

# An Indonesian Marine Bacterium, *Pseudoalteromonas rubra*, Produces Antimicrobial Prodiginine Pigments

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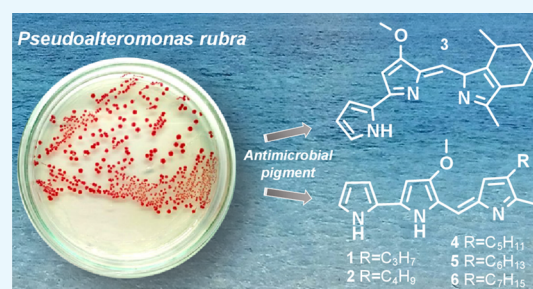


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**ABSTRACT:** Red pigmented marine bacteria, *Pseudoalteromonas rubra* strains PS1 and SB14, were isolated from two sampling locations in different ecosystems on Alor Island, Indonesia, and cultured in the laboratory. We analyzed the 16S rRNA gene sequences and examined the pigment composition and found that both strains produced cycloprodigiosin (3), prodigiosin (4), and 2-methyl-3-hexyl-prodiginine (5) as major compounds. In addition, we detected three minor compounds: prodigiosin derivatives 2-methyl-3-propyl prodiginine (1), 2-methyl-3-butyl prodiginine (2), and 2-methyl-3-heptyl-prodiginine (6). To our knowledge, this is the first report that *P. rubra* synthesizes not only prodigiosin and cycloprodigiosin but also four prodigiosin derivatives that differ in the length of the alkyl chain. The antimicrobial activity of cycloprodigiosin, prodigiosin, and 2-methyl-3-hexyl-prodiginine was examined by a disk-diffusion test against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, and *Candida albicans*. We found that, at a concentration of 20  $\mu\text{g}/\text{mL}$ , cycloprodigiosin showed the greatest inhibition ( $25.1 \pm 0.55$  mm) against *S. aureus*.



## INTRODUCTION

Antimicrobial agents are drugs generally obtained from microorganisms that are crucial to the prevention and treatment of bacterial infection. However, the use of antibiotics in medicine, which is too frequent and inappropriate, contributes to the rapid spread of antibiotic resistance.<sup>1</sup> Antimicrobial resistance (AMR) poses a serious global problem. A previous report from the World Health Organization (WHO) combined available global data on AMR in seven common bacterial pathogens<sup>2</sup> and highlighted a lack of systematic data on AMR in Southeast Asia.<sup>3</sup> Nevertheless, it was reported that nosocomial infections caused by antimicrobial-resistant bacteria resulted in 38,481 deaths in Thailand.<sup>4</sup> New drugs are needed because of decreases in drug efficacy and the need to combat antibiotic resistance. Marine microorganisms represent a significant source of new drugs for development due to their rich biodiversity and genetic capacity to produce unique metabolites.

Prodiginines are a family of microbial red pigments produced as secondary metabolites by many bacterial species.<sup>5–7</sup> There are two types of prodiginines:<sup>7,8</sup> linear alkyl side chain-containing compounds, such as prodigiosin and undecylprodigiosin, and cyclic compounds, such as cycloprodigiosin, metacycloprodigiosin, prodigiosin R1, and streptorubin B. Prodiginines have received considerable interest because of their numerous biological activities, including antimicrobial,<sup>9</sup> anticancer,<sup>10,11</sup> antitumor,<sup>12</sup> and

anti-inflammatory activities.<sup>13</sup> Recent studies have shown that the antimicrobial effect of prodigiosin on bacterial physiology was concentration-dependent. In *Escherichia coli* cells treated with prodigiosin above the minimum inhibitory concentration (MIC), no significant DNA damage or cytoplasmic membrane disintegration was observed.<sup>14</sup> However, prodigiosin was observed to be responsible for plasma membrane leakage.<sup>15</sup>

Although prodiginines were extracted from the terrestrial bacteria *Serratia marcescens*<sup>16,17</sup> early on, these compounds were later obtained from several bacteria in the genera *Pseudomonas*, *Pseudoalteromonas*, *Hahella*, *Vibrio*, and *Zooshikella*, which were isolated from different marine habitats.<sup>18–20</sup> The genus *Pseudoalteromonas* is a group of widespread marine bacteria. There are three species from this genus, *Pseudoalteromonas (P.) rubra*, *P. denitrificans*, and *P. bacteriolytica*, which are known to produce prodigiosin.<sup>21–23</sup>

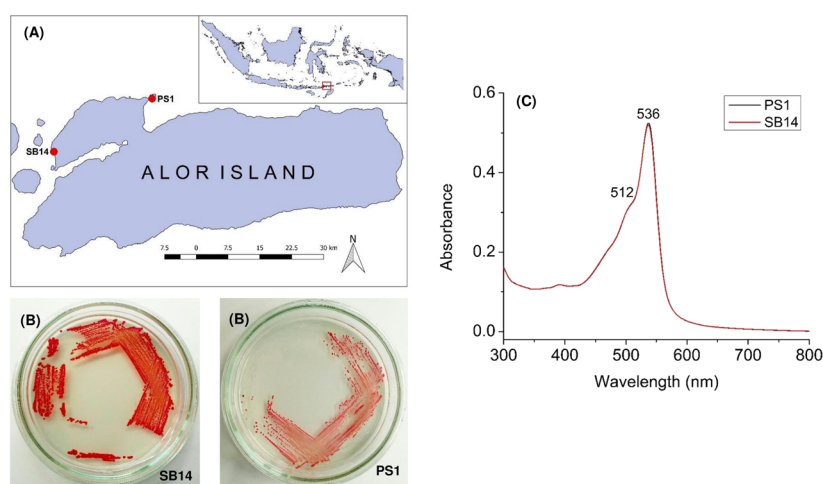
Currently, of the 200 *Pseudoalteromonas* genomes submitted to the National Center for Biotechnology Information (NCBI),<sup>61</sup> there are nine genomes that are code for biosynthetic enzymes that responsible for synthesize prodigi-

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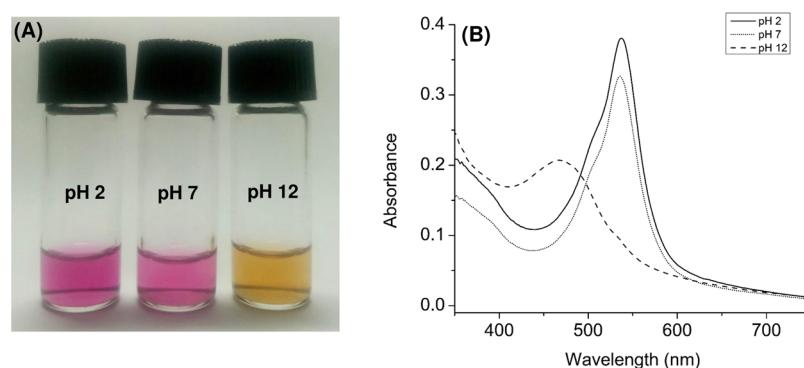
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**Figure 1.** (A) Sampling locations on Alor Island; inset shows the Indonesian Archipelago. (B) Two isolated strains, PS1 and SB14. (C) UV–Vis absorption spectra of their crude pigment extracts in methanol. (Photos were taken by E. Setiyono.)



**Figure 2.** (A) Differences in the color of prodiginine in acidic (pH 2), neutral (pH 7), and alkaline (pH 12) conditions. (B) Associated UV–Vis absorption spectra. (Photos were taken by E. Setiyono.)

nines: one belongs to *P. denitrificans*, and eight belong to *P. rubra*.<sup>24</sup> *P. rubra* strain ATCC 29570 (formerly *Alteromonas rubra* NCMB 1890) isolated from the Mediterranean Sea is a Gram-negative, red, and motile marine bacterium that has antibiotic activity against *Staphylococcus epidermidis*.<sup>21</sup> This strain has been reported to synthesize prodiginosin; however, there is little information on the prodiginosin itself, except for absorption spectra of the crude pigment extract. Later, fractionation of a prodiginosin extract was conducted using a high-performance liquid chromatography (HPLC) system. Several prodiginosins were separated, but there was no detailed compound identification provided. Gerber and Gauthier<sup>25</sup> found a new prodiginosin-like pigment from *P. rubra* with a molecular mass of ( $m/z$ ) 321 that is known as cycloprodiginosin. Over more than three decades, other types of prodiginosin derivatives, 2-methyl-3-butyl-prodiginine, 2-methyl-3-hexyl-prodiginine, and 2-methyl-3-heptyl-prodiginine, were identified in the marine bacterium *Pseudoalteromonas* sp. 1020R, which has 99% homology to *P. rubra*.<sup>26</sup> Strain 1020R produced only prodiginosin and its derivatives with different alkyl chains. Until now, there have been no reports on a *P. rubra* strain that contains cycloprodiginosin, except for the originally identified *P. rubra* strain (ATCC 29570). The previous studies indicate that the strains of *P. rubra* are compositionally heterogeneous in terms of prodiginosins, and information concerning prodiginosins composition in each

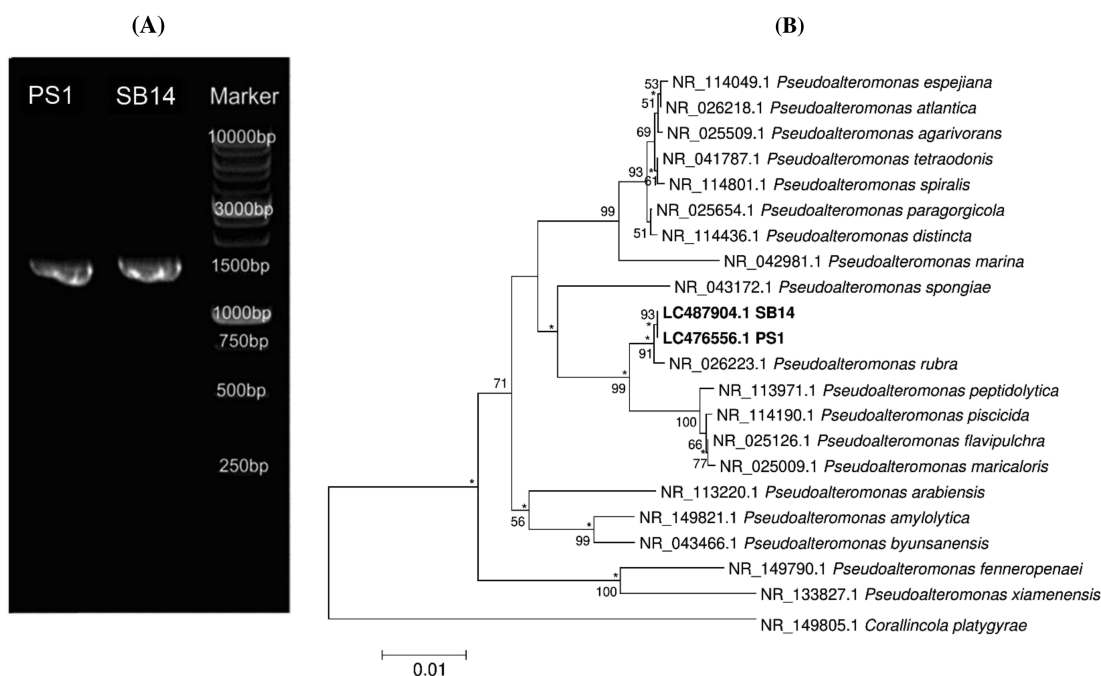
strain is important to distinguish subspecies among the strains of *P. rubra*.

Recently, we isolated two new marine bacterial strains that have UV–Vis spectral characteristics of prodiginosins. The strains were designated *P. rubra* PS1 and *P. rubra* SB14 according to a 16S rRNA sequence analysis and blast search, which revealed more than 99% similarity to *P. rubra* ATCC 29570. The prodiginosins were successfully separated and identified by reversed-phase (RP)-HPLC, and the purified compounds were identified by electrospray ionization-mass spectrometry (ESI-MS/MS). Here, we report the bacterial isolation and the determination of the prodiginosin composition and the antimicrobial activity of three types of prodiginosins isolated from *P. rubra* strain PS1 against pathogenic microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, and *Candida albicans*.

## RESULTS AND DISCUSSION

### Bacterial Isolation and Spectrophotometric Analysis.

Two strains were successfully isolated from different habitats. Strain PS1 was isolated from seawater in a habitat dominated by seagrasses and mammals such as *Dugong dugon* in Sika Island. Strain SB14 was isolated from seawater in a habitat of a healthy coral reef ecosystem in Sebanjar Beach (Figure 1A,B). The UV–Vis absorption spectra of the methanol extract of strains PS1 and SB14 were characterized by a broad band in the 400–600 nm wavelength range with an absorption



**Figure 3.** (A) 16S rRNA gene PCR amplicons on 0.8% agarose gel of *P. rubra* strains PS1 and SB14. (B) Phylogeny of strains PS1 and SB14, the type strains of recognized species in the genus *Pseudoalteromonas*, and representatives of related taxa. *Corallincola platygyrae* was used as an outgroup. Only bootstrap values >50% (expressed as percentages of 1000 replications) are shown at branch points. Asterisks indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.01 substitutions per nucleotide position.

maximum ( $\lambda_{\max}$ ) at 536 nm and a shoulder at 512 nm (Figure 1C). These spectra were identical to the UV–Vis absorption spectrum of prodiginine pigments.<sup>27,28</sup> The UV–Vis spectra of prodiginine were also recorded in 95% MeOH at different pH values. Under acidic conditions (pH 2, adjusted with 0.01 N HCl), its color was red with  $\lambda_{\max}$  at 536 nm. Under neutral conditions, the color changed to pink, and the intensity decreased. Under alkali conditions (pH 12, adjusted with 0.01 NaOH), it was orange, and the spectra shifted to the left with  $\lambda_{\max}$  at 465 nm (Figure 2), indicating deprotonation of the nitrogen atoms on the three conjugated pyrrole rings by NaOH.<sup>29</sup> These spectral changes are in line with the ones reported by Song et al.<sup>30</sup>

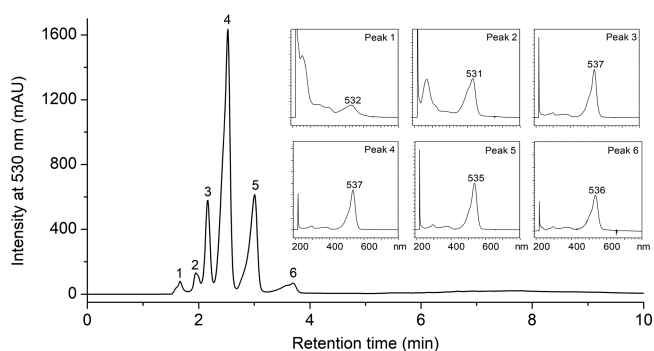
**Molecular Identification of Bacteria.** The 16S rRNA gene sequences of strains PS1 and SB14 comprised 1294 and 1343 bp, respectively. The nucleotide data from both strains have been deposited in the DNA Data Bank of Japan (DDBJ; <https://ddbj.nig.ac.jp>) with accession numbers LC476556 and LC487904 for strains PS1 and SB14, respectively. The similarity of the 16S rRNA genes of these strains was calculated using the nucleotide BLAST program (NCBI) for highly similar sequences (megablast). A comparative study of the 16S rRNA gene sequences showed that strains PS1 and SB14 shared 99.46 and 99.48% sequence similarity, respectively, with *P. rubra* strain ATCC 29570. In the phylogeny constructed as a neighbor-joining tree, strains PS1 and SB14 were grouped with *P. rubra* strain ATCC 29570, with a bootstrap resampling value of 91% (Figure 3). The relationship of strains PS1 and SB14 with *P. rubra* strain ATCC 29570 was also determined in trees constructed with the maximum-likelihood and maximum-parsimony algorithms, and the recovered nodes are marked by asterisks in Figure 3 below.

At the time of writing this report, there were 45 strains of *P. rubra*, including our two strains, which were isolated from

different sources, that have been deposited in the NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) (Table S1, Supporting Information). *P. rubra* has been discovered throughout the region spanning the Indian Pacific to Atlantic Oceans. However, most strains have been found in the Indo-Pacific area (Figure S1, Supporting Information). The first *P. rubra* strain identified with prodiginine production capability was isolated from seawater.<sup>21</sup> The 45 strains were isolated from various habitats: 14 from seawater, 7 from coral, 5 from macroalgae, 4 from nudibranchs and mussels, and 1 each from sediment, protist, fish, leaf, and larval stone (Figure S2, Supporting Information).

#### HPLC Analysis of Prodiginine and Its Derivatives.

Prodiginine and its derivatives from the pigment extracts of *P. rubra* strains PS1 and SB14 were separated on a  $C_8$  column. Both strains have the same prodiginine composition based on their HPLC chromatograms. The HPLC separation profile contains six well-resolved compounds (Figure 4).

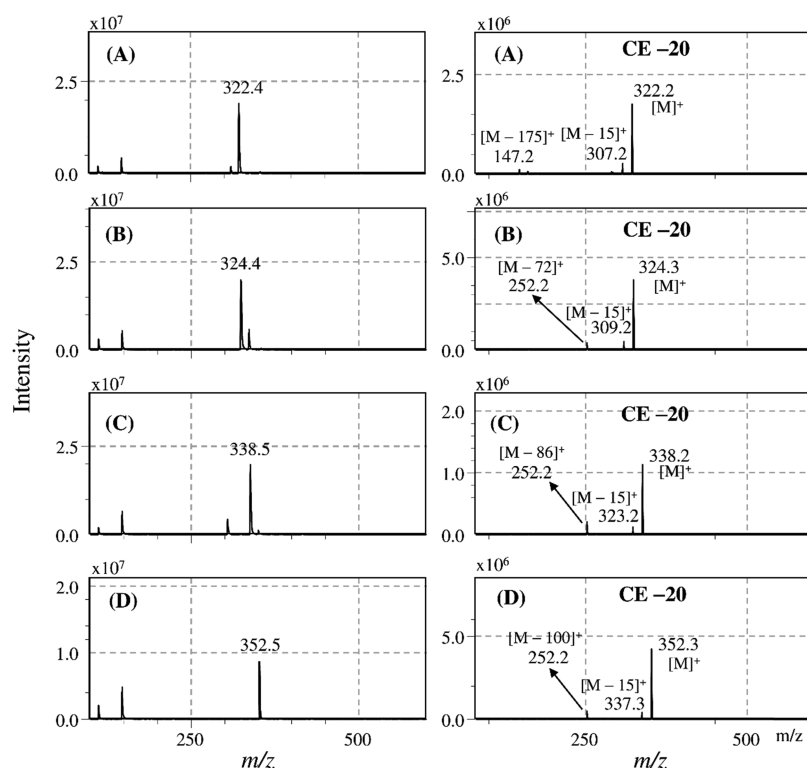


**Figure 4.** HPLC chromatogram of the crude pigment extract of *P. rubra* strain PS1 and the UV–Vis spectra of the eluent peaks.

Table 1. Identification of Prodiginine Pigments in *P. rubra* Strain PS1<sup>a</sup>

peak #	identification	$t_R$ (min)	$\lambda_{max}$ (nm)	molecular ion ( $m/z$ )	product ion ( $m/z$ )	CE (V)	compound formula
1	2-methyl-3-propyl prodiginine	1.66	532	296.1 [M + H] <sup>+</sup>	92.0 [M - 204] <sup>+</sup>	-35	C <sub>18</sub> H <sub>21</sub> N <sub>3</sub> O
					252.2 [M - 44] <sup>+</sup>	-30	
					281.0 [M - 15] <sup>+</sup>	-20	
2	2-methyl-3-butyl prodiginine	1.94	531	310.2 [M + H] <sup>+</sup>	92.1 [M - 218] <sup>+</sup>	-49	C <sub>19</sub> H <sub>23</sub> N <sub>3</sub> O
					252.2 [M - 58] <sup>+</sup>	-31	
					295.3 [M - 15] <sup>+</sup>	-22	
3	cycloprodiginosin	2.16	537	322.4 [M + H] <sup>+</sup>	147.2 [M - 175] <sup>+</sup>	-32	C <sub>20</sub> H <sub>23</sub> N <sub>3</sub> O
					292.2 [M - 30] <sup>+</sup>	-33	
					307.2 [M - 15] <sup>+</sup>	-24	
4	prodiginosin	2.52	537	324.4 [M + H] <sup>+</sup>	92.2 [M - 232] <sup>+</sup>	-53	C <sub>20</sub> H <sub>25</sub> N <sub>3</sub> O
					252.1 [M - 72] <sup>+</sup>	-33	
					309.5 [M - 15] <sup>+</sup>	-23	
5	2-methyl-3-hexyl prodiginine	3.00	535	338.5 [M + H] <sup>+</sup>	92.2 [M - 246] <sup>+</sup>	-51	C <sub>21</sub> H <sub>27</sub> N <sub>3</sub> O
					252.1 [M - 86] <sup>+</sup>	-33	
					323.2 [M - 15] <sup>+</sup>	-23	
6	2-methyl-3-heptyl prodiginine	3.69	536	352.5 [M + H] <sup>+</sup>	92.2 [M - 260] <sup>+</sup>	-52	C <sub>22</sub> H <sub>29</sub> N <sub>3</sub> O
					252.2 [M - 100] <sup>+</sup>	-34	
					337.3 [M - 15] <sup>+</sup>	-25	

<sup>a</sup>Note: Product ions were obtained at the optimized collision energy (CE) determined by multiple reaction monitoring (MRM).



**Figure 5.** ESI-MS/MS analysis of the purified compounds. Full Q1 scan (left) and product ion scan (right) mass spectra of (A) cycloprodiginosin, (B) prodiginosin, (C) 2-methyl-3-hexyl-prodiginine, and (D) 2-methyl-3-heptyl-prodiginine.

The first two compounds, peaks #1 and #2, are minor compounds eluted at retention times ( $t_R$ ) of 1.66 and 1.94 min with  $\lambda_{max}$  at 532 and 531 nm, respectively. The third compound (peak #3) eluted at a  $t_R$  of 2.16 min with  $\lambda_{max}$  at 537 nm. The fourth compound (peak #4) eluted at a slightly more nonpolar  $t_R$  than peak #3 and was a major compound with a  $t_R$  of 2.52 min and  $\lambda_{max}$  at 537 nm. The last two compounds (peaks #5 and #6) had a  $t_R$  of 3.00 and 3.69 min and  $\lambda_{max}$  at 535 and 536 nm, respectively. Next, the six compounds (peaks #1, #2, #3, #4, #5, and #6) were analyzed in highly purified samples (purity of >97%) by ESI-MS/MS

and assigned as follows with descriptions below: 2-methyl-3-propyl prodiginine (peak #1), 2-methyl-3-butyl prodiginine (peak #2), cycloprodiginosin (peak #3), prodiginosin (peak #4), 2-methyl-3-hexyl prodiginine (peak #5), and 2-methyl-3-heptyl prodiginine (peak #6). The identification of prodiginosin and four derivatives was confirmed by the linear relationship between the log capacity factor ( $k'$ ) of the pigments and the number of the carbon atoms in the alkyl side chain of the pigment molecule (Figure S3, Supporting Information), as shown previously for esterifying alcohols in chlorophylls<sup>31</sup> and



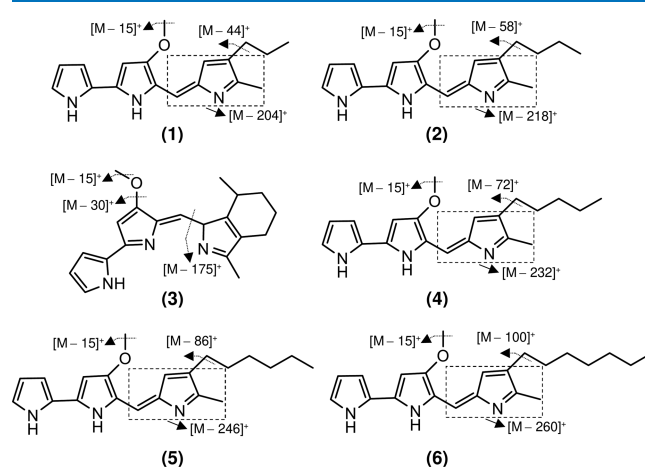
hydroxyl moieties in  $\beta$ -carotene congeners.<sup>32</sup> The characteristics of the purified pigments are listed in Table 1.

### MS/MS Analysis of Prodigiosin and Its Derivatives.

The six purified compounds were analyzed by ESI-MS/MS to determine the molecular ions and fragment ions. The full Q1 scan mass spectrum of peak #3 showed a molecular ion at a mass-to-charge ratio ( $m/z$ ) of 322.4  $[M + H]^+$  (Figure 5A, left). Further study of the product ion scan mass spectrum with a CE of  $-20$  V showed a molecular ion at  $m/z$  322.2  $[M + H]^+$  and product ions at  $m/z$  307.2  $[M - 15]^+$  and 147.2  $[M - 175]^+$  (Figure 5A, right), indicating loss of a methyl group  $[CH_3]$  and  $C_{10}H_9N_2O$ , respectively. The UV-Vis absorption (Figure 4) and mass (Figure 5A) spectra obtained for purified peak #3 were consistent with those of the cycloprodigiosins reported by Gerber and Gauthier<sup>25</sup> and Lee et al.,<sup>33</sup> which include an immunosuppressant,<sup>34</sup> anticancer compound,<sup>35</sup> and antimalarial drug.<sup>36</sup> Next, a major compound, peak #4, was analyzed by mass spectrometry (Figure 5B). The mass spectra from the Q1 scan showed a molecular ion at  $m/z$  324.4  $[M + H]^+$  and two product ions at  $m/z$  252.2  $[M - 72]^+$  and 309.2  $[M - 15]^+$  (Figure 5B, right). Comparison of the molecular and product ions with those of standard prodigiosin hydrochloride revealed the same mass spectra (Figure S4, Supporting Information). The mass spectra and absorption maxima of the purified sample were consistent with those of prodigiosin (2-methyl-3-pentyl-prodiginine).<sup>33</sup> Therefore, the major compound, peak #4 at 2.52 min, was identified as prodigiosin. Based on the molecular ion and product ions, peaks #5 and #6 were identified as 2-methyl-3-hexyl-prodiginine ( $m/z$  338.5  $[M + H]^+$ ) (Figure 5C) and 2-methyl-3-heptyl-prodiginine ( $m/z$  352.5  $[M + H]^+$ ) (Figure 5D), respectively, which were also reported in the marine bacterium *Hahella chejuensis* KCTC 2396.<sup>37,38</sup> The minor compounds, peaks #1 and #2, respectively, were identified as 2-methyl-3-propyl prodiginine ( $m/z$  296.1  $[M + H]^+$ ) and 2-methyl-3-butyl prodiginine ( $m/z$  310.2  $[M + H]^+$ ) (Figure S5, Supporting Information), respectively. To our knowledge, this is the first report on the presence of four derivatives of prodigiosin with different alkyl side chains in *P. rubra* in addition to prodigiosin and cycloprodigiosin. These findings largely rely on recently developed MS techniques, such as single ion monitoring (SIM) and MRM, which are based on the selection of a precursor ion and one or more characteristic fragment ions and are powerful tools for determining the structure of target compounds.

In this study, MS analysis was carried out in positive mode, and product ions were almost all obtained at a CE of  $-20$  V. Interestingly, prodigiosin and its analogs showed similar fragmentations, with losses of a methyl group at  $m/z$   $[M - 15]^+$  and alkyl chains at  $m/z$   $[M - 44]^+$ ,  $[M - 58]^+$ ,  $[M - 72]^+$ ,  $[M - 86]^+$ , and  $[M - 100]^+$ , respectively, for compounds containing propyl, butyl, pentyl, hexyl, and heptyl side chains (Figure 5 and Figure S5). The complete alkyl chains were lost as shown in agreement to the previously studies by Wang et al. and Lee et al.<sup>26,33</sup> The complete alkyl chain might be removed due to strong ESI ionization technique from the prodigiosins compound instead remains as allylic carbocation such as benzylic ions. To obtain candidate product ions, we conducted MRM to determine the optimum CE (Table 1). At a CE of  $-33$  V, an additional product ion was found for cycloprodigiosin at  $m/z$  292.15  $[M - 30]^+$ , indicating loss of a methyl group and one oxygen atom  $[CH_3 + O - H]^+$ . Additionally, the product ion at  $m/z$  92 that was found for

prodigiosin and its derivatives with different alkyl chain sides due to loss of cyclopentadienecarbonitrile  $[C_6H_5N_2 + H]^+$  was obtained at a CE of  $-51$  to  $-53$  V. The optimal CE for the loss of methyl and alkyl groups was  $-23$  to  $-25$  V and  $-33$  to  $-34$  V, respectively. Thus, the CE is a key factor, and the MRM method used in this study to determine the optimum CE is ideal for analyzing candidate product ions (Figure 6).



**Figure 6.** Molecular structure and fragmentation of identified molecules at different CEs based on optimized product ions using MRM. For the optimum CE values, see Table 1. 1, 2-methyl-3-propyl prodiginine; 2, 2-methyl-3-butyl prodiginine; 3, cycloprodigiosin; 4, prodigiosin; 5, 2-methyl-3-hexyl prodiginine; 6, 2-methyl-3-heptyl prodiginine.

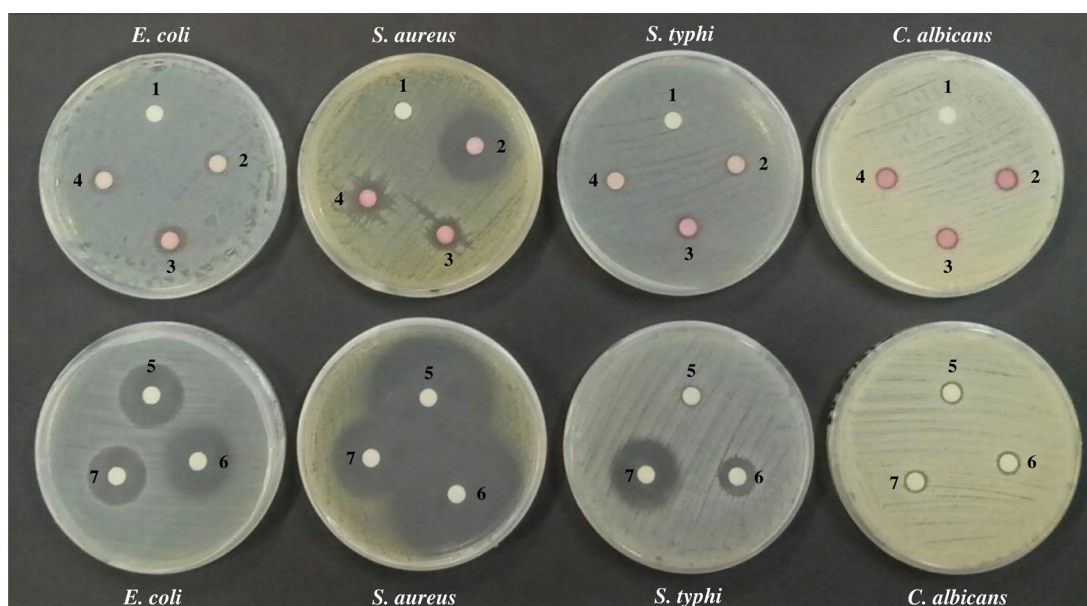
**Antimicrobial Activity.** The antimicrobial activities of cycloprodigiosin, prodigiosin, and 2-methyl-3-hexyl prodiginine compared to the standard antibiotic amoxicillin, ampicillin, and chloramphenicol are presented in Table 2 and Figure 7.

As shown in Table 2, cycloprodigiosin, prodigiosin, and 2-methyl-3-hexyl prodiginine exhibited antimicrobial activity against all tested pathogenic organisms. The inhibition zone varied from 9.0 to 10.5 mm when applied to *E. coli*. A smaller clear zone of 8.1–8.5 mm was obtained against *S. typhi*. An interesting result was obtained when the pigments were applied to *S. aureus*: a significant inhibition zone from 10.1 to 25.1 mm. In this case, cycloprodigiosin showed greater activity than prodigiosin and 2-methyl-3-hexyl prodiginine. The pigments also showed antifungal activity (7.9–8.2 mm) against *C. albicans*. There was no clear zone in the negative control (DMSO). However, the positive controls amoxicillin, ampicillin, and chloramphenicol demonstrated susceptibility to *E. coli*, *S. typhi*, and *S. aureus*. On the other hand, *C. albicans* was resistant to all antibiotics with resulting inhibition zone only around 8.3–8.6 mm. The bioactivities of cycloprodigiosin and prodigiosin from the marine bacterium *Zooshikella rubidus* S1-1 have been studied by Lee et al.<sup>33</sup> Disks containing 50  $\mu$ g of these compounds produced an inhibition zone of approximately 8.0 mm against *Salmonella typhimurium*, *S. aureus*, *E. coli*, *Bacillus subtilis*, and *Candida albicans*.<sup>33</sup> Another experiment by Lapenda et al.<sup>9</sup> showed that 300  $\mu$ g of prodigiosin from *Serratia marcescens* produced significant inhibition zones against *S. aureus* (35 mm), *Enterococcus faecalis* (22 mm), and *Streptococcus pyogenes* (14 mm). However, no inhibition zones were found against *E. coli*, *Pseudomonas aeruginosa*, or *Acinetobacter*.<sup>9</sup> In our case,

**Table 2.** Antimicrobial Activity (mm) of Cycloprodigiosin, Prodigiosin, and 2-Methyl-3-hexyl Prodiginine Detected in the Disk Diffusion Test<sup>a</sup>

compound	inhibition zone (mm)			
	<i>E. coli</i>	<i>S. aureus</i>	<i>S. typhi</i>	<i>C. albicans</i>
cycloprodigiosin	9.0 ± 0.49	25.1 ± 0.55	8.1 ± 0.03	7.9 ± 0.07
prodigiosin	10.5 ± 0.9	11.6 ± 0.28	8.5 ± 0.13	8.2 ± 0.09
2-methyl-3-hexyl prodiginine	9.1 ± 0.49	10.1 ± 0.44	8.1 ± 0.09	7.9 ± 0.06
amoxicillin	23.4 ± 0.26	41.5 ± 0.26	8.2 ± 0.17	8.6 ± 0.12
ampicillin	24.1 ± 0.20	45.7 ± 0.21	13.4 ± 0.20	8.6 ± 0.07
chloramphenicol	20.8 ± 0.32	27.3 ± 0.18	23.5 ± 0.20	8.3 ± 0.06

<sup>a</sup>The data used are averages of triplicate measurement ± the standard error (±SE).



**Figure 7.** Antimicrobial activity of cycloprodigiosin (2), prodigiosin (3), and 2-methyl-3-hexyl prodiginine (4) compared to the standard antibiotic amoxicillin (5), ampicillin (6), and chloramphenicol (7). DMSO as a negative control is marked with number 1. (Photos were taken by E. Setiyono.)

cycloprodigiosin, prodigiosin, and 2-methyl-3-hexyl prodiginine at a concentration of 20  $\mu\text{g}/\text{mL}$  caused clear zones around the disks, suggesting that these compounds are sensitive to *E. coli*, *S. typhi*, *S. aureus*, and *C. albicans*. Bioactive prodiginine-producing marine bacteria comprise a wide variety of genera, including *Beneckeia*,<sup>38</sup> *Pseudovibrio*,<sup>39</sup> *Vibrio*,<sup>40–42,59</sup> *Streptomyces*,<sup>5,43–45,60</sup> *Pseudomonas*,<sup>46</sup> *Pseudoalteromonas*,<sup>21,23,26,47</sup> *Haehella*,<sup>48,49,58</sup> and *Zooshikella*.<sup>33,50,51</sup> However, the prodiginine types and composition differ among them, as shown in Table S2, Supporting Information. Our isolated bacteria, *P. rubra* strains PS1 and SB14, are included in one of these genera. *Pseudoalteromonas* species appear to have a wide habitat, as described above. As shown in this study, strains of *P. rubra* can be easily isolated from a variety of marine environments and cultured by standard manipulation techniques; therefore, they might be a potential source for the production of bioactive prodigiosin and its derivatives.

## EXPERIMENTAL SECTION

**Sampling Location.** Sampling was conducted on Alor Island, Eastern Indonesia. It is a tropical island located on the eastern tip of the Nusa Tenggara Islands at 8°15'S and 124°45'E. The island is bordered by the Flores Sea and the Banda Sea in the north, the Ombai Strait in the south (separating it from Timor Island), and the Pantar Strait in the

west. Alor Island is one of the two main islands in the Alor Regency, East Nusa Tenggara Province, Indonesia. Two isolated strains were collected from two different locations with different ecosystems: Sika Island coast and Sebanjar Beach (Figure 1). Seawater was sampled in the surface and the bottom layer (depths of 0 and 6 m). Samples were collected in 50 mL sterile tubes and placed in a cold box immediately. Samples were then brought to the Ma Chung Research Center for Photosynthetic Pigments for bacterial isolation. A map of sampling locations was created using QGIS version 2.18.19 and the ESRI continent base map.

**Bacterial Isolation and Purification.** Up to 35  $\mu\text{L}$  of seawater was spread directly into Petri dishes containing Zobell agar medium (0.5 g of yeast, 2.5 g of peptone, and 13 g of Bacto agar in 1 L of seawater) and incubated for 3 days at 35 °C. After 3 days of incubation, the bacterial colonies on the Petri dishes were observed. The red pigmented bacterium was purified by the streak plate method and cultured in new medium for 3 days at 32 °C. Purification of the pigmented bacterium was based on its color, shape, margin, elevation, and size. The purification was performed repeatedly until the colony was pure. The pure bacterium was then stored at 10 °C until further study.

**Bacterial Culture.** The pure bacterium was cultured on the same medium as that used in the bacterial isolation. However,

the culturing was carried out in a shaking incubator (New Brunswick Scientific Excella E24, Edison, NJ, USA) at 32 °C for 24 h. Red pigment production is very rapid. After 24 h, the pigment begins to fade. The cells of the bacterium were scraped, collected, and placed in 25 mL plastic tubes and then precipitated by centrifugation at 10,000 rpm for 10 min at 4 °C, and the collected cells were stored in a freezer at −30 °C until use.

**DNA Extraction.** DNA extraction was carried out by the Chelex method.<sup>52</sup> The bacterial cells were inoculated into a mixture of 100  $\mu$ L of ddH<sub>2</sub>O and 1 mL of 0.5% saponin (w/v) in 1 $\times$  PBS and incubated overnight. The precipitate was separated and collected by centrifugation at 12,000 rpm for 10 min. Subsequently, 100  $\mu$ L of ddH<sub>2</sub>O and 50  $\mu$ L of 20% Chelex 100 (w/v) were mixed until the precipitate dissolved. The resulting solution was boiled for 10 min and vortexed for 5 min. Then, the mixture containing bacterial DNA was centrifuged at 12,000 rpm for 10 min and stored at −20 °C until use. The bacterial DNA concentrations were quantified and qualified by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). The concentration of 1  $\mu$ L of DNA was analyzed, and the DNA purity was calculated as the 260/280 nm ratio.

**PCR Amplification of the 16S rRNA Gene Sequence.** The universal primers 27F (5'-AGAGTTTGTATCMTGGCT-CAG-3') and 1492R (5'-TACGGTTAACCTTGTTAC-GACTT-3') in a PCR mixture were used in this study. For analysis of 16S rRNA gene sequences, GoTaq Green Mix Promega (Promega, Madison, Wisconsin, USA) (25  $\mu$ L) was used to amplify DNA. The PCR mixture consisted of GoTaq Green Master Mix Promega (25  $\mu$ L), primer 27F (0.5–5  $\mu$ L), primer 1492R (0.5–5  $\mu$ L), DNA extract (1–5  $\mu$ L), and nuclease-free water (50  $\mu$ L). PCR was conducted in an MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, California, USA). The cycling conditions were as follows: initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 7 min.<sup>53</sup> The PCR products were analyzed using agarose 0.8% gel electrophoresis, and the results were visualized by a UVIDoc HD5 system (UVITEC, Cambridge, UK). The amount of DNA ladder per lane and the sample volume loaded per lane were 0.2  $\mu$ g and 1  $\mu$ L, respectively.

**DNA Sequencing.** DNA sequencing was performed using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and PCR sequencing was performed using Big Dye Terminator v.3.1. The sequencing analysis was carried out using an ABI 3130XL system (Applied Biosystem, Carlsbad, California, USA). The sequencing data for the amplified 16S rRNA genes from each bacterium were deposited in the GenBank database of the NCBI (<http://www.ncbi.nlm.nih.gov>). The sequencing data were inserted into the Advanced BLAST search program to identify the sequences of any closely related organisms. The 16S rRNA sequencing data of the bacteria were also deposited in the DDBJ.

**Phylogenetic Analysis.** The results of DNA sequencing were aligned using ClustalW Multiple Alignment. MEGA 6 software was used for phylogenetic analysis. The phylogenetic trees were determined by the neighbor-joining method with Kimura's two-parameter model. The tree topology was evaluated by bootstrap analysis of the neighbor-joining method based on 1000 resamplings.<sup>54</sup>

**Pigment Extraction.** The collected cells were homogenized in a mixture of methanol and acetone (7:3, v/v, 1 mL of mixture per 0.1 g of cells) by vortexing five times (1 min of vortexing, 1 min on ice) and then disrupted by sonication in pulse mode with 60% amplitude and 10 s on/30 s off for 10 min (QSonica, Newtown, Connecticut, USA). A few grains of CaCO<sub>3</sub> and sodium ascorbate were added to the mixture to prevent pigment degradation via oxidation. The pigment extract was separated from the cell debris by centrifugation at 19,230g for 5 min at 4 °C (Kubota 6500 centrifuge, Tokyo, Japan). The resulting extract was collected and dried using a rotary evaporator at 100 rpm and 35 °C in a water bath (Heidolph Laborota 4010 digital, Schwabach, Germany). The dried pigment extract was stored at −30 °C until use.

**Spectrophotometric Analysis.** The UV–Vis absorption spectra of crude pigment extracts were recorded by a UV–Vis 1700 spectrophotometer (Shimadzu, Kyoto, Japan). Methanol was used for background subtraction in all measurements. The dried pigment extract was diluted in methanol and measured at a wavelength ( $\lambda$ ) of 200–1100 nm. Subsequently, the obtained data were analyzed using OriginPro 8.5.1 software (OriginLab, Northampton, Massachusetts, USA).

**HPLC Analysis and Purification of Prodiginines and Their Derivatives.** The pigments were separated and purified by preparative HPLC (Shimadzu preparative UFLC, Kyoto, Japan) and analyzed by analytical HPLC (Shimadzu analytical-UFLC, Kyoto, Japan) using a Symmetry C<sub>8</sub> column (150  $\times$  4.6 mm, 3.5  $\mu$ m particle size, 100 Å pore size) (Waters, Milford, MA, USA). The HPLC-grade solvents (MERCK, Darmstadt, Germany) were degassed for 5 min with ultrasonication prior to use. The mobile phases used consisted of two solvents. Solvent A was methanol (MeOH), and solvent B was 1% formic acid in water (H<sub>2</sub>O). The elution gradient was 70% A for min 0–2, 85% A for min 2–4, and 100% A for or min 4–10. The flow rate was 1 mL/min with a column oven temperature of 29 °C. The pigments were detected with a diode array detector (Shimadzu SPD M20A, 190–800 nm) at  $\lambda$  530 nm. The prodigiosin pigment standard (from *Serratia marcescens*, >98% purity, CAS number: 56144-17-3) was obtained from Sigma-Aldrich (MERCK).

**MS/MS Analysis.** The highly purified compounds (>97% purity) were analyzed by ESI-MS with a triple quadrupole mass spectrometer (LCMS-8030, Shimadzu). Determination of the pigments was based on their MS data and spectral properties. The simple MS method was used with isocratic HPLC elution by 0.1% formic acid in a mixture of methanol (90%) and water (10%) for 2 min at a flow rate of 0.3 mL/min without a column. The DL temperature was 200 °C, the nebulizing gas flow rate was 3 L/min, the heat block temperature was 350 °C, the drying gas flow rate was 15 L/min, the column oven temperature was 30 °C, and the cooler temperature was 5 °C. Prodiginine was detected at a wavelength ( $\lambda$ ) of 530 nm. The initial identification of precursor ions was conducted by using a Q1Q3 scan in positive and negative modes at  $m/z$  50 to 600 using an event time of 0.1 s and a scan speed of 6000 u/s. The precursor ions were then fragmented in product ion scan mode by using various CEs. Pigment identification was based on the molecular mass of the precursor from the Q1Q3 scan and the product ions and on the product ions optimized by the MRM method. Identification of the pure compounds was performed according to the precursor ion, product ions, and SIM data. The chemical structures of the identified compounds were



drawn using ChemDraw software version 12.0.2 (PerkinElmer, Inc., Massachusetts, USA).

**Pigment Identification.** The pigments were identified by HPLC and MS/MS analyses based on the chromatographic, that is, retention time ( $t_R$ ); spectrophotometric, that is, spectral shape and maximal absorption wavelength ( $\lambda_{max}$ ); and mass, that is, precursor and fragment ions, properties compared to those of reference compounds.<sup>21,23,25,35,36,50</sup> The prodigiosin in *P. rubra* PS1 and SB14 was compared to a prodigiosin standard (Sigma-Aldrich, MERCK). The data consisted of a full Q1 scan and product ion scans at the optimized CE of the prodigiosin standard in the LabSolution MS Library (Shimadzu). The prodigiosin from *P. rubra* PS1 and SB14 was isolated and purified to >97% purity. Then, the isolated prodigiosin was identified by comparison of the recorded chromatographic and spectral data with the data on the prodigiosin standard stored in the library using LabSolution LCMS version 5.4 (Shimadzu). The software compared retention times and aligned MS/MS data to calculate a match factor and produced a degree of similarity between spectra. Cycloprodigiosin, 2-methyl-3-propyl prodiginine, 2-methyl-3-butyl prodiginine, 2-methyl-3-hexyl-prodiginine, and 2-methyl-3-heptyl-prodiginine were analyzed on the basis of MS and fragmentation data.

**Antimicrobial Activity.** The antimicrobial activity was determined by the disk-diffusion technique according to the Kirby-Bauer disk diffusion susceptibility test protocol.<sup>55</sup> Prodigiosin, cycloprodigiosin, and 2-methyl-3-hexyl prodiginine were used as antimicrobial agents against three pathogenic bacteria: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Salmonella typhi*, from clinical isolation and one pathogenic yeast, *Candida albicans* ATCC 10231. Briefly, 90 mm dishes were filled with 15 mL of Mueller-Hinton agar (MHA) to a depth of 4 mm for bacteria and Sabouraud dextrose agar (SDA) for yeast. The pathogenic microorganisms were swabbed on the dishes following the McFarland turbidity standard (0.5) using sterile cotton swabs. Blank disks were placed on the surface of the medium, and each antimicrobial agent (20  $\mu\text{g/mL}$ ) was injected into the disks. DMSO was used as a negative control. Ten micrograms of amoxicillin, ampicillin, and chloramphenicol were applied as positive controls. Dishes were inverted and incubated at 37 °C for 24 h. The inhibition zone surrounding disks was measured, as was the disk diameter. The prodigiosin concentration was determined by absorption in acidified ethanol (4% v/v of 1 N HCl) with a molar extinction coefficient  $\epsilon_{335}$  of  $139,800 \pm 5100 \text{ M}^{-1} \text{ cm}^{-1}$ .<sup>56,57</sup>

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.9b04322>.

*P. rubra* strains that have been deposited in the NCBI (Table S1); the distribution map from 45 strains of *P. rubra* that have been found until now (Figure S1); the number of isolated *P. rubra* found from different sources (Figure S2); the plot of log  $k'$  value of 2-methyl-3-propyl-prodiginine ( $k' = -0.605$ ), 2-methyl-3-butyl-prodiginine ( $k' = -0.339$ ), prodigiosin ( $k' = -0.048$ ), 2-methyl-3-hexyl-prodiginine ( $k' = 0.099$ ), and 2-methyl-3-heptyl-prodiginine ( $k' = 0.249$ ) versus the number of the carbon atoms of the alkyl side chains of

the pigment molecule showed a linear line with  $R^2 = 0.9755$  (Figure S3); the comparison mass spectra of standard prodigiosin hydrochloride and purified pigment peak #4 from *P. rubra* strain PS1 (Figure S4); ESI-MS/MS analysis of the purified compounds (Figure S5); and prodigiosin-producing marine bacteria (Table S2) (PDF)

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### Notes

The authors declare no competing financial interest.

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