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

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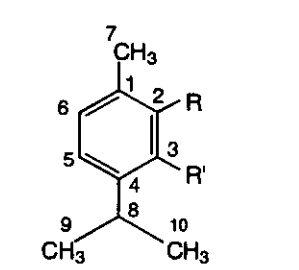
Abstract ---- Upon the fractionation guided by piscicidal activity using the Medaka (*Oryzias latipes*), three piscicidal substances were isolated from the root of *Inula nervosa*, and were characterized as thymol and its derivatives based on the spectral analyses including 2D nmr techniques. A new piscicide,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

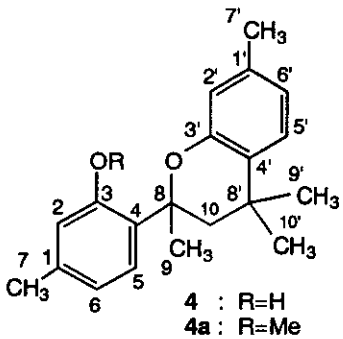
Piscicidal activity has been shown to be useful as a primary screening for biologically active substances from natural sources, as revealed by the fact that piscicides often exhibit other biological activities; for examples, insecticidal,[1] anti-tumor promotion[2] and aphrodisiac[3] activities for rotenoids[4], tumor promotion[5] 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-H_2O 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 $CHCl_3$-acetone followed by preparative tlc (*n*-hexane-$CHCl_3$-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**), $[\alpha]_D \pm 0^o$ ($CHCl_3$), was obtained as a colorless oil, and its EI-ms showed the molecular ion peak at *m/z* 296, corresponding to the molecular formula $C_{20}H_{24}O_2$. The ^1H nmr spectrum (500

MHz, $CDCl_3$) of **4** disclosed five tertiary methyl signals at δ1.18, 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 D_2O). 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 ^{13}C nmr spectrum (126 MHz, $CDCl_3$) exhibited 20 carbon signals which comprise twelve sp^2 and eight sp^3 carbon resonances including those due to five methyl and one methylene group (Table 1). The ^1H-^{13}C COSY spectrum of **4** indicated that two sp^3 carbons are quaternay, 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 $FeCl_3$-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 $(Me)_2SO_4$-K_2CO_3. The chemical shift (δ 8.16) of the hyroxyl 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-^{13}C 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.





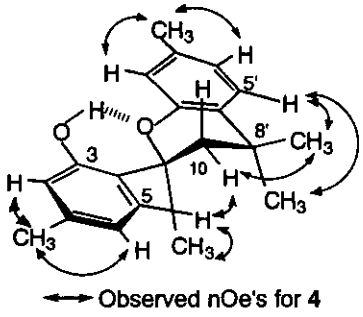


Table 1. Nmr data for thymol (**1**) and inulavosin (**4**) in $CDCl_3$

	1	**4**[a]		
	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 (TLm) after 24 hr,[6] 1.3 μg/ml], while thymol (**1**) and its isobutylate (**2**) showed weak activities (TLm 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 *Staphyllococcus aureus* and *Pseudomonas aeroginosa* 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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13. Details of the antibacterial activity will be published elsewhere.

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