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Research Article

SYNTHESIS AND BIOLOGICAL EVALUATION OF PSEUDOSTELLARIN-A ANALOGS

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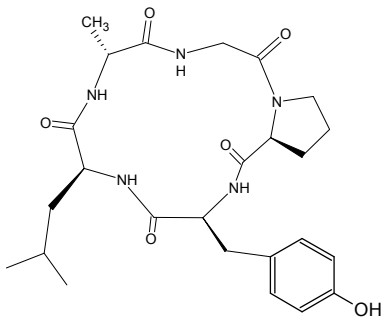
ABSTRACT

N-methylated analog of pseudostellarin-A was synthesized by coupling tertiary butyloxycarbonyl(Boc-) group protected amino acids with the amino acid methyl ester hydrochlorides using N,N'-Dicyclohexylcarbodiimide coupling agent. The structural elucidation of the synthesized compound was performed using FTIR, NMR, FABMASS and Elemental analysis. The synthesized compound was screened for the antifungal and antihelmintic properties. The synthesized N-methylated compound found to contain potent antihelmintic activity against earthworms (*Eudrilus eugeniea*) compared to standard mebendazole.

Keywords: 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 Family: *Caryophyllaceae* by Itokawa et al.[1-3] The structure of pseudostellarin-A consists of one tyrosine, one leucine, one alanine, one glycine and one proline units: cyclo[Tyr-Leu-Ala-Gly-Pro]. The synthesis of pseudostellarin-A was carried out by Himaja et al.[4] and the synthesized product was reported to show good anthelmintic activity as well as moderate antifungal activity.



Keeping in view of the wide biological application exhibited by various natural cyclic peptide cyclic 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 order to carry out the synthesis, the cyclic pentapeptide was disconnected into two dipeptide units and a single amino acid unit. The Boc-amino acids were coupled with the amino acid methyl ester hydrochlorides by dicyclohexylcarbodiimide (DCC) as the coupling agent.

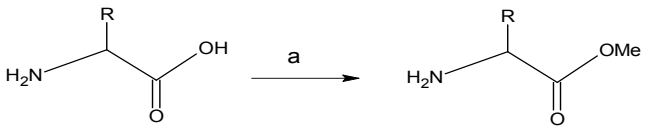
MATERIALS AND METHODS

All the reactions requiring anhydrous conditions were conducted in a dry apparatus and were magnetically stirred unless otherwise stated. Organic extracts were dried over anhydrous sodium sulphate. Melting points were determined by capillary method and were uncorrected. IR spectra were recorded on Jasco FT/IR-5300 IR spectrometer using a thin film supported on KBr pellets for solids and chloroform as a solvent for semisolids. ^{1}H NMR spectra were recorded on Bruker AC NMR spectrometer (300 MHz). FABMASS spectra were recorded at room temperature on a Joel Sx 102/DA-6000 mass spectrometer using xenon as the carrier gas. M-nitro benzyl alcohol was used as the matrix.

General Scheme of Peptide Synthesis

Protection of Carboxyl Group:

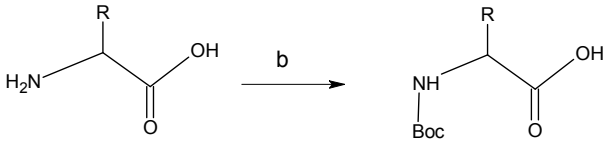
The carboxyl groups are generally protected as methyl, ethyl or benzyl esters. Methyl ester carboxyl protections were carried out by modifying the method given by Webb et al[5, 6].



a → $SOCl_2$, MeOH, Reflux, 8-10h.

Protection of Amino Group:

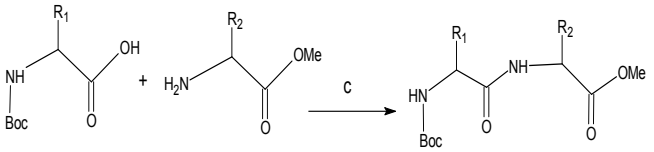
The tertiary butyloxycarbonyl (Boc-) group is one of the most widely used amino protecting groups because of its affordable cost, ease of availability and its selective removable property. The Boc-amino acids were prepared by the following route[7].



b → $(Boc)_{2O}$, 1N NaOH, isopropanol, RT, 2hr

Preparation of the Dipeptides:

Of the various reported methods for peptide coupling, we adopted the methods proposed by Bodanszky[8] and M. M. Joulie[9]. From these procedures we derived our own more convenient method for the peptide coupling reactions. The dipeptides were prepared by using Boc-amino acid and amino acid methyl ester hydrochloride, TEA and DCC.



c → DCC, $CHCl_3$, TEA, RT, 24h.

Preparation of the Pentapeptide:

The pentapeptide was prepared from one dipeptide unit and one tripeptide unit after appropriate deprotection at the desired functional groups. The Boc-group was deprotected by trifluoroacetic acid (TFA) and the ester group was removed by hydrolysis with LiOH. Deprotected dipeptide and tripeptide units were coupled using the procedure similar to that of the dipeptide coupling.

Cyclisation of the Linear Pentapeptides:

Cyclisation of the linear Pentapeptide unit was carried out by the p-nitrophenyl ester method of Bodanszky[8] with certain modifications.

The ester group of the linear fragment was removed with LiOH and the p-nitrophenyl ester group was introduced by stirring the deprotected pentapeptide in $CHCl_3$ with p-nitrophenol. 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 triethyl amine was added and the reaction mixture was kept at 0°C for 7 days. Washed several times with saturated $NaHCO_3$ until the byproduct, p-nitrophenol was completely removed, finally washed with 5% HCl and distilled water dried over anhydrous Na_2SO_4 and evaporated in vacuum to get the cyclised product. The crude product was purified by recrystalized from $CHCl_3$.

Evaluation of Antifungal Activity

The synthesized N-methylated pseudostellarin-A analog was screened for the antifungal activity by screening the compound for its minimum inhibitory concentration (MIC) using cup plate method[10] .The minimum inhibitory concentration of 250 µg/ml was obtained by preparing a solution containing 25mg/ml of test sample in dimethylformamide.

To the sterile Petri plate 20 ml of sterile Sabouraud's agar medium was poured and was inoculated with diluted suspension of test organism, *Candida albicans*. Wells were made in the medium and 10µl of solution of the test samples was filled into the wells. The samples were placed in refrigerator for 1 hr for diffusion and then incubated at 30°C for 48 hrs. After incubation the growth inhibition zones around the disks were observed. The diameter of the zone of inhibition is directly proportional to the activity of the substance. The inhibitory zone of the test sample was compared with the inhibitory zone of the standard antifungal drug Fluconazole.

Evaluation of Anthelmintic Activity

Anthelmintics are therapeutic agents used to destroy parasitic worms or remove them from the infected host. Anthelmintic activity studies were carried out aganist earthworms (*Eudrilus eugeniea)* by Garg's method[11].

Sample suspensions were prepared by triturating the compounds with 12.5% tween 80 and distilled water and the resultant mixture was stirred using a mechanical stirrer for 30 minutes. The resulting suspensions were then diluted to contain 100 mg in 5ml of the test samples. Standard Mebendazole was also prepared with the same concentration in a similar way.

Five earthworms of similar sizes were placed in a petri plate of 4 inches diameter containing 50ml of suspension of the test standard drugs (Mebendazole) at RT. The test compound and the control were also prepared in a similar way and the death time was ascertained by placing the earthworms in warm water at 50°C, which stimulated the movement if the worm was alive. The results of Anthelmintic Activity against *Eudrilus eugeniea a*re shown in Table 3.

RESULTS AND DISCUSSION

Table 1: Physical Data of the Cyclic Pentapeptides

Cyclised product	Physical state	% yeild
Cyclo[Tyr-(N-Me)Leu-Ala-Gly-Pro]	Semisolid mass	60.59

Spectral Data

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

^{1}H NMR(300MHz, $CDCl_3$): δ 8.1(1H, br. s, -NH), 7.7(1H, br. s, -NH), 6.8(1H, br. s, -NH), 7.1(1H, s, -OH), 7.1-6.9(4H, m, Ar-H of Tyr), 4.8(2H, m, α-H), 4.6-4.4(2H, m, α-H), 3.9-3.7(2H, m, α-H of Gly), 3.7-3.5(5H, m, CH_3 of Ala and β-CH_2 of Tyr), 3.4-3.2(2H, m, N-CH_2 of Pro), 3.2-2.9(4H,N-CH_2, β-CH_2 of Pro) 2.2(3H, s, N-CH_3), 2.4-2.2(2H, m, β-CH_2 of

Leu), 2.0-1.0(9H, m, γ-CH_2 of Pro, -$C(CH_3)_2$ of Leu).**IR ($CHCl_3$)**: 3676.9(m, -OH), 3327.5(br. s, -NH Stretch), 3017.6(s, Aromatic -CH Stretch), 2931.8(s, -CH Stretch),1759.9(s, C=O Stretch of ester), 1665 (s, C=O Stretch of amide),1511.9(s, -NH bend), 1454.2(s, CH bend), 1216.6 (s, -C-N Stretch) cm^{-1}.**FABMASS:** m/z = 516.**Elemental Analysis:** Found (Calcd) %C: 62.03 (60.57), %N:12.55(13.58).

Table 2: Data of Antifungal Activity

SI. No	Compounds	Diameter of zone of inhibition(mm)
1.	6	15
2.	Fluconazole	22
3.	DMF (Control)	0

Table 3: Data of the Anthelmintic Activity

Sl. No.	Compounds	Concentration (mg)	Mean paralysing time (min) ± S.E.	Mean death time (min) ± S.E.
1.	6	100	4.00 ± 0.98	4.55 ± 0.53
2.	Mebendazole	100	4.10 ± 0.32	5.54 ± 0.34
3.	Control	–	N.E.	N.E.

'S.E.' represents Standard Error, 'N.E.' indicates No Effect

The synthesized N-methylated analog of pseudostellarin-A was evaluated for their antifungal and antihelmintic activities. The compound showed moderate antifungal activity against fungi viz. *Candida albicans*, in comparison to the standard drug Fluconazole. N-methylated pseudostellarin-A analog was found to exhibit potent anthelmintic activity against earthworms viz. *Eudrilus eugenia*. The synthesized N-methlyated analog was found to be more active as compared to the standard drug Mebendazole.

CONCLUSION

The N-methylated analog of pseudostellarin-A was designed keeping in view of the of the wide biological application of natural cyclic peptides based on literature survey. The N-methylated pseudostellarin-A analog was synthesized with good yield using solution phase synthesis method. The synthesized N-methlyated analog was found to be a potent anthelmintic compound as compared to the standard drug Mebendazole. Thus the further studies on biological role of N-alkylated cyclic peptide analogs might help in identifying lead molecules with potent activities.

ACKNOWLEDGEMENT

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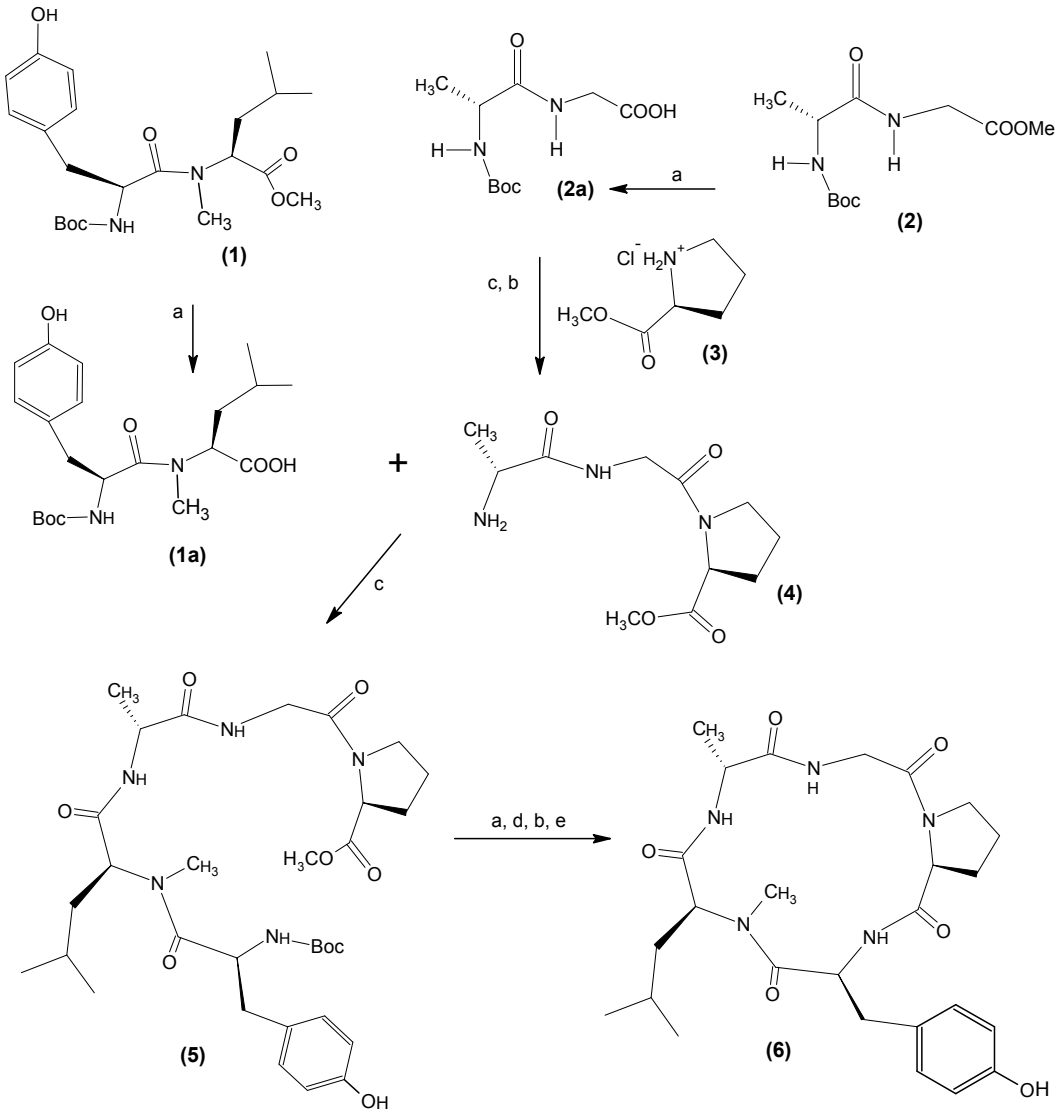
Scheme 1: Synthesis of Cyclo [Tyr-(N-Me)Leu-Ala-Gly-Pro] Pseudostellarin-A(6)



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, $CHCl_3$,

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