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Diaporthichalasin, a novel CYP3A4 inhibitor from an endophytic *Diaporthe* sp.

Surachai Pornpakakul,[a,*] Sophon Roengsumran,[a] Suchon Deechangvipart,[a] Amorn Petsom,[a] Nongnuch Muangsin,[a] Nattaya Ngamrojnavanich,[a] Nongluksna Sriubolmas,[b] Narongsak Chaichit[c] and Tomihisa Ohta[d]

[a]*Department of Chemistry, Faculty of Science, Chulalongkorn University, Phyathai Road, Bangkok 10330, Thailand*
[b]*Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road, Bangkok 10330, Thailand*
[c]*Department of Physics, Faculty of Science and Technology, Thammasart University, Rangsit, Pathum Thani 12121, Thailand*
[d]*Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan*

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Abstract—A novel CYP3A4 inhibitor, diaporthichalasin, together with pycnidione were isolated from an endophytic fungus, *Diaporthe* sp. Their structures were elucidated on the basis of spectral data and the structure of diaporthichalasin was confirmed by X-ray crystallographic analysis. Diaporthichalasin exhibited significantly potent inhibition of CYP3A4 with an IC_{50} value of 0.626 μM, while the IC_{50} value of pycnidione was 465 μM.

1. Introduction

Cytochrome P450 (CYP) enzymes have been mainly expressed in liver microsomes and are recognized to be responsible for drug metabolism, carcinogenesis, and degradation of xenobiotics. These enzymes constitute three families including CYP1, CYP2, and CYP3, which play an important role in the biosynthesis of lipids, steroids, and other secondary metabolites.[1] CYP3A4 is the most abundant enzyme in human liver microsomes,[2] metabolizing over 50% of drugs biotransformed by this enzyme.[3,4]

In an ongoing search for bioactive compounds of endophytic fungi, we discovered a novel compound, diaporthichalasin (**1**), and a known compound, pycnidione (**2**), isolated from the filamentous fungus *Diaporthe* sp. Bkk3,[5] an endophyte of *Croton sublyratus* leaves collected from Bangkok. We report herein the isolation, structure elucidation, and biological activities of both compounds.

Fermentation was carried out as follows. Micelia of the fungus growing on malt extract agar was inoculated in a 250 mL Erlenmeyer flask containing 100 mL of malt extract broth culture medium (malt extract 2%, glucose 2%, and bacterial peptone 0.1%) and cultured statically at room temperature for 60 days. Twenty liters of whole broth was filtered through filter paper (Whatman no. 1) to separate the broth supernatant and mycelia. The mycelia were extracted with hexane, dichloromethane and methanol, respectively. The dichloromethane extract was evaporated under reduced pressure to afford a brown viscous crude material (10 g), which was subjected to silica gel column chromatography and eluted with hexane–dichloromethane and dichloromethane–methanol in a stepwise fashion. Similar fractions were combined on the basis of TLC with detection by UV light and vanillin/H_2SO_4 reagent.

Compound **1** was crystallized from the combined fractions eluted with dichloromethane–methanol (97:3) during evaporation of the solvent and obtained after filtration as a white solid (1.5 g). The solid fraction

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* Corresponding author. Tel.: +66 2 2187637/66 81 5620555; fax: +66 2 2187598; e-mail: psuracha@chula.ac.th

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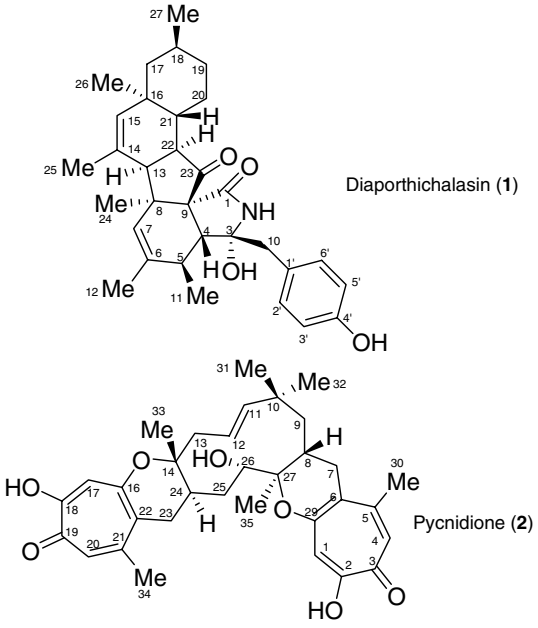
obtained from elution with dichloromethane–methanol (98:2) was washed with diethyl ether to give **2** as a pale orange solid (250 mg).



Compound **1** had a molecular formula of $C_{32}H_{41}NO_4$ as established by HRESIMS.[6] ^1H and ^{13}C NMR data presented in Table 1 indicated the presence of a *para*-substituted benzene ring, six methyl groups, two double bonds, and two carbonyl groups. Since the 13 degrees of unsaturation were accounted for, it was implied that **1** should contain five additional rings. The HMBC experiment (Table 1) with the assistance of COSY, TOCSY, and NOESY (Fig. 1) led to the structure of **1**. The large coupling constant of H-22 with H-21 ($J_{H\text{-}22/H\text{-}21} \approx 12$ Hz) and the observed NOEs between H-22 and H-13 and between H-13 and the methyl protons of C-24 in the NOESY experiment suggested that H-22 was axially orientated and occupied the same face as H-13 and the methyl protons of C-24. The observed NOEs between H-4 and the methyl protons of C-11 and between H-4 and H-10 in the NOESY experiment revealed that they occupied the same faces. Due to non-observation of NOEs between H-21 and the methyl protons of C-26 and between the methyl protons of C-26 and the methyl protons of C-27, it was suggested that the methyl protons of C-26 were on the opposite face to H-21 and the methyl protons of C-27. The configurations of **1** were thereby established except for the configuration at C-9. Fortunately, compound **1** could be crystallized from acetonitrile in the presence of a small amount of water and the complete relative configurations of **1** were finally established by X-ray crystallographic analysis (Fig. 2).[7] It revealed that compound **1** was a novel compound, diaporthichalasin, which was an isomer of the previous known phomopsichalasin,[8] an antibacterial, isolated from an endophytic *Phomopsis* sp. Further support for the difference of diaporthichalasin and phomopsichalasin came from the fact that diaporthichalasin showed a different specific optical rotation with a strong negative optical rotation, $[\alpha]_D^{20}$ −135 (*c* 0.14, MeOH), compared with phomopsichalasin, $[\alpha]_D^{25}$ −7.16[8] and diaporthichalasin exhibited no antimicrobial activity against *Bacillus subtilis* ATCC 6633,

Table 1. NMR spectral data[a] for **1** in DMSO-d_6

Position	δ_C	δ_H (mult; *J*, Hz)	HMBC (H→C)
1	174.83	—	
2	—	8.58 (s)	1, 3, 4, 9
3	87.99	—	
4	49.27	2.47 (s)	3, 5, 6, 8, 9, 11, 23
5	28.82	2.03 (m)	3, 4, 6, 7, 9, 11
6	134.77	—	
7	126.13	5.08 (s)	5, 8, 9, 12, 13
8	43.64	—	
9	63.62	—	
10	44.07	2.86 (s)	3, 4, 1′, 2′, 6′
11	20.04	0.71 (d, 7.2)	4, 5, 6
12	22.44	1.58 (s)	5, 6, 7
13	50.14	2.72 (d, 8.0)	7, 8, 14, 15, 21, 22, 24
14	128.13	—	
15	137.95	5.36 (s)	13, 16, 17, 21, 25
16	35.56	—	
17	47.80	0.58 (dd, 12.4 and 12.0)	16, 18, 19, 26, 27
		1.41 (br d, 12.8)	16
18	26.72	1.54 (m)	
19	35.37	0.46 (br q, 12.4)	
		1.62 (br d, 9.2)	
20	22.99	0.98 (br q, 12.4)	
		1.37 (m)	
21	40.34	1.32 (dd, 12.8 and 13.2)	26
22	48.75	2.03 (dd, 12 and 8.4)	8, 9, 13, 14, 16, 21
23	218.95	—	
24	25.49	1.47 (s)	7, 8, 9, 13
25	24.98	1.82 (s)	13, 14, 15
26	19.52	0.74 (s)	15, 16, 17, 21
27	22.66	0.75 (d, 7.6)	17, 18, 19
1′	126.69	—	
2′ and 6′	131.60	7.09 (d, 8.4)	10, 3′, 4′
3′ and 5′	114.83	6.67 (d, 8.4)	1′, 4′
4′	155.93	—	
OH	—	5.63 (s)	
OH	—	9.26 (br s)	

[a] Data were obtained at 400 MHz for ^1H and 100 MHz for ^{13}C NMR with chemical shifts (δ) in ppm and were referenced to residual solvent signals with resonances at δ_H 2.54 and at δ_C 39.52.

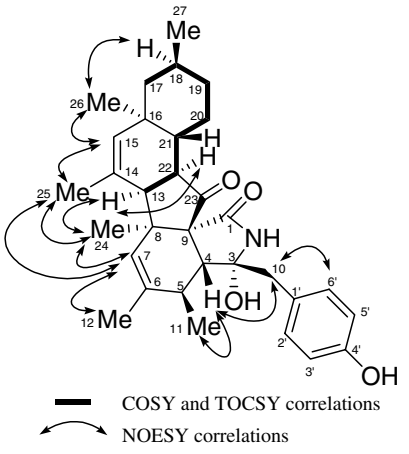


Figure 1. COSY, TOCSY, and NOESY correlations.

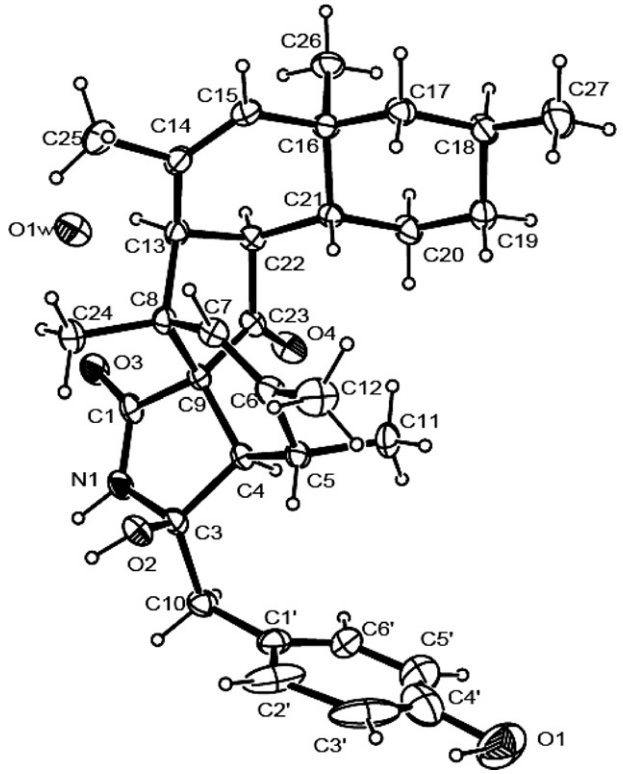
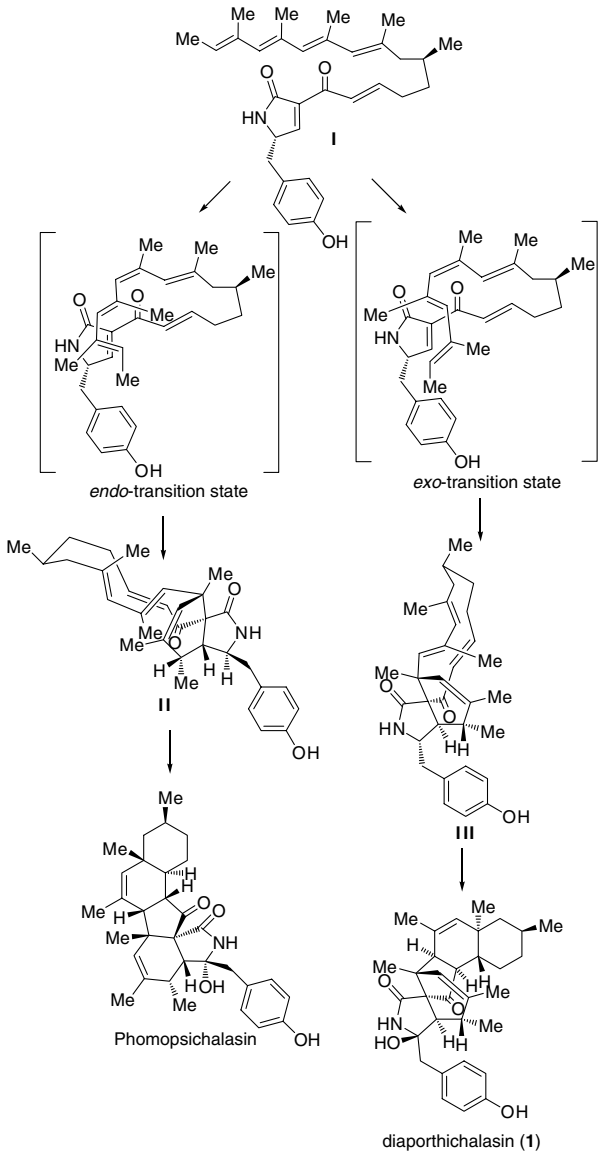


Figure 2. X-ray crystal structure of diaporthichalasin (**1**).

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231 at <125 μg/mL using microtiter plate broth dilution assay.

In a general biosynthetic pathway of cytochalasins, Vederas and Tamm suggested that cytochalasin-type metabolites are constructed from an amino acid and a polyketide unit, c.f. **I**.[9] On the basis of cytochalasin biosynthesis, the phenol moiety in **1** is derived from tyrosine and the fused rings of **1** are created by double Diels–Alder closure.[10,11] The perhydroisoindole moiety of diaporthichalasin (**1**) is thought to arise through an *exo*-selective intramolecular Diels–Alder reaction similar to chaetochalasin A[12] while the perhydroisoindole moiety of phomopsichalasin is thought to arise through an *endo*-selective intramolecular Diels–Alder reaction. The Diels–Alder reactions of putative **II** and **III** provide the cycloadducts, which then undergo hydroxylation to give phomopsichalasin and diaporthichalasin (**1**), respectively (Scheme 1).

The mass spectral data (HRESIMS) of **2** indicated the molecular formula of $C_{33}H_{40}O_7$. 1D and 2D NMR spectra,[13] including COSY, TOCSY, HSQC, and HMBC, which established the structure of **2**. The coupling constant of H-25 with H-26 ($J \approx 11$ Hz) suggested that they were axially oriented. In the NOESY experiment, the cross peak observed between H-8 and H-26 and between H-26 and the methyl protons of C-33 revealed that they were on the same faces. Since no NOEs were observed between H-8 and the methyl protons of C-35 and between H-24 and the methyl protons of C-33, this



Scheme 1. Proposed biosynthetic pathway for diaporthichalasin (**1**) and phomopsichalasin.

indicated that both pyran rings were trans-fused to the 11-membered ring and therefore compound **2** should have the same structure as pycnidione, isolated from *Phoma* sp. as a stromelysin inhibitor[14] and from the fungus OS-F69284 (ATCC 74390).[15]

CYP3A4 activity was monitored by nifedipine oxidation with expressed human CYP3A4. Ketokonazole was used as positive control and exhibited inhibition of CYP3A4 with an IC_{50} value of 0.11 μM. Compound **1** exhibited significantly potent inhibition of CYP3A4 with an IC_{50} value of 0.626 μM, while the IC_{50} value of **2** was 465 μM.

2. Assay of CYP inhibition[16]

CYP activity was based on nifedipine oxidation. Various amounts (0–10 μM, final concentration) of samples in

1 μL of DMSO were added to 192 μL of a solution containing 100 mM phosphate buffer (pH 7.4) containing 50 μM nifedipine (Wako Pure Chemical Industries, Ltd. (Osaka, Japan)), 5 mM glucose-6-phosphate (Oriental Yeast Co., Ltd. (Tokyo, Japan)), 0.5 mM β-$NADP^+$ (Oriental Yeast Co., Ltd.), 0.5 mM $MgCl_2$, and 4.3 μg/mL glucose-6-phosphate dehydrogenase (Oriental Yeast Co., Ltd.) and incubated at 37 °C for 5 min. CYP3A4 (Gentest Co. (Woburn, MA)) was also preincubated in 7 μL of the buffer at 37 °C for 5 min and added to the sample solution. After incubation at 37 °C for 1 h, the reaction was quenched by the addition of 100 μL of MeOH. After adding 3.7 μg of 6-methoxycarbonyl-5-methyl-7-(2-nitrophenyl)-4,7-dihydrofuro[3,4-*b*]-pyridin-1-(3*H*)-one in 1 μL of DMSO as an internal standard, the reaction mixture was extracted with 1 mL of ether, and the ether layer was evaporated. The residue was dissolved in 100 μL of MeOH, and an aliquot (20 μL) was analyzed by reverse-phase HPLC (column, TSK-gel ODS-120T, 4.6 mm i.d. × 150 mm; mobile phase, 64% MeOH–H_2O; flow rate, 1.0 mL/min; detection, UV 254 nm); retention times: 2.9 min for the internal standard, 4.0 min for the nifedipine metabolite (nifedipine pyridine), and 5.5 min for nifedipine. The value of IC_{50}, the concentration required for 50% inhibition of CYP3A4 activity, was calculated from the data of duplicate measurements.

Acknowledgements

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Supplementary data

Supplementary data (for the isolated natural products) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.11.102.

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5. Endophytic fungus isolate Bkk3 was identified based on fungal morphology and analysis of the DNA sequence of the ITS region of ribosomal RNA gene. The fungus grew on PDA as a white filamentous fungus colony. On banana leaf agar it did not develop any fruiting body or conidia after cultivation for 2 months at 25 °C. The DNA sequence of ITS1-5.8S-ITS2 of Bkk3 has been submitted to GenBank with accession numbers of DQ435767. A GenBank search for similar sequences to its ITS region revealed *Diaporthe phaseolorum* as the closest match with 96% sequence identity. Phylogenetic analysis with 19 known close species hit (*Diaporthe* and *Phomopsis*, its anamorph, 90–96% sequence identities) using maximum parsimony placed it in the same clade with *D. phaseolorum* with 80% bootstrap support. These results suggested that Bkk3 should be a filamentous fungus *Diaporthe* sp.
6. Diaporthichalasin **1**: mp 169–170 °C; $[\alpha]_D^{20}$ −135 (*c* 0.14, MeOH); λ_{max} (MeOH) (log ε) 233 (3.51) and 277 (3.04) nm; ν_{max} (KBr) 3409, 3339, 2948, 2361, 1722, 1687, 1509, 1448, 1378, 1226, 1104, 1026, 857, 770 cm^{-1}; ESI-TOF/MS *m*/*z* 526.2927 $[M+Na]^+$ calcd for $C_{32}H_{41}NO_4Na$ 526.2933.
7. Crystal data of **1** were obtained using a BRUKER SMART CCD diffractometer, MoK_α radiation (λ = 0.71073 Å), graphite monochromator, $C_{32}H_{41}NO_4$, monoclinic, space group $P2_1$, unit cell dimensions $a = 9.3143(1)$ Å, $b = 9.4664(1)$ Å, $c = 16.7682(2)$ Å, $\beta = 998.87(2)°$, $V = 1460.85(3)$ $Å^3$, $D_{calcd} = 1.186$ g/cm^3, $Z = 2$, $F(000) = 564$, $\mu = 0.079$ mm^{-1}. Data were collected at 293(2) K using ω–2θ scans in the ranges $\theta = 1.23$–$30.33°$. A total of 10,710 reflections was collected, 5745 were unique ($R_{int} = 0.0132$). The structure was refined by full-matrix least-squares on F^2. The non hydrogen atoms were refined anisotropically. Hydrogen atoms were located in difference Fourier maps and refined isotropically. The final refinement $[I > 2\sigma(I)]$ gave $R_1 = 0.0659$, $wR_2 = 0.1932$. Crystallographic data (excluding structure factors) of **1** has been deposited with the Cambridge Crystallographic Data Center as supplementary publication number CCDC 619406. Copies of this information may be obtained free of charge from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-1223-336-033; email: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk).
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13. Pycnidione **2**: mp 286 °C (decomp); $[\alpha]_D^{20}$ +317 (*c* 0.045, $CHCl_3$); λ_{max} (MeOH) (log ε) 260 and 363 (4.58 and 4.23) nm; ν_{max} (KBr) 3860, 3734, 3669, 3608, 3161, 2948, 1700, 1630, 1591, 1523, 1443, 1388, 1280, 1154, 1084, 993, 897, 774, 667 cm^{-1}; δ_H ($CDCl_3$, 400 MHz) 0.78 (1H, dd, $J = 4$ and 14.4 Hz, H-9), 1.07 (3H, s, H_3-31), 1.10 (3H, s, H_3-32), 1.14 (3H, s, H_3-35), 1.41 (3H, s, H_3-33), 1.54 (1H, dd, $J = 11.6$ and 12.0 Hz, H-25), 1.79 (1H, d, $J = 15.2$ Hz, H-9), 1.82 (1H, br s, H-8), 2.20 (1H, m, H-24), 2.23 (1H, m, H-25), 2.37 (1H, d, $J = 16$ Hz, H-7), 2.38 (3H, s, H_3-30), 2.41 (3H, s, H_3-34), 2.42 (1H, dd, $J = 4$ and 17.2 Hz, H-23), 2.52 (1H, dd, $J = 10.8$ and 13.2 Hz, H-13), 2.73 (1H, br dd, $J = 2.4$ and 13.2 Hz, H-13), 2.85 (1H, dd, $J = 5.2$ and 17.2 Hz, H-7), 3.38 (1H, dd, $J = 13.6$ and 17.2 Hz, H-23), 4.22 (1H, d, $J = 11.2$ Hz, H-26), 5.68 (1H, ddd, $J = 4.4$, 10.4 and 15.6 Hz, H-12), 5.80 (1H, d, $J = 16.8$ Hz, H-11), 6.92 (1H, s, H-1), 6.95 (1H, s, H-17), 7.13 (1H, s, H-20) and 7.16 (1H, s, H-4);

δ_C ($CDCl_3$, 100 MHz) 15.94 (C-35), 19.19 (C-33), 27.0 (C-32), 27.28 (C-34), 27.34 (C-30), 29.06 (C-31), 30.04 (C-25), 31.81 (C-8), 32.86 (C-23), 34.18 (C-7), 34.82 (C-10), 41.28 (C-24), 46.13 (C-13), 46.41 (C-9), 70.64 (C-26), 80.35 (C-14), 81.84 (C-27), 112.3 (C-1), 113.32 (C-17), 118.48 (C-6), 122.27 (C-22), 124.58 (C-20), 125.48 (C-4), 125.71 (C-12), 143.89 (C-11), 150.37 (C-21), 151.31 (C-5), 159.33 (C-29), 160.13 (C-16), 162.71 (C-2), 163.00 (C-18), 172.47 (C-19) and 173.20 (C-3); ESI-TOF/MS *m*/*z* 549.2844 $[M+H]^+$ calcd for $C_{33}H_{41}O_7$ 549.2852.

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