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Synthesis, DFT Study, and Antitumor Activity of Some New Heterocyclic Compounds Incorporating Isoxazole Moiety

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Thiazolidin-4-one derivative **3** was synthesized by the transformation of chloroacetamide derivative **2** with NH_4SCN.The condensation of **3** with *p*-anisaldehyde afforded the corresponding arylidene derivative **4**. Also, the alkylation of chloroacetamide derivative **2** with different heterocyclic compounds was investigated. Annulation of 5-amino-3-methylisoxazole (**1**) with α-halocarbonyl compounds **12** and **14** furnished pyrrolo[3,2-d]isoxazole and isoxazolo[5,4-b]azepin-6-one derivatives **13** and **15**, respectively, while reaction of **1** with 1-chloro-4-(chloromethyl)benzene gave the monoalkylated product **17**. The newly synthesized compounds were screened for their antitumor activity, and the geometry optimizations are in a good agreement with the experimentally observed data.

Keywords: Isoxazole; Chloroacetamide; Thiazolidin-4-one; Alkylation; In vitro antitumor activity.

INTRODUCTION

There are numerous biologically active molecules with five membered rings containing two heteroatoms, among which is isoxazole. It is an important heterocyclic unit that has been widely used as a key building block for the synthesis of biologically active compounds. The isoxazole nucleus and its derivatives are attracting much attention in the fields of pharmacology and medicine[1,2] because of their various biological functions, including antitumor,[3] anti-HIV,[4] antifungal,[5] anticonvulsant,[6] analgesic,[7] herbicidal,[8] and cestoidal activities.[9] Isoxazole derivatives are also employed in the treatment of leprosy[10] and diabetes,[11–14] and have useful activities in conditions like schizophrenia, hypertension, and Alzheimer's disease, and in leflunomide (a disease-modifying antirheumatic drug, DMARD), valdecoxib (a COX-2 inhibitor), and zonisamide (an anticonvulsant). The active metabolite of leflunomide, A771726, is known to inhibit the tumor necrosis factor (TNF)-α and interleukin (IL)-1α from Kupffer cells in vitro.[15] In addition, leflunomide is used to inhibit the production of TNF-α, IL-1α, and IL-6 induced by bacterial lipopolysaccharides (LPSs) in peritoneal macrophages in rats with adjuvant arthritis.[16] Thiazolidin-4-one derivatives are important compounds because of their broad range of biological activities[17–23] such as antifungal, antitumor, antibacterial, antitubercular, anticonvulsant, antihistaminic, anti-inflammatory, antiviral, and cardiovascular.[24–27] Isoxazoles functionalized with an additional nitrogen-containing group have found many applications;[28a] thus it was considered of value to synthesize some new heterocyclic derivatives incorporating two active moieties in a single molecular framework to evaluate their biological activities. Recently, a number of different quantum mechanical theories have been advanced, for example, the density functional theory (DFT). It is a computational quantum mechanical modeling method used for studying organic compounds. The DFT method is used as a descriptor of chemical reactivity, orbitals, and the energy bandgap.[28b] By considering the above facts and their increasing importance in the pharmaceutical and biological fields, and in continuation of our program,[29–31] this work has been devoted to developing new classes of heterocyclic systems that incorporate the isoxazole nucleus to explore their potential antitumor activity.

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RESULTS AND DISCUSSION

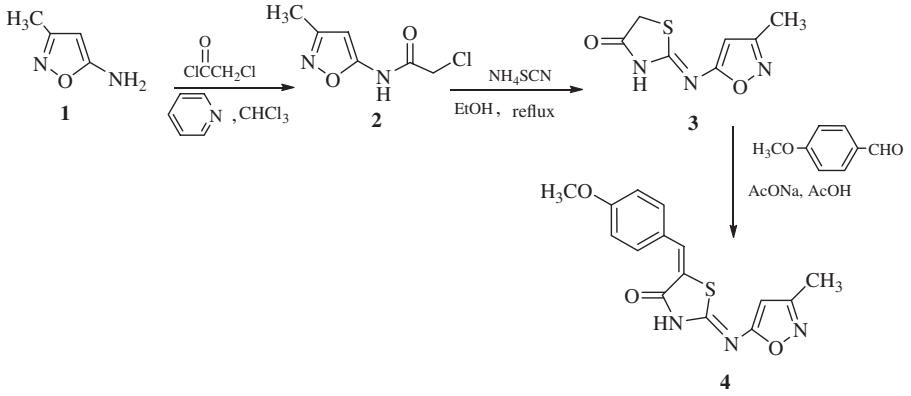
Chemistry

5-Amino-3-methylisoxazole (**1**) was reacted with chloroacetylchloride, furnishing the corresponding 2-chloro-*N*-(3-methylisoxazol-5-yl)acetamide (**2**) by a modified procedure of Uyeo Shojiro *et al.* (Scheme 1).[32] Compound **2** was treated with ammonium thiocyanate in refluxing ethanol, affording 2-(3-methylisoxazol-5-ylimino)thiazolidin-4-one (**3**). Compound **3** was synthesized through intramolecular cyclization and the Dimroth-like rearrangements of **3′′** under appropriate reaction conditions.[33,34] Its IR spectrum showed bands at 3126 and 1724 cm^{-1} due to (N–H) and (C=O amide), respectively. The lactam structure of **3** was confirmed on the basis of its ^{1}H NMR spectrum, which displayed a singlet signal at δ 4.00 ppm assignable to CH_2 protons and another singlet signal at δ 11.50 ppm of the NHCO proton. The mass spectrum of **3** showed a molecular ion peak at *m*/*z* 197 (M^+, 82.3%) and a base peak at *m*/*z* 82. It has been reported that the insertion of arylidene moieties at the thiazolidin-4-one ring enhanced its biological activity.[35–37] Prompted by these findings and as part of our efforts to discover potentially active new antitumor agents, we report here the condensation of **3** with *p*-anisaldehye in acetic acid and sodium acetate to give 5-(4-methoxybenzylidene)-2-(3-methylisoxazol-5-ylimino)thiazolidin-4-one (**4**). The ^{1}H NMR spectrum of **4** exhibits singlet signals for three protons at δ 3.80 corresponding to the (OCH_3) group, a doublet at 7.08 (J = 8.34 Hz) for two aromatic protons, a doublet at 7.85 for two aromatic protons (J = 8.33 Hz) and at 7.68 corresponding to ethylenic proton, and a singlet signal at δ 11.50 ppm for the NHCO proton. The MS spectrum showed a molecular ion peak at *m*/*z* 315 (M^+, 28.0%) and a base peak at 151. Furthermore, the signals that appeared in ^{13}C NMR of compound **4** are quite compatible with its structure. The postulated mechanism for the formation of compound **3** is shown in Scheme 2.

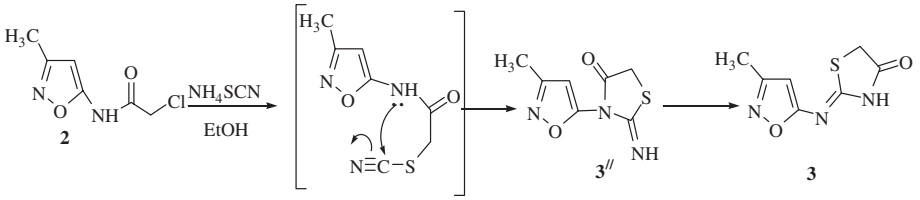
Furthermore, 2-cyano-*N*-(3-methyl-isoxazol-5-yl) acetamide (**5**) was synthesized by treating **2** with potassium cyanide in dimethylformamide (Scheme 3). Its IR spectrum exhibits bands at 3494 cm^{-1} (NH), at 2229 cm^{-1} for (CN), and at 1727 cm^{-1} for (C=O). Its ^{1}H NMR spectrum showed singlet signals at δ at 3.64 for (CH_2) and at 9.70 ppm for NH. The MS spectrum showed a molecular ion peak at *m*/*z* 165 (M^+, 22.4%) and a base peak at *m*/*z* 80.

In addition to increasing the potent activity of 2-chloro-*N*-(3-methylisoxazol-5-yl)acetamide (**2**) as an anticancer agent,[38,39] we built upon it other heterocylic moieties of potential activity via the transformation of **2** with different heterocyclic systems containing nucleophilic centers to afford binary heterocycles. Therefore, the alkylation of **2**with 5-amino-1,3,4-thiadiazole-2-thiol (**6**) in dry acetone gave α-(5-amino-1,3,4-thiadiazol-2-ylthio)-*N*-(3-methylisoxazol-5-yl)acetamide (**7**). Its IR spectrum showed bands at 3407, 3267, and 3164 $cm^{-1}$for (NH) and (NH_2) and at 1655 cm^{-1} for (C=O). Its ^{1}H NMR spectrum showed five singlet signals corresponding to CH_3, CH_2, isoxazole-CH, NH_2, and NH at δ 2.17, 4.02, 6.11, 7.40, and 11.81 ppm, respectively. Its MS indicated a molecular ion peak at *m*/*z* 271 (M^+, 0.9%) and a base peak at *m*/*z* 40.

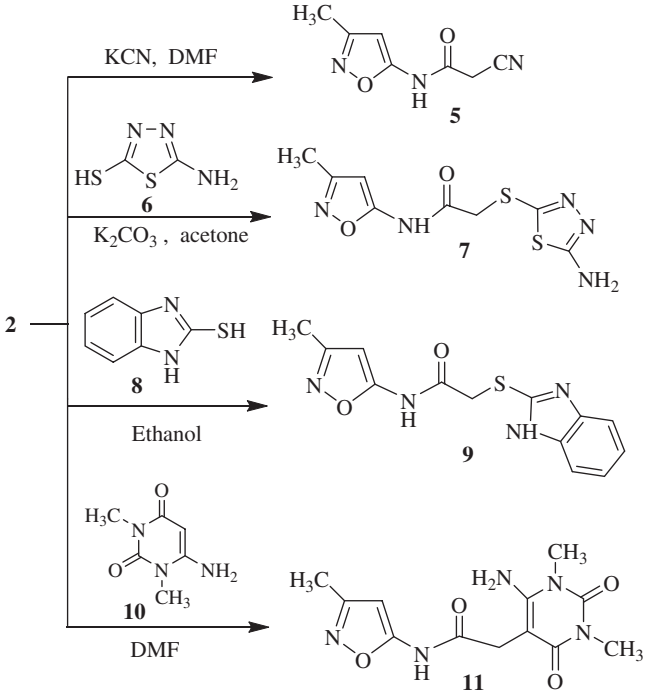
In a similar manner, when **2** was reacted with 1*H*–benzo[d]imidazole-2-thiol (**8**) in ethanol and triethylamine,



Scheme 1. Synthesis of thiazolidinone derivatves **3** and **4**.



Scheme 2. Plausible mechanism of the formation of compound **3**.



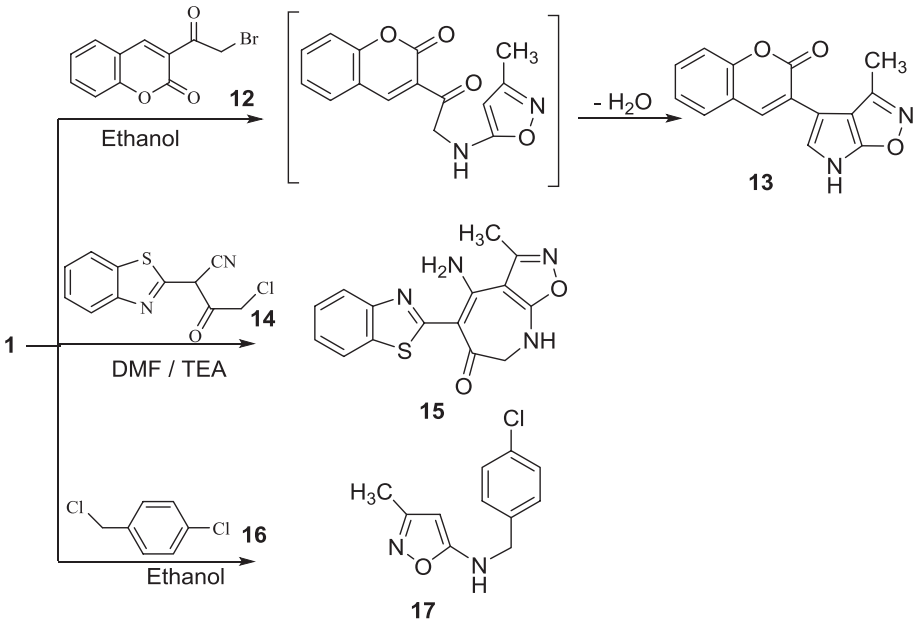
Scheme 3. Synthesis of bioactive isoxazoles **5**, **7**, **9**, and **11**.

it furnished 2-(1*H*–benzo[d]imidazol-2-ylthio)-*N*-(3-methylisoxazol-5-yl)acetamide (**9**) (Scheme 3). The IR spectrum exhibits bands at 3201 and 3149 cm^{-1} for two (NH) and at 1691 cm^{-1} for (C=O). Its ^1H NMR spectrum exhibits five singlet signals corresponding to CH_3, CH_2, isoxazole-CH, NH, and NH C=O at δ 2.27, 4.01, 6.21, 11.20, and 12.76, respectively, and shows muliplets for four aromatic protons at 6.93–7.83 ppm. Its MS shows a molecular ion peak at *m*/*z* 288 (M^+, 14%) and a base peak at *m*/*z* 200.

Also, 6-aminouracil has many medical and biological activities. In the light of these, our study was extended to the investigation of the reactivity of **2** with 6-aminouracil (**10**) in DMF, which gave **11**. The ^1H NMR spectrum of compound **11** revealed three singlet signals at 2.13 (CH_3 isoxazole) and 3.2 and 3.4 (for two CH_3 pyrimidines), and singlet signals at 2.81 and 11.05 ppm for CH_2 and NHCO protons, respectively.

Coumarins are important heterocyclic compounds with great applications in medicinal chemistry; this core structure can be found in compounds with diverse biological and medicinal applications.[40] In this context, we investigated the reaction of 5-amino-3-methylisoxazole (**1**) as a 1,3-binucleophile with electrophilic compounds such as 3-bromoacetylcoumarin **12** in ethanol under reflux, which furnished 3-(3-methyl-6*H*-pyrrolo[3,2-*d*] isoxazol-4-yl)-2*H*–chromen-2-one (**13**). Formation of compound **13** is assumed to proceed via nucleophilic displacement of the bromide to give an *N*-alkylated intermediate, which subsequently undergoes tautomerism with the elimination of water. IR spectrum of **13** showed bands at 3412 cm^{-1} for NH and at 1720 cm^{-1} for C=O. In the ^1H NMR spectrum of **13**, three singlet signals appeared at 5.52, 6.51, and 8.60 ppm for NH, CH-pyrole, and CH-coumarin, respectively. Its MS spectrum showed good agreement with its structure, with a molecular ion peak at *m*/*z* 267 (M^+ + H, 1.8%) and a base peak at *m*/*z* 78.

Furthermore, continuation of our work on the synthesis of biologically interesting heterocyclic compounds containing the fused azepine heterocyclic ring, synthesized by refluxing **1** with 2-(benzo[*d*]thiazol-2-yl)-4-chloro-3-oxobutyronitrile (**14**) in DMF and triethylamine (Scheme 4), led to the formation of 4-amino-5-(benzo[*d*] thiazol-2-yl)-3-methyl-7,8-dihydro-6*H*-isoxazolo[5,4-*b*]azepin-6-one (**15**). Formation of compound **15** is assumed to proceed via the displacement of the chlorine of **14** with the amino group of isoxazole followed by the attack of C-4 of isoxazole, which is the more nucleophilic carbon to the cyano group, affording **15**. Its structure was elucidated by IR and ^1H NMR (see the Experimental section).The carbon skeleton of compound **15** was assigned using the ^{13}C NMR spectrum. Finally, the reaction of **1** with 1-chloro-4-(chloromethyl)benzene (**16**) in methanol led to the formation of 5-*N*-(4-chlorobenzyl)amino-3-methylisoxazole (**17**). The ^1H NMR spectrum of compound **17** displayed two singlet signals at 4.60 and 4.80 for CH_2 and NH ppm,



Scheme 4. Alkylation of 5-amino-3-methylisoxazole (**1**).

respectively, and also two doublets at 7.34 (2H, d) and 7.81 (2H, d), which were readily assigned to the aromatic protons. Its mass spectrum showed a molecular ion peak at *m/z* 222 (M^+, 14.8%) and a base peak at *m/z* 149.

The structures of all the newly synthesized compounds were established on the basis of their microanalytical data as shown in the experimental section.

BIOLOGICAL ACTIVIY

Antitumor testing

Eight of the new synthesized isoxazole derivatives were tested for cytotoxicity against well-known established tumor cell model Ehrlich ascite cells (EACs) in vitro.[41–43] The ED_{100}, ED_{50}, ED_{25}, and IC50 values of the active compounds are summarized in Table 1. The data show clearly that compounds **7**, **9**, and **11** have the highest cytotoxic activity in comparison to the well-known cytotoxic antitumor agents such as 5-fluorouracil (5-FU). However, compounds **13** and **17** were only moderately potent **c**ompared to the other compounds. But compounds **3**, **4,** and **15** showed low activities.

Structure–activity relationship of antitumor activities

By comparing the results obtained for the investigated compounds with their structures, the following

Table 1. In vitro cytotoxicity of isoxazole derivatives (Ehrlich ascites cells dead %)

		% Death		
Compound no.	IC_{50} μg	ED_{100} μg/mL	ED_{50} μg/mL	ED_{25} μg/mL
5-FU	32.21	96.1	67.0	42.9
3	74.21	22.0	13.3	6.5
4	80.43	17.0	11.4	6.1
7	**11.52**	96.3	65.7	41.5
9	30.53	96.4	68.4	44.8
11	**8.54**	97.6	68.3	44.2
13	52.65	70.2	39.0	28.8
15	98.43	38.0	17.0	10.0
17	**48.32**	80.5	41.4	21.5

IC_{50} is the inhibitive concentration. ED_{100}, ED_{50}, and ED_{25} are the effective doses at 25, 50, and 100 μL, respectively, of the compounds used. Dead % refers to the percentage of dead tumor cells. 5-Fu is 5-fluorouracil, a well-known cytotoxic agent.

Table 2. Binding energies and frontier orbital energies obtained from DFT

Compound	Binding energy (eV)	Total energy (eV)	HOMO (eV)	LUMO (eV)	$E_{(HOMO-LUMO)}$
7 anti	−117.47872	−41 662.46368	−5.489	−1.854	−3.635
9 anti	−156.25778	−34 574.68075	−5.582	−2.016	−3.566
11 anti	−169.96766	−28 338.16501	−5.224	−1.558	−3.666

structure–activity relationships (SARs) were postulated: (1) isoxazole derivatives **7**, **9**, and **11** were more potent than 5-Fu, which may be attributed to the presence of the 1,3,4-thiadiazole, benzo[d]imidazole, and pyrimidine-2,4(1*H*,3*H*)-dione moieties, respectively; (2) compounds **13**, **17**, and **15** showed moderate activity, which may be due to the presence of coumarin, *p*-chlorophenyl and azepine moieties, respectively; and (3) finally, isoxazole derivatives **3** and **4** had low activity compared to that of the other compounds.

MOLECULAR MODELING

Geometry optimization

Geometry optimization of the molecular structures of the different compounds was carried out using DFT via cluster calculations using the DMOL3 program[44] in the Materials Studio package[45] in order to investigate their stability relative to each other. The values obtained for the main quantum parameters are summarized in Table 2. According to the frontier molecular orbital theory, chemical reactivity is a function of the interaction between the HOMO and LUMO levels of the reacting species.[24b] HOMO represents the ability to donate an electron, whereas LUMO as an electron acceptor represents the ability to obtain an electron. The energy gap between E_{HOMO} and E_{LUMO} of the molecules is an important parameter as a function of the reactivity of molecules. Moreover, the experimental results for the newly synthesized compounds were in agreement with the theoretical data. By determining the energy gap ($E_{HOMO} - E_{LUMO}$), one gets an indication of the biological activity. Molecules with a small energy gap are more polarized and reactive than hard ones because they easily offer electrons to an acceptor. Also, the low values of the energy gap may be due to the groups or moieties entering those molecules. Therefore, the energy gaps for the three compounds **7**, **9**, and **11** (Figures 1–3) with the highest antitumor activity were determined. The results, indicated in Table 2 show that compound **11** has the lowest energy gap, which means the ease of charge transfer in **11**, which enhanced its biological activity compared to compounds **7** and **9.** Also, the isolated compound **11** showed large values of binding energy than compounds **7** and **9**, which improved the stability of **11**.[46] Moreover, the energy difference between the frontier orbitals ($E_{HOMO} - E_{LUMO}$) was found to be higher in the negative value in the case of compound **7** than in compound **9**, so the former's cytotoxic activity as antitumor agent is higher than that of compound **9.**

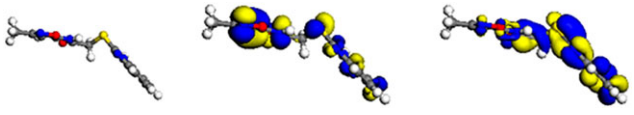
CONCLUSIONS

The objective of the present study was to synthesize and investigate the antitumor activity of some novel isoxazole skeletons bearing other heterocyclic moieties, starting from 5-amino-3-methylisoxazole. The data showed that compounds **7**, **9**, and **11** are the most potent compounds with higher cytotoxic activity than even 5-FU. The data reported here may be a helpful guide for medicinal chemists who are working in this area.

EXPERIMENTAL

Materials and methods

All melting points are in Celcius (uncorrected) and were determined by a Gallenkamp electric melting point apparatus. Elemental analyses were carried out



Compound 9 | 9 HOMO | 9 LUMO

Fig. 1. Molecular geometry and calculated HOMO and LUMO of compound **9** anti.

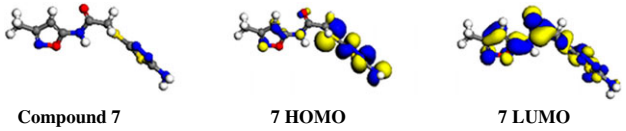


Fig. 2. Molecular geometry and calculated HOMO and LUMO of compound **7** anti.

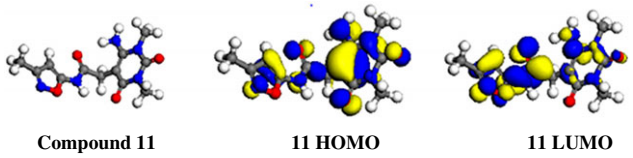


Fig. 3. Molecular geometry and calculated HOMO and LUMO of compound **11** anti.

at the Micro analytical Center, Faculty of Science, Cairo University. IR spectra were recorded (in KB, cm^{-1}) on a Mattson 5000 FTIR spectrophotometer at the Micro analytical Center, Faculty of Science, Mansoura University. ^1H NMR spectra were measured on a Varian spectrometer at 400 MHz, using TMS as internal reference and DMSO-d_6 or $CDCl_3$ as solvent at the National Research Center, Cairo. ^{13}C NMR Spectra were acquired on a JEOL ECX-400 spectrometer operating at 100 MHz at room temperature in $CDCl_3$ and DMSO-d_6 using a 5 mm probe at the Chemistry department, School of Engineering and Science, University of Jacobs, Bremen, Germany. The chemical shifts (δ) are reported in parts per million and referenced to the residual solvent peak. Mass spectra were recorded on a Kratos (70 eV) MS instrument and/or a Varian MAT 311 mass spectrometer at Micro analytical Center, Cairo University. Reaction mixtures were monitored by thin-layer chromatography (TLC) using EM science silica gel-coated plates with visualization by irradiation with an ultraviolet lamp. Fetal bovine serum was from GIBCO, UK, and the RPMI-1640 medium from Sigma chemical Co. (St. Louis, MO, USA). Biological testing was carried out at Drug Department, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt. Ehrlich cells (Ehrlich ascites carcinoma, EAC) were derived from the ascetic fluid of diseased mice (the cells were purchased from the National Cancer institute, Cairo, Egypt).

***2*-Chloro-*N*-(3-methylisoxazol-5-yl)acetamide (2).** To a solution of **1** (0.49 g, 5 mmol) in chloroform (10 mL) containing pyridine (0.49 g, 5 mmol) was added choloracetyl chloride (0.56 g, 5 mmol), with the temperature maintained at 0–10°C during addition. The reaction mixture was stirred for 1 h and then poured into cold water. The formed precipitate was filtered off, washed with water, and recrystallized from ethanol to give **2**. White crystals; 99% yield; mp 131–132°C (ethanol) (Lit. mp [32] =130–132°C); R_f = 0.5 [pet. Ether (40–60)/ethyl acetate (1:3)].

(*Z*)-2-(3-Methylisoxazol-5-ylimino)thiazolidin-4-one (3). A mixture of ***2*-**chloro-*N*-(3-methylisoxazol-5-yl)acetamide (**2**) (0.87 g, 5 mmol) and ammonium thiocyanate (0.76 g, 0.1 mol) in absolute ethanol (50 mL) was refluxed for 5 h, and then the reaction mixture was allowed to stand overnight at room temperature. The precipitate formed was filtered off, washed with water, and recrystallized from ethanol to give **3**. White crystals; 80% yield; mp 255–257°C; R_f = 0.3 [pet. Ether (40–60)/ethyl acetate (1:3)]; IR (cm^{-1}): 3126 (NH), 1724 (CO), 1624 (C=N); ^1H NMR (DMSO-d_6) δ: 2.31 (s, 3H, CH_3), 4.00 (s, 2H, CH_2), 6.11 (s, 1H, isoxazole-CH), 11.50 (s, 1H, NHCO);MS: (EI, 70 eV) *m/z* (%) = 199 (M^+ + 2, 0.5), 198 (M^+ + H, 4.7), 197 (M^+, 82.3), 143 (9.2), 142 (2.7), 124 (36.9), 115 (8.5), 98 (4.4), 97 (2.3), 82 (100.0, base peak), 69 (29.6), 68 (10.8); Anal. Calcd. for $C_7H_7N_3O_2S$ (197.21): C, 42.63; H, 3.58; N, 21.31; Found: C, 42.65; H, 3.65; N, 21.29.

(2*Z*,5*E*)-5-(4-Methoxybenzylidene)-2-(3-methylisoxazol-5-ylimino)thiazolidin-4-one (4). A mixture of **3** (0.79 g, 4 mmol) in acetic acid (35 mL) was buffered

with sodium acetate (8 mmol) followed by the addition of anisaldehyde (0.73 mL, 6 mmol). The solution was refluxed for 2 h, then allowed to stand overnight, and the precipitate formed was filtered off and washed with ethanol. The resulting crude product was purified by recrystallization from ethanol tofurnish **4**: Yellow crystals; 85% yield; mp 225–227°C; R_f = 0.3 [pet. Ether (40–60)/ethyl acetate (1:3)]; IR (cm^{-1}): 3120 (NH), 3056, 2966 (CH, str.), 1724 (C=O), 1644 (C=N), 1616 (C=C); ^{1}H NMR (DMSO-d_6) δ: 2.19 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 6.03 (s, 1H, isoxazole-CH), 7.08 (d, J = 8.34 Hz, 2H, aromatic-CH), 7.85 (d, J = 8.33 Hz, 2H, aromatic-CH), 7.68 (s, 1H, CH = C), 11.50 (s, 1H, NHCO); ^{13}C NMR (DMSO-d_6), δ: 12.22 (CH_3), 56.03 (CH_3-O), 93.54 (CH-isoxazole), 115.52, 115.90, 119.69, 121.98, 125.98, 132.27 (C=C), 132.77 (CH = C), 156.95 (C-N), 161.75 (C-O), 161.73 (C=N), 167.41 (N = C-CH_3), 168.16 (C=O); MS: (EI, 70 eV) m/z (%) = 315 (M^+, 28.0), 314 (M^+– 1, 14.4), 257 (26.2), 241 (17.6), 211 (37.1), 197 (16.7), 174 (19.9), 151 (100.0, base peak), 137 (16.4), 105 (24.4), 78 (24.6); Anal. Calcd. for $C_{15}H_{13}N_3O_3S$ (315.35): C, 57.13; H, 4.16;N, 13.33; Found: 57.15; H, 4.17; N, 13.34.

***2*-Cyano-*N*-(3-methylisoxazol-5-yl)acetamide (5)**. Compound **2** (0.56 g, 5 mmol) was dissolved in dimethylformamide (5 mL), and to the resulting clear solution was added potassium cyanide (0.36 g, 5.5 mmol) in water (1 mL) while stirring. Addition was controlled so that the temperature of the reaction mixture was maintained at 70°C. After complete addition of the cyanide solution, stirring was continued for a further 15 min. Then the reaction mixture was cooled and added to an ice-cold solution of conc. Hydrochloric acid (0.5 mL) in water (100 mL). The precipitate formed was filtered off, washed with water, dried, and recrystallized from ethanol to give **5**. Yellow crystals; 80% yield; mp 120–121°C; R_f = 0.3 [pet. Ether (40–60)/ethyl acetate (1:3)]; IR: (cm^{-1}) 3494 (NH), 2954, 2929 (CH, str.), 2229 (CN), 1727 (C=O), 1621 (C=N); ^{1}H NMR ($CDCl_3$) δ: 2.27 (s, 3H, CH_3), 3.64 (s, 2H, CH_2), 6.24 (s, 1H, isoxazole-CH), 9.70 (s, 1H, NH); MS: (EI, 70 eV) m/z (%) = 167 (M^++ 2, 1.6), 165 (M^+, 22.4), 149 (2.5), 129 (2.9), 123 (2.5), 115 (2.0), 91 (8.9), 80 (100.0, base peak); Anal. Calcd. for $C_7H_7N_3O_2$ (165.15): C, 50.91; H, 4.27;N, 25.44; Found: C, 50.87; H, 4.25; N, 25.42.

2-(5-Amino-1,3,4-thiadiazol-2-ylthio)-*N*-(3-methylisoxazol-5-yl) acetamide (7). A mixture of **2** (0.56 g, 4 mmol), anhydrous potassium carbonate (0.55 g, 4 mmol), and 5-amino-1,3,4-thiadiazole-2-thiol (**6**) (0.532 g, 4 mmol) in dry acetone (20 mL) was heated under reflux for 6 h, and then the reaction mixture was allowed to stand at room temperature overnight. The formed precipitate was collected by filtration, washed with ethanol, and recrystallized from benzene to furnished **7**: White crystals; 60% yield; mp 215–217°C; R_f = 0.3 [pet. Ether (40–60)/ethyl acetate (1:3)]; IR (cm^{-1}): (3407, 3267 (NH_2), 3164 (NH), 1655 (C=O), 1645, 1608 (C=N), 1217, 1062 (C–S–C); ^{1}H NMR (DMSO-d_6) δ: 2.17 (s, 3H, CH_3), 4.02 (s, 2H, CH_2), 6.11 (s, 1H, isoxazole-CH), 7.90 (s, 2H, NH_2), 11.81 (s, 1H, NH); MS: (EI, 70 eV) m/z (%) = 272 (M^++ H, 0.53), 271 (M^+, 0.9), 270 (M^+– 1, 4.1), 174 (85.67), 100 (12.3), 98 (55.0), 97 (14.8), 82 (57.2), 40 (100.0, base peak); Anal. Calcd. for $C_8H_9N_5O_2S_2$ (271.31): C, 35.41; H, 3.34;N, 25.81; Found: C, 35.39; H, 3.33; N, 25.78.

2-(1*H*–Benzo[d]imidazol-2-ylthio)-N-(3-methylisoxazol-5-yl)acetamide (9). A mixture of **2** (0.56 g, 4 mmol) and 1*H*–benzo[d]imidazole-2-thiol (**8**) (0.4 g, 4 mmol) in dioxane (20 mL) was heated under reflux for 3 h, and then the reaction mixture was allowed to stand at room temperature overnight. The formed precipitate was collected by filtration, washed with ethanol, and recrystallized from ethanol to give **9**: Black crystals; 90% yield; mp 180–181°C; R_f = 0.5 [pet. Ether (40–60)/ethyl acetate (3:4)]; IR: (cm^{-1}) 3201, 3149 (2NH), 2908, 2815 (CH, str.), 1691 (C=O), 1616 (C=N); ^{1}H NMR (DMSO-d_6) δ: 2.27 (s, 3H, CH_3), 4.01 (s, 2H, CH_2), 6.21 (s, 1H, isoxazole-CH), 6.93–7.83 (m, 4H, Aromatic-CH), 11.20 (s, 1H, NH), 12.76 (s, 1H, NH C=O); MS: (EI, 70 eV) m/z (%) = 288 (M^+, 14), 216 (25.2), 200 (100.0, base peak), 199 (20.5), 102 (9.8), 90 (9.0);Anal. Calcd. for $C_{13}H_{12}N_4O_2S$ (288.32): C, 54.16; H, 4.20; N, 19.43; Found: C, 54.15; H, 4.22; N, 19.40.

2-(6-Amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-(3-methylisoxazol-5-yl)acetamide (11). A mixture of **2** (0.56 g, 4 mmol) and 6-amino-1,3-dimethyl-pyrimidine-2,4-(1*H*,3*H*)-dione (**10**) (0.62 g, 4 mmol) in DMF (50 mL) was heated under reflux for 3 h, and then the reaction mixture was allowed to stand overnight. The precipitate formed

was collected by filtration, washed with ethanol, and then recrystallized from ethanol to afford **11.** Yellow crystals; 70% yield; mp 220–222°C; R_f = 0.2 [pet. Ether (40–60): ethyl acetate (1:4)]; IR (cm^{-1}): 3397, 3351 (NH_2), 2996, 2950 (CH, str.), 1660 (C=O), 1610 (C=C); ^{1}H NMR (DMSO-d_6) δ: 2.13 (s, 3H, CH_3-isoxazole), 2.81(s, 2H, CH_2), 3.2 (s, 3H, CH_3 pyrimidine), 3.40 (s, 3H, CH_3-pyrimidine), 6.12 (s, 1H, isoxazole-CH), 6.90 (s, 2H, NH_2), 11.05 (s, 1H, NHCO); MS: (EI, 70 eV) *m/z* (%) = 295 (M^++2, 1.1), 292 (M^+ − H, 1.8), 211 (2.2), 155 (16.1), 82 (20.5), 44 (100.0, base peak); Anal. Calcd. for $C_{12}H_{15}N_5O_4$ (293.28): C, 49.14; H, 5.16; N, 23.88; Found: C, 49.13; H, 5.15; N, 23.86.

3-(3-Methyl-6*H*-pyrrolo[3,2-*d*]isoxazol-4-yl)-2*H*–chromen-2-one (13). A mixture of **1** (0.49 g, 5 mmol) and 3-bromoacetyl coumarin (**12**) (1.33 g, 5 mmol) was refluxed in ethanol (50 mL) for 5 h. The reaction mixture was allowed to stand overnight at room temperature. The resultant solid (hydrobromide) was separated by filtration. The free base was obtained by neutralization with sodium bicarbonate solution to give the solid product. It was washed with water and then recrystallized from ethanol to give **13**: Red crystals; 66% yield; mp 161–162°C (ethanol); R_f = 0.6 [pet. Ether (40–60): ethyl acetate (1:3)]; IR (cm^{-1}): 3412 (NH), 2970, 2925 (CH, str.), 1720 (CO), 1607 (C=C); ^{1}H NMR ($CDCl_3$) δ: 2.11 (s, 2H, CH_3), 5.52 (s, 1H, NH),6.51 (s, 1H, CH-pyrole), 7.41–7.91 (m, 4H, aromatic-CH), 8.60 (s, 1H, CH-coumarin); MS (EI, 70 eV) *m/z* (%) = 268 (M^+ + 2, 3.9), 267 (M^+ + 1, 1.8), 205 (2.6), 173 (15.3), 168 (1.3), 145 (3.9), 121 (80.0), 80 (16.6), 79 (38.4), 78 (100.0, base peak); Anal. Calcd. for $C_{15}H_{10}N_2O_3$ (266.25): C, 67.67; H, 3.79; N, 10.52; Found: C, 67.64; H, 3.78; 10.54.

4-Amino-5-(benzo[*d*]thiazol-2-yl)-3-methyl-7,8-dihydro-6*H*-isoxazolo [5,4-*b*]azepin-6-one (15). A mixture of **1** (0.49 g, 5 mmol) and 2-(benzo[*d*]thiazol-2-yl)-4-chloro-3-oxobutyronitrile (**14**) (1.25 g, 5 mmol) in DMF (10 mL) and triethylamine (three drops) was refluxed for 3 h. The reaction mixture was allowed to stand overnight at room temperature to give brown crystals, which was collected by filtration and recrystallization from DMF to afford 1**5**: Brown crystals; 60% yield; mp 290–292°C; R_f = 0.6 [pet. Ether (40–60)/ethyl acetate (1:3)]; IR (cm^{-1}): 3409, 3400 (NH_2), 1695 (C=O), 1614 (C=N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 2.30 (s, 3H, CH_3), 4.54 (s, 2H, CH_2), 6.43 (s, 2H, NH_2), 7.13–7.99 (m, 4H, aromatic-CH), 8.30 (s, 1H, NH); ^{13}CNMR (DMSO-d_6) δ: 22.24 (CH_3), 55.58, 113.14, 113.14, 114.79 (C=N), 121.39, 121.42, 124.55, 124.82, 127.08 (C–S), 128.78, 145.54 (C–N), 148.96, 167.56, and 191.20 (C=O); Anal. Calcd. for $C_{15}H_{12}N_4O_2S$ (312.35): C, 57.68; H, 3.87;N, 17.94; Found: C, 57.70; H, 3.88; N, 17.95.

***N*-(4-Chlorobenzyl)-3-methylisoxazol-5-amine (17).** A mixture of **1** (0.49 g, 5 mmol) and 1-chloro-4-(chloromethyl) benzene (**16**) (0.80 g, 5 mmol) in absolute ethanol (50 mL) was heated under reflux for 3 h, and then the reaction mixture was allowed to stand overnight. The precipitate formed was collected by filtration, washed with ethanol, and then recrystallized from ethanol to afford **17**: Yellow crystals; 57% yield; mp > 300°C; R_f = 0.5 [pet. Ether (40–60)/ethyl acetate (2:5)]; IR (cm^{-1}): 3139 (NH), 2823 (CH, str.), 1533 (C–N) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 2.10 (s, 3H, CH_3), 4.60 (s, 2H, CH_2N), 4.80 (s, 1H, NH), 6.31 (s, 1H, isoxazole-CH), 7.34 (d, *J* = 9.01 Hz, 2H, aromatic-CH), 7.81 (d, *J* = 8.97 Hz, 2H, aromatic-CH); MS: (EI, 70 eV) *m/z* (%) = 223 (M^+ + 1, 6.6), 222 (M^+, 14.8), 207 (8.6), 112 (56.6), 111 (9.07), 126 (20.5), 141 (18.2), 149 (100.0, base peak), 123 (44.5), 121 (31.7), 97 (12.9), 96 (24.0), 73 (21.1), 67 (65.8); Anal. Calcd. for $C_{11}H_{11}ClN_2O$ (222.67): C, 59.33; H, 4.98;N, 12.58; Found: C, 59.29; H, 4.97; N, 12.56.

Antitumor activity using Ehrlich ascites in vitro assay

Different concentrations of the tested compounds were prepared (100, 50, and 25 μg/mL, DMSO). Ascites fluid from the peritoneal cavity of the donor animal (containing Ehrlich cells) was aseptically aspirated. The cells were grown partly floating and partly attached in a suspension culture in RPMI 1640 medium, supplemented with 10% fetal bovine serum. They were maintained at 37°C in a humidified atmosphere with 5% CO_2 for 2 h. The viability of the cells was determined by the microscopic examination using a hemocytometer and using trypan blue stain (stains only the dead cells).[47,48]

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