Synthesis and Biological Activity of β-Endorphin and Analogues. Additional Evidence for Multiple Opiate Receptors

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The solid-phase synthesis of highly purified sheep β-endorphin (β_s-endorphin) and three analogues, including [D-Ala^2]-, [D-Ala^2,MePhe^4]-, and [D-Ala^2,MePhe^4,Met(O)^5]-β_s-endorphins, is reported. Synthetic β_s-endorphin was found identical with the natural hormone by several analytical criteria as well as by three biological tests. Relative potencies of the synthetic compounds were estimated by their ability to inhibit the electrically evoked contractions of the guinea pig ileum and of the rat vas deferens and to compete with tritiated naloxone for opiate receptors on rat brain homogenates. Introduction of D-Ala in position 2 of β_s-endorphin induces a 58% increase in its activity with the rat vas deferens bioassay and a slight decrease in its activity with the two other bioassays. Replacement of Phe^4 in [D-Ala^2]-β_s-endorphin by N^{α}-methylphenylalanine results in a respective 2.6- and 1.4-fold increase in the activity of β_s-endorphin with the rat vas deferens and [^{3}H]naloxone binding tests, whereas a dramatic decrease in the activity was found with the guinea pig ileum bioassay. Further substitution of [D-Ala^2,MePhe^4]-β_s-endorphin, introducing Met(O) in position 5, does not induce any additional change in the biological activity of the parent peptide. The heterogeneity of responses caused by a specific modification in β_s-endorphin strongly suggests the existence of more than one type of receptor for the opioid peptide.

Since the discovery of endogenous morphine-like peptides, the enkephalins[1,2] and the endorphins,[3-5] much work has been accomplished to design analogues of enkephalin with stronger activity and longer lasting effects. However, relatively little work has yet been done with the larger peptide, β-endorphin, which was found to be the most potent, naturally occurring opioid peptide in producing analgesia[4,6,7] and hypothermia[8] when injected centrally.

Recently, Roemer et al.[9] have found that simultaneous introduction of D-Ala, MePhe, and Met(O)-ol, respectively, in positions 2, 4, and 5 of [Met]enkephalin, leads to a tremendous increase in its analgesic activity. In order to see if the biological activity of β-endorphin can be enhanced, we introduced similar modifications in its structure and tested these analogues in three different biological preparations. The results, herein reported, suggest the existence of more than one type of receptor for β-endorphin.

Results and Discussion

Among the various recent improvements of the techniques for solid-phase peptide synthesis,[12] the use of preformed symmetrical anhydrides of *tert*-butyloxycarbonylamino acids for the coupling step proved to be successful for the synthesis of relatively large peptides such as β-LPH-(42–91)[13] and, more recently, β-endorphins.[14,15] However, in order to avoid any possibility of degradation of the preformed anhydrides of *tert*-butyloxycarbonylamino acids, application of this technique to the automatic solid-phase peptide synthesizer necessitates the refrigeration of the standing reactive materials. In the present studies, the complete synthesis of β_s-endorphin and analogues was accomplished automatically on a solid-phase peptide synthesizer, equipped with refrigerated (−5 °C) amino acid reservoirs.

After their purification by gel filtration on Sephadex G-10, chromatography on CMC, and partition chromatography on Sephadex G-50, the synthetic peptides were obtained in good yields (25% based on the starting resin) and found homogeneous when analyzed separately by thin-layer chromatography, paper electrophoresis, partition chromatography (Table I), and gel electrophoresis. Synthetic β_s-endorphin was identical with the natural hormone as shown by thin-layer chromatography (R_f 0.45, BPAW; R_f 0.077, BAW) and gel electrophoresis (Figure 1). Amino acid analysis of acid digests[18] gave values in accordance with those expected (Table II). It may be noted that the different analogues can be distinguished from each other by partition chromatography on Sephadex G-50 (Table I). This further emphasizes the efficiency of partition chromatography[17] in separating closely related β-endorphin molecules.[18]

The opiate activities of the synthetic peptides were measured by their ability to inhibit the binding of [^{3}H]-naloxone to rat brain homogenates[19] and the electrically

Table I. R_f Values of Synthetic β_s-Endorphin and Analogues in Partition Chromatography (PC), Thin-Layer Chromatography (TLC), and Paper Electrophoresis (PE)

prepn of β_s-endorphin	PC[a]	TLC[b] BPAW	TLC[b] BAW	PE[c]
natural		0.45	0.077	
synthetic	0.20	0.45	0.077	0.47
[D-Ala^2]-	0.35	0.44	0.096	0.47
[D-Ala^2,MePhe^4]-	0.38	0.46	0.085	0.47
[D-Ala^2,MePhe^4,Met(O)^5]-	0.21	0.42	0.061	0.47

[a] Sephadex G-50; solvent system, 1-butanol–pyridine–0.6 M ammonium acetate in 0.1% aqueous acetic acid (5:3:11, v/v). [b] Solvent systems: BPAW, 1-butanol–pyridine–acetic acid–water (6.0:6.0:1.2:4.8); BAW, 1-butanol–acetic acid–water (4:3:3), ninhydrin detection. [c] 400 V for 4 h, ninhydrin detection.

Table II. Amino Acid Analysis of Acid Hydrolysates of Synthetic β_s-Endorphin and Analogues

amino acid	β_s-endorphin	β_s-endorphin analogue [D-Ala^2]	[D-Ala^2,MePhe^4]	[D-Ala^2,MePhe^4,Met(O)^5]
Asp	2.2	2.0	2.1	2.0
Thr	2.8	2.7	2.9	3.0
Ser	1.8	1.9	1.9	2.1
Pro	0.9	1.0	1.1	1.0
Glu	3.0	3.1	2.9	3.0
Gly	3.0	2.0	2.2	2.2
Ala	2.2	3.1	3.1	3.2
Val	1.1	0.9	0.9	1.1
Met(O)				0.8
Met	1.0	1.0	0.9	
Ile	1.8	1.8	1.7	1.8
Leu	2.2	2.0	2.0	2.1
Tyr	0.8	0.9	1.0	1.0
Phe	1.9	2.0	1.2	1.1
MePhe			0.9	1.0
His	1.2	1.1	1.1	1.2
Lys	5.0	5.1	5.0	5.1

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Table III. Relative Inhibitory Potencies of Natural and Synthetic β_s-Endorphin and Analogues on the Binding of [^{3}H]Naloxone to Rat Brain Homogenates and on the Electrically Induced Contractions of the Rat Vas Deferens and Guinea Pig Ileum

	[^{3}H]naloxone		rat vas deferens		guinea pig ileum	
prepn of β_s-endorphin	ID_{50},[a] nM	rel potency,[b] %	ID_{50},[a] nM	rel potency,[b] %	ID_{50},[a] nM	rel potency,[b] %
natural	10 ± 0.5	100	130 ± 17	100	27 ± 2.4	100
synthetic	10 ± 1.5	100	130 ± 12	100	27 ± 3.0	100
[D-Ala^2]-	12 ± 1.0	84	82 ± 9	158	29 ± 2.5	93
[D-Ala^2,MePhe^4]-	7 ± 1.0	143	50 ± 10	260	260 ± 22	10
[D-Ala^2,MePhe^4,Met(O)^5]-	7 ± 0.8	143	52 ± 8	250	280 ± 18	10

[a] Concentration which gives half-maximal response. Values are mean ± SEM of three sets of duplicates for the [^{3}H]-naloxone binding test and of six determinations for the two other biological assays. Experiments were performed as described under the Experimental Section. [b] Relative to natural β_s-endorphin.

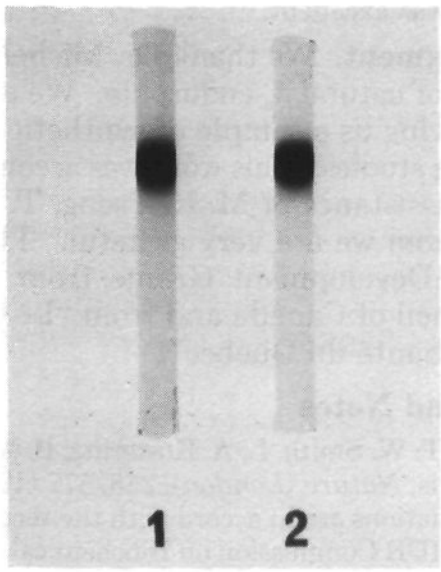

Figure 1. Disc electrophoresis of natural (1) and synthetic (2) β_s-endorphin on polyacrylamide gel at pH 4.5. Detection with Amido-Schwarz dye.

induced contractions of the guinea pig ileum[20] and of the rat vas deferens.[21,22] We have recently demonstrated that the rat vas deferens provides a specific bioassay for the endorphins since it is specifically and exclusively sensitive to the naturally occurring opioid peptides: enkephalins, β-endorphin, and related peptide compounds.[21]

Table III shows the relative potencies of β_s-endorphin and analogues as calculated by their ID_{50} values, e.g., the concentration of the peptide which gives half-maximal response. The activity of synthetic β_s-endorphin was found identical with that of the natural hormone by the three different biological tests. However, it might be important to notice that the ID_{50} value, as calculated for the rat vas deferens preparation (130 nM), is approximately one order of magnitude higher than that for the [^{3}H]naloxone binding test (10 nM) and five times higher than that for the guinea pig ileum assay (27 nM).

Introduction of D-Ala in position 2 of β_s-endorphin induced a small decrease in the activity of the parent peptide as measured by the guinea pig ileum assay (relative potency of 93% compared to the parent peptide, Table III) and the [^{3}H]naloxone binding test (relative potency of 84%). It has previously been shown that the resistance of the Tyr-Gly bond of opioid peptides to enzymatic degradation is essential for their biological activity.[23,24] However, the Tyr-Gly bond in β-endorphin, contrary to enkephalin, was found already quite resistant to the action of aminopeptidases,[25] and that may partly explain why the introduction of D-Ala in position 2 of β_s-endorphin does not increase its biological activity. On the other hand, with the rat vas deferens preparation, [D-Ala^2]-β_s-endorphin was 1.58-fold more potent than β_s-endorphin either indicating a higher level of metabolism in this tissue or a distinct structural requirement for β-endorphin (Table III).

When Phe^4 of [D-Ala^2]-β_s-endorphin was replaced by N^α-methylphenylalanine, a 90% reduction of the activity, as compared with β_s-endorphin, was obtained with the guinea pig ileum assay, whereas respective 1.43- and 2.6-fold increases were found in the activities with the [^{3}H]naloxone binding test and the rat vas deferens bioassay. It is very interesting to notice that a modification in the structure of β-endorphin can produce opposite effects on its biological activities depending upon the choice of the assay. These data raise two possibilities: (1) the existence of several modes of interaction of β-endorphin with its analgesic receptor, as already proposed by Portoghese[26,27] for different series of narcotic analgesics, or (2) the existence of multiple opiate receptors for β-endorphin.[26-29] Additional support for the latter concept is brought by the previous observations that the guinea pig ileum contains a morphine-type receptor for β-endorphin,[29] whereas no or very few receptors for morphine have been found in the rat vas deferens[21,22] even though this preparation is highly sensitive to β-endorphin.

Further modification of [D-Ala^2,MePhe^4]-β_s-endorphin, introducing Met(O) in position 5, did not change any of its biological activities (Table III). It is worth noticing that the same modification introduced in enkephalin induced a large increase in its analgesic activity.[9] However, it is possible that the oxidation of the methionine residue in enkephalin increases its resistance against the action of carboxypeptidases. As the methionine residue in β-endorphin is already hindered inside the molecule, protection against the action of carboxypeptidases is no more necessary, and this could explain why the introduction of Met(O) is position 5 of [D-Ala^2,MePhe^4]-β_s-endorphin does not increase its biological activities (Table III).

Lord et al.[25] have recently demonstrated the multiplicity of receptors for endogenous opioid peptides, and they proposed to classify them according to their specific sensitivity to known opiate agonists[26] such as μ for morphine, κ for ketocyclazocine, or σ for *N*-allylcyclazocine. However, as we have previously shown,[21] the rat vas deferens is only sensitive to endogenous opioid peptides, excluding other narcotic analgesics acting on μ, κ, or σ receptors. It appears from our results that the structural requirements in β-endorphin for opiate activity in the rat vas deferens are different from those found in the guinea pig ileum, and these differences cannot be related to any known opiate receptor such as μ, κ, or σ.[21] The rat brain homogenates seem to contain both types of receptors, that is, those related to the opiate receptor in the rat vas deferens and those related to the opiate receptor in the guinea pig ileum. Indeed, with the [^{3}H]naloxone binding test, some modifications in β_s-endorphin such as the introduction of D-Ala in position 2 reduce its affinity as found with the guinea pig ileum bioassay, whereas other modifications such as N-methylation of Phe^4 increase its ac-

tivity as found with the rat vas deferens bioassay (Table III). The fact that the affinity of β-endorphin for one specific opiate receptor can be selectively increased, whereas it is decreased for other opiate receptors, may find some application in the selective stimulation of one or another physiological function of β-endorphin.

Experimental Section

Thin-layer chromatography of synthetic peptides was run on silica gel with the following solvent systems: BPAW, 1-butanol-pyridine-acetic acid-water (6.0:6.0:1.2:4.8); BAW, 1-butanol-acetic acid-water (4:3:3). Gel electrophoresis, paper electrophoresis, amino acid analyses, chromatography on CMC, and partition chromatography[17] on Sephadex G-50 were performed as described.[14] Benzhydrylamine resin (0.35 mequiv/g) and *tert*-butyloxycarbonylamino acids were purchased from Bachem Fine Chemical Inc. The purity of the *tert*-butyloxycarbonylamino acids was verified by thin-layer chromatography in a solvent system of methanol–chloroform (1:1) before use.

Protected β_s-Endorphin Benzhydrylamine Resin. *N*-*tert*-Butyloxycarbonyl-α-benzyl-L-glutamylbenzhydrylamine resin was prepared and found to yield glutamine upon treatment with liquid HF.[14] Starting with 0.85 g of the resin (0.3 mmol), synthesis of protected β_s-endorphin benzhydrylamine resin was performed by the use of preformed symmetrical anhydrides of *tert*-butyloxycarbonylamino acids[12] as described previously[14] with the following exceptions: (a) synthesis was performed in a Beckman Model 990B peptide synthesizer equipped with refrigerated (−5 °C) amino acid reservoirs (St-Pierre et al., unpublished results); (b) two treatments with 5% diisopropylethylamine were used for neutralization; (c) DMF was added to the solution of symmetrical anhydrides of *tert*-butyloxycarbonylamino acids to a final concentration of 10% in order to avoid the crystallization of the preformed anhydrides. The asparagine residues were introduced by *p*-nitrophenyl ester coupling.[30] Side-chain protecting groups were Lys and Tyr, *o*-Br-Z; His, Boc;[21] Arg, tosyl;[31] Asp and Glu, OBzl; Thr and Ser, Bzl. After removal of the last N^{α}-Boc protecting group with 50% F_3AcOH in methylene chloride,[32] the finished peptide was dried in vacuo over P_2O_5 overnight to yield 2.8 g.

β_s-Endorphin. A sample (1 g) of the protected peptide resin was treated with liquid HF (ca. 15 mL) in the presence of anisole (2 mL) for 30 min at −20 °C and then at 0 °C for 1 h. HF was removed in vacuo and the residue was washed with ether and ethyl acetate before the extraction of the peptide with 50% acetic acid (25 mL). This material was then purified by chromatography on Sephadex G-10, CMC, and partition chromatography on Sephadex G-50 in the solvent system 1-butanol–pyridine–0.6 M ammonium acetate in 0.1% acetic acid (5:3:11), as described previously,[14] to yield 92 mg of highly purified β_s-endorphin (ca. 25% yield based on the starting resin).

Amino acid analysis of an acid hydrolysate of the purified material (0.5-mg sample) gave values in accordance with those expected (Table II). Thin-layer chromatography of 50-μg samples gave a single spot (ninhydrin detection) in two different solvent systems (Table I). A single band was obtained on polyacrylamide gel electrophoresis (75-μg sample) which corresponded to natural β_s-endorphin (Figure 1). Partition chromatography of a 4-mg sample on a Sephadex G-50 column (0.8 × 45 cm; holdup volume, 10.7 mL) gave a sharp peak (Folin–Lowry reaction[33]) with an R_f value of 0.20 (Table I). On paper electrophoresis, the synthetic material migrated as a single spot (ninhydrin detection, Table I) with an R_f value of 0.47 (compared to Lys).

[D-Ala^2]-, [D-Ala^2,MePhe^4]-, and [D-Ala^2,MePhe^4,Met(O)^5]-β_s-endorphins. Synthesis and purification of [D-Ala^2]-, [D-Ala^2,MePhe^4]-, and [D-Ala^2,MePhe^4,Met(O)^5]-β_s-endorphins were achieved following the same procedures as those already described for the synthesis of β_s-endorphin. Characterizations of these products were also obtained by amino acid analysis (Table II), thin-layer chromatography, gel electrophoresis, paper electrophoresis, and partition chromatography on Sephadex G-50 (Table I).

Biological Assays. The opiate binding assay was based on the ability of the synthetic peptides to inhibit [^{3}H]naloxone binding to rat brain homogenates as described.[19] Assays were performed at 25 °C for 30 min with 2-mL aliquots of the tissue homogenate (ca. 1 mg wet weight) and 0.7 mol of [^{3}H]naloxone (25 Ci/mmol from New England Nuclear). The ID_{50} values were derived from log-probit plots of three to five concentrations of the compounds, each representing the means of three separate sets of duplicates.

The depression of electrically induced contractions of the guinea pig ileum and rat vas deferens was measured as described.[20] The ID_{50} values were obtained from log-probit plots of five increasing concentrations, each representing the means from six different tissues. There was no tachyphylaxia with enkephalins and nonpeptidic narcotic analgesics, but, in some instances, with the guinea pig ileum assay, β-endorphin and analogues could cause some tachyphylaxia if injected 30 min apart at the same dose. In order to eliminate this possibility, the same tissue was subjected to increasing concentrations of only one compound. Then, the reproducibility was excellent.

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(2-*exo*-3-*endo*)-2-Aryltropane-3-carboxylic Esters, a New Class of Narcotic Antagonists

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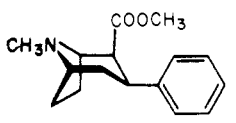
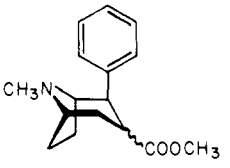
Tropanes **4** bearing an exo aromatic group on carbon-2, an *endo*-carbomethoxy group on carbon-3, and either a methyl group or a hydrogen on the nitrogen were found to be narcotic antagonists which were devoid of demonstrable analgesic activity. The activity resided in the 1*S* enantiomer. Compound **4** (R = *m*-hydroxyphenyl) showed an AD_{50} of 0.37 mg/kg sc and 1.8 mg/kg po (rats) as an antagonist in the Harris–Pierson modification of the D'Amour–Smith test. The tropane esters for this study were prepared by a Grignard reaction which gave essentially complete 1,4-addition in the absence of copper salts. Nearly equal quantities of esters epimeric at carbon-3 were formed.

In an earlier paper[1] we described some 3-*exo*-phenyltropane-2-*exo*-carboxylic esters (exemplified by **1** with



1



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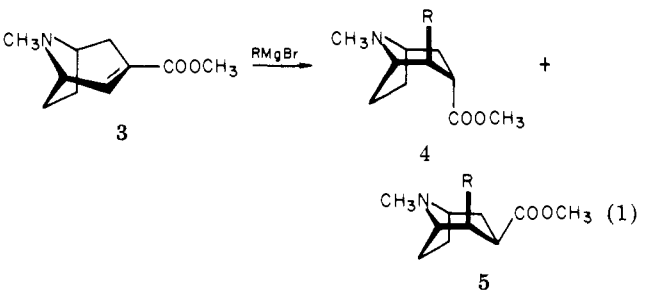
absolute configuration as drawn) which are powerful, orally active, central nervous system stimulants. Extremely restrictive configurational requirements for this activity were evident in this rigid molecule since activity was lost when the ester group was moved into an endo configuration or moved to the 4-exo position (the enantiomer of **1**) or when the ethylene bridge was removed.

We now report on a series of tropane esters, **2** (Table I), wherein the aromatic ring occupies the 2-exo position and the ester is in the 3 position. Those members which contain a 3-endo ester function are narcotic antagonists without accompanying analgesic activity, whereas their 3-exo counterparts are completely devoid of this activity. The present paper details this narcotic antagonism (Table II). Some hypoglycemic and analgesic activity associated with the 3-exo esters of this series is described separately in an accompanying paper.[2]

There is difficulty inherent in representing *R* and *S* enantiomers with a single general formula. The hypoglycemic esters referred to in the previous paragraph have the 1*R* configuration as drawn in **2**, whereas the active narcotic antagonists reported in the present paper have a 1*S* configuration (opposite that of **2**). Although most of the compounds in the present paper are racemates and either enantiomer could be used for general representation, the choice was made to show hereafter the representation of the enantiomers related to the narcotic antagonists. 1*S* configurations are drawn.

Chemistry. The desired esters were prepared by the reaction of Grignard reagents with unsaturated ester **3**[3] according to eq 1. As in the analogous series of esters **1**,[1]



3 4 5 (1)

the presence of copper salts was not necessary in order to obtain 1,4-addition to this conjugated system. Further reaction of the primary products with RMgBr was minimized by adopting a reaction temperature of −20 to −25 °C and using ether rather than tetrahydrofuran as the solvent. Esters **4** and **5** were formed in nearly equal amounts.

Equilibration of this mixture with $NaOCH_3$ in CH_3OH shifted the ratio to about 1:15 of **4**–**5**, thus establishing the relationship of the two products as epimers and allowing configurational assignment. In the present series the conversion of endo ester **4** to exo ester **5** was a high-yield procedure in contrast to the esters of type **1** where β-elimination of the nitrogen could occur.[1]

Separation of esters **4** and **5** was accomplished by fractional crystallization and/or column and plate chromatography. Generally the 3-exo ester showed the larger R_f value in *i*-$PrNH_2$–Et_2O–pentane mixtures. However, the mixture of thienyl esters **36** was not resolvable by TLC. The 3-exo esters were readily recognizable by NMR since the aromatic ring was close enough to the ester function to shift the OCH_3 signal upfield by about 0.3 ppm from the OCH_3 signal positions seen in the 3-endo ester series. These peak positions are recorded in Table I.[4] Shift studies on these epimers using $Eu(fod)_3$ furnished limited

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