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NOTE

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Malabaricone C from *Myristica cinnamomea* Exhibits Anti-Quorum Sensing Activity

Yee Meng Chong,[†] Wai Fong Yin,[†] Chia Yong Ho,[†] Mohamad Rais Mustafa,[‡] A. Hamid A. Hadi,[‡] Khalijah Awang,[‡] Putri Narrima,[‡] Chong-Lek Koh,[§] David R. Appleton,[‡,⊥] and Kok-Gan Chan*[,†]

[†]Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

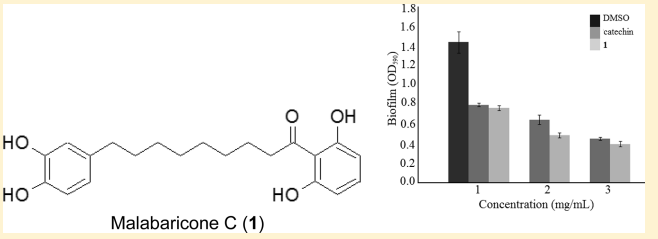
[‡]Centre for Natural Products Research and Drug Development, Department of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

[§]Natural Sciences and Science Education AG, National Institute of Education, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Singapore

[⊥]Sime Darby Technology Center Sdn. Bhd., 1st Floor, Block B, UPM-MTDC Technology Center III, Lebuh Silikon, Universiti Putra Malaysia, Serdang, 43400 Selangor, Malaysia

Supporting Information

ABSTRACT: A methanol-soluble extract of the bark of *Myristica cinnamomea* was found to exhibit anti-quorum sensing activity, and subsequent bioassay-guided isolation led to the identification of the active compound malabaricone C (**1**). Compound **1** inhibited violacein production by *Chromobacterium violaceum* CV026 when grown in the presence of a cognate signaling molecule, *N*-3-oxohexanoyl-homoserine lactone. Furthermore, **1** inhibited the quorum sensing-regulated pyocyanin production and biofilm formation in *Pseudomonas aeruginosa* PAO1. These results suggest that the anti-quorum sensing activity of **1** and related molecules should be investigated further.



The incidence of multi-drug-resistant pathogenic bacteria is increasing worldwide, and this has rendered current antibiotic treatment regimes ineffective in many cases. Consequently, the development of novel therapeutic measures is needed urgently.[1] A promising approach is to target bacterial cell-to-cell communication, commonly known as quorum sensing, as the basis of anti-pathogenic drug treatment.

Bacterial quorum sensing usually involves the production of small, diffusible signal molecules known as autoinducers.[2] When a threshold level is reached, the autoinducer will bind to its cognate receptor[3] to form a complex, which will in turn regulate the expression of target genes, particularly those responsible for virulence.[3−5] Several signaling molecules have been identified, including *N*-acyl homoserine lactone derivatives and small post-translationally processed peptides in Gram-negative and Gram-positive bacteria, respectively.[6−8] Interruption of quorum sensing, also known as quorum quenching, can be achieved by targeting the signaling molecule itself,[9−11] the transcriptional activator,[12] or the autoinducer synthase.[13] Thus, anti-quorum sensing treatment may be a plausible way to attenuate bacterial virulence without killing the pathogens, and this may reduce the acquisition of drug resistance by the bacteria.[14] The first anti-quorum sensing compound, efficient in reducing bacterial virulence determinants, is a halogenated furanone isolated from the red marine alga *Delisea pulchra*.[12] Subsequently, other anti-quorum sensing compounds have been reported in higher plants.[15]

Our group embarked on a study to discover anti-quorum sensing compounds from Malaysian plants. Plants of the genus of *Myristica* have been extensively used in folk medicine in Asia, including Malaysia.[16] Acylphenols, including malabaricones B and C, extracted from *Myristica* spp. have been reported to exhibit antimicrobial, cytotoxic, and nematocidal activities and healing properties for gastric ulcers.[17−21] We report here the in vitro anti-quorum sensing activity of malabaricone C, purified from the MeOH extract of the bark of *Myristica cinnamomea* King (Myristicaceae).[22]

Initially we tested the anti-quorum sensing activity of the *M. cinnamomea* bark MeOH extract by growing the biosensor *Chromobacterium violaceum* CV026 overnight in the presence of *N*-3-oxohexanoyl-homoserine lactone (0.25 mg/mL) and the extract or DMSO, as the solvent. No antibacterial effect of the extract or DMSO was observed, as judged by the cell viability (data not shown). However, the bark MeOH extract inhibited violacein production by *C. violaceum* CV026, indicating anti-quorum sensing activity. Subsequent profiling of the *M. cinnamomea* bark MeOH extract using LC-MS coupled to an off-line quorum sensing assay, followed by bioassay-guided fractionation, led to the isolation of **1** as the major bioactive constituent (1.9% dry weight, >95% purity by HPLC). Compound **1** was identified as

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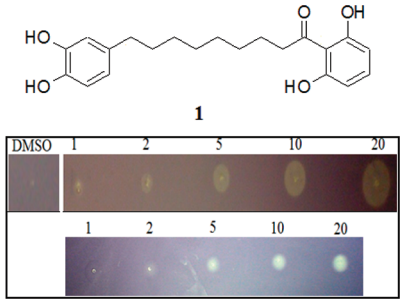

Figure 1. Screening of anti-quorum sensing activity of **1** using *C. violaceum* CV026. Compound **1** (upper panel) or (+)-catechin (positive control, lower panel) was spotted at 1, 2, 5, 10, and 20 μg (labeled as 1, 2, 5, 10, and 20, respectively) onto a lawn of *N*-3-oxohexanoyl-homoserine lactone-induced *C. violaceum* CV026. Halo formation indicates anti-quorum sensing activity. DMSO (solvent) served as a negative control.

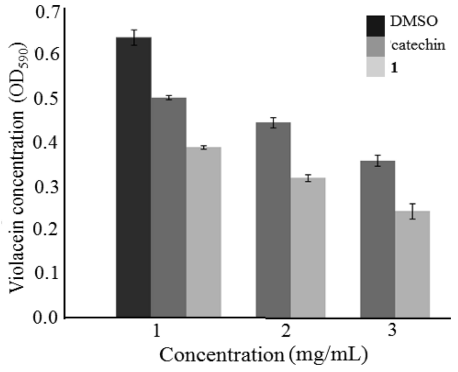

Figure 2. Quantitative analysis of violacein inhibition by **1**. Compound **1** (light gray) was added to *N*-3-oxohexanoyl-homoserine lactone-induced *C. violaceum* CV026 cells to 1, 2, or 3 mg/mL (as labeled on the horizontal axis). DMSO (black) and (+)-catechin (dark gray) served as negative and positive controls, respectively. Violacein was extracted and expressed as absorbance at 590 nm (OD_{590} at vertical axis). The bars represent standard errors of the means.

malabaricone C (Figure 1) by LC-HRMS and NMR analyses, in comparison with literature data.[17,20]

Violacein production by *N*-3-oxohexanoyl-homoserine lactone-induced *C. violaceum* CV026 is a quorum sensing-mediated phenotype.[23] Compound **1** inhibited violacein production in this assay, with the most pronounced inhibition of quorum sensing activity at 3 mg/mL (Figure 2).

Pseudomonas aeruginosa PAO1 is one of the most intensely studied opportunistic human pathogens. It causes infections of the blood, skin, eye, gut, respiratory system, genitourinary tract, and burn wounds. In particular, it can colonize lung tissues of cystic fibrosis patients, resulting in chronic pulmonary damage.[24] The free-radical-generating pigment pyocyanin is one of the major virulence determinants of *P. aeruginosa* PAO1.[24] The anti-quorum sensing activity of **1** was validated further by measuring its effect on pyocyanin production in *P. aeruginosa* PAO1. Figure 3A shows that **1** decreased *P. aeruginosa* PAO1 pyocyanin production when administered at concentrations $\geq$ 1 mg/mL. Concurrently, **1** did not exhibit adverse effects on the viability of *P. aeruginosa* PAO1.

Biofilm formation is considered as a pathogenicity trait during chronic infection such as cystic fibrosis.[24] Bacteria present within biofilms have characteristics distinct from those of the free-living,

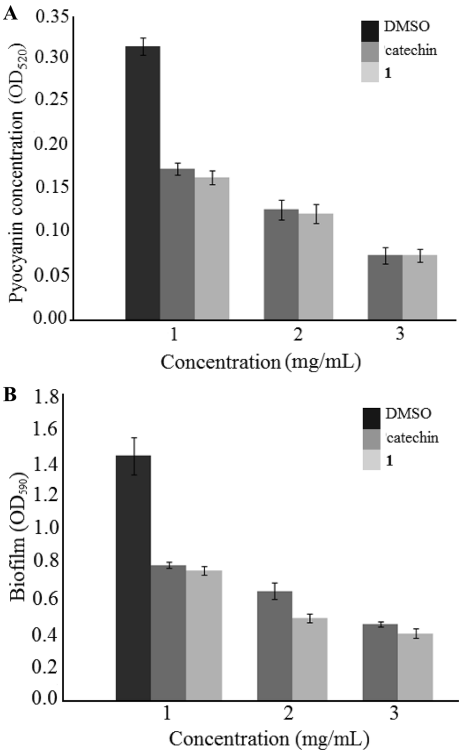

Figure 3. Inhibition of pyocyanin production (A) and biofilm formation (B) in *P. aeruginosa* PAO1 by **1**. Pyocyanin production and biofilm formation were monitored at 520 and 590 nm (OD_{520} and OD_{590}), respectively. For both assays, **1** (light gray) was added to *P. aeruginosa* PAO1 cell cultures at 1, 2, or 3 mg/mL. DMSO (control, black) and (+)-catechin (dark gray) served as negative and positive controls, respectively. The bars represent standard errors of the means.

planktonic bacteria of the same species, particularly their antibiotic susceptibility. Most pathogenic bacteria including *P. aeruginosa* PAO1 can form biofilms.[24] We investigated the ability of **1** to inhibit quorum sensing-regulated biofilm formation by *P. aeruginosa* PAO1 in order to further validate its potential as an anti-quorum sensing agent. When **1** was administered at 1, 2, and 3 mg/mL, the corresponding *P. aeruginosa* PAO1 biofilm formations were reduced by 44.9%, 63.0%, and 69.4%, respectively (Figure 3B), as compared to a control. The biofilm formation inhibition effect of **1** was similar to that of (+)-catechin, the positive control used.

P. aeruginosa possesses two *N*-acylhomoserine lactone-dependent quorum sensing systems, namely, the *rhl* and *las* systems.[24] The two virulence factors selected in this work, pyocyanin production and biofilm formation, are regulated by *P. aeruginosa rhl* and *las* quorum-sensing systems, respectively.[25] The *rhl* system regulates pyocyanin production through the *rhl* autoinducer *N*-butyryl-homoserine lactone and two minor autoinducers, *N*-hexanoyl-homoserine lactone and *N*-3-oxohexanoyl-homoserine lactone.[25] However, the *las* system is involved in the maturation of *P. aeruginosa* biofilm via *las* autoinducer *N*-3-oxododecanoyl-homoserine lactone.[25] It appears that **1** was effective at inhibiting both the *lasR*- and *rhlR*-regulated virulence determinants in *P. aeruginosa* PAO1, suggesting broad-spectrum effects.

A *C. violaceum* CV026 LuxR homologue receptor, namely, CviR, binds only to short-chain *N*-acylhomoserine lactones and, in particular, *N*-hexanoyl-homoserine lactone.[23] Therefore, in

the bioassay involving *C. violaceum* CV026, **1** may have modulated the interaction of *C. violaceum* CV026 CviR receptor to its cognate signaling molecules. Compound **1** is unlikely to interfere with the synthesis of autoinducers in *C. violaceum* CV026 because CV026 has a defective *luxI* synthase gene.[23] We verified that **1** did not inhibit autoinducer production in *P. aeruginosa* PAO1 (data not shown). Hence, **1** is unlikely to be able to interact with LuxI homologues (autoinducer synthase) of *P. aeruginosa* PAO1. Our data collectively suggest that **1** interfered with bacterial quorum sensing by interacting with LuxR but not LuxI homologues. However, more work is required to confirm this hypothesis.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were recorded on a JEOL ECA400 NMR spectrometer, and chemical shifts were referenced to the residual nondeuterated solvent signal. Analytical reversed-phase LC-MS and HRMS analyses were performed on a Shimadzu UFLC-IT-TOF system using a Waters Xbridge C_{18} column (2.2 × 50 mm, 2.5 μm) at 40 °C and a flow rate of 0.5 mL/min. The eluent flow was split 1:4 post-UV detector using a passive splitter between the mass spectrometer and a Gilson FC204 fraction collector. Fractions were collected every 30 s into a 96-well microtiter plate for biological assays. A linear gradient from 90:10 H_2O (0.1% formic acid)−MeCN (0.1% formic acid) to 100% MeCN (0.1% formic acid) over 7 min was used for the chromatographic resolution. Preparative HPLC was performed on a Gilson GX-281/322/156 system.

Plant Material. *Myristica cinnamomea* was collected at the Labis Forest Reserve, Bekok, Johor, Malaysia, in August 2003 and identified by a botanist. A voucher specimen (KL-5043) was deposited at the Herbarium of CENAR, Department of Chemistry, Faculty of Science, University of Malaya.

Extraction and Isolation. Dried, powdered bark material of *M. cinnamomea* (50 g) was extracted with methanol, filtered, and dried in vacuo to yield a crude extract (14.7 g). The extract was prepared in DMSO to a concentration of 50 mg/mL and diluted with water/buffer for biological screening. Analytical LC-MS analysis of the bark MeOH extract revealed that compounds eluted at retention times of 4.5−5.5 min were responsible for the anti-quorum sensing activity detected. The bark extract (300 mg) in methanol (2 mL) was then fractionated by preparative HPLC equipped with a Waters Novapak RPC_{18} column (25 × 100 mm, 6 μm, plus 10 mm guard) using water (0.1% formic acid)−MeCN (0.1% formic acid) as solvents and a flow rate of 12 mL/min with the following gradient: 0−5 min 20% MeCN, 5−10 min 20−40% MeCN, 10−65 min 40−80% MeCN to yield **1** (t_R of 36 min, >95% purity by HPLC, 17.3 mg, 1.9% dry weight). Malabaricone C (**1**) was obtained as a white, amorphous powder; (−)-HRESIMS m/z 357.1714 $[M - H]^-$ (calcd for $C_{21}H_{25}O_5$, 357.1707). This compound exhibited ^1H and ^{13}C NMR data consistent with literature values.[17,20]

Anti-Quorum Sensing Activity. An overnight culture of *C. violaceum* CV026 (in LB broth, 28 °C, 220 rpm) was adjusted to an OD_{600} of 0.8 before inoculation into 300 mL of molten LB agar supplemented with *N*-3-oxohexanoyl-homoserine lactone (0.25 mg/mL) (Sigma, St. Louis, MO). Compound **1** was spotted at 1, 2, 5, 10, or 20 μg onto LB agar seeded with *N*-3-oxohexanoyl-homoserine lactone-induced *C. violaceum* CV026. DMSO was also spotted as negative control. The plate was incubated overnight at 28 °C. Inhibition of violacein production by *N*-3-oxohexanoyl-homoserine lactone-induced *C. violaceum* CV026 by **1** indicated the anti-quorum sensing activity of **1**, and the results were digitally recorded. (+)-Catechin [(2*R*,3*S*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol], which has a significant negative effect on the production of pyocyanin, elastase, and biofilm,[26] was used as positive control for all quorum sensing inhibition assays in *C. violaceum* CV026 and *P. aeruginosa* PAO1. (+)-Catechin was purchased from Sigma (Sigma, St. Louis, MO) and dissolved in DMSO.

Quantitative Analysis of Violacein Production. An overnight culture of *C. violaceum* CV026 was adjusted to an OD_{600} of 1.2 before it was transferred to a 96-well plate (100 μL aliquots) followed by the additions of **1** to 1, 2, or 3 mg/mL and *N*-3-oxohexanoyl-homoserine lactone to 0.25 mg/mL. DMSO and (+)-catechin were included as negative and positive controls, respectively. The microplate was incubated for 16 h at 28 °C followed by drying at 60 °C. Next, 100 μL of DMSO was added to each well to dissolve the dried violacein for 2 h at 28 °C with shaking. The absorbance of each well was measured at 590 nm. The experiments were performed in triplicate.

Pyocyanin Assay. An overnight culture of *P. aeruginosa* PAO1 was diluted with LB medium to an OD_{600} of 0.1, and the diluted culture was supplemented with **1** at 1, 2, or 3 mg/mL. DMSO and (+)-catechin were included as negative and positive controls, respectively. After overnight incubation at 37 °C, the cell culture (5 mL) was mixed with 3 mL of chloroform, and the organic layer was mixed thoroughly with 1 mL of 0.2 M hydrochloric acid. The organic layer was collected by centrifugation (2000*g*), and the absorbance was measured at 520 nm. The experiments were performed in triplicate.

Biofilm Assay. An overnight culture of *P. aeruginosa* PAO1 was diluted with LB medium to an OD_{600} of 0.1 and further grown at 37 °C in LB medium containing 0.5% (w/v) glucose (filter-sterilized, pore size of 0.22 μm) and **1** at 1, 2, or 3 mg/mL. Cells grown in the presence of DMSO and (+)-catechin served as negative and positive controls, respectively. To facilitate biofilm formation, *P. aeruginosa* PAO1 cells were incubated statically for 18 h at 37 °C in sterile plastic tubes (50 mL volume). Planktonic bacteria were discarded, and the tubes were air-dried for 15 min and stained with 1 mL of 1% (v/v) crystal violet for 45 min. The stained biofilms were washed again with sterile distilled water followed by the addition of 2 mL of ethanol (95%, v/v). The resulting solution (100 μL) was transferred to a microtiter well, and the absorbance determined at 590 nm. The experiments were performed in triplicate.

ASSOCIATED CONTENT

Supporting Information. ^1H and ^{13}C NMR spectra and LC chromatogram of **1** are available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +60 3 7967 5162. Fax: +60 3 7967 4509. E-mail: kokgan@um.edu.my.

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