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•Research article•

Four new diphenyl ether derivatives from a mangrove endophytic fungus *Epicoccum sorghinum*

ZHU Jun-Jie, HUANG Qi-Sen, LIU Sheng-Quan, DING Wei-Jia, XIONG Ya-Hong*, LI Chun-Yuan*

Key Laboratory for Biobased Materials and Energy of Ministry of Education, College of Materials and Energy, South China Agricultural University, Guangzhou 510642, China

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[ABSTRACT] Four new diphenyl ethers, named epicoccethers K−N (**1**−**4**), were purified from the fermentation medium of a fungus *Epicoccum sorghinum* derived from *Myoporum bontioides*, and identified through HR-ESI-MS and NMR spectral analysis. Except that compound **1** showed moderate antifungal activity against *Penicillium italicum* and *Fusarium graminearum*, the other three compounds showed stronger activity against them than triadimefon. All of them showed moderate or weak antibacterial activity towards *Staphylococcus aureus* and *Escherichia coli* with O6 and O78 serotypes except that **3** was inactive to *E. coli* O6.

[KEY WORDS] *Epicoccum sorghinum*; Antimicrobial activity; Diphenyl ether; Secondary metabolite
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Introduction

Nowadays, marine fungi with high adaptability to extreme ocean environments become one of the most important sources of new drug leads [1-4]. Among them, *Epicoccum sorghinum* has produced metabolites with multifunction including anti-inflammation, antiplatelet aggregation, antiangiogenesis and cytotoxicity against triple-negative breast cancer cells [5, 6]. During our effort to search for novel antimicrobial compounds from different endophytes [7-9], a fungus of *Epicoccum sorghinum* L28 purified from *Myoporum bontioides* A. Gray collected in mangrove was chemically investigated. Herein, the isolation, identification and antimicrobial evaluation of four new diphenyl ethers (**1**−**4**) (Fig. 1) are reported.

Materials and Methods

Instruments, reagents and chemicals

HR-ESI-MS were evaluated by a LCMS-IT-TOF (Shimadzu Corporation, Tokyo, Japan) mass spectrometer. NMR spectra were recorded on a Bruker AV600 spectrometer (Bruker Biospin GmbH Corporation, Karlsruhe, Germany) with tetramethylsilane (TMS) as a reference. Column chromatography (CC) was carried out using 50−80 μm silica gel (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China), and Sephadex LH-20 (Merck company, Darmstadt, Germany). HPLC was performed on an Elite system with a P230p pump and a UV230II wavelength detector (Elite Analytical Instrument Co., Ltd., Dalian, China) using a C_{18} column (250 mm × 10 mm, 5 μm, H&E Co., Ltd., Beijing, China). All solvents were of analytical grade except methanol (HPLC grade).

Microbial materials

E. sorghinum L28 was isolated from a semi-mangrove plant *M. bontioides* in Leizhou Peninsula, China, in June 2018, and then identified according to its Internal Transcribed Spacer (ITS) rRNA sequence (No. MZ378789 in GenBank) [10]. The strain and the pathogens *Penicillium italicum*, *Fusarium graminearum*, *Escherichia coli* with O6 serotype (*E. Coli* O6), *Escherichia coli* with O78 serotype (*E. Coli* O78) and *Staphylococcus aureus* were acquired and stored in College of Materials and Energy, South China Agricultural University (Guangzhou, China).

Cultivation, extraction, separation and spectroscopic data

After initial fermentation on PDA medium, the scale-up culture of *E. sorghinum* L28 were performed in 42 × 500 mL Erlenmeyer flasks without rotation at 27 °C for 25 days, using liquid medium (250 mL water, 5 g glucose, and 0.6 g tryptone). The obtained fermentation product was extracted

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[*Corresponding author] Tel: 86-20-85280319, E-mails: xiongyahong@scau.edu.cn (XIONG Ya-Hong); chunyuanli@scau.edu.cn (LI Chun-Yuan)
These authors have no conflict of interest to declare.

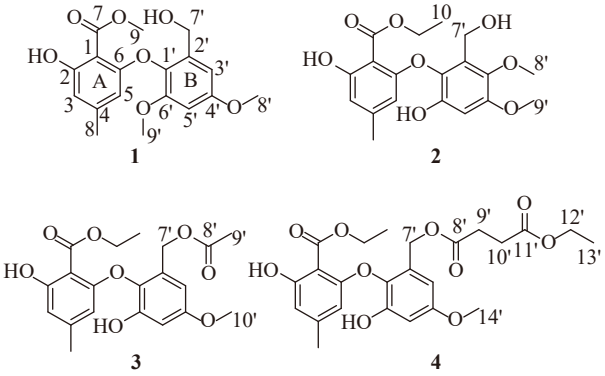

Fig. 1 Structures of compounds 1–4

using ethyl acetate (EtOAc) for three times, and then the solvent was evaporated to obtain a black crude extract. The extract (30.6 g) was transferred to CC (55 cm × 6 cm) and partitioned into seven fractions (Fr. A to Fr. G) through gradient elution using the mixture of petroleum ether (PE) : EtOAc (*V/V*, 21 : 1, 7 : 1, 3 : 1, 1 : 1, 1 : 3, 1 : 7, 1 : 21). Fr. C (6.2 g) was then partitioned into twenty-three fractions (Fr. C1 to Fr. C23) on CC (45 cm × 2.5 cm) eluted with PE : EtOAc (*V/V*, 21 : 1, 7 : 1, 3 : 1, 1 : 1) according to the TLC properties. Fr. C7 (0.73 g) was subjected to Sephadex LH-20 CC (55 cm × 1.0 cm, MeOH) to get thirteen subfractions (Fr. C7.1 to Fr. C7.13). Fr. C7.8 (9.5 mg) was further purified by HPLC eluted with MeOH : H_2O (*V/V*, 70 : 30 to 90 : 10, 3.0 mL·min^{-1}, 20 min) to obtain compound **1** (5.6 mg, t_R 16.6 min). Fr. C9 (0.97 g) was separated on Sephadex LH-20 CC (68 cm × 1.0 cm, MeOH) to give fifteen subfractions (Fr. C9.1 to Fr. C9.15). Fr. C9.11 (13.3 mg) was further separated by HPLC with MeOH : H_2O (*V/V*, 40 : 60 to 100 : 0, 3.0 mL·min^{-1}, 60 min) to get products **3** (4.8 mg, t_R 37.1 min) and **4** (3.4 mg, t_R 40.9 min). Fr. D (8.1 g) was then partitioned into nineteen fractions (Fr. D1 to Fr. D19) on CC (52 cm × 2.5 cm) eluted with PE : EtOAc (*V/V*, 7 : 1, 3 : 1, 1 : 1, 1 : 3) with respect to the TLC properties. Fr. D9 (0.53 g) was partitioned on Sephadex LH-20 CC (68 cm × 1.0 cm, MeOH) to get seventeen subfractions (Fr. D9.1 to Fr. D9.17). Fr. D9.10 (8.7 mg) was subjected to HPLC eluted with MeOH : H_2O (*V/V*, 60 : 40 to 80 : 20, 3.0 mL·min^{-1}, 20 min) to afford **2** (5.2 mg, t_R 19.1 min).

Epicoccether K (**1**). Colorless oil; HR-ESI-MS *m/z* 347.1130 $[M - H]^-$ (Calcd. for $C_{18}H_{19}O_7$, 347.1131); IR (KBr) ν_{max} 3489, 2954, 1730, 1659, 1606, 1488, 1440, 1207, 1155, 1067, 827 cm^{-1}; UV (MeOH) λ_{max} (log ε): 207 (4.51), 255 (3.60), 296 (2.12) nm; ^{1}H and ^{13}C NMR (Table 1).

Epicoccether L (**2**). Colorless oil; HR-ESI-MS *m/z* 379.1390 $[M + H]^+$ (Calcd. for $C_{19}H_{23}O_8$, 379.1393); IR (KBr) ν_{max} 3420, 2934, 1657, 1621, 1489, 1453, 1310, 1252, 1205, 1078, 1003, 828 cm^{-1}; UV (MeOH) λ_{max} (log ε): 208 (4.55), 256 (3.41), 297 (2.33) nm; ^{1}H and ^{13}C NMR (Table 2).

Epicoccether M (**3**). White solid; HR-ESI-MS *m/z* 391.1391 $[M + H]^+$ (Calcd. for $C_{20}H_{23}O_8$, 391.1393); IR (KBr) ν_{max} 3430, 2927, 1730, 1655, 1621, 1452, 1311, 1256, 1207, 1155, 1064, 830 cm^{-1}; UV (MeOH) λ_{max} (log ε): 205 (4.65), 255 (3.37), 286 (2.01), 317 (1.97) nm; ^{1}H and ^{13}C NMR (Table 1).

Epicoccether N (**4**). Orange oil; HR-ESI-MS *m/z* 477.1758 $[M + H]^+$ (Calcd. for $C_{24}H_{29}O_{10}$, 477.1761); IR (KBr) ν_{max} 3430, 2912, 1735, 1658, 1587, 1451, 1217, 1083, 831 cm^{-1}; UV (MeOH) λ_{max} (log ε): 203 (4.51), 257 (3.35), 289 (2.13), 313 (2.03) nm; ^{1}H and ^{13}C NMR (Table 1).

Antimicrobial evaluation

Antimicrobial activities against two phytopathogenic fungi *P. italicum*, and *F. graminearum*, and three pathogenic bacteria *E. coli* O6, *E. coli* O78 and *S. aureus* were evaluated by the dilution method, according to previous reports [6]. Triadimefon (for fungi) and cefradine (for bacteria) (Aladdin Bio-Chem Tech., Co., Shanghai, China) were used as the positive controls, while PDB : 5% DMSO : H_2O 50 : 50 (*V* : *V*) (the solvent) was the negative control.

Results and Discussion

Epicoccether K (**1**) possessed a molecular formula of $C_{18}H_{20}O_7$ as shown in HR-ESI-MS at *m/z* 347.1130 ($[M - H]^-$, Calcd. 347.1131). There were two groups of meta-positioned aromatic protons at δ_H 6.40 (1H, d, 1.8 Hz, H-3), 5.86 (1H, 1.8 Hz, H-5), and at δ_H 6.61 (1H, d, 3.0 Hz, H-3′), 6.78 (1H, d, 3.0 Hz, H-5′) in the ^{1}H NMR spectrum of **1** (Table 1), indicating two 1,2,4,6-tetrasubstituted benzenes in **1**. The ^{1}H NMR data also included three methoxys at δ_H 3.83 (H-8′), 3.74 (H-9′) and 3.93 (H-9), a chelated phenolic hydroxy at δ_H 11.19 (2-OH), an aromatic methyl at δ_H 2.12 (H-8), a hydroxymethyl at δ_H 4.53 (H-7′), and a hydroxy at δ_H 4.14 (7′-OH). The ^{13}C NMR (Table 1) and HSQC spectra displayed signals for twelve aromatic carbons, of which four were methines (δ_c 111.5, C-3; δ_c 106.2, C-5; δ_c 104.3, C-3′; and δ_c 99.7, C-5′) and the others (δ_c 102.1, C-1; δ_c 163.5, C-2; δ_c 146.9, C-4; δ_c 160.2, C-6; δ_c 134.1, C-1′; δ_c 137.8, C-2′; δ_c 158.9, C-4′; and δ_c 153.6, C-6′) were quaternary carbons. Moreover, an ester carbonyl (δ_c 172.0, C-7), a hydroxymethyl (δ_c 59.7, C-7), an aromatic methyl (δ_c 22.0, C-8) and three methoxy (δ_c 52.8, C-9; δ_c 55.9, C-8′; and δ_c 56.4, C-9′) carbons were observed. These data were much similar to methyl barceloneate acquired from *Penicillium albocoremium* [11], except for one more methoxy (C-9′) and one less hydroxyl in **1**. HMBC correlation from H-9′ to C-6′ indicated that the methoxy, rather than the hydroxyl, was located at C-6′ in **1**, which was different from methyl barceloneate. Thus, the structure of **1** was established and then confirmed by comprehensive analysis of the HMBC correlations (Fig. 2).

Epicoccether L (**2**) possessed a molecular formula of $C_{19}H_{22}O_8$ (nine degrees of unsaturation) as shown in HR-ESI-MS at *m/z* 379.1390 ($[M + H]^+$, Calcd. 379.1393). There were two meta-positioned aromatic protons at δ_H 6.52 (1H, d, 1.8 Hz, H-3), 6.03 (1H, d, 1.8 Hz, H-5), and another aromatic proton at δ_H 6.63 (1H, s, H-5′) in the ^{1}H NMR spectrum of **1** (Table 1). Moreover, the ^{13}C NMR spectrum showed twelve olefinic and one ester carbonyl carbon signals. These findings suggested that **2** contained two benzenes, where one was 1,2,4,6-tetrasubstituted, and the other was pentasubstituted. A

Table 1 ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data of compounds **1–4**

No.	**1**[a]		**2**[b]		**3**[a]		**4**[a]	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
1		102.1		101.6		102.8		102.8
2		163.5		162.6		163.1		163.1
2-OH	11.19 (s)		10.74 (s)		11.15 (s)		11.11 (s)	
3	6.40 (d, 1.8)	111.5	6.52 (d, 1.8)	112.8	6.43 (d, 1.8)	111.7	6.42 (d, 1.8)	111.7
4		146.9		147.1		146.5		146.6
5	5.86 (d, 1.8)	106.2	6.03 (d, 1.8)	106.7	5.96 (d, 1.8)	106.7	5.96 (d, 1.8)	106.6
6		160.2		158.2		160.0		160.0
7		172.0		169.7		170.5		171.3
8	2.12 (s)	22.0	2.18 (s)	22.2	2.16 (s)	21.9	2.16 (s)	21.9
9	3.93 (s)	52.8	4.52 (q, 7.2)	62.5	1.37 (t, 7.2)	14.5	1.37 (t, 7.2)	14.5
10			1.42 (t, 7.2)	14.3	4.42 (q, 7.2)	62.2	4.42 (q, 7.2)	62.2
1′		134.1		132.4		134.3		134.3
2′		137.8		128.5		131.8		131.8
3′	6.78 (d, 3.0)	104.3		140.9	6.57 (d, 3.0)	106.6	6.57 (d, 3.0)	106.3
4′		158.9		151.3		158.7		158.7
5′	6.61 (d, 3.0)	99.7	6.63 (s)	100.9	6.59 (d, 3.0)	103.4	6.59 (d, 3.0)	103.4
6′		153.6		145.3		151.5		151.5
6′-OH			6.06 (s)					
7′	4.53 (d, 5.4)	59.7	4.59 (s)	56.2	5.00 (s)	61.9	5.05 (s)	62.0
7′-OH	4.14 (t, 5.4)							
8′	3.84 (s)	55.9	3.89 (s)	61.9		171.3		172.3
9′	3.74 (s)	56.4	3.87 (s)	56.2	1.86 (s)	20.5	2.47 (m)	27.9
10′					3.80 (s)	55.8	2.47 (m)	27.9
11′								172.6
12′							4.06 (q, 7.2)	60.9
13′							1.18 (t, 7.2)	14.5
14′							3.80	56.0

[a]Measured in CD_3COCD_3; [b]Measured in $CDCl_3$

Table 2 **Antifungal and antibacterial activities of compounds 1–4**

Compound	*P. italicum*	*F. graminearum*	*E. coli* O6	*E. coli* O78	*S. aureus*
1	100	200	25	100	50
2	50	50	50	200	50
3	50	100	> 200	100	100
4	25	50	200	25	100
Triadimefon[Ⅰ]	50	150	NT	NT	NT
Cefradine[Ⅱ]	NT	NT	3.125	12.5	1.0

[Ⅰ] Positive control toward fungi; [Ⅱ] Bacterial positive control

contrastive analysis of the ^{13}C NMR data for **2** and **1** (Table 1) showed that the structural unit of benzene ring A in **2** was almost consistent with that of **1**. However, the $-COOCH_3$ moiety at C-1 in **1** was replaced by $-COOCH_2CH_3$ in **2**, which was revealed by ^{1}H-^{1}H COSY correlation between H-9 and H-10 and HMBC correlation from H-9 to C-7. For the benzene ring B in **2**, two methoxys (δ_H 3.87, H-9′ and δ_H 3.89, H-8′), an aromatic hydroxymethyl (δ_H 4.59, H-7′), and a hydroxy (δ_H 6.06, 6′-OH) were visible in the ^{1}H NMR spectrum (Table 1). HMBC correlations from H-7′ to C-1′, C-2′ and C-3′, from H-8′ to C-3′, from 6′-OH to C-1′, C-5′ and C-6′, from H-5′ to C-3′ and C-4′, and from H-9′ to C-4′, placed them at their own positions as shown in Fig. 2, while implied

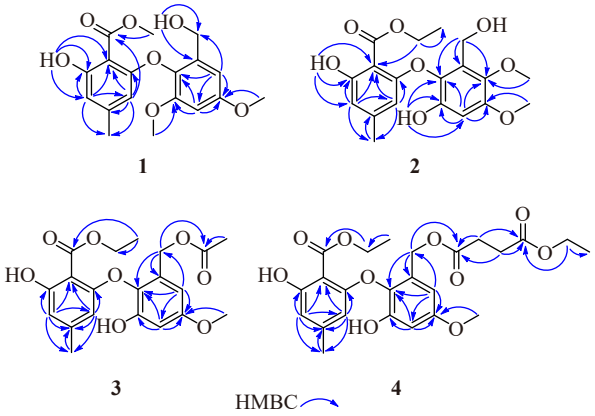

Fig. 2 Key HMBC of compounds 1–4

the connectivity of C-1′ with the oxygen atom at C-6.

Epicoccether M (**3**) possessed a molecular formula of $C_{20}H_{22}O_8$ as shown in HR-ESI-MS at *m/z* 391.1391 ([M + H]$^+$, Calcd. 391.1393). A contrastive analysis of the NMR data for **3** and **2** (Table 1) showed that the A ring in **2** was completely retained in **3**. Comparison of the remaining data of **3** with those of barceloneic acid A produced by a fungus of *Phoma* sp. [12], indicated that the B ring of **3** had one more acetyl group (δ_H 1.86, H-9′; δ_c 171.3, C-8′, 20.5, C-9′) than that of barceloneic acid A. HMBC correlations from H-7′ (δ_H 5.0) to C-8′, along with the chemical shift of C-8′, suggested that this acetyl group was connected to the oxygen atom attached on C-7′ to form an acetoxy group. Accordingly, the structure was determined, and then its NMR data was completely assigned (Table 1) by analysis of the HMBC correlations (Fig. 2).

Epicoccether N (**4**) possessed a molecular formula of $C_{24}H_{28}O_{10}$ shown by HR-ESI-MS at *m/z* 477.1758 ([M + H]$^+$, Calcd. 477.1761). A contrastive analysis of the NMR data for **4** and **3** (Table 1) showed that the A ring in **4** was identical to that in **3**. Comparing the remaining NMR data of **4** with those of barceloneic acid A [12], indicated that the B rings of theirs were quite similar. However, the former had one more ethoxy group (δ_H 1.18, H-13′, δ_c 14.5, C-13′; δ_H 4.06, H-12′, δ_c 60.9, C-12′), two more carbonyl carbons (δ_c 172.3, C-8′; δ_c 172.6, C-11′) and two more methenes (δ_H 2.47, H-9′ and H-10′; δ_c 27.9, C-9′ and C-10′), which formed a substitute –$COCH_2CH_2COOCH_2CH_3$ revealed by HMBC correlations from H-12′ to C-11′, and from H-9′ and H-10′ to C-8′ and C-11′. HMBC correlations from H-7′ to C-8′ indicated that this substituent was located at 7′-O atom (Fig. 2).

Except that **1** showed moderate antifungal activity against *Penicillium italicum* and *Fusarium graminearum*, the other three compounds showed stronger activity against them than the positive control triadimefon. Moreover, the activity of **2** and **4** against *F. graminearum* was three times that of triadimefon, and the activity of **4** against *P. italicum* was twice that of triadimefon. For antibacterial assay, all compounds showed moderate or weak activity (MICs 25−200 μg·mL^{-1}) towards *S. aureus*, *E. coli* (O6 serotype), and *E. coli* (O78 serotype) except that **3** was inactive to *E. coli* (O6 serotype) (MIC > 200 μg·mL^{-1}). These results provide new candidates of antimicrobial leads, especially antifungal ones against the pathogens listed above.

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