

Synthesis and biological evaluation of some new N⁴-substituted isatin-3-thiosemicarbazones

HUMAYUN PERVEZ¹, ZAHID HUSSAIN CHOCHAN¹, MUHAMMAD RAMZAN¹,
FAIZ-UL-HASSAN NASIM², & KHALID MOHAMMED KHAN³

¹Department of Chemistry, Bahauddin Zakariya University, Multan 60800, Pakistan, ²Department of Chemistry, The Islamia University of Bahawalpur, Bahawalpur, Pakistan, and ³H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

(Received 14 April 2008; accepted 6 May 2008)

Abstract

A new series of 12 N⁴-substituted isatin-3-thiosemicarbazones **2a–l** has been synthesized, characterized and screened for *in vitro* cytotoxic, phytotoxic and urease inhibitory effects. All the compounds proved to be active in the brine shrimp bioassay; **2a**, **2b**, **2d**, **2f** and **2h–l** exhibited a high degree of cytotoxic activity ($LD_{50} = 1.10 \times 10^{-5} \text{ M} - 3.10 \times 10^{-5} \text{ M}$). In urease-inhibition assay, compounds **2a**, **2b**, **2e**, **2f**, **2h–j** and **2l** proved to be potent inhibitors displaying relatively much greater inhibition of the enzyme with IC_{50} values ranging from 20.6 μM to 50.6 μM . Amongst these, **2a** and **2f** were found to be the most potent ones exhibiting pronounced inhibition with IC_{50} value 20.6 μM . All the synthetic compounds showed weak to moderate (10–40%) phytotoxicity at the highest tested concentration (500 $\mu\text{g/mL}$) indicating their usefulness as inhibitors of soil ureases.

Keywords: *Isatin, thiosemicarbazones, cytotoxicity, phytotoxicity, urease inhibition, soil ureases inhibitors*

Introduction

The biological activities of isatin and its derivatives have been known for a long time. Isatin itself exhibited a range of actions such as CNS-MAO inhibition, sedative, anticonvulsant and anxiogenic activities [1]. Similarly, isatin derivatives are known to possess a wide spectrum of pharmacological properties including anthelmintic, antibacterial, anticonvulsant, anti-fungal, antineoplastic, antiviral, cysticidal, herbicidal, hypotensive and enzymatic inhibition [1–4]. Among these, isatins-derived thiosemicarbazones have raised considerable interest [5–12]. Stimulated by this and in continuation of our drug discovery programme [13–18], we have introduced very recently a number of N⁴-substituted isatin-3-thiosemicarbazones as urease inhibitors [19,20]. Urease, a nickel-dependent metalloenzyme, catalyzes the hydrolysis of urea in plants, algae, fungi and several microorganisms in the final step of organic nitrogen mineralization to produce

ammonia and carbamate [21]. The carbamate then spontaneously hydrolyses, at physiological pH, to form bicarbonate and a second molecule of ammonia [22–25]. The hydrolysis of the reaction products results in an abrupt net increase in pH, the major cause for the negative effects of the action of urease both for human and animal health, and for agriculture.

Urease serves as a virulence factor in certain human and animal infections of the urinary and gastrointestinal tracts, being involved in the development of kidney stones, urinary catheter encrustation, pyelonephritis, peptic ulceration, ammonia encephalopathy, hepatic coma and urinary tracts infections [22,25,26]. Several classes of molecules including hydroxamic acids may be an answer for the treatment of these conditions [27]. However, earlier studies [22,25, 28,29] show many side effects, including depressed bone marrow biosynthesis and inhibition of DNA

Correspondence: H. Pervez, Department of Chemistry, Bahauddin Zakariya University, Multan 60800, Pakistan.
E-mail: pdhpervez@hotmail.com

synthesis, and high doses have shown to be teratogenic. Therefore, need for the development of more potent and non- or less toxic urease inhibitors is evident and continues to be of a considerable interest.

In an agricultural setting, high soil bacterial ureases activity causes significant environmental and economic problems by releasing abnormally large amounts of ammonia into the atmosphere during urea fertilization. This further induces damage to germinating seeds, seedlings and young plants primarily by depriving them of their nutrition by the essential nutrient and secondly by ammonia toxicity, increasing the pH of the soil [30–33]. Urease inhibitors have been proposed to control urea hydrolysis in soil [22,34–39]. Particularly, phosphoramides have received considerable attention as urease inhibitors [37,39]. Studies have shown that urease inhibitors can be combined with fertilizers in small amounts to increase the overall efficiency of nitrogen utilization [38]. However, it has been found that the inhibitors can induce some phytotoxicity at higher concentrations [40]. These findings have emphasized the need to search for some efficient urease inhibitors, which could be combined with fertilizers in small amounts, so as to reduce environmental pollution and increase activity of urea nitrogen uptake by plants.

The exact knowledge with regard to the regions of urease involved in the binding of the substrates or inhibitors is a starting point in designing effective inhibitors capable of complementing all the structural requirements for a close interaction. In all the enzyme-inhibitor complexes examined so far, the urease active site is found to have pseudooctahedral and paramagnetic dinuclear nickel ions. It has been unambiguously proved by spectroscopic and X-ray crystallographic studies that hydroxamic acids (HXA) and phosphorodiamidates (PPD) represent the classes of synthetic inhibitors, which are good metal chelators and their mechanism of interaction involves binding to the metal ions of the active site of enzyme. Nonetheless, regardless of the class of the compounds, it is reported [41] that a few functionalities with electronegative atoms such as oxygen, nitrogen and sulphur act either as bidentate (mostly), tridentate (rarely), or as ligand chelator to form octahedral complexes with the two slightly distorted octahedral nickel ions of the enzyme. Prompted by these findings, we very recently synthesized [19] a number of isatin-thiosemicarbazones (derivatives of thiourea, a substrate-like urease inhibitor) and tested them as urease inhibitors [20], as they were expected to act as chelating agents coordinating with the two nickel ions of the enzyme through carbonyloxygen, imino nitrogen and thiolato sulphur atoms, thus inhibiting its activity. Some of these thiosemicarbazones showed promising activity and are proposed to be potential candidates for orally effective therapeutic agents used for the treatment of certain clinical conditions caused by bacterial ureases.

The present work is an extension to such studies and is mainly based on the optimization of new urease inhibitors with enhanced efficacy. It deals with the synthesis and evaluation of urease inhibitory properties of a new series of 12 N⁴-substituted isatin-3-thiosemicarbazones and describes the effects of the nature of phenyl group at N⁴ (modified by placement of one, two or three substituents about the ring) on the cytotoxic, phytotoxic and urease inhibitory properties of these compounds.

Materials and methods

General

All reagents and solvents were used as obtained from the suppliers or recrystallized/redistilled as necessary. Melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Elemental analyses were performed on a Leco CHNS-9320 elemental analyzer. Infrared spectra (KBr discs) were run on Shimadzu Prestige-21 FT-IR spectrometer. The ¹H-NMR spectra were recorded in CDCl₃ and C₂D₆SO on Bruker (Rhenistetten-Forchheim, Germany) AM 300 and AM 400 spectrometers operating at 300 MHz and 400 MHz, respectively, using TMS as an internal standard. ¹H chemical shifts are reported in δ (ppm) and coupling constants in Hz. The electron impact mass spectra (EIMS) were determined with a Finnigan MAT-312 and a JEOL MSRoute mass spectrometer. The progress of the reaction and the purity of the products were checked on TLC plates coated with Merck silica gel 60 GF₂₅₄ and the spots were visualized under ultraviolet light at 254 and 366 nm and/or spraying with iodine vapour. *In vitro* biological testing of the synthesized compounds was made at the Department of Chemistry, The Islamia University of Bahawalpur, Pakistan and Dr. Panjwani Center for Molecular Medicine & Drug Research, H.E.J. Research Institute of Chemistry, University of Karachi, Pakistan.

Synthesis

General procedure for the preparation of isatin-thiosemicarbazones (2a-l). To a hot solution of isatin (0.005 mol) in ethanol (10 mL) containing a few drops of glacial acetic acid was added the appropriate thiosemicarbazide (0.005 mol) dissolved in ethanol (10 mL) under stirring. The reaction mixture was then refluxed for 2 h. The crystalline solid formed during refluxing was collected by suction filtration. Thorough washing with hot ethanol followed by ether afforded the desired compounds **2a-l** in pure form.

N-(2-Fluorophenyl)-2-(2-oxo-1,2-dihydro-3H-indol-3-ylidene)-1-hydrazinecarbothioamide (2a). Yield 76% as yellow crystals; m.p. 232–234°C (d); IR (KBr,

cm^{-1}): 3311, 3186, 3147 (NH stretching), 1691 (C=O), 1618 (C=N), 1531 (NH bending), 1163 (C=S); $^1\text{H-NMR}$ (CDCl_3 , δ , ppm): 6.91 (d, $\mathcal{J} = 7.9$ Hz, 1H, indole C₇-H), 7.01-7.20 (m, 4H, indole C₅-H, C₆-H and phenyl C₄-H, C₅-H), 7.30-7.37 (m, 2H, phenyl C₃-H, C₆-H), 7.62 (d, $\mathcal{J} = 7.5$ Hz, 1H, indole C₄-H), 8.57 (s, 1H, CS-NH), 9.69 (s, 1H, indole NH), 12.86 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 316 ($[\text{M}^+] + 2$, 3), 315 ($[\text{M}^+] + 1$, 8), 314 ($[\text{M}^+]$, 42), 286 (100), 161 (19), 153 (13), 150 (16), 147 (3), 134 (18), 132 (16), 118 (13), 104 (15), 91 (6), 77 (6); (Found: C, 57.44; H, 3.51; N, 17.84. Calc. for $\text{C}_{15}\text{H}_{11}\text{FN}_4\text{OS}$: C, 57.32; H, 3.50; N, 17.83%).

N-(3-Fluorophenyl)-2-(2-oxo-1,2-dihydro-3H-indol-3-ylidene)-1-hydrazinecarbothioamide (**2b**). Yield 71% as light orange crystals; m.p. 240°C (d); IR (KBr, cm^{-1}): 3282, 3246 (NH stretching), 1695 (C=O), 1593 (C=N), 1535 (NH bending), 1145 (C=S); $^1\text{H-NMR}$ (DMSO, δ , ppm): 6.92 (d, $\mathcal{J} = 7.8$ Hz, 1H, indole C₇-H), 7.07-7.13 (m, 2H, indole C₅-H, phenyl C₅-H), 7.34-7.51 (m, 2H, indole C₆-H, phenyl C₆-H), 7.59-7.63 (m, 2H, phenyl C₂-H, C₄-H), 7.75 (d, $\mathcal{J} = 7.4$ Hz, 1H, indole C₄-H), 10.86 (s, 1H, CS-NH), 11.26 (s, 1H, indole NH), 12.85 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 314 ($[\text{M}^+]$, 37), 285 (100), 203 (5), 161 (25), 160 (4), 153 (19), 150 (30), 147 (3), 145 (14), 132 (30), 118 (34), 104 (73), 91 (14), 77 (25); (Found: C, 57.42; H, 3.51; N, 17.82. Calc. for $\text{C}_{15}\text{H}_{11}\text{FN}_4\text{OS}$: C, 57.32; H, 3.50; N, 17.83%).

2-(2-Oxo-1,2-dihydro-3H-indol-3-ylidene)-*N*-[2-(trifluoromethoxy)phenyl]-1-hydrazinecarbothioamide (**2c**). Yield 80% as orange crystals; m.p. 244°C (d); IR (KBr, cm^{-1}): 3320, 3200, 3110 (NH stretching), 1705 (C=O), 1605 (C=N), 1541 (NH bending), 1193 (C=S); $^1\text{H-NMR}$ (CDCl_3 , δ , ppm): 6.95 (d, $\mathcal{J} = 7.8$ Hz, 1H, indole C₇-H), 7.14-7.24 (m, 2H, indole C₅-H, phenyl C₄-H), 7.34-7.42 (m, 3H, indole C₆-H, phenyl C₅-H, C₆-H), 7.60 (d, $\mathcal{J} = 7.5$ Hz, 1H, indole C₄-H), 7.89 (s, 1H, phenyl C₃-H), 8.98 (s, 1H, CS-NH), 10.13 (s, 1H, indole NH), 12.85 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 380 ($[\text{M}^+]$, 3), 352 (100), 295 (94), 219 (53), 203 (17), 161 (29), 144 (34), 134 (18), 104 (17), 91 (11), 77 (9), 69 (12); (Found: C, 50.70; H, 2.90; N, 14.73. Calc. for $\text{C}_{16}\text{H}_{11}\text{F}_3\text{N}_4\text{O}_2\text{S}$: C, 50.53; H, 2.89; N, 14.74%).

2-(2-Oxo-1,2-dihydro-3H-indol-3-ylidene)-*N*-[4-(trifluoromethoxy)phenyl]-1-hydrazinecarbothioamide (**2d**). Yield 69% as yellow crystals; m.p. 228°C (d); IR (KBr, cm^{-1}): 3310, 3244 (NH stretching), 1695 (C=O), 1615 (C=N), 1544 (NH bending), 1157 (C=S); $^1\text{H-NMR}$ (CDCl_3 , δ , ppm): 6.92 (d, $\mathcal{J} = 7.8$ Hz, 1H, indole C₇-H), 7.11 (t, $\mathcal{J} = 7.6$ Hz, 1H, indole C₅-H), 7.26 (d (overlapped with the solvent), 2H, phenyl C₂-H, C₆-H), 7.35 (t, $\mathcal{J} = 7.6$ Hz, 1H, indole C₆-H), 7.61 (d,

$\mathcal{J} = 7.5$ Hz, 1H, indole C₄-H), 7.75 (d, $\mathcal{J} = 8.8$ Hz, 2H, phenyl C₃-H, C₅-H), 7.98 (s, 1H, CS-NH), 9.46 (s, 1H, indole NH), 12.87 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 382 ($[\text{M}^+] + 2$, 7), 381 ($[\text{M}^+] + 1$, 18), 380 ($[\text{M}^+]$, 88), 352 (100), 295 (10), 220 (35), 177 (56), 161 (49), 150 (62), 147 (17), 132 (48), 118 (34), 104 (44), 91 (11), 77 (15), 65 (5); (Found: C, 50.65; H, 2.90; N, 14.74. Calc. for $\text{C}_{16}\text{H}_{11}\text{F}_3\text{N}_4\text{O}_2\text{S}$: C, 50.53; H, 2.89; N, 14.74%).

2-(2-Oxo-1,2-dihydro-3H-indol-3-ylidene)-*N*-[2-(trifluoromethyl)phenyl]-1-hydrazinecarbothioamide (**2e**). Yield 83% as yellow crystals; m.p. 256°C (d); IR (KBr, cm^{-1}): 3350, 3250, 3150 (NH stretching), 1683 (C=O), 1589 (C=N), 1521 (NH bending), 1159 (C=S); $^1\text{H-NMR}$ (CDCl_3 , δ , ppm): 6.94 (d, $\mathcal{J} = 8.1$ Hz, 1H, indole C₇-H), 7.16 (t, $\mathcal{J} = 7.5$ Hz, 1H, indole C₅-H), 7.34-7.42 (m, 2H, indole C₆-H, phenyl C₄-H), 7.61-7.69 (m, 3H, phenyl C₃-H, C₅-H, C₆-H), 7.72 (d, $\mathcal{J} = 7.5$ Hz, 1H, indole C₄-H), 8.49 (s, 1H, CS-NH), 9.96 (s, 1H, indole NH), 12.87 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 366 ($[\text{M}^+] + 2$, 5), 365 ($[\text{M}^+] + 1$, 15), 364 ($[\text{M}^+]$, 71), 336 (100), 295 (5), 203 (26), 184 (69), 161 (50), 160 (15), 150 (24), 147 (7), 132 (43), 118 (23), 104 (34), 91 (10), 77 (12), 65 (3); (Found: C, 52.90; H, 3.03; N, 15.39. Calc. for $\text{C}_{16}\text{H}_{11}\text{F}_3\text{N}_4\text{OS}$: C, 52.75; H, 3.02; N, 15.38%).

2-(2-Oxo-1,2-dihydro-3H-indol-3-ylidene)-*N*-[3-(trifluoromethyl)phenyl]-1-hydrazinecarbothioamide (**2f**). Yield 55% as deep orange crystals; m.p. 228°C (d); IR (KBr, cm^{-1}): 3261, 3244 (NH stretching), 1695 (C=O), 1598 (C=N), 1544 (NH bending), 1165 (C=S); $^1\text{H-NMR}$ (CDCl_3 , δ , ppm): 6.97 (d, $\mathcal{J} = 7.8$ Hz, 1H, indole C₇-H), 7.17 (ddd, $\mathcal{J} = 7.5$, 7.5, 0.6 Hz, 1H, indole C₅-H), 7.71 (ddd, $\mathcal{J} = 7.8$, 7.8, 1.2 Hz, 1H, phenyl C₅-H), 7.52-7.60 (m, 2H, indole C₆-H, phenyl C₆-H), 7.67 (d, $\mathcal{J} = 7.8$ Hz, 1H, indole C₄-H), 7.99 (br.s, 2H, phenyl C₂-H, C₄-H), 8.10 (s, 1H, CS-NH), 9.59 (s, 1H, indole NH), 12.93 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 364 ($[\text{M}^+]$, 27), 336 (100), 219 (4), 203 (18), 161 (37), 160 (12), 150 (29), 147 (4), 145 (56), 134 (2), 132 (34), 118 (24), 104 (45), 91 (12), 77 (22), 65 (6), 51 (15); (Found: C, 52.88; H, 3.03; N, 15.38. Calc. for $\text{C}_{16}\text{H}_{11}\text{F}_3\text{N}_4\text{OS}$: C, 52.75; H, 3.02; N, 15.38%).

2-(2-Oxo-1,2-dihydro-3H-indol-3-ylidene)-*N*-[4-(trifluoromethyl)phenyl]-1-hydrazinecarbothioamide (**2g**). Yield 84% as yellow crystals; m.p. 270°C (d); IR (KBr, cm^{-1}): 3284, 3244, 3184, 3124 (NH stretching), 1691 (C=O), 1600 (C=N), 1544 (NH bending), 1163 (C=S); $^1\text{H-NMR}$ (CDCl_3 , δ , ppm): 6.91-7.16 (m, 3H, indole C₇-H, phenyl C₂-H, C₆-H), 7.27-7.67 (m, 4H, indole C₅-H, C₆-H and phenyl C₃-H, C₅-H), 7.91 (d, $\mathcal{J} = 8.2$ Hz, 1H, indole C₄-H), 8.50 (s, 1H, CS-NH), 9.59 (s, 1H, indole NH), 12.91 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 365 ($[\text{M}^+] + 1$,

5), 364 ($[M^+]$, 26), 336 (100), 219 (4), 203 (24), 161 (40), 160 (12), 150 (32), 147 (3), 145 (59), 133 (21), 132 (38), 118 (32), 104 (55), 91 (19), 77 (28), 65 (7), 51 (20); (Found: C, 52.91; H, 3.01; N, 15.37. Calc. for $C_{16}H_{11}F_3N_4OS$: C, 52.75; H, 3.02; N, 15.38%).

N-(2,4-Difluorophenyl)-2-(2-oxo-1,2-dihydro-3H-indol-3-ylidene)-1-hydrazinecarbothioamide (**2h**). Yield 68% as deep yellow crystals; m.p. 252°C (d); IR (KBr, cm^{-1}): 3282, 3244 (NH stretching), 1695 (C=O), 1618 (C=N), 1544 (NH bending), 1159 (C=S); 1H -NMR ($CDCl_3$, δ , ppm): 6.89-6.95 (m, 3H, indole C₇-H, phenyl C₅-H, C₆-H), 7.12 (t, $f = 7.6$ Hz, 1H, indole C₅-H), 7.36 (t, $f = 7.7$ Hz, 1H, indole C₆-H), 7.58 (s, 1H, phenyl C₃-H), 7.61 (d, $f = 7.6$ Hz, 1H, indole C₄-H), 8.37 (s, 1H, CS-NH), 9.48 (s, 1H, indole NH), 12.88 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 334 ($[M^+]$ + 2, 3), 333 ($[M^+]$ + 1, 6), 332 ($[M^+]$, 22), 304 (100), 171 (44), 161 (25), 150 (10), 147 (8), 132 (13), 118 (25), 104 (22), 91 (9), 77 (10), 65 (5); (Found: C, 54.35; H, 3.02; N, 16.86. Calc. for $C_{15}H_{10}F_2N_4OS$: C, 54.22; H, 3.01; N, 16.87%).

N-(2,5-Difluorophenyl)-2-(2-oxo-1,2-dihydro-3H-indol-3-ylidene)-1-hydrazinecarbothioamide (**2i**). Yield 69% as deep yellow crystals; m.p. 256°C (d); IR (KBr, cm^{-1}): 3307, 3246 (NH stretching), 1695 (C=O), 1620 (C=N), 1544 (NH bending), 1165 (C=S); 1H -NMR (DMSO, δ , ppm): 6.94 (d, $f = 7.8$ Hz, 1H, indole C₇-H), 7.12 (t, $f = 7.5$ Hz, 1H, indole C₅-H), 7.23-7.52 (m, 4H, indole C₆-H, phenyl C₃-H, C₄-H, C₆-H), 7.68 (d, $f = 7.5$ Hz, 1H, indole C₄-H), 10.73 (s, 1H, CS-NH), 11.28 (s, 1H, indole NH), 12.92 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 334 ($[M^+]$ + 2, 3), 333 ($[M^+]$ + 1, 8), 332 ($[M^+]$, 38), 304 (100), 203 (8), 171 (54), 161 (27), 154 (60), 144 (41), 129 (32), 118 (26), 104 (15), 91 (8), 76 (8), 63 (12); (Found: C, 54.37; H, 3.02; N, 16.86. Calc. for $C_{15}H_{10}F_2N_4OS$: C, 54.22; H, 3.01; N, 16.87%).

N-(2,6-Difluorophenyl)-2-(2-oxo-1,2-dihydro-3H-indol-3-ylidene)-1-hydrazinecarbothioamide (**2j**). Yield 69% as deep yellow crystals; m.p. 250°C (d); IR (KBr, cm^{-1}): 3350, 3235 (NH stretching), 1695 (C=O), 1620 (C=N), 1557 (NH bending), 1182 (C=S); 1H -NMR (DMSO, δ , ppm): 6.94 (d, $f = 7.8$ Hz, 1H, indole C₇-H), 7.12 (ddd, $f = 7.5, 7.5, 0.6$ Hz, 1H, indole C₅-H), 7.25 (t, $f = 8.1$ Hz, 1H, phenyl C₄-H), 7.39 (ddd, $f = 7.8, 7.8, 1.2$ Hz, 1H, indole C₆-H), 7.46-7.52 (m, 2H, phenyl C₃-H, C₅-H), 7.67 (d, $f = 7.5$ Hz, 1H, indole C₄-H), 10.57 (s, 1H, CS-NH), 11.28 (s, 1H, indole NH), 12.96 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 334 ($[M^+]$ + 2, 6), 333 ($[M^+]$ + 1, 18), 332 ($[M^+]$, 92), 304 (100), 171 (29), 161 (39), 150 (49), 147 (6), 132 (54), 118 (37), 104 (50), 91 (11), 77 (19), 65 (4); (Found: C, 54.38; H, 3.02; N, 16.85. Calc. for $C_{15}H_{10}F_2N_4OS$: C, 54.22; H, 3.01; N, 16.87%).

N-(3,5-Difluorophenyl)-2-(2-oxo-1,2-dihydro-3H-indol-3-ylidene)-1-hydrazinecarbothioamide (**2k**). Yield 84% as orange crystals; m.p. 270°C (d); IR (KBr, cm^{-1}): 3304, 3203, 3188 (NH stretching), 1699 (C=O), 1604 (C=N), 1548 (NH bending), 1155 (C=S); 1H -NMR (DMSO, δ , ppm): 6.93 (d, $f = 7.8$ Hz, 1H, indole C₇-H), 7.09-7.18 (m, 2H, indole C₅-H, phenyl C₄-H), 7.38 (ddd, $f = 7.8, 7.8, 1.1$ Hz, 1H, indole C₆-H), 7.56, 7.58 (2d, $f = 2.2$ Hz, 2H, phenyl C₂-H, C₆-H), 7.75 (d, $f = 7.4$ Hz, 1H, indole C₄-H), 10.89 (s, 1H, CS-NH), 11.28 (s, 1H, indole NH), 12.91 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 333 ($[M^+]$ + 1, 7), 332 ($[M^+]$, 48), 304 (100), 187 (5), 171 (19), 161 (19), 160 (4), 150 (20), 147 (2), 145 (22), 133 (13), 132 (30), 118 (26), 104 (57), 91 (14), 77 (37), 51 (28); (Found: C, 54.40; H, 3.02; N, 16.86. Calc. for $C_{15}H_{10}F_2N_4OS$: C, 54.22; H, 3.01; N, 16.87%).

2-(2-Oxo-1,2-dihydro-3H-indol-3-ylidene)-*N*-(2,4,6-trichlorophenyl)-1-hydrazinecarbothioamide (**2l**). Yield 81% as light orange crystals; m.p. 261°C (d); IR (KBr, cm^{-1}): 3300, 3143, 3132 (NH stretching), 1683 (C=O), 1585 (C=N), 1527 (NH bending), 1168 (C=S); 1H -NMR (DMSO, δ , ppm): 6.93 (d, $f = 7.8$ Hz, 1H, indole C₇-H), 7.10 (t, $f = 7.5$ Hz, 1H, indole C₅-H), 7.38 (t, $f = 7.6$ Hz, 1H, indole C₆-H), 7.67 (d, $f = 7.1$ Hz, 1H, indole C₄-H), 7.82 (s, 2H, phenyl C₃-H, C₅-H), 10.80 (s, 1H, CS-NH), 11.24 (s, 1H, indole NH), 12.95 (s, 1H, N-NH); EIMS (70 eV) m/z (%): 400 ($[M^+]$ + 2, 3), 399 ($[M^+]$ + 1, 2), 398 ($[M^+]$, 7), 371 (6), 297 (1), 244 (10), 196 (56), 138 (95), 111 (100), 102 (43), 89 (50), 75 (83), 63 (23), 50 (41); (Found: C, 45.20; H, 2.25; N, 14.03. Calc. for $C_{15}H_9Cl_3N_4OS$: C, 45.06; H, 2.25; N, 14.02%).

Biological testing

Cytotoxicity (in vitro). Brine shrimp (*Artemia salina* leach) eggs were hatched in a shallow rectangular plastic dish (22 × 32 cm) filled with artificial sea water, which was prepared with a commercial salt mixture (Instant Ocean, Aquarium System, Inc., Mentor, Ohio, USA) and double-distilled water. An unequal partition was made in the plastic dish with the help of a perforated device. Approximately 50 mg of eggs were sprinkled into the large compartment, which was darkened, while the smaller compartment was opened to ordinary light. After two days, nauplii were collected by a pipette from the lighted side. A sample of the test compound was prepared by dissolving 2 mg of each compound in 2 mL of methanol. From this stock solution, 500, 50 and 5 μ L were transferred to 9 vials, three for each dilution, and one vial was kept as control having 2 mL of methanol. The solvent was allowed to evaporate overnight. After two days, when shrimp larvae were ready, 1 mL of sea water and 10

shrimps were added to each vial (30 shrimps/dilution) and the volume was adjusted with sea water to 5 mL per vial. After 24 h, the number of survivors was counted. Data were analyzed by a Finney computer program to determine the LD₅₀ values [42].

Phytotoxicity (in vitro). This bioassay was performed according to a modified protocol [43]. The test compounds were incorporated with sterilized E-medium at different concentrations i.e. 5, 50 and 500 µg/mL in MeOH. Sterilized conical flasks were inoculated with compounds of desired concentrations prepared from the stock solution and allowed to evaporate overnight. Each flask was inoculated with 20 mL of sterilized E-medium and ten *Lemna aequinocalis* W&L, each containing a rosette of three fronds. Other flasks were supplemented with MeOH serving as negative control and a reference inhibitor i.e. paraquat serving as positive control. Treatments were replicated three times and flasks incubated at 30°C in Fisons Fi-Totron 600 H growth cabinet for 7d, 9000 lux light intensity, 56 ± 10 rh (relative humidity), and 12 h day length. Growth of *Lemna aequinocalis* in compound-containing flask was determined by counting the number of fronds per dose and growth inhibition was calculated with reference to negative control.

Urease inhibitory activity (in vitro). Reaction mixtures comprising 25 µL of enzyme (Jack bean urease) solution and 55 µL of buffers containing 100 mM urea were incubated with 5 µL of test compounds (0.1 mM concentration) at 30°C for 15 min in 96-well plates. Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn [44]. Briefly, 45 µL each of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 µL of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Device, USA). All reactions were performed in triplicate in a final volume of 200 µL. The results (change in absorbance per min) were processed by using SoftMax Pro software (Molecular Device, USA). All the assays were performed at pH 8.2 (0.01 M K₂HPO₄ · 3H₂O, 1 mM EDTA and 0.01 M LiCl). Percentage inhibitions were calculated from the formula 100-(OD_{testwell}/OD_{control}) × 100. Thiourea was used as the standard inhibitor of urease.

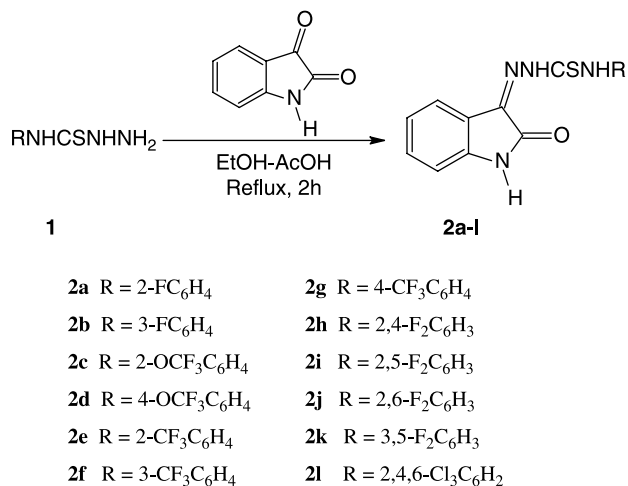
Results and discussion

The present work describes the synthesis and *in vitro* determination of the cytotoxic, phytotoxic and urease inhibitory effects of twelve new N⁴-substituted isatin-3-thiosemicarbazones **2a-1**.

Chemistry

The synthesis of isatin-thiosemicarbazones was straightforward. A mixture of isatin, appropriate thiosemicarbazide and ethanol containing a few drops of glacial acetic acid was heated under reflux for 2 h (Scheme 1). The crystalline solid formed during heating in each case was filtered. Thorough washing with hot ethanol followed by ether provided the desired compounds **2a-1** in moderate to good yields (55–84%).

The synthesized thiosemicarbazones were identified by elemental analysis, IR, ¹H-NMR and EI mass spectra. Satisfactory elemental analysis (± 0.4% of calculated values) was obtained for all compounds. The IR spectra of **2** showed two separate bands resulting from the NH stretchings of indole and thioamide functions in the 3350–3200 and 3188–3124 cm⁻¹ regions. The lactam C=O, azomethine C=N and thioamide C=S stretchings were observed in the 1705–1683, 1620–1585 and 1193–1145 cm⁻¹ regions, respectively [45–47]. The ¹H-NMR spectra of **2** exhibited three singlets at δ 7.98–10.89, δ 9.42–11.95 and δ 12.75–12.96 for the thiosemicarbazone N⁴-H, indole NH and thiosemicarbazone N²-H, respectively [45,48,49]. The indole C₇-H appeared as doublet at δ 6.87–6.97, while the indole C₅-H and C₆-H appeared at δ 7.10–7.17 and 7.35–7.39, respectively, as a triplet or a doublet of doublet. Indole C₄-H, experienced a deshielding effect due to the inductive effect of the C=N function and resonated as a doublet at δ 7.60–7.91 [49–51]. In certain cases, however, overlapping of the first three signals, particularly that of indole C₅-H and C₆-H were observed as multiplets. These signals appeared in combination with different aromatic protons of the N⁴-substituents. The EI mass spectra of **2** showed molecular ions of different intensity, which confirmed their molecular weights. The major fragmentation pathway involved the cleavage of the exocyclic N-N,



Scheme 1. Synthesis of title compounds.

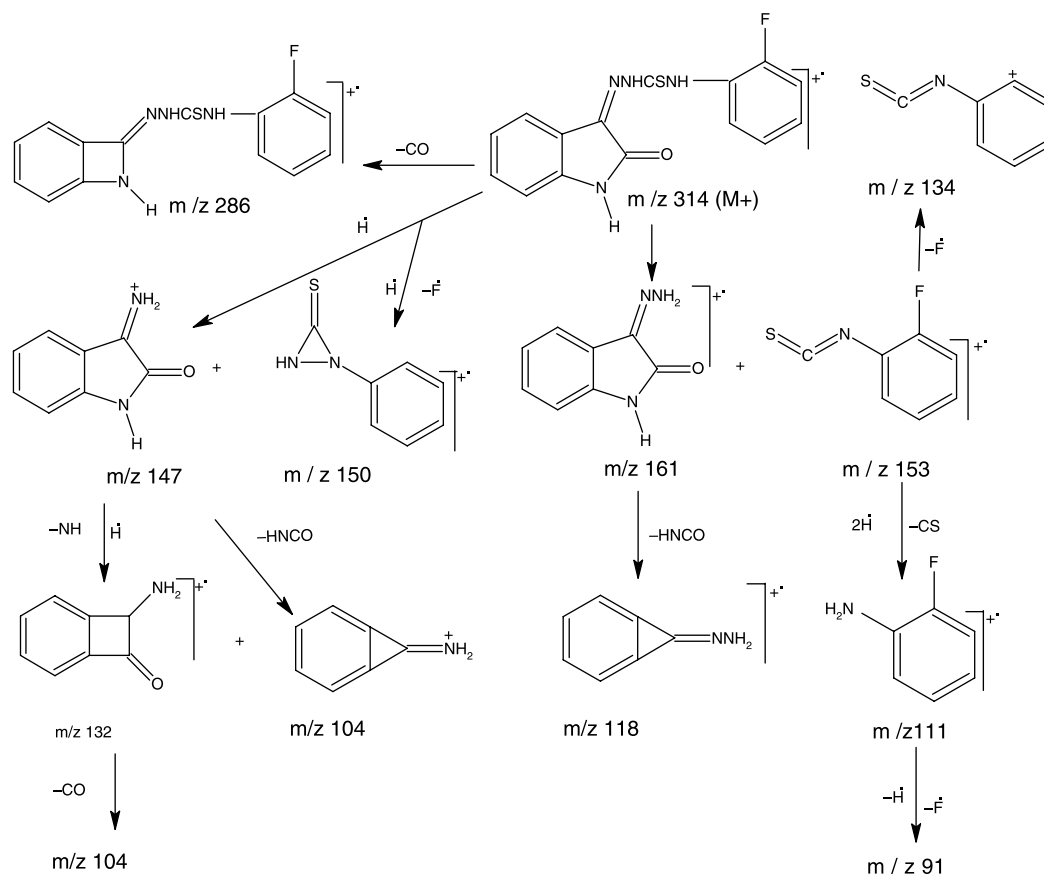


Figure 1. The proposed fragmentation pattern of the compound **2a**.

NH-CS and endocyclic NH-CO bonds. The proposed fragmentation pattern of **2a** is depicted in Figure 1.

Biological testing

Cytotoxicity (in vitro). The synthetic thiosemicarbazones **2a-l** having one, two or three substituents about the phenyl ring substituted at N⁴ were tested for their cytotoxicity by the brine shrimp bioassay. All the compounds of this series displayed promising cytotoxicity ($LD_{50} = 1.10 \times 10^{-5} \text{ M} - 1.36 \times 10^{-4} \text{ M}$) against *Artemia salina* (Table I). The remaining compound **2m** with no substituent in the phenyl ring gave a value of $LD_{50} > 3.38 \times 10^{-4} \text{ M}$ in our earlier assay [20] and, therefore, can be considered to be almost inactive. It may be noted that compound **2l**, the only chloro-substituted compound tested in the assay, showed maximum activity ($LD_{50} = 1.10 \times 10^{-5} \text{ M}$) and, therefore, proved to be the most potent compound in the present series. Next potent was **2b** ($LD_{50} = 1.60 \times 10^{-5} \text{ M}$) having a fluoro substituent at *meta* position of the phenyl ring. Compound **2a** with fluoro substituent at *ortho* position was found to be relatively less potent, giving a value of $LD_{50} = 3.10 \times 10^{-5} \text{ M}$. To the contrary, all the difluoro-substituted compounds **2h-k**, regardless of the positions of substitution in the phenyl ring, displayed about the

same values ($LD_{50} = 2.00$ or $2.10 \times 10^{-5} \text{ M}$). In the case of trifluoromethyl-substituted compounds **2e-g**, the *meta*-substituted compound **2f** showed about six-fold larger cytotoxicity ($LD_{50} = 2.20 \times 10^{-5} \text{ M}$) than the *ortho*-substituted **2e** ($LD_{50} = 1.36 \times 10^{-4} \text{ M}$) and five-fold larger cytotoxicity than the *para*-substituted **2g** ($LD_{50} = 1.17 \times 10^{-4} \text{ M}$). Similarly, amongst the two trifluoromethoxy-substituted compounds tested in the assay, compound **2d** with a *para* substituent displayed about seven-fold larger cytotoxicity ($LD_{50} = 1.80 \times 10^{-5} \text{ M}$) than the *ortho*-substituted

Table I. Brine shrimp bioassay for compounds **2a-l**.

Compounds	LD_{50} (M)
2a	3.10×10^{-5}
2b	1.60×10^{-5}
2c	1.21×10^{-4}
2d	1.80×10^{-5}
2e	1.36×10^{-4}
2f	2.20×10^{-5}
2g	1.17×10^{-4}
2h	2.00×10^{-5}
2i	2.10×10^{-5}
2j	2.00×10^{-5}
2k	2.10×10^{-5}
2l	1.10×10^{-5}
2m [19,20]	$> 3.38 \times 10^{-4}$

2c (LD₅₀ = 1.21 × 10⁻⁴ M). These structure-activity relationships may serve as a basis for chemical modifications aimed at the development of certain cytotoxic agents of clinical interest.

Phytotoxicity (in vitro). All the presently synthesized thiosemicarbazones **2a-1** were further screened for their phytotoxic effects at three different concentrations i.e. 500, 50 and 5 µg/mL. Compound **2m**, the synthesis of which has been reported elsewhere [19], serves as a reference point to evaluate the effects of substituents about the phenyl ring on the inhibition potential of these synthetic compounds. At the highest concentration tested (500 µg/mL), compounds **2b-m** exhibited weak or non-significant (10–30%) plant growth inhibition, whereas **2a** displayed moderate (40%) inhibition (Table II). On the other hand, at the lowest concentration (5 µg/mL), eight compounds i.e. **2a-c**, **2e**, **2f**, **2h**, **2i** and **2m**, out of the thirteen compounds tested, showed no plant growth inhibition, whereas the rest i.e. **2d**, **2g** and **2j-l** exhibited non-significant (5–15%) inhibition. From the results obtained in this assay, it may thus be concluded that our compounds, in general, displayed either non-significant or no plant growth inhibition at much higher levels of concentration as compared to the standard paraquat, which showed 100% inhibition at a concentration of 0.015 µg/mL. Moreover, the percent inhibition values revealed that the type and position of the substituents did not affect the phytotoxic activity to a larger extent. In deed, very small difference in activity of certain compounds was observed, particularly at the lowest level of concentration (5 µg/mL).

Urease inhibitor (in vitro). All the synthesized thiosemicarbazones **2a-1** were also subjected to

urease inhibitory studies. The results collected in Table III revealed that as compared to compound **2m** with no substituent in the phenyl ring, substitution of one, two or three inductively electron-withdrawing substituents at different positions of the phenyl ring either induced or enhanced the urease inhibitory activity of the compounds. This conclusion receives support from the results obtained by us in an earlier assay [20]. For example, compounds **2c** and **2d** having trifluoromethoxy substituents at *ortho* and *para* positions, and **2f** and **2g** with trifluoromethyl substituents at *meta* and *para* positions are found to show enhanced activity (40.7%, 49.6%, 78.2% and 44.6%, respectively) when compared with the corresponding compounds having methoxy- and methyl- substituents exhibiting 12.85%, 28.67%, 13.98% and 7.58% inhibition, respectively. Much pronounced enhancement was observed in the case of **2f** (13.98% → 78.2%). Similarly, compound **2e** with trifluoromethyl group at the *ortho* position displayed 63.1% inhibition of the enzyme, whereas the corresponding compound having methyl substituent was found to exhibit no inhibitory activity at the tested concentration (100 µM). This indicated that the thiosemicarbazone moiety having electron-withdrawing substituents about the phenyl ring substituted at N⁴ might interfere with the enzyme activity more efficiently. Of the twelve compounds **2a-1** tested in this assay, eight i.e. **2a**, **2b**, **2e**, **2f**, **2h-j** and **2l** proved to be potent inhibitors demonstrating relatively a higher degree of urease inhibitory activity with IC₅₀ values ranging from 20.6 µM to 50.6 µM. Compounds **2a** and **2f** showed pronounced urease inhibition with IC₅₀ value 20.6 µM, which was even better than the standard thiourea (IC₅₀ value 21.0 µM) and may act as lead compounds. However, in comparison to compound **2a**, compound **2b** with a fluoro substituent at *meta* position of the phenyl ring

Table II. Percent growth inhibition of *Lemna aquivalis* by compounds **2a-1** at different concentrations.*

Compound	500	50	5
	(µg/mL) (% G.I.†)	(µg/mL) (% G.I.†)	(µg/mL) (% G.I.†)
2a	40	25	00
2b	10	5	00
2c	10	05	00
2d	15	10	05
2e	15	10	00
2f	10	05	00
2g	15	10	05
2h	10	05	00
2i	10	05	00
2j	25	15	10
2k	30	25	15
2l	20	15	10
2m [19]	10	05	00

* The reference compound paraquat shows 100% growth inhibition at a concentration of 0.015 µg/mL; † G.I.: growth inhibition.

Table III. Inhibition of Jack bean urease by compounds **2a-1**.

Compounds	Percentage (%) of inhibition	
	at 100 µM	IC ₅₀ ± SEM (µM)
2a	84.9	20.6 ± 0.5
2b	57.3	50.6 ± 2.2
2c	40.7	
2d	49.6	
2e	63.1	33.1 ± 0.35
2f	78.2	20.6 ± 0.07
2g	44.6	
2h	71.9	47.6 ± 0.4
2i	66.6	47.9 ± 0.96
2j	92.0	33.1 ± 0.35
2k	38.8	
2l	79.6	28.6 ± 1.8
2m [19,20*]	NA	
Thiourea †		21.0 ± 0.01

* Screened against human urease; NA: no inhibitory activity; † Standard inhibitor of urease enzyme.

exhibited much enhanced IC_{50} value (50.6 μM). This clearly indicated that **2b** as compared to **2a** interfered with the enzyme activity differently, resulting into decreased inhibitory potential. Compound **2l** having three chloro substituents at *ortho* and *para* positions of the phenyl ring also exhibited much better inhibition of the enzyme (79.6%) with an IC_{50} value 28.6 μM . However, in our earlier assay [20], compounds having only one chloro substituent at *ortho* or *para* position of the phenyl ring exhibited relatively much lower inhibition (43.66% and 25.58%, respectively). Chlorine is both electron-donating by resonance effect (+M) and electron-withdrawing by inductive effect (-I). Much higher percentage of inhibition exhibited by compound **2l** in the present assay clearly indicates that the ultimate or overall electro-withdrawing effect of the three chloro substituents increases the inhibitory potential of the compound to a much larger extent. Compounds **2e** and **2j** having one trifluoromethyl and two fluoro substituents at *ortho* positions of the phenyl ring, respectively, were also proved to be potent inhibitors in the present series, as they displayed relatively much greater activity with IC_{50} value 31.1 μM . However, these compounds were found to be less potent than compounds **2f** and **2a** having trifluoromethyl and fluoro substituents at *meta* and *ortho* positions of the phenyl ring, respectively. These results indicate that steric hindrance, particularly in the case of **2e** plays an important role in decreasing the inhibitory potential of the compound. Amongst the rest difluoro-substituted compounds **2h** and **2i**, compound **2h** having substituents at the *ortho* and *para* positions exhibited slightly more inhibition (71.9%) with an IC_{50} value 47.6 μM . Compared to this, **2i** having substituents at the *ortho* and *meta* positions demonstrated 66.6% inhibition with an IC_{50} value 47.9 μM .

Ureases obtained from different sources contain, in addition to two Lewis acid nickel ions, one to three protein subunits present in varied stoichiometric ratios [25]. A urease inhibitor can, therefore, interfere with the enzymatic activity by interacting either with the metal ions or the protein component. Sulphenamide, for example, has been reported to inhibit activity of the *H. pylori* urease by interacting with the sulphhydryl (S-H) group present in its protein components [52,53]. Many mechanisms, including competitive and non-competitive are known to be involved in the interaction of an inhibitor with an enzyme. Though the exact mechanism of urease inhibition by our test compounds **2a-l** is not known, it is intriguing to carry out investigations with regard to detailed kinetics of such interaction. Apparently, these compounds employ a mechanism of action by exploiting a common transition catalysis state and acting as chelating agents to form octahedral complexes with the two slightly distorted octahedral nickel ions of the enzyme. Further, since our test compounds showed

non-significant phytotoxic activity at the highest tested concentration, therefore, they could be combined with fertilizers in small amounts for inhibiting the soil ureases, thus increasing the overall efficiency of nitrogen utilization.

In summary, we have demonstrated the potential of N^4 -substituted isatin-3-thiosemicarbazones to exhibit selected biological activities i.e. cytotoxicity, phytotoxicity and more importantly the urease inhibitory activity. All the synthesized thiosemicarbazones proved to be active in brine shrimp bioassay; most of them exhibited promising cytotoxic activity. In urease-inhibition bioassay, eight out of twelve tested compounds showed a high degree of activity; two of these i.e. **2a** and **2f** demonstrated pronounced urease inhibition and may act as lead compounds. Extensive mechanism-based studies are required to contribute to the better understanding of the mechanism of action of these compounds. In phytotoxicity assay, almost all the synthetic compounds demonstrated non-significant activity at the highest tested concentration, thus inviting attention to their usefulness as potent inhibitors of soil ureases. Structure-activity relationship studies revealed that the type and position of the substituents about the phenyl ring substituted at N^4 of the thiosemicarbazone moiety did not affect the phytotoxic activity to a larger extent. Based on the data presented in Tables I, II and III, and in terms of further development and structure-activity relationship studies, combination of the substitution at position-1 and/or -5 of the isatin scaffold with attachment of different aryl groups at N^4 of the thiosemicarbazone moiety is considered worth pursuing. Work in this regard along with extended structure-activity relationship studies will be reported in the near future.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

- [1] Pandeya SN, Smitha S, Jyoti M, Sridhar SK. Biological activities of isatin and its derivatives. *Acta Pharm* 2005;55: 27–46 (and references therein).
- [2] da Silva JFM, Garden SJ, Pinto AC. The chemistry of isatins: A review from 1975 to 1999. *J Braz Chem Soc* 2001;12: 273–324 (and references therein).
- [3] Beauchard A, Ferandin Y, Frere S, Lozach O, Blairvacq M, Meijer L, Thierry V, Besson T. Synthesis of novel 5-substituted indirubins as protein kinases inhibitors. *Bioorg Med Chem* 2006;14:6434–6443.
- [4] Hyatt JL, Moak T, Hatfield JM, Tsurkan L, Edwards CC, Wierdl M, Danks MK, Wadkins RM, Potter PM. Selective inhibition of carboxylesterases by isatins, indole-2,3-diones. *J Med Chem* 2007;50:1876–1885.
- [5] Karali N. Synthesis and primary cytotoxicity evaluation of new 5-nitroindole-2, 3-dione derivatives. *Eur J Med Chem* 2002;37:909–918.
- [6] Chiyanzu I, Hansell E, Gut J, Rosenthal PJ, McKerrow JH, Chibale K. Synthesis and evaluation of isatins and

- thiosemicarbazone derivatives against cruzain falcipain-2 and rhodesain. *Bioorg Med Chem Lett* 2003;13:3527–3530.
- [7] Bal TR, Anand B, Yogeewari P, Sriram D. Synthesis and evaluation of anti-HIV activity of isatin β -thiosemicarbazone derivatives. *Bioorg Med Chem Lett* 2005;15:4451–4455.
- [8] Chiyanzu I, Clarkson C, Smith PJ, Lehman J, Gut J, Rosenthal PJ, Chibale K. Design, synthesis and anti-plasmodial evaluation *in vitro* of new 4-aminoquinoline isatin derivatives. *Bioorg Med Chem* 2005;13:3249–3261.
- [9] Rai A, Sengupta SK, Pandey OP. Lanthanum (III) and praseodymium (III) complexes with isatin thiosemicarbazones. *Spectrochim Acta* 2005;61A:2761–2765.
- [10] Terzioglu N, Karali N, Gursoy A, Pannecouque C, Leysen P, Paeshuysse J, Neyts J, de Clercq E. Synthesis and primary antiviral activity evaluation of 3-hydrazono-5-nitro-2-indolone derivatives. *Arkivoc* 2006;109–118.
- [11] Quenelle DC, Keith KA, Kern ER. *In vitro* and *vivo* evaluation of isatin- β -thiosemicarbazone and marboran against vaccinia and cowpox virus infections. *Antiviral Res* 2006;71:24–30.
- [12] Karki SS, Thota S, Darj SY, Balzarini J, de Clercq E. Synthesis, anticancer and cytotoxic activities of some mononuclear Ru(II) compounds. *Bioorg Med Chem* 2007;15:6632–6641.
- [13] Chohan ZH, Pervez H, Rauf A, Scozzafava A, Supuran CT. Antibacterial Co(II), Cu (II), Ni (II) and Zi (II) complexes of thiadiazole-derived furanyl, thiophenyl and pyrrolyl Schiff bases. *J Enz Inhib Med Chem* 2002;17:117–122.
- [14] Chohan ZH, Pervez H, Khan KM, Rauf A, Supuran CT. Binding of transition metal ions [cobalt, copper, nickel and zinc] with furanyl-, thiophenyl-, pyrrolyl-, salicylyl- and pyridyl-derived cephalaxins as potent antibacterial agents. *J Enz Inhib Med Chem* 2004;19:51–56.
- [15] Chohan ZH, Pervez H, Khan KM, Rauf A, Maharvi GM, Supuran CT. Antifungal cobalt (II), copper (II), nickel (II) and zinc (II) complexes of furanyl-, thiophenyl-, pyrrolyl-, salicylyl- and pyridyl-derived cephalaxins. *J Enz Inhib Med Chem* 2004;19:85–90.
- [16] Chohan ZH, Pervez H, Khan KM, Rauf A, Maharvi GM, Supuran CT. Antifungal mono- and di- substituted symmetrical and unsymmetrical tirazine-derived Schiff bases and their transition metal complexes. *J Enz Inhib Med Chem* 2004;19:161–168.
- [17] Chohan ZH, Pervez H, Khan KM, Rauf A, Supuran CT. Isatin-derived antibacterial and antifungal compounds and their transition metal complexes. *J Enz Inhib Med Chem* 2004;19:417–423.
- [18] Chohan ZH, Pervez H, Rauf A, Khan KM, Supuran CT. Antibacterial cobalt (II), copper (II), nickel (II) and zinc (II) complexes of mercaptothiadiazole- derived furanyl, thienyl, pyrrolyl, salicylyl and pyridinyl Schiff bases. *J Enz Inhib Med Chem* 2006;21:193–201.
- [19] Pervez H, Iqbal MS, Tahir MY, Choudhary MI, Khan KM. Synthesis of some N⁴-substituted isatin-3-thiosemicarbazones. *Nat Prod Res* 2007;21:1178–1186.
- [20] Pervez H, Iqbal MS, Tahir MY, Nasim FH, Choudhary MI, Khan KM. *In vitro* cytotoxic, antibacterial, antifungal and urease inhibitory activities of some N⁴-substituted isatin-3-thiosemicarbazones. *J Enz Inhib Med Chem* 2007; iFirst:1–7 10.1080/14756360701746179.
- [21] Hausinger RP. Nickel utilization by microorganisms. *Microbiol Rev* 1987;51:22–46.
- [22] Mobley HLT, Hausinger RP. Microbial urease: Significance, regulation and molecular characterization. *Microbiol Rev* 1989;53:85–108.
- [23] Hausinger RP. *Biochemistry of nickel*. New York: Plenum; 1993. p 23–57.
- [24] Andrews RK, Blakeley RL, Zerner B. In: Lancaster Jr. LR, editor. *The bioinorganic chemistry of nickel*. New York: VCH; 1988. p 141–165.
- [25] Mobley HLT, Island MD, Hausinger RP. Molecular biology of microbial ureases. *Microbiol Rev* 1995;59:451–480.
- [26] Collins CM, D’Orazio SEF. *Helicobacter pylori* nickel binding protein. *Mol Microbiol* 1993;9:907–913.
- [27] Ciurli S, Benini S, Rypniewski WR, Wilson KS, Miletti S, Mangani S. Structural properties of the nickel ions in urease: Novel insights into the catalytic and inhibition mechanism. *Coord Chem Rev* 1999;190–192:331–355 (and references therein).
- [28] Griffith DP. Struvite stones. *Kidney Int* 1978;13:372–382.
- [29] Sim E, Jones A, Stanley L. Acetohydroxamate, a urease inhibitor, inhibits the covalent binding reaction of complement proteins C₃ and C₄. *Acta Pharmacol Toxicol* 1985;57:304–306.
- [30] Radel RJ, Gautney J, Peters GE. Urease inhibitor developments. In: Bock B, Kissel D, editors. *Ammonia volatilization from urea fertilizers*. Muscle Shoals, AL: Tennessee Valley Authority; 1988. p 111–136.
- [31] Peters GE, Radel RJ, Medina RJ. Synthesis of phenoxycyclo-triphosphazatrienes. *J Agric Food Chem* 1988;36:384–390.
- [32] Gasser JKR. Urea as fertilizer. *Soils Fert* 1964;27:175–180.
- [33] Tomlinson TE. Urea: Agronomic applications. *Proc Fert Soc* 1970;113:1–76.
- [34] Sahrawat KL. Control of urea hydrolysis and nitrification in soils by chemicals: Properties and problems. *Plant Soil* 1980;57:335–352.
- [35] Mulvaney RL, Bremner JM. Control of urease transformation in soils. *Soil Biochem* 1981;5:153–196.
- [36] Hauck RD. In: Hauck RD, editor. *Nitrogen in crop production*. Madison, WI: American Society of Agronomy; 1984. p 551–560.
- [37] Martens DA, Bremner JM. Effectiveness of phosphoroamides for retardation of urea hydrolysis in soils. *Soil Sci Soc Am J* 1984;48:302–305.
- [38] Bremner JM, Krogmeier MJ. Elimination of the adverse effects of urea fertilizer on seed germination, seedling growth, and early plant growth in soil. *Proc Natl Acad Sci USA* 1988;85:4601–4604.
- [39] McCarthy GW, Bremner JM, Lee JS. Inhibition of plant and microbial ureases by phosphoroamides. *Plant Soil* 1990;127:269–283.
- [40] Krogmeier MJ, McCarthy GW, Bremner JM. Potential phytotoxicity associated with the use of soil urease inhibitors. *Proc Natl Acad Sci USA* 1989;86:1110–1112.
- [41] Amtul Z, Rahman AU, Siddiqui RA, Choudhry MI. Chemistry and mechanism of urease inhibition. *Curr Med Chem* 2002;9:1323–1348 (and references therein).
- [42] Finney DJ. *Probit analysis*. 3rd ed. London: Cambridge University Press; 1971.
- [43] McLaughlin JL, Chang CJ, Smith DL. In: Rahman AU, editor. *Studies in natural products chemistry; Bench-top bioassays for the discovery of products: Structure and chemistry (Part B)*. Amsterdam: Elsevier; 1991. p 383.
- [44] Weatherburn MW. Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem* 1967;39:971–974.
- [45] Omar AME, Eshba NH, Salama HM. Synthesis of some substituted isatin- β -thiosemicarbazones and isatin- β -hydrazonothiazoline derivatives as potential antiviral and antimicrobial agents. *Arch Pharm (Weinheim)* 1984;317:701–709.
- [46] Petrov I, Grupce O, Stafilov T. The nitrogen–hydrogen stretching region of some imides and thioimides. *J Mol Struct* 1986;142:275–278.
- [47] Naumov P, Anastasova F. Experimental and theoretical vibrational study of isatin, its 5-(NO₂, F, Cl, Br, I, CH₃)

- analogues and the isatinato anion. *Spectrochim Acta* 2001; 57A:469–481.
- [48] Karali N, Gursoy A. Synthesis and anticonvulsant activity of some new thiosemicarbazone and 4-thiazolidone derivatives bearing an isatin moiety. *Farmaco* 1994;49:819–822.
- [49] Laatsch H, Thomson RH, Cox PJ. Spectroscopic properties of violacein and related compounds: Crystal structure of tetramethyl violacein. *J Chem Soc Perkin Trans 2* 1984: 1331–1339.
- [50] Eshba NH, Salama HM, Labouta IM, Omar AME. Synthesis of some substituted 1,2,4-triazino [5,6-b] indole derivatives as potential antiviral anticancer agents. *Pharmazie* 1987;42: 664–666.
- [51] Baron ML, Martin LL, Rae ID, Simmonds PM, Woolcock ML. Relaxation processes in aromatic methyl groups. II. Methyl-methyl nuclear overhauser enhancements. *Aust J Chem* 1990;43:741–747.
- [52] Nagata K, Satoh H, Iwahi T, Shimoyama T, Tamura T. Potent inhibitory action of the gastric proton pump inhibitor lansoprazole against urease activity of *Helicobacter pylori*: Unique action selective for *H. pylori* cells. *Antimicrob Agents Chemother* 1993;37:769–774.
- [53] Kuhler TC, Fryklund J, Bergman NA, Weilitz J, Lee A, Larsson H. Structure-activity relationship of omeprazole and analogues as *Helicobacter pylori* urease inhibitor. *J Med Chem* 1995;38:4906–4916.