JOURNAL OF NATURAL PRODUCTS
Article
Cite This: J. Nat. Prod. 2019, 82, 694−701
pubs.acs.org/jnp

Cytotoxic Activity of Riccardin and Perrottetin Derivatives from the Liverwort *Lunularia cruciata*

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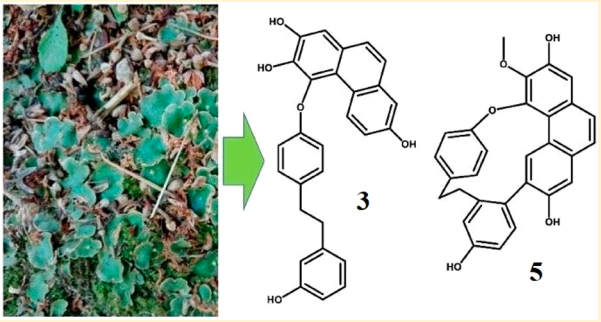
Supporting Information

ABSTRACT: Seven new bisbibenzyls (**1**−**7**) were isolated from the methanol extract of the liverwort *Lunularia cruciata* along with one previously known bibenzyl and five known bisbibenzyls. The structures of compounds **1**−**7** were elucidated on the basis of the spectroscopic data. These newly isolated bisbibenzyls may be divided into two groups, the acyclic bisbibenzyls, perrottetins (**1**−**3**), and the cyclic analogues, riccardins (**4**−**7**). Besides standard perrottetin and riccardin structures (**1** and **4**, respectively), they contain phenanthrene (**3** and **5**), dihydrophenanthrene (**2**), and quinone moieties (**6** and **7**), rarely found in natural products. The new compounds **3** and **5**, as well as the known riccardin G, exhibited cytotoxic activity against the A549 lung cancer cell line with IC_{50} values of 5.0, 5.0, and 2.5 μM, respectively.



The liverworts (Marchantiophyta) are rich sources of bisbibenzyls and their derivatives. So far, more than 60 macrocyclic and acyclic bisbibenzyls have been isolated from various liverwort species.[1,2] Generally, they are active as antimicrobial, antioxidant, antitrypanosomal, antiviral, and cytotoxic agents. In addition, they possess farnesoid X-receptor (FXR) receptor activity, liver-X-activity, muscle relaxing and calcium inhibitory, nitric oxide production inhibitory, tubulin inhibitory, and cardiotonic activities.[2]

Lunularia cruciata (L.) Dumort. ex Lindb. (Lunulariaceae) is a thalloid liverwort characterized by crescent-shaped cups that contain gemmae (small vegetative propagules), used for asexual reproduction. The thallus surface of this liverwort is dotted with tiny pores that are framed by pentagon-shaped boundaries. It is a dioecious plant with thalli that are 5−10 mm wide and up to 2.5 cm long and is irregularly dichotomously branched. *L. cruciata* is the sole species in the genus *Lunularia*.[3] Chemical analysis of the Japanese *L. cruciata* has revealed the presence of lunularin, lunularic acid, bisbibenzyls, perrottetin F, and its derivatives.[2,4] Jockovic et al. noted the presence of two flavonoids, luteolin-7-*O*-glucoside and quercetin, in the methanol extract of European *L. cruciata*.[5] The crude extracts of *L. cruciata* showed antimicrobial activity against selected pathogenic bacteria.[6,7] Lunularin isolated from a vascular plant, *Melampyrum pratense*, inhibited E-selectin and interleukin-8 expression after stimulation with lipopolysaccharide, suggesting its potential anti-inflammatory activity.[8] Perrottetin F showed anti-influenza PA endonuclease activity.[9] Riccardin C exhibited cytotoxicity for a prostate cancer cell line and anti-MRSA activity.[10,11] Riccardins C and F were identified as an LXRa agonist/LXRb antagonist and an LXRa antagonist, respectively.[12] Riccardin D, a demethylated derivative of riccardin G, has shown inhibitory effects on several different types of human cancer cells.[13−17]

Reported herein are the isolation and structure elucidation of seven new chemical constituents of *L. cruciata*. Three of them belong to perrottetin-type (**1**−**3**) and four are riccardin-type bisbibenzyls (**4**−**7**). Additionally, six previously reported compounds, one bibenzyl and five bisbibenzyls, were also isolated. Cytotoxic activity of all of the isolated compounds against two human cell lines, A549 (lung carcinoma) and MRC5 (healthy lung fibroblasts), was also investigated.

RESULTS AND DISCUSSION

The methanol extract of *L. cruciata* was fractionated by a combination of silica gel CC, preparative silica gel TLC, and semipreparative reversed-phase HPLC to afford seven new bisbibenzyls (**1**−**7**), one known bibenzyl (lunularin), and five known bisbibenzyls (perrottetins E and F and riccardins C, F, and G, respectively). Lunularin was found to be a constituent

Received: May 15, 2018
Published: March 8, 2019

DOI: 10.1021/acs.jnatprod.8b00390
J. Nat. Prod. 2019, 82, 694−701

Table 1. ^{1}H and ^{13}C NMR Data of Compounds 1−3 Recorded in CD_3OD (500 MHz for ^{1}H and 125 MHz for ^{13}C)[a]

	1		2		3	
position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	157.4		155.0		157.1	
2	118.5	6.73 d (9.0)	115.6	6.76 d (9.0)	116.2	6.74 d (9.0)
3	130.6	7.07 d (9.0)	127.9	7.01 d (9.0)	130.5	7.02 d (9.0)
4	137.4		134.6		136.6	
5	130.6	7.07 d (9.0)	127.9	7.01 d (9.0)	130.5	7.02 d (9.0)
6	118.5	6.73 d (9.0)	115.6	6.76 d (9.0)	116.2	6.74 d (9.0)
7	38.3	2.82 m	35.5	2.75 m	38.3	2.75 m
8	39.4	2.80 m	36.5	2.71 m	39.4	2.75 m
9	144.8		141.9		144.9	
10	116.5	6.62 t (2.0)	113.7	6.51 brs	116.4	6.60 m
11	158.5		155.6		158.4	
12	113.9	6.58 m[b]	111.0	6.49 dd (8.0; 2.0)	113.9	6.55 m
13	130.4	7.04 t (7.5)	127.5	6.95 t (8.0)	130.3	7.00 t (8.0)
14	121.0	6.64 dt (7.5)	118.1	6.55 m[c]	121.0	6.57 m
1′	137.6		146.0		141.2	
2′	145.7		141.0		139.9	
3′	111.6	6.13 d (2.0)	118.1	6.59 s	120.4	
4′	133.7		126.9		127.5	
5′	110.3	6.56 d (2.0)	129.9		110.8	7.18 s
6′	148.1		109.7	7.14 s	147.1	
7′	86.4	4.12 t (7.5)	26.7	2.55 m	127.9	7.49 d (9.0)
8′	45.5	2.70 dd (13.0; 7.5)	28.0	2.62 m	125.9	7.38 d (9.0)
		2.98 dd (13.0; 7.5)				
9′	141.1		137.2		135.4	
10′	117.6	6.47 brt	113.0	6.55 brs[c]	112.4	6.87 d (2.5)
11′	158.2		155.1		155.9	
12′	114.2	6.56 m[b]	112.1	6.60 dd (8.0; 2.0)	117.3	6.87 dd (9.5; 2.5)
13′	130.2	6.99 t (7.5)	123.0	7.40 d (8.0)	129.5	8.95 d (9.5)
14′	122.1	6.50 dt (7.5)	124.6		123.9	
OMe-7′	56.8	3.15 s				

[a]J values are given in parentheses. [b,c]Signals overlapped.

of celery[18] and *Morus* species among vascular plants,[19] but is mainly distributed in the Hepaticae.[20] It was detected in the liverwort *Conocephalum conicum* as a decarboxylated product of lunularic acid.[21] Perrottetins E and F are bisbibenzyls isolated initially from the liverwort *Radula perrottetii*,[22] with riccardin C obtained from *Reboulia hemisphaerica*,[23] riccardin F from *Blasia pusilla*,[24] and riccardin G from *Marchantia chenopoda*.[25] Subsequently, these compounds have been found in a several more liverwort species,[1,2] but all these six known compounds are now being reported for the first time in *L. cruciata*. The assignment of NMR data of **1**−**7** (Tables 1 and 2) was based on 2D NMR methods, as well as comparison to those of the known co-occurrinig bisbibenzyls.[21−25]

Compound **1**, isolated as a brown amorphous substance, was found to possess the molecular formula $C_{29}H_{27}O_6$, on the basis of the $[M - H]^-$ molecular ion peak at m/z 471.1827 of its negative HRESIMS and from the ^{13}C NMR spectrum, as shown in Table 1. The UV bands at 272 and 282 nm and the IR absorption bands at 3422, 1609, and 1593 cm^{-1} indicated the presence of a phenolic moiety in **1**. The ^{1}H NMR chemical shifts and coupling constants of the protons of the A-, B-, and D-rings of **1** were very similar to those of perrottetin F.[22] The major differences between the spectra of these compounds were due to an additional three-proton singlet of a methoxy group (δ_H, 3.15; δ_C, 56.8) and one-proton triplet signal (δ_H, 4.12, H-7′) bonded to an oxygenated carbon. The 2D NMR data revealed their position at C-7′, as observed by the COSY correlations H-7′/H-8′a, H-8′b, the HMBC cross-peaks H-3′, H-5′, H-8′/C-7′, and the NOE correlations OMe-7′/H-7′, H-5′, H-3′ (Figures S5, S8, and S11, Supporting Information). Thus, the structure of the new bisbibenzyl, **1**, was established as 7′-methoxyperrottetin F. The absolute configuration at C-7′ remains to be clarified. There is a possibility that **1** could be an artifact of 7′-hydroxyperrottetin F, which was transformed to the methoxy derivative during the isolation procedure, as seen for the bisbibenzyl marchantin G.[1]

Compound **2** was isolated as an amorphous violet substance. Its negative-mode HRESIMS exhibited a $[M - H]^-$ molecular ion peak at m/z 423.1610 and, together with the ^{13}C NMR spectrum, showed a molecular formula of $C_{28}H_{24}O_4$. The UV bands at 278, 294 (sh), and 320 nm were characteristic for an extended conjugation. The ^{1}H NMR spectrum of **2** was similar to that of perrottetin F[22] and compound **1**, with the protons of the A- and B-rings exhibiting the same pattern. The molecular formula, when compared to that of **1**, indicated one additional unsaturation or a ring, i.e, direct coupling between the C- and D-rings. When comparing the ^{1}H NMR spectrum of **2** and **1**, a one-proton singlet at δ_H 6.59 appeared instead of the characteristic doublet (J = 2.0 Hz) for H-3′ (Table 1). A strong HMBC correlation of this proton to C-7′ (Figure S22, Supporting Information) confirmed its assignment as H-3′. At the same time, a singlet at δ_H 7.14 could be assigned to H-6′. The HMBC cross-peaks between H-12′, H-10′, H-6′/C-14′ and H-13′, H-3′/C-5′ and the strong NOE correlations of H-

DOI: 10.1021/acs.jnatprod.8b00390
J. Nat. Prod. 2019, 82, 694−701

Table 2. ^{1}H and ^{13}C NMR Data of Compounds 4−7 (500 MHz for ^{1}H and 125 MHz for ^{13}C)[a]

	4[b]		5[b]		6[b]		7[b]	
position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	154.7		155.7		153.7		153.5	
2	123.3	6.66−6.75 m[g]	113.3	5.72 dd (8.5; 2.0)	122.4[c]	6.56 dd (8.0; 2.0)[c]	122.6[c]	6.77 d (8.0)
3	130.6	6.94 m[c,h]	130.9	6.35 dd (8.5; 2.0)	129.1[d]	7.11 dd (8.0; 2.0)[d]	129.3[d]	6.94 d (8.0)[c]
4	141.5		135.0		140.0		139.6	
5	130.6	6.66−6.75 m[c,g]	128.5	6.25 dd (8.5; 2.0)	130.7[d]	6.68 dd (8.0; 2.0)[d]	129.8[d]	7.05 d (8.0)[c]
6	123.3	6.66−6.75 m[g]	117.6	6.80 dd (8.5; 2.0)	122.8[c]	6.98 dd (8.0; 2.0)[c]	122.8[c]	6.84 d (8.0)
7	38.7	2.53 m	38.1	2. 64 td (13.0; 3.5)	37.8	2.80 m[g]	34.4	2.66 m
		2.64 m		2. 86 dt (13.0; 3.5)		3.12 m[h]		2.84 m
8	36.6	2.73 m	37.8	2.13 td (13.0; 3.5)	34.1	2.75 m[g]	30.1	2.76 m
		3.01 m		2.94 dt (13.0; 3.5)		3.06 m[h]		2.92 m
9	144.8		142.3		142.6		145.6	
10	117.7	6.86 d (2.5)	116.4	6.88 d (2.5)	121.9	6.98 d (8.0)	188.3	
11	158.0		156.5		130.5	7.28 t (8.0)	137.2[f]	6.88 d (9.0)[g]
12	114.3	6.67 dd (7.5; 2.5)	127.8	6.70 dd (8.5; 2.5)	112.6	6.72 d (8.0)	136.8[f]	6.88 d (9.0)[g]
13	133.8	6.94 d (7.5)[h]	132.5	6.67 t (8.5)	155.0		186.8	
14	131.3		129.8		121.1		142.4	
1′	136.1		141.7		147.4		147.2	
2′	154.0		144.2		148.8		148.9	
3′	110.6	4.83 d (2.0)	116.3		117.1	5.78 d (2.0)	116.7	5.38 d (2.0)
4′	138.4		132.5		133.0		133.9	
5′	110.8	6.35 (2.0)	109.5	7.20 s	122.1	6.81 dd (8.5; 2.0)	121.8	6.77 dd (8.0; 2.0)
6′	151.7		148.5		112.9	6.96 d (8.5)	112.1	6.89 d (8.0)[g]
7′	39.4	2.85 m	127.0	7.50 d (9.0)	31.6	2.50 ddd (14.0; 12.0; 2.0)	37.1	2.65 m
		2.93 m				2.90 ddd (14.0; 7.0; 2.0)		2.79 m
8′	39.0	2.53 m	126.1	7.41 d (9.0)	33.1	2.04 ddd (14.0; 12.0; 2.0)	37.9	2.49 m
		2.64 m				3.06 m[h]		2.81 m
9′	142.4		130.4		146.0		144.4	
10′	121.9	6.11 dd (7.5; 1.5)	121.0		188.0		121.4	6.53 dd (8.5; 2.0)
11′	133.6	6.74 (7.5)	130.0	7.21 s	135.4	6.48 s	129.8	6.77 d (8.0)
12′	127.4		113.8		122.1		118.0	
13′	154.8		150.8		185.7		152.7	
14′	117.1	6.28 d (1.5)	110.2	6.98 s	135.7	5.96 s	118.1	6.14 d (2.0)
OMe-1′	61.5	3.87 s	62.1	4.02 s	55.9	3.93 s	56.3	3.94 s

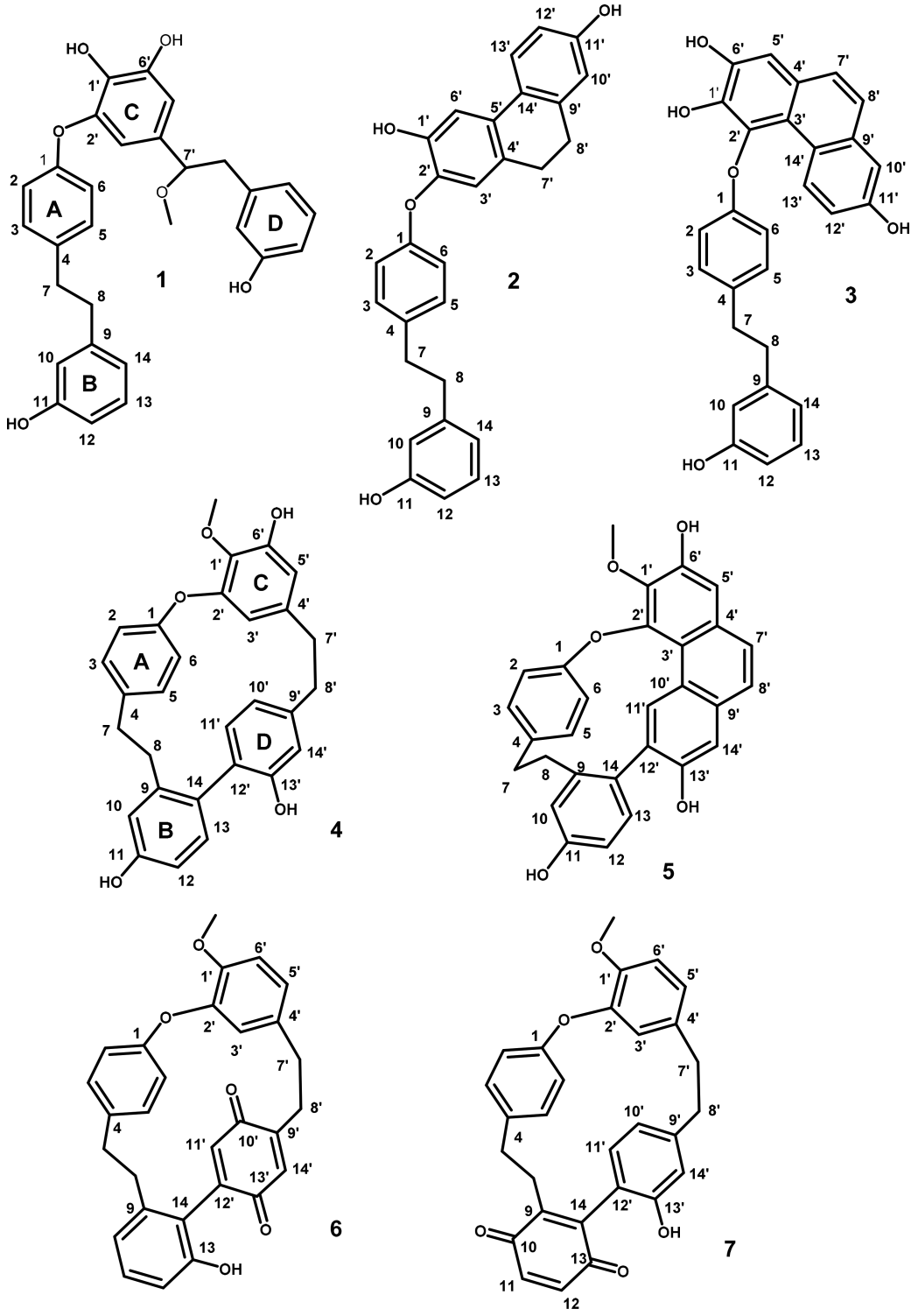
[a]J values are given in parentheses. [b]Compounds **4** and **6** were recorded in CD_3OD, compound **5** in the mixture $CD_3OD/CDCl_3$, and compound **7** in $CDCl_3$. [c,d,e,f]Signals are interchangable. [g,h]Signals overlapped.

6′/H-13′ and H-3′/H-6, H-7′ supported a dihydrophenanthrene structure of the C- and D-rings in a perrottetin skeleton, indicating **2** is an intramolecular C-5′−C-14′ coupling product of perrottetin E (Figures 1 and S22, S19, and S20, Supporting Information). Similar dihydrophenanthrene bisbibenzyl derivatives, 2′-(11-hydroxy-1-bibenzyloxy)-1′-methoxy-6′,10′,11′-trihydroxy-7′,8′-dihydrophenanthrene and 2′-(10,11-dihydroxy-1-bibenzyloxy)-1′-methoxy-6′,10′,11′-trihydroxy-7′,8′-dihydrophenanthrene, were found in the methanol extract of the Ecuadorian liverwort *Frullania convoluta,* along with lunularin and lunularic acid.[26]

Compound **3** was isolated as an amorphous violet substance. Its negative-mode HRESIMS exhibited a $[M - H]^-$ molecular ion peak at m/z 437.1405, which together with the ^{13}C NMR spectrum was consistent with the molecular formula $C_{28}H_{22}O_5$. The UV bands at 256, 272 (sh), 284 (sh), 308 (sh), 348, and 362 nm were characteristic for the occurrence of more extended conjugation than found in **2**. This was in agreement with the molecular formula, which revealed one additional degree of unsaturation in comparison to that in **2**. The ^{1}H NMR spectrum of **3** exhibited the presence of aliphatic signals of only four protons belonging to a benzylic bridge between the A- and B-rings, similar to that of **1** and **2** (Figure S26, Supporting Information). The protons of the D-ring (H-10′, H-12′, and H-13′), for which the connectivity was established according to the COSY correlations (Figure S29, Supporting Information), exhibited a coupling pattern typical for a 1,3,4-trisubstituted benzene ring, and their signals were shifted significantly toward lower fields, especially the one found at δ_H 8.94 (d, J = 9.0 Hz, H-13′). This was due to the combination of the strong resonance and anisotropic effect of the C-ring (Figure S25, Supporting Information). The mutual COSY correlation of the two doublets found at δ_H 7.40 (H-7′) and 7.50 (H-8′) and their NOE correlations H-7′/H-5′ and H-8′/H-10′ referred to the phenanthrene moiety including the C- and D-rings. The strong NOE correlation H-13′/H-6 of the A-ring confirmed the proximity of the A- and D-rings as a consequence of the phenanthrene substructure (Figures S29, S30, Supporting Information). The H-5′ singlet (δ_H 7.18) exhibited a NOE correlation with H-7′ (δ_H 7.49), which, together with the HMBC cross-peaks H-5′/C-1′, C-3′, C-7′ confirmed the position of the C-ring protons (Figures S30, S33, Supporting Information). The HSQC spectrum and remaining HMBC correlations (Figures S31−S34, Supporting Information) indicated a new phenanthrene-perrottetin-type

DOI: 10.1021/acs.jnatprod.8b00390
J. Nat. Prod. 2019, 82, 694−701

Chart 1



structure with an intramolecular C-3′−C-14′ coupled perrottetin F unit dehydrogenated at C-7′ and C-8′ (Figure 1).

Compound **4** was obtained as a brown amorphous solid. Its molecular formula, $C_{29}H_{26}O_5$, was deduced by the $[M - H]^-$ molecular ion peak at m/z 453.1716 in its negative-mode HRESIMS in combination with the ^{13}C NMR spectrum. The presence of an aromatic system in **4** was also supported by the UV absorption bands at 252 (sh), 284 and 292 nm (sh) and IR band at 3214 cm^{-1}. The chemical shifts and coupling constants of the ^1H NMR spectrum of **4** resembled those of riccardin F except for the C-ring, indicating that compound **4** possesses an additional hydroxy group in the C-ring of a riccardin F skeleton (Figure S45, Supporting Information). This supposition was confirmed by the following spectroscopic data. The ^1H NMR spectrum of **4** exhibited a one-proton doublet (δ_H 4.83, J = 2.0 Hz), characteristic for H-3′, which appeared at very high field due to the anisotropic effect of the A-ring, as previously observed in cyclic bisbibenzyls of the riccardin and

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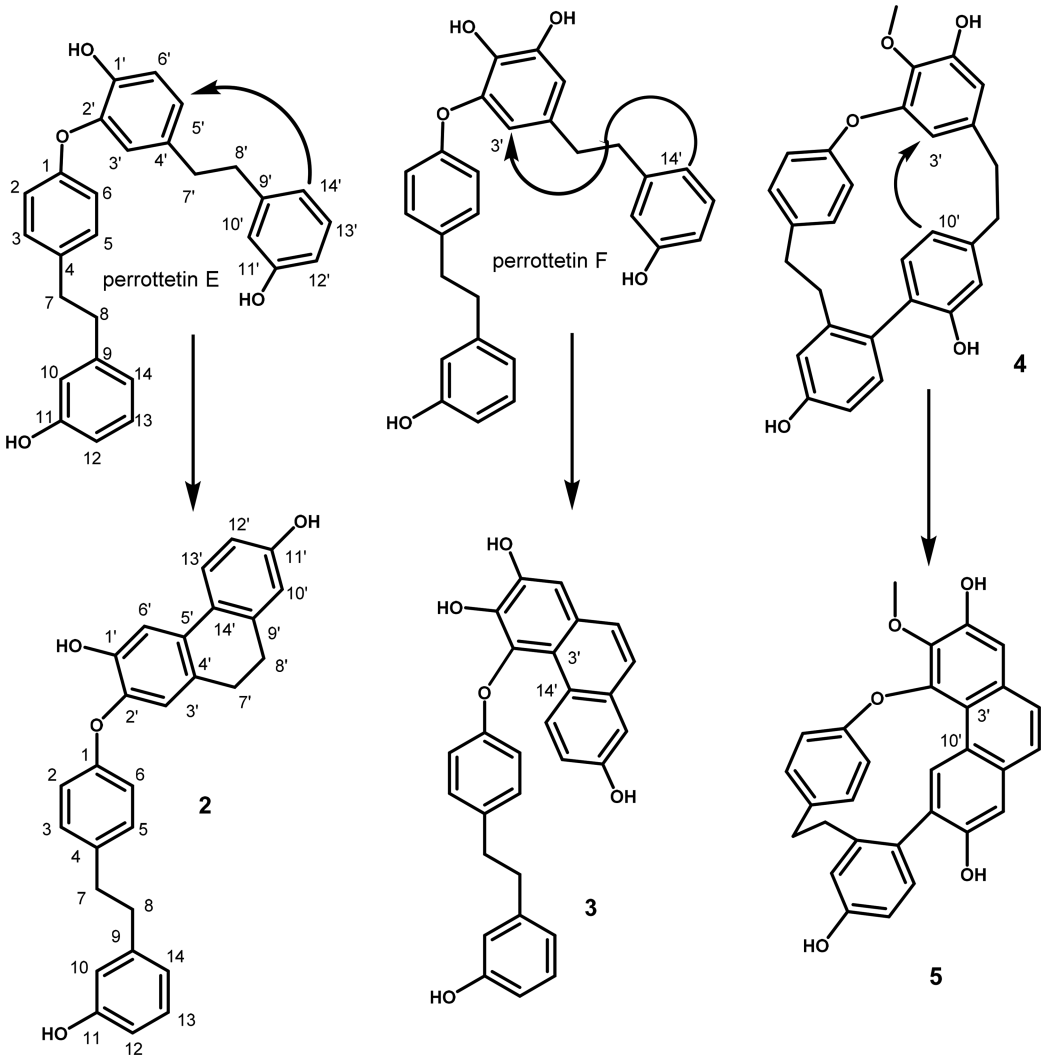

Figure 1. Proposed intramolecular oxidative coupling connections involved in the formation of compounds **2**, **3**, and **5**.

marchantin series (Figure S35, Supporting Information). The COSY correlation H-3′/H-5′ and the HMBC correlations H-3′/C-5′, C-1′; H-5′, OMe-1′/C-1′ revealed the presence of a methoxy group at C-1′ (the same as that in riccardin F), while the presence of two doublets (δ_H 4.83 and 6.35, H-3′ and H-5′, respectively) with couplings of 2.0 Hz indicated an additional OH substituent at C-6′ of ring C. This was confirmed by the HMBC correlation at H-5′/C-6′ (Figures S39, S43, Supporting Information). Thus, the structure of **4** was established as 6′-hydroxyriccardin F, a new derivative of riccardin F.

Compound **5**, a violet solid, was found to have the molecular formula $C_{29}H_{22}O_5$, according to a negative-mode HRESIMS with a $[M - H]^-$ molecular ion peak at m/z 449.1402 as well as the ^{13}C NMR spectrum. Similar to **3**, the UV bands at 225, 270, 318 (sh), 352, and 370 nm and the IR absorptions at 3429 and 1615 cm^{-1} suggested the presence of a phenolic group and extended conjugation. The almost identical chemical shifts of two doublets of H-7′ and H-8′ and a singlet of H-5′ with those of the same protons in **3**, together with similarity of the UV data, supported the occurrence of a phenanthrene moiety in **5** incorporating the C- and D-rings. This was confirmed by the HMBC correlations between H-8′/C-10′, C-14′, C-9′ as well as H-7′/C-3′, C-4′, C-5′, C-9′ and the NOESY cross-peaks of H-14′/H-8′ and of H-7′/H-5′ (Figures S48 , S51, and S54, Supporting Information). Additionally, the HMBC correlations between H-11′, H-10/C-14 and H-13′, H-14/C-12 confirmed a riccardin skeleton for **5**, the same as in **4**. The strong HMBC correlations OMe-1′, H-5′/C-1′ confirmed the position of the methoxy group at C-1′, and since the proton signal of H-5′ was a singlet, it was deduced that an additional OH group is located at C-6′. This assumption was also confirmed by the HMBC correlation of H-5′/C-6′ (Figures S54 and S55, Supporting Information). Analyses of the COSY, NOESY, and HMBC spectra showed that compound **5** has the same substitution pattern of the A- and B-rings as seen in the riccardin series. Contrary to **4**, the proton signals of the A-ring appeared as sharp doublets of doublets, suggesting that compound **5** has a strained structure due to the riccardin-phenanthrene moiety. The strong anisotropic effect of a phenanthrene moiety explained the very low chemical shifts observed for H-8b and H-7b (Figure S47, Supporting Information). In comparison to **4**, it could be proposed that **5** occurs from intramolecular phenolic oxidative coupling (cyclization) of **4** (6′-hydroxyriccardin F) via the connection of C-10′ to C-3′, followed by dehydrogenation at C-7′ and C-8′. For **3**, a twisted coupling of C-14′ to C-3′ in perrottetin F occurs (Figure 1). The difference between H-2 and H-6, as well as H-3 and H-5, in the ^1H NMR spectrum was determined

DOI: 10.1021/acs.jnatprod.8b00390
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by the strong H-11′/H-6 and the weak H-11′/H-5 NOE correlation. Thus, compound **5** represents a new secondary metabolite very similar to the phenanthrene bisbibenzyl angustatin A, which was isolated from the Chinese liverwort *Asterella angusta*,[27] and to the highly strained cavicularin, isolated from the Japanese liverwort *Cavicularia densa*, resulting from the intramolecular cyclization in riccardin C.[1]

Compound **6**, an amorphous orange compound, gave the molecular formula $C_{29}H_{24}O_5$, as established by the negative-mode HRESIMS ($[M - H]^-$ ion peak at m/z 451.1556) and the ^{13}C NMR spectrum. The NMR spectroscopic data of **6** resembled those of riccardin G, except for the presence of a *para*-quinone moiety, as confirmed by the strong UV absorption bands at 234, 285, 320, 352, and 370 nm, the IR absorptions at 1611, 1581, and 1655 cm^{-1}, and the presence of two signals for carbonyl carbon atoms (δ_C 185.7 and 188.0) in the ^{13}C NMR spectrum. The connectivity between the C-ring and the *para*-quinone D-ring in **6** was established by the NOE correlations H-3′, H-5′/C-7′ and the HMBC cross-peaks H-7′, H-11′/C-9′ and H-8′/C-10′, C-14′. The location of the *para*-quinone group was determined by the very low chemical shifts of H-8′b and H-7′b that appeared in the ^1H NMR spectrum, because of the diamagnetic anisotropic effect of the C-10′ carbonyl group. A methoxy group apparent at δ_H 3.93 was ascribed to the C-1′ position according to the HMBC cross-peaks OMe-1′, H-3′, H-5′/C-1′ and the NOE correlation H-6′/OMe-1′ (Figures S62, S66, and S67, Supporting Information). The triplet found at δ_H 7.28 (J = 8.0 Hz) of H-11 was connected by COSY correlations to the doublet of H-10 and the doublet of doublets of H-12. According to the HMBC correlations, especially H-11′/C-14, a riccardin-type connection between C-14 and C-12′ of the B- and D-ring was determined. The protons of the A-ring, instead of the characteristic AA′BB′ pattern typical for a *para*-disubstitued benzene ring, exhibited two pairs of doublets of doublets. This was in accordance with the restricted mobility of the A-ring, leading to the chemical nonequivalence of protons H-2 and H-6, as well as of H-3 and H-5. Moreover, the occurrence of positive correlations in phase-sensitive NOESY cross-peaks H-2/H-6 and H-3/H-5 (Figure S63, Supporting Information) indicated a slow equilibrium between two equivalent conformations of the A-ring (Figure 2). HMBC and NOE correlations distinguished the pairs of protons H-2/H-6 to H-3/H-5 (HMBC: H-2, H-6/C-4; H-3, H-5/C-1; NOE: H-2, H-6/H-3′), but an exact determination of the positions of their signals was not determined. Based on the spectroscopic data described above, the structure of **6** was proposed as that of a new secondary metabolite, riccardin G 10′,13′-paraquinone.

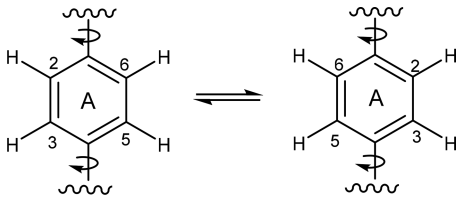

Figure 2. A-ring in **6** and **7** slowly exchanging between two equivalent conformations.

Compound **7**, an amorphous yellow solid, gave the same molecular formula, $C_{29}H_{24}O_5$ ($[M - H]^-$ ion peak at m/z 451.1541; identified by negative-mode HRESIMS), as that of **6**. The UV (236, 285, 35, and 370 nm), IR (1640 cm^{-1}), and the ^1H and ^{13}C NMR spectra were similar to those of **6**. Contrary to **6**, compound **7** was not completely soluble in CD_3OD and its NMR spectra were recorded in $CDCl_3$ and in a combination of $CDCl_3$ and CD_3OD. The ^{13}C NMR spectrum of **7** also revealed two carbonyl carbon atom signals (δ_C 186.8 and 188.3, Table 2, Figure S70, Supporting Information). The observed HMBC (H-7/C-3, C-5, C-9 and H-8/C-10) and COSY correlations (H-7/H-8) were used to connect these two carbonyls with the protons of a benzyl "bridge" (H_2-7 and H_2-8) between the A- and B-rings, suggesting a quinone moiety in the B-ring. HMBC correlations observed between H_2-8, H-12/C-10 and H-11/C-13 (Figures S72, S76, S78, and S79, Supporting Information) confirmed this assignment. The position of the methoxy group (3H-singlet, δ_H 3.94) was assigned to C-1′ by the same correlations as shown for **6**. A characteristic cyclic bisbibenzyl doublet of H-3′ found at δ_H 5.38 (J = 2.0 Hz) exhibited HMBC cross-peaks H-3′/C-1′, C-5′, C-7′ and together with the COSY H-5′/H-6′ correlation revealed a 1′,2′,4′-substitution pattern of the C-ring (Figures S72, S76, and S78, Supporting Information). The HMBC correlations H-10′/C-8′, C-12′, C-14′ and H-14′/C-8′, C-10′, C-12′ supported a 9′,12′,13′-substitution pattern of the D-ring. The strong HMBC correlation between H-11′/C-14 supported a riccardin-type C-12′−C-14 connection in **7**. The same rationale for the restricted mobility of the-A-ring (as for **6**) was also valid for **7**. The H-2 and H-6, and H-3 and H-5 pairs of protons are chemically nonequivalent. The occurrence of positive cross-peaks in phase-sensitive NOESY H-2/H-6 and H-3/H-5 (Figure S73, Supporting Information) indicated a slow equilibrium between two equivalent conformations of the A-ring (Figure 2). Consequently, the structure of **7** was characterized as riccardin G 10,13-*para*-quinone. Similar bisbibenzyl C-10,13 *para*-quinones, isoriccardinquinones A and B, with a C-12−C-11′ biphenyl linkage and the same type of *para*-quinone, marchantiaquinone, with a C-14−O−C-11′ linkage, have been found in the liverworts *Marchantia paleacea* and *Reboulia hemisphaerica*, respectively.[1]

It is noteworthy that most of the newly isolated bisbibenzyls, although without stereocenters (all except **1**), possess optical activity: compound **1** exhibited $[\alpha]^{22}_D$ −3.3 (*c* 1.0, MeOH), **2** $[\alpha]^{22}_D$ +2.0 (*c* 1.0, MeOH), **4** $[\alpha]^{22}_D$ +6.0 (*c* 1.0, MeOH), **5** $[\alpha]^{22}_D$ −33.0 (*c* 1.0, MeOH), **6** $[\alpha]^{22}_D$ −8.0 (*c* 1.0, MeOH), and **7** $[\alpha]^{22}_D$ −1.0 (*c* 1.0, MeOH). Compound **5** exhibited the highest value of −33.0, although there is no chiral center in this molecule, suggesting a highly strained conformation. This phenomenon suggested that compound **5** has both planar and axial chirality, as has been seen in cavicularin.[1]

The methanol extract of *L. cruciata* was not cytotoxic against either the A549 or MRC5 cell line even at a concentration of 200 μg/mL (results not shown). The IC_{50} values of all isolated compounds against two different cell lines were between 3 and 200 μM, without pronounced selectivity between normal and cancer cell lines except for **2** and **1**, with SI indices 6 and 4, respectively (Table S2, Supporting Information). The most potent cytotoxicity was exhibited by the known riccardin G, with IC_{50} values of 2.5 μM for A549 and 7.5 μM for MRC5 cells, and the new compounds **5**, with IC_{50} values of 5.0 μM for A549 and 3.0 μM for MRC5 cells, and **3**, with IC_{50} values of 5.0 μM for both A549 and MRC5 cells. Cisplatin used as the positive control exhibited IC_{50} values of 2.5 μM for A549 cells and 3.5 μM for MRC5 cells. Both **5** and **3** possess a phenanthrene moiety, and further investigation of cytotoxic activity of synthetic bisbibenzyls with phenanthrene moieties

DOI: 10.1021/acs.jnatprod.8b00390
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should be considered. All other compounds were less active or inactive (IC_{50} = 10 μM or >10 μM) for both cell lines (Table S2, Supporting Information).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Analytical Autopol IV automatic polarimeter with methanol as solvent, and the compound concentration used was 1.00 mg/mL. UV spectra were recorded on a GBC Cintra UV/vis spectrometer with methanol as solvent in the concentration range 3−9 × 10^{-5} M. IR spectra were obtained on a Thermoscientific Nicolet 6700 FT-IR spectrometer. NMR spectra were recorded on a Bruker Avance III 500 spectrometer at 500.26 MHz for ^{1}H and 125.80 MHz for ^{13}C, with CD_3OD as a solvent. HRESIMS data were obtained on an Agilent 6210 time-of-flight LC/MS system equipped with an ESI interface (ESITOFMS). The solvent was methanol, and the mobile phase was 0.2% HCOOH/CH_3CN, 1:1, 0.2 mL/min. The ESI was operated in a negative mode, and nitrogen was used as the drying gas (12 L/min) and nebulizing gas at 350 °C (45 psi). The OCT RF voltage was set to 250 V, and the capillary voltage was set to 4.0 kV. The voltages applied to the fragmentor and skimmer were 140 and 60 V, respectively. Scanning was performed from m/z 100 to 1500. Column chromatography (CC) was performed on silica gel 60 (0.063−0.200 mm, Merck). Analytical TLC was carried out on silica gel 60 GF254 20 × 20 cm plates, with a layer thickness of 0.25 mm (Merck). Preparative TLC was carried out on silica gel 60 GF254 (<0.063 mm, Merck)-packed 20 × 20 cm plates, thickness 0.75 mm. Semipreparative HPLC separation was performed on an Agilent Instrument 1100 series equipped with a DAD (Agilent Technologies, G1315C). The column used was a Zorbax Eclipse XDB C_{18} (9.4 mm × 250 mm, 5 μm). Solvents for HPLC separation were of chromatographic grade. Solvents for CC were freshly distilled.

Plant Material. The plant material was collected in Cesena, Italy, in a city park below the fortress (Rocca Malatestiana, coordinates: 44.136195° N 12.239885° E), in September 2016, and was identified by one of the authors (M.V.). The samples were dried at room temperature. A voucher specimen (No. 17290) has been deposited in the Herbarium at the Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade (BEOU), Belgrade, Serbia.

Extraction and Isolation. The air-dried liverwort *Lunularia cruciata* (100 g) was milled and extracted initially with *n*-hexane and then with CH_3OH (4 × 1 L, 48 h) at room temperature, using an ultrasonic bath. After evaporation of the solvents, the crude methanol extract (10.3 g) was fractionated by silica gel CC (700 × 50 mm) using gradient elution with *n*-hexane/ethyl acetate mixtures having increasing percentages of ethyl acetate (Table S1, Supporting Information). Similar fractions (volumes 20−25 mL) were combined according to TLC and further separated first by preparative silica gel TLC (elution systems different ratios CH_2Cl_2/MeOH) and later by semipreparative HPLC using a C_{18} column and a DAD (254, 280 nm). The eluent was H_2O/CH_3CN and the flow rate 4 mL/min. The basic HPLC program was as follows: 0−12 min, 30% CH_3CN; 12−25 min, 30−100% CH_3CN; 25−30 min, 100% CH_3CN. After separation by preparative silica gel TLC (with CH_2Cl_2/MeOH, 96:4) and additional separation by semipreparative HPLC, 7 mg of riccardin F (t_R 23.2 min), 5 mg of riccardin G (t_R 24.2 min), 2 mg of **6** (t_R 23.4 min), and 6 mg of **7** (t_R 23.7 min) were isolated from collected fractions 211−225. From the combined fractions 226−232 and their different subfractions obtained after preparative TLC (with CH_2Cl_2/MeOH, 96:4) and semipreparative HPLC, 42 mg of lunularin (t_R 17.7 min), 33 mg of perrottetin E (t_R 21.9 min), 278 mg of perrottetin F (t_R 20.5 min), 3 mg of **2** (t_R 19.2 min), 5 mg of **5** (t_R 20.0 min), 3 mg of **3** (t_R 21.4 min), and 8 mg of riccardin C (t_R 21.7 min) were obtained. Compound **1** was isolated in the amount of 2 mg from the combined fractions 233−245 in the same manner (t_R 20.9 min). Prior to the investigation of the biological activity, the purity of all compounds was checked by HPLC-DAD at 280 nm and by NMR spectroscopy. The purity was 98% and greater for all isolated compounds.

Compound ***1:*** brown, amorphous solid; $[\alpha]^{22}_D$ −3.3 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 220 (4.20), 272 (3.86), 282 sh (3.84) nm; IR (KBr) ν_{max} 3422, 2927, 2856, 1609, 1600, 1593, 1455, 1340, 1216, 1167, 1078, 1027, 979 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 1; HRESIMS m/z 471.1827 $[M - H]^-$ (calcd for $C_{29}H_{28}O_6$ − H, 471.1813).

Compound ***2:*** pale violet, amorphous solid; $[\alpha]^{22}_D$ +2.0 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.27), 278 (3.94), 294 sh (3.68), 320 (3.68) nm; IR (KBr) ν_{max} 3451, 3033, 2929, 2855, 1610, 1504, 1474, 1455, 1354, 1257, 1235, 1166, 1128, 1105, 1017, 964, 890, 855, 821 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 1; HRESIMS m/z 423.1610 $[M - H]^-$ (calcd for $C_{28}H_{24}O_4$ − H, 423.1602).

Compound ***3:*** violet, amorphous solid; $[\alpha]^{22}_D$ 0 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 224 (4.46), 256 (4.69), 272 sh (4.44), 284 sh (4.27), 308 sh (3.76), 344 (3.22), 362 (3.27) nm; IR (KBr) ν_{max} 3374, 2925, 2854, 1614, 1588, 1504, 1474, 1456, 1434, 1365, 1276, 1228, 1164, 1119, 1033, 1012, 963, 868 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 1; HRESIMS m/z 437.1405 $[M - H]^-$ (calcd for $C_{28}H_{22}O_5$ − H, 437.1394).

Compound ***4:*** brown, amorphous solid; $[\alpha]^{22}_D$ +6.0 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.75), 252 sh (4.06), 284 (3.90), 292 sh (3.83) nm; IR (KBr) ν_{max} 3241, 3030, 2934, 2860, 2838, 1650, 1606, 1590, 1564, 1505, 1435, 1351, 1291, 1217, 1168, 1042, 998, 852, 835, 814 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 2; HRESIMS m/z 453.1716 $[M - H]^-$ (calcd for $C_{29}H_{26}O_5$ − H, 453.1707).

Compound ***5:*** violet, amorphous solid; $[\alpha]^{22}_D$ −33.0 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 225 (4.42), 270 (4.51), 318 sh (3.84), 352 (3.20), 370 (3.23) nm; IR (KBr) ν_{max} 3429, 2925, 2855, 1615, 1606, 1559, 1501, 1464, 1445, 1426, 1410, 1383, 1354, 1287, 1220, 1178, 1164, 1123, 1104, 1041, 1021, 998, 867, 822 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 2; HRESIMS m/z 449.1402 $[M - H]^-$ (calcd for $C_{29}H_{22}O_5$ − H, 449.1394).

Compound ***6:*** orange, amorphous solid; $[\alpha]^{22}_D$ −8.0 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 236 (4.44), 255 sh (4.34), 264 sh (4.29), 285 (3.94), 320 (3.16), 352 (3.20), 370 (3.23) nm; IR (KBr) ν_{max} 3445, 3271, 3028, 2927, 2854, 1655, 1631, 1611, 1581, 1515, 1506, 1462, 1447, 1421, 1340, 1264, 1234, 1212, 1165, 1128, 1100, 1018, 976, 853, 788, 753 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 2; HRESIMS m/z 451.1556 $[M - H]^-$ (calcd for $C_{29}H_{24}O_5$ − H, 451.1551).

Compound ***7:*** orange, amorphous solid; $[\alpha]^{22}_D$ −1.0 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 238 (4.34), 258 sh (4.24), 282 (4.01), 392 (3.35) nm; IR (KBr) ν_{max} 3463, 3058, 3033, 2933, 2855, 1654, 1616, 1590, 1514, 1443, 1424, 1334, 1297, 1265, 1232, 1216, 1166, 1129, 1071, 1019, 937, 844 cm^{-1}; ^{1}H NMR and ^{13}C NMR see Table 2; HRESIMS m/z 451.1541 $[M - H]^-$ (calcd for $C_{29}H_{24}O_5$ − H, 451.1551).

Cytotoxicity Assays. Cytotoxicity (antiproliferative activity) of the plant methanol extract and the isolated pure compounds was determined using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.[28] Briefly, A549 (human lung carcinoma, obtained from ATCC) and MRC5 cells (human lung fibroblast, obtained from ATCC) were plated in a 96-well flat-bottom plate at a concentration of 1 × 10^4 cells per well, grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C, and maintained as monolayer cultures in RPMI-1640 medium, supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, and 10% (v/v) fetal bovine serum (FBS). After a 24 h incubation period of MRC5 and A549 cells, medium containing increasing concentrations of each tested compound (μM) was added to the cells. Control cultures received the solvent DMSO, and blank wells contained 200 μL of growth medium. Cisplatin (*cis*-diammineplatinum(II) dichloride; Sigma-Aldrich) was used as a positive control. After 24 h of incubation, the MTT reduction assay was carried out and cell proliferation was determined from the absorbance at 540 nm on a Tecan Infinite 200 Pro multiplate reader (Tecan Group, Männedorf, Switzerland). The MTT assay was

DOI: 10.1021/acs.jnatprod.8b00390
J. Nat. Prod. 2019, 82, 694−701

performed three times in quadruplicate for each test compound concentration, and the results are presented as a percentage of the control (untreated cells) that was arbitrarily set to 100%.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00390.

NMR spectra of the isolated compounds and additional figures and tables (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported financially by the Ministry of Education, Science and Technological Development of Republic of Serbia, Project Nos. 172053, 173029, and 173048. The authors thank Y. Okamoto (Tokushima Bunri University, Tokushima, Japan) for technical assistance with the MS measurements.

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DOI: 10.1021/acs.jnatprod.8b00390
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