ISSN 2348-232X

Unique Research Journal of Chemistry

Available online: www.ujconline.net

Research Article

SYNTHESIS AND BIOLOGICAL EVALUATION OF CYCLO(NITRO) PROCTOLIN ANALOGS

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Received: 24-07-2015; Revised: 22-08-2015; Accepted: 20-09-2015

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ABSTRACT

Proctolin is a neuropeptide present in insects and crustaceans. It is a potent stimulator in the contraction of a number of visceral and skeletal muscles in insects. It is also referred to as a neuromodulator. N-methylated analog of Cyclo(nitro)proctolin was synthesized using solution phase peptide synthesis. N,N'-Dicyclohexyl carbodiimide was used as the coupling reagent. The structural elucidation of the synthesized compound was carried out using by IR, ^{1}H NMR, FABMASS and elemental analysis. The compound was evaluated for antimicrobial and anthelmintic activities.

Keywords: Proctolin, neuropeptide, neuromodulator, Cyclo(nitro)proctolin, N,N'-Dicyclohexyl carbodiimide.

INTRODUCTION

Pharmaceutical chemistry has always been a vital part of highly integrated and multidisciplinary process of various drug development. However, the increasing prevalence of diseases requires increasing drug therapy based on greater understanding of novel pharmacological principles. Also the descending efficacy and increasing side-effects as well as adverse effects has urged the development of better drugs.Peptides have emerged 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 are among the most powerful bactericidal antibiotics. Many of them have been isolated from natural sources like marine animals and culture filtrates. Cyclic structures have been observed in many peptide antibiotics such as Gramicidin, Bacitracin, Polymyxin B, Colistin, Viomycin.[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.

Cyclisation of peptides reduces the flexibility of the linear molecules and stabilizes secondary structure of peptides. Antimicrobial peptides interact with membranes and may be cytotoxic as a result of 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 less, therefore synthesis of these compounds in laboratories has been attemoted. Antimicrobial evaluation of these compounds gave good results.

Proctolin, a linear pentapeptide: [Arg-Tyr-Leu-Pro-Thr] 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 consists 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)proctolinis as follows:

Cyclo[(N-Me, O-Me)Tyr-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.

These 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 cyclic pentapeptides..

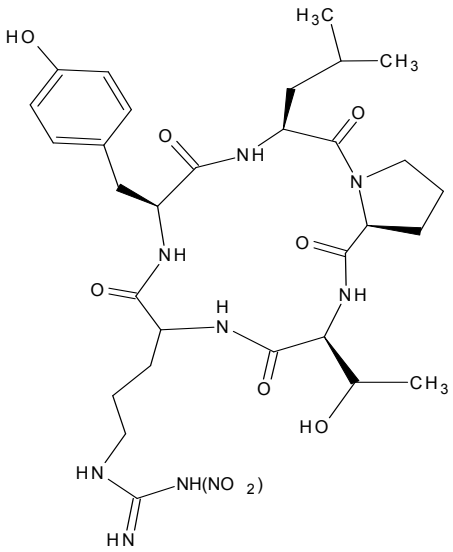


Figure 1: Cyclo(Nitro)Proctolin

MATERIALS AND METHODS

Commercially available regents and analytical grade solvents were used without further purification. Anhydrous condition for all the reactions were conducted in dried apparatus. All the reactions were magnetically stirred unless otherwise stated. Organic extracts were dried over anhydrous sodium sulphate. 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 were recorded.

General Method of Peptide Synthesis:

Peptides are formed by coupling of two or more amino acids. This requires the protection of the amino group of one amino acid and the carboxylic group of the other. Protecting groups have to be chosen carefully so that they can be easily introduced, to be chemically stable under the conditions of peptide bond formation, to protect the adjacent chiral centre from racemisation 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 of an amino acid was protected using Boc, following the method proposed by Belagali.et.al.[3]

Protection of carboxylic group of amino acids:

The carboxyl group of an amino acid was protected by converting the acid group to ester. This was done following the method proposed by Webb.et.al.[4]

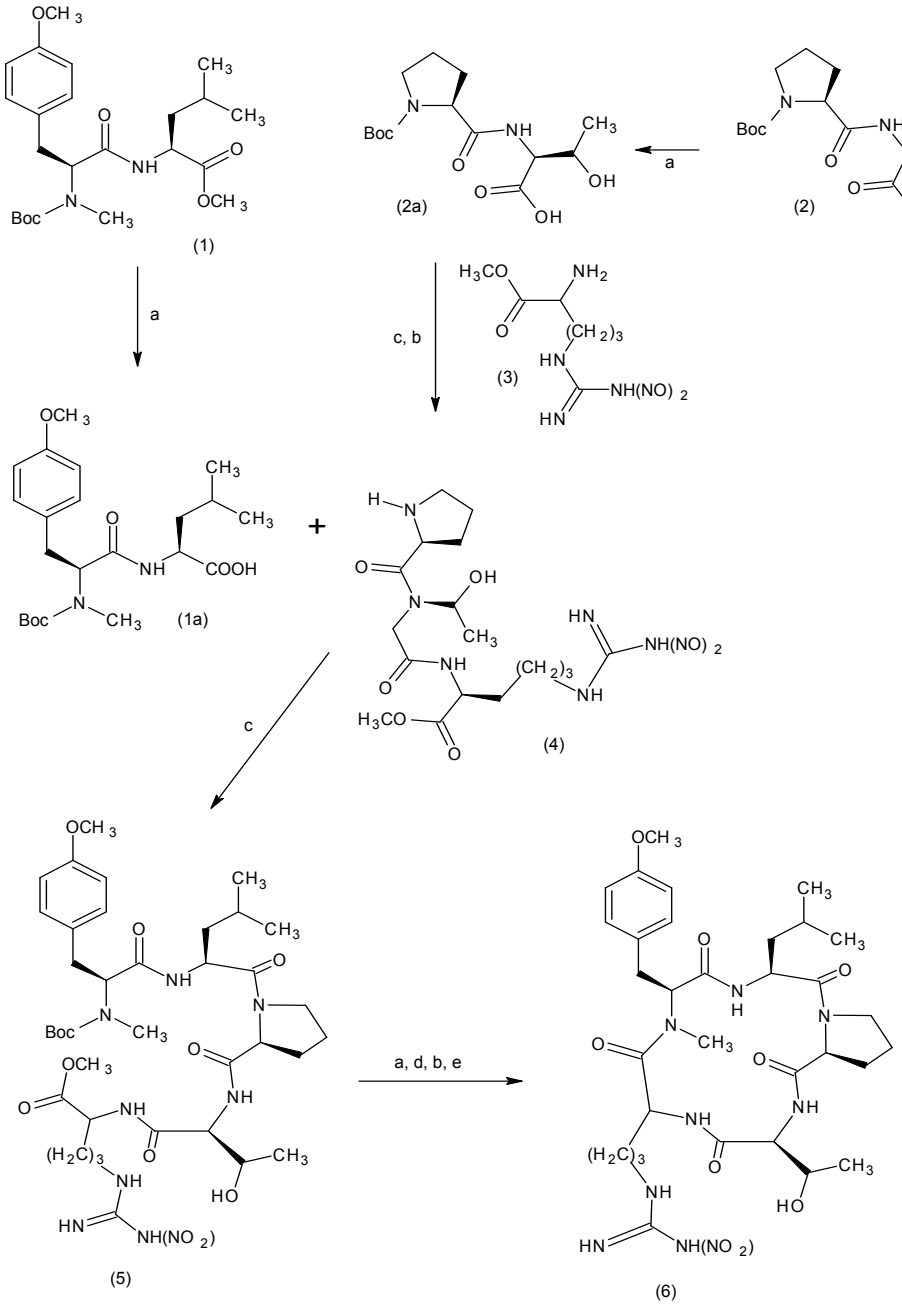
Preparation of peptides:

Of the various reported methods for peptide coupling, the methods proposed by Bodanszky and M. M. Joulie were 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'-Dicyclohexyl carbodiimide. To increase the 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 of 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. To remove the unreacted p-nitrophenol completelythe reaction mixture was washed several times with saturated sodium bicarbonate. 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 moisture the reaction mixture was dried over anhydrous Na_2SO_4 and evaporated in vacuum to get the cyclised product. The crude product was purified by recrystallisation from $CHCl_3$.

Design And Synthesis Of Cyclo(nitro)Proctolin Analog :



a = LiOH, THF:H_2O(1:1), RT, 1 hr
b = TFA, NMM, RT, 1 hr
c = DCC, NMM,$CHCl_3$, RT, 36 hr
d = pnp, $CHCl_3$ RT, 12 hr
e = NMM, $CHCl_3$, 7 days, 0 ^{0}C

Scheme: Synthesis of Cyclo[(N-Me,O-Me)Tyr-Leu-Pro-Thr-(nitro)Arg]

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 sample was filled into the cups and placed in the refrigerator for 1 hr for diffusion and then incubated at 30°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 were compared with that produced by the standard antifungal drug, Fluconazole.Sample was tested at 250 µg level. To obtain this, sample solution containing 25 mg/ml of the test sample was dissolved in sterile dimethylformamide (DMF) and 10 µl each of the sample and standard was added onto each cup using a micropipette.

Evaluation of Anthelmintic Activity:

Anthelmintic activity studies were carried out against earthworms (*Eudriluseugeniea)* by Garg's method. Suspension of the sample was prepared by triturating the sample with 12.5% Tween 80 and distilled water and the resultant mixture was 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 petriplateof 4 inches diameter containing 50ml of suspension of the test standard drug (Mebendazole) at RT. 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 petriplates 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 results of anthelmintic activity against *Eudrilus eugeniea* were tabulated.

RESULTS AND DISCUSSION

Table 1: Physical Data of the Cyclic Pentapeptides

Cyclised product	Physical state	% yeild
Cyclo[(N-Me,O-Me)Tyr-Leu-Pro-Thr-(nitro)Arg]	Semisolid mass	46.23

Spectral Analysis:

1) Compound 3- Cyclo [(N-Me,O-Me)Tyrosyl-Leucyl-Prolyl-Threonyl-(nitro)Arginine]:

^{1}H NMR(300MHz, $CDCl_3$):δ 8.1 (1H, br. s, -NH), 8.0(1H, br. s, -NH), 7.2-7.0(2H, m-H of Tyr), 6.9(1H, br. s, -NH), 4.8-4.6(1H, m, α-H), 4.5-4.4(1H, s, α-H), 3.9-3.7(2H, m, α-H), 3.75(3H, s, OCH_3), 3.6(2H, t, β-CH_2 of Arg), 3.4(2H, m, β-CH_2 of Tyr), 3.0-2.9(2H, m, β-CH_2 of Arg), 2.9-2.85(2H, s, β-CH_2 of Leu), 2.8(3H, s, N-CH_3), 2.1(4H, m, CH_2-CH_2- of Pro), 2.0(3H, m, NH of Arg), 1.8(15H, m, -CH_3 of Thr, -$C(CH_3)_2$ of Leu, CH_2-CH_2-CH_2- of Arg).

IR ($CHCl_3$): 3780.9(m, -OH Stretch), 3328.8(br. s, -NH Stretch), 2923.5(s, Aromatic -CH Stretch), 2854.0 (s, -CH Stretch), 1661.8(s, C=O Stretch of amide),1560 (s, Asymmetric N-O Stretch),1516.9(s,-NHbend),1459.6(s,-CH bend),1370.8 (s, Symmetric N-O Stretch), 1247.8 (s, C-N Stretch) cm^{-1}.

FABMASS: m/z = 704

Elemental Analysis:Found (Calcd) % C: 53.09 (52.64), %N: 19.87 (19.05)

Biological evaluation:

Antimicrobial activity:

The synthesized compounds possess less antibacterial activity, but they 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 18mm when 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	18
2.	Fluconazole	22
3.	DMF (Control)	0

Anthelmintic Activity:

The N-methylated analog of cyclo(nitro)proctolin was found to exhibit potent anthelmintic activity against earthworms viz. *Eudrilus eugenia.* The synthesized compounds 6 was found to be more active as compared to the standard drug, Mebendazole.

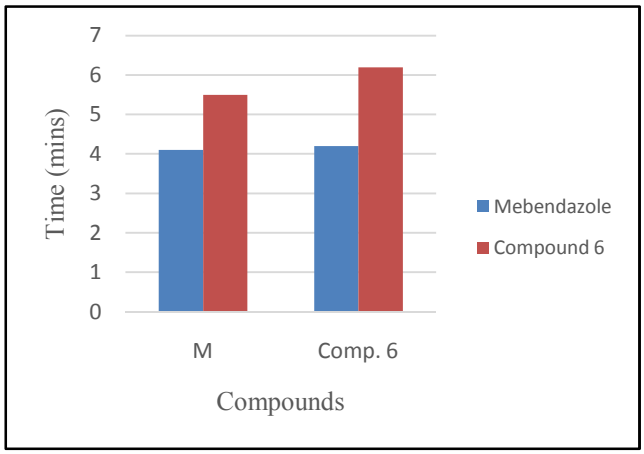


Figure 1: Comparison of anthelmintic activities of compounds

CONCLUSION

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

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 compoundwas found topossess less antibacterial activitybut was moderately active against fungi upto 125µg. Sensitivity testing was carried out by cup plate method at 250µg level.*Candida albicans* was selected as the test organism.

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

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

ACKNOWLEDGEMENT

The authors are thankful to Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences and VIT University for providing the necessary facilities and support.

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Source of support: Nil, Conflict of interest: None Declared