Synthesis of diketopiperazines containing prolinyl unit – cyclo(L-prolinyl-L-leucine), cyclo(L-prolinyl-L-isoleucine) and cyclo(L-tryptophyl-L-proline)

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Diketopiperazines cyclo(L-prolinyl-L-isoleucine) 4a, cyclo(L-prolinyl-L-leucine) 4b and cyclo(L-tryptophyl-L-proline) 6 were prepared from their respective suitably protected amino acid derivatives by standard peptide chemistry. Cyclo(L-(4-hydroxyprolinyl)-L-phenylalanine) 3, 4a and cyclo(L-prolinyl-L-tyrosine) 5 were tested for their antibacterial activity.

2,5-Diketopiperazines, formed by cyclization of dipeptides, are interesting compounds because of their important biological properties. Diketopiperazines have been isolated from plants mainly marine sponges, microorganisms as well as from higher animals. 2,5-Diketopiperazines are also obtained as by-products during the process of acidic or enzymatic digestion of proteinous stuff using mammalian or bacterial proteases.

Suzuki et al. synthesized a number of analogs cyclo(Tyr-Arg) 1 and have studied their analgesic properties in mice. Many of these diketopiperazines contain proline and hydroxyproline and most of them exhibit important biological properties. Recently, the diketopiperazines, cyclo[L-(4-hydroxyproline)-L-leucine], cyclo[L-(4-hydroxyproline)-D-leucine], 2a and 2b and cyclo[L-(4-hydroxyproline)-L-phenylalanine] 8 have been isolated from Palythoa sp., a marine bacterium, and are shown to stimulate plant growth. Herein we report the preparation of diketopiperazines 4a, 4b and 6 and the biological activity of 3, 4a and 5 (Chart 1).

Results and discussion

The target molecules were individually synthesized using the plan depicted in Schemes 1 and 2 beginning with the appropriate amino acids. The amino group of the L-proline was first protected using benzylchloroformate in alkaline medium. L-Isoleucine and L-leucine were converted into their corresponding ester hydrochloride by refluxing the amino acid in absolute ethanol in the presence of thionyl chloride. The ester hydrochlorides obtained were washed with K₂CO₃ to obtain the pure ethyl esters 7a and 7b. The use of concentrated sulfuric acid as catalyst provided the desired esters in lower yields (45%).

Coupling of protected amino acids were accomplished with diecylohexylcarbodiimide (DCC) in dry DCM at room temperature to give dipeptides 8a and 8b.

Prior to cyclization of the dipeptides, the prolinylamino group was deprotected using transfer hydrogenation by refluxing the dipeptide in MeOH in the presence of Pd-C and cyclohexene afforded the N-deprotected products 9a and 9b. The peaks corresponding to the benzylic protons of the Z-group at 5.73 and 5.1 were absent in the ¹H NMR spectra of 9a and 9b, confirming that the Z-group had been successfully removed.

The final step of the reaction involved the cyclization of the dipeptides 9a and 9b. This was first attempted using DMAP in refluxing toluene but the reaction was unsuccessful. Use of higher boiling solvents such as xylene did
not improve the situation. Refluxing the dipeptide in xylene in the presence of NaH as base also did not yield the targeted molecules since after work up, the $^1$H NMR spectrum showed the presence of the ester peaks (δ 4.1 and 1.1). In the light of these results, it was decided to conduct the cyclization via saponification of the ester. The latter reaction was successfully accomplished using lithium hydroxide since the $^1$H NMR spectrum of the reaction mixture showed the absence of the ethyl ester peaks. However, due presumably to the high solubility of the resulting ammonium salt in water, the work-up using dilute mineral acid proved to be very difficult.

Refluxing the dipeptide 9a at 180° in ethylene glycol using a catalytic amount of potassium carbonate finally yielded the cyclo(l-prolinyl-l-isoleucine) 4a. The same procedure yielded also the isomer 4b (Scheme 1).

For the diketopiperazine 6 the α-NH of tryptophan was protected as the BOC group using BOC anhydride in the presence of triethylamine (TEA). Coupling of the BOC-protected tryptophan 10 with proline ethyl ester 11 using DCC and 1,1-dihydroxybenzotriazole in the presence of TEA yielded the dipeptide 12.

Treatment of the dipeptide 12 with TFA gave the deprotected dipeptide 13. The absence of the intense singlet at δ 1.1 ppm confirmed that BOC was effectively removed. The dipeptide 13 underwent cyclization by refluxing in ethylene glycol using catalytic amount of K$_2$CO$_3$ to give the diketopiperazine 6 (Scheme 2).

Antibacterial screening of all the compounds were carried out, using the Gram-positive bacterium Staphylococcus aureus, and the Gram-negative bacteria, Escherichia coli, Pseudomonas aeruginosa and Salmonella typhimurium. Blank tests showed that DMSO and water used in the preparation of the test solutions did not affect the growth of the microorganisms. Antibacterial activity of the different diketopiperazines 3, 4a and 5 was evaluated at concentration of 1600 μg ml$^{-1}$. Cetyltrimethylammonium bromide (CTBA) was used as control. The diketopiperazine 3 containing a hydroxyl group was found active against P. aeruginosa and S. aureus. The other dipeptides were found inactive. Hence, antibacterial activity of 3 was further studied using different concentrations (1600, 800, 400, 200 and 100 μg ml$^{-1}$) in DMSO and the zone of inhibition was measured. At a concentration of 1600 μg ml$^{-1}$, 3 inhibited the growth of P. aeruginosa with an inhibition zone of 15.5 mm while it inhibited the growth of S. aureus with an inhibition zone of 10.5 mm. CTBA inhibited the growth of both the bacteria with inhibition zone of 20 mm. 3 inhibited the activity of S. aureus at a minimum concentration of 400 μg ml$^{-1}$ (7.0 mm) while it was still active P. aeruginosa at a concentration of 100 μg ml$^{-1}$ (9.5 mm).

**Experimental**

$^1$H and $^{13}$C spectra (CDCl$_3$) were recorded on a Brucker Spectrospin instrument at 250 MHz with TMS as internal standard and IR spectra on a Mattson Genesis II series FTIR spectrophotometer. Chromatography refers to the 'flash column' technique over silica gel (70–230 mesh). TLC was carried out on plates pre-coated with silica and visualized by exposure to iodine vapour. C, H, N were analyzed on a LECO CHNS-932 analyzer. All chemicals were of A.R. grade except methanol, which was of GPR grade. Methanol
was distilled and kept over molecular sieves.

N-(Benzyloxy carbonyl)-L-proline: To a ice-cold (0°) solution of L-proline (1.5 g, 13.05 mmol) dissolved in 4 M NaOH (6.3 ml, 27.41 mmol, 2.1 eq), benzyl chloroformate (2.26 g, 15.66 mmol, 1.3 eq) was added dropwise over a period of 20 min with stirring while maintaining the temperature at 0°. The mixture was further stirred for 2 h with gradual warming to room temperature while monitoring the progress of the reaction by TLC (methanol/glacial acetic acid, 19:1, Rf 0.6). The reaction mixture was then diluted with water (10 ml) and extracted with ethyl ether (2 x 10 ml). The resulting aqueous phase was acidified with HCl (1:1). The liberated oil was extracted with ether (5 x 10 ml) and dried over anhyd. sodium sulfate. Evaporation of the solvent gave the product as a very pale yellow viscous liquid (2.6 g, 80%).

Method A: To a stirred, ice-cold suspension of isoleucine (1.5 g, 11.45 mmol) in absolute ethanol (50 ml), thionyl chloride (2.1 g, 17.17 mmol, 1.275 eq) was added dropwise over a period of 20 min with stirring while maintaining the temperature at 0°. The mixture was further stirred for 2 h with gradual warming to room temperature while monitoring the progress of the reaction by TLC (methanol/glacial acetic acid, 19:1, Rf 0.6). The reaction mixture was then diluted with water (10 ml) and extracted with ethyl ether (2 x 10 ml). The resulting aqueous phase was acidified with HCl (1:1). The liberated oil was extracted with ether (5 x 10 ml) and dried over anhyd. sodium sulfate. Evaporation of the solvent gave viscous liquid (2.6 g, 80%).

Isoleucine ethyl ester 7a. Method A: To a stirred, ice-cold suspension of isoleucine (1.5 g, 11.45 mmol) in absolute ethanol (50 ml), thionyl chloride (2.1 g, 17.17 mmol, 1.275 ml, 1.5 eq) was added dropwise and the mixture was refluxed for 3.5 h. The resulting pale yellow solution when evaporated in vacuo gave an oily liquid. It was mixed with DCM (10 ml) and the isoleucine ester was neutralized by adding aq. NaHCO₃ (4 x 5 ml). The organic layer was dried over anhyd. sodium sulfate. Evaporation of the solvent gave viscous yellow-brown liquid (1.183 g, 65%); ν max 3400 (NH), 1693 and 1702 cm⁻¹ (CO); δ H 7.0-7.3 (5H, m, aromatic CH), 5.1 (2H, m), benzyllic CH₂, 4.5 (1H, m, CH), 3.5 (2H, m, CH₂), 1.9-2.18 (4H, m, 2 x CH₂).

Method B: To a stirred, ice-cold suspension of isoleucine (1.5 g, 11.45 mmol) in absolute ethanol (50 ml), thionyl chloride (2.1 g, 17.17 mmol, 1.275 ml, 1.5 eq) was added dropwise and the mixture was refluxed for 3.5 h. The resulting pale yellow solution was concentrated in vacuo and the residue was dissolved in DCM (10 ml). The mixture was further stirred for 2 h and the solvent was evaporated. The resulting oil was extracted with ether (5 x 1 ml). The organic layer was dried over anhyd. sodium sulfate (Na₂SO₄) and evaporated to give a viscous yellow substance (2.6 g, 80%).

Isoleucine ethyl ester 7b: To a stirred, ice-cold suspension of isoleucine (1.5 g, 11.45 mmol) in absolute ethanol (15 ml) and catalytic amount of activated palladium on carbon. The mixture was refluxed for 5 h, then diluted with water (10 ml) and extracted with ethyl acetate (5 x 1 ml). The organic layer was dried (Na₂SO₄) and evaporated to give a viscous yellow-brown paste. The crude product was purified by column chromatography (EtOAc) and hexane, 2:1 (15 ml). Evaporation of the solvent yielded the desired dipeptide as a viscous oil, which solidified on cooling (3.39 g, 85%); ν max 3372 (NH), 1732 (CO), 1688 cm⁻¹ (CO); δ H 7.0-7.3 (5H, m), 6.4 (1H, bs, NH), 5.1 (2H, s), 4.5 (11H, bs), 4.4 (1H, bs), 4.2 (2H, bs), 3.5 (2H, m), 1.7-1.9 (51H, m), 1.1-1.3 (5H, m), 0.9 (6H, m); δ C 180, 171.6, 128.5, 128.0, 127.8, 67.3, 61.1, 58.9, 56, 49, 37.8, 35, 29, 25.1, 15.4, 14.2, 11.6 ppm. N-(Benzyloxy carbonyl)-L-proline-L-isoleucine ethyl ester 8b: Yield 60%; δ H 7.1-7.3 (5H, m), 5.1 (2H, s), 4.3 (2H, m), 4.1 (2H, q, J = 7 Hz), 3.5 (2H, m), 2.1-2.3 (4H, m), 1.5-1.6 (3H, t), 1.15 (3H, t, J = 7 Hz), 0.9 (6H, m).

Prolinyl-L-isoleucine ethyl ester 9a: To a solution of the protected peptide 8a (3.39 g, 8.692 mmol) in methanol (20 ml) were added cyclohexene (10 ml) and catalytic amount of activated palladium on carbon. The mixture was refluxed for 5 h, then purified by column chromatography by eluting first with EtOAc then with methanol. Evaporation of the methanol fraction gave a viscous yellow substance (1.99 g, 90%); ν max 3314-3462 (NH) 1665 (CO amide), 1738 cm⁻¹ (CO ester); δ H 5.3 (1H, NH), 4.5 (1H, dd, J = 7 Hz, 5 Hz), 4.3 (2H, q, J = 6 Hz), 3.8 (1H, dd, J = 7.3 Hz, J = 5 Hz), 2.9-3.0 (2H, m), 1.9-2.0 (3H, m), 1.7 (2H, m), 1.4 (1H, m), 1.2 (3H, t, J = 7 Hz), 1.1 (1H, m), 0.9 (6H, m). Prolinyl-L-isoleucine ethyl ester 9b: Yield 50%; δ H 4.5 (1H, m), 4.1 (2H, q, J = 7 Hz), 3.9 (1H, m), 3.0 (2H, t, J = 7 Hz), 2.2 (1H, m), 1.8-2.0 (2H, m), 1.5-1.6 (2H, m), 1.2 (3H, t, J = 7 Hz), 1.1 (2H, m), 0.9 (6H, d, J = 7.25 Hz).

Cyclo(L-prolinyl-L-isoleucine) 4a: To a solution of N-deprotected peptide 9a (1.99 g, 7.800 mmol) in ethylene glycol (15 ml), potassium carbonate (1 g, 1 eq) was added. The mixture was refluxed for 5 h, then diluted with water (15 ml) and extracted with ethyl acetate (5 x 10 ml). The organic layer was dried (Na₂SO₄) and evaporated to give a reddish-brown paste. The crude product was purified by column chromatography (EtOAc) to a brownish paste (0.716 g, 46%) in 30% overall yield starting from L-proline: δ H 4.0 (1H, m), 3.6 (1H, m), 3.5 (1H, m), 3.4 (1H, m), 2.3 (1H, m), 1.8 (4H, m), 1.4 (1H, m), 1.1 (1H, m), 0.9 (6H, m); δ C 174.6, 169, 63, 60, 58.9, 46, 26, 16, 15, 11 ppm; [α]Daising 4.426 (CHCl₃). Cyclo(L-prolinyl-L-isoleucine) 4b: Yield 52%; δ H 4.0 (1H, t, J = 7.3 Hz), 3.8 (1H, t, J = 7.3 Hz), 3.5 (2H, t), 1.9-2.3 (5H, m), 1.5 (1H, m), 1.1 (1H, m), 0.9 (6H, m).
N-t-Butyloxycarbonyltryptophan 10: To a stirred suspension of tryptophan (0.5 g, 2.5 mmol) in methanol, TEA (5 mL) and 

\( \text{Boc}_2O \) (0.53 g) were added. The reaction mixture was stirred for 24 h at room temperature. The reaction was monitored by TLC (EtOAc: Hex, 1:1, \( R_f = 0.86 \)). Methanol was then evaporated and a yellow paste was obtained. A minimum amount of water was added to the reaction mixture and extracted with diethyl ether (2 \times 25 mL), washed with brine (20 mL), dried (\( \text{Na}_2\text{SO}_4 \)) and on evaporation a yellow paste was obtained (0.60 g, 80%); \( \nu_{\text{max}} \) 3300 (NH), 1710, 1690 cm\(^{-1}\) (CO, ester), 1690 cm\(^{-1}\) (CO, amide); \( \delta_H \) 8.1 (1H, s), 7.5 (4H, m), 4.4 (1H, m), 4.0 (2H, q), 3.2 (2H, d), 1.5 (3H, t), 1.1 (9H, s).

l-Prolinyl ethyl ester 11: To a stirred, ice-cold suspension of proline (1.7 g, 0.015 mol) in absolute ethanol (50 mL), thionyl chloride (2.73 g, 1.7 mL, 1.5 eq) was added dropwise and the mixture was refluxed for 3-4 h. The reaction mixture was then allowed to stand overnight and the ethanol was evaporated in vacuo to leave proline ester hydrochloride as a viscous colourless liquid, which was stirred with dry diethyl ether. The ether was removed by evaporation leaving a viscous colourless liquid (2.49 g, 90%); \( \nu_{\text{max}} \) 1730 cm\(^{-1}\) (CO); \( \delta_H \) 4.3 (1H, m), 4.0 (2H, q, \( J \) 7 Hz), 2.6 (2H, m), 1.9 (4H, t), 1.4 (3H, t, \( J \) 7 Hz). The resulting l-prolinyl ethyl ester hydrochloride (3.4 g) was dissolved in water and washed with a saturated solution of potassium carbonate and extracted with diethyl ether (2 x 25 mL), thionyl chloride (2.73 g, 1.7 ml, 1.5 eq) was added to it and the product was extracted with DCM. The organic extract was evaporated and a yellow paste was obtained (0.26 g, 30%); \( \delta_H \) 7.5 (5H, m, ArH), 4.2 (2H, m), 3.7 (2H, m), 2.5 (1H, m), 1.7 (4H, m), \( m/z \) (Cl) 312 (M + \( \text{NH}_4^+ \)), 284 (M + H\(^+\)), 120 (base peak).

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