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SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF NEW QUATERNARY AMMONIUM SALTS BEARING 1,2,4-TRIAZOLE MOIETIES DERIVED FROM THREE NATURAL AMINO ACIDS

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Abstract

Secondary quaternary ammonium salts (QAS) derived from three natural amino acids (L-Leucine, L-Phenylalanine and L-Methionine) bearing 1,2,4-triazole and acetic acid moieties were synthesized and characterized by IR, ^{1}H and ^{13}C NMR. The antimicrobial properties of the synthesized compounds were screened for their preliminary in vitro antibacterial and antifungal activity against a panel of standard strains of pathogenic microorganisms including three gram-positive bacteria, three gram-negative bacteria, and two registered yeast species. The results obtained from the antimicrobial activity showed that all the synthesized compounds displayed varying degrees of inhibition against the tested microorganisms. It was concluded that the compounds 6c possess good activity against gram-positive bacteria compound 6b showed moderate activity, compounds 6(a-c) displayed remarkable and significant anti-yeast activities.

INTRODUCTION

It is well-known in the literature that, Quaternary ammoniums salts (QAS), shows a wide variety of activities [1-4] such as antibacterial [5], antifungal [6], antimalarial [7], anti-virus [8], Biocides [9], antibiotic [10], anticancer [11], and antibody-drug [12]. Amino acids are necessary for human and animal species [13] and many essential biological processes [14]. They are classified into essential and non-essential amino acids based on their synthesis in humans [15], they can also participate as bioactive molecules in nutrition metabolism [16]. Since amino acids are widely used as raw materials for the chemical preparation of environmentally friendly surfactants [17], therefore amino acids may act as starting material for the synthesis of various derivatives of 1,2,4-triazole [18]. Similarly, the literature revealed that compounds containing a 1,2,4-triazole ring in their structure exhibit diverse applications in pharmacological [19] and other medicinal uses [20], the combination of different functional groups frame may lead to compounds with interesting biological profiles[21]. Prompted by these investigations, we report here the synthesis and evaluating the antimicrobial activities of new compounds derived from three natural amino acids (L-Leucine, L-Phenylalanine, and L-Methionine) incorporated heterocycles like 1,2,4- triazole with attached secondary quaternary ammonium salts group with acetic acid moieties.

MATERIALS AND METHODS

Materials

All the chemicals and reagents were obtained from Sigma Aldrich and Biochem. Melting points were measured using BUCHI 540 apparatus and are uncorrected, IR spectra were recorded as potassium bromide pellets on a Shimadzu 8300

spectrophotometer ($\bar{\upsilon}$ max in cm^{-1}), The ^1H and ^{13}C NMR spectra were recorded in D_2O on a Bruker AM, NMR spectrometer (300 MHz) using TMS as internal standard (δ in ppm). Proceeding of reactions and checking the purity of the compounds were made by TLC on silica gel supplied by MERCK, iodine was used for visualization.

Methods

General Procedure for the Preparation of Amino Acid Methyl Esters 2(a-c)

Amino acid methyl esters were prepared according to the literature [22], into a solution of the corresponding amino acid 1(a-c) (0.018 mole) in absolute methanol and concentrated sulfuric acid (2 ml) was heated at 80 ℃ in oil bath. Reaction followed with TLC to obtain the desired compound. The excess of acid neutralized with sodium bicarbonate, washed with methylene chloride. Solvent evaporated and the product collected.

Leucine methyl ester **(2a)**: R*f*= 0.23($CHCl_3$); yield 72%; mp129-130˚C; IR (KBr υ max cm^{-1}): 3421 (N-H), 1747 (C=O), 1006 (C-O-C).

Phenylalanine methyl ester **(2b)**: R*f*=0.8($CHCl_3$); yield 84%; mp164-165˚C; IR (KBr υ max cm^{-1}): 3485 (N-H), 3030 (=C-H), 1747 (C=O), 1562 (C=C), 1008 (C-O-C).

Methionine methyl ester **(2c)**: R*f*=0.66($CHCl_3$/MeOH 4/1); yield 67%; mp234 -235˚C; IR (KBr υ max cm^{-1}): 3414(N-H), 1743 (C=O), 1016 (C-O-C), 802 (C-S-C).

General Procedure for the Preparation of Amino Acid Hydrazide 3(a-c)

Amino acid methyl esters **2(a-c)** (0.011 mole) were refluxed with hydrazine hydrate (0.1 mole, 3 ml) in absolute ethanol for 4-5 hours. Reactions were monitored by TLC. Mixtures were cooled down, the solvent evaporated, and the products collected.

Leucine acid hydrazide **(3a)**: R*f*=0.71($CHCl_3$); yield 84%; thick syrup; IR (KBr υ max cm^{-1}): 3419 (N-H), 1625 (C=O).

Phenylalanine acid hydrazide **(3b)**: R*f*=0.69 ($CHCl_3$/MeOH 4/1); yield 92%; thick syrup; IR (KBr υ maxcm^{-1}): 3303 (N-H), 3055 (=C-H), 1618 (C=O), 1510 (C=C), 1093 (C-N).

Methionine acid hydrazide **(3c)**: R*f*=0.38($CHCl_3$); yield 87%; mp104-105˚C;IR (KBr υ max cm^{-1}): 3419 (N-H), 1685 (C=O), 1112 (C-N), 619 (C-S-C).

General Procedure for the Synthesis of 5-Substituted-1, 2, 4-triazoles-2-thione 4(a-c)

A mixture of the appropriate acid hydrazide of amino acid (0.011 mole), ammonium thiocyanate (0.82g, 0.011 mole), and hydrochloric acid (5 mL) in ethanol (50 mL) was refluxed for 8h in an oil bath. After cooling, the product was filtered and this intermediate (1.85 g, 0.011 mole) was refluxed in 10 % sodium hydroxide solution (5 mL) for 6 h. The resulting solution was cooled and filtered. The filtrate was acidified with hydrochloric acid to pH 5-6.

5-(1-amino-3-methylbutyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione **(4a)**:R*f* = 0.45 ($CHCl_3$); yield 87%; Yellow color gel; IR (KBr υ max cm^{-1}): 3429 (N-H), 1625 (C=N), 1230 (C=S), 1128 (C-N).

^{1}H-NMR (300MHz, D_2O), δ (ppm): 5.6 (4H, CH-N<u>H</u>$_2$, S=C-N<u>H</u>-, C-N<u>H</u>-C); 3.3 (1H, C<u>H</u>-NH_2); 1.7 (1H, C<u>H</u>(CH_3)$_2$); 0.9 (6H, CH(C<u>H</u>$_3$)$_2$).

^{13}C-NMR (300MHz, D_2O), δ (ppm): 173.00 (<u>C</u>=N), 170.66 (<u>C</u>= S), 59.06 (<u>C</u>H-NH_2), 55.75 (CH-<u>C</u>H$_2$-); 22.90 (CH-(<u>C</u>H$_3$)$_2$).

5-(1-amino-2-phenylethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione **(4b)**:

R*f*=0.33 ($CHCl_3$/MeOH 3/1); yield 78%; mp164-165˚C; IR (KBr υ max cm^{-1}): 3440 (N-H), 1624 (C=N), 1400(C= C), 1309 (C=S), 1168.8 (C-N).

^{1}H-NMR (300MHz, D_2O), δ (ppm): 8.5 -7.8 (1H, C<u>H</u>-arm); 4.7 (1H, -N<u>H</u>$_2$, C-N<u>H</u>-C, -N<u>H</u>-C=S); 4.2 (1H, C<u>H</u>-NH_2); 3.1 (2H, C<u>H</u>$_2$-Ar).

^{13}C-NMR (300MHz, D_2O), δ (ppm): 180.02 (<u>C</u>=S); 171.15 (<u>C</u>=N); 163.59-128.49 (<u>C</u>H arm); 57.86 (<u>C</u>H-NH_2); 37.96 (<u>C</u>H$_2$-arm).

5-[1-amino-3-(methylthio)propyl]-2,4-dihydro-3H-1,2,4-triazole-3-thione **(4c)**:

R*f* = 0.80 ($CHCl_3$); yield 89%; Light yellow gel; IR (KBr υ max cm^{-1}): 3413 (N-H), 1624 (C=N), 1321 (C=S), 1114.8 (C-N), 867.9 (C-S-C).

^{1}H-NMR (300MHz, D_2O), δ (ppm): 4.8 (4H, N<u>H</u>-C=S, C-N<u>H</u>-C, -N<u>H</u>$_2$); 3.5 (1H, C<u>H</u>-NH_2); 1.8 (2H, –C<u>H</u>$_2$-S); 1.1 (2H, –S-C<u>H</u>$_3$).

^{13}C-NMR (300MHz, D_2O), δ (ppm): 161.75 (<u>C</u>=S); 145.34 (<u>C</u>=N); 54.28 (<u>C</u>H-NH_2); 33.20 (CH-<u>C</u>H$_2$); 31.83 (<u>C</u>H$_2$-S); 14.02 (–S-<u>C</u>H$_3$).

General Procedure for the Synthesis of 5-Substituted 1,2,4-triazole -2-methylthio 5(a-c)

A mixture of the corresponding thione compound 5(a-c) (0.01 mole) was refluxed with an equimolar quantity of sodium hydroxide (0.4 g, 0.01 mole), and methyl iodide (1.3 g, 0.01 mole) in absolute ethanol for 6 h. After cooling to room temperature, the solvent was removed under reduced pressure and then the residue was collected.

3-methyl-1-[5-(methylthio)-4H-1,2,4-triazol-3-yl]butan-1-amine **(5a)**:

R*f* = 0.46 ($CHCl_3$/MeOH 9/1); yield 79%; yellow dough; IR (KBr υ max cm^{-1}): 3427 (N-H), 1639 (C=N), 846 (C-S-C).

^{1}H-NMR (300MHz, D_2O), δ (ppm): 5.6 (3H, –C- N<u>H</u>-C, CH-N<u>H</u>$_2$); 3.3 (1H, C<u>H</u>-NH_2); 2.4 (3H, –S-C<u>H</u>$_3$); 1.7 (1H, C<u>H</u>(CH_3)$_2$); 0.9 (6H, CH(C<u>H</u>$_3$)$_2$).

^{13}C-NMR (300MHz, D_2O), δ (ppm): 173.00 (<u>C</u>=N), 170.66 (<u>C</u>=N), 59.06 (<u>C</u>H-NH_2), 55.75 (C10); 46.99 (CH-(<u>C</u>H$_3$)$_2$), 22.90 (-S-<u>C</u>H$_3$).

1-[5-(methylthio)-4H-1,2,4-triazol-3-yl]-2-phenylethanamine **(5b)**:

R*f* = 0.35 ($CHCl_3$/MeOH 3/1); yield 80%; brown gel; IR (KBr υ max cm^{-1}): 3440 (N-H), 1627 (C=N), 1461 (C=C), 759.9 (C-S-C).
^{1}H-NMR (300MHz, D_2O), δ (ppm): 7.2 (1H, C<u>H</u>arm); 6.4 (3H, C-N<u>H</u>-C -N<u>H</u>$_2$); 3.2 (1H, C<u>H</u>–NH_2); 2.8 (2H, –C<u>H</u>$_2$-Ar); 2.4 (3H, -S-C<u>H</u>$_3$).
^{13}C-NMR (300MHz, D_2O), δ (ppm): 163.74-150.67 (<u>C</u>=N); 129.37 (Carm); 50.90 (<u>C</u>H–NH_2); 38.15 (<u>C</u>H_2–Ar); 24.20 (-S-<u>C</u>H_3).
3-(methylthio)-1-[5-(methylthio)-4H-1,2,4-triazol-3-yl]propan-1-amine **(5c)**:
R*f* = 0.41 ($CHCl_3$/MeOH 2/1); yield 77%; brown dough; IR (KBr υ max cm^{-1}): 3413.8 (N-H), 1624.0 (C=N), 1321.1 (C=S), 1114.8 (C-N), 867.9 (C-S-C).
^{1}H-NMR (300MHz, D_2O), δ (ppm): 4.7 (1H, –N<u>H</u>$_2$, C-N<u>H</u>-C); 3.9 (1H, C<u>H</u>-NH_2); 3.5 (3H, –S-C<u>H</u>$_3$); 2.1 (2H, –C<u>H</u>$_2$-S), 2.0 (2H, CH-C<u>H</u>$_2$); 1.8 (3H, –S-C<u>H</u>$_3$).
^{13}C-NMR (300MHz, D_2O), δ (ppm): 166.58 (<u>C</u>=S); 135.59 (<u>C</u>=N); 59.31 (<u>C</u>H-NH_2); 49.67 (CH-<u>C</u>H_2); 46.68 (-<u>C</u>H_2-S-); 20.40 (–S-<u>C</u>H_3); 18.40 (–S-<u>C</u>H_3).

General Procedure for the Synthesis of 5-Substituted -1,2,4-triazole -2-methylthio N-carboxymethyl ammonium bromides 6(a-c)

The ammonium salt compounds 6(a-c) were obtained by *N*-alkylation reaction with an equimolar quantity of compounds 5(a-c) (0.0042 mole) with Bromoacetic acid (0.0042 mole 0.58 g) in dry acetone (50ml), this mixture was refluxed for 8 h. After that, the reaction mixture was cooled to room temperature and the solvent was evaporated under reduced pressure, an oily product appeared. The crude product was washed with diethyl ether several times.
N-(carboxymethyl)-3-methyl-1-[5-(methylthio)-4*H*-1,2,4-triazol-3-yl]butan-1-ammonium bromide (6a):
R*f* = 0.5 ($CHCl_3$/MeOH 2/1); yield 51%; brown gel; IR (KBr υ max cm^{-1}): 3415 (N-H), 2927 (C-H), 1722.3 (C=O), 1627.8 (C=N), 894.9 (C-S-C).
^{1}H-NMR (300MHz, D_2O), δ (ppm): 8.3 (3H, O<u>H</u>, CH-N<u>H</u>$_2^+$, C-N<u>H</u>-C); 4.8 (1H, C<u>H</u>-NH_2); 4.6 (2H, C<u>H</u>$_2$-COOH); 3.1 (3H, –S-C<u>H</u>$_3$); 1.8 (2H, CH-C<u>H</u>$_2$); 0.9 (6H, CH-(C<u>H</u>$_3$)$_2$).
^{13}C-NMR (300MHz, D_2O), δ (ppm): 171.43 (<u>C</u>=O), 162.55 (<u>C</u>=N), 64.29 (<u>C</u>H-NH_2), 38.12 (-<u>C</u>H_2-COOH); 26.88 (CH-(<u>C</u>H_3)$_2$), 21.74 (-S-<u>C</u>H_3).
N-(carboxymethyl)-1-[5-(methylthio)-4H-1,2,4-triazol-3-yl]-2-phenylethan ammonium bromide (6b):
R*f* = 0.58 ($CHCl_3$/MeOH 4/1); yield 65%; brown gel; IR (KBr υ max cm^{-1}):
3505.5 (O-H), 2947 (C-H), 1741.6 (C=O), 1596.9 (C=N), 1411.8 (C=C), 854.4 (C-S-C).
^{1}H-NMR (300MHz, D_2O), δ (ppm): 8.3 (4H, OH, C-N<u>H</u>-C, –N<u>H</u>$_2^+$); 7.4-7.3 (1H, C<u>H</u>arm); 4.7 (1H, –C<u>H</u>-NH_2^+); 3.9 (2H, C<u>H</u>$_2$COOH), 3.7 (2H, C<u>H</u>$_2$-Ar), 2.2 (3H, S-C<u>H</u>$_3$).
^{13}C-NMR (300MHz, D_2O), δ (ppm): 178.15 (<u>C</u>=O); 175.36-170.19 (<u>C</u>=N); 129.40-127.97 (<u>C</u>Harm); 63.31 (<u>C</u>H-NH_2^+); 59.89 (<u>C</u>H_2-COOH); 36.54 (-<u>C</u>H_2-Ar); 16.37 (-S-<u>C</u>H_3).
N-(carboxymethyl)-3-(methylthio)-1-[5-(methylthio)-4H-1,2,4-triazol-3-yl]propan-1- ammonium bromide (6c):
R*f* = 0.38 ($CHCl_3$/MeOH 4/1); yield 56%; Light brown gel; IR (KBr υ max cm^{-1}): 3411 (O-H), 2939.(C-H), 1598 (C=N), 1712 (C=O), 831 (C-S-C).
^{1}H-NMR (300MHz, D_2O), δ (ppm): 8.3 (4H, O<u>H</u>, C-N<u>H</u>-C, CH-N<u>H</u>$_2^+$); 4.6 (1H, C<u>H</u>-NH_2^+); 4.2 (2H, C<u>H</u>$_2$COOH); 3.2 (2H, C<u>H</u>$_2$-S-), 2.5 (2H, CH-C<u>H</u>$_2$); 2.5 (3H, –S-C<u>H</u>$_3$); 2.0 (3H, –S-C<u>H</u>$_3$).
^{13}C-NMR (300MHz, D_2O), δ (ppm): 175.33 (<u>C</u>=N); 166.48 (<u>C</u>=O); 140.17 (<u>C</u>=N); 60.93 (<u>C</u>H-NH_2); 42.16 (–<u>C</u>H_2COOH); 32.79 (CH-<u>C</u>H_2); 30.42 (<u>C</u>H_2-S-); 15.86 (–S-<u>C</u>H_3); 15.43 (–S-<u>C</u>H_3).

Biological Activity

The prepared compounds have been studied for their antimicrobial activity in vitro against three tested bacteria *Enterococcus faecalis* ATCC 49452, *Bacillus subtilis,* ATCC 6633, *Bacillus cereus* ATCC 11778 as gram-positive bacteria and *Escherichia coli* ATCC 25933, *Campylobacter fetus* ATCC 27374, *Enterobacter cloacae* ATCC 13047 as gram-negative bacteria and two fungi *Candida albicans* ATCC 10231 and *Candida albicans* IP 444. The microorganisms were provided from the collection of pure cultures of the Laboratory of Biotoxicology, Pharmacognosy, and Biological recovery of plants, University of Dr. Moulay Taher, Saida.

Antibacterial Activity

In vitro antibacterial activity of compounds were tested to six bacterial strains, three Gram-positive and three Gram-negative bacteria, DMSO was used as a diluting solvent for the tested compounds. A stock solution of each test compound (100 mg / mL) was prepared in DMSO following a series of dilutions in the concentration range of 10, 5, 2.5, 1.5, 0.625, 0.3125, 0.156, 0.078 and 0.039 mg/mL were prepared. The inoculates were standardized according to the 0.5 McFarland scale and subjected to dilution in Mueller-Hinton Broth after reading at a length of 600 nm. A broth microdilution method [23] was used to determine the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the tested compounds. Fresh cultures of bacteria obtained by inoculating bacteria in Muller-Hinton broth. After incubation at 35 - 37°C for 18 - 24 hr, the plates were inspected visually for inhibition of bacterial growth, the minimum inhibitory concentration (MIC) was noted by seeing the lowest concentration of the tested compounds at which there was no visible growth.

To evaluate the minimal bactericidal concentration (MBC) of tested compounds, MBC is defined as the lowest compound's concentration that inhibits at least 99.9% of the bacterial cell as compared to the colony count of the starting inoculums.

Anti-yeast Activities

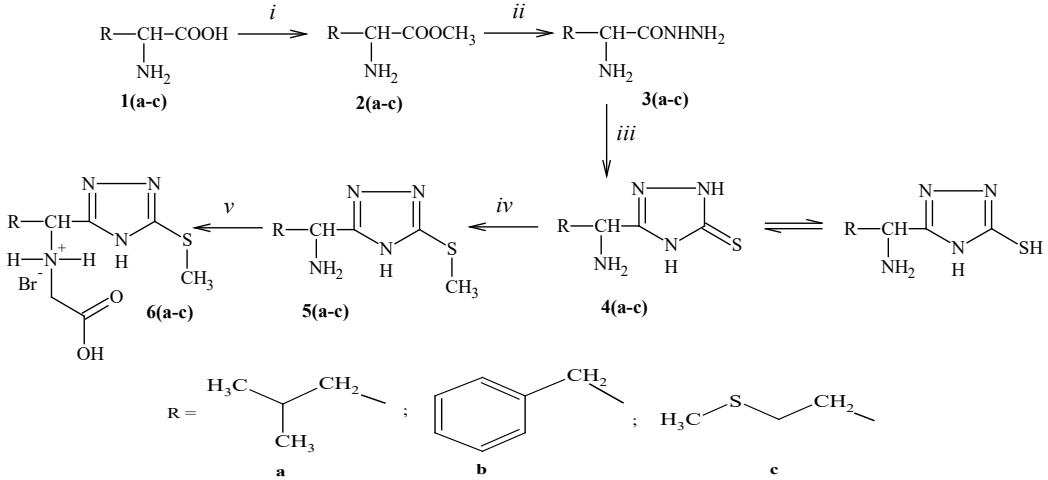
Compounds were characterized in vitro for anti-yeast activities against two registered yeast species, *Candida albicans* ATCC 10231 and *Candida albicans* IPP 444, each sample was sown by subculturing on Sabouraud agar and stored at 4 °C, readings were taken after incubations at 25 °C for 2-5 days. A reference method for broth dilution antifungal susceptibility testing of yeasts [23] was used to determine the minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC).

RESULTS AND DISCUSSION

Synthesis

The target compounds **6(a-c)** were synthesized via multi-step as illustrated in Scheme 1.

In the first step amino acid methyl ester **2(a-c)** were synthesized using starting amino acid in methanol and concentrated sulfuric acid in good yields (67-87%). Esters then converted to acid hydrazides using hydrazine in ethanol. The hydrazides were converted to substitute 1,2,4-triazole-2-thione **4(a-c)** using ammonium thiocyanate and ethanolic Hydrochloric acid, the compounds were alkylated with methyl iodide in ethanolic sodium hydroxide to give the S-methyl derivative **5(a-c)**. Compound **5(a-c)** was quaternized with bromoacetic acid to form the final products **6(a-c)**.



Scheme 1 Synthetic route to the title compounds **6(a-c)**. Reagents*: i)* CH_3OH and concentrated H_2SO_4, *ii*) $NH_2NH_2.H_2O$ and C_2H_5OH, *iii*) NH_4SCN, HCl and KOH *iv*) CH_3I, NaOH and C_2H_5OH, *v*) $BrCH_2COOH$ and acetone.

Antibacterial activity

The antibacterial activity of compounds **6(a-c)** was tested by the broth microdilution method to determine the minimum inhibitory concentrations (MIC) and the minimum bactericidal concentrations (MBC) against the reference strain. The test was carried out in triplicates and the values of MIC and MBC results of the tested compounds are summarized in Table 1.

Table 1. Antibacterial MIC and MBC of the tested compounds **6(a-c)**.

Compounds	Values of MIC/MBC (mg/mL)											
	Gram-positive bacteria						Gram-negative bacteria					
	Enterococcus faecalis ATCC 49452		*Bacillus subtilis* ATCC 6633		*Bacillus cereus* ATCC 11778		*Escherichia coli* ATCC 25933		*Campylobacter fetus* ATCC 27374		*Enterobacter cloacae* ATCC 13047	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
6a	ND	ND	2.5	5	5	10	ND	ND	ND	ND	10	ND
6b	10	ND	2.5	10	ND	ND	10	ND	5	10	2.5	10
6c	1.5	2.5	1.5	1.5	1.5	2.5	5	10	ND	ND	5	10

Samples with MIC>10 mg/ml were considered not determined (ND)

The results of antibacterial activity showed that, compound 6c showed activity against all bacterial strains, except against *Campylobacter fetus*. Other compounds 6b showed moderate activity against *Bacillus subtilis, Campylobacter fetus,* and *Enterobacter cloacae,* however, compounds 6a were active against *Bacillus subtilis* and *Bacillus cereus,*

moderate activity was observed against *Enterobacter cloacae.*

The antibacterial action is sometimes partial and after a decrease in the number of bacteria, it is observed an increase in bacterial growth. This phenomenon called rebound may be due to instability of the in vitro antibacterial agent at a heterogeneity of the bacterial population that may contain genotypically more resistant bacteria than the general population or induction of enzymes conferring bacterial resistance to antibacterial extract [24].

Anti-yeast Activities

The newly synthesized compounds **6(a-c)** were screened *in vitro* for their anti-yeast activities, against two registered yeast species of Candida albicans. The test was performed twice, and the average values of MIC and MFC are summarized in Table 2.

Table 2. Anti-yeast MIC and MFC of the tested compounds **6(a-c)**.

Compounds	Species of yeast MIC /MFC µg mL^{-1}			
	Candida albicans ATCC 10231		*Candida albicans* IP 444	
	MIC	MFC	MIC	MFC
6a	5	5	1.5	5
6b	2.5	2.5	10	10
6c	10	10	5	5

The results of anti-yeast activity studies reveal that the tested compounds exhibited significant Anti-yeast activities; compounds **6b** showed the highest activity against *Candida albicans* ATCC 10231 and compound **6a** displayed good activity against *Candida albicans* IP 444. The antifungal action is due to an increase in the permeability of the plasma membrane followed by a rupture of the latter leading to a leakage of the cytoplasmic content and therefore the death of the cell. Indeed, compounds containing various organic groups react with membrane enzymes and degrade the plasma membrane of yeasts [25].

CONCLUSION

In conclusion, we have synthesized, new QAS compounds containing 5-membered heterocyclic ring system moiety especially 1,2,4- triazoles derivatives of three natural amino acids (L-Leucine, L-Phenylalanine, and L-Methionine) attached to secondary ammonium bromide group were successfully synthesized and characterized. Physical and antibacterial, anti-yeast properties were studied. Biological screening experiments have demonstrated that several compounds possess promising antimicrobial potential against some tested microorganisms. The newly synthesized compounds were prepared with an objective of developing better antimicrobial activity, and future investigations are necessary to evaluate antimycobacterial, antiviral, antiparasitic, and anticancer activity.

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CONFLICT OF INTEREST

Authors declare that there was no conflict of interest regarding the publication of this manuscript.

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