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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Design, synthesis and in vitro antimicrobial activity of novel phenylbenzamido-aminothiazole-based azasterol mimics

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Abstract Phenylbenzamide framework as a mimic of the azasterol structure was investigated by synthesizing 4-phenylbenzamido-2-aminothiazole **4**, and evaluating its MIC against *Escherichia coli*, *Enterobacter cloacae*, *Bacillus licheniformis* and *Mycobacterium tuberculosis* (MTB) $H_{37}Rv$ as well as antifungal activity against three test phytopathogenic fungi. Further, the bioisosterism strategy was implemented to synthesize a series of azo-derivatives of **4** (**6a**–**6j**). All the azo-compounds were screened for their antibacterial and antifungal activity. The electronic (UV–vis) absorption characteristics of the final compounds were examined. Resazurin-mediated microtitre plate-antibacterial assay was implemented for first time on these azo-derivatives. Compounds **4** and **6b** had significant antibacterial activity. For the compound **4**, MIC against *Escherichia coli* is 7.8×10^{-3} mg/mL and MIC against *Mycobacterium tuberculosis* (MTB) $H_{37}Rv$ is 16 μg/mL were identified. Compounds **4**, **6d**, **6g** and **6h** showed excellent antifungal activity, when compared to the standard nistatin, against three test phytopathogenic fungi.

Keywords Antimicrobial activity · Azasterol mimic · Azo-derivatives · 2-Aminothiazole · Bioisosterism

Introduction

The growing interest in the thiazole chemistry lies in designing new compounds and their wide range of pharmaceutical, biological and medicinal applications. The thiazole ring is a key structural motif present in numerous marine alkaloids (Davyt and Serra, 2010), natural bioactive compounds like vitamin-B1 (thiamine), thiamine pyrophosphate (TPP, a coenzyme important in respiration in the Krebs cycle), epothilones and the large family of macrocyclic thiopeptide antibiotics, thiostrepton and micrococcin P1 (Dondoni, 2010). Moreover, thiazole ring is found in penicillins (broad spectrum antibiotics), sulfathiazole (antimicrobial drug), ritonavir (antiretroviral drug), abafungin (antifungal drug) and tiazofurin (antineoplastic drug). However, highly active condensed thiazoles are still essentially in the development stage (Smirnova *et al.*, 2006). In addition, aminothiazole derivatives were well explored as potent pan-Src kinase inhibitors (Das *et al.*, 2006a), selective Itk inhibitors (Das *et al.*, 2006b), anticancer agents (Al-Said *et al.*, 2011; Luzina and Popov, 2009), in reducing ulcerogenicity (Verma *et al.*, 2010), antimicrobial agents (Pattan *et al.*, 2009; Argyropoulou *et al.*, 2009), and novel adenosine receptor antagonists (van Tilburg *et al.*, 2001).

Azasterols, nitrogen-containing sterol compounds, were shown to exhibit excellent antifungal and anitparasitic activity (Oehlschlager and Czyzewska, 1992; Ator *et al.*, 1989; Khabnadideh *et al.*, 2000). Mimicking the azasterols particularly by replacing the B and C rings by an amide group and A and D rings by a phenyl ring were shown to

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exhibit moderate activity against the parasitic infections, which are caused by the trypanosomatid family, by inhibiting sterol biosynthesis in the parasites (Gigante *et al.*, 2009; Gros *et al.*, 2006a, b; Lorente *et al.*, 2005, 2004; Magaraci *et al.*, 2003). Further developments in this area required the modification of side chain that is present on the azasterol moiety.

Benzamide derivatives showed a wide range of biological activities including potentially active against multi drug resistant tuberculosis (MDR-TB) (Krátký *et al.*, 2010), histone deacetylase inhibitors (Nagaoka *et al.*, 2006; Andrews *et al.*, 2008), VEGFR tyrosine kinase inhibitors (Nakamura *et al.*, 2006), spasmolytic activity (Brunhofer *et al.*, 2008), potassium channel activators (Calderone *et al.*, 2006a, 2006b) and CSF-1R inhibitors in the cancer treatment (Scott *et al.*, 2008). Many typical retinoids were also synthesized (Hashimoto and Miyachi, 2005) having benzamide core structure.

On the other hand, dyes in the development of drugs and pharmaceuticals were recently reviewed (Wainwright, 2008). It describes suramin (Germanin) which is still a front line treatment for Human African trypanosomiasis (HAT) having four repeating units of benzamide core structure. Suramin was developed by replacement of the azo (–N=N–) linkages with urea (–NH–CO–NH–) and carboxamide (–CO–NH–) groups in the trypan dyes. A number of antimalarial drugs, antipsychotics, antihistamines, and antidepressants were also derived from methylene blue (Wainwright, 2008). Similarly, an azo-dye lead was modified for the development of KDR kinase inhibitors (Bilodeau *et al.*, 2004). In addition to this, some natural dyes (Singh *et al.*, 2005) and synthetic dyes (Bondock *et al.*, 2007) have inherent antimicrobial activity. Thiazole-based azo-derivatives also found wide applications in the dyeing of synthetic fibres, polyester fabrics (Metwally *et al.*, 2004; Maradiya and Patel, 2003; Khalil *et al.*, 2010). These observations prompted us to synthesize benzamide containing, as azasterol (antiparasitic) mimic (Gigante *et al.*, 2010), (Fig. 1) aminothiazole-ligated azo-derivatives as novel antibacterial and antifungal agents. It is important to mention that a bioisosterism strategy (Xu and Zeng, 2010; Lima and Barreiro, 2005) (Fig. 2) was implemented to design and synthesis of these azo-derivatives.

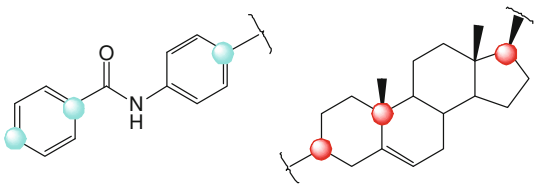


Fig. 1 Benzamide moiety as a mimic of azasterol core

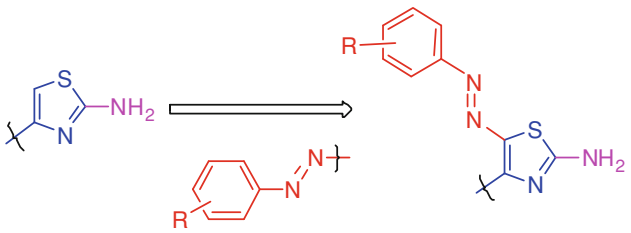


Fig. 2 Bioisosterism strategy towards the design of novel azo-compounds

While evaluating the antibacterial assay by either agar diffusion or broth dilution methods precipitation of the test compounds was found to be a major problem which is probably due to the pH incompatibility or hydrophobic nature of the azo-compounds. Thus, microtitre plate-based antibacterial assay was used.

Experimental

General

TLC was run on silica gel 60 F_{254} precoated aluminium plates (Merck) and NMR spectra were recorded at 25 °C on a Bruker Avance III 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) or 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) instrument with $CDCl_3$ or DMSO-d_6 as solvent. Chemical shifts are given in δ (ppm) and coupling constants (*J*) in hertz, multiplicity has given as s (singlet), d (double), t (triplet), m (multiplet) and brs (broad singlet). IR spectra were recorded in KBr discs on a JASCO FT/IR-5300. Elemental analyses were recorded on a Thermo Finnigan Flash EA 1112 analyzer. Mass spectra were recorded on Shimadzu-LCMS-2010A mass spectrometer. UV–vis absorption spectra were recorded on Perkin Elmer Lambda-35 spectrometer at the wavelength of maximum absorption (λ_{max}) in DMSO. Melting points were measured in open capillary tubes on a Buchi Melting Point B-540 apparatus and were uncorrected.

Synthesis

*Procedure for the preparation of N-(4-acetylphenyl)benzamide (**3**)*

p-Aminoacetophenone **1** (5.0 gm, 37.0 mmol) was dissolved in DMF (25 mL) and diluted with of CCl_4 (100 mL). To this solution, triethylamine (15.4 mL, 0.1 mol) was added under stirring. Then benzoylchloride **2** (6.4 mL, 55.0 mmol) was added dropwise at 25 °C under vigorous stirring. The reaction mixture was refluxed for 2 h

and then CCl_4 was distiled completely. After allowing the mixture to cool to 25 °C, the mixture was poured into 10 % $NaHCO_3$ solution (200 mL), and the precipitated solid was filtered. After drying, the solid compound was thoroughly washed with diethyl ether to give pure compound **3** as a light brown colour solid; yield 89 %; mp. 200–202 °C; IR (KBr): 3352, 1674, 1599, 1523, 1404, 1275, 1182, 829, 717 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): δ 8.06 (s, 1H), 8.01 (d, $J = 8.5$ Hz, 2H), 7.90 (d, $J = 7.5$ Hz, 2H), 7.79 (d, $J = 8.5$ Hz, 2H), 7.60 (t, $J = 7.5$ Hz, 1H), 7.53 (t, $J = 8.0$ Hz, 2H), 2.62 (s, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 196.9, 165.8, 142.3, 134.4, 133.1, 132.2, 129.8, 128.9, 127.1, 119.3, 26.4. LCMS (EI): *m/z* 240.15 (M^++1).

*Procedure for the preparation of N-(4-(2-aminothiazol-4-yl)phenyl)benzamide (**4**)*

A mixture of *N*-(4-acetylphenyl)benzamide **3** (5.0 gm, 20.0 mmol), thiourea (4.7 gm, 62.0 mmol) and iodine (7.9 gm, 31.0 mmol) in ethanol (25 mL) was refluxed for 8 h. The crude reaction mixture was allowed to 25 °C and poured into water (100 mL). The solution was basified with conc., NH_4OH then filtered the precipitated solid. The solid compound was again washed with aqueous sodium thiosulfate solution to remove excess iodine and then with water and dried. The crude product was taken in chloroform (20 mL) to get slurry which was stirred for 30 min and then filtered and dried under vacuum to give pure compound **4** as a white solid; yield 70 %; mp. 220–223 °C; IR (KBr): 3435, 1647, 1591, 1518, 1406, 1327, 1257, 1039, 829, 690 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.31 (s, 1H), 7.96 (d, $J = 8.0$ Hz, 2H), 7.79 (brs, 4H), 7.54–7.61 (m, 3H), 7.04 (s, 2H), 6.94 (s, 1H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 168.6, 165.9, 150.1, 138.6, 135.4, 132.0, 130.9, 128.8, 128.1, 126.2, 120.6, 100.9. LCMS (EI): *m/z* 296.15 (M^++1). Anal. Calcd. for $C_{16}H_{13}N_3OS$: C, 65.06; H, 4.44; N, 14.23. Found: C, 65.16; H, 4.49; N, 14.12.

*General procedure for synthesis of benzamide containing 2-aminothiazole based azo-derivatives (**6a–6j**)*

*N-(4-(2-amino-5-(phenyldiazenyl)thiazol-4-yl)phenyl)benzamide (**6a**)* A solution of sodium nitrite (93 mg, 1.3 mmol) in water (2 mL) was gradually added to a cooled (0 °C) solution of the aniline (93 mg, 1 mmol) in conc., HCl (1.0 mL) and stirred for 20 min to get the diazonium salt solution. This solution was added with continuous stirring to a cooled (0 °C) solution of *N*-(4-(2-aminothiazol-4-yl)phenyl)benzamide **4** (0.3 gm, 1 mmol) in ethanol (45 mL) and sodium acetate (83 mg, 10 mmol). The reaction mixture was stirred at 0–5 °C for 2 h and then diluted with cold water. The precipitated solid was collected by filtration and washed with water and dried. Trituration of this solid with toluene (2/3 times) to remove the moisture provided pure compound **6a** as an orange colour solid; yield 60 %; mp. 254–257 °C; IR (KBr): 3466, 1639, 1593, 1504, 1321, 686, 516 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.49 (s, 1H), 8.41 (s, 2H), 8.25 (d, $J = 8.0$ Hz, 2H), 7.68–8.00 (m, 4H), 7.49–7.66 (m, 7H), 7.37–7.39 (m, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 170.0, 166.2, 155.6, 153.0, 140.9, 139.5, 135.2, 132.2, 131.0, 129.8, 129.4, 129.1, 128.9, 128.1, 122.1, 120.3. LCMS (EI): *m/z* 400 (M^++1). Anal. Calcd. for $C_{22}H_{17}N_5OS$: C, 66.15; H, 4.29; N, 17.53. Found: C, 66.21; H, 4.31; N, 17.45.

*N-(4-(2-amino-5-((4-methoxyphenyl)diazenyl)thiazol-4-yl)phenyl)benzamide (**6b**)* Red colour solid; yield 56 %; mp. 210–212 °C; IR (KBr): 2916, 1653, 1597, 1520, 1319, 1248, 1145, 1022, 833 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.47 (s, 1H), 8.24 (s, 2H), 8.23 (d, $J = 8.8$ Hz, 2H), 7.93-7.99 (m, 4H), 7.54–7.66 (m, 5H), 7.08 (d, $J = 8.8$ Hz, 2H), 3.83 (s, 3H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 169.1, 166.2, 160.5, 153.1, 147.0, 140.7, 139.6, 135.2, 132.2, 130.7, 129.3, 128.9, 128.2, 123.8, 120.3, 115.1, 55.9. LCMS (EI): *m/z* 428 (M^+-1). Anal. Calcd. for $C_{23}H_{19}N_5O_2S$: C, 64.32; H, 4.46; N, 16.31. Found: C, 64.45; H, 4.39; N, 16.25.

*N-(4-(2-amino-5-(p-tolyldiazenyl)thiazol-4-yl)phenyl)benzamide (**6c**)* Dark brown solid; yield 58 %; mp. 220–223 °C; IR (KBr): 1631, 1599, 1516, 1325, 1257, 1186, 1113, 823, 750, 698 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.49 (s, 1H), 8.38 (s, 2H), 8.24 (d, $J = 8.0$ Hz, 2H), 7.94-8.00 (m, 4H), 7.55–7.63 (m, 5H), 7.32 (d, $J = 8.0$ Hz, 2H), 2.37 (s, 3H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 170.1, 166.3, 150.1, 141.3, 139.3, 138.8, 135.1, 132.2, 131.0, 130.4, 128.9, 128.2, 121.9, 120.3, 21.4. LCMS (EI): *m/z* 412 (M^+-1). Anal. Calcd. for $C_{23}H_{19}N_5OS$: C, 66.81; H, 4.63; N, 16.94. Found: C, 66.75; H, 4.66; N, 16.87.

*N-(4-(2-amino-5-((2-chlorophenyl)diazenyl)thiazol-4-yl)phenyl)benzamide (**6d**)* Brick red colour solid; yield 81 %; mp. 245–247 °C; IR (KBr): 3287, 1641, 1595, 1514, 1494, 1408, 1319, 1242, 1194, 839, 690 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.49 (s, 1H), 8.59 (s, 2H), 8.24 (d, $J = 8.4$ Hz, 2H), 7.96 (t, $J = 7.2$ Hz, 4H), 7.52–7.63 (m, 5H), 7.41 (t, $J = 7.6$ Hz, 1H), 7.31 (t, $J = 7.6$ Hz, 1H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 171.0, 166.3, 156.9, 148.8, 141.4, 140.3, 135.2, 132.2, 131.8, 131.1, 130.9, 129.8, 128.9, 128.5, 128.2, 120.3, 118.3. LCMS (EI): *m/z* 432.10 (M^+−2). Anal. Calcd. for $C_{22}H_{16}ClN_5OS$: C, 60.90; H, 3.72; N, 16.14. Found: C, 60.81; H, 3.76; N, 16.25.

*N-(4-(2-amino-5-((3-chlorophenyl)diazenyl)thiazol-4-yl)phenyl)benzamide (**6e**)* Orange colour solid; yield 83 %; mp. 262–264 °C; IR (KBr): 3290, 1643, 1591, 1529, 1406, 1319, 1278, 1182, 1147, 690 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.51 (s, 1H), 8.61 (s, 2H), 8.24 (d, $J = 12.0$ Hz, 2H), 7.98 (t, $J = 8.0$ Hz, 4H), 7.53–7.63 (m, 6H), 7.40 (d, $J = 8.0$ Hz, 1H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 170.8, 166.3, 157.5, 154.3, 141.3, 139.2, 135.2, 134.4, 132.2, 131.5, 131.2, 129.1, 128.9, 128.2, 121.4, 120.8, 120.2. LCMS (EI): *m/z* 432.20 (M^+-2). Anal. Calcd. for $C_{22}H_{16}ClN_5OS$: C, 60.90; H, 3.72; N, 16.14. Found: C, 60.96; H, 3.69; N, 16.07.

*N-(4-(2-amino-5-((4-chlorophenyl)diazenyl)thiazol-4-yl)phenyl)benzamide (**6f**)* Orange colour solid; yield 79 %; mp. 286–288 °C; IR (KBr): 3288, 1641, 1589, 1518, 1498, 1408, 1313, 1082, 825, 688 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.49 (s, 1H), 8.51 (s, 2H), 8.24 (d, $J = 8.8$ Hz, 2H), 7.95-7.99 (m, 4H), 7.54–7.67 (m, 7H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 170.4, 166.3, 156.5, 151.7, 141.1, 139.4, 135.2, 133.0, 132.2, 131.1, 129.8, 129.2, 128.9, 128.2, 123.6, 120.2. LCMS (EI): *m/z* 434.20 (M^+). Anal. Calcd. for $C_{22}H_{16}ClN_5OS$: C, 60.90; H, 3.72; N, 16.14. Found: C, 60.85; H, 3.68; N, 16.21.

*N-(4-(2-amino-5-((4-bromophenyl)diazenyl)thiazol-4-yl)phenyl)benzamide (**6g**)* Light brown colour solid; yield 57 %; mp. 276–279 °C; IR (KBr): 3288, 1641, 1591, 1498, 1406, 1313, 1257, 1005, 823, 688 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.50 (s, 1H), 8.53 (s, 2H), 8.24 (d, $J = 8.8$ Hz, 2H), 7.97 (t, $J = 9.6$ Hz, 4H), 7.69 (d, $J = 8.4$ Hz, 2H), 7.57–7.63 (m, 5H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 170.5, 166.3, 156.6, 152.0, 141.2, 139.4, 135.2, 132.8, 132.2, 131.1, 129.2, 128.9, 128.2, 123.9, 121.6, 120.2. LCMS (EI): *m/z* 478.25 (M^+). Anal. Calcd. for $C_{22}H_{16}BrN_5OS$: C, 55.24; H, 3.37; N, 14.64. Found: C, 55.31; H, 3.41; N, 14.55.

*N-(4-(2-amino-5-((2-nitrophenyl)diazenyl)thiazol-4-yl)phenyl)benzamide (**6h**)* Dark brown colour solid; yield 80 %; mp. 212–214 °C; IR (KBr): 3310, 1649, 1591, 1523, 1317, 1238, 1186, 844, 738, 688, 513 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.52 (s, 1H), 8.80 (s, 2H), 8.22 (d, $J = 8.0$ Hz, 2H), 7.97 (brs, 4H), 7.88 (d, $J = 8.0$ Hz, 1H), 7.71 (d, $J = 4.0$ Hz, 2H), 7.53–7.61 (m, 4H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 172.0, 166.3, 159.3, 146.6, 144.7, 141.8, 140.0, 135.2, 133.3, 132.2, 131.3, 128.9, 128.7, 128.5, 128.2, 124.1, 120.2, 119.4. LCMS (EI): *m/z* 445.25 (M^++1). Anal. Calcd. for $C_{22}H_{16}N_6O_3S$: C, 59.45; H, 3.63; N, 18.91. Found: C, 59.31; H, 3.61; N, 18.79.

*N-(4-(2-amino-5-((3-nitrophenyl)diazenyl)thiazol-4-yl)phenyl)benzamide (**6i**)* Brick red colour solid; yield 61 %; mp. 275–277 °C; IR (KBr): 3410, 1655, 1649, 1597, 1520, 1309, 1255, 1186, 688 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.54 (s, 1H), 8.76 (s, 2H), 8.32 (s, 1H), 8.27–8.29 (m, 2H), 8.16 (d, $J = 8.4$ Hz, 1H), 8.07 (d, $J = 7.6$hz, 1H), 7.99–8.01 (m, 4H), 7.77–7.80 (m, 1H), 7.57–7.64 (m, 3H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 171.4, 166.3, 158.8, 153.8, 149.2, 141.6, 139.1, 135.2, 132.2, 131.3, 131.2, 128.9, 128.6, 128.2, 122.4, 120.2, 115.2. LCMS (EI): *m/z* 445.25 (M^++1). Anal. Calcd. for $C_{22}H_{16}N_6O_3S$: C, 59.45; H, 3.63; N, 18.91. Found: C, 59.56; H, 3.59; N, 18.79.

*N-(4-(2-amino-5-((4-nitrophenyl)diazenyl)thiazol-4-yl)phenyl)benzamide (**6j**)* Violet colour solid; yield 75 %; mp. 286–289 °C; IR (KBr): 3288, 1649, 1587, 1508, 1408, 1292, 1246, 1190, 1145, 1097, 688 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.56 (s, 1H), 8.94 (s, 2H), 8.28–8.34 (m, 4H), 7.98–8.01 (m, 4H), 7.79 (d, $J = 9.2$ Hz, 2H), 7.55–7.65 (m, 3H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 172.2, 166.4, 160.3, 157.4, 145.9, 141.9, 140.2, 135.2, 132.2, 131.6, 128.9, 128.7, 128.2, 125.6, 122.4, 120.3. LCMS (EI): *m/z* 443.30 (M^+−1). Anal. Calcd. for $C_{22}H_{16}N_6O_3S$: C, 59.45; H, 3.63; N, 18.91. Found: C, 59.54; H, 3.68; N, 19.07.

Antibacterial screening test for azo-derivatives: determination of minimum inhibitory concentration (MIC) using modified resazurin assay

Two Gram-negative bacteria *Escherichia coli*, *Enterobacter cloacae* and a Gram-positive *Bacillus licheniformis* were used as test cultures to screen the azo-derivatives for antibacterial activity.

Preparation of bacterial culture

Using aseptic techniques, a single colony was transferred into a 100 mL bottle of isosensitest broth capped and placed in an incubator overnight at 37 °C. After 12–18 h of incubation, using aseptic preparation and the aid of a centrifuge, a clean sample of bacteria was prepared. The broth was spun down using a centrifuge set at 4000 rpm for 5 min with appropriate aseptic precautions. The supernatant was discarded into an appropriately labelled contaminated waste beaker. The pellet was resuspended using 20 mL of sterile normal saline and centrifuged again at 4000 rpm for 5 min. This step was repeated until the supernatant was clear. The pellet was then suspended in 20 mL of sterile normal saline and the optical density was recorded at 500 nm, and serial dilutions were carried out with appropriate aseptic techniques until the optical density was in the range of 0.5–1.0. For each test bacterium, a cell

concentration of 5×10^5 CFU/mL was used for antibacterial assay.

Preparation of resazurin solution

The resazurin solution was prepared by dissolving a 67.5 mg tablet in 10 mL of sterile distiled water. A vortex mixer was used to ensure that it was a well dissolved and homogenous solution.

Preparation of the plates

Plates were prepared under aseptic conditions. A sterile 96 well plate was labelled. A volume of 100 μL of test material DMSO (stock concentration of 2 mg/mL for test compounds and 2.5 mg/mL for chloramphenicol) was pipetted into the first row of the plate. To all other wells, 50 μL of nutrient broth or normal saline was added. Serial dilutions were performed using a multichannel pipette. Tips were discarded after use such that each well had 50 μL of the test material in serially descending concentrations. To each well, 10 μL of resazurin indicator solution was added. Using a pipette, 30 μL of 3.3 × strength isosensitised broth was added to each well to ensure that the final volume was single strength of the nutrient broth. Finally, 10 μL of bacterial suspension (5×10^6 cfu/mL) was added to each well to achieve a concentration of 5×10^5 cfu/mL. Each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated. Each plate had a set of controls: a column with a broad-spectrum antibiotic as positive control (chloramphenicol in serial dilution), a column with all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution adding 10 μL of nutrient broth instead. The plates were prepared in triplicate, and placed in an incubator set at 37 °C for 18–24 h. The colour change was then assessed visually. Any colour changes from purple to pink or colourless were recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC value. The average of three values was calculated and that was the MIC for the test material and bacterial strain.

Fungal spore germination inhibition assay using haemocytometer

Spore isolation

Antifungal activity for the pure synthetic compounds was estimated using fungal spore germination inhibition assay. Pure cultures of the test fungi, *Alternaria alternata*, *Curvularia lunata* and *Fusarium oxysporum* were used for spore isolation. Fungal cultures were grown on petri plates filled with potato dextrose agar (PDA). After 10 days of incubation at 28 °C, fungal mycelia were removed with sterile glass slide and resuspended in autoclaved MQ water. The resulting suspension was filtered aseptically through sterilized muslin cloth. Counting of spores was done using haemocytometer. The filtrate was adjusted with sterile water to a spore count of 2.3×10^4/mL, 3.8×10^4/mL, 2.5×10^4/mL for *A. alternate*, *C. Lunata* and *F. oxysporum*, respectively, and stored at 4 °C.

Procedure

To test the antifungal effect, a reaction containing 100 μL of the pure compound + 50 μL of PDB (4X) + 50 μL of respective spores were incubated at 28 °C for 24 h. Appropriate controls like PDB + spores alone, 100 μL of DMSO + 50 μL of PDB (4X) + 50 μL of respective spores, and a positive control containing 40 μL of nystatin + 50 μL of PDB (4X) + 50 μL of respective spores + 60 μL of MQ water were used. After 24-h incubation, spores were observed under compound microscope and counting was done using haemocytometer.

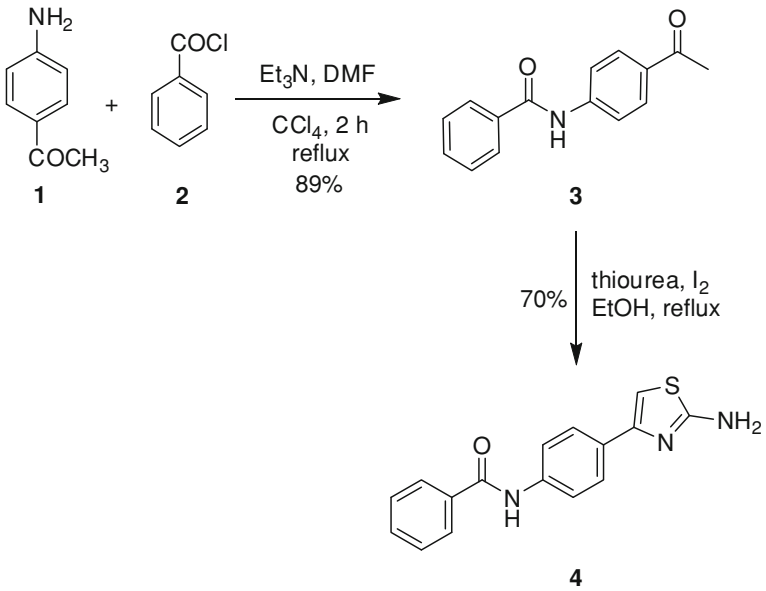
Results and discussion

Chemistry

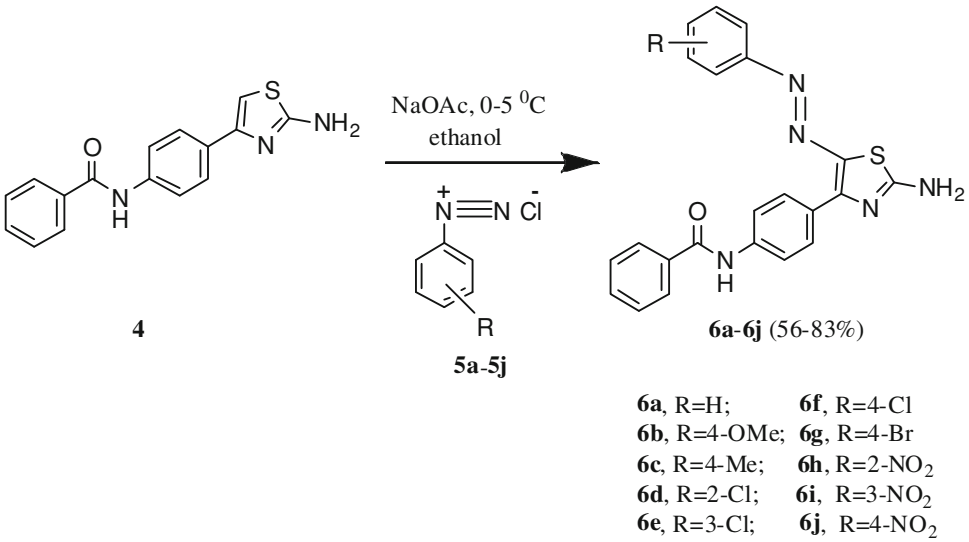
The precursor for diazo-coupling reaction was synthesised starting from 4-aminoacetophenone **1**. As shown in Scheme 1, amidation of benzoyl chloride **2** with amine **1** in CCl_4 using Et_3N and dimethyl formamide under reflux conditions provided the *N*-(4-acetylphenyl)benzamide **3** in 89 % yield. 2-aminothiazole moiety was made by adopting a one-step protocol (Dodson and King, 1945; King and Hlavacek, 1950). Thus, reaction of ketone **3** with thiourea (2 eq) and iodine (1 eq) in ethanol under reflux provided the 2-aminothiazole functionalised phenyl benzamide **4** in good yield (70 %).

Towards the synthesis of target diazo-compounds, a series of diazonium salts possessing electron releasing groups (ERG) and electron-withdrawing groups (EWG) were synthesised (**5a–5j**) by diazotisation of the corresponding aniline derivatives. Coupling of these diazonium salts with 2-amino thiazole **4** provided the diazo-derivatives **6a-6j** in excellent yield. The regioselective azo coupling at 5th position to the 2-aminothiazole ring was confirmed by observing the absence of singlet at δ 6.94 ppm in the ^{1}H NMR and δ 100.9 ppm in ^{13}C NMR spectrum, which are corresponding to the 5-CH of 2-amino thiazole ring in compound **4**, of the diazo-derivatives **6a–6j**. Better yields were obtained when amines possessing either chloro or nitro groups (Scheme 2).

Scheme 1 Synthesis of phenylbenzamide linked 2-aminothiazole bioisosteres



Scheme 2 Synthesis of phenylbenzamido-aminothiazole based azasterol mimics



6a, R=H; **6f**, R=4-Cl
6b, R=4-OMe; **6g**, R=4-Br
6c, R=4-Me; **6h**, R=2-NO_2
6d, R=2-Cl; **6i**, R=3-NO_2
6e, R=3-Cl; **6j**, R=4-NO_2

In the ^{1}H NMR of all the synthesized compounds, a broad singlet at δ 10.31–10.56 ppm corresponding to the benzamide (–CONH) proton and another singlet at δ 7.04 ppm assigning to NH_2 group on thiazole ring of compound **4** was shifted to down field with chemical shift value ranging from δ 8.24 to 8.94 ppm for the azo-compounds **6a–6j**. Further, the ^{13}C NMR spectra showed chemical shifts at δ 168.6–172.2 ppm equivalent to the thiazole carbon having NH_2 group. The ^{13}C NMR peaks of amide carbonyl were found to be between δ 165.9–166.4 ppm. The thiazole carbon that is attached to the phenylbenzamide was found to appear between δ 150.1–160.3 ppm. All the final products were also confirmed by taking LCMS. Finally, the elemental analysis results were within ±0.4 % of the theoretical values assures the purity of the final products for bioactivity studies.

The electronic (UV–vis) absorption spectra of the compounds **6a–6j** were recorded at a wavelength of maximum absorption λ_{max} (nm) in DMSO of 10^{-5} M solutions and are listed in Table 1. It was observed in general that there is an intense band at a λ_{max} ranging from 478 to 537 nm on the absorption spectra. As seen in, Fig. 3, generally, electron-withdrawing nitro groups in all positions for compounds **6h**, **6i**, **6j** and 2-chloro of compound **6d** cause bathochromic (red) shifts. For the compound **6j**, the bathochromic shift and hyperchromic shift of the 4-NO_2 group is particularly large. The electron-donating methoxy group cause hypochromic shift for the compound **6b**.

Table 1 Influence of the substituents on λ_{max} (nm) of the azo-derivatives (**6a–6j**)

Compound	*R*	λ_{max} (nm)[a]
6a	H	478
6b	4-OMe	487
6c	4-Me	479
6d	2-Cl	492
6e	3-Cl	486
6f	4-Cl	487
6g	4-Br	487
6h	2-NO_2	505
6i	3-NO_2	493
6j	4-NO_2	537

[a] Absorption maxima in DMSO

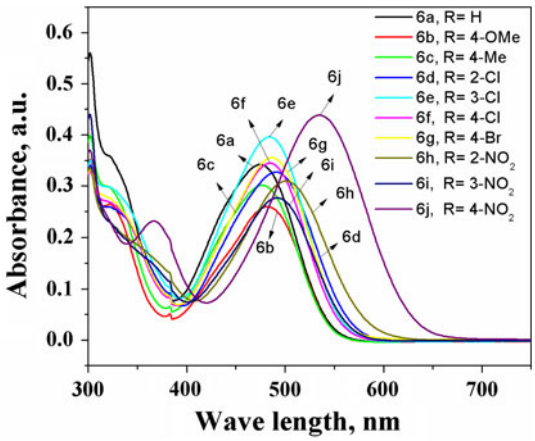
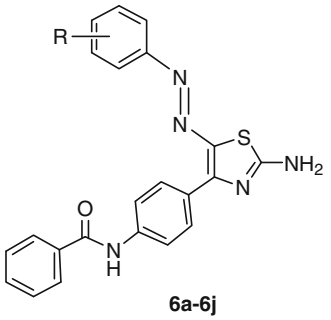


Fig. 3 Influence of the substituents effect on UV absorption spectra of azo-derivatives (**6a–6j**) in DMSO

Antimicrobial activity

Interestingly, compound **4** was found to be potent against *Mycobacterium tuberculosis* (MTB) $H_{37}Rv$ with minimum inhibitory concentration (MIC) 16 μg/mL. The MIC value of compound **4** was obtained by the Microplate Alamar Blue Assay (Franzblau *et al.*, 1998) (MABA) after 5 days of incubation at 37 °C. The experiment was done in duplicate and was repeated twice to check the reproducibility of the MIC value and also confirmed in the BacT/ALERT®MP Mycobacterium detection system (bioMérieux, France). The newly synthesized diazo-derivatives were screened against two Gram-negative bacteria *Escherichia coli*, *Enterobacter cloacae* and one Gram-positive bacterium *Bacillus licheniformis* for their in vitro antibacterial activity and assess the MIC. The antibacterial activity was evaluated using microtitre plate-based antibacterial assay (Sarker *et al.*, 2007) incorporating

Table 2 Antibacterial activity data of synthesized compounds (**4**, **6a–6j**): MIC (mg/mL) determination using modified resazurin assay



6a-6j

Compound No.	*R*	Gram-negative		Gram-positive
		E.coli	*E. cloacae*	*B. licheniformis*
4	–	**7.8×10^{-3}**	2.5×10^{-1}	1.25×10^{-1}
6a	H	**6.25×10^{-2}**	2.5×10^{-1}	2.5×10^{-1}
6b	4-OMe	**7.8×10^{-3}**	2.5×10^{-1}	1.25×10^{-1}
6c	4-Me	1.25×10^{-1}	2.5×10^{-1}	1.25×10^{-1}
6d	2-Cl	1.25×10^{-1}	2.5×10^{-1}	1.25×10^{-1}
6e	3-Cl	2.5×10^{-1}	2.5×10^{-1}	1.25×10^{-1}
6f	4-Cl	2.5×10^{-1}	2.5×10^{-1}	1.25×10^{-1}
6g	4-Br	2.5×10^{-1}	2.5×10^{-1}	2.5×10^{-1}
6h	2-NO_2	1.25×10^{-1}	2.5×10^{-1}	1.25×10^{-1}
6i	3-NO_2	**6.25×10^{-2}**	2.5×10^{-1}	**6.25×10^{-2}**
6j	4-NO_2	**6.25×10^{-2}**	**1.25×10^{-1}**	**3.125×10^{-2}**
Chloramphenicol	–	3.9×10^{-3}	3.9×10^{-3}	3.9×10^{-3}

Bold values indicate the best antibacterial and anti-fungal activities

resazurin as an indicator of cell growth with chloramphenicol as standard drug. As shown in Table 2, the MIC values of the synthesized compounds are generally within the range of 7.8×10^{-3}–2.5×10^{-1} mg/mL against all tested microorganisms. Compounds **4**, **6b** (R = 4-OMe) showed excellent activity and compounds **6a**, **6i** and **6j** exhibited good activity against *E. coli*. Compounds **6i**, **6j** also showed very good activity against Gram-positive bacteria *B. licheniformis*. Out of all azo-compounds, **6j** (R = 4-NO_2) was found to exhibit significant activity against all three test bacteria.

Antifungal activity was estimated using fungal spore germination inhibition assay (Singh and Chhatpar, 2011). *Alternaria alternata*, *Curvularia lunata* and *Fusarium oxysporum* were used in this test by taking nystatin as standard antifungal drug. The results of antifungal screening of the synthesized compounds and standard drug are given in Table 3. Compounds **4**, **6d**, **6g** and **6h** were 1.31, 1.80, 1.11 and 1.71 multiple times, respectively, better than reference standard nystatin, against *A. alternata*.

Table 3 Antifungal activity data of synthesized compounds (**4**, **6a–6j**) using fungal spore germination inhibition assay

Compound	*Alternaria alternata*		*Curvularia lunata*		*Fusarium oxysporum*	
	% of inhibition	No. of spores ungerminated	% of inhibition	No. of spores ungerminated	% of inhibition	No. of spores ungerminated
4	**131.25**	**10.5**	**106.9**	**7.7**	66.7	3.4
6d	**180**	**14.4**	**173.6**	**12.5**	**117.6**	**6**
6g	**111.25**	**8.9**	93	6.7	**207.8**	**10.6**
6h	**171.25**	**13.7**	87.5	6.3	**149**	**7.6**
6j	–	NA	84.7	6.1	70.6	3.6
Nystatin	100	8	100	7.2	100	5.1

NA not active, nystatin considered as 100 % inhibition activity, Compounds **6a**, **6b**, **6c**, **6e**, **6f** and **6i** were not active for antifungal assay
Bold values indicate the best antibacterial and anti-fungal activities

Compounds **4**, **6d** were 1.06, 1.73 multiple times better than nystatin, against *C. lunata*. Compounds **6d**, **6g** and **6h** (R = 2-Cl, 4-Br and 2-NO_2 respectively) were 1.17, 2.07 and 1.49 multiple times, respectively, better than standard, against *F. oxysporum*.

Conclusions

In conclusion, a series of ten novel benzamide linked 2-aminothiazole-based azo-derivatives were synthesized. Substituent's influence on the wavelength of maximum absorption has been studied. When, R = 4-NO_2 for the compound **6j**, the bathochromic and hyperchromic shifts are particularly large. Our study demonstrated clearly that, compounds **4**, **6a**, **6b**, **6i** and **6j** had significant antibacterial activity. An efficient resazurin-mediated microtitre plate-based antibacterial assay was implemented. Further, for compound **4** with MIC against *E. coli* is 7.8×10^{-3} mg/mL and MIC against MTB $H_{37}Rv$ is 16 μg/mL were identified. Compounds **4**, **6d**, **6g** and **6h** were also found to be highly potent antifungal agents. The biological potency of this series would render them attractive leads for further exploration.

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Conflict of interest The authors have declared no conflict of interest.

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