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ENZYME INHIBITION STUDIES OF OXINDOLE ALKALOIDS FROM *ISATIS COSTATA*

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Abstract – Costinones A (**1**) and B (**2**), the new oxindole alkaloids have been isolated from *Isatis costata* along with indirubin (**3**). Their structures have been assigned on the basis of spaectroscopic data. Both the compounds inhibited lipoxygenase and butyrylcholinesterase enzymes non-competitively in concentration-dependent fashion with promising inhibitory potentials.

INTRODUCTION

The genus *Isatis*, belonging to the family Brassicaseae, comprises 50 species mainly distributed in Irano-Turanian region. In Pakistan it is represented by seven species.[1] *Isatis tinctoria* is used in Chinese folk and modern medicine.[2] "Ban-Lan-Gen" is one of the most commonly used traditional Chinese medicines for antipyretic, anti-inflammatory, antiviral and detoxifying purposes. Its original source was considered to be the dried roots of three plants, *Isatis indigotica*, *Isatis tinctoria* and *Strobilanthes cusia*.[3,4] In a recent nationwide investigation, the roots of *I. indigotica* have been identified as the main source of "Ban-Lan-Gen" and recorded in Chinese Pharmacopoeia (1990 edn).[5] The ethano pharmacological importance of the genus *Isatis* prompted us to investigate the chemical constituents of *Isatis costata*, which is an annual or biennial herb, found in northern part of Pakistan. No phytochemical work has so far been carried out on this species. Herein we report the isolation and structural elucidation of costinones A (**1**) and B (**2**), the new oxindole alkaloids along with indirubin (**3**).[6]

Lipoxygenase (LOX, EC 1.13.11.12) are key enzymes in the biosynthesis of variety of bioregulatory compounds such as hydroxyeicosatetraenoic acids (HETEs), leukotrienes, lipoxins and hepoxylines.[7] It has been found that these LOX products play a role in a variety of disorders such as bronchial asthma, inflammation.[8] and tumor angiogenesis.[9] 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 the compounds (**1**) and (**2**) showed significant LOX inhibitory potential.

Cholinesterase are implicated as key biological players in Alzheimer's disease (AD), which makes them logical targets for inhibitory therapeutics. It has been found that butyrylcholinesterase inhibition may be help in the treatment of Alzheimer's disease (AD) and related dementias.[10] Thus the search for new cholinesterase inhibitors appears to be a promising approach to develop potential drugs for the treatment of AD. The isolated alkaloids (**1)** and (**2)** displayed moderate activity against butyrylcholinesterase enzyme.

RESULTS AND DISCUSSION

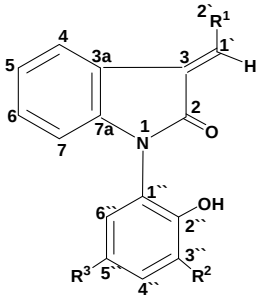
The ethanolic extract of *Isatis costata* was partitioned in between EtOAc and water. Alkaloids liberated from the aqueous fraction with 10% NH_4OH were extracted out with CH_2Cl_2.Column chromatography of CH_2Cl_2 fraction provided two new oxindole alkaloids named as costinones A (**1**) and B (**2**), as described in the EXPERIMENTAL.

Costinone A (**1**) was obtained as pale yellow crystals, mp 207–208 ^{O}C (EtOH). The HREIMS spectrum showed molecular ion at *m/z* 327.0742, consistent with the molecular formula $C_{17}H_{13}NO_6$. The IR spectrum indicated the presence of hydroxyl (3470 cm^{-1}), conjugated ketone (1690 cm^{-1}) and aromatic ring (1600, 1575, 1510 cm^{-1}). The UV spectrum showed absorption maxima at 207, 230 and 275 nm suggesting the presence of an oxindole chromophore.[11]

The ^{1}H NMR spectrum was characteristics of disubstituted indoles (δ 7.59, dd, J = 7.9 and 1.5 Hz, H-4; δ 7.09, dd, J = 8.6 and 1.5 Hz, H-7; δ 7.51, ddd, J = 8.6, 7.2 and 1.5 Hz, H-6; δ 7.39, ddd, J = 7.9, 7.2 and 1.5 Hz, H-5). In addition it showed a vinylic proton at δ 7.00 (H-1`) as singlet and a methoxy group at δ 3.56. The presence of tetra-substituted phenyl ring could be inferred by the presence of two *meta* coupled aromatic protons at δ 6.50 (d, J = 2.0 Hz, H-4``) and $\delta$ 6.79 (d, $J$ = 2.0 Hz, H-6``). On acetylation with acetic anhydride in pyridine **1** gave a tri-*O*-acetyl derivative (M^+ 453) whose IR spectrum did not show the presence of indolic N-H. This provided evidence for the attachment of trisubstituted phenyl moiety to the indolic nitrogen which could further be substantiated by the characteristics fragment at *m/z* 201 $(M-C_6H_6O_3)^+$. The HMBC correlations were also in complete agreement to the presence of trihydroxy phenyl moiety at indolic nitrogen.

The ^{13}C NMR spectrum (BB and DEPT) showed 17 signals comprising of one methyl, seven methine and nine quaternary carbons. The signals at δ 170.1 and 167.6 could be assigned to amide and ester carbonyl carbons. The olefinic carbons resonated at δ 136.4 and 123.5, respectively. The 12 signals ranging from δ 158.4-99.3 were due to aromatic carbons. The methoxy carbon resonated at δ 53.0.

The above spectral data were consistent with oxindole type alkaloids with additional carbomethoxymethylidene and a trihydroxyphenyl moieties attached to the indolic nitrogen. This was confirmed by HMBC correlations in which the vinylic proton at δ 7.00 showed ^{2}J correlations with C-3 (δ 136.4) and ^{3}J correlations with C-2 (δ 170.1) and C-3a (δ 125.2). The methoxy protons at δ 3.56 showed ^{3}J correlation with C-2` (167.6). The geometry of the double bond was assigned on the basis of chemical shifts of the olefinic proton and C-1`. Winterfeldt, *et al.*[12] have reported that the exocyclic vinylic proton in the α-methylene lactam system is more shielded in the *E* geometry because of a large paramagnetic anisotropic effect from lactam carbonyl group in the *cis* position and resonates comparatively downfield (δ 6.79) than the *Z* isomer (δ 5.86). Same is true for C-1` which is more shielded in the case of *E* isomers and resonates ~ 4 ppm downfield at δ 123.5 in the ^{13}C NMR spectrum.[13]. Since these values were fully consistent with *E* geometry, the structure of costinone A (**1**) could be assigned as (*E*) methyl 2-[2-oxo-1-(2``, 3``,5``- trihydroxyphenyl)-1, 2-dihydro-3*H*-indol-3-ylidene]acetate.



1 R^1 = CO_2Me; R^2 = R^3 = OH

2 R^1 = OMe; R^2 = H; R^3 = CO_2Me

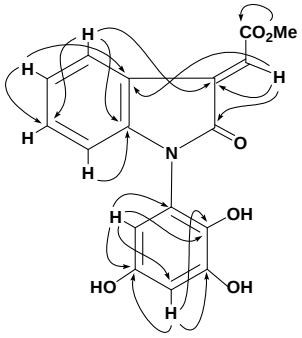


Figure 1 Important HMBC correlations of **1**

Costinone B (**2**) formed pale yellow crystals, mp 195–197 ^{O}C, $[\alpha]_D$ +99.7^{o}. The molecular formula was established by HREIMS spectrum showing molecular ion at *m/z* 325.0947. The UV and IR spectra were very similar to those of **1.** The ^{1}H and ^{13}C NMR spectra showed notable difference in the substituted phenyl moiety attached to the indolic nitrogen. This moiety was now found to be trisubstituted, the three aromatic protons forming ABX type coupling pattern at δ 8.13 (d, J = 1.6 Hz, H-6``), 8.00 (dd, $J$ = 7.9, 1.6 Hz, H-4``) and 7.15 (d, J = 7.9 Hz, H-3``). Another difference was the presence of methoxymethylidene group. This was evident from HMBC spectrum in which the methoxy protons at δ 3.95 showed ^{3}J correlation with C-1` (149.8). In ^{13}C NMR spectrum, most of the peaks were similar to

those of **1** except the resonances due to aromatic substituent at the indolic nitrogen. The former now showed the carbonyl carbon of the methyl carboxylate group at δ 165.7. Moreover, compared to **1**, the signal of C-3`` shifted upfield to δ 117.0 due to absence of oxygen substituent and the signal of C-5`` shifted upfield to 125.2 ppm due to now presence of carboxyl group. The presences of 2``-hydroxy-5``-carbomethoxyphenyl group was also confirmed by EIMS spectrum , the latter showing the characteristics fragment at *m/z* 173 (M^+–$C_8H_8O_3$).The HMBC correlations were in exact agreement to the assigned structure of costinone B (**2**) as methyl 4-hydroxy-3-{3-[(*E*)-methoxymethylidene]-2-oxo-2,3-dihydro-1*H*-indol-1-yl}benzoate.

Table-1. ^{1}H and ^{13}C NMR spectral data (CD_3OD) for compounds (**1-2**)

Costinone A (**1**)			Costinone B (**2**)	
Position	**^{13}C**	**^{1}H ppm (J, Hz)**	**^{13}C**	**^{1}H ppm (J, Hz)**
1	-	-	-	-
2	170.1	-	169.9	-
3	136.4	-	104.7	-
3a	125.2	-	120.1	-
4	120.5	7.59(1H, dd, 7.9,1.5)	123.3	7.67 (1H, dd, 7.8, 1.7)
5	120.1	7.39 (1H, ddd, 7.9,7.2, 1.5)	118.7	7.35 (1H, ddd, 7.8, 7.1, 1.7)
6	132.3	7.51(1H, ddd, 8.6, 7.2, 1.5)	133.1	7.41 (1H, ddd, 8.5, 7.1, 1.7)
7	113.9	7.09 (1H, dd, 8.6, 1.5)	111.0	7.10 (1H, dd, 8.5, 1.7)
7a	147.7	-	147.9	-
1`	123.5	7.00 (1H, s)	149.8	6.80 (s)
2`	167.6	-	61.3	3.95 (3H, s)
3`	53.0	3.56 (3H, s)	-	-
1``	128.0	-	130.4	-
2``	130.1	-	152.7	
3``	150.2	-	117.0	7.15(1H, d, 7.9)
4``	99.3	6.50 (1H, d, 2.0)	130.1	8.00 (1H, dd, 7.9, 1.6)
5``	158.4	-	125.2	-
6``	104.1	6.79 (1H, d, 2.0)	123.9	8.13 (1H, d, 1.6)
1```` C=O	-	-	165.7	-
2```` OCH_3	-	-	52.6	3.90 (3H, s)

Costinones A (**1**) and B (**2**), inhibited LOX and BChE enzymes non-competitively as in both cases there was a decrease in V_{max} without any subsequent effect on the affinity (K_m values) of the LOX or BChE enzyme towards the substrate (linoleic acid or BTCh), respectively. In other words, inhibitor and the substrate bind randomly and independently at the different sites of LOX or BChE. It indicates that

inhibition depends only on the concentration of inhibitor and dissociation constant (K_i). In case of BChE inhibition, compound (**1**) (K_i = 20.2 ± 0.02 μM) has higher inhibitory potential than **2** (K_i = 46.3 ± 0.1 μM) probably due to the change of functional group from trihydroxyphenyl to hydroxyl carbomethyoxyphenyl group. In case of LOX inhibition trihydroxybenzoyl moiety in compound (**1**) **(**K_i = 16.5±0.04 μM) seems to play key role, while change in substitution pattern of phenyl moiety in **2** decreased the activity (K_i = 28.6 ± 0.04 μM).

Table-2. Steady-state inhibition of BChE and LOX enzymes by compounds (**1**) and (**2**).

	BChE			**LOX**		
Compounds	***IC*$_{50}$ ± SEM[μM]**	**K_i ± SEM[μM]**	**Type of Inhibition**	***IC*$_{50}$ ± SEM[μM]**	**K_i ± SEM[μM]**	**Type of Inhibition**
Costinone A (**1**)	22.5±0.02	20.2±0.1	NC	15.5±0.01	16.5±0.04	NC
Costinone B (**2**)	45.9±0.05	46.3±0.1	NC	30.5±0.01	28.6±0.1	NC
Galnathamine*	8.5±0.01	8.0±0.03	NC	-	-	-
Baicalein[#]	-	-	-	22.5±0.05	20.1±0.03	MT

Key: NC = Non-competitive, MT= Mixed-type,
*Standard inhibitor of BChE, [#]Standard inhibitor of LOX, **)[a]** Standard mean error of three assays, **)[b]** Dissociation constant or inhibition constant (determined from non-linear regression analysis by Dixon plot and secondary Lineweaver-Burk plot at various concentrations of **1-2**).

CONCLUSION

In conclusion, our search for lipoxygenase and BChE inhibitory constituents from *I. costata* has resulted in the isolation of new oxindole alkaloids which may find use in treatment inflammation, asthama, aging, tumor, angiogenesis, cancer and Alzheimer's disease. However, further *in vivo* study would help in exploring the pharmacological properties of these compounds.

EXPERIMENTAL

General: Optical rotations were taken on a JASCO DIP-360 digital polarimeter. IR spectra were measured on a JASCO 302-A spectrophotometer in $CHCl_3$. UV spectra were obtained on a Hitachi UV-3200 spectrophotometer. NMR spectra were run on a Bruker instrument. Chemical shifts δ are shown in ppm relative to TMS as internal standard and coupling constant *J* are described in Hz. EI, FAB, and HREI-MS spectra were recorded on a JEOL JMS-HX-110 and JMS-DA-500 mass spectrometers. Silca gel 230-400 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 material was collected in April 2004 from N. W. F. P Swat and identified as *Isatis costata* by Dr. Ghosia Lutfullah, Centre of Biotechnology, University of Peshawar,

Pakistan. A voucher specimen (BPU-105) is deposited in the herbarium of the Department of Botany, University of Peshawar, Peshawar, Pakistan.

Extraction and isolation: The shade-dried whole plants (17 kg) were chopped and extracted thrice with EtOH (60 L) at rt for 96 h. The methanolic extract was evaporated *in vacuo* to give a dark greenish residue (400 g), which was partitioned between EtOAc and water. The water fraction was basified with 10% NH_4OH and extracted with CH_2Cl_2. The CH_2Cl_2 fraction (40 g) was subjected to column chromatography eluting with *n*-hexane-EtOAc in increasing order of polarity to obtain six fractions. The fraction obtained from *n*-hexane-EtOAc (5:5) was rechromatographed over silica gel using *n*-hexane-EtOAc (8:2-3:7) as solvent systems to afford two successive fractions. The second fraction was further purified by column chromatography over silica gel using *n*-hexane-EtOAc (5:5) as eluent to afford the compound (**1**) (19 mg). The compound (**2**) (16 mg) was purified by column chromatography of the first fraction using solvent system *n*-hexane-EtOAc (6:4). The fraction obtained from *n*-hexane-EtOAc (6:4) was rechromatographed over silica gel using *n*-hexane-EtOAc (8.5:1.5-5:5) as an eluent to afford two successive fractions. The second fraction on purification by column chromatography over silica gel provided the known compound (**3**) by using *n*-hexane- EtOAc (7:3) as eluent.

Costinone A (1): Pale yellow crystals, mp 207-208 ^{O}C (EtOH), $[\alpha]_D$ +110.0^o (c = 0.12 MeOH); UV λ_{max} (MeOH) nm (log ε) 275 (2.25), 230 (3.95), 207 (4.21); IR ν_{max} cm^{-1}: 3470, 1720, 1690, 1600, 1575,1510; EI-MS *m/z* $[M]^+$ 327 (27), 267 (100), 143 (45), 126 (37), 98 (17), 90 (60), 60 (35), 28 (30), 27 (12); HREIMS calcd for $C_{17}H_{13}NO_6$: 327.0742. Found: 327.0739. Data of ^{1}H and ^{13}C NMR spectra are shown in Table 1.

Costinone B (2): Pale yellow crystals, mp 195-197 ^{O}C (EtOH); $[\alpha]_D$ +99.7^o (c = 0.12 MeOH); UV λ_{max} (MeOH) nm (log ε) 279 (2.50), 230 (4.10), 210 (4.47); IR ν_{max} cm^{-1}: 3460, 1715, 1687, 1610, 1560, 1505; EI-MS *m/z* $[M]^+$ 325 (21), 293 (100), 152 (41), 143 (47), 115 (50), 98 (15), 90 (57); HREIMS calcd for $C_{18}H_{15}NO_5$: 325.0950. Found: 325.0947. Data of ^{1}H and ^{13}C NMR spectra are shown in Table 1.

Lipoxygenase inhibition assay

LOX inhibiting activity was measured by modifying the spectrophotometric method developed by *A. L. Tappel*.[14] LOX (1.13.11.12) type I-B (Soybean) and linoleic acid was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. The assay conditions and protocol was the same as described in our previous article.[15] All the kinetic experiments were performed in 96-well micro-titre plates by using *SpectraMax 384plus* (Molecular Devices, USA).

Cholinesterase inhibition assay

Horse-serum BChE, butyrylthiocholine chloride, 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB), and galanthamine were purchased from Sigma (St. Louis, MO, USA). Buffer and other chemicals were of analytical grade. BChE inhibiting activity was measured by the spectrophotometric method developed by *Ellman et al*.[16] All the inhibition studies were performed in 96-well microtiter-plates using *SpectraMax 340* (Molecular Devices, CA, USA). Assay protocol and conditions were the same, as mentioned previously.[17]

According to Ellman *et al.*[16] since the extinction coefficient of the yellow anion is known, the rate of the enzymatic reaction was determined by the following equation:

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

Determination of kinetic parameters

The concentration of test-compounds that inhibited the hydrolysis of substrate ((butyrylthiocholine) or oxidation of substrate (linoleic acid) by 50% (IC_{50}) was determined by monitoring the effect of various concentrations of the inhibitors in the assays on the inhibition values. The IC_{50} (inhibitor conc. that inhibits 50% activity of BChE and LOX) values were then calculated using the EZ-Fit Enzyme Kinetics program (*Perrella Scientific Inc., Amherst*, USA).

Dissociation constant/inhibition constant (K_i) was determined by the interpretation of Dixon plot.[18] Lineweaver-Burk plot.[19] and their secondary replots using initial velocities obtained over a substrate concentration range between 0.05-0.2 mM for butyrylthiocholine chloride (BTCh) and linoleic acid.

Non-linear regression equations were used to determine the values of K_I, K_m and V_{max} in the Lineweaver-Burk plot and Dixon plots. The K_i values [dissociation constant/inhibition constant of LOX-inhibitor BChE-inhibitor complex into free LOX or BChE and inhibitor were determined graphically by Dixon plot and Lineweaver-Burk plots; firstly, $1/V_{maxapp}$ was calculated at each intersection points of lines of every inhibitor concentration on y-axis of the Lineweaver-Burk plot and then replotted against various concentrations of inhibitor. Secondly, the slope of each line of inhibitor concentration on Lineweaver-Burk plot was plotted against inhibitor concentrations. Then replotting slope at various concentrations of inhibitor, K_i was the intercept on x-axis.

Statistical analysis

Graphs were plotted using GraFit program. Values of the correlation coefficients, slopes, intercepts and their standard errors were obtained by the linear regression analysis using the same program. The

correlation for all the lines of all graphs was found >0.99. Each point in the constructed graphs represents the mean of three experiments.

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