BIOORGANIC CHEMISTRY 8, 255–262 (1979)

Enkephalin Analogs Modified in the Aromatic Ring of the N-Terminal Tyrosine Residue

WIESŁAW A. KLIŚ, ELEONORA NAWROCKA, AND IGNACY Z. SIEMION,

Institute of Chemistry, Wrocław University, 50–383 Wrocław, Poland

AND

ANDRZEJ PLECH, ZDZISŁAW STACHURA, AND ZBIGNIEW S. HERMAN

Department of Pharmacology, Silesian Academy of Medicine, 41-808 Zabrze, Poland

Received September 12, 1978

Two analogs of Leu^5-enkephalin, (1-*O*-methyltyrosine,5-leucine)-enkephalin and [1-(3′-amino)-tyrosine,5-leucine]-enkephalin, were synthesized by classical methods. Both analogs show high biological potency after injection into the lateral brain ventricle of the rat. In both cases substitution of the Tyr residue of enkephalin leads to a pronounced prolongation of analgesic action, as compared with the unsubstituted peptide.

INTRODUCTION

After isolation of endogenous polypetides with opioid-like activity from brains of various animals it was suggested that these substances might function as neuromodulators or neurotransmitters (*1*). This hypothesis is now supported by the regional distribution (*2*) and neuronal localization (*3*) of enkephalins, their stereospecific binding to brain receptors (*4*), their action on the electrical activity of isolated neuronal units (*5*), and their effects on rat motility (*6*). Beluzzi *et al.* (*7*) were the first to report an analgesic effect of Met^5- and Leu^5-enkephalin after intracerebroventricular injection, using the tail-flick procedure. This claim was followed by other reports, of which many confirmed (*8*, *9*), but some failed to confirm the analgesic activity (*10*). The failure to demonstrate such activity may have been due to inadequate attention to the very short time course of action, due to rapid degradation. The enkephalin system has been proposed to be a natural endogenous ligand for the opiate receptor in brain, since morphine-like properties of these compounds were observed (*11–15*).

The unique biological properties of these pentapeptides toward the opiate receptors of the central nervous system stimulated the search for structural analogs of enkephalins with new pharmacological properties. So far the synthesis of an enormous number of enkephalin analogs has been reported in the literature. The modifications of the enkephalin structure depend mainly on shortening (*16–18*) or lengthening (*17*) of the peptide chain. Among the analogs, the most interesting ones seem to be those with an additional Tyr residue on the N-terminal end and an additional Leu residue on the C-terminal end.

0045-2068/79/020255-09$02.00/0

The important conclusion arising from these investigations was that the minimal structural unit capable of binding to brain receptors and eliciting biological response is the tetrapeptide Tyr-Gly-Gly-Phe (*17, 19, 20*). Another group of analogs with modified C-terminal carboxyl group (*16*) has also been investigated. Finally, analogs with individual amino acid residues substituted by other amino acids have also been obtained (*18, 21–24*). The most promising analogs are those with a D-amino acid in place of Gly-2 (*21–23*), a proline (*22*), its amide (*23*), or thiazolidine-4-carboxylic acid (*24*)—in place of Leu-5.

The topographical analogy between enkephalins and morphine was discussed recently by Gorin and Marshall (*25*). In their opinion this analogy depends primarily on the correspondence between the tyramine moiety in the morphine molecule and the phenolic ring and terminal amino group of Tyr-1 residue in the peptide. It is also possible that the aromatic ring of the Phe-4 residue in the peptide corresponds to atoms C-5 and C-6 of the C-ring of morphine. In the opinion of these authors the side chain in the highly potent C-bridged morphine analogs, the oripavines, corresponds to Met-5 or Leu-5 side chains, and not with side chain of Phe-4.

Basing our ideas on the topographical analogy between enkephalin and morphine, we intended to investigate the question whether similar substitutions in the aromatic ring of Tyr-1 residue on the one hand, and in the aromatic ring of morphine on the other, will evoke similar changes in biological activity. In this connection we synthesized two analogs of 5-leucine-enkephalin (I): (1-*O*-methyltyrosine, 5-leucine)-enkephalin (II), and [1-(3′-amino)-tyrosine,5-leucine]-enkephalin (III): Substance II corresponds to codeine,

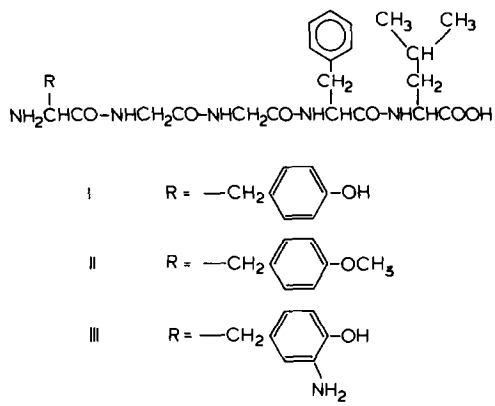
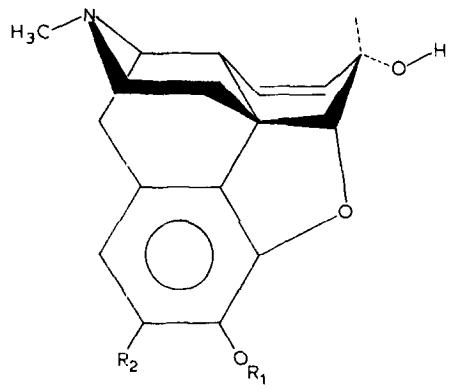


FIG. 1. Leu^5-enkephalin and its analogs.

and substance III to 2-amino-morphine. Both these morphine derivatives show a drastic decrease of analgesic potency, as compared with that of morphine (*26–28*).

The problem of the search for active enkephalin analogs, based on the indicated topographical similarities between enkephalin and morphine, was not systematically reported in the literature. However, it must be noted that Ling and Guillemin (*16*) have investigated a (1-*O*-methyltyrosine,5-methionine)-enkephalin. According to these authors, this analog exhibits low *in vitro* biological activity. Very recently a similar approach in the study of structure–activity relationship of enkephalins was reported by



$-R_1 = -R_2 = -H$ morphine

$-R_1 = -CH_3, -R_2 = -H$ codeine

$-R_1 = -H, -R_2 = -NH_2$ 2-aminomorphine

FIG. 2. Morphine and its analogs.

Schiller *et al.* (*29*), who also investigated, among other enkephalin analogs, (1-*O*-methyltyrosine,5-methionine)-enkephalin. From spectrofluorimetric data obtained for Trp[4]-Met[5]-enkephalin they concluded that the distance between the aromatic rings in enkephalins corresponds to that predicted by the beta-bend model for peptide conformation. In contrast to the conclusion of Gorin and Marshall (*25*), and in agreement with this distance determination, they suggested that the side chain in oripavine molecule corresponds with Phe-4 residue of the peptide.

MATERIALS AND METHODS

(1-*O*-methyltyrosine,5-leucine)-enkephalin (II) and [1-(3′-amino)-tyrosine,5-leucine]-enkephalin (III) were synthesized according to the (3 + 2) scheme. The starting compounds needed for the synthesis were obtained by known methods: L-3′-nitrotyrosine according to Greenstein and Winitz (*30*), L-*O*-methyltyrosine according to Siedel *et al.* (*31*), glycyl-glycine ethyl ester hydrochloride according to Kader and Stirling (*32*), *N*-*t*-butyloxycarbonyl-L-phenylalanine according to Schnabel (*33*), and L-leucine benzyl ester *p*-toluenosulphonate according to Zervas *et al.* (*34*). In the syntheses of tripeptide fragments the DCCI or azide methods were used. The pentapeptide sequence was obtained by azide coupling. Details of the syntheses will be given in the patent description. The data for intermediate products are summarized in Table 1. The final compounds were characterized by the following data: (1-*O*-methyltyrosine,5-leucine)-enkephalin (II) monoacetate, mp 188–191°C, $[\alpha]_{546}$ + 25.7 (c 1, methanol). *Anal.* Calcd for $C_{31}H_{43}O_9N_5$: N, 11.1. Found: N, 11.4. Amino acid analysis: OMe–$Tyr_{1.0}Gly_{2.0}Phe_{1.0}Leu_{1.0}$. [1-(3′-amino)-tyrosine,5-leucine]-enkephalin monoacetate, (III) mp 162–166°C, $[\alpha]_{546}$ −8.0 (c 1, methanol); *Anal.* Calcd for $C_{30}H_{42}O_9N_6$: N, 13.3. Found: N, 12.9. Amino acid analysis: NH_2–$Tyr_{0.9}Gly_{2.0}Phe_{1.1}Leu_{1.0}$.

TABLE 1

DATA FOR INTERMEDIATES IN THE SYNTHESIS OF II AND III

Compound	Mp °C	Methanol $[\alpha]_{546}$ c1	Analysis					
			Found (%)			Calculated (%)		
			C	H	N	C	H	N
Z-(OMe)Tyr-Gly-Gly-OEt	124–126	+2.5	61.0	6.4	8.7	61.1	6.2	8.9
Z-(OMe)Tyr-Gly-Gly-$NHNH_2$	193–196	−30.1 (DMF)			14.3			14.8
Boc-Phe-Leu-OBzl	85.5–86.5	−25.0	69.1	7.7	6.0	69.2	7.7	6.0
Z-(OMe)Tyr-Gly-Gly-Phe-Leu-OBzl	177–179	−26.7 (DMF)	66.8	6.6	8.9	66.6	6.5	8.8
(NO_2)Tyr-OMe × HCl	107–109	+1.1	43.3	4.7	10.5	43.5	4.7	10.1
Z-(NO_2)Tyr-$NHNH_2$	188–191		54.7	4.7	14.9	54.5	4.9	15.0
Z-(NO_2)Tyr-OMe	100–101	−1.0	57.9	4.9	7.4	57.8	4.9	7.5
Z-(NO_2)Tyr-Gly-Gly-OEt	116–119	−4.0	55.1	5.4	11.3	55.0	5.2	11.2
Z-(NO_2)Tyr-Gly-Gly-$NHNH_2$	189–194				17.3			17.2
Z-(NO_2)Tyr-Gly-Gly-Phe-Leu-OBzl	162–166	+23.0 (DMF)	63.0	5.5	10.4	62.6	5.8	10.2

Leu-enkephalin was synthesized and kindly donated by Dr K. Medzihradszky from Institute of Organic Chemistry, Eötvös University, Budapest.

Biological experiments were carried out on male Wistar rats from Central Animal Farm of Silesian Academy of Medicine (220–250 g). Analgesic activity of the substances was measured by reproducible and specific hot-plate procedure described by O'Callaghan and Holtzman (*35*). A licking of the fore or hind paws was used as the endpoint for the determination of response latencies recorded to the nearest 0.1 sec. If a latency time was 30 sec the rat was removed from the hot plate to avoid heat burn and in this case the latency time was assumed as 30 sec.

Compounds II, or III, or Leu^5-enkephalin were dissolved in 0.9% sodium chloride solution and injected into the lateral brain ventricle according to the method described elsewhere (*36*). The correctness of the place of injection was checked visually after the killing of each animal. Substances examined were injected in a dose of 100 μg or in a dose of 200 μg in 20 μl of solution. Corresponding control animals were injected with the same volume of the solvent. Analgesic activity was measured several times during 1 hr after injection, as indicated in the figures. As an additional control, morphine hydrochloride was injected intracerebroventricularly to two groups of 10 rats each, using a dose of 10 and 100 μg. The effects were observed during 1 hr. Results were statistically elaborated using t test of Student. In another series of experiments, substances II or III were injected intraperitoneally as a suspension in 0.9% NaCl with the addition of 20% of tragacanth, in a dose of 50 100 or 200 mg/kg. Analgesic activity was measured 30 min, 1, 2, and 3 hr after injection.

RESULTS AND DISCUSSION

Morphine hydrochloride in a dose of 10 and 100 μg increased the latency period to 25 and above 30 sec, respectively (12 sec if only the solvent was injected). The behavior

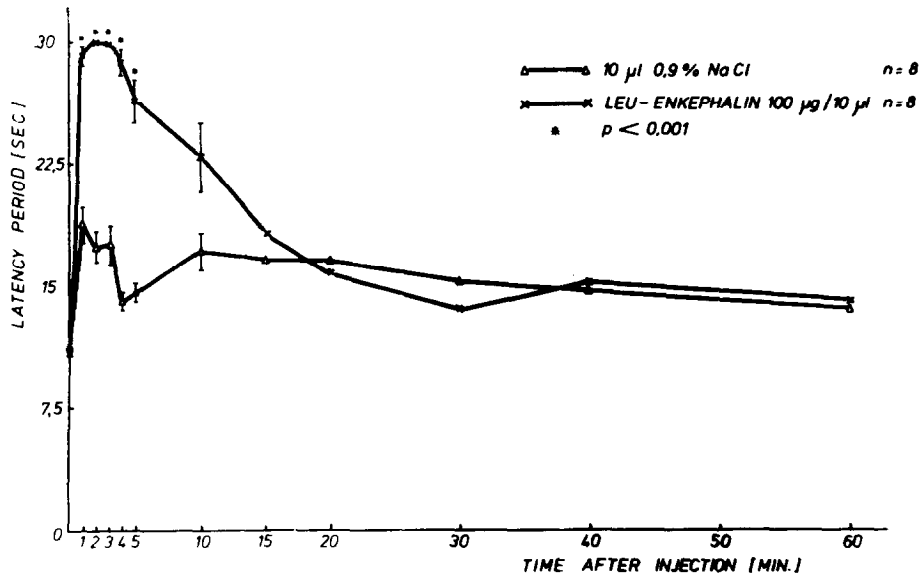


FIG. 3. Analgesic activity of Leu[5]-enkephalin (I) after intracerebroventricular injection.

of animals was controlled 10, 15, 20, 30, 45, and 60 min after injection. Leu-enkephalin in a dose of 100 µg induced obvious analgesic activity of 5-min duration. Analog II (200 µg) injected intracerebroventricularly caused analgesia of 10-min duration. Intracerebroventricular injection of analog III in a dose of 100 or 200 µg induced a dose-dependent analgesia which persisted 20 min after higher dose. The intensity of analgesic

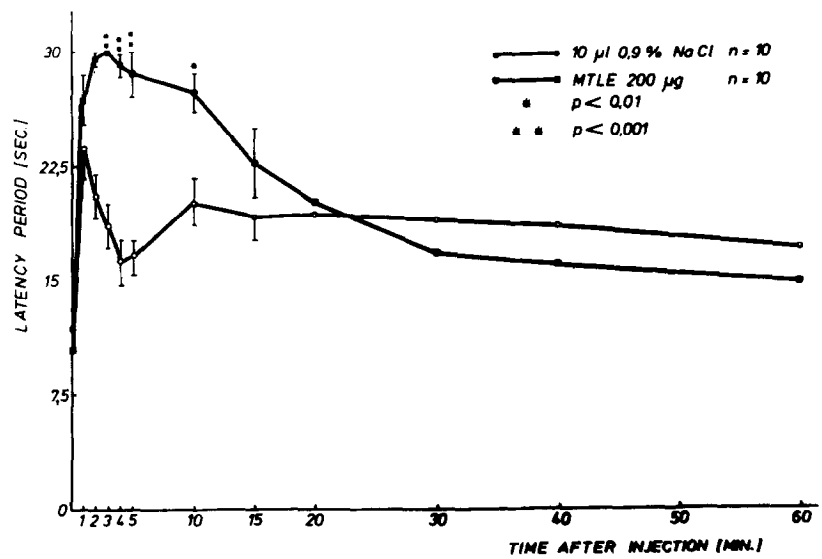


FIG. 4. Analgesic activity of (1-*O*-methyltyrosine,5-leucine)-enkephalin (II), (MTLE) after intracerebroventricular injection.

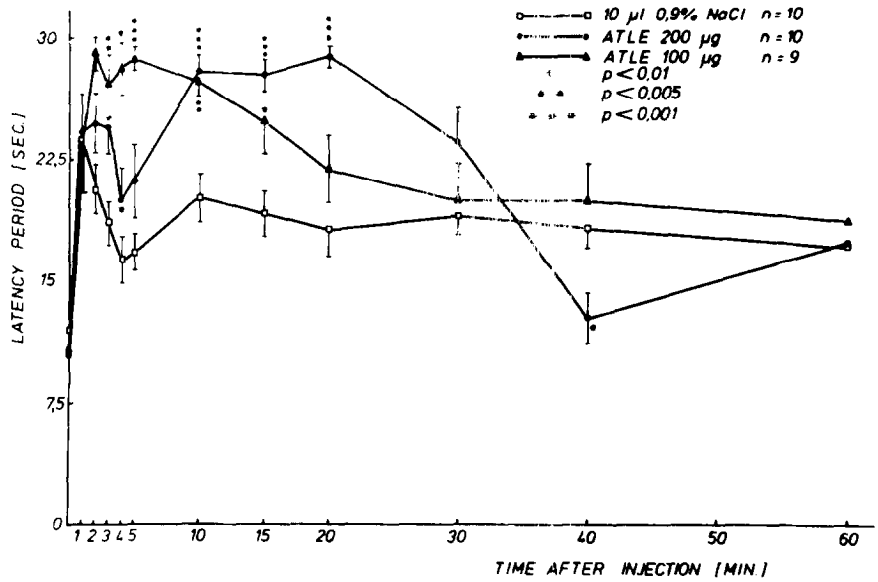


FIG. 5. Analgesic activity of [1-[3'-amino)-tyrosine,5-leucine]-enkephalin (III, ATLE) after intracerebroventricular injection.

activity of all three examined substances was very similar. Analogs II and III injected intraperitoneally in a dose of 50, 100, or 200 mg/kg had no analgesic effect.

Enkephalins induce analgesia and depression of motor activity in rats and mice. These effects are, however, very short lasting and require the administration of large amounts of peptide (about 100–200 µg per rat). This limited action is probably due to the rapid inactivation of the compound resulting from cleavage of the Tyr^1–Gly^2 peptide bond by brain endopeptidase (*37*, *38*). Conclusive evidence concerning this possibility was obtained by studies with the protected analogs, D-Ala^2-Met^5-enkephalinamide (*39*) or D-Met^2, Pro^5-enkephalinamide (*40*). Results of our experiments confirmed the short-lasting analgesic effect of Leu^5-enkephalin applied intracerebroventricularly in a dose of 100 µg. Both analogs of this enkephalin, especially analog III, possess a distinctly longer analgesic than I. These results suggest that they are less rapidly metabolized by brain tissue than Leu-enkephalin. The lack of analgesic activity for both analogs II and III after intraperitoneal injection indicates that these compounds, introduced in this way might be quickly inactivated or do not penetrate the blood brain barrier.

Our results do not reveal direct correspondence between the biological activities of similarly substituted morphine and enkephalin derivatives. However, our observations confirm the importance of the Tyr-1 residue of the peptide for its biological activity, because its chemical modification results in a pronounce biological effect. It seems that the modification of the Tyr-1 residue presents interesting possibilities for the synthesis of enkephalin analogs.

It must be also noted that our results differ from those presented by Ling and Guillemin (*16*) and Schiller *et al.* (*29*) for (1-*O*-methyltryosine,5-methionine)-enkephalin, which possessed only very little *in vitro* activity. However, Ling and Guillemin (*16*) have studied the peptide activity with guinea pig ileum, and Schiller *et al.*

(*29*) determined the activity of the sample by means of the mouse vas deferens test, and not by direct administration of the peptide solution into the brain of the experimental animal. It seems that the discrepancy between our results and literature data results from the different conditions of biological testing.

We would like to acknowledge that the results of Schiller *et al.* (*29*) rendered the hypothesis of the topographical analogy between morphine and enkephalin very probable, because the biological effects observed after chemical modification of the peptide (O-methylation, or N-methylation of N-terminal Tyr-1) were in good agreement with this hypothesis. Our results suggest however that this promising hypothesis of a morphine–enkephalin structural relationship must be regarded with caution and needs further support.

ACKNOWLEDGMENT

This work was supported in part by the Polish Academy of Sciences Grant Nr MR I. 12.

REFERENCES

1. J. HUGHES, T. W. SMITH, H. W. KOSTERLITZ, L. A. FOTHERGILL, B. A. MORGAN, AND H. R. MORRIS, *Nature* (*London*) **258**, 577 (1975).
2. R. SIMANTOV, J. KUHAR, G. W. PASTERNAK, AND S. H. SNYDER, *Brain Res.* **106**, 189 (1967).
3. R. ELDE, T. HOKFELT, O. JOHANSSON, AND L. TERENIUS, *Neuroscience* **1**, 349 (1976).
4. R. SIMANTOV AND S. H. SYNDER, *Proc. Nat. Acad. Sci. U.S.A.* **73**, 2515 (1976).
5. R. C. A. FREDERICKSON AND F. H. NORRIS, *Science* **194**, 440 (1976).
6. F. BLOOM, D. SEGAL, N. LING, AND R. GUILLEMIN, *Science* **194**, 630 (1976).
7. J. D. BELLUZZI, N. GRANT, V. GARSKY, D. SARANTAKIS, C. D. WISE, AND L. STEIN, *Nature* (*London*) **260**, 625 (1976).
8. J-K. CHANG, B. T. W. FONG, A. PERT, AND C. B. PERT, *Life Sci.* **18**, 1473 (1976).
9. L. GRÁF, J. I. SZÉKELY, A. Z. RÓNAI, Z. DUNAI-KOVACS, AND S. BAJUSZ, Nature (*London*) **263**, 240 (1976).
10. W. FELDBERG AND D. G. SMYTH, *J. Physiol.* **260**, 30P (1976).
11. L. TERENIUS AND A. WAHLSTRÖM, *Acta Pharmacol. Toxicol. Suppl.* **35**, 55 (1974).
12. J. HUGHES, *Brain Res.* **88**, 295 (1975).
13. G. W. PASTERNAK, R. GOODMAN, AND S. H. SNYDER, *Life Sci.* **16**, 1765 (1975).
14. G. W. PASTERNAK, R. SIMANTOV, AND S. H. SNYDER, *Mol. Pharmacol.* **12**, 504 (1976).
15. H. N. BHARGAVA, *Eur. J. Pharmacol.* **41**, 81 (1977).
16. N. LING AND R. GUILLEMIN, *Proc. Nat. Acad. Sci. U.S.A.* **73**, 3308 (1976).
17. L. TERENIUS, A. WAHLSTRÖM, G. LINDENBERG, S. KARLSSON, AND U. RAGNARSSON, *Biochem. Biophys. Res. Commun.* **71**, 175 (1976).
18. N. S. AGARWAL, V. J. HRUBY, R. KATZ, W. KLEE, AND M. NIERENBERG, *Biochem. Biophys. Res. Commun.* **76**, 129 (1977).
19. B. A. MORGAN, C. F. C. SMITH, A. A. WATERFIELD, J. HUGHES, AND H. W. KOSTERLITZ, *J. Pharm. Pharmacol.* **28**, 660 (1976).
20. L. H. LAZARUS, N. LING, AND R. GUILLEMIN, *Proc. Nat. Acad. Sci. U.S.A.* **73**, 2156 (1976).
21. E. T. WEI, L. F. TSENG, H. H. LOH, AND C. H. LI, *LIfe Sci.* **18**, 321 (1977).
22. S. BAJUSZ, A. Z. RONAI, J. I. SZÉKELY, L. GRÁF, Z. DUNAI-KOVAĆS, AND I. BERZETEI, *FEBS Lett.* **76**, 91 (1977).
23. S. BAJUSZ, A. Z. RONAI, H. I. SZÉKELY, L. GRÁF, Z. DUNAI-KOVAĆS, AND I. BERZETEI, *Acta Biochim. Biophys. Acad. Sci Hung.* **11**, 305 (1976).

24. D. YAMASHIRO, L-F. TSENG, AND C. H. LI, *Biochem. Biophys. Res. Commun.* **78**, 1124 (1977).
25. F. A. GORIN AND G. R. MARSHALL, *Proc. Nat. Acad. Sci. U.S.A.* **74**, 5179 (1977).
26. O. J. BRAENDEN, N. B. EDDY, AND H. HALBACH, *Bull. World Health Org.* **13**, 937 (1955).
27. B. M. COX AND M. WEINSTOCK, *Brit. J. Pharmacol. Chemother.* **27**, 81 (1966).
28. N. B. EDDY, H. HALBACH, AND O. J. BRAENDEN, *Bull. World Health Org.* **14**, 353 (1956).
29. P. W. SCHILLER, C. F. YAM, AND M. LIS, *Biochemistry* **16**, 1831 (1977).
30. J. P. GREENSTEIN AND M. WINITZ, "Chemistry of Amino Acids," 1st ed., Vol. 3, pp. 2285–2286. Wiley, New York, 1961.
31. W. SIEDDEL, K. STURM, AND R. GEIGER, *Chem. Ber.* **96**, 1438 (1963).
32. A. T. KADER AND C. J. M. STIRLING, *J. Chem. Soc.* 258 (1964).
33. E. SCHNABEL, *Ann.* **702**, 188 (1967).
34. L. ZERVAS, M. WINITZ, AND J. P. GREENSTEIN, *J. Org. Chem.* **22**, 1515 (1957).
35. J. P. O'CALLAGHAN AND S. G. HOLTZMAN, *J. Pharmacol. Exp. Ther.* **192**, 497 (1975).
36. Z. S. HERMAN, *Psychopharmacologia Ber.* **16**, 369 (1970).
37. Y. F. JACQUET AND N. MARKS, *Science* **194**, 632 (1976).
38. J. L. MEEK, H-Y. T. YANG, AND E. COSTA, *Neuropharmacology* **16**, 151 (1977).
39. C. B. PERT, A. PERT, J-K. CHANG, AND B. T. W. FONG, *Science* **194**, 330 (1976).
40. J. I. SZÉKELY, A. Z. RONAI, Z. DUNAI-KOVACS, E. MIGLÉCZ, I. BERZETEI, S. BAJUSZ, AND L. GRÁF, *Eur. J. Pharmacol.* **43**, 293 (1977).