Cholinesterase Inhibitory Constituents from *Onosma hispida*

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Hispidone, a new flavanone, has been isolated from *Onosma hispida* and assigned the structure (2*S*)-5,2′-dihydroxy-7,4′,5′-trimethoxyflavanone (1) by spectroscopic methods. In addition, (2*S*)-5,2′-dihydroxy-7,5′-dimethoxyflavanone (2), benzoic acid (3), and 4-hydroxy benzoic acid (4) are also reported for the first time from this species.

Key words *Onosma hispida*; Boraginaceae; flavanone, cholinesterase inhibitor

The genus *Onosma* (Boraginaceae) comprises about 85 species, occurring mainly in Iran and westward to Syria, Turkey, and Europe.[1)] It is represented in Pakistan by eight species. A literature survey revealed that very little phytochemical work has been carried out on the genus *Onosma* and only some naphthaquinones,[2)] alkaloids[3)] and phenolic compounds[4)] have so far been reported. *Onosma hispida* WALL is perennial herb up to 70 cm tall with a prominent taproot and is widely distributed in northern areas of Pakistan. Medicinally, the plant has cooling, laxative, anthelmintic, and alexipharmic effects and is also used in treating diseases of the eye, disorders of the blood, bronchitis, and abdominal pain.[5)] The plant is used as a dye[6)] and is also utilized as a substitute for alkanet (nchusa tinctoria). Our preliminary pharmacological screening of the methanolic extract revealed inhibitory activity against cholinesterase enzymes. This prompted us to carry out phytochemical studies on this plant. We here report the isolation and structure elucidation of a new flavanone, called hispidone (**1**), along with (2*S*)-5,2′-dihydroxy-7,5′-dimethoxyflavanone (**2**),[7)] benzoic acid (**3**),[8)] and 4-hydroxy benzoic acid (**4**),[9)] isolated for the first time from this species. This is the second report of the isolation of compound **2**, following its earlier isolation from *Eupatorium odoratum*.[7)]

According to the cholinergic hypothesis, memory impairment in patients with senile dementia of the Alzheimer type results from a deficiency in cholinergic function in the brain.[10,11)] Hence the most promising therapeutic strategy for activating central cholinergic function has been the use of cholinergomimetic agents. The enzyme acetyl cholinesterase (AChE) has long been an attractive target for rational drug design and the discovery of mechanism-based inhibitors for the treatment of Alzheimer disease (AD). The aim of administering acetylcholinesterase inhibitors is to boost the endogenous levels of acetylcholine in the brains of AD patients, thereby increasing cholinergic neurotransmission. Recently, it has also been found that butyryl cholinesterase (BChE) inhibition may be an effective tool for the treatment of AD and related dementias.[12)] In the course of this investigation we evaluated compounds **1** and **2** to determine their enzyme inhibitory activity against AChE and BChE. Both compounds were found to be potent cholinesterase inhibitors and inhibited enzymes in a concentration-dependent manner with the IC_{50} values 11.6 and 28.0 μM against AChE and 15.7 and 7.9 μM against BChE, respectively.

Result and Discussions

The methanolic extract of shade-dried whole plant material (10 kg) of *O. hispida* was evaporated *in vacuo*, suspended in H_2O, and successively partitioned with hexane, EtOAc, and BuOH. As a result of a series of column chromatographic techniques compounds **1**—**4** were isolated as described in Experimental.

Hispidone (**1**) was isolated as colorless crystalline solid. The molecular formula was assigned to be $C_{18}H_{18}O_7$ by high-resolution electron-impact (HR-EI)-MS showing an $[M]^+$ ion at *m/z* 346.1050 (Calcd for $C_{18}H_{18}O_7$, 346.1052). The IR spectrum revealed the presence of hydroxyl groups (3452 cm^{-1}), a methoxyl group (1588, 1112 cm^{-1}), and an α,β-unsaturated carbonyl group (1666 cm^{-1}). The UV absorption band at λ_{max} 287 nm suggested it to be flavanone.[13)] The ^{1}H-NMR spectrum provided signals of functional groups including a chelated hydroxyl group (δ 12.09, 1H, s), methoxyl groups [δ 3.78 (3H, s, MeO-7), 3.81 (3H, s, MeO-5′), 3.83 (3H, s, MeO-4′)], and a dihydropyrone moiety in the flavanone structure showing a characteristic ABX-type coupling pattern [δ 2.81 (1H, dd, J=17.2, 3.2 Hz) 3.20 (1H, dd, J=17.2, 13.4 Hz) (H_2-3), 5.72 (1H, dd, J=13.4, 3.2 Hz, H-2)] and two super imposable signals at δ 6.05 (d, J=2.0 Hz, H-8) and 6.07 (d, J=2.0 Hz, H-6) of *meta*-substituted ring A. The two aromatic proton singlets of ring B appeared at δ 6.53 (H-3′) and δ 7.21 (H-6′) on a 2′,4′,5′ trioxygenated substituted ring B.[14)] The broad band and distortionless enhancement by polarization transfer ^{13}C-NMR spectra of **1** corroborated the presence of three methyl, one methylene, five methane, and nine quaternary carbons. The signals at δ 75.8 and δ 43.2 were typical of C-2 and C-3 carbons of the flavanone skeleton. EI-MS gave a distinct peak at *m/z* 346 $[M]^+$ and further two fragment ions were observed at *m/z* 167 ($[A+H]^+$) and *m/z* 180 ($[B_3]^+$) which were caused by retro-Diels–Alder fragmentation, confirming the one hydroxyl and methoxyl group in ring A, and one hydroxyl and two methoxyl groups in ring B of the flavanone skeleton.[14)] The position of the hydroxyl and methoxyl groups was further confirmed by heteronuclear multiple-quantum coherence (HMBC) and nuclear Overhauser effect correlations[14)] (Fig. 1). Dihydroflavanones are reported to have the S configuration at C-2,[15)] which could be confirmed by the CD spectrum which showed similar Cotton effects as reported in the literature.[15,16)] On the basis of the above evidence, the structure of **1** was determined to be 2*S*-(5,2′-dihydroxy-7,4′,5′-trimethoxyflavanone.

Compounds **1** and **2** were subjected to enzyme inhibition

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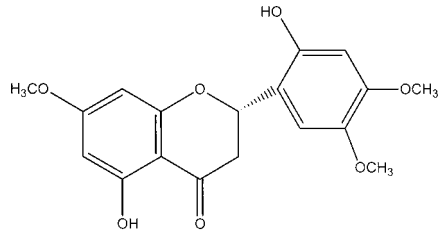

1

Fig. 1

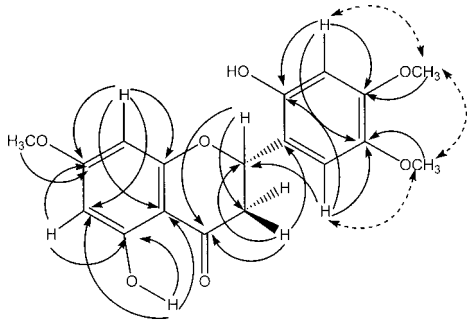

Fig. 2

Table 1. *In Vitro* Quantitative Inhibition of AChE and BChE by Compounds **1** and **2**

Compounds	AChE IC_{50} (μM)	BChE IC_{50} (μM)
1	11.6±0.6	15.7±2.0
2	28.0±5.0	7.9±1.6
Galanthamine	32.2±2.5	163.0±5.0

IC_{50} values are the mean±standard error mean (S.E.M.) of three assays.

screening. Compound **2** was more selective for BChE, whereas compound **1** showed significant inhibitory action against AChE but the galanthamine positive control was approximately 5 times more potent against AChE (Table 1).

Experimental

General Optical rotations were measured on a JASCO DIP-360 digital polarimeter. IR spectral data were measured using a JASCO 302-A spectrophotometer with $CHCl_3$. UV spectra were obtained on a Hitachi UV-3200 spectrophotometer and NMR spectra on Bruker spectrophotometers, at 400 MHz. Chemical shifts δ in ppm relative to $SiMe_4$ as an internal standard and coupling constant J are given in Hz. EI-, FAB-, and HR-EIMS were recorded on JEOL JMS-HX-110 and JMS-DA-500 mass spectrometers, *m/z* (ret. int). Silica gel 60, 200—440 mesh (E. Merck), was used for column chromatography. Silica gel plates (Si 60 F_{254}, E. Merck) were used for TLC.

Plant Material The whole plant of *O. hispida* WALL (Boraginceae) was collected from Swat (Pakistan) in 2002 and identified by Dr. Jahndar Shah, Plant Taxonomist, Islamia College Peshawar. A voucher specimen was deposited in the herbarium of the University of Peshawar.

Extraction and Isolation The shade-dried plant (10 kg) was chopped and soaked in MeOH for 10 d, extracted three times at room temperature in the same solvent and filtered. The filtrate was evaporated *in vacuo* to give a dark-greenish residue (500 g), which was suspended in water and partitioned successively with hexane, $CHCl_3$, EtOAc, and BuOH to obtain hexane-soluble, $CHCl_3$-soluble, EtOAc-soluble, and BuOH-soluble fractions, respectively. The $CHCl_3$-soluble fraction was subjected to column chromatography using hexane–$CHCl_3$ in increasing order of polarity to give six fractions. The major fraction obtained from hexane–$CHCl_3$ (6 : 4) was further purified by column chromatography on silica gel using hexane–$CHCl_3$ (4 : 6) as a solvent system to afford compound **2** (14 mg). The fraction obtained from hexane–$CHCl_3$ (1 : 1) was subjected to column chromatography on silica gel using hexane–$CHCl_3$ (3 : 7) to obtain crystals of compound **1** (8 mg), which were finally washed with hexane to remove colored impurities. Compounds **3** (20 mg) and **4** (16 mg) were obtained through elution with hexane–$CHCl_3$ (8 : 2) followed by further purification through column chromatography over silica gel using hexane–EtOAc (9 : 1) as the eluent.

Hispidone (**1**): Crystalline solid, mp 192—193 °C. ^{1}H-NMR (acetone-d_6, 400 MHz): δ 12.09 (1H, s, OH-5), 7.21 (1H, s, H-6′), 6.53 (1H, s, H-3′), 6.07 (1H, d, J=2.0 Hz, H-6), 6.05 (1H, d, J=2.0 Hz, H-8), 5.72 (1H, dd, J=13.4, 3.2 Hz, H-2), 3.83 (3H, s, MeO-4′), 3.81 (3H, s, MeO-5′), 3.78 (3H, s, MeO-7), 3.20 (1H, dd, J=17.2, 13.4 Hz, H-3_{ax}), 2.81 (1H, dd, J=17.2, 3.2 Hz, H-3_{eq}). ^{13}C-NMR (acetone-d_6, 100 MHz) δ: 197.6 (s, C-4), 167.5 (s, C-7), 164.4 (s, C-5), 163.1 (s, C-9), 150.2 (s, C-4′), 147.2 (s, C-2′), 144.6 (C-5′), 117.2 (s, C-1′), 113.6 (d, C-6′), 104.9 (d, C-3′), 103.3 (s, C-10), 95.4 (d, C-6), 94.8 (d, C-8), 75.8 (d, C-2), 57.3, 56.7 (q, MeO-5′ and 4′), 55.2 (q, MeO-7), 43.2 (t, C-3). UV λ_{max} (MeOH) nm (log ε): 287 (4.31), 340 sh (3.82). CD: $\Delta\varepsilon_{282}$ −5.8, $\Delta\varepsilon_{310}$ +2.9. FAB-MS (positive) *m/z*: 347 $[M+H]^+$. EI-MS *m/z* 346 $[M]^+$ (70), 315 (8), 180 (64), 167 (100). IR ν_{max} ($CHCl_3$) cm^{-1}: 3452, 2932, 1666, 1638, 1588, 1112. $[\alpha]_D^{25}$ −8.5° (c=0.11, MeOH).

Compound **2**: Crystalline solid, mp 192—193 °C. $[\alpha]_D^{25}$=−8.5° (c=0.11, MeOH). HR-EI-MS *m/z*: 316.0939 (Calcd for $C_{17}H_{16}O_6$, 316.0946). The spectral data were in complete agreement with those reported in the literature for (2*S*)-5,2′-dihydroxy-7,5′-dimethoxyflavanone.[7]

***In Vitro* Cholinesterase Inhibition Assay** AChE- and BChE-inhibiting activities were measured by slightly modifying the spectrophotometric method developed by Ellman *et al.*[17] Electric-eel AChE (type VI-S, Sigma), and horse-serum BChE (Sigma) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma), respectively, were used as substrates of the reaction. 5,5′-Dithiobis [2-nitrobenzoic acid] (DTNB, Sigma) was used for the measurement of cholinesterase activity. A mixture of 140 μl of 0.1 mM sodium phosphate buffer (pH 8.0), 10 μl of DTNB, 20 μl of test compound solution, and 20 μl of AChE or BChE solution was incubated for 15 min at 25 °C. The reaction was then initiated by the addition of 10 μl of acetylthiocholine or butyrylthiocholine, respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively, at a wavelength of 412 nm. Test compounds and the control were dissolved in 5% EtOH. All the reactions were performed in triplicate.

Estimation of IC_{50} Values The concentrations of test compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC_{50}) were determined by monitoring the effect of increasing concentrations of these compounds in assays of the inhibition values. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, MA, U.S.A.).

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