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ORIGINAL ARTICLE

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Indole-3-carbaldehyde: a tyrosinase inhibitor from fungus YL185

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Abstract Indole-3-carbaldehyde (**1**) was isolated as a tyrosinase inhibitor from the ethyl acetate-soluble fraction of extracellular fluids of unknown fungus YL185. The partial sequencing data of 18S ribosomal DNA (18S rDNA) indicate that this isolate belongs to the family Polyporaceae or Corticiaceae sensu lato. Indole-3-carbaldehyde inhibited the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by mushroom tyrosinase with a 50% inhibitory concentration (IC_{50}) of 1.3 mM and showed inhibitory activity on melanin production in B16 melanoma cells. The aldehyde group of **1** plays an important role in eliciting tyrosinase inhibitory activity.

Key words Tyrosinase · Melanin · Inhibitor · Indole-3-carbaldehyde · Fungus

Introduction

Tyrosinase (EC 1.14.18.1; PPO) is known to be a key enzyme for melanin biosynthesis in plants, microorganisms, and mammalian cells.[1] This enzyme catalyzes two reactions: hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity), which in turn are polymerized to brown, red, or black pigments.[1] Many tyrosinase inhibitors have been tested in cosmetics and pharmaceuticals as a way to prevent overproduction of melanin in epidermal layers.[2] Also, tyrosinase is one of the most important key enzymes in the insect molting process,[3] and investigation of its inhibitors may be important for finding alternative insect control agents.[4] Melanin formation is considered deleterious to the color quality of plant-derived food, and prevention of this browning reaction has always been a challenge to food scientists.[5] This broadens the possible use of such tyrosinase inhibitors as food additives in addition to insect control agents and whitening agents. Furthermore, it has been reported that tyrosinase could be central to dopamine neurotoxicity and contribute to the neurodegeneration associated with Parkinson's disease.[6] Tyrosinase is the main enzyme involved in the enzymatic browning of mushrooms, which is responsible for sensory quality loss and loss of nutrient quality. These observations led us to focus on an exploration of tyrosinase inhibitors.

As fungi are increasingly being investigated for biologically active secondary metabolites, we undertook a screening program for tyrosinase inhibitory fungal metabolites. As a result, an extract of an unknown fungus, YL185, has been found to have tyrosinase inhibitory activity. In this study we partially characterized this unknown fungus YL185 by phylogenetic analysis using partial sequencing of the 18S rDNA. We detected an active compound in the extract of YL185 culture and examined its structural criteria for enhancing inhibitory activity.

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Materials and method

Reagents

The chemicals used were L-tyrosine, L-DOPA, ethylenediaminetetraacetic acid (EDTA), cetyltrimethylammonium bromide (CTAB), polyvinylpyrrolidone (PVP) (Wako, Osaka, Japan); mushroom tyrosinase (5350 U/mg), dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA); theophylline, indole-3-carbinol (**2**) (Sigma-Aldrich Chemle Gmbh, Steinhein, Germany); kojic acid, indole-3-carbaldehyde (**1**) (Tokyo Kasei Kogyo, Tokyo, Japan); fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA); Eagle's minimal essential media (EMEM)

(Nissui, Tokyo, Japan); trypsin (Invitrogen, Tokyo, Japan); RNase A (TaKaRa BIO, Shiga, Japan); potato-dextrose agar (PDA), malt extract (Difco, Detroit, MI, USA).

Fungi

Fungus, strain YL185, was isolated from decayed wood in Okinawa Prefecture, Japan. The fungus was maintained on PDA slants.

Enzyme assays

Although mushroom tyrosinase differs somewhat from other sources, this fungal source was used for the present experiment because of its ready available. It should be noted that the commercial tyrosinase was reported to contain numerous proteins in addition to tyrosinase[7] but was used without purification. The temperature was controlled at 25°C using an Ecoline E100 circulating bath (Lauda Co., Germany) with a heater and digital thermometer. The reaction was started by adding the enzyme. Although tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, the assay was carried out in air-saturated solution. Kojic acid was used as a positive control.[2] The sample was first dissolved in DMSO and used for the actual experiment at 30-fold dilution. Controls (without inhibitor) containing DMSO at that concentration were routinely prepared. The assay was performed as previous described.[8] First, 333 μl of 2.5 mM L-DOPA solution was mixed with 600 μl of 0.1 M phosphate buffer ($Na_2HPO_4 \cdot 12H_2O$-$NaH_2PO_4 \cdot 2H_2O$) (pH 6.5) and incubated at 25°C. Then 33 μl of the sample solution and 33 μl of the aqueous solution of mushroom tyrosinase (1380 U/ml) were added to the mixture, and we immediately measured the initial rate of linear increase in optical density at 475 nm on the basis of dopachrome formation, using a V530 spectrophotometer (Jasco, Japan). The extent of inhibition by adding the samples is expressed as the concentration necessary for 50% inhibition (IC_{50}).

B16 melanoma cells assay

B16 melanoma 4A5 cells (Riken Cell Bank, Japan) were grown in plastic tissue culture flasks or plates in EMEM supplemented with 10% FBS and theophylline 0.09 mg/ml. Cells were incubated at 37°C in an atmosphere of 5% CO_2. Confluent cultures were rinsed in phosphate-buffered saline (PBS) and removed from the plastic containers using 0.25% trypsin/EDTA. The cells were placed in 24-well plastic culture plates at a density of 1×10^5 cells/well and incubated for 24 h in medium prior to treatment with the sample. After 24 h, the medium was replaced with 990 μl of flesh medium. To this was added 10 μl of DMSO with or without the test compound (control). This procedure was repeated every other day, and the remaining adherent cells were assayed on day 4 (see below). Thus, the cells were continuously exposed to the test compounds for 4 days.[9]

Determination of melanin content

The melanin content of cells after treatment was determined as follows. After removing the medium and washing cells with PBS, the cells were lysed by adding 1.0 ml of 1 N NaOH. The crude cell extracts were assayed using a microplate reader (Bio-Tek, USA) at 405 nm to determine the melanin content. Results from samples were analyzed as a percent of the control culture.[9] The data were analyzed by Student's *t*-test, with differences of $P < 0.05$ being regarded as significant.

Cell viability and proliferation

The MTT assay was used to determine cellular viability and proliferation after cells were treated by samples for 4 days as described.[10] The degree of cell growth was determined by means of the MTT assay. MTT was dissolved in PBS at a concentration of 5 mg/ml, filtered through a 0.22-μm membrane filter (cellulose acetate, DISMIC-13 cp; Advantec, Tokyo, Japan), and added at a concentration of 5% (v/v) to the culture medium. The culture plate was further incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 4 h. After removing the medium, 1.0 ml of 0.04 N HCl was added, and the absorbance was measured at 570 nm relative to 640 nm. The data were analyzed by Student's *t*-test, with differences of $P < 0.05$ being regarded as significant.

Phylogenetic analysis

Phylogenetic analysis was performed as previously described.[11] For DNA extraction, mycelia were grown on PDA or a 2% (w/v) malt extract and 1.5% (w/v) agar plate. The mycelia were placed with liquid nitrogen in a mortar and ground with a pestle into a fine powder. The mycelium powder was transferred to another mortar and ground with CTAB2 buffer [2% (w/v) CTAB; 1.4 M NaCl; 0.1 M Tris-HCl; 0.1% (v/v) β-mercaptoethanol; 20 mM EDTA; 2% (w/v) PVP; pH 9.0] with RNase A 50 μg/ml. The mixture was put into a 1.5-ml tube and incubated at 65°C for 30 min. The lysate was extracted with the same volume of chloroform twice. The same volume of isopropanol was added to the aqueous layer to precipitate DNA. Precipitated pellets were dissolved in sterilized MilliQ water at 4°C overnight. The mixture was further centrifuged at $\geq$10000 *g* for 15 min, and the supernatant was used. Polymerase chain reaction (PCR) amplification was performed for the 18S rDNA using the primers EukNS20F (5′-TGTAGTCATATGCTTGTCTC AA-3′) and EukNS1750R (5′-TCCTCTAAATGACCAA GTTTG-3′).[12] Amplifications were performed using TaKaRa EX-Taq DNA polymerase (TaKaRa BIO) in a thermal cycler (PC801; Astec, Japan). PCR reactions consisted of an initial denaturation at 94°C for 1.5 min, 32 cycles of amplification, and a final extension at 72°C for 5 min. Each amplification cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. A negative control using MilliQ water instead of

Fig. 1. Partial 18S rDNA sequence of YL185

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AAAGATTAAG CCATGCATGT CTAAGTATAA ACAAGTTTGT ACTGTGAAAC TGCGAATGGC  60
TCATTAAATC AGTTATAGTT TATTTGATGG TGCTTTACTA CATGGATAAC TGTGGTAATT 120
CTAGAGCTAA TACATGCAAT CAAGCCCCGA CTTCTGGAAG GGGTGTATTT ATTAGATAAA 180
AAACCAACGC GGTTCGCCGC TCCCTTGGTG ATTCATAATA ACTTCTCGAA TCGCATGGCC 240
TTGTGCCGGC GATGCTTCAT TCAAATATCT GCCCTATCAA CTTTCGATGG TAGGATAGAG 300
GCCTACCATG GTTTCAACGG GTAACGGGGA ATAAGGGTTC GATTCCGGAG AGGGAGCCTG 360
AGAAACGGCT ACCACATCCA AGGAAGGCAG CAGGCGCGCA AATTACCCAA TCCCGACACG 420
GGGAGGTAGT GACAATAAAT AACAATATAG GGCTCTTTCG GGTCTTATAA TTGGAATGAG 480
TACAATTTAA ATCTCTTAAC GAGGAACAAT TGGAGGGCAA GTCTGGTGCC AGCAGCCGCG 540
GTAATTCCAG CTCCAATAGC GTATATTAAA GTTGTTGCAG TTAAAAAGCT CGTAGTTGAA 600
CTTCAGACCT GGCTGGGCGG TCTGCCTAAC GGTATGTACT GTCCGGCTGG GTCTTACCTC 660
TTGGTGAGCC GGCATGCCCT TCACTGGGTG TGTCGGGGAA CCAGGACTTT TACCTTGAGA 720
AAATTAGAGT GTTCAAAGCA GGCTTATGCC CGAATACATT AGCATGGAAT AATAAAATAG 780
GACGTGCGGT TCTATTTTGT TGGTTTCTAG AGTCGCCGTA ATGATTAATA GGGATAGTTG 840
GGGGCATTAG TATTCCGTTG CTAGAGGTGA AATTCTTGGA TTTACGGAAG ACTAACTATT 900
GCGAAAGCAT TTGCCAAGGA TGTTTTCATT AATCAAGAAC GAAGGTTAGG GGATCGAAAA 960
CGATCAGATA CCGTTGTAGT CTTAACAGTA AACTATGCCG ACTAGGGATC GGGCGAACTC 1020
AATTTGATGT GTCGCTCGGC ACCTTACGAG AAATCAAAGT CTTTGGGTTC TGGGGGGAGT 1080
ATGGTCGCAA GGCTGAAACT TAAAGGAATT GACGGAAGGG CACCACCAGG TGTGGAGCCT 1140
GCGGCTTAAT TTGACTCAAC ACGGGGAAAC TCACCAGGTC CAGACATGAC TAGGATTGAC 1200
AGATTGATAG CTCTTTCATG ATTTTATGGG TGGTGGTGCA TGGCCGTTCT TAGTTGGTGG 1260
AGTGATTTGT CTGGTTAATT CCGATAACGA ACGAGACCTT AACCTGCTAA ATAGCCAGGC 1320
CGGCTTTTGC TGGTCGCCGG CTTCTTAGAG GGACTGTCTG CGTCTAGCAG ACGGAAGTTT 1380
GAGGCAATAA CAGGTCTGTG ATGCCCTTAG ATGTTCTGGG CCGCACGCGC GCTACACTGA 1440
CAGAGCCAGC GAGTTTTTTT CCTTGGCCGG AAGGTCTGGG TAATCTTGTG AAACTCTGTC 1500
GTGCTGGGGA TAGAGCATTG CAATTATTGC TCTTCAACGA GGAATACCTA GTAAGCGTGA 1560
GTCATCAGCT CGCGTTGATT ACGTCCCTGC CCTTTGTACA CACCGCCCGT CGCTACTACC 1620
GATTGAATGG CTTAGTGAGG TGTTGGGATT GGCTTCGGGG AGCCGGCAAC GGCACCCTGT 1680
TGCTGAGAAC TTCATCAAAC TTGGTCATTT AGAGGAAGTA AAAGTCGTAA CAAGGTT     1737
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DNA was set up for each experiment. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Japan). Direct sequencing of PCR products was conducted for both strands using the ABI PRIZM 377 Genetic Analyzer (Applied Biosystems, Japan). The sequence reaction was conducted using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Amplified 18S rDNA fragments were sequenced with the following primers: EukNS600R (5′-ATACGCTATTG GAGCTGGAA-3′); EukNS581F (5′-TCCCAGCTCCAA TAGCGTAT-3′); EukNS1165R (5′-CCTGGTGGTGCC CTTCC-3′); and EukNS1149F (5′-GGAAGGGCACCAC CAGG -3′). Sequences were edited with the GENETYX-MAC 9.0 program (Software Development Co., Japan). The partial 18S sequence of YL185 is shown in Fig. 1. It was subjected to the BLAST Search (http://www.ncbi.nlm.nih.gov/blast/index.html) to determine the phylogenetic relations as the first step. BLAST Search was provided by the National Center for Biotechnology Information. Some of the sequence data used in this study were obtained from the DNA Data Bank of Japan (DDBJ), GenBank and the European Molecular Biology Laboratory (EMBL). All accession numbers of the sequence are shown in Fig. 2. Alignment was conducted using CLUSTAL X version 1.63b[13] (using parameter sets as follows: gap opening 10.00, gap extension 0.05, delay divergent sequences (%) 40, DNA transition weight 0.5; the "use negative matrix" was off, and we used IUB DNA weight matrix), and SeqPup 0.6f.[14] All insert-deletion, ambiguity alignment, gaps, and N were omitted from multiple alignment after alignment was complete. Phylogenetic relations were determined using the "bootstrap N-J [neighbor-joining (NJ) method[15]] tree" program in CLUSTAL X. To evaluate the strength of support for the branches of the NJ trees, 100 replications of bootstrap[16] analysis were performed. The heterobasidiomycete "jerry fungi" (*Auricularia* and *Dacrymyces*) were chosen as the outgroup based on the work of Hibbett et al.[17,18] The tree was displayed using TreeView PPC 1.6.6.[19]

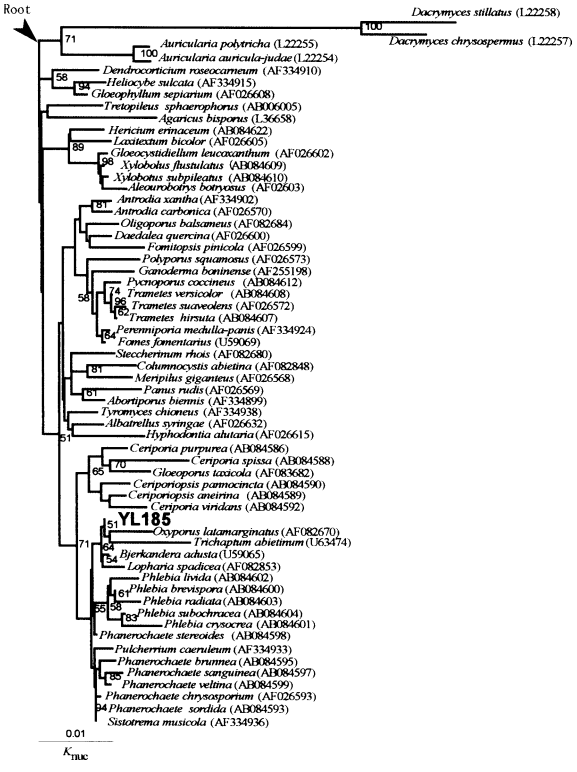
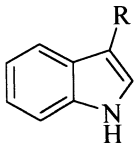


Fig. 2. Neighbor-joining tree derived from the partial 18S rDNA sequence. *Arrowhead* indicates the root. The tree was rooted by the outgroup using the sequence of heterobasidiomycete "jerry fungi." Bootstrap values ≥ 50% are indicated. Bar indicates 0.01 *K*nuc in nucleotide sequences. Accession numbers of the DDBJ/GenBank/EMBL are shown in parentheses

Characterization of tyrosinase inhibitor from YL185

An Erlenmeyer flask containing 200 ml PMY (peptone 5 g/l, malt extract 5 g/l, yeast extract 2 g/l, glucose 15 g/l) was inoculated with 10 agar plugs obtained from 5-day-old PDA plates of fungus YL185. An incubation was carried out at 30°C and 150 rpm until mycelia grew well, after which the culture was divided into mycelia and extracellular fluid. The mycelia were homogenated, suspended in water, and parti-



R= CHO: Indole-3-carbaldehyde (**1**)
R= CH_2OH: Indole-3-carbinol (**2**)

Fig. 3. Structures of indole compounds

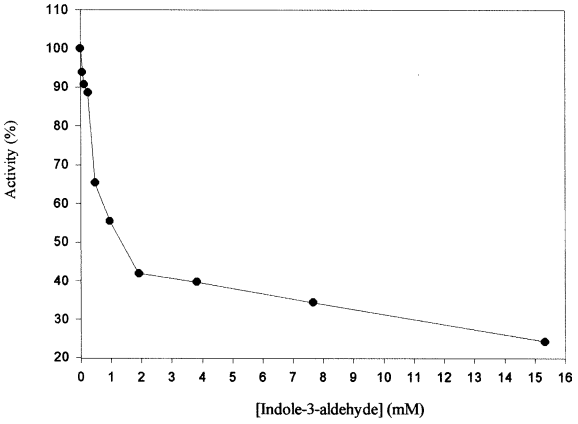


Fig. 4. Effects of indole-3-carbaldehyde on the activity of mushroom tyrosinase for the catalysis of L-3,4-dihydroxyphenylalanine (L-DOPA)

tioned with ethyl acetate successively to give an ethyl acetate-soluble fraction and an aqueous fraction. Neither mycelial fraction showed tyrosinase inhibitory activity at 400 μg/ml. The extracellular fluid was partitioned with ethyl acetate to give ethyl acetate-soluble and aqueous fractions, which showed 38% inhibition or no inhibition of tyrosinase, respectively, at 400 μg/ml. For isolating active compound from the ethyl acetate-soluble extracellular fluid, scale-up cultivation was performed as follows. It should be noted that the ethyl acetate extract of the PMY medium itself showed no inhibitory activity on tyrosinase. The ethyl acetate-soluble fraction (3.5 g) from extracellular fluid of YL185 culture (27.2 L), which showed tyrosinase inhibitory activity as described above, was chromatographed on SiO_2 (700 g) into 42 fractions, eluted with a gradient of increasing amounts of ethyl acetate in *n*-hexane followed by methanol [ethyl acetate: *n*-hexane 1:2, 1.5 L (fractions 1–5) $\rightarrow$ 1:1, 1.5 L (fractions 6–18) $\rightarrow$ 2:1, 1.5 L (fractions 19–35) $\rightarrow$ ethyl acetate, 1.5 L (fractions 36–41) $\rightarrow$ methanol, 1.5 L (fraction 42)]. Of these, fractions 26 and 27, which showed tyrosinase inhibitory activity (44% inhibition at 100 μg/ml), were combined (110 mg), and a part of this combined fraction was further chromatographed by reverse-phased preparative high-performance liquid chromatography (HPLC) (Inertsil PREP-ODS: 20 mm i.d. $\times$ 250 mm) using MeOH/H_2O (30:70) at 12 ml/min to afford the main active principle (3.0 mg) as white powder. This active principle was identified as indole-3-carbaldehyde (**1**) (Fig. 3) by comparing of the spectroscopic data with those of an authentic sample. It should be noted that small amounts of some compounds other than **1** were detected in fractions 26 and 27 but were not characterized because of the small quantities.

Results and discussion

On searching for naturally occurring bioactive compounds from fungal metabolites, we found that the ethyl acetate-soluble fraction of PMY culture of the unknown strain YL185 had tyrosinase inhibitory activity, with 38% inhibition at 400 μg/ml. The fungus is usually identified from its morphological features when basidiomata can be obtained easily. However, we could not obtain basidiomata of the strain YL185 despite all attempts. Recently, phylogenetic analysis using the partial sequence of 18S rDNA has been widely carried out.[17,18,20–22] 18S rDNA was also known for the region, which show a good phylogenetic relation with the morphological classification at the level of genus or upper classification level.[23] Therefore, the phylogenetic analysis using the partial sequence of the 18S rDNA[11] has been applied to characterize the unknown fungus YL185. As the first step, the partial 18S rDNA sequence of YL185 (about 1700 bp) was subjected to BLAST Search to determine the phylogenetic relations. Based on the results of BLAST Search, the 18S rDNA sequences of related basidiomycetes were collected from DDBJ/GenBank/EMBL and our data. A phylogenic tree was obtained from overlapping data sets of the 18S rDNA. There were 235 informative sites in this data set. The Bootstrap NJ analysis tree is shown in Fig. 2. As the result, strain YL185 is related to the genuses *Oxiporus* (current family Schizoporaceae[24]), *Trichaputum*, *Bjerkandera* (current family Hapalopilaceae[24]), and *Lopharia* (current family Phanerochaeteceae[24]). Hibbett and Donoghue[22] defined these genuses as members of group 5. Except for *Lopharia*, the genuses are traditionally classified in the Polyporaceae.[25,26] Group 5 also includes *Phanerochaete* (current family Phanerochaeteceae[24]), *Pulcherricium* (Corticiaceae[24]), *Sistotrema* (current family Sistotremaceae[24]), and *Phlebia* (current family Meruliaceae[24]), which are traditionally classified in the Corticiaceae sensu lato.[25,27] Clade of group 5 (including *Oxiporus*, *Trichaputum*, *Bjerkandera*, *Lopharia*, *Phanerochaete*, *Pulcherricium*, *Sistotrema*, *Phlebia*) was supported with the boot-strap value 71/100. Strain YL185 includes Polyporaceae lineage in group 5, but the bootstrap value for branch of the Polyporaceae lineage and the Corticiaceae lineage is low (<50, not shown). Therefore, it is unclear whether strain YL185 is classified in Polyporaceae or Corticiaceae sensu lato. In this study, strain YL185 was not identified completely, but it was strongly inferred that it belongs to family Polyporaceae or Corticiaceae sensu lato according to Hibbett and Donoghue.[22]

Indole-3-carbaldehyde (**1**) (Fig. 3), an inhibitor, was isolated from YL185 cultivation on mushroom tyrosinase. Inhibition of the enzyme by **1** was concentration-dependent, as shown in Fig. 4. As the concentration of **1** increased, the

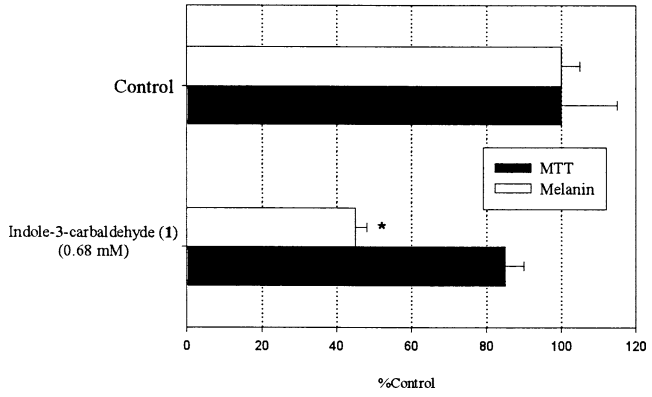


Fig. 5. Effect of indole-3-carbaldehyde (**1**) on melanin production and cell growth using B16 melanoma cells. Each value represents the mean ± SD ($n = 3$). *MTT*, 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide. $*P < 0.05$ compared with the corresponding control

remaining enzyme activity was decreased. The IC_{50} of **1** was 1.3 mM using L-DOPA. It should be noted that kojic acid, which is known to be a potent tyrosinase inhibitor,[2] had an IC_{50} of 0.02 mM.

The melanin biosynthesis inhibitory effect of **1** has been examined using B16 melanoma cells. As shown in Fig. 5, compound **1** inhibited melanin synthesis at 0.68 mM without significant cytotoxicity. These results suggest that **1** is a melanin biosynthesis inhibitor on B16 melanoma cells, possibly by inhibiting tyrosinase activity.

The reason for the tyrosinase inhibitory activity exerted by **1** is still unclear, but at least in part it can be explained as previously reported by Kubo and Kinst-Hori.[28] Aldehydes are generally protein-reactive compounds and are known to react with biologically important nucleophilic groups such as sulfhydryl, amino, or hydroxyl groups. Formation of a Schiff base with a primary amino group in the enzyme is likely, as the aromatic nucleus is known to stabilize it by conjugation. Thus, **1** may form a Schiff base with a primary amine group in the enzyme. Also, it is known that **1** reacts with various secondary amines.[29,30] It has been reported to react with imidazole or 4-methylimidazole to produce corresponding conjugated enamines.[29,30] Several studies have been performed with tyrosinase, thereby revealing conserved histidines with imidazole moieties, which are important for Cu binding and enzymatic activity.[31] Therefore, **1** might bind to histidine residue at active sites in tyrosinase, resulting in inhibitory activity. To further support the above proposal, indole-3-carbinol (**2**) was studied. As expected, **2** no longer exhibited potent inhibitory activity (Fig. 3, Table 1).

It is known that *o*-quinones are highly reactive and have bacteriostatic activity.[32] The browning reaction catalyzed by tyrosinase may be involved in a defensive mechanism against bacterial infection.[33] Tyrosinase inhibitors such as **1** could play a role in vivo as endogenous regulators of the *o*-quinone concentration and melanin pigmentation formed in YL185 with varying degrees of efficiency depending on the microenvironmental conditions of the fungus. Also, indole-3-carbaldehyde is related to the biosynthesis and metabolism of indoleacetic acid. For instance, it was reported that peroxidase catalyzed transformation of indole-3-acetaldehyde to indole-3-carbaldehyde.[34]

Table 1. Inhibitory effect on mushroom tyrosinase by indole compounds

Compound	IC_{50} (mM)
Indole-3-carbaldehyde (**1**)	1.3
Indole-3-carbinol (**2**)	>50[a]

IC_{50}, inhibitory concentration of 50%
[a] Unable to establish an IC_{50} value owing to the solubility problem

We are now further investigating which moiety in **1** causes tyrosinase inhibitory activity and its mode of action. Knowledge of the regulatory control of tyrosinase activity on a molecular basis may provide a more rational, scientific approach to designing a safe, effective tyrosinase inhibitor.

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