

Presence of isoquinoline alkaloids in molluscan ganglia

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Abstract

Tetrahydropapaveroline and reticuline, isoquinoline alkaloids, were purified and identified in the pedal ganglia of the marine mollusk *Mytilus edulis*.

OBJECTIVES: To determine the presence of tetrahydropapaveroline and reticuline, isoquinoline alkaloids, in animal tissues previously shown to express opiate receptors.

METHODS AND RESULTS: This was achieved by high performance liquid chromatography coupled with electrochemical detection and finally identified by electro-spray ionization quadrupole time of flight mass spectrometry (Q-TOF). The expression of these two tetrahydroisoquinoline alkaloids in the animals neural tissue is at the level of 2.5 ± 0.64 ng/ganglion and 1.8 ± 0.52 ng/ganglion, respectively.

CONCLUSION: This finding suggests that morphine biosynthesis may occur in *Mytilus* neural tissues, and that the biosynthesis pathway may be similar to that reported in the poppy plant.

Introduction

Tetrahydroisoquinoline alkaloids (TIQs) belong to a group of naturally occurring pharmacologically active compounds. They are taken up, stored, and released from nerve terminals, and interfere with normal catecholaminergic transport and storage systems [1,2]. Among these TIQs, tetrahydropapaveroline (THP) and reticuline are particularly interesting because of their involvement in neural function and in morphine biosynthesis, respectively. THP is an endogenous 1-benzyltetrahydroisoquinoline previously found in both plant and animal tissues, including human tissues [3,4]. It is synthesized by a non-enzymatic Pictet-Spengler reaction of dopamine with 3,4-dihydroxyphenyl-acetaldehyde [5,6].

There is a large body of evidence that other opiate alkaloids, such as morphine, thebaine, and codeine, exist in vertebrates [7,8,9]. In invertebrates, specifically *Mytilus edulis*, the presence of morphine, morphine 6 glucuronide, morphine 3 glucuronide and codeine have been reported [10,11,12,13]. Taken together these reports suggest that animals may have the ability to synthesize opiate alkaloids.

At the present time, there is no documentation on the presence of TIQs in invertebrates. Indeed, if morphine is made in these tissues, its precursors should be identified. We now demonstrate the presence of THP and reticuline in mollusk ganglionic tissue by way of high pressure liquid chromatography (HPLC) coupled to electrochemical detection and nano-flow electrospray ionization, double-quadrupole orthogonal-acceleration, and time of flight mass spectrometry (Q-TOF MS) determination.

Materials and methods

Mytilus edulis were collected directly from the seashore in Mattituck (Long Island, New York) and kept under laboratory conditions as previously described [14]. For these experiments one hundred animals were placed and maintained in artificial seawater at 24°C. For the biochemical analysis, groups of 10 animals had their pedal ganglia excised on ice. Each experiment was repeated three times.

Extraction

Extraction experiments using internal or external THP and reticuline standards were performed in different rooms to avoid contamination of the biological samples. Single-use siliconized tubes were used to prevent the loss of compounds. Ten dissected ganglia were grouped together and were extensively washed with phosphate buffered saline (PBS); 0.01M NaCl; 0.132 mM, NH_4HCO_3 ; pH 7.2) prior to extraction (3 times in 1 ml). Tissues were weighed and homogenized in 1N HCl (0.1g/ml) with sonic dismembrator (Fisher Scientific, USA). The resulting homogenates were extracted with 5 ml chloroform/isopropanol 9:1. After 5 min at room temperature, homogenates were centrifuged at 3000 rpm for 15 min. The supernatant was collected

and dried with a Centrivap Console (Labconco, Kansas City, Missouri). The dried extract was then dissolved in 0.1% trifluoroacetic acid (TFA) water before solid phase extraction. Samples were loaded on a Sep-Pak Plus C-18 cartridge (Waters, Milford, MA) previously activated with 100% acetonitrile and washed with 0.1% TFA-water. THP and reticuline elution were performed with a 15% acetonitrile solution (water/acetonitrile/TFA, 84.9%: 15%: 0.1%, v/v/v). Eluted sample was dried with a Centrivap Console and dissolved in water prior to HPLC analysis.

HPLC and electrochemical detection

The HPLC analyses were performed with a Waters 626 pump (Waters, Milford, MA) and a C-18 Unijet microbore column (BAS). A flow splitter (BAS) was used to provide the low volumetric flow-rates required for the microbore column. The split ratio was 1/9. Operating the pump at 0.5 ml/min, yielded a microbore column flow-rate of approximately 50 $\mu\text{l}/\text{min}$. The injection volume was 5 μl . Sample detection was performed with an amperometric detector LC-4C (BAS, West Lafayette, Indiana). The microbore column was coupled directly to the detector cell to minimize the dead volume. The electrochemical detection system employed a glassy carbon-working electrode (3mm), a 0.02 Hz filter (500mV; range 10 nA) and a 16- μm gasket. The chromatographic system was controlled by Waters Millennium³² Chromatography Manager V3.2 software and the chromatograms were integrated with Chromatograph software [15].

Standard curves were prepared by adding different amounts of THP and reticuline (0.1–20 ng) to 1 N HCl and conducting the solid phase extraction in triplicate. The derivatives were analyzed by HPLC. The standard curves were generated by least squares linear regression.

THP and reticuline in the tissues were analyzed by HPLC in the following manner: the mobile phases were: Buffer A: 10 mM sodium chloride, 0.5 mM EDTA, 100 mM sodium Acetate, pH 5.0; Buffer B: 10 mM sodium chloride, 0.5 mM EDTA, 100 mM sodium Acetate, 50% acetonitrile, pH 5.0. The injection volume was 5 μl . The running conditions were: from 0 min 0% buffer B; 10 min, 5% buffer B; at 25 min 50% buffer B; at 30 min 100% buffer B. Both buffers A and B were filtered through a Waters 0.22 μm filter and the temperature of the whole system was maintained at 25°C. Several HPLC purifications procedures, including the use of negative tissues, i.e., mantle, were performed between each sample to prevent residual alkaloids contamination. Furthermore, the fraction of blank chromatography corresponding to the elution of the morphine was checked by Q-TOF mass spectrometry analysis, confirming that no alkaloids remained in the system. HPLC analyses of the incubating sea water and mussel mantle tissue were used as controls and they yielded negative data.

Mass Spectrometry

Q-TOF-MS offers an effective way of demonstrating endogenous alkaloids in biological tissues [16,17,18]. A commercial Q-TOF-MS system (Micromass, Manchester, U.K.) was used to test the HPLC fractions. One μl of acetonitril/water/formic acid (50:49:1, v/v/v) containing the sample was loaded in a gold-coated capillary (micromass F-type needle). This sample was sprayed at a flow rate of 30 nl/min, giving an extended analysis time during which we acquired an MS spectrum, as well as several MS/MS spectra. During MS/MS, or tandem mass spectrometry, fragmentations are generated from a selected precursor ion by collision-induced dissociation (CID). Due to the fact that not all ions fragment with the same efficiency, the collision energy is typically varied between 25 and 35 V so that the parent ion is fragmented into a satisfying number of different daughter ions. Needle voltage was set at 950 V; and cone voltage was set at 30V. The instrument was operated in the positive mode.

Results

In specific HPLC fractions, compounds with identical retention times to those of authentic THP and reticuline external standards were consistently detected in runs using five different samples of ganglion extractions (*Fig. 1*). The system of electrochemical detection could detect over 0.1 ng of THP and 0.15 ng of reticuline, respectively (*Fig. 1B* inset). The mean concentration of THP and reticuline in the five ganglia samples, determined by extrapolation from the peak areas calculated for the external standards, were 2.5 ± 0.64 ng/ganglion and 1.8 ± 0.52 ng/ganglion, respectively.

In Q-TOF-MS, the molecular mass attributed to the single-charged THP from the nerve tissue, 288.30 da (*Fig. 2A*), was virtually identical to the corresponding value for the authentic standard (*Fig. 2B*) and the theoretical value (288.32da). Fragmentation analysis showed THP from the ganglia has the identical fragments (*Fig. 2C*) compared with the standard (*Fig. 2D*).

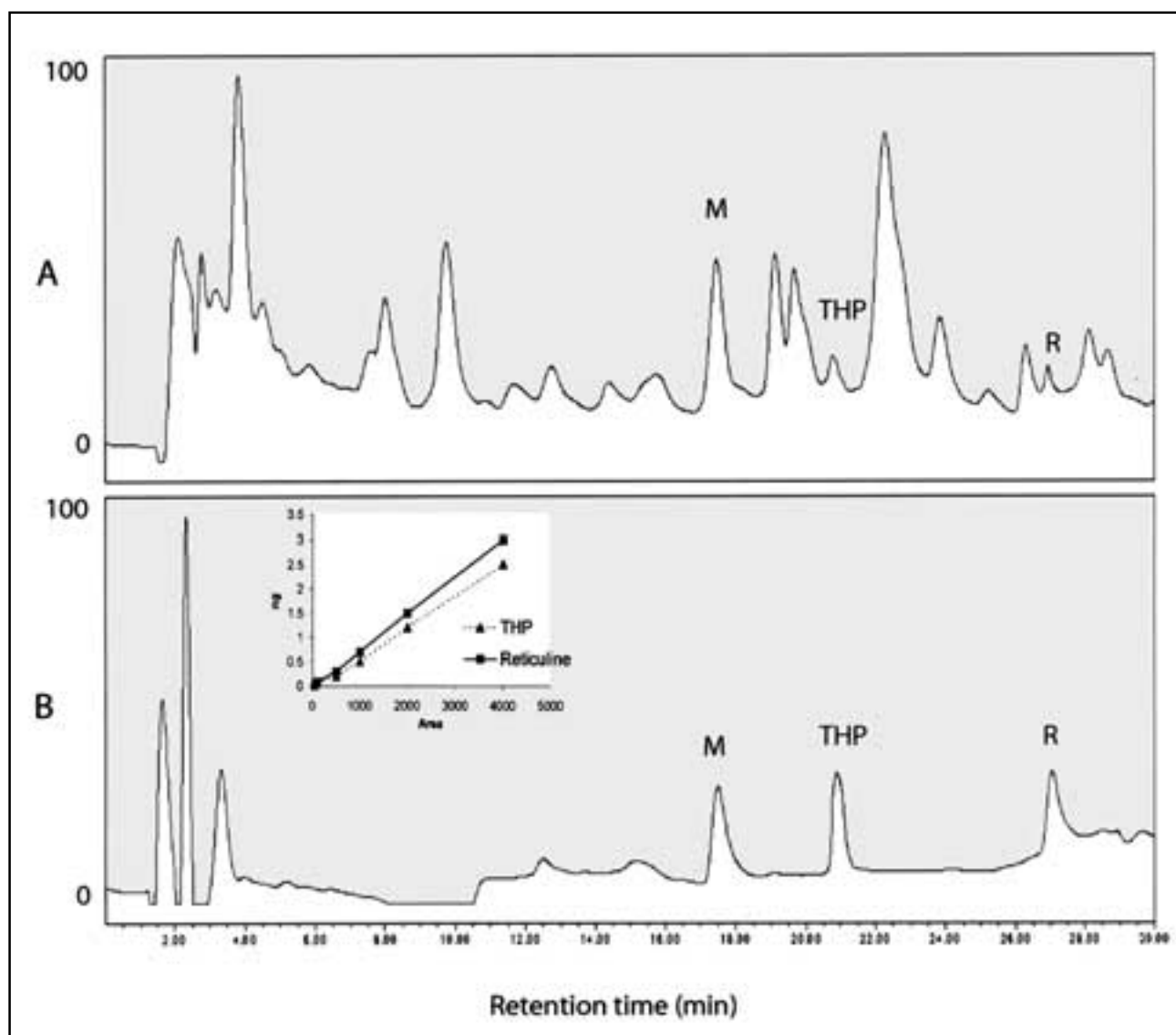


Figure 1. HPLC purification of alkaloids in the pedal ganglia of *Mytilus edulis*, **A.** Extraction of 20 ganglia; labeling indicates peaks corresponding to specific alkaloid standards found in **B.** HPLC run of authentic alkaloid standards (30 ng/ each). M, morphine; THP, tetrahydropapaveroline; R, reticuline. Inset, detection limit of THP and reticuline.

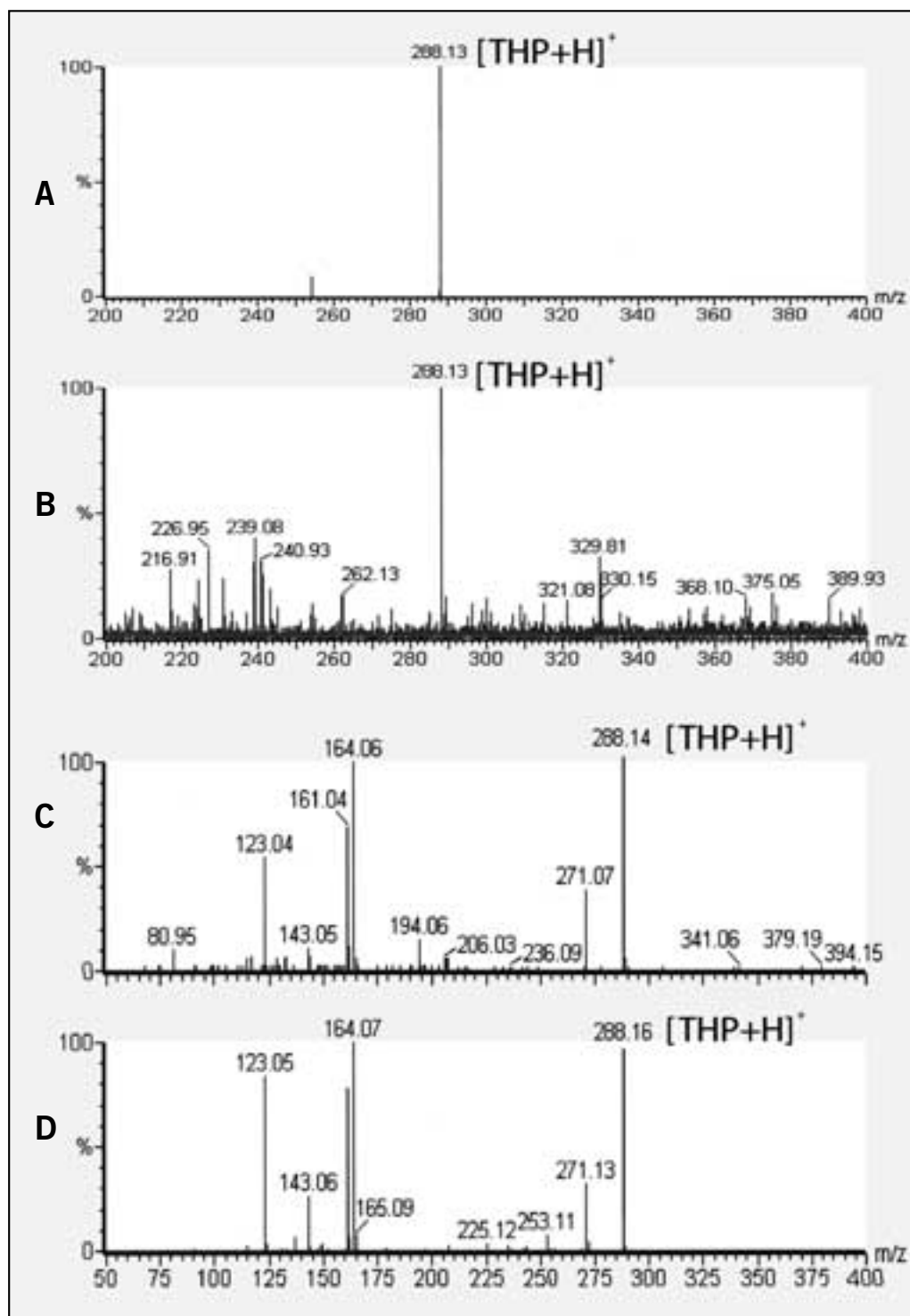


Figure 2. Q-TOF MS determination of THP. **A**, authentic THP standard using MS mode. **B**, 20 ganglionic extraction from HPLC fraction noted in Fig.1A, MS mode. **C**, Fragmentation analysis of authentic THP molecule. **D**, Fragmentation of molecule with the identical mass of THP from the ganglia extraction.

HPLC fractions corresponding to reticuline in the ganglia showed the molecular mass of 330.17da (Fig.3B). This value is identical to the authentic standard (Fig.3A) and the theoretical value. Fragmentation of ganglia and the reticuline standard using CID yielded identical fragments (Fig.3D) as the authentic standard (Fig.3C). The sum of the molecular weight of the two fragments (137.05da, 192.09da) shown in Fig.3D is identical to the reticuline molecule 329.20da, demonstrating its molecular identity.

Discussion

We demonstrate the presence of both THP and reticuline in the pedal ganglia of *Mytilus edulis*. This is the first report on the detection of these TIQs in invertebrate tissues. This work supports previous studies which suggest that morphine is an endogenous signal molecule since morphine, morphine 6 glucuronide and codeine also have been found and identified in *Mytilus* nervous tissues [10,13]. Supporting the

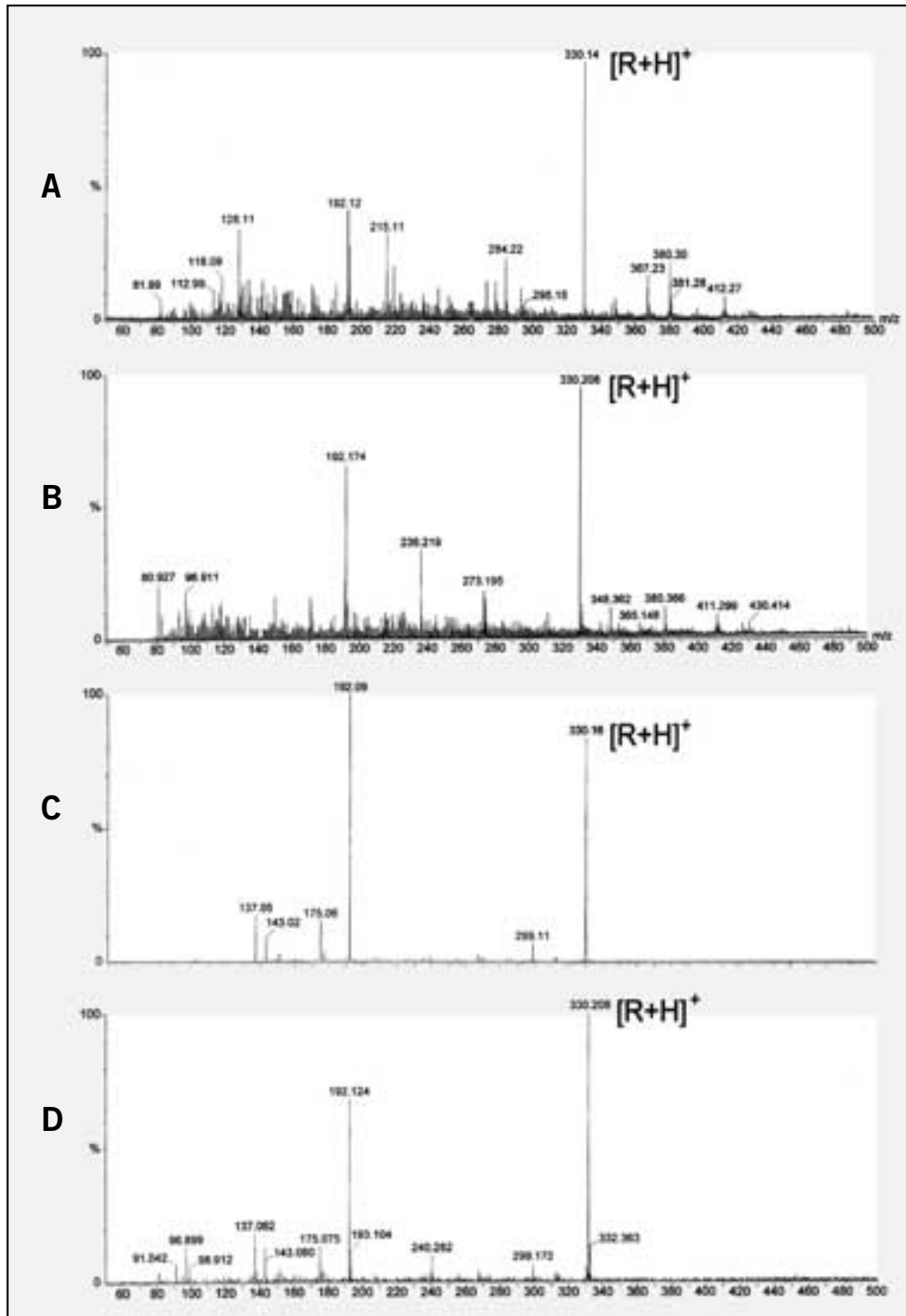


Figure 3. Q-TOF mass spectrometry determination of Reticuline. **A**, Authentic reticuline standard. **B**, Extraction (20 ganglia) from HPLC fraction. **C**, Fragmentation analysis of authentic reticuline molecule. **D**, Fragmentation of molecule with the identical mass of reticuline from the ganglia extraction.

presence of endogenous morphine in this tissue, is the study demonstrating μ opiate receptor expression in *Mytilus* [19]. Here, this receptor has been demonstrated to be coupled to nitric oxide release in an opioid peptide insensitive and opiate alkaloid selective manner [20,21]. Taken together, it appears that isoquinoline alkaloids are present in invertebrates and that morphine biosynthesis has the potential to occur.

The biosynthesis of morphine in plants begins with the tyrosine metabolites dopamine and 3,4-dihydroxyphenylacetaldehyde and proceeds via THP and reticuline, the latter undergoing the key conversion in the pathway to form the morphinan compound salutaridin. This compound is then converted by oxidation, demethylation, and reduction via thebaine and codeine to morphine [22]. Furthermore, in pig liver, cytochrome P450, an enzyme that is a highly regional and stereose-

lective, catalyzes the conversion of reticuline to salutaridine [23]. Additionally, the conversion of codeine, thebaine, and salutaridine to morphine has been demonstrated in rat tissue, including the brain, suggesting morphine synthesis is possible [7,24,25,26].

In this regard, experiments by Zenk *et al* showed that (S)-norcoclaurine, not THP derived from an enzymatic and stereospecific condensation of dopamine with acetaldehyde, is the first isoquinoline in the biosynthesis of reticuline [22]. Since this data was obtained from plants, it cannot be excluded that another pathway for morphine synthesis exists in animals. Our results strongly support the hypothesis that morphine synthesis may occur in animal tissues and that morphine's biosynthesis may be similar to that reported in the poppy plant. In this regard, intermediates of morphine synthesis may be obtained from an organism's diet and later converted to morphine in animal tissues where it can serve as a signal molecule [12,27].

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