Hongoquercins, New Antibacterial Agents from the Fungus LL-23G227: Fermentation and Biological Activity

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Two new antibiotics, hongoquercins A and B, were isolated from fermentation extracts of the unidentified fungus LL-23G227. In the optimum medium, titers of the A and B components reached approximately 2.1 g/liter and 0.02 g/liter, respectively. The optimum temperature for antibiotic production was approximately 22°C. Growth was delayed at 15°C but appeared to reach higher levels than was observed at 22°C. Addition of dextrose to growth media increased hongoquercin B production. Hongoquercin A exhibited moderate activity against Gram-positive bacteria. Mechanistic studies conducted in an *E. coli imp* strain suggested membrane damage as the primary mode of bactericidal action. These compounds also lysed human red blood cells, suggesting a similar mode of action on eukaryotic cells.

The increasing incidence of antibiotic resistance among Gram-positive pathogens has lead to the appearance of recalcitrant or untreatable bacterial infections. In response, many pharmaceutical companies have initiated or expanded programs to discover more effective antibiotics. During our screening of microbial extracts for antibacterial activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*, the fungal culture LL-23G227 (Fig. 1) was selected for further study. This isolate was of particular interest because preliminary efforts to classify it were unsuccessful, suggesting that it may be taxonomically rare. Furthermore, this strain was isolated in Costa Rica, a location known for its extraordinary biodiversity. The bioactive components were identified as the new metabolites hongoquercins A and B (Fig. 2).[1] These compounds appear related to a class of sesquiterpene-substituted phenols commonly found in sponges[2] and a few brown algae.[3] Similar fungal metabolites reported in the literature are siccanin[4] and presiccanochromenate.[5] This paper describes the production and biological activities of hongoquercins A and B.

Fig. 1. Photograph of culture LL-23G227.

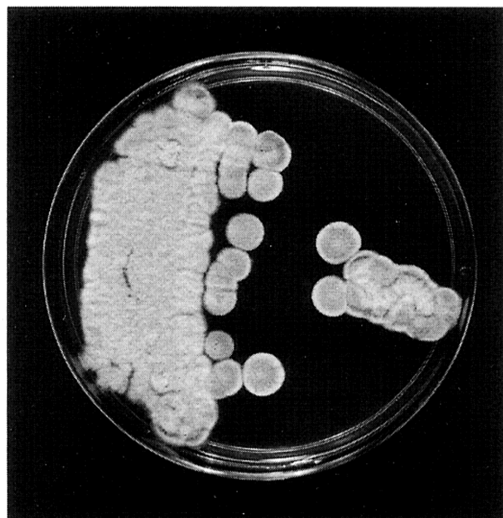
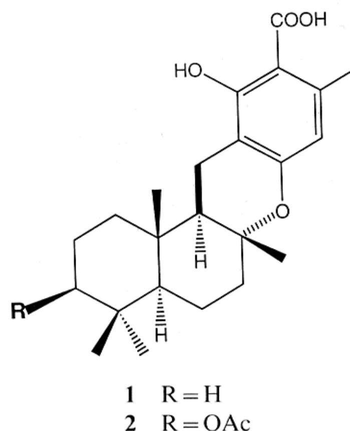


Fig. 2. Structures of hongoquercins A (**1**) and B (**2**).



Results and Discussion

Production of Hongoquercins A (1) and B (2)

During the initial screening of extracts obtained from solid and liquid fermentations, a solid cracked corn medium was found to be superior to the other media for production of antimicrobial activity. On this medium, accumulation of **1** peaked by day 3, reaching titers of 78 mg per 240 grams of cracked corn (1 Fernbach flask equivalent). In contrast, titers of **2** increased steadily through day 7, reaching levels of approximately 29 mg per 240 grams of cracked corn.

To produce larger quantities of the hongoquercins, various liquid media were tested. In the selected medium CSO-5, titers of **1** and **2** began to accumulate after 48 hours, reaching levels of 2.1 g/liter and 0.02 g/liter, respectively, after approximately 156 hours (Fig. 4). In contrast, cell mass reached maximum levels within 84 hours, indicating that in this medium, secondary metabolite production occurred during a distinct idiophase.

Preliminary studies suggested that hongoquercin B accumulation increased with the addition of dextrose to the fermentation medium. To further study the effects of dextrose on hongoquercin production, LL-23G227 was grown in medium SO-11 (previously shown to produce relatively high titers of **1** and **2**) with glycerol (2%) or dextrose (3%) as sole carbohydrate supplement. Replacement of dextrose with glycerol decreased

Fig. 3. Hongoquercin production in cracked corn medium.

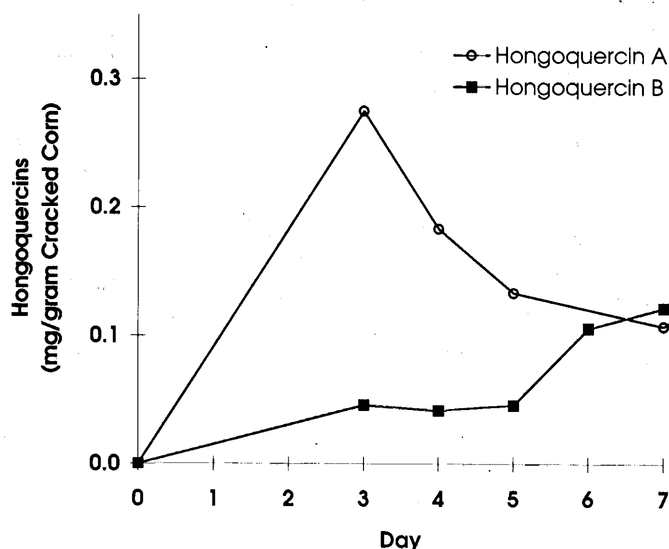


Fig. 4. Growth and production of hongoquercin in CSO-5 medium.

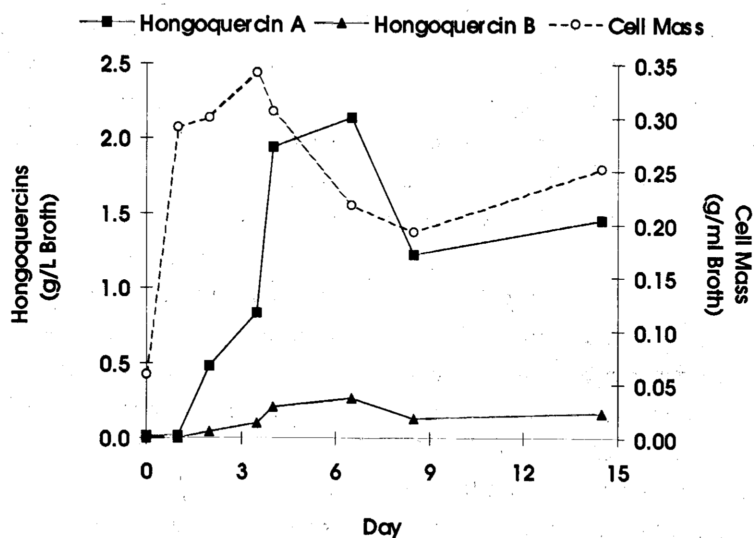
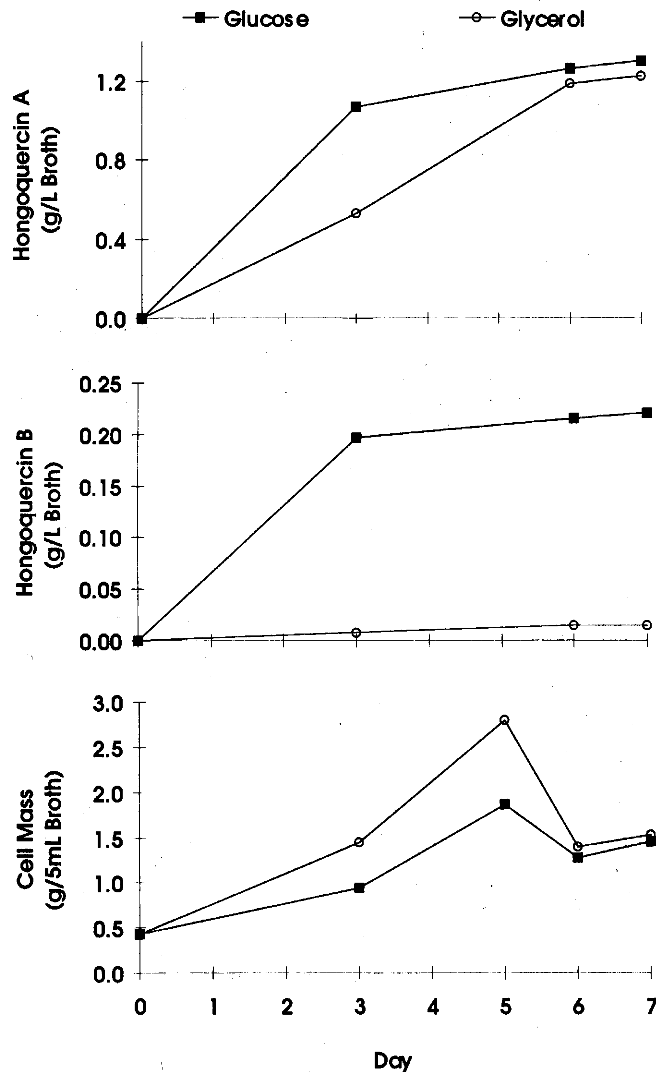


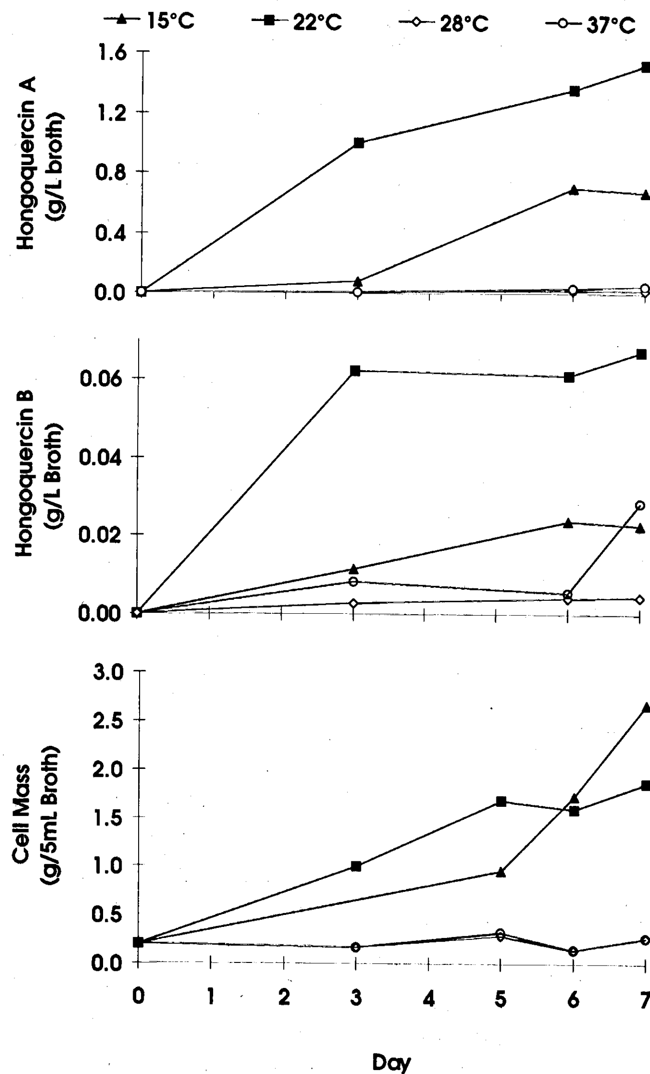
Fig. 5. Effects of glucose or glycerol on growth and hongoquercin production.



hongoquercin B approximately 10-fold but had little effect on the production of hongoquercin A (Fig. 5). Because hongoquercins A and B differ only in an acetate group, we speculate that metabolism of dextrose may increase the intracellular pools of acetate or acetyl CoA, stimulating increased production of hongoquercin B. Surprisingly, glycerol appears to have stimulated cell growth (Fig. 5), a factor which could contribute to the depletion of acetate pools, and the decreased synthesis of hongoquercin B.

The effect of temperature on secondary metabolite production in microorganisms is well documented.[6,7] To identify the optimum temperature range for growth and hongoquercin production, fermentations in CSO-5 medium were incubated at 15°C, 22°C, 28°C and 37°C. The optimum temperature for hongoquercin production was 22°C (Fig. 6). In contrast, although the growth rate at 22°C initially exceeded that at 15°C, the amount of cell mass accumulating at 15°C ultimately appeared to be greater. Growth and hongoquercin production were not observed at 28°C, suggesting a preference by LL-23G227 for relatively cool growth conditions. The annual temperature of the San Jose province of Costa Rica, the region from which culture LL-23G227 was isolated, ranges from approximately 14°C to 27°C, and averages 23°C. The ability of this culture to grow at 15°C and 22°C, but not at 28°C is consistent with the temperature ranges of its niche. Interestingly, the fermentation temperature (22°C) which supported production of higher hongoquercin titers coincided with the average annual temperature in San Jose. Similar results linking the temperature of an isolate's natural environment and the preferred temperature for secondary metabolite production were recently reported for the marine fungus

Fig. 6. Temperature optimum for growth and hongoquercin production.



Hypoxylon oceanicum.[8] This potential relationship between the average annual temperature of an isolate's niche and the optimum temperature for secondary metabolite production warrants further study.

Biological and Mechanistic Activity

The antibacterial activity of hongoquercin A is summarized in Table 1. It exhibited moderate activity against Gram-positive bacteria, but was inactive against Gram-negative organisms and *Candida albicans*. It exhibited good activity against *E. coli imp* but not against wild-type *E. coli* strains, suggesting that this compound has difficulty passing through the normal Gram-negative outer cell membrane. Hongoquercin B was generally inactive against all test organisms, but it did show some activity against *E. coli imp* and a strain of *B. subtilis* (MIC, 16 and 32 μg/ml, respectively; data not shown). Both compounds were inactive in medium containing 5% sheep's blood, suggesting strong serum binding of these compounds (data not shown).

Inhibition of DNA, RNA, and protein synthesis was determined by measuring the incorporation of ^{3}H-Tdr, ^{3}H-Udr, and ^{3}H-AA, respectively, into TCA-precipitable material of a logarithmic-phase culture of *E. coli imp*. Control drugs affected the anticipated macromolecular processes (Table 2). Treatment with **1** for 5~10 minutes inhibited the uptake and the incorporation of all three radiolabeled precursors. Inhibition patterns were similar to those of polymyxin B, suggesting an instant disruption of membrane integrity. The lysis of human RBCs (hRBC) and the leakage of intracellular potassium from bacteria further support the membrane-damaging effects of **1** (Table 3). Hongoquercin B was much less active against bacterial isolates.

Consistent with this lack of antibacterial activity, it also failed to induce leakage of intracellular potassium from *E. coli imp* and to cause hRBC lysis.

Experimental

Isolation of Organism

Culture LL-23G227 was isolated by MYCOsearch (MYCOsearch Natural Products Discovery Center, OSI Pharmaceuticals Inc., 4727 University Drive, Suite 400, Durham, NC 27707) from leaf litter in the San Jose Province of Costa Rica. Taxonomically, the fungus is unidentified, producing sterile, nonsporulating colonies (Fig. 1) with dematiaceous mycelia and hyphae diameters ranging from 2 μm to more than 5 μm.

Table 1. Antibacterial activity [MIC (MBC), μg/ml] of hongoquercin A.

Organism	Hongo-quercin A	Penicillin G
Bacillus subtilis 327	0.50 (1)	≤0.06
Staphylococcus aureus 375	2 (4)	≤0.06
Staphylococcus aureus 310	2 (8)	64
Enterococcus faecalis 422	2 (8)	≤0.06
Enterococcus faecium 379	2 (8)	32
Escherichia coli imp 389	4 (8)	1
Escherichia coli 81	>128	16
Klebsiella pneumoniae 425	>128	32
Proteus mirabilis 99	>128	16
Pseudomonas aeruginosa 339	>128	>128
Candida albicans 54	>128	>128

Broth microdilution method; medium, TSB for Ef422 & 379, YMB for Ca54, and MHB for all other organisms; inoculum, $1 \sim 5 \times 10^5$ cfu/ml; incubation, 35°C for 18 hours. Final concentration of DMSO in the medium was 2.5%, which did not interfere with growth of the organism.

Fermentation

Seed: Culture LL-23G227 was inoculated into 50 ml of potato dextrose medium in a 250 ml Erlenmeyer flask and incubated for 7 days at 22°C and 200 rpm. The culture was then transferred to second stage potato dextrose seed flasks and incubated as above for 8 days.

Production: Solid medium fermentations were performed with cracked corn/yeast extract medium in 250 ml Erlenmeyer flasks (12 g cracked corn with 5 ml 0.1% yeast extract in water). Flasks were inoculated with 2.5 ml of seed and incubated at 22°C for 7 days. Cultures were extracted with 30 ml of methanol and were analyzed by HPLC. Hongoquercins A and B were quantified by comparison of peak areas with those of purified standards. The time course for the production of hongoquercins A and B in the cracked corn medium was determined by inoculating replicate flasks and extracting the contents of a flask at each time point.

Liquid fermentations were performed with CSO-5 medium (1% dextrose, 2% cornstarch, 1.5% molasses, 0.5% bacto peptone, 0.5% soy flour, 0.2% yeast extract, 0.1% calcium carbonate, pH 7) in 250 ml Erlenmeyer flasks (50 ml medium), 2.8 liter Fernbach flasks (1 liter medium) or 10 liter and 30 liter fermentors. All vessels were inoculated with a 5% volume of seed per volume

Table 2. Effect of hongoquercin A and reference antimicrobial agents on radiolabeled precursor uptake and incorporation into macromolecules in *E. coli imp*.

Compound	Conc. (μg/ml)	% ^{3}H-Tdr		% ^{3}H-Udr		% ^{3}H-AA	
		Uptake	Incorp.	Uptake	Incorp.	Uptake	Incorp.
Hongoquercin A	2	91	85	91	81	95	79
	4	84	82	90	81	65	64
	8	77	27	46	28	31	32
	16	40	7	11	7	42	14
	32	30	2	1	1	33	11
	64	1	1	0	0	18	13
Ciprofloxacin	0.25	57	13	103	57	105	93
Rifampicin	0.25	159	90	46	3	91	37
Chloramphenicol	8	112	90	109	92	45	16
Polymyxin B	8	3	1	7	2	22	3

Data represent % of untreated control after 5 minutes drug pretreatment and 5 minutes pulse labeling.
Uptake = total radiolabeled precursor remaining in the cells after an instant saline wash.
Incorp. = precursor incorporated into TCA-precipitable material.
^{3}H-Tdr, ^{3}H-Udr and ^{3}H-AA are tritiated thymidine, uridine and amino acids, respectively.

Table 3. Membrane-damaging effects on human RBC and *E. coli imp*.

Compound	μg/ml	RBC lysis (%)[a]	K-Leakage (%)[a]
Hongoquercin A	64	97	37
	32	97	nt
	16	97	nt
	8	2	nt
Hongoquercin B	64	1	5
	32	0	0
Polymyxin B	32	4	60
Amphotericin B	4	60	nt
	2	10	nt
	1	1	nt

[a] Drug treatment periods for RBC lysis and potassium leakage from *E. coli* were 2 and 1 hour, respectively.
nt, not tested.

production medium and were incubated at 22°C. Flask fermentations were incubated at 200 rpm, 2 in. orbit for 7~14 days. Fermentors (10 liter or 30 liter) were incubated at 500 rpm with 1 liter air/liter medium/minute sparged into the vessel and were harvested after 3~5 days. Samples were prepared for analysis by lyophilization of the whole broth and extraction with methanol; the extracts were analyzed by HPLC as described below. The time course for the production of hongoquercins A and B in CSO-5 or SO-11 (3% dextrose, 1.5% soy flour, 0.2% sodium chloride, 0.1% calcium carbonate) media was performed in duplicate 250 ml Erlenmeyer flasks and was monitored by repeated sampling of flasks over time.

HPLC Determinations

Analytical HPLC was carried out on a Hewlett-Packard HP1090 equipped with a diode array detector using a VYDAC ODS column (5 μ, 4.6 mm i.d. × 250 mm). The column was eluted with 80% CH_3CN/20% water/0.1% TFA at a flow rate of 1.0 ml per minute, and monitored at a wavelength of 270 nm. The retention times of **1** and **2** were 6.4 and 14.6 minutes, respectively.

In Vitro Susceptibility Testing

In vitro antibacterial activity was determined by the broth microdilution method.[9,10] Briefly, 100 μl of exponentially growing cells in minimal broth or Mueller-Hinton broth (10^5 CFU/ml) were treated with 0.002 μg/ml to 128 μg/ml of hongoquercin A or B (final concentration). The MIC was defined as the lowest concentration of antibiotic which prevented turbidity after 18 hours of incubation at 37°C. Minimum bactericidal concentration (MBC) was defined as the lowest concentration of antimicrobial agent that killed 99.99% of the bacteria.

Incorporation of Radiolabeled Precursors

Macromolecular synthesis in *E. coli imp* was studied by measuring the incorporation of the appropriate radiolabeled precursors into trichloroacetic acid (TCA)-precipitable material.[10] *E. coli imp* was grown at 37°C, 200 rpm in modified minimal medium (50 ml medium/250 ml Erlenmeyer flask) to an A_{450} of 0.20. Aliquots of 100 μl were dispensed into microtiter wells containing 5 μl of antibiotic, and the plates were incubated for 5 minutes at 37°C with vigorous agitation. Cells were pulse-labeled for 5 minutes by adding one of the following radiolabeled precursors at the indicated final concentrations: ^{3}H-Tdr, 0.5 μCi/ml with 0.1 μg/ml of unlabeled thymidine/ml; ^{3}H-Udr, 0.5 μCi with 0.5 μg unlabeled uridine/ml; or ^{3}H-AA, 10 μCi/ml. To determine specific incorporation into DNA, RNA, and protein, 100 μl of chilled (4°C) TCA (10%) supplemented with 0.5 mg of unlabeled precursors per ml was added to each well, and the plate was immediately refrigerated for 1 hour. The precipitate was collected on a glass fiber filter (Wallac filtermat B, Wallac 1205-404) using a Skatron 96-well cell harvester (Model 11050) programmed for a 3 second prewet with chilled DI water, a 12 second wash with 5% chilled TCA and a 5 second drying cycle. To assess the effects of the drugs on cellular uptake of radiolabeled precursors, the addition of TCA to the microtiter plate was eliminated, and the contents of each well were harvested onto a glass fiber filter by the Skatron 96-well cell harvester programmed for a 3 second prewet with chilled DI water, a 10 second wash with chilled normal saline (0.9% NaCl in DI water), and a 5 second drying cycle. Filter mats were dried for 7 minutes at high power in a microwave oven (Quasar, 700 Watts), solid scintillant (MeltilexB, Pharmacia 1205-402) was applied, and the isotope that was retained on the filter was quantified in an LKB Betaplate scintillation counter (Wallac 1205). The levels of incorporation of ^{3}H-Tdr, ^{3}H-Udr, and ^{3}H-AA are expressed as a percent of the untreated control.

Effect on Intracellular Potassium of *E. coli imp*

Effects on intracellular potassium in *E. coli imp* were studied in a saline buffer (10 mM Hepes buffer containing 150 mM NaCl and 0.1 mM KCl, pH 7.0).[11] A log-phase culture was washed twice with saline buffer, and the pellet

was resuspended in the same buffer to an OD_{600} of 2.00. One ml of the bacterial suspension was treated with the test compounds at various concentrations for 1 hour, after which the cells were pelleted by centrifugation (at $10,000 \times g$ for 2 minutes). The resulting supernatant was diluted 1 : 10 in HPLC grade water and was analyzed for potassium ion by atomic absorption spectrophotometry (Instrumentation Laboratories 551). For the determination of the total potassium level, 1 ml of the culture was hydrolyzed in 2 M sulfuric acid (100°C, for 1 hour), chilled for 1 hour, and centrifuged (at $10,000 \times g$ for 2 minutes). The supernatant was then diluted 1 : 10 and analyzed for potassium ion.

Lysis of Human RBC

One ml of freshly pooled blood was centrifuged (at $10,000 \times g$ for 2 minutes), the pellet was washed four times with normal saline by repeated resuspension and centrifugation, and then the pellet was resuspended in 1 ml of RBC buffer (10 mM Na-phosphate + 150 mM NaCl + 1 mM $MgCl_2$, pH 7.4).[12] Twenty five microliters of the RBC suspension was added to microfuge tubes containing 1 ml of drug solution (final concentration ranging from 1 ~ 128 μg/ml) prepared in duplicate in RBC buffer. After 2 hours of treatment, the tubes were centrifuged (at $10,000 \times g$ for 2 minutes) and the absorbance (at 540 nm) of the supernatant was measured. For 100% lysis, 25 μl of RBC suspension was added into 1 ml of water.

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