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RESEARCH ARTICLE

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Synthesis of chiral pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides with tyrosinase and urease inhibitory activity

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ABSTRACT

A new series of sulfonamide derivatives of pyrazolo[4,3-*e*][1,2,4]triazine with chiral amino group has been synthesized and characterized. The compounds were tested for their tyrosinase and urease inhibitory activity. Evaluation of prepared derivatives demonstrated that compounds **(8b)** and **(8j)** are most potent mushroom tyrosinase inhibitors whereas all of the obtained compounds showed higher urease inhibitory activity than the standard thiourea. The compounds (**8a**), (**8f**) and (**8i**) exhibited excellent enzyme inhibitory activity with IC_{50} 0.037, 0.044 and 0.042 μM, respectively, while IC_{50} of thiourea is 20.9 μM.

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Pyrazolo[4,3-*e*][1,2,4]triazine; sulfonamides; tyrosinase inhibitors; urease inhibitors

Introduction

Sulfonamides constitute an important class of drugs. They possess various types of pharmacological activities such as antibacterial, high-ceiling diuretic, hypoglycemic, antithyroid, anti-inflammatory and antiglaucoma[1–9]. Moreover, numerous sulfonamides were found to act as tyrosinase and urease inhibitors[10–12].

Tyrosinase (EC 1.14.18.1) is a multifunctional copper containing enzyme from oxidase family, widely distributed in animals, plants and microorganisms[13]. It catalyzes two distinct reactions of melanin synthesis, the hydroxylation of L-tyrosine by monophenolase action and the oxidation of L-DOPA (3,4-dihydroxyphenylalanine) to o-dopaquinone[14]. Tyrosinase catalyzes melanin biosynthesis which plays a crucial role in protecting the skin from solar radiations. Melanin can serve as a photoprotector and is responsible for the pigmentation and color patterns of mammalian skin. It has been reported that effective tyrosinase inhibitors could ameliorate skin hyperpigmentation, neurodegenerative diseases, and could potentially improve cancer remedies[15,16]. However, when exposed to excessive ultraviolet light, melanin produced in excess causes various skin disorders[17].

Urease (EC 3.5.1.5) is a nickel containing enzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide or carbamate by at least 10[14] over the spontaneous reaction[18]. It is of our interest to focus on the discovery of novel urease inhibitors as it is already known that these compounds play a crucial part in the therapies both human and plants disorders. In agriculture, high urease activity during urea fertilization causes significant environmental and economic problems by releasing abnormally large amounts of ammonia into the atmosphere[19,20]. Moreover, it contributes to the development of peptic ulcers, pyelonephritis, kidney stones, hepatic encephalopathy, hepatic coma urolithiasis and urinary catheter encrustation[21].

Our current work continuing our interest in the synthesis of new sulfonamide derivatives of pyrazolo[4,3-*e*][1,2,4]triazine as new inhibitors of tyrosinase and urease enzymes. The synthesis pathway leading to the title compounds is depicted in Scheme 1.

Experimental

Chemistry

Materials and methods

All chemicals used were of reagent grade quality and were used as received without any further purification and were obtained from Sigma-Aldrich. Melting points were determined on a Mel-Temp apparatus and are uncorrected. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). The chemical shift values are expressed in ppm (part per million) with TMS as internal reference. The relative integrals of peak areas agreed with those expected for the assigned structures. Molecular weight of final compounds were assessed by electrospray ionization mass spectrometry (ESI/MS) on a Agilent Technologies 6538 UHD Accurate Mass Q-TOF LC/MS. Elemental compositions are within ±0.4% of the calculated values. For preparation and spectroscopic data of compounds **2–7** and **8a**, **8b**, **8 h**, **8i** see literature[22–24].

Synthesis of sulfonamides 8a–j

A mixture of sulfonyl chloride **7** (100 mg, 0.29 mmol) and amine (100 mg, 1 mmol) in anhydrous acetonitrile (5 mL) was stirred

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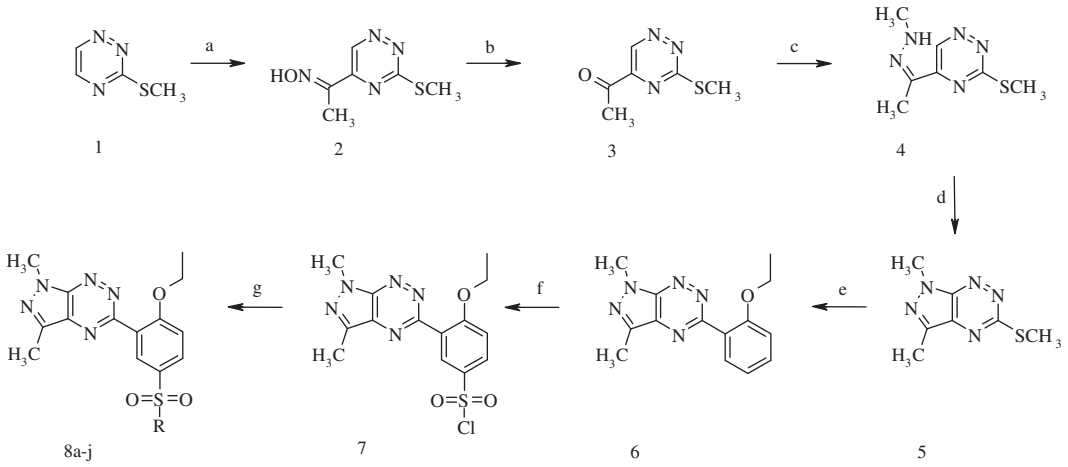

8a : R = (*S*)-NHCH(CH$_3$)CH$_2$OH
8b : R = (*R*)-NHCH(CH$_3$)CH$_2$OH
8c : R = (*S*)-NHCH(CH$_2$OH)CH(CH$_3$)CH$_3$
8d : R = (*R*)-NHCH(CH$_2$OH)CH(CH$_3$)CH$_3$
8e : R = (*S*)-NHCH$_2$CH(OH)CH$_3$
8f : R = (*R*)-NHCH$_2$CH(OH)CH$_3$
8g : R = (*S*)-NHCH$_2$CH(OH)CH$_2$OH
8h : R = (*R*)-NHCH$_2$CH(OH)CH$_2$OH
8i : R = (*S*)-NHCH(CH$_2$OH)CH$_2$CH(CH$_3$)CH$_3$
8j : R = (*R*)-NHCH(CH$_2$OH)CH$_2$CH(CH$_3$)CH$_3$

Scheme 1. Synthetic pathway to the sulfonamides **8a–i**. Reagents and conditions: (a) $CH_3CH_2NO_2$, KOH, DMSO, 2 h, 80–86%; (b) $Na_2S_2O_4$, H_2O/dioxane, rt, 12 h, 55–65%; (c) CH_3NH–NH_2, PTSA, EtOH, rt, 1 h, 50–55%; (d) method A: 10% HCl, EtOH, reflux, 1 h, 58–61%; method B: PTSA, 140 °C, 1 min, 61%; (e) ethoxyphenylboronic acid, $Pd(PPh_3)_4$, CuMeSal, THF, Ar, reflux, overnight, 75–80%; (f); $ClSO_3H$, 0 °C to rt, 2 h, 75–95%; (g) appropriate amine, anhydrous MeCN, rt, overnight, 72–93%.

overnight at room temperature, and then the reaction mixture was concentrated *in vacuo* to afford the crude sulfonamide, as a yellow solid[2,24]. The residue was purified on silica gel using a mixture of CH_2Cl_2:EtOH (25:1) as eluent to give the titled compounds as a yellow solid.

(*S*)-3-(1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxy-*N*-(1-hydroxy-propan-2-yl) benzenesulfonamide (8a)

Yield 92%, mp 132–136 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.09 (d, 3H, J = 6.4 Hz), 1.35 (t, 3H, J = 6.8 Hz), 2.63 (bs, 1H, OH, exchanged with D_20), 2.70 (s, 3H), 3.39–3.46 (m, 2H), 3.56 (d, 1H, J = 7.6 Hz), 4.19 (q, 2H, J = 6.4 Hz), 4.31 (s, 3H), 5.11 (d, 1H, J = 6.0 Hz, NH, exchanged with D_2O), 7.14 (d, 1H, J = 9.2 Hz), 7.98 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.4 Hz), 8.28 (d, 1H, J = 2.4 Hz).^{13}C-NMR ($CDCl_3$) δ: 11.0, 14.4, 17.9, 34.8, 51.5, 64.9, 65.9, 112.7, 126.9, 130.4, 131.3, 132.1, 134.5, 142.2, 146.7, 158.4, 160.4. HRMS (ESI, *m/z*) calcd. for $C_{17}H_{22}N_6O_4S$ [M+] 406.1423. Found [M+] 406.1427. Anal. calcd. for $C_{17}H_{22}N_6O_4S$: C, 50.23; H, 5.46; N, 20.68. Found: C, 50.00; H, 5.49; N, 20.50.

(*R*)-3-(1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxy-*N*-(1-hydroxy-propan-2-yl) benzenesulfonamide (8b)

Yield 89%, mp 132–136 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.09 (d, 3H, J = 6.4 Hz), 1.34 (t, 3H, J = 6.8 Hz), 2.69 (s, 3H), 2.84 (bs, 1H, OH, exchanged with D_20), 3.38–3.45 (m, 2H), 3.55 (d, 1H, J = 7.6 Hz), 4.18 (q, 2H, J = 6.4 Hz), 4.32 (s, 3H), 5.24 (d, 1H, J = 6.4 Hz, NH, exchanged with D_2O), 7.13 (d, 1H, J = 8.8 Hz), 7.98 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.27 (d, 1H, J = 2.4 Hz). ^{13}C-NMR ($CDCl_3$) δ: 11.0, 14.4, 17.8, 34.8, 51.5, 64.9, 65.9, 112.7, 126.9, 130.4, 131.3, 132.1, 134.5, 142.1, 146.7, 158.4, 160.4. HRMS (ESI, *m/z*) calcd. for $C_{17}H_{22}N_6O_4S$ [M+] 406.1423. Found [M+] 406.1426. Anal. calcd. for $C_{17}H_{22}N_6O_4S$: C, 50.23; H, 5.46; N, 20.68. Found: C, 49.89; H, 5.49; N, 20.55.

(*S*)-3-(1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxy-*N*-(1-hydroxy-3-methylbutan-2-yl)benzenesulfonamide (8c)

Yield 91%, mp 115–120 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.83 (dd, 6H, J_1 = 6.8 Hz, J_2 = 2.8 Hz), 1.34 (t, 3H, J = 6.8 Hz), 1.82 (s, 1H, J = 6.8 Hz), 2.69 (s, 3H), 3.07–3.11 (m, 1H), 3.52–3.55 (m, 2H), 4.17 (q, 2H, J = 6.8 Hz), 4.32 (s, 3H), 5.23 (d, 1H, J = 8.4 Hz, NH, exchanged with D_2O), 7.11 (d, 1H, J = 8.8 Hz), 7.96 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.8 Hz), 8.27 (d, 1H, J = 2.8 Hz). ^{13}C-NMR ($CDCl_3$) δ: 11.0, 14.4, 18.5, 19.1, 29.6, 34.8, 60.9, 62.5, 64.9, 112.6, 126.7, 130.4, 131.4, 132.4, 134.5, 142.1, 146.6, 158.4, 160.3. HRMS (ESI, *m/z*) calcd. for $C_{19}H_{26}N_6O_4S$ [M+] 434.1736 Found [M+] 434.1741. Anal. calcd. for $C_{19}H_{26}N_6O_4S$: C, 52.51; H, 6.03; N, 19.35. Found: C, 52.18; H, 6.06; N, 19.22.

(*R*)-3-(1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxy-*N*-(1-hydroxy-3-methylbutan-2-yl) benzenesulfonamide (8d)

Yield 72%, mp 115–120 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.84 (dd, 6H, J_1 = 6.8 Hz, J_2 = 3.6 Hz), 1.35 (t, 3H, J = 6.8 Hz), 1.82 (s, 1H, J = 6.8 Hz), 2.70 (s, 3H), 3.08–3.11 (m, 1H), 3.50–3.58 (m, 2H), 4.18 (q, 2H, J = 6.8 Hz), 4.32 (s, 3H), 5.16 (d, 1H, J = 8.8 Hz, NH, exchanged with D_2O), 7.11 (d, 1H, J = 8.8 Hz), 7.96 (dd, 1H, J_1 = 8.8 Hz, J_2 = 2.4 Hz), 8.28 (d, 1H, J = 2.4 Hz). ^{13}C-NMR ($CDCl_3$) δ: 11.0, 14.4, 18.5, 19.1, 29.6, 34.8, 60.9, 62.5, 64.9, 112.6, 126.8, 130.4, 131.4, 132.4, 134.5, 142.2, 146.7, 158.4, 160.3. HRMS (ESI, *m/z*) calcd.

for $C_{19}H_{26}N_6O_4S$ [M+] 434.1736 Found [M+] 434.1741. Anal. calcd. for $C_{19}H_{26}N_6O_4S$: C, 52.51; H, 6.03; N, 19.35. Found: C, 52.18; H, 6.10; N, 19.15.

(*S*)-3-(1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxy-*N*-(2-hydroxy-propyl)-benzenesulfonamide (8e)

Yield 90%, mp 132–135 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.13 (d, 3H, $J = 6.4$ Hz), 1.34 (t, 3H, $J = 6.8$ Hz), 2.67 (bs, 1H, OH exchanged with D_2O), 2.70 (s, 3H), 2.79–2.86 (m, 1H), 3.05–3.11 (m, 1H), 3.86–3.94 (m, 1H), 4.17 (q, 2H, $J = 6.8$ Hz), 4.32 (s, 3H), 5.35 (t, 1H, $J = 6.0$ Hz, NH, exchanged with D_2O), 7.13 (d, 1H, $J = 8.8$ Hz), 7.95 (dd, 1H, $J_1 = 8.8$ Hz, $J_2 = 2.4$ Hz), 8.24 (d, 1H, $J = 2.4$ Hz). ^{13}C-NMR ($CDCl_3$) δ: 11.0, 14.4, 20.5, 34.8, 50.1, 64.9, 66.3, 112.8, 127.0, 130.4, 131.2, 131.3, 134.5, 142.1, 146.7, 158.4, 160.4. HRMS (ESI, *m/z*) calcd. for $C_{17}H_{22}N_6O_4S$ [M+] 406.1423 Found [M+] 406.1426. Anal. calcd. for $C_{17}H_{22}N_6O_4S$: C, 50.23; H, 5.46; N, 20.68. Found: C, 49.89; H, 5.52; N, 20.53.

(*R*)-3-(1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxy-*N*-(2-hydroxy-propyl)-benzenesulfonamide (8f)

Yield 90%, mp 132–135 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.13 (d, 3H, $J = 6.4$ Hz), 1.34 (t, 3H, $J = 6.8$ Hz), 2.59 (bs, 1H, OH exchanged with D_2O), 2.70 (s, 3H), 2.79–2.86 (m, 1H), 3.05–3.11 (m, 1H), 3.86–3.94 (m, 1H), 4.18 (q, 2H, $J = 6.8$ Hz), 4.32 (s, 3H), 5.29 (bs, 1H, NH, exchanged with D_2O), 7.13 (d, 1H, $J = 8.8$ Hz), 7.95 (dd, 1H, $J_1 = 8.8$ Hz, $J_2 = 2.4$ Hz), 8.24 (d, 1H, $J = 2.4$ Hz). ^{13}C-NMR ($CDCl_3$) δ: 11.0, 14.4, 20.6, 34.8, 50.1, 64.9, 66.4, 112.8, 127.0, 130.4, 131.3, 131.3, 134.5, 142.1, 146.8, 158.4, 160.5. HRMS (ESI, *m/z*) calcd. for $C_{17}H_{22}N_6O_4S$ [M+] 406.1423 Found [M+] 406.1425. Anal. calcd. for $C_{17}H_{22}N_6O_4S$: C, 50.23; H, 5.46; N, 20.68. Found: C, 49.91; H, 5.56; N, 20.48.

(*S*)-*N*-(2,3-dihydroxypropyl)-3-(1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxybenzenesulfonamide (8g)

Yield 78%, mp 117–121 °C. ^{1}H-NMR (MeOH-d_4) δ: 1.29–1.33 (m, 5H), 2.68 (s, 3H), 2.85–2.90 (m, 1H), 3.04–3.08 (m, 1H), 3.49 (t, 2H, $J = 6.0$ Hz), 3.67 (m, 1H), 4.22 (q, 2H, $J = 6.8$ Hz), 4.31 (s, 3H), 7.36 (d, 1H, $J = 8.8$ Hz), 8.01 (dd, 1H, $J_1 = 8.8$ Hz, $J_2 = 2.4$ Hz), 8.17 (d, 1H, $J = 2.4$ Hz). ^{13}C-NMR (DMSO) δ: 11.1, 14.7, 35.0, 46.4, 63.9, 65.0, 70.7, 113.9, 127.1, 130.4, 130.6, 132.4, 134.4, 141.5, 147.0, 158.4, 159.9. HRMS (ESI, *m/z*) calcd. for $C_{17}H_{22}N_6O_5S$ [M+] 422.1372 Found [M+] 406.1375. Anal. calcd. for $C_{17}H_{22}N_6O_5S$: C, 48.33; H, 5.25; N, 19.90. Found: C, 48.05; H, 5.29; N, 19.77.

(*R*)-*N*-(2,3-dihydroxypropyl)-3-(1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxybenzenesulfonamide (8h)

Yield 79%, mp 117–121 °C. ^{1}H-NMR (MeOH-d_4) δ: 1.29–1.33 (m, 5H), 2.69 (s, 3H), 2.85–2.90 (m, 1H), 3.04–3.08 (m, 1H), 3.49 (t, 2H, $J = 6.0$ Hz), 3.66 (m, 1H), 4.22 (q, 2H, $J = 6.8$ Hz), 4.31 (s, 3H), 7.36 (d, 1H, $J = 8.8$ Hz), 8.01 (dd, 1H, $J_1 = 8.8$ Hz, $J_2 = 2.4$ Hz), 8.17 (d, 1H, $J = 2.4$ Hz). ^{13}C-NMR (MeOH-d_4) δ: 20.2, 23.8, 44.1, 55.5, 72.9, 74.1, 79.8, 123.0, 136.1, 139.4, 139.6, 141.5, 143.4, 150.5, 156.1, 167.4, 169.0. HRMS (ESI, *m/z*) calcd. for $C_{17}H_{22}N_6O_5S$ [M+] 422.1372 Found [M+] 406.1376. Anal. calcd. for $C_{17}H_{22}N_6O_5S$: C, 48.33; H, 5.25; N, 19.89. Found: C, 48.14; H, 5.35; N, 19.77.

(*S*)-3-(1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxy-*N*-(1-hydroxy-4-methylpentan-2-yl)benzenesulfonamide (8i)

Yield 91%, mp 112–116 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.73 (d, 3H, $J = 6.8$ Hz), 0.81 (d, 3H, $J = 6.8$ Hz), 1.27–1.29 (m, 1H), 1.32–1.38 (m, 4H), 1.56–1.60 (m, 1H), 2.70 (s, 3H), 3.36–3.39 (m, 1H), 3.41–3.45 (m, 1H), 3.54–3.57 (m, 1H), 4.19 (q, 2H, $J = 6.8$ Hz), 4.33 (s, 3H), 5.03 (d, 1H, $J = 8.0$ Hz, NH, exchanged with D_2O), 7.13 (d, 1H, J = 8.8 Hz), 7.98 (dd, 1H, $J_1 = 8.8$ Hz, $J_2 = 2.4$ Hz), 8.30 (d, 1H, $J = 2.4$ Hz). ^{13}C-NMR ($CDCl_3$) δ: 11.0, 14.4, 21.9, 22.7, 24.3, 34.8, 41.1, 53.8, 64.7, 64.9, 112.7, 127.0, 130.4, 131.4, 132.4, 134.5, 142.2, 146.7, 158.3, 160.4. HRMS (ESI, *m/z*) calcd. for $C_{20}H_{28}N_6O_4S$ [M+] 448.1893 Found [M+] 448.1899. Anal. calcd. for $C_{20}H_{28}N_6O_4S$: C, 53.55; H, 6.29; N, 18.74. Found: C, 53.33; H, 6.39; N, 18.63.

(*R*)-3-(1,3-dimethyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazin-5-yl)-4-ethoxy-*N*-(1-hydroxy-4-methylpentan-2-yl)benzenesulfonamide (8j)

Yield 93%, mp 112–116 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.70 (d, 3H, $J = 6.4$ Hz), 0.78 (d, 3H, $J = 6.4$ Hz), 1.25–1.27 (m, 1H), 1.31–1.36 (m, 4H), 1.54–1.60 (m, 1H), 2.67 (s, 3H), 3.10 (bs, 1H, OH), 3.32–3.42 (m, 2H), 3.52–3.57 (m, 1H), 4.18 (q, 2H, $J = 6.8$ Hz), 4.30 (s, 3H), 5.34 (d, 1H, $J = 8.0$ Hz, NH, exchanged with D_2O), 7.11 (d, 1H, $J = 8.8$ Hz), 7.96 (dd, 1H, $J_1 = 8.8$ Hz, $J_2 = 2.4$ Hz), 8.27 (d, 1H, $J = 2.4$ Hz). ^{13}C-NMR ($CDCl_3$) δ: 11.0, 14.4, 21.9, 22.7, 24.3, 34.8, 41.1, 53.8, 64.7, 64.9, 112.7, 127.0, 130.4, 131.4, 132.4, 134.5, 142.2, 146.7, 158.3, 160.4. HRMS (ESI, *m/z*) calcd. for $C_{20}H_{28}N_6O_4S$ [M+] 448.1893 Found [M+] 448.1894. Anal. calcd. for $C_{20}H_{28}N_6O_4S$: C, 53.55; H, 6.29; N, 18.74. Found: C, 53.21; H, 6.33; N, 18.59.

Pharmacology

Reagents

Mushroom tyrosinase (EC 1.14.18.1), urease from Jack bean (EC 3.5.1.5), L-DOPA (3,4-dihydroxyphenylalanine), Kojic acid, thiourea, EDTA, sodium nitroprusside and active chloride, were purchased from Sigma (St. Louis, MO, USA). Stock solutions of the reducing substrates were prepared in phosphate buffer (20 mM, pH 6.8).

Mushroom tyrosinase inhibition assay

The mushroom tyrosinase (EC 1.14.18.1) (Sigma Chemical Co.) was used for *in vitro* bioassays as described previously with some modifications.[25,26] Briefly, 140 µL of phosphate buffer (20 mM, pH 6.8), 20 µL of mushroom tyrosinase (30 U/mL) and 20 µL of the inhibitor solution were placed in the wells of a 96-well micro plate. After pre-incubation for 10 min at room temperature, 20 µL of L-DOPA (3,4-dihydroxyphenylalanine) (0.85 mM) was added and the plate was further incubated at 25 °C for 20 min. Subsequently the absorbance of dopachrome was measured at 475 nm using a micro plate reader ($OPTI_{Max}$, Tunable). Kojic acid was used as a reference inhibitor and for negative tyrosinase inhibitor phosphate buffer was used instead of the inhibitor solution. The extent of inhibition by the test compounds was expressed as the percentage of concentration necessary to achieve 50% inhibition (IC_{50}). Each concentration was analyzed in three independent experiments run in triplicate. The IC_{50} values determined by the data analysis and graphing software Origin 8.6, 64-bit (OriginLab Corporation, Northampton, MA).

Urease inhibition assay

The urease activity was determined by measuring amount of ammonia produced with indophenols method described by Weatherburn[27]. The reaction mixtures, comprising 20 μL of enzyme (Jack bean urease, 5 U/mL) and 20 μL of test compounds in 50 μL buffer (100 mM urea, 0.01 M K_2HPO_4, 1 mM EDTA and 0.01 M $LiCl_2$, pH 8.2), were incubated for 30 min at 37 °C in 96-well plate. Briefly, 50 μL each of phenol reagents (1%, w/v phenol and 0.005%, w/v sodium nitroprusside) and 50 μL of alkali reagent (0.5%, w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The absorbance at 625 nm was measured after 10 min, using a microplate reader ($OPTI_{Max}$, Tunable). All reactions were performed in triplicate. The urease inhibition activities were calculated according to the following formula:

$$\text{Urease inhibition activity } (\%) = \left(OD_{control} - OD_{sample} \times 100\right)/OD_{control}$$

Where $OD_{control}$ and OD_{sample} represents the optical densities in the absence and presence of sample, respectively. Thiourea was used as the standard inhibitor for urease.

Kinetic study

Lineweaver–Burk plots of 1/absorbance versus 1/L-DOPA and 1/absorbance versus 1/Urea were used to determine the type of enzyme inhibition. A series of experiments were performed to determine the inhibition kinetics by following method[18,28,29]. Different inhibitor concentrations of compound **8b** with 0, 15, 30, 61, 123 and 247 μM, **8j** with 0, 14, 28 and 55.8 μM, respectively were used in case of mushroom tyrosinase inhibition. Substrate L-DOPA concentration was between 0.062 and 2 mM in all tyrosinase kinetic study. Pre-incubation and measurement time was the same as discussed in mushroom tyrosinase inhibition assay protocol. Maximal initial velocity was determined from initial linear portion of absorbance up to five minutes after addition of enzyme at a 30 s interval. The inhibition type on the enzyme was assayed by Lineweaver–Burk plots of inverse of velocities (1/*V*) versus inverse of substrate concentration 1/[S] mM^{-1}, and the inhibition constant *Ki* was determined by two methods, by secondary replot of 1/*V* (y-intercept of Lineweaver–Burk plot) versus inhibitor concentrations and by Dixon plot of inverse of velocities (1/*V*) versus inhibitor concentrations.

Urease inhibition was measured by varying the concentration of urea in the presence of different concentrations (0, 0.125, 0.25 and 0.5 μM) of compound **8a.** Inhibitory constant (*Ki*) was determined as the intersection on the X-axis of the secondary replot of 1/*V* (y-intercept of Lineweaver–Burk plot) versus inhibitor concentrations and by Dixon plot of inverse of velocities (1/*V*) versus inhibitor concentrations. Briefly the urea concentration was changed from 3.12 to 100 mM for urease kinetics studies and remaining procedure was same as describes in urease inhibition assay protocol. Urease activity was determined by measuring ammonia production using the indophenol method as reported previously[30]. The results (change in absorbance per min) were processed by using SoftMaxPro software(Molecular Devices Corporation Sunnyvale, CA) and a Dixon plot was constructed by plotting (1/*V*) versus inhibitor concentration [I].

Results and discussion

Chemistry

The synthesis of target sulfonamides **8a–i** was achieved by a convenient multiple procedure starting from 3-methylsulfanyl-1,2,4-triazine (**1**) as shown in Scheme 1. Briefly, in the first step the reaction of **1** with nitroethane in KOH/DMSO mixture at room temperature gave appropriate oxime **2** as a main product[22], which was readily transformed into ketone **3** in good yield[23]. Compound **3** was subjected to the reaction with methylhydrazine in the presence of acidic media according to standard procedure to give suitable hydrazone **4** as key intermediate for the preparation of 1*H*-pyrazolo[4,3-*e*][1,2,4]triazine derivative **5**. The hydrazone **4** could be converted into derivative **5** under conventional heating (10% HCl, EtOH, reflux, 1 h)[31] or under solvent free reaction conditions according to our previous published procedure[32]. Using Guillaumet and co-worker's method[33] for the palladium-catalyzed cross-coupling reaction of 3-methylsulfanyl-1,2,4-triazine with boronic acid derivatives we have reacted 5-methylsulfanyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazines **5** with 2-ethoxyphenylboronic acid in the presence of copper (I) 3-methylsalicylate to obtain derivative **6** in excellent yield. Chlorosulfonylation reaction of compound **6** in neat chlorosulfonic acid at 0 °C proceeded smoothly and selectively at the 5′-position of the phenyl ring to give the desired product **7** in excellent yield. The chlorosulfonyl derivative **7** was readily coupled with appropriate pure enantiomeric amines in acetonitrile at room temperature to produce the final chiral sulfonamides **8a–i** in high yield.

Pharmacology

Effect of inhibitors on tyrosinase and urease

Tyrosinase assay All chiral sulfonamides **8a–j** are structurally similar and showed comparable inhibitory effects on mushroom tyrosinase [IC_{50} in the range of 27.9–40.17 μM]. Weaker inhibitors were derivatives **8i** (IC_{50} = 40.17 μM), **8e** (IC_{50} = 39.75 μM), and **8c** (IC_{50} = 39.00 μM) with (*S*)-(+)-leucinol, (*S*)-(+)-1-amino-3-propanol and (*S*)-(+)-2-amino-3-methyl-1-butanol moieties in sulfonamide group. The most active inhibitor in the tested group was compound **8j** (IC_{50} = 27.90 μM) which contains (*R*)-(−)-leucinol substituent (Table 1).

Tyrosinase contains one binuclear copper complex in the catalytic center and the catechol hydroxyl groups of the substrate are suggested to be bound to the copper atoms during the enzymatic oxidation process. Phenolic hydroxyl groups are generally capable to coordinate copper atoms causing inhibition of the enzyme in competition to the catechol substrate. However, our previous study[10] showed that inhibitors with NH group in the sulfonamide part (as piperazinyl, homopiperazinyl) are more active as they reduce the enzyme activity of tyrosinase to 29.5%–33.5%. It was suggested, that NH or free NH_2 group are involved in the inhibition effect of tyrosinase activity. Our suggestion was confirmed by

Table 1. The inhibitory effects of compounds **8a–8j** on mushroom tyrosinase and urease.

Compounds code	Mushroom tyrosinase IC_{50} (μM)	Urease IC_{50} (μM)
8a	38.46 ± 0.42	**0.037±0.003**
8b	**30.76±3.56**	0.084 ± 0.020
8c	39.00 ± 1.34	0.078 ± 0.020
8d	38.15 ± 1.68	0.064 ± 0.020
8e	39.75 ± 1.92	0.076 ± 0.007
8f	34.72 ± 2.01	**0.044±0.130**
8g	36.49 ± 1.75	0.063 ± 0.016
8h	32.46 ± 1.87	0.080 ± 0.014
8i	40.17 ± 1.09	**0.042±0.012**
8j	**27.9±2.43**	0.080 ± 0.015
Kojic Acid	16.69 ± 2.8	–
Thio-Urea	–	20.7 ± 0.45

Bolded values represent the most active derivatives.

the activity of some inhibitor with two NH groups that reduced enzyme activity of tyrosinase to 20.2%.

The inhibition of the compound **8j** may arise with strong participation OH group due to its conformation. However, other tested derivatives with OH were only weak tyrosinase inhibitors. Therefore, we suggest that primary binding to one copper atom should be strengthened by additional interactions between the inhibitor e.g. π electrons of the ring and another copper atom or the amino acid residues of the enzyme's active site to accomplish the inhibition.

Urease assay The synthesized pirazolotriazine-sulfonamides (**8a–j**) have been evaluated for their inhibitory effects on urease. All of the synthesized compounds showed better inhibitory activity with IC_{50} ranging from 0.037 to 0.084 μM than thiourea with IC_{50} of 20.7 μM. The structures of amino alcohols in sulfonamide group were varied to study their role in enzyme inhibitory activity. The excellent activity was shown by derivatives (**8a**) and (**8i**) with IC_{50} 0.037 and 0.042 μM, respectively. In case of compound (**8a**) the presence of 2-hydroxy-1-methyl ethaneamine moiety with S configuration at sulfonamide group has central importance in urease inhibitory activity in the group of tested derivatives. This moiety in compound (**8a**) is responsible for its most potent urease inhibitory activity. On the other hand, isomeric derivative (**8b**) with *R*-configuration in sulfonamide part showed the lowest activity in the group of obtained compounds although it exhibited higher urease inhibitory activity than standard thiourea.

Mechanism underlying inhibitory effect of compounds (8a), (8b) and (8j)

Since **(8b)** and **(8j)** against mushroom tyrosinase and **(8a)** against urease enzyme were the most potent inhibitors, we therefore further study mechanism underlying their inhibitory effect. The kinetic studies of the enzyme by the Lineweaver–Burk plot of (1/*V*) versus 1/[S] in the presence of different concentrations of inhibitors **(8b),**

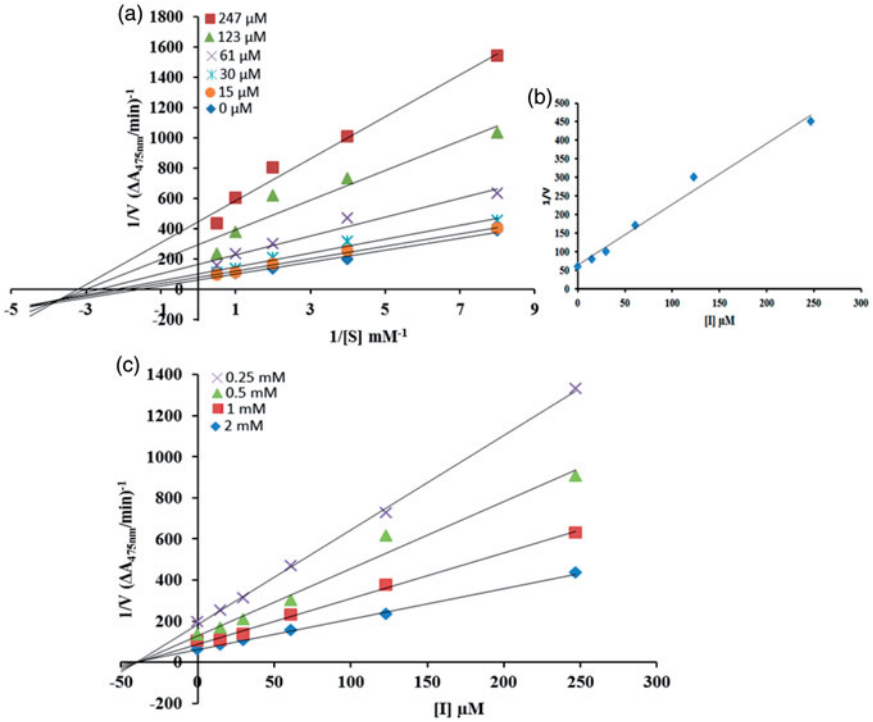

Figure 1. a) Lineweaver–Burk plots for the inhibition of mushroom tyrosinase in the presence of compound (**8b**). Concentrations of (**8b**) were 0, 15, 30, 61, 123 and 247 μM, respectively. Substrate L-DOPA Concentrations were 0.125, 0.25, 0.5, 1 and 2 mM, respectively. b) The secondary replot of the Lineweaver–Burk plot, 1/V (y-intercept) of a) versus various concentrations of (**8b**). c) The Dixon plot of the reciprocal of the initial velocities versus various concentrations of compound (**8b**) at fixed substrate concentration.

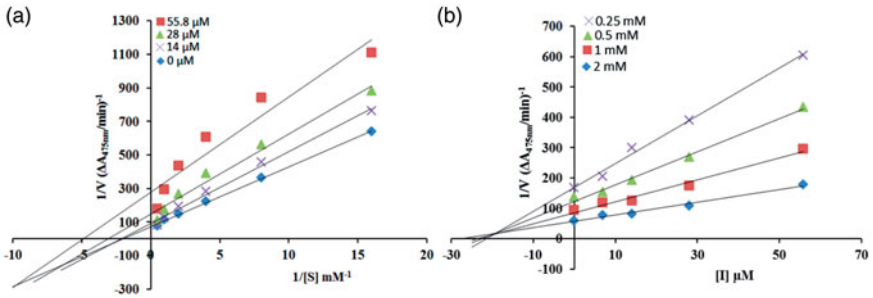

Figure 2. a) Lineweaver–Burk plots for the inhibition of mushroom tyrosinase in the presence of compound (**8j**). Concentrations of (**8j**) were 0, 14, 28 and 55.8 μM, respectively. Substrate L-DOPA Concentrations were 0.062, 0.125, 0.25, 0.5, 1 and 2 mM, respectively. b) The Dixon plot of the reciprocal of initial velocities versus various concentrations of compound (**8j**) at fixed substrate concentration.

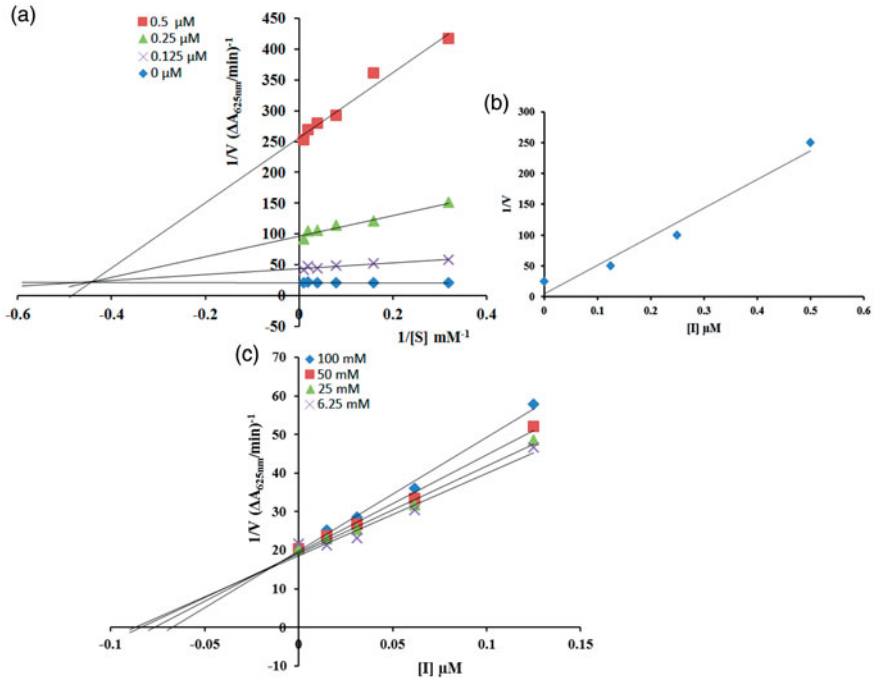

Figure 3. a) Double reciprocal Lineweaver–Burk plots for the inhibition of Jack bean urease in the presence of compound (**8a**). Concentrations of (**8a**) were 0, 0.125, 0.25 and 0.5 μM, respectively. Substrate urea concentrations were 3.12, 6.25, 12.5, 25, 50 and 100 mM, respectively. b) The secondary replot of the Lineweaver–Burk plot, 1/V (y-intercept) of a) versus various concentrations of (**8a**). c) The Dixon plot of the reciprocal of the initial velocities versus various concentrations of compound (**8a**) at fixed substrate concentration.

Table 2. Inhibitory effect of compound **8b** and **8j** on mushroom tyrosinase activity and of **8a** on urease activity.

	Enzyme			
	Mushroom tyrosinase		Urease	
Compound	Ki (μM)	Type of inhibition	Ki (μM)	Type of inhibition
8a	–	–	0.01	Mixed type
8b	40	Noncompetitive	–	–
8j	20	Mixed type	–	–

Ki is enzyme inhibition constant.
– is not determined.

(8j) and **(8a)** which gave a series of straight lines as shown in Figures 1–3(a). Furthermore, inhibition constant (*Ki*) were determined to gain insightful pathway by secondary replots and Dixon plots as shown in Figures 1–3(b,c). The results showed that compound **(8b)** behaved as a noncompetitive inhibitor (Figure 1), which means that it could combine with both the free enzyme and the enzyme-substrate complex. Thus, the interaction between **(8b)** and the enzyme was independent from the interaction between the substrate and the enzyme, and the inhibitor did not change substrate-enzyme affinity. With increasing concentrations, a series of lines was revealed with a common intercept on the 1/[S] axis, but with different slopes. On the other hand in Dixon plot **(8b)** was consistent with the characteristic patterns of noncompetitive inhibition with *Ki* value 40 μM and compound **(8j)** show mixed type inhibition with *Ki* value 20 μM as shown in Figure 2(a,b). In case of compound **(8a)** whose kinetic mechanism was studied against urease, by increasing the concentration of substrate (urea) gave family of straight lines, all of which intersected within the second quadrant. The analysis showed that V_{max} decreased with increasing K_m in the presence of increasing concentrations of **(8a)** This behavior of compound **(8a)**, indicated that it is a mixed type inhibitor with respect to the substrate urea with *Ki* value 0.01 μM as shown in Figure 3(a–c). The results of inhibition type and inhibition constants are summarized in Table 2.

Conclusions

We have described facile and efficient method for the preparation of new chiral 1*H*-pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides from simple available starting materials. The sulfonamides (**8a–j**) have been synthesized to validate their role in tyrosine and urease inhibitory activity. The most potent inhibitory activity against tyrosinase was displayed by compounds (**8b**) and (**8j**) with IC_{50} 30.76 and 27.90 μM, respectively. All of the obtained derivatives showed higher urease inhibitory activity than the standard thiourea. The kinetic analysis exhibited that compounds (**8b**) is noncompetitive inhibitor while (**8j**) is a mixed type inhibitor of tyrosinase and (**8a**) is a mixed type inhibitor of urease. According to the systematic investigation it could be deduced that pyrazolotriazine sulfonamides are a promising urease inhibitors for treatment of the urease related diseases.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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References

1. Gadad AK. Mahajanshetti CS, Nimbalkar S, Raichurkar A. Synthesis and antibacterial activity of some 5-guanylhydrazone/thiocyanato-6-arylimidazo[2,1-b]-1,3, 4-thiadiazole-2-sulfonamide derivatives. Eur J Med Chem 2000;9:853–7.
2. Mojzych M, Bielawska A, Bielawski K, et al. Pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides as carbonic anhydrase inhibitors with antitumor activity. Bioorg Med Chem 2014;22:2643–7.
3. Zani F, Vicini P. Antimicrobial activity of some 1,2-benzisothiazoles having a benzenesulfonamide moiety. Arch Pharm 1998;331:219–23.
4. Brzozowski Z, S1awinski J, Saczewski F, et al. Carbonic anhydrase inhibitors: synthesis and inhibition of the human cytosolic isozymes I and II and transmembrane isozymes IX, XII (cancer-associated) and XIV with 4-substituted 3-pyridinesulfonamides. Eur J Med Chem 2010;45:2396–404.
5. Renzi G, Scozzafava A, Supuran CT. Carbonic anhydrase inhibitors: topical sulfonamide antiglaucoma agents incorporating secondary amine moieties. Bioorg Med Chem Lett 2000;10:673–6.
6. Maren TH. Carbonic anhydrase: chemistry, physiology, and inhibition. Physiol Rev 1967;47:595–781.
7. Maren TH. Relatons between structure and biological activity of sulfonamides. Ann Rev Pharmacol Toxicol 1976;16:309–27.
8. Drew J. Drug discovery: a historical perspective. Science 2000;287:1960–194.
9. Li JJ, Anderson D, Burton EG, et al. 1,2-Diarylcyclopentenes as selective cyclooxygenase-2 inhibitors and orally active anti-inflammatory agents. J Med Chem 1995;38:4570–8.
10. Mojzych M, Dolashki A, Voelter W. Synthesis of pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides, novel Sildenafil analogs with tyrosinase inhibitory activity. Bioorg Med Chem 2014;22:6616–24.
11. Khan KM, Maharvi GM, Perveen S, et al. Synthesis of methyl ether analogues of sildenafil (*Viagra*®) possessing tyrosinase inhibitory potential. Chem Biodivers 2005;2:470–6.
12. Saeed A, Mahmood S-u, Rafiq M, et al. Iminothiazoline-sulfonamide hybrids as jack bean urease inhibitors; Synthesis, kinetic mechanism and computational molecular modeling. Chem Biol Drug Des 2016;87:434–43.
13. Gawande SS, Warangkar SC, Bandgar BP, Khobragade CN. Synthesis of new heterocyclic hybrids based on pyrazole and thiazolidinone scaffolds as potent inhibitors of tyrosinase. Bioorg Med Chem 2013;21:2772–7.
14. Chen Z, Cai D, Mou D, et al. Design, synthesis and biological evaluation of hydroxy- or methoxy-substituted 5-benzylidene(thio) barbiturates as novel tyrosinase inhibitors. Bioorg Med Chem 2014;22:3279–84.
15. Chan C–F, Lai S–T, Guo Y–C, Chen M–J. Inhibitory effects of novel synthetic methimazole derivatives on mushroom tyrosinase and melanogenesisOriginal. Bioorg Med Chem 2014;22:2809–15.
16. Yoshimori A, Oyama T, Takahashi S, et al. Structure–activity relationships of the thujaplicins for inhibition of human tyrosinase. Bioorg Med Chem 2014;22:6193–200.
17. Komori Y, Imai M, Yamauchi T, et al. Effect of p-aminophenols on tyrosinase activity. Bioorg Med Chem 2014;22:3994–4000.
18. Xiao Z-P, Peng Z-Y, Dong J-J, et al. Synthesis, molecular docking and kinetic properties of β-hydroxy-β-phenylpropionyl-hydroxamic acids as *Helicobacter pylori* urease inhibitors. Eur J Med Chem 2013;68:212–21.
19. Khan KM, Naz F, Taha M, et al. Synthesis and in vitro urease inhibitory activity of N,N′-disubstituted thioureas. Eur J Med Chem 2014;74:314–23.
20. Chen W, Li Y, Cui Y, et al. Synthesis, molecular docking and biological evaluation of Schiff base transition metal complexes as potential urease inhibitors. Eur J Med Chem 2010;45:4473.
21. Khan KM, Rahim F, Khan A, et al. Synthesis and structure–activity relationship of thiobarbituric acid derivatives as potent inhibitors of urease. Bioorg Med Chem 2014;22:4119–23.
22. Rykowski A, Makosza M. Reaction of 1,2,4-triazines with nitronate anions, direct nucleophilic acylation of 1,2,4-triazines. Tetrahedron Lett 1984;25:4795–6.
23. Rykowski A, Lipinska T. A concise route to a key intermediate in the total synthesis of sempervirine. Commun 1996;26:4409–14.
24. Mojzych M, Kubacka M, Mogilski S, et al. Relaxant effects of selected sildenafil analogues in the rat aorta. J Enzyme Inhib Med Chem 2015;31:381–8.
25. Seo WD, Ryu YB, Curtis-Long MJ, et al. Evaluation of anti-pigmentary effect of synthetic sulfonylamino chalcone. Eur J Med Chem 2010;45:2010–17.
26. Ashraf Z, Rafiq M, Seo S-Y, et al. Design, synthesis and bioevaluation of novel umbelliferone analogues as potential mushroom tyrosinase inhibitors. J Enzyme Inhib Med Chem 2015;30:874–3.
27. Weatherburn MW. Phenol-hypochlorite reaction for determination of ammonia. Anal Chem 1967; 39:971–4.
28. Chiari ME, Vera DMA, Palacios SM, Carpinella MC. Tyrosinase inhibitory activity of a 6-isoprenoid-substituted flavanone isolated from Dalea elegans. Bioorg Med Chem 2011;19:3474–82.
29. Ghani U, Ullah N. New potent inhibitors of tyrosinase: novel clues to binding of 1,3,4-thiadiazole-2(3H)-thiones, 1,3,4-oxadiazole-2(3H)-thiones, 4-amino-1,2,4-triazole-5(4H)-thiones, and substituted hydrazides to the dicopper active site. Bioorg Med Chem 2010;18:4042–8.
30. Amtul Z, Kausar N, Follmer C, et al. Cysteine based novel noncompetitive inhibitors of urease(s)-distinctive inhibition susceptibility of microbial and plant ureases. Bioorg Med Chem 2006; 16:6737–44.
31. Rykowski A, Mojzych M, Karczmarzyk Z. A new synthesis of pyrazolo[4,3-e][1,2,4]triazines via acid promoted ring closure of the phenylhydrazones of 5-acyl-1,2,4-triazines. Heterocycles 2000;53:2175–85.
32. Mojzych M, Rykowski A. Transformations of phenylhydrazones of 5-acyl-1,2,4-triazines to pyrazolo[4,3-e][1,2,4]triazines or 4-cyanopyrazole. J Heterocycl Chem 2007;44:1003–7.
33. Alphonse F-A, Suzenet F, Keromnes A, et al. Palladium-catalyzed 3-thiomethyltriazine-boronic acid cross coupling: easy access to 3-substituted-1,2,4-triazines. Synlett 2002;3:447–50.