Full Papers

Antinociceptive Effects of 14-Membered Cyclopeptide Alkaloids

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The analgesic potential of six 14-membered-ring cyclopeptide alkaloids, namely, franganine (**1**), discarine B (**2**), scutianines B (**3**), C (**4**), and D (**5**), and adouetine X (**6**), have been investigated. Among the compounds tested, only franganine (**1**) and adouetine X (**6**) produced antinociceptive effects in a mouse model of acute pain, without inducing undesirable side effects. Furthermore, compound **6** also exhibited a pronounced analgesic effect in a chronic neuropathic pain model in mice. It has been found that adouetine X (**6**) can decrease the activities of Ca^{2+}-ATPase and Na^+/K^+-ATPase in vitro. Thus, the present findings have demonstrated that adouetine X (**6**) is a promising analgesic agent.

Cyclopeptide alkaloids are defined as polyamidic basic compounds of plant origin embodying a *p*- or *m*-ansa structure.[1] These compounds are found mainly in the Rhamnaceae and have been classified by different methods. However, the most common classification method is according to the size of the macrocycle, as either 13- 14-, or 15-membered cyclic ether rings.[2] Some studies have showed that these compounds may function as ionophores in plants and may be involved in the process of nutrient and metal absorption, but the real physiological role of cyclopeptide alkaloids in plants is still unknown.[1,2] Studies of their biological properties have been hampered due to the low amounts present in the plants of origin of these compounds and a lack of practical synthetic methods.[1] Thus, few biological activities have been reported for this group of substances, including antibacterial, antifungal, antiplasmodial, sedative, and immunostimulant activities.[1]

The 14-membered ring class has the most representative compounds and is the largest subgroup of cyclopeptide alkaloids.[2] This class includes almost all the scutianine-, discarine-, and sanjoine-related compounds. Scutianines B (**3**) and D (**5**) and discarine B (**2**), but not scutianine C (**4**) and franganine (**1**), showed modest antibacterial activity.[3,4] Sanjoinine A exhibited sedative, hypnotic, and analgesic properties, and it is reported to be an effective inhibitor of calmodulin-induced activation of Ca^{2+}-ATPase and a modulator of the γ-amino butyric acid (GABA) system.[5−8]

The search for new analgesic compounds with few undesirable effects and good efficacy in chronic painful conditions is potentially very important. Neuropathy-associated pain has become a major clinical problem, so the search for new drug molecules to alleviate this intractable pain is now an interesting strategy.[9] Therefore, in the present study, we have investigated the potential antinociceptive effect of six 14-membered cyclopeptide alkaloids in mice as well as their possible mechanism of action and ability to develop undesirable effects.

Results and Discussion

The tail-flick test is a simple pain model that uses an acute noxious stimulus to screen new analgesic drugs, usually substances that act on the central nervous system, such as opioid agonists.[10]

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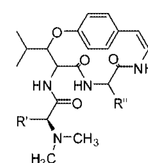

	R'	R''
Franganine (**1**)	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$
Discarine B (**2**)	$CH(CH_3)CH_2CH_3$	$CH_2C_8H_6N$
Scutianine B (**3**)	$CH_2C_6H_5$	$CH_2C_6H_5$
Scutianine C (**4**)	$CH(CH_3)CH_2CH_3$	$CH_2C_6H_5$
Scutianine D (**5**)	$CH_2C_6H_5$	$CH(OH)C_6H_5$
Adouetine X (**6**)	$CH_2CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$

Since only small amounts of cyclopeptide alkaloids are available for in vivo studies, restricting the possibility of systemic administration, we injected cyclopeptide alkaloids by the intrathecal (i.t.) route because it requires less compound than systemic routes. In addition, i.t. administration is used clinically to deliver analgesic drugs, and the spinal cord is an important modulator in pain pathways.[11] The i.t. injection of adouetine X (**6**), franganine (**1**), scutianine B (**3**), and discarine B (**2**) (10 nmol/site) produced antinociception in the mice tail-flick test, 15 and 60 min after administration (Figure 1). Scutianine D (**5**) or vehicle did not change tail-flick latency at any time tested. On the other hand, i.t. administration of scutianine C (**4**) (10 nmol/site) produced hyperalgesia in the tail-flick test, 15 and 60 min after injection (Figure 1). Among the cyclopeptide alkaloids tested, adouetine X (**6**) was more effective in producing antinociception and increased latencies in the tail-flick test by about 70%. The opposite action of some cyclopeptide alkaloids in the tail-flick test (i.e., induction of analgesia or hyperalgesia) is in accordance with Lee and co-workers, who demonstrated that the 13-membered cyclopeptide alkaloids paliurines A and F acted as sedatives in mice and the closely related alkaloid nummularine H acted as a stimulant under identical conditions, indicating the significant effect of minor structural modifications on the pharmacological actions of cyclopeptide alkaloids.[12] In addition, cyclopeptide alkaloids could interact with some putative neurotransmitter

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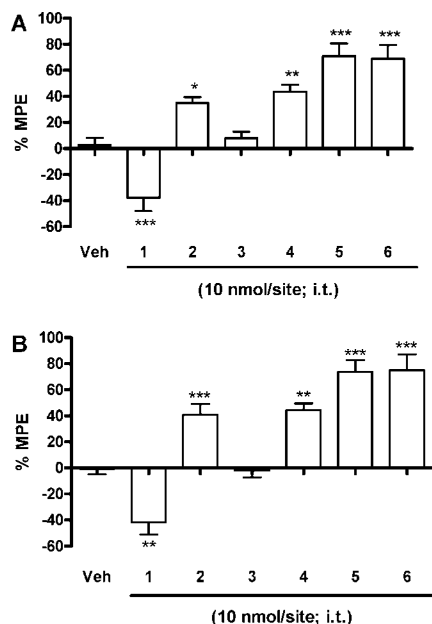

Figure 1. Effects of cyclopeptide alkaloid administration (10 nmol/site; i.t.) in the tail-flick test. The tail-flick response (% MPE) was measured 15 min (A) and 60 min (B) after the injection in mice ($n = 7-8$). Data are expressed as means ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one way ANOVA followed by SNK post hoc test.

systems of the central nervous system (CNS) that modulate nociception in an opposite manner.

As the interaction with glutamate receptors seems to contribute to mechanisms underlying antinociceptive or hyperalgesic actions of some compounds,[13] and glutamate is the major excitatory transmitter in the CNS, we investigated the role of glutamate receptors in the antinociceptive action of cyclopeptide alkaloids. In the spinal cord, agonists of glutamate receptors produce nociception, while antagonists might produce analgesia.[13] However, the cyclopeptide alkaloids tested cannot change the specific binding of [^{3}H]-L-glutamate to cerebral plasma membranes in mice, showing that the interaction with glutamate receptors is not necessary for the actions of these compounds in nociception (Table 1). Furthermore, another important neurotransmitter system in the CNS is the GABAergic system, which has opposite actions compared to the glutamatergic system. The action of γ-aminobutyric acid (GABA) is an important factor for modulation of pain. In this way, GABA receptor agonists and antagonists usually function as analgesic or hyperalgesic drugs, respectively.[14] It is known that the 14-membered cyclopeptide alkaloid sanjoinine A stimulates the GABAergic system.[7] However, the involvement of GABA receptors in the antinociceptive or hyperalgesic actions of the cyclopeptide alkaloids tested must be further determined.

Certain drugs can provide false-positive responses in the tail-flick test because they may induce sedation, motor activity impairment, or body temperature alteration.[10] Knowing that some cyclopeptide alkaloids can induce a sedative effect,[12] we assessed the undesirable effects of discarine B (**2**), scutianines B (**3**), C (**4**), and D (**5**), franganine (**1**), and adouetine X (**6**) in mice. The open-field test is sensitive to compounds that cause sedation or motor impairment.[15] Discarine B (**2**) and scutianine C (**4**) (10 nmol/site) decreased the number of crossings, while scutianine B (**3**) (10 nmol/site) was able to decrease the number of rearings and crossings in the open-field test. However, franganine (**1**), scutianine D (**5**), and adouetine X (**6**) did not change the motor activity in mice 15 min after injection (Table 2). Moreover, adouetine X (**6**) did not alter motor activity 60 or 120 min after its intrathecal administration (Table 3). Intrathecal administration of discarine B (**2**) (10 nmol/site) produced a hypothermic effect in mice 15 and 60 min after its administration. No other cyclopeptide alkaloid tested was able to alter mice body temperature (Table 2). Taken together, these data suggest that for some cyclopeptide alkaloids [namely, discarine B (**2**) and scutianines B (**3**) and C (**4**)], the analgesic effects observed in the tail-flick test might be false positives since they also alter mice motor activity in the open-field test or body temperature. On the other hand, franganine (**1**) and adouetine X (**6**) seem to produce a pure analgesic effect, as they did not change the spontaneous locomotion or body temperature.

Thus, since adouetine X (**6**) showed no undesirable effects and shows good efficacy at producing antinociception, the antinociceptive action of this compound was further evaluated. The ID_{50} value assessed for adouetine X (**6**) was 2.47 (2.25−2.69) nmol/site at 15 min in the tail-flick test in mice. The time−response curve of the antinociceptive effect of adouetine X (**6**) (10 nmol/site) showed that the antinociceptive effect of this compound started at 0.25 h and lasted up to 1 h after treatment (Figure 2). Next, we investigated the effect of adouetine X (**6**) in neuropathic pain. Injury to a peripheral nerve often results in a chronic neuropathic pain condition that is characterized by abnormal painful hypersensitivity, such as allodynia (pain responses to non-noxious stimuli).[9] This type of chronic pain differs substantially from acute pain not only in terms of the persistence of pain but also with regard to the maladaptive changes at various levels of the nervous system.[16] Thus, the available analgesic drugs often have limited therapeutic value in the management of neuropathic pain, and they may even represent a risk to the patient due to their common side effects.[9] Therefore, the development of safe and efficacious drugs to treat chronic pain is a priority. We observed that partial ligation of the sciatic nerve in mice induced a significant allodynia (characterized as a decrease in mechanical threshold in response to von Frey hair filaments) 7 days after the nerve injury compared to the sham-operated group. Adouetine X (**6**) (10 nmol/site; i.t.) was markedly effective in reducing this mechanical allodynia induced by nerve injury in the operated group from 0.5 to 1 h after treatment (Figure 3). Moreover, adouetine X (**6**) did not alter the detection of normal mechanical stimuli assessed in sham-operated mice (Figure 3). These findings are certainly relevant, considering that chronic pain is difficult to treat since it is quite resistant to most of the analgesic drugs currently available on the market.[9]

Finally, we attempted to find a target responsible for the antinociceptive action of adouetine X (**6**). Previous findings have demonstrated that the 14-membered cyclopeptide alkaloids sanjoinine-A and sanjoinine-G2, along with the synthetic derivatives, sanjoinine AH-1 and sanjoinine-A dialdehyde, effectively inhibit calmodulin-induced activation of Ca^{2+}-ATPase.[5,6] Thus we investigated the possible effect of adouetine X (**6**) in distinct Mg^{2+}-dependent ATPase activities (Ca^{2+}-ATPase or Na^+/K^+-ATPase). Adouetine X (**6**) was able to decrease the Ca^{2+}-ATPase activity, with inhibition values of 68.5 ± 9.9% and 91.8 ± 6.5% for concentrations of 3 and 10 μM, respectively. Adouetine X (**6**), at a concentration of 10 μM, but not 3 μM, also reduced the activity of Na^+/K^+-ATPase, with 92.5 ± 5.6% inhibition (Figure 4). Thus, adouetine X (**6**) seems to be a potent inhibitor, since the classical Na^+/K^+-ATPase inhibitor ouabain reduces about 30% of the enzyme

Table 1. Effects of Cyclopeptide Alkaloids Tested (**1**−**6**) in the Displacement of [^{3}H]-L-Glutamate Binding to Mice Brain Membranes[a]

vehicle	**1**	**2**	**3**	**4**	**5**	**6**
107.1 ± 0.1	122.7 ± 8.3	102.2 ± 1.8	101.1 ± 3.2	94.8 ± 3.2	94.4 ± 14.6	111.8 ± 16.2

[a] The results are presented as means ± SEM of at least two separate displacements.

Table 2. Effect of Treatments with Cyclopeptide Alkaloids (10 nmol/site; i.t.) after 5 min on Spontaneous Activity (number of crossings and rearings) in the Open-Field Test and after 20 or 60 min on Rectal Temperature (°C) in Mice[a]

compound	crossings	rearings	rectal temperature (20 min)	rectal temperature (60 min)
vehicle	120.0 ± 8.0	19.7 ± 1.9	0.16 ± 0.1	−0.31 ± 0.09
scutianine C (**4**)	74 ± 4.9*	17.6 ± 2.7	−0.23 ± 0.11	−0.40 ± 0.15
scutianine D (**5**)	120.8 ± 14.4	31.6 ± 7.3	−0.08 ± 0.22	−0.26 ± 0.12
scutianine B (**3**)	78.0 ± 9.4*	10 ± 1.9*	0.16 ± 0.44	−0.45 ± 0.49
discarine B (**2**)	70.0 ± 10.1*	13.5 ± 3.2	−0.37 ± 0.09*	−0.94 ± 0.19*
franganine (**1**)	113.3 ± 10.8	19.2 ± 3.0	−0.2 ± 0.12	−0.11 ± 0.13
adouetine X (**6**)	106.7 ± 10.1	18.17 ± 2.1	−0.08 ± 0.12	−0.20 ± 0.11

[a] Values represent means ± SEM for 7 or 8 animals in each group. *$p < 0.05$; Student's t test.

Table 3. Effect of Treatment with Adouetine X (**6**) (10 nmol/site; i.t.) after 60 and 120 min on Spontaneous Activity (number of crossings and rearings) in the Open-Field Test in Mice[a]

	crossings		rearings	
time (min)	vehicle	adouetine X (6)	vehicle	adouetine X (6)
60	51.8 ± 5.3	39.8 ± 3.1	7.0 ± 0.7	6.0 ± 1.8
120	22.1 ± 5.2	31.3 ± 4.2	3.8 ± 1.0	4.5 ± 2.1

[a] Values represent means ± SEM for 6 animals in each group.

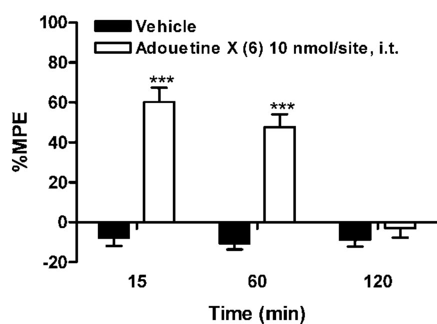

Figure 2. Time course of the antinociceptive effect of adouetine X (**6**) after intrathecal administration (10 nmol/site) in the tail-flick test in mice ($n = 7$ or 8). Data are expressed as means ± SEM (% MPE). ***$p < 0.001$, two-way ANOVA followed by Bonferroni post hoc test.

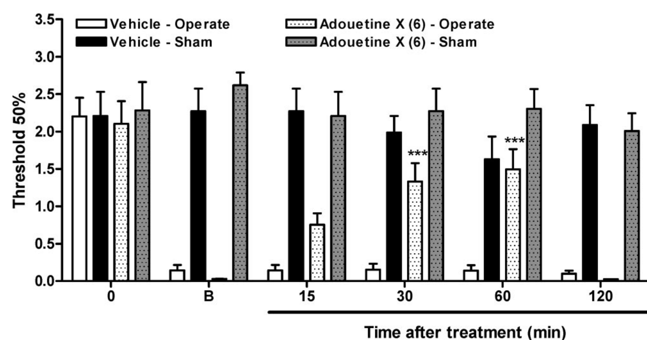

Figure 3. Mechanical sensitivity to von Frey hair filament stimulation of the right hindpaw in sham-operated and operated animals in partial ligation of the sciatic nerve (PLSN). Mice were treated with adouetine X (**6**) (10 nmol/site, i.t.) or vehicle at different intervals of time after drug treatment. Data are expressed as means ± SEM (% MPE). Significantly different from control values: ***$p < 0.001$ (two-way analysis of variance followed by Bonferroni post hoc test).

activity at 10 μM in synaptosomes.[17] It has been demonstrated that pro-nociceptive mediators can stimulate plasma membrane Ca^{2+}-ATPase, keeping the intracellular Ca^{2+} concentration low and increasing the excitability of sensory neurons that conduct the nociceptive stimulus to the CNS.[18] Thus, the inhibition of plasma membrane Ca^{2+}-ATPase in sensory neurons could produce analgesia. Additionally, i.t. injection of Na^+/K^+ ATPase inhibitors, such

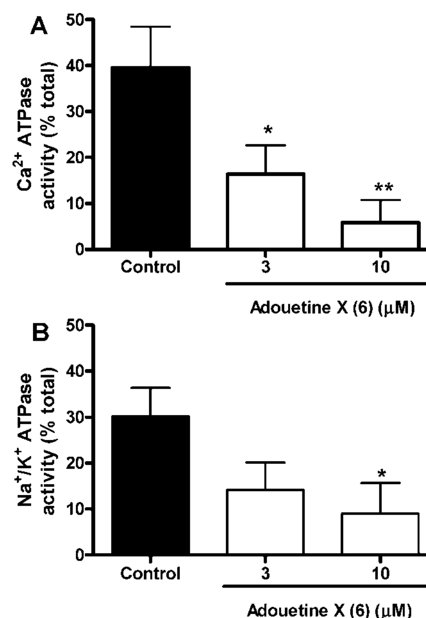

Figure 4. Ca^{2+}-ATPase (A) and Na^+/K^+-ATPase (B) activity in the presence of 3 or 10 μM adouetine X (**6**). Values represent means ± SEM (% total activity) of determinations made in six separate experiments. Significantly different from control values: **$p < 0.01$, *$p < 0.05$ (one-way ANOVA followed by SNK post hoc test).

as ouabain, can produce an antinociceptive effect in the tail-flick test in rodents.[19] Moreover, adouetine X (**6**) could act indirectly on ATPases through the inhibition of Ca^{2+}/calmodulin-dependent protein kinase. In fact, plasma membrane Ca^{2+}-ATPase and Na^+/K^+ ATPase are modulated by Ca^{2+}/calmodulin-dependent protein kinase,[20,21] and some cyclopeptide alkaloids can cause inhibition of Ca^{2+}/calmodulin-dependent protein kinase II.[5] A putative action of adouetine X (**6**) on Ca^{2+}/calmodulin-dependent protein kinase could also explain its antinociceptive effect on neuropathic pain. In fact an increased activity of Ca^{2+}/calmodulin-dependent protein kinase II was observed in the spinal cord after peripheral nerve injury. Moreover the intrathecal administration of an inhibitor of calcium calmodulin-dependent kinase II attenuated the development of mechanical allodynia in rats.[22,23] Further studies must be carried out to assess the effect of adouetine X (**6**) on Ca^{2+}/calmodulin-dependent protein kinase activity.

In summary, we have found that adouetine X (**6**) showed an analgesic effect in acute and chronic neuropathic pain models, without inducing detectable undesirable side effects. The effect of adouetine X (**6**) seems to be related to the inhibition of Ca^{2+}-ATPase and Na^+/K^+ ATPase. These findings suggest that the cyclopeptide alkaloid adouetine X (**6**) is an interesting prototype analgesic drug.

Experimental Section

General Experimental Procedures. Melting points were determined in an MQAPF-301 melting point apparatus and are uncorrected. Optical rotations were taken on a Perkin-Elmer 341 digital polarimeter. NMR

spectra were acquired on a Bruker DPX-400 operating at 400 and 100 MHz, for ^{1}H and ^{13}C, respectively. Chemical shifts are given in ppm using TMS as an internal standard. Mass spectra MS: 70 eV. FAB-MS were obtained on a VG analytical 70±150-S mass spectrometer equipped with a FAB ion source from a 3-nitrobenzyl alcohol matrix.

Plant Materials. The root bark of *Discaria americana* Gill. & Hook. (Rhamnaceae) and *Scutia buxifolia* Reissek (Rhamnaceae) was collected in São Sepé, Brazil (29°45′30″ S, 54°20′33″ W) in December 2000 and January 2003 and authenticated by Prof. Renato Zachia and Prof. Adelino Alvarez Filho, Department of Botany, Universidade Federal de Santa Maria, RS Brazil, where the specimen samples (SMDB 2688, SMD-B146) were deposited, respectively. The roots of *Melochia chamaedrys* A. St.-Hil. (Sterculiaceae) were collected in February−May 2001 in the state of Rio Grande do Sul, Brazil, and authenticated by Prof. Thais S. C. Dorow, Department of Botany, Universidade Federal de Santa Maria, RS, Brazil, where a specimen sample (SMDB9262) was deposited at the herbarium of the Federal University of Santa Maria.

Extraction and Isolation. The alkaloids franganine (**1**) and discarine B (**2**) were isolated from the roots of *D. americana* (Rhamnaceae), whereas scutianines B (**3**), C (**4**), and D (**5**) were isolated from the roots of *S. buxifolia* (Rhamnaceae), and adoutine X (**6**) was isolated from the roots of *M. chamaedrys* (Sterculiaceae), as described previously by Morel et al.,[24−26] respectively.

Animals. Adult male Swiss mice (25−35 g; $n = 6$ or 7) were used in all experiments. The animals were housed in groups of 20 to a cage at controlled temperature (22 ± 1 °C) with a 12 h light/dark cycle and with standard laboratory chow and tap water ad libitum. Each animal was used only once. The experiments reported in this study were carried out in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals.[27] The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of treatment using the tested compounds.

Treatment. Test compounds were injected intrathecally (i.t.) into the spinal column between L5 and L6 of unanesthetized mice, as reported previously.[28] The dose used for the animal treatment was 10 nmol/site (5 μL) for all compounds, and the vehicle used was 1% DMSO (dimethylsulfoxide) plus 1% Tween 80 in PBS. The dose of 10 nmol/site was used because it was the largest amount of compound that could be diluted. The rectal temperature, tail-flick reaction time, and locomotor activity were measured in each group of animals, as described earlier.[29]

Locomotor Activity. To evaluate possible nonspecific muscle relaxant or sedative effects of the test compounds, the spontaneous locomotor performance was measured in the open-field test.[15] The apparatus was a rectangular arena (28 × 18 × 12 cm) with the floor divided into 18 equal squares. The number of areas crossed with all paws and the number of rearing responses were recorded. Mice were placed in an open-field 5 min after injection of the test compounds (10 nmol/site), and the spontaneous activity was measured during the next 10 min period.

Rectal Temperature. Since many drugs can change body temperature, the rectal temperature was measured to evaluate if the test compounds were capable of causing any variation in this parameter.[30] Baseline temperatures were recorded before test drug administration, measuring approximately 37−38 °C for each mouse. The body temperature was measured again 15 and 60 min after the administration of each test compound (10 nmol/site) was tested and Δ°C was used to calculate the change in body temperature (Δ°C = test temperature − baseline temperature).

Tail-Flick Test. In order to evaluate the possible antinociceptive (analgesic) effect of the compounds tested, the tail-flick reaction time to a heat stimulus was used. The warm-water tail immersion test was performed according to a method described previously,[31] using a water bath with the temperature maintained at 48 °C. Before vehicle or test compound administration (10 nmol/site; i.t.), the baseline latency period (6−7 s) was determined. At 15 and 60 min after treatment with control or test compounds, tail-flick latency was reassessed, and differences in relation to the baseline values were calculated. An 18 s maximum latency was used to avoid tissue damage. Antinociception was expressed as percentage of maximum possible effect (MPE), which was calculated as %MPE = [(test latency control latency)/(10 − control latency)] × 100. Percent MPE was calculated for each mouse using at least six mice per test compound.

Determination of Time Course and Potency for Antinociception Caused by Adouetine X (6). To evaluate the time course of the antinociceptive effect of adouetine X (**6**), a dose of 10 nmol/site (i.t.) was used. The baseline latency (6−7 s) was measured before the injection of the compound. At 0.25, 1, and 2 h after the administration of adouetine X (**6**), the latency was reassessed and the MPE (%) was calculated for each time. In addition, the rectal temperature and locomotor activity were observed at 0.25, 1, and 2 h after the administration of adouetine X (**6**) in the same animals.

The ID_{50} value for adouetine X (**6**) was measured by the up-and-down method in the tail-flick test.[32] The up-and-down method is a procedure that has been confirmed to reduce the number of animals needed to determine LD_{50} values without compromising reliability. Subjects were evaluated sequentially such that the results of each animal determined the dose that the subsequent animal received. The first animal was given a dose of drug that was close to the expected ED_{50} and then evaluated in the tail-flick test. If the %MPE value exceeded 50%, then the dose for the next animal was incrementally decreased (e.g., decreased by log dose of 0.301). Alternatively, if the tail-flick latency did not exceed the 50% MPE criterion, then the dose was incrementally increased (e.g., increased by log dose of 0.301). The first dose tested was 10 nmol/site. If the animal showed a MPE higher than 50%, the dose was decreased logarithmically. However, when the MPE was lower than 50%, the dose was increased. Six animals were used to determine the ID_{50} value for this compound. The ID_{50} value was calculated from appropriate tables given by Dixon.[33] A confidence limit (C.L.) of 95% was determined by the following equation: 95% C.L. = dose increment × ($\sqrt{2/n}$) × 1.96. In addition, tree vehicle-treated subjects were evaluated to control for handling and stress factors.

Partial Sciatic Nerve Ligation. A partial nerve ligation of the sciatic nerve (PLSN) was produced by tightly ligating a common sciatic nerve of the left hindpaw under deep anesthesia.[34] Mice were anaesthetized intraperitoneally (i.p.) using a mixture of ketamine (90 mg/kg) and xylazine (3 mg/kg). The same surgical procedure was used for sham mice except for nerve ligation. The experiments were conducted 1 week after the surgery. The operated and sham animals were treated with adouetine X (**6**) (10 nmol/site) or vehicle. The mechanical allodynia was evaluated at different time intervals (0.25, 0.5, 1, and 2 h) as a reduction in mechanical threshold to withdrawal using von Frey hair filaments, as described before.[35]

[^{3}H]-Glutamate Binding Assay. Cerebral cortices obtained from mice were used for membrane preparation, carried out as described before.[36] Sodium-independent [^{3}H]-L-glutamate binding to cerebral plasma membranes was investigated as described previously.[37] Briefly, membranes were incubated in a 0.5 mL reaction mixture containing 50 mM Tris-acetate buffer (pH 7.4), 40 nM [^{3}H]-L-glutamate, and 10 μM of each test compound (dissolved in water and 1% DMSO). Incubation was carried out at 30 °C for 30 min, and the reaction was stopped by filtration using GF/B glass microfiber filters. Dried filters were transferred to Eppendorf tubes containing scintillation fluid, and the radioactivity was determined with a Packard scintillation spectrometer at 40−45% efficiency. Specific binding was calculated as the difference between total binding and nonspecific binding, which was measured in the presence of a 10 000-fold excess (4 mM) of unlabeled L-glutamate. The assays were performed in triplicate, and the results represent the combined data from three individual experiments. [^{3}H]-L-Glutamate was purchased from Amersham (São Paulo, Brazil). Protein concentration was measured using bovine serum albumin as standard.[38]

ATPase Activity Assay. The ATPase activity was estimated by a method adapted from Mark et al.[39] and Fighera et al.[40] The enzyme activity was determined by measuring the amount of inorganic phosphate (Pi) liberated from ATP during the incubation of the synaptosomal fraction. Synaptosomes were isolated from mice brains by a method adapted from Rodrigues et al.[41] Protein concentration was assayed by the method described previously[38] using bovine serum albumin as a standard. The method allows the quantification of distinct Mg^{2+}-dependent ATPase activities (Na^{+}/K^{+}-ATPase or Ca^{2+}-ATPase activity) in the same sample. Activities were measured in triplicate in covered 96-well microtiter plates at 37 °C. Fifty microliters of assay buffer (5 mM HEPES, 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.1) was placed in each plate after 50 μL of another assay buffer (5 mM Hepes and 320 mM sacarose) containing 1 μg of membrane protein was added to each well. The Na^{+}/K^{+}-ATPase activity was determined by subtracting the ouabain (0.2 mM)-sensitive activity

from the overall Mg^{2+}-ATPase activity level, and the percentage of activity in relation to the total was calculated from that observed for the control and adouetine X (**6**) (3 and 10 μM). The Ca^{2+}-ATPase activity was determined by subtracting the activity measured in the presence of Ca^{2+} and ouabain from that determined in the absence of Ca^{2+} (no added Ca^{2+} plus 0.1 mM EGTA) and the presence of ouabain from that observed for the control and adouetine X (**6**) (3 and 10 μM). The plate was preincubated at 37 °C for 10 min, and the assay was started with the addition of 50 μL of adenosine triophosphate (ATP) to a final concentration of 3 mM, making the final reaction volume 160 μL. After 60 min, the reaction was terminated by the addition of 50 μL of 5.7% ammonium molybdate in 6 N H_2SO_4 plus 10 μL of 8% acid ascorbic. The level of inorganic phosphate present, quantified by the colorimetric method of Fiske and Subbarow,[42] was used as a measure of ATPase activity. Plates were read on a Fisher Biotech Microkinetics Reader BT 2000 at 630 nm. The absorbance values obtained were converted to activity values by linear regression using a standard curve of sodium monobasic phosphate that was included in the assay procedure. Ouabain, adenosine triophosphate, and ammonium molybdate ascorbic acid were purchased from Sigma (St. Louis, MO). Values reported represent the mean and SD of at least six separate experiments.

Statistical Analysis. The results of antinociceptive activity and the side effects were expressed as means ± SEM, which are reported as geometric means accompanied by their respective 95% confidence limits. Data were analyzed by Student's *t* test and one-way or two-way analysis of variance (ANOVA). Post hoc tests (Student−Newman−Keuls, SNK) were carried out when appropriate. The level of significance was set at $p < 0.05$.

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Supporting Information Available: Physical and NMR spectroscopic data for the cyclopeptide alkaloids used in this study. This information is available free of charge via the Internet at http://pubs.acs.org.

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