Supplementary Information

Discovery of Novel Antifungal Scaffolds by Structural Simplification of Natural Product Sampangine

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1. Experimental protocols of biological assays

Strains, culture and agents. All strains were routinely grown in YPD (1% yeast extract, 2% peptone and 2% dextrose) liquid medium at 30°C in a shaking incubator. For in vitro experiments, 6.4 mg/mL compound in DMSO was used as a stock and added to the culture suspensions to obtain the required concentrations. Other mediums used included YPD + 10% (vol/vol) Fetal Bovine Serum (FBS), Spider medium, Lee's medium (pH = 6.8), Synthetic Low-Ammonium Dextrose (SLAD) medium and RPMI 1640 medium.

Antifungal susceptibility testing. Antifungal susceptibility test was carried out in 96-well microtiter plates (Greiner, Germany) using the broth microdilution protocol of the Clinical and Laboratory Standards Institute methods (M27-S4) with a few modifications. Briefly, the initial concentration of fungal suspension in RPMI 1640 medium was $10^3$ CFU/ml, and different concentrations of compound were added to the suspension. Plates were incubated at 35°C for 24 h. The growth inhibition was determined by spectrophotometer, and optical density was measured at 630 nm. Each strain was tested in triplicate. MIC$_{80}$ were determined as the lowest concentration of the drugs that inhibited growth by 80%.

Time-kill curve assay. Exponentially growing *C. albicans* cells were washed with PBS, then resuspended with RPMI 1640 medium to $1 \times 10^6$ cells/ml and divided into different bottles. Different concentrations of compound were added to the *C. albicans* suspensions. The samples were cultured at 30°C under constant shaking (200 rpm). Portions of cell suspensions were withdrawn at designated time points (0, 2, 4, 6 and 8
h). Serial dilutions were plated on YPD agar to determine the CFU/ml of the cell suspensions. CFUs were determined after incubation for 48 h at 30°C. Three independent experiments were performed.

**In vitro biofilm formation assay.** Biofilm formation assay was performed in a 96-well tissue culture plate (Corning, cat. no. 3599) by seeding with 100 μl cell suspensions (1.0 × 10^6 cells/ml) in RPMI 1640 medium, and incubating them statically at 37°C. After 90 min adhesion, the medium was aspirated, non-adherent cells were removed, and fresh medium was added. The plate was further incubated at 37°C for 24 h until formation of mature biofilms. A semiquantitative measure of the formed biofilms was calculated using an XTT reduction assay. To detect the effect of compound on the formation of biofilms, different concentrations of compound were added to the fresh RPMI 1640 after 90 min adhesion and incubated at 37°C for 24 h. To detect the effect of the drugs on mature biofilms, different concentrations of compound were added after 24-h incubation with the mature biofilms formed, and the plates were incubated at 37°C for further 24 h.

**Scanning electron microscopy (SEM).** SEM was performed to investigate the ultrastructure of biofilms. Glass disks coated with poly-L-lysine hydrobromide (Sigma, cat. no. P6282) were used to develop biofilms. The disks were inoculated with *C. albicans*, incubated statically at 37°C for 90 min to allow adhesion. After removing non-adherent cells, the disks were incubated with fresh RPMI 1640 medium at 37°C for 24 h. For compound treatment groups, compound was added with the fresh RPMI 1640 medium after 90 min adhesion. Biofilms were washed and placed in
a fixative consisting of 2% (v/v) glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.2) for 2 h. The samples were rinsed twice in cacodylate buffer, garnished with 1% osmic acid for 2 h, dehydrated in an ascending ethanol series, treated with hexamethyl-disilazane (Polyscience Europe GmbH, Eppelheim, Germany) and dried overnight. The specimens were coated with gold and observed through a Philips XL-30 SEM (Philips, The Netherlands) in high vacuum mode.

**Cellular surface hydrophobicity (CSH) assay.** *C. albicans* CSH was measured by water-hydrocarbon two-phase assay. Briefly, the formed *C. albicans* biofilms were removed from the flask surface to obtain a cell suspension (OD$_{600}$ = 1.0 in YPD medium). Then 1.2 ml suspension was pipetted into a clean glass tube and overlaid with 0.3 ml octane. The mixture was vortexed for 3 min and then stood at room temperature for another 3 min for phase separation. Then OD$_{600}$ of the aqueous phase was determined. OD$_{600}$ for the group without the octane overlay was used as the control. Three repeats were performed for each group. Relative hydrophobicity was obtained as [(OD$_{600}$ of the control minus OD$_{600}$ after octane overlay)/OD$_{600}$ of the control] $\times$ 100%.

**Antifungal effect evaluation using a *C. elegans*–*C. albicans* infection model.** *C. elegans* was first infected by *C. albicans*. Briefly, *C. elegans* glp-4; sek-1 adult nematodes were added to the center of *C. albicans* SC5314 lawns on BHI kanamycin (45 µg/mL) agar plates and incubated at 25°C for 4 h to allow infections. Worms were washed four times with sterile M9. Thirty worms were then pipetted into each well of 12-well tissue culture plates (Corning, USA) containing 2 ml of liquid medium (80%
M9, 20% BHI) and kanamycin (45 µg/mL). For compounds treatment groups, compound was added at 4 µg/mL or 16 µg/mL. 16 µg/mL FLC treatment group was set as the positive control, and the DMSO solvent group was set as the negative control. Worms were scored daily and dead worms were removed from the assay. Survival was examined by using the Kaplan-Meier method and differences were determined by using the log-rank test (STATA 6; STATA, College Station, TX). A P value of, 0.05 was considered statistically significant.

2. Chemical synthesis and structural characterization of the target compounds

Experimental Protocols and Structural Characterization of Target

**General Methods:** Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 400 spectrometer with TMS as an internal standard. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and Hz, respectively. High-resolution mass spectrometry data were collected on a Kratos Concept mass spectrometer. TLC analysis was carried out on silica gel plates GF254 (Qingdao Haiyang Chemical, China). Silica gel column chromatography was performed with Silica gel 60 G (Qingdao Haiyang Chemical, China). Commercial solvents were used without any pretreatment.
8-Methyl-2-nitrothieno[2,3-g]quinoline-4,9-dione (ZG-20-07). Compound 2a (15.2 g, 0.1 mol) was solved in con. H$_2$SO$_4$ (150 mL). After cooling to 0 °C, KNO$_3$ (12 g, 0.12 mol, 1.2 eq) was added in portion and the mixture was stirred at 0 °C for 1 h and added dropwise into the ice water (100 mL). The resulting mixture was extracted with EtOAc (100 mL × 3), washed by NaHCO$_3$ solution (100 mL × 2), dried over Na$_2$SO$_4$, and filtrated. The solvent was removed under reduced pressure to afford compound 2b as a pale yellow solid (19.1 g, yield 90.2%), which can be used directly in the next step without further purification. $^1$H NMR (400 MHz, CDCl$_3$) δ: 2.30 (q, $J = 6.27$ Hz, 2H), 2.60-2.67 (m, 2H), 3.08 (t, $J = 6.02$ Hz, 2H), 8.16 (s, 1H). Following our previous procedure, key intermediate 2e was obtained as a yellow solid (yield 85.3%). $^1$H NMR (400 MHz, CDCl$_3$) δ: 7.55 (s, 1H), 8.32 (s, 1H).

A solution of compound (E)-2-((E)-but-2-en-1-ylidene)-1,1-dimethylhydrazine (560 mg, 5.0 mmol) in anhydrous EtOH (5 mL) was added dropwise to a solution of 2e (717 mg, 2.5 mmol) in anhydrous EtOH (30 mL) at 0 °C. Then, sodium bicarbonate (425 mg, 5.0 mmol) was added in one portion. The mixture was then heated to 40 °C and stirred for 3 h. After cooling to room temperature, the mixture was poured into H$_2$O (50 mL) and extracted with CH$_2$Cl$_2$ (3 × 50 mL). The combined organic layers were washed with H$_2$O (3 × 25 mL), dried over Na$_2$SO$_4$, filtered and concentrated to dryness. The residue was purified by flash silica gel chromatography using EtOAc/Hexane (v/v = 1:3) as eluants to give compound ZG-20-07 (565 mg, yield 82.4%) as a yellow solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ: 8.89(d, $J = 4.64$ Hz, 1H), 8.50 (s, 1H), 7.75(d, $J = 4.76$, 1H), 2.79 (s, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ:
To a solution of 5-bromo-2-nitrobenzo[b]thiophene-4,7-dione (92 mg, 0.37 mmol) in anhrous EtOH (2 mL) was added pyrrolidine (27 mg, 0.37 mmol, 1 eq) at -25 °C. After stirring at the same temperature for 20 min, the reaction was quenched by cold water (2 mL). The reaction mixture was extracted with DCM (20 mL × 3) and the organic layer was washed with brine (20 mL × 3), dried over MgSO$_4$ and concentrated to give a crude product, which was further purified by column chromatography. Notably, two regioisomers ZG-20-41 and ZG-20-41(2) were obtained, which was purified by prep-HPLC. Preparative HPLC was carried out using a C18 reverse phase column (250 × 21.2 mm, 4μm; Phenomenex Synergi) with a linear gradient of 0–100% CH$_3$CN in 0.05% aqueous HCl over 20 min at 40 °C at a flow rate of 25.0 mL min. The eluents were collected at RT = 9.5 min for ZG-20-41 and at RT = 11.5 min for ZG-20-41(2).

2-Nitro-5-(pyrrolidin-1-yl)benzo[b]thiophene-4,7-dione (ZG-20-41). Yellow solid (14.3 mg, yield 32.6%). 1H NMR (400 MHz, CDCl$_3$) d: 8.17 (s, 1H), 5.61 (s, 1H), 2.03 (br s, 4H), 1.26 (br s, 4H); 13C NMR (100 MHz, CDCl$_3$) d: 177.45, 177.30, 156.93, 148.65, 142.74, 141.62, 125.32, 103.74, 51.44, 29.71. HRMS (ESI) m/z: 275.2172.
2-Nitro-6-(pyrrolidin-1-yl)benzo[b]thiophene-4,7-dione (ZG-20-41(2)). Brown solid (15.3 mg, yield 34.9%). 1H NMR (400 MHz, CDCl3) δ: 8.21 (s, 1H), 5.73 (s, 1H), 2.06 (br. s, 4H), 1.23 (br s, 4H); 13C NMR (100 MHz, CDCl3) δ: 177.15, 175.62, 157.30, 149.12, 142.23, 135.79, 125.55, 103.97, 59.85, 31.05. HRMS (ESI) m/z: calcd for C12H10N2O4S [M+H]+ 279.2103, Found 279.2102.
3. Structure-activity relationship of ZG-20-07 (MIC80, µg/mL)

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4. Effects of compounds ZG-20-07 and ZG-20-41 on biofilm formation imaged by scanning electron microscopy (SEM)

**Fig. S1** (A, B) Effects of different concentrations of compounds ZG-20-07 and ZG-20-41 on biofilm formation imaged by SEM. Images in the dashed boxes
are enlarged and the enlarged images are shown right.

5. Effects of compounds ZG-20-07 and ZG-20-41 on yeast-to-hypha morphological transition of *C. albicans*

Fig. S2 (A, B) Effects of different concentrations of compounds ZG-20-07 and ZG-20-41 on yeast-to-hypha morphological transition of *C. albicans*. Several hypha-inducing media were used, including YPD+FBS, Lee, Spider, SLAD, and RPMI1640. The cellular morphology was photographed after incubation at 37°C for 3 h.
References