β-Endorphin: Replacement of tyrosine in position 27 by tryptophan increases analgesic potency—preparation and properties of the 2-nitrophenylsulfenyl derivative

(peptide synthesis/mouse tail-flick method/opiate receptor-binding assay)

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ABSTRACT   An analog of human β-endorphin with trypto-
phan in position 27 has been synthesized by the solid-phase
method. Bioassay of the analog showed that it had almost 4 times
the analgesic potency of the parent hormone but only 68% of the
opiate receptor-binding activity. This peptide is the most potent
analgesic among the known synthetic analogs of β-endorphin. The
2-nitrophenylsulfenyl derivative of the analog has been prepared
and shown to have a lower analgesic potency and a higher opiate
receptor-binding activity than the parent compound.

All known β-endorphins, (β-EPs) isolated from pituitary glands
of various species have 31 amino acids and similar primary struc-
ture (1–3). In residue position 27, the amino acid is either ty-
rosine or histidine (Fig. 1). Since there is no tryptophan in the
β-EP molecule, it appeared of interest to synthesize an analog
that has tryptophan in position 27. Moreover, photoreactive 2-
nitro-4(5)-azidophenylsulfenyl derivatives of tryptophan-con-
taining peptides have recently been described (4). If the analog
that has tryptophan in position 27 is biologically active, it will
be useful to study the characteristics of the β-EB receptor by
using these photoaffinity labeling reagents. In this communi-
cation, we describe the synthesis and properties of the analog
and its 2-nitrophenylsulfenyl derivative.

EXPERIMENTAL PROCEDURES

Aminomethyl-resin was prepared as described by Mitchell et
al. (5) to give a load of 0.286 mmol/g by picric acid titration (6).

tert-Butoxycarbonyl(benzyl)glutamyl-4-(oxymethyl)phenyl-
acetic acid. This compound (Boc, *tert*-butoxycarbonyl; Bzl,
benzyl) was prepared as described for derivatives of other amino
acids (5). The cesium salt of Boc-Glu(Bzl)-OH (3.2 mmol) (7) was
allowed to react with 4-(bromomethyl)-phenylacetic acid phen-
acyl ester (3.2 mmol) in dimethylformamide (15 ml) for 2 hr at
24°C to give 1.607 g (83%) of a crystalline product: mp 38–40°C.
After removal of the phenacyl group, the resulting product
(0.985 g) was recrystallized twice from dichloromethane/pe-
troleum ether to give a final yield of 0.74 g (59% based on phen-
acyl ester intermediate): mp 119-120°C; TLC (chloroform/
acetic acid, 15:1) R_F 0.77 (single spot with Cl_2/tolidine detec-
tion); $[\alpha]_D^{24}$ −17.6° (*c* 2, methanol). Analysis: Calculated for
$C_{26}H_{31}NO_8$ (485.53): C, 64.32; H, 6.44; N, 2.88. Found: C,
64.28; H, 6.33; N, 2.84.

Boc-Glu(Bzl)-4-(oxymethyl)-phenylacetamidomethyl (Pam)-
resin. Boc-Glu(Bzl)-4-(oxymethyl)phenylacetic acid (0.60 g,
1.24 mmol) was dissolved in CH_2Cl_2 (5 ml) and mixed at 0°C
with 1.0 ml of a solution of dicyclohexylcarbodiimide (0.60

mmol) in CH_2Cl_2. After 15 min at 0°C and 10 min with warming
to 24°C, the mixture was filtered and the filtrate was added to
aminomethyl-resin (2.1 g holding 11 ml of CH_2Cl_2). The mixture
was stirred for 1 hr at 24°C and then treated with an additional
0.30 mmol of dicyclohexylcarbodiimide for 2 hr. The mixture
was filtered and the resin was washed with CH_2Cl_2 (three 25-
ml portions) and absolute ethanol (three 25-ml portions). The
yield of dried product was 2.23 g; its amine content was 0.4 nmol/
g by picric acid titration (6).

Protected Human β-[Trp²⁷]Endorphinyl-4-(oxymethyl)-Pam-
resin. A sample (673 mg, 0.18 mmole) of Boc-Glu(Bzl)-4-(oxy-
methyl)-Pam-resin in a Beckman 990 peptide synthesizer was
carried through the schedules described for the synthesis of
human β-EP (β$_h$-EP) (8) with the following exceptions: (*i*) the
use of trifluoroethanol for coupling was omitted and (*ii*) Boc-
Trp(formyl)-OH (1.5 mmol) (9) was dissolved in a mixture of
dimethylformamide (0.5 ml) and CH_2Cl_2 (2.0 ml) before car-
rying out coupling by the symmetrical anhydride procedure.
After removal of the last Boc group, the yield of protected pep-
tide resin was 1.494 g.

β$_h$-[Trp²⁷]EP. Protected peptide resin (710 mg, correspond-
ing to 86 μmol of starting load) was treated with liquid HF and
processed through Sephadex G-10 as described (8) to give 268
mg of product. For deformylation (10), this material was dis-
solved in water (30 ml) and taken to pH 11.3 with 1 M NaOH.
After 1.5 min, the solution was adjusted to pH 5.1 with glacial
acetic acid and subjected to chromatography on CM-cellulose
at 24°C in a 2.0 × 48 cm column initially equilibrated with 0.01
M NH₄OAc, pH 4.5. A gradient was effected through a 500-ml
constant volume mixing chamber containing the initial buffer
by introduction of 0.4 M NH₄OAc. Collection of eighty 10-ml
fractions gave two major peaks (tubes 38 and 46) and two minor
ones (tubes 33 and 58), a pattern that is more complex that those
consistently observed for analogs of β-EP. The inference that
this result was due to side reactions in the deformylation step
(9) was confirmed by the simple pattern given by chromatog-
raphy on CM-cellulose of material that had not been defor-
mylated (one major peak followed by one minor peak). In this
manner, the peak centered in tube 46 was tentatively identified
as corresponding to the desired product (yield, 80.5 mg). Final
purification was achieved by partition chromatography on
Sephadex G-50 in a 2.56 × 45 cm column eluting with 1-butanol/
pyridine/acetic acid/water, 500:300:1:1000 (vol/vol) and col-
lecting 4.4-ml fractions. The product eluted with R_F 0.4 and
isolation by lyophilization gave 25 mg of highly purified β$_h$-
[Trp²⁷]EP (ca. 8% yield based on starting aminomethyl-resin).

Abbreviations: β-EP, β-endorphin; β$_h$-EP, human β-EP; Boc, *tert*-bu-
toxycarbonyl; Bzl, benzyl; Pam-resin, phenylacetamidomethyl-resin;
NPS-Cl, 2-nitrophenylsulfenyl chloride.

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                        1            5                   10                  15
Human:      H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-

Camel, bovine, ovine:   H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu

Porcine:    H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-

Equine:     H-Tyr-Gly-Gly-Phe-Met-Ser-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-

Rat:        H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-

Turkey:     H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-His-Ser-Gln-Met-Pro-Leu-Leu-Thr-Leu-

Ostrich:    H-Tyr-Gly-Gly-Phe-Met-Ser-Ser-Glu-Arg-Gly-Arg-Ala-Pro-Leu-Val-Thr-Leu-
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                    20                  25                  30
Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH

Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln-OH

Phe-Lys-Asn-Ala-Ile-Val-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln-OH

Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln-OH

Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Val-His-Lys-Lys-Gly-Gln-OH

Phe-Lys-Asn-Ala-Ile-Val-Lys-Ser-Ala-Tyr-Lys-Lys-Gly-Gln-OH

Phe-Lys-Asn-Ala-Ile-Val-Lys-Ser-Ala-Tyr-Lys-Lys-Gly-Gln-OH
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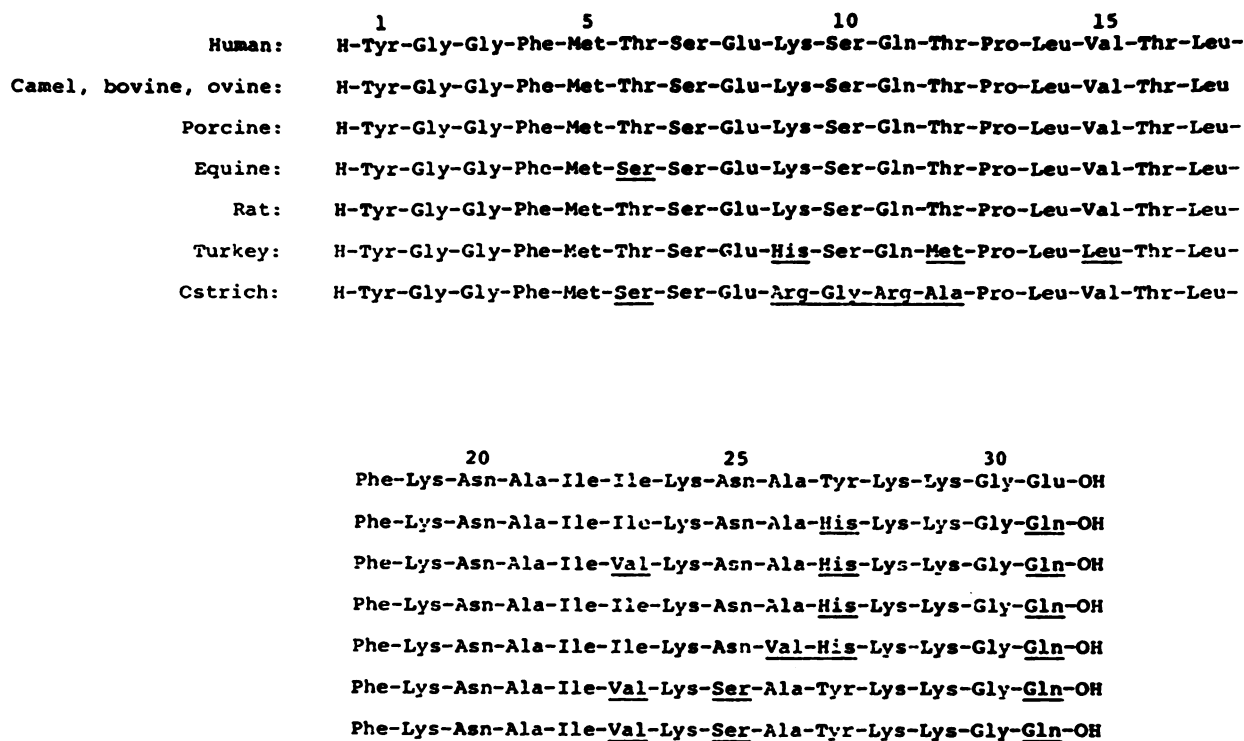
FIG. 1.   Amino acid sequences of β-EP from various species.

It was homogeneous in TLC (silica gel) in 1-butanol/pyridine/acetic acid/H$_2$O (5:5:1:4) (R_F 0.65) and on paper electrophoresis on Whatman 3 MM (400 V, 5 hr) at pH 3.7 and 6.7 (R_F 0.55 and 0.40, respectively, relative to lysine). In all cases 50 μg of peptide was spotted and detection was by ninhydrin and Cl$_2$/tolidine.

Amino acid analysis of a 24-hr HCl hydrolysate gave (theoretical values in parentheses) aspartic acid, 2.10 (two); threonine 2.70 (three); serine, 1.67 (two); glutamic acid, 3.04 (three); proline, 0.98 (one); glycine, 2.95 (three); alanine, 2.17 (two); valine, 1.07 (one); methionine, 0.82 (one); isoleucine, 1.50 (two); leucine, 2.15 (two); tyrosine, 0.89 (one); phenylalanine, 1.91 (two); lysine, 5.08 (five); tryptophan, destroyed (the low value for isoleucine is accounted for by the acid-resistant Ile-Ile moiety). Amino acid analysis of an enzymic digest (trypsin/chymotrypsin followed by leucine aminopeptidase) gave aspartic acid, 0.22 (zero); threonine/serine/asparagine/glutamine, 7.35 (eight); glutamic acid, 2.17 (two); proline, 0.62 (one); glycine, 2.84 (three); alanine, 2.33 (two); valine, 1.16 (one); methionine, 0.99 (one); isoleucine, 1.86 (two); leucine, 1.90 (two); tyrosine, 1.06 (one); phenylalanine, 2.06 (two); lysine, 5.05 (five); tryptophane, 1.00 (one). Our current commercial batch of leucine aminopeptidase releases proline poorly.

Reaction of β$_h$-[Trp²⁷]-EP with 2-Nitrophenylsulfenyl Chloride. 2-Nitrophenylsulfenyl chloride (NPS-Cl) from Eastman was recrystallized as described (11). In a typical experiment, the synthetic analog (8 mg) was dissolved in 0.5 ml of 0.1 M HOAc and 22 mg of NPS-Cl in 0.5 ml of glacial HOAc was added with continuous stirring. After 1 hr at 22°C, the product was separated from excess reagent by gel filtration on a Sephadex G-10 column (1.2 × 26 cm) previously equilibrated with 0.2 M HOAc. The peptide was recovered by lyophilization (yield, 6 mg).

To estimate the extent of modification, the NPS derivative was dissolved in 80% HOAc (1.0 mg/ml) and the amount of NPS

chromophore was determined spectrophotometrically (ε, 4000 at 365 mμ) according to Scoffone *et al.* (11).

Bioassay. The opiate receptor-binding assay was carried out as described (12) using rat brain membrane preparations with tritiated β$_h$-EP (13) as primary ligand and synthetic β$_h$-EP as standard competing ligand (8). Analgesic potency was estimated by the tail-flick method (14) using groups of 8–10 mice per dose as described (15).

RESULTS AND DISCUSSION

Solid-phase synthesis (16) of β$_h$-[Trp²⁷]EP was carried out by using the Pam-resin described by Mitchell *et al.* (5) with tryptophan protected by the formyl group (9, 17). In all other respects, synthesis and purification were carried out essentially as described for the preparation of β$_h$-EP (8). The analog was

Table 1. Amino acid composition of β$_h$-[NPS-Trp²⁷]EP

Amino acid	β$_h$-[NPS-Trp²⁷]EP	β$_h$-[Trp²⁷]EP	Theoretical
Lysine	4.8	5.4	5
Phenylalanine	2.0	2.0	2
Tyrosine	0.9	1.0	1
Leucine	2.1	2.1	2
Isoleucine*	1.4	1.3	2
Methionine	1.0	1.0	1
Valine	1.0	1.0	1
Alanine	1.9	1.9	2
Glycine	2.9	2.8	3
Proline	1.0	1.0	1
Glutamic acid	2.9	2.9	3
Serine†	1.6	1.6	2
Threonine†	2.8	2.7	3
Aspartic acid	2.0	2.0	2

* The Ile-Ile bond is known for resistance to 24-hr acid hydrolysis.
† Values not corrected for destruction.

Table 2. Analgesic potency and opiate receptor-binding activity of synthetic β_h-[Trp^{27}]EP and its NPS derivative

	Analgesic potency (A)		Opiate receptor-binding activity (B)		
Peptide	AD$_{50}$, μg per mouse	Rela-tive*	IC$_{50}$, nM	Rela-tive	B/A
β_h-EP	0.104	100	0.615	100	1.00
β_h-[Trp^{27}]EP	0.028	371	0.905	68	0.18
β_h-[NPS-Trp^{27}]EP	0.168	62	0.200	260	4.20

AD$_{50}$, Mean analgesic dose.
* On a mol/mol basis.

obtained in highly purified form, as studies on the partition properties of β-EP and its analogs have shown (18). The relatively low yield (ca. 8%) as compared with that of the parent hormone (ca. 32%) in an earlier synthesis (8) can probably be accounted for in part by side reactions occurring during the removal of the formyl group (9). Such side reactions, which involve transfer of a formyl group to an amino group, would be expected to be more serious in a peptide containing a large number of lysine residues as β_h-EP.

The reaction of NPS-Cl with β_h-[Trp^{27}]EP in 50% HOAc was found to alkylate quantitatively the tryptophan residue as determined spectrophotometrically at 365 mμ (1.0 mol of NPS/mol of derivative). To ascertain that only the tryptophan residue was modified by NPS-Cl, the derivative was submitted to amino acid analysis after acid analysis. The results indicate that the amino acid contents of the derivative and β_h-[Trp^{27}]EP are identical (Table 1).

The biological activities of the two analogs are presented in Table 2. β_h-[Trp^{27}]EP is the most potent analgesic peptide
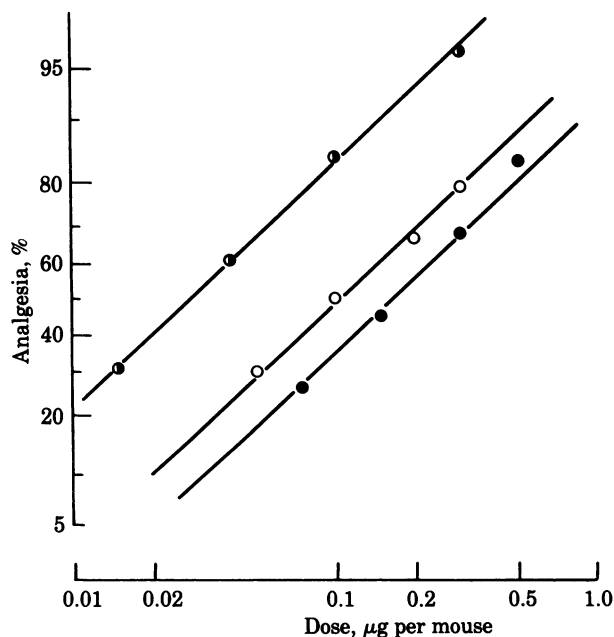

FIG. 2. Logarithmic probit dose–response relationship of antinociceptive effect produced by intracerebroventricular injection of β_h-EP and analogs. After control latency was obtained, groups of eight mice were injected and the tail-flick response was determined at 0.25, 0.50, and 1 hr. ○, β_h-EP; ◑, β_h-[Trp^{27}]EP; ●, β_h[NPS-Trp^{27}]EP.

among the various synthetic analogs of β-EP tested. It exhibits nearly 4 times the analgesic potency of the parent molecule (Fig. 2). However, it possesses only 68% of the opiate receptor-binding activity in comparison with that of β_h-EP. Thus, the ratio of binding activity to analgesic potency is 0.18, the lowest value ever obtained.

Modification of β_h-[Trp^{27}]EP with NPS-Cl decreased the analgesic potency to 1/6 that of the parent compound and increased the binding activity 4-fold. In comparison with β_h-EP, β_h-[NPS-Trp^{27}]EP possesses 62% analgesic potency. Addition of the NPS group in the tryptophan residue may cause steric hindrance, which could reduce analgesic potency, but increases the hydrophobicity, which enhances the opiate receptor-binding activity.

It has recently been reported (19) that replacement of glutamic acid-8 by glutamine increases the analgesic potency of β_h-EP 2.7-fold. The data presented here show that replacement of tyrosine-27 by tryptophan elevates the potency even more (Table 2). Earlier studies indicate that residue position 27 in β_h-EP can be replaced by histidine (20) or phenylalanine (21, 22) with moderate increase of analgesic potency. It may be possible to obtain a very potent analgesic analog by modification of residue positions 8 and 27 in the β_h-EP molecule.

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