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A kind of potential food additive produced by *Streptomyces coelicolor*: Characteristics of blue pigment and identification of a novel compound, λ-actinorhodin

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Abstract

The blue pigment produced by *Streptomyces coelicolor* 100 with a yield as high as 3 g/l is a mixture of 10 components. The structure of one of the components was identified and it is a new actinorhodin analogue, named as λ-actinorhodin. The natural pigment can be dissolved in alkaline water solution and a number of organic solvents in common use. The color of a water solution of the pigment changes with pH value. The pigment is stable to light, heat and food additives in common use, and resistant to oxidants and reducers under acidic conditions and to reducers under alkaline conditions. Most inspected metal ions hardly affected pigment stability except for Fe^{2+} at high concentration and Pb^{2+}. The pigment is nontoxic with $LD_{50} > 15{,}000$ mg/kg in an acute toxicity trial. The good characteristics of the pigment make it potential useful in the food processing industry as an additive.
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Keywords: Actinorhodin; λ-actinorhodin; Food additive; Pigment; Blue pigment; *Streptomyces coelicolor*

1. Introduction

Edible colors are divided into two kinds, synthetic and natural. Synthetic colors have been widely used in the food industry for many years. It has been found, in recent years, that all of the synthetic colors are hardly nutrients, and most of them are toxic to different extents and some are potentially carcinogenic. With the understanding of the harmfulness of the synthetic colors, natural pigments are being increasingly emphasized. By their natural character, safety and use as additives, natural pigments have reached commercial potential (Pszczolla, 1998).

For edible natural pigments, their solubility, coloring, stability and safety are important indices of their applicability in the food industry. The solubility of pigments relies on the polarity and charges carried on the pigment molecules and the property of the solvent. Coloring of pigments depends on the polarity and ionic intensity of the solution. Complicated mechanisms of fade and disorder of natural pigments involve molecular polymerization, isomerization and degradation with comprehensive effects of pH value, light, temperature, moisture content, oxygen and metal ions. Investigations of these factors are important for improving the stability of blue pigments and rationally applying them in food processing.

Streptomyces coelicolor is a kind of actinomyces which can synthesize blue pigments. One of the products, actinorhodin, is an antibiotic produced by *S. coelicolor* A3(2) and can inhibit most Gram-positive bacteria such as *Staphylococcus aureus* at a relatively high concentration (Wright & Hopwood, 1976), but it has no

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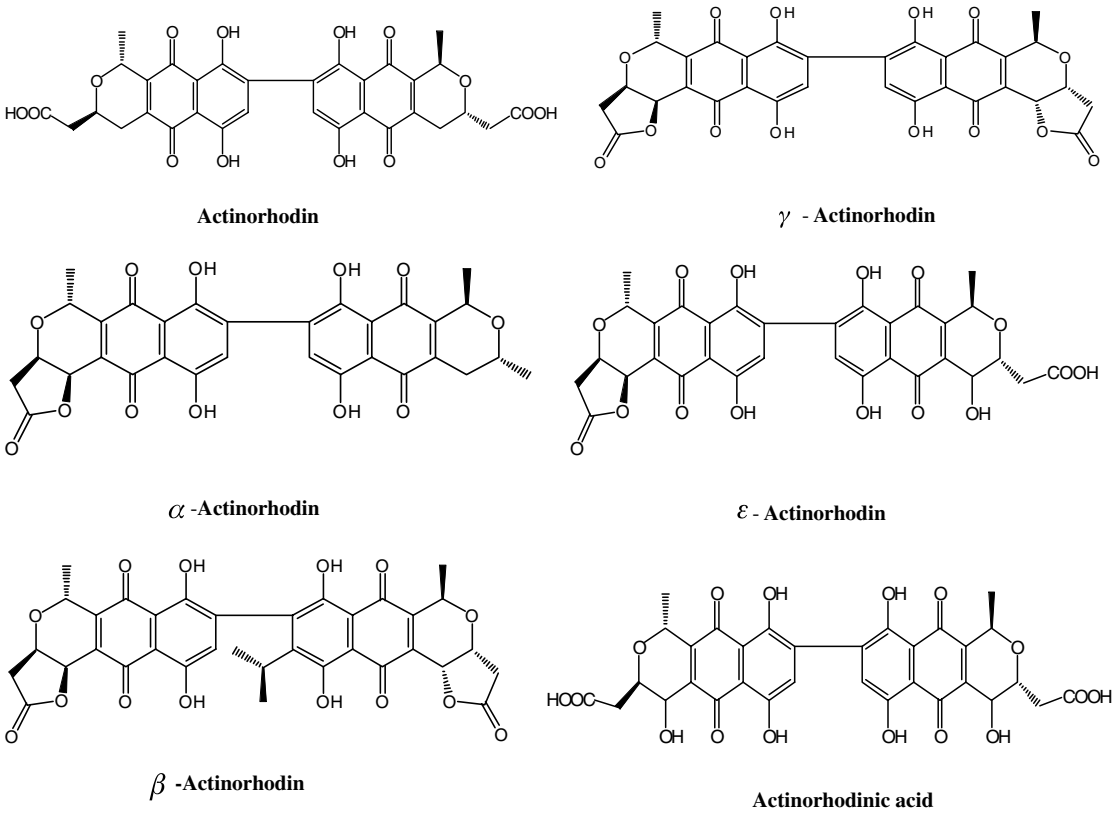

Fig. 1. The structures of actinorhodin and its known analogues.

effects on Gram-negative bacteria such as *Escherichia coli*. It is a pH indicator, turning red below pH 8.5 and blue above. The biosynthetic pathway of actinorhodin was revealed (Cole & Hopwood, 1987) and some analogues of actinorhodin, including α-, β-, γ-, ε-actinorhodin and actinorhodinic acid, were also reported (Bystrykh et al., 1996) (Fig. 1). Fermentative production of actinorhodin-related blue pigments was studied extensively (Bystrykh et al., 1996; Doull & Vining, 1990; Elibol & Mavituna, 1998, 1999; Hobbs, Frazer, & Gardner, 1990).

In this work, a strain of *S. coelicolor* was found to be able to produce a kind of blue pigment, which showed some properties similar to actinorhodin. The solubility, coloring change, stability, and toxicity of the blue pigment were studied. One component was separated and identified as a new compound.

2. Materials and methods

2.1. Strain, media and fermentation conditions

S. coelicolor 100 was screened from soil and identified (Zhang, Chen, & Ji, 1999).

Slant medium contained 20 g mannitol, 20 g soybean flour, and 20 g agar in 1 l of tap water, pH 7.5. Seed medium contained 20 g soluble starch, 2 g beef extract, 0.5 g NaCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.01 g $FeSO_4 \cdot 7H_2O$ in 1 l of tap water, pH 7.5. Fermentation medium contained 20 g mannitol, 20 g soybean flour, 0.5 g NaCl, 2.28 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$, and 0.01 g $FeSO_4 \cdot 7H_2O$ in 1 l of tap water, pH 7.5. In the experiment, spore suspensions were spread onto slant medium agar plates and incubated for 7–10 days at 30 °C to allow for sporulation. To prepare vegetative inoculum, two loops of spores from a plate culture on slant medium agar were added to 30 ml seed medium in a 250-ml flask. The culture was incubated for 24 h at 30 °C on a rotary shaker (200 rpm). The washed mycelium, resuspended in the same volume of water, was used as the inoculum. Three litres of fermentation medium in a 5-l fermentor were inoculated, at 10% (v/v) level, with mycelium culture and incubated for 7 days at 30 °C, pH 7.2–7.5, with aeration at 3 l/min and agitation at 300 rpm.

2.2. Refinement of blue pigment from fermentation broth

Fermentation broth was adjusted to pH 12 with 2 M NaOH, and strongly agitated for 25 min, and centrifuged (4000*g*, 15 min). The supernatant was collected and adjusted to pH 2 with 2 M HCl. After centrifuging, the amaranth sediment, as crude blue pigment, was har-

vested and dried in a vacuum. A yield of about 3 g blue pigment per litre of fermentation broth was achieved.

2.3. Pigment measurement and its residual ratio

The maximum absorption wavelength of pigment solution is pH-dependent and pigment content in solution can be measured by optical density (OD). Except where shown otherwise, OD values were determined at 588 nm and pH 9. The pigment residual ratio (%) is defined as the ratio of OD value after treating to that before treating.

2.4. HPLC analysis and isolation of blue pigment

The blue pigments were analysed on a HP 1100 series with auto-injector (25 μl). A Kromasil ODS C-18 column, 4.6×250 mm (5 μm), was used at 30 °C, with UWD at 260 and 530 nm. The following gradient elution (1 ml/min) with solvent A (water containing 1% HAc) and solvent B (acetonitrile) was used: during the initial 6 min, from 50% to 50% B; during the following 19 min, from 50% to 75% B.

The component of the blue pigments was prepared by semi-preparative HPLC on a Kromasil ODS C-18 column, 10×250 mm (10 μm) at 30 °C with a flow rate of 2 ml/min and detection at 260 nm, and eluted with a gradient, during the initial 25 min, from 60% to 80% B and during the following 5 min, from 80% to 100% B.

2.5. NMR and MS analysis

1D and 2D spectra were recorded in DMSO-d_6 (0.45 ml) with TFA (0.05 ml) and TMS as internal standard on a Bruker AVANCE 500 instrument.

TOFMS was measured with a Micromass LCT mass instrument.

3. Results and discussion

3.1. Solubility and coloring of blue pigment

The blue pigment was soluble in alkaline water solution and organic solvents such as acetone, dimethyl sulfoxide, acetic nitrile, acetoacetate, methanol and chloroform, but insoluble in petroleum ether. The solubility of the pigment decreased with pH value, and the pigment precipitated almost completely at pH values below 2.

The color of a water solution of blue pigment changed with pH value. The pigment became red in acidic conditions and was gaudier at lower pH. It was amaranth color at pH 7–8, blue above pH 8, and the blue color became dark with pH increase. Its property of changing color with pH value will help in food processing and other applications.

Table 1
Effect of pH value and solvent on color and λ_{max} of pigment solution

Solvents or pH	Color	λ_{max} (nm)
Water, pH 3	Red	484
Water, pH 5	Red	498
Water, pH 7	Amaranth	519
Water, pH 9	Blue	588
Water, pH 12	Deep blue	623
Methanol	Red	530
Ethanol	Red	526
Acetone	Red	530

Solvent and pH value exerted important effects on the color and maximum absorption wavelength (λ_{max}) of the pigment. When the pigment was dissolved in water with different pH values or other solvents, the solutions assumed different colors and spectral scanning showed their different absorption wavelengths (Table 1). Corresponding to the change in color, the absorption wavelength increased with pH value. The color and absorption wavelength show little change in organic solvents.

3.2. Stability of the pigment

The pigment is relatively stable under natural light. Its solution buffered at pH 7 was laid in an indoor natural light or an indoor dark place for 30 days, and optical density at 519 nm, as residual ratio of pigment (A%), was measured. There were slight declines of residual ratios of pigment in the two conditions, which were dependent on light strength. The residual ratio of pigment in indoor natural light for 30 days was above 80%. Assuming that the decline of residual ratio of pigment follows first order kinetics, that is

$$\frac{dA}{dt} = -kA.$$

The decline coefficient, k, is a measurement of pigment stability, and its values are 8.1×10^{-3} and 5.2×10^{-3} d^{-1} under indoor natural light and in indoor dark place, respectively.

After the pigment was dissolved in water solutions buffered at different pH values, such as pH 3, 5, 7, 9, 12 and the solutions were irradiated with ultraviolet rays (15 W light and 30 cm distance), the residual ratios of pigment were measured. The results showed that the pigment is stable under ultraviolet rays at various pH values. The decline coefficients, k, were 3.7×10^{-4}, 3.5×10^{-4}, 1.8×10^{-4}, 4.2×10^{-4} and 5.1×10^{-4} min^{-1} at pH 3, 5, 7, 9 and 12, respectively.

Temperature is an important factor for natural pigment stability. The residual ratios of pigment were obtained at different temperatures by treating its water

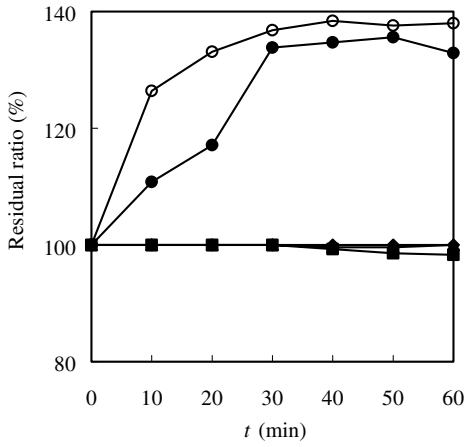

Fig. 2. Stability of pigment solution at pH 7 and different temperatures (♦ 20, ■ 40, ▲ 60, ● 80, ○ 100 °C).

solution at pH 7 for 1 h in the water bath with the temperatures of 20, 40, 60, 80 and 100 °C (Fig. 2). Below 60 °C, there were little changes in residual ratios of pigment. However, unexpectedly, the colors of pigment solutions became deeper and the residual ratios of pigment increased during the treating at higher temperatures. When the solution was treated for 30 min at 80 or 100 °C, its color increased by about 35%. This could be explained by pigment analogues that were changeable into one another, which was verified by later chromatographic analysis. The pigment was of a the nature suitable for food processing.

Natural pigments can usually be oxidized or reduced to alter their chemical structures. The resistance of the pigment to chemical oxidization and reduction was investigated with H_2O_2 as oxidizer and Na_2SO_3 as reducer. After adding the pigment to H_2O_2 and Na_2SO_3 solutions of different concentrations and treating for 2 h at 20 °C, the residual ratios of pigment were measured. Under acidic conditions, the pigment was stable in H_2O_2 solution, which implied its good resistance to oxidization. However, the pigment was not resistant to H_2O_2 in alkaline conditions, and the resistance of the pigment to oxidization decreased with increase of H_2O_2 concentration and pH value. On other hand, there was little effect of Na_2SO_3 on pigment stability, which shows its good resistance to reduction. Therefore, contact with oxidizer in alkaline conditions should be avoided when refining, processing and using the pigment.

Vitamin C and sodium benzoate are the additives in common use in the food industry. Vitamin C is an antioxidant because of its good reducing property, and sodium benzoate is a good preservative. Their influences on the stability of pigment solution were investigated by adding each of them to the solution and storing for 2 h at room temperature. There was no change in the color of pigment solutions at different pH values after adding vitamin C, and the residual ratios of pigment were above 97.5% except for that of 95.6% at pH 3. Similarly, after adding sodium benzoate to the pigment solution at different pH values, the residual ratios of pigment were close to 100% (data not shown). This indicates that the pigment is resistant to the effects of vitamin C and sodium benzoate.

The effects of metal ions on pigment stability were also observed by dissolving pigment in the buffer of pH 7, adding individual metal ion solution to it, and measuring the residual ratio of pigment after 1 h. It was found that most metal ions did not obviously influence the stability of pigment. Mg^{2+} could add color of the pigment solution by 12% and oppositely, Al^{3+} cut color of the pigment solution by 12%. Pb^{2+} could combine with the pigment to form sediment. The sediment is resoluble by adding 0.25% HCl. With this property, the pigment could be purified by successive operations of sedimenting by Pb^{2+}, centrifugation, discarding supernatant and resuspension of sediment in 0.25% HCl in alcohol. This is similar in principle to the $PbAc_2$ sediment method that was anciently used in the isolation of soluble plant pigments. The main question in the method is pollution by lead. In our experiment, the isolation process was designed, based on the fact that the pigment solubility is low in acidic solution and the pigment precipitates completely at pH 2. The pigment denatured at 5 mM Fe^{2+} solution. Furthermore, the effect of Fe^{2+} concentration on pigment stability was studied. Fe^{2+} would still result in the denaturation of pigment, even though, at 0.5 mM. However, the effect of Fe^{2+} was feeble when its concentration lowered to below 0.05 mM.

3.3. Mouse acute toxicity trial

Toxicity investigation of a substance falls into three categories in toxicology: acute, short-term, and long-term toxicity. The main objective of the acute toxicity trial is to measure the half lethal dose of tested substance (LD_{50}).

The experiment was carried out according to Evaluation Regulation of Food Safety in China (GB15193). A preliminary acute toxicity trial was done at first to obtain the range of LD_{50} for the new pigment.

In the acute toxicity trial of the pigment, 40 mice of the Kunming species were selected with average body weight of 20 ± 2 g. The mice were divided equally into two groups according to trial dose, 1500 and 15,000 mg/kg, with sex proportions of half and half. After orally taking the pigment by mouse weight, they were observed for 14 days.

During the observation period, no death occurred for the mice that were given the natural blue pigment at a dosage of 1500 mg/kg orally once. But, for the mice that orally took 15,000 mg/kg pigment once, two mice died at

the 2nd day, and one mouse died on each the 3rd and 13th day. It was found, in autopsy, that the death of four mice during 14 days derived from small intestine hemorrhage and necrosis.

In practical administration, with the poor solubility of pigment in CMC solution, it was possible for the mouse group, at the dosage of 15,000 mg/kg, to receive an overdose once by oral gavages. We wondered whether the intestine necrosis of mice did not derive from a genuine toxic reaction of the pigment. Thus, another complete acute toxicity trial was designed. In the experiment, 70 mice were divided equally into seven groups for the different pigment doses of 0, 463.6, 1000, 2155.1, 4633.4, 10,000, and 15,000 mg/kg according to Horn's method by oral administration twice a day. During a 14-day trial, no mouse died in any dose group. It was verified that the mouse death in the preliminary experiment resulted from taking an overdose pigment once by oral gavages. Thus, the pigment could be classified as a non-toxic substance according to the general toxicity standard.

3.4. HPLC analysis and preparation of blue pigments

The crude blue pigment was dissolved in acetonitrile and analysed by a HPLC (HP 1100) at 530 and 260 nm (Fig. 3).

It was found that the blue pigment produced by *S. coelicolor* 100 included 10 components (Bp1, 2, 3, 4, 5, 6, 7, 8, 9 and 10). Two components, Bp7 and Bp10 were obtained by semi-preparative HPLC. Bp7 was found to be unstable, therefore Bp10 was chosen for identification.

3.5. Identification of compound Bp10

TOFMS showed a molecular weight of 926 by two ion peaks $[M+H]^+$ and $[M+Na]^+$ observed at *m/z* 927 and 949, respectively. Element analysis gave a composition of C 57.06%, H 5.00% and O 37.94%. Therefore, a molecular formula of $C_{44}H_{46}O_{22}$ was deduced for compound Bp10.

Three keto-carbon signals at δ 183.6, 183.2, and 175.5 in ^{13}C NMR were observed, suggesting this compound

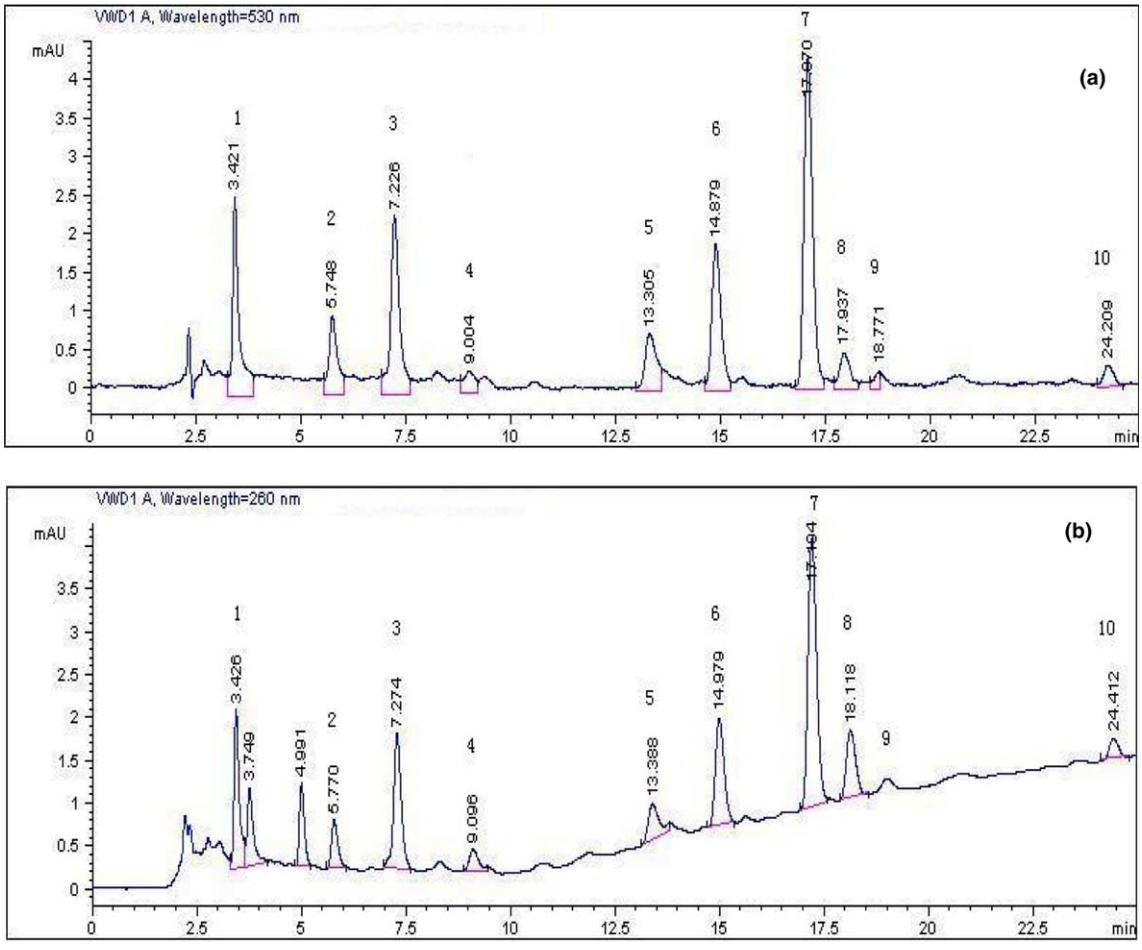

Fig. 3. HPLC chromatograms of blue pigment at 530 nm (a) and 260 nm (b).

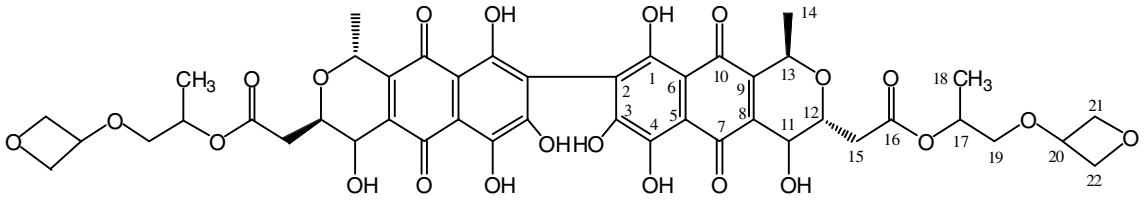

Fig. 4. The structure of compound Bp10.

to have a similar skeleton to actinorhodin (Bystrykh et al., 1996; Gorst-Allman, Rudd, Chang, & Floss, 1981). Also, nine other unsaturated carbon signals (δ 141.3, 116.8, 141.0, 141.0, 112.9, 115.7, 140.8 and 127.6) were in close agreement with the skeleton. DEPT analysis showed that all these signals were of quaternary carbon, and no phenyl proton signal was observed in ^{1}H NMR, which indicated that a hydroxy or alkyl group was linked to C-3. No alkyl signal correlated to C-3 was found in 2D spectra. Therefore, it could be concluded that a hydroxy group was at C-3.

Two proton signals, at δ 5.56 and 5.25, correlated with C-8 (δ 140.8) and C-9 (δ 127.6) in HMBC spectra, corresponded to two carbon signals at δ 69.4 and 66.5 in HMQC spectra, which should be assigned to C-11 or C-13. The 14-CH_3 (δ 1.58) correlated with the CH signal at δ 5.25 with a coupling constant of 6.7 Hz in ^{1}H–^{1}H COSY. So the signal at δ 66.5 was assigned to C-13 and δ 69.4 to C-11. 11-H (δ 5.56) correlated with 12-H (δ 4.90) in ^{1}H–^{1}H COSY, from which the chemical shift of C-12 was assigned to δ 65.5 in HMQC spectra. Similarly, the proton signal (δ 2.55, 3.34) and carbon signals at C-15 (δ 36.9) could be assigned on the basis of 2D spectra, which was further proved by the correlation of 12-CH and 15-CH_2 with 16-C in HMBC spectra. Some carbon signals of the side chain at δ 72.0–75.0 were observed in ^{13}C NMR. On the basis of its molecular formula and 2D spectra, the structure of compound Bp10 was identified (Fig. 4) and all signals of proton and carbon were assigned accordingly (Table 2). For describing the new actinorhodin analogue, we named it as λ-actinorhodin.

Table 2
^{1}H NMR (500 MHz, δ_H) and ^{13}C NMR (125 MHz, δ_C) spectral data for compound Bp10 [d_6-DMSO–TFA (9:1)]

Position	δ_C	δ_H
1	141.3 (s)	
2	116.8 (s)	
3	141.0 (s)	
4	141.0 (s)	
5	112.9 (s)	
6	115.7 (s)	
7	183.2 (s)	
8	140.8 (s)	
9	127.6 (s)	
10	183.6 (s)	
11	69.4 (d)	5.56 (1H, m)
12	65.5 (d)	4.90 (1H, m)
13	66.5 (d)	5.25 (1H, dd, J = 6.7 Hz)
14	17.7 (q)	1.58 (3H, d, J = 6.7 Hz)
15	36.9 (t)	2.55 (1H, m), 3.34 (1H, m)
16	175.5 (s)	
17	74.8 (d)	3.24 (1H, m)
18	17.4 (q)	1.06 (3H, m)
19	72.4 (t)	3.28–3.53 (2H, m)
20	74.8 (d)	3.51 (1H, m)
21	72.4 (t)	3.28–3.53 (2H, m)
22	72.4 (t)	3.28–3.53 (2H, m)

Bp10 (λ-actinorhodin) was obtained as an amaranthine powder (acetonitrile). UV λ_{max}^{MeOH}: 260 and 532 nm; IR ν_{max} (KBr): 3448, 2950, 2926, 2871, 1779, 1631, 1584, 1413, 1373, 1268, 1193, 1115, 897, 752 cm^{-1}; TOFMS m/z 927 $[M+H]^+$, 949 $[M+Na]^+$. Element composition: C 57.06%, H 5.00% and O 37.94%.

4. Conclusions

The blue pigment from the fermentation broth of *S. coelicolor* 100 with a yield as high as 3 g/l is a mixture of 10 components. It is soluble in alkaline water solution and a number of organic solvents in common use except for petroleum ether. The color of the pigment solution changed with pH value, from red at pH < 7,through amaranth at pH 7–8, to blue at pH ⩾ 8. The pigment was stable to light and heat, and resistant to oxidants and reducers under acidic conditions and to reducers under alkaline conditions. No evident influences of food additives in common use, such as vitamin C and sodium benzoate, on pigment stability were observed. Most inspected metal ions hardly effected pigment stability except for Fe^{2+} at concentrations higher than 0.5 mM and Pb^{2+}. An acute toxicity trial verified that the pigment was nontoxic with LD_{50} > 15,000 mg/kg. On the basis of its molecular formula and 2D spectra, the structure of compound Bp10 was identified and it is a new actinorhodin analogue, named as λ-actinorhodin.

The good characteristics of the pigment give it potential for the food processing industry as an additive. It can be used to make some colorful beverages and cakes. In a broad sense, it is also a safe ingredient in lipstick to color blue.

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