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The Depigmenting Activities of Hydroxyl Carboxamide Derivatives Containing Hydrophobic Moiety

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Skin pigmentation results from an increase in either the number of melanocytes or the activity of tyrosinase, where the latter is responsible for melanin synthesis.[1] Tyrosinase, a copper-containing enzyme, is widely distributed in mammals and fungi. In mammals, tyrosinase is involved in the hydroxylation of tyrosine to DOPA and the oxidation of DOPA to dopaquinone.[2] Dopaquinone further undergoes complex reactions to yield melanin. Melanin plays a significant role in protecting the skin against the harmful effects of UV-radiation. However, abnormal melanin production, observed in the form of melasma, freckles, and age spots, can cause esthetic problems. Therefore, inhibitors of tyrosinase have become increasingly important in the medicinal and cosmetic industries.[3] Various polyphenolic compounds, such as hydroquinone,[4] catechol,[5] and resorcinol,[6] have been reported to exhibit a potent depigmenting activity by directly inhibiting tyrosinase. Recently, we synthesized rucinol derivatives containing the adamantane moiety and evaluated their depigmenting activities.[7] We introduced adamantane moiety considering cell penetration. As expected, 4-adamantantyl resorcinol **2** showed more potent depigmenting activity than rucinol **1**. We also found that 2,4-dihydroxy benzamide (**3**) exhibited a potent depigmenting activity in a cell-based assay without direct inhibition of tyrosinase activity. The structure of **3** consists of three parts; a resorcinol group, an amide linkage, and an adamantane moiety. In this study, we synthesized hydroxyl carboxamide derivatives containing adamantane moiety (**4a-4f**) and containing cyclohexane moiety (**5a-5f**), and we evaluated their cytotoxicity, depigmentation, tyrosinase inhibitory, and radical scavenging activities.

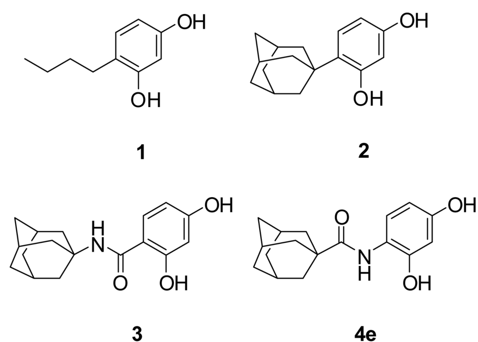
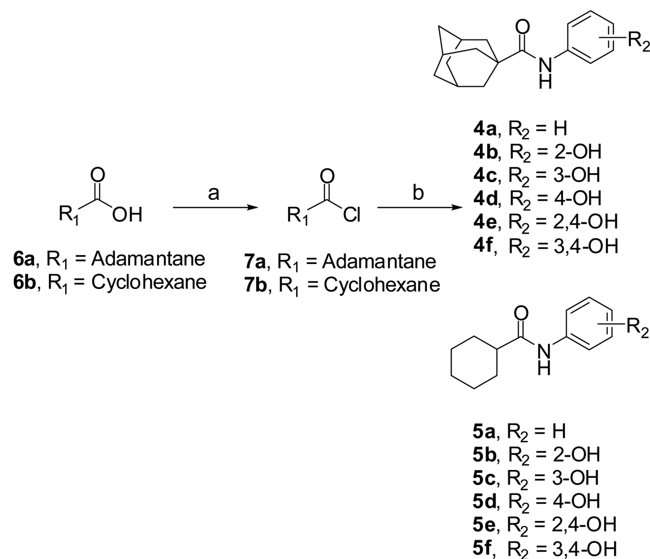


Figure 1. Structures of depigmenting agents.

The synthetic pathways of hydroxyl carboxamide derivatives containing adamantane and cyclohexane moieties are shown in Scheme 1. 1-Adamantanecarboxylic acid (**6a**) and cyclohexanecarboxylic acid (**6b**) were refluxed in thionyl chloride to afford acid chlorides (**7a** and **7b**). These acid chlorides were reacted immediately with various anilines and triethylamine in methylene chloride to produce the corresponding carboxamide derivatives (**4a-4f** and **5a-5f**).

The cytotoxicity of the compounds was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The degree of depigmenting activity was determined using a melanin content assay. The results are listed in Table 1. Depigmenting activity (IC_{50} > 50 μM) and



Scheme 1. Reaction conditions: (a) $SOCl_2$, reflux; (b) anilines, triethylamine, methylene chloride.

Table 1. Depigmentation and cytotoxicity of resorcinol derivatives (**2** and **3**), hydroxyl carboxamide derivatives (**4a**-**4f** and **5a**-**5f**), and hydroquinone

Compound	Inhibition of melanin formation IC_{50}[a]	Cytotoxicity IC_{50}[a]	ClogP[b]
2	7.93 μM	37.36 μM	-
3	25.35 μM	75.81 μM	-
4a	> 50 μM	> 100 μM	3.82
4b	9.43 μM	81.00 μM	3.94
4c	> 50 μM	57.24 μM	3.71
4d	4.61 μM	67.12 μM	3.71
4e	18.70 μM	72.08 μM	3.27
4f	6.37 μM	39.85 μM	3.11
5a	> 50 μM	> 100 μM	3.19
5b	39.44 μM	> 100 μM	2.75
5c	> 50 μM	> 100 μM	2.52
5d	7.69 μM	71.11 μM	2.52
5e	12.66 μM	61.20 μM	2.08
5f	8.78 μM	59.62 μM	1.92
Hydroquinone	4.01 μM	7.5 μM	-

[a]Values were determined from the logarithmic concentration-inhibition curves and are given as the mean values of the results of 3 experiments. [b]Clog P values are obtained from Chem Draw 12.0 V. – : Not examined

Table 2. Tyrosinase inhibitory activity and antioxidant activity of resorcinol derivatives (**2** and **3**), hydroxyl carboxamide derivatives (**4a**-**4f** and **5a**-**5f**), and hydroquinone

Compound	Tyrosinase inhibitory activity IC_{50}[a]	Antioxidant activity IC_{50}[a]
2	1.35 μM	81.22 μM
3	> 200 μM	> 300 μM
4a	> 200 μM	> 300 μM
4b	> 200 μM	> 300 μM
4c	> 200 μM	> 300 μM
4d	> 200 μM	> 300 μM
4e	> 200 μM	5.83 μM
4f	> 200 μM	4.87 μM
5a	> 200 μM	> 300 μM
5b	> 200 μM	> 300 μM
5c	> 200 μM	> 300 μM
5d	> 200 μM	> 300 μM
5e	> 200 μM	6.02 μM
5f	> 200 μM	8.03 μM
Hydroquinone	10.01 μM	-

[a]Values were determined from the logarithmic concentration-inhibition curves and are given as the mean values of the results of three experiments. – : Not examined

cytotoxicity (IC_{50} > 100 μM) were not observed for **4a**, which did not contain any hydroxyl group. Compound **4b** (IC_{50} = 9.43 μM), which contained the 2-hydroxyl group, and **4d** (IC_{50} = 4.61 μM), which contained the 4-hydroxyl group, showed potent depigmenting activities. However, **4c**, which contained the 3-hydroxyl group (IC_{50} > 50 μM), did not show any depigmenting activity. The dihydroxyl compounds (**4e** and **4f**) also showed potent depigmenting activity. The inhibitory effects of **4e** (IC_{50} = 18.70 μM) and **4f** (IC_{50} = 6.37 μM) were slightly lower than that of **4d**. Among the tested compounds containing adamantane moiety, *N*-(4-hydroxyphenyl)adamantane carboxamide (**4d**) showed the most potent inhibitory activity. Its activity (IC_{50} = 4.61 μM) was comparable to that of hydroquinone (IC_{50} = 4.01 μM). Next, we substituted the adamantane moiety with a cyclohexane group to determine the importance of hydrophobic character and bulkiness. The hydrophobicity and bulkiness of the cyclohexane group was less than that of the adamantane moiety. The cytotoxicity and depigmenting activity tendencies of **5a-5f** were similar to those of **4a-4f**. In the cyclohexanecarboxamide derivatives, *N*-(4-hydroxyphenyl)-cyclohexanecarboxamide (**5d**) exhibited the most potent activity (IC_{50} = 7.69 μM). From these results, we conclude that 4-hydroxyphenyl moiety can be an effective pharmacophore for depigmentation.

Because tyrosinase catalyzes an oxidation reaction, the antioxidants may exhibit depigmenting activity by inhibiting the oxidation step without directly interacting with tyrosinase.[8] Vitamin C exhibited potent tyrosinase inhibitory activity[9] and this activity may be due to the reducing ability of vitamin C. Further, we evaluated the inhibition of tyrosinase and the antioxidant activity of carboxamide derivatives. The tyrosinase inhibitory activity was determined using a mushroom tyrosinase assay, whereas the antioxidant activity was determined using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The results are listed in Table 2.

In the tyrosinase assay, 4-adamantyl resorcinol (**2**) exhibited potent inhibitory activity (IC_{50} = 1.35 μM). However, there was no inhibitory activity in the case of compound **3**. In addition, **4a-4f** and **5a-5f** also did not exhibit any inhibitory activities. From these results, we conclude that the introduction of amide and carboxamide linkages negatively influenced on the binding in tyrosinase. In the DPPH assay, dihydroxyl compounds (**4e** and **4f**) exhibited inhibitory activity (IC_{50} = 5.83 μM and IC_{50} = 4.87 μM, respectively). The radical scavenging activities of **5a-5f** were similar to those of **4a-4f**, and the dihydroxyl compounds (**5e** and **5f**) showed inhibitory activity (IC_{50} = 6.02 μM and IC_{50} = 8.03 μM, respectively). In the radical scavenging activity, only carboxamide derivatives (**4e**, **4f**, **5e**, and **5f**) containing dihydroxyl group exhibited inhibitory activity which is in contrast to benzamide derivative (**3**).

In conclusion, we synthesized a series of hydroxyl carboxamide derivatives containing the adamantane moiety (**4a-4f**) and cyclohexane moiety (**5a-5f**). First, we evaluated their depigmenting activities. We found that monohydroxyl derivatives (**4b**, **4d**, **5b**, and **5d**) and dihydroxyl derivatives (**4e**, **4f**, **5e**, and **5f**) significantly suppressed cellular melanin formation without inhibiting tyrosinase activity. This study revealed that the depigmenting activity of carboxamide derivatives may not be related to the direct inhibition of tyrosinase. Among the derivatives, **4d** (4-hydroxyl compound) containing the adamantane moiety showed stronger inhibitory activities (IC_{50} = 4.61 μM) than hydroquinone (IC_{50} = 4.01 μM). In the DPPH assay, only dihydroxyl derivatives (**4e**, **4f**, **5e**, and **5f**) showed radical scavenging activities. Recently,

the depigmenting mechanism of a dihydroxy benzamide derivative has been elucidated.[10] Furthermore, ongoing studies are being conducted to clarify the detailed depigmenting mechanism of hydroxyl carboxamide derivatives.

Experimental Section

***N*-Phenyladamantanecarboxamide (4a).** 1-Adamantanecarboxylic acid **6a** (9.0 g, 0.05 mol) was dissolved in 100 mL of $SOCl_2$ at 0 °C and refluxed for 1 h. The $SOCl_2$ was removed *in vacuo*, and crude acid chloride was dissolved in CH_2Cl_2 (50 mL). To a solution of aniline (4.6 g, 0.05 mol) and triethylamine (6.1 g, 0.06 mol) in methylene chloride (200 mL) was added prepared acid chloride solution. The reaction mixture was stirred for 4 h at room temperature. The reaction mixture was concentrated *in vacuo* and the residue was extracted with ethyl acetate (350 mL), washed with water. The organic layer was dried with anhydrous $MgSO_4$ and concentrated to give a crude product. The resultant was purified with column chromatography using ethyl acetate and hexane to give a *N*-phenyladamantanecarboxamide (**4a)** (10.3g) in 81% yields.

^{1}H NMR (300 MHz, DMSO-d_6) δ 9.07 (s, 1H), 7.65 (d, 2H, J = 8.1Hz), 7.26 (m, 2H), 7.01 (m, 1H), 2.01 (s, 3H), 1.90 (s, 6H), 1.70 (s, 6H). ^{13}C-NMR (125 MHz, DMSO-d_6) 175.9, 139.3, 128.3, 123.1, 120.3, 40.9, 38.3, 36.0, 27.7. ESI MS, *m/e* 256 $[M+H]^+$.

***N*-(2-Hydroxyphenyl)adamantanecarboxamide (4b).** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.90 (s, 1H), 8.45 (s, 1H), 7.84 (d, 1H, J = 7.8 Hz), 6.93 (m, 2H), 6.79 (m, 1H), 2.02 (s, 3H), 1.89 (s, 6H), 1.70 (s, 6H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 175.6, 147.4, 126.4, 124.2, 121.3, 119.0, 115.3, 40.9, 38.5, 35.9, 27.5. ESI MS: (*m/e*) 272 $[M+H]^+$.

***N*-(3-Hydroxyphenyl)adamantanecarboxamide (4c).** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.28 (s, 1H), 8.94 (s, 1H), 7.24 (s, 1H), 7.01 (m, 2H), 6.42 (m, 1H), 2.00 (s, 3H), 1.88 (s, 6H), 1.69 (s, 6H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 175.7, 157.3, 140.3, 128.8, 110.9, 110.1, 107.3, 40.8, 38.2, 35.8, 27.6. ESI MS: (*m/e*) 272 $[M+H]^+$.

***N*-(4-Hydroxyphenyl)adamantanecarboxamide (4d).** ^{1}H NMR (300MHz, DMSO-d_6) δ 9.18 (s, 1H), 8.85 (s, 1H), 7.37 (d, 2H, J = 8.4 Hz), 6.64 (d, 2H, J = 8.4 Hz), 1.99 (s, 3H), 1.87 (s, 6H), 1.69 (s, 6H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 175.4, 153.3, 130.8, 122.3, 114.7, 40.6, 38.0, 36.1, 27.7. ESI MS: (*m/e*) 272 $[M+H]^+$.

***N*-(2,4-Dihydroxyphenyl)adamantanecarboxamide (4e).** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.57 (s, 1H), 9.12 (s, 1H), 8.36 (s, 1H), 7.39 (d, 1H, J = 8.4 Hz), 6.31 (s, 1H), 6.15 (d, 1H, J = 8.4 Hz), 2.00 (s, 3H), 1.87 (s, 6H), 1.69 (s, 6H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 175.5, 154.7, 149.2, 123.3, 118.1, 105.7, 103..0, 40.5, 38.6, 35.9, 27.6. ESI MS, *m/e* 288 $[M+H]^+$.

***N*-(3,4-Dihydroxyphenyl)adamantanecarboxamide (4f).** ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.73 (s, 1H), 8.65 (bs, 2H), 7.13 (s, 1H), 6.79 (d, 1H, J = 8.4 Hz), 6.58 (d, 1H, J = 8.4 Hz), 1.99 (s, 3H), 1.86 (s, 6H), 1.68 (s, 6H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 175.1, 144.5, 141.1, 131.2, 114.8, 111.6, 109.2. ESI MS, *m/e* 288 $[M+H]^+$.

***N*-Phenylcyclohexanecarboxamide (5a).** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.76 (s, 1H), 7.64 (d, 2H, J = 8.1 Hz), 7.28 (m, 2H), 7.02 (m, 1H), 2.31 (m, 1H), 1.61-1.83 (m, 5H), 1.19-1.47 (m, 5H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 174.1, 139.4, 128.5, 119.0, 44.7, 29.0, 25.34, 15.18. ESI MS, *m/e* 204 $[M+H]^+$.

***N*-(2-Hydroxyphenyl)cyclohexanecarboxamide (5b).** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.80 (s, 1H), 9.13 (s, 1H), 7.67 (d, 2H, J = 8.1 Hz), 6.92 (m, 1H), 6.85 (d, 1H, J = 8.1 Hz), 6.72 (m, 1H), 2.45 (m, 1H), 1.60-1.83 (m, 5H), 1.21-1.48 (m, 5H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 174.8, 147.7, 126.4, 124.5, 122.0, 118.9, 115.9, 44.2, 29.2, 25.33, 25.11. ESI MS, *m/e* 220 $[M+H]^+$.

***N*-(3-Hydroxyphenyl)cyclohexanecarboxamide (5c).** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.62 (s, 1H), 9.12 (s, 1H), 7.17 (s, 1H), 6.99 (m, 2H), 6.40 (d, 2H, J = 8.1 Hz), 2.29 (m, 1H), 1.60-1.82 (m, 5H), 1.21-1.46 (m, 5H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 174.0, 157.4, 140.4, 129.0, 109.90, 109.82, 106.2, 44.7, 29.0, 25.33, 25.17. ESI MS, *m/e* 220 $[M+H]^+$.

***N*-(4-Hydroxyphenyl)cyclohexanecarboxamide (5d).** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.48 (s, 1H), 9.09 (s, 1H), 7.36 (d, 2H, J = 8.7 Hz), 6.65 (d, 2H, J = 8.7 Hz), 2.31 (m, 1H), 1.62-1.81 (m, 5H), 1.20-1.46 (m, 5H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.4, 152.9, 131.1, 120.7, 114.8, 44.6, 29.1, 25.37, 25.21. ESI MS, *m/e* 220 $[M+H]^+$.

***N*-(2,4-Dihydroxyphenyl)cyclohexanecarboxamide (5e).** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.60 (s, 1H), 9.09 (bs, 1H), 9.06 (s, 1H), 7.22 (d, 1H, J = 8.4 Hz), 6.28 (s, 1H), 6.15 (d, 1H, J = 8.4 Hz), 2.49 (m, 1H), 1.60-1.80 (m, 5H), 1.02-1.49 (m, 5H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 174.5, 155.0, 149.5, 123.7, 118.1, 105.8, 103.4, 44.0, 29.2, 25.3, 25.1. ESI MS, *m/e* 234 $[M-H]^+$.

***N*-(3,4-Dihydroxyphenyl)cyclohexanecarboxamide (5f).** ^{1}H NMR (300 MHz, DMSO-d_6) 9.38 (s, 1H), 8.84 (s, 1H), 8.48 (s, 1H), 7.12 (s, 1H), 6.79 (d, 1H, J = 8.1 Hz), 6.58 (d, 1H, J = 8.1 Hz), 2.23 (m, 1H), 1.65-1.80 (m, 5H), 1.03-1.49 (m, 5H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.38, 144.7, 140.8, 131.5, 115.1, 110.2, 107.8, 44.6, 29.1, 25.37, 25.22. ESI MS, *m/e* 234 $[M-H]^+$.

Cell Culture. Murine melan-a melanocytes were originally derived from C57BL/6 J (black, a/a) mice, a kind gift from Prof. Dorothy C. Bennett (St. George's Hospital, London, U.K.). Melan-a cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 200 nM of phorbol 12-myristate 13-acetate (TPA) at 37 °C in 10% CO_2. The culture medium was changed twice every week, and the cells were subcultured once a week.

Measurements of Cell Viability. To evaluate the effects of carboxamide derivatives on cell viability, the percentages of viable melan-a cells were determined using a modified crystal violet assay. After removing the medium from each well, the cells were washed with PBS and stained with 0.1% crystal violet in 10 % ethanol for 5 min at room temperature. The cells were then rinsed four times with distilled water,

and crystal violet retained by adherent cells was extracted with 95% ethanol at room temperature for 10 min. Crystal violet absorption was measured at 590 nm (Molecular Devices Co., Sunnyvale, CA, U.S.A.).

Measurements of Melanin Content. The melanin content was measured using the method reported by Hosoi *et al.* with a slight modification. The cells (2×10^5 cells/mL) were seeded into 24-well plates and the test compounds were added in triplicate. The medium was changed daily and after 4 d of culture, the cells were lysed with 1 mL of 1 N NaOH. Then 200 μL of each crude cell extract was transferred into 96-well plates. The relative melanin content was measured at 400 nm with a microplate reader (Molecular Devices).

Mushroom Tyrosinase Assay. Mushroom tyrosinase, L-tyrosine were purchased from Sigma Chemical. The reaction mixture for mushroom tyrosinase activity consisted of 150 μL of 0.1 M phosphate buffer (pH 6.5), 3 μL of sample solution, 8 μL of mushroom tyrosinase (2,100 unit/mL, 0.05 M phosphate buffer at pH 6.5), and 36 μL of 1.5 mM L-tyrosine. Tyrosinase activity was determined by reading the optical density at 490 nm on a microplate reader (Bio-Rad 3550, Richnmond, CA, U.S.A.) after incubation for 20 min at 37 °C. The inhibitory activity of the sample was expressed as the concentration that inhibits 50% of the enzyme activity (IC_{50}).

DPPH Assay. DPPH radical-scavenging assay was carried out using the following procedure. The reaction mixture containing various concentrations of the test samples and DPPH methanolic solution (0.2 mM) was incubated at room temperature for 30 min and the absorbance was measured at 517 nm. The scavenging activity was expressed as a percent compared to control DPPH solution (100%).

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