ANALOGUES OF THE CHOLECYSTOKININ OCTAPEPTIDE MODIFIED IN THE CENTRAL PART OF THE MOLECULE*

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In a series of analogues of the cholecystokinin octapeptide (CCK-8) the amino acid residues were gradually modified by substituting Gly by Pro in position 4, Trp by His in position 5, Met by Cle in position 6, or the Gly residue was inserted between Tyr and Met in positions 2 and 3 of the peptide chain, and in the case of the cholecystokinin heptapeptide (CCK-7) the Met residues were substituted by Nle or Aib. These peptides were investigated from the point of view of their biological potency in the peripheral and central region. From the results of the biological tests it follows that the modifications carried out in these analogues and in their N^{α}-Boc derivatives mean a suppression of the investigated biological activities by 2—3 orders of magnitude (at a maximum dose of the tested substance of $2 . 10^{-2}$ mg per animal). This means that a disturbance of the assumed biologically active conformation of CCK-8, connected with a considerable decrease of the biological potency of the molecule, takes place not only after introduction of the side chain into its centre (substitution of Gly^4), but also after the modification of the side chains of the amino acids or by extension of the backbone in further positions around this central amino acid.

Cholecystokinin (CCK) is a linear peptide hormone isolated for the first time from gastrointestinal tract of the hog, stimulating the contraction of the gall bladder and pancreatic secretion (for review see[1,2]). Among other physiological effects mainly its function in the control of food intake should be mentioned[3]. A number of cholecystokinin sequences of various lengths was also found later in the brain. Among them CCK-8** (*Ia*) occurs most frequently. This is a carboxy terminal octapeptide of the original sequence of 33 amino acids, which has an equally broad spectrum of biological effects as the intact hormone in the peripheral region and as a neurotransmitter in the central nervous system[5-10]. Therefore the structural

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** The nomenclature and the symbols of amino acids and peptides obey the published recommendations[4]. In addition to the common symbols we use the following symbols: Cle 1-aminocyclopentanecarboxylic acid (cycloleucine), Aib 2-aminoisobutyric acid. The amino acids used in this study are of the L-configuration.

modifications of CCK-8 in various parts of the molecule may be interesting from the point of view of their influence on the spectrum of biological activities in consequence of the change of the assumed conformation of the molecule[11–14]. From the proposed model of the biologically active conformation of CCK-8 it followed that the changes of the amino acid residues in the central part of the molecule may have the greatest effect on its agonistic effects, either in the sense of an absolute decrease of the potency of the peptide or the dissociation of the effects. This was confirmed, for example, by the studies in which the glycine residue was substituted by an amino acid with a side chain or with a methylated amino group[15].

In this paper we focussed our attention just on this part of the CCK-8 molecule and investigated the biological properties of its selected analogues, prepared by fragment condensation, catalysed with papain[16]. When substituting the glycine residue in position 4 by proline (*Ib*) we also started from the results of the conformational analysis of proteins, according to which these two amino acids change the helical arrangement of the basic peptide chain. We were further interested in finding how some biological properties of CCK-8 would be affected by exchanging the tryptophan side chain in position 5 for the more basic, but less sterically demanding histidine chain (*Ic*). In the case of the third analogue the methionine residue in position 6, was substituted by cycloleucine (*Id*) and in the fourth by 2-aminoisobutyric acid under simultaneous substitution of Met in position 3 by the isosteric norleucine (*Ie*). We wanted to determine the tolerance of the CCK receptors to the change of the length and the orientation of the side chain in position 6, caused by its shortening and by the increase of its steric volume in the immediate vicinity of the basic peptide chain. In the last analogue the residue of aspartic acid was also omitted at its amino terminus, so that it may in fact be considered an analogue of CCK-7. On the other hand the prolonged analogue *If* was investigated, in which we had inserted a glycine residue between tyrosine and methionine in positions 2 and 3. In addition to the above mentioned analogues their partially protected intermediates *IIb*–*IIf* were also tested, derived from the basic sequence *IIa*, the synthesis of which has been described in a preceding paper[16].

Ia: H-Asp—Tyr(SO_3H)—Met-Gly-Trp-Met-Asp-Phe-NH_2

$$\overset{1}{\text{Boc-Asp}}(\text{OBu}^t)\text{-}\overset{2}{\text{Tyr}}(SO_3^-.Na^+)\text{-}\overset{3}{\text{Met}}\text{-}\overset{4}{\text{Gly}}\text{-}\overset{5}{\text{Trp}}\text{-}\overset{6}{\text{Met}}\text{-}\overset{7}{\text{Asp}}\text{-}\overset{8}{\text{Phe}}\text{-NH}_2$$

Ib, *IIb*: [Pro^4], *Ic*, *IIc*: [His^5], *Id*, *IId*: [Cle^6]

Ie, *IIe*: [des-Asp^1, Nle^3, Aib^6], *If*, *IIf*: [endo-Gly^{2a}]

The analogues *Ib*–*If* were prepared from compounds *IIb*–*IIf*. Their N-tert-butyloxycarbonyl and β-tert-butyl ester protecting groups were split off in an inert

atmosphere at low temperature under the effect of aqueous trifluoroacetic acid in the presence of the scavengers, thioglycolic acid and dithiothreitol.

The analogues *Ib* and *Ic* were purified by preparative HPLC and the analogues *Id*—*If* by means of preparative paper electrophoresis which afforded homogeneous products, as shown by their subsequent HPLC analysis. The biological activity of peptides *Id*—*If* and *IIb*—*IIf* was assayed in anorectic, analgetic, sedative and guinea pig gall bladder tests.

EXPERIMENTAL

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), 1-butanol–acetic acid–water (4 : 1 : 1) (S2), 1-butanol–pyridine–acetic acid–water (15 : 10 : : 3 : 6) (S3). Samples for amino acid analysis were hydrolyzed by 6M-HCl at 110°C for 20 h. Trp containing samples were hydrolyzed under the same conditions with 4% thioglycolic acid added. Samples containing O^4-sulfotyrosine were hydrolyzed with 0·2M-$Ba(OH)_2$ at the same temperature for 20 h and the barium hydroxide was removed by introduction of gaseous CO_2. The amino acid analyses were carried out on a Durrum D-500 amino acid analyzer (Durrum Instrum. Corp., Palo Alto, U.S.A.). Mass spectrometry with FAB or SIMS technique was used for determination of M^+ of the corresponding peptides (VG Analytical, England). For HPLC either a Spectra Physics SP 8700 instrument with an SP 8400 UV detector and an SP 4100 integrator or a Jasco HPLC pump BIP-I with a Biotronik UV Detector BT 3035 (Japan Spectroscopic Co., Ltd.) were used. The analytical HPLC was carried out on a 15 × 0·4 cm column packed with Separon SIX C-18 (7 μm) or on a Vydac column of the same size (The Separations Group, Hesperia, U.S.A.), flow rate 42 ml/h, detection at 222 nm, mobile phase methanol or acetonitrile with 0·05% aqueous trifluoroacetic acid. Preparative HPLC was done on a 25 × 0·8 cm column packed with the same stationary phases, flow rate 180 ml/h, mobile phase a mixture of methanol with 0·05M ammonium acetate buffer pH 6·7. Preparative electrophoresis was carried out in a moist chamber on a Whatman 3MM paper (20 V/cm) in a pyridine–acetate buffer pH 6·7 for 60 min. Detection of peptides on paper strips was carried out with ninhydrin. On the basis of this detection the peptide was eluted from the ninhydrin non-sprayed part of the paper by 0·05M ammonium acetate buffer pH 6·7 and the solution was freeze dried. The synthesis and physical properties of the peptides *IIb*—*IIf* are described elsewhere[16].

The Splitting Off of the N-Tert-butyloxycarbonyl and β-Tert-butyl Ester Protecting Groups in Protected Peptides *IIb*—*IIf*

The protected peptide (0·1 mmol) was slowly stirred at 0°C under nitrogen or argon for 4 h with a mixture of trifluoroacetic acid (2·6 ml), water (120 μl), thioglycolic acid (24 μl) and dithiothreitol (24 mg). After deprotection of peptides *IIb* and *IIc* ether was added to the reaction mixture and the precipitated trifluoroacetate was separated by filtration. After dissolution in 0·05M ammonium acetate buffer of pH 6·7 it was purified by HPLC. When deprotection of much smaller amounts of protected peptides *IId*—*IIf* was carried out, the reaction mixture was diluted with 0·05M ammonium acetate buffer after 4 h and the solution lyophilized and the product purified by preparative paper electrophoresis.

Purification of analogues Ib and Ic (*Table* I): After elimination of protecting groups the analogues *Ib* and *Ic* were dissolved in 0·05M ammonium acetate buffer and purified by preparative HPLC, using the following gradient: 0·05M ammonium acetate of pH 6·7 – 25% MeOH (10 min), 50% MeOH (30 min), 75% MeOH (10 min), detection at 254 and 280 nm. The required peptides were eluted approximately 5 min after attaining a 50% MeOH concentration in the mobile phase.

Purification of analogues Id—If (*Table* I): The lyophilizate of the crude peptide (*Id—If*) was dissolved in a mixture of water and methanol 1 : 1 and the solution was applied onto the central line of a sheet of Whatman 3MM paper (30 × 11 cm) along its whole width. Electrophoresis was then carried out as in the above section. The strip of paper containing the required peptide (*Id—If*) was extracted with 0·05M ammonium acetate buffer pH 6·7 and the extract was lyophilized.

Tests of Biological Activity

Guinea pig gall bladder test according to[18]: the guinea pig was decapitated and its gall bladder was extracted and cut longitudinally to a strip which was put into a temperated bath (37°C) and bubbled through with O_2–CO_2, where it was fixed onto a magnetoelectric recorder. The compounds were tested in concentrations from $1 . 10^{-10}$ to $3 . 10^{-6}$ mol/l and the activity (concentration) was compared with that of standard solutions of CCK-8 or caerulein.

TABLE I

Analytical data on analogues *Ib—If*

Compound Yield, %	Formula M.w./(M + H)$^+$	R_T[a] min	Amino acid analysis[b]				Electrophoretic mobility		TLC
			Asp Trp	Tyr Phe	Met Cle	Gly Aib	$E^{2,4}_{Gly}$ $E^{5,7}_{Glu}$	$E^{2,4}_{His}$ $E^{5,7}_{Pic}$	
Ib	$C_{52}H_{67}N_{10}O_{16}S_3$	17·2	2·19	0·96	1·84	1·05[c]	0·17	0·14	0·37 (S1)
33	1 184·3/1 184[d]	50[e]	0·99	0·97	—	—	0·69	0·45	0·38 (S2)
Ic	$C_{44}H_{59}N_{11}O_{16}S_3$	9·62	2·17	0·97	1·85	1·01	0·34	0·28	0·18 (S1)
32	1 094·2/1 095[d]	40[e]	1·00[f]	1·00	—	—	0·32	0·24	1·11 (S2)
Id	$C_{50}H_{62}N_{10}O_{16}S_2$	7·91	2·02	0·97	0·92	1·00	0·13	0·10	0·42 (S1)
38	1·123·3/1 123[d]	30[g]	0·88	1·04	1·01	—	0·68	0·47	0·41 (S2)
Ie	$C_{45}H_{57}N_9O_{13}S$	7·10	1·05	0·98	1·04[h]	1·02	0·43	0·28	0·30 (S1)
43	964·1/964[d]	30[g]	0·91	0·99	—	1·02	0·56	0·38	0·20 (S2)
If	$C_{51}H_{65}N_{11}O_{17}S_3$	12·04	2·04	0·93	1·89	1·97	0·12	0·10	0·37 (S1)
35	1 200·3/1 200[d]	50[e]	0·94	1·00	—	—	0·62	0·36	0·34 (S2)

[a] HPLC retention times; [b] Hydrolysis with thioglycolic acid, after alkaline hydrolysis only Tyr(SO_3) was detected; [c] Pro; [d] A peak corresponding to a mass without SO_3 was also detected; [e] Mobile phase: % of methanol/0·05% trifluoroacetic acid; [f] His; [g] Mobile phase: % of acetonitrile/0·05% trifluoroacetic acid; [h] Nle.

The analgetic activity[19,20] was determined on mice in the hot-plate test after i.p. administration and the prolongation of the time of licking and jumping was measured in comparison with controls. The compounds were tested in doses of 0·1—20 μg per mouse.

The sedative effect[19,20] was evaluated in a small open field test, i.e. the mouse was put into a glass cylinder 10 minutes after i.p. application and its exploratory activity was observed. The number of rearings in 5 minute observation period served as a measure. The compounds were tested in 0·01—20 μg doses per mouse.

The anorectic activity was followed on mice as described in ref.[21]. Shortly, over 5 days the mice had free access to food from 8·00 to 16·00 h. On the sixth day these mice, having fasted for 16 h, were injected i.p. with the tested compounds and 10 min after application a weighed amount of food was given to them. Then, at 30 min intervals, the food was withdrawn, weighed and fresh weighed food was added. The amount of food eaten was thus followed for 4 h. The compounds were tested in doses of 0·01—20 μg per mouse.

The compounds were always tested first for anorectic activity, because in this test the behaviour of the animals tested may be observed during the first 40 min after application. It was found that the mice which differed distinctly in their behaviour during this period also displayed measurable activities in the sedative and analgetic test. For reasons of time and expense the analgetic and sedative test was carried out only in cases when the mice showed abnormal behaviour in the anorectic test, in comparison with controls (application of physiological solution).

RESULTS AND DISCUSSION

The structural modifications which were carried out caused in all tested analogues a dramatic decrease of biological potency (Table II). In consequence of the substitution of glycine in position 4 by proline, peptides *Ib* and *IIb* have measurable only a gall bladder activity (3 orders of magnitude lower), while neither anorectic nor CNS activity could be observed at the doses applied. This result is in agreement with earlier observations[15] when the glycine residue was substituted by amino acids with an aliphatic side chain (Ala, Aib), and the corresponding analogues also had a distinctly suppressed biological potency. In principle this is in agreement with the results of American authors who have prepared and tested this analogue parallelly[17]. Hence, it is evident that the receptors in corresponding peripheral organs and in the brain require only a certain spatial arrangement of the CCK-8 molecule for their interaction, with which the presence of the side chain in this position might interfere. Therefore the proline residue most probably disturbs the assumed structure of the β-turn in the amino terminal part of the analogue molecule[15] and it fixes such a conformation as is incompatible with the requirements of the receptors considered. From this it follows that the glycine residue plays one of the key roles in CCK-8 conformation and its substitution disturbs the spatial orientation of the functional groups contained in the link to the CCK receptors.

The substitution of the tryptophan residue in position 5 by histidine led to a complete loss of biological activity both in analogue *Ic* and in its protected derivative *IIc* (the maximum applied dose was 0·8 mg/kg of the animal), which may indicate the indispensability of the aromatic tryptophan side-chain for the interaction of the

CCK-8 molecule in the β-turn arrangement of its carboxy terminal part with the receptor system.

The substitution of the methionine residue in position 6 by non-coded amino acids, cycloleucine and 2-aminoisobutyric acid resulted in a considerable decrease of peripheral and suppression of central biological activity, both in analogues *Id* and *Ie* and in their protected precursors *IId* and *IIe*. When considering the fact that in compound *IIe* the original analogue Boc[desAsp^1, Nle^3]CCK-8 (isosteric Nle instead of Met in position 3) is comparably as active as Boc-CCK-8, then the decrease in biological activity of this peptide can only be accounted for by the presence of Aib in position 6. A complete suppression of the activity in the corresponding analogue *Ie* may be caused by the cumulation of the effect of substitution in position 6 and a further shortening of the molecule at the amino terminal by the Boc group. It is evident that the presence of both achiral sterically demanding amino acids with a branching at the α-carbon (hence, with the frame of the peptide backbone) represent a considerable obstacle for the formation of the conformational arrangement of the carboxyl terminal part of the molecule (of the assumed β- or γ-turn) acceptable for the interaction with the receptors. Consequently, analogue *Id*, containing cycloleucine in position 6 is slightly recognized only by the gall bladder receptor, and its protected derivative *IId* negligibly by receptors which trigger the anorectic activity. It is evident that in the substitution of methionine in position 6 by cycloleucine or

TABLE II
Biological activities of CCK-8 analogues *Ib*—*If* and their protected derivatives *IIb*—*IIf*

Peptide	Activity[a]			
	GP[b]	ANO[c]	SED[d]	ANA[e]
Pro^4]CCK-8 (*Ib*)	0·02	0	NT[f]	NT
[His^5]CCK-8 (*Ic*)	0[g]	0	0	0
[Cle^6]CCK-8 (*Id*)	1	0	NT	NT
[desAsp^1,Nle^3,Aib^6]CCK-8 (*Ie*)	0	0	NT	NT
[endo-Gly^{2a}]CCK-8 (*If*)	0	0	NT	NT
[Boc-Asp(OBu^t)^1,Pro^4]CCK-8 (*IIb*)	0·1	0	0	0
[Boc-Asp(OBu^t)^1,His^5]CCK-8 (*IIc*)	0	0	NT	NT
[Boc-Asp(OBu^t)^1,Cle^6]CCK-8 (*IId*)	0	0·2	NT	NT
[Boc-Tyr(SO$_3$)^1,Nle^2,Aib^5]CCK-7 (*IIe*)	1·5	1	0	0
[Boc-Asp(OBu^t)^1,endo-Gly^{2a}]CCK-8 (*IIf*)	1	1	0·3	0·3

[a] in % of CCK-8; [b] Guinea pig gall bladder contractions; [c] Anorectic activity (mice); [d] Sedative effect (mice); [e] Analgetic activity (mice); [f] Not tested; [g] 0 means no activity up to the concentration $3 . 10^{-6}$ mol/l or a dose 0·8 mg/kg in the in vitro and in vivo tests, respectively.

2-aminoisobutyric acid the insufficient lenght of the side chains of these non-coded amino acids is also operative, if compared with methionine or norleucine.

The insertion of a further glycine residue into the peptide chain between tyrosine in position 2 and methionine in position 3 in the original CCK-8 molecule (peptides *If* and *IIf*) was also connected with a distinct suppression of the activities by 2–3 orders of magnitude. This is probably a proof that this modification of the structure again interferes with the folded structure of the amino terminal part of the molecule forming the β-turn, fixed by the H-bond between the amide proton of glycine in position 4 and the amide carbonyl of Asp in position 1, in which just the residues of tyrosine and methionine in position 2 and 3 participate.

These results confirm the assumptions of considerable intolerance of the receptor systems both in the peripheral and central regions to the changes of the conformational arrangement of the CCK-8 molecule, caused by structural modifications which affect the peptide backbone directly (analogues *Ib*, *Id–If* and their protected derivatives *IIb*, *IId–IIf*). In the case of the substitution of the tryptophan residue in position 5 by histidine (analogue *Ic* and its derivative *IIc*) the explanation of the loss of any biological potency is more complex and the interpretation may be based both on the assumption of the interaction of the side chain with further aromatic systems in the molecule (Tyr-2 or Phe-8) and the important effect of this interaction on the fixation of the assumed folded structures in both parts of the molecule, or it may be assumed that the interaction of the side chain of tryptophan itself with the receptor site is indispensable for triggering the particular biological activities, taking into account that the imidazole system of histidine in the particular analogue (*Ic*) is not compatible with the above mentioned interactions.

REFERENCES

1. Jorpes J. E., Mutt V. in: *Secretin, Cholecystokinin, Pancreozymin and Gastrin* (J. E. Jorpes and V. Mutt., Eds.), p. 1. Springer, Berlin 1973.
2. Mutt V. in: *Gastrointestinal Hormones* (G. B. J. Glass, Ed.), p. 169. Raven, New York 1980.
3. Antin J., Gibbs J., Smith G. P.: Physiol. Behav. *20*, 67 (1978).
4. *Nomenclature and Symbolism for Amino Acids and Peptides. Recommendation 1983.* Eur. J. Biochem. *138*, 9 (1984).
5. Jensen R. T., Lemp G. F., Gardner J. D.: J. Biol. Chem. *257*, 5554 (1982).
6. Vanderhaeghen J. J., Signeau J. C., Gepts W.: Nature *221*, 557 (1975).
7. Pluscec J., Sheehan J. T., Sabo E. F., Williams J. A., Kocy O., Ondetti M. A.: J. Med. Chem. *13*, 349 (1970).
8. Saito A., Sankaran H., Goldfine I. D., Williams J. A.: Science *208*, 1155 (1980).
9. Morley J. E.: Life Sci. *30*, 479 (1982).
10. Woods S. C., West D. B., Stein L. J., McKay L. D., Lotter E. C., Porte S. C., Kenney N. J., Porte D.: Diabetologia *20*, 305 (1981).
11. Durieux C., Belleney J., Lallemand J. Y., Roques B. P., Fournié-Zaluski M. C.: Biochem. Biophys. Res. Commun. *114*, 705 (1983).

12. Fournié-Zaluski M. C., Belleney J., Durieux C., Gacel G., Roques B. P., Begue D., Manant I., Lux B., Gerard D.: Ann. N. Y. Acad. Sci. *448*, 598 (1983).
13. Fournié-Zaluski M. C., Belleney J., Lux B., Durieux C., Gerard D., Gacel G., Maigret B., Roques B. P.: Biochemistry *25*, 3778 (1986).
14. Loomis R. E., Lee Ping-Cheung, Tseng Ching-Chung: Biochim. Biophys. Acta *911*, 168 (1987).
15. Gacel G., Durieux C., Fellion E., Fournié-Zaluski M. C., Bégué D., Menant I., Rossignol P., Roques B. P. in: *Proc. 18th Europ. Pept. Symp.* (U. Ragnarssen, Ed.), p. 383. Almquist Wiksel Int., Stockholm 1984.
16. Čeřovský V., Pírková J., Majer P., Slaninová J., Hlaváček J.: Collect. Czech. Chem. Commun. *55*, 314 (1990).
17. Rosamond J. D., Comstock J. M., Thomas N. J., Clark A. M., Blosser J. C., Simmons R. D., Gawlak D. L., Loss M. E., Augello-Vaisey S. J., Spatola A. E., Benovitz D. E. in: *Proc. 10th Amer. Pept. Symp.* (G. R. Marshall, Ed.), p. 610, ESCOM, Leiden 1988.
18. Lonovics J., Guzman G., Devitt P., Hajtmancik K. E., Suddith R. L., Rayford P. L., Thompson J. C.: Gastroenterology *79*, 817 (1980).
19. Zetler G.: Eur. J. Pharmacol. *66*, 137 (1980).
20. Zetler G.: Eur. J. Pharmacol. *92*, 151 (1983).
21. Čeřovský V., Hlaváček J., Slaninová J., Jošt K.: Collect. Czech. Chem. Commun. *53*, 1086 (1988).

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