Cucurbitane Triterpenoids from *Momordica charantia* and Their Cytoprotective Activity in *tert*-Butyl Hydroperoxide-Induced Hepatotoxicity of HepG2 Cells

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Two novel pentanorcucurbitane triterpenes, 22-hydroxy-23,24,25,26,27-pentanorcucurbit-5-en-3-one (1) and 3,7-dioxo-23,24,25,26,27-pentanorcucurbit-5-en-22-oic acid (2) together with a new trinorcucurbitane triterpene, 25,26,27-trinorcucurbit-5-ene-3,7,23-trione (3) were isolated from the methyl alcohol extract of the stems of *Momordica charantia*. The structures of the new compounds were elucidated by spectroscopic methods. Compounds 2 and 3 showed potent cytoprotective activity in *tert*-butyl hydroperoxide (*t*-BHP)-induced hepatotoxicity of HepG2 cells.

Key words Chinese herb; *Momordica charantia*; Cucurbitaceae; pentanorcucurbitane; trinorcucurbitane; cytoprotective

Momordica charantia L., a slender-stemmed tendril climber, belongs to the family Cucurbitaceae and is commonly known as bitter gourd or bitter melon. It is wildly cultivated as a vegetable crop in tropical and subtropical areas, including Asia, East Africa, and South America. Tissues of this plant have extensively been used as folk medicine for the treatment of diabetes and diseases of liver in Taiwan. The previous pharmacological studies have demonstrated that the extracts or constituents of tissues of *M. charantia* possess anti-diabetic and anti-inflammatory activities.[1—3] The Cucurbitaceae plants have been recognized as rich sources of cucurbitane-type triterpenoids possessing anti-diabetic activity.[3] More than seventy cucurbitane-type triterpenoids have been identified from the fruits,[3—10] seeds,[11,12] roots,[13] leaves and vines[14,15] of *M. charantia*. As part of our program aimed at the discovery of the cucurbitane-type triterpenes from *M. charantia* originated in Taiwan, we have reported the isolation and structure elucidation of 21 cucurbitane-type triterpenoids from the MeOH extract of the stems of this plant.[16—18] In our continuing investigation on the same extract, we further isolated two novel pentanorcucurbitane triterpenes, 22-hydroxy-23,24,25,26,27-pentanorcucurbit-5-en-3-one (**1**) and 3,7-dioxo-23,24,25,26,27-pentanorcucurbit-5-en-22-oic acid (**2**), as well as a novel trinorcucurbitane triterpene, 25,26,27-trinorcucurbit-5-ene-3,7,23-trione (**3**) (Fig. 1). In this paper, we describe the isolation and structure elucidation of compounds **1**—**3** and the cytoprotective activities of compounds **2** and **3** in *tert*-butyl hydroperoxide (*t*-BHP)-induced hepatotoxicity of HepG2 cells.

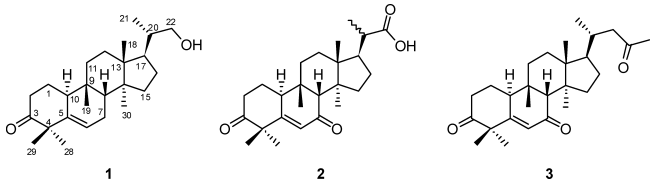

Fig. 1. Structures of Compounds **1**—**3** from *M. charantia*

Results and Discussion

Compound **1** was obtained as a white amorphous powder. The IR spectrum suggested the presence of hydroxy (3422 cm^{-1}), double bond (3075, 1654 cm^{-1}), and isolated ketone (1708 cm^{-1}) absorptions. The ^{1}H- and ^{13}C-NMR spectra of **1** (Table 1) contained signals for five methyl singlets [δ_H 0.84, 0.86, 0.87, 1.19, 1.22 (3H each, s)], one methyl doublet [δ_H 1.00 (3H, d, J=5.6 Hz)], a trisubstituted double bond [δ_H 5.64 (1H, m); δ_C 120.3 (d), 142.5 (s)], and an ABX splitting pattern of secondary alcohol [3.31 (1H, d, J=6.4, 10.4 Hz), 3.62 (1H, d, J=2.4, 10.4 Hz); δ_C 68.2 (t)]. The ^{13}C-NMR spectrum of **1** revealed 25 carbon signals, which were assigned by the distortionless enhancement by polarization transfer (DEPT) experiments as six aliphatic methyl, seven aliphatic methylene, four aliphatic methine, four aliphatic quaternary, one oxygenated methylene, one olefinic methine, one quaternary olefinic, and one isolated ketone carbonyl carbons. Its high resolution electron impact mass spectrum (HR-EI-MS) exhibited a molecular ion at m/z 372.3048, consistent with the molecular formula, $C_{25}H_{40}O_2$, which indicated six degrees of unsaturation. Two degrees of unsaturation were attributable to a trisubstituted olefin and a ketone group, and the remaining four units of unsaturation were accounted for the tetracyclic skeleton. On the basis of the fact that the major tetracyclic triterpenoids presenting in the genus *Momordica* plants are cucurbitane-type triterpenes, compound **1** was tentatively proposed to be a 23,24,25,26,27-pentanorcucurbitacin derivative. By comparison of the ^{1}H- and ^{13}C-NMR data with those of the known compound, (23*E*)-25-hydroxycucurbita-5,23-diene-3,7-dione,[16] indicated that both compounds exhibited identical structure in rings A—D of the tetracyclic skeleton and the ^{13}C-NMR signals of C-23—C-27 of the side chain were absent in **1**. Thus, compound **1** was considered as a 23,24,25,26,27-pentanorcucurbitacin triterpene. The planar structure of **1** was constructed by the ^{1}H–^{1}H correlated spectroscopy (COSY), heteronuclear multiple-quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC) spectra. The HMBC cor-

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Table 1. ^{1}H- and ^{13}C-NMR Data for **1**—**3** (400, 100 MHz in $CDCl_3$)

Position	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	25.7	1.49 m, 2.01 m	23.6	1.65 m, 2.12 m	23.6	1.64 m, 2.12 m
2	38.6	2.39 m, 2.58 m	38.1	2.52 m, 2.62 m	38.2	2.52 m, 2.62 m
3	215.2		211.4		211.8	
4	50.9		51.5		51.5	
5	142.5		167.8		168.0	
6	120.3	5.64 m	125.4	6.15 d (2.4)	125.4	6.14 d (2.0)
7	24.5	1.86 m, 2.38 m	202.3		202.3	
8	43.2	1.78 m	59.0	2.43 s	59.2	2.42 s
9	34.9		36.8		36.8	
10	38.5	2.55 m	41.1	2.92 ddd (2.0, 4.0, 11.6)	41.3	2.89 m
11	32.1	1.50 m, 1.70 m	31.2	1.55 m, 1.84 m	31.3	1.58 m, 1.80 m
12	30.2	1.45 m, 1.55 m	29.8	1.58 m, 1.89 m	29.8	1.62 m, 1.78 m
13	46.3		45.9		46.0	
14	49.0		48.4		48.7	
15	34.9	1.16 m, 1.26 m	34.6	1.14 m, 1.58 m	34.6	1.12 m, 1.58 m
16	27.3	1.32 m, 1.88 m	26.7	1.41 m, 1.91 m	28.0	1.52 m, 1.84 m
17	46.8	1.60 m	46.7	1.97 m	49.8	1.50 m
18	15.5	0.87 s	15.7	0.90 s	15.5	0.92 s
19	27.2	0.86 s	27.2	0.94 s	27.3	0.93 s
20	39.0	1.68 m	42.2	2.50 m	32.9	2.06 m
21	16.8	1.00 d (5.6)	17.3	1.21 d (6.8)	19.9	0.89 d (6.4)
22	68.2	3.31 dd (6.4, 10.4), 3.62 dd (2.4, 10.4)	180.9		51.1	2.16 m, 2.48 m
23					209.4	
24					30.8	2.10 s
28	28.6	1.19 s	28.4	1.31 s	28.5	1.33 s
29	22.6	1.22 s	23.0	1.34 s	23.2	1.31 s
30	17.7	0.84 s	17.9	0.91 s	18.1	0.87 s

relations between H-1 (δ_H 1.49, 2.01)/C-3 (δ_C 215.2), C-5 (δ_C 142.5), C-10 (δ_C 38.5); H-6 (δ_H 5.64)/C-4 (δ_C 50.9), C-5, C-7 (δ_C 24.5), C-8 (δ_C 43.2), C-10 (Fig. 2) confirmed that the isolated ketone and the trisubstituted double bond were located at C-3 and C-5,6 positions, respectively. The hydroxy group was attached on C-22 was assured by the HMBC correlations between H-22 (δ_H 3.31, 3.62)/C-17 (δ_C 46.8), C-20 (δ_C 39.0), C-21 (δ_C 16.8) (Fig. 2). The relative configurations of sterogenic C-atoms in the tetracyclic rings were determined by significant nuclear Overhauser effect (NOE) correlations between H-8 (δ_H 1.78)/Me-18 (δ_H 0.87), Me-19 (δ_H 0.86); H-10/Me-28 (δ_H 1.19), Me-30 (δ_H 0.84); H-17 (δ_H 1.60)/Me-30 (δ_H 0.84) in the nuclear Overhauser enhancement exchange spectroscopy (NOESY) spectrum (Fig. 3). The NOESY correlations between H-20 (δ_H 1.68)/Me-18; Me-21 (δ_H 1.00)/H-12 (δ_H 1.55); H-17 (δ_H 1.60)/H-22 (δ_H 3.31, 3.62) allowed us to assure the configuration at C-20 as *R*.[19—21] Furthermore, the electron impact mass spectrum (EI-MS) of **1** showed the fragment ion at *m*/*z* 313 $[M-C_3H_7O$ (side chain)$]^+$ derived from the loss of side chain by the cleavage of C-17/C-20 bond. In addition, the fragment ion at *m*/*z* 222 $[M-C_{10}H_{14}O]^+$ was corresponded to retro-Diels-Alder (RDA) cleavage between A and B rings. From the above evidence, compound **1** was determined as 22-hydroxy-23,24,25,26,27-pentanorcucurbit-5-en-3-one, namely pentanorcucurbitacin A. Complete ^{1}H- and ^{13}C-NMR chemical shifts were established by ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY spectra.

The HR-EI-MS of **2** showed a molecular ion at *m*/*z* 400.2606, which corresponded to the molecular formula, $C_{25}H_{36}O_4$, indicating eight degrees of unsaturation. The IR

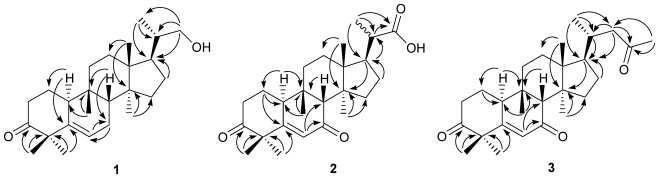

Fig. 2. Main HMBC Correlations of **1**—**3**

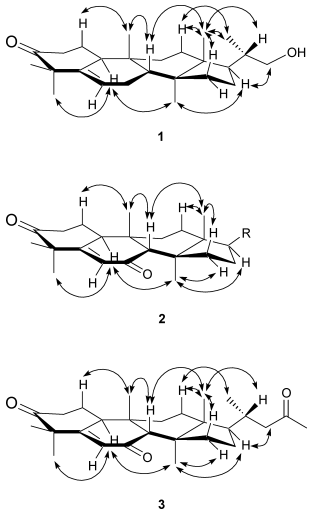

Fig. 3. Main NOESY Correlations of **1**—**3**

spectrum indicated the presence of an isolated ketone, a carboxylic acid, a conjugated ketone, and a conjugated double bond. The presence of an α,β-unsaturated ketone system was further confirmed by the significant UV absorption at

247 nm. The ^{1}H- and ^{13}C-NMR spectra of **2** (Table 1) displayed signals for five methyl singlets [δ_H 0.90, 0.91, 0.94, 1.31, 1.34 (3H each, s)], one set of α,β-unsaturated carbonyl system [δ_H 6.15 (1H, d, J=2.4 Hz); δ_C 125.4 (d), 167.8 (s), 202.3 (s)]. The ^{13}C-NMR spectrum of **2** revealed 25 resonances. The ^{13}C-NMR data of **2** showed close resemblance with those of the known compound, (23*E*)-25-hydroxycucurbita-5,23-diene-3,7-dione,[16] indicated that both compounds exhibited identical structure in rings A—D of the tetracyclic skeleton and the ^{13}C-NMR signals of C-23—C-27 of the side chain were absent in **2**. Compound **2** was tentatively proposed to exhibit a basic skeleton of 23,24,25,26,27-pentanorcucurbitacin. In turn, the downfield methine signal at δ_H 2.50 (H-20) as well as the HMBC correlations between H-17 (δ_H 1.97)/C-20 (δ_C 42.2); H-20 (δ_H 2.50)/C-17 (δ_C 46.7); H-20/C-22 (δ_C 180.9); Me-21 (δ_H 1.21)/C-22 (Fig. 2) confirmed that the carboxylic acid group was located at C-22 position. The relative configurations of sterogenic C-atoms in the tetracyclic rings were determined by significant NOE correlations between H-8 (δ_H 2.43)/Me-18 (δ_H 0.90), Me-19 (δ_H 0.94); H-10 (δ_H 2.92)/Me-28 (δ_H 1.31), Me-30 (δ_H 0.91); H-17 (δ_H 1.97)/Me-30 (δ_H 0.91) in the NOESY spectrum (Fig. 3). The configuration at C-20 was considered as a racemic center due to locating at α-position of carbonyl functionality. The EI-MS spectrum of **2** showed the fragment ion at *m/z* 327 $[M-C_3H_5O_2$ (side chain)$]^+$ derived from the loss of side chain by the cleavage of C-17 and C-20 bond. In addition, the fragment ion at *m/z* 164 $[M-C_{15}H_{24}O_2]^+$ was corresponded to flavone like RDA cleavage. Thus, compound **2** was elucidated as 3,7-dioxo-23,24,25,26,27-pentanorcucurbit-5-en-22-oic acid, namely pentanorcucurbitacin B.

Compound **3** was obtained as a white amorphous powder. The molecular formula was determined as $C_{27}H_{40}O_3$ from the HR-EI-MS $[M]^+$ *m/z* 412.2981. In the UV spectrum, a significant absorption maximum at 246 nm suggested the presence of an α,β-unsaturated ketone. The IR spectrum displayed absorption bands for a conjugated ketone, a conjugated double, and an isolated cyclohexanone. The ^{1}H- and ^{13}C-NMR spectra of **3** (Table 1) exhibited signals for the presence of five methyl singlets [δ_H 0.87, 0.92, 0.93, 1.31, 1.33 (3H each, s)], a secondary methyl [δ_H 0.89 (3H, d, J=6.4 Hz)], an acetyl methyl [δ_H 2.10 (s)], and one α,β-unsaturated carbonyl system [δ_H 6.14 (1H, d, J=2.0 Hz); δ_C 125.4 (d), 168.0 (s), 202.3 (s)]. In turn, the ^{13}C-NMR spectrum of **3** revealed 27 carbon signals. By comparison of the ^{1}H- and ^{13}C-NMR data with those of the known compound, (23*E*)-cucurbita-5,24-diene-3,7,23-trione,[17] indicated that both compounds exhibited identical structure in rings A—D and the C-25—C-27 NMR signals of side chain were absent in **3**. Thus, compound **3** was considered as a 25,26,27-trinorcucurbitacin triterpene with a C_5 side chain containing an isolated ketone functionality. The NMR signals for an acetyl methyl [δ_H 2.10 (s); δ_C 30.8 (s)] as well as the HMBC correlations between H-20 (δ_H 2.06)/C-23 (δ_C 209.4); Me-21 (δ_H 0.89)/C-22 (51.1); H-24 (δ_H 2.10)/C-23 confirmed that the isolated ketone was located at C-23 (Fig. 2). The EI-MS spectrum of **3** showed the fragment ion at *m/z* 369 $[M-COCH_3]^+$ derived from the loss of an acetyl group. The relative configurations of sterogenic C-atoms in the tetracyclic rings were determined by significant NOE correlations between H-8/Me-18 (δ_H 0.92), Me-19 (δ_H 0.93); H-10/Me-28 (δ_H 1.33), Me-30 (δ_H 0.87); H-17 (δ_H 1.50)/Me-30 in the NOESY spectrum (Fig. 3). The configuration at C-20 was determined as *R* on the basis of the NOESY correlations between H-20 (δ_H 2.06)/Me-18; Me-21 (δ_H 0.89)/H-12 (δ_H 1.62); H-22 (δ_H 2.16)/H-17 (δ_H 1.50).[19—21] From the above evidence, compound **3** was characterized as 25,26,27-trinorcucurbit-5-ene-3,7,23-trione. Complete ^{1}H- and ^{13}C-NMR chemical shifts were established by ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY spectra.

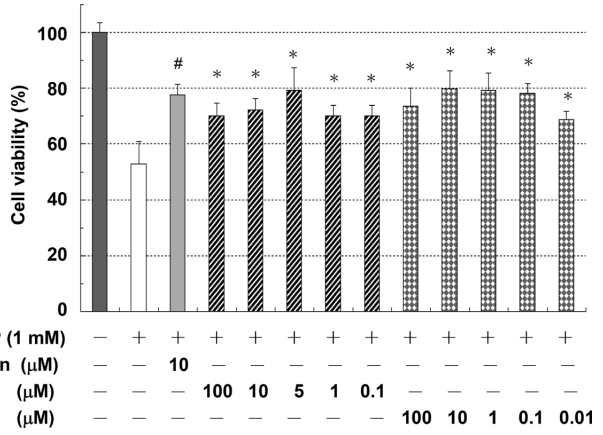

Fig. 4. Cytoprotective Effect of **2** and **3** on *t*-BHP-Toxicified HepG2 Cells

Statistically significant compared with normal data ($p<0.05$). * Statistically significant compared with *t*-BHP control data ($p<0.05$).

Compounds **2** and **3** were tested for their cytotoxic activity toward human hepatoma HepG2 cells with fluorouracil (5-FU) as a positive control (IC_{50}=1.9 μM) by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method based on the described procedures.[18] The two compounds were not cytotoxic against the HepG2 cell line up to 100 μM. The cytoprotective effects of compounds **2** and **3** on HepG2 cells injured by *tert*-butyl hydroperoxide (*t*-BHP) were performed in an assay described earlier.[22] The pretreatment of compounds **2** and **3** on HepG2 cells inhibited *t*-BHP-induced cytotoxicity. Compound **2** at a dose of 5 μM protected the *t*-BHP-induced cytotoxicity of HepG2 to 55.2% of control group. Compound **3** at a dose of 10 μM protected the *t*-BHP-induced cytotoxicity of HepG2 to 56.9% of control group. The protective effects of compounds **2** and **3** were similar to that of silybin, a commercial agent, which protected the *t*-BHP-induced cytotoxicity of HepG2 to 52.5% of control group at a dose of 10 μM (Fig. 4).

Experimental

General Experimental Procedures Optical rotations were measured on a JASCO DIP-180 digital spectropolarimeter. UV spectra were measured in MeOH using a Shimadzu UV-1601PC spectrophotometer. IR spectra were obtained on a Nicolet 510P FT-IR spectrometer. NMR spectra were recorded in $CDCl_3$ at room temperature on a Varian Mercury plus 400 NMR spectrometer, and the solvent resonance was used as internal shift reference [tetramethyl silane (TMS) as standard]. The 2D NMR spectra were recorded by using standard pulse sequences. EI-MS and HR-EI-MS were recorded on Finnigan TSQ-700 and JEOL SX-102A mass spectrometers, respectively. TLC was carried out on silica gel 60 F_{254} plates (Merck, Germany). Column chromatography was performed on silica gel (230—400 mesh ASTM, Merck). HPLC was performed on a Hitachi L-7000 chromatograph with a Lichrosorb Si gel 60 (5 μm) column (250×10 mm).

Plant Material The stems of *Momordica charantia* were collected in Pingtung County, Taiwan in July, 2003. Identification of the voucher specimens was done by Prof. Sheng-Zehn Yang, Curator of Herbarium, National

Pingtung University of Science and Technology. A voucher specimen (no. 2013) was deposited at the Herbarium of National Pingtung University of Science and Technology.

Extraction and Isolation The air-dried stems (18 kg) of *M. charantia* were extracted with MeOH (3×30 l) at room temperature (7 d each). The combined MeOH extract was evaporated under reduced pressure to afford a black residue, which was suspended in H_2O (3 l), and then partitioned sequentially, using EtOAc and *n*-BuOH (3×2 l) as solvent. The EtOAc fraction (386 g) was chromatographed on a silica gel column (120×10 cm), using solvent mixtures of *n*-hexane and EtOAc with increasing polarity as eluents. Eleven fractions were collected as follows: 1 [5000 ml, *n*-hexane], 2 [4000 ml, *n*-hexane–EtOAc (49 : 1)], 3 [4000 ml, *n*-hexane–EtOAc (19 : 1)], 4 [4000 ml, *n*-hexane–EtOAc (9 : 1)], 5 [4000 ml, *n*-hexane–EtOAc (17 : 3)], 6 [4000 ml, *n*-hexane–EtOAc (8 : 2)], 7 [4000 ml, *n*-hexane–EtOAc (7 : 3)], 8 [3000 ml, *n*-hexane–EtOAc (5 : 5)], 9 [3000 ml, *n*-hexane–EtOAc (4 : 6)], 10 [3000 ml, *n*-hexane–EtOAc (2 : 8)], and 11 (6000 ml, EtOAc). Fraction 6 was further chromatographed on a silica gel column (5×45 cm), eluted with CH_2Cl_2–EtOAc (8 : 1 to 0 : 1) to give seven fractions (each about 700 ml), 6A—6G. Fr. 6E was subjected to column chromatographed over silica gel eluted with *n*-hexane–CH_2Cl_2–EtOAc (5 : 3 : 1) and semipreparative HPLC eluted with *n*-hexane–EtOAc (7 : 3) to yield **1** (1.1 mg). Fraction 7 was further chromatographed on a silica gel column (5×45 cm), eluted with CH_2Cl_2–EtOAc (8 : 1 to 0 : 1) to resolve into seven fractions (each about 600 ml), 7A—7G. Fr. 7C was subjected to column chromatography over silica gel eluted with CH_2Cl_2–EtOAc (15 : 1) and semipreparative HPLC eluted with *n*-hexane–EtOAc (7 : 3) to yield **3** (6 mg). Fraction 8 was further purified through a silica gel column (5×45 cm), eluted with CH_2Cl_2–EtOAc (7 : 1) to obtain six fractions (each about 500 ml), 8A—8F. Fr. 8F was subjected to column chromatographed over Si gel eluted with *n*-hexane–CH_2Cl_2–EtOAc (3 : 3 : 1) and semipreparative HPLC eluted with CH_2Cl_2–EtOAc (3 : 2) to yield **2** (4.5 mg).

22-Hydroxy-23,24,25,26,27-pentanorcucurbit-5-en-3-one (**1**): Amorphous white powder; $[\alpha]_D^{25}$ +36.7 (c=0.10, MeOH); ^{1}H- and ^{13}C-NMR data, see Table 1; IR (KBr) ν_{max} 3422, 3075, 2950, 2872, 1708, 1654, 1465, 1382, 1270, 1031, 977, 734, 705 cm^{-1}; EI-MS *m*/*z*: 372 $[M]^+$ (1), 357 (2), 354 (1), 313 (1), 271 (3), 222 (88), 207 (52), 189 (19), 163 (100), 133 (30), 123 (58), 105 (62), 91 (60); HR-EI-MS *m*/*z*: 372.3048 (Calcd for $C_{25}H_{40}O_2$ 372.3030).

3,7-Dioxo-23,24,25,26,27-pentanorcucurbit-5-en-22-oic Acid (**2**): Amorphous white powder; $[\alpha]_D^{25}$ +38.7 (c=0.36, MeOH); ^{1}H- and ^{13}C-NMR data, see Table 1; IR (KBr) ν_{max} 3427, 3072, 2950, 2872, 1693, 1645, 1460, 1377, 1260, 1216, 1124, 1036, 886, 842 cm^{-1}; UV (MeOH) λ_{max} (log ε) 247 (3.53) nm; EI-MS *m*/*z*: 400 (M^+, 100), 385 (68), 372 (19), 357 (15), 339 (20), 327 (14), 311 (21), 263 (30), 205 (34), 164 (45), 136 (38), 121 (20); HR-EI-MS *m*/*z*: 400.2606 (Calcd for $C_{25}H_{36}O_4$ 400.2614).

25,26,27-Trinorcucurbit-5-ene-3,7,23-trione (**3**): Amorphous white powder; $[\alpha]_D^{25}$ +86.2 (c=0.37, MeOH); ^{1}H- and ^{13}C-NMR data, see Table 1; IR (KBr) cm^{-1}: 3041, 2955, 2882, 1718, 1645, 1616, 1465, 1382, 1353, 1294, 1250, 1158, 886; UV (MeOH) λ_{max} (log ε) 203 (4.21), 246 (3.55) nm; EI-MS *m*/*z*: 412 $[M]^+$ (4), 369 (7), 355 (16), 339 (8), 205 (24), 149 (25), 136 (42), 121 (100), 107 (69), 93 (89), 79 (76), 67 (73); HR-EI-MS *m*/*z*: 412.2981 (Calcd for $C_{27}H_{40}O_3$ 412.2978).

Cytoprotective Assay HepG2 cells (hepatocellular carcinoma cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine 2 mM, 1% penicillin/streptomycin (penicillin 10000 U/ml and streptomycin 10 mg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C. The protective effect of compounds **2** and **3** on HepG2 cells injured by *t*-BHP was measured using the MTT colorimetric assay based on the described procedures with some modifications.[22] Briefly, HepG2 cells were plated on a 96-well plate with 1×10^4 cells per well. The cells were treated with different concentrations of test compounds. After preincubated for 2 h, the cultured media were changed to the media containing *t*-BHP (100 mM), incubated for 3 h and then rinsed with phosphate-buffered saline. Subsequently, the wells were incubated with the MTT (100 μl/well concentrated at 5 mg/ml) at 37 °C for 4 h. After removing the supernatant, 200 μl of dimethyl sulfoxide (DMSO) was added to redissolve the formazan crystals. Absorbance at 550 nm was measured to estimate survived cells.

Statistic Analysis The significance of various treatments was determined by the Student's *t*-test. The results were expressed as mean±S.E.M. Differents were considered significant if $p<0.05$.

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References

1) Rathi S. S., Grover J. K., Vats V., *Phytother. Res.*, **16**, 236—243 (2002).
2) Kobori M., Nakayama H., Fukushima K., Ohnishi-Kameyama M., Ono H., Fukushima T., Akimoto Y., Masumoto S., Yukizaki C., Hoshi Y., Deguchi T., Yoshida M., *J. Agric. Food Chem.*, **56**, 4004—4011 (2008).
3) Harinantenaina L., Tanaka M., Takaoka S., Oda M., Mogami O., Uchida M., Asakawa Y., *Chem. Pharm. Bull.*, **54**, 1017—1021 (2006).
4) Fatope M. O., Takeda Y., Yamashita H., Okabe H., Yamauchi T., *J. Nat. Prod.*, **53**, 1491—1497 (1990).
5) Kimura Y., Akihisa T., Yuasa N., Ukiya M., Suzuki T., Toriyama M., Motohashi S., Tokuda H., *J. Nat. Prod.*, **68**, 807—809 (2005).
6) Nakamura S., Murakami T., Nakamura J., Kobayashi H., Matsuda H., Yoshikawa M., *Chem. Pharm. Bull.*, **54**, 1545—1550 (2006).
7) Li Q. Y., Chen H. B., Liu Z. M., Wang B., Zhao Y. Y., *Magn. Reson. Chem.*, **45**, 451—456 (2007).
8) Akihisa T., Higo N., Tokuda H., Ukiya M., Akazawa H., Tochigi Y., Kimura Y., Suzuki T., Nishino H., *J. Nat. Prod.*, **70**, 1233—1239 (2007).
9) Matsuda H., Nakamura S., Murakami T., Yoshikawa M., *Heterocycles*, **71**, 331—341 (2007).
10) Liu Y., Ali Z., Khan I. A., *Planta Med.*, **74**, 1291—1294 (2008).
11) Miyahara Y., Okabe H., Yamauchi T., *Chem. Pharm. Bull.*, **29**, 1561—1566 (1981).
12) Okabe H., Miyahara Y., Yamauchi T., Miyahara K., Kawasaki T., *Chem. Pharm. Bull.*, **28**, 2753—2762 (1980).
13) Chen J. C., Tian R. R., Qiu M. H., Lu L., Zheng Y. T., Zhang Z. Q., *Phytochemistry*, **69**, 1043—1048 (2008).
14) Yasuda M., Iwamoto M., Okabe H., Yamauchi T., *Chem. Pharm. Bull.*, **32**, 2044—2047 (1984).
15) Chen J. C., Liu W. Q., Lu L., Qiu M. H., Zheng Y. T., Yang L. M., Zhang X. M., Zhou L., Li Z. R., *Phytochemistry*, **70**, 133—140 (2009).
16) Chang C. I., Chen C. R., Liao Y. W., Cheng H. L., Chen Y. C., Chou C. H., *J. Nat. Prod.*, **69**, 1168—1171 (2006).
17) Chang C. I., Chen C. R., Liao Y. W., Cheng H. L., Chen Y. C., Chou C. H., *J. Nat. Prod.*, **71**, 1327—1330 (2008).
18) Chang C. I., Chen C. R., Liao Y. W., Shih W. L., Cheng H. L., Tzeng C. Y., Li J. W., Kung M. T., *Chem. Pharm. Bull.*, **58**, 225—229 (2010).
19) Antonov A. S., Afiyatullov S. S., Kalinovsky A. I., Ponomarenko L. P., Dmitrenok P. S., Aminin D. L., Agafonova I. G., Stonik V. A., *J. Nat. Prod.*, **66**, 1082—1088 (2003).
20) Antonov A. S., Kalinovsky A. I., Stonik V. A., Afiyatullov S. S., Aminin D. L., Dmitrenok P. S., Mollo E., Cimino G., *J. Nat. Prod.*, **70**, 169—178 (2007).
21) Mansoor T. A., Hong J., Lee C. O., Bae S. J., Im K. S., Jung J. H., *J. Nat. Prod.*, **68**, 331—336 (2005).
22) Lee H. U., Bae E. A., Han M. J., Kim D. H., *Biol. Pharm. Bull.*, **28**, 1992—1994 (2005).