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Synthesis and Bioevaluation of 4,5,6,7-Tetrahydrobenzo[*d*]isoxazole Derivatives as Melanogenesis Inhibitors

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Melanin, the main component of skin color, is synthesized in the melanosomes of melanocytes through a physiological process called melanogenesis and then transferred to neighboring keratinocytes.[1] Melanin absorbs high-energy UV radiation and transforms it into the thermal energy. In this way, melanin plays a protective role against UV-induced DNA damage.[2] However, abnormal skin pigmentation is a major dermatological concern. Abnormal production of melanin causes hypopigmentation or hyperpigmentation such as vitiligo, melasma, age spot, and freckle.[3] In addition, it has been reported that alteration in melanin production is observed in melanoma and Parkinson's disease.[4] Although key factors including melanocortin 1 receptor (MC1R), Microphthalmia associated transcription factor (MITF) and tyrosinase have been found, the molecular mechanism of melanogenesis is not yet fully understood. Potent chemical regulators for melanogenesis could be a useful tool to elucidate the complex mechanisms. In particular, the regulation of dyschromias is of growing interest, and further options for the treatment of hyperpigmentation are in great demand across Asia.[5] Such a demand has led to the discovery of a variety of natural or synthetic chemicals to regulate melanin biosynthesis (Figure 1).[6] Most of the reported melanogenesis inhibitors decrease tyrosinase activity, and a few small molecules such as arbutin[7] and kojic acid[8] are available on the market as skin lightening agents. However, arbutin has shown insufficient activity and kojic acid has toxicity issue.[9] We have reported cyclohepta[*d*]isoxazole derivatives as novel melanogenesis inhibitors that decrease the expression of tyrosinase.[10]

Investigation of the Structure-activity relationship (SAR)

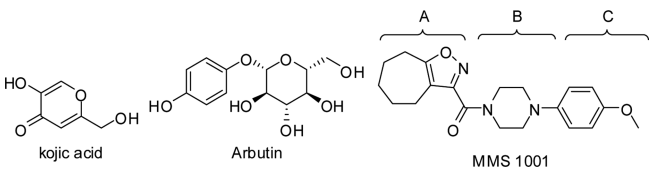
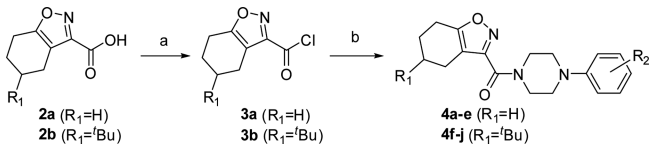
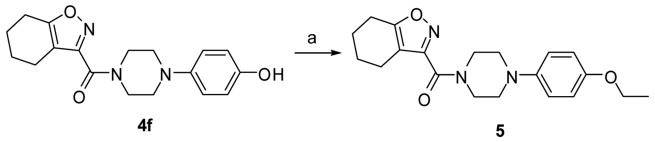


Figure 1. Representative melanin biosynthesis inhibitors and a validated hit compound (MMS1001)[10] as a down-regulator of tyrosinase expression.

[a]These authors contributed equally to this work.



Scheme 1. (a) oxalyl chloride, DMF (cat.), CH_2Cl_2, rt, 2 h; (b) arylpiperazines, Et_3N, THF, rt, 1 h, 60-79% for 2 steps.



Scheme 2. (a) EtI, K_2CO_3, DMF, 80 °C, overnight, 75%.

has been explored through modification of regions B and C, which led to the identification of potent melanogensis inhibitors in our previous study (Figure 1).[10b] In order to explore and further extend the SAR of this series of melanogenesis inhibitors, a variety of the ring contracted derivatives were synthesized. Herein, we describe the synthesis and melanogenesis activity of 4,5,6,7-tetrahydrobenzo[*d*]isoxazole derivatives.

Compounds **4a-j** were synthesized by amide coupling of arylpiperazines with 4,5,6,7-tetrahydrobenzo[*d*]isoxazole-3-carboxylic acid chloride in the presence of triethylamine in dry THF. The acid chlorides **3a-b** were obtained by reaction of commercially available 4,5,6,7-tetrahydrobenzo[*d*]isoxazole-3-carboxylic acid with oxalyl chloride and a catalytic amount of DMF in dichloromethane[11] (Scheme 1). *O*-alkylation of **4f** with iodoethane gave compound **5** (Scheme 2). Melanogenesis activity of melanin synthesis inhibition is shown in Table 1. The *para*-methoxy derivative **4b** exhibited slightly greater potency than the phenol **4a**. However, cytotoxicity was observed at 10 μM. A shift of the methoxy group on the aryl group from the *para* to *ortho* position (**4c**) greatly reduced activity. The introduction of trifluoromethyl at *para* position (**4d**) resulted in almost complete loss of potency at even 10 μM. The *para*-fluoro substituent (**4j**) did not improve activity. Next, we synthesized compounds removing *tert*-butyl group in region A. Phenol **4f** retained activity at 1 μM but showed cytotoxicity at 10 μM.

Table 1. Inhibitory activity of the compounds on α-MSH induced melanogenesis in mouse melanoma cell line B16F10

Compound	R_1	R_2	% inhibition[a]		
			50 μM	10 μM	1 μM
4a	*t*-Bu	*p*-OH		toxic[b]	37
4b	*t*-Bu	*p*-OMe		toxic[b]	49
4c	*t*-Bu	*o*-OMe		14	6
4d	*t*-Bu	*p*-CF_3		13	< 5
4e	*t*-Bu	*p*-F		34	< 5
4f	H	*p*-OH		toxic[b]	32
4g	H	*p*-OMe		50	32
4h	H	*o*-OMe		46	7
4i	H	*p*-CF_3		62	21
4j	H	*p*-F		toxic[b]	17
5	H	*p*-OEt		19	< 5
MMS1001				53	36
Kojic acid	-	-	29		
Arbutin	-	-	27		

[a]Melanin contents were quantified by measuring absorbance at 400 nm and were normalized by the number of viable cells. [b]The number of viable cells decreased to less than 80% of the control. Viability of cells was measured using crystal violet staining.[10a]

Compound **4g** exhibited activity as good as MMS1001 at both 10 μM and 1 μM without cytotoxicity. *para*-Trifluoromethyl compound **4i** showed excellent potency, in contrast to **4d**, indicating that each may be recognized by the target in a different binding mode according to the presence or absence of *tert*-butyl group. Unfortunately, *para*-ethoxy compound **5** did not show significant activity while the corresponding cyclohepta[*d*]isoxazole derivative was highly potent.[10b] The *ortho*-methoxy compound **4h** showed notable activity at 10 μM, but poor activity at 1 μM. As reported in previous study, melanogenesis activity of this scaffold seems to be sensitive to a subtle substituent changes. Taken together, our data suggest that hydroxyl or small alkoxy substituents at the *para* position, except in the case of **4i**, typically promote melanogenesis inhibition in this class of compounds. In summary, we have synthesized 4,5,6,7-tetrahydrobenzo[*d*] isoxazole derivatives and tested the ability of the these molecules to act as melanogenesis inhibitors through potential down-regulation of tyrosinase expression. Compounds **4g**, **4h**, and **4i** were far more potent than the well-known compounds, arbutin and kojic acid, without intrinsic cytotoxicity. Therefore, we believe that they deserve further development as novel melanogenesis inhibitors.

Experimental

General Procedure for Synthesis of 3a-b. To a solution of 4,5,6,7-tetrahydrobenzo[*d*] isoxazole-3-carboxylic acids **2a-b** (1.0 equiv.), oxalyl chloride (3.0 equiv.) in dichloromethane, was added DMF (0.01 equiv.). The mixture was stirred for 2 h at room temperature. The reaction mixture was concentrated in rotary evaporator, dried in *vacuo*. The crude product was used next step without further purification.

General Procedure for Synthesis of 4a-j. To a solution of acid chloride **3a-b** (1.0 equiv.), triethylamine (3.0 equiv.) in dry THF, was added arylpiperazines (1.2 equiv.) in dry THF dropwise. The mixture was stirred for 1 h at room temperature. The reaction mixture was diluted with EtOAc, washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by flash column chromatography on silica gel (EtOAC: hexanes = 1:1-1:5) to yield the desired products (**4a-j**).

Compound 4a: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.82 (m, 4H), 3.90 (m, 4H), 3.11 (s, 4H), 3.02 (m, 2H), 2.59 (m, 1H), 2.35 (m, 1H), 2.11 (m, 1H), 1.34 (m, 2H), 0.95 (s, 9H).

Compound 4b: ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.90-6.93 (m, 2H), 6.84-6.87 (m, 2H), 3.87-4.01 (m, 4H), 3.77 (s, 3H), 3.10-3.18 (m, 4H), 2.96-3.06 (m, 2H), 2.53-2.61 (m, 1H), 2.30-2.37 (m, 1H), 2.00-2.13 (m, 1H), 1.28-1.42 (m, 2H), 0.96 (s, 9H).

Compound 4c: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.01-7.06 (m, 1H), 6.87-6.94 (m, 3H), 3.91-4.02 (m, 4H), 3.89 (s, 3H), 3.09-3.17 (m, 4H), 2.96-3.06 (m, 2H), 2.53-2.61 (m, 1H), 2.31-2.38 (m, 1H), 2.06-2.13 (m, 1H), 1.27-1.42 (m, 2H), 0.96 (s, 9H).

Compound 4d: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.52 (d, *J* = 8.6 Hz, 2H), 6.95 (d, *J* = 8.6 Hz, 2H), 3.92 (m, 4H), 3.37 (s, 4H), 3.03 (m, 2H), 2.56 (m, 1H), 2.35 (m, 1H), 2.13 (m, 1H), 1.34 (m, 2H), 0.96 (s, 9H).

Compound 4e: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.94 (m, 4H), 3.93 (m, 4H), 3.17 (s, 4H), 3.02 (m, 2H), 2.59 (m, 1H), 2.35 (m, 1H), 2.11 (m, 1H), 1.37 (m, 2H), 0.97 (s, 9H).

Compound 4f: ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.80-6.84 (m, 2H), 6.73-6.76 (m, 2H), 3.82-3.92 (m, 4H), 3.07 (s, 4H), 2.68-2.77 (m, 4H), 2.17 (s, 1H), 1.69-1.82 (m, 4H).

Compound 4g: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81-6.95 (m, 4H), 3.92 (s, 4H), 3.78 (s, 3H), 3.18 (s, 4H), 2.69-2.84 (m, 4H), 1.69-1.87 (m, 4H).

Compound 4h: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.00-7.09 (m, 1H), 6.86-6.95 (m, 3H), 3.86-4.02 (m, 4H), 3.89 (s, 3H), 3.13 (s, 4H), 2.71-2.83 (m, 4H), 1.69-1.88 (m, 4H).

Compound 4i: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.52 (d, *J* = 8.5 Hz, 2H), 6.95 (d, *J* = 8.5 Hz, 2H), 3.95 (m, 4H), 3.37 (m, 4H), 2.79 (m, 4H), 1.80 (m, 4H).

Compound 4j: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.99 (m, 2H), 6.90 (m, 2H), 3.93 (m, 4H), 3.17 (m, 4H), 1.06 (m, 4H), 1.79 (m, 4H).

Compound 5: A mixture of **4f** (20 mg, 0.061 mmol), EtI (19 mg, 0.122 mmol) and K_2CO_3 (84 mg, 0.61 mmol) in DMF (1 mL) was stirred overnight at 80 °C. the solids were filtered off and the filtrate was evaporated to give a residue. The residue was purified by flash column chromatography on silica gel (EtOAc:hexanes = 1:3) to afford 16 mg of compound **5** (75%) as a yellowish solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.87 (m, 4H), 3.98 (m, 6H), 3.13 (s, 4H), 2.77 (m, 4H), 1.79 (m, 4H), 1.39 (t, *J* = 7.0 Hz, 3H).

Measurement of Melanin Content. Extracellular melanin release was measured as described previously.[10] Briefly, melanin biosynthesis inhibitory activity of synthesized com-

pounds was evaluated for α-melanocyte-stimulating hormone (α-MSH) induced melanogenesis in mouse melanoma cell line B16F10. The melanoma cells were treated with compounds (1 μM, 10 μM) in the presence of α-MSH (1 μM) and incubated for 3 days. Absorbance was measured at 400 nm to quantify the melanin content in culture media and was normalized by the number of viable cells. The cells were then counted using a hemocytometer. Melanin production was expressed as the percentage of α-MSH-treated controls.

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