CHOLECYSTOKININ HEPTAPEPTIDE ANALOGUES WITH MULTIPLE MODIFICATION IN PEPTIDE CHAIN*

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Dedicated to Professor Otto Wichterle on the occasion of his 80th birthday.

Using solid phase synthesis we prepared the cholecystokinin fragment Boc-CCK-7 (Boc-Tyr($SO_3^-Na^+$)-Met-Gly-Trp-Met-Asp-Phe-NH_2) *Ia* and its seven analogues *Ib* – *Ih*. In the analogues *Ib* and *Ic* the Met residue in the carboxyterminal part of the molecule was substituted for L- or D-$PheMe_3$. In the analogues *Id* and *Ie* with Phe residue substituted by L- or D-$PheMe_3$ the Neo was inserted in the place of this Met residue and in the analogues *If* and *Ig*, in addition to $PheMe_3$ substitution in the carboxyterminus, both Met residues were replaced for Neo. This dual substitution for Met residues was also applied in the analogue *Ih* with coded Phe residue in the C-terminus.

Cholecystokinin (CCK)** is a linear polypeptide consisting of 33 amino acid residues** which stimulates the gastrointestinal motility and gall bladder contractions as well as pancreatic amylase secretion[2,3]. Beside further physiological effects, CCK is involved in the food intake control[4]. Some of its fragments have been identified in gastrointestinal tract as well as in brain of several animal species[5-9]. Structure–activity relationship studies[10,11] have shown that the C-terminal octa- and heptapeptides (CCK-8 and CCK-7) reproduce the entire range of biological activities of CCK. Beside CCK-8 itself, particularly N-protected CCK-7 derivatives exhibit high CCK potency[11-15].

Therefore, we synthesized Boc-CCK-7, [Boc-Tyr($SO_3^-Na^+$)-Met-Gly-Trp-Met-Asp-Phe-NH_2] (*Ia*) and its analogues *Ib* – *Ih* with replaced Met and Phe residues in positions

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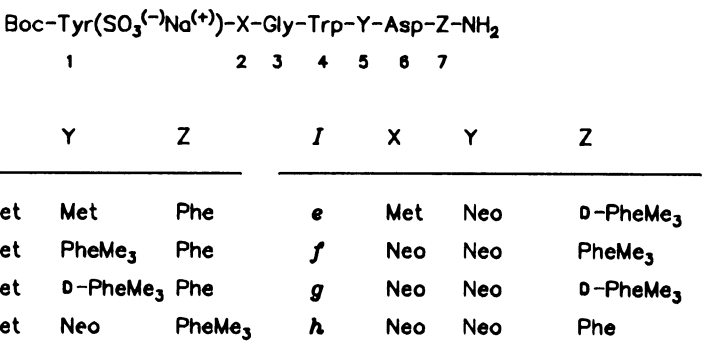
**The nomenclature and symbols of amino acids and peptides obey the published recommendations[1]. In addition following abbreviations were used: Neo, neopentylglycine; $PheMe_3$, 2,4,6-trimethylphenylalanine.

2, 5 and 7 for non coded Neo and $PheMe_3$ ones. The results of the biological activity assay and the structure–activity relationships in this series are described and discussed elsewhere[16,17].

Peptides *Ia – Ih* were prepared by combined solid phase–solution procedure. In the first stage of the synthesis the carboxyterminal hexapeptides were built on *p*-methylbenzhydrylamine (p-MBHA) resin using *N*-hydroxybenzotriazole (HOBt) esters of the corresponding *tert*-butyloxycarbonyl (Boc) amino acids[18 - 22] in dimethylformamide (DMF), prepared in situ by *N,N'*-dicyclohexylcarbodiimide (DCC) mediated reaction. The Boc group was cleaved by 50% trifluoroacetic acid (TFA)–anisole mixture in dichloromethane (DCM) and neutralization was carried out by 5% *N,N'*-diisopropylethylamine (DIEA) in DCM. Progress in peptide synthesis was followed by the ninhydrin[23] and bromophenol blue[24] tests.

After splitting off the hexapeptides from the resin by HF/ethanedithiol and their coupling to *N*-hydroxysuccinimide (HOSuc) ester of Boc-Tyr-OH in DMF, the corresponding Boc-heptapeptides were sulfated using SO_3-pyridine complex and compounds were purified and diastereoisomers separated by HPLC.



EXPERIMENTAL

Diastereoisomeric peptides were separated on a reversed phase Vydac HPLC column using gradient 0 – 50% of methanol (30 min) and 50 – 70% of methanol (50 min) in 0.05 M ammonium acetate, pH 6.7. Their determination was carried out by modified procedure of Toth et al.[25]. The corresponding analogue (0.3 mg) from HPLC separation was hydrolyzed in 6 M HCl at 110 °C for 20 h. The solution was evaporated in vacuo, the solid dissolved in water and spotted to CHIRALPLATE (Macherey–Nagel, Germany) together with the methylated phenylalanine which was either intact or was in advance incubated with 1% solution of L-amino acid oxidase (Snake venom from *Crotalux atrox*, Serva) in 0.1 M TRIS buffer (pH 7.5) (20 μl) in oxygen filled, tightly closed tube for 24 h at 38 °C. Also the standards of the other amino acids presented in the analogue were spotted. After elution of

the plate in the system CH_3CN–H_2O–methanol (4 : 1 : 1) and comparison of the all of R_F values for spotted samples, the D- or L-enantiomer of the $PheMe_3$ containing analogue could be determined. Samples for amino acid analysis were hydrolyzed by 6 M HCl at 110 °C for 20 h with 4% thioglycolic acid added. Samples containing O^4-sulfotyrosine were also hydrolyzed with 0.2 M $Ba(OH)_2$ at the same temperature for 20 h and the excess of 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 Instruments, U.S.A.).

Mass spectroscopy with FAB 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 25 × 0.4 cm Vydac column (The Separations Group, Hesperia, U.S.A.), flow rate 60 ml/h, detection at 222 nm, mobile phase a mixture of methanol with 0.05% aqueous TFA.

Peptides *Ia* – *Ih*

A mixture of the Boc-Phe-OH (compounds *Ia*, *Ib*, *Ic*, *Ih*) or Boc-D,L-$PheMe_3$-OH (compounds *Id*, *Ie*, *If*, *Ig*) (1.5 mmol), p-MBHA resin (0.5 mmol/g, 1.0 g), HOBt (0.20 g, 1.5 mmol) and DCC (0.31 g, 1.5 mmol) in DMF (10 ml) was stirred for 6 h at room temperature in a reaction vessel of a home made solid phase synthesizer and the solvent was then filtered off. The Boc-Phe-p-MBHA or Boc-D,L-$PheMe_3$-p-MBHA resin was washed with DCM (3 × 20 ml), 2-propanol (3 × 20 ml) and DCM (3 × 20 ml). Unreacted amino groups on the resin were acetylated with a mixture: acetic anhydride (2 ml)–DIEA (3 ml)–DCM (25 ml) for 1 h at room temperature. The Boc group was split off by stirring with a mixture: 50% TFA–9% anisole–1% ethanedithiol in DCM for 5 and 30 min. TFA.H-Phe-p-MBHA and TFA.D,L-H-$PheMe_3$-p-MBHA resins were neutralized by 5% DIEA in DCM (2 × 20 ml), 2 × 5 min. The synthetic cycle was completed by washing the amino acid resins with DCM (5 × 20 ml) and was repeated with 3 equivalents (1.5 mmol) of Boc-Asp(OBzl)-OH. Then, in the case of H-Asp(OBzl)-Phe-p-MBHA resin the cycle was repeated with the same amount of Boc-Met-OH (peptide *Ia*), Boc-D,L-$PheMe_3$-OH (peptides *Ib*, *Ic*) or Boc-Neo (peptide *Ih*); in the case of H-Asp(OBzl)-D,L-$PheMe_3$-p-MBHA resin with the same amount of Boc-Neo-OH (peptides *Id*, *Ie*, *If*, *Ig*). The corresponding tripeptide-resins were acylated successively by Boc-Trp-OH, Boc-Gly-OH and Boc-Met-OH (peptides *Ia*, *Ib*, *Ic*, *Id*, *Ie*) or Boc-Neo-OH (peptides *If*, *Ig*, *Ih*) using above described synthetic cycle.

Boc-hexapeptide resins were then treated with a liquid HF (20 ml) in the presence of ethanedithiol (1 ml) and indole (100 mg) at 0 °C for 1 h and after evaporation of the HF the cleaved and deprotected hexapeptide amides were washed out from the resin by means of 50% (3 × 20 ml) and 6% (3 × 20 ml) acetic acid, respectively. The combined acetic acid washings were extracted by ethyl acetate (3 × 150 ml), diluted with water (100 ml) and freeze dried.

In all cases the lyophilizate was dissolved in DMF (20 ml) and acylated with Boc-Tyr-OSuc (0.57 g, 1.5 mmol) at room temperature in the presence of DIEA (0.26 ml; 1.5 mmol) for 48 h. Dimethylformamide was evaporated, the residual oil triturated with water until it precipitated and the solid product was filtered off, washed with water and dried in desiccator over solid KOH.

To the solution of the Boc-heptapeptide amide (0.08 g) in DMF (1 ml) and pyridine (1 ml) a complex of SO_3–pyridine (0.80 g) was added and the mixtures were stirred under nitrogen for 48 h at room temperature. The solvents were evaporated, the sulfated peptide was dissolved in an ice cooled 0.5 M $NaHCO_3$ (50 ml) and the solution freeze dried. Methanol (50 ml) was added to the lyophilizate, the precipitated salt removed by filtration and the solvent evaporated at room temperature. O-Sulfate esters of the corresponding Boc-heptapeptides *Ia* – *Ih* were purified and sep-

arated by the reverse phase HPLC using gradient 0 – 50% of methanol (30 min) and 50 – 70% of methanol (50 min) in 0.05 M ammonium acetate, pH 6.7. For analytical HPLC separation conditions see Table I.

TABLE I
Analytical data on CCK analogues *Ia – Ih*

Compound	Formula M.w. (M^+)[a]	k'[b]	Amino acid analysis[c]			
			Asp	Tyr	Met	Gly
			Trp	Phe	Neo	$PheMe_3$
Ia	$C_{50}H_{63}N_9O_{15}S_3Na_2$	3.82	1.02	0.97	1.84	1.05
	1 172.3 (1 071)		0.91	1.02	–	–
Ib	$C_{57}H_{69}N_9O_{15}S_2Na_2$	5.41	1.01	0.96	0.98	1.02
	1 230.4 (1 129)		0.90	1.04	–	1.00
Ic	[d]	7.06	1.04	0.95	0.93	1.01
			0.91	1.02	–	1.00
Id	$C_{55}H_{73}N_9O_{15}S_2Na_2$	6.03	1.03	1.05	0.92	1.03
	1 210.4 (1 109)		0.87	–	1.00	0.98
Ie	[d]	7.28	1.04	0.99	0.91	1.03
			0.87	–	1.00	1.01
If	$C_{57}H_{77}N_9O_{15}SNa_2$	6.51	1.01	0.93	–	1.04
	1 206.4 (1 105)		0.93	–	2.00	0.99
Ig	[d]	7.57	1.02	0.94	–	1.02
			0.92	–	2.00	1.01
Ih	$C_{54}H_{71}N_9O_{15}SNa_2$	5.97	1.03	0.99	–	1.01
	1 164.3 (1 063)		0.93	1.02	2.00	–

[a] Peak corresponding to a mass without Boc (101) was detected as M^+. [b] HPLC capacity factor was determined at mobile phase 70% of methanol in 0.05% trifluoroacetic acid. [c] Hydrolysis with thioglycolic acid, after alkaline hydrolysis only Tyr(SO_3) was detected. [d] The same molecular weight as for L-Phe methylated derivative containing analogue was confirmed by FAB MS.

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