Synthetic and Systems Biotechnology 4 (2019) 173–179

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: http://www.keaipublishing.com/synbio

Original Research Article

Brocaeloid D, a novel compound isolated from a wheat pathogenic fungus, *Microdochium majus* 99049

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ARTICLE INFO

Keywords:
Wheat pathogenic fungus
Anticancer
Antibacterial
Natural product

ABSTRACT

Microbes serve as the most important resource for drug discovery. During our screening for bioactive compounds from our natural products library, a pathogenic fungus, *Microdochium majus* strain 99049, from wheat was selected for further investigation. A new alkaloid named brocaeloid D (**1**), together with six previously characterized compounds (**2–7**) were identified. Compound **1** belongs to 4-oxoquinoline with C-2 reversed prenylation and a succinimide substructure. All the structures of these newly isolated compounds were determined by different means in spectroscopic experiments. The absolute configurations of **1** was further deduced from comparison of its CD spectrum with that of known compound **2**. The bioactivities of these identified compounds were evaluated against several pathogenic microorganisms and cancer cell lines. Compounds **1–5** showed activity against HUH-7 human hepatoma cells with IC_{50} values of 80 μg/mL. Compound **6** showed mild activity against HeLa cells (IC_{50} = 51.9 μg/mL), weak anti-MTB activity (MIC = 80 μg/mL), and moderate anti-MRSA activity (MIC = 25 μg/mL), and compound **7** showed weak anti-MRSA activity (MIC = 100 μg/mL).

1. Introduction

The public health threat of antibiotic resistance has gained attention globally because of the occurrence of several untreatable infections [1,2]. In particular, the combination of widespread multidrug resistance caused by methicillin-resistant *Staphylococcus aureus* (MRSA) [3,4], multidrug-resistant Tuberculosis (MDR-TB), and extensively drug-resistant Tuberculosis (XDR-TB) has resulted in major illnesses and deaths world-wide [5,6]. To combat severe antibiotic resistance, different strategies have been proposed. For instance, high quality, comprehensive, and real-time surveillance data are essential to reduce the burden of antibiotic resistance [7]. However, in the present situation, the lack of new antibiotic drugs presents a grave public health challenge [8].

Natural products and their derivatives, which offer compounds shaped by natural selection, are the main source of our current antibiotic drugs [9,10]. Historically, microbial natural products have been the major source of such novel antibiotics [6,11–15]. Notably, actinomycetes [16,17] and fungi [18–21] have provided fruitful sources of pharmaceutically useful drugs. In particular, fungi isolated from extreme environments, such as polar glaciers, deep-sea locations, deserts, and from plants have provided potential sources of many useful antibiotics [22–27].

Fungi are a rich source of natural products, particularly phytopathogenic fungi which may produce toxic substances with antimicrobial potential. Cimmino et al. [28] reviewed the literature from 2007 through 2015 on fungal phytotoxins, which are generally secondary metabolites which play important roles in the induction of

Peer review under responsibility of KeAi Communications Co., Ltd.
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https://doi.org/10.1016/j.synbio.2019.09.001
Received 11 July 2019; Received in revised form 10 September 2019; Accepted 15 September 2019

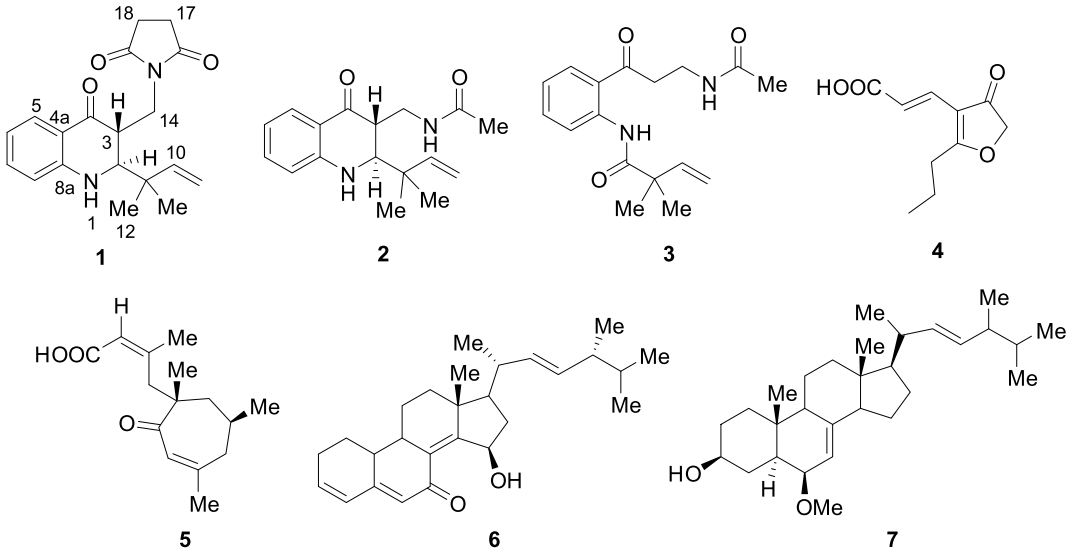


Fig. 1. Structures of compounds **1**–**7**.

disease symptoms or interfere with host plant physiological processes. The use of phytotoxins produced by phytopathogenic fungi against weeds is an ecofriendly tool as the basis of natural and presumably safe bioherbicides. Phytoalexins which are synthesized and accumulate in plants after their exposure to pathogenic microorganisms, are another type of secondary metabolite that has potential applications. These types of compounds have been studied as promising antifungal, anticancer, and plant disease control agents [29–32]. Ruszkowska and Wróbel [33] reported on a group of indole-derived phytoalexins containing at least one sulfur atom in the side chain or among rings, showing diverse biological activities. In recent years, there has been a significant increase in the isolation of bioactive secondary metabolites from plant pathogenic fungi [34–36].

In this study, we report on bioactive compounds from a wheat pathogenic fungus, *Microdochium majus* strain 99049, based on a bioassay-guided isolation approach. A new alkaloid derivative named brocaeloid D (**1**), was found together with six previously characterized compounds (Fig. 1), including brocaeloid A (**2**) [37], brocaeloid B (**3**) [37], cillifuranone (**4**) [38], leptosphaeric acid (**5**) [39], 15β-hydroxyl-(22*E*, 24*R*)-ergosta-3,5,8,22-tetraen-one (**6**) [40], and (22*E*, 24*R*)-6β-methoxyergosta-7,22-diene-3β,5α-diol (**7**) [41]. The bioactivity of these identified compounds was evaluated against *S. aureus*, MRSA, MTB, HUH-7 human hepatoma cells, HeLa cells and, A549 lung carcinoma cells.

2. Materials and methods

2.1. General experimental details

The CD spectrum was recorded on a JASCO J-815 Spectropolarimeter, using $CHCl_3$ as solvent. The NMR spectra were obtained on a Bruker Avance DRX500 spectrometer. Chemical shifts were calibrated internally against residual solvent signals (DMSO-d_6: δ_C 39.5, δ_H 2.50; Acetone-d_6: δ_C 206.7, 29.9, δ_H 2.05). HRESIMS measurements were obtained on Agilent 1200 HPLC/6520 QTOF-MS. Sephadex LH-20 (GE) was used for purification. HPLC was performed using Agilent 1200 Series HPLC controlled using ChemStation Rev.B.02.01.

2.2. Characterization and identification of fungal strain MM99049

Microdochium majus strain 99049 (MM99049) was isolated from *Triticum aestivum* grown in Atwood, Ontario, Canada [42]. It was cultured on 2% potato dextrose agar (PDA, Fisher Scientific) at 20 °C. The fungus was identified by sequencing of the internal transcribed spacer (ITS) region and comparison to the GenBank database.

The primer pair ITS1 (5″-TCCGTAGGTGAACCTGCGG-3″) and ITS4 (5″-TCCTCCGCTTATTGATATGC-3″) was used to amplify the ITS region of MM99049. PCR amplification (25 μL final volume containing 0.4 μL each primer, 2.5 μL 10 × buffer, 2.5 μL 2.5 nM dNTP, 0.4 μL rTap polymerase, and 1 μL DNA template) of the ITS sequence was performed on an ABI PCR Thermal Cycler with the initial denaturation at 94 °C for 5 min, 25 cycles of denaturation (94 °C 1 min), annealing (55 °C 1 min), and elongation (72 °C 45 s), and a final elongation at 72 °C for 10 min. Highly similar sequences were obtained from GenBank, and a phylogenetic tree was constructed using MEGA 5.0 software.

2.3. Compound extraction and isolation

The fungal strain was cultured on PDA at 20 °C for 7 days. Agar plugs were cut into small cubes 0.5 cm across under aseptic conditions, and five pieces were placed in each of three Erlenmeyer flasks (250 mL), each containing 50 mL PD broth. The broth had final pH adjusted to 5.1 ± 0.2 with 6 N HCl after autoclaving at 121 °C for 15 min. Three of these flasks of the inoculated media were incubated at 20 °C on a rotary shaker at 200 rpm for 5 days to prepare the seed culture. Large-scale fermentation was carried out in 30 Erlenmeyer flasks (1000 mL), each containing 160 g rice, which had been soaked in 200 mL distilled H_2O overnight before autoclaving at 15 psi for 30 min. After cooling to room temperature, each flask was inoculated with 5.0 mL spore suspension and grown at 20 °C for 45 days.

The fermented rice substrate was extracted repeatedly with ethyl acetate (4 × 500 mL) at room temperature. The organic solvent was evaporated to dryness under vacuum to afford the crude extract (15.6 g), which was fractionated by silica gel column chromatography (8 × 38 cm) eluted with a gradient of n-hexane-ethyl acetate (v/v 100:0, 80:1, 50:1, 32:1, 24:1, 20:1, 15:1, 13:1, 10:1, 9:1, 4:1, 1:1), followed by dichloromethane-methanol (100:0, 5:1, 1:1, 0:100) to give 16 fractions (Frs. 1–16) based on thin layer chromatography (TLC) analysis (8 × 6 cm, n-hexane-ethyl acetate, v/v 9:1). Fr. 5 (0.165 g) was subjected to Sephadex LH-20 (2.5 × 50 cm, CH_2Cl_2/MeOH 1:1) to give seven subfractions (Frs. 5.1–5.7). Fr. 5.3 (57.8 mg) was further purified by reverse phase-high performance liquid chromatography (RP-HPLC) (Agilent ZORBAX, SB-C3, 5 μm column, 9.4 × 250 mm) using 43% ACN in H_2O to obtain compound **4** (7.0 mg, t_R = 16.74 min). Fr. 14 (0.257 g)

was subjected to Sephadex LH-20 (2.5 × 50 cm, CH_2Cl_2/MeOH 1:1) to give seven subfractions (Frs. 14.1–14.8). Fr. 14.4 (98.8 mg) was further purified by RP-HPLC (Agilent ZORBAX, SB-C3, 5 μm column, 9.4 × 250 mm) using 45% acetonitrile in water to afford compound **5** (5.0 mg, t_R = 24.35 min). Fr.15 (2.235 g) was subjected to Sephadex LH-20 (2.5 × 50 cm, CH_2Cl_2/MeOH 1:1) to give seven subfractions (Frs. 15.1–15.6). Fr. 15.3 (125.8 mg) was further purified by RP-HPLC (Agilent ZORBAX, SB-C3, 5 μm column, 9.4 × 250 mm) using 37% ACN in water to obtain compounds **1** (2.7 mg, t_R = 24.35 min), **2** (4.3 mg, t_R = 20.81 min), and **3** (5.0 mg, t_R = 24.47 min). Fr.16 (7.65 g) was subjected to Sephadex LH-20 (2.5 × 50 cm, CH_2Cl_2/MeOH 1:1) to give seven subfractions (Frs. 16.1–16.7). Fr. 16.3 (185.8 mg) was further purified by RP-HPLC (Agilent Eclipse, XDB-C8, 5 μm column, 9.4 × 250 mm) using 37% ACN in water to obtain compounds **6** (5.0 mg, t_R = 30.12 min) and **7** (2.7 mg, t_R = 38.43 min).

2.4. Bioactivity tests

2.4.1. General antimicrobial assays

Antibacterial assays were performed following the Antimicrobial Susceptibility Testing Standards outlined by the Clinical and Laboratory Standards Institute using the bacteria *Staphylococcus aureus* (ATCC 6538), MRSA (clinical strain from Chaoyang Hospital, Beijing, China), and *Candida albicans* (SC5314). For each organism, a loopful of glycerol stock previously stored at −80 °C was streaked on an LB agar plate and incubated at 37 °C overnight, a single colony was selected and streaked across an LB-agar plate, and the colony was incubated overnight at 37 °C. An isolated colony was then picked out, suspended in Mueller–Hinton broth, and grown to approximately 1 × 10^4 cfu/mL. A twofold serial dilution of each bio-assay compound to be tested (4000–31.25 μg/mL in DMSO) was prepared, and an aliquot of each dilution (2 μL) was added to a 96-well flat bottom microtiter plate (Greiner). Vancomycin and ciprofloxacin were used as positive controls and DMSO as the negative control. An aliquot (78 μL) of the bacterial suspension was then added to each well (to give final concentrations of 100 to 7.8 μg/mL in 2.5% DMSO), and the plates were incubated at 37 °C aerobically for 16 h. Finally, the optical density of each well at 600 nm was measured with an EnVision 2103 Multilabel Plate Reader (PerkinElmer Life Sciences). MIC values were defined as the minimum concentration of compound that inhibited visible bacterial growth. All the experiments were performed in triplicate.

2.4.2. Anti-MTB assays

The anti-MTB H37Rv assays utilize constitutive GFP expression (pUV3583c-GFP) with direct readout of fluorescence as a measure of bacterial growth (Cowley and Av-Gay 2001). The *in vitro* activity of compounds against MTB H37Rv was determined in a 96-well plate as previously described [43]. *M. tuberculosis* H37Rv was grown at 37 °C to mid-log phase in Middlebrook 7H9 broth (Becton Dickinson) supplemented with 10% Oleic Albumin Dextrose Catalase (OADC) enrichment (Becton Dickinson), 0.05% Tween 80, and 0.2% glycerol. The culture was then diluted with culture medium to OD_{600} = 0.025.

For primary screening, 78 μL of the bacterial suspension were added to each well of the flat-bottom 96-well microplates, followed by adding 2 μL of each compound (in DMSO) separately. The compounds were serially diluted two-fold in each column. Isoniazid with two-fold serial dilution concentrations from 800 to 6.25 ng/mL served as positive controls and DMSO as negative control. The *M. tuberculosis* H37Rv plates were incubated at 37 °C for 10 days. GFP fluorescence was measured with a Multilabel Plate Reader using the bottom read mode, with excitation at 485 nm and emission at 535 nm. MIC was defined here as the minimum concentration of drug that inhibited more than 90% of bacterial growth assessed by fluorescence value.

2.4.3. Cancer cell assays

Human cancer cells were obtained from BeNa Culture Collection. These human cancer cell lines were used: HUH-7 human hepatoma cell (BNCC337690), HeLa cell (BNCC342309), and A549 lung carcinoma cell (BNCC337696). These cancer cell lines were seeded into a 96-well microplate at the density of 5 × 10^3 per well. The plates were cultured at 37 °C in 5% CO_2 for adherence. Serial dilutions of each compound in DMSO were placed into 96-well microplates. The plates were cultured at 37 °C in 5% CO_2 for 44 h. An aliquot of 15 μL of MTT was added into each well and the plates were cultured at 37 °C in 5% CO_2 for 4 h. DMSO (150 μL) was added to each cell. The plates were vibrated for 15 min, and read at 570 nm [44].

2.5. Accession number

Microdochium majus strain 99049 has been deposited at the China General Microbiological Culture Collection Center with an accession number 3.19156. The ITS nucleotide sequence of strain MM99049 has been submitted to GenBank with an accession number MH698506.

3. Results

3.1. Fungal identification

After incubation at 20 °C for 7 days, strain MM99049 formed pure colonies on PDA. No obvious diffusible pigments were produced. Comparison with GenBank sequences, and a phylogenetic tree (Fig. 2) based on ITS sequences indicated that MM99049 is a member of the species *Microdochium majus*. In the tree, *Selenodriella cubensis* was selected as the outgroup because it is a sister taxon to *Microdochium*, and one of the few other genera placed in Microdochiaceae. Strain MM99049 has been deposited at the China General Microbiological Culture Collection Center (accession number 3.19156), and its ITS nucleotide sequence has been deposited in NCBI under GenBank

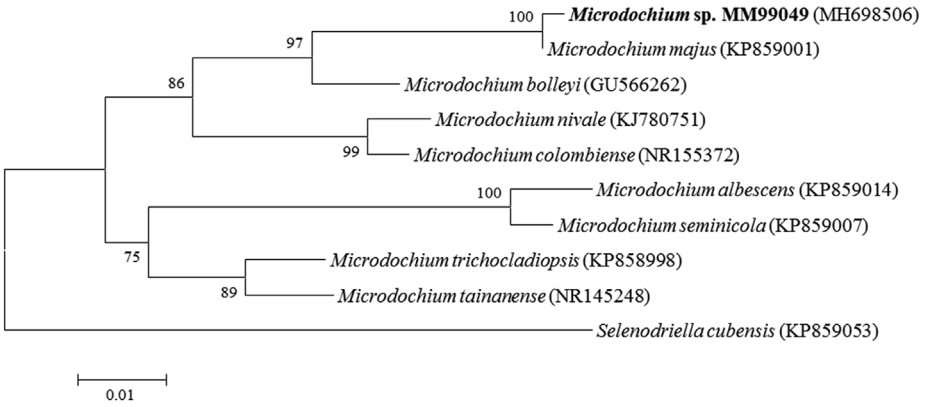


Fig. 2. Neighbor-joining tree of *Microdochium majus* strain 99049 based on rDNA ITS sequences constructed using the neighbor-joining algorithm in MEGA 5.0. Numbers at nodes indicate levels of bootstrap support (in percent) based on 1000 resampled datasets; only values > 50% are shown. GenBank accession numbers are given in parentheses. Bar = 0.01 nucleotide substitutions per site. *Selenodriella cubensis* was chosen as the outgroup.

Table 1
The ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), and 2D NMR data of **1** in acetone-d_6 (J in Hz).

Pos.	δ_C, mult	δ_H, mult (J in Hz)	COSY (^{1}H-^{1}H)	HMBC (^{1}H-^{13}C)
1	NH-	–	–	–
2	62.1, CH	3.21 s	3	3, 4, 8a, 10, 13, 14
3	44.9, CH	3.01 t (8.0)	2,14	4, 14
4	193.5, C	–	–	–
4a	117.5, C	–	–	–
5	127.7, CH	7.49 dd (7.9, 1.6)	6	4, 7, 8a
6	115.6, CH	6.81 d (8.5)	5,7	4a
7	136.3, CH	7.24 td (8.5, 1.6)	6,8	5,8a
8	116.6, CH	6.51 t (7.9)	7	5
8a	151.4, C	–	–	–
9	44.1, C	–	–	–
10	145.5, CH	5.70 dd (17.5, 10.5)	11	13
11a	113.8, CH_2	4.93 dd (17.5, 1.0)	10	9, 10
11b	–	4.87 dd (10.5, 1.0)	–	–
12	24.8, CH_3	1.01 s	–	2, 9, 10, 13
13	23.3, CH_3	0.94 s	–	2, 9, 10, 12
14a	41.1, CH_2	3.71 dd (13.3, 8.3)	–	2, 3, 4, 16, 19
14b	–	3.61 dd (13.3, 6.8)	–	–
15	N	–	–	–
16	177.9, C	–	–	–
17	28.8, CH_2	2.64 s	–	16, 18, 19
18	28.8, CH_2	2.64 s	–	17, 18, 19
19	177.6, C	–	–	–

accession number MH698506.

3.2. Structure elucidation

The ethyl acetate (EtOAc) extract of strain MM99049 was subjected to a silica gel column and was further purified by a combination of column chromatography on silica gel, Sephadex LH-20, and HPLC preparation on a RP-18 column (Agilent ZORBAX, SB-C3, 5 μm, 9.4 × 250 mm; Agilent Eclipse, XDB-C8, 5 μm, 9.4 × 250 mm) to obtain a new compound, **1**, and six characterized compounds **2–7**.

Brocaeloid D (**1**) was initially obtained as a light-green amorphous powder. Its molecular formula was determined to be $C_{19}H_{23}O_3N_2$ (11 degrees of unsaturation) on the basis of HRESIMS ion at m/z 327.1755 (calcd. for $[M + H]^+$, 327.1703) (Fig. S8). Combined analysis of its ^{1}H NMR, ^{13}C NMR and HSQC spectroscopic data (Figs. S1–3; Table 1) revealed the presence of two methyl groups, four methylenes, seven methines, and three quaternary carbon signals, and three carbonyl groups. The NMR spectroscopic (Figs. S1–6) data of **1** were very similar to those of brocaeloid A (**2**), a 4-oxoquinoline with C-2 reversed prenylation [37], except for three additional signals at δ_C 28.6 (C-17), 28.6 (C-18), and 177.6 (C-19) and one absent methyl group in the ^{13}C NMR spectra of **1**. Thus, the substructure 2,3-dihydroquinoline-4(1H)-one with a isoprene group attached on C-2 can be established by HMBC correlations of H-2/C-10, H-2/C-13, and H-12/C-2 (Fig. 3). In addition, **1** exhibited two more degrees of unsaturation than **2**. This extra structure unit on **1** could be determined to be a succinimide substructure by its HMBC correlations of H-17/C-18, H-17/C-19, and H-18/C16. The connectivity of succinimide substructure and 2,3-dihydroquinoline-4(1*H*)-one moiety could be deduced by the HMBC

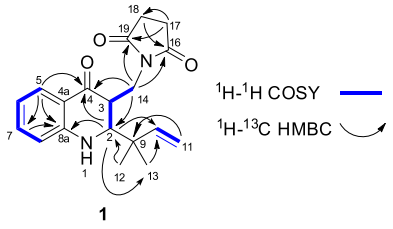


Fig. 3. Key ^{1}H-^{1}H COSY (bold line) and HMBC (single arrow) correlations of **1**.

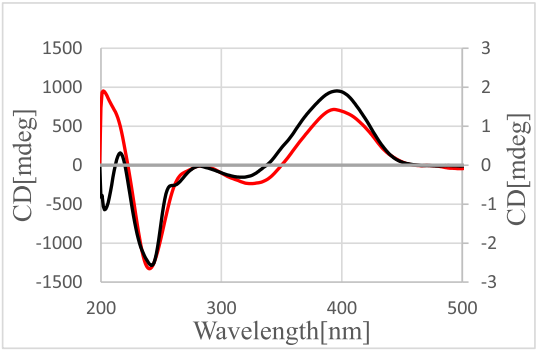


Fig. 4. The experimental CD spectra of compounds **1** (red) and **2** (black) in MeOH.

correlations (Fig. 3). From the HMBC spectrum of **1**, the lower field protons at δ_H 3.71 (dd, J = 13.3, 8.3 Hz) and 3.61 (dd, J = 13.3, 6.8 Hz) showed correlations with carbons at δ_C 62.1 (C-2), 193.5 (C-4), 177.9 (C-16), and 177.9 (C-19). The planar structure of **1** was thus established as shown in Fig. 1. Compound **1** has two chirality centers, implying four possible stereoisomers, and the relative and absolute configurations of **1** were deduced by comparison of its NMR and CD data (Fig. 4) with those of brocaeloid A (**2**). The CD spectra of **1** and **2** both showed a positive Cotton effect at 384 nm and a negative Cotton effect at 323 nm, suggesting a 2*S* and 3*R* configurations for C-2 and C-3, respectively, of both **1** and **2**. All the signals of **1** were assigned unambiguously by 1D and 2D NMR spectra (Figs. S1–8; Table 1).

The structures of six known compounds, brocaeloid A (**2**) [37], brocaeloid B (**3**) [37], cillifuranone (**4**) [38], leptosphaeric acid (**5**) [39], 15β-hydroxyl-(22*E*, 24*R*)-ergosta-3,5,8,22-tetraen-one (**6**) [40], and (22*E*, 24*R*)-6β-methoxyergosta-7,22-diene-3β,5α-diol (**7**) [41], were determined based on their HRESIMS, ^{1}H NMR, and ^{13}C NMR data (Figs. S9–29) and by comparison with those of previous reports.

3.3. Biological activity

The bioactivities of these compounds were evaluated against *S. aureus*, MRSA, *M. tuberculosis* H37Rv (MTB), *C. albicans*, HUH-7 human hepatoma cell, HeLa cell, and A549 lung carcinoma cell. As shown in Table 2, compounds **1–5** showed activity against HUH-7 human hepatoma cell with IC_{50} values at 80 μg/mL, but did not show activity against other tested pathogenic microorganisms or cancer cell lines. Compound **6** was active against HeLa cells with IC_{50} value of 51.9 μg/mL. In addition, **6** also showed weak anti-MTB with an MIC value at 80 μg/mL. Among all these isolated compounds, **6** showed moderate anti-MRSA activity with an MIC value of 25 μg/mL, while **7** showed weak anti-MRSA activity (MIC = 100 μg/mL).

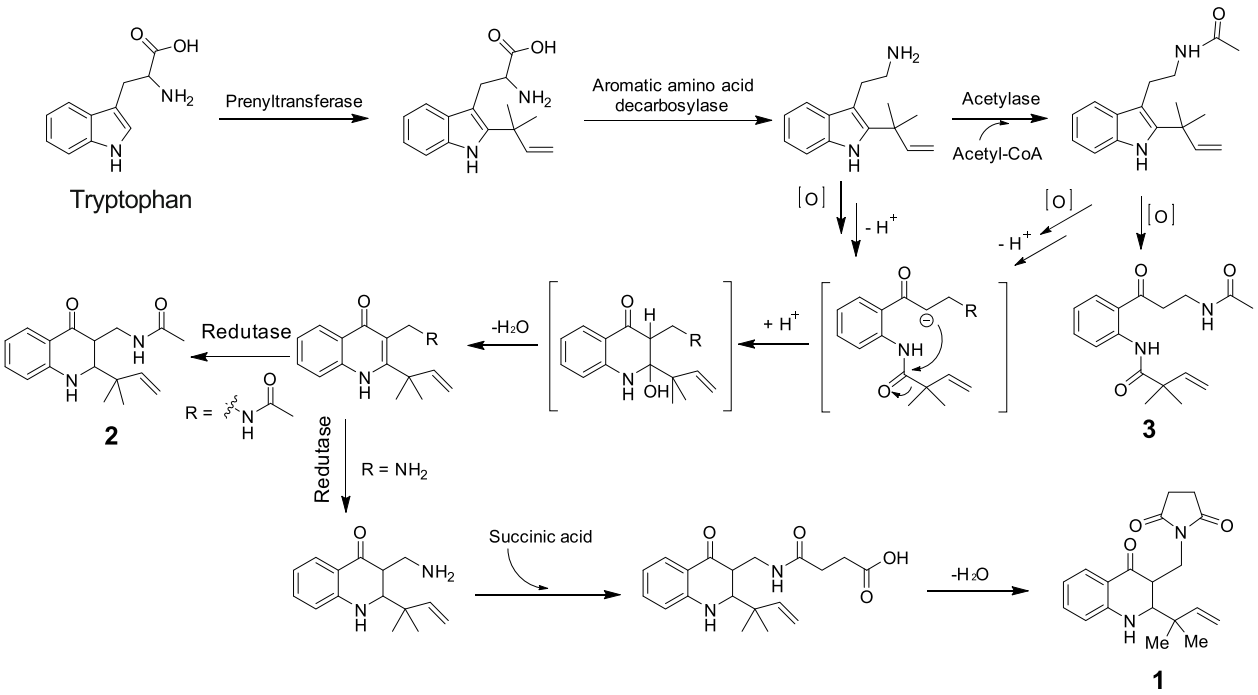
4. Discussion and conclusion

The classical methods for natural product discovery have mostly relied on crude extracts from cultured isolates followed by successive rounds of bioactivity-guided fractionation and identification of the purified compounds for further development into potent drugs [45]. However, because of the de-replication of known compounds, the discovery ratio of new natural products (NPs) from microorganisms has decreased in recent years. Thus, finding new strategies to overcome the reduction of NPs and subsequently discovery of new bioactivities is a major research challenge. Attention to discovery of novel biologically active natural products has shifted from terrestrial microbes to poorly explored microbial resources, such as deep sea marine habitats [15,27], deserts [25,46], endophytes, and other microbial associates of uncommon plant species [23,24,47]. Over more than a decade, endophytic fungi have been explored as “biofactories” of novel bioactive

Table 2
In vitro cytotoxicity of compounds **1–7**.

Organism	Minimum inhibitory concentration (μg/mL) for pathogens and IC_{50} (μg/mL) for cell lines							
	Control	1	2	3	4	5	6	7
MTB[d]	0.025[a]	> 80	> 80	> 80	> 80	> 80	80	> 80
MRSA[e]	0.625[b]	> 100	> 100	> 100	> 100	> 100	25.0	100
SA[f]	0.625[b]	> 100	> 100	> 100	> 100	> 100	100	> 100
CA[g]	0.016[c]	> 100	> 100	> 100	> 100	> 100	100	> 100
HUH-7 cell	NT[h]	80	80	80	80	80	> 100	> 100
HeLa cell	NT[h]	> 100	> 100	> 100	> 100	> 100	51.9	> 80
A549 cell	NT[h]	> 100	> 80	> 100	> 80	> 80	> 80	> 80

[a] Isoniazid.
[b] Vancomycins.
[c] Ketoconazole.
[d] *Mycobacterium tuberculosis* H37Rv (ATCC 27294).
[e] MRSA, Methicillin-resistant *Staphylococcus aureus* (clinical isolate 309).
[f] *Staphyloccocus aureus* (ATCC 6538).
[g] *Candida albicans* (SC5314).
[h] Not tested.



Scheme 1. Plausible biosynthetic pathways for compounds **1–3**.

compounds, exerting antibacterial activity ranging from moderate to powerful against bacterial strains resistant to currently used antibiotics [48]. Phytopathogens may also be a source of novel therapeutic compounds because they produce secondary metabolites in their battle against the plant host, as well as compounds to fend off competing micro-organisms.

Many classes of active compounds have been isolated from the genus *Microdochium*, such as antifungal and antibacterial monocerin and isocoumarin derivatives, antifungal and antialgal isocoumarin derivatives [49], chlorofusin [50], and human toxins [51]. However, investigation of the chemical and biological capacities of this genus and their effects on human diseases has yet to be uncovered. In addition, exponentially increasing activity on fungal genome sequencing and bioinformatics analyses of the secondary metabolites from fungi have revealed significant potentials leads for mining novel natural products from this source [52,53].

Our study on the wheat pathogen *M. majus* isolate 99049 extends the range of bioactive chemical entities from the genus *Microdochium*. Compound **1** belongs to the 4-oxoquinoline family with C-2 reversed prenylation, which contains a succinimide substructure [37]. Compound **1** and its biogenetic analogs (**2** and **3**) are probably generated by a common biosynthesis pathway from a tryptophan precursor (Scheme 1) [37,54]. However, compound **1** with the succinimide substructure has not yet been reported and the introduction of one molecule, succinic acid, onto the intermediate is proposed, followed by dehydrogenation to give the final product **1** (Scheme 1). In conclusion, phytopathogenic fungi are potential source of novel compounds with potential new biosynthetic pathways. In the future, more attention should be paid to discovery of novel compounds from such fungi, and the results obtained suggest that compounds **1–5** possess anti-cancer activity which should be explored further for drug development.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was partially supported by the grants from the National Natural Science Foundation of China (31430002, 81573341, 21877038, 31720103901, 31320103911), Taishan Scholarship, Open Project Funding of the State Key Laboratory of Bioreactor Engineering, the 111 Project (B18022), National Key R&D Program of China 2017YFE0108200, and the Fundamental Research Funds for the Central Universities (22221818014). Discovery and isolation of *Microdochium majus* strain 99049 was supported by the Natural Sciences and Engineering Research Council of Canada funding to T. Hsiang.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2019.09.001.

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