International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.06 pp 316-321, 2016

Synthesis of Cyclo[tyrosyl-(N-Me)leucinyl-prolyl-threonyl-(nitro)Arginine]:A potent Anthelmintic Agent against *Eudrillus eugeniae*

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Abstract: Proctolin is a neuropeptide which exists in insects and crustaceans. It is a effective stimulator in the contraction of a number of visceral and skeletal muscles in insects. It is also referred to as a neuromodulator. Solution phase peptide synthesiswas employed to synthesize N-methylated analog of Cyclo(nitro)proctolin, Cyclo[Tyrosyl-(N-Me)Leucinyl-Prolyl-Threonyl-(nitro) Arginine] using N, N'–Dicyclohexylcarbodiimide as the coupling reagent. The synthesized compound was characterized by IR, ^{1}H NMR, FABMASS and elemental analysis. The compound was evaluated for anthelmintic and antimicrobial activities.

Keywords: Proctolin, neuropeptide, neuromodulator, Cyclo(nitro) proctolin, N, N'–Dicyclohexylcarbodiimide.

Introduction

Pharmaceutical chemistry has played a vital role inmultidisciplinary and highly integrated process of various drug development. However, a greater understanding of novel pharmacological principles is required to encounter the increasing prevalence of diseases. Also the decreasing efficacy and increasing side-effects as well as adverse effects has urged the development of better drugs. Peptides have developed as an important class of organic compounds in the late nineteenth century with the discovery of pituitary hormones. Most of the polypeptide antibiotics have cyclic structures and complex structures. And most of them are resistant to animal and plant proteases. Cyclopeptide antibiotics constitutes among the most powerful bactericidal antibiotics. Many of them have been isolated from natural sources like marine animals and culture filtrates. Many peptide antibiotics such as Gramicidin, Bacitracin, Polymyxin B, Colistin andViomycin have been observed to consist of cyclic structures.[1] However, the size of the cyclic rings of these peptides is too large to generate conformationally constrained structures. Thus smaller cyclic structures have been incorporated into numerous bioactive peptides leading to highly potent and selective analogs.

The flexibility of the linear molecules is reduced by cyclisation of peptides and it also stabilizes the secondary structure of peptides. Antimicrobial peptides interact with membranes and may result in the disturbance of the bacterial inner or outer membranes. The methylation of N- atom eliminates the hydrogen on the N- atom. The N-methylated peptide antibiotics are found to possess enhanced activity as compared to the unmethylated forms. The inherent medicinal properties of cyclic peptides promoted scientists to isolate these compounds from natural sources. But the quantities obtained from natural sources is very scarce, therefore synthesis of these compounds in laboratories has been attempted. Antimicrobial evaluation of these compounds gave good results.

Proctolin, a linear pentapeptide: [Arg-Tyr-Leu-Pro-Thr] was isolated from the cockroach, *Periplaneta Americana* (L), in 1975.[2] The synthesis of Proctolin was carried out by the same group in 1977. The Proctolin analog, cyclo(nitro)proctolin was synthesized by Boja et al.[4] The synthesized product showed good anthelmintic activity as well as good antimicrobial activity. A structure of cyclo(nitro)Proctolin comprises of one tyrosine, one leucine, one

proline, one threonine and one (nitro)arginine units: cyclo[Tyr-Leu-Pro-Thr-(nitro)Arg].Keeping in view the significance of N-methylated analogs of cyclic peptides showing potent antimicrobial activity, design and synthesis of N-methylated analog of cyclo(nitro)proctolin was attempted.The newly designed N-methylated analog of cyclo(nitro)proctolin is as follows:

Cyclo[Tyr-(N-Me)Leu-Pro-Thr-(nitro)Arg].In order to carry out the synthesis, the cyclic pentapeptide was disconnected into two dipeptide units and a single amino acid unit. The dipeptides were prepared from the respective protected amino acids and were coupled, after appropriate deprotection to get the pentapeptide,which was finally cyclised by p-nitrophenyl ester method using high-dilution technique to get the desired cyclic pentapeptide.

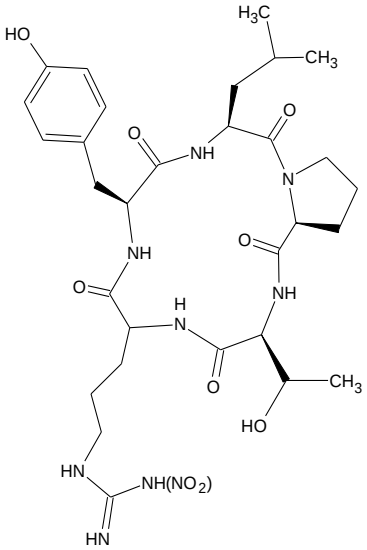


Fig.1 Cyclo(Nitro)Proctolin

Experimental

Analytical grade solvents and commercially available regents and were used without further purification. All the reactions were conducted in dried apparatus to maintain anhydrous conditions. All the reactions were magnetically stirred unless otherwise stated. Organic extracts were dried over anhydrous sodiumsulphate.Melting points were determined by capillary method. IR spectra were recorded on FTIR spectrometer using a thin film support on KBr pellets. The values are reported as υ_{max} (cm^{1}). ^{1}H NMR spectra was recorded on ^{1}H NMR Brucker JOEL (400MHz) NMR spectrometer. FAB Mass spectra was recorded.

Design and Synthesis Of Cyclo[Tyr-(N-Me)Leu-Pro-Thr-(nitro)Arg]

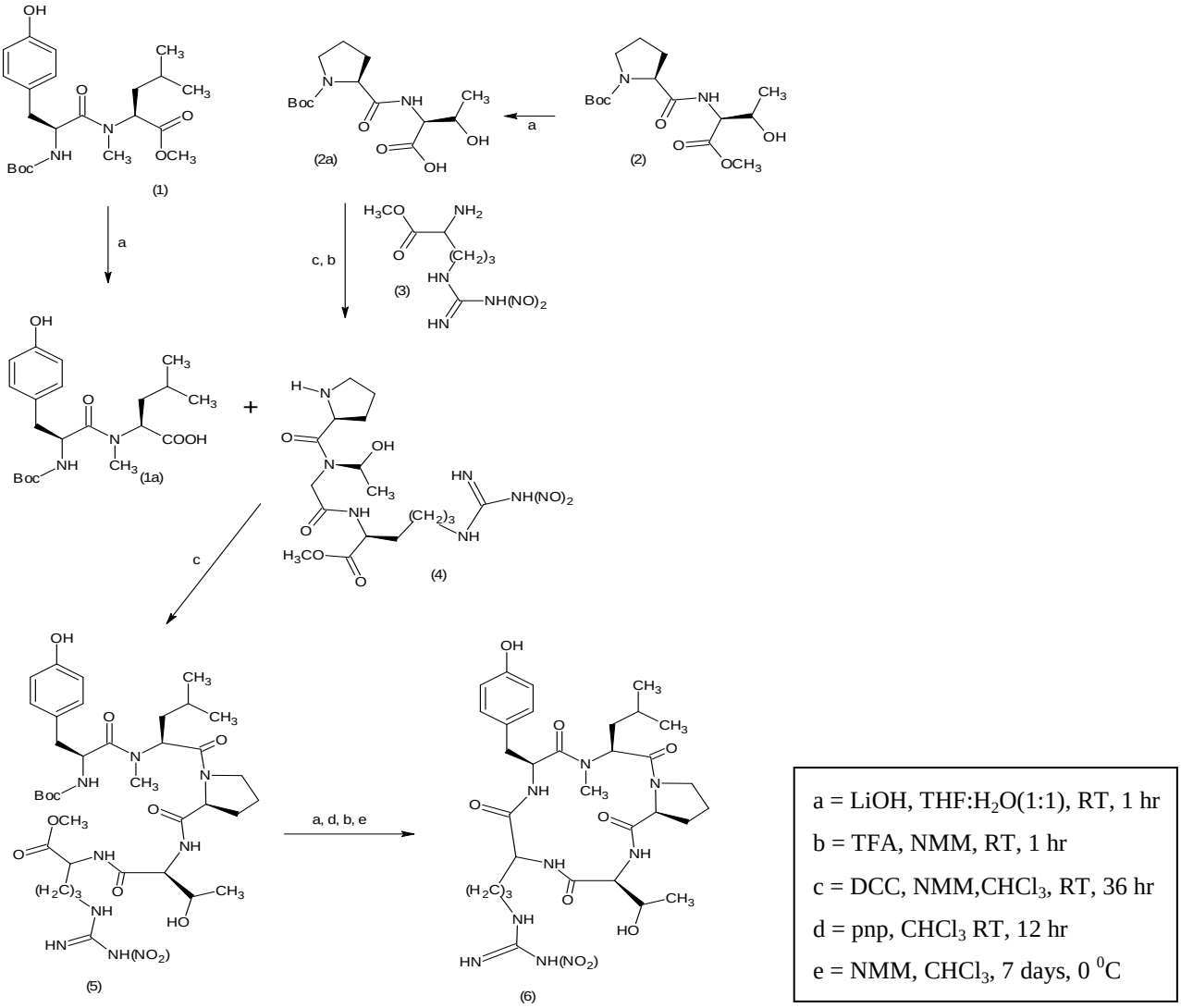


Fig. 2. Scheme For the synthesis of Cyclo[Tyr-(N-Me)Leu-Pro-Thr-(nitro)Arg]

General Method of Peptide Synthesis

Peptides are designed by coupling of two or more amino acids. Thisinvolves the protection of the amino group of one amino acid and the carboxylic group of the other. Protecting groups have to be chosencautiously so that they can be easily introduced, to protect the adjacent chiral centre from racemisation, to be chemically stable under the conditions of peptide bond formation and finally they must be easily removable under mild conditions at the end or at intermediate phase in the peptide synthesis.

Protection of the Amino Group

The amino group protection was carried out using Boc, following the method proposed by Belagali.et.al.[3]

Protection of carboxylic group of amino acids

The carboxyl group protection was carried out by converting the acid group to ester. This was done following the method proposed by Webb.et.al.[4]

Preparation of peptides

There are various reported methods for peptide coupling, however, the methods proposed by Bodanszky and M. M. Jouliewere adopted, which was convenient and useful. The dipeptides were prepared by coupling Boc-amino acid and amino acid methyl ester hydrochloride using triethylamine and N,N'-Dicyclohexylcarbodiimide. To obtain the desired length of the peptide chain the carboxylic group was deprotected and another amino acid methyl ester was coupled to the dipeptide. This process was repeated until the desired pentapeptide chain was obtained.[5-10]

Cyclisation of the linearpepntapeptide

The linear pentapeptide unit was cyclised by the p-nitrophenyl ester method proposed by Bodanszky[71] with certain modifications. The carboxyl group of the linear fragment was deprotected with lithium hydroxide 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 sodium bicarbonate to remove the unreacted p-nitrophenol completely. The Boc- group was removed by trifluoroacetic acid using the standard procedure. To the deprotected linear pentapeptide a catalytic amount of triethylamine was added and the reaction mixture was kept at 0°C for 7 days. The reaction mixture was washed several times with saturated $NaHCO_3$ until the byproduct, p-nitrophenol was removed completely. Finally it was washed with 5% HCl and distilled water. To remove any traces of moisturethe reaction mixture was dried over anhydrous Na_2SO_4 and evaporated in vacuum to get the cyclised product. The crude product was purified byrecrystallisation from $CHCl_3$.

Evaluation of Antimicrobial Activity

The synthesized compound was evaluated for antibacterial, antifungal and anthelmintic activities.The compound showed negligible activity against microbes.

Determination of Antifungal Activity

Antifungal activity of the synthesized compound was determined by cup plate method. 20 ml of sterile Sabouraud's agar medium was poured into a sterile petriplate and was inoculated on its surface with a suitably diluted suspension of test organism, *Candida albicans*. Cups were made in the medium by cutting circular pieces of agar medium using a sterile 6mm cork borer. 10µl of solution of the test samples was filled into the cups and placed in the refrigerator for 1 hr for diffusion and then incubated at 30^{o}C for 48 hrs. After incubation, the plates were observed for the growth of inhibition zones around the disks. The diameter of the zone of inhibition is proportional to the antimicrobial activity of the substance. The diameters of the zone of inhibition was compared with that produced by the standard antifungal drug, Fluconazole.The sample was tested at 250 µg level. To obtain this, sample solution containing 25 mg/ml of the test sample was prepared in sterile dimethylformamide (DMF) and 10 µl of the solution was added onto each cup using a micropipette.

Evaluation of Anthelmintic Activity

Anthelmintic activity studies was carried out against earthworms (*Eudriluseugeniea)* by Garg's method. Suspension of the sample was prepared by triturating the samples with 12.5% Tween 80 and distilled water and the resultant mixtures were stirred using a mechanical stirrer for 30 minutes. The resulting suspension was used for the activity studies. The suspension was diluted to contain 100 mg in 5ml of the test sample. The same concentration of the standard drug, Mebendazole was also prepared in a similar way.

Five earthworms of similar sizes were placed in a petriplate of 4 inches diameter containing 50ml of suspension of the test standard drugs (Mebendazole) at room temperature. Another set of five earth- worms was kept as control in 50ml suspension of distilled water and 12.5% Tween 80.

50ml of the suspension of the test compound was added into separate petriplate containing five earthworms. The time required for the paralysis and death of the worms was noted. The death time was ascertained by placing the earthworms in warm water at 50°C, which stimulated the movement if the worm was alive.[11]

The result of anthelmintic activity against *Eudriluseugeniea*was tabulated.

Results

Cyclo[Tyr-(N-Me)Leu-Pro-Thr-(nitro)Arg] was obtained as a semi-solid mass with 55.40% yield.

Table 1: Physical Data of the Cyclic Pentapeptides

Sl. No.	Cyclised product	Physical state	% yeild
1.	Cyclo[Tyr-(N-Me)Leu-Pro-Thr-(nitro)Arg]	Semi-solid mass	55.40

Spectral Analysis-

1) Compound 1- Cyclo[Tyrosyl-(N-Me)Leucinyl-Prolyl-Threonyl-(nitro) Arginine]:

^{1}H NMR(300MHz, $CDCl_3$):δ 7.8 (1H, br. s, -NH), 7.1(1H, s, -OH), 7.1-6.9(4H, m.Ar-H), 6.9-6.8(2H, m, -NH), 4.7(2H, m, α-H), 4.4(2H, m, α-H), 3.5-3.3(5H, m, β-CH_2 of Tyr, Arg, β-CH of Thr), 2.2(3H, s, N-CH_3), 2.1-

1.8(12H, m, , β-CH_2 of Leu, CH_2-CH_2- of Pro, N-CH_2 of Arg, 3(-NH) of Arg, 1.8-1.1(9H, m, -CH_3 of Thr, -$CH(CH_3)_2$ of Leu).

IR ($CHCl_3$): 3672.0(m, -OH Stretch), 3325.8(br. s, -NH Stretch), 3140(s, Aromatic -CH Stretch), 2861.1(s, -CH Stretch), 1760(s, C=O Stretch of ester),1669.9(s, C=O Stretch of amide), 1512.2(s, Asymmetric N-O Stretch),1457.4(s, C-H bend), 1392.9(s, Symmetric N-O Stretch), 1220.5 (s, C-N Stretch) cm^{-1}.

FABMASS: m/z = 690

Elemental Analysis:Found (Calcd) % C: 52.19 (51.92), % N: 20.12 (19.46)

Biological evaluation-

Antimicrobial activity

The synthesized compoundpossesses less antibacterial activity, but showed moderate antifungal activity against fungi viz. *Candida albicans*, in comparison to the standard drug Fluconazole.

Antifungal Activity

The synthesized compound showed moderate inhibition by inhibiting fungal growth till fourth dilution.

Table 2: Minimum Inhibitory Concentration for Antifungal Activity

Dilution→ / Compd No.↓	I	II	III	IV	V	VI	VII	VIII
6	–	–	–	–	+	+	+	+
Fluconazole	–	–	–	–	–	–	–	+

Antifungal Activity of the compounds against *C. albicans*

The synthesized compound showed moderate activity with a zone of inhibition of 16mmwhen compared to the standard drug which produced 22mm inhibition zone.

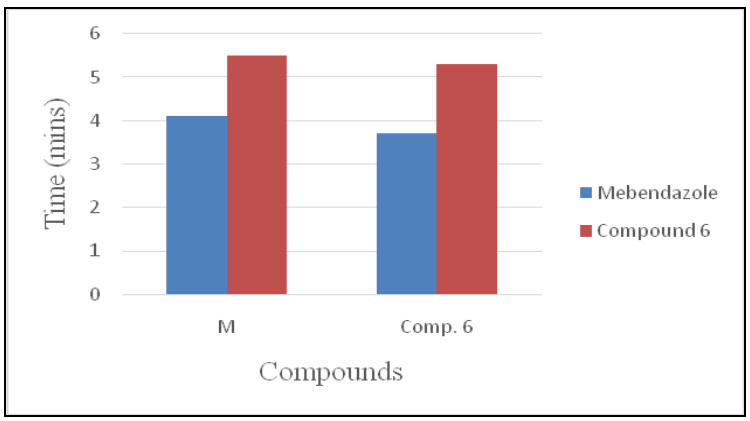
Table 3: Data of Antifungal Activity of the compounds against *C. albicans*

Sl. No.	Compound No.	Diameter of zone of inhibition (mm)
1.	6	16
2.	Fluconazole	22
3.	DMF (Control)	0

Anthelmintic Activity

The N-methylated analog of cyclo(nitro)proctolin, Cyclo[Tyrosyl-(N-Me)Leucinyl-Prolyl-Threonyl-(nitro) Arginine] was found to exhibit potent anthelmintic activity against earthworms viz. *Eudrilus eugenia.* The synthesized compound was found to be more active as compared to the standard drug, Mebendazole.

Fig.1: Anthelmintic activity of Cyclo[Tyrosyl-(N-Me)Leucinyl-Prolyl-Threonyl-(nitro) Arginine]



Discussion

The N-methylated analog of cyclo(nitro)proctolin was designed based on literature survey and the properties of cyclic peptide antibiotics.

The analog was synthesised by solution phase peptide synthesis with satisfactory yield and was characterised by IR, ^{1}H NMR, FABMASS and elemental analysis.

The synthesised compound was evaluated for antibacterial and antifungal activities (MIC) from 1000μg to 8μg. The compound was found topossess less antibacterial activitybut was moderately active against fungi upto 125μg. Sensitivity testing was carried out by cup plate method at 250μg level against*Candida albicans*.

Anthelmintic activity was carried out at 100 mg level against *Eudrilus eugeniea*. The compoundexhibited potent anthelmintic activity.

Further studies of the biological activity on cyclic pentapeptidesmay be carried out which may reveal interesting activities like immunosuppressive activity,cytotoxic andtyrosinase inhibitory activity that usually presented by N-alkylated cyclic peptides.

Acknowledgement

The authors are thankful to VIT-DST-SAIF, VIT University and NitteGulabiShetty Memorial Institute of Pharmaceutical Sciences for providing the necessary facilities and support.

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