THE DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF THE PEPTIDE DERIVATIVES CONTAINING GUANIDINE MOIETY WITH 5-CHLORO-THIOPHENE-2-CARBOXYLIC ACID CONJUGATES

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ABSTRACT

A new series of dipeptide derivatives with 5-chloro-thiophene-2-carboxylic acid conjugates have been designed and synthesized using solid-phase peptide synthesis. The synthesized dipeptide derivatives were characterized by using spectroscopic tools, like $^1$H and $^{13}$C NMR, IR, and mass and elemental analysis. Additionally, the obtained target dipeptide derivatives were evaluated for in vitro antimicrobial activity. The antibacterial activity of the target compounds was evaluated against four bacteria (two Gram-positive; Streptococcus pyogenes and Staphylococcus aureus, two Gram-negative; Escherichia coli and Pseudomonas aeruginosa). The screened analogs showed good antibacterial activity. Noteworthily, among them, Thiophene-Tyr-Arg-OH derivative exhibited excellent activity against Escherichia coli (MIC=15 µg/mL) as compared to standard drug ampicillin. The antifungal activity was evaluated against two fungi (Aspergillus niger and Candida albicans). The screened analogs also showed good antifungal activity.

Keywords: Heterocyclic Hybrids, Thiophene Conjugates, Solid Phase Peptide Synthesis, Dipeptides, Antibacterial Activity, Antifungal Activity.
better cell membrane permeability than small molecule drugs, which is a critical criterion for passive absorption in the gastrointestinal tract.

In the light of the above facts, we have designed the synthesis of a new series of dipeptide derivatives with 5-chloro-thiophene-2-carboxylic acid at N terminus and Arginine at the C terminus (Fig.-2) and evaluated their antimicrobial activities. Moreover, we have compared the observed antibacterial and antifungal activities of the synthesized dipeptides with the standard drugs Ampicillin and Nystatin, respectively. We chose the solid phase peptide synthesis (SPPS) route for the synthesis purpose because it is simple, highly efficient, involves easy purification, rapidly generates the linear peptides, the synthesized intermediates do not require isolation and high-speed method as compared to the traditional solution-phase peptide synthesis.33

**EXPERIMENTAL**

**Material and Methods**
The resin was procured from Merck. All the amino acids used are L and Fmoc protected and were procured from Sichuan China. Melting points were obtained by an open capillary method and are uncorrected. The coupling and deprotection reactions were monitored by the Kaiser test. Compounds were purified by preparative HPLC or precipitation using ethyl acetate and hexane mixtures. IR spectra were recorded on Nicolet impact 400 FT/IR spectrometer using KBr pressed pellet technique. MASS spectra were recorded on Shimadzu LC-MS (at 70 eV) Mass Spectrometer. 1H and 13C NMR spectra were recorded on Bruker Neo 400 MHz NMR spectrometer using DMSO- d6 solvent.

**General Procedure for the Synthesis of Intermediates la-j**
The 2-chlorotrityl chloride (2-CTC) resin (substitution 1.0 mmol/g) was added to the peptide synthesizer, washed with 10 volumes of dichloromethane (DCM) and drained. Further, added 10 volumes of DCM and stirred for 1h (swelling) then drained. Dissolved Fmoc-Arg(pbf)-OH (3 eq.) in dry DCM (10 ml/gm) and added diisopropylethylamine (DIPEA) (4 eq.). Added the amino acid solution to the resin. Agitated the reaction mixture vigorously for 2 h. Filtered and washed the peptidyl resin twice with DCM and then twice with dimethylformamide (DMF). Capped the unreacted functional group of resin by using DCM, methanol and DIPEA (85:10:5). Loading percentage monitored by UV absorption in the range of 300 nm.
General Procedure for the Synthesis of Intermediate 5a-j

The standard SPPS method was applied with Fmoc/tBu/boc protocol. Fmoc-Arg(Pbf)-2-CTC resin (loading 1 mmol/g) was placed into a reaction vessel and allowed to swell in DMF (20 v with respect to initial weight of resin); the resin had a loading of 3 mmol, DMF 6 v with respect to initial weight of resin), 3 eq. of HBTU (3 mmol) and 6 eq. of DIPEA (6 mmol). After swelling, the peptides were synthesized using CSBio automated peptide synthesizer by repetition of the following steps: (1) Fmoc deprotection performed (wash) with a solution of 20% piperidine in DMF (v/v) two times; 5 minutes and 10 minutes (10 v with respect to the initial weight of resin). The reaction completion was monitored by the Kaiser test (the blue color solution was observed, comply). After completion of the synthesis, the peptidyl resin was filtered and then washed with DMF (×5). The reaction was monitored using Kaiser test (colorless beads and solution were observed, comply) and the peptidyl resin was filtered and then washed with DMF (×5).
General Procedure for the Synthesis of Intermediate 6a-j
The protected peptidyl resin was treated with a mixture of TFA:TIS:H₂O (90:5:5) (10 mL/gm) at 20 °C for 3 h. The mixture was precipitated with DIPE (50 volume), filtered and washed with 3x10 volume diisopropylether (DIPE). The filter cake was dried under a vacuum at room temperature to obtain the crude.

Compound-6a (5-chlorothiophene-2-carbonyl)-D-serylarginine
79.0% yield; mp 186-192 °C; ^1H NMR (400 MHz, DMSO-d₆, δ ppm): 12.64 (s, 1H), 8.62 (s, 1H), 8.27 (s, 1H), 8.02, (d, 1H), 7.76 (s, 1H), 6.80 (d, 1H), 6.62 (s, 2H), 4.91 (s, 1H), 4.54 (t, 1H), 4.20 (t, 1H), 3.91 (d, 2H), 3.35 (m, 2H), 1.77 (m, 2H), 1.55 (m, 3H); ^13C NMR (100 MHz, DMSO-d₆, δ ppm): 173.8, 170.2, 160.7, 157.2, 139.2, 133.5, 129.2, 128.5, 72.5, 61.9, 56.5, 40.7, 28.6, 25.5; LCMS (ESI): m/z = 406.85 [M⁺ 100%]. Anal. Calcd. for C₁₅H₂₀ClN₃O₅S: C, 41.43; H, 4.97; Cl, 8.73; N, 17.26; O, 19.71; S, 7.90; Found C, 41.40; H, 4.95; Cl, 8.77; N, 17.20; O, 19.76; S, 7.93 %.

Compound-6b (5-chlorothiophene-2-carbonyl)-D-methionylarginine
66.7% yield; mp 184-190 °C; ^1H NMR (400 MHz, DMSO-d₆, δ ppm): 174.6, 171.0, 161.2, 158.0, 140.6, 138.2, 135.8, 129.7, 56.3, 56.0, 41.5, 31.5, 29.6, 29.2, 24.5, 15.3; LCMS (ESI): m/z = 450.91 [M⁺ 100%]. Anal. Calcd. for C₁₅H₂₀ClN₃O₅S₂: C, 42.71; H, 5.38; Cl, 7.88; N, 15.56; O, 14.22; S, 14.25; Found C, 42.70; H, 5.39; Cl, 7.89; N, 15.55; O, 14.21; S, 14.26%.

Compound-6c (5-chlorothiophene-2-carbonyl)-D-cysteinylarginine
65.2% yield; mp 202-206 °C; ^1H NMR (400 MHz, DMSO-d₆, δ ppm): 12.65(s, 1H), 8.76 (s, 1H), 8.31 (s, 1H), 8.07 (d, 1H), 7.85 (s, 1H), 6.85 (d, 1H), 6.64(s, 2H), 4.53 (t, 1H), 4.49 (t, 1H), 3.33 (t, 2H), 2.49 (s, 1H), 2.02-2.60 (m, 4H), 2.05 (s, 3H), 1.75 (t, 2H), 1.50 (m, 2H); ^13C NMR (100 MHz, DMSO-d₆, δ ppm): 174.6, 171.0, 161.2, 158.0, 140.6, 138.2, 135.8, 129.7, 56.3, 56.0, 41.5, 31.5, 29.6, 29.2, 24.5, 15.3; LCMS (ESI): m/z = 422.90 [M⁺ 100%]. Anal. Calcd. For C₁₅H₂₀ClN₃O₅S₂: C, 39.85; H, 4.78; Cl, 8.40; N, 16.60; O, 15.17; S, 15.20; Found: C, 39.74; H, 4.68; Cl, 8.39; N, 16.50; O, 15.11; S, 15.58%.

Compound-6d (5-chlorothiophene-2-carbonyl)-D-tyrosinylarginine
69.2% yield; mp 201-205 °C; ^1H NMR (400 MHz, DMSO-d₆, δ ppm): 12.65 (s, 1H), 9.09 (s, 1H), 8.82 (s, 1H), 8.59 (s, 1H), 8.12 (d, 1H), 7.85 (s, 1H), 6.92 (d, 1H), 6.72 (s, 2H), 6.30-6.90 (d, 4H), 4.93 (t, 1H), 4.62 (t, 1H), 3.42 (2H), 3.37 (t, 2H), 2.54 (s, 1H), 1.74 (m, 2H), 1.50 (m, 2H); ^13C NMR (100 MHz, DMSO-d₆, δ ppm): 174.6, 171.5, 161.3, 158.1, 155.6, 140.0, 138.3, 135.9, 130.2, 129.7, 129.1, 115.7, 56.9, 56.2, 41.5, 37.9, 29.6, 24.6; LCMS (ESI): m/z = 482.95 [M⁺ 100%]. Anal. Calcd. for C₂₀H₂₅ClN₃O₅S: C, 49.84; H, 5.02; Cl, 7.36; N, 14.53; O, 16.60; S, 6.65; Found: C, 49.82; H, 5.04; Cl, 7.35; N, 14.54; O, 16.61; S, 6.64.

Compound-6e (4R)-5-((1-carboxy-4-guanidinobutyl)amino)-4-(5-chlorothiophene-2 carboxamido)-5-oxopentanoic acid
62.9% yield; mp 184-190 °C; ^1H NMR (400 MHz, DMSO-d₆, δ ppm): 12.61-12.01 (s, 2H), 8.78 (s, 1H), 8.32 (s, 1H), 8.01 (d, 1H), 7.81 (s, 1H), 6.88 (d, 1H), 6.60 (s, 2H), 4.46(t, 1H), 4.22 (t, 1H), 3.30 (m, 2H), 2.49 (s,1H), 2.33 (t, 2H), 2.06 (m, 2H), 1.71 (m, 2H), 1.51 (m, 2H); ^13C NMR (100 MHz, DMSO-d₆, δ ppm): 174.43, 173.70, 171.76, 160.67, 157.33, 139.04, 133.68, 129.16, 128.50, 72.50, 53.01, 52.11, 49.13, 30.84, 28.41, 25.66; LCMS (ESI): m/z = 448.89 [M⁺ 100%]. Anal. Calcd. for C₁₆H₂₂ClN₅O₅S: C, 42.91; H, 4.95; Cl, 7.91; N, 15.64; O, 21.43; S, 7.16; Found: C, 42.82; H, 4.91; Cl, 7.93; N, 15.55; O, 21.33; S, 7.46%.

Compound-6f (5-chlorothiophene-2-carbonyl)lysylarginine
66.7% yield; mp 192-196 °C; ^1H NMR (400 MHz, DMSO-d₆, δ ppm): 12.11 (s, 1H), 8.67 (s, 1H), 8.32 (s, 1H), 8.81 (d, 2H), 7.81 (s, 1H), 6.80 (d, 1H), 6.62 (s, 2H), 4.55 (t, 1H), 4.21 (s, 1H), 3.43 (t, 2H), 2.49
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(72x38) PEPTIDE DERIVATIVES CONTAINING GUANIDINE MOIETY

(72x76) screened for their antifungal activity in triplicates against the bacteria at different concentrations of 1000, 500, 250, 200 µg/mL. The strains used were newly procured and stored under appropriate conditions. The compounds were diluted and tested. Muller Hinton Broth dilution method was used for the antibacterial assay. 10 µg/mL of the compounds which were found to be effective in inhibition were further screened for their antibacterial activity in triplicates against the bacteria at different concentrations of 500, 250, 200 µg/mL.

**Compound-6g (5-chlorothiophene-2-carbonyl)-D-alanylgarginine**

69.4% yield; mp 180-185 °C; 1H NMR (400 MHz, DMSO-d6, δ, ppm): 12.64 (s, 1H), 8.77 (s, 1H), 8.32 (s, 1H), 8.07 (d, 1H), 7.84 (s, 1H), 6.85 (d, 1H), 6.66 (s, 2H), 4.65 (m, 1H), 4.53 (m, 1H), 3.34 (t, 2H), 2.5 (s, 1H), 1.7 (m, 2H), 1.50 (m, 2H), 1.47 (d, 3H); 13C NMR (100 MHz, DMSO-d6, δ, ppm): 174.7, 171.7, 161.3, 158.0, 140.7, 138.3, 135.9, 129.8, 56.1, 53.6, 41.6, 29.7, 24.6, 17.9; LCMS (ESI): m/z = 390.86 [M+ 100%]. Anal. Calcd. for C17H22ClN6O3S: C, 43.13; H, 5.17; Cl, 9.09; N, 17.96; O, 16.42; S, 8.22; Found: C, 43.12; H, 5.18; Cl, 9.08; N, 17.97; O, 16.42; S, 8.22%.

**Biological Activity**

**Antibacterial Activity Assay**

The strains used were newly procured and stored under appropriate conditions. The compounds were screened for their antibacterial activity in triplicates against the bacteria at different concentrations of 1000, 500, 250, 200 µg/mL. The compounds which were found to be effective in inhibition were further diluted and tested. Muller Hinton Broth dilution method was used for the antibacterial assay. 10 µg/mL of the compounds which were found to be effective in inhibition were further diluted.

**Antifungal Activity Assay**

The strains used were newly procured and stored under appropriate conditions. The compounds were screened for their antifungal activity in triplicates against the bacteria at different concentrations of 1000, 500, 250, 200 µg/mL. The compounds which were found to be effective in inhibition were further diluted.
and tested. Muller Hinton Broth dilution method was used for the antifungal assay. In this study, for fungal growth, Sabouraud’s dextrose broth was used at 28 °C in aerobic conditions for 48 hours. Nystatin was used as a standard for comparison purposes.

RESULTS AND DISCUSSION

Chemistry
A new series of dipeptides derivatives with 5-chloro-thiophene-2-carboxylic acid conjugates 6a-j was synthesized using the solid-phase peptide synthesis method. The SPPS route was employed for the synthesis purpose as it is simple, highly efficient, involves easy purification, rapidly generates the linear peptides, the synthesized intermediates do not require isolation and high-speed method as compared to traditional solution-phase peptide synthesis. The first step involves an attachment of Fmoc-Arg(pbf)-OH to the CTC resin using DIPEA in DCM (Scheme-1). Loading percentage monitored by UV absorption in the range of 300 nm.

The structures of 6a-j were characterized by 1H NMR, 13C NMR, IR and mass spectroscopic techniques. The IR spectra displayed the characteristic absorption bands at 3540-3598, 1610-1690 and 3303-3404 cm⁻¹ for -COOH, -CONH₂, and -N-H stretching, respectively. 1H NMR spectrum of compounds 6a-j showed the predictable aromatic signals, i.e., two singlets at 1.31-1.60 and 8.65-9.80 ppm for protons of N-H and -COOH, respectively.

The complete physical date of the dipeptide derivatives 6a-j is incorporated in Table-1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>M.F.</th>
<th>M.W.</th>
<th>M.P.(°C)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6a</td>
<td>-CH₂-OH</td>
<td>C₁₄H₂₀Cl₂N₅O₅S</td>
<td>405.85</td>
<td>186-192 °C</td>
<td>70.10%</td>
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<tr>
<td>6b</td>
<td>-(CH₂)₃-S-CH₃</td>
<td>C₁₆H₂₂Cl₂N₅O₅S</td>
<td>449.91</td>
<td>182-189 °C</td>
<td>77.00%</td>
</tr>
<tr>
<td>6c</td>
<td>-CH₂-SH</td>
<td>C₁₄H₂₀Cl₂N₅O₅S</td>
<td>421.9</td>
<td>186-192 °C</td>
<td>65.20%</td>
</tr>
<tr>
<td>6d</td>
<td>-CH₂-C₂H₄-OH</td>
<td>C₁₅H₂₂Cl₂N₅O₈S</td>
<td>481.95</td>
<td>201-205 °C</td>
<td>69.20%</td>
</tr>
<tr>
<td>6e</td>
<td>-(CH₂)₃-COOH</td>
<td>C₁₄H₂₀Cl₂N₅O₅S</td>
<td>447.89</td>
<td>184-190 °C</td>
<td>62.90%</td>
</tr>
<tr>
<td>6f</td>
<td>-(CH₂)₃-NH₂</td>
<td>C₁₄H₂₀Cl₂N₅O₈S</td>
<td>447.9</td>
<td>192-196 °C</td>
<td>66.70%</td>
</tr>
<tr>
<td>6g</td>
<td>-CH₃</td>
<td>C₁₄H₂₀Cl₂N₅O₅S</td>
<td>389.86</td>
<td>180-185 °C</td>
<td>69.40%</td>
</tr>
<tr>
<td>6h</td>
<td>-(CH₂)₃-</td>
<td>C₁₆H₂₀Cl₂N₅O₅S</td>
<td>415.89</td>
<td>182-187 °C</td>
<td>74.10%</td>
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<tr>
<td>6i</td>
<td>-CH₂(=O)-CH₂</td>
<td>C₁₄H₂₀Cl₂N₅O₅S</td>
<td>419.88</td>
<td>187-191 °C</td>
<td>70.40%</td>
</tr>
<tr>
<td>6j</td>
<td>-CH₂-C₂H₄</td>
<td>C₂₀H₂₆Cl₂N₅O₈S</td>
<td>465.95</td>
<td>202-207 °C</td>
<td>66.10%</td>
</tr>
</tbody>
</table>

Table-1: The Complete Physical Data of Compounds 6a-j

Biological Evaluation (In vitro Antimicrobial activity)

In vitro antimicrobial activities (MIC) of all the newly synthesized dipeptides 6a-j were evaluated by the conventional Broth microdilution method. The antibacterial activity was evaluated against four bacteria (two Gram-positive; Staphylococcus aureus (S. a.) MTCC 96 and Streptococcus pyogenes (S. p.) MTCC 442, two Gram-negative; Escherichia coli MTCC 443 (E. c.) and Pseudomonas aeruginosa (P. a.) MTCC 1688) and the results were tabulated in Table-2. Among the screened analogs, dipeptides 6c, 6d, 6f and 6i showed good antibacterial activity compared with the standard drug ampicillin. Additionally, the derivative 6e showed excellent antibacterial activity against Escherichia coli (MIC=15 µg/mL) compared with the standard drug ampicillin (MIC=100 µg/mL) (Fig.-3).

Additionally, in vitro antifungal activity of all the newly synthesized dipeptides 6a-j was evaluated against two fungal strains, Candida albicans (C. a.) MTCC 227 and Aspergillus niger (A. n.) MTCC 282 and the results were tabulated in Table-2. The dipeptide derivatives 6a and 6e-j showed good antifungal activity compared with the standard Nystatin (Fig.-4).
Table 2: The Results of In-vitro Antibacterial and Antifungal Screenings of Compounds 6a-j

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MIC (Anti-bacterial Activity) in µg/mL</th>
<th>MIC (Anti-fungical Activity) in µg/mL</th>
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<tbody>
<tr>
<td>6a</td>
<td>125</td>
<td>150</td>
</tr>
<tr>
<td>6b</td>
<td>150</td>
<td>200</td>
</tr>
<tr>
<td>6c</td>
<td>50</td>
<td>150</td>
</tr>
<tr>
<td>6d</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>6e</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>6f</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>6g</td>
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<td>6h</td>
<td>250</td>
<td>150</td>
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<td>6i</td>
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<td>100</td>
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<td>6j</td>
<td>150</td>
<td>200</td>
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<tr>
<td>Ampicillin (std-1)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Nystatin (std-2)</td>
<td>-</td>
<td>-</td>
</tr>
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</table>
In this research, we have designed and synthesized a new series of dipeptide derivatives with 5-chlorothiophene-2-carboxylic acid conjugates 6a-j. The characterization of the synthesized derivatives was carried out by spectroscopic techniques (1H & 13C NMR, IR and mass) and elemental analysis. Furthermore, with the anticipation of creating novel therapeutics aiding as effective antimicrobial agents, the obtained dipeptides were evaluated for their in vitro antimicrobial activities. The dipeptide derivatives 6c, 6d, 6f and 6i exhibited good antibacterial activity, while 6e showed an excellent activity compared to the standard drug ampicillin. Additionally, the dipeptide derivatives 6a and 6e-j showed good antifungal activity compared with the standard Nystatin. The research work described in this article lays out a vision for the evolution of novel antimicrobial agents which are efficacious over a diversified span of pathogenic strains. Moreover, this research work sheds a light on the future progress of hybrids of heterocycles and peptides with antimicrobial properties.

ACKNOWLEDGEMENT

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CONCLUSION


Fig.-3: The In vitro Antibacterial Activities of dipeptides 6a-j against the Bacteria Strains E. c., P. a., S. a. and S. p. and their comparison with the Standard Drug Ampicillin

Std-2: Nystatin C. a.: Candida albicans, A. n.: Aspergillus niger

Fig.-4: The In vitro Antifungal Activities of dipeptides 6a-j against the Fungal Strains C. a. and A. n. and their comparison with the Standard Drug Nystatin
REFERENCES


[RJC-6907/2021]