Isolation of Onosmins A and B, Lipoxygenase Inhibitors from *Onosma hispida*

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Onosmins A (1) and B (2), lipoxygenase inhibitors, have been isolated from *Onosma hispida*. Their structures were established as 2-[(4-methylbenzyl)amino]benzoic acid (1) and methyl 2-[(4-methylbenzyl)amino]benzoate (2) through spectroscopic studies, including 2D-NMR. The known compounds apigenin (3), 6,4′-dimethoxy-3,5,7-trihydroxyflavone (4), 6,7-dimethoxy-3,5,4′-trihydroxyflavone (5) and apigenin 7-*O*-β-D-glucoside (6) are also reported for the first time from this species. Compounds (1) and (2) inhibited lipoxygenase (LOX, EC 1.13.11.12) enzyme in a concentration-dependent fashion with IC_{50} values of 24.0 and 36.2 μM, respectively. Lineweaver–Burk as well as Dixon plots and their secondary replots indicated that the nature of inhibition was purely a non-competitive type, with K_i values 22.0 μM and 31.1, respectively.

Key words *Onosma hispida*; Boraginaceae; lipoxygenase inhibiting constituent; kinetics

The genus *Onosma* belongs to the family Boraginaceae. It consists of 85 species, occurring mainly in Iran and westward to Syria, Turkey and Europe. It is represented in Pakistan by 8 species.[1] *Onosma hispida* Wall. is a perennial herb up to 70 cm tall with a prominent taproot, it is widely distributed in the northern areas of Pakistan. The plant has cooling, laxative, anthalminitic and alexipharmic effects, and is also used to treat diseases of the eye, derangements of the blood, bronchitis, abdominal pain, stangury, thirst, itch, leucoderma, fever, wounds, piles and in urinary calculi.[2] Its flowers are prescribed as a stimulant and cardiac tonic in rheumatism and diseases of heart.[3] The plant is used as a dye for wool, hair and also imparts a rich red colour to medicinal oil and fats.[4]

Previously, aliphatic ketone,[5] lipids,[6] naphthazarins,[7] alkaloids,[8] shikonin,[9] phenolic constituents[10] and naphthoquinones[11] have been reported from the genus *Onosma*. Our previous work on *O. hispida* has resulted in the isolation of cholinesterase inhibitory flavanone.[12] However, the crude ethanolic extract and the subsequent hexane fraction also showed lipoxygenase inhibitory activity. This prompted us to carry out further phytochemical studies on this plant. Herein we report the isolation and structural elucidation of onosmins A (1) and B (2), lipoxygenase inhibitors, along with the known compounds apigenin (3),[13] 6,4′-dimethoxy-3,5,7-trihydroxyflavone (4),[14] 6,7-dimethoxy-3,5,4′-trihydroxyflavone (5)[15] and apigenin 7-*O*-β-D-glucoside (6).[16]

Lipoxygenases (LOX, EC 1.13.11.12) constitute a family of non-haem iron containing dioxygenases that are widely distributed in animals and plants. The non-haem iron serves as a catalytic center for the stereo- and regio-specific dioxygenation of select carbon atoms in polyunsaturated fatty acids containing a 1,4-pentadiene motif. Linoleic acid is the primary substrate of the plant LOXs, while the mammalian isozymes mainly catalyze the metabolism of arachidonic acid. In mammalian cells, LOX are key enzymes in the biosynthesis of many bioregulatory compounds such as hydroxyeicosatetraenoic acids (HETEs), leukotrienes, lipoxins and hepoxylines.

It has been found that these LOX products play a role in a variety of disorders such as bronchial asthma and inflammation,[17] and also have a profound influence on the development of several human cancers.[18] LOX are therefore a potential target for the rational drug design and discovery of mechanism-based inhibitors for the treatment of bronchial asthma, inflammation, cancer and autoimmune diseases. Compounds **1** and **2** showed significant inhibitory activity against lipoxygenase enzyme.

Results and Discussion

The ethanolic extract of the shade-dried whole plant material (10 kg) of *O. hispida* was evaporated *in vacuo*, then the residue was suspended in H_2O and successively partitioned with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH. Onosmins A (**1**) and B (**2**) were isolated from the *n*-hexane-soluble fraction, while compounds **3**—**6** were obtained from the $CHCl_3$-soluble fraction of *O. hispida* by successive use of column chromatography.

Onosmin A (**1**) was isolated as a white amorphous solid. The molecular ion peak at *m/z* 241.1100 in the high resolution electron-impact (HR-EI)-MS indicated its molecular formula to be $C_{15}H_{15}NO_2$ (Calcd 241.1103). The UV spectrum in MeOH showed λ_{max} at 343 nm (log ε 3.78). Absorption bands in the IR spectrum of **1** suggested the presence of a carboxylic acid (1680 cm^{-1}), amine (3450 cm^{-1}) and aromatic ring system (1610, 1500, 1460 cm^{-1}). It gave effervescence with dilute sodium carbonate solution, confirming the presence of a carboxylic acid group.

Distortionless enhancement by polarization transfer (DEPT) experiments revealed the presence of one methyl, one methylene, six methine and five non-protonated carbons. The downfield signal at δ 168.7 could be assigned to the acid carbonyl carbon. The methyl and methylene carbons were observed at δ 20.3 and δ 49.1, respectively. The signals at δ 148.9, 138.5, 135.9, 133.7, 130.3, 130.3×2, 127.2×2, 116.9, 113.5 and 111.2 showed the presence of aromatic rings. In the ^{1}H-NMR there were eight aromatic protons which could be assigned to 1,2- and 1,4-disubstituted phenyl rings on the

basis of ^{1}H–^{1}H COSY spectrum and the coupling patterns. The ^{1}H-NMR displayed a pair of *ortho*-coupled A$_2$B$_2$ type signals at δ 7.04 and δ 6.29 (each 2H, d, J=8.1 Hz), indicating the presence of a 1,4-disubstituted benzene ring. It also showed a dd at δ 6.90 (J=7.9, 1.5 Hz, H-6), dd at δ 8.0 (J=8.6, 1.5 Hz, H-3), and a pair of ddd at δ 7.27 (J=8.6, 7.2, 1.5 Hz, H-4) and δ 6.67 (J=7.9, 7.2, 1.5 Hz, H-5), respectively, confirming the presence of a 1,2-disubstituted benzene ring. The signals at δ 2.28 (3H, s), 4.46 (2H, br s) and 8.30 (1H, br s) could be assignable to aromatic methyl, benzylic protons and an amine, respectively. In the heteronuclear multiple-bond coherence (HMBC) spectrum (Fig. 1), the benzylic protons (H-7′, δ 4.46) showed ^2J correlation with C-1′ (δ 138.5) and ^3J correlations with C-2′, C-6′ (δ 127.2×2) and C-2 (δ 148.9), thus confirming that the secondary amine nitrogen combined with a benzyl group and a phenyl ring. The H-6 at δ 6.90 showed ^3J correlations with C-2 (δ 148.9), C-4 (δ 133.7) and C-7 (δ 168.7). The methyl protons at δ 2.28 showed ^2J correlation with C-4′ (δ 135.9) and ^3J correlations with C-3′, C-5′ (δ 130.3×2), respectively. On the basis of these, onosmin A (1) was assigned the structure 2-[(4-methylbenzyl)amino]benzoic acid.

Onosmin B (2) was also obtained as a white amorphous solid. It was assigned the molecular formula C$_{16}$H$_{17}$NO$_2$ (m/z 255.1255, Calcd 255.1259) by HR-EI-MS. The UV spectrum in MeOH showed λ_{max} at 353 nm (log ε 4.11). It showed the presence of an ester (1720 cm^{-1}), amine (3470 cm^{-1}) and aromatic ring system (1600, 1510, 1455 cm^{-1}) in its IR spectrum. The ^{1}H- and ^{13}C-NMR spectra were found to be similar to those of 1 (Experimental), except for an additional signal

due to a methoxyl group at δ 3.90 and at δ 51.6, respectively. The absence of a free carboxylic group indicated that 2 is the corresponding methyl ester of 1. Conclusive evidence was provided by HMBC experiments. Besides the interactions previously described for 1, a ^3J correlation was observed by carbomethoxy protons at δ 3.90 and C-7 (δ 169.1). Thus, onosmin B (2) was assigned the structure methyl 2-[(4-methylbenzyl)amino]benzoate. Although 1 and 2 are a rare

1. R = H
2. R = CH$_3$
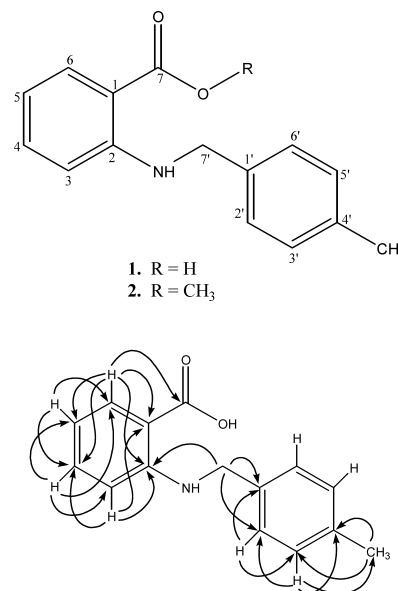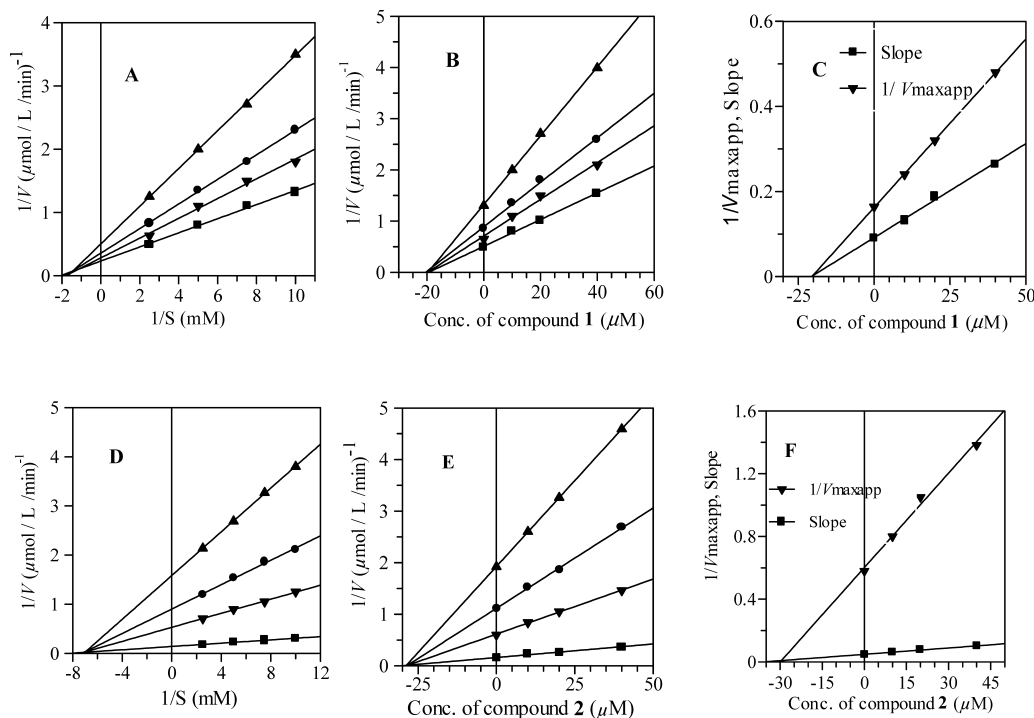

Fig. 1. Important HMBC Correlations of 1

Fig. 2. Steady-State Inhibition of Lipoxygenase Enzyme by Compounds 1 and 2

(A) is the Lineweaver–Burk plot of the reciprocal of initial velocities *versus* the reciprocal of four fixed linoleic acid (substrate) concentrations in the absence (■) and presence of 10 μM (▼), 20 μM (●), 40 μM (▲) of compound 1. (B) is the Dixon plot at four fixed linoleic acid concentrations, (■) 0.2 mM, (▼) 0.1 mM, (●) 0.066 mM and (▲) 0.05 mM. (C) is the secondary replots of the Lineweaver–Burk plot, 1/V_{maxapp} or slope *versus* various concentrations of compound 1. (D) is the Lineweaver–Burk plot of the reciprocal of initial velocities *versus* reciprocal of four fixed linoleic acid (substrate) concentrations in the absence (■) and presence of 10 μM (▼), 20 μM (●), 40 μM (▲) of compound 2. (E) is the Dixon plot at four fixed linoleic acid concentrations, (■) 0.2 mM, (▼) 0.1 mM, (●) 0.066 mM and (▲) 0.05 mM. (F) is the secondary replots of the Lineweaver–Burk plot, 1/V_{maxapp} or slope *versus* various concentrations of compound 2. The correlation coefficient for all the lines of all the graphs was >0.99. Each point in the graphs represents the mean of three experiments.

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

Compounds	$IC_{50} \pm$ S.E.M.[b] [μM]	$K_i{}^{a)} \pm$ S.E.M. [μM]	Type of inhibition
1	24.2 ± 0.04	22.0 ± 0.1	Non-competitive
2	36.0 ± 0.03	31.1 ± 0.05	Non-competitive
Bacalein[c]	22.0 ± 0.05	18.0 ± 0.02	Mixed-type

a) K_i (dissociation constant or inhibition constant) was determined from nonlinear regression analysis by Dixon plot and secondary Lineweaver–Burk plot at various concentrations of **1**, **2**. (Each point in Lineweaver–Burk and Dixon plots and their replots represents the mean of three determinations.) *b)* Standard mean error of 3—5 assays. *c)* Positive control used in assays.

class of compounds which have not previously been isolated from the genus *Onosma*, related compounds have been reported from natural resources.[19—21] Moreover, these are genuine natural products, as their presence has been confirmed in two further batches of fresh plant material.

Compounds **1** and **2** inhibited LOX enzyme in a concentration-dependent manner with K_i values 22.0 and 31.1 μM and IC_{50} values 24.2 and 36.0 μM, respectively (Table 1). K_i values were calculated in three ways: first, the slopes of each line in the Lineweaver–Burk plot were plotted against different concentrations of **1** and **2**. Secondly, the $1/V_{\text{maxapp}}$ was calculated by plotting different fixed concentrations of linoleic acid versus ΔV in the presence of different fixed concentrations of **1** and **2**, and then K_i was calculated as an intercept on the *x*-axis by plotting different concentrations of **1** and **2** *versus* $1/V_{\text{maxapp}}$. In the third method, K_i was directly measured from the Dixon plot as an intercept on the *x*-axis. Determination of the inhibition type is critical for identifying the mechanism of inhibition and the sites of inhibitor binding. Lineweaver–Burk and Dixon plots and their replots indicated pure non-competitive type inhibition of **1** and **2** against LOX enzyme. In other words, we can say that **1** and **2** and linoleic acid bind randomly and independently at the different sites of LOX. This indicates that the inhibition depends only on the concentrations of **1** and **2** and the dissociation constant (K_i).

From the results (Table 1), it is clear that **1** containing a benzoic acid pharmachophore possesses stronger ($K_i = 22.0 \mu$M) binding strength against LOX than **2** containing methyl benzoate moiety ($K_i = 31.1 \mu$M). The higher inhibitory potential of **1** may also be due to the presence of –COOH, which has a stronger electron withdrawing effect than –COOCH$_3$, which can thus convert the active state Fe^{2+} of LOX into inactive Fe^{3+} more efficiently.

Conclusion

In conclusion, our search for lipoxygenase inhibitory constituents from *O. hispida* has resulted in the isolation of new compounds which may find use in treating inflammation, asthma, aging, tumor angiogenesis and cancer. However, further *in vivo* study would help in exploring the pharmacological properties of these compounds.

Experimental

General Experimental Procedure IR spectra were recorded on a 460 Shimadzu spectrometer. EI-MS was recorded on JMS-HX-110 with a data system and on JMS-DA500 mass spectrometers. The ^{1}H- and ^{13}C-NMR, HMQC, and HMBC spectra were recorded on Bruker NMR spectrometers operating at 400 MHz for ^{1}H- and 100 MHz for ^{13}C-NMR, respectively. The chemical shift values are reported in ppm (δ) units, and the coupling constants (J) are in Hz. Aluminum sheets precoated with silica gel 60 F$_{254}$ (20×20 cm, 0.2 mm thick; E-Merck) were used for TLC, and silica gel

(230—400 mesh E. Merck) was used for column chromatography.

Plant Material *Onosma hispida* WALL. (Boraginaceae), whole plant was collected from Swat (Pakistan) in 2002 and identified by Dr. Jahander Shah, Plant Taxonomist, Islamia College Peshawar. A voucher specimen (No. BPU-55) has been deposited in the herbarium of the University of Peshawar.

Extraction and Isolation The dried whole plant material (10 kg) was extracted three times with ethanol at room temperature. The ethanol extract was evaporated under reduced pressure to afford a dark-greenish residue which was suspended in water and successively extracted with *n*-hexane, CHCl$_3$, EtOAc and *n*-BuOH. The *n*-hexane fraction was subjected to column chromatography, and eluted with *n*-hexane–CHCl$_3$ in increasing order of polarity to provide four fractions. The fraction obtained from *n*-hexane–CHCl$_3$ (6:4) was rechromatographed over flash silica gel using *n*-hexane–CHCl$_3$ (9:1—1:1) as a solvent system to give two successive fractions. The second fraction afforded compound **1** (10 mg) by silica gel column chromatography using *n*-hexane–CHCl$_3$ (8:2) as an eluent. The first fraction, which was a mixture of two compounds, was purified through column chromatography over silica gel using *n*-hexane–CHCl$_3$ (9:1) to afford compound **2** (13 mg). The CHCl$_3$ fraction was subjected to column chromatography over silica gel, then successively eluted with *n*-hexane–CHCl$_3$ in increasing order of polarity to obtain six fractions. The fraction which was eluted with *n*-hexane–CHCl$_3$ (3:7) showed two major and two minor spots on TLC. It was further subjected to column chromatography (silica gel) using *n*-hexane–CHCl$_3$ (1:1) to afford compound **3** (12 mg), and *n*-hexane–chloroform (4:6) to obtain compound **4** (15 mg). The fraction which was eluted with *n*-hexane–CHCl$_3$ (1:9) showed two major spots on TLC. It was further subjected to column chromatography (silica gel) using *n*-hexane–CHCl$_3$ (3:7) as an eluent to afford compound **5** (14 mg), and *n*-hexane–CHCl$_3$ (1:9) to provide **6** (18 mg), respectively.

Onosmin A (**1**): White amorphous solid. mp 185—187 °C. UV λ_{max} (MeOH): 343, 284 nm. IR ν_{max} (KBr): 3450, 1680, 1610, 1500, 1460 cm^{-1}. ^{1}H-NMR (CDCl$_3$, 400 MHz) δ: 8.30 (1H, br s, N–H), 8.0 (1H, dd, $J=8.6$, 1.5 Hz, H-3), 7.27 (1H, ddd, $J=8.6$, 7.2, 1.5 Hz, H-4), 7.04 (2H, d, $J=8.1$ Hz, H-2′,6′), 6.90 (1H, dd, $J=7.9$, 1.5 Hz, H-6), 6.67 (1H, ddd, $J=7.9$, 7.2, 1.5 Hz, H-5), 6.29 (2H, d, $J=8.1$ Hz, H-3′,5′), 4.46 (2H, br s, H$_2$-7′), 2.28 (3H, s, CH$_3$-4′). ^{13}C-NMR (CDCl$_3$, 100 MHz) δ: 168.7 (C-7), 148.9 (C-2), 138.5 (C-1′), 135.9 (C-4′), 133.7 (C-4), 130.3 (C-3′,5′), 130.0 (C-3), 127.2 (C-2′,6′), 116.9 (C-5), 113.5 (C-6), 111.2 (C-1), 49.1 (C-7′), 20.3 (CH$_3$-4′). EI-MS *m/z*: 241 [M$^+$], 196, 136, 121, 105, 45. HR-EI-MS *m/z*: 241.1100 [M$^+$] (C$_{15}$H$_{15}$NO$_2$ Calcd 241.1103).

Onosmin B (**2**): White amorphous solid. mp 137—140 °C. UV λ_{max} (MeOH): 353, 275 nm. IR ν_{max} (KBr): 3470, 1720, 1600, 1510, 1455 cm^{-1}. ^{1}H-NMR (CDCl$_3$ 400 MHz) δ: 8.10 (1H, br s, N–H), 7.90 (1H, dd, $J=8.0$, 1.6 Hz, H-3), 7.24 (1H, ddd, $J=8.0$, 7.6, 1.6 Hz, H-4), 7.06 (2H, d, $J=8.0$ Hz, H-2′,6′), 6.70 (1H, dd, $J=7.9$, 1.5 Hz, H-6), 6.65 (1H, ddd, $J=7.9$, 7.6, 1.6 Hz, H-5), 6.26 (2H, d, $J=8.0$ Hz, H-3′,5′), 4.48 (2H, br s, H$_2$-7′), 3.90 (3H, s, OCH$_3$-7), 2.30 (3H, s, CH$_3$-4′). ^{13}C-NMR (CDCl$_3$, 100 MHz) δ: 169.1 (C-7), 149.4 (C-2), 138.7 (C-1′), 135.3 (C-4′), 134.1 (C-4), 131.4 (C-3), 130.7 (C-3′,5′), 126.5 (C-2′,6′), 116.0 (C-5), 113.6 (C-6), 110.7 (C-1), 51.6 (OCH$_3$), 48.9 (C-7′), 20.6 (CH$_3$-4′). EI-MS *m/z*: 255 [M$^+$], 196, 150, 134, 105, 59. HR-EI-MS *m/z*: 255.1255 [M$^+$] (C$_{16}$H$_{17}$NO$_2$ Calcd 255.1259).

Lipoxygenase Inhibition Assay LOX inhibiting activity was measured by modifying the spectrophotometric method developed by Tappel.[22] Lipoxygenase (1.13.11.12) type I-B (Soybean) and linoleic acid was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade. The assay conditions and protocol were the same as described in our previous article.[23] All kinetic study was performed in 96-well microtitre plates using SpectraMax 384plus (Molecular Devices, CA, U.S.A.). The IC_{50} values were then calculated using the EZ-Fit Enzyme kinetics program

(Perrella Scientific Inc., Amherst, MA, U.S.A.). The percentage (%) inhibition was calculated as follows $(E-S)/E \times 100$, where E is the activity of the enzyme without test compound and S is the activity of enzyme with test compound.

Determination of Kinetic Parameters Dissociation constant/inhibition constant (K_i) was determined by the interpretation of a Dixon plot,[24] Lineweaver–Burk plot,[25] and their secondary replots using initial velocities obtained over a substrate (linoleic acid) concentration range between 0.05—0.2 mM. The dependence of V_{max}/K_m and V_{max} on **1**—**2** [I] is given by:

$$V_{max}/K_m = \left(\frac{(V_{max}/K_m)K_I}{K_I+[I]} \right) \Rightarrow I = \frac{K_I}{K_I+[I]} \Rightarrow K_I = K_I+[I]$$

Statistical Analysis Graphs were plotted using a GraFit program.[26] Values of the correlation coefficients, slopes, intercepts and their standard errors were obtained by linear regression analysis using the same program. Correlation for all the lines of all graphs was found to be >0.99. Each point in the constructed graphs represents the mean of three experiments.

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