# Isolation and Characterization of Cytotoxic Diterpenoid Isomers from Propolis

Tetsuya Matsuno[a], Yasuyuki Matsumoto[b], Masahiro Saito[b] and Junji Morikawa[b]

[a] National Institute of Health, Gakuen, Musashimurayama, Tokyo 208, Japan
[b] Eiken Chemical Co., Ltd. 1-33-8 Hongo, Bunkyo-ku, Tokyo 113, Japan

Z. Naturforsch. **52c,** 702–704 (1997);
received March 27/May 12, 1997

Propolis, Clerodane Diterpenoid, Naphthalene Carboxylic Acid Derivative, Tumor Cells, Cytotoxicity

The methanol extract of Brazilian propolis was fractionated by HPLC, based on HuH13 (human hepatocellular carcinoma) cell cytotoxicity assay. Two isomers of diterpenoid with a molecular formula of $C_{20}H_{30}O_3$ (MW: 318.46) were isolated. The structures of these colorless compounds were determined as clerodane diterpenoids (I, 15-oxo-3, 13*Z*-kolavadien-17-oic acid; II, 15-oxo-3*Z*, 13*E*-kolavadien-17-oic acid).

## Introduction

Propolis is a resinous material gathered by honey bees from the buds and burk of certain trees and plants, and used inside their hives. It has been used as a folk remedy for treating various ailments mainly in Europe. It contains a variety of compounds including phenolic compounds like flavonoids, cinnamic acid, its derivatives, caffeic acid, its derivatives, aldehydes, ketones etc. (Greenaway *et al.,* 1991).

Ethyl ether extracts of propolis had previously been demonstrated to be cytotoxic to KB and HeLa cell lines (Hladoń *et al.,* 1980). Grunberger *et al.* (1988) isolated and characterized one such biologically active component as caffeic acid phenethyl ester. Matsuno (1995) isolated a new clerodane diterpenoid which showed preferential cytotoxicity to tumor cells. This compound suppressed skin tumorigenesis induced by topical application of 7,12-dimethylbenz(a)anthracene on mouse back skin (Mitamura *et al.,* 1996).

Reprint requests to Dr. T. Matsuno,
Institute of Cancer Research, 701 West 168th Street, New York, NY 10032, USA.
Telefax: (212) 305-5328.

We investigated chemical constituents of Brazilian propolis which showed tumoricidal activities. In the present paper we report the isolation and characterization of diterpenoid isomers.

## Results and Discussion

The structures of these colorless compounds were determined as being isomers of clerodane diterpenoid (**I**, 15-oxo-3, 13*Z*-kolavadien-17-oic acid (13*Z*-symphyoreticulic acid); **II**, 15-oxo-3*Z*, 13*E*-kolavadien-17-oic acid (13*E*-symphyoreticulic acid) as shown in Fig. 1) with the molecular formula $C_{20}H_{30}O_3$ (MW: 318.46) deduced from the following data.

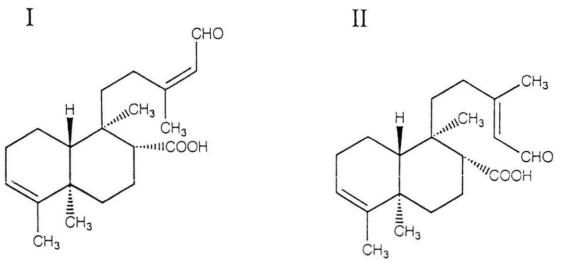


Fig. 1. **I**, 15-oxo-3, 13*Z*-kolavadien-17-oic acid (13*Z*-symphyoreticulic acid); **II**, 15-oxo-3*Z*, 13*E*-kolavadien-17-oic acid (13*E*-symphyoreticulic acid).

Mass spectrum *m/e*: 318 $[M]^+$ (by EIMS method, 70 eV).

$^1$H NMR (400 MHz, $CDCl_3$): internal standard TMS, δ (ppm): I, 0.96 (3H, S, 20-H), 1.08 (3H, S, 19-H), 1.45 (1H, bd, *J*=12.0, 10-H), 1.60 (3H, bs, 18-H), 2.00 (3H, d, *J*=1.0, 16-H), 2.64, 2.46 (2H, dt, *J*=12.3, 4.1, 12-H), 5.22 (1H, bs, 3-H), 5.85 (1H, dd, *J*=8.0, 1.0, 14-H), 9.93 (1H, d, *J*=8.0, 15-H), II, 0.96 (3H, S, 20-H), 1.06 (3H, S, 19-H), 1.40 (1H, bd, *J*= 11.7 Hz, 10-H), 1.60 (3H, bs, 18-H), 1.98, 2.31 (2H, m, dt, *J*-13.2, 3.7 Hz, 12-H), 2.21 (3H, d, *J*=1.2 Hz, 16H), 5.21 (1H, bs, 3-H), 5.90 (1H, dd, *J*=8.1, 1.2 Hz, 14-H), 9.97 (1H, d, *J*=8.1, 15-H).

The oily substance (which also showed the cytotoxicity) shown in Fig. 2 had similar chromatographic behavior with **I** and **II** on the column of Inertsil SIL.

From spectral data it was estimated that these compounds (**I** and **II**) are identical to those present in *Jungermannia infusca* (Toyota *et al.,* 1989).

0939–5075/97/0900–0702 $ 06.00  N

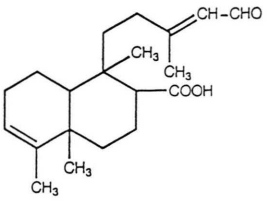


Fig. 2.

Table. Cytotoxicity of the compounds **I** and **II** with cultured cells.

| Cell lines | $ID_{50}$ [$\mu$g/ml]* |
|---|---|
| HuH13 | 18 |
| HeLa | 23 |
| KB | 22 |

* Mean of triplicate experiments. The oily substance showed the similar cytotoxicity.

These compounds (**I, II** and the oily substance) retarded the growth of HuH13 (human hepatocellular carcinoma), KB, HeLa and HLC-2 (human lung carcinoma) cells and damaged them. $ID_{50}$ of these compounds to these cell lines was around 20–30 μg/ml as shown in the Table. When cells were damaged they swelled and appeared more granulated with a rougher surface. Time to cell death by exposure of the cells to the compounds was inversely related to dose.

## Experimental section

*Isolation.* 100 g of Brazilian propolis (mixture of the collection from hives located in various districts of Brazil including São Paulo, Paraná and Santa Catalina etc.) was homogenized by stirring at room temperature with ×5 volumes of MeOH. To the supernatant water was added (10% (v/v)) and the precipitate formed was removed by low speed centrifugation. To the supernatant (495 ml) equal volume of ethyl acetate and 300 ml of water were added and mixed. The upper layer was collected followed by evaporation *in vacuo* by a rotary evaporator. The extract obtained (approximately 20 g) was dissolved in 200 ml of MeOH and filtered through Nylon Membrane (Type M NYL, Whatman, 0.2 μm) and was subsequently applied to the HPLC columns.

*Preparative HPLC:* ODS 80 Tm (TOSO), column size: 55×300 mm, detection: UV 254 nm, eluent: 70 to 100% (v/v) linear gradient of MeOH, flow rate: 20 ml/min. Solvent of collected fractions (eluted by approximately 95% MeOH, retention time: around 120 min) was evaporated by a rotary evaporator.

The extract was dissolved in chloroform ad applied to semi-preparative HPLC column and eluted by chloroform.

*Semi-preparative HPLC:* Inertsil SIL (GL Science), column size: 10×250 mm, detection: UV 254 nm, flow rate, 10 ml/min. Two main fractions (retention time: 9.5–10.5 min and 10.5–11.5 min, respectively) were obtained. After evaporation the material was dissolved in chloroform, followed by final HPLC chromatography. Two peaks (**I** and **II**) were obtained.

*Final semi-preparative HPLC:* GPC-H2001 (Shodex, Showadenko), column size: 20×500 mm, detection: UV 254 nm, eluent: chloroform, flow rate: 10 ml/min, retention time of **I**: around 20 min, retention time of **II**: around 19 min. The final yield was 40 mg (**I**) and 50 mg (**II**), respectively.

Cell culture and cytotoxicity of the compounds were performed as described previously (Matsuno, 1991; 1995).

Greenaway W., May J., Scaysbrook T. and Whatley F. R. (1991), Identification by gas chromatography-mas spectrometry of 150 compounds in propolis. Z. Naturforsch. **46c,** 111–121.

Grunberger D., Banerjee R., Eisinger K., Oltz E. M., Efros L., Caldwell M., Esevez V. and Nakanishi K. (1988), Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester. Experientia **44,** 230–232.

Hladoń B., Bylka W., Ellnain-Wojtaszek M., Skrzypczak L. (1980), *In vitro* studies on the cytostatic activity of propolis extract. Arzneim.-Forsch./Drug Res. **30,** 1847–1848.

Matsuno T. (1991), Pathway of glutamate oxidation and its regulation in the HuH13 line of human hepatoma cells. J. Cell. Physiol. **148,** 290–294.

Matsuno T. (1995), A new clerodane diterpenoid isolated from propolis. Z. Naturforsch. **50c,** 93–97.

Mitamura T., Matsuno T., Sakamoto S., Maemura M., Kudo H., Suzuki S., Kuwa K., Yoshimura S., Sassa S., Nakayama T. and Nagasawa H. (1996), Effect of a new clerodane diterpenoid isolated from propolis on chemically induced skin tumors in mice. Anticancer Res. **16,** 2669–2672.

Toyota M., Nagashima F. and Asakawa Y. (1989), Clerodane, kaurane and labdane diterpenoids from the liverwort *Jungermannia infusca.* Phytochemistry **28,** 3415–3419.