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Microsphaerins A–D, four novel benzophenone dimers with activity against MRSA from the fungus *Microsphaeropsis* sp.

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ABSTRACT

Bioassay-guided isolation using an MRSA whole cell assay yielded four novel benzophenone dimers, microsphaerins A–D (**1**–**4**), from two Singapore isolates of the soil anamorph *Microsphaeropsis* sp. The structures of **1**–**4** were elucidated on the basis of spectral data and the structures of **1** and **4** were confirmed by X-ray crystallographic analyses.

1. Introduction

Over the last 20 years, microbial antibiotic resistance has become a major human health problem. This was exemplified in a recent report that indicated 1598 out of 8987 people infected with methicillin-resistant *Staphylococcus aureus* (MRSA) died in U.S. hospitals from July 2004 to December 2005.[1] In addition, it was shown that MRSA is rapidly moving from a hospital setting toward a community based disease.[1,2] Hence, there is a urgent need to discover new classes of antibiotics with novel mechanisms of action to treat multi-drug resistant bacterial infections,[3–5] which has coincided with many pharmaceutical companies reducing or ceasing their antimicrobial research and development efforts.[6,7] Finding novel antibiotics suitable for drug development is difficult, and this is demonstrated by a recent review that describes only a limited success identifying novel antibacterial leads using a genomics based platform.[8] Although there are still a significant number of compounds undergoing antibacterial clinical evaluations, most are based upon known antibiotic classes with established mechanisms of action.[9–12]

As part of our search for novel antibacterial agents with activity against MRSA, two strains of the anamorphic fungus *Microsphaeropsis*, F2076 and F2078, which were collected in Singapore from same lake sediment, were found to be active in a broth based high-throughput assay. Bioassay-guided isolation led to the identification of four novel benzophenone dimers, microsphaerins A–D (**1**–**4**) that displayed antibacterial activities against MRSA in the low μM range.

2. Structure elucidation of microsphaerins A–D (1–4)

The mycelial-derived MeOH extracts from 10 L fermentations of *Microsphaeropsis* sp. (strains F2076 and F2078) were separated by solvent partition and preparative C_{18} reversed-phase HPLC. Microsphaerin B (**2**) (250 mg), microsphaerin C (**3**) (12 mg), and microsphaerin D (**4**) (3 mg) were isolated from F2076, while microsphaerin A (**1**) (1 g) and microsphaerin D (**4**) (200 mg) were isolated from F2078.

Microsphaerin A (**1**) was obtained as a yellow, crystalline solid that displayed absorption maxima at λ_{max} 348, 288, and 209 in the UV spectrum, which indicated the presence of an extended chromophore. The molecular formula of **1**, $C_{30}H_{26}O_{10}$, was established from the quasimolecular ion peak at *m*/*z* 545.1445 $[M-H]^-$ in the (−)-HRESIMS. The ^{13}C NMR spectrum of **1** (Table 1) in CD_3OD

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Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of microsphaerins A (**1**) and B (**4**) in CD_3OD, and microsphaerins C (**2**) and D (**3**) in $CDCl_3$

No.	**1**		**2**		**3**		**4**	
	^{13}C δ	^{1}H δ, m, J	^{13}C δ	^{1}H δ, m, J	^{13}C δ	^{1}H δ, m, J	^{13}C δ	^{1}H δ, m, J
1	142.9	—	172.8	—	170.1	—	141.0	—
1-OH				14.73, s		13.82, s		
2	126.5	—	48.7	3.50, d, 9	50.5	3.41, d, 9	129.0	—
3	117.5	6.70, s	46.6	3.77, d, 9	52.0	4.07, d, 9	131.1	—
4	150.6	—	204.8	—	202.6	—	147.9	—
5	121.9	—	80.7		79.6	—	122.5	—
5-CH_3	11.6	2.01, s	26.0	1.59, s	27.3	1.71, s	12.2	1.93, s
6	136.4	—	106.0	—	107.4	—	135.3	—
7	203.3	—	187.6	—	189.5	—	203.6	—
8	110.8	—	104.5	—	105.7	—	110.7	—
9	163.8	—	157.9	—	159.1	—	163.9	—
10	109.0	6.15, s	110.7	6.43, br s	111.0	6.45, br s	108.9	6.11, s
11	150.5	—	152.7	—	152.6	—	150.7	—
11-CH_3	22.1	2.19, s	23.5	2.31, s	23.5	2.32, s	22.2	2.18, s
12	109.0	6.15, s	112.1	6.39, br s	110.7	6.38, br s	108.9	6.11, s
13	163.8	—	162.9	—	159.1	—	163.9	—
13-OH				11.10, s		11.18, s		
1′	142.9	—	175.2	—	175.3	—	183.3	—
1′-OH				13.00, s		12.97, s		
2′	126.5	—	41.9	3.54, d, 4	45.1	3.32, d, 4	43.9	3.85, d, 4
3′	117.5	6.70, s	37.8	1.89, dd, 4, 13, 2.35, d, 13	36.7	2.17, dd, 4, 13, 2.39, d, 13	47.9	2.45, dd, 4, 11, 2.61, d, 11
4′	150.6	—	84.0	—	84.4	—	85.6	—
4′-OH				4.57, s		2.93, s		
5′	121.9	—	85.4	—	84.6	—	83.9	—
5′-CH_3	11.6	2.01, s	21.9	1.66, s	22.6	1.64, s	21.3	1.73, s
6′	136.4	—	106.5	—	105.3	—	104.7	—
7′	203.3	—	188.9	—	188.5	—	187.3	—
8′	110.8	—	105.9	—	105.9	—	106.0	—
9′	163.8	—	159.5	—	158.2	—	159.7	—
10′	109.0	6.15, s	112.1	6.37, s	112.1	6.38, s	110.5	6.22, s
11′	150.5	—	151.6	—	151.6	—	150.6	—
11′-CH_3	22.1	2.19, s	23.6	2.31, s	23.6	2.30, s	22.3	2.23, s
12′	109.0	6.15, s	111.9	6.37, br s	112.0	6.38, br s	110.9	6.26, s
13′	163.8	—	163.1	—	163.1	—	162.5	—
13′-OH				11.21, br s		11.21, br s		

displayed 15 carbon signals, corresponding to 1 carbonyl, 9 quaternary aromatic carbons, 3 aromatic methines, and 2 aromatic methyl groups, while the ^{1}H NMR spectrum of **1** showed signals for two aromatic methyl (δ_H 2.01 and 2.19) and three aromatic methines (δ_H 6.15, 2H, s and 6.70, 1H, s). These data indicated that microsphaerin A (**1**) had an axis of symmetry. Analysis of the ^{1}H NMR, ^{13}C NMR, COSY, HSQC, and HMBC experiments showed the presence of a benzophenone unit. Connection of the functional groups in **1** was achieved on the basis of HMBC correlations from H-10 to C-7, C-8 and C-9, from H-12 to C-7, C-8 and C-10, from H-3 to C-1, C-2 and C-5, and from 5-CH_3 to C-4, C-5 and C-6, in establishing the benzophenone unit with attachment through either C-2 or C-3. An HMBC correlation between H-3 and C-5 suggested that the benzophenone units were joined through C-2 and an X-ray analysis[§] of crystals of microsphaerin A (**1**) grown from mixtures of $CHCl_3$ and MeOH confirmed the proposed structure (Fig. 1).

The molecular formula of microsphaerin B (**2**), $C_{30}H_{26}O_{10}$, which was determined by HRESIMS, was identical with microsphaerin A (**1**). However, the ^{13}C NMR spectrum of **2** displayed 30 signals that was assigned to 4 methyl, 1 methylene, 3 methine, and 4 aromatic methine groups, using a multiplicity-edited HSQC experiment,

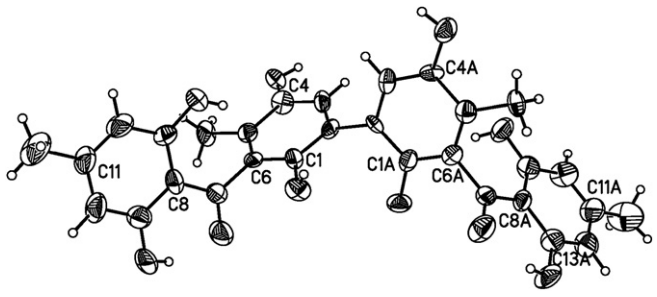
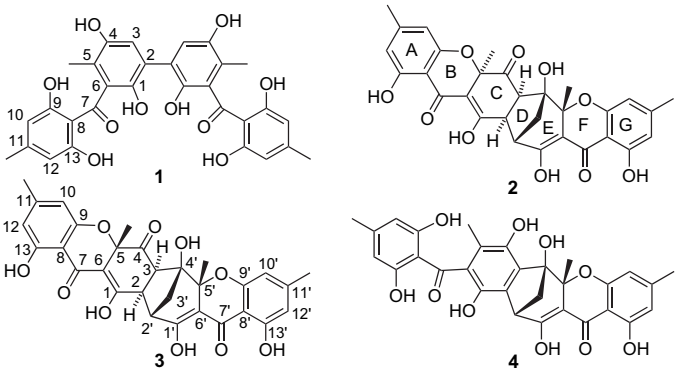


Figure 1. The ORTEP drawings of microsphaerin A (**1**) with thermal ellipsoids drawn at 50% probability.



[§] The X-ray structures of microsphaerin A (**1**) and D (**4**) have rather large *R*-factors and, as a consequence, care is needed when using these data for molecular modeling purposes.

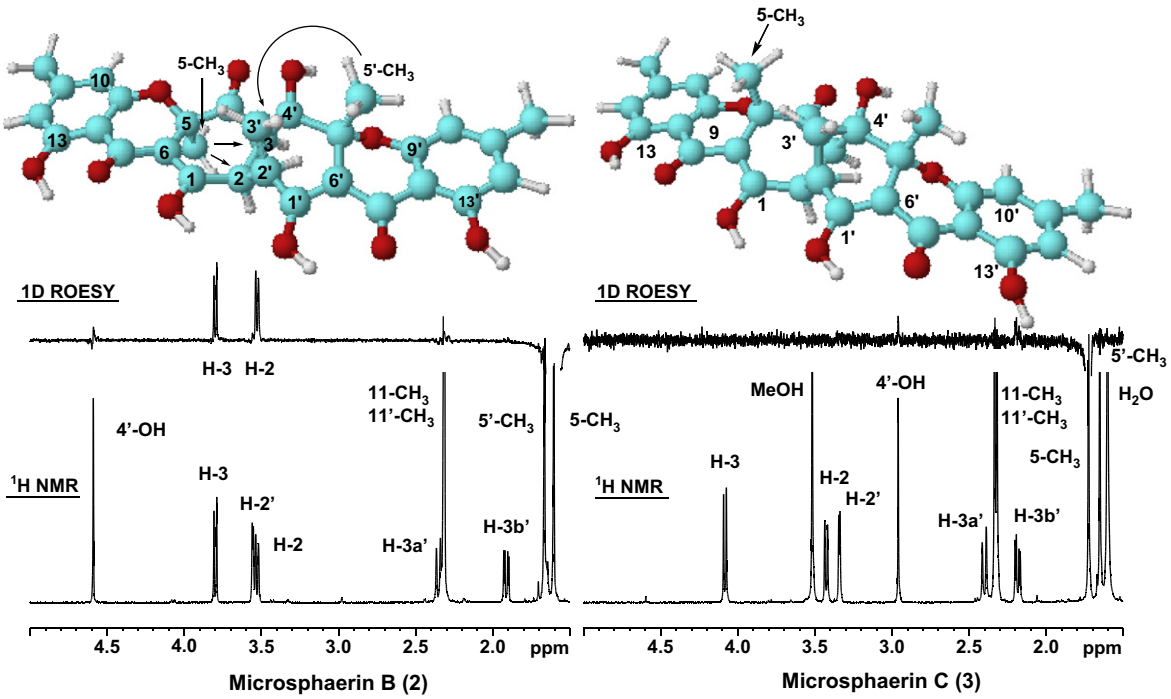


Figure 2. Structures, 1D ROESY spectra (selective refocusing of 5-CH_3) and ^{1}H NMR spectra of microsphaerins B (**2**) and C (**3**) (note: small amounts of equilibrium product in ^{1}H NMR spectra).

while the remaining 18 were assigned as 15 sp^2 and 3 sp^3 hybridized quaternary carbons (Table 1). The ^{1}H and COSY spectra indicated the presence of a CH–CH group (δ_H 3.50, d, J 9 Hz, 3.77, d, J 9 Hz), a CH_2–CH group (δ_H 1.89, dd, J 4, 13 Hz, 2.35, d, J 13 Hz, 3.54, d, J 4 Hz), four methyl singlets (δ_H 1.59, 1.66, 2.31, and 2.31) and two 1,3,4,5-tetrasubstituted phenyl groups (δ_H 6.37, s, 2H; 6.39, s, 1H; 6.43, s, 1H) (Table 1). In addition, five hydroxyl resonances were observed in the ^{1}H NMR spectrum of **2**, which were assigned as two chelated enols (δ_H 14.73, s; 13.00, s), two phenolic protons (δ_H 11.21, br s; 11.10, s), and one hydroxyl group (δ_H 4.57, s). HMBC correlations from 5′-CH_3 to C-4′, C-5′ and C-6′, 5-CH_3 to C-4, C-5 and C-6, and 1-OH to C-1, C-2 and C-6 established the structure for the C, D, and E rings. The structure of the A and G rings was determined to be 1,3,4,5-tetrasubstituted benzenes using $^2J_{CH}$ and $^3J_{CH}$ HMBC correlations from 13-OH, 13′-OH, H-10, H-10′, H-12, H-12′, 11-CH_3, and 11′-CH_3 to carbon resonances in this system. The $^4J_{CH}$ HMBC correlations observed from H-10 and H-10′ to carbonyl resonances at δ_C 187.6 (C-7) and 188.9 (C-7′), respectively, required C-7 and C-7′ keto linkages between C-6/C-8 and C-6′/C-8′. The ^{13}C NMR shifts assigned to C-5 (δ_C 80.7), C-5′ (δ_C 85.4), C-9 (δ_C 157.9), and C-9′ (δ_C 159.5) were consistent with ether linkages between C-5/C-9 and C-5′/C-9′ and the planar structure of **2** was determined. The relative stereochemistry of microsphaerin B (**2**) was determined by a combination of ^{1}H/^{1}H coupling constant analysis and 1D ROESY NMR experiments. The key piece of evidence permitting the establishment of the relative stereochemistry between H-2, H-3, H-2′, H-3′$_{a,b}$, and 4′-OH was the observation that H-2 had a 9 Hz coupling with H-3 but no coupling with H-2′, which requires dihedral angles of approximately 90° and 0° between H-2/H-2′ and H-2/H-3, respectively. That is only possible if there is a cis-ring junction between rings C and D and α orientations were arbitrarily assigned to H-2 and H-3. As a consequence of the cis-orientation of the C and D ring junction, the 4′-OH group must be in an α orientation for the D and E rings to be joined as a bicyclo[3.2.1]oct-2-ene ring system. The 5′-CH_3 was proposed to have a β orientation as no ROESY interactions were observed between 5′-CH_3 and H-2 and H-3, which also is consistent with the relative configuration proposed for **3**. ROESY interactions between 5-CH_3 and H-2 and H-3 (Fig. 2) indicated that 5-CH_3 was in the α orientation and the relative configuration of **2** was determined as shown.

Microsphaerin C (**3**) was obtained as an optically active, yellow powder, which had the same molecular formula ($C_{30}H_{26}O_{10}$) as **1** and **2**. Examination of the ^{1}H, ^{13}C, COSY, multiplicity-edited HSQC, and HMBC NMR spectra of **3** indicated that both **2** and **3** had the same planar structure. The ^{1}H/^{1}H coupling constants of H-2, H-3, H-2′, and H-3′ in **3** were identical to **2** and the lack of ROESY

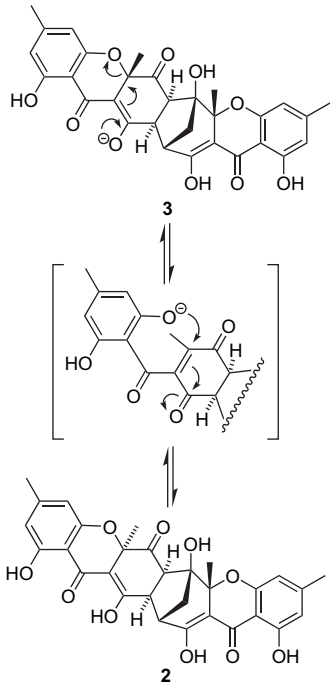


Figure 3. Proposed mechanism for interconversion of microsphaerins B (**2**) and C (**3**).

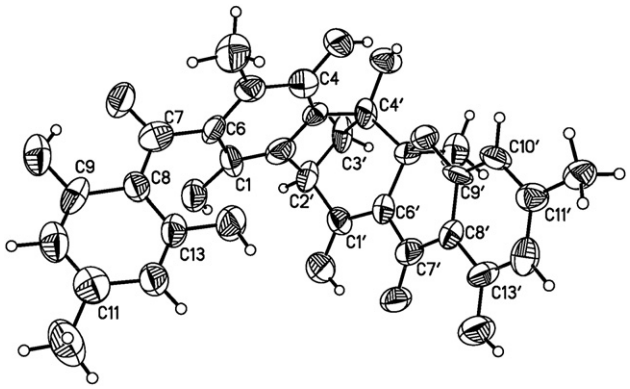


Figure 4. The ORTEP drawings of microsphaerin D (**4**) with thermal ellipsoids drawn at 50% probability.

interactions from 5′-CH_3 suggested that the relative stereochemistry of the D and E rings was the same for **2** and **3**. Weak ROESY interactions were observed for microsphaerin C (**3**) from 5-CH_3 to H-3b' and 4'-OH (Fig. 2) that suggested 5-CH_3 had a β orientation, which was the opposite of **2**.

Microsphaerin B (**2**) and microsphaerin C (**3**) were found to slowly interconvert. The interconversion of **2** and **3** (Fig. 3) most likely proceeds in an analogous way to that proposed for the beticolins[13] and other compounds such as the recently reported antifungal parnafungins.[14]

Microsphaerin D (**4**) was obtained as yellow, monoclinic crystals and had the same molecular formula as **1**–**3**. The analysis of the ^1H and ^{13}C NMR spectra (Table 1) using COSY, multiplicity-edited HSQC, and HMBC allowed full assignment of the spectral data of **4** and revealed the presence of two fused monomeric moieties. The first was a benzophenone system similar to that present in **1**, while the other moiety present in **4** was reminiscent of the D, E, F, and G rings of **2** and **3** except for the double bond between C-2 and C-3. A crystal was obtained in the mixed solvent of MeOH containing $CHCl_3$ and the proposed structure was confirmed by single-crystal X-ray crystallographic analysis (Fig. 4). The crystal structure also allowed the relative configuration of the D and E rings to be determined, which was consistent with that proposed for **2** and **3**.

3. Biosynthetic considerations

Microsphaerins A–D (**1**–**4**) are related in structure to the xanthoquinodins (e.g., xanthoquinodin B2 (**5**) and A1 (**6**)),[15,16] beticolins (e.g., beticolin 1 (**7**) and 6 (**8**)),[13,17,18] and acremonidins (e.g., acremonidin B (**9**)).[19] The microsphaerins could be formed by dimerization of two benzophenone units to form microsphaerin A (**1**), which undergoes further cyclization to form tetrahydroxanthones in the cases of **2**, **3**, and **4**. The xanthoquinodins and beticolins are most likely formed from dimerization of diversonolic ester (**10**)[20] and an anthraquinone such as helminthrosporin (**11**),[21] while acremonidins are most likely formed from dimerization of the benzophenone acremeonidin E (**12**) and helminthrosporin (**11**). The absolute configuration of microsphaerins B–D (**2**–**4**) was not been determined in this study and only the absolute configuration of beticolins has been determined to date using X-ray crystallography.[13] It is interesting to note that two morphologically identical fungi obtained from the same lake sediment produced different compounds using identical fermentation conditions.

4. Biological activities of microsphaerin A–D (1–4)

Microsphaerins A–D (**1**–**4**) had IC_{90} values 3, 3, 5, and 1 μM, respectively, when measured in an MRSA whole cell assay and **4** was chosen for further profiling against Gram-positive, Gram-negative bacteria, and cell lines (Table 2). Microsphaerin D (**4**) displayed good activity against various Gram-positive bacteria but was inactive against Gram-negative bacteria except for *Klebsiella pneumoniae*. However, **4** displayed only a 10-fold window of antibacterial activity compared to cytotoxicity and, as a consequence, no further development as an antibacterial agent was undertaken.

5. Experimental

5.1. General experimental procedures

Optical rotations were recorded on a JASCO DIP-1000 digital polarimeter. UV spectra were obtained on a Pharmacia Biotech Ultrospec 2000 and IR spectra on a Perkin–Elmer BioRad FTIR spectrophotometer using single reflection Diamond ATR crystal plates. NMR spectra were collected on a Bruker Avance DRX-500 NMR spectrometer, using 5-mm BBI (^1H, G-COSY, multiplicity-

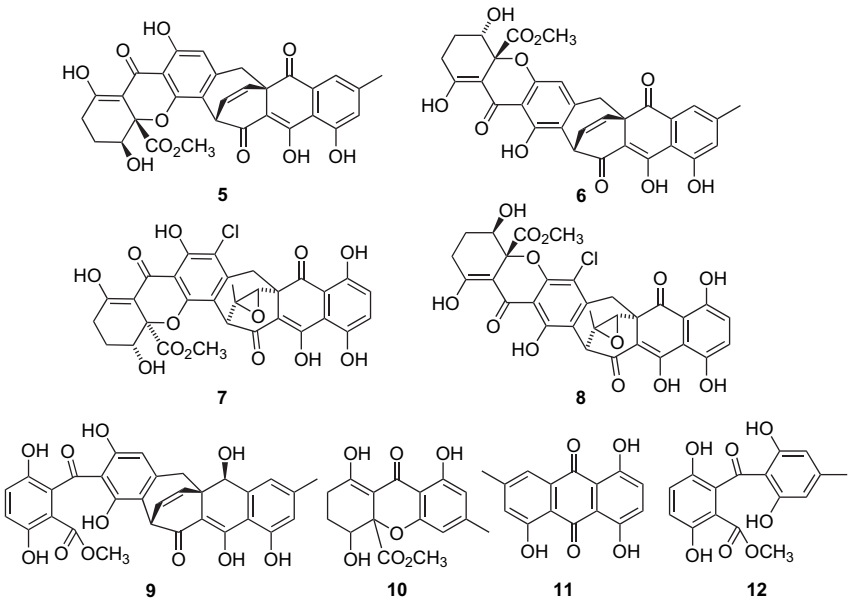


Table 2
Antibacterial and cytotoxicity biological activity of microsphaerin D (**4**)

Screen type	Screen	Activity[a] (μM)
Gram-positive bacteria	*Staphylococcus aureus* (ATCC 25923)	1.3
	MRSA (ATCC 33591)	1.0
	Enterococcus faecalis (ATCC 51299)	1.3
	Streptococcus pneumoniae (ATCC 49619)	3.6
	Bacillus subtilis (ATCC 6633)	3.0
Gram-negative bacteria	*Escherichia coli* (ATCC 25922)	>50
	Klebsiella pneumoniae (ATCC 10031)	>50
	Moraxella catarrhalis (ATCC 49143)	1.3
	Haemophilus influenzae (ATCC 49247)	>50
	Pseudomonas aurogenosa (ATCC 27853)	>50
Mammalian cells	Chinese hamster ovary cells (CHO)	9
	Hepatoblastoma cell line (HepG2)	25
	Human lung fibroblasts (MRC5)	13
	Human embryonic kidney cells (HEK293)	20

[a] IC_{90} for bacterial assays and IC_{50} for mammalian cell assays. All assays were run in triplicate.

edited G-HSQC, and G-HMBC spectra) or BBO (^{13}C spectra) probeheads equipped with z-gradients. Spectra were calibrated to residual protonated solvent signals (CD_2HOD δ_H 3.30 and CD_3OD δ_C 49.0; $CHCl_3$ δ_H 7.27 and $CDCl_3$ δ_C 78.0). HRESIMS values were collected on a Bruker Daltonics-micrOTOF-Q mass spectrometer, using sodium trifluoroacetate as an internal standard. Preparative HPLC was performed on a Gilson system complete with UniPoint software, 170 DAD detector, dual 306 pumps, 811C dynamic mixer, Gilson 202 fraction collector, and a Rheodyne 7125 injector with a 5 mL injection loop. The HPLC column was a Waters C_{18} Novapak cartridge column (40×100 mm) and solvent A was 0.1% HCOOH in CH_3CN and solvent B was 0.1% HCOOH in H_2O.

5.2. Fungal isolation, taxonomy, and fermentation

5.2.1. Fungal isolation and characterization

Both strains were isolated from lake sediment in Singapore and have been deposited in the MerLion Pharmaceuticals culture collection as F2076 and F2078. The strains were grown on MEA and sporulated profusely within 10–15 days of inoculation. Morphological characterization was undertaken using dissected pycnidia, mounted in lactoglycerol or water and observed using an Olympus BX50 microscope. Both strains were identified initially as members of the anamorphic genus *Chaetophoma*. However, due to the difficulty of comparing taxonomic characters derived from strains growing on agar with the literature based on characters from natural substrates, molecular characterization also was undertaken in an attempt to assist phylogenetic placement.

5.2.2. LSU rDNA sequencing

DNA was extracted from the fungal cells by placing approximately a loopful of aerial hyphae (with at least one pycnidia and associated conidia) into a nuclease free, microcentrifuge tube containing 200 μL of PrepMan Ultra sample preparation reagent (Applied Biosystems). Tubes were vortexed for 10–15 s and placed in a heating block for 10 min at 100 °C. Samples were centrifuged at 14,000 rpm for 3 min and 150 mL was transferred to a new tube and stored at −20 °C for future use. Prior to storage a 1:100 dilution working stock was prepared.

PCR was used to amplify the DNA preparation by combining 15 μL of diluted genomic DNA with 15 μL of MasterMix (Applied Biosystems) and undertaken using the following conditions: initial denaturation 95 °C for 10 min; 35 cycles of 95 °C for 10 s, 53 °C for 30 s, 72 °C for 1 min; final extension at 72 °C for 10 min. The presence of PCR products was determined using 2% agarose gel containing SBYR Safe (Invitrogen Life Technologies) as the staining agent. The PCR products were treated with ExoSap-IT (USB) to remove excess primers and nucleotides with an enzyme activation step of 37 °C for 15 min, followed by an inactivation step at 80 °C for 15 min.

Next, cycle sequencing was performed using MicroSeq sequencing module (forward and reverse modules) using the following conditions: 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min. The prepared DNA was washed with sodium acetate and ethanol to remove dyes, air dried and sequenced by a third party (first base) using an ABI Prism Big Dye Terminator Cycle Sequencing Kit (Perkin–Elmer).

5.2.3. Sequence data analysis

Sequences generated from the two strains (F2076 and F2078) were used to perform a BLAST search to determine if possible taxonomic matches were present in the GenBank database. Prior to phylogenetic analysis ambiguous positions at the beginning and end of the sequences were eliminated to optimize alignment. Whilst the GenBank search did not provide any matches with a high degree of sequence similarity, the sequences with the greatest similarity were within or consistent with the ascomycete order Pleosporales. One of the BLAST matches was a strain identified as *Microsphaeropsis arundinis* (Ahmad) Sutton, which has a sequence similarity with our strains of 94%. Whilst these strains are unlikely to be conspecific with *M. arundinis*, the level of sequence similarity, combined with appropriate morphology,[22] makes *Microsphaeropsis* an appropriate generic placement. Further molecular work would be needed to confirm the taxonomy, including comparison with numerous type strains, but this is beyond the scope of the current research.

5.2.4. Fermentation

F2076 and F2078 were sub-cultured on malt extract agar (CM057B, Oxoid) for 7 days at 24 °C. This was used to inoculate 250 mL Erlenmeyer flasks each containing 50 mL of seed medium composed of 0.4% glucose, 1% malt extract, and 0.4% yeast extract. The pH of the medium was adjusted to 5.5 prior to sterilization and autoclaved at 121 °C for 30 min. The seed flasks were incubated for 5 days at 24 °C on a rotary shaker with a 50 mm orbit, at 200 rpm. Seed culture (5 mL) was used to inoculate 50 mL of liquid medium in a 250 mL flask. The liquid medium was composed of 0.4% yeast extract, 2% glucose, and 2% oatmeal, the pH was adjusted to 7.5 prior to sterilization and autoclaved at 121 °C for 30 min. The fermentation was carried out for 9 days at 24 °C, on an orbital shaker with a 50 mm orbit at 200 rpm.

5.3. Extraction and isolation

The culture broths (200×50 mL, total 10 L) of *Microsphaeropsis* sp. (F2076) were combined and centrifuged to separate the supernatant and the mycelia. The combined mycelia were shaken overnight two times with MeOH (2 L), filtered and an equal volume of CH_2Cl_2 and H_2O was added to the MeOH. The organic layer from the CH_2Cl_2/H_2O/MeOH (1:1:1) solvent partition was evaporated to dryness using rotary evaporation and separated by C_{18} reversed-phase preparative HPLC (gradient elution: solvent A/B 55:45→60:40 over 40 min, 60:40→70:30 over 30 min and 70:30→100:0 over 20 min; flow rate 12 mL/min) to give microsphaerin D (**4**) (3 mg), microsphaerin C (**3**) (12 mg), and microsphaerin B (**2**) (250 mg). The mycelia from a 10 L fermentation of *Microsphaeropsis* sp. (F2078) was extracted and separated in an identical manner to give microsphaerin A (**1**) (1 g) and microsphaerin D (**4**) (200 mg).

*5.3.1. Microsphaerin A (**1**)*

Yellow, monoclinic crystals; UV (MeOH) λ_{max} (log ε) 209 (4.88), 288 (4.64), and 348 (3.97) nm; IR (Diamond ATR) ν_{max} cm^{-1} 1621, 1576; ^1H NMR (CD_3OD, 500 MHz) δ_H 6.70 (2H, s, H-3, H-3′), 6.15 (4H, s, H-10, H-12, H-10′, H-12′), 2.19 (6H, s, 11-CH_3, 11′-CH_3), 2.01 (6H, s, 5-CH_3, 5′-CH_3); ^{13}C NMR (CD_3OD, 125 MHz) δ_C 203.3 (C-7, C-7′), 163.8 (C-9, C-9′, C-13, C-13′), 150.6 (C-4, C-4′), 150.5 (C-11, C-11′), 142.9 (C-1, C-1′), 136.4 (C-6, C-6′), 126.5 (C-2, C-2′), 121.9 (C-5, C-5′), 117.5 (C-3, C-3′), 110.8 (C-8, C-8′), 109.0 (C-10, C-10′, C-12, C-12′), 22.1 (11-CH_3, 11′-CH_3), 11.6 (5-CH_3, 5-CH_3); HRMS ((−)-ESI): M−H^-, found: 545.1445. $C_{30}H_{25}O_{10}$ requires 545.1447. Crystal data of microsphaerin A dihydrate: $C_{30}H_{26}O_{10}\cdot 2(O)$, M=578.51, monoclinic, space group $C2/c$, a=21.586(4), b=12.776(3), c=12.620(3), β=95.11(3), V=3466.4(12) Å^3, Z=4, D_c=1.109 g cm^{-3}, T=180 K, F(000)=1208, λ=0.71073 Å, μ=0.087 mm^{-1}, $R1$=0.2719 for 2717 $F_o>2\sigma(F_o)$.

*5.3.2. Microsphaerin B (**2**)*

Yellow powder; $[\alpha]_D$ +226 (c 1.44, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 209 (4.65), 284 (4.03), and 342 (4.54) nm; IR (Diamond ATR) ν_{max} cm^{-1} 1718, 1644, 1615, 1576; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 14.73 (1H, s, 1-O*H*), 13.00 (1H, s, 1′-O*H*), 11.21 (1H, br s, 13′-O*H*), 11.10 (1H, s, 13-O*H*), 6.43 (1H, br s, H-10), 6.39 (1H, br s, H-12), 6.37 (2H, s, H-10′, H-12′), 4.57 (1H, s, 4′-O*H*), 3.77 (1H, d, J 9 Hz, H-3), 3.54 (1H, d, J 4 Hz, H-2′), 3.50 (1H, d, J 9 Hz, H-2), 2.35 (1H, d, J 13 Hz, H-3′$_b$), 2.31 (3H, s, 11-CH_3), 2.31 (3H, s, 11″-CH_3), 1.89 (1H, dd, J 13, 4 Hz, H-3′$_a$), 1.66 (3H, s, 5′-CH_3), 1.59 (3H, s, 5-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 204.8 (C-4), 188.9 (C-7′), 187.6 (C-7), 175.2 (C-1′), 172.8 (C-1), 163.1 (C-13′), 162.9 (C-13), 159.5 (C-9′), 157.9 (C-9), 152.7 (C-11), 151.6 (C-11′), 112.1 (C-12), 112.1 (C-10′), 111.9 (C-12′), 110.7 (C-10), 106.5 (C-6′), 106.0 (C-6), 105.9 (C-8′), 104.5 (C-8), 85.4 (C-5′), 84.0 (C-4′), 80.7 (C-5), 48.7 (C-2), 46.6 (C-3), 41.9 (C-2′), 37.8 (C-3′), 26.0 (5-CH_3), 23.6 (11′-CH_3), 23.5 (11-CH_3), 21.9 (5′-CH_3); HRMS ((−)-ESI): M−H^-, found: 545.1436. $C_{30}H_{25}O_{10}$ requires 545.1447.

*5.3.3. Microsphaerin C (**3**)*

Yellow powder; $[\alpha]_D$ +194 (c 0.125, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 209 (4.67), 284 (4.07), and 342 (4.53) nm; IR (Diamond ATR) ν_{max} cm^{-1} 1721, 1644, 1621, 1576; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 13.82 (1H, s, 1-O*H*), 12.97 (1H, s, 1′-O*H*), 11.18 (1H, s, 13-O*H*), 6.45 (1H, br s, H-10), 6.38 (1H, s, H-10′), 6.38 (1H, br s, H-12), 6.38 (1H, br s, H-12′), 4.07 (1H, d, J 9 Hz, H-3), 3.41 (1H, d, J 9 Hz, H-2), 3.32 (1H, d, J 4 Hz, H-2′), 2.93 (1H, s, 4′-O*H*), 2.39 (1H, d, J 13 Hz, H-3′$_b$), 2.32 (3H, s, 11-CH_3), 2.30 (3H, s, 11′-CH_3), 2.17 (1H, dd, J 4, 13 Hz, H-3′$_a$), 1.71 (3H, s, 5-CH_3), 1.64 (3H, s, 5′-CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 202.6 (C-4), 189.5 (C-7), 188.5 (C-7′), 175.3 (C-1′), 170.1 (C-1), 163.1 (C-13′), 159.1 (C-9, C-13), 158.2 (C-9′), 152.6 (C-11), 151.6 (C-11′), 112.1 (C-10′), 112.0 (C-12′), 111.0 (C-10), 110.7 (C-12), 107.4 (C-6), 105.9 (C-8′), 105.7 (C-8), 105.3 (C-6′), 84.6 (C-5′), 84.4 (C-4′), 79.6 (C-5), 52.0 (C-3), 50.5 (C-2), 45.1 (C-2′), 36.7 (C-3′), 27.3 (5-CH_3), 23.6 (1′-CH_3), 23.5 (11-CH_3), 22.6 (5′-CH_3); HRMS ((−)-ESI): M−H^-, found: 545.1438. $C_{30}H_{25}O_{10}$ requires 545.1447.

*5.3.4. Microsphaerin D (**4**)*

Yellow monoclinic crystals; $[\alpha]_D$ −457 (c 0.49, MeOH); UV (MeOH) λ_{max} (log ε) 209 (4.76), 284 (4.45), and 347 (4.35) nm; IR (Diamond ATR) ν_{max} cm^{-1} 1636, 1605, 1557; ^1H NMR (CD_3OD, 500 MHz) δ_H 6.26 (1H, s, H-12′), 6.22 (1H, s, H-10′), 6.11 (2H, s, H-10, H-12), 3.85 (1H, d, J 4 Hz, H-2′), 2.61 (1H, d, J 11 Hz, H-3′$_b$), 2.45 (1H, dd, J 4, 11 Hz, H-3′$_a$), 2.23 (3H, s, 11′-CH_3), 2.18 (3H, s, 11-CH_3), 1.93 (3H, s, 5-CH_3), 1.73 (3H, s, 5′-CH_3); ^{13}C NMR (CD_3OD, 125 MHz) δ_C 203.6 (C-7), 187.3 (C-7′), 183.3 (C-1′), 163.9 (C-9, C-13), 162.5 (C-13′), 159.7 (C-9′), 150.7 (C-11), 150.6 (C-11′), 147.9 (C-4), 141.0 (C-1), 135.3 (C-6), 131.1 (C-3), 129.0 (C-2), 122.5 (C-5), 110.9 (C-12′), 110.7 (C-8), 110.5 (C-10′), 108.9 (C-10, C-12), 106.0 (C-8′), 104.7 (C-6′), 85.6 (C-4′), 83.9 (C-5′), 47.9 (C-3′), 43.9 (C-2′), 22.3 (11′-CH_3), 22.2 (11-CH_3), 21.3 (5′-CH_3), 12.2 (5-CH_3); HRMS ((−)-ESI): M−H^-, found: 545.1448. $C_{30}H_{25}O_{10}$ requires 545.1447. Crystal data of microsphaerin D hemihydrate: $2(C_{30}H_{26}O_{10})\cdot O$, M=1109.02, monoclinic, space group $P2$, a=11.558(4), b=7.903(3), c=15.549(6), β=91.722(5), V=1419.7(9) Å^3, Z=1, D_c=1.297 g cm^{-3}, T=180 K, F(000)=580, λ=0.71073 Å, μ=0.099 mm^{-1}, $R1$=0.1715 for 2350 $F_o>2\sigma(F_o)$.

5.4. X-ray crystallography

Single crystal X-ray diffraction was used for determining the solid-state structure of the microsphaerins A (**1**) and D (**4**).[23] X-ray reflections were collected on a Rigaku Saturn CCD area detector with graphite monochromated Mo Kα radiation (λ=0.71073 Å). Data were collected and processed using CrystalClear (Rigaku) software. Structures were solved and refined by direct methods using SHELX[24] and SHELX-TL[25] programs. Compound **1** crystallizes as dihydrate and crystals of compound **4** were found to be a hemihydrate. Refinement of coordinates and anisotropic thermal parameters of non-hydrogen atoms were carried out by the full-matrix least-squares refinement. Attempts to locate the hydrogen atoms of water molecules from the Fourier difference map were not successful. All hydroxyl and C–H hydrogens were fixed at the idealized positions. Poor crystal quality of **1** and **4** prevented refinement to better R-values.

5.5. Biological assays

5.5.1. Bacterial whole cell assays

An overnight culture of *S. aureus* ATCC 33591 (MRSA) was diluted 100× with fresh Mueller–Hinton (MH) broth and allowed to reach the log phase of growth at 100 rpm, 37 °C. The bacterial culture was diluted with fresh MH broth to obtain a final inoculum size of 2.5×10^5 cfu/mL; 90 μL of the adjusted inoculum was dispensed into 96-well U-bottom polystyrene plates containing 10 μL of test compounds in 12.5% aqueous DMSO. The plates were incubated for 18–20 h at 37 °C. Following initial incubation, 50 μL of 0.032% resazurin in MH broth was added to each well. Plates were then incubated at 37 °C for 30 min before measuring fluorescence at excitation 530 nm and emission 590 nm. Other whole cell assays were run using a similar procedure.

5.5.2. Mammalian cell cytotoxicity assay

Chinese hamster ovary (CHO) cells were seeded at a density of 12,000 cells/well in 100 μL of Ham's F12 medium supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Plates were incubated overnight at 37 °C in the presence of 5% carbon dioxide. On day 2 the growth medium was replaced with 90 μL of Ham's F12 medium supplemented with 2% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and 10 μL of the test compounds (suspended in 12.5% aqueous DMSO) was then added to the wells. After 24 h incubation, 25 μL of 0.05% resazurin was added to each well and the plates incubated for another 1 h before measuring fluorescence at excitation 530 nm and emission 590 nm. Other cytotoxicity assays were run using a similar procedure.

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