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Terpenoids and Meroterpenoids from Cultures of Two Grass-Associated Species of *Amylosporus* (Basidiomycota)

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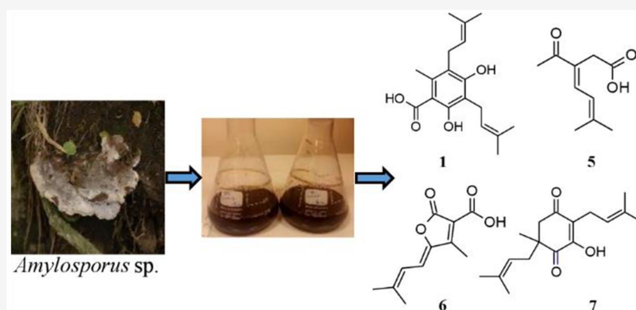
ABSTRACT: An investigation of the chemical components of the fermentation extract of two cultures of *Amylosporus* cf. *graminicola* and *Amylosporus* cf. *campbelii* from Cuba and Zimbabwe, respectively, led to the isolation of seven previously undescribed secondary metabolites for which we proposed the trivial names amylosporanes A−G (**1**−**7**) along with the known compounds orsellinic acid (**11**), colletorin D acid (**12**), colletorin B (**13**), colletochlorin B (**14**), and the β-lactam cyclo-(*S*-Pro-*R*-Leu) (**15**). Three additional compounds (**8**−**10**) previously unknown from a fungal source were also characterized for the first time, and two of them were assigned the trivial names amylosporanes H−I (**8**−**9**) while the other was identified as cannabigerorcinic acid (**10**). The structures of the isolated compounds were determined based on their high-resolution electrospray ionization mass spectrometry (HR-ESIMS) spectra and an extensive analysis of their 1D and 2D NMR spectroscopic data. Based on literature searches, we hypothesized that a majority of the isolated metabolites have orsellinic acid (**11**) as a biosynthetic precursor following a combined route of mevalonate-associated and orsellinic acid-associated pathways. Colletochlorin B (**14**), the only compound possessing chlorine in its structure, exhibited significant activity against *Bacillus subtilis* (minimum inhibitory concentration, 2 μg/mL), stronger than that of oxytetracycline, and significant cytotoxicity against A431 cells with an IC_{50} value of 4.6 μM.



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In the search for novel bioactive compounds with potential for therapy of infectious diseases or other beneficial properties, cultures of tropical African Basidiomycota have recently been found to be a good source.[1,2] In particular, not only African relatives of the traditional edible and medicinal Asian mushrooms such as *Sanghuangporus* and *Laetiporus*[3,4] but also rare and endemic tropical species of genera that have never been studied before for bioactive constituents, such as *Heimiomyces*,[5] have recently yielded interesting secondary metabolites. This study describes the evaluation of *Amylosporus* cf. *campbelii* and *Amylosporus* cf. *graminicola* (following Ryvarden and Johansen 1980, Ryvarden 1973) that were isolated from specimens collected in Zimbabwe and Cuba, respectively. *Amylosporus* was erected by Ryvarden (1973) to accommodate a polypore that is associated with grasses. It currently includes 12 species, which were all described from Asia and Neotropical America.[6] The genus belongs to the order Russulales and the family Bondarzewiaceae, and no secondary metabolites have hitherto been reported from any of its species.[7] Extracts of the strains of the *Amylosporus* spp. showed moderate antibacterial effects in our screening for novel anti-infective metabolites along with a unique HPLC/DAD-MS metabolite profile, and we selected these fungi for an evaluation of the active principles.

RESULTS AND DISCUSSION

Submerged mycelial cultures of the basidiomycete *Amylosporus* sp. and purification of the extracts using preparative reverse-phase (RP) chromatography led to the isolation of 15 compounds among which seven turned out to be new to science and were assigned the trivial names amylosporanes A−G (**1**−**7**), and five known metabolites were identified as orsellinic acid (**11**), colletorin D acid (**12**), colletorin B (**13**), colletochlorin B (**14**), and the β-lactam cyclo-(*S*-Pro-*R*-Leu) (**15**). Three additional compounds (**8**−**10**) so far unknown from a fungal source were also characterized in the present work, and two of them were assigned the trivial names amylosporanes H and I (**8** and **9**) while compound **10** was identified as cannabigerorcinic acid.

Compound **1** was obtained as a yellow oil, and its molecular formula was established as $C_{18}H_{24}O_4$ (7 degrees of unsaturation) based on its high-resolution electrospray ionization mass spectrometry (HR-ESIMS; positive mode)

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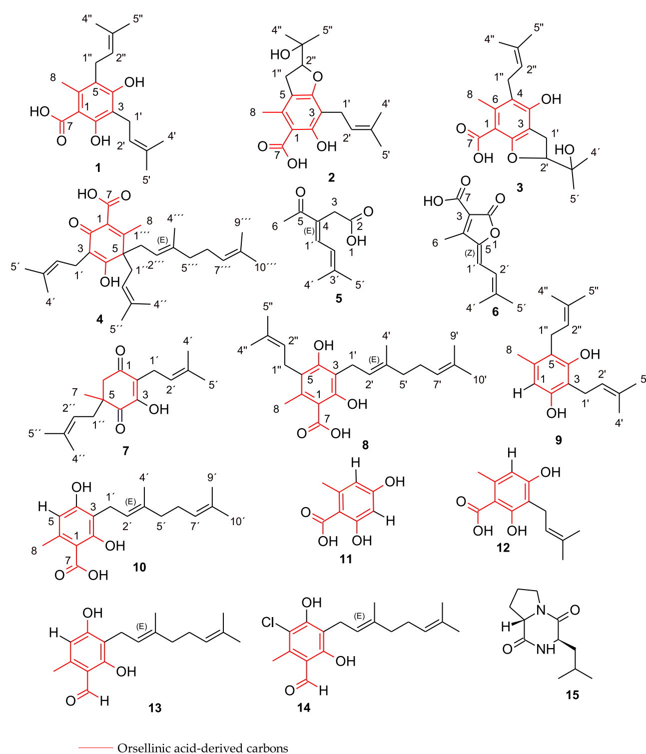

data. The ^{1}H NMR spectrum showed signals that integrated for 21 protons. Those protons were further identified as four vinyl methyl groups H-4′, H-4″, H-5′, and H-5″ at δ 1.77 (3H, s), δ 1.77 (3H, s), δ 1.67 (3H, d, J = 1.2 Hz), and δ 1.68 (3H, d, J = 0.9 Hz), respectively; two allylic methylene groups H-1′ and H-1″ at δ 3.34 (4H, br d, J = 7.0 Hz); two olefinic protons H-2′ and H-2″ at δ 5.16 (1H, dddd, J = 7.0, 5.6, 2.8, 1.4 Hz) and δ 5.00 (1H, ddt, J = 7.2, 5.9, 1.2 Hz), respectively; as well as one methyl group H-8 at δ 2.44 (3H, s) (Table 1). The ^{1}H−^{1}H COSY correlations observed between H-1′ and H-2′/H-4′/H-5′, H-2′, and H-4′/H-5′ along with COSY correlations of H-1″ to H-2″/H-4″/H-5″ and H-2″ to H-4″/H-5″ allowed the identification of two prenyl side chains (Figure 1). The ^{13}C NMR spectrum exhibited signals of 18 carbon atoms in agreement with the established molecular formula. Those carbons were further identified by an extensive analysis of the 1D NMR spectra coupled with the ^{1}H−^{13}C HSQC spectrum showing signals corresponding to one carboxylic acid at δ 176.3 (C-7); six aromatic carbons at δ 107.8 (C-1), δ 161.4 (C-2), δ 114.1 (C-3), δ 158.6 (C-4), δ 121.5 (C-5), and δ 139.3 (C-6); two olefinic carbons at δ 124.1 (C-2′) and δ 124.8 (C-2″); as well as seven sp^{3}-hybridized carbons including 5 methyl groups C-4′, C-4″, C-5′, C-5″, and C-8 at δ 18.1, δ 18.1, δ 26.1, δ 26.0, and δ 18.8, respectively, and two methylene groups C-1′ and C-1″ at δ 23.4 and δ 26.4, respectively. The existence of six aromatic carbons on the ^{13}C NMR spectrum and the absence of aromatic protons on the ^{1}H NMR spectrum suggested the presence of a hexasubstituted aromatic nucleus. The substituents on the aromatic nucleus were identified as two prenyl units, one methyl group, one

Table 1. ^{13}C and ^{1}H NMR Data of Compounds 1−4 in Methanol-d_4 (δ in ppm)

	1[a]		2[a]		3[b]		4[a]	
no.	δ_C, type	δ_H [J (Hz)]	δ_C, type	δ_H [J (Hz)]	δ_C, type	δ_H [J (Hz)]	δ_C, type	δ_H [J (Hz)]
1	107.8, C		108.1, C		110.4, C		130.8, C[c]	
2	161.4, C		163.6, C		159.0, C		182.8, C[c]	
3	114.1, C		109.1, C		111.7, C		117.5, C	
4	158.6, C		162.5, C		154.4, C		n.o., C	
5	121.5, C		119.3, C		122.0, C		55.5, C	
6	139.3, C		136.6, C		136.9, C		162.8, C	
7	176.3, C		177.9, C		172.5, C		171.8, C	
8	18.8, CH_3	2.44, s	19.9, CH_3	2.48, s	17.2, CH_3	2.27, s	17.8, CH_3	2.23, s
1′	23.4, CH_2	3.34, br d (7.0)[d]	23.6, CH_2	3.22, m	29.2, CH_2	3.08, dd (8.8, 4.3)	22.1, CH_2	3.04, br d (6.9)
2′	124.1, CH	5.16, dddd (7.0, 5.6, 2.8, 1.4)	124.1, CH	5.25, m	91.4, CH	4.62, t (8.9)	123.7, CH	4.99, m[d]
3′	132.5, C		131.7, C		72.7, C		132.4, C	
4′	18.1, CH_3	1.77, s	18.1, CH_3	1.75, s	26.0, CH_3	1.29, s	18.2, CH_3	1.71, s
5′	26.1, CH_3	1.67, d (1.2)	26.1, CH_3	1.65, s	25.2, CH_3	1.21, s	26.0, CH_3	1.63, s
1″	26.4, CH_2	3.34, br d (7.0)[d]	30.8, CH_2	3.05, d (8.5)	26.0, CH_2	3.31, m[d]	37.5, CH_2	2.79, m;[d] 2.50, m[d]
2″	124.8, CH	5.00, ddt (7.2, 5.9, 1.2)	90.6, CH	4.58, t (8.5)	124.6, CH_3	5.00, m	119.3, CH	4.65, m[d]
3″	131.9, C		72.8, C		131.6, C		135.6, C	
4″	18.1, CH_3	1.77, s	26.2, CH_3	1.25, s	18.1, CH_3	1.76, d (0.9)	18.4, CH_3	1.6, s
5″	26.0, CH_3	1.68, d (0.9)	24.5, CH_3	1.20, s	26.0, CH_3	1.66, d (1.1)	26.1, CH_3	1.56, s
1‴							37.5, CH_2	2.79, m;[d] 2.50, m[d]
2‴							119.1, CH	4.65, m[d]
3‴							139.4, C	
4‴							16.8, CH_3	1.59, s
5‴							40.9, CH_2	1.84, t (7.0)
6‴							27.9, CH_2	1.93, m
7‴							125.3, CH	4.99, m[d]
8‴							132.4, C	
9‴							17.9, CH_3	1.54, s
10‴							26.1, CH_3	1.63, s

[a] ^{1}H 500 MHz, ^{13}C 125 MHz. [b] ^{1}H 700 MHz, ^{13}C 175 MHz, n.o.: not observed. [c] Chemical shift read from HMBC. [d] Overlapped.

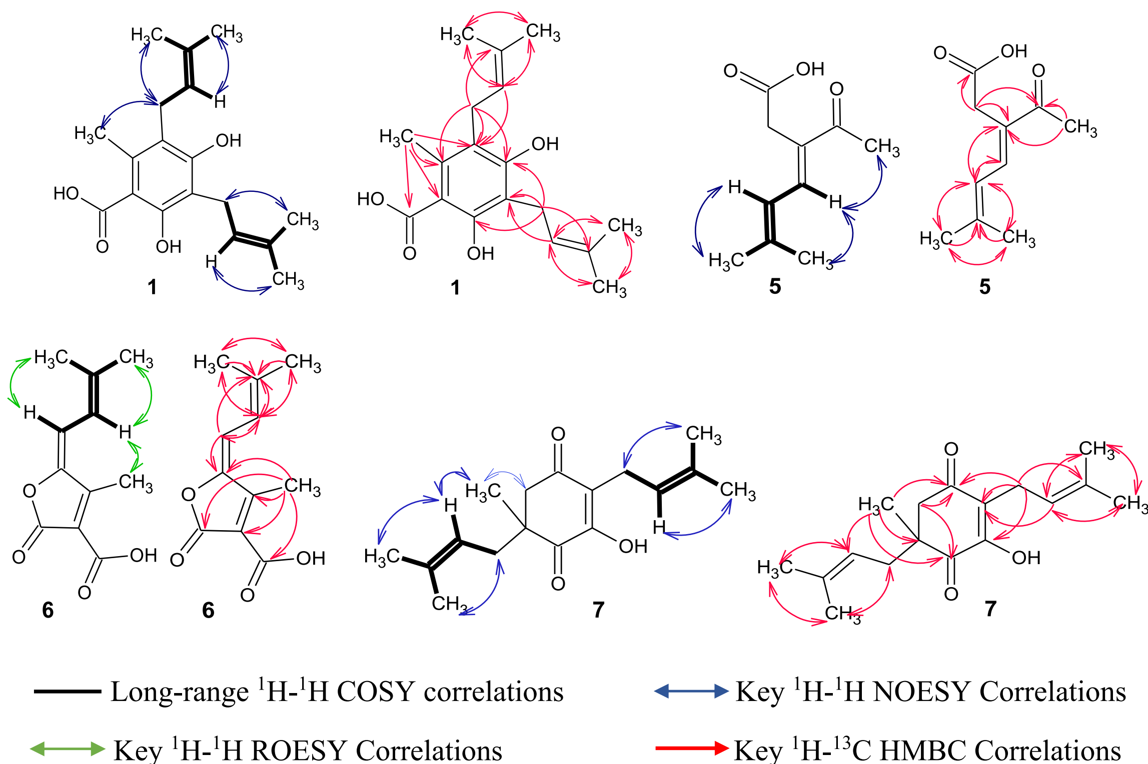

Figure 1. Key HMBC, COSY, NOESY, and ROESY correlations of compounds **1** and **5**−**7**.

carboxylic acid group, and two hydroxy groups based on a careful analysis of its 1D NMR and 2D NMR data including COSY, HSQC, NOESY, and HMBC spectra, which confirmed the proton and carbon assignments of the prenyl side chains. The connectivity of the substituents to the aromatic nucleus was revealed by a comprehensive analysis of its ^{1}H−^{13}C HMBC and ^{1}H−^{1}H NOESY spectra (Figure 1). Thus, the HMBC correlations between the signal at δ 2.44 (H-8) with C-7 (δ 176.3)/C-1 (δ 107.8)/C-5 (δ 121.5)/C-6 (δ 139.3) indicated that the methyl group (C-8) and carboxylic acid group (C-7) were, respectively, attached to C-6 and C-1 in the aromatic nucleus leading to a methyl benzoic acid moiety. The connectivity of the methyl group C-8 at C-6 was further confirmed on the ^{1}H−^{1}H NOESY spectrum where a correlation between H-8 and H-1″ was observed. The chemical shifts of δ 161.4 and δ 158.6 observed for C-2 and C-4, respectively, suggested that they were both linked to a hydroxy group. The position of the first prenyl group placed at C-3 of the methylbenzoic acid moiety was determined by HMBC correlations of H-1′ (δ 3.34) to C-3 (δ 114.1)/C-4 (δ 158.6)/C-2 (δ 161.4), whereas the second prenyl side chain was located as a benzene substituent at C-5 in agreement with HMBC correlations between H-1″ (δ 3.34) and C-5 (δ 121.5)/C-6 (δ 139.3)/C-4 (δ 158.6). Moreover, the ^{1}H−^{1}H NOESY correlations of H-4′ with H-1′ and H-4″ with H-1″ confirmed the *cis* positioning of the methyl groups C-4′ and C-4″ with the respective allylic methylene groups C-1′ and C-1″, indicating unambiguously their *trans* positioning with the respective neighboring olefinic protons H-2′ and H-2″. On the other hand, the *cis* positioning of the aforementioned olefinic protons with the methyl groups C-5′ and C-5″ was clearly evidenced by ^{1}H−^{1}H NOESY correlations of H-5′ with H-2′ and H-5″ with H-2″. All of these data allowed a full assignment for all hydrogens and carbons and established the structure of compound **1** as 2,4-dihydroxy-6-methyl-3,5-bis(3-methylbut-2-en-1-yl)benzoic acid named amylosporane A.

Compound **2** was obtained as a yellow oil, and its HR-ESIMS indicated a molecular formula of $C_{18}H_{24}O_5$ determined by the $[M + H]^+$ ion at m/z 321.1697 and the $[M + Na]^+$ ion at m/z 343.1516. An analysis of its 1D and 2D NMR data showed similarities with compound **1**, indicating that compounds **2** and **1** were closely related. The difference that occurred was possibly the epoxidation of the $\Delta^{2'',3''}$ double bond followed by a cyclization leading to the formation of a furan, as previously postulated by Dallery et al., who have discovered similar compounds.[8] This was confirmed with its ^{1}H NMR spectrum where the signal of the olefinic proton at δ 5.00 (1H, ddt, J = 7.2, 5.9, 1.2 Hz; attributed to H-2″ in compound **1**) was absent, and the resonance of the signal of an oxymethylene at δ 4.58 (1H, t, J = 8.5 Hz) corresponding to H-2″ in compound **2** was recorded instead (Table 1). On its ^{13}C NMR spectrum, the presence of signals in the high field region at δ 90.6 (C-2″) and δ 72.8 (C-3″) recorded for compound **2** instead of signals in the low field region at δ 124.8 (C-2″) and δ 131.9 (C-3″) observed for compound **1** further confirmed this assumption. Moreover, the assignment and the positioning of the furan in compound **2** was evidenced by the ^{1}H−^{1}H COSY correlation between H-1″ (δ 3.05, d, J = 8.5 Hz, 2H) and H-2″ (δ 4.58, t, J = 8.5 Hz, 1H) as well as HBMC correlations of H-2″ (δ 4.58, t, J = 8.5 Hz, 1H) to C-5 (δ 119.3) and C-4 (δ 162.5) and H-1″ (3.05, d, J = 8.5 Hz, 2H) to C-2″ (δ 90.6), C-4 (δ 162.5), C-5 (δ 119.3), and C-6 (δ 136.6). The ^{1}H−^{1}H ROESY correlation observed between H-8 and H-1″ also contributed to the positioning of this furan, confirming that the prenyl unit involved in the formation of the furan was the one next to the methyl group C-8 and that it is attached at C-5 of the aromatic nucleus. In addition, the low specific rotation (−0.49, c 0.41) obtained for this compound

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Table 2. ^{13}C and ^{1}H NMR Data of Compounds 5−7 in Methanol-d_4 (δ in ppm)

	5[a]		6[a]		7[b]	
no.	δ_C, type	δ_H [J (Hz)]	δ_C, type	δ_H [J (Hz)]	δ_C, type	δ_H [J (Hz)]
1					199.3, C	
2	175.3, C		167.9, C		127.0, C	
3	31.8, CH_2	3.41, s	117.0, C		157.4, C	
4	132.7, C		162.3, C		200.4, C	
5	201.5, C		148.0, C		49.5, C[c]	
6	25.4, CH_3	2.39, s	11.9, CH_3	2.51, s	49.5, CH_2[c]	2.64, d (16.4); 2.72, d (16.4)
7			164.8, C		24.5, CH_3	1.23, s
1′	140.0, CH	7.59, d (11.6)	114.6, CH	6.66, d (11.8)	23.4, CH_2	3.08, d (7.31)
2′	122.4, CH	6.24, dt (11.6, 1.3)	120.2, CH	6.43, dt (11.9, 1.3)	121.4, CH	5.12, m
3′	150.0, C		150.4, C		134.0, C	
4′	19.2, CH_3	1.98, s	19.3, CH_3	2.00, s	18.1, CH_3	1.74, s
5′	27.3, CH_3	1.96, s	27.3, CH_3	1.99, s	26.3, CH_3	1.66, s
1″					40.0, CH_2	2.25, dd (14.0, 8.39); 2.36, dd (14.0, 7.31)
2″					120.0, CH	5.02, m
3″					137.3, C	
4″					18.0, CH_3	1.56, s
5″					26.1, CH_3	1.68, s

[a] ^{1}H 500 MH_Z, ^{13}C 125 MHz. [b] ^{1}H 700 MH_Z, ^{13}C 175 MH_Z. [c] Overlapped with solvent peak.

indicated that the oxidative cyclization of **1** into **2** is not stereoselective, thus revealing that **2** occurs as a mixture of enantiomers. This hypothesis was confirmed on its ECD spectrum (Figure S18) where no Cotton effect was observed. Therefore, the structure of compound **2** was established as 6-hydroxy-2-(2-hydroxypropan-2-yl)-4-methyl-7-(3-methylbut-2-en-1-yl)-2,3-dihydro-1-benzofuran-5-carboxylic acid named amylosporane B.

The molecular formula of compound **3**, also isolated as a yellow oil, was deduced to be $C_{18}H_{24}O_5$ from the HR-ESIMS data. Compound **3** had the same molecular formula as compound **2**, and both are related congeners of compound **1** as determined from an analysis of their 1D and 2D NMR data. A comparison of the NMR data of compounds **3** and **2** revealed that they display high similarities. The only difference was the prenyl and the hydroxy group involved during the epoxidation and the formation of the furan ring in compound **1**. Unlike compound **2** where the prenyl unit attached at C-5 and the hydroxy group placed at C-4 of the benzoic acid moiety in compound **1** were involved in the formation of the furan, in compound **3**, rather, epoxidation[8] occurred on the $\Delta^{2',3'}$ double bond belonging to the prenyl side chain attached at C-3. Moreover, the hydroxy group connected at C-2 of the aromatic nucleus in compound **1** was the one involved in the formation of the furan. This assumption was confirmed on the HMBC spectrum where correlations between H-1′ (3.08, dd, J = 8.8, 4.3 Hz, 2H) and C-2 (δ 159.0)/C-3 (δ 111.7) were observed in compound **3** instead of correlations between H-1″ (3.05, d, J = 8.5 Hz) and C-5 (δ 119.3)/C-6 (δ 136.6) shown by compound **2**. The assignment and the positioning of the furan ring on the aromatic nucleus were further evidenced by the homonuclear $^{1}H-^{1}H$ COSY correlation of H-1′ (3.08, dd, J = 8.8, 4.3 Hz, 2H) to H-2′ (δ 4.62, t, J = 8.9 Hz, 1H) as well as the heteronuclear $^{1}H-^{13}C$ HMBC correlations between H-2′ (δ 4.62, t, J = 8.9 Hz, 1H) and C-1′ (δ 29.2)/C-3 (δ 111.7)/C-2 (δ 159.0) and H-1′ (3.08, dd, J = 8.82, 4.30, 2H) and C-2′ (δ 91.4)/C-4 (δ 154.4).

From the aforementioned results, the planar structure of compound **3** was unambiguously established as 4-hydroxy-2-(2-hydroxypropan-2-yl)-6-methyl-5-(3-methylbut-2-en-1-yl)-2,3-dihydro-1-benzofuran-7-carboxylic acid named amylosporane C. Due to the paucity of the sample, we could not investigate the absolute configuration of this compound.

Compound **4** was isolated as a brownish oil, and the molecular formula $C_{28}H_{40}O_4$ (9 degrees of unsaturation) was assigned to it by an analysis of its HR-ESIMS data. The IR spectrum (Figure S36) provided evidence of a hydroxy group (3443 cm^{-1}, OH), a carboxylic acid group (2969 cm^{-1}, OH; and 1722 cm^{-1}, C=O), and a conjugated ketone group (1670 cm^{-1}, C=O). An analysis of 1D and 2D NMR spectroscopic data of compound **4** revealed its similarity to compound **1**. The main difference was the oxidation of the OH-bearing carbon C-2 (δ 161.4) in compound **1**, which turned into a conjugated ketone group occurring at δ 182.8 (C-2) in compound **4**. In addition, the resonance of signals attributable to an additional prenylated side chain was observed in compound **4**. This side chain was identified as a geranyl unit on the basis of a combined analysis of ^{1}H NMR, $^{1}H-^{1}H$ COSY, and $^{1}H-^{13}C$ HSQC spectra where resonances of signals corresponding to three vinyl methyl at δ 1.59 (s, H-4‴), δ 1.54 (s, H-9‴), and δ 1.63 (3H, s, H-10‴); three methylene at δ 2.79 (m, H-1‴a), δ 2.50 (m, H-1‴b), δ 1.84 (t, J = 7.0 Hz, H-5‴), and δ 1.93 (m, H-6‴); and two olefinic protons at δ 4.65 (m, H-2‴) and δ 4.99 (m, H-7‴) were observed. The heteronuclear $^{1}H-^{13}C$ HMBC and the homonuclear $^{1}H-^{1}H$ NOESY correlations confirmed the assignment of protons and carbons of the geranyl side chain. The *E*-geometry of the asymmetrically substituted $\Delta^{2''',3'''}$ double bond was determined based on the carbon chemical shift of δ 16.83 observed for C-4‴[9] as well as NOESY correlations of H-4‴ with H-1‴ and of H-2‴ with H-5‴. Moreover, the oxidation of the OH-bearing carbon C-2 in compound **1** which turned into a conjugated ketone group in compound **4** justified the significant downfield shift (+22.94 ppm) of the C-1 carbon which resonates at δ 130.8 in compound **4** instead of δ 107.8 as in compound **1**. In addition, the conjugated system resulting from this oxidation affected the shielding (+32 ppm) of the sp^2-hybridized carbon C-6, the chemical shift of which appears at δ 162.8 in compound **4** instead of δ 139.3 in compound **1**. The presence of the signal in the high field region at δ 55.5 (C-5), corresponding to an

sp^3-hybridized quaternary carbon recorded in compound **4** instead of the signal in the low field region at δ 121.9 (C-5) showed in compound **1**, was in good agreement with the oxidation that occurred. The positioning of the geranyl side chain in compound **4** placed at C-5 was confirmed by HMBC correlations between H-1‴a, H-1‴b, and C-5/C-6. Although the ^{13}C NMR spectrum of compound **4** depicted 25 carbons instead of 28 as shown by the molecular formula, the resonance of additional carbons at δ 182.8 and δ 130.8 attributable to C-2 and C-1, respectively, was observed on the HMBC spectrum, which showed ^{3}J correlations of H-8 to C-1 and of H-1″ to C-2. However, the signal of carbon C-4 could not be observed, and this is probably due to the tautomerization that can occur between the hydroxy group at C-4 and the ketone group at C-2 (Figure S3). The low specific rotation (1.25, *c* 0.319) and the absence of a Cotton effect in the ECD spectrum (Figure S37) suggested that **4** occurs as a racemic mixture. Based on the above-mentioned data, compound **4** was therefore identified as a new polyprenylated orsellinic acid derivative and named amylosporane D.

Compound **5**, isolated as a yellow oil, was assigned the molecular formula $C_{10}H_{14}O_3$ (4 degrees of unsaturation) on the basis of its HR-ESIMS data. The ^{1}H NMR spectrum exhibited resonance attributed to two olefinic protons at δ 7.59 (d, *J* = 11.6 Hz, H-1′) and δ 6.24 (dt, *J* = 11.6, 1.3 Hz, H-2′); one allylic methylene group at δ 3.41 (s, H-3); and three methyl group singlets at δ 2.39 (s, H-6), δ 1.98 (s, H-4′), and δ 1.96 (s, H-5′). The ^{13}C NMR spectrum showed signals of 10 carbons. Those carbons were further identified as one carboxylic acid carbon at δ 175.3 (C-2); one ketone carbon at δ 201.5 (C-5); two olefinic carbons at δ 140.0 (C-1′) and δ 122.4 (C-2′); two sp^2-hybridized carbons attributed to C-4 and C-3′ at δ 132.7 and δ 150.0, respectively; as well as four sp^3-hybridized carbons including one methylene carbon at δ 31.8 (C-3) and three methyl carbons at δ 25.4 (C-6), δ 19.2 (C-4′), and δ 27.3 (C-5′) based on its 1D NMR data (Table 2) in combination with the ^{1}H−^{13}C HSQC spectrum. The presence of a prenyl group was evidenced not only on its ^{1}H−^{1}H COSY spectrum through correlations between H-2′ (δ 6.24) and H-1′ (δ 7.59)/H-4′ (δ 1.98)/H-5′ (δ 1.96) but also on its ^{1}H−^{13}C HMBC spectrum, which displayed correlations between H-2′ and C-4′/C-5′/C-1′ (Figure 2). The connectivity of the prenyl side chain with the other fragment of the molecule was performed by an analysis of its ^{1}H−^{13}C HMBC and ^{1}H−^{1}H NOESY spectrum. Therefore, the correlations shown by the ^{1}H−^{13}C HMBC spectrum between H-2′ and C-4, H-1′ and C-3/C-5/C-3′/C-2′/C-2, and H-6 and C-5/C-4/C-1′ as well as correlations of H-3 with C-2/C-4/C-5 confirmed the gross structure obtained for compound **4**. The E-geometry of the $\Delta^{1',4}$ double bond was assigned based on the ^{1}H−^{1}H NOESY correlation observed between H-6 (δ 2.39, s) and H-1′ (δ 7.59, d, *J* = 11.6 Hz) as well as the correlation of H-2′ (δ 6.24, dt, *J* = 11.6, 1.3 Hz) with H-3 (δ 3.41, s) (Figure 2). The *cis* positioning of the methyl group C-4′ with the olefinic carbon C-1′ was evidenced on the same spectrum where the NOESY correlation of H-4′ with H-1′ was observed. All of these data allowed the identification of compound **5** as (3E)-3-acetyl-6-methylhepta-3,5-dienoic acid, named amylosporane E.

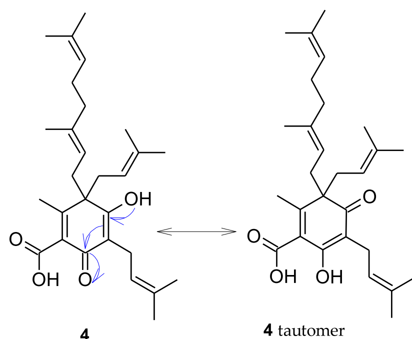

Figure 2. Possible tautomerization of compound **4**.

Compound **6** was obtained as a yellow amorphous solid, and its molecular formula $C_{11}H_{12}O_4$ was determined by HR-ESIMS data, corresponding to 6 degrees of unsaturation. The ^{1}H NMR spectrum displayed signals of two olefinic protons at δ 6.66 (d, *J* = 11.8 Hz, H-1′) and δ 6.43 (dt, *J* = 11.9 Hz, 1.3, H-2′) and three methyl group singlets at δ 2.51 (s, H-6), δ 2.00 (s, H-4′), and δ 1.99 (s, H-5′). The ^{1}H−^{1}H COSY spectrum of compound **6** showed spin−spin coupling systems between H-1′ and H-2′/H-4′/H-5′ and H-2′ and H-4′/H-5′ suggesting the presence of a prenyl group (Figure 2). Eleven carbons were recorded by ^{13}C NMR and further identified based on a combined analysis of its 1D NMR (Table 2) and ^{1}H−^{13}C HSQC spectra as one carboxylic acid carbon (C-7) at δ 164.8; two olefinic carbons at δ 120.2 (C-2′) and δ 114.6 (C-1′); three methyl carbons at δ 11.9 (C-6), δ 19.3 (C-4′), and δ 27.3 (C-5′); and five nonprotonated sp^2-hybridized carbons at δ 167.9 (C-2), δ 117.0 (C-3), δ 162.3 (C-4), δ 148.0 (C-5), and δ 150.4 (C-3′). The resonance of signals at δ 167.9 (C-2), δ 117.0 (C-3), δ 162.3 (C-4), δ 148.0 (C-5), and δ 164.8 (C-7) suggested the existence of a furanone carboxylic acid moiety. The linkage positions of the substituents on the furanone were determined by the ^{1}H−^{13}C HMBC spectrum, in which correlations of H-1′ (δ 6.66) to C-5 (δ 148.0)/C-4 (δ 162.3)/C-2′ (120.2)/C-3′ (δ 150.4), H-2′ (δ 6.43) to C-5 (δ 148.0)/C-1′ (δ 114.6)/C-4′ (δ 19.3)/C-5′ (δ 27.3), and H-6 (δ 2.51) to C-4 (δ 162.3)/C-5 (δ148.0)/C-3 (δ 117.0)/C-2 (δ 167.9) confirmed the connectivity of the prenyl unit at C-5 and the attachment of the methyl group at C-4 of the furan (Figure 1). The positioning of the carboxylic acid group attached at C-3 of the furan nucleus was clearly evidenced by the ^{1}H−^{13}C HMBC correlation observed between H-6 (δ 2.51, s) and C-7 (δ 164.8). Moreover, the ^{1}H−^{1}H ROESY correlation observed between H-6 (δ 2.51, s) and H-1′ (δ 6.66, d, *J* = 11.8 Hz) was in favor of the Z-configuration for the $\Delta^{1',5}$ double bond, and the ^{1}H−^{1}H ROESY correlation of H-1′ to H-4′ confirmed the *cis* position of the methyl group at C-4′ with the olefinic proton at C-1′. Therefore, the structure of compound **6** was determined as (5Z)-4-methyl-5-(3-methylbut-2-en-1-ylidene)-2-oxo-2,5-dihydrofuran-3-carboxylic acid and named amylosporane F.

Compound **7** was isolated as beige needlelike crystals from both mycelia and supernatant. Its HR-ESIMS spectrum revealed a molecular formula of $C_{17}H_{24}O_3$ (6 degrees of unsaturations) based on the existence of the ion peaks $[M + H]^+$ at *m*/*z* 277.1795 and $[M + Na]^+$ at *m*/*z* 299.1616. The ^{1}H NMR spectrum showed signals of two olefinic protons resonating at δ 5.12 (m, H-2′) and δ 5.02 (m, H-2″) as well as signals of five methyl group singlets at δ 1.23 (s, H-7), δ 1.74 (s, H-4′), δ 1.66 (s, H-5′), δ 1.56 (s, H-4″), and δ 1.68 (s, H-5″). The signal of one allylic methylene group doublet at δ 3.08 (d, *J* = 7.31 Hz, H-1′) as well as signals of diastereotopic protons H-1″ and H-6 at δ 2.36 (dd, *J* = 14.0 Hz, 7.31, H-1″a), δ 2.25 (dd, *J* = 14.0 Hz, 8.39, H-1″b), δ 2.72 (d, *J* = 16.4 Hz,

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Table 3. ^{13}C and ^{1}H NMR Data of Compounds 8−10 in Methanol-d_4 (δ in ppm)

no.	8[b]		9[b]		10[a]	
	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	108.4, C		110.5, CH	6.19, s	106.0, C	
2	161.2, C		154.3, C		164.7, C	
3	114.0, C		114.9, C		114.0, C	
4	158.4, C		154.4, C		161.0, C	
5	121.4, C		120.2, C		111.4, CH	6.18, s
6	139.2, C		135.7, C		141.9, C	
7	176.3, C				176.0, C	
8	18.7, CH_3	2.45, s	20.0, CH_3	2.12, s	24.4, CH_3	2.46, s
1′	23.3, CH_2	3.35, br s[c]	23.7, CH_2	3.31, m[c]	22.9, CH_2	3.26, d (7.02)
2′	124.2, CH	5.17, td (6.99, 1.08)	124.9, CH	5.19, tt (7.04, 1.34)	124.5, CH	5.21, m
3′	136.3, C		131.9, C		135.1, C	
4′	16.5, CH_3	1.77, s	18.1, CH_3	1.76, s	16.4, CH_3	1.75, s
5′	41.1, CH_2	1.98, t (7.53)	26.1, CH_3	1.66, d (0.86)	41.1, CH_2	1.94, t (7.93)
6′	27.8, CH_2	2.06, m			27.9, CH_2	2.05, m
7′	125.6, CH	5.06, m			125.7, CH	5.06, tt (7.13, 1.34)
8′	132.3, C				132.1, C	
9′	17.9, CH_3	1.56, s			17.8, CH_3	1.55, s
10′	26.0, CH_3	1.61, s			26.0, CH_3	1.61, s
1″	26.4, CH_2	3.35, br s[c]	26.4, CH_2	3.25, br d (6.67)		
2″	124.9, CH	5.00, m	125.2, CH	5.03, tt (6.72, 1.45)		
3″	131.8, C		131.4, C			
4″	18.1, CH_3	1.77, s	18.1, CH_3	1.75, s		
5″	26.0, CH_3	1.68, s	26.0, CH_3	1.67, d (1.08)		

[a] ^{1}H 500 MHz, ^{13}C 125 MHz. [b] ^{1}H 700 MHz, ^{13}C 175 MHz. [c] Overlapped.

H-6a), and δ 2.64 (d, J = 16.4 Hz, H-6b) were also recorded (Table 2). The homonuclear ^{1}H−^{1}H COSY spectrum clearly indicated the presence of two prenyl moieties in which correlations of H-1′ to H-2′/H-4′/H-5′ and of H-2′ to H-4′/H-5′ were recorded for the first prenyl side chain, and correlations between H-1″ and H-2″/H-4″/H-5″ and H-2″ and H-4″/H-5″ were observed for the second (Figure 1). The ^{13}C NMR spectrum exhibited signals of 17 carbons further identified by a combined analysis of 1D NMR and ^{1}H−^{13}C HSQC spectra as two ketone carbonyl carbons C-1 (δ 199.3) and C-4 (δ 200.4); two olefinic carbons C-2′ (δ 121.4) and C-2″ (δ 120.0); two allylic methylene carbons C-1′ (δ 23.4) and C-1″ (δ 40.0); one methylene carbon C-6 at δ 49.5; five methyl groups C-4′ (δ 18.1), C-5′ (δ 26.3), C-4″ (δ 18.0), C-5″ (δ 26.1), and C-7 (δ 24.5); one quaternary sp^3-hybridized carbon C-5 at δ 49.5; and four nonprotonated sp^2-hybridized carbons C-2 (δ 127.0), C-3 (δ 157.4), C-3′ (δ 134.0), and C-3″ (δ 137.3). The resonances of signals at δ 199.3 (C-1) and δ 200.4 (C-4) corresponding to the signals of the two carbonyl groups were indicative of a quinonoid nucleus.[9]

The respective positions of the four substituents on the quinonoid nucleus were deduced from the long-range ^{1}H−^{13}C HMBC experiment and ^{1}H−^{1}H NOESY which further confirmed the attributions of ^{1}H and ^{13}C signals of the prenyl chains (Figure 2). Therefore, the ^{3}J ^{1}H−^{13}C HMBC correlation observed between the allylic methylene H-1′ and the ketone carbonyl carbon C-1 indicated the connectivity of the first prenyl chain at C-2. Further confirmation of this position was evidenced by the ^{3}J ^{1}H−^{13}C HMBC correlation of H-1′ to the OH-bearing carbon at C-3 (δ 157.24), indicating subsequently a C-3 position for the hydroxy group. The second prenyl unit on the other hand was attached at C-5. Its position was confirmed by a ^{3}J ^{1}H−^{13}C HMBC correlation of the allylic methylene H-1″ with the carbonyl group at δ 200.4 (C-4) as well as ^{3}J correlations of H-1″ with the methylene C-6 and the methyl C-7 subsequently indicating the position of the methyl group at C-5. The position of the methyl group C-7 placed at C-5 was also clearly evidenced by ^{3}J ^{1}H−^{13}C HMBC correlations between H-7 and C-1″/C-4/C-6. Moreover, the geminal position of the prenyl unit and the methyl group was substantiated by ^{1}H−^{1}H NOESY correlations between the methyl group C-7, the prenyl methylene C-1″, and the methylene C-6. The NOESY correlations of H-7 with H-1″a/H-1″b/H-6a/H-6b also provided information about the molecular conformation, suggesting the equatorial position of the methyl group C-7. In addition, the NOESY correlations observed between H-4″ and H-1″a/H-1″b and H-4′ and H-1′ confirmed the *cis* position of the methyl groups C-4″ and C-4′ with the methylene groups C-1″ and C-1′, respectively, asserting unambiguously the *cis* position of the methyl groups C-5″ and C-5′ with the olefinic protons H-2″ and H-2′, respectively. This assertion was clearly evidenced on the ^{1}H−^{1}H NOESY spectrum where correlations of H-5″ with H-2″ and H-5′ with C-2′ were recorded. The very low specific optical rotation (0.65, c 0.49) as well as the absence of a Cotton effect on its ECD spectrum (Figure S65) indicated that **7** was isolated as a racemic mixture. Therefore the structure of compound **7** was established as 3-hydroxy-5-methyl-2,5-bis(3-methylbut-2-en-1-yl)cyclohex-2-ene-1,4-dione named amylosporane G.

Compounds **8**−**10** were characterized by using 1D and 2D NMR spectroscopic data in combination with their HR-ESIMS spectra (Figures S67−S92). Literature searches in Sci-finder identified them as already known; however, to the best of our knowledge, they have never been isolated from a fungal source, and the NMR data of compounds **8** and **10** have never been reported.

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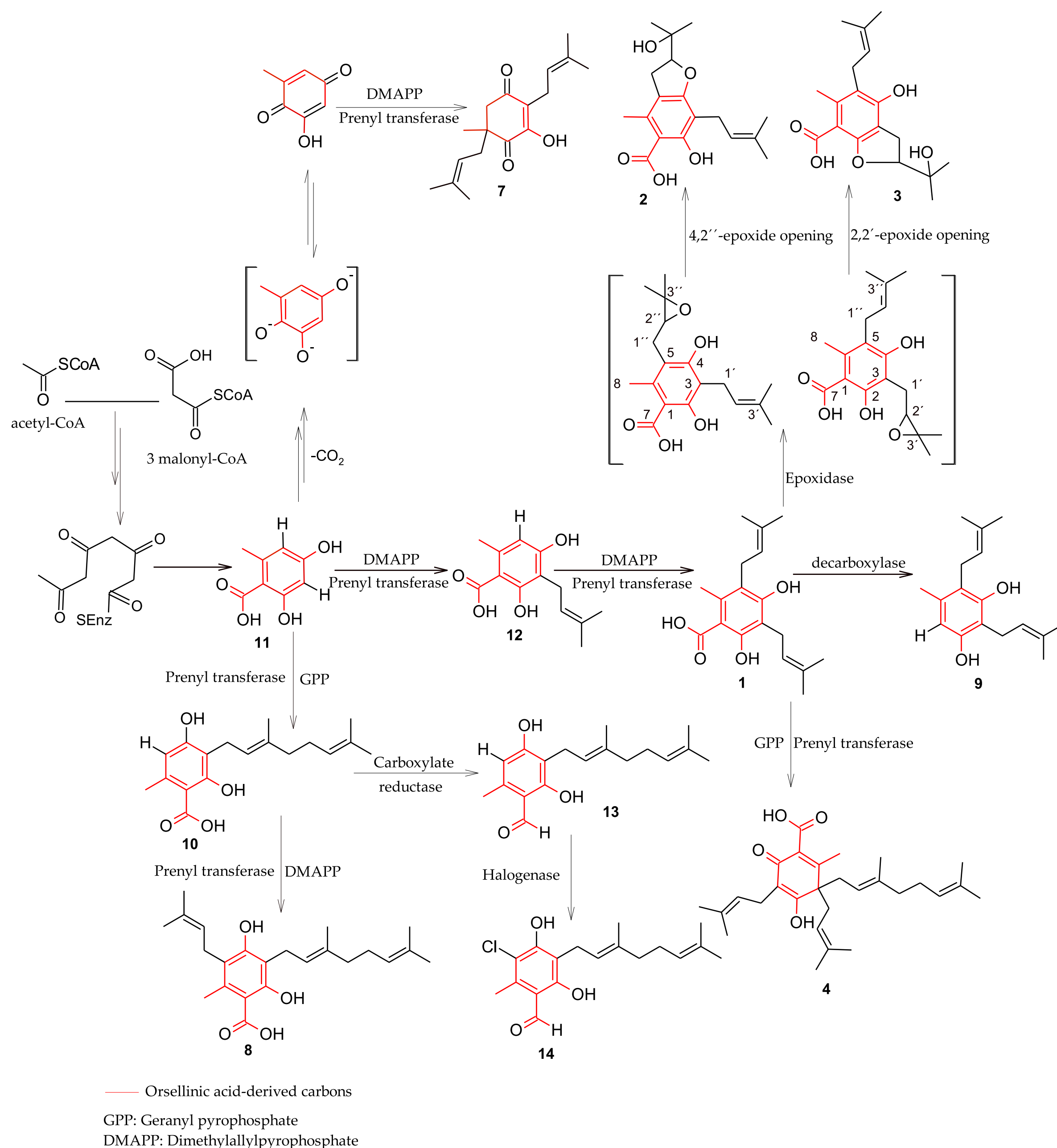
Scheme 1. Hypothesized Biogenetic Pathway of Compounds 1−4 and 7−14



Compound **8**, for which we assigned the trivial name amylosporane H, was obtained as a yellow oil and was previously detected by UPLC-DAD-MS as a possible acyclic precursor during the elucidation of the biosynthetic pathways of prenylated benzopyrans in the plant *Peperomia obtusifolia*.[10] However, to the best of our knowledge, it has never been isolated. Therefore, this is the first report for the isolation and structural characterization of this compound (Table 3).

Compound **9**, isolated as a yellow oil, was previously identified as a di-isoprenoid derivative of orcinol during the biogenetic-type synthesis of orcinol with 2-methyl-3-buten-2-ol;[11] however, it has never been isolated. Here, the isolation of this compound is reported for the first time from a natural source, and it was assigned the trivial name amylosporane I. Its ^{1}H and ^{13}C NMR spectroscopic data are reported in Table 3.

Compound **10**, named cannabigerorcinic acid, was isolated as a brownish oil. It was identified in previous studies as a minor phytocannabinoid derivative found in *Cannabis sativa* and resulting from the enzymatic reaction of orsellinic acid and geranyl pyrophosphate (GPP)[12] catalyzed by a non-natural aromatic prenyltransferase. However, to the best of our knowledge, it has never been isolated and characterized. Therefore, its ^{1}H and ^{13}C NMR data are reported for the first time in the present work (Table 3).

From a spectroscopic analysis and HR-ESIMS data and by comparison with the literature data, compounds **11**−**15** were identified: orsellinic acid (**11**),[13] colletorin D acid (**12**),[8] colletorin B (**13**),[14] colletochlorin B (**14**),[14] and cyclo-(*S*-Pro-*R*-Leu) (**15**).[15] The structure of compound **15** was confirmed to be cyclo-(*S*-Pro-*R*-Leu) not only based on its spectroscopic

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Table 4. Minimum Inhibitory Concentrations (MICs) of Compounds 1, 4, 9, 10, 13, and 14 against Tested Microorganisms

	MIC (μg/mL)						
test organism	1	4	9	10	13	14	positive control
Bacillus subtilis DSM 10	66.7		33.3	66.7	4.2	2.1	3.33
Candida albicans DSM 1665							33.3[a]
Chromobacterium violaceum DSM 30191			n.t.[b]				1.7[c]
Escherichia coli DSM 1116							8.3[c]
Micrococcus luteus DSM 1790		66.7	n.t.		33.33		0.83[c]
Mucor hiemalis DSM 2656	66.7		33.3		16.67	33.3	16.7[a]
Mycobacterium smegmatis ATCC 700084			n.t.				1.7[d]
Pichia anomala DSM 6766			n.t.				16.7[a]
Pseudomonas aeruginosa PA14			n.t.				0.4[e]
Rhodoturula glutinis DSM 10134			n.t.				4.2
Schizosaccharomyces pombe DSM 70572			n.t.				16.7[a]
Staphylococcus aureus DSM 346	66.7	33.33	33.33	66.7	8.33	1.04	0.4[c]

[a]Nystatin 1 mg/mL. [b]n.t.: not tested. Empty cells: no inhibition. [c]Oxytetracycline 1 mg/mL. Starting concentrations for the antimicrobial assay were 66.7 μg/mL. [d]Kanamycin 1 mg/mL. [e]Gentamicin 1 mg/mL.

analysis but also by a comparison of its optical rotation value (−87, *c* 0.51) with that reported in the literature.[15]

From a biosynthetic standpoint, *Amylosporus* cf. *campbelii* and *Amylosporus* cf. *graminicola* produce several prenylated compounds derived from orsellinic acid (**11**) which is considered as a first intermediate metabolite in the biosynthesis of aromatic compounds in fungi.[16] By undergoing several modifications such as prenylation, reduction, epoxidation, cyclization, decarboxylation, and halogenation, orsellinic acid leads to metabolites **1**−**4**, **7**−**10**, and **12**−**14** (Scheme 1). Although all of the reactions depicted in Scheme 1 are common in fungal secondary metabolite biosynthesis pathways and were reported in the proposed biosynthesis of the colletorin/colletochlorin family in the ascomycete *Colletotrichum higginsianum*,[8] it still remains uncertain that the genes encoding the enzymes involved in the biochemical route responsible for the biosynthesis of these secondary metabolites are the same within different fungal phyla. Considering that, the biosynthesis pathways of a given compound can vary from one organism to another as clearly illustrated by the gibberellin synthesis pathways in plants, fungi, and bacteria, which have evolved independently.[17] We can therefore only hypothesize that compounds **1**−**4**, **8**−**10**, and **12**−**14** isolated from the two *Amylosporus* strains might be synthesized through a combined route of the mevalonate pathway and the orsellinic acid associated pathway. This assumption requires further investigation as an in-depth biochemical investigation was not conducted, and the enzymes involved in the biosynthesis of these meroterpenoids remain unknown. In addition, the biosynthesis of compound **7** (possessing a quinone nucleus) seems to involve orsellinic acid as a main precursor. The methylcyclohexenone moiety in the core structure of **7** might originally be derived from orsellinic acid, following the same biosynthetic route as proposed for the synthesis of antroquinonols in *Antrodia cinnamomea*.[16] This also supports our hypothesis that orsellinic acid is an important precursor indispensable for the biosynthesis of meroterpenoids (**1**−**4**, **7**−**10**, and **12**−**14**) isolated from *Amylosporus* spp.

Antimicrobial and Cytotoxic Activity. The isolated metabolites except compounds **3** and **12** were evaluated for their antimicrobial activity. Minimum inhibitory concentration (MIC) values showed that only compounds **1**, **4**, **9**, **10**, **13**, and **14** were active against some of the organisms tested at a concentration of ≤66.7 μg/mL (Table 4), whereas the remaining compounds were inactive under test conditions (Table S1).

Furthermore, the ability of all isolated compounds (except **3** and **12**) to inhibit the proliferation of mammalian cell lines was examined. Five compounds showed activity against the cell lines tested (Table 5) whereas the others were inactive (Table S2). Colletochlorin B (**14**), the only compound possessing chlorine in its structure, showed the strongest activity against all cell lines tested with half maximal inhibitory concentration (IC_{50}) values in the range 4.6−20.2 μM. This is in agreement with previous studies that demonstrated cytotoxic activity of chlorinated colletorin derivatives.[14]

Table 5. Cytotoxic Effect (IC_{50}) of Compounds 1, 4, 9, 12, and 13 against Several Cancer Cell Lines

	IC_{50} (μM)					
cell line	1	4	9	13	14	epothilone B
L929	59.2	50.0	53.8	66.0	18.0	1.4×10^{-3}
KB3.1	42.7	10.2	57.7	62.5	7.7	6.9×10^{-5}
PC-3	n.t.[a]	10.0	n.t.	n.t.	5.3	7.1×10^{-5}
MCF-7	n.t.	12.9	n.t.	n.t.	4.9	8.1×10^{-5}
SKOV-3	n.t.	8.4	n.t.	n.t.	5.6	2.9×10^{-4}
A431	n.t.	15.4	n.t.	n.t.	4.6	6.7×10^{-5}
A549	n.t.	27.3	n.t.	n.t.	20.2	8.8×10^{-5}

[a]n.t.: not tested. Epothilone B 1 mg/mL. Starting concentrations for the cytotoxicity assay were 37 μg/mL.

EXPERIMENTAL SECTION

General Experimental Procedure. Electrospray ionization mass (ESI-MS) spectra were recorded with an UltiMate 3000 Series uHPLC instrument (Thermo Fischer Scientific, Waltman, MA) utilizing a C_{18} Acquity UPLC BEH column (2.1 × 50 mm, 1.7 μm; Waters, Milford, MA) connected to an amaZon speed ESI-Iontrap-MS instrument (Bruker, Billerica, MA). HPLC parameters were set as follows: solvent A, H_2O + 0.1% formic acid; solvent B, acetonitrile (ACN) + 0.1% formic acid; gradient, 5% B for 0.5 min increasing to 100% B in 19.5 min and then isocratic conditions at 100% B for 5 min, a flow rate of 0.6 mL/min, and a diode-array detection (DAD) of 190−600 nm. HR-ESIMS (high-resolution electrospray ionization mass spectrometry) spectra were recorded with an Agilent 1200 Infinity Series HPLC−UV system [Agilent Technologies, Santa Clara, CA; column 2.1 × 50 mm, 1.7 μm, C_{18} Acquity UPLC BEH

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(Waters); solvent A, H_2O + 0.1% formic acid; solvent B, ACN + 0.1% formic acid; gradient, 5% B for 0.5 min increasing to 100% B in 19.5 min and then maintaining 100% B for 5 min, flow rate 0.6 mL/min, UV/vis detection 200−640 nm] connected to a MaXis ESI-TOF mass spectrometer (Bruker) (scan range 100−2500 *m/z*, capillary voltage 4500 V, dry temperature 200 °C). A final purification of bioactive compounds was conducted using an Agilent 1100 preparative HPLC instrument (Agilent, Waldbronn, Germany). All chemicals were purchased from either AppiChem GmbH (Darmstadt, Germany) or Carl Roth GmbH (Karlsruhe, Germany). The highest-grade solvents utilized for HPLC runs were obtained from Merck Co. (Darmstadt, Germany). Optical rotations were recorded in methanol (Uvasol, Merck, Darmstadt, Germany) by using an Anton Paar MCP-150 polarimeter (Seelze, Germany) at 20 °C. UV/vis spectra were recorded using methanol (Uvasol, Merck, Darmstadt, Germany) with a Shimadzu UV/vis 2450 spectrophotometer (Kyoto, Japan). ECD spectra were obtained on a J-815 spectropolarimeter (JASCO, Pfungstadt, Germany). Nuclear magnetic resonance (NMR) spectra were recorded with an Avance III 500 spectrometer (Bruker, ^1H NMR, 500 MHz; and ^{13}C NMR, 125 MHz) and an Ascend 700 spectrometer with a 5 mm TXI cryoprobe (Bruker, ^1H NMR, 700 MHz; and ^{13}C NMR, 175 MHz). IR spectra were recorded with a PerkinElmer FT-IR spectrophotometer equipped with a Universal ATR sampling accessory.

Fungal Material. The following strains of *Amylosporus* were studied: *Amylosporus* cf. *campbelii* MUCL 41576. Zimbabwe, Matabeleland North, near Victoria falls, emerging from soil, among grasses. leg. C. Decock, Jan. 1996; *Amylosporus* cf. *graminicola* MUCL44090. Cuba, Sancti Spiritus, Falcon, emerging from soil, among grasses, leg. C. Decock, Aug. 2002.

The specimens and corresponding cultures are deposited at MUCL, Louvain-la-Neuve, Belgium (https://bccm.belspo.be/about-us/bccm-mucl).

Fermentation and Extraction. *Amylosporus* strains MUCL 41576 and MUCL 44090 were cultured on liquid Q6 1/2 medium for 3 months until glucose depletion. Glucose levels were checked regularly using Fehling's strips (Bayer Diastix Harnzuckerstreifen). Using a cork borer of 5 mm diameter, regular agar plugs were selected to inoculate the 5 L fermentation cultures composed of 25 sterile 500 mL Erlenmeyer flasks filled with 200 mL of medium. Preliminary cultures were prepared on oatmeal agar plates for around 7 days until sufficient agar masses were produced to start the liquid culture inoculation. Occasional samples were collected during the batch fermentation, and LCMS runs were conducted to detect an upsurge of secondary metabolite formation. Sterilized Q6 1/2 medium composed of cotton seed flour 5 g/L, glycerol 10 g/L, and D-glucose 2.5 g/L was selected based on previous optimization studies carried out in our laboratory.[18] The medium was prepared, and the pH was adjusted to 7.2; the medium was then autoclaved at 121 °C for 20 min. During the entire fermentation period, flasks were kept rotating at 140 rpm in the dark at 23 °C on a rotary shaker. Three days after glucose consumption and stagnation of secondary metabolite formation as revealed by HPLC analysis, mycelial extraction began with subsequent separation from the supernatant, vacuum filtration, and acetone extraction in an ultrasonic bath. The supernatant was extracted with Amberlite XAD-16N (Sigma-Aldrich, Darmstadt, Germany), 40 g per L, which was acetone extracted three times in an ultrasonic bath at 40 °C for half an hour; additionally, acetone portions were collected, and the solvent was evaporated and the remaining aqueous part extracted with ethyl acetate.[19] The ethyl acetate organic portion was dried over anhydrous sodium sulfate and concentrated to yield 0.8 g of a brownish black solid. The mycelia were extracted with acetone but were found to yield little of the target compounds and mainly fatty acids. Therefore, the extract was discarded.

Isolation of Compounds 1−15. Isolation procedures were carried out for the supernatant part using reversed phase HPLC (Gilson, PLC 2020, Middleton) by employing the C_{18} stationary phase in a VP-Nucleodur column 100-5 (Macherey-Nagel, 250 mm × 40 mm, 7 μm).[1] UV detection allowed the visualization of compounds at 210, 254, and 356 nm; the elution gradient comprised deionized water (Millipore, Milli-Q) and acetonitrile (ACN). Eight fractions were collected according to peak intensity using an elution gradient ranging from 15% to 100% ACN in 70 min. Fractions F1 and F2 were eluted by an increasing gradient of 15−40% ACN for 20 min and from 40% to 100% in 3 min followed by isocratic conditions at 100% ACN for 4 min. A Kromasil C_{18} preparative column of dimensions 250 mm × 20 mm (7 μm C18) was employed to give compounds **14** (5.33 mg) and **4** (5.04 mg) from F1 and compounds **10** (26 mg) and **11** (2.02 mg) from F2 through isocratic elution of 100% ACN for 15 min. Compounds **2** (2 mg), **5** (20 mg), and **6** (26 mg) were obtained from fractions F3 and F4 with a gradient elution of 35−76% ACN for 25 min; subsequently, a gradient shift was conducted from 76% to 100% ACN in 5 min, and a hold was maintained at 100% ACN for another 5 min. Fractions F5−F8, were combined together and purified to yield eight compounds, **8** (4.5 mg), **1** (3.66 mg), **15** (3.5 mg), **7** (4.6 mg), **9** (4.7 mg), **3** (1.35 mg), **13** (3.66 mg), and **12** (2 mg), using the gradient 50−80% ACN B for 25 min followed by a second gradient from 80% to 100% ACN in 3 min before running 100% ACN for 5 min. All isolated compounds, unless otherwise stated, were run on preparative HPLC using a Kromasil C_{18} column in 100% ACN for 15 min to attain the best chemical purity.

Antimicrobial Assay. All of the isolated metabolites except compounds **3** and **12** were evaluated for their antimicrobial activity against a panel of fungal microorganisms (*Pichia anomala*, *Schizosaccharomyces pombe*, *Mucor hiemalis*, *Candida albicans*, and *Rhodotorula glutinis*), Gram positive-bacteria (*Micrococcus luteus*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Mycobacterium smegmatis*), and Gram-negative bacteria (*Chromobacterium violaceum*, *Escherichia coli*, and *Pseudomonas aeruginosa*). An assessment of the antimicrobial activity was determined using a serial dilution assay in 96-well plates, and the MIC was calculated according to our established protocol.[20,21] Basically, the compounds dissolved in methanol with a concentration of 1 mg/mL were diluted in the range 66.7−0.52 μg/mL and incubated with the test organisms overnight. The next day, growth of the test organisms was visually observed, and the MIC was determined at the lowest concentration where a clear zone (no growth of the test organisms) was observed. Methanol, being the compounds' dissolving solvent, was also used as a negative control. Oxytetracycline, ciprofloxacin, gentamicin, and kanamycin were used as a positive control against bacteria whereas nystatin was used against fungi. A detailed experimental procedure can be found in the SI.

Cytotoxicity Assay. The in vitro cytotoxicity (IC_{50}) of the isolated metabolites except compounds **3** and **12** against several mammalian cell lines (human endocervical adenocarcinoma KB 3.1, breast cancer MCF-7, lung cancer A549, ovary cancer SK-OV-3, prostate cancer PC-3, squamous cancer A431, and mouse fibroblasts L929) was determined by a colorimetric tetrazolium dye MTT assay[22] using epothilone B as a positive control in accordance to our previously reported experimental procedure.[20,23] A detailed protocol can be found in the SI.

*Amylosporane A (**1**).* Yellow oil. UV (MeOH, c = 0.01 mg/mL) λ_{max} (log ε) 200 (4.23), 220 (4.29), 256 (3.72), 302 (3.48) nm. HR-ESIMS *m/z* 327.1567 $[M + Na]^+$; *m/z* 631.3243 $[2M + Na]^+$; *m/z* 287.1640 $[M + H - H_2O]^+$; *m/z* 305.1747 $[M + H]^+$ (calcd for $C_{18}H_{25}O_4$, 305.1747). For NMR data, see Table 1.

*Amylosporane B (**2**).* Yellow oil. $[\alpha]^{20}_D$ = −0.49 (c 0.41, MeOH). UV (MeOH, c = 0.01 mg/mL) λ_{max} (log ε) 215 (4.23), 260 (3.69), 304 (3.49) nm. HR-ESIMS *m/z* 343.1516 $[M + Na]^+$; *m/z* 663.3143 $[2M + Na]^+$; *m/z* 303.1590 $[M + H - H_2O]^+$; *m/z* 321.1697 $[M + H]^+$ (calcd for $C_{18}H_{25}O_5$, 321.1697). For NMR data, see Table 1.

*Amylosporane C (**3**).* Yellow oil. HR-ESIMS *m/z* 343.1518 $[M + Na]^+$; *m/z* 663.3144 $[2M + Na]^+$; *m/z* 303.1591 $[M + H - H_2O]^+$; *m/z* 321.1697 $[M + H]^+$ (calcd for $C_{18}H_{25}O_5$, 321.1697). For NMR data, see Table 1.

*Amylosporane D (**4**).* Brownish oil. $[\alpha]^{20}_D$ = 1.25 (c 0.319, MeOH). UV (MeOH, c = 0.01 mg/mL) λ_{max} (log ε) 201 (4.22), 341 (3.28) nm. HR-ESIMS *m/z* 463.2818 $[M + Na]^+$; *m/z* 903.5762 $[2M + Na]^+$; *m/z* 423.2897 $[M + H - H_2O]^+$; *m/z* 441. 2998 $[M + H]^+$ (calcd for $C_{28}H_{41}O_4$, 441.2999). For NMR data, see Table 1.

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*Amylosporane E (**5**)*. Yellow oil. UV (MeOH, c = 0.01 mg/mL) λ_{max} (log ε) 201 (3.9), 289 (3.96) nm. HR-ESIMS m/z 205.0835 [M + Na]$^+$; m/z 165.0910 [M + H − H_2O]$^+$; m/z 183.1014 [M + H]$^+$ (calcd for $C_{10}H_{15}O_3$, 183.1016). For NMR data, see Table 2.

*Amylosporane F (**6**)*. Yellow amorphous solid. UV (MeOH, c = 0.02 mg/mL) λ_{max} (log ε) 201 (3.75), 284 (3.55), 355 (3.76) nm. HR-ESIMS m/z 231.0623 [M + Na]$^+$; m/z 191.0698 [M + H − H_2O]$^+$; m/z 209.0804 [M + H]$^+$ (calcd for $C_{11}H_{13}O_4$, 209.0808). For NMR data, see Table 2.

*Amylosporane G (**7**)*. Beige needlelike crystal. $[\alpha]^{20}_D$ = 0.65 (c 0.49, MeOH). UV (MeOH, c = 0.01 mg/mL) λ_{max} (log ε) 201 (4.11), 294 (3.74), 341 (3.55) nm. HR-ESIMS m/z 299.1616 [M + Na]$^+$; m/z 277.1795 [M + H]$^+$ (calcd for $C_{17}H_{25}O_3$, 277.1798). For NMR data, see Table 2.

*Amylosporane H (**8**)*. Yellow oil. UV (MeOH, c = 0.01 mg/mL) λ_{max} (log ε) 201 (4.46), 248 (4.18), 306 (3.96) nm. HR-ESIMS m/z 395.2191 [M + Na]$^+$; m/z 767.4492 [2M + Na]$^+$; m/z 355.2266 [M + H − H_2O]$^+$; m/z 373.2372 [M + H]$^+$ (calcd for $C_{23}H_{33}O_4$, 373.2373). For NMR data, see Table 3.

*Amylosporane I (**9**)*. Yellow oil. UV (MeOH, c = 0.01 mg/mL) λ_{max} (log ε) 205 (4.14), 248 (3.99), 306 (3.74) nm. HR-ESIMS m/z 283.1847 [M + Na]$^+$; m/z 261.1847 [M + H]$^+$ (calcd for $C_{17}H_{25}O_2$, 261.1849). For NMR data, see Table 3.

*Cannabigerorcinic Acid (**10**)*. Brownish oil. UV (MeOH, c = 0.01 mg/mL) λ_{max} (log ε) 201 (4.25), 219 (4.22), 248 (4.11), 306 (3.86) nm. HR-ESIMS m/z 327.1564 [M + Na]$^+$; m/z 631.3230 [2M + Na]$^+$; m/z 287.1639 [M + H − H_2O]$^+$; m/z 305.1744 [M + H]$^+$ (calcd for $C_{18}H_{25}O_4$, 305.1747). For NMR data, see Table 3.

*Orsellinic Acid (**11**)*. Yellow oil. HR-ESIMS m/z 191.0314 [M + Na]$^+$; m/z 359.0623 [2M + Na]$^+$; m/z 151.0390 [M + H − H_2O]$^+$; m/z 169.0495 [M + H]$^+$ (calcd for $C_8H_9O_4$, 169.0495). ^{1}H NMR data (MeOH-d_4, 500 MHz): δ_H 6.19 (dd, J = 2.44 Hz, 0.76 Hz, 1H, H-5), δ_H 6.14 (d, J = 2.44 Hz, 1H, H-3), δ_H 2.48 (s, 3H, H-8).[13]

*Colletorin D Acid (**12**)*. Yellow oil. HR-ESIMS m/z 259.0942 [M + Na]$^+$; m/z 495.1884 [2M + Na]$^+$; m/z 219.1014 [M + H − H_2O]$^+$; m/z 237.1119 [M + H]$^+$ (calcd for $C_{13}H_{17}O_4$, 237.1121). ^{1}H NMR data (MeOH-d_4, 500 MHz): δ_H 6.19 (s, 1H, H-5), δ_H 5.20 (tt, J = 7.32, 1.22 Hz, 1H, H-2′), δ_H 3.25 (d, J = 7.17, 2H, H-1′), δ_H 2.48 (s, 3H, H-8), δ_H 1.75 (s, 3H, H-4′), δ_H 1.65 (s, 3H, H-5′).[8]

*Colletorin B (**13**)*. Pale brown oil. HR-ESIMS m/z 311.1617 [M + Na]$^+$; m/z 289.1798 [M + H]$^+$ (calcd for $C_{18}H_{25}O_3$, 289.1798). ^{1}H NMR data (MeOH-d_4, 500 MHz): δ_H 10.04 (s, 1H, H-7), δ_H 6.22 (s, 1H, H-5), δ_H 5.19 (td, J = 7.26, 1.07 Hz, 1H, H-2′), δ_H 5.05 (tt, J = 7.06, 1.26 Hz, 1H, H-7′), δ_H 3.25 (d, J = 7.32, 2H, H-1′), δ_H 2.47 (s, 3H, H-8), δ_H 2.04 (q, J = 7.38, 2H, H-6′), δ_H 1.94 (t, J = 7.48, 2H, H-5′), δ_H 1.75 (s, 3H, H-4′), δ_H 1.60 (s, 3H, H-10′), δ_H 1.55 (s, 3H, H-9′).[14]

*Colletochlorin B (**14**)*. Pale brown oil. HR-ESIMS m/z 345.1230 [M + Na]$^+$; m/z 347.1204 [M + 2 + Na]$^+$; m/z 325.1383 [M + 2 + H]$^+$; m/z 323.1410 [M + H]$^+$ (calcd for $C_{18}H_{24}ClO_3$, 323.1408). ^{1}H NMR data (MeOH-d_4, 500 MHz): δ_H 10.16 (s, 1H, H-7), δ_H 5.19 (br t, J = 7.17 Hz, 1H, H-2′), δ_H 5.03 (br t, J = 7.10 Hz, 1H, H-7′), δ_H 3.35 (d, J = 7.17, 2H, H-1′), δ_H 2.60 (s, 3H, H-8), δ_H 2.05 (q, J = 7.17, 2H, H-6′), δ_H 1.95 (t, J = 7.32, 2H, H-5′), δ_H 1.77 (s, 3H, H-4′), δ_H 1.59 (s, 3H, H-10′), δ_H 1.54 (s, 3H, H-9′).[14]

*Cyclo-(S-Pro-R-Leu) (**15**)*. Pale yellow crystal. $[\alpha]^{20}_D$ = −87 (c 0.51, MeOH), HR-ESIMS m/z 233.1258 [M + Na]$^+$; m/z 413.2635 [2M + Na]$^+$; m/z 193.0833 [M + H − H_2O]$^+$; m/z 211.1441 [M + H]$^+$ (calcd for $C_{11}H_{19}N_2O_2$, 211.1441). ^{1}H NMR data (MeOH-d_4, 500 MHz): δ_H 4.26 (m, 1H, H-6), δ_H 4.13 (m, 1H, H-9), δ_H 3.51 (m, 2H, H-3), δ_H 2.30 (m, 1H, H-5a), δ_H 2.02 (m, 1H, H-5b), δ_H 2.02 (m, 1H, H-4a), δ_H 1.92 (m, 1H, H-4b), δ_H 1.92 (m, 1H, H-10a), δ_H 1.87 (m, 1H, H-11), δ_H 1.52 (m, 1H, H-10b), δ_H 0.97 (d, J = 3.05, 3H, H-12), δ_H 0.95 (d, J = 2.9, 3H, H-13).[15]

CONCLUSION

The potential of basidiomycetes as a prolific source for the discovery of new biologically active secondary metabolites has been reported for decades. The present study was the first on the secondary metabolism of the basidiomycete genus *Amylosporus* and led to the isolation of 15 compounds, seven of which were found to constitute novel natural products. Most of them were identified as polyprenylated orsellinic acid derivatives. So far, this class of compounds has only been isolated from the pathogenic ascomycete *Colletotrichum* sp. and were never before found in basidiomycetes. The present study therefore demonstrated that a chemical and biological evaluation of the potential of basidiomycetes from underexploited regions like the African tropics and the Caribbean remains of great value for the discovery of new bioactive secondary metabolites.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.1c00975.

HR-ESIMS, ESIMS, and 1D and 2D NMR spectra of compounds **1−10**; UV/vis spectra of compounds **1−2** and **4−10**; ECD spectrum of compounds **2**, **4**, and **7**; IR spectrum of compound **4**; HR-ESIMS, ESIMS, and ^{1}H NMR spectra of compounds **11−15**; and antimicrobial assay and cytotoxicity assay protocols (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Sandargo, B.; Chepkirui, C.; Cheng, T.; Chaverra-Muñoz, L.; Thongbai, B.; Stadler, M.; Hüttel, S. *Biotechnol. Adv.* **2019**, *37*, 107344.

(2) Gressler, M.; Löhr, N. A.; Schäfer, T.; Lawrinowitz, S.; Seibold, P. S.; Hoffmeister, D. *Nat. Prod. Rep.* **2021**, *38*, 702−722.

(3) Cheng, T.; Chepkirui, C.; Decock, C.; Matasyoh, J. C.; Stadler, M. *J. Nat. Prod.* **2019**, *82*, 1283−1291.

(4) Chepkirui, C.; Matasyoh, J. C.; Decock, C.; Stadler, M. *Phytochem. Lett.* **2017**, *20*, 106−110.

(5) Cheng, T.; Chepkirui, C.; Decock, C.; Matasyoh, J. C.; Stadler, M. *J. Nat. Prod.* **2020**, *83*, 2501−2507.

(6) Ryvarden, L. *Norw. J. Bot.* **1973**, *20*, 1−5.

(7) Wijayawardene, N. N.; Hyde, K. D.; Al-Ani, L. K. T.; Tedersoo, L.; Haelewaters, D.; Rajeshkumar, K. C.; Deng, C.; et al. *Mycosphere* **2020**, *11*, 1060−1456.

(8) Dallery, J. F.; Le Goff, G.; Adelin, E.; Iorga, B. I.; Pigné, S.; O'Connell, R. J.; Ouazzani, J. *J. Nat. Prod.* **2019**, *82*, 813−822.

(9) Mahiou, V.; Roblot, F.; Hocquemiller, R.; Cavé, A.; Rojas De Arias, A.; Inchausti, A.; Yaluff, G.; Fournet, A. *J. Nat. Prod.* **1996**, *59*, 694−697.

(10) Batista, A. N. L.; Santos-Pinto, J. R. A. D.; Batista, J. M. J.; Souza-Moreira, T. M.; Santoni, M. M.; Zanelli, C. F.; Kato, M. J.; López, S. N.; Palma, M. S.; Furlan, M. T. *J. Nat. Prod.* **2017**, *80*, 1275−1286.

(11) Manners, G.; Jurd, L.; Stevens, K. *Tetrahedron* **1972**, *28*, 2949−2959.

(12) Noble, M. A.; Hoff, K. G.; Komor, R. S. WIPO Patent WO/2021/046367 A2, 11 March 2021.

(13) Van Eijk, G. W. *Antonie van Leeuwenhoek* **1969**, *35*, 497−504.

(14) Gutiérrez, M.; Theoduloz, C.; Rodríguez, J.; Lolas, M.; Schmeda-Hirschmann, G. *J. Agric. Food. Chem.* **2005**, *53*, 7701−7708.

(15) Adamczeski, M.; Reed, A. R.; Crews, P. *J. Nat. Prod.* **1995**, *58*, 201−208.

(16) Yu, P. W.; Cho, T. Y.; Liou, R. F.; Tzean, S. S.; Lee, T. H. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 4701−4711.

(17) Salazar-Cerezo, S.; Martínez-Montiel, N.; García-Sánchez, J.; Pérez-Y-Terrón, R.; Martínez-Contreras, R. D. *Microbiol. Res.* **2018**, *208*, 85−98.

(18) Moussa, A. Y.; Lambert, C.; Stradal, T.; Ashrafi, S.; Maier, W.; Stadler, M.; Helaly, S. E. *Antibiotics* **2020**, *9*, 132.

(19) Chepkirui, C.; Richter, C.; Matasyoh, J. C.; Stadler, M. *Phytochemistry* **2016**, *132*, 95−101.

(20) Becker, K.; Wessel, A. C.; Luangsa-Ard, J. J.; Stadler, M. *Biomolecules* **2020**, *10*, 805.

(21) Matio Kemkuignou, B.; Treiber, L.; Zeng, H.; Schrey, H.; Schobert, R.; Stadler, M. *Molecules* **2020**, *25*, 5497.

(22) Mosmann, T. *J. Immunol. Methods.* **1983**, *65*, 55−63.

(23) Primahana, G.; Narmani, A.; Surup, F.; Teponno, R. B.; Arzanlou, M.; Stadler, M. *Biomolecules* **2021**, *11*, 783.