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Lipoxygenase Inhibitory Constituents from *Indigofera oblongifolia*

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Indigin, alkylated xanthene (**1**) and indigoferic acid (**2**) have been isolated from the chloroform soluble fraction of *Indigofera oblongifolia*, along with β-sitosterol (**3**) and 3-hydroxybenzoic acid (**4**), which are reported for the first time from this species. Their structures were determined through spectroscopic techniques. Both the new compounds **1** and **2** showed significant activity against enzyme lipoxygenase, while **2** further showed moderate inhibition against BChE.

Key words: *Indigofera oblongifolia*, Fabaceae, Lipoxygenase inhibition

INTRODUCTION

Indigofera oblongifolia Forsk belongs to the family Fabaceae and is widely distributed in Asia and Africa. Its leaves are used in folk medicines in urinary tract infection, urolithiasis, cough and skin infection (Ali, 2001). Later these were also reported to posses antimicrobial activity (Dahot, 1999). No phytochemical work on this species has so far been reported. A methanolic extract of the whole plant showed strong cytotoxicity in brine shrimp lethality test. On further fractionation the major cytotoxicity was located in $CHCl_3$ fraction. This prompted us to carry out phytochemical studies on the $CHCl_3$ fraction of *I. oblongifolia* which resulted in the isolation and structure elucidation of indigin, a novel alkylated xanthene (**1**) and indigoferic acid, the fatty acid ester of *p*-hydroxy(*E*)-cinnamic acid (**2**), respectively. In addition β-sitosterol and 3-hydroxybenzoic acid have also been reported from this species for the first time.

MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured on a JASCO DIP-360 polarimeter. Melting points were determined on Buchi melting point apparatus and are uncorrected. UV spectra were recorded on Hitachi U-3200 spectrophotometer. IR spectra were recorded on FTIR-8900 Shimadzu spectrometer. The ^{1}H-, ^{13}C-NMR, HMQC, and HMBC spectra were recorded on Bruker spectrometers operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C-NMR, respectively. The chemical shift values are reported in ppm (δ) units and the coupling constants (*J*) are in Hz. MS and HR-MS were obtained on a JMS-HX-110 with a data system on JMS-DA 500 mass spectrometers. Aluminum sheets precoated with silica gel 60 F_{254} (20×20 cm, 0.2 mm thick; E-Merck) were used for TLC and flash silica gel (230-400 mesh) was used for column chromatography. Visualization of the TLC plates was carried out under UV at 254 and 366 nm and also by spraying ceric sulphate reagent with heating.

Materials

The whole plant of *I. oblongifolia* Forsk (Fabaceae) was collected in May 2002 from Hyderabad (Pakistan) and identified by Dr. G. R. Bhatti, Plant Taxonomist, Department of Botany, Shah Abdul Latif University, Khairpur, where a voucher specimen (SM 1130) has been deposited.

Extraction and isolation

Whole plant of *I oblongifolia* Forsk (was shade dried, ground and extracted with methanol (3×60 L). The combined methanolic extract (1 kg) was partitioned between

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n-hexane and water. The aqueous fraction further extracted out with chloroform and ethylacetate. The $CHCl_3$ fraction (50 g) was subjected to column chromatography over silica gel using *n*-hexane-$CHCl_3$, $CHCl_3$, $CHCl_3$-MeOH, and MeOH as eluents in increasing order of polarity. The fraction obtained from *n*-hexane : $CHCl_3$ (3.5:6.5) was a mixture of two components which were separated by column chromatography over silica gel using solvent system *n*-hexane : EtOAc (1:1) to afford compounds 3-hydroxy benzoic acid (27 mg) from the top fractions and **2** (18 mg) from the tail fractions. The fraction obtained from *n*-hexane : $CHCl_3$ (2:8) was also a mixture of two components which were separated by column chromatography using solvent system *n*-hexane : EtOAc (2.8 : 7.2), collecting 10 mL fraction in each case, to obtain compound β-sitosterol (43 mg) from fractions 20-30 and **1** (16 mg) from fractions 60-70.

The structure of known compounds was done by spectroscopic studies and by comparison of their physical and spectral data with those reported in literature (Bernard, 1977), (Aldrich Library of ^{13}C- and ^{1}H-NMR Spectra, 1992).

Indigin (1)

White gummy solid, UV λ_{max}^{MeOH} nm: 225, 269, 318; IR ν_{max}^{KBR} cm^{-1}: 3583, 2960, 2835, 1628, 1435, 1235, 1065, 997, 935. $[\alpha]_D^{25}$ –69.5(c = 0.025); HREIMS *m/z*: 440.3640 (calcd. for $C_{30}H_{48}O_2$: 440.3654); ^{1}H-NMR (500 MHz, $CDCl_3$+CD_3OD; 9.5:0.5) δ: 1.02 (3H, s, H-13), 1.22-1.27 (30H, br s, H-14-H-29), 1.55 (1H, m, H-1), 1.70 (1H, m, H-1), 2.19 (2H, m, H-2), 2.20 (1H, m, H-9), 2.70 (1H, m, H-9), 3.60 (2H, t, *J* = 6.8 Hz, H_2-29), 4.33 (1H, m, H-4a), 5.30 (1H, m, H-4), 6.80 (1H, m, H-5), 7.02 (2H, m, H-6 and H-7); 7.10 (1H, m, H-8), ^{13}C-NMR (125 MHz, $CDCl_3$ + CD_3OD) δ: 31.9 (C-1), 22.7 (C-2), 146.5 (C-3), 119.0 (C-4), 77.2 (C-4a), 116.1 (C-5), 127.1 (C-6), 119.5 (C-7), 129.1 (C-8), 33.0 (C-9), 29.9 (C-9a), 152.8 (C-11), 121.5 (C-12), 17.0 (C-13), 29.9 (C-14), 24.9-29.7 (C-15-C-27), 33.6 (C-28), and 62.1 (C-29).

Indigoferic acid (2)

White amorphous powder, m.p. 70–72°C. UV $\lambda_{max}^{CHCl_3}$ nm: 204, 226, 307; IR ν_{max}^{KBR} cm^{-1}: 2925, 2286, 1700, 1615. HREIMS *m/z*: 472.3535 (calcd. for $C_{30}H_{48}O_4$: 472.3552); ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.88 (3H, t, *J* = 6.4 Hz, H-21"), 1.25-1.27 (34H, br s, H-4"-H-20"), 1.64 (2H, m, H-3"), 2.40 (2H, t, *J* = 7.2 Hz, H-2"), 6.29 (1H, d, *J* = 15.8 Hz H-2'), 6.79 (2H, d, *J* = 8.2 Hz, H-2 and H-6), 7.44 (2H, d, *J* = 8.2 Hz, H-3 and H-5), 7.57 (1H, d, *J* = 15.8 Hz, H-1'). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 162.5 (C-1), 115.7 (C-2), 130.9 (C-3), 126.1 (C-4), 130.9 (C-5), 115.7 (C-6), 146.1 (C-1'), 115.6 (C-2'), 171.2 (C-3'), 177.1 (C-1"), 34.0 (C-2"), 25.1 (C-3"), 29.1–30.6 (C-4"–C-20"), 14.5 (C-21").

Methanolysis of 2

Sodium metal (0.5 g) was added to the freshly distilled dry methanol (25 mL) with stirring in an ice bath. When all the sodium metal was dissolved, **2** (5 mg) was added and left overnight at r.t. under nitrogen. Methanol was removed and the yellow gum thus obtained was diluted with water, and extracted with ethyl acetate. The organic layer was washed several times till neutral pH, dried over anhydrous sodium sulphate and freed from solvent. The GCMS of the resulting fatty acid methyl ester gave $[M]^+$ peak at *m/z* 340 corresponding to the formula $C_{22}H_{44}O_2$ [methyl heneicosanoate (b.p .207-208°C; lit. b.p 207°C)].

In vitro enzyme inhibition activity

Lipoxygenase inhibition assay

LOX inhibiting activity was measured by modifying the spectrophotometric method developed by *A. L. Tappel* (Tappel, 1962). 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 was the same as described previously (N. Mukhtar *et al.*, 2004). All the inhibition study was performed in 96-well micro-titre plates by using *SpectraMax 384plus* (Molecular Devices, U.S.A.).

Cholinesterase inhibition assay

Electric-eel (*Torpedo califomica*) AChE (type VI-S), horse-serum BChE, acetylthiocholine iodide, butyrylthiocholine chloride, 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB), galanthamine and eserine were purchased from Sigma (St. Louis, MO, U.S.A.). Buffer and other chemicals were of analytical grade. AChE and BChE inhibiting activities were measured by the spectrophotometric method developed by *Ellman et al.* (Ellman *et al.*, 1961). All the inhibition studies were performed in 96-well microtiter-plates using *SpectraMax 340* (Molecular Devices, CA, U.S.A.). Assay protocol and conditions were the same, as mentioned previously (Riaz *et al.*, 2004).

According to Ellman *et al* [Ellman *et al.*, 1961) since the extinction coefficient of the yellow anion is known, the rate of the enzymatic reaction can be determined by the following equation:

$$\text{Rate(mol/L/min.)} = \frac{\text{Change in absorbance/min.}}{13{,}600}$$

Determination of IC_{50} values

The percentage (%) inhibition was calculated as follows (E – S)/E×100, where E is the activity of the enzyme without test compound and S is the activity of enzyme with test compound. The concentrations of test compounds that inhibited the hydrolysis of substrate (butyrylthiocholine)

Table I. *In vitro* quantitative inhibition of BChE and LOX by compounds **1** and **2**

Compounds	IC_{50} ± SEM[b] [μM]	
	LOX	BChE
1	36.5 ± 0.05	-
2	49.5 ± 0.01	29.5 ± 0.01
Baicalein[c]	22.6 ± 0.02	-
Galanthamine[d]		8.5 ± 0.01

[b] Standard mean error of five assays
[d] Positive control used in BChE and AChE inhibiting assays
[d] Positive control used in LOX inhibiting assay

and oxidation of substrate (linoleic acid) by 50% (IC_{50}) were determined by monitoring the effect of various concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (*Perrella Scientific Inc., Amherst*, U.S.A.).

Both the compounds displayed significant inhibitory potential against LOX enzyme with IC_{50} values (36.5 ± 0.05) and (49.5.5 ± 0.1) μM, respectively. Compound **2** was found active against BChE with IC_{50} (29.5 ± 0.01) μM, while compound **1** was found inactive against BChE. Compounds **1** and **2** showed no activity against AChE enzyme (Table I).

RESULTS AND DISCUSSION

Indigin was isolated as colorless gummy solid from the $CHCl_3$ fraction of *I. oblongifolia*. The molecular formula was deduced from the HREIMS as $C_{30}H_{48}O_2$. The UV spectrum gave λ_{max} at 241, 277, and 285 nm. The IR spectrum showed a band at 3583 cm^{-1} for hydroxyl group and another band at 1628 cm^{-1} for the olefinic bond. The nuclear magnetic resonance spectrum (^{1}H-NMR) showed the presence of an ortho disubsituted benzene ring [δ 7.10 (1H, m), δ 7.02 (2H, m) and δ 6.80 (1H, m)]. The multiplet at δ 5.50 could be assigned to the proton of a trisubsituted double bond. A methyl group on a quaternary carbon resonated at δ 1.02. The signal of oxygenated methylene protons was observed at δ 3.60 and a broad signal from δ 1.22 to 1.27 (30H) confirmed the presence of fifteen methylene groups. These evidences revealed a xanthene type structure for indigin (**1**). This was further confirmed by EIMS which showed fragmentation pattern similar to 3-isopropyl-9a-methyl-1,2,4a,9a-tetrahydroxanthene (Anam, 1997), the former only differing from latter in having a C-16 chain instead of isopropyl group. The rearranged peaks at *m/z* 334 and 108 represented the fission of the molecule at C-9-C-9a and C-4a-O bonds into two fragments, the latter fragment bearing phenol-methylene. The peak at *m/z* 319 generated by the loss of C-13 methyl group from the *m/z* 334 fragment. Cleavage of the C-9–C-9a and C-11-O bonds gave the rearrangement peak at *m/z* 91 while similar cleavage at C-9–C-12 resulted in the formation of the *m/z* 93 fragment. The presence of a benzene ring was further confirmed by the peak at *m/z* 77. All these evidences showed the presence of primary alcoholic group in the C-16 alkyl chain. The analysis of the ^{1}H-NMR spectrum using COSY correlations permitted a complete map of the coupling scale of all the protons present in the molecule to be traced in an unambiguous manner. The ^{13}C-NMR spectrum of compound **1** showed signals of xanthene nucleus at δ 31.9 (C-1), 22.7 (C-2), 146.5 (C-3), 119.0 (C-4), 77.2 (C-4a), 116.1 (C-5), 127.1 (C-6), 119.5 (C-7), 129.1 (C-8), 33.0 (C-9), 29.9 (C-9a), 152.8 (C-11), 121.5 (C-12), and 17.0 (C-13). All these signals were assigned on the basis of 2D HMQC and also by comparing the data with uvarisesquiterpene (Muhammad *et al.*, 1980). The oxymethylene carbons of the side chain resonated at δ 62.1 and two further methylenes being observed at δ 33.6 and 29.9, respectively. The rest of the methylenes were observed between δ 24.9–29.7. The remaining problem was to assign the position of the side chain. Comparison of ^{13}C-NMR of compound **1** with that of δ-selinene (Schulte *et al.*, 1982) suggested the position of the olefinic bond and the side chain to be at C-3. The reported (Schulte *et al.*, 1982) C-3 chemical shift of δ-selinene is δ 146.85 whereas it was δ 146.5 for compound **1**. On the basis of these evidences indigin (**1**) was assigned the structure 3-(1'-hydroxydecanyl)-9a-methyl-1,2,4a,9a-tetrahydroxanthene. The HMBC correlation (Fig. 2) was in complete agreement to the assigned structure. The stereochemical determination of compound **1** was made possible by nuclear overhauser effect (nOe) between the protons of the CH_3-13 and H-4a. Selective irradiation of the methyl group CH_3-13 produced a significant increase in the intensity of the protons H-4a, H-4 and H-9β. This indicated proximity between CH_3-13 and H-4a. The stereostructure of **1** is, therefore, of the *cis* type.

Indigoferic acid (**2**) was isolated as an amorphous powder and its molecular formula was deduced by HREIMS as $C_{30}H_{48}O_4$. It gave effervescence with aqueous sodium bicarbonate showing the presence of free carboxylic group. The IR spectrum showed band at 1700 cm^{-1} for the carboxylic group and another band at 1615 cm^{-1} for the olefinic bond. The UV spectrum showed λ_{max} at 204, 226, and 307 nm. The ^{1}H-NMR spectrum showed the presence

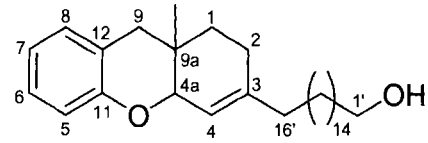

Fig. 1. Structure of compound **1**

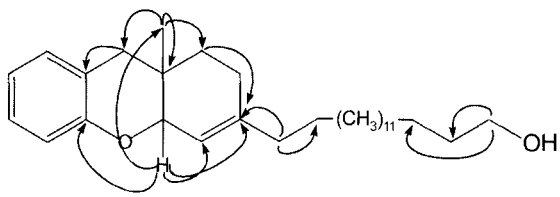

Fig. 2. Important HMBC correlations of compound **1**

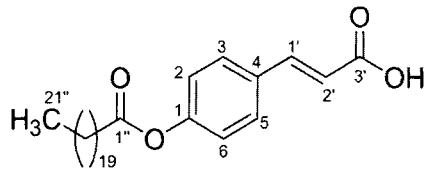

Fig. 3. Structure of compound **2**

of *para*-subsituted (*E*)-cinnamic acid moiety [olefinic protons at δ 7.57 (1H, d, *J* = 15.8 Hz) and 6.29 (1H, d, *J* = 15.8 Hz), aromatic protons forming AA BB pattern with signals at δ 7.44 (2H, d, *J* = 8.2 Hz) and 6.79 (2H d, *J* = 8.2 Hz)]. In addition it showed a terminal methyl at δ 0.88 (3H, t, *J* = 6.4 Hz) and a broad signal for methylene groups at δ 1.29 (38H). In ^{13}C-NMR spectrum the signals at δ 177.1 and 171.2 could be attributed to the ester and carboxylic groups, respectively. The olefinic carbons were observed at δ 146.1 and 115.6, while the quaternary aromatic carbons appeared at δ 162.5 and 126.1, respectively. All these evidences showed that indigoferic acid is a derivative of *p*-hydroxy-(*E*)-cinnamic acid with phenolic group being esterified with C-21 fatty acid (Fig. 3). Conclusive evidence to the assigned structure was provided by methanolysis which yielded the methyl ester of the corresponding acid showing M^+ peak at *m/z* 340 in GCMS. The HMBC correlations were also in complete agreement to the assigned structure.

Lipoxygenases (LOX, EC 1.13.11.12) constitute a family of non-haem iron containing dioxygenases that are widely distributed in animals and plants. It has been found that these LOX products play a role in a variety of disorders such as bronchial asthma, inflammation (Steinhilber, 1999) and tumor angiogenesis (Ding *et al.*, 2001). LOXs are therefore potential target for the rational drug design and discovery of mechanism–based inhibitors for the treatment of bronchial asthma, inflammation, cancer and autoimmune diseases. Both compounds **1** and **2** showed significant activity against lipoxygenase (Table I).

The role of BChE (E.C 3.1.1.8) in normal aging and brain diseases is still elusive. It has been found that BChE is found in significantly higher quantities in Alzheimer's plaques than in plaques of normal age-related non-demented brains (Yu *et al.*, 1999). The compound **2** showed moderate activity against BChE while **1** was inactive (Table I). Moreover, both **1** and **2** were inactive against AChE.

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