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Characterizing and Evaluating the Pharmacological Potentials of Synthesized Cyclic Tetra Peptides: Antioxidant, COX Inhibitory, and Antimicrobial Perspectives

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ABSTRACT:

This study aimed to synthesize and characterize cyclic tetrapeptides (CTPs) with potential pharmacological activities. The cyclic tetrapeptides were synthesized using N-methylmorpholine (NMM) as a base to facilitate the efficient cyclization of linear tetrapeptide segments. The percentage yields ranged from 77.5% to 81.6%, and the Rf values for CTP-1 and CTP-2 were 0.74 and 0.76, respectively. The synthesized CTPs were characterized by IR and 1H NMR spectroscopy. The disappearance of specific absorption bands and singlets corresponding to ester and tert-butyl groups confirmed successful cyclization. Antimicrobial activity was evaluated against *Staphylococcus aureus* and *Escherichia coli* by measuring the zones of inhibition (ZOI) at various concentrations. CTP-1 and CTP-2 demonstrated significant antimicrobial activity, with CTP-2 showing higher efficacy than Ciprofloxacin at 500 µg/mL. The COX-1 and COX-2 inhibitory assays indicated that both CTP-1 and CTP-2 exhibited significant inhibition at 250 µg/mL, suggesting strong anti-inflammatory properties. Additionally, the ABTS radical decolorization assay revealed concentration-dependent scavenging activity, with IC50 values comparable to quercetin and vitamin C. In conclusion, the synthesized cyclic tetrapeptides exhibited promising antimicrobial, anti-inflammatory, and antioxidant activities, highlighting their potential as therapeutic agents and warranting further pharmacological exploration.

Keyword: Peptide, Cyclic tetrapeptide, Antibacterial, ABTS radical, Cyclooxygenase

1. Introduction

Peptides, short chains of amino acids linked by peptide bonds, play crucial roles in various biological processes. They serve as hormones, neurotransmitters, growth factors, and antibiotics, among other functions. Peptides can be linear or cyclic, with the latter having unique structural and functional advantages. Cyclic peptides, characterized by their ring structure formed through head-to-tail cyclization or through side chain-to-side chain links, have garnered significant interest in research and therapeutic applications due to their enhanced stability, specificity, and bioavailability. Linear peptides are often susceptible to enzymatic degradation and conformational flexibility, which can limit their therapeutic efficacy. Cyclic peptides, however, exhibit increased resistance to proteolytic enzymes due to their constrained cyclic structure. This structural rigidity not only enhances their stability but also improves their binding affinity and selectivity towards target molecules (Zhao et al., 2024, Zhang et al., 2024,

Tran et al., 2024, Jiang et al., 2024, Ji et al., 2024). The constrained conformation of cyclic peptides restricts their spatial arrangement, often leading to a higher degree of specificity in their interactions with biological targets. The synthesis of cyclic peptides can be achieved through various chemical and enzymatic methods. Chemical synthesis typically involves the use of solid-phase peptide synthesis (SPPS), followed by cyclization in solution. The choice of cyclization strategy, such as head-to-tail, side chain-to-side chain, or backbone cyclization, depends on the desired properties and target applications. Enzymatic cyclization, utilizing enzymes like transpeptidases or cyclases, offers a more environmentally friendly approach and can provide high regio- and stereoselectivity (Tran et al., 2024, Jafarzadeh et al., 2024).

One of the primary advantages of cyclic peptides over linear peptides is their enhanced metabolic stability. This stability results from their resistance to proteolytic degradation, making them suitable for oral administration and increasing their half-life in vivo. Additionally, the rigid structure of cyclic peptides reduces the entropic penalty upon binding to target proteins, leading to improved binding affinity and specificity (Abdalla and McGaw, 2018, Poongavanam et al., 2024, Mehta, 2024, Liu et al., 2024, Lin et al., 2024, Lee et al., 2024). Cyclic peptides have shown promise in a wide range of therapeutic applications. They have been investigated as potential drugs for treating cancer, infectious diseases, autoimmune disorders, and cardiovascular diseases. For instance, the cyclic peptide drug cyclosporine is widely used as an immunosuppressant to prevent organ rejection in transplant patients. Another notable example is the cyclic peptide antibiotic daptomycin, effective against multi-drug-resistant Gram-positive bacteria. The synthesis of cyclic peptides is driven by their unique structural and functional advantages over linear peptides (Dahiya et al., 2019). Cyclic peptides exhibit increased stability due to their constrained cyclic structure, which protects them from proteolytic degradation. This enhanced stability translates to a longer half-life and improved bioavailability, making them suitable for therapeutic applications. The rigid conformation of cyclic peptides allows for high specificity and affinity towards target molecules, reducing the likelihood of off-target interactions and potential side effects. This specificity is crucial in pharmacological applications, where precision in targeting diseased cells or pathogens is essential. Cyclic peptides have demonstrated significant biological activities, including antimicrobial, anti-inflammatory, and antioxidant properties. These activities make them promising candidates for treating a variety of conditions, such as infections, inflammatory diseases, and oxidative stress-related disorders. For example, cyclic peptides like cyclosporine and daptomycin have proven effective as immunosuppressants and antibiotics, respectively. Synthesizing cyclic peptides enables the exploration of their structure-activity relationships, providing insights into designing more potent and selective therapeutic agents (Wang et al., 2019, Poongavanam et al., 2024, Mehta, 2024, Liu et al., 2024, Lin et al., 2024, Lee et al., 2024). By evaluating their pharmacological activities, researchers can identify potential drug candidates with superior efficacy and safety profiles, ultimately contributing to the development of novel therapeutic strategies. Taking into account all this information and data, this present research work was designed to synthesize cyclic tetrapeptides and perform characterization followed by pharmacological evaluation. As part of the battery of pharmacological evaluation tests, antimicrobial activity, cyclooxygenase inhibition and free radical scavenging were evaluated.

2. Materials and Methods

Chemical, Reagents and Instruments

The drug samples, which included Indomethacin, Amphotericin B, and Ciprofloxacin, were given as gifts by pharmaceutical companies. Every other chemical and reagent utilised in the

synthesis and assessment procedures was obtained solely from authorised suppliers and was of both synthetic and analytical quality.

Physico-chemical properties and characterizations

Using silica gel G plates and thin-layer chromatography (TLC), all of the chemical reactions were investigated. The developing solvent system used was a 8:2 mixture of methanol and chloroform. In a firmly closed room, brown patches appeared upon exposure to iodine vapors. Using a melting point device and an open capillary approach, the melting points of the synthesized compounds were ascertained. The Fourier-transform infrared (FTIR) spectra of the compounds, prepared in potassium bromide (KBr) pellets, were recorded on a FTIR-RXI spectrophotometer (PERKIN ELMER). The proton nuclear magnetic resonance (^1H-NMR) spectral data of the synthesized compounds were documented with the assistance of a NMR spectrophotometer (Bruker). These measurements were conducted in deuterium-substituted chloroform using tetramethylsilane (TMS) as the internal standard, with chemical shifts reported in delta (δ) parts per million (ppm).

Synthetic Procedure for Linear Peptide Fragments

Synthesis of Boc-amino acids (1)

L-Tyrosine (3.62 g, 0.02 mol) was dissolved in a solution of 1 mol/L sodium hydroxide (NaOH, 20 mL) and isopropanol (20 mL). To this mixture, di-tert-butyl dicarbonate (Boc2O, 6 mL, 0.026 mol) in isopropanol (10 mL) was added. Subsequently, an additional 20 mL of 1 mol/L NaOH was introduced into the solution. The resulting mixture was stirred at room temperature for 2 hours. Upon completion of the reaction, the mixture was washed with light petroleum ether (boiling point 40–60°C, 20 mL) to remove impurities. The aqueous layer was then acidified to pH 3.0 using 1 mol/L sulfuric acid (H2SO4). The acidic solution was extracted three times with chloroform (3 × 20 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure to yield the crude Boc-Tyrosine product. The crude product was subsequently crystallized from a mixture of chloroform and petroleum ether (boiling point 40–60°C) to obtain pure Boc-Tyrosine (1) (Dahiya et al., 2006). In a similar manner, pure pro (1c), pure glycine (1b), and pure leucine (1a) were each synthesised.

Synthetic procedure for L-amino acid methyl ester hydrochlorides (2)

To prepare L-amino acid methyl ester hydrochlorides, 1.4 mL of thionyl chloride (0.02 mol) was added slowly to 100 mL of methanol at 0°C. Once the thionyl chloride was fully incorporated, 1.78 g of L-Alanine (0.02 mol) was introduced into the solution. The mixture was then refluxed for 8-10 hours at a temperature of 100-110°C. After the reflux period, the resulting mixture was evaporated to remove the solvent. The excess dimethylsulfite was removed by triturating the residue with ether at 0°C. The crude solid obtained was crystallized using a mixture of methanol and ether at 0°C. This process yielded pure L-Alanine methyl ester hydrochloride (2). Following this procedure, other L-amino acid methyl ester hydrochlorides were synthesized similarly:

- L-Serine methyl ester hydrochloride (2a)
- L-Histidine methyl ester hydrochloride (2b)
- L-Glutamic acid methyl ester hydrochloride (2c)

Synthesis of Boc-dipeptide methyl esters (3)

To synthesize Boc-dipeptide methyl esters, 2.3 mL of N-methylmorpholine (NMM, 0.021 mol) was added to a mixture of 1.05 g of compound 2 (0.01 mol) in 20 mL of chloroform (CHCl3) at 0°C. The resulting mixture was stirred for 15 minutes. Subsequently, 2.81 g of compound 1

(0.01 mol) in 20 mL of CHCl3 was added. To this mixture, 2.1 g of dicyclohexylcarbodiimide (DCC, 0.01 mol) was introduced with continuous stirring. The mixture was stirred for 36 hours. After this period, the resulting mixture was filtered, and the residue was washed with 30 mL of CHCl3. The filtrate was washed sequentially with 5% sodium bicarbonate (NaHCO3) solution and 25 mL of saturated sodium chloride (NaCl) solution. The organic layer was dried over anhydrous sodium sulfate (Na2SO4), filtered, and evaporated under vacuum. The crude product was crystallized from a mixture of chloroform and petroleum ether (boiling point 40–60°C) and kept at 0°C to obtain pure Boc-Tyr-Ala-OMe (3). Following this procedure, other Boc-dipeptide methyl esters were synthesized similarly:

- Boc-Leu-Ser-OMe (3a)
- Boc-Gly-His-OMe (3b)
- Boc-Pro-Glu-OMe (3c)

Deprotection of dipeptide at carboxyl end (**4**)

To deprotect the dipeptide at the carboxyl end, 0.36 g of lithium hydroxide (LiOH) was added to a solution of 3.66 g of compound 3 (0.01 mol) in a 1:1 mixture of tetrahydrofuran (THF) and water (36 mL) at 0-5°C. The reaction mixture was then stirred at room temperature for 1 hour. Following the reaction, the solution was acidified to pH 3.5 using 0.5 mol/L sulfuric acid (H2SO4). The aqueous layer was extracted three times with 25 mL portions of diethyl ether. The combined organic extracts were dried over anhydrous sodium sulfate (Na2SO4) and concentrated under reduced pressure. The crude product was crystallized from a mixture of methanol and ether to obtain pure Boc-Tyr-Ala-OH (4). Following this procedure, Boc-Gly-His-OH (4a) was synthesized similarly.

Deprotection of dipeptide at amino group (**5**)

To deprotect the dipeptide at the amino group, 3.32 g of compound 3a (0.004 mol) was dissolved in 35 mL of chloroform (CHCl3). To this solution, 0.91 g of trifluoroacetic acid (TFA, 0.008 mol) was added, and the mixture was stirred at room temperature for 1 hour. This reaction yielded Leu-Ser-OMe (5a). Following this procedure, the following compounds were synthesized in the same manner:

- Leu-Ser-OMe (5b)
- Boc-Pro-Glu-OMe (5c)

Synthesis of Boc-tetrapeptide methyl esters (6)

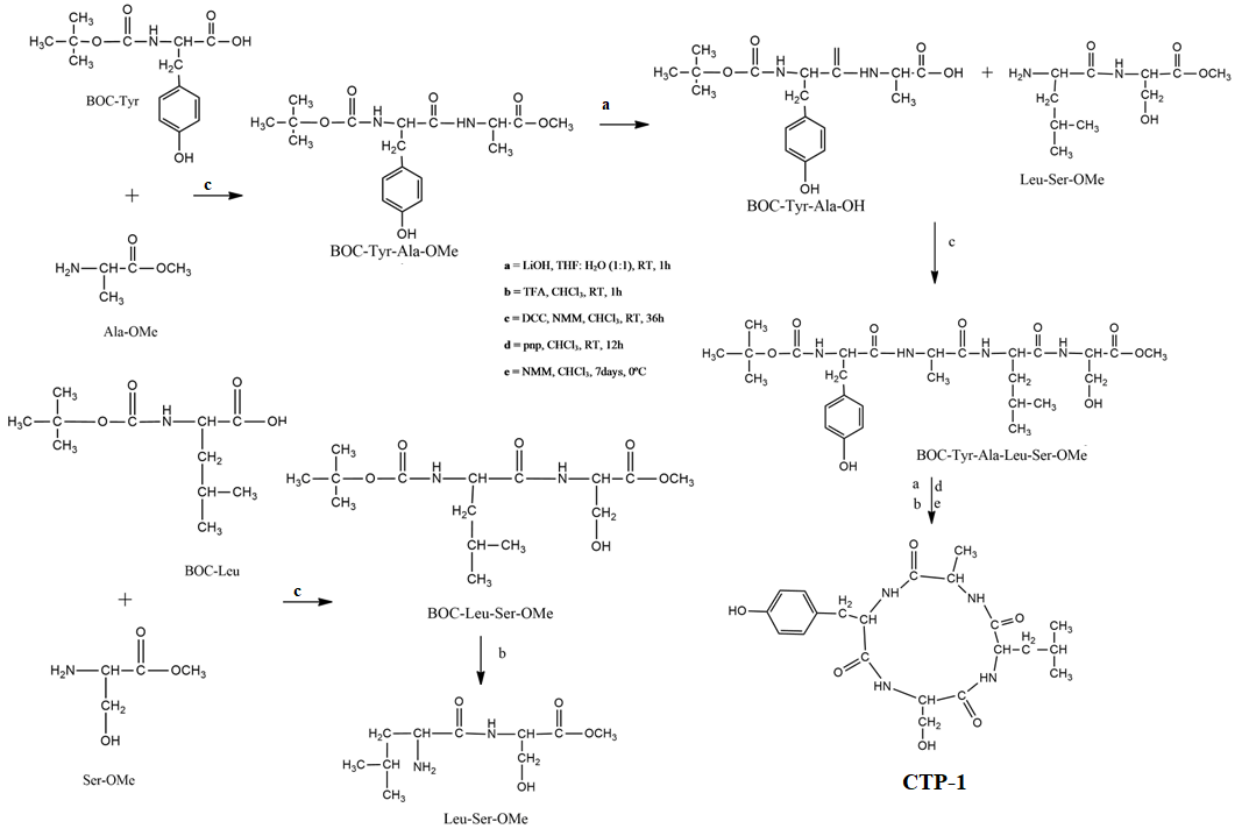
To synthesize Boc-tetrapeptide methyl esters, 2.3 mL of N-methylmorpholine (NMM, 0.021 mol) was added to a mixture of 3.52 g of Boc-Tyr-Ala-OMe (3) (0.01 mol) in 20 mL of chloroform (CHCl3) at 0°C. The resulting mixture was stirred for 15 minutes. Then, 2.16 g of Leu-Ser-OMe (5a) (0.01 mol) in 20 mL of CHCl3 was added to the mixture. To this, 2.1 g of dicyclohexylcarbodiimide (DCC, 0.01 mol) was added with continuous stirring. The mixture was stirred for 36 hours. After the stirring period, the resulting mixture was filtered, and the residue was washed with 30 mL of CHCl3. The filtrate was then washed with 5% sodium bicarbonate (NaHCO3) solution, followed by 25 mL of saturated sodium chloride (NaCl) solution. The organic layer was dried over anhydrous sodium sulfate (Na2SO4), filtered, and evaporated under vacuum. The crude product was crystallized from a mixture of chloroform and petroleum ether (boiling point 40–60°C) and kept at 0°C to obtain pure Boc-Tyr-Ala-Leu-Ser-OMe (6). Similarly, the following Boc-tetrapeptide methyl esters were synthesized using the same procedure:

- Boc-Gly-His-Pro-Glu-OMe (6b)

Synthesis of cyclic tetrapeptides (6a and 7)

To synthesize cyclo(Tyr-Ala-Leu-Ser-OMe) (6a), 5.50 g of Boc-Tyr-Ala-Leu-Ser-OMe (6) (0.005 mol) was dissolved in 50 mL of chloroform at 0-5°C. To this solution, 0.94 g of p-nitrophenol (0.0067 mol) was added, and the mixture was stirred for 12 hours at room temperature. The resulting solution was then filtered, and the filtrate was washed with three portions (3 × 15 mL) of 10% sodium bicarbonate (NaHCO3) solution until the excess p-nitrophenol was removed. The filtrate was then washed with two portions (2 × 10 mL) of 5% hydrochloric acid solution to obtain Boc-Tyr-Ala-Leu-Ser-O-pnp (6b). Next, 4.54 g of compound 6b (0.004 mol) was dissolved in 35 mL of chloroform, and 0.91 g of trifluoroacetic acid (TFA, 0.008 mol) was added. The mixture was stirred for 1 hour at room temperature. The filtrate was washed with two portions (2 × 25 mL) of 10% NaHCO3 solution. The organic layer was dried over anhydrous sodium sulfate (Na2SO4) to prepare Tyr-Ala-Leu-Ser-O-pnp (6c). Compound 6c was then dissolved in 25 mL of chloroform, and 2.3 mL of N-methylmorpholine (NMM, 0.021 mol) was added. The reaction mixture was kept at 0°C for 7-8 days. The resulting mixture was washed with 10% NaHCO3 solution until the p-nitrophenol byproduct was completely removed, followed by washing with three portions (3 × 15 mL) of 5% hydrochloric acid. The organic layer was dried over anhydrous Na2SO4. Finally, chloroform was distilled off, and the crude cyclized product was crystallized from a mixture of chloroform and hexane to yield pure cyclo (Tyr-Ala-Leu-Ser) (6a). Similarly, utilizing compound 6b, Cyclo (Gly-His-Pro-Glu) (7a) as the pure cyclic tetrapeptides was obtained. The synthetic pathways for the newly synthesized tetracyclopeptides are illustrated in Figures 1, and 2.

Figure 1. Pathway for synthesis of cyclic tetrapeptide (CTP-1)



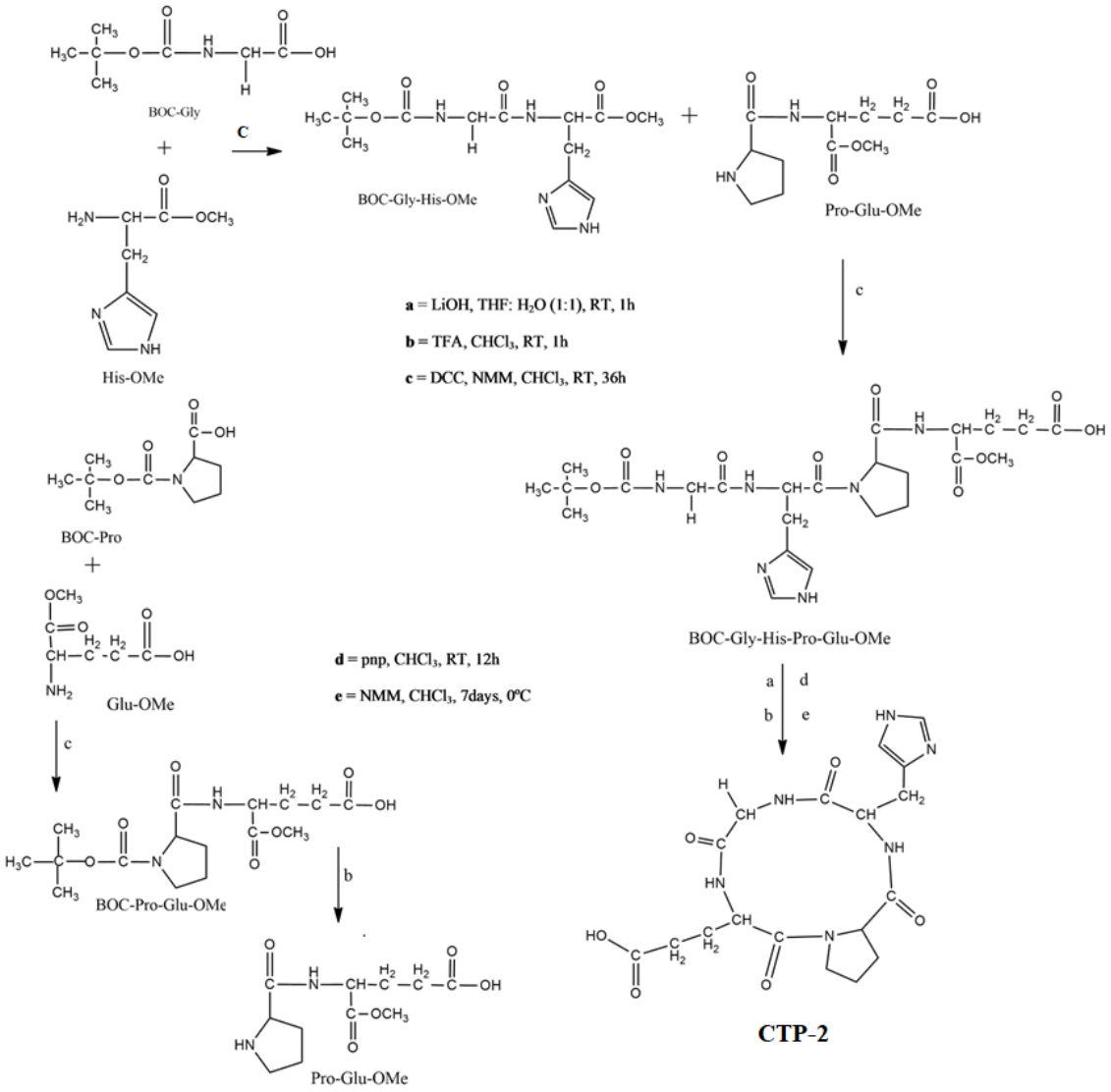
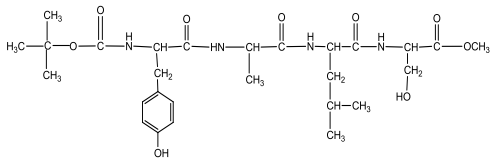
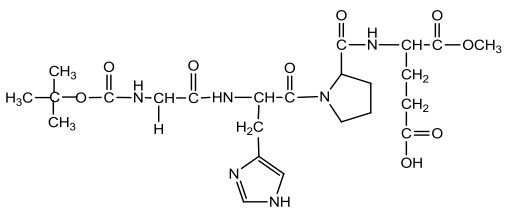


Figure 2. Pathway for synthesis of cyclic tetrapeptide (CTP-2)

Table 1. Physical Characterization of Synthesized Boc-Tetrapeptide methyl esters (Linear Tetrapeptides)

S. No	Boc-tetrapeptide methyl ester	Structure	R_f Value $CHCl_3$: MeOH (8:2)	% age yield
1.	Boc-Tyr-Ala-Leu-Ser-OMe		0.66	78.9 %
2.	Boc-Gly-His-Pro-Glu-OMe		0.84	86.8 %

Evaluation of Pharmacological Activities

The synthesized peptides were evaluated pharmacologically for their antimicrobial, cox inhibitory and antioxidant potential**.**

Antimicrobial Activity

Microorganisms

The microbiological strains used to assess the antibacterial activities were *Escherichia coli* and *Staphylococcus aureus*. Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* microorganisms were used to test the antibacterial activity. From "The Microbial Type Culture Collection and Gene Bank," all of the strains were sorted (MTCC). Additionally, the bacteria were cultivated on nutritional agar slants. Before being used, the cultures were well-preserved at 4 °C and were maintained by periodically subculturing them.

Table 2. Specifics and growing environments for the investigated microorganisms.

Standard Antibiotic	Solvent used	MTCC No.	Micro-organisms	Strain	Incubation time	Temp
Ciprofloxacin	Chloroform	737	*Staphylococcus aureus*	*gram +ve*	24 h	37˚C
Ciprofloxacin	Chloroform	1687	*Escherichia coli*	*gram -ve*	24h	30˚C

Screening for antibacterial and antifungal activity

Antibacterial activity used the agar-well diffusion technique. Reconstituted cyclic tetrapeptide samples in dimethylsulphoxide (DMSO) yielded a range of quantities (50, 100, 200, 250, and 500 µg/ml). The test microorganisms were introduced into the culture medium by gently preparing 0.5 ml of the fresh cultures (24 hours) (To assess the antibacterial activity, bacterial cultures were adjusted to #0.5 McFarland turbidity standards). The culture was created in sterile Petri plates with 20 millilitres of melting sterile agar that had been chilled to between 30 and 37 °C. After the material solidified, four 6 mm-diameter wells were created using a sterile borer. One hundred microliters of both the test and the blank were added to each well. According to table 2 above, the antimicrobial assay plates were incubated at 30-37 °C for 24 hours. The conventional antibiotic listed in table 2 above were used as positive antibacterial controls. The diameter of the zones of inhibition surrounding each well, including the well diameter, was recorded as the antibacterial activity level. The average diameter of the inhibitory zone was measured for each test, which was conducted in triplicate (Mukherjee et al., 1995, Kataki et al., 2010).

Minimum Inhibitory Concentration (MIC) determination

The peptide samples were exposed to the MIC test evaluation after demonstrating antibacterial activity in the agar-well diffusion technique. The established technique previously mentioned was used to determine the minimum inhibitory concentration (MIC) of the tetrapeptides for each test microorganism in triplicate (Salama and Marraiki, 2010, Kataki, 2010)**.**

Cyclooxygenase -1 (COX-1) and cyclooxygenase -2 (COX-2) assays

The COX-1 assay was conducted using the previously published method (Redl et al., 1994, Aguilar et al., 2002). 190 µl of 0.1 M Tris-HCL, 18 µl of L-adrenaline-D-hydrogentartrate, and 10 µl of hematine were combined with 10 µl of the sample solution. After adding COX-1 (0.2 units) to the mixture and letting it sit for 5 minutes, 5 µl of arachidonic acid was added. To end the incubation at 37 °C, 10% formic acid (10 µl) was added after 20 minutes. Afterwards, the concentration of PGE 2 was determined using the R and D systems PGE 2 enzyme-

immunoassay. The COX-2 assay was carried out using the previously described methodology (Aguilar et al., 2002, Redl et al., 1994). A total of 190 µl of 0.1 M Tris-HCL buffer, 18 µl of L-adrenaline-D-hydrogentartrate, 10 µl of disodium edetate (Na2-EDTA), and 10 µl of hematine were combined with 10 µl of the sample solution. After that, the mixture was pre-incubated for five minutes with 0.2 units of COX-2 added. 5 µl of arachidonic acid were then added. After 20 minutes, the incubation at 37 o C was stopped by adding 10 µl of 10% formic acid. PGE2-enzyme-immunoassay (R and D systems) was ultimately utilized to determine the PGE 2 concentration.

2, 2′-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical decolorization assay
Water was used to dissolve ABTS to a concentration of 7 mM. The final concentration of 2.45 mM potassium persulfate was added to the ABTS stock solution to create ABTSη. The combination was then left to stand in the dark at room temperature for 12 to 16 hours before being used. To test the samples, phosphate-buffered saline (5 mM; pH 7.4) was added to the ABTS· stock solution until the absorbance at 734 nm reached 0.70. Five minutes after the initial mixing, 1.0 ml of diluted ABTS· was added to 20 µl of sample, and the absorbance reading was obtained (Re et al., 1998). The percentage of ABTS· -scavenging for this activity is determined using the formula below:
% ABTS·-scavenging activity = [Control absorbance – Sample absorbance] / [Control absorbance] × 100

Statistical analysis
The findings were expressed as mean (n = 6) ± SD. GraphPad Prism software was used to perform post hoc "Dunnett's Multiple Comparison Test" after one-way analysis of variance (ANOVA) for all statistical analyses. "p" values were deemed statistically significant if they were less than 0.05.

3. Results and Discussions

Synthesis and characterization of cyclic tetrapeptides (CTPs)
The cyclic tetrapeptides (CTPs) were synthesized efficiently with percent yield ranging from 77.5 to 81.6% (Table 3). The experiments revealed NMM as an effective base for the efficient and successful cyclization of the linear tetrapeptide segments. Rf values for synthesized CTP-1 and CTP-2 were found to be as 0.74 and 0.76 respectively. The process of Cyclization was achieved successfully as supported by the vanishing of absorption bands at 1765, 1212, 2820 and 1385, 1370 cm−1 due to C=O str, C–O str, ester, C-H, str OCH_3 and C–H, *tert*-butyl groups in the IR spectra of CTP-1. Cyclization was supported by the vanishing of absorption bands at 1739, 1215, 2850 and 1400, 1350 cm^{-1} due to C=O str, C–O str, ester, C-H, str OCH_3 and C–H *tert*-butyl groups in the IR spectra of CTP-2**.** The ^{1}H NMR spectrum also demonstrated successful Cyclization following the efficient formation of the cyclotetrapeptides as evident by vanishing of singlets, matching to three protons of the methyl ester group and nine protons of the tert-butyl group of Boc in the ^{1}H NMR spectrum. The spectral data were tabulated and presented in table 4. The spectra of cyclotetrapeptides are shown in Figure 4-5.

Table 3. Melting point, percentage yield and R_f values of the synthetic cyclic tetrapeptides

Serial No.	Compound Code	Melting Point	% Yield	TLC (R_f value) $CHCl_3$: MeOH
1	CTP-1	167-172	81.6	0.74
2	CTP-2	173-177	77.5	0.76

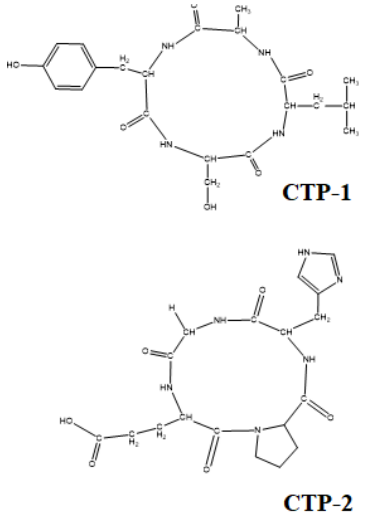


Figure 3. Structures of cyclic tetrapeptides (CTP-1 and CTP-2)

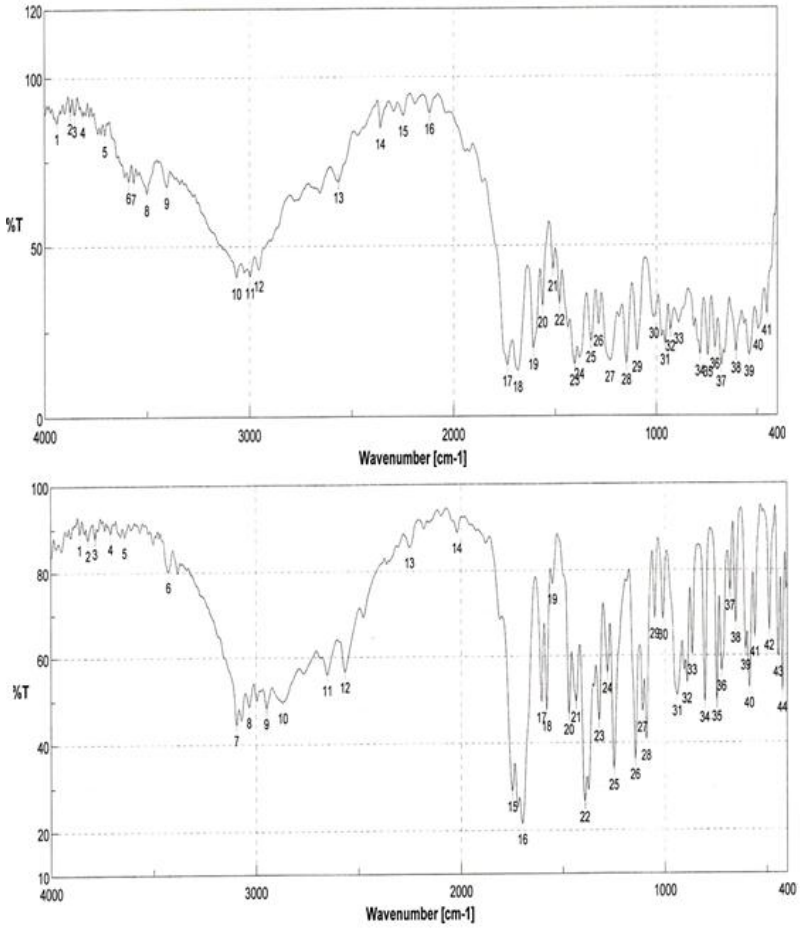


Figure 4. FTIR Spectra of CTP-1and CTP-2

Figure 5. Mass Spectra of CTP-1 and CTP-2

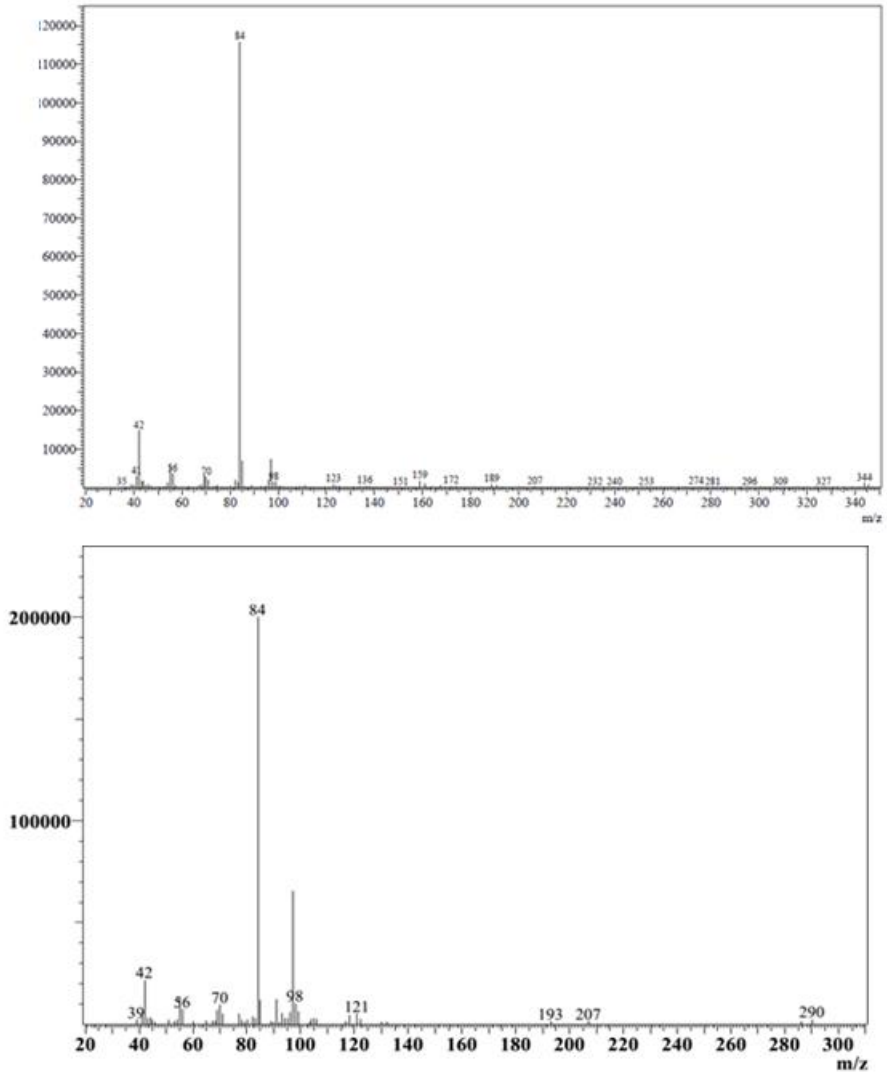


Table 4. Spectral interpretations and data for the cyclic tetrapeptides

Compound	IR Value (υ cm^{-1})	Mass
CTP-1	3510 (OH, stretching, alcoholic), 3320 (N–H stretching, amide), 3060 (C–H stretching, aromatic ring) and, 2950 (C–H stretching, asymmetric, CH2), 2850 (C–H stretching, asymmetric, CH), 1650 (C=O stretching, 2° amide),1594, 1466 (C=C ring stretching), 1545 (N–H bend, 2° amide), 1225 cm-1 (C–O stretching, Tyrosine).	ESI-MS: m/z 437.82 [M+H]+

CTP-2	3330 (N–H stretching, amide), 3040 (O-H, streching, COOH), 2900 (C–H stretching, asymmetric, CH), 2990, 3000 (C–H stretching, cyclic CH2 and CH), 2850 (C–H stretching, symmetric, CH3), 1730 (C=O stretching, COOH), 1660 (C=N stretching), 1633 (C=O stretching, 2° amide), 1555 (N–H bend, 2° amide),1320 (N–H bend), 1100 (C-N stretching, amide)	ESI-MS: m/z 431.11 [M+H]+
CTP-2	**^{1}H NMR ($CDCl_3$) δ**	
	10.81 (s, 1H, COOH), 8.35 (br. s, 3H, NH, amide), 7.70 (s, 1H, NH's, imz ring), 7.35 (s, 1H, imz ring), 7.32 (s, 1H, imz ring), 4.71-4.61 (m, 1H, α-H, His), 3.88 (s, 2H, Gly), 3.85-3.92 (m, 1H, α-H, Pro), 3.41-3.35 (m, 2H, Pro), 2.89-2.93 (d, 2H, His), 1.55-1.76 (m, 4H, β-H's, and γ -H's, Pro).	

Biological and Pharmacological activity

Antimicrobial activity

Zone of inhibition was used to quantify the cyclic tetrapeptides' antimicrobial activity (ZOI). The reference standard medication (50µg/ml) and the compounds under test (50, 100, 200, 250, and 500 µg/ml) were diluted. In table 5-6, the antimicrobial screening findings are compiled and displayed. The ZOI clearly shows that CTP-1 and CTP-2 have strong antibacterial action against *Escherichia coli* and *Staphylococcus aureus*. These antibacterial outcomes are on par with those of the common medication Ciprofloxacin. With the exception of CTP 2, which shown greater activity than standard Ciprofloxacin at 500 against Escherichia coli and Staphylococcus aureus, all CTPs demonstrated lower significant antibacterial activity equivalent to that of conventional Ciprofloxacin. In summary, every CTP exhibited an increase in activity that was dependent on concentration.

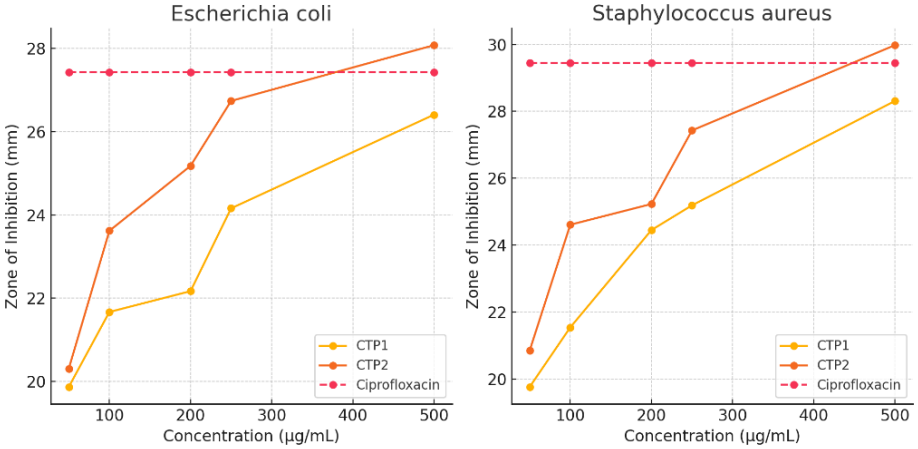
Table 5. Shows antibacterial activity of synthesized compounds against *Escherichia coli* in terms of ZOI are as follow:

Escherichia coli		
Conc. (µg/mL)	**Zone of inhibition (mm)**	
	Cyclic Tetrapeptides	
	CTP-1	**CTP-2**
50	19.87	20.31
100	21.67	23.62
200	22.17	25.18
250	24.16	26.74
500	26.41	28.08
DMSO	0	0
Ciprofloxacin (50µg/mL)	27.43	27.15

Table 6. Shows antibacterial activity of synthesized compounds against *Staphylococcus aureus* terms of ZOI are as follow:

Staphylococcus aureus		
Conc. (µg/mL)	**Zone of inhibition (mm)**	
	Cyclic Tetrapeptides	
	CTP-1	**CTP-2**
50	19.76	20.85
100	21.54	24.61
200	24.45	25.23
250	25.19	27.43
500	28.31	29.98
DMSO	0	0
Ciprofloxacin (50 µg/mL)	29.45	29.66

Figure 6. Antibacterial activity of synthesized peptides (CTP-1 and CTP-2) against *Escherichia coli* and *Staphylococcus aureus* terms of ZOI



Cyclooxygenase -1 (COX-1) and cyclooxygenase -2 (COX-2) assays

In the arachidonic acid pathway inhibition of cyclooxygenase (COX-1 and COX-2) enzymes were also found to be linked with the anti-inflammatory activity. Therefore, in the present study the CTP-1 and CTP-2 were also evaluated for their possible COX inhibitory capacity at three concentration levels (150, 200, 250 µg/ml). Interestingly, CTP-1 and CTP-2 exhibited a significant inhibition of cyclooxygenase- 1 and -2 at 250 µg/ml concentration (Table 7).

Table 7. Cyclooxygenase -1 (COX-1) and cyclooxygenase -2 (COX-2) inhibitory activity of CTP-1 and CTP-2 at 250 µg/ml concentration (values are expressed as mean ± SD; *n*=3)

Drugs	**% Inhibition**	
	COX-1	**COX-2**
CTP-1	72.32 ± 1.18	77.52 ± 1.26
CTP-2	78.38 ± 1.13	81.73 ± 1.15
Indomethacin	86.29 ± 1.22	89.54 ± 1.13

2, 2'-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical decolorization assay

The CTP-1 and CTP-2 produced a concentration dependent scavenging of $ABTS^{\cdot+}$ radical. The calculated IC_{50} values of the peptides, Quercetin and vitamin C were found to be 118.57 µg/ml, 114.21 ± 1.09 µg/ml, 81.94 ± 1.01 µg/ml and 112.24 ± 1.02 µg/ml, respectively (Table 8).

Table 8. Scavenging activity of CTP-1 and CTP-2 against ABTS radicals (values are expressed as mean ± SD; *n*=3)

Drugs	**Inhibitory concentration (IC_{50}) (µg/ml)**
	ABTS radical
CTP-1	118.57 ± 1.31
CTP-2	114.21 ± 1.09
Quercetin	81.94 ± 1.01
Vitamin C	112.24 ± 1.02

4. Conclusions

The synthesis and characterization of cyclic tetrapeptides (CTPs) demonstrated high efficiency with yields ranging from 77.5% to 81.6%. NMM was identified as an effective base for cyclizing the linear tetrapeptide segments. The Rf values for CTP-1 and CTP-2 were 0.74 and 0.76, respectively, indicating successful cyclization. This was confirmed by the disappearance of specific absorption bands in the IR spectra, such as those corresponding to C=O, C–O, ester, C-H, and tert-butyl groups. The 1H NMR spectra further supported successful cyclization, evidenced by the absence of singlets matching the methyl ester and tert-butyl protons. In terms of biological and pharmacological activities, the antimicrobial activity of the cyclic tetrapeptides was evaluated using the zone of inhibition (ZOI) method. Both CTP-1 and CTP-2 exhibited significant antimicrobial activity against Staphylococcus aureus and Escherichia coli, with CTP-2 showing higher activity than Ciprofloxacin at 500 µg/mL. This indicates a concentration-dependent increase in activity for all CTPs. The COX-1 and COX-2 inhibitory assays revealed that both CTP-1 and CTP-2 exhibited significant inhibition at a concentration of 250 µg/mL, suggesting their potential anti-inflammatory properties. Additionally, the ABTS radical decolorization assay demonstrated that both peptides exhibited concentration-dependent scavenging activity, with IC50 values comparable to quercetin and vitamin C. Overall, the synthesized cyclic tetrapeptides showed promising antimicrobial, anti-inflammatory, and antioxidant activities, making them potential candidates for further pharmacological studies.

List of Abbreviations

°C Degree Centigrade
mol moles
gm Gram
h Hours
mL Milliliter
mol L-1 Mol per litre
m.p. Melting point
b.p. Boiling point
DCC Dicyclohexylcarbodiimide
NMM N-methylmorpholine
$CHCl_3$ Chloroform
THF Tetrahydrofuran
TEA Triethylamine
TFA Trifluoroacetic acid
Boc2O Di-tert-butylpyrocarbonate
NaCl Sodium chloride
NaOH Sodium hydroxide
FT-IR Fourier transform infrared spectroscopy

[1]HNMR Nuclear Magnetic resonance spectroscopy

Conflict Of Interest

The authors declare that there is no conflict of interest in the manuscript.

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