A New Benzo-γ-pyran Derivative Isolated from Propolis

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Introduction

Propolis is a glue-like substance prepared by honeybees from plant materials including their own secretion. It has been employed as a folk remedy for treating various ailments. Propolis extract is alleged to exhibit a broad spectrum of activities including antibiotic, antiinflammation, anti-oxidant and tumor cell arresting properties.

Ethanol extracts of propolis had previously been demonstrated to be cytotoxic to the human oral epidermoid carcinoma (KB) and HeLa cell lines (Hladon et al., 1980). Guided by Ltk−cell growth inhibition assay, Grunberger et al. (1988) isolated and characterized a biologically active component which showed preferential cytotoxicity to tumor cells as caffeic acid phenethyl ester. Matsuno (1995) isolated a new clerodane diterpenoid which showed preferential cytotoxicity to tumor cells versus normal ones. Recently, other cytotoxic substances were isolated from propolis and characterized as diterpenoid isomers (13Z and 13E- ecliptan and tumor cell arresting properties.

Oral epidermoid carcinoma (KB) and HeLa cell lines (Hladori et al., 1997a; Matsuno et al., 1997b). Guided by HuH 13 cell cytotoxicity assay, a compound, named PM-3, was obtained as colorless needles, mp 113–115 °C. It was sparingly soluble in acidic and neutral water, practically insoluble in n-hexane and petroleum ether, slightly soluble in basic water, methanol, ethanol, acetone, ether and soluble in acetoneitrile, chloroform, ethyl acetate, dimethyl sulfoxide and dimethyl formamide.

Results and Discussion

Guided by HuH 13 cell cytotoxicity assay, a compound, named PM-3, was obtained as colorless needles, mp 113–115 °C. It was sparingly soluble in acidic and neutral water, practically insoluble in n-hexane and petroleum ether, slightly soluble in basic water, methanol, ethanol, acetone, ether and soluble in acetoneitrile, chloroform, ethyl acetate, dimethyl sulfoxide and dimethyl formamide.

The structure of this compound was determined from the following data.


2) 1H-NMR (400 MHz, CDCl3, internal standard TMS) δ (ppm): 1.44 (6H, s, 10-H, 11-H), 1.73 (3H, s, 16-H), 1.75 (3H, s, 15-H), 3.27 (2H, d, J = 7.5, 12-H), 5.27 (1H, m, 13-H), 5.66 (1H, d, J = 9.9, 8-H), 6.27 (1H, d, J = 15.8, 18-H), 6.32 (1H, d, J = 9.9, 7-H), 7.05 (1H, d, J = 2.1, 2-H), 7.19 (1H, d, J = 2.1, 6-H), 7.68 (1H, d, J = 15.8, 17-H), 11.9 (1H, bs, 19-H).

3) 13C-NMR (400 MHz, CDCl3, internal standard TMS) δ (ppm): 126.2, 124.4, 121.0, 153.2, 122.0, 132.6, 25.8, 17.8, 147.2, 114.1, 173.2.

The chemical shift of carbons were determined by (1) DEPT 45, 90 and 135; (2) HMOC and HMBC. Individual carbons were assigned by 2D-NMR. DEPT 13C-NMR confirmed eleven carbons with one or three protons attached (C2, C6, C7, C8, C10, C11, C13, C15, C16, C17, C18) and one carbon with two protons attached (C12).

In the present paper we report the isolation and characterization of a new benzopyran derivative which has cytotoxic activity.

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These data led us to determine the structure of PMS-3 as a benzopyran derivative, as shown in Fig. 1a (assignment of each carbon is tentatively according to that of artepillin C).

Previously we isolated artepillin C (Bohlmann and Jakupovic, 1979; Bohlmann et al., 1981) (Fig. 1b) from propolis and chemically synthesized it (Matsuno et al., 1997b). Fig. 1 clearly shows that these compounds are closely related structurally. Comparison of the 1H-NMR signals of PM-3 and artepillin C revealed that there were different signals due to 7 and 8-H of PM-3, suggesting that PM-3 is a cyclization product of artepillin C (coupling of 9C to O of 4C). This was confirmed since PM-3 was chemically synthesized from artepillin C essentially according to the oxidative cyclization procedure described by Anand and Jain (1972).

There are many papers on the derivatives or analogues of benzopyran (Cooke and Down, 1970; Huneck et al., 1986; Yamato, 1992). However, this compound including its biological activities has not been reported yet.

These data, therefore, led us to determine the structure of PM-3 as a new benzopyran derivative, properly, 3-[2-dimethyl-8-(3-methyl-2-butenyl) benzopyran]-6-propenoic acid.

The cytotoxicity of PM-3 is shown in Table I. PM-3 retarded the growth of HuH13 (human hepatocellular carcinoma), HeLa, KB (human oral epidermoid carcinoma) and HLC-2 (human lung carcinoma) cells and damaged them with ID50 around 20 µg/ml. Time to cell death by exposure of the cells to the compound was inversely related to dose. However, in contrast to artepillin C, PM-3 required much longer incubation time to induce the cell damage. There may be a possibility, therefore, that PM-3 acquires its cytotoxicity after alteration of its structure either in the incubation media and/or in the culture cells during incubation period. We have isolated the isomer of this compound (Hirota et al., unpublished data). The cytotoxic activity of PM-3 and its analogues is under investigation in relation to their chemical structures.

### Experimental Section

#### Extraction and isolation

100 g of Brazilian propolis (mixed product collected from hives located in various districts of Brazil including São Paulo, Paraná and Santa Catarina etc) was homogenized and extracted by stirring at room temp, with MeOH. To the extract was added H2O (10% (v/v)) and the precipitate formed was removed by low speed centrifugation. To the supernatant equal volume of of ethyl acetate and half volume of distilled water were added and mixed. The upper layer was collected, followed by evaporation of the solvent by rotary evaporator. The extract was dissolved in MeOH and filtered through nylon membrane (Type M COOH
NYL, Whatman, 0.2 µm) and used for isolation by means of preparative HPLC.

Preparative HPLC. HPLC system: ODS 80 T M (Toso), column: 55 x 300 mm with 20 ml sample loop, detection: UV 210 nm, elution: linear gradient of MeOH (70–100% (v/v)), flow: 20 ml/min. The eluates (fraction B, eluted by ca. 95% MeOH, retention time: around 120 min) were evaporated to dryness by rotary evaporator.

Semi-preparative HPLC. The extract was dissolved in chloroform and applied to an Inertsil SIL column (GL Science, 10 x 250 mm), followed by elution with CHCl₃ (retention time: 5.5–6 min). Detection: UV 210 nm, flow: 10 ml/min. The eluates were dried in a rotary evaporator, dissolved in CHCl₃, and final purification was achieved by molecular sieve HPLC chromatography.

Final semi-preparative HPLC. Column: GPC-H 2000 (Shodex, Showadenko, 20 x 500 mm), eluant: CHCl₃, detection: UV 210 nm, flow: 3.5 ml/min, retention time: around 20 min. The final yield was 15 mg.

Cell cultures

Cell culture was performed essentially as described previously (Matsuno, 1995). After plating and incubation of the cells in a 96-well micro plate overnight, they were incubated with added PM-3 for 1, 3 and 5 days, respectively. To the culture media MTS was added and the cells were incubated for 24 h followed by measurement of the absorbance of the formazan formed at 490 nm as described (Matsuno, 1997b).

Chemical synthesis of PM-3 from artepillin C

Artepillin C was dissolved in dry CH₂Cl₂ and stirred with equal amount of 2,3-dichloro-5,6-bicyano-benzoquinone for 1 h at room temp. The insoluble residue was filtered and the filtrate evaporated to dryness. The resulting solid was applied to silica gel gel column and eluted with n-hexane-acetone. The compound (mp 113–115 °C) was recrystallized from AcOEt-n-hexane in ca. 25% yield.

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Note added in proof:

The presence of this benzopyran derivative in Brazilian propolis has recently been reported (Boudourova-Krasteva et al. (1987), Z. Naturforsch. 52C, 676–679; Banskota et al. (1998), J. Natural Products 61, 896–900).