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Synthesis and in vitro antibacterial evaluation of 6-substituted 4-amino-pyrazolo[3,4-*d*]pyrimidines

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Abstract Pyrazolo[3,4-*d*]pyrimidines are one of the most important classes of fused heterocyclic compounds which exhibit a broad range of biological and medicinal properties. They are known as anticancer, antifungal, antibacterial, antiviral and anti-inflammatory agents. In this study, some new 6-substituted 4-amino-pyrazolo[3,4-*d*]pyrimidine derivatives were prepared *via* reaction of 5-amino-3-methyl-1-phenyl-1*H*-pyrazole-4-carbonitrile with various nitriles in the presence of sodium ethoxide as catalyst. The inhibitory properties of synthesized compounds were studied according to CLSI guidelines against some pathogenic bacteria including four gram-positive strains (*Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis subsp. spizizenii*) and three gram-negative strains (*Pseudomonas aeruginosa*, *Shigella flexneri* and *Salmonella enterica subsp. enterica*). The antibacterial effects of all derivatives were compared with those of antibiotics belonging to different classes. The values were reported as inhibition zone diameter (IZD), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The effect of substituents on the biological activity of derivatives was discussed as well. The inhibitory effect of compound **6a**, was shown to be the most, with MIC values in the range of 32–4096 μg/mL. Since most of the synthesized compounds were effective against *Streptococcus pyogenes* and *Pseudomonas aeruginosa*, they can be considered as inhibitors of these two bacteria.

Keywords Antibacterial effects · Pyrazolo[3,4-*d*]pyrimidines · Nitriles · Cyclocondensation · Sodium ethoxide catalyst

Introduction

Pyrazolo[3,4-*d*]pyrimidine core is founded in many natural products and biologically active compounds. A vast number of studies in the field of cancer showed the inhibitory activity of pyrazolo[3,4-*d*]pyrimidines against protein kinase enzymes (Laurenzana et al. 2016; Verschueren et al. 2017). Allopurinol (1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one) was known as an anticancer agent and inhibitor of xanthine oxidase (Irwig and Krakoff 1966; Kumar et al. 2011). It was also used to treat gout and hyperuricemia. The derivatives of pyrazolo[3,4-*d*]pyrimidin-4-one were described as adenosine deaminase inhibitors by La Motta et al. (2009). Anti-HSV-1 activity of some pyrazolo[3,4-*d*]pyrimidine derivatives was examined using plaque reduction infectivity assay by Rashad et al. (2009). In a research project, the specific absorption rate (SAR) and activity of substituted 4-amino-1*H*-pyrazolo[3,4-*d*]pyrimidines were measured against insulin-like growth factor-1 receptor (IGF1R), epidermal growth factor receptor (EGFR) and receptor tyrosine-protein kinase 2 (ErbB2) by Wang et al. (2010).

A wide range of pharmacological properties such as anti-inflammatory and analgesic (Kadry 2014), anti-tubercular (Trivedi et al. 2012) antitumor and antiparasitic (Kandeel et al. 2013; Ma et al. 2016) and herbicidal

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activities (Liu et al. 2007) were also described in the literatures.

There are several approaches reported in the synthesis of pyrazolo[3,4-*d*]pyrimidines, for instance: reaction of 5-amino-pyrazole-4-carbohydrazides with formamide and hydrazine hydrate under microwave irradiation (Todorovic et al. 2011), or with urea and decaline (Ghorab et al. 2010) or with benzoyl chloride derivatives and triethylorthoformate (Daidone et al. 2002). There are reports on the reaction of ethyl 5-amino-1*H*-pyrazole-4-carboxylates with hydrazine hydrate and triethylorthoformate (Ghorab et al. 2010) and also with formamide or benzoyl isothiocyanate (Carraro et al. 2006) as well as reaction of 4-hydrazinylpyrimidine derivatives with isocyanates (Bhuyan et al. 2002). It is also observed that this ring system could form in the reaction of *t*-butyl carbazate with 4-chloropyrimidine-5-carbonitrile (Soth et al. 2011) or treatment of 5-aminopyrazole derivatives with formamide in the presence of phosphorous tribromide (Huang et al. 2012).

One of the common starting materials in the synthesis of pyrazolo[3,4-*d*]pyrimidines is 5-amino-4-cyanopyrazoles (Quintela et al. 2001; Song et al. 2011; Rahmouni et al. 2014; Agrebi et al. 2014; Tintori et al. 2015; Siebeneicher et al. 2016). Reaction of these compounds with nitriles under various conditions is reported (Hanefeld et al. 1996; Smith et al. 2007; Davoodnia et al. 2012). Among which, EtONa(or K) in ethanol is less costly and more appropriate in comparison to other available methods (Hanefeld et al. 1996; Salaheldin et al. 2009).

Due to the biologically importance of the pyrazolo[3,4-*d*]pyrimidine skeleton especially as antimicrobial agents (Burch 1968; Eweas et al. 2012; Khobragade et al. 2010), we focused on the synthesis of some novel 6-substituted 4-amino-pyrazolo[3,4-*d*]pyrimidines as potential prodrugs (Vignaroli et al. 2013) using ethanolic sodium ethoxide solution. In vitro inhibitory effects of the synthesized compounds against seven gram-positive and -negative bacterial strains were evaluated *via* measuring inhibition zone diameter (IZD), and determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The effects of substituent variation on the antibacterial activity of derivatives were discussed as well.

Experimental

Materials

Melting points were recorded on a Kruss type KSP1N melting point meter and are uncorrected. The IR spectra were determined on Bruker Tensor-27 FT-IR spectrometer in KBr pellets. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker FT-NMR Ultra Shield-400 spectrometer at 400 and 100 MHz, respectively, in DMSO-d_6 with $SiMe_4$ (^{1}H and ^{13}C) internal standard. Elemental analyses were performed for C, H and N on a Thermo Finnigan Flash EA microanalyzer.

Synthesis of 5-amino-3-methyl-1-phenyl-1H-pyrazole-4-carbonitrile (4)

Compound **4** was prepared according to the procedure reported in the literature (Allouche et al. 2013), as follows: two drops of acetic acid was added to a solution of malononitrile (**1**) (0.01 mol, 0.66 g), triethyl orthoacetate (**2**) (0.01 mol, 1.62 g) and phenylhydrazine (**3**) (0.01 mol, 1.07 g) in ethanol (10 mL), and the solution was heated to reflux for 12 h. After cooling the reaction mixture to room temperature, the precipitated product was collected by filtration, washed sequentially with water (10 mL) and cold ethanol (10 mL). The crude product was recrystallized from ethanol to afford pure pyrazole **4**. White crystals, yield (1.68 g, 85%), mp 128–129 °C. ^{1}H NMR (Acetone-d_6), δ: 2.13 (s, 3H, Me), 6.04 (br, 2H, NH_2), 7.32–7.51 (m, 5H, Ph). ^{13}C NMR (Acetone-d_6), δ: 11.47 (Me), 76.82 [C(4)], 115.34 (C≡N), 123.76 [C(2′), C(6′)], 126.87 [C(4′)], 128.93 [C(3′), C(5′)], 139.04 [C(1′)], 148.79 [C(3)], 150.45 [C(5)]. IR (ν/cm^{-1}): 3337, 3232 (NH_2), 2208 (C≡N), 1644 (C=N). Anal. Calcd. for $C_{11}H_{10}N_4$ (%): C, 66.65; H, 5.09; N, 28.26. Found (%): C, 66.69; H, 5.07; N, 28.24.

General procedure for the preparation of 6-substituted 4-amino-pyrazolo[3,4-d]pyrimidines 6a–g

Clean metallic sodium (0.01 mol, 0.23 g) was added portion wise during at least 5 min to a stirred and cooled (ice-water bath) absolute ethanol (20 mL). Aminopyrazole **4** (0.01 mol, 1.98 g) and nitriles **5a–g** (**5a**: 0.02 mol, 0.41 g; **5b**: 0.01 mol, 1.03 g; **5c**: 0.01 mol, 1.17 g; **5d**: 0.01 mol, 1.82 g; **5e**: 0.01 mol, 1.48 g; **5f**: 0.01 mol, 1.38 g; **5g**: 0.01 mol, 1.72 g) were then added to a sodium ethoxide solution. The resulting solution or mixture was heated to reflux for 8–12 h (**6a**: 12 h, **6b**: 11 h, **6c**: 12 h, **6d**: 10 h, **6e**: 8 h, **6f**: 10 h, **6g**: 8 h). Let, the reaction mixture was cooled to room temperature, the precipitated product was filtered off, and washed, respectively, with water (10 mL) and ethanol (10 mL). The desired product was dried in air and recrystallized from methanol to provide analytically pure derivatives **6a–g**.

3,6-Dimethyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (6a) Bright yellow powder, yield (1.79 g, 75%), mp 268–270 °C. ^{1}H NMR, δ: 2.37 (s, 3H, $Me_{pyrimidine}$), 2.50 (s, 3H, $Me_{pyrazole}$), 7.32 (t, 2H, J = 7.4 Hz, NH_2), 7.52 (t, 3H, J = 7.7 Hz, 3′-H, 4′-H, 5′-H), 8.07 (d, 2H,

J = 8.0 Hz, 2′-H, 6′-H). ^{13}C NMR, δ: 13.89 ($Me_{pyrazole}$), 22.80 ($Me_{pyrimidine}$), 98.91 [C(3a)], 120.63 [C(2′), C(6′)], 125.79 [C(4′)], 129.47 [C(3′), C(5′)], 139.26 [C(1′)], 143.11 [C(3)], 154.10 [C(7a)], 160.43 [C(4)], 166.10 [C(6)]. IR (ν/cm^{-1}): 3494 (NH_2), 1676 (C=N). Anal. Calcd. for $C_{13}H_{13}N_5$ (%): C, 65.25; H, 5.48; N, 29.27. Found (%): C, 65.17; H, 5.49; N, 29.34.

*3-Methyl-1,6-diphenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**6b**)* Yellow powder, yield (1.66 g, 55%), mp 227–228 °C. ^{1}H NMR, δ: 2.67 (s, 3H, Me), 7.32 (t, 2H, J = 7.3 Hz, NH_2), 7.52 (m, 3H, 3″-H, 4″-H, 5″-H), 7.58 (t, 3H, J = 7.7 Hz, 3′-H, 4′-H, 5′-H), 8.32 (d, 2H, J = 7.9 Hz, 2′-H, 6′-H), 8.45 (m, 2H, 2″-H, 6″-H). ^{13}C NMR, δ: 15.00 (Me), 99.63 [C(3a)], 120.61 [C(2′), C(6′)], 125.90 [C(4′)], 128.56 [C(2″), C(6″)], 128.80 [C(3″), C(5″)], 129.61 [C(3′), C(5′)], 130.88 [C(4″)], 138.39 [C(1″)], 139.62 [C(1′)], 143.39 [C(3)], 155.94 [C(7a)], 158.97 [C(4)], 162.12 [C(6)]. IR (ν/cm^{-1}): 3497, 3312 (NH_2), 1650 (C=N). Anal. Calcd. for $C_{18}H_{15}N_5$ (%): C, 71.74; H, 5.02; N, 23.24. Found (%): C, 71.64; H, 5.04; N, 23.32.

*3-Methyl-1-phenyl-6-p-tolyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**6c**)* White powder, yield (2.21 g, 70%), mp 218–219 °C. ^{1}H NMR, δ: 2.40 (s, 3H, $Me_{benzene}$), 2.66 (s, 3H, $Me_{pyrazole}$), 7.31 (t, 2H, J = 8.2 Hz, NH_2), 7.34 (d, 2H, J = 8.1 Hz, 3″-H, 5″-H), 7.58 (t, 3H, J = 7.7 Hz, 3′-H, 4′-H, 5′-H), 8.31 (d, 2H, J = 7.9 Hz, 2′-H, 6′-H), 8.34 (d, 2H, J = 8.1 Hz, 2″-H, 6″-H). ^{13}C NMR, δ: 14.99 ($Me_{pyrazole}$), 21.52 ($Me_{benzene}$), 99.53 [C(3a)], 120.58 [C(2′), C(6′)], 125.85 [C(4′)], 128.56 [C(2″), C(6″)], 129.41 [C(3′), C(5′)], 129.61 [C(3″), C(5″)], 135.71 [C(4″)], 139.65 [C(1′)], 140.58 [C(1″)], 143.35 [C(3)], 156.00 [C(7a)], 158.99 [C(4)], 162.20 [C(6)]. IR (ν/cm^{-1}): 3497, 3313 (NH_2), 1655 (C=N). Anal Calcd. for $C_{19}H_{17}N_5$ (%): C, 72.36; H, 5.43; N, 22.21. Found (%): C, 72.27; H, 5.46; N, 22.27.

*6-(4-Bromophenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**6d**)* White powder, yield (2.77 g, 73%), mp 212–213 °C. ^{1}H NMR, δ: 2.66 (s, 3H, Me), 7.33 (t, 2H, J = 7.4 Hz, NH_2), 7.57 (t, 3H, J = 7.6 Hz, 3′-H, 4′-H, 5′-H),7.73 (d, 2H, J = 8.5 Hz, 3″-H, 5″-H), 8.27 (d, 2H, J = 7.7 Hz, 2′-H, 6′-H), 8.37 (d, 2H, J = 8.5 Hz, 2″-H, 6″-H). ^{13}C NMR, δ: 14.97 (Me), 99.68 [C(3a)], 120.72 [C(2′), C(6′)], 124.63 [C(4″)], 125.99 [C(4′)], 129.61 [C(3′), C(5′)], 130.51 [C(2″), C(6″)], 131.81 [C(3″), C(5″)], 137.62 [C(1″)], 139.51 [C(1′)], 143.42 [C(3)], 155.76 [C(7a)], 158.96 [C(4)], 161.15 [C(6)]. IR (ν/cm^{-1}): 3497, 3318 (NH_2), 1653 (C=N). Anal Calcd. for $C_{18}H_{14}BrN_5$ (%): C, 56.86; H, 3.71; N, 18.42; Br, 21.01. Found (%): C, 56.91; H, 3.77; N, 18.38; Br, 20.94.

*3-Methyl-6-(4-nitrophenyl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**6e**)* Orange powder, yield (2.66 g, 77%), decomp. 264–266 °C. ^{1}H NMR, δ: 2.68 (s, 3H, Me), 7.55–7.62 (m, 3H, 3′-H, 4′-H, 5′-H), 7.60 (t, 2H, J = 7.6 Hz, NH_2), 8.05 (d, 2H, J = 8.6 Hz 2″-H, 6″-H), 8.10–8.14 (m, 2H, 2′-H, 6′-H), 8.35 (d, 2H, J = 8.6 Hz, 3″-H, 5″-H). ^{13}C NMR, δ: 14.98 (Me), 9,9.80 [C(3a)], 120.77 [C(2′), C(6′)], 125.86 [C(4′)], 129.68 [C(3′), C(5′)], 133.69 [C(2″), C(6″)], 135.36 [C(3″), C(5″)], 139.46 [C(1′)], 140.44 [C(1″)], 143.47 [C(3)], 149.58 [C(4″)], 153.75 [C(7a)], 159.06 [C(4)], 167.43 [C(6)]. IR (ν/cm^{-1}): 3373, 3178 (NH_2), 1657 (C=N). Anal Calcd. for $C_{18}H_{14}N_6O_2$ (%): C, 62.42; H, 4.07; N, 24.27; O, 9.24. Found (%): C, 62.37; H, 4.01; N, 24.21; O, 9.32.

*6-(4-Chlorophenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**6f**)* White powder, yield (1.71 g, 51%), mp 195–196 °C. ^{1}H NMR, δ: 2.66 (s, 3H, Me), 7.33 (t, 2H, J = 7.4 Hz, NH_2), 7.56–7.62 (m, 5H, 3′-H, 4′-H, 5′-H, 3″-H, 5″-H), 8.27–8.29 (m, 2H, 2′-H, 6′-H), 8.42–8.45 (m, 2H, 2″-H, 6″-H). ^{13}C NMR, δ: 14.97 (Me), 99.64 [C(3a)], 120.71 [C(2′), C(6′)], 126.01 [C(4′)], 128.90 [C(2″), C(6″)], 129.62 [C(3′), C(5′)], 130.24 [C(3″), C(5″)], 135.68 [C(4″)], 137.24 [C(1″)], 139.51 [C(1′)], 143.42 [C(3)], 155.76 [C(7a)], 158.96 [C(4)], 161.04 [C(6)]. IR (ν/cm^{-1}): 3497, 3311 (NH_2), 1650 (C=N). Anal Calcd. for $C_{18}H_{14}ClN_5$ (%): C, 64.38; H, 4.20; N, 20.86; Cl, 10.56. Found (%): C, 64.32; H, 4.13; N, 20.90; Cl, 10.65.

*6-(2,4-Dichlorophenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**6g**)* Yellow powder, yield (2.37 g, 64%), mp 192–194 °C. ^{1}H NMR, δ: 2.68 (s, 3H, Me), 7.29 (t, 2H, J = 7.3 Hz, NH_2), 7.49–7.57 (m, 4H, 3′-H, 4′-H, 5′-H, 3″-H), 7.74-7.79 (m, 2H, 5″-H, 6″-H), 8.21 (d, 2H, J = 7.9 Hz, 2′-H, 6′-H). ^{13}C NMR, δ: 14.97 (Me), 99.39 [C(3a)], 120.70 [C(2′), C(6′)], 126.11 [C(4′)], 127.62 [C(5″)], 129.55 [C(3′), C(5′)], 129.95 [C(6″)], 133.04 [C(3″)], 133.20 [C(2″)], 134.40 [C(4″)], 137.97 [C(1″)], 139.31 [C(1′)], 143.47 [C(3)], 154.98 [C(7a)], 158.95 [C(4)], 162.08 [C(6)]. IR (ν/cm^{-1}): 3491, 3308 (NH_2), 1647 (C=N). Anal Calcd. for $C_{18}H_{13}Cl_2N_5$ (%): C, 58.39; H, 3.54; N, 18.92; Cl, 19.15. Found (%): C, 58.32; H, 3.62; N, 20.90; Cl, 18.84.

Antibacterial activity

Solution of all derivatives and antibiotics were, respectively, prepared in 10% DMSO and double-distilled water at initial concentrations of 9011 and 17.6 μg/mL. All tests were repeated three times and the results were expressed as the average of three independent experiments.

Preparation of the bacterial suspension

Gram-negative bacterial strains including *Pseudomonas aeruginosa* (PTCC 1310), *Shigella flexneri* (PTCC 1234), *Salmonella enterica subsp. enterica* (PTCC 1709), and gram-positive bacterial strains including *Streptococcus pyogenes* (PTCC 1447), *Staphylococcus aureus* (PTCC 1189), *Bacillus cereus* (PTCC 1665) and *Bacillus subtilis subsp. spizizenii* (PTCC 1023) were prepared from the Persian Type Culture Collection (PTCC), Tehran, Iran. All bacteria were cultured on nutrient agar (HiMedia, India), and then incubated for 24 h at 37 °C under normal atmospheric air. Finally, in sterile conditions, a bacterial suspension with concentration of 0.5 McFarland (1.5×10^8 CFU/ml) in Müller-Hinton broth (HiMedia, India) was obtained spectrophotometrically, which used as a storage source. Antibacterial activities were tested according to Clinical and Laboratory Standards Institute (CLSI) broth microdilution and disk diffusion methods described in guidelines M07-A9, M26-A and M02-A11 (Balouiri et al. 2016) with a slight modification.

Measurement of inhibition zone diameters

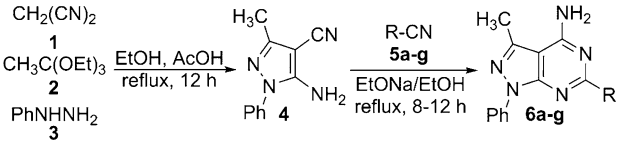
100 µL of a bacterial suspension of the storage source was spread on Müller-Hinton agar (HiMedia, India). Some sterile blank discs were placed on this culture medium at appropriate intervals. 10 µL of initial concentrations for derivatives and antibiotics were poured onto these disks, IZD values were measured by calliper after incubation at 37 °C for 24 h under mentioned conditions.

Determination of the minimum inhibitory concentration (MIC)

100 µL of Müller-Hinton broth medium was added to all the wells in each row. Then, 100 µL of initial solution of derivatives in 10% DMSO as a solvent was added to the first well. After mixing, serial twofold dilutions were continued to the eighth well. Finally, 10 µL of bacterial suspension was added to each well. The same procedure was applied to the antibiotics. As a result, the concentration range of derivatives and antibiotics were, respectively, 4096–32 and 8–0.063 µg/mL. The plates were incubated with shaking at 100 rpm at 37 °C for 24 h under similar conditions. The MICs were determined as the lowest concentration of derivatives or antibiotics which resulted in no visible bacterial growth.

Determination of the minimum bactericidal concentration (MBC)

Samples of all wells that showed no growth in the MIC test, were cultured in Müller-Hinton agar medium, which then, were incubated at 37 °C for 24 h under the conditions listed previously. The MBC was identified as the lowest concentration of derivatives or antibiotics at which no bacteria survived.



6a, R = CH_3; **6b**, R = C_6H_5; **6c**, R = 4-CH_3-C_6H_4; **6d**, R = 4-Br-C_6H_4; **6e**, R = 4-NO_2-C_6H_4; **6f**, R = 4-Cl-C_6H_4; **6g**, R = 2,4-di-Cl-C_6H_3

Scheme 1 Synthesis of pyrazolo[3,4-*d*]pyrimidines 6a–g

Results and discussion

Chemistry

In this study, a pyrazole core was used as the starting material for the preparation of pyrazolo[3,4-*d*]pyrimidines **6a–g**. 5-Amino-3-methyl-1-phenyl-1*H*-pyrazole-4-carbonitrile (**4**) was synthesized in one-pot procedure from the reaction of malononitrile (**1**), triethyl orthoacetate (**2**) and phenylhydrazine (**3**). Then, compound **4** was reacted with various nitriles **5a–g** under reflux conditions in EtONa/EtOH which afforded products **6a–g** (Scheme 1).

According to the results recorded in the experimental section, there is no significant relationship between yields and reactivity of reagents.

The reaction is carried out in the presence of sodium ethoxide as the catalyst. Probably, formation of strong hydrogen bond between the amine hydrogens on pyrazole ring and the oxygen of sodium ethoxide increased the nucleophilicity and reactivity of the amino group. Unfortunately, all our efforts to use potassium carbonate and glycerol as a basic deep eutectic media were unsuccessful (Naser et al. 2013).

Antibacterial effects

Antibacterial effects of synthesized derivatives and the antibiotics (penicillin, ampicillin, cefazolin, gentamicin, tetracycline, azithromycin and nalidixic acid) were evaluated against seven pathogenic bacteria from different genera and presented as IZD, MIC and MBC values in Table 1.

Most of the compounds have the ability to inhibit the growth of gram-positive bacterium *Streptococcus pyogenes* and gram-negative bacterium *Pseudomonas aeruginosa*. On the basis of the spread of inhibitory properties and the MIC values, derivatives were ranked in the following order **6a** > **6f** > **6c** > **6d** > **6e** > **6g** > **6b**, and based on the substituents at 6-position were arranged by

Table 1 IZD, MIC and MBC values of synthesized derivatives and antibiotics against some bacteria

Bacteria	1189[a]			1310[b]			1665[c]			1447[d]			1023[e]			1709[f]			1234[g]		
Products	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC
6a	14.43	1024	4096	14.25	512	2048	13.62	1024	4096	14.24	32	64	15.66	32	32	12.61	1024	4096	–	–	–
6b	–	–	–	12.18	512	2048	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6c	–	–	–	15.54	512	2048	–	–	–	8.41	512	1024	8.78	32	64	–	–	–	–	–	–
6d	–	–	–	15.23	512	2048	–	–	–	10.76	32	256	–	–	–	–	–	–	–	–	–
6e	–	–	–	10.44	512	2048	–	–	–	15.44	256	512	–	–	–	–	–	–	–	–	–
6f	–	–	–	11.68	1024	4096	–	–	–	16.33	512	2048	–	–	–	11.34	1024	4096	12.84	1024	4096
6g	–	–	–	17.37	1024	4096	–	–	–	12.26	512	1024	–	–	–	–	–	–	–	–	–
Penicillin	18.32	8	32	–	–	–	–	–	–	22.61	0.25	0.5	35.56	8	16	14.03	4	16	18.28	8	16
Ampicillin	17.03	8	32	–	–	–	–	–	–	30.06	4	8	34.18	8	16	26.24	8	16	30.63	8	32
Cefazolin	21.24	8	8	–	–	–	–	–	–	21.64	4	8	34.33	0.5	1	23.25	2	2	21.88	2	2
Gentamicin	22.19	1	1	25.90	0.063	0.063	25.51	0.25	4	14.19	2	2	28.57	0.063	0.063	23.84	8	8	19.46	2	8
Tetracycline	–	–	–	15.23	0.063	0.125	12.64	0.25	0.5	–	–	–	–	–	–	–	–	–	–	–	–
Azithromycin	–	–	–	15.80	0.063	0.125	18.54	1	2	14.78	4	8	–	–	–	–	–	–	–	–	–
Nalidixic acid	–	–	–	16.90	0.063	0.125	–	–	–	–	–	–	8.19	1	2	–	–	–	–	–	–

IZD (mm), MIC (μg/mL), MBC (μg/mL)

(-): Not detected noticeable antibacterial effect at the initial concentrations

Bacterial strains: [a]*Staphylococcus aureus*, [b]*Pseudomonas aeruginosa*, [c]*Bacillus cereus*, [d]*Streptococcus pyogenes*, [e]*Bacillus subtilis subsp. spizizenii*, [f]*Salmonella enterica subsp. enterica*, [g]*Shigella flexneri*

the order alkyl > monosubstituted phenyl > disubstituted phenyl > phenyl. Compound **6a** was the most broad-spectrum antibacterial agent and showed inhibitory effects against six bacterial strains. Pyrimidine ring in derivative **6a** unlike others contains an aliphatic substituent (CH_3) at 6-position. Among aryl-substituted derivatives **6b–g**, inhibitory properties of compound **6f** including 4-chlorophenyl substituent at the same position was more significant. Such effect is probably due to the presence of a chlorine as an antimicrobial agent. Unexpectedly, the presence of residual chlorine in product **6g** reduced antibacterial activities. A more remarkable reduction was also observed in 6-phenyl substituted heterocycle **6b**, which was the only effective against *Pseudomonas aeruginosa*. In this study, some common antibiotics including penicillin, ampicillin, cefazolin, gentamicin, tetracycline, azithromycin and nalidixic acid from penicillin, penicillin combination, cephalosporin, aminoglycoside, tetracycline, macrolide and quinolone classes were used as positive controls. No inhibitory activity against *Pseudomonas aeruginosa* and *Bacillus cereus* was observed from β-lactam antibiotics (the first three medicines) unlike other antibiotics. Gentamycin was effective on all bacterial strains tested. It seems that tetracycline and nalidixic acid, which blocked the growth of only two pathogens, were not proper options for the treatment of infections caused by bacteria listed above.

Conclusions

In this research project, some novel 6-substituted 4-aminopyrazolo[3,4-*d*]pyrimidine derivatives were synthesized as potential antibacterial agents in a two-step procedure from cyclocondensation of 5-amino-3-methyl-1-phenyl-1*H*-pyrazole-4-carbonitrile with different nitriles in EtONa/EtOH. Antimicrobial activities of derivatives were evaluated against a variety of gram-positive and -negative pathogenic bacteria. Finally, the inhibitory effects of derivatives against some bacteria especially *Streptococcus pyogenes* and *Pseudomonas aeruginosa* were shown. Based on the broad-spectrum antibacterial activities of derivatives specially heterocycle **6a**, the effect of substituent changes at positions 1, 3 and 6 on the antimicrobial or other biological properties of these prodrugs can be studied in future experiments.

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