54. [D-Ala^2, Phe (p-NO_2)^4, Leu^5]Enkephalin Amide and N^{α}-[D-Ala^2, Leu^5]-Enkephalyl-N^{ε}-Acetyl-Lysine Amide: Synthesis and Biological Properties of Prospective Enkephalin Cooperative-Affinity and Photoaffinity Labels

Preliminary Communication[1])

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Summary

As a study preliminary to attempts at photoaffinity and cooperative affinity labelling of cells and cell membrane vesicles bearing opiate receptors, the title compounds were prepared and tested by rat brain membrane binding assays and by a modified guinea-pig ileum bio-assay. The potency of the two compounds in both systems was considerably greater than that of the standard peptide, [Leu^5]enkephalin, justifying further work with these and similar compounds.

Continuing our work on photoaffinity labelling with peptides containing p-nitro- and p-azidophenylalanine [1] and on the characterization and isolation of receptor-bearing cell membrane vesicles with cooperative affinity labelling methods [2], we are engaged in a joint venture to apply these and other [3] methods to the study of opiate receptors.

Opiate receptors of the central nervous system as a general class of receptor that binds morphine and related agonists and antagonists as well as the endorphins (of which the enkephalins are the shortest peptides) [4] are presently in the focus of interest in the field of molecular neuroendocrinology. Their (differential? [5]) isolation would be a great help for investigating the mechanism of neuromodulation by

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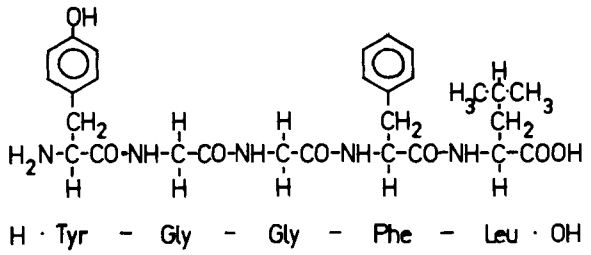
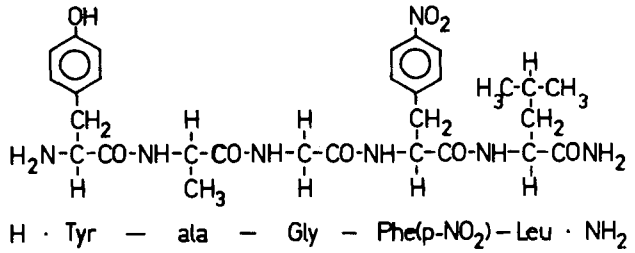
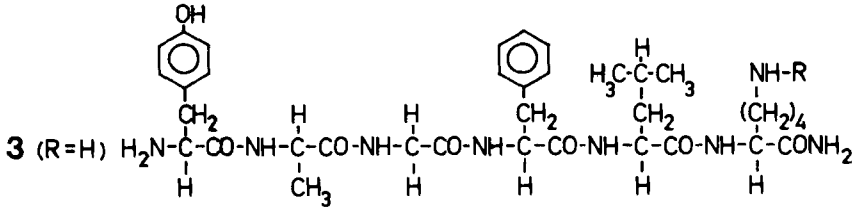


1 H · Tyr – Gly – Gly – Phe – Leu · OH



2 H · Tyr – ala – Gly – Phe(p-NO_2) – Leu · NH_2



3 (R=H) H · Tyr – ala – Gly – Phe – Leu – Lys · NH_2

3a R=CO·CH$_3$ **3b** R=CO·CH$_2$Br **3c** R=CO·(CH$_2$)$_5$-N(CO)$_2$

the endorphins, which appears to play a great role in pain perception, psychic functions, and psychic disorders [6].

As a preliminary to this work, we are studying enkephalin analogues containing modifications in the Phe^4 position [7], and also *C*-terminal extensions of the molecule that may allow for convenient covalent attachment to tobacco mosaic virus [2]. In order to be useful for our purpose, such compounds must bind to the opiate receptors and be either biological agonists or antagonists of the neuromodulator function. The two peptides chosen for a first round of investigations are [ala^2, Phe(p-NO_2)^4, Leu^5]-enkephalin amide (**2**) and N^{α}-[Leu^5]enkephalyl-N^{ε}-acetyl-lysine amide (**3a**) (**3a** is a model for the corresponding bromoacetyl (**3b**) and 6-maleimidohexanoyl (**3c**) derivatives that can be attached to tobacco mosaic virus [2]). They were compared with the naturally occurring [Leu^5]enkephalin (**1**) [8].

β-Turns connecting two stretches of antiparallel peptide chains in the hydrogen-bonded β-pleated-sheet structure were described many years ago [9] and their stabilization by the presence of D-amino acids implied [10]. The physical basis of the

phenomenon was studied later, and the name β-turn suggested [11]. A β-turn in [Leu^5]enkephalin (**1**) involving the two glycine residues was demonstrated in the crystal structure [12] and is most probably also present in solution [13]. A stabilization of the β-turn by the introduction of D-alanine into position 2 might partly explain the enhanced potency of [ala^2,Met^5]enkephalin amide and other [ala^2]-enkephalin analogues [14] in various assay systems. Perhaps the protection against proteolytic enzymes offered by D-alanine^2 and by the *C*-terminal amide group also contributes to the enhancement of the apparent potency of these analogues. In any case, it was because of this enhancement that we chose to include ala^2 and *C*-terminal amide as features of peptides **2** and **3a–3c**.

Materials and methods. – [Leu^5]enkephalin (**1**) was prepared both by the solid-phase technique and in homogeneous solution. Analogue **2** was synthesized from BOC · Tyr-ala-Gly · OH and H · Phe(*p*-NO_2)-Leu · NH_2 by classical procedures and was obtained as a pure compound. Analogues **3** were prepared from BOC · Tyr-ala-Gly · OH and H · Phe-Leu-Lys(Z) · NH_2: condensation led to BOC · Tyr-ala-Gly-Phe-Leu-Lys(Z) · NH_2, from which the benzyloxycarbonyl group was removed by catalytic hydrogenolysis. In the ensuing BOC · Tyr-ala-Gly-Phe-Leu-Lys · NH_2, the ε-amino group of lysine can easily be substituted by acetyl, bromoacetyl, or 6-maleimidohexanoyl; removal of *t*-butoxycarbonyl by mild acidolysis yielded **3a**, **3b** and **3c**. A detailed account of these syntheses is in preparation.

Binding assays were carried out as previously reported [7]. [Leu^5]enkephalin was included in each binding experiment as reference compound. The guinea-pig ileum bio-assay was performed according to a recently published procedure [3a]. The compounds were tested with three pieces of ileum from two different animals.

Results. – The results of a representative rat brain membrane binding assay are shown in *Figure 1*. Parallel log-dose/displacement curves are observed for **2**, **3a** and **1** with IC50 [7] values of $2.0 \cdot 10^{-9}$ M, $1.9 \cdot 10^{-8}$ M, and $5.3 \cdot 10^{-8}$ M, respectively. The average potency ratios relative to **1** obtained from three independent binding experiments are indicated in the *Table*.

In *Figure 2*, the log-dose/response curves for the inhibition of electrically invoked contractions of guinea-pig ileum are presented. The IC50 values of $3.7 \cdot 10^{-9}$ M, $2.7 \cdot 10^{-8}$ M and $8.4 \cdot 10^{-8}$ M obtained for **2**, **3a** and **1**, respectively, correlate well with the binding assay data, as revealed by comparison of the corresponding potency ratios *(Table)*.

Table. *In vitro opiate activities of enkephalin analogues*

Analogue	Potency ratio relative to [Leu^5]enkephalin (**1**)	
	Receptor binding assay[a])	Guinea-pig ileum bio-assay
2	30.9±4.4	22.7
3a	3.9±0.1	3.6
1	1	1

[a]) Mean of three experiments ± SEM.

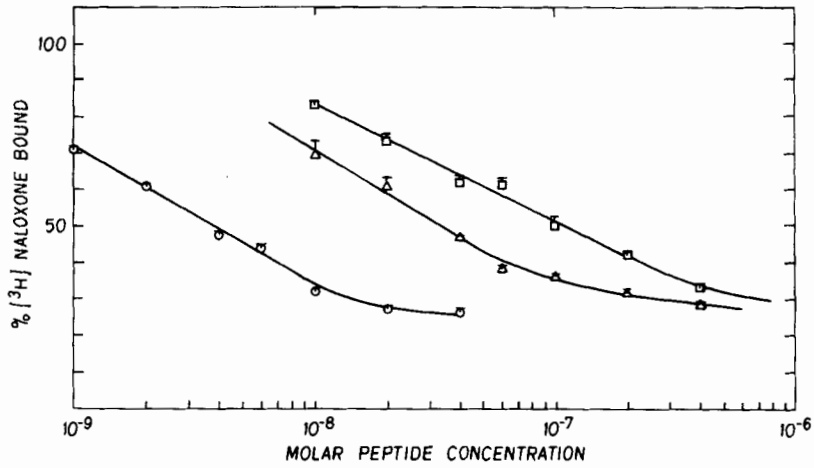


Fig. 1. *Displacement of [^{3}H]naloxone by* **2** (○———○), **3a** (△———△), *and* **1** (□———□) *from rat brain membrane binding sites. Each point represents the mean of three measurements ± SEM*

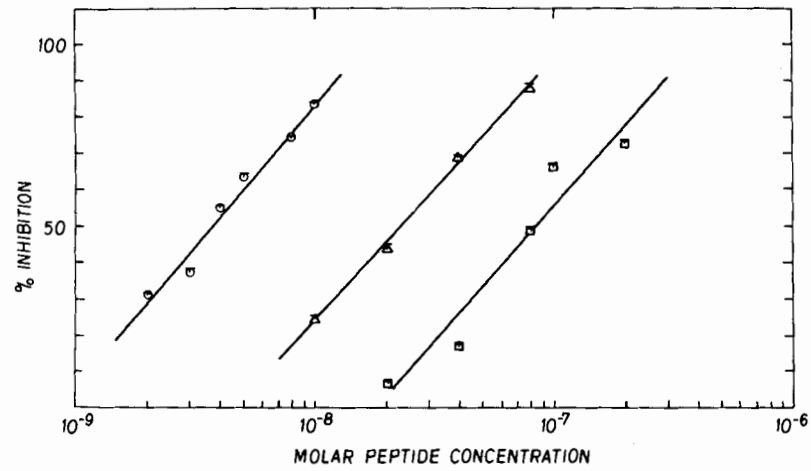


Fig. 2. *Log-dose/response curves for* **2** (○———○), **3a** (△———△), *and* **1** (□———□) *in the guinea-pig ileum assay. Each point represents the mean of 6 to 10 measurements ± SEM*

Discussion. – The analogues **3a** and **2** were found to be roughly 3 and 25 times more active in the binding and bio-assay than [Leu^5]enkephalin (**1**). [ala^2, Met^5]-Enkephalin amide was reported to be bound 'almost as tightly' to opiate receptor preparations as [Met^5]enkephalin [14b], or 1.2–1.5 times as tightly [14a]; however in the biological assays, the D-Ala compounds were much more active (about 10-fold in the vas deferens assay [14a]). On the assumption that the Leu^5-series behaves comparably to the Met^5-series, our results would seem to indicate that the nitro group and the N^{ε}-acetyl-lysine group contribute considerably to the binding force (enhancing it roughly 20- and 2-fold, respectively). The fact that analogues of

[Leu^5]enkephalin with replacements in the Phe^4 position can enhance the binding without destroying biological activity (the [carboranylalanine^4]analogue showed enhanced binding [7] and no loss of biological activity [15]) might imply a certain 'address function' [16] of the *C*-terminal half of the molecule, and a 'message function' [16] of the *N*-terminal part which bears a certain resemblance to morphine and morphine analogues [3].

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