Cytotoxic Acridone Alkaloids from the Stem Bark of *Citrus maxima*

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A new acridone alkaloid, 5-hydroxynoracronycine alcohol (**4**), along with six known acridone alkaloids, glycocitrine-I (**1**), 5-hydroxynoracronycine (**2**), citrussinine-I (**3**), grandisine-I (**5**), natsucitrine-II (**6**), and citracridone-III (**7**), were isolated from the stem bark of *Citrus maxima* f. buntan. Their structures were established on the basis of spectroscopic methods. Compounds **1-4** and **7** were found to be cytotoxic on two tumor cell lines.

Keywords: *Citrus maxima*; Stem bark; Aqueous portion; Acridone alkaloid; Anti-tumor activity.

INTRODUCTION

The occurrence of many kinds of acridone alkaloids from *Citrus* plants has been reported. These compounds are reported to show inhibitory effects on EBV-EA for anti-tumor promoting activity,¹ and cytotoxic,² antiviral,³ and antimalarial⁴ activities. Recently, acridones have attracted broad attention as components of photosensitizers used in photodynamic therapy, a newly introduced cancer treatment.^{5,6} The antitumor activities of a series of naturally occurring acridone alkaloids have been studied by determining their antiproliferative activities on several leukemia cell lines, including HL-60, the human promyelocytic leukemia cell line, and L-1210, the murine leukemia cell line.⁷⁻¹¹

In this paper, we wish to report the isolation and structural elucidation of a new acridone alkaloid isolated from the stem bark of *Citrus maxima*, in addition to six known acridone alkaloids. Among these compounds, five acridone alkaloids (**1-4** and **7**) were examined for their antiproliferative effects on several types of cancer cell lines—HepG2, KB, H2058, COLO 201, HA22T, and Chang liver cells.

RESULTS AND DISCUSSION

The concentrated methanolic extract of the stem bark of *C. maxima* was treated with water. The aqueous portion was chromatographed on a Dianion HP-20 column by eluting with water followed by methanol. The methanol eluate was

rechromatographed over a Sephadex LH-20 column eluting with methanol, which resulted in the isolation of a new acridone alkaloid, 5-hydroxynoracronycine alcohol (**4**), along with six known acridone alkaloids, glycocitrine-I (**1**),¹² 5-hydroxynoracronycine (**2**),^{13,14} citrussinine-I (**3**),¹⁵ grandisine-I (**5**),¹⁶ natsucitrine-II (**6**),¹⁷ and citracridone-III (**7**).¹⁸ The structures of these compounds were identified by spectroscopic data (¹H NMR, ¹³C NMR, and 2D NMR) and by comparing with literature data (Fig. 1).

Compound **4** was isolated as yellow powder and exhibited a molecular formula C₁₉H₁₇NO₅ by HREIMS. The absorption bands at 3364 and 1625 cm⁻¹ in the IR spectrum together with downfield signal at δ 14.42 (1H, s) in the ¹H NMR spectrum revealed the presence of one intramolecular hydrogen-bonded hydroxyl group. The ¹H NMR spectrum of **4** was similar to that of **2**¹³ except for the presence of a CH₂OH group and the disappearance of a methyl group. It also showed an ABC pattern of signals at δ 7.33 (1H, d, *J* = 7.6 Hz), 7.21

Fig. 1. Structures of acridone alkaloids from *C. maxima*.

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(1H, t, $J = 7.6$ Hz), and 7.78 (1H, d, $J = 7.6$ Hz) due to H-6, H-7, and H-8, respectively. The AB type signals at δ 5.66 and 6.82 (1H each, d, $J = 10$ Hz), a methyl singlet at δ 1.47, and oxymethylene signals at δ 3.64 and 3.76 (1H each, d, $J = 12$ Hz) revealed the presence of a 2-hydroxymethyl-2-methylpyran ring attached to ring C. A sharp singlet at δ 3.84 (3H) was attributed to an *N*-methyl group. In the ^{13}C NMR spectrum of **4**, signals of the *N*-methyl carbon and the C-13 carbon in the 2-hydroxymethyl-2-methylpyran ring appeared at δ 49.1 and 80.6, respectively. In the HMBC spectrum (Fig. 2), the correlations between the methyl signal at δ_{H} 1.47 (H-14) and carbon signals at δ_{C} 67.5 (C-15), 80.6 (C-13), and 121.9 (C-12) confirmed that the CH_2OH group was located at C-13. This clearly indicated that compound **4** was produced by the hydroxylation of **2**. From the above data, the structure of 5-hydroxynoracronycine alcohol (**4**) was established as shown.

Compounds **1-4**, and **7** were tested for cytotoxicity *in vitro* on HepG2, KB, H2058, COLO 201, HA22T, and Chang liver cell lines. These five alkaloids showed cytotoxic effects ($\text{IC}_{50} < 50 \mu\text{M}$) on HepG2 and KB cell lines, while they did not exhibit cytotoxic activity ($\text{IC}_{50} > 100 \mu\text{M}$) on H2058, COLO 201, HA22T, and Chang liver cell lines. Among these alkaloids, compound **4** was most cytotoxic to the KB epidermoid carcinoma cell line ($\text{IC}_{50} = 19.5 \mu\text{M}$), and compound **7** was most cytotoxic to HepG2 hepatoma cell line ($\text{IC}_{50} = 17.0 \mu\text{M}$).

EXPERIMENTAL SECTION

General Experimental Procedures

Melting points were recorded on a Yanaco MP-I3 micro melting point apparatus and are uncorrected. NMR spectra were recorded at 500 MHz for ^1H and 125 MHz for ^{13}C on a Varian Unity Inova-500 spectrometer. FEBMS and HRFEBMS spectra were obtained using Finnigan MAT GCQ and Finnigan MAT 95S spectrometers, respectively. Reversed-phase HPLC was performed on a Hewlett-Packard series 1100 pump system equipped with a Hewlett-Packard UV/VIS detector.

Fig. 2. Major HMBC correlations of compound **4**.

Plant Material

The buntan shaddock was cultivated at Hwa-Lien District Agricultural Improvement Station, Hwa-Lien County, Taiwan. The stem bark was collected in June, 2002, pulverized and dried at 45 °C. A voucher specimen was deposited in the Laboratory of Plant Nutrition, Department of Agricultural Chemistry, National Taiwan University, Taiwan.

Extraction and Isolation

The oven-dried (45 °C) stem bark powder of *C. maxima* (20 kg) was refluxed three times in methanol (120 L $\times$ 3). The MeOH extract was concentrated under vacuum and the concentrated extract (500 mL) was treated with water (5 L). The aqueous portion was chromatographed on a Diaion HP-20 column (11 $\times$ 60 cm) with water (8 L) followed by methanol (6 L). The methanol eluate was subjected to Sephadex LH-20 column chromatography (7 $\times$ 60 cm) with methanol (4.5 L). Fractions were collected in 200 mL portions and pooled according to their TLC profile. Four fractions (1-4) were collected. Five known compounds, glycoctrine-I (**1**, 11.2 mg), 5-hydroxynoracronycine (**2**, 9.3 mg), citrusine-I (**3**, 86.6 mg), grandisine-I (**5**, 1.2 mg), and natsucitrine-II (**6**, 2.0 mg), along with a new compound **4** (3.7 mg) were obtained from Fr. 3 by Sephadex LH-20 column eluting with MeOH. Fr. 4 was also subjected to Sephadex LH-20 column to give citracridone-III (**7**, 10 mg).

5-Hydroxynoracronycine alcohol (**4**)

Yellow powder, mp 162–163 °C; UV_{max} (MeOH): 266 (log ϵ , 4.34), 283 (4.21); IR bands (KBr) ν_{max} 3364, 2917, 1625, 1567, 1372 cm^{-1} ; ^1H NMR (500 MHz, acetone- d_6): δ 1.47 (3H, s, H-14), 3.64 (1H, d, $J = 12$ Hz, H-15), 3.76 (1H, d, $J = 12$ Hz, H-15), 3.84 (3H, s, N-CH₃), 5.66 (1H, d, $J = 10$ Hz, H-12), 6.10 (1H, s, H-2), 6.82 (1H, d, $J = 10$ Hz, H-11), 7.21 (1H, t, $J = 7.6$ Hz, H-7), 7.33 (1H, d, $J = 7.6$ Hz, H-6), 7.78 (1H, d, $J = 7.6$ Hz, H-8), 14.42 (1H, br, OH at C-1); ^{13}C NMR (125 MHz, acetone- d_6): δ 22.5 (C-14), 49.1 (N-CH₃), 67.5 (C-15), 80.6 (C-13), 98.2 (C-2), 103.3 (C-4), 107.6 (C-9a), 117.1 (C-8), 120.9 (C-6), 121.9 (C-12), 123.0 (C-11), 124.2 (C-7), 125.8 (C-8a), 138.1 (C-10a), 148.8 (C-4a), 149.4 (C-5), 162.2 (C-3), 165.5 (C-1), 182.9 (C-9); FABMS m/z 339; HRFABMS m/z 339.1097 (calcd for $\text{C}_{19}\text{H}_{17}\text{NO}_5$, 339.1101).

Cytotoxic Activity Against Tumor Cells¹⁹

Four human tumor cell lines used in this study were provided by the Cell Bank of Veterans General Hospital, Taipei, Taiwan. They were malignant melanoma cell line (H2058),

colon adenocarcinoma cell line (COLO 201), and hepatoma cell lines (HA22T, HepG2). An epidermoid carcinoma cell line (KB; CCRC 60017) and a non-tumor cell line (Chang liver cells; CCRC 60024) were purchased from the Food Industry Research and Development Institute (FIRDI, Taiwan).

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay was performed on 96-well plates. The assay is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to give a blue formazan product that could be measured spectrophotometrically. Tumor cells ($1-1.5 \times 10^4$ cells/mL) were inoculated in each well and the plates were incubated overnight at 37 °C and in 5% CO₂. Twenty-four hours after seeding, 200 µL treated or non-treated compounds in various concentrations were added and the plates were incubated for 2 days. At day 3, 20 µL of MTT solution (5 mg/mL) per well was added to each cultured medium. After four hours incubation, the medium was discarded and formazan blue formed in the cells was resolved by adding 100 µL of DMSO. The plates were read on a Dynatech MR5000 Microelisa reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. Cytotoxicity was expressed as 50% inhibitory concentration (IC₅₀) of cell growth.

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