Stress-Driven Discovery of Novel Cryptic Antibiotics from a Marine Fungus Penicillium sp. BB1122

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Standard laboratory cultures have long been known to hinder activation of specific gene clusters which in turn hamper production of secondary metabolites with unique properties due to lack of innovation or the inability to trigger cryptic gene clusters’ expression. Due to challenges related to the avoidance of the isolation of replicated metabolites, resistance-developing pathogens are to be addressed by the scientific community worldwide in order to progress with novel and potent compounds which could further be developed in the future for pharmaceutical usage. This study reports the isolation of novel cryptic antibiotics from a marine fungus Penicillium sp. BB1122 collected from Zhoushan coast by applying the “metal-stress” strategy, here referring to the heavy metal cobalt (6 mM). High-performance liquid chromatography-guided isolation of four novel and four known compounds belonging to the polyketide class has been carried out where their relative as well as absolute configurations have been determined using spectroscopic analysis techniques as well as by the comparison of theoretically calculated ECD spectrum and the experimental ECD spectrum, respectively. The structures of novel compounds 7 and 8 represent the first example of 2,5-dioxabicyclo[2.2.1]heptane pyrone backbone bearing a migrated polyene chain. The novel compounds 7, 8, and 5 exhibited impressive antibiotic properties against methicillin resistant Staphylococcus aureus (MRSA) with MIC value of around 0.5 and 1 µg/mL, respectively. Moreover, the new compounds 1, 7, and 8 displayed potent antibiotic activities with MIC values of around 4 µg/mL against the pathogenic Pseudomonas aeruginosa. Moreover, the MBC of the different potent compounds ranged from 1 to 128 µg/mL against MRSA, P. aeruginosa, and Klebsiella pneumoniae. In addition, the cytotoxic activities were also evaluated where new antibiotics 7 and 8 were not obviously harmful toward normal liver cell lines LO2, showing IC50 values above 100 µg/mL. As a consequence, the results from this study unveiled that cobalt stress is an effective strategy to discover novel antibiotics from microorganisms.

Keywords: marine fungus, metal-stress strategy, spectroscopic techniques, polyketide, antibiotics

INTRODUCTION

The marine biosphere is known to be a treasure of unique, priceless, and potent biologically active natural products which originate from both marine flora and fauna (Ammerman et al., 1984; Kang et al., 2015). Marine organisms usually strive under specific conditions like temperature, pressure, dissolved oxygen, and nutrient availability, thus leading to the generation of a structural miscellany
and biochemical distinctiveness of secondary metabolites compared to terrestrial ones (Aneiros and Garateix, 2004; Bhatnagar and Kim, 2010; Barboza et al., 2012; Martin et al., 2013; Tsoukalas et al., 2014; Rangnekar and Khan, 2015). As such, reports by Blunt et al. (2017) unveiled a total of 14,637 compounds were seen during the past years. Moreover, owing to the lack of valid resources leading to resistance capacities. Therefore, urgent innovative techniques are a must to isolate and elucidate potent novel compounds were seen during the past years. Moreover, owing to the evolutionary concept, pathogens had the ability to rapidly mutate resulting in the amendment of their genetic materials leading to resistance capacities. Therefore, urgent innovative techniques are a must to isolate and elucidate potent novel candidates with antibiotic properties. According to Shi et al. (2015), generation of natural products can be intensified by specific chelating capabilities, distinct enzymatic reactions and biochemical distinctiveness of secondary metabolites. The isolation and structure elucidation of several isolated novel polyketides compounds are discussed followed by their antibiotic activity screening against meticillin resistant Staphylococcus aureus (MRSA), Pseudomonas aeruginosa, and Klebsiella pneumoniae.

**MATERIALS AND METHODS**

**General Experimental Procedures**
The high-performance liquid chromatography (HPLC) system used was composed of a Waters 717 plus Autosampler, a Waters 600 Controller, a Waters 996 Photodiode Array Detector, and a Waters Millog workstation (Waters, Shinagawa, Tokyo, Japan). Optical rotations were measured in methanol on a PerkinElmer-341 polarimeter. The IR spectra were run on a NicoletAvatar-360FT-IR spectrometer. H NMR (500 MHz) and 13C NMR (125 MHz) spectra were measured at 25°C in a Bruker AVANCE DMX 500 NMR spectrometer with TMS as an internal standard. CD spectra were measured on a JASCO J-715 (JASCO) spectropolarimeter. UV spectra were also recorded in methanol on a Shimadzu UV2550. ESIMS were recorded on an Agilent 6460 Triple Quad LCMS. Preparative HPLC was performed on a ChuangXinTongHeng system equipped with a Venusil MP-C18 column (10 mm × 250 mm, Agela Technologies, Tianjin, China). The organic solvents used in chromatographic separation were of analytical grade purchased from Sayfo Technology (Tianjin China) and chromatographic grade for HPLC analysis purchased from Tedia, United States. Deionized water was prepared by reverse osmosis Milli-Q water (18 MW; Millipore, Bedford, MA, United States) and used for all solutions and dilutions. Agar powder for plate culture and cobalt chloride was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

**Isolation, Normal and Metal-Stress Cultivation of the Fungal Strain**

**Isolation of the Fungal Strain**
The fungus was isolated from marine sediment collected along Zhoushan coast, China in January 2016. The latter was grown on PDA agar, consisting of 100 g potato lixivium, 10 g dextrose, 35 g sea salt, and 15 g agar powder per liter. During this study, the fungus strain was viewed under the microscope where the conidiophore was seen as being branched. Conidia were glued with phialides which in turn were attached at the end of conidiophores (Houbraken and Samson, 2011; Samson et al., 2011). According to the morphology of the strain as well as on the basis of the ITS 18S fragment, Penicillium sp. BB1122 was ascribed to the fungus (see Supplementary Table S2).

**Normal and Metal-Stress Cultivation**
The normal cultivation of Penicillium sp. BB1122 was carried out in 500 mL flasks containing 200 mL liquid PDB for 14
days at 28°C under static conditions. Then the metal-stress method was applied using six different metals namely cobalt, manganese, chromium, nickel, zinc, and cadmium with original concentrations 100, 200, 400, and 800 μM each, respectively. The mycelium was removed and culture broth was extracted with an equal volume of ethyl acetate. Due to the unchanged HPLC profile of the normal and stressed cultures, the concentrations of the different metals were increased where the final optimal stress conditions was determined as 6 mM cobalt ion concentration for stress culture based on the HPLC profile of the extract and the peak areas of the stress-induced products. Moreover, both the normal and cobalt-stressed cultures were tested for their antibacterial capacities which enhanced our decision to enlarge 6 mM cobalt culture due to the stressed culture’s effective inhibitory abilities.

Fermentation in Large Scale

*Penicillium* sp. BB1122 was inoculated onto agar plates containing PDA medium. After incubation for 14 days at 28°C, the pre-culture was used for inoculation in two 500 mL flasks containing 200 mL liquid medium as parent bottles. The 30 L of fermentation broth was extracted with ethyl acetate. After evaporation of the solvent the crude extract was dissolved in methanol. Analytical reversed phase HPLC-UV experiments were performed using a C18 column (sepax Amethyst C18-H, 100 mm 3.00 mm) applying a water/methanol gradient from 20% methanol to 100% methanol in 30 min, maintaining 100% methanol for 10 min, flow rate 0.8 mL/min on a LC20A system (Shimadzu, Kyoto, Japan) equipped with a Prominence CBM-20A/20 Alite controller, SPD-20A UV detector, and Prominence CTO-20A column oven. The stressed-induced compounds were isolated by preparative HPLC, which was carried out using a HPLC-UV system (P3000 type high pressure infusion pump, UV3000 type ultraviolet/visible light detector, Rheodyne 7725 I manual sampling valve, sepax C18, 150 mm 80 mm, column). Based on the results of the analytical HPLC analysis, gradient phase of 40–100% methanol was applied for 2 h for isolation of compounds 1–8 at a flow rate of 10 mL/min.

**Neocitreoviridin (1):** yellowish syrup; [α]23D + 22.3 (c 0.25, CHCl3); UV (MeOH) λ_max (log ε) 250 (4.02), 301 (3.92) nm; IR ν_max 3415, 1711, 1656, 1631, 1561, 1453, 1400, 1286, 1253, 1213, 1144, 1089, 1020, 938, 811 cm⁻¹; 1H NMR and 13C NMR, see Table 1; HR-TOF-MS m/z 425.1919 [M+Na]+ (calcd. for C23H29NaO6, 425.1935).

**10Z-isocitreoviridinol (5):** yellowish syrup; [α]23D − 17.2 (c 0.18, CHCl3); UV (MeOH) λ_max (log ε) 268 (3.98), 360 (3.95) nm; IR ν_max 3399, 2969, 1708, 1655, 1629, 1560, 1452, 1400, 1252, 1144, 1089, 1020, 938, 811 cm⁻¹; 1H NMR and 13C NMR, see Table 2; HR-TOF-MS m/z 419.2049 [M+H]+ (calcd. for C23H31O7, 419.2064).

**Penicillistressol (7):** yellowish syrup; [α]24D − 35.9 (c 0.20, CHCl3); UV (MeOH) λ_max (log ε) 274 (3.90) nm; IR ν_max 3150, 1711, 1608, 1560, 1515, 1402, 1256, 1169 cm⁻¹; 1H NMR and 13C NMR, see Table 3; HR-TOF-MS m/z 419.2045 [M+H]+ (calcd. for C23H31O7, 419.2064).

**Isopenicillistressol (8):** yellowish syrup; [α]24D − 42.3 (c 0.10, CHCl3); UV (MeOH) λ_max (log ε) 274 (3.93) nm; IR ν_max 3415, 1711, 1656, 1631, 1561, 1453, 1400, 1286, 1253, 1213, 1090, 1063, 1020, 939, 812 cm⁻¹; 1H NMR and 13C NMR, see Table 3; HR-TOF-MS m/z 419.2048 [M+H]+ (calcd. for C23H31O7, 419.2064) (see Supplementary Material).

### Computation Section

The geometry was optimized starting from initial conformations, with DFT calculations at the B3LYP/6-31+G(d) level using the Gaussian 09 program. Frequency analysis was done at the same level of theory to verify that these optimized structures are real minima on the potential energy surface. Time-dependent DFT calculations were performed on the lowest-energy conformations for each configuration using 30 excited states and under the methanol solution. ECD spectra

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**Table 1** | 1H NMR data (500 MHz, δ in ppm, J in Hz), 13C NMR data (125 MHz, δ in ppm) for compounds 1 and 2 in CDCl3.

| Position | 1 (CDCl3)  | 2 (CDCl3) |
|----------|------------|-----------|
|          | δC         | δH (J in Hz) | δC         | δH (J in Hz) |
| 1        | 12.46, CH3  | 1.19d (6.36) | 12.26, CH3  | 1.18d (6.10) |
| 2        | 77.51, CH   | 3.85*       | 77.50, CH   | 3.84*       |
| 3        | 81.26, C    |             | 80.79, C    |             |
| 4        | 84.74, CH   | 3.99s       | 85.60, CH   | 3.98s       |
| 5        | 86.50, C    |             | 84.10, C    |             |
| 6        | 148.17, CH  | 5.72s       | 141.41, CH  | 5.50s       |
| 7        | 136.29, C   |             | 136.11, C   |             |
| 8        | 138.49, CH  | 6.37*       | 140.84, CH  | 6.36*       |
| 9        | 127.04, CH  | 6.35*       | 127.75, CH  | 6.34*       |
| 10       | 139.35, CH  | 6.59dd (14.53, 11.04) | 138.71, CH  | 6.51*       |
| 11       | 130.26, CH  | 6.32*       | 130.96, CH  | 6.47dd (16.51, 12.11) |
| 12       | 136.47, CH  | 7.20*       | 134.07, CH  | 7.18dd (14.54, 11.57) |
| 13       | 119.15, CH  | 6.34*       | 118.43, CH  | 6.39*       |
| 14       | 154.93, C   |             | 154.60, C   |             |
| 15       | 108.51, C   |             | 107.84, C   |             |
| 16       | 171.39, C   |             | 170.85, C   |             |
| 17       | 88.82, CH   | 5.57s       | 88.40, CH   | 5.50s       |
| 18       | 164.97, C   |             | 164.19, C   |             |
| 19       | 17.49, CH3  | 1.20s       | 17.37, CH3  | 1.22s       |
| 20       | 22.66, CH3  | 1.39s       | 21.27, CH3  | 1.38s       |
| 21       | 14.15, CH3  | 1.94s       | 13.30, CH3  | 1.92s       |
| 22       | 9.11, CH3   | 1.98s       | 9.18, CH3   | 1.96s       |
| 23       | 56.57, CH3  | 3.85s       | 56.16, CH3  | 3.84s       |

*Peaks are overlapped.
with a starting concentration of 512 standard. Tetracycline was dissolved in Mueller-Hinton broth inoculum density of 5

Each pathogenic culture was then diluted in 0.9% saline to an

[CMCC(B)46117] were cultured and left overnight to grow.

Peaks are overlapped.

were generated using the program SpecDis by applying a Gaussian band shape with 0.2 eV width, from dipole-length rotational strengths (Frisch et al., 2009; Xie et al., 2011, 2012; Bruhn et al., 2015).

**Antibiotic Activity Screening of Stress Metabolites**

The conventional broth dilution assay was used to evaluate the antibacterial activities. Three clinical pathogens namely MRSA, P. aeruginosa [CMCC(B)10104], and K. pneumoniae [CMCC(B)46117] were cultured and left overnight to grow. Each pathogenic culture was then diluted in 0.9% saline to an inoculum density of 5 × 10⁸ cfu by comparison with a McFarland standard. Tetracycline was dissolved in Mueller–Hinton broth with a starting concentration of 512 µg/mL and was used as positive control whereas methanol was used as negative control. A total of 125 µL of MHB was distributed into the 96-well plates. A total of 125 µL of the different samples were dispensed into well 1 and serially diluted across the plate. Ultimately, addition of 125 µL of the bacterial inoculum was performed and the plates were incubated at 37°C for 18 h. The bacteriostatic abilities of the compounds were noted as MBCs which was equal to the lowest concentration of the samples which killed 99.9% of the original inoculum (Clinical and Laboratory Standards Institute, 2014).

**Cytotoxicity Assay**

Human liver cell line LO2 was obtained from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences). LO2 cells were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 units/mL streptomycin. All the cells were grown in a humidified cell incubator with an atmosphere of 5% CO₂ at 37°C and subcultured with 0.25% trypsin + 0.02% EDTA. MTT, dimethyl sulfoxide (DMSO), ribonuclease (RNase), propidium iodide (PI), Annexin V-FITC/PI kit were obtained from Sigma. RPMI 1640, DMEM, FBS were purchased from Gibco. Hoechst, trypsin, and the pan caspase inhibitor z-VAD-FMK were obtained from

### Table 2 | 1H NMR data (500 MHz, δ in ppm, J in Hz), 13C NMR data (125 MHz, δ in ppm) for compounds 5 and 6 in MeOD.

| Position | 5 (MeOD) | 6 (MeOD) |
|----------|----------|----------|
|          | δC       | δH (J in Hz) | δC       | δH (J in Hz) |
| 1        | 13.49, CH₃ | 1.6d (6.43) | 13.49, CH₃ | 1.17* |
| 2        | 80.97, CH₃ | 4.04* | 80.24, CH | 4.08* |
| 3        | 84.76, C | 86.00, C | 84.96, C | |
| 4        | 78.51, CH₃ | 3.98s | 75.85, CH | 3.99s |
| 5        | 84.50, C | 84.96, C |  |
| 6        | 80.97, CH₃ | 3.60s | 82.25, CH | 3.75s |
| 7        | 79.82, C | 79.26, C |  |
| 8        | 148.53, CH₃ | 6.10* | 147.54, CH | 6.21* |
| 9        | 128.71, CH₃ | 6.41* | 127.36, CH | 6.39* |
| 10       | 138.87, CH₃ | 6.57* | 139.53, CH | 6.59* |
| 11       | 132.49, CH₃ | 6.49* | 132.00, CH | 6.46* |
| 12       | 137.05, CH₃ | 7.14dd (14.98, 10.99) | 137.12, CH | 7.12dd (15.39, 11.41) |
| 13       | 120.36, CH₃ | 6.55* | 120.01, CH | 6.54* |
| 14       | 155.90, C | 156.00, C |  |
| 15       | 109.82, C | 109.61, C |  |
| 16       | 173.12, C | 173.10, C |  |
| 17       | 89.14, CH₃ | 5.62s | 89.06, CH | 5.60s |
| 18       | 166.37, C | 166.37, C |  |
| 19       | 17.52, CH₃ | 1.27s | 16.70, CH | 1.30s |
| 20       | 18.65, CH₃ | 1.29s | 17.29, CH | 1.30s |
| 21       | 26.72, CH₃ | 1.26s | 27.88, CH | 1.35s |
| 22       | 8.87, CH₃ | 2.00s | 8.89, CH₃ | 1.99s |
| 23       | 57.28, CH₃ | 3.90s | 57.27, CH₃ | 3.89s |

*Peaks are overlapped.

### Table 3 | 1H NMR data (500 MHz, δ in ppm, J in Hz), 13C NMR data (125 MHz, δ in ppm) for compounds 7 and 8 in MeOD.

| Position | 7 (MeOD) | 8 (MeOD) |
|----------|----------|----------|
|          | δC       | δH (J in Hz) | δC       | δH (J in Hz) |
| 1        | 13.56, CH₃ | 1.13* | 13.03, CH₃ | 1.21* |
| 2        | 16.69, CH | 1.25* | 13.43, CH | 1.27* |
| 3        | 85.19, C | 80.27, C |  |
| 4        | 78.70, CH | 3.70s | 78.51, CH | 3.69s |
| 5        | 87.96, C | 85.90, C |  |
| 6        | 91.10, CH | 4.18s | 89.28, CH | 4.17s |
| 7        | 137.70, C | 136.55, C |  |
| 8        | 128.70, CH | 6.18d (11.39) | 127.55, CH | 6.18d (10.90) |
| 9        | 130.99, CH | 6.54* | 130.86, CH | 6.56* |
| 10       | 133.62, CH | 6.28* | 131.39, CH | 6.30* |
| 11       | 134.34, CH | 6.47* | 133.49, CH | 6.46d (14.88, 10.81) |
| 12       | 131.54, CH | 5.91dd (15.10, 6.83) | 128.72, CH | 5.90* |
| 13       | 70.30, CH | 5.24d (6.85) | 70.13, CH | 5.24d (6.96) |
| 14       | 160.41, C | 160.25, C |  |
| 15       | 109.64, C | 109.50, C |  |
| 16       | 173.37, C | 173.22, C |  |
| 17       | 89.42, CH | 5.62s | 88.01, CH | 5.62s |
| 18       | 166.85, C | 166.72, C |  |
| 19       | 13.16, CH₃ | 1.27s | 12.77, CH₃ | 1.20s |
| 20       | 13.81, CH₃ | 1.16s | 15.42, CH₃ | 1.13s |
| 21       | 15.56, CH₃ | 1.78s | 13.69, CH₃ | 1.77s |
| 22       | 8.78, CH₃ | 2.00s | 8.67, CH₃ | 2.00s |
| 23       | 57.47, CH₃ | 3.90s | 57.35, CH₃ | 3.82s |

*Peaks are overlapped.
Beyotime Institute of Biotechnology. Compounds were dissolved in DMSO and diluted to the proper concentrations before use, with the concentration of DMSO kept below 0.1% in all assays. Cell viability was measured by the MTT method as previously described, with some modifications (Jiang et al., 2013). Briefly, cells were seeded in 96-well microtiter plates at a density of $5 \times 10^3$ cells/well for 24 h. After drug treatment for the indicated times, cells were incubated with MTT (0.5 mg/mL) for 4 h. The formazan precipitate was dissolved in 150 $\mu$L DMSO, and the absorbance was detected at 490 nm with a Sunrise microplate reader (Tecan Group Ltd.). DMSO (0.1%) was used as negative control for all assays. Each test was performed in triplicate.

RESULTS AND DISCUSSION

During this study, cobalt was chosen as the elicitor to stress the fungus which boosted up its antibacterial exhibition in comparison to the normal growing strain. To prove this hypothesis, the MIC of the cobalt-stressed extract was tested against the three pathogens namely MRSA, P. aeruginosa, and K. pneumoniae where it was revealed to be much lower (0.5 $\mu$g/mL) compared to the normal culture extract with much higher MIC value which was considered negligible.

Moreover, based on the difference of the HPLC profile between the normal culture (blue) and the cobalt-stressed culture (pink), eight metabolites which as shown in Figure 1 have been successfully isolated, among which four are novel and four are known compounds. In this study, our focus pointed out on the semi-polar compounds which were easily identified as new peaks popping out compared to their absence in the normal culture. As such, enlargement of cobalt-stressed culture was performed following the HPLC-guided isolation which allowed the isolation of the stress metabolites by application of the preparative HPLC (C18) followed by the elucidation and confirmation of a new citreoviridin analog (1), a new polynye pyrone polyketide with a 2,6-dioxabicyclo[3,2,1]octane ring (5) and two new polynye pyrone polyketides with 2,5-dioxabicyclo[2,2,1]heptane ring (7 and 8) together with four known compounds with the help of spectroscopic analysis techniques like 1D, 2D NMR and HRMS (Figure 2).

Novel Compounds Elucidation

Compound 1

Compound 1 was isolated as a yellowish syrup. The molecular formula was determined to be $C_{23}H_{30}O_{6}$ by analysis of the HR-TOF-MS ion peaks at $m/z$ 425.1919 [M+Na]$^+$ (calcd. 425.1935), $m/z$ 403.2102 [M+H]$^+$ (calcd. 403.2115), and $m/z$ 827.3964 [2M+Na]$^+$ (calcd. 827.3977). The $^1$H and $^{13}$C NMR of 1 (Table 1) showed similar chemical shifts and same multiplicities for 23 carbon atoms of a tetrahydrofuran ring, an $\alpha$-pyrone ring and an octa-1,3,5,7-tetraene with a minor difference indicating that compound 1 supposedly possess the basic structure of $\alpha$-pyrone type polyketide (da Rocha et al., 2015). Naturally occurring $\alpha$-pyrone type polyketides can be categorized into two main classes: verrucosidin analogs and citreoviridin analogs.
FIGURE 2 | Structures of the isolated stress metabolites 1–8. HPLC-guided isolation of four novel compounds corresponding to a citreoviridin analog (1), a new polyene pyrone polyketide with a 2,6-dioxabicyclo[3,2,1]octane ring (5) and two new polyene pyrone polyketides with 2,5-dioxabicyclo[2,2,1]heptane ring (7 and 8) together with four known compounds epicitsreoviridinol (4), citreoviridinol (6), epicitreoviridinol (3), and citreoviridin (2) were isolated from cobalt-stressed Penicillium sp. BB1122.

Both classes of molecules contain two distinct domains: α-pyrone ring and a tetrahydrofuran ring connected by an olefinic chain (Pan et al., 2016). Both polyketides analogs exist in nature with the verrucosidinol analogs found in the majority of structures. The main differences lie in the olefinic chain where there are three methyl groups on the olefinic chain of verrucosidinol...
analogs whereas only one methyl group is found on the olefinic chain of citreoviridin analogs. The characteristic olefinic proton signals at δH 5.72 (s, H-6), 6.37 (H-8), 6.35 (H-9), 6.59 (dd, J = 14.53, 11.04 Hz, H-10), 6.32 (H-11), 7.20 (H-12), 6.34 (H-13), and a methyl protons signal at δH 1.94 (s, H-21) in the 1H NMR spectrum excluded the possibility of the existence of verrucoosidinol analogs. This influence was further confirmed by the lack of the substructure of oxirane ring in I. The diagnostic sequence of H8 = H9 = H10 = H11 = H12 = H13 deduced from the cross peak of H-9/H-10, H-10/H-11, H-11/H-12, H-12/H-13 in the 1H-1H COSY of I demonstrated the presence of citreoviridin backbone. To clarify the connection of three domains and the positions of two hydroxyls, five methyls, one carbonyl, and a methoxy group in the supposed citreoviridin backbone, detailed 2D NMR experiments were carried out. The olefinic protons signals at δH 5.57 (s, H-17) showed long range correlations with lactone carbonyl carbon signal at δC 164.97 (C, C-18) and δC 108.51 (C, C-15) and δC 171.39 (C, C-16), among which the olefinic C-16 displayed HMBC cross peak of OCH3/C-16, positioning the carbonyl at C-18 and OCH3 at C-16 of the α-pyrene ring. The Me-22 was located at C-15 owing to the long range correlation between δH 1.98 (s, Me-22) and δC 154.93 (C, C-14), 119.15 (CH, C-13) in the HMBC spectrum of 1. The HMBC cross peak of Me-21/C-7 and Me-21/C-6 and NOESY cross peak of H-6/Me-21 were observed, exhibiting that Me-21 was assigned to C-7 on the olefinic ring in molecule 1. Two oxygenated tertiary carbons at δC 77.51 (CH, C-2), 84.74 (CH, C-4) and two oxygenated quaternary carbons at δC 81.26 (C, C-3), 86.50 (C, C-5) were observed in the 13C NMR spectrum of 1, revealing the presence of a highly oxygenated tetrahydrofuran ring. Three methyl groups on the tetrahydrofuran ring positioned at C-2, C-3, and C-5 were deducted from the observation of long range correlations from proton signal at δH 1.19 (d, J = 6.36 Hz, Me-1) to carbon resonance at δC 77.51 (CH, C-2), 81.26 (C, C-3), and correlations from proton resonance at δH 1.20 (s, Me-19) to carbon signals at δC 77.51 (CH, C-2), 84.74 (CH, C-4), and correlations from the proton signals at δH 1.39 (s, Me-20) to carbon resonance at δC 84.74 (CH, C-4), 86.50 (C, C-5). Two hydroxyl groups were inferred from the oxygenated carbon resonance feature of C-3 and C-4 in the 13C NMR spectrum of I with assistance to the molecular formula C23H30O6. The α-pyrene ring and the olefinic chain were bridged by C13–C14 bond deducted from the HMBC analysis of the cross peaks from hydrogen signal at δH 6.34 (H-13) to carbon resonances at δC 154.93 (C, C-14), 108.50 (C, C-15) and hydrogen signal at δH 1.98 (s, Me-22) to carbon signal at δC 86.50 (C, C-5) proved the linkage of C5–C6 between the tetrahydrofuran ring and the olefinic chain, which was also confirmed by NOESY cross peak C-6/C-4 in compound 1. From the above analysis, the planar structure was elucidated as drawn in Figure 3 which appeared the same as citreoviridin. Detailed analysis of NOESY experiment (Figure 4) proved that the geometry of I of C6=C7 double bond was different from the E configuration of C6=C7 in citreoviridin. The diagnostic NOESY correlation from proton signal at δH 5.72 (s, H-6) to proton signal at δH 1.94 (s, Me-21) demonstrated Z configuration of C6=C7 which is the first example in nature. The relative configuration at C-5 was proved to be different from that of the known compound citreoviridin. In the NOESY spectrum of I, the proton resonance at δH 1.39 (s, Me-20) showed cross peak with proton signal at δH 3.99 (s, H-4) indicating that the olefinic chain at C-5 and the hydroxyl at C-4 were on the same side as β-oriented as opposed to the α-oriented of the olefinic chain in the known compound citreoviridin. The hydroxyl at C-3 and the methyl at C-2 were on the same side due to the observation of the NOESY cross peak of Me-19/H-2, and at the same time Me-19 showed no correlation with H-4 indicating an α-oriented Me-1 and β-oriented Me-19.

To further confirm the new structures, ESI-MS/MS experiment of compound 1 was carried out. The positive and negative modes of compound I were achieved by MS2 experiments (see Supplementary Material). The positive ion ESI mass spectrum of compound gave the major [M+H]+ ion at m/z 403.2101. As shown in Figure 5, the fragmentation of this precursor ion yielded an interesting product ion at m/z 315.1586, which was attributed to the elimination of a neutral molecule C4H3O2 (88 Da) from the precursor ion m/z 403.2101. The product ions at m/z 297.1478 and 285.1474 were generated by the loss of 18 and 30 Da, which were reasonably assigned as the elimination of H2O and formaldehyde (CH2O), respectively. Furthermore, neutral loss of 146 Da (assigned to C11H14) to...
produce fragment ion at m/z 139.0851 was also observed. The negative ion ESI mass spectrum of the compound gave the major [M-H]⁻ ion at m/z 401.1984. The fragment ion at m/z 300.1371 was generated by the loss of 101 Da, which was consistent with the presence of the hydroxyl residue at C-3 position with continuous loss of methyl radical (15 Da) to yield a product ion at m/z 285.1122.

Determination of the absolute configuration of 1 by simulation of the ECD spectrum was performed. GAUSSIAN 09 was run to optimize the minimum energy geometries of two conformers using DFT at the B3LYP/6-31+G(d,p) level in the gas phase. The ECD spectra calculation were simulated using the time-dependent density functional theory (TDDFT) employing the B3LYP functional at the B3LYP/6-31+G(d,p) level in methanol (Frisch et al., 2009; Xie et al., 2011, 2012; Bruhn et al., 2015). SpecDis was used to draw the calculated ECD curves with a σ of 0.2 eV. The calculated ECD spectrum was seen to be identical to the experimental ECD spectrum where the absolute configuration of 1 was determined as 2S, 3S, 4S, 5S (Figure 6). Compound 1, being a new polyketide was thus termed as neocitreoviridin (Figure 2).

**Compound 7**

Polyene α-pyrene polyketide mycotoxins, a group of compounds attracted lots of attention because of their novel structures as well as biological properties (Wang et al., 2005) whose structure possess a conjugate pyrone system and a 2,6-dioxabicyclo[3.2.1]octane ring moieties. Two main structure features can be categorized as citreoviridin analogs and aurovertin analogs (Nishiyama et al., 1987; Wang et al., 2005). The only difference between the two classes lies in that the 2,6-dioxabicyclo[3.2.1]octane ring is attached by ethyl group in the first category namely citreoviridin analogs while methyl group is attached in aurovertin analogs. Aurovertins have been extensively studied whereas although citreoviridin (four naturally occurring compounds have been isolated so far), were isolated during the year 1970–1980, the ¹H and ¹³C NMR have not been assigned and their absolute configurations have not been achieved. During this study, three known citreoviridin analogs namely citreoviridinol (6), epicitreoviridinol (3), and epissocitreoviridinol (4) (Nishiyama et al., 1987; Wang et al., 2005) and one new citreoviridin analog namely 10Z-isocitreoviridinol (5) were isolated as stressed metabolites. Their NMR assignments and the absolute configurations were achieved for the first time.

Compound 7 (Figure 2) was obtained as yellowish syrup. The molecular formula of 7 was determined to be C₃₀H₃₀O₇ on the basis of HR-TOF-MS ion peak at m/z 419.2045 [M+H]⁺ (calcd. 419.2064). The ¹H and ¹³C NMR spectra of 7 (Table 3) showed similar chemical shifts for seven carbon atoms of α-pyrene moiety as compared to the new compound 1 and known compounds citreoviridinol (6), epicitreoviridinol (3), and epissocitreoviridinol (4) indicating that compound 7 is an α-pyrene polyketide. However, the polyene olefinic chain was proved to be different from those of citreoviridinol, aurovertin, and citreoviridin analogs (Wang et al., 2005; da Rocha et al., 2015) based on 1D and 2D NMR data of 7. The unique proton signal at δH 5.24 (d, J = 6.85 Hz, H-13) in the ¹H NMR spectrum of 7 differentiated its structure from all the naturally occurring citreoviridinol, aurovertin, and citreoviridin analogs where their olefinic chain is directly attached on an α-pyrene unit. The substitute of hydroxyl group on C-13 enabled the migration of the polyene chain. To determine the polyene migration, detailed 2D NMR experiments were carried out. The olefinic chain in the molecule 7 was linked to α-pyrene ring by a oxygenated carbon which was confirmed by the ¹H,¹H COSY sequence of C8–C9 = C10–C11 = C12–CO and long range correlations from proton signal at δH 5.24 (d, J = 6.85 Hz, H-13) to the olefinic carbon signal δC 131.54 (CH, C-12) in the polyene chain and the olefinic carbon signal δC 160.41 (C, C-14) in the α-pyrene ring. This is the first example of the polyene migration phenomenon in the α-pyrene polyketide. Because of the migration of the double bonds, the 2,6-dioxabicyclo[3.2.1]octane ring system was rearranged. The rearrangement of the ring system in the other side of the polyene chain of compound 7 was determined mainly by the analysis of HMBC and NOESY spectra. Since one double bond was migrated to C-7, the octane ring that was linked by oxygen to C-7 was opened on the linkage between oxygen atom and C-7. In spite of the octane ring opening, the NOESY correlations from the proton signal at δH 3.70 (s, H-4)
FIGURE 5 | The plausible key MS² fragmentation pathway of compound 1 was confirmed by ESI-MS/MS analysis in positive and negative modes. During positive mode, the fragmentation of 1 yielded a product ion at m/z 315.1586 which in turn lost 18 Da followed by loss of 30 Da and ultimately neutral loss of 146 Da was seen. During negative mode, fragment ion at m/z 300.1371 was generated by the loss of 101 Da followed by continuous loss of methyl radical.

to proton signal at $\delta_H$ 1.78 (s, Me-21) and olefinic proton signal at $\delta_H$ 6.18 (d, $J = 11.39$ Hz, H-8) indicated the closeness between them. Thus, it was hypothesized that the octane ring was condensed, which was confirmed by the analysis of the molecular formula $C_{23}H_{30}O_7$. In the opened octane ring, there were two oxygenated carbons namely C-4 and C-6, holding the possibility to condense the ring with oxygenated C-3. However, the orientation of C₄-O bond at C-4 was proved to point to
the opposite side of C3-O bond owing to the NOESY cross peaks of the proton resonance at δH 3.70 (s, H-4) to the proton resonance at δH 6.18 (d, J = 11.39 Hz, H-8) and of the proton resonance at δH 3.70 (s, H-4) to the proton resonance at δH 1.78 (s, Me-21). This inference ruled out the possibility of rearranged ring between C-4 and C-3 by an oxygen atom bridge, leaving the only possibility to rearrange the 2,6-dioxabicyclo[3.2.1]octane ring into 2,5-dioxabicyclo[2.2.1]heptane ring bridged by O atom between C-6 and C-3. From the above established data, compound 7 was assigned as a pyrone polyketide with a 2,5-dioxabicyclo[2.2.1]heptane ring and a α-pyrone connected by a migrated polyene chain. The three main domains were constructed by the detailed analysis of 2D NMR data. The olefinic chain was deduced to connect the heptane ring through C6–C7 from the characteristic cross peaks Me-21/C-6, H-6/C-7, and H-6/C-8. The long range correlation from methyl proton signal...
at $\delta_{H}$ 1.78 (s, Me-21) to carbon signal at $\delta_{C}$ 128.70 (CH, C-8) indicated that Me-21 stayed at C-7 as those of citreoviridinols (Nishiyama et al., 1987; Forbes et al., 1991). Three methyl groups Me-1, Me-19, and Me-20 were positioned at C-2, C-3, and C-5, respectively on the 2,5-dioxabicyclo[2.2.1]heptane ring, which was inferred from the HMBC cross peaks of Me-1/C-2, Me-19/C-3, and Me-20/C-5. A hydroxyl group was placed on C-4 based on long range correlation from the proton signal at $\delta_{H}$ 3.70 (s, H-4) to carbon signal at $\delta_{C}$ 85.19 (C, C-3). The long range correlation from the proton resonance at $\delta_{H}$ 5.91 (dd, $J = 15.10$, 6.83 Hz, H-12) and the proton resonance at $\delta_{H}$ 5.24 (d, $J = 6.85$ Hz, H-13) in the polyene chain to the olefinic carbon signal at $\delta_{C}$ 160.41 (C, C-14) connected the polyene chain to the pyrone ring by an oxygenated C-13 on which a hydroxyl was placed. From the above analysis, the planar structure of 7 was elucidated as a new polyene pyrone polyketide as drawn in Figure 7. The relative configuration of 7 was determined by NOESY experiment. The olefinic chain was assigned as $\beta$-oriented due to the observation of the NOESY cross peaks of H-4/Me-21 and H-4/H-8 allowing the determination of the relative configuration of 7. (B) The diagnostic NOESY cross peak of H-4/H-6 as well as the NOESY cross peak of H-4/Me-19 demonstrated the configuration of 8, which was different from 7.

Compound 8 (Figure 2), a yellowish syrup, was isolated as a mixture with compound 7. The HRMS (TOF) exhibited an ion peak at $m/z$ 419.2048 [M+H]$^+$ (calcd. 419.2064), corresponding to the molecular formula C$_{23}$H$_{30}$O$_7$. With the help of 2D NMR data of the mixture and the NMR data of the pure compound 7, the proton signals and carbon signals were explicitly singled out (Table 3). The NMR data of compounds 8 and 7 were in very close agreement to each other with minor difference at C-5 ($\delta_{C}$ 85.90), C-6 ($\delta_{C}$ 89.28), C-20 ($\delta_{C}$ 15.42), C-21 ($\delta_{C}$ 13.69) and 5 ppm difference at C-3 ($\delta_{C}$ 80.27) in their $^{13}$C NMR spectra. Detailed
1D and 2D NMR experiments allowed the complete assignment of the planar structure of 8 which appeared to be the same as compound 7 (Figure 7). It was implied that the configuration of 8 in certain chiral centers was different between 8 and 7. In order to clarify the difference between the structure features of the two compounds, detailed NOESY experiments were carried out. The hydroxyl group at C-4 was oriented toward the opposite side of the polyene chain as that in 7 due to the observation of NOESY cross peak H-4/Me-19 and H-4/H-6. The diagnostic cross peak of H-4/Me-21 and H-4/H-8 in 7 demonstrated the configuration of 8 was different from 7 with the olefinic chain being β-oriented in 8 (Figure 8). The new compound was given the trivial name isopenicillstressol.

To further confirm the new structures, ESI-MS/MS experiment of compounds 7 and 8 was carried out. The positive mode of 7 and 8 was achieved by MS² experiments with the precursor ion [M+H]^+ ion at m/z 419. The characteristic ion at m/z 275.1261 was produced from precursor ion by the loss of C_7H_8O_3 (140 Da) followed by double bonds fragmentation.

**Compound 5**

Compound 5 was obtained as a yellowish syrup. The molecular formula C_{23}H_{30}O_{7} was determined by analysis of the HR-TOF-MS ion peak at m/z 419,2049 [M+H]^+ (calcd. 419,2064). The formula was supported by $^{13}$C NMR data which indicated 9 degrees of unsaturation. In the $^1$H and $^{13}$C NMR spectrum of 5, important characteristics signals such as methyl signals [δ<sub>H</sub> 1.16 (d, J = 6.43 Hz, Me-1); δ<sub>H</sub> 1.27 (s, Me-19); δ<sub>H</sub> 1.29 (s, Me-20); δ<sub>H</sub> 1.26 (s, Me-21); δ<sub>H</sub> 2.00 (s, Me-22); δ<sub>H</sub> 3.90 (s, Me-23)] as well as conjugate olefinic pattern in the $^{1}$H COSY (H-8/H-9, H-9/H-10, H-10/H-11, H-11/H-12, H-12/H-13) are indicative of a citreoviridinol backbone possessing a 2,6-dioxabicyclo[3.2.1]octane attached to a γ-pyrone through a conjugated olefinic chain. Further detailed analysis of 2D NMR data and comparison of the NMR data with known citreoviridinol analogs in literature (Forbes et al., 1991; Wang et al., 2005) strongly suggested that the structure of compound 5 was highly similar to that of isocitreoviridinol (Figure 10). The NOESY cross peak from the proton signal at δ<sub>H</sub> 3.98 (s, H-4) in the octane ring unit to the olefinic proton at δ<sub>H</sub> 6.10 (H-8) revealed that the olefinic chain was β-oriented and a boat conformation of the octane was adopted because of the close distance between C-8 and C-4. The hydroxyl group at C-6 was deduced to be β-oriented from the analysis of a diagnostic cross peak from the proton signal at δ<sub>H</sub> 3.60 (s, H-6) to the proton signal at δ<sub>H</sub> 1.26 (s, Me-21). Me-19

![Chemical Structure of Compound 7](image_url)
displayed NOESY correlation with H-2 indicating an α-oriented Me-1. All above analysis matched well to the structure of the known compound isocitreoviridinol (Nishiyama et al., 1985). However, detailed analysis of the conjugated olefinic chain with NOESY experiment demonstrated that the configuration of the double bond C10=C11 was Z configuration as opposed to E configuration in isocitreoviridinol (Figure 11). Therefore, 5 was elucidated as a new compound named as 10Z-isocitreoviridinol.

In order to determine the stereochemistry of compound 5, the theoretically calculated ECD spectrum and the experimental ECD spectrum were compared. The minimum energy geometries of two conformers were optimized using DFT at the B3LYP/6-31+G(d,p) level in the gaseous phase (Frisch et al., 2009; Xie et al., 2011, 2012; Bruhn et al., 2015). The calculated ECD spectrum was seen to be similar with the experimental ECD spectrum (Figure 6) due to the negative and positive Cotton effects (CEs) leading to the thorough configuration of 5. Therefore the absolute configuration of 5 was determined as 2S, 3R, 4S, 5R, 6S, 7R (Figure 2).

**Known Compounds Elucidation**

Furthermore, known compounds episiocitreoviridinol (4), citreoviridinol (6), epicitreoviridinol (3), and citreoviridin (2) (Figure 2) were determined by detailed 1D and 2D NMR data analysis as well as comparison of the data in literature (Tables 1, 2, 4). Since, known compounds episiocitreoviridinol (4), citreoviridinol (6), epicitreoviridinol (3) possessed the same planar structure, NOESY experiments were applied to elucidate their structures as shown in Figure 12.

The relative configuration of the known compound citreoviridin was also confirmed by NOESY experiments as shown in Figure 4.

The absolute configurations of the known compounds episiocitreoviridinol (4) and citreoviridinol (6), epicitreoviridinol (3) were determined by simulation of the ECD spectra. GAUSSIAN 09 program was employed to optimize the minimum geometries of two conformers DFT at the B3LYP/6-31+G(d) level in the gas phase. The ECD spectra calculation were simulated using the time-dependent density functional theory (TDDFT) using the B3LYP functional at the B3LYP/6-31+G(d) level in methanol. The calculated ECD curves were drawn by using SpecDis with a σ of 0.2 eV (Frisch et al., 2009; Xie et al., 2011, 2012; Bruhn et al., 2015). As such, the absolute configurations of episiocitreoviridinol (4), citreoviridinol (6), and epicitreoviridinol (3) were determined as 2S, 3R, 4S, 5R, 6R, 7R and 2R, 3S, 4R, 5S, 6S, 7R and 2R, 3S, 4R, 5S, 6R, 7R, respectively (Figure 6).

### Biological Activity, Cytotoxicity, and Possible Mode of Action

The isolated novel and known compounds were tested for their bacteriostatic as well as bactericidal abilities against three pathogenic bacteria namely MRSA, *P. aeruginosa*, and *K. pneumoniae*. The ESKAPE pathogens were used in this study due to fact that they are the main cause of nosocomial infections worldwide.

The novel compounds 7 and 8 exhibited impressive antibiotic abilities with MIC value of around 0.5 μg/mL followed by the novel compound 5 as well as the known compounds 3 and 6 which also showed potent antibiotic capacities with MIC values of 1 and 4 μg/mL against MRSA, respectively. Moreover, compounds 1, 7, and 8 displayed strong antibiotic activities with MIC values of around 4 followed by MIC values of 8 for compounds 2 and 5 against *P. aeruginosa*. However, lower antibacterial activities were noted for compounds 1, 2, and 4 against MRSA and compounds 3, 4, and 6 against *P. aeruginosa* as well as compounds 1, 2, 5, 7, and 8 against *K. pneumoniae* with MIC values of around 64 μg/mL. The positive control tetracycline displayed MIC values of around 2 and 8 μg/mL against MRSA and *P. aeruginosa*, respectively. The MBCs of the different potent compounds ranged from 1 to 128 μg/mL against MRSA, *P. aeruginosa*, and *K. pneumoniae*. The MIC and MBC values of the isolated compounds surpassing 128 μg/mL against the three pathogens were considered as “negligible capacity” (Supplementary Table S1).

Moreover, due to the lack of the amount of the different isolates, only compounds 7 and 8 were evaluated for their cytotoxicity. As a result, the new antibiotics 7 and 8 were not obviously harmful toward normal liver cell lines LO2, showing IC50 values above 100 μg/mL.

### Table 4

| Position | 3 (CDCl3) | 4 (CDCl3) |
|----------|-----------|-----------|
|          | δC        | δH (J in Hz) | δC        | δH (J in Hz) |
| 1        | 13.09, CH3 | 1.20d (6.38) | 13.18, CH3 | 1.19d (6.34) |
| 2        | 78.07, CH | 4.02* | 79.54, CH | 4.08* |
| 3        | 84.01, C  | 82.97, C | 85.33, CH | 3.98s |
| 4        | 74.61, CH | 3.98s | 75.33, CH | 3.98s |
| 5        | 83.09, C  | 82.62, C | 80.29, CH | 3.61s |
| 6        | 82.77, CH | 3.75s | 78.18, C  | 3.75s |
| 7        | 78.42, C  | 78.18, C | 78.18, C  | 78.18, C |
| 8        | 141.93, CH | 6.22* | 146.64, CH | 5.99d (15.33) |
| 9        | 128.81, CH | 6.39* | 128.14, CH | 6.38* |
| 10       | 137.80, CH | 6.53* | 137.25, CH | 6.49* |
| 11       | 131.27, CH | 6.48* | 131.53, CH | 6.43* |
| 12       | 135.99, CH | 7.16dd (14.99, 11.21) | 135.84, CH | 7.16dd (15.07, 11.09) |
| 13       | 119.02, CH | 6.51* | 119.22, CH | 6.46* |
| 14       | 154.56, C  | 154.52, C | 150.86, C  | 150.86, C |
| 15       | 108.12, C  | 108.36, C | 171.10, C  | 171.10, C |
| 16       | 170.99, C  | 171.10, C | 171.10, C  | 171.10, C |
| 17       | 88.66, CH | 5.55s | 88.72, CH | 5.60s |
| 18       | 164.43, C  | 164.63, C | 164.63, C  | 164.63, C |
| 19       | 16.15, CH3 | 1.25s | 17.28, CH3 | 1.25s |
| 20       | 16.92, CH3 | 1.33s | 18.57, CH3 | 1.34s |
| 21       | 31.57, CH3 | 1.33s | 26.50, CH3 | 1.28s |
| 22       | 8.85, CH3  | 1.97s | 8.85, CH3  | 1.98s |
| 23       | 56.27, CH3 | 3.85s | 56.31, CH3 | 3.86s |

*Peaks are overlapped.*
Various factors such as the difficulty of entering pathogenic cells where Gram-negative bacteria being famous for their tenacious outer membrane and cytoplasmic membrane as well as their efflux pump or the inactivation or alteration of the target in both Gram-positive and Gram-negative bacteria (Singh and Barett, 2006) have made it challenging to find potent antimicrobials. For instance, one among the mechanisms of β-lactams was revealed as being able to interrupt the homeostatic biosynthesis of cell walls. They showed the capacity of restricting peptide bond formation which are usually catalyzed by transpeptidase enzymes, thus hindering the cross-bonding of peptidoglycan units in pathogenic cell walls (Tipper and Strominger, 1965; Wise and Park, 1965) whereas quinolones/fluoroquinolone are known to constrain DNA gyrase or topoisomerase II and IV which are vital for strand rejoining (Chen et al., 1996; Drlica et al., 2008). On the other hand, macrolides displayed the ability of impeding protein synthesis by linking to the 50S bacterial ribosomal subunit leading to the restriction of the attachment of the peptidyl to the tRNA to the next amino acid by peptidyl transferase (Menninger and Otto, 1982; Vannuffel and Cocito, 1996; Kohanski et al., 2010).

The compounds isolated in this study belong to the α-pyrene class of antibiotics (Tupin et al., 2009) where the structures of the new antibiotics 7, 8, and 5 bear similarities to myxopyronins which possess an α-pyrene and polyene units. In a previous study, myxopyronins were suggested as being RNA synthesis inhibitors. The main target of myxopyronins was the bacterial RNA polymerase (RNAP) which consists of large subunits of β and β′, giving RNAP a jaw-like shape. It was revealed that myxopyronin had the ability of binding to the switch-2 region of the RNAP β′ subunit, which restricted the open promoter complex formation. Moreover, it was also shown that myxopyronin had greater chance of hampering transcription at an early initiation step, most probably before the addition of the promoter DNA (Tupin et al., 2009). Thus, it could be hypothesized that the compounds exhibiting antibiotic capacities might have targeted the RNAP, which impeded interactions between RNAP and DNA suppressing the formation of an active open promoter complex during transcription as seen for myxopyronin in a study carried out by (Belogurov et al., 2008). Another study carried out by Mukhopadhyay et al. (2008), showed that myxopyronin had an impact on the opening and closing of the RNAP clamp, which avert the downstream DNA.
segment from entering the RNAP cleft. As a whole, it is plausible to postulate that the potent compounds isolated in this study could be prominent transcription inhibitors in pathogens. As a consequence, Gram-positive and Gram-negative bacteria have specific targets which need to be examined fastidiously and addressed by promising compounds exhibiting anti-pathogenic activities where the concept of “synergy between antibiotics” might also be an effective recourse for human health benefits.

CONCLUSION

Antibiotics have long been on the first row in fighting against microbial infections. Due to evolution of pathogens leading to antibiotic resistance, researchers are encouraged to focus on the isolation of antibacterial compounds, their mechanisms of action as well as their biosynthetic pathway which have been neglected for quite some time. Microorganisms dwelling in harsh conditions like the hydrothermal vents bearing very high temperatures or the arctic regions with temperatures sometimes below 0°C, are exceptional places with unlimited promises to secondary metabolites with antimicrobial capacities. Conventional techniques as applied before should be improved to yield potent metabolites with innovative pharmaceutical abilities. Henceforth, culturing the unculturable by applying different concept like optimization of laboratory conditions, coculture techniques or making use of various elicitors like ecological signals and chemical cues should be addressed to unveil the production of cryptic antibiotics (Zhu et al., 2014). For instance, microbes can be challenged and triggered to generate enthralling and priceless natural products. As demonstrated in this study, heavy metal strategy being used as an elicitor, here referring to the heavy metal cobalt which showed a distinct HPLC profile when compared to the normal one, is proven to have an impact on the secondary metabolism of the fungus strain Penicillium sp. BB1122, thus producing four novel compounds which revealed potent antibiotic properties against MRSA and P. aeruginosa. The bioactivity results obtained in this study revealed the remarkable ability of the novel compounds to expose their potential as antibacterials. Moreover, in-depth studies are required to scrutinize the action mechanism of the potent compounds against the ESKAPE pathogens which may bring to light some resistance secrets of clinical pathogens. As such, searching for effective elicitors may have a great impact on marine microbes which in turn lead to the generation of unexpected though potent natural products. This study may pave the way in researching new techniques for the isolation of novel secondary metabolites which can afterward be associated with cutting-edge molecular techniques to unlock cryptic biosynthetic gene clusters to streamline the discovery of novel pharmaceutically important compounds. As a whole, the ocean may be considered as a unique, untapped scaffold of marine drugs which can be considered as an exclusive shield against infectious diseases.

AUTHOR CONTRIBUTIONS

The experimental work was designed and performed by BA under the supervision of BW who is the corresponding author. XW and SH contributed analysis tools. BN, CP, NA, and BW wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01450/full#supplementary-material

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