ORIGINAL ARTICLE

Isolation and structure elucidation of new phthalide and phthalane derivatives, isolated as antimicrobial agents from *Emericella* sp. IFM57991

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Three new phthalide derivatives, emefuranones A₁, A₂ and B (1–3); six new phthalane derivatives, emefuran A, B₁, B₂, C₁, C₂ and D (4–9); three new farnesylated phthalide derivatives, farnesylemefuranones A–C (10–12); xylarinol C (13); and emericelloxide (14), along with four known compounds (dustanin, sorbicillin, aspergillodiol and xylarinol A), were isolated from the culture extracts of *Emericella* sp. IFM57991. Structures of 1–14 were elucidated on the basis of spectroscopic analysis and chemical evidence. Compounds 4–7 and 13 showed moderate antibacterial activities against *Bacillus subtilis*.

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INTRODUCTION

Ascomycete fungi of the genus *Emericella* are well known for their ability to produce diverse bioactive compounds. Our research group previously has isolated multiple secondary metabolites (some with bioactivities) from species of this genus, with examples including emestrin (a novel antifungal macrocyclic epidthiodioxopiperazine) from *E. striata*^{1,2}; emesterones A–B³ (18,22-cyclosterols with antifungal and cytotoxic activities) and emethallicins A–F^{4–6} (epitetrathiodioxopiperazine derivatives that are potent inhibitors of mast cell histamine release) from *E. heterothallica*; variecolol, variecolactone and variecolin⁷ (sesterterpene inhibitors of angiotensin II receptor binding), epurpurins A–C^{8,9} (dicyanide derivatives), and emindoles PA–PC¹⁰ (indoloditerpenes) from *E. purpurea*; falconensins A–N^{11–15} (azaphilone derivatives with anti-inflammatory activity), falconensones A–B^{16–19} (yellow pigments with antioxidant activity) and differentiation and apoptosis inducers from *E. falconensis* and *E. fruiticulosa*; and nidulalin A (a dihydroxanthone derivative, subsequently reported to have DNA topoisomerase II inhibitor activity^{20,21}) and nidulalin B (a benzophenone derivative, subsequently reported to have immunosuppressive activity²¹) from *E. nidulans*.²²

During our studies in search of bioactive compounds from fungi, we have isolated the interesting new fungal strain, IFM57991, belonging to the genus *Emericella*. From the culture extract of this fungus, we have isolated three new isobenzofuranone derivatives, emefuranones A₁, A₂ and B (1–3); six new isobenzofuran derivatives, emefuran A, B₁, B₂, C₁, C₂ and D (4–9); three new farnesylated isobenzofuranone derivatives, farnesylemefuranones A–C (10–12); xylarinol C (13); and emericelloxide (14), along with four known compounds (dustanin,²³ sorbicillin,²⁴ aspergillodiol²⁵ and xylarinol A²⁶; Figure 1).

In this paper, the isolation, structure elucidation and biological activities of these compounds are described.

RESULTS AND DISCUSSION

Planar structure of emefuranones (1–3) and emefurans (4–9)

The molecular formulae of 1–9 are summarized in Supplementary Table S1. The ¹H- and ¹³C-NMR spectra of 1–9 are shown in Table 1. A detailed analysis of the ¹H-NMR spectra of 1–9 revealed that these compounds share *o*-couplings phenyl proton signals, two singlet methyl proton signals, an olefin proton signal and methylene proton signals coupled above the olefin proton. ¹H–¹H COSY spectra showed the correlation between 5-H and 6-H in all 9 compounds (1–9), and the correlation between 1''-H₂ and 2''-H was observed in 1 and 3–9. The HMBC from 4''-H₃/5''-H₃ to C-2'', C-3'' and C-5''/C-4'', from 1''-H₂ to C-3a and C-5, from H-5 to C-3a, C-7, and C-1'', and from 6-H to C-4, C-7, and C-7a were observed in the partial structures of 1–9, respectively. In addition, the ¹H–¹H COSY spectrum of 2 did not detect the correlation between 1''-H₂ and 2''-H, but a HMBC correlation from 2''-H to C-4 was shown. On the basis of the above data, we postulated that compounds 1–9 have a prenylated hydroxyphenyl moiety as part of their common partial structure (Figure 2).

The molecular formulae of both 1 and 2 were established as C₂₁H₃₀O₅, and each compound had 7 degrees of unsaturation. The ¹H-NMR spectra of 1 and 2 each showed a primary methyl proton signal (1: δ 0.85 (t, *J* = 6.2 Hz), 2: δ 0.85 (t, *J* = 6.6 Hz) each), three methine proton signals including two oxygenated ones, five methylene proton signals and the peaks derived from the common moiety (Table 1). The ¹³C-NMR spectra of 1 and 2 both showed a characteristic carbonyl carbon signal (Table 1). The benzofuranone

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Figure 1 New compounds **1–14** and their derivatives from *Emericella* sp. IFM57991.

moiety in **1** and **2** was confirmed based on the correlations of HMBC spectra in **1** and **2** from 3-H to C-1, C-3a and C-7a (Figure 2). The ¹H-¹H COSY spectrum of **1** showed the continuous correlation between 3-H and 5'-H₂, and that of **2** showed the continuous correlation between 3-H and 4'-H₂ (Figure 2). The correlations of the HMBC spectrum of **1** were observed from 7'-H₂ to C-5' and C-6' and from 8'-H₃ to C-6' and C-7', and the correlations of the HMBC spectrum of **2** were observed from 7'-H₂ to C-5' and C-6' and from 8'-H₃ to C-6' (Figure 2). These results suggested that the planar structure of **1** and **2** was established as 3-(2,3-dihydroxyoctyl)-7-hydroxy-4-(3-methylbut-2-en-1-yl)-(3H)-isobenzofuran-1-one. Furthermore, **1** and **2** were hypothesized to be diastereomers based on the differences in melting point and optical rotation values when compared with each other (Supplementary Table S1).

The molecular formula of emefuranone B (**3**) was determined to be C₁₅H₁₆O₅ by HR-EI-MS (Supplementary Table S1). Comparison of the ¹³C-NMR spectrum of **3** with that of **1** revealed that the peaks belonging to the dihydroxyoctyl moiety in **1** were absent from **3**, whereas peaks corresponding to the C-2' carbonyl group (δ_C 170.8) and methylene group in **3** were absent from **1** (Table 1). These results and analysis of two-dimensional (2D)-NMR spectra established the structure of **3** as 2-[4-hydroxy-7-(3-methylbut-2-en-1-yl)-3-oxo-1,3-dihydroisobenzofuran-1-yl] acetic acid (Figure 2).

The molecular formula of emefuran A (**4**) was deduced as C₂₁H₃₂O₄, that is, the molecule had one fewer oxygen atom and

two more hydrogen atoms than **1**, as judged by HR-CI-MS (Supplementary Table S1). The ¹H- and ¹³C-NMR data of **4** were similar to those of **1**, except for that of the C-1 (**4**: δ 69.3, **1**: δ 168.1), and a new oxymethylene (δ_H 4.83 and δ_H 4.89) present in **4** (Table 1). These facts suggested that **4** was a 1-deoxy derivative of **1**. Moreover, based on the detailed analysis of 2D-NMR spectra in **4**, the structure of **4** was determined to be 3-(2,3-dihydroxyoctyl)-7-hydroxy-4-(3-methylbut-2-enyl)-(3H)-isobenzofuran (Figure 2).

Emefurans B₁ (**5**) and B₂ (**6**) were separated by preparative HPLC on a silica gel column, with respective retention time (*t_R*) values of 14.5 and 20.0 min. The molecular formula of these compounds was hypothesized as C₂₁H₃₀O₄ by HR-EI-MS and HR-CI-MS (Supplementary Table S1), and the ¹H- and ¹³C-NMR spectra of **5** and **6** were very similar to each other (Table 1). Moreover, the ¹H- and ¹³C-NMR spectra of **5** and **6** also were similar to those of **4**, except for that of the C-2' (δ 70.8 in **4**, δ 211.0 in both **5** and **6**), and the absence of 4's methine signal at 2'-H (δ 3.52) from **5** and **6** (Table 1). Based on the above results and the detailed analysis of the 2D-NMR spectra of **5** and **6**, it was hypothesized that **5** and **6** have the same planar structures, and that the two compounds were C-2' oxygenated derivatives of **4** (Figure 2).

Emefurans C₁ (**7**) and C₂ (**8**) were determined to have the same molecular formulae (C₂₃H₃₂O₅) by HR-EI-MS and HR-CI-MS, respectively (Supplementary Table S1), and the ¹H- and ¹³C-NMR spectra of **7** and **8** were similar to each other. Furthermore, optical

Table 1 ¹H- and ¹³C-NMR data of 1–9 in DMSO-*d*₆

Emefuranone A ₁		Emefuranone A ₂		Emefuranone B (3)		Emefuran A (4)		Emefuran B ₁ (5)		Emefuran B ₂ (6)		Emefuran c ₁ (7)		Emefuran c ₂ (8)		Emefuran d (9)	
(1)		(2)		(3)		(4)		(5)		(6)		(7)		(8)		(9)	
No.	δ _C	δ _H (J in Hz)	δ _C	δ _H (J in Hz)	δ _C	δ _H (J in Hz)	δ _C	δ _H (J in Hz)	δ _C	δ _H (J in Hz)	δ _C	δ _H (J in Hz)	δ _C	δ _H (J in Hz)	δ _C	δ _H (J in Hz)	
1	168.1	168.7	167.8	167.8	69.3	4.83 (d 12.4), 4.89 (d 12.4)	69.7	4.82 (d 12.6), 4.91 (d 12.4)	69.7	4.81 (d 12.2), 4.91 (d 12.2)	69.7	4.82 (d 12.1), 4.91 (d 12.3)	69.8	4.82 (d 12.4), 4.94 (d 12.4)	69.7	4.83 (d 12.4), 4.94 (dd 12.4, 2.5)	
3	76.9	5.61 (brd 10.4)	77.2	5.62 (brd 10.6)	80.1	5.40 (brd 10.2)	78.8	5.63 (brd 9.3)	79.0	5.62 (brd 9.6)	78.6	5.61 (brd 9.7)	78.6	5.58 (brd 9.6)	80.2	5.54 (dt 9.7, 2.2)	
3a	149.9	150.4	148.0	148.0	142.7	142.7	141.1	141.1	141.1	141.1	140.7	140.7	140.7	140.6	140.6	140.6	
4	125.4	125.6	125.5	125.5	124.7	124.7	124.8	124.8	124.8	124.8	124.9	124.9	124.9	124.9	124.9	124.9	
5	136.3	7.27 (d 8.3)	136.7	7.25 (d 8.3)	128.7	6.82 (d 8.1)	129.0	6.85 (d 8.1)	129.0	6.85 (d 8.0)	129.1	6.86 (d 8.0)	129.1	6.86 (d 8.1)	129.0	6.85 (d 8.2)	
6	116.0	6.83 (d 8.3)	116.6	6.83 (d 8.3)	114.1	6.60 (d 8.1)	114.4	6.62 (d 8.1)	114.4	6.62 (d 8.0)	114.5	6.63 (d 8.0)	114.5	6.63 (d 8.1)	114.5	6.62 (d 8.2)	
7	155.1	156.0	155.1	155.1	149.5	149.5	149.6	149.6	149.7	149.7	149.7	149.7	149.7	149.6	149.6	149.6	
7a	110.9	111.2	111.2	111.2	124.5	124.5	124.7	124.7	124.6	124.6	124.7	124.7	124.7	124.8	124.8	124.8	
1'	37.5	1.37 (m), 2.08 (t 12.6)	38.8	1.58 (m), 1.96 (t 12.7)	38.0	1.56 (m), 1.60 (brd 10.2)	43.0	2.74 (d 16.7), 2.91 (dd 16.7, 9.3)	42.7	2.70 (d 16.5), 2.88 (dd 16.5, 9.6)	43.4	2.37 (d 16.7), 2.91 (dd 16.7, 9.7)	42.9	2.62 (d 16.9), 2.90 (dd 16.9, 9.6)	40.2	2.30 (dd 15.5, 2.2)	
2'	69.8	3.61 (brd 9.5)	71.1	3.48 (brt 8.4)	70.8	3.52 (dt 9.2, 5.2)	211.0	16.7, 9.3	211.0	16.5, 9.6	204.3	16.7, 9.7	204.6	16.9, 9.6	172.0	172.0	
3'	73.2	3.23 (m)	74.4	3.19 (m)	74.1	3.20 (brs)	76.3	3.93 (brs)	76.5	3.92 (brs)	78.1	4.98 (dd 7.7, 4.1)	78.3	4.98 (dd 12.0, 5.2)	78.3	4.98 (dd 12.0, 5.2)	
4'	31.6	1.23 (m), 1.40 (m)	33.2	1.23 (m), 1.52 (m)	32.5	1.24 (m), 1.68 (m)	32.8	1.43 (m), 1.60 (m)	32.6	1.43 (m), 1.56 (m)	28.9	1.63 (m), 1.80 (m)	29.2	1.63 (m), 1.75 (m)	29.2	1.63 (m), 1.75 (m)	
5'	25.3	1.22 (m), 1.40 (m)	25.5	1.23 (m), 1.44 (m)	25.1	1.23 (m), 1.45 (m)	24.3	1.32 (m)	24.2	1.32 (m)	24.2	1.32 (m)	24.1	1.25 (m)	24.1	1.25 (m)	
6'	31.5	1.22 (m)	32.0	1.23 (m)	31.5	1.24 (m)	31.1	1.23 (m)	31.1	1.22 (m)	30.8	1.26 (m)	30.8	1.23 (m)	30.8	1.23 (m)	
7'	22.1	1.27 (m)	22.6	1.27 (m)	22.1	1.27 (m)	22.1	1.26 (m)	22.0	1.25 (m)	21.8	1.26 (m)	21.8	1.25 (m)	21.8	1.25 (m)	
8'	13.9	0.85 (t 6.2)	14.4	0.85 (t 6.6)	13.9	0.86 (t 7.1)	13.8	0.86 (t 6.6, 5.4)	13.9	0.85 (t 6.5, 5.3)	13.8	0.85 (t 5.3)	13.8	0.85 (brt 6.3, 5.1)	13.8	0.85 (brt 6.3, 5.1)	
1''	28.9	3.23 (d 6.6)	29.4	3.23 (m)	29.1	3.25 (t 8.2)	29.8	3.13 (brt 7.1)	30.1	3.13 (brt 6.6)	30.1	3.13 (brs)	30.1	3.12 (brt 6.3)	30.1	3.12 (dd 15.6, 7.3), 3.19 (dd 15.6, 7.3)	
2''	122.2	5.19 (t 6.6)	122.6	5.20 (t 6.0)	121.8	5.18 (brt 6.3)	123.1	5.18 (t 7.1)	122.9	5.14 (t 6.6)	122.9	5.14 (t 6.5)	122.8	5.12 (t 6.6)	122.8	5.15 (brt 7.3)	
3''	132.4	133.0	132.8	132.8	131.3	131.3	131.6	131.6	131.6	131.6	131.7	131.7	131.6	131.6	131.6	131.6	
4''	25.4	1.68 (s)	26.0	1.69 (s)	25.4	1.70 (s)	25.5	1.68 (s)	25.4	1.68 (s)	25.4	1.68 (s)	25.4	1.67 (s)	25.4	1.68 (s)	
5''	17.8	1.66 (s)	18.3	1.68 (s)	17.7	1.66 (s)	17.6	1.63 (s)	17.6	1.63 (s)	17.6	1.62 (s)	17.6	1.62 (s)	17.6	1.65 (s)	
3'-COCH ₃																	
3'-COCH ₃

Figure 2 Selected ¹H-¹H COSY and HMBC correlations of 1-9.

rotation values and CD spectra of 7 and 8 suggested that these compounds were diastereomeric with each other. Comparison of the ¹H- and ¹³C-NMR spectra between 7 and 5, and between 8 and 6, revealed a lower field shift of a methine proton at C-3' position (from δ_{H} 3.93 in 5 and δ_{H} 3.92 in 6 to δ_{H} 4.98 in both 7 and 8) and the new appearance of an acetyl signal (7: δ_{H} 2.07/ δ_{C} 20.4 and δ_{C} 170.0; 8: δ_{H} 2.07/ δ_{C} 20.4 and δ_{C} 169.9) (Table 1). Based on the above results and a detailed analysis of the 2D-NMR spectra of 7 and 8, it was suggested that 7 and 8 had the same planar structures, and that the two were acetylated derivatives of 5 and 6, respectively (Figure 2).

The molecular formula of emefuran D (9) was confirmed as C₁₅H₁₈O₄ by HR-EI-MS (Supplementary Table S1). The ¹H- and ¹³C-NMR spectra of 9 were similar to those of 3, except for the C-1 (δ_{C} 60.7 in 9, δ_{C} 167.8 in 3) (Table 1). Therefore, 9 was a reductant of 3 at the C-1 position. Detailed analysis of the 2D-NMR spectra confirmed that 9 was the 1-deoxy derivative of 3 (Figure 2).

Stereochemistry of emefuranones (1-3) and emefurans (4-9)

The absolute configurations at the C-2' and C-3' positions in 1, 2, and 4 were determined by the method of Riguera *et al.*, employing a technique that used the α -methoxy- α -(trifluoromethyl)-phenylacetyl chloride (MTPA-Cl) reagent.^{27,28} The differences in chemical shift values ($\Delta\delta = \delta_{\text{S}} - \delta_{\text{R}}$) between diastereomeric MTPA esters (1a and 1b, 2a and 2b, 4a and 4b) were calculated to determine the absolute configuration at the C-2' and C-3' positions (Supplementary Figure S1). From the resulting calculated values, it was determined that the stereochemistry at the C-2' and C-3' positions in 1, 2 and 4 were all in the S configurations. The absolute configurations at the C-3

position in 1 and 2 were assigned by comparison of the CD spectra of 1 and 2 with that of spiroxaline.²⁹ A cotton effect at 220 nm of 2 showed the same chirality as that of spiroxaline, and the CD spectrum of 1 displayed the opposite chirality compared with that of spiroxaline and 2 (Supplementary Figure S2). Therefore, it was determined that the absolute configuration at the C-3 position in 1 and 2 was S and R configuration, respectively.

The absolute configuration at the C-3' position in 5 and 6 was determined to be S configuration based on the modified Mosher's method³⁰ (Supplementary Figure S3). To determine the absolute configurations at C-3 in 5 and 6, these compounds were used as substrates for attempted reaction via the oxidation cleavage and phenylglycine methyl ester (PGME) method.³¹ These compounds were treated with KIO₄ to obtain the cleavage products 15 and 16, and 15 and 16 were treated with (R)- and (S)- PGME to produce the amide derivatives (15a, 15b, 16a and 16b) (Supplementary Figure S4). The absolute configuration at the C-3 positions in 15 and 16 was determined to be R and S, respectively, based on the results of the differences in chemical shift value of the PGME amide of 15 and 16 ($\Delta\text{values} = \delta_{\text{R}} - \delta_{\text{S}}$). Therefore, the absolute configuration of the C-3 position in 5 and 6 was inferred as R and S, respectively.

The CD curve of 4 was similar to that of 5, and showed opposite orientation to that of 6. Therefore, the absolute configuration at C-3 in 4 was inferred as the S configuration (Supplementary Figure S5).

To determine the stereochemistry of 7 and 8, 5-8 were acetylated with acetic anhydride. ¹H-NMR and CD spectra of acetylated 7 were the same as those of the acetylated 5, and those of acetylated 8 were the same as those of acetylated 6. Therefore, we inferred that the C-3

and C-3' positions of **7** were in the same *S* configuration as **5**, and that those of **8** were in the same stereochemistry as **6** (C-3 and C-3' with *R* and *S* configuration, respectively).

The CD spectra and optical rotations of emefranone B (**3**) and emefuran D (**9**) showed values of near zero. Treatment of **3** and **9** with (*S*)-PGME produced the PGME amide derivatives, and the results of HPLC analysis with these derivatives showed that the (*S*)-PGME derivatives for **3** and **9** each included two peaks (derivative of **3**: $t_R = 23.2$ and 25.1 min; derivative of **9**: $t_R = 27.3$ and 28.8 min) on their HPLC chromatograms (Supplementary Figure S6). These facts were consistent with the inference that **3** and **9** were racemic compounds.

Structure of farnesylemefuranones A–C (**10**–**12**)

Farnesylemefuranone A (**10**) was obtained as a colorless amorphous powder. The molecular formula was determined to be $C_{24}H_{32}O_6$ on the basis of HR-ESI-MS (Supplementary Table S1). The 1H -NMR spectrum showed an aromatic proton signal (δ 6.99, s), two olefin proton signals, two oxymethylene signals, a methoxy proton signal, two singlet methyl proton signals, two doublet methyl proton signals, three sp^3 methylene proton signals and a sp^3 methine proton signal

(Table 2). The ^{13}C -NMR and HSQC (heteronuclear single quantum coherence) spectra assigned two carbonyl carbons (δ 214.7 and δ 171.4), 10 sp^2 carbons, a methoxy carbon (δ 56.3), four methyl carbons (δ 18.3, δ 18.3, δ 16.4 and δ 16.1), six sp^3 methylene carbons including two oxygenated one (δ 69.7 and δ 67.5) and a sp^3 methine carbon (δ 40.8) (Table 2). The four partial structures (H-2' to H-3', H-5' to H-7', H-9' to H-10' and H-12' to H-14') were deduced from analysis of the 1H - 1H COSY spectrum (Figure 3). The HMBC correlations showed the presence of an isobenzofuranone moiety and a farnesyl moiety in **12**. Furthermore, we observed HMBC correlations from H₂-2' (δ 4.68) to the quaternary sp^2 carbon at the C-5 position of an isobenzofuranone moiety via an ether linkage, and from the methoxy proton (δ 3.91) to the quaternary sp^2 carbon at the C-6 position. These results indicated that the farnesyl moiety and methoxy group were connected to the C-5 and C-6 positions of the isobenzofuranone moiety, respectively (Figure 3). The stereochemistry of the olefin group in **10** was elucidated using selected NOESY correlations, and all double bonds were determined to be in the *E* configuration (Supplementary Figure S7). Therefore, the structure of **10** was established as shown in Figure 1.

The molecular formulae of farnesylemefuranone B (**11**) and C (**12**) were determined to be $C_{23}H_{30}O_6$ and $C_{23}H_{32}O_7$ by HR-EI-MS and HR-ESI-MS, respectively. Comparison of the 1H - and ^{13}C -NMR spectra of **11** and **12** with those of **10** showed the presence of an isobenzofuranone moiety in **11** and **12**. However, the methoxy group in **10** was absent from both **11** and **12** (Table 2). Compound **11** had five sp^2 carbons, including a carbonyl carbon (δ 215.9), when compared with **10**. Instead of the carbonyl carbon (δ 214.7) and methine carbon (δ 40.8) seen in **10**, two oxymethines (δ_C 78.1 and δ_C 73.2) were observed in **12**. Therefore, **11** and **12** were inferred to be farnesylated isobenzofuranones. Based on the above results and detailed analysis of 2D NMR spectra (Figure 2), the planar structure of **11** and **12** was determined as shown in Figure 1. The stereochemistries of the olefin groups in **11** and **12** were elucidated using selected NOESY and NOE correlations and the coupling constant, and all double bonds were determined to be in the *E* configuration (Supplementary Figure S7). The absolute configuration at C-11' in **12** was elucidated to be *S* using the Mosher's method (Supplementary Figure S8).

Structure of xylarinol C (**13**) and emericelloxide (**14**)

Xylarinol C (**13**) was obtained as pale yellow amorphous solid. The molecular formula of **13** was determined as $C_{15}H_{16}O_3$ by HR-EI-MS. The 2D-NMR spectral data of **13** indicated the presence of an isoprenyl group and a 9-hydroxybenzo[c]oxepin-3(*1H*)-one skeleton similar to that of xylarinol A (Figure 1). Furthermore, the HMBC correlation from the H-1' (δ 3.37) to the C-6 position (δ 131.8) on the benzene ring of **13** indicated that the isoprenyl group was connected to the C-6 position of the 9-hydroxybenzo[c]oxepin-3(*1H*)-one skeleton (Figure 4). Therefore, we inferred that xylarinol C (**13**) was a 9-isoprenyl derivative of xylarinol A.

The molecular formula of emericelloxide (**14**) was determined as $C_{21}H_{26}O_3$ by HR-ESI-MS; the 1H -NMR spectrum of **14** showed *o*-couplings phenyl proton signals [δ 6.67 ($J = 8.4$ Hz), and δ 6.89 ($J = 8.4$ Hz)], three olefinic proton signals (δ 5.14, δ 6.15 and δ 6.72), an oxygenated methine proton signal (δ 4.95), five methylene proton signals, a primary methyl proton signal [δ 0.87 (3H, t, $J = 6.8$ Hz)] and two singlet methyl proton signals. The ^{13}C -NMR spectrum of **14** showed a carbonyl carbon signal, 10 sp^2 carbon signals, two oxygenated carbon signals and eight sp^3 carbon signals. These results suggested that the partial structure of **14** included a prenylated

Table 2 1H - and ^{13}C -NMR data of farnesylemefuranones A (**10**)-C (**12**) in $CDCl_3$

No.	Farnesylemefuranone A (10)		Farnesylemefuranone B (11)		Farnesylemefuranone C (12)	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	171.4		172.0		171.8	
3	67.5	5.21(s)	67.9	5.20 (s)	67.8	5.20 (s)
3a	121.1		121.2		121.2	
4	144.3		143.9		143.8	
5	139.0		139.1		139.1	
6	154.2		151.1		151.0	
6-OMe	56.3	3.91 (s)				
7	99.7	6.99 (s)	104.1	7.02 (s)	104.1	7.00 (s)
7a	126.1		125.5		125.6	
2'	69.7	4.68 (d 7.3)	71.6	4.25 (m)	71.8	4.21 (m)
3'	119.2	5.44 (brt 7.3)	40.1	2.47 (d 7.3)	39.8	2.42 (t 5.8)
4'	143.6		132.5		132.1	
5'	39.4	2.02 (m)	128.1	5.99 (d 10.8)	127.7	5.34 (t 6.7)
6'	26.0	2.03 (m)	124.8	6.21 (dd 14.8,10.8)	25.8	2.21 (m)
7'	124.0	5.05 (brt 5.8)	139.6	5.46 (dd 14.8,8.4)	39.2	2.13 (m)
8'	134.4		36.9	2.18 (brt 6.8)	138.3	
9'	33.3	2.22 (t 7.6)	30.6	1.52 (dd 14.4,7.5)	121.2	5.22 (t 6.8)
				1.66 (dd 14.2,8.2)		
10'	38.8	2.53 (t 7.6)	38.2	2.43 (d 7.6)	30.4	2.15 (m)
11'	214.7		215.9		78.1	3.40 (dd 9.5,3.6)
12'	40.8	2.60 (hept 7.0)	40.8	2.61 (hept 7.1)	73.2	
13'	18.3	1.07 (d 7.0)	18.3	1.08 (d 7.0)	26.4	1.21 (s)
14'	18.3	1.07 (d 7.0)	18.3	1.08 (d 7.0)	23.5	1.18 (s)
15'	16.1	1.58 (s)	20.8	1.02 (d 6.9)	16.1	1.63 (s)
16'	16.4	1.63 (s)	16.5	1.79 (s)	16.2	1.68 (s)

Figure 3 Selected ¹H-¹H COSY and HMBC correlations of farnesylemefuranones A (10)–C (12).

Figure 4 Selected ¹H-¹H COSY and HMBC correlations of xylarinol C (13).

hydroxyphenyl moiety, although **14** lacked the furanone and furan moieties of **1**–**9**. Detailed analysis of the 2D-NMR spectra indicated that the structure of **14** was 2-hexanoyl-7-hydroxy-4-(3-methylbut-2-en-1-yl)-1,2-dihydronaphth[1,2-b]oxirene (Figure 4).

Antifungal and antibacterial activities and cytotoxicity were tested for **1**–**9**, **13**, **16**, **17**, dustanin and sorbicillin. Compounds **4**–**7** and **13**, **16** and **17** showed weak inhibition of *B. subtilis* (respective inhibition zones: 8.5, 10, 9, 9, 13, 9 and 9 mm in 250 µg per disk). Cytotoxicity and antifungal activity were not observed at concentrations of 50 µM and 250 µg per disk, respectively.

Isobenzofuranone derivatives, emefuranones A₁, A₂, B (**1**–**3**), isobenzofuran derivatives emefurans A, B₁, B₂, C₁, C₂, D (**4**–**9**), and farnesylemefuranones A–C (**10**–**12**), xylarinol C (**13**) and emericelloxide (**14**) were isolated, along with known compounds dustanin, sorbicillin, aspergillodiol and xylarinol A from the culture extract of *Emericella* sp. IFM57991. Compounds **4**–**7**, **13**, **16** and **17**, showed moderate antibacterial activity against *B. subtilis* and no activities against *Escherichia coli*.

MATERIALS AND METHODS

General procedure

Melting points were measured on a Yanagimoto micro-melting point apparatus (Yanaco New Science Inc., Kyoto, Japan). Optical rotations were measured on a JASCO DIP-1000 (JASCO Co., Ltd., Tokyo, Japan). CD spectra were obtained with a JASCO J-820 spectrophotometer (JASCO Co., Ltd.). UV spectra were recorded on an Amersham Biosciences, Ultrospec 2100 spectropolarimeter (GE Healthcare Japan Corp., Tokyo, Japan). Measurements of IR

spectra were performed with a JASCO FT/IR-4100 spectrometer on a KBr cell (JASCO Co., Ltd.). NMR spectra (both **1** and **2D**) were obtained on a Bruker AVANCE-400 (400.13 MHz for ¹H and 100.61 MHz for ¹³C) spectrometer (Bruker Bio Spin K. K., Kanagawa, Japan). Multiplicity of signals is abbreviated as follows: s, singlet; d, doublet; dd, doublet of doublet; dt, doublet of triplets; t, triplet; q, quartet; m, multiplet; br, broad. EI and CI-MS spectra were obtained from a JEOL JMS-600W spectrometer (JEOL Ltd., Tokyo, Japan). ESI-MS spectra were obtained from a JEOL T100LP spectrometer (JEOL Ltd.).

Fungal strain

The fungal strain IFM57991 was obtained from the Medical Mycology Research Center, Chiba University, Japan. Colonies, which were pale brown in color, grew moderately on potato dextrose agar and produced curved hull cells. However, conidiogenesis and sexual spore formation were not observed, precluding morphological identification of the fungus at the species-level. DNA sequence analysis of the β-tubulin-encoding gene identified the strain as *Emericella* sp.

Culture, extraction and isolation

Culture medium consisted of moist rice, autoclaved at 120 °C for 20 min before inoculation. *Emericella* sp. IFM57991 was inoculated into each of 25 Roux flasks (containing 150 g of moist rice per flask) and cultured at 25 °C for 21 days. The fermented rice was extracted with MeOH and the organic layer was evaporated. The resultant extract was suspended in H₂O and extracted with EtOAc, and then the organic layer was evaporated in vacuo. The EtOAc extract (11.3 g) was suspended in *n*-hexane and extracted with MeCN and then the MeCN layer was evaporated in vacuo. The MeCN extract (4.5 g) was chromatographed using a Sephadex LH-20 column (solvent system: hexane-CHCl₃ (1:4; 200 ml), CHCl₃-acetone (3:2; 200 ml), (1:4; 200 ml), acetone (200 ml) and MeOH (500 ml) to yield 11 fractions.

The third fraction was suspended in MeOH and filtered. The residual solid was confirmed to contain dustanin (200 mg). The filtrate was purified by middle-pressure liquid chromatography (MPLC) on a silica gel column (φ25x250, YAMAZEN, Osaka, Japan) using CHCl₃-acetone (15:1) followed by further purification by high-performance liquid chromatography (HPLC) on a silica gel column (φ10x250, GL science, Tokyo, Japan); elution with hexane-EtOAc (5:1) yielded farnesylemefuranone A (**10**; 3 mg).

The fourth fraction was suspended in MeOH and filtered. The residue was confirmed to contain emefuranone A₁ (**1**; 40 mg). The filtrate was chromatographed by MPLC on a silica gel column using CHCl₃-acetone (10:1) followed by further purification by HPLC on a silica gel column. Elution with benzene-EtOAc (12:1) and then CHCl₃-acetone (25:1) yielded emefuran C₂ (**8**; 3 mg); subsequent elution with CHCl₃-acetone (20:1) followed by CHCl₃-EtOAc (4:1) yielded emefuranone A₂ (**2**; 3 mg); and subsequent elution with hexane-EtOAc (4:1) yielded emericelloxide (**14**; 4 mg).

The fifth fraction was suspended by MeOH and filtered. The residue was confirmed to contain emefuranone A₁ (**1**; 30 mg). The filtrate was purified by MPLC on a silica gel column using benzene-acetone (5:1) followed by further purification by HPLC on a silica gel column; elution with CHCl₃-acetone (15:1) yielded emefuran C₁ (**7**; 10 mg).

The sixth fraction was chromatographed by MPLC on a silica gel column using CHCl₃-MeOH (10:1) followed by further purification by HPLC on a silica gel column. Elution with benzene-acetone (15:1) yielded sorbicillin (10 mg), xylarinol C (**13**; 3 mg), emefuran B₁ (**5**; 25 mg) and emefuran B₂ (**6**; 30 mg); subsequent elution with CHCl₃-acetone (5:1) and CHCl₃-EtOAc (5:1) yielded aspergillodiol (3 mg) and emefuran A (**4**; 4 mg); and subsequent elution with CHCl₃-EtOAc (5:1) yielded farnesylemefuranone B (**11**; 2 mg).

The seventh fraction was purified by MPLC on a silica gel column using hexane-acetone (2:1) and benzene-acetone (4:1) followed by further purification by HPLC on a silica gel column; elution with CHCl₃-MeOH (10:1) yielded emefuranone B (**3**; 3 mg). The residue was chromatographed by HPLC on a silica gel column; elution with CHCl₃-acetone (5:1) and CHCl₃-EtOAc (5:1) yielded farnesylemefuranone C (**12**; 2 mg).

The eighth fraction was chromatographed and purified by HPLC on a silica gel column using *n*-hexane-acetone (3:2) and benzene-acetone (4:1) to give emefuran D (**9**; 4 mg). The residue was purified by MPLC on a silica gel

column using CHCl₃-MeOH (15:1) followed by further purification by HPLC on a silica gel column; elution with CHCl₃-EtOAc (5:1) and CHCl₃-acetone (15:1) yielded xylariol A (2 mg).

Physicochemical properties of 1–14

The physicochemical properties of new compounds (1–14) are summarized in Supplementary Table S1, and the ¹H- and ¹³C-NMR spectral data of 1–12 are shown in Tables 1 and 2.

Xylariol C (13)

¹H-NMR (DMSO-*d*₆) δ 1.71 (3H, s, H₃-4'), 1.73 (3H, s, H₃-5'), 3.37 (2H, d, *J* = 7.1 Hz, H-1'), 5.15 (2H, t, *J* = 7.1 Hz, H-2'), 5.22 (2H, brs, H-1), 6.34 (1H, d, *J* = 12.3 Hz, H-4), 6.89 (1H, d, *J* = 8.4 Hz, H-8), 7.12 (1H, d, *J* = 8.4 Hz, H-7) and 7.46 (1H, d, *J* = 12.3 Hz, H-5). ¹³C NMR (MeOD) δ 16.6 (C-5'), 24.4 (C-4'), 31.1 (C-1'), 61.6 (C-1), 116.7 (C-8), 121.1 (C-4), 122.2 (C-9a), 122.6 (C-2'), 130.8 (C-7), 131.8 (C-6), 132.2 (C-3'), 134.7 (C-5a), 138.6 (C-5), 150.8 (C-9) and 169.8 (C-3).

Emericeloxide (14)

¹H-NMR (DMSO-*d*₆) δ 0.87 (3H, t, *J* = 6.8 Hz, 6'-H), 1.26 (2H, m, 4'-H₂), 1.28 (2H, m, 5'-H₂), 1.45 (2H, m, 3'-H₂), 1.67 (3H, s, 4''-H₃), 1.69 (3H, s, 5''-H₃), 2.57 (1H, dt, *J* = 17.8, 7.6 Hz, 2'-H₂), 2.84 (1H, dt, *J* = 17.8, 7.6 Hz, 2'-H₂), 3.26 (2H, t, *J* = 7.4 Hz, 1''-H₂), 4.95 (1H, s, 1-H), 5.14 (1H, t, *J* = 7.4 Hz, 2''-H), 5.50 (1H, s, 8-OH), 6.15 (1H, d, *J* = 10.1 Hz, 3-H), 6.67 (1H, d, *J* = 8.4 Hz, 7-H), 6.72 (1H, d, *J* = 10.1 Hz, 4-H) and 6.89 (1H, d, *J* = 8.4 Hz, 6-H). ¹³C-NMR (DMSO-*d*₆) δ 13.9 (C-6'), 17.7 (C-5''), 22.0 (C-5'), 22.8 (C-3'), 25.5 (C-4''), 30.3 (C-1''), 30.9 (C-4'), 35.9 (C-2'), 66.4 (C-1), 75.6 (C-2), 115.4 (C-7), 120.7 (C-8a), 124.0 (C-2''), 125.3 (C-4), 127.7 (C-3), 127.8 (C-5), 129.4 (C-6), 130.0 (C-4a), 130.6 (C-3''), 153.8 (C-8) and 210.1 (C-1'). The chemical shift of C-3 may be exchanged with C-5.

Esterification of emefuranone A₁ (1), A₂ (2), emefuran A (4), B₁ (5) and B₂ (6) with MTPA-Cl

Well-dried samples (1: 2 mg, 2: 1 mg, 4: 1 mg, 5: 2 mg, 6: 2 mg) were dissolved in pyridine-*d*₅ (1: 200 μl, others: 300 μl) and added to (R)-(-)-MTPA-Cl (150 μl) or (S)-(+)-MTPA-Cl (150 μl) in each NMR tube (φ 2.5 mm). Then, the NMR tubes were held for 15 h at 50 °C. The assignment was made on the basis of ¹H-NMR spectrum.

Oxidative cleavage of emefuran B₁ (5) or emefuran B₂ (6)

Emefuran B₁ (5: 6 mg) or emefuran B₂ (6: 5 mg) were stirred with KIO₄ (15 mg) in H₂O (0.5 ml) and dioxane (0.5 ml) for 12 h at 50 °C. Then, H₂O (10 ml) was added, the suspension was extracted with diethyl ether, and the ether layer was evaporated. After concentration in vacuo, the organic layer was chromatographed and purified by HPLC on silica gel column using hexane-acetone (3:2) to give 15 (1 mg) or 16 (0.5 mg).

Compound 15

Colorless amorphous powder. ESI-MS *m/z*: 285 (M+Na)⁺. UV λ_{max} (MeOH) nm (log ε): 213 (4.6), 221 (4.2) and 280 (3.4). CD (MeOH) Δε (nm): 6.1 (209), -1.5 (231), -0.6 (276). H-NMR (CDCl₃) δ 1.70 (3H, s, 5''-H), 1.73 (3H, s, 4''-H), 2.61 (1H, dd, *J* = 15.8, 9.6 Hz, 1'-H), 2.89 (1H, dd, *J* = 15.8, 2.2 Hz, 1'-H), 3.22 (2H, t, *J* = 7.1 Hz, 1''-H), 5.11 (1H, d, *J* = 12.4 Hz, 1-H), 5.18 (1H, m, 2''-H), 5.20 (2H, m, 1-H), 5.73 (1H, brd, *J* = 9.6 Hz, 3-H), 6.64 (1H, d, *J* = 8.2 Hz, 6-H) and 6.96 (1H, d, *J* = 8.2 Hz, 5-H).

Compound 16

Colorless amorphous powder. ESI-MS *m/z*: 285 (M+Na)⁺. UV λ_{max} (MeOH) nm (log ε): 212 (4.3), 223 (4.2) and 277 (3.5). CD (MeOH) Δε (nm): -4.4 (212), 1.4 (230), 0.6 (276). H-NMR (CDCl₃) δ 1.70 (3H, s, 5''-H), 1.73 (3H, s, 4''-H), 2.61 (1H, dd, *J* = 15.8, 9.6 Hz, 1'-H), 2.89 (1H, dd, *J* = 15.8, 2.2 Hz, 1'-H), 3.22 (2H, t, *J* = 7.1 Hz, 1''-H), 5.11 (1H, d, *J* = 12.4 Hz, 1-H), 5.18 (1H, m, 2''-H), 5.20 (2H, m, 1-H), 5.73 (1H, brd, *J* = 9.6 Hz, 3-H), 6.64 (1H, d, *J* = 8.2 Hz, 6-H) and 6.96 (1H, d, *J* = 8.2 Hz, 5-H).

Acetylation of emefuran B₁ (5), emefuran B₂ (6), emefuran C₁ (7) and emefuran C₂ (8)

Emefuran B₁ (5: 2 mg), emefuran B₂ (6: 2 mg), emefuran C₁ (7: 2 mg) or emefuran C₂ (8: 2 mg) was dissolved in pyridine (300 μl) in each Erlenmeyer flask. Acetic anhydride (200 μl) then was added to each flask and reactions were held at room temperature overnight. After the reaction, the reactants were added to H₂O and extracted with EtOAc. The organic layer was evaporated, and then purified by HPLC on a silica gel column using CHCl₃-EtOAc (40:1) to obtain 17 (each 1.2 and 0.7 mg) from 5 and 7, or to obtain 18 (1.7 and 0.7 mg) from 6 and 8.

Compound 17

Colorless amorphous powder. ESI-TOF-MS *m/z*: 453.2292 [(M+Na)⁺ 453.2253 for C₂₅H₃₄O₆Na]. UV λ_{max} (MeOH) nm (log ε): 206 (4.6), 219 (4.3). CD (MeOH) Δε (nm): 2.1 (206), -1.1 (229), 0.7 (277). H-NMR (DMSO-*d*₆) δ 0.86 (3H, t, *J* = 6.7 Hz, 8'-H), 1.17–1.38 (6H, m, 5'-, 6'-, 7'-H), 1.58–1.77 (2H, m, 4'-H), 1.65 (3H, s, 5''-H), 1.70 (3H, s, 4''-H), 2.08 (3H, s, 3'-OAc), 2.25 (3H, s, 7-OAc), 2.76 (1H, dd, *J* = 17.2, 1.6 Hz, 1'-H), 2.98 (1H, dd, *J* = 17.2, 9.6 Hz, 1'-H), 3.25 (2H, d, *J* = 7.0 Hz, 1''-H), 4.80 (1H, d, *J* = 12.8 Hz, 1-H), 4.90 (1H, dd, *J* = 12.8, 2.3 Hz, 1-H), 4.99 (1H, dd, *J* = 8.5, 3.8 Hz, 3'-H), 5.17 (1H, t, *J* = 7.1 Hz, 2''-H), 5.69 (1H, dt, *J* = 9.6, 1.6 Hz, 3-H), 6.99 (1H, d, *J* = 8.2 Hz, 6-H), 7.10 (1H, d, *J* = 8.2 Hz, 5-H).

Compound 18

Colorless amorphous powder. ESI-TOF-MS *m/z*: 453.2291 [(M+Na)⁺, 453.2253 for C₂₅H₃₄O₆Na]. UV λ_{max} (MeOH) nm (log ε): 206 (4.4), 219 (4.1). CD (MeOH) Δε (nm): -2.5 (202), 1.7 (229), 1.4 (289). H-NMR (DMSO-*d*₆) δ 0.85 (3H, t, *J* = 6.7 Hz, 8'-H), 1.21–1.34 (6H, m, 5'-, 6'-, 7'-H), 1.62–1.78 (2H, m, 4'-H), 1.64 (3H, s, 5''-H), 1.70 (3H, s, 4''-H), 2.07 (3H, s, 3'-OAc), 2.25 (3H, s, 7-OAc), 2.70 (1H, dd, *J* = 17.1, 2.0 Hz, 1'-H), 2.96 (1H, dd, *J* = 17.1, 9.2 Hz, 1'-H), 3.23 (2H, d, *J* = 6.7 Hz, 1''-H), 4.80 (1H, d, *J* = 12.9 Hz, 1-H), 4.91 (1H, dd, *J* = 12.9, 2.4 Hz, 1-H), 4.98 (1H, dd, *J* = 8.2, 4.5 Hz, 3'-H), 5.17 (1H, brt, *J* = 6.7 Hz, 2''-H), 5.67 (1H, brd, *J* = 9.2 Hz, 3-H), 6.99 (1H, d, *J* = 8.1 Hz, 6-H) and 7.10 (1H, d, *J* = 8.1 Hz, 5-H).

Amidation of emefuranone B (3) with PGME-Cl

Emefuranone B (3: 1 mg) was treated with (S)-(+)-PGME-Cl (20 mg), PyBOP (22 mg), HOBT (13.0 mg) and NMM (100 μl) in DMF (1 ml) at room temperature for 20 h. EtOAc and 6% HCl aqueous solution (25 ml) were added; the EtOAc layer was washed with saturated NaHCO₃ aqueous solution (25 ml); and the resulting EtOAc layer was added to 4% NaCl aqueous solution (25 ml) and partitioned. The organic layer was desiccated with anhydrous sodium sulfate. After filtration and evaporation, the extract was chromatographed by HPLC on an ODS column (φ10x250, GL science, Japan) using 60% MeOH.

Amidation of emefuran D (9) with PGME-Cl

Emefuran D (9: 1 mg) was treated with (S)-(+)-PGME-Cl (20 mg), PyBOP (22 mg), HOBT (13.0 mg) and NMM (100 μl) in DMF (1 ml) at room temperature for 20 h. EtOAc and 6% HCl aqueous solution (25 ml) were added, and the EtOAc layer was washed with saturated NaHCO₃ aqueous solution (25 ml); the resulting EtOAc layer was added to 4% NaCl aqueous solution (25 ml), and partitioned. The organic layer was desiccated with anhydrous sodium sulfate. After filtration and evaporation, the extract was chromatographed by HPLC on an octadecylsilyl (ODS) column using 60% MeOH.

Cytotoxicity assay

Cytotoxicity assays were performed by a modification of the previously described method³². Cells were distributed into 96-well microplates at 4000 cells per well and allowed to attach for 4–6 h. Then, cells were grown to 80% confluency in respective media as follows: A549 human lung cancer cells and Hela human cervical cancer cells were incubated in Dulbecco's modified Eagle's medium (Invitrogen Co., Ltd., Carlsbad, CA, USA); LNCap human prostate adenocarcinoma cells were incubated in RPMI-1640 medium (Wako Pure

Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum, penicillin G (100 U ml⁻¹), streptomycin (100 mg ml⁻¹) and amphotericin B (0.25 mg ml⁻¹). Then, media were supplemented with the 419 indicated concentrations of the test compounds for 48–72 h. Cell proliferation was 420 measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) to count living 421 cells by combination staining with WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium) and 1-methoxy PMS (1-methoxy-5-methylphenazinium methanesulfate). Briefly, the medium was removed and replaced with 10 ml per well of Cell Counting Kit-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), and the plates were incubated for 4 h. Cell number was determined by scanning with a Bio-Rad Model Q4 550 microplate reader at 450 nm (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Antifungal assay

Antifungal assays were performed by the paper disc method using pathogenic filamentous fungi, *Aspergillus fumigatus* IFM 41243 and *A. niger* IFM 41398, and pathogenic yeasts, *Candida albicans* IFM 40009 and *Cryptococcus neoformans* ATCC 90112. Test substances, dissolved in CHCl₃, were added to paper discs (8 mm), which then were dried and placed on the assay plates. The plates were incubated at 25 °C for 48–72 h and the diameters of the inhibitory zones were measured.

Antibacterial assay

Antibacterial assays were performed by the paper disc method against *Bacillus subtilis* ATCC6633 and *Escherichia coli* JCM1640. Test substances, dissolved in CHCl₃, were added to paper discs (8 mm), which then were dried and placed on the assay plates. The plates were incubated at 34 °C for 24 h and the diameters of the inhibitory zones were measured.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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