Sorbicillinoid analogs with cytotoxic and selective anti-Aspergillus activities from Scytalidium album

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As part of an ongoing project to explore filamentous fungi for anticancer and antibiotic leads, 11 compounds were isolated and identified from an organic extract of the fungus Scytalidium album (MSX51631) using bioactivity-directed fractionation against human cancer cell lines. Of these, eight compounds were a series of sorbicillinoid analogs (1–8), of which four were new (scabucillin A (2), scalbucillin B (3), scalbucillin C (6) and scalbucillin D (8)), two were phthalides (9–10) and one was naphthalenone (11). Compounds (1–11) were tested in the MDA-MB-435 (melanoma) and SW-620 (colon) cancer cell lines. Compound 1 was the most potent with IC50 values of 1.5 and 0.5 μM, followed by compound 5 with IC50 values of 2.3 and 2.5 μM at 72 h. Compound 1 showed a 48-h IC50 value of 3.1 μM when tested against the lymphocytic leukemia cell line OSU-CLL, while the nearly identical compound 5 had almost no activity in this assay. Compounds 1 and 5 showed selective and equipotent activity against Aspergillus niger with minimum IC values of 0.05 and 0.04 μg ml−1 (0.20 and 0.16 μM), respectively. The in vitro hemolytic activity against sheep erythrocytes of compounds 1 and 5 was investigated and were found to provoke 10% hemolysis at 52.5 and 45.0 μg ml−1, respectively, indicative of a promising safety factor.

INTRODUCTION

Bioactivity-directed purification studies of filamentous fungi from the Mycosynthetix library, representing over 55,000 accessions, had yielded structurally diverse cytotoxic secondary metabolites.1–6 As part of these, the organic extract of a solid-phase culture of a fungus identified as Scytalidium album (MSX51631), which was isolated in 1990 from Cobb County, GA, USA, showed promising cytotoxic and selective anti-Aspergillus activities from Scytalidium album

RESULTS AND DISCUSSION

Two solid-substrate large-scale cultures of the fungus S. album were extracted with 1:1 CHCl3–MeOH and partitioned with water. The vacuum-dried organic extract was then defatted with 1:1 CH3CN/MeOH-hexane. The resulting CH3CN/MeOH-soluble extract showed cytotoxic activity against the SW-620 and MDA-MB-435 cancer cell lines (~83% and 71% inhibition of cell growth when tested at 95% purity as evidenced by UPLC (Supplementary Figure S1)).

A series of eight sorbicillinoid analogs (1–8), of which four were new, all possessing the carbon skeleton of the known antibiotic, sorbicillin,6 were isolated and identified in this study (Figure 1). The NMR, HR-MS and UV data identified 1 (13.87 mg) and 5 (3.25 mg) as the known 5′-formyl-2′-hydroxyl-4′-methoxy-(E,E)-sorbophenone isolates were also evaluated in an array of bacteria and fungi. The hemolytic activity of compounds 1, 3 and 5 was measured against sheep red blood cells, so as to probe a safety factor with respect to the promising anti-Aspergillus activity of these three compounds.

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and 5′-formyl-2′-hydroxy-4′-methoxy-(E)-4-hexenophenone, respectively. Both of these were isolated in 1973 from the fungus *Scytalidium* sp. FY strain. In an analogous manner, compounds 4 (5.89 mg) and 7 (8.21 mg) were identified as the known 1-(2′-hydroxy-4′-methoxy-5′-methylphenyl)-E-E-2,4-hexadien-1-one and 1-(2′-hydroxy-4′-methoxy-5′-hydroxymethylphenyl)-E-4-hexen-1-one, respectively. Both compounds 4 and 7 were isolated in 2006 from a fungicidal isolate of the genus *Phaeoacremonium*.

Characterization data for these known isolates compared favorably with the literature (see Supplementary Figure S2 for 1H and 13C NMR spectra of compounds 1, 4, 5, and 7, respectively).

Four new sorbicillinoid analogs (2, 3, 6 and 8) were isolated as well, and their structures were identified by comparison of HR-MS and NMR data with each other and with structurally related known compounds. Compound 2 (1.77 mg) was obtained as a yellow powder. The molecular formula was determined as C15H18O5 by HR-ESIMS and analysis of 1H, 13C and edited-HSQC NMR data (Tables 1 and 2; Supplementary Figure S3), establishing an index of hydrogen deficiency of 8. Inspection of the HR-MS and NMR data suggested 2 as a sorbicillinanalog with structural similarity with 1. For example, compound 2 showed the characteristic C1′-C6′ sorbyl side chain, as noted by two conjugated doublets with coupling constants consistent with an E, E configuration (δH 2.81/3.3 and δH 4.0/5.5 = 14.6 Hz) that were conjugated with a ketone carbonyl (δC 192.8) that was further conjugated with an aryl group. Additional similarities included NMR signals characteristic of six aromatic carbons and two singlet aromatic protons, suggesting a 1,2,4,5-tetrasubstituted benzene ring with two aromatic protons para to each other, a methoxy group and a hydrogen-bonded phenolic proton. All of the benzene ring substituents were confirmed by HMBC correlations (Figure 2). Key differences between compounds 1 and 2 were the replacement of the singlet aldehyde proton and the carbonyl carbon in 1 (δH/δC 10.27/187.7) by a broad exchangeable proton and a carbonylic acid carbon in 2 (δH/δC 10.05/164.3). These data, along with an extra oxygen atom in 2 relative to 1, as supported by a 16 a.m.u. difference in the HR-MS data, suggested that the aldehyde substituent at C-5′ of the benzene ring in 1 was replaced by a carbonylic acid group in 2. The trivial name scalbucillin A was ascribed to 2.

Compound 3 (1.10 mg) was obtained as a yellow powder with a molecular formula of C14H14O5 as determined by HR-ESIMS and analysis of 1H NMR, 13C NMR and edited-HSQC NMR data (Tables 1 and 2; Supplementary Figure S4). Inspection of the NMR data suggested compound 3 as a sorbicillinoid analog with structural similarity to 7. However, compound 3 lacked the four aliphatic protons H2-2 and H-3 (δH 2.98 and 2.41) that were replaced by two olefinic protons (δH 6.95 and 7.49) with a coupling constant consistent with an E configuration (δH 2.98/1.43 = 15.0 Hz). Conjugation of the C2-C3 double bond with the ketone carbonyl resulted in an upfield shift of the chemical shift of C1 in 3 relative to 7. Further examination of the NMR data, including COSY and HMBC spectra (Figure 2), yielded the structure of 3, which was ascribed the trivial name scalbucillin B.

Compound 6 (9.97 mg) was obtained as a white powder. The chemical formula was determined as C15H18O5 by HR-ESIMS and analysis of 1H, 13C and edited-HSQC NMR data (Tables 1 and 2; Supplementary Figure S7). Examining the NMR data suggested a sorbicillinoid analog with structural similarity to 5 (Table 1). As in 5, compound 6 showed NMR signals consistent with a side chain containing two olefinic protons with a coupling constant that supported an E configuration (δH 2.98/1.43 = 15.5 Hz). A chemical shift of the ketone carbonyl of 204.7 p.p.m. indicated the lack of conjugation with the double bond. Furthermore, the presence of a three-proton doublet of doublets (δH 1.65, dd, δC 70.3, 1.2 Hz) with a chemical shift and coupling constants consistent with an allylic position established the side chain of compound 6 (Figure 1). Additional similarities included NMR data characteristic of a 1,2,4,5-tetrasubstituted benzene ring with two sharp singlet aromatic protons, indicating positions para to each other. A sharp singlet proton (δH 13.05), which did not show single-bond conjugation to a carbon as evidenced from the edited-HSQC NMR experiment, indicated a phenolic proton ortho to the side chain and hydrogen bonded with the carbonyl carbon. Key differences between compounds 5 and 6 include replacement of the aldehyde proton in 5 (δH 10.26) by a methoxy functionality in 6 (δH/δC 3.88/52.2) and replacement of the aldehyde carbon (δC 187.5) by an ester functionality (δC 165.4). These data, along with a 30 a.m.u. difference in the HR-MS data of compounds 5 and 6, indicated replacement of the aldehyde in 5 by a methyl ester in 6. The trivial name scalbucillin C was ascribed to compound 6.

Compound 8 (2.47 mg), which was obtained as a white powder, had a molecular formula of C15H18O5 as determined by HR-ESIMS and analysis of 1H, 13C and edited-HSQC NMR data (Tables 1 and 2; Supplementary Figure S9). The NMR data of 8 indicated a sorbicillinoid analog with structural similarity to 4. However, 8 lacked the H-2 and H-3 olefinic protons, which were replaced by four aliphatic protons (δH 2.95 and 2.40 for H2-2 and H2-3, respectively). This resulted in a downfield shift of C1 in 8 (δC 204.2), relative to that in 4 (δC 192.3), as the latter conjugates with the C2-C3 double bond. Further analysis of the NMR data, including COSY and HMBC experiments (Figure 2), yielded the structure of 8, which was ascribed the trivial name scalbucillin D.

In addition to compounds 1-8, two known phthalides (5,7-dimethoxypthalide (9)11,12 and 4,6-dimethoxyphthalide (10))13,14 and one known naphthalene (isosclerone (11))15 were isolated and identified by comparison of NMR, HR-MS and optical rotation data (for 11) with the literature. This is the first report of 10 from a fungal source.

The cytotoxicity of compounds (1–11) was measured against MDA-MB-435 and SW-620 cancer cell lines. Compound 1 was the most potent with IC_{50} values (concentrations that inhibit growth by 50% relative to untreated cells) of 1.5 and 0.5 μM, respectively, followed by compound 5, with IC_{50} values of 2.3 and 2.5 μM, respectively.

Figure 1 Structure of compounds (1–11) isolated from *S. album.*
isolated previously from the fungus *S. album* lacked significant inhibitory activity when tested against 9KB as reported by the National Institutes of Health (personal communication with Dr David Newman, National Cancer Institute).

Table 1 1H NMR data for compounds 2, 3, 6 and 8 (in CDCl₃, 700 MHz for 2 and 3 and 500 MHz for 6 and 8, chemical shifts in p.p.m., coupling constants in Hz)

|    | δH, Mult. (J in Hz) | δH, Mult. (J in Hz) | δH, Mult. (J in Hz) | δH, Mult. (J in Hz) |
|----|-------------------|-------------------|-------------------|-------------------|
| 2  | 7.05, d (14.6)    | 6.95, d (15.0)    | 3.02, dd (7.5)    | 2.95, dd (8.0)    |
| 3  | 7.54, dd          | 7.49, dd (15.0)   | 2.41, ddd (7.5)   | 2.40, ddd (8.0)   |
| 4  | 6.39, m           | 6.34, m           | 5.49, dtq (15.5)  | 5.49, dtq (15.5)  |
| 5  | 6.38, dq          | 6.31, dq (15.0)   | 5.50, dq (15.5)   | 5.50, dq (15.5)   |
| 6  | 1.94, d (5.1)     | 1.92, dd (6.4)    | 1.65, dd (6.3)    | 1.65, dd (5.2)    |
| 1' | —                 | —                 | 1.2                | 1.2               |
| 2' | —                 | —                 | —                 | —                 |
| 3' | 6.58, s           | 6.46, s           | 6.47, s           | 6.37, s           |
| 4' | —                 | —                 | —                 | —                 |
| 5' | —                 | —                 | —                 | —                 |
| 6' | 8.74, s           | 7.71, s           | 8.37, s           | 7.44, s           |
| 7' | 4.12, s           | 3.90, s           | 3.88, s           | 3.84, s           |
| 8' | 3.93, s           | —                 | —                 | —                 |
| 9' | 13.87, s          | 13.58, s          | 13.05, s          | 12.79, s          |
| 7'-OH | 10.05, bs | 2.02, t (6.1) | — | — |

Abbreviation: Mult., multiplicity.

Table 2 13C NMR data for compounds 2, 3, 6 and 8 (in CDCl₃, 175 MHz for 2 and 3, and 125 MHz for 6 and 8, chemical shifts in p.p.m.)

|    | δC       | δC       | δC       | δC       |
|----|----------|----------|----------|----------|
| 2  | 192.8    | 192.4    | 204.7    | 204.2    |
| 3  | 120.6    | 121.4    | 38.0     | 38.0     |
| 4  | 146.9    | 145.1    | 27.2     | 27.5     |
| 5  | 130.4    | 130.4    | 129.2    | 129.6    |
| 6  | 143.5    | 141.8    | 126.6    | 126.2    |
| 1' | 19.1     | 19.0     | 18.0     | 18.0     |
| 2' | 115.1    | 113.3    | 112.9    | 112.7    |
| 3' | 169.6    | 166.5    | 167.9    | 164.0    |
| 4' | 100.5    | 99.7     | 100.5    | 99.0     |
| 5' | 163.1    | 163.8    | 165.5    | 164.3    |
| 6' | 109.2    | 120.7    | 111.6    | 118.1    |
| 7' | 137.2    | 129.9    | 135.9    | 131.1    |
| 8' | 164.3    | 61.2     | 165.4    | 15.8     |
| 9' | 57.2     | 55.8     | 52.2     | 55.8     |

Followed by compound 3 with an MIC value of 0.60 µg ml⁻¹ (2.42 µM). Relative to amphotericin B, a clinically used agent that was used as a positive control, compounds 1 and 5 were more potent by more than two orders of magnitude. A hemolytic assay was used to assess the toxicity of compounds 1, 3 and 5 toward sheep red blood cells in vitro. Compounds 1, 3 and 5 resulted in 10% hemolysis of sheep red blood cells at 52.5, 37.5 and 45.0 µg ml⁻¹, respectively, with safety factors (calculated as the 10% hemolysis concentration over MIC) of 1050, 62.5 and 1125, respectively (Table 4). Antifungal activities in disk assays against *A. fumigatus* and *Fusarium verticillioides* of compounds 1 and 7 were reported previously by Reátegui et al. In the current study, compound 7 showed no activity against the tested fungal strains.

**CONCLUSIONS**

A series of eight sorbicillinoid analogs (1–8), of which four were new, along with two phthalides (9–10), and one naphthalene (11), were isolated from the fungus *S. album* (MSX51631). Although the sorbicillinoid family encompasses over 50 members, only four sorbicillin-type phenolics, as described by Harmed et al., have been isolated previously from natural sources. This study served to double that to eight related analogs; it is not clear, however, if these compounds are produced by the same biosynthetic route that leads to sorbicillin.

This report expands significantly upon the structure–activity relationship of these compounds in cytotoxicity assays, establishing the importance of the aldehyde moiety at the 5’ position and the conjugated sorbyl side chain. Whether the aldehyde was oxidized to the carboxylic acid (such as compound 2) or reduced to the primary alcohol (such as compound 3), the growth inhibitory activity was diminished, suggesting the reaction of the aldehyde-containing compounds with DNA, RNA or proteins to form Schiff base adducts. Similarly, when the aldehyde was retained but the sorbyl side chain was reduced, as in compound 5 versus 1, the growth inhibitory activity was reduced by 1.5- to 5-fold in solid tumor cell lines, and at least 10-fold in a B-lymphocytic cell line (IC₅₀ of 5 not reached in those cells). This is in clear contrast to the activities of compounds 1 and 5.
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Compounds C. albicans C. neoformans A. niger
10 nM in SW-620 cells, which had 48% and 30% viable cells, respectively.
to provoke 10% hemolysis.
for the biological activity of compound difference suggests that the saturated sorbyl side chain is important
5
4
6
7.9 16.0
3

Table 4 Antifungal and hemolytic activity of compounds 1, 3 and 5

| Compound | C. albicans (μg ml⁻¹) | C. neoformans (μg ml⁻¹) | A. niger (μg ml⁻¹) | 10% Hemolysis factor |
|----------|----------------------|------------------------|-------------------|---------------------|
| 1        | >5 3                 | >53                    | 0.05              | 52.5               | 1050               |
| 3        | >38                  | >38                    | 0.60              | 37.5               | 62.5               |
| 5        | >45                  | >45                    | 0.04              | 45.0               | 1125               |
| Amphotericin B¹ | 16                  | 8                      | 31                | 62.5               | 2.0                |
| Triton X-100⁰ | NT                  | NT                     | NT                | 0.40               | NA                 |

Abbreviations: NA, not available; NT, not tested.¹ Hemolytic activity is the concentration required to provoke 10% hemolysis.
²Safety factor was calculated as the concentration inducing 10% hemolysis over MIC against A. niger.
³Positive controls.

The discovery of strong, narrow-spectrum anti-A. niger activity underscores the utility of subjecting natural products to as wide a screen as possible to detect biological activity. Since Aspergillus species are highly invasive to human beings and continue to present safety challenges for agricultural products and food, the exploration of novel structures for potential application in these settings is a high priority.¹⁻⁸⁻¹³ The exceptional potency and apparent selectivity of several of the compounds isolated here strongly supports further exploration of this class of secondary metabolites.

METHODS

General
UV spectra were obtained using a Varian Cary 100 Bio UV-Vis spectrophotometer (Varian, Walnut Creek, CA, USA). NMR data were collected using either a JEOL ECA-500 NMR spectrometer operating at 500 MHz for ¹H and 125 MHz for ¹³C, or a JEOL ECS-400 NMR spectrometer operating at 400 MHz for ¹H and 100 MHz for ¹³C, and equipped with a high-sensitivity JEOL Royal probe and a 24-slot autosampler (both from JEOL, Tokyo, Japan), or an Agilent 700 MHz NMR spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA), equipped with a cryoprobe, operating at 700 MHz for ¹H and 175 MHz for ¹³C. Residual solvent signals were utilized for referencing. High resolution mass spectra (HR-MS) were obtained using a Thermo LTQ Orbitrap XL mass spectrometer equipped with an ESI source (Thermo Fisher Scientific, San Jose, CA, USA). A Waters Acquity UPLC system (Waters, Milford, MA, USA) utilizing a Waters BEH C₁₈ column (1.7 μm; 50 × 2.1 mm) was used to check the purity of the isolated compounds with data collected and analyzed using Empower software (Waters Corp., Milford, MA, USA). Phenomenex Gemini-NX C₁₈ analytical (5 μm; 250 × 4.6 mm), preparative (5 μm; 250 × 21.2 mm) and semipreparative (5 μm; 250 × 10.0 mm) columns (all from Phenomenex, Torrance, CA, USA), and a YMC ODS-A analytical (5 μm; 150 × 4.6 mm), preparative (5 μm; 250 × 20 mm) and semipreparative (5 μm; 250 × 10.0 mm) columns (all from YMC America, Allentown, PA, USA) were used on a Varian Prostar HPLC system equipped with ProStar 210 pumps and a Prostar 335 photodiode array detector, with data collected and analyzed using Galaxie Chromatography Workstation software (version 1.9.3.2, Varian). Flash chromatography was conducted on a Teledyne ISCO CombiFlash RF using Silica Gold columns and monitored by UV and evaporative light-scattering detectors (both from Teledyne Isco, Lincoln, NE, USA).

Fungal strain isolation and identification

MycoSynthex fungal strain MSX51631 was collected in 1990 from Cobb County, GA, USA. Molecular techniques were used to identify the strain by sequencing the nuclear internal transcribed spacers and intervening 5.8S gene along with the D1/D2 regions of the adjacent nuclear ribosomal large subunit. DNA extraction, PCR amplification, sequencing and phylogenetic analyses were conducted as described previously. The ITS region was used for species-level identification using the pairwise sequence alignment option on the Fungal Barcoding webpage (http://www.fungalbarcoding.org/). This function allows performing online alignment between an input (query) sequence and the available sequences in the database, which comprise different curated global fungal databases, including, Q-bank Fungi database (http://www.q-bank.eu/fungi/); CBS-KNAW Fungal Biodiversity Centre (http://www.cbs.knaw.nl/collections); Molecular Mycology Research Laboratory (http://www.mycofungloglab.org/); Portuguese Yeast Culture Collection (http://pycc.bio-aware.q-bank.eu/fungi/); CBS-KNAW Fungal Biodiversity Centre (http://www.cbs.knaw.nl/); NCBI GenBank. The search returns the best-matching pairwise (local) or global alignments of two query sequences. Identifications were performed in a monophasic way in which sequences were compared to produce a global similarity or probability coefficient between the unknown sequence and the reference sequence database (http://www.fungalbarcoding.org/). Based on the results of the pairwise sequence alignment, the closest genus and species that matched the ITS sequence of the strain MSX51631 were representatives against A. niger in which they are effectively equipotent. This difference suggests that the saturated sorbyl side chain is important for the biological activity of compound 1 only in mammalian cells. This interesting observation may not only provide clues as to mechanism of action of this class of agents, it also suggests that compound 5 may be a potential candidate for development as an antifungal, as it may not elicit substantial negative effects in normal human cells. This hypothesis was supported by the relatively high safety factor of compound 5 (as well as 1) with regards to hemolysis of sheep red blood cells. In addition to compounds 1 and 5, compound 3 also showed selective potency against A. niger, with a similarly low toxicity toward sheep red blood cells in vitro but a lower safety factor (that is, higher MIC).
Supplementary Figure S13). Therefore, the strain MSX51631 was identified by pairwise sequence alignment search on the Fungal Barcoding webpage (98%), gaps = 0. A combined ITS-LSU sequence was deposited in the GenBank (accession no. KJ772505).

Fermentation, extraction and isolation
Storage conditions, fermentation and extraction procedure of the fungal strain MSX51631 were as described previously. In brief, cultures of strain MSX51631 were grown in 2.8-l Fernbach flasks (Corning, Corning, NY, USA) containing 150 g rice and 300 ml H2O. A seed culture grown in YISS medium was used as an inoculum. Following incubation at 22 °C for 14 days, the solid culture was extracted by adding a 500 ml mixture of 1:1 MeOH–CHCl3. The culture was cut into small pieces using a spatula and shaken for about 16 h at ~125 r.p.m. at room temperature (rt), followed by vacuum filtration. The remaining residues were washed with 100 ml of 1:1 MeOH–CHCl3. To the filtrate, 900 ml CHCl3 and 150 ml H2O were added; the mixture was stirred for 30 min and then transferred into a separatory funnel. The bottom layer was drawn off and evaporated to dryness. The dried organic extract was re-constituted in 100 ml of 1:1 MeOH–CH3CN and 100 ml of hexanes. The biphase solution was shaken vigorously and then transferred to a separatory funnel. The MeOH–CH3CN layer was drawn off and evaporated to dryness under vacuum. The defatted material (~750 mg) was dissolved in a mixture of CHCl3–MeOH, adsorbed onto Celite 545 and fractionated via flash chromatography using a gradient solvent system of hexane–CHCl3–MeOH at a 30 m/min flow rate and 61.0 column volumes over 34.1 min to afford five fractions. Fraction 2 (~315.97 mg) was subjected to preparative reversed-phase HPLC over a Phenomenex Gemini-NX C18 preparative column using a gradient system of 50:50 to 60:40 of CH3OH–H2O (acidiﬁed with 0.1% formic acid) at a flow rate of 21 ml/min to yield thirteen sub-fractions. Sub-fractions 2 and 7 yielded compounds (Microtest 96, Falcon, Swedesboro, NJ, USA) and incubated overnight (37 °C in 5% CO2). Samples dissolved in DMSO were then diluted and added to the appropriate wells (concentrations: 25, 5, 1, 0.2 and 0.04 μg ml−1); total volume: 100 μl; DMSO: 0.5%). The cells were incubated in the presence of test substance for 72 h at 37 °C and evaluated for viability with a commercial absorbance assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). Activity was expressed by molarity relative to the negative (solvent) control. The positive control was vinblastine tested at 1 μM in MDA-MB-435 cells (48% viability) and 10 μM in SW-620 cells (30% viability).

Compounds 1 and 5 were also tested against the B-lymphocytic cell line OSU-CLL7 using CellTiter 96 reagents according to the manufacturer’s instructions (Promega). The 48-h IC50 of compound 1 was calculated using Prism software (GraphPad, La Jolla, CA, USA).

Antimicrobial assay
The antimicrobial activity of compounds (1–11) against a panel of bacteria and fungi were measured as described previously. In brief, strains of Escherichia coli strain C (ATCC 13706), Staphylococcus aureus (ATCC 6538) and Mycobacterium smegmatis (ATCC 607) were obtained from the ATCC, Saccharomyces cerevisiae, Candida albicans, Cryptococcus neoformans, A. niger and Micrococcus luteus strains were obtained from the Virginia Tech Microbiology teaching culture collection. Colonies of E. coli, S. aureus, M. luteus, S. cerevisiae, C. albicans and C. neoformans were grown on 1/10-strength Brain Heart Infusion Broth (BBL Microbiology Systems, Cockeysville, MD, USA) containing 0.2% (w/v) sucrose (BHB+5) and 1.5% (w/v) agar. M. smegmatis

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of S. album (isotype sequence; CBS 372.65; identities = 482/485 = 99.3%, gaps = 0), the other closest matches included those of Ascomyctea sp. olr1m237 (AY787735; identities = 470/470 = 100%), gaps = 0, Ascomyctea sp. olr1m349 (HM036638; identities = 454/454 = 100%), gaps = 0. Pairwise sequence alignment was also performed with combined internal transcribed spacer-large subunit (ITS-LSU) region, which suggested that the closest match of the query sequence was S. album (isotype sequence; CBS 372.65; identities = 927/942 = 98%), gaps = 6 (0.7%).

A combined ITS-LSU rDNA complete linkage tree was generated using pairwise sequence alignment search on the Fungal Barcoding webpage (Supplementary Figure S13). Therefore, the strain MSX51631 was identiﬁed as having afﬁnities to S. album L. Beyer & Klingström. (Helotiales, Leotiomycetidae, Leotiomycetes, Pezizomycotina and Ascomycota). The combined ITS-LSU sequence was deposited in the GenBank (accession no. KJ772505).
was grown on Middlebrook 7H10 agar (BBL Microbiology Systems) and A. niger on potato dextrose agar (PDA; BBL Microbiology Systems). Streaked plates were incubated at 37 °C for 3–7 days, except for that of A. niger, which was incubated in the dark at 30 °C. A single colony for each microbe, except A. niger, was used to inoculate 5 ml of 1/10-strength BHIB+S (E. coli, M. luteus, M. luteus, S. aureus), Middlebrook 7H9 broth (M. smegmatis) or yeast extract peptone maltose broth (S. cerevisiae, C. albicans and S. neoformans) and incubated at 37 °C (S. cerevisiae and M. luteus) at 30 °C for 4–7 days. After growth, the resulting broth cultures were diluted with buffered saline gelatin (gelatin (0.1 g l⁻¹), NaCl (8.5 g l⁻¹), KH₂PO₄ (0.3 g l⁻¹), Na₂HPO₄ (0.6 g l⁻¹)) to equal the turbidity of a No. 1 McFarland standard. Broth cultures were streaked on plate count agar (BBL Microbiology Systems) and incubated for 3–4 days at 37 °C, so as to check for viability and contamination. Plates for M. luteus and S. cerevisiae were grown at 30 °C. Spores of A. niger were scraped from the surface of the PDA and suspended in 5 ml of 1/10-strength BHIB+S, and that suspension was transferred to a sterile test tube. The turbidity was adjusted to be equivalent to that of a No. 1 McFarland standard by dilution with buffered saline gelatin. Those spore suspensions were streaked on PDA and incubated at 37 °C for 3–4 days, so as to confirm viability and lack of contamination.

The MICs of the isolated compounds were measured by broth microdilution in 96-well microtitre plates. A twofold dilution series of the compounds was prepared in 96-well microtitre plates in a 50 μl volume of 1/10-strength BHIB+S, and the dilution series was inoculated with 50 μl of each cell suspension. The resulting inoculated dilution series were incubated at either 30 or 37 °C (same as growth temperature), and growth, as noted by turbidity, was scored visually and recorded on the fourth day. MIC of each cell suspension. The resulting inoculated dilution series were incubated for 3 days at 37 °C, to con firm the absence of turbidity compared with the drug-free control.

Hemolytic assay

Hemolytic activity of compounds 1, 3 and 5 was measured against sheep red blood cells as described elsewhere.1,2

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)