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Short communication

Synthesis and biological evaluation of Pseudostellarin D

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Abstract – Pseudostellarin D, a natural cyclic heptapeptide, was successfully synthesized and characterized by IR, ^{1}H NMR, ^{13}C NMR, FABMS and elemental analysis. The synthesized compound was evaluated for antibacterial, antifungal, anti-inflammatory and anthelmintic activities. © Elsevier, Paris

Pseudostellarin D / tyrosinase / *Pseudostellaria heterophylla* / cyclic peptide / cyclic heptapeptide / antibacterial / antifungal / anti-inflammatory / anthelmintic

1. Introduction

Peptides, in particular cyclic peptides, are among the various organic compounds that have received the most attention during the last three decades due to their unique structures and biological activities. Recently Hiroshi Morita et al. [1] reported the isolation of a new potent tyrosinase inhibitory cyclic peptide, Pseudostellarin D, from the roots of *Pseudostellaria heterophylla*. In continuation of our work on synthesizing natural cyclic peptides and screening for their biological activities [2], an attempt was made towards the synthesis of Pseudostellarin D. The synthesised product was further evaluated for antimicrobial, anti-inflammatory and anthelmintic activities, keeping in view the wide range of activities exhibited by various natural cyclic peptides.

2. Chemistry

The title compound (**7**) was prepared by the method shown in *scheme 1*. Pseudostellarin D, a cyclic heptapeptide, is cyclo[Gly-Gly-Tyr-Pro-Leu-Ile-Leu]. In order to carry out the synthesis, the molecule was split into three dipeptide units: Boc-Gly-Gly-OMe (**1**), Boc-Pro-Leu-OMe (**3**), Boc-Ile-Leu-OMe (**4**) and a single amino acid unit Tyr-OMe. The dipeptides were synthesised by coupling Boc-amino acids with the respective amino acid methyl ester hydrochorides using DCC and triethylamine. The ester group of the dipeptide (**1**) was removed with LiOH and the deprotected dipeptide was coupled with Tyr-OMe to get the tripeptide Boc-Gly-Gly-Tyr-OMe (**2**). The ester group of the dipeptide (**3**) was removed with LiOH and the Boc-group of the dipeptide (**4**) was removed with trifluoroacetic acid (TFA). Both the deprotected units were coupled to get the tetrapeptide Boc-Pro-Leu-Ile-Leu-OMe (**5**). The tetrapeptide (**5**) was then coupled with the tripeptide (**2**) after proper deprotection to get the heptapeptide Boc-Gly-Gly-Tyr-Pro-Leu-Ile-Leu-OMe (**6**). Finally, cyclization of the linear heptapeptide (**6**) was carried out by the p-nitrophenyl ester method to get the cyclic heptapeptide Pseudostellarin D. The physical constants of compound (**7**) are listed in *table I*. The newly synthesized compound was analysed for C, H, N and the structure was confirmed on the basis of IR, NMR and mass spectrum. Characteristic IR absorption bands of -CONH- moiety were present in the cyclized products. ^{1}H NMR spectrum of the cyclized product clearly indicates the presence of all respective amino acid moieties. Moreover, the ^{1}H NMR and ^{13}C NMR spectra of the cyclized product are in good agreement with the

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Boc-Gly-OH + H-Gly-OMe
↓a
Boc-Gly-Gly-OMe
↓b
Boc-Gly-Gly-OH + H-Tyr-OMe
(1) ↓a
Boc-Gly-Gly-Tyr-OMe
(2)

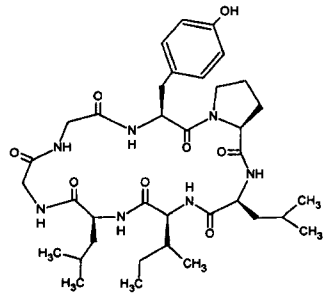
Boc-Pro-OH + H-Leu-OMe → ↓a → Boc-Pro-Leu-OMe → ↓b (3) → Boc-Pro-Leu-OH

Boc-Ile-OH + H-Leu-OMe → ↓a → Boc-Ile-Leu-OMe → ↓c (4) → H-Ile-Leu-OMe

Boc-Pro-Leu-OH + H-Ile-Leu-OMe
↓a
Boc-Pro-Leu-Ile-Leu-OMe
↓c (5)

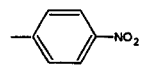
Boc-Gly-Gly-Tyr-OMe
↓b (2)

Boc-Gly-Gly-Tyr-OH + H-Pro-Leu-Ile-Leu-OMe
↓a
Boc-Gly-Gly-Tyr-Pro-Leu-Ile-Leu-OMe
↓b, d (6)
Boc-Gly-Gly-Tyr-Pro-Leu-Ile-Leu-OPNP
↓c, e



Pseudostellarin D
(7)

where

PNP = 

a : $CHCl_3$, DCC, Et_3N, 24 hrs, RT
b : THF : H_2O (1:1), LiOH, 1hr, RT
c : CF_3COOH/ $CHCl_3$, 1 hr, RT
d : p-nitrophenol, DCC, $CHCl_3$, 12 hrs, RT
e : $CHCl_3$, Pyridine, 10 days, 0°C

Scheme 1.

Table I. Physical data of Pseudostellarin D.

Compound	m.p. °C (lit. m.p.)	Yield %	Formula
7	176–178 (177–179)	76.4	$C_{36}H_{55}N_7O_8$

results shown in the literature for the naturally isolated compound. The absence of peaks corresponding to the p-nitrophenyl ester group in the IR, ^1H NMR, ^{13}C NMR and mass spectrum of the cyclic peptide clearly indicate the cyclization of the linear segment.

The mass spectrum of Pseudostellarin D showed the $(M^+ + 1)$ peak at m/z 714 which is consistent with the molecular formula $C_{36}H_{55}N_7O_8$.

3. Pharmacology

The synthesized compound Pseudostellarin D was evaluated for its antibacterial, antifungal, anti-inflammatory and anthelmintic activity. The antibacterial and antifungal activities were carried out by the disc diffusion method [3] and the results are summarized in *table II*. The anti-inflammatory activity was carried out according to the method of Winter et al. [4] and the results are presented in *table III*. The anthelmintic activity was carried out by Garg's method [5] and the results are given in *table IV*.

Pseudostellarin D was crystallized from $CHCl_3$/n-hexane and was analysed for C, H and N; the results had a maximum deviation ± 0.4% from the theoretical values.

In conclusion, it may be stated that the cyclic heptapeptide Pseudostellarin D can be synthesized successfully in the laboratory using the solution phase method for peptide synthesis (and p-nitrophenyl ester method for cyclization). The results from antimicrobial and pharmacological studies show that Pseudostellarin D is a biologically active cyclic peptide. These studies give extra

Table II. Antimicrobial activity of Pseudostellarin D.

Compound	Diameter of zone of inhibition (in mm)					
	P. aeruginosa	*E. coli*	*B. subtilis*	*S. aureus*	*C. albicans*	*A. niger*
7	16	13	16	16	–	–
Penicillin	11	12	18	18	–	–
Griseofulvin	–	–	–	–	20	18
DMSO (Control)	–	–	–	–	–	–

– indicates no activity.

Table III. Anti-inflammatory activity of Pseudostellarin D.

Compound	increase in paw volume (mL) ± S.E.	% inhibition of oedema
7	0.75 ± 0.03	17.58
Ibuprofen	0.55 ± 0.03	39.56
control	0.91 ± 0.03	–

Table IV. Anthelmintic activity of Pseudostellarin D.

Compound	conc. (mg)	Mean paralysing time (min) ± S.E.	Mean death time (min) ± S.E.
7	100	98.84 ± 2.10	128.81 ± 2.04
	200	89.75 ± 1.87	112.32 ± 2.12
Mebendazole	100	17.44 ± 0.09	54.4 ± 1.08
	200	12.14 ± 1.02	31.04 ± 1.09
control	–	–	–

evidence for the intense biological activities and medicinal importance of the cyclic peptides.

4. Experimental protocols

4.1. Chemical methods

The melting points were determined in open capillaries and are uncorrected. IR spectra were recorded on a Perkin-Elmer IR spectrometer and ^1H NMR spectra were recorded on a 90 MHz spectrometer using $CDCl_3$ as a solvent and TMS as internal standard. Purity of all the compounds was checked by the TLC on silica gel G plates.

4.1.1. Starting materials

For the protection of the amino group of the L-amino acids, di-tertiary butyl pyrocarbonate (Boc-O-Boc) was used. The carboxyl group of L-amino acids was protected by esterification. The Bodanszky procedure [6] with modifications [7] was used for the synthesis of the peptides. Boc-group was removed by stirring the Boc-amino acid/peptide (1 mmol) with CF_3COOH (2 mmols) in $CHCl_3$ (15mL) for 1 h at RT and the ester group was removed by stirring the amino acid/peptide methyl ester (1 mmol) with LiOH (1.5 mmol) in (1:1) THF: H_2O (16 mL) for 1 h at room temperature. Finally by employing p-nitrophenyl ester method [8], the cyclization was successfully carried out.

4.1.2 General procedure for peptide coupling

Amino acid/peptide methyl ester hydrochloride (10.0 mmol) was dissolved in dichloromethane (20 mL). To this, triethylamine (4 mL, 28.7 mmol) was added at 0 °C and the reaction mixture was stirred for 15 min. Boc-amino acid (10.0 mmol) in $CHCl_3$ (20 mL) and DCC (10.0 mmol) were added with stirring. After 24 h stirring, the reaction mixture was filtered and the filtrate was washed with 5% $NaHCO_3$ (20 mL) and saturated NaCl (20 mL) solutions. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum. To remove the traces of the dicyclohexylurea (DCU), the product was dissolved in a minimum amount of chloroform and cooled to 0 °C. The crystallized DCU was removed by filtration. To the filtrate, petroleum ether was added at 0 °C to recrystallize the pure product. Using the above procedure, the following Boc-peptide methyl esters were prepared.

4.1.2.1. Boc-Glycyl-Glycine methyl ester (**1**)

Colourless viscous liquid, % yield: 71.8; Anal $C_{10}H_{18}N_2O_5$ [% C found (calcd.): 48.76 (48.75), % H found (calcd.): 7.28 (7.31), % N found (calcd.): 11.40 (11.38)]. The IR spectrum provided the following characteristic absorption bands (KBr, cm^{-1}). 3 350 (br. s, –NH), 2 934 (s, CH), 1 701 (m, –C=O ester), 1 675 (s, C=O amide), 1 228 (s, C–N). The NMR spectrum provided the following characteristic chemical shifts ($CDCl_3$): δ 5.5 (br. s, 2H, NH), 3.9 (m, 4H, α-CH), 3.75 (s, 3H, OCH_3), 1.4 (s, 9H, ^{t}Boc).

4.1.2.2. Boc-Glycyl-Glycyl-Tyrosine methyl ester (**2**)

Brown semisolid mass, % yield: 71.5, $[\alpha]_D$: + 7.8°, Anal $C_{19}H_{27}N_3O_7$ [% C found (calcd.): 67.47 (67.40), % H found (calcd.): 8.07 (8.03), % N found (calcd.): 12.18 (12.50)]. The IR spectrum showed the following characteristic absorption bands (KBr, cm^{-1}). 3 320 (br, s, –NH), 2 925 (s, –CH), 1 705 (s, C=O ester), 1 685 (s, C=O amide), 1 650 (br, s, C=O amide), 1 450 (s, C–N), The NMR spectrum provided the following characteristic chemical shifts ($CDCl_3$): δ 8.8–8.6 (br. s, 2H,

NH), 8.2–8.1 (br. s, 1H, NH), 7.0 (d, 2H, C_3 & C_5–H, J = 7.8 Hz), 6.9 (d, 2H, C_2 & C_6–H, J = 7.8 Hz), 4.6–4.3 (m, 1H, α-CH), 4.2–4.0 (m, 4H, α-CH), 3.75 (s, 3H, OCH_3), 3.3–3.2 (m, 2H, β-CH_2), 1.4 (s, 9H, ^{t}Boc).

4.1.2.3. Boc-Prolyl-Leucine methyl ester (**3**)

White crystals, m.p. 93 °C, % yield: 75.4, Anal $C_{17}H_{30}N_2O_5$ [% C found (calcd.): 59.58 (59.60), % H found (calcd.): 8.78 (8.78), % N found (calcd.): 8.15 (8.15)]. The IR spectrum showed the following characteristic absorption bands (KBr, cm^{-1}). 3 250 (br, s, –NH), 2 950 (s, –CH), 1 710 (s, C=O ester), 1 700 (br, s, C=O amide), 1 620 (s, C=O amide), 1 420 (s, C–N). The NMR spectrum provided the following characteristic chemical shifts ($CDCl_3$): δ 6.4 (br. s, 1H, NH), 4.6–4.4 (m, 1H, α-CH), 4.3–4.1 (m, 1H, α-CH), 3.7 (s, 3H, OCH_3), 3.5–3.2 (m, 2H, N–CH_2), 2.0–1.6 (m, 6H, CH_2–CH_2 & CH_2), 1.45 (s, 9H, ^{t}Boc), 1.3–1.1 (m, 1H, CH), 0.95 (d, 6H, -$(CH_3)_2$, J = 6.0 Hz).

4.1.2.4. Boc-Isoleucyl-Leucine methyl ester (**4**)

White crystals, m.p. 70 °C, % yield: 76.3, Anal. $C_{18}H_{34}N_2O_5$ [% C found (calcd.): 60.30 (60.38), % H found (calcd.): 9.46 (9.49), % N found (calcd.): 7.78 (7.81)]. The IR spectrum showed the following characteristic absorption bands (KBr, cm^{-1}). 3 540 (br, s, –NH), 2 975 (s, –CH), 1 735 (s, C=O ester), 1 675 (s, C=O amide), 1 660 (s, C=O amide), 1 340 (s, C–N). The NMR spectrum provided the following characteristic chemical shifts ($CDCl_3$): δ 6.6 (br. s, 1H, NH), 5.3 (br. s, 1H, NH), 4.6–4.5 (m, 1H, α-CH), 4.3–4.1 (m, 1H, α-CH), 3.7 (s, 3H, OCH_3), 2.0–1.8 (m, 4H, –CH_2), 1.5 (s, 9H, ^{t}Boc), 1.3–1.1 (m, 2H, 2–CH), 1.0 (doublet overlapped with triplet, 12H, $4CH_3$).

4.1.2.5. Boc-Prolyl-Leucyl-Isoleucyl-Leucine methyl ester (**5**)

Brown semisolid mass, % yield: 73.0, $[\alpha]_D$: + 12.5°, Anal $C_{29}H_{52}N_4O_7$ [% C found (calcd.): 61.20 (61.20), % H found (calcd.): 9.12 (9.15), % N found (calcd.): 9.86 (9.85)]. The IR spectrum showed the following characteristic absorption bands (KBr, cm^{-1}). 3 245 (br, s, –NH), 2 935 (s, –CH), 1 740 (s, C=O ester), 1 690 (s, C=O amide), 1 675 (br. s, C=O amide), 1 650 (br. s, C=O amide), 1 350 (s, C–N). The NMR spectrum provided the following characteristic chemical shifts ($CDCl_3$): δ 6.9–6.8 (br. s, 1H, NH), 6.4–6.2 (br. s, 1H, NH), 4.8–4.6 (m, 2H, α-CH), 4.4–4.3 (m, 1H, α-CH), 4.2–4.0 (m, 1H, α-CH), 3.75 (s, 3H, OCH_3), 3.5–3.1 (m, 2H, N–CH_2), 2.3–1.5 (m, 10H, CH_2–CH_2, CH_2 & CH_2–CH_2), 1.45 (s, 9H, ^{t}Boc), 1.3–1.1 (m, 3H, CH), 0.95 (doublet overlapped with triplet, 18H, $6CH_3$).

4.1.2.6. Boc-Glycyl-Glycyl-Tyrosinyl-Prolyl-Leucyl-Isoleucyl-Leucine methyl ester (**6**)

Yellow semisolid mass, % yield: 62.8, $[\alpha]_D$: –61.5°, Anal $C_{42}H_{67}N_7O_{11}$ [% C found (calcd.): 59.62 (59.65), % H found (calcd.): 7.91(7.93), % N found (calcd.): 11.62 (11.60)]. The IR spectrum showed the following characteristic absorption bands (KBr, cm^{-1}). 3 315 (br, s, –NH), 2 930 (s, –CH), 1 730 (s, C=O ester), 1 695 (s, C=O amide), 1 670 (br. s, C=O amide), 1 645 (s, C=O amide), 1 325 (s, C–N). The NMR spectrum provided the following characteristic chemical shifts ($CDCl_3$): δ 8.8–8.6 (br. s, 2H, NH), 8.2–7.9 (br. s, 4H, NH), 6.9 (d, 2H, C_3 & C_5–H, J = 8.5 Hz), 6.7 (d, 2H, C_2 & C_6–H, J = 8.5 Hz), 4.6–4.3 (m, 5H, α-CH), 4.2–4.0 (m, 4H, α-CH), 3.75 (s, 3H, OCH_3), 3.65–3.3 (m, 2H, N–CH_2), 3.1–3.0 (m, 2H, β-CH_2), 2.2–1.6 (m, 10H, CH_2–CH_2, CH_2, CH_2 & CH_2), 1.45 (s, 9H, ^{t}Boc), 1.3–1.1 (m, 3H, –CH), 0.95 (doublet overlapped with triplet, 18H, $6CH_3$).

4.1.2.7. Pseudostellarin D (**7**)

p-Nitrophenyl ester method:

The ester group of the linear segment (2 mmol) was removed with LiOH (3 mmol) and the p-nitrophenyl ester group was introduced using the following procedure:

The Boc-peptide (1.5 mmol) was dissolved in $CHCl_3$ (15 mL) at 0 °C. Then p-nitrophenol (0.27 g, 2 mmol), DCC (0.214 g, 1.5 mmol) were added and stirred for 12 h at room temperature. The reaction mixture was filtered and the filtrate was washed with $NaHCO_3$ solution (10%) until excess of p-nitrophenol was removed and finally washed with 5 % HCl (5 mL) to get Boc-peptide-PNP ester.

To the above Boc-peptide-PNP ester (1.2 mmol) in $CHCl_3$ (15 mL), CF_3COOH (0.274 g, 2.4 mmol) was added, stirred for 1 h at room temperature and washed with 10% $NaHCO_3$ solution. The organic layer was dried over anhydrous Na_2SO_4. To the peptide-PNP ester in $CHCl_3$ (20 mL), pyridine (1.4 mL, 2 mmol) was added and kept at 0 °C for 10 d. The reaction mixture was washed with 10% $NaHCO_3$ until the byproduct p-nitrophenol was removed completely and finally washed with 5% HCl (5 mL). The organic layer was dried over anhydrous Na_2SO_4. Chloroform and pyridine were distilled off to get the crude product of the cyclized compound, which was then recrystallized from $CHCl_3$/n-hexane. Pale yellow solid, m.p. 176–178 °C (lit. 177–179 °C), % yield 76.4, $[\alpha]_D$: –65.5° (lit. –64.8°), Anal $C_{36}H_{55}N_7O_8$ [% C found (calcd.): 60.61 (60.59), % H found (calcd.): 7.70 (7.71), % N found (calcd.): 15.69 (15.71)]. The IR spectrum showed the following characteristic absorption bands (KBr, cm^{-1}).

3 610 (br, s, –OH), 3 165 (br, s, –NH), 2 980 (s, –CH), 1 715 (br. s, C=O ester), 1 685 (s, C=O amide), 1 670 (s, C=O amide), 1 645 (s, C=O amide), 1 330 (br. s, C–N). The NMR spectrum provided the following characteristic chemical shifts ($CDCl_3$): δ 8.8–8.6 (br. s, 2H, NH), 8.2–7.9 (br. s, 4H, NH), 6.9 (d, 2H, C_3 & C_5–H, J = 8.5 Hz), 6.7 (d, 2H, C_2 & C_6–H, J = 8.5 Hz), 4.6–4.3 (m, 5H, α–CH), 4.25–4.0 (m, 4H, α-CH), 3.65–3.3 (m, 2H, N–CH_2), 3.1–3.0 (m, 2H, β-CH_2), 2.2–1.6 (m, 10H, CH_2–CH_2, CH_2, CH_2 & CH_2), 1.3–1.1 (m, 3H, –CH), 0.95 (doublet overlapped with triplet, 18H, $6CH_3$). ^{13}C NMR (DMSO-D_6, 25.4 MHz): 172.0 (s), 171.7 (s), 171.5 (s), 171.0 (s), 170.5 (s), 168.6 (s), 167.0 (s), 155.5 (s), 130.2 (d), 128.0 (s), 114.3 (d), 61.9 (d), 56.3 (d), 55.0 (d), 54.5 (d), 51.6 (d), 45.2 (t), 43.1 (t), 42.6 (t), 40.5 (t), 39.7 (t), 39.0 (t), 38.3 (d), 29.8 (t), 24.8 (t), 24.3 (d), 24.0 (d), 23.6 (t), 23.0 (q), 22.5 (q), 22.0 (q), 20.7 (q), 15.0 (q), 10.8 (q). The FAB mass spectrum showed the (M^+ + 1) peak at m/z 714.4.

4.2. Biological methods

4.2.1. Evaluation of antimicrobial activity

Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa and *Escherichia coli* were cultivated in nutrient broth medium. *Candida albicans* and *Aspergillus niger* were cultured in Fluid Sabraud's medium.

Evaluation of the antimicrobial activity of all compounds was initially performed employing the disc diffusion technique [3]. Each compound was tested at a level of 50 μg/disc using dimethyl sulfoxide (DMSO) as the solvent. Penicillin and griseofulvin were used as standard antibiotics against bacterial and fungal species at 10 μg and 25 μg per disc, respectively. The diameter of the zone of inhibition was measured after 24 h incubation at 37 °C *(table II)*.

4.2.2. Evaluation of anti-inflammatory activity

Winter's hind paw method [4] was used in the present study for the evaluation of the anti-inflammatory activity. Carragenin (an irritant) at a concentration of 1mg/mL was injected subcutaneously into the hind paw of the rat to produce the oedema. Different groups of animals were administered with standard NSAID (Ibuprofen), test samples and the vehicle used for the preparation of samples. The increase in paw volume was measured before and after 3 h of administration and the results were compared *(table III)*.

4.2.3. Evaluation of anthelmintic activity

Anthelmintic activity studies were carried out against earth worms (*Pontoscolex corethrusus*) by Garg's method [5]. Suspensions of the samples were prepared by triturating the samples with tween 80 and distilled water and the resultant mixtures were stirred using a mechanical stirrer for 30 min. The resulting suspensions were used for the activity studies. The suspensions were diluted to contain 0.1% and 0.2% w/v of the test compound. 0.1% and 0.2% w/v suspensions of the standard drug mebendazole were also prepared in a similar way. Six earth worms of similar sizes were placed in petri plates of 4 inches diameter containing 50 mL each of suspension of the test and standard drug (mebendazole) at room temperature. Another set of six earth worms were kept as control in 50 mL suspension of distilled water and 0.5% tween 80.

50 mL each of the suspensions of the test compounds were added into separate petri plates containing six earth worms in each. The time required for the paralysis and death of the worms were noted. The death time was ascertained by placing the earth worms in warm water at 50 °C which stimulated the movement if the worm was alive *(table IV)*.

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