Nonprenylated Rotenoids, a New Class of Potent Breast Cancer Resistance Protein Inhibitors

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Two rotenoids isolated from *Boerhaavia diffusa* (Nyctaginaceae), boeravinones G (**1**) and H (**2**), have been found to potently inhibit the drug efflux activity of breast cancer resistance protein (BCRP/ABCG2), a multidrug transporter responsible for cancer cell resistance to chemotherapy. The isolation of nine additional rotenoid derivatives (**3**−**11**), including the new boeravinones I (**10**) and J (**11**), from the extract of *B. diffusa* roots allowed us to establish structure−activity relationships toward inhibition of BCRP-mediated drug transport activity. The results show the positive roles of a methoxy group at position 6 of ring B and the absence of a substituent at position 10, and the requirement for a 6a/12a double bond between rings B and C. In contrast, both contraction of ring B, to give a coumaronochromone (**11**), and tetrasubstitution of ring D appeared to be detrimental for the inhibitory potency. The present study provides the first data on the BCRP-inhibiting activity of rotenoid derivatives, indicating boeravinones as a new class of interesting BCRP inhibitors.

Introduction

Breast cancer resistance protein (BCRP/ABCG2) is a recently discovered multidrug transporter of cancer cells,[1−3] belonging to the ABC ("ATP-Binding Cassette") superfamily of membrane proteins.[4] It is overexpressed in many types of tumors, as acute myeloid leukaemia, even before any chemotherapeutic treatment.[5] BCRP is able to efflux a wide spectrum of antitumor drugs, including mitoxantrone, camptothecin derivatives, methotrexate, and anthracyclines,[6] similarly to the first discovered and well characterized transporter P-glycoprotein.[7] However, BCRP is rather insensitive to most P-glycoprotein inhibitors, such as verapamil, valspodar (PSC833), and zosuquidar trihydrochloride (LY335979),[8,9] and BCRP inhibitors thus far proposed still remain unsatisfactory, as pointed out in a recent review.[10] The mycotoxin fumitremorgin C was found to be efficient[11] but highly neurotoxic, while its chemical derivatives appeared to be much less effective.[12] Elacridar (GF120918), commonly used as a reference compound, is in fact at least 40-fold more efficient on P-glycoprotein.[13] On the other hand, very recently, we have found that some hydrophobic flavones, such as tectochrysin and 6-prenylchrysin, are potent and specific BCRP inhibitors, characterized by low intrinsic cytotoxicity,[14] which makes them potential candidates for future clinical trials.

As a part of an ongoing research program aimed at the isolation, structural characterization, and pharmacological evaluation of multidrug resistance modulators from terrestrial plants,[15,16] we have recently started the phytochemical analysis of *Boerhaavia diffusa* (Nyctaginaceae). This plant is one of the most popular herbal remedies in the Indian Ayurvedic medicine, that prescribes its roots as a diuretic and analgesic and to improve the functions of kidney and liver.[17] We have already reported on the in vitro spasmolytic action of *B. diffusa* roots extract[18] and on the isolation of two new rotenoid derivatives from this source, named boeravinones G (**1**) and H (**2**) (Figure 1).[19] A careful reexamination of the organic extract obtained from *B. diffusa* roots has now resulted in the isolation of eight additional compounds of this class (**3**−**11**), two of which, named boeravinone I (**10**) and boeravinone J (**11**), being new compounds (Figure 1). In particular, boeravinone J (**11**), belongs to the rare class of coumaronochromones, whose skeleton differs from that of rotenoids in having a five-membered ring B.

All the secondary metabolites obtained from this investigation (**1**−**11**) have been assayed for the inhibition of the multidrug transporter BCRP. Boeravinones G (**1**) and H (**2**) resulted in highly potent inhibitors of BCRP-mediated mitoxantrone efflux in transfected cells; furthermore, analysis of the activities obtained for the entire library of compounds **1**−**11** allowed the determination of structure−activity relationships, which complement those previously established for flavonoids.[14] The present study provides the first data on the BCRP-inhibiting activity of rotenoid derivatives.

Isolation and Structural Elucidation of Rotenoids. Roots of *Boerhaavia diffusa* were exhaustively extracted with methanol and the obtained extracts were subjected to Kupchan partitioning to obtain four different fractions (*n*-hexane, CCl_4, $CHCl_3$, *n*-BuOH). Preliminary spectroscopic analysis showed that both CCl_4 and $CHCl_3$ fractions contained rotenoids and, therefore, these fractions were combined and further separated. Sequential application of silica gel column chromatography and HPLC as detailed in the Experimental Section led to the isolation of boeravinone G (**1**, 8.8 mg),[19] boeravinone H (**2**, 6.4 mg),[19] 6-*O*-demethylboeravinone H (**3**, 2.4 mg),[20] boeravinone A (**4**, 12.3 mg),[21] boeravinone B (**5**, 6.2 mg),[21] boeravinone E (**6**, 8.4 mg),[22] coccineone B (**7**, 3.9 mg),[23] boeravinone C (**8**, 5.1 mg),[24]

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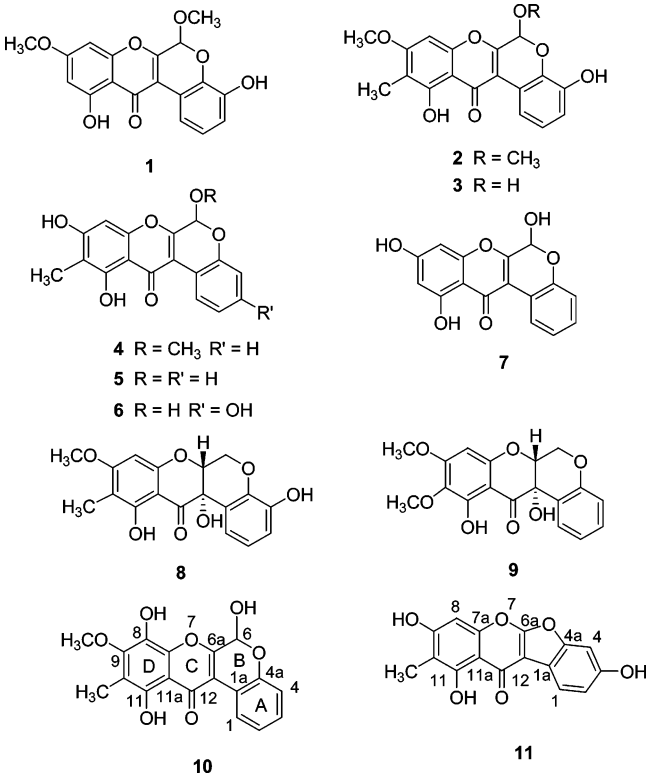


Figure 1. Chemical structure of compounds isolated from *Boerhaavia diffusa*.

Table 1. ^{13}C and ^{1}H NMR Data of Boeravinones I (**10**) and J (**11**) Recorded in CD_3OD

	10		11	
pos	δ_C (mult.)	δ_H (mult., J in Hz)	δ_C (mult.)	δ_H (mult., J in Hz)
1	126.8 (CH)	8.75 (d, 7.5)	121.2 (CH)	7.81 (d, 7.0)
1a	116.4 (C)		113.5 (C)	
2	122.4 (CH)	7.02 (t, 7.5)	113.9 (CH)	6.93 (d, 7.0)
3	128.0 (CH)	7.19 (t, 7.5)	158.9 (C)	
4	115.8 (CH)	6.97 (d, 7.5)	99.3 (CH)	7.01 (bs)
4a	147.3 (C)		149.6 (C)	
6	95.4 (CH)	6.12 (s)	-	
6a	153.9 (C)		161.5 (C)	
7a	143.5 (C)		155.1 (C)	
8	123.9 (C)		94.1 (CH)	6.48 (s)
9	153.1 (C)		164.5 (C)	
9-OMe	56.2 (CH_3)	3.80 (s)	-	
10	106.8 (C)		108.3 (C)	
10-Me	10.1 (CH_3)	2.01 (s)	9.3 (CH_3)	2.08 (s)
11	163.8 (C)		160.9 (C)	
11a	107.7 (C)		106.4 (C)	
12	179.0 (C)		178.2 (C)	
12a	110.0 (C)		99.0 (C)	

coccineone E (**9**, 7.9 mg),[25] and the new boeravinones I (**10**, 1.3 mg) and J (**11**, 1.1 mg) in the pure state (Figure 1). The structures of the known compounds **1**−**9** were deduced by comparing their spectroscopic data with those reported in the literature,[19−25] while the structures of the new compounds **10** and **11** have been deduced as follows.

The molecular formula of boeravinone I (**10**, Figure 1), a pale-yellow amorphous solid, was inferred by HR-FABMS as $C_{18}H_{14}O_7$. The ^{1}H NMR spectrum of **10** (CD_3OD, Table 1) showed only seven signals: five methine resonances (one singlet and four multiplets) in the region between δ_H 6.10 and 8.80, a methyl resonance at δ_H 2.01 and a methoxy resonance at δ_H 3.80. The four multiplets of the ^{1}H NMR spectrum were assigned unambiguously to four aromatic methines arranged in sequence: the 2D COSY spectrum confirmed that the triplets

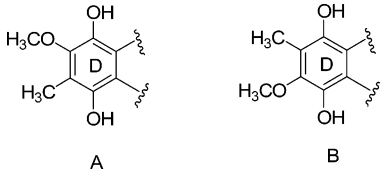


Figure 2. Alternative candidate structures for ring D of boeravinone I (**10**).

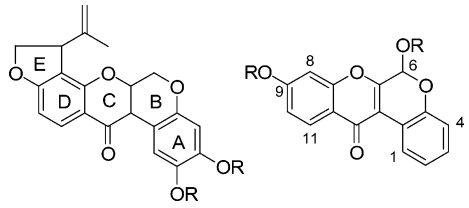
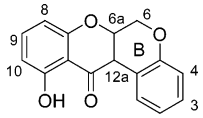


Figure 3. Left: General structure of rotenoids from Leguminosae and Fabaceae. Right: General structure of rotenoids from Nyctaginaceae.

at δ_H 7.02 and 7.19 (both J = 7.5 Hz) were mutually coupled and *ortho*-coupled with the doublets at δ_H 8.75 (J = 7.5 Hz) and 6.97 (J = 7.5 Hz), respectively. The ^{13}C NMR spectrum (CD_3OD, Table 1) of **10** showed the resonances of the *O*-methyl carbon at δ_C 56.2, of the *C*-methyl carbon at δ_C 10.1, and of 16 carbons in the low-field region. The 2D NMR HMQC and HMBC spectra were used to analyze monodimensional data and to guide the assembly of the rotenoid skeleton of boeravinone I (**10**). In particular, the HMQC spectrum of **10** allowed the association of seven carbons with the protons that they directly link (the remaining 11 carbons are unprotonated), indicating the hemiacetal nature of the singlet at δ_H 6.12 (the corresponding carbon resonates at δ_C 95.4). The $^{2,3}J_{H-C}$ correlations, detected through the 2D HMBC spectrum of **10**, between the hemiacetal proton at δ_H 6.12 (H-6) and C-4a (δ_C 147.3), C-6a (δ_C 153.9), and C-12a (δ_C 110.0), and those of the signal at δ_H 8.75 (H-1) with C-12a, C-4a and C-1a (δ_C 116.4), corroborated by comparison with data reported for other boeravinones,[18−25] allowed us to elucidate the structures of rings A-C. Therefore, in accordance with the molecular formula, ring D must accommodate two hydroxyl groups, a methyl group, and a methoxy group on the four available positions. The following information proved to be useful to correctly accommodate these groups on ring D: i) the bathochromic shift of band I, obtained in the UV spectrum of **10** upon addition of $AlCl_3$ and $AlCl_3$/HCl was diagnostic for the presence of hydroxy substitution at C-11;[26] ii) the absence of bathochromic shift of band I in the UV spectrum after addition of $NaOAc/H_3BO_3$ excluded the presence of an *ortho*-dihydroxy system;[26] iii) the strong NOE correlation between the methoxy and the methyl protons is only compatible with the placement of these two groups on adjacent carbons of ring D. These data allowed the unambiguous location of the two hydroxyl groups at C-8 and C-11, respectively, leaving only two candidate structures: 8,11-dihydroxy-9-methoxy-10-methyl (A) or the isomeric 8,11-dihydroxy-10-methoxy-9-methyl (B) (Figure 2). However, an accurate analysis of the ^{13}C NMR data obtained for ring D carbons of **10** allowed us to discard the latter hypothesis. In particular, taking into account the marked shielding effect of −OH or −OR substituents on *ortho* and *para* positions of a phenyl ring, the relatively high-field resonance of the CH_3 linking carbon (δ_C 106.8) can be rationalized only by assuming the presence of oxygenated substituents at the three *o*,*o*,*p* positions (as in structure A). In the alternative structure B (Figure 2), the ^{13}C NMR resonance of the CH_3 linking carbon would have been at least 10−15 ppm higher. Further support to the structure A comes from the

Table 2. Efficiencies of Rotenoids To Inhibit BCRP-Mediated Mitoxantrone Efflux Leading to the Drug Accumulation[a]



	substituents								
compound	3	4	6	8	9	10	6a/12a	concentration used (μM)	% maximal mitoxantrone accumulation[b]
boeravinone G (**1**)	H	OH	OMe	H	OMe	H	DB[c]	5	92 ± 6.5
boeravinone H (**2**)	H	OH	OMe	H	OMe	Me	DB	5	68 ± 6.1
boeravinone E (**6**)	OH	H	OH	H	OH	Me	DB	10	56 ± 5.0
boeravinone B (**5**)	H	H	OH	H	OH	Me	DB	10	55 ± 5.8
boeravinone C (**8**)	H	OH	H	H	OMe	Me	12a-OH	10	31 ± 4.2
coccineone B (**7**)	H	H	OH	H	OH	H	DB	10	29 ± 5.3
boeravinone A (**4**)	H	H	OMe	H	OH	Me	DB	10	27 ± 5.1
coccineone E (**9**)	H	H	H	H	OMe	OMe	12a-OH	10	15 ± 5.2
compound **3**	H	OH	OH	H	OMe	Me	DB	20	15 ± 3.1
boeravinone I (**10**)	H	H	OH	OH	OMe	Me	DB	20	12 ± 5.4
boeravinone J (**11**)	OH	H	contracted ring B	H	OH	Me	DB	20	15 ± 3.1

[a] Each *Boerhaavia diffusa* compound was added, at the indicated concentration, to BCRP-transfected and control cells incubated with mitoxantrone, and residual fluorescence was monitored by flow cytometry as detailed in the Experimental Section. [b] The inhibitor-dependent mitoxantrone accumulation was expressed as the mean of three independent experiments ± SD. [c] DB indicates double bond.

deshielded resonance of C-11 (δ_C 163.8), which excludes the presence of the methoxy group at the *ortho* position (as in structure B). Therefore, the structure of boeravinone I (**10**) can be completely defined as reported in Figure 1. Compound **10** exhibited $[\alpha]_D = 0$ and, thus, as in the previously isolated boeravinones,[18−25] it is racemic at the single chiral center, C-6. Boeravinone J (**11**, Figure 1), a yellow amorphous solid, gave a pseudomolecular ion peak at *m*/*z* 297 in the negative-ion ESI mass spectrum, while HR-FAB mass spectrum indicated the molecular formula $C_{16}H_{10}O_6$. ^{1}H NMR spectrum of **11** (Table 1) was extremely poor of signals, showing only four resonances in the aromatic region (two doublets and two singlets from δ_H 6.40 to 7.90) and a methyl singlet at δ_H 2.10. Inspection of ^{13}C NMR spectrum of boeravinone J (Table 1)[27] indicated the presence of a methyl carbon resonance (δ_C 9.3) and of 15 resonances in the low-field region of the spectrum. Four of these carbons were protonated and were associated to the relevant proton signals through inspection of the HMQC spectrum. The above data, compared to those of other *Boerhaavia* rotenoids, showed that **11** lacks of one of the carbons of the rotenoid skeleton, that was easily identified as the acetal carbon at C-6 of ring B.[27] Accordingly, the pattern of ^{13}C NMR resonances of **11** was in good agreement with those reported for coumaronochromones,[28] rare rotenoid analogues showing a contracted ring B due to the lack of C-6. The series of^{2,3} J_H−C correlations detected in the 2D HMBC spectrum of **11** confirmed the presence of a coumaronochromone skeleton and contributed to the correct placement of the substituents. In particular, correlations of the signal at δ_H 7.81 (H-1) with C-12a (δ_C 99.0), C-1a (δ_C 113.5), and with the oxygenated carbons C-4a (δ_C 99.3) and C-3 (δ_C 158.9), and correlations of H-4 (δ_H 7.01, broad singlet) with C-1a and C-4a allowed us to individuate the A-C rings skeleton. In addition, taking into account that H-1 (doublet, $J = 7.0$ Hz) is coupled with an hydrogen atom at the *ortho* position, the OH group must be located at C-3. Consequently, two hydroxyls and a methyl group should be linked at ring D. As for boeravinone I (**10**), also in the case of boeravinone J (**11**), measurement of UV spectra after addition of $AlCl_3$, $AlCl_3$/HCl, and $NaOAc/H_3BO_3$ indicated the presence of a hydroxy substitution at C-11 and excluded the presence of an *ortho*-dihydroxy system.[25] Analysis of a phase-sensitive HMBC experiment[29] provided valuable information to place the two remaining groups on ring D: the obtained pattern of correlations was in agreement with placement of the hydroxyl group at C-9 and of the methyl group at C-10. Indeed, relatively large correlations (≥7 Hz, indicative of ^3J_H−C, in planar systems) were detected for H-8/C-11a (δ_C 106.4) and H-8/C-10 (δ_C 108.3), while correlations of H-8 with the three oxygenated carbons at δ_C 155.1, δ_C 164.5, and δ_C 160.9 were relatively small (≤2 Hz, indicative of ^2J_H−C or ^4J_H−C, in planar systems). Further support to assign the correct resonances to ring D carbon atoms and to determine the structure of boeravinone J (**11**), came from comparison with data reported in the literature for boeravinones A (**4**), B (**5**), and E (**6**) (Figure 1), that share with **11** the structure of rings C and D. Indeed, ^{13}C NMR resonances reported for ring D carbons of **4**−**6**[21,22] are practically identical to parallel value detected for boeravinone J (**11**).

BCRP inhibition. Rotenoids are isoflavonoid derivatives typically found as secondary metabolites of Leguminosae and Fabaceae plants, and, compared to tricyclic isoflavonoids, their peculiar structural features are: i) an additional oxygenated ring (ring B) and, almost invariably, ii) a 2,3-disubstitution on ring A and iii) a prenyl group attached at position 8, commonly cyclized to form a five- or a six-membered ring (ring E) (Figure 3). These compounds are frequently toxic through inhibition of mitochondrial electron transport chain at complex I;[30] however, a study aimed at establishing the "toxophore" of the rotenoid molecules revealed that both the prenyl-derived ring and the dimethoxy substitution on ring A are essential requirements.[31] Accordingly, the restricted group of rotenoids isolated from plants belonging to Nyctaginaceae, as those described in the present study, which lack the isoprenoid residue on ring D and show a monosubstituted or an unsubstituted ring A (Figure 3), were shown to be noncytotoxic ($IC_{50} > 20$ μM on several cell lines, data not shown).

Rotenoids isolated from *B. diffusa* (**1**−**11**) were assayed for inhibition of the BCRP activity by flow cytometry on HEK-293 human cells transfected by wild-type (R482) BCRP and exposed to the antitumor drug mitoxantrone.[14] Table 2 shows the efficiency of **1**−**11** to inhibit BCRP-mediated mitoxantrone efflux, leading to the drug accumulation. Boeravinones G (**1**) and H (**2**) induced a high accumulation of mitoxantrone at the

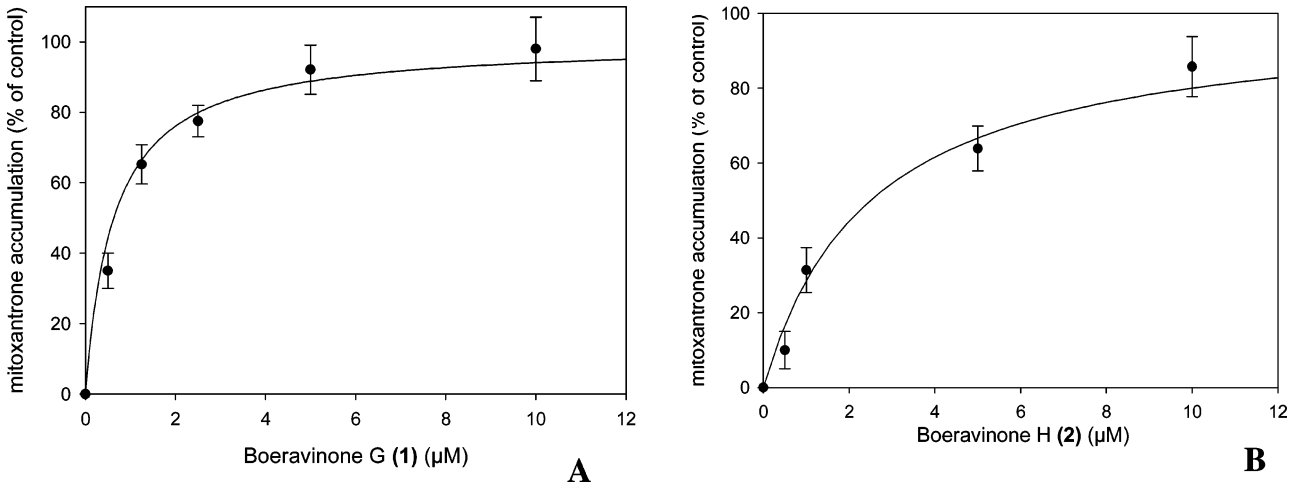


Figure 4. Concentration-dependent inhibition by boeravinones G (**1**) and H (**2**) of BCRP-mediated mitoxantrone efflux. BCRP-transfected HEK-293 cells were incubated as in Table 2 in the presence of increasing concentrations of either boeravinone G (**1**, panel A) or H (**2**, panel B). The data obtained from three separate experiments are indicated as the mean ± SD. The IC_{50} was determined graphically as the concentration producing half-maximal mitoxantrone accumulation.

lowest concentration tested, through a strong inhibition of the BCRP drug-efflux activity. For boeravinone G (**1**), the nearly complete effect approached that produced by elacridar (GF120918), a commonly used BCRP inhibitor, taken here as a reference. A concentration dependence study (Figure 4) allowed the determination of IC_{50} values of 0.7 ± 0.07 μM and 2.5 ± 0.47 μM for **1** and **2**, respectively. Most of the remaining boeravinones (**4**−**8**) were less active and required higher concentrations to produce appreciable inhibition, while compound **3** and the new **10** and **11** displayed only a marginal inhibitory activity (Table 2).

The close relationship among the structures of the tested compounds allowed us to draw structure−activity relationships, identifying the following substituent positive effects: (i) the absence of a methyl substituent at position 10 in **1** as compared to **2**; (ii) the preference for a methoxy group over a hydroxyl group at position 6 (**2** *versus* **3**). It is to be noted that when position 4 was unsubstitued and a hydroxyl replaced the methoxy group at both positions 9 and 6, the two above effects i and ii appeared to be reversed (**5** *versus* **7** and **5** *versus* **4,** respectively); (iii) an intact ring B, since its contraction to a five-membered ring giving a coumaronochromone derivative was clearly detrimental (**6** *versus* **11**). In contrast, a hydroxyl group at position 3 appeared to be neutral (**6** *versus* **5**). Although compounds **8** and **9** had no direct counterpart, it seemed that loss of the double bond $\Delta^{6a(12a)}$ between rings B and C, and consequently, of the molecular planarity was deleterious for activity. Interestingly, in our previous work,[14] we found a similar effect, at the same position, for flavanones/flavones. Analogously, the tetrasubstitution of ring D, present in compound **10**, appears to be responsible for a marked decrease in activity.

Interestingly, the complete "western" moiety (including rings C and D) of **1**, the best rotenoid identified here, is identical to that of tectochrysin (**12**), the best unprenylated flavonoid recently tested under the same experimental conditions (Figure 5).[14] However, boeravinone G (**1**) is more potent than tectochrysin, with an IC_{50} of 0.7 μM (this work) as compared to 3.0 μM,[14] which highlights the role of the "eastern" moiety of the molecule, stabilized here by the additional heterocyclic and substituted ring B (Figure 5). Since introduction of a noncyclized prenyl group at C-6 in chrysin derivatives (corresponding to C-10 in boeravinones) was found to increase the inhibitory efficiency (IC_{50} = 0.3 μM for 6-prenylchrysin),[14] we may hope

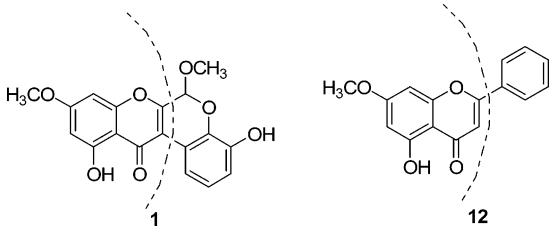


Figure 5. Comparison between the structures of boeravinone G (**1**, IC_{50} = 0.7 μM) and tectochrysin (**12**, IC_{50} = 3.0 μM).

to further improve the boeravinone G potency by studying related noncyclized prenylated derivatives.

Conclusion

The present investigation allowed the identification of non-prenylated rotenoids from *Boerhaavia diffusa* (boeravinones) as a new class of promising BCRP (ABCG2) inhibitors. In particular, boeravinones G (**1**) and H (**2**) were identified as being particularly potent. Hopefully, taking advantage of parallel information obtained for flavone derivatives, chemical modifications on their simple planar structures could pave the way for the development of even more potent MDR modulators. Finally, the lack of cytotoxicity of these compounds makes them good candidates for in vivo tests as adjuvants in antitumor chemotherapy.

Experimental Section

General Methods. Optical rotations (MeOH) were measured on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (λ = 589 nm) and a 10-cm microcell. UV spectra (MeOH) were recorded on a Beckman spectrometer. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on a Varian INOVA spectrometer; chemical shifts were referenced to the residual solvent signal (CD_3OD: δ_D = 3.32, δ_C = 49.0). The multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. One-bond heteronuclear ^{1}H−^{13}C connectivities were determined with the HMQC experiment. Two- and three-bond ^{1}H−^{13}C connectivities were determined by HMBC experiments optimized for a $^{2,3}J$ of 7 Hz. Heteronuclear coupling constant were qualitatively evaluated by using PS-HMBC.[29] Low-resolution electrospray (negative ions) spectra were performed LCQ Finnigan MAT mass spectrometer; low- and high-resolution FABMS (glycerol matrix) were performed on a VG Prospec Fisons mass spectrometer. Medium-pressure liquid chromatography (MPLC) was performed using a Büchi 861

apparatus using a silica gel (230−400 mesh) column. HPLC separations in isocratic mode were achieved on a Beckman apparatus equipped with a refractive index detector and with Phenomenex LUNA (250 × 4.6 mm) silica (5 μm) columns.

Plant Material: Extraction and Isolation. *Boerhaavia diffusa* roots were collected in Jammu, India, in June 2003, and the plant was identified by Dr. R. Longo (Carlo Sessa S.p.A., Milan). A voucher specimen (no. 22.02) is deposited in the Herbarium of Carlo Sessa S.p.A. (Milan, Italy). Roots of *B. diffusa* (22 kg, dry weight) were extracted (5 × 3 L) with methanol at room temperature for 1 h. Evaporation of the pooled extracts left a brown material (15.4 g) that was subjected to a modified Kupchan partition scheme, as previously described,[19] obtaining CCl_4 (2.4 g), $CHCl_3$ (3.5 g), and *n*-BuOH (8.8 g) fractions. The CCl_4 and $CHCl_3$ fractions were combined and then chromatographed by MPLC on a silica gel (230−400 mesh) column (750 × 25 mm), using a linear gradient system (400 mL for each solvent) from *n*-hexane to EtOAc to MeOH−EtOAc (1:1). All obtained fractions were subjected to preliminary spectroscopic investigation and those apparently containing rotenoids were further separated by HPLC. The first fraction (*n*-hexane−EtOAc, 8:2) was purified by HPLC on an analytical column (250 × 4.6 mm) using *n*-hexane−EtOAc 85:15 as eluent, flow rate 1.0 mL/min, affording coccineone E (**9**, 7.9 mg). The fraction eluted with hexane/EtOAc 7:3 was purified by HPLC on an analytical column using hexane/EtOAc 75:25 as eluent, flow rate 1.0 mL/min, obtaining boeravinone G (**1**, 8.8 mg), boeravinone H (**2**, 6.4 mg), 6-*O*-demethylboeravinone H (**3**, **2**.4 mg), boeravinone A (**4**, 12.3), and boeravinone B (**5**, 6.2 mg). The fraction eluted with hexane/EtOAc 6:4 was purified by HPLC on an analytical column using hexane/EtOAc 65:35 as eluent, flow rate 1.0 mL/min, obtaining boeravinone E (**6**, 8.4 mg). The fraction eluted with *n*-hexane−EtOAc, 1:1, was purified by HPLC on an analytical column (250 × 4.6 mm) using *n*-hexane−EtOAc 55:45 as eluent, flow rate 1.0 mL/min, affording the new compounds boeravinone I (**10**, 1.3 mg) and boeravinone J (**11**, 1.1 mg). The fraction eluted with *n*-hexane−EtOAc 4:6 was purified by HPLC on an analytical column using *n*-hexane-EtOAc 4:6 as eluent, flow rate 0.8 mL/min, yielding boeravinone C (**8**, 5.1 mg). Finally, the fraction eluted with EtOAc−*n*-hexane 7:3 was further purified by HPLC (EtOAc−*n*-hexane, 6:4, flow rate 0.8 mL/min,) yielding coccineone B (**7**, 3.9 mg).

Boeravinone I (10): Pale yellow amorphous solid; $[\alpha]^{25}_D$ 0 (*c* 0.01, MeOH); UV (CH_3OH): λ_{max} (log ϵ) 330 nm (3.85), 274 nm (4.50); UV (CH_3OH + $AlCl_3$): λ_{max} 363, 280 nm; UV (CH_3OH + $AlCl_3$/HCl): λ_{max} 371, 283 nm; UV (CH_3OH + $NaOAc/H_3BO_3$): λ_{max} 331, 274 nm; ^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD): see Table 1. ESIMS (negative-ion) *m*/*z* 341 $[M - H]^-$; HRFABMS (negative ions): found *m*/*z* 341.0666 (calcd for $C_{18}H_{13}O_7$, *m*/*z* 341.0661).

Boeravinone J (11): Yellow amorphous solid; UV (CH_3OH): λ_{max} (log ϵ) 340 nm (3.40), 276 nm (4.40); UV (CH_3OH + $AlCl_3$): λ_{max} 374, 283 nm; UV (CH_3OH + $AlCl_3$/HCl): λ_{max} 382, 289 nm; UV (CH_3OH + $NaOAc/H_3BO_3$): λ_{max} 340, 276 nm; ^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD): see Table 1. ESIMS (negative-ion) *m*/*z* 297 $[M - H]^-$; HRFABMS (negative ions) found *m*/*z* 297.0405 (calcd for $C_{16}H_9O_6$, *m*/*z* 297.0399).

Biological Activity. The inhibitory activity of boeravinones was assayed by flow cytometry. HEK-293 human cells transfected by wild-type (R482) BCRP were exposed to 5 μM mitoxantrone for 30 min at 37 °C, in the absence or presence of inhibitor added as a dimethyl sulfoxide solution (0.5% final concentration), washed in PBS, and further incubated for 60 min with the same inhibitor concentration in mitoxantrone-free medium. Residual intracellular drug fluorescence was monitored with a FACscan flow cytometer (Becton Dickinson, Moutain View, CA). The maximal fluorescence (100%) was the difference between mean fluorescence of control cells (transfected with empty vector) and BCRP-transfected ones incubated with substrate but without inhibitor. The same maximal fluorescence with the latter cells was obtained in the presence of 5 μM ELACRIDAR, taken as a reference inhibitor of BCRP-mediated drug efflux. The addition of boeravinones did not significantly modify the fluorescence of control cells, and cells without drug were taken as an autofluorescence control.

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Supporting Information Available: ^{1}H NMR spectra for compounds **1**−**11** and purity criteria for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Doyle, L. A.; Yang, W.; Abruzzo, L. V; Krogmann, T.; Gao, Y.; Rishi, A. K.; Ross, D. D. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 15665−15670.

(2) Allikmets, R.; Schriml, L. M.; Hutchinson, A.; Romano-Spica, V.; Dean, M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res.* **1998**, *58*, 5337−5339.

(3) Miyake, K.; Mickley, L.; Litman, T.; Zhan, Z.; Robey, R.; Cristensen, B.; Brangi, M.; Greenberger, L.; Dean, M.; Fojo, T.; Bates, S. E. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: Demonstration of homology to ABC transport genes. *Cancer Res.* **1999**, *59*, 8−13.

(4) Klein, I.; Sarkadi, B.; Varadi, A. An inventory of the human ABC proteins. *Biochim. Biophys. Acta* **1999**, *1461*, 237−262.

(5) Diestra, J. E.; Scheffer, G. L.; Catala, I.; Maliepaard, M.; Schellens, J. H.; Scheper, R. J.; Germa-Lluch, J. R.; Izquierdo, M. A. Frequent expression of the multi-drug resistance-associated protein BCRP/MXR/ABCP/ ABCG2 in human tumours detected by the BXP-21 monoclonal antibody in paraffin-embedded material. *J. Pathol.* **2002**, *198*, 213−219.

(6) Sarkadi, B.; Ozvegy-Laczka, C.; Nemet, K.; Varadi, A. ABCG-2, a transporter for all seasons. *FEBS Lett.* **2004**, *567*, 116−120.

(7) Endicott, J. A.; Ling, V. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu. Rev. Biochem.* **1989**, *58*, 137−171.

(8) Sikic, B. I.; Fisher, G. A.; Lum, B. L.; Halsey, J.; Beketic-Oreskovic, L.; Chen, G. Modulation and prevention of multidrug resistance by inhibitors of P-glycoprotein. *Cancer Chemother Pharmacol.* **1997**, *40*, 13−19.

(9) Shepard, R. L.; Cao, J.; Starling, J. J.; Dantzig, A. H. Modulation of P-glycoprotein but not MRP1- or BCRP-mediated drug resistance by LY335979. *Int. J. Cancer* **2003**, *103*, 121−125.

(10) Ahmed-Belkacem, A.; Pozza, A.; Macalou, S.; Perez-Victoria, J. M.; Boumendjel, A.; Di Pietro, A. Inhibitors of cancer cell multidrug resistance mediated by breast cancer resistance protein (BCRP/ABCG2). *Anti-Cancer Drugs* **2006**, *17*, 239−243.

(11) Rabindran, S. K.; Ross, D. D.; Doyle, L. A.; Yang, W.; Greenberger, L. M. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res.* **2000**, *60*, 47−50.

(12) Allen, J. D.; van Loevezijn, A.; Lakhai, J. M.; van der Valk, M.; van Tellingen, O.; Reid, G.; Schellens, J. H.; Koomen, G. J.; Schinkel, A. H. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Mol. Cancer Ther.* **2002**, *1*, 417−425.

(13) de Bruin, M.; Miyake, K.; Litman, T.; Robey, R.; Bates, S. E. Reversal of resistance by GF120918 in cell lines expressing the ABC half-transporter, MXR. *Cancer Lett.* **1999**, *46*, 117−126.

(14) Ahmed-Belkacem, A.; Pozza, A.; Munoz-Martinez, F.; Bates, S. E.; Castanys, S.; Gamarro, F.; Di Pietro, A.; Perez-Victoria, J. M. Flavonoid Structure-Activity Studies Identify 6-Prenylchrysin and Tectochrysin as Potent and Specific Inhibitors of Breast Cancer Resistance Protein ABCG2. *Cancer Res.* **2005**, *65*, 4852−4860.

(15) Corea, G.; Fattorusso, E.; Lanzotti, V.; Taglialatela-Scafati, O.; Appendino, G.; Ballero, M.; Simon, P.; Dumontet, C.; Di Pietro, A. Jatrophane Diterpenes as P-Glycoprotein Inhibitors. First Insights of Structure-Activity Relationships and Discovery of a New, Powerful Lead. *J. Med. Chem.* **2003**, *46*, 3395−3402.
(16) Corea, G.; Fattorusso, E.; Lanzotti, V.; Motti, R.; Simon, P.-N.; Dumontet, C.; Di Pietro, A. Jatrophane Diterpenes as Modulators of Multidrug Resistance. Advances of Structure-Activity Relationships and Discovery of the Potent Lead Pepluanin A. *J. Med. Chem.* **2004**, *47*, 988−992.
(17) Lad, V. *The Complete Book of Ayurvedic Home Remedies*; Three Rivers Press: New York, 1999.
(18) Borrelli, F.; Ascione, V.; Capasso, R.; Izzo, A. A.; Fattorusso, E.; Taglialatela-Scafati, O. Spasmolytic Effects of Nonprenylated Rotenoid Constituents of *Boerhaavia diffusa* Roots. *J. Nat. Prod.* **2006**, *69*, 903−906.
(19) Borrelli, F.; Milic, N.; Ascione, V.; Capasso, R.; Izzo, A. A.; Capasso, F.; Petrucci, F.; Valente, R.; Fattorusso, E.; Taglialatela-Scafati, O. Isolation of new rotenoids from *Boerhaavia diffusa* and evaluation of their effect on intestinal motility. *Planta Med.* **2005**, *71*, 928−932.
(20) Yang, S.-W.; Ubillas, R.; McAlpine, J.; Stafford, A.; Ecker, D. M.; Talbot, M. K.; Rogers, B. Three new phenolic compounds from a manipulated plant cell culture, *Mirabilis jalapa*. *J. Nat. Prod.* **2001**, *64*, 313−317.
(21) Kadota, S.; Lami, N.; Tezuka, Y.; Kikuchi, T. Constituents of the Roots of *Boerhaavia diffusa* L. Examination of sterols and structure of new rotenoids, boeravinones A and B. *Chem. Pharm. Bull.* **1989**, *37*, 3214−3220.
(22) Lami, N.; Kadota, S.; Kikuchi, T. Constituents of the roots of *Boerhaavia diffusa* L. Isolation and structure determination of boeravinones D, E, and F. *Chem. Pharm. Bull.* **1991**, *39*, 1863−5.
(23) Ferrari, F.; Messana, I.; Sant'Ana, E. G. Two new isoflavonoids from *Boerhaavia coccinea*. *J. Nat. Prod.* **1991**, *54*, 597−98.
(24) Kadota, S.; Lami, N.; Tezuka, Y.; Kikuchi, T. Structure and NMR spectra of boeravinone C, a new rotenoid analog from *Boerhaavia diffusa* Linn. *Chem. Pharm. Bull.* **1988**, *36*, 2289−92.
(25) Santos, A. S.; Caetano, L. C.; Sant'Ana, E. G. A 12a-hydroxyrotenoid from roots of *Boerhaavia coccinea*. *Phytochemistry* **1998**, *49*, 255−258.
(26) (a) Mabry, T. J.; Markham, K. R.; Thomas, M. B. *The Systematic Identification of Flavonoids*; Springer: New York, 1970. (b) Jurd, L.; Geissman, T. A. Absorption spectra of metal complexes of flavanoid compounds. *J. Org. Chem.* **1956**, *21*, 1395−1401.
(27) We decided to number boeravinone J (**11**) by using the same numbering system adopted for the rotenoid skeleton. Although, in this case, position 6 must be skipped, there is the advantage of an easy comparison of ^{1}H and ^{13}C NMR resonances of boeravinone J (**11**) with those of the corresponding nuclei of boeravinone I (**10**) and of the other boeravinones.[17−24]
(28) Hanawa, F.; Tahara, S.; Mizutani, J. Retro-Diels-Alder fragmentation of coumaronochromones and complete assignments of ^{13}C NMR data for lupinalbin A and ayamenin B. *Heterocycles* **1991**, *8*, 1563−1570.
(29) Matsumori, N.; Murata, M.; Tachibana, K. Conformational analysis of natural products using long-range carbon-proton coupling constants: three dimensional structure of okadaic acid in solution. *Tetrahedron* **1995**, *51*, 12229−38.
(30) Gutman, M.; Singer, T. P.; Casida, J. E. Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. XVII. Reaction sites of piericidin A and rotenone. *J. Biol. Chem.* **1970**, *245*, 1992−1997.
(31) Crombie, L.; Josephs, J. L.; Cayley, J.; Larkin, J.; Weston, J. B. The rotenoid core structure: Modifications to define the requirements of the toxophore. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 13−16.

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