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Novel stilbenes isolated from the root bark of *Ekebergia benguelensis*

Daniel Chávez,[a,†] Hee-Byung Chai,[a] Tangai E. Chagwedera,[b] Qi Gao,[c] Norman R. Farnsworth,[a] Geoffrey A. Cordell,[a] John M. Pezzuto[a] and A. Douglas Kinghorn[a,*]

[a]*Program for Collaborative Research in the Pharmaceutical Sciences and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612, USA*

[b]*Department of Pharmacy, University of Zimbabwe, Harare, Zimbabwe*

[c]*Bristol-Myers Squibb, Pharmaceutical Research Institute, Wallingford, CT 06492, USA*

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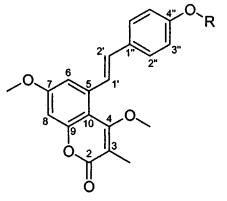
Abstract—Four novel stilbenes (**1**–**4**) were isolated from the root bark of *Ekebergia benguelensis*. The structures were established by spectroscopic methods including 2D NMR analysis. The structure of **1**, a new stilbene–coumarin hybrid representing a novel skeleton, was confirmed by single X-ray analysis. Compounds **1**–**4** were evaluated against a panel of human cancer cell lines.

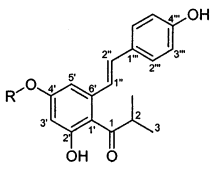
As part of an ongoing collaborative search for novel antineoplastic agents derived from plants, the root bark of *Ekebergia benguelensis* C. DC. (Meliaceae), collected in Zimbabwe, was investigated.[1] In a previous study on the root bark of *E. benguelensis* collected in Tanzania, Jonker et al. isolated a coumarin, two oxygenated squalene derivatives, and two triterpenes.[2] We report herein the bioassay-guided chromatographic separation of a $CHCl_3$-soluble extract of *E. benguelensis*, which has led to the isolation of four novel stilbenes (**1**–**4**). Of the new compounds, **1** proved to be the only isolate with threshold cytotoxic activity (Lu1, $ED_{50}=5.1$ μg/mL), while its glucoside (**2**) was isolated as a non-cytotoxic constituent. These compounds represent a novel carbon skeleton having a new stilbene–coumarin nucleus. The structure of **1** was confirmed by X-ray crystallographic analysis. Compounds **3** and **4** are resveratrol derivatives with an isobutyryl substituent.

A methanolic extract (26 g) of the air-dried root bark of *E. benguelensis*[3] (1.2 kg) was partitioned with petroleum ether and $CHCl_3$ to afford petroleum ether (3.1 g) and $CHCl_3$ (5.5 g) residues. Bioassay-guided chromatographic separation of the $CHCl_3$ extract, using the Lu1 human lung cancer cell line (ED_{50}, 16.0 μg/mL) to monitor cytotoxicity, led to the isolation of four novel stilbenes [(**1**[4] (40 mg, 0.0033% w/w), **2**[4] (20 mg, 0.0017% w/w), **3**[4] (174 mg, 0.0145% w/w), and **4**[4] (4.5 mg 0.0004% w/w)]. Additionally, two known compounds were isolated and identified by comparison of their published spectroscopic data as 5-(4-hydroxyphenethenyl)-4,7-dimethoxycoumarin[5] (28 mg, 0.0023% w/w) and betulinic acid[6] (12 mg, 0.0010% w/w). In this paper, we report the isolation and structural characterization of **1**–**4**.

Compound **1** was isolated as yellow needles. Its molecular formula was deduced as $C_{20}H_{18}O_5$ by HREIMS. Its IR spectrum showed absorptions for hydroxyl (3300 cm^{-1}), carbonyl (1698 cm^{-1}), and aromatic (1597 cm^{-1}) functionalities. The ^1H NMR spectrum (Table 1) showed signals of a *trans* double bond at δ_H 8.01/7.11 ($J=16.1$ Hz), an A_2B_2 system at δ_H 7.69/7.27 ($J=8.5$ Hz) typical for a *para*-substituted phenyl ring, and an AB system with *meta*-correlated protons at δ_H 7.16/



1 R = H
2 R = β-Glc



3 R= CH_3
4 R= H

* Corresponding author. Tel.: (312) 996-0914; fax: (312) 996-7107; e-mail: kinghorn@uic.edu

† Present address: Centro de Graduados e Investigación, Instituto Tecnológico de Tijuana, Apdo. Postal 1166, Tijuana, BC 22000, Mexico.

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Table 1. NMR data and HMBC correlations for **1** and **2**[a]

Position	**1**		**2**		HMBC[b]
	^{1}H	^{13}C	^{1}H	^{13}C	
2		163.7		163.7	
3		111.5		111.6	
4		166.1		166.0	
5		138.4		138.0	
6	7.16 d (2.3)	111.6	7.14 d (2.5)	117.7	7, 8, 10, 1′
7		163.7		161.9	
8	6.90 d (2.3)	100.3	6.94 d (2.5)	100.5	6, 7, 9, 10
9		155.7		155.7	
10		108.8		108.9	
1′	8.01 d (16.1)	125.2	8.00 d (16.1)	126.7	5, 6, 10, 1″
2′	7.11 d (16.1)	132.4	7.05 d (16.1)	131.7	5, 1″, 2″, 6″
1″		129.1		131.8	
2″, 6″	7.69 d (8.5)	128.9	7.65 d (8.7)	128.4	2′, 3″, 4″, 5″
3″, 5″	7.27 d (8.5)	116.9	6.47 d (8.7)	117.4	1″, 2″, 4″, 6″
4″		159.5		158.7	
C-3 Me	2.17 s	10.4	2.18 s	10.4	2, 3, 4
C-4 OMe	3.62 s	60.3	3.59 s	60.3	4
C-7 OMe	3.79 s	55.8	3.78 s	55.8	7
C-4″ OH	11.94 s	–			
1‴			5.74 d (7.1)	102.1	4″
2‴			4.38–4.44 m	74.9	
3‴			4.38–4.44 m	78.6	
4‴			4.38–4.44 m	71.3	
5‴			4.20 m	79.1	
6a‴			4.61 dd (12.0, 1.9)	62.4	
6b‴			4.45 dd (12.0, 5.1)		

[a] ^{1}H NMR, 300 MHz; ^{13}C NMR, 75 MHz, pyridine-d_5; data in ppm (J in Hz).
[b] HMBC correlations for compounds **1** and **2**.

6.90 ($J=2.3$ Hz). These chemical shifts were observed in the HMQC spectrum to correlate with their ^{13}C NMR signals at δ_C 125.2/132.4, 128.9/116.9, and 111.6/100.3, respectively, supporting a stilbene type of nucleus.[7–9] This compound showed NMR data very similar to values published for 5-(4-hydroxyphenethenyl)-4,7-dimethoxycoumarin, a compound previously isolated from *Monotes engleri*[5] and also isolated in the present study. The only differences observed were signals for a methine carbon at $\delta_{H/C}$ 5.81/88.9 (C-3) instead of a methyl group at $\delta_{H/C}$ 2.17/10.4, as in compound **1**.[10] Careful analysis of the HMBC spectrum allowed a stilbene–coumarin skeleton for **1** to be proposed. The cross-peaks observed for the *meta*-correlated aromatic protons (δ 7.16/6.90) and the methoxy signal at δ_H 3.79 with the carbon resonance at δ_C 163.7 enabled the methoxy group to be placed at C-7 and the hydroxy group at C-4‴. Thus, **1** was assigned as the new compound 5-[(1*E*)-2-(4-hydroxyphenyl)ethenyl]-4,7-dimethoxy-3-methyl-2*H*-1-benzopyran-2-one. In order to support the proposed structure of **1**, an X-ray analysis was performed on a single crystal obtained from pyridine/CH_2Cl_2. The crystal was found to be a 1:1 pyridine solvate of compound **1**,[11] and confirmed the novel carbon skeleton suggested (Fig. 1).

Compound **2** was obtained as a white amorphous powder. Its molecular formula was determined as $C_{26}H_{28}O_{10}$ by HRFABMS (positive mode). The NMR data indicated clearly that this compound is a glycoside of **1**. Signals for the sugar moiety at δ_H 5.74 (H-1‴), 4.61 (H-6a‴), 4.45 (H-6a‴), 4.38–4.44 (3H, H-2‴, H-3‴, H-4‴), and 4.20 (H-5‴) and its correlated signals in the HMQC spectrum at δ_C 102.1 (C-1‴), 79.1 (C-5‴), 78.6 (C-3‴), 74.9 (C-2‴), 71.3 (C-4‴) and 62.4 (C-6‴) indicated the presence of a glucose moiety.[12] Treatment of **2** with β-D-glucosidase led to the aglycone, which showed identical spectroscopic properties to compound **1**. The sugar unit was determined as glucose by co-TLC with an authentic sample. Analysis of the HMBC spectrum showed correlations for the anomeric proton (δ_H 5.74) as well as H-2″/6″ (δ_H 7.65) and H-3″/5″ (δ_H 6.47) with δ_C 158.7, indicating that glucose is attached to C-4″. Thus, compound **2** was characterized as 5-[(1*E*)-2-(4β-D-glucopyranosyloxyphenyl)ethenyl]-4,7-dimethoxy-3-methyl-2*H*-1-benzopyran-2-one.

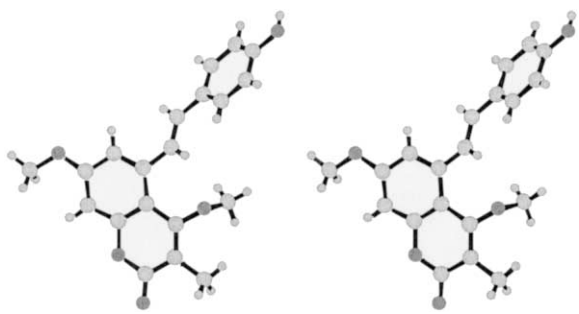

Figure 1. X-Ray crystallographic stereoviews of **1**.

Compound **3** was a major component of the $CHCl_3$ extract. Its molecular formula was established as $C_{19}H_{20}O_4$ by HREIMS. The ^1H NMR data (Table 2) indicated a stilbenoid nucleus[7–9] having a *trans* double bond at δ_H 7.13 (H-1″)/6.77 (H-2″), an A_2B_2 system for the *para*-substituted phenyl ring at δ_H 7.35/6.88 ($J=8.5$ Hz), and an AB system with *meta*-correlated protons at δ_H 6.55/6.39 ($J=2.5$ Hz). Additional coupled protons at δ_H 3.58 (H-2) and 1.15 (6H), confirming an AB_6 system ($J=6.7$ Hz), were in agreement with an isopropyl moiety. Finally, a signal for a methoxy group at δ_H 3.83 and a hydroxy signal at δ_H 12.29 that disappeared with D_2O were observed. The presence of a chelated proton at δ_H 12.29 and HMBC correlations for the protons in the isopropyl unit with a carbonyl at δ_C 212.8 led to the conclusion that an isobutyryl moiety was attached to the aromatic ring *ortho* to a hydroxyl group. Additional HMBC correlations for the methoxy group and the *meta*-correlated aromatic protons with the C-4′ signal at δ_C 164.3 enabled the placements of the methoxy group at C-4′ and the remaining hydroxyl group at C-4‴. Thus, **3** was assigned as 1-{2-hydroxy-6-[(1*E*)-2-(4-hydroxyphenyl)ethenyl]-4-methoxyphenyl}-2-methyl-1-propanone.

Compound **4** was isolated as a yellow amorphous powder. The NMR data were very similar to those of **3**, with the only difference observed being the lack of the methoxy group. The molecular formula determined as $C_{18}H_{18}O_4$ by HREIMS showed a difference of 14 amu, suggesting that **4** is the demethoxy derivative of **3**. Permethylation of **4** with diazomethane gave a compound with an identical ^1H NMR spectrum and R_f on TLC to the permethylated derivative of **3**, confirming the structure of **4**. Therefore, **4** was determined as 1-{2,4-dihydroxy-6-[(1*E*)-2-(4-hydroxyphenyl)ethenyl]-phenyl}-2-methyl-1-propanone.

Isolates **1**–**4** were evaluated against a panel of human cancer cell lines.[13] Compound **1** showed marginal cytotoxicity against a Lu1 cell line (human lung cancer, $ED_{50}=5.1$ μg/mL), whereas glucoside **2** was inactive (ED_{50} >20 μg/mL). The most susceptible cell lines with **3** and **4** were KB (human oral epidermoid carcinoma, $ED_{50}=9.9$ μg/mL) and LNCaP (hormone-dependent human prostate cancer, $ED_{50}=7.5$ μg/mL), respectively. We have previously reported the selective cytotoxicity of 5-(4-hydroxyphenethenyl)-4,7-dimethoxycoumarin and betulinic acid against the LNCaP (hor-

Table 2. NMR data for **3** and **4** and HMBC correlations for **3**[a]

Position	**3**[b]			**4**[c]	
	^1H	^{13}C	HMBC	^1H	^{13}C
1		212.8			211.8
2	3.58 sept. (6.7)	39.3	3, 4	3.50 sept. (6.8)	40.8
3, C-2 CH_3	1.15 d (6.7)	19.7	2	1.11 d (6.8)	19.3
1′		113.8			117.1
2′		164.1			161.9
3′	6.39 d (2.5)	99.9	1′, 2′, 4′, 5′	6.33 d (2.2)	102.5
4′		164.3			158.6
5′	6.55 d (2.5)	108.4	1′, 3′, 4′, 1″	6.65 d (2.2)	107.3
6′		142.4			141.9
1″	7.13 d (15.9)	125.8	1′, 5′, 6′, 1‴	7.13 d (16.0)	125.5
2″	6.77 d (15.9)	132.3	6′, 2‴, 6‴	6.89 d (16.0)	132.2
1‴		129.2			129.6
2‴, 6‴	7.35 d (8.5)	128.2	2″, 3‴, 4‴, 5‴	7.41 d (8.6)	129.0
3‴, 5‴	6.88 d (8.5)	115.9	2‴, 4‴, 6‴	6.86 d (8.6)	116.5
4‴		156.3			158.6
C-4′ OCH_3	3.83 s	55.5	4′		
C-2′ OH	12.29 s		1′, 2′, 3′		

[a] ^1H NMR, 300 MHz; ^{13}C NMR, 75 MHz; data in ppm (J in Hz).
[b] In $CDCl_3$.
[c] In acetone-d_6.

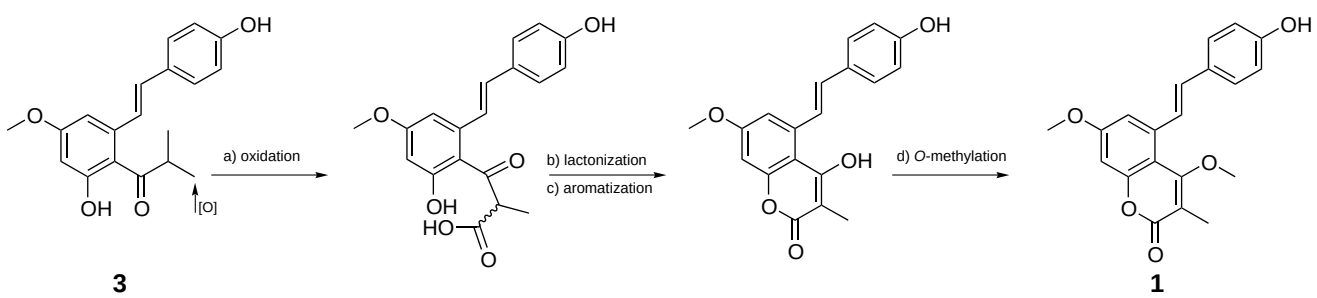

Figure 2. Postulated biogenesis of **1**.

mone-dependent human prostate cancer) and Mel-2 (human melanoma) cell lines, respectively.[5,14]

Based on the structures of the compounds isolated, it is proposed that the carbon skeleton of compound **1** is biosynthesized from **3**, as shown in Fig. 2. This hypothesis relies on the fact that compound **3** with a stilbene nucleus was isolated in large amounts and may be the precursor of **1**. If this is the case, then compound **1** should be considered a stilbene derivative, which undergoes subsequent modifications to the stilbene–coumarin type nucleus.

Acknowledgements

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3. This is a recollection of an initial sample deposited in the Field Museum of Natural History, Chicago, IL, under the reference number A1849.
4. Compound **1**: Yellow needle crystals. Mp 192–194°C; UV (MeOH) λ_{max} (log ε) 207 (4.41), 220 (4.38), 293 (4.31), 334 (4.46) nm; IR (film) ν_{max} 3373, 2940, 2869, 1684, 1599, 1540, 1508, 1337, 1233, 1156 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS (70 eV) m/z [M]$^+$ 338 (100), 310 (7), 295 (8), 231 (26), 218 (8), 190 (10), 107 (10); HREIMS m/z [M]$^+$ 338.1152 (calcd for $C_{20}H_{18}O_5$ 338.1154). Compound **2**: White amorphous powder. Mp 123–126°C; $[\alpha]_D^{20}$ +9.0 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.22), 228 sh (3.04), 290 (2.85), 327 (2.96) nm; IR (film) ν_{max} 3334, 2918, 2850, 1650, 1540, 1467, 1076 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; FABMS (glycerol) m/z [M+H]$^+$ 501 (4), 460 (3), 338 (13), 277 (20), 105 (100); HRFABMS (glycerol) m/z [M+H]$^+$ 501.1762 (calcd for $C_{26}H_{29}O_{10}$ 501.1761). Compound **3**: Reddish amorphous powder. Mp 173–175°C; UV (MeOH) λ_{max} (log ε) 207 (4.28), 230 sh (4.10), 280 (4.18), 309 sh (4.26), 321 (4.28) nm; IR (film) ν_{max} 3338, 2958, 2927, 1605, 1512, 1479, 1381, 1361, 1208, 1150 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 2; EIMS (70 eV) m/z [M]$^+$ 312 (41), 297 (7), 269 (100), 241 (9); HREIMS m/z [M]$^+$ 312.1371 (calcd for $C_{19}H_{20}O_4$ 312.1362). Compound **4**: Pale yellow amorphous powder. Mp 80–72°C; UV (MeOH) λ_{max} (log ε) 209 (4.34), 230 sh (4.14), 279 (4.20), 318 (4.25) nm; IR (film) ν_{max} 3416, 2960, 2925, 1598, 1510, 1480, 1350, 1200, 1169 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 2; EIMS (70 eV) m/z [M]$^+$ 298 (41), 255 (100), 227 (9); HREIMS m/z 298.1205 (calcd for $C_{18}H_{18}O_4$ 298.1200).
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11. X-Ray crystal structure analysis of compound **1**: Crystal data: $C_{20}H_{18}O_5 \cdot C_5H_5N$, monoclinic, space group: $P2_1/c$, $a=12.0355(4)$, $b=10.8991(7)$, $c=16.6204(8)$ Å, $\beta=103.043(4)°$, $V=2124.0(2)$ Å^3, $Z=4$, $d_x=1.304$ g cm^{-3}, Cu Kα radiation, absorption coefficient $\mu=0.71$ mm^{-1}. A colorless plate crystal of dimensions 0.15×0.20×0.40 mm was used for X-ray measurements at 295 K on an Enraf–Nonius CAD4 diffractometer with a graphite monochromator. The total number of reflections measured was 4595, of which 3497 were consider to be observed ($I4\sigma$). The absorption correction was 0.76–1.00 (T_{min}–T_{max}). The structure was solved by direct methods and refined by full-matrix least-squares.[15] Final agreement factors were $R(F^2)=0.081$; $wR(F^2)=0.180$, where $w=1/[\sigma^2(F_o^2)+(0.123P)]$, $S=1.057$.
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