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Production of Oleanene Triterpenes by *Streptomyces*

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Three oleanene triterpenes have been isolated from the culture broth of *Streptomyces* strain H 1082-MY 15. Two of them were identified as known soybean sapogenols, soyasapogenols E and B. The third compound, a new triterpene, was shown to be 24-hydroxyolean-12-ene-3,22-dione by spectroscopic analysis and chemical correlation.

Keywords——*Streptomyces*; taxonomy; oleanene triterpene; soyasapogenol E; soyasapogenol B; 24-hydroxyolean-12-ene-3,22-dione; chemical correlation

In our screening for cholesterol esterase produced by microbes, the primary screening was performed by examining silica gel thin layer chromatograms of the incubation mixture of culture filtrate and cholesterol linolate.[1] Cholesterol linolate or its hydrolysis product cholesterol gave a characteristics purple or pink color on the thin layer chromatograms when they were heated after being sprayed with 40% sulfuric acid. In the course of the above screening, an unidentified *Streptomyces* species was found to produce two substances which afforded a bright blue to green color on the thin layer chromatograms, and were different from cholesterol linolate or cholesterol. These substances, named carbazomycins A and B, had a carbazole skeleton and showed antimicrobial activity.[2,3] Their structures were determined by spectroscopic[3] and X-ray crystallographic[4] analysis, and furthermore, the taxonomy of the carbazomycin-producing microorganism and the mechanism of biosynthesis of carbazomycin B have just been reported.[5]

On the other hand, another *Streptomyces* species, strain H 1082-MY 15, was shown to produce three substances different from cholesterol linolate, cholesterol or carbazomycins on thin layer chromatography (TLC) in the course of similar screening for cholesterol esterase. These substances were tentatively designated as compounds I (**1**), II (**2**), and III (**3**). The major component, compound I (**1**), and the minor component, compound III (**3**), have been proved to be oleanene triterpenes, soyasapogenols E and B, respectively. The second major component, compound II (**2**), was different from the known soyasapogenols[6–8] and its structure has been determined to be 24-hydroxyolean-12-ene-3,22-dione by spectroscopic and chemical means, in particular by chemical correlation with soyasapogenol B. In this paper, we describe the characterization of the producing microorganism, the fermentation, and the extraction, purification and structural determination of compounds I, II, and III.

Characterization of the Producing Microorganism

Strain H 1082-MY 15 was isolated from a soil sample collected at Abashiri City, Hokkaido, Japan. The characteristics of the microorganism were studied in accordance with the methods described for the International Streptomyces Project (ISP) by Shirling and Gottlieb[9] and by Waksman.[10] Color names and hue numbers were determined according to the "Guide to Color Standard"[11] and "Color Harmony Manual."[12]

The cultural characteristics of strain H 1082-MY 15 are shown in Table I. The aerial mycelium was brownish-gray to light brownish-gray on inorganic salts-starch agar (ISP

medium No. 4). The substrate mycelium was pale yellow to pale yellowish-brown on yeast-malt extract agar (ISP medium No. 2), oatmeal agar (ISP medium No. 3), inorganic salts-starch agar and glycerol asparagine agar (ISP medium No. 5). No soluble pigment was produced on any of the media tested.

Physiological characteristics of the microorganism are listed in Table II. Melanoid pigments were produced on tryptone-yeast extract broth (ISP medium No. 1), peptone-yeast

TABLE I. Cultural Characteristics of Strain H 1082-MY 15

	Growth	Aerial mycelium	Reverse side of colony	Soluble pigment
Yeast extract-malt extract agar (ISP-medium 2)	Good	Very poor white	Pale yellowish-brown (2 gc)	None
Oatmeal agar (ISP-medium 3)	Moderate	None	Pale yellow (2 ea)	None
Inorganic salts-starch agar (ISP-medium 4)	Moderate	Moderate Brownish-gray to light brownish-gray (3 fe)	Pale yellow (2 ea)	None
Glycerol-asparagine agar (ISP-medium 5)	Poor	None	Pale yellow (2 ca)	None

Hue numbers in parentheses were determined according to the "Color Harmony Manual."[12]

TABLE II. Physiological Characteristics of Strain H 1082-MY 15

Melanin production	
Tyrosine agar (ISP-medium 7)	Positive
Peptone-yeast extract iron agar (ISP-medium 6)	Positive
Tryptone-yeast extract broth (ISP-medium 1)	Positive
Starch hydrolysis	Positive
Gelatin liquefaction	Positive
Skim milk	
Coagulation (37 °C)	Positive
Peptonization (37 °C)	Negative
Cell wall type	I (L,L-Diaminopimelic acid)
Carbon utilization	
D-Glucose	++
D-Xylose	++
L-Arabinose	+
L-Rhamnose	++
D-Fructose	+
Raffinose	++
D-Mannitol	++
Isoinositol	++
Sucrose	++
Cellulose	−

++, good utilization; +, fair utilization; −, no utilization.

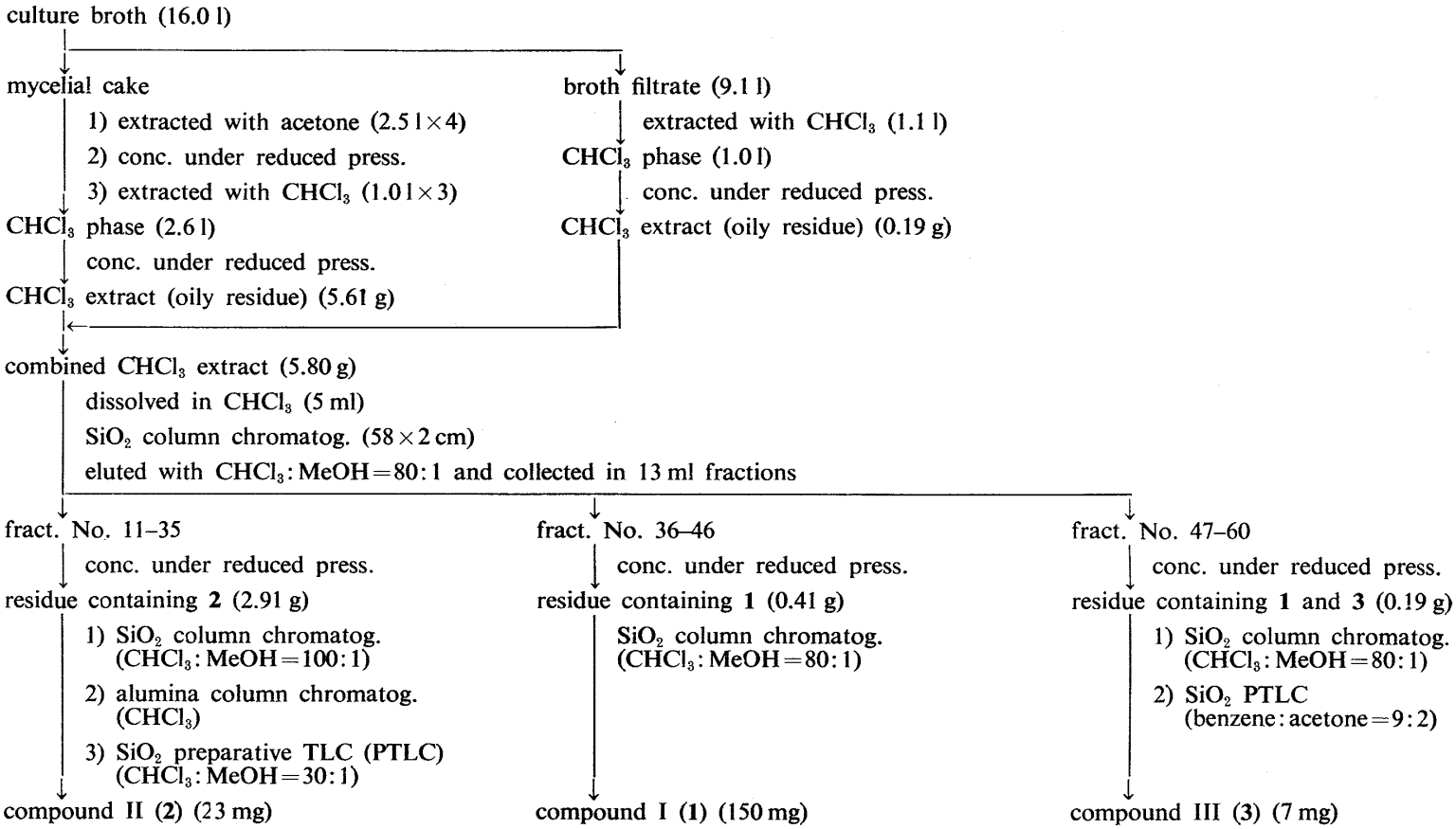


Chart 1. Extraction and Purification of Compounds I, II, and III

extract iron agar (ISP medium No. 6) and tyrosine agar (ISP medium No. 7). Starch hydrolysis, gelatin liquefaction and milk coagulation tests were positive. LL-Diaminopimelic acid was found in the acid hydrolysate of whole cells (cell wall, type I).[13]

D-Glucose, D-xylose, L-arabinose, D-fructose, raffinose, mannitol, *meso*-inositol and sucrose, but not cellulose, supported growth as the sole carbon source in the basal medium of Pridham and Gottlieb[14] as shown in Table II.

Sporophores were seen to be spiral under a microscope and spore surfaces were seen to be smooth under an electronmicroscope.

Thus, strain H 1082-MY 15 is considered to be a member of the color series gray of *Streptomyces*;[15] melanoid pigments are produced; spore wall ornamentation is smooth, and the spore chain is spiral.

Fermentation

Streptomyces strain H 1082-MY 15 was cultured to prepare a seed inoculum in shaking flasks, each containing 100 ml of an inoculation medium composed of 1.0% maltose and 0.4% yeast extract, at 27 °C for 48 h on a reciprocal shaker (amplitude 7 cm, 140 strokes per minute). Portions of the seed inoculum (each 2 ml) were used to inoculate shaking flasks each containing 100 ml of a production medium composed of 1.5% soluble starch, 1.0% glucose, 2.0% Nissin soyameal, 0.5% Ebios (dried yeast distributed by Tanabe Pharmaceutical Co., Ltd.), 0.25% NaCl and 0.3% $CaCO_3$ (pH 7.6 before sterilization) and the culture was grown at 27 °C on a rotary shaker (amplitude 7 cm, 180 strokes per minute) for 92 h.

Isolation and Purification

The compounds in the broth filtrate were extracted with chloroform and those in the mycelial cake were extracted with acetone. The procedures of extraction and purification are shown in Chart 1. The *Rf* values and color development of compounds I, II, and III on TLC are listed in Table III.

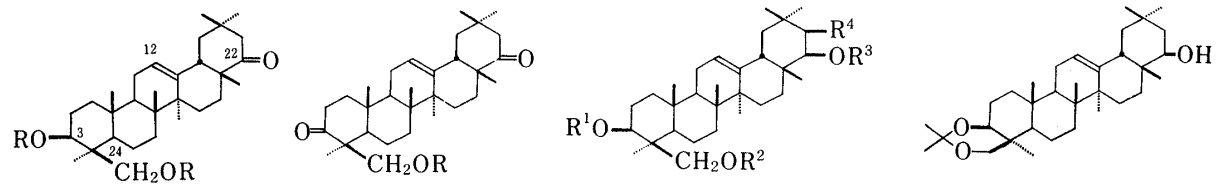
Structural Studies of Compounds I, II, and III

The major component, compound I (**1**), was obtained as colorless prisms, mp 257—260 °C (from $CHCl_3$–MeOH), $[\alpha]_D^{27}$ +28.5° (c=0.93, $CHCl_3$), M^+ at m/z 456 ($C_{30}H_{48}O_3$), and gave a positive Liebermann–Burchard color test for triterpenes. It showed hydroxyl and ketone bands (3270, 1705 cm^{-1}) in its infrared (IR) spectrum and did not show any ultraviolet (UV) absorption maximum above 215 nm. The proton magnetic resonance (^{1}H-NMR) spectrum of **1** in $CDCl_3$ disclosed the presence of seven tertiary methyls (δ 0.86, 0.90, 0.94, 1.23, and 1.24, each 3H, s; 0.99, 6H, s), one primary alcohol group (δ 3.33 and 4.19, 2H, ABq, J=12 Hz), one secondary alcohol group (δ 3.43, 1H, t-like), one olefinic proton having two allylic protons (δ 5.28, 1H, t-like) and two hydroxyl protons (δ 3.3—3.4, 2H, br) which disappeared on addition of D_2O. Acetylation of **1** with acetic anhydride and pyridine yielded a diacetate (**4**), colorless needles, mp 248—251 °C, $[\alpha]_D^{18}$ +27.9° (c=1.0, $CHCl_3$). The IR spectrum of **4** showed bands due to esters (1738, 1728, and 1256 cm^{-1}) and a ketone group

TABLE III. *Rf* Values of **1**, **2** and **3** on TLC

Solvent	3	1	2
Benzene : AcOEt = 7 : 3	0.21	0.23	0.37
AcOEt	0.66	0.72	0.80
Benzene : acetone = 9 : 2	0.30	0.34	0.46
$CHCl_3$: MeOH = 20 : 1	0.34	0.42	0.56

Color development (by spraying 10% H_2SO_4 followed by heating): **1**, bright violet; **2**, faint pink → green; **3**, brown.



compound I (**1**) : R=H (=soyasapogenol E)
4 : R=Ac

compound II (**2**) : R=H
7 : R=Tr

compound III (**3**) : $R^1=R^2=R^3=R^4=H$ (=soyasapogenol B)
6 : R^2=Tr, $R^1=R^3=R^4=H$
soyasapogenol A (**8**) : $R^1=R^2=R^3=H$, $R^4=OH$

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Chart 2

(1700 cm^{-1}). Its mass spectrum (MS) gave the molecular ion peak at *m/z* 540, which corresponded to the molecular formula $C_{34}H_{52}O_5$. In the ^{1}H-NMR spectrum of **4**, the signals of two new acetoxyl groups (δ 2.04 and 2.06, both 3H, s) appeared, and the methylene signals assignable to an acetylated primary alcohol (δ 4.13 and 4.40, 2H, ABq, J=12 Hz) and the methine signal due to an acetylated secondary alcohol group (δ 4.61, 1H, t-like) were all highly deshielded by the acetylation on going from **1** to **4**, while the other proton signals of **4** were observed at almost the same positions as those of the corresponding protons of **1**.

From these findings, **1** was suggested to be a triterpene having an oleanene skeleton. The reported chemicals and spectral data, especially ^{1}H-NMR data, for soyasapogenol E[8,16,17] and its diacetate[8] were very similar to those of **1** and **4**, respectively, although the reported melting point of soyasapogenol E diacetate (mp 234—236 °C)[8] was lower than that of **4** (mp 248—251 °C). However, an authentic sample of soyasapogenol E was not available. Thus, we converted soyasapogenol B (**3**), one of the main aglycones of soybean saponins,[17,19] to soyasapogenol E by oxidation of the secondary alcohol at C_{22} into a ketone; this is a modification of the method described by Smith *et al.*[7] Compound I (**1**) obtained from strain H 1082-MY 15 was identical with soyasapogenol E obtained above by comparison of their TLC, ^{1}H-NMR and IR (KBr) properties and by mixed mp determination.

Compound II (**2**) was obtained as colorless small plates, mp 196—201 °C, $[\alpha]_D^{26}$ +34.8° (c=0.64, $CHCl_3$), M^+ at *m/z* 454 ($C_{40}H_{46}O_3$). Its IR spectrum was very similar to that of **1**, and also showed hydroxyl and ketone bands (3500, 3400, 1700 (broad) cm^{-1}). The ^{1}H-NMR spectrum of **2** was also very similar to that of **1**. It showed the signals of seven tertiary methyls (δ 0.87, 1.25, 1.27, each 3H, s; 1.02, 12H, s), one primary alcohol group (–C<u>H</u>$_2$OH) (δ 3.50 and 4.00, 2H, ABq, J=12 Hz) and one olefinic proton having allylic protons (δ 5.35, 1H, t-like), while a signal due to a secondary alcohol group (>C<u>H</u>–OH) which was observed at δ 3.43 in the spectrum of **1** was missing in the spectrum of **2**. From the above data, compound II (**2**) was presumed to be the 3-oxo derivative of **1**, namely, 24-hydroxyolean-12-ene-3,22-dione.

In order to confirm the structure of **2**, we selectively oxidized the hydroxyl groups at C-3 and C-22 of soyasapogenol B (**3**) to obtain the 3,22-dioxo derivative. Thus, we first prepared 24-trityl-soyasapogenol B (**6**)[17] by treating soyasapogenol B with triphenylmethyl chloride in pyridine. Then, **6** was oxidized with CrO_3–pyridine complex to afford the 3,22-dioxo-24-trityl derivative (**7**) of **3**, the structure of which was ascertained as follows. The MS of **7** gave the molecular ion peak at *m/z* 696, corresponding to the molecular formula, $C_{49}H_{60}O_3$. Its ^{1}H-NMR spectrum exhibited signals due to one triphenylmethyl group, but did not show any methine proton signal of secondary alcohols. This compound (**7**) was then detritylated by heating with diluted hydrochloric acid in methanol and dichloromethane to yield 24-hydroxyolean-12-ene-3,22-dione, which was unambiguously shown to be identical with our compound II (**2**) by comparison of thier TLC, ^{1}H-NMR and IR (KBr) spectra and by mixed

TABLE IV. Relative Amounts of Compounds I, II, III and Soyasapogenols

	Extract of culture broth	Hydrolysate of the soyasaponin
Compound I (**1**) (=soyasapogenol E)	++ (0.34)	± (0.34)
Compound II (**2**)	+ (0.46)	−
Compound III (**3**) (=soyasapogenol B)	± (0.30)	++ (0.30)
Soyasapogenol A (**8**)	−	++ (0.21)

Rf values on TLC developed with benzene : acetone = 9 : 2 are listed in parentheses.
++, a large amount; +, a moderate amount; ±, trace; −, none.

mp determination.

The minor component, compound III (**3**), was obtained as colorless fine needles, mp 242—244 °C, and gave the same *Rf* value and the same color development as soyasapogenol B on TLC. The identity of **3** and soyasapogenol B was established by mixed mp determination and IR and TLC comparisons.

As far as we know, no triterpenes have been reported as products of microorganisms belonging to Actinomycetales. Thus, we presume that compounds I (=soyasapogenol E), II (24-hydroxyolean-12-ene-3,22-dione) and III (=soyasapogenol B) might all be derived from soybean saponins existing in Nissin soyameal, which is soybean powder defatted, roasted and used as an N-source for the production medium. We examined by TLC the constituents of the hydrolysate of soybean saponin which we obtained from Nissin soyameal by the reported method.[20] In Table IV, quantitative comparisons between the contents of **1**, **2**, **3** and soyasapogenol A (**8**) in the extract of the cultured broth and those in the hydrolysate of the saponin are shown. Soyasapogenol E, the major component of the broth, was a trace component of the hydrolysate, and compound II (**2**), the second major component of the broth, could not be detected in the hydrolysate. Therefore, the soybean saponins in Nissin soyameal presumably underwent hydrolysis and/or oxidation by enzymes produced by *Streptomyces* strain H 1082-MY 15 to afford compound I (=soyasapogenol E) (**1**) (in this case, hydrolysis and oxidation), compound II (24-hydroxyolean-12-ene-3,22-dione) (**2**) (hydrolysis and oxidation), and compound III (=soyasapogenol) B (**3**) (hydrolysis).

Experimental

All melting points were taken on a Yanagimoto micro melting point apparatus and are uncorrected. The UV spectra were obtained on a Hitachi 124 spectrophotometer in methanol and the IR spectra were recorded on a Shimadzu IR-408 spectrophotometer in KBr tablets. The ^{1}H-NMR spectra were recorded on a Hitachi R-40 spectrometer at 90 MHz using tetramethylsilane (TMS) as an internal standard. The MS were taken on a JEOL JMS-01SG-2 mass spectrometer. TLC was performed on Merck silica gel 60 F_{254} pre-coated TLC plates and spots on TLC were detected by spraying the plates with 10% H_2SO_4 and heating.

Compound I (1)——Colorless prisms, mp 257—260 °C (from $CHCl_3$–MeOH), $[\alpha]_D^{27}$ +28.5° (c=0.93, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3270 (OH), 1705 (six-membered ketone). UV (MeOH): transparent above 210 nm. ^{1}H-NMR ($CDCl_3$): as described in the text. MS: M^+ m/z 456.

Compound I Diacetate (4)——Acetylation of **1** (40 mg) with Ac_2O (1 ml) and pyridine (1 ml) at room temperature overnight followed by the usual work-up gave a product, which was recrystallized from EtOH to afford colorless needles of **4** (40 mg), mp 248—251 °C (lit.[8]: mp 234—236 °C), $[\alpha]_D^{18}$ +27.9° (c=1.0, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: as given in the text. ^{1}H-NMR ($CDCl_3$): as described in the text. MS: M^+ m/z 540.

Conversion of Soyasapogenol B (3) to Soyasapogenol E (1)——Soyasapogenol B (100 mg) was transformed to the acetonide by treatment with 2,2-dimethoxypropane (7 mg) and *p*-toluenesulfonic acid monohydrate (30 mg) in dry acetone (4.5 ml) at room temperature for 10 h, and usual work-up followed by purification by chromatography gave colorless prisms of soyasapogenol B acetonide (**5**) (86 mg), mp 160—163 °C, $[\alpha]_D^{20}$ +78.6° (c=0.28, MeOH).

The acetonide thus obtained (**5**) (80 mg) was oxidized with CrO_3–pyridine complex in a usual manner and the keto-acetonide (60 mg) obtained was hydrolyzed with 2% HCl–MeOH followed by purification to yield colorless needles of soyasapogenol E (**1**), 42 mg, mp 246—249 °C. This product was proved to identical with compound I (**1**) by TLC, IR (KBr) and ^{1}H-NMR comparisons and by mixted mp determination.

Tritylation of Soyasapogenol B (3′) Followed by Oxidation Giving 7——1) A solution of **3** (87 mg) in dry pyridine (4 ml) was treated with trityl chloride (140 mg) and the mixture was stirred under reflux for 12 h. More trityl chloride (100 mg) was then added to the reaction mixture, which was stirred under reflux for a further 12 h. After work-up in a usual manner, the product was chromatographed on neutral alumina to give 24-*O*-tritylsoyasapogenol B (**6**) (77 mg), which showed a single spot on TLC.

2) Oxidation: A solution of the trityl derivative (**6**) (70 mg) in pyridine (5 ml) was treated with CrO_3–pyridine complex (CrO_3 135 mg, pyridine 2 ml) at room temperature for 6 h. Work-up in a usual manner gave a product which was purified first by column chromatography on neutral alumina eluted with benzene–acetone (10 : 1→5 : 1), then by preparative TLC (Merck silica gel 60 F_{254} pre-coated TLC plates) developed with benzene to give the 3,22-dioxo-24-trityl derivative (**7**) of soyasapogenol B (55 mg). The structure of this product was substantiated by ^{1}H-NMR and MS analysis. ^{1}H-NMR ($CDCl_3$, δ): 0.69, 0.85 (3H each), 0.99 (6H), 1.18, 1.27, 1.41 (3H each) (in total, seven *tert*-methyls), 3.16, 3.55 (2H, ABq, J=10 Hz, $-C_{(24)}\underline{H}_2OTr$), 7.2—7.4 (15H, trityl). MS: M^+ *m/z* 696.

Acid Hydrolysis of 7 Giving 3,22-Dioxo-24-hydroxyolean-12-ene (2)——A mixture of **7** (50 mg) in 1% HCl–MeOH (4 ml) was heated at 80 °C for 30 min, then neutralized with Amberlite IRA-400 (HCO_3^- form) and filtered. The filtrate and washings were evaporated and the residue obtained was purified with column chromatography over Si gel eluted with a mixture of benzene–acetone (24 : 1→21 : 1) to afford colorless crystals. Recrystallization from $CHCl_3$–MeOH mixture gave colorless needles of 3,22-dioxo-24-hydroxyolean-12-ene, which was shown to be identical with compound II (**2**) by mixed mp determination and IR (KBr) and TLC comparisons.

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