Structure–Activity Studies of Dermorphin. Synthesis and Some Pharmacological Data of Dermorphin and Its 1-Substituted Analogues

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Dermorphin and its analogues substituted at position 1 by *N*-acetyltyrosine, *O*-methyltyrosine, phenylalanine, D-phenylalanine, or alanine were obtained by solid-phase peptide synthesis. Their pharmacological effects were studied in vitro by the guinea pig ileum method and in vivo by the hot plate method, and the results were compared with those of morphine. The most pronounced activity was shown for dermorphin. A radioreceptor study showed a moderate affinity of dermorphin and its Tyr(Me)[1] analogue for the opiate receptor sites from striatal homogenates.

Dermorphin was recently isolated from the skin of South American frogs, its structure was determined, and the synthesis of the peptide was performed.[1-3] This very potent heptapeptide with opiate-like activity has the following sequence of amino acids:

H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2
1 2 3 4 5 6 7

Together with this peptide, in the skin of the South American frogs of the *Phyllomedusa* species, its analogue was found in which the proline residue was replaced by hydroxyproline. Regarding the source from which the peptides were isolated, it was astonishing to find D-alanine in position 2 of the amino acid sequence.

In order to investigate the relationship between the structure of dermorphin and its pharmacological properties, we synthesized the parent peptide and five analogues in which the tyrosine residue in position 1 was replaced by *N*-acetyltyrosine, *O*-methyltyrosine, phenylalanine, D-phenylalanine, and alanine.

Results

Synthesis. Both dermorphin and its analogues were obtained by peptide synthesis on a solid support.[4-6] The amino groups were protected with the *tert*-butyloxycarbonyl group, while the hydroxyl groups of tyrosine and serine were given benzyl protection. The protected serine was attached to the resin, and the peptide bonds were formed either by using dicyclohexylcarbodiimide or by means of symmetric anhydrides. The synthesized peptides were removed from the resin by ammonolysis. Dermorphin and its analogues were purified by gel filtration on Sephadex G-15 after deprotection, which was accomplished first by acidolysis and then by hydrogenation over Pd catalyst. The results of the syntheses are summarized in Table I.

Biological Activity. Dermorphin and its analogues were tested by the guinea pig ileum,[7] hot plate,[8] and radioreceptor[9] methods.

The results of the guinea pig ileum (gpi) test are presented in Table II. The most pronounced inhibitory action was obtained for dermorphin. Its inhibitory potency (IC_{50} = 5.2 ± 0.62 × 10^{-11})[10] was greater than that of morphine (IC_{50} = 7.5 ± 1.6 × 10^{-8} M) and [D-Ala^2]Met-enkephalinamide (IC_{50} = 2.5 ± 0.54 × 10^{-9} M). Other dermorphin analogues also inhibited the twitches but at higher concentration. The inhibitory action of all compounds was completely reversed by the specific opiate-receptor antagonist, naloxone, at a concentration 2 × 10^{-6} M.

The results of the hot plate bioassay are presented in Table III. All investigated dermorphin analogues, with the exception of Ac-Tyr^1- and D-Phe^1-dermorphin, produced dose-dependent analgesia in mice after intraperitoneal administration. Moreover, the analgesic activity of dermorphin is greater than that produced by morphine on a molar ratio basis. The analgesic activity of all investigated peptides was completely abolished by subcutaneous injection of naloxone (3 × 10^{-6} M/kg, 5 min prior to the testing procedure).

Dermorphin and its analogue, Tyr(Me)^1-dermorphin, produced pronounced displacement of specifically bound [^{3}H]naloxone from striatal homogenates (Figure 1). At a concentration of 10^{-5} M these peptides displaced about 50% of the labeled naloxone from the opiate receptor sites in the rat striatum. Moreover, Tyr(Me)^1-dermorphin showed the highest affinity in this test. On the other hand, Ac-Tyr^1-, D-Phe^1-, and Ala^1-dermorphins showed rather low affinity for the opiate receptor sites as compared to dermorphin. However, Phe^1-dermorphin produced a moderate displacement of [^{3}H]naloxone, which was smaller than that of dermorphin and Tyr(Me)^1-dermorphin but greater than that of the remaining analogues.

Discussion

In all hitherto known opiate-like peptides (enkephalins,[12] endorphins,[13,14] kyotorphin,[15] α-neoendorphin,[16] dynor-

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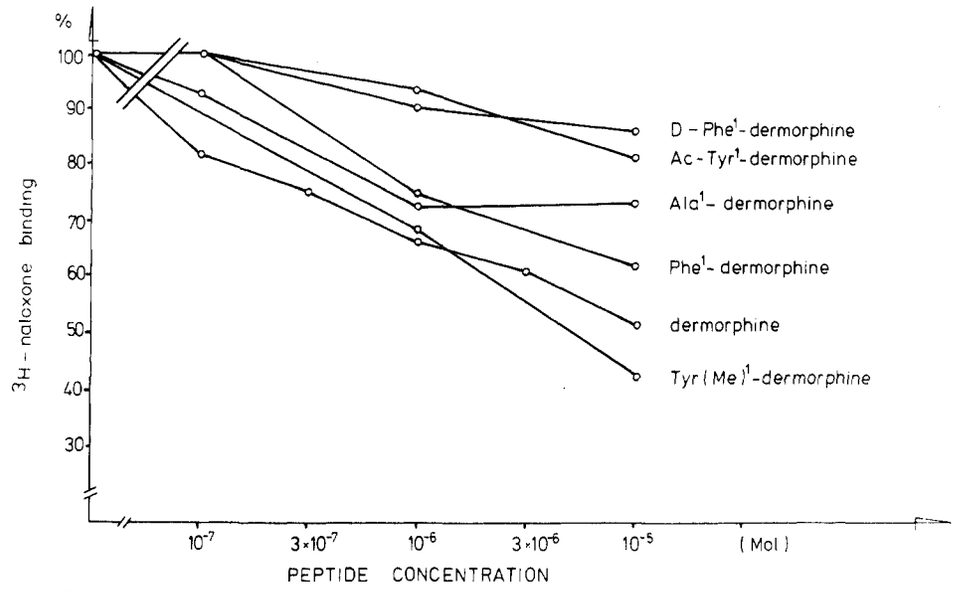

Figure 1. Displacement of [^{3}H]naloxone by increasing amounts of the unlabeled ligands in straital homogenates from rats. Abscissa: concentration of unlabeled ligands. Ordinates: inhibition of [^{3}H]naloxone binding (percent of total).

Table I. Properties of Dermorphin and Its Analogues

peptide	total yield of pure product, %	$[\alpha]^{20}_D$, deg (*c* 1, 1 N AcOH)	TLC R_f the following solvent systems[a] A	B	C	D
dermorphin	20.3	+4.95	0.60	0.78	0.81	0.49
Tyr(Me)^1-dermorphin	17.1	+7.6	0.76	0.86	0.90	0.70
Ac-Tyr^1-dermorphin	29.2	+3.2	0.69	0.81	0.83	0.63
Phe^1-dermorphin	29.0	+3.5	0.58	0.73	0.80	0.51
D-Phe^1-dermorphin	9.1	−6.0	0.57	0.74	0.81	0.50
Ala^1-dermorphin	24.1	−9.0	0.39	0.68	0.71	0.29

[a] Solvent systems: A, 1-butanol-acetic acid-water (4:1:1, v/v), B, 1-butanol-acetic acid-water-pyridine (15:3:12:10, v/v), C, 1-butanol-ethyl acetate-acetic acid-water (1:1:1:1, v/v), D, 1-butanol-acetic acid-water (4:1:5, upper phase, v/v).

Table II. Inhibition of Electrically Evoked Twitches in the Guinea Pig Ileum Preparation Produced by Dermorphin and Its Analogues[a]

peptide	rel potencies to morphine
dermorphin	1440
Tyr(Me)^1-dermorphin	40.5
Ac-Tyr^1-dermorphin	0.0044
Phe^1-dermorphin	0.026
D-Phe^1-dermorphin	0.0042
Ala^1-dermorphin	0.0017
morphine	1
[D-Ala^2,Met^5]-enkephalinamide	30

[a] Dose-response curves were constructed on the basis of action of three to four concentrations. The effect of each concentration was calculated as an average of at least four independent determinations.

Table III. ED_{50} Values with 95% Confidence Limits for Dermorphin and Its Analogues in Mice[a]

peptide	ED_{50}, mol/kg
dermorphin	9.5×10^{-7} (6.3-12.1)
Tyr(Me)^1-dermorphin	6.0×10^{-5} (0.4-9.8)
Ac-Tyr^1-dermorphin	$>10^{-3}$
Phe^1-dermorphin	1.55×10^{-5} (0.5-11.2)
D-Phe^1-dermorphin	$>10^{-3}$
Ala^1-dermorphin	6.1×10^{-5} (2.0-8.3)
morphine	1.7×10^{-6} (1.1-2.2)

[a] ED_{50} values were calculated on the basis of dose-response curves (the analgesic action of at least four doses of each compound was investigated). Each dose was tested for at least 10 animals.

phin,[17] casomorphin,[18] and dermorphins[2,3]) the tyrosine residue occurs in position 1. This prompted us to synthesize the corresponding analogues to investigate the influence of the tyrosine residue in position 1 of dermorphin on the agonistic activity and opiate-receptor affinity.

Our results indicated that dermorphin and some of its analogues possess opiate agonistic activity both in vitro and in vivo. In the electrically stimulated guinea pig ileum preparation, the most pronounced opiate agonistic activity was shown for the nonmodified dermorphin. The action of this peptide was stronger than that of its analogues, as well as morphine and [D-Ala^2]Met-enkephalinamide. On the other hand, radioreceptor studies showed higher affinity of both dermorphin and its Tyr(Me)^1 analogue for the opiate receptor sites from striatal homogenates than the remaining analogues (Figure 1). The Tyr(Me)^1 analogue showed higher affinity to the opiate receptors than the parent peptide.

Moreover, investigated peptides, with the exceptions of Ac-Tyr^1- and D-Phe^1-dermorphins, showed analgesic activity in mice after systemic intraperitoneal administration. Also, in this case the most pronounced analgesic activity, greater than that produced by morphine, was observed for

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the nonmodified dermorphin. It might be concluded that dermorphin, as well as some of its analogues, can easily penetrate the brain after systemic administration. Since it was completely abolished by naloxone, the analgesic activity produced by these peptides is due to their direct action on opiate receptors in the brain.

Experimental Section

Peptide Synthesis. Dermorphin and its analogues were obtained by solid-phase peptide synthesis.[4-6] Chloromethylated polymer (Bio-Beads S-X-1, Bio-Rad Laboratories) containing 1.34 mequiv of Cl/g was used. It was esterified[19] with the cesium salt of Boc-Ser(Bzl)-OH to an extent of 0.63 mmol/g. *tert*-Butyloxycarbonyl-protected amino acids were prepared according to Grzonka.[20] The peptide-resin was checked for unreacted amino groups by the ninhydrin test.[21] The purity of peptides was demonstrated by TLC on silica gel plates (Merck, G-60) with the following solvent systems: (A) *n*-BuOH–AcOH–H_2O (4:1:1, v/v); (B) *n*-BuOH–AcOH–H_2O–pyridine (15:3:12:10, v/v); (C) *n*-BuOH–H_2O–AcOH–AcOEt (1:1:1:1, v/v); (D) *n*-BuOH–AcOH–H_2O (4:1:5, v/v, upper phase). The spots of the peptides were detected with the iodine vapors and ninhydrin.

Boc-Ser(Bzl)-resin (1). Cs_2CO_3 (3.26 g, 10 mmol) in water (7 mL) was added to the solution of Boc-Ser(Bzl)-OH (5.9, 20 mmol) in EtOH (15 mL). The solution was degased and evaporated to dryness, the residual water was removed by azeotropic distillation with toluene, and the solution was finally dried in vacuo over P_2O_5. The crystalline cesium salt of Boc-Ser(Bzl)-OH was dissolved in DMF (150 mL) and added to the chloromethylated resin (18 g). The suspension was stirred for 24 h at 50 °C and then filtered, and the filtrate was washed three times with DMF, DMF–water (9:1), DMF–MeOH (1:1), MeOH, and CH_2Cl_2 and dried in vacuo. The yield was 23.17 g (0.63 mmol of protected serine per gram of resin).

Boc-D-Ala-Phe-Gly-Tyr(Bzl)-Pro-Ser(Bzl)-resin (2). Boc-Ser(Bzl)-resin (1; 7.93 g, 5 mmol) was subjected to five cycles of deprotection, neutralization, and coupling. A cycle for incorporation of each amino acid residue into the growing peptide chain involved the following steps: (1) three washings with CH_2Cl_2; (2) one washing with CH_2Cl_2–EtOH (1:1); (3) three washings with EtOH; (4) one wasing with EtOH–AcOH (1:1); (5) three washings with AcOH; (6) removal of the Boc group by treatment with 1.3 N HCl/AcOH (1 × 5 min, 1 × 25 min); (7) three washings with AcOH; (8) one washing with AcOH–EtOH (1:1); (9) three washings with EtOH; (10) one washing with EtOH–CH_2Cl_2 (1:1); (11) three washings with CH_2Cl_2; (12) neutralization with 10% NEt_3 in CH_2Cl_2 (1 × 5 min, 1 × 10 min); (13) three washings with CH_2Cl_2; (14) equilibration with 3 equiv of the appropriate Boc-protected amino acid in CH_2Cl_2 for 5 min, followed by addition of dicyclohexylcarbodiimide (3 equiv) and reacting for 4 h; (15) three washings with CH_2Cl_2; (16) repeated coupling with Boc-protected amino acid and DCCI (step 14) for 18 h. After completion of the synthesis, the protected 6-peptide resin was washed five times with the CH_2Cl_2, EtOH, and MeOH solvents and then dried: yield 10.87 g (91% based on substituted Ser).

Boc-Tyr(Bzl)-D-Ala-Phe-Gly-Tyr(Bzl)-Pro-Ser(Bzl)-resin (3). The foregoing 6-peptide resin (2; 1.35 g, 0.62 mmol) was converted in one cycle of solid-phase peptide synthesis with Boc-Tyr(Bzl)-OH to yield the 7-peptide resin (3; 1.50 g, 71%).

H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 (4, Dermorphin). To the protected 7-peptide resin (3; 1.5 g) suspended in MeOH (20 mL) and chilled to −70 °C, ammonia (20 mL) was distilled. The flask was well stoppered and left for 48 h at room temperature. The ammonia and MeOH were then evaporated, and the residue was extracted with hot MeOH. The peptide was precipitated with ethyl ether and filtered, and the filtrate was dried in vacuo over P_2O_5. The protected peptide was dissolved in 1.3 N HCl/AcOH (5 mL) and precipitated with ethyl ether after 30 min. The product was filtered, and the filtrate was washed with ethyl ether, dissolved in a MeOH–AcOH (5:1) mixture, and hydrogenolyzed over 10% Pd/C for 24 h. The catalyst was filtered off, the free peptide was precipitated with AcOEt and filtered, and the filtrate was dried over P_2O_5. The yield was 335 mg. The crude peptide was subjected to gel filtration on a Sephadex G-15 column (160 × 1.8 cm) in 1 N AcOH. Dermorphin (77.2 mg, 20.3%) was isolated by lyophilization from fractions comprising a single symmetrical peak and was found to be chromatographically pure: $[\alpha]^{20}_D$ +4.95° (*c* 1, 1 N AcOH); $[\alpha]^{20}_D$ +5.7° (*c* 1, MeOH, in the presence of equimolar quantity of CF_3COOH); lit.[1] $[\alpha]^{23}_D$ for the trifluoroacetate +5.5° (*c* 1, MeOH); TLC R_f (A) 0.60, R_f (B) 0.70, R_f (C) 0.81, R_f (D) 0.49. Amino acid analysis: Tyr, 1.93; Ala, 1.01; Phe, 1.02; Gly, 1.00; Pro, 0.96; Ser, 0.97.

***N*-Ac-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 (5).** A single cycle of deprotection, neutralization, and coupling with Boc-Tyr(Bzl)-OH converted the 6-peptide resin (2; 1.35 g, 0.62 mmol) to the protected 7-peptide resin. The *tert*-butyloxycarbonyl group was removed with 1.3 N HCl/AcOH, and after neutralization of the free amino group of tyrosine, it was acetylated on the resin with acetic anhydride (0.18 mL, 1.86 mmol) in the presence of NEt_3 (0.26 mL, 1.86 mmol). The peptide was split from the resin by ammonolysis. The benzyl groups were removed from the protected amide of the 7-peptide (614 mg) by catalytic hydrogenation. The crude analogue (432 mg) was purified on a Sephadex G-15 column with 1 N AcOH as eluent. The yield of pure N^1-acetyldermorphin was 155 mg (29.2%): $[\alpha]^{20}_D$ +3.2° (*c* 1, 1 N AcOH); TLC R_f (A) 0.65, R_f (B) 0.81, R_f (C) 0.83, R_f (D) 0.63. Amino acid analysis: Tyr, 1.97; Ala, 1.00; Phe, 1.03; Gly, 1.00; Pro, 0.95; Ser, 0.98.

H-Tyr(Me)-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 (6). This peptide was obtained in the same manner as described for 3, except that Z-Tyr(Me)-OH[22] was used in the coupling step. After ammonolysis, the amide of the 7-peptide (616 mg) was deprotected by catalytic hydrogenation. The crude analogue (250 mg) was purified by gel filtration on Sephadex G-15 and lyophilized. The yield of the pure analogue was 90 mg (17.1%); $[\alpha]^{20}_D$ +7.6° (*c* 1, 1 N AcOH); TLC R_f (A) 0.76, R_f (B) 0.86, R_f (C) 0.90, R_f (D) 0.70. Amino acid analysis: Tyr, 0.94; Ala, 1.02; Phe, 1.02; Gly, 1.00; Pro, 0.99; Ser, 0.96.

Other Analogues of Dermorphin Substituted at Position 1. Analogues of dermorphin substituted at position 1 by the L-alanine, L-phenylalanine, and D-phenylalanine residues were obtained as described above for dermorphin (4). The results are summarized in Table I.

Pharmacological Assays. The coaxially, electrically stimulated guinea pig ileum preparation was used for estimation of the opiate receptor agonistic properties of the investigated dermorphins according to the method described by Kosterlitz and Watt.[7] The effects of dermorphins were compared with those of other well-known opiate agonists, morphine and [D-Ala^2]Met-enkephalinamide.

The analgesic potency of the peptides was estimated in Swiss male mice weighing 20–22 g. In the hot-plate test,[8] the latency of hind-paw licking was taken as the nociceptive response. ED_{50} values and the 95% confidence limits were calculated by the methods of Tyers[23] and Finney.[24] Drugs were dissolved in doubly distilled water and injected at a dose of 0.2 mL/20 g of body weight.

The affinity of peptides to the opiate receptor sites in vitro was studied by testing their ability to inhibit the specific binding of the opiate antagonist [^{3}H]naloxone to striatal membranes of rats according to Członkowski et al.[9]

Acknowledgment. This work was supported in part by the Polish Academy of Sciences (Grant MR-I.12.1.6.3).

Registry No. 4, 77614-16-5; 5, 86129-31-9; 6, 86146-54-5; Phe^1-dermorphin, 85635-39-8; D-Phe^1-dermorphin, 86129-32-0; Ala^1-dermorphin, 86129-33-1; Boc-Ser(Bzl)-OH, 23680-31-1; Boc-Ser(Bzl)-OH·Cs, 62361-26-6; Boc-Tyr(Bzl)-OH, 2130-96-3; Z-Tyr(Me)-OH, 17554-34-6.

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