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Phytotoxicity and anti-phytopathogenic activities of marine-derived fungi and their secondary metabolites†

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To find new pesticides for agricultural use, 133 fungal strains were isolated from coastal marine habitats, from which 37 independent isolates were identified, belonging to 20 genera in nine orders, and the diversity of the isolated fungi were discussed. The phytotoxicity and anti-phytopathogenic fungal and bacterial activities of these 37 extracts, and two previously isolated fungal extracts were evaluated, displaying different levels of bioactivity. Based on the bioactive and chemical screening, an *Alternaria* sp. (P8) strain, which showed prominent bioactivity and contained abundant secondary metabolites was selected for further chemical investigation; one new compound, a benzopyranone (**1**), and seven known compounds (**2–8**) were obtained. Their structures were determined by analysing extensive NMR spectroscopic data and ECD comparisons. Compounds **1**, **2**, and **6–8** showed obvious phytotoxicity, especially against amaranth, and compound **1** also showed potent antifungal activity toward *Alternaria brassicicola*. To the best of our knowledge, this is the first report of the phytotoxicity of marine-derived fungi and their secondary metabolites. These studies should provide the foundation for future research into the use of such fungal extracts to combat weeds and diseases in agriculture.

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1 Introduction

It has been estimated that at least 10% of global food production is lost to plant diseases; among these, weeds, plant pathogenic fungi, and bacteria are three major pests causing drastic loss in yield.¹ Conventional chemicals have brought many benefits to mankind in the agricultural area, but their toxicity to both humans and animals has always been a concern.² Therefore, new pesticides with high efficacy and safety need to be discovered and developed to replace the conventional ones.³

Natural products (NPs) play an important role in the search for new pesticides. According to the literature, NPs accounted for the majority (35.7%) of new active ingredient registrations

with the Environmental Protection Agency (EPA) from 1997 to 2010.⁴ As a major component of NPs, microbial-produced bio-pesticides have been very successful in the past, with such products as bialaphos, spinosyns, blasticidin, destruxins, basianolide, and isarolides.⁵ However, after more than half a century of combinatorial chemistry research of terrestrial microorganisms, a declining number of new compounds have been found, which has prompted scientists to find new bio-pesticides from marine microorganisms. During the last two decades, marine-derived fungi have gained much attention among all marine organisms, as they can produce large numbers of novel biological compounds, including polyketides, meroterpenoids, terpenoids, peptides, alkaloids, and steroids, with antimicrobial, anticancer, antiviral, anti-inflammatory, antioxidant, and insecticidal activities.⁶ However, most of the bioactive compounds were utilized for medical, rather than agricultural applications. Hence, there is tremendous potential to find biopesticides from marine-derived fungi.

During our previous study of the agricultural applications of marine-derived fungi, 24 out of 31 fungal strains displayed obvious anti-phytopathogenic activities, and four bioactive compounds were isolated from two fungal strains.⁷ Due to the potential applications of marine-derived fungi for plant protection which few groups focused on, it is necessary to continue studying marine-derived fungi and their secondary metabolites that have agricultural bioactivities. In the present study, the isolation, identification, and bioactivity screening

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† Electronic supplementary information (ESI) available: Table S1: Identification and phylogenetic affiliations of the isolated marine-derived fungal strains, Table S2: antifungal activity against spore germination of the marine-derived fungal extracts (10.0 mg mL⁻¹), Table S3: antifungal activity against mycelial growth (10.0 mg mL⁻¹) of plant pathogenic fungi, Table S4: Antibacterial activity of the marine-derived fungal extracts, Fig. S1: the sources of the isolate marine-derived fungal strains, Fig. S2–S8: ¹H NMR, ¹³C NMR, COSY, HMQC, HMBC, 1D NOE, and HRESIMS spectra of compound **1**. See DOI: 10.1039/c8ra08047j

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(phytotoxic and anti-phytopathogenic activities) of marine-derived fungi are reported, and the structure elucidation, as well as biological activities of isolated compounds from one selected strain are presented and discussed.

2 Results and discussion

2.1 Diversity of the isolated marine-derived fungi

Marine fungal strains have been obtained from nearly every possible marine habitat, including marine plants (most notably algae and mangrove plants), marine invertebrates (e.g., sponges, corals, ascidians, holothurians, bivalves, and crustaceans), vertebrates (mainly fish), and inorganic matter (sediments and sea water).⁸ In this study, a total of 133 fungal strains were isolated from marine plants (algae and mangroves), animals (fish, crab and starfish), sediments, and seawater, which were collected from two coastal marine habitats in Qingdao and Haikou, China, in May, 2017. Among these, 88 fungal strains were from Haikou and 45 were from Qingdao. The mangrove-derived fungi were the dominant group that we obtained (59 strains), accounting for 44% in this study, followed by 37 animal-derived (28%), 20 alga-derived (15%), 15 sediment-derived (11%), and two seawater-derived (2%) strains (Fig. S1†).

It was reported that there were 530 species in 321 genera of filamentous marine fungi, among which 424 species were within Ascomycota (in 251 genera), 94 species were anamorphic fungi (in 61 genera), and 12 species were within Basidiomycota (in nine genera). These data apply to those marine taxa that can be isolated or cultivated by classical microbial techniques, and that have been studied in detail and taxonomically described by marine mycologists; the overall figures are expected to be much higher.⁸ Among our isolated marine-derived fungi, according to the morphotypes, strains of *Penicillium* sp. and *Aspergillus* sp. accounted for a large proportion of the 45 isolates from the Qingdao intertidal zone, while *Trichoderma* sp. strains seemed to be the predominant fungi of the 88 isolates from the Hainan coastal habitat. Redundant isolates were excluded by analysis of their morphological characteristics and 37 independent strains were selected for sequencing and identification according to ITS sequences. Based on the sequences deposited into the National Center for Biotechnology Information, 36 strains belonged to the phylum Ascomycota, including eight taxonomic orders: Botryosphaerales, Diaporthales, Eurotiales, Glomerellales, Hypocreales, Mucorales, Pleosporales, and Xylariales (Fig. 1 and Table S1†). Particularly, one representative strain was grouped in the genus *Poitrasia*, of the order Mucorales and phylum Mucoromycota, which was the first reported *Poitrasia* sp. strain from marine environments. Isolates of Eurotiales (38%), Hypocreales (27%), and Pleosporales (16%) were dominant. According to the phylogenetic analysis, all identified fungi belonged to 20 genera, among which *Penicillium* sp. (five strains), *Trichoderma* sp. (four strains), *Talaromyces* sp. (four strains), *Aspergillus* sp. (three strains), and *Fusarium* sp. (three strains) were the dominant groups, accounting for 51.4% in this study; the remaining genera occurred as singletons or doubletons.

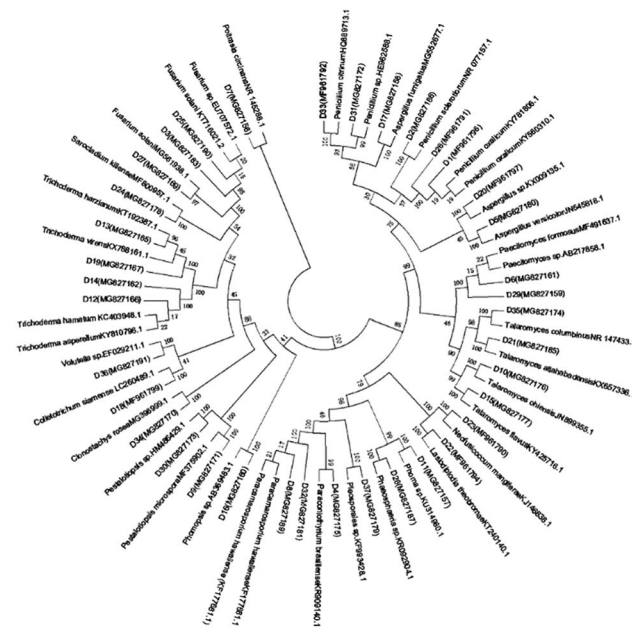


Fig. 1 Neighbor-joining phylogenetic tree of 37 representative marine-derived fungi based on 18S rRNA gene sequences.

2.2 Exploring for the bioactivities of marine-derived fungal extracts

2.2.1 Phytotoxicity of the marine-derived fungal extracts.

Natural products have had a significant impact on the development of a number of conventional pesticides. However, this has not been the case for herbicides. Although many natural products exhibit potent phytotoxicity against seed germination or germ tube elongation,⁹ only one class of natural product-derived herbicide, the triketones, has been registered since 1997, according to the EPA.⁴ Marine fungi have been a hotspot for the study of new biopesticides and medicines, as they can produce secondary metabolites with novel structures and potential bioactivities.¹⁰ However, few studies have focused on their agricultural applications, especially the herbicidal effect of marine fungi. As far as we know, there has been no research studying the phytotoxicity of marine fungi and their secondary metabolites. Therefore, searching for phytotoxic compounds from marine-derived fungi holds promise.

In the present study, 37 newly isolated marine fungi and two fungal strains that we had previously obtained and showed to have potent agricultural biological activity, were tested for their phytotoxicity toward seedling growth and leaf health. The effects of the marine fungal extracts toward seedling growth of amaranth (*Amaranthus retroflexus* L.) and lettuce (*Lactuca sativa*) are shown in Table 1. Two fungal extracts (D2 and D3) significantly inhibited the seed germination of amaranth at 1.0 mg mL⁻¹, while no significant difference was found between the control and most of the fungal extracts for the germination of lettuce seeds. However, despite germination not being inhibited noticeably, the marine fungal isolates showed excellent inhibition of the root and hypocotyl growth of the two tested plants. Fifteen fungal extracts displayed obvious phytotoxicity toward



Table 1 Phytotoxicity of marine fungal extracts (1.0 mg mL⁻¹) toward seedling growth of amaranth and lettuce^a

Strains	Root length (mm)		Hypocotyl length (mm)	
	Amaranth	Lettuce	Amaranth	Lettuce
D1	6.47 ± 0.86	5.73 ± 0.70	4.70 ± 1.07	—
D2	0.00 ± 0.00	—	0.00 ± 0.00	—
D3	0.00 ± 0.00	—	0.00 ± 0.00	—
D5	7.55 ± 1.24	—	4.15 ± 0.56	—
D12	0.00 ± 0.00	—	5.70 ± 0.79	—
D13	5.15 ± 0.34	6.65 ± 1.42	—	—
D14	2.30 ± 0.27	2.40 ± 0.07	4.55 ± 1.28	—
D20	5.45 ± 0.59	—	—	—
D21	9.10 ± 1.00	—	—	—
D22	3.20 ± 1.92	—	2.15 ± 1.46	—
D25	4.40 ± 0.55	—	4.50 ± 1.42	—
D35	9.25 ± 1.06	—	—	—
D36	8.55 ± 1.86	—	—	—
P8	7.55 ± 0.81	0.00 ± 0.00	0.00 ± 0.00	—
P18	10.00 ± 1.17	—	5.20 ± 0.47	—
gp	0.00 ± 0.00	0.00 ± 0.00	2.10 ± 0.10	4.40 ± 0.73
ck	22.05 ± 1.75	19.35 ± 1.12	9.45 ± 0.40	11.11 ± 2.39

^a “gp” was glyphosate. The length < 2.0 mm was regarded as no germination. “—” means no obvious effect on seedling growth.

seedling growth, among which D2, D3, D12, D14, D22, and P8 were the most effective since they showed the strongest inhibition of the root or hypocotyl growth of at least one of the two tested plants. It was obvious that the effect of the marine fungal isolates on seedling growth was greater with amaranth than with lettuce, and the phytotoxicity was stronger toward root growth than toward hypocotyl growth. The genera *Trichoderma* was the most effective fungi as all three strains (D12, D13, and D14) showed potent phytotoxicity toward radicle and embryonal elongations. It is well-known that fungi such as *Alternaria* sp., *Fusarium* sp., and *Colletotrichum* sp. can produce phytotoxins that have considerable potential as models to develop herbicides with new modes of action unrelated to those in current use. *Trichoderma* spp. have been studied as biopesticides and biofertilizers due to their abilities to protect crops from plant pathogens and promote vegetative growth. However, products for weed control from *Trichoderma* spp. are commercially limited and the few known studies in this area are restricted to *T. virens*.¹¹ Therefore, our report provides a new area for the application of *Trichoderma* sp. in agriculture.

The leaf spray experiments were performed on amaranth and Chinese pennisetum (*Pennisetum alopecuroides* (L.) Spreng.) in pot assays. The development of necrotic lesions after 3–7 days of treatment with 39 marine fungal extracts indicated that 10 strains showed obvious phytotoxicity toward amaranth, while six ones were effective on Chinese pennisetum (Table 2). It is notable that D5 and P8 showed phytotoxicity toward amaranth leaves that was as strong as the positive control glyphosate, and D5 still showed phytotoxicity when its concentration was decreased to 1.0 mg mL⁻¹ (Fig. 2). Of importance, the phytotoxicity manifested within three days, which was faster than that of glyphosate, indicating that these fungi could produce

Table 2 Phytotoxicity of marine fungal extracts (10.0 mg mL⁻¹) toward amaranth and Chinese pennisetum by leaf spray assays^a

Strains	Chinese pennisetum	
	Amaranth	Chinese pennisetum
D1	2	0
D3	1	0
D5	4	0
D6	1	2
D7	1	1
D10	2	1
D13	1	0
D17	0	1
D20	3	3
P8	4	3
P18	3	0
gp	4	3

^a 0 means no effect (control), 1 was inhibition rate <50%, 2 was 50% < inhibition rate < 70%, 3 was 70% < inhibition rate < 90%, and 4 was inhibition rate >90%.

secondary metabolites with potent phytotoxicity. Amaranth was much more sensitive to the marine fungal extracts than was Chinese pennisetum, and the newer leaves were more sensitive than the older ones were. Further investigation on the phytotoxicity toward amaranth and Chinese pennisetum leaves was carried out by leaf puncture assays at a low concentration of 1.0 mg mL⁻¹. Only one fungal isolate (D5) induced necrotic lesions on amaranth at 20 µg per droplet 3 days after its application, demonstrating great potential of being developed as a bioherbicide (Fig. 2).

From the results above, it was obvious that seven fungal strains (D1, D3, D5, D13, D20, P8, and P18) showed phytotoxicity against not only seedling growth, but also leaf health. To the best of our knowledge, we are exploring the phytotoxicity of marine-derived fungi and the genera *Poitrasia*, *Talaromyces*, and *Volutella* for the first time.

2.2.2 Antifungal activity of the marine-derived fungal extracts. The 37 fungal isolates and two previously obtained fungal strains (P8 and P18) were tested for their antifungal

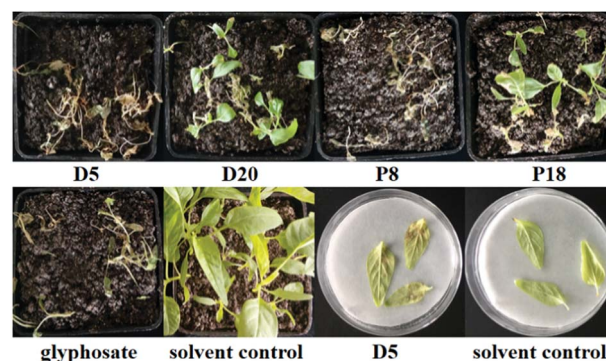


Fig. 2 Phytotoxicity of four marine-derived fungal extracts D5, D20, P8, and P18 (10.0 mg mL⁻¹, 5 mL per pot) on amaranth by leaf spray assays, and D5 (1.0 mg mL⁻¹, 20 µL) on amaranth by leaf puncture assays.



activity by observing their ability to inhibit spore germination and mycelial growth of *Alternaria alternata* (Fries) Keissler, *A. brassicicola*, *Aspergillus niger* van. Tiegh, *Diaporthe medusaea* Nitschke, and *Pestalotia theae*. Of the 39 fungal extracts assayed, nine (23.1%) showed antifungal activity against spore germination, while 10 (25.6%) showed good inhibition (>50%) at 10.0 mg mL⁻¹ against mycelial growth of at least one of the phytopathogenic fungi tested (Tables S2, S3,† and Fig. 3). It should be noted that nearly all of the fungal isolates could inhibit the mycelial growth of at least one phytopathogenic fungus to varying degrees, but only the isolates with greater than 50% inhibition were considered bioactive. In general, the marine fungal extracts were most active against *A. brassicicola* and *D. medusaea* when considering both mycelium growth and spore inhibition. Fungi classified within the genera of *Penicillium* and *Trichoderma* contributed the greatest to the antifungal activity, as three strains (D1, D2, and D26) out of five *Penicillium* sp. and three (D12, D13, and D14) out of four *Trichoderma* sp. displayed obvious antifungal activity. D12, D14, and D27 exhibited the most distinct antifungal activity as they could inhibit the spore germination or mycelial growth of at least five phytopathogenic fungi, suggesting that these strains could produce interesting and useful antifungal compounds.

2.2.3 Antibacterial activity of the marine-derived fungal extracts. Marine fungal sources have been very prolific producers of thousands of natural antibiotics. More than half of all antibiotics are produced by actinomycetes, 10–15% by non-filamentous bacteria, and ~20% by filamentous fungi.¹² In this study, the 37 fungal extracts (the other two ones P8 and P18 have been tested before) of the fermented fungi were evaluated for their activity against the plant bacterial pathogens, *Acidovorax avenae*, *Pseudomonas syringae* pv. *lachrymans*, and

Ralstonia solanacearum. Fourteen fungi showed varying degrees of antibacterial activity at 10.0 mg mL⁻¹, representing 37.9% of the fermented strains (Table S4†), while only three strains exhibited prominent activity when the test concentration was reduced to 1.0 mg mL⁻¹ (Fig. 4). The antibacterial activity toward the target bacterial strain, *P. syringae* pv. *lachrymans*, was the most remarkable, which was consistent with our previous study.⁷ D1 and D21 were the most effective fungi and their extracts exhibited distinct antibacterial activity against both *P. syringae* pv. *lachrymans* and *A. avenae*, which was much stronger than that of the positive control, streptomycin sulfate. It was reported previously that the dominant genera in antimicrobial compound-producing marine fungi were the *Aspergillus* sp. and *Penicillium* sp.,¹³ and our present study supported that conclusion. In particular, this was the first report of the antibacterial activity of the *Poitrasia* sp. fungi, to the best of our knowledge. Our study shows that continuing investigations into these marine-derived fungi represent promising strategies to identify molecules with potent antibacterial activity.

2.3 Investigation of the bioactive compounds from *Alternaria* sp. P8

We previously reported the antibacterial activity of a marine-derived fungus *Alternaria* sp. P8 and its two bioactive secondary metabolites.⁷ In the present study, the potent phytotoxicity and antifungal activity of this fungus were also discovered. This suggests a broad agricultural bioactivity spectrum of P8, which warranted further investigation. Fifty Erlenmeyer flasks of fungi were cultivated in solid medium. Chemical investigations of the P8 extracts led to the isolation of one new benzopyranone, (+)-(2*S*,3*R*,4*aR*)-altenuene (1), and seven known compounds, (+)-isoaltenuene (2),¹⁴ alternariol (3),¹⁵ altenuisole (4),¹⁶ alternariol 9-methyl ether (5),¹⁵ altertoxin I (6),¹⁷ stemphyrylenol (7),⁷ and alterpyrenol (8) (Fig. 5).⁷

Compound 1 was isolated as a white, amorphous powder and assigned the molecular formula C₁₅H₁₆O₆ by HRESIMS, indicating seven degrees of unsaturation. The ¹H NMR spectrum displayed signals for three aromatic or olefinic protons at δ_H 6.75 (d, *J* = 2.0 Hz), 6.50 (d, *J* = 2.0 Hz), and 6.30 (d, *J* = 3.5 Hz); two oxymethines at δ_H 3.95 (t, *J* = 3.5 Hz) and 3.70 (dt, *J* = 7.5, 3.5 Hz); one methoxy group at δ_H 3.86 (s); one set of nonequivalent methylene protons at δ_H 2.26 (dd, *J* = 14.0, 3.5 Hz) and 1.95 (dd, *J* = 14.0, 7.5 Hz); and one methyl group at δ_H 1.47 (s). The ¹³C NMR and distortion less enhancement by

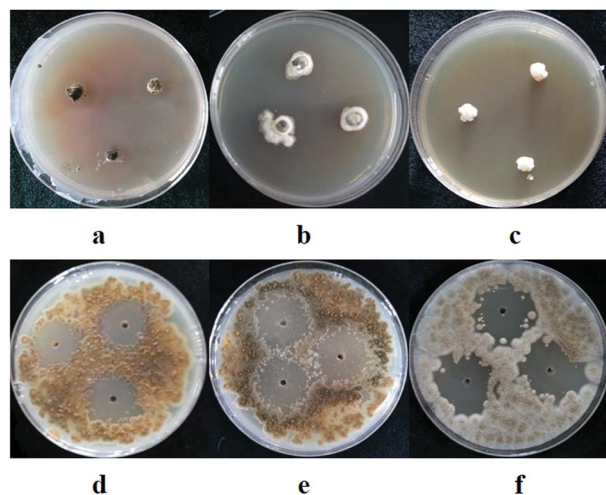


Fig. 3 Antifungal activity of marine-derived fungal extracts (10.0 mg mL⁻¹). (a) Extracts of D1 against *A. brassicicola* by mycelium growth rate method; (b) extracts of D1 against *A. alternata* by mycelium growth rate method; (c) extracts of D12 against *D. medusaea* by mycelium growth rate method; (d) extracts of D26 against *A. brassicicola* by agar diffusion method; (e) extracts of D27 against *A. brassicicola* by agar diffusion method; (f) extracts of D27 against *P. theae* by agar diffusion method.

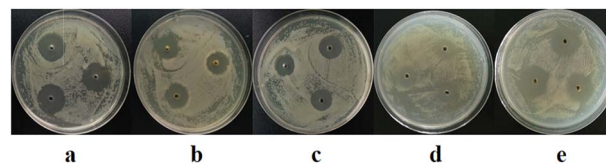


Fig. 4 Antibacterial activity of marine-derived fungal extracts (10.0 mg mL⁻¹). (a) Extracts of D1 against *P. syringae* pv. *lachrymans*; (b) extracts of D21 against *P. syringae* pv. *lachrymans*; (c) extracts of D26 against *P. syringae* pv. *lachrymans*; (d) extracts of D1 against *A. avenae*; (e) extracts of D21 against *A. avenae*.



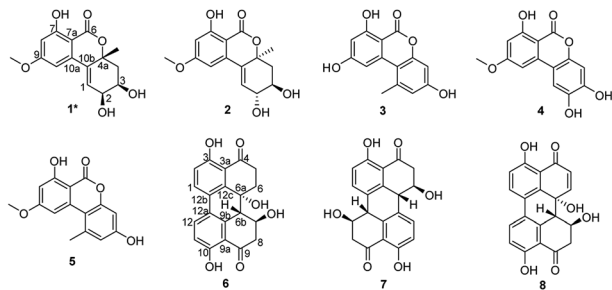


Fig. 5 Chemical structures of compounds 1–8.

polarization transfer (DEPT) spectra showed resonances for one ester carbonyl (δ_C 168.2), eight aromatic or olefinic carbon atoms (δ_C 165.8, 163.0, 139.2, 131.8, 131.0, 102.3, 100.9, and 100.0), one sp^3 oxygenated quaternary carbon (δ_C 81.2), two oxymethines (δ_C 69.5 and 68.8), one methoxyl group (δ_C 55.8), one methylene (δ_C 38.5) and one methyl group (δ_C 27.4). These spectroscopic features, in addition to the 2D NMR data, suggested that **1** belonged to the benzopyranone family and is very similar to altenuene, which was isolated from an endolichenic fungal strain, *Nigrospora sphaerica* (No. 83-1-1-2), cultured from tissues of the lichen, *Parmelinella wallichiana* (Taylor) Elix & Hale collected from the Zixi Mountains of the Yunnan Province of China.¹⁴

The relative configuration of **1** was deduced by *J*-based configurational analysis and 1D nuclear Overhauser effect (NOE) correlations. The 1H - 1H coupling constants of H-2 (δ_H 3.95, t, J = 3.5 Hz) and H-3 (δ_H 3.69, dt, J = 7.5, 3.5 Hz) indicated a *cis*-relationship of these two protons. The signal for Ha-4 (δ_H 1.95) showed one vicinal coupling constant (J = 7.5 Hz) characteristic of axial-axial-type coupling, leading to the assignment of an axial orientation for Ha-4 and H-3. In the NOE difference spectrum, irradiation of 4a-CH₃ enhanced the resonance of Ha-4, indicating a *cis* relationship between them. This evidence suggested that 4a-CH₃ and H-2/H-3 were on the opposite face of the molecule. The absolute configuration of **1** was determined by ECD analysis. The negative first Cotton effect at 279 nm ($\Delta\epsilon$ -41.95) and the positive second one at 232 nm ($\Delta\epsilon$ +71.88) (Fig. 6) in the ECD spectrum indicated a 4a*R*-configuration.¹⁴ Thus, the absolute configuration of **1** was determined to be 2*S*,3*R*,4a*R*.

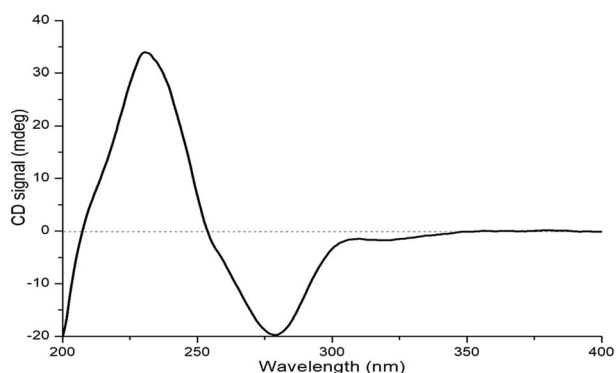


Fig. 6 ECD spectrum of compound 1.

In our previous study, we found that compound **8** exhibited potent antibacterial activity against *Clavibacter michiganensis* and **7** displayed obvious antifungal activity against *A. brassicicola* and *P. theae*. In the present study, the phytotoxicity of **1–8**, and the antifungal and antibacterial activities of **1–6** were determined. Compounds **1**, **2**, and **6–8** showed obvious phytotoxicity against the seedling growth of amaranth and lettuce at 200 ppm (Table 3). The perylenequinones (**6–8**) were more bioactive than the benzopyranones (**1** and **2**) were, since they could inhibit seed germination at 200 ppm and still display strong phytotoxicity when their concentration decreased to 50 ppm (Table 4). It seemed that the isolated compounds inhibited the seedling growth of lettuce less than that of amaranth, and their effects on root elongation were much more obvious than their effects on hypocotyl elongation. Because no phytotoxicity of **3–5** was observed, preliminary analysis of the structure–activity relationships of the benzopyranones (**1–5**) indicated that the replacement of benzene by cyclohexene could positively influence phytotoxicity. Compound **1** also showed potent antifungal activity toward *A. brassicicola*, with a minimum inhibitory concentration (MIC) of 125 $\mu g mL^{-1}$, which was equivalent to that of the positive control carbendazim. Compound **6** displayed moderate antifungal activity against *D. medusaea*, with an MIC of 62.5 $\mu g mL^{-1}$, compared to 31.3 $\mu g mL^{-1}$ for carbendazim. Compounds **1–6** exhibited no obvious antibacterial activity.

3 Experimental section

3.1 Fungal materials, isolation, identification, and fermentation

The marine-derived fungi in this study were isolated from seawater, sediments, animals, and algae collected from coastal marine habitats of the Yellow Sea, Qingdao, and South China Sea, Haikou, China, during May, 2017. These samples were immediately processed for isolation and purification of fungi, using potato dextrose agar (PDA) culture media with a salinity of 3%, according to the method we previously described.⁷ The isolated fungi were transferred into cryogenic vials containing potato dextrose water (PDW) culture media and glycerol (v/v = 3 : 1), and were deposited at the Marine Agriculture Research

Table 3 Phytotoxicity of compounds **1**, **2**, and **6–8** (200 ppm) toward seedling growth^a

Strains	Root length (mm)		Hypocotyl length (mm)	
	Amaranth	Lettuce	Amaranth	Lettuce
1	4.65 ± 0.53	19.30 ± 3.06	4.96 ± 0.45	6.65 ± 1.14
2	4.30 ± 0.10	14.01 ± 1.77	7.42 ± 0.27	7.78 ± 0.87
6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
7	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
8	0.00 ± 0.00	4.45 ± 0.43	0.00 ± 0.00	4.60 ± 0.34
gP	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
H ₂ O	12.08 ± 0.43	21.00 ± 1.07	7.34 ± 0.26	9.00 ± 0.54

^a Values means ± SD. The length < 2.0 mm was regarded as no germination.



Table 4 Phytotoxicity of compounds **1**, **2**, and **6–8** (50 ppm) toward seedling growth^a

Strains	Root length (mm)		Hypocotyl length (mm)	
	Amaranth	Lettuce	Amaranth	Lettuce
6	0.00 ± 0.00	0.00 ± 0.00	4.15 ± 0.19	0.00 ± 0.00
7	0.00 ± 0.00	2.25 ± 0.26	4.90 ± 0.45	3.45 ± 0.26
8	0.00 ± 0.00	7.90 ± 0.41	6.00 ± 0.39	5.07 ± 0.21
gp	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
H ₂ O	12.08 ± 0.43	21.00 ± 1.07	7.34 ± 0.26	9.00 ± 0.54

^a Values means ± SD. The length < 2.0 mm was regarded as no germination.

Center, Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao, China. The obtained fungi were identified according to morphological characteristics and a molecular protocol by amplification and DNA sequencing of the internal transcribed spacer (ITS) region of the rRNA gene, as well as phylogenetic analysis.

The separated marine-derived fungi were cultivated in PDW culture media with 3% salinity in three, 1 L Erlenmeyer flasks (each containing 400 mL of culture broth) at 28 °C without shaking for 40 days. The pooled cultures (1.2 L) were filtered to separate the broth from the mycelia. The mycelia were extracted three times with CH₂Cl₂ : MeOH (1 : 1, v/v), and the solution was concentrated under reduced pressure to afford a residue, which was extracted with EtOAc three times. Then, the broth was extracted three times with an equal volume of EtOAc, and the mycelia extracts were combined and concentrated under vacuum to afford a total extract.

3.2 Bioassays of the marine-derived fungal extracts

3.2.1 Phytotoxicity assays. The phytotoxicity of the marine-derived fungal isolates were assayed using seedling growth, leaf spray, and leaf puncture experiments.

In the seedling growth assays, seeds of two dicotyledons, amaranth and lettuce were used. The procedure was conducted according to a published protocol with slight modifications.¹⁸ The plant seeds were soaked in sterile water for 5 h and in 0.3% NaClO for 15 min, then were rinsed three times with sterile water. The extracts were dissolved in methanol to a final concentration of 1.0 mg mL⁻¹, and 200 µL per well was added to 24-well plats containing filter paper at the bottom of the wells (Whatman no. 1). Glyphosate and methanol were used as a positive control and solvent control, respectively. After the methanol evaporated, five seeds were placed in each well of the 24-well plates, and 200 µL of sterile water per well was added. The plates were then incubated at 28 °C for 96 h with a cycle of 12 h light and 12 h dark. The lengths of the roots and hypocotyls were recorded.

The leaf spray assays were performed using the leaves of amaranth and Chinese pennisetum, according to a previously described method, with modifications.¹⁹ Plastic pots (10 cm diameter) were filled with nutrient soil bought from Shouguang Wode Nursery Substrate Co. Ltd., Weifang, China. Twenty seeds

were planted in each pot, and after seeding, the pots were placed in trays without drainage holes and watered from the bottom. The plants were grown in an illuminating incubator at 28 °C with 12 h illumination and 12 h darkness. Fourteen-day-old amaranth (3–4 true leaf stage) and 7 day-old Chinese pennisetum (10–12 cm high) were used for experiments. The fungal extracts were dissolved in 4% MeOH with 0.25% surfactant (pesticide emulsifier 1602) to final concentrations of 10.0, 5.0, and 1.0 mg mL⁻¹. Glyphosate and 4% MeOH were used as a positive control and solvent control, respectively. The solutions (5.0 mL) were sprayed on the plants and a qualitative estimation of phytotoxicity was obtained by using a visual rating scale from 0 to 4, where 0 was no effect (solvent control), 1 was an inhibition <50%, 2 was 50% < inhibition < 70%, 3 was 70% < inhibition < 90%, and 4 was inhibition >90%.

The leaf puncture assays were carried out using the leaves of amaranth and Chinese pennisetum by a previously described method.²⁰ The fungal extracts were dissolved in 4% MeOH to a final concentration of 1.0 mg mL⁻¹. A droplet (20 µL) of solution was applied to detached leaves previously punctured with a needle. Glyphosate and 4% MeOH were used as a positive control and solvent control, respectively. The leaves were kept in a moistened chamber under continuous fluorescent lighting. Symptoms were estimated visually 3–7 days after droplet application.

3.2.2 Antifungal assays. The antifungal activity of the extracts against five plant pathogenic fungi, including *A. alternata* (Fries) Keissler, *A. brassicicola*, *D. medusaea* Nitschke, and *P. theae* was evaluated by the mycelium growth rate method with slight modification.²¹ The fungal extracts were dissolved in dimethyl sulfoxide (DMSO) to generate a final concentration of 10.0 mg mL⁻¹, and 100 µL was then spread on each Petri dish (diameter = 9 cm) containing 20 mL PDA media. A mycelial agar disk (diameter = 4 mm) of each plant pathogenic fungus from the edge of 72 h-old culture was placed in the center of the Petri dish. The dish was incubated at 28 °C for 72 h. Prochloraz and DMSO were employed as a positive control and solvent control, respectively. Then, the diameter of each colony was measured by making two measurements at right angles. The inhibitions of growth were calculated using the following formula, where *D* represents the average colony diameter of the tested extracts, *D*_{ck} represents the average colony diameter of the solvent control, and 4 is the diameter of the original mycelial agar disk (in mm).

$$\text{Inhibition of growth (\%)} = [1 - (D - 4)/(D_{ck} - 4)] \times 100$$

The antifungal activity of the EtOAc extracts against the plant pathogenic fungi, *A. alternata* (Fries) Keissler, *A. brassicicola*, *A. niger* van. Tiegh, and *P. theae*, was also tested using an agar diffusion method, with modifications.²² Briefly, the spores of the fungi were adjusted to 1 × 10⁵ CFU mL⁻¹, and 100 µL suspensions were then spread on Petri dishes (diameter = 9 cm) containing 20 mL PDA media. Three wells (diameter = 6 mm each) were created in this pathogen-embedded agar with sterile glass tubes. The lyophilized EtOAc extracts were dissolved in



DMSO to a final concentration of 10.0 mg mL⁻¹ and 10 µL of the solutions was added to each well on the plates. Prochloraz and DMSO were employed as a positive control and solvent control, respectively. The Petri dishes were incubated at 28 °C for 72 h in darkness and the diameter of each zone of inhibition was measured.

3.2.3 Antibacterial assays. The antibacterial activity of the EtOAc extracts against three plant pathogenic bacteria, namely *A. avenae*, *P. syringae* pv. *lachrymans*, and *R. solanacearum*, was evaluated by an agar diffusion method that previously reported.^{7,23}

3.3 Isolation and structure elucidation of compounds from *Alternaria* sp. P8

Optical rotations were measured on a Jasco P-1020 digital polarimeter (Jasco, Inc., Easton, MD, USA), and UV spectra were recorded on a Techcomp UV2310II spectrophotometer (Techcomp, Ltd., Shanghai, China). Electronic circular dichroism (ECD) spectra were obtained on a Jasco J-815-150S circular dichroism spectrometer (Jasco, Inc., Tokyo, Japan). NMR spectra were recorded on an Agilent DD2 500 MHz NMR spectrometer (500 MHz for ¹H and 125 MHz for ¹³C; Agilent Technologies, Santa Clara, CA, USA) using tetramethylsilane as an internal standard. Electrospray ionization mass spectrometry (ESIMS) and high-resolution electrospray ionization mass spectrometry (HRESIMS) spectra were obtained with a Micro-mass Q-TOF spectrometer and Thermo Scientific LTQ Orbitrap XL spectrometer. Semi-preparative HPLC was performed on a Waters C18 (5 µm, 10 × 250 mm) column using a Waters 2695 separation module equipped with a Waters 2996 photodiode array (PDA) detector (Waters, MA, USA). Silica gel (200–300 mesh; Qing Dao Hai Yang Chemical Group Co., Qingdao, China), octadecylsilyl-silica gel (45–60 µm; Merck, Darmstadt, Germany), and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) were used for column chromatography. Compounds were monitored by thin layer chromatography (TLC) (G60, F-254; Yan Tai Zi Fu Chemical Group Co., Yantai, China), and spots were visualized by heating the silica gel plates after spraying with 12% H₂SO₄ in H₂O containing saturated vanillins.

The fungal strain *Alternaria* sp. P8 was fermented on rice media in 50 Erlenmeyer flasks (each containing 80 g rice and 120 mL H₂O) at 28 °C for 40 days. The culture medium was extracted three times with CH₂Cl₂ : MeOH (1 : 1, v/v), and the pooled solutions were concentrated under reduced pressure to afford a residue, which was extracted with EtOAc three times. The pooled EtOAc extracts were concentrated under reduced pressure and subjected to vacuum liquid chromatography (VLC) on silica gel. An eluent gradient of EtOAc in petroleum ether (0/100 to 100/0, v/v) followed by MeOH in EtOAc (0–100%) was used to give five fractions (Fr. 1–Fr. 5). Fr. 3 was first subjected to silica gel column chromatography (mobile phase, 30% EtOAc in petroleum ether), and then separated by octadecyl-silica column, eluting with 30–60% MeOH in H₂O, to obtain Fr. 3-1–Fr. 3-3. Fr. 3-2 was applied to Sephadex LH-20 column chromatography (mobile phase, CH₂Cl₂ : MeOH, 1 : 1, v/v), followed by purification on HPLC with 60% MeOH to afford 5 (8.9 mg).

Fr. 4 was fractionated on silica gel column chromatography using a gradient elution with petroleum ether : EtOAc to afford three subfractions (Fr. 4-1–Fr. 4-4). Fr. 4-3 was applied to an octadecyl-silica column, eluting with 50% MeOH and then was purified by Sephadex LH-20 column chromatography (mobile phase, CH₂Cl₂ : MeOH, 1 : 1, v/v) to obtain three subfractions (Fr. 4-3-1–4-3-3). Fr. 4-3-2 was purified on HPLC with 25% MeCN in H₂O to give 6 (15.3 mg), 7 (8.5 mg), and 8 (11.2 mg), while Fr. 4-3-3 was purified by semipreparative HPLC, eluting with 50% MeOH to give 3 (14.6 mg) and 4 (5.1 mg). Fr. 4-4 was purified on HPLC with 35% MeCN to give 1 (17.2 mg) and 2 (18.9 mg).

(+)-(2*S*,3*R*,4*aR*)-Altenuene (1): white, amorphous powder; [α]_D²⁰ –6.4° (c 0.10, MeOH); UV (MeOH) λ_{max} (log ϵ) 240 (4.80), 278 (4.33), 319 (4.10) nm; ECD (0.14 mM, MeOH) λ_{max} ($\Delta\epsilon$) 232 (+71.88), 279 (–41.95) nm; ¹H NMR (DMSO-*d*₆, 500 MHz) δ 11.30 (s, 7-OH), 6.75 (1H, d, *J* = 2.0 Hz, H-10), 6.54 (1H, d, *J* = 2.0 Hz, H-8), 6.30 (1H, d, *J* = 3.5 Hz, H-1), 3.95 (1H, t, *J* = 3.5 Hz, H-2), 3.86 (3H, s, 9-OCH₃), 3.69 (1H, dt, *J* = 7.5, 3.5 Hz, H-3), 2.26 (1H, dd, *J* = 14.0, 3.5 Hz, H-4), 3.69 (1H, dt, *J* = 7.5, 3.5 Hz, H-3), 1.95 (1H, dd, *J* = 14.0, 7.5 Hz, H-4), 1.47 (1H, s, *J* = 14.0, 7.5 Hz, 4a-CH₃); ¹³C NMR (DMSO-*d*₆, 125 MHz) δ 168.2 (C, C-6), 165.8 (C, C-9), 163.0 (C, C-7), 139.2 (C, C-10a), 131.8 (C, C-10b), 131.0 (CH, C-1), 102.3 (CH, C-10), 100.9 (CH, C-8), 100.0 (C, C-7a), 81.2 (C, C-4a), 69.5 (CH, C-2), 68.8 (CH, C-3), 55.9 (CH₃, 9-OCH₃), 38.5 (CH₂, C-4), 27.4 (CH₃, 4a-CH₃); HRESIMS *m/z* 291.0878 [M – H][–] (calcd for C₁₅H₁₅O₆, 291.0874).

3.4 Bioassays of compounds 1–8

The phytotoxicity of 1–8 against seed germination and seedling growth of amaranth and lettuce (200 and 50 ppm) was conducted as mentioned above. The antibacterial and antifungal activities were evaluated by a conventional broth-dilution assay according to the National Center for Clinical Laboratory Standards recommendations, with modifications as we previously reported.⁷

4 Conclusions

In summary, 37 marine-derived fungi were identified from 133 strains that were isolated from several marine biotopes. The diversity of the obtained fungi was investigated, leading to the discovery of the first marine-derived *Poitrasia* sp. strain. The phytotoxicity, as well as the anti-phytopathogenic fungal and bacterial activities of the fungal extracts were tested, and one strain, *Alternaria* sp. P8, was selected for chemical investigation according to its bioactivity. This resulted in the isolation of eight compounds, including one new compound, which exhibited potent phytotoxicity, antifungal activity, and antibacterial activity. The phytotoxicity of marine-derived fungi in this study is very interesting, since this is the first report, to the best of our knowledge, on the discovery and evaluation of the phytotoxic efficacy of marine-derived fungi. As few studies focus on agricultural bioactivities, our results suggest that exploring the application of marine-derived fungi in agriculture represents a promising strategy for discovering new biopesticides.



Conflicts of interest

There are no conflicts to declare.

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