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# Pseudodeflectusin, a novel isochroman derivative from *Aspergillus pseudodeflectus* a parasite of the sea weed, *Sargassum fusiform*, as a selective human cancer cytotoxin

Akitsu Ogawa,[a] Chikako Murakami,[b,c] Shinji Kamisuki,[a] Isoko Kuriyama,[c] Hiromi Yoshida,[c,d] Fumio Sugawara[a,e] and Yoshiyuki Mizushina[c,d,*]

[a]*Department of Applied Biological Science, Tokyo University of Science, Noda, Chiba 278-8510, Japan*
[b]*Department of Interactable Diseases, International Medical Center of Japan, Shinjuku-ku, Tokyo 162-8655, Japan*
[c]*Laboratory of Food and Nutritional Sciences, Department of Nutritional Science, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan*
[d]*High Technology Research Center, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan*
[e]*Frontier Research Center for Genomic Drug Discovery, Tokyo University of Science, Noda, Chiba 278-8510, Japan*

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**Abstract**—A new isochroman derivative named pseudodeflectusin was isolated from a culture broth of *Aspergillus pseudodeflectus*. The structure was determined by spectroscopic means as 9-hydroxy-7-methyl-2-(methylethylidine)-furano[3,2-*H*]isochroman-3-one. This compound exhibited cytotoxicity for several human cancer cell lines from the stomach (NUGC-3), cervix (HeLa-S3), and peripheral blood (HL-60), but did not affect those from the lung (A549) or colon (DLD-1). The $LD_{50}$ value of this compound for HL-60 cells was 39 μM.


## 1. Introduction

Historically, terrestrial fungi have been a rich source of pharmaceutically important compounds. More recently, these studies have expanded to include marine species.[1] In our natural products isolation program for the construction of a small molecule library, we isolated a new isochroman derivative as a fungal metabolite isolated from a culture broth of *Aspergillus pseudodeflectus*. A sea weed, *Sargassum fusiform*, was collected in the Miura Peninsula and washed with sterilized water. After cutting it into small pieces (about 5 mm diameter), it was put onto a potato dextrose agar plate and incubated for five days at 28 °C in the dark. A single colony was arbitrarily picked and transferred to fresh culture medium, and was incubated at room temperature in the dark for three weeks. Two compounds were purified from an extract of the culture with $CH_2Cl_2$ on a silica gel column. One of them was identified as 7-methyl-2-(1-methylethylethlidene)-furo[3,2-*H*]isoquinoline-3-one (compound **1**, Chart 1) by spectrometric means and a comparison of physicochemical properties. Another isolate was identified as a new compound and the structure was deduced to be 9-hydroxy-7-methyl-2-(methylethylidine)-furano[3,2-*H*]isochroman-3-one (pseudodeflectusin, compound **2**, Chart 1). We describe here the structural determination and the

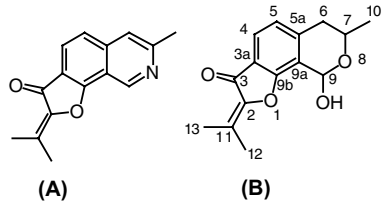


**Chart 1.** Structure of compound **1** (left) and **2** (pseudodeflectusin, right).

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* Corresponding author. Tel.: +81-78-974-1551x3232; fax: +81-78-974-5689; e-mail: mizushin@nutr.kobegakuin.ac.jp

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cytotoxicity of this compound against several human cancer cell lines.

## 2. Results and discussion

### 2.1. Isolation and cultivation of fungus

The fungal strain, Hiji005 was isolated from sea alga that had been collected in the Miura Peninsula, Kanagawa, Japan. Under sterile conditions, the alga was suspended in 3.6% NaCl solution. The fungal was selected by culturing the suspension onto potato dextrose agar plates (Difco) and subsequently transferring the mycerial tips several times. Culture was incubated at room temperature. Hiji005 was identified as *A. pseudodeflectus* by NCIMB Japan Co., Ltd. A small agar plug was then transferred into a 2 L Erlenmeyer Flask containing 1 L of a culture of 24 g potato dextrose broth (Difco). Cultures of Hiji005 (4 L) were grown for three weeks without shaking in the dark.

### 2.2. Extraction and isolation of isochroman compounds

The fungal mycelia were removed from the culture broth by filtering them through cheesecloth. The filtrate was extracted with $CH_2Cl_2$. The organic layer was evaporated in vacuo to obtain 442.3 mg crude residue. This crude extract was separated by silica gel column chromatography with hexane–EtOAc (4:1–1:4) to give 7.4 mg compound **1** as slightly pale needles and 282.6 mg mixture. The mixture was twice chromatographed on silica gel with $CHCl_3$–methanol (19:1) and $CHCl_3$–methanol (99:1–95:5) to yield 1.2 mg compound **2** as colorless needles.

### 2.3. Structural determination of isochroman compounds

The molecular formula of compound **1** was determined with a positive high resolution electro spray ionization mass spectrometer (HR-ESIMS) spectrum as $C_{15}H_{13}NO_2$ ($m/z$ found 240.1047 $[M+H]^+$, calcd for $C_{15}H_{14}NO_2$: 240.1019). From analyses of the NMR spectra and mass spectrum, the structure of compound **1** was identified as TMC-120B, which had been isolated from *Aspergillus urtus* as described by Kohno et al.[2]

The molecular formula of compound **2** was determined with a positive HR-ESIMS spectrum as $C_{15}H_{16}O_4$ ($m/z$ found 283.0950 $[M+Na]^+$, calcd for $C_{15}H_{16}O_4Na$: 283.0940). Analyses of the $^{13}C$, $^1H$, DEPT, and HMQC spectra identified three methyl, one methylene, four methine, and seven quaternary carbons as listed Table 1. In the HMBC spectrum, $^1H$ of two methyl groups at $\delta$ 2.12 ppm and $\delta$ 2.36 showed correlations with two olefinic carbons (C-2, C-11) at $\delta$ 132.3 ppm and $\delta$ 145.2, which suggested the presence of $C{=}C(-CH_3)_2$. A couple of *ortho* protons suggested by the coupling constant $^3J_{H4-H5}$ of 8.0 Hz were assigned to be H-4 and H-5, respectively. An aromatic ring was assigned by characteristic correlation including H-4 at $\delta$ 7.61 ppm to C-5a at $\delta$ 143.8 and the oxygen bearing C-9b at $\delta$ 162.0 as well as H-5 at $\delta$ 6.88 to C-3a at $\delta$ 122.0 and C-9a at $\delta$ 119.5. A proton of H-4 at $\delta$ 7.61 ppm also correlated with C-3 at $\delta$ 183.2. The chemical shift of C-3 at $\delta$ 183.2 ppm suggested O=C–C=C (C-3, C-2, and C-11, respectively) as the partial structure. Chemical shifts observed on the NMR spectra of compound **2** were almost similar to those of compound **1** except C-6, C-9, C-10, H-6, H-7, H-9, H-10, and OH. The assignment of COSY (H–H) spectrum suggested the partial structure of $CH_3$–CH(O)–$CH_2$– to be due to C-10, C-7, and C-6, respectively. This partial structure connected to the aromatic ring from the HMBC correlation between H-6 at $\delta$ 2.73 ppm and $\delta$ 2.77 and two aromatic carbons (C-5 at $\delta$ 122.7 ppm and C-5a at $\delta$ 143.8). The HMBC spectrum, which was recorded in acetone-$d_6$, showed correlation with the signal at $\delta$ 5.83 ppm (assigned to be OH) to C-9 at $\delta$ 87.8 and C-9a at $\delta$ 122.2. The COSY spectrum in acetone-$d_6$ showed correlation with OH at $\delta$ 5.83 ppm and H-9 at $\delta$ 6.18. In $CDCl_3$, the correlation between the proton of OH at 3.07 and H-9 at 6.27 was not observed. From the above correlations and the chemical shifts of C-9 at $\delta$ 87.7 ppm and H-9 at $\delta$ 6.27, a hemiacetal group was indicated. Therefore the structure of compound **2** was established to be 9-hydroxy-7-methyl-2-(methylethylidine)-furano[3,2-*H*]isochroman-3-one, which was named pseudodeflectusin (Fig. 1). Determination of the absolute configuration is underway.

**Table 1.** $^1H$ and $^{13}C$ NMR spectral data of compound **2** (pseudodeflectusin)

| | $\delta_H$ ($J$ Hz) | $\delta_C$ |
|---|---|---|
| 2 | | 145.2 |
| 3 | | 183.2 |
| 3a | | 122.0 |
| 4 | 7.61 d (8.0) | 123.8 |
| 5 | 6.88 d (8.0) | 122.7 |
| 5a | | 143.8 |
| 6 | 2.77 dd (17.4, 3.8) | 36.0 |
| | 2.73 dd (17.4, 10.4) | |
| 7 | 4.46 ddq (10.4, 6.2, 3.8) | 62.9 |
| 9 | 6.27 d (4.0) | 87.8 |
| 9a | | 119.5 |
| 9b | | 162.0 |
| 10 | 1.39 d (6.2) | 21.1 |
| 11 | | 132.3 |
| 12 | 2.12 s | 20.3 |
| 13 | 2.36 s | 17.4 |
| OH | 3.07 d (4.0) | |

Recorded in $CDCl_3$ for TMS as an internal standard and chemical shifts are expressed as $\delta$ ppm. s: singlet, d: doublet, dd: doublet of doublets, q: quartet.

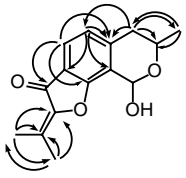


**Figure 1.** HMBC NMR experiment of compound **2** (pseudodeflectusin).

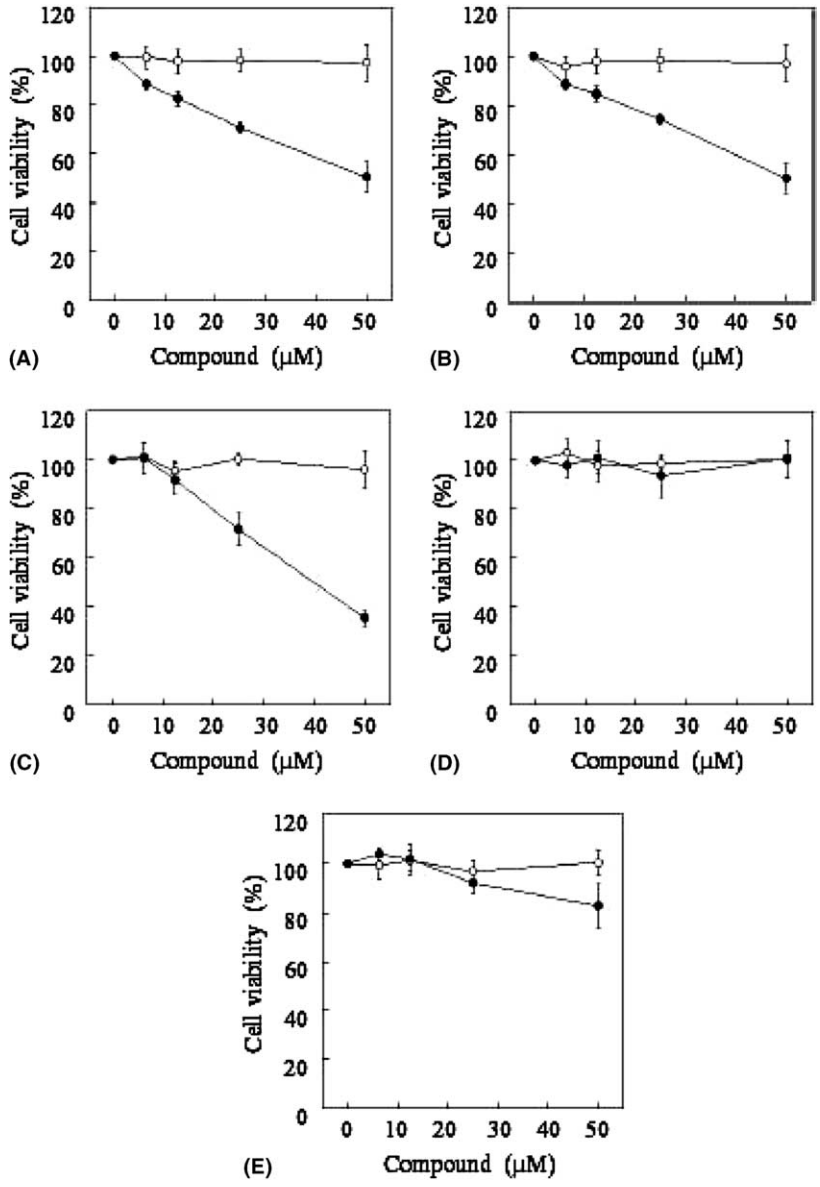


**Figure 2.** Human cancer cell growth inhibition by compound **2** (pseudodeflectusin). Dose–response curves of growth inhibition of the stomach cancer cell line, NUGC-3 (A), cervix cancer cell line, HeLa-S3 (B), peripheral blood cancer cell line, HL-60 (C), lung cancer cell line, A549 (D), and colon cancer cell line, DLD-1 (E) by compounds **1** (white circle) and **2** (black circle). The survival rate was determined by MTT assay. Data are expressed as the mean ± SD ($n = 4$).

### 2.4. Effect of compounds 1 and 2 on cultured human cancer cells

As shown in Figure 2, pseudodeflectusin (compound **2**) showed a dose-dependent inhibitory effect on cell growth with the NUGC-3 human stomach cancer cell line, the HeLa human cervix cancer cell line and the HL-60 human peripheral blood cancer cell line. The concentrations of **2** required for the $LD_{50}$ were 49, 47, and 39 μM, respectively. On the other hand, **2** did not suppress growth of the A549 human lung cancer cell line and the DLD-1 human colon cell line. These results suggested that **2** selectively inhibited certain human cancer cell lines, but did not have an effect on flattened epithelium cells such as A549 and DLD-1. Compound **1**, which is an analogue of **2**, did not inhibit human cancer cell growth. Structural differences between **1** and **2** such as the presence of nitrogen atom and OH group at carbon position 9 might be important for inhibition.

Leakage of lactate dehydrogenase (LDH) was examined to evaluate the cytotoxicity of **2**. As shown in Fig. 3, **2** treatment resulted in a marked increase in LDH leakage compared to control HeLa cells. Additionally, the contents of intracellular glutathione (GSH) decreased with **2** treatment (Fig. 4). GSH confers resistance to apoptotic agents, including chemotherapy drugs.[3] Thus, these results suggested that **2** induced depletion of the intracellular GSH content, which consequently significantly decreased cell viability. However, the cause of the selective activity against cell viability remains unclear and will be studied in the future.

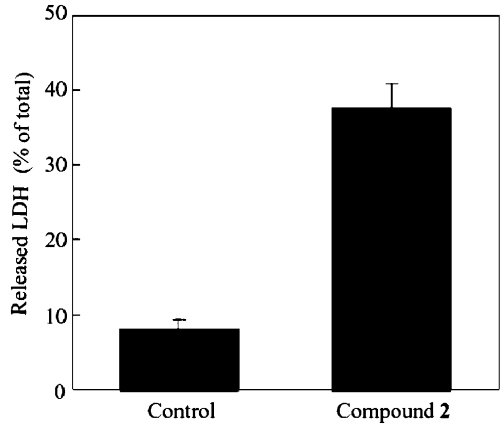


**Figure 3.** The cytotoxicity of compound **2** (pseudodeflectusin). HeLa-S3, human cervix cancer cells were treated with 50 μM of compound **2** for 48 h. The cytotoxicity of compound **2** was examined by measuring the leakage of LDH before and after the treatment of compound **2**. Data are expressed as the mean ± SD ($n = 4$).

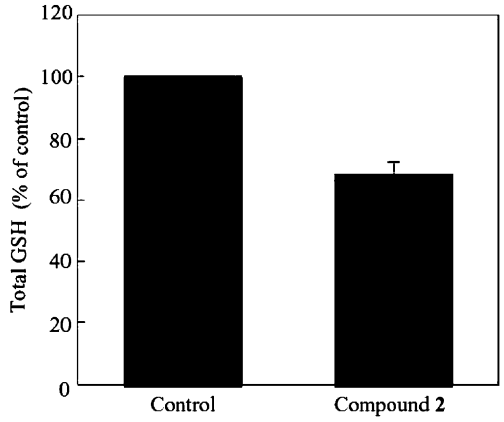


**Figure 4.** The total GSH content. HeLa-S3 cells were treated with 50 μM of compound **2** (pseudodeflectusin) for 48 h. The content of GSH in the cells was determined before and after the treatment of compound **2**. Data are expressed as the mean ± SD ($n = 4$).

Compounds **1** and **2** were investigated for the ability to inhibit of mammalian DNA metabolic enzymes such as DNA polymerases, terminal deoxynucleotidyl transferases, telomerases, reverse transcriptases, DNA topoisomerases, RNA polymerases, polynucleotide kinases, and deoxyribonucleases. These compounds did not inhibit any of these enzymes activities (data not shown).

## 3. Experimental section

### 3.1. Investigation of cell growth inhibition on human cancer cells

For investigation of the in vivo effects of the purified compounds, a human gastric cancer cell line, NUGC-3, a human epitheloid carcinoma cell line, HeLa-S3, a human promyelocytic leukemia cell line, HL-60, a human adenocarcinoma of lung cancer cell line, A549, and a human adenocarcinoma of colon cancer cell line, DLD-1, were obtained from the Health Science Research Bank (Osaka, Japan). The cells were routinely cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 unit/mL penicillin, and 1.6 mg/mL $NaHCO_3$. The cells were routinely cultured at 37 °C in standard medium in a humidified atmosphere of 5% $CO_2$-95% air. The cytotoxicity of the compounds was investigated as follows. High concentrations (10 mM) of the compounds were dissolved in DMSO and stocked. Approximately $3 \times 10^5$ cells per well were inoculated in 96-well microplates, then the compound stock solution was diluted to various concentrations and applied to each well. After incubation for 48 h, the survival rate was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.[4]

### 3.2. Cytotoxicity assay

Total GSH in cells were analyzed by an enzymatic recycling method according to Baker et al.[5] The leakage of LDH was measured by a commercial kit (LDH-Cytotoxicity Test, Wako Pure Chemical Industries) according to the manufacturer's instruction manual.

### 3.3. Structure determination

**3.3.1. 7-Methyl-2-(1-methylethylidene)-furo[3,2-*H*]isoquinoline-3-one (1).** Slightly pale needles; mp 175–177 °C (MeOH); IR (film) $\nu_{max}$ 1696, 1655, 1628, 1575, 1502, 1430, 1373, 1314, 1285, 1162, 1106, 922, 870, 835 $cm^{-1}$; HR-ESIMS $m/z$ found 240.1047 $[M+H]^+$, calcd for $C_{15}H_{14}NO_2$: 240.1019; $^1H$ NMR (400 MHz, $CDCl_3$): 9.57 (1H, s), 7.82 (1H, d, $J = 8.5$ Hz), 7.55 (1H, s), 7.38 (1H, d, $J = 8.5$ Hz), 2.76 (3H, s), 2.46 (3H, d, $J = 0.6$ Hz), 2.26 (3H, d, $J = 0.6$ Hz); $^{13}C$ NMR (100 MHz, $CDCl_3$): 182.2, 164.0, 156.7, 146.2, 145.6, 141.3, 133.8, 124.2, 120.6, 119.5, 119.3, 114.6, 24.7, 20.4, 17.5.

**3.3.2. Pseudodeflectusin: 9-hydroxy-7-methyl-2-(methylethylidine)-furano[3,2-*H*]isochroman-3-one (2).** Colorless needles; mp 179–180 °C (THF–hexane, 19:1); $[\alpha]_D^{23.2}$ +11 ($c$ 0.18, MeOH); IR (film) $\nu_{max}$ 3338, 3020, 1690, 1645, 1607, 1437, 1289, 1119, 1080, 1022, 855, 669 $cm^{-1}$; HR-ESIMS $m/z$ found 283.0950 $[M+Na]^+$, calcd for $C_{15}H_{17}O_4Na$: 283.0940; $^{13}C$ and $^1H$ data, see Table 1.

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