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Byelyankacin: A Novel Melanogenesis Inhibitor Produced by *Enterobacter* sp. B20

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Abstract A novel melanogenesis inhibitor, byelyankacin (**1**), was isolated from the fermentation broth of a bacterial strain. The producing organism, designated B20, was identified as a member of the genus *Enterobacter* based on taxonomic characteristics. **1** was obtained as a white powder from the culture medium by solvent extraction and serial chromatographic purification. The structure of **1** was determined as (*E*)-4-(2-isocyanovinyl)phenyl α-L-rhamnopyranoside on the basis of spectroscopic data. **1** potently inhibited mushroom tyrosinase and melanogenesis of B16-2D2 melanoma cells with IC_{50} value of 2.1 nM and 30 nM, respectively.

Keywords byelyankacin, melanogenesis inhibitor, mushroom tyrosinase, B16-2D2 melanoma cell, *Enterobacter* sp.

Melanogenesis occurs within melanocytes and results from the melanin pigment in cells. Melanocytes, neural crest-derived cells residing at the basal layer of epidermis have specialized lysosome-like organelles, termed melanosomes, which contain several enzymes that mediate the production of melanin [1]. Tyrosinase catalyzes the initial two rate-limiting reactions of this process, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and its subsequent oxidation to DOPAquinone [2, 3]. Then, tyrosinase-related proteins (TRP-1 and TRP-2) are involved in the processing of DOPAquinone to melanin polymers [4, 5]. Recently, a number of tyrosinase inhibitors from natural products have been used in cosmetics as skin-whitening agents [6~8]. Accordingly, we have screened a wide range of fermented broths for specific inhibitory effects on melanin production by melanocytes. Previously, we have reported screening program for melanogenesis inhibitors from microorganisms, using the larval haemolymph of the silkworm, *Bombyx mori*, in which we have reported melanoxadin [9] and melanoxazal [10] from the fermented broth of fungal strains. On the other hand, in the course of our screening for inhibitors of melannogenesis using mushroom tyrosinase and B16-2D2 melanoma cells, we discovered a new inhibitor, which we named byelyankacin (**1**) (Fig. 1). **1** was produced in the fermentation broth of strain B20, which was isolated from a soil sample collected in Iriomote island, Okinawa, Japan. This strain (B20) was identified as *Enterobacter* sp. from morphological and chemical characteristics, and named *Enterobacter* sp. B20. Then, the characteristics of the strain B20 were compared with the known species of *Enterobacter* described in Bergey's Manual of Systematic Bacteriology [11] and a microscopic picture is depicted in Fig. 2. In this

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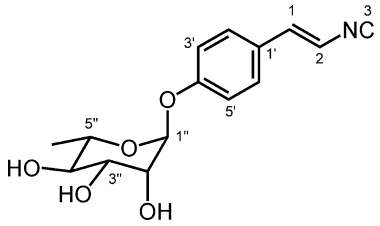

Fig. 1 Structure of byelyankacin (**1**).

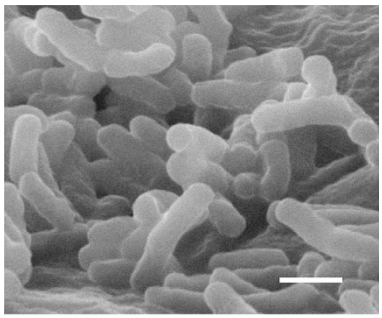

Fig. 2 Scanning electron micrograph of *Enterobacter* sp. B20.

communication, we preliminarily report the production, isolation, structure and biological activities of **1**.

The producing strain identified as *Enterobacter* sp. B20 was found by screening a total of 2970 bacterial strains. This strain has been deposited at the International Patent Organism Depositary, the National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan, as FERM BP-8266. A slant culture of the strain B20 grown on Bennett agar was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 1.0%, potato starch 2.0%, polypeptone 0.5%, yeast extract 0.5% and $CaCO_3$ 0.4%. The pH was adjusted to 7.0 before sterilization. Incubation was done on a rotary shaker (220 rpm) for 3 days at 28°C. Three milliliters of the seed culture was transferred into a 500-ml Erlenmeyer flask containing 150 ml of the production medium (glycerol 4.0%, corn steep liquor 3.0%, $NaNO_3$ 0.4%, $CaCO_3$ 0.2% and $MgSO_4 \cdot 7H_2O$ 0.02%, pH 5.5). The fermentation was carried out on a rotary shaker (220 rpm) for 5 days at 28°C. The fermentation broth (20 liters) was centrifuged and the mycelial cake was extracted with 80% Me_2CO. After removal of Me_2CO, the aqueous solution was extracted twice with EtOAc. The supernatant was extracted with EtOAc. The EtOAc extract from the mycelium and the supernatant were combined and concentrated *in vacuo* to give an oily residue (8.4 g). The oily substance was subjected to silica gel chromatography and eluted with a stepwise gradient of $CHCl_3$ - MeOH (100 : 1, 20 : 1 and 10 : 1). The fraction eluted by $CHCl_3$ - MeOH (10 : 1) was concentrated *in vacuo* to give 1.2 g of crude powder. The powder was applied to an OASIS® HLB extraction cartridge (Waters, 6 g/3 cc) eluting with MeOH, followed by Sephadex LH-20 column (MeOH) to afford an active powder (289 mg). The powder was further purified by a preparative HPLC (column: Senshu Pak PEGASIL ODS, 20 mm×250 mm, Senshu Scientific Co., Ltd.) and developed with 30% acetonitrile (flow rate: 5.0 ml/min; detection, UV 210 nm) to give 110 mg of **1**, as a white powder. The molecular formula of **1** was established as $C_{15}H_{17}NO_5$ [found *m/z* 292.1187 $(M+H)^+$, calcd. 292.1185 for $C_{15}H_{18}NO_5$], on the basis of high-resolution FAB-MS and NMR spectral analyses. The physico-chemical properties of **1** were as follows: $[\alpha]_D^{25}$ −142 (*c* 0.1, MeOH), UV λ_{max}^{MeOH} nm (ε) 210 (15,200), 221 (15,250) and 281 (26,100), IR ν_{max} (KBr) cm^{-1} 3413, 2925, 2119, 1645, 1604, 1510, 1452, 1384, 1321, 1241, 1180, 1124, 1062, 1020, 983. **1** was soluble in DMSO, MeOH, EtOAc and H_2O but insoluble in $CHCl_3$, ether and *n*-hexane. The structure of **1** was determined by the analyses of 2D NMR experiments such as COSY, NOESY and HMBC. The ^{13}C-NMR spectrum of **1** showed 15 carbon signals, which were assigned to one methyl, eleven methines and three quaternary carbons by a DEPT experiment. The ^1H-^1H COSY spectrum of **1** revealed the presence of three partial structures: a trans olefin, 1,4-disubstituted benzene and a rhamnose moiety as shown in Fig. 1. Additionally, the observation of a characteristic absorption at 2119 cm^{-1} in the IR spectrum and ^{13}C-NMR signal at δ_C 164.7 indicated clearly the presence of an isonitrile group attached to sp^2 carbon. The ^{13}C-NMR spectrum of **1** is shown in Fig. 3. The ^1H and ^{13}C chemical shifts assignments (in CD_3OD) for **1** are indicated below: C-1 (δ_C 137.6/δ_H 6.99), C-2 (δ_C 110.6/δ_H 6.52), C-3 (δ_C 164.7), C-1′ (δ_C 128.3), C-2′ and 6′ (δ_C 129.5/δ_H 7.40), C-3′ and 5′ (δ_C 117.8/δ_H 7.06), C-4′ (δ_C 159.0), C-1″ (δ_C 99.7/δ_H 5.45), C-2″ (δ_C 71.9/δ_H 3.98), C-3″ (δ_C 72.2/δ_H 3.82), C-4″ (δ_C 73.7/δ_H 3.45), C-5″ (δ_C 70.8/δ_H 3.57), C-6″ (δ_C 18.0/δ_H 1.20). From these spectrometric data, the structure of **1** was elucidated to be (*E*)-4-(2-isocyanovinyl)phenyl α-L-rhamnopyranoside, named byelyankacin.

Mushroom tyrosinase activity was determined by modified Pomeranz method [12]. In brief, the sample was mixed with the assay medium, consisting of 0.05 ml of L-tyrosine solution (2.5 mM), 0.15 ml of 100 mM sodium phosphate buffer (pH 6.8) in 96-well micro-plates (Corning, New York, USA), and then, with 0.05 ml of mushroom tyrosinase solution (600 U/ml). Heat-inactivated mushroom tyrosinase was used as a control. The plates

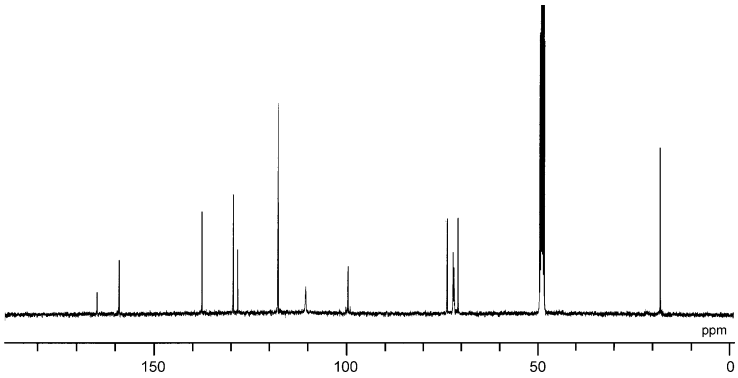

Fig. 3 ^{13}C-NMR spectrum of byelyankacin in CD_3OD (400 MHz).

were incubated at room temperature for 10 minutes, and the absorbance at 475 nm was measured with a micro-plate reader (BioRad 3550, Hercules, CA). Also, the inhibitory effect of **1** on melanogenesis was studied biochemically using a highly pigmented clone B16-2D2 mouse melanoma cells [13]. B16-2D2 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, without phenol red at 37°C in an atmosphere of 95% air and 5.0% CO_2. B16-2D2 cells were seeded at 10^4 cells in 100 μl of growth medium in 96-well micro-plates. After one day, byelyankacin was added to the culture, and cells were further incubated for 5 days. The supernatant of the culture was removed and the cells were lysed in 1.0% Triton X-100, 0.1 N NaOH solution for 20 minutes at room temperature. To calculate the melanin concentration, we measured the absorbance of the supernatants and lysates at 475 nm and compared it with the standard curve of synthetic melanin. The cytotoxicity of the sample was determined by measuring the protein content of the lysates by the method of Bradford protein colorimetric assay kit (BioRad, Hercules, CA).

The inhibitory effects of **1** on mushroom tyrosinase using tyrosine as a substrate, melanogenesis-suppressing activity and cyotoxicity on B16-2D2 mouse melanoma cells are shown in Table 1. **1** showed strong inhibitory activity against mushroom tyrosinase with a 50% inhibitory concentration (IC_{50}) of 2.1 nM. It was 5,200, 11,000, 71,000 and 190,000 times more active, respectively, than hydroquinone, kojic acid, arbutin and ascorbic acid which are now being used as depigmentation agents. The IC_{50} of **1** against melanogenesis of B16-2D2 cells was 30 nM, and that against protein synthesis of the cells was 3.1 mM. These results suggest that byelyankacin is a highly safe tyrosinase inhibitor. Tyrosinase found in plant, animal and fungi tissue frequently differ with respect to their primary structure, size, glycosylation pattern and activation characteristics. However, all tyrosinases have in common a binuclear type 3 copper center within their active site [14]. **1** possesses an isocyanide functionality. It is known that isocyanide binds to copper [15]. Thus, **1** may inhibit tyrosinase activity through binding of copper within the active site. The details on the mode of action and further evaluation of **1** will be reported later.

Table 1 The inhibitory effects of byelyankacin (**1**) on mushroom tyrosinase, melanogenesis-suppressing activity and cell toxicity on B16-2D2 mouse melanoma cells

Inhibitor	IC_{50} (M)		
	Mushroom tyrosinase	B16-2D2 mouse melanoma	
		Melanogenesis	Protein
Byelyankacin	2.1×10^{-9}	3.0×10^{-8}	3.1×10^{-3}
Hydroquinone	1.1×10^{-5}	4.5×10^{-5}	7.2×10^{-5}
Kojic acid	2.3×10^{-5}	3.1×10^{-3}	$>5.0\times10^{-3}$
Arbutin	1.5×10^{-4}	1.2×10^{-4}	5.2×10^{-4}
L-Ascorbic acid	4.0×10^{-4}	3.2×10^{-3}	n.d.

n.d.: not determined.

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