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A Quinolone Alkaloid, from the Aleurone Layer of *Oryza sativa* cv. *Mihyangbyo*, Inhibits Growth of Cultured Human Leukemia Cell

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Abstract

***Oryza sativa* cv. *Mihyangbyo* is one of several recently developed varieties of rice; characterized by high levels of aromatic components, which may increase its sensory and nutritional properties. In conjunction with our continuing investigation of bioactive components of improved grain varieties, a quinolone alkaloid was isolated from the *n*-butanol soluble fraction of the aleurone layer of *Oryza sativa* cv. *Mihyangbyo* (Gramineae) through activity-guided fractionation and isolation. The compound exhibited moderate antineoplastic activity in a human leukemia cell line (U937) with an IC_{50} value of 118.1 μg/mL, based on the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell proliferation assay. The chemical structure of the functional compound was determined, based on physical and spectroscopic characteristics.**

Key words: *Oryza sativa* cv. *Mihyangbyo*, Gramineae, alkaloid, 4-carbomethoxy-6-methoxy-2-quinolone, human leukemia cell, MTT assay, 2D NMR

INTRODUCTION

Rice (*Oryza sativa* L. Gramineae) is the major crop in many Asian countries. The development of new varieties of rice, produced by genetic engineering, has the potential to improve yield, organoleptic acceptability, and nutritional value. *Oryza sativa* cv. *Mihyangbyo* is an aromatic rice developed for enhanced flavor and aroma by the National Crop Experiment Station, Rural Development Administration, Korea.

Two antimicrobial substances in rice hull have been isolated and identified as 4-hydroxybenzoic acid and *trans* 4-hydroxycinnamic acid, with IC_{50} concentrations of 100~170 and 160 μg/mL, respectively (1). Recently, a new quinolone alkaloid, 4-carbomethoxy-6-hydroxy-2-quinolone from the aleurone layer of *Oryza sativa* cv. *Heugjinmi*, has been shown to have moderate antioxidative activity (IC_{50}; 36.4 μg/mL) using a 1,1-diphenyl-2-picrylhydrazyl free radical scavenging assay (2).

The alkaloids, which comprise the largest single class of secondary plant substances, are nitrogenous bases with one more nitrogen atoms, which are usually incorporated into a cyclic structure. Alkaloids are often toxic to man and many have dramatic physiological activities; hence their wide use in medicine (3). A simple, but by no means infallible, test for alkaloids in fresh leaf or fruit material is the bitter taste they often impart to the tongue.

There is overwhelming evidence, from experimental and epidemiological studies, that most common forms of human cancers are related to environmental and life-style factors, such as diet; and that susceptibility to those factors may be increased by specific genetic risk factors (4).

As part of a continuing study on cytotoxic components in foods (5-9), a quinolone alkaloid, that is moderately cytotoxic to a human leukemia cell line, was isolated from the *n*-butanol soluble fraction of the aleurone layer of *Oryza sativa* cv. *Mihyangbyo*, using the bioassay-guided fractionation and isolation method.

MATERIALS AND METHODS

Experimental Section

General experimental procedures: Melting point was measured on a Mitamura-Riken melting point apparatus, and was uncorrected. The UV and IR spectra were recorded on a Hitachi 3100 UV-vis and JASCO FT-IR-5300 spectrophotometer, respectively. EI-MS was obtained on a Hewelett Packard Model 5985B GC/MS spectrometer. ^{1}H and ^{13}C NMR spectra were measured, with TMS as an internal standard, using a Bruker CXP-500 spectrometer operating at 500 and 125 MHz, respectively. ^{1}H-^{1}H COSY, DEPT, ^{1}H-^{13}C HMQC and ^{1}H-^{13}C HMBC NMR experiments were also conducted on the same instrument.

Chemicals: 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), propidium iodide and RNAse were purchased from the Sigma Chemical Co. (St. Louis,

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MO, USA). All other chemicals were purchased from commercial sources and were of the highest purity available.

Plant material: The aleurone layer of *Oryza sativa* cv. *Mihyangbyo* was supplied by the National Crop Experiment Station, Rural Development Administration (RDA), Suwon, Gyeonggi-do, Korea, in March 2000. A voucher specimen has been deposited at the RDA.

Extraction and isolation: The dried and ground aleurone layer of *Oryza sativa* cv. *Mihyangbyo* (1.0 kg) was extracted with ethyl alcohol-H_2O (9:1 *vol/vol*) three times by maceration. The resultant extract was combined and concentrated to dryness *in vacuo* at 60°C. The dried ethanol extract was partitioned with *n*-hexane to yield a dried *n*-hexane residue (2.8 g). An ethanol extract was then suspended in H_2O and partitioned with ethyl acetate, and *n*-butanol to give, upon drying, ethyl acetate (3.7 g) and *n*-butanol soluble residues (2.6 g), and an aqueous partition (4.9 g). Fractionation of the *n*-butanol partition of the aleurone layer of *Oryza sativa* cv. *Mihyangbyo* was carried out using an open silica gel column with a $CHCl_3$-MeOH gradient system (98:2→90:10). The eluates, with similar TLC profiles, were combined into pooled fractions. Fractions from F009 to F016 were subjected to repeated fractionation using Sephadex LH-20 size-exclusion chromatography (MeOH) yielding 14.0 mg of a pure substance determined as compound 1.

Biological activities

Cell culture: U937 (human monocytic leukemia cell line) was cultured at 37°C in 5 % CO_2 in RPMI 1640 containing 2 mM glutamine, 10 % heat-inactivated fetal calf serum, penicillin (100 units/mL) and streptomycin (100 g/mL).

Cell viability assay: The effect of plant fractions on the viability of U937 cells was determined by MTT assay (5). Cells at the exponential phase were collected and transferred into each well (about 104～105 cells in a 180 μL/well). The cells were incubated for various times (up to 96 hr) in the presence of various amounts of fractions (0～200 μg/mL) in a total reaction volume of 200 μL. Fifty μL of a 2 mg/mL MTT solution was then added to each well (0.1 mg/well). After incubating for 4 hr, the plates were centrifuged at 800×g for 5 min and supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μL of DMSO and the A540 was read on a scanning multi-well spectrophotometer (Molecular Device Co., Sunnyvale, CA). All experiments were performed in triplicate.

4-Carbomethoxy-6-methoxy-2-quinolone (1): M.p.>320°C, pale yellow plates (MeOH); UV_{max} (MeOH) (log ε): 248 (4.7), 287 (4.2), 382 (4.0) nm; IR (KBr) ν_{max}: 3362 (NH), 1698, 1660 (CO), 1625 (CN) cm^{-1}; EI-MS (70 eV) *m/z* 233 $[M]^+$ (23), 202 $[M - OCH_3]^+$ (100), 174 $[M - OCH_3 - CO]^+$ (46), 146 $[M - OCH_3 - CO - CO]^+$ (19); ^1H NMR (500 MHz, DMSO-d_6), ^{13}C NMR (125 MHz, DMSO-d_6), HMQC and HMBC data, see Table 1.

Methylation of 4-carbomethoxy-6-hydroxy-2-quinolone: To a solution of compound 2 (2) (5.0 mg) in methanol (1.8 mL) was added $(CH_3)_2SO_4$ (2.0 mL) and anhydrous K_2CO_3 (1.0 mg), and stirred for 5 hr at 50°C. After dilution with water, the reaction mixture was extracted with chloroform. The chloroform layer was washed with water, dried with Na_2SO_4, evaporated, and purified by recrystallization with methanol to give pale yellow powder : m.p.>320°C, pale yellow powder (MeOH); UV_{max} (MeOH) (log ε): 242 (4.7), 281 (4.1), 382 (4.0) nm; IR (KBr) ν_{max}: 3365 (OH, NH), 1708, 1660 (CO), 1625 (CN) cm^{-1}; EI-MS (70 eV) *m/z* 233 $[M]^+$ (23), 202 $[M - OCH_3]^+$ (100), 174 $[M - OCH_3 - CO]^+$ (46), 146 $[M - OCH_3 - CO - CO]^+$ (19); ^1H NMR (500 MHz, DMSO-d_6), ^{13}C NMR (125 MHz, DMSO-d_6) data were identical with compound 1.

RESULTS AND DISCUSSION

Isolation and identification of compound

Compound 1 was isolated as pale yellow plates, mp >320, gave an $[M]^+$ at *m/z* 233 in the EI-MS spectrum, indicating

Table 1. ^1H NMR, ^{13}C NMR and HMBC spectral data of compound 1[1] (DMSO-d_6)

Position (mult., *J* in Hz)	δ H	δ C[2]	^1H-^{13}C correlations
2		160.1 (s)	
3	6.88 (s)	124.2 (d)	C-2, C-4, C-10, 4-COO
4		116.2 (s)	
5	7.49 (d, 2.6)	109.5 (d)	C-4, C-6, C-7, C-9, C-10
6		59.6 (s)	
7	7.08 (dd, 2.6, 8.9)	120.6 (d)	C-5, C-6, C-8
8	7.24 (d, 8.9)	116.6 (d)	C-2, C-4, C-5, C-6, C-7, C-9, C-10
9		138.9 (s)	
10		132.8 (s)	
COO		165.9 (s)	
OCH_3	3.92 (s)	52.7 (q)	C-5, 4-COO
	3.59 (s)		C-5, C-6, C-8
NH	11.95 (s)		C-4

[1]^1H NMR and ^{13}C NMR spectra were run in DMSO-d_6 at 500 and 125 MHz, respectively. Chemical shifts are shown in the scale with *J* values in parentheses. [2]Multiplicity from DEPT experiment; s=singlet, d=doublet, q=quartet.

the molecular formula $C_{12}H_{11}NO_4$. Compound 1 had UV absorption bands at 248, 287 and 382 nm; and carbonyl absorption at 1698 and 1660 cm^{-1}, and amide absorption at 1625 cm^{-1} in its IR spectra, suggesting the presence of a 2-quinolone skeleton (2). The IR bands at 1625, and the signal at δ 11.95 in the ^1H NMR spectrum infer the presence of an amide group in the molecule. The ^1H NMR spectrum contained a singlet of one proton at δ 6.88 for H-3, and two singlets of three protons at δ 3.92 and δ 3.59 for two methoxyl group protons. The HMBC spectrum revealed the correlation between the methoxyl group protons and the carboxyl carbon at δ 165.9, which revealed the presence of a carbomethoxyl moiety at C-4 of the 2-quinolone nucleus. The *meta*-coupled doublet at δ 7.49 (J = 2.6 Hz) in the de-shielded position of the aromatic protons, correlated with a quaternary carbon at δ 116.2 could be assigned to H-5 (Table 1). On the basis of the above spectroscopic evidence, the structure of compound 1 was determined to be 4-carbomethoxy-6-methoxy-2-quinolone (Fig. 1 and 2).

Biological activity of isolated compound 1

Cultured cell-based assays were used to evaluate the cytotoxic potential of extracts, solvent-soluble subfractions, and the isolated compound against the growth of the human leukemia cell line, U937. Compound 1 showed cytotoxic activity in a dose dependent manner. The ethanol extract of the aleurone layer of *Oryza sativa* cv. *Mihyangbyo* exhibited a mild cytotoxic activity, with IC_{50} values of 149.1 μg/mL based on the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell proliferation assay. The *n*-butanol soluble fraction exhibited moderate cytotoxic activity, with IC_{50} values of 105.0 μg/mL against U937 cell. Compound 1 was isolated from F009 to F016 subfractions through the bioassay-guided isolation method. The 118.1 μg/mL 50 % inhibitory concentration (IC_{50}) of compound 1 on U937 cell demonstrated that it has moderate cytotoxic activity (Table 2). The structure and stereochemistry of the isolated compound was determined by physicochemical and spectroscopic experiments. In conclusion, biologically active phytochemicals, including 4-carbomethoxy-6-methoxy-2-quinolone, in the aleurone layer of *Oryza sativa* cv. *Mihyangbyo* can be predicted to prevent/reduce some carcinogenic processes. These data indicate that high quality crops developed through genetic engineering can be used, not only to meet increasing demands on production, but also for potential functional properties.

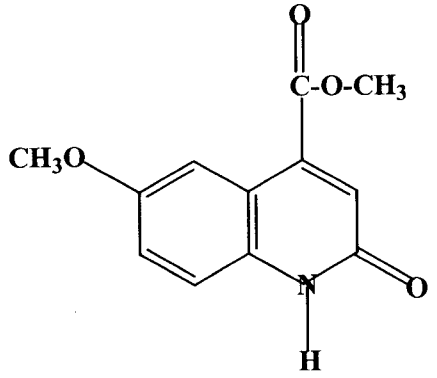

Fig. 1. Structure of compound 1.

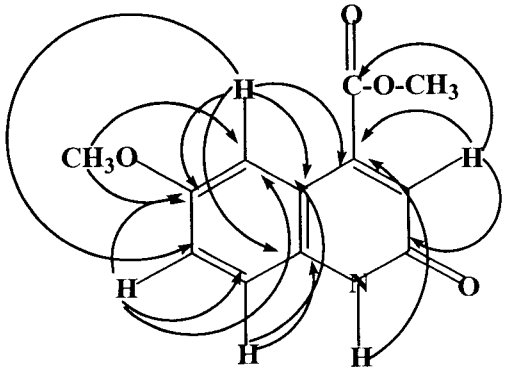

Fig. 2. HMBC correlations of compound 1.

Table 2. Cytotoxicity (IC_{50}, μg/mL)[1] of each samples against in human leukemia cell (U937)

Samples	Yield (g) (%)	Cytotoxicity[1]
EtOH extract	109.0 (10.9)	149.1
n-Butanol soluble fraction	2.6 (0.26)	105.0
Compound 1	0.014 (0.0014)	118.1

[1]The 50% inhibitory concentration (IC_{50}) was measured by MTT assay after 3-day culture and values were the mean of triplicate experiments.

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