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Research Article

SOLUTION PHASE SYNTHESIS AND ANTIMICROBIAL EVALUATION OF N-METHYLATED D-ANALOG OF AMIDOMYCIN

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ABSTRACT

N-methylated D-analog of Amidomycin, was synthesized by solution phase peptide synthesis using ethyl-3-dimethylaminopropylcarbodiimide (EDC) as the coupling agent and triethylamine (TEA) as the base. The structure of the compound was confirmed by IR, ^{1}H NMR, ^{13}C 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. All compounds were found to be active against both bacteria and fungi from 25-100µg.

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

INTRODUCTION

Amidomycin, a cyclic octapeptide, was produced by a *Streptomyces* species. It was first isolated in the year 1957[1]. Synthesis of amidomycin was first carried out in the year 1963[2]. 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 analogs 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 N-atom eliminates the hydrogen, responsible for cleavage of peptide bonds. The hydrogen bonding pattern of N-methylated amino acids is different from that of unmethylated forms.[3-5]. N-methylated cyclic peptides are 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 analog of amidomycin have been designed containing one N-methyl-D-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 ethyl-3-dimethylaminopropylcarbodiimide (EDC) as the coupling agent and triethylamine (TEA) as the base to get the protected dipeptides. The dipeptides were appropriately deprotected 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-butylpyrocarbonate, trifluoroacetic acid and triethylamine were obtained from Spectrochem Ltd. Mumbai. EDC, 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 ν_{max} (cm^{-1}). ^{1}H NMR spectra were recorded on Bruker JOEL (400MHz) NMR

spectrometer. 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. 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 deprotection were done by standard procedures[6-9].

N-methylation of Boc-D-Valine:

Boc-D-Val (0.663 g, 2.0 mmol) was dissolved in dry THF (20 ml) and cooled to 0^0C. 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. NH_4Cl (10 ml), 20% $Na_2S_2O_3$ solution and sat. NaCl solution (10 ml). Organic layer was dried and concentrated. Using the above method following N-methyl-D-Val was prepared.

Preparation of Dipeptides:

Amino acid methyl ester hydrochloride (10 mmol) was dissolved in chloroform ($CHCl_3$) (20 ml). To this, TEA (4 ml, 28.7 mmol) was added at 0°C and the reaction mixture was stirred for 15 mins.

Boc-amino acid (10 mmol) in $CHCl_3$ (20 ml) and EDC (10 mmol) were added with stirring. After 12 hrs, the reaction mixture was filtered and the residue was washed with $CHCI_3$ (30 ml) and added to the filtrate. The filtrate was washed with 5% $NaHCO_3$ (20 ml), 5% HCl (20 ml) and distilled H_2O (20 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated in a vacuum. The residue was purified by recrystallization from $CHCl_3$. 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 EDC/TEA to get the protected tetrapeptide by the procedure similar to that of the dipeptides. Boc-L-[Val-Val-Val-Val]-OMe and Boc-N-methyl-D-Val-L-[Val-Val-Val]-OMe were synthesized in this manner.

Preparation of linear octapeptide:

The Boc-group of the tetrapeptides Boc-L-[Val-Val-Val-Val]-OMe was removed and the ester group of the tetrapeptide Boc-L-Val-L-Val-N-methyl-D-Val-L-Val-OMe was deprotected. Both the deprotected 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 [6] 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 hrs in $CHCl_3$ with p-nitrophenol at 0^oC.

The reaction mixture was washed several times with saturated Sodium bi carbonate 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, $CHCl_3$ and pyridine was added and the reaction mixture was kept at 0^oC for 10 days. The mixture was finally washed with 5% HCl, dried and evaporated in vacuum to get the cyclised product, which was then recrystallized from $CHCl_3$/n-hexane (Scheme 1).

Determination of Minimum Inhibitory Concentration (MIC):

The MIC of the cyclic peptide was determined by the serial tube dilution technique[10-12] 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, Asperigillus flavus* and *Asperigillus fumigatus*).

4 mg of the sample was dissolved in 2ml of sterile dimethyl formamide (DMF) to obtain stock solution having concentration of 200µg/ml. In serial dilution technique, 1ml prepared stock solution was transferred to test tube containing 1ml nutrient broth medium for bacterial cultures and 1ml Potato Dextrose Broth (PDB) for fungal cultures to give concentration 100µg/ml from which 1ml was transferred to another test tube containing 1ml of broth medium to give concentration 50µg/ml and so on up to concentration 6.25µg/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 37^0C for 24 hours and tubes inoculated with fungal cultures were incubated aerobically at 25^0C for 48 hours. The tubes were observed for the presence/absence of growth.

Concentration of the solutions of the Test Compound:

Concentration of the stock solution = 4 mg/2 ml (200µg/ml)

Tube number	I	II	III	IV	V	VI
Concentration µg/ml	6.25	12.5	25	50	100	200

RESULTS AND DISCUSSION

Physical Data and Spectral Analysis:

Cyclo-N-methyl-D-Val-L-[Val-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);

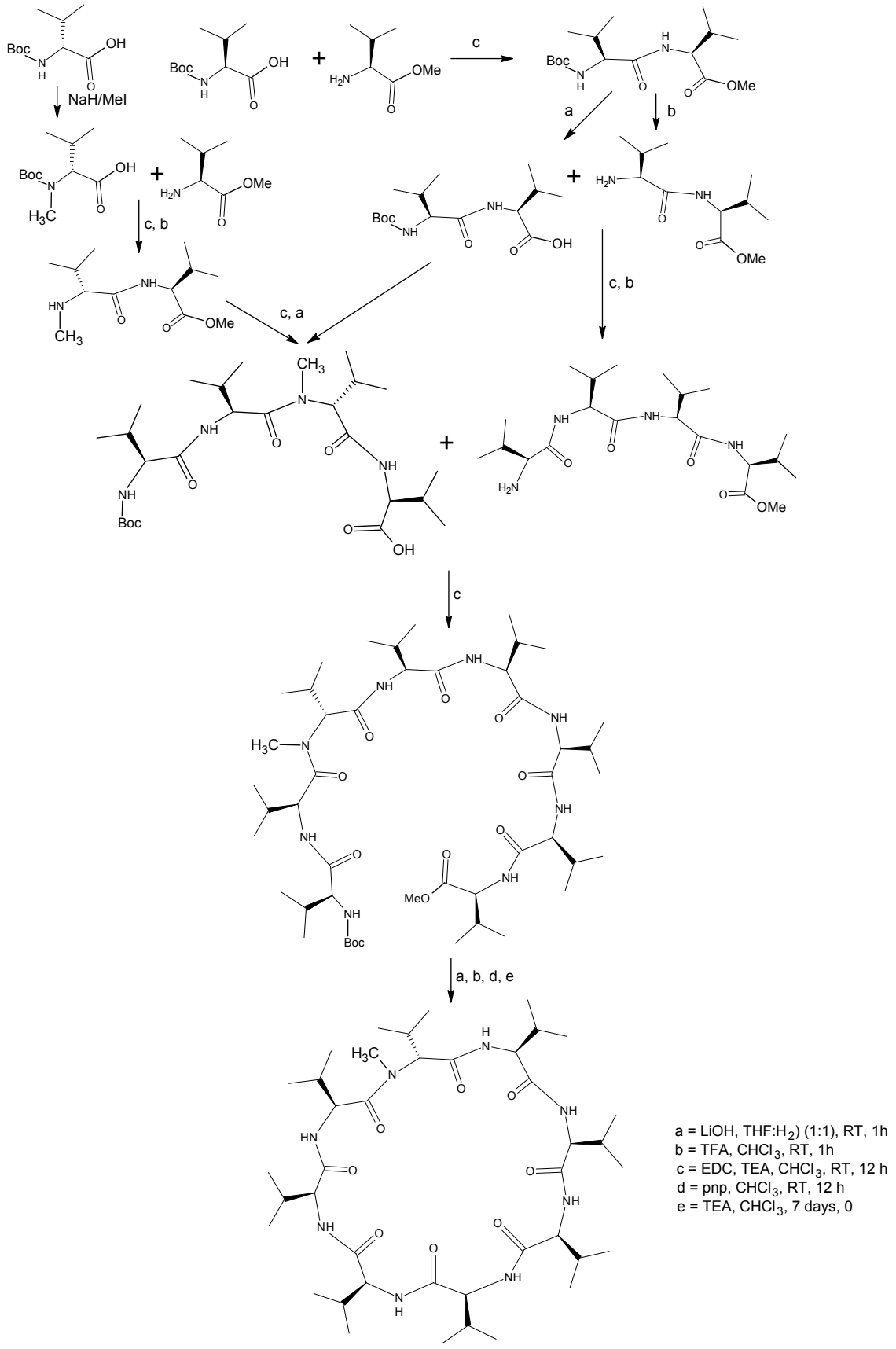
^{1}H NMR spectrum (δ, ppm): 8.05(1H, br. s, -NH), 7.4(3H, br. s, -NH), 6.9(1H, br.s, -NH), 6.45(3H, br. s, -NH), 4.6(2H, m, α-H), 4.4(1H, m, α-H), 4.25(2H, m, α-H), 4.1(2H, m, α-H), 4.0(1H, m, α-H), 2.2(3H, s, -NCH_3), 1.4-1.0(8H, m, β-H), 1.0-0.9(48H, m, -CH_3);

^{13}C NMR: (75.467MHz, $CDCl_3$): 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. = $C_{41}H_{74}N_8O_8$, M. W. = 809, Found (Cal) %C: 64.43 (65.13), %N: 9.97 (10.0).

Scheme 1



Cyclo-N-methyl-D-Val-L-[Val-Val-Val-Val-Val-Val-Val]

Minimum Inhibitory Concentration (MIC):
The synthesized cyclic peptides were evaluated for antibacterial and antifungal activities (MIC) from 25µg to 200µg. The cyclic peptide showed activity from 50µg to 200µg against all bacterial and fungal strains. The results of the MIC are given in Table 1 and Table 2.

Table 1: Minimum Inhibitory Concentration for Antibacterial Activity

Comp. No↓	Presence/absence of growth																							
Organism→	*S. aureus*						*B. subtilis*						*E. coli*						*P. aeruginosa*					
Dilution→	I	II	III	IV	V	VI	I	II	III	IV	V	VI	I	II	III	IV	V	VI	I	II	III	IV	V	VI
CP-2	–	–	+	+	+	+	–	–	+	+	+	+	–	–	+	+	+	+	–	–	+	+	+	+

'+' indicates presence of growth, ' –' indicates absence of growth

Table 2: Minimum inhibitory concentration for antifungal activity

Compd. No.↓	Presence/absence of growth																	
Organism →	*C. albicans*						*A. flavus*						*A. fumigatus*					
Dilution →	I	II	III	IV	V	VI	I	II	III	IV	V	VI	I	II	III	IV	V	VI
CP-2	–	–	+	+	+	+	–	–	+	+	+	+	–	–	+	+	+	+

'+' 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, FABMASS and elemental analysis. The compounds were screened for the Minimum Inhibitory Concentration (MIC) against four strains of bacteria and three strains of fungi and both the compounds were found to be active against both bacteria and fungi from 25-200µg.

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