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Synthesis and biological activity of hydroxy substituted phenyl-benzo[*d*]thiazole analogues for antityrosinase activity in B16 cells

Young Mi Ha [a], Ji Young Park [b], Yun Jung Park [b], Daeui Park [a], Yeon Ja Choi [a], Ji Min Kim [a], Eun Kyeong Lee [a,c], Yu Kyeong Han [a], Jin-Ah Kim [b], Ji Yeon Lee [b], Hyung Ryong Moon [b,*], Hae Young Chung [a,*]

[a] *Molecular Inflammation Research Center for Aging Intervention (MRCA), College of Pharmacy, Pusan National University, Kumjeong-Gu, Busan 609-735, Republic of Korea*
[b] *Laboratory of Medicinal Chemistry, College of Pharmacy, Pusan National University, Busan 609-735, Republic of Korea*
[c] *Research Center, Dongnam Institute of Radiological and Medical Sciences, Busan 619-953, Republic of Korea*

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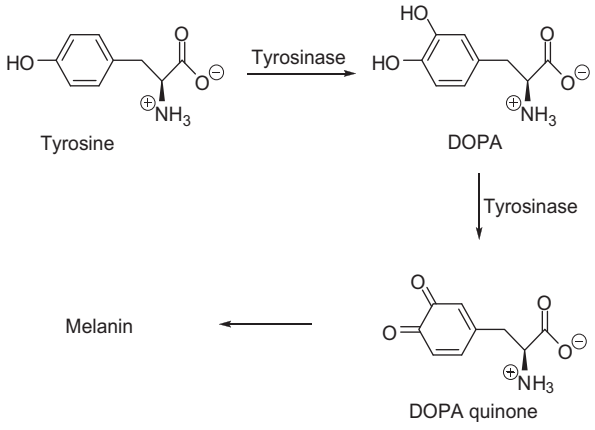
ABSTRACT

In this study, we synthesized hydroxy and/or alkoxy substituted phenyl-benzo[*d*]thiazole derivatives using substituted benzaldehydes and 2-aminothiophenol in MeOH. The structures of these compounds were established by ^{1}H and ^{13}C NMR and mass spectral analyzes. All synthesized compounds were evaluated for their mushroom tyrosinase inhibition activity. Out the 12 generated compounds, **2a** and **2d** exhibited much higher tyrosinase inhibition activity (45.36–73.07% and 49.94–94.17% at 0.01–20 μM, respectively) than kojic acid (9.29–50.80% at 1.25–20 μM), a positive control.

The cytotoxicity of **2a** and **2d** was evaluated using B16 cells and the compounds were found to be non-toxic. Compounds **2a** and **2d** were also demonstrated to be potent mushroom tyrosinase inhibitors, displaying IC_{50} values of 1.14 ± 0.48 and 0.01 ± 0.0002 μM, respectively, compared with kojic acid, which has an IC_{50} value of 18.45 ± 0.17 μM. We also predicted the tertiary structure of tyrosinase, simulated the docking with compounds **2a** and **2d** and confirmed that the compounds strongly interact with mushroom tyrosinase residues. Kinetic plots showed that **2a** and **2d** are competitive tyrosinase inhibitors. Substitutions with a hydroxy group at R^3 or both R^3 and R^1 of the phenyl ring indicated that these groups play a major role in the high binding affinity to tyrosinase. We further found that compounds **2a** and **2d** inhibit melanin production and tyrosinase activity in B16 cells. These results may assist in the development of new potent tyrosinase inhibitors against hyperpigmentation.

Tyrosinase, a copper containing metalloenzyme, is one of the enzymes responsible for skin pigmentation in mammals and is the rate-determining enzyme that catalyzing the first two steps in the melanin-biosynthesis pathway: 3′-hydroxylation of L-tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine) and oxidation of L-DOPA to DOPA quinone (Scheme 1).[1] Most melanin-biosynthesis inhibitors are phenol or catechol analogues, which are structurally similar to tyrosinase substrate, tyrosine or L-DOPA.[2] However, *N*-phenylthiourea (Fig. 1) has been reported to inhibit a catechol oxidase enzyme that belonging to the tyrosinase type-3 copper protein group because its sulfur atom is bound to both copper ions of the enzyme in the active site.[3,4] On the basis of these findings, we considered that it might be interesting to synthesize hydroxyl and/or alkoxy substituted phenyl-benzo[*d*]thiazole derivatives (Fig. 1), in which the sulfur atom of the benzothiazole ring can act as a chelator of copper ions in the active site of tyrosinase, similar to *N*-phenylthiourea. Additionally, because the nitrogen atom of the substituted phenyl-benzo[*d*]thiazole derivatives can



Scheme 1. Pathway for melanin biosynthesis.

* Corresponding authors. Tel.: +82 51 510 2814; fax: +82 51 518 2821 (H.Y.C.); tel.: +82 51 510 2815; fax: +82 51 513 6754 (H.R.M.).
E-mail addresses: mhr108@pusan.ac.kr (H.R. Moon), hyjung@pusan.ac.kr (H.Y. Chung).

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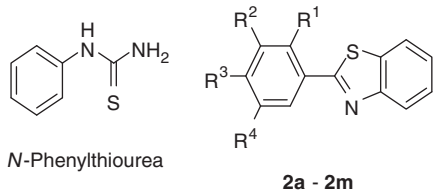
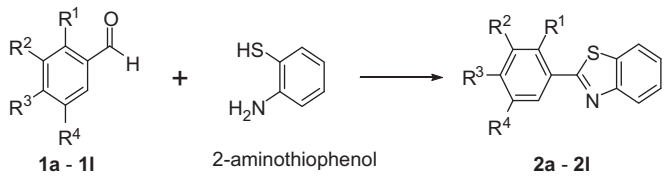


Figure 1. Chemical structures of *N*-phenylthiourea and substituted phenyl-benzo[*d*] thiazole analogues (**2a**–**2l**).

form a quaternary ammonium ion at physiological pH, similar to tyrosine and DOPA, the nitrogen atom might act as a positive center capable of interacting with anionic or partially anionic groups of amino acid residues existing in the tyrosinase active site. Therefore, hydroxy and/or alkoxy substituted phenyl-benzo[*d*]thiazole derivatives might serve as potent competitive tyrosinase inhibitors. The target compounds, substituted phenyl-benzo[*d*]thiazole analogues,[5–7] were prepared by stirring substituted benzaldehydes and 2-aminothiophenol in MeOH (Scheme 2). The structures of the synthesized compounds were determined by ^{1}H and ^{13}C NMR and mass spectral analyzes.

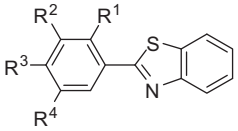
The inhibitory activities of the synthesized compounds were examined using mushroom tyrosinase as described previously with minor modification.[8] The inhibition of mushroom tyrosinase using the synthesized compounds is summarized in Table 1. Our results showed that 2′,4′-dihydroxyphenyl-benzo[*d*]thiazole (**2d**) exhibited the most potent inhibitory activity against mushroom tyrosinase, with 94.17% inhibition at 20 μM. *4′-Hydroxyphenyl-benzo[d]thiazole (**2a**)* and 4′-hydroxy-3′-methoxy-phenyl-benzo[*d*]thiazole (**2g**) lagged behind **2d** in decreasing order of inhibitory potency, with 73.07% and 49.76% inhibition at 20 μM, respectively. Mushroom tyrosinase inhibition with compounds **2a** and **2d** was more potent than that obtained with a reference tyrosinase inhibitor, kojic acid, which exhibited 50.80% inhibition at a concentration of 20 μM. Compounds **2c**, **2e**, **2f**, and **2i**–**2l** exhibited moderate mushroom tyrosinase inhibition, and the remaining compounds, **2b** and **2h**, showed no or low inhibitory activity against mushroom tyrosinase. It is notable that the benzo[*d*]thiazole analogue, **2d**, with a 2′,4′-dihydroxyphenyl scaffold, has a more potent inhibitory effect against mushroom tyrosinase than **2e** with a catechol (3′,4′-dihydroxyphenyl) scaffold, even though compound **2e** with catechol is structurally more similar to DOPA, one of substrates for tyrosinase (Scheme 1). This indicates that a 2′,4′-dihydroxyphenyl scaffold can exhibit more potent inhibitory activity against tyrosinase than a catechol scaffold, depending on the remaining template (e.g., benzo[*d*]thiazole) and excluding the phenyl scaffold.

In this study, we investigated in greater detail the bioactivities of compounds **2a** and **2d**, which exhibit more potent activity than kojic acid. As shown in Table 2, kojic acid, **2a**, and **2d** were found to inhibit mushroom tyrosinase activity in a concentration-dependent manner. The data showed that compounds **2a** (IC_{50} = 1.14 μM) and **2d** (IC_{50} = 0.01 μM) are more potent inhibitors of mushroom tyrosinase than kojic acid (IC_{50} = 18.45 μM), which



Scheme 2. Synthesis of the target compounds, substituted phenyl-benzo[*d*]thiazole analogues, **2a**–**2l**. Reagents and conditions: MeOH, RT, 5–96 h, 15.2–82.6%.

Table 1
Substitution pattern and mushroom tyrosinase inhibition of the synthesized substituted-phenyl-benzo[*d*]thiazole analogues **2a**–**2l**



Compound	R^1	R^2	R^3	R^4	Tyrosinase inhibition[a] (%)
2a	H	H	OH	H	73.07 ± 0.27
2b	OH	H	H	H	−3.91 ± 0.18
2c	H	H	OMe	H	33.02 ± 0.16
2d	OH	H	OH	H	94.17 ± 2.29
2e	H	OH	OH	H	36.10 ± 0.33
2f	H	OH	OMe	H	35.04 ± 0.61
2g	H	OMe	OH	H	49.76 ± 0.30
2h	H	OEt	OH	H	18.42 ± 0.20
2i	OMe	H	OMe	H	31.52 ± 0.18
2j	H	OMe	OMe	H	42.48 ± 0.65
2k	H	OMe	OH	OMe	38.49 ± 0.20
2l	H	OMe	OMe	OMe	36.32 ± 0.23
Kojic acid					50.80 ± 0.57

[a] Tyrosinase inhibition was measured using L-tyrosine as the substrate at 20 μM. Values represent the means ± S.E. of three experiments.

Table 2
Inhibitory effects of compounds kojic acid, **2a**, and **2d** on mushroom tyrosinase activity

Compound	Concn[a] (μM)	Tyrosinase inhibition (%)	IC_{50}[b] (μM)
Kojic acid	0.31	−0.98 ± 0.49	18.45 ± 0.17
	1.25	9.29 ± 0.37	
	5	30.75 ± 0.80	
	20	50.80 ± 0.57	
2a	0.01	45.36 ± 0.38	1.14 ± 0.48
	0.08	46.18 ± 0.47	
	0.31	47.97 ± 0.40	
	1.25	54.67 ± 1.75	
	5	57.87 ± 0.71	
	20	73.07 ± 0.27	
2d	0.01	49.94 ± 0.07	0.01 ± 0.0002
	0.08	70.75 ± 0.27	
	0.31	80.88 ± 0.48	
	1.25	85.60 ± 2.31	
	5	87.73 ± 0.53	
	20	94.17 ± 2.29	

[a] Values represent the means ± S.E. of three experiments.
[b] 50% inhibitory concentration (IC_{50}).

was used as a reference compound. We predicted the tertiary structure of tyrosinase and simulated docking to compounds **2a** and **2d**. Using DOCK6, we searched for tyrosinase residues that may bind to **2a** and **2d**. We found that the most important expected binding residues interacting with compounds **2a** were VAL 93, PRO 96, THR 97, TRP 239, LEU 297, TRP 300, SER 301, TRP 319, PRO 323, ASP 324, GLY 328, and LYS 329, and **2d** were VAL 93, PRO 96, THR 97, TRP 239, LEU 297, SER 301, ASP 324, GLY 328, LYS 329, and THR 350, according to DOCK6 (Fig. 2). Residues were located within 3 Å of the ligand. Docking simulation provided support for the slope–parabolic mixed-type inhibition observed, as this type of inhibition is generally observed when there are multiple-inhibitor binding sites. Docking results showed *that compound **2a** ($-30.33\ kcal{\cdot}mol^{-1}$) combines with mushroom tyrosinase more strongly than compound **2d** ($-27.37\ kcal{\cdot}mol^{-1}$).*

To explore the mechanism of inhibition, we examined the kinetic behaviors of tyrosinase activity in the presence of compounds **2a** and **2d**. The data are shown in Figure 3. The mode of enzyme

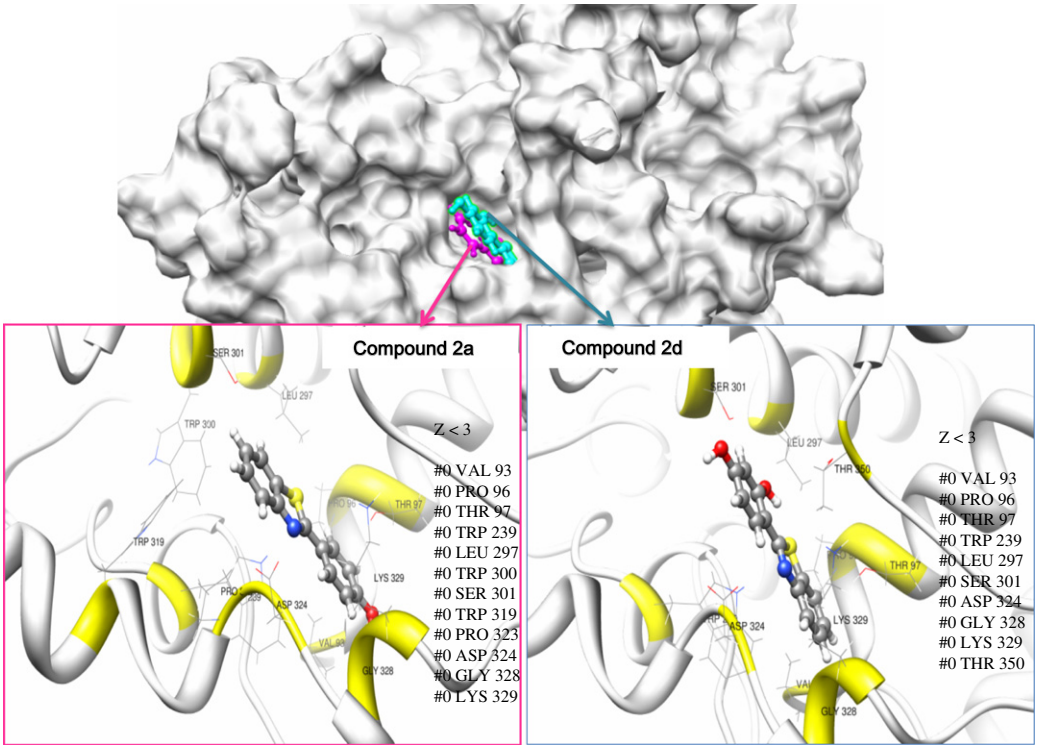


Figure 2. Computational structure prediction for mushroom tyrosinase and docking simulation with compounds **2a** and **2d**. Predicted 3D structure of mushroom tyrosinase. The boxes indicate compound **2a** or **2d** binding sites with tyrosinase residues. The yellow area indicates the tyrosinse–ligand interactions (Z <3).

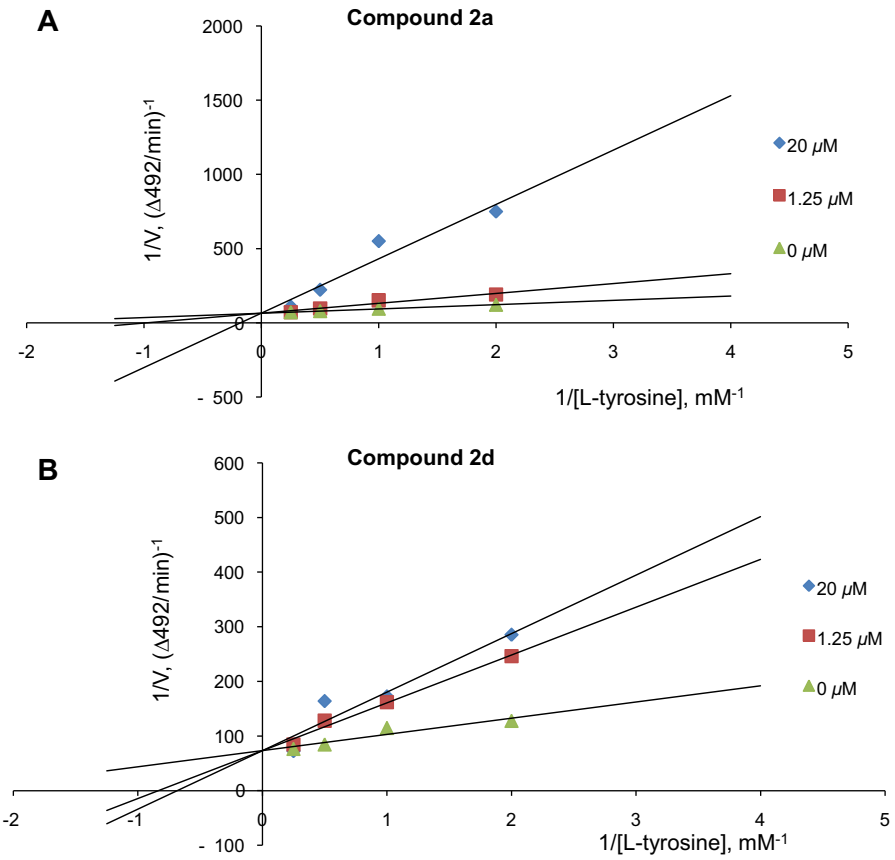


Figure 3. Lineweaver–Burk plot of mushroom tyrosinase. Data were obtained as mean values of 1/V, the inverse of the absorbance increase at a wavelength of 492 nm per min (ΔA_{492} min^{-1}), of three independent tests with different concentrations of L-tyrosine as a substrate. Enzyme inhibitors are indicated as follows: 1.25 μM (square), 20.0 μM (diamond), and no of compound **2a** or **2d** (triangle).

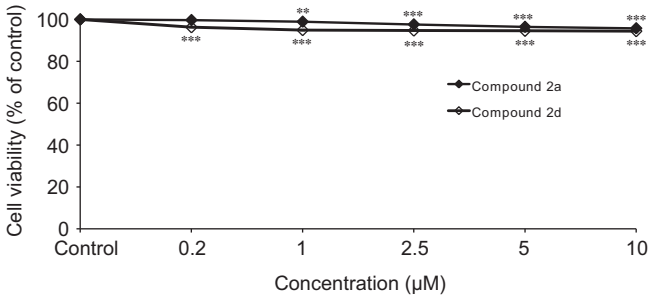


Figure 4. Effect of compounds **2a** and **2d** on B16 cell viability. Cells were treated with varying doses of compound **2a** or **2d** (0–10 μM) and examined using an MTT assay. Data are expressed as a percentage of the control. $^{**}p$ <0.01 and $^{***}p$ <0.001, compared with the untreated control.

inhibition was determined by Lineweaver–Burk plot analysis, in which the inhibitory kinetics of **2a** exhibited K_i values of 5.67×10^{-7} M and 1.70×10^{-6} M at 1.25 and 20 μM, respectively, whereas those of **2d** exhibited K_i values of 6.73×10^{-7} M and 7.70×10^{-6} M at 1.25 and 20 μM of **2d**. Mushroom tyrosinase activity in the presence of compound **2a** at 1.25 and 20 μM, respectively. Mushroom tyrosinase activity in the presence of compound **2a** at 1.25 and 20 μM exhibited K_m values of 1.02 and 5.65 mM, respectively, and the same V_{max} value of 1.54×10^{-2}. In the presence of compound **2d**, mushroom tyrosinase activity at 1.25 and 20 μM exhibited K_m values of 1.16 and 1.46 mM, respectively, and the same V_{max} value of 1.37×10^{-2}. Accompanying the increase in L-tyrosine concentration there was a dose-dependent increase in the K_m value of mushroom tyrosinase without a change in the value of V_{max}, suggesting that both compounds **2a** and **2d** are competitive inhibitors of mushroom tyrosinase. Thus, compounds **2a** and **2d** might exert enzymatic inhibitory activities by binding to the copper active site of mushroom tyrosinase.

To evaluate the cytotoxic effects of compounds **2a** and **2d**, we used the murine B16F10 melanoma cell line (B16 cells). The effects of compounds **2a** and **2d** on cell viability are presented in Figure 4. At doses of 0.2, 1.0, 2.5, 5.0, and 10.0 μM of compounds **2a** or **2d** for 48 h, cell viability was 99.70%, 98.92%, 97.63%, 96.46%, and 95.77% for compound **2a**, and 96.28%, 94.92%, 94.70%, 94.57%, and 94.41% for compound **2d**, respectively, compared with a control. Thus, neither compound **2a** nor **2d** is cytotoxic to B16 cells in the concentration range of 0.2–10 μM.

To assess the inhibitory effects of compounds **2a** and **2d** on melanogenesis, we quantified the melanin content of B16 cells treated with compounds **2a** and **2d** (Fig. 5). The melanin content of B16 cells after treatment with compound **2a** in the presence of 100 nM α-melanocyte-stimulating hormone (α-MSH) decreased dose-dependently, showing 201.43% at 0.2 μM, 187.61% at 1.0 μM, 128.97% at 2.5 μM, 104.05% at 5.0 μM, and 97.14% at 10.0 μM of **2a** (Fig. 5A), compared with the 100 nM α-MSH-only-treated group (242.34%) and the control group (100%). Similarly, cells treated with compound **2d**, exhibited melanin contents of 223.81% at 0.2 μM, 216.91% at 1.0 μM, 189.85% at 2.5 μM, 140.97% at 5.0 μM, and 130.66% at 10.0 μM (Fig. 5B), compared with 100 nM α-MSH-only-treated group (240.13%) and the control group (100%). On the basis of the cytotoxicity results (Fig. 4), inhibition of melanogenesis appears to be due neither to cytotoxic effects nor to cell growth inhibition by compounds **2a** and **2d**. The suppression of melanogenesis by compounds **2a** and **2d** could be

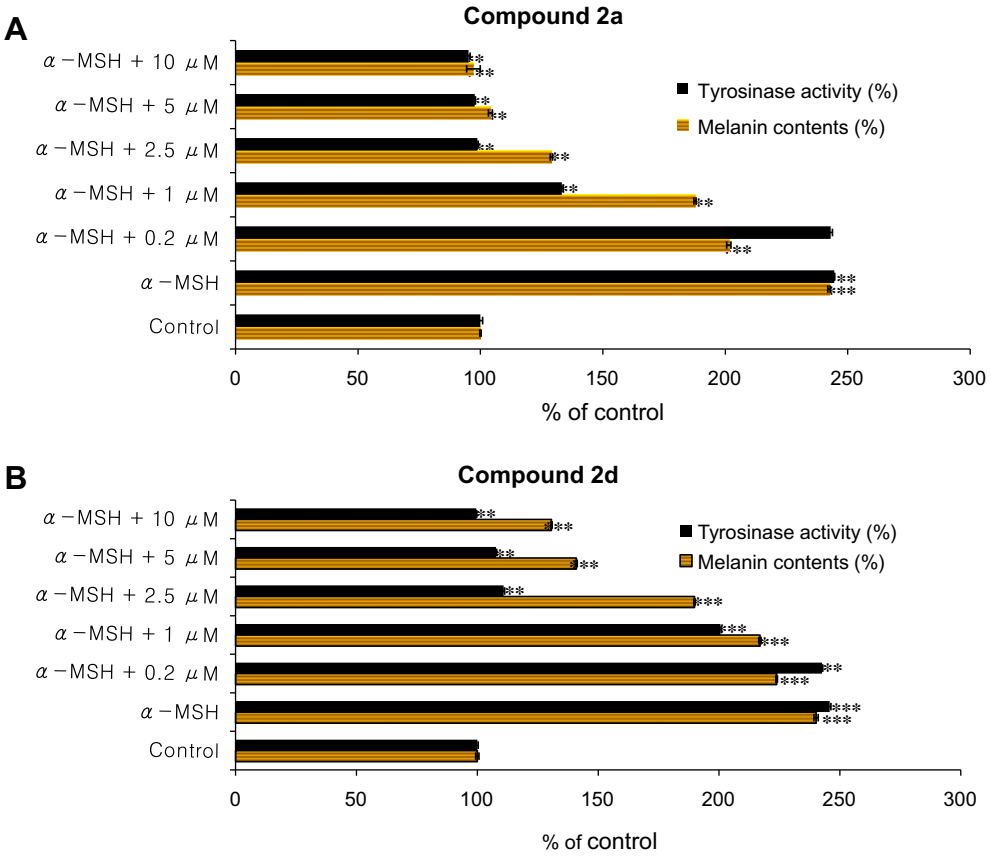


Figure 5. Inhibitory effect of compound **2a** (A) or **2d** (B) after treatment with 100 nM α-MSH in B16 cells. Melanin contents were measured at 405 nm. Values represent the mean ± S.E. of three experiments. Data are expressed as a percentage of the control. $^{**}p$ <0.01 and $^{***}p$ <0.001, compared with the 100 nM α-MSH-treated group. Effect of compound **2a** or **2d** on tyrosinase activity: In the presence of 100 nM α-MSH, B16 cells were treated with varying doses of compound **2a** or **2d** (0.2–10.0 μM) for 24 h. Results are expressed as the percentage of control and each column represents the mean ± S.E. of three determinations. $^{***}p$ <0.001, compared with the 100 nM α-MSH-treated group.

attributed to their inhibition of murine-derived tyrosinase. Although compound **2a** (IC_{50} = 1.14 μM) is a less potent inhibitor of mushroom tyrosinase than compound **2d** (IC_{50} = 0.01 μM), as shown in Table 2, **2a** is a more potent inhibitor of melanogenesis than **2d** in B16 cells.

To analyze the mechanisms by which compounds **2a** and **2d** inhibit melanin biosynthesis, we examined the inhibitory effects of these compounds on cellular tyrosinase activity using murine-derived tyrosinase. After incubation for 24 h with compound **2a** or **2d**, mushroom-derived tyrosinase activities were 242.99% at 0.2 μM, 133.27% at 1.0 μM, 98.78% at 2.5 μM, 97.66% at 5.0 μM, and 95.17% at 10.0 μM of **2a** (Fig. 5A), compared with the 100 nM α-MSH-only-treated group (244.46%) and the control group (100%), and were 242.63% at 0.2 μM, 200.52% at 1.0 μM, 110.84% at 2.5 μM, 107.73% at 5.0 μM, and 99.59% at 10.0 μM of **2d** (Fig. 5B), compared with the 100 nM α-MSH-only-treated group (245.72%) and the control group (100%). Interestingly, contrary to results with mushroom tyrosinase, compound **2a** showed more potent inhibitory activity against murine-derived tyrosinase and more potent inhibition of melanogenesis in B16 cells than **2d**.

In summary, among substituted phenyl-benzo[*d*]thiazole analogues, compounds **2a** and **2d** were found to be approximately 16-fold and 1845-fold, respectively, more potent than kojic acid, which is often used as a positive reference compound. Docking simulation with **2a** and **2d** confirmed that they bind strongly to mushroom tyrosinase. Both **2a** and **2d** were identified as competitive inhibitors of mushroom tyrosinase in a kinetic study. The lack of **2a** and **2d** cytotoxicity indicates that the suppression of melanogenesis by compound **2a** or **2d** can be attributed to the inhibition of murine tyrosinase. These data suggest that both **2a** and **2d** have strong de-pigmenting activities without discernible cytotoxicity in a B16 cell system. These results provide the strongest evidence to date that the 2′,4′-dihydroxyphenyl and 4′-hydroxyphenyl scaffolds among substituted phenyl-benzo[*d*]thiazole analogues exhibit more potent inhibitory activities against tyrosinase than the catechol scaffold, which depends on the remaining template excluding the phenyl scaffold. Both **2a** and **2d** are promising candidates for the development of safe pharmacological or cosmetic agents that have potent inhibitory effects against tyrosinase activity and melanin biosynthesis.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.064.

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