Cyclopentabenzofuran Lignan Protein Synthesis Inhibitors from *Aglaia odorata*

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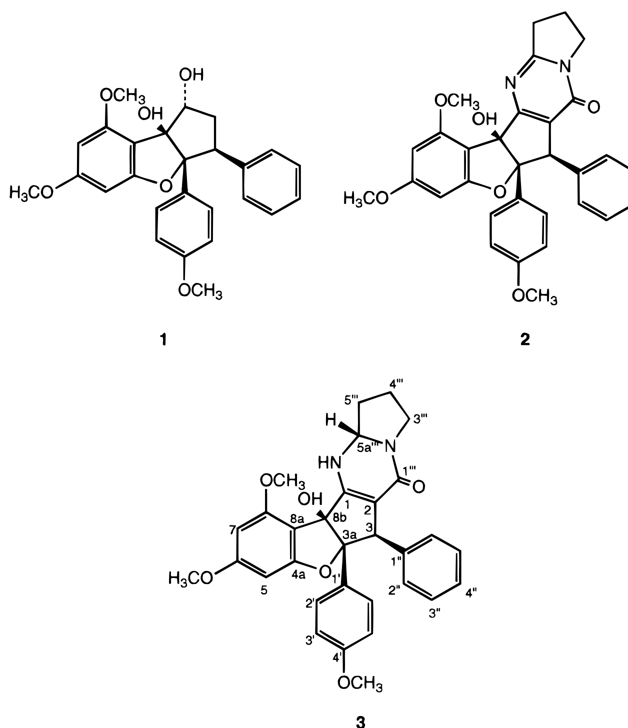
In the course of screening for Ras function inhibitors, rocaglaol (**1**) and the related compounds, the known pyrimidinone (**2**) and the novel aglaiastatin (**3**), were isolated from a $CHCl_3$ extract of the leaves of *Aglaia odorata*. The structure of **3** was elucidated as a novel cyclopentabenzofuran on the basis of its NMR spectroscopic data and by X-ray crystallographic analysis. These compounds (**1**−**3**) were potent inhibitors of the growth of K-*ras*-NRK cells, with IC_{50} values of 1−10 ng/mL, and induced normal morphology in K-*ras*-NRK cells at 10−30 ng/mL. They also specifically inhibited protein synthesis. Aglaiastatin (**3**) was slightly more potent than **1** and **2** in inhibiting cell growth. Aglaiastatin (**3**) reduced the amount of Ras, possibly by inhibiting its *de novo* synthesis.

Ras is a member of a family of small GTP-binding proteins and plays an important role in various signaling pathways for cell growth, differentiation, and transformation.[1,2] Activation of the *ras* proto-oncogene is found in about 20% of all human neoplasms. It is especially common in pancreatic carcinoma (about 80%) and colon carcinoma (about 40%).[3] Inhibition of *ras* functions should help to suppress activated *ras*-expressing neoplasms.

We have been screening tropical plant extracts as inhibitors of Ras function using the index of morphological change in K-*ras*-expressing cells. As a result, we have determined damnacanthal,[4] conophylline,[5] and conophyllidine (unpublished results) to be inhibitors that induced normal flat morphology in K-ras^{ts}-NRK and K-*ras*-NRK cells.



Results and Discussion

In this paper, we describe potent protein synthesis inhibitors from the leaves of *Aglaia odorato*—namely, the known compound rocaglaol (**1**),[6] a related pyrimidinone (**2**),[7] and the novel aglaiastatin (**3**). They all induced flat conversion in K-*ras*-NRK cells.

The molecular weight of **3** ($C_{31}H_{30}N_2O_6$) was only two hydrogen atoms more than that of **2**. The ^{13}C-NMR spectrum of **3** shown in Table 1 was therefore similar to that of **2**; however, in **3** one carbon signal was absent in the lowfield region (δ_C 70.3) being present. The proton of this methine gave spin couplings with the H-5‴ methylene proton in the $^1H-^1H$ COSY spectrum. Moreover, a ninhydrin reaction was positive for **3**, whereas it was negative for **2**. Accordingly, the double bond of C=N in **2** was reduced in **3**. Compound **3** was crystallized from $MeOH-H_2O$, and then the relative configuration was obtained by X-ray crystallographic analysis, as shown in Figure 1.

Compounds **1**−**3** induced flat morphology and cytoskeletal organization in K-*ras*-NRK cells at 10−30 ng/mL. The morphological change was apparent 1 day after the addition of these substances, and the change was most prominent after 3 days. The induced flat morphology was reversed by 1 day after the removal of each compound. However, they did not markedly change the morphology of NRK cells. Compounds **1**−**3** potently inhibited the growth of various cell lines, with IC_{50} values in the 1−10 ng/mL region, as shown in Table 2. The inhibitory effect of **3** was the most potent among the three compounds tested. The inhibitory effect of these lignans on the growth of cells, however, was not specific to *ras*-expressing cells, inasmuch as the IC_{50} values for *ras*-expressing cells were not much different from those for control cells.

As for the mechanism underlying the morphological change, compounds **1**−**3** were shown to inhibit protein synthesis specifically, as shown in Figure 2, whereas they did not inhibit DNA and RNA synthesis. Incubation of K-*ras*-NRK cells with **3** induced a significant decrease of the cellular Ras proteins as shown in Figure

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Table 1. ^{1}H-(500 MHz) and ^{13}C-NMR (125 MHz) Data (J in Hz) of Aglaiastatin (**3**) in $CDCl_3$[a]

position	δ_H	δ_C	position	δ_H	δ_C
1		157.2	1′		127.2
2		106.5	2′,6′	7.13 (2H, d, J = 9.0)	128.3
3	4.60 (1H, s)	57.3	3′,5′	6.58 (2H, d, J = 9.0)	112.5
3a		105.1	4′		158.5
4a		161.1	4′-OCH_3	3.66 (3H, s)	55.0
5	6.28 (1H, d, J = 2.0)	89.4	1″		138.8
6		164.0	2″,6″	7.10 (2H, d, J = 7.4)	128.5
7	6.08 (1H, d, J = 2.0)	92.8	3″,5″	7.03 (2H, dd, J = 7.4, 7.4)	127.4
8		157.5	4″	6.95 (1H, dd, J = 7.4, 7.4)	126.0
8a		107.2	1‴		162.3
8b		88.2	3‴	3.48 (1H, m)	44.0
6-OCH_3	3.83 (3H, s)	55.5[b]		3.62 (1H, m)	
8-OCH_3	3.83 (3H, s)	55.8[b]	4‴	1.94−2.06 (2H, m)	22.8
8b-OH	1.97 (1H, br s)		5‴	1.97−2.06 (1H, m)	32.5
				2.47 (1H, m)	
			5a‴	5.23 (1H, m)	70.3
			6‴-NH	5.24 (1H, s)	

[a] Chemical shifts in ppm with TMS an internal standard. [b] These assignments may be interchanged.

Table 2. Inhibition of Cell Growth by Compiounds **1**−**3**

cell line	Inhibition[a] **1**	**2**	**3**
K-*ras*-NRK	5.8	81.0	2.8
K-*ras*-NIH3T3	7.0	8.9	5.6
H-*ras*-NIH3T3	5.3	7.8	1.0
N-*ras*-NIH3T3	7.5	8.0	1.8
NRK	10.0	9.8	2.1
NIH3T3	2.0	7.6	4.1

[a] Data are expressed as IC_{50} values (ng/mL).

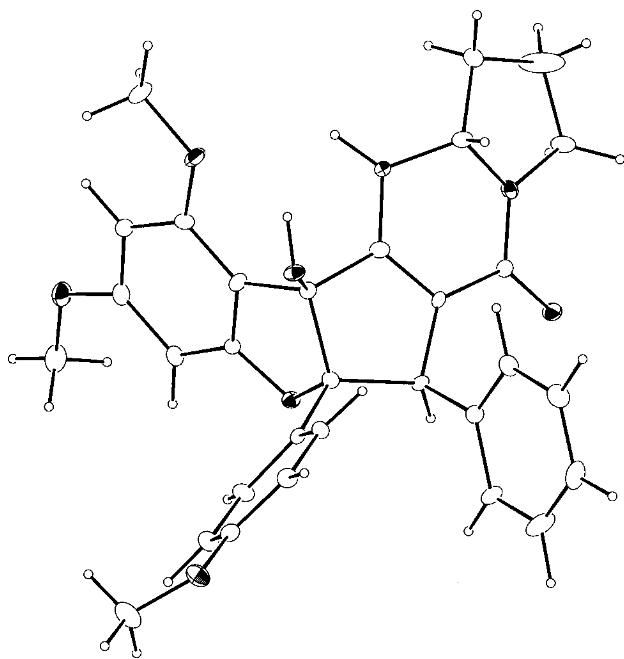

Figure 1. ORTEP[13] drawing of **3** (no absolute configuration is implied).

3. It is possible that the fast turnover of Ras was rather selectively inhibited by the protein synthesis inhibitor. At least partly, flat conversion of the cells might be due to the lowered Ras level, although aglaiastatin and related compounds should also inhibit the syntheses of other fast turnover proteins. Cycloheximide, a protein synthesis inhibitor, was also reported to induce flat conversion in *ras*-expressing cells.[8]

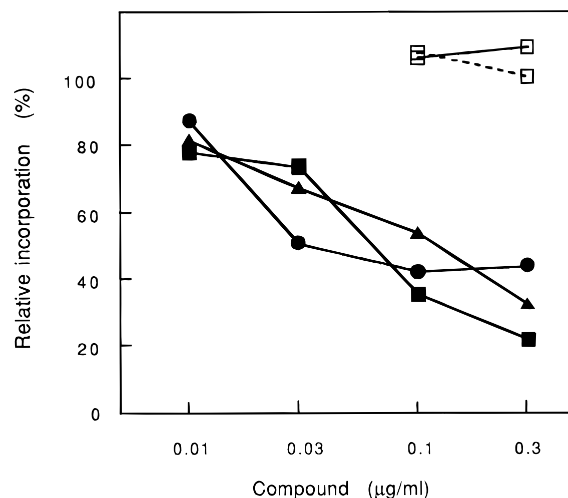

Figure 2. Inhibition of protein synthesis in K-*ras*-NRK cells by rocalgaol (**1**), the pyrimidinone compound (**2**), and aglaiastatin (**3**). K-*ras*-NRK cells incubated at 37 °C were labeled with [^{3}H] leucine for 1 h in the presence of **1** (●), **2** (▲), or **3** (■), and then the radioactivity of the acid-insoluble fraction was counted. The cells wee also labeled with [^{3}H]thymidine (□—□) or [^{3}H]uridine □···□) for 1 h in the presence of **3**. Values are means of duplicate determinations.

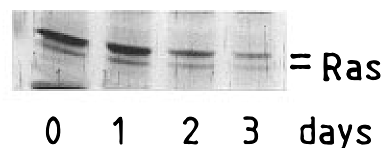

Figure 3. Decrease of Ras level by aglaiastatin (**3**) in K-*ras*-NRK cells. The cells were incubated with 2.5 ng/mL of **3** for indicated periods and lysed. The cell lysate was subjected to electrophoresis, blotted to the nitrocellulose membrane, and reacted with anti-Ras antibody. The same amount (5 μg/lane) of total proteins was applied for each lane. The result is a representative of triplicate experiments.

From *Aglaia odorata*, rocaglamide[9,10] and odorinol,[11,12] which possess related structures, have been isolated, and they showed antitumor activity against P-388 lymphocytic leukemia in vivo. Aglaiastatin (**3**) may also be expected to have antitumor activity, especially on K-*ras*-expressing tumors.

Experimental Section

General Experimental Procedures. The mp was determined with a Yanagimoto MP-3 micro-melting point apparatus and was uncorrected. The UV spectrum was measured on a Tokyo photoelectric ANA-72V spectrophotometer. The IR spectrum was measured on a JASCO FT-IR 5300 spectrometer. The FABMS was obtained on a JEOL JMS-SX 102 mass spectrometer. The NMR spectra were recorded on JEOL JNM-EX 500 NMR spectrometer. The optical rotation was measured by a Perkin-Elmer 241 polarimeter.

Biological screening was conducted using the index of morphological change in K-*ras*-NRK cells (normal rat kidney cells transformed by Kirsten sarcoma virus). Compounds that induce flat morphology were screened and purified. The cells (2 × 10^3/0.2 mL) were seeded into 96-well plates and incubated at 37 °C for 24 h. Then, 2 μL of plant extract (2 mg/mL in 10% Me_2CO) were added, and the cell morphology was examined each day for 3 days.

Plant Material. Leaves of *Aglaia odorata* Lour. (Meliaceae) were collected in Thailand near Khon Kaen

in February 1993, and January, May, and September 1994. The plant was identified by Prof. T. Kowithayakorn, Plant Science Department, Faculty of Agriculture, Khon Kaen University, where a voucher specimen is maintained.

Extraction and Isolation. Leaves of *Aglaia odorata* (100 g dry wt) were extracted with $CHCl_3$ to give 3.9 g of a residue. The $CHCl_3$ extract (3.9 g) was chromatographed on a Si gel column using a mixture of toluene−Me_2CO as eluent, and two active fractions eluted with toluene−Me_2CO [(16:1, fraction 1) and (3:1−1:1, fraction 2)] were obtained, yielding 387.6 mg and 639.2 mg, respectively.

Fraction 1 was dissolved in 80% aqueous MeOH, and the soluble fraction was applied to a reversed-phase Si gel column. The active fraction then eluted with 80% aqueous MeOH. The active material was dissolved in MeOH and chromatographed over a Toyopearl HW-40 column developed with MeOH. The active fractions were combined and subjected to preparative HPLC. The column was developed with MeOH−H_2O (65:35), and the combined active fractions were concentrated to dryness to give 4.6 mg of purified **1**. Compound **1** was identified as rocaglaol by comparison with published physical spectral data.[6]

Fraction 2 was dissolved in MeOH and chromatographed over a Toyopearl HW-40 column developed with MeOH. The active material was dissolved in 70% aqueous MeOH, applied to a reversed-phase Si gel column, and then eluted with 70% aqueous MeOH. The active fractions were combined and subjected to preparative HPLC (column, Pegasil ODS [20 × 250 mm, Senshu Science Co., Tokyo, Japan]; flow rate, 5 mL/min; detection, UV 210 nm). The column was developed with MeOH−H_2O (65:35), and two active fractions were separated; and each fraction was concentrated to dryness. Thus, purified **2** and **3** were obtained (5.2 mg and 2.1 mg, respectively). Compound **2** was identified as a reported pyrimidinone by comparison with published physical and spectral data.[7]

Aglaiastatin (3). Compound **3** was obtained as colorless powder and was soluble in MeOH and $CHCl_3$, but only slightly soluble in hexane and H_2O. The R_f values in the solvent systems $CHCl_3$−MeOH (20:1) and toluene−Me_2CO (1:1) were 0.42 and 0.49, respectively: mp 157−160 °C; $[\alpha]^{23}{}_D$ +59.7° (*c* 0.3, MeOH); UV (MeOH) λ max (ϵ) 281 (4163), 304 (4480) nm; IR (KBr) ν max 3445, 2924, 2850, 1624, 1514, 1425, 1250, 1221, 1200, 1148, 1115, 1034 cm^{-1}; ^1H; and ^{13}C-NMR data, see Table 1, FABMS *m/z* 527 $[M + H]^+$. anal. Calcd for $C_{31}H_{30}N_2O_6{\cdot}1.5H_2O$: C, 67.26; H, 6.01; N, 5.06. Found: C, 67.19; H, 6.07; N, 4.78.

X-ray Crystallography of 3.[14] Needle crystals were grown from a MeOH−H_2O mixture. Crystallographic data: $C_{31}H_{30}O_6N_2{\cdot}CH_3OH$, MW 558.63, orthorhombic, $P2_12_12_1$, $a = 19.990(10)$ Å, $b = 20.964(9)$ Å, $c = 6.843(8)$ Å; $V = 2868(4)$ Å^3, $Z = 4$, $D_x = 1.294$ g cm^{-3}, μ (Mo Kα) δ 0.086 mm^{-1}. The X-ray intensities up to $2\theta = 50°$ were measured on Rigaku AFC-5 four-circle diffractometer with graphite-monochromatized Mo Kα radiation. Non-hydrogen atoms were refined anisotropically, and hydrogen atoms were introduced except for MeOH. Final *R* is 0.069 for 1699 reflections. The absolute structure was not determined, with only relative stereochemistry being corroborated.

Macromolecular Synthesis. The cells (3 × 10^4) were seeded in 24-well plates and incubated overnight. The medium was then removed, and 1 mL of DMEM containing 1 μCi of [methyl-^{3}H]thymidine (46 Ci/mmol), [5-^{3}H]uridine (26 Ci/mmol), or L-[4,5-^{3}H]leucine (85 Ci/mmol) was added to each well. After a 1-h incubation with the test chemical, the cells were treated with 0.5 mL of 10% TCA for 10 min at 4 °C and washed twice with 0.5 mL of 10% TCA. Then the cells were lysed with 0.5 mL of 0.5% NaOH for 30 min at 37 °C, and the radioactivity was counted.

Immunoblotting. K-*ras*-NRK cells (1 × 10^6) plated in 60-mm dishes were treated with **3** for the indicated period. The cells were scraped off and lysed in 200 μL of RIPA buffer (25 mM HEPES, 1.5% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 50 mM NaF, 100 μM sodium vanadate, 1 mM PMSF, 0.1 mg/mL leupeptin) for 60 min at 4 °C. After centrifugation, the supernatant was added by 100 μL of 42 mM Tris−HCl containing 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, and 0.002% bromophenol blue, and the lysate was boiled for 5 min. The lysate was electrophoresed on a 12.5% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane. Nonspecific sites were blocked with 6% calf serum for 1 h at room temperature. The membranes were then treated with 5 mL of 200 ng/mL anti-Ras antibody (Y13-259 Oncogene Science, Inc. Cambridge, MA) overnight, and incubated with biotinylated anti-rat IgG linked with alkaline phosphatase-streptavidin for 20 min at room temperature. Then the membrane was treated with 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and nitroblue tetrazolium.

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