SYNTHESIS OF N-METHYLATED ANALOG OF AMIDOMYCIN: A POTENT ANTIMICROBIAL CYCLOHEPTAPEPTIDE

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ABSTRACT
N-methylated analog of Amidomycin, was synthesized by solution phase peptide synthesis using dicyclohexyl carbodiimide (DCC) as the coupling agent and triethylamine (TEA) as the base. The structure of the compound was confirmed by IR, 1H NMR, 13C NMR, FABMASS and elemental analysis. The synthesized cyclic peptide was evaluated for Minimum Inhibitory Concentration (MIC) against four strains of bacteria and three strains of fungi. It was found to be active against both bacteria and fungi from 50-100 μg.

Keywords: Amidomycin, solution phase peptide synthesis, DCC, MIC.

INTRODUCTION
Amidomycin, a cyclic depsipeptide, was produced by a Streptomyces species. In the year 1957, Vining L.C. et al.1 first isolated it and elucidated its structure. Synthesis of amidomycin was carried out by Shemyakin M. M. et al. in the year 19632. It is an antibiotic which is primarily active against yeasts. However, no further studies were carried out on Amidomycin because of the complexity of the synthesis of the alternate amide and ester linkages and usage of D-amino acid which is very costly. Hence simple analogues were designed with only amide linkages, thus making synthesis easier and cost effective by incorporating L-amino acids. N-methylated amino acids are commonly found in naturally occurring peptide antibiotics. The methylation of nitrogen atom eliminates the hydrogen, responsible for cleavage of peptide bonds. The hydrogen bonding pattern of peptide containing these amino acids is different from that of unmethylated forms.3-5 N-methylated cyclic peptides is found to possess cytotoxic and antimicrobial activity. A review of the structures of cyclic peptides exhibiting antimicrobial activity showed presence of at least one D-amino acid and/or N-methylated amino acid units in the molecule. Hence the cyclic octapeptide analogue of amidomycin have been designed containing one N-methyl-L-valine and rest L-valine units. In order to carry out the synthesis, the cyclic octapeptide was disconnected into four dipeptide units. The dipeptides were prepared from the respective protected amino acids. The amino group was protected with tertiary butyloxycarbonyl (Boc-) group and the carboxyl group was protected by converting it into the methyl ester. The Boc-amino acids were coupled with the amino acid methyl ester hydrochlorides by dicyclohexyl carbodiimide (DCC) as the coupling agent and triethylamine (TEA) as the base to get the protected dipeptides. The dipeptides were appropriately de protected and coupled to get the octapeptides, which were finally cyclised by p-nitrophenyl ester method using high dilution technique to get the cyclic octapeptide.

MATERIALS AND METHODS
All the reactions requiring anhydrous conditions 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 and were uncorrected. Amino acids, di-tert-butylpyrocatearone, trifluoroacetic acid and triethylamine were obtained from Spectrochem Ltd, Mumbai, India. DCC, Diethyl ether, Methanol and Chloroform was obtained from AVRA. 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. The values are reported as υmax (cm-1).

1H NMR spectra were recorded on Bruker JOEL (400 MHz) NMR spectrometer. The spectra were obtained in CDCl3 and the chemical shift values are reported as values in ppm relative to TMS (δ = 0) as internal standard. FABMASS spectra were recorded on a Joel Sx 102/DA-6000 mass spectrometer using xenon as the carrier gas. The spectra were recorded at room temperature; m-nitrobenzyl alcohol was used as the matrix. The protection of amino and carboxyl group and their de protection were done by standard procedures.6,9

N-methylation of Boc-amino acid
Boc-amino acid (0.663 g, 2.0 mmol) was dissolved in dry THF (20 ml) and cooled to 0°C. To this NaH (2.88 g, 12 mmol) and MeI (0.852 g, 6.0 mmol) were added and stirred overnight at room temperature. The reaction mixture was diluted with ether (20 ml), washed with sat. NH4Cl (10 ml), 20% Na2S2O3 solution and sat. NaCl solution (10 ml); Organic layer was dried and concentrated. Using the above method following N-methyl amino acid was prepared.

Preparation of Dipeptides
Amino acid methyl ester hydrochloride (10 mmol) was dissolved in chloroform (CHCl3) (20 ml). To this, TEA (4 ml, 28.7 mmol) was added at 0°C and the reaction mixture was stirred for 15 minutes. Boc-amino acid (10 mmol) in CHCl3...
(20 ml) and DCC (10 mmol) were added with stirring. After 36 h, the reaction mixture was filtered and the residue was washed with CHCl₃ (30 ml) and added to the filtrate. The filtrate was washed with 5 % NaHCO₃ (20 ml), 5 % HCl (20 ml) and distilled H₂O (20 ml). The organic layer was dried over anhydrous Na₂SO₄, filtered and evaporated in a vacuum. The residue was purified by re-crystallization from CHCl₃.

Boc-L-Val-Val-OMe and Boc-N-methyl-L-Val-L-Val-OMe was prepared in this manner.

Preparation of Tetrapeptides
The deprotected dipeptide units were coupled using DCC/TEA to get the protected tetrapeptide by the procedure similar to that of the dipeptides. Boc-L-[Val-Val-Val]-OMe and Boc-N-methyl-L-Val-L-[Val-Val]-OMe were synthesized in this manner.

Preparation of linear octapeptide
The Boc-group of the tetrapeptides Boc-L-[Val-Val-Val]-OMe was removed and the ester group of the tetrapeptide Boc-L-Val-L-Val-N-methyl-L-Val-L-Val-OMe was de protected. Both the de protected units were coupled to get the linear octapeptide.

Preparation of Cyclic octapeptide
The cyclisation of the linear octapeptide unit was carried out by the p-nitrophenyl ester with certain modifications. The ester group of the linear fragment was removed and the p-nitrophenyl ester group was introduced by stirring it for 12 h in CHCl₃ with p-nitrophenol at 0°C. The reaction mixture was washed several times with saturated NaHCO₃ until the unreacted p-nitrophenol was removed completely and washed with 5 % HCl to get Boc-peptide-pnp ester. The Boc-group was also removed, CHCl₃ and pyridine was added and the reaction mixture was kept at 0°C for 10 days. The mixture was finally washed with 5 % HCl, dried and evaporated in vacuum to get the cyclised product, which was then re-crystallized from CHCl₃/n-hexane (Scheme 1).

Scheme 1
Determination of Minimum Inhibitory Concentration (MIC)
The MIC of the cyclic peptide was determined by the serial tube dilution technique\(^{16-12}\) against two strains of Gram positive bacteria (Staphylococcus aureus and Bacillus subtilis), two strains of Gram negative bacteria (Escherichia coli and Pseudomonas aeruginosa) and three strains of fungi (Candida albicans, Aspergillus flavus and Aspergillus fumigatus). 4 mg of the sample was dissolved in 2 ml of sterile dimethyl formamide (DMF) to obtain stock solution having concentration of 200 µg/ml. In serial dilution technique, 1 ml prepared stock solution was transferred to test tube containing 1 ml nutrient broth medium for bacterial cultures and 1 ml Potato Dextrose Broth (PDB) for fungal cultures to give concentration 100 µg/ml from which 1 ml was transferred to another test tube containing 1 ml of broth medium to give concentration 50 µg/ml and so on up to concentration 6.25 µg/ml. After preparation of suspension of test organisms (10 organisms per ml), 1 drop of suspension (0.02 ml) was added to each broth dilution; a positive control was prepared in a similar way except that the test compound was not added. A negative control was prepared without the test compound and the test organisms. Tubes inoculated with bacterial cultures were incubated aerobically at 37°C for 24 hours and tubes inoculated with fungal cultures were incubated aerobically at 25°C for 48 hours. The tubes were observed for the presence/absence of growth.

Concentration of the solutions of the Test Compound

<table>
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<tr>
<th>Tube number</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
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<td>Concentration µg/ml</td>
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<td>12.5</td>
<td>25</td>
<td>50</td>
<td>100</td>
<td>200</td>
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</table>

RESULTS AND DISCUSSION
Physical Data and Spectral Analysis
Cyclo-N-methyl-L-Val-L-[Val-Val-Val-Val-Val-Val]
Yield 81.35 %; light brown semisolid; IR spectrum (ν/cm\(^{-1}\)):
3287.4 cm\(^{-1}\) (br. s, -NH Stretch), 2932 cm\(^{-1}\) (s, -CH stretch),
1647.3 cm\(^{-1}\) (s, C=O stretch), 1557 cm\(^{-1}\) (s, -NH bend),
1452.8 cm\(^{-1}\) (s, NH bend), \(^1\)H NMR spectrum (δ, ppm):
8.05(1H, br. s, -NH), 7.4(3H, br. s, -NH), 6.9(1H, br.s, -NH),
6.45(3H, br. s, -NH), 4.6(2H, m, α-H), 4.4(1H, m, α-H),
4.25(2H, m, α-H), 4.1(2H, m, α-H), 4.0(1H, m, α-H),
2.2(3H, s, -NCH\(_3\)), 1.4-1.0(8H, m, β-H), 1.0-0.9(48H, m, -CH\(_3\)); \(^1\)C NMR: (75.46MHz, CDCl\(_3\)): 170.4(C=O of Val),
59.9(α-C), 52.7(α-C), 52.0 (α-C), 48.7(α-C), 33.8 (β-C),
33.5 (β-C), 32.8 (β-C), 32.2 (β-C), 31.0 (β-C), 30.5 (β-C),
30.2 (β-C), 29.6 (β-C), 25.9 (CH\(_3\)), 25.6 (CH\(_3\)), 25.4 (CH\(_3\)),
25.2 (CH\(_3\)), 24.9 (CH\(_3\)), 24.7 (CH\(_3\)), 19.2 (CH\(_3\)), 18.8 (CH\(_3\)),
17.8 (CH\(_3\)); FABMASS: m/z (M + 1) = 809; Elemental Analysis: M. F. = C\(_{41}\)H\(_{21}\)N\(_{6}\)O\(_8\), M. W. = 809, Found (Cal) %
C: 64.43 (65.13), % N: 9.97 (10.0).

Minimum Inhibitory Concentration (MIC)
The synthesized cyclic peptides were evaluated for antibacterial and antifungal activities (MIC) from 200 µg to 6.25 µg. The cyclic peptide showed activity from 50 µg to 200 µg against all bacterial and fungal strains. The results of the MIC are given in Table 1 and Table 2.

<table>
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<tr>
<th>Compd. No(\uparrow)</th>
<th>Presence/absence of growth</th>
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<td><strong>S. aureus</strong></td>
</tr>
<tr>
<td><strong>Dilution</strong></td>
<td>I</td>
</tr>
<tr>
<td>CP-2</td>
<td>–</td>
</tr>
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</table>

*+* indicates presence of growth, ‘–’ indicates absence of growth

<table>
<thead>
<tr>
<th>Compd. No(\uparrow)</th>
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<tbody>
<tr>
<td><strong>Organism</strong></td>
<td><strong>C. albicans</strong></td>
</tr>
<tr>
<td><strong>Dilution</strong></td>
<td>I</td>
</tr>
<tr>
<td>CP-2</td>
<td>–</td>
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</tbody>
</table>

*+* indicates presence of growth, ‘–’ indicates absence of growth

CONCLUSION
The N-methylated cyclic octapeptide could be conveniently and efficiently synthesized by the prescribed scheme with good yields. The structure of the cyclic peptide was confirmed by IR, \(^1\)H NMR, \(^13\)C NMR, FAB MASS and elemental analysis. The compounds were screened for the Minimum Inhibitory Concentration (MIC) against four strains of bacteria and three strains of fungi and it was found to be active against both bacteria and fungi from 50-200 µg.

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REFERENCES

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