Mushroom Tyrosinase Inhibition Activity of Some Chromones

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Currently, aloesin is used in the cosmetic industry as a whitening agent because it inhibits tyrosinase activity. Aloesin is a C-glycosylated chromone compound isolated from aloe, and it is difficult to synthesize because of C-glycosyl moiety in the molecule. The purpose of this study is to search for a new chromone compound which is easy to synthesize and which posesses stronger tyrosinase inhibitory activity than aloesin. Fourteen chromone derivatives were synthesized and screened for their mushroom-tyrosinase inhibitory activity. 5-Methyl-7-methoxy-2-(2′-benzyl-3′-oxobutyl)chromone (15) showed the strongest activity among tested compounds. Its activity was not only stronger than aloesin, but also stronger than arbutin and kojic acid. The kinetic analysis revealed a competitive inhibition of 15 with tyrosinase for the L-tyrosine binding site.

Key words aloesin; chromone; tyrosinase inhibitory activity; dopachrome assay; competitive inhibition

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase enzyme widely distributed in nature, which can be found in fungi, and in higher plants and animals.[1)] It catalyzes the conversion of tyrosine to dopa, dopaquinone, and subsequent autopolymerization to melanin.[2)] Tyrosinase inhibitor has been used as a whitening agent or antihyperpigment agent because of its ability to suppress dermal-melanin production. Many scientists are working to isolate tyrosinase inhibitors from natural products. Arbutin,[3)] kojic acid,[4)] and hydroquinones[5)] have been reported to have inhibitory activity. They had been widely used in cosmetic industry as whitening composition. However kojic acid and arbutin have been failed to demonstrate the inhibitory activity of pigmentation in intact melanocytes or in clinical trial.[6)] Hydroquinones are considered to be cytotoxic to melanocytes and potentially mutagenic to mammalian cells.[6)] Since the most widely used compounds failed to demonstrate the clinical efficacy, there is a strong need to develop a new tyrosinase inhibitor that is clinically active.

Aloe (Liliaceae) is a perennial evergreen, herbaceous plant or tropical woody plant. More than 360 species are known in the world. It has long-been used in folk medicine for the treatment of burns and dermatitis. It is also widely used in the cosmetic industry as a moisturizing agent and skin-whitening composition. Recently aloesin (**1**) was reported as a tyrosinase-inhibitory principle to this plant,[7—9)] and it is now used as a whitening composition in cosmetics. Recently one of Korean research group demonstrated clinical efficacy of aloesin.[10)] Currently, aloesin is purified from aloe, however this purification method involves a complex and time-consuming process. Aloesin is difficult to synthesize due to its C-glycosyl moiety.

In this study, we attempted to locate a tyrosinase inhibitory chromone compound which possess more potent tyrosinase-inhibitory activity than aloesin, and which is easier to synthesize.

Results and Discussion

Aloesin is difficult to synthesize because of the C-glycosyl moiety in the molecule. Since the purpose of this study was to search for a new compound that is easy to synthesize, our first goal centered around whether or not a chromone skeleton without C-glycoside has tyrosinase inhibition activity. Consequently, we synthesized compound **2** that has no C-glycosyl moiety, and then we evaluated its activity. Fortunately, compound **2** exhibited stronger inhibition activity (IC_{50}= 0.75 mM) than aloesin (**1**) itself (IC_{50}=0.90 mM).

Our next concern was the alkylation of hydroxyl group at the 7-C-position. The protection and deprotection process of hydroxyl group at the 7-C-position in the synthetic pathway of **2**, reduced the overall yield. Therefore, alkylation of hydroxyl group at the 7-C-position is beneficial since the deprotection process is unnecessary. We synthesized C_1–C_4 alkyl ether derivatives (compounds **3**—**9**), and their activity was examined. The longer alkyl group showed weaker inhibition activity. For example, methyl (**3**), ethyl (**4**), propyl (**5**), and *n*-butyl (**9**) ether compounds exhibited IC_{50} values of 0.07, 0.21, 0.67, and 2.2 mM, respectively. 1-Methylpropyl and 2-methylpropyl derivatives (**7**, **8**) showed stronger inhibitory activity than did the *n*-butyl compound (**9**).

The extension of the terminal alkyl group at the 2-C-position had little effect on the activity. Namely, methyl (**3**), ethyl (**10**), isopropyl-propyl (**11**), and propyl (**12**) derivatives showed IC_{50} of 0.07, 0.06, 0.07 and 0.08 mM, respectively. Modification of the bridge between the chromone ring and the terminal alkyl group had a great effect on the activity. The ethylene derivative (**13**) showed IC_{50} of 0.04 mM, while the methylene derivative (**3**) showed 0.07 mM. The propylene derivative (**14**), however, exhibited decreased activity (IC_{50}=0.09 mM). The phenylethylene derivative (**15**) exhibited stronger activity (IC_{50}=0.03 mM) than did the **13** and **14**.

The mode of inhibition of **15** was studied using L-tyrosine as a substrate. The Lineweaver–Burk plot[11)] showed that **15** inhibits tyrosinase activity in a competitive manner with a K_i value of 2.7×10^{-5} M (Fig. 3). Other deglycosylated compounds (**3**, **10**—**14**) are also exhibited competitive inhibition (data not shown). It is interesting that the chromone C-glycosides, *i.e.* aloesin and 2″-feruloylaloesin, are noncompetitive inhibitors,[7,12)] while the structurally similar **15** is a competitive inhibitor.

Compound **15** exhibited the strongest activity among the 15 chromone compounds. It is a stronger inhibitor than not only aloesin, but also arbutin and kojic acid. Furthermore, it is easy to synthesize, and more hydrophobic than aloesin,

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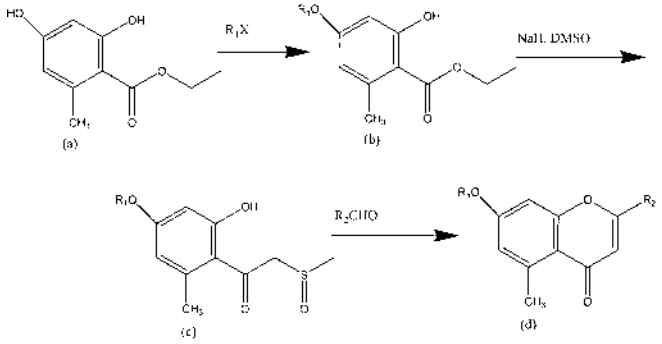


Fig. 1. Synthesis of Chromone Derivatives

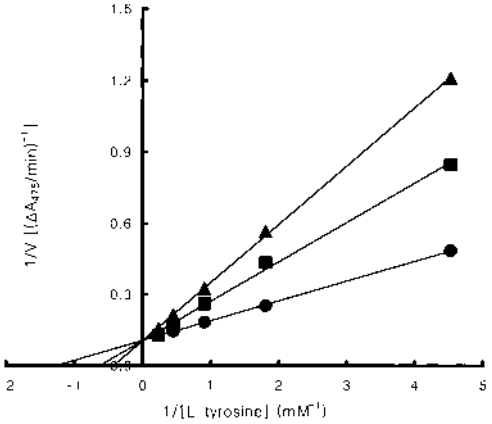
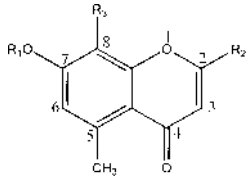


Fig. 2. Lineweaver–Burk Plots Showing the Reciprocal of the Velocity ($1/v$) of the Mushroom–Tyrosinase Reaction *versus* the Reciprocal of the Substrate Concentration ($1/S$) with L-Tyrosine as the Substrate

This result shows the rate of the tyrosinase reaction in the presence of **15** (0.03 mM ■, 0.05 mM ▲) and in the absence of **15** (●).

Table 1. Tyrosinase Inhibition Activity of Chromone Derivatives



Compound No.	R_1	R_2	R_3	IC_{50}[a] (mM)
Aloesin (**1**)	H	CH_2COCH_3	Glc	0.90
2	H	CH_2COCH_3	H	0.75
3	CH_3	CH_2COCH_3	H	0.07
4	CH_2CH_3	CH_2COCH_3	H	0.21
5	$CH_2CH_2CH_3$	CH_2COCH_3	H	0.67
6	$CH(CH_3)_2$	CH_2COCH_3	H	0.91
7	$CH_2CH(CH_3)_2$	CH_2COCH_3	H	1.0
8	$CH(CH_3)CH_2CH_3$	CH_2COCH_3	H	1.4
9	$CH_2CH_2CH_2CH_3$	CH_2COCH_3	H	2.2
10	CH_3	$CH_2COCH_2CH_3$	H	0.06
11	CH_3	$CH_2COCH(CH_3)_2$	H	0.07
12	CH_3	$CH_2COCH_2CH_2CH_3$	H	0.08
13	CH_3	$CH_2CH_2COCH_3$	H	0.04
14	CH_3	$CH_2CH_2CH_2COCH_3$	H	0.09
15	CH_3	$CH_2CH(C_6H_5)COCH_3$	H	0.03
Kojic acid				0.09
Arbutin				0.19

a) The results are from the three concurrent readings and each S.D. was usually within 2% of the mean.

which may be beneficial since it can easily penetrate the epidermis.

Conclusion

In conclusion, deglycosylated compound of aloesin showed stronger tyrosinase inhibitory activity. Methylation of hydroxyl group at the 7-C position increased tyrosinase inhibitory activity. Extension of terminal alkyl group at the 2-C position affected little on the activity. Modification of bridge between chromone ring and terminal alkyl group affected much on the activity. 5-Methyl-7-methoxy-2-(2′-benzyl-3′-oxobutyl)chromone (**15**) exhibited the strongest inhibitory activity among tested compounds. Its activity was stronger than aloesin, arbtin and kojic acid. Compound **15** showed competitive inhibition kinetics.

Experimantal

Chemicals Mushroom tyrosinase, L-tyrosine, kojic acid, and arbutin were purchased from the Sigma Chemical Co. (St. Louis, U.S.A.). Aloesin was isolated from the leaves of *A. vera* in our laboratory.[13] Other chemicals for the synthesis were purchased from the Aldrich Chemical Co. (Wisconsin, U.S.A.).

Synthesis of Chromone Derivatives Compounds **2** and **3** were directly synthesized by Gramatica's method.[14] Compounds **4**—**15** were synthesized by a modified Gramatica's method as follows (Fig. 1): Ethyl-2,4-dihydroxy-6-methylbenzoate (a) was coupled with an alkyl halide taking advantage of a strong intramolecular hydrogen bond between the ethoxycarbonyl group and the hydroxyl group in the ortho-position. Obtained ethyl 2-hydroxy-4-alkoxy-6-methylbenzoate (b) was treated with dimethyl sulfoxide (DMSO)/NaH to yield ethyl 2′-hydroxy-4′-alkoxy-6′-methyl-2-(methylsulfinyl)acetophenone (c) as the key intermediates. (c) was allowed to react with aldehyde in toluene under reflux, which yielded chromone derivatives (d). Fourteen kinds of chromone derivatives were synthesized (Table 1).

5-Methyl-7-hydroxy-2-(2′-oxopropyl)chromone (**2**): Amorphous powder; mp 150—152 °C; Mass (electron impact (EI)) *m/z*: 232 (72) [M^+], 190 (100), 151 (48). ^{1}H-NMR (CD_3OD) δ: 2.26 (3H, s, CO–C$\underline{H}_3$), 2.71 (3H, s, Ar–C$\underline{H}_3$), 6.08 (1H, s, 3-H), 6.63 (2H, d, J=3.18 Hz, 6-H, 8-H).

5-Methyl-7-methoxy-2-(2′-oxopropyl)chromone (**3**): Amorphous powder; mp 90—92 °C; Mass (EI) *m/z*: 246 (90) [M^+], 204 (100), 175 (18), 165 (46). ^{1}H-NMR (CD_3OD) δ: 2.26 (3H, s, CO–C$\underline{H}_3$), 2.71 (3H, s, Ar–C$\underline{H}_3$), 3.87 (3H, s, –OC$\underline{H}_3$), 6.08 (1H, s, 3-H), 6.63 (2H, d, J=3.30 Hz, 6-H, 8-H).

5-Methyl-7-ethoxy-2-(2′-oxopropyl)chromone (**4**): Amorphous powder; mp 48—50 °C; Mass (EI) *m/z*: 260 (91) [M^+], 218 (100), 190 (27), 161 (12), 151 (14). ^{1}H-NMR (CD_3OD) δ: 1.42 (3H, t, J=6.95 Hz, –OCH$_2$C$\underline{H}_3$), 2.29 (3H, s, CO–C$\underline{H}_3$), 2.77 (3H, s, Ar–C$\underline{H}_3$), 4.11 (2H, q, J=3.48 Hz, –OC$\underline{H}_2$CH$_3$), 6.14 (1H, s, 3-H), 6.83 (1H, d, 6-H), 6.92 (1H, d, J=2.46 Hz, 8-H).

5-Methyl-7-propoxy-2-(2′-oxopropyl)chromone (**5**): Brownish yellow oil; Mass (EI) *m/z*: 274 (100) [M^+], 232 (78), 190 (70), 161 (18), 151 (17), 57 (12). ^{1}H-NMR (CD_3OD) δ: 1.02 (3H, t, J=7.42 Hz, –OCH$_2$CH$_2$C$\underline{H}_3$), 1.79 (2H, m, –OCH$_2$C$\underline{H}_2$CH$_3$), 2.22 (3H, s, CO–C$\underline{H}_3$), 2.75 (3H, s, Ar–C$\underline{H}_3$), 4.11 (2H, t, J=4.45 Hz, –OC$\underline{H}_2$CH$_2$CH$_3$), 6.12 (1H, s, 3-H), 6.77 (1H, d, J=2.43 Hz, 6-H), 6.82 (1H, d, J=2.70 Hz, 8-H).

5-Methyl-7-isopropoxy-2-(2′-oxopropyl)chromone (**6**): Brownish yellow oil; Mass (EI) *m/z*: 274 (55) [M^+], 232 (21), 190 (100), 161 (11), 151 (14). ^{1}H-NMR (CD_3OD) δ: 1.33 (6H, d, J=5.85 Hz, –OCH(C$\underline{H}_3$)$_2$), 2.22 (3H, s, CO–C$\underline{H}_3$), 2.74 (3H, s, Ar–C$\underline{H}_3$), 4.70 (1H, m, OC$\underline{H}$(CH$_3$)$_2$), 6.12 (1H, s, 3-H), 6.72 (1H, d, J=2.46 Hz, 6-H), 6.80 (1H, d, J=2.67 Hz, 8-H).

5-Methyl-7-(β-methylpropoxy)-2-(2′-oxopropyl)chromone (**7**): Brownish yellow oil; Mass (EI) *m/z*: 288 (91) [M^+], 262 (25), 246 (18), 232 (26), 190 (100), 151 (19), 57 (18). ^{1}H-NMR (CD_3OD) δ: 1.02 (6H, d, J=6.60 Hz, –OCH$_2$CH(C$\underline{H}_3$)$_2$), 2.08 (1H, m, –OCH$_2$C$\underline{H}$(CH$_3$)$_2$), 2.27 (3H, s, CO–C$\underline{H}_3$), 2.75 (3H, s, Ar–C$\underline{H}_3$), 3.82 (2H, d, J=6.33 Hz, –OC$\underline{H}_2$CH(CH$_3$)$_2$), 6.12 (1H, s, 3-H), 6.77 (1H, d, J=2.06 Hz, 6-H), 6.82 (1H, d, J=2.46 Hz, 8-H).

5-Methyl-7-(α-methylpropoxy)-2-(2′-oxopropyl)chromone (**8**): Brownish yellow oil; Mass (EI) *m/z*: 288 (64) [M^+], 232 (37), 190 (100), 161 (12), 151 (14). ^{1}H-NMR (CD_3OD) δ: 0.98 (3H, t, J=7.44 Hz, –OCH(CH$_3$)CH$_2$C$\underline{H}_3$), 1.30 (3H, d, J=5.85 Hz, –OCH(C$\underline{H}_3$)CH$_2$CH$_3$), 1.70 (2H, m, –OCH(CH$_3$)C$\underline{H}_2$CH$_3$), 2.37 (3H, s, CO–C$\underline{H}_3$), 2.74 (3H, s, Ar–C$\underline{H}_3$), 4.50 (1H, m, –OC$\underline{H}$(CH$_3$)CH$_2$CH$_3$), 6.12 (1H, s, 3-H), 6.74 (1H, d, J=1.68 Hz, 6-H), 6.80 (1H, d, J=2.67 Hz, 8-H).

5-Methyl-7-butoxy-2-(2′-oxopropyl)chromone (**9**): Brownish yellow oil; Mass (EI) *m/z*: 288 (100) [M^+], 246 (54), 232 (9), 190 (68), 161 (12), 151 (14). ^{1}H-NMR (CD_3OD) δ: 1.01 (3H, t, J=7.44 Hz, –$OCH_2CH_2CH_2C\underline{H}_3$), 1.58 (2H, m, –$OCH_2CH_2C\underline{H}_2CH_3$), 1.81 (2H, m, –$OCH_2C\underline{H}_2CH_2CH_3$), 2.26 (3H, s, CO–C$\underline{H}_3$), 2.74 (3H, s, Ar–C$\underline{H}_3$), 4.10 (2H, t, J=6.70 Hz, –$OC\underline{H}_2CH_2CH_2CH_3$), 6.12 (1H, s, 3-H), 6.75 (1H, d, J=2.22 Hz, 6-H), 6.82 (1H, d, J=2.46 Hz, 8-H).

5-Methyl-7-methoxy-2-(2′-oxobutyl)chromone (**10**): Amorphous powder; mp 78—82 °C; Mass (EI) *m/z*: 260 (43) [M^+], 204 (100), 175 (15), 165 (42), 57 (45). ^{1}H-NMR (CD_3OD) δ: 1.06 (3H, t, J=7.23 Hz, –$COCH_2C\underline{H}_3$), 2.63 (2H, q, J=3.29 Hz, –$COC\underline{H}_2CH_3$), 2.74 (3H, s, Ar–C$\underline{H}_3$), 3.86 (3H, s, –O$C\underline{H}_3$), 6.12 (1H, s, 3-H), 6.76 (1H, d, J=3.30 Hz, 6-H), 6.82 (1H, d, J=4.39 Hz, 8-H).

5-Methyl-7-methoxy-2-(2′-oxo-3′-methylbutyl)chromone (**11**): Amorphous powder; mp 46—48 °C; Mass (EI) *m/z*: 274 (53) [M^+], 204 (100), 175 (18), 165 (30), 71 (25). ^{1}H-NMR (CD_3OD) δ: 1.14 (6H, d, J=2.94 Hz, –$OCH(C\underline{H}_3)_2$), 2.67 (1H, m, –$OC\underline{H}(CH_3)_2$), 2.71 (3H, s, Ar–C$\underline{H}_3$), 3.86 (3H, s, –O$C\underline{H}_3$), 6.11 (1H, s, 3-H), 6.74 (1H, d, J=5.33 Hz, 6-H), 6.79 (1H, d, J=6.35 Hz, 8-H).

5-Methyl-7-methoxy-2-(2′-oxopentyl)chromone (**12**): Amorphous powder; mp 68—72 °C; Mass (EI) *m/z*: 274 (45) [M^+], 204 (100), 175 (18), 165 (30), 71 (25). ^{1}H-NMR (CD_3OD) δ: 0.93 (3H, t, J=7.23 Hz, –$COCH_2CH_2C\underline{H}_3$), 1.61 (2H, m, –$COCH_2C\underline{H}_2CH_3$), 2.46 (3H, t, J=7.18 Hz, –$COC\underline{H}_2CH_2CH_3$), 2.72 (3H, s, Ar–C$\underline{H}_3$), 3.85 (3H, s, –O$C\underline{H}_3$), 6.11 (1H, s, 3-H), 6.74 (1H, d, J=2.23 Hz, 6-H), 6.79 (1H, d, J=2.46 Hz, 8-H).

5-Methyl-7-methoxy-2-(3′-oxobutyl)chromone (**13**): Amorphous powder; mp 110—112 °C; Mass (EI) *m/z*: 260 (98) [M^+], 217 (100), 189 (15), 175 (23), 165 (37). ^{1}H-NMR (CD_3OD) δ: 2.19 (3H, s, –$COC\underline{H}_3$), 2.72 (3H, s, Ar–C$\underline{H}_3$), 2.84 (2H, t, J=4.51 Hz, –$COC\underline{H}_2CH_2$), 2.95 (2H, t, J=4.39 Hz, –$COCH_2C\underline{H}_2$), 3.87 (3H, s, –O$C\underline{H}_3$), 6.03 (1H, s, 3-H), 6.74 (1H, d, J=2.65 Hz, 6-H), 6.83 (1H, d, J=2.55 Hz, 8-H).

5-Methyl-7-methoxy-2-(4′-oxopentyl)chromone (**14**): Amorphous powder; mp 54—56 °C; Mass (EI) *m/z*: 274 (95) [M^+], 231 (86), 216 (100), 204 (36), 188 (60), 173 (50), 165 (32). ^{1}H-NMR (CD_3OD) δ: 1.99 (2H, t, J=7.30 Hz, –$COCH_2CH_2C\underline{H}_2$), 2.12 (3H, s, –$COC\underline{H}_3$), 2.53 (4H, m, –$COC\underline{H}_2CH_2CH_2$), 2.77 (3H, s Ar–C$\underline{H}_3$), 3.83 (3H, s, –O$C\underline{H}_3$), 5.98 (1H, s, 3-H), 6.64 (1H, d, J=2.05 Hz, 6-H), 6.74 (1H, d, J=2.14 Hz, 8-H).

5-Methyl-7-methoxy-2-(2′-benzyl-3′-oxobutyl)chromone (**15**): Brownish yellow oil; Mass (EI) *m/z*: 336 (20) [M^+], 294 (95), 266 (40), 175 (88), 165 (26), 91 (100), 65 (25). ^{1}H-NMR (CD_3OD) δ: 2.20 (3H, s, –$COC\underline{H}_3$), 2.68 (3H, s, Ar–C$\underline{H}_3$), 2.87 (1H, m, –$COC\underline{H}(C_6H_5)CH_2$), 3.01 (2H, d, J=7.20 Hz, –$COCH(C_6H_5)C\underline{H}_2$), 3.84 (3H, s, –O$C\underline{H}_3$), 5.93 (1H, s, 3-H), 6.66 (1H, d, J=2.36 Hz, 6-H), 6.78 (1H, d, J=2.43 Hz, 8-H), 7.23 (5H, m, –$COCH(C_6\underline{H}_5)CH_2$).

Enzymatic Assay of Tyrosinase Activity Tyrosinase-inhibition activity was determined by the modified dopachrome method using L-tyrosine as a substrate.[2] Forty microliters of mushroom tyrosinase solution (48 units/ml), 40 μl of 0.1 mg/ml of L-tyrosine in 1/15 M potassium phosphate buffer (pH 6.8), 80 μl of 1/15 M potassium phosphate buffer (pH 6.8), and 40 μl of sample in 5% DMSO solution were added to a 96-well microplate. The assay mixture was incubated at 37 °C for 30 min. Instead of a sample in 5% DMSO solution, 5% DMSO solution was added to a blank solution. Before and after incubation, the amount of dopachrome produced in the reaction mixture was measured at 475 nm in a microplate reader. The percentage of inhibition of tyrosinase activity was calculated as follows:

$$\text{inhibition (\%)} = \frac{(A-B)-(C-D)}{A-B} \times 100$$

A: absorbance of blank solution after incubation
B: absorbance of blank solution before incubation
C: absorbance of sample solution after incubation
D: absorbance of sample solution before incubation

Kinetic Study Inhibition kinetic was studied by the Lineweaver and Burk (double-reciprocal) plot method.[11] Experiments were conducted with the same protocol used in the "Enzymatic assay of tyrosinase activity," except for the concentration of L-tyrosine. Reaction velocities (V) were measured with (0.03, 0.05 mM) and without an inhibitor on the linear part of the kinetics (initial rates) where the amount of substrate is not the limiting parameter. The results were analyzed according to the Lineweaver and Burk plot method that allows the determination of the Michaelis constant (K_m) and maximum velocity (V_m). The inhibition constant (K_i) was calculated as follows, where [I] and [S] are the concentration of inhibitor and substrate, respectively.

$$\frac{1}{V} = \frac{K_m}{V_m} \cdot \left(1 + \frac{[I]}{K_i}\right) \cdot \frac{1}{[S]} + \frac{1}{V_m}$$

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