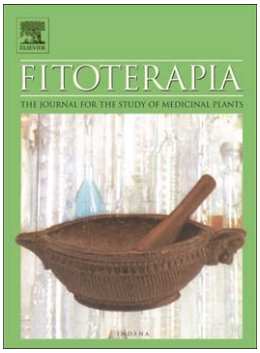


Two new *ent*-kaurane-type diterpene glycosides from zucchini (*Cucurbita pepo* L.) seeds

Takashi Kikuchi, Hiromi Ando, Ken-ichiro Maekawa, Hiroki Arie, Takeshi Yamada, Reiko Tanaka

PII: S0367-326X(15)30093-9
DOI: doi: 10.1016/j.fitote.2015.09.019
Reference: FITOTE 3272

To appear in: *Fitoterapia*

Received date: 5 August 2015
Revised date: 17 September 2015
Accepted date: 18 September 2015

Please cite this article as: Takashi Kikuchi, Hiromi Ando, Ken-ichiro Maekawa, Hiroki Arie, Takeshi Yamada, Reiko Tanaka, Two new *ent*-kaurane-type diterpene glycosides from zucchini (*Cucurbita pepo* L.) seeds, *Fitoterapia* (2015), doi: 10.1016/j.fitote.2015.09.019

Two New *ent*-Kaurane-type Diterpene Glycosides from Zucchini (*Cucurbita pepo* L.) Seeds

Takashi Kikuchi, Hiromi Ando, Ken-ichiro Maekawa, Hiroki Arie, Takeshi Yamada, Reiko Tanaka*

Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan

Key words: *Cucurbita pepo L.*; *ent*-Kaurane-type diterpene; Glycoside; Inhibitory activity on nitric oxide production

Corresponding author. Tel./Fax: +81-72-690-1084; e-mail: tanakar@gly.oups.ac.jp

Abstract

Two new *ent*-kaurane diterpene glycosides; 12α-(β-D-glucopyranosyloxy)-7β-hydroxykaurenolide (**1**) and 7β-(β-D-glucopyranosyloxy)-12α-hydroxykaurenolide (**2**), a new steroid; (24*S*)-stigmasta-7,22*E*,25-trien-3-one (**12**), and known compounds (**3**−**11**, **13**−**14**) were isolated from zucchini (*Cucurbita pepo* L.) seeds. The absolute structures of **1** and **2** were determined by acid hydrolysis and application of a modified Moscher's method. Furthermore, isolated compounds (**1**−**14**), and a derivative, **1a**, were evaluated for their inhibitory effects on macrophage activation by an inhibitory assay of nitric oxide (NO) production.

1. Introduction

Pumpkins, including *Cucurbita moschata*, *C. pepo*, and *C. maxima*, are gourd squashes of the genus *Cucurbita* and the family Cucurbitaceae. *Cucurbita moschata* seeds have been used as an anthelmintic [1], and *Cucurbita pepo* seeds, as an anthelmintic and diuretic [2]. Previous studies reported the isolation of 3-*p*-aminobenzoyl multiflorane-type triterpenes: 3-*O*-*p*-aminobenzoyl-29-*O*-benzoylmultifrora-8-ene-3α,7β,29-triol, 3-*O*-*p*-aminobenzoyl-29-*O*-benzoylmultifrora-7,9(11)-diene-3α,29-diol, 7-epi zucchini factor A, and debenzoyl zucchini factor B, from *C. pepo* seeds [3, 4]. We recently isolated five malutiflorane-type triterpenoids, including three new compounds; 3α-*p*-nitrobenzoylmultiflora-7:9(11)-diene-29-benzoate, 3α-acetoxymultiflora-7:9(11)-diene-29-benzoate, and 3α-acetoxymultiflora-5(6):7: 9(11)-triene-29-benzoate, from *C. pepo* seeds, and evaluated their cytotoxic activities in cancer cell lines as well as their inhibitory activities on melanogenesis [5]. In a continuing study to explore new compounds possessing biological activities from *C. pepo* seeds, we herein isolated two new *ent*-kaurane diterpene glycosides; 12α-(β-D-glucopyranosyloxy)-7β-hydroxy-kaurenolide (**1**) and 7β-(β-D-glucopyranosyloxy)-12α-hydroxy-kaurenolide (**2**), and a new steroid; (24*S*)-stigmasta-7,22*E*,25-trien-3-one (**12**), along with known compounds (**3**−**11**, and **13**−**14**). Furthermore, isolated compounds (**1**−**14**), and a derivative, **1a**, were evaluated for their inhibitory effects on macrophage activation by an inhibitory assay of nitric oxide (NO) production in RAW264.7 mouse macrophages stimulated by lipopolysaccharide (LPS).

2. Experimental

2.1. General Experimental Procedures

The following chemicals and reagents were purchased: fetal bovine serum (FBS) from *Invitrogen Co.* (Carlsbad, CA, U.S.A.), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) from *Sigma-Aldrich Japan Co.* (Tokyo, Japan), Dulbecco's modified Eagle's medium (D-MEM), and antibiotics from *Nacalai Tesque, Inc.* (Kyoto, Japan). All other chemicals and reagents were of analytical grade. Melting points were determined on a Yanagimoto micro-melting point apparatus and were uncorrected. Optical rotations were measured with a JASCO DIP-1000 digital polarimeter. IR spectra were recorded on a Perkin-Elmer 1720X FTIR spectrophotometer. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded on an Agilent vnmrs600 in CD_3OD and $CDCl_3$ with tetramethylsilane as the internal standard. HR-FAB-MS was recorded on a JEOL JMS-7000 mass spectrometer. Column chromatography (CC): Diaion HP-20 (*Mitsubishi Chemical Co.*, Tokyo, Japan), Sephadex LH-20 (*GE healthcare Bio-Sciences AB, Uppsala, Sweden*), and octadecyl silica gel (ODS; Chromatorex-ODS, 100–200 mesh; *Fuji Silysia Chemical, Ltd.*, Aichi, Japan). HPLC was carried out on an ODS column [*Cosmosil 5C18-PAQ column* (Nacalai Tesque, Inc., Kyoto, Japan), 25 cm × 20 mm i.d.] at 35ºC with MeCN / H_2O [15 : 85 (HPLC system *I*), 20 : 80 (HPLC system *II*), 25 : 75 (HPLC system *III*), flow rate 4.0 ml/min], *Cosmosil 5C18-MS-II* (Nacalai Tesque, Inc., Kyoto, Japan), 25 cm × 20 mm i.d.] at 35ºC {MeCN / H_2O [10 : 90 (HPLC system *IV*), 20 : 80 (HPLC system *V*)], and $(CH_3)_2CO$ / H_2O [10 : 1 (HPLC system *VI*)], flow rate 4.0 ml/min}, and SiO_2 column [*Cosmosil 5SL-II column* (Nacalai

Tesque, Inc., Kyoto, Japan), 25 cm × 20 mm i.d.] at 35ºC with hexane / EtOAc [10 : 1 (flow rate 8.0 ml/min) (HPLC system *VII*), 5 : 1 (flow rate 4.0 ml/min) (HPLC system *VIII*)].

2.2. Plant Material

The seeds of *Cucurbita pepo*, produced in USA, were purchased from TAKADA SEEDS Co., Ltd. in 2011. A voucher specimen was deposited in the Herbarium of the Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences.

2.3. Extraction and Isolation

The seeds of *cucurbita pepo* L. (20 kg) were subjected to extraction with MeOH under reflux (1 week, 4 times). The MeOH extract (710 g) was then partitioned between Et_2O and H_2O, followed by BuOH and H_2O.

The Et_2O fraction (Fr.) (410 g) was subjected to SiO_2 CC [SiO_2 (6 kg)] resulting in 8 fractions, Ea—Eh [CH_3Cl / EtOAc (1 : 0)→(5 : 1)→(0 : 1) and MeOH], followed by Ed (59 g), eluted with CH_3Cl, was chromatographed by SiO_2 (1 kg) to yield 7 fractions, Ed1—Ed7 [CH_3Cl / MeOH (1 : 0)→(10 : 1)→(1 : 1)→(0 : 1)]. Of these, Fr. Ed2 (28 g), eluted with $CHCl_3$, was subjected to re-chromatography by SiO_2 (1 kg) to give 25 fractions, Ed2-1—Ed2-25 [hexane / EtOAc (10 : 1)→(5 : 1)→(2 : 1)→(1 : 1)→(0 : 1)]. SiO_2 CC (12 g) of Ed2-21 (324.4 mg), eluted with hexane / EtOAc (2 : 1), gave **13** (50.00 mg) with hexane / EtOAc (5 : 1). Preparative HPLC (HPLC system *VII*) of Ed2-7 (10.4 mg), eluted with hexane / EtOAc (10 : 1), gave **12** (1.30 mg; t_R 17.0 min). Ed3 (10 g), eluted with CH_3Cl, was subjected to re-chromatography by SiO_2 (400 g) to give 43 fractions, Ed3-1—Ed2-43 [hexane / EtOAc (10 : 1)→(3 : 1)→(1 : 1)→(0 : 1)

and MeOH]. Preparative HPLC (HPLC system *VIII*) of Ed3-28 (206.4 mg), eluted with hexane / EtOAc (10 : 1), gave **14** (3.31 mg; t_R 81.5 min).

The BuOH Fr. (40 g) was subjected to Diaion HP-20 CC [Diaion HP-20 (600 g)] resulting in H_2O Fr. (17 g), MeOH Fr. (17 g), and $(CH_3)_2CO$ Fr. (169 mg). The MeOH Fr. was chromatographed by ODS (50 g) to yield to 10 fractions, Ma—Mj [MeOH / H_2O (1 : 1)→(7 : 3)→(9 : 1)→(1:0)]. Of these, Fr. Ma (11 g), eluted with MeOH / H_2O (1 : 1), was subjected to re-chromatography with ODS (50 g) to give 16 fractions, Ma1—Ma16 [MeCN / H_2O (1 : 9)→(2 : 8)→(3 : 7)→(4 : 6)→(1 : 0)]. Fr. Ma2 (4643.86 mg), eluted with MeCN / H_2O (1 : 9), was separated by sephadex LH-20 CC [sephadex LH-20 (40 g)] to afford 13 fractions, Ma2-1—Ma2-13 (H_2O→MeOH). Preparative HPLC of Ma2-6 (84.47 mg)(HPLC system *I*), eluted with H_2O, gave **6** (17.59 mg; t_R 86.6 min) and **10** (3.27 mg; t_R 116.8 min). Fr. Ma3 (1040.96 mg), eluted with MeCN / H_2O (1 : 9), was separated by sephadex LH-20 CC [sephadex LH-20 (40 g)] to afford 21 fractions, Ma3-1—Ma3-21 (H_2O→MeOH). Preparative HPLC of Ma3-11 (42.49 mg)(HPLC system *I*), eluted with MeOH, gave **9** (4.96 mg; t_R 110.0 min). Fr. Ma5 (1051.17 mg), eluted with MeCN / H_2O (2 : 8), was separated by preparative HPLC (HPLC system *II*) to give **5** (12.79 mg; t_R 28.2 min), **3** (48.79 mg; t_R 32.5 min), **4** (26.30 mg; t_R 33.3 min), and **1** (12.83 mg; t_R 40.3 min). Fr. Ma6 (192.50 mg), eluted with MeCN / H_2O (2 : 8), was separated by preparative HPLC (HPLC system *IV*) to give fifteen fractions, Ma6-1—Ma6-15. Ma6-13 was identified as **7** (8.60 mg; t_R 51.4 min). Fr. Ma6-7 (32.1 mg) was purified with re-preparative HPLC (HPLC system *III*) to give **1** (24.87 mg) and **8** (4.73 mg; t_R 49.4 min). Ma7 (638.86 mg), eluted with MeCN / H_2O (2 : 8), was separated by sephadex LH-20 CC [sephadex LH-20 (30 g)] to afford 13 fractions, Ma7-1—Ma7-12 (H_2O→MeOH). Preparative HPLC of Ma7-3 (89.8

mg)(HPLC system *III*), eluted with H_2O, gave **2** (3.76 mg; t_R 24.2 min). Preparative HPLC of Ma7-11 (105.9 mg)(HPLC system *V*), eluted with MeOH, gave **11** (1.05 mg; t_R 89.7 min).

2.4. Acid Hydrolysis and Determination of Sugar Configurations

The determination of absolute sugar configurations was carried out according to a method reported previously with slight modifications [6]. A solution of **1** (13.39 mg) in 10% HCl_{aq} (1 mL) was heated at 90°C for 5 days. The mixture was extracted with EtOAc (3 times). The combined organic layers were evapd. *in vacuo* to furnish a crude product. The crude product was subjected to CC (SiO_2), yielding **1a** (3.33 mg). The H_2O layer was concentrated to dryness. The residue was then dissolved in pyridine (0.1 mL) and stirring with L-cysteine methyl ester hydrochloride (0.5 mg) at 60 °C for 1 h. The reaction mixture was treated with *o*-tolylisothiocyanate, and heated at 60 °C for 1 h. The reaction mixture was analyzed by reversed-phase HPLC [column: *Cosmosil 5C_{18}-PAQ column* (Nacalai Tesque, Inc., Kyoto, Japan), 250 × 4.6 mm i.d. (5 μm); mobile phase: MeCN−H_2O (2:8, v/v) in 1% AcOH; detection: refractive index; flow rate: 1.0 mL/min; column temperature: 35 °C] to identify the derivatives of D-glucose by comparison of their retention times with those of authentic samples (t_R: D-glucose 28.7 min, L-glucose 26.0 min). The acid hydrolysis of **1** (18.83 mg) was performed again to give **1a** (2.38 mg). The acid hydrolysis of **2** (1.57 mg) was conducted in a similar manner as described above, and its EtOAc layer gave **1a** (0.36 mg). The H_2O layer afforded D-glucose derivative by derivatization with L-cysteine methyl ester hydrochloride and *o*-tolylisothiocyanate, followed by HPLC analysis.

2.5. Preparation of (S)- and (R)- MTPA Esters from **1a**

(−)-MTPA-Cl (100 mg) was added to a solution of **1a** (2.37 mg) in pyridine. The mixture was stirred at r.t. for 18 h, poured into H_2O, and then extracted with CH_2Cl_2. The organic layer was evapd. *in vacuo* to give a crude product. The crude product was subjected to CC [SiO_2, hexane-EtOAc (5 : 1)] to yield **1b**, which was the (*S*)-MTPA ester of **1a** (2.12 mg). **1c** (2.01 mg), which was the (*R*)-MTPA ester of **1a**, was prepared from **1a** (2.48 mg) and (+)-MTPA-Cl (100 mg) in a similar manner as described above.

2.6. Oxidation of **13**

13 (10.9 mg) in CH_2Cl_2 (5 mL) was added to a solution of pyridium chlorochromate (15.8 mg) and SiO_2 (15.8 mg) in CH_2Cl_2 (5 mL) . The mixture was stirred at r.t. for 4 h, and then filtrated over SiO_2. The filtrate was evapd. *in vacuo* to furnish a crude product. Preparative HPLC of the crude extract gave **12** (3.89 mg; t_R 43.3 min)(HPLC system *VI*).

*2.7.12α-(β-D-glucopyranosyloxy)-7β-hydroxykaurenolide (**1**)*

Amorphous solid, $[\alpha]_D^{21}$ −20.5 (*c* = 0.26, EtOH); IR ν_{max}^{KBr} cm^{-1}: 3434, 2933, 2869, 1754, 1514, 1450, 1370, 1209, 1079; FAB-MS *m/z* :517 $[M+Na]^+$, 495 $[M+H]^+$; HR-FAB-MS *m/z*: 517.2416 (calcd for 517.2414: $C_{26}H_{38}O_9Na$)

*2.8. 7β-(β-D-glucopyranosyloxy)-12α-hydroxykaurenolide (**2**)*

Amorphous solid, $[\alpha]_D^{19}$; −9.4 (*c* = 0.045, EtOH); IR ν_{max}^{KBr} cm^{-1}: 3411, 2923, 2876, 1756, 1649, 1080, 1025; FAB-MS *m/z*: 517 $[M+Na]^+$, 495 $[M+H]^+$; HR-FAB-MS *m/z*: 517.2412 (calcd for 517.2414: $C_{26}H_{38}O_9Na$)

*2.9. 16,17-dihydro-7β-hydroxy-12-oxokaurenolide (**1a**)*

Amorphous solid; $[\alpha]_D^{23}$ −79.8 (*c* = 0.11, EtOH); IR ν_{max}^{KBr} cm^{-1}: 3463, 2954, 2882, 1759, 1699, 1452, 1196, 1064; EI-MS *m/z* : 332 $[M]^+$ (100), 314 (9), 288 (10), 273 (21), 255 (30), 165 (25), 137 (45), 109 (74); HR-EI-MS *m/z*: 332.1984 (calcd for 332.1987: $C_{20}H_{28}O_4$)

*2.10. (24S)-stigmasta-7,22E,25-trien-3-one (**12**).*

$[\alpha]_D^{22}$ +17.9 (*c* = 0.57, $CHCl_3$); IR ν_{max}^{KBr} cm^{-1}: 2963, 2868, 1718, 1645, 1445, 1382, 967, 885; EIMS *m/z* (rel. int.): 408 (21) [M]+, 379 (21), 325 (3), 298 (19), 269 (100), 229 (8); HREIMS *m/z*: 408.3392 [M]+ (calcd for 408.3392: $C_{29}H_{44}O$)

2.11. Cell culture

The RAW264.7 cell line was grown in D-MEM medium, which was supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). Cells were incubated at 37°C in a 5% CO_2 humidified incubator.

2.12. Cytotoxicity assay

Cytotoxicity assays were performed according to a method reported previously [7] Briefly, RAW264.7 cells (5×10^4 cells in 100 μL), which were pre-incubated for 24h on a 96-well microplate, were treated for 24 h with test samples dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 30, 10, 3 μM, and 1 μM, and MTT solution was added. After a 3 h incubation, 20% SDS containing 0.1 M HCl was added

to dissolve the formazan produced by the cells. The absorbance of each well was read at 570 nm using a microplate reader.

2.13. Inhibitory assay of NO production

An inhibitory assay of NO production was performed according to a method reported previously [8] with slight modifications. Briefly, RAW264.7 cells (5×10^4 cells in 100 μL) were seeded on 96-well microplates and incubated for 24h. D-MEM (100 μL) containing the test samples (final concentration of 30, 10, 3, or 1 μM) dissolved in DMSO (final concentration 0.2 %) and LPS (final concentration of 5 μg / mL) was added. After cells had been treated for 24 h, the supernatant of the cultured medium (150 μL) was transferred to a 96-well microplate, and 75 μL of 0.1 % *N*-(1-naphtyl)ethylenediamine in H_2O and 75 μL of 1 % sulfanilamide in 5 % phosphoric acid were then added. After being incubated for 30 min, the absorbance of each well was read at 570 nm using a microplate reader. The optical density of vehicle control cells was assumed to be 100%.

3. Results and discussion

Compounds **1**−**13** were isolated from the MeOH extract of *C. pepo* seeds (Fig. 1). Compound **1** had an $[M+Na]^+$ ion in the HR-FAB-MS at *m/z* 517.2416, which was consistent with the molecular formula $C_{26}H_{38}O_9Na$ (calcd 517.2414). Its IR spectrum showed absorption that indicated a hydroxy group (3434 cm^{-1}) and a γ-lactone (1754 cm^{-1}). ^1H and ^{13}C NMR spectra indicated two tertiary methyls [δ_H 0.88 (s), 1.28 (s)], three oxymethines [δ_H 3.82 (brt), 4.30 (d), 4.63 (t); δ_C 72.3 (d), 84.5 (d), 85.3 (d)], an

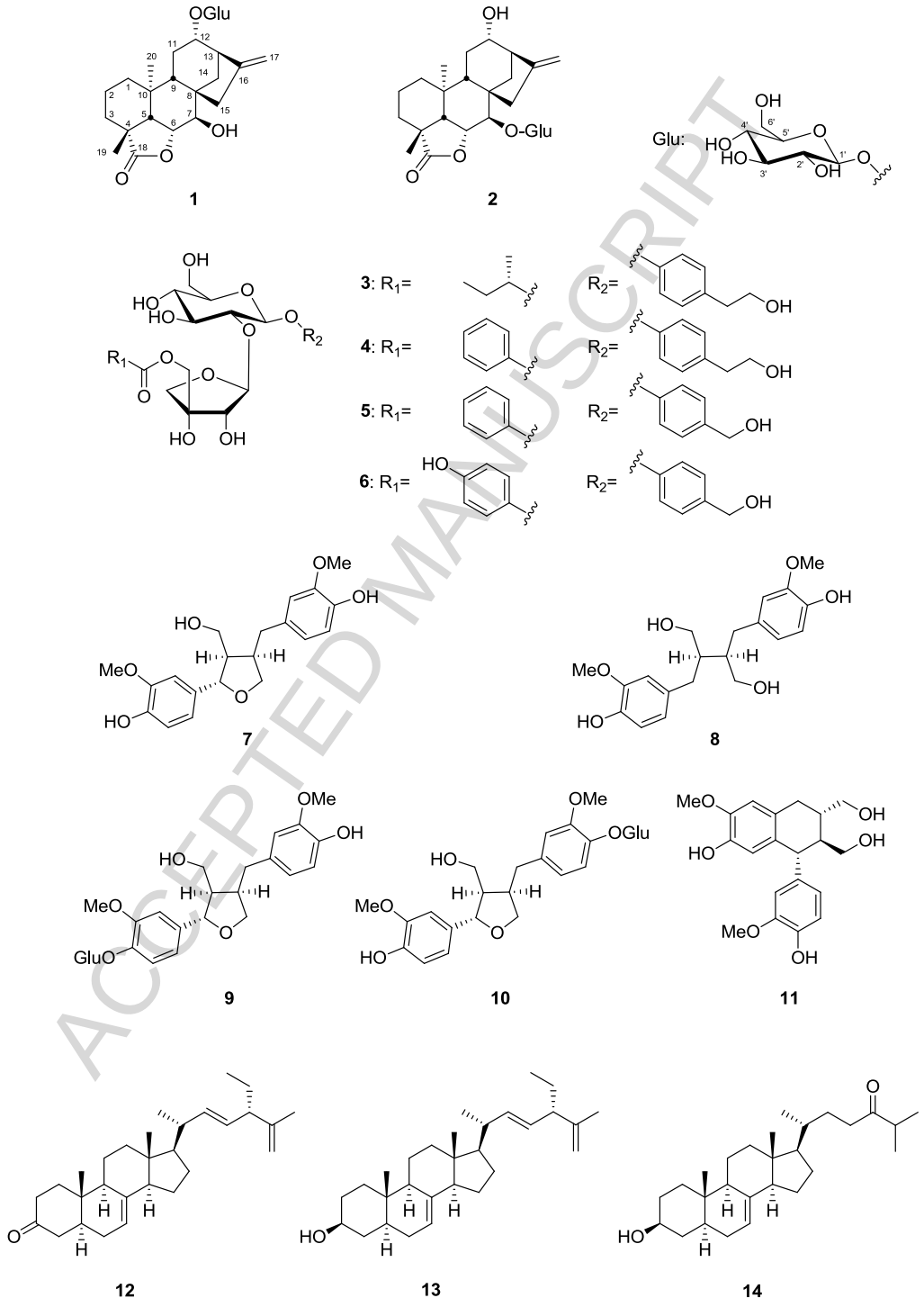


Fig 1. Structures of compounds **1**−**14** from *C. pepo* seeds.

Table 1

^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Spectroscopic Data of Compounds **1**—**3**[a].

		1[b]				**2**[b]				**1a**[c]			
No.		δ_H	(*J* in Hz)	δ_C		δ_H	(*J* in Hz)	δ_C		δ_H	(*J* in Hz)	δ_C	
1	α	1.58 m		38.4	t	1.58 dt	(5.0, 12.6)	38.2	t	1.54 m		37.0	t
	β	1.04 m				1.01 m				1.01 m			
2	α	1.56 m	(2H)	18.4	t	1.53 m	(2H)	18.7	t	1.54 m		16.9	t
	β									1.63 m			
3	α	2.04 m		29.3	t	2.09 dt	(5.3, 14.3)	29.8	t	2.09 m		27.7	t
	β	1.42 m				1.41 ddd	(6.7, 9.9, 14.3)			1.45 m			
4				43.1	s			42.9	s			41.6	s
5		1.89 d	(6.5)	53.0	d	1.91 d	(6.2)	53.2	d	1.79 d	(6.7)	52.6	d
6	β	4.63 t	(6.5)	85.3	d	4.96 t	(6.2)	84.2	d	4.67 t	(6.7)	83.3	d
7	α	4.30 d	(6.5)	72.3	d	4.36 d	(6.2)	83.1	d	4.43 d	(6.7)	71.5	d
8				46.7	s			46.3	s			46.3	s
9		1.01 dd	(4.1, 14.7)	52.2	d	1.03 m		52.1	d	1.36 dd	(5.3, 13.8)	51.4	d
10				35.2	s			34.9	s			34.2	s
11	α	1.40 m		27.1	t	1.23 m		28.4	t	2.24 dd	(5.3, 16.5)	35.8	t
	β	1.85 m				1.79 m				2.33 dd	(13.8, 16.5)		
12		3.82 brt	(8.2)	84.5	d	3.66 m		77.3	d			214.7	s
13		2.75 brd	(5.0)	46.0	d	2.49 d	(5.3)	49.6	d	2.27 dd	(1.2, 4.7)	55.2	d
14	α	1.72 m	(2H)	33.0	t	1.76 m		32.5	t	1.65 dd	(1.2, 12.6)	33.7	t
	β					2.02 dd	(4.7, 11.8)			2.09 dd	(4.7, 12.6)		
15	α	2.60 dt	(2.3, 14.7)	43.4	t	2.76 brd	(14.6)	44.5	t	1.74 dd	(7.6, 11.7)	42.6	t
	β	1.71 m				1.78 m				1.37 m			
16				155.6	s			155.9	s	2.11 m		36.0	d
17	A	5.02 brs		110.0	t	4.98 brs		109.4	t	1.16 d	(7.0)	22.6	q
	B	5.09 brs				5.09 brs							
18				184.9	s			184.2	s			181.8	s
19		1.28 s		25.9	q	1.26 s		26.0	q	1.32 s		25.6	q
20		0.88 s		20.9	q	0.86 s		20.2	q	0.92 s		20.0	q
1'		4.44 d	(8.0)	102.5	d	4.43 d	(8.0)	106.0	d				
2'		3.16 dd	(8.0, 9.1)	75.1	d	3.23 dd	(8.0, 9.1)	75.6	d				
3'		3.37 m		78.2	d	3.35 t	(9.1)	78.5	d				
4'		3.28 m		71.8	d	3.30 m		71.8	d				
5'		3.28 m		78.1	d	3.30 m		77.5	d				
6'	A	3.66 m		62.8	t	3.66 m		63.0	t				
	B	3.86 dd	(1.5, 12.0)			3.83 dd	(2.1, 11.4)						

[a]Assignments were based on ^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY spectroscopic data.

[b]Measured in CD_3OD.

[c]Measured in $CDCl_3$.

exo-methylene [δ_H 5.02 (brs), 5.09 (brs); δ_C 110.0 (t), 155.6 (s)], and a terminal glucose [δ_H 4.44 (d); δ_C 62.8 (t), 71.8 (d), 75.1 (d), 78.1 (d), 78.2 (d), 102.5 (d)]. The configuration of glucose at C-1′ was determined to be β based on the the coupling constants of H-1′ [δ_H 4.44 (d), $J_{1',2'}$=8.0 Hz]. The following correlations were observed in the HMBC spectrum; Me-19 [δ_H 1.28 (s)]/C-3, C-4, C-5, and C-18 [δ_C 184.9 (s)]; Me-20 [δ_H 0.88 (s)]/C-1, C-5, C-9, and C-10; H-6 [δ_H 4.63 (t)]/C-5 and C-7 [δ_C 72.3 (d)] ; H-7 [δ_H 4.30 (d)]/C-8; H-13 / C-12 [δ_C 84.5 (d)] and C-16 [δ_C 155.6 (s)]; H_2-17 [δ_H 5.02 (brs), 5.09 (brs)]/C-13 and C-15; and H-1' [δ_H 4.44 (d)]/C-12 (Fig. 2). In the ^{1}H-^{1}H COSY spectrum, the correlations of H_2-1−H_2-2−H_2-3; H-5−H-6−H-7; and H_2-11−H-12; H-13−H_2-14 were observed (Fig. 2).

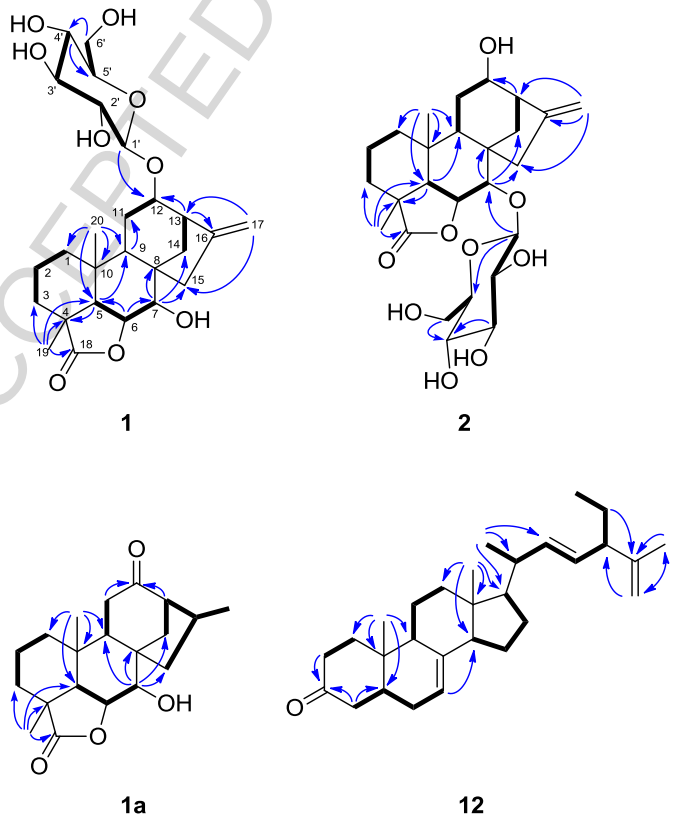


Fig. 2. Key HMBC (⟶) and ^{1}H-^{1}H COSY (▬) correlations of compounds **1**–**2**, **1a**, and **12**.

These results indicated that **1** was a kaurane-type diterpene or its enantiomer, an *ent*-kaurane-type, as shown in Figure 2. The relative structures of the 6, 7, and 12 positions were characterized by NOESY experiments, which observed the following correlations; H-6β/Me-19; H-7α/Me-20; and H-12β/H-17B (Fig. 3). The acid hydrolysis of **1** was performed to determine the configurations of glucose and aglycon (Fig. 4).

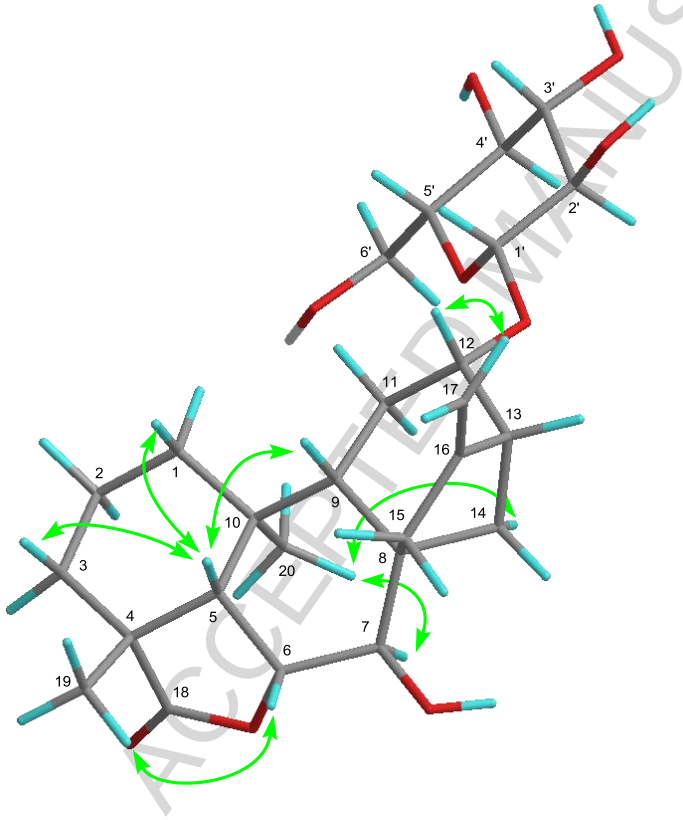


Fig. 3. Selected NOE correlations of compound **1.**

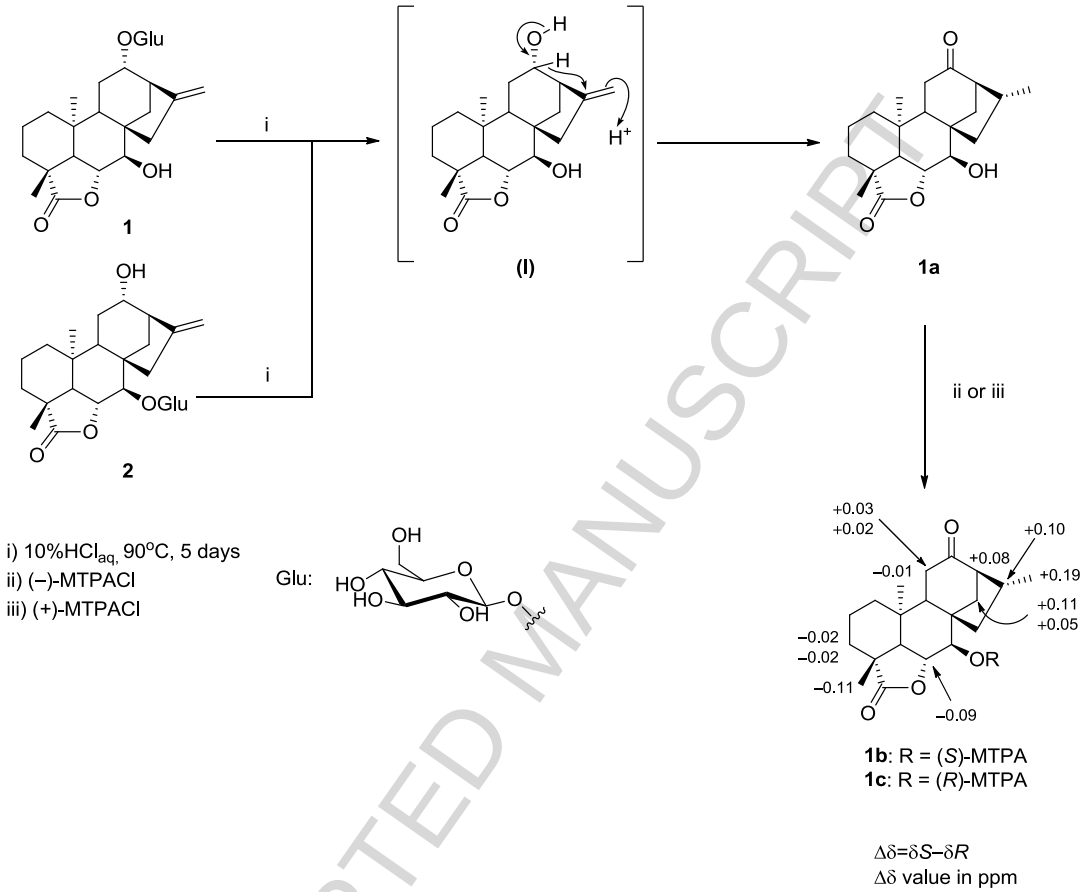


Fig. 4. Acid hydrolysis of compounds **1** and **2**, and application of a modified Moscher's method for **1a.**

Acid hydrolysis by 10% HCl yielded an unexpected 12-oxo product, **1a**, and D-glucose. The formation of **1a** from **1** was inferred as follows: (I) was produced from **1** by acid hydrolysis. π bond at 16 and 17 positions in (I) attacked H^+ ion. And then H^- ion from C-12 attacked carbocation at C-16, followed by carbonyl group generation at C-12 gave **1a**. D-glucose was identified by derivatization with L-cysteine methyl ester hydrochloride and *o*-tolylisothiocyanate, followed by a HPLC analysis [6]. The relative structures of the 6 and 7 positions in **1a** were the same as those in **1** because the following NOE correlations were observed; H-6β/Me-19 and Me-20/H-7α and H-14$_{pro\text{-}R}$

(Fig. 5). The absolute configuration of **1a** was characterized by the application of a modified Moscher's method (Fig. 4). The $\Delta\delta$ ($\delta S-\delta R$) values for H-6 (−0.09), Me-19 (−0.11), and Me-20 (−0.01) were negative, while those of H-13 (+0.08), H-16 (+0.10), and Me-17 (+0.19) were positive (Fig. 4). Therefore **1a**, and its substance, **1** possessed a 7β-hydroxy group, and **1** was established as the 6α,7β,12α-trihydroxy-*ent*-kaur-16-en-18-oic acid γ-lactone 12-*O*-β-D-glucopyranoside (Table 1).

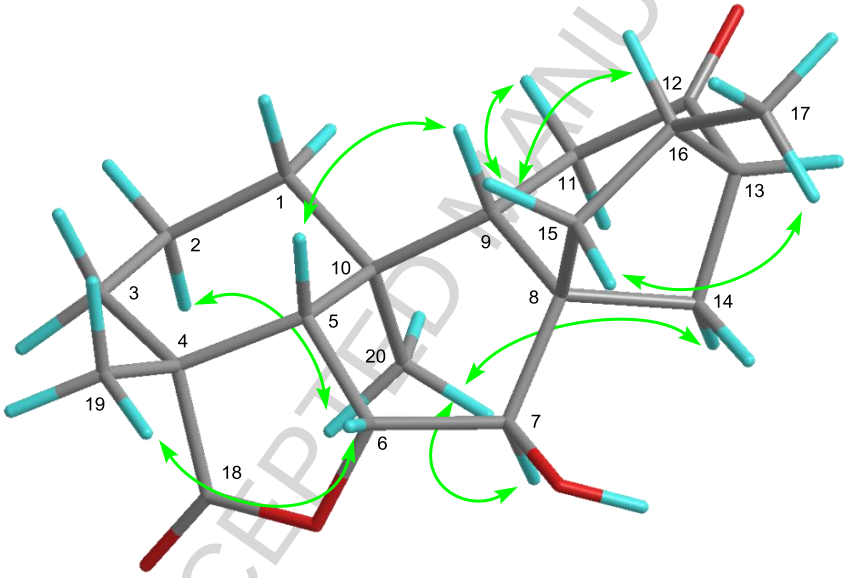


Fig. 5. Selected NOE correlations of compound **1.**

Compound **2** had an $[M+Na]^+$ ion in the HR-FAB-MS at *m/z* 517.2412, which was consistent with the molecular formula $C_{26}H_{38}O_9Na$ (calcd 517.2415), similar to **1**. Its IR spectrum showed absorption, which indicated a hydroxy group (3411 cm^{-1}) and a γ-lactone (1756 cm^{-1}). ^1H and ^{13}C NMR spectra indicated that the structure of **2** was similar to that of **1**, except for a 7β-hyhdroxy group [δ_H 4.30 (d); δ_C 72.3 (d) in **1**] and 12α-(β-glucopyranosyloxy) group [δ_H 3.82 (brt); δ_C 84.5 (d) in **1**], as well as the presence of a 7β-(β-glucopyranosyloxy) group [δ_H 4.36 (d); δ_C 83.1 (d) in **2**] and

12α-hydroxy group [δ_H 3.66 (m); δ_C 77.3 (d) in **2**]. These results were confirmed by HMBC and ^{1}H-^{1}H COSY experiments (Fig. 2). The acid hydrolysis of **2** gave the same products as those of **1**, namely **1a** (Fig. 4), and D-glucose. D-glucose was identified by derivatization with L-cysteine methyl ester hydrochloride and *o*-tolylisothiocyanate, followed by a HPLC analysis [6]. Therefore the absolute configuration of **2** was the same as that of **1**, and **2** was established as the 6α,7β,12α-trihydroxy-*ent*-kaur-16-en-18-oic acid γ-lactone 7-*O*-β-D-glucopyranoside (Fig. 1).

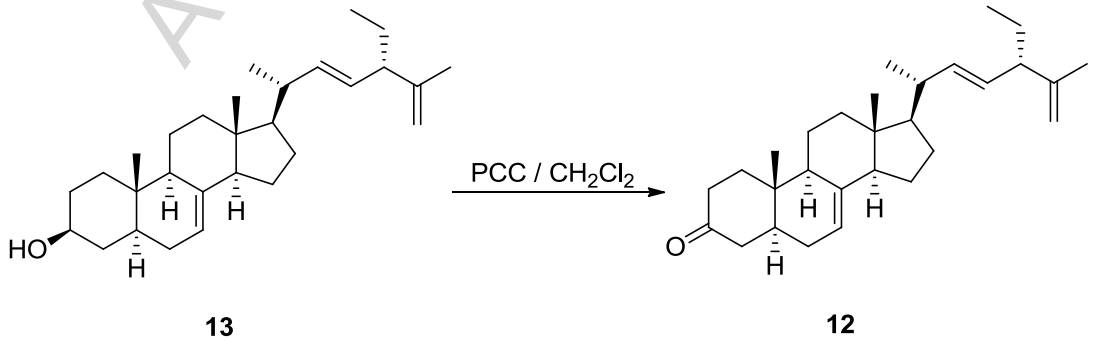
Compound **12** had an $[M]^+$ ion in the HR-EI-MS at *m/z* 408.3392, which was consistent with the molecular formula $C_{29}H_{44}O$ (calcd 408.3392). Its IR spectrum showed the presence of a carbonyl group (1718 cm^{-1}). ^{1}H and ^{13}C NMR spectra indicated that the presence of three tertiary methyls [δ_H 0.58 (s), 1.02 (s), 1.65 (s)], a secondary methyl [δ_H 1.03 (d)], a primary methyl [δ_H 0.84 (t)], a trisubstituted olefin [δ_H 5.18 (m); δ_C 117.0 (d), 139.5 (s)], a disubstituted olefin [δ_H 5.19 (dd), 5.25 (dd); δ_C 130.3 (d), 136.9 (d)], an exo-methylene [δ_H 4.71 (2H, m); δ_C 109.6 (t), 148.6 (s)], and a ketone [δ_C 212.0 (s)] (Table 2). The ^{1}H and ^{13}C NMR signals of **12** were similar to those of the known compound **13**, except for the absence of a hydroxy group and existence of a carbonyl group at C-3 [δ_C 212.0 (s)]. This was confirmed by HMBC and ^{1}H-^{1}H COSY experiments (Fig. 2). Furthermore, the oxidation of **13** by PCC gave **12** (Fig. 6). Therefore, compound **12** was established as (24*S*)-stigmasta-7,22*E*,25-trien-3-one.

Table 2

NMR spectral data for **12** in $CDCl_3$.[a]

No.		δ_H	(*J* in Hz)	δ_C		No.		δ_H	(*J* in Hz)	δ_C	
1	α	1.46 m		38.8	t	15	α	1.51 m		23.0	t
	β	2.13 ddd	(2.6, 5.8, 13.2)				β	1.41 m			
2	α	2.28 m		38.1	t	16		1.75 m	(2H)	28.3	t
	β	2.43 m				17		1.29 m		55.8	d
3				212.0	s	18		0.58 s		12.1	q
4		2.23 m	(2H)	44.2	t	19		1.02 s		12.4	q
5		1.81 m		42.9	d	20		2.04 m		40.5	d
6	α	1.26 m		30.0	t	21		1.03 d	(6.8)	20.9	d
	β	1.83 m				22		5.25 dd	(8.2, 15.3)	136.9	d
7		5.18 m		117.0	d	23		5.19 dd	(8.4, 15.3)	130.3	d
8				139.5	s	24		2.43 m		52.0	d
9		1.75 m		48.8	d	25				148.6	s
10				34.4	s	26		4.71 m	(2H)	109.6	t
11		1.63 m	(2H)	21.7	t	27		1.65 s		20.2	q
12	α	1.27 m		39.3	t	28	A	1.41 m		25.7	t
	β	2.04 m					B	1.48 m			
13				43.3	s	29		0.84 t	(7.4)	12.1	q
14		1.83 m		55.0	d						

[a]Assignments were based on ^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY spectroscopic data.



13 **12**

Fig. 6. Preparation of **12** by the oxidation of **13**.

Known compounds were identified as cucurbitoside F (**3**) [9], cucurbitoside A (**4**) [10], cucurbitoside C (**5**) [10], cucurbitoside D (**6**) [10], (+)-lariciresinol (**7**) [11], (−)-secoisolariciresinol (**8**) [11], lariciresinol-4′-*O*-β-D-glucoside (**9**) [12], lariciresinol-4-*O*-β-D-glucoside (**10**) [12], isolariciresinol (**11**) [13], (24*S*)-stigmasta-7,22*E*,25-trien-3β-ol (**13**) [14], and 3β-hydroxycholest-7-en-24-one (**14**) [15] by comparisons with spectroscopic data of those previously reported.

Macrophages may be a potential therapeutic target for inflammatory diseases [16]. Activated macrophages release pro-inflammatory mediators, such as NO, reactive oxygen, interleukin-1 beta, tumor necrosis factor-alpha, and other inflammatory mediators, which play important roles in biological defense. However, the overexpression of these mediators had been implicated in diseases such as osteoarthritis, rheumatoid arthritis, and diabetes because the increased production of pro-inflammatory mediators has been shown to induce severe or chronic inflammation [16]. Isolated compounds (**1**−**14**), the derivative, **1a**, and L-NMMA, which was a NO synthase inhibitor and used as a positive control, were evaluated for macrophage activation by the inhibitory assay of NO production in RAW264.7 mouse macrophages stimulated by LPS. Among the compounds tested, 3β-hydroxycholest-7-en-24-one (**14**) exhibited inhibitory effects on macrophage activation at 3−30 μM (produced NO: 81.3% at 3 μM, 61.2% at 10 μM, and 33.9% at 30 μM). The IC_{50} value of **14** (IC_{50} 15.5 μM) was superior to those of L-NMMA (IC_{50} 23.9 μM). Compound **14** exhibited low cytotoxicity at 30 μM (cell viability: 85.9% at 30 μM), but not at effective concentrations, namely 3 and 10 μM. Compounds **1**−**13** and **1a** did not exhibit inhibitory effects on macrophage activation (IC_{50} >30 μM). These results suggested that compound **14** has potential as an anti-inflammatory disease agent.

Table 3

Inhibitory Effects of Macrophage Activation by Compound **14** and L-NMMA from *Cucurbita pepo* L. Seeds.[a]

	NO produced % (Cell viability %)				IC_{50}
	1 μM	3 μM	10 μM	30 μM	(μM)
14	104.3±4.5	81.3±4.5	61.2±4.0**	33.9±2.6**	15.5
	(96.1±0.8)	(97.8±0.4)	(94.6±1.3)	(84.9±0.8)	
L-NMMA	93.3±2.2	91.4±0.8	68.9±4.5	43.1±1.1	23.9
	(101.5±0.9)	(101.9±0.4)	(98.5±0.9)	(109.4±0.5)	

[a] NO Produced (%) and cell viability (%) were determined based on the absorbance at 570 nm by comparisons with values for DMSO (100%). Each value represents the mean ± standard error (S.E.) of four determinations. The concentration of DMSO in the sample solution was 2 μl/ml. Significant differences from the vehicle control group were shown as **: $p<0.01$, and *$p<0.05$.
[b] Positive control.

Acknowledgments

We thank Dr. Katsuhiko Minoura and Dr. Mihoyo Fujitake (Osaka University of Pharmaceutical Sciences) for the NMR and MS measurements.

1 Okada M., Ed. *Newly Revised Illustrated Medicinal Plants of World*; Hokuryukan Pablishing Co., ltd.: Tokyo, 2002; p 514.

2 Chevallier A., The Encyclopedia of Medicinal Plants; Seibundo Shinkosha Pablishing Co., ltd.: Tokyo, 2000; p 194.

3 Appendino G, Jakupovic J, Belloro E, Marchesini A. Secondary metabolites from edible plants and spices. Part 4. Multiflorane triterpenoid esters from pumpkin. An unexpected extrafolic source of PABA. Phytochemistry 1999; 51: 1021—6.

4 Appendino G, Jakupovic J, Belloro E, Marchesini A. Triterpenoid *p*-aminobenzoates from the seeds of zucchini. Fitoterapia 2000; 71: 258—63.

5 Tanaka R, Kikuchi T, Nakasuji S, Ue Y, Shuto D, Igarashi K, Okada R, Yamada T. Novel 3α-*p*-nitrobenzoylmultiflora-7:9(11)-diene-29-benzoate and two new triterpenoids from the Seeds of zucchini (*Cucurbita pepo* L.). Molecules 2013; 18: 7448—59.

6 Tanaka, T., Nakashima, T., Ueda, T., Tomii, K., Kouno, I. Facile discrimination of aldose enantiomers by reversed-phase HPLC. Chem Pharm Bull 2007; 55: 899—901.

7 Yamada, T., Muroga, Y., Jinno, M., Kajimoto, T., Usami, Y., Numata. A., Tanaka, R. New class azaphilone produced by a marine fish-derived Chaetomium globosum. The stereochemistry and biological activities. Bioorg Med Chem 2011; 19: 4106—13.

8 Matsui Y, Kikuchi T, Inoue T, Muraoka O, Yamada T, Tanaka R, Carapanolides J-L from the seeds of Carapa guianensis (andiroba) and their effects on LPS-activated NO production. Molecules 2014; 19: 17130-40.

9 Li W, Koike K, Tatsuzaki M, Koide A, Nikaido T. Cucurbitosides F-M, Acylated Phenolic Glycosides from the Seeds of *Cucurbita pepo.* J Nat Prod 2005**;** 68: 1754—7.

10 Koike K, Li W, Liu L, Hata E, and Nikaido T. New Phenolic Glycosides from the Seeds of *Cucurbita moschata*. Chem Pharm Bull 2005; 53: 225—8.

11 Xie L-H, Akao T, Hamasaki K, Deyama T, Hattori M, Biotransformation of pinoresinol diglucoside to mammalian lignans by human intestinal microflora, and isolation of Enterococcus faecalis strain PDG-1 responsible for the transformation of (+)-pinoresinol to (+)-lariciresinol. Chem Pharm Bull 2003; 51: 508—15.

12 Sugiyama M, Kikuchi M, Characterization of lariciresinol glucosides from

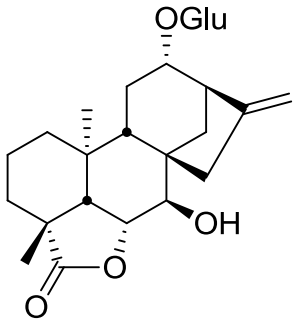
Osmanthus asiaticus. Heterocycles 1993; 36; 117—21.

13 Urones J G, De Pascual Teresa J, Sanchez Marcos I, Diez Martin D, *ent*-Isolariciresinol in *Reseda suffruticosa*. Phytochemistry 1987; 26: 1540—1.

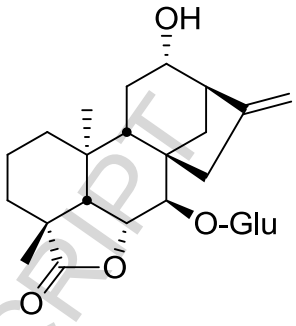
14 Rodriguez J B, Gros E G, Bertoni M H, Cattaneo P, The sterols of Cucurbita moschata ("calabacita") seed oil. Lipids 1996; 31: 1205—8.

15 Dittrich S, Bracher F. Traceless bond construction via rearrangement of *N*-Boc-*N*-allylhydrazones giving 1,1-disubstituted olefins. Tetrahedron, 2015; 71: 2530—9.

16. Zhang Y, Liu C, Dong B, Ma X, Hou L, Cao X, Wang C. Anti-inflammatory Activity and Mechanism of Surfactin in Lipopolysaccharide-Activated Macrophages. Inflammation 2015; 38: 756-64.



1



2

Graphical abstract

Conflict of interest

The authors have no conflict of interest directly relevant to the content of this article.