*Journal of Natural Products*
*Vol. 49, No. 2, pp. 340-342, Mar-Apr 1986*

# THE ICHTHYOTOXIC PRINCIPLES OF *ZANTHOXYLUM CLAVA-HERCULIS*

KOPPAKA V. RAO and RICHARD DAVIES

*Department of Medicinal Chemistry, College of Pharmacy, Box J-4, J. Hillis Miller Health Center, University of Florida, Gainesville, Florida 32610*

The lignoid compound asarinin and the insecticidal and sialogogic compound neoherculin were isolated earlier from the bark of *Zanthoxylum clava-herculis* L. (Rutaceae) (1-3). In our study of bioactive plants, we found the extract of the bark to be highly toxic to fish, and fractionation was undertaken based on this activity. After a preliminary solvent partition, the extract was chromatographed on silica gel and five crystalline components (A-E) were isolated. Of these, the last two (D and E) were found to be responsible for the toxicity of the extract.

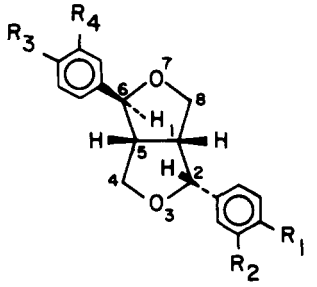
Fractions A and B were isomeric with the formula $C_{20}H_{18}O_6$ ($M^+$ 354) and were shown to be identical with (−)-asarinin (**1**) and (−)-sesamin (**2**), respectively. Both of these belong to the 3,7-dioxabicyclo [3,3,0] octane type lignans, with **1** being the axial/equatorial isomer and **2**, the diequatorial isomer. (−)-Sesamin has been found in a variety of plants, including some species of *Zanthoxylum*, but has not been isolated from *Z. clava-herculis* previously (4,5).

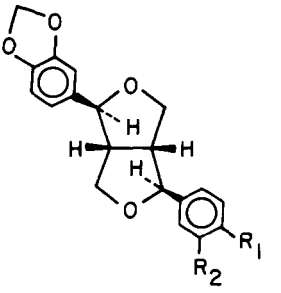
Fraction C, $C_{25}H_{28}O_6$ ($M^+$ 424) showed color reactions and uv spectral characteristics very similar to those of **1** and **2**, which indicated that it belonged to the same general class of lignoids. The $^1$H-nmr spectrum (300 MHz) showed some signals very similar to those shown by (−)-asarinin in both chemical shifts and splitting patterns. However, there were additional signals not found in the spectrum of **1**, such as, a methoxyl, two methyl groups on an unsaturated carbon (δ 1.75), and an allylic ether (δ 4.58, d, 2H and 5.94, t, 1H). These data suggested a general structural similarity with **2** but with one of the methylenedioxy groups of **2** replaced by a methoxyl and an O-$CH_2$-$CH=C(CH_3)_2$ function. In support of this view, the mass spectrum showed strong peaks at *m/z* 356 (M-$C_5H_8$) and 69 ($C_5H_9$).

Two compounds of this structure have been isolated so far: xanthoxylol-γ, γ-dimethylallyl ether (**3**) and piperitol-γ, γ-dimethylallyl ether (**4**), both from *Zanthoxylum piperitum* (4), but the physical properties of Fraction C were different from those of **3** and **4**. Structure **5** was assigned to the present compound through the elucidation of the stereochemistry of the ring system and establishment of the relative positions of the two aryl substituents.

On the basis of prior $^1$H-nmr spectral comparison of **1** and **2**, including decoupling studies, it was possible to make the following assignments of the signals observed in the spectrum of **5**: δ1.75, d, 2×3′-$CH_3$; 2.87, q, H-5; 3.32, m, H-1/H-8e; 3.84, m, H-4a/H-8a; 3.89, s, $OCH_3$; 4.12, d, H-4e; 4.42, d, H-6; 4.58, d, H-1′; 4.86, d, H-2; 5.53, t, H-2′; 5.94, d, O-$CH_2$-O; 6.85, m, 6 arom. H. The equatorial proton 4e is located very close to the aromatic ring, and this explains its significant deshielding. Irradiation at δ 2.87 (H-5) caused a d↦s change in the signal at 4.42 (H-6) and a partial collapse of the signal at 3.84 (H-4a/H-8a). Irradiation of the multiplet at 3.32 (H-1/H-8e) caused a d↦s change of the signal at 4.80 (H-2). Similarly, irradiation at 3.84 (H-4a/H-8e) caused a d↦s change at 4.12 (H-4e).

The spectral data thus established an axial/equatorial stereochemistry for **5**. One can, therefore, eliminate **3** from consideration because it has the





1 $R_1R_2=R_3R_4=O-CH_2-O$

2 $R_1R_2=OCH_2O$

3 $R_1=OCH_2CH=C(CH_3)_2$; $R_2=OCH_3$; $R_3R_4=OCH_2O$

4 $R_1=OCH_2CH=C(CH_3)_2$; $R_2=OCH_3$

5 $R_1R_2=OCH_2O$; $R_3=OCH_2CH=C(CH_3)_2$; $R_4=OCH_3$

diequatorial configuration. Although **4** does have the same axial/equatorial stereochemistry, its physical properties distinguish it from **5**. There is yet another member of this group, known as (−)-pluviatilol, which is a position isomer of xanthoxylol; hence, it also has the same axial/equatorial configuration (5). Fraction C is thus assigned the structure of γ,γ-dimethylallyl ether of (−)-pluviatilol (**5**).

Fraction D, $C_{16}H_{25}NO$, the major component, was shown to be neoherculin and to be the major ichthyotoxic principle of the extract with an $IC_{50}$ of $2\times10^{-7}$M when tested according to the procedure of Stephan (6).

Fraction E, $C_{19}H_{17}NO_3$ showed spectral properties corresponding to the aporphine alkaloids, and its identity with *N*-acetylanonaine was established based on these data. This compound has been isolated from a variety of plant sources, such as *Magnolia obovata* (7) and *Liriodendron tulipifera* (8) but not from *Z. clava-herculis*. *N*-acetylanonaine was also found to be toxic to fish with an $IC_{50}$ of $3\times10^{-4}$M.

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were obtained on a Fisher-Johns hot stage apparatus and are uncorrected. The following instruments/conditions were used for the spectra recorded: uv, Beckman model 35, EtOH; ir, Beckman Acculab-3, KBr pellet; nmr, Varian T-60, Nicolet Instrument Corp., NJ, 300 spectrometer and NIC-1180 E data system, $CDCl_3$ with TMS as the internal standard; ms, Perkin-Elmer/Hitachi RMU-6E, direct probe. Tlc was carried out using Merck silica gel H, PF360, 254 and column chromatography using Fisher silica gel 0.063 mm.

ISOLATION.—The plant material collected in the Gainesville, Florida, area was identified at the University of Florida herbarium where a voucher sample was preserved. Ground bark (2 kg) was extracted three times with 95% EtOH at 20°. The concentrated extract was suspended in $H_2O$ (1 liter) and extracted with $3\times300$ ml of $Et_2O$. The solvent extract was concentrated and partitioned between (4:1) MeOH-$H_2O$ (500 ml) and $C_6H_{14}$ (500 ml). The lower layer was diluted with an equal volume of $H_2O$ and extracted with $C_6H_6$ ($2\times500$ ml). The concentrated $C_6H_6$ extract was applied to a column of silica gel (250 g) in $C_6H_6$-$C_6H_{14}$ (1:1). Elution was with the same solvent, followed by $C_6H_6$ and 2-5% $Me_2CO$ in $C_6H_6$. The fractions were examined by tlc, combined into groups, concentrated, and the products crystallized.

(−) Asarinin (**1**): crystallized from $C_6H_{14}$, mp 120-121° [lit. 121° (2)]; $[\alpha]^{20}D$ −120°; $M^+$ 354.

(−) Sesamin (**2**): crystallized from $C_6H_{14}$, mp 123-124° [lit. 124° (5)]; $[\alpha]^{20}D$ −69° [lit. −68°, (5)], $M^+$ 354.

(−) Pluviatilol γ,γ-dimethylallyl ether (**5**): crystallized from $C_6H_{14}$, mp 119-120°, $[\alpha]^{20}D$ −116°; uv 234, 285 nm; ir 1470, 1430, 1230, 1210, 1120, 1060, 1010, 960 $cm^{-1}$; $^{13}C$ nmr 18.22, 25.82, 50.17, 54.63, 55.96, 65.87, 69.77, 71.01, 71.02, 82.09, 87.67, 101.03, 106.54, 108.14, 109.23, 113.09, 117.63, 119.53, 120.05, 130.99, 135.21, 137.50, 147.31, 147.94, 149.39; ms *m/z* 424, 356, 325, 233, 232, 231, 221, 206, 205, 204, 203, 194, 189, 180, 178, 174, 163, 161, 152, 149, 137, 135, 69, Anal. calc. for $C_{25}H_{28}O_6$: C, 70.74; H, 6.65. Found: C, 70.79; H, 6.62.

Neoherculin: crystallized from $C_6H_{14}$ as colorless needles, mp 68-70° [lit. 70° (3)]; uv λ max 258, 270, 280 nm.

*N*-Acetylanonaine: Crystallized from MeOH, mp 230-231° [lit. 229-230° (7)]; $[\alpha]^{20}D$ −356°, $M^+$ 307.

TOXICITY TESTS ON FISH.—The fish (*Pimephales promelas*, Cypronidae), commonly known as Missouri minnows or fat heads, were purchased from a local bait shop and equilibrated to the new surroundings for 24 h before being used. They were kept in deionized $H_2O$, previously adjusted to pH 6.5 with phosphate buffer at 23°. Sufficient oxygen was provided, and the animals were deprived of food during the test period.

Groups of ten fish were placed in containers with different concentrations of the test drug. The percentage mortality was recorded every 30 min for 2 h. The $LC_{50}$ (M/L) and its 95% confidence limits were determined from the percentage mortality data after 2 h of exposure as described by Stephan (6).

## ACKNOWLEDGMENTS

The authors are happy to express appreciation to Dr. David W. Hall for the identification of the plant, Dr. Roy King for the mass spectra, and Prof. Raymond J. Bergeron for the 300 MHz nmr spectra.

## LITERATURE CITED

1. H. Dieterle and K. Schwengler, *Archiv. Pharm.*, **277**, 33 (1939).
2. J. Naquette, J.L. Ponsset, A. Cane, P. Delaveau, and R.R. Paris, *Ann. Pharm. Franc.*, **31**, 49 (1973).
3. L. Crombie, *Nature*, **174**, 833 (1954).
4. F. Abe, S. Yakara, K. Kubo, G. Nonaka, H. Okabe, and I. Nishioka, *Chem. Pharm. Bull.*, **22**, 2650 (1974).
5. K. Freudenberg and G.S. Sidhu, *Tetrahedron Lett.*, 3 (1960).
6. C.E. Stephan, "Aquatic Toxicology and Hazard Evaluation, ASTM STP. Ed. by F.L. Mayer and J.L. Hasmelink, 1977, p. 65.
7. Y. Shashida, R. Sugiyama, S. Iwasaki, H. Shinomura, H. Ikotawa, and M. Fujita, *J. Pharm. Soc. Jpn.*, **96** 659 (1976); *Chem. Abs.*, **88**, 3043u (1978).
8. C.D. Hufford, *Phytochemistry*, **15**, 1169 (1976).

*Received 14 August 1985*