Cyclopenta[*b*]benzofurans from *Aglaia* Species with Pronounced Antifungal Activity against Rice Blast Fungus (*Pyricularia grisea*)

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Eight flavaglines, six cyclopenta[*b*]benzofurans, a cyclopenta[*bc*]benzopyran, and a benzo[*b*]oxepine, together with an aromatic butyrolactone were isolated from *Aglaia odorata*, *A. elaeagnoidea*, and *A. edulis* (Meliaceae) and tested against the three plant pathogens *Pyricularia grisea*, *Fusarium avenaceum*, and *Alternaria citri* for antifungal properties. Using the microdilution technique linked with digital image analysis of germ tubes, the benzofurans displayed strong activity, whereas the benzopyran, benzoxepine, and butyrolactone were inactive at the highest concentration tested. *P. grisea*, responsible for rice blast disease, was the most susceptible fungus against all benzofurans, with rocaglaol as the most active derivative. Based on EC_{50}, EC_{90}, and MIC values, the antifungal activity of rocaglaol was clearly higher than of the reference compounds, blasticidin S and Benlate.

Keywords: *Cyclopenta[b]benzofurans; cyclopenta[bc]benzopyrans; benzo[b]oxepines; flavaglines; rocaglaol; aglafolin; pannellin; rocaglamide; aglaroxin A; desmethyl rocaglamide; Meliaceae; Aglaia; antifungal; microdilution; Pyricularia grisea; Alternaria citri; Fusarium avenaceum*

INTRODUCTION

The formation of cyclopenta[*b*]benzofurans represents a typical chemical trend in the genus *Aglaia* of the family Meliaceae. Together with structurally related benzo[*bc*]pyrans and benzo[*b*]oxepines they constitute a distinct class of natural products recently named flavaglines (Brader et al., 1998; Bacher et al., 1999). Several derivatives were shown to possess very high insecticidal activity (Ishibashi et al., 1993; Janprasert et al., 1993; Satasook et al., 1994; Nugroho et al., 1997a,b; Brader et al., 1998) as well as significant cytotoxicity in many different cancer cell lines (King et al., 1982; Wu et al., 1997; Cui et al., 1997). They also specifically can inhibit protein synthesis (Ohse et al., 1996) or completely block platelet aggregation (Wu et al., 1997). With respect to these different bioactivities, comparatively less is known about antifungal properties of flavaglines. Up to now only one observation has been reported based on bioautography on TLC plates (Homans and Fuchs, 1970) using *Cladosporium cucumerinum* Ellis & Arthur as test fungus (Fuzzati et al., 1996). In this case the flavagline ester aglafolin (**2**) inhibited the growth of the fungus at 2.5 μg whereas the reference compound, propiconazole, was already active at 0.01 μg.

In the course of our joint research project with the Kasetsart University in Bangkok, Thailand, a broad-based phytochemical screening within Rutaceae (Greger et al., 1996; Vajrodaya et al., 1998) and Meliaceae (Brader et al., 1998; Bacher et al., 1999) linked with bioassays of the corresponding crude extracts was carried out to discover new biologically active compounds. In this connection special attention was focused on extracts with strong antifungal activity against *Pyricularia grisea* (Cooke) Sacc. (teleomorph, *Magnaporthe grisea* Barr), the causative fungus of rice blast disease. This is one of the most important diseases of rice, resulting in yield losses ranging from 50% to 90% of the expected crop (Agrios, 1997). Although crude extracts of *Aglaia* species did not show clear inhibition zones in our current bioautographic screening on TLC plates against the routine test fungus *Cladosporium herbarum* (Pers.: Fr.) Link, some of them displayed very high antifungal activity against *P. grisea* in germ tube inhibition tests in microwells. Consequently, a more detailed screening in microwell plates was essential to discover active samples of different *Aglaia* species as well as to establish the different activities of isolated compounds.

Based on germ tube inhibition tests in 2-fold serial broth dilutions (microdilution) six flavaglines, isolated from *A. odorata* Lour., *A. edulis* (Roxb.) Wall., and *A. elaeagnoidea* (A. Juss.) Benth. were shown to possess strong antifungal activity against *P. grisea*. The already known rocaglaol (**1**) (Ishibashi et al., 1993) exhibited the highest activity with an EC_{50} value as low as 0.01 μg mL^{-1} and a minimal inhibitory concentration (MIC) at 1.6 μg mL^{-1}; both values are lower than those of the widely used commercial fungicides Benlate and blasticidin S (Table 1). In the present paper we describe the different antifungal activities of eight flavaglines and the probably related aglalactone (**9**) (Brader et al., 1998) against *P. grisea* as well as against two other plant pathogens, *Alternaria citri* Ellis & Pierce emend. Bliss & Fawcett and *Fusarium avenaceum* (Corda: Fr.) Sacc., also known as agressive fungi causing various diseases, e.g., the alternaria rot in citrus fruits (Brown and Eckert, 1988) and fusarium head blight in different

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Table 1. Germ Tube Inhibition Effect of Compounds 1−9 against Three Plant Pathogenic Microfungi[a]

	Pyricularia grisea			*Fusarium avenaceum*			*Alternaria citri*		
μg mL^{-1}	EC_{50} (95% FL)	EC_{90} (95% FL)	MIC	EC_{50} (95% FL)	EC_{90} (95% FL)	MIC	EC_{50} (95% FL)	EC_{90} (95% FL)	MIC
1	0.01 (0.006−0.02)	0.3 (0.2−0.6)	1.6	0.4 (0.2−0.6)	9 (5−19)	25	6 (3−9)	24 (15−83)	50
2	0.05 (0.02−0.15)	8 (2−73)	3	1.6 (0.9−2.8)	38 (18−129)	100	44 (22−115)	171 (77−2232)	200
3	0.06 (0.01−0.24)	171 (17−8917)	50	11 (3−157)	>200	200	>200	>200	>200
4	0.9 (0.5−1.7)	35 (14−166)	25	8 (4−14)	66 (32−160)	200	>200	>200	>200
5	1.4 0.2−6)	>200	100	nd	nd	nd	nd	nd	nd
6	7 (4−15)	146 (50−1780)	100	nd	nd	nd	nd	nd	nd
7	>200	>200	>200	nd	nd	nd	nd	nd	nd
8	>200	>200	>200	nd	nd	nd	nd	nd	nd
9	>200	>200	>200	nd	nd	nd	nd	nd	nd
Benlate[b]	0.06 (0.02−0.2)	84 (10−3404)	200	0.02 (0.002−0.1)	>200	100	>200	>200	>200
blasticidin *S*[b]	13 (5−56)	>200	>200	0.4 (0.2−0.7)	7 (4−19)	25	6 (3−12)	53 (24−280)	50

[a] 100 μL stock solution (2 mg of test compound/250 μL of acetone/4.75 mL of 4% malt extract [w/v], emulgated with 0.2% Tween 80) were 2-fold microdiluted in 4% malt extract broth (w/v); negative control, stock solution without test compound. 50 μL of spore suspension (10^4 cfu, 4% malt extract broth [w/v]) were added per well. After incubation for 16 h at room-temperature growth was stopped by adding 10 μL of lactophenol blue to each well. Mycelial size was determined by pixel counts after capturing images of 10 germinated spores per well on a hard disk. Probit-log estimates were calculated from a concentration range of 200−0.1 μg mL^{-1} for **4**−**9**, and blasticidin S, and of 200−0.000 02 μg mL^{-1} for compounds **1**−**3**, and Benlate. [b] Positive control; FL, fiducial limits; MIC (minimum inhibitory concentration), lowest concentration showing no conidiospore germination; nd, not determined.

grain crops (Bottalico, 1998). The fungitoxic properties were calculated by image analysis of germinated conidiospores.

MATERIALS AND METHODS

General Experimental Procedures. The following equipment was used: UV, Hewlett-Packard, 8452 A diode array spectrophotometer; IR, Perkin-Elmer, 16PC FT-IR; HPLC, Hewlett-Packard 1090II, UV-diode array detection at 230 nm, Hypersil BDS C18 5 μm, 250 × 4 mm; microscopy, Olympus SZH10 stereo microscope equipped with an Olympus DF Plan 2× lens and a Sony DXC-C1MDP video camera. Fungal culture media, potato glucose agar, and malt extract broth were supplied by Merck.

Plant Material. Leaves, stem, and root bark of *Aglaia* species were collected separately: (a) *A. elaeagnoidea* (HG14, HG16, HG18) from southeast Thailand, Chantaburi, Khao Soi Dao; (b) *A. edulis* (HG515) from southwest Thailand, Prachuap Khiri Khan, Thap Sakae; (c) *A. odorata* (HG501) from southeast Thailand, Rayong, Ko Samet. Voucher specimens are deposited at the Herbarium of the Institute of Botany, University of Vienna (WU).

Extraction and Isolation. Dried parts of *Aglaia* species were ground and extracted with MeOH at room temperature for 3 days, filtered, and concentrated. The aqueous residue was extracted with $CHCl_3$. The obtained fraction was roughly chromatographed on silica (Merck Si 60, 0.2−0.5 mm, 600 × 20 mm column), using hexane/EtOAc mixtures with EtOAc increasing from 15% to 100% and finally 100% MeOH, and further by preparative MPLC (400 × 40 mm column, Merck Lichroprep Si 60, 25−40 μm, UV detection, 254 nm) using a step-gradient elution of 30% and 50% EtOAc in hexane and 100% EtOAc. Preparative TLC (Merck, Si gel 60, 0.5 mm) was used to finally purify the compounds (compare Brader et al., 1998; Bacher et al., 1999).

Rocaglaol (1), Aglafolin (2), Rocaglamide (4), and Desmethyl Rocaglamide (6). A portion (1000 mg) of the $CHCl_3$ fraction from 23 g of dried root bark of *A. odorata* was roughly separated by column chromatography (Si gel). Elution with 30% EtOAc in hexane and 100% EtOAc afforded a mixture (326 mg) containing **1**, **2**, and **4**; finally with 100% EtOAc and MeOH it afforded a mixture (172 mg) containing **4** and **6** (detected by HPLC). Further separation of the first mixture (326 mg) by MPLC with 30% EtOAc in hexane yielded a fraction with 15 mg of pure rocaglaol (**1**) and a fraction containing **1** and **2**. Preparative TLC (CH_2Cl_2:EtOAc:MeOH = 40:56:4) of the latter fraction gave 4 mg of rocaglaol (**1**) and 10 mg of aglafolin (**2**). The MPLC fraction with 100% EtOAc afforded 10 mg of rocaglamide (**4**). MPLC separation of the second mixture (172 mg) with 100% EtOAc yielded various fractions containing **4** and a fraction with higher retention time containing **6**. All fractions were combined and purified by preparative TLC as mentioned above, to give again 20 mg of rocaglamide (**4**) and 3.5 mg of desmethyl rocaglamide (**6**). Chromatographic, optical, and spectroscopic data of all compounds were consistent with those reported previously (King et al., 1982; Ishibashi et al., 1993; Janprasert et al., 1993; Fuzzati et al., 1996).

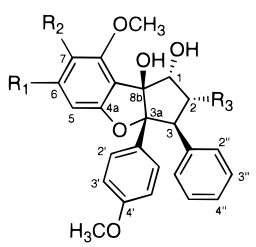
Pannellin (3), Aglaroxin A (5), Thapoxepine A (7), and Thapsakinacetate A (8). Dried root bark (263 g) of *A. edulis* afforded 3 mg of pannellin (**3**), 24 mg of aglaroxin A (**5**), 41 mg of thapoxepine A (**7**), and 22 mg of thapsakinacetate A (**8**) as described in Bacher et al. (1999).

Aglalactone (9). Dried stem bark (200 g) of *A. elaeagnoidea* afforded 2 mg of aglalactone (**9**) as described in Brader et al. (1998).

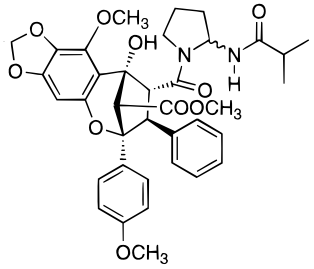
Fungicides. Benlate (50% benomyl) was obtained from Du Pont (USA) and blasticidin S was obtained from ICN Biomedicals (USA) for positive controls.

Preparation of Stock Inoculum Suspensions. Authentic samples of test fungi are deposited at the culture collection of the Institute of Applied Microbiology, Agricultural University of Vienna (VIAM,) and determined by R. A. Samson, Centraalbureau voor Schimmelcultures Baarn-Delft, Netherlands (CBS). *Pyricularia grisea* (VIAM MA1627), provided by Ms. Arunee Surin, Rice Disease Research Group, Plant Pathology and Microbiology Division, Ministry of Agriculture, Bangkok (originally isolated from rice grown near Saraburi, central Thailand), *Alternaria citri* (CBS 192.81; VIAM MA1628), and *Fusarium avenaceum* (VIAM MA1512) were grown on 4% potato glucose agar (w/v) at room temperature. Conidiospores were harvested after either 14 days (*A. citri*, *P. grisea*) or 3 days (*F. avenaceum*) and suspended in a 0.9% NaCl solution (w/v) containing 5% DMSO (v/v). They were stored either in liquid nitrogen (*A. citri*, *F. avenaceum*) or in a refrigerator at 4 °C (*P. grisea*). The number of colony forming units (cfu) was determined by spreading 20 μL of the 10-times microdiluted suspension in a Petri dish and by counting germinated spores per dilution.

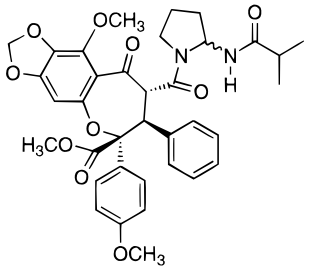
Antifungal Bioassay. Pure compounds (2 mg) were dissolved in acetone p.a. (250 μL) and mixed with 4% malt extract broth (w/v) (4.75 mL) containing 0.2% Tween 80 as emulgator to give a concentration of 400 μg mL^{-1} in stock solution. Microdilution was performed with sterile disposable microtitration multiwell plates (96 U-shaped wells) modified after Espinel-Ingroff et al. (1992) and Wilson et al. (1997). Each compound was diluted 2-fold with a 50 μL multichannel pipet and tested in duplicate. Spore suspensions were adjusted to approximately 1 × 10^4 cfu mL^{-1} with 4% malt extract broth (w/v) (Gehrt et al., 1995; Espinel-Ingroff et al., 1997), from which 50 μL was dispensed into each well to achieve final concentrations of 200−0.1 μg mL^{-1} for each compound tested. The multiwell plates were incubated 16 h in darkness at room



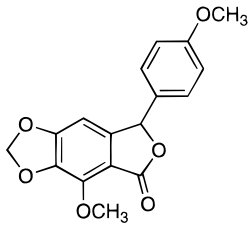
	R_1	R_2	R_3
rocaglaol (**1**)	OCH_3	H	H
aglafolin (**2**)	OCH_3	H	$C(=O)-OCH_3$
pannellin (**3**)	$O-CH_2-O$		$C(=O)-OCH_3$
rocaglamide (**4**)	OCH_3	H	$C(=O)-N(CH_3)_2$
aglaroxin A (**5**)	$O-CH_2-O$		$C(=O)-N(CH_3)_2$
desmethyl rocaglamide (**6**)	OCH_3	H	$C(=O)-NH(CH_3)$



thapsakinacetate A (**7**)



thapoxepine A (**8**)



aglalactone (**9**)

Figure 1. Molecular structures of compounds **1**−**9**. On the basis of more detailed NMR experiments (Seger et al., 2000; *Monatsh. Chem*.) the originally published structure of **9** (Brader et al., 1998) had to be corrected.

temperature, and then the fungal growth was stopped by adding 10 μL of lactophenol blue solution (Merck) to each well. To assess the different germ tube development in the wells, images of 10 germinated spores per well were captured to hard disk and the germ tube sizes were calculated in pixels (NIH Image 1.60, National Institute of Health, public domain). Scarceness of test compounds permitted only one replicate. Highly active compounds were further diluted to 0.000 02 μg mL^{-1}.

Data Analysis. Minimum inhibitory concentration (MIC) was determined as the lowest compound concentration completely inhibiting spore germination as outlined in the National Committee for Clinical Laboratory Standards (NCCLS) method M27-A (1997). EC_{50} and EC_{90} values were calculated by probit-log analysis as described for a quantitative assay (Roberts and Boyce, 1972).

RESULTS AND DISCUSSION

Eight flavaglines (**1**−**8**) isolated from *Aglaia odorata*, *A. elaeagnoidea*, and *A. edulis* were tested for bioactivity against three plant pathogenic fungi, *Pyricularia grisea*, *Alternaria citri*, and *Fusarium avenaceum*, using the spore germination inhibition assay in microwells. As shown in Table 1, six cyclopenta[*b*]benzofuran derivatives (**1**−**6**) exhibited clear antifungal activity against *P. grisea*, whereas the cyclopenta[*bc*]benzopyran **7**, the benzo[*b*]oxepine **8**, and the probably remote related aglalactone (**9**) did not show fungicidal properties at 200 μg mL^{-1}. Particularly low EC_{50}, EC_{90}, and MIC values against all three pathogens were found in rocaglaol (**1**) which can be distinguished from the other active benzofurans (**2**−**6**) by a loss of substitution at position C-2. The positive controls, Benlate (50% benomyl) and blasticidin S, showed variable activities against the three fungi with the exception of *A. citri*, which appeared to be largely resistant against Benlate.

P. grisea was shown to be the fungus most susceptible to all active flavaglines, of which rocaglaol (**1**) with an EC_{50} of 0.01 μg mL^{-1}, EC_{90} of 0.3 μg mL^{-1}, and a MIC of 1.6 μg mL^{-1} is by far the most active derivative. In this case, the narrow 95% fiducial limits of the probit estimates characterize a clear-cut sigmoidal dose−activity relationship (Figure 2). Substitution at C-2 in compounds **2**−**6** obviously leads to a decrease of activity (Table 1). It is interesting to note that aglafoline (**2**) and pannellin (**3**) show an EC_{50} value similar to rocaglaol (**1**), but EC_{90} and MIC values are significantly higher. In contrast to rocaglaol (**1**), the action profile of aglafolin (**2**) has a higher end point range. In this case, however, a sigmoidal dose−response relation is still perceptible (Figure 2A). On the other hand, pannellin (**3**) shows a trailing end point range at 3−12 μg mL^{-1} (log concentration 0.8−1.4), thus causing rather large fiducial limits of EC_{90} (Figure 2A, Table 1). The obvious inconsistencies between EC_{90} and MIC values can be explained by different ways of interpreting data. Whereas the MIC was already obtained by visual determination of total spore inhibition, EC_{90} values were derived from calculations based on probit-log estimations of the various responses of 10 different spores per concentration. Comparing flavagline **2** with **3** and **4** with **5**, it is evident that the dioxomethylene bridge between C-6 and C-7 further diminishes antifungal activity (Table 1). Moreover, in *P. grisea* the pair of esters **2** and **3** were more active than the corresponding pair of amides **4** and **5**. Demethylation of amide nitrogen also reduces activity leading to the less active benzofuran derivative desmethyl rocaglamide (**6**).

To get more information about the antifungal spectrum of flavaglines, the most active benzofurans **1**−**4** were also tested against *F. avenaceum* and *A. citri*. In this case only compounds **1** and **2** exerted effects on both fungi, whereas **3** and **4** were only active against *F. avenaceum*. In *A. citri*, however, **3** and **4** did not show any antifungal action at concentrations of 200 μg mL^{-1} (Table 1). The lower susceptibility of *A. citri* against flavaglines is also clearly expressed by an EC_{50} of 6 μg mL^{-1} for rocaglaol (**1**), which is 600 times higher than for *P. grisea*. With respect to these different susceptibilities, it deserves special interest that the positive control blasticidin S, a metabolite of *Streptomyces griseochro-*

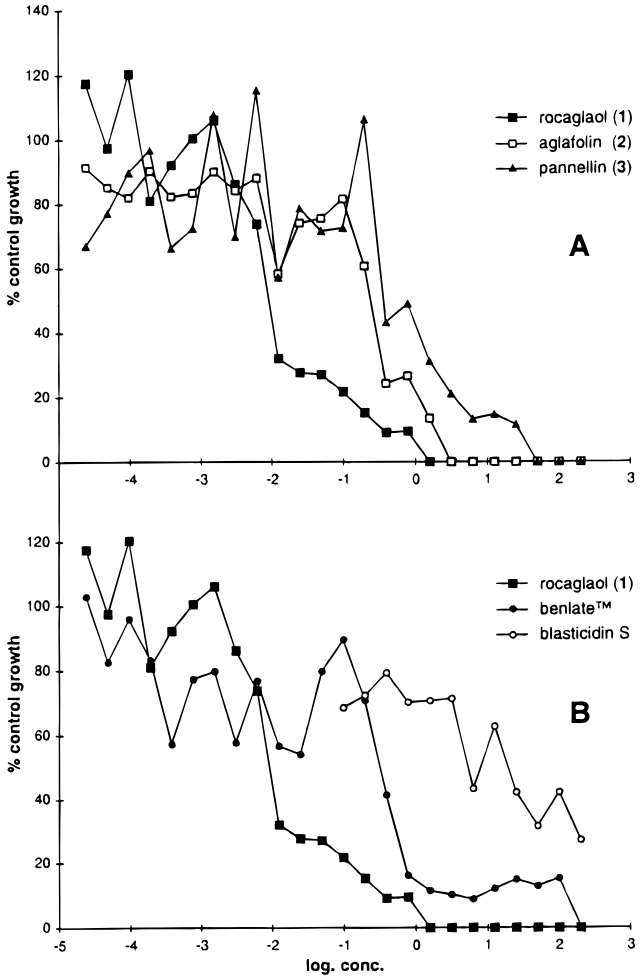


Figure 2. Quantitative germ tube inhibition effects of (**A**) aglafoline (**2**) and pannellin (**3**), and (**B**) Benlate and blasticidin S on *P. grisea* in comparison to the most active rocaglaol (**1**). Test compound range: 0.000 02−200 μg mL^{-1} except blasticidin S (0.1−200 μg mL^{-1}). Mean of control growth represents 100%. Growth values are the means across 10 germ tubes per concentration.

mogenes (Takeuchi et al., 1958), showed a reverse effect being less active against *P. grisea* than against *F. avenaceum* and *A. citri*. On the other hand, this compound was the first successfully used fungicide in Japan because of its potent curative effect on rice blast (Yamaguchi, 1995). The development of this compound into a commercial product was spurred by reports on strong action against mycelial growth of *P. grisea* (Misato et al., 1959). Compared with rocaglaol (**1**), however, blasticidin S is clearly less active against *P. grisea* (Table 1, Figure 2B). The second positive control, Benlate, is also applied against rice blast (Agrios, 1997). The major component of Benlate, benomyl, is a broad-spectrum benzimidazole fungicide and acts by binding to tubulin and subsequent meiosis and mitosis inhibition (Davidse and Ishii, 1995). In our tests it displayed EC_{50} values similar to those of rocaglaol (**1**), but was clearly less active on the EC_{90} and MIC level (Table 1, Figure 2B). Moreover, it was completely inactive against *A. citri* at 200 μg mL^{-1}. Regarding the already known resistances of *Alternaria* against benomyl (Gutter and Yanko, 1971), the observed activity of rocaglaol (**1**) against *A. citri* again demonstrates the exceptional antifungal capacity of this compound. This high fungitoxicity was unexpected since in recent insect bioassays against larvae of the cotton leaf worm (*Spodoptera littoralis*, Noctuidae) rocaglaol (**1**) was shown to be the least active derivative out of five cyclopenta[*b*]benzofurans. In accordance with antifungal effects, the benzopyran (**7**) and benzoxepine derivative (**8**) also did not show marked insect toxicity against *S. littoralis* (Bacher et al., 1999). With respect to the lower antifungal capacities of the commercial fungicides blasticidin S and Benlate, and the already known resistance of *P. grisea* to blasticidin S (Sakurai et al., 1975; Ryu et al., 1983), rocaglaol (**1**) highly offers itself for further in vivo studies to determine its actual value as an anti-rice blast agent in the field.

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