ANALOGUES OF CHOLECYSTOKININ METHYLATED IN THE SIDE CHAIN OF CARBOXYTERMINAL PHENYLALANINE*

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Received April 18, 1991
Accepted May 20, 1991

In the course of our study on cholecystokinin (CCK) a series of Boc-CCK-7 analogues was synthesized. Their carboxyterminal part was modified by phenylalanine derivatives containing 2 or 4 and 2,6 or 2,4,6 methylated aromatic side-chain. During the synthesis, the racemic phenylalanine derivatives were used and peptides containing either L- or D- methylated phenylalanine were separated using a preparative HPLC. Gall bladder contraction, anorectic, sedative and analgetic bioassays of these analogues revealed that all of them behaved as CCK-8 agonists. While the analogues containing L-form of the methylated phenylalanines had almost the same potency (80%—130%) in comparison to CCK-8, the presence of the D-form decreased the biological activity of corresponding analogues to 8—62% of the CCK-8 potency. These results are in agreement with the suggestion that phenylalanine residue in C-terminus takes part in biological activity transduction only.

Cholecystokinin (CCK)** is a linear polypeptide hormone first isolated by Ivy and Oldberg[2] from hog intestine and found to stimulate gastrointestinal motility and gall bladder contractions as well as pancreatic amylase secretion[3]. In addition to other physiological effects CCK also seems to control food intake[4]. Several molecular forms of cholecystokinin were found in the brain of human and several animal species, its carboxyterminal octapeptide CCK-8 being the most abundant[5-13]. The CCK-8 exhibits multiple biological activities as a hormone in the periphery and as a neurotransmitter and neuromodulator in the central nervous system (analgesia, sedation, thermoregulation effect etc.). Some authors[14-16] have observed that

* Part CCXXIV in the series Amino Acids and Peptides; Part CCXXIII: Collect. Czech. Chem. Commun. *56*, 2209 (1991).

** The nomenclature and symbols of amino acids and peptides obey the published recommendations[1]. In addition to common symbols we use the following symbols: Bzl, benzyl; For, formyl; *p*-MBHA resin, *p*-methylbenzhydrylamine resin; HOBt, 1-hydroxybenzotriazol; DCC, dicyclohexylcarbodiimide; Boc, tert.butyloxycarbonyl; DMF, dimethylformamide; DCM, dichloromethane; TFA, trifluoroacetic acid; DIEA, diisopropylethylamin; OSuc, N-hydroxysuccinimide.

modification of the phenylalanine residue at the C-terminus have caused interesting changes in the biological potency of this molecule. The structure variation in this residue and inversion of its configuration at α-carbon affected mostly a transduction of biological potency of the receptor bound CCK-8 analogues. Hence, an extinction[17], an antagonism[18] or a change of the intrinsic agonistic activity[19] occurred as a result of space structure deviation in the complex hormone–receptor. Interestingly, an introduction of N-methylphenylalanine residue (backbone modification) to this position yielded an analogue with nearly the same biological potency compared to CCK-8 suggesting enhanced enzymic stability[16]. Hruby et al.[20] also prepared cholecystokinin octapeptide analogues with a β-carbon methylated phenylalanine, however, this modification has concerned of replacement of the Met residue in position 2.

The carboxyterminal heptapeptide of cholecystokinin (CCK-7) was described to possess the same spectrum of biological activities as the CCK. Biological potency of this short peptide sequence is even increased when α-amino group is protected by acyl or urethane type groups and potency of such shortened forms of CCK analogues can be compared to that of CCK-8 (refs[12,21–23]). Several papers on structure–activity relationship of N-protected cholecystokinin heptapeptide analogues and results on carboxyterminal Phe residue modification have therefore stimulated us to prepare a series of Boc-CCK-7 (*Ia*) analogues in which this residue was substituted for its derivatives methylated in their side-chain. In the synthesis, we utilized racemic phenylalanines containing methyl groups in positions (i) 2, (ii) 2, 6 (ref.[24]), (iii) 4 and (iv) 2, 4, 6 (refs[25,26]) of their aromatic nucleus. The compounds were used in solid phase/solution couplings to prepare the corresponding Boc-CCK-7 analogues (*Ib*–*Ii*) (for a preliminary communication see ref.[27]).

TABLE I

HPLC conditions for separation of Boc-CCK-7 analogues *Ib*–*Ii*

Peptide	Retention times[a]		Gradient conditions[b] (flow 1 ml/min)
	L-	D-	
[Phe(2-Me)^7] (*Ib*, *Ic*)	14·37	15·40	60–100% MeOH, 20 min
[Phe(4-Me)^7] (*Id*, *Ie*)	19·05	20·10	60–100% MeOH, 30 min
[Phe(2,6-diMe)^7] (*If*, *Ig*)	21·64	22·82	50–100% MeOH, 30 min
[Phe(2,4,6-triMe)^7] (*Ih*, *Ii*)	23·65	24·55	50–100% MeOH, 30 min

[a] In minutes. [b] Vydac analytical column 25 × 0·4 cm from Separations Group, Hesperia, CA, U.S.A.; Spectra Physics SP 8800 HPLC pump with UV/VIS detector SP 8450 and integrator SP 4290; gradient % of MeOH in 0·05% TFA.

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

Ia, X = Phe

Ib, X = Phe(2-Me) *Ic*, X = D-Phe(2-Me)
Id, X = Phe(4-Me) *Ie*, X = D-Phe(4-Me)
If, X = Phe(2,6-diMe) *Ig*, X = D-Phe(2,6-diMe)
Ih, X = Phe(2,4,6-triMe) *Ii*, X = D-Phe(2,4,6-triMe)

In the first stage of the synthesis, the carboxyterminal hexapeptides were built on *p*-MBHA resin using HOBt esters of the corresponding Boc-amino acids in DMF, prepared "in situ" by DCC mediated reaction. The Boc group was cleaved by 50%

TABLE II
Analytical data on analogues of Boc-CCK-7 (*Ib*–*Ii*)

Compound	Formula M.w./M^+	TLC[a]	Amino acid analysis[b]		
			Tyr Trp	Met Asp	Gly Phe(X)[c]
[Phe(2-Me)^7]	$C_{51}H_{65}N_9O_{15}S_3Na_2$	0·55	0·96	1·89	1·00
(*Ib*)	1 186·3/1 085[d]		0·89	1·02	1·03
[D-Phe(2-Me)^7]	[e]	0·44	0·97	1·92	1·00
(*Ic*)			0·88	0·99	1·02
[Phe(4-Me)^7	[e]	0·56	0·95	1·91	1·00
(*Id*)			0·91	1·04	1·02
[D-Phe(4-Me)^7]	[e]	0·48	0·96	1·84	1·00
(*Ie*)			0·89	1·02	1·04
[Phe(2,6-diMe)^7]	$C_{52}H_{67}N_9O_{15}S_3Na_2$	0·53	0·96	2·01	1·00
(*If*)	1 200·4/1 099[d]		0·85	1·04	1·01
[D-Phe(2,6-diMe)^7]	[e]	0·39	0·99	1·94	1·00
(*Ig*)			0·87	1·06	1·03
[Phe(2,4,6-triMe)^7]	$C_{53}H_{69}N_9O_{15}S_3Na_2$	0·44	0·96	1·92	1·00
(*Ih*)	1 214·4/1 113[d]		0·91	1·03	1·01
[D-Phe(2,4,6-triMe)^7]	[e]	0·31	0·97	1·89	1·00
(*Ii*)			0·92	1·05	0·99

[a] R_F value (CH_3CN–H_2O-methanol, 4 : 1 : 1) of the methylated Phe derivative after a hydrolysis of corresponding analogue by 6M-HCl at 110°C for 20 h; [b] hydrolysis with thioglycolic acid; after alkaline hydrolysis only Tyr(SO_3) was detected; [c] methylated derivative of Phe; [d] peak corresponding to a mass without Boc (101) was detected as M^+; [e] the same molecular weight as for L-Phe methylated derivative containing analogue was confirmed by M^+.

TFA/anisole mixture in DCM and neutralization was carried out by 5% DIEA in DCM. After splitting off the hexapeptides from the resin by HF/ethanedithiol and their coupling to Boc-Tyr-OSuc in DMF, the corresponding Boc-heptapeptides were sulfated using SO_3–pyridine complex. The diastereoisomeric peptides were separated on a reversed phase HPLC column (Tables I and II). The L- and D- forms of the methylated Phe in the peptide peaks obtained were determined after acidic hydrolysis of the samples either using L-amino acid oxidase treatment followed by conventional amino acid analysis or by TLC on chiral plates[28].

The biological activity of analogues *Ib*–*Ii* (Table III) was determined in four tests using guinea pig gall bladder preparation[29] and mouse satiety[30], sedation and analgesia[31,32] assays. As it was stated in the previous papers[33,34] the sedation and analgetic effects were assesed only when the anorectic assay revealed a deviated behaviour of the experimental animals (e.g. a decreased motility or/and general flabbiness of mice).

EXPERIMENTAL

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.) Mass spectroscopy with FAB technique was used for determination of M^+ of the corresponding peptides (VG Analytical, England). For HPLC a Spectra Physics instrument

TABLE III
Biological activities (in % of CCK-8) of CCK analogues *Ia*–*Ii*

Peptide	GPGB[a]	Anor.[b]	Sed.[c]	Anal.[d]
Boc-CCK-7 (*Ia*)	50	100	100	100
Boc-[Phe(2-Me)^7]CCK-7 (*Ib*)	100	133	100	100
Boc-[D-Phe(2-Me)^7]CCK-7 (*Ic*)	27	62	—[e]	—[e]
Boc-[Phe(4-Me)^7]CCK-7 (*Id*)	80	100	100	100
Boc-[D-Phe(4-Me)^7]CCK-7 (*Ie*)	8	20	—[e]	—[e]
Boc-[Phe(2,6-diMe)^7]CCK-7 (*If*)	100	100	100	100
Boc-[D-Phe(2,6-diMe)^7]CCK-7 (*Ig*)	40	50	—[e]	—[e]
Boc-[Phe(2,4,6-triMe)^7]CCK-7 (*Ih*)	100	100	100	—[e]
Boc-[D-Phe(2,4,6-triMe)^7]CCK-7 (*Ii*)	23	50	50	—[e]

[a] Guinea pig gall bladder contractions[29]; [b] anorectic activity[30]; [c] sedative effect (mice)[31]; [d] analgetic activity (mice)[32]; [e] not tested.

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 methanol with 0·05% aqueous TFA. The 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, detection at 280 nm. N^{α}-Boc-protected amino acids were prepared in the laboratory following published methods[35–39]. Just before use, all amino acid derivatives were tested for homogeneity by thin-layer chromatography (Silufol plates, Kavalier, Czechoslovakia) in the 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). Detection was with ninhydrin or by the chlorination method. Solvents were evaporated on a rotary evaporator (bath temperature 30°C) "in vacuo"; DMF was evaporated at the same temperature "in vacuo" (150 Pa). Progress in peptide synthesis was followed by the ninhydrin[40] and bromophenol blue[41] tests.

The Synthesis of Peptides *Ib—Ii* on Polymer Support (Table II)

A mixture of the methylated Boc-Phe derivative (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 resin was washed with DCM (3 × 20 ml), 2-propanol (3 × 20 ml) and DCM (3 × 20 ml) and remains of the amino groups on the resin were acetylated with a mixture: acetic anhydride (2 ml)–DIEA (3 ml)–DCM (25 ml) for 2 h at room temperature. Than the Boc group was split off by stirring with a mixture: 50% TFA–10% anisole in DCM for 5 and 30 min and TFA.H-Phe-*p*-MBHA resin was neutralized by 5% DIEA in DCM (2 × 20 ml), 5 min. The synthetic cycle was completed by washing the H-Phe-*p*-MBHA resin with DCM (5 × 20 ml) and was repeated with 3 equivalents (1·5 mmol) of Boc-Asp(OBzl)OH, Boc-Met-OH, Boc-Trp(For)OH, Boc-Gly-OH and Boc-Met-OH. The Boc-hexapeptide resin was then treated with a liquid HF (20 ml) in the presence of ethanedithiol (1 ml) at 0°C for 1 h and after evaporation of HF the cleaved and deprotected hexapeptide amide was 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. This material was dissolved in DMF (20 ml) and acylated using Boc-Tyr-OSuc (0·57 g; 1·5 mmol) at room temperature in the presence of DIEA (0·26 ml; 1·5 mmol), 48 h. The DMF was evaporated, the residual oil was triturated with a water until it precipitated and the solid product was filtered off, washed with the 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) was added a complex of pyridine–SO_3 (0·80 g) and the mixture was stirred under nitrogen for 24 h at room temperature. The solvents were evaporated, the sulfated peptide was dissolved in an ice cooled 0·5M-$NaHCO_3$ (50 ml) and the solution was freeze dried. A methanol was added to the lyophilizate, the precipitated salt removed by filtration and the solvent evaporated at room temperature. Sulfate esters of the corresponding Boc-heptapeptides *Ib—Ii* were purified and separated by the reverse phase HPLC using gradient 0—50% of methanol (30 min) and 50—70% of methanol (50 min) in 0·1M ammonium acetate, pH 6·7. For analytical HPLC separation conditions see Table I.

Determination of Methylated Phenylalanines Configuration in Analogues *Ib—Ii*

The determination was carried out by modified procedure of Toth et al.[27]. The analogue (0·4 mg) from HPLC separation was hydrolysed in 6M-HCl at 110°C for 20 h. The solution was evapo-

rated in vacuo, one part of the hydrolysate was dissolved in 0·1M TRIS buffer (pH 7·5) (20 μl) and 1% solution (20 μl) of L-amino acid oxidase (Snake venom from Crotalux atrox, Serva) was added. The tube was filled with oxygen, tightly closed and incubated for 24 h at 38°C. After 24 h new enzyme was added and the incubation was continued for another 24 h. This reaction mixture was subjected to amino acid analysis which revealed the presence (D-enantiomer) or absence (L-enantiomer) of corresponding methylated phenylalanine in the analogue. While this procedure was satisfactory in the case of Phe(2-Me) and Phe(4-Me) enantiomers determination, the peaks of Phe(2,4-diMe) and Phe(2,4,6-triMe) derivatives were not accessible for measurement due to the interference with Trp peak in the spectrum. Therefore the second part of the hydrolysate non treated with enzyme was spotted directly to CHIRALPLATE(Macherey–Nagel GmbH, Düren, Germany) together with the corresponding methylated phenylalanine which was either intact or was treated by the same way like the analogue and also standards of the other amino acids presented in the analogue. 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 methylated phenylalanine) containing analogue could be determined.

RESULTS AND DISCUSSION

The biological activities of analogues *Ib*–*Ii* are summarized in Table III. As it can be seen, these peptides behave as full agonists of CCK-8. The analogues containing L- form of methylated phenylalanines (*Ib*, *Id*, *If*, *Ih*) exhibit almost the same potency (80–130%) as the standart compound in all of the bioassays. These results suggest that methylation of the phenylalanine side-chain does not interfere with the interaction peptide–receptor since this sterical hindrance does not change a conformation of the peptide recognized by receptors. As a result, no significant dissociation of the biological activities could be observed in the L-series. It means that both types of CCK receptors considered in the periphery (A) and central nervous system (B) do not discriminate between the Phe residue and its methylated derivatives placed in the C-terminus of the molecule. Thus, in agreement with previous suggestion[14], we can suppose this position to take part in the biological activity transduction only. Moreover, the process of transduction is able to tolerate and accept in full the L-isomer of the methylated Phe residues in the carboxyterminal part of the corresponding analogues.

As to the analogues modified in the C-terminus with the D-isomers of the methylated derivatives of Phe (*Ic*, *Ie*, *Ig*, *Ii*), we have observed decreased biological activities (8–62% of the potency of CCK-8) in all tests. Compared to CCK-8, especially the guinea pig gall bladder contraction potency was more decreased in the case of analogues *Ie* and *Ii* with *para*- methylated Phe residues: Phe(4-Me) and Phe(2,4,6-triMe) than in the case of those containing *ortho*- methylated Phe residues (*Ic*, *Ig*): Phe (2-Me) or Phe (2,6-diMe).

Taking in consideration that the C-terminal Phe residue or its derivatives in CCK are not engaged in a recognition process but in the biological activity transduction only we can suppose that the differences in structure and/or chirality in this position

will apply after a binding of the corresponding analogue to the receptor. In this secondary process also hydrophobic interactions might play an important role because the increased lipophility of the methylated Phe residues does not decrease the activity similarly to the analogue with aliphatic side-chain amino acid residue (Leu) in this position which exhibited the agonistic activity, too[14].

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Translated by the author (J.H.).