SYNTHESIS AND BIOLOGICAL PROPERTIES OF CHOLECYSTOKININ HEPTAPEPTIDE ANALOGUES CONTAINING D- AND L-FORMS OF TERT-LEUCINE OR NEOPENTYLGLYCINE IN POSITION 5*

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Using solution or solid-phase synthesis we prepared the cholecystokinin fragment Boc-CCK-7 (Boc-Tyr-(SO_3^-.Na^+)-Met-Gly-Trp-Met-Asp-PheNH_2) and its four analogues in which the methionine moiety (Met) in the carboxy-terminal part is replaced by tert-leucine (Tle) or neopentylglycine (Neo) residue or D-enantiomers of these non-coded amino acids. These structural modifications led to reduction of the studied biological activities (gall bladder contraction, anorectic activity, analgetic and sedation activity) of all prepared analogues except Boc[Neo^5]-CCK-7 which, being less analgetically active, retains full gall bladder and sedation activity of CCK-8. Moreover, its anorectic activity is substantially higher (400%). This analogue is very interesting particularly for its selectively increased (4×) anorectic effect compared with that of CCK-8.

Cholecystokinin (CCK)** is a peptide consisting of 33 amino acid residues. It was isolated for the first time by Ivy and Oldberg[2] from hog intestine where it stimulates the gastrointestinal motility and gall bladder contractions as well as pancreatic amylase secretion[3,4]. Beside further physiological effects, CCK is involved in the food intake control.[5] Some of its fragments have been identified in gastrointestinal tract as well as in brain of several animal species[6-10]. Structure–activity relationship studies have shown[11,12] that the C-terminal hepta- and octapeptides (CCK-8 and CCK-7) reproduce the entire range of biological activities of CCK (refs[11,12]). Beside CCK-8 itself, particularly the N-protected CCK-7 derivatives exhibit high CCK potency[12-14].

Therefore, we synthesized Boc-CCK-7, (Boc-Tyr(SO_3^-.Na^+)-Met-Gly-Trp-Met-Asp-Phe-NH_2) (*Ia*) and its analogues *Ib*–*Ie* (Table I) in which we focussed on

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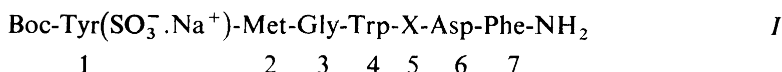
** The nomenclature and symbols of amino acids and peptides obey the published recommendations[1]. In addition to common symbols we use the following symbols: Neo, neopentylglycine, Tle, tert-leucine.

substitution in position 5 which, according to the results of ^{1}H NMR spectroscopy, fluorescence transfer and energy calculations[15,16], is one of the key sites for the C-terminal folding around the sequence Gly-Trp-Met-Asp, indispensable for the biological activity. As shown by previous studies, the CCK receptors in both the peripheral and central region are sensitive to changes in this folded conformation caused by steric hindrance in the vicinity of the peptide backbone (replacement of methionine in position 5 with cycloleucine or 2-aminoisobutyric acid)[17], or to the length, bulk and lipophilicity of the side chain[11,18]. Of all the so far described substitutions, only Nle proved to be fully isosteric with Met because the corresponding analogues exhibited equal biologic activity[19–21].

In the present communication we have investigated the effect on the gall bladder, anorectic, sedation and analgetic activity of CCK analogues in which the methionine in position 5 is replaced by non-coded amino acids with various number of carbon atoms in a lipophilic, branched side chain: tert-leucine, neopentylglycine and their D-enantiomers.



Boc-Tyr(SO_3^-.Na^+)-Met-Gly-Trp-X-Asp-Phe-NH_2 *I*
1 2 3 4 5 6 7

Ia, X = Met *Ib*, X = Neo *Ic*, X = D-Neo
Id, X = Tle *Ie*, X = D-Tle

II, Boc-Neo-Asp-Phe-NH_2
III, Boc-Trp-Neo-Asp-Phe-NH_2
IV, Boc-Met-Gly-Trp-Neo-Asp-Phe-NH_2

In the preparation of peptides *Ia*–*Ie* we started from phenylalanine, amide-bonded to *p*-methylbenzhydrylamine resin, and built the peptide chain up to the hexapeptide stage, using 1-hydroxybenzotriazole (HOBt) esters of the corresponding N-tert-butyloxycarbonyl (Boc) amino acids in dimethylformamide (DMF). In each step the Boc protecting group was cleaved off with 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) in the presence of anisole. The formed TFA salt was neutralized with 5% N,N-diisopropylethylamine (DIEA) in DCM. The hexapeptide amides were cleaved from the resin by action of liquid hydrogen fluoride in the presence of ethanedithiol, acylated with N-hydroxysuccinimidyl ester of Boc-tyrosine (Boc-Tyr-OSuc) in DMF and the formed Boc-heptapeptides were directly sulfated with SO_3-pyridine complex. Peptides *Ia* and *Ib* were also prepared by the solution synthesis[22,23] from Asp-Phe-NH_2 (refs[24–26]) which had been first acylated with N-hydroxysuccinimidy lesters of Boc-Met or Boc-Neo (ref.[27]) in the presence of triethylamine (TEA). After removal of the amino-protecting Boc group with TFA

in the presence of ethanedithiol, the obtained tripeptide amides were further acylated with N-hydroxysuccinimidyl ester of Boc-Trp in the presence of DIEA. The amino--protecting group was removed by treatment with TFA containing ethanedithiol with indole as scavenger and the coresponding tetrapeptides were subjected to fragment condensation with pentafluorophenyl ester of Boc-Met-Gly (ref.[23]) in the presence of DIEA. The amino-protecting Boc group was again removed as described above and the corresponding hexapeptide amides were acylated with N-hydroxysuccinimidyl ester of Boc-Tyr in the presence of DIEA. As in the preceding preparation, the amides of Boc-heptapeptides were sulfated with SO_3-pyridine complex.

Peptides *Ia*–*Ie* were purified by preparative HPLC and their effect on stimulation of guinea pig gall bladder contraction and their anorectic, analgetic and sedation activities were investigated.

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)

TABLE I

Data on CCK analogues *Ia*–*Ie* synthesized in this paper by SPPS

Compound	Formula M.w./M$^+$	k^a	TLC		Amino acid analysis[b]			
			S1 S3	S2 S4	Asp Trp	Tyr Phe	Met Tle	Gly Neo
Ia	$C_{50}H_{63}N_9O_{15}S_3Na_2$ 1 172·3/1 071[c]	1·08	0·43 0·44	0·45 0·43	1·02 0·91	0·97 1·02	1·84 —	1·05 —
Ib	$C_{52}H_{67}N_9O_{15}S_2Na_2$ 1 168·4/1 067[c]	2·25	0·67 0·72	0·53 0·57	1·03 0·89	0·99 0·97	0·97 —	1·05 1·00
Ic	[d]	1·88	0·67 0·68	0·45 0·62	1·05 0·90	0·96 1·02	0·98 —	1·03 1·00
Id	$C_{51}H_{65}N_9O_{15}S_2Na_2$ 1 154·1/1 053[c]	1·13	0·70 0·67	0·44 0·60	1·01 0·89	0·98 1·02	1·01 1·00	1·04 —
Ie	[d]	1·10	0·69 0·65	0·47 0·62	1·03 0·93	0·97 1·04	0·96 1·00	1·03 —

[a] Mobile phase 65% of methanol in ammonium acetate buffer of pH 6·6; [b] hydrolysis with thioglycolic acid, after alkaline hydrolysis only Tyr(SO_3) was detected; [c] peak corresponding to a mass without Boc (101) was detected as M$^+$; [d] the same molecular weight was confirmed by FAB MS.

(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). 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 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 CO_2. The amino acid analyses were performed on a Durrum D-500 amino acid analyzer (Durrum Instrum. Corp., Palo Alto, U.S.A.). Optical rotations were determined on a Perkin–Elmer 41 MCA polarimeter. Mass spectroscopy with FAB or SIMS technique was used for determination of M^+ of the corresponding peptides (VG Analytical, England). For HPLC a Spectra Physics instrument with an SP 8800 pump, an SP 8450 UV detector and an SP 4290 integrator was used. The analytical HPLC was carried out on a 15 × 0·4 cm Vydac column (The Separations Group, Hesperia, U.S.A.), flow rate 42 ml/h, detection at 222 nm, mobile phase methanol with 0·05% aqueous trifluoroacetic acid. Preparative HPLC was done on 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 6·7.

Tert-butyloxycarbonylneopentylglycyl-aspartyl-phenylalanine Amide (*II*)

To a solution of Boc-Neo-OSuc (0·28 g) in DMF (5 ml) was added TEA (0·3 ml) and a solution of Asp-Phe-NH_2 hydrobromide (0·3 g; prepared by reaction of the corresponding N-benzyloxycarbonyl derivative[24–26] with 34% HBr in acetic acid at room temperature for 15 min, addition of ether to the reaction mixture and decantation) in DMF (5 ml). After stirring for 2 days at room temperature, the solvent was evaporated and the dry residue was dissolved in ethyl acetate, washed with 20% solution of citric acid and dried over anhydrous Na_2SO_4. The solution was filtered, the solvent evaporated and the remaining oil crystallized on addition of ether; yield 0·38 g (83·8%), m.p. 204–205°C. Amino acid analysis: Neo 1·00, Asp 0·93, Phe 0·98. For $C_{25}H_{38}N_4O_7$ (507·6) calculated: 59·16% C, 7·74% H, 11·04% N; found: 58·92% C, 7·79% H, 10·79% N.

Tert-butyloxycarbonyltryptophyl-neopentylglycyl-aspartyl-phenylalanine Amide (*III*)

TFA.Neo-Asp-Phe-NH_2 (0·21 g; prepared from the corresponding Boc derivative by treatment with TFA at room temperature for 30 min, addition of ether and decantation) was dissolved in DMF (5 ml) and acylated with Boc-Trp-OSuc (0·16 g) in the presence of DIEA (0·07 ml) at room temperature for 48 h. The solvent was evaporated, the oily residue was triturated with water until it solidified, filtered, washed with water and dried in a desiccator, yielding 0·25 g (93%) of product, m.p. 199–201°C. $[\alpha]_D$ −30·9° (*c* 2, methanol). Amino acid analysis: Trp 1·03, Neo 1·00, Asp 1·06, Phe 1·00. HPLC analysis (60% methanol–0·05% trifluoroacetic acid): k = = 3·6. For $C_{36}H_{48}N_6O_8$ (692·8) calculated: 62·41% C, 6·98% H, 12·13% N; found: 62·58% C, 7·11% H, 11·96% N.

N-Tert-butyloxycarbonylmethionyl-glycyl-tryptophyl-neopentylglycyl-aspartyl--phenylalanine Amide (*IV*)

A mixture of TFA. Trp-Neo-Asp-Phe-NH_2 (0·12 g; obtained from the corresponding Boc derivative as described for compound *III*), Boc-Met-Gly-OPfp (ref.[23], 0·09 g), DMF (3 ml) and DIEA (0·05 ml) was stirred at room temperature for 48 h. After evaporation of the solvent, the material was triturated with water until it solidified, filtered, washed with water and ethyl acetate and

dried; yield 0·12 g (82%) of product, m.p. 201–204°C, $[\alpha]_D$ −29·4° (*c* 0·2, methanol). Amino acid analysis: Asp 1·00, Gly 0·97, Met 1·00, Phe 0·97, Trp 0·83, Neo 1·02, HPLC analysis (70% methanol–0·05% trifluoroacetic acid): $k = 5{\cdot}9$. For $C_{43}H_{60}N_8O_{10}S$ (881·1) calculated: 58·62% C, 6·86% H, 12·72% N, 3·64% S; found: 58·86% C, 6·72% H, 12·53% N, 3·81% S.

N-Tert-butyloxycarbonyl-O-sulfotyrosyl-methionyl-glycyl-tryptophyl-methionyl--aspartyl-phenylalanine Amide (*Ia*)

A mixture of Boc-Tyr-OSuc (0·04 g) and DIEA (0·05 ml) was stirred with TFA. Met-Gly-Trp--Met-Asp-Phe-NH_2 (0·10 g; prepared by removing the Boc protecting group from the corresponding hexapeptide[22,23] as described in the preparation of *III*) in DMF (4 ml) at room temperature for 56 h. The reaction mixture was evaporated to dryness and the residue triturated with water until it solidified. The protected heptapeptide amide was filtered, washed with water, dried (0·08 g) and dissolved in DMF (1 ml) and pyridine (1 ml). This solution was stirred with pyridine–SO_3 complex (0·80 g) for 24 h at room temperature under nitrogen. After evaporation of the solvents, the residue was dissolved in 0·5M $NaHCO_3$ at 0°C and the solution was freeze--dried. The residue was mixed with methanol and the separated salt was collected. The peptide *Ia* was purified by HPLC (gradient 0–70% methanol in 0·1M ammonium acetate, pH 6·7, 70 min); yield 0·026 g of pure peptide. Amino acid analysis: Asp 1·07, Gly 1·11, Met 1·94, Phe 0·98, Tyr 0·93, Trp 0·87. After alkaline hydrolysis the corresponding content of O-sulfotyrosine was estimated; no traces of Tyr were found. Molecular weight, determined by mass spectrometry, corresponded to a molecule $C_{50}H_{63}N_9O_{15}S_3Na_2$ (1 172·3), from which the Boc group (101) had been split off: 1 071.

N-Tert-butyloxycarbonyl-O-sulfotyrosyl-methionyl-glycyl-tryptophyl-neopentyl--glycyl-aspartyl-phenylalanine Amide (*Ib*)

Using the same procedure as described for compound *Ia*, Boc-Tyr-OSuc (0·04 g) was reacted with TFA. Met-Gly-Trp-Neo-Asp-Phe-NH_2 (0·10 g; prepared by removal of the Boc protecting group in peptide *IV* as described for compound *III*). The obtained protected heptapeptide amide (0·08 g, 77%) was further sulfated by the procedure described for peptide *Ia* to give the desired analogue *Ib* (0·023 g). Amino acid composition: Asp 1·04, Gly 1·10, Met 0·94, Neo 1·00, Phe 0·98, Tyr 0·98, Trp 0·88. No traces of Tyr were detected after alkaline hydrolysis of the peptide. Molecular weight found by mass spectrometry (1 067) corresponded to that of a molecule $C_{52}H_{67}N_9O_{15}S_2Na_2$ (1 168·4) devoid of the Boc group.

Preparation of Peptides *Ia*–*Ie* on Polymer Resin (Table I)

Boc-Phe (0·40 g) was coupled to a *p*-methylbenzhydrylamine resin (PMBHA) (0·5 mmol g^{-1}, 1·0 g) in the presence of HOBt (0·20 g) and dicyclohexylcarbodiimide (DCC, 0·31 g) in DMF (10 ml). After stirring for 6 h, the liquid part of the reaction mixture was filtered off, the Boc--Phe-PMBHA resin was washed with 20 ml of DCM (3×), 2-propanol (3×) and DCM (3×) and the unreacted amino groups (if any) were acetylated by treatment with a mixture of acetic anhydride (2 ml) and DIEA (3 ml) in DCM (25 ml) for 2 h. The Boc group was removed by two successive treatments with 50% TFA in DCM in the presence of anisole (10%) (5 and 30 min) and after washing with DCM (3 × 20 ml) the obtained TFA. Phe-PMBHA resin was neutralized with 10% DIEA in DCM (20 ml) (2 × 5 min). In the end of the cycle, the Phe-PMBHA resin was washed with DCM (5 × 20 ml) and the whole cycle was repeated with 3 equivalents (1·5 mmol) of Boc-Asp (OBzl), Boc-X, where X = Met, Neo or D-Neo (ref.[27]), Tle or D-Tle

(ref.[26]), and Boc-Trp(For), Boc-Gly and Boc-Met. Then the corresponding hexapeptide amides were cleaved off from the resin by treatment with liquid HF (10 ml) in the presence of ethane-dithiol (1 ml) at 0°C for 1 h, HF was distilled from the reaction vessel, the reaction mixture was washed with ethyl acetate (3 × 50 ml) and the hexapeptides were extracted from the resin with 50% acetic acid and water (3 × 50 ml). After freeze-drying of the solution, the products were dissolved in DMF (20 ml) and condensed with Boc-Tyr-OSuc (0·57 g, 1·5 mmol) at room temperature in the presence of DIEA for 48 h. The solvent was distilled off and the residue was triturated with water until it solidified, the solid was filtered, washed with water, dried in a desiccator over solid KOH and sulfated as described in the solution synthesis of the analogue *Ib*. The same procedure was applied to the purification of peptides *Ia*–*Ie* by HPLC.

Biological Activity Assays

The guinea-pig gall bladder contraction ability and the sedation and analgetic effects in mice were estimated using the described methods[29–31]. For the anorectic effect determination a test has been elaborated[22] based on comparison of the amount of solid food taken by 5-membered groups of 18 h starving mice, that had been given intraperitoneally either the peptide in physiological solution or only the physiological solution (control). The amount of the food taken was followed in 30 min intervals to 240 min after application. During the first 40 min also deviations in the behaviour, compared with the control animals, were followed. As already mentioned in our previous paper[17], the sedation and analgetic effects were tested and quantitatively evaluated only in cases when in the anorectic effect test the mice behaved differently from the control (e.g. lower motility or an overall flabbiness).

RESULTS AND DISCUSSION

The biological tests show that replacement of Met in position 5 of CCK-7 (position 31 in CCK) by non-coded amino acids with branched side chain (Tle and Neo and their D-isomers) results predominantly in markedly different biological activity compared with that of CCK-8 or Boc-CCK-7 (Table II). In this position, the tert-

TABLE II

Biological activities (% of CCK-8 activity) of CCK analogues *Ia*–*Ie*

Peptide	Gall bladder[a]	Anorexy[b]	Sedation[c]	Analgesy[d]
Boc-CCK-7 (*Ia*)	50	100	NT[e]	NT
Boc-[Neo[5]]CCK-7 (*Ib*)	100*	400	100	20–100
Boc-[D-Neo[5]]CCK-7 (*Ic*)	0·4	0[f]	NT	NT
Boc-[Tle[5]]CCK-7 (*Id*)	1	0[f]	NT	NT
Boc-[D-Tle[5]]CCK-7 (*Ie*)	0·1	0[f]	NT	NT

[a] Guinea-pig gall bladder contractions[27]; [b] anorectic activity (mice)[22]; [c] sedation effect (mice)[28] [d] analgetic activity (mice)[29]; [e] not tested; [f] 0 means no activity up to the concentration $3 . 10^{-6}$ mol l^{-1}, or dose 0·8 mg kg^{-1} in the in vitro and in vivo test, respectively.

-butyl side chain in Tle represents a sterically bulky structure interfering with the peptide backbone arrangement (β-turn) necessary for recognition of the molecule by the corresponding receptors; this interference follows from the proximity of the branching to the backbone. On the other hand, the Tle side chain is probably too short to be able to participate efficiently in the assumed interaction with the hydrophobic site of the receptor, as is the case of the side chain of Met in the parent peptide or Nle in its fully active analogue. Thus, in this case the introduction of tert-leucine indeed results in the expected dissociation of the biological activity, the analogue *Id* interacting only with peripheral receptors of the type A (as e.g. in the case of the oxytocine molecule[32], where Tle is this subtle steric element contributing to such a conformation that is differently recognized by the uterotonic and mammalian gland receptors); however this activity is reduced by 2 orders of magnitude.

A substantially different profile of biological activities results from substitution with neopentylglycine. Compared with the Tle compound, the extension of the side chain by mere CH_2 group in the analogue *Ib* enhanced the GP gall bladder activity by two orders of magnitude (to the original CCK-8 value) and its anorectic activity was four times higher than that of CCK-8. This case thus represents a substantially more pronounced trend of dissociation between gall bladder and anorectic activity than that we have observed with the parent peptide of this series, Boc-CCK-7 (*Ia*). Unlike the other analogues tested (*Ic*–*Ie*), compound *Ib* reduces the motility and induces an overall flabbiness of the experimental animals. A study of CNS-activities of this compound has shown the same level of sedation activity as CCK-8 and a somewhat lower analgetic effect. It seems thus that the analogue *Ib* is one of the most active anorectics known so far, with an unincreased gall bladder or CNS activity, that have been obtained as a result of a mere simple substitution of an amino acid moiety in the molecule[33,34]. The observed dissociation may indicate heterogeneity of peripheral A receptors (gall bladder activity) and receptors mediating the anorectic activity. The anorectic action is probably mediated by an effect on the appetite control centers in the hypothalamus[35]. The magnitude of the biological effect of analogue *Ib* determines the length of the hydrophobic amino acid side chain in position 5 (i.e. three atoms) that is necessary for preserving the biological potency of CCK. At the same time, it is shown how, under this condition, the intensity of interaction of the molecule with different receptor types can be influenced by a subtle structural modification (i.e. chain branching) in this position.

The generally reduced biological potency of analogues *Ic* and *Ie*, containing in position 5 instead of Met the D-Tle and D-Neo residues, respectively, is in principle in accord with the model of biologically active conformation of carboxy-terminal hepta- and octapeptide fragments of CCK[15,16], and its conditions for a correct, by receptors recognized, orientation of the molecule. The presence of the D-isomers interfers with the original folded arrangement of the C-terminal part of these peptides where Trp and Met represent edges of the assumed β-turn. Consequently, the re-

quired strong hydrophobic interactions of the D-Tle or D-Neo side chains with the corresponding receptor site, and transduction of biological effects, are not possible.

The results of this structure-activity study have proved that a change of absolute configuration in the position of the Met moiety in the carboxy-terminal part of CCK-8 strongly reduces the biological potencies of the corresponding analogues and that this position is also extraordinarily important for recognition of the molecule by different receptor types.

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ERRATA:

The Interactions of Tensides with Calconalide I
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Collect. Czech. Chem. Commun. *56*, 2100 (1991), p. 2105, Fig. 4:
For log c_{tens} read $-\log c_{tens}$.