Full Paper

Synthesis and Antioxidant Activity of a New Class of Bis and Tris Heterocycles

Venkatapuram Padmavathi, Bhumireddy Chinnachennaiahgari Venkatesh, Akkarapalli Muralikrishna, and Adivireddy Padmaja

Department of Chemistry, Sri Venkateswara University, Tirupati, Andhra Pradesh, India

A series of novel heterocycles, bisbenzoxazolyl/benzothiazolyl/benzimidazolyl pyrazoles/isoxazoles/pyrimidines were synthesized and evaluated for their antioxidant activity. The bisbenzoxazolyl-isoxazole **10** exhibited good antioxidant activity when compared with the standard ascorbic acid.

Keywords: Antioxidant activity / Benzimidazolyl pyrazoles / Benzothiazolyl / Bisbenzoxazolyl / Cyclocondensation / Isoxazoles / Ketenedithiolates / Pyrimidines

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Introduction

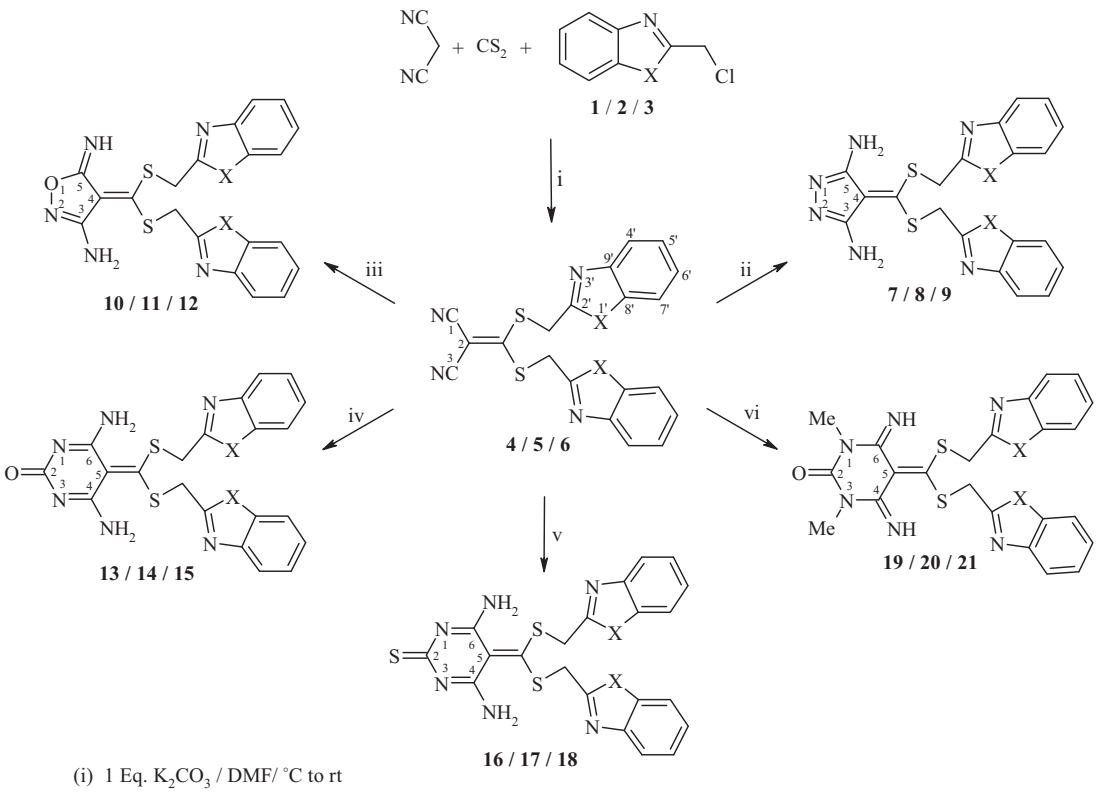
Heterocycles are well known for their wide range of biological properties. Pyrimidine derivatives gained importance in medicinal chemistry for their therapeutic applications [1, 2]. In fact pyrimidine forms an integral part of a large number of pharmocologically important compounds like thymine, riboflavin, purine bases and sulfadiazine [3–7], etc. Pyrazole and isoxazole derivatives show a wide range of activities including antiinflammatory [8], anticancer [9], antibacterial [10], antiviral [11], antidiabetic [12], and antimicrobial [13]. Besides, isoxazole analogs of curcuminoids exhibit antioxidant activity [14]. They are therefore attractive building blocks for pharmaceutical research (e.g., Celebrex, Viagra, Acomplia, Valdecoxib, Celecoxib). The benzoxazole ring is one of the most common heterocycles in medicinal chemistry and plays an important role in designing new drugs. Substituted benzoxazoles possess diverse chemotherapeutic activities viz., antimicrobial [15–19], antiviral [20], and antitumor [21, 22]. Benzothiazole derivatives have industrial applications as antioxidants [23], vulcanization accelerators, etc. [24]. The benzimidazole moiety exists in many biologically active natural products and synthetic compounds [25, 26]. Some of them exhibit clinical value toward breast cancer [27–30], leukemia [31, 32], tumor cells [33, 34], etc. Thus the literature reflects that there is a constant strive to develop a variety of heterocycles of pharmacological potency. In this context, we prepared a new class of tris heterocycles viz., bisbenzoxazolyl/benzothiazolyl/benzimidazolyl pyrazoles/isoxazoles/pyrimidines exploiting ketene dithiolates as synthetic intermediates and studied their antioxidant properties.

Correspondence: Venkatapuram Padmavathi, Department of Chemistry, Sri Venkateswara University, Tirupati 517 502, Andhra Pradesh, India.
E-mail: vkpuram2001@yahoo.com
Fax: +91-877-2249532

Results and discussion

Chemistry

The general synthetic pathway for the synthesis of bis and tris heterocycles is depicted in Scheme 1. The *gem* dicyano compounds 2-(bis((benzo[*d*]oxazol-2′-yl)methylthio)methylene)-malononitrile (**4**), 2-(bis((benzo[*d*]thiazol-2′-yl)methylthio)-methylene)malononitrile (**5**), and 2-(bis((1′*H*-benzo[*d*]imidazol-2′-yl)methylthio)methylene)malononitrile (**6**) were prepared by a one pot reaction of malononitrile, carbon disulfide, and 2-(chloromethyl)benzooxazole (**1**)/2-(chloromethyl)benzothiazole (**2**)/2-(chloromethyl)-1*H*-benzimidazole (**3**) in the presence of K_2CO_3 in DMF (Scheme 1). The compounds **1** and **2** were obtained by the irradiation of 2-aminophenol/2-aminothiophenol and chloroacetyl chloride for 10 min at a power of 500 W [35, 36]. However, **3** was prepared by treating *o*-phenylenediamine with chloroacetic acid in the presence of 5 N HCl [37]. The ^{1}H NMR spectra of **4**, **5**, and **6** displayed a singlet at δ 4.41, 4.37, and 4.35 ppm due to methylene protons. The ^{13}C NMR spectra of **4** exhibited signals at δ 29.4, 83.5, 111.4, 182.8; **5** at 29.0, 83.8, 112.7, 183.5; and **6** at 28.9, 82.9, 111.8, 183.0 ppm due to CH_2, ($(NC)_2C$=C), CN, and (C=*CS*(S)), respectively.



Scheme 1. Synthesis of bis and tris heterocycles.

The reaction of **4**/**5**/**6** with hydrazine hydrate in the presence of piperidine and ethanol resulted in 4-(bis((benzo[*d*]oxazol-2′-yl)methylthio)methylene)-4*H*-pyrazole-3,5-diamine (**7**), 4-(bis((benzo[*d*]thiazol-2′-yl)methylthio)methylene)-4*H*-pyrazole-3,5-diamine (**8**), and 4-(bis((1′*H*-benzo[*d*]imidazol-2′-yl)methylthio)methylene)-4*H*-pyrazole-3,5-diamine (**9**). The ^{1}H NMR spectra of **7**, **8**, and **9** displayed a singlet at δ 4.37, 4.42, and 4.39 due to methylene protons and another broad singlet at 5.74, 5.76, and 5.81 ppm due to NH_2. In addition, **9** exhibited a broad singlet at 11.87 ppm due to NH of benzimidazole. The signals due to NH_2 and NH disappeared when D_2O was added. Similarly, 4-(bis((benzo[*d*]oxazol-2′-yl)methylthio)methylene)-4,5-dihydro-5-iminoisoxazol-3-amine (**10**), 4-(bis((benzo[*d*]thiazol-2′-yl)methylthio)methylene)-4,5-dihydro-5-iminoisoxazol-3-amine (**11**), and 4-(bis((1′*H*-benzo[*d*]imidazol-2′-yl)methylthio)methylene)-4,5-dihydro-5-iminoisoxazol-3-amine (**12**) were obtained by treating **4**/**5**/**6** with hydroxylamine hydrochloride. The ^{1}H NMR spectra of **10**, **11**, and **12** displayed a singlet at δ 4.40, 4.36, and 4.43 due to methylene protons. Two broad singlets were observed at 5.77, 9.86 in **10**, at 5.65, 9.72 in **11**, and at 5.80, 9.98 ppm in **12**, which were accounted for NH_2 and =NH and disappeared when D_2O was added. Likewise, the reaction of **4**/**5**/**6** with urea produced 4,6-diamino-5-(bis((benzo[*d*]oxazol-2′-yl)methylthio)methylene)pyrimidin-2(5*H*)-one (**13**), 4,6-diamino-5-(bis((benzo[*d*]thiazol-2′-yl)methylthio)methylene)pyrimidin-2(5*H*)-one (**14**), and 4,6-diamino-5-(bis((1′*H*-benzo[*d*]imidazol-2′-yl)methylthio)methylene)pyrimidin-2(5*H*)-one (**15**). Adopting similar methodology, 4,6-diamino-5-(bis((benzo[*d*]oxazol-2′-yl)methylthio)methylene)pyrimidine-2(5*H*)-thione (**16**), 4,6-diamino-5-(bis((benzo[*d*]thiazol-2′-yl)methylthio)methylene) pyrimidine-2(5*H*)-thione (**17**), and 4,6-diamino-5-(bis((1′*H*-benzo[*d*]imidazol-2′-yl)methylthio)methylene)pyrimidine-2(5*H*)-thione (**18**) were prepared by the reaction of **4**/**5**/**6** with thiourea. The ^{1}H NMR spectra of **13**–**18** showed a singlet ~4.35 due to methylene protons and a broad singlet ~5.80 ppm due to NH_2. The compounds **15** and **18** presented another broad singlet ~12.90 due to NH of benzimidazole.

Table 1. The *in vitro* antioxidant activity of **4–21** in DPPH method

Compound	Concentration			
	50 μg/mL	75 μg/mL	100 μg/mL	IC_{50} μmol/mL
4	52.43 ± 1.42	54.31 ± 1.21	58.71 ± 1.38	0.117 ± 1.29
5	–	–	–	–
6	–	–	–	–
7	64.10 ± 0.94	66.12 ± 0.86	67.42 ± 1.01	0.089 ± 0.46
8	49.32 ± 1.75	50.92 ± 1.42	51.23 ± 1.84	0.108 ± 1.80
9	–	–	–	–
10	75.40 ± 0.37	76.31 ± 0.65	79.12 ± 0.71	0.075 ± 0.16
11	53.45 ± 1.31	56.32 ± 1.87	58.81 ± 1.55	0.097 ± 1.13
12	–	–	–	–
13	57.31 ± 1.03	59.14 ± 1.41	61.24 ± 1.32	0.094 ± 0.78
14	–	–	–	–
15	–	–	–	–
16	56.71 ± 0.89	58.30 ± 1.26	61.10 ± 1.06	0.092 ± 0.69
17	–	–	–	–
18	–	–	–	–
19	68.23 ± 0.37	69.18 ± 0.16	71.31 ± 0.25	0.074 ± 0.20
20	–	–	–	–
21	–	–	–	–
Ascorbic acid	77.15 ± 0.15	80.98 ± 0.38	83.82 ± 0.54	0.183 ± 0.06
Blank	–	–	–	–

(–) Showed no scavenging activity. Values were the means of three replicates ± SD.

The signals of NH_2 and NH disappeared when D_2O was added. In the same manner, 5-(bis((benzo[*d*]oxazol-2′-yl)methylthio)-methylene)-tetrahydro-4,6-diimino-1,3-dimethylpyrimidin-2(1*H*)-one (**19**), 5-(bis((benzo[*d*]thiazol-2′-yl)methylthio)methylene)-tetrahydro-4,6-diimino-1,3-dimethylpyrimidin-2(1*H*)-one (**20**), and 5-(bis((1′*H*-benzo[*d*]-imidazol-2′-yl)methylthio)methylene)-tetrahydro-4,6-diimino-1,3-dimethylpyrimidin-2(1*H*)-one (**21**) were obtained by treating **4**/**5**/**6** with *N*,*N*′-dimethylurea (Scheme 1). The ^{1}H NMR spectra of **19**, **20**, and **21** displayed two singlets at δ 4.37, 4.32, 4.45 and 2.74, 2.68, 2.70 due to methylene and *N*-Me protons. A broad singlet was observed at 9.78, 9.81, and 9.89 ppm due to =NH which disappeared when D_2O was added.

Biological results

Antioxidant activity

The compounds **4**–**21** were tested for antioxidant property by 2,2,-diphenyl-1-picrylhydrazyl (DPPH) [38, 39], nitric oxide (NO) [40, 41], and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) [42] methods.

The observed data on the antioxidant activity of the compounds and control drug are shown in Tables 1–3. In this study our goal is to identify the efficient heterocyclic moiety for antioxidant activity. Amongst the *gem* dicyano compounds, bisbenzoxazolyl derivative **4** was active while bisbenzothiazolyl (**5**) and bisbenzimidazolyl (**6**) compounds were inactive. However, in the tris heterocyclic systems, compounds having pyrazolyl and isoxazolyl derivatives, **7** and **10** showed good radical scavenging activity in all the three methods when compared with the standard ascorbic acid. On the other hand, compounds **8** and **11** showed moderate activity

Table 2. The *in vitro* antioxidant activity of **4–21** in NO method

Compound	Concentration		
	50 μg/mL	75 μg/mL	100 μg/mL
4	59.57 ± 1.89	61.54 ± 1.54	64.93 ± 1.66
5	–	–	–
6	–	–	–
7	72.00 ± 1.01	74.10 ± 1.04	78.44 ± 1.12
8	56.21 ± 1.67	57.57 ± 1.42	59.95 ± 1.74
9	–	–	–
10	84.22 ± 1.86	87.51 ± 1.54	89.85 ± 1.71
11	61.13 ± 1.24	62.20 ± 1.02	65.15 ± 1.08
12	–	–	–
13	63.09 ± 1.02	65.41 ± 1.42	68.34 ± 1.32
14	–	–	–
15	–	–	–
16	61.19 ± 1.34	63.32 ± 1.15	65.26 ± 1.23
17	–	–	–
18	–	–	–
19	75.29 ± 0.79	77.34 ± 1.12	80.05 ± 0.96
20	–	–	–
21	–	–	–
Ascorbic acid	86.02 ± 0.91	89.46 ± 1.31	91.53 ± 1.22
Blank	–	–	–

(–) Showed no scavenging activity. Values were the means of three replicates ± SD.

Table 3. The *in vitro* antioxidant activity of **4–21** in ABTS method

Compound	Concentration		
	50 μg/mL	75 μg/mL	100 μg/mL
4	17.21 ± 1.54	18.82 ± 1.87	20.53 ± 1.95
5	–	–	–
6	–	–	–
7	25.61 ± 1.54	26.17 ± 1.45	27.48 ± 1.75
8	16.31 ± 1.71	17.14 ± 1.85	19.39 ± 1.97
9	–	–	–
10	27.40 ± 1.10	28.00 ± 1.54	28.74 ± 1.34
11	17.33 ± 0.89	19.42 ± 0.68	20.56 ± 1.02
12	–	–	–
13	19.43 ± 1.35	20.93 ± 1.57	21.55 ± 1.79
14	–	–	–
15	–	–	–
16	18.72 ± 1.54	20.32 ± 1.63	21.82 ± 1.48
17	–	–	–
18	–	–	–
19	26.32 ± 1.28	27.74 ± 1.76	28.04 ± 1.14
20	–	–	–
21	–	–	–
Ascorbic acid	28.80 ± 0.86	29.20 ± 1.25	29.70 ± 0.98
Blank	–	–	–

(–) Showed no scavenging activity. Values were the means of three replicates ± SD.

while **9** and **12** were inactive. Amongst compounds having pyrimidine derivatives, compound **19** exhibited higher activity when compared with compounds **13** and **16**. This may be due to the presence of electron donating methyl substituents and also non-quinoline like heterocycle. The compounds **14**, **15**, **17**, **18**, **20**, and **21** were inactive. This indicates that compounds with benzoxazolyl unit exhibited greater activity than those having benzothiazolyl and benzimidazolyl units. The presence of other heterocyclic rings pyrazoles and isoxazoles enhances the activity. Further the results exemplified that the compound having the benzoxazolyl unit in combination with isoxazole (**10**) is the most powerful antioxidant agent.

Conclusion

In summary, we prepared a novel series of bisbenzoxazolyl/benzothiazolyl/benzimidazolyl pyrazoles/isoxazoles/pyrimidines adopting simple and versatile synthetic methodologies and identified bis benzoxazolyl isoxazole derivative as potential antioxidant agent.

Experimental

Chemistry

Melting points were determined in open capillaries on a Mel-Temp apparatus and are uncorrected. The purity of the compounds was checked by TLC (silica gel H, BDH, ethyl acetate/hexane, 1:3). The IR spectra were recorded on a Thermo Nicolet IR 200 FT-IR spectrometer as KBr pellets and the wave numbers are given in cm^{-1}. The ^1H NMR spectra were recorded in DMSO-d_6 on a Bruker-400 spectrometer (400 MHz). The ^{13}C NMR spectra were recorded in DMSO-d_6 on a Bruker spectrometer operating at 100 MHz. All chemical shifts are reported in δ (ppm) using TMS as an internal standard. The mass spectra were recorded on Jeol JMS-D 300 and Finnigan Mat 1210 B at 70 eV with an emission current of 100 μA. The microanalyses were performed on a Perkin-Elmer 240C elemental analyzer. The antioxidant property was carried out by using a Shimadzu UV-2450 spectrophotometer. The compounds 2-(chloromethyl)benzoxazole (**1**), 2-(chloromethyl)benzothiazole (**2**), and 2-(chloromethyl)-1*H*-benzimidazole (**3**) were prepared by the literature procedure [35–37].

*General procedure for the synthesis of 2-(bis((benzo[d]-oxazol-2′-yl)methylthio)methylene)malononitrile (**4**)/2-(bis((benzo[d]thiazol-2′-yl)methylthio)methylene)-malononitrile (**5**)/2-(bis-((1′H-benzo[d]imidazol-2′-yl)-methylthio)methylene)malononitrile (**6**)*

To a solution of dry potassium carbonate (1.38 g, 10 mmol) in DMF (2 mL), malononitrile (0.66 g, 10 mmol) in DMF (1 mL) followed by carbon disulfide (0.90 mL, 15 mmol) was added dropwise under vigorous stirring. After 30 min, the reaction mixture was cooled to 0°C and to this 2-(chloromethyl)benzoxazole (**1**)/2-(chloromethyl)benzothiazole (**2**)/2-(chloromethyl)-1*H*-benzimidazole (**3**) (20 mmol) in DMF (5 mL) was added in 20–30 min. The reaction mixture was further stirred for 1 h at room temperature and poured into cold water. The precipitate was collected, dried, and recrystallized from aqueous methanol.

*2-(Bis((benzo[d]oxazol-2′-yl)methylthio)methylene)-malononitrile **4***

Yellow solid in 69%; m.p.: 169–171°C; IR (KBr) υ_{max} (cm^{-1}): 1605 (C=N), 1635 (C=C), 2253 (C≡N); ^1H NMR (DMSO-d_6) δ (ppm): 4.41 (s, 4H, CH_2), 7.19–7.42 (m, 8H, Ar–H); ^{13}C NMR (DMSO-d_6) δ (ppm): 29.4 (CH_2), 83.5 ($(NC)_2C$=C), 111.4 (CN), 163.5 (C-2′), 182.8 (C=*C*S(S)), 117.1, 120.6, 122.2, 124.6, 139.5, 149.0 (aromatic carbons); MS (*m*/*z*): 404.33 [$M^{+\bullet}$]. Anal. Calcd. for $C_{20}H_{12}N_4O_2S_2$: C 59.39, H 2.99, N 13.85; Found: C 59.44, H 2.95, N 13.93%.

*2-(Bis((benzo[d]thiazol-2′-yl)methylthio)methylene)-malononitrile **5***

Yellow solid in 66%; m.p.: 186–188°C; IR (KBr) υ_{max} (cm^{-1}): 1610 (C=N), 1631 (C=C), 2248 (C≡N); ^1H NMR (DMSO-d_6) δ (ppm): 4.37 (s, 4H, CH_2), 7.46–8.01 (m, 8H, Ar–H); ^{13}C NMR (DMSO-d_6) δ (ppm): 29.0 (CH_2), 83.8 ($(NC)_2C$=C), 112.7 (CN), 169.1 (C-2′), 183.5 (C=*C*S(S)), 120.4, 121.8, 124.3, 126.1, 135.2, 151.4 (aromatic carbons); MS (*m*/*z*): 436.51 [$M^{+\bullet}$]. Anal. Calcd. for $C_{20}H_{12}N_4S_4$: C 55.02, H 2.77, N 12.83; Found: C 55.09, H 2.78, N 12.78%.

*2-(Bis((1′H-benzo[d]imidazol-2′-yl)methylthio)methylene)-malononitrile **6***

Yellow solid in 74%; m.p.: 200–202°C; IR (KBr) υ_{max} (cm^{-1}): 1603 (C=N), 1633 (C=C), 2245 (C≡N), 3280 (NH); ^1H NMR (DMSO-d_6) δ (ppm): 4.35 (s, 4H, CH_2), 7.28–7.53 (m, 8H, Ar–H), 12.78 (bs, 2H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 28.9 (CH_2), 82.9 ($(NC)_2C$=C), 111.8 (CN), 150.4 (C-2′), 183.0 (C=*C*S(S)), 119.1, 121.4, 122.6, 127.9, 131.7, 137.5 (aromatic carbons); MS (*m*/*z*): 402.32 [$M^{+\bullet}$]. Anal. Calcd. for $C_{20}H_{14}N_6S_2$: C 59.68, H 3.51, N 20.88; Found: C 59.65, H 3.49, N 20.97%.

*General procedure for the synthesis of 4-(bis((benzo[d]-oxazol-2′-yl)methylthio)methylene)-4H-pyrazole-3,5-diamine (**7**)/4-(bis((benzo[d]thiazol-2′-yl)methylthio)-methylene)-4H-pyrazole-3,5-diamine (**8**)/4-(bis((1′H-benzo[d]imidazol-2′-yl)methylthio)methylene)-4H-pyrazole-3,5-diamine (**9**)*

A solution of **4**/**5**/**6** (1 mmol), hydrazine hydrate (0.05 mL, 1.2 mmol), and piperidine (3 mL) in ethanol (10 mL) was heated to reflux for 6–8 h. After completion of the reaction, it was cooled and poured into ice-cold water containing conc. HCl. The solid obtained was filtered, dried and recrystallized from 2-propanol.

*4-(Bis((benzo[d]oxazol-2′-yl)methylthio)methylene)-4H-pyrazole-3,5-diamine **7***

Pale yellow solid in 80%; m.p.: 194–196°C; IR (KBr) υ_{max} (cm^{-1}): 1610 (C=N), 1625 (C=C), 3320, 3415 (NH_2); ^{1}H NMR (DMSO-d_6) δ (ppm): 4.37 (s, 4H, CH_2), 5.74 (bs, 4H, NH_2), 7.23–7.46 (m, 8H, Ar–H); ^{13}C NMR (DMSO-d_6) δ (ppm): 28.6 (CH_2), 102.4 (C-4), 163.1 (C-2′), 164.2 (C-3 & C-5), 174.8 (C=*C*S(S)), 117.5, 121.4, 123.1, 125.6, 138.6, 149.8 (aromatic carbons); MS (*m*/*z*): 436.32 [$M^{+\bullet}$]. Anal. Calcd. for $C_{20}H_{16}N_6O_2S_2$: C 55.03, H 3.69, N 19.25; Found: C 55.09, H 3.73, N 19.34%.

*4-(Bis((benzo[d]thiazol-2′-yl)methylthio)methylene)-4H-pyrazole-3,5-diamine **8***

Pale yellow solid in 88%; m.p.: 214–216°C; IR (KBr) υ_{max} (cm^{-1}): 1612 (C=N), 1630 (C=C), 3325, 3419 (NH_2); ^{1}H NMR (DMSO-d_6) δ (ppm): 4.42 (s, 4H, CH_2), 5.76 (bs, 4H, NH_2), 7.51–8.18 (m, 8H, Ar–H); ^{13}C NMR (DMSO-d_6) δ (ppm): 29.2 (CH_2), 101.8 (C-4), 163.5 (C-3 & C-5), 168.6 (C-2′), 175.2 (C=*C*S(S)), 121.9, 122.4, 124.9, 127.2, 134.7, 152.1 (aromatic carbons); MS (*m*/*z*): 468.49 [$M^{+\bullet}$]. Anal. Calcd. for $C_{20}H_{16}N_6S_4$: C 51.26, H 3.44, N 17.93; Found: C 51.33, H 3.43, N 18.00%.

*4-(Bis((1′H-benzo[d]imidazol-2′-yl)methylthio)methylene)-4H-pyrazole-3,5-diamine **9***

Pale yellow solid in 82%; m.p.: 225–227°C; IR (KBr) υ_{max} (cm^{-1}): 1609 (C=N), 1631 (C=C), 3295 (NH), 3331, 3428 (NH_2); ^{1}H NMR (DMSO-d_6) δ (ppm): 4.39 (s, 4H, CH_2), 5.81 (bs, 4H, NH_2), 7.26–7.60 (m, 8H, Ar–H), 11.87 (bs, 2H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 29.5 (CH_2), 102.6 (C-4), 151.2 (C-2′), 163.9 (C-3 & C-5), 174.5 (C=*C*S(S)), 119.9, 122.0, 123.9, 128.7, 131.4, 136.3 (aromatic carbons); MS (*m*/*z*): 434.51 [$M^{+\bullet}$]. Anal. Calcd. for $C_{20}H_{18}N_8S_2$: C 55.28, H 4.18, N 25.79; Found: C 55.23, H 4.21, N 25.90%.

*General procedure for the synthesis of 4-(bis((benzo[d]-oxazol-2′-yl)methylthio)methylene)-4,5-dihydro-5-iminoisoxazol-3-amine (**10**)/4-(bis((benzo[d]thiazol-2′-yl)methylthio)methylene)-4,5-dihydro-5-iminoisoxazol-3-amine (**11**)/4-(bis((1′H-benzo[d]imidazol-2′-yl)methylthio)-methylene)-4,5-dihydro-5-iminoisoxazol-3-amine (**12**)*

A mixture of **4**/**5**/**6** (1 mmol), hydroxylamine hydrochloride (0.07 g, 1.1 mmol), piperidine (3 mL), and ethanol (10 mL) was heated to reflux for 7–9 h. It was cooled and poured into ice-cold water containing conc. HCl. The solid separated was collected, dried, and recrystallized from 2-propanol.

*4-(Bis((benzo[d]oxazol-2′-yl)methylthio)methylene)-4,5-dihydro-5-iminoisoxazol-3-amine **10***

Pale yellow solid in 85%; m.p.: 191–193°C; IR (KBr) υ_{max} (cm^{-1}): 1612 (C=N), 1628 (C=C), 3312 (NH), 3320, 3418 (NH_2); ^{1}H NMR (DMSO-d_6) δ (ppm): 4.40 (s, 4H, CH_2), 5.77 (bs, 2H, NH_2), 7.18–7.52 (m, 8H, Ar–H), 9.86 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 28.9 (CH_2), 104.2 (C-4), 162.1 (C-2′), 165.5, 167.2 (C-3, C-5), 176.0 (C=*C*S(S)), 118.1, 122.8, 124.5, 126.7, 139.1, 149.6 (aromatic carbons); MS (*m*/*z*): 437.28 [$M^{+\bullet}$]. Anal. Calcd. for $C_{20}H_{15}N_5O_3S_2$: C 54.91, H 3.46, N 16.01; Found: C 54.98, H 3.44, N 15.96%.

*4-(Bis((benzo[d]thiazol-2′-yl)methylthio)methylene)-4,5-dihydro-5-iminoisoxazol-3-amine **11***

Pale yellow solid in 84%; m.p.: 218–220°C; IR (KBr) υ_{max} (cm^{-1}): 1608 (C=N), 1626 (C=C), 3315 (NH), 3331, 3426 (NH_2); ^{1}H NMR (DMSO-d_6) δ (ppm): 4.36 (s, 4H, CH_2), 5.65 (bs, 2H, NH_2), 7.67–8.14 (m, 8H, Ar–H), 9.72 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 29.4 (CH_2), 103.7 (C-4), 164.5, 166.0 (C-3, C-5), 167.4 (C-2′), 175.6 (C=*C*S(S)), 120.9, 122.3, 125.9, 127.8, 134.3, 151.7 (aromatic carbons); MS (*m*/*z*): 469.37 [$M^{+\bullet}$]. Anal. Calcd. for $C_{20}H_{15}N_5OS_4$: C 51.15, H 3.22, N 14.91; Found: C 51.21, H 3.23, N 14.97%.

*4-(Bis((1′H-benzo[d]imidazol-2′-yl)methylthio)methylene)-4,5-dihydro-5-iminoisoxazol-3-amine **12***

Pale yellow solid in 89%; m.p.: 226–228°C; IR (KBr) υ_{max} (cm^{-1}): 1603 (C=N), 1630 (C=C), 3298 (NH), 3327, 3434 (NH_2); ^{1}H NMR (DMSO-d_6) δ (ppm): 4.43 (s, 4H, CH_2), 5.80 (bs, 2H, NH_2), 7.25–7.76 (m, 8H, Ar–H), 9.98 (bs, 1H, NH), 12.74 (bs, 2H, NH); ^{13}C NMR (DMSO-d_6) δ [ppm]: 28.6 (CH_2), 103.4 (C-4), 150.5 (C-2′), 165.8, 167.9 (C-3, C-5), 175.8 (C=*C*S(S)), 117.2, 120.5, 122.3, 126.1, 131.1, 136.9 (aromatic carbons); MS (*m*/*z*): 435.39 [$M^{+\bullet}$]. Anal. Calcd. for $C_{20}H_{17}N_7OS_2$: C 55.16, H 3.93, N 22.51; Found: C 55.20, H 3.96, N 22.59%.

*General procedure for the synthesis of 4,6-diamino-5-(bis((benzo[d]oxazol-2′-yl)methylthio)methylene)-pyrimidin-2(5H)-one (**13**)/4,6-diamino-5-(bis((benzo[d]-thiazol-2′-yl)methylthio)methylene)pyrimidin-2(5H)-one (**14**)/4,6-diamino-5-(bis((1′H-benzo[d]imidazol-2′-yl)-methylthio)methylene)pyrimidin-2(5H)-one (**15**)*

To an equimolar (1 mmol) mixture of compound **4**/**5**/**6** and urea, piperidine (3 mL) and ethanol (10 mL) were added and heated to reflux for 8–10 h. The contents of the flask were cooled and poured into ice-cold water containing conc. HCl. The solid separated was filtered, dried, and purified by recrystallization from 2-propanol.

*4,6-Diamino-5-(bis((benzo[d]oxazol-2′-yl)-methylthio)methylene)pyrimidin-2(5H)-one **13***

Yellow solid in 83%; m.p.: 210–212°C; IR (KBr) υ_{max} (cm^{-1}): 1612 (C=N), 1626 (C=C), 1675 (*C*O–N), 3334, 3447 (NH_2); ^{1}H NMR (DMSO-d_6) δ (ppm): 4.32 (s, 4H, CH_2), 5.78 (bs, 4H, NH_2), 7.18–7.58 (m, 8H, Ar–H); ^{13}C NMR (DMSO-d_6) δ (ppm): 30.1 (CH_2), 101.2 (C-5), 159.3 (C-2), 162.6 (C-2′), 164.3 (C-4 & C-6), 174.4 (C=*C*S(S)), 119.8, 123.4, 125.0, 127.2, 139.2, 148.9 (aromatic carbons); MS (*m*/*z*): 464.43 [$M^{+\bullet}$]. Anal. Calcd. for $C_{21}H_{16}N_6O_3S_2$: C 54.30, H 3.47, N 18.09; Found: C 54.27, H 3.46, N, 18.14%.

4,6-Diamino-5-(bis((benzo[d]thiazol-2′-yl)-methylthio)methylene)pyrimidin-2(5H)-one ***14***

Yellow solid in 76%; m.p.: 235–237°C; IR (KBr) υ_{max} (cm^{-1}): 1608 (C=N), 1628 (C=C), 1664 (*CO*–N), 3329, 3427 (NH_2); ^1H NMR (DMSO-d_6) δ (ppm): 4.40 (s, 4H, CH_2), 5.89 (bs, 4H, NH_2), 7.55–8.03 (m, 8H, Ar–H); ^{13}C NMR (DMSO-d_6) δ (ppm): 30.6 (CH_2), 102.3 (C-5), 160.2 (C-2), 167.5 (C-2′), 165.5 (C-4 & C-6), 174.7 (C=*CS*(S)), 120.4, 121.8, 124.3, 126.1, 134.9, 151.6 (aromatic carbons); MS (*m*/*z*): 496.61 [$M^{+\bullet}$]. Anal. Calcd. for $C_{21}H_{16}N_6OS_4$: C 50.79, H 3.25, N 16.92; Found: C 50.85, H 3.26, N 16.99%.

4,6-Diamino-5-(bis((1′H-benzo[d]imidazol-2′-yl)-methylthio)methylene)pyrimidin-2(5H)-one ***15***

Yellow solid in 80%; m.p.: 246–248°C; IR (KBr) υ_{max} (cm^{-1}): 1604 (C=N), 1631 (C=C), 1668 (*CO*–N), 3305 (NH), 3331, 3446 (NH_2); ^1H NMR (DMSO-d_6) δ (ppm): 4.34 (s, 4H, CH_2), 5.80 (bs, 4H, NH_2), 7.28–7.67 (m, 8H, Ar–H), 12.93 (bs, 2H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 29.8 (CH_2), 101.2 (C-5), 149.9 (C-2′), 159.8 (C-2), 164.9 (C-4 & C-6), 173.8 (C = *CS*(S)), 119.7, 121.4, 123.5, 127.9, 131.9, 136.7 (aromatic carbons); MS (*m*/*z*): 462.45 [$M^{+\bullet}$]. Anal. Calcd. for $C_{21}H_{18}N_8OS_2$: C 54.53, H 3.92, N 24.23; Found: C 54.58, H 3.94, N, 24.34%.

*General procedure for the synthesis of 4,6-diamino-5-(bis((benzo[d]oxazol-2′-yl)methylthio)methylene)-pyrimidine-2(5H)-thione (**16**)/4,6-diamino-5-(bis((benzo[d]-thiazol-2′-yl)methylthio)methylene)pyrimidine-2(5H)-thione (**17**)/4,6-diamino-5-(bis((1′H-benzo[d]imidazol-2′-yl)methylthio)methylene)pyrimidine-2(5H)-thione (**18**)*

A mixture of **4**/**5**/**6** (2 mmol), thiourea (0.15 g, 2 mmol), piperidine (4 mL), and ethanol (15 mL) was heated to reflux for 12–15 h. The reaction mixture was cooled and poured into ice-cold water containing conc. HCl. The solid separated was filtered on a Buchner funnel, dried, and recrystallized from 2-propanol.

4,6-Diamino-5-(bis((benzo[d]oxazol-2′-yl)methylthio)-methylene)pyrimidine-2(5H)-thione ***16***

Pale yellow solid in 73%; m.p.: 215–217°C; IR (KBr) υ_{max} (cm^{-1}): 1487 (C=S), 1611 (C=N), 1629 (C = C), 3319, 3437 (NH_2); ^1H NMR (DMSO-d_6) δ (ppm): 4.42 (s, 4H, CH_2), 5.72 (bs, 4H, NH_2), 7.24–7.46 (m, 8H, Ar–H); ^{13}C NMR (DMSO-d_6) δ (ppm): 31.3 (CH_2), 102.1 (C-5), 162.8 (C-2′), 164.3 (C-4 & C-6), 172.1 (C-2), 173.5 (C=*CS*(S)), 119.2, 122.6, 123.9, 125.7, 139.9, 148.7 (aromatic carbons); MS (*m*/*z*): 480.51 [$M^{+\bullet}$]. Anal. Calcd. for $C_{21}H_{16}N_6O_2S_3$: C 52.48, H 3.36, N 17.49; Found: C 52.45, H 3.33, N 17.45%.

4,6-Diamino-5-(bis((benzo[d]thiazol-2′-yl)methylthio)-methylene)pyrimidine-2(5H)-thione ***17***

Pale yellow solid in 77%; m.p.: 240–242°C; IR (KBr) υ_{max} (cm^{-1}): 1490 (C=S), 1613 (C=N), 1632 (C=C), 3324, 3441 (NH_2); ^1H NMR (DMSO-d_6) δ (ppm): 4.36 (s, 4H, CH_2), 5.83 (bs, 4H, NH_2), 7.49–8.08 (m, 8H, Ar–H); ^{13}C NMR (DMSO-d_6) δ (ppm): 32.5 (CH_2), 101.8 (C-5), 163.7 (C-4 & C-6), 168.7 (C-2′), 172.6 (C-2), 174.4 (C = *CS*(S)), 119.2, 122.6, 124.3, 126.5 134.7, 151.2 (aromatic carbons); MS (*m*/*z*): 512.63 [$M^{+\bullet}$]. Anal. Calcd. for $C_{21}H_{16}N_6S_5$: C 49.19, H 3.15, N 16.39; Found: C 49.25, H 3.17, N 16.46%.

4,6-Diamino-5-(bis((1′H-benzo[d]imidazol-2′-yl)-methylthio)methylene)pyrimidine-2(5H)-thione ***18***

Pale yellow solid in 86%; m.p.: 266–268°C; IR (KBr) υ_{max} (cm^{-1}): 1495 (C=S), 1607 (C=N), 1626 (C=C), 3282 (NH), 3323, 3443 (NH_2); ^1H NMR (DMSO-d_6) δ (ppm): 4.39 (s, 4H, CH_2), 5.69 (bs, 4H, NH_2), 7.27–7.59 (m, 8H, Ar–H), 12.83 (bs, 2H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 31.9 (CH_2), 102.5 (C-5), 151.5 (C-2′), 164.1 (C-4 & C-6), 173.2 (C-2), 173.9 (C=*CS*(S)), 119.9, 123.9, 124.6, 127.3, 131.1, 136.9 (aromatic carbons); MS (*m*/*z*): 478.47 [$M^{+\bullet}$]. Anal. Calcd. for $C_{21}H_{18}N_8S_3$: C 52.70, H 3.79, N 23.41; Found: C 52.74, H 3.82, N 23.49%.

*General procedure for the synthesis of 5-(bis((benzo[d]-oxazol-2′-yl)methylthio)methylene)-tetrahydro-4,6-diimino-1,3-dimethylpyrimidin-2(1H)-one (**19**)/5-(bis((benzo[d]thiazol-2′-yl)methylthio)methylene)-tetrahydro-4,6-diimino-1,3-dimethylpyrimidin-2(1H)-one (**20**)/5-(bis((1′H-benzo[d]imidazol-2′-yl)methylthio)-methylene)-tetrahydro-4,6-diimino-1,3-dimethyl-pyrimidin-2(1H)-one (**21**)*

The compound **4**/**5**/**6** (1 mmol), *N*,*N*′-dimethylurea (0.08 g, 1 mmol), piperidine (3 mL), and ethanol (10 mL) were heated to reflux for 9–11 h. The contents of the flask were cooled and poured into ice-cold water containing conc. HCl. The solid separated was filtered, dried and purified by recrystallization from 2-propanol.

5-(Bis((benzo[d]oxazol-2′-yl)methylthio)methylene)-tetrahydro-4,6-diimino-1,3-dimethylpyrimidin-2(1H)-one ***19***

Yellow solid in 70%; m.p.: 200–202°C; IR (KBr) υ_{max} (cm^{-1}): 1605 (C=N), 1628 (C=C), 1665 (*CO*–N), 3293 (NH); ^1H NMR (DMSO-d_6) δ (ppm): 2.74 (s, 6H, N–CH_3), 4.37 (s, 4H, CH_2), 7.21–7.39 (m, 8H, Ar–H), 9.78 (bs, 2H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 27.2 (N–CH_3), 30.5 (CH_2), 101.2 (C-5), 155.2 (C-4 & C-6), 158.4 (C-2), 163.1 (C-2′), 174.2 (C=*CS*(S)), 119.6, 123.8, 124.5, 125.9, 139.5, 149.7 (aromatic carbons); MS (*m*/*z*): 492.35 [$M^{+\bullet}$]. Anal. Calcd. for $C_{23}H_{20}N_6O_3S_2$: C 56.08, H 4.09, N 17.06; Found: C 56.14, H 4.11, N 17.02%.

5-(Bis((benzo[d]thiazol-2′-yl)methylthio)methylene)-tetrahydro-4,6-diimino-1,3-dimethylpyrimidin-2(1H)-one ***20***

Yellow solid in 72%; m.p.: 228–230°C; IR (KBr) υ_{max} (cm^{-1}): 1608 (C=N), 1625 (C=C), 1662 (*CO*–N), 3276 (NH); ^1H NMR (DMSO-d_6) δ (ppm): 2.68 (s, 6H, N–CH_3), 4.32 (s, 4H, CH_2), 7.46–7.98 (m, 8H, Ar–H), 9.81 (bs, 2H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 28.3 (N–CH_3), 29.7 (CH_2), 101.7 (C-5), 154.7 (C-4 & C-6), 157.6 (C-2), 168.5 (C-2′), 173.8 (C=*CS*(S)), 119.5, 123.1, 125.2, 126.8, 135.1, 151.7 (aromatic carbons); MS (*m*/*z*): 524.46 [$M^{+\bullet}$]. Anal. Calcd. for $C_{23}H_{20}N_6OS_4$: C 52.65, H 3.84, N 16.02; Found: C 52.62, H 3.83, N 16.08%.

5-(Bis((1′H-benzo[d]imidazol-2′-yl)methylthio)methylene)-tetrahydro-4,6-diimino-1,3-dimethylpyrimidin-2(1H)-one ***21***

Yellow solid in 75%; m.p.: 251–253°C; IR (KBr) υ_{max} (cm^{-1}): 1611 (C=N), 1627 (C=C), 1667 (*CO*–N), 3285 (NH), 3318 (NH); ^1H NMR (DMSO-d_6) δ (ppm): 2.70 (s, 6H, N–CH_3), 4.45 (s, 4H, CH_2), 7.25–7.42

(m, 8H, Ar–H), 9.89 (bs, 2H, NH), 12.88 (bs, 2H, NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 27.8 (N–CH_3), 31.0 (CH_2), 102.1 (C-5), 150.8 (C-2′), 155.3 (C-4 & C-6), 156.9 (C-2), 175.1 (C=*C*S(S)), 120.2, 122.5, 125.3, 127.7, 132.1, 137.2 (aromatic carbons); MS (*m*/*z*): 490.39 [$M^{+\bullet}$]. Anal. Calcd. for $C_{23}H_{22}N_8OS_2$: C 56.31, H 4.52, N 22.84; Found: C 56.35, H 4.55, N 22.93%.

Biological assays

Antioxidant activity

The compounds **4–21** were evaluated for antioxidant property by DPPH, NO, and ABTS methods.

DPPH radical scavenging activity

The hydrogen atom or electron donation ability of the compounds was measured from the bleaching of the purple colored methanol solution of DPPH radical. The spectrophotometric assay uses the stable radical DPPH as a reagent. To 4 mL of 0.004% w/v methanol solution of DPPH, 1 mL of various concentrations of the test compounds (50, 75, and 100 μg/mL) in methanol were added. After a 30 min incubation period at room temperature, the absorbance was read against blank at 517 nm. Ascorbic acid was used as the standard. The percent of inhibition (*I*%) of free radical production from DPPH was calculated by the following equation

$$I\% = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{blank}}\right] \times 100$$

where A_{control} is the absorbance of the control reaction (containing methanolic DPPH and Ascorbic acid), A_{sample} the absorbance of the test compound (containing methanolic DPPH and test compound), and A_{blank} is the absorbance of the blank (containing only methanolic DPPH). Tests were carried out in triplicate.

Nitric oxide (NO) scavenging activity

NO scavenging activity was measured by slightly modified methods of Green *et al*. and Marcocci *et al*. [40, 41]. NO radicals were generated from sodium nitroprusside. 1 mL of sodium nitroprusside (10 mM) and 1.5 mL of phosphate buffer saline (0.2 M, pH 7.4) were added to different concentrations (50, 75, and 100 μg/mL) of the test compounds and incubated for 150 min at 25°C. After incubation, 1 mL of the reaction mixture was treated with 1 mL of Griess reagent (1% sulfanilamide, 2% H_3PO_4, and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromatophore was measured at 546 nm. Ascorbic acid was used as standard. NO scavenging activity was calculated by the following equation

$$\%\,\text{of scavenging} = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{blank}}\right] \times 100$$

where A_{control} is the absorbance of the control reaction (containing all reagents and ascorbic acid), A_{sample} the absorbance of the test compound (containing all reagents and test compound), and A_{blank} is the absorbance of the blank (containing only reagents).

ABTS radical scavenging activity

The antioxidant activity of the test compounds and standard (ascorbic acid) were assessed on the basis of the radical scavenging effect of the stable ABTS free radical. The $ABTS^{\bullet+}$ solution was prepared by mixing 0.02 mol of ABTS salt with 0.01 mol of potassium persulfate in 25 mL of distilled water. The solution was kept at room temperature in the dark for 16 h before use. Then the $ABTS^{\bullet+}$ solution was diluted with methanol in order to obtain an absorbance between 0.7 and 0.9 at 734 nm using the spectrophotometer. Fresh $ABTS^{\bullet+}$ solutions were prepared for each assay. To 50, 75, and 100 μg/mL of each test compounds and standard, 1 mL of $ABTS^{\bullet+}$ solution was added and allowed to react for 2 h in dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. The corresponding blank reading was also taken and the results in percentage were expressed as the ratio of absorbance decrease at 734 nm and the absorbance of $ABTS^{\bullet+}$ solution in the absence of test compounds.

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