

INULAVOSIN, A NEW THYMOL DIMER WITH PISCICIDAL ACTIVITY FROM *INULA NERVOSA*

Takashi Yoshida,^{*,a} Kazuko Mori^a, and Guangxin He^b

Faculty of Pharmaceutical Sciences, Okayama University,^a Tsushima, Okayama 700, Japan and Yunnan Institute for Drug Control,^b Kunming, China

Abstract ---- Upon the fractionation guided by piscicidal activity using the Medaka (*Oryzias latipes*), three piscicidal substances were isolated from the root of *Inula nervosa*, and were characterized as thymol and its derivatives based on the spectral analyses including 2D nmr techniques. A new piscicide, named inulavosin, was a thymol dimer with a new heterocyclic skeleton.

Piscicidal activity has been shown to be useful as a primary screening for biologically active substances from natural sources, as revealed by the fact that piscicides often exhibit other biological activities; for examples, insecticidal,¹ anti-tumor promotion² and aphrodisiac³ activities for rotenoids⁴, tumor promotion⁵ for phorbol-type diterpenoid,⁶ and antifungal activity for some quinones.^{7,8} Upon the bioassay-guided fractionation using the Medaka (killie-fish; *Oryzias latipes*), we isolated a new piscicidal component named inulavosin (**4**) and two related compounds (**1** and **2**) from the roots of *Inula nervosa* (Compositae). This communication deals with the characterization of these active constituents.

The pulverized dried roots of *I. nervosa* collected at Kunming in China were homogenized in an aqueous acetone (acetone-H₂O 7:3) at room temperature. The concentrated aqueous solution was extracted with ether. The ether extract which showed a potent piscicidal activity was subjected to column chromatography over silicic acid using CHCl₃-acetone followed by preparative tlc (*n*-hexane-CHCl₃-actone 5:2:1) to give three active compounds [**1** (0.043%), **2** (0.019%) and **4** (0.006%)]. Compounds (**1** and **2**) were characterized as thymol and its isobutylate,⁹ respectively, on the basis of the spectral analysis. The identity of **2** was confirmed by direct comparison of the spectral data with those of authentic specimen prepared from thymol (**1**) and isobutylic anhydride.

Inulavosin (**4**), [α]_D ± 0° (CHCl₃), was obtained as a colorless oil, and its EI-ms showed the molecular ion peak at *m/z* 296, corresponding to the molecular formula C₂₀H₂₄O₂. The ¹H nmr spectrum (500

MHz, CDCl₃) of **4** disclosed five tertiary methyl signals at δ 1.18, 1.42, 1.69, 2.27 and 2.30, the latter two of which were assigned to aromatic methyl groups by analogy of their chemical shifts to that (δ 2.31) of **1**. The presence of an isolated methylene group and a hydroxyl group was also indicated by the signals at δ 2.06 and 2.54 (each 1H, d, $J=14.5$ Hz) and 8.16 (1H, s, disappearing on addition of D₂O). Two pairs of ABX-type signals [δ 7.17, 7.03 (each 1H, d, $J=8$ Hz), 6.81, 6.65 (each 1H, dd, $J=8, 1.5$ Hz), 6.75 and 6.67 (each 1H, d, $J=1.5$ Hz)] were observed in the aromatic region, indicating the presence of two 1,3,4 (or 1,2,4)-trisubstituted benzene nuclei in the molecule. The ¹³C nmr spectrum (126 MHz, CDCl₃) exhibited 20 carbon signals which comprise twelve sp² and eight sp³ carbon resonances including those due to five methyl and one methylene group (Table 1). The ¹H-¹³C COSY spectrum of **4** indicated that two sp³ carbons are quaternary, one of which should bear an oxygen atom (δ 81.7). Among the aromatic carbon signals, two at δ 150.2 and 154.5 were similarly attributable to oxygen-bearing carbons. These nmr data, along with the uv absorption [λ_{max} (EtOH) 278 nm (log ϵ 3.65), 286 (3.62)] similar to that of **1**, suggested that inulavosin is a dimer of thymol [or isothymol (**3**)] or its equivalent, biogenetically formed by C-C coupling between C-10 of **1** (or **3**) and C-8 of another molecule of **1** (or **3**). The presence of a phenolic hydroxyl group in **4** was indicated by a positive coloration with FeCl₃-pyridine reagent,¹⁰ and by production of a monomethyl ether (**4a**) [m/z 310 (M)⁺; δ_{H} 3.84 (OMe)], upon methylation with diazomethane or (Me)₂SO₄-K₂CO₃. The chemical shift (δ 8.16) of the hydroxyl proton signal in the ¹H nmr spectrum of **4** suggests that the hydroxyl group should form hydrogen bond with nearby oxygen atom. The structure **4** was thus proposed for inulavosin which was substantiated by comparison of its spectral data with those of **1**, and by the ¹H-¹³C long-range shift correlations (Table 1). The aromatic methyl signal at δ 2.27 correlated through three-bond couplings with the carbon signals at δ 118.3 and 123.3, which were assigned to C-2' and C-6', respectively, by heteronuclear shift correlation spectrum. Similarly, the methyl signal at δ 2.30 showed correlations with the carbon resonances at δ 118.5 (C-2) and 120.6 (C-6). These observations clearly indicated that **4** isn't an isothymol dimer, but a thymol dimer, which was further confirmed by nOe's between the aromatic methyl protons (H-7, 7') and *meta*-coupled protons (H-2, 6 and 2', 6') in the ROESY spectrum. Two thymol nuclei in **4** were confirmed to be linked through the methylene group on the basis of the three-bond couplings between the methylene carbon (C-10) and the aliphatic methyl proton signals (C-9, C-9' and C-10' methyls). The other long-range correlations summarized in Table 1 support the structure (**4**). The structure of inulavosin was thus represented by the formula (**4**). The lack of optical activity for **4** suggests that the dimerization and/or oxidation at C-8 of **1** leading to **4** might occur in a non-enzymatic manner. Although thymol and its derivatives are known to distribute widely in the Labiatae and Compositae families,¹¹ inulavosin (**4**) is the first example of dimeric derivative of thymol.

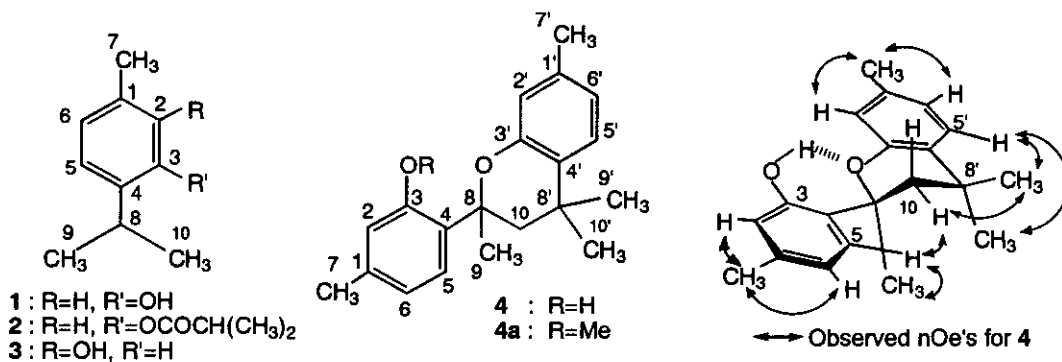


Table 1. Nmr data for thymol (**1**) and inulavosin (**4**) in CDCl₃

	1		4a	
	δ_C	δ_C	δ_H	
			Proton coupled via one bond	Proton coupled via two or three bond
C-1 (1')	136.6	137.5 (139.1)		H-7 (H-7') H-5 (H-5')
C-2 (2')	116.0	118.5 (118.3)	6.67 (6.75) H-2 (2')	H-7 (H-7')
C-3 (3')	152.5	150.2 (154.5)		H-5 (H-5')
C-4 (4')	131.3	127.0 (129.1)		H-9 (H-9', 10')
C-5 (5')	126.2	126.6 (126.8)	7.03 (7.17) H-5 (5')	
C-6 (6')	121.6	120.6 (123.3)	6.65 (6.81) H-6 (6')	H-2 (H-2') H-7 (H-7')
C-7 (7')	20.8	21.1 (21.0)	2.30 (2.27) H-7 (7')	
C-8	26.7	81.7		H-9, H-10
C-8'		31.0		H-10, H-9' H-10'
C-9	22.7	28.4	1.69 H-9	
C-9'		32.4	1.42 H-9'	
C-10	22.7	47.9	2.06, 2.54 H-10	H-9, H-9' H-10'
C-10'		33.3	1.18 H-10'	

a) Numbering for **4** was temporarily based on that of **1** in view of the characterization as thymol dimer.

The piscicidal activities of the compounds (1, 2 and 4) were evaluated by the toxicity against the Medaka (*Oryzias latipes*).⁶ Inulavosin (4) exhibited a potent piscicidal activity [median tolerance limit (TL_m) after 24 hr,⁶ 1.3 µg/ml], while thymol (1) and its isobutylate (2) showed weak activities (TL_m 10 and 30 µg/ml, respectively). As thymol is well known as a bactericide and an antiseptic,¹² antibacterial activity of compounds (2 and 4) were also tested. Inulavosin (4) showed a significant antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* at a concentration of 10 µg/ml and 100 µg/ml, respectively. These activities were stronger than that of thymol (1) for each bacterium at the same concentration.¹³

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REFERENCES AND NOTES

1. C. Teedale, *East Afr. Med.*, 1954, **31**, 351.
2. L. Layengi, I. S. Lee, W. Mar, H. H. S. Fong, J. M. Pezzuto, and A. D. Kinghorn, *Phytochemistry*, 1994, **36**, 1523.
3. J. Kokwaro, Medicinal Plants of East Africa, East Africa Literature Bureau, Nairobi, Kenya, 1976.
4. H. J. Arnold and M. Gulumian, *J. Ethnopharmacol.*, 1984, **12**, 35.
5. Y. Ito, S. Yanase, H. Tokuda, M. Kishishita, H. Ohigashi, M. Hirota, and K. Koshimizu, *Cancer Lett.*, 1983, **18**, 87.
6. T. Okuda, T. Yoshida, S. Koike, and N. Toh, *Phytochemistry*, 1975, **14**, 509.
7. S. D. Rosa, A. D. Giulio, and C. Iodice, *J. Nat. Prod.*, 1994, **57**, 1711.
8. E. Gomez, O. D. Cruz-Giron, A. A. Cruz, B. S. Joshi, V. Chittawong, and D. H. Miles, *J. Nat. Prod.*, 1989, **52**, 649.
9. W. Rinn, *Planta Medica*, 1970, **18**, 147.
10. Japanese Pharmacopoeia, 12th ed., General Notices, 1991, p. D-265, B-520, Hirokawa Publishing Co.
11. F. Bohlmann, P. K. Mahanta, J. Jakupovic, R. C. Rastogi, and A. Natsu, *Phytochemistry*, 1978, **17**, 1165, and literatures cited therein.
12. T. Z. Liu, *Clin. Chem.*, 1979, **25**, 336.
13. Details of the antibacterial activity will be published elsewhere.