Sauroine, an Alkaloid from *Huperzia saururus* with Activity in Wistar Rats in Electrophysiological and Behavioral Assays Related to Memory Retention

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The present study describes the effects of sauroine (**1**), the main alkaloid obtained from *Huperzia saururus*, on memory retention and learning. To evaluate this, electrophysiological experiments and behavioral tests (step down) were performed on male Wistar rats. The results showed that **1** improved memory retention in the step-down test, significantly increasing hippocampal plasticity. Thus, **1** seems to be a constituent responsible for the activity claimed in folk medicine for *H. saururus* in Argentina.

With the aim of studying the chemical properties of species that have been used in Argentinean folk medicine, an investigation was conducted on *Huperzia saururus* (Lam.) Trev. (Lycopodeaceae). This species is commonly known as "cola de quirquincho", for which the aerial parts are consumed as an infusion or decoction because of their claimed folk use as an aphrodisiac or for an improved memory effect. This species grows primarily in South America from the north of Peru to Argentina, as well as in Southern Africa, Madagascar, and the Mascarenes.[1] In Argentina, it can be found from the northwestern region (Jujuy, Salta, and Catamarca) to the central part of the country (San Luis, Córdoba, and Buenos Aires), at high altitudes.[2]

Previous chemical studies of *H. saururus* collected in Pampa de Achala (Córdoba), Argentina, showed the presence of eight alkaloids in a purified alkaloid extract,[3,4] with sauroine (7α,8-*endo*-dihydroxylycopodine, **1**) being the predominant alkaloid in this extract.[5] Compound **1** belongs to the *Lycopodium* group of alkaloids. Some *Lycopodium* alkaloids show activity as acetylcholinesterase inhibitors,[6] and a few have an effect on learning and memory; among them is huperzine A, first isolated from *H. serrata.*[7]
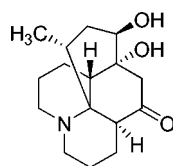
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Information storage in the CNS is thought to involve changes in synaptic efficiency that occur in response to a particular pattern of activity. One of these is long-term potentiation (LTP) in the hippocampus.[8] LTP, a model of activity-dependent synaptic plasticity, has been proposed as a cellular mechanism for at least some types of learning and memory.[9,10]

The glutamate receptor type NMDA (*N*-methyl-D-aspartate) is known to be involved in the induction of LTP; however other neurotransmitters can modulate glutamatergic transmission.[11] There is a cholinergic hypothesis that links the central cholinergic circuit,
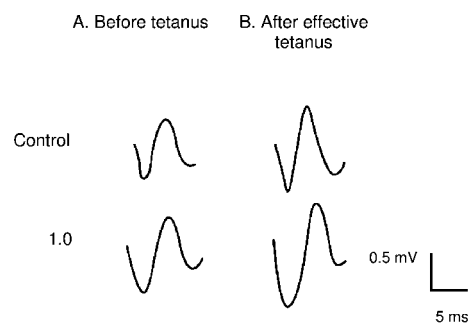

Figure 1. Oscilloscope-modified photographs corresponding to typical averaged field potentials, recorded from the granule cell layer of the *dentate gyrus* following stimulation of the perforant path (PP) for control and sauroine group (1.0 μg/mL): (A) before tetanus and (B) after an effective tetanus. Calibration bars represent 5 ms and 0.5 mV.

including the septohippocampal, with storage mechanisms of long-term memory. It is noteworthy that drugs enhancing the strength of synaptic plasticity could in turn improve the storage of certain types of memory, representing a potential therapeutic alternative for neurodegenerative diseases such as Alzheimer's disease (AD).[12,13]

After isolation and purification using chromatographic techniques, sauroine (**1**) was used for various assays on rat hippocampal slices. Figure 1A shows an example of a characteristic electrophysiological field response evoked in the granule cell layer of the *dentate gyrus* after single-pulse stimulation in the perforant path in control and in sauroine (**1**)-treated slices. This consisted of a gradual positive-going field excitatory postsynaptic potential (EPSP). The EPSP reflects synaptic currents at perforant path−dentate granule cell synapses in the *stratum moleculare*. In Figure 1B, the increased amplitude of fEPSP after an effective tetanus can be seen.

One-way ANOVA on the threshold frequency to elicit LTP revealed a significant interaction [$F(1,9) = 81.04$, $p < 0.00$] between the effects of **1** and controls (Ctrol). Newman−Keuls pairwise comparisons of means tests showed that when the slices were perfused with **1** (1.0 μg/mL), an increment in the hippocampal synaptic plasticity was observed, measured as a diminution of the threshold to generate LTP. The perfused slices ($n = 6$) exhibited an average threshold of 21.66 ± 7.10 Hz, while for Ctrol ($n = 5$) the average was 100.00 ± 14.14 (Figure 2). This increase in hippocampal synaptic plasticity observed in slices perfused with **1** can be interpreted as an improved effect on memory.

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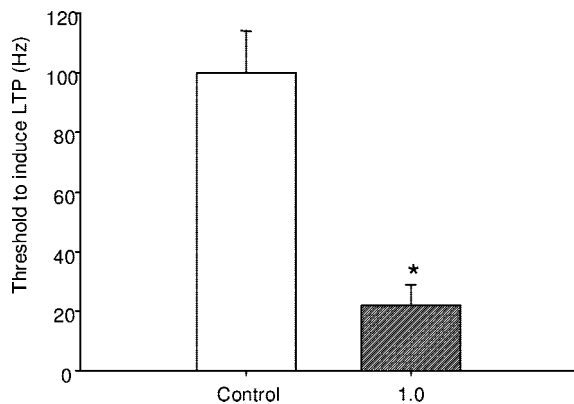† Departamento de Farmacia.
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Figure 2. Threshold to LTP induction in hippocampal *dentate gyrus*. Bars represent means and vertical bars ± SEM ($n = 5$ or 6 animals/group, $*p < 0.0004$).
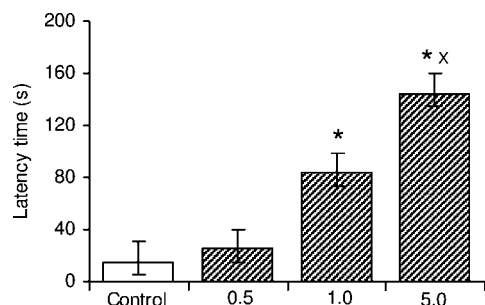

Figure 3. Effect of hippocampal sauroine (**1**) administration on long-term memory retention in rats. The animals were injected with different doses of sauroine (0.5, 1.0, or 5.0 ng/rat) or ACSF (Control). The latency of time (in seconds) was quantified. The results are expressed as a median (interquartile range) ($n = 7-9$ animals/group, * = significant differences in comparison to control animals, $p < 0.05$, × = significant differences in comparison to animals with sauroine (**1**) 5.0 ng/rat administration, $p < 0.05$).

Figure 3 shows latency time as an index of memory retention after the treatments. Thus, the animals injected with **1** at doses of 1 or 5 ng/rat exhibited a significant dose-dependent increase on long-term memory retention compared with control animals [injected with artificial cerebrospinal fluid (ACSF)] [H (31,3) = 28.88, $p < 0.00$]. The intrahippocampal administration of **1** improved performance in this behavioral task, which assesses learning and memory. It has been demonstrated that the hippocampus seems to be the principal structure involved in this memory test. However, it is known that in several memory aversive paradigms (such as step down), the amygdala also play a critical role; thus, the participation of the latter in the mediation of the CNS effects of **1** cannot be discarded.

Previous findings related to crude alkaloid extract of *H. saururus* showed that it exhibited a marked and selective activity as an acetylcholinesterase inhibitor,[3] as well as facilitation of LTP in rat hippocampal slices[14] and an increase in memory retention.[15] In consequence, the present results suggest that **1** is an effective contributor of this extract in enhancing memory retention, with the experimental conditions used.

Experimental Section

General Experimental Procedures. One- and two-dimensional ^1H and ^{13}C NMR spectra were measured on a 400 MHz Bruker Advance II NMR spectrometer, using $CDCl_3$ as solvent. Column chromatography was performed on Sephadex LH-20 and G-10 (Pharmacia). TLC was carried out on precoated silica gel GF_{254} plates (Merck), and alkaloids were revealed under UV light and by spraying the plates with Dragendorff's reagent. Electrophysiological experiments were con-

ducted using a BSC-BU Harvard Apparatus. In these experiments, temperature was controlled by a TC-202A Harvard Apparatus. Tetanus was applied by an A310 Accupulser pulse generator (World Precision Instruments Inc.). Ketamine HCl (Vetanarcol) and xylazine (Kensol) were purchased from Laboratorios König S.A, Buenos Aires, Argentina. Intrahippocampal injections were carried out using a 10 μL Hamilton syringe connected by Pe-10 polyethylene tubing to a 30-gauge needle extending it 0.75 mm beyond a steel guide cannula.

Plant Material. Aerial parts of *H. saururus* were collected in Pampa de Achala, San Alberto Department, Province of Córdoba, Argentina, in November 2001, and identified by Dr. Gloria Barboza, Instituto Multidisciplinario de Biología Vegetal, Universidad Nacional de Córdoba. A voucher specimen was deposited at the herbarium of the Museo Botánico de Córdoba (CORD) as CORD 684.

Extraction and Isolation. Aerial parts of *H. saururus* (2.45 kg) were dried, ground, and then alkalinized with NaOH reduced to a powder (200 g). This mixture was hydrated with distilled water until pH 12 and extracted with $CHCl_3$ using a Soxhlet extractor. The organic solvent was evaporated under reduced pressure until acquiring half of its original volume. This crude total extract (77 g) was partitioned twice with 0.01 N HCl to pH 2. The acidic aqueous extracts were combined and then alkalinized with 0.1 N NaOH to pH 12 and subsequently partitioned with $CHCl_3$ with a liquid−liquid extractor. The chloroform extract obtained (3.95 g) was purified by passage over Sephadex LH-20 in a glass column, employing $CHCl_3$−EtOH (1:1) as the mobile phase. All fractions positive to Dragendorff's reagent were combined and evaporated under reduced pressure to yield 2.41 g of an alkaloid extract. This residue was submitted to gel filtration on Sephadex LH-20, and acetone was used as mobile phase (1500 mL) to afford four major fractions. Fraction 3 (463.3 mg) was subjected to Sephadex G_{10} column chromatography and eluted with 5% EtOH. Fraction 3.2 afforded **1**, which was purified by preparative TLC on silica gel GF_{254}, eluted with cyclohexane−diethylamine (1:1), yielding 19.6 mg of pure compound. The identification of **1** was developed by spectroscopic techniques[4] and compared with an authentic sample previously obtained by our laboratory.

Animals. All procedures performed in the present study were conducted according to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals as approved by the College of Chemistry, Animal Care and Use Committee, Universidad Nacional de Córdoba.

Electrophysiological Assay. Male Wistar rats 60−75 days old and weighing 190−275 g were used. Animals were housed in groups of five in their home boxes and kept under a 12:12 L/D cycle (lights on at 7 a.m.) and at regular temperature conditions (22 ± 1 °C). Food and water were available ad libitum.

Electrophysiological Procedures. Animals were sedated with a 50:50 mixture of CO_2−O_2 and then sacrificed by cervical dislocation, with their brains being removed for hippocampal electrophysiological assays. Electrophysiological experiments were carried out using the in vitro hippocampal slice preparation. Briefly, rats were sacrificed between 11:00 and 12.00 a.m. to prevent variations caused by circadian rhythms or nonspecific stressors.[16,17] The hippocampal formation was dissected, and transverse slices approximately 400 μm thick were placed in a recording chamber, perfused with standard saturated Krebs solution (NaCl 124.3 mM, KCl 4.9 mM, MgSO$_4$·7H$_2$O 1.3 mM, H$_2$KPO$_4$ 1.25 mM, HNaCO$_3$ 25.6 mM, glucose 10.4 mM, CaCl$_2$·2H$_2$O 2.3 mM) with 95% O_2 and 5% CO_2. The rate of perfusion was 1.6 mL/min, while the bathing solution temperature was kept at 28 °C. A stimulating electrode made of two 50 μm-diameter insulated and twisted wires (except for the cut ends) was placed in the perforant path, and then a recording microelectrode made with a micropipette (tip 10−20 μm) was inserted in the dentate granule cell body layer. Only slices showing a stable response were included in this electrophysiological study. Ten field potentials that responded to the stimuli were sampled at 0.2 Hz, averaged online using a computer, and the data thus obtained were stored for further analysis. Once no further changes were observed in the amplitude of the response, over 20 min, the intensity of the electrical stimulus to the perforant path was set at a value that would elicit spikes of approximately 30% of the maximum response. The long-term potentiation eliciting frequency threshold was then determined. Tetanus, consisting of a train of pulses (0.5 ms) of 2 s duration and with increasing frequency, was delivered to the slice at intervals that ranged from 20 min up to 45 min, starting with a 5 Hz tetanus, for which the intensity increased with each train to 10, 20, 40, 60, 80, 100, 120, up

to 200 Hz. Fifteen to 20 min after receiving tetanus, a new averaged response was recorded; when long-term potentiation was not observed, another tetanus at the next highest frequency was applied. Long-term potentiation was considered to have occurred when the amplitude of the evoked field potential recorded after the tetanus had risen by at least 30% and had persisted 60 min. Once long-term potentiation was achieved, no further tetanus was given.

In experiments in which the generation of LTP was possible, we proceeded to use another slice perfused with standard saturated Krebs solution containing saurorine chlorhydrate (1 μg/mL). After 20 min, the same protocol for the generation of LTP was used, recording in these cases in the presence of the saturated solution containing saurorine.

Statistics. The experimental data were analyzed by one-way ANOVA, followed by Newman−Keuls pairwise comparisons of means ($p \leq 0.05$ represents a significant difference between groups).

Behavioral Task: Step-Down Test. Animals. The experiments were performed on male Wistar rats (270−300 g) with food and water ad libitum. The colony room was maintained under controlled temperature (21−23 °C) and light (12 h light, 12 h dark). The animals were placed in individual cages.

Experimental Procedure. The animals were separated in two groups. These were control group, where the rats received artificial cerebrospinal fluid (ACSF) into the hippocampus, and the **1**-treated group, where the animals received three doses of 0.5, 1.0, or 5.0 ng/rat of **1** into the hippocampus. Each animal was used in only one experiment. The number of animals ranged between 7 and 9 per treatment.

Surgery. Animals were anesthetized with 55 mg/kg ketamine HCl and 11 mg/kg xylazine and placed in a stereotaxic apparatus. The rats were implanted bilaterally into the hippocampus (Hi) with steel guide cannulas, according to the atlas of Paxinos and Watson.[18] The coordinates relative to bregma were as follows: anterior, −4.3 mm; lateral, ± 4.0 mm; and vertical, −3.4 mm for CA1 Hi. Cannulas were fixed to the skull surface with dental acrylic cement. During the 7-day recovery period, the animals were handled daily to habituate them to the injection procedures. They were injected with **1** or ACSF. Each infusion of 0.5 μL per side was delivered over a 1 min span.

Histology. At the end of the experiments, the animals were immediately killed by decapitation. Methylene blue injection (0.5 mL) was used to confirm the correct injection site. The cannula's position was assessed histologically on frozen brain slices (−20 °C). Only results obtained from animals in which the tips of the cannulas were placed into the Hi were included in this study.

Step-Down Test (Inhibitory Avoidance). Rats were subjected to one trial of step-down inhibitory avoidance. The training apparatus was a 50 × 25 × 25 cm plastic box with a 2.5 cm height, 7.0 cm width, and a platform on the left of the training box apparatus. The floor of the apparatus was made of parallel 0.1 cm caliber stainless steel bars spaced 1.0 cm apart from each other. The animals were placed on the platform. Latency to step down, placing the four paws on the grid, was measured. In the training session, immediately upon stepping down,

the rats received a 0.4 mA, 2 s scrambled shock to the foot and were then immediately removed from the training box and placed in their home cages. The session test was procedurally identical except that no shock was given. A ceiling of 180 s was imposed on the retention test measures. Latency time was taken as a measure of memory retention. The retention test was carried out 24 h after training in order to measure long-term memory retention.

Statistics. Since the variables being analyzed do not follow a normal distribution and its variance does not fulfill the assumption of homoscedasticity, data of inhibitory avoidance are expressed as medians (interquartile range) and analyzed by nonparametric tests (Mann−Whitney or Kruskal−Wallis); $p < 0.05$ was accepted as a statistically significant value.

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