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# Characterization and pharmacological actions of tecostanine, an alkaloid of *Tecoma stans*

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*Received July 19, 2002, accepted September 18, 2002*

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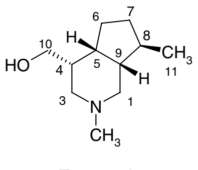
*Pharmazie 58: 140–142 (2003)*

Tecostanine (**1**) was isolated from *Tecoma stans* leaves. Its sterochemistry was elucidated as well as its antihyperglycemic activity and its affinity to opioid and nicotinic receptors. The oxalate salt of **1** did not significantly affect blood glucose levels in normoglycaemic and hyperglycaemic rats. It did not appear to interact with opioid receptors (mu type) and showed only moderate affinity to the nicotinic receptor.

## 1. Introduction

*Tecoma stans* (L.) Juss. ex Kunth (Bignoniaceae) is a plant traditionally used in Mexico for the control of diabetes, dysenteric diseases, anorexia, hepatic problems and for its analgesic action. According to these folk reports, it was shown recently that the plant extract is able to reduce the area under glucose tolerance curve in rabbits [1]. Moreover, the narcotic and anestethic activity of flowers of *Tecoma stans*, as well the anti-inflammatory and antinociceptive activity of alcoholic extracts of pods and flowers of another species, *Tecoma sambucifolia*, have been reported [2]. It is known that this plant contains monoterpene alkaloids; two of these, tecostanine (**1**) and tecomine, isolated from *Tecoma stans* growing in Egypt, are reported among the substances responsible for the hypoglycaemic activity of the plant [3, 4]; their mechanism of action was not elucidated, but it seems to need a minimum of active β-cells of the pancreas for the action [3]. Thus, due to the interest in new antidiabetic agents, we decided to isolate **1** from *Tecoma stans* leaves, determine its hitherto unknown stereochemistry and reinvestigate its hypoglycaemic action. Moreover, since *Tecoma stans* exerts several pharmacological actions and other monoterpene alkaloids like skytanthine (alkaloid of *Skytanthus acutus*), structurally related to tecostanine, shows Straub-type symptoms and psychotropic activity similar to nicotine after i.p. administration to mice [5], we also investigated the affinity of **1** to the opioid and the nicotinic receptors.



Tecostanine

**1**

## 2. Investigations, results and discussion

The structure of **1** was confirmed by $^1H$-$^1H$ COSY experiments and $^{13}C$ NMR studies. Its $^1H$ NMR spectrum (Table) shows a signal for two methyl groups at δ 0.99 (d, J = 8.0 Hz) and at δ 2.24 (N−CH$_3$), respectively, 2 carbinolic protons at δ 3.57 (dd, J = 7.6, 10.6 Hz) and at δ 3.45 (dd, J = 6.2, 10.6 Hz) which suggest the presence of a oxymethylene derivative. Its $^{13}C$ NMR spectrum (Table) shows resonances for 11 carbons. In addition, the DEPT experiments show absorption for 5 methylenes, 4 methynes and 2 methyl groups at δ 22.9 and at δ 46.7, respectively, the latter confirming the presence of a N−CH$_3$. Assignments in the $^1H$ NMR spectrum were assisted by $^1H$-$^1H$ COSY experiments. Thus the double doublet centered at δ 3.45 showed a cross peak with a proton at δ 3.57 (dd) and a multiplet at δ 2.19 characterizing the resonances of $H_{10\alpha}$ $H_{10\beta}$ and $H_4$. $H_4$ showed a cross peak with $H_{3eq}$ centered at δ 2.75 (dd, J = 2.8, 11.2 Hz), with $H_{3ax}$ at δ 1.65 (t, J = 11.2 Hz), as well as

**Table: $^1H$ and $^{13}C$ NMR spectral data of 1**[a]

| Carbon | $^{13}C$ δ (mult.) | $^1H$ δ (mult., J = Hz) |
|---|---|---|
| 1 | 64.9 (t) | Eq 2.60 (ddd, 2.0, 6.0, 12.0)$^{*1ax, 9}$. Ax 1.55 (m) |
| 3 | 58.1 (t) | Eq 2.75 (dd, 2.8, 11.2)$^{*3ax, 4}$. Ax 1.65 (t)$^{*4}$ |
| 4 | 36.7 (d) | 2.19 (m)$^{*5}$ |
| 5 | 36.4 (d) | 1.52 (m)$^{*6eq}$ |
| 6 | 32.1 (t) | Eq 1.95 (m)$^{*6ax}$. Ax 1.15 (m) |
| 7 | 22.6 (t) | Eq 1.53 (m)$^{*7ax}$. Ax 1.15 (m) |
| 8 | 39.8 (d) | 1.63 (m)$^{*11,7eq}$ |
| 9 | 46.8 (d) | 1.86 (m)$^{*1eq}$ |
| 10 | 54.1 (t) | α 3.45 (dd, 6.2, 10.6); β 3.57 (dd, 7.6, 10.6)$^{*4}$ |
| 11 | 22.9 (q) | 0.99 (d, 8.0) |
| N−CH$_3$ | 46.7 (q) | 2.24 (s) |

[a] Data obtained by $^1H$-$^1H$ COSY experiments. Solvent: CDCl$_3$
* = cross peak = proton correlated

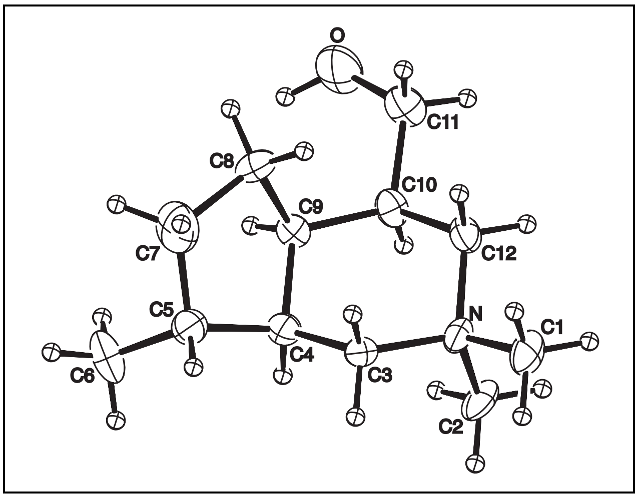


Fig.: X-ray crystal structure of **2**

with $H_5$ (δ 1.52, m). On the other hand, the signal between $H_5$ and a multiplet at δ 1.95 permits the characterization of $H_{6eq}$.The $H_8$ signal was identified by the cross peak with a methyl group at δ 0.98 (d, J = 8.0). In addition it was also characterized the $H_{7ax}$ signal at δ 1.15.

The absolute stereochemistry of **1** was determined by single crystal x-ray diffraction analysis of the corresponding methiodide (**2**). The absolute configurations to the four chiral centers present in the cation are *R* to C5, *S* to C4, *S* to C9, and *R* to C10 (Fig.).

The activity of **1** in the form of its oxalate salt (**3**) on glycemia was assessed in normoglycaemic and hyperglycaemic rats. It appears that **3** at the doses of 50 and 150 mg/kg does not significantly affect blood glucose levels in normoglycemic or hyperglycaemic rats. No effect of **3** could be demonstrated on insulin levels in conditions in which insulin secretion is hyperstimulated. These results are not in agreement with a previous report, in which tecostanine was shown to be effective i.v. at the dose of 20 mg/kg and orally at the dose of 50 mg/kg, as hypoglycaemic agent in rabbits [3, 4], probably owing to a different sensitivity between the two different species to the compound.

The effect of **3** on opioid receptors was studied by measuring the intracellular accumulation of cyclic AMP (cAMP) in SH-SY5Y cells, a human neuroblastoma cell line which express functional opioid receptors mainly of mu type [6, 7]. **3** does not affect the FSK-induced cAMP increase either at 1 μM or 1 mM concentrations; likewise **3** does not appear to interact with opioid receptors (mu type) or with their intracellular signal pathways since it is ineffective in altering the inhibitory action of morphine on cAMP accumulation.

In the pharmacological model conducted, tecostanine **1** shows only a moderate affinity at the nicotinic receptor (Ki = 3.16 μM).

## 3. Experimental

### 3.1. Material and apparatus

Melting points were determined on a Büchi apparatus (uncorr). Optical rotation was obtained on a Perkin-Elmer mod. 241 spectropolarimeter; IR spectrum was recorded on a Perkin-Elmer FT-IR spectrophotometer mod. 1600 (Nujol). $^1H$ and $^{13}C$ NMR Spectra were recorded on a Bruker AMX-200 spectrophotometer. Chemical shifts values are shown in ppm with TMS as an internal standard. MS were recorded on a Finnigan Mat SSQ 710 equipped with a gas chromatograph Varian 3400. X-ray crystallography data were collected on a Enraf-Nonius CAD4 diffractometer. Microanalysis was carried out in the Microanalysis Laboratory of the Dipartimento di Scienze Farmaceutiche, Modena University.

### 3.2. Extraction

Leaves of *Tecoma stans* (L.) Juss. ex Kunth (Bignoniaceae) growing in Cairo, Egypt, collected during July 1999 were used. The plant was identified by Prof. Hammouda. A voucher specimen (LC2002/01) was deposited in the Herbarium of the Dipartimento di Scienze Farmaceutiche, Università di Modena e Reggio Emilia. The dried leaves were extracted (Soxhlet) with diethyl ether/$NH_3$ 15% (10%). The organic layer was then extracted with 4N HCl; the aqueous phase was then washed with ether, basified with $NH_3$ 30% and extracted with $CH_2Cl_2$ (2 × 100 mL). The organic layer was then dried ($Na_2SO_4$) and the solvent removed under reduced pressure. Tecostanine (**1**) was then obtained by means of column chromatography (silica gel 70–230 mesh) with a gradient of $CH_2Cl_2$ and increasing quantity of methanol (from 0% to 40%). **1** was eluted at 10% methanol (mobile phase $CH_2Cl_2/CH_3OH/NH_3$ 89.5/10/0.5; $R_f$ 0.29). For X-rays studies and biological assays, **1** was transformed in its methiodide **2** and oxalate **3**, respectively.

#### 3.2.1. Tecostanine (1)

Pale yellow, amorphous solid; yield 1.55 g, m.p. 80 °C (82 °C [8]); $[\alpha]\ ^{25}/_D$ +6° (c 2.00, $CHCl_3$) (0°, [8]); EIMS m/z183 [$M^+$] (60), 182 (100), 152 (40), 138 (30), 100 (40), 58 (80).

#### 3.2.2. Tecostanine methiodide (2)

Mp 243–246 °C (methanol); IR (Nujol) ($cm^{-1}$): 3424; $^1H$ NMR (DMSO-$d_6$, 200 MHz) δ 4.80 (1 H, t), 3.50–3.10 (4 H, m), 3.15 (3 H, s), 3.05 (3 H, s), 2.90 (1 H, t), 2.40–1.90 (5 H, m), 1.80–1.45 (3 H, m), 1.10 (1 H, m), 1.00 (3 H, d).

#### 3.2.3. Tecostanine .0.5 $(COOH)_2$ (3)

Mp 113–116 °C (methanol/diethyl ether); $^1H$ NMR (DMSO-$d_6$, 200 MHz) δ 3.45–3.05 (4 H, m), 2.73 (3 H, s), 2.80–2.50 (2 H, m), 2.40–2.20 (2 H, m), 2.10–1.85 (2 H, m), 1.75–1.45 (3 H, m), 1.14 (1 H, m), 0.98 (3 H, d); *anal.* C, 63.44%, H 9.80%, N, 6.01%, calcd for $C_{12}H_{22}NO_3$, C 63.13%, H 9.71%, N 6.13%.

### 3.3. X-Ray crystallography

$C_{12}H_{24}NO^+ \cdot I^-$; colourless, air stable crystals of **2**, grown by slow evaporation of ethanol solutions. The selected one had the approximate dimensions 0.30 × 0.25 × 0.22 mm. All measurements were carried out at room temperature. Unit cell dimensions were obtained from least-squares fit to the setting angles of 25 automatically centred reflections in the 7–16° θ range. Crystals are orthorhombic, $P2_12_12_1$ space group, whit a = 9.068(2), b = 9.256(2), c = 16.671(3) Å, and V = 1399.3(5) $Å^3$. For Z = 4 the calculated density is 1.544 $Mg/m^3$. A total of 2546 reflections were collected in the 2.34–27.96° θ range in $\omega - 2\theta$ scan mode. Data were averaged to 2367 unique reflections ($R_{int} = 0.0189$), of which 1490 had $I \geq 2\sigma(I)$. Intensities were corrected for Lorentz and polarization effects, and an empirical absorption correction based on the ψ scan was applied to data ($\mu = 2.268\ mm^{-1}$; $0.999 \geq T_{factor} \geq 0.811$). The structure was solved by direct methods (SHELX86 program) and was refined through full- matrix least-squares calculations based on $F^2$ (SHEXL93) for all unique reflections [9, 10]. All non-hydrogen atoms were refined anisotropically. All but one (located in ΔF map) hydrogen atoms were constrained to ride in ideal positions on their bonded atoms, at bond distance from 0.96 to 0.98 Å. Their thermal parameters were set 1.2 times $U_{eq}$ of bonded atoms. Least-squares refinements of 357 parameters led to final R1 and wR2 values of 0.0363 and 0.0885, respectively, for reflections with $I \geq 2\sigma(I)$; corresponding values for all intensity data were 0.0738 and 0.0991. The absolute configuration was determined by refinement on Flack χ parameter, whose final value was 0.00(5). Largest peak and hole in a final ΔF map were 0.717 and −0.682 $e \cdot Å^{-3}$, respectively [11].

### 3.4. Pharmacology

#### 3.4.1. Glycaemia and insulin evaluations

Adult male Sprague Dawly rats 150–175 g were used in all the experiments. Normoglycaemia: animals fed a normal pellet diet were used. Groups of 5 animals were treated orally (gavage) either with vehicle (0.5 ml of saline) or with 50 and 150 mg/kg of (**3**) dissolved in the same amount of saline. Before treatment (time 0) and after 1, 3 and 6 h 1 ml of blood was withdrawn by intracardiac puncture under light ether anestesia. Glucose levels were immediately tested (Glucotrend plus strips system, Roche) and the remaining blood was allowed to clot then centrifuged and stored at −20 °C until assayed for insulin by radioimmunoassay (Ultrasensitive Rat Insulin ELISA, DRG Diagnostics, Marburg, Germany). Hyperglycaemia: glucose (2 g/kg) was injected intraperitoneally 30 min before each blood sampling. The remaining experimental procedure was carried out as described above.

### *3.4.2. Interaction with opioid receptors*

Cell cultures: opioid receptor-expressing human SH-SY5Y neuroblastoma cells were kindly provided by Dr. June Biedler, Memorial Sloan-Kettering Cancer Center, New York) [6, 12]. The cells were grown at 37 °C (in a humidified $CO_2$ incubator) in MEM containing non-essential aminoacids, 1 mM sodium pyruvate, 100 µg streptomycin/ml, 100 UI penicillin/ml and 10 mg/l of phenol red (Biochrom KG, Berlin, Germany). The medium was supplemented with 10% FBS (FBS, Gibco, Grand Island, NY). Confluent cells were harvested with trypsin solution and $1 \times 10^6$ cells were seeded in 57 $cm^2$ dishes. The medium was replaced at 2-day intervals. cAMP accumulation: SH-SY5Y cells were plated in 24-wells plates ($60 \times 10^3$ cells/well), at 2nd and 4th day of culture the cells were treated with 10 µM retinoic acid [13]. On day 8 of the culture, the cells were washed with 1 ml of MEM (prewarmed at 37 °C) and preincubated for 15 min with the phosphodiesterase inhibitor IBMX (0.5 mM) (Sigma Chemicals, St. Louis, MO). This was followed by a 15-min incubation period at 37 °C in MEM in the presence of **3**, Forskolin (Sigma Chemicals, St. Louis, MO) and morphine sulfate, made from 1000× concentrated ethanolic solution. Intracellular cAMP accumulation was measured by means of a commercial assay kit (Pharmacia Amersham, Milano) after ethanolic extraction of the cells according to manufacturers' instructions. All the samples (run in sestuplicate from two independent experiments) were assayed for protein content using a microassay with human serum albumin as a standard [14]. No variations of total protein/well were detected in all the experimental groups.

### *3.4.3. Nicotinic binding assay*

Membrane preparations: rat cerebral cortical membranes were purchased from ABS Inc. (Wilmington, DE). Prior to use, the frozen membrane pellets were slowly thawed, washed and resuspended in 30 volumes of assay buffer (composition, mM: Tris HCl, 50; NaCl, 120; KCl, 5; $MgCl_2$, 1 and $CaCl_2$, 2.5; pH 7.4 at 4 °C). The homogenate was centrifuged at 45,000 × g for 20 min at 4 °C and the pellet resuspended in ice-cold buffer. Binding: binding conditions were as previously described [15]. Samples containing 150–200 µg of protein, 0.7 nM $[^3H](-)$-cytisine (30 Ci/mmol), and the various concentrations of the nAChR modulators were incubated in a final volume of 500 µL for 75 min at 4 °C in triplicate. Non-specific binding was determined in the presence of 10 µM $(-)$-nicotine.

Acknowledgements: We thank Prof. Dr. M. Hammouda, Department of Botany, University College for Girls, Ain Shams University, Heliopolis, Cairo, Egypt, for kindly providing the plant material.

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