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Some novel peptides containing a modified pyrazolopyrimidine moiety: design, synthesis, and in vitro antibacterial screening

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Abstract

Numerous peptide drugs are currently undergoing advanced phases of clinical testing to determine their efficacy in combating antibiotic-resistant bacterial pathogens. Our aim was to prepare some novel peptides containing a modified pyrazolopyrimidine moiety and assess their activity against a set of selected bacteria in comparison to a widely used antibiotic, ciprofloxacin. In this study, eight new peptide compounds incorporating a modified pyrazolopyrimidine moiety were synthesized. Our results revealed that compounds **4** and **5**, which contained only the pyrazolopyrimidine scaffold were less active than the peptide-conjugated pyrazolopyrimidines **10**, **11**, **13**, **14**, **15,** and **17**. The antibacterial activities of the eight novel compounds **4**, **5**, **10**, **11**, **13**, **14**, **15**, and **17** were evaluated against a panel of bacterial strains. All the novel compounds exhibited potent antibacterial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* strains compared to the reference antibiotic ciprofloxacin. The tested *Escherichia coli* strain displayed resistance against the newly synthesized compounds. Moreover, *P. aeruginosa* strain displayed resistance against ciprofloxacin and six of the newly synthesized compounds. Compounds **15** and **17** effectively inhibited the growth of the *P. aeruginosa* strain at MIC $\geq$ 1 µg/mL. Our results are encouraging and urge additional biological and pharmacological screening of the most active compounds against drug-resistant microbial strains.

Keywords Antibacterial activity, Peptides, Pyrazolpyrimidines

Introduction

According to the World Health Organization (WHO), antimicrobial resistance is "a serious threat that is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country". The resistance of *Streptococcus pneumonia* and *Escherichia coli* to third-generation cephalosporins and fluoroquinolones are only a few of many examples of antimicrobial resistance highlighted in the WHO reports. Previously, when fluoroquinolones were introduced for the first time in the 1980s, they were effective against urinary tract infections (UTIs) caused by *E. coli*, with almost no resistance. Currently, in many countries around the world, fluoroquinolones are no longer reliable for treating more than

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50% of patients. This exemplifies the growing emergence of resistance against one of the most effective antibiotics in curing UTIs [1].

As purine analogs, pyrazolopyrimidines are of substantial importance in the chemical and pharmaceutical industries [2–4]. Many pyrazolopyrimidine derivatives have been shown to possess antiviral [5], antibacterial [6], anti-inflammatory [7], and potential antineoplastic activities [8], as the same as thienopyrimidine [9]. Pyrazolo[3,4-*d*] pyrimidine heterocyclic core is continuously attracting the attention of medicinal chemists due to its remarkable pharmacological properties. These compounds were designed and synthesized as potent and selective kinase inhibitors [10], antileishmanial [11], antibacterial [12], antiviral [13], anticancer [13–17] agents, and adenosine A_{2A} receptor antagonists [8].

Since pharmacological treatment carries some secondary effects, several studies have focused on non-pharmacological treatments to improve the lives of Alzheimer's disease patients [18]. However, further studies are still required to prevent deterioration and treat Alzheimer's disease. One of those studies showed that pyrazolopyrimidines have been found effective for the treatment of neurodegenerative diseases and its crystal structure with the microtubule affinity regulating kinase 4 (MARK4) that plays an essential role in the tau-assisted regulation of microtubule dynamics [19]. Targeting the modulation of MARK4 activity is an effective strategy for the therapeutic intervention of Alzheimer's disease [20].

Peptide drugs, on the other hand, have emerged as a promising chemical class that is suitable for producing semi-synthetic medications. The therapeutic use of peptides has developed throughout time and continues to evolve as drug development and treatment paradigms change. Peptide drug development has expanded beyond its conventional emphasis on endogenous human peptides to embrace a larger spectrum of structures discovered via medicinal chemistry or other natural sources. Those medications are already being tested for their efficacy in the treatment of certain central nervous system illnesses, cancer, and inflammation, as well as for their potency as antimicrobials and enzyme inhibitors [21]. To date, over 60 peptide drugs have been authorized in the United States, Europe, and Japan, with another 150 in active clinical development and 260 in human clinical trials [22]. A great future is expected for antimicrobial peptides.

According to some reports, protein degradation by proteolytic enzymes does not generate biological waste and constitutes no hazard. This is because many proteases (>500), that cut proteins into peptide fragments, could potentially be altered under pathological conditions. Additionally, some of the cleavage products of larger proteins were proven to have specific, and sometimes unexpected, reactions against human pathogens [23].

It seems likely that humans harbor important peptide immune modulators and effectors. In human peptide libraries, a dozen of therapeutically intriguing peptides with antimicrobial and anti- or pro-viral activity have been identified [23]. Antimicrobial peptides (AMPs) kill microbial pathogens directly by disrupting the physical integrity of the microbial membrane, increasing membrane permeabilization, inducing membrane destabilization, crossing the membrane into the cytoplasm of bacteria to act on intracellular targets, and accumulating on the membrane surface causing tension in the bilayer, which eventually leads to membrane disruption and micelle formation. Additionally, AMPs display indirect antimicrobial activity, which helps the host clear bacteria by modulating the host's innate immune responses, which include chemotaxis stimulation, modulation of immune cell differentiation, adaptive immunity initiation, suppression of toll-like receptors (TLR)—and/or cytokine-mediated production of proinflammatory cytokines, and anti-endotoxin activity [24, 25].

Due to their wide range of pharmacological activity, five and six-membered heterocyclic nitrogen-containing systems such as pyrazole, imidazole, triazoles, thiazolidine, pyrazolidine, pyrimidine, and pyridine were the area of our previous research. However, in the current work, we focused on pyrimidine derivatives that are considered to be important for medicinal drugs as well. Because pyrimidine is a basic nucleus in DNA and RNA, it has been found to be associated with diverse biological activities. Moreover, we used broth dilution as a method of screening rather than the disk-diffusion method in addition to expanding the range of tested bacterial strains to screen the biological activities of the newly synthesized compounds which were designed with the hypothesis that conjugation of the Pyrazolo[3,4-*d*] pyrimidine heterocyclic core and peptides might significantly amplify the biological activities of both sides. The results were promising for compounds **13**, **14**, **15,** and **17** (Fig. 1).

We aim to proceed a future in vivo studies for screening further biological activities of the newly synthesized compounds on one of the preclinical models for human diseases in drug development like dogs [26].

Results and Discussion

Using absolute ethanol as a solvent in the presence of acetic acid (AcOH) as a catalyst, ethoxymethylene malononitrile and phenylhydrazine (**1**) were refluxed to give a 58% yield of ethyl 5-amino-1-phenyl-1*H*-pyrazole-4-carboxylate (**2**). 80% formamide ($HCONH_2$) was used to reflux the amine derivative **2** to form a 65% yield

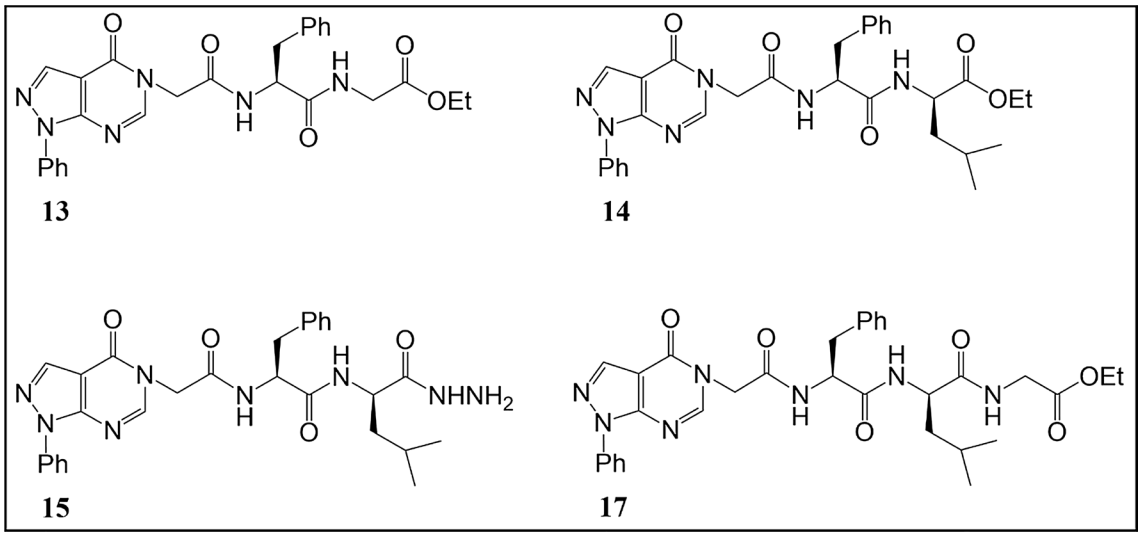

Fig. 1 Chemical structures for compounds **13**, **14**, **15,** and **17**

of 1-phenyl-1,5-dihydro-4*H*-pyrazolo[3,4-*d*] pyrimidin-4-one (**3**). Reflux of **3** with ethyl chloroacetate in dry acetone and anhydrous potassium carbonate gave an 88% yield of Ethyl 2-(4-oxo-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*] pyrimidin-5-yl) acetate (**4**). A three-hour reflux of a solution of **4** in absolute ethanol and hydrazine hydrate ($N_2H_4{\cdot}H_2O$) yielded a 95% colorless powder yield of 2-(4-oxo-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*] pyrimidin-5-yl) acetohydrazide (**5**). At − 10 °C, the treatment of the corresponding amino acid with thionyl chloride ($SOCl_2$) in absolute ethanol followed by the addition of absolute ethanol was the method to get amino acid ethyl ester hydrochlorides (**6–8**). Small and non-polar (Gly) amino acids are used in flexible linkers to allow the joining of proteins or protein domains that require a certain degree of movement or interaction [27]. The peptide linkers are able to increase stability/folding, increase expression, improve biological activity, allow targeting, and/or alter pharmacokinetic properties. As the optimum hydrophobicity is expected to show the highest antimicrobial activity [28], phenylalanine and leucine can achieve an optimal hydrophobic ratio (HR) which is the percentage of a hydrophobic amino acid (Ile, Val, Leu, Phe, Cys, Met, Ala, Trp) in the peptide chain. Hence, glycine, phenylalanine, and leucine have been chosen in the current study to elongate the peptide chain due to the role of glycine in maximizing the degree of movement and interaction, and the hydrophobicity of phenylalanine and leucine.

The elongation of the peptide chain is as the following sequence: Phe, Phe-Gly, Phe-Gly-Leu, and Phe-Gly-Leu-Gly. Moreover, the Phe-Gly-Leu-Gly sequence is a mimic of the enzyme-degradable peptide linker Gly-Phe-Leu-Gly that represents a target for most peptide-drug conjugates to incorporate with. This is because of its stability in plasma and susceptibility to cleavage by lysosomal proteases after endocytosis [29].

The treatment of the acid hydrazide **5** with acetic acid and 1*N* hydrochloric acid (HCl) followed by cooling at − 5 °C gave acetyl azide **9** after the addition of sodium nitrite ($NaNO_2$). In ethyl acetate ($CH_3COOC_2H_5$) containing triethylamine (Et_3N) at 0 °C, a treatment of **9** with *L*-phenylalanine ethyl ester hydrochloride (**7**) was performed to give a product that has been purified by recrystallization from ethanol to give an 80% yield of Ethyl (2-(4-oxo-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*] pyrimidin-5-yl) acetyl)-*L*-phenylalaninate **(10).**

Three hours of heating under reflux in ethanol for a mixture of **10** and $N_2H_4{\cdot}H_2O$ afforded a 90% yield of (*S*)-*N*-(1-hydrazineyl-1-oxo-3-phenylpropan-2-yl)-2-(4-oxo-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*] pyrimidin-5-yl) acetamide **(11).**

The treatment of **11** with AcOH and 1*N* HCl followed by cooling at − 5 °C gave acid azide **12** after the addition of $NaNO_2$. In $CH_3COOC_2H_5$ containing Et_3N at 0 °C, a treatment of **12** with glycine ethyl ester hydrochloride (**6**) was performed to give a product that has been purified by recrystallization from ethanol to give a 75% yield of Ethyl (2-(4-oxo-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*] pyrimidin-5-yl) acetyl)-*L*-phenylalanylglycinate **(13)**.

The treatment of acid azide **12** with *L*-leucine ethyl ester hydrochloride (**8**) in $CH_3COOC_2H_5$ containing Et_3N at 0 °C gave a 77% yield of ethyl (2-(4-oxo-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*] pyrimidin-5-yl) acetyl)-*L*-phenylalanyl-*D*-leucinate (**14**) after purification by recrystallization from ethanol.

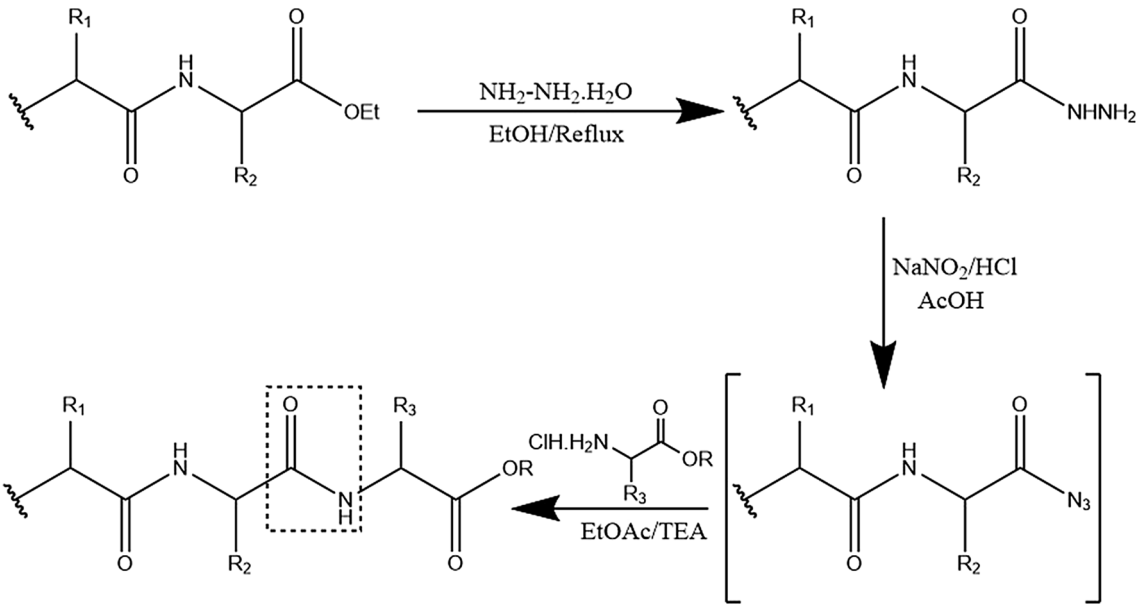

Fig. 2 Installation mechanism of new amino acid ethyl hydrochloride on pyrazolopyrimidine moiety

Three hours of heating under reflux for a mixture of the ester **14** and $N_2H_4{\cdot}H_2O$ in ethanol afforded an 88% yield of (*S*)-*N*-((*R*)-1-hydrazineyl-4-methyl-1-oxopentan-2-yl)-2-(2-(4-oxo-1-phenyl-1,4-dihydro-5*H*-pyrazolo[3,4-*d*] pyrimidin-5-yl) acetamido)-3-phenylpropanamide **(15)**.

The treatment of the acid hydrazide **15** with AcOH and 1*N* HCl followed by cooling at − 5 °C gave acid azide **16** after the addition of $NaNO_2$. In $CH_3COOC_2H_5$ containing Et_3N at 0 °C, a treatment of **16** with glycine ethyl ester hydrochloride (**6**) was performed to give a product that has been purified by recrystallization from ethanol to give an 80% yield of ethyl 2-{(*R*)-4-methyl-2-[(*S*)-2-(2-(4-oxo-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-5(4*H*)-yl) acetamido)-3-phenylpropanamido] pentanamido] acetate (**17**).

The mechanism of installing amino acid ethyl ester hydrochlorides on pyrazolopyrimidine moiety is illustrated in Fig. 2.

The structures of **4, 5, 10, 11, 13, 14, 15,** and **17** were elucidated by infrared spectroscopy (IR spectroscopy), fast atom bombardment mass spectrometry (FAB-MS) in addition to elemental analysis. We depended on FAB-MS as a technique for the analysis of protein sequence and structure. FAB-MS technique gives details of the peptide sequence and post-translational modifications such as N-terminal acylation, glycosylation, phosphorylation, and disulfide bridging. Additionally, a combination of interpreting the functional group region (3600–1200 cm^{-1}) provided by IR and comparing the fingerprint region (1200–600 cm^{-1}) with those in spectral libraries provides, in many cases, sufficient evidence to positively identify a compound side by side with composition elemental elucidation by elemental analysis [30]. All reactions are illustrated in Fig. 3. Physical properties, structural data, and elemental analysis of the newly synthesized derivatives are shown in Table 1. Screening for antibacterial activities of the newly synthesized derivatives has been performed against two gram-positive and two gram-negative bacterial strains as demonstrated in Table 2.

The Minimum Inhibitory Concentrations (MICs) values of the screened compounds are shown in Table 2. According to the Clinical Laboratory Standard Institute's (CLSI) most recent standards for MIC breakpoints [31], all newly synthesized compounds tested in this study displayed potent antibacterial effects against *Staphylococcus aureus* ATCC 29213, *E. faecalis* ATCC 29212, and *E. coli* ATCC 25922 with MIC < 1 µg/mL. In contrast, the *Pseudomonas aeruginosa* ATCC 27853 was found resistant to ciprofloxacin (MIC = 3 µg/mL), which is beyond the CLSI resistance breakpoint of fluoroquinolones (MIC ≥ 2 µg/mL). Fluoroquinolones-resistant *P. aeruginosa* was frequently associated with UTIs worldwide. It was discovered that 36.4% of UTIs associated *P. aeruginosa* were resistant to fluoroquinolones [32]. Similar findings were previously reported in another study [33]. Interestingly in this study, the new compounds **17** and **15** were found effective against the ciprofloxacin-resistant *P. aeruginosa* strain with MICs ≤ 1 µg/mL.

Moreover, lower concentrations of compounds **17, 13**, **15,** and **14** than those of ciprofloxacin were found inhibitory to the panel of tested bacteria except *E. coli*

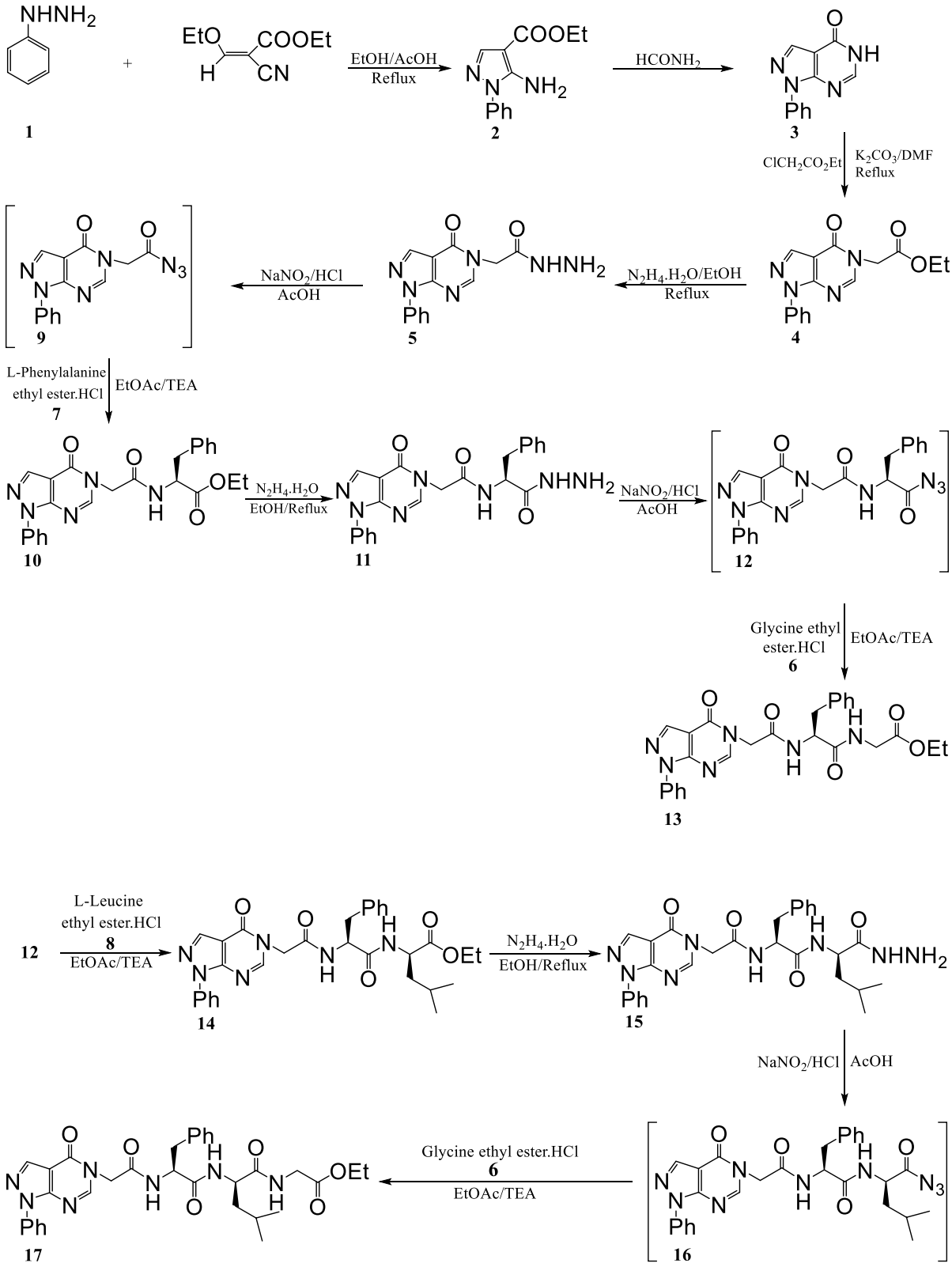

Fig. 3 Synthesis of new peptides incorporated with pyrazolopyrimidine moiety

Table 1 Physical properties, structural data, and elemental analysis for the synthesized compounds

Compound no	mp (°C)	yield (%)	Molecular formula	MW (g/mol)	Elemental analysis: Found (Calculated)		
					C	H	N
4	270–272	88	$C_{15}H_{14}N_4O_3$	298.3	60.40 (60.22)	4.73 (4.55)	18.78 (18.66)
5	> 300	95	$C_{13}H_{12}N_6O_2$	284.28	54.93 (54.76)	4.25 (4.13)	29.56 (29.44)
10	190–192	80	$C_{24}H_{23}N_5O_4$	445.48	64.71 (64.61)	5.20 (5.07)	15.72 (15.65)
11	179–181	90	$C_{22}H_{21}N_7O_3$	431.46	61.24 (61.12)	4.91 (4.83)	22.73 (22.66)
13	170–172	75	$C_{26}H_{26}N_6O_5$	502.53	62.14 (62.03)	5.21 (5.13)	16.72 (16.61)
14	190–192	77	$C_{30}H_{34}N_6O_5$	558.64	64.5 (64.37)	6.13 (6.03)	15.04 (14.91)
15	> 300	88	$C_{28}H_{32}N_8O_4$	544.62	61.75 (61.59)	5.92 (5.81)	20.58 (20.41)
17	255–257	80	$C_{32}H_{37}N_7O_6$	615.69	62.43 (62.33)	6.06 (5.90)	15.92 (15.8)

Mp: melting point, MW: molecular weight, C: Carbon, H: Hydrogen, N: Nitrogen

Table 2 MIC (µg/mL) of the newly synthetized compounds against different bacterial species

Compound	MIC (µg/mL)[a]			
	Gram-positive bacteria		Gram-negative bacteria	
	Staphylococcus aureus **ATCC 29213**	***Enterococcus faecalis*** **ATCC 29212**	***Escherichia coli*** **ATCC 25922**	***Pseudomonas aeruginosa*** **ATCC 27853**
Ciprofloxacin	0.2	0.4	0.02	3
4	0.3	0.6	0.6	4
5	0.25	0.6	0.8	3.5
10	0.08	0.2	0.16	2
11	0.05	0.31	0.25	2
13	0.02	0.01	0.13	2
14	0.03	0.01	0.13	2.5
15	0.02	0.1	0.1	1
17	0.01	0.08	0.11	0.9

[a] MIC, minimum inhibitory concentration

(Table 2). The Peptides-Pyrazolopyrimidine compounds were found more effective than one of the most effective traditional antibiotics. The detailed MICs of the synthesized compounds against different bacterial species are shown in Table 2.

In an adoption of the idea of synergism, a conjugation of the pyrazolo[3,4-*d*] pyrimidine heterocyclic core and peptides shows an increase in the antibacterial activities of the newly synthesized peptides with an elongated sequence of amino acids ethyl ester HCl like compounds **13**, **14**, and **17**. Also, the hydrophobicity of glycine provided an amplified antibacterial activity against *S. aureus, E. faecalis, E. coli,* and *P. aeruginosa*. This was obvious for compound **10**.

By comparing the antibacterial activities of the newly synthesized compounds with ciprofloxacin, the results have shown that compounds **17**, **13, 15,** and **14** have the highest activity against *S. aureus* followed by compounds **11** and **10**. Compounds **13, 14, 17,** and **15** are the most active against *E. faecalis* followed by compounds **10** and **11**. Compounds **17** and **15** have a potent inhibitory effect against *P. aeruginosa* followed by compounds **10**, **11**, **13,** and **14**. In contrast, the least antimicrobial activity of tested compounds was encountered against *E. coli* strain ATCC 25922.

Hydrazides have been shown to have antimicrobial properties [34], which can be explained by the ability of hydrazides same as hydrazines to induce DNA alterations [35]. DNA modification has the potential to inhibit bacterial growth by interfering with bacterial replication and protein synthesis [36]. Furthermore, the negative charges of fused peptides aid in electrostatic binding with bacterial cell walls. The hydrophobic group of the peptide aids in bacterial cell penetration, resulting in membrane disruption and bacterial cell death [25]. Furthermore, recent research showed enhanced antibacterial activity of antimicrobial compounds when conjugated to a delivery system composed of cell-adhesive peptides and gold

nanoparticles [37]. Hence, these novel compounds could be a potential alternative to overcome the evolving antimicrobial resistance of UTIs-causing bacteria. The main limitation of the current study was the time-consuming mechanisms as a result of utilizing routine organic solvents, in addition to relatively lower yields of pure products in comparison to green synthesis with ionic liquids. Using green nano-catalyst, such as cellulose-based nanobiocomposite, in the synthesis of pyrimidine derivatives can be more sustainable, as it can reduce the energy and time of reactions besides increasing the yield of product compounds [38]. However, the main point of strength is that we were able to elucidate all the produced compounds using different spectroscopic tools.

In conclusion, in the current study, we generated some novel peptide compounds incorporated with pyrazolopyrimidine moiety by conjugating pyrazolo[3,4-*d*] pyrimidine heterocyclic core and some known amino acids ethyl ester hydrochlorides. The addition of hydrazine hydrate results in the formation of hydrazides **5**, **11**, and **15**. Moreover, with the addition of Phe, Phe-Gly, Phe-Gly-Leu, and Phe-Gly-Leu-Gly, novel peptides **10**, **13**, **14**, and **17** were formed. Our findings revealed that compound **17** has the highest antibacterial potency, followed by compounds **13, 15,** and **14** against most of the microorganisms employed as reference cultures. In addition, our results demonstrated antibacterial activities for compounds **10** and **11** against most of the used reference cultures of bacteria. Presenting novel, structurally elucidated, and biologically active compounds like the current eight compounds is a significant contribution to human knowledge. So, future work is needed to synthesize more elongated peptide chain compounds using *L*-amino acids ethyl ester hydrochlorides **6**, **7, 8,** and other amino acids derivatives employing nano-catalyst in a green synthesis, as well as assessing their antimicrobial activities.

Materials and methods

Synthetic methods, analytical, and spectral data

The melting points of the newly synthesized derivatives, which were uncorrected, were measured by a Büchi melting point apparatus. A Bruker-Vector22 spectrometer (Bruker, Bremen, Germany) recorded the IR spectra (KBr). ^{1}H NMR was recorded by a Varian Gemini spectrometer (300 MHz, DMSO-d_6) with tetramethylsilane (TMS) as an internal reference. The coupling constants (*J* values) are given in Hertz (Hz) and the chemical shifts are indicated in δ scale (ppm) relative to tetramethylsilane (TMS) which was used as a reference. Analytical thin-layer chromatography (TLC) was performed to monitor the progress of the reactions using aluminum silica gel 60 F_{245} plates (Merck, Darmstadt, Germany) [9].

Experimental

Ethyl 5-amino-1-phenyl-1H-*pyrazole-4-carboxylate (2)*

A 0.01 mol of phenylhydrazine (**1**) and 3–5 drops of AcOH were added to a solution of 0.01 mol of ethoxymethylene malononitrile in 50 mL ethanol. A five-hour reflux for the mixture was monitored by TLC (silica gel) with CH_2C1_2/CH_3OH (9:1) showing the disappearance of phenylhydrazine. Cooling to room temperature was allowed for the reaction mixture followed by collecting the precipitate by filtration, drying, and recrystallization from ethanol (Fig. 4A).

1-phenyl-1,5-dihydro-*4H*-pyrazolo[3,4-*d*]pyrimidin-4-one (3)

A 0.01 mol of ethyl 5-amino-1-phenyl-1*H*-pyrazole-4-carboxylate (**2**) was added to 10 mL $HCONH_2$ 80%. A 10 h reflux of the reaction mixture followed by pouring onto cold water led to the collection of the solid product **3** by filtration, several times washing with water, drying, and recrystallization from dimethylformamide (DMF) (Fig. 4B).

Ethyl 2-(4-oxo-1-phenyl-1,4-dihydro-*5H*-pyrazolo[3,4-*d*] pyrimidin-5-yl)acetate (4)

A 0.01 mol (1.4 g) of ethyl chloroacetate was added to a solution of 0.01 mol (2.12 g) of **3** in 30 mL dry Dimethylformamide and 0.01 mol (1.38 g) of anhydrous potassium carbonate. A six-hour heating under reflux for the reaction mixture followed by pouring on crushed ice, filtration, and recrystallization from ethanol produced

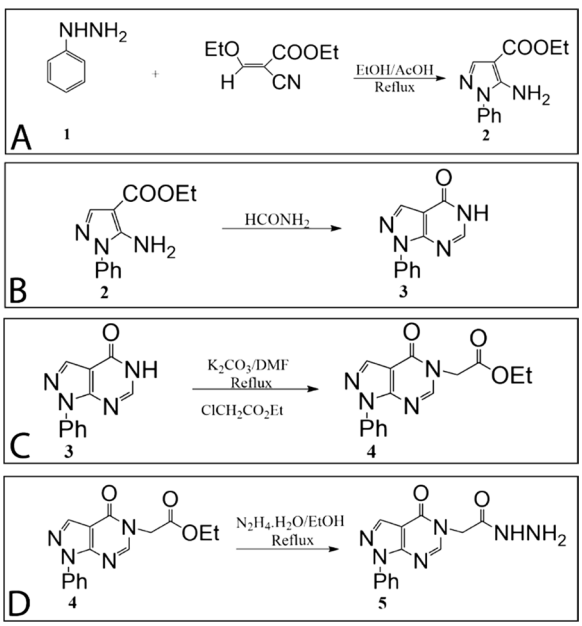

Fig. 4 Synthesis of the compounds **2**, **3**, **4,** and **5**

an 88% yield of the corresponding ester **4**, m.p. 270–272 °C (Fig. 4C).

I.R, FAB-MS in addition to elemental analysis were performed to elucidate the structure of **4**.

(i) IR spectrum showed absorption band at = 1753, (C=O), 1669 cm-1. (C=O), 1596 cm-1 (C=N).
(ii) FAB-MS (positive mode, NBOH-NaI-matrix): *m/z* = 299 [MH^+]. 321 [MNa^+].
(iii) Anal. Calcd. For $C_{15}H_{14}N_4O_3$ (298.3); C, 60.40; H, 4.73; N. 18.78. Found: C, 60.22; H, 4.55; N, 18.66.

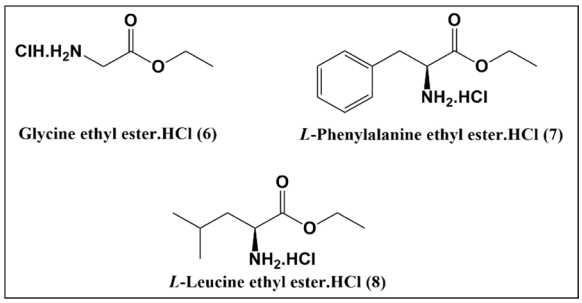

Fig. 5 Structures of the compounds **6**, **7**, and **8**

2-(4-oxo-1-phenyl-1,4-dihydro-*5H*-pyrazolo[3,4-*d*]pyrimidin-5-yl)acetohydrazide (5)

A three-hour reflux in 30 mL absolute ethanol for 0.01 mol (2.98 g) solution of the respective ester **4** and 0.03 mol (1.5 g) hydrazine hydrate followed by removing the solvent under reduced pressure, collecting the remaining precipitate, drying, and recrystallization from ethanol afforded a 95% yield of a pale-yellow powder of the acid hydrazide **5**, m.p. > 300 °C (Fig. 4D).

I.R, ^{1}H-NMR, and FAB-MS in addition to elemental analysis were performed to elucidate the structure of the acid hydrazide **5**.

(i) IR spectrum showed absorption band at = 3325 cm^{-1} (NH_2, NH), 1674 cm^{-1} (2xC=O), 1615 cm^{-1} (C=N).
(ii) ^{1}H-NMR (300 MHz, DMSO-*d6*): δ 3.51 (s, 2H, CH_2), 7.21 (s, 1H, Ar–H), 7.61 (s, 1H, Ar–H), 7.93 (m, 2H, Ar–H), 8.20–8.40 (m, 2H, Ar–H), 8.77 (m, 1H, Ar–H), 12.92 (brs, 3H, NH_2, NH) ppm.
(iii) FAB-MS (positive mode, NBOH-NaI-matrix): *m/z* = 285 [MH^+], 307 [MNa^+]. (iv) Anal. Calcd. For $C_{13}H_{12}N_6O_2$ (284.28); C, 54.93; H, 4.25; N, 29.56. Found: C, 54.76; H, 4.13; N, 29.44.

Amino acids ethyl ester hydrochloride (6–8)

A 0.01 mol of an amino acid (glycine, *L*-phenylalanine, and/or *L*-leucine) was added to 100 mL of absolute ethanol and the mixture was cooled to −10 °C, then 0.11 mol (7.9 mL) pure $SOCl_2$ was added in drops. By maintaining the reaction mixture at −5 °C temperature during the addition process and continue stirring for another 3 h, the material was completely soluble. The obtained mixture was kept at room temperature for 24 h. The solvent was extracted *in vacuo* and a fraction of absolute ethanol was added and reevaporated to obtain the corresponding amino acid ethyl ester hydrochlorides (Fig. 5).

Ethyl (2-(4-oxo-1-phenyl-1,4-dihydro-*5H*-pyrazolo[3,4-*d*]pyrimidin-5-yl)acetyl)-*L*-phenylalaninate (10)

A 0.80 mol of solution of **5** in 6 mL AcOH, 3 mL 1*N* HCl, and 25 mL H_2O were cooled in an ice bath at −5 °C. A 12.60 mol of $NaNO_2$ (0.87 g) was added with stirring in 3 mL cold H_2O. The yellow syrup of 2-(4-oxo-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidin-5(4*H*)-yl) acetyl azide (**9**) was obtained after 15 min of continuous stirring at −5 °C. Taking the azide in 30 mL cold ethyl acetate, washing with 30 mL $NaHCO_3$ (3%), 30 mL H_2O, and dried Na_2SO_4 preceded 20 min stirring at 0 °C and filtration of a 0.90 mol solution of *L*-phenylalanine ethyl ester hydrochloride (**7**) in 20 mL ethyl acetate containing 0.2 mL of Et_3N. The filtrate was added to the azide solution. Before washing, the mixture was settled at −5 °C for 12 h and kept for extra 12 h at room temperature. Subsequently, the mixture was washed with 30 mL of 0.5*N* HCl, 30 mL of $NaHCO_3$ (3%), 30 mL of H_2O, and finally dried Na_2SO_4. Evaporation of the filtrate under reduced pressure and purification of the residue by recrystallization from ethanol afforded an 80% yield of a dark brown powder of the corresponding peptide **10**, m.p. 190–192 °C (Fig. 6A).

I.R and FAB-MS in addition to elemental analysis were performed to elucidate the structure of **10**.

(i) IR spectrum showed absorption band at = 3429 (NH), 1750 (C=O), 1639 (C=O).
(ii) FAB-MS (positive mode, NBOH-NaI-matrix): *m/z* = 246 [MH^+], 268 [MNa^+].
(iii) Anal. Calcd. For $C_{24}H_{23}N_5O_4$ (445.48); C, 64.71; H, 5.20; N, 15.72. Found: C, 64.61; H, 5.07; N, 15.65.

(S)-N-(1-hydrazineyl-1-oxo-3-phenylpropan-2-yl)-2-(4-oxo-1-phenyl-1,4-dihydro-*5H*-pyrazolo[3,4-*d*]pyrimidin-5-yl)acetamide (11)

A three-hour reflux for a solution of 0.01 mol of **10** in 30 mL absolute ethanol and 0.03 mol (1.5 mL) N_2H_4. H_2O was followed by collecting the precipitate under

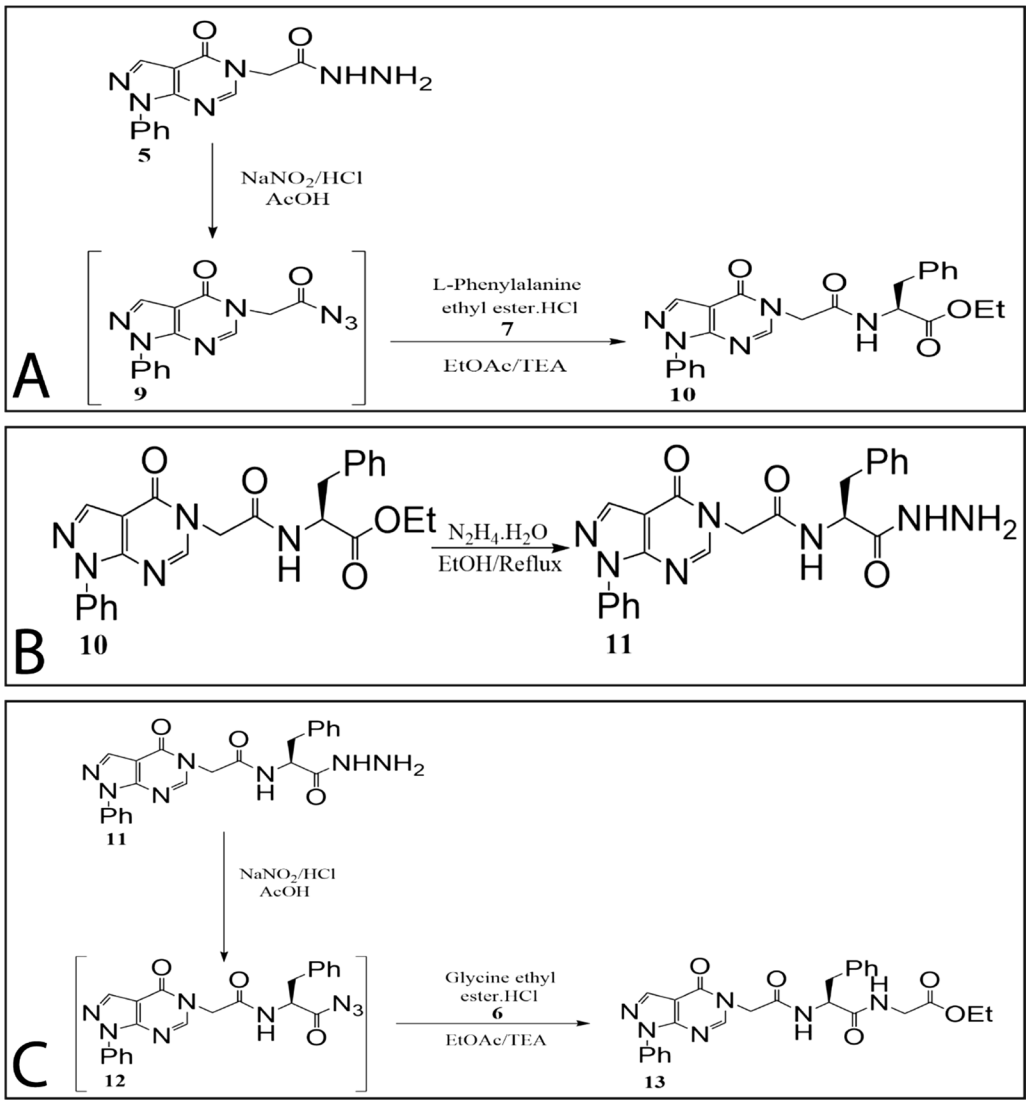

Fig. 6 Synthesis of compounds **10**, **11,** and **13**

reduced pressure by removing the solvent, drying, and recrystallization from ethanol gave a 90% yield of a black powder of the acid hydrazide **11**, m.p. 179–181 °C [39] (Fig. 6B).

I.R and FAB-MS in addition to elemental analysis were performed to elucidate the structure of **11.**

(i) IR spectrum showed absorption band at = 3417 (NH_2, NH), 1635 (C=O).

(ii) FAB-MS (positive mode, NBOH-NaI-matrix): $m/z = 432$ [MH^+], 454 [MNa^+].

(iii) Anal. Calcd. For $C_{22}H_{21}N_7O_3$ (431.46); C, 61.24; H, 4.91; N, 22.73. Found: C, 61.12; H, 4.83; N, 22.66.

Ethyl (2-(4-oxo-1-phenyl-1,4-dihydro-*5H*-pyrazolo[3,4-*d*] pyrimidin-5-yl)acetyl)-*L* phenylalanylglycinate (13)

A 0.80 mol of solution of **11** in 6 mL AcOH, 3 mL 1*N* HCl, and 25 mL H_2O were cooled in an ice bath at -5 °C. A 12.60 mol of $NaNO_2$ (0.87 g) was added with stirring in 3 mL cold H_2O. The yellow syrup of (*S*)-2-(2-(4-oxo-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidin-5(4*H*)-yl) acetamido)-3-phenylpropanoyl azide (**12**) was obtained after 15 min of continuous stirring at −5 °C. Taking the azide in 30 mL cold ethyl acetate, washing with 30 mL $NaHCO_3$ (3%), 30 mL H_2O, and dried Na_2SO_4 preceded 20 min stirring at 0 °C and filtration of a 0.90 mol solution of Glycine ethyl

ester hydrochloride (**6**) in 20 mL ethyl acetate containing 0.2 mL of Et_3N. The filtrate was added to the azide solution. Before washing, the mixture was settled at −5 °C for 12 h and kept for extra 12 h at room temperature. Subsequently, the mixture was washed with 30 mL of 0.5*N* HCl, 30 mL of $NaHCO_3$ (3%), 30 mL of H_2O, and finally dried Na_2SO_4. Evaporation of the filtrate under reduced pressure and purification of the residue by recrystallization from ethanol afforded a 75% yield of a dark brown foam of the corresponding peptide **13**, m.p. 170–172 °C (Fig. 6C).

I.R and FAB-MS in addition to elemental analysis were performed to elucidate the structure of **13**.

(i) IR spectrum showed absorption band at = 3433 (2xNH), 1739, (C=O), 1668 (C=O).
(ii) FAB-MS (positive mode, NBOH-NaI-matrix): *m/z* = 503 [MH^+], 525 [MNa^+].
(iii) Anal. Calcd. For $C_{26}H_{26}N_6O_5$ (502.53); C, 62.14; H, 5.21; N, 16.72. Found: C, 62.03; H, 5.13; N, 16.61.

Ethyl (2-(4-oxo-1-phenyl-1,4-dihydro-*5H*-pyrazolo[3,4-*d*] pyrimidin-5-yl)acetyl)-*L*-phenylalanyl-*D*-leucinate (14)

The azide **12** was taken in 30 mL cold ethyl acetate, washed with 30 mL $NaHCO_3$, 30 mL H_2O, and dried Na_2SO_4. After filtration of a 0.90 mol solution of *L*-leucine ester hydrochloride (**8**) in 20 mL ethyl acetate containing 0.2 mL of Et_3N, the filtrate was added to the azide solution. Before washing, the mixture was settled at −5 °C for 12 h and kept for extra 12 h at room temperature. Subsequently, the mixture was washed with 30 mL of 0.5*N* HCl, 30 mL of $NaHCO_3$ (3%), 30 mL of H_2O, and finally dried Na_2SO_4. Evaporation of the filtrate under reduced pressure and purification of the residue by recrystallization from ethanol afforded a 77% yield of a white powder of the corresponding peptide **14**, m.p. 190–192 °C (Fig. 7A).

I.R and FAB-MS in addition to elemental analysis were performed to elucidate the structure of **14.**

(i) IR spectrum showed absorption band at = 3434 (2xNH), 1740, (C=O), 1669 (C=O).
(ii) FAB-MS (positive mode, NBOH-NaI-matrix): *m/z* = 559 [MH^+], 581 [MNa^+].
(iii) Anal. Calcd. For $C_{30}H_{34}N_6O_5$ (558.64); C, 64.50; H, 6.13; N, 15.04. Found: C, 64.37; H, 6.03; N, 14.91.

(S)-N-((R)-1-hydrazineyl-4-methyl-1-oxo-pentan-2-yl)-2-(2-(4-oxo-1-phenyl-1,4-dihydro-*5H*-pyrazolo[3,4-*d*]pyrimidin-5-yl) acetamido)-3-phenylpropanamide (15)

A 3-h reflux for a solution of 0.01 mol of **14** in 30 mL absolute ethanol and 0.03 mol (1.5 mL) $N_2H_4{\cdot}H_2O$ was followed by collecting the precipitate under reduced pressure by removing the solvent, drying, and recrystallization from ethanol gave an 88% yield of a pale brown powder of the acid hydrazide **15**, m.p. > 300 °C (Fig. 7B).

I.R and FAB-MS in addition to elemental analysis were performed to elucidate the structure of **15**.

(i) IR spectrum showed absorption band at = 3339–3294 (NH_2, 3xNH), 1660 (C=O).
(ii) FAB-MS (positive mode, NBOH-NaI-matrix): *m/z* = 545 [MH^+], 567 [MNa^+].
(iii) Anal. Calcd. For $C_{28}H_{32}N_8O_4$ (544.62); C, 61.75; H, 5.92; N, 20.58. Found: C, 61.59; H, 5.81; N, 20.41.

Ethyl (2-(4-oxo-1-phenyl-1,4-dihydro-*5H*-pyrazolo[3,4-*d*] pyrimidin-5-yl)acetyl)-*L*-phenylalanyl-*D*-leucylglycinate (17)

A 0.80 mol of solution of **15** in 6 mL AcOH, 3 mL 1*N* HCl, and 25 mL H_2O were cooled in an ice bath at −5 °C. A 12.60 mol of $NaNO_2$ (0.87 g) was added with stirring in 3 mL cold H_2O. The yellow syrup of (*R*)-4-methyl-2-{(*S*)-2-[2-(4-oxo-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidin-5(4*H*)-yl) acetamido]-3-phenyl- propanamido] pentanoyl azide **(16)** was obtained after 15 min of continuous stirring at −5 °C. Taking the azide in 30 mL cold ethyl acetate, washing with 30 mL $NaHCO_3$ (3%), 30 mL H_2O, and dried Na_2SO_4 preceded 20 min stirring at 0 °C and filtration of a 0.90 mol solution of glycine ester hydrochloride (**6**) in 20 mL ethyl acetate containing 0.2 mL of Et_3N. The filtrate was added to the azide solution. Before washing, the mixture was settled at −5 °C for 12 h and kept for extra 12 h at room temperature. Subsequently, the mixture was washed with 30 mL of 0.5*N* HCl, 30 mL of $NaHCO_3$ (3%), 30 mL of H_2O, and finally dried Na_2SO_4. Evaporation of the filtrate under reduced pressure and purification of the residue by recrystallization from ethanol afforded an 80% yield of a white powder of the corresponding peptide **17**, m.p. 255–257 °C (Fig. 7C).

I.R, ^{1}H-NMR, and FAB-MS in addition to elemental analysis were performed to elucidate the structure of **17**.

(i) IR spectrum showed absorption band at = 3433 (3xNH), 1748, (C=O), 1643 (C=O).
(ii) ^{1}H-NMR (300 MHz, DMSO-*d6*): δ 0.90 (d, 6H, *J* = 4.5 Hz, 2xCH_3), 1.30 (t, 3H, *J* = 5.0 Hz, CH_3), 1.51 (m, 1H, CH), 1.82 (m,2H, CH_2), 3.50 (m, 2H, CH_2), 4.22 (m, 4H, 2xCH_2), 4.55 (m, 2H,CH_2), 4.99 (m, 3H, CH, CH_2), 7.40–7.52 (m, 2H, Ar–H), 7.60–7.80 (m, 3H, Ar–H), 7.90–8.30 (m, 5H, Ar–H, 3xNH) ppm.
(iii) FAB-MS (positive mode, NBOH-NaI-matrix): *m/z* = 616, [MH^+], 638 [MNa^+].

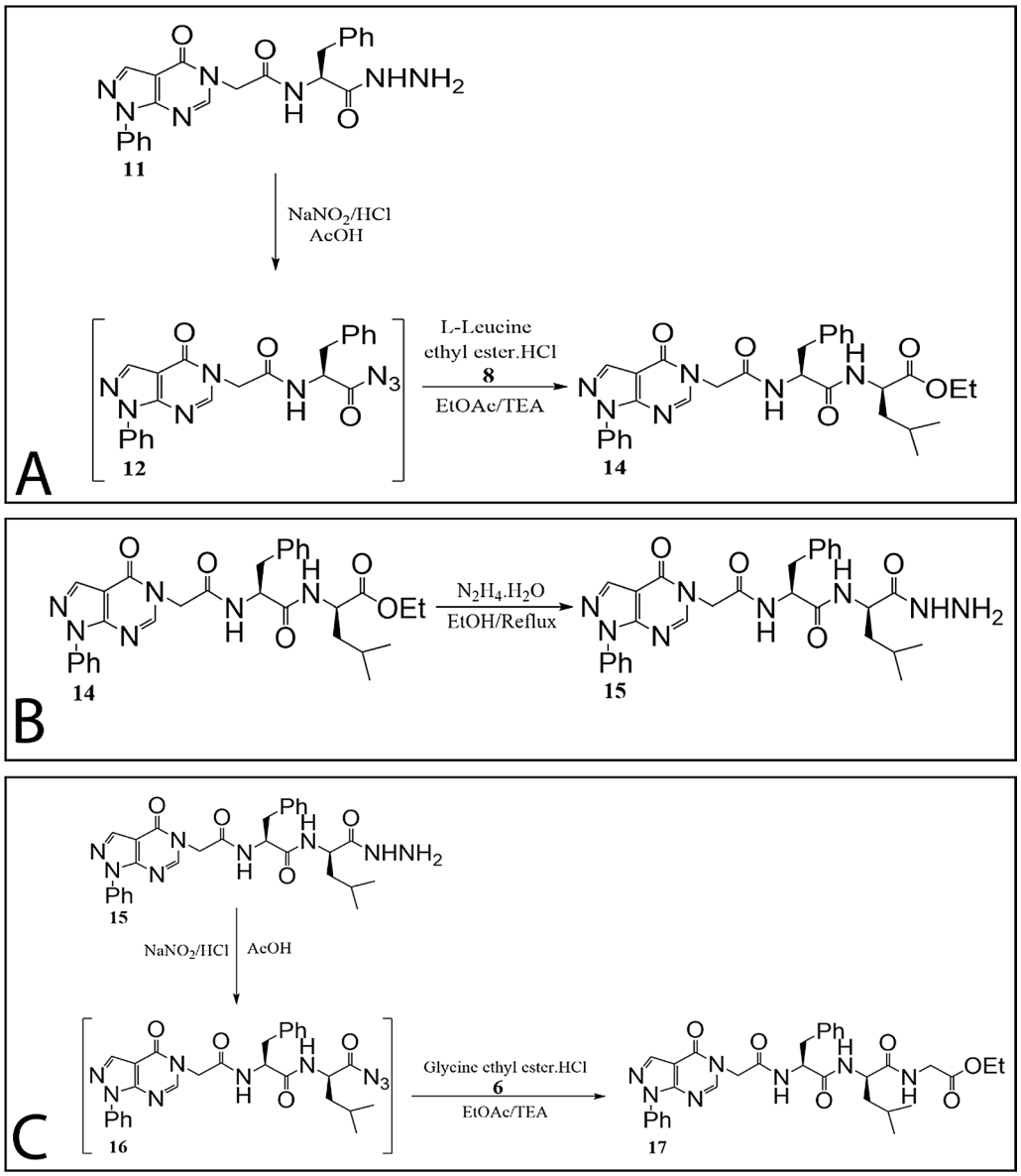

Fig. 7 Synthesis of compounds **14**, **15,** and **17**

Antimicrobial screening

Microorganisms' culture

The newly synthesized derivatives were assessed for their antimicrobial activity using the broth microdilution method against *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922 bacterial strains. The broth microdilution method was performed as previously elucidated [1]. Strains were incubated for 18–24 h. at 37 °C in Mueller–Hinton Broth (MHB) (Oxoid Chemical Co., UK). The culture was diluted using sterile MHB to a turbidity equivalent to 0.5 MacFarland turbidity standard (~ 10^8 CFU/mL. The bacterial suspension was further diluted to a concentration of 10^6 CFU/mL.

Preparation of antimicrobial solution

Four stock solutions were prepared from each of the newly synthesized compounds by dissolving in 10% DMSO/water solution to concentrations of 14, 16, 20, and 25 µg/mL. The stock solutions were transferred to microtiter plates and serial twofold dilution was carried out using 10% DMSO solution to make 10 subsequent dilutions (50 µL) from each stock solution. Each well-received 50 µL of bacterial inoculum (final inoculum

concentration approx. 5×10^5 CFU/mL). Ciprofloxacin was used as a control antibiotic. Growth and sterility control wells were prepared for each plate. The plates were incubated for 24 h at 37 °C. Bacterial inhibition was reported in wells that show no turbidity compared to the growth control. The Minimum Inhibitory Concentration (MIC) of each compound was recorded in the least concentration well that showed bacterial inhibition.

Abbreviations

WHO	World Health Organization
UTIs	Urinary tract infections
MARK4	Microtubule affinity regulating kinase 4
AMPs	Antimicrobial peptides
TLR	Toll-like receptors
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
HR	Hydrophobic ratio
Gly	Glycine
Ile	Isoleucine
Val	Valine
Leu	Leucine
Phe	Phenylalanine
Cys	Cysteine
Met	Methionine
Ala	Alanine
Trp	Tryptophan
IR	Infrared spectroscopy
FAB-MS	Fast atom bombardment mass spectroscopy
AcOH	Acetic acid
$HCONH_2$	Formamide
$N_2H_4{\cdot}H_2O$	Hydrazine hydrate
$SOCl_2$	Thionyl chloride
HCl	Hydrochloric acid
$CH_3COOC_2H_5$	Ethyl acetate
Et_3N	Triethylamine
Mp	Melting point
MW	Molecular weight
C	Carbon
H	Hydrogen
N	Nitrogen
CLSI	Clinical Laboratory Standard Institute's
MIC	Minimum inhibitory concentration
TMS	Tetramethylsilane
Hz	Hertz
TLC	Thin-layer chromatography
DMF	Dimethylformamide
MHB	Mueller–Hinton Broth

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Author contributions
Mohamed Ge. Z. and Adel A.-H.A. designed and performed both the experiments and the study. Mahmoud Ge. Z. and Abdul-Raouf A. performed antimicrobial screening. Einas Y. and Amina M. prepared and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and analysed during the current study are available in the Biological Magnetic Resonance Bank (BMRB) repository, https://bmrbig.org/released/bmrbig43

Declarations

Ethics approval and consent to participate
All procedures performed in this study don't involve any human subjects, human data or tissue, or animals.

Competing interests
The authors declare that they have NO affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

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