Antifungal Agents from the Roots of *Cudrania cochinchinensis* against *Candida, Cryptococcus,* and *Aspergillus* Species

Toshio Fukai,*[,†] Makiko Yonekawa,[†] Ai-Jun Hou,[†,‡] Taro Nomura,[†] Han-Dong Sun,[§] and Jun Uno[⊥]

School of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan, School of Pharmacy, Fundan University, 138 Yi Xue Yuan Road, Shanghai 200032, People's Republic of China (PRC), Laboratory of Phytochemistry, Kunming Institute of Botany, Academia Sinica, Kunming 650204, Yunnan, PRC, and Division of Molecular Biology and Therapeutics, Research Center for Pathogenic Fungi and Microbial Toxicoses (RCPFMT), Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba, Chiba 260-8673, Japan

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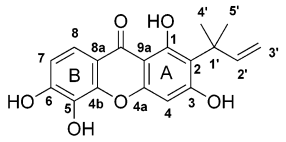
Bioassay-guided fractionation resulted in the isolation of four antifungal agents from the roots of *Cudrania cochinchinensis*. Two of these were new compounds, cudraxanthone S [**1**, 1,3,5,6-tetrahydroxy-2-(1,1-dimethyl-2-propenyl)xanthone] and cudraflavanone B (**2**, 2′,4′,5,7-tetrahydroxy-6-prenylflavanone). The remaining two compounds were known compounds, toxyloxanthone C (**3**) and wighteone (**4**). Among these compounds, **1**, **3**, and **4** exhibited antifungal activities against *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *A. nidulans* (MICs = 2−8 μg/mL). Compounds **1** and **3** also showed antifungal activity against *Candida glabrata* (MICs = 4−8 μg/mL).

The incidences of infections by opportunistic fungi are increasing, especially in patients whose immune systems are compromised by AIDS, cancer, diabetes, age, and other causes. Many antifungal compounds have been identified, but safe and effective antifungal drugs have not yet been developed because of the high degree of similarity between fungi and mammalian cells.[1] Therefore, amphotericin B (AMPH), which was developed many years ago,[2] is still widely used in treatment for deep-seated mycoses despite its serious side effects. Azole group antifungal agents, miconazole (MCZ), ketoconazole, fluconazole, and itraconazole, are also used clinically.[3] The side effects of these antibiotics are relatively weak, but these medicines have nephrotoxicity and hepatotoxicity and cause vomiting and impotence. This has consequently resulted in a strong demand for drugs that have much weaker side effects. Medicinal plants that have been used for a long time may be good sources of safe antifungal agents. The water or Chinese liquor extract of *Cudrania cochinchinensis* Lour. (Moraceae) is used for the treatment of gonorrhea, rheumatism, jaundice, hepatitis, boils, scabies, and bruising in traditional Chinese medicine. There is no report on side effects of this liquid medicine, but warnings are given against prescribing to pregnant women.[4] In the course of our continuous program for discovery of antifungal agents from natural sources, four antifungal phenols with an isoprenoid group were isolated from Chinese *C. cochinchinensis*. These antifungal agents showed selective activity against several human pathogenic fungi.

Benzene and ethyl acetate (EtOAc) solubles from an ethanol (EtOH) extract of the roots of *C. cochinchinensis* showed antifungal activity against *Candida albicans* by a disk diffusion method. Bioassay-guided fractionation of the EtOAc-soluble portion resulted in the isolation of two new compounds, cudraxanthone S (**1**) and cudraflavanone B (**2**), and two known compounds, toxyloxanthone C (**3**)[5] and wighteone (**4**),[6] along with a nonactive compound (+)-aromadendrin.[7]



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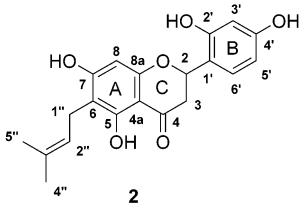

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Cudraxanthone S (**1**) decomposed at 162 °C and gave a positive reaction with $FeCl_3$ reagent by TLC. The compound was assigned a molecular formula of $C_{18}H_{16}O_6$ as inferred from its HR-MALDI-TOF-MS and NMR data. UV data suggested that **1** should have a 1,3,5,6-tetraoxygenated xanthone skeleton.[8] The ^{1}H NMR spectrum of **1** contained signals of four hydroxyl groups in which one was hydrogen-bonded (δ 14.23), *ortho*-coupled aromatic signals (B ring), an aromatic singlet signal (A ring), and a set of signals due to a 1,1-dimethyl-2-propenyl group. The chemical shift of the hydrogen-bonded OH indicated the isoprenoid group occurred at the C-2 position.[8] The ^{13}C NMR signals of **1** were assigned with its HMQC and HMBC spectra (data not shown). The chemical shifts of the A ring carbons (C-1−C-4, C-4a, and C-9a) of **1** resembled those of cudraxanthone P,[9] having the same A ring, and those of the B ring (C-5−C-8, C-4b, and C-8a) resembled those of macluraxanthone, with the same B ring (see Supporting Information). Furthermore, in the HMBC spectrum, the olefinic proton signal at δ 6.36 (H-2′) showed a cross-peak with C-2, also indicating that the group was located at C-2. Thus, cudraxanthone S was concluded to be 1,3,5,6-tetrahydroxy-2-(1,1-dimethyl-2-propenyl)xanthone (**1**).

Cudraflavanone B (**2**), $C_{20}H_{20}O_6$, $[\alpha]_D$ ± 0°, gave a positive reaction with $FeCl_3$ reagent by TLC. Its UV spectrum indicated that **2** was a flavanone or an isoflavanone. The NMR spectra showed characteristic signals of a flavanone together with a signal of an aromatic proton

* To whom correspondence should be addressed. Tel: (+81)-47-472-1792. Fax: (+81)-47-476-6195. E-mail: fukai@phar.toho-u.ac.jp.
† Toho University.
‡ Fundan University.
§ Kunming Institute of Botany.
⊥ Chiba University.

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(A ring), AXY type aromatic protons (B ring), a set of signals indicating a 3-methyl-2-butenyl (prenyl) group, and protons of a hydrogen-bonded OH and three normal OH groups. The chemical shift of 5-OH (δ 12.49) indicated that C-6 of **2** was substituted with the prenyl group.[9] In the ^{13}C NMR spectrum signals were assigned with HMQC and HMBC spectra (data not shown), the signals of the ring A (C-4a, C-5−C-8, and C-8a) resembled those of 6-prenylericiodictyol (3′,4′,5,7-tetrahydroxy-6-prenylflavanone),[9] and those of the B ring (C-1′−C-6′) were similar to those of leachianone G (2′,4′,5,7-tetrahydroxy-8-prenylflavanone) (see Supporting Information). In the HMBC spectrum, the methylene signal of the prenyl group (H_2-1″) showed cross-peaks with C-6 and C-5. Consequently, cudraflavanone B was elucidated as 2′,4′,5,7-tetrahydroxy-6-prenylflavanone (**2**).

Previously, we isolated 27 phenolic compounds from the benzene-soluble portion of an EtOH extract of *C. cochinchinensis*.[8] This portion also showed antifungal activity using a disk diffusion method in the present investigation [diameter of inhibition zone (DIZ) = 9 mm]. On our prescreening of antifungal compounds obtained from these active fractions (benzene- and EtOAc-soluble portions), eight compounds, **1**, **2**, **3**, **4**, 1,3,7-trihydroxy-2-prenylxanthone (**5**),[10] cudraphenones C and D (**6**),[8] and cudranone,[11] were active compounds (DIZs = 9−11 mm). Minimum inhibitory concentrations (MICs) of 12 phenolic compounds (the eight active compounds and some related compounds) from the plant against human pathogenic fungi, *Candida*, *Cryptococcus*, and *Aspergillus* species, were determined (see Supporting Information).

Compound **1** exhibited antifungal activities against *Candida glabrata*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *A. nidulans* (MICs = 2−4 μg/mL). The activities of **3**, a cyclic derivative of **1**, against these strains were slightly weaker than those of **1** (MICs = 8 μg/mL). The 1,3,7-trihydroxyxanthone with a hydrophobic group but without OHs at C-5 and C-6 (**5**) exhibited no activity against these microorganisms. The 1,5-dihydroxyxanthone with a hydrophobic group (6-deoxyjacareubin) also showed no activity against these strains (MICs > 128 μg/mL) with the exception of *C. neoformans* (MIC = 32 μg/mL). Recently, antifungal xanthones were isolated from *Calophyllum caledonicum*[12] and *Tovomita krukovii*.[13] These compounds exhibit antifungal activities against *A. fumigatus* and/or *C. albicans* and have a hydrophobic group on the A ring and a hydroxyl group at C-5 or C-6. It is likely that antifungal xanthones from plants require a hydrophobic group on ring A and three or four OHs in which one or two OHs must be at C-5 and/or C-6. Benzophenones are considered to be biosynthetic precursors of xanthones.[14] The benzophenones with two hydrophobic groups and three or four OHs (**6** and cudraphenone B) exhibited antifungal activities against *C. neoformans*, *A. fumigatus*, and *A. nidulans* (MICs = 2−16 μg/mL).

Flavanone **2** showed only weak antifungal activity against some species (see Supporting Information). The isoflavone with a hydrophobic group on ring A (**4**) was the most potent of the 12 compounds, with MICs for two *Aspergillus* species (MICs = 2−4 μg/L) and *C. neoformans* (MIC = 4 μg/mL). On the other hand, the isoflavone without a hydrophobic group (3′-*O*-methylorobol) showed no antifungal effect against all fungi investigated here. Previously, it was reported that flavones with two hydrophobic groups (morusin and kuwanon C, which are more hydrophobic than **4**) exhibit no antifungal activity except against *Trichophyton mentagrophytes* and *Microsporum gypseum* (MICs = 12.5−25 μg/mL).[15]

Experimental Section

General Experimental Procedures. AMPH and MCZ were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Sigma (St. Louis, MO), respectively. α-Cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) was used as the matrix of HR-MALDI-TOF-MS, and calibration was performed with two peaks of the matrix, *m/z* 190.05042 $[M + H]^+$ and *m/z* 379.09301 $[2M + H]^+$. The other general procedures, instruments, and chemicals were as described in our previous paper.[8,16]

Plant Material and Extraction. The roots of *C. cochinchinensis* Lour. (Moraceae) were collected in Xishuangbanna, Yunnan, PRC, in July 1998, and air-dried. The identity of plant material was verified by Prof. Zhong-Wen Lin (Kunming Institute of Botany), and a voucher specimen (KIB 98-7-20 Lin) was deposited in the Herbarium of the Department of Taxonomy, Kunming Institute of Botany, Academia Sinica. The dried and powdered roots (8.7 kg) were extracted with EtOH under reflux three times and filtered. The filtrate was evaporated in vacuo to give a residue (1 kg), which was suspended in water and partitioned successively with *n*-hexane, C_6H_6, EtOAC, and *n*-BuOH. The EtOAC-soluble portion investigated here was the same as that described previously.[8]

Isolation. The EtOAc-soluble portion (182 g) of the EtOH extract (DIZ = 13 mm) was subjected to column chromatography (CC) over silica gel eluted with a mixture of *n*-hexane and acetone to yield fractions 1−5. The following isolation procedure was guided by antifungal activity against *C. albicans* with a disk diffusion method (DDM), in which a paper disk was dipped directly into the eluted solution of each fraction of CC without concentration. Fraction 2 (*n*-hexane−acetone, 3:1; 93.7 g) was rechromatographed over silica gel (benzene−EtOAc, column 2) to yield fractions 2.1−2.20. Fraction 2.4 (benzene−EtOAc, 5:1; 8.3 g) was subject to CC over silica gel [first column, benzene−chloroform ($CHCl_3$); second column, *n*-hexane−EtOAc] to yield cudraxanthone S (**1**, 48 mg), toxyloxanthone C (**3**, 34 mg),[8] and wighteone (**4**, 3 mg).[8] From fraction 2.5 (15.5 g), 76 mg of (+)-aromadendrin,[6] $[\alpha]^{24}_D$ + 10.7° (*c* 0.33, MeOH), was obtained with crystallization from $CHCl_3$−EtOAc. The mother liquor was chromatographed over silica gel [first column, benzene−acetone; second column, *n*-hexane−$CHCl_3$; third column, benzene (saturated with H_2O)−MeOH; fourth column, $CHCl_3$ only], followed by ODS CC (MeOH−H_2O, 4:1) to give cudraflavanone B (**2**, 11 mg). The known compounds were identified by comparison of their spectral data with those reported previously (see Supporting Information).

Cudraxanthone S (1): granule (acetone); mp 162 °C (dec); $FeCl_3$ reaction on TLC plates, blue green; UV (MeOH) λ_{max} (log ϵ) 202 (4.24), 252 (4.53), 284 (3.88), 327 (4.24) nm; ^{1}H NMR (acetone-d_6, 400 MHz) δ 14.23 (1H, s, OH-1), 8.8−9.5 (3H, br, OH), 7.61 (1H, d, *J* = 9 Hz, H-8), 6.93 (1H, d, *J* = 9 Hz, H-7), 6.43 (1H, s, H-4), 6.36 (1H, dd, *J* = 10, 17 Hz, H-2′), 6.94 (1H, dd, *J* = 1, 17 Hz, H-3′), 4.84 (1H, dd, *J* = 1, 12 Hz, H-3′), 1.61 (6H, s, H_3-4′ and H_3-5′); ^{13}C NMR (acetone-d_6, 100 MHz) δ 181.4 (C, C-9), 164.6 (C, C-3), 163.9 (C, C-1), 156.4 (C, C-4a), 151.9 (C, C-6), 150.8 (CH, C-2′), 146.5 (C, C-4b), 133.5 (C, C-5), 117.5 (CH, C-8), 115.6 (C, C-2), 114.7 (C, C-8a), 113.5 (CH, C-7), 108.6 (CH_2, C-3′), 103.1 (C, C-9a), 95.2 (CH, C-4), 41.6 (C, C-1′), 29.2 (CH_3, C-4′ and C-5′); HRMALDITOFMS *m/z* 329.1063 $[M + H]^+$ (calcd for $C_{18}H_{17}O_6$, 329.1025).

Cudraflavanone B (2): resin (acetone−*n*-hexane); $[\alpha]^{24}_D$ ±0° (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (4.58), 225 (sh) (4.34), 291 (4.19), 335 (3.44) nm; ^{1}H NMR (acetone-d_6, 400 MHz) δ 12.49 (1H, s, OH-5), 9.4−8.3 (3H, br, OH), 7.29 (1H, d, *J* = 8 Hz, H-6′), 6.46 (1H, d, *J* = 2 Hz, H-3′), 6.41 (1H, dd, *J* = 2, 8 Hz, H-5′), 5.76 (1H, dd, *J* = 3, 13 Hz, H-2), 5.67 (1H, s, H-8), 5.23 (1H, m, H-2″), 3.24 (2H, br d, *J* = 7 Hz, H_2-1″), 3.13 (1H, dd, *J* = 3, 17 Hz, H-3), 2.68 (1H, dd, *J* = 13, 17 Hz, H-3), 1.73 (3H, br s, H_3-5″), 1.63 (3H, br s, H_3-4″); ^{13}C NMR (acetone-d_6, 100 MHz) δ 197.8 (C, C-4), 164.8 (C, C-7), 162.5

(C, C-5), 162.3 (C, C-8a), 159.5 (C, C-4′), 156.3 (C, C-2′), 131.1 (C, C-3″), 129.0 (CH, C-6′), 123.7 (CH, C-2″), 117.6 (C, C-1′), 108.9 (C, C-6), 107.9 (CH, C-5′), 103.5 (C and CH, C-4a and C-3′), 95.3 (CH, C-8), 75.3 (CH, C-2), 42.8 (CH_2, C-3), 25.8 (CH_3, C-4″), 21.5 (CH_2, C-1″), 17.8 (CH_3, C-5″); HRMALDITOFMS *m*/*z* 357.1336 $[M + H]^+$ (calcd for $C_{20}H_{21}O_6$, 357.1338).

Test Microorganisms. Strains of IFM (the collection of the former Institute of Food Microbiology, present RCPFMT) were derived from the collection of RCPRMT (*Aspergillus fumigatus* IFM 41374, *A. fumigatus* IFM 41236, *A. nidulans* IFM 5369), and *Candida albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, and *Cryptococcus neoformans* ATCC 90112 were from American Type Culture Collection (Rockville, MD). Yeasts were maintained on modified Sabouraud's dextrose agar slant (2% glucose, 1% peptone, and 1.5% agar) (SDA) or potato dextrose agar (Difco Laboratories, Detroit, MI) slant (PDA). Filamentous fungi were also maintained on PDA.

Disk Diffusion Method (DDM). Standard samples were dissolved in MeOH at 100 μg/mL (AMPH) and 10 μg/mL (MCZ). A paper disk (1.5 mm thick, 8 mm diameter, Tokyo Roshi, Tokyo, Japan) was dipped into the solution, and then the disk was air-dried on a filter paper on a clean bench. The inoculum amount of the stock culture of *C. albicans* ATCC 90028, preincubated in YPD-Broth (Difco), used for the DDM was adjusted by measuring the diameter of inhibition zone (DIZ) of the standard samples as follow: DIZs of AMPH and MCZ were 22 and 14 mm, respectively, when incubated on Yeast Morphology Agar (Difco) for 48 h at 30 °C. Each test sample was dissolved in MeOH at 400 mg of fraction/mL and 10 mg of pure compound/mL, and the amounts of the sample absorbed on a disk were approximately 15 and 0.4 mg/disk, respectively. Diameters of inhibition zone (DIZ) of the positive controls on the screening were 18 mm (AMPH, 100 μg/mL) and 15 mm (MCZ, 10 μg/mL).

MIC Measurement. Broth microdilution was according to the method of the National Committee for Clinical Laboratory Standards with a little modification as described previously.[17] All compounds were dissolved in dimethyl sulfoxide, and the final concentration of the solvents was less than 1%. The stock solution (12.8 mg/mL) was diluted with RPMI-1640 medium with a serial 2-fold dilution to concentrations from 0.128 to 128.0 μg/mL. Details of the procedure are given in the Supporting Information.

Supporting Information Available: Table of antifungal activity (MICs), ^{13}C NMR data of compounds related to **1** and **2**, data of known compounds, structures of tested compounds, and details of the MIC measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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