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Effect of melanin produced by a recombinant *Escherichia coli* on antibacterial activity of antibiotics

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A recombinant plasmid, pYL-1, containing a tyrosinase gene whose expression is under the control of a phage T5 promoter and 2 *lac* operators, was constructed. *Escherichia coli* JM109 harboring pYL-1 was used for production of bacterial melanin. A simple procedure for the isolation and purification of melanin was developed. The ultraviolet (UV)-visible light absorption spectra of melanin prepared by chemical synthesis and derived from different organisms, including bacteria, a plant and an animal source, were determined. Melanins produced by both bacteria and chemical synthesis showed a steady increase of absorption at wavelengths of UV light ranging from approximately 200-400 nm, while melanin derived either from plant or animal sources showed an additional discrete absorption peak at wavelength 280 nm upon a similar steady increase of absorption. This additional absorption peak could be due to the presence of protein-bound melanins in animal and plant sources while a free form of melanin was obtained from bacteria and chemical synthesis. Analysis of the effect of bacterial melanin on the activity of antibiotics against *E. coli* revealed that the activities of polymyxin B, kanamycin, tetracycline, and ampicillin were markedly reduced in the presence of melanin, whereas the activity of norfloxacin was not affected. The reduction of the antibacterial activity may result directly from the interaction of antibiotics with melanin. However, the mechanism of this interaction remains to be demonstrated.

Key words: Anti-bacterial agents, *Escherichia coli*, melanin, microbial sensitivity tests, plasmids

Melanin is an endogenous, non-hemoglobin-derived, brown-black pigment that is widely produced by animals, plants and microorganisms [1-8]. Melanins are produced in the form of granules usually composed of a mixture of macromolecular polymers while some of them may be bound to proteins. Formed from L-tyrosine and L-dopa precursors, the chemical units which predominate in melanin are of the indole type [9].

In *Streptomyces antibioticus*, the critical step in melanin biogenesis is the catalytic oxidation of tyrosine by the enzyme tyrosinase [9]. The tyrosinase gene encoding tyrosinase has been cloned and used as a genetic marker of the cloning vector pIJ702 [10].

The light absorption properties of melanin have been shown to be involved in several biologic functions, such as photoreceptor shielding, thermoregulation, and photoprotection [9,11]. In addition, melanin has been shown to be a powerful cation chelator, a free radical sink [11], and a potential target for anti-melanoma therapy [12]. Several studies showed that melanins were capable of binding chemicals and drugs [13-15]. Production of melanin is also a common property of pathogenic bacteria [1,16]; thus, it is possible that melanin is produced by pathogenic bacteria as a defense system which reduces the susceptibility of bacteria to antibiotics.

In this study, we constructed a recombinant plasmid, pYL-1, and examined the expression of tyrosinase in *Escherichia coli*. Procedures for the production, isolation and purification of melanin produced by bacteria and plants were also developed. The ultraviolet (UV)-visible light absorption spectra of melanin from different sources and the effect of melanin on the susceptibility of *E. coli* to various antibiotics were also examined.

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Materials and Methods

Microorganisms and plasmid

E. coli JM109, *Streptomyces lividans* 66 (*SL*66) and plasmid pIJ702 were obtained from the Culture Collection and Research Center, Hsin-Chu, Taiwan. Plasmid pQE32 was purchased from Qiagen (Germany).

Chemicals and medium

Chemically synthetic melanin, animal melanin (*Sepia officinalis*), ampicillin, kanamycin, norfloxacin, polymyxin B, and tetracycline were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Luria-Bertani (LB) medium (Difco, USA), isopropylthio-β-D-galactoside (IPTG) [Gibco-BRL, USA], and cupric chloride (Difco) were used in culturing *E. coli* and in melanin production.

Construction of plasmid pYL-1

The methods used for DNA isolation, restriction enzyme digestion, transformation, and other manipulation were as described by Sambrook and Gething [17]. All enzymes, buffers and the conditions used were as recommended by the suppliers. Both original circular pQE32 and pIJ702 plasmid were linearized by digestion with *Sph*I restriction enzyme. Recombinant plasmid pYL-1 was constructed by ligating the *Sph*I-digested pIJ702 with the *Sph*I-digested pQE32 to create a hybrid plasmid pYL-1 (Fig. 1). In this recombinant plasmid, the expression of tyrosinase gene is under the control of phage T5 promoter and two *lac* operators. To examine the expression of tyrosinase gene, *E. coli* JM109 harboring plasmid pYL-1 (*E. coli* JM109/pYL-1) was cultured on an LB agar plate supplemented with 0.1% tyrosine, 0.0017% cupric chloride, and 0.36 Mm IPTG and incubated at 37°C. Production of melanin, a black pigment, indicated the expression of tyrosinase gene.

Production and isolation of melanin

In the production of bacterial melanin, *E. coli* JM109/pYL-1 was inoculated into 5 mL of LB broth containing 100 μg/mL ampicillin and incubated at 37°C. After overnight incubation, 5 mL of the seed culture was transferred into 1 liter of LB broth supplemented with 0.1% tyrosine, 0.0017% cupric chloride, and 0.36 Mm IPTG and then incubated at 37°C on a shaker at 150 rpm for 2 days. The procedures used for isolation and purification of melanin produced by bacteria are shown in Fig. 2. At the end of incubation, the culture medium was centrifuged to remove the cells, and melanin was precipitated from the supernatant by adjusting the pH to 3.0 with 5N HCl. The precipitated melanin was re-dissolved in distilled water at pH 8.0, and further purified by Pharmacia Sephadex LH-20 column liquid chromatography (Sephadex LH-20) according to the method of Granath and Kvist [18]. Plant melanin was extracted from an herb, *Mensona procumbens Hemsl,* purchased from a medicinal herb store in Taipei, by boiling in alkaline water (pH 8.0) followed by purification by the same procedure described above for bacterial melanin.

UV absorption spectrum of melanin

To determine the UV-visible light absorption spectrum of melanin, 0.02 mg of melanin produced by different sources (chemical synthesis, bacteria, plant, and animal) was dissolved in alkaline distilled water at pH 8.0, and the resulting solution was scanned with a spectrophotometer (Spectronic-Genesys, USA) to determine its absorption spectra of UV-visible light at wavelength ranging from 200 to 1000 nm.

Effect of melanin on the susceptibility of *E. coli* to antibiotics

Antimicrobial activity of antibiotics against *E. coli* in the presence of melanin was determined based on minimal inhibitory concentrations (MICs) by a series dilution method in 2-mL volumes of Mueller-Hinton broth (Difco) containing various amounts of bacterial melanin ranging from 0.01% to 0.04% (w/v). To prepare the inocula of the test organism, colonies of *E. coli* JM109 were selected from LB agar plate and transferred to a tube containing 5 mL of LB broth. After overnight incubation at 37°C, the cell concentration was adjusted to an inoculum of 10^5 colony forming units for each tube. All cultures were incubated at 37°C for 20 h. The MIC was defined as the lowest concentration (μg/mL) of antibiotic that could completely inhibit the growth of the test organism.

Results

Construction of recombinant plasmid pYL-1 and melanin production in *E. coli*

The recombinant plasmid pYL-1 containing tyrosinase gene was successfully constructed by ligating the *Sph*I-digested pIJ702 with the *Sph*I-digested pQE32 such that the tyrosinase gene can be expressed under the control of phage T5 promoter and 2 *lac* operators.

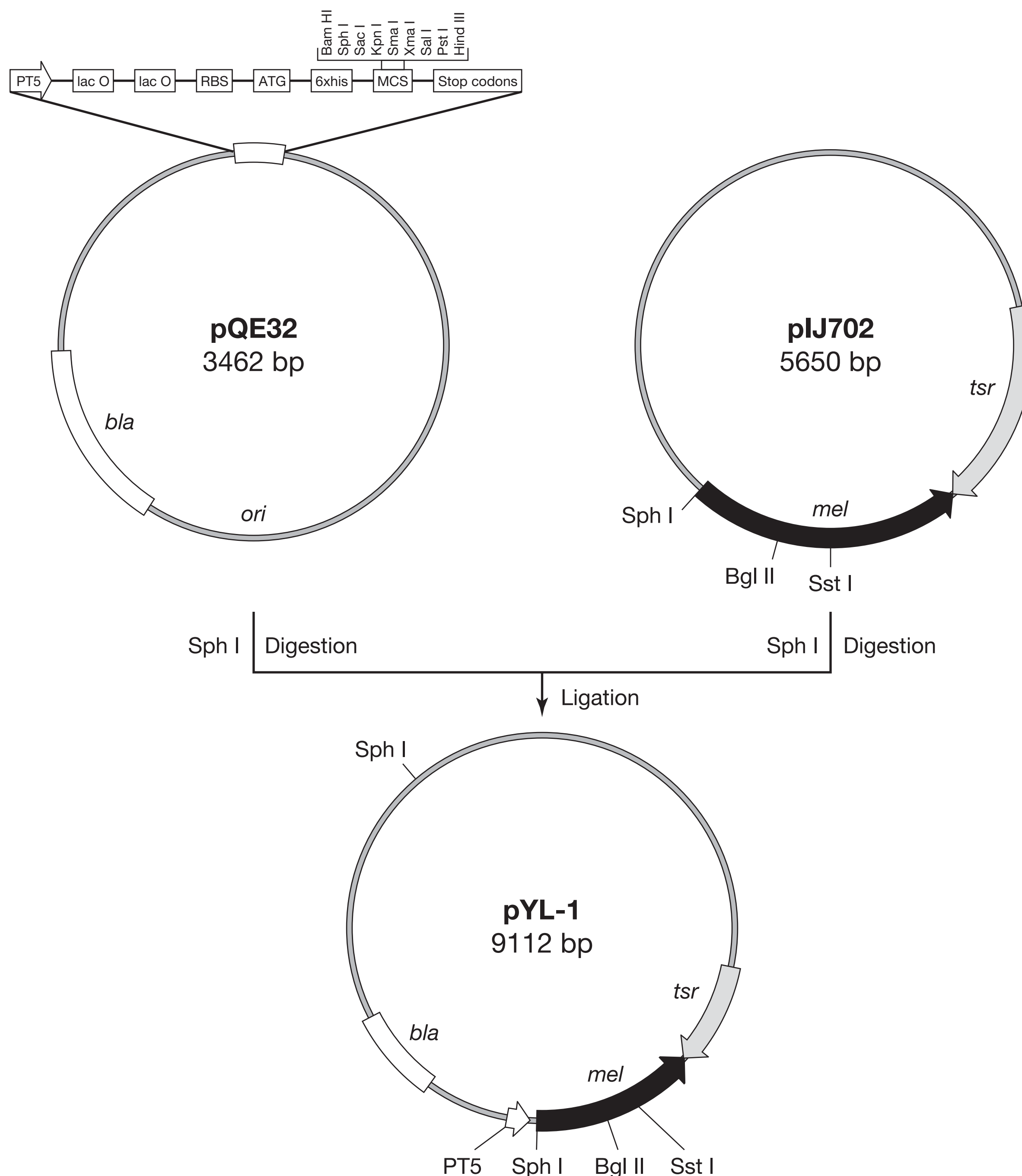

Fig. 1. Construction of recombinant plasmid pYL-1. Both pQE32 and pIJ702 plasmid were digested by *Sph*I restriction enzyme to generate linear plasmid DNA and joined together by ligation to create the recombinant plasmid pYL-1. Only relevant restriction sites are shown.

The gene product was translationally fused to a tag sequence coding for 6 × histidine polypeptide (Fig. 1). For regulating the expression of tyrosinase gene in *E. coli*, a double *lac* operator system was used to effectively block protein synthesis in the presence of *lac* repressor, allowing the expression of tyrosinase gene to be induced by the addition of IPTG. In contrast to those colonies of *E. coli* JM109 harboring plasmid pQE32 (*E. coli* JM109/pQE32) and *E. coli* JM109 alone after overnight incubation at 37°C, dark-brownish colonies of *E. coli* JM109/pYL-1 appeared on the LB agar supplemented with 1% tyrosine, 0.0017% cupric chloride, and 0.36 mM IPTG (Fig. 3), indicating the expression of tyrosinase gene in *E. coli* cells.

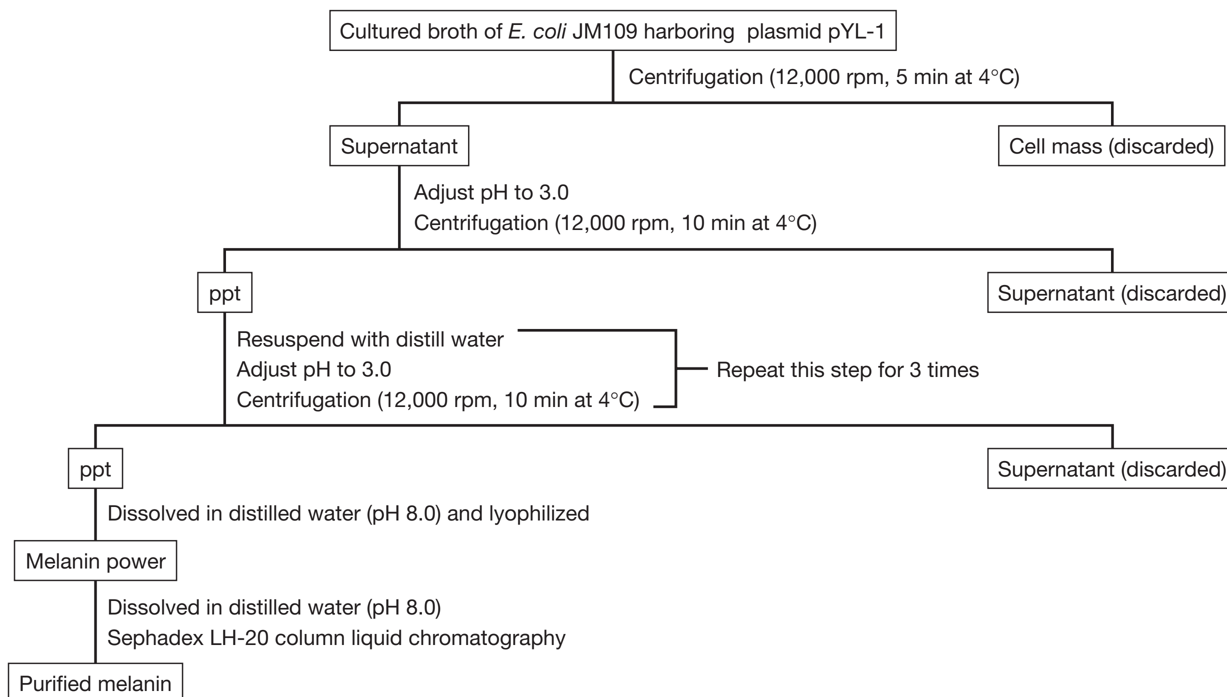

Fig. 2. Procedures for isolation and purification of melanin from cultured broth of *Escherichia coli* JM109 harboring plasmid pYL-1. ppt = precipitate.

Production, isolation and purification of melanin

The maximal production of melanin by *E. coli* JM109/pYL-1 was obtained at 2 days of culture (data not shown). After isolation by acidic precipitation and purification by gel filtration, the yield of bacterial melanin was calculated to be about 0.4 g per liter of fermented culture. The purified major component of bacterial melanin showed a single band in Sephadex LH-20 (data not shown).

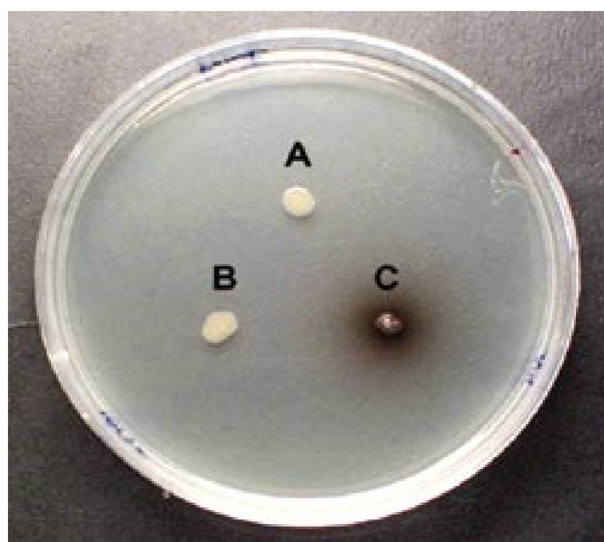

Fig. 3. Melanin production on Luria-Bertani agar plate supplemented with 0.1% tyrosine, 0.0017% cupric chloride and 0.36 mM isopropylthio-β-D-galactoside. (A) *Escherichia coli* JM109 alone. (B) *E. coli* JM109 harboring plasmid pQE32. (C) *E. coli* JM109 harboring plasmid pYL-1. All of the cultures were incubated at 37°C for 48 h.

Ultraviolet visible spectroscopy of melanin

As shown in Fig. 4, both commercially synthetic melanin and bacterial melanin showed a steadily increasing absorption at wavelengths ranging approximately from 200 to 400 nm; however, both animal and plant melanins exhibited an additional absorption peak at wavelength 280 nm compared to those of bacterial and the chemically synthetic melanins.

Interference of bacterial melanin with antimicrobial activity

The effects of melanin on the susceptibility of bacteria to 5 different antibiotics was examined. In the presence of melanin, the activities of polymyxin B, kanamycin, tetracycline, and ampicillin were markedly reduced in a dose-dependent manner (Table 1), whereas there was no effect of melanin on the activity of norfloxacin. These results indicate that the interaction between bacterial melanin and some classes of antibiotics may result in a reduction of their antimicrobial activities.

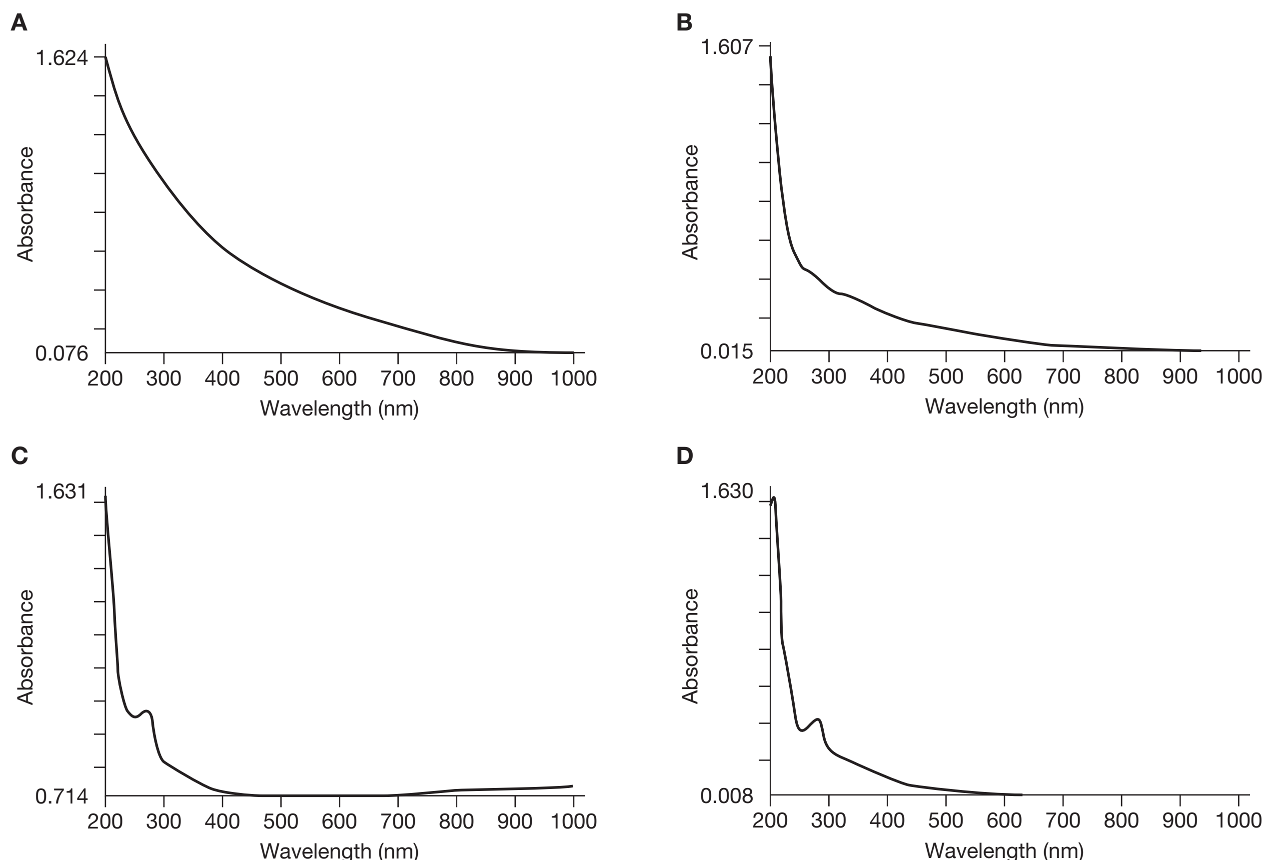

Fig. 4. Ultraviolet-visible light absorption spectra of melanins produced by: (A) commercial synthesis; (B) bacteria; (C) animal (*Sepia officinalis*); and (D) plant (*Mesona procumbens Hemsl*).

Discussion

The novel characteristics of the constructed recombinant plasmid pYL-1, such as its capability to replicate in both *E. coli* and *SL*66 (data not shown) with a convenient cloning marker (tyrosinase gene) for insertional or replaceable inactivation and 2 selection markers (thiostrepton and ampicillin-resistant gene), make pYL-1 a useful shuttle cloning vector. However, plasmid pYL-1 cannot express tyrosinase in *SL*66 (data not shown) because the *E. coli* T5 promoter responsible for driving the transcription of tyrosinase gene in pYL-1 cannot be recognized by the transcription system of *SL*66 [18]. It has been shown that melanin could be efficiently produced in *E. coli* JM109/pYL-1 containing the tyrosinase gene of *S. antibioticus*.

Melanins derived from different sources including plant, animal, bacteria, and chemical synthesis exhibit good solubility in neutral and basic aqueous solution, but very poor solubility in acidic aqueous solution and in most organic solvents [10]. Thus, for determination of the UV-visible absorption spectra in this study, melanins derived from different sources were dissolved in alkaline distilled water at pH 8.0. The bacterial and the chemically synthesized melanins showed a steadily increasing end-absorption with no abrupt peak

Table 1. Effect of bacterial melanin on antimicrobial activity of antibiotics[a]

Melanin concentration (%)	Minimum inhibitory concentration (µg/mL)				
	Polymyxin B	Kanamycin	Tetracycline	Ampicillin	Norfloxacin
0.04	0.78	25	6.25	6.25	0.10
0.02	0.78	25	3.13	3.13	0.10
0.01	0.39	12.5	1.56	3.13	0.10
0	<0.10	3.13	0.78	1.56	0.10

[a]*Escherichia coli* JM109 was used as test organism.

at the wavelengths of UV-visible light (200-400 nm), indicating that they may exist in a free form. Proteins specifically exhibit a maximum absorption peak at 280 nm, explaining our finding that melanins from plant and animal sources exhibited an additional absorption peak at wavelength 280 nm apart from the steady end-absorption observed in bacterially and synthetically derived melanins, suggesting that the discrete absorption could derive from a protein moiety bound to the melanins.

Table 1 shows that the antibacterial activity of polymyxin B, kanamycin, tetracycline, and ampicillin against *E. coli* was markedly reduced in the presence of bacterial melanin. This result suggests that these organisms might have formed a melanin-bound complex that diminished the antibacterial activity. However, this effect on activity was not observed for norfloxacin. The reduction of antibacterial activity in the presence of melanin could be due to interaction of the affected antibiotics with melanin via NH_2 groups. Aminoglycoside antibiotics like kanamycin have been reported to bind tightly to melanin [13-15]. Fukuda and Sasaki [20] reported that the highest melanin binding ratio was seen in aminoglycosides, whereas cephalosporins and fluoroquinolones had relatively low melanin binding ratios and melanin-bound aminoglycosides showed a reduction in their anti-mcirobial activity with *Bacillus subtilis* and *E. coli*. Kunin [21] suggested that the binding capacity of aminoglycosides for melanin may correlate with the number of free amino groups. Our results support this hypothesis, since polymyxin B, kanamycin, tetracycline, and ampicillin possess free amino groups ranging from 5, 4, 1, and 1, respectively, whereas norfloxacin has no free amino group (Fig. 5). Gomez and Nosanchuk

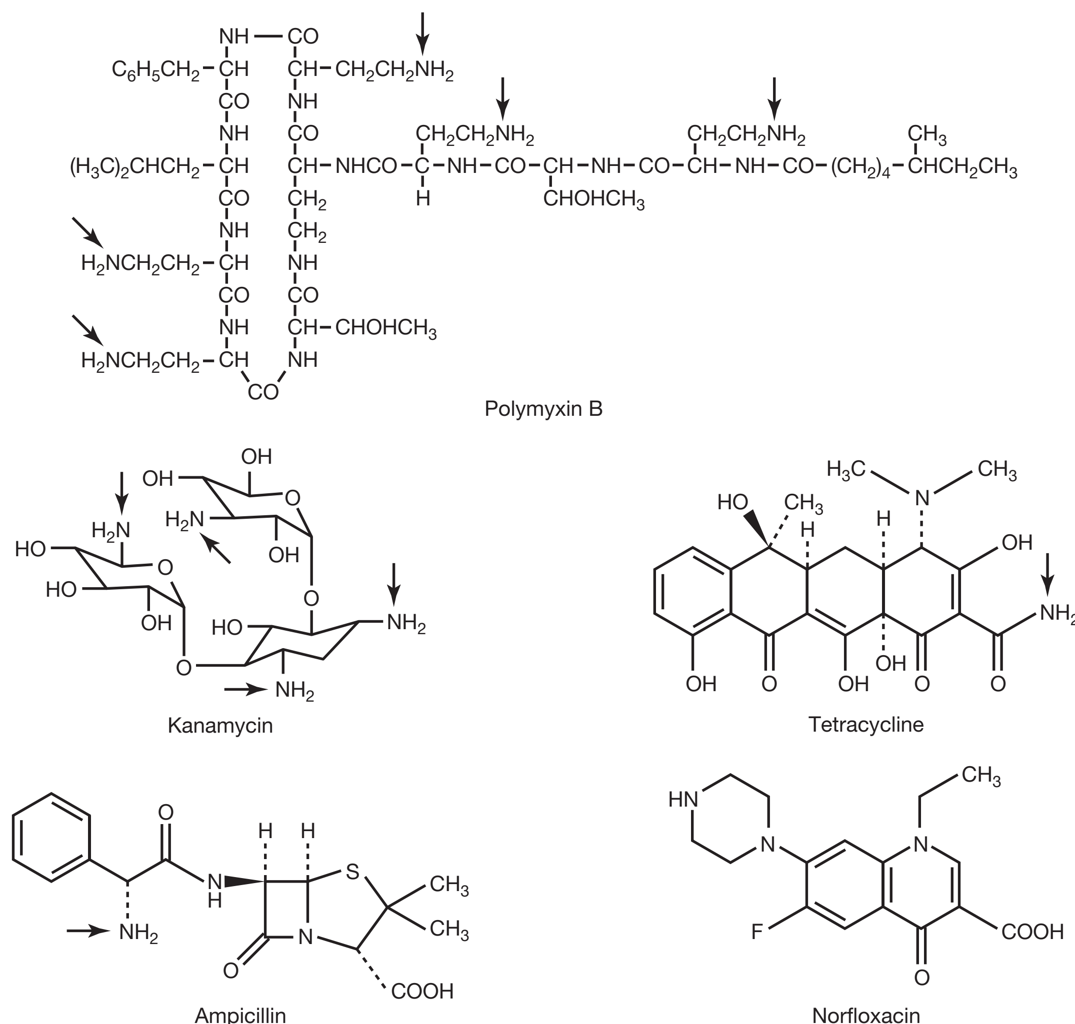

Fig. 5. Chemical structures of polymyxin B, kanamycin, tetracycline and ampicillin but not norfloxacin show free amino groups as indicated by arrows.

[22] also reported that *Cryptococcus neoformans* and *Histoplasma capsulatum* yeast cells could bind amphotericin B and caspofungin, thereby reducing the fungicidal activity of these agents. Thus, we conclude that the observed reduction of the antimicrobial activity of polymyxin B, tetracycline, kanamycin and ampicillin in this study was a direct result of the interaction of these agents with melanin. The relevance of the present observations to in vivo conditions remains unclear, especially since melanin is normally sequestered in intracellular organelles; however, it seems possible that this phenomenon could interfere with the activity of antimicrobial chemotherapy in melanin-containing bacteria, fungi and tissues.

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