Bisindigotin, a TCDD Antagonist from the Chinese Medicinal Herb *Isatis indigotica*

Xiao Y. Wei,[†,‡] Chung Y. Leung,[†] Chris K. C. Wong,[†] Xiao L. Shen,[†,§] Ricky N. S. Wong,[†] Zong W. Cai,[⊥] and Nai K. Mak*,[†]

Department of Biology, Hong Kong Baptist University, Kowloog Tong, Hong Kong, People's Republic of China, South China Botanical Garden, Chinese Academy of Sciences, Leyiju, Guangzhou 510650, People's Republic of China, Guangzhou University of Traditional Chinese Medicine, Jichang Road, Guangzhou 510405, People's Republic of China, and Department of Chemistry, Hong Kong Baptist University, Kowloog Tong, Hong Kong, People's Republic of China

Received October 25, 2004

A new indigoid derivative, bisindigotin (**1**), with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-antagonistic activity was isolated from the ethanol extract of the Chinese medicinal herb *Isatis indigotica*. Its structure was determined by spectroscopic methods. In the human HepG2 hepatoma cell model, **1** (50 nM to 2 μM) was found to dose-dependently inhibit TCDD-induced ethoxyresorufin *O*-deethylase (EROD) activity.

Compounds with antagonistic activity against 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced toxicity have been recently isolated and identified in various plant species. α-Naphthoflavone, a recently identified natural product from the root exudates of *Acroptilon repens* (Russian knapweed),[1] has been shown to inhibit TCDD-induced ethoxyresorufin *O*-deethylase (EROD) activity in rat hepatoma cells.[2] Other structural classes of phytochemicals with TCDD antagonistic activity have also been described. Dibenzoylmethane (DBM), a naturally occurring phytochemical, was found to prevent TCDD-induced EROD activity in human HepG2 cells.[3] In this study, we report the isolation and structural determination of a new TCDD antagonistic compound, bisindigotin (**1**), from the Chinese medicinal herb *Isatis indigotica*. This herbal plant has long been used as a folk medicine in China for treatment of viral diseases and diseases with inflammatory nature.[4] However, the medicinal application of *I. indigotica* in other diseases has not been examined. The identification of **1** as an inhibitor of TCDD-induced EROD activity in this herbal plant may enable us to further evaluate the use of *I. indigotica* in the research of antidotes for TCDD intoxication.

The EtOH extract of the powdered, dry leaves of *I. indigotica* was successively fractionated with petroleum ether and $CHCl_3$. The $CHCl_3$-soluble fraction was separated by column chromatography over silica gel to yield bisindigotin (**1**) along with two known compounds, indirubin and indigotin (indigo),[5,6] which were identified by co-TLC with the authentic samples.

Bisindigotin (**1**) was obtained as a deep purple amorphous powder. The molecular formula was determined as $C_{32}H_{18}N_4O_2$ from the HRTOFMS, which exhibited a pseudomolecular ion peak $[M + H]^+$ at *m/z* 491.1516 (calcd 491.1508). The IR spectrum showed absorptions for NH groups (3390 cm^{-1}) and carbonyl groups (1716 cm^{-1}). The UV spectrum exhibited maximum absorptions at 227, 261, 352, and 568 nm. The ^{13}C NMR spectrum (Table 1) gave the signals for two carbonyls (δ 198.2 and 180.0), 16 aromatic methines, and 14 aromatic quaternary carbons.

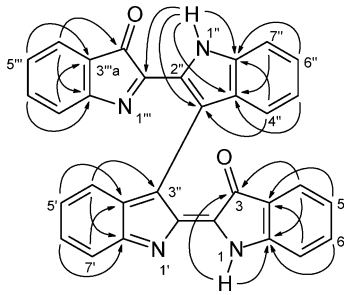


Figure 1. Main HMBC correlations of **1**.

The ^1H NMR spectrum of **1** (Table 1) showed eight broad doublets [δ 8.90, 8.28, 8.26, 7.74, 7.72, 7.04, 6.96, and 6.68 (each 1H, J = 8.0 Hz)] and seven broad triplets [δ 7.96, 7.43, 7.31, 7.13, 7.11, 6.79 (each 1H, J = 8.0 Hz), and 7.78 (2H, J = 8.0 Hz)], and these signals were attributed to four 1,2-disubstituted phenyl groups by analysis of the $^1H-^1H$ COSY spectrum. The spectrum also contained the proton signals for a chelated NH group at δ 12.09 (1H, br s) and a free NH group at δ 8.68 (1H, br s). The above evidence together with the $^{13}C-^1H$ COSY spectrum and HMBC (Figure 1) suggested that **1** is comprised of four indole moieties.[7,8] The two NH protons at δ 12.09 and 8.68 could be assigned to H-1″ and H-1, respectively, because the NOE interactions between the former and H-7″ and between the latter and H-7 were observed in the NOESY spectrum of **1**. The two carbonyls were located at C-3 and C-3‴, respectively, on the basis of the HMBC spectrum, in which the correlations were observed from the carbonyl carbon at δ 198.2 to H-4 and H-1 and from the higher field carbonyl carbon at δ 180.0 to H-4‴. Acetylation of **1** afforded a monoacetamide, 1-acetylbisindigotin (**2**). The ^{13}C NMR spectrum of **2** (Table 1) was closely similar to that of **1** except that the signals for an acetyl group were present and the signals of C-3 and C-7a were upfield shifted, while the signals of C-2, C-5, and C-7 downfield shifted when compared to **1**. This indicated the attachment of the acetyl group to N-1 in **2** and supported the presence of H-1 in **1**. The connection between C-2″ and C-2″ was indicated by the existence of a hydrogen bond between the oxygen of C-3‴ carbonyl and the hydrogen of H-1″, which was evidenced by the downfield shift of H-1″ in the ^1H NMR spectrum of **1** relative to those of indirubin and indigotin.[7,8] Ethylization of **1** afforded a 1-ethyl derivative, 1-ethylbis-

* To whom correspondence should be addressed. Tel: (852) 3411-7059. Fax: (852) 3411-5995. E-mail: nkmak@hkbu.edu.hk.
[†] Department of Biology, Hong Kong Baptist University.
[‡] South China Botanical Garden, Chinese Academy of Sciences.
[§] Guangzhou University of Traditional Chinese Medicine.
[⊥] Department of Chemistry, Hong Kong Baptist University.

10.1021/np049662i CCC: $30.25
Published on Web 02/16/2005

Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1**–**3**[a]

	1		**2**		**3**	
position	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	8.68 br s					
2		127.8		136.1		136.5
3		198.2		195.0		198.3
3a		117.3		120.0		117.1
4	7.72 br d (8.0)	124.4	7.98 br d (8.0)	125.2	7.75 br d (8.0)	125.2
5	7.11 br t (8.0)	120.3	7.64 br t (8.0)	127.2	7.10 br t (8.0)	120.7
6	7.78 br t (8.0)	139.4	8.17 br t (8.0)	141.1	7.89 br t (8.0)	141.4
7	7.04 br d (8.0)	112.1	8.68 br d (8.0)	118.5	7.14 br d (8.0)	109.7
7a		160.1		152.9		159.6
2′		138.4		139.6		139.3
3′		151.3		151.2		151.8
3′a		125.8		126.6		126.3
4′	8.28 br d (8.0)	122.6	8.32 br d (8.0)	124.1	8.28 br d (8.0)	123.7
5′	7.31 br t (7.6)	122.6	7.39 br t (7.6)	124.6	7.36 br t (7.6)	124.2
6′	7.43 br t (8.0)	132.2	7.48 br t (8.0)	134.0	7.50 br t (8.0)	133.7
7′	6.96 br d (8.0)	111.8	6.65 br d (8.0)	111.2	6.86 br d (8.0)	112.3
7′a		144.3		143.4		144.6
1″	12.09 br s		12.42 br s		12.42 br s	
2″		114.1		115.4		114.8
3″		108.5		106.6		107.1
3″a		122.3		122.1		122.7
4″	6.68 br d (8.0)	118.5	6.40 br d (8.0)	118.0	6.51 br d (8.0)	118.9
5″	6.79 br t (8.0)	120.0	6.80 br t (8.0)	121.7	6.82 br t (8.0)	121.4
6″	7.13 br t (8.0)	123.4	7.16 br t (8.0)	124.8	7.15 br t (8.0)	124.6
7″	7.74 br d (8.0)	113.1	7.81 br d (8.0)	114.6	7.77 br d (8.0)	114.4
7″a		138.6		139.8		139.5
2‴		131.0		131.9		132.6
3‴		180.0		180.8		180.8
3‴a		133.5		133.6		132.7
4‴	8.90 br d (8.0)	130.6	8.86 br d (8.0)	131.4	8.84 br d (8.0)	131.3
5‴	7.78 br t (8.0)	129.6	7.87 br t (8.0)	131.2	7.87 br t (8.0)	131.0
6‴	7.96 br t (8.0)	132.6	8.04 br t (8.0)	134.2	8.02 br t (8.0)	134.2
7‴	8.26 br d (8.0)	135.6	8.27 br d (8.0)	136.4	8.26 br d (8.0)	136.4
7‴a		145.0		145.7		145.6
*C*OCH$_3$				169.8		
CO*C*H$_3$			1.76 s	22.8		
*C*H$_2$CH$_3$					3.12 m, 3.28 m	37.8
CH$_2$*C*H$_3$					0.65 t (7.0)	13.8

[a] Solvent for **1**: mixture of DMSO-d_6, THF-d_8, and $CDCl_3$ (1:1:1). Solvent for **2** and **3**: mixture of DMSO-d_6 and THF-d_8 (1:1).

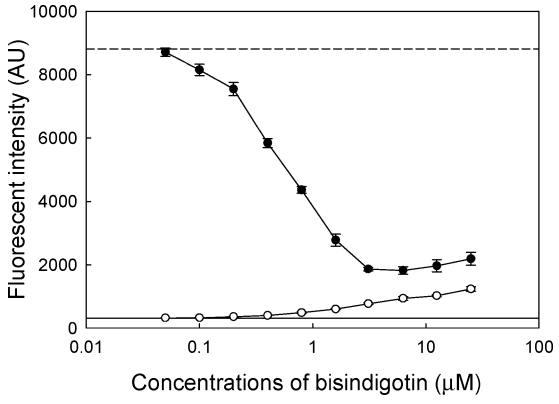


Figure 2. Inhibition of TCDD-induced EROD activity by bisindigotin (**1**): (○) bisindigotin; (●) TCDD (0.5 nM) + bisindigotin; (- - -) 0.5 nM TCDD; and (—) Hep G2 alone. Each point is reported as mean [(AU, arbitary units) ± SD (n = 4); P < 0.05]. Note: At the concentration range of 0–100 μM, bisindigotin alone was not cytotoxic to the HepG2 cells.

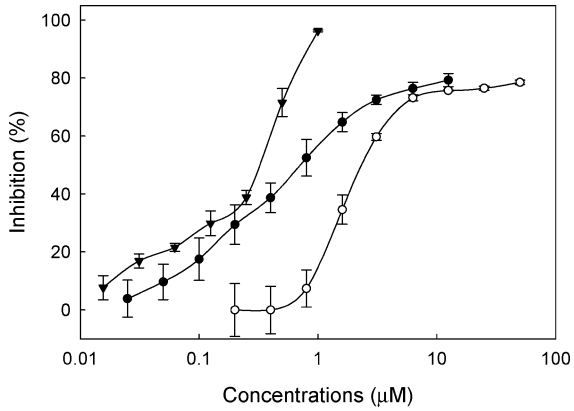
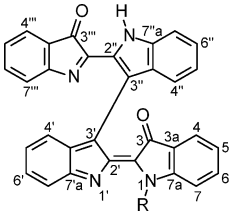


Figure 3. Inhibition of TCDD-induced EROD activity in HepG2 cells by α-naphthoflavone (▼), bisindigotin (**1**) (●), and acetylbisindigotin (**2**) (○) on TCDD-treated HepG2 cells. Experimental conditions were the same as those shown in Figure 2.

indigotin (**3**). The observation of an NOE interaction between the methyl protons of the Et-1 group and H-7′ in the NOESY spectrum of **3** indicated the linkage between C-2 and C-2′ via a double bond and a *Z* configuration of the double bond. Further, the linkage between C-3′ and C-3″ was readily derived, as it was the only possibility remaining. Thus, the structure of **1** was determined as shown.

The effect of **1** on TCDD-induced EROD activity was evaluated using the human HepG2 hepatoma cells. A dose-dependent (0.1–5 μM) inhibition of TCDD-induced EROD activity in the HepG2 cells was observed at 24 h after treatment (Figure 2). The 50% of inhibition of EROD activity (IC_{50}) was 0.8 μM. In the control experiment, the inhibitory activity of **1** was compared with the known antagonist α-naphthoflavone[1] and the derivative 1-acetyl-bisindigotin (**2**) (Figure 3). The IC_{50} of **1** (0.8 μM) was

slightly higher than α-naphthoflavone (0.32 μM). However, the activity of **1** was reduced after acetylation (IC_{50} = 3 μM). This result indicated that the chemical modification of **1** might modify the biological activity of **1**. It is noted that indirubin, a known indigoid alkaloid closely related to **1**, has previously been reported to function as an agonist for TCDD-induced activities.[9]



1 R = H
2 R = Ac
3 R = Et

Experimental Section

General Experimental Procedures. The UV spectra were recorded in THF on a Perkin-Elmer Lambda 35 UV−vis spectrophotometer. The IR spectrum was measured in KBr on a WQF-410 FT-IR spectrophotometer. The ^1H (400 MHz), ^{13}C (100 MHz), and 2D NMR spectra were recorded on a Bruker DRX-400 spectrometer with TMS as an internal standard. The HRTOFMS measurement was performed on an API Q-STAR Pulsari Q-TOF mass spectrometer in the positive-ion mode. The ESIMS data were taken on an API 2000 LC/MS/MS system. For column chromatography, Si gel 60 (200−300 mesh, Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China) and Develosil ODS (10 μm, Nomura Chemical Co. Ltd., Japan) were used. TLC was performed on precoated plates (Kieselgel 60F_{254}, Merck) and developed using a mixture of *n*-hexane−THF (2:1).

Plant Material. The leaves of *Isatis indigotica* Fort. were supplied by Anguo TCM Material Company, Anguo Town, Anguo County, Hebei Province, People's Republic of China, in June 2001, and authenticated by one of the coauthors, X.L.S. A voucher specimen (WXY0101) has been deposited at the herbarium of South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, People's Republic of China.

Extraction and Isolation. The powdered, air-dried leaves (2.0 kg) of *I. indigotica* were extracted with 95% EtOH (8 L × 5) by percolation at room temperature. The EtOH percolate was concentrated in vacuo to small volume (1 L). This concentracted EtOH solution was mixed with 1 kg of silica gel, and the mixture was dried in vacuo. The dried mixture was packed in a column and eluted sequentially with petroleum ether (bp 60−90 °C, 10 L) and $CHCl_3$ (8 L). The $CHCl_3$ eluent, upon evaporation, yielded a purple-brown syrup (40 g). This syrup was subjected to column chromatography over silica gel eluted with a petroleum ether−$CHCl_3$−EtOAc (5:4:1) mixture to yield **1** (100 mg) along with indirubin (150 mg) and indigo (130 mg).

Bisindigotin (1): deep purple amorphous powder; UV (THF) λ_{max} (log ϵ) 227 (4.94), 261 (4.78), 352 (4.62), 568 (4.38) nm; IR (KBr) ν_{max} 3390 (NH), 1716 (C=O), 1614, 1591, 1574, 1560, 1549, 1521, 1481, and 1469 (aromatic rings) cm^{-1}; ^1H (400 MHz) and ^{13}C (100 MHz) NMR, see Tables 1; ESIMS (positive-ion mode) *m/z* 491 $[M + H]^+$, 462 $[M - CO]^+$, 434 $[M - 2 \times CO]^+$; HRTOFMS (positive ion mode) *m/z* 491.1516 $[M + H]^+$ (calcd for $C_{32}H_{19}N_4O_2$, 491.1508).

Preparation of 1-Acetylbisindigotin (2). To a borosilicate glass vial with a cap were added 30 mg of compound **1**, 5 mL of pyridine, 2 mL of acetic acid anhydrous, and 200 mg of TsOH. The vial was tightly sealed with the cap and allowed to stand at 90 °C for 6 h. After being cooled to room temperature, the reaction mixture was poured into 50 mL of water. Filtration of the aqueous solution afforded a deep purple solid. This solid was subjected to passage over an ODS column and eluted with MeOH−H_2O (9:1). The purple eluent was collected and concentrated to dryness to afford **2** (15 mg) as a deep purple powder: UV (THF) λ_{max} (log ϵ) 261 (4.73), 346 (4.60), 561 (4.28) nm; ^1H (400 MHz) and ^{13}C (100 MHz) NMR data, see Table 1; ESIMS (positive-ion mode) *m/z* 533 $[M + H]^+$, 518 $[M + H - Me]^+$, 462 $[M + H - Ac - CO]^+$.

Preparation of 1-Ethylbisindigotin (3). Compound **1** (50 mg) was dissolved in 10 mL of THF. To the solution were added 10 mL of 15% aqueous NaOH, 100 mg of hexadecyltrimethylammonium bromide, and 0.4 mL of ethyl bromide. The reaction mixture was stirred at room temperature for 48 h. The THF layer was washed with the saturated aqueous NaCl, then dried with anhydrous Na_2SO_4, and concentrated to dryness. The residue was applied to column chromatography over silica gel eluted with petroleum ether−THF (2:1). The red eluent, after concentration, afforded a deep purple residue. The residue was subjected to further column chromatography over ODS eluted with MeOH−H_2O (9:1) to yield **3** (7 mg) as a deep purple powder: UV (THF) λ_{max} (log ϵ) 261 (4.77), 346 (4.63), 563 (4.31) nm; ^1H (400 MHz) and ^{13}C (100 MHz) NMR data, see Tables 1; ESIMS (positive-ion mode) *m/z* 557 $[M + K]^+$, 541 $[M + Na]^+$, 519 $[M + H]^+$, 461 $[M - Et - CO]^+$.

EROD Assay. EROD activity (7-ethoxyresorufin-*O*-deethylase) was determined as described previously.[10,11] Briefly, HepG2 cells (2×10^4 cells/well) were seeded in wells of a 96-well plate. The cells were then treated with various concentrations of compound **1** and TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, Cambridge Isotope Laboratories, Inc.) and incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h. After incubation, the culture medium was replaced with 100 μL of fresh medium containing 8 μM 7-thoxyresorufin (Sigma) and 10 μM dicumarol (Sigma). The mixture was then incubated at 37 °C for 1 h. Afterward the medium was transferred to a new 96-well plate and mixed with 130 μL of absolute ethanol. Resorufin-associated fluorescence was measured using a multiwell fluorescence reader (FluroStar), with excitaion/emission wavelength of 530/590 nm. Protein content was measured by a Bio-Rad protein assay kit. The total amount of cellular protein was used for data normalization after EROD assay.

Acknowledgment. This work was supported by a Group Research−Central Allocation and an earmarked research grant (project number HKBU 2036/00M) from the Research Grant Council of Hong Kong.

References and Notes

(1) Stermitz, F. R.; Bais, H. P.; Foderaro, T. A.; Vivanco, J. M. *Phytochemistry* **2003**, *64*, 493−497.
(2) Merchant, M.; Arellano, L.; Safe, S. *Arch. Biochem. Biophys.* **1990**, *281*, 84−89.
(3) MacDonald, C. J.; Ciolino, H. P.; Yeh, G. C. *Cancer Res.* **2001**, *61*, 3919−3924.
(4) Jiangsu New Medical College In *Dictionary of Traditional Chinese Medicines*; Shanghai Science and Technology Press, 1986.
(5) Fearon, W. R.; Boggust, W. A. *Biochem. J.* **1950**, *46*, 62−67.
(6) Friedmann, E.; Marrian, D. H.; Perutz, M. F. *Biochim. Biophys. Acta* **1950**, *5*, 45−52.
(7) Blanz, J.; Ehninger, G.; Zeller, K.-P. *Res. Comm. Chem. Pathol. Pharmacol*. **1989**, *64*, 146−156.
(8) Hosoe, T.; Nozawa, K.; Kawahara, N.; Fukushima, K.; Nishimura, K.; Miyaji, M.; Kawai, K. *Mycopathologia* **1999**, *146*, 9−12.
(9) Sugihara, K.; Kitamura, S.; Yamada, T.; Okayama, T.; Ohta, S.; Yamashita, K.; Yasuda, M.; Fujii-Kuriyama, Y.; Saeki, K.; Matsui, S.; Matsuda T. *Biochem. Biophys. Res. Commun.* **2004**, *318*, 571−578.
(10) Lai, K. P.; Li, W.; Xu, Y.; Wong, M. H.; Wong, C. K. *Environ. Res*. **2004**, *96*, 88−94.
(11) Lai, K. P.; Wong, M. H.; Wong, C. K. *Toxicol. Sci*. **2004**, *78*, 41−49.

NP049662I