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New analogues of leucine-methionine-enkephalin†

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Abstract Nine analogues of the opioid pentapeptides leucine-/ methionine-enkephalinamide, involving replacement of amino acid at position 5 or amino acids at positions 2 and 5, have been synthesized by the solid phase method using mainly 9-fluorenylmethyloxycarbonyl amino acid trichlorophenyl esters in the presence of 1-hydroxybenzotriazole, the solid support being the Merrifield resin. All the analogues were effective in inhibiting the electri cally stimulated contractions of the guinea pig ileum (*in vitro*) and one of them, tyrosyl-D-norvalyl-glycyl-phenylalanyl-methioninamide was found to be about 82 times more active than morphine. They also exhibited analgesic activity as well as antidiarrhoeal activity in mice (*in vivo*).

Keywords. Fmoc-amino acid active esters; Merrifield resin; enkephalin analogues; biological activity; structure-activity studies.

Introduction

As part of our study of structure-activity relationships, a few analogues of enkephalin, resulting from the single or multiple replacements of amino acids at positions 1, 2 and 5 of the natural sequence, Tyr-Gly-Gly-Phe-Leu/Met, were reported by us earlier (Sivanandaiah *et al.,* 1985). In continuation of these studies, two analogues were obtained by replacement of the amino acid at position 5 only and 7 more by replacement of amino acids at both positions 2 and 5. The biological activities of these analogues were studied.

Materials and methods

All the amino acids used except glycine are of L-configuration unless otherwise specified. Melting points were determined using Leitz-Wetzlar melting point apparatus and are uncorrected. Thin-layer chromatography was carried out on silica gel G (G. Merck, Darmstadt, West Germany) plates using the solvent system, chloroform: methanol: acetic acid (40:5:5), and the R_f value is designated as R_f (CMA). 9-Fluorenylmethyloxycarbonyl derivatives (Fmoc) (amino acids) were obtained by the method of Chang *et al.* (1980) and their active esters according to Sivanandaiah and Gurusiddappa (1984). The completion of condensation and deprotection were monitored by Kaiser's test (Kaiser *et al.,* 1970).

The analogues (table 1) have been synthesized by the solid-phase method (Merrifield, 1963) employing the conventional Merrifield resin (G. Merck,

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Abbreviations used: Fmoc, 9-Fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; Boc, butoxycarbonyl; OTcp, trichlorophenyl ester; GPI, guinea pig ileum; Eth, ethionine; D-Nva, D-norvaline; Nle, norleucine.

Table 1. List of peptides synthesized.

Peptide no.	Sequence
I	Tyr-Gly-Gly-Phe-D-Nva-NH_2
II	Tyr-D-Phe-Gly-Phe-D-Nva-NH_2
III	Tyr-D-Ala-Gly-Phe-D-Nva-NH_2
IV	Tyr-D-Val-Gly-Phe-D-Nva-NH_2
V	Tyr-Gly-Gly-Phe-Eth-NH_2
VI	Tyr-D-Ala-Gly-Phe-Eth-NH_2
VII	Tyr-D-Met-Gly-Phe-Eth-NH_2
VIII	Tyr-D-Ser-Gly-Phe-D-Met-NH_2
IX	Tyr-D-Nva-Gly-Phe-D-Met-NH_2

Darmstadt, West Germany). The peptide chain was built using Fmoc amino acid active esters in the presence of 1-hydroxybenzotriazole (HOBt) following the protocol reported earlier (Sivanandaiah *et al.,* 1985). Fmoc group was removed by treatment with the less expensive diethylamine instead of the usually employed piperidine. The introduction of Ser, however, was effected by dicyclohexylcarbodiimide/ HOBt method. The N-terminal amino acid Tyr was introduced as butoxycarbonyl (Boc)-Tyr-trichlorophenyl ester (OTcp). The protected pentapeptides were then released from the resin by ammonolysis and the Boc group was cleaved from the protected peptides by treatment with 98% formic acid in the presence of anisole. The peptide salts were neutralised by treatment with IRA-400.

The synthetic peptides (I-IX) were assayed for their ability to inhibit the electrically induced contractions of the guinea pig ileum (GPI) (Kosterlitz and Watt, 1964). The analgesic and antidiarrhoeal activities of the analogues were studied in albino mice using Eddy's hot plate test (Eddy, 1932) and charcoal meal test (Lenaerts, 1974), respectively.

Results and discussion

The time required for the completion of coupling of each amino acid is given in table 2 and the yields of protected and free pentapeptides are given in table 3. The physical constants, analytical data and results of amino acid analyses are listed in tables 4 and 5. The relative potencies (GPI, analgesic and antidiarrhoeal) of these analogues are shown in tables 6 and 7.

Table 2. Reaction time for each amino acid residue.

Amino acid residue	Time (min)
Fmoc-Phe-OTcp	60
Fmoc-Gly-OTcp	60
Fmoc-D-Phe-OTcp	60
Fmoc-D-Ala-OTcp	50
Fmoc-D-Val-OTcp	90
Fmoc-D-Met-OTcp	90
Fmoc-D-Ser (DCC/HOBt)	120
Fmoc-D-Nva-OTcp	75
Boc-Tyr-OTcp	70

Table 3. Yields of protected and free peptides.

Peptide	Yields (%)	
	X = Boc*	X = H
X-Tyr-Gly-Gly-Phe-D-Nva-NH_2	75	83
X-Tyr-D-Phe-Gly-Phe-D-Nva-NH_2	73	82
X-Tyr-D-Ala-Gly-Phe-D-Nva-NH_2	74	83
X-Tyr-D-Val-Gly-Phe-D-Nva-NH_2	75	81
X-Tyr-Gly-Gly-Phe-Eth-NH_2	68	78
X-Tyr-D-Ala-Gly-Phe-Eth-NH_2	71	80
X-Tyr-D-Met-Gly-Phe-Eth-NH_2	65	77
X-Tyr-D-Ser-Gly-Phe-D-Met-NH_2	74	78
X-Tyr-D-Nva-Gly-Phe-D-Met-NH_2	72	79

*Overall yield based on the amount of amino acid (D-Nva Eth or D-Met) esterified to the resin.

GPI assay

The biological activities of these analogues reveal (table 6) that substitution of the amino acid at position 5 of enkephalin-amides by ethionine (Eth) or D-norvaline (D-Nva) leads to loss of activity (I and V). In accordance with earlier observations (Morley, 1980), the introduction of a D-amino acid residue in place of Gly^2 causes a marked increase in potency. Substitution by D-Phe, D-Ala and D-Val at position 2 of D-Nva^5-enkephalinamide (I) increases the activity 10 to 20-fold, whereas substitution by D-Ala and D-Met at position 2 of Eth^5-enkephalinamide (V) leads to 27 and 93-fold increase in potency, respectively. Similarly, in the case of D-Met^5-enkephalinamide which has a potency of only 0·105 relative to Met-enkephalin, substitution by D-Ser/D-Nva at position 2 leads to 24/789-fold increase in potency. However, it is apparent from the above results and other available data that the magnitude of increase in activity depends on the nature of the C-terminal part of the molecule. Accordingly, [D-Ala^2, Eth^5] -enkephalinamide (VI) is more active than the corresponding Pro^5-enkephalinamide but less active than [D-Ala^2, Nva^5]-enkephalinamide; further, [D-Met^2, Eth^5] -enkephalinamide (VII) is more active than both the corresponding Nva^5- and Pro^5-enkephalinamides. The incorporation of D-Nva in place of Leu^5 in D-Phe^2-, D-Ala^2- and D-Val^2-leucine enkephalinamides causes decrease in their activity. In the case of D-Ser^2 analogue, substitution by D-Met at position 5 leads to a 10-fold increase in activity; this peptide, however, is slightly less active than the Nle^5 analogue. The spatial orientation of the side chain is also crucial for activity as suggested by the low activity of [D-Ala^2, D-Nva^5]-enkephalinamide compared to [D-Ala^2, Nva^5]-enkephalinamide. Among the 9 synthetic analogues now reported in this paper, [D-Nva^2, D-Met^5]-enkephalinamide (IX) has been found to be the most potent. It is 82·82 times more active than morphine whereas D-Met^5-enkephalinamide has an activity of only 0·105 relative to Met-enkephalin.

Analgesic activity

The analgesic activity of the analogues was studied in albino mice by intravenous administration through the caudal vein at a dose of 15 mg/kg body weight. The

Table 4. Physical constants and analytical data of protected peptides.

Peptides	MP (°C)	R_fC	$[\alpha]_D^{25}$ (c, 1; DMF)	Molecular formula	Elemental analysis	%C	%H	%N
Boc-Tyr-Gly-Gly-Phe-D-Nva-NH_2	158–160	0·32	−13°	$C_{32}H_{44}N_6O_8$	Calcd.	60·0	6·88	13·13
					Found	59·75	6·68	13·29
Boc-Tyr-D-Phe-Gly-Phe-D-Nva-NH_2	140–142	0·51	−3°	$C_{39}H_{50}N_6O_8$	Calcd.	64·11	6·85	11·51
					Found	64·03	6·95	11·40
Boc-Tyr-D-Ala-Gly-Phe-D-Nva-NH_2	164–166	0·33	−17°	$C_{3_2}H_{46}N_6O_8$	Calcd.	60·55	7·03	12·84
					Found	60·36	7·23	12·62
Boc-Tyr-D-Val-Gly-Phe-D-Nva-NH_2	186–188	0·55	−20°	$C_{35}H_{50}N_6O_8$	Calcd.	61·59	7·33	12·32
					Found	61·39	7·22	12·46
Boc-Tyr-Gly-Gly-Phe-Eth-NH_2	166–168	0·41	−18°	$C_{33}H_{46}N_6O_8S$	Calcd.	57·73	6·71	12·25
					Found	57·43	6·70	12·19
Boc-Tyr-D-Ala-Gly-Phe-Eth-NH_2	172–174	0·47	−16°	$C_{34}H_{48}N_6O_8S$	Calcd.	58·29	6·86	12·00
					Found	58·42	6·64	12·23
Boc-Tyr-D-Met-Gly-Phe-Eth-NH_2	148–150	0·42	−6°	$C_{36}H_{52}N_6O_8S_2$	Calcd.	56·84	6·84	11·05
					Found	56·62	6·66	11·16
Boc-Tyr-D-Ser-Gly-Phe-D-Met-NH_2	154–155	0·67	+2°	$C_{33}H_{46}N_6O_9S$	Calcd.	56·41	6·55	11·97
					Found	56·43	6·75	11·79
Boc-Tyr-D-Nva-Gly-Phe-D-Met-NH_2	206–208	0·52	−8°	$C_{34}H_{48}N_6O_8S$	Calcd.	58·82	7·00	11·76
					Found	58·88	6·94	11·79

DMF, Dimethylformamide.

Table 5. Amino acid composition of protected peptides.

Peptide	Amino acid ratios								
	Tyr	Gly	Phe	Nva	Ala	Val	Eth	Met	Ser
Boc-Tyr-Gly-Gly-Phe-D-Nva-NH_2	1·00	2·04	1·08	0·88	—	—	—	—	—
Boc-Tyr-D-Phe-Gly-Phe-D-Nva-NH_2	1·02	1·15	1·93	0·90	—	—	—	—	—
Boc-Tyr-D-Ala-Gly-Phe-D-Nva-NH_2	0·92	1·02	1·00	1·06	0·96	—	—	—	—
Boc-Tyr-D-Val-Gly-Phe-D-Nva-NH_2	1·04	1·10	0·94	0·95	—	0·97	—	—	—
Boc-Tyr-Gly-Gly-Phe-Eth-NH_2	0·91	2·12	0·96	—	—	—	0·95	—	—
Boc-Tyr-D-Ala-Gly-Phe-Eth-NH_2	0·89	1·09	0·93	—	1·08	—	0·94	—	—
Boc-Tyr-D-Met-Gly-Phe-Eth-NH_2	1·06	1·14	0·98	—	—	—	0·91	0·90	—
Boc-Tyr-D-Nva-Gly-Phe-D-Met-NH_2	1·08	1·11	1·00	0·93	—	—	—	0·92	—
Boc-Tyr-D-Ser-Gly-Phe-D-Met-NH_2	1·09	1·05	1·04	—	—	—	—	0·99	0·95

Table 6. Relative GPI potencies of enkephalin analogues.

Name of the compound	Relative GPI potency
Morphine sulphate	1
Tyr-Gly-Gly-Phe-D-Nva-NH_2 (I)	0·0226
Tyr-D-Phe-Gly-Phe-D-Nva-NH_2 (II)	0·3816
Tyr-D-Ala-Gly-Phe-D-Nva-NH_2 (III)	0·2969
Tyr-D-Val-Gly-Phe-D-Nva-NH_2 (IV)	0·1955
Tyr-Gly-Gly-Phe-Leu-NH_2	0·21[a]
Tyr-D-Phe-Gly-Phe-Leu-NH_2	2·38[a]
Tyr-D-Ala-Gly-Phe-Leu-NH_2	2·10[a]
Tyr-D-Val-Gly-Phe-Leu-NH_2	0·28[a]
Tyr-Gly-Gly-Phe-Eth-NH_2 (V)	0·0917
Tyr-D-Ala-Gly-Phe-Eth-NH_2 (VI)	2·4908
Tyr-D-Met-Gly-Phe-Eth-NH_2 (VII)	8·5416
Tyr-D-Ala-Gly-Phe-Pro-NH_2	1·60[b]
Tyr-D-Ala-Gly-Phe-Nva-NH_2	3·20[b]
Tyr-D-Met-Gly-Phe-Pro-NH_2	5·71[b]
Tyr-D-Met-Gly-Phe-Nva-NH_2	7·27[b]
Tyr-Gly-Gly-Phe-D-Met-NH_2	0·105[c,d]
Tyr-D-Ser-Gly-Phe-D-Met-NH_2 (VIII)	2·4827
Tyr-D-Nva-Gly-Phe-D-Met-NH_2 (IX)	82·8192
Tyr-D-Ser-Gly-Phe-Leu-NH_2	0·26[a]
Tyr-D-Ser-Gly-Phe-Nle-NH_2	3·10[a]

[a]Sivanandaiah et al. (1985). [b]Mathur (1981). [c]Morley (1980). [d]Potency relative to Met-enkephalin (=1).

Table 7. Relative analgesic and antidiarrhoeal potencies of enkephalin analogues.

	Relative Potency	
Name of the compound	Analgesic activity	Antidiarrhoeal activity
Morphine sulphate	1	1
Tyr-Gly-Gly-Phe-Met	0·003[a]	
Tyr-Gly-Gly-Phe-D-Nva-NH_2 (I)	0·6500	1·2132
Tyr-D-Phe-Gly-Phe-D-Nva-NH_2 (II)	0·5833	0·8410
Tyr-D-Ala-Gly-Phe-D-Nva-NH_2 (III)	0·5610	0·9814
Tyr-D-Val-Gly-Phe-D-Nva-NH_2 (IV)	0·3433	1·0086
Tyr-Gly-Gly-Phe-Eth-NH_2 (V)	0·5626	0·6744
Tyr-D-Ala-Gly-Phe-Eth-NH_2 (VI)	0·5053	1·0963
Tyr-D-Met-Gly-Phe-Eth-NH_2 (VII)	0·6166	0·9631
Tyr-D-Ser-Gly-Phe-D-Met-NH_2 (VIII)	0·6610	0·7419
Tyr-D-Nva-Gly-Phe-D-Met-NH_2 (IX)	0·5553	0·8098
Tyr-D-Ala-Gly-Phe-D-Met-NH_2	16·6[a]	
Tyr-D-Ala-Gly-Phe-Met-NH_2	0·43[a]	

[a]Frederickson(I977).
Bioassays were performed at the Government College of Pharmacy, Bangalore.

potencies were compared to that of morphine and all the synthetic analogues possess 50–66% of the potency of morphine. By this method Met-enkephalin has been found to have an activity of only 0·003 relative to morphine but its analogue, [D-Ala^2, D-Met^5]-enkephalinamide has been reported to be 16·6 times more active. When we

modified the latter analogue by incorporating D-Ser or D-Nva in place of D-Ala the activity decreased considerably. Further, we also observed that the introduction of Eth in place of Met in [D-Ala^2 Met^5]-enkephalinamide (reported to have an activity of 0·43 relative to morphine) increases the activity slightly. Among the 9 synthetic analogues now reported, [D-Ser^2, D-Met^5]-enkephalinamide (VIII) has been found to be the most potent by this route of administration.

Antidiarrhoeal activity

Intraperitoneal administration at a dose of 30 mg/kg showed good antidiarrhoeal activity. Among the 9 analogues tested, peptides I, IV and VI are more potent whereas peptides III and VII are almost equipotent compared to morphine. Analogue I, the most potent in this assay, is the least potent in the GPI assay (table 7).

Conclusion

From the above results, it can be seen that a definite relationship seems to exist between the analgesic activity of the peptide and its potency in the GPI, and this has been observed by others also. Thus, the analogues (VI-IX) with high potency in the GPI show good analgesic activity as well.

Some enkephalin analogues, which are far less active compared to morphine in the GPI assay, show much higher potency as antidiarrhoeal agents, thus implying that the receptors involved in the antidiarrhoeal activity of enkephalins may be different from those involved in the GPI assay. This has also been observed by others; the peptide, Tyr-Ile-Asn-Met-Leu, with a structure considerably different from that of enkephalin, has proved to be an effective antidiarrhoeal agent (Morley, 1980).

References

Chang, C. D., Waki, M., Ahmad, M., Meienhofer, J., Lundell, E. O. and Haug, J. D. (1980) *Int. J. Peptide Protein Res.,* **15,** 59.

Eddy, N. S. (1932) *J. Pharmacol,* **45,** 339.

Frederickson, C. A. (1977) *Life Sci.,* **21,** 22.

Kaiser, E., Colescott, R. R., Bossinger, C. D. and Cook, P. I. (1970) *Anal. Biochem.,* **34,** 595.

Kosterlitz, H. W. and Watt, A. J. (1964) *Br. J. Pharmacol,* **33,** 266.

Lenaerts, F. M. (1974) in *Synthetic antidiarrhoeal drugs* (eds V. B. William and Harbanslar) (New York: Marcel Decker) p. 66.

Mathur, K. B. (1981) in *Current status of centrally acting peptides* (ed. B. N. Dhawan) (Oxford: Pergmon Press) p. 37.

Merrifield, R. B. (1963) *J. Am. Chem. Soc,* **85,** 2149.

Morley, J. S. (1980) *Annu. Rev. Pharmacol. Toxicol,* **20,** 81.

Sivanandaiah, K. M. and Gurusiddappa, S. (1984) *Indian J. Chem.,* **B23,** 372.

Sivanandaiah, K. M. Gurusiddappa, S. and Rangaraju, N. S. (1985) *J. Biosci*, **8,** 263