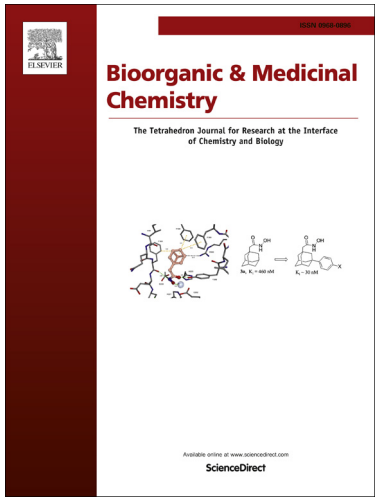

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Design, synthesis and biological evaluation of hydroxy- or methoxy-substituted 5-benzylidene(thio) barbiturates as novel tyrosinase inhibitors

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Abstract

Here a new class of hydroxy- or methoxy-substituted 5-benzylidene(thio)barbiturates were designed, synthesized and their inhibitory effects on the diphenolase activity of mushroom tyrosinase were evaluated. The results showed that several compounds had more potent tyrosinase inhibitory activities than the widely used tyrosinase inhibitor kojic acid (IC_{50} = 18.25 μM). In particular, 3',4'-dihydroxylated **1e** was found to be the most potent inhibitor with IC_{50} value of 1.52 μM. The inhibition mechanism analysis revealed that the potential compounds **1e** and **2e** exhibited such inhibitory effects on tyrosinase by acting as the irreversible inhibitors. Structure-activity relationships' (SARs) analysis also suggested that further development of such compounds might be of interest.

Keywords: 5-Benzylidene(thio)barbiturate; Tyrosinase inhibitor; SARs; Inhibition mechanism.

1. Introduction

Tyrosinase (EC 1.14.18.1), also known as polyphenoloxidase (PPO), is a copper-containing bifunctional enzyme widely distributed in nature. It catalyzes two distinct reactions of melanin synthesis, the hydroxylation of L-tyrosine by monophenolase action and the oxidation of L-DOPA to the corresponding *o*-dopaquinone by diphenolase action.[1] Melanin synthesis was responsible for the undesired enzymatic browning of fruits and vegetables and resulted in a loss of nutritional and market values.[2] The production of abnormal melanin pigmentation (melasma, freckles, ephelide, sneile lentigines, etc) was a serious esthetic problem in human beings.[3] Moreover, tyrosinase was found to be involved the molting process of insects[4] and contributed to the neurodegeneration associated with Parkinson's disease.[5] Therefore, tyrosinase inhibitors have become increasingly important in food industry as well as in the medicinal and cosmetic products due to decreasing the excessive accumulation of pigmentation resulting from the enzyme action.

To date, many efforts have been spent in the search for effective and safe tyrosinase inhibitors, and a large number of naturally occurring and synthetic tyrosinase inhibitors have been extensively reported.[6-7] However, only few of them are put into practical use due to their weak individual activities or safety concerns. Undoubtedly, it is still necessary to search and develop novel tyrosinase inhibitors with better activity and lower side effect.

Among all the known tyrosinase inhibitors, polyphenol derivatives have attracted considerable attention due to their favorable interaction with the hydrophobic protein pocket surrounding the binuclear copper active site of tyrosinase,[8] such as *N*-hydroxycinnamoylphenalkyl amides,[9] hydroxysubstituted benzaldoximes,[10] aurones,[11] chalcones,[12] flavanones,[13] resveratrols and their analogs,[14] benzoate ester derivatives,[15] and pyrazole derivatives.[16] Recently, our groups also demonstrated a series of 4-*o*-substituted 5-benzylidene barbiturate and thiobarbiturate derivatives as potential tyrosinase inhibitors.[17] Inhibition mechanism analysis revealed that such type of compounds, similar to N-hydroxy-N'-phenylthiourea, N-hydroxy-N'-phenylurea[18] and thiosemicarbazide derivatives,[19] were able to efficiently complex the two copper ions in the active site of tyrosinase.

Taking advantage of above information, we speculated that coupling products of proper hydroxy-substituted benzaldehydes with barbituric or thiobarbituric acid could not only complex

the binuclear copper active site of tyrosinase but also interact with the hydrophobic enzyme pocket, and thus, leading to increasing inhibitor-enzyme binding affinity and improving inhibitory effects on mushroom tyrosinase. Based on these, in our continuing interest to develop new potential tryosinase inbibitors, here a series of new, low-cost and easy-to-prepare 5-benzylidene(thio)barbiturates bearing hydroxyl groups on phenyl ring were designed, synthesized and their inhibitory effects on the diphenolase activitiy of mushroom tyrosinase were evaluated. Besides, to verify the importance of hydroxyl group moiety, the corresponding methoxy-substituted analogues were investigated. Furthermore, their SARs were discussed and the inhibition mechanism of selected compounds was studied.

2. Experimental section

2.1. General

Melting points were determined on a WRS-1B digital instrument without correction. NMR spectra were recorded on a Varian Mercury-Plus 300 spectrometers in $CDCl_3$ or DMSO-d_6 at 25 ^oC. All chemical shifts (d) are quoted in parts per million downfield from TMS and coupling constants (J) are given in Hertz. Mass spectra were obtained from VG ZAB-HS, LCMS-2010A or LCQ DECA XP spectrometer. Infrared (IR) spectra were measured on VECTOR 22 spectrometer using a potassium bromide (KBr) disk, scanning from 400 to 4000 cm^{-1}. All reactions were monitored by TLC and spots were visualized with UV light or iodine. All commercially available reagents and solvents were used without further purification. Mushroom tyrosinase (specific activity of the enzyme is 6680 U/mg) and L-DOPA (L-3,4-dhydroxyphenylalanine) were purchased from Sigma Chemical Co..

2.2. Chemistry

General procedure for the synthesis of 1b-l and 2b-l: To a solution of barbituric acid or thiobarbituric acid (1.0 mmol) in hot ethanol (20 mL), the corresponding aromatic aldehyde (1.0 mmol) was added. The reaction mixture was heated 2 h at 80 ^oC and cooled to room temperature. The precipitated solid was filtered, washed with ethanol and dried under vacuum. The crude product was recrystallized from *N*, *N*-dimethylformamide (DMF) and water. Compound **1a** and **2a** have been synthesized and characterized in our previous report.[17b]

2.2.1. 5-(2-Hydroxybenzyliden)pyrimidine-2,4,6(1H, 3H, 5H)trione (1b)

Yellow powder was obtained (0.20 g, 84%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3434, 3209, 3090, 2858, 1701, 1533, 1457, 1364, 1273, 774; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.25 (s, 1H, NH), 11.14 (s, 1H, NH), 10.95 (s, 1H, OH), 7.34-7.05 (m, 5H, CH, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 164.21, 162.31, 156.06, 157.23, 150.19, 137.43, 133.21, 124.34, 119.09, 117.68, 116.52; FAB-MS m/z (%): 232 (100) (M).

2.2.2. 5-(3-Hydroxybenzyliden)pyrimidine-2,4,6(1H, 3H, 5H)-trione (1c)

Yellow powder was obtained (0.20 g, 85%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3302, 1736, 1684, 1567, 1404, 1319, 1280, 796; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.33 (s, 1H, NH), 11.18 (s, 1H, NH), 9.68 (s, 1H, OH), 8.15 (s, 1H, CH), 7.63 (s, 1H, ArH), 7.43-7.40 (d, J = 7.5Hz, 1H, ArH), 7.26-7.22 (t, J = 7.5Hz, 1H, ArH), 6.94-6.91 (m, 1H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 164.21, 162.31, 156.06, 157.23, 150.19, 137.43, 133.21, 124.34, 119.09, 117.68, 116.52; ESI-MS m/z (%): 231 (75) (M-1).

2.2.3. 5-(2,4-Dihydroxybenzyliden)pyrimidine-2,4,6(1H, 3H, 5H)-trione (1d)

Yellow powder was obtained (0.20 g, 81%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3455, 3364, 1731, 1674, 1556, 1404, 1309, 1289, 795; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.61 (s, 1H, NH), 11.26 (s, 1H, NH), 8.15 (m, 1H, CH), 7.93-7.89 (m, 1H, ArH), 7.00-6.95 (m, 2H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 163.92, 162.16, 157.16, 150.95, 150.29, 145.70, 133.94, 116.45, 113.14, 109.82, 103.27; FAB-MS m/z (%): 248 (100) (M).

2.2.4. 5-(3,4-Dihydroxybenzyliden)pyrimidine-2,4,6(1H, 3H, 5H)-trione (1e)

Yellow powder was obtained (0.21 g, 83%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3469, 3251, 1727, 1663, 1545, 1506, 1403, 1295, 794; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.18 (s, 1H, NH), 11.06 (s, 1H, NH), 8.17 (s, 1H, CH), 8.08 (s, 1H, ArH), 7.62-7.59 (m, 1H, ArH), 6.84-6.81 (m, 1H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 163.90, 162.27, 158.23, 155.15, 152.10, 150.43, 134.57, 125.36, 118.92, 115.33, 112.43; EI-MS m/z (%): 248 (100) (M), 231 (24), 204 (50), 188 (25), 161 (23), 134 (25), 88 (10); HRMS: calculated (248.0433), found (248.0422).

2.2.5. 5-(3-Hydroxyl-4-methoxybenzyliden)pyrimidine-2,4,6(1H, 3H, 5H)-trione (1f)

Yellow powder was obtained (0.24 g, 90%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3247, 3090, 1729, 1673, 1553, 1508, 1444, 1274, 1147, 795, 520; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.21 (s, 1H,

NH), 11.08 (s, 1H, NH), 9.36 (s, 1H, OH), 8.12-8.08 (m, 2H, CH, ArH), 7.72-7.68 (m, 1H, ArH), 7.04-7.02 (m, 1H, ArH), 3.87 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 164.46, 162.57, 156.06, 153.38, 150.67, 146.26, 130.73, 125.88, 120.93, 115.52, 111.77, 56.26; ESI-MS m/z (%): 261 (20) (M-1).

2.2.6. 5-(3-Methoxyl-4-hydroxybenzyliden)pyrimidine-2,4,6(1H, 3H, 5H)-trione (1g)

Yellow powder was obtained (0.21 g, 78%), mp >250 ^oC; IR (KBr, cm^{-1}) υ: 3272, 2863, 1746, 1663, 1540, 1499, 1407, 1309, 1281, 1176, 825, 523; ^1H NMR (DMSO-d_6, 300 MHz) δ: 11.20 (s, 1H, NH), 11.08 (s, 1H, NH), 10.48 (s, 1H, OH), 8.43 (s, 1H, CH), 8.20 (s, 1H, ArH), 7.80-7.76 (d, *J* = 10.8Hz, 1H, ArH), 6.90-6.86 (d, *J* = 10.8Hz, 1H, ArH), 3.82 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 164.45, 162.58, 156.02, 152.50, 151.66, 146.29, 130.66, 125.95, 120.92, 115.58, 112.50, 56.43; ESI-MS m/z (%): 261 (100) (M-1).

2.2.7. 5-(3,4,5-Trimethoxybenzyliden)pyrimidine-2,4,6(1H, 3H, 5H)-trione (1h)

Yellow powder was obtained (0.23 g, 76%), mp >250 ^oC; IR (KBr, cm^{-1}) υ: 3265, 2894, 1730, 1667, 1544, 1505, 1355, 1278, 1169, 792; ^1H NMR (DMSO-d_6, 300 MHz) δ: 11.35 (s, 1H, NH), 11.23 (s, 1H, NH), 8.24 (s, 1H, CH), 7.82 (s, 2H, ArH), 3.81 (s, 6H, OCH_3), 3.77 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 164.31, 162.69, 155.78, 152.54(2C), 150.74, 142.54, 128.17, 117.92, 113.26 (2C), 61.04, 56.76 (2C); ESI-MS m/z (%): 337 (67) (M+CH_3OH).

2.2.8. 5-(4-Methoxybenzyliden)pyrimidine-2,4,6(1H, 3H, 5H)-trione (1i)

Yellow powder was obtained (0.23 g, 93%), mp >250 ^oC; IR (KBr, cm^{-1}) υ: 3251, 3066, 2895, 1738, 1695, 1651, 1601, 1562, 1522, 1431, 1269, 869; ^1H NMR (DMSO-d_6, 300 MHz) δ: 11.29 (s, 1H, NH), 11.17 (s, 1H, NH), 8.37-8.33 (d, *J* = 9.0Hz, 2H, ArH), 8.22 (s, 1H, CH), 7.06-7.03 (d, *J* = 9.0Hz, 2H, ArH), 3.86 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 164.47, 164.00, 162.72, 155.59, 150.80, 138.11 (2C), 125.78, 116.12, 114.57 (2C), 56.44; ESI-MS m/z (%): 277 (27) (M+CH_3OH).

2.2.9. 5-(3,4,5-Trihydroxybenzyliden)pyrimidine-2,4,6(1H, 3H, 5H)-trione (1j)

Yellow powder was obtained (0.18 g, 68%), mp >250 ^oC; IR (KBr, cm^{-1}) υ: 3215, 1687, 1661, 1524, 1503, 1218, 792; ^1H NMR (DMSO-d_6, 300 MHz) δ: 11.16 (s, 1H, NH), 11.03 (s, 1H, NH), 7.98 (s, 1H, CH), 7.51 (s, 2H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 164.81, 162.72, 156.99, 150.83, 145.80 (2C), 141.67, 123.54, 116.27 (2C), 114.34; ESI-MS m/z (%): 263 (47) (M-1).

2.2.10. 5-(2,4,6-Trihydroxybenzyliden)pyrimidine-2,4,6(1H, 3H, 5H)-trione (1k)

Yellow powder was obtained (0.16 g, 61%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3447, 3148, 1681, 1656, 1531, 1496, 1220, 811; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.14 (s, 1H, NH), 11.05 (s, 1H, NH), 8.10 (s, 1H, CH), 7.56 (s, 2H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 164.82, 162.90, 157.04, 150.79 (2C), 145.71, 142.33, 118.45, 115.53, 111.06 (2C); ESI-MS m/z (%): 263 (24) (M-1).

2.2.11. 5-(3,4,5-Trihydroxybenzyliden)pyrimidine-2,4,6(1H, 3H, 5H)-trione (1l)

Yellow powder was obtained (0.19 g, 70%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3450, 3220, 1677, 1645, 1520, 1499, 1231, 1108, 687; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.23 (s, 1H, NH), 11.10 (s, 1H, NH), 10.95 (s, 1H, OH), 10.60 (s, 1H, OH), 9.83 (s, 1H, OH), 8.77 (s, 1H, CH), 8.32-8.28 (d, J = 6.0Hz, 1H, ArH), 7.46-7.42 (d, J = 9.0Hz, 1H, ArH), 6.39-6.35 (d, J = 9.0Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 165.21, 163.05, 153.96, 152.20, 151.05, 150.93, 132.53, 126.87, 113.92, 112.52, 108.07; ESI-MS m/z (%): 263 (53) (M-1).

2.2.12. 5-(2-Hydroxybenzyliden)-2-thioxo-dihyropyrimidine-4,6(1H, 5H)-dione (2b)

Yellow powder was obtained (0.20 g, 83%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3427, 3134, 1655, 1558, 1457, 1382, 1276, 1134, 761; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 13.28 (s, 1H, NH), 12.27 (s, 1H, NH), 7.19 (m, 2H, CH, ArH), 7.09 (m, 2H, ArH), 6.98-6.96 (m, 1H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.57, 162.41, 160.11, 158.41, 150.39, 133.40, 131.72, 122.33, 119.59, 117.86, 115.25; ESI-MS m/z (%): 247 (78) (M-1).

2.2.13. 5-(3-Hydroxybenzyliden)-2-thioxo-dihyropyrimidine-4,6(1H, 5H)-dione (2c)

Yellow powder was obtained (0.24 g, 87%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3235, 1664, 1561, 1458, 1377, 1280, 812; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.42 (s, 1H, NH), 12.31 (s, 1H, NH), 9.75 (s, 1H, OH), 8.15 (s, 1H, CH), 7.68 (s, 1H, ArH), 7.48-7.44 (d, J = 7.8Hz, 1H, ArH), 7.29-7.24 (t, J = 7.8Hz, 1H, ArH), 6.97-6.93 (dd, J_1 = 7.8Hz, J_2 = 2.4Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 179.53, 162.43, 160.02, 157.55, 156.30, 134.30, 129.86, 126.04, 120.09, 117.13, 113.21; ESI-MS m/z (%): 247 (100) (M-1).

2.2.14. 5-(2,4-Dihydroxybenzyliden)-2-thioxo-dihyropyrimidine-4,6(1H, 5H)-trione (2d)

Yellow powder was obtained (0.19 g, 73%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3037, 1710, 1673, 1594, 1466, 1384, 1261, 1125, 851, 793; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.58 (s, 1H, NH),

11.24 (s, 1H, NH), 8.80 (m, 1H, CH), 7.92-7.88 (m, 1H, ArH), 6.95 (m, 2H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 179.14, 162.37, 160.17, 159.89, 159.75, 152.34, 131.12, 120.60, 111.03, 109.42, 105.20; FAB-MS m/z (%): 264 (100) (M).

2.2.15. 5-(3,4-Dihydroxybenzyliden)-2-thioxo-dihyropyrimidine-4,6(1H, 5H)-dione (2e)

Yellow powder was obtained (0.21 g, 80%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3447, 3120, 2908, 1650, 1575, 1505, 1443, 1279, 1144, 1003, 695; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.26 (s, 1H, NH), 12.18 (s, 1H, NH), 8.27-8.26 (d, J_1 = 2.1Hz, 1H, ArH), 8.12 (s, 1H, CH), 7.66-7.62 (dd, J_1 = 2.1Hz, J_2=8.7Hz, 1H, ArH), 6.86-6.83 (d, J_2 = 8.7Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.62, 163.10, 160.59, 157.74, 153.85, 145.60, 132.96, 125.16, 122.07, 116.22, 114.32; EI-MS m/z (%): 264 (100) (M), 247 (30), 204 (25), 166 (55), 134 (33); HRMS: calculated (2464.0422), found (264.0196).

2.2.16. 5-(3-Dydroxyl-4-methoxybenzyliden)-2-thioxodihyropyrimidine-4,6(1H, 5H)-dione (2f)

Yellow powder was obtained (0.23 g, 88%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3447, 3120, 1650, 1575, 1505, 1442, 1317, 1279, 1188, 1144, 1002, 482; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.29 (s, 1H, NH), 12.20 (s, 1H, NH), 8.15 (m, 2H, CH, ArH), 7.76 (m, 1H, ArH), 7.08-7.04 (m, 1H, ArH), 3.88 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.41, 162.20, 160.36, 153.38, 150.58, 148.65, 130.98, 125.01, 120.93, 118.20, 111.55, 56.70; EI-MS m/z (%): 278 (100) (M), 261 (20), 180 (35), 161(23), 133 (25), 105 (24).

2.2.17. 5-(3-Methoxyl-4-hydroxybenzyliden)-2-thioxodihyropyrimidine-4,6(1H, 5H) -dione (2g)

Yellow powder was obtained (0.20 g, 74%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3519, 3116, 2910, 1652, 1503, 1430, 1395, 1283, 1170, 1026, 960, 871, 786, 525; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.29 (s, 1H, NH), 12.19 (s, 1H, NH), 10.67 (s, 1H, OH), 8.46 (s, 1H, CH), 8.22 (s, 1H, ArH), 7.86 (m, 1H, ArH), 6.89 (m, 1H, ArH), 3.82 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.27, 162.19, 160.86, 155.31, 150.44, 146.94, 130.88, 123.12, 120.52, 118.86, 112.70, 56.53; EI-MS m/z (%): 278 (100) (M), 261 (20), 218 (17), 180 (35), 105 (16).

2.2.18. 5-(3,4,5-Trimethoxybenzyliden)-2-thioxodihydropyrimidine-4,6(1H, 5H)-dione (2h)

Yellow powder was obtained (0.23 g, 71%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3118, 2907, 1655,

1505, 1442, 1385, 1278, 1169, 1009, 783; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.43 (s, 1H, NH), 12.32 (s, 1H, NH), 8.25 (s, 1H, CH), 7.87 (s, 2H, ArH), 3.82 (s, 6H, OCH_3), 3.79 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.81, 162.61, 160.48, 152.55 (2C), 143.10, 128.22, 117.93, 113.62 (2C), 109.98, 61.12 (2C), 56.82; ESI-MS m/z (%): 353 (100) (M+Na).

2.2.19. 5-(4-Methoxybenzyliden)-2-thioxo-dihydropyrimidine-4,6(1H, 5H)-dione (2i)

Yellow powder was obtained (0.25 g, 94%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3187, 2913, 1665, 1502, 1443, 1274, 870, 629; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.37 (s, 1H, NH), 12.27 (s, 1H, NH), 8.42-8.38 (d, *J* = 9.0Hz, 2H, ArH), 8.24 (s, 1H, CH), 7.08-7.04 (d, *J* = 9.0Hz, 2H, ArH), 3.87 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.81, 164.54, 162.80, 160.55, 156.56, 138.60 (2C), 125.92, 116.25, 114.77 (2C), 56.57; ESI-MS m/z (%): 293 (30) (M+CH_3OH).

2.2.20. 5-(3,4,5-Trihydroxybenzyliden)-2-thioxodihydropyrimidine-4,6(1H, 5H)-dione (2j)

Yellow powder was obtained (0.17 g, 62%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3457, 3164, 1663, 1503, 1445, 1278, 804; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.25 (m, 2H, NH), 9.91 (s, 1H, OH), 9.53 (s, 2H, OH), 7.98 (s, 1H, CH), 7.58 (s, 2H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.58, 162.83, 160.67, 150.81, 145.94 (2C), 142.77, 123.79, 116.77 (2C), 114.32; ESI-MS m/z (%): 279 (100) (M-1).

2.2.21. 5-(2,4,6-Trihydroxybenzyliden)-2-thioxodihydropyrimidine-4,6(1H, 5H)-dione (2k)

Yellow powder was obtained (0.15 g, 57%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3449, 3124, 1656, 1505, 1446, 1280, 798; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.24 (m, 2H, NH), 8.13 (s, 1H, CH), 7.58 (s, 2H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.46, 162.50, 160.39 151.04 (2C), 144.69, 143.54, 119.78, 115.87, 113.64 (2C); ESI-MS m/z (%): 279 (65) (M-1).

2.2.22. 5-(3,4,5-Trihydroxybenzyliden)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (2l)

Yellow powder was obtained (0.16 g, 63%), mp >250 ^{o}C; IR (KBr, cm^{-1}) υ: 3451, 3143, 1649, 1507, 1457, 1291, 1121, 761, 687; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.52 (m, 2H, NH), 10.12 (m, 1H, OH), 8.89 (m, 2H, CH, ArH), 7.52-7.48 (d, *J* = 8.4Hz, 1H, ArH), 7.07-7.04 (d, *J* = 8.4Hz, 1H, ArH), 6.39-6.35 (d, *J* = 9.0Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 178.61, 163.19, 161.22, 159.86, 153.44, 144.21, 133.57, 124.32, 116.49, 115.09, 110.27; ESI-MS m/z (%): 279 (31) (M-1).

2.3. Biology

Tyrosinase inhibition assay was performed as described by our groups[17] with some slight modifications. Briefly, all the synthesized compounds were screened for the diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. 5-Benzylidene(thio)barbiturates were first dissolved in DMSO, and the final concentration of DMSO in the test solution was 1.0%. Phosphate buffer, pH 6.8, was used to dilute the DMSO stock solution of test compounds. Thirty units of mushroom tyrosinase (0.5 mg/ml) was first pre-incubated with the compounds, in 50 mM phosphate buffer (pH = 6.8), for 10 min at 25 ^{o}C. Then the L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm of formation of the DOPAchrome for 1 min. The measurement was performed in triplicate for each concentration and averaged before further calculation. IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of the dose-response curves. As a control, the IC_{50} of kojic acid was also measured.

3. Results and Discussion

3.1. Synthesis

5-Benzylidene(thio)barbiturates were prepared through the well-known Knoevenegal condensation of the corresponding benzaldehydes and barbituric or thiobarbituric acid as described previously.[17,20] Briefly, a number of commercially available benzaldehydes were reacted with barbituric or thiobarbituric acid in hot ethanol for 2 h to afford 5-benzylidenebarbiturate **1a-l** and 5-benzylidenethiobarbiturate **2a-l** in good yields (Scheme 1).

3.2. Biological activity

3.2.1. Inhibitory effects on the diphenolase activity of mushroom tyrosinase

Our previous work have showed that such coupling products **1a** and **2a** bearing a hydroxyl substituent at position-4' of A-ring had excellent inhibitory activities on mushroom tyrosinase with IC_{50} values of 13.98 μM and 14.49 μM, respectively.[17b] Inspired by this, we sought to probe the position and number of hydroxyl substituent for the influence of tyrosinase inhibitory activity. It was noteworthy that such expansion of 5-benzylidene(thio)barbiturates has not been reported so far. Therefore, here the inhibition of our synthetic hydroxy- or methoxyl-substituted 5-benzylidene(thio)barbiturates on the mushroom tyrosinase was investigated and compared with

kojic acid, which is a widely used skin-whitening material in cosmetic industry. As shown in Table 1, parent compounds barbituric acid, thiobarbituric acid and 3,4-dihydroxybenzaldehyde showed no activities at the concentration of 100 μM. As predicted, many condensation products of barbituric acid or thiobarbituric acid and hydroxybenzaldehydes displayed potent tyrosinase inhibitory activities. Especially, the obtained compounds **1d** (IC_{50} = 17.15 μM), **1e** (IC_{50} = 1.52 μM), **1g** (IC_{50} = 5.50 μM), **1j** (IC_{50} = 5.51 μM), **2e** (IC_{50} = 6.10 μM), **2g** (IC_{50} = 7.80 μM) and **2j** (IC_{50} = 7.14 μM) exhibited more potent tyrosinase inhibitory activities than the reference inhibitor kojic acid (IC_{50} = 18.25 μM). These results showed that the introduction of 5-benzylidene substructures was crucial for presenting the inhibitory effect on tyrosinase.

Compared with compounds **1a** and **2a**, unfortunately, the results from Table 1 showed that our synthesized compounds **1b** and **2b** having a hydroxyl group at position-2' and **1c** and **2c** having a hydroxyl group at position-3' of A-ring displayed a loss of activity (less than 20% inhibition at a concentration of 100 μM). Interestingly, incorporation of additional hydroxyl group onto position-3' of **1a** to afford the most potent compound **1e** with IC_{50} value of 1.52 μM, and its thiobarbiturate analogue **2e** (IC_{50} = 6.10 μM) also showed significant inhibitory activity. Compared with **1a**, compound **1d** bearing an additional hydroxyl group at positon-2' of A-ring exhibited comparable activity, whereas its thiobarbiturate analogue **2d** showed no inhibitiory activity on tyrosinase. Replacement of hydroxyl group at position-3' of **1e** and **2e** with a methoxy group to give **1g** (IC_{50} = 5.50 μM) and **2g** (IC_{50} = 7.80 μM) led to a reduction in potency. Similar findings were also observed at position-4' of A-ring (**1a** *vs* **1i**, **2a** *vs* **2i**, **1e** *vs* **1f** and **2e** *vs* **2f**). These results suggested that 4'-hydroxyl group might be absolutely necessarily and 3'-hydroxyl group facilitated their inhibitory activities while 3' or 4'-methoxy group was unfavorable.

To further investigate the effect of the number and position of hydroxyl substituents on A-ring, 3', 4', 5'-trihydroxlated **1j** and **2j**, 2', 4', 6'-trihydroxlated **1k** and **2k**, 2',3',4' -trihydroxlated **1l** and **2l** were examined for the tyrosinase inhibitory activity. As shown in **Table 1**, the inhibitory effects of **1j** (IC_{50} = 5.51 μM) and **2j** (IC_{50} = 7.14 μM) were slightly lower than that of 3',4'-dihydroxylated **1e** and **2e**. Compound **2l** (IC_{50} = 95.83 μM) showed weak inhibitory activity, whereas **1k**, **2k** and **1l** exhibited a completely loss of activity. Replacement of hydroxyl groups at position-3', 4', 5' of A-ring with methoxy substituents to afford **1h** and **2h** also showed no activity.

These data further revealed that 4’-hydroxyl group might played a very vital role in determining inhibitory effects on mushroom tyrosinase and methoxy group was not advantageous for the inhibitory potency.

Furthermore, 3’, 4’-dihydroxylated **1e** and **2e** showed more potent activities than 3’, 4’, 5’-trihydroxylated **1j** and **2j**, 2’, 4’, 6’-trihydroxylated **1k** and **2k**, and 2’, 3’, 4’-trihydroxylated **1l** and **2l,** suggesting that the third substituent might hinder the correct docking of the inhibitor to the active site of tyrosinase.

3.2.2. Inhibitory mechanism of compound 1e and 2e on mushroom tyrosinase

The inhibition mechanism on mushroom tyrosinase by **1e** for the oxidation of L-DOPA was investigated. Fig 1A showed the relationship between enzyme activity and its concentration in the presence of **1e**. The plots of the remaining enzyme activity (*V*) versus the concentrations of enzyme at different inhibitor concentrations ([*E*]) gave a family of parallel straight lines with the same slopes, indicating that the inhibitory effect of **1e** on the tyrosinase was irreversible. Identical research was carried out on **2e**, and same conclusion was drawn that **2e** was irreversible inhibitor (Fig. 1B). These results suggested that 5-benzylidene(thio)barbiturates effectively inhibited the enzyme by binding to its binuclear active site irreversibly.

On the basis of the crystallographic structure of tyrosinase and our obtained results, a possible binding mode was postulated (Fig. 2). As shown in Fig. 2, the barbiturate moiety could fit the binuclear copper active site well because the oxygen and/or sulfur atom formed strong chelation with the binuclear copper in the active center of tyrosinase. Such interaction acted like a bridge to link 5-benzylidene moiety bearing the hydroxyl or methoxyl group and the hydrophobic protein pocket in tyrosinase, and thus facilitating them to interact. Moreover, the additional hydrogen bonding interaction might occur between hydroxyl groups and some key bioactive amino acids existed in the hydrophobic domain,[8,21] since the above SARs analysis have disclosed that 5-benzylidene(thio)barbiturates bearing the hydroxyl substituent showed more potent tyrosinase inhibitory activities than those bearing the methoxyl substituent.

4. Conclusion

In summary, here we have developed a series of hydroxy- or methoxyl-substituted 5-benzylidene(thio)barbiturates from simple chemicals as novel tyrosinase inhibitors. The results

showed that several compounds had more potent tyrosinase inhibitory activities than the widely used tyrosinase inhibitor kojic acid. Especially, compound **1e** exhibited the most potent tyrosinase inhibitory activity with IC_{50} value of 1.52 μM. SARs analysis indicated (1) the introduction of 5-benzylidene substructures was crucial for the inhibition of tyrosinase activity; (2) the number and position of hydroxyl substituent on the phenyl ring played a vital role in determining inhibitory effects; (3) the 4-hydroxyl group of phenyl ring might be absolutely necessarily. The inhibition mechanism analysis revealed that 5-benzylidene(thio)barbiturates exhibited such inhibitory effects on tyrosinase by acting as the irreversible inhibitors. All the results suggested that these molecules can serve as the interesting candidates for the treatment of tyrosinase-related disorders and as the lead for the development of new and potent tyrosinase inhibitors. However, it should be noted that, from a clinical point of view, this model using mushroom tyrosinase was not sufficient for evaluating active molecules destined for human use.[6,11a] Therefore, further investigations of these potent compounds using human tyrosinase and a human melanoma cell line are underway in our laboratory, and the research results will be reported in due course.

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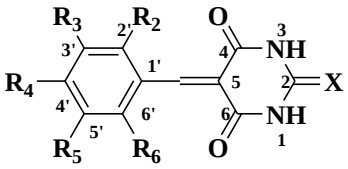
Table 1 Structures and inhibitory activity against mushroom tyrosinase (diphenolase) of 5-benzylidene(thio)barbiturates **1a-l** and **2a-l**.



Compd	R_2,	R_3,	R_4,	R_5,	R_6,	X	IC_{50} (μM)[a]
1a	H	H	OH	H	H	O	13.98 ±1.65[b]
1b	OH	H	H	H	H	O	NI[c]
1c	H	OH	H	H	H	O	NI
1d	OH	H	OH	H	H	O	17.15 ±1.56
1e	H	OH	OH	H	H	O	1.52 ±0.25
1f	H	OH	OMe	H	H	O	NI
1g	H	OMe	OH	H	H	O	5.50 ±0.64
1h	H	OMe	OMe	OMe	H	O	NI
1i	H	H	OMe	H	H	O	100.0 ±5.5
1j	H	OH	OH	OH	H	O	5.51 ±0.48
1k	OH	H	OH	H	OH	O	NI
1l	OH	OH	OH	H	H	O	NI
2a	H	H	OH	H	H	S	14.49 ±1.21[b]
2b	OH	H	H	H	H	S	NI
2c	H	OH	H	H	H	S	NI
2d	OH	H	OH	H	H	S	NI
2e	H	OH	OH	H	H	S	6.10 ±0.76
2f	H	OH	OMe	H	H	S	NI
2g	H	OMe	OH	H	H	S	7.80 ±0.83
2h	H	OMe	OMe	OMe	H	S	NI
2i	H	H	OMe	H	H	S	70.12 ±1.92
2j	H	OH	OH	OH	H	S	7.14
2k	OH	H	OH	H	OH	S	NI
2l	OH	OH	OH	H	H	S	95.83 ±2.25
Barbituric acid							NI
Thiobarbituric acid							NI
3,4-Dihydroxybenzaldehyde							NI
Kojic acid							18.25 ±1.85

[a] Assay performed using mushroom tyrosinase. Values are means of three different experiments. IC_{50} = mean ± S.E.M., S.E.M.: standard error of mean.

[b] The data have been reported in reference 17b.

[c] No obvious inhibition at a concentration of 100 μM (<20%).

Fig. 1 The inhibitory effects of **1e** and **2e** on mushroom tyrosinase for the catalysis of L-DOPA (A and B). The concentrations of compound **1e** for curves 1-4 were 0, 1.0, 1.5 and 2.0 μM, respectively. The concentrations of compound **2e** for curves 1-3 were 0, 5.0 and 10.0 μM, respectively.

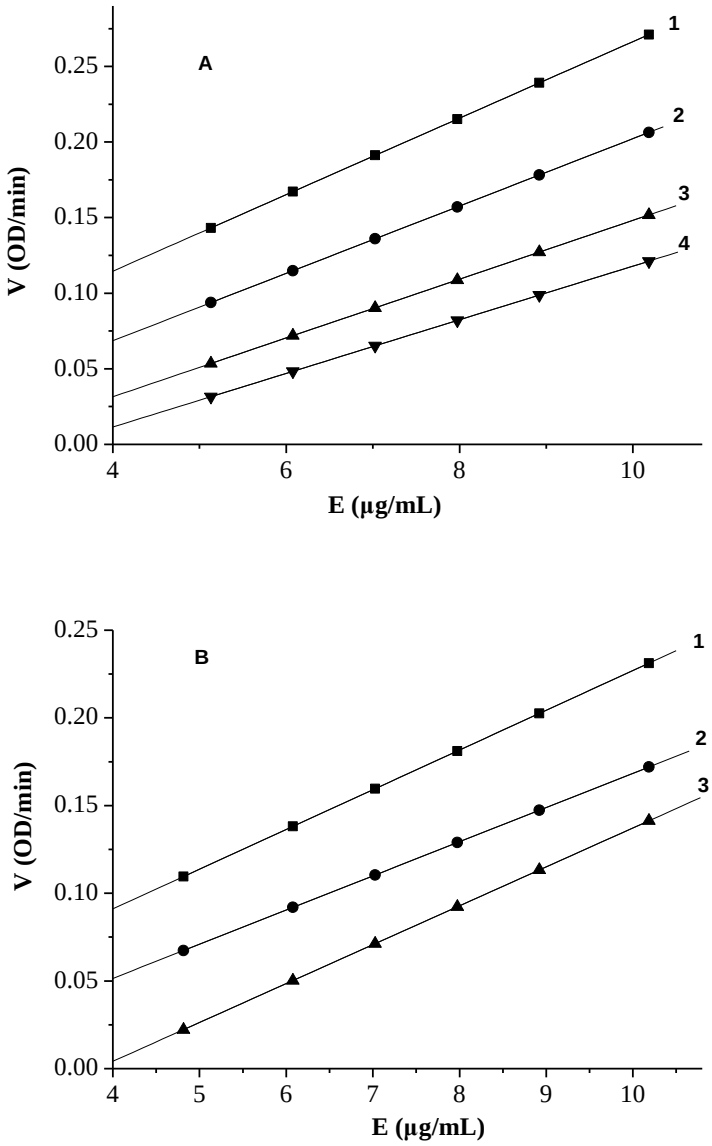
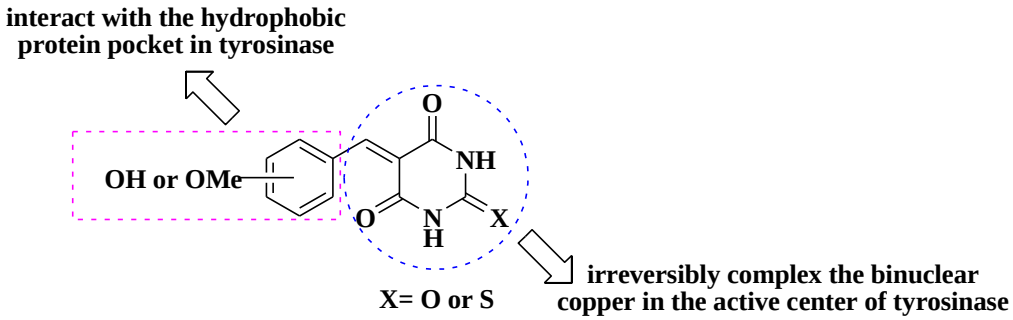
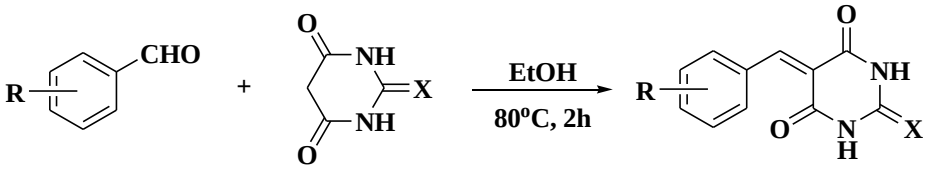


Fig. 2 Postulated binding mode.



Scheme 1 Synthesis of compounds **1a-l** and **2a-l**.

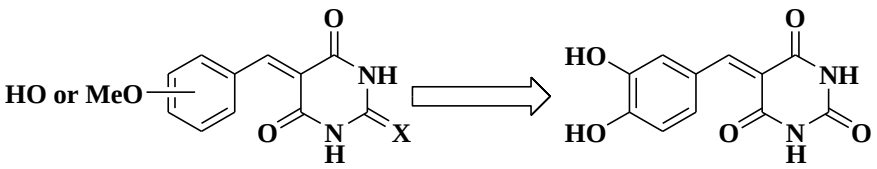


a~l

A: X=O
B: X=S

1a~l: X=O
2a~l: X=S

Graphical Abstract



X=O, 1a-l
X=S, 2a-l

1e
IC_{50} = 1.52 μM

A series of hydroxy- or methoxy-substituted 5-benzylidene (thio)barbiturate derivatives were designed, synthesized, and evaluated as novel tyrosinase inhibitors. Compounds **1e** were found to be the most potent inhibitor. SARs were discussed and the inhibition mechanism of selected compounds **1e** and **2e** was also investigated.