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SYNTHESIS OF GLABRIN A AND ITS N-METHYLATED ANALOG AS POTENT INSECTICIDAL AGENTS

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ABSTRACT
Two new cyclohexapeptides, Glabrin A and Glabrin B were isolated from the seeds of *Annona glabra*. The cyclohexapeptides show simple architectures with highly repeated residue units, which showed moderate antifungal and weak antitumor activities *in vitro*. The Glabrin A and its N-Methylated analogs were synthesized and were evaluated for their biological activity. The N-methylated peptide antibiotics are found to possess enhanced activity as compared to the unmethylated forms. The structure of the new compounds was confirmed by IR, ^{1}HNMR and Mass spectroscopy. The synthesized compounds were tested for their anthelmintic activity and insecticidal activity.
Keywords: Glabrin A, cyclohexapeptide, solution phase peptide synthesis, anthelmintic activity and insecticidal activity.

INTRODUCTION

Cyclopeptide antibiotics are among the most powerful bactericidal antibiotics. Many of them have been isolated from natural sources like marine and culture filtrates. Cyclic structures have been observed in many native peptides such as Somatostatin, Oxytocin, Cyclosporine, Calcitonin and peptide antibiotics such as Gramicidin, Bacitracin, Polymyxin B, Colistin and Viomycin. Interaction of peptides with membrane is an important requirement for most antimicrobial peptides[1-4]. The inherent medicinal properties of cyclic peptides prompted scientists to isolate these compounds from natural sources. Since only minute quantities are obtained from natural sources, attempts have been made for the synthesis of these compounds in laboratories and antimicrobial evaluation of these compounds gave good results. N-methylated amino acids are commonly found in naturally occuring peptide antibiotics. The methylation of N-atom eliminates the hydrogen, responsible for cleavage of peptide bond. The hydrogen bonding pattern of peptide containing these amino acids is different from that of unmethylated peptides. Studies have shown that N-methylated peptide antibiotics are found to possess enhanced activity as compared to unmethylated forms[5,6].
Two new cyclohexapeptides, including Glabrin A and Glabrin B were isolated by Li Chao-Ming *et al*[7] from the seeds of Annona glabra. The cyclohexapeptides show surprisingly simple architectures with highly repeated residue units, which showed moderate antifungal and weak antitumor activities *in vitro*. An attempt is made to synthesize Glabrin A and its N-methylated analog using solution phase technique in peptide synthesis (Scheme I). The synthesized compounds will be evaluated for anthelmintic, antifungal, antibacterial properties.

MATERIALS AND METHODS

All the reactions requiring anhydrous conditions were conducted in flame dried apparatus. Solvents and reagents were purified by standard methods. All the reactions were magnetically stirred unless otherwise stated. Boc-amino acids, amino acid methyl ester hydrochlorides were prepared by standard procedures[8]. N-methylated amino acids were prepared using NaH/CH_3I by Bentoin method[9]. Organic extracts were dried over anhydrous solution sulphate. Melting points were determined by capillary method and were uncorrected. Amino acids, di-tert-butylpyrocarbonate, trifluoroacetic acid, Imidazole and N-methyl morpholine were obtained, from Spectrochem Ltd. Mumbai. IR spectra were recorded on Perkin Elmer FT/IR spectrometer using a thin film supported on KBr pellets for solids and chloroform as a solvent for semisolids. The values are reported as ν_{max} (cm^{-1}). ^{1}H NMR spectra were recorded on Bruker Advance II 400 NMR spectrometer (400 MHz). The spectra were obtained in $CDCl_3$ and the chemical shift values are reported as values in ppm relative to TMS (δ =0) as internal standard. Multiplicities were described using the abbreviations: s= singlet, d = doublet, m= multiplet and br = broad. Mass spectra were recorded on a Jeol Sx-102 mass spectrometer using xenon as the carrier gas. The spectra were recorded at room temperature.

Preparation of Dipeptides: Amino acid methyl ester hydrochloride (20 mmol) was dissolved in chloroform (20 ml). To this, NMM (2.2 ml, 20 mmol) was added at 0°C and the reaction mixture was stirred for 15 mins. Boc-amino acid (20 mmol) in $CHCl_3$ (20 ml) and DIPC (20 mmol) were added with stirring. After 36 hrs, the reaction mixture was filtered and the residue was washed with $CHCl_3$ (30 ml) and added to the filtrate. The filtrate was washed with 5% $NaHCO_3$ (20ml) and 5%HCl (20ml) solutions. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum. To remove the traces of the diisopropylurea (DIPU), the product was dissolved in minimum amount of chloroform and cooled to 0°C. The crystallized DIPU was removed by filtration. Petroleum ether was added to the filtrate at 0°C to recrystallize the pure product. Using the above method the following Boc-dipeptide methyl esters were prepared.
Boc-Leu-Val-OMe and Boc-Leu-(N-Me)Val-OMe.

Preparation of the Tetrapeptide: The tetrapeptide was prepared from two corresponding deprotected dipeptide units using the procedure similar to that of the dipeptide

coupling. By using the above method, Boc-Ile-Tyr-Pro-Gly-OMe a tetrapeptide was prepared.

Preparation of linear hexapeptide: The hexapeptide unit synthesized by coupling the corresponding deprotected tetrapeptide and dipeptide using the procedure similar to that of the dipeptide. By using the above procedure following hexapeptide were prepared:

Boc-Ile-Tyr-Pro-Gly-Leu-Val-OMe and Boc-Ile-Tyr-Pro-Gly-Leu-(N-Me)Val-OMe.

Preparation of Cyclic hexapeptide: The cyclisation of the linear hexapeptide unit was carried out by the p-nitrophenyl ester method of Bodanszky[8] with certain modifications. The ester group of the linear segment was removed with LiOH and the *p*-nitrophenol ester group was introduced using the following procedure:

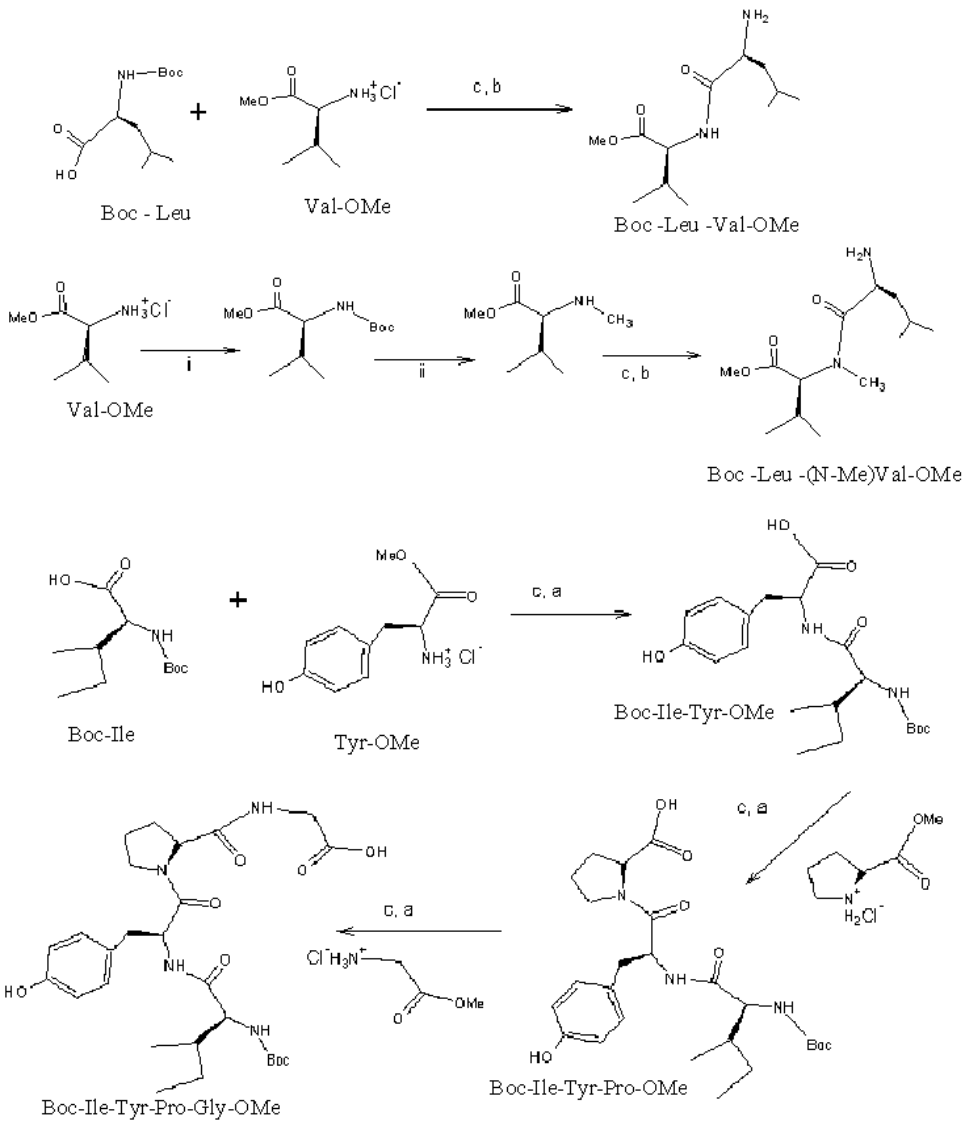
The Boc-peptide carboxylic acid (1.5 mmol) was dissolved in $CHCl_3$ (15 ml) at 0^0C. Then DIPC (1.89gm, 15mmol) and *p*-nitrophenol was added (0.27 g, 2 mmol) and stirred for 12 hours at room temperature. The reaction mixture was filtered and the filtrate was washed with $NaHCO_3$ solution (10%) until 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 hour at room temperature and washed with 10% $NaHCO_3$ solution. The organic layer was dried over anhydrous Na_2SO_4. To the Boc-deprotected peptide-pnp-ester in $CHCl_3$ (15 ml), NMM (1.4 ml, 2mmol.) was added and kept at RT for 3days. 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.

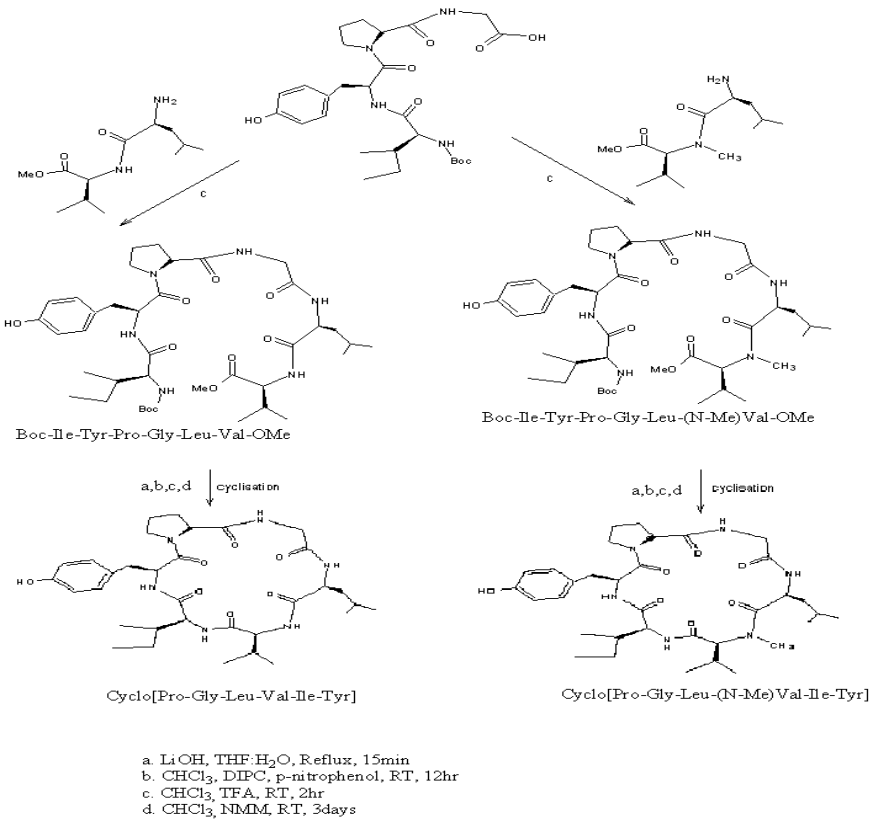
By using the above procedure following cyclic peptides were prepared

Cyclo [Pro-Gly-Leu-Val-Ile-Tyr] and Cyclo [Pro-Gly-Leu-(N-Me)Val-Ile-Tyr]

The overall synthesis for compound 1 and 2 are mentioned in Scheme I .

Scheme I





a. LiOH, THF:H_2O, Reflux, 15min
b. $CHCl_3$, DIPC, p-nitrophenol, RT, 12hr
c. $CHCl_3$, TFA, RT, 2hr
d. $CHCl_3$, NMM, RT, 3days

Biological Evaluation

Evaluation of Anthelmintic Activity: Anthelmintics are therapeutic agents used to destroy parasitic worms or remove them from the infected host. The ultimate test of anthelmintic activity is the ability of a chemical agent to eliminate the worms from a specifically parasitized animal with a minimum of toxic effect to the host. A suitable *invitro* test can be considered as a useful screening method, although *in vivo* screening methods provide a natural environment for the studies.

General Procedure: Anthelmintic activity studies were carried out against earthworms *(Eudrilus eugeniea)* by Garg's method[10]. Suspensions of the samples were prepared by triturating the samples with 15% Tween 80 and distilled water and the resultant mixtures were stirred using a mechanical stirrer for 30 mins. The resulting suspensions were used for the activity studies. The suspensions were diluted to contain 100 mg in 20ml of the test samples. Standard drug, Mebendazole was also prepared with the same concentration in a similar way. Earthworm was placed in a beaker containing 20ml of suspension of the test standard drugs (Mebendazole) at RT. Another set of earth worm was kept as control in 20ml suspension of distilled water and 15% Tween 80. 20ml each of the suspensions of the test compounds were added into separate beaker containing one earthworm in each. 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. The results of Anthelmintic Activity of the newly synthesized compounds are given in Table 1.

Evaluation of Insecticidal Activity

Insecticides are pesticides used against insects. A suitable *invitro* test can be considered as a useful screening method.

General Procedure: Insecticidal studies of the synthesized compounds were carried out against termites (*Coptotermes formasanus*) by Morita *et. al* method[11].

Watt Mann filter paper was first cut according to the inner diameter of the Petri plate, 100mg of test compounds were dissolved in chloroform (2ml) and were poured uniformly on the Petri plates fitted with filter paper. Standard drug (Chloropyrifos) solution was also prepared in similar way and poured in the Petri plate. For control only the solvent was poured on the filter paper placed in plate. Five termites were placed in each of the Petri plate and covered with lid, wet cotton was attached to the upper lid. Set up was kept undisturbed and death time was noted. The results of insecticidal activity of the newly synthesized compounds are given in Table 2 .

Table 1: Data of Anthelmintic Activity

Sl. No	Compound Name	Conc. of Compound (Mg)	Paralyzing Time (Mins. Secs)	Death Time (Mins. Secs)
1	Compound I (1)	100	75.09	81.60
2	Compound II (2)	100	67.55	70.55
3	Mebendazole	100	53.25	55.00
4	Control	-	-	-

Table 2: Data of Insecticidal Activity

Sl. No.	Compound Name	Conc. of Compound (Mg)	Death Time (Hrs.Mins)
1.	Compound 1	100	2.55
2.	Compound 2	100	2.35
3.	Chloropyrifos	100	2.45

RESULTS AND DISCUSSION

The two compounds, Compound I and its N-Methyl analog could be conveniently and efficiently synthesized by prescribed Scheme I with good yields. The newly synthesized compounds were characterized by IR, ^{1}H NMR and Mass spectroscopy. The detailed spectral data are as follows.

Cyclic hexapeptide (1)

IR ($CHCl_3$): 3430.5 (-NH stretching.), 2928.3 (aliph.-CH stretching.), 1639.2 (C=O stretching of amide), 1540.3 (-NH bending), .cm^{-1}

Mass Spectroscopy: in m/z: Cyclo [Pro-Gly-Leu-Val-Ile-Tyr]

Molecular ion peak was observed at m/z 647.6 corresponds to the molecular formula : $C_{33}H_{52}N_6O_7$

^{1}H NMR (300MHz, $CHCl_3$): δ 7.8–7.2 (9H, m, Aromatic H, NH-), 4.2 (7H, m, α-H, Gly α-H), 1.4-1.0 (10H, m, β-Hs), 1.0–0.4 (22H, m, γ-CH_3 of Val, Leu, Ile and Pro).

Cyclic hexapeptide 2

IR ($CHCl_3$): 3330.66 (-NH stretching.), 2931.08 (aliph.-CH stretching.), 1630.26 (C=O stretching of amide), 1529. (-NH bending), cm^{-1}.

Mass in m/z: Molecular ion peak was observed at m/z 647.6

^{1}HNMR (300MHz, $CHCl_3$): δs 7.8–7.2 (9H, m, Aromatic H, NH-), 4.4 (7H, m, α-H, Gly α-H), 1.4-1.0 (10H, m, β-Hs), 1.0–0.4 (22H, m, γ-CH_3 of Val, Leu, Ile and Pro).

The spectral data revealed the formation of linear hexapeptide and its N-Methylated analog. All the synthesized compounds were subjected to anthelmintic activity (Table 1) by Garg *et.al* method and insecticidal activity (Table 2) by Morita *et. al* The compounds exhibited weak anthelmintic activity against *Eudrillus Eugeniea* at the concentration of 100mg/20ml of test samples, similar to the standard drug, Mebendazole. The synthesized compounds showed moderate activity against *Coptotermes Formosanus* at the concentration of 100mg/2ml of test samples, as compared to the standard Chloropyrifos.

CONCLUSION

The two compounds could be conveniently and efficiently synthesized by prescribed Scheme with good yields by solution phase peptide synthesis. The newly synthesized compounds showed weak anthelmintic activity compared to that of standard drug Mebendazole. Compound 1 and 2 were tested for insecticidal activity and exhibited significant activity in comparison to the standard drug Chloropyrifos.

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