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Full Paper

Isatinones A and B, New Antifungal Oxindole Alkaloids from *Isatis costata*

Itrat Fatima [1], Ijaz Ahmad [2], Itrat Anis[1], Abdul Malik [1,*] and Nighat Afza [3]

[1] International Centre for Chemical Sciences, HEJ Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan
[2] Medicinal Botanic Centre, PCSIR Labs. Complex Peshawar, Peshawar, N.W.F.P, Pakistan
[3] Pharmaceutical Research Centre, PCSIR Labs. Complex, Karachi-75270, Pakistan

[*]Author to whom correspondence should be addressed; e-mail: abdul.malik@iccs.edu;
Tel: (+92) 21-4824926; Fax: (+92) 21-4819018

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Abstract: Two new oxindole alkaloids isatinone A (**1**) and B (**2**) have been isolated from *Isatis costata*, along with the known trisindoline. Their structures have been assigned on the basis of spectroscopic techniques and chemical studies. Both new compounds showed significant antifungal activity.

Keywords: *Isatis costata*; Brassicaseae; oxindole alkaloids; structure elucidation; antifungal activity

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Introduction

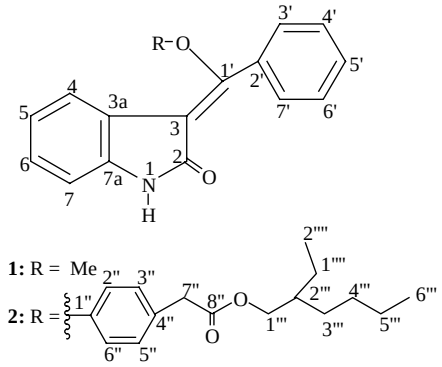
The genus *Isatis*, belonging to the family Brassicaseae, comprises 50 species, mainly distributed in the Irano-Turanian region. In Pakistan it is represented by seven species [1]. *Isatis tinctoria* or woad is a common plant cultivated throughout the centuries to produce the blue dye indigo. Nowadays, woad is also 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, antimicrobial 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]. Now the roots of *Isatis indigotica* have been

identified as the main source of "Ban-Lan-Gen" and recorded as such in Chinese Pharmacopoeia (1990 edn.) [5]. The ethno-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 parts of Pakistan. Herein we report the isolation and structural elucidation of isatinones A (**1**) and B (**2**), along with the known trimeric oxindole alkaloid trisindoline [6]. Both alkaloids **1** and **2** showed significant antifungal activity against various strains.

Results and Discussion

The ethanolic extract of *Isatis costata* was partitioned between EtOAc and water. Alkaloids librated from the aqueous fraction with 10% NH_4OH were extracted with CH_2Cl_2. Column chromatography of the CH_2Cl_2 fraction provided the known alkaloid trisindoline, along with two new oxindole alkaloids which we have named isatinones A (**1**) and B (**2**). These compounds have been assigned the structures shown in Figure 1, as described below.

Figure 1. Structures of isatinone A (**1**) and B (**2**).

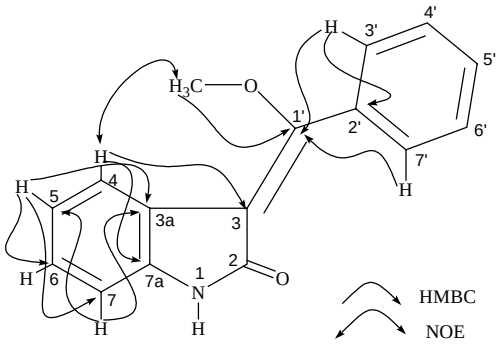


Isatinone A (**1**) was a pale yellow amorphous solid, mp 178-179°C. The molecular formula $C_{16}H_{13}NO_2$ was established its the HR-EI-MS spectrum, which showed a molecular ion peak at *m/z* 251.0946 (calc. for $C_{16}H_{13}NO_2$: 251.0943). The IR spectrum indicated the presence of an amide carbonyl (1685 cm^{-1}) and an aromatic ring (1610, 1560, 1450 cm^{-1}). The UV spectrum showed absorption maxima at 208, 231 and 270 nm, suggesting the presence of an oxindole chromophore [7]. The ^{1}H-NMR spectrum was characteristic of disubstituted indoles [δ 7.54 (1H, dd, *J*=7.9, 1.5 Hz, H_4), 7.19 (1H, ddd, *J*=8.4, 7.1, 1.5 Hz, H_6), 7.08 (1H, ddd, *J*=7.9, 7.1, 1.0 Hz, H_5), and 6.86 (1H, dd, *J*=8.4, 1.0 Hz, H_7)]. In addition, it showed an indolic amide NH singlet at δ 12.7. The presence of a mono-substituted phenyl ring could be inferred by the ^{1}H-NMR spectrum, which showed the appropriate signals in the aromatic region [δ 7.02 (2H, m), 7.34 (2H, m), and 7.35 (1H, m)]. In addition it showed the presence of signals due to a methoxyl group at δ 3.87 (3H, s).

The ^{13}C-NMR spectrum (BB and DEPT) showed sixteen signals, comprising of one methyl, nine methine and five quaternary carbons. The downfield signals at δ 167.1 could be assigned to the carbonyl carbon of an amide. The olefinic carbons resonated at δ 148.7 and δ 103.1, respectively. The other 12 signals ranging from δ 144.0-115.1 were due to aromatic carbons, while the methoxy carbon resonated at δ 56.7. The above spectral data was consistent with an oxindole type alkaloid with additional phenyl and methoxyl moieties. Since the presence of a disubstituted indolic moiety has already been established, therefore the only location for these groups is the exocyclic olefinic carbon

of a methylidene moiety. The structure was not only supported by ^{1}H–^{1}H COSY spectrum, but also by HMBC correlations (Figure 2) in which the H-4 proton at δ 7.54 showed ^{2}J correlations with C-3a (δ_C 124.0) and ^{3}J correlations with C-7a (δ_C 144.0) as well as C-3 (δ_C 103.1). The proton at δ_H 7.02 (H-3') showed ^{3}J correlations with C-1' (δ_C 148.7) and ^{2}J correlations with C-2' (δ_C 141.1). The methoxyl protons at δ_H 3.87 showed ^{3}J correlation with C-1' (δ_C 148.7). The geometry of the double bond was assigned on the basis of chemical shifts of the olefinic carbons. Thielke *et al.* [8] have reported that the olefinic carbon in the α-methylene lactam system is less shielded in the *Z* than in the *E* geometry because of a large paramagnetic anisotropy effect from the lactam carbonyl group. The values of olefinic carbons were consistent with theoretically calculated values for the *E* geometry and showed very close agreement to those of costinone B, reported in the literature [9]. This was further confirmed by NOE correlation between δ_H 7.54 (H-4) and protons of the methyl group at δ_H 3.87 [10]. The assignments of ^{13}C-NMR signals were facilitated by HMQC spectrum and found in complete agreement to the assigned structure of isatinone A (**1**) as 3-[(E)-methoxy (phenyl) methylidene]-1,3-dihydro-*2H*-indol-2-one.

Figure 2. Important HMBC and NoE correlations of isatinone A (**1**).



Isatinone B (**2**) was isolated as a pale yellow amorphous solid, mp 189-191°C. The molecular formula $C_{31}H_{33}NO_4$ was determined by negative ion HRFABMS, which showed a pseudomolecular ion peak at *m/z* 482.2328 (calc. for $C_{31}H_{32}NO_4$: 482.2331). The UV and IR spectra were very similar to those of **1**, except the presence of additional absorptions due to the ester moiety. The ^{1}H- and ^{13}C-NMR spectra were also found to be similar to those of **1**, except for the replacement of the methoxyl group by a 2-ethylhexyl phenylacetic acid ester moiety.

The ^{13}C-NMR spectrum (BB and DEPT) showed thirty-one signals, comprising of two methyl, six methylene, fourteen methine and nine quaternary carbons. The signals at δ_C 169.3 and 165.3 could be assigned to carbonyl carbons of ester and amide, respectively. The methylidene olefinic carbons resonated at δ_C 149.4 and 103.9, respectively. The other signals ranging from δ_C 144.4–115.0 were due to aromatic carbons. The oxymethylene carbon resonated at δ_C 69.1, while signals of five methylene groups were observed from δ_C 49.6–24.0. The two terminal methyl groups resonated at δ_C 14.4 and 11.4, respectively.

The ^{1}H-NMR displayed a pair of *ortho*-coupled AA'XX' type signals at δ_H 7.06 and δ_H 6.49 (each 2H, d, *J*=8.2 Hz), indicating the presence of an additional 1, 4-disubstituted benzene ring. The signal of the oxymethylene protons was observed at δ_H 4.20, while another methylene group was observed at δ_H 3.40 (s). The unresolved multiplets at δ_H 1.31 and δ_H 1.54, integrating for four protons each, respectively, were due to four further methylenes. In addition, it showed an aliphatic methine signal at

δ_H 1.70 (m) and a pair of three proton triplets for the terminal methyl groups at δ 0.83 (*J*=6.4 Hz) and δ 0.90 (*J*=6.3 Hz), respectively.

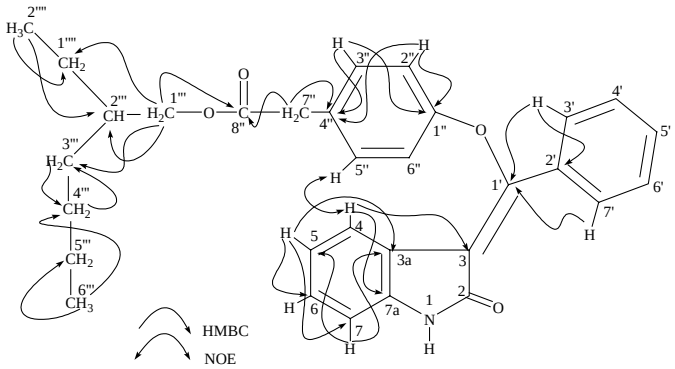
Table 1. Correlated ^{1}H-NMR and COSY spectral data (CD_3OD) of isatinone A (**1**) and isatinone B (**2**).

1			**2**		
C/H	**δ H**	**^{1}H-^{1}H COSY**	**C/H**	**δ H**	**^{1}H-^{1}H COSY**
1	12.7 (1H, s)		1	12.7 (1H, s)	
2			2		
3			3		
3a			3a		
4	7.54 (1H, dd, 7.9, 1.5)	H-5, H-6	4	7.20 (1H, dd, 8.0, 1.5)	H-5, H-6
5	7.08 (1H, ddd, 7.9, 7.1, 1.0)	H-4, H-6	5	7.08 (1H, ddd, 8.0,7.0, 1.5)	H-4, H-6
6	7.19 (1H, ddd, 8.4, 7.1, 1.5)	H-5, H-7	6	7.61 (1H, ddd, 8.4, 7.0, 1.5)	H-5, H-7
7	6.86 (1H, dd, 8.4, 1.0)	H-6, H-5	7	6.89 (1H, dd, 8.4, 1.5)	H-6, H-5
7a			7a		
1'			1'		
2'			2'		
3'	7.02 (1H, m)	H-4', H-5'	3'	7.64 (1H, m)	H-4', H-5'
4'	7.34 (1H, m)	H-3', H-5'	4'	7.36 (1H, m)	H-3', H-5'
5'	7.35 (1H, m)	H-4', H-6'	5'	7.36 (1H, m)	H-4', H-6'
6'	7.34 (1H, m)	H-5', H-7'	6'	7.36 (1H, m)	H-5', H-7'
7'	7.02 (1H, m)	H-6', H-5'	7'	7.64 (1H, m)	H-6', H-5'
OCH_3	3.87 (1H, s)		1"		
			2"	7.06 (1H, d, 8.2)	H-3"
			3"	6.49 (1H, d, 8.2)	H-2"
			4"		
			5"	6.49 (1H, d, 8.2)	H-6"
			6"	7.06 (1H, d, 8.2)	H-5"
			7"	3.41 (2H, s)	
			8"		
			1'"	4.20 (2H, m)	H-2'"
			2'"	1.70 (1H, m)	H-1'", H-3'", H-1""
			3'"	1.65 (2H, m)	H-2'", H-4'"
			4'"	1.54 (2H, m)	H-3'", H-5'"
			5'"	1.31 (2H, m)	H-4'", H-6'"
			6'"	0.83 (3H, t, 6.4)	H-5'"
			1""	1.33 (2H, m)	H-2'", H-2""
			2""	0.90 (3H, t, 6.3)	H-1""

The structure was confirmed by a series of ^{1}H-^{1}H COSY (Table 1) and HMBC correlations (Figure 3). In addition to the usual correlations due to indolic and phenyl moieties, it further showed ^2J correlation of H-2" at δ 7.06 with C-1" (δ_C 141.0) and ^3J correlation with C-4" (δ_C 124.1). The signal at δ_H 3.41 (H_2-7") showed ^2J correlations with C-4" (δ_C 124.1) and C-8" (δ_C 169.3). The oxymethylene protons at δ_H 4.20 (H-1'") showed ^3J correlations with C-8" (δ_C 169.3); C-3'" (δ_C 31.6); C-1"" (δ_C 24.0) and ^2J correlation with C-2'" (δ_C 40.2). The terminal methyl groups at δ_H 11.4 and δ_H 14.4 showed ^2J correlations with C-5'" (δ_C 24.9) and C-1"" (δ_C 24.0), respectively. The *E* geometry was assigned by comparing the chemical shifts of C-3 and C-1' in the ^{13}C-NMR spectrum, which showed close resemblance to those of **1**. It could further be confirmed by the presence of NOE correlations between

H-4 at δ_H 7.20 and the protons of the substituted phenyl moiety at δ_H 7.06 (Figure 3). The assignments of ^{1}H- and ^{13}C-NMR signals were facilitated by ^{1}H–^{1}H COSY and HMQC spectra and found in complete agreement with the assigned structure of isatinone B (**2**) as 2-ethylhexyl 2-{4-[2-oxo-1, 2-dihydro-*3H*-indol-3-ylidene) (phenyl) methoxy] phenyl} acetate.

Figure 2. Inportant HMBC and NoE correlations of isatinone B (**2**).



The known alkaloid trisindoline was identified by comparison of its spectroscopic characteristics with those reported in the literature [6].

Biological Activity

The antifungal activities of both **1** and **2** were determined by the agar tube dilution method and significant activity was observed against *Trichophyton schoen leinii*, *Aspergillus niger*, *Candida albicans*, *Trichophyton simii*, and *Macrophomina phaseolina*.

Table 2. *In vitro* fungicidal bioassay of crude extract and Isatinones A (**1**) and B (**2**).

Name of fungus	**Inhibition (%) of crude extract**	**Inhibition (%)**		**Standard drugs**	**Inhibition (%) of Standard drugs**
		1	**2**		
Trichophyton schoen leinii	71.4	70.0	81.2	Miconazole	90
				Ketoconazole	90
Aspergillus niger	50.1	68.0	78.0	Amphotericin-B	100
Pseudallescheria boydri	39.4	55.7	59.5	Miconazole	90
				Ketoconazole	90
Candida albicans	48	69.1	70.3	Nystatin	90
Microsporum canis	34	15.5	25.0	Miconazole	100
				Ketoconazole	100
Trichophyton mentagrophytes	53	60.0	50.7	Miconazole	100
				Ketoconazole	100
Trichophyton simii	67.5	77.0	80.4	Miconazole	100
Fusarium solani var. lycopersici (tomato)	12	2	8	Benlate	100
Macrophomina phaseolina	56	71.0	75.1	Benlate	100
				Nabam	
Rhizoctonia solani	60.2	50.0	54.0	Benlate	100

Conclusions

In summary, the isolation of two novel antifungal oxindole alkaloids named isatinone A and B and the known alkaloid trisindoline from *I. costata* has been achieved and their structures elucidated with the help of spectroscopic techniques.

Experimental

General

Optical rotations were recorded on a JASCO DIP-360 digital polarimeter. IR spectra were measured on a JASCO 302-A spectrophotometer in $CHCl_3$. UV spectra was obtained on a Hitachi UV-3200 spectrophotometer. NMR spectra were run on an AMX-400 Bruker instrument. Chemical shifts δ are shown in ppm relative to TMS as internal standard and coupling constant *J* are given in Hz. EI-, FAB-, and HREIMS were recorded on a JEOL JMS-HX-110 and JMS-DA-500 mass spectrometers. Silica 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* C. A. Mey 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 plant (17 kg) was chopped up and extracted three times with EtOH (60 L) at room temperature for 96 h. The ethanolic extract was evaporated *in vacuo* to give a dark greenish residue (400 g), which was partitioned between EtOAc and water. The aqueous fraction was made basic with 10% NH_4OH and the liberated bases extracted with CH_2Cl_2. The CH_2Cl_2 fraction (40 g) was subjected to column chromatography eluting with *n*-hexane-EtOAc mixtures in increasing order of polarity to afford six fractions F_1-F_6. Silica gel column chromatography of fraction F_2 (eluted with 7:3 *n*-hexane-EtOAc) and elution with mixtures of *n*-hexane-EtOAc provided fractions F_{2A} (7:3) and fraction F_{2B} (5:5), respectively. Slow evaporation of fraction F_{2A} deposited pale yellow crystals of isatinone A (**1**, 11 mg). The fraction F_{2B} was rechromatographed over silica gel, again eluting with *n*-hexane-EtOAc mixtures. The eluent obtained from 3:7 *n*-hexane EtOAc provided isatinone B (**2**, 17 mg). The fraction F_3 obtained from *n*-hexane-EtOAc (6:4) was rechromatographed over silica gel using mixtures *n*-hexane-EtOAc (8:2 → 3:7) as solvent to afford two successive fractions, the first of which, further on purification by column chromatography over silica gel and elution with 7:3 *n*-hexane-EtOAc afforded trisindoline (25 mg).

3-[(E)-methoxyphenylmethylidene]-1,3-dihydro-2H-indol-2-one (*Isatinone A*, **1**): $C_{16}H_{13}NO_2$; pale yellow amorphous solid; mp 178-179°C; UV (MeOH) λ_{max} 208, 231, 270 nm; IR ν_{max} (KBr): 3301, 1680, 1600, 1565, 1460 cm^{-1}; ^{13}C-NMR δ: 167.1 (C-2), 103.1 (C-3), 124.0 (C-3a), 119.8 (C-4), 123.3 (C-5), 129.4 (C-6), 115.1 (C-7), 144.0 (C-7a), 148.7 (C-1'), 141.1 (C-2'), 124.5 (C-3'), 130.5 (C-4'), 130.8 (C-5'), 130.5 (C-6'), 124.5 (C-7'), 56.7 (OCH_3); EIMS, *m/z* 251 $[M]^+$, 236, 209, 160, 131, 117, 92, 77; HREIMS: 251.0946 (calcd for $C_{16}H_{13}NO_2$, 251.0943). Complete assignments of ^{1}H-NMR and ^{1}H-^{1}H COSY data for **1** are described in Table 1. Important HMBC and NOE correlations are illustrated in Figure 2.

2-Ethylhexyl 2-{4-[2-oxo-1,2-dihydro-3H-indol-3-ylidene) (phenylmethoxy]phenyl} acetate (*Isatinone B*, **2**): $C_{31}H_{33}NO_4$; pale yellow amorphous solid; mp 189–191°C; $[\alpha]_D^{18}$ +89.7˚ (*c*. 0.02, MeOH); UV (MeOH) λ_{max} 205, 232, 275 nm; IR ν_{max} (KBr): 3305, 1685, 1715, 1610, 1560, 1450 cm^{-1}; ^{13}C-NMR δ: 165.3 (C-2), 103.9 (C-3), 124.1 (C-3a), 119.8 (C-4), 123.1 (C-5), 129.8 (C-6), 115.0 (C-7), 144.4 (C-7a), 149.4 (C-1'), 133.6 (C-2'), 129.4 (C-3'), 130.5 (C-4'), 130.9 (C-5'), 130.5 (C-6'), 129.4 (C-7'), 141.0 (C-1''), 115.0 (C-2''), 130.5 (C-3''), 124.1 (C-4''), 130.5 (C-5''), 115.0 (C-6''), 49.6 (C-7''), 169.3 (C-8''), 69.1 (C-1'''), 40.2 (C-2'''), 31.6 (C-3'''), 30.1 (C-4'''), 24.9 (C-5'''), 11.4 (C-6'''), 24.0 (C-1''''), 14.4 (C-2''''); EIMS, *m/z* 483 $[M]^+$, 349, 321, 311, 293, 236, 160, 131, 116, 91, 77. Negative HRFABMS: 482.2328 (calcd 482.2331 for $C_{31}H_{32}NO_4$). Complete assignments of ^{1}H-NMR and ^{1}H-^{1}H COSY data for **2** are described in Table 1. Important HMBC and NOE correlations are illustrated in Figure 3.

Trisindoline: Colorless amorphous solid; UV (MeOH) λ_{max} 290, 280, 274, 254, 219 nm; IR ν_{max} (KBr): 3200, 1705, 1472 cm^{-1}; HREIMS: 363.1371 (calcd for $C_{24}H_{17}N_3O$, 363.1401); ^{13}C- and ^{1}H-NMR data were identical with those reported in the literature [6].

Bioassays

The antifungal bioassay was performed on human, animal and plant pathogens. The crude extracts, compounds **1** and **2** and the standard drugs (each at a concentration of 400 μg/mL of Sabourd Dextose Agar) were subjected to antifungal activity assays against *Trichophyton schoen leinii* ATCC 22775, *Aspergillus niger* ATCC 1015, *Pseudallescheria boydri* ATCC 44330, *Candida albicans* ATCC 10231, *Microsporum canis* ATCC 36299, *Trichophyton mentagrophytes* ATCC 28185, *Trichophyton simii* ATCC 25923, *Fusarium solan* ATCC 36031, *Macrophomina phaseolina* ATCC 53789, *Rhizoctonia solani* ATCC 76131, according to the established protocol [11]. The compounds **1** and **2** showed significant activity against *Trichophyton schoen leinii*, *Aspergillus niger, Candida albicans, Trichophyton simii, Macrophomina phaseolina*; moderate activity against *Pseudallescheria boydri, Trichophyton mentagrophytes, Rhizoctonia solani*, and weak activity against *Microsporum canis* and *Fusarium solani* (Table 2). It is important to note that compound **2** was more potent **1**, which is probably be due to the presence of the ester moiety.

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Sample Availability: Samples of the compounds are available from the corresponding author.

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