Chapter 2

bis-Thiourea derivatives of dipeptides: Synthesis and biology

The work presented in this chapter are published in:


2.1. Introduction

Antibiotics are the most prescribed drugs in the world since from their discovery. However, the developed new drugs cannot surrender bacterial pathogens unconditionally because of rapid development of resistance against first effective drugs. Hence continuing search for new class of molecules such as the aminoglycosides, macrolides and glycopeptides as well as by the chemical modification of previously existing drugs which may inhibit bacterial pathogens by different mode of actions. Unfortunately, there is no assurance that the development of new antimicrobial drugs can keep pace with the ability of bacterial pathogens to develop resistance [1, 2]. Therefore, it is necessary for effective curing of bacterial disease which stimulates the research into design and synthesis of novel antimicrobial molecules with different mode of action.

In this chapter, we have directed our systematic efforts towards the development of novel bis-thiourea derivatives of dipeptides (KD/ kD/ KW/ kW) conjugated to 6-fluoro-3-(piperidin-4-yl)benzo[d]isoxazole (Het A) and evaluated their antimicrobial, antioxidant and anti-inflammatory activities. The chemistry and biology are described here under.

2.2. Experimental

2.2.1. Materials and methods

Capital and small single letter code for amino acids indicate L and D configurations respectively whereas for three letters code, we have used, for example, Lys and lys representing L- and D-configurations respectively. Amino acids, EDCI, HOBt and TFA were purchased from Advanced Chem. Tech. (Louisville, Kentucky, USA). Phenyl iso(thio)cyanates, isonipecotic acid, IBCF, NMM, DPPH, DMPD and
ABTS were purchased from Sigma Aldrich (India). All solvents and reagents used for the synthesis were of analytical grade. Silica gel (60-120 mesh) for column chromatography was purchased from Merck Pvt. Ltd., (Mumbai, India). Progress of the reaction was monitored by TLC using silica gel coated on glass plates with the solvent system comprising chloroform/ methanol/ acetic acid in the ratio 98:02:03 \((R_f^a)\) and 95:05:03 \((R_f^b)\). The compounds on the TLC plates were detected by iodine vapors. Melting points were determined on a Superfit melting point apparatus (India) and are uncorrected. FT-IR studies were performed using a PerkinElmer Spectrum Version 10.03.09 (PIKE Technologies). \(^1\)H NMR (400 MHz) and \(^13\)C NMR (100 MHz) spectra were recorded on an Agilent Technologies (USA) using DMSO-\(d_6\) as solvent and TMS as standard. High resolution mass spectrometric analysis was performed on a Bruker MicroTOF QII mass spectrometer in positive mode.

2.2.2. Synthesis

2.2.2.1. Synthesis of Het A

Synthesis of N-formyl isonipecotic acid (1)

The synthesis of Het A involves three steps and is represented in Scheme 2.1., the procedure for which is described below.

Formic acid (175.5 mL, 4.62 mol) and acetic anhydride (438.5 mL, 4.62 mol) were heated to 55–60 °C for 1 h and gradually cooled to 0–5 °C. Isonipecotic acid (200 g, 0.77 mol) was added in portion to the mixed anhydride at the same temperature. Reaction was maintained for 14 h and concentrated the crude under vacuum. To the residue, 300 mL of 2-propanol was added and filtered under cool conditions (6–8 °C) to obtain the pure compound 1 (114 g, 95%). M.P. 134–137 °C (lit.136-138 °C) [3], FTIR, ATR (cm\(^{-1}\)), 1716, 1631.
Chapter 2

Synthesis of 2,4-difluorobenzoyl-4-(1-formyl)piperidine (2)

*N*-Formyl isonipecotic acid (1) (50 g, 0.318 mol) was added to a solution of thionyl chloride (45.24 mL, 0.636 mol) and dichloromethane (50 mL), the reaction mixture was refluxed at 35–40 °C for about 3 h. The reaction was monitored by TLC (chloroform/methanol; 8:2). After completion of the reaction, excess thionyl chloride and solvent were distilled off completely. The mixture was cooled to rt, added dichloromethane (50 mL) and kept under nitrogen atmosphere. A mixture of 1,3-difluorobenzene (37.49 mL, 0.382 mol), aluminium chloride (74.31 g, 0.55 mol) and dichloromethane (50 mL) was cooled to −5 °C. To this acid chloride was added dropwise for about 1 h and the reaction mass was kept for another 4 h. After completion of the reaction, the reaction mixture was quenched with ice-cold water (600 mL) containing dil. HCl (10 mL) and extracted with dichloromethane thrice (each 100 mL). The organic layer was washed with 5% sodium bicarbonate solution and again with water. It was concentrated and to the residue was added n-hexane (75 mL) at rt. The reaction mixture was filtered at 10-15 °C to get 2 (44 g, 88%). M.P. 65-68 °C (lit. 64-66 °C) [3], FTIR, ATR (cm⁻¹), 1712, 1656.

Conversion of 2 to Het A

Compound 2 (25 g, 0.098 mol) was added to a stirred solution of hydroxylammonium sulfate (12.16 g, 0.074 mol), methanol (100 mL) and KOH flakes (22.17 g, 0.39 mol). The mixture was refluxed for 6–8 h, cooled to rt and 25 mL of demineralised water was added and refluxed again for 2 h. After completion of the reaction, the contents were concentrated under vacuum, allowed to attain rt and demineralised water (400 mL) was added. Het A was extracted with dichloromethane (100 mL x 2), the organic layer was concentrated and to this was added methanol (50 mL) and conc. HCl (20 mL). The pure compound Het A (21.8 g) was obtained by
filtering the reaction mass at 5–8 °C. M.P. 300-303 °C (Lit. 302-306 °C) [3], Yield=86%.

Reagents and conditions: i) Ac₂O, HCOOH; ii) SOCl₂, AlCl₃, 1,3-difluorobenzene; iii) KOH, (NH₂OH)₂·H₂SO₄, Conc. HCl/MeOH

Scheme 2.1. Synthetic route for Het A

2.2.2.2. Synthesis of Boc-Lys(2-ClZ)-Asp(OBzl)-OBzl (4), Boc-lys(2-ClZ)-Asp(OBzl)-OBzl (5), Boc-Lys(2-ClZ)-Trp-OMe (16) and Boc-lys(2-ClZ)-Trp-OMe (17)

The schemes for the synthesis of dipeptides, deprotection, conjugation to Het A and finally convertion to thiourea derivatives are presented in Scheme 2.2. and Scheme 2.3. The experimental chemistry is described below.

Boc-Lys(2-ClZ)-OH/ Boc-lys(2-ClZ)-OH (4.15 g, 10 mmol) was dissolved in acetonitrile separately (40 mL) and cooled to 0 °C was added NMM (1.09 mL, 10 mmol). This was further cooled to -15±1 °C and IBCF (1.43 mL, 11 mmol) was added drop-wise over a period of 15 min under stirring while maintaining the temperature at -15±1 °C and divided into two parts. The reaction mixture was stirred for an additional 10 min and a pre-cooled solution of Asp(OBzl)-OBzl.PTSA salt (4.85 g, 10 mmol)/ Trp-OMe.HCl salt (2.54 g, 10 mmol) and NMM (1.09 mL, 10 mmol) in DMF (48 mL/ 25 mL) was added slowly. After 20 min, pH of the solution was adjusted to 8 by the addition of NMM and the reaction mixture was stirred overnight at rt. The solvent was removed under reduced pressure and residue was poured into about 200
mL ice-cold 90% saturated KHCO₃ solution and stirred for 30 min. The precipitated product was taken into CHCl₃ and washed sequentially with 5% NaHCO₃ solution (2x50 mL), water (2x50 mL), 0.1N cold HCl solution (2x50 mL) and finally brine (2x50 mL). The crude compounds were recrystalliized from ether/ petroleum ether to get desired products 4, 5, 16 and 17.

2.2.2.3. Synthesis of Boc-Lys(2-ClZ)-Asp (6), Boc-lys(2-ClZ)-Asp (7), Boc-Lys(2-ClZ)-Trp (18) and Boc-lys(2-ClZ)-Trp (19)

*Saponification:* To a solution of 4, 5, 16 and 17 (8.4 mmol) in methanol (10 mL/g of compound) separately was added 1N NaOH (4 eq. for 4, 5 and 2 eq. for 16, 17) and stirred for 2 h at rt. Completion of the reaction was monitored by TLC and the solvent was evaporated, cooled, neutralized with cold 1N HCl, extracted with chloroform, washed with cold 1N HCl followed by water and dried over anhydrous Na₂SO₄. The solvent was removed under reduced pressure, triturated with ether, filtered and dried to obtain 6, 7, 18 and 19.

2.2.2.4. General procedure for the conjugation of 6, 7, 18 and 19 with Het A

To a cooled solution of 6, 7, 18 and 19 (7.5 mmol) and HOBt (2 eq. for 6, 7 and 1 eq. for 18, 19) in DMF separately (10 mL/g of peptide) was added NMM (2 eq. for 6, 7 and 1 eq. for 18, 19). EDCI (2.4 eq. for 6, 7 and 1.2 eq. for 18, 19) was added under stirring while maintaining the temperature at 0 °C. The reaction mixture was stirred for an additional 10 min and a pre-cooled solution of Het A (2 eq. for 6, 7 and 1 eq. for 18, 19) and NMM (2 eq. for 6, 7 and 1 eq. for 18, 19) in DMF (10 mL/g) was added slowly. After 20 min, pH of the solution was adjusted to 8 by the addition of NMM and the reaction mixture was stirred overnight at rt. DMF was removed under reduced pressure and the residue was poured into about 200 mL ice-cold 90% saturated KHCO₃ solution and stirred for 30 min. The precipitated product was taken
into CHCl$_3$ and washed sequentially with 5% NaHCO$_3$ solution (2x50 mL), water (2x50 mL), 0.1N cold HCl solution (2x50 mL) and finally brine (2x50 mL). The obtained crude were recrystallized from ether/ petroleum ether to get desired products 8, 9, 20 and 21.

2.2.2.5. General procedure for the synthesis of bis-thiourea derivatives (10-15 & 22-27)

*Hydrogenolysis (removal of 2-ClZ of Lys):* A solution of 8/ 9/ 20 and 21 (0.42 mmol) in methanol (10 mL/g of peptide) was stirred with 10% Pd on carbon (100 mg) and HCOONH$_4$ (53 mg, 2 eq.) for 4 h at rt. After the reaction was completed (monitored by TLC), reaction mixture was filtered through celite to remove the catalyst and the filtrate was concentrated and taken into chloroform. The organic layer was washed with 50% saturated brine solution (2x20 mL), dried over anhydrous Na$_2$SO$_4$, and the solvent was removed under reduced pressure.

*Deprotection of Boc:* Boc was removed by stirring the above compounds with TFA (10 mL/g of peptide) for 45 min at rt. After the reaction was completed, TFA was evaporated, triturated with dry ether, filtered and dried to obtain TFA.H-Lys(H.TFA)-Asp(Het A)-Het A, TFA.H-lys(H.TFA)-Asp(Het A)-Het A, TFA.H-Lys(H.TFA)-Trp-Het A and TFA.H-lys(H.TFA)-Trp-Het A.

*Formation of thioureas:* To cold solution of the above TFA salts (0.33 mmol each) in DMF (10 mL/g of compound) separately was added NMM (1.32 mmol, 4 eq.). To this solution respective substituted phenyl isothiocyanates (0.79 mmol, 2.4 eq.) was added drop-wise while maintaining the temperature at 0 °C. The reaction mixture was stirred for 8 h slowly warming to rt. DMF was removed under reduced pressure and the residue was poured into about 20 mL ice-cold 90% saturated KHCO$_3$ solution and stirred for 15 min. The precipitated compound was extracted into
chloroform and washed sequentially with 5% NaHCO₃ solution (2x20 mL), water (2x20 mL), 0.1N cold HCl solution (2x20 mL) followed by brine. The organic layer was dried over anhydrous Na₂SO₄ and the solvent was removed under reduced pressure. The crude product was purified on column chromatography using a mixture of chloroform and methanol in the ratio 9:1 as eluting system to get thioureas of KD/kD-conjugates (10-15) and KW/kW-conjugates (22-27). The physical and spectroscopic data of all the synthesized compounds are provided in Table 2.1.
Reagents and conditions: i) IBCF, NMM, -15±1 °C to rt; ii) 1N NaOH/ MeOH, rt; iii) EDCI/ HOBt, NMM, 0 °C to rt; iv) Pd–C (10%)/ HCOONH₄; v) TFA, 45 min, rt; vi) NMM, DMF, R–C₆H₄–N=C=S, 0 °C to rt, where R=H, OCH₃, F

Scheme 2.2. Schematic representation of the synthesis of bis-thiourea derivatives of KD/ kD-conjugates
Reagents and conditions: i) IBCF, NMM, -15±1 °C to rt; ii) 1N NaOH/ MeOH, rt; iii) EDCI/ HOBt, NMM, 0 °C to rt; iv) Pd–C (10%)/ HCOONH₄; v) TFA, 45 min, rt; vi) NMM, DMF, R–C₆H₄=N=C=S, 0 °C to rt, where R=H, OCH₃, F

Scheme 2.3. Schematic representation of the synthesis of bis-thiourea derivatives of KW/ kW-conjugates
### Table 2.1. Physical and spectroscopic data of dipeptides and their conjugates

<p>| No. | Color | ( R_f ) Values | ( R_f^a ) | ( R_f^b ) | Yield | M.P. °C | Theoretical Mol. Wt. | Actual Mass ( M^+/(M+1) ) | Molecular Formula | FTIR, ATR (cm(^{-1})) | (^1)H NMR (DMSO-(d_6), δ ppm) | (^1^3)C NMR (DMSO-(d_6), δ ppm) |
|-----|-------|------------------|----------|----------|-------|--------|----------------------|-----------------------------|-----------------|------------------|-------------------|------------------|------------------|
| 4   | White | 0.65             | 0.83     | 88.5     | 97-99 | 709.2766| 710.3890            | ( \text{C}<em>{37}\text{H}</em>{44}\text{ClN}_3\text{O}<em>9 ) | 3300-3150 (NH), 1735-1703 (CO) | Lys = 1.31-1.34 (6H, m, ( \delta</em>{\beta,\gamma,\delta})CH(_2)), 1.36 (9H, s, Boc), 2.88-2.95 (2H, m, ( \delta)CH(_2)), 3.89-3.90 (1H, q, ( \delta)CH), 5.08 (2H, s, benzyl-CH(_2)), 7.31-7.48 (4H, m, ArH), 7.48-7.49 (1H, m, ( \delta)NH), 8.31-8.35 (1H, d, ( \delta)NH); Asp = 2.76-2.82 (2H, d, ( \delta)CH(<em>2)), 4.74-4.76 (1H, q, ( \delta)CH), 5.07 (4H, s, benzyl-CH(<em>2)), 6.82-6.84 (1H, d, ( \delta)NH), 7.31-7.48 (10H, m, ArH) | 172.79, 170.93, 170.28, 156.23, 155.77, 135.67, 136.24, 136.11, 135.08, 132.76, 130.18, 130.14, 129.73, 128.88, 128.83, 128.51, 128.43, 128.25, 127.77, 78.44, 66.79, 66.39, 63.01, 54.49, 48.95, 46.22, 32.03, 29.52, 28.64, 23.17 |
|     |       | (S)-Dibenzyl ( 2-(\text{(S)-2-((}\text{tert-butoxycarbonyl)amino)\text{-6-(((2-chlorobenzyl)oxy)carbonyl)amino} \text{)hexanamido}) \text{succinate} ) | | | | | | | | |
| 5   | White | 0.64             | 0.83     | 89.0     | 97-99 | 709.2766| 710.3890            | ( \text{C}</em>{37}\text{H}</em>{44}\text{ClN}_3\text{O}<em>9 ) | 3300-3150 (NH), 1735-1703 (CO) | Lys = 1.31-1.35 (6H, m, ( \delta</em>{\beta,\gamma,\delta})CH(_2)), 1.37 (9H, s, Boc), 2.85-2.94 (2H, m, ( \delta)CH(_2)), 3.88-3.90 (1H, q, ( \delta)CH), 5.08 (2H, s, benzyl-CH(_2)), 7.31-7.48 (4H, m, ArH), 7.48-7.49 (1H, m, ( \delta)NH), 8.31-8.35 (1H, d, ( \delta)NH); Asp = 2.76-2.82 (2H, d, ( \delta)CH(_2)), 4.74-4.76 (1H, q, ( \delta)CH), 5.07 (4H, s, benzyl-CH(_2)), 6.82-6.84 (1H, d, ( \delta)NH), 7.31-7.48 (10H, m, ArH) | 172.80, 170.91, 170.29, 156.22, 155.74, 136.24, 136.12, 135.07, 132.78, 130.22, 130.15, 129.72, 128.87, 128.84, 128.55, 128.42, 128.25, 127.72, 78.43, 66.79, 66.42, 63.05, 54.42, 48.92, 36.28, 32.10, 29.51, 28.63, 23.19 |</p>
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(S)-2-((S)-2-((tert-Butoycarbonyl)amino)-6-(((2-chlorobenzyloxy)carbonylamino)hexanamido)succinic acid

![Chemical structure](image)

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(S)-2-((R)-2-((tert-Butoycarbonyl)amino)-6-(((2-chlorobenzyloxy)carbonylamino)hexanamido)succinic acid

![Chemical structure](image)
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<td>(S)-N-((S)-1,4-bis(4-(6-Fluorobenzol[d]isoxazol-3-yl)piperidine-1-yl)-1,4-dioxobutan-2-yl)-2,6-bis(3-phenylthioureido)hexanamide</td>
<td>C\textsubscript{48}H\textsubscript{51}F\textsubscript{2}N\textsubscript{9}O\textsubscript{5}S\textsubscript{2}</td>
<td>3320-3100 (NH), 1730-1705 (CO), 1525-1510 (CS)</td>
<td>Thiourea = 6.81-8.06 (10H, m, ArH), 9.48 (1H, br s, -^{6}NH), 9.80-9.83 (1H, m, -^{6}NH), 12.25 (2H, s, NH); Lys = 1.24-1.54 (6H, m, -^{6}CH\textsubscript{2}), 4.33-4.36 (2H, m, -^{6}CH\textsubscript{2}), 4.89 (1H, br s, -^{6}CH); Asp = 2.67-2.81 (2H, d, -^{6}CH\textsubscript{2}), 5.14 (1H, br s, -^{6}CH), 8.66-8.68 (1H, m, -^{6}NH), Heterocycle = 1.54-1.90 (8H, m, -CH\textsubscript{2}), 2.99-3.01 (2H, m, -CH), 3.17-4.04 (8H, m, -CH\textsubscript{2}), 6.81-8.06 (6H, m, ArH)</td>
<td>179.45, 179.20, 172.05, 168.82, 167.21, 165.45, 163.79, 163.65, 134.09, 133.24, 131.91, 126.32, 125.70, 117.25, 114.92, 107.42, 107.23, 104.90, 104.52, 57.16, 44.72, 43.29, 35.25, 33.18, 29.03, 25.50, 22.72, 21.56</td>
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<td>C\textsubscript{50}H\textsubscript{55}F\textsubscript{2}N\textsubscript{9}O\textsubscript{5}S\textsubscript{2}</td>
<td>3320-3100 (NH), 1730-1705 (CO), 1520-1505 (CS)</td>
<td>Thiourea = 3.88 (6H, s, -^{6}OCH\textsubscript{3}), 6.77-8.06 (8H, m, ArH), 9.25-9.31 (1H, m, -^{6}NH), 9.61-9.64 (1H, m, -^{6}NH), 12.22 (2H, s, NH); Lys = 1.22-1.39 (6H, m, -^{6}CH\textsubscript{2}), 4.34-4.36 (2H, m, -^{6}CH\textsubscript{2}), 4.87 (1H, br s, -^{6}CH); Asp = 2.67-2.82 (2H, d, -^{6}CH\textsubscript{2}), 5.13 (1H, br s, -^{6}CH), 8.64-8.68 (1H, m, -^{6}NH), Heterocycle = 1.52-1.81 (8H, m, -CH\textsubscript{2}), 2.98-3.00 (2H, m, -CH), 3.17-4.04 (8H, m, -CH\textsubscript{2}), 6.77-8.06 (6H, m, ArH)</td>
<td>181.01, 179.45, 171.95, 168.85, 167.22, 165.75, 163.83, 163.55, 156.87, 134.12, 132.94, 131.95, 126.32, 125.82, 118.25, 114.04, 107.35, 107.33, 105.12, 104.62, 57.28, 55.64, 44.02, 43.34, 35.30, 33.17, 29.04, 25.52, 22.59, 21.50</td>
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<td>C$<em>{48}$H$</em>{49}$F$<em>{4}$N$</em>{9}$O$<em>{5}$S$</em>{2}$</td>
<td>3320-3100 (NH), 1730-1705 (CO), 1520-1505 (CS)</td>
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<td>C_{50}H_{33}F_{2}N_{9}O_{7}S_{2}</td>
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<td>181.05, 179.52, 171.90, 168.84, 167.34, 165.95, 163.42, 163.12, 156.80, 134.13, 132.46, 131.31, 126.15, 125.18, 118.40, 114.09, 107.43, 107.30, 105.10, 104.65, 57.29, 55.58, 44.16, 43.47, 35.20, 33.12, 29.18, 25.76, 22.24, 21.54</td>
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<td>181.21, 180.70, 172.20, 168.45, 167.92, 165.20, 163.96, 163.52, 163.20, 134.27, 133.18, 131.80, 126.09, 125.60, 117.32, 114.75, 107.20, 107.14, 104.28, 104.10, 58.20, 43.05, 42.23, 35.76, 33.18, 29.44, 25.35, 22.68, 21.20</td>
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<td>tert-butyl 2-Chlorobenzyl ((S)-6-(((S)-1-((6-fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl))-3-(1H-indol-3-yl)-1-oxopropan-2-yl)amino)-6-oxohexane-1,5-diyl)dicarbamate</td>
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C_{42}H_{48}ClF_{6}N_{6}O_{7}
\]

3310-3150 (NH), 1730-1700 (CO)

Lys = 1.31-1.84 (6H, m, 2\(\beta\gamma\delta\) CH\(_2\)), 1.34 (9H, s, Boc), 2.91-2.95 (2H, m, 2\(\alpha\) CH\(_2\)), 4.32-4.39 (1H, m, 2\(\alpha\) CH\(_2\)), 5.03 (2H, s, benzyl-CH\(_2\)), 6.91-7.70 (4H, m, ArH), 7.89-8.05 (1H, m, 3\(\alpha\)NH), 8.14-8.27 (1H, d, 3\(\alpha\)NH); Trp = 2.70-2.93 (2H, m, 2\(\beta\) CH\(_2\)), 5.02-5.03 (1H, m, 3\(\alpha\) CH\(_2\)), 6.84-6.91 (1H, m, 3\(\alpha\)NH), 6.91-7.70 (5H, m, ArH), 10.85 (1H, s, NH), Heterocycle = 1.86-1.92 (4H, m, -CH\(_2\)), 2.46-2.67 (1H, m, -CH), 3.04-3.89 (4H, m, -CH\(_2\)), 6.91-7.70 (3H, m, ArH)

172.06, 170.17, 169.76, 165.28, 163.38, 162.81, 161.10, 160.84, 156.20, 155.72, 136.50, 135.07, 132.71, 130.00, 129.64, 127.78, 127.66, 124.19, 124.08, 123.97, 121.29, 118.78, 118.59, 118.49, 117.41, 113.01, 112.76, 111.76, 110.12, 109.93, 97.82, 97.55, 78.50, 54.89, 49.45, 49.07, 45.24, 41.90, 41.59, 33.65, 33.54, 32.09, 30.66, 29.95, 29.71, 29.45, 29.04, 28.58, 28.42, 23.17

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\[
C_{42}H_{48}ClF_{6}N_{6}O_{7}
\]

3310-3150 (NH), 1730-1700 (CO)

Lys = 1.30-1.82 (6H, m, 2\(\beta\gamma\delta\) CH\(_2\)), 1.36 (9H, s, Boc), 2.91-2.98 (2H, m, 2\(\alpha\) CH\(_2\)), 4.30-4.32 (1H, m, 3\(\alpha\) CH\(_2\)), 5.03 (2H, s, benzyl-CH\(_2\)), 6.90-7.72 (4H, m, ArH), 7.89-8.03 (1H, m, 3\(\alpha\)NH), 8.12-8.25 (1H, d, 3\(\alpha\)NH); Trp = 2.70-2.92 (2H, m, 2\(\beta\) CH\(_2\)), 5.02-5.04 (1H, m, 3\(\alpha\) CH\(_2\)), 6.84-6.91 (1H, m, 3\(\alpha\)NH), 6.92-7.71 (5H, m, ArH), 10.84 (1H, s, NH), Heterocycle = 1.84-1.94 (4H, m, -CH\(_2\)), 2.49-2.68 (1H, m, -CH), 3.02-3.88 (4H, m, -CH\(_2\)), 6.91-7.70 (3H, m, ArH)

172.26, 170.28, 169.70, 165.65, 163.34, 162.83, 161.25, 160.65, 156.18, 155.38, 136.56, 135.19, 132.35, 130.09, 129.63, 127.69, 127.37, 124.24, 124.05, 123.90, 121.37, 118.98, 118.70, 118.50, 117.26, 113.09, 112.70, 111.59, 110.10, 109.92, 97.80, 97.65, 78.58, 62.95, 54.64, 49.54, 49.19, 45.58, 41.91, 41.69, 33.93, 33.55, 32.01, 30.69, 29.95, 29.76, 29.40, 29.16, 28.63, 28.68, 23.10
Chapter 2

Brown 0.58 0.70 75.0 119-123 804.3040 805.9471

(S)-N-((S)-1-(4-(6-Fluorobenzofl)isoaxol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2,6-bis(3-phenylthioureido)hexanamide

C_{43}H_{55}FN_{8}O_{5}S_{2}

3320-3110 (NH), 1730-1700 (CO), 1550-1510 (CS)

Thiourea = 6.75-7.72 (10H, m, ArH), 8.56-8.66 (1H, m, -NH), 9.39-9.43 (1H, m, -NH), 9.72-9.76 (1H, s, NH), 12.20 (1H, s, NH); Lys = 1.16-1.31 (6H, m, -CH_{2}), 4.29-4.33 (2H, m, -CH_{2}), 4.98 (1H, br s, -CH); Trp = 2.61-2.84 (2H, d, -CH_{2}), 4.98 (1H, br s, -CH), 6.75-7.72 (5H, m, ArH), 7.91-7.97 (1H, m, -NH), 10.80 (1H, s, NH); Heterocycle = 1.50-1.94 (4H, m, -CH_{2}), 2.99-3.01 (1H, m, -CH), 3.17-4.04 (4H, m, -CH_{2}), 6.75-7.72 (3H, m, ArH)

180.82, 180.43, 171.38, 169.95, 169.67, 167.90, 165.37, 163.54, 139.82, 136.51, 133.88, 129.84, 127.72, 124.41, 124.03, 123.40, 121.30, 118.80, 118.58, 117.27, 111.79, 110.29, 110.03, 107.58, 107.36, 104.82, 104.58, 63.46, 60.85, 57.10, 56.91, 49.73, 44.86, 44.25, 43.78, 43.50, 41.47, 41.28, 40.66, 32.88, 32.35, 31.95, 30.08, 29.39, 29.05, 28.84, 28.52, 28.11, 22.78

White 0.51 0.64 73.0 124-126 864.3251 865.9445

(S)-N-((S)-1-(4-(6-Fluorobenzofl)isoaxol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2,6-bis(3-(4-methoxyphenyl)thioureido)hexanamide

C_{43}H_{49}FN_{8}O_{5}S_{2}

3320-3110 (NH), 1730-1700 (CO), 1540-1510 (CS)

Thiourea = 3.72 (6H, s, -OCH_{3}), 6.75-7.52 (8H, m, ArH), 8.50-8.55 (1H, m, -NH), 9.20-9.23 (1H, m, -NH), 9.53-9.59 (1H, s, NH), 12.23 (1H, s, NH); Lys = 1.19-1.78 (6H, m, -CH_{2}), 4.28-4.36 (2H, m, -CH_{2}), 4.95 (1H, br s, -CH); Trp = 2.60-2.82 (2H, d, -CH_{2}), 4.95 (1H, br s, -CH), 6.75-7.52 (5H, m, ArH), 7.91-7.94 (1H, m, -NH), 10.82 (1H, s, NH); Heterocycle = 1.52-1.96 (4H, m, -CH_{2}), 2.95-3.02 (1H, m, -CH), 3.18-4.05 (4H, m, -CH_{2}), 6.75-7.52 (3H, m, ArH)

181.10, 180.75, 171.41, 171.24, 169.93, 169.62, 167.87, 165.85, 163.55, 163.69, 156.87, 136.50, 133.89, 132.31, 127.71, 126.21, 125.47, 124.14, 124.03, 121.30, 118.80, 118.60, 118.46, 117.27, 117.10, 114.32, 111.86, 111.79, 110.28, 110.03, 107.60, 107.36, 105.82, 104.58, 57.19, 55.64, 49.71, 44.87, 44.31, 43.78, 43.51, 41.27, 32.91, 28.97, 28.52, 28.34, 28.14, 22.71
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<td>(S)-N-((S)-1-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2,6-bis(3-(4-fluorophenyl)thioureido)hexanamide</td>
<td>C_{43}H_{43}F_{3}N_{8}O_{3}S_{2}</td>
<td>3320-3110 (NH), 1730-1700 (CO), 1560-1520 (CS)</td>
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<td>Thiourea = 6.75-7.76 (8H, m, ArH), 8.26-8.48 (1H, m, -^2^2NH), 9.28-9.30 (1H, m, -^3^3NH), 9.69-9.72 (1H, s, NH), 12.22 (1H, s, NH); Lys = 1.19-1.74 (6H, m, -^β,γ,δ^CH_{2}), 4.28-4.34 (2H, m, -^ε^CH_{2}), 4.95 (1H, br s, -^α^CH); Trp = 2.62-2.83 (2H, d, -^β^CH_{2}), 5.00 (1H, br s, -^α^CH), 6.75-7.76 (5H, m, ArH), 7.91-7.97 (1H, m, -^6^6NH), 10.81 (1H, s, NH), Heterocycle = 1.54-1.97 (4H, m, -^CH_{2}), 2.94-3.04 (1H, m, -^CH), 3.15-4.06 (4H, m, -^CH_{2}), 6.75-7.76 (3H, m, ArH)</td>
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<td>C_{43}H_{43}F_{3}N_{8}O_{3}S_{2}</td>
<td>3320-3110 (NH), 1730-1700 (CO), 1550-1510 (CS)</td>
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<td>Thiourea = 6.72-7.74 (10H, m, ArH), 8.55-8.66 (1H, m, -^2^2NH), 9.40-9.43 (1H, m, -^2^2NH), 9.70-9.75 (1H, s, NH), 12.21 (1H, s, NH); Lys = 1.17-1.35 (6H, m, -^β,γ,δ^CH_{2}), 4.28-4.35 (2H, m, -^ε^CH_{2}), 4.95 (1H, br s, -^α^CH); Trp = 2.62-2.83 (2H, d, -^β^CH_{2}), 4.97 (1H, br s, -^α^CH), 6.72-7.74 (5H, m, ArH), 7.90-7.95 (1H, m, -^6^6NH), 10.82 (1H, s, NH), Heterocycle = 1.52-1.94 (4H, m, -^CH_{2}), 2.98-3.02 (1H, m, -^CH), 3.18-4.05 (4H, m, -^CH_{2}), 6.72-7.74 (3H, m, ArH)</td>
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<td>26</td>
<td>(R)-N-((S)-1-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2,6-bis(3-(4-methoxyphenyl)thioureido)hexanamide</td>
<td>C$<em>{45}$H$</em>{49}$FN$<em>{3}$O$</em>{5}$S$_{2}$</td>
<td>3320-3110 (NH), 1730-1700 (CO), 1540-1510 (CS)</td>
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<td>Thiourea = 3.74 (6H, s, -OCH$<em>{3}$), 6.74-7.52 (8H, m, ArH), 8.52-8.56 (1H, m, -NH), 9.20-9.23 (1H, m, -$^{4}$NH), 9.52-9.57 (1H, s, NH), 12.22 (1H, s, NH); Lys = 1.20-1.79 (6H, m, $^{1}$-CH$</em>{2}$), 4.29-4.36 (2H, m, $^{2}$-CH$_{2}$), 4.96 (1H, br s, $^{4}$CH)</td>
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<td>Trp = 2.62-2.81 (2H, d, $^{18}$-CH$<em>{2}$), 4.95 (1H, br s, $^{1}$-CH), 6.74-7.52 (5H, m, ArH), 7.91-7.93 (1H, m, -NH), 10.84 (1H, s, NH), Heterocycle = 1.50-1.90 (4H, m, -CH$</em>{2}$), 2.90-3.01 (1H, m, -CH), 3.14-4.06 (4H, m, -CH$_{2}$), 6.74-7.52 (3H, m, ArH)</td>
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<td>181.18, 180.72, 171.54, 171.18, 169.84, 169.45, 167.76, 165.36, 165.35, 163.85, 163.52, 156.88, 136.55, 133.90, 132.34, 127.72, 126.28, 125.65, 124.19, 124.11, 121.29, 118.85, 118.69, 118.46, 117.20, 117.19, 114.32, 111.80, 111.79, 110.28, 110.19, 107.53, 107.28, 104.76, 104.63, 57.29, 55.56, 49.76, 44.82, 44.65, 43.23, 43.13, 41.19, 32.18, 28.53, 28.19, 28.09, 28.01, 22.73</td>
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<td>(R)-N-((S)-1-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2,6-bis(3-(4-fluorophenyl)thioureido)hexanamide</td>
<td>C$<em>{46}$H$</em>{43}$F$<em>{3}$N$</em>{8}$O$<em>{3}$S$</em>{2}$</td>
<td>3320-3110 (NH), 1730-1700 (CO), 1560-1520 (CS)</td>
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<td>Thiourea = 6.79-7.80 (8H, m, ArH), 8.24-8.43 (1H, m, -NH), 9.25-9.32 (1H, m, -NH), 9.70-9.73 (1H, s, NH), 12.20 (1H, s, NH); Lys = 1.18-1.73 (6H, m, $^{1}$-CH$<em>{2}$), 4.30-4.35 (2H, m, $^{2}$-CH$</em>{2}$), 4.98 (1H, br s, $^{4}$CH)</td>
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<td>Trp = 2.63-2.84 (2H, d, $^{18}$-CH$<em>{2}$), 5.01 (1H, br s, $^{4}$CH), 6.79-7.80 (5H, m, ArH), 7.92-7.96 (1H, m, -NH), 10.84 (1H, s, NH), Heterocycle = 1.55-1.96 (4H, m, -CH$</em>{2}$), 2.93-3.05 (1H, m, -CH), 3.14-4.06 (4H, m, -CH$_{2}$), 6.79-7.74 (3H, m, ArH)</td>
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<td>181.20, 180.25, 171.40, 171.26, 169.86, 169.53, 167.96, 165.26, 163.75, 160.56, 158.98, 136.85, 136.49, 133.80, 127.96, 125.96, 125.18, 124.10, 121.28, 118.65, 118.56, 118.43, 117.29, 115.64, 115.39, 111.98, 110.28, 107.65, 107.30, 104.89, 104.59, 57.65, 49.72, 49.59, 44.86, 44.23, 43.98, 43.53, 41.40, 41.29, 40.86, 33.09, 32.65, 29.10, 28.65, 28.23, 28.18, 28.08, 22.77</td>
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2.3. Biological studies

2.3.1. Antibacterial activity

The *in vitro* antibacterial activity was evaluated against human pathogens of both Gram-negative bacteria *Escherichia coli* (*E*. *coli*) and Gram-positive bacteria *Staphylococcus aureus* (*S*. *aureus*) by agar well diffusion method [4].

2.3.1.1. Materials and methods

The following materials were used for the testing:

a) Nutrient agar
b) Sterilized petridishes, pipettes and beakers
c) Sterilized 6 mm cork borer
d) 18-24 h old growth culture in nutrient broth
e) Sterilized test tubes containing solutions of test compounds in desired concentration

2.3.1.2. Preparation of media

Nutrient agar media was prepared by dissolving agar (40 g), bacteriological peptone (1 g), beef extract (5 g) and sodium chloride (5 g) in distilled water (1000 mL). The solution was adjusted to pH 7.0 to 7.4 by using NaOH solution (40%, appx 0.25 mL for 100 mL of nutrient broth) and then sterilized for 30 min at 15 lbs pressure in an autoclave.

2.3.1.3. Preparation of sub culture

One day prior to test, the microorganisms were inoculated into the sterilized nutrient broth and incubated at 37 °C for 24 h. On the day of testing, organisms were
sub-cultured into sterile nutrient broth. After incubating for 3 h, the growth thus obtained was used as inoculums for the test.

2.3.1.4. Sterilization of media and glass wares

The media used in the present study was sterilized in conical flask of suitable capacity by autoclaving at 15 lbs pressure for about 20 min. The cork borers, petridishes, test tubes and pipettes were sterilized in hot air oven at 160 °C for an h.

2.3.1.5. Procedure for antibacterial assay

Prior to the evaluation, the organisms were inoculated into sterile nutrient broth and incubated at 37 °C for 24 h. At the time of testing, bacteria were subcultured into 25 mL broth and maintained under laboratory condition for the growth of the organisms. The test compounds were prepared similar to drug concentration by dissolving 10 mg of samples in 10 mL of DMSO to get concentration of 1 mg/mL and further diluted to 50 µg/mL concentration. About 15-20 mL of liquefied nutrient agar media was prepared, sterilized and poured into sterile petri plates under aseptic conditions. The media was allowed to solidify, after solidification, with the help of cork borer (6 mm diameter) wells were made. The plates were inoculated with the test organism using spread plate method. The wells were filled with 0.1 mL of test solutions, positive control (streptomycin) and a negative control (DMSO). The plates were incubated at 37 °C for 24 h after which the zone of inhibition (in mm) was measured.

2.3.2. Antifungal activity

The *in vitro* antifungal activity was evaluated against two fungal species namely *Aspergillus niger* (*A. niger*) and *Fusarium moniliforme* (*F. moniliforme*) by agar well diffusion method [5].
2.3.2.1. Materials and methods

The following materials were used for the testing:

a) Sabourauds agar
b) Sterilized petridishes, pipettes and beakers
c) Sterilized 6 mm cork borer
d) Sterilized inoculation loops
e) 16-18 h old growth culture in Sabourauds agar
f) Sterile test tubes for the preparation of test compounds in desired concentration

2.3.2.2. Preparation of media

The media used for antifungal activity is also known as Sabourauds glucose agar media which was prepared by dissolving glucose (40 g), peptone (10 g) and agar (20 g) in 1000 mL distilled water. This solution was sterilized for 30 min and adjusted the pH to 7.4 using sterilized sodium hydroxide solution (40%, appx 0.1 mL for 100 mL of broth).

2.3.2.3. Preparation of sub-culture

Two days prior to the testing, the organisms were sub-cultured into sterile nutrient broth. After incubating the same for two days the growth obtained was used as inoculums for the test.

2.3.2.4. Sterilization of media

The culture media used was sterilized in an Erlenmeyer flask of suitable capacity in an autoclave at 15 lbs pressure for 45 min. The cork borer, petridishes and test tubes were sterilized in a hot air oven at 160 °C for 1 h.
2.3.2.5. Preparation of test samples

All the synthesized compounds and the standard, bavistin were dissolved in DMSO to get the respective stock solutions of concentration 200 µg/mL. For each antifungal assay, 50 µL of the stock solution was added to standard disc (6 mm diameter) at the center of the petriplate (10 µg/disc). DMSO was taken as negative control where as standard drug bavistin was used as the positive control.

2.3.2.6. Procedure for antifungal activity

Prior to the determination of antifungal activity, the fungi were subcultured on a sterile broth solution and the test compounds and bavistin were prepared by dissolving 10 mg of samples in 10 mL of DMSO to get concentration of 1 mg/mL and further diluted to 50 µg/mL concentration. The nutrient agar media was prepared, sterilized and poured onto sterile petri plate and was allowed for solidification. Fungal cultures were point inoculated onto a sterile media, with the help of cork borer wells and were filled with 0.1 mL bavistin, DMSO and test compounds. The plates were incubated at rt for 3-4 days and the zone of inhibition (in mm) was measured.

2.3.3. Antioxidant activities

2.3.3.1. DPPH assay

The radical scavenging activity of DPPH free radicals by synthesized compounds was determined according to the reported method [6]. Briefly, 500 µg/mL of test compounds was mixed at different concentrations (25, 50, 100, 200 and 300 µg/mL) with 1 mL of 0.1 mM DPPH in methanol solution and 450 µL of 50 mM Tris HCl buffer (pH 7.4), methanol (50 µL) only was used as the experimental control. After 30 min of incubation at rt, the reduction in the number of DPPH free radicals
was measured by reading the absorbance at 517 nm. AA and GA were used as standards. Percent inhibition was calculated from the following equation:

\[
\text{% Inhibition} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \right) \times 100
\]

2.3.3.2. DMPD assay

The DMPD radical scavenging ability of synthesized compounds was determined by the Fogliano et al., method [7] with slight modifications by Gulcin [8]. This assay is based on the capacity of the compounds to inhibit DMPD\(^{+}\) cation radical formation. Briefly, 105 mg of DMPD was dissolved in 5 mL of distilled water. Then, 1 mL of this solution was added to 100 mL of 0.1 M acetate buffer (pH 5.3). DMPD\(^{+}\) was produced by adding 0.3 mL ferric chloride (0.05 M) to this solution. Different concentrations of standard antioxidants or synthesized compounds (25, 50, 100, 200 and 300 µg/mL) were added, and the total volume was adjusted to 1 mL with distilled water. One mL of the DMPD\(^{+}\) solution was directly added to the reaction mixture. The reaction mixture was incubated in the dark for 15 min and the absorbance was measured at 505 nm.

\[
\text{% Inhibition} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \right) \times 100
\]

2.3.3.3. ABTS assay

The ability of the test sample to scavenge ABTS\(^{+}\) radical cation was determined according to the literature method with slight modifications [9]. The ABTS\(^{+}\) radical cation was pre-generated by mixing 7 mM ABTS\(^{+}\) stock solutions with 2.45 mM potassium persulfate (final concentration) and incubating for 12–16 h
in the dark at rt until the reaction was complete and the absorbance was stable. The absorbance of the ABTS$^+$ solution was equilibrated to 0.70 (± 0.02) by diluting with distilled water at rt, then 2 mL was mixed with different concentration of the test sample (25, 50, 100, 200, and 300 µg/mL) and the absorbance was measured at 734 nm after 6 min. The scavenging capability of ABTS$^+$ radical was calculated using the following equation:

$$\text{ABTS}^+ \text{ scavenging effect (\%)} = \left[ \frac{(A_c - A_s)}{A_c} \right] \times 100$$

where, $A_c$ is the initial concentration of the ABTS$^+$ and $A_s$ is the absorbance of the remaining concentration of ABTS$^+$ in the presence of compounds.

### 2.3.4. Anti-inflammatory activity

#### 2.3.4.1. Human erythrocyte suspension

The blood was collected in an EDTA coated tube from a healthy volunteer who had not taken any NSAIDs for 2 weeks prior to the experiment. The blood sample washed with 0.9% saline and centrifuged to 3000 rpm at 10 min for three times. Further, the blood suspension washed with 40% (v/v) of 0.9% saline made using isotonic phosphate buffer which was composed of 154 mM NaCl in 10 mM sodium phosphate buffer at pH 7.4 used as stock erythrocyte suspension.

#### 2.3.4.2. Hypotonic solution-induced haemolysis

The percentage inhibition of haemolysis activity of the synthesized compounds was measured following reported method [10] with slight modification. The test sample containing different concentrations of sample (25, 50, 100, 200 and 300 µg/mL) and 0.5 mL of stock erythrocyte suspension mixed with 5 mL of hypotonic solution (50 mM NaCl in 10 mM sodium phosphate buffer at pH 7.4). The control consists of 0.5 mL of stock erythrocyte suspension mixed with 5 mL of
hypotonic buffer solution. The standard drugs IM and IP were tested similar to test concentration. The test and control samples were incubated at rt for 10 min and centrifuged at 3000 rpm. The supernatant was collected and measured the absorbance at 540 nm spectrophotometrically. The percentage inhibition of haemolysis was calculated from the following equation.

\[
\text{% Inhibition of haemolysis} = \left(\frac{A_1 - A_2}{A_1}\right) \times 100
\]

Where:
\(A_1\) = Absorbance of hypotonic buffered solution alone
\(A_2\) = Absorbance of test/standard sample in hypotonic solution

2.4. Molecular docking studies

Maestro 9.3.5 version of the Schrodinger software suite, 2011 is used to obtain binding interaction of molecules with targeted site. The 3D crystallographic structure of proteins (PDB ID: 1JIJ, 1KZN, 2VF5, 2HCK and 1CX2) was retrieved from Protein Data Bank (www.rcsb.org/pdb). The lowest energy states of ligand with combination of all stereoisomers were achieved using LigPrep program and it was optimized by force field OPLS-2005 (Optimized Potential for Liquid Simulations). The protein structures were pre-processed, modified and refined by Protein Preparation Wizard. Further, it was minimized by OPLS-2005 force field. The protein and ligand interaction performed by generation of receptor grid in the target site of protein by GLIDE. Depending on the extent of docking the G scores/docking scores were produced it will determine the best fitted ligand to target protein.
2.5. Results and discussion

2.5.1. Chemistry

Four dipeptides were synthesized by classical solution phase method using Boc chemistry. The peptides were designed in such a way that they had both L and D configured amino acids since the latter have found to exert promising results in biological activity [11]. We chose Lys/lys and Asp since these are trifunctional amino acids wherein further modification are possible at two positions as against bifunctional amino acids. Further, Trp was used at the C-terminus keeping in mind the diverse biological applications and also our previous experience [12]. These peptides were then conjugated to Het A using EDCI/ HOBr as coupling agent and NMM as base. The temporary protecting groups at N$_{\alpha}$ and N$_{\varepsilon}$ of Lys/lys were removed either by hydrogenolysis or TFA treatment and converted into bis-thiourea derivatives by reacting with different substituted phenyl isothiocyanates in presence of NMM, a base. The yields of the compounds were found to be good and verified the structures by M.P., $^1$H NMR, $^{13}$C NMR and mass spectrometric techniques. The NMR and mass data were found to be in good agreement with the structures assigned. Further the synthetic peptides were incubated in alkaline solution at 37 °C for 24 h and observed that there was no change in their composition, properties and structure as revealed by spectroscopic data (not shown). This clearly demonstrated that the synthetic peptides are stable at physiological conditions.

2.5.2. Biology

2.5.2.1. Antimicrobial activity

The results obtained as zone of inhibition (mm) are presented in Table 2.2. It is observed that all the synthesized molecules exhibited the same trend of activity
with respect to both antibacterial and antifungal properties. The four dipeptides (4, 5, 16 and 17) exhibited moderate activity. When benzyl/methyl ester attached at the C-terminus was removed (6, 7, 18 and 19), there was a slight enhancement in the activity. This may be due to the increase in the polarity of the compounds which would help the molecule to penetrate more through the cell membrane of microbes and thereby inactivate them [13]. Further, attachment to Het A (8, 9, 10 and 11) enhanced the activity indicating that conjugation plays an important role in the activity. A drastic enhancement in the activity was observed when the above intermediates were converted into bis-thiourea derivatives. It was found that the increase in activity depends largely on the presence of substituent on the phenyl ring.

Among the derivatives synthesized, compounds bearing EWG (F) have exhibited high activity compared to the presence of electron donating moiety (OCH$_3$). The presence of fluorine in the molecules increase the lipophilicity and thus enhance the rate of cell penetration that leads to inhibition of the active site in the receptor [14]. The compounds without any substitution (i.e., presence of only -H) showed least activity than the substituted counterparts. The Trp containing dipeptides and their analogues (16-27) were found to be more potent antimicrobials than their corresponding Asp containing dipeptides and their analogues (4-15). This may be due to more hydrophobicity, aromatic indole ring and acid-base character of Trp [15]. Further, the dipeptide derivatives possessing D-Lys were found to be more active than the L-Lys confirming the importance of configuration in biological activities. The graphical representation of antibacterial and antifungal activities of the synthesized compounds are shown in Fig. 2.1. and 2.2. respectively.
2.5.2.2. Antioxidant activity

*In vitro* antioxidant activities of all the synthesized compounds were evaluated by DPPH, DMPD and ABTS cation radical assays. The IC$_{50}$ values, the effective concentration at which 50% of the radicals were scavenged, were calculated to evaluate the antioxidant activities. A lower IC$_{50}$ value indicated greater antioxidant activity. IC$_{50}$ values of lower than µg/mL usually implied effective activities in antioxidant properties [16]. The IC$_{50}$ of the standards were also determined for comparison. The results are tabulated in Table 2.3.

All the synthesized compounds showed antioxidant activities. The intermediates such as, protected dipeptides (4, 5, 16 and 17), deprotected dipeptides (6, 7, 18 and 19) and Het A exhibited radical scavenging activity in all the three assays at higher concentrations. Their conjugation (8, 9, 20 and 21) improved the activity significantly. Further, a substantial increase in the activity was observed when the conjugates were converted into *bis*-thiourea derivatives. This increase in activity depends on the presence of different substituents on the phenyl ring of thiourea derivatives.

Compounds 11, 14, 23 and 26 showed excellent radical scavenging activities with IC$_{50}$ values 40, 30, 25 and 20 µg/mL respectively in DPPH assay much better than the standards AA (55 µg/mL) and GA (50 µg/mL). In DMPD radical scavenging assay, the compounds 11, 14, 23 and 26 showed potent antioxidant activity with IC$_{50}$ values 45, 35, 30 and 25 µg/mL, respectively, which is much better than the commercial standards AA (IC$_{50}$ = 60 µg/mL) and GA (IC$_{50}$ = 55 µg/mL). The compounds 11, 14, 23 and 26 also exhibited striking antioxidant activity with IC$_{50}$ values 50, 35, 25 and 20 µg/mL, respectively, which is better than the standards AA (IC$_{50}$ = 50 µg/mL) and GA (IC$_{50}$ = 55 µg/mL) in ABTS$^+$ assay. The compounds
having OCH₃ (electron releasing) group in phenyl ring (11, 14, 23 and 26) were found to be most potent antioxidants which is also in accordance with earlier observations [17-20]. The compounds 10, 13, 22 and 25 without any substituents on the phenyl ring of thiourea derivatives and the compounds 12, 15, 24 and 27 with F, an electron withdrawing group on the phenyl ring of thiourea derivatives showed moderate antioxidant activity.

The antioxidant activity of the synthesized molecules also depends on the nature of the amino acids present. The analogues of KW/ kW-conjugates (16-27) were found to be more potent antioxidants than KD/ kD-conjugates (4-15). This may be due to high oxygen radical absorbance capacity of Trp i.e. the presence of indole ring and aromatic nature [21-23]. Further, the dipeptide derivatives with D-configuration (lys) were found to be more potent than the dipeptide derivatives with L-configuration (Lys) confirming the importance of configuration in biological activities [24, 25]. The graphical representation of antioxidant activity of synthesized compounds is provided in Fig. 2.3.

### 2.5.2.3. Anti-inflammatory activity

All the synthesized compounds were evaluated for in vitro anti-inflammatory activity using known literature procedure using human erythrocytes [10]. A substantial number of compounds have been identified to exhibit excellent to moderate inhibitory activity compared to standard drugs, IM and IP. IC₅₀ values of all the compounds are tabulated in Table 2.3.

All the synthesized compounds showed anti-inflammatory activities. The dipeptides (4, 5, 16 and 17) showed IC₅₀ of 260 µg/mL, 240 µg/mL, 130 µg/mL and 100 µg/mL respectively. The removal of carboxyl protection of dipeptides (6, 7, 18 and 19) led to slight increase in the activity and the IC₅₀ was 130 µg/mL, 105 µg/mL,
120 µg/mL and 90 µg/mL respectively. Which is agreement with our earlier observation [12]. The conjugation of these dipeptides with Het A (8, 9, 20 and 21) increased the anti-inflammatory activity to a greater extent and the IC50 = 90 µg/mL, 85 µg/mL, 75 µg/mL and 65 µg/mL respectively. The drastic enhancement in activity resulted from their conversion into bis-thiourea derivatives. The increase in activity depends on the presence of substituent on the phenyl ring.

The presence of EWG (F) in the phenyl ring of thiourea moiety favors the anti-inflammatory activity (12, 15, 24 and 27) and the IC50 was found to be 40 µg/mL, 35 µg/mL, 35 µg/mL and 25 µg/mL respectively. The presence of EDG (OCH3) in the phenyl ring decreased the activity (11, 14, 23 and 26) and the IC50 was found to be 120 µg/mL, 100 µg/mL, 100 µg/mL and 80 µg/mL respectively. The phenyl ring without any substitution (only H) showed less anti-inflammatory activity (10, 13, 22 and 25) and the IC50 was 80 µg/mL, 70 µg/mL, 65 µg/mL and 60 µg/mL respectively. These electronic effects were also well correlated with earlier results [26, 27]. Another interesting aspect of these results is that the Trp containing dipeptides and their analogues (16-27) were found to be more potent anti-inflammatory agents than Asp containing dipeptides and their analogues (4-15). This indicated that presence of aromatic and hydrophobic Trp is responsible for biological activities [12]. Further, the dipeptide derivatives with D-Lys was found to be more potent than the L-configuration confirming the importance of configuration in biological activities. The graphical representation of anti-inflammatory activity of synthesized compounds is provided in Fig. 2.4.

2.5.3. Molecular docking studies

In order to gain insight into the exact binding location of ligand and protein, the active molecules (10-15 and 22-27) including standards were subjected to
molecular docking with active site of *S. aureus* TyrRS (PDB ID: 1JIJ) and COX-2 (PDB ID: 1CX2) showed good binding interaction and G score.

The potentiality of compounds bind to the active site of protein was ranked based on glide score/docking score (Table 2.2. and 2.3.). The docking results of antimicrobial and anti-inflammatory studies revealed that the molecules (10-15 and 22-27) having binding affinity towards target protein with G score/docking score ranging from -7.8 to -6.5 and -6.7 to -5.3 respectively. Among all docked ligand the compound 27 with highest G score -7.8 and -6.7 may be considered as a good inhibitor of 1JIJ and 1CX2 proteins. The binding modes (2D and 3D) of compound 27 were illustrated in Fig. 2.5. and 2.6. With 1JIJ protein the NH group of thiourea moiety of compound 27 has two hydrogen bond interactions with Asp 195 and carbonyl group of Trp has hydrogen bond interaction with Lys 84. In addition, it showed π-π stacking interaction with His 47 and π-cation interaction with Arg 88. Furthermore, with 1CX2 protein the NH groups of thiourea moiety of 27 having four hydrogen bond interactions with Asp 124 and Asp 87. Along with this it has π-π stacking interaction with Lys 118 and π-cation interaction with Phe 30. Based on these results, it was concluded that the NH group of thiourea moiety, carbonyl group of Trp and indole side chain of Trp were responsible for interaction with active site of protein.
Table 2.2. Antimicrobial activity and docking score of bis-thiourea derivatives of dipeptide conjugated Het A.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Antibacterial activity (Zone of inhibition in mm)a</th>
<th>Antifungal activity (Zone of inhibition in mm)a</th>
<th>docking score with 1JJ</th>
</tr>
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<td>F. moniliforme</td>
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<td>BS</td>
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*aValues are mean of three determinations, the range of which are <5% of the mean in all cases; SM = Streptomycin; BS = Bavistin; '-' : no activity/ not analyzed
Table 2.3. Antioxidant and anti-inflammatory activities and docking score of the synthesized bis-thiourea derivatives of dipeptides conjugated to Het A.

<table>
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<tr>
<th>Entry</th>
<th>Antioxidant activity (IC$_{50}$ µg/mL)$^a$</th>
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$^a$ Values are mean of three determinations, the range of which are <5% of the mean in all cases; AA = Ascorbic acid; GA = Gallic acid; IM = Indomethacin; IP = Ibuprofen; ‘-’: no activity/ not analyzed
Fig. 2.1. Graphical representation of antibacterial activity of synthesized compounds against *E. coli* and *S. aureus*.

Fig. 2.2. Graphical representation of antifungal activity of synthesized compounds against *F. moniliforme* and *A. niger*. 
Fig. 2.3. Graphical representation of antioxidant activity of synthesized compounds

Fig. 2.4. Graphical representation of anti-inflammatory activity of synthesized compounds
Fig. 2.5. 2D and 3D images of compound 27 with 1IJJ protein

Fig. 2.6. 2D and 3D images of compound 27 with 1CX2 protein
2.6. Conclusion

In summary, we have studied the antimicrobial, antioxidant and anti-inflammatory activities of synthesized molecules and compared the biological assay data with molecular docking studies. The results revealed that compounds 12, 15, 24 and 27 containing EWG (F) in the phenyl ring of the thiourea moiety exhibited excellent antimicrobial and anti-inflammatory activities and showed good binding interaction with active site of 1JIJ and 1CX2 proteins. Compounds 11, 14, 23 and 26 EDG (OCH₃) in phenyl ring of the thiourea moiety showed excellent antioxidant properties. Furthermore, from docking study we have strong evidence that configuration of amino acids in dipeptide and its analogs play a significant role in the biological activities.
2.7. $^1$H, $^{13}$C NMR and mass spectra of the representative compounds

Fig. 2.7. $^1$H NMR spectrum of (S)-Methyl 2-((S)-2-((tert-butoxycarbonyl)amino)-6-(((2-chlorobenzyl)oxy)carbonyl)amino)hexanamido)-3-(1H-indol-3-yl)propanoate
(Compound 16)
Fig. 2.8. $^{13}$C NMR spectrum of (S)-Methyl 2-((S)-2-((tert-butoxycarbonyl)amino)-6-(((2-chlorobenzyl)oxy)carbonyl)amino)hexanamido)-3-(1H-indol-3-yl)propanoate
(Compound 16)
Fig. 2.9. Mass spectrum of (S)-Methyl 2-((S)-2-((tert-butoxycarbonyl)amino)-6-(((2-chlorobenzyl)oxy)carbonyl)amino)hexanamido)-3-(1H-indol-3-yl)propanoate  
(Compound 16)
Fig. 2.10. $^1$H NMR spectrum of tert-butyl 2-Chlorobenzyl ((S)-6-(((S)-1-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)amino)-6-oxohexane-1,5-diyl)dicarbamate

(Compound 20)
Fig. 2.11. $^{13}$C NMR spectrum of tert-butyl 2-Chlorobenzyl ((S)-6-(((S)-1-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)amino)-6-oxohexane-1,5-diyl)dicarbamate (Compound 20)
Fig. 2.12. Mass spectrum of tert-butyl 2-Chlorobenzyl ((S)-6-(((S)-1-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)amino)-6-oxohexane-1,5-diyl)dicarbamate (Compound 20)
Fig. 2.13. $^1$H NMR spectrum of (S)-N-(S)-1-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2,6-bis(3-phenylthioureido)hexanamide (Compound 22)
Fig. 2.14. $^{13}$C NMR spectrum of (S)-N-((S)-1-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2,6-bis(3-phenylthioureido)hexanamide
(Compound 22)
Fig. 2.15. Mass spectrum of (S)-N-((S)-1-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2,6-bis(3-phenylthioureido)hexanamide
(Compound 22)
Fig. 2.16. $^1$H NMR spectrum of (S)-N-((S)-1-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2,6-bis(3-(4-methoxyphenyl)thioureido)hexanamide
(Compound 23)
Fig. 2.17. $^{13}$C NMR spectrum of (S)-N-((S)-1-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)-3-((1H-indol-3-yl)-1-oxopropan-2-yl)-2,6-bis(3-(4-methoxyphenyl)thioureido)hexanamide

(Compound 23)
Fig. 2.18. Mass spectrum of (S)-N-((S)-1-(4-(6-Fluorobenzodisoxazol-3-yl)piperidin-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-2,6-bis(3-(4-methoxyphenyl)thioareido)hexanamide (Compound 23)
2.8. References