ter the third appearance of the tumor, bilateral ovariectomy also resulted in regression, suggesting that the tumor was endocrine dependent (*6*).

The observations made on the single animal appeared to justify our initial assumption that analog **1** acted through a suppression of ovarian steroid function. We therefore sought confirmatory evidence through the use of the DMBA-induced rat mammary tumor system (*7*). In this experiment we administered the carcinogen as described (*7*). However, all animals were started on treatment on the same day, 5 months after a single administration of DMBA. At this point all animals bearing tumors were stratified according to three criteria: body weight, time after the appearance of the first tumor, and total tumor volume per animal. Allocation of animals into groups was done on the basis of random selection from this stratification. Results of the experiment are shown in Table 1. It is evident that both the group treated with analog **1** and the group subjected to ovariectomy showed significant tumor regression when compared to controls.

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2. The structure of analog **1** is as follows: pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHC_2H_5. Thus the -Gly-NH_2[10] in gonadotropin releasing hormone has been replaced by -NHC_2H_5. The abbreviations for the amino acid residues are as follows: pGlu, pyroglutamic acid; His, histidine; Trp, tryptophan; Ser, serine; Tyr, tyrosine; Leu, leucine; Arg, arginine; and Pro, proline.
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[D-Ala^2]-Met-Enkephalinamide: A Potent, Long-Lasting Synthetic Pentapeptide Analgesic

Abstract. *[D-Ala^2]-Met-enkephalinamide (DALA), a synthetic enkephalin analog designed by in vitro analysis, binds to opiate receptors almost as tightly as methionine-enkephalin. Since it is not susceptible to degradation by brain enzymes, low doses (5 to 10 micrograms) cause profound, long-lasting, morphine-like analgesia when microinjected into rat brain.*

Opiate receptors are stereospecific components of vertebrate synaptic membranes distributed heterogeneously in the mammalian nervous system. Morphine and other opiates initiate their pharmacological effects by complexing reversibly with these receptors, which have been indentified (*1*) and studied extensively with isotopically labeled opiates (*2*). A number of endogenous peptides, which appear to serve as natural ligands for opiate receptors, have been shown to exist in brain (*3*), pituitary (*4*), human cerebrospinal fluid (*5*), and human blood (*6*). The first of these to be identified, enkephalin, is a mixture of two pentapeptides (Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu) (*7*) extracted from porcine brain by Hughes and Kosterlitz and their colleagues (*8*), and also from calf brain (*9*). While enkephalin has potent and readily demonstrable morphine-like effects on the guinea pig ileum and mouse vas deferens (*8*), extremely high quantities (120 to 200 μg) are required to elicit analgesia when microinjected into rat cerebral ventricles (*10*) and brain (*11*). Thus, Met-enkephalin is at least 50-fold weaker than morphine as an analgesic, despite the fact that it has about half of the affinity of morphine for rat brain opiate receptors (*11*). Indeed, Met-enkephalin's mild analgesia dissipates completely in several minutes (*11*), even when injected directly into active brain sites where morphine induces analgesia which persists for several hours. This apparent discrepancy is explained by the finding that enkephalin's affinity for opiate receptors is rapidly and efficiently destroyed by brain enzymatic activity (*11*).

We now report the synthesis of a novel pentapeptide, designed and detected by in vitro analysis, which elicits a potent, long-lasting analgesia.

Affinity for opiate receptors was assessed by the inhibition of stereospecific [^{3}H]naloxone binding to rat brain membranes, as originally described (*12*), with minor modifications designed to avoid the enzymatic destruction of peptides which occurs at warm temperatures (*11*). Analgesia was assessed by the "tail-flick" procedure (*13*), the "flinch-jump" task (*14*), and by subjectively evaluating the animals' reactions to pinches of the limbs with forceps. In the "tail-flick" task, a high-intensity light was focused on a rat's tail, which had been blackened with a felt-tip pen, and the time that it took an animal to remove its tail from this radiant heat was measured to the nearest 0.5 second. In the "flinch-jump" task, the shock intensity at which an animal jumped and squealed was quantified 15 minutes after injection (immediately after testing the tail-flick). Morphine and peptides were dissolved in pyrogen-free water and microinjected in a volume of 1 μl through permanent indwelling cannulae located in the periaqueductal gray region of the midbrain (*11*). This brain region mediates the analgesic actions of opiates in rodents (*15*) and primates (*16*). Peptides were synthesized by the solid phase method with the use of a Beckman 990 synthesizer, and peptide content was determined by amino acid analysis after demonstration of purity by thin-layer chromatography in three solvent systems and electrophoresis (*17*).

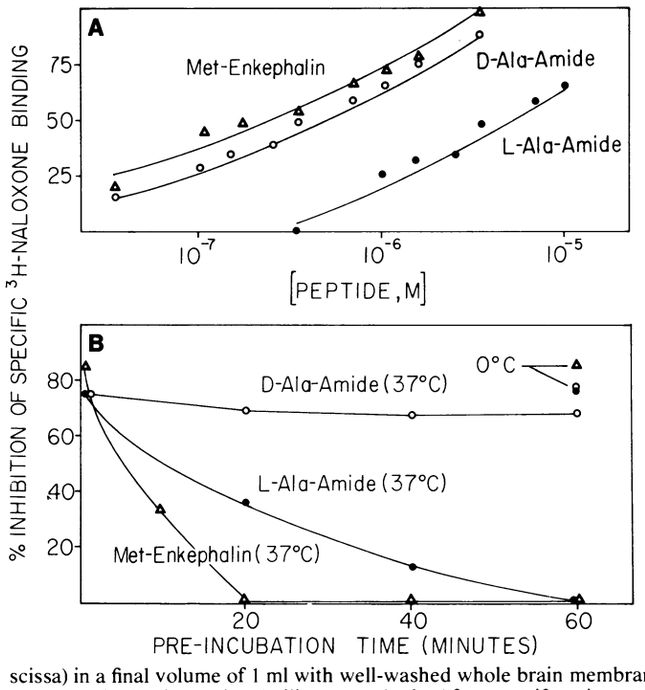
We have demonstrated earlier that several substitutions of enkephalin's amino terminal tyrosine residue (Tyr^1) result in severe losses of opiate receptor affinity of two to three orders of magnitude (*11*). We reasoned that cleavage of the Tyr^1-Gly^2 peptide bond would thus be a likely mechanism for rapid enzymatic inactivation of enkephalin, so that enzyme-resistant analogs must contain substitutions that protect this bond. Substitution of Met-enkephalinamide at the 2-position with D-alanine results in a peptide which almost completely retains its affinity for opiate receptors ($K_D = 4 \times 10^{-7}$ to $5 \times 10^{-7} M$) (Fig. 1A). Substitution in the same position with the "natural" enantiomer, L-alanine, results in about a tenfold weaker receptor affinity. These values reflect actual affinity for receptors since enzymatic degradation is avoided by the low temperature employed (*11*). The slope of the line describing inhibition of [^{3}H]naloxone binding as a function of peptide concentration is the same as that characteristic of morphine, the benzomorphans, and all other opiates previously examined (*12*, *18*). A drug's affinity for opiate receptors in vitro is closely predictive of its analgesic po-

tency in vivo when metabolism and distribution are taken into account (*12*, *18*).

Susceptibility of peptides to proteolytic enzyme degradation was assessed by preliminary incubation with well-washed rat brain membranes for various times at 0° and 37°C (Fig. 1B). Subsequent analysis suggests that the D-alanine analog is completely resistant to receptor affinity loss produced by particulate brain enzymes. This is exceptional since a number of synthetic analogs previously evaluated in this fashion were highly sensitive to the brain proteases (*11*). Met-enkephalin is rapidly degraded in vitro so that its ability to bind to opiate receptors is completely lost after 20 minutes of incubation. Hughes (*3*) has shown that enkephalin's activity in the guinea pig ileum is destroyed by preliminary incubation with ileal strips. [L-Ala^2]-Met-enkephalinamide is also readily degraded at 37°C, indicating that it is the D-Ala^2 substitution rather than the terminal amide which bestows enzyme resistance. Incubation of all peptides for 1 hour at 0°C with membranes or 37°C with buffer has no significant effect on subsequent receptor inhibition.

Examination of the analgesic potency of [D-Ala^2]-Met-enkephalinamide (DALA) in vivo was assessed after in vitro analysis had suggested that DALA might have potent, long-lasting analgesic properties. DALA (5 μg) elicited maximal analgesia 15 minutes after microinjection; this analgesic effect slowly disappeared and returned to baseline by 3 hours after injection (Fig. 2). Injection of 10 μg of DALA followed a similar time course, but still caused a significant increase in tail-flick latency 3 hours after microinjection. Analgesia induced by 5 μg of morphine persisted for about the same number of hours as that induced by 10 μg of DALA, but was more intense at 1 and 2 hours after injection (tail-flick latencies of 10.1 ± 0.68 and 8.3 ± 0.8 seconds, n = 11). Rats that received a preliminary injection of the opiate antagonist naloxone (2 mg/kg, intraperitoneally) 15 minutes before microinjection of DALA (10 μg) showed almost complete reversal of analgesia (10.6 ± 0.72 to 6.8 ± 0.84 seconds, n = 6; $P < .01$). As was described (*11*) and shown in Fig. 2, Met-enkephalin (120 μg) elicited only a 4-second increase in tail-flick latency. This mild analgesia disappeared 5 minutes after injection. [L-Ala^2]-Met-enkephalinamide, as would be predicted from its weaker affinity and enzyme sensitivity, failed to elicit a significant alteration in tail-flick latency (n = 6). In the flinch-jump test, 10 μg of DALA (n = 11) and 10 μg of morphine (n = 10) increased jump-squeal thresholds from 0.62 to 0.90 ma and from 0.54 to 1.02 ma, respectively ($P < .01$ for both comparisons). At this time, moreover, both morphine- and DALA-treated rats failed to vocalize or show distress to pinches of the hind limbs with forceps.

It seems conclusive that the difficulty of eliciting analgesia with enkephalin is due to rapid cleavage of its Tyr^1-Gly^2 peptide bond. [D-Ala^2]-Met-enkephalinamide, its very similar but nondegradable analog, is shown here to be a potent analgesic in three rat tasks; since DALA is almost as potent and long-lasting as morphine, it provides a useful tool for studying behavioral effects of opiate peptides. In particular, the notion that a more "natural" opiate should be less able to activate mechanisms that produce tolerance and physical dependence can be experimentally examined. DALA (50 mg/kg) fails to elicit analgesia after intravenous injection (n = 2), presum-



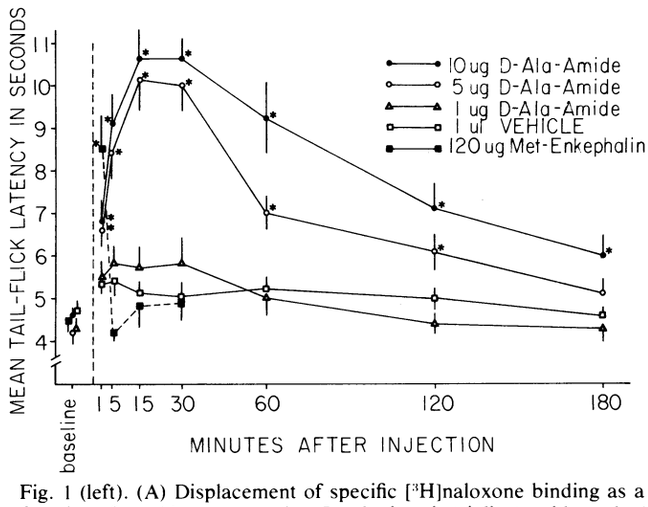


Fig. 1 (left). (A) Displacement of specific [^{3}H]naloxone binding as a function of peptide concentration. Incubations in triplicate with washed brain membranes were conducted at 0°C for 1 hour in 100 mM NaCl, 0.05M tris buffer (pH 7.0 at 25°C) in a final volume of 0.5 ml with 1.3 nM [^{3}H]naloxone (*11*). Specific binding, which was about 90 percent of the total binding, was 2363 ± 166 count/min. (B) Inactivation of opiate peptides by preliminary incubation with brain membranes at 37°C. Peptides were incubated for stated times (abscissa) in a final volume of 1 ml with well-washed whole brain membranes (25 mg original wet weight). Incubation was terminated by immersing test tubes for 2 minutes in a boiling water bath. After centrifugation at 3000g, duplicate portions (100 to 300 μl) of the supernatant were assessed for opiate receptor inhibitory activity, as described above. Initial peptide concentrations for Met-enkephalin (Tyr-Gly-Gly-Phe-Met), DALA (Tyr-D-Ala-Gly-Phe-Met-amide), and L-Ala (Tyr-L-Ala-Gly-Phe-Met-amide) were, respectively, $1 \times 10^{-5}M$, $1 \times 10^{-5}M$, and $8 \times 10^{-5}M$. Fig. 2 (right). Changes in tail-flick latency after injections of [D-Ala^2]-Met-enkephalinamide into the periaqueductal gray matter. Animals were tested for analgesia after injection of either 1, 5, or 10 μg of DALA or 1 μl of H_2O (vehicle). The tests were made only at the intervals indicated on the abscissa. Baseline response levels were determined approximately 1 hour prior to injection. A trial was automatically terminated if a rat failed to remove its tail within 12 seconds. The drugs were administered in a counterbalanced order separated by 5 days. Vertical lines indicate the standard error of the mean, the asterisk indicates $P < .05$ for comparisons of DALA against vehicle control after a significant treatment effect ($P < .01$) was found with a repeated measures analysis of variance. A common logarithmic conversion was used to normalize the data prior to analyses. Data for Met-enkephalin are plotted from (*11*). For Met-enkephalin, the asterisk indicates $P < .05$ for comparisons of postinjection responses against baseline levels.

ably because it fails to cross the blood-brain barrier. With appropriate chemical modification, DALA could, conceivably, give rise to a totally synthetic opiate peptide analgesic which would be active after systemic administration.

It has long been known (*19*) that introduction of unnatural, D-amino acids into peptides can convey resistance to degradative enzymes. This principle has recently been applied to produce a potent analog of luteinizing hormone releasing hormone (LHRH) by substitution of Gly^6 with D-Ala^6 (*20*), resulting in resistance to enzymatic degradation (*21*). Presumably, the methyl group of the D-alanine side chain of DALA is positioned so that opiate receptor recognition is relatively unhindered while enzymatic access to the critical Tyr^1-Gly^2 bond is blocked. In support of this explanation, a number of additional enzyme-resistant DALA analogs have been synthesized which contain position-2 substitutions by other D-amino acids, L-proline and sarcosine (*22*).

Note added in proof: Also, a recent report of chemical analysis of enzymatic breakdown products of enkephalin (*23*) suggests that cleavage of the Tyr^1-Gly^2 amide bond is the initial deactivation step.

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Ascorbic Acid as a Factor Controlling the Development of Cyanide-Insensitive Respiration

Abstract. *Lycorine, an inhibitor of ascorbic acid biosynthesis, prevents the elicitation of KCN-insensitive respiration in aerated potato tuber slices. Ascorbic acid administration prevents the lycorine effect. Because KCN-insensitive respiration, which takes place during the aerobic incubation of potato tuber slices, specifically depends on the synthesis of new proteins, we suggest that ascorbic acid could be required to carry out a synthetic process.*

While the role of many vitamins in cell metabolism is now recognized at the molecular level, a considerable amount of uncertainty still exists with regard to that of vitamin C (ascorbic acid). Our work suggests an alternative approach to the definition of the role of ascorbic acid (AA) in cell metabolism.

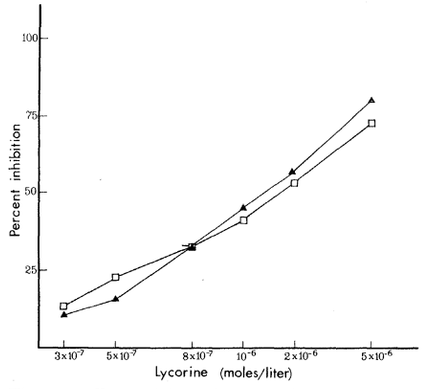

Fig. 1. Effect of lycorine on biosynthesis of ascorbic acid (AA) (□) and on the development of KCN-insensitive respiration (▲). The AA biosynthesis was evaluated as described (*1*, *15*).

Lycorine, an alkaloid extracted from Amarillidaceae, is a quite specific inhibitor of AA biosynthesis (*1*). The physiological effects induced by lycorine in both plants (*2*) and animals (*3*) are probably due to this peculiar action. Therefore lycorine appears to be a good tool to determine what metabolic reactions in the cell are directly related to AA variations. We present data showing that the development of cyanide-insensitive respiration is a process closely controlled by AA.

Potato tuber slices aerobically maintained show a strong increment of oxygen uptake within a day after cutting, and the respiration becomes relatively insensitive to carbon monoxide and to 1 m*M* cyanide (*4*). The rate of respiration in "fresh" slices (that is, slices used 30 minutes after cutting) is roughly 20 to 30 μl of O_2 per hour per gram of fresh tissue and rises in "activated" slices (*5*) (those used after a day's aeration) to 100 to 120