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Research Article

Synthesis and Biological Evaluation of Kojic Acid Derivatives Containing 1,2,4-triazole as Potent Tyrosinase Inhibitors

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A series of 5-substituted-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole derivatives were synthesized by nucleophilic substitution reaction of 5-hydroxy-2-chloromethyl -4H-pyran-4-one with 5-substituted-3-mercapto-4-amino-1,2,4-triazole, and their inhibitory effects on mushroom tyrosinase were evaluated. The results indicated that most of the synthesized compounds exhibited significant inhibitory activity. Specifically, 5-(4-chlorophenyl)-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6j) exhibited the most potent tyrosinase inhibitory activity with IC_{50} value of 4.50 ± 0.34 μM. The kinetic studies of the compound (6j) demonstrated that the inhibitory effects of the compound on the tyrosinase were belonging to competitive inhibitors. Meanwhile, the structure–activity relationship was also discussed.

Key words: 1,2,4-triazole, kojic acid derivatives, mushroom tyrosinase, tyrosinase inhibitors

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Melanogenesis is a physiological process resulting in the production of melanin pigment, which plays an important role in the prevention of sun-induced skin injury (1). Although the melanin production in human skin is a major defense mechanism against UV light, excessive accumulations of epidermal pigmentation can cause various hyperpigmentation disorders, such as freckle, senile lentigines, and melasma. Tyrosinase (polyphenol oxidase, EC 1.14.18.1) is a multifunctional copper-containing enzyme that is widely distributed in micro-organisms, animals, and plants (2). It can catalyze two distinct reactions during the biosynthesis of melanin, involving the hydroxylation of L-tyrosine to L-dopa and the oxidation of L-dopa to dopaquinone, which is highly reactive and can polymerize spontaneously to form melanin (3). Therefore, the regulation of melanin synthesis via the inhibition of tyrosinase is an important research topic (4). In clinical usage, tyrosinase inhibitors are used for treatments of dermatological disorders related to melanin hyperaccumulation and are essential in cosmetics for depigmentation (5,6), such as age spots and freckle, caused by the accumulation of an excessive level of epidermal pigmentation (7). Inhibition of tyrosinase is equally important commercially. In most fruits and vegetables, the enzyme is responsible for undesired browning that takes place during senescence or damage during postharvest handling, leading to faster degradation and shorter shelf life (8). Therefore, inhibition of tyrosinase is desirable to control browning and reduce economic losses. Accordingly, the development of safe and effective tyrosinase inhibitors is of great concern in clinical medicine and cosmetic industries.

Kojic acid (**1**) is produced by various bacteria and fungi, such as *Penicillium* and *Aspergillus*, and is widely used as a skin-whitening agent because it can inhibit tyrosinase (9). However, its inhibiting effect and storage properties are inadequate for use in cosmetics. Thus, many kojic acid derivatives have been synthesized, usually by modifying the C-7 hydroxyl group to form a variety of new derivatives. Triazole is the core structural motif in a variety of different compounds in medicinal chemistry and has been reported to exhibit a broad range of biological properties, including enzyme inhibition (10,11), antimicrobial (12), antinociceptive (13), and anti-inflammatory activities (14). Studies by Usman Ghani *et al.* (15) have demonstrated that 5-substituted-3-mercapto-4-amino-1,2,4-triazole (**4**) derivatives have promising inhibitory activities of tyrosinase. Therefore, in view of exploring new, potent, and safer inhibitors of tyrosinase, we designed a novel compounds by combining structures of two putative tyrosinase inhibitors, kojic acid (**1**) and 5-substituted-3-mercapto-4-amino-1,2,4-triazole (**4**), to

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form 5-substituted-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole derivatives (**6**) (Figure 1).

Methods and Materials

Chemical reagents and instruments

Melting points were determined on a SGW X-4 melting point apparatus and were uncorrected. IR spectra were recorded on a PE-2000 spectrometer in KBr pellets and are reported in cm^{-1}. All NMR spectra were recorded on a Bruker AV-II 500 MHz NMR spectrometer, operating at 500 MHz for 1H, and 125 MHz for ^{13}C, TMS was used as an internal reference for ^{1}H and ^{13}C chemical shifts, and DMSO-d_6 were used as solvent. Mass spectra were collected on a Waters Xevo Q-TOF HRMS instrument. Tyrosinase, L-3,4-dihydroxyphenylalanine (L-DOPA), and kojic acid were purchased from Sigma-Aldrich Chemical Co (Shanghai, China). Other chemicals were purchased from Aladdin Reagent and Sinopharm Chemical Reagent and were used without further purification.

Synthesis of 5-substituted-3-mercapto-4-amino-1,2,4-triazoles (4)

According to the literature method (16), appropriate acylhydrazine (5.0 mmol) was dissolved in 75 mL absolute alcohol containing KOH (7.5 mmol) at room temperature followed by carbon disulfide (7.5 mmol) and the resulting mixture was stirred at room temperature for 15 h. The precipitated solid was filtered and washed with anhydrous ethanol. The crude product potassium salt **3** (2.0 mmol) was dissolved in hot water (20 mL) and treated with 80% hydrazine hydrate (6.0 mmol). Reaction mixture was refluxed until the reaction mixture changed its color to green. A white solid was precipitated by diluting with cold water (100 mL) and acidifying with concentrated hydrochloric acid. After filtering, the solid was washed with cold water (20 mL) and recrystallized from ethanol to give the pure compound **4**.

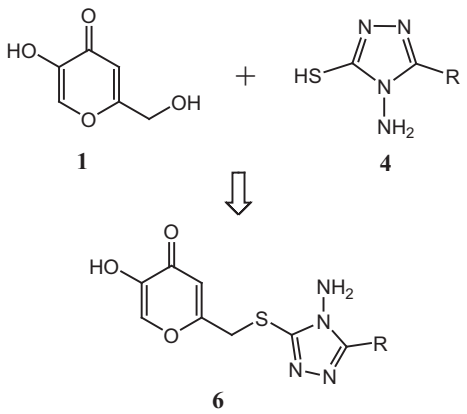


Figure 1: Design of the new tyrosinase inhibitors.

Synthesis of 5-hydroxy-2-chloromethyl-4H-pyran-4-one (5)

Kojic acid (1.42 g, 10 mmol) was added to thionyl chloride (100 mL) in ice bath. The reaction mixture was stirred at room temperature overnight. The formation of white solid was filtered, washed with CH_2Cl_2, and afforded the desired compounds **5**.

General procedure for synthesis of the target compounds (6a-l)

A mixture of 5-substituted-3-mercapto-4-amino-1,2,4-triazoles **4** (1 mmol) and triethylamine (1.1 mmol) was dissolved in DMF (10 mL) in a round-bottomed flask equipped with a reflux condenser. Then, the 5-hydroxy-2-chloromethyl-4H-pyran-4-one **5** (1.1 mmol) was then slowly added dropwise to the mixture, and the reaction solution was heated to 80 °C on a water bath for approximately 12 h. The completion of reaction was monitored by TLC. After removing half of the solvent, 30 mL of water was poured into the flask and the precipitate formed was filtered, which was recrystallized in ethanol and water to obtain the target compounds.

5-methyl-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6a). White powder; yield 52.0%; mp. 175–176 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.29 (s, 3H, CH_3), 4.26 (s, 2H, CH_2), 5.59 (s, 2H, NH_2), 6.37 (s, 1H, CH), 8.03 (s, 1H, CH), 9.12 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 9.86, 31.96, 112.76, 139.82, 145.77, 149.16, 153.85, 163.47, 173.71; IR(KBr) ν: 3318, 3187, 2981, 1612, 1584, 1461, 1383, 1266, 1179, 1143, 1105, 1064, 1019, 958 cm^{-1}; MS (ESI, *m/z*): 277 $(M + Na)^+$. HRMS (ESI, *m/z*) calcd for $[C_9H_{10}N_4NaO_3S]^+$ $(M + Na)^+$ 277.0371, found 277.0367.

5-ethyl-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6b). White powder; yield 55.0%; mp. 181–182 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 1.20 (t, J = 7.5 Hz, 3H, CH_3), 2.66 (q, J = 7.0 Hz, 2H, CH_2), 4.26 (s, 2H, CH_2), 5.90 (s, 2H, NH_2), 6.37 (s, 1H, CH), 8.02 (s, 1H, CH), 9.13 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 10.88, 17.25, 31.97, 112.71, 139.76, 145.75, 149.23, 157.67, 163.43, 173.64; IR(KBr) ν: 3309, 3190, 2970, 2914, 2873, 1635, 1581, 1530, 1424, 1388, 1323, 1286, 1246, 1207, 1183, 1131, 1048, 963 cm^{-1}; MS (ESI, *m/z*): MS (ESI, *m/z*): 291 $(M + Na)^+$. HRMS (ESI, *m/z*) calcd for $[C_{10}H_{12}N_4NaO_3S]^+$ $(M + Na)^+$ 291.0528, found 291.0527.

5-propyl-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6c). White powder; yield 48.0%; mp. 179–180 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 0.93 (t, J = 7.5 Hz, 3H, CH_3), 1.65–1.70 (m, 2H, CH_2), 2.63 (t,

J = 7.5 Hz, 2H, CH_2), 4.26 (s, 2H, CH_2), 5.88 (s, 2H, NH_2), 6.37 (s, 1H, CH), 8.02 (s, 1H, CH), 9.11 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 13.68, 19.60, 25.56, 32.08, 112.71, 139.77, 145.79, 149.14, 156.56, 163.43, 173.66; IR(KBr) ν: 3317, 3189, 2965, 2932, 2873, 1636, 1606, 1582, 1527, 1459, 1421, 1384, 1322, 1286, 1206, 1155, 1132, 942 cm^{-1}; MS (ESI, *m/z*): MS (ESI, *m/z*): 305 (M + Na) $^+$. HRMS (ESI, *m/z*) calcd for $[C_{11}H_{14}N_4NaO_3S]^+$ (M + Na)$^+$ 305.0684, found 305.0675.

5-phenyl-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6d). White powder; yield 51.2%; mp. 205–206 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.36 (s, 2H, CH_2), 6.20 (s, 2H, NH_2), 6.46 (s, 1H, CH), 7.51–7.53 (m, 3H, PhH), 7.98–8.00 (m, 2H, PhH), 8.06 (s, 1H, CH), 9.15 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 32.06, 112.84, 126.73, 127.78, 128.53, 129.78, 139.85, 145.82, 151.85, 154.43, 163.36, 173.69; IR(KBr) ν: 3318, 3179, 3035, 1649, 1583, 1508, 1475, 1455, 1436, 1413, 1339, 1285, 1236, 1214, 1169, 1158, 1135 cm^{-1}; MS (ESI, *m/z*): 317 (M+H) $^+$. HRMS (ESI, *m/z*) calcd for $[C_{14}H_{13}N_4O_3S]^+$ (M+H)$^+$ 317.0703, found 317.0712.

5-(4-methylphenyl)-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6e). Gray powder; yield 42.6%; mp. 201–202 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.37 (s, 3H, CH_3), 4.35 (s, 2H, CH_2), 6.18 (s, 2H, NH_2), 6.45 (s, 1H, CH), 7.32 (d, J = 8.0 Hz, 2H, PhH), 7.88 (d, J = 8.5 Hz, 2H, PhH), 8.06 (s, 1H, CH), 9.16 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 20.99, 32.07, 112.85, 123.93, 127.69, 129.11, 139.50, 139.87, 145.84, 151.60, 154.47, 163.48, 173.71; IR(KBr) ν: 3437, 3168, 3031, 2968, 2753, 1632, 1582, 1460, 1383, 1247, 1217 cm^{-1}; MS (ESI, *m/z*): 331 (M + H) $^+$. HRMS (ESI, *m/z*) calcd for $[C_{15}H_{15}N_4O_3S]^+$ (M + H)$^+$ 331.0859, found 331.0857.

5-(3-methylphenyl)-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6f). Brown powder; yield 39.5%; mp. 170–171 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.37 (s, 3H, CH_3), 4.35 (s, 2H, CH_2), 6.19 (s, 2H, NH_2), 6.45 (s, 1H, CH), 7.31–7.41 (m, 2H, PhH), 7.77–7.80 (m, 2H, PhH), 8.08 (s, 1H, CH), 9.17 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 21.02, 32.03, 112.84, 124.94, 126.64, 128.28, 128.44, 130.39, 137.79, 139.86, 145.84, 151.74, 154.52, 163.37, 173.70; IR(KBr) ν: 3432, 3168, 3028, 2960, 2812, 1638, 1580, 1459, 1386, 1241, 1221 cm^{-1}; MS (ESI, *m/z*): 331 (M + H) $^+$. HRMS (ESI, *m/z*) calcd for $[C_{15}H_{15}N_4O_3S]^+$ (M + H)$^+$ 331.0859, found 331.0855.

5-(2-methylphenyl)-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6g). Brown powder; yield 38.2%; mp. 173–174 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.24 (s, 3H, CH_3), 4.35 (s, 2H, CH_2), 5.95 (s, 2H, NH_2), 6.43 (s, 1H, CH), 7.29–7.32 (m, 1H, PhH), 7.36 (d, J = 7.5 Hz, 1H, PhH), 7.40–7.43 (m, 1H, PhH), 7.47 (d, J = 7.5 Hz, 1H, PhH), 8.07 (s, 1H, CH), 9.20 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 19.86, 32.29, 112.94, 125.55, 126.35, 129.86, 130.30, 130.39, 138.02, 139.92, 145.86, 150.40, 155.22, 163.33, 173.71; IR(KBr) ν: 3412, 3208, 3028, 2962, 2816, 1640, 1580, 1470, 1380, 1241, 1200 cm^{-1}; MS (ESI, *m/z*): 331 (M + H) $^+$. HRMS (ESI, *m/z*) calcd for $[C_{15}H_{15}N_4O_3S]^+$ (M + H)$^+$ 331.0859, found 331.0855.

5-(4-methoxylphenyl)-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6h). Brown powder; yield 36.5%; mp. 223–224 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 3.81 (s, 3H, CH_3), 4.34 (s, 2H, CH_2), 6.16 (s, 2H, NH_2), 6.44 (s, 1H, CH), 7.07 (d, J = 9.0 Hz, 2H, PhH), 7.94 (d, J = 8.5 Hz, 2H, PhH), 8.06 (s, 1H, CH), 9.16 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 32.11, 55.32, 112.83, 113.98, 119.12, 129.31, 139.85, 145.82, 151.23, 154.28, 160.39, 163.40, 173.69; IR(KBr) ν: 3448,3244, 3091, 2938, 2836, 1648, 1589, 1480, 1379, 1249, 1213 cm^{-1}; MS (ESI, *m/z*): 347 (M + H) $^+$. HRMS (ESI, *m/z*) calcd for $[C_{15}H_{15}N_4O_4S]^+$ (M + H)$^+$ 347.0809, found 347.0802.

5-(2-methoxylphenyl)-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6i). Brown powder; yield 40.8%; mp. 116–117 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 3.82 (s, 3H, CH_3), 4.35 (s, 2H, CH_2), 5.74 (s, 2H, NH_2), 6.45 (s, 1H, CH), 7.09 (t, J = 8.5 Hz, 1H, PhH), 7.19 (d, J = 8.5 Hz, 1H, PhH), 7.41–7.56 (m, 2H, PhH), 8.06 (s, 1H, CH), 9.19 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 31.85, 55.85, 112.88, 115.52, 120.59, 131.51, 132.05, 139.86, 145.83, 150.52, 154.44, 156.96, 162.37, 163.42, 173.71; IR(KBr) ν: 3542, 3316, 3070, 2945, 2774, 1646, 1581, 1471, 1449, 1408, 1295, 1224 cm^{-1}; MS (ESI, *m/z*): 347 (M + H) $^+$. HRMS (ESI, *m/z*) calcd for $[C_{15}H_{15}N_4O_4S]^+$ (M + H)$^+$ 347.0809, found 347.0806.

5-(4-chlorophenyl)-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6j). Brown powder; yield 43.5%; mp. 206–207 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.52 (s, 2H, CH_2), 5.74(s, 2H, NH_2), 6.51 (s, 1H, CH), 7.66 (d, J = 9.0 Hz, 2H, PhH), 7.97 (d, J = 8.5 Hz, 2H, PhH), 8.09 (s, 1H, CH), 9.25 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 33.11, 113.33, 121.84, 128.31, 129.64, 136.90, 140.09, 145.95, 162.22, 162.50, 164.90, 173.62; IR(KBr) ν: 3438, 3058, 2969, 2940, 2891, 2671, 1640, 1481, 1429, 1390, 1388, 1151 cm^{-1}; MS (ESI, *m/z*): 351 (M + H) $^+$. HRMS (ESI, *m/z*) calcd for $[C_{14}H_{12}ClN_4O_3S]^+$ (M + H)$^+$ 351.0313, found 351.0321.

5-(2-chlorophenyl)-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6k). Yellow powder; yield 49.5%; mp. 147–148 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.37 (s, 2H, CH_2), 5.96 (s, 2H, NH_2), 6.44 (s, 1H, CH), 7.47–7.51 (m, 1H, PhH), 7.56–7.59 (m, 2H, PhH), 7.63–7.65 (m, 1H, PhH), 8.07 (s, 1H, CH), 9.22 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 32.34, 113.03, 126.21, 127.19, 129.73, 131.96, 132.50, 133.50, 139.95, 145.89, 150.91, 153.88, 163.24, 173.73; IR(KBr) ν: 3420, 3078,

2977, 2941, 2881, 2677, 1646, 1475, 1449, 1397, 1383, 1171 cm^{-1}; MS (ESI, *m/z*): 351 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{14}H_{12}ClN_4O_3S]^+$ $(M + H)^+$ 351.0313, found 351.0315.

5-(2-bromophenyl)-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (6l). Brown powder; yield 43.0%; mp. 160–161 °C; ^1H NMR (DMSO-d_6, 500 MHz) δ: 4.36 (s, 2H, CH_2), 5.91 (s, 2H, NH_2), 6.42 (s, 1H, CH), 7.47–7.54 (m, 3H, PhH), 7.79 (d, J = 8.0 Hz, 1H, PhH), 8.07 (s, 1H, CH), 9.14 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 32.42, 113.01, 123.49, 127.61, 128.31, 132.06, 132.61, 132.78, 139.93, 145.86, 150.57, 155.01, 163.16, 173.69; IR(KBr) ν: 3428, 3038, 2949, 2920, 2893, 2682, 1641, 1480, 1429, 1397, 1388, 1162 cm^{-1}; MS (ESI, *m/z*): 394 $(M + H)^+$. HRMS (ESI, *m/z*) calcd for $[C_{14}H_{12}BrN_4O_3S]^+$ $(M + H)^+$ 394.9808, found 394.9805.

Tyrosinase inhibition assay

Tyrosinase inhibition assays were performed according to a modified method described by Kubo (17).

The synthesized compounds were tested for diphenolase inhibitory activity of tyrosinase using L-DOPA (dihydroxyphenylalanine) as substrate. Mushroom tyrosinase (EC 1. 14. 18. 1, Sigma Product T3824 with an activity of 3320 U/mg) was used for the bioassay. All the compounds were dissolved in DMSO and its final concentration in the reaction mixture was 3.0%. Thirty units of mushroom tyrosinase (0.5 mg/mL) were first preincubated with the compounds, in 50 mM phosphate buffer (pH 6.8) for 20 min at 30 °C. The L-DOPA (final concn 0.5 mM) was added to the mixture, and the enzyme reaction was continuously monitored by measuring the change in absorbance at 475 nm of formation of the dopachrome for 1 min. The activity was expressed as the sample concentration that gave a 50% inhibition in the enzyme activity (IC_{50}). The percent of inhibition of tyrosinase was calculated as follows:

Percent inhibition (%) = [(B - S)/B] × 100

Here, the B and S represent absorbance values for the blank and samples, respectively. All the experiments were carried out at least in triplicate and averaged. The data were expressed as mean ± SE and were evaluated by one-way analysis of variance (ANOVA) followed by a *post hoc* test, or *t*-test using SPSS 19.0. (IBM, Armonk, NY, USA) $p < 0.05$ was considered to be significant. Kojic acid was used as standard inhibitors for the tyrosinase.

Results and discussion

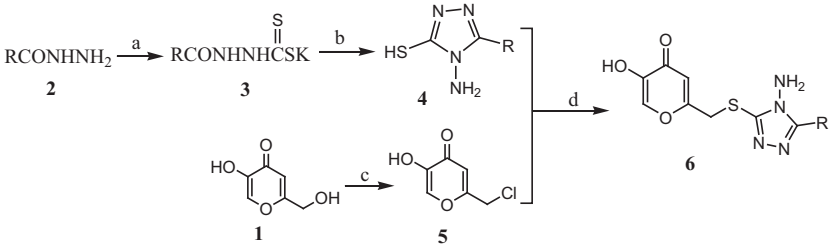
Chemistry

The synthetic procedures employed to obtain the target compounds 5-substituted-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole derivatives (**6**) are depicted in Scheme 1. The raw materials 5-substituted-3-mercapto-4-amino-1,2,4-triazoles (**4**) and 5-hydroxy-2-chloromethyl-4H-pyran-4-one (**5**) were prepared according to the literature method (16). And then, the compounds (**4**) reacted with compounds (**5**) by nucleophilic substitution reaction in DMF medium in the presence of Et_3N to give 5-substituted-3-[5-hydroxy-4-pyrone-2-yl-methymerca-pto] -4-amino-1,2,4-triazole derivatives **6a-6l** with good yields**.** The structures of all the newly synthesized compounds **6a-6l** were confirmed by ^1H NMR, ^{13}C NMR, HRMS, and IR. Their solubility was also surveyed; these newly synthesized compounds could be dissolved in DMF, DMSO, and CH_3OH, but they could not be dissolved in CH_2Cl_2 and H_2O.

Tyrosinase inhibitory activity

For evaluating the tyrosinase inhibitory activity, all the synthesized compounds were subjected to tyrosinase inhibition assay with L-DOPA as substrate using kojic acid as the positive control, according to the typical assay protocol developed by Hearing (18) with some slight modifications. Figure 2 showed that the remaining enzyme activity rapidly decreased with the increasing concentrations of compound **6j**.

The IC_{50} values of 5-substituted-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole derivatives (**6**) against tyrosinase were summarized in Table 1. From the screening results in Table 1, it was observed that most of the synthesized compounds exhibited potent inhibition on mushroom tyrosinase with IC_{50} values ranged from 4.50 ± 0.34 to 91.03 ± 2.19 μM. Specially, compounds **6d, 6e, 6f, 6g, 6h, 6i, 6j, 6k, and 6l** displayed higher inhibitory activities on mushroom tyrosinase *in vitro* than



Scheme 1: General synthesis of compounds (**6a**–**6l**). Reagents and conditions: (a) CS_2/KOH, ethanol; (b) $NH_2NH_2.H_2O$ (80%), H_2O; reflux 3 h; (c) $SOCl_2$, stir 24 h; (d) Et_3N, 80 °C, reflux 12 h.

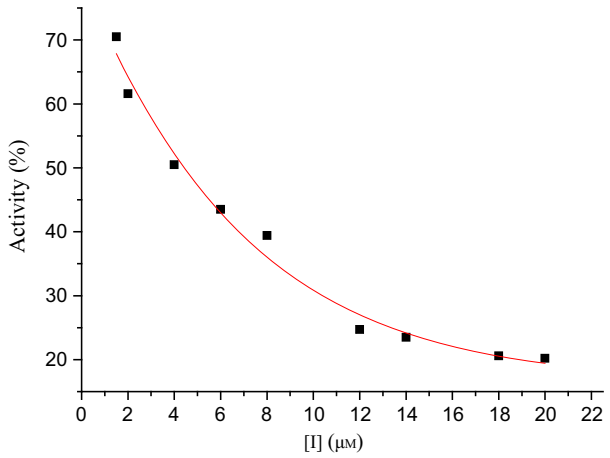


Figure 2: Effect of compound **6j** on the diphenolase activity of mushroom tyrosinase for the catalysis of L-DOPA at 30 °C.

Table 1: Tyrosinase inhibitory activities of the synthesized compounds

Compounds	R	IC_{50} (±SE, μM)[a]
6a	CH_3	91.03 ± 2.19
6b	CH_2CH_3	25.71 ± 0.46
6c	$CH_2CH_2CH_3$	28.16 ± 1.03
6d	Ph	7.45 ± 0.31
6e	p-CH_3Ph	6.25 ± 0.62
6f	m-CH_3Ph	6.82 ± 0.48
6g	o-CH_3Ph	7.12 ± 1.21
6h	p-CH_3OPh	11.20 ± 0.39
6i	o-CH_3OPh	13.21 ± 0.79
6j	p-ClPh	4.50 ± 0.34
6k	o-ClPh	5.35 ± 0.15
6l	o-BrPh	5.98 ± 0.42
Kojic Acid[b]		19.00 ± 0.75

[a]IC50 values: the concentration of the inhibitor required to produce 50% inhibition of tyrosinase, each value represents the mean ± SE of three experiments.
[b]Standard inhibitors of the tyrosinase.

the reference standard inhibitor kojic acid. Although compounds **6a**, **6b,** and **6c** have the inhibitory activities on mushroom tyrosinase, their inhibitory activities were less than kojic acid, this may indicate that compounds bearing benzene ring on 1,2,4-triazole could exhibit significant increases in activity. Among the compounds **6d–6l,** compounds **6j**, **6k**, and **6l** demonstrated the most potent inhibitory activities. In particular, compound **6j** exhibited greater inhibitory activities with IC_{50} 4.50 ± 0.34 μM.

From the inhibitory activities on mushroom tyrosinase, preliminary structure–activity relationships of the synthesized compounds were achieved. In general, compared with compounds **6a–6l**, the aromatic substituents on 1,2,4-triazole ring had great effect on the inhibitory activities, for example, the compounds **6d, 6e, 6f, 6g, 6h, 6i, 6j, 6k, and 6l** displayed the more potent inhibitory activities than the compounds with aliphatic hydrocarbon on 1,2,4-triazole ring (**6a**, **6b,** and **6c)**. Among these compounds, compounds with halogen substituents (**6j, 6k,** and **6l**) on the benzene ring showed stronger inhibitory activities than the compound with methyl, methoxyl, and no substituent on the benzene ring (**6d, 6e, 6f, 6g, 6h,** and **6i**). Furthermore, the inhibitory activities of chlorine-substituted compounds were more potent than bromine-substituted compounds. This shows that the electron-withdrawing groups were a beneficial effect on the inhibitory activities of these compounds. The results of the structure–activity relationships indicated that the aromatic substituents on 1,2,4-triazole ring at position 3 enhanced the inhibitory activities, and chloro- or bromo-substituted benzene rings were found to be the most favorable for the inhibitory activities, which will encourage us to further design more potent inhibitor of tyrosinase.

Inhibitory mechanism

Among the tested target compounds, 5-(4-chlorophenyl)-3-[5-hydroxy-4-pyrone-2-yl-methymercapto]-4-amino-1,2,4-triazole (**6j**) showed the highest inhibitory activity, and hence, we carried out the kinetic analysis of **6j** for tyrosinase inhibition using L-DOPA as a substrate. The Lineweaver–Burk plots for the inhibition of tyrosinase by **6j** were obtained with variable concentrations of **6j** and the substrate (Figure 3). The intersection of these lines on the vertical axis indicated that **6j** was a competitive inhibitor of tyrosinase with respect to L-DOPA as a substrate; therefore, we speculated that this type of compound could enter into the active center of tyrosinase.

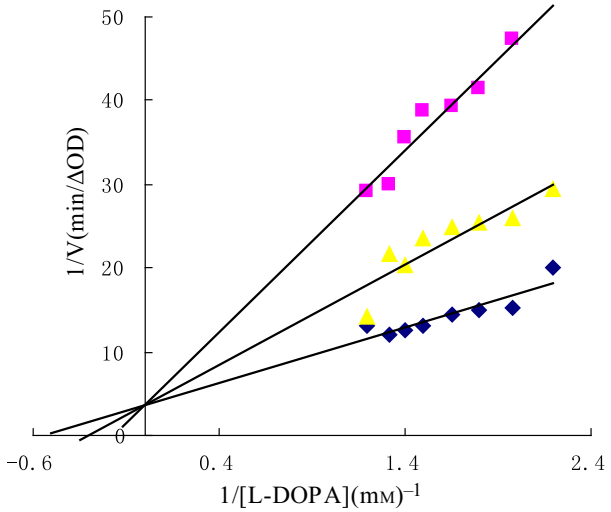


Figure 3: Lineweaver–Burk plots for the inhibition of Mushroom tyrosinase by compound **6j**. Concentrations of compound **6j** for curves were (◆) 0 μM, (▲) 2.0 μM, and (■) 4.0 μM respectively.

Conclusions

In conclusion, a series of novel 5-substituted-3-[5-hydroxy-4-pyrone-2-yl-methylmercapto]-4-amino-1,2,4-diazole derivatives (**6a-6l**) have been synthesized, and their inhibitory activity on mushroom tyrosinase has been evaluated. The results showed most of them displayed higher inhibitory activity on tyrosinase than kojic acid (**1**). Among the compounds synthesized, compound **6j** showed the strongest inhibitory activity with IC_{50} value of 4.50 ± 0.34 μM. The inhibition kinetics analyzed by Lineweaver–Burk plots revealed that compound **6j** acted as a competitive inhibitor of mushroom tyrosinase. Preliminary structure–activity relationships (SARs) analysis indicated that (i) aromatic substituent moiety might play an important role in determining their inhibitory activities, and (ii) chlorine atom at the 4th position of benzene ring might be beneficial to improve the inhibitory activities of tyrosinase. The work will contribute to further understanding of the mechanism of tyrosinase inhibition and developing effective drugs against tyrosinase born illness.

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