Callistemenonone A, a novel dearomatic dibenzofuran-type acylphloroglucinol with antimicrobial activity from *Callistemon viminalis*

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A new acylphloroglucinol with a novel architecture including an unprecedented dearomatic dibenzofuran core, named callistemenonone A (1), was isolated from the leaves of *Callistemon viminalis* (Myrtaceae). The structure was fully characterized on the basis of extensive spectroscopic analysis, including UV, HRESIMS, as well as 1D and 2D NMR spectral data (HSQC, HMBC, and ROESY). The deduced structure represents the first example of a natural dibenzofuran with two phenyl moieties coupling through tertiary hydroxy and ketal carbons. A plausible biogenetic pathway involving oxidative coupling and dearomatization as key steps is proposed to account for the biosynthesis of this novel class of dibenzofuran. Moreover, antimicrobial assays, in conjunction with the time-killing and biophysical studies, revealed that 1 exerted potent bactericidal activity against a panel of methicillin resistant pathogenic microbes with a unique mechanism.

The genus *Callistemon* belongs to the family Myrtaceae, and is comprised of over 30 species with most being woody, aromatic, and ornamental trees or shrubs endemic to Australia¹. *Callistemon viminalis* (Sol. ex Gaertn.) G. Don (commonly known as red bottlebrush) is an Australian native green shrub with leaves and stems reputed to possess a variety of biological effects, including antidiabetic², antimicrobial, anti-inflammatory, antistaphylococal, antithrombin³–⁶, and nematicidal, larvicidal, as well as pupicidal activities⁷. It was introduced into China some decades ago. Presently, it is widely cultivated in southern China as an ornamental tree and is adopted into Chinese folk medicine to treat colds and arthralgia⁸.

Plants in the genus *Callistemon* are known for their enriched phloroglucinol content, which is responsible for a variety of the biological activities of these herbal medicines⁹–¹². Previous phytochemical studies on different parts of *C. viminalis* showed the presence of flavonoids⁹, triterpenoids⁹, tannins, and phloroglucinol derivatives¹⁰–¹¹. In a continuation of our efforts to discover new antibacterial constituents from the plants of family Myrtaceae¹²–¹⁷, the ethanolic extract of *C. viminalis* was found to exhibit in vitro antibacterial activity with a MIC value of 100μg/mL against MRSA JCSC 2172. Antibacterial activity guided isolation has now led to the isolation of a new potent (MIC 20μg/mL, anti-MRSA JCSC 2172) acylphloroglucinol with an unprecedented dearomatic dibenzofuran skeleton, designated as callistemenonone A (1) (Fig. 1). Herein, the isolation, structural elucidation, bioassay, and antibacterial mechanism of 1 are described.

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Results and Discussion

*C. viminalis* leaves were extracted at room temperature with 95% EtOH, and the resulting pooled crude extract was concentrated under reduced pressure and then partitioned to afford *n*-hexane-soluble, EtOAc-soluble, and aqueous fractions. The *n*-hexane and EtOAc-soluble fraction were combined and subjected to repeated silica gel column chromatography to obtain 1 as a yellowish oil.

**Figure 1.** Structure of callistemenonone A (1).

| position | δ<sub>H</sub> (125 MHz) | δ<sub>C</sub> (500 MHz) | HMBC correlations          |
|----------|------------------------|------------------------|-----------------------------|
| 1′       | 191.1 s                |                        |                             |
| 2′       | 82.8 s                 |                        |                             |
| 3′       | 108.4 s                |                        |                             |
| 4′ a 4′b | 42.2 t                 | 3.11 (d, 16.3), 3.26   | C-2′, C-3′, C-5′, C-6′      |
| 5′       | 191.9 s                |                        |                             |
| 6′       | 110.3 s                |                        |                             |
| 7′       | 200.1 s                |                        |                             |
| 8′       | 45.6 t                 | 2.77 (dd, 7.3, 13.4), 2.39 | C-7′, C-9′, C-10′, C-11′     |
| 9′       | 26.5 d                 | 1.83 (m)               | C-7′, C-8′, C-10′, C-11′     |
| 10′      | 22.3 q                 | 0.75 (d, 6.8)          | C-8′, C-9′, C-11′           |
| 11′      | 22.4 q                 | 0.80 (d, 6.8)          | C-8′, C-9′, C-10′           |
| 3′-OMe   | 56.1 q                 | 3.85 (s)               | C-3                         |
| 3′′-OMe  | 55.9 q                 | 3.59 (s)               | C-3′                        |
| 2-OH     | 4.60 (s)               |                        |                             |
| 5-OH     | 13.7 (s)               |                        | C-4, C-5, C-6               |

**Table 1.** ¹H and ¹³C NMR data of 1 in CDCl₃ (δ in ppm, J in Hz).
The 1H–1H COSY spectrum revealed the presence of two spin-coupling fragments (Fig. 2), namely, a (C-8/C-9/C-10/C-11) and b (C-8'/C-9'/C-10'/C-11'). The key HMBC cross-peaks (Fig. 2) observed in the coupling fragment a were H-10 to C-8, C-9, and C-11; H-11 to C-8, C-9, and C-10; H-9 to C-7; and H-8 to C-6. These correlations confirmed the presence of an isovaleryl group. Moreover, the HMBC correlations of the 5-OH to C-4, C-6, of H-4 to C-2, and C-6, as well as of the 3-Ome to C-3 indicated the presence of a penta-substituted benzene moiety. Furthermore, the HMBC cross peak between H-8 to C-6 suggested a connection of the isovaleryl group to C-6 of the benzene moiety, which led to the construction of a typical phloroglucinol unit I, which was characteristically found in the genus Callistemon. Similarly, the HMBC correlations of H-3-10 to C-3' and C-9, and C-11; of H-11 to C-8', C-9', and C-10'; of H-9 to C-7', and of H-8' to C-6', together with key HMBC correlations of H-4' to C-3', of C-2' and C-6', as well as of the 3'-Ome to C-3', confirmed the other sub-structure portion as II (Fig. 2).

Although the HMBC experiment failed to provide direct and conclusive evidence for the linkage between the structural fragments I and II due to the absence of a suitable correlate proton, assembly of the two moieties was achieved through a connection of C-1-O-C-3' which led to the presence of a furan ring and, together with portions I and II, accounted for the nine double bond equivalents. Compound 1 might be an equimolar mixture of the two enantiomers based on its zero optical rotation value. The relative configuration of 1 was deduced from an analysis of the ROESY correlations (Fig. 3). The key ROE correlations between the 2′-OH/H-4a' and the 3′-Ome/H-4a indicated that the 2′-OH and 3′-Ome were cofacial. Therefore, the structure was determined as I, and given the trivial name callistemenonone A, representing the first member of a new family of phloroglucinol derivatives with an unprecedented dearomatic dibenzofuran core combining an α,β-triketone with a phloroglucinol unit.

A biotransformation pathway (Fig. 4) was proposed to account for the biogenesis of callistemenonone A (1). Briefly, isovaleryl-CoA (2) and malonyl-CoA (3) would undergo PKS (polyketide synthase)-catalyzed condensation to yield the acylphloroglucinol 4, which has previously been reported from plants of the Myrtaceae family. Oxidative coupling leads to the putative key precursor 6 through the radical intermediate 5. Further oxidative dearamatization of 6 would generate the intermediate 7, leading to construction of the unique tertiary hydroxyl carbon and the formation of the dibenzofuran architecture in callistemenonone A (1) through Michael addition.

The antimicrobial activity of I was evaluated towards a panel of Gram-positive bacteria, including Staphylococcus aureus strain (CMCC 26003), Bacillus cereus strain (CMCC 63302) and several strains of methicillin-resistant S. aureus MRSA JCSC 2172, MRSA JCSC 4469, and MRSA JCSC 4744 using standard MIC method and following the CLSI guidelines. As a result, compound 1 was shown to possess potent antibacterial activity against these Gram-positive bacteria with MICs and MBCs ranging from 5 to 80μg/mL as presented in Table 2. The potency is 3–60 fold less than that the positive control substance vancomycin (Van). Callistemenonone A (1) was inactive towards the Gram-negative bacteria Escherichia coli (ATCC 8739), presumably due to its relative inability to penetrate the permeability barrier presented by the outer membrane and/or the action of efflux transporters.

In order to understand the actions of callistemenonone A (1) at the morphologic level and to shed light on the mechanism underlying the antimicrobial activity against the clinical MRSA isolate JCSC 2172, a mechanistic investigation was initiated combining morphological, biochemical, and biophysical studies. As a result, time-kill kinetic experiments dosing with 1 × MIC revealed that 1 exhibited rapid, in vitro bactericidal activity (killing > 90% within 4 h) and complete bactericidal effect, with a sharp reduction in CFU of 99.99%, after 24 h exposure.
Figure 3. The key ROESY correlations (H→H) of 1.

Figure 4. Proposed biogenesis of callistemenonone A (1).

Table 2. In vitro antibacterial activities of callistemenonone A (1) against bacteria strains. *Positive control; Van = vancomycin; ND: not determined.
However, in contrast to vancomycin, concentration-dependent experiment of 1 with 8 × MIC did not illustrate a dose-response effect.

Further biophysical studies using fluorescence probes for membrane potential were performed to determine whether 1 caused cytoplasm membrane depolarization. As shown in Fig. 5B, after treating the MRSA JCSC 2172 cells with serial concentrations of 1 (0.5 × MIC, 1 × MIC, 2 × MIC, 4 × MIC, 8 × MIC), a sharp increase in the fluorescence was observed, suggesting that the favorable bactericidal action of 1 could be attributed to a significant dissipation of the membrane potential due to depolarization. However, the SYTOX green assay indicated that the bactericidal action of 1 was independent of membrane disruption, because there was no obvious increase of fluorescence detected when the MRSA suspension was treated even with 8 × MIC of 1 for up to 4 h. In contrast, the positive control melittin caused an unambiguous increase in fluorescence within 60 s (Fig. 5C) and in the whole concentrations range from 0.5 to 8 MIC after 4 h (Fig. 5D). These results were a strong indication that the bactericidal action of 1 was independent of membrane disruption.

Moreover, the aforementioned results were in good agreement with the morphological study using scanning electron microscopy, wherein 1 failed to disrupt the cytoplasmic membrane structure of MRSA JCSC 2172 cells following treatment with 8 × MIC of 1 for 4 h. As shown in Fig. 6, the morphology of the treated cells remained intact with smooth spheres comparable to those of the untreated control cells. Based on the results of membrane depolarization and membrane disruption assay, in conjunction with the time-killing study (killing > 90% after 4 h), it can be concluded that callistemenone A (1) acts rapidly as a bactericidal antibiotic by disturbing the bacterial membrane potential without causing membrane disruption.
Melittin, the positive control used in several experiments, is a lytic and highly toxic peptide. It could be interesting to see if melittin and 1 in combination can lead to synergize the potency of 1 without undue toxicity from melittin. The synergism of 1 and melittin was investigated according to the Bliss independence model. Synergy value (0.025) was calculated using the formula as mentioned in Materials and Methods, this synergy value (0.025) demonstrated a very little synergistic relationship between 1 and melittin against MRSA.

In summary, these experiments have described herein the isolation, structural elucidation, and biological evaluation of a novel acylphloroglucinol named callistemenonone A (1), which was isolated from the leaves of *Callistemon viminalis* (Myrtaceae). The metabolite represents a new type of dimeric acylphloroglucinol with an unprecedented dearomatic dibenzofuran core which is biogenetically formed through tertiary hydroxy and ketal carbons. The oxidative coupling reaction and dearomatization were proposed to be key biotransformation steps for its biogenesis. The investigation of antibacterial mechanism, in conjunction with the time-killing study, revealed that 1 acted rapidly as a bactericidal agent by disturbing the bacterial membrane potential without causing membrane disruption. This represents a very favorable bactericidal mechanism which differs from any other antibiotic agent available either in the clinic or in development, rendering it a very promising candidate for further extensive investigation through medicinal chemistry, with an aim towards new antibiotic drug discovery.

**Experimental**

**General procedures.** Optical rotation was measured on a Perkin-Elmer 341 polarimeter (Perkin-Elmer, Boston, MA, USA). UV spectra were recorded in MeOH on a Perkin-Elmer Lambda 35 UV-vis spectrophotometer (Perkin-Elmer, Boston, MA, USA). 1D and 2D NMR spectra were recorded on a Bruker Advance-500 spectrometer with TMS as internal standard (Bruker BioSpin AG, Fallanden, Switzerland). HRESIMS data were obtained on a Bruker Bio TOF IIIQ mass spectrometer (Bruker Daltonics, Billerica, MA, USA). All solvents were analytical grade (Shanghai Chemical Plant, Shanghai, China). Silica gel (200–300 mesh) was used for column chromatography, and precoated silica gel GF254 plates (Qingdao Haiyang Chemical Plant, Qingdao, China) were used for TLC analysis. C18 reversed-phase silica gel (150–300 mesh, Merck), MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Industries Ltd.), and Sephadex LH-20 gel (Amersham Biosciences) were also used for column chromatography. TLC spots were visualized under UV light and by dipping into 5% H2SO4 in alcohol, followed by heating. In the biological and biophysical experiments, the solvent used to dissolve 1 were DMSO.

**Plant material.** The leaves of *C. viminalis* were collected from the South China Botanical Garden, Chinese Academy of Sciences in March 2015 and identified by Dr. Yun-Fei Deng of SCBG. A voucher specimen was deposited at the Laboratory of Natural Product Chemistry Biology, SCBG.

**Extraction and isolation.** The chopped fresh leaves of *C. viminalis* (15.0 kg) were extracted with 95% EtOH (30 L × 3) at room temperature for 24 hours. The pooled solvents were evaporated under reduced pressure to yield a brown syrupy residue (3.0 kg), which was suspended in H2O (3 L) and extracted successively with n-hexane (3 L × 3) and EtOAc (3 L × 3) to afford n-hexane- and EtOAc-soluble fractions. Most of spots of the two parts were similarity, which were detected by Thin layer chromatography (TLC). So n-hexane- and EtOAc-soluble fractions (2.2 kg) were combined and subjected to silica gel column chromatography eluting with n-hexane/EtOAc with increasing polarity from 20:1 → 0:1 to yield six sub-fractions (A-F). Sub-fraction C (32.0 g) was chromatographed on a silica gel column with a gradient of CHCl3/MeOH (100:1 to 20:1) to yield three fractions (C1–C3). Sub-fraction C2 (3 g) was subjected to RP-C18 column chromatography (diameter: 60 cm, length: 254 mm; particle size: 150–200 mesh) and eluted with MeOH/H2O (90:10, 95:5, 100:0) to obtain three further sub-fractions (C21 to C23). Sub-fraction C21 (400 mg) was subjected to CC on Sephadex LH-20 gel (CHCl3/MeOH, 1:1, v/v) and further purified by repeated silica gel column chromatography (n-hexane/EtOAc, 20:1 → 1:1) to yield 1 (25 mg).
Callistemenonene A (1): Yellowish oil; [α]$_D^{20}$ = 0 (c 0.20, MeOH). UV (MeOH) λ$_{max}$/nm (log ε) 238.7 (0.83), 281.0 (0.98); $^1$H (300 MHz) and $^{13}$C (125 MHz) NMR data, see Table 1; negative ESIMS m/z 461 [M – H]$^-$; HRESIMS m/z 461.1819 [M – H]$^-$ (for C$_{24}$H$_{30}$O$_{9}$, calcld. 461.1810).

**Antibacterial assay.** Compounds were dissolved in DMSO. The minimum inhibitory concentration (MIC) evaluation was carried out in 96-well plate according to the standard microdilution method. The minimum bactericidal concentration (MBC) was determined by sub-culturing 100 μL bacterial suspensions from the wells on the TSA plates. The plates were incubated for 24 h before the MBCs were determined. The MBC was categorized as the concentration where ≥ 99.9% reduction in bacterial cell count was observed. Test strains were S. aureus (CMCC 26003), B. cereus (CMCC 63302), and E. coli (ATCC 8739) which were purchased from Guangdong Microbiology Culture Center (Guangzhou, China). MRSA (JCSC 2172), MRSA (JCSC 4469), MRSA (JCSC 4744) were provided by T. Ito and K. Hiramatsu.

**Time killing assay.** An overnight culture of cells (MRSA JCSC 2172) was adjusted in 0.85% NaCl buffer to obtain a bacterial suspension with 10$^6$ to 10$^8$ CFU/mL. The inoculate was treated with various concentrations (1 × and 8 × MIC) of callistemenonene A (1) and vancomycin. The mixtures were incubated at 37°C. Culture aliquots were removed at 0 h, 0.5 h, 2 h, 4 h, 8 h, and 24 h, then they were serially diluted to 10$^6$–10$^8$ times and 100 μL 10-fold serially diluted suspensions were plated on TSA plates. The TSA plates were incubated at 37°C for 24 h, colonies were counted to calculate surviving cfu/mL. Bactericidal activity was defined as a ≥ 3-log10 CFU/mL decrease, in comparison with the baseline, after 24 h of incubation.

**Cytoplasmic membrane depolarization Assay.** The effect of callistemenonene A on the membrane potential of MRSA JCSC 2172 cells was investigated using a modified version of the method described by Wu and Hancock. Briefly, cultures of MRSA (JCSC 2172) were grown to exponential phase and then harvested. Bacteria were suspended and washed with buffer solution (5 mM HEPES at pH 7) until an optical density OD 0.1 at 600 nm (OD$_{600}$) was obtained. The cell suspension was then incubated with 0.4 μM 3,3-dipropylthiacyclobacaine (DiSC$_3$-5) for 20 min at 37°C with shaking. Then, 100 mM KCl was added to the buffer to balance the chemical potential of K$^+$ inside and outside the cells. The desired concentration of callistemenonene A (1) was added into a stirred cuvette, and the change in fluorescence was monitored at an excitation wavelength of 660 nm and an emission wavelength of 675 nm. DMSO and vancomycin were used as the negative controls with no effect on the depolarization. Experiments were repeated at least three times and found to be reproducible. Data from an individual experiment are presented.

**SYTOX green assay.** The protocol was based on the method of Rathinakumar et al. Briefly, MRSA (JCSC 2172) cells were suspended in 0.85% NaCl buffer until an OD$_{600}$ of 0.2 was obtained. The bacterial suspension was incubated with serial concentrations of callistemenonene A (1), respectively for 4 h, and then incubated with 31 μM of SYTOX green for 5 min. The fluorescence was measured at an excitation wavelength of 504 nm and an emission wavelength of 523 nm. Melittin, a cell lytic factor, was used as the positive control. Experiments were repeated at least three times and found to be reproducible. Data from an individual experiment are presented.

**Scanning electron microscopy.** For the observations using scanning electron microscopy, exponential-phase bacteria were treated with the compound at 8 × MIC for 4 h at 37°C. The cells were washed twice, suspended in PBS, and prefixed in 0.1 M phosphate buffer (pH 7.2) containing 2% glutaraldehyde and 2.5% paraformaldehyde overnight. After washing six times with 0.1 M phosphate buffer, the samples were post-fixed in 1% osmium tetroxide for 2 h. After washing another three times with 0.1 M phosphate buffer, the samples were dehydrated through a graded ethanol series and subjected to freeze-drying (JFD-310, JEOL, Tokyo, Japan). Samples were then mounted on stubs and coated with gold in a sputter coater (JFC-1600, JEOL, Tokyo, Japan), and then examined and photographed under a scanning electron microscope (JSM-6360LV, JEOL, Tokyo, Japan).

**Synergistic assay.** The synergy of 1 in combination with melittin was investigated in vitro against MRSA2172 using the Bliss independence model as described in previous reports. Briefly, bacterial strains were incubated with a sub-inhibitory concentration of simvastatin and control antimicrobials for 12 h and the degree of synergy was calculated using the formula: Synergy (S) was calculated using the formula: S = (f$_{A0}$/f$_{0B}$) (f$_{0A}$/f$_{00}$) – (f$_{A0}$/f$_{00}$). The parameter f$_{A0}$ refers to the optical density of the bacteria grown in the presence of 1 and melittin; parameters f$_{0A}$ and f$_{0B}$ refer to the bacterial growth rate in the presence of 1 alone and melittin alone, respectively; the parameter f$_{00}$ refers to the bacterial growth in the absence of drugs. Degree of synergy (S) values corresponds to the following cut-offs: Zero indicates neutral, values above zero (positive value) represents synergism, and values below zero (negative values) correspond to antagonism. Drug combinations with higher positive value represents high degree of synergism.

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