

Cytotoxic Metabolites from the Fungal Endophyte *Alternaria* sp. and Their Subsequent Detection in Its Host Plant *Polygonum senegalense*

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From the Egyptian medicinal plant *Polygonum senegalense* the fungal endophyte *Alternaria* sp. was isolated. Extracts of the fungus grown either in liquid culture or on solid rice media exhibited cytotoxic activity when tested *in vitro* against L5178Y cells. Chromatographic separation of the extracts yielded 15 natural products, out of which seven were new compounds, with both fungal extracts differing considerably with regard to their secondary metabolites. Compounds **1**, **2**, **3**, **6**, and **7** showed cytotoxic activity with EC₅₀ values ranging from 1.7 to 7.8 μg/mL. When analyzed *in vitro* for their inhibitory potential against 24 different protein kinases, compounds **1–3**, **5–8**, and **15** inhibited several of these enzymes (IC₅₀ values 0.22–9.8 μg/mL). Interestingly, compounds **1**, **3**, and **6** were also identified as constituents of an extract derived from healthy leaves of the host plant *P. senegalense*, thereby indicating that the production of natural products by the endophyte proceeds also under *in situ* conditions within the plant host.

Endophytes are microorganisms that colonize living, internal tissues of plants without causing any immediate, overtly negative effects.¹ Their relationships with host plants range from symbiotic to slightly pathogenic. Both types of associations are apparently asymptomatic and inconspicuous.² It has been hypothesized that the interaction between fungal endophytes and their host plants is characterized by a finely tuned equilibrium between fungal virulence and plant defense, in which disease symptoms may develop if the association is disturbed by either a weakening of plant defense or an increase in fungal virulence.³ Moreover, endophytic fungi are thought to interact mutualistically with their host plant, so that the host plant provides nutrients to the endophyte, which in turn produces bioactive substances to enhance the growth and competitiveness of the host in nature.^{4–6} Accordingly, endophytes have been identified as a prolific source of a wide array of new pharmacologically active secondary metabolites that might prove suitable for specific medicinal or agrochemical applications.⁷

In this study, we have investigated the endophytic fungal strain *Alternaria* sp., which was isolated from the leaves of the traditional medicinal plant *Polygonum senegalense* growing in Egypt. External application of an extract obtained from fresh leaves of this plant is reported in folk medicine to be effective in treating skin troubles.⁸ Furthermore, species of *Polygonum* are known in traditional medicine for their diuretic, cholagic, antihemorrhagic, and antiseptic properties.⁹

Alternaria species have a widespread distribution. Many species are plant pathogens, which cause both pre- and postharvest decay.

A number of metabolites of polyketide origin, including α-dibenzopyrones such as alternariol (**1**) and its 5-*O*-methyl ether (**3**), also known as djalonenzone, have been isolated previously from *Alternaria* spp. Several of these metabolites are reported to be toxic to mammalian systems. In this context, it is interesting to note that recently the estrogenic potential of alternariol (**1**), as well as its inhibitory effect on cell proliferation by interference with the cell cycle, was reported, as observed by flow cytometry and microscopy of cultured mammalian cells.¹⁰

Results and Discussion

From extracts of *Alternaria* sp. grown in liquid Wickerham medium were obtained new sulfated derivatives of alternariol and its monomethyl ethers (**2** and **4**) as well as the known compounds alternariol (**1**), alternariol 5-*O*-methyl ether (**3**), altenusin (**6**), 2,5-dimethyl-7-hydroxychromone (**13**), tenuazonic acid (**14**), and altertoxin I (**15**). When grown alternatively on solid rice medium, four new compounds were afforded, identified as 3'-hydroxyalternariol 5-*O*-methyl ether (**5**), desmethylaltenusin (**7**), alterlactone (**8**), and alternaric acid (**10**), and, in addition to **1**, **3**, and **6**, the also known talaroflavone (**9**) and altenuene (**11**). Furthermore, a new stereoisomer of altenuene was isolated from the fungus grown on solid rice medium, namely, 4'-epialtenuene (**12**).

The EtOAc extract of liquid cultures of *Alternaria* sp. was partitioned between *n*-hexane and 90% MeOH. Column chromatography of the 90% MeOH-soluble portion of the organic broth extract on Sephadex LH-20 yielded six compounds (**1**, **3**, **6**, **13**, **14**, and **15**). These compounds were identified on the basis of their UV, ¹H NMR, ¹³C NMR, and mass spectrometric data and comparison with published data as alternariol (**1**),¹¹ alternariol 5-*O*-methyl ether (**3**),¹² altenusin (**6**),¹³ talaroflavone (**9**),¹⁴ 2,5-dimethyl-7-hydroxychromone (**13**),¹⁵ tenuazonic acid (**14**),¹⁶ and altertoxin I (**15**).^{17–19}

From the *n*-butanol portion two rather polar compounds (**2** and **4**) were obtained by column chromatography on Sephadex LH-20. Compound **2** was isolated as reddish-white needles. Its UV spectrum showed considerable similarity to UV spectra typical for alternariol derivatives. The HRESI/MS exhibited a peak at *m/z* 339.0170 [M + H]⁺, indicating a molecular formula of C₁₄H₁₀O₈S. The ¹H, ¹³C,

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Table 1. Cytotoxicity Assay for *Alternaria* Extracts and Isolated Compounds

sample tested	L5178Y growth in % (@ 10 $\mu\text{g/mL}$)	EC ₅₀ ($\mu\text{g/mL}$)
<i>Alternaria</i> liquid <i>n</i> -BuOH	99.8	
<i>Alternaria</i> liquid EtOAc	52.8	
<i>Alternaria</i> rice EtOAc	0.1	
1	3.7	1.7
2	0.9	4.5
3	1.8	7.8
4	89.0	
5	47.1	
6	1.2	6.8
7	0.8	6.2
8	11.8	
9	92.9	
10	101.2	
11 + 12	94.0	
13	95.0	
14	57.9	
15	77.3	
kahalalide F (positive control)		6.3

HMBC, and NOE NMR data (Tables 2 and 3) were consistent with the carbon skeleton of alternariol (**1**), except for the prominent downfield shifts observed for H-4, C-4, H-6, and C-6, together with a pronounced upfield shift of C-5, which were indicative of a sulfate substituent at C-5.²⁰ This assumption was confirmed by a conspicuous fragment ion corresponding to the loss of 80 amu in the mass spectrum of **2** and the hypsochromic shift in the UV spectrum of **2** compared to that of **1**, which is attributed to the electron-withdrawing effect of the sulfate group.²¹ Thus, compound **2** was identified as the new natural product alternariol 5-*O*-sulfate.

The molecular formula of a second new compound (**4**) was determined as C₁₅H₁₂O₈S, on the basis of the [M + H]⁺ signal at *m/z* 353.0320 in the HRESI/MS, thus revealing an increase in the molecular weight of 14 amu compared to **2**. Comparison of the spectroscopic properties of **4** with those of **2** and **3** (Tables 2 and 3) in conjunction with the observed HMBC correlations indicated the presence of the same carbon framework as in alternariol 5-*O*-methyl ether (**3**). Similar differences in the UV, NMR, and mass spectra of **4** and **3** to those described above for **2** and **1** led to the identification of **4** as the new natural product alternariol 5-*O*-methyl ether-4'-*O*-sulfate.

The 90% MeOH-soluble material from the solid rice culture of *Alternaria* sp. was fractionated by vacuum-liquid chromatography (VLC) on silica gel. Subsequent purification of the fractions was performed by preparative HPLC, affording seven compounds (**5**, **7–9**, **10**, **11**, and **12**).

The HRESI/MS of the new compound **5** showed a pseudomolecular ion at *m/z* 289.0720 ([M + H]⁺), indicative of a molecular formula of C₁₅H₁₂O₆ and thus having one additional oxygen atom as compared to **3**. Compound **5** was isolated as violet needles, displaying a UV spectrum that had a close similarity to that of **3**. The ¹H NMR spectrum (Table 2) likewise resembled that of **3** except for the absence of one *meta*-coupled pair of aromatic protons and the presence of an aromatic proton singlet at δ 6.82 assigned for H-5'. In the HMBC spectrum (Table 2), this proton signal was found to correlate to C-1', C-3', C-4', and CH₃-6' with an upfield shift observed for C-4', C-5', and C-6' and a downfield shift for C-3' compared to the corresponding chemical shifts in **1–4**. Accordingly, **5** was identified as the new natural product 3'-hydroxyalternariol 5-*O*-methyl ether.

Compound **7** was isolated as a viscous, reddish oil. The HRESI/MS exhibited a prominent peak at *m/z* 299.0520 [M + Na]⁺, indicating a molecular formula of C₁₄H₁₂O₆, consistent with the loss of CH₂ compared to altenuin (**6**). Both compounds showed similar UV, NMR (Table 4), and MS data, except for the lack of the methoxyl group in **7**. Hence, **7** was identified as desmethylaltenuin, representing a new natural product.

The molecular formula of compound **8** was determined as C₁₅H₁₂O₆ on the basis of its pseudomolecular ion [M + H]⁺ at *m/z* 289.0700 obtained by HRESI/MS. The ¹³C NMR spectrum (Table 6) displayed one methoxyl group, 12 aromatic carbons, a carbonyl function, and an oxygenated methylene group corresponding to a pair of doublets at δ_{H} 4.80 and 4.85 (*J* = 11.1 Hz) in the ¹H NMR spectrum (Table 6). A ³*J* HMBC correlation of these protons with the carbonyl carbon (C-7) indicated that the lactone ring in **8** was not formed through connection of the carbonyl carbon to the phenolic hydroxyl group, as in the previously discussed alternariol derivatives, but instead was formed through a hydroxylated methyl group to yield an additional seven-membered ring. Two *meta*-coupled hydrogens, observed in the ¹H NMR spectrum at δ 6.45 and 6.50 (each doublet, *J* = 2.2 Hz), were assigned to H-9 and H-11, respectively. In the ROESY spectrum, both protons showed correlations (Table 6) to the methoxyl group, indicating its location at C-10. In addition, the correlations observed from H-9 and H-11 to C-7a in the HMBC spectrum (Table 6), as well as the chelated nature of OH-8 deduced from its appearance as a sharp signal at δ_{H} 10.21, confirmed the attachment of the aromatic ring to the lactone ring at C-7a/C11a. Furthermore, two hydroxyl substituents were placed in an *ortho*-position at C-2 and C-3 on the basis of the chemical shift values of the respective carbon atoms appearing at δ_{C} 146.6 and 145.9, respectively. The respective neighboring protons, H-1 and H-4, observed at δ 7.03 and 6.90 in the ¹H NMR spectrum, respectively, were assigned from their HMBC correlations, in turn, to C-3 and C-2. Furthermore, H-4 showed correlations to the methylene group in both the ROESY and the HMBC spectrum as well as HMBC correlations to C-4a and C-11b. On the other hand, a prominent NOE between H-1 and H-11 as well as the HMBC correlations of H-11 to C-11b and those of H-1 to C-4a, C-11a, and C-11b indicated the connection of the two aromatic rings through the C-11a–C-11b bond. The dibenzooxepinone skeleton thus established was further confirmed by comparing the NMR data of **8** to those reported for ulocladol²² and graphisactone D.²³ Both compounds differ from **8** in having an additional hydroxyl function at C-1 as well as methoxyl groups at C-3 (both) and C-8 (graphisactone D). Accordingly, **8** was identified as a new natural product, for which the name alterlactone is proposed. It is worth mentioning that this is only the third report of a compound featuring this carbon skeleton from nature. Biogenetically it should be derived from altenuin (**6**) by hydroxylation at the aromatic methyl group, followed by lactone ring closure with the carboxyl function at the adjacent aromatic ring system.

The HRESI/MS of the new compound **10** showed the pseudomolecular ion [M + Na]⁺ at *m/z* 301.0700, providing the molecular formula C₁₄H₁₄O₆. The ¹H NMR spectrum (Table 4) displayed *meta*-coupled hydrogens at δ 6.40 and 6.07 (each as a doublet, *J* = 2.5 Hz) corresponding to H-6 and H-4, respectively, and a methoxyl group at δ 3.78, thus resembling the ¹H NMR data observed for the aromatic rings of metabolites **6**, **7**, and **9**. Similar to talaroflavone (**9**), a secondary alcoholic group was detected as a carbinolic hydrogen at δ_{H} 4.38 and the corresponding ¹³C NMR signal at δ 73.2, which was found to be part of a saturated spin system by coupling to two methylene hydrogens at δ_{H} 2.49 (brd, *J* = 17.0 Hz) and 3.00 (dd, *J* = 6.9, 17.0 Hz) (Table 4). A singlet at δ 1.99 (3H) in the ¹H NMR spectrum with a corresponding ¹³C NMR signal at δ 17.8 indicated the presence of a vinyl methyl group. This was located as being adjacent to the methylene protons on the basis of the long-range COSY correlations observed for CH₃-2' to CH₂-3' as well as similar correlations appearing in the ROESY spectrum (Table 4). The ROESY spectrum showed also strong correlations between the CH₃-2' group and H-4, as well as between the methoxyl signal and both H-4 and H-6. Furthermore, interpretation of the HMBC spectrum (Table 4) revealed correlations of CH₃-2' to C-1', C-2', and C-3' as well as H-3' to C-5', confirming the cyclopentenone substructure. This was corroborated by the cor-

Table 2. ¹HNMR and HMBC Data of **1**, **2**, **4**, and **5** at 500 MHz

position	1^a		2^a		4^a		5^b	
	δ_{H} mult. (J Hz)	HMBC	δ_{H} mult. (J Hz)	HMBC	δ_{H} mult. (J Hz)	HMBC	δ_{H} mult. (J Hz)	HMBC
4	6.32 d (2.0)	2,3,6	6.86 d (2.2)	2,3,5,6	6.52 d (2.5)	2,3,6	6.63 d (1.8)	2,3,5,6
6	7.20 d (2.0)	2,4,5,1'	7.83 d (1.8)	2,4,5	7.27 d (2.5)	2,4,5,1'	7.30 d (1.8)	2,4,5,1'
3'	6.55 d (2.5)	1',2',4',5'	6.57 d (1.8)	1',2',4',5'	7.14 d (2.5)	2',4',5'		
5'	6.65 d (2.5)	CH ₃ , 1',3',4'	6.67 d (2.2)	CH ₃ , 1',3',4'	7.08 d (2.5)	CH ₃ , 1',3',4'	6.82 s	CH ₃ , 1',3',4'
CH ₃	2.71 s	1',5',6'	2.75 s	1',5',6'	2.74 s	1',5',6'	2.74 s	1',5',6'
OCH ₃					3.84 s	5	3.99 s	5

^a Recorded in MeOH-*d*₄. ^b Recorded in DMF-*d*₇.

Table 3. ¹³C NMR Data of **1**, **2**, **4**, and **5** at 125 MHz

position	1^a	2^a	4^a	5^b
	δ_{C}	$\delta_{\text{C}}^{\text{c}}$	δ_{C}	δ_{C}
1	139.8	n.d.	138.8	139.5
2	99.1	101.6	100.5	99.2
3	166.1	160.1	165.8	165.4
4	101.9	106	100.9	99.7
5	166.8	160.1	168.1	167.3
6	105.4	108	105.9	104.2
7	166.8	n.d.	n.d.	165.8
1'	110.9	109.7	115.1	110.1
2'	154.4	153.2	153.6	142.3
3'	102.7	101.3	108.8	132.4
4'	159.8	159	154.5	148.2
5'	118.5	117.6	122.9	117.7
6'	140	139	139.5	127.3
CH ₃	25.8	24.6	25.7	24.9
OCH ₃			56.3	56.3

^a Recorded in MeOH-*d*₄. ^b Recorded in DMF-*d*₇. ^c Derived from HMBC spectrum (sample decomposed before ¹³C NMR was acquired). n.d.: not detected (due to missing correlations or low sample quantity, respectively).

relation observed from H-4 to C-1' in the HMBC spectrum, which also established the C-3–C-1' bond, linking both rings.

In order to determine the absolute configuration of the metabolite, the modified Mosher procedure was applied.²⁴ The observed shift differences between the (*S*)-MTPA ester and its (*R*)-MTPA ester epimer²⁵ allowed the assignment of the chiral center at C-4' to the *R* configuration, as shown in **10** (Table 5). The compound was identified as a new natural product, for which the name alternarienic acid is proposed.

Compounds **11** and **12** were obtained as an inseparable mixture forming a viscous, yellow oil. Their HRESI/MS showed a [M + H]⁺ signal at *m/z* 293.1020, indicating the molecular formula C₁₅H₁₆O₆. The major compound in the fraction was identified as altenuene (**11**) by comparison of its UV, ¹H NMR, ¹³C NMR, and mass spectrometric data with those published previously.²⁶ An in-depth analysis of the observed coupling patterns revealed that altenuene (**11**) adopted a half-chair conformation with a near antiperiplanar relationship between H-3'ax and H-4'ax and a pseudoequatorial orientation of the C-5' hydroxyl group,²⁶ which was in accordance with reported X-ray crystallographic data.²⁷

In contrast, the ¹H NMR spectrum of **12** (Table 7) showed similar ¹H NMR data for the aromatic portion of the compound, but significantly different resonances and coupling patterns for the CH₂-3' methylene protons at δ 2.15 (br t, *J* = 12.3 Hz) and 2.25 (dd, *J* = 11.9 and 3.7 Hz), which could be explained by the methylene group situated adjacent to an equatorial 4'-hydroxyl group. This, taken together with the strong ROESY correlations between the 2'-methyl group, one of the methylene protons (δ_{H} 2.25), H-4' (δ_{H} 3.73), and H-5' (δ_{H} 4.20), indicated the adoption of a half-chair conformation and the placement of the 4'-hydroxyl group in the equatorial position. Thus, the relative stereochemistry was assigned as shown in **12**, which permitted the compound to be identified as the previously unreported 4'-epialtenuene. All remaining NMR spectroscopical data were in accordance with this conclusion (Table 7). Assignment of all signals belonging to **12** was possible due to

the lower amount of **12** present in the mixture with **11** (1:2). It was not possible, however, to determine the absolute stereochemistry as the compounds were obtained as an inseparable mixture. Moreover, the compounds were found to be optically inactive, probably due to their racemic nature, as previously reported in the literature for **11**.²⁷

Chemical screening indicated a difference between *Alternaria* extracts obtained from liquid Wickerham medium and solid rice cultures. The extract obtained from liquid cultures featured alternariol (**1**) and tenuazonic acid (**14**) as main components, whereas altenuin (**6**) was the major substance detected in the extract from solid rice cultures, with no detectable amounts of tenuazonic acid.

These differences resulted in a clear difference with regard to the cytotoxic activity of both extracts against L5178Y mouse lymphoma cells. The EtOAc extract obtained from solid rice cultures was more active than the corresponding extract obtained from liquid cultures (Table 1).

Recently, alternariol (**1**) was reported to inhibit cell proliferation by interference with the cell cycle.¹⁰ The results of our cytotoxicity assay of the isolated alternariol derivatives from the endophytic fungus *Alternaria* sp. (Table 1) indicated that all alternariol derivatives exhibited activity toward the L5178Y mouse lymphoma cells, except for alternariol 5-*O*-methyl ether-4'-*O*-sulfate (**4**) and 3'-hydroxyalternariol 5-*O*-methyl ether (**5**). This suggests that the free hydroxyl group at C-4', present in all bioactive alternariol derivatives encountered in this study (**1**–**3**), plays an important role in mediating cytotoxicity since substitution of this functional group decreased the activity significantly. Furthermore, the presence of an additional hydroxyl group at C-3' resulted in a clear reduction of activity. The most active compound detected was alternariol (**1**), with an EC₅₀ value of 1.7 $\mu\text{g}/\text{mL}$. Alternariol 5-*O*-sulfate (**2**) and alternariol 5-*O*-methyl ether (**3**) showed EC₅₀ values of 4.5 and 7.8 $\mu\text{g}/\text{mL}$, respectively. This indicated that substitution of OH-5 results in a slight decrease of activity, with the derivative with sulfate substitution (**2**) being more active than the methoxyl-substituted derivative (**3**). On the other hand, both altenuin (**6**) and desmethylaltenuin (**7**) showed almost identical activities, with EC₅₀ values of 6.8 and 6.2 $\mu\text{g}/\text{mL}$, indicating that substitution of OH-5 had only a negligible effect on the activity of these nonlactonized precursors to the alternariol-type compounds. Comparison of the altenuin-type compounds with the alternariol derivatives indicated that the presence of a lactone ring increased the cytotoxic activity. In contrast, formation of the lactone ring through the aromatic hydroxymethyl group resulted only in moderate activity as found for alterlactone (**8**), which caused inhibition of cell growth to 11.8% when assayed at a dose of 10 $\mu\text{g}/\text{mL}$. Compounds with only one phenyl ring such as **9**–**12** were inactive in the cytotoxicity test.

Interestingly, testing of all compounds at a dose of 1 $\mu\text{g}/\text{mL}$ in biochemical protein kinase activity assays revealed a similar pattern of activity to that found in the MTT assay for L5178Y cells (Table 8). Eleven of the tested compounds inhibited the activity of at least one of the 24 kinases that were investigated by at least 40%. For each of these compounds IC₅₀ values against all 24 protein kinases were determined. Alternariol (**1**) (Figure 1) and its derivatives **2** and **3** inhibited protein kinases ARK5, Aurora A, Aurora B, b-RAF,

Table 4. NMR Data of Compounds **7** and **10** at 500 (¹H) and 125 (¹³C) MHz (in MeOH-*d*₄)

position	7			10			
	δ_{H} mult. (J Hz)	HMBC	δ_{C}	δ_{H} mult. (J Hz)	ROESY	HMBC	δ_{C}
1			148.3				164.3
2			106.5				109.9
3			163				137.3
4	6.25 d (2.2)	2,3,5,6	102.3	6.07 d (2.5)	OCH ₃ ,CH ₃	2,5,6,1'	110.2
5			165.7				164.3
6	6.03 d (2.2)	2,4,1'	112.1	6.40 d (2.5)	OCH ₃	2,4,5,7	101.4
7							166
1'			135.8				142.5
2'	6.46 s	3',4',6',1	116.6				165.2
3'			143.2	A 3.00 dd (6.9,17.3) B 2.49 brd (17.0)	CH ₃ CH ₃	2',4' 1',2',4',5'	41.8
4'			144.7	4.38 dd (2.8, 6.3)			73.2
5'	6.55 s	CH ₃ ,1',3',4'	117.2				209
6'			127.3				
CH ₃	1.90 s	1',5',6',3'	19.3	1.99 s		1',2',3',5'	17.8
OCH ₃				3.78 s		5	55.8

Table 5. Chemical Shift Difference between the (*S*)-MTPA and (*R*)-MTPA Esters of **10**

position	chemical shift (δ_{H} , in C ₅ D ₅ N, at 500 MHz)			Δ $\delta_{\text{S}} - \delta_{\text{R}}$
	10	(<i>S</i>)-MTPA ester	(<i>R</i>)-MTPA ester	
3'A	3.0503	3.3192	3.3166	0.0026
3'B	2.8074	2.8994	2.707	0.1924
CH ₃	1.9447	1.9352	1.932	0.0032

Table 6. NMR Data of Compound **8** at 500 (¹H) and 125 (¹³C) MHz (in DMSO-*d*₆)

position	δ_{H} mult. (J Hz)	ROESY	HMBC	δ_{C}
1	7.03 s	11	3,4a,11a,11b	115.5
2				146.6
3				145.9
4	6.90 s	5	2,3,5,11b,4a	115.5
4a				140
5	A 4.85 d (11.0) B 4.80 d (11.3)	4	4,7,11b	67.8
7				168.7
7a				109.5
8				159.9
9		OCH ₃	7a,8,10,11	100.8
10	6.45 d (2.2)			162.2
11		OCH ₃ ,1	7a,9,10,11b	105
11a	6.50 d (2.2)			126.6
11b				129.8
OCH ₃		9,11	10	55.4
2-OH	3.81 s			
3-OH	9.37 brs ^a			
8-OH	9.47 brs ^a			
	10.21 brs			

^a Assignments may be interchanged.

IGF1-R, VEGF-R2, VEGF-R3, FLT3, PDGF-Rbeta, and SAK with an IC₅₀ below 1 × 10⁻⁶ g/mL, in accordance with their cytotoxic activity. Moreover, altenusin (**6**) and desmethylaltenusin (**7**) showed slightly weaker inhibition of protein kinases with IC₅₀ values below 1 × 10⁻⁵ g/mL, in agreement with a recent report of altenusin as a potent inhibitor of pp60c-Src tyrosine kinase.²⁸ 3'-Hydroxyalternariol 5-*O*-methyl ether (**5**) and altertoxin I (**15**) were inhibitory to protein kinases in a similar manner to alternariol (**1**) in spite of their observed lack of activity in the MTT assay. On the other hand **9**, **10**, and **11/12** exhibited IC₅₀ values of only 1 × 10⁻⁵ g/mL or below against the various kinases selected, which corresponds to their lack of activity in the MTT assay when tested at a dose of 10 μg/mL. With the exception of 3'-hydroxyalternariol 5-*O*-methyl ether (**5**) and altertoxin I (**15**), this similar pattern of activity indicates that the inhibition of protein kinases may be one of the major mechanisms contributing to the cytotoxic activity of alternariol- and altenusin-type compounds.

Table 7. NMR Data of Compound **12** at 500 (¹H) and 125 (¹³C) MHz (in MeOH-*d*₄)

position	δ_{H} mult. (J Hz)	ROESY	HMBC	δ_{C}
1				139
2				101.2
3				165.4
4	6.46 d (2.2)		2,3,5,6	102
5				167.9
6	6.63 d (2.2)	6'	4,5,1'	103.7
7				169.6
1'				134.2
2'				83.5
3'α	2.15 brt (12.3)	5'β	CH ₃ ,2',4',5'	44.5
3'β	2.25 dd (11.9, 3.7)	CH ₃ ,5'β	CH ₃ ,1',2',4',5'	
4'	3.73 ddd (12.3, 8.2, 3.7)			72
5'	4.20 dd (8.2, 2.5)		1',4',6'	74.2
6'	6.17 d (2.5)	3'α,3'β	2',4',1	130.4
CH ₃	1.54 s	6	1',2',3'	26.6
OCH ₃	3.85 s	3'β	5	56.3

Since alternariol (**1**) showed the highest *in vitro* inhibitory potency (Figure 1), we tested this compound in cellular phosphorylation assays for Aurora B, FLT3, IGF1-R, and VEGF-R2. However, the activity of none of these four kinases was significantly inhibited (IC₅₀ > 1 × 10⁻⁶ g/mL) in these assays (data not shown). Since alternariol efficiently inhibited proliferation of L5178Y lymphoma cells, it is unlikely that the lack of activity in the cellular phosphorylation assays is due to the inability of the compound to penetrate the cell membrane. Therefore, inhibition of cell proliferation is very likely not caused by inhibition of Aurora B, FLT3, IGF1-R, or VEGF-R2. It is, however, possible that inhibition of other kinases that have not been tested or interference with targets unrelated to protein kinases by alternariol is the molecular basis for the observed inhibition of cell proliferation.

The crude MeOH extract of the fungal host plant *P. senegalense* was fractionated over Diaion HP-20, and the resulting fractions were analyzed by LC-MS for the presence of the identified fungal metabolites. Alternariol 5-*O*-methyl ether (**3**) could be detected unequivocally in fraction 4 (obtained by elution with 100% MeOH) of the host plant (data not shown). Similar results were obtained for altenusin (**6**) and alternariol (**1**), occurring in fractions 2 (50% MeOH/H₂O) and 3 (75% MeOH/H₂O), respectively (data not shown). Interestingly, these substances were produced both in liquid cultures of *Alternaria* sp. and in cultures grown on solid rice medium, while any of the compounds obtained from only one of the two cultures were not detected in any of the host plant fractions. These results suggest the production, albeit in minor quantities,²⁹ of alternariol-type metabolites by the endophytic fungus under *in situ* conditions within the tissues of healthy host plants, implying their possible contribution to the mutualistic interaction between

Table 8. IC₅₀ Values of Selected Compounds against 24 Different Protein Kinases^a

cpd	AKT1	ARK5	Aurora-A	Aurora-B	B-RAF-VE	CDK2/CycA	CDK4/CycD1	COT	EGF-R	EPHB4	ERBB2	FAK
1	n.a.	9.5×10^{-7}	7.3×10^{-7}	4.2×10^{-7}	1.6×10^{-6}	3.3×10^{-6}	2.4×10^{-6}	8.0×10^{-6}	5.5×10^{-6}	n.a.	9.4×10^{-6}	2.4×10^{-6}
2	n.a.	7.5×10^{-7}	3.9×10^{-7}	2.2×10^{-7}	7.4×10^{-7}	8.4×10^{-7}	2.7×10^{-6}	7.5×10^{-6}	4.2×10^{-6}	3.1×10^{-6}	8.4×10^{-6}	2.1×10^{-6}
3	7.9×10^{-6}	4.0×10^{-6}	5.0×10^{-6}	1.3×10^{-6}	4.8×10^{-6}	3.6×10^{-6}	2.1×10^{-6}	2.6×10^{-6}	1.7×10^{-6}	1.4×10^{-6}	4.5×10^{-6}	8.9×10^{-6}
5	5.3×10^{-6}	3.5×10^{-7}	1.5×10^{-6}	1.1×10^{-6}	1.5×10^{-6}	2.4×10^{-6}	1.7×10^{-6}	2.3×10^{-6}	1.1×10^{-6}	1.2×10^{-6}	2.7×10^{-6}	4.9×10^{-6}
6	n.a.	6.6×10^{-6}	1.8×10^{-6}	1.1×10^{-6}	3.3×10^{-6}	n.a.	4.5×10^{-6}	8.8×10^{-6}	9.8×10^{-6}	9.4×10^{-6}	n.a.	n.a.
7	n.a.	2.6×10^{-6}	2.7×10^{-6}	1.5×10^{-6}	7.4×10^{-6}	n.a.	4.4×10^{-6}	9.7×10^{-6}	4.2×10^{-6}	5.9×10^{-6}	n.a.	n.a.
8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
10	n.a.	4.0×10^{-6}	4.4×10^{-6}	3.0×10^{-6}	9.0×10^{-6}	n.a.	n.a.	n.a.	6.5×10^{-6}	n.a.	n.a.	n.a.
11 + 12	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
15	n.a.	2.2×10^{-6}	1.7×10^{-6}	9.8×10^{-7}	6.7×10^{-6}	n.a.	6.4×10^{-6}	5.5×10^{-6}	3.5×10^{-6}	8.9×10^{-6}	n.a.	7.3×10^{-6}

cpd	IGF1-R	SRC	VEGF-R2	VEGF-R3	FLT3	INS-R	MET	PDGFR-beta	PLK1	CK2-alpha1	SAK	TIE2
1	3.4×10^{-6}	4.4×10^{-6}	9.2×10^{-7}	1.3×10^{-6}	4.3×10^{-7}	2.1×10^{-6}	5.4×10^{-6}	8.9×10^{-7}	6.9×10^{-6}	2.0×10^{-6}	9.6×10^{-7}	7.0×10^{-6}
2	4.9×10^{-6}	5.6×10^{-6}	7.0×10^{-7}	1.7×10^{-6}	3.3×10^{-7}	2.9×10^{-6}	7.6×10^{-6}	1.3×10^{-6}	4.4×10^{-6}	2.0×10^{-6}	3.8×10^{-7}	6.8×10^{-6}
3	1.7×10^{-6}	1.2×10^{-6}	1.1×10^{-6}	9.6×10^{-7}	1.0×10^{-6}	7.9×10^{-6}	n.a.	1.8×10^{-6}	8.2×10^{-6}	n.a.	2.5×10^{-6}	3.5×10^{-6}
5	9.1×10^{-7}	1.4×10^{-6}	6.4×10^{-7}	3.9×10^{-7}	5.3×10^{-7}	1.3×10^{-6}	1.7×10^{-6}	1.8×10^{-6}	5.7×10^{-6}	n.a.	8.3×10^{-7}	1.4×10^{-6}
6	4.9×10^{-6}	8.3×10^{-6}	5.0×10^{-6}	n.a.	1.6×10^{-6}	5.6×10^{-6}	4.3×10^{-6}	5.9×10^{-6}	7.1×10^{-6}	n.a.	3.2×10^{-6}	9.8×10^{-6}
7	2.4×10^{-6}	4.7×10^{-6}	3.8×10^{-6}	6.8×10^{-6}	1.8×10^{-6}	3.0×10^{-6}	2.5×10^{-6}	5.8×10^{-6}	n.a.	n.a.	1.7×10^{-6}	3.8×10^{-6}
8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
10	3.6×10^{-6}	4.6×10^{-6}	5.1×10^{-6}	3.7×10^{-6}	6.6×10^{-6}	8.1×10^{-6}	9.4×10^{-6}	n.a.	n.a.	n.a.	2.5×10^{-6}	n.a.
11 + 12	n.a.	n.a.	n.a.	n.a.	7.0×10^{-6}	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
15	1.7×10^{-6}	2.1×10^{-6}	1.4×10^{-6}	2.8×10^{-6}	6.6×10^{-7}	9.4×10^{-6}	7.0×10^{-6}	7.6×10^{-6}	n.a.	n.a.	1.7×10^{-6}	8.8×10^{-6}

^a Inhibitory potential of compounds at various concentrations were determined in biochemical protein kinase activity assays. Listed are IC₅₀ values in g/mL. Italic IC₅₀ values are below 1×10^{-6} g/mL. n.a.: not active, i.e., IC₅₀ > 1×10^{-5} g/mL.

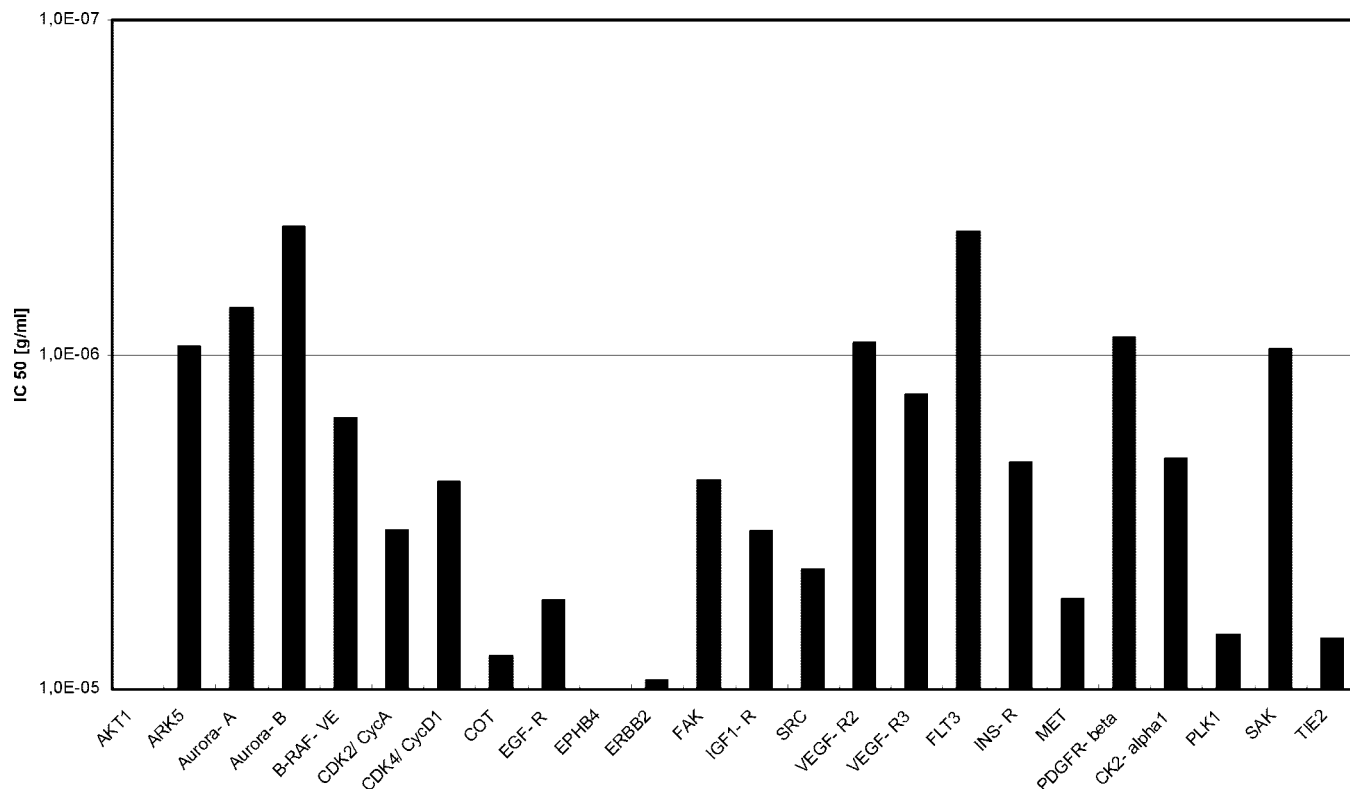
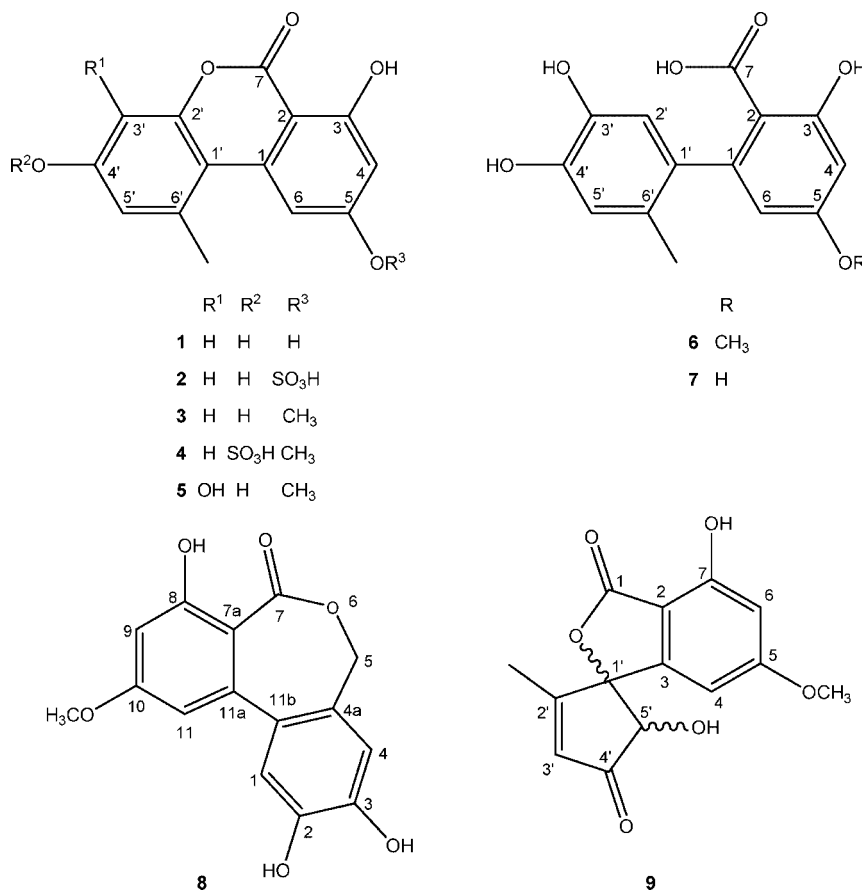


Figure 1. Inhibitory profile against 24 protein kinases of alternariol (1). Effects of compound in biochemical *in vitro* protein kinases assay were tested at various compound concentrations. Depicted are IC₅₀ values in g/mL on a logarithmic scale, starting from an activity threshold of 1 × 10⁻⁵ g/mL.

Chart 1



endophyte and host and proving a contribution of the fungal endophyte to the chemical composition of the host plant. It is worth

mentioning that, apart from a few studies that reported the isolation of typical fungal metabolites from plant sources, for example,

alternariol 5-*O*-methyl ether (**3**) from *Anthocleista djalonenis*,¹² 2,5-dimethyl-7-hydroxychromone (**13**) from *Polygonum cuspidatum*,³⁰ *Hypericum perforatum*,³¹ and Rhei Rhizoma,¹⁵ as well as aureonitol, a common metabolite of *Chaetomium* species, from an extract of *Helichrysum aureo-nitens*,³² the presence of secondary metabolites of endophytic fungi in the same host plant individuals from which the fungal cultures were originally isolated has only rarely been reported. Recently, it was demonstrated that *Neotyphodium uncinatum*, a common endophyte of *Festuca pratensis*, has the full biosynthetic capacity for some of the most common loline alkaloids, which were formerly found exclusively in endophyte-infected grasses.³³ Intensive studies of grass–endophyte associations also indicated that these physiologically active alkaloids protect the host plants from grazing livestock and invertebrate herbivores as well as pathogenic microorganisms. The alkaloids also inhibit germination and growth of other competing grasses and increase plant growth and seed production.^{6,34–36} Moreover, endophyte-infected grasses usually possess increased tolerance to drought and aluminum toxicity.³⁷

The present study adds a further example of the unequivocal detection of fungal metabolites in the host plant of the respective endophyte. It remains to be investigated whether the occurrence of alternariol and structurally related derivatives is ecologically advantageous to *P. senegalense*, as shown previously for endophyte-infected grasses.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Perkin-Elmer-241 MC polarimeter. ¹H and ¹³C NMR spectra were recorded on Bruker ARX 500 or AVANCE DMX 600 NMR spectrometers. ESIMS was conducted on a Finnigan LCQ Deca mass spectrometer, and HRESIMS were obtained on a Micromass Qtof 2 mass spectrometer. Solvents were distilled before use, and spectral grade solvents were used for spectroscopic measurements. HPLC analysis was performed using a HPLC (Dionex P580) system coupled to a photodiode array detector (UVD340S). Routine detection was at 235, 254, 280, and 340 nm. The separation column (125 × 4 mm, L × i.d.) was pre-filled with Eurospher-10 C₁₈ (Knauer, Germany), and the following gradient was used (MeOH, 0.02% H₃PO₄ in H₂O): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100% MeOH.

Fungal Material. The fungus *Alternaria* sp. was isolated from fresh healthy leaves of *Polygonum senegalense* Meisn. (Polygonaceae) growing in the wild. The plant was collected in April 2004 near Alexandria, Egypt. A voucher specimen (code no. 234) was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University. Following surface sterilization with 70% EtOH for 1 min the leaf samples were rinsed in sterile water. To distinguish the remaining epiphytic fungi from endophytic fungi, an imprint of the leaf surface on biomalt agar was performed. Small tissue samples from inside the leaves were cut aseptically and pressed onto agar plates containing an antibiotic to suppress bacterial growth (composition of isolation medium: 15 g/L malt extract, 15 g/L agar, and 0.2 g/L chloramphenicol in distilled water, pH 7.4–7.8, adjusted with 10% NaOH or 36.5% HCl). After incubation at room temperature, the fungal strain under investigation was found to grow exclusively out of the leaf tissue, but not on the agar plates taken from the imprint of the leaf surface. From the growing cultures pure strains of *Alternaria* sp. were isolated by repeated re-inoculation on malt agar plates.

Identification of Fungal Cultures. The fungus (strain no. II2L4) was identified using a molecular biological protocol by DNA amplification and sequencing of the ITS region as described previously.³⁸ This fungal strain was identified as *Alternaria* sp.; however, due to the lack of similar sequences in GenBank, identification of the strain to the species level was not possible. A voucher strain is kept at the one of the authors' laboratory (P.P.).

Cultivation. Mass growth of the fungus for the isolation and identification of new metabolites was carried out in Erlenmeyer flasks (1 L each). The fungus was grown in liquid Wickerham medium (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, distilled water added up to 1000 mL, pH 7.2–7.4, adjusted with 10% NaOH or 36.5% HCl, liquid medium/flask = 300 mL, 20 flasks) or on rice solid medium

(to 100 g commercially available rice was added 100 mL of distilled water and kept overnight prior to autoclaving, 6 flasks) at room temperature under static conditions for 21 or 30 days, respectively.

Extraction and Isolation. For the extraction of natural products from the liquid culture, mycelia and culture filtrates were collected and successively extracted with EtOAc and *n*-BuOH. The EtOAc portion was then taken to dryness and partitioned between *n*-hexane and 90% MeOH. The 90% MeOH and *n*-BuOH crude fractions (0.6 and 0.9 g, respectively) were chromatographed over Sephadex LH-20 using MeOH as solvent system. TLC plates with silica gel F₂₅₄ (Merck, Darmstadt, Germany) were used for monitoring the fractions using CH₂Cl₂/MeOH (95:5, 90:10, 85:15, and 80:20) as well as CH₂Cl₂/MeOH/EtOAc (90:10:5 and 80:20:10). Detection was carried out under UV light at 254 and 366 nm or by spraying the plates with anisaldehyde reagent. The rice culture was extracted with EtOAc. The crude extract obtained was dried and partitioned between *n*-hexane and 90% MeOH. The 90% MeOH-soluble material (1.2 g) was then fractionated by vacuum-liquid chromatography (VLC) on silica gel 60 using CH₂Cl₂/MeOH gradient elution. Further purification was achieved by preparative HPLC (Varian, PrepStar 218) on a Microsorb 60-8 C₁₈ column (250 × 21.4 mm, L × i.d.) using the following gradient (MeCN/H₂O): 0 min, 10% MeCN; 5 min, 10% MeCN; 35 min, 100% MeCN; 45 min, 100% MeCN. Yields of compounds were as follows: **1** 203.9 mg (from liquid culture, 189.0 mg; from rice culture, 14.9 mg), **2** 60.9 mg, **3** 239 mg (from liquid culture, 14 mg; from rice culture, 225 mg), **4** 5.9 mg, **5** 5.8 mg, **6** 378 mg (from liquid culture, 25 mg; from rice culture, 353 mg), **7** 5.6 mg, **8** 8.4 mg, **9** 5.0 mg, **10** 16.9 mg, **11** + **12** 18.8 mg, **13** 4.6 mg, **14** 101.2 mg, and **15** 3.5 mg.

Alternariol 5-*O*-sulfate (2**):** reddish-white needles; UV λ_{max} (PDA) 215, 250, 288, 345 nm; ¹H NMR, see Table 2; ¹³C NMR, see Table 3; ESIMS positive *m/z* 339 [M + H]⁺ (22), 259 [M + H – SO₃]⁺ (100), negative *m/z* 337 [M – H][–] (100), 257 [M – H – SO₃][–] (37); HRESIMS *m/z* 339.0170 [M + H]⁺ (calcd for C₁₄H₁₁O₈S, 339.0169).

Alternariol 5-*O*-methyl ether-4'-*O*-sulfate (4**):** reddish-white needles; UV λ_{max} (PDA) 203, 254, 285, 337 nm; ¹H NMR, see Table 2; ¹³C NMR, see Table 3; ESIMS positive *m/z* 353 [M + H]⁺ (12), 273 [M + H – SO₃]⁺ (100), negative *m/z* 351 [M – H][–] (100), 271 [M – H – SO₃][–] (45); HRESIMS *m/z* 353.0320 [M + H]⁺ (calcd for C₁₅H₁₃O₈S, 353.0326).

3-Hydroxyalternariol 5-*O*-methyl ether (5**):** violet needles; UV λ_{max} (PDA) 203, 236, 260, 340 nm; ¹H NMR, see Table 2; ¹³C NMR, see Table 3; ESIMS positive *m/z* 289 [M + H]⁺ (100), negative *m/z* 287 [M – H][–] (100), 272 [M – CH₃ – H][–] (39); HRESIMS *m/z* 289.0720 [M + H]⁺ (calcd for C₁₅H₁₃O₆, 289.0707).

Desmethyaltenuenol (7**):** viscous, reddish oil; UV λ_{max} (PDA) 203, 224, 255, 293 nm; ¹H and ¹³C NMR, see Table 4; ESIMS negative *m/z* 275 [M – H][–] (100), 232 [M – H – CO₂][–] (98); HRESIMS *m/z* 299.0520 [M + Na]⁺ (calcd for C₁₄H₁₂NaO₆, 299.0526).

Alterlactone (8**):** white flakes; UV λ_{max} (PDA) 206, 221, 254 nm; ¹H and ¹³C NMR, see Table 6; ESIMS positive *m/z* 599 [2M + Na]⁺ (93), 289 [M + H]⁺ (71), 271 (100) [M + H – H₂O]⁺, negative *m/z* 287 [M – H][–] (100), 243 [M – H – CO₂][–] (82), 229 [M – H – CO₂ – CH₃][–] (64), HRESIMS *m/z* 289.0700 [M + H]⁺ (calcd for C₁₅H₁₃O₆, 289.0707).

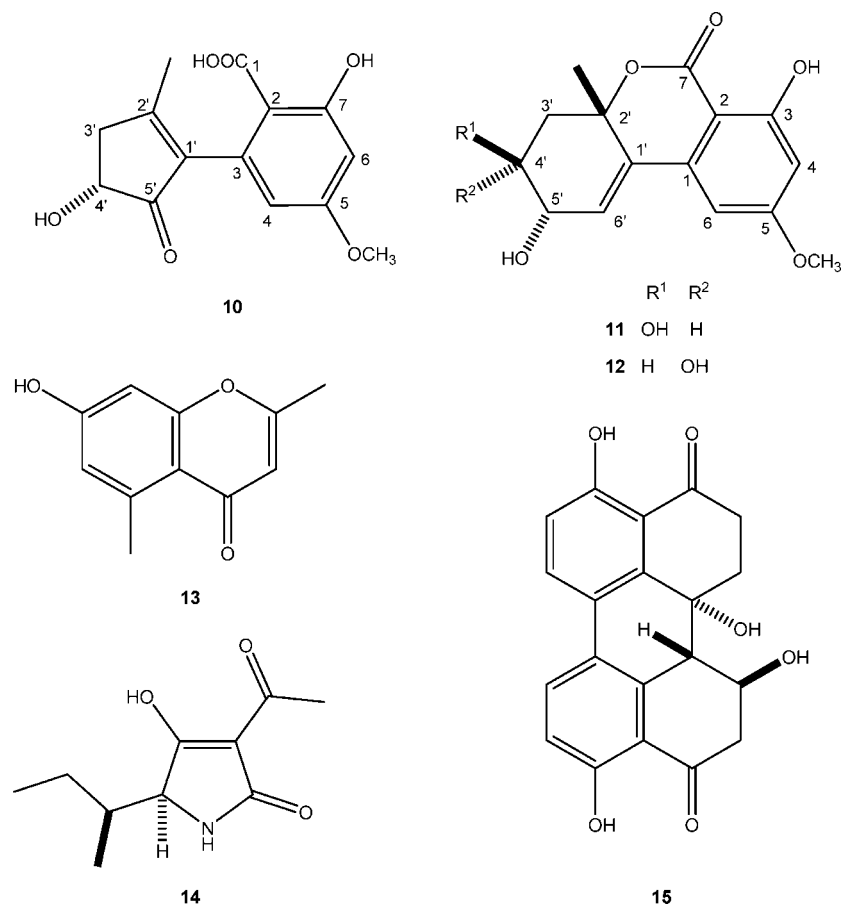
Alternarienic Acid (10**):** viscous, yellow oil; [α]_D²⁰ +75 (c 1.0, MeOH); UV λ_{max} (PDA) 218, 260 (sh), 302 nm; ¹H and ¹³C NMR, see Table 4; ESIMS positive *m/z* 301 [M + Na]⁺ (53), 279 [M + H]⁺ (82), 261 [M + H – H₂O]⁺ (100), negative *m/z* 277 [M – H][–] (100), 233 [M – H – CO₂][–] (80); HRESIMS *m/z* 301.0700 [M + Na]⁺ (calcd for C₁₄H₁₄NaO₆, 301.0683).

4'-Epialtenuene (12**):** inseparable 1:2 mixture with altenuene (**11**); viscous, yellow oil; [α]_D²⁰ 0; UV λ_{max} (PDA) 241, 281, 318 nm; ¹H and ¹³C NMR, see Table 7; ESIMS positive *m/z* 293 [M + H]⁺ (75), 257 [M + H – 2H₂O]⁺ (100), negative *m/z* 291 [M – H][–] (100); HRESIMS *m/z* 293.1020 [M + H]⁺ (calcd for C₁₅H₁₇O₆, 293.1020).

Cell Proliferation Assay. Cytotoxicity was tested against L5178Y mouse lymphoma cells using a microculture tetrazolium (MTT) assay and compared to that of untreated controls as described previously.³⁹ All experiments were carried out in triplicate and repeated three times. As controls, media with 0.1% EGMME/DMSO were included in the experiments. The depsipeptide kahalalide F isolated from *Elysia grandifolia*³⁹ was used as positive control.

Biochemical Protein Kinase Activity Assay. All biochemical protein kinase activity assays were performed in 96-well FlashPlates from Perkin-Elmer/NEN (Boston, MA) in a 50 μL reaction volume.

Chart 2



The reaction cocktail contained 20 μL of assay buffer, 5 μL of ATP solution (in H_2O), 5 μL of test compound (in 10% DMSO), 10 μL of substrate, and 10 μL of purified recombinant protein kinase. The assay for all enzymes contained 60 mM HEPES-NaOH, pH 7.5, 3 mM MgCl_2 , 3 mM MnCl_2 , 3 μM Na-orthovanadate, 1.2 mM DTT, 50 $\mu\text{g}/\text{mL}$ PEG₂₀₀₀₀, and 1 μM [γ -³³P]-ATP (ca. 5×10^5 cpm per well). The following substrates were used: GSK3(14–27), AKT1; tetra(LR-RWSLG), Aurora A, B; MEK1 KM, B-Raf; histone H1, CDK2/CycA; Rb-CTF, CDK4/CycD1; Poly(Glu,Tyr)_{4:1}, EGF-R, EPHB4, ERBB2, FAK, IGF1-R, SRC, VEGF-R2, VEGF-R3, Tie2; Poly(Ala,Glu,Lys,Tyr), FLT3, INS-R, Met, PDGF-Rb; casein, PLK1, CK2a1. Autophosphorylation was measured for ARK5, COT, and SAK.

Cellular Phosphorylation Assays. The assays were performed in 48-well plates using human colon cancer cell line HT29 (Aurora B), murine embryonic fibroblasts stably transfected with either FLT3 and IGF1-R, respectively, and the human endothelial cell line HUE (VEGF-R2). All cells were pretreated with 10 μM staurosporine for 90 min to reduce basal level phosphorylation. Incubation of cells with the test compounds for 90 min at 37 °C was followed by treatment with either calyculin A (HT29, 30 min), FLT3-ligand (MEF FLT3, 5 min), IGF1 (MEF IGF1R, 3 min), or VEGF₁₆₅ (HUE, 7 min). Subsequently cells were lysed and lysates were transferred to 96-well plates precoated with capture antibody. Detection of autophosphorylated kinases (FLT3, IGF1-R, VEGF-R2) and phosphorylated substrate (Aurora B), respectively, was done using anti-P-His H3 (Aurora B assay) or antiphosphotyrosine antibody PY99.

Chiral Derivatization. The reaction was performed according to a convenient Mosher ester method described in the literature.²⁴ The compounds (2×1 mg of each) were transferred into NMR tubes and dried under vacuum. The samples were dissolved in deuterated pyridine (0.5 mL), and both (*R*)- and (*S*)-MTPA chloride were added separately into the NMR tubes immediately under a N_2 gas stream. The reagent was added in the ratio of 0.14 mM reagent to 0.10 mM of the compound.⁴⁰ The NMR tubes were shaken carefully to mix the samples and MTPA chloride evenly. The reaction NMR tubes were permitted to stand at room temperature and monitored by ¹H NMR spectroscopy

until the reaction was found to be complete. ¹H–¹H COSY was measured to confirm the assignment of the signals.

Extraction and Fractionation of Plant Material. The plant sample (*P. senegalense* stems, leaves, and flowers; total of 2 g dry wt) was frozen at –80 °C followed by freeze-drying. The freeze-dried sample was ground and extracted with 90% MeOH overnight with shaking, and the resulting extract was dried. The dried residue was subjected to partitioning between *n*-hexane and 90% MeOH. The 90% MeOH-soluble fraction was fractionated over Diaion HP-20 using $\text{H}_2\text{O}/\text{MeOH}$ and MeOH/acetone gradient elution.

LC-MS Analysis of *Alternaria* Metabolites in *Polygonum senegalense* Fractions. Fractions of *P. senegalense* were analyzed by HPLC and LC-MS. The obtained chromatograms were searched for the specific molecular weights (mass chromatograms or extracted ion chromatograms, isolation width set to ± 0.5 amu around the nominal mass) of the compounds isolated from *Alternaria* sp. cultures. Co-elution studies with the corresponding pure metabolites and the respective plant fractions were carried out, and retention times, molecular ion of the target compounds, and MS and MS/MS data were compared. Specifically, the following pseudomolecular ions, each corresponding to the respective $[\text{M} - \text{H}]^-$, were used for successful detection of secondary metabolites in the host plant extract: alternariol (**1**), *m/z* 257; alternariol 5-*O*-methyl ether (**3**), *m/z* 271; alternusin (**6**), *m/z* 273.

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