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ORIGINAL PAPER

Pyrenes and pyrendiones from *Uvaria lucida*

Masataka Moriyasu · Sousuke Takeuchi · Momoyo Ichimaru · Noriyshi Nakatani · Yumi Nishiyama · Atsushi Kato · Simon G. Mathenge · Francis D. Juma · Patrick B. ChaloMutiso

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Abstract A chemical investigation of the chloroform extract of the roots of *Uvaria ludida* Benth. (Annonaceae), an important African traditional medicine, led to the isolation of six new compounds; three pyrenes, 2-hydroxy-1,8-dimethoxypyrene (**1**), 8-methoxy-1,2-methylenedioxypyrene (**2**), and 7-hydroxy-8-methoxy-1,2-methylenedioxypyrene (**3**), two pyrenediones, 2-hydroxy-1,8-pyrenedione (**4**) and 2-methoxy-1,8-pyrenedione (**5**), and a sesquiterpene, (−)-10-oxo-isodauc-3-en-15-oic acid (**6**), together with eight known compounds (**7**–**14**). The structural elucidation by spectroscopic studies of the compounds isolated is described. While pyrenes did not exhibit strong cytotoxicity against human promyelocytic leukemia HL-60 cells, pyrenediones showed strong cytotoxicity. The IC_{50} of **4** was 70 ng mL^{-1}, which was close to that of etoposide (IC_{50} = 60 ng mL^{-1}).

Keywords *Uvaria lucida* · Annonaceae · Pyrenes · Pyrenediones · Cytotoxicity against HL-60

M. Moriyasu (✉) · S. Takeuchi · M. Ichimaru · N. Nakatani · Y. Nishiyama · A. Kato
Department of Natural Medicinal Chemistry, Kobe pharmaceutical University, 4-19-1, Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan
e-mail: moriyasu@kobepharma-u.ac.jp

S. G. Mathenge · P. B. ChaloMutiso
Department of Natural Medicinal Chemistry of Nairobi, P.O. Box 30197, Nairobi, Kenya

F. D. Juma
Department of Medicine, University of Nairobi, P.O. Box 30588, Nairobi, Kenya

Introduction

Uvaria lucida Benth (Annonaceae) is usually an erect or struggling shrub, 1.2–4.5 m tall [1], and is distributed in East Africa. This is an important medicinal plant in Kenya and its root has been used as a remedy for stomach and bowel troubles, especially for diarrhea and vomiting. In previous phytochemical studies of this plant, various compounds such as 2-hydroxybenzylchalcones, 2-hydroxybenzylflavanones, bissesquiterpenes and alkaloids were isolated [2–4]. In addition, two pyrenes, 2-hydroxy-1,7,8-trimethoxypyrene and 2,7-dihydroxy-1,8-dimethoxypyrene, which are very rare compounds in the plant kingdom, were also reported [2]. As part of our research for novel and antitumor compounds from African medicinal plants, extracts of several solvents of the branch of *U. lucida* were assessed for cytotoxicity toward human promyelocytic leukemia HL-60 cells. Extraction was carried out with petroleum ether, chloroform and methanol successively. We studied the constituents of chloroform extracts since it showed strong cytotoxic activity toward HL-60 cells.

Results and discussion

Following HPLC analysis of chloroform extract of the branch of *U. lucida*, many peaks were recognized. By a combination of column chromatography, preparative HPLC and preparative TLC, six new compounds (**1**–**6**) and eight known compounds (**7**–**14**) were isolated (Fig. 1). Two pyrenes, 2-hydroxy-1,7,8-trimethoxypyrene (**7**) and 2,7-dihydroxy-1,8-dimethoxypyrene (**8**), which had already been isolated from this plant [2], were also obtained in the present study. Three new compounds (**1**–**3**) and two known pyrenes (**7**, **8**) showed similar UV spectra having two

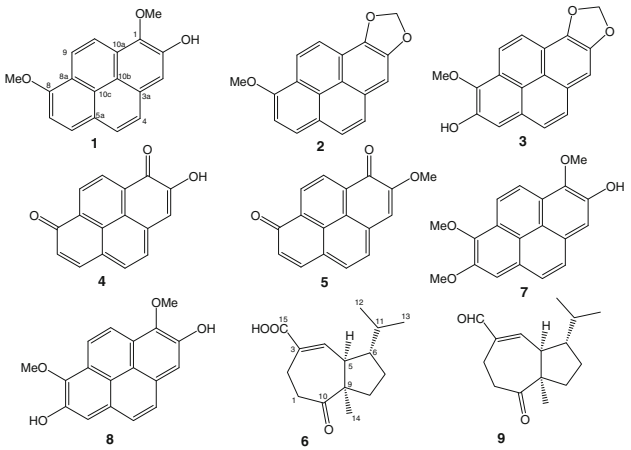

Fig. 1 Structures of new compounds **1–6** and their related compounds

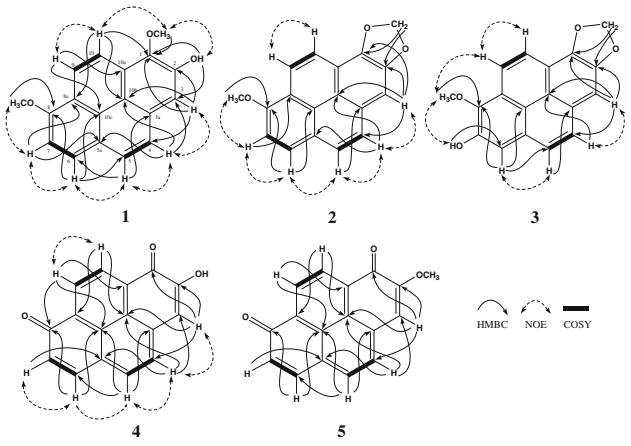

Fig. 2 COSY, HMBC and NOE correlations of **1–5**

absorption maxima near 252 nm and 330–360 (broad) measured with a photodiode-array detector. NMR data (^{1}H- and ^{13}C-NMR) of **1**, **2** and **3** also resembled those of **7** and **8**. These results suggest that compounds 1–3 should be pyrenes. Compound **1** was obtained as a yellow amorphous, and had a molecular formula of $C_{18}H_{14}O_3$ based on HREIMS. The IR bands indicated the presence of a hydroxyl group (3300 cm^{-1}, broad). In ^{1}H-NMR spectra, three sets of adjacent aromatic methine protons evidenced by COSY, a singlet methine proton, two methoxyl groups and a hydroxyl group, were found. The connection of five aromatic methines, H-3 (δ_H 7.70), H-4 (δ_H 7.75), H-5 (δ_H 7.88), H-6 (δ_H 8.07), and H-7 (δ_H 7.49) was proved by COSY and NOE, as depicted in Fig. 2. Because NOE was observed between H-7 and one of the methoxyl groups (δ_H 4.11), this methoxyl group was assigned as OCH_3-8. Similarly, the connection of residual adjacent methines, H-9 (δ_H 8.48) and H-10 (δ_H 8.15), another methoxyl group, OCH_3-1 (δ_H 4.17), and a hydroxyl group, OH-2 (δ_H 6.09) was confirmed by COSY and NOE (Fig. 2). HMBC correlations were observed from OH-2 to C-1 and C-3, which confirmed the structure shown in Fig. 2. All other HMBC correlations also supported the structure.

Compound **2** was obtained as a yellow amorphous, and had a molecular formula of $C_{18}H_{12}O_3$ based on HREIMS. Both UV and NMR data of **2** resembled those of **1**, but a hydroxyl group was not found from the IR spectrum. Similar to **1**, three sets of adjacent aromatic methines and a singlet methine were found. In addition, signals of a methoxyl group (δ_H 4.19) and singlet methylene appeared in a rather lower field (δ_H 6.30), which is attributable to a methylenedioxy group. The connection of five methines and a methoxyl group (H-3, δ_H 7.58; H-4, δ_H 7.78; H-5, δ_H 7.85; H-6, δ_H 8.07; H-7, δ_H 7.51; OCH_3-8, δ_H 4.19) was disclosed from 2D NMR in a manner similar to **1**. The position of two residual methines and the methylenedioxy group was determined from COSY and HMBC as follows. HMBC correlations from H-3 to two quaternary carbons (δ_C 141.0 and δ_C 145.7) confirmed that the methylenedioxy group should bind to C-1 and C-2. This was also supported from the fact that HMBC correlations were observed from the methylene proton to the quaternary carbons. Consequently, the residual methines should bind to C-9 and C-10. All other HMBC correlations in Fig. 2 supported this structure.

Compound **3** was also obtained as a yellow amorphous, and had a molecular formula of $C_{18}H_{12}O_4$ based on HREIMS. UV spectra resembled those of compound **2**. NMR data, including 2D-NMR data, also resembled those of compound **2**, except that the number of adjacent methines was not three sets but two, and instead, two singlet methines appeared. A broad singlet proton attributable to a hydroxyl group (δ_H 6.03) appeared, and the IR bands also indicated the presence of a hydroxyl group (3300 cm^{-1}, broad). These results suggest that one of the methines should be replaced with a hydroxyl group. 2D-NMR (COSY, NOE and HMBC) shown in Fig. 2 disclosed the connection of H-3 (δ_H 7.61), H-4 (δ_H 7.87), H-5 (δ_H 7.82), H-6 (δ_H 7.73), and OH-7 (δ_H 6.03). Similar to compound **2**, the methylenedioxy group should bind to C-1 and C-2. The connection of OH-7, OCH_3-8 (δ_H 4.10), H-9 (δ_H 8.12) and H-10 (δ_H 8.01) was determined from NOE shown in Fig. 2.

Two new compounds (**4** and **5**) were deep purple and both had three absorption maxima near 260, 360 (broad), and 540 nm (broad) measured with a photodiode-array detector. The molecular formula of **4** was determined as $C_{16}H_8O_3$ based on HREIMS. The IR bands indicated the presence of a hydroxyl group (3450 cm^{-1}, broad) and the carbonyl groups (1645 and 1622 cm^{-1}). NMR data (^{1}H- and ^{13}C-NMR) resembled that of pyrenes mentioned above to a considerable extent, and three sets of adjacent aromatic

methines and a singlet methine were found. In ^{13}C-NMR, signals of two quaternary carbons attributable to carbonyl carbons appeared in a lower field (δ_C 185.2 and δ_C 179.9). This suggests that compound **4** will be a pyrendione which is very rare in the plant kingdom. The connection of five methines was determined from COSY and NOE shown in Fig. 2, which were as follows: H-3 (δ_H 6.97), H-4 (δ_H 7.51), H-5 (δ_H 7.56), H-6 (δ_H 7.70), and H-7 (δ_H 6.59). Since an HMBC correlation was observed from H-6 to one of the carbonyl carbons (δ_C 185.2), this carbon was assigned as C8. The remaining adjacent methines should bind to H-9 and H-10. The HMBC correlation from H-9 (δ_H 8.61) to C-8 also supports this. HMBC correlations were observed from H-3 to two quaternary carbons (δ_C 180.6 and δ_C 149.6). Considering the conjugated structure of the pyrene ring, the remaining carbonyl oxygen should bind to C-1, and consequently the hydroxyl group to C-2. All HMBC data shown in Fig. 2 support the structure.

The molecular formula of compound **5** was determined as $C_{17}H_{10}O_3$ based on HREIMS. Both UV-visible spectra and NMR data were very similar to those of **4**. Different from **4**, a hydroxyl group was not found from IR, and instead, a signal of a methoxyl group appeared in NMR. All data suggest that a hydroxyl group in compound **4** should be changed to the methoxyl group in compound **5**. All HMBC correlations in Fig. 2 also supported this structure.

Compound **6** was obtained as a colorless oil, $[\alpha]_D$ −4.7°, and had a molecular formula of $C_{15}H_{22}O_3$ based on HREIMS. In this work, we isolated known compound (**9**) whose NMR data quite resembled that of **6**. The structure of **9** was disclosed as (−)-10-oxo-isodauc-3-en-15-al by NMR (^{1}H-, ^{13}C-, and 2D-NMR), which was already isolated from *Chromolaena laevigata* [5] and *Conza linifolia* [6]. Spectral data of **9** including $[\alpha]_D$, agreed with the reported one [7]. We measured the 2D-NMR spectra of **9**, and confirmed that the relative configuration proposed [5] was correct. The absolute configuration of **9** was not proved yet. Similar to **9**, compound **6** contains two carbonyl groups, as evidenced by ^{13}C-NMR. One of the carbonyl carbons in **9** attributable to a formyl group [C-15, δ_C 192.7; H-15, δ_H 9.35 (s)] disappeared in **6**. Instead, a quaternary carbonyl carbon appeared in a higher field (C-15, δ_C 168.6). Comparing the molecular formula of **6** and **9**, the formyl group in **9** should be changed to carboxylic acid in **6**. To confirm this, we derived **6** to its methyl ester (**6-Me**) by CH_2N_2. The molecular formulae of the reaction product agreed with the methyl derivative of **6**, and signals of the methyl group appeared [δ_C 51.2; δ_H 3.72 (3H, s)]. Thus, the structure of **6** was determined. The 2D-NMR data (HMBC, NOESY) in Fig. 3 indicates that the relative configuration of **6** is the same as that of **9**.

The other five known compounds were identified as chromolevane dione (**10**) [5], 4-hydroxy-3-methoxycinnamaldehyde (**11**) [8], syringealdehyde (**12**), (+)-15-oxo-α-cadinol (**13**) [9, 10], and vanillic acid (**14**).

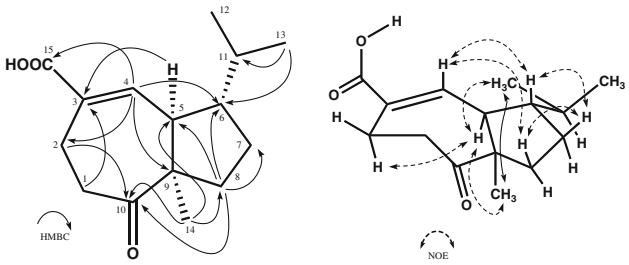

Fig. 3 HMBC and NOE correlations of **6**

The cytotoxicity of ten isolated compounds (**1**–**5**, **7**, and **9**–**12**) was tested by the WST-8 assay [11] against the growth of human promyelocytic leukemia HL-60 cells. Among these, **10** (IC_{50} 8.8 μg mL^{-1}) and **11** (IC_{50} 13.5 μg mL^{-1}) showed some cytotoxicity. Among six pyrenes and pyrendiones tested, two pyrendiones showed strong cytotoxicity, while the cytotoxicity of four pyrenes was not so strong ($IC_{50} > 20$ μg mL^{-1}). The cytotoxicity of 2-hydroxy-1,8-pyrenedione (**4**) and 2-methoxy-1,8-pyrenedione (**5**) was 0.070 and 4.4 μg mL^{-1}, respectively. The cytotoxicity of **4** was comparable to that of etoposide (0.060 μg mL^{-1}). Morphological examination by a fluorescent microscope [12] showed that the cells treated with **4** (0.040 and 0.080 μg mL^{-1}) clearly exhibited significant morphological changes, i.e., chromosomal condensation and nuclear degradation, which is indicative of apoptotic cell death.

Experimental

General experimental procedures

IR spectra were measured with a Shimadzu 8200 FT-IR spectrometer. EIMS, HREIMS, SIMS and HRSIMS were taken with a Hitachi M-4100 spectrometer. All NMR experiments were performed on a VXR-500 spectrometer (Varian) equipped with 5 mm ^{1}H and ^{13}C probes operating at 499.84 and 125.7 MHz, respectively. Chemical shifts were referenced to internal TMS. For HPLC analysis, a PU-980 Intelligent HPLC pump (Jasco) and an SPD-M 10A VP photodiode array detector (Shimadzu) were used. For preparative HPLC, a PU-1580 Intelligent pump (JASCO) and a UV-2077 Plus 4-λ Intelligent UV/VIS detector (JASCO) were combined. Kieselgel 60 (60–230 mesh, Merck) was used for column chromatography. The following reagents and instruments were obtained from the companies indicated: RPMI-1640 medium with L-glutamine (Nacalai Tesque); fetal bovine serum (FBS) (Sigma); WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-

Table 1 ^{1}H- and ^{13}C-NMR spectral data of new compounds (**1**)–(**5**) in $CDCl_3$

	1			**2**				**3**				**4**				**5**				
	δ_H		J (Hz)	δ_C	δ_H		J (Hz)	δ_C	δ_H		J (Hz)	δ_C	δ_H		J (Hz)	δ_C	δ_H		J (Hz)	δ_C
1				140.2				141.0				142.0				179.9				180.6
2				147.1				145.7				145.1				153.6				149.6
3	7.70	s		111.1	7.58	s		104.4	7.61	s		105.2	6.74	s		112.3	6.97	s		113.8
3a				129.7				127.4				127.0				132.5				132.7
4	7.75	d	8.0	124.2	7.78	d	8.5	124.7	7.87	d	8.5	126.0	7.50	d	7.0	128.9	7.51	d	7.0	130.1
5	7.88	d	8.0	127.0	7.85	d	8.5	125.5	7.82	d	8.5	124.7	7.58	d	7.0	131.7	7.56	d	7.0	131.8
5a				124.4				125.2				[a]				129.0				129.5
6	8.07	d	8.5	126.0	8.07	d	9.0	125.8	7.73	s		111.4	7.62	d	9.5	141.9	7.70	d	9.5	142.0
7	7.49	d	8.5	107.4	7.51	d	9.0	108.1				153.2	6.60	d	9.5	128.5	6.59	d	9.5	128.4
8				153.9				153.2				140.7				185.2				185.2
8a				119.1				119.6				[a]				132.9				133.4
9	8.48	d	9.5	121.9	8.38	d	9.5	121.9	8.12	d	9.0	120.7	8.61	d	8.0	129.2	8.61	d	8.0	128.9
10	8.15	d	9.5	119.1	7.94	d	9.5	118.4	8.01	d	9.0	120.3	8.69	d	8.0	129.9	8.69	d	8.0	130.1
10a				126.0				120.5				119.1				132.3				130.7
10b				120.7				121.6				121.3				125.3				125.4
10c				124.7				126.6				126.7				127.6				127.8
1,2-OCH_2O-					6.30			101.8	6.31			101.9								
1-OMe	4.17	s		62.5																
2-OMe													3.97	s		55.9				
8-OMe	4.11	s		56.2				56.2	4.10	s		62.4								
2-OH	6.09	br-s																		
7-OH									6.03	br-s										

d in ppm from TMS, J coupling constants

[a] Could not be read

(2,4-disulfophenyl)-2H-tetrazolium, mono-sodium salt) (Cell Counting Kit-8, Dojindo); and 96-well microplates (Iwaki Scitech). Absorbance was measured using a Microplate Luminometer Anthos Lucy 2 (Aloka). Morphological changes of cells were observed by a IX70 fluorescent microscope (Olympus).

Plant material

Branches of *U. lucia* were collected in the Kaloleni district of Kenya in 1995. The samples were identified and authenticated by Mr. S. G. Mathenge, Department of Botany, University of Nairobi, Nairobi, Kenya. Voucher specimens are deposited at both Kobe Pharmaceutical University and the University of Nairobi.

Extraction and isolation

The dried and finely cut branches (1013 g) of *U. lucida* were extracted successively with hot petroleum ether (3L × 3), hot $CHC1_3$ (3L × 3) and hot MeOH (3L × 3). Each combined filtrate was evaporated in vacuo until dryness to yield a syrupy mass. The yields were 14.3 g (petroleum ether), 22.2 g ($CHC1_3$), and 53.4 g (MeOH), respectively. The crude $CHCl_3$ extracts (7.3 g), which showed strong cytotoxicity towards HL-60 cells, was chromatographed on silica gel ($CHCl_3$–acetone–MeOH) and fractionated into eleven fractions (Fr-I to Fr-XI). Each fraction was analyzed by HPLC on an ODS column (Cosmosil 5C18 AR-II, 5 μm, 150 × 6 mm i.d., Nacalai Tesque) with the mobile phase composed of two mixed solvents, (A) 0.2 M $NaC1O_4$: 60% $HC1O_4$ = 1000:0.2 and (B) CH_3CN with gradient elution. Several peaks appeared in Fr-II (yield 0.07 g), Fr-IV (0.43 g), Fr-V (0.04 g), Fr-VI (0.43 g), Fr-VIII (0.61 g), and Fr-IX (0.40 g), and these fractions were purified further by the combination of prep. HPLC and prep. TLC. Other five fractions showed no remarkable peak, and we did not treat them further. Prep. HPLC was carried out on a larger ODS column (Cosmosil 5C18-ARII, 5 μm, 250 × 20 mm i.d., Nacalai Tesque) under the mobile phases mentioned above [(A) and (B)] with gradient elutions. Thus, 14 compounds **1**–**14** were purified. The yields of each compound obtained in this study were as follows: **1** (2.0 mg, Fr-IV), **2** (1.8 mg, Fr-II),

Table 2 ^{1}H- and ^{13}C-NMR spectral data of (**6**), (**6-Me**), and (**9**) in $CDCl_3$

	6				**6-Me**				**9**			
	δ_H		*J* (Hz)	δ_C	δ_H		*J* (Hz)	δ_C	δ_H		*J* (Hz)	δ_C
1	2.44	m		39.5	2.46	m		39.6	2.44	m		38.9
	2.81	m			2.83	m			2.71	m		
2	2.60	m		22.7	2.58	m		22.9	2.47	m		19.7[a]
	2.81	m			2.83	m			2.75	m		
3				132.7				133.6				143.8
4	6.97	d	5.0	151.0	6.84	d	5.0	149.0	6.63	d	5.5	158.6
5	2.41	dd	8.5, 5.0	52.8	2.39	dd	8.5, 5.0	52.6	2.52	dd	8.0, 5.5	53.2[b]
6	1.80	m		55.8	1.78	m		55.9	1.83	m		55.4[c]
7	1.40	m		27.0	1.39	m		27.0	1.43	m		26.8[d]
	1.85	m			1.84	m			1.87	m		
8	1.40	m		34.9	1.39	m		34.9	1.41	m		35.2[e]
	2.13	m			2.11	m			2.22	m		
9				59.4				59.3				59.7
10				212.2				212.2				212.2
11	1.61			32.9	1.61			33.0	1.65			32.4
12	0.93	d	7.0	19.9	0.92	d	6.5	19.9	0.95	d	6.5	21.9
13	0.92	d	7.0	21.9	0.93	d	6.5	22.0	0.94	d	6.5	19.5
14	1.31	s		25.0	1.30	s		24.9	1.33	s		25.0
15				168.6		s		166.8	9.35	s		192.7
COOMe					3.72	s		51.2				

[a] Assigned as C-7 in Ref. [6]

[b] Assigned as C-6 in Ref. [6]

[c] Assigned as C-5 in Ref. [6]

[d] Assigned as C-8 in Ref. [6]

[e] Assigned as C-2 in Ref. [6]

3 (1.1 mg, Fr-IV), **4** (11.7 mg, Fr-VI), **5** (1.4 mg, Fr-V and Fr-VI), **6** (2.9 mg, Fr-IX), 7 (1.0 mg, Fr-IV), **8** (3.7 mg, Fr-VIII), **9** (2.4 mg, Fr-IV), **10** (2.4 mg, Fr-V), **11** (1.2 mg, Fr-VIII), **12** (6.3 mg, Fr-IV), **13** (1.1 mg, Fr-VI), and **14** (0.5 mg, Fr-VI).

2-Hydroxy-1,8-dimethoxypyrene (**1**) Yellow amorphous resin. IR (KBr) cm^{-1}: 3,300 (OH), 1591, 1313, 1251, 1091, 1000, HREIMS (positive ion mode) *m/z*: 278.0959 $[M]^+$ (calcd for $C_{18}H_{14}O_3$, 278.0943), EIMS (positive ion mode) *m/z* (rel. int.): 278 $[M]^+$ (94), 263 (100), ^{1}H- and ^{13}C-NMR (Table 1).

8-Methoxy-1,2-methylenedioxypyrene (**2**) Yellow amorphous resin. IR (KBr) cm^{-1}: 1597, 1510, 1461, 1444, 1336, 1253, 1136, HREIMS (positive ion mode) *m/z*: 276.0782 $[M]^+$ (calcd for $C_{18}H_{12}O_3$, 276.0786), EIMS (positive ion mode) *m/z* (rel. int.): 276 $[M]^+$ (100), 261 (99), ^{1}H- and ^{13}C-NMR (Table 1).

7-Hydroxy-8-methoxy-1,2-methylenedioxypyrene (**3**) Yellow amorphous resin. IR (KBr) cm^{-1}: 3,300 (OH), 1602, 1508, 1460, 1444, 1336, 1251, 1193, HREIMS (positive ion mode) *m/z*: 292.0741 $[M]^+$ [calcd for $C_{18}H_{12}O_4$, 292.0736), EIMS (positive ion mode) *m/z* (rel. int.]: 292 $[M]^+$ (100), 277 (93), 263 (59), ^{1}H- and ^{13}C-NMR (Table 1).

2-Hydroxy-1,8-pyrenedione (**4**) Purple amorphous resin. IR (KBr) cm^{-1}: 3,450 (OH), 1633 (C=O), 1587, 1500, 1465, 1355, HREIMS (positive ion mode) *m/z*: 248.0487 $[M]^+$ (calcd for $C_{16}H_8O_3$, 248.0473), EIMS (positive ion mode) *m/z* (rel. int.): 245 $[M]^+$ (100), 220 (37), ^{1}H- and ^{13}C-NMR (Table 1).

2-Methoxy-1,8-pyrenedione (**5**) Purple amorphous resin. IR (KBr) cm^{-1}: 1645 (C=O), 1622 (C=O), 1595, 1348, 1282, 1249, HREIMS (positive ion mode) *m/z*: 262.0623 $[M]^+$ (calcd for $C_{17}H_{10}O_3$, 262.0630), EIMS (positive ion mode) *m/z* (rel. int.): 262 $[M]^+$ (100), ^{1}H- and ^{13}C-NMR (Table 1).

(−)-10-oxo-isodauc-3-en-15-oic acid (**6**) Colorless oil. $[\alpha]_D$ −4.7° (*c* 0.09, $CHC1_3$), IR (KBr) cm^{-1}: 1701 (C=O), 1675 (C=O), 1465, 1380, 1282, 1263, HREIMS (positive ion mode) *m/z*: 250.1580 $[M]^+$ (calcd for $C_{15}H_{22}O_3$, 250.1569), EIMS (positive ion mode) *m/z* (rel. int.): 250 $[M]^+$ (42), 207 (93), 167 (100), ^{1}H- and ^{13}C-NMR (Table 2).

Methylation of **6** *p*-toluensulfonyl-*N*-methyl-*N*-nitrosoamide (2 g) was dissolved in Et_2O (10 mL), and NaOH (0.5 g) was dissolved in 1 mL of water and added 10 mL of MeOH. These two solutions were mixed. The resulting CH_2N_2 gas was introduced into the solution of **6** (2.4 mg) in MeOH for 24 h. After removing MeOH the reaction product (**6-Me**, 2.7 mg) was obtained.

6-Me yellow oil. $[\alpha]_D$ −4.9° (*c* 0.26, $CHC1_3$), IR (KBr) cm^{-1}: 1715 (C=O), 1464, 1377, 1273, HREIMS (positive ion mode) *m/z*: 264.1726 $[M]^+$ (calcd for $C_{16}H_{24}O_3$, 264.1725), EIMS (positive ion mode) *m/z* (rel. int.): 264 $[M]^+$ (42), 221 (93), 189 (100), ^{1}H- and ^{13}C-NMR (Table 2).

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