The First Glycosides Isolated from Propolis: Diterpene Rhamnosides

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Two diterpene glycosides, *ent-8*(17)-labden-15-O-α-L-rhamnoside and *ent*-8(17)-labden-15-O-(3′-O-acetyl)-α-L-rhamnoside (new natural compounds) were isolated from propolis from El Salvador. The compounds showed significant antibacterial activity and moderate toxicity to *Artemia salina* nauplii. These are the first glycosides reported in bee glue.

Introduction

Propolis (bee glue) is a sticky dark-coloured material that honeybees collect from living plants, mix it with wax and use in construction and adaptation of their nests. It has been known as a remedy since ancient times and is still used in folk medicine (Ghisalberti, 1979), in "bio-cosmetics", "health foods" and for numerous further purposes (Matsuda, 1994, Wollenweber and Buchmann, 1997). Tropical samples from different locations have shown significant differences to propolis from temperate zones, as well as between themselves (Marcucci and Bankova, 1999; Bankova *et al.*, 2000). For this reason, tropical bee glue has recently become a subject of increasing interest for chemists and biologists and turned out to be a source of new biologically active compounds (Banskota *et al.*, 2000; Claus *et al.*, 2000, Hirota *et al.*, 2000; Kimoto *et al.*, 1998). In this paper we report the isolation and characterization of two diterpene glycosides from propolis originating from Central America, and their biological activity. These are the first glycosides reported in bee glue.

Experimental

Propolis was collected in the Eastern region of El Salvador, in January 2000.

Extraction of propolis

Propolis (50 g) was cut into small pieces and extracted with 70% ethanol (1:10, w:v) at room temperature for 24 h. A small part of this extract (10 ml) was evaporated to dryness and subjected to biological tests. The ethanol extract was concentrated *in vacuo* and extracted successively with *n*-hexane (3 times) and with ethyl ether (3 times). The hexane extract was evaporated to give 9.0 g dry residue and the ethyl ether extract gave13.0 g dry residue after evaporation.

Isolation of compounds

The *n*-hexane extract was subjected to column chromatography on silica gel with an acetone – *n*-hexane gradient to produce several fractions. After repeated column chromatography and preparative TLC on silica gel, *n*-hexane – methylethylketone as the mobile phase, compounds **1** and **2** were isolated.

ent-8(17)-labden-15-O-α-L-rhamnopyranoside (**1**). Colorless oil, 78 mg; $[\alpha]_D^{20}$ −50.7° (c = 0.006). MS, ^{1}H- and ^{13}C-NMR spectra identical with literature data (Fukuyama *et al.*, 1999).

ent-8(17)-labden-15-O-(3′-O-acetyl)-α-L-rhamnopyranoside (**2**). Colorless oil, 55 mg, $[\alpha]_D^{20}$ −41.7° (c = 0.008), 480 (M^+., 3%), 420 (1%), 291 (10%), 189 (100%), 137 (48%), 42 (55%). ^{1}H-NMR (250 MHz, CDCl3): δ 0.68 (3H, s, CH_3-20), 0.80 (3H, s, CH_3-19), 0.87 (3H, s, CH_3-18), 0.90 (3H, d, J = 6.2 Hz, CH_3-16), 1.10 (1H, m, H-5), 1.12 91H, m, H-

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3), 1.33 (3H, d, J = 6.0Hz, CH_3-6′), 1.35 (1H, m, H-3), 1.50 (2H, m, H-2), 1.60 (2H, m, H-6), 1.75 (2H, m, H-1), 1.95 (1H, m, H-7β), 2.18 (3H, s, CH_3-OAc), 2.35 (1H, m, H-7α), 3.43 (1H, m, H-15), 3.68 (1H, m, H-5′), 3.70 (1H, m, H-4′), 3.72 (1H, m, H-15), 4.00 (1H, br s, H-2′), 4.49 (1H, d, J = 1.4Hz, H-17), 4.74 (1H, d, J = 1.7Hz, H-1′), 4.81 (1H, d, J = 1.4Hz, H-17). For ^{13}C-NMR – see Table I.

Antibacterial activity

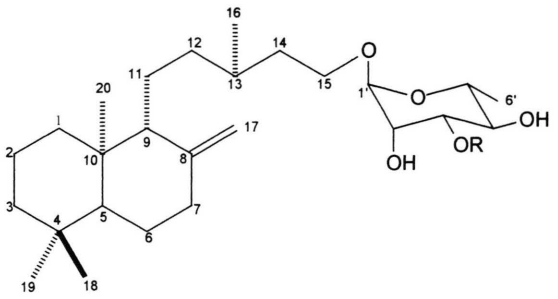
For the investigation of the antibacterial and antifungal activity, the agar cup method (Spooner and Sykes, 1972) was used with test strains *Staphylococcus aureus* 209 (obtained from the Bulgarian Type Culture Collection, Institute for State Control of Drugs, Sofia), *Escherichia coli* WF+ (obtained from the Collection of ZIMET, Central Institute of Microbiology and Experimental Therapy, Jena, Germany) and *Candida albicans* 562 (obtained from the Bulgarian Type Culture Collection, institute for State Control of Drugs, Sofia). An inhibitory zone with a diameter less than 10 mm corresponds to lack of activity (10 mm is the diameter of the cup). 0.1 ml of test solution containing 0.4 mg of each substance in ethanol was applied to every cup (concentration of the test solution 4 mg/ml).

Cytotoxicity assay

Brine shrimp eggs obtained locally (Petrov, Sofia) were hatched following the procedure of Soils *et al.* (1993). *Artemia salina* (nauplii) lethality (Soils *et al.,* 1993) was determined using caffeic acid phenethyl ester (CAPE) as active reference compound. Concentrations of 1000, 100, 10 and 1 ppm were used. 10 *A. salina* per concentration plus control. The activity of the total extract and of the individual compounds was measured.

Results and Discussion

The *n*-hexane fraction from the ethanolic extract of the investigated propolis sample was subjected to repeated column chromatography and preparative TLC on silica gel and afforded two compounds (**1** and **2**). Their structures were determined by EIMS, ^{1}H and ^{13}C NMR spectra, DEPT, COSY, HMQC, HMBC.



1 R=H
2 R=Ac

The mass spectrum of compound **1** showed a M^+ ion peak of low intensity at *m/z* 438. The ^{13}C-NMR spectrum (Table I), including DEPT experiments, disclosed 26 carbon signals. The relatively low-field sp^3 methine carbon signal at δ_C 99.8 and the corresponding proton signal (HMQC) at δ_H 4.76 were suggestive of the presence of an anomeric center in a glycoside moiety. This suggestion

Table I. ^{13}C NMR data of **1** and **2**.[a]

Position	δC	
	1	**2**
1	39.1 t	39.1 t
2	19.4 t	19.4 t
3	42.2 t	42.2 t
4	33.6 s	33.6 s
5	55.5 d	55.5 d
6	24.4 t	24.4 t
7	38.4 t	38.4 t
8	148.8 s	148.9 s
9	57.1 d	57.1 d
10	39.7 s	39.7 s
11	20.8 t	20.8 t
12	36.1 t	36.0 t
13	30.4 d	30.3 d
14	36.6 t	36.6 t
15	66.1 t	66.2 t
16	19.6 q	19.5 q
17	106.2 d	106.2 d
18β	33.5 q	33.6 q
19α	21.7 q	21.7 q
20	14.4 q	14.4 q
1′	99.8 d	99.4 d
2′	71.1 d	69.8 d
3′	71.7 d	75.0 d
4′	72.8 d	71.6 d
5′	68.0 d	68.5 d
6′	17.5 q	17.5 q
CH_3C=O	–	21.2 q
CH_3 **C**=O	–	171.8 s

[a] ^{13}C-NMR were measured at 62.9 MHz in $CDCl_3$.

was supported by the presence of four more oxygenated methine carbons. The methyl carbon signal at δ_C 17.5 and the corresponding three-proton doublet at δ_H 1.32 indicated that the sugar was a 6-deoxy hexose. The coupling constant of the anomeric proton H-1′, J = 1.3 Hz is characteristic for α-L-rhamnose (Khatuntseva *et al.*, 1996). Detailed NMR analysis, including the long-range coupling networks observed between the methyl and olefinic protons with the adjacent carbons in a HMBC experiment (Fig. 1.), identified the aglycone as 8(17)-labden-15-ol. Comparison with published spectral data (MS, ^{1}H- and ^{13}C-NMR) confirmed that the structure of **1** was *ent*-8(17)-labden-α-L-rhamnopyranoside, recently found in Brazilian *Mimosa hostilis* (Fukuyama *et al.*, 1999).

The spectral characteristics of **2** were similar to those of **1**. The difference between the two compounds appeared to be the presence of one additional acetyl group in **2** (this was obvious from the NMR and MS spectral data, Table I and Experimental). Significant differences were observed in the chemical shifts of some of the carbon atom signals belonging to the sugar moiety (Table I). The downfield shift of the C-3′ carbon signal (+3.3 ppm) and the upfield shifts of the C-2′ (−1.3 ppm) and C-4′ (−1.2 ppm) carbon signals unambiguously determined the position of the acetyloxy group at C-3′. Compound **2** was identified as *ent*-8(17)-labden-15-O-(3′-O-acetyl)-α-L-rhamnopyranoside, a new natural compound.

Both rhamnosides showed good activity against *Staphylococcus aureus*, which was significantly higher than that of the total extract (Table II). No activity against *Escherichia coli* and *Candida albicans* was observed. The new labdenol glycosides exhibited moderate activity against *Artemia salina* nauplii (Table II). Obviously, **2** is partially responsible for the toxicity of the ethanol extract.

This is the first isolation of glycosides from a propolis sample. It is well known that bees "manufacture" propolis using substances actively secreted by plants as well as substances exuded from wounds in plants: lipophilic materials on leaves and leaf buds, gums, resins, latices etc. (Crane, 1987). Normally, such lipophilic excretions do not contain glycosides but labdane xylosides have been isolated from the resinous leaf exudate of *Haplopappus* species (Urzua *et al.*, 1995). The plant source of the above-described rhamnosides remains unclear. The identification of **1** and **2** in some plant exudate will be an indication that this exudate is one of the sources of propolis in El Salvador.

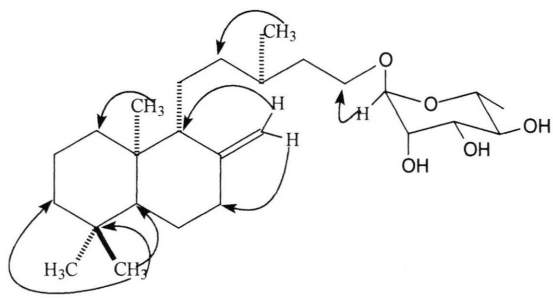


Fig. 1. Important HMBC correlations in **1**.

Table II. Biological activity of propolis extract and isolated compounds.

Sample	Cytotoxicity assay	Antibacterial activity against *S. aureus* [a]
	LC_{50} ± SD [mg/ml] [b]	Diameter of the inhibitory zone ± SD [mm] [b]
Alcohol extract	39±9	12±1
1	39±25	21±1
2	15±7	20.3±0.6
CAPE	0.45±0.05	–

[a] Concentration of the test solution 4 mg/ml.
[b] Mean of three measurements.

Bankova V., De Castro S. L. and Marcucci M. C. (2000), Propolis: recent advances in chemistry and plant origin. Apidologie **31**, 3–15.

Banskota A. Tezuka Y., Midorikawa K., Matsushige K. and Kadota Sh. (2000), Two novel cytotoxic benzofuran derivatives from Brazilian propolis. J. Nat. Prod. **63**, 1277–1279.

Claus R., Kinscherf Ch., Bonaterra G., Basnet P., Metz J. and Deigner H.-P. (2000), Antiapoptotic effects of propolis extract and propol on human macrophages exposed to minimally modified low density lipoprotein. Arzneim.-Forsch./Drug Res. **50**, 373–379.

Crane E. (1988), Beekeeping: Science, Practice and World Recources. Heinemann, London, pp. 367–372.

Fukuyama Y., Yokoyama R., Ohsaki A., Takahashi H. and Minami H. (1999), An example of the co-occurrence of enantiomeric labdane-type diterpenes in leaves of *Mimosa hostilis*. Chem. Pharm. Bull. **47**, 454–455.

Ghisalberti, E. (1979), Propolis: a review. Bee World **60,** 59–84.

Hirota M., Matsuno T., Fujiwara T., Sugiyama H. and Mineshita S. (2000), Enhanced cytotoxicity in a Z-photoisomer of a benzopyran derivative of propolis. J. Nat. Prod. **63**, 366–370.

Khatuntseva E. A., Shashkov A. S and Nifant'ev N. E. (1996), Synthesis and investigation of the NMR spectra and the conformation of branched oligosaccharides. 16. Synthesis of 3,4-di-O-glycosilated derivatives of methyl-α-L-rhamnopyranoside. Bioorg. Khim. **22**, 376–382.

Kimoto T., Arai Sh., Kohguchi M., Aga M., Nomura Yu., Micaleff M., Kurimoto M. and Mito K. (1998), Apoptosis and suppression of tumor growth by artepillin C extracted from Brazilian propolis. Cancer Detection Prevention **22**, 506–515.

Marcucci M. C. and Bankova V. S. (1999), Chemical composition, plant origin and biological activity of Brazilian propolis. Curr. Topics Phytochem. **2**, 115–123.

Matsuda Sh. (1994), Propolis – health care food. Foods and Food Ingred J Japan **160**, 64–73.

Soils P. N., Wright C. W., Anderson A. M., Gupta M. P. and Phillipson J. D. (1993), A microwell cytotoxicity assay using *Artemia salina* (brine shrimp), Planta Med. **59**, 250 –252.

Spooner F. D and Sykes G. (1972) Laboratory assessment of antibacterial activity. In: Methods in Microbiology (Norris J. R. and Ribbons D. W., Eds.), Vol. 7B. Academic Press, London, New York, pp. 216–217.

Urzua A., Tojo E. and Soto J. (1995), A diterpene xyloside from the resinous exudate of *Haplopappus diplopappus*. Phytochemistry **38**, 555–556.

Wollenweber E. and Buchmann S. L. (1997), Feral honey bees in the Sonoran Desert: propolis sources other than poplar (*Populus* spp.). Z. Naturforsch. **52c**, 530–535.