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Complex marine natural products as potential epigenetic and production regulators of antibiotics from a marine *Pseudomonas aeruginosa*

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Abstract

Marine microbes are capable of producing secondary metabolites for defense and competition. Factors exerting an impact on secondary metabolite production of microbial communities included bioactive natural products and co-culturing. These external influences may have practical applications such as increased yields or the generation of new metabolites from otherwise silent genes in addition to reducing or limiting the production of undesirable metabolites. In this paper, we discuss the metabolic profiles of a marine *Pseudomonas aeruginosa* in the presence of a number of potential chemical epigenetic regulators, adjusting carbon sources and co-culturing with other microbes to induce a competitive response. As a result of these stressors certain groups of antibiotics or antimalarial agents were increased most notably when treating *P. aeruginosa* with sceptrin and co-culturing with another *Pseudomonas* sp. An interesting cross-talking event between these two *Pseudomonas* species when cultured together and exposed to sceptrin was observed.

Keywords

Quinolone, Rhamnolipid, Phenazine, Modifier, Co-culture

Introduction

Marine microbes are capable of producing distinct secondary metabolite profiles based on their need for defense, competition or a number of other environmental factors [9]. The biosynthesis of active metabolites from microbes can be economical and environmentally friendly hence the discovery of bioactive substances from microbes is appealing to the pharmaceutical and agrochemical industries as a whole. The most common approach for discovery of bioactive secondary metabolites from microbes is screening microbes for biological activities. However, a major challenge to this approach is the cultivation of microbes under laboratory conditions. Even under the best of circumstances, these experiments fail to emulate environmental conditions such as competition, diverse nutrient sources, extreme temperature variations, and other epigenetic factors. The result is an artificial environment for producing secondary metabolites which may cause the silencing of certain genetic pathways. To address this problem, strategies of using stimuli were utilized to more closely mimic the natural environment under potential stressful conditions. Under specific stresses, microbes can respond to stimuli, thus increasing the yield of certain

secondary metabolites or producing new metabolites. Cichewicz's research group identified several new metabolites by treating the fungus *Cladosporium cladosporioides* with histone deacetylase or DNA methyltransferase inhibitors [25]. This study provided evidence that the ecological responses could be attributed to the chemical activation of gene expression corresponding to the secondary metabolite biosynthetic gene clusters. While many epigenetic studies focus on direct DNA methylation inhibitors, other chemical stressors, such as antibiotics, can induce an epigenetic response [1]. Fenical's research group reported an increased production of emericellamides A and B when co-culturing the fungus *Emericella* sp. with an actinomycete *Salinispora arenicola* [17]. In this case, biochemical communication between microorganisms represents more complicated interactions in microbial communities. These interactions resulting in the change of metabolic production in live microbial colonies and communities can be visualized today using MS imaging and chemical networking [24].

In 2009, marine sediment microbes were collected from the Texas Gulf Coast. Based on general antimicrobial and antimalarial activities screening of their extracts, 17 antibiotic producing microbes were identified. From one of the microbes, YPD1C, which was characterized as *Pseudomonas aeruginosa*, seven rhamnolipids, two derivatives of phenazine and six 2-alkyl-4-quinolones were isolated. This isolation allowed us to understand the metabolic profile of biologically active secondary metabolites from *P. aeruginosa*, and provided the basis for comparison to identify the differences in metabolite production when treating *P. aeruginosa* with different modifiers and external stimuli. In the world of bacteria, stimuli can change bacterial metabolic profile by affecting DNA methylation [5], transcription initiation [22], quorum sensing [7], kinase signaling [16] or carbon and nitrogen metabolism [15]. In this paper, impacts on antibiotic production of *P. aeruginosa* were discussed using epigenetic modifiers including kinase inhibitors manzamine A, kahalalide F, and sceptrin, a cell transport regulator ilimaquinone; adjustment to growth factors such as carbon sources and temperature or co-culturing with four other antibiotic producing microbes (YPD1A, YPD1D, YPD5A, and YPD5C) also collected from the Gulf of Mexico in the fall of 2009. Interestingly, an improvement of antibiotic production was observed in a mixed microbial community with the presence of the chemical modifier sceptrin.

Methods

Microbial collection and isolation

On October 24, 2009, a sediment collection was made in a tributary leading into West Galveston Bay (N29 13.349° W95 12.992°). Sediment was collected from the bay floor using a Petite Ponar grabber and scooped into a 50 mL conical tube. The volume of collected sediment was diluted with sterile filtered artificial sea water (Instant Ocean, following the manufactures instructions) to approximately 1.5 times the original volume. The sediment was mixed by agitation and heat shocked at 55°C for 8 minutes. The sample was allowed to rest until the sediment had settled. Volumes of 300 and 150 µL of supernatant were plated on Yeast Peptone Dextrose (10/20/20 g/L, YPD) media with 50 µg/mL of ampicillin. The microbes were allowed to grow at room temperature until colonies appeared. Once colonies grew, individual colonies were chosen and streaked onto secondary plates containing no antibiotics, but still supplemented with 2% Instant Ocean. These microbes were again grown at room temperature until colonies appeared. Using these plates, pure colonies were isolated and glycerol stocked at -80°C for preservation and large scale growth.

16S rRNA

YPD1A, YPD1C, YPD1D, YPD5A, and YPD5C were grown in supplemented YPD media at 30°C and 200 rpm overnight. DNA was isolated using MoBio Ultrapure DNA purification system and following the manufacturer's instructions. 16S DNA was amplified by PCR using the published 17f and 1492r universal bacteria 16s primers. The resulting amplified DNA was cloned into the pCR2.1 vector using a topoTA (Invitrogen) ligation kit. Plasmids were purified and subsequently sequenced using the T7 and M13 sites in pCR2.1. Analysis was conducted by comparison of acquired sequences of the five microorganisms with NCBI's BLAST.

Large scale growth and extraction

Eight cultures of YPD1C isolate (500 mL per each) were grown using YPD media supplemented with 2% Instant Ocean in 2.5 L Fernbach flasks incubated at 30°C and 250 rpm for 7 days. The cultures were sonicated for 20 min and then centrifuged for 10 min at 3600 rpm in 250 mL portions. The supernatant was decanted into a flask and the cell pellet was discarded. Approximately 160 mL of HP-20 resin was added to the supernatant. The flask was again placed in the incubator and left overnight at 30°C and 250 rpm.

The HP-20 resin was extracted using a Buchner funnel. The media was discarded. The HP-20 was treated with 1 L of EtOH followed by 500 mL of EtOAc and extractions were dried under vacuum. The extracts were assayed to assess biological activity.

Compound isolation

The EtOH extract of YPD1C cultures (20 g) were loaded onto an HP-20 column and eluted with 100% H₂O, H₂O-MeOH (75:25, 50:50, 25:75), 100% MeOH, and MeOH-EtOAc (50:50). The last two fractions and EtOAc extract of YPD1C culture (2 g) acquired from the large scale extraction previously were combined. Further purifications were conducted on a silica VLC flash column eluted with a gradient of hexanes-EtOAc (100:0, 75:25, 50:50, 25:75, 0:100), EtOAc-MeOH (75:25, 50:50, 25:75, 0:100), and MeOH-H₂O (50:50) to afford ten subfractions. Active subfractions based on a *Bacillus cereus* disc diffusion assay were subjected to HPLC on C18 (250 mm×20.2 mm, 5 µm), C18 (250 mm×10 mm, 5 µm), or C8 (250 mm×10 mm, 5 µm) columns using gradients of MeOH with 0.05% HCOOH-H₂O with 0.05% HCOOH (from 20:80 to 100:0), or MeCN-H₂O (from 28:72 to 100:0).

Treatments of *P. aeruginosa* (YPD1C)

YPD1C was cultured at different temperatures with different carbon sources (glucose, sodium acetate, oil mix, starch, and citric acid), nitrogen source (NH₄Cl, NaNO₃, NaNO₂, urea, and peptone), chemical regulators (scepttrin, kahalalide F, manzamine A, and ilimaquinone) and co-cultured with the four other antibiotic producing microbes (YPD1A, YPD1D, YPD5A, and YPD5C). Considering the extensive number of experiments required, an orthogonal design was used to minimize the sample size. Each sample was processed in duplicate (SI-Tab. 1). Cultures of the YPD1C isolate (50 mL) were grown using different media supplemented with 2% Instant Ocean mix in 250 mL flasks incubated at 15–45°C and 250 rpm for 3–7 days. YPD1C grown in YPD media at 30°C and 250 rpm for 7 days was used as a positive control, while four regulators prepared in YPD media respectively were used as negative controls. Prior to harvest and OD₆₀₀ value was recorded to compare biomass. All the cultures were sonicated for 20 min and centrifuged for 10 min at 3600 rpm. The supernatant was extracted with 100 mL of EtOAc (×3). The organic layers were combined for further bioassays and fingerprint analysis.

Fingerprint setup of each extract

Each extract was dissolved in 5 mL MeOH and filtered. The volume of 10 μ L of each sample was eluted on a C8 column (4.6 mm $\times$ 250 mm, 5 μ m) using a gradient of MeOH (with 0.05% HCOOH) – H₂O (with 0.05% HCOOH) from 40:60 to 100:0 over 45 minutes at flow rate of 0.6 mL/min. A TOF-MS detector was used to acquire fingerprints on positive mode. A fingerprint of each extract was compared.

Disc diffusion assay

Disc Diffusion Assay (DDA) provided a rapid and reliable method to screen fractions during purification. A concentration limitation experiment was performed with each active extract to determine the minimal amount needed to observe a zone of inhibition. This minimal amount of each column fraction was added to a 6 mm filter paper disc. *Bacillus cereus* cells suspended in water (no more than 250 μ L) were evenly plated onto antibiotic media 2 (Difco). The discs were added to the plate with a control of 50 μ g of kanamycin and allowed to grow at 30°C overnight. Activity was determined as a clear zone of inhibition. Relative activity between fractions could be determined based on the radius of inhibition.

Antimicrobial assay

All organisms were obtained from the American Type Culture Collection (Manassas, VA) and included the fungi *Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, *Aspergillus fumigatus* ATCC 204305, and the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591 (MRSA), *Escherichia coli* ATCC 35218, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI (formerly NCCLS) methods. *M. intracellulare* was tested using a modified method. Samples were serially-diluted in 20% DMSO/saline and transferred in duplicate to 96 well flat-bottom microplates. Microbial inocula were prepared by correcting the OD₆₃₀ of microbe suspensions in the incubation broth. DMSO was used as the negative control. Drug controls [ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and amphotericin B (ICN Biomedicals, Ohio) for fungi] were included in each assay. All organisms were read at either 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) or 544ex/590em, (*M. intracellulare*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany) prior to and after incubation. Samples are tested in duplicate at one concentration (50 μ g/mL) and percent inhibitions are calculated relative to blank and growth controls. Samples showing 50% inhibition in at least one test organism are selected for dose response (secondary) studies at 20, 4 and 0.8 μ g/mL. If samples from the secondary assay showing an IC₅₀ of <2 are retested in duplicate at 50, 10, 2, 0.4, 0.08, 0.016 μ g/mL.

Antimalarial assay

Antimalarial activity was determined *in vitro* against chloroquine sensitive (D6, Sierra Leone) and resistant (W2, Indo China) strains of *Plasmodium falciparum* by measuring plasmodial LDH activity. Chloroquine and artemisinin were used as the positive controls. Test compounds were dissolved in DMSO (2 mg/mL). A 200 μ L suspension of *P. falciparum* culture (2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 μ g/mL amikacin) was added to the 96-well plate containing 10 μ L of serially diluted samples. The plate was flushed with a gas mixture of 90% N₂, 5% O₂, and 5% CO₂ and incubated at 37 °C for 72 h in a modular incubation chamber. Plasmodial LDH activity was determined by using Malstat™ reagent (Flow Inc., Portland, OR) [14]. The incubation mixture (20 μ L) was mixed with 100 μ L of the Malstat™ reagent and incubated for 30 min. A 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) (20 μ L) was then added, and the plate was further incubated for 1 h in the dark. The

reaction was stopped by adding 100 μ L of a 5% acetic acid solution. The plate was read at 650 nm. The *in vitro* cytotoxicity to mammalian cells (vero cells) was also tested to determine the selectivity index of antimalarial activity of compounds. Cells were seeded at a density of 25,000 cells/well and incubated for 24 h. Serially diluted samples were added and incubated for 48 h. The number of viable cells was determined by Neutral Red assay [3]. Doxorubicin was used as a positive control. IC₅₀ values were obtained from dose response curves.

Results

Identification of active compounds

Biological evaluation revealed that fractions 3, 4 and 6 (hexanes-EtOAc 50:50, 25:75, and EtOAc-MeOH 75:25, respectively) inhibit growth of *B. cereus* (SI-Fig. 1) in DDA. Further purification yielded 15 known compounds (Fig. 1). Fractions 3 and 4 were purified by HPLC yielding two characterized active compounds 1-phenazine-carboxamide (**1**, SI-Fig. 2) and 1-phenazinecarboxylic acid (**2**, SI-Fig. 3). Fraction 6 was eluted using LH-20 with DCM-MeOH (50:50) producing 10 sub-fractions, in which Fr6-4, Fr6-6 and Fr6-7 were active in the *B. cereus* DDA. Fr6-4 was further purified by HPLC, yielding six pure compounds. Fr6-4-7-6 was identified as 2-heptyl-4(1*H*)-quinolone (**3**, SI-Fig. 4) using LC-MS, ¹H, ¹³C, and HMBC NMR spectra. Structures of the remaining five compounds were determined to be known compounds 1-hydroxy-2-heptyl-4-quinolone (**4**, SI-Fig. 5), 2-Octyl-4(1*H*)-quinolone (**5**, SI-Fig. 6) and 2-nonyl-4(1*H*)-quinolone (**6**, SI-Fig. 7), 2-(2-nonenyl)-4(1*H*)-quinolone (**7**, SI-Fig. 8), and 2-(1-nonenyl)-4(1*H*)-quinolone (**8**, SI-Fig. 9) using LC-MS and HMQC overlay experiments. Further isolations of Fr6-6 and Fr6-7 were conducted using a C8 (250mm×10mm, 5 μ m) column to yield seven known rhamnolipids: Rha-C₁₀-C₁₀ (**9**, SI-Fig. 10), Rha-Rha-C₁₀-C₁₀ (**10**, SI-Fig. 11), Rha-Rha-C₁₀-C_{12:1} (**11**, SI-Fig. 12), Rha-Rha-C₁₀-C₁₂ (**12**, SI-Fig. 13), Rha-C₁₀-C_{12:1} (**13**, SI-Fig. 14), Rha-C₁₀-C₁₂ (**14**, SI-Fig. 15), Rha-C₈-C₁₀ (**15**, SI-Fig. 16). Two D-rhamnose and two α -hydroxy fatty acid moieties were identified by comparing characteristic spin systems from the 1D and 2D ¹H COSY spectra and optical rotation values with the literature [8, 20].

Activities of isolated compounds

Compounds **1–3**, **6**, and **8–15** were submitted for antimicrobial and antimalarial activities (Tab.1). Compounds **4**, **5**, and **7** were not assayed due to the low yields. Compound **1**, **2**, **11**, and **12** showed antifungal activity against *C. neoformans* with an IC₅₀ of 6.99, 1.30, 15.67, 5.82 μ g/mL, respectively; **13** and **14** show antibacterial activity against *S. aureus* with IC₅₀ values of 19.88, 5.70 μ g/mL, MRSA with IC₅₀ at 18.89, 12.33 μ g/mL, respectively; **1** and **8** also demonstrated activity against *M. intracellulare* with IC₅₀ values of 17.48, 20.00 μ g/mL, respectively. Compounds **3**, **6**, **8**, and **15** displayed antimalarial activity with IC₅₀ at 4.30, 0.78, 0.13, 0.26 μ g/mL in D6 clone, and 3.30, 0.92, 0.14, 0.36 μ g/mL in W2 clone, respectively. They have no cytotoxicity against Vero cells. This was the first report regarding antimalarial activity of these molecules. The potency and safety of **6** and **8** suggest a favorable potential in treating malaria.

Identification of microorganisms

BLAST analysis demonstrated YPD1C was 99% identical to *Pseudomonas aeruginosa*. YPD1A and YPD1D were determined to be unique *Bacillus* sp., YPD5A and YPD5C were two *Pseudomonas* sp..

Activities of extracts under different treatments

Extract activity was normalized by comparison with the activity of the regulator control in each bioassay and comparing the OD₆₀₀ values to account for biomass, thus providing an accurate assessment. Direct sum in an orthogonal design was used to determine the best condition for each modifying factor. As a result, the best condition to produce antimicrobial compounds was oil mix (50% corn oil, 50% olive oil) as the carbon source, peptone as a nitrogen source, and incubation at 30°C for 11 days (SI-Tab. 2). However, extracts under most conditions were less active when compared to positive control. Interestingly, when treating YPD1C with scep trin or co-culturing with YPD5A, the best antimalarial activity was observed. Treatments with manzamine A, kahalaide F and ilimiquinone proved to inhibit growth in initial experiments and were not further pursued. YPD5A produced the most significant result in co-culture experiment and the focus shifted to co-culture with this strain.

To further determine the significance of scep trin and YPD5A on the antibiotic production from YPD1C, a second batch of experiments was designed using YPD media, at 30°C and 250 rpm for 7 days, different concentration of scep trin, with/without YPD5A. OD₆₀₀ values were measured to test viability of the microbe in each condition and give a relative biomass between experiments. Antimicrobial and antimalarial activities were tested for all the extracts (Tab. 2).

Extracts from YPD5A (T7-T9) did not have antimicrobial and antimalarial activities, while extracts with the presence of YPD1C (T1-T3, T5-T6) displayed activities in all assays. The activity data (T1-T6) was analyzed with analysis of variance (ANOVA). As a result, all the activities showed significant difference between conditions ($P < 0.01$). To further determine which treatment was responsible for the major difference, a Dunnett's test was applied by comparison of treatment groups (T2-T6) with a positive control group (T1). In the groups singly treated with scep trin or co-culturing (T2, T3 in all activities, and T4 in antimicrobial activities), there was no significance observed ($P > 0.05$). However, when treating scep trin and co-culturing with YPD5A, a significant enhancement of all activities was observed (T5, $P < 0.05$; T6, $P < 0.01$; Fig. 2–3). The antimicrobial activities of YPD5A (T7-T9) decreased significantly when adding 5 μM scep trin (T9, $P < 0.05$). Meanwhile, the OD₆₀₀ value of T9 dropped more than five times and was equivalent to media controls. It was unclear if this decline was due to a bacteriostatic or a bacteriocidal effect; however, at 1 μM there was no effect on growth. This lack of growth would also result in lack of antibiotic production.

The total ions chromatograms (TICs) of YPD5A extracts (T7-T9) displayed different profiles from those of YPD1C extracts (Fig. 4 and 5). The MS analysis confirmed that YPD5A did not produce the same active metabolites as YPD1C. This remained consistent with the activity result presented above. The TICs of T1, T5, and T6 showed an increase in secondary metabolite production, especially phenazines (R_t 10–15 min) and quinolones (R_t 18–26 min) (Fig. 5). To further determine the variation of each class of metabolites, extracted ions chromatograms (EICs) were compared by extracting ions at $m/z=224$, 225, 244, 260, 270, 272 (Fig. 6), 651, 505, 677, 679, 531, and 533 (Fig. 7) corresponding to compounds **1**, **2**, **3**, **4**, **7**, **8**, **6**, **10**, **9**, **11**, **12**, **13**, and **14**, respectively. It was noticed that the yield of compounds **1**, **2**, **3**, **4**, **6**, **8**, **11**, **12**, and **14** increased in T5 and T6. Moreover, metabolites in quinolones area (R_t 18–26 min) with $m/z=298$ displayed a high intensity. Based on the MS data and retention time, it could contain two more methylenes than compound **7** or **8**. Some other signals need to be further identified. According to the activities, compounds **1**, **2**, **11**, and **12** were responsible for the antifungal activity, compounds **1**, **8**, and **14** for the antibacterial activity, while compounds **3**, **6**, and **8** for the antimalarial activity. The comparison of fingerprint complemented the observed bioactivity and our statistical analysis.

Discussion

Role of yeast extract in media

As the result of orthogonal experimentation, the best condition to produce antimicrobial compounds was oil mix (50% corn oil, 50% olive oil) as the carbon source, and peptone as a nitrogen source. However, antibiotic production under the given condition was far less than that of YPD medium. YPD consists of yeast extract, peptone, and dextrose, among which only yeast extract was not added during the media modifications. Hence, we propose that one or more components of yeast extract play a key role in the high yield of antibiotic production. Yeast extract is a processed by extracting yeast cell contents. There was evidence that small RNAs from yeast extract present in media could change bacterial protein profiles, thus influencing bacterial adaption [18]. Yeast extract may serve both as a nutrient in growth media in addition to a stimulus for *P. aeruginosa*, thus inducing antibiotic production; however, due to the complex nature of yeast extract it was not added to maintain control of the media constitution.

Roles of regulators

Epigenetic refers to heritable changes from parent to progeny. In bacteria, DNA methylation is a major component of epigenetic inheritance and induction of the changes is invoked by use of methylase inhibitors [4]. There is potential for other stressing molecules, such as antibiotics, to also induce heritable changes in the phenotype [1]. The potential of kinase inhibitors was tested in this experiment using marine natural products. Manzamine A has been identified as a potent GSK-3 β inhibitor [12]. Kahalalide F was shown to down-regulate the ErbB3 and inhibit Akt signaling [11]. Both of these compounds are cytotoxic. When the regulator controls were tested, both manzamine A and kahalalide F displayed inhibition on all microbes. Cultures treated with manzamine A and kahalalide F lacked growth during fermentation, indicating that the compounds were cytotoxic to the bacteria. At non-inhibitory concentrations these may induce an epigenetic response.

Sceptrin has diverse activities including antifungal, antibacterial, and antihistaminic. It has shown to be an inhibitor of somatostatin and cell motility [6]. Based on our experimental data, the addition of sceptrin does not have a significant influence on YPD1C. However, it does affect the vitality of another *Pseudomonas* sp., YPD5A. The fingerprint comparison of T7-T9 suggested a different metabolic profile after treating with 5 μ M sceptrin (Fig. 5). These could be degradation products of sceptrin or other metabolites released from YPD5A as part of a stress induced stringent response. The data indicated that YPD5A was able to trigger a series of responses in YPD1C under stress conditions [23]. The exact mechanism of this interaction is unclear and includes potential cross talk between microbes, recognition of cellular components of lysed YPD5A, and degradation of sceptrin.

Mixed fermentation

Usually, microbes within the same genus are able to consume the same nutrients for their survival. When co-culturing, there could be potential competition for nutrients [19, 21]. In our experiment, another *Pseudomonas* sp., YPD5A, was co-cultured with YPD1C. A comparison of activities as well as OD₆₀₀ values between T1 and T4 suggested the vitality of both YPD1C and YPD5A were weakened after co-culturing due to an insufficient of nutrient.

Chemical communications between microbes can induce a series of interactions between species. The data clearly indicated that YPD1C co-cultured with only sceptrin or YPD5A was not sufficient to equal the results of culturing all three components together. We have also established that the concentration used in this co-culture was sufficient to inhibit growth

of YPD5A. This suggests several possible roles in the co-culture. One option is that YPD5A released a signaling molecule when exposed to 5 μ M scep trin and this molecule induces production of secondary metabolites in YPD1C. Another option is a much more complex cross-talk mechanism between the two stains involving a combination of quorum sensing molecules and scep trin or derivatives thereof. Modulation of quorum sensing (QS) in *P. aeruginosa* has been well studied. Natural products such as tobramycin [2] and lyngbyoic acid [13] were reported as regulators of QS signaling. Quinolones have been implicated in QS regulation [10]. An increase in OD₆₀₀ as well as quinolone concentration implied a relationship between the antibiotic production of the species and microbial competition between species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Structures of antibiotics isolated from *P. aeruginosa*

Fig. 2.
Antimicrobial activities of YPD1C extracts with different treatments
*: $P < 0.05$; **: $P < 0.01$ as the results of Dunnett's test.

Fig. 3.

Antimalarial activity of YPD1C extracts with different treatments

ND: IC₅₀ was not tested due to less than 10% of inhibition in the primary assay

*: $P < 0.05$; **: $P < 0.01$ as the results of Dunnett's test.

Fig. 4.
TIC comparison of T7–T9

Fig. 5.
TIC comparison of T1, T5–T6

Fig. 6.
Quinolones EIC comparison of T1, T5–T6

Fig. 7.
Rhamnolipids EIC comparison of T1, T5–T6

Table 1

Antimicrobial and antimalarial activities of isolated compounds 1–15

Antifungal (IC50, µg/mL)					Antibacterial (IC50, µg/mL)			Antimalarial (IC50, µg/mL)		
<i>C. albicans</i>	<i>C. glabrata</i>	<i>C. krusei</i>	<i>A. fumigatus</i>	<i>C. neoformans</i>	<i>S. aureus</i>	MRSA	<i>E. Coli</i>	<i>M. intracellulare</i>	D6	W2
1 ND	ND	ND	ND	6.99	ND	ND	ND	17.48	ND	ND
2 ND	ND	ND	ND	1.30	ND	ND	ND	ND	ND	ND
3 ND	ND	ND	ND	ND	ND	ND	ND	ND	0.78	0.92
6 ND	ND	ND	ND	ND	ND	ND	ND	ND	0.13	0.14
8 ND	ND	ND	ND	ND	ND	ND	ND	20.00	0.26	0.36
9 ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
10 ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
11 ND	ND	ND	ND	15.67	ND	ND	ND	ND	ND	ND
12 ND	ND	ND	ND	5.82	ND	ND	ND	ND	ND	ND
13 ND	ND	ND	ND	ND	19.88	18.89	ND	ND	ND	ND
14 ND	ND	ND	ND	ND	5.70	12.33	ND	ND	ND	ND
15 ND	ND	ND	ND	ND	ND	ND	ND	ND	4.30	3.30

ND: IC₅₀ was not tested due to less than 50% of inhibition was observed in the primary assays.

Table 2

Antimicrobial and antimalarial activities of *P. aeruginosa* with epigenetic regulations (n=3)

Media	Temp. (°C)	Days	Sceptrin (μM)	Microorganism	OD ₆₀₀	Antifungal (% Inh.)			Antibacterial (% Inh.)			Antimalarial (IC ₅₀ , μg/mL)		
						<i>C. neoformans</i>			<i>S. aureus</i>			MRSA		
C1	YPD	---	---	---	0.60±0.004			7.72±0.96	8.98±1.04	9.17±1.16	ND	ND	ND	ND
C2	YPD	---	5	---	0.63±0.006			9.15±0.58	8.04±0.88	8.71±1.74	ND	ND	ND	ND
T1	YPD	30	---	YPD1C	4.11±0.008			18.1±4.62	55.2±5.25	57.6±3.84	7.25±0.78	9.15±1.20		
T2	YPD	30	1	YPD1C	4.28±0.017			18.8±8.81	44.8±11.8	51.7±12.8	5.95±0.21	8.45±1.48		
T3	YPD	30	5	YPD1C	4.30±0.072			24.4±6.89	38.3±8.29	41.4±9.27	5.43±1.25	6.80±2.09		
T4	YPD	30	---	YPD1C+5A	3.56±0.061			6.45±1.36	6.39±2.82	15.1±4.63	ND	ND		
T5	YPD	30	1	YPD1C+5A	3.95±0.066			45.1±12.6	65.6±16.2	81.9±13.1	2.80±1.17	3.41±0.86		
T6	YPD	30	5	YPD1C+5A	4.12±0.005			63.5±4.43	94.1±2.49	94.3±4.00	0.89±0.10	1.10±0.14		
T7	YPD	30	---	YPD5A	3.59±0.147			13.4±1.89	11.4±2.12	17.5±1.92	ND	ND		
T8	YPD	30	1	YPD5A	3.59±0.101			11.7±5.46	8.43±3.50	15.3±1.40	ND	ND		
T9	YPD	30	5	YPD5A	0.68±0.026			7.21±3.04	3.35±1.02	7.10±2.53	ND	ND		

ND: IC₅₀ was not tested due to less than 10% of inhibition was observed in the primary assay.