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Tyrosol from marine Fungi, a novel Quorum sensing inhibitor against *Chromobacterium violaceum* and *Pseudomonas aeruginosa*

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ABSTRACT

An ethyl acetate extracts isolated from a marine fungal strain, *Penicillium chrysogenum* DXY-1, obtained from marine sediments surrounding the East Sea, was found to exhibit anti-quorum sensing (anti-QS) activity. Interestingly, a novel active compound was identified as tyrosol by the purification and structural characterization. At a concentration of 0.5 mg/mL, tyrosol decreased QS-regulated violacein production in *Chromobacterium violaceum* CV026 by 53.5% and decreased QS-regulated pyocyanin production, elastase activity and proteolytic activity in *Pseudomonas aeruginosa* PA01 by 63.3%, 57.8% and 9.9%, respectively. SEM images showed that tyrosol inhibited biofilm formation in *P. aeruginosa* PA01 without having any effect on bacterial growth. Molecular docking results revealed that the natural signal molecule C_6HSL and tyrosol bound to different receptor pockets of CviR, and tyrosol inhibited the QS activity of CviR in *C. violaceum* by binding to the DNA-binding domain and blocking pathogenic gene expression. All the data suggest that tyrosol may act as a potential inhibitor of the QS systems to solve the looming crisis of bacterial resistance. We believe that there are other active compounds with relatively high anti-QS activity or synergistic inhibitory effects on QS in the crude extract, which warrants further research.

1. Introduction

Since the early 20th century, antibiotics have been used as primary drugs that can resist infections [1,2]. However, with the continued emergence of superbugs and untreatable infections, antibiotic resistance may become a leading concern [1–3]. In addition, antimicrobial agents obtained for classic targets and by conventional screening methods cannot fundamentally address this concern. As such, there is a clear need to identify new targets and develop antimicrobial agents with new mechanisms of action [4,5]. Quorum sensing (QS) is a process of bacterial communication that relies on extracellular signaling molecules called autoinducers [6,7]. In recent years, QS has been shown to regulate the survival and pathogenicity of bacteria [8], which has attracted the attention of pharmacologists and made QS an important target for the development of new antibacterial drugs [9]. These QS inhibitors do not kill bacteria or inhibit bacterial growth and can quench QS-regulated pathogenic behaviors [7–10], such as toxin production, biofilm formation, swarming and secretion, which results in the loss of bacterial ability to cause disease and does not easily induce drug-resistant mutations. This mechanism of action of antimicrobial agents is greatly different from traditional antibiotics. Therefore, QS-targeted antimicrobial drugs are regarded as ideal antibacterial drugs of the future [9,11,12].

Oceans are rich in microbial resources [13,14]. Moreover, owing to their relatively unique high-pressure, high-salt, low-temperature, hypoxic, and oligotrophic ecological environments, oceans provide marine microorganisms with unique metabolic mechanisms, greatly increasing the probability of the discovery of novel lead compounds. Many chemical drugs with good therapeutic effects have been developed from secondary metabolites of marine microorganisms, indicating that marine microorganisms are important resources for natural drugs [14]. Therefore, researchers are using bacterial QS systems targets to screen QS inhibitors (QSIs) from the metabolites of marine microorganisms to develop new antibacterial drugs [15–19]. Recently, our

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group reported that a diketopiperazine factor from *Rheinheimera aquimaris* QSI02 exhibits anti-QS activity [20]. The diketopiperazine factor was identified as cyclo (Trp-Ser), which may act as a potential inhibitor of the QS systems of both *Chromobacterium violaceum* CV026 and *Pseudomonas aeruginosa* PA01. Marine fungi, which are organisms with especially high "creation coefficients", have the advantages of ease of cultivation, rapid reproduction and metabolites with complex structures and unique activities [13,14,21], which are good candidates for QSI screening.

Herein, we isolated marine fungi strains from marine sediments surrounding the East Sea collected in Taiwan Strait (China) and tested the anti-QS activity of purified extracts by treatment of the *C. violaceum* CV026 biosensor system with the ethyl acetate extract containing fungal metabolites. A marine fungal strain, named DXY-1, with anti-QS activity was screened out. By rDNA-ITS sequence comparison and analysis of morphological characteristics, this strain was identified as *Penicillium chrysogenum* and named *P. chrysogenum* DXY-1. Next, the chemical components of *P. chrysogenum* DXY-1 fermentation broth were studied in detail. Using gel column chromatography and preparative HPLC, the methanol solution of the crude extract was separated into several fractions. One fraction with anti-QS activity, as tested by the violacein assay, was identified as tyrosol, a monomeric compound, by UV and NMR spectroscopy. Tyrosol is a known natural compound, obtained from a variety of sources [22–24], but to the best of our knowledge, the isolation of tyrosol from *Penicillium chrysogenum* has not been reported to date. The inhibitory effects of tyrosol on *P. aeruginosa* has been reported [25], here the effects of tyrosol on both QS-regulated violacein production in *C. violaceum* and QS-regulated virulence factor production (pyocyanin, elastase and proteolytic enzymes) in *P. aeruginosa* were studied. Additionally, biofilm formation was also assayed. To understand the interactions between the QSI, namely, tyrosol, and receptor proteins, molecular docking analysis was conducted.

2. Results and discussion

2.1. Isolation and structure elucidation of OSIs from P. Chrysogenum DXY-1

Based on the violacein assay (Fig. 1), a marine fungal strain with anti-QS activity obtained from marine sediment surrounding the East Sea, collected in Taiwan Strait (China), was isolated. This strain, named DXY-1, was cultured in potato dextrose agar (PDA) medium at 28 °C for 3 days. The colony diameter reached approximately 42 mm; the surface was flocculent; the colour was dark green; and the colony grew radially (Fig. 2A). Moreover, we used bio-microscopy to observe the morphological characteristics of the mycelia and spores. As shown in Fig. 2B, the mycelia had transverse septa; the conidiophores were long and straight; the tops were broom-like conidial chains; the spores were easily detached; and the dispersed individual spores were globular. In addition, in combination with rDNA-ITS sequence comparison (Figs. S1 and S2), DXY-1 was identified as *Penicillium chrysogenum* [26,27] and named *P. chrysogenum* DXY-1.

Next, to isolate active compounds with good efficacy and novel structures from the metabolites, *P. chrysogenum* DXY-1 was cultured in submerged fermentation on a large scale. Following extraction, 6.5 g of ethyl acetate extract was isolated from 30 L of broth (see Material and methods for details). Based on the violacein assay, the compound with anti-QS activity was further purified by using repeated silica gel column purification, Sephadex LH-20-based purification, and preparative HPLC. The structure of this compound was first investigated based on UV–vis absorption curves (Fig. S3). The characteristic absorption peak of benzene appeared at approximately 275 nm, and the peak at 220 nm represented the K absorption band formed by the conjugated double bond system, which indicates that the compound contains a benzene ring or/and additional double bonds. These results were confirmed by NMR spectroscopy (Figs. S4 and S5, Material and methods). Based on the above UV and NMR spectroscopic analyses and by comparison with the literatures [22–24], this compound was identified as tyrosol (Fig. 3).

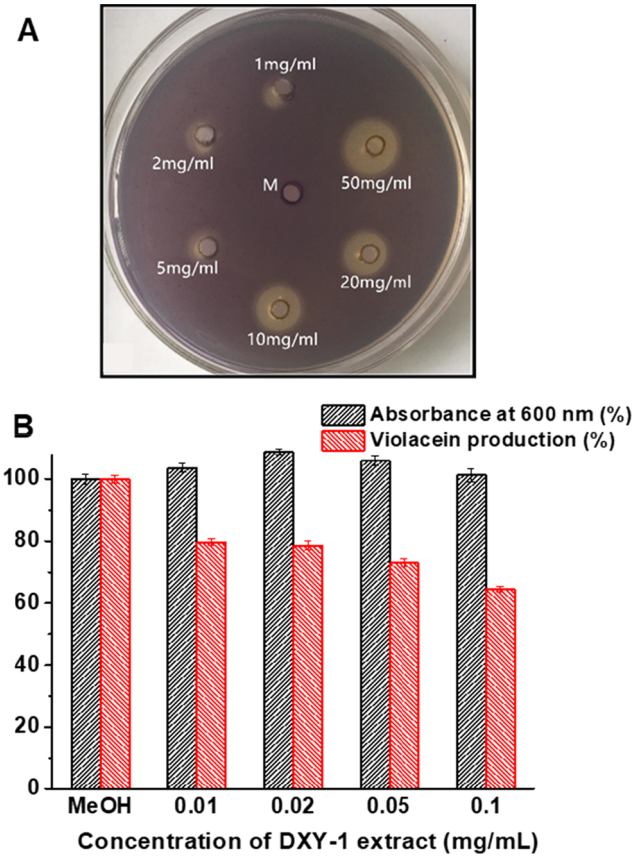

Fig. 1. (A) Antibacterial disc assay in *C. violaceum* CV026 by the crude extract of DXY-1 metabolites; (B) Effects of the crude extract of DXY-1 metabolites on violacein production in *C. violaceum* CV026 and the growth of *C. violaceum* CV026. Values are presented as the mean ± SD; n = 3. MeOH was used as a negative control.

2.2. Anti-QS activity of tyrosol

To evaluate the efficacy of tyrosol as an antimicrobial agent, *C. violaceum* CV026 and *P. aeruginosa* PA01 were exposed to the compound.

2.2.1. Inhibition of QS-regulated violacein production in C. Violaceum

C. violaceum CV026 is a deletion mutant that lacks the signal molecule synthase gene *cviI*. Therefore, in *C. violaceum* CV026, QS-regulated violacein production is induced by the exogenous natural signal molecule C_6HSL and inhibited by QSI [28,29]. Thus, the anti-QS activity of tyrosol could be determined by checking violacein production. After incubation for 12 h, the colour of the *C. violaceum* CV026 bacterial culture solutions gradually faded with increasing concentrations of tyrosol (Fig. S6), which indicates that violacein production decreased [28,29]. Moreover, violacein production was quantitatively measured by using a microplate reader at a wavelength of 585 nm. In addition, the absorbance at 600 nm was measured to evaluate cell growth. As shown in Fig. 4, at a concentration of 0.5 mg/mL, tyrosol inhibited violacein production by up to 53.5% compared to the negative control, methanol (MeOH). Additionally, tyrosol had little effect on the growth of *C. violaceum* CV026. These results demonstrate that tyrosol effectively inhibits QS-regulated violacein production at concentrations ranging from 0.1 to 0.5 mg/mL in the presence of the exogenous natural signal molecule C_6HSL.

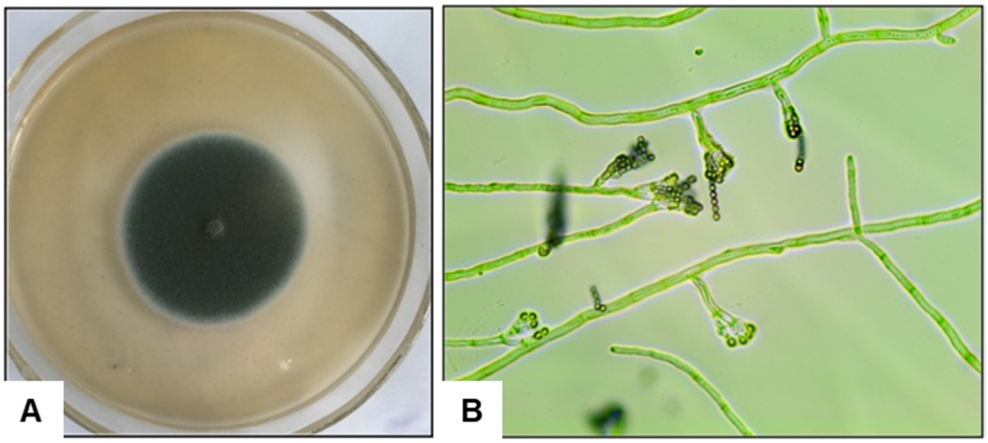

Fig. 2. Colony morphology (A) and mycelial morphology (B) of DXY-1, which was cultured in PDA medium containing 2% w/v glucose; 2% agar; 20% potatoes; 0.01% chloramphenicol per 100 mL seawater at 28 °C for 3 days.

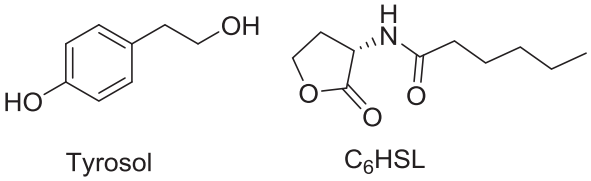

Fig. 3. Structures of compounds.

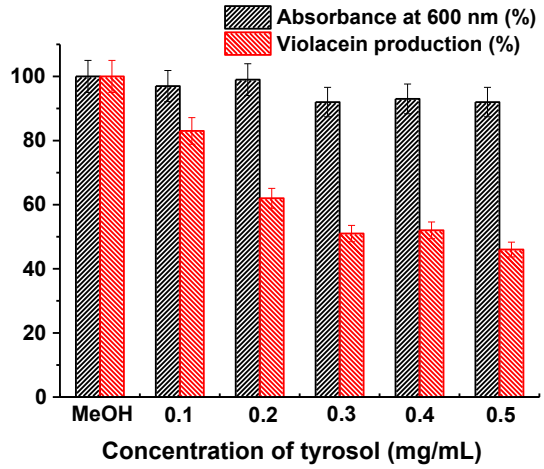

Fig. 4. Effects of tyrosol on the growth of *C. violaceum* CV026 and violacein production in *C. violaceum* CV026. Values are presented as the mean ± SD; n = 3. MeOH was used as a negative control.

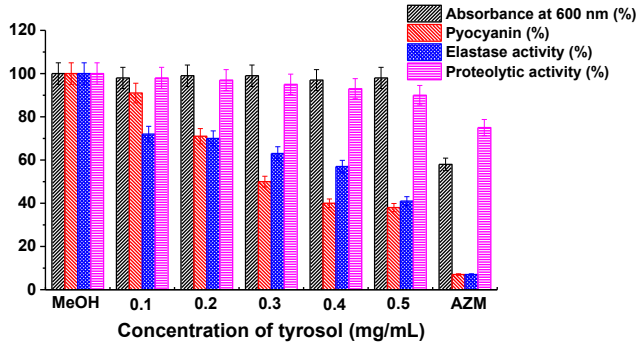

Fig. 5. Effects of tyrosol on the growth, pyocyanin production, elastase activity and proteolytic activity of *P. aeruginosa* PA01. Values are presented as the mean ± SD; n = 3. MeOH and AZM (0.2 mg/mL) were used as negative and positive controls, respectively.

2.2.2. Inhibition of QS-regulated virulence factors and biofilm formation in *P. aeruginosa*

P. aeruginosa is an opportunistic human pathogen [25,30–32]. It is responsible for many life-threatening conditions and infectious diseases, including chronic wounds, urinary tract infections, and respiratory tract infections, notably causing cystic fibrosis in hospitalized patients. *P. aeruginosa* infections are difficult to treat by using antibiotics. QS-regulated virulence factors and biofilm formation play important roles in survival and invasion. The main virulence factors secreted by *P. aeruginosa* include pyocyanin, elastase and proteases [25,32,33]. Therefore, by examining pyocyanin production, elastase activity, proteolytic activity and biofilm formation in *P. aeruginosa* PA01, the anti-QS activity of tyrosol could be determined. As shown in Fig. 5, compared to the negative control, at a concentration of 0.5 mg/mL, tyrosol inhibited violacein production by up to 63.3%, elastase activity by 57.8%, and proteolytic activity by 9.9% without having any effect on cell growth. At a concentration of 0.2 mg/mL, the positive control azithromycin (AZM)[33,34] not only effectively inhibited virulence factor production but also inhibited the growth of *P. aeruginosa* PA01 by up to 44.8%, leading readily to increased antibiotic resistance. In addition, the effect of tyrosol on biofilm formation was confirmed by scanning electron microscopy (SEM). After incubation with MeOH for 48 h, the *P. aeruginosa* PA01 biofilms exhibited compact surfaces coating the intact rod-like cells (Fig. 6A). In contrast with AZM, the cells appeared granular, which indicated that AZM could induce distortion of *P. aeruginosa* A01 by membrane blebbing, lysis of the membrane, and loosening of the outer membrane after lysis and effectively kill the bacteria (Fig. 6B). Bacterial cells treated with tyrosol showed normal morphology with distinct, intact inner and outer membranes, and a thin, uniform periplasmic space. But the biofilms were destroyed significantly, which also eliminated the basis of pathogenicity generation (Fig. 6C). Based on these results, we infer that tyrosol effectively inhibits virulence factor production and biofilm formation in *P. aeruginosa* PA01 without interfering its growth at concentrations ranging from 0.1 to 0.5 mg/mL. These results demonstrate that tyrosol not only efficiently inhibited QS-regulated violacein production in *C. violaceum* CV026 but also suppressed QS-regulated pyocyanin production, elastase activity, proteolytic activity and biofilm formation in *P. aeruginosa* PA01.

2.3. Molecular docking analysis

Furthermore, to analyse the mechanism of action of tyrosol, docking studies [35] were performed to determine the possibility of binding

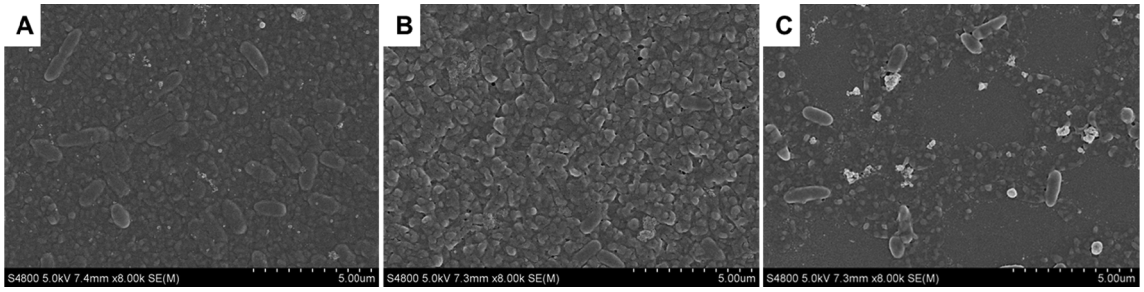

Fig. 6. SEM images of *P. aeruginosa* PA01 biofilms after 48 h of incubation (scale bar: 5.00 μm). (A) Negative control with MeOH; (B) positive control with AZM; (C) 0.5 mg/mL tyrosol.

Table 1
Details of the docked complex of the 3QP1 receptor protein with the natural ligand C_6HSL and tyrosol.

Molecule	Binding energy (kcal/mol)	Hydrogen bonding interactions	Key hydrophobic interactions
Natural ligand C_6HSL	−6.86	Asp97, Trp84, Tyr80, Ser155	Leu100, Leu85, Ile99, Tyr88, Trp111, Phe126
Tyrosol	−5.42	Glu112, Gly138, Arg163	Ser137, Met110, Arg159, Gly158

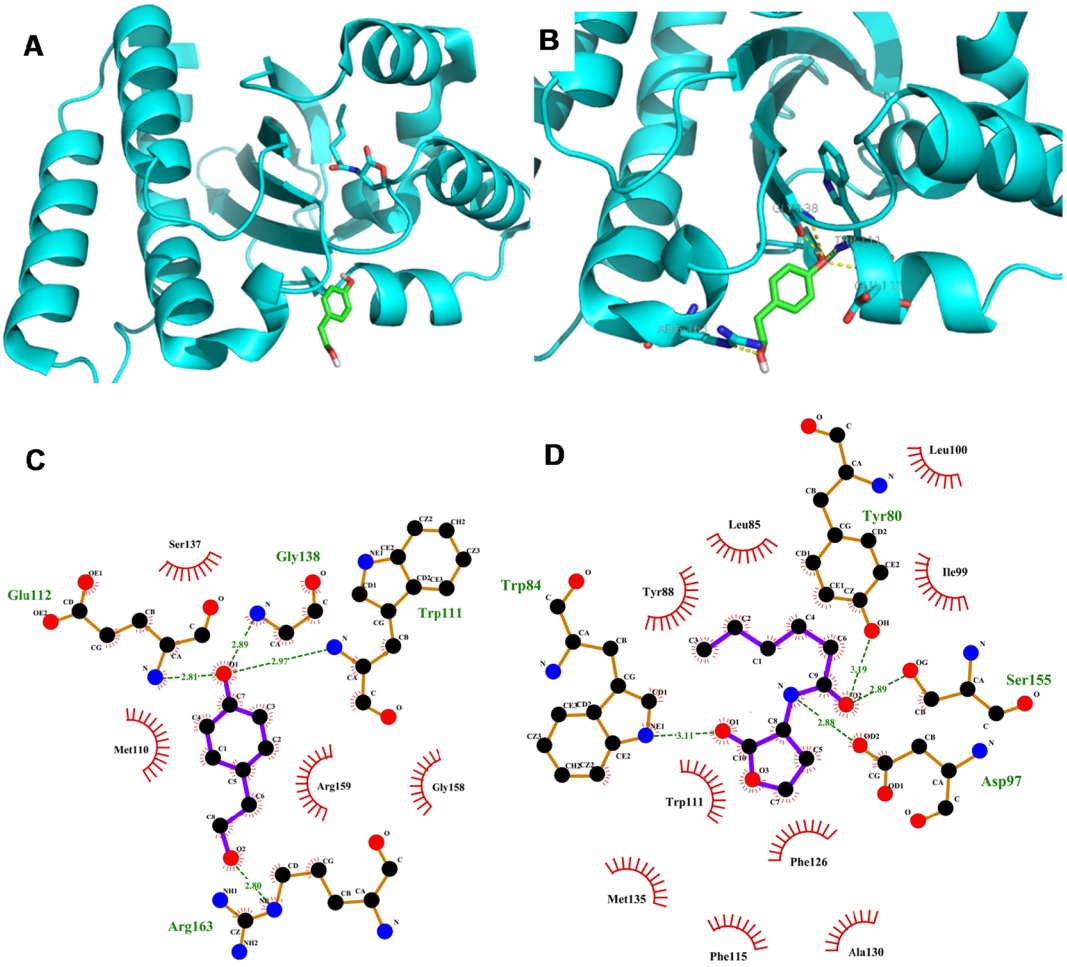

Fig. 7. Docked complex of the 3QP1 receptor protein with tyrosol and C_6HSL. (A) Docked conformations of the natural ligand C_6HSL, shown as blue sticks, and tyrosol, shown as green sticks, in the active site of 3QP1. (B) Analysis of docked tyrosol bound to 3QP1, showing the key interactions in the binding pocket. The hydrogen bonds are shown as yellow dotted lines. (C) LigPlot of tyrosol bound to 3QP1, showing the key hydrophobic interactions. (D) LigPlot of C_6HSL bound to 3QP1, showing the key hydrophobic interactions.

interactions between tyrosol and QS receptors. The crystal structure of the QS signal receptor CviR (PDB ID, 3QP1) from *C. violaceum* was used for the docking calculations. As a control, docking of the natural ligand C_6HSL (Fig. 3) in CviR was performed, and the interactions observed were highly consistent with those reported in X-ray structures [29,35] (Fig. S7), indicating that the docking method was credible. The docking results are shown in Table 1 and Fig. 7. The analysis using Autodock 4.2.6 program suggested that the docking energy of tyrosol and CviR

(−5.42 kcal/mol) was slightly higher than that of the natural ligand C_6HSL and CviR (-6.86 kcal/mol). However, via PyMol software analysis, it is proved that C_6HSL and tyrosol bound to different receptor pockets of CviR (Fig. 7A). Interactions between ligands and the target protein were evaluated by PyMol and LigPlot$^+$ software. As shown in Table 1, Fig. S7B and Fig. 7B, 7C, 7D, C_6HSL and tyrosol bound to 3QP1 with entirely different hydrogen bonding interactions and key hydrophobic interactions. The binding site of tyrosol was present in the DNA-binding domain of CviR [29,35], which resulted in changes in the conformation of the active receptor protein. The corresponding DNA could not bind to the protein; the relevant gene expression could not be induced; and violacein could not be synthesized. In other words, CviR-tyrosol binding changed the active receptor protein conformation, preventing the normal transcription of downstream genes and thus blocking the disease-causing behaviours regulated by QS. Via this mechanism of action, tyrosol inhibited the QS system in *C. violaceum*.

3. Conclusion

The data obtained in this study suggest that tyrosol, isolated from *P. chrysogenum* DXY-1, a marine fungal strain obtained from marine sediments surrounding the East Sea, collected in Taiwan Strait (China), can be used as a QS inhibitor against *C. violaceum* and *P. aeruginosa*. The docking results demonstrate that tyrosol inhibits the QS system of CviR in *C. violaceum* by binding to the DNA-binding domain and blocking pathogenic gene expression. To the best of our knowledge, this study is the first to report the isolation of tyrosol, with anti-QS activity, from *P. chrysogenum*.

Notably, at the same concentration, the anti-QS activity of tyrosol was not better than that of the crude extract (Fig. 1 and Fig. 4). Therefore, we believe that there are other active compounds with relatively high anti-QS activity or synergistic inhibitory effects on QS in the crude extract, which warrants further research.

4. Material and methods

4.1. Bacterial strains and culture conditions

C. violaceum CV026 and *P. aeruginosa* PA01 were used in this study. Each isolate was cultured for 12 h in Luria Bertani (LB) medium containing 0.5% w/v yeast extract, 1% w/v tryptophan, 1.0% agar, and 0.5% w/v NaCl.

4.2. Fermentation and extraction of P. chrysogenum DXY-1

The strain *P. chrysogenum* DXY-1 was obtained from marine sediment surrounding the East Sea collected in Taiwan Strait (China). Fermentation was conducted in an Erlenmeyer flask (1000 mL) with 400 mL of defined medium composed of 40 g of glucose, 10 g of peptone and 5 g of NaCl dissolved in 1 L of seawater. The flasks were incubated on a rotary shaker at 28 °C at 150 rpm. After 7 days of cultivation, 30 L of fermentation broth was obtained. The mycelium pellets in the fermentation broth were disrupted by using a homogenizer, and then, the supernatant was extracted three times with an equal volume of ethyl acetate and concentrated *in vacuo* to obtain 6.5 g of crude extract.

4.3. Isolation and structural elucidation

The crude extract was dissolved with methanol (MeOH) and then filtered through a 0.22-μm organic-phase filter membrane. The methanol solution of the crude extract was separated into 13 fractions (fractions 1-13) using a gel column chromatography system (Sephadex LH-20) with methanol as the eluent. Each fraction was tested by the violacein assay, and the target fraction was identified as fraction 6. Then, fraction 6 was purified by preparative HPLC using a C18 reversed-phase column (ZORBAX SB-Aq, 4.6 × 150 mm, 5 μm). The mobile phase was methanol:water = 25:75 (v/v), and the flow rate was 0.8 mL/min. One compound, a white amorphous powder, was obtained and identified as tyrosol. The UV–vis spectrum was recorded on a microplate reader (M2e; Molecular Devices, Sunnyvale, CA, USA). The data obtained from NMR spectroscopy were as follows. ^{1}H NMR (400 MHz, d_6-DMSO, δ, ppm, TMS): 9.11 (s, 1H, ArO*H*), 6.99 (d, 2H, J = 8.4 Hz, Ar*H*), 6.66 (d, 2H, J = 8.4 Hz, Ar*H*), 4.55 (s, 1H, $ArCH_2CH_2O\mathit{H}$), 3.51 (m, 2H, $ArCH_2\mathit{C}H_2OH$), 2.60 (t, 2H, $ArCH_2CH_2OH$). ^{13}C NMR (100 MHz, d_6-DMSO, δ, ppm): 155.8 (ArOH), 130.0 (Ar), 129.8 (Ar), 115.3 (Ar), 62.9 ($ArCH_2\mathit{C}H_2OH$), 38.6 ($ArCH_2CH_2OH$). This compound exhibited ^{1}H and ^{13}C NMR data that were consistent with literature values [22–24].

4.4. Violacein assay

The effects of the ethyl acetate extract, separated fractions and tyrosol on violacein production were quantified as previously described [20,36]. The specific experimental steps were as follows: 200 μL of *C. violaceum* CV026, 100 μL of the signal molecule C_6HSL and 200 μL of the test material (MeOH or tyrosol solution) were added into 20 mL of LB medium. Following incubation at 28 °C with shaking overnight, 1 mL of culture from each flask was removed and placed into a tube and centrifuged at 12,000 r/min for 10 min to obtain insoluble violacein and cells. The supernatant was discarded, and 1 mL of DMSO was added to the tube. The solution was thoroughly vortexed for a few minutes and centrifuged at 12,000 r/min for 10 min to remove the insoluble cells. The absorbance was measured with a microplate reader (M2e; Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 585 nm. The obtained bacterial cells were re-suspended in 1 mL of sterile water to evaluate cell growth by measuring the optical density (OD) at 600 nm. Values are presented as the mean ± SD; n = 3.

4.5. Pyocyanin assay

The effect of tyrosol on pyocyanin production was quantified as previously described [20,37]. The specific experimental steps were as follows: *P. aeruginosa* PA01 was inoculated into LB medium at an initial OD_{600} of 0.05 and cultured with tyrosol, MeOH or azithromycin (AZM) at 37 °C for 12 h. After centrifugation to remove the cells, 5 mL of the supernatant was extracted using 3 mL of chloroform ($CHCl_3$), and the organic layer was transferred into a new Eppendorf tube and mixed with 1 mL of 0.2 M HCl. The water layer was collected by centrifugation. The absorbance was measured with a microplate reader (M2e; Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 520 nm. Values are presented as the mean ± SD; n = 3.

4.6. Elastase activity assay

Elastase activity was determined using a previously reported method [20,37]. The specific experimental steps were as follows: *P. aeruginosa* PA01 was inoculated into LB medium at an initial OD_{600} of 0.05 and cultured with tyrosol, MeOH or AZM at 37 °C for 12 h. After centrifugation to remove the cells, the top layer (100 μL) was collected by filtration using a 0.22-μm nylon filter, and then 10 mg of elastin-Congo red (ECR) and 900 μL of Na_2HPO_4 buffer (pH = 7.0) were added. After incubation at 37 °C for 2 h, the samples were centrifuged to remove insoluble ECR. The absorbance was measured with a microplate reader (M2e; Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 495 nm. Values are presented as the mean ± SD; n = 3.

4.7. Proteolytic activity assay

Proteolytic activity was determined using a previously reported method [37]. The specific experimental steps were as follows: *P. aeruginosa* PA01 was inoculated into LB medium at an initial OD_{600} of 0.05 and cultured with tyrosol, MeOH or AZM at 37 °C for 12 h. After

centrifugation to remove the cells, the top layer (100 μL) was collected by filtration using a 0.22-μm nylon filter, and then, 400 μL of 50 mM K_2HPO_4 buffer (pH = 7.0) containing 0.8% azo casein was added. After incubation at 30 °C for 3 h, 1.5 M HCl was added, and the reaction was placed for 10 min in an ice bath. The supernatant was collected by centrifugation, and then, 0.5 mol/L NaOH was added. The samples to be tested were obtained by centrifugation again. The absorbance was measured with a microplate reader (M2e; Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 440 nm. Values are presented as the mean ± SD; n = 3.

4.8. Biofilm assay

The effect of tyrosol on biofilm formation was further confirmed with SEM (S-4800, Hitachi, Japan). The specific experimental steps were as follows: 3 mL of *P. aeruginosa* PA01 (OD_{600} = 0.05) was cultured with tyrosol, MeOH or AZM in a 6-well plate. A sterile glass slide was placed at the bottom of each well, and the plate was incubated at 37 °C for 48 h. Then, the non-adherent cells were removed by washing with phosphate-buffered saline (PBS). After being fixed with 2.5% glutaraldehyde and coated with gold, the glass slides were used for SEM.

4.9. Docking studies

All computations were carried out by a series of software programs including ChemBioOffice 2010, Autodock 4.2.6, LigPlot$^+$ v.1.4 and PyMol 1.5.0.3 [20,38–40]. The natural ligand C_6HSL and studied molecule tyrosol were first drawn by ChemBioOffice software, and the energies were minimized with ChemBio3D Ultra 12.0. The crystal structure of *C. violaceum* CviR receptor protein was downloaded from the protein data bank (PDB) with the ID code of 3QP1 and used as target receptor protein [29]. Firstly, the water molecules and co-crystallized ligand of 3QP1 were eliminated. The protein was then modified by adding polar hydrogens and Compute Gasteiger charges. The grid box was settled to whole receptor involved in the active site, and the docking parameter files were created using the Lamarckian genetic algorithm (GA) before the program was run. The number of GA runs was set to 100 replications and the other settings were set as defaults. The results of the docking computations were ranked by binding energy. The conformations and interactions between ligand and the target protein were visualized by PyMol molecular graphic system and LigPlot$^+$.

Declaration of Competing Interest

The authors declare no competing financial interest.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103140.

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