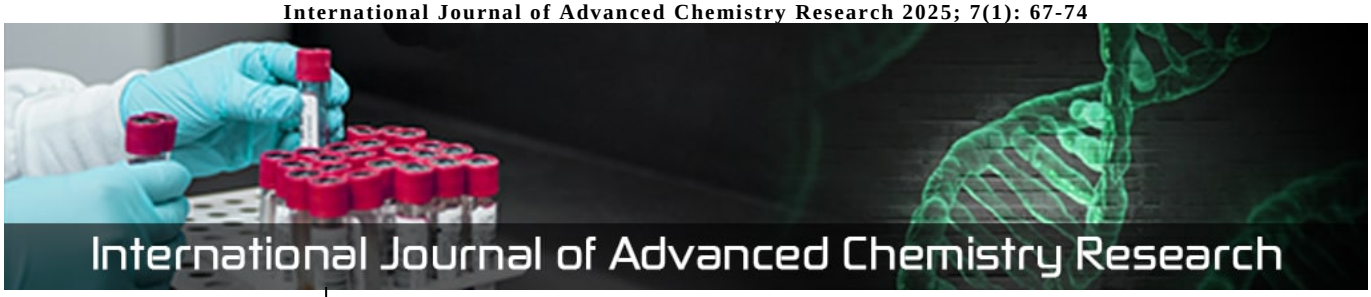

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Conformational analysis of pipecolic acid substituted collagen model peptides

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Abstract
Collagen model peptides, $(Xaa\text{-}Yaa\text{-}Gly)_{10}$ have been used to understand the propensity of the amino acid in Xaa nad Yaa positions. Five membered cyclic imino acid, proline (Pro), have found to be most investigated model. In this investigation Pipecolic acid (Pip), a six member cyclic imino acid and a higher homologue of Pro has been used for sysnthesis of $(Pro\text{-}Pip\text{-}Gly)_{10}$, $(Pip\text{-}Pro\text{-}Gly)_{10}$, and $(Pip\text{-}Pip\text{-}Gly)_{10}$ have been synthesized, characterised and compared with $(Pro\text{-}Pro\text{-}Gly)_{10}$ for their physical property such as solubility. Their triple helix formation characteristics at 4 °C has been investigated by circular dichroism (CD) spectroscopy. It has been found that Pip has higher propensity for Yaa position compared to Xaa position in triple helix conformations.

Keywords: Collagen, model peptides, triple helix, pipecolic acid

1. Introduction
Collagen fibers within connective tissues were first identified in the 19th century.(Ranvier, 1870) Nageotte demonstrated that acid-solubilized collagen could form fibers, a finding corroborated by X-ray diffraction and microscopy.(EASTOE, 1955) [6] In 1938, Astbury proposed an initial structural model featuring trans and cis peptide units (Astbury, 1938) [1], while in 1951, Pauling and Corey introduced a model comprising three coaxial helices.(PAULING & COREY, 1951a, 1951b) [22] Subsequently, two single helical models were developed: one exhibiting 10/3-helical symmetry with a 31 Å repeat, and another displaying 7/2-helical symmetry with a 21 Å repeat.(Cohen & Bear, 1953) [5] Ramachandran and Kartha later proposed a triple helical structure characterized by nine amino acids per unit cell, based on the polyproline-II conformation with three-fold symmetry.(Ramachandran & Kartha, 1955a, 1955b) [24] Naturally occurring collagen fibers, which contain approximately 1000 residues per chain, exhibit resistance to crystallization, thereby constraining X-ray analysis. Synthetic peptides have facilitated the examination of collagen structures. The consistency of collagen across species permits evolutionary investigations. Studies of human collagens have identified the Proline-Hydroxyproline-Glycine (Pro-Hyp-Gly) sequence as the most stabilizing triplet.(Ramshaw *et al*., 1998) [26]
$(Pro\text{-}Pro\text{-}Gly)_{20}$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ Have ben synthesised (Sakakibara *et al*., 1968) [30] These collagen model peptides, notably (Pro-Pro-Gly)10 and (Pro-Hyp-Gly)10, have significantly contributed to the understanding of collagen structure. These repeated tripeptide models are referred to as homomers. Several collagen model peptides have been crystallized, including (Pro-Pro-Gly)10, (Pro-Hyp-Gly)10, and Gly→ Alanine.(Berisio *et al*., 2002; Kenjiokuyama *et al*., 1999; Kramer *et al*., 1998) [3, 11, 12] Among these, (Pro-Pro-Gly)10 was the first collagen-like system to be synthesized and crystallized. This peptide continues to be of significant interest, serving as a benchmark for examining the effects of pyrrolidine ring substituents on the stability of the triple helix.(Berisio *et al*., 2002) [3] The structure was refined to a resolution of 1.3 Å using crystals cultivated under microgravity conditions and analyzed with synchrotron radiation.(Berisio *et al*., 2000) [4]
Amino acid substitutions at the Xaa and Yaa positions include Hyp, 4-fluroproline, (Flp), Methylproline (Mep), and 4-aminoproline (Amp). Peptides incorporating Hyp, such as $(Pro\text{-}Hyp\text{-}Gly)_{10}$, facilitate the investigation of collagen hydroxylation. Raines synthesized (Pro-

4R-Flp-Gly)$_{10}$, (Pro-4S-Flp-Gly)$_{10}$, and (4S-Flp-4R-Gly)$_{10}$ to examine electronegative effects.(Shoulders & Raines, 2009) [31] Babu and Ganesh demonstrated that 4(R)-Amp collagen mimetics exhibit greater pH-dependent stability than Hyp, attributed to the protonation effects of the amino group.(Babu & Ganesh, 2001 [2]; Research involving azetidine carboxylic acid (Aze) indicated that (Gly-Pro-Aze) forms triple helices analogous to poly(Gly-Pro-Pro), albeit with reduced stability. Both (G1y-Aze-Pro)n and poly(Gly-Aze-Aze) formed stable yet geometrically distinct helices, with Aze chains displaying increased flexibility. (Fowden & Richmond, 1963; Zagari *et al*., 1990) [7, 38] These findings imply that modelling the effects of ring conformation entropy on angles and hydrophobicity, alongside pipecolic acid, could elucidate stability factors.

Pipecolic acid (Pip), also referred to as (S)-Piperidine-2-carboxylic acid, is a proline analogue characterized by a six-membered piperidine ring. (Figure 1)

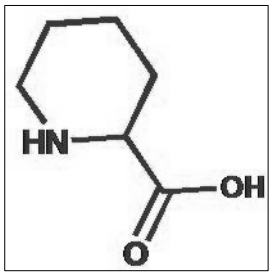

Fig 1: Structure of Pipecolic acid

The incorporation of Pip influences the structural properties of collagen by altering the torsion angles in model peptides, thereby affecting the hydration of the PPII helix. Pip is naturally present in legumes(Grobbelaar & Steward, 1953) [10], Neurospora (Schweet *et al*., 1954), cerebrospinal fluid (Lam *et al*., 1984), blood (Gatfield *et al*., 1968), urine (Lam *et al*., 1984), and the rat brain (Nishio & Segawa, 1983) [20], and it activates aminobutyric acid receptors in neonatal chicks (Takagi *et al*., 2003) [35]. It is an essential component of peptide mimetics found in immunosuppressants such as FK506 (Tanaka *et al*., 1987) [36], bupivacaine, VX710, demethoxyrapamycin, and trapoxins.

Pip has been utilized as a substitute for proline in structure-activity studies of peptides. The substitution of proteinogenic amino acids with cyclic imino acids has been employed in structure-activity investigations and for the development of peptidomimetics with enhanced pharmacological profiles. A pentapeptide containing Pip influences the thermodynamic properties of the interaction between peptidyl prolyl cis-trans isomerase Pin1 and its substrate.

2. Experimental

2.1 Synthesis of Pipecolic acid

Various methodologies are available for the synthesis of pipecolic acid (Pip), encompassing chemoenzymatic techniques (Nazabadioko *et al*., 1998) [18], asymmetric induction (Roos & Dastlik, 2003) [29], and catalytic processes (Ginesta *et al*., 2002). A photocatalytic transformation employing TiO_2 or CdS offers an environmentally friendly approach, yielding ammonia as a by-product, albeit with limited efficiency. (Myers *et al*., 1997) [17] In the chemoenzymatic pathway developed by Watanabe, diethyl acetamidomalonate undergoes alkylation with dibromobutane, facilitated by sodium ethoxide as the alkylating agent.(Watanabe *et al*., 2005) [37] The resultant compound is subsequently hydrolyzed using sodium hydroxide, followed by decarboxylation to produce a monoester. Further hydrolysis yields acetyl-DL-2-amino-6-bromohexanoic acid. The racemic mixture is resolved using aminoacylase, with the L-enantiomer undergoing cyclization to form L-pipecolic acid, which is subsequently converted into derivatives such as Fmoc-L-Pip-OH, Boc-L-Pip-OH, and HCl·H-L-Pip-OBzl **(**Scheme 1).

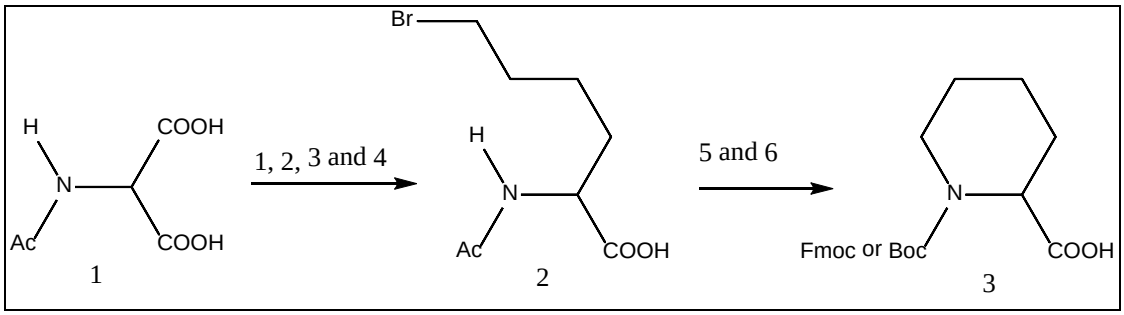

Fig 1: Synthesis of Compound 3

Scheme 1. Synthesis Pip and their derivatives. 1) (i) EtOH, sodium ethoxide, reflux, 30 min; (ii) 1,4-dibromobutane, reflux, 5 h, 2) NaOH aq, EtOH, 0˚C, 3 h, 3) toluene, reflux, 3 h, 4) NaOH aq, EtOH, 0˚C, 3 h, 5) L-aminoacylase, $CoCl_2$.6 H_2O, pH 7, 38˚C, 24 h, 6) Boc_2O, Et_3N, Dioxane or Fmoc-OSu, Na_2CO_3, dioxane-H_2O

2.2 Synthesis of tripeptides

Unless otherwise specified, all solvents and reagents were of reagent grade and utilized without further purification. 1-Hydroxybenzotriazole (HOBt), N,N'-Dicyclohexylcarbodiimide (DCC), O-(Benzotriazole-N,N,N',N'-tetramethyluronium) hexafluorophosphate (HBTU), N,N-Diisopropylethylamine (DIEA) were used to synthesis of tripeptide fragment (Figure 2). Flash chromatography was conducted using silica gel 60 (230-400 mesh) with the indicated solvents as eluents. All compounds were routinely analyzed using thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC). TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F254), with spot visualization achieved through UV light exposure. Analytical HPLC was conducted on a Hitachi instrument equipped with a Chromolith Performance RP-18e column. The mobile phases employed were A: H2O with 0.1% TFA, and B: CH_3CN with 0.1% TFA, utilizing a solvent gradient from A to B over 15 minutes, with detection at 220 nm and a flow rate of 2 mL/min. Fast atom bombardment (FAB)

mass spectra and high-resolution mass spectra (HRMS) were obtained using a JEOL JMS-SX 102A instrument. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 500 MHz spectrometer. Unless otherwise noted, all NMR spectra were recorded in CDCl3 solutions with tetramethylsilane (TMS) as the internal standard. All 1H chemical shifts are reported in parts per million (s = singlet; d = doublet; t = triplet; m = multiplet).

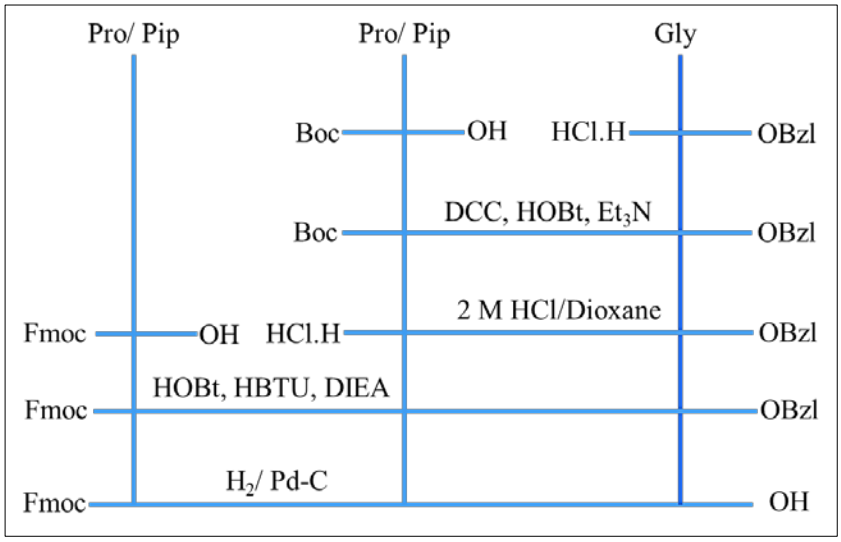

Fig 2: General Scheme of preparation of tripeptides; Fmoc-L-Pro-L-Pro-Gly-OH, Fmoc-L-Pro-L-Pip-Gly-OH, Fmoc-L-Pip-L-Pro-Gly-OH and Fmoc-L-Pip-L-Pip-Gly-OH.

2.2.1 Fmoc-L-Pro-L-Pro-Gly-OH

To a cooled solution of HCl.H-Gly-OBzl (4.2 g, 20 mmol) in DMF (40 mL), triethylamine (2.84 mL, 20 mmol), Boc-L-Pro-OH (4.3 g, 20 mmol), HOBt·H_2O (3.06 g, 20 mmol), and DCC (5 g, 22 mmol) were added. The mixture was then stirred overnight at room temperature. After completion of the reaction, DMF was evaporated, and the residue was dissolved in ethyl acetate. The DCU was removed by filtration, and the filtrate was successively washed with 10% citric acid, 4% sodium bicarbonate, and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to an oily residue, which was purified by silica gel chromatography using a mixture of chloroform and methanol (97:3) to yield Boc-L-Pro-Gly-OBzl (6.15 g, 85%) as a white foam. (HPLC, rt 10.49 min) MALDI TOF MS, $[M+Na]^+$ 385.77 for $C_{19}H_{26}N_2NaO_5$ (calcd 385.17).

Boc-L-Pro-Gly-OBzl (6.97 g, 12 mmol) was dissolved in 2 M HCl/dioxane (120 mL), and the mixture was kept at room temperature for 2 h. The reaction was monitored using TLC. After completion of the reaction, HCl/dioxane was evaporated. The residue was washed with diethyl ether and pumped to obtain HCl.H-L-Pro-Gly-OBzl as a white powder (3.57 g, 100%). To a cooled solution of HCl.H-L-Pro-Gly-OBzl (3.57 g, 7 mmol) in DMF (35 mL), Fmoc-L-Pro-OH (1.04 g, 12 mmol), HOBt·H_2O (1.83 g, 12 mmol), HBTU (4.5 g, 12 mmol), and DIEA (5.94 mL, 36 mmol) were added and stirred for 4 h at room temperature. After completion of the reaction, DMF was evaporated, and the residue was dissolved in ethyl acetate and successively washed with 10% citric acid, 4% sodium bicarbonate and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to an oily residue, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to obtain Fmoc-L-Pro-L-Pro-Gly-OBzl (5.5 g, 76%) as white foam, HPLC, rt 7.46 min. MALDI TOF MS, $[M+H]^+$ 582.56 for $C_{34}H_{36}N_3O_6$ (calcd. 582.26).

Fmoc-L-Pro-L-Pro-Gly-OBzl (5.5 g, 9.2 mmol) was dissolved in methanol (75 mL), Pd-C (450 mg) was added, and the mixture was stirred under a hydrogen atmosphere overnight. The reaction was monitored using TLC and HPLC. After completion of the reaction, Pd-C was filtered and methanol was evaporated. The product was purified by crystallization using ether-petroleum ether (1:3 v/v) to yield Fmoc-L-Pro-L-Pro-Gly-OH (4.5 g, 98%) as a white solid. HPLC, rt 5.38 min. HR-FAB MS, $[M+H]^+$ 491.2056 for $C_{27}H_{30}N_3O_6$ (calcd 491.2135).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.99- 8.04 (m, 1H), 7.99-7.30 (m, 8H), 4.49- 4.23 (m, 5H), 3.79 (dd, J_1 = 17 Hz, J_2 = 7 Hz, 2H), 3.43- 3.33 (m, 4H), 2.19-1.98, (m, 4H), 1.93 - 1.73 (m, 4H).

2.2.2 Fmoc-L-Pro-L-Pip-Gly-OH

To a cooled solution of HCl. H-Gly-OBzl (5.5 g, 24 mmol) in DMF (60 mL), triethylamine (3.4 mL, 24 mmol), Boc-L-Pip-OH (1.29 g, 5.5 mmol), HOBt·H_2O (3.67 g, 24 mmol), and DCC (6 g, 28.8 mmol) were added. The mixture was then stirred overnight at room temperature. After completion of the reaction, DMF was evaporated, and the residue was dissolved in ethyl acetate. The DCU was removed by filtration, and the filtrate was successively washed with 10% citric acid, 4% sodium bicarbonate, and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to an oily residue, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Boc-L-Pip-Gly-OBzl (7.3 g, 81%) as a white foam. HPLC, rt 7.32 min. MALDI TOF MS, $[M+H]^+$ 377.21 for $C_{20}H_{30}N_2O_5$ (calcd 377.19)

Boc-L-Pip-Gly-OBzl (2.9 g, 7.7 mmol) was dissolved in 4 M HCl/dioxane (50 mL), and the mixture was kept at room temperature for 40 min. The reaction was monitored using TLC. After completion of the reaction, HCl/dioxane was evaporated. The residue was washed with diethyl ether and pumped to obtain HCl.H-L-Pip-Gly-OBzl as a white powder (2.4 g, 100%). To a cooled solution of HCl.H-L-Pip-Gly-OBzl (2.4 g, 7.7 mmol) in DMF (20 mL), Fmoc-L-Pro-OH (2.7 g, 8 mmol), HOBt·H_2O (1.23 mg, 8 mmol), HBTU

(3.03 g, 8 mmol), and DIEA (4.1 mL, 24 mmol) were added and stirred for 4 h. After completion of the reaction, DMF was evaporated, and the residue was dissolved in ethyl acetate and successively washed with 10% citric acid, 4% sodium bicarbonate and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to an oily residue, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to obtain Fmoc-L-Pro-L-Pip-Gly-OBzl (3 g, 72%) as white foam, HPLC, rt 7.32 min. HR-FAB MS, $[M+H]^+$ 596.2755 for $C_{35}H_{38}O_6N_3$ (calcd. 596.2761)

Fmoc-L-Pro-L-Pip-Gly-OBzl (3 g, 5 mmol) was dissolved in methanol (25 mL), Pd-C (250 mg) was added, and the mixture was stirred under a hydrogen atmosphere for 10 h. The reaction was monitored using TLC and HPLC. After completion of the reaction, Pd-C was filtered off, and methanol was evaporated. The product was purified by crystallization using ether-petroleum ether (1:3 v/v) to yield Fmoc-L-Pro-L-Pip-Gly-OH (2.3 g, 91%) as a white solid. HPLC, rt 7.96 min. HR-FAB MS, $[M+H]^+$ 506.2278 for $C_{28}H_{32}O_6N_3$ (calcd. 506.2291).

^{1}H NMR (500 MHz, DMSO-d_6): δ 7.99- 8.04 (m, 1H), 7.99- 7.30 (m, 8H), 4.49- 4.23 (m, 5H), 3.79 (dd, J_1 = 17 Hz, J_2 = 7 Hz, 2H), 3.43- 3.33 (m, 4H), 2.19-1.98, (m, 4H), 1.93 - 1.73 (m, 4H).

2.2.3 Fmoc-L-Pip-L-Pro-Gly-OH

Boc-L-Pro-Gly-OBzl (365 g, 1 mmol) was dissolved in 2 M HCl/dioxane (10 mL) and the mixture was kept at room temperature for 2 h. The reaction was monitored using TLC. After completion of the reaction, HCl/dioxane was evaporated. The residue was washed with diethyl ether and pumped to obtain HCl.H-L-Pro-Gly-OBzl as a white powder (300 mg, 100%). To a cooled solution of HCl.H-L-Pro-Gly-OBzl (300 mg, 1 mmol) in DMF (3 mL), Fmoc-L-Pip-OH (0.351 mg, 1 mmol), $HOBt·H_2O$ (153 mg, 1 mmol), HBTU (379 mg 1 mmol), and DIEA (0.50 mL, 3 mmol) were added and stirred for 4 h at room temperature. After completion of the reaction, DMF was evaporated, and the residue was dissolved in ethyl acetate and successively washed with 10% citric acid, 4% sodium bicarbonate and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to an oily residue, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to obtain Fmoc-L-Pip-L-Pro-Gly-OBzl (475 mg, 82%) as white foam, HPLC, rt 7.46 min. HR-FAB MS, $[M+H]^+$ 596.2762 for $C_{35}H_{38}O_6N_3$ (calcd. 596.2761).

Fmoc-L-Pip-L-Pro-Gly-OBzl (475 mg, 1 mmol) was dissolved in MeOH (5 mL). Pd-C (50 mg) was added, and the mixture was stirred overnight under a hydrogen atmosphere. The reaction was monitored using TLC and HPLC. After completion of the reaction, Pd-C was filtered off, and methanol was evaporated. The product was purified by crystallization using ether-petroleum ether (1:3 v/v) to yield Fmoc-L-Pip-L-Pro-Gly-OH (410 mg, 97%) as a white solid. HPLC, rt 6.13 min. HR-FAB MS, $[M+H]^+$ 506.2275 for $C_{28}H_{32}O_6N_3$ (calcd. 506.2291).

^{1}H NMR (500 MHz, DMSO-d_6): δ 7.92- 7.83 (m, 3H), 7.67- 7.51 (m, 2H), 7.45- 7.45 (2H), 7.36- 3.32 (m, 2H), 4.81- 4.78 (m, 1H), 4.59- 4.54 (m, 1H), 4.42- 4.24 (m, 3H), 3.80- 3.75 (m, 1H), 3.71 (dd, J_1=17 Hz, J_2= 6Hz, 2H), 3.66- 3.57 (m, 1H), 3.54- 3.47 (m, 1H), 3.30- 3.29 (m, 1H), 2.08- 1.92 (m, 2H), 1.88- 1.77 (m, 4H), 1.73- 1.3 (m, 1H), 1.62- 1.55 (m, 1H), 1.50- 1.42 (m, 1H), 1.33- 1.25 (m, 1H).

2.2.4 Fmoc-L-Pip-L-Pip-Gly-OH

Boc-L-Pip-Gly-OBzl (3.3 g, 8.7 mmol) was dissolved in 4 M HCl/dioxane (60 mL), and the mixture was maintained at room temperature for 40 min. The reaction was monitored using TLC. After completion of the reaction, HCl/dioxane was evaporated. The residue was washed with diethyl ether and pumped to obtain HCl.H-L-Pip-Gly-OBzl as a white powder (2.7 g, 100%). To a cooled solution of HCl.H-L-Pip-Gly-OBzl (2.3 g, 7.4 mmol) in DMF (20 mL), Fmoc-L-Pip-OH (2.6 g, 7.4 mmol), $HOBt·H_2O$ (1.23 g, 8 mmol), HBTU (3.03 g, 8 mmol), and DIEA (4.1 mL, 24 mmol) were added and stirred for 4 h at room temperature. After completion of the reaction, DMF was evaporated, and the residue was dissolved in ethyl acetate and successively washed with 10% citric acid, 4% sodium bicarbonate and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to an oily residue, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to obtain Fmoc-L-Pip-L-Pip-Gly-OBzl (3.3 g, 73%) as white foam, HPLC, rt 10.49 min. HR-FAB MS, $[M+H]^+$ 610.3018 for $C_{36}H_{40}O_6N_3$ (calcd. 610.2917).

Fmoc-L-Pip-L-Pip-Gly-OBzl (3.1 g, 5.1 mmol) was dissolved in methanol (30 mL). Pd-C (250 mg) was added, and the mixture was stirred under a hydrogen atmosphere for 10 h. The reaction was monitored using TLC and HPLC. After completion of the reaction, Pd-C was filtered off, and methanol was evaporated. The product was purified by crystallization using ether-petroleum ether (1:3 v/v) to yield Fmoc-L-Pip-L-Pip-Gly-OH (2.4 g, 91%) as a white solid. HPLC, rt 9.26 min. HR-FAB MS, $[M+H]^+$ 520.2499 for $C_{29}H_{34}O_6N_3$ (calcd. 520.2448).

Fmoc-L-Pip-L-Pip-Gly-OBzl (3.1 g, 5.1 mmol) was dissolved in methanol (30 mL). Pd-C (250 mg) was added, and the mixture was stirred under a hydrogen atmosphere for 10 h. The reaction was monitored using TLC and HPLC. After completion of the reaction, Pd-C was filtered off, and methanol was evaporated. The product was purified by crystallization using ether-petroleum ether (1:3 v/v) to yield Fmoc-L-Pip-L-Pip-Gly-OH (2.4 g, 91%) as a white solid. HPLC, rt 9.26 min. HR-FAB MS, $[M+H]^+$ 520.2499 for $C_{29}H_{34}O_6N_3$ (calcd. 520.2448).

^{1}H NMR (500 MHz, DMSO-d_6): δ 7.93- 7.89 (m, 3H), 7.65- 7.58 (m, 2H), 7.44- 7.41 (m, 2H), 7.35- 7.32 (m, 2H), 5.10- 4.98 (m, 1H), 4.40- 4.25 (m 4H), 3.74 (dd, J_1 = 9.3 Hz, J_2 = 5.8 Hz, 2H), 3.24- 3.17 (m, 4 H), 2.15 -1.83 (m, 4H), 1.64 - 1.19 (m, 8H).

2.3 Synthesis of segments for solid phase synthesis

Barlos resin has proven to be the most significant development in solid-phase peptide synthesis (SPPS)-linker technology. Compared to benzyl alcohol-based resins, the attachment of α-amino acids to Barlos resin is devoid of racemization. The presence of imino acid-induced β-turns in peptides complicates the coupling of subsequent residues or the elongation of the peptide chain. To mitigate this issue, Barlos resin was employed. The trityl linker facilitates the attachment of a diverse array of bifunctional building blocks, including amino acids, amino alcohols, diols, and diamines. The functionalized trityl group exhibits stability

against nucleophiles, a requisite condition for solid-phase peptide synthesis (SPPS). Product cleavage occurs under mild conditions due to the high stability of trityl cations. Standard cleavage conditions include HOAc/TFE/DCM or diluted TFA in methylene chloride, although TFA vapor or HCl gas can also be directly applied to the dry bead. Due to these advantages, Barlos resin has been increasingly utilized for the synthesis of collagen model peptides. In this study, it served as a solid support for peptide synthesis.

Fmoc derivatives of the tripeptide residues were used for coupling. This strategy employs Fmoc as the protective group and piperidine for deprotection. As the peptide chains grow, they have the potential to interact with each other; therefore, the surface concentration of peptides on the solid support was kept low. Using this strategy segments Fmoc-L-Pro-L-Pro-Gly-OH, Fmoc-Pip-Pip-Gly-OH, Fmoc-L-Pip-Pro-Gly-OH and Fmoc-Pro-Pip-Gly-OH were anchored on Barlos resin with to obtain tripeptide segments with low surface concentration (0.13-0.20 mmol/g). Manual peptide synthesis can be accomplished using a fritted-filter reaction vessel. Fmoc deprotection after each amino acid coupling was accomplished using 20% piperidine in DMF. An amino acid is coupled to the free N-terminal of the peptide using a coupling mixture such as the protected amino acid (3 eq), PyBOP (3 eq), HOBt (3 eq), and DIPEA (6 eq) in 1:1 DCM:DMF until the resin is negative for the free amine test. Another popular set of coupling conditions is amino acid (4.4 eq), HBTU (4 eq), N, N-diisopropylethylamine (DIPEA) (8 eq), and DMF. In this case, only two equivalents of tripeptide residues were applied for coupling with one equivalent of HOBt, one equivalent of HBTU, and 2.9 equivalents of DIEA. The progress of the amino acid coupling reactions was monitored using p-chloranil. The p-chloranil solution turns the resin beads dark black or blue in the presence of a primary amine if acetaldehyde is used as the solvent or in the presence of a secondary amine if acetone is used instead; the beads remain colorless or pale yellow otherwise. In addition, from time to time, a small part of the resin was removed, and the fragments were cleaved and analyzed by mass spectrometry. Matrix-assisted laser desorption onization (MALDI) time-of-flight (TOF) MS is particularly useful for verifying and improving peptide synthesis. After reaching the desired length the peptides were cleaved from the solid support by the mixture of AcOH/TFE/CH_2Cl_2 (2:2:6). The peptides were precipitated using *tert.*-butylmethylether and finally purified by semi-preparative RP-HPLC. Peptides were characterized by HPLC and MALDI-TOF MS.

3. Results and Discussion

3.1 Solubility of peptide models

Differences in the peptides were observed during the preliminary physical examination. Different types of solvents have been used to dissolve collagen model peptides. They were dissolved in water, MeOH, 10% AcOH, or buffered solutions as per the requirements of the experiments. Phosphate-buffered solution (PBS), pH 7, simulates the ionic environment of a biological system. It contained 0.20 g KCl, 0.20 g KH_2PO_4, 8.0 g NaCI, 2.16 g and $NaHPO_4.7H_2O$ in 1.00 L H_2O. This buffer was used to dissolve Pip-containing model peptides. Model Peptides demonstrated differences in their solubility in PBS. Whereas the reference peptide and model peptide with one Pip substitution in every tripeptide unit were soluble, the peptide with all Pro residues substituted with Pip was insoluble in water. This cannot be attributed solely to changes in the hydrophobicity of the peptide. A similar model peptide with Methylproline (Mep) in place of Pro, $(4R\text{-}Mep\text{-}4S\text{-}Mep\text{-}Gly)_7$ was also soluble in water. In the Mep residue, the number of C atoms was similar to that of Pip.(Shoulders & Raines, 2009) [31]

The incorporation of Pip residues into peptides results in two primary differences: (1) alterations in ring conformations and (2) variations in torsion angles. Proline, in particular, demonstrates distinct torsion angles and puckering at the Xaa and Yaa positions.

The conformational characteristics of proline residues in collagen are determined by their backbone torsion angles (Φ, Ψ, and ω) and the puckering mode of the pyrrolidine ring. The torsion angles, specifically Φ (rotation about the N-Cα bond), Ψ (rotation about the Cα-C bond), and ω (rotation about the peptide bond), restrict the orientation of the peptide backbones. Furthermore, the pyrrolidine ring of proline can assume distinct puckering modes, namely Cγ-endo (“Down”) and Cγ-exo (“Up”). Within the collagen triple helix, proline residues at the Xaa position preferentially adopt the Cγ-endo conformation, whereas those at the Yaa position predominantly assume the Cγ-exo conformation. This alternating ring puckering is essential for stabilizing the triple helical structure by minimizing steric strain and facilitating favorable interchain interactions. The experimentally observed torsion angles and puckering modes of the proline residues at the Xaa and Yaa positions. (Table 1)

Table 1: Torsion angles and puckering conformations of proline residues at the Xaa and Yaa positions of collagen.

Position	Φ (°)	Ψ (°)	ω (°)	Puckering
Xaa	-77.94	166.13	175.83	Down
Yaa	-60.26	163.44	179.69	Up

Pip demonstrates a preference for the chair conformation; however, its capacity for variation in the torsion angle remains undetermined. This indicates that while a single substitution at the Xaa and Yaa positions maintains similarity with the reference peptide, simultaneous substitutions at both positions result in a significant alteration in the peptide's properties.

Peptides comprising 50% to 75% hydrophobic residues are typically insoluble or only partially soluble in aqueous solutions. In such instances, it is advisable to initially dissolve these peptides in a minimal volume of stronger solvents, such as DMF, acetonitrile, isopropyl alcohol, ethanol, acetic acid, 4-8 M GdnHCl or urea, DMSO (provided the sequence does not contain C, W, or M), and other similar organic solvents. Subsequently, the solution should be gradually introduced into a stirred aqueous buffer solution. In this context, (Pip-Pip-Gly)10 was dissolved in a minimal amount of TFE and then diluted with PBS to yield a solution in 4% TFE/PBS.

3.2 Conformational analysis

Various analytical techniques have been employed to study collagen and its model peptides, including X-ray crystallography (Berisio *et al.*, 2000) [4], electron microscopy (Olsen *et al.*, 1971), sedimentation equilibrium methods (Sakakibara *et al.*, 1968) [30], NMR spectroscopy (Long *et al.*, 1992) [15], Raman (Renugopalakrishnan *et al.*, 1985) [28]

and infrared spectroscopy (Lazarev *et al*., 1978) [14], differential scanning calorimetry (Slatter *et al*., 2003), fluorescence spectroscopy (Simon-Lukasik *et al*., 2003), and neutron scattering (Middendorf *et al*., 1995) [16]. Each of these methods provides unique structural and dynamic insights. Nevertheless, many of these techniques present experimental challenges and are not always suitable for large-scale or routine analyses. In contrast, Circular Dichroism (CD) spectroscopy has emerged as a rapid, sensitive, and versatile method that requires minimal sample quantities and facilitates systematic exploration of solvent conditions, pH, temperature, and cofactors. (Figure 3) Consequently, CD spectroscopy is the most widely utilized technique for examining the secondary structure and thermal stability of collagen and its model peptides.(Nishihara & Doty, 1958) [19]

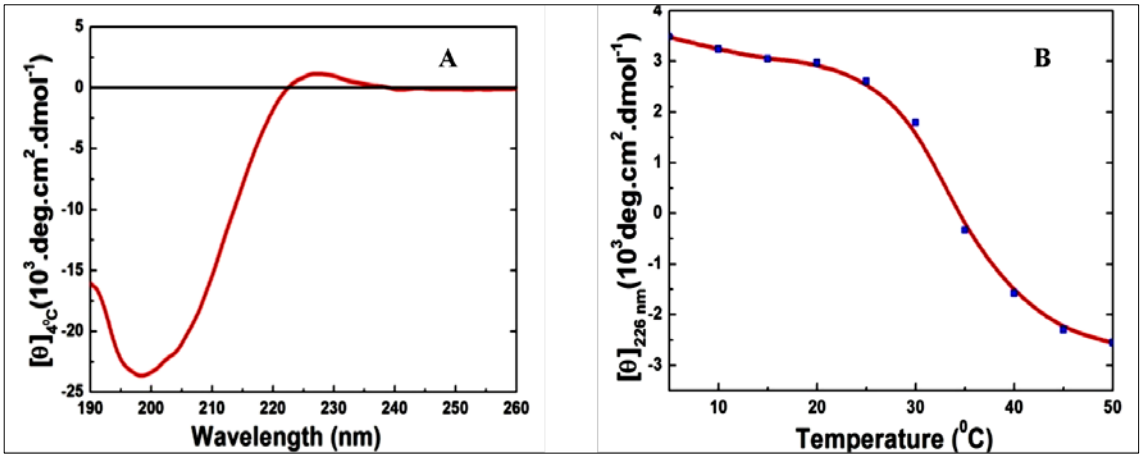

Fig 3: A). CD spectra of (Pro-Pro-Gly)10 at 4°C in PBS (pH 7) and B) its thermal denaturation curve.

The formation of triple helix structures is a complex, multistep process, complicated by the multimeric nature, the correct register of the Gly-Xaa--Yaa sequence, and the high imino acid content. Type I collagen is capable of refolding into a native triple helix *in vitro*; however, this process necessitates extensive time and precise temperature control to ensure correct chain registration. *In vivo*, molecules are synthesized as procollagen, with N- and C-terminal globular propeptides flanking the $(Gly\text{-}Xaa\text{-}Yaa)_n$ domain. The C-propeptides self-associate to facilitate chain selection and trimerization, with C-terminal tethering aiding in the correct chain register for triple-helix nucleation. The triple helix conformation propagates from the C- to the N-terminus. *In vitro* collagen model peptides exhibit unfolded states with approximately 10% *cis* peptide bonds, with propagation limited by *cis*-trans isomerization required to form the native triple helix.

An equilibrium is established between the unfolded and folded states of the helix. The unfolding of the associated helix without the correct register, followed by refolding into the correct register, occurs at low temperatures. The solution was maintained at a low temperature to achieve equilibration. The selection of the medium influences the formation of the triple helix. The presence of free amine and carboxylic acid groups at the C and N terminals destabilizes the triple helical association. Under acidic or basic conditions, the C or N terminals become protonated, thereby reducing electrostatic repulsion. Collagen model peptides exhibit stability at both low and high pH values. A 0.1 M phosphate buffer solution (PBS) was employed to investigate Pip incorporation in collagen model peptides. Solutions were maintained at 4 °C for 48 hours prior to analysis via CD spectroscopy. (Figure 4)

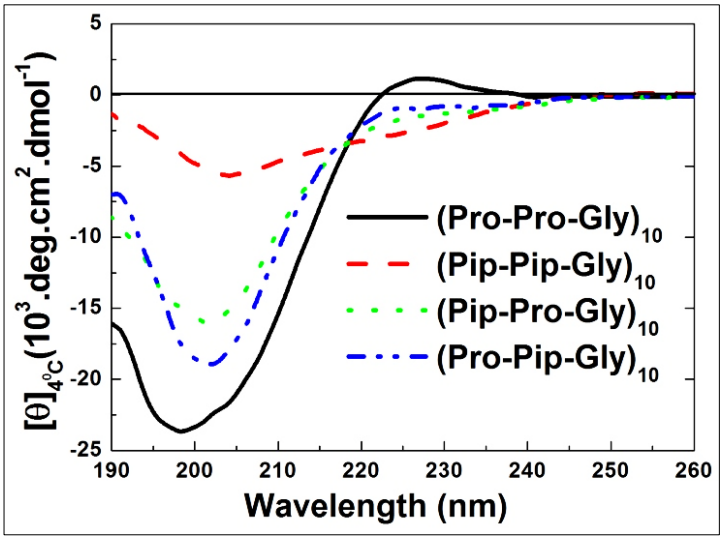

Fig 4: Compared CD spectra of collagen model peptides at 4°C in PBS (pH 7).

The reference model peptide, $(Pro\text{-}Pro\text{-}Gly)_{10}$, exhibited characteristic circular dichroism (CD) spectra of collagen, marked by significant negative ellipticity at 200 nm and positive ellipticity at 225 nm. In contrast, peptides containing Pip did not display positive ellipticity, and the intensity of negative ellipticity was reduced compared to the reference model peptides. These findings are presented in. Positive ellipticity is indicative of the right-handed superhelical conformation achieved by collagen model peptides, which also promotes triple helical association. The absence of positive ellipticity near 225 nm suggests a similar conformation in Pip-containing model peptides.

All samples exhibit negative ellipticity, a characteristic feature of the polyproline-II (PPII) structure that indicates the left-handed helicity of the peptide. Notable differences are observed in the negative minima, including a shift in minima towards higher wavelengths and a reduction in ellipticity.

The observed shift in the ellipticity minima suggests that the incorporation of Pip alters the conformation from the PPII type of $(Pro\text{-}Pro\text{-}Gly)_{10}$. This observation requires further analysis for confirmation. The reduction in the minima indicates a decrease in the degree of left-handed helicity. The substitution of all Pip residues results in only residual helicity in the model peptide, as exemplified by $(Pip\text{-}Pip\text{-}Gly)_{10}$. A notable feature of these CD spectra is the variation in the ellipticity pattern between $(Pro\text{-}Pip\text{-}Gly)_{10}$ and $(Pip\text{-}Pro\text{-}Gly)_{10}$, clearly demonstrating that the two positions are not equivalent for Pip substitution. The $(Pro\text{-}Pip\text{-}Gly)_{10}$ exhibits deeper negative maxima compared to $(Pip\text{-}Pro\text{-}Gly)_{10}$, further indicating the difference in conformational change due to positional variation. It is also evident that the deviation in conformation of $(Pip\text{-}Pip\text{-}Gly)_{10}$ from that of $(Pro\text{-}Pro\text{-}Gly)_{10}$ is more significantly influenced by Pip in the Xaa position than in the Yaa position. Additionally, the destabilization observed in $(Pip\text{-}Pip\text{-}Gly)_{10}$ is not merely an additive effect of the destabilization in $(Pip\text{-}Pro\text{-}Gly)_{10}$ and $(Pro\text{-}Pip\text{-}Gly)_{10}$, but also involves additional interactions between two adjacent Pip residues.

4. Conclusion

In the investigation of collagen model peptides, the substitution of Pro with Pip in the sequence $(Pro\text{-}Pro\text{-}Gly)_{10}$ has demonstrated that the imino acid positions Xaa and Yaa contribute distinctly to the stability of the helix. Specifically, replacing Pip at the Xaa position resulted in a more pronounced negative impact on helicity compared to the Yaa position. Furthermore, when both positions were substituted concurrently, significant alterations were observed, including reduced solubility and changes in the circular dichroism (CD) spectral pattern.

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