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Constituents of Rosaceous Plants. I. Structures of New Triterpenoids from *Cowania mexicana*

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In our search for possible anti-tumor-promoters, we carried out an investigation of the leaves and branches of *Cowania mexicana* D. DON (Rosaceae). Two new cucurbitane type triterpenes, 15-oxo-cucurbitacin F (3) and 15-oxo-23,24-dihydrocucurbitacin F (4), were isolated together with cucurbitacin F (1) and 23,24-dihydrocucurbitacin F (2). These triterpenes were inhibitors of Epstein-Barr virus early antigen activation induced by 12-*O*-tetradecanoylphorbol-13-acetate, a well-known tumor-promoter. The structures of 3 and 4 were determined from 2D-NMR spectral data and difference NOE experiments.

Keywords *Cowania mexicana*; 15-oxo-cucurbitacin F; 15-oxo-23,24-dihydrocucurbitacin F; cucurbitacin F; 23,24-dihydrocucurbitacin F; Rosaceae

In a primary random screening of many plants and crude drugs for possible anti-tumor-promoters, the leaves and branches of *Cowania mexicana* D. DON (Rosaceae) showed strong inhibitory activity on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a well-known tumor-promoter.[1] The branches and leaves of this plant have traditionally been used by American Indians as a cough suppressant and a remedy for respiratory disease.[2] There is no report, so far, of any systematic research on the constituents of *C. mexicana*. In this paper, we describe the isolation of triterpenoids from this plant and their structural elucidation.

Results and Discussion

The leaves and branches of *C. mexicana* were extracted with MeOH. Then each MeOH extract was extracted with *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH. These extracts were submitted to a primary screening for inhibitory effects on EBV-EA activation, and the CH_2Cl_2 and EtOAc extracts showed strong activity in this model. Four cucurbitacins (**1**—**4**) were isolated from the active extracts, together with oleanolic acid, (−)-epicatechin and 3-*O*-*β*-D-glucopyranosyl-*β*-sitosterol, using various chromatographic techniques as shown in the experimental section.

Compound **1** was obtained as colorless needles and identified as cucurbitacin F by comparison with previously reported ^{1}H- and ^{13}C-NMR spectral data.[3] Further, triacetate (**5**) was derived from **1**, and also identified by the

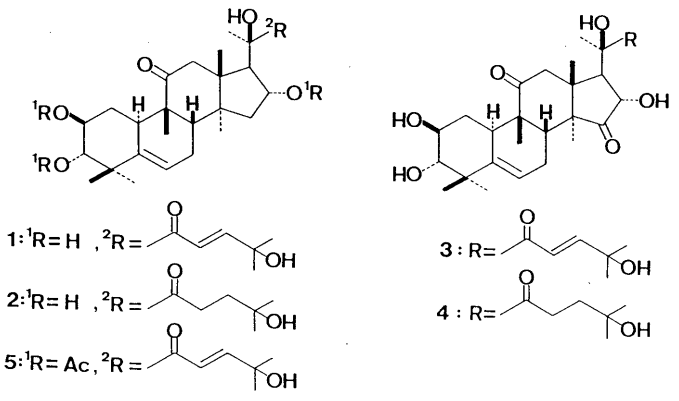

Chart 1

TABLE I. ^{13}C-NMR Chemical Shifts of Cucurbitacins **1**—**4**, (ppm) from TMS in Pyridine-d_5

	1	2	3	4		1	2	3	4
C-1	34.67	34.58	34.56	34.57	C-16	70.50	70.26	72.46	72.30
C-2	71.00	70.92	70.83	70.85	C-17	59.16	58.65	53.94	53.43
C-3	81.43	80.04	81.32	81.30	C-18	20.43	20.31	21.48	21.30
C-4	42.81	42.73	42.83	42.82	C-19	19.10	19.06	19.64	19.64
C-5	142.48	142.35	142.52	142.51	C-20	79.29	81.33	79.03	79.87
C-6	118.70	118.62	118.84	118.86	C-21	25.50	25.39	26.22	26.01
C-7	24.19	24.06	22.91	22.88	C-22	204.26	215.97	204.11	215.98
C-8	34.40	34.31	34.56	34.57	C-23	120.82	32.59	120.11	32.96
C-9	48.84	48.61	49.34	49.34	C-24	155.58	38.39	156.36	38.39
C-10	43.16	43.00	37.97	37.93	C-25	70.24	68.95	70.32	69.03
C-11	213.17	213.10	211.92	211.85	C-26	29.97	29.98	29.93	30.08
C-12	49.23	49.18	47.16	47.17	C-27	29.74	29.71	29.78	29.90
C-13	48.65	48.73	45.79	45.75	C-28	20.53	20.25	13.71	13.82
C-14	51.15	51.00	55.33	55.39	C-29	22.51	22.40	22.49	22.49
C-15	46.50	46.32	216.10	216.04	C-30	25.45	25.31	25.41	25.40

reported spectral data.[3] Compound **2** was obtained as a colorless powder and identified as 23,24-dihydrocucurbitacin F by direct comparison with an authentic sample.[4]

Compound **3**, $C_{30}H_{44}O_8$, mp 223—226 °C, was obtained as colorless needles. In the IR spectrum (1630 cm^{-1}), **3** showed the presence of an α,β-unsaturated carbonyl group. In addition, the ^{13}C-NMR spectrum and the distortionless enhancement by polarization transfer (DEPT) experiments of **3** showed the presence of three carbonyl carbons (δ 204.11, 211.92, 216.10), four olefinic carbons (δ 118.84, 120.11, 142.52, 156.36), three secondary alcohols (δ 70.83, 72.46, 81.32), two tertiary alcohols (δ 79.03 and 70.32) and eight tertiary methyl carbons (δ 13.71, 19.64, 21.48, 22.49, 25.41, 26.22, 29.78, 29.93). The ^1H-NMR spectrum of **3** showed the presence of trans olefinic protons at δ 7.65 (1H, d, J=15.2 Hz) and δ 7.56 (1H, d, J=15.2 Hz). Comparison of ^1H- and ^{13}C-NMR spectral data of **3** and cucurbitacin F (**1**) showed that the A-B ring residue and the side-chain at C-17 were similar. A detailed structural elucidation of **3** was carried out using two dimensional (2D)-NMR spectra and difference nuclear Overhauser effect (NOE) experiments. All the proton and carbon signals of **3** could be assigned using ^1H–^1H correlation spectroscopy (COSY), DEPT experiments, ^1H–^{13}C COSY spectral data as shown in Tables I and II. To confirm the connectivities of the partial structure, the ^1H–^{13}C long range COSY of **3** was measured and significant correlations are indicated by arrows in Fig. 1. The carbonyl carbon at δ 211.92 (C-11)

TABLE II. ^1H-NMR Chemical Shifts of Compounds **3** and **4**, (ppm) from TMS in Pyridine-d_5

	3	4
1β-H	1.56 (d, J=12.2)	1.55 (d, J=12.0)
1α-H	2.72 (br d, J=12.2)	1.72 (d, J=12.0)
2-H	4.15 (t like)	4.14 (m)
3-H	3.43 (d, J=8.3)	3.42 (d, J=8.4)
6-H	5.75 (br d, J=5.2)	5.75 (br s)
7α-H	2.51 (dd, J=19.5, 7.8)	2.51 (dd, J=19.2, 8.0)
7β-H	3.22 (dd, J=19.5, 5.2)	3.22 (dd, J=19.2, 5.2)
8-H	2.38 (br d, J=7.8)	2.39 (br d, J=8.1)
10-H	2.40[a]	2.40[a]
12-βH	3.00 (d, J=14.6)	2.97 (d, J=14.6)
12-αH	3.40 (d, J=14.6)	3.44 (d, J=14.6)
16-H	4.75 (dd, J=8.3, 5.9)	4.67 (br d, J=8.5)
17-H	3.12 (d, J=8.3)	3.12 (d, J=8.5)
18-Me	1.23 (3H, s)	1.24 (6H, s)
19-Me	1.24 (3H, s)	
21-Me	1.69 (3H, s)	1.67 (3H, s)
23-H	7.65 (d, J=15.2)	2.25 (2H, m)
24-H	7.56 (d, J=15.2)	3.46 (m)
26,27-Me	1.46 (6H, s)	1.40 (6H, s)
28-Me	1.62 (3H, s)	1.64 (3H, s)
29-Me	1.26 (3H, s)	1.28 (3H, s)
30-Me	1.48 (3H, s)	1.46 (3H, s)

a) Coupling pattern and J values of these signals were not clear, because these signals overlapped the signal of 8-H.

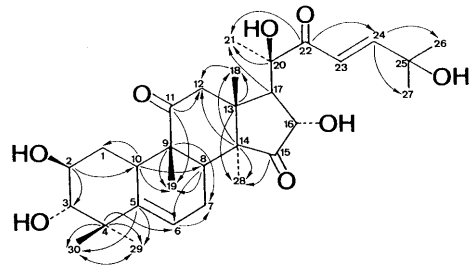

Fig. 1. Correlation (^{13}C ^1H) in ^1H–^{13}C Long-Range COSY Spectrum of **3**

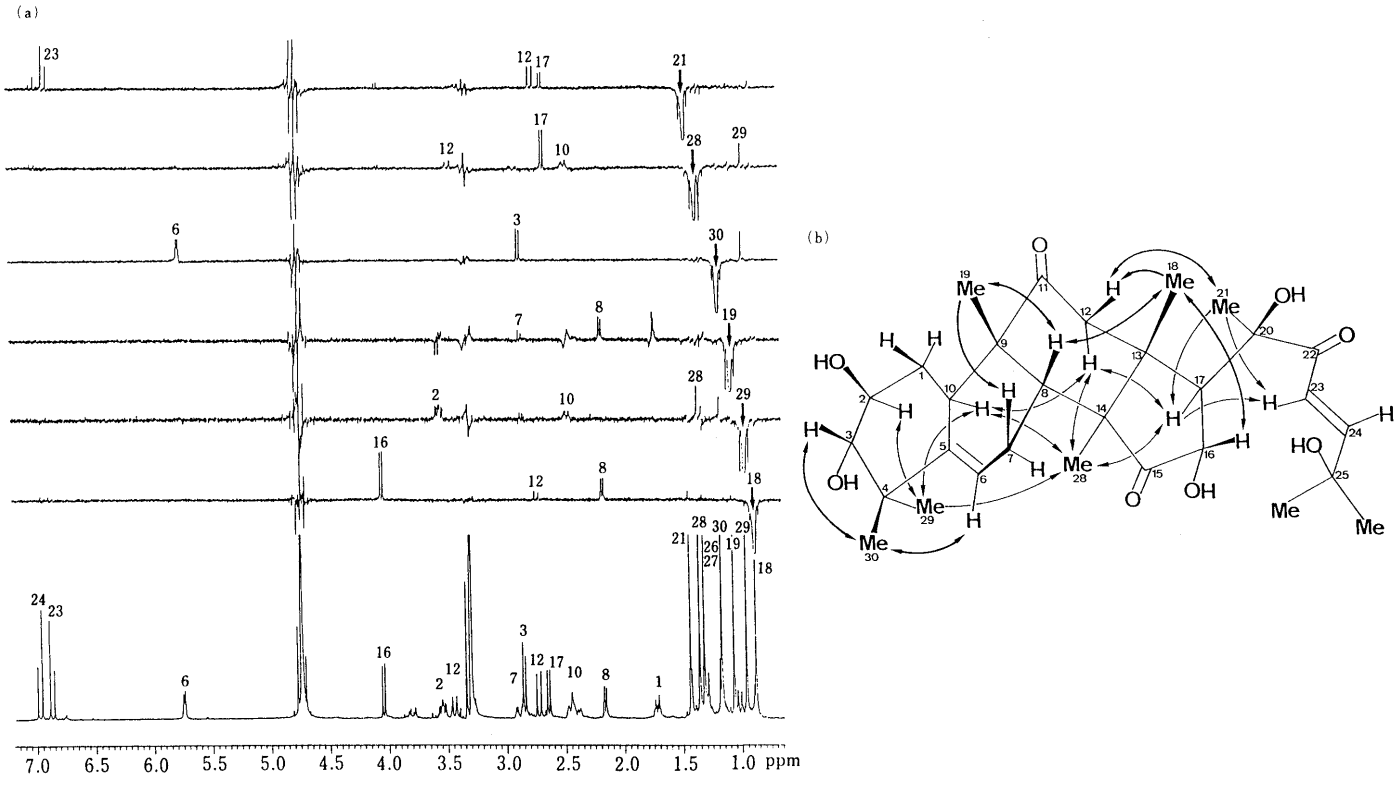

Fig. 2(a). A Part of the Difference NOE Spectra of **3** in CD_3OD
(b). Significant Enhancement of Signal Intensity by Difference NOE Experiments of **3** in CD_3OD

is correlated with the methyl protons at δ 1.24 (19-Me) and with one of the methylene protons at δ 3.00 (12β-H). The quaternary carbon at δ 55.33 (C-14) is correlated with the methyl protons at δ 1.62 (28-Me) and 1.23 (18-Me), and with one of the methylene protons at δ 3.00 (12β-H). The carbonyl carbon at δ 216.10 (C-15) is correlated with the methyl protons at δ 1.62 (28-Me). The quaternary carbon at δ 49.34 (C-9) and the olefinic carbon at δ 118.84 (C-6) are correlated with one of the methylene protons at δ 3.22 (7β-H). Further, the differenc NOE spectra (in CD_3OD) of **3** were measured in order to confirm the relative stereochemistry. Irradiation of the signal of 18-methyl protons enhanced the signal intensities of two methine protons (8-H and 16-H) and one of the methylene protons (12β-H). Correspondingly, irradiation of both the signals of 8-H and 16-H enhanced the signal intensity of the 18-methyl protons. Irradiation of the signal of the 28-methyl protons enhanced the signal intensities of two methine protons (10-H and 17-H) and one of the methylene protons (12α-H). Also, correspondingly, irradiation of the signals of 10-H, 12α-H and 17-H enhanced the signal intensity of the 28-methyl protons. Irradiation of the signal of 21-methyl protons enhanced the signal intensities of 17-H, 23-H and one of the methylene protons (12β-H). Some significant NOE results are indicated by arrows in Fig. 2(a, b). From these results, the structure of the new compound **3** isolated from *C. mexicana* is characterized as 15-oxo-cucurbitacin F.

Compound **4**, $C_{30}H_{46}O_8$, mp 207—209 °C, was obtained as a colorless powder. Comparison of ^{1}H- and ^{13}C-NMR spectral data of **4** and **3**, suggested that compound **4** has the same cucurbitan skeleton as 15-oxo-cucurbitacin F (**3**). In the ^{13}C-NMR spectrum of **4**, the signal of the carbonyl carbon at δ 215.98 (C-22) shifted to lower field compared with that of **3** at δ 204.11, and two methylene carbons at δ 32.96 (C-23) and 38.39 (C-24) appeared instead of a pair of olefinic carbons at δ 120.11 (C-23) and 156.36 (C-24) in **3**. Further, in the ^{1}H- and ^{13}C-NMR spectra of **4**, the proton and carbon signals of the side-chain at the C-17 position are similar to those of **2**. Therefore, **4** is the dihydro derivative of **3**, just as **2** is to **1**. All proton and carbon signals of **4** were assigned using ^{1}H–^{1}H and ^{1}H–^{13}C COSY spectral data and DEPT experiments as shown in Tables I and II. From these results, the structure of compound **4** was characterized as 15-oxo-23,24-dihydrocucurbitacin F.

Although many cucurbitacin triterpenoids have been isolated from the plants, belonging to the species Cucurbitaceae,[5] Elaeocarpaceae,[3a] Primulaceae,[3b] and Cruciferae,[3b] this report is the first example of the isolation and structural elucidation of cucurbitacins from rosaceaous plants.

Of these four compounds, **1** and **2** showed significant inhibitory effects on EBV-EA activation (more than 60% inhibition at a 5×10^2 mol ratio of compound/TPA).[6] The two-stage carcinogenesis tests *in vivo*[7] of these compounds are now in progress.

Experimental

Melting points were determined with a Yanagimoto micro melting point apparatus, and are uncorrected. IR spectra were recorded on a Shimadzu IR-408 spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian XL-300 instrument using tetramethylsilane as an internal standard. 2D-NMR and difference NOE spectra were recorded on a JEOL JNM GX-400 spectrometer. Optical rotations were measured on a JASCO DIP-181 digital polarimeter. Preparative HPLC was carried out on a Nihon Bunseki Kogyo LC-09 using a gel-permeation chromatography column (300 mm × 2, solvent: MeOH, flow rate: 5 ml/min) with UV (254 nm) and refractive index (RI) detectors.

Plant Materials The leaves and branches of *C. mexicana* were collected at Utah, U.S.A. in May, 1992. The plant used in the present study was identified by one of us (J. R. Estes) and voucher specimens were deposited in the herbarium of Kyoto Pharmaceutical University.

Extraction and Isolation The fresh leaves (1.2 kg) and branches (1.2 kg) of *C. mexicana* were air-dried, cut and separately exhaustively extracted with hot MeOH. The solvent was removed *in vacuo*, leaving a dark brown syrup in both cases (281 g from leaves and 190 g from branches). Each syrup was suspended in water and extracted with *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH saturated with water, in that order. Each organic layer from the leaves was evaporated *in vacuo* to give an oily residue (*n*-hexane 43.1 g, CH_2Cl_2 58.0 g, EtOAc 34.4 g and *n*-BuOH 45.2 g), and each organic layer from the branches was evaporated to give an oily residue (*n*-hexane 28.3 g, CH_2Cl_2 38.5 g, EtOAc 25.8 g and *n*-BuOH 34.7 g).

Part of the EtOAc extract (20.0 g) of the leaves was chromatographed on silica gel, eluting with $CHCl_3$, $CHCl_3$–MeOH (95 : 5) and $CHCl_3$–MeOH (90 : 10), to give 9 separated fractions (Nos. 1 to 9). From fraction No. 1, oleanolic acid was isolated and identified by comparison with an authentic sample.[8] Fraction No. 4 (1.0 g) was sujected to column chromatography on silica gel to afford the crude cucurbitacins fraction (540 mg). This crude fraction was subjected to preparative HPLC to give compounds **1** (248 mg), **2** (18 mg), **3** (30 mg) and **4** (25 mg). From fraction No. 6, (−)-epicatechin was isolated and identified by comparison with an authentic sample.[9]

Compound **1**: Colorless needles, mp 198—200 °C (from MeOH) (lit. mp 249—253 °C,[3a] 174—178 °C[3c]), $[\alpha]_D^{26}$ +42.5° (c = 1.05, MeOH) (lit.[3a] value +28°), was idntified as cucurbitacin F by comparison with reported spectral data.[3a,b]

Compound **2**: Colorless powder, mp 144—147 °C (from MeOH) (lit. mp 146—148 °C), $[\alpha]_D^{27}$ +55.3° (c = 0.66, MeOH) (lit.[3a] value +41.2°), was directly identified by comparison with an authentic sample of 23,24-dihydrocucurbitacin F (TLC behavior and IR, ^{1}H- and ^{13}C-NMR spectra).[4a]

Compound **3**: Colorless needles, mp 223—226 °C (from MeOH), $[\alpha]_D^{26}$ +57.5° (c = 0.43, MeOH). IR (KBr) cm^{-1}: 3400 (OH), 1735 (>C=O), 1685 (>C=O), 1630 (C=C−C=O). ^{1}H-NMR (CD_3OD) δ: 6.97 (1H, d, J = 15.5 Hz, 24-H), 6.86 (1H, d, J = 15.5 Hz, 23-H), 5.74 (1H, d, J = 5.5 Hz, 6-H), 4.04 (1H, d, J = 8.8 Hz, 16-H), 3.54 (1H, m, 2α-H), 3.44 (1H, d, J = 14.6 Hz, 12α-H), 2.90 (1H, m, 7β-H), 2.84 (1H, d, J = 9.3 Hz, 3β-H), 2.72 (1H, d, J = 14.6 Hz, 12β-H), 2.64 (1H, d, J = 8.8 Hz, 17-H), 2.44 (1H, m, 10-H), 2.39 (1H, d, J = 2.7 Hz, 7α-H), 2.16 (1H, d, J = 7.5 Hz, 8-H), 1.72 (1H, m, 1β-H), 1.43 (3H, s, 21-Me), 1.36 (3H, s, 28-Me), 1.32 (6H, s, 26-, 27-Me), 1.17 (3H, s, 30-Me), 1.06 (3H, s, 19-Me), 1.02 (1H, m, 1α-H), 0.96 (3H, s, 29-Me), 0.88 (3H, s, 18-Me). *Anal.* Calcd for $C_{30}H_{50}O_{11} \cdot 3H_2O$: C, 61.41; H, 8.59. Found: C, 61.36; H, 8.37. ^{1}H- and ^{13}C-NMR (in pyridine-d_5): Given in Tables I and II.

Compound **4**: Colorless needles, mp 207—209 °C (from MeOH), $[\alpha]_D^{26}$ +65.2° (c = 0.54, MeOH). IR (KBr) cm^{-1}: 3450 (OH), 1740 (>C=O), 1690 (>C=O). *Anal.* Calcd for $C_{30}H_{46}O_8 \cdot 1.5H_2O$: C, 64.14; H, 8.79. Found: C, 64.13; H, 8.88. ^{1}H- and ^{13}C-NMR (in pyridine-d_5): Given in Tables I and II.

Compounds **1**, **2**, **3** and **4** were also isolated from the active extracts, CH_2Cl_2 extract of leaves and CH_2Cl_2 and EtOAc extracts of branches, by a method similar to that described above. The compound, 3-*O*-β-D-glucopyranosyl-β-sitosterol, was isolated from the CH_2Cl_2 extracts of the leaves and branches and identified by comparison with an authentic sample.[8] The total yields of these cucurbitacins from the dried material are as follows: cucurbitacin F (**1**, 405 mg, 0.034% from branches; 953 mg, 0.079% from leaves), 23,24-dihydrocucurbitacin F (**2**, 18 mg, 0.002%; 90 mg, 0.008%), 15-oxo-cucurbitacin F (**3**, 61 mg, 0.005%); 72 mg, 0.006%) and 15-oxo-23,24-dihydrocucurbitacin F (**4**, 112 mg, 0.009%; 64 mg, 0.005%).

Acetylation of 1 Compound (**1**, 30 mg) was treated with Ac_2O–pyridine (1 : 1, 3 ml) at room temperature overnight. Work-up in the usual manner afforded a triacetate (**5**, 18 mg) as an amorphous powder. Compound **5** was identified as 2,3,16-tri-acetylcucurbitacin F by comparison with reported spectral data.[3a]

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