A NEW SCREENING METHOD FOR MELANIN BIOSYNTHESIS INHIBITORS USING *STREPTOMYCES BIKINIENSIS*

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A novel screening method for melanin biosynthesis inhibitors using *Streptomyces bikiniensis* NRRL B-1049 as the indicator organism has been developed.

Several known compounds, including tyrosinase inhibitors, were found to inhibit melanin production of *S. bikiniensis* on agar plates. This screening method was applied to fermentation broths of actinomycetes and several cultures with melanin biosynthesis inhibitory activity were found.

Melanin synthesis inhibitors such as 4-hydroxyanisole and hydroquinone are used topically for treatment of localized hyper-pigmentation in humans such as lentigo, nevus, ephelis, post-inflammatory state and melanoma of pregnancy.

Tyrosinase (phenol oxidase)[1] is known to be a key enzyme for melanin biosynthesis in plants, microorganisms and mammalian cells. Most of the known tyrosinase inhibitors such as 4-hydroxyanisole, 5-hydroxyindole and hydroquinone showed potent activity by this screening method.

In our fermentation broth screening, feldamycin[2] and its related compound BU-3619E (BMY-28566)[3] have been discovered by this method. A depsipeptide antibiotic, virginiamycin, and a tetracycline analog were also found to be active in this screening. The inhibitory activity of these compounds to mushroom tyrosinase[4] and melanin formation of B16 melanoma cells were also examined in parallel with the new method.

This paper describes the assay method for melanin biosynthesis inhibitors using *Streptomyces bikiniensis* as the indicator organism and the subsequent screening results.

Materials and Methods

Selection of the Indicator Organism

There are many melanin-producing species in the genus *Streptomyces*.[5] Six strains, namely *Streptomyces phaeochromogenes* NRRL B-1248, *Streptomyces antibioticus* NRRL B-546, *S. bikiniensis* NRRL B-1049, *Streptomyces chartreusis* NRRL B-2287, *Streptomyces venezuelae* NIHJ 39 and *Streptomyces lividans* 3131, were examined for production of melanin and sensitivity to inhibitors using peptone-yeast extract-iron agar (medium ISP No. 6) and tyrosine agar (medium ISP No. 7). 4-Hydroxyanisole was used as a reference compound. From them, *S. bikiniensis* NRRL B-1049 was chosen as the indicator organism for this screening method because of the appearance of a clear melanin-inhibition zone on agar against a dark background of melanin production.

Determination of Inhibition of Melanin Production of *S. bikiniensis*

A preserved culture of *S. bikiniensis* NRRL B-1049 was inoculated on a Papavizas' VDYA agar slant which contained V-8 juice (Campbell Soup Co.) 200 ml, glucose 2 g, yeast extract (Difco) 2 g, $CaCO_3$ 1 g, agar (Difco) 20 g and distilled water 800 ml, the pH being adjusted to 7.2 before autoclaving.

After incubation at 28°C for 2 weeks, 2 ml of sterile water was added onto the slant culture and the spore mass formed on the aerial mycelium was scraped with an inoculating loop. The spore suspension thus obtained was transferred to a sterile test tube. Agar medium ISP No. 7 (40 ml) supplemented with Bacto-yeast extract (Difco) 0.2%, was poured into Petri dishes (90 mm i.d.). After solidification, 0.4 ml of the spore suspension of *S. bikiniensis* was added to the agar plate and spread over the agar surface uniformly with a glass hockey bar.

Table 1. Inhibitory activity of 4-hydroxyanisole to melanin formation by *Streptomyces bikiniensis* NRRL B-1049.

Concentration (μg/ml)	Inhibition zone (mm)
1,000	42
500	30
250	24
125	18
62.5	0
31.3	0

Medium: ISP No. 7 + yeast extract 0.2%.
Incubation: 28°C, 48 hours.

After drying of the agar surface, a paper disc (8 mm diameter) soaked with sample solution was placed on the agar plate. The plate was incubated at 28°C for 48 hours; the resulting zone (mm i.d.) of inhibition of melanin formation was measured from the reverse side of the plate. 4-Hydroxyanisole was used as a reference standard (Table 1).

Inhibition of Tyrosinase

The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5) 2.3 ml, 1.5 mM L-tyrosine solution 0.4 ml and 2,000 U/ml mushroom tyrosinase (Sigma), in 0.05 M phosphate buffer (pH 6.5) 0.1 ml. A sample solution (0.2 ml) was added to the reaction mixture and incubated at 25°C for 10 minutes. The optical density at 470 nm was measured by a spectrophotometer. The inhibitory activity of the sample was expressed as the concentration which inhibits 50% of the enzyme activity (IC_{50}).

Inhibition of Melanin Synthesis in B16 Melanoma Cells

Growing cells of B16 melanoma were suspended in 3.6 ml of EAGLE's minimum essential medium containing 10% fetal calf serum (FCS) at 3×10^3 cells/ml. The cell suspension (4 ml) was poured into plastic dishes (60 mm i.d.) and the cells were allowed overnight to completely adhere to the dish. Test samples dissolved in sterile water (0.4 ml) were added to the dish and incubated at 37°C for 6 days in a CO_2 incubator with an atmosphere containing 5% CO_2. During the incubation, the culture medium was renewed once with fresh medium containing the same sample solution. After 6-days incubation, the adherent cells were washed with phosphate-buffered saline (PBS) and detached from the dish by trypsinization. The cells were collected in a test tube and washed twice with PBS. The number was determined by a Coulter cell counter.

In order to extract melanin from the B16 melanoma cells, 1.0 ml of the cell suspension was shaken with an equal volume of a mixture of 10% DMSO and 1 N NaOH and stored at room temperature for 10 minutes. After centrifugation at 3,000 rpm for 10 minutes, the optical density at 470 nm of the resulting supernatant was measured by a spectrophotometer.

Results and Discussion

Recently, KAHN and ANDRAWIS[6] reported that tropolone and some other copper chelators such as ascorbate, cysteine and 2-mercaptobenzothiazole inhibit tyrosinase. Kojic acid[7] has been also reported to have the same activity.

To test the assay systems inhibition of melanin formation in *S. bikiniensis* and of mushroom tyrosinase were investigated using fourteen tyrosinase inhibitiors. In this experiment, compounds which strongly inhibit tyrosinase also inhibit melanin formation by *S. bikiniensis*, but compounds with weak inhibitory activity against tyrosinase did not show any inhibition of melanin formation by *S. bikiniensis* (Table 2).

The biosynthetic pathway of melanin formation is illustrated in Fig. 1. Tyrosinase is the sole key enzyme for melanin biosynthesis, having a role in oxidation from tyrosine to dopa and dopa to dopa

Table 2. Inhibitory activity of tyrosinase inhibitors to melanin formation of *Streptomyces bikiniensis* and to mushroom tyrosinase.

Compound	*S. bikiniensis* NRRL B-1049		Mushroom tyrosinase
	Concentration (μg/ml)	Inhibition zone (mm)	IC_{50} (μg/ml)
4-Hydroxyanisole	1,000	40	15.0
5-Hydroxyindole	1,000	25	0.7
Hydroxyquinone	1,000	42	1.5
Tropolone	1,500	31	0.02
5-Hydroxytropolone	1,500	20	5.0
7-Hydroxytropolone	1,500	25	0.15
3,7-Dihydroxytropolone[12]	1,500	33	1.0
Kojic acid	1,000	0	15.0
L-Cysteine	1,500	0	15.0
Sodium azide	1,500	0	30.0
Sodium ascorbate	1,500	0	150.0
Sodium thiosulfate	1,500	0	30.0
2-Mercaptobenzothiazole	1,500	25	0.2
EDTA	1,500	25	200.0

Medium: ISP No. 7+Bacto-yeast extract 0.2%.
Incubation: 28°C, 48 hours.

Fig. 1. Biosynthetic pathway of melanin formation.

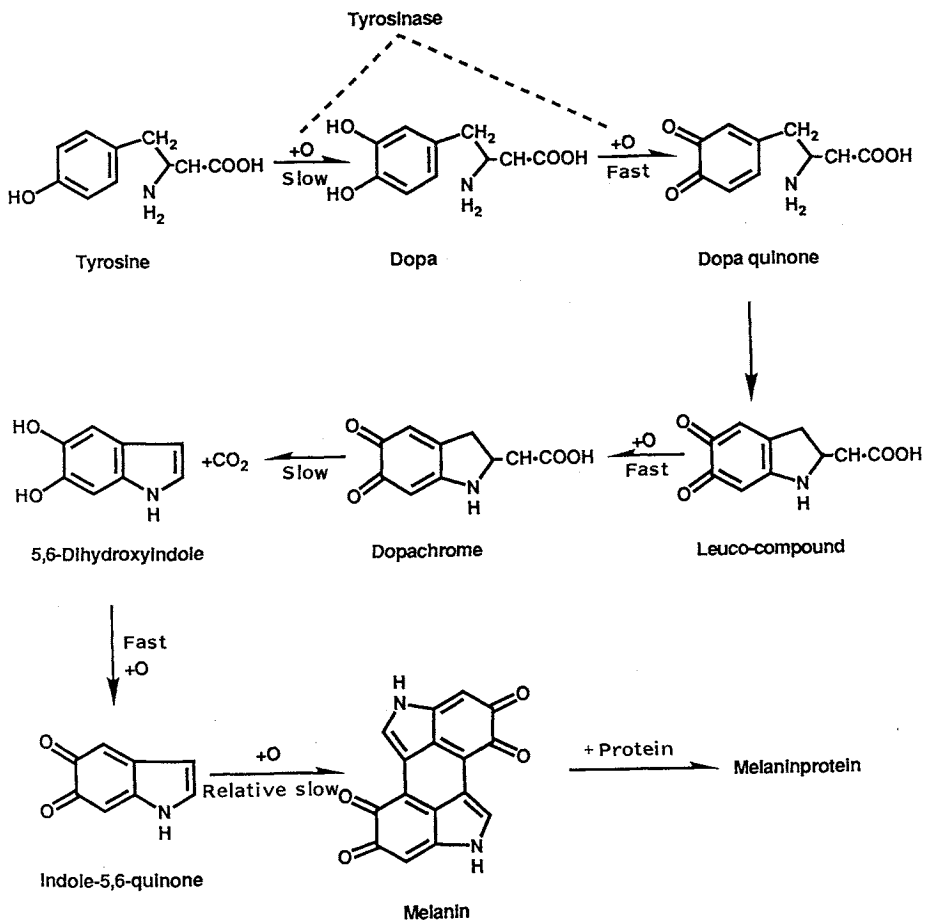

Table 3. Activity of compounds screened by *Streptomyces bikiniensis* and related compounds.

Compound	*S. bikiniensis* NRRL B-1049	B16 Melanoma	Mushroom tyrosinase
	Inhibition zone (mm)[a]	IC_{50} (μg/ml)	IC_{50} (μg/ml)
Virginiamycin M	26	2.0	>200
Virginiamycin S	48	3.2	>200
2-Acetyl-2-decarboxamidooxytetracycline	18	6.0	>200
Mikamycin B	42	3.2	>200
Etamycin	44	0.5	>200
Tetracycline	31	<6.3	>200
Oxytetracycline	21	2.0	>200
Feldamycin	34	20.0	>200
BU-3619E	32	30.0	>200

[a] Concentration of 250 μg/ml.

quinone. However, depsipeptide antibiotics such as virginiamycin,[8] mikamycin[9] and etamycin[10] and the tetracycline group antibiotics also showed inhibitory activity to melanin production by *S. bikiniensis*, but they did not show any inhibitory activity against mushroom tyrosinase (Table 3).

These results indicated that the depsipeptide antibiotics and tetracyclines probably inhibit production of tyrosinase in *S. bikiniensis* but do not inhibit the enzymatic reaction of tyrosinase.

Approximately 2,000 actinomycetes cultures were screened using *S. bikiniensis* as the indicator organism. Five active culture were obtained. Four of them yielded products identical with virginiamycins M and S,[8] feldemycin[2] and 2-acetyl-2-decarboxamidooxytetracycline[11] by physico-chemical properties and spectral data.

An active culture designated *Streptomyces clavifer* strain N924-2 has been found to produce a new compound named BU-3619E (BMY-28566). The detail of this compound and taxonomy of the producing organism will be described in separate paper.[3] These antibiotics were found to exhibit inhibitory activity to melanin formation in both *S. bikiniensis* and B16 melanoma cells but did not inhibit mushroom tyrosinase.

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