International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.9, No.6, pp 250-256, 2016

Synthesis and Biological Evaluation of Cyclo [(N-Me, O-Me) Tyr-Leu-Ala-Gly-Pro] a Pseudostellarin-A Analog

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Abstract : N-methylated analog of pseudostellarin-A was synthesized by solution phase peptide synthesis using dicyclohexylcarbodiimide as a coupling reagent in the presence of a base. The structure of the compound was confirmed by IR, ^{1}H NMR, ^{13}C NMR, FABMASS and elemental analysis. The synthesized compound was screened for antifungal and anthelmintic activity. The N-methylated Cyclo [(N-Me, O-Me) Tyr-Leu-Ala-Gly-Pro] compound showed potent anthelmintic against *Eudrilus eugeniea* compared to the standard mebendazole. The N-methylated Cyclopentapeptide compound was also found to contain moderate antifungal activity.
Key words: N-Methylmorpholine, p-Nitrophenyl ester, Dicyclohexylcarbodiimides, Pseudostellarin-A.

Introduction

Pseudostellarin-A is a cyclic peptide isolated from the roots of *Pseudostellaria heterophylla*, belonging to the *Caryophyllaceae* family[1-3]. Pseudostellarin-A, a cyclic pentapeptide consists of one tyrosine, one leucine, one alanine, one glycine and one proline units: cyclo[Tyr-Leu-Ala-Gly-Pro]. Previously Himaja et al [4] reported the antimicrobial and anthelmintic activity of the synthesized pseudostellarin-A. The various biological applications of naturally derived cyclic peptides such as Oxytocin, Calcitonin, Polymixin B and Gramicidine highlighted peptides to be important source with wide medical applications[5, 6]. Moreover, the N-methylated amino acids, which are found in naturally occurring peptides are reported for their wide pharmacological applications like anti-malarial, anti-microbial activities and as antibiotics[7].

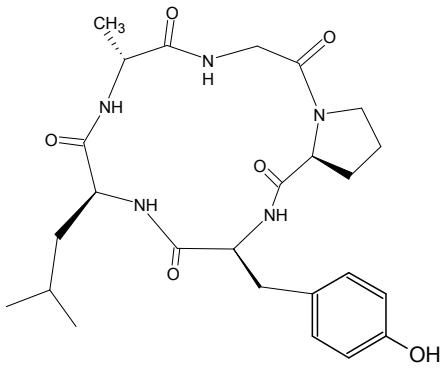


Fig 1: **Structure of pseudostellarin-A**

Studies report that the N-methylated peptides contain enhanced activity compared to unmethylated forms[8, 9]. Hence, keeping in view, the wide range of activities exhibited by various natural cyclic peptide and N-methylated peptide analogs and with emphasis on introducing conformational constraints and reduced biodegradability of peptides by enzymes; N-methylated analog of pseudostellarin-A was synthesized and evaluated for the antifungal and anthelmintic activities. In continuation to the previous reports and biological importance of Pseudostellarin, an attempt was made towards the synthesis of N-methylated pseudostellarin-A analog. The synthesized compound was evaluated for the anthelmintic and antifungal activities. The cyclic pentapeptide was disconnected in to one dipeptide unit and a tripeptide unit. The synthesis was performed by solution phase method using dicyclohexylcarbodiimide (DCC) as the coupling agent and cyclisation carried out by p-nitrophenyl ester method.

Experimental section

Chemicals and instruments[15-25]

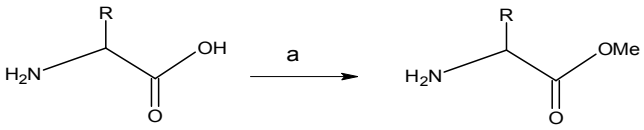
All reactions requiring anhydrous condition were performed in clean dry apparatus and were magnetically stirred unless otherwise stated. Melting point was determined by capillary method and was uncorrected. An IR spectrum was obtained using Jasco FT/IR-5300 IR spectrometer ^{1}H NMR analysis was performed using Bruker AC NMR spectrometer (300 MHz). A FABMASS spectrum was obtained on a Joel Sx 102/DA-6000 mass spectrometer using xenon as the carrier gas.

General scheme of peptide synthesis

Protection of carboxyl group

The protection of carboxyl group was performed by modified procedure described below. The carboxyl groups were protected by converting them to methyl esters by the following method (Scheme1).

a $\rightarrow$ $SOCl_2$, MeOH, Reflux, 8-10h.

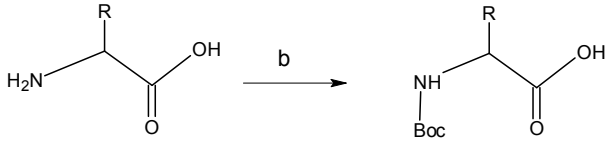


Scheme 1: Synthesis of amino acid carboxylic ester

Protection of the amino group

The tertiary butyloxycarbonyl (Boc-) group is a commonly used amino protecting group, which is known for its affordable cost, ease of availability and its selectivity. The Boc-amino acids were prepared by the following method (Scheme 2).

b $\rightarrow$ (Boc)2O, 1N NaOH, isopropanol, RT, 2hr



Scheme 2: Synthesis of Boc-amino acid

N-methylation of Boc-amino acid

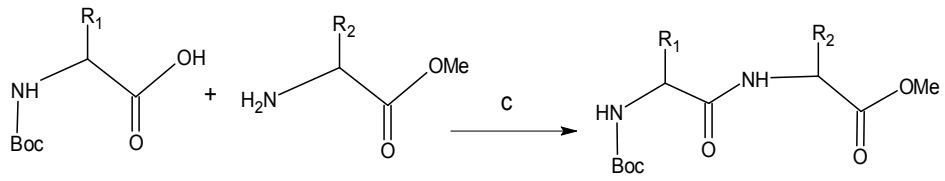
The Boc-Tyr amino acid was dissolved in dry THF and cooled to 0°C. The methylation was carried out by the reaction of activated sodium hydride (12mmol) and methyl iodide (6mmol) with the amino acid. The solution was stirred for 4 hrs at room temperature to obtain the product (1a). The reaction mixture was washed

with saturated NH_4Cl, 20% $Na_2S_2O_2$ solution and NaCl solution. The organic layer was concentrated using a rotatory evaporator at reduced pressure to obtain the final product[10].

Synthesis of dipeptides

The dipeptides were synthesized by the peptide coupling method proposed by Bodanszky [11] with minor modifications. The Boc-amino acid and amino acid methyl ester hydrochloride of equimolar concentration were coupled using DCC as a coupling reagent and triethylamine as a base.

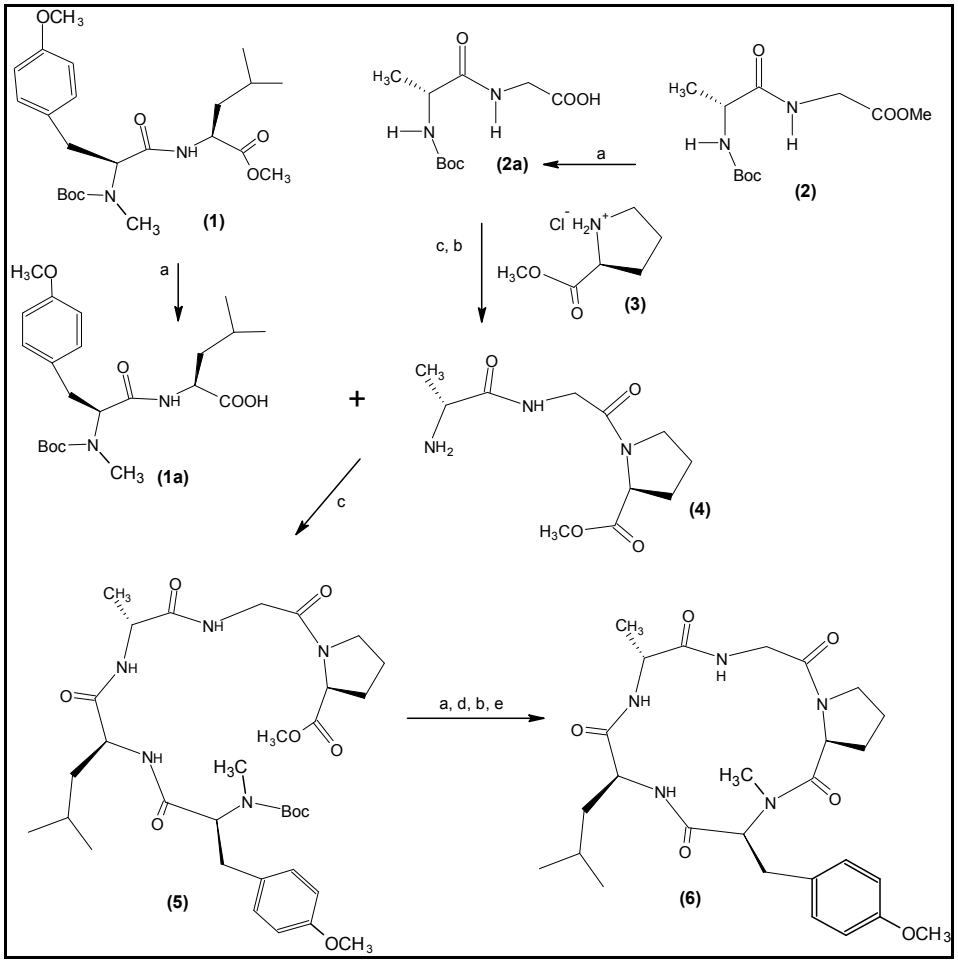
c → DCC, $CHCl_3$, TEA, RT, 24h.



Scheme 3: Synthesis of Dipeptide

Synthesis of linear pentapeptide

The pentapeptide linear sequence was synthesized by coupling one dipeptide unit ((N-Me, O-Me) Tyr-Leu) and a tripeptide sequence (Ala-Gly-Pro) after appropriate deprotection of the desired functional groups. Trifluroacetic acid was used for the deprotection of Boc- group and deprotection of ester group was performed by hydrolysis catalyzed by LiOH. The coupling was performed by the similar procedure carried out for dipeptide synthesis using DCC as a coupling reagent.



a = LiOH, THF: H_2O (1:1), RT, 1 hr;

b = TFA, NMM, RT, 1 hr;

c = DCC, NMM, $CHCl_3$, RT, 24 hr;

d = pnp, $CHCl_3$ RT, 12 hr;

e = NMM, $CHCl_3$, 7 days, 0 ^{0}C

Scheme 4: Synthesis of Cyclo [(N-Me, O-Me) Tyr-Leu-Ala-Gly-Pro] Pseudostellarin-A(6)

Cyclisation of the pentapeptide

The cyclisation of the linear pentapeptide ((N-Me, O-Me) Tyr-Leu-Ala-Gly-Pro) unit was performed by the p-nitrophenyl ester method with certain modifications [12]. The ester group of the linear pentapeptide sequence was deprotected using LiOH. The p-nitro phenol group was introduced by stirring the deprotected pentapeptide in $CHCl_3$ with p-nitrophenol and the reaction mixture was washed several times with saturated $NaHCO_3$ until the unreacted p-nitrophenol was removed completely. The Boc- group was removed by TFA using the standard procedure. To the deprotected linear fragment a catalytic amount of triethylamine was added and the reaction mixture was kept at 0°C for 7 days. Once the reaction was complete the obtained product was washed several times with saturated $NaHCO_3$ until the byproduct p-nitrophenol was completely removed. Further, the product was finally washed with 5% HCl and distilled water to obtain the final product which was dried over anhydrous Na_2SO_4 and evaporated under vacuum to get the cyclised product. The crude product was purified by recrystallization.

Evaluation of Antifungal Activity

The antifungal activity of the synthesized N-methylated pseudostellarin-A analog was performed by agar well diffusion method[13]. To the sterile petri plates containing 20 ml of sabouraud's agar medium was streaked with suspensions of *Candida albicans*. The test sample of 25mg/ml in dimethylformamide was added to the wells made in the gel medium inoculated with test organism. Similar, process was performed for the reference drug fluconazole and control. All the samples were incubated at 30^{o}C for 48 hrs and after incubation the growth inhibitory zone around the wells were measured. The diameter of the zone of inhibition is said to be directly proportional to the antifungal activity of the samples.

Evaluation of anthelmintic Activity

The anthelmintic property of the N-methylated pseudostellarin-A analog was performed by Garg's method[14]. The test sample was prepared by triturating the sample with 12.5% tween 80 and distilled water mixture, which was followed by 30 min of stirring using a mechanical stirrer. The standard drug (Mebendazole) of 100 mg concentration for every 5 ml of the solution to be tested was also prepared in a similar way.

Five earthworms (*Eudrilus eugeniea)* of similar size were placed in to 250 ml beaker containing 50 ml of test, standard and control solution solutions. The death time of the sample was determined by transferring the earth worms to beakers containing warm water (50°C) and tested for the movement of the organism if alive. The mean death time of the organism was studied by checking if no movement that confirming the death of the organism. The results of anthelmintic activity of the sample are shown in Table 3.

Results and discussion

The synthesized N-methylated analog was conventionally synthesized by solution phase method with good percentage yield. The synthesized compound was characterized by IR, 1H NMR, FABMASS and elemental analysis, which confirms the formation of N-methylated analog of Pseudostellarin-A. The physical data (Table 1) and spectra data of the synthesized compound is described below;

Table 1: Physical Data of the Cyclic Pentapeptide

Sl. No.	Cyclised product	Physical state	% yeild
1.	Cyclo[(N-Me,O-Me)Tyr-Leu-Ala-Gly-Pro)	Semisolid mass	57.00

Spectral data

1) Cyclo[(N-Me, O-Me)Tyrosyl-Leucyl-Alanyl-Glycyl-Proline] (6)

^{1}H NMR(300MHz, $CDCl_3$): δ 8.2 (1H, br. s, -NH), 8.1(1H, br. s, -NH), 7.2-6.9(4H, m, Ar-H of Tyr), 4.8-4.6(1H, m, α-H), 4.4-4.2(2H, m, α-H), 4.1-3.9(2H, m, α-H of Gly), 3.8-3.6(5H, m, CH_3 of Ala and β-CH_2 of Tyr), 3.75(3H, s, OCH_3), 3.6-3.4(2H, m, N-CH_2 of Pro), 2.8(3H, s, N-CH_3), 2.3-2.2(2H, m, β-CH_2 of Leu), 2.0-1.0(10H, m, CH_2-CH_2- of Pro, -$C(CH_3)_2$ of Leu).**IR ($CHCl_3$)**:3326.5(br. s, -NH Stretch), 2926.5(s, Aromatic-CH Stretch), 2855.6(s, -CH Stretch),1750 (s, C=O Stretch of ester),1661.9(s, C=O Stretch Of amide), 1514.7(s, -NH bend), 1455(s, CH bend), 1248.8 (s -CN Stretch) cm^{-1}. **FABMASS:** m/z = 529, (M+2). **Elemental Analysis:** Found (Calcd) %C: 61.79(61.23), % N: 12.93(13.22).

Table 2: Antifungal Activity of synthesized compound

SI.No	Compounds	Diameter of zone of inhibition(mm)
1	6	18
2	Fluconazole	22
2	DMF (Control)	0

The synthesized N-methylated analog of pseudostellarin-A was evaluated for their antifungal and anthelmintic activities. The compound showed moderate antifungal activity against fungi viz. *Candida albicans*, in comparison to the standard drug. The zone of inhibition of synthesized compound and the standard drug was comparably moderate with that of standard Fluconazole (Table 2).

The anthelmintic property of the synthesized was calculated by the analysis of the paralysis time and death time of the earthworms. N-methylated analog of pseudostellarin-A was also found to exhibit potent anthelmintic activity against earthworm's viz. *Eudrilus eugenia.* Compound 6 with the mean paralyzing time of (2.15 ± 0.69) and mean death time of (2.55 ± 0.24) was found to be more active as compared to the standard drug Mebendazole which was found to contain greater mean paralyzing (4.10 ± 0.32) and death time (4.54 ± 0.34).

Table 3: Anthelmintic activity of synthesized compound

Sl. No.	Compounds	Concentration (mg)	Mean paralyzing time (min) ± S.E.	Mean death time (min) ± S.E.
1.	6	100	2.15 ± 0.69	2.55 ± 0.24
2.	Mebendazole	100	4.10 ± 0.32	4.54 ± 0.34
3.	Control	–	N.E.	N.E.

Conclusion

The N-methylated pseudostellarin-A was successfully synthesized by solution phase method with good yield. The characteristic IR, ^{1}H NMR, FABMASS and elemental analysis data confirmed the formation of the N-methylated cyclic pentapeptide compound. The compound was found to contain potent anthelmintic property as compared to the existing drug Mebendazole. Thus the further studies on biological activity of the N-alkylated cyclic peptide analogs would help in identifying lead molecules with wide pharmacological applications.

Acknowledgement

The authors are thankful to VIT-DST SAIF, Vellore and Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences for providing the necessary facilities and support.

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