5 ml). After incubation at 40°C for 20 h the solution was taken down and the residue was dissolved in 0.1M-NaOH (35 ml). The solution was adjusted to pH 7 with 0.1M-NaOH, washed with ethyl acetate and freeze-dried. The lyophilisate was triturated with methanol, filtered and the solvent was evaporated. The residue was triturated with dioxane, the insoluble material was filtered off, the filtrate was evaporated and the residue was crystallized from ether, affording 245 mg (84%) of product, m.p. 134–136°C. *R_F* 0.55 (S1), 0.39 (S2), 0.54 (S3), 0.63 (S4), *k'* = 0.40; [α]_D -3.6° (c 0.8, methanol). Amino acid analysis after alkaline

hydrolysis: Tyr(SO₃⁻) 1.20, Asp 1.00, no traces of Tyr. For C₂₃H₃₃N₂NaO₁₁S.0.5 H₂O (577.6) calculated: 47.82% C, 5.93% H, 4.85% N; found: 47.91% C, 5.36% H, 4.69% N.

Benzyloxycarbonylaspartyl-phenylalanine Amide (XIa)

Calcium chloride (5 mg) and thermolysin (5 mg) were added to a solution of phenylalanine amide hydrochloride (900 mg; 4.5 mmol) and benzyloxycarbonylaspartic acid (535 mg; 2 mmol) in 0.4M-NaOH (10 ml) which had been adjusted to pH 7.0 with 4M-NaOH. The mixture was incubated at 38°C for 20 h. The separated precipitate was washed with 1M-HCl and water and dried. Washing with light petroleum afforded 690 mg (83%) of product, m.p. 209–211°C. *R_F* 0.81 (S1), 0.15 (S2), 0.76 (S3), 0.56 (S4), *k'* = 1.00; [α]_D -40.2° (c 0.5, dimethylformamide). Amino acid analysis: Asp 1.00, Phe 1.05. For C₂₁H₂₃N₃O₆ (413.4) calculated: 61.01% C, 5.61% H, 10.16% N; found: 60.78% C, 5.55% H, 10.23% N. Reported¹⁶ m.p. 214–216°C and [α]_D -36.6° (c 1, dimethylformamide).

Tert-butyloxycarbonylmethionyl-glycyl-tryptophan Methyl Ester (VIa)

Hydroxybenzotriazole (0.9 g; 6.6 mmol) was added to a solution of tert-butyloxycarbonylmethionyl-glycine (1.84 g; 6 mmol) and tryptophan methyl ester hydrochloride (1.68 g; 6.6 mmol) in dimethylformamide (35 ml) and the mixture was cooled to below -10°C. The solution was made alkaline with N-ethylpiperidine and dicyclohexylcarbodiimide (1.36 g; 6.6 mmol) was added. After stirring at -10°C to -5°C for 1 h and at room temperature overnight the precipitated dicyclohexylurea was removed, dimethylformamide evaporated and the residue dissolved in ethyl acetate. The organic solution was washed successively with an HSO₄⁻ buffer pH 2, water, 5% NaHCO₃, water, dried over sodium sulfate and the solvent was evaporated. The residue was crystallized from ether-light petroleum to give 2.54 g (83%) of the product, m.p. 72–74°C. *R_F* 0.81 (S1), 0.70 (S2), 0.78 (S3), 0.79 (S4), *k'* = 3.05; [α]_D +2.6° (c 0.3, dimethylformamide). Amino acid analysis: Gly 1.06, Met 0.88, Trp 1.06. For C₂₄H₃₄N₄O₆S (506.6) calculated: 56.90% C, 6.76% H, 11.06% N; found: 57.50% C, 7.01%, 10.86% N.

Tert-butyloxycarbonylmethionyl-glycyl-tryptophan (VIb)

α-Chymotrypsin (10 mg) was added to a solution of tert-butyloxycarbonylmethionyl-glycyl-tryptophan methyl ester (510 mg; 1 mmol) in a mixture of methanol (10 ml) and 0.2M carbonate-bicarbonate buffer pH 9 (10 ml) and the mixture was stirred at room temperature for 2 h (after 1 h another portion (10 mg) of the enzyme was added). Methanol was evaporated, the aqueous solution was acidified with cold 0.1M-HCl (20 ml) and the product was taken up in ethyl acetate (when the product, precipitated after acidification, was isolated by filtration and washing with water, the yield was lower). The organic solution was washed with water, dried over sodium sulfate and the solvent evaporated. The residue was crystallized from ether-light petroleum to afford 390 mg (79%) of product, m.p. 103–105°C. On recrystallization of a sample from ether the melting point rose to 149–151°C. *R_F* 0.83 (S1), 0.32 (S2), 0.79 (S3), 0.67 (S4), *k'* = 1.86; [α]_D +2.2° (c 0.3, dimethylformamide). Amino acid analysis: Gly 1.05, Met 1.01, Trp 0.94. I or C₂₃H₃₂N₄O₆S (492.6) calculated: 56.08% C, 6.54% H, 11.37% N; found: 55.52% C, 6.45% H, 11.30% N.

Tert-butyloxycarbonylmethionyl-glycyl-tryptophyl-methionyl-aspartyl-phenylalanine Amide (IVa)

"Complex" of pentafluorophenol with dicyclohexylcarbodiimide (420 mg; 0.55 mmol) was added to a solution of tert-butyloxycarbonylmethionyl-tryptophan (247 mg; 0.5 mmol) in dimethyl-

formamide (2 ml) and the mixture was stirred at room temperature for 4 h. After removal of dicyclohexylurea the solution was added to a suspension of methionyl-aspartyl-phenylalanine amide (206 mg; 0.5 mmol) ($k' = 0.38$, $E_{\text{Gly}}^{2,4} = 0.88$, $E_{\text{His}}^{5,7} = 0.0$) in dimethylformamide (6 ml) and the mixture was stirred at room temperature for 2 h (the mixture became homogeneous after 50 min). Dimethylformamide was evaporated, the residue was triturated with ether, washed with ether, ethyl acetate, an HSO_4^- buffer pH 2, water, and dried. Crystallization from methanol-ether afforded 330 mg (75%) of the product, m.p. 187–188°C. R_F 0.86 (S1), 0.40 (S2), 0.78 (S3), 0.70 (S4), $k' = 5.15$; $[\alpha]_{\text{D}} -31.1^\circ$ (c 0.3; dimethylformamide). Amino acid analysis: Asp 1.09, Gly 0.98, Met 1.94, Phe 0.98, Trp 0.84. For $\text{C}_{41}\text{H}_{56}\text{N}_8\text{O}_{10}\text{S}_2 \cdot \text{H}_2\text{O}$ (903.1) calculated: 54.52% C, 6.47% H, 12.41% N; found: 54.59% C, 6.26% H, 12.53% N. Reported^{11,12} m.p. 184–185°C, $[\alpha]_{\text{D}} -19.7^\circ$ (c 1.4, dimethylformamide) and m.p. 200–201°C, $[\alpha]_{\text{D}} -30.3^\circ$ (c 2, dimethylformamide), respectively.

Disodium Salt of Tert-butyloxycarbonyl- β -tert-butyloxyaspartyl-

-O⁴-sulfotyrosyl-methionyl-glycyl-tryptophyl-methionyl-aspartyl-phenylalanine Amide (II)

Ethylenediaminetetraacetic acid (0.6 mg) and dithiothreitol (1 mg) were added to a solution of sodium salt of tert-butyloxycarbonyl- β -tert-butyloxyaspartyl-O⁴-sulfotyrosine methyl ester (114 mg; 0.2 mmol) and methionyl-glycyl-tryptophyl-methionyl-aspartyl-phenylalanine amide trifluoroacetate (90 mg; 0.1 mmol) ($k' = 1.23$, $E_{\text{Gly}}^{2,4} = 0.58$, $E_{\text{His}}^{5,7} = 0.0$) in a mixture of methanol (0.4 ml) and 0.2M carbonate-bicarbonate buffer, pH 9.5 (1.6 ml) and the solution was adjusted to pH 9.1 with 0.5M-NaOH. Papain (0.5 mg) in water (10 μl), containing 1 mmol l⁻¹ ethylenediaminetetraacetic acid and dithiothreitol, was added. After incubation at 30°C for 20 min the reaction was quenched by addition of methanol (1.2 ml) and rapid cooling. The mixture was filtered through a column of Dowex 50 (4 ml) in 50% aqueous methanol, the filtrate was neutralized to pH 7 with 0.1M-NaOH and freeze-dried, affording 165 mg of material from which the octapeptide II was isolated by preparative HPLC. The fraction, containing compound II, was taken down, freeze-dried and triturated with ether to afford 57 mg (42%) of the product, m.p. 188–190°C. R_F 0.77 (S1), 0.36 (S2), 0.71 (S3), 0.68 (S4), $k' = 3.50$, $[\alpha]_{\text{D}} -25.3^\circ$ (c 0.4; dimethylformamide). Amino acid analysis: Asp 2.05, Gly 0.97, Met 1.84, Tyr 0.93, Phe 1.00, Trp 0.96, after alkaline hydrolysis: Tyr(SO₃⁻) 0.93, Gly 1.00, no traces of Tyr. For $\text{C}_{58}\text{H}_{76}\text{O}_{18}\text{N}_{10}\text{Na}_2\text{O}_{18}\text{S}_3 \cdot 3\text{H}_2\text{O}$ (1398) calculated: 49.82% C, 5.91% H, 10.02% N; found: 49.28% C, 5.88% H, 10.65% N. Reported³ m.p. 215–217°C, $[\alpha]_{\text{D}} -22.5^\circ$ (c 1, dimethylformamide) (for unpurified product).

CCK-8 (I)

Octapeptide II (10 mg) was dissolved in a mixture of trifluoroacetic acid (180 μl), water (20 μl), thioglycolic acid (2 μl), and dithiothreitol (2 mg) at 0°C. After standing at 0°C under nitrogen for 3 h, the product was precipitated with ether and purified by preparative HPLC; yield 2.9 mg (34%) of a lyophilisate that was chromatographically and electrophoretically identical with a commercial sample of CCK-8 (R_F 0.47 (S1), 0.07 (S2), 0.49 (S3), 0.54 (S4), $k' = 0.52$, $E_{\text{Gly}}^{2,4} = 0.00$, $E_{\text{Glu}}^{5,7} = 0.64$) and exhibited the same biological properties.

Biological Activity Assays

The ability to stimulate guinea-pig gall-bladder contractions was tested according to ref.¹⁷, the sedative and analgetic activity was determined as described^{18,19} and the anorectic effect was tested in an assay elaborated by us²⁰ and based on the amount of solid food taken by five-

-membered groups of starving (18 h) mice during the given time interval (40 min) after i.p. application of the peptide (or physiological solution as control).

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