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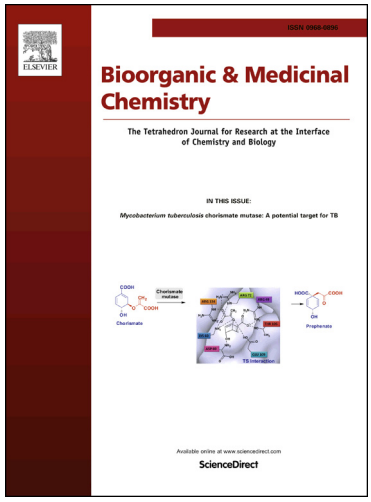

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Metabolite diversification by cultivation of the endophytic fungus *Dothideomycete* sp. in halogen containing media: Cultivation of terrestrial fungus in seawater

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ABSTRACT:

The endophytic fungus, *Dothideomycete* sp. CRI7, isolated from the terrestrial plant, *Tiliacora triandra*, was salt tolerant, capable of growing in the culture medium prepared from seawater; salts in seawater did not have any effects on the fungal growth. Metabolite productions of the fungus CRI7 cultivated in media prepared from seawater (MSW), prepared from deionized water supplemented with potassium bromide (MKBr) or potassium iodide (MKI), and prepared from deionized water (MDW) were investigated. It was found that the cultivation of the fungus CRI7 in MKBr and MSW enabled the fungus to produce nine new metabolites (**1**-**9**). The production of an azaphilone, austdiol (**10**), of the fungus CRI7 grown in MDW was 0.04g/L, which was much lower than that grown in MSW, MKBr, and MKI media which provided the yields of 0.5, 0.9, and 1.2 g/L, respectively, indicating that halogen salts significantly enhanced the production of the polyketide **10**. The cultivation of terrestrial fungi in media containing halogen salts could therefore be useful for the metabolite diversification by one strain-many compounds (OSMAC) approach. Moreover, the isolated polyketides had significant biosynthetic relationship, suggesting that the cultivation of fungi in halogen containing media could provide the insights into certain polyketide biosynthesis. One of the isolated compounds exhibited antibacterial activity with the MIC value of 100 μg/mL.

Keywords: *Dothideomycete* sp.; One strain-many compounds (OSMAC); Endophytic fungi; Metabolite diversification; Antibacterial activity

1. Introduction

Endophytic fungi are rich sources of bioactive compounds, which have diverse chemical skeletons and biological activities of pharmaceutical interests.[1-5] In 2012, we isolated the endophytic fungus *Dothideomycete* sp. CRI7 from a Thai medicinal plant, *Tiliacora triandra*; this fungus produced a novel tricyclic polyketide together with its biosynthetic precursor azaphilone derivatives.[6] The fungus *Dothideomycete* sp. CRI7 was exceptionally sensitive to sources of potato and malt extract used for the preparation of culture media. Subsequently, we employed the one strain-many compounds (OSMAC) method by changing the carbon sources which enabled the fungus CRI7 to produce new polyketides, azaphilones, and an isochromanone.[7] Previously, we used the OSMAC approach for the biohalogenation of natural depsidones produced by the marine-derived fungus *Aspergillus unguis*, simply by cultivating the fungus in media containing different halogen salts such as potassium bromide (KBr) and potassium iodide (KI).[8] Moreover, the cultivation of the fungus in media containing KBr also provided insights into depsidone biosynthesis.[8] Recently, Yamazaki and co-workers also demonstrated that the marine-derived fungus *Trichoderma* sp. could produce new halogenated epidithiodiketopiperazines when cultivating the fungus in freshwater media supplemented with sodium halides.[9] Therefore, cultivation of marine-derived fungi in halogen containing media proved to be useful for the production of new fungal metabolites. These works encouraged us to investigate the effects of halogen salts in endophytic fungi aiming at the production of new metabolites. However, fungal endophytes are mostly isolated from terrestrial plants, not from the marine origins, and therefore they may not tolerate high concentrations of salts. As mentioned earlier, the endophytic fungus *Dothideomycete* sp. CRI7 was sensitive to the carbon sources, and thus producing new metabolites after changing the carbon sources;[7] it is therefore challenging to investigate the effects of halogen salts on the metabolite production of this fungus. The fungus *Dothideomycete* sp. CRI7 was isolated from the plant, *T. triandra*, collected from Nakhonsawan Province, Thailand. Geographically, Nakhonsawan Province is far from the sea, i.e., more than 300 kilometers from the sea, and this area does not have saline and alkaline soils. Surprisingly, the fungus *Dothideomycete* sp. CRI7 isolated from the terrestrial plant was exceptionally tolerant to salt as the fungus could grow in the medium prepared from seawater, instead of freshwater. The salt tolerance ability enabled us to cultivate the fungus CRI7 in seawater and halogen containing media, leading to the production of nine new polyketides (**1**-**9**) (Figure 1). Herein we report the isolation and characterization of new

metabolites (**1**-**9**) from the terrestrial endophytic fungus *Dothideomycete* sp. CRI7, which was cultivated in seawater and halogen containing media.

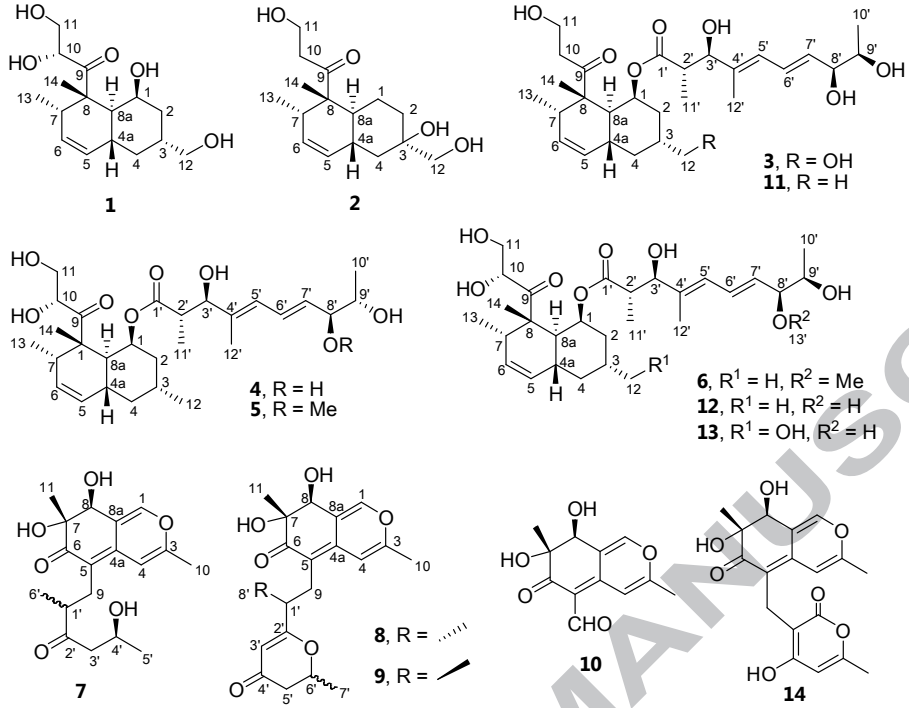


Figure 1. Metabolites isolated from the endophytic fungus *Dothideomycete* sp. CRI7.

2. Results and discussion

2.1. Production of fungal metabolites in media containing seawater, KBr, and KI

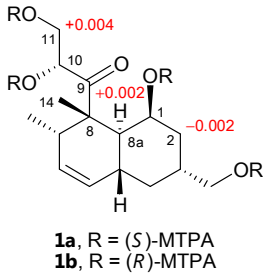
It was found that the terrestrial fungus *Dothideomycete* sp. CRI7 was salt tolerant and capable of growing in seawater with 30-33 ppt salinity using a modified Czpeck medium. Note that seawater did not show any negative effects on the growth rate of the fungus CRI7; similar growth rates were observed for the fungus grown in the media prepared from deionized water (MDW) and seawater (MSW). Since the fungus CRI7 could grow in seawater, we were able to cultivate the fungus in Czpeck medium prepared from deionized water supplemented with potassium bromide (MKBr) or potassium iodide (MKI). HPLC chromatograms of the fungal metabolite profiles grown in the media MKBr, MSW, MKI, and MDW revealed that the media MKBr and MSW significantly gave good metabolite profiles

because some metabolites could clearly be observed on the HPLC chromatograms (Supplementary Information, Figure S1). Previously, when the fungus CRI7 grown in Czpeck medium prepared from deionized water, it produced polyketides, for example, austdiol (**10**), calbistrins F-H (**11**-**13**), and dothideomynones A-C.[7] In the present study, the fungus CRI7 grown in the MKBr was found to produce not only known compounds (**10**-**14**) but also new compounds, which were named as calbistrins I-K (**1**-**3**) and dothideomynones D-F (**7**-**9**) (Figure 1). Large amount (4.76 g; 72.6 %) of austdiol (**10**) was obtained from 6.55 g of a crude extract of the MKBr medium (5L). The fungus CRI7 grown in a modified Czpeck medium prepared from seawater (MSW) was found to produce known compounds (**10**-**14**) and new metabolites, namely calbistrins L-N (**4**-**6**). Austdiol (**10**) (2.65 g; 48.0 %) was obtained from 5.51 g of a crude broth extract of the MSW medium (5 L). However, the fungus CRI7 grown in the MKI medium was not rich in the metabolite production, therefore we cultivated the fungus in 10 L of the MKI medium in order to study the compound profile. Austdiol (**10**) and a new compound, dothideomynone E (**8**), were found as major metabolites in the MKI medium. Again, large amount (12.92 g; 70.8 %) of austdiol (**10**) was obtained from 18.23 g of a crude extract of the MKI medium. Interestingly, 5 L of freshwater (MDW) gave only 0.89 g (0.2 g/L) of a crude extract that contained 0.2 g of austdiol (**10**) (0.04g/L). The MSW, MKBr, and MKI culture media provided a crude extract of 1.1, 1.3, 1.8 g/L, respectively, with respective yields for austdiol (**10**) of 0.5, 0.9, and 1.2 g/L. Note that the yields of austdiol (**10**) from the MSW (0.5 g/L), MKBr (0.9 g/L), and MKI (1.2 g/L) media were 12.5, 22.5, and 30.0 times more than that from the MDW (0.04g/L) medium. These results indicated that halogens, i.e., chloride, bromide, and iodide, clearly enhanced the yields of a crude extract and the polyketide austdiol (**10**) (gram scale production). Therefore, halogens may influence genes (and/or) enzymes involving the polyketide biosynthesis. Previously, seawater in culture media was found to enhance the production of antimicrobial compounds in marine fungi; when the concentration of seawater increased, the production of antimicrobial metabolites was improved.[10] To our knowledge, there have been no reports on the observation of metabolite profile when culturing the terrestrial fungi in seawater, and this is the first report demonstrating that halogen salts enhanced the production of secondary metabolites of fungi.

2.2. Characterization of new fungal metabolites (1-9)

Calbistrin I (**1**) was obtained as yellow amorphous solid, and its molecular formula was assigned as $C_{16}H_{26}O_5$ (by HRESI-MS). The ^{1}H NMR spectrum of **1** indicated the

presence of two methyl protons at δ_H 0.73 and 1.56, four methylene protons which resonate between δ_H 0.82-3.83, six sp^3 methine protons between δ_H 1.66-4.53, and two sp^2 methine protons at δ_H 5.41 and 5.50 (Table 1). The ^{13}C NMR and HSQC spectra indicated the presence of 16 carbons in **1**, including eight methine, four methylene, two methyl, and two quaternary carbons. The carbons at δ_C 130.0 and 131.3 were sp^2 methine carbons, while four oxygenated carbons were observed at δ_C 65.2, 66.4, 67.9, and 75.1 (Table 1). The ^1H-^1H COSY spectrum of **1** established the fragment of H-8a/H-1/H_2-2/H-3/H_2-4/H-4a/H-5/H-6/H-7/H_3-13, and it also showed the correlations of H-4a/H-8a,H-3/H_2-12; and H-10/H_2-11. The HMBC spectrum of **1** showed the correlations from both H-7 and H-8a to C-8; and from H-8a, H-10, H_2-11 and H_3-14 to C-9. The coupling constant $J_{4a,8a}$ of *ca* 11 Hz indicated a *trans* relationship between H-4a and H-8a. The NOESY spectrum of **1** exhibited correlations between H-8a and H-1 and between H-8a and H_3-13, implying that H-8a, H-1, and H_3-13 were in the same plane. The NOESY correlations were observed between H-3/H-4a and between H-4a/H_3-14, suggesting that these protons were co-planar. These NMR data established the structure of calbistrin I (**1**), whose structure was identical to the core structure of calbistrin H (**13**), previously isolated from the same fungus.[7] However, the C-10 stereochemistry of calbistrin H (**13**) was not previously addressed.[7] We next tried to determine the absolute configuratuion of C-1 and C-10 in **1** using the Mosher ester procedure carried out in NMR tubes that was formerly described by Kinghorn and co-workers.[11] Mosher's (MTPA) esters **1a** and **1b** were prepared, and the $\Delta\delta$ values (δ_S–δ_R) indicated that the absolute configuration of C-1 in compound **1** was *S* (Figure 2). Although the $\Delta\delta$ values for C-9 and C-8 could not be obtained because of the absence of protons at these positions, the $\Delta\delta$ value at C-11 implied that the absolute configuration of C-10 was *R*. Note that the 10*R* absolute configuration of the known polyketide, calbistrin H (**13**),[7] was also established accordingly. Upon these data, the structure of calbistrin I (**1**) was established as shown in Figure 1.



1a, R = (*S*)-MTPA
1b, R = (*R*)-MTPA

Figure 2. $\Delta\delta$ (δ_S-δ_R) of (*S*)- and (*R*)-MTPA esters (**1a** and **1b**) of compound **1**.

Calbistrin J (**2**), obtained as colorless oil, had the molecular formula $C_{16}H_{26}O_4$ (by HRESI-MS). The ^{1}H NMR of **2** shared a great deal of similarity to that of compound **1** (Table 1). Analysis of the ^{1}H and ^{13}C NMR data, as well as the molecular formula obtained from the HRESI-MS, suggested that compound **1** was a derivative of **2**. Analysis of 2D NMR spectra revealed that two oxygenated methines (at C-1 and C-10) in **1** were replaced by a methylene group in **2**. The 2D NMR spectra also indicated that the C-3 methine in **1** (δ_C 35.2) was transformed to an oxygenated quaternary carbon (δ_C 72.0) in **2**. Calbistrin J (**2**) should share the same biosynthetic origin as that of calbistrin I (**1**), therefore they should have the same absolute configuration.

Table 1 ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectroscopic data (acetone-d_6) for compounds **1** and **2**.

Position	**1**		**2**	
	δ_C, type	δ_H, (*J* in Hz)	δ_C, type	δ_H, (*J* in Hz)
1	66.4, CH	4.22 (br s)	22.9, CH_2	1.34 (m); 1.50 (m)
2	39.1, CH_2	1.20 (td,13.0, 2.3); 1.74 (dq, 13.4, 3.6)	35.1, CH_2	1.46 (dd, 10.5, 3.7); 1.60 (m)
3	35.2, CH	2.07 (m)	72.0, qC	-
4	37.5, CH_2	0.82 (q, 12.3); 1.93 (dq, 12.5, 3.3)	41.6, CH_2	1.24 (t, 11.8); 1.71 (dt, 12.9, 2.8)
4a	31.2, CH	2.40 (t, 11.6)	33.7, CH	2.24 (m)
5	131.3, CH	5.41 (d, 10.1)	130.5, CH	5.33 (d, 9.9)
6	130.0, CH	5.50 (ddd, 9.9, 4.7, 2.3)	130.8, CH	5.54 (ddd, 9.9, 4.8, 2.7)
7	42.2, CH	2.20 (q, 6.3)	40.2, CH	2.18 (m)
8	52.5, qC	-	52.5, qC	-
8a	43.3, CH	1.66 (d, 11.1)	39.8, CH	1.59 (m)
9	214.6, qC	-	213.3, qC	-
10	75.1, CH	4.53 (dd, 5.4, 3.3)	42.3, CH_2	2.73 (qt, 12.0, 6.0)
11	65.2, CH_2	3.64 (dd, 11.7, 5.5); 3.83 (dd, 11.7, 3.2)	57.8, CH_2	3.77 (m)
12	67.9, CH_2	3.36 (dd, 6.0, 2.6)	71.9, CH_2	3.32 (br s)
13	19.2, CH_3	0.73 (d, 7.0)	19.0, CH_3	0.76 (d, 7.0)
14	18.6, CH_3	1.56 (s)	17.6, CH_3	1.24 (s)
1-OH	-	-	-	3.52
10-OH	-	-	-	3.16

Calbistrin K (**3**), a yellow amorphous solid, had the molecular formula of $C_{28}H_{44}O_8$ (by HRESI-MS). Analysis of NMR data (Table 2) indicated that **3** shared the same core structure as that in **1** and **2**. The fragment of H-8a/H-1/H_2-2/H-3/H_2-4/H-4a/H-5/H-6/H-7/H_3-13 in **3** was established by the ^{1}H-^{1}H COSY correlations. The ^{1}H-^{1}H COSY spectrum of **3** also showed the correlations of H-4a/H-8a, H-3/H_2-12, and H_2-10/H_2-11. The ^{1}H and ^{13}C

NMR data revealed that an oxygenated C-10 in **1** was replaced by a methylene group in **3**. Careful analysis of NMR data revealed that **3** had the same side chain as that for calbistrins F-H (**11**-**13**) previously isolated from the same fungus.[7] The side chain fragments of H_3-11'/H-2'/H-3' and H-5'/H-6'/H-7'/H-8'/H-9'/H_3-10' were constructed by the ^{1}H-^{1}H COSY correlations, while the HMBC correlations were observed from H_3-11', H-2', and H-3' to C-1'; H-5' to C-3'; H-6' to C-4'; and H_3-12' to C-3', C-4', and C-5'; these data established the structure of the side chain as 3,8,9-trihydroxy-2,4-dimethyldeca-4,6-dienoic acid. The side chain was linked, through the ester linkage at C-1/C-1', by the HMBC correlation of H-1 to C-1'. The coupling constant ($J_{6',7'}$ = 15.1 Hz) of H-6' and H-7' and the NOESY correlations between H-5' and H-7' and between H-6' and H_3-12' suggested the *E* geometry of H-4'/H-5' and H-6'/H-7' double bonds in **3**. The coupling constants of $J_{2',3'}$ = 9.8 Hz and $J_{8',9'}$ = 4.2 Hz were similar to those of the side chain of calbistrin F (**11**), therefore the absolute configurations at C-2', C-3', C-8', and C-9' in **3** were the same as that for calbistrin F (**11**).[7] On the basis of these data, the structure of calbistrin K (**3**) was established as shown.

Calbistrin L (**4**) was obtained as a yellow amorphous solid, and had a molecular formula of $C_{28}H_{44}O_8$ (by HRESI-MS). Its ^{1}H and ^{13}C NMR data were similar to those of calbistrin K (**3**). Analysis of NMR data revealed that the C-10 methylene and C-12 oxygenated methylene in **3** were replaced by the oxygenated methine (C-10) and methyl group (C-12) in **4**, respectively. The NMR data of the side chain of calbistrin L (**4**) were similar to that of calbistrin K (**3**), except the signals of the positions at 8' and 9'. The ^{13}C NMR resonances of C-8' (δ_C 76.8) and C-9' (δ_C 71.1) for **3** were significantly different from that for **4** (C-8', δ_C 71.3; C-9', δ_C 77.6), implying that the C-8'/C-9' configuration of **4** was different from that of **3**. The coupling constant $J_{8',9'}$ of 6.3 Hz for **4** was larger than that of **3** ($J_{8',9'}$ = 4.2 Hz; with the *syn* H-8'/H-9' relationship), therefore the *anti* configuration was proposed for the H-8'/H-9' position in **4**. The multiplicity on the ^{1}H NMR spectrum of H-9' in **3** was multiplet (*m*) with the 9'*R* configuration, while that in **4** was quintet (*quin*) with the coupling constant of 6.3 Hz (Table 2), implying that **4** had *anti* H-8'/H-9' relationship with the 9'*S* configuration. The structure of calbistrin L (**4**) was therefore established by these spectroscopic data.

Table 2 ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectroscopic data (acetone-d_6) for compounds **3** and **4**.

Position	**3**		**4**	
	δ_C, type	δ_H, (J in Hz)	δ_C, type	δ_H, (J in Hz)
1	70.7, CH	5.45 (br s)	71.1, CH	5.37 (br s)
2	35.0, CH_2	1.23 (m)	40.5, CH_2	1.09 (m); 1.91 (m)
3	36.0, CH	1.96 (m)	27.8, CH	1.87 (m)
4	37.1, CH_2	0.90 (m); 1.96 (m)	42.7, CH_2	0.90 (m); 1.87 (m)
4a	32.7, CH	2.43 (td, 11.7, 0.9)	32.8, CH	2.43 (t, 11.6)
5	130.6, CH	5.45 (d, 9.8)	130.5, CH	5.42 (d, 9.9)
6	130.3, CH	5.56 (ddd, 9.8, 5.1, 2.4)	130.2, CH	5.54 (ddd, 9.9, 5.0, 2.5)
7	42.4, CH	2.15 (q, 6.5)	42.5, CH	2.23 (qd, 6.8, 1.4)
8	52.0, qC	-	52.0, qC	-
8a	42.0, CH	1.82 (d, 11.7)	41.8,CH	1.83 (dd, 11.6, 1.0)
9	213.4, qC	-	214.2, qC	-
10	42.1, CH_2	2.70 (dt, 15.6, 5.9)	75.1, CH	4.45 (br s)
11	57.7, CH_2	3.73 (m)	65.2, CH_2	3.55 (m); 3.65 (m)
12	67.6, CH_2	3.38 (d, 5.4)	22.3, CH_3	0.88 (d, 6.2)
13	18.9, CH_3	0.75 (d, 7.0)	19.1, CH_3	0.72 (d, 7.0)
14	19.5, CH_3	1.37 (s)	18.2, CH_3	1.46 (s)
1'	175.2, qC	-	175.5, qC	-
2'	45.0, CH	2.58 (dq, 9.8, 7.1)	45.3, CH	2.57 (dq, 9.6, 7.2)
3'	80.4, CH	4.12 (dd, 9.8, 3.2)	80.5, CH	4.11 (dd, 9.6, 3.1)
4'	137.8, qC	-	138.0, qC	-
5'	128.1, CH	6.01 (d, 11.0)	128.3, CH	6.02 (d, 11.2)
6'	127.1,CH	6.52 (ddd, 15.1, 11.0, 0.98)	127.6, CH	6.55 (ddd, 15.2, 11.2, 1.2)
7'	135.0, CH	5.79 (dd, 15.1, 6.2)	135.0, CH	5.73 (dd, 15.2, 6.4)
8'	76.8, CH	4.01 (t, 4.2)	71.3, CH	3.89 (t, 6.3)
9'	71.1, CH	3.66 (m)	77.6, CH	3.87 (quin, 6.3)
10'	18.7, CH_3	1.08 (d, 6.3)	19.2, CH_3	1.07 (d, 6.3)
11'	14.7, CH_3	0.94 (d, 7.0)	14.8, CH_3	0.91 (d, 7.2)
12'	11.3, CH_3	1.72 (s)	11.3, CH_3	1.72 (s)
3'-OH	-	3.48* (br s)	-	-
8'-OH	-	2.87* (br s)	-	-
9'-OH	-	3.89* (br s)	-	-

*Assignment may be interchanged in the same column.

Calbistrin M (**5**) had a molecular formula, $C_{29}H_{46}O_8$, as deduced from the HRESI-MS. In general, calbistrin M (**5**) had similar ^{1}H and ^{13}C NMR spectra to those of calbistrin L (**4**), except an additional signal of a methyl group (δ_H 3.25, s; δ_C 56.6) in **5** (Table 3). The ^{13}C NMR resonance at C-8' of **5** (δ_C 87.5) was *ca* 16 ppm downfield shifted from that of **4** (δ_C 71.3), suggesting that the methoxy group located at C-8' in **5**. The HMBC correlation from H_3-13' to C-8' confirmed the methoxy position in **5**. The coupling constant $J_{8',9'}$ of 7.8 Hz suggested the *anti* relationship for the H-8'/H-9' position in **5**. Upon these data, the structure of calbistrin M (**5**) was established as shown in Figure 1.

Table 3 ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectroscopic data (acetone-d_6) for compounds **5** and **6**.

Position	**5**		**6**	
	δ_C, type	δ_H, (J in Hz)	δ_C, type	δ_H, (J in Hz)
1	71.0, CH	5.38 (br s)	71.1, CH	5.38 (br s)
2	40.4, CH_2	1.18 (m); 1.91 (m)	40.5, CH_2	1.03 (m); 1.87 (m)
3	27.7, CH	1.89 (m)	27.8,CH	1.87 (m)
4	42.3, CH_2	0.88 (m); 1.87 (m)	42.5, CH_2	0.88 (m); 1.87 (m)
4a	32.7, CH	2.43 (t, 11.2)	32.9, CH	2.43 (t, 11.8)
5	130.4, CH	5.42 (d, 9.9)	130.6, CH	5.42 (d, 9.8)
6	130.1, CH	5.54 (ddd, 9.9, 5.0, 2.4)	131.7, CH	5.53 (dd, 15.1, 7.6)
7	42.5, CH	2.26 (q, 4.7)	42.7, CH	2.27 (t, 7.4)
8	51.9, qC	-	52.0, qC	-
8a	41.7, CH	1.83 (d, 12.2)	41.9, CH	1.87 (d, 11.3)
9	214.1, qC	-	214.2, qC	-
10	75.0, CH	4.45 (br s)	75.1, CH	4.45 (br s)
11	65.1, CH_2	3.64 (m), 3.70 (m)	65.2, CH_2	3.62 (dd, 11.9, 5.5); 3.71 (m)
12	22.3, CH_3	0.88 (d, 6.8)	22.3, CH_3	0,88 (d, 6.2)
13	19.1, CH_3	0.74 (d, 7.0)	19.1, CH_3	0.74 (d, 7.0)
14	18.1, CH_3	1.47 (s)	18.2, CH_3	1.46 (s)
1'	175.3, qC	-	175.4, qC	-
2'	45.1, CH	2.57 (dq, 9.8, 7.1)	45.3, CH	2.57 (dq, 9.7, 7.1)
3'	80.5, CH	4.16 (d, 9.8)	80.4, CH	4.13 (dd, 9.8, 2.5)
4'	138.4, qC	-	138.9, qC	-
5'	128.1, CH	6.06 (d, 10.9)	127.9, CH	6.06 (d, 11.0)
6'	130.5, CH	6.52 (dd, 15.3, 10.9)	130.6, CH	6.57 (dd, 15.0, 11.0)
7'	132.1, CH	5.62 (dd, 15.3, 8.0)	130.2, CH	5.53 (dd, 15.0, 7.6)
8'	87.5, CH	3.49 (dd, 7.8, 4.6)	88.2, CH	3.42 (t, 5.3)
9'	69.9, CH	3.70 (m)	70.0, CH	3.60 (m)
10'	19.1, CH_3	1.07 (d, 6.4)	18.9, CH_3	1.03 (d, 6.3)
11'	14.8, CH_3	0.93 (d, 7.1)	14.8, CH_3	0.93 (d, 7.1)
12'	11.2, CH_3	1.75 (s)	11.4, CH_3	1.75 (s)
13'	56.6, CH_3	3.25 (s)	56.7, CH_3	3.25 (s)

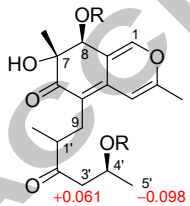
Calbistrin N (**6**) had the same molecular formula, $C_{29}H_{46}O_8$, as that of **5**. Both **5** and **6** were obtained from the reversed phase HPLC separation, and the retention times (t_R) for **5** and **6** were 43.9 and 45.1 min, respectively. ^{1}H and ^{13}C NMR spectra of **6** showed the signal for a methoxy group (δ_H 3.25, s; δ_C 56.7) (Table 3). Calbistrin N (**6**) had similar ^{1}H and ^{13}C NMR data to that of calbistrin G (**12**) (Figure 1), which was previously separated from the same fungus.[7] Analysis of ^{1}H and ^{13}C NMR spectra revealed that **6** had an additional methoxy group when compared to **12**; the HMBC correlation from H_3-13' to C-8' indicated the methoxy group being attached at C-8' in **6**. The coupling constant $J_{8',9'}$ of 5.3 Hz in **6** suggested the *syn* relationship for the H-8'/H-9' position in **6**, which was similar to that of calbistrin G (**12**).[7] Therefore, calbistrin N (**6**) was identified as a methoxy derivative of **12**.

Dothideomynone D (**7**), a yellow amorphous solid, had the molecular formula, $C_{18}H_{24}O_6$ (by HRESI-MS). The ^{1}H NMR spectrum (Table 4) of **7** showed signals of four methyl groups at δ_H 0.96, 1.05, 1.12, and 2.24, two methylene groups at δ_H 2.61 and 2.67/2.28, three sp^3 methine protons at δ_H 2.74, 4.15, and 4.44, and two sp^2 methine protons at δ_H 6.39 and 7.38. The ^{13}C NMR spectrum had 18 lines attributable to five methine, two methylene, four methyl, and seven non hydrogen-bearing carbons (by DEPT spectra). The three downfield sp^3 carbons at δ_C 64.4, 73.1, and 76.5 were of oxygenated sp^3 carbons, while that of δ_C 199.2 and 213.9 were of ketones in **7**. The ^{1}H-^{1}H COSY spectrum of **7** displayed allylic couplings of H-4/H_3-10 and H-1/H-8. The HMBC spectrum of **7** showed the correlations from H-1 to C-3, C-4a, C-8 and C-8a; H-4 to C-3, C-4a, C-5, C-8a, and C-10; H-8 to C-4a, C-7, C-8a, and C-11; H_3-10 to C-3, C-4 and C-4a (a four-bond correlation); and H_3-11 to C-6, C-7, and C-8. The ^{1}H-^{1}H COSY and HMBC correlations mentioned above established the partial structure from C-1 to C-11 that was similar to that of the known azaphilones, austdiol (**10**) and dothideomynone B (**14**) (Figure 1).[7] The ^{1}H-^{1}H COSY spectrum of **7** established the fragments of H_2-9/H-1'/H_3-6' and H_2-3'/H-4'/H_3-5', while the HMBC showed the correlations from both H_2-9 and H_3-6' to C-2' and from H_2-9 to C-4a and C-6. These data established the structure of a side chain in **7**, which was substituted at C-5 of **7**, similar to that of dothideomynone B (**14**) (Figure 1).[7] The core structure of **7** should have the same absolute configuration at C-7 and C-8 as that of austdiol (**10**) and dothideomynone B (**14**) since these polyketides might share the same biosynthetic origin. Mosher's method carried out in NMR tubes[11] was used to determine the absolute configuration of a chiral secondary alcohol at C-4'. The $\Delta\delta$ of (*S*)- and (*R*)-MTPA esters (**7a** and **7b**) indicated the 4'*S* configuration in **7** (Figure 3). Unfortunately, the C-1' configuration in **7** could not be determined based on available spectroscopic data. Upon these spectroscopic data, the structure of dothideomynone D (**7**) was secured.

Table 4 ^{1}H and ^{13}C NMR spectroscopic data (acetone-d_6) for compounds **7**-**9**.

Position	**7**[a]		**8**[b]		**9**[b]	
	δ_C, type	δ_H, (J in Hz)	δ_C, type	δ_H, (J in Hz)	δ_C, type	δ_H, (J in Hz)
1	144.9, CH	7.38 (s)	144.9, CH	7.36 (s)	144.9, CH	7.38 (s)
2	-	-	-	-	-	-
3	160.1, qC	-	159.9, qC	-	160.3, qC	-
4	104.7, CH	6.39 (s)	105.0, CH	-	104.6, CH	6.38 (s)
4a	143.1, qC	-	143.0, qC	6.38 (s)	143.1, qC	-
5	112.7, qC	-	113.0, qC	-	112.8, qC	-
6	199.2, qC	-	199.1, qC	-	199.1, qC	-
7	76.5, qC	-	76.7, qC	-	76.5, qC	-
8	73.1, CH	4.44 (s)	73.2, CH	-	73.3, CH	4.40 (s)
8a	121.3, qC	-	121.4, qC	4.41 (s)	121.5, qC	-
9	27.9, CH_2	2.67 (m); 2.28 (m)	29.8, CH_2	-	29.8, CH_2	2.34 (dd, 13.3, 6.0); 2.67 (dd, 13.3, 9.0)
10	19.6, CH_3	2.24 (s)	19.7, CH_3	2.22 (s)	19.6, CH_3	2.22 (s)
11	19.2, CH_3	1.05 (s)	19.4, CH_3	1.02 (s)	19.4, CH_3	1.03 (s)
1'	46.4, CH	2.74 (m)	39.2, CH	2.51 (m)	40.0, CH	2.54 (q, 6.6)
2'	213.9, qC	-	180.1, qC	-	180.2, qC	-
3'	51.0, CH_2	2.61 (d, 6.2)	103.9, CH	5.06 (s)	103.9, CH	5.05 (s)
4'	64.4, CH	4.15 (m)	192.3, qC	-	192.8, qC	-
5'	23.6, CH_3	1.12 (dd, 6.2, 1.3)	43.4, CH_2	2.47 (dd, 4.3, 0.7)	43.2, CH_2	2.27 (m)
6'	15.8, CH_3	0.96 (dd, 6.9, 1.9)	76.7, CH	4.45 (tdd, 12.7, 6.4, 4.2)	76.5, CH	4.38 (dq, 11.6, 5.4)
7'	-	-	20.7, CH_3	1.45 (d, 6.3)	20.4, CH_3	1.42 (d, 6.3)
8'	-	-	18.0, CH_3	1.10 (d, 6.5)	17.9, CH_3	1.13 (d, 6.9)
7-OH	-	4.81* (br s)	-	4.78 (br s)*	-	4.81 (br s)*
8-OH	-	4.23* (br s)	-	4.20 (br s)*	-	4.22 (br s)*
4'-OH	-	3.68* (br s)	-	-	-	-

[a] ^{1}H NMR data recorded at 300 MHz, and ^{13}C NMR data recorded at 75 MHz.
[b] ^{1}H NMR data recorded at 600 MHz, and ^{13}C NMR data recorded at 150 MHz.
*Assignment may be interchanged in the same column.



7a, R = (*S*)-MTPA
7b, R = (*R*)-MTPA

Figure 3. $\Delta\delta$ (δ_S-δ_R) of (*S*)- and (*R*)-MTPA esters (**7a** and **7b**) of compound **7**.

Dothideomynone E (**8**) had the molecular formula of $C_{20}H_{24}O_6$, as deduced from HRESI-MS. In general, the ^{1}H NMR signals of **8** were similar to those of **7**, and the ^{13}C NMR resonances of C-1 to C-11 in **8** were close to that of **7** (Table 4). These data suggested that compounds **8** and **7** shared the same core structure, but they had different side chain. The molecular formula obtained from the HRESI-MS, together with the information from the ^{13}C NMR spectrum, indicated that the side chain of **8** had two more carbons than that of **7**. The ^{1}H-^{1}H COSY spectrum of **8** established the fragments of H_2-9/H-1'/H_3-8' and H_2-5'/H-6'/H_3-7', while the HMBC spectrum showed the correlations from H_2-9 to C-2', C-4a, and C-6; H_3-8' to C-2'; H-3' to C-1', C-2' and C-5'; and H_2-5' to C-4'. The ^{13}C NMR resonance (δ_C 192.3) of the ketone (C-4') of the side chain in **8** was 21.6 ppm shifted upfield when compared to that of **7** (δ_C 213.9; C-2'), implying that **8** had a conjugated ketone with a ring system, not a linear side chain. The typical ^{13}C NMR resonances for the ketone carbon of the 2,3-dihydropyran-4-one unit are *ca* 192.8-193.7 ppm.[12] The NMR chemical shifts of the oxygenated atoms C-6' (δ_H 4.45; δ_C 76.7) and C-2' (δ_C 180.1), together with the molecular formula of $C_{20}H_{24}O_6$ obtained from the MS data, implied that C-2' was bridged at C-6' through the ether bond, forming the 2,3-dihydropyran-4-one unit in **8**. The NOESY correlation of H-3' and H_3-8' was observed, showing the proximity among these protons. On the basis of these spectroscopic data, the gross structure of **8** was established. Considering the biosynthetic analogy, the absolute configuration at C-7 and C-8 in **8** should be the same as that of the known azaphilones (**10** and **14**) (Figure 1). The NOESY spectrum of **8** showed the correlation between H-3' and H_3-11, implying that the conformation of **8** allowed the two protons being in close proximity. After the MMFF94 energy minimization using ChemBio3D Ultra software, the proposed conformation of **8** is depicted in Figure 4, showing the close proximity between H-3' and H_3-11 with the tentative 1'*R* configuration. However, the configuration of C-6' could not be addressed based on the available spectroscopic data.

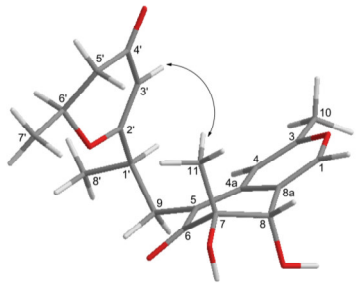


Figure 4. The conformation of **8** after MMFF94 energy minimization, showing the close proximity of H-3' and H_3-11.

Dothideomynone F (**9**) had the same molecular formula, $C_{20}H_{24}O_6$, as that of **8**. The ^1H and ^{13}C NMR data (Table 4) of **9** were nearly identical to that of **8**, and analysis of 2D NMR data revealed that both **9** and **8** had the same gross structure. The NOESY spectrum of **9** did not show the correlation between H-3' and H_3-11, while that of **8** displayed the correlations between the two protons; therefore the C-1' configuration of **9** should be different from that of **8**. Accordingly, the C-1' configuration of **9** was proposed to be *S*. Upon these data, dothideomynone F (**9**) was identified as the 1'-epimer of dothideomynone E (**8**).

2.3. Biosynthetic relationship of the isolated compounds

It is worth mentioning that new compounds isolated from MKBr and MSW media had biosynthetic relationship. Acetyl-CoA and malonyl-CoA should be the building blocks of the polyketides from the fungus *Dothideomycete* sp.; acetyl-CoA acts as the starter unit, while malonyl-CoA is the extender unit (Figure 5). The condensation of acetyl-CoA and malonyl-CoA gives the linear intermediate which in turn undergoes cyclization to give the structures of the intermediate **A** or dothideomynone D (**7**). Calbistrins I (**1**) and J (**2**) may be derived from the intermediate **A**. Enzymatic oxidation of the intermediate **A** at C-3 by certain enzyme could lead to the structure of calbistrin J (**2**), while the oxidation at C-1 and C-10 of the intermediate **A** could give rise to the formation of calbistrin I (**1**), which is in turn esterified with the side chain to give calbistrin H (**13**) (Figure 5). Dothideomynone D (**7**) is a potential biosynthetic precursor of dothideomynones E (**8**), F (**9**), and B (**14**). Oxidation of the C-6' methyl group of **7** to a carboxylic acid, concomitantly with the oxidation of the C-4' alcohol, could lead to the formation of the intermediate **B**, which in turn undergoes the keto-enol tautomerization to give the intermediate **C** (Figure 5). Finally, lactonization between the C-6' carboxylic acid and the C-4' alcohol of the intermediate **C** could give rise to dothideomynone B (**14**) (Figure 5). The biosynthesis of dothideomynone E (**8**) or F (**9**) may start with the oxidation of the C-4' alcohol in dothideomynone D (**7**) to form the intermediate **D**, which in turn undergoes the two-carbon chain extension process using malonyl-CoA as an extender, a common step by the enzyme polyketide synthase during the polyketide biosynthesis, to give the intermediate **E** (Figure 5). The reduction at the C-6' ketone of the intermediate **E** could give rise to the intermediate **F**. Cyclization at C-2'/C-6', through the ether bond formation of the intermediate **F**, could give rise to the structure of dothideomynone E (**8**) or F (**9**) (Figure 5). Therefore, the cultivation of the terrestrial fungus *Dothideomycete* sp. CRI7 in halogen containing media (bromide in MKBr and chloride in MSW media) not only gave new

compounds, but also shed some lights on the biosynthetic information of certain polyketides. Previously, cultivation of the marine-derived fungus *Aspergillus unguis* in halogen containing media also provided insights into the biosynthesis of depsidones, and some biosynthetic intermediates were co-isolated with new depsidone derivatives.[8] It is known that little changes in cultivation parameters, i.e., nutrients in culture media and addition of enzyme inhibitors, could lead to increase of the number of secondary metabolites produced by fungi or bacteria; this method is collectively known as the OSMAC technique.[13] Judging from the results obtained from this study, as well as those from the previous studies,[8] the cultivation of microorganisms in halogen containing media proves to be useful for the OSMAC technique.

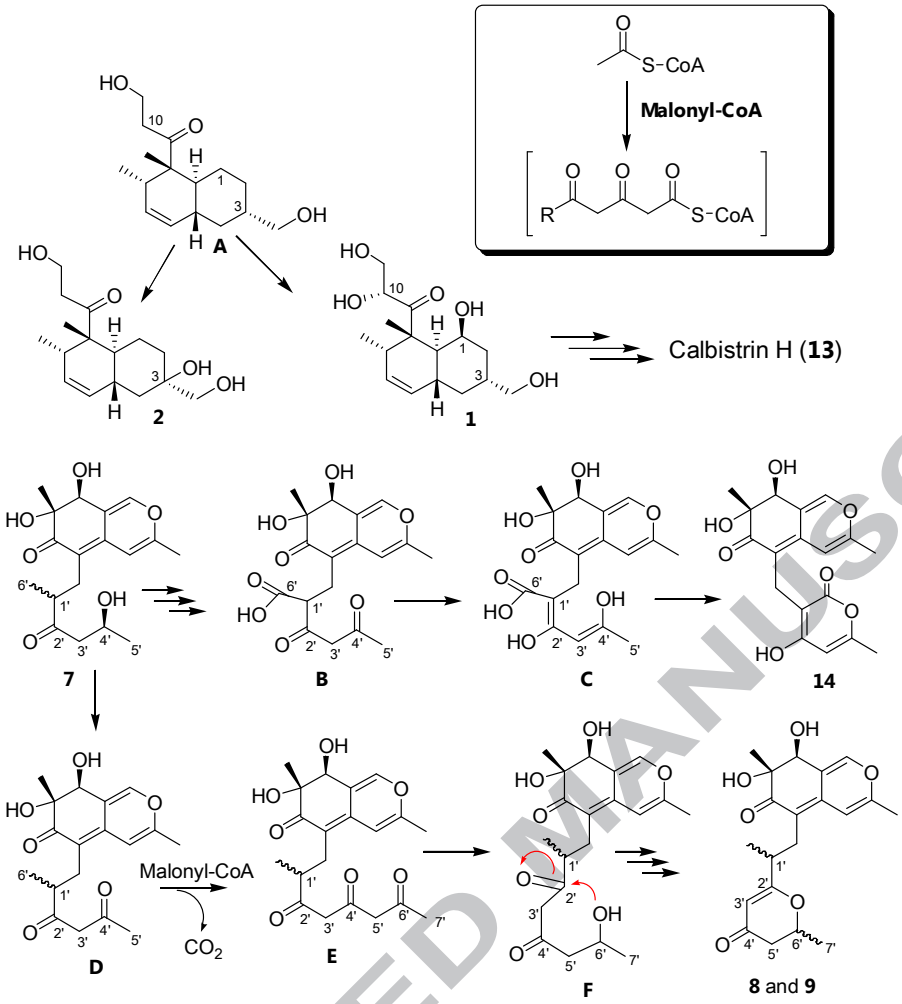


Figure 5. Proposed biosynthetic relationships among the polyketides isolated from the fungus *Dothideomycete* sp.

2.4. Biological activities of the isolated compounds

Some compounds isolated from the endophytic fungus *Dothideomycete* sp. CRI7 were evaluated for biological activities, i.e., cytotoxic, antibacterial, and antifungal activities. Antibacterial and antifungal activities of compounds **1-9** were evaluated against Gram-

positive and Gram-negative bacteria, as well as *Candida albicans*, using a broth microdilution method. Among the compounds tested, only compound **6** exhibited antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* with a minimum inhibitory concentration (MIC) value of 100 μg/mL. However, none of the compounds exhibited antifungal activity. Previously, calbistrin derivatives, calbistrins A and B, were reported to display potent antifungal activity with the MIC values of 0.78 and 3.12 μg/mL, respectively,[14] and therefore they were received attentions from organic chemists. Accordingly, the total synthesis of calbistrin A was achieved by Tatsuta and co-workers.[15] Compounds **3**, **4**, and **7**-**9** were assessed for their cytotoxic activity against MOLT-3, HepG2, HuCCA-1, and A549 cell lines, however they did not show the activity toward these cancer cell lines. Calbistrins I (**1**) and J (**2**) are structurally related to aspermytin A and decumbenone C which showed biological activities; aspermytin A could induce neurite outgrowth in pheochromocytoma cells,[16] while decumbenone C exhibited potent cytotoxic activity.[17] Unfortunately, the cytotoxic activity of **1** and **2** could not be evaluated due to the limited amounts of **1** and **2** obtained from the isolation.

3. Conclusions

This is the first demonstration on the cultivation of the terrestrial endophytic fungus in seawater. The salt tolerance ability of the fungus *Dothideomycete* sp. CRI7 enabled us to culture the fungus in media prepared from seawater, as well as in media supplemented with halogen salts (KBr or KI). Halogen salts (NaCl in seawater, KBr, and KI) were found to enhance the production of polyketides, and the multigram production (up to *ca* 12 g) of austdiol (**10**) was obtained from the fungus grown in the media containing halogen salts. Nine new polyketides (**1**-**9**) were obtained from the fungus grown in media containing seawater and KBr, and these metabolites have biosynthetic relationships. Therefore, the cultivation of the terrestrial fungus in media containing halogens not only gave new metabolites but also gave information for the biosynthetic pathways of certain polyketides. Note that the endophytic fungus *Dothideomycete* sp. was isolated from the root of the plant, *T. triandra*, which was collected from the area with hot and drought weather (Nakhonsawan Province, Thailand) during summer period. The temperature in the summer of this area could be up to 45 °C, but the temperature of the soil, where the plant root was collected, might be more than 45 °C because of the heat accumulation in soil. We speculated that the fungus *Dothideomycete* sp. might adapt itself at the cellular levels in order to survive under the hot

and drought weather during summer period, and such adaptation could lead to the salt tolerance ability. It might be possible that endophytic fungi associated with the plant hosts grown in hot and drought areas may have the salt tolerance ability. Therefore, these fungal sources are of interest for the production of bioactive secondary metabolites, e.g., the cultivation of these fungi in seawater and halogen containing media.

4. Experimental section

4.1. General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. UV spectra were obtained from a Shimadzu UV-1700 PharmaSpect UV–Vis spectrophotometer. IR spectra were acquired with a Perkin Elmer Spectrum ONE spectrophotometer using Attenuated Total Reflectance (ATR) technique. NMR spectra were recorded either on a Bruker Avance 300 (^{1}H at 300 MHz, ^{13}C at 75 MHz) or on Bruker Avance 600 (^{1}H at 600 MHz, ^{13}C at 150 MHz). HRESI-MS spectra were obtained from a Bruker MicroTOF mass spectrometer. High performance liquid chromatography (HPLC) was carried out using Waters 1525 binary module machine equipped with Waters 2998 photodiode array detector. Column chromatography (CC) was packed with Sephadex LH-20 (GE Health Care Bio-Sciences AB). Malt extract for the preparation of culture media was purchased from Scharlau, S.L., Barcelona, Spain.

4.2. Fungal material and culture media

The endophytic fungus was isolated from the roots of a Thai medicinal plant, *Tiliacora triandra*, and identified as *Dothideomycete* sp. (no.CRI7) using a molecular method; the DNA sequence of 18S-ITS1-5.8S-ITS2 rRNA gene region was submitted to Gene Bank with an accession number of JQ867364.[6] The fungus was deposited at Chulabhorn Research Institute, Thailand.

The MDW, MSW, MKBr, and MKI culture media were prepared based on the modified Czapek malt media which contained malt extract and $NaNO_3$.[18] The MDW culture medium contained malt extract (30 g) and $NaNO_3$ (2 g) in 1 L of deionized water. The MSW culture medium was composed of malt extract (30 g) and $NaNO_3$ (2 g) in 1 L of seawater. Seawater was collected from Bang Saen, Chonburi Province, Thailand, and it had 33% salinity, as measured by a salinity refractometer. The MKBr culture medium contained 30 g of malt extract, 2 g of $NaNO_3$, and 53 g of KBr in 1L of deionized water, while the MKI medium contained 30 g of malt extract, 2 g of $NaNO_3$, and 74 g of KI in 1L of deionized

water. The 33% salinity for both MKBr and MKI media was obtained with such formulation (measured by a salinity refractometer), which was similar to that of the seawater medium.

4.3. Extraction and isolation of fungal metabolites

The fungus *Dothideomycete* sp. CRI7 was cultivated in 1 L Erlenmeyer flasks, each containing 250 mL of MKBr, MSW, MKI, and MDW (total culture medium = 5 L for MKBr, MSW and MDW or 10 L for MKI). The fungus was incubated for 21 days, which was then filtered to separate broth and mycelia. Broth of each culture medium was extracted with an equal volume of EtOAc (3 times) to obtain a crude extract.

A crude broth extract (6.55 g) of the MKBr medium was dissolved in MeOH. The residue, which was insoluble in MeOH, was identified as austdiol (**10**) (4.76 g). The MeOH soluble portion of a crude extract was fractionated using Sephadex LH-20 (3 x 100 cm) CC, eluted with MeOH, to obtain fourteen fractions (A1-A14). Fractions A5 and A6, which showed similar TLC patterns and ^{1}H NMR spectra, were combined to give 395.3 mg of a material, which was further separated by C18 reversed phase HPLC (RP-HPLC), eluted with MeOH:H_2O (72:28), to yield compounds **3** (50.4 mg, t_R 8.2 min), **11** (65.1 mg, t_R 31.4 min), **12** (122.5 mg, t_R 26.9 min), and **13** (17.5 mg, t_R 7.3 min). Fractions A7, A8, and A9, which had similar TLC patterns and ^{1}H NMR spectra, were combined to give 640.8 mg of a material, which was further separated by C18 RP-HPLC, eluted with MeOH:H_2O (50:50), to give compounds **7** (65.7 mg, t_R 14.4 min), **8** (34.2 mg, t_R 24.7 min), and **9** (29.2 mg, t_R 28.2 min). A fraction with the t_R of 12 min, which was obtained from C18 RP-HPLC separation, was further purified by C18 RP-HPLC using MeCN:H_2O (30:70) as the solvent system, yielding compounds **1** (20.5 mg, t_R 7.92 min) and **2** (17.3 mg, t_R 8.91 min). The fractions A12, A13, and A14 were combined to give 162.4 mg of a material, which was further purified with C18 RP-HPLC using MeOH:H_2O (30:70) as the solvent system to yield dothideomynone B (**14**) (24.6 mg, t_R 31.2 min) and compound **10** (21.8 mg, t_R 15.3 min).

A crude extract (5.51g) from the MSW medium was suspended in MeOH, and the residue, which was insoluble in MeOH, was identified as austdiol (**10**) (2.65 g). The MeOH soluble portion of a crude extract was fractionated using Sephadex LH-20 (3 x 100 cm) CC, eluted with MeOH, to obtain eleven fractions (B1-B11). Fractions B6 and B7, which showed similar TLC patterns and ^{1}H NMR spectra, were combined to give 583.7 mg of a material, which was further separated by C18 RP-HPLC using MeOH:H_2O (72:28) as the solvent system to yield compounds **4** (15.0 mg, t_R 25.5 min), **5** (14.1 mg, t_R 43.9 min), and **6** (8.4 mg, t_R 45.1 min). Similarly, fractions B10-B11 were combined and separated by C18 PR-HPLC,

using MeOH:H_2O (30:70) as the solvent system, yielding dothideomynone B (**14**) (12.7 mg, t_R 33.0 min) and compound **10** (10.1 mg, t_R 15.9 min).

4.4. Spectroscopic data of compounds

4.4.1. Calbistrin I (1)

Yellow amorphous solid; $[\alpha]^{29}_D$ +106.24 (*c* 1.7, MeOH); UV (EtOH) λ_{max} (log ε): 202.0 (3.03) nm; IR (UATR) ν_{max} 3384, 2924, 2854, 1699, 1453, 1401, 1378, 1263, 1160, 1062, 1028, 998, 903, 769, 731 cm^{-1}; HRESI-MS: *m/z* 321.1683 $(M+Na)^+$, calcd *m/z* 321.1678 for $C_{16}H_{26}O_5Na$; ^1H and ^{13}C NMR spectroscopic data, see Table 1.

4.4.2. Calbistrin J (2)

Colorless oil; $[\alpha]^{29}_D$ +104.81 (*c* 1.6, MeOH); UV (EtOH) λ_{max} (log ε) 202.0 (3.03) nm; IR (UATR) ν_{max} 3382, 2926, 2881, 1696, 1457, 1380, 1355, 1316, 1264, 1181, 1141, 1112, 1045, 978, 944, 904, 810, 773, 733 cm^{-1}; HRESI-MS: *m/z* 305.1727 $(M+Na)^+$, calcd *m/z* 305.1729 for $C_{16}H_{26}O_4Na$; ^1H and ^{13}C NMR spectroscopic data, see Table 1.

4.4.3. Calbistrin K (3)

Yellow amorphous solid; $[\alpha]^{27}_D$ +24.44 (*c* 5.5, acetone); UV (EtOH) λ_{max} (log ε) 296.5 (3.04), 240.0 (3.78) nm; IR (UATR) ν_{max} 3360, 2911, 2852, 1706, 1659, 1631, 1454, 1378, 1261, 1180, 1037, 971, 733 cm^{-1}; HRESI-MS: *m/z* 531.2924 $(M+Na)^+$, calcd *m/z* 531.2934 for $C_{28}H_{44}O_8Na$; ^1H and ^{13}C NMR spectroscopic data, see Table 2.

4.4.4. Calbistrin L (4)

Yellow amorphous solid; $[\alpha]^{30}_D$ +66.31 (*c* 3.2, acetone); UV (EtOH) λ_{max} (log ε) 340.0 (2.22), 275.5 (2.93), 240.0 (4.25) nm; IR (UATR) ν_{max} 3420, 2952, 2925, 2154, 1702, 1456, 1379, 1302, 1265, 1180, 1162, 1121, 1057, 1007, 977, 925, 883, 819, 775, 734, 703 cm^{-1}; HRESI-MS: *m/z* 531.2938 $(M+Na)^+$, calcd *m/z* 531.2934 for $C_{28}H_{44}O_8Na$; ^1H and ^{13}C NMR spectroscopic data, see Table 2.

4.4.5. Calbistrin M (5)

Yellow amorphous solid; $[\alpha]^{29}_D$ +80.71 (*c* 3.4, acetone); UV (EtOH) λ_{max} (log ε) 293.5 (2.42), 240.5 (4.22) nm; IR (UATR) ν_{max} 3421, 2925, 2868, 2367, 2144, 1706, 1603, 1456, 1378, 1308, 1253, 1179, 1162, 1067, 895, 730 cm^{-1}; HRESI-MS: *m/z* 545.3104 $(M+Na)^+$, calcd *m/z* 545.3090 for $C_{29}H_{46}O_8Na$; ^1H and ^{13}C NMR spectroscopic data, see Table 3.

4.4.6. Calbistrin N (6)

Yellow amorphous solid; $[\alpha]^{29}_D$ +116.94 (*c* 1.6, acetone); UV (EtOH) λ_{max} (log ε) 363.5 (1.84), 339.5 (2.06), 240.0 (3.95) nm; IR (UATR) ν_{max} 3422, 2924, 2854, 2154, 1710,

1456, 1378, 1253, 1178, 1162, 1079, 1008, 971, 927, 889, 774, 730 cm^{-1}; HRESI-MS: *m/z* 545.3069 $(M+Na)^+$, calcd *m/z* 545.3090 for $C_{29}H_{46}O_8Na$; ^1H and ^{13}C NMR spectroscopic data, see Table 3.

4.4.7. Dothideomynone D (7)

Yellow amorphous solid; $[\alpha]^{27}_D$ +191.41 (*c* 7.2, acetone); UV (EtOH) λ_{max} (log ε) 357.0 (3.58), 235.0 (3.30) nm; IR (UATR) ν_{max} 3421, 2967, 2926, 2349, 2137, 1699, 1609, 1379, 1290, 1153, 1098 cm^{-1}; HRESI-MS: *m/z* 359.1471 $(M+Na)^+$, calcd *m/z* 359.1471 for $C_{18}H_{24}O_6Na$; ^1H and ^{13}C NMR spectroscopic data, see Table 4.

4.4.8. Dothideomynone E (8)

Yellow amorphous solid; $[\alpha]^{27}_D$ +92.69 (*c* 4.9, MeOH); UV (EtOH) λ_{max} (log ε) 358.0 (4.18), 260.0 (3.90), 237.5 (3.78) nm; IR (UATR) ν_{max} 3393, 2921, 2851, 1643, 1595, 1546, 1444, 1408, 1291, 1269, 1164, 1096, 1038, 1002, 965, 922, 881, 831, 721 cm^{-1}; HRESI-MS: *m/z* 383.1463 $(M+Na)^+$, calcd *m/z* 383.1471 for $C_{20}H_{24}O_6Na$; ^1H and ^{13}C NMR spectroscopic data, see Table 4.

4.4.9. Dothideomynone F (9)

Yellow amorphous solid; $[\alpha]^{27}_D$ +181.87 (*c* 4.7, MeOH); UV (EtOH) λ_{max} (log ε) 356.5 (4.22), 263.5 (4.09), 237.0 (3.92) nm; IR (UATR) ν_{max} 3422, 2977, 2928, 2856, 1655, 1595, 1545, 1445, 1387, 1267, 1164, 1095, 1040, 1001, 967, 948, 922, 875, 828, 733, 702 cm^{-1}; HRESI-MS: *m/z* 383.1474 $(M+Na)^+$, calcd *m/z* 383.1471 for $C_{20}H_{24}O_6Na$; ^1H and ^{13}C NMR spectroscopic data, see Table 4.

4.5. Preparation of (*R*)– and (*S*)–MTPA esters of compounds 1 and 7

The Mosher ester procedure carried out in NMR tubes previously described by Kinghorn and co-workers[11] was employed for the determination of the secondary alcohol in **1** and **7**. Compound **1** (1.0 mg) was transferred into an NMR tube, and it was completely dried under vacuum. Pyridine-d_5 (0.5 mL) and (*R*)-(−)-α -methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride (4 µL) were then added into an NMR tube, and the reaction mixture was shaken carefully to mix compound **1** with (*R*)-MTPA chloride evenly. The reaction mixture in an NMR tube was permitted to stand at room temperature and monitored every 2 h by observing the ^1H NMR spectrum of the mixture. The reaction was complete after 6 h, giving (*S*)-MTPA ester derivative (**1a**). ^1H NMR data of the **1a** were obtained directly from the reaction in an NMR tube. In the same manner described for the preparation of **1a**, another portion of compound **1** (1.0 mg) was reacted with (*S*)-MTPA chloride in an NMR tube to yield (*R*)-MTPA ester derivative (**1b**).

(*S*)-MTPA ester **1a**: ^{1}H NMR (pyridine-d_5, 300 MHz) δ 7.22–7.59 (20H, m, aromatic signals of MTPA), 5.59 (m, H-5, H-6), 5.13 (dd, *J* = 6.1, 3.4 Hz, H-10), 4.93 (br s, H-1), 4.47 (dd, *J* = 11.5, 3.6 Hz, H-11a), 4.25 (dd, *J* = 11.1, 6 Hz, H-11b), 3.85 (each 3H, OCH_3 of MTPA), 3.77 (d, *J* = 6.3 Hz, 2H-12), 3.69 (m, Ha-4), 2.86 (t, *J* = 10.4 Hz, H-4a), 2.68 (m, H-7), 2.27 (m, H-2a), 2.15 (d, *J* = 12 Hz, H-8a), 1.99 (s, 3H-14), 1.93 (d, *J* = 7.5 Hz, H-3), 1.61 (m, H-2b), 1.18 (d, *J* = 12.3 Hz, Hb-4), 1.09 (d, *J* = 6.9 Hz, 3H-13).

(*R*)-MTPA ester **1b**: ^{1}H NMR (pyridine-d_5, 300 MHz) δ 7.22–7.59 (20H, m, aromatic signals of MTPA), 5.59 (m, H-5, H-6), 5.13 (dd, *J* = 6.3, 3.6 Hz, H-10), 4.93 (br s, H-1), 4.46 (dd, *J* = 11.5, 3.4 Hz, H-11a), 4.24 (dd, *J* = 11.5, 6.3 Hz, H-11b), 3.84 (each 3H, OCH_3 of MTPA), 3.77 (d, *J* = 6 Hz, 2H-12), 3.67 (m, H-4b), 2.87 (t, *J* = 10.8 Hz, H-4a), 2.67 (m, H-7), 2.27 (m, H-2a), 2.15 (d, *J* = 11.7 Hz, H-8a), 1.99 (s, 3H-14), 1.94 (m, H-3), 1.61 (m, H-2b), 1.57 (m, H-4b), 1.18 (d, *J* = 12.6 Hz, H-4c), 1.1 (d, *J* = 6.9 Hz , 3H-13).

Preparation of the (*S*)- and (*R*)-MTPA esters **7a** and **7b** was carried out in the same manner as that for **1a** and **1b** mentioned above. A portion of compound **7** (1.0 mg) was individually reacted with (*R*)- and (*S*)-MTPA chloride to give (*S*)- and (*R*)-MTPA esters **7a** and **7b**, respectively.

(*S*)-MTPA ester **7a**: ^{1}H NMR (pyridine-d_5, 300 MHz) δ 7.22–7.59 (15H, m, aromatic signals of MTPA), 6.86 (m, H-1), 6.48 (d, *J* = 1.5 Hz, H-4), 5.86 (m, H-8), 5.01 (m, H-4'), 3.66 (d, *J* = 6.3 Hz, H-1'), 3.11 - 2.87 (m, 2H-9), 2.64 (m, 2H-3'), 1.98 (s, 3H-10), 1.28 (d, *J* = 6.3 Hz, 3H-5'), 1.13 (s, 3H-11), 1.10 (d, *J* = 4.6 Hz, 3H-6').

(*R*)-MTPA ester **7b**: ^{1}H NMR (pyridine-d_5, 300 MHz) δ 7.22–7.61 (15H, m, aromatic signals of MTPA), 6.37 (m, H-1), 6.28 (d, *J* = 1.5 Hz, H-4), 5.90 (m, H-8), 5.01 (m, H-4'), 3.62 (m, H-1'), 2.94 - 2.74 (m, 2H-9), 2.58 (m, 2H-3'), 2.02 (s, 3H-10), 1.38 (d, *J* = 6.3 Hz, 3H-5'), 1.07 (m, 3H-11), 1.01 (m, 3H-6').

4.6. Bioassay for antibacterial and antifungal activities

A broth microdilution method was used for the evaluation of antibacterial and antifungal activities of the isolated compounds.[19] The isolated compounds were tested on the microorganisms including Gram-positive bacteria (*Bacillus cereus*, *Enterococcus faecalis, Staphylococcus aureus*, and *Staphylococcus epidermidis*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Shigella flexneri*), and *Candida albicans*. Briefly, the tested compounds were dissolved in DMSO and a two-fold serial dilution in 96-well plates was carried out. Bacterial and fungal suspensions were prepared in normal saline solution and adjusted to a turbidity of the 0.5 with the McFarland

standard. The final concentration of DMSO did not exceed 0.5% (v/v). The plates were incubated at 36 °C for 20 h, and the absorbance was measured at 600 nm to determine the minimum inhibitory concentration (MIC) value of the tested compounds. For the antibacterial activity, chloramphenicol and tetracycline were used as the standard drugs. Chloramphenicol exhibited antibacterial activity against *B. cereus*, *S. aureus,* and *S. epidermidis* with the MIC value of 0.78 µg/mL, while tetracycline had respective MIC values of 6.25, 0.78, and 1.56 µg/mL toward *B. cereus*, *S. aureus,* and *S. epidermidis*, respectively. Amphotericin B was used as the standard antifungal drug, exhibiting the MIC value of 0.78 µg/mL. Note that the MIC is defined as the lowest concentration of the compound that inhibits the growth of microorganism.

4.7. Bioassay for cytotoxic activity

Adhesive cell lines including HuCCA-1, HepG2, and A549 cancer cells, as well as non-adhesive MOLT-3 cell line, were used for cytotoxic activity testing. The activity toward the adhesive cell lines was assessed using the MTT assay,[20] while that for non-adhesive MOLT-3 cell line was tested with the XTT assay.[21] Etoposide and doxorubicin were used as the reference drugs. Etoposide exhibited cytotoxic activity against MOLT-3 and HepG2 cell lines with respective IC_{50} values of 0.04 and 24.32 µg/mL, while doxorubicin showed the activity toward HepG2, HuCCA-1, and A549 cell lines with the IC_{50} values of 0.22, 0.33, and 0.27 µg/mL, respectively.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at ….

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Highlights:

- The terrestrial fungus *Dothideomycete* sp. was used for OSMAC approach.
- The terrestrial fungus was grown in media containing seawater and halogens.
- Nine new compounds were obtained from the fungus grown in seawater and KBr.
- Metabolites obtained from halogen containing media had biosynthetic relationships.
- Cultivation of terrestrial fungi in seawater could be useful for OSMAC approach.

Graphical Abstract:

Metabolite diversification by cultivation of the endophytic fungus *Dothideomycete* sp. in halogen containing media: Cultivation of terrestrial fungus in seawater

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