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β-Sitosteryl-D-glucoside from the Olive Tree (*Olea europaea* LINNE; Oleaceae) as a Feeding Stimulant toward the Olive Weevil (*Dyscerus perforatus*)

Emiko Kadowaki[a], Yasuhiro Yoshida[b], Naomichi Baba and Shuhei Nakajima

(Department of Bioresources Chemistry)

Guided by a feeding stimulant activity test on the olive weevil (*Dyscerus perforatus*), a feeding stimulant was isolated from the crude methanol extract of olive tree (*Olea europaea*). Based on the spectral data and the literature survey, it was identified as β-sitosteryl-D-glucoside.

Key words : Olive weevil, Olive, Feeding stimulant, β-Sitosteryl-D-glucoside

Introduction

The olive weevil [*Dyscerus perforatus* (ROELOFS); Coleopetera; Curculionidae] is a native species in Japan and now the most serious pest of the olive trees. Originally, this weevil seemed to colonise *Ligustrum japonicum* Thunb. and *L. obtusifolium* Sieb. et Zucc, both of which belong to the same oleacea family as olive. However, when olive trees were introduced to Japan in 1908, the weevils immediately attacked the plants and soon preferred them to the former hosts. Unlike in the former hosts, where the weevils live in a low population density, it is extraordinary high in the case of olive trees and the subsequent assault becomes seriously damaging for the host plant.

During the course of our study on the relationship between olive trees and olive weevils, we came to be interested in the possible chemical constituents that are responsible for host selection and attraction of the olive weevil to this plant. Previously, we reported that a secoiridoid glucoside, oleuropein, and some lignans, (−)-olivil and (+)-1-acetoxypinoresinol, from the olive tree stimulated the feeding habit of the weevil[1,2]. In this study, we found a steroidal glucoside as another feeding stimulant component in the olive tree. Here, we describe the isolation, characterization and activity of this feeding stimulant.

Materials and Methods

General procedure. All the NMR experiments were conducted on a Varian VXR500 (500 MHz for ^{1}H, 125 MHz for ^{13}C) spectrometer. GCMS (Automass 20, JEOL) analyses in the electron impact ionization (EI, 70 eV) were performed on a DB-1 column (ϕ0.25 mm × 30 m), using a programme from 70 °C (3 min) to 250 °C (70 min) at 10 °C min^{-1}. GC-FID (G-3000, HITACHI) was performed on the TC-1701 column (ϕ0.25 mm × 30 m), using a programme from 110 °C to 280 °C (5 min) at 5 °C min^{-1}. IR (KBr) spectrum was measured on a JASCO FT/IR-5000 spectrometer and optical rotation was taken on a JASCO Dip-360 digital polarimeter.

Insect material. The weevils were field collected as newly emerged adults from infested olive trees in July 2001. Male and female weevils were separately reared in plastic containers (27 cm × 20 cm × 13 cm) with a piece of young olive branch (*ca*. 20 cm length, ϕ5 mm) and wet cotton under gregarious conditions at 25 °C, with a 12L : 12D photoperiod.

Plant material. Olive trees (*Nevadillo Blanco*) were obtained from Nippon Olive Co., Ltd. in April 2001. They were cut in pieces for extraction.

Extraction and isolation of the plant material. Olive trunks (9.1 kg) were extracted with MeOH (26.4 L) at room temperature for 7 days. The extract was filtered, concentrated under reduced pressure and partitioned first with hexane (500 ml × 3) then with EtOAc (500 ml × 3). The EtOAc soluble fraction (160 g) was separated by column chromatography using silica gel 60 (Nacalai Tesque, 230-400 mesh), eluted successively with hexane (100 %) → hexane : EtOAc = 7 : 3 → 1 : 1 → EtOAc (100 %) → MeOH (100 %). The feeding stimulatory activity was found in the MeOH (100 %) fraction. In this manner the fraction was further

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a) *Graduate School of Natural Science and Technology, Okayama University, Tsushimanaka, Okayama 700-8530, Japan.*
b) *Nippon Olive Co., Ltd. 3911-10 Ushimado, Ushimado-cho, Oku-gun, Okayama 701-4394, Japan.*

separated by silica gel column and eluted with $CHCl_3$: MeOH = 4:1, followed by another silica gel column and eluted with $CHCl_3$:MeOH = 10:0.5 → 10:1, to afford an active fraction (Fr. 111, 220 mg). After evaporation of the solvent to near dryness and recrystallization from EtOAc, the active compound was obtained from the fraction as colorless needles (35 mg).

Active compound. Mp. not determined (dec. at 225 °C); $[\alpha]_D^{20}$ −44.0° (*c* 0.68, pyridine); IR ν max (KBr) cm^{-1}: 3400 (−OH), 2938 (C–H), 1462 (C=C), 1077 (C–O); NMRδ_H (500 MHz, *pyridine-d_5*); 0.66 (3H, s), 0.86 (3H, d, *J* = 6.0 Hz), 0.87 (3H, d, *J* = 6.0 Hz), 089 (3H, s), 0.92 (2H, m), 0.93 (3H, s), 0.98 (5H, m), 1.10 (2H, m), 1.23 (2H, m), 1.26 (4H, m), 1.38 (5H, m), 1.50 (1H, m), 1.54 (2H, m), 1.68 (1H, m), 1.71 (1H, m), 1.73 (1H, m), 1.87 (2H, m), 1.97 (1H, br, d, *J* = 12.5 Hz), 2.13 (1H, m), 2.47 (1H, t, *J* = 12.0 Hz), 2.74 (1H, dt, *J* = 12.0, 2.1 Hz), 3.97 (2H, m), 4.07 (1H, t, *J* = 7.6 Hz), 4.30 (2H, m), 4.43 (1H, dd, *J* = 11.6, 5.1 Hz), 4.58 (1H, d, *J* = 11.6 Hz), 5.06 (1H, d, *J* = 7.6 Hz), 5.35 (1H, t, *J* = 3.0 Hz); NMRδ_C (125 MHz, *pyridine-d_5*); 12.0, 12.2, 19.0, 19.2, 19.4, 20.0, 21.3, 23.4, 24.5, 26.4, 28.5, 29.4, 30.2, 32.0, 32.2, 34.2, 36.4, 36.9, 37.5, 39.3, 39.9, 42.5, 46.0, 50.3, 56.2, 56.8, 62.8, 71.7, 75.4, 78.1, 78.5, 78.6, 102.6, 121.9, 140.9.

Hydrolysis of active compound and sugar analysis. The active compound (10 mg) was hydrolysed with 1 M HCl in MeOH at 60 °C for 7 hr. Then, the solvent was evaporated and the residue was suspended in 1 M HCl and heated at 60 °C for 15 hr. After neutralization with $NaHCO_3$, the products were distributed between H_2O–$CHCl_3$. The aqueous layer was freeze-dried and trimethylsilylated with Tri-Sil Reagent (PIERCE, 100 μl) for 5 min at room temperature. After concentration under N_2 stream, the residue was dissolved in *n*-hexane and analysed by GC under the conditions as described above. The retention time (t_R: 20.56 min) of the trimethylsilylated sugar from the active compound was compared with that of an authentic sample (trimethylsilylated β-D-glucose, t_R: 20.64 min).

On the other hand, the $CHCl_3$ layer was purified by column chromatography using silica gel 60 and eluted with hexane: EtOAc = 4:1. The combined fractions gave 4.9 mg of aglycone which was analysed by GCMS. Aglycone (β-sitosterol); GCEIMS *m/z* (rel. int.): 414 $[M]^+$ (23), 381 (25), 303 (39), 255 (38), 213 (100).

Bioassay. For preconditioning, male and female insects were released in separate Petri dishes (ϕ40 mm) containing moistened paper disks (Advantec Toyo No. 2, ϕ5 mm) and were given distilled water every 12 hr for 24 hr. After the 24 hours starvation period, the moistened paper disks were replaced with paper disks containing sucrose and the active compound. The paper disks were prepared as follows: A methanol solution of a sample (1 mg/ml) and an aqueous solution of sucrose (5 mg/10 μl) were applied (sample 50 μg/disk, sucrose 5 mg/disk) uniformly to the surface of the paper disks and then the disks were air dried. A paper disk with MeOH and sucrose was used as a control. Then the Petri dishes were kept at 25 °C with a 12L: 12D photoperiod for 48 hr. Distilled water 20 μl/disk was added to each disk every 12 hr. Each test was repeated ten times. The extent of feeding responses was evaluated by measuring the bitten area of the disk. Thus, the assessment was expressed by a score from zero to + 3, in which zero is the case of no biting, + 1 is for a bitten track being found, + 2 is for less than 50 % bitten and + 3 is for more than 50 %. The feeding stimulative activity is defined as $[(A-B)/(+3 \times C)] \times 100\,\%$), which A = the total score of the sample disks, B = the total score of the control disks, and C = number of insects used. The results of the test were analyzed by Students' t-test.

Results and Discussion

The purification of the active compound was guided by feeding stimulant activity test (see Materials and Methods) on olive weevil. After extraction of olive tree with methanol, the methanol extract was evaporated and successively partitioned with hexane/water and EtOAc/water. The EtOAc-soluble fraction exhibited feeding stimulant activity. The active compound was isolated as colorless needles by further chromatographic separation of the fraction.

The ^{13}C-NMR spectrum of the active compound showed 35 carbon signals, including the signals corresponding to two olefinic carbons at δ121.9 (C-6) and δ140.9 (C-5). Furthermore, the ^{1}H-NMR spectrum exhibited one olefinic proton signal at δ5.35 (H-6), two angular methyl groups at δ0.89 (s, H-18), δ0.93 (s, H-19), an isopropyl (δ0.86 (H-26), 0.87 (H-27), 1.68 (H-25)) and an ethyl (δ0.66 (H-29), 1.26 (H-28)) groups (Fig. 1).

The active compound did not show any absorption maximum in the UV spectrum. However, strong absorption due to many hydroxyl groups (3400 cm^{-1})in its IR spectrum and the signals in the ^{1}H-NMR (δ3.97 (H-5'), 4.07 (H-2'), 4.30 (H-3', 4'), 4.43 (H-6'α), 4.58 (H-6'β), 5.06 (H-1')) and ^{13}C-NMR (δ62.8 (C-6'), 71.7 (C-4'), 75.4 (C-2'), 78.1 (C-5'), 78.6 (C-3'), 102.6 (C-1')) spectra suggested that the compound was a steroidal glycoside[4].

After the acidic hydrolysis of the active compound, the GCEIMS spectrum of the aglycone gave the molecular ion $[M]^+$ at *m/z* 414 (molecular formula:

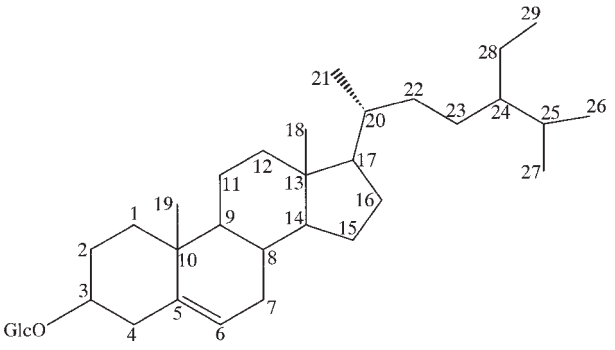

Fig. 1 Structure of active compound.

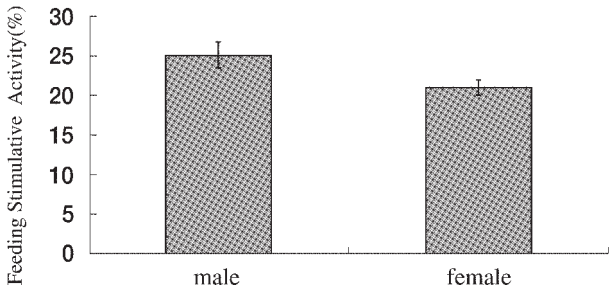

Fig. 2 Effect of the active compound on the feeding stimulative activity of male and female weevils.
Bars are mean±SE (n=50).

$C_{29}H_{50}O$). In addition, the fragmentation pattern in the mass spectrum and the chemical shifts in the ^{13}C-NMR spectrum resembled those of β-sitosterol[3]. The GC retention time of trimethylsilylated sugar and the coupling constant ($J_{1',2'}=7.6$ Hz) of the anomeric proton indicated that the sugar moiety of the active compound was β-D-glucosyl. These data, therefore, led us to elucidate the structure of the active compound to be β-sitosteryl-D-glucoside.

Figure 2 exhibits the feeding response of the active compound on olive weevils. It shows moderate feeding stimulant activity for both males (*ca.*25%) and females (*ca.*21%), respectively. The difference between the responses of males and females was not significant ($p < 0.05$, t-test).

β-Sitosterol is known as one of the biting factors for silkworm[5] and it is also an important intermediate in cholesterol biosynthesis of insects[6]. Therefore, it was suggested that this compound may play an important role for their behavior, host selection and attraction. In addition, the activity of the compound discovered in the present study suggests use in stimulant on bait for rounding up the weevils in one place.

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オリーブに含まれるオリーブアナアキゾウムシ摂食刺激物質 β-sitosteryl-D-glucoside

門脇英美子[a)]・吉田　靖弘[b)]・馬場　直道・中島　修平
（生物資源化学講座）

オリーブアナアキゾウムシは，モクセイ科のオリーブに多数寄生し甚大な被害を与えるため，オリーブ栽培上の深刻な問題となっている．我々は，これまでオリーブのメタノール抽出物から，オリーブアナアキゾウムシの摂食刺激成分として，雌雄に活性を示すセコイリドイド配糖体１種と，雌に特異的に活性を持つ２種のリグナン類を得た．さらに今回，同じメタノール抽出物から，活性物質としてステロイド配糖体である β-sitosteryl-D-glucoside を得た．この成分は雌雄に対してほぼ同等の摂食刺激活性を示した．

a) 岡山大学大学院自然科学研究科
b) 日本オリーブ株式会社