Accepted Manuscript

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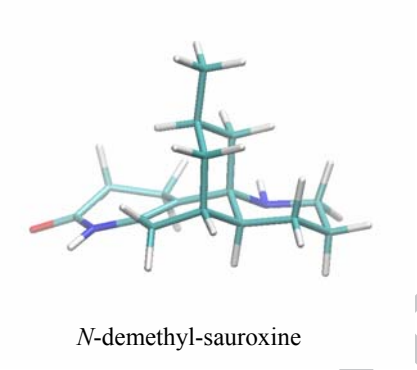
PII: S0040-4039(13)01217-3
DOI: http://dx.doi.org/10.1016/j.tetlet.2013.07.068
Reference: TETL 43266

To appear in: *Tetrahedron Letters*

Received Date: 1 June 2013
Revised Date: 7 July 2013
Accepted Date: 9 July 2013

Please cite this article as: Vallejo, M.G., Ortega, M.G., Cabrera, J.L., Agnese, A.M., *N*-demethyl-sauroxine, a novel Lycodine Group alkaloid from *Huperzia saururus*, *Tetrahedron Letters* (2013), doi: http://dx.doi.org/10.1016/j.tetlet.2013.07.068

GRAPHICAL ABSTRACT



N-demethyl-sauroxine

N-demethyl-sauroxine, a novel Lycodine Group alkaloid from *Huperzia saururus*

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The present study describes the isolation and identification of *N*-demethyl-sauroxine, a novel Lycopodium alkaloid obtained from *Huperzia saururus* (Lam.) Trevis. (Lycopodiaceae). Its structure and relative stereochemistry were elucidated on the basis of its spectral data and chemical correlations. Additionally, acetylcholinesterase inhibitory activity was evaluated (IC_{50} = 209.6 ± 1.1 μM). The structure of the already identified alkaloid sauroxine was also re-validated through two dimensional NMR data.

Keywords: Huperzia saururus; *N*-demethyl-sauroxine; acetylcholinesterase inhibition; sauroxine NMR two dimensional data.

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Huperzia saururus (Lam.) Trevis. ("cola de quirquincho", Lycopodiaceae) is a native Argentine species, used in the ethnomedicine as aphrodisiac[1] and memory improver.[2] As a consequence of decoctions consuming, cases of intoxication were detected, with manifestations such as nauseas, vomits, abortion, even comma as it is explained by the Health Ministry in Argentina.[1,3]

Regarding chemical studies, we have identified *Lycopodium* alkaloids as the main compounds being sauroine and sauroxine (**1**) the majority alkaloids.[4,5] According to the hypothetic cholinergic stimulation implied by the adverse effects, biological assays developed with the alkaloid extract (AE) showed an important inhibitory action on the acetylcholinesterase enzyme (AChE).[4] Also it was demonstrated that AE exerts a facilitation of both, the long term potentiaton (LTP) induction on hippocampal slices[6] and the memory retention according to Step-down Test results.[7] Besides, sauroine has similar properties in relation to the memory phenomenon, but it has not an inhibitory effect on AChE.[5,8]

In this paper, the isolation and structure elucidation of a novel *Lycopodium* alkaloid (**2**) is described. The obtaining of the new compound through a semi-synthesis strategy was also proposed, employing sauroxine, the second major alkaloid in *H. saururus,* as a substrate.

Additionally, due to the limited existing data of sauroxine,[9,10] its structure was re-validated.

PREFERRED POSITION FOR CHEMICAL STRUCTURES

Aerial parts of *H. saururus* dried and ground (2.45 kg) were alkalinized with 0.1 M NaOH (pH 12) and extracted by soxhlet with $ClCH_3$ as solvent. 77 g of extract were obtained. This was solubilized with pH 2 HCl solution, then alkalinized until pH 12 with 0.1 N NaOH and partitioned with $ClCH_3$ by using a liquid-liquid extractor. The organic phase, with a yield of 3.95 g, was purified by Sephadex LH-20 using $ClCH_3$/EtOH (1:1) as mobile phase. All the fractions that were positive to the Dragendorff`s reagent were combined (2.21 g). This fraction was re-

submitted to Sephadex LH_{20}, but acetone was used as mobile phase. Fraction 3 was purified by CC using Sephadex G-10 with H_2O/EtOH (95:5). By posterior purification of the third fraction by TLC using ciclohexane/$ClCH_3$/diethylamine (5:4:1) as mobile phase, **1** (9.7 mg, 0.00039%) was obtained; fraction 4 was purified in the same way but using ciclohexane/diethylamine (1:1) as mobile phase. Three bands were obtained, and the first one was purified by TLC with Cl_2CH_2/MeOH (4:1) leading to *N*-demethyl-sauroxine (**2,** 1.9 mg, 0.0000775%). It is noteworthy that this alkaloid was also obtained by using other extraction methods, and other extractant such as H_20, both at acid and neutral pH (data not shown), thus dismissing the possibility that **2** could be an artefact.

Semi-synthesis of **2** was carried out by means of an adaptation of the Polonovsky reaction.[11] **1** (9.1 mg) was solubilized in MeOH (200 µL) and maintained at 0ºC. Then, H_2O_2 (20 µL) was added drop by drop. Reaction was left to develop at 25ºC, through 6 h. Later, little amounts of MnO_2 were added to quenching the H_2O_2 excess, and the necessary amount of MnO_2 was detected with KI-starch paper. This suspension was filtered by Celite® and taken to dryness, then solubilized in MeOH (200 µL) and, HCl 6 M was added until pH 2. Solution was taken to dryness again. Thus, sauroxine *N*-oxide hydrochloride was obtained (10.7 mg). This was dissolved in MeOH (200 µL) and $FeSO_4$•$7H_2O$ (2 equiv) was added. This second step of reaction was developed as well at 25ºC through 30 min. The solution was taken to dryness and solubilized with EDTA 0.1 M at pH 10 and then it was partitioned with Cl_3CH ten times. Organic fractions were reunited and $MgSO_4$ (1.2 mg) was added. After filtration, it was taken to dryness. Thus, a mixture of **1** and **2** was obtained.

Both alkaloids were immediately separated by TLC, with ciclohexane/Cl_3CH/dietilamine (5:4:1) as mobile phase. This way, 1.2 mg of **2** were obtained. Semi-synthetic and natural compounds were compared chromatographically by TLC.

Sauroxine (**1**). The structure of **1** was re-validated by the 2D NMR (^{1}H-^{1}H COSY, HMQC, HMBC, NOESY) data. The ^{1}H-^{1}H COSY and TOCSY (Figure 1) revealed the connectivities of

C-2 to C-3, C-6 to C-7 and C-8, C-9 to C-11, and C-14 to C-16. The HMBC data (Figure 1) confirmed the α-pyridone ring: H-2 showed correlations with C-1, C-4 and C-5, H-3 with C-2 and C-4, and N_{α}-H with C-4. Connection to the B ring is showed through the following correlations: H-6 with C-4 and C-5, and H-7 with C-4 and C-5. Connectivities in B ring were H-6 to C-7, C-8 and C-12, H-7 to C-12 and C-13, and H-8 to C-6, C-7 and C-12. Piperidine C ring was constituted according to the correlations between H-9 with C-11 and C-13, H-10 with C-12, H-11 with C-9 and C-10, and H-12 with C-11. Finally, D ring correlations were H-7 with C-15, H-14 with C-15, H-16 with C-8, C-14 and C-15, and H-14 with C-4. NOESY correlations (Figure 1) allowed the statement of the stereochemistry of **1**. The most important were the existing between H-12 and H-6b, and H-3a.

Around thirty *Lycopodium* alkaloids belong to the Lycodine Class, where C/D mostly exhibit the *trans* configuration[12], α-obscurine being one example.[13] Differently, **1** is an stereoisomer of α-obscurine and for this reason it is also called 12-epi-α-obscurine. In 1974, Nakashima et al., showed differences in ^{13}C NMR spectrum between the carbon signals at C-8 and C-14 for **1** and α-obscurine[10]. In the present work, we improve the analysis showing these differences through 2D NMR, supporting the unique conformation of C/D ring in **1** and **2**.

N-demethyl-sauroxine[14] (**2**). The IR absortions implied the presence of a ketone carbonyl group (1665.80 cm^{-1}), an amide and amine group at 3223 cm^{-1} and 3382 cm^{-1}, respectively. **2** showed a molecular ion at m/z 260 (12.5 %) consistent with the formula $C_{16}H_{25}N_2O$ deduced from the HRESIMS (found: 261.1973; calcd; 261.1967). The base peak at m/z 203 (100 %) indicates the loss of the bridge ring (C-8, C-14, C-15, and C-16), plus a hydrogen atom from C-12 suggesting that **2** possess a lycodine-type skeleton[15].

The ^{13}C analysis and HSQC-DEPT spectra indicated 16 carbon signals and showed the presence of only one methyl carbon, three methine carbons, eight methylene carbons, and four quaternary carbons. The carbon signals at δ_C= 170.8 and δ_C= 59.5 correspond to the carbonyl group (C-1) and the quaternary function (C-13).The signals at $\delta_{C\text{-}4}$= 110.6 and $\delta_{C\text{-}5}$= 133.6

correspond to the double bond C-C. HMBC experiment established that those signals have not correlations with any proton (Table 1). Comparing the ^{13}C NMR spectrum of **1** and **2,** the most important differences were one less carbon signal and the absence of the signal belonging to the methyl group (N_β-CH_3) of **1**. Furthermore, there is an increase of $\delta_{C\text{-}10}$= 18.6 (22.8), and $\delta_{C\text{-}12}$= 32.6 (40.1) signals, and a decrease of $\delta_{C\text{-}9}$= 47.6 (39.9) and $\delta_{C\text{-}11}$= 24.9 (22.4). All this is consistent with the effect observed in lycodine, in comparison with *N*-methyl-lycodine, where the *N*-methyl group is axially oriented as well.[10]

The ^1H-^1H COSY and TOCSY (Figure 2) revealed the connectivities of C-2 to C-3, C-6 to C-7 and C-8, C-9 to C-11, and C-14 to C-16. The HMBC data (Figure 2) led to confirm the α-pyridone ring: H-2 showed correlations with C-1, C-3 and C-4, H-3 with C-4 and C-5, and N_α-H with C-4. Connection to the B ring is showed through the following correlations: H-6 with C-4 and C-5. Connectivities in B ring were H-6 to C-7, C-8 and C-12. Connectivity in the D ring was H-8 to C-12. Finally, D ring correlations were H-14 with C-4, C-13 and C-15, H-16 with C-8, C-14 and C-15.

NOESY correlations (Figure 2) allowed the statement of the stereochemistry of **2**. The most important were those existing between H-12 and H-6b, and H3a. Together with the previous spectroscopic data, we can determine the C/D ring configuration as *cis*, and therefore, **1** and **2** are the only two Lycodine Group alkaloids having this conformation.

Semi-synthetic **2** was obtained as an amorphous solid, at a very low yield. Nevertheless, some important signals were detected in the ^1H NMR spectrum such as the methyl group in C-16 and again, the absence of a methyl group in N_β compared to **1**. In addition, several carbon signals were identified: C-16 (22.1), C-2 (30.8), C-7 (33.8) and C-12 (42.6). As in the natural product, these chemical shifts are different from those observed in **1**.

As it was previously mentioned, the purified alkaloid extract of *H. saururus* was previously evaluated in our lab in relation to its effect on the acetylcholinesterase enzyme. As the results obtained exhibited a strong inhibitory effect (IC_{50}= 0.58 μg/mL)[4], we evaluated some of the

purified alkaloids as well, searching for the one with the best results. Thus, sauroine showed no inhibitory effect[5], sauroxine had an IC_{50} = 8.9 ± 0.4 μg/mL (32.3 μM)[17], 6-hydroxylycopodine showed an IC_{50} = 78.1 ± 3,5 μg/mL (298.8 ± 13.3 μM)[17], and in the present paper we evaluated the same activity for **2**. An IC_{50} = 54.5 μg/mL (209.6 ± 1.1 μM) was obtained. As it can be seen, hitherto no compound has similar inhibitory activity to the extract, so we postulate a possible synergistic effect among the alkaloids present.

Acknowledgements

Authors are grateful to Dr. G. Barboza for the species identification; Dr. Gloria Bonetto (UNC) and Dr. Dan Borchardt (University of California) for carrying out the NMR experiments, and Lic. José Borioni and Dr. Marcelo Puiatti (UNC) for 3D figures and to SeCyT - Universidad Nacional de Córdoba (05/C375), ANPCyT BID – PICT 1576, and CONICET D32/10 for the grants.

Supplementary data

Supplementary data associated to this article can be found in the online version, at…

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14. *N*-demethyl-sauroxine{**2**, very pale yellow oil; $[\alpha]_D^{26}$ +17.46 (c 0.32, MeOH)}, IR (BrK) ν_{max} 3383, 3223, 2929, 1741 and 1666 cm^{-1}.

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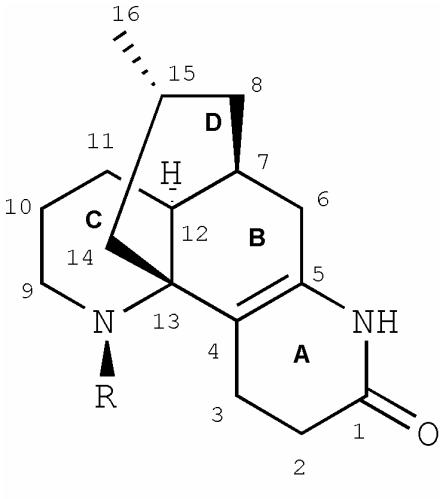
Table 1. NMR data of *N*-demethyl-sauroxine

position	δ_C [a]	δ_H [b] (multi, *J* in MHz)
1	170.8 (C)	---
2a	30.5 (CH_2)	2.42 (1H, br t, 7.7)
2b		2.68 (1H, br t, 7.7)
3a	19.7 (CH_2)	2.52-2,59 (1H, m)
3b		2.75 (1H, br t, 10.1)
4	110.6 (C)	---
5	133.5 (C)	---
6a	34.4 (CH_2)	1.84 (1H,d, 18,8)
6b		2.60 (1H, dt, 18.8; 4,3)
7	32.7 (CH)	2.06-2.11 (1H, m)
8a	35.5 (CH_2)	1.31-1.36 (1H, m)
8b		1.42 (1H, br d, 13,3)
9a	39.9 (CH_2)	3.02 (1H, t, 12.3, 3.0)
9b		3.19 (1H, d, 12.3)
10a	22.4 (CH_2)	1.50-157 (1H, m)
10b		1.82 (1H, br d, 12.2)
11	22.8 (CH_2)	1.88-2.02 (1H, m)
12	40.1 (CH)	2.03-2.10 (1H, m)
13	59.5 (C)	---
14	32.2 (CH_2)	1.65-1.73 (1H, m)
15	26.2 (CH)	1.72 (1H, m)
16	21.7 (CH_3)	0.95 (3H, d, 5.7)

[a] 100 MHz, $CDCl_3$
[b] 400 MHz, $CDCl_3$



1: R = Me
2: R = H

Figure 1.Selected 2D NMR correlations for **1**.

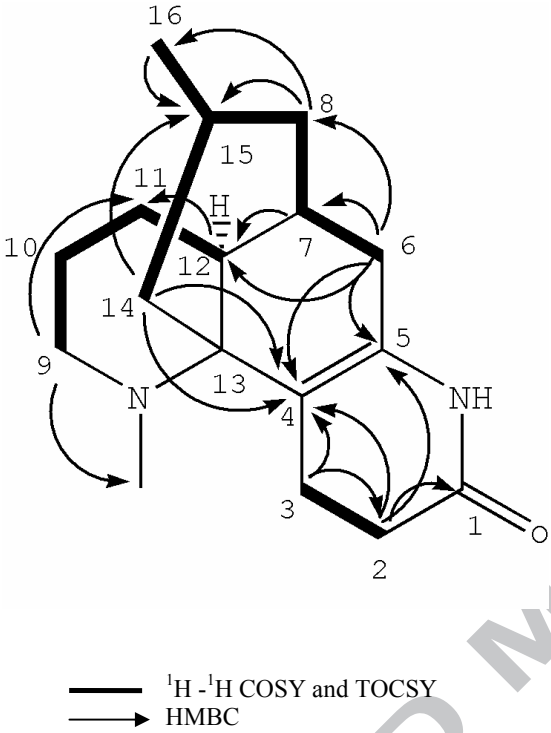
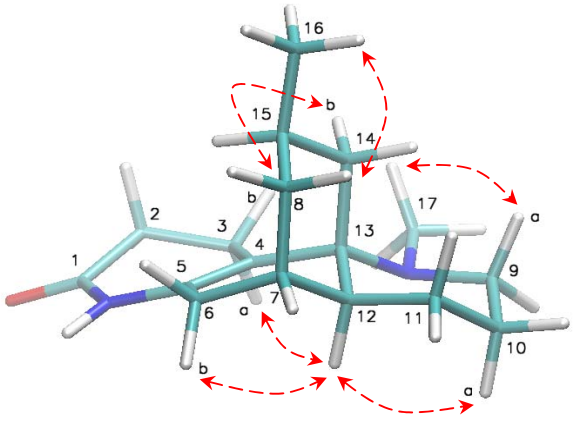
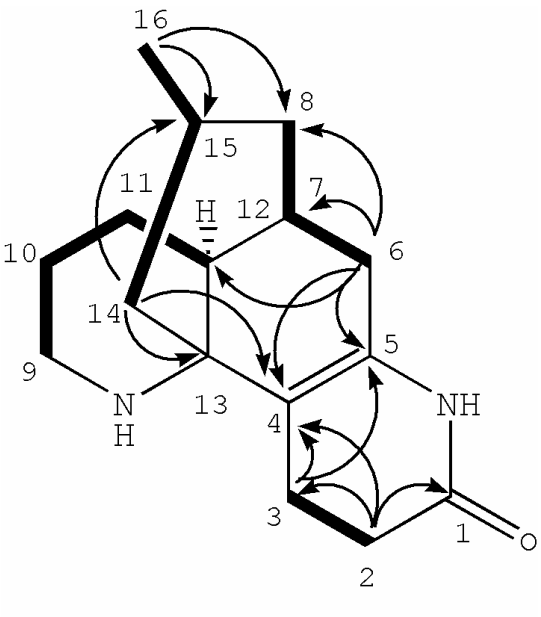


Figure 2. Selected NOESY correlations for **1**.



NOESY

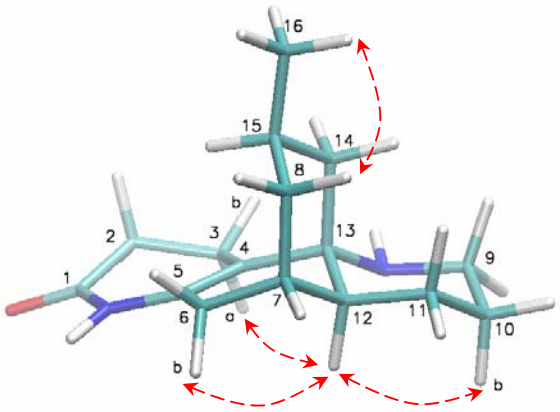
Figure 3. Selected 2D NMR correlations for **2**.



^{1}H -^{1}H COSY and TOCSY

HMBC

Figure 4. Selected NOESY correlations for **2**.



NOESY