Efficient identification of fungal antimicrobial principles by tandem MS and NMR database

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Abstract

The continuous re-isolation of the known and non-applicable compounds that is time-consuming and wasting resources is still a critical problem in the discovery of bioactive entities from natural resources.

To efficiently address the problem, high performance liquid chromatography-diode array detector-microfractionation (HPLC-DAD-microfractionation) guided by disk agar diffusion assay was developed, and the active compounds were further identified using the tandem mass spectrometry (MS/MS)-based molecular networking. Of 150 fungal strains screened, the methanolic extracts of Phoma herbarum PPM7487, Cryptosporiopsis ericae PPM7405, and Albifimbria verrucaria PPM945 exhibited potent antimicrobial activity against Candida albicans SC5314 and Cryptococcus neoformans H99 in the preliminary agar diffusion assay. The concept of OSMAC (one strain many compounds) was employed in the fungal cultures in order to enrich the diversity of the 2nd metabolites in this study. HPLC coupled with off-line bioactivity-directed profiling of the extracts enabled a precise localization of the compounds responsible for the conspicuous antimicrobial activity. The purified active compounds were identified based mainly on MS/MS database, and further supported by 13C nuclear magnetic resonance (NMR) spectral data compared to the literatures. In addition to nineteen known compounds, a new trichothecene derivative 1, namely trichoverrin D, was isolated and identified through this protocol. The antifungal activities of all the pure isolates were evaluated, and the structure activity relationships were also inferred. This report has demonstrated the combination of HPLC microfractionation and MS/MS coupled by NMR spectral dereplication for speeding up the antimicrobial natural products discovery process.

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1. Introduction

Natural products (NPs) along with some synthesized drugs as inspired by the scaffolds of natural products are undoubtedly useful for discovering pharmaceutical leads [1−5]. However, after the great strides made in high-throughput screening (HTS) and synthetic compound libraries, natural product screening has ever been paused in some pharmaceutical companies since it requires special know-how, and is time-consuming for identifying active compounds and laborious [6]. Additionally, it is often criticized for resulting in the continuous re-isolation of previously known compounds in these processes. Thus, in NPs research, either as part of bioactivity-guided isolation studies for drug discovery purposes or in the frame of metabolomic investigation for biomarker identification, the secondary metabolites in crude extracts of various origins have to be characterized efficiently. That means it is essential to develop an assay system coupled with an optimized natural product library and to use high-level analytical techniques for dereplication.

The term “dereplication” was first coined in the CRC Handbook of Antibiotic Compounds published in 1980 to refer to recognizing and eliminating from considering those active substances already studied [7,8]. Thereafter, dereplication has been regarded as a key process of discovering new pharmacologically active substances in mixtures of natural products. During the early period, many systematic dereplication methods for crude extracts in combination with biological screening have been developed using LC-UV-(MS) [9,10]. A recent advance of dereplication techniques has been the MS/MS-based molecular networks (MN) algorithm [11,12]. The MS/MS spectra-associated platform is based on mass fragmentation patterns with the underlying concept that structurally related molecules will fragment in similar ways to give analogous patterns. This tool, available on the Global Natural Products Social Molecular Networking (GNPS at http://gnps.ucsd.edu) web site, allows users to include their structurally annotated data sets into a publicly available database, thereby enabling compound dereplication. In addition, new molecules that are related to known substances in the database can rapidly be assigned to specific structural families so as to accelerate the discovery and characterization process.

In this work, as part of a continuing search for antimicrobial agents from natural resources, the methanolic extracts of small scale solid-state fermentation of 150 fungal strains were primarily screened against Candida albicans, Cryptococcus neoformans, Escherichia coli, and Staphylococcus aureus by agar-based diffusion assay. Three fungal strains, Phoma herbarum PPM7487, Cryptosporiopsis ericae PPM7405, and Albinumbria verrucaria PPM945, showed potent antifungal activity among 150 fungal strains in a preliminary screening. Using off-line activity-based HPLC-DAD profiling of their methanolic extracts coupled with molecular networking of tandem MS, database of ChembioFinder 12.0, and online database of $^{13}$C NMR spectra enabled to precise localization and identification of the compounds responsible for the antimicrobial activity of the fungal strains. This report herein describes the efficient isolation and identification of one new trichothecone type sesquiterpene derivative along with nineteen known compounds with antimicrobial activities by employing above methods.

2. Experimental section

2.1. General experimental procedures

Optical rotations and UV were measured on a JASCO P-2000 polarimeter (Tokyo, Japan) and Thermo UV−Visible Heλios α Spectrophotometer (Bellefonte, CA, USA), respectively. $^1$H and $^{13}$C NMR were acquired on a Bruker DRX-500 SB spectrometer (Ettingen, Germany). Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to the Dionex UltiMate™ 3000 RSLCnano UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) via an electrospray ionization (ESI) source was used in this study for high-resolution mass spectrometry. IR spectra were recorded on a JASCO FT/IR 4100 spectrometer (Tokyo, Japan). L-7100 pump and L-7455 Diode array detector (Hitachi, Tokyo, Japan) were used in HPLC analysis and purification.

2.2. Fungal stains and pathogenic strains used for screening of antimicrobial agents

One hundred and fifty fungal strains were collected and isolated from Taiwan, and were identified by one of us (SST) and deposited at the Department of Plant Pathology and Microbiology, National Taiwan University, Taipei, Taiwan. Four pathogenic strains, C. albicans SC3514, C. neoformans H99, E. coli K12, and S. aureus ATCC29213 were used for preliminary antimicrobial screening.

2.3. Cultivation and fermentation of the bioactive fungal strains

The mycelia of P. herbarum PPM7487, C. ericae PPM7405, and A. verrucaria PPM945 were inoculated into 500 mL erlenmeyer flask, each containing 50 g brown rice (Santacruz, Taiwan) and 1.2 g Bacto™ Malt Extract (in 40 mL deionized water). The fermentation was conducted with aeration at 25 °C for 10 days. During the same period, the OSMAC approach was also performed by using various cereals including brown rice, millet, coix seed, quinoa, soybean, and sesame as media in order to enhance the chemical diversity of the three fungal strains.

2.4. HPLC-DAD microfractionation coupled with agar-based disk diffusion assay

The microfractionation of methanolic extracts of P. herbarum PPM7487, C. ericae PPM7405, and A. verrucaria PPM945 were performed on a reversed-phase analytical column (Phenomenex Kinetex C18, 5 μm, 4.6 i.d. × 250 mm, Torrance, CA, USA). The flow rate was set at 1.0 mL/min, and the injection volume...
for the crude extracts at the concentration of 100 ppm was 10.0 μL. The elution system used was H2O (A) and MeCN (B), with 0.1% formic acid in H2O. The gradient program was 1%–20% B in 20 min, then 35%–100% B from 20 to 55 min, and keeping at 100% B till 70 min. C. albicans SC3514 and C. neoformans H99 were grown overnight at 30 °C, and 0.1 OD600 (in 100 μL) was spread on the surface of YPD medium containing 1.5% agar. Paper discs (6 mm), each containing 20 μg of all of the compounds collected from the peaks in HPLC microfractionation of the crude extracts, were placed on the surface of the medium. The plates were incubated at 37 °C for 48 h, then measured the inhibition zones.

2.5. HRESIMS, tandem MS analysis, generation of the molecular networking and ChemBioFinder 12.0 library search

The metabolite profiling of three bioactive fungal strains P. herbarum PPM7487, C. ericae PPM7405, and A. verrucaria PPM945 were performed by a Dionex UltiMate™ 3000 RSLCnano UHPLC system (Thermo Fisher Scientific) coupled with Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) equipped with an electrospray interface. The foundation of the present UHPLC-HRESIMS-MS/MS dereplication procedure was the construction of a database for the identification of the bioactive secondary metabolites from fungi via recording full-scan (high-resolution) mass spectra and MS/MS spectra in positive ionization (ESI) modes (Table S1). All the Thermo Xcalibur RAW data were converted to the general format mzXML, a text-based format used to represent mass spectrometry data describing the scan number, precursor m/z, and the m/z and intensity of each ion observed in MS/MS, using ReAdW version 4.3.1. Then, data treatment, generation of the MN, and the dereplicate process were performed using GNPS web site by importing the mzXML format files and optimizing the networks by the software Cytoscape version 3.4.0. On the other hand, the generated nodes of MN by precursor mass were annotated and matched against the ChemBioFinder 12.0 database of fungal metabolites (Table S1). The 13C NMR data which demonstrated high resolution of all carbon signals of all the isolates were acquired for supporting the identification of the compounds and precise determination of the configurations among stereoisomers in comparison with the literatures (Table S2). Additionally, two dimensional NMR data were used to fully elucidate the structure of the new compound.

2.6. Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The pure chemical entities isolated in this study were evaluated the antimicrobial activity against four pathogenic fungal strains including C. albicans SC3514, C. albicans 12–99 (azole-resistant isolate), C. albicans 89 (echinocandin-resistant isolate), and C. neoformans H99. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were evaluated by the Clinical and Laboratory Standards Institute (CLSI) M27-A3 guidelines [13]. Testing was done in RPMI-1640, buffered to pH 7.0 with 0.165 M 3-(N-morpholino) propane sulfonic acid. Yeast strains were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) overnight at 30 °C with shaking, and washed twice with deionized H2O. The OD600 was measured and each strain was diluted to 1 OD600/mL. This inoculum was diluted to 0.0005 OD600/mL in RPMI-1640. Then, 98 μL of the strain culture was added to each well in a 96 well plate format. Two μL of serially diluted drugs were added to the wells, yielding a final volume of 100 μL per well. The two times concentrations of compounds which ranged from 0.125 to 64 μg/mL were added across the plate with the highest to the lowest from row one to ten. The plates were incubated 48 h for C. albicans isolates and 72 h for C. neoformans at 35 °C, and observed the presence or absence of visible growth. The in vitro drug studies were performed at least twice. The minimum inhibitory concentration (MIC) of drug was defined as the clear well with the lowest concentration of each drug. The minimum fungicidal concentration (MFC) were determined by transferring 3 μL of solution from MIC-assay well to drug-free solid YPD plates. The viability of fungal cells was checked after the plates were incubated overnight for C. albicans isolates and 48 h for C. neoformans at 30 °C.

3. Results and discussions

3.1. HPLC-DAD microfractionation and off-line antimicrobial assay

Of 150 fungal strains tested, only P. herbarum PPM7487, C. ericae PPM7405, and A. verrucaria PPM945 exhibited significant anti-fungal activities against C. albicans and/or C. neoformans, and all the strains were inactive toward E. coli and S. aureus in the preliminary antimicrobial screening (Fig. 1). In order to track the antifungal principles from the HPLC-DAD chromatograms of P. herbarum PPM7487, C. ericae PPM7405, and A. verrucaria PPM945, microfractions were thus collected by peaks (1, 2, 7, 8, 10, 13–16) or time intervals if peaks were overlapped (Fig. 2A), and evaluated the antifungal activity of every microfractions by off-line agar-based disc diffusion assay. The overlapped peaks were further purified by repeated isocratic HPLC to give 4–6, 11–12, and 17–20 (Fig. 2A). In an attempt to enrich the

![Fig. 1 – Agar-based disc diffusion assay of the methanolic extracts of three fungal strains P. herbarum PPM7487, C. ericae PPM7405, and A. verrucaria PPM945. Penicillium and ketoconazole were used as positive controls. Each disc was added 40 μL of fungal methanolic extracts (100 ppm) or positive controls (10 ppm), and dried before tests.](image-url)
Fig. 2 – (A) The microfractions 1–20 performed by RPLC-DAD profiling (UV 254 nm detection). The overlapped microfractions were expanded using isocratic systems as indicated by arrows. (B) RPLC-DAD microfractionation in combination with offline agar-based disc diffusion assay used for efficiently tracking the antimicrobial principles from fungal extracts obtained by OSMAC approaches (e.g. cultivated in millet versus in brown rice). Ketoconazole was used as a positive control. Each disc was added 40 μL of collected microfractions (500 ppm), fungal methanolic extract (100 ppm), or positive control (10 ppm), and dried before tests.
diversity of the 2nd metabolites, the cereals used in culture media of the three fungal strains were varied. As a result, compounds 3 and 9 exhibiting significant antifungal activities against both C. albicans and C. neoformans were isolated from A. verrucaria PPM945 cultivated in millet in addition to above 18 compounds isolated from the fungal strains cultured in brown rice (Fig. 2B).

3.2. Molecular networking and identification of antimicrobial principles based on MS and 13C NMR spectra

The precursor ions and fragments data of the antimicrobial principles were used for constructing MN by GNPS and further optimized by Cytoscape version 3.4.0 [14,15]. This approach has been employed successfully for clustering compounds sharing similar MS/MS data, and has been recently implemented as an efficient tool for the dereplication of natural products initially. In the present case, MN revealed four clusters with different structural features based mainly on the MS/MS data analysis using GNPS. Furthermore, utilizing the precursor ions of all the isolates in the MN to match against a ChemBioFinder 12.0 database of fungal metabolites for approaching the particular chemical skeleton among the cluster linkages facilitated the constituent investigation of the three fungal strains to verify the occurrence of new as well as valuable chemical entities. There were one node of aliphatic lactone glycoside family (pink), three nodes of the cyclohexapeptide family (green), four nodes of O-linked glycosylated polyketide family (green) together with twelve nodes of trichothecene family (blue) in four clusters of MN (Fig. 3). Although cross searches of the corresponding structural information in the ChemBioFinder 12.0 using twenty precursor ion MS data and genus Phoma, Cryptosporiopsis, and Albifimbria as keywords yielded 86 proposed structures without any MS2 data in literatures for further identification except compounds 10 and 11 (Table S1). Compounds 10 and 11 were identified initially using MS2 interpretation when compared to literatures [16]. Targeted isolation and purification of all the analogs were still required for confirming the MS-based assignments by 13C NMR interpretations (Table S2). Thus, the structures of known compounds 2–20 were shown at Fig. 4.

3.3. Structural elucidation of trichoverrin D (1)

Compound 1, an amorphous white powder, was determined to have a molecular formula of C31H43O10, as evidenced by its 13C NMR spectrum and HRESIMS analysis. The IR absorptions at 3610, 1706, and 1645 cm\(^{-1}\) indicated the presence of a hydroxy, a conjugated ester carbonyl, and an olefinic group, respectively, which was further confirmed by a UV \(\lambda_{\text{max}}\) at 220 and 261 nm [17]. The \(^1H\) NMR spectrum (CDCl3, 500 MHz) of 1 demonstrated conspicuous resonances corresponding to two three-proton singlets at \(\delta_H 1.69\) (3H, s, H-16) and 2.17 (3H, s, H-6’) for two methyls attached to olefinic carbons, a three-proton doublet at \(\delta_H 1.17\) (3H, d, \(J = 6.4\) Hz, H-8’) connected with a carboxylic carbon, and a three-proton singlet at \(\delta_H 2.01\) (3H, d, J = 3.7 Hz, H-7’) borne by a carboxylic proton. The 13C NMR (CDCl3, 125 MHz) in combination with the HSQC spectrum of 1 revealed 31 carbon signals corresponding to five methyls at \(\delta_C 7.0\) (C-14), 18.8 (C-8’0), 18.9 (C-8’0), 20.9 (C-7’0), and 23.2 (C-16), seven methylenes at \(\delta_C 21.4\) (C-7), 27.9 (C-8), 36.9 (C-9), 42.6 (C-10), 48.5 (C-11), 52.4 (C-12), 69.3 (C-13), 72.0 (C-15), and 120.9 (C-3), two methine carbons at \(\delta_C 70.9\) (C-2), 117.7 (C-4), a quaternary carbon at \(\delta_C 139.5\) (C-5’), and twelve oxygenated carbons at \(\delta_C 164.5\) (C-1), 167.2 (C-6’), 173.5 (C-19), 174.0 (C-20), 175.8 (C-21), 177.7 (C-23), 187.8 (C-24), 189.9 (C-25), 202.7 (C-17), 205.4 (C-18), and 206.9 (C-22).
Fig. 4 – Chemical structures of 1–20 identified in this report.
forms based on the J values of H-2/H-3 (J = 11.7 Hz) and H-4/H-5 (J = 15.5 Hz), respectively. Further comparisons of the spectral data of 1 with those of 13'-acetyltrichoverrin B [18], 1 was almost compatible with 13'-acetyltrichoverrin B except the configurations of their OH-6' and OH-7'. The relative configurations of the 6',7'-diols were deduced to be three based on the large mutually coupled \(^1\)H NMR spectral data of 6.2 Hz in contrast with that of the erythro form with J \(= 4.0-5.5\) Hz of 13'-acetyltrichoverrin B [18]. Compound 1 (J125 = \(-77.5\) (c 0.4, MeOH)) and its 5'-deacetyl analog trichoverrin A (J125 = \(-21.5\) (c 0.3, MeOH)) sharing the same sign of the optical rotational values, thus the absolute configurations of 6',7'-diols of 1 were determined to be both S-forms [18]. Accordingly, the structure of 1 was well established as shown in Fig. 4, and named trichoverrin D.

### 3.4. Evaluation of antimicrobial activity and structure-activity relationship

As shown in Table 2, both 12 and 16 showed the lowest minimum inhibition concentration (MIC) at 0.5 µg/mL against C. albicans SC5314, and 16 with an MFC value of 0.5 µg/mL exhibited more potent fungicidal activity than 12 against C. albicans SC5314. The minimum inhibition concentration (MIC) of 2, 4, 7, 8, 10, 11, 13–15, and 17–19 ranged from 1 to 8 µg/mL, and only 2, 14, 15, and 17–19 exhibited significant fungicidal activity with MFC values of 1–8 µg/mL. However, only compounds 14, 16–18, and 20 retained significant fungicidal activity against the C. albicans 12–99 (an azole-resistant isolate) with MFC values ranging from 4 to 8 µg/mL, and as far as I know, this is the first time to describe the antifungal activities of 18 and 20 against C. albicans. Only compounds 10 and 12 showed slight inhibitory activity with an MFC value of 64 µg/mL against C. albicans 89 (an echinocandin-resistant isolate). The minimum inhibition concentration (MIC) of 2–4 and 8–13 against C. neoformans H99 ranged from 2 to 8 µg/mL, and only 3, 8, 11–13 exhibited significant fungicidal activity with MFC values of 4–8 µg/mL against the same fungal strain. Among these, compounds 4, 9, and 12 showed the lowest minimum inhibition concentration (MIC) at 2 µg/mL against C. neoformans H99, and 12 with an MFC value of 4 µg/mL exhibited more potent fungicidal activity against C. neoformans H99 than those of 4 and 9. Taken together, compounds 2–4 and 8–12 exhibited significant antifungal activities against both C. albicans SC5314 and C. neoformans H99, and this is the first paper to announce their antifungal activities against C. neoformans. Concerning the structural features of trichothecene derivatives 2, 4, 5, and 10–12, it was found the carbonyl group instead of an ethanol functionality at C-6' of compounds 10–12 with the most potent antifungal activities against C. albicans and/or C. neoformans seemed to play a crucial role for their bioactivity. Considering the structures of compounds 6–8, 8 with the strongest activity indicated no substitution at C-4' could be the key functionality. Moreover, the acetoxy attached at C-4' of compound 7 instead of a hydroxy at C-4' of compound 6 seemed to exhibit more antifungal activity against both C. albicans and C. neoformans.

| Table 1 — \(^1\)H and \(^{13}\)C NMR data for compound 1. |
| Positions | \(^1\)H \(^{13}\)C |
| 1 | 3.84 d (5.1) | 79.0 |
| 2 | 2.01 m | 36.9 |
| 3 | 2.57 ddd (15.6, 7.8, 5.1) | 75.1 |
| 4 | 5.89 dd (7.8, 3.4) | 48.8 |
| 5 | 1.98 m | 43.2 |
| 6 | 1.69 m | 21.4 |
| 7 | 2.01 m | 27.9 |
| 8 | 1.98 m | 140.9 |
| 9 | 5.43 brd (5.6) | 18.5 |
| 10 | 3.97 brd (5.6) | 66.8 |
| 11 | 2.16 m | 65.9 |
| 12 | 2.85 d (3.7) | 48.2 |
| 13 | 3.16 d (3.7) | 7.0 |
| 14 | 0.81 s | 63.0 |
| 15 | 4.02 d (12.2) | 23.2 |
| 16 | 4.21 d (12.2) | 165.7 |
| 17 | 1.69 s | 118.3 |
| 18 | 5.66 d (11.7) | 143.4 |
| 19 | 6.57 dd (11.7, 11.3) | 147.9 |
| 20 | 7.58 ddd (15.2, 15.1, 11.1) | 142.0 |
| 21 | 6.03 dd (15.5, 5.9) | 76.6 |
| 22 | 4.01 m | 70.5 |
| 23 | 3.66 dq (6.4, 6.2) | 18.9 |
| 24 | 1.74 d (6.4) | 165.8 |
| 25 | 5.72 brs | 117.1 |
| 26 | 2.45 m | 156.3 |
| 27 | 4.20 m | 39.5 |
| 28 | 4.20 m | 61.9 |
| 29 | 2.17 s | 18.8 |
| 30 | 2.17 s | 171.3 |
| 31 | 2.01 s | 20.9 |

\( ^1\)H NMR (500 MHz, Chloroform-d) spectroscopic data (δ in ppm, mutl. (J in Hz)).

\(^{13}\)C NMR (125 MHz, Chloroform-d) spectroscopic data (δ in ppm).
4. Conclusion

Future investigations on natural products, performing an effective platform to deal with the critical problems, such as taking long time to continuously re-isolate the known or worthless components, would be definitely important. In this study, we adopted bioassay-guided HPLC-DAD-micro-fractionation together with molecular networking and database dereplication to disclose precisely the antimicrobial principles from P. herbarum PPM7487, C. ericae PPM7405, and A. verrucaria PPM945 as efficient as possible. Twenty chemical entities classified as four skeletal types were isolated and identified based on this method. Of these, 2–4 and 8–12 exhibited potent antifungal activities against C. neoformans H99, which have not been mentioned elsewhere. In addition, the targeted isolation strategy accompanied with the bioassay platform make the finding of the bioactive chemical entities much easier, and that could ordinarily be the key step for the new drug development.

Conflicts of interest

The authors declare that there are no potential conflicts of interest.

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Appendix A. Supplementary data

Original MS data and 13C NMR data of compounds 2–20 and 1D and 2D NMR spectra of compound 1 are available as Supporting Information.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfda.2019.06.003.

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Table 2 – Antifungal activities of compounds 1–20 against Candida albicans and Cryptococcus neoformans.

| Compounds | MIC (µg/mL) | MFC (µg/mL) |
|-----------|-------------|-------------|
|           | C. albicans SC5314 | C. neoformans H99 | C. albicans 12-99 | C. albicans 89 | C. albicans SC5314 | C. neoformans H99 | C. albicans 12-99 | C. albicans 89 |
| 1         | > 64        | 64          | > 64          | > 64          | > 64        | 64          | > 64          | > 64          |
| 2         | 8           | 8           | 64            | 32            | 8           | 64          | > 64          | > 64          |
| 3         | 4           | 8           | 32            | 32            | 32          | 8           | > 64          | > 64          |
| 4         | 2           | 2           | > 64          | > 64          | > 64        | > 64        | > 64          | > 64          |
| 5         | 16          | 16          | > 64          | > 64          | > 64        | > 64        | > 64          | > 64          |
| 6         | 2           | > 64        | > 64          | > 64          | > 64        | > 64        | > 64          | > 64          |
| 7         | 1           | 4           | > 64          | > 64          | > 64        | > 64        | > 64          | > 64          |
| 8         | 2           | 2           | > 64          | > 64          | > 64        | > 64        | > 64          | > 64          |
| 9         | 1           | 4           | 8             | 8             | 8           | 8           | > 64          | > 64          |
| 10        | 1           | 4           | 8             | 16            | 8           | 8           | > 64          | > 64          |
| 11        | 0.5         | 2           | 4             | 8             | 8           | 8           | > 64          | > 64          |
| 12        | 1           | 4           | > 64          | > 64          | > 64        | > 64        | > 64          | > 64          |
| 13        | 1           | > 64        | 2             | > 64          | > 64        | > 64        | > 64          | > 64          |
| 14        | 1           | > 64        | 1             | > 64          | > 64        | > 64        | > 64          | > 64          |
| 15        | 0.5         | > 64        | 1             | > 64          | > 64        | > 64        | > 64          | > 64          |
| 16        | 4           | 64          | 4             | > 64          | > 64        | > 64        | > 64          | > 64          |
| 17        | 4           | 32          | 4             | > 64          | > 64        | > 64        | > 64          | > 64          |
| 18        | 4           | 64          | 64            | > 64          | > 64        | > 64        | > 64          | > 64          |
| 19        | 4           | 64          | 64            | > 64          | > 64        | > 64        | > 64          | > 64          |
| 20        | 32          | > 64        | 4             | > 64          | > 64        | > 64        | > 64          | > 64          |

| a | MIC: minimum inhibitory concentration (lowest concentration that allows no detectable growth). |
| b | MFC: minimum fungicidal concentration (the lowest concentration that kills the fungus), which was determined by transferring 3 × 103 CFU/mL of C. albicans 89 into four replicate wells with no visible growth to fresh drug-free YPD solid agar plate and incubated at 30 °C for overnight or 48 h. The highest test concentration for compounds 1–20 was 20 µg/mL. |
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