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ORIGINAL ARTICLE

Mullinamides A and B, new cyclopeptides produced by the Ruth Mullins coal mine fire isolate *Streptomyces* sp. RM-27-46

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Two new cyclopeptides, mullinamides A [cyclo-(-L-Gly-L-Glu-L-Val-L-Ile-L-Pro-)] and B [cyclo-(-L-Glu-L-Met-L-Pro-)] were isolated from the crude extract of terrestrial *Streptomyces* sp. RM-27-46 along with the three known cyclopeptides surugamide A [cyclo-(-L-Ile-D-Ile-L-Lys-L-Ile-D-Phe-D-Leu-L-Ile-D-Ala-)], cyclo-(-L-Pro-L-Phe-) and cyclo-(-L-Pro-L-Leu-). The structures of the new compounds were elucidated by the cumulative analyses of NMR spectroscopy and HRMS. Although mullinamides A and B displayed no appreciable antimicrobial/fungal activity or cytotoxicity, this study highlights the first reported antibacterial activity of surugamide A.
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INTRODUCTION

Coal fires, initiated either via natural ignition or human intervention, can persist uncontrolled for decades in underground coal mines, coal waste piles and unmined coal beds. They occur worldwide in all coal-bearing geographies and produce substantial greenhouse gas and toxic emissions, which, in the case of underground coal fires, are exhausted through thermal vents and seams.[1–4] As such, these exhaust ports and adjacent environments offer highly unique soil/atmospheric conditions likely to impact upon the metabolic output of terrestrial actinomycetes. Within this context, we have focused upon two underground coal mine fire sites in eastern Kentucky (Ruth Mullins and Truman Shepard)[5–8] and this effort has facilitated the discovery of a range of new secondary metabolites from corresponding *Streptomyces* strains including the recently reported unique tetracyclic polyketide ruthmycin.[9–13] As an extension of this ongoing effort, herein, we describe the isolation, structure elucidation and preliminary biological activity assessment of five cyclic peptides (Figure 1) from the previously unreported Ruth Mullins isolate *Streptomyces* sp. RM-27-46. Notably, two of the cyclic peptides, mullinamides A [cyclo-(-L-Gly-L-Glu-L-Val-L-Ile-L-Pro-)] (**1**) and B [cyclo-(-L-Glu-L-Met-L-Pro-)] (**2**), are new and a third, surugamide A, is a cyclic octapeptide recently reported as a metabolite of a marine *Streptomyces* isolated near Japan.[14] Although surugamides were previously found to weakly inhibit caspase *in vitro*, the current study is also the first to divulge the moderate antibacterial activity of surugamide A (*Staphylococcus aureus* MIC of 10 μM).

RESULTS AND DISCUSSION

Compound **1** was obtained as white powder (Table 1). The molecular formula of **1** was established as $C_{23}H_{37}N_5O_7$ on the basis of HR-ESI-MS (Table 1) and NMR data, indicating eight degrees of unsaturation. The proton NMR (Table 2) of **1** in dimethyl sulfoxide (DMSO)-d_6 displayed typical features of a peptide-derived compound, including four amide protons at δ_H 8.07 (t, 5.4), 8.00 (d, 6.8), 7.87 (d, 8.7) and 7.84 (d, 8.3), and five α-protons at δ_H 4.42, 4.19, 4.12, 4.09, 4.00 and 3.80 p.p.m. The ^{13}C-NMR of **1** (Table 2) was also consistent with a peptidic nature with six amide/acid carbonyls (δ_C 181.7, 175.5, 174.6 (two overlapped carbon signals), 174.0, 169.5) and five α-carbon signals (δ_C 61.5, 59.4, 59.0, 58.3, 42.7). The 1D (^{1}H and ^{13}C) and 2D (COSY, TOCSY, HSQC and HMBC) NMR data revealed that **1** was composed of five amino acid residues. Specifically, the independent spin system of the type $XCHCH_2CH_2X'$ suggested the presence of a glutamic acid unit, whereas the spin systems of $XCHCH(CH_3)_2$, $XCHCH(CH_3)CH_2CH_3$ and $XCHCH_2CH_2CH_2X'$ identified from TOCSY experiments were indicative of valine, isoleucine and proline residues. One remaining CH_2 group, which lacked correlations with other protons in the COSY and TOCSY spectra, suggested the presence of a glycine moiety. Five carbonyl signals and the proline

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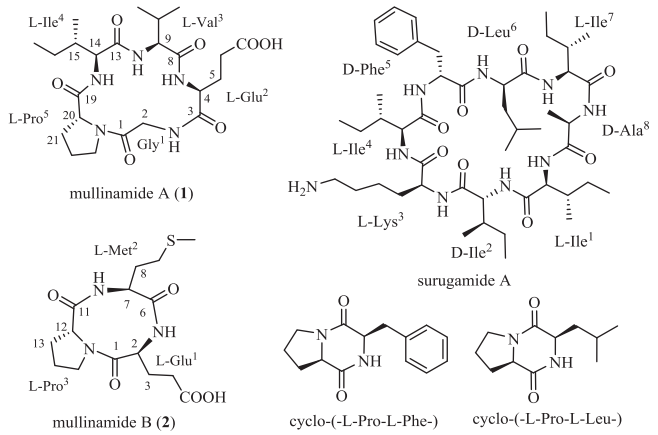

Figure 1 Structures of compounds isolated from *Streptomyces* sp. RM-27-46.

Table 1 Physico-chemical properties of mullinamides A (1) and B (2)

	*Mullinamide A (**1**)*	*Mullinamide B (**2**)*
Molecular formula	$C_{23}H_{37}N_5O_7$	$C_{15}H_{23}N_3O_5S$
Appearance	White powder	White powder
UV	End absorption	End absorption
$[\alpha]_D^{25}$	−36.9° (*c* 2.5, MeOH)	−10.6° (*c* 0.3, MeOH)
(+)-ESI-MS: *m/z*	496 $[M+H]^+$, 991 $[2\ M+H]^+$	358 $[M+H]^+$, 715 $[2\ M+H]^+$
(−)-ESI-MS: *m/z*	494 $[M-H]^-$	—
(+)-HR-ESI-MS: *m/z*	496.2767 $[M+H]^+$, 518.2590 $[M+Na]^+$	358.1434 $[M+H]^+$,
Calcd.	496.2771 for $C_{23}H_{38}N_5O_7$ $[M+H]^+$, 518.2591 for $C_{23}H_{37}N_5O_7Na$ $[M+Na]^+$	358.1437 for $C_{15}H_{24}N_3O_5S$ $[M+H]^+$
(−)-HR-ESI-MS: *m/z*	494.2627 $[M-H]^-$	356.1287 $[M-H]^-$
Calcd.	494.2615 for $C_{23}H_{36}N_5O_7$ $[M-H]^-$	356.1280 for $C_{15}H_{22}N_3O_5S$ $[M-H]^-$

ring accounted for seven of the requisite eight degrees of unsaturation as dictated by the molecular formula, suggesting a cyclic form. Key connections among the five amino acid residues were established via HMBC correlations (Figure 2): 2-NH (δ_H 8.07) to C-2 (δ_C 42.7) and C-3 (δ_C 175.5); 4-NH (δ_H 8.00) to C-3 (δ_C 175.5) and C-8 (δ_C 174.6); 9-NH (δ_H 7.84) to C-8 (δ_C 174.6) and C-13 (δ_C 174.0); 14-NH (δ_H 7.87) to C-13 (δ_C 174.0) and C-19 (δ_C 174.6); and H-23 (δ_H 3.43, 3.52) to C-1 (δ_C 169.5). Importantly, these connections established the sequence **1** as cyclo-(-Gly-Glu-Val-Ile-Pro-). Subsequent Marfey's analysis[15] of the total hydrolysate of **1** using 6 N HCl overnight revealed the building blocks of **1** to be proteinogenic amino acids (L-Glu, L-Ile, L-Pro and L-Val). Thus, the structure of **1** was assigned as cyclo-(-L-Gly-L-Glu-L-Val-L-Ile-L-Pro-) and, as a new cyclopeptide, was named mullinamide A in reference to its point of origin.

Compound **2** was obtained as a white powder and its molecular formula was deduced as $C_{15}H_{23}N_3O_5S$ from its HR-ESI-MS at *m/z* 358.1434 $[M+H]^+$ (Calcd. 358.1437 for $C_{15}H_{24}N_3O_5S$ $[M+H]^+$), Table 1. The ^{1}H NMR spectrum (Table 2) of **2** also revealed peptide features with three α-protons at δ_H 4.64, 4.24 and 4.05 and two amide protons at δ_H 8.28 and 7.80 p.p.m. The planar structure of **2** was similarly established by 1D and 2D NMR analysis (Figure 2) to be cyclo-(-Glu-Met-Pro-), and the absolute amino acid configurations subseqeuntly identified by the total hydrolysis of **2** using 6 N HCl, followed by Marfey's analysis method as described for **1**. On the basis of the cumulative data, compound **2** was identified as cyclo-(-L-Glu-L-Met-L-Pro-), and, as a new cyclopeptide, was named mullinamide B in reference to its point of origin.

Three additional compounds were also isolated from *Streptomyces* sp. RM-27-46 and identified as surugamide A [cyclo-(-L-Ile-D-Ile-L-

Table 2 1H and ^{13}C-NMR data for mullinamides A (1) and B (2)

Position	δ_H[a] *(J in Hz)*	δ_H[b] *(J in Hz)*	δ_C[c], *type*
*Mullinamide A (**1**)*			
Gly			
1			169.5, qC
2a	3.97, d (16.8)	3.80, dd (5.8, 17.1)	42.7, CH_2
2b	4.16, d (16.8)	4.00, dd (4.9, 17.1)	
NH		8.07, t (5.4)	
Glu			
3			175.5, qC
4	4.22, m	4.12, m	58.3, CH
5	2.11, 2.48, m	2.42, m	26.8, CH_2
6	2.32, 2.42, m	2.04, 2.12, m	30.6, CH_2
7			181.7, qC
NH		8.00, d (6.8)	
Val			
8			174.6, qC
9	4.32, m	4.09, m	59.0, CH
10	2.18, m	2.01, m	31.9, CH
11	0.96[d]	0.94[d]	18.4, CH_3
12	0.96[d]	0.94[d]	19.7, CH_3
NH		7.84, d (8.3)	
Ile			
13			174.0, qC
14	4.25, m	4.19, m	59.4, CH
15	1.86, m	1.70, m	38.0, CH
16	1.20, 1.60, m	1.06, 1.45, m	25.8, CH_2
17	0.90, t (7.6, 7.2)	0.80, t (7.4)	11.4, CH_3
18	0.96[d]	0.93[d]	16.0, CH_3
NH		7.87, d (8.7)	
Pro			
19			174.6, qC
20	4.49, m	4.42, m	61.5, CH
21	1.94, 2.19, m	1.75, 1.96, m	30.7, CH_2
22	2.00, m	1.86, m	26.1, CH_2
23	3.61, m	3.43, 3.52, m	47.9, CH_2
*Mullinamide B (**2**)*			
Glu			
1			175.3, qC
2	4.22, m	4.05, m	57.8, CH
3	2.29, 2.45, m	2.16, m	26.7, CH_2
4	2.06, 2.38, m	1.84, m	30.9, CH_2
5			181.7, qC
NH		7.80, d (4.6)	
Met			
6			175.1, qC
7	4.81, m	4.64, m	51.7, CH
8	1.96, 2.08, m	1.80, m	31.9, CH_2
9	2.63, m	2.52, m	30.6, CH_2
10	2.11, s	2.03, s	15.4, CH_3
NH		8.28, d (6.7)	
Pro			
11			172.4, qC
12	4.45, m	4.24, m	60.5, CH
13	2.02, 2.29, m	1.81, 2.15, m	30.3, CH_2
14	2.06, m	1.90, m	26.0, CH_2
15	3.75, 3.87, m	3.59, 3.68	48.7, CH_2

[a]measured in CD_3OD, 400 MHz.
[b]measured in DMSO-d_6, 500 MHz.
[c]measured in CD_3OD, 100 MHz.
[d]overlapped.

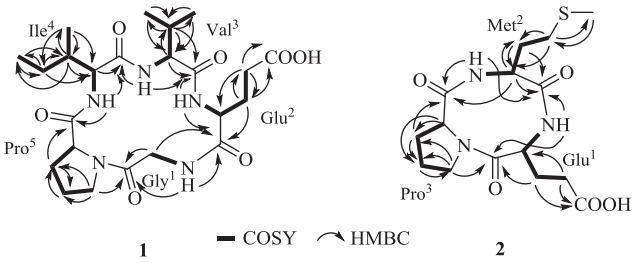

Figure 2 1H, 1H-COSY (▬) and selected HMBC (→) correlations of mullinamides **a** (**1**) and **b** (**2**).

Lys-L-Ile-D-Phe-D-Leu-L-Ile-D-Ala-)],[14] cyclo-(-L-Pro-L-Phe-)[16,17] and cyclo-(-L-Pro-L- Leu-)[17] based upon correlation of cumulative NMR and HRMS data to that reported in the literature.

Mullinamides A (**1**) and B (**2**) displayed no antibacterial (*Staphylococcus aureus* ATCC 6538, *Salmonella enterica* ATCC 10708) or antifungal (against *Saccharomyces cerevisiae* ATCC 204508) activities at or below 100 μM and no cytotoxicity against human cancer cell lines (A549 non-small cell lung, PC3 prostate) at or below 10 μM. In contrast, surugamide A (previously only reported to inhibit bovine cathepsin B with an IC_{50} of 21 μM)[14] displayed moderate growth inhibition against *Staphylococcus aureus* (MIC of 10 μM). Although the biosynthetic loci encoding surugamides were previously noted to be widely distributed among marine *Streptomyces* species collected near Japan,[14] the current study suggests surugamide production may be a much broader phenomenon than previously appreciated.

MATERIALS AND METHODS

General experimental procedures

Optical rotation was recorded on a Jasco DIP-370 Digital Polarimeter (Jasco, Easton, MD, USA). All NMR data were recorded at 500 MHz or 400 MHz for ^1H and 100 MHz for ^{13}C with Varian Inova NMR spectrometers (Agilent, Santa Clara, CA, USA). LC-MS was conducted with a Waters 2695 LC module (Waters, Milford, MA, USA), equipped with a micromass ZQ and a Symmetry Anal C_{18} column (4.6 × 250 mm, 5 μm; Waters). HR-ESI-MS spectra were recorded on an AB SCIEX Triple TOF 5600 System (AB Sciex, Framingham, MA, USA). HPLC analyses were performed on an Agilent 1260 system equipped with a photodiode array detector) detector and a Phenomenex C_{18} column (250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA). Semipreparative HPLC separation was performed on a Varian Prostar 210 HPLC system (Agilent) equipped with a photodiode array detector detector 330 using a Supelco DiscoveryBio wide pore C_{18} column (250 × 21.2 mm, 10 μm; flow rate, 8 ml min^{-1}; Sigma-Aldrich, St Louis, MO, USA). All solvents used were of American Chemical Society grade and purchased from Pharmco-AAPER (Brookfield, CT, USA). Sephadex LH-20 (25 ~ 100 μm) was purchased from GE Healthcare (Little Chalfont, UK). C_{18}-functionalised silica gel (40 ~ 63 μm) was purchased from Material Harvest (Cambridge, UK). AmberliteXAD16N resin (20–60 mesh) was purchased from Sigma-Aldrich. TLC silica gel plates (60 F_{254}) were purchased from EMD Chemicals (Darmstadt, Germany).

Isolation of *Streptomyces* sp. RM-27-46

The soil sample was collected from the Ruth Mullins underground coal mine fire site, Perry County, KY (coordinates: N 37° 18.725′ and W 83° 10.3335′). For strain purification, 0.5 g of soil sample was suspended in 1.0 ml of sterile water and the suspension was heated at 75 °C for 10 min to eliminate non-sporulating bacteria. Following serial dilution (10^{-1}, 10^{-2}, 10^{-3}) of the suspension with sterile water, a 100 μl aliquot was spread on oatmeal agar (Difco, Ref: 255210, Becton, Dickinson and Company, Sparks, MD, USA) and on ISP4 agar (Difco, Ref: 277210, Becton, Dickinson and Company) plates supplemented with nalidixic acid (75 μg ml^{-1}) and cycloheximide (50 μg ml^{-1}). A number of sporulating bacterial colonies were observed after a week of incubation at 28 °C and each colony was subsequently streaked on a M_2-agar plate (glucose (4.0 g), malt extract (10.0 g), yeast extract (4.0 g), $CaCO_3$ (1.0 g) and agar (15.0 g) were dissolved in 1 l of demineralized water). Individual bacterial colonies were isolated from the second generation plates. AntiBase[18] comparison of HPLC–HRMS profiles of the culture extracts of 46 actinomycete strains isolated from a single soil sample collected near a thermal vent associated with the Ruth Mullins underground coal mine fire indicated that one of these, *Streptomyces* sp. RM-27-46, was capable of unique metabolite production.

Identification of *Streptomyces* sp. RM-27-46

Genomic DNA was isolated from a fully grown colony using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Rockford, IL, USA). The partial 16S rRNA gene fragment was amplified using universal primers (27F, 5′-AGAGTTTGATCMTGGCTCAG-3′; 1492R, 5′-GGTTACCTTGTTACGACT T-3′)[19] and Phusion High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) and was gel-purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). The amplified fragment (1311 bp) displayed 99% identity (BLAST search) to the 16S rRNA gene sequence of *Streptomyces exfoliatus* strain NBRC 13475. The sequence of 16S rRNA has been deposited in the NCBI nucleotide database under accession number KF732715.

Host fermentation and metabolite extraction, isolation and purification

Streptomyces sp. RM-27-46 was cultivated on M_2-agar plates at 28 °C for 7 days. Chunks of agar with the fully grown strain were used to inoculate five 250 ml Erlenmeyer flasks, each containing 50 ml of medium A (glucose (10.0 g), yeast extract (5.0 g), soluble starch (20.0 g), peptone (5.0 g), NaCl (4.0 g), K_2HPO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5) and $CaCO_3$ (2 g) were dissolved in 1 l of demineralized water). Individual cultures were grown at 28 °C with shaking (200 r.p.m.) for 3 days and subsequently used as seed cultures for the scale-up fermentation. The seed cultures were used to inoculate 100 Erlenmeyer flasks (250 ml) each containing 100 ml of medium A. The fermentation (10 l) was continued for 7 days at 28 °C with 200 r.p.m. agitation. The obtained culture broth was centrifuged at 5000 r.p.m. for 30 min. The biomass (mycelium) was extracted with MeOH (3 × 500 ml) and then the recovered organics were evaporated *in vacuo* at 40 °C to yield 5.1 g of crude extract. The supernatant was mixed with 3% (w/v) XAD-16 resin and stired overnight, followed by filtration. The resin was washed with water (3 × 600 ml) and then extracted with MeOH until the eluant was colorless. The methanol extract was subsequently evaporated to afford 15.6 g of crude extract. Both extracts (obtained from the biomass and supernatant) revealed a similar metabolite profile based upon HPLC and TLC analysis and were therefore combined (20.7 g) before further isolation. The crude extract was then subjected to a silica gel column chromatography (15 × 8 cm, 250 g) eluted with a gradient of $CHCl_3$-MeOH (100:0–0:100) to yield eight fractions, A–H. Fraction B (850 mg) was subjected to Sephadex LH-20 column followed by semipreparative HPLC (mobile phase: 5–35% aqueous CH_3CN over 25 min, 8 ml min^{-1}) to yield 24.0 mg of cyclo-(-L-Pro-L-Phe-; retention time: 15.0 min) and 15.0 mg of cyclo-(-L-Pro-L-Leu-; retention time: 9.1 min). Fraction D (320 mg) was first subjected to a Sephadex LH-20 column (MeOH) to obtain four subfractions, D-1—D-4. Subfraction D-2 (102 mg) was resolved by semipreparative HPLC (mobile phase: 10–35% aqueous CH_3CN in 25 min, flow rate: 8 ml min^{-1}) to yield reginamide A (14.0 mg; retention time: 11.4 min). Compound **2** (3 mg, retention time: 10.2 min) was isolated as a white powder from the subfraction D-3 (52.0 mg) also via semipreparative HPLC (mobile phase: 20–60% aqueous CH_3CN over 25 min, 8 ml min^{-1}). Fraction E (200 mg) was subjected to a Sephadex LH-20 column (50 × 2 cm) using methanol to elute target compounds. The major fraction obtained from the Sephadex LH-20 column was dried and then further purified using semipreparative HPLC (mobile phase: 10–35% aqueous CH_3CN in 25 min, flow rate: 8 ml min^{-1}) to yield compound **1** (25.0 mg, retention time: 11.6 min). On the basis of HPLC and TLC, the remaining fractions (A, C, and F −H) and subfractions lacked additional metabolites of interest and were therefore excluded from further consideration.

Determination of amino acid configuration

The absolute configuration of each amino acid residue was determined following Marfey's method.[15] Specifically, compounds **1** or **2** (each 1.0 mg) were hydrolyzed in 6 N HCl (1 ml) at 110 °C for 14 h. After drying under nitrogen, the corresponding hydrolysate was dissolved in 2 ml of EtOAc-H_2O (1:1). The aqueous layer was dried in vacuo, to which a solution of 1% Marfey's reagent in acetone (200 µl) was subsequently added, followed by 1 M $NaHCO_3$ (50 µl). The reaction was heated to 40 °C for 1 h, cooled to room temperature and acidified with 2 N HCl (25 µl). The reaction mixture was diluted with MeOH (0.5 ml) and analyzed by HPLC using the following gradient: 0–55 min, linear gradient from 10–55% CH_3CN in 50 mM triethylammonium phosphate buffer (Phenomenex C_{18} column, 250 × 4.6 mm, 5 µm; 1 ml min^{-1}; 430 nm). Derivatized standards were prepared from the authentic D- and L-glutamic acid, valine, isoleucine, proline and methionine (50 µl of a 50 mM stock) following an identical procedure. The retention times for Marfey's derivatives of authentic D-Glu, L-Glu, D-Val, L-Val, D-Ile, L-Ile, D-Pro, L-Pro, D-Met and L-Met were 24.7, 22.6, 36.8, 31.8, 41.2, 36.1, 28.5, 26.3, 35.0 and 30.4 min, respectively. Those for the Marfey's derivatives of Glu, Val, Ile and Pro in the hydrolysate of **1** were 22.5 (L-Glu), 31.7 (L-Val), 36.1 (L-Ile) and 26.2 (L-Pro)min, respectively. Those of **2** were 22.6 (L-Glu), 30.3 (L-Met), 26.2 (L-Pro) min, respectively.

Cell viability assay

A resazurin-based cytotoxicity assay, also known as AlamarBlue assay, was used to assess the cytotoxicity of agents against the human lung non-small cell carcinoma cell line A549 and human prostate cancer cell lines PC3 where the degree of cytotoxicity was based upon residual metabolic activity as assessed via reduction of resazurin (7-hydroxy-10-oxido-phenoxazin-10-ium-3-one) to its fluorescent product resorufin. A549 and PC3 cells, purchased from ATCC (Manassas, VA, USA), were grown in Dulbecco's Modified Eagle Medium/F-12 Kaighn's modification and minimum essential media/Earle's balanced salt solution media, respectively (Thermo scientific HyClone, Logan, UT, USA), with 10% heat-inactivated fetal bovine serum, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM L-glutamine. Cells were seeded at a density of 2×10^3 cells per well onto 96-well culture plates with a clear bottom (Corning, NY, USA), incubated 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 and were exposed to test agents for 2 days (positive controls: 1.5 mM hydrogen peroxide, 10 µg ml^{-1} actinomycin D). Resazurin (150 µM final concentration) was subsequently added to each well and the plates were shaken briefly for 10 seconds and were incubated for another 3 h at 37 °C to allow viable cells to convert resazurin into resorufin. The fluorescence intensity for resorufin was detected on a scanning microplate spectrofluorometer FLUOstar Omega (BMG LABTECH GmbH, Ortenberg, Germany) using an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The assay was repeated in 3 independent experimental replications. In each replication, the emission of fluorescence of resorufin values in treated cells were normalized to, and expressed as a percent of, the mean resorufin emission values of untreated control (metabolically active cells; 100%, all cells are viable).

Antibacterial and antifungal activity tests

Antibacterial assay. The protocol used for the determination of MIC was based upon minor modifications of previously published protocol.[20] Two bacterial strains (*Staphylococcus aureus* (ATCC 6538) and *Salmonella enterica* (ATCC 10708)) were used as model strains for the assay. Single colonies from each strain were grown in 5 ml of tryptic soy broth (BD 211825) and nutrient broth (BD 234000) media, respectively, and the cultures were allowed to grow overnight at 37 °C with shaking at 220 r.p.m. The overnight culture for each strain was diluted to OD_{600} 0.8–1.1 and 150 µl of the diluted culture was added to each well of a sterile clear polystyrene 96-well plate (TPP, Fisher Scientific, Pittsburgh, PA, USA). Test agents were dissolved in DMSO, serially diluted and aliquots added to the plate-based wells (kanamycin, positive control; DMSO, vehicle control). Plates were incubated at 37 °C for 16 h with shaking (150 r.p.m.). Samples (final concentrations 1–60 µM) and controls were tested in triplicate. The OD_{600} was subsequently measured using a scanning microplate spectrofluorometer FLUOstar Omega (BMG Labtech, Cary, NC, USA). The acquired OD_{600} values were normalized to the negative control wells (as 100% viability). The minimal concentration of the tested sample that caused growth inhibition was recorded as the MIC.

Antifungal assay. The yeast *Saccharomyces cerevisiae* (ATCC 204508) was used in a broth microdilution antifungal assay. The assay was performed in a 96-well plate using the CLSI (formerly NCCLS) guidelines.[21] Individual colonies from an overnight plate were used to innoculate 5 ml YAPD (ATCC medium number 1069) and incubated overnight at 37 °C. The overnight culture was diluted to OD_{600} of 0.1 using sterile YAPD and 150 µl of the diluted culture was transferred to each well of a sterile clear polystyrene 96-well plate (TPP, Fisher Scientific). Test agents were dissolved in DMSO, serially diluted and aliquots added to the plate-based wells (amphotericin B, positive control; DMSO, vehicle control). The plate was incubated at 37 °C for 16 h with shaking (200 r.p.m.) and the OD_{600} was subsequently measured using a scanning microplate spectrofluorometer FLUOstar Omega (BMG Labtech). The acquired OD_{600} values were normalized to the negative control wells (as 100% viability). The minimal concentration of the tested sample that caused growth inhibition was recorded as the MIC. Samples (final concentrations 1–60 µM) and controls were tested in triplicate.

CONFLICT OF INTEREST

JST is a co-founder of Centrose (Madison, WI, USA). The remaining authors declare no conflict of interest.

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