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Synthetic Studies on Enkephalin Analogs. II.[1,2] Enhanced Analgesic Activity of H-Tyr-D-Ala-Gly-Phe-NHNH-CO-CH_2CH_3 following N-Methylation of Tyr and Phe

Susumu Shinagawa, Masahiko Fujino,* Harumitsu Ishii, and Kiyohisa Kawai

Central Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka, 532, Japan

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In order to study the effect of N-methylation of a potent enkephalin analog, H–Tyr–D-Ala–Gly–Phe–NHNH–CO–CH_2CH_3, on analgesic activity, six new analogs were synthesized in which one or more of amino acid residues and the acyl-hydrazide constituting the tetrapeptide acyl-hydrazide were N-methylated. N-Methylation of both Tyr at position 1 and Phe at position 4 of the analog led to a derivative which was twice as potent as morphine. On the other hand, N-methylation of D-Ala at position 2, Gly at position 3 or NHNH–CO–CH_2CH_3 at position 5 markedly decreased the analgesic potency. Five analogs with a modified tyrosine residue at position 1 of the tetrapeptide acyl-hydrazide were also synthesized in order to assess the role of the N-terminal Tyr residue in the biological activity.

Keywords——enkephalin analog; analgesia; tetrapeptide; acyl-hydrazide; N-methylation; morphine; structure-activity relations

In the preceding paper,[1] we reported the synthesis and analgesic activity of enkephalin-like tetrapeptide derivatives and found that H–Tyr–D-Ala–Gly–Phe–NHNH–CO–R (R= lower alkyl) was half as potent as morphine after intravenous or subcutaneous injection in the mouse hot-plate test. Next, we synthesized six tetrapeptide acyl-hydrazides in which one or more of the amino acid residues and acyl-hydrazide part were N-methylated, in order to study the effect of N-methylation on analgesic activity. This series of analogs was: H–MeTyr–D-Ala–Gly–Phe–NHNH–CO–CH_2CH_3 (I), H–Tyr–D-MeAla–Gly–Phe–NHNH–CO–CH_2CH_3 (II), H–Tyr–D-Ala–Sar–Phe–NHNH–CO–CH_2CH_3 (III), H–Tyr–D-Ala–Gly–MePhe–NHNH–CO–CH_2CH_3 (IV), H–Tyr–D-Ala–Gly–Phe–N(CH_3)NH–CO–CH_2CH_3 (V), H–MeTyr–D-Ala–Gly–MePhe–NHNH–CO–CH_2CH_3 (VI). It was interesting to examine the activity of these analogs because Roemer *et al.*[3,4] reported that N-methylation of Phe of [D-Ala^2, Met(O)-ol^5]-enkephalin resulted in a large increase of activity.

This paper also deals with the synthesis and activity of five analogs with a modified Tyr residue at position 1 of the tetrapeptide acyl-hydrazide; these were prepared in order to obtain information on the structure-activity relations in the N-terminal part. The following analogs were synthesized: H–Tyr–Tyr–D-Ala–Gly–Phe–NHNH–CO–$CH_2CH_2CH_3$ (VII), H–Arg–Tyr–D-Ala–Gly–Phe–NHNH–CO–$CH_2CH_2CH_3$ (VIII), H–Lys–Tyr–D-Ala–Gly–Phe–NHNH–CO–$CH_2CH_2CH_3$ (IX), H–β–Ala–Tyr–D-Ala–Gly–Phe–NHNH–CO–$CH_2CH_2CH_3$ (X), H–Tyr($COCH_3$)–D-Ala–Gly–Phe–NHNH–CO–$CH_2CH_2CH_3$ (XI). Since N-substitution of the N-terminal Tyr of Met-enkephalin by Tyr, Arg, or Lys yields a hexapeptide which retains significant activity in *in vitro* assay,[5] we though it interesting to examine the *in vivo* activity of the above compounds (analogs VII—X). The analog VIII is especially interesting as β-lipotropin has an Arg residue at position 60, which precedes the sequence -Tyr^{61}–Gly–Gly–Phe–Met-.[6] Synthesis of the analog XI was planned by analogy to the well-known opiate heroin which is prepared by acetylation of two hydroxyl groups of morphine and is five times more active than morphine. Thus, acetylation of the hydroxyl group of N-terminal Tyr might be promising.

Table I. Physicochemical Properties of Intermediates

Compound No.	Structure	mp (°C)	$[\alpha]_D$ in DMF (temp., conc.)	TLC[a) Rf^1	Formula	Analysis (%) Found C	Found H	Found N	Calcd C	Calcd H	Calcd N
1	Z-MeTyr(Bu^t)-OH	94—95	−54.0°(21, 0.48)	0.61	$C_{22}H_{27}NO_5$	68.52	6.96	3.64	68.55	7.06	3.63
2	Z-D-Ala-Gly-OH	128—129	−1.4°(24, 0.55)	0.25	$C_{13}H_{16}N_2O_5$	56.42	5.70	9.66	55.71	5.75	10.00
3	Z-Phe-NHNH-$COCH_2CH_3$	194—196	−21.2°(21, 0.43)	0.62	$C_{20}H_{23}N_3O_4$	65.21	6.50	11.01	65.02	6.28	11.38
4	Z-MeTyr(Bu^t)-D-Ala-Gly-OH	91—96	−52.9°(21, 0.41)	0.45	$C_{27}H_{35}N_3O_7$	63.59	7.11	7.82	63.14	6.87	8.18
5	Z-MeTyr(Bu^t)-D-Ala-Gly-Phe-NHNH-CO-CH_2CH_3	143—144	−43.7°(21, 0.40)	0.51	$C_{39}H_{50}N_6O_8$	64.25	7.08	11.49	64.09	6.90	11.50
6	Z-Tyr-D-MeAla-Gly-OH	85—88	+46.2°(21, 0.40)	0.20	$C_{23}H_{27}N_3O_7$	60.61	6.08	8.88	60.38	5.95	9.19
7	Z-Tyr-D-MeAla-Gly-Phe-NHNH-CO-CH_2CH_3	139—140	+26.7°(21, 0.48)	0.40	$C_{35}H_{42}N_6O_8$	62.01	6.62	12.07	62.30	6.27	12.46
8	Z-Sar-Phe-NHNH-CO-CH_2CH_3	165—166	−2.6°(25, 0.47)	0.49	$C_{23}H_{28}N_4O_5$	62.92	6.52	12.43	62.71	6.41	12.72
9	Z-D-Ala-Sar-Phe-NHNH-CO-CH_2CH_3	147—148	−10.9°(25, 0.43)	0.50	$C_{26}H_{33}N_5O_6$	60.89	6.75	13.43	61.04	6.50	13.69
10	Z-Tyr-D-Ala-Sar-Phe-NHNH-CO-CH_2CH_3	179—180	−27.0°(25, 0.50)	0.43	$C_{35}H_{42}N_6O_8$	61.82	6.26	12.32	62.30	6.27	12.46
11	Z-Tyr-D-Ala-Gly-MePhe-OMe	103—104	−17.2°(23, 0.31)	0.68	$C_{33}H_{38}N_4O_8$	64.30	6.20	8.99	64.06	6.19	9.06
12	Z-Tyr-D-Ala-Gly-MePhe-$NHNH_2$	95—96 (dec.)	−26.8°(23, 0.50)	0.29	$C_{32}H_{38}N_6O_7$	61.85	6.28	13.09	62.12	6.19	13.59
13	Z-Tyr-D-Ala-Gly-MePhe-NHNH-CO-CH_2CH_3	139—141 (dec.)	−32.0°(24, 0.43)	0.35	$C_{35}H_{42}N_6O_8 \cdot 3H_2O$	57.81	6.30	11.65	57.68	6.63	11.53
14	Z-Phe-N(CH_3)-NH_2	120—121	+36.0°(22, 0.38)	0.60	$C_{18}H_{21}N_3O_3$	66.88	6.88	12.91	66.03	6.47	12.84
15	Z-Phe-N(CH_3)NH-$COCH_2CH_3$	91—92	+102.4°(22, 0.50)	0.60	$C_{21}H_{25}N_3O_4$	66.02	6.57	10.65	65.78	6.57	10.96
16	Z-Tyr-D-Ala-Gly-Phe-N(CH_3)NH-CO-CH_2CH_3	115—117	+30.0°(22, 0.50)	0.28	$C_{35}H_{42}N_6O_8 \cdot 1/2HO_2$	61.90	6.88	11.57	61.48	6.34	11.28
17	Z-MeTyr(Bu^t)-D-Ala-Gly-MePhe-NHNH-CO-CH_2CH_3	110—112 (dec.)	−54.7°(25, 0.45)	0.61	$C_{40}H_{52}N_6O_8$	64.28	7.47	11.04	64.50	7.04	11.28
18	Z-Tyr-Tyr-D-Ala-Gly-Phe-NHNH-CO-$CH_2CH_2CH_3$	204—206	−29.0°(21, 0.50)	0.31	$C_{44}H_{51}N_7O_{10} \cdot 1/2H_2O$	62.43	6.35	11.58	62.39	6.18	11.58
19	Z-Arg(NO_2)-Tyr-D-Ala-Gly-Phe-NHNH-CO-$CH_2CH_2CH_3$	166—167	−18.4°(21, 0.38)	0.20	$C_{41}H_{53}N_{11}O_{11}$	56.30	6.22	16.73	56.22	6.09	17.55
20	Z-Lys(Z)-Tyr-D-Ala-Gly-Phe-NHNH-CO-$CH_2CH_2CH_3$	209—211	−26.8°(21, 0.50)	0.53	$C_{49}H_{60}N_8O_{11}$	65.21	6.45	11.88	62.80	6.45	11.96
21	Z-β-Ala-Tyr-D-Ala-Gly-Phe-NHNH-CO-$CH_2CH_2CH_3$	229—231	−12.6°(22, 0.50)	0.25	$C_{38}H_{47}N_7O_9 \cdot 1/2H_2O$	60.22	6.37	12.72	60.47	6.54	12.99
22	Z-Tyr($COCH_3$)-D-Ala-Gly-Phe-NHNH-CO-$CH_2CH_2CH_3$	196—199	−22.7°(23, 0.31)	0.49	$C_{37}H_{44}N_6O_9$	61.52	6.17	11.62	62.00	6.19	11.72

a) See "Experimental."

All the analogs were synthesized by the solution method. Z-N-Methyl-amino acids were prepared by the NaH/CH_3I method, which was reported to give optically pure N-methyl-amino acids by Benoiton *et al.*[7] Analogs I and II were synthesized by a route similar to route A described in the previous paper,[1] with Z-MeTyr(Bu^t)-D-Ala-Gly-OH (**4**) and Z-Tyr-D-MeAla-Gly-OH (**6**) instead of Z-Tyr-D-Ala-Gly-OH, respectively. For the synthesis of the analog III, Z-Tyr-D-Ala-Sar-Phe-NHNH-CO-CH_2CH_3 (**10**) was synthesized by the stepwise elongation method using HONB-active esters.[8] For the synthesis of the analog IV, Z-Tyr-D-Ala-Gly-OH was condensed with H-MePhe-OMe by the DCC-HONB method[8] and the resulting methyl ester was treated with $NH_2NH_2 \cdot H_2O$ to yield Z-Tyr-D-Ala-Gly-MePhe-$NHNH_2$ (**12**). The compound **12** was acylated with propionic acid by the HOBT-DCC method[9] to give the protected peptide, which was deblocked by hydrogenation. Analog V was obtained by a route similar to route A[1] using Z-Tyr-D-Ala-Gly-OH[1] and Z-Phe-N-(CH_3)NH-CO-CH_2CH_3 (**15**), which was prepared the reaction of Z-Phe-ONB[8] with NH_2NH-CH_3, followed by acylation with anhydrous propionic acid.

For the synthesis of analogs VII, VIII, IX and X, the corresponding Z-amino acid was condensed with H-Tyr-D-Ala-Gly-Phe-NHNH-CO-$CH_2CH_2CH_3$[1] by the HONB-active ester method.[8] The resulting protected pentapeptide was deblocked by hydrogenolysis over Pd-black catalyst and purified by Sephadex LH-20 gel filtration to give the desired compound. Analog XI was prepared by acetylation with anhydrous acetic acid of Z-Tyr-D-Ala-Gly-Phe-NHNH-CO-$CH_2CH_2CH_3$,[1] followed by hydrogenation.

All the analogs thus obtained were chromatographically homogeneous and gave the expected amino acid ratios. The propionyl or *n*-butyryl moiety of the acyl-hydrazide part and the N-methyl group of N-methyl amino acid residues were confirmed by nuclear magnetic

Table II. Physicochemical Properties of Synthetic Analogs

Analog I:	$[\alpha]_D^{27}$ +31.5° (c=0.22, MeOH), Rf^2=0.19; Rf^3=0.42; Rf^4=0.49.[a] Amino acid analysis: Gly 1.00; Ala 0.81; MeTyr 0.71; Phe 1.28 (84%).[b] ^{1}H-NMR(D_2O), δ: 2.72 (3H, s, N-C<u>H</u>$_3$ of MeTyr).
Analog II:	$[\alpha]_D^{21}$ +46.0° (c=0.22, MeOH), Rf^2=0.20; Rf^3=0.40; Rf^4=0.50. Amino acid analysis: Gly 1.00; Tyr 1.05; Phe 1.06 (81%). ^{1}H-NMR(D_2O), δ: 2.98 (3H, s, N-C<u>H</u>$_3$ of MeAla).
Analog III:	$[\alpha]_D^{25}$ +6.7° (c=0.39, MeOH), Rf^2=0.21; Rf^3=0.39; Rf^4=0.48. Amino acid analysis: Ala 1.00; Tyr 0.86; Phe 1.00 (86%). ^{1}H-NMR (D_2O), δ: 2.94 (3H, s, N-C<u>H</u>$_3$ of Sar).
Analog IV:	$[\alpha]_D^{24}$ −4.1° (c=0.27, MeOH), Rf^2=0.12; Rf^3=0.42; Rf^4=0.60. Amino acid analysis: Gly 1.00; Ala 1.03; Tyr 1.24; MePhe 0.96 (80%). ^{1}H-NMR (D_2O), δ: 2.95 (3H, s, N-C<u>H</u>$_3$ of MePhe).
Analog V:	$[\alpha]_D^{22}$ +98.2° (c=0.36, MeOH), Rf^2=0.20; Rf^3=0.36; Rf^4 =0.51. Amino acid analysis: Gly 1.00; Ala 1.01; Tyr 0.10[c]; Phe 0.94 (82%). ^{1}H-NMR (D_2O), δ: 3.18 [3H, s, N(C<u>H</u>$_3$)-NH-CO]
Analog VI:	$[\alpha]_D^{23}$ +14.4° (c=0.25, MeOH), Rf^2=0.21; Rf^3=0.52; Rf^4=0.60. Amino acid analysis: Gly 1.00; Ala 0.89; MeTyr 0.71; MePhe 0.98 (84%). ^{1}H-NMR (D_2O), δ: 2.72 (3H, s, N-C<u>H</u>$_3$ of MeTyr), 2.95 (3H, s, N-C<u>H</u>$_3$ of MePhe).
Analog VII:	$[\alpha]_D^{21}$ +1.3° (c=0.31, MeOH), Rf^2=0.15; Rf^3=0.54; Rf^4=0.59. Amino acid analysis: Gly 1.00; Ala 0.90; Tyr 1.63; Phe 0.88 (84%).
Analog VIII:	$[\alpha]_D^{21}$ +16.3° (c=0.32, MeOH), Rf^3=0.19; Rf^4=0.44; Rf^5=0.69. Amino acid analysis: Gly 1.00; Ala 0.83; Tyr 0.74; Phe 0.78; Arg 0.70 (83%).
Analog IX:	$[\alpha]_D^{21}$ +18.8° (c=0.36, MeOH), Rf^3=0.21; Rf^4=0.44; Rf^5=0.70. Amino acid analysis: Gly 1.00; Ala 1.00; Tyr 0.91; Phe 0.98; Lys 0.94 (82%).
Analog X:	$[\alpha]_D^{22}$ +20.0° (c=0.33, MeOH), Rf^2=0.10; Rf^3=0.30; Rf^4=0.58. Amino acid analysis: Gly 1.00; Ala 0.99; Tyr 0.96; Phe 1.00; β-Ala 1.06 (81%).
Analog XI:	$[\alpha]_D^{23}$ +25.3° (c=0.33, MeOH), Rf^2=0.23; Rf^3=0.57; Rf^4=0.63. Amino acid analysis: Gly 1.00; Ala 0.95; Tyr 0.88; Phe 0.98 (80%). ^{1}H-NMR (D_2O), δ: 2.45 (3H, s, Tyr(CO-C<u>H</u>$_3$)].

a) Solvent systems used for TLC are described in "Experimental."
b) Average recovery. c) See ref. 14.

resonance (NMR) spectral measurements. The physicochemical properties of intermediates and analogs of enkephalin are listed in Tables I and II, respectively.

The analgesic activities of the eleven newly synthesized analogs were measured in the same way as described previously,[1] and compared with that of the parent peptide, FK-33-824[3,4] or the Bajusz compound (H–Tyr–D-Met–Gly–Phe–Pro–NH_2).[10]

Table III. Analgesic Activity of N-Methylated Tetrapeptide Acyl-hydrazides

Compound No.	Structure	Relative potency[a]	
		i.v.	*s.c.*
I	H–MeTyr–D-Ala–Gly–Phe–NHNH–$COCH_2CH_3$		0.5
II	H–Tyr–D-MeAla–Gly–Phe–NHNH–$COCH_2CH_3$		0.025
III	H–Tyr–D-Ala–Sar–Phe–NHNH–$COCH_2CH_3$		<0.05
IV	H–Tyr–D-Ala–Gly–MePhe–NHNH–$COCH_2CH_3$		1.0
V	H–Tyr–D-Ala–Gly–Phe–N(CH_3)NH–$COCH_2CH_3$		<0.05
VI	H–MeTyr–D-Ala–Gly–MePhe–NHNH–$COCH_2CH_3$		2.0
	H–Tyr–D-Ala–Gly–Phe–NHNH–$COCH_2CH_3$[b]	0.5	0.5
	H–Tyr–D-Met–Gly–Phe–Pro–NH_2[c]		0.5
	H–Tyr–D-Ala–Gly–MePhe–Met(O)–ol[d]	2.5	4.0

a) Morphine=1; minimum effective dose of morphine·HCl=0.5 mg/kg (weight/weight). *b*) See ref. 1. *c*) The Bajusz compound, see ref. 10. *d*) FK-33-824, see refs. 3 and 4.

As shown in Table III, N-methylation of the fourth amino acid residue, Phe, of H–Tyr–D-Ala–Gly–Phe–NHNH–CO–CH_2CH_3 enhanced the activity to twice that of the parent peptide. N-Methylation of both Tyr and Phe of the tetrapeptide acyl-hydrazide led to a derivative which was twice as potent as morphine. The increase in activity upon N-methylation at position 4 may be largely, if not totally, due to protection from deactivation by enzymatic cleavage of the –Gly–Phe– amide bond.[11]

In contrast, N-methylation of D-Ala at position 2 or Gly at position 3 or NHNH–CO–CH_2CH_3 at position 5 markedly decreased the analgesic potency. The decrease of activity upon N^1-methylation of the hydrazide part of H–Tyr–D-Ala–Gly–Phe–N^1H–N^2H–CO–CH_2CH_3 is particularly interesting, because N^2-alkylation of the hydrazide part resulted in about the same potency as that of the parent peptide, as described in the preceding paper.[1] The remarkable loss of activity that resulted from the above N-methylation at position 2, 3 or 5 was presumably due to destruction of the conformation required for a peptide-receptor interaction. In other words, intact hydrogens in the first, second and fourth amide bonds are necessary for optimal analgesic activity.

Table IV. Analgesic Activities of Tetrapeptide Acyl-hydrazides with a Modified Tyrosine Residue

Analog No.	H–X–D-Ala–Gly–Phe–NHNH–CO–$CH_2CH_2CH_3$	Relative potency[a] (morphine=1) in *s.c.*
VII	Tyr–Tyr	0.25
VIII	Arg–Tyr	0.50
IX	Lys–Tyr	0.50
X	β-Ala–Tyr	<0.05
XI	Tyr($COCH_3$)	0.25
	Tyr[b]	0.50

a) Minimum effective dose of morphine·HCl=0.5 mg/kg (weight/weight).
b) See ref. 1.

Table IV indicates that addition of Tyr, Arg or Lys to the N-terminus of H–Tyr–D-Ala–Gly–Phe–NHNH–CO–$CH_2CH_2CH_3$ resulted in a pentapeptide which was approximately as active as the parent molecule, while addition of β-Ala resulted in complete loss of activity. However, it was reported that N-substitution of Tyr at position 1 of Met-enkephalin by Tyr, Arg or Lys resulted in a hexapeptide which was three or four times less potent than Met-enkephalin in the *in vitro* test.[5] The discrepancy between the *in vitro* and *in vivo* results may be interpretated by considering that *in vivo* the X–Tyr (X=Tyr, Arg, Lys) amide bond is easily cleaved by plasma and tissue enzymes to produce a biologically potent tetrapeptide acyl-hydrazide. This view was indirectly supported by the inactivity of the analog X, whose β–Ala–Tyr amide bond is stable to enzymes. Similar results have recently been reported by Pless *et al.*, who found that addition of D-Phe, but not Phe, to the N-terminus of H–Tyr–D-Ala–Gly–MePhe–Met(O)-ol reduced the analgesic activity.[12]

Acetylation of the tyrosine hydroxyl group did not increase the potency, in contrast to the case of heroin, and resulted in an analog with about 50% activity, suggesting transformation of the analog XI to the parent peptide *in vivo*. This result is in agreement with the recent study by Kiso *et al.*[13] on H–Tyr–D-Met(O)–Gly–Phe–ol.

Experimental

General experimental methods employed are essentially the same as described in the preceding paper. Amino acid analyses were run on a Hitachi KLA-3B or Hitachi 835 amino acid analyzer. When MePhe or MeTyr was present in hydrolysates, they were detected on the latter analyzer, and the recovery could be calculated as well as that of other amino acids. Thin layer chromatography was performed on silica gel (precoated silica gel plate 60 F_{254}, Merck). The solvents employed were: Rf^1, $CHCl_3$–MeOH–AcOH (9: 1: 0.5); Rf^2, $CHCl_3$–MeOH–AcOH (8: 2: 0.5); Rf^3, AcOEt–pyridine–AcOH–H_2O (60: 20: 6: 11); Rf^4, *n*-butanol–AcOH–H_2O (4: 1: 1); Rf^5, AcOEt–*n*-butanol–AcOH–H_2O (1: 1: 1: 1).

Z-MeTyr(Bu^t)-OH (1)——Z–Tyr(Bu^t)–OH·DCHA[15] (30.4 g) was dissolved in AcOEt (200 ml), and the solution was washed with 5% aqueous citric acid and water, then dried over anhydr. Na_2SO_4 and concentrated. The residue and CH_3I (20.6 ml) were dissolved in THF (150 ml), and NaH dispersion (7.3 g) was added to the solution under stirring at 0°C. After the suspension had been stirred at room temperature for 24 h, AcOEt (100 ml) and H_2O (15 ml) were added to this mixture. The solvent was evaporated, ether was added and the evaporation was repeated. The residue was dissolved in AcOEt (200 ml). The AcOEt solution was washed with 5% aqueous citric acid and H_2O, dried (Na_2SO_4), and concentrated. The residue was crystallized from AcOEt–pet. ether; yield, 13.5 g (64%), mp 94—95°C, $[\alpha]_D^{21}$ −54.0° (c=0.48, DMF), Rf^1 =0.61. *Anal.* Calcd for $C_{22}H_{27}NO_5$: C, 68.55; H, 7.06; N, 3.63. Found: C, 68.52; H, 6.96; N, 3.64.

Z-D-Ala-Gly-OH (2)——Z–D-Ala–OH (22.3 g) and HONB (19.7 g) were dissolved in THF (100 ml), and DCC (22.6 g) was added under cooling at 0°C. After the mixture had been stirred for 4 h, the DC-urea precipitate was filtered off. The filtrate was combined with a mixture of glycine (7.5 g) and $NaHCO_3$ (7.5 g) in H_2O (40 ml), and the whole was stirred at room temperature overnight. The THF was evaporated, and the residue was taken up in AcOEt (200 ml). The AcOEt solution was washed with 1 N HCl (100 ml) and H_2O, and dried over anhydr. Na_2SO_4. The AcOEt was evaporated and the resulting residue was crystallized from pet. ether and recrystallized from AcOEt–pet. ether; yield, 21.0 g (75%), mp 128—129°C, $[\alpha]_D^{24}$ −1.4° (c=0.55, DMF). Rf^1=0.25. *Anal.* Calcd for $C_{13}H_{16}N_2O_5$: C, 55.71; H, 5.75; N, 10.00. Found: C, 56.42; H, 5.70; N, 9.66.

Z-Tyr-D-Ala-Gly-MePhe-OMe (11)——Z–MePhe–OH[7] (1.8 g) was dissolved in MeOH (20 ml), and then 5 N HCl–dioxane (2 ml) was added. After the solution had been allowed to stand at room temperature overnight, the solvent was evaporated and the residue was extracted with AcOEt (100 ml). The extract was washed with H_2O and dried over anhydr. Na_2SO_4. The AcOEt was evaporated and the residue was dissolved in MeOH (50 ml) and hydrogenated over Pd-black catalyst. The catalyst was filtered off, and the filtrate was evaporated to dryness to give H–MePhe–OMe as an oil, which was dissolved in DMF (20 ml). Z–Tyr–D-Ala–Gly–OH[1] (1.55 g) and HONB (0.75 g) were added to the solution, then DCC (0.80 g) was added under cooling at 0°C. The mixture was stirred at 0°C for 5 h and at room temperature overnight. The DC-urea precipitate was filtered off and filtrate was evaporated to dryness. The residue was treated in the usual manner (extracted with AcOEt, washed with 5% $NaHCO_3$, 0.1 N HCl and H_2O, dried over anhydr. Na_2SO_4, and concentrated). The resulting residue was treated with ether, collected by filtration and purified by reprecipitation from MeOH–ether; yield, 1.2 g (56%), mp 103—104°C, $[\alpha]_D^{23}$ −17.2° (c=0.31, MeOH), Rf^1=0.68. *Anal.* Calcd for $C_{33}H_{38}N_4O_8$: C, 64.06; H, 6.19; N, 9.06. Found: C, 64.30; H, 6.20; N, 8.99.

Z-Tyr-D-Ala-Gly-MePhe-NHNH$_2$ (12)——The compound **11** (1.0 g) was dissolved in MeOH (20 ml), and $NH_2NH_2 \cdot H_2O$ (0.5 ml) was added to the solution. The mixture was allowed to stand at room temperature

overnight, then the MeOH was evaporated. The residue was treated with ether to give a powder, which was reprecipitated from MeOH–ether; yield, 0.82 g (82%), mp 95—96°C (dec.), $[\alpha]_D^{23}$ −26.8° (c=0.50, DMF), Rf^1=0.29. *Anal.* Calcd for $C_{32}H_{38}N_6O_7$: C, 62.12; H, 6.19; N, 13.59. Found: C, 61.85; H, 6.28; N, 13.09.

Z-Tyr-D-Ala-Gly-MePhe-NHNH-CO-CH_2CH_3 (13)——The compound **12** (426 mg) was dissolved in DMF (5 ml), and propionic acid (0.07 ml), HOBT (130 mg) and DCC (200 mg) were added under cooling. The mixture was stirred at 0°C for 5 h then at room temperature overnight. The insolubles were filtered off, then the filtrate was evaporated to dryness and the residue was treated in the usual manner. The resulting residue was treated with ether and collected by filtration; yield, 410 mg (87%), mp 139—141°C (dec.), $[\alpha]_D^{24}$ −32.0° (c=0.43, DMF), Rf^1=0.35. *Anal.* Calcd for $C_{35}H_{42}N_6O_8\cdot 3H_2O$: C, 57.68; H, 6.63; N, 11.53. Found: C, 57.81; H, 6.30; N, 11.65.

Z-Phe-N(CH_3)-NH_2 (14)——Z–Phe–ONB[8] (6.9 g) and NH_2NH–CH_3 (0.8 ml) were dissolved in THF (20 ml) and the mixture was stirred at room temperature for 4 h. The THF was evaporated and the residue was dissolved in 3% MeOH–$CHCl_3$ (15 ml). The solution was applied to a column (silica gel, 80 g), which was eluted with the same solvent. The desired fractions (110—220 ml) were combined and evaporated to dryness to give crystals, which were collected by filtration; yield, 1.8 g (37%), mp 120—121°C, $[\alpha]_D^{22}$ +36.0° (c=0.38, DMF), Rf^1=0.60. *Anal.* Calcd for $C_{18}H_{21}N_3O_3$: C, 66.03; H, 6.47; N, 12.84. Found: C, 66.88; H, 6.88; N, 12.91. ^{1}H-NMR ($CDCl_3$), δ: 3.04 [3H, s, CO–N($C\underline{H}_3$)–NH_2], 3.34 [2H, s, CO–N(CH_3)–N$\underline{H}_2$]. On the other hand, Z–Phe–NHNH–CH_3 was obtained from other fractions (350—430 ml) from the above chromatography, ^{1}H-NMR ($CDCl_3$), δ: 2.44 [3H, s, CO–NHNH–C$\underline{H}_3$].

Z-Phe-N(CH_3)-NH-CO-CH_2CH_3 (15)——The compound **14** (1.3 g) was dissolved in THF (50 ml), then anhydrous propionic acid (0.58 ml) and TEA (0.6 ml) were added at 0°C. The whole was stirred at room temperature for 4 h, then the THF was evaporated and the residue was dissolved in AcOEt (100 ml). The AcOEt solution was washed with water, dried over anhydr. Na_2SO_4 and then concentrated. The resulting crystals were collected by filtration and recrystallized from AcOEt; yield, 1.10 g (78%), mp 91—92°C, $[\alpha]_D^{22}$ +102.4° (c=0.50, DMF), Rf^1=0.60. *Anal.* Calcd for $C_{21}H_{25}N_3O_4$: C, 65.78; H, 6.57; N, 10.96. Found: C, 66.02; H, 6.57; N, 10.65.

Z-MeTyr(Bu^t)-D-Ala-Gly-MePhe-NHNH-CO-CH_2CH_3 (17)——Z–MePhe–OMe (1.1 g), which was prepared as described for the synthesis of the compound **11**, was dissolved in MeOH (50 ml) and catalytic reduction was carried out with Pd-black catalyst. The catalyst was filtered off and the filtrate was evaporated to dryness. The resulting residue was dissolved in DMF (10 ml). On the other hand, **2** (0.92 g) and HONB (0.71 g) were dissolved in DMF (10 ml), and DCC (0.75 g) was added under cooling. The mixture was stirred at 0°C for 4 h, and the DC-urea precipitate was filtered off. The filtrate was combined with the above-mentioned amine component and the mixture was stirred at room temperature overnight. The DMF was evaporated and the residue was treated in the usual manner. The resulting oil (2.5 g) was dissolved in MeOH (20 ml), then $NH_2NH_2\cdot H_2O$ (1.5 ml) was added. The solution was allowed to stand overnight, then the MeOH was evaporated. The residue was treated in the usual manner to give Z–D-Ala–Gly–MePhe–$NHNH_2$ as an oil, which was dissolved in THF (20 ml). To this solution, propionic acid (0.19 ml), HOBT (0.38 g) and DCC (0.45 g) were added at 0°C. The mixture was stirred at 0°C for 4 h then at room temperature overnight. The insolubles were filtered off and the filtrate was evaporated to dryness. The residue was treated in the usual manner and the oil obtained was dissolved in MeOH (50 ml). The catalytic hydrogenation was carried out with Pd-black. The catalyst was filtered off and the filtrate was evaporated to dryness. The residue was dissolved in THF (10 ml). On the other hand, **1** (0.73 g) was dissolved in THF (10 ml) and HONB (0.41 g) and DCC (0.43 g) were added under cooling at 0°C. After being stirred at 0°C for 4 h, the mixture was filtered to remove the DC-urea precipitate. The filtrate was combined with the above mentioned amine component and the mixture was stirred at room temperature overnight. The THF was evaporated and the residue was treated in the usual manner. The resulting residue was treated with ether to give a powder, which was purified by reprecipitation from MeOH–ether; yield, 1.2 g (86%), mp 110—112°C (dec.), $[\alpha]_D^{25}$ −54.7° (c=0.45, DMF), Rf^1=0.61. *Anal.* Calcd for $C_{40}H_{52}N_6O_8$: C, 64.50; H, 7.04; N, 11.28. Found: C, 64.28; H, 7.47; N, 11.04.

Z-Tyr-Tyr-D-Ala-Gly-Phe-NHNH-CO-$CH_2CH_2CH_3$ (18)——TEA (0.11 ml) and Z–Tyr–ONB (360 mg) were added to a mixture of H–Tyr-D-Ala–Gly–Phe–NHNH–CO–$CH_2CH_2CH_3\cdot$HCl[1] (403 mg) in DMF (10 ml). The whole was stirred at room temperature overnight, then the DMF was evaporated and the residue was treated with ether to give a powder, which was purified by reprecipitation from *n*-butanol–AcOEt; yield, 530 mg (90%), mp 204—206°C, $[\alpha]_D^{21}$ −29.0° (c=0.50, DMF), Rf^1=0.31. *Anal.* Calcd for $C_{44}H_{51}N_7O_{10}\cdot 1/2\ H_2O$: C, 62.39; H, 6.18; N, 11.58. Found: C, 62.43; H, 6.35; N, 11.58.

Synthesis of Tetrapeptide Analogs of Enkephalin. H-Tyr-D-Ala-Gly-MePhe-NHNH-CO-CH_2CH_3 (IV) ——The compound **13** (0.20 g) was dissolved in MeOH (50 ml) and hydrogenated over Pd-black. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in a small amount of 1 N aqueous acetic acid and applied to a column (2.5×120 cm) of Sephadex LH-20, which was eluted with the same solvent. The fractions from 290 ml through 315 ml were combined and lyophilized; yield, 110 mg, $[\alpha]_D^{24}$ −4.1° (c=0.27), Rf^2=0.12; Rf^3=0.42; Rf^4=0.60. Amino acid analysis: Gly 1.00; Ala 1.03; Tyr 1.24; MePhe 0.96 (average recovery 80%). ^{1}H-NMR (D_2O), δ: 1.15 (3H, t, CO–CH_2–C$\underline{H}_3$), 2.36 (2H, q, CO–C$\underline{H}_2CH_3$), 2.95 (3H, s, N–C$\underline{H}_3$ of MePhe).

H-MeTyr-D-Ala-Gly-MePhe-NHNH-CO-CH_2CH_3 (VI)——The compound **17** (0.53 g) was catalytically reduced, treated with TFA, and purified by gel filtration in the same manner as described for the synthesis of the analog IV to give the desired product; yield, 150 mg, $[\alpha]_D^{23}$ +14.4° (c=0.25, MeOH), Rf^2=0.21; Rf^3=0.52; Rf^4=0.60. Amino acid analysis: Gly 1.00; Ala 0.89; MeTyr 0.71; MePhe 0.98 (average recovery 84%). ^{1}H-NMR (D_2O), δ: 1.15 (3H, t, CO–$CH_2C\underline{H}_3$), 2.36 (2H, q, CO–$C\underline{H}_2CH_3$), 2.72 (3H, s, N–$C\underline{H}_3$ of MeTyr), 2.95 (3H, s, N–$C\underline{H}_3$ of MePhe).

H-Tyr-Tyr-D-Ala-Gly-Phe-NHNH-CO-$CH_2CH_2CH_3$ (VII)——The compound **18** (0.38 g) was deblocked in a similar manner to that described for the synthesis of the analog IV. The resulting crude peptide was dissolved in a small amount of 1 N aqueous acetic acid. The solution was applied to a column (2.5 × 120 cm) of Sephadex LH-20, which was eluted with the same solvent. The fractions from 370 ml through 390 ml were combined and lyophilized; yield, 150 mg, $[\alpha]_D^{21}$ +1.3° (c=0.31, MeOH), Rf^2=0.15; Rf^3=0.54; Rf^4=0.59. Amino acid analysis: Gly, 1.00; Ala 0.90; Tyr 1.63; Phe 0.88 (average recovery 84%).

Analogs I, II and V were synthesized essentially in the same manner as described for the synthesis of H–Tyr-D-Ala–Gly–Phe–NHNH–CO–$CH_2CH_2CH_3$.[1] Analog III was synthesized using Z–Tyr-D-Ala–Sar–Phe–NHNH–CO–CH_2CH_3 (**10**), which was prepared by the stepwise elongation method starting from H–Phe–NHNH–CO–CH_2CH_3. Analogs VIII, IX and X were synthesized in the same way as described for the synthesis of the analog VII. Physicochemical properties of intermediates and analogs are shown in Tables I and II, respectively.

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References and Notes

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