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# Mutation of Bacterium *Vibrio gazogenes* for Selective Preparation of Colorants

**Farzaneh Alihosseini**,
Div. of Textile and Clothing, University of California, Davis, CA 95616

**Jozsef Lango**,
Dept. of Molecular Biosciences, University of California, Davis, CA 95616

**Kou-San Ju**,
Dept. of Microbiology, University of California, Davis, CA 95616

**Bruce D. Hammock**, and
Dept. of Entomology, University of California, Davis, CA 95616

**Gang Sun**
Div. of Textile and Clothing, University of California, Davis, CA 95616

## Abstract

A novel marine bacterium strain effectively produced prodiginine type pigments. These colorants could dye wool, silk and synthetic fabrics such as polyester and polyacrylic and also show antibacterial properties against *Escherichia coli* and *Staphylococcus aureus* bacteria on the dyed products. Methyl nitrosoguanidine was used as a mutation agent to increase the genetic diversity and the production yield of the bacteria of the family of *Vibrio gazogenes*. The analysis of the mutated samples showed that two new main colorants as well as three previously found ones were produced. Liquid chromatography electro spray ionization mass spectrometry (LC-ESI-MS) and nuclear magnetic resonance (NMR) spectroscopic techniques were used to elucidate the structures of the newly produced colorants. Mass measurements revealed that the colorants C1, C2, C3, C4 have molecular masses of 321, 323, 351, and 295 Da. One unstable colorant C5 with molecular mass of 309 Da was detected as well. The mutated bacteria strains increased the yield of pigment production by about 81% and produced prodigiosin in 97% purity. The antibiotic activities of pure colorants are discussed as well. Based on their bio-activity and excellent dyeing capabilities, these colorants could be employed in cosmetic and textile industries.

### Keywords

antibacterial pigment; prodigiosin; textiles; mass spectrometry; natural dye

## Introduction

Prodiginines are the secondary metabolites produced by different bacteria genera including *Serratia marcescens, Pescudomonas magneslorubra, Vibrio marcescens, Vibrio gazogenes, and Streptomyces coelicolor*.[1–5] These groups of natural compounds belong to a family of



Correspondence concerning this article should be addressed to G. Sun at gysun@ucdavis.edu.

pyrrole red pigments. They have a common 4-methoxy, 2–2 bipyrrole ring systems, and their variation is based on different alkyl side chains on their C-pyrrole ring (Figure 1).

The alkyl side chains are different in length and their positions on the C-ring, and also they can represent cyclic and acyclic variations.[1] The main pigment component of this group is prodigiosin with a —$C_5H_{11}$ side chain. Cycloprodigiosin and metacycloprodigiosin are examples of cyclic forms.[6–10] Prodigiosin has been reported having unique antibiotic properties, such as strong bactericidal activity against gram positive bacteria, protozoa, and pathogenic fungi. In the past decade they were found to have promising immuno-suppressive activities at nontoxic doses and have also shown potent apoptotic affects on human cancer cell and therefore, are considered as a valuable area of further research.[11–13]

Searching for new renewable, environmental friendly dyes led us to look for fungi and bacteria as new sources. We have previously reported that a new isolated marine bacterium close to family of *Vibrio gazogenes* was capable of producing prodigiosin and cycloprodigiosin. The pigments had ability to dye wool, nylon, acrylic and polyester and also showed antibacterial activities. The thus dyed fabrics demonstrated strong antimicrobial ability against both gram positive bacterium *S. aureus* and gram negative bacterium *E. coli*.[1] The biocidal activities of prodigiosins have been already identified *in vitro* conditions; however, there was no report of showing the same properties on solid substrates such as fabrics. These colorants with strong dyeing and antibacterial activity could have broad applications in textile and cosmetic industries. Thus, it seems necessary to look for prodiginine derivatives with different colors.

It has been reported that different bacteria could produce various pigmented analogs of prodigiosin.[7] The total amount of these pigments and their relative ratio is a function of the type of bacteria, growth media, pH, and temperature. It is often very difficult to purify them due to their very similar chemical and physical properties. Considering the industrial outlook, it is necessary to find bacteria strains that can produce higher yields of relatively pure pigments. The aim of this work was to screen genetically diverse bacteria to produce new pigments and specifically target the bacteria for individual pigment production with enhanced yields. Generating bacterial strains which are able to produce a pure pigment in high yield would be of great importance because it can reduce the difficulty, time, and energy necessary in purification processes. As a mutating agent, 1-methyl-3-nitro-l-nitrosoguanidine was employed in this study.

## Material and Methods

### Chemical mutagenesis of KSJ45

Wild type bacteria KSJ45 was grown in 3 mL seawater (SW) rich media overnight at 28°. Cells were harvested by centrifugation, then resuspended in 3mL of half-strength of SW-rich media, and divided into two 1.5 mL samples. One crystal of 1-methyl-3-nitro-l-nitrosoguanidine (~1 mg) was added to one sample of resuspended cells. After incubation at room temperature for 2 hours, cells from each sample were harvested by centrifugation and washed three times with SW-base. 50 μL of serial dilutions of sample were plated onto SW-rich media agar, and the plates were incubated at 28°C for 4 days. Different mutated strains were named as M1, M2 and so on. Based on the colony color of the resulting strains, 14 of them were selected, and their pigment products were analyzed.

### Preparation of prodiginine mutants

Mutants of KSJ45 were grown in 50 mL of SW-rich media in 250 mL Erlenmeyer flasks at 28°C, 200 RPM. The growth of the bacteria was measured using spectrometry at visible light ($\lambda_{max}$ of 660 nm). When cultures reached $OD_{660}$: 1.5, cells were harvested by centrifugation.

The pigments were extracted from the cells twice with 50 mL of methanol using a rotary shaker, at room temperature for 4 hours, in the dark. Extracts were combined and stored in the dark at −20°C until chemical analysis.

### Purification of prodiginines

The crude methanol-extracts were filtered (Whatman, GF/A, 15 cm, England) to remove any residual biomass and then concentrated by using a rotary evaporator (Type R-114, Buchi Rotavapor, Germany). The extraction was followed by a chloroform–water liquid–liquid extraction to remove hydrophilic impurities. The organic phase, containing the prodiginines, was concentrated again by using a rotary evaporator. The dried pigments were reconstituted in methanol. The final step of purification was achieved by HPLC using Phenomenex Luna C-18[2] semipreparative column (250 mm × 10 mm, 5 μ) (Phenomenex, Torrance, CA). The separation was performed by using water (A) and acetonitrile/methanol (1:1) (B) mobile phases, and a gradient elution program at 3 mL/min with the following parameters: 0–25 min 15–100% B (linear gradient), 25–35 min 100% B, and 35–40 min 15% B to re-equilibrating the column. Fractions containing targeted compounds were combined and concentrated by solvent evaporation.

### Identification of prodiginines—structure analytical methods and technology

Nuclear magnetic resonance (NMR), liquid-chromatography mass spectrometry (LC-MS), and Fourier transform mass spectrometry (FT-MS) structure elucidation methods were applied to characterize and identify the purified compounds. The instrumentation and analytical methods used are described in details in our previous report.[1]

### Inhibition zone assay

Strains of *Escherichia coli (E. coli*, k-12) or *Staphylococcus aureus* (*S. aureus*, ATCC 12600) were grown in 10 mL nutrient broth (approximate formula per liter: beef extract, 3.0 g; peptone, 5.0 g; distilled water, 1000 mL; final pH 6.8 ± 0.2) at $10^5$–$10^6$ CFU/mL overnight as an indicator culture. Serial dilutions of the indicator cultures using TBS (Tris-buffered saline) from $10^{-1}$ to $10^{-6}$ were prepared, and 50 μL of $10^{-1}$ to $10^{-6}$ dilutions of each separated compound was spread onto an agar plate. An aliquot (5 μL) of the above dilution was spotted onto a sterile 0.5 cm filter paper disk. Methanol (5 μL) on a filter disk was used as a control. The solvent was completely evaporated off the disk. Three disks including one control and two compounds were placed onto the LB agar plates spread with the indicator cultures. Plates were then incubated at the 37°C for 24 hours, and zones of inhibition were measured after 24 hours (the length of distance away from the filter disk where there is no growth). 50 μL of $10^{-1}$ to $10^{-6}$ dilutions of original bacteria were paced onto a separate set of agar plates and incubated overnight. The number of the colonies per plate for each dilution was counted to determine the concentration of original overnight bacteria (CFU/mL).[14]

### Growth inhibition assay

The test compound (5 μL in methanol) was placed into five empty and sterile test tubes. 5 μL of the solvent alone (no test compound) was placed into a second set of five tubes as a control. Solvent was evaporated completely out the test tubes. One milliliter of the $10^{-3}$ to $10^{-7}$ dilutions of *E. coli* (K-12) or *S. aureus* (ATCC 12600) was added into each set of test tubes. Fifty microliter from each tube was placed onto a separate agar plate. After 1 hour another 50 μL from each test tube was placed onto a new agar plate. All plates were incubated at 37°C for 24 hours. The numbers of colonies were counted from all plates and were compared to determine the inhibition of bacterial growth of the samples, based on the following equation:

$$\% \text{ inhibition} = \text{CFU/mL with compound} / \text{CFU/mL without compound (control)}.$$

## Results and Discussion

Subsequent to mutation, 14 colonies with different colors were selected and grown on the SW-rich media separately, and named as M1, M2 (Mutant 1, Mutant 2) and so on [Picture 1].

The prodigiosin pigments are produced in the last stage of bacterial growth. It is not well understood why they are produced by the specific bacteria, however, it has been suggested15 that they are produced to consume the toxic intermediates which have been produced by bacteria in early growth stages and have not been used. It is assumed that about 10 enzymes participate in different steps to incorporate these intermediates and produce the pigments.[15] The final step is the enzymatic coupling of rings A and B, methoxybi-pyrrolecarboxaldehyde (MBC) with ring C, MP (methylpen-tylpyrrole), so any interruption or changes in these steps can affect the pigment production by the bacteria.

Some mutants were colorless, indicating that the pigment production was blocked probably in the initial or intermediate stages of the process. Lack of pigment production in some mutants could be due to deficiency of the crucial enzymes.[15,16] Mutants with different colors indicate that they either contain different pigments or different ratios of pigments. In addition, some extracts of mutants had deeper color, possibly due to the higher yield of pigment production while the others had very low amount of colorants. The results in Table 1 illustrate that 5 main pigments and some minor pigments have been found. The colorants with molecular masses of 321 Da (C1), 323 Da (C2), and 351 Da (C3) were identical to the ones produced by the un-mutated bacteria, where prodigiosin was identified as the main colorant and two other minor prodiginine analogs of C1 and C3 were confirmed as well.[1] The mutated samples produced two new pigments with molecular mass of 309 Da (C5) and 295 Da (C4), and their structures were elucidated with the above specified analytical techniques. The initial LC-MS study proved that M62 contains all the colorants in the highest yield, so it was used for separation of constituents and their structural elucidation study.

### Structural identification of the new pigments (C4, C5)

The UV-vis spectrum of the newly produced component C4, which has an LC retention time of 31.1 min, revealed an absorption maximum at 533 nm in methanol solution. This component showed an accurate molecular ion $(M + H)^+$ mass of *m/z* 296.1771 Th, which supports the structure of a neutral molecule with elementary composition of $C_{18}H_{21}N_3O$ ($\Delta m$ = 0.8 mDa). This compound had a similar fragmentation pattern (Figure 2) compared to the prodigiosin with a fragment ion of *m/z* 252 Th, which was related to the loss of an alkyl side chain — $C_3H_7$), and a fragment ion of *m/z* 264 Th for the loss of a methyl group.[1] To support the structure of compound C4, $^1$H NMR and $^{13}$C NMR analyzes along with H-COSY and HMQC were carried out. The $^1$H and $^{13}$C NMR chemical shifts and signal assignments (Table 2) were in agreement with the chemical shifts of prodigiosin, except for absence of two methyl group in the side chain. The structure has been found identical to prodigiosin with a shorter aliphatic side chain.

Signals in the very low field in $^1$H NMR spectrum (~12.5ppm, two signals) showed the presence of N—H protons of the pyrrole rings (Table 2). COSY NMR data clearly indicated that the NH1 proton interacted with the protons on ring A (C2, C3, C4), while the NH6 group had correlation with both protons on C8 and C14 which is an evidence for alternating protons between N group of ring B and C.

The second new pigment (C5) was found in mutated samples with an LC retention time of 42.0 min and UV-vis absorption maximum at 500 nm (Figure 3a). ESI-MS/MS technique was used to characterize the pigment. This C5 component showed a molecular ion mass $[M + H]^+$ of *m/z* 310 Th (Figure 3b). These data are similar to the well known norprodigiosin mass spectral behavior.[17] Due to the instability of the pigment exposed by light, oxygen (air) and/or acidic condition of extraction, it is necessary to develop a unique analytical and separation method to collect the required quantity of unmodified components for NMR characterizations.

## New minor colorants C6 with molecular mass of 309 Da

In sample M62, two products with an identical molecular mass of 309 Da were detected. These components appeared in both negative and positive modes, what is characteristic for the prodigiosin analog compounds. The accurate mass measurements gave identical elementary composition of $C_{19}H_{23}N_3O$ with an ~30 nm UV maximum deviation, and more than 9 minute of LC retention time difference. These analytical data confirmed that these components are constitutional (structural) isomers, components C5 and C6 (Figure 4). C5 with $\lambda_{max}$ of 502 nm was already identified with a structure similar to prodigiosin except having one hydroxyl group instead of methoxy group in the ring B (Figure 3). The other structure gave the same fragmentation pattern similar to prodigiosin with a molecular mass with 14 Da lower than prodigiosin and maximum absorbance at 526 nm. The structure was identified similar to prodigiosin with shorter side chain (Figure 5c).

## Colorant C1 (cycloprodigiosin)

The structures of minor colorants were previously predicted[1] based on mass fragmentation analysis, but the amounts of the isolated colorants were not sufficient for confirmatory NMR studies. The mutated bacteria produced larger amounts of these colorants, thus, we were able to separate and collect sufficient amounts of pure constituents for NMR analysis and complete the structure elucidation of colorants with molecular masses of 321 Da (C1) and 351 Da (C3). The $^1H$ and $^{13}C$ NMR data for these components are shown in Table 2. The $^1H$ aromatic chemical shifts for cycloprodigiosin are almost identical to that of prodigiosin in the rings A and B. Absence of H14 in ring C is related to the formation of ring between positions 14 and 15 in cycloprodigiosin. $^{13}C$ NMR spectra showed 20 carbons, which were recognized by DEPT 90 and DEPT 135 as two —$CH_3$, one —$OCH_3$, 5 aromatic CH, 3 aliphatic $CH_2$ and one aliphatic CH and 8 quaternary C atoms. HMQC data confirmed that one $CH_3$ group is in correlation with C16 in ring C. Instead of triplet —$CH_3$ group for the terminal group in the side chain of prodigiosin, this compound showed a doublet in position 22 [δ 1.28 (d, 3H)] which affirms that they have vicinal position to a CH group rather than $CH_2$ in prodigiosin. The COSY NMR spectra showed correlation between this —$CH_3$ with singlet proton on C21 and proved that there is a —$CH_3$ group on the aliphatic ring.

The $^1H$ NMR spectrum proved that the two protons on C18 are not equivalent (they have different chemical shifts) while they were assigned to the same carbon based on HSQC NMR spectra (Figure 6b). $^1H$, H COSY revealed the coupling between both protons (Figure 6a). The same result was achieved for protons on C20 (non equivalent geminal protons), but protons on C19 had no difference in their chemical shifts. These data support that the aliphatic ring is not completely planar thus two conformers exist providing different chemical environment for protons on C18 and C20 which can explain the different chemical shifts of the geminal protons. Representations of the conformers of cycloprodigiosin (Figure 6c) are given by Spartan molecular modeling program (Wavefunction, Irvine, CA).

## Colorant C3

NMR study of a colorant with molecular mass of 351 Da showed that the proton chemical shifts are almost identical to the prodigiosin, with the difference of two extra methylene groups

in the aliphatic area [δ1.29 (m, 2H), and δ 1.25 (m, 2H)]. The results of the ESI MS/MS experiments in product mode showed a very similar decomposition pattern to the fragmentation of prodigiosin. EI-MS fragmentation of prodigiosin (C2) and component C3 showed that both compounds have the same core structure with a fragment ion mass of *m/z* 266 Th, and the difference is the length of the side chains (Figures 5a,b). EI GC/MS data demonstrated that the colorant C3 has two isomers with retention times of 63.9 and 64.7 min, with identical EI mass spectra.

### New minor colorant C7 with molecular mass of 337 Da

Besides the five main pigments, a component C7 was detected at a very low level. It had a maximum absorption at λ: 535 and eluted in 37.6 min retention time, which is between the elution time of prodigiosin (35.3 min) and C3 (39.7 min). Mass spectral analysis showed a parent ion $[M + H]^+$ of *m/z* 338 Th, and the accurate mass measurement supports an elemental composition of $C_{21}H_{27}N_3O$, ($\Delta m$ = 3.2 mDa), which is 14 Da higher than the molecular mass of the prodigiosin. The MS/MS spectra showed a deduction of 15 Da related to a methyl lost, a finger print for prodiginine analogs. The fragment ion of *m/z* 252 Th revealed that the core structure is exactly the same as prodigiosin, indicating the presence of a new prodiginine with one methyl group more than prodigiosin on the side chain. Complete structural elucidation needs further NMR study which was not applicable to this sample due to low abundance of component.

### Oxidized components

The results in Table 1 also show that components $C4_{ox}$, $C1_{ox}$, $C2_{ox}$, $C3_{ox}$, $C5_{ox}$ with accurate molecular masses of 311.1656, 337.1775, 339.1953, 367.2240, and 325.1746 Da have elemental compositions of $C_{18}H_{21}N_3O_2$, $C_{20}H_{23}N_3O$, $C_{20}H_{25}N_3O$, $C_{22}H_{29}N_3O$, $C_{19}H_{23}N_3O_2$, respectively. Thus, they contain one more oxygen (16 Da) than the related colorant components, C4 (295 Da), C1 (321 Da), C2 (323 Da), C3 (351 Da), and C5 (309 Da). An addition of oxygen atom implies that the given components have been oxidized. Some studies reported photo oxidation on prodigiosin analog compounds,[18] and it was assumed that photo-oxidation of prodigiosin could lead to an additional OH group on the adequate pyrrole rings. Thus, it seems that all of the colorants in extracts of mutant strains have been fully or partially oxidized. These strains clearly contained more oxidation products than the original strain; however, in this phase of the study the mechanism of this process cannot be confirmed.

### Pigment profile of different mutants

In addition of producing new colorants, selected mutant pigments resulted in different yields and the ratio of produced structures. The yield was calculated based on UV absorption at 534 nm (ε = 70,000) which is applicable for most of prodiginine analogs.[19] The results in Table 3 show that the strain M62 has produced the highest amount of colorants, which was 81% more than those produced by the wild type bacterium.

To estimate the accuracy of the yield measurements it is necessary to consider the following possible sources of errors; (a) to extract all the pigments it is necessary to acidify the extraction solution to increase the efficiency of the hydrophobic pigments and move them into organic phase, but it might destroy or change the structure of unstable components; (b) all the measurements were done at $\lambda_{max}$ 534 nm, (which is the average maximum for the most of the pigments) while the maximum absorption of colorant C5 is 500 nm.[17,20] Considering the above mentioned sources of inaccuracy in pigment percentage and yield measurement, the data should be considered only as good estimates.

Estimated quantitative results for pigment ratios in different mutants were measured using LC-UV-vis-MS analytical method (Table 3). The most important findings were related to mutants

M58 and M39. As shown in Table 2, the mutant M58 was able to produce about 97% of prodigiosin as the main constituent. This is the only strain that did not produce any cyclic form of prodigiosin with molecular mass of 321 Da (C1). In the extract 3.5% of colorant C5 with a molecular mass of 309 Da was also detected. This result is important as it shows that the mutation has the ability to produce pure compounds and potentially eliminate the time-consuming and expensive purification processes. The M39 shows different results from other mutants, which produced Cycloprodigiosin (C1) with 57%, as the main pigment, followed by 41% of prodigiosin and 2% of C5. This is the only mutant where the production of prodigiosin was suppressed.

Evaluating the pigment production of mutants M32, M48 and M62 revealed that these samples produced the highest amount of colorant C5 among the mutants, while two mutants, M29 and M54 produced small traces of C5 (Table 3). These two extracts had a bright pink color while the other extracts with more C5 had deep orange color. Two remaining pigments C4, C3 with molecular masses of 295 Da and 351 Da were found in the smallest amount in all the mutants. The maximum percentage of colorant C3 (molecular mass of 351 Da) was 5%, observed in M51. There have been several previous efforts to generate diverse bacterial strains and identify sites of the blockaid of biosynthesis of prodigiosin using ultraviolet radiation and cross feeding techniques.[15,21,22]

### Antibacterial properties of different mutants

Inhibition zone test was carried out to determine whether prodiginine analogs have an inhibitory effect on growth *in vitro* of gram positive and gram negative bacteria. Prodiginines were not effective against *E. coli*, but they showed inhibition zones against *S. aureus*. The difference in antimicrobial efficacy among prodiginines was not significant; however component C4 (molecular mass of 295 Da) was more effective than others. The order of effectiveness was: C4 (4.5 mm) > C2 (4 mm) > C3 (3 mm) revealing that compounds with shorter side chains had higher activity. Low polarity of compounds with longer side chain probably prevents their movement into the media and decreases the inhibition zone size. Cycloprodigiosin (C1), a cyclic form pigment, showed the lowest effectiveness against *S. aureus*(2 mm). To optimize the effective concentrations, three dilutions of prodigiosin (C2) were tested. By increasing the concentration from 0.15 μM to 3 μM, the inhibition zone was significantly increased from 2 to 7mm.

To further study the effect of pigments on the viability of bacteria, the growth inhibition test was applied. A significant dose dependent decrease in the number of vial cells for prodigiosin (C2) was observed with an $IC_{50}$ of 0.15 μM (Table 4). The dose of 1.5 μM of all pigments was able to inhibit growth of bacteria by more than 90% after 12 hrs. Cycloprodigiosin (C1) was the most effective compound against *S. aureus* in bacterial growth tests while it did not show significant efficacy in the inhibition zone test. The results for the other pigments were in agreement with inhibition zone test revealing that the length of side chain has negative effect on antibacterial property of the prodiginines.

## Conclusion

To increase yield and improve selectivity of pigment production as well as to possibly find new pigments, the bacteria KSJ45 was mutated by using 1-methyl-3-nitro-l-nitrosoguanidine. The results have shown that mutated samples produced two new colorants of norprodigiosin (C5) and propylprodigiosin (C4), in addition to three previously found components of prodigiosin (C2), cycloprodigiosin (C1), and heptylprodigiosin (C3). Quantitative analysis by HPLC showed that one of the mutants is able to produce prodigiosin in 97% purity. This result is important in commercial applications simplifying the purification process. The results have shown also that another mutant could possibly produce cycloprodigiosin in a high volume

(56% of total colorants). The yield of pigment production was increased in average by 81% by mutation. All of the colorants found belong to the family of prodigiosin with a core structure of three pyrrole rings, thus, they are expected to have the same antibacterial activity. All colorants showed inhibition zone of growth for gram positive bacteria (*S. aureus*) in 1.5 dose of 1.5 μM of all pigments was able to inhibit growth of bacteria by M concentration. The results confirmed that longer alkyl side chain can reduce the antibacterial properties of colorants in the assays used.

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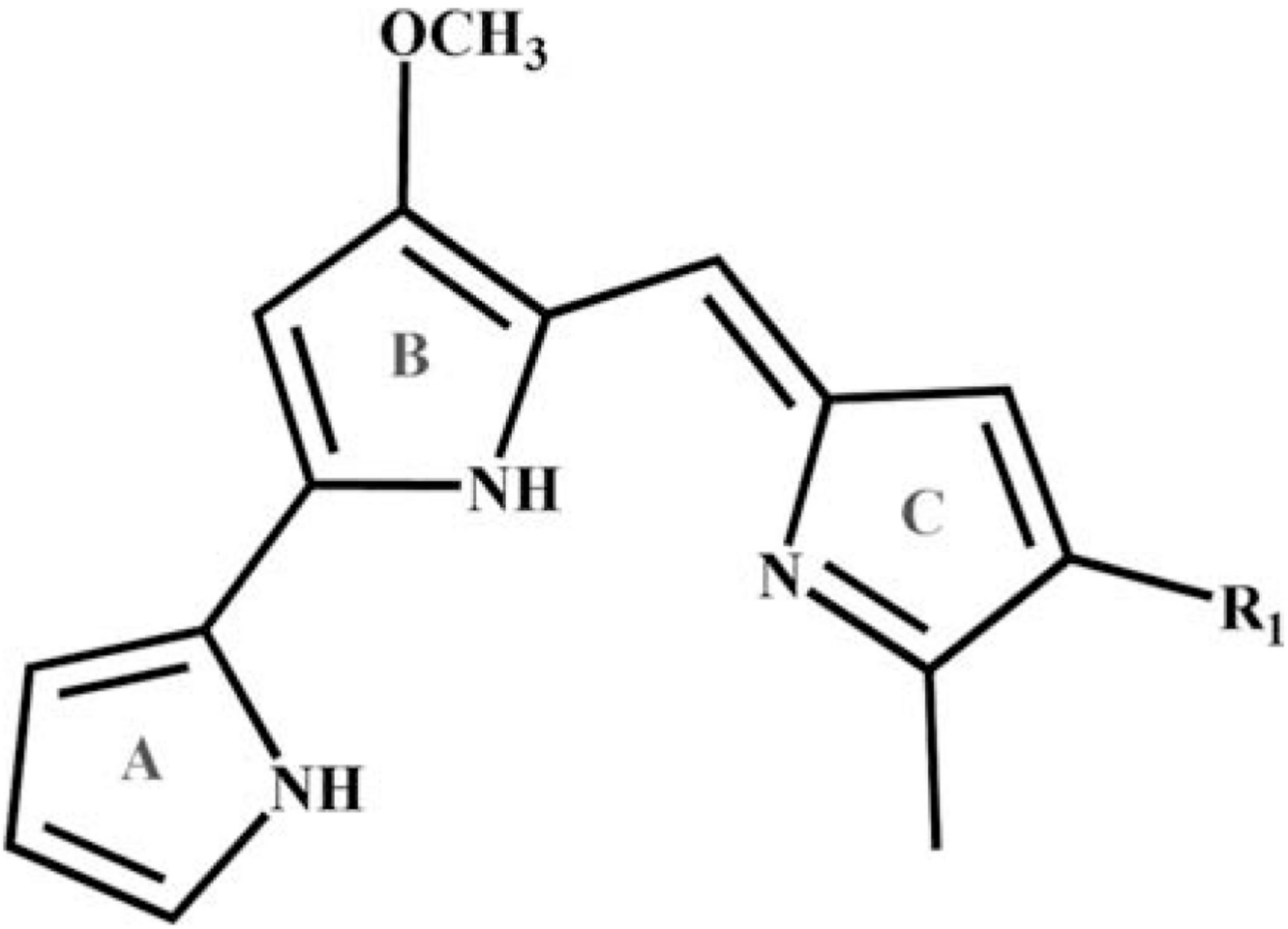


**Figure 1.**
Chemical structure of prodigiosin (where R1 is *n*-pentyl).

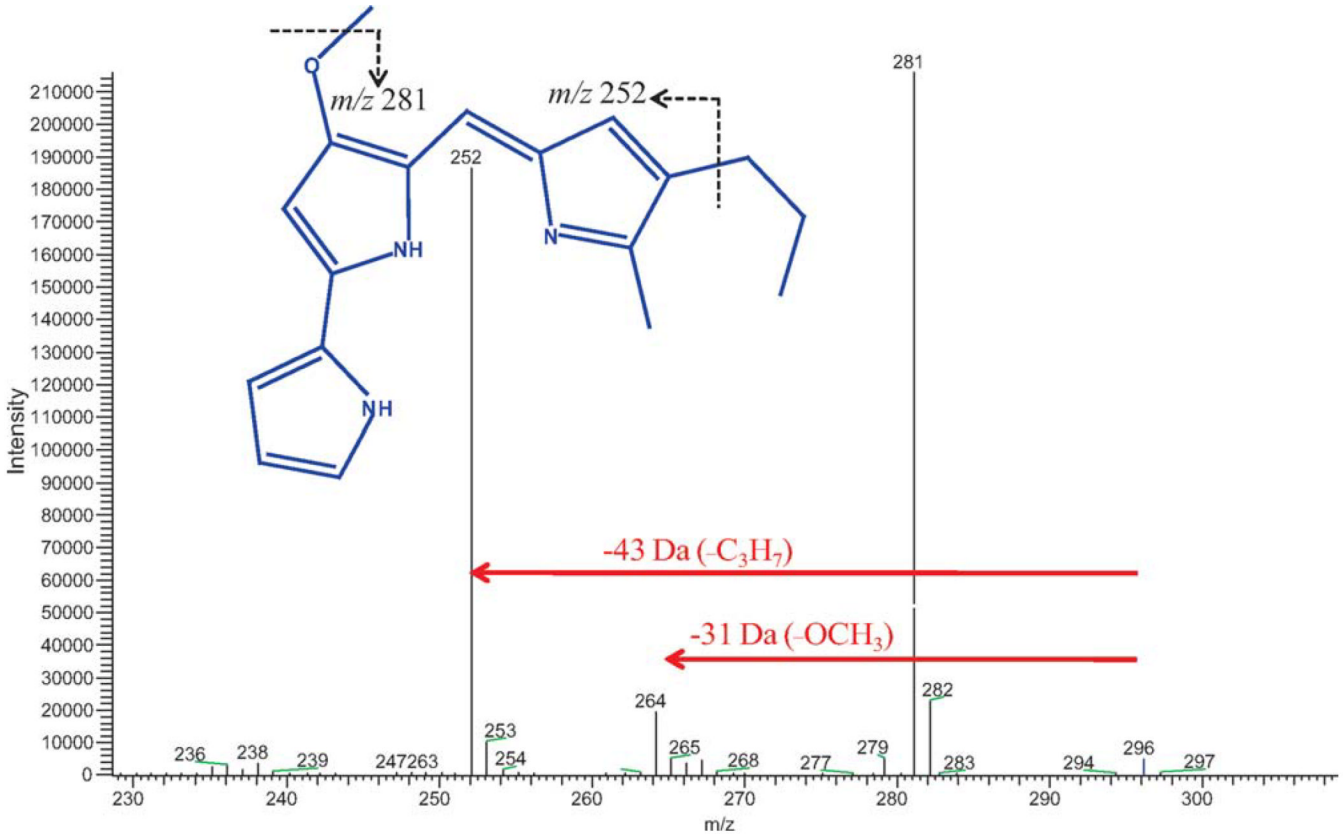


**Figure 2.**
MS/MS spectrum of the molecular ion [$(M + H)^+$ = 296 Th] of component C4 in product mode.

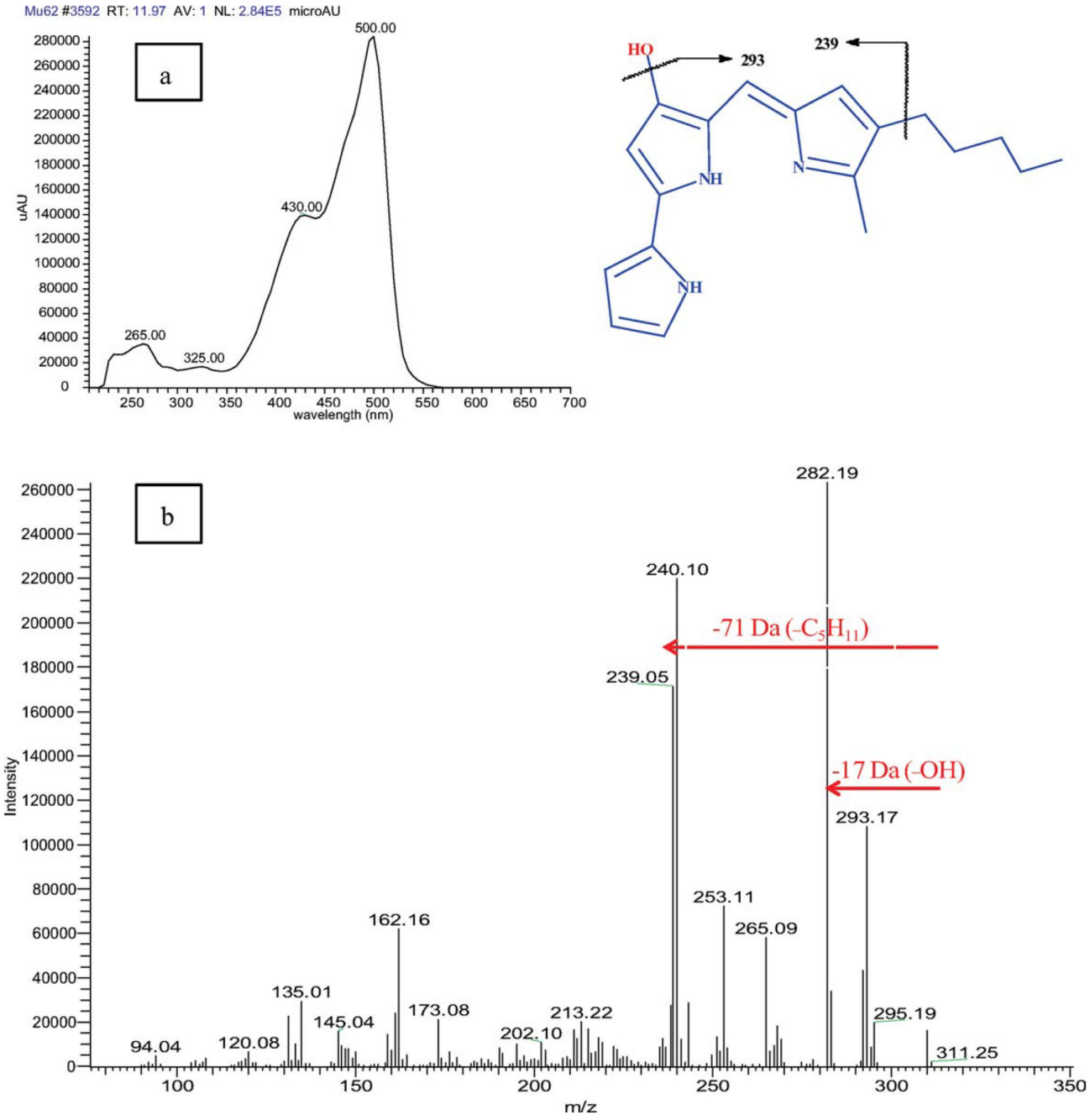


**Figure 3.**
Structure of component C5 from mutated sample M62, (a) UV-vis spectrum, (b) MS/MS spectrum of the molecular ion [$(M + H)^+$ = 310 Th].

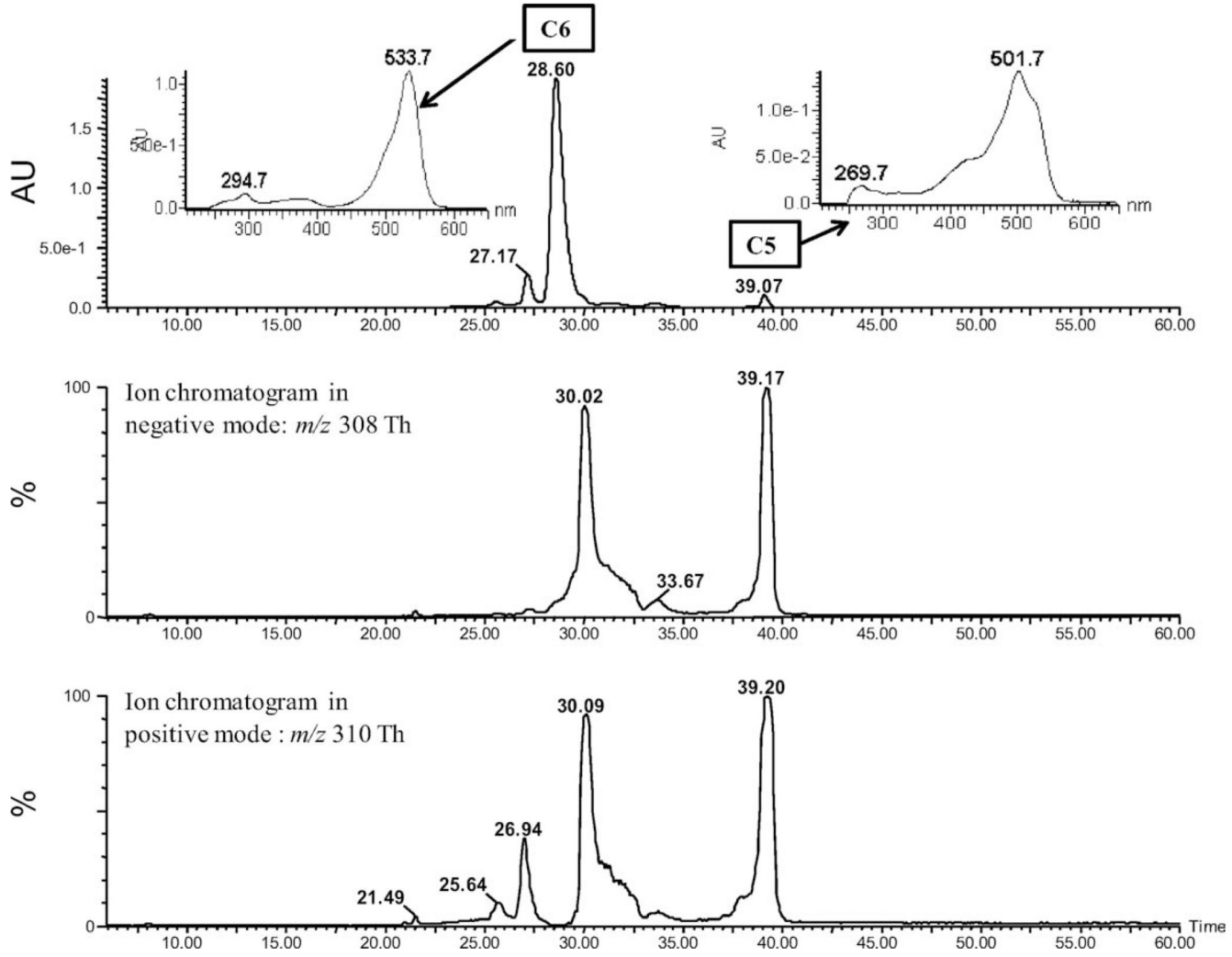


**Figure 4.**
UV-vis trace, UV-Vis spectra and ion chromatograms of compounds C5 (detected at 28.6 min) and C6 (detected at 39.07 min) in negative and positive mode.

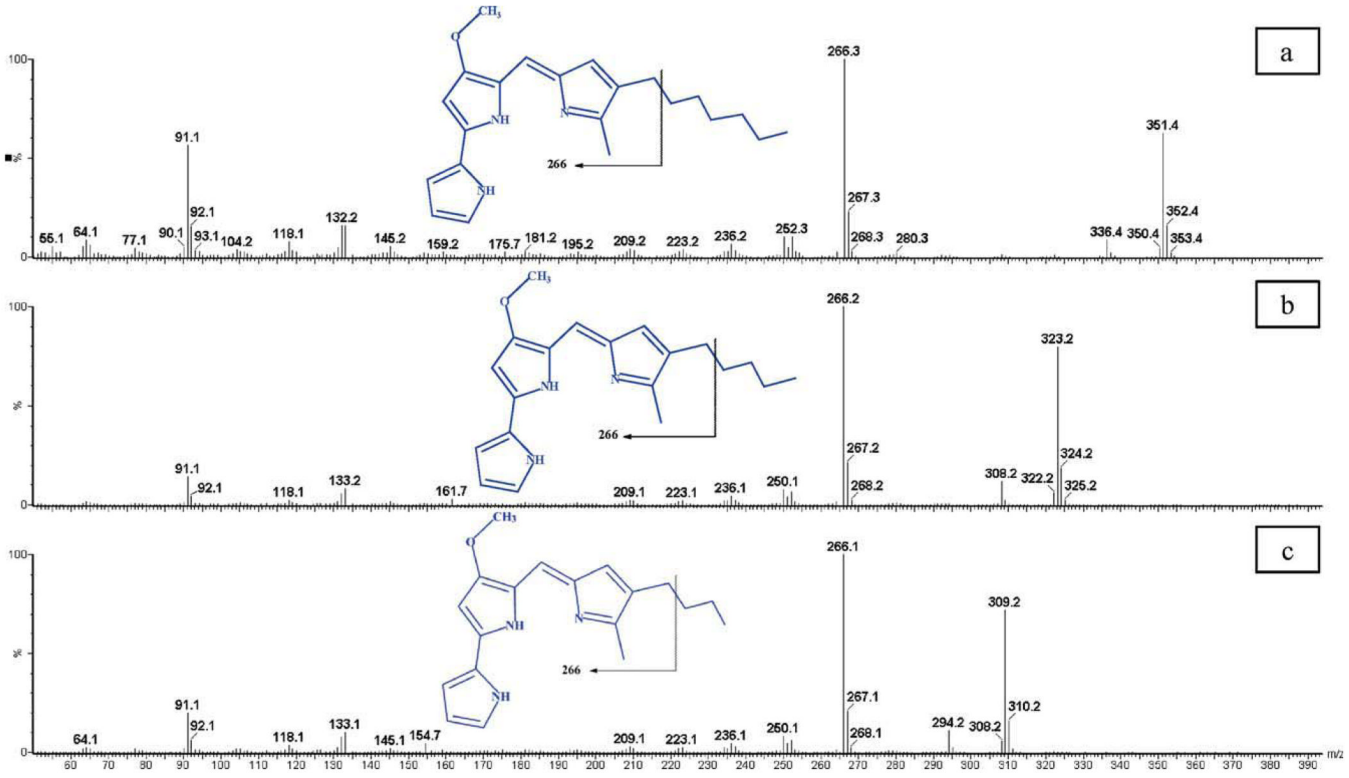


**Figure 5.**
EI-MS spectra of components (a) C3 (351Da), (b) C2 (323 Da), (c) C6 (309 Da).

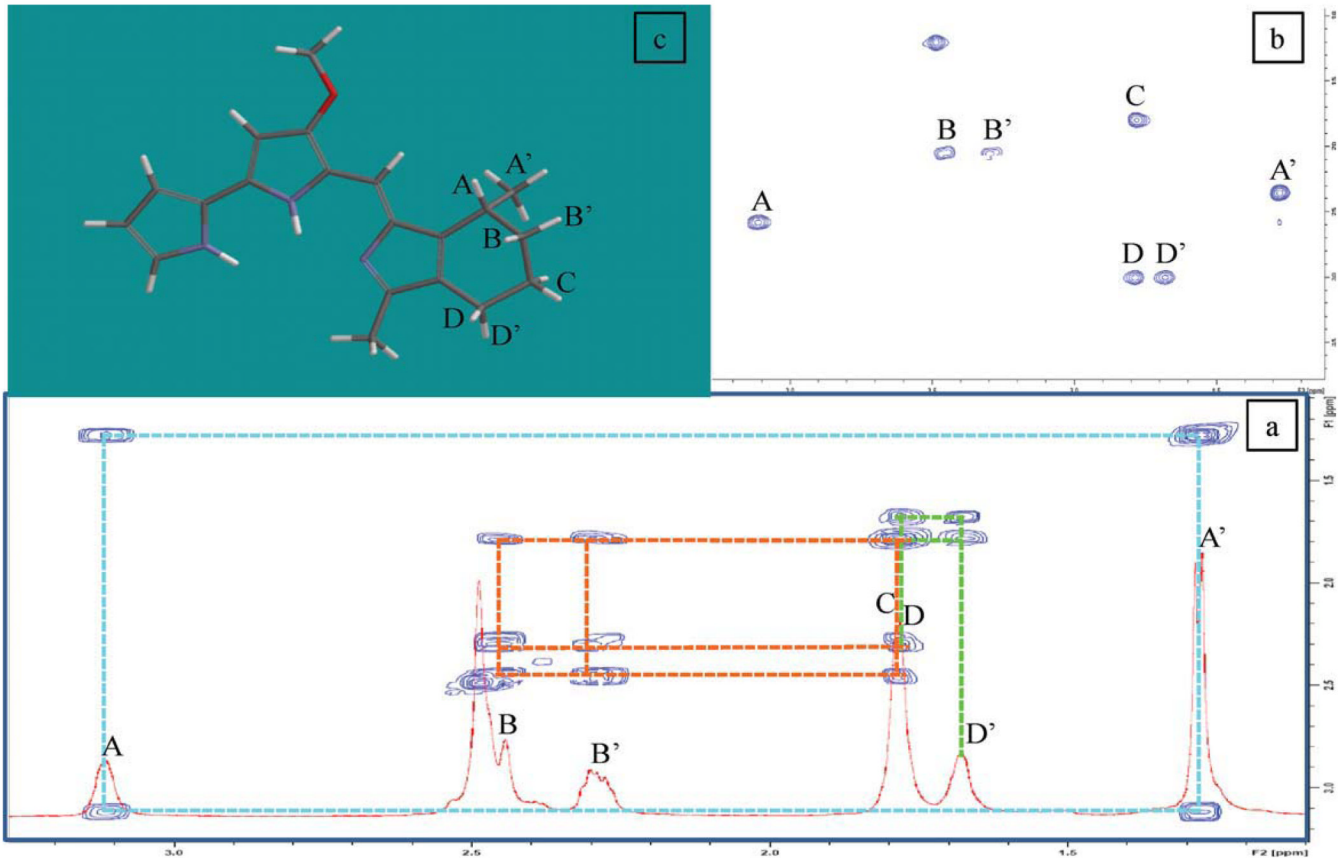


**Figure 6.**
(a) COSY NMR, (b) HSQC spectra, (c) 2D molecular structure of component (C1).

**Picture 1.**
Methanolic extract of different mutants of bacteria KSJ45.

**Table 1**

Properties of Prodiginine Analogue Pigments Produced by Mutated Bacteria

| Component ID | Accurate Mass (Da) | Elementary Composition | Mass Measurement Error (mDa) | LC Retention Time (min) | UV Max (nm) | GC/MS Retention Time (min) | Observed Mass GC/MS (Da) | GC/MS Retention Time of Derivative | Observed Molecular Mass of the Derivative | Derivatization Degree |
|---|---|---|---|---|---|---|---|---|---|---|
| C1 | 321.1833 | $C_{20}H_{23}N_3O$ | 0.8 | 32.9 | 537.7 | 63.0 | 321.2 | 62.6 | 393.3 | 1 |
| C1(ox)* | 337.1775 | $C_{20}H_{23}N_3O_2$ | −1.6 | 33.0 | – | – | – | – | – | – |
| C2 | 323.2096 | $C_{20}H_{25}N_3O$ | −0.7 | 35.3 | 534.7 | 61.7 | 323.2 | 61.6 | 395.2 | 1 |
| | | | | | | | | 62.9 | 467.2 | 2 |
| C2(ox)* | 339.1953 | $C_{20}H_{25}N_3O_2$ | 0.6 | 35.5 | – | – | – | – | – | – |
| C3α | 351.2303 | $C_{22}H_{29}N_3O$ | −0.8 | 39.7 | 534.7 | 63.9 | 351.4 | 64.0 | 423.3 | 1 |
| C3β | | | | | | 64.7 | 351.4 | 64.8 | 423.3 | 1 |
| C3(ox)* | 367.2240 | $C_{22}H_{29}N_3O_2$ | −2.0 | 39.8 | – | – | – | – | – | – |
| C4 | 295.1693 | $C_{18}H_{21}N_3O$ | 0.8 | 29.5 | 533.7 | 57.7 | 295.2 | 58.1 | 367.2 | 1 |
| C4(ox)* | 311.1656 | $C_{18}H_{21}N_3O_2$ | 2.2 | 30.1 | – | – | – | – | – | – |
| C5 | 309.1841 | $C_{19}H_{23}N_3O$ | 1.9 | 30.1 | 532.7 | 59.6 | 309.2 | – | – | – |
| C6 | 309.1841 | $C_{19}H_{23}N_3O$ | 0.5 | 42.0 | 500.7 | – | – | – | – | – |
| C6(ox)* | 325.1746 | $C_{19}H_{23}N_3O_2$ | 4.0 | 42.2 | – | | | | | |
| C7 | 337.2154 | $C_{21}H_{27}N_3O$ | 3.2 | 37.6 | 535.0 | – | – | – | – | – |

*Oxidized forms of prodiginine components.

**Table 2**

$^{1}H$ and $^{13}C$ NMR Data of Prodiginine Pigments of Bacterial Strain M62

| | C1 (321 Da) | | C2 (323 Da) | | C4 (295 Da) | | C3 (351 Da) | |
|---|---|---|---|---|---|---|---|---|
| **Assign** | **($^{1}H$)** | **σ ($^{13}C$)** | **($^{1}H$)** | **σ ($^{13}C$)** | **($^{1}H$)** | **σ ($^{13}C$)** | **($^{1}H$)** | **σ ($^{13}C$)** |
| NH1 | 12.48 (b, 1H) | | 12.44 (b, 1H) | | 12.54 (b, 1H) | | | |
| NH6 | 12.59 (b, 2H) | | 12.54 (b, 2H) | | 12.69 (b, 2H) | | | 165.92 |
| C2 | 7.19 (m, 1H) | 126.01 | 7.23 (m, 1H) | 127.15 | 7.24 (m, 1H) | 127.13 | 7.28 (m, 1H) | 125.65 |
| C3 | 6.33 (m, 1H) | 111.29 | 6.35 (m, 1H) | 111.81 | 6.36 (m, 1H) | 111.83 | 6.36 (m, 1H) | 93.15 |
| C4 | 6.87 (m, 1H) | 115.77 | 6.92 (m, 1H) | 117.24 | 6.93 (m, 1H) | 117.11 | | |
| C5 | – | 122.47 | – | 122.08 | – | 122.2 | | 121.76 |
| C7 | – | 146.88 | – | 147.78 | – | 147.6 | | 207.2 |
| C8 | 6.08 (s, 1H) | 92.51 | 6.08 (s, 1H) | 92.94 | 6.08 (s, 1H) | 92.87 | 6.08 (s, 1H) | 58.65 |
| C9 | – | 164.85 | – | 165.83 | – | 165.74 | | 169.37 |
| C10 | – | 118.99 | – | 120.72 | – | 120.6 | | 115.86 |
| C11 | 3.99 (s, 3H) | 58.56 | 4.01 (s, 3H) | 58.72 | 4.01 (s, 3H) | 58.81 | 4.01 (m, 1H) | 12.36 |
| C12 | 6.99 (s, 1H) | 112.86 | 6.95 (s, 1H) | 115.99 | 6.96 (s, 1H) | 116.16 | 6.93 (s, 2H) | 117.56 |
| C13 | – | 122.9 | – | 125.18 | – | 125.07 | | 122.32 |
| C14 | – | 145.62 | 6.69 (s, 1H) | 128.50 | 6.69 (s, 1H) | 128.62 | 6.66 (m, 1H) | 111.85 |
| C15 | – | 123.69 | – | 128.57 | – | 126.8 | | 127.91 |
| C16 | – | 146.12 | – | 146.86 | – | 147.00 | | |
| C17 | 2.49 (s, 3H) | 12.26 | 2.76 (s, 3H) | 12.41 | 2.54 (s, 3H) | 12.59 | 2.43 (s, 3H) | 25.41 |
| C18 | 1–1.68 (m, 1H) 2–1.78 (m, 1H) | 30.26 | 2.39 (t, 2H) | 25.29 | 2.37 (t, 2H) | 27.46 | 2.39 (t, 2H) | 29.69 |
| C19 | 1.78 (m, 2H) | 18.21 | 1.53 (tt, 2H) | 29.68 | 1.56 (tt, 2H) | 23.41 | 1.53 (m, 2H) | 29.69 |
| C20 | 1–2.29 (m, 1H) 2–2.44 (m, 1H) | 20.72 | 1.30 (m, 2H) | 22.49 | 0.94 (t, 3H) | 13.83 | | 22.71 |
| C21 | 3.11 (s, 1H) | 26.04 | 1.32 (m, 2H) | 29.78 | – | – | 1.31 (m, 2H) | 30.25 |
| C22 | 1.28 (d, 3H) | 23.81 | 0.89 (t, 3H) | 14.01 | – | – | 1.29 (m, 2H) | 14.5 |
| C23 | – | – | – | – | – | – | 1.25 (m, 2H) | |
| C24 | – | – | – | – | – | – | 0.88 (t, 3H) | 12.36 |

**Table 3**

Yield and Percentage of Pigments Produced by 14 Different Bacterial Strains Analyzed by HPLC (Methanol Extracts Without Acidity)

| | C1 (321 Da) | C2 (323 Da) | C3 (351 Da) | C4 (295 Da) | C5 (309 Da) | Concentration (mg/L) |
|---|---|---|---|---|---|---|
| M21 | 40.9 | 57.6 | 0.0 | 0 | 1.5 | 24 |
| M26 | 30.6 | 58.1 | 4.4 | 0 | 6.9 | 33 |
| M29 | 19.6 | 78.0 | 2.5 | 0 | 0.0 | 120 |
| M32 | 4.5 | 82.5 | 0.5 | 0 | 12.4 | 22 |
| M39 | 57.1 | 40.9 | 0.0 | 0 | 2.0 | 12 |
| M42 | 13.4 | 83.2 | 0.0 | 1.9 | 1.3 | 68 |
| M47 | 12.9 | 83.9 | 0.0 | 1.0 | 2.1 | 78 |
| M48 | 8.3 | 75.1 | 0.0 | 1.1 | 15.4 | 330 |
| M51 | 11.1 | 75.4 | 4.9 | 0 | 8.6 | 38 |
| M53 | 10.5 | 77.8 | 2.1 | 0 | 9.7 | 110 |
| M54 | 10.5 | 88.8 | 0.0 | 0 | 0.7 | 70 |
| M58 | 0.0 | 96.5 | 0.0 | 0 | 3.5 | 240 |
| M62 | 10.2 | 72.9 | 0.0 | 1.3 | 15.6 | 380 |
| Wild | 4.1 | 83.1 | 3.9 | 0 | 8.9 | 210 |

## Table 4

Growth Inhibition Efficacies of Prodiginine Analogs Against *S. aureus* with $10^5$ CFU/mL Concentration after Different Times

| | Percentage Reduction of Bacteria *S. aureus* in Different Time Contact | | |
|---|---|---|---|
| **Pigments (1.5 μM)** | **15 min** | **1 hr** | **12 hr** |
| Prodigiosin (C2, 0.15 μM) | 52.0 | 61.2 | 74.4 |
| Prodigiosin (C2, 3.0 μM) | 66.0 | 67.1 | 95.5 |
| Prodigiosin (C2, 1.5 μM) | 62.1 | 64.5 | 91.6 |
| Propylprodigiosin (C4) | 43.0 | 59.7 | 99.7 |
| Heptylprodigiosin (C3) | 51.6 | 61.2 | 93.3 |
| Cycloprodigiosin (C1) | 66.7 | 70.0 | 99.99 |