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Gentisyl Alcohol Derivatives from the Marine-Derived Fungus *Penicillium terrestre*

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Nine new gentisyl alcohol derivatives, namely, the trimeric terrestrol A (**8**), dimeric terrestrols B−H (**1**–**7**), and a monomeric derivative (**12**), together with four known analogues (**9**–**11**, **13**) were isolated from the marine-derived fungus *Penicillium terrestre*. The structures of the new compounds were elucidated by spectroscopic methods including one- and two-dimensional NMR as well as low- and high-resolution mass spectrometric analysis. These new compounds (**1**–**8**, **12**) showed cytotoxic effects on HL-60, MOLT-4, BEL-7402, and A-549 cell lines with IC_{50} values in the range 5–65 μM. Compound **6** also showed moderate inhibitory activity against protein tyrosine kinases (Src and KDR). Furthermore, all new compounds exhibited moderate radical scavenging activity against DPPH with IC_{50} values in the range 2.6–8.5 μM.

It is known that some fungal and bacterial strains have the potential to produce diverse secondary metabolites, and exploring the biosynthetic potential of these strains is a very successful strategy for finding novel secondary metabolites.[1,2] In the course of our search for new anticancer compounds of marine microorganisms from Chinese coastal waters, more than 30 active microbial strains were investigated through a bioassay-guided isolation procedure, and some secondary metabolites with novel structures and interesting activities were found in some of these strains. Recently, we experimented with new culture conditions for these strains in an attempt to find new anticancer compounds. The secondary metabolite patterns of the 30 active strains under different culture conditions were analyzed by chemical screening. Among these strains, the metabolite pattern of a fungal strain identified as *Penicillium terrestre* C. N. Jenson exhibited TLC and HPLC profiles very distinct from those of the original strain,[3–5] from which two new cytotoxic active polyketides and sorbicillin analogues had been isolated. Consequently, we selected this strain for further chemical investigation. The investigation resulted in the isolation of eight new gentisyl alcohol polymers, terrestrols A−H (**1**–**8**), a monomer (**12**), and four known analogues (**9**–**11**, **13**)[6–8] (Figure 1). The new compounds were evaluated for cytotoxic effects on HL-60, MOLT-4, BEL-7402, and A-549 cell lines, protein tyrosine kinases (Src, KDR), and DPPH. All compounds showed cytotoxic activities (IC_{50} 5–65 μM) and antioxidant activities (IC_{50} 2.6–8.5 μM). Compound **6** also showed moderate inhibitory activity to protein tyrosine kinases.

A significantly smaller number of gentisyl alcohol polymers compared to the related monomers have been reported from microorganisms. This paper represents the first report of a gentisyl alcohol trimer from nature. A variety of bis-,[9–11] tri-,[12,13] and tetra-brominated[14,15] benzyl polymers and their monomers[9,11] have been isolated from marine red alga,[10,12,13,16,17] green alga,[11,14,15] and brown alga,[9] and many of these compounds were reported to exhibit antimicrobial,[11] antioxidant,[10,18] feeding deterrent,[17] enzymatic inhibitory,[14–16] and cytotoxic activities.[9,13]

In this paper we report the isolation, structural elucidation, and bioactivities of compounds **1**–**13**.

Results and Discussion

The bioactive EtOAc extract of *P. terrestre* was chromatographed on Si gel and Sephadex LH-20 columns and finally purified by reversed-phase HPLC to afford compounds **1**–**13**. NMR and MS analyses indicated that 10 (**1**–**8**, **12**, **13**) were new natural products, although compound **13** has been previously reported as a synthetic product. The remaining three compounds were identified to have known structures (**9**–**11**).

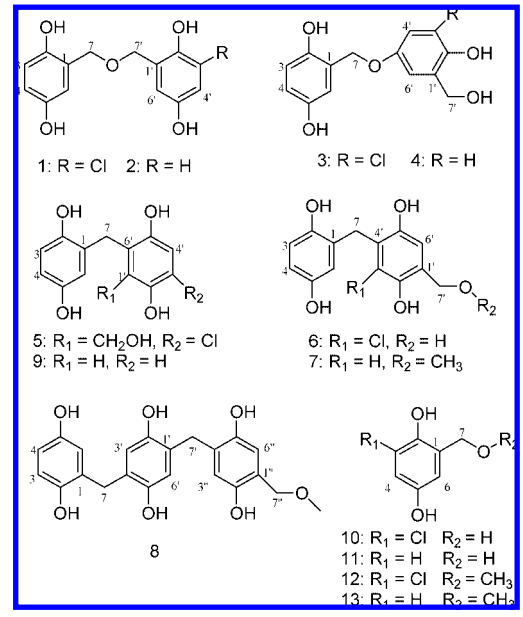

Figure 1. Structures of compounds **1**–**13**.

Compound **1** was obtained as a brown gum. The IR spectrum showed a strong yet broadened absorption band for hydroxy groups at 3257 cm^{-1} and characteristic absorption bands for aromatic rings at 1609 and 1465 cm^{-1}.[9,12,13] The negative ESIMS spectrum exhibited a characteristic chlorinated quasi-molecular ion peak cluster at *m/z* 295/297 (3:1) ([M − H]$^-$), and the molecular formula was determined as $C_{14}H_{13}ClO_5$ by negative HRESIMS at *m/z* 295.0372 ([M − H]$^-$; calcd for $C_{14}H_{12}{}^{35}ClO_5$, 295.0373), which was further corroborated by the NMR data. The δ 6.0–7.0 region of the ^{1}H NMR spectrum of **1** (Table 1) showed resonances for three aromatic protons of the 1,2,4-trisubstituted phenyl ring A, at δ 6.72 (d, J = 2.8 Hz, H-6), 6.49 (dd, J = 8.7, 2.8 Hz, H-4), and 6.61 (d, J = 8.7 Hz, H-3), as well as two *meta*-coupled aromatic protons at δ 6.74 (d, J = 2.8 Hz, H-6′) and 6.66 (d, J = 2.8 Hz, H-4′) of ring B. In addition, the region between δ 9.5 and 8.5 had four exchangeable protons, and the region between δ 4.5 and 4.0 had two broadened proton signals (δ 4.45, s, 2H; δ 4.49, s, 2H).

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Table 1. ^{1}H NMR Data of Compounds **1**–**8**, **12**, and **13** in DMSO-d_6[a]

	1	**2**	**3**	**4**	**5**	**6**
position	δ_H (*J*/Hz)	δ_H (*J*/Hz)	δ_H (*J*/Hz)	δ_H (*J*/Hz)	δ_H (*J*/Hz)	δ_H (*J*/Hz)
3	6.61 (d, 8.7)	6.60 (d, 8.7)	6.65 (d, 8.7)	6.63 (d, 8.7)	6.57 (d, 8.7)	6.59 (d, 8.2)
4	6.49 (dd, 8.7, 2.8)	6.49 (dd, 8.7, 2.8)	6.53 (dd, 8.7, 2.8)	6.51 (dd, 8.7, 3.2)	6.33 (dd, 8.7, 2.8)	6.35 (dd, 8.2, 2.8)
6	6.72 (d, 2.8)	6.74 (d, 2.8)	6.70 (d, 2.8)	6.73 (d, 3.2)	5.93 (d, 2.8)	5.94 (d, 2.8)
7	4.45 (2H, s)	4.44 (2H, s)	4.89 (2H, s)	4.86 (2H, s)	3.78 (2H, s)	3.84 (2H, s)
3′/3″				6.65 (overlap)		
4′/4″	6.66 (d, 2.8)		6.86 (d, 3.2)	6.65 (overlap)	6.80 (s)	
6′/6″	6.74 (d, 2.8)		6.92 (d, 3.2)	6.93 (d, 1.4)		6.88 (s)
7′/7″	4.49 (2H, s)		4.50 (2H, s)	4.44 (2H, s)	4.42 (2H, s)	4.52 (2H, d, 5.0)
2-OH	8.80 (s)	8.71 (s)	8.97 (s)	8.90 (s)	8.72 (s)	8.69 (s)
5-OH	8.70 (s)	8.67 (s)	8.73 (s)	8.69 (s)	8.37 (s)	8.39 (s)
2′-OH	8.51 (s)		8.58 (s)	8.87 (s)	8.82 (s)	8.34 (s)
5′-OH	9.23 (s)				9.09 (s)	9.11 (s)
7′-OH			5.29 (s)	4.99 (s)	5.30 (s)	5.22 (t, 5.0)
CH_3						

	7	**8**		**12**	**13**
position	δ_H (*J*/Hz)	δ_H (*J*/Hz)	δ_H (*J*/Hz)	δ_H (*J*/Hz)	δ_H (*J*/Hz)
3	6.58 (d, 8.3)	6.58 (d, 8.3)			6.62 (d, 8.7)
4	6.38 (dd, 8.3, 2.7)	6.38 (dd, 8.3, 3.2)		6.65 (d, 3.0)	6.51 (dd, 8.7, 3.2)
6	6.34 (d, 2.7)	6.36 (d, 3.2)		6.66 (d, 3.0)	6.67 (d, 3.2)
7	3.61 (2H, s)	3.59 (2H, s)		4.36 (2H, s)	4.31 (2H, s)
3′/3″	6.39 (s)	6.40 (s)	6.39 (s)		
4′/4″					
6′/6″	6.68 (s)	6.38 (s)	6.67 (s)		
7′/7″	4.26 (2H, s)	3.59 (2H, s)	4.26 (2H, s)		
2-OH	8.56 (s)	8.58 (s)		9.20 (s)	8.70 (s)
5-OH	8.52 (s)	8.50 (s)		8.51 (s)	8.66 (s)
2′-OH	8.56 (s)	8.46 (s)	8.55 (s)		
5′-OH	8.62 (s)	8.47 (s)	8.60 (s)		
7′-OH					
CH_3	3.26 (s)		3.26 (s)	3.29 (s)	3.28 (s)

[a] Spectra were recorded at 600 MHz for ^{1}H using TMS as internal standard.

Table 2. ^{13}C NMR Data of Compounds **1**–**8**, **12**, and **13** in DMSO-d_6[a]

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**		**12**	**13**
position	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C
1	125.1 qC	125.5 qC	123.6 qC	124.3 qC	127.4 qC	126.7 qC	127.8 qC	127.9 qC		128.8 qC	125.3 qC
2	147.1 qC	147.0 qC	147.2 qC	147.1 qC	147.1 qC	147.5 qC	147.4 qC	147.4 qC		142.4 qC	147.3 qC
3	115.5 CH	115.5 CH	115.8 CH	115.7 CH	114.9 CH	114.9 CH	115.3 CH	115.3 CH		121.2 qC	115.7 CH
4	114.4 CH	114.3 CH	115.1 CH	114.8 CH	112.5 CH	112.5 CH	113.0 CH	112.9 CH		114.5 CH	114.6 CH
5	149.7 qC	149.8 qC	149.8 qC	149.8 qC	149.7 qC	149.7 qC	149.7 qC	149.7 qC		150.6 qC	149.8 qC
6	115.0 CH	114.8 CH	115.2 CH	115.0 CH	114.8 CH	114.6 CH	116.7 CH	116.8 CH		114.0 CH	115.2 CH
7	67.1 CH_2	66.8 CH_2	65.0 CH_2	64.8 CH_2	24.7 CH_2	26.8 CH_2	29.1 CH_2	29.0 CH_2		69.1 CH_2	68.9 CH_2
1′/1″	129.1 qC		132.7 qC	129.6 qC	129.0 qC	129.7 qC	122.4 qC	125.0 qC	122.2 qC		
2′/2″	142.4 qC		143.2 qC	147.8 qC	144.3 qC	142.1 qC	147.1 qC	147.3 qC	147.1 qC		
3′/3″	121.0 qC		120.6 qC	115.0 CH	118.3 qC	122.5 qC	116.6 CH	116.4 CH	116.6 CH		
4′/4″	114.5 CH		113.0 CH	112.9 CH	114.5 CH	122.4 qC	126.6 qC	124.9 qC	126.8 qC		
5′/5″	150.6 qC		151.8 qC	151.4 qC	148.7 qC	149.1 qC	147.3 qC	147.3 qC	147.2 qC		
6′/6″	113.8 CH		112.8 CH	113.6 CH	125.0 qC	112.3 CH	114.9 CH	116.5 CH	114.9 CH		
7′/7″	67.2 CH_2		58.8 CH_2	58.2 CH_2	57.0 CH_2	58.9 CH_2	68.6 CH_2	28.8 CH_2	68.7 CH_2		
CH_3							57.5 CH_3		57.5 CH_3	57.8 CH_3	57.7 CH_3

[a] Spectra were recorded at 150 MHz for ^{13}C using TMS as internal standard.

The ^{13}C NMR and DEPT spectra of **1** (Table 2) displayed 14 carbons assignable to two benzylic methylene carbons that were oxygenated and two benzene rings. Four of the carbons in these two benzene rings were oxygenated (δ > 142 ppm).[9,12,13] The phenolic hydroxy groups were *para*-substituted on the basis of the HMBC correlations of 2-OH with C-1, 5-OH with C-4, C-5, and C-6, and 5′-OH with C-4′, C-5′, and C-6′. These data indicated that **1** was a gentisyl alcohol dimer, which was confirmed by key HMBC correlations (Figure 2) of CH_2-7 with C-1, C-2, C-6, and C-7′, and CH_2-7′ with C-1′, C-2′, C-6′, and C-7, implying that two benzyl moieties were connected through an ether bond between C-7 and C-7′. Further analysis and comparison of chemical shifts of aromatic carbons with the known compound **10** (δ_C 120.7, s, C-3′) (see Experimental Section) indicated that C-3′ (δ_C 121.0, s) in **1** was also chlorinated, consistent with the molecular formula.

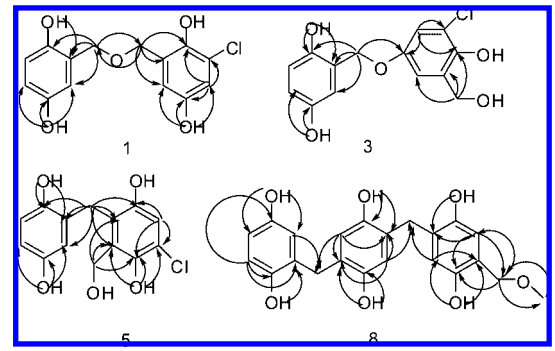

Figure 2. Key HMBC correlations of compounds **1**, **3**, **5**, and **8**.

Thus compound **1** was established to be 2-chloro-6-[(2,5-dihydroxybenzyloxy)methyl]benzene-1,4-diol.

Compound **2** was obtained as a brown gum and showed IR absorptions similar to those of **1**. Its molecular formula of $C_{14}H_{14}O_5$ was established by negative HRESIMS (*m*/*z* 261.0766, $[M - H]^-$, calcd for $C_{14}H_{13}O_5$, 261.0763). However, the NMR spectra of **2** showed only half the number of signals as deduced from its molecular formula. The ^1H and ^{13}C NMR data of **2** (Tables 1 and 2) were closely comparable to those of **11** (except for the absence of OH-7), including one 1,2,4-trisubstituted phenyl moiety, which indicated that **2** was a symmetric homodimeric ether comprising two gentisyl alcohol units. This was in agreement with the COSY and HMBC data. Therefore, the structure of compound **2** is 2,2′-oxybis(methylene)dibenzene-1,4-diol.

Compound **3** was obtained as a brown gum. The molecular formula of **3** was determined as $C_{14}H_{13}ClO_5$ by HRESIMS (*m*/*z*295.0370, $[M - H]^-$), which was the same as **1**. Its IR spectrum (KBr) showed absorption bands for hydroxy groups (3309 cm^{-1}) and aromatic rings (1595, 1482 cm^{-1}). Analysis and comparison of 1D NMR of **3** with those of **1** (Tables 1 and 2) suggested that, like **1**, **3** also possessed a trisubstituted and a tetrasubstituted benzyl group. Instead of the phenolic 5′-OH in **1**, an exchangeable proton at δ 5.29 (s, 7′-OH) was observed, implying that **3** was also a dimer consisting of a gentisyl alcohol unit and a chlorogentisyl alcohol unit, which were connected differently compared to **1**. Furthermore, the HMBC correlations (Figure 2) of CH_2-7 with C-1, C-2, C-6, and C-5′ indicated that the two units were connected through an ether bond between C-7 and C-5′. Thus compound **3** was established to be 2-[{3-chloro-4-hydroxy-5-(hydroxymethyl)phenoxy}methyl]-benzene-1,4-diol.

Compound **4** was obtained as a brown gum and showed IR absorptions similar to those of **2**. The HRESIMS of **4** (*m*/*z* 261.0769, $[M - H]^-$) suggested that it was an isomer of **2**, with a molecular formula of $C_{14}H_{14}O_5$. On the basis of comparison of the ^1H and ^{13}C NMR data of **4** with those of **3** (Tables 1 and 2), a 1,2,4-trisubstituted benzyl system was found to remain in **4**, but another trisubstituted benzyl group in **4** replaced the tetrasubstituted benzyl group in **3**. Since the molecular mass of **4** was 34 Da lower than that of **3** and the chemical shift of C-3′ appeared at higher field in **4** (δ_C 115.0), it showed that an aromatic proton in **4** replaced a chlorine in **3**. Since resonances for OH-5′ and OH-7 were absent in the ^1H NMR spectrum, it indicated that **4** was an asymmetric homodimer, consisting of two gentisyl alcohol units, and the two monomers were connected at C-7 and C-5′ through an ether linkage, which was confirmed by the HMBC correlations of H-7 with C-1, C-2, C-6, and C-5′. Thus compound **4** was established to be 2-[{4-hydroxy-3-(hydroxymethyl)phenoxy}methyl]benzene-1,4-diol.

Compound **5** was obtained as a brown gum and showed IR absorptions similar to those of **1**. The HRESIMS of compound **5** (*m*/*z* 295.0368, $[M - H]^-$) suggested that it was an isomer of compounds **1** and **3**, with a molecular formula of $C_{14}H_{13}ClO_5$.On the basis of comparison of the 1D NMR data of **5** with those of **1** (Tables 1 and 2), a 1,2,4-trisubstituted phenyl ring A and four phenolic hydroxy groups were evident in **5**. The spectrum also contained a singlet at δ 6.80 (s, H-4′) and an extra exchangeable proton at δ 5.30 (s, 7′-OH). In addition, one of the deshielded methylene groups in **1** was replaced by a shielded methylene group (δ_H 3.78, 2H, s; δ_C 24.7) in **5**. The above data suggested that **5** was also a dimer consisting of a gentisyl alcohol unit and a chlorogentisyl alcohol unit, but the two monomers were connected through a carbon−carbon bond between C-7 and C-6′, which was further confirmed by the HMBC correlations (Figure 2) of H-7 with C-1, C-2, C-6, C-1′, C-5′, and C-6′. Thus compound **5** was established to be 5-chloro-2-(2,5-dihydroxybenzyl)-3-(hydroxymethyl)benzene-1,4-diol.

Compound **6** was obtained as a brown gum and showed IR absorptions similar to those of **1**. The HRESIMS of compound **6** (*m*/*z* 295.0372, $[M - H]^-$) suggested that it was an isomer of compounds **1**, **3**, and **5**, with a molecular formula of $C_{14}H_{13}ClO_5$. The ^1H and ^{13}C NMR data of **6** were similar to those of **5** (Tables 1 and 2) and also included a 1,2,4-trisubstituted phenyl ring A, a pentasubstituted phenyl ring B, one deshielded methylene group, one shielded methylene group, four phenolic hydroxy groups, and an alcoholic hydroxy function. These data suggested that **6** contained a similar dimer structure consisting of a gentisyl alcohol unit and a chlorogentisyl alcohol unit, but with a different connection. Furthermore, the HMBC correlations of H-7 with C-1, C-2, C-6, C-3′, C-4′, and C-5′ indicated that the two monomers were connected by C-7 and C-4′ through a carbon−carbon bond in **6**. Thus compound **6** was established as 3-chloro-2-(2,5-dihydroxybenzyl)-5-(hydroxymethyl)benzene-1,4-diol.

Compound **7** was obtained as a brown gum and showed IR absorptions similar to those of **1**. The molecular formula of **7** was determined as $C_{15}H_{16}O_5$ by HRESIMS (*m*/*z* 275.0913, $[M - H]^-$). On the basis of comparison of the 1D NMR data of **7** with those of **6**, the pentasubstituted phenyl ring B in **6** was replaced by the tetrasubstituted phenyl group in **7**, which contained two protons at δ 6.68 (s, H-6′) and 6.39 (s, H-3′) and an *O*-methyl group (3.26, s) replacing the 7′-OH in **6**. Because the molecular mass of **7** was 20 Da lower than that of **6**, taking into account the 1D NMR data, it was implied that **7** was a dechlorinated and methylated product of **6**. This was also supported by the key HMBC correlations of H-7 with C-1, C-2, C-6, C-3′, C-4′, and C-5′ and of H-7′ with C-1′, C-2′, C-6′, and OCH_3. Thus compound **7** was established to be 2-(2,5-dihydroxybenzyl)-5-(methoxymethyl)benzene-1,4-diol.

Compound **8** was obtained as a brown gum and showed IR absorptions similar to those of **1**. The molecular formula of **8** was determined as $C_{22}H_{22}O_7$ by HRESIMS (*m*/*z* 397.1272, $[M - H]^-$). The ^1H and ^{13}C NMR data (Tables 1 and 2) indicated that ring A and ring C of **8** were the same as **7**, and this was confirmed by the HMBC data (Figure 2). However, **8** had six more protons (two phenolic hydroxy groups, two aromatic protons, and one shielded methylene) in the ^1H NMR spectrum and seven more carbons (six aromatic carbons including two oxygenated ones and one nonoxygenated methylene carbon) in the ^{13}C NMR spectrum. In conjunction with the MS data, it suggested that **8** was composed of three monomeric units. Because of the near overlay of C-1′ (125.0) and C-4′ (124.9), C-2′ (147.3) and C-5′ (147.3), C-3′ (116.4) and C-6′ (116.5), and H-3′ (6.40 s) and H-6′ (6.38 s), ring B was symmetrical, which was further supported by the HMBC correlations of OH-2′ with C-1′ and C-2′, OH-5′ with C-4′ and C-5′, H-3′ with C-1′, C-4′, and C-7, and H-6′ with C-1′, C-4′, and C-7′. Furthermore, the HMBC correlations of H-6 with C-7, H-3′ with C-7, H-6′ with C-7′, and H-3″ with C-7′ also revealed that the three moieties were connected through carbon−carbon bonds between C-7 and C-4′ and between C-7′ and C-4″, respectively. Thus, the structure of compound **8** was established to be 2-[2,5-dihydroxy-4-(methoxymethyl)benzyl]-5-(2,5-dihydroxybenzyl)benzene-1,4-diol.

Compound **12**, a colorless amorphous powder, had the molecular formula $C_8H_9ClO_3$ as determined by HRESIMS at *m*/*z* 187.0165 ($[M - H]^-$, calcd 187.0162). The ^1H and ^{13}C NMR spectra of **12** were very similar to those of **10** except for the presence of one *O*-methyl group (δ_H 3.29, 3H, s; δ_C 57.8), indicating the replacement of the alcoholic hydroxy group in **10** by OCH_3 in **12**. Thus the structure of **12** was established as a methylated derivative of **10**.

Compounds **1**–**8**, **12**, and **13** were tested for cytotoxic effects on HL-60 and MOLT-4 cell lines by the MTT method[19] and A-549 and BEL-7402 cell lines by the SRB method.[20] All evaluated compounds showed cytotoxicity toward the four cancer cell lines (Table 3). Furthermore, when the 10 compounds were assayed for their inhibitive activity against protein tyrosine kinases by ELISA, only compound **6** showed moderate activity (Table 5).

Gentisyl alcohol and 3-chlorogentisyl alcohol are common in microorganisms, but their dimers and trimers are rare. Trimers of

Table 3. Cytotoxicity of Compounds **1**– **8**, **12**, and **13**

	cytotoxicity (IC_{50}, μM)			
compound	HL-60	MOLT-4	A-549	BEL-7402
1	6.1	5.8	18.3	62.3
2	5.5	5.6	18.2	57.3
3	5.3	5.5	14.3	38.5
4	54.7	6.4	9.6	59.0
5	55.0	58.1	13.8	63.2
6	5.1	6.5	5.7	6.0
7	6.3	5.8	33.8	61.9
8	33.3	5.5	23.5	57.0
12	6.7	64.7	56.5	58.1
13	58.9	86.2	>100	>100

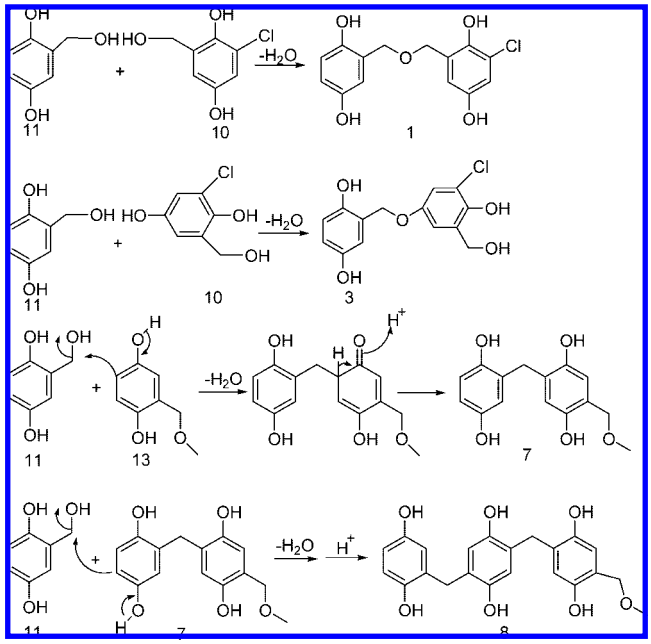

Figure 3. Possible biogenetic pathways of compounds **1**, **3**, **7**, and **8**.

Table 4. DPPH Radical Scavenging Activity of Compounds **1**–**8**, **12**, and **13**

compound	IC_{50} (μM)	compound	IC_{50} (μM)
1	4.3	**6**	4.1
2	4.6	**7**	6.3
3	4.4	**8**	2.6
4	6.2	**12**	8.5
5	5.2	**13**	9.8
AA[a]	17.4		

[a] AA = ascorbic acid (positive control).

gentisyl alcohol are unprecedented in nature. A possible biogenetic pathway of dimers and trimers may be through dehydration of the gentisyl alcohol and its derivatives, which includes three types (Figure 3): dehydration involving the two benzylic hydroxy groups (**1**, **2**); dehydration involving the alcoholic hydroxy group and a 5′-phenolic hydroxy group (**3**, **4**); dehydration involving the alcoholic hydroxy group and an aromatic proton (**5**–**9**).[11]

Compounds **1**–**8**, **12**, and **13** were also evaluated for their radical scavenging activity against DPPH.[21] As shown in Table 4, all of the new compounds showed moderate activity (ascorbic acid as positive control, IC_{50} 17.4 μM).

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were recorded on a Nicolet Nexus 470 spectrophotometer in KBr discs. ^{1}H, ^{13}C NMR, DEPT, and 2D NMR spectra were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as an internal standard, and chemical shifts were recorded as δ values. ESIMS were measured on a Q-TOF Ultima Global GAA076 LC mass spectrometer. Semipreparative HPLC was performed using an ODS column [YMC-pack ODS (A), 10 × 250 mm, 5 μm, 4 mL/min].

Table 5. PTK Inhibitory Activity of Compound **6**

		inhibitory rate (%)	
compound	concentration (μM)	Src	KDR
6	10	35.9	31.8
PP2[a]	1	89.4	
SU11248[b]	10		82.1

[a] PP2, positive control of Src. [b] SU11248, positive control of KDR.

Fungal Material. The fungus *P. terrestre* was isolated from marine sediments collected in Jiaozhou Bay, China. It was identified by Professor C. X. Fang, China Center for Type Culture Collection (identification number: 2004021), and the voucher specimen is preserved in the China Center for Type Culture Collection (patent depositary number: CCTCC M 204077). Working stocks were prepared on potato dextrose agar slants stored at 4 °C.

Fermentation and Extraction. The fungus was cultured under static conditions at 24 °C for 30 days in 200 1000-mL conical flasks containing the liquid medium (300 mL/flask) composed of soytone (0.1%), soluble starch (1.0%), and seawater (60%). The fermented whole broth (60 L) was filtered through cheese cloth to separate it into supernatant and mycelia. The former, which showed strong radical scavenging activity against DPPH, was extracted three times with EtOAc to give an EtOAc solution and concentrated under reduced pressure to give a crude extract (20.0 g).

Purification. The crude extract (20.0 g) was applied to a Si gel (200−300 mesh) column packed in petroleum ether. The column was eluted in gradient ratios with petroleum ether−$CHCl_3$ and then with $CHCl_3$−MeOH. The active fraction that was eluted with $CHCl_3$−MeOH (10:1) was further separated into five subfractions by Sephadex LH-20 using MeOH as the eluting solvent. Subfraction 2 was further purified by PHPLC (35% MeOH, 4.0 mL/min), to give compound **8** (2.8 mg). Subfraction 3 was further purified by PHPLC (30% MeOH, 4.0 mL/min) to yield compounds **1** (6.2 mg), **3** (5.3 mg), **6** (4.9 mg), and **7** (4.5 mg). Subfraction 4 was further purified by PHPLC (25% MeOH, 4.0 mL/min) to yield compounds **2** (6.7 mg), **4** (5.1 mg), **5** (5.8 mg), and **9** (6.5 mg). Subfraction 5 was further purified by PHPLC (20% MeOH, 4.0 mL/min) to yield compounds **10** (30.2 mg), **11** (25.8 mg), **12** (9.8 mg), and **13** (18.7 mg).

Biological Assays. In the MTT assay, the cell lines were grown in RPMI-1640 supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Two hundred microliters of those cell suspensions at a density of 5×10^4 cell mL^{-1} was plated in 96-well microtiter plates and incubated for 24 h at the above condition. Then 2 μL of the test compound solution (in MeOH) at different concentrations was added to each well and further incubated for 72 h in the same condition. Twenty microliters of the MTT solution (5 mg/mL in IPMI-1640 medium) was added to each well and incubated for 4 h. Then 150 μL of an old medium containing MTT was gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a Spectra Max Plus plate reader at 540 nm.

In the SRB assay, 200 μL of the cell suspensions was plated in 96-well plates at a density of 2×10^5 cell mL^{-1}. Then 2 μL of the test compound solutions (in MeOH) at different concentrations was added to each well, and the culture was further incubated for 24 h. Following drug exposure, the cells were fixed with 12% trichloroacetic acid and the cell layer was stained with 0.4% SRB. The absorbance of SRB solution was measured at 515 nm. Dose−response curves were generated, and the IC_{50} values, the concentration of compound required to inhibit cell proliferation by 50%, were calculated from the linear portion of the log dose−response curves.

ELISA. The tyrosine kinase activities were determined in 96-well ELISA plates precoated with 20 μg/mL Poly (Glu, Tyr)$_{4:1}$ (Sigma, St. Louis, MO). Briefly, 80 μL of 5 μM ATP solution diluted in kinase reaction buffer (50 mM HEPES pH 7.4, 20 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.2 mM Na_3VO_4, 1 mM DTT) was added to each well. Various concentrations of compounds diluted in 10 μL of 1% DMSO (v/v) were added to each reaction well, with 1% DMSO (v/v) used as the negative control. Experiments at each concentration were performed in duplicate.

The kinase reaction was initiated by the addition of purified tyrosine kinase proteins diluted in 10 μL of kinase reaction buffer solution. After incubation for 1 h at 37 °C, the plate was washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (T-PBS). Next, 100 μL of anti-phosphotyrosine (PY99) antibody (1:500 dilution) diluted in T-PBS containing 5 mg/mL BSA was added. After 30 min incubation at 37 °C, the plate was washed three times. Horseradish peroxidase-conjugated goat anti-mouse IgG (100 μL) diluted 1:2000 in T-PBS containing 5 mg/mL BSA was added. The plate was reincubated at 37 °C for 30 min and washed as before. Finally, 100 μL of a solution containing 0.03% H_2O_2 and 2 mg/mL *o*-phenylenediamine in 0.1 M citrate buffer, pH 5.5, was added, and samples were incubated at room temperature until color emerged. The reaction was terminated by the addition of 50 μL of 2 M H_2SO_4, and the plate was read using a multiwell spectrophotometer (VERSAmax, Molecular Devices, Sunnyvale, CA) at 490 nm. The inhibition rate (%) was calculated using the following equation: $[1 - (A_{490}/A_{490}\ \text{control})] \times 100\%$. IC_{50} values were calculated from the inhibition curves.

In the DPPH scavenging assay, samples to be tested were dissolved in MeOH and the solution (160 μL) was dispensed into wells of a 96-well microtiter tray. Forty microliters of the DPPH solution in MeOH (1.5×10^{-4}) was added to each well. The mixture was shaken and left to stand for 30 min. After the reaction, absorbance was measured at 520 nm, and the percent inhibition was calculated. IC_{50} values denote the concentration of sample required to scavenge 50% of the DPPH free radicals.

Compound 1: brown gum; UV (MeOH) λ_{max} (log ϵ) 298 (3.40) nm; IR (KBr) ν_{max} 3257, 1609, 1465, 1359, 1202, 986 cm^{-1}; ^1H and ^{13}C NMR (Tables 1 and 2); HRESIMS *m/z* 295.0372 $[M - H]^-$ (calcd for $C_{14}H_{12}ClO_5$, 295.0373).

Compound 2: brown gum; UV (MeOH) λ_{max} (log ϵ) 298 (3.51) nm; IR (KBr) ν_{max} 3226, 1623, 1496, 1462, 1352, 1202, 1004 cm^{-1}; ^1H and ^{13}C NMR (Tables 1 and 2); HRESIMS *m/z* 261.0766 $[M - H]^-$ (calcd for $C_{14}H_{13}O_5$, 261.0763).

Compound 3: brown gum; UV (MeOH) λ_{max} (log ϵ) 297 (3.59) nm; IR (KBr) ν_{max} 3309, 1595, 1482, 1400, 1195, 1004 cm^{-1}; ^1H and ^{13}C NMR (Tables 1 and 2); HRESIMS *m/z* 295.0370 $[M - H]^-$ (calcd for $C_{14}H_{12}ClO_5$, 295.0373).

Compound 4: brown gum; UV (MeOH) λ_{max} (log ϵ) 295 (3.48) nm; IR (KBr) ν_{max} 3254, 1616, 1503, 1445, 1366, 1205, 1004 cm^{-1}; ^1H and ^{13}C NMR (Tables 1 and 2); HRESIMS *m/z* 261.0769 $[M - H]^-$ (calcd for $C_{14}H_{13}O_5$, 261.0763).

Compound 5: brown gum; UV (MeOH) λ_{max} (log ϵ) 300 (3.52) nm; IR (KBr) ν_{max} 3254, 1602, 1458, 1294, 1215, 993 cm^{-1}; ^1H and ^{13}C NMR (Tables 1 and 2); HRESIMS *m/z* 295.0368 $[M - H]^-$ (calcd for $C_{14}H_{12}ClO_5$, 295.0373).

Compound 6: brown gum; UV (MeOH) λ_{max} (log ϵ) 299 (3.64) nm; IR (KBr) ν_{max} 3237, 1616, 1434, 1356, 1202, 997 cm^{-1}; ^1H and ^{13}C NMR (Tables 1 and 2); HRESIMS *m/z* 295.0372 $[M - H]^-$ (calcd for $C_{14}H_{12}ClO_5$, 295.0373).

Compound 7: brown gum; UV (MeOH) λ_{max} (log ϵ) 298 (3.40) nm; IR (KBr) ν_{max} 3250, 1599, 1448, 1359, 1205, 1000 cm^{-1}; ^1H and ^{13}C NMR (Tables 1 and 2); HRESIMS *m/z* 275.0913 $[M - H]^-$ (calcd for $C_{15}H_{15}O_5$, 275.0919).

Compound 8: brown gum; UV (MeOH) λ_{max} (log ϵ) 299 (3.91) nm; IR (KBr) ν_{max} 3254, 1619, 1499, 1438, 1356, 1198, 986 cm^{-1}; ^1H and ^{13}C NMR (Tables 1 and 2); HRESIMS *m/z* 397.1272 $[M - H]^-$ (calcd for $C_{22}H_{21}O_7$, 397.1287).

Compound 10: colorless, amorphous powder; ^1H NMR (d_6-DMSO, 600 MHz) δ 9.12 (s, 2-OH), 8.41 (s, 5-OH), 6.75 (d, 2.8, H-6), 6.62 (d, 2.8, H-4), 5.22 (t, 5.5, 7-OH), 4.48 (2H, d, 5.5, H-7); ^{13}C NMR (d_6-DMSO, 150 MHz) δ 150.6 (qC, C-5), 141.9 (qC, C-2), 132.8 (qC, C-1), 120.7 (qC, C-3), 113.6 (CH, C-4), 113.0 (CH, C-6), 58.9 (CH_2, C-7); ESIMS *m/z* 173 $[M - H]^-$.

Compound 11: colorless, amorphous powder; ^1H NMR (d_6-DMSO, 600 MHz) δ 8.59 (s, 2-OH), 8.57 (s, 5-OH), 6.76 (d, 3.2, H-6), 6.57 (d, 8.2, H-3), 6.45 (dd, 8.2, 3.2, H-4), 4.94 (t, 5.5, 7-OH), 4.43 (2H, d, 5.5, H-7); ^{13}C NMR (d_6-DMSO, 150 MHz) δ 149.9 (qC, C-5), 146.5 (qC, C-2), 129.5 (qC, C-1), 115.3 (CH, C-3), 114.2 (CH, C-6), 113.5 (CH, C-4), 58.5 (CH_2, C-7); EIMS *m/z* 140 $[M]^+$.

Compound 12: colorless, amorphous powder; UV (MeOH) λ_{max} (log ϵ) 298 (3.45) nm; IR (KBr) ν_{max} 3361, 1603, 1467, 1323, 1193, 1085 cm^{-1}; ^1H and ^{13}C NMR (Tables 1 and 2); HRESIMS *m/z* 187.0165 $[M - H]^-$ (calcd for $C_8H_8ClO_3$, 187.0162).

Compound 13: colorless, amorphous powder; UV (MeOH) λ_{max} (log ϵ) 297 (3.40) nm; IR (KBr) ν_{max} 3364, 1623, 1464, 1361, 1206, 1080 cm^{-1}; ^1H and ^{13}C NMR (Tables 1 and 2); EIMS *m/z* 154 $[M]^+$.

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