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Research Article

SYNTHESIS AND ANTIMICROBIAL EVALUATION OF SOME AMIDOMYCIN ANALOGS

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ABSTRACT

Two analogs of Amidomycin, was synthesized by solution phase peptide synthesis using dicyclohexylcarbodiimide (DCC) as the coupling agent and triethylamine (TEA) as the base. Configurational change of D- to L- on Valine was made on Amidomycin to derive the compounds 1) Cyclo-L-[Val-Val-Val-Val-Val-Val-Val-Val] and 2) Cyclo-D-Val-L-[Val- Val-Val-Val-Val-Val-Val]. The structure of these compounds was confirmed by IR, ^{1}H NMR, ^{13}C NMR, FABMASS and elemental analysis. The synthesized cyclic peptides were evaluated for Minimum Inhibitory Concentration (MIC) against four strains of bacteria and three strains of fungi. All compounds were found to be active against bacteria from 25-200µg and against fungi from 50-200µg.

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

INTRODUCTION

Peptides belongs to the important classes of organic compounds with many biological activities like antifungal, antibacterial,[1,2] antioxidant, anthelmintic, antitubercular, and anti-inflammatory activities.[3-5] The main goal in peptide synthesis is the development of approaches to design peptide ligands with specific chemical, physical and biological activities. Peptides ligands generally act by interaction with acceptor or receptor molecules like enzymes, hormones, neurotransmitters, etc.[6,7]

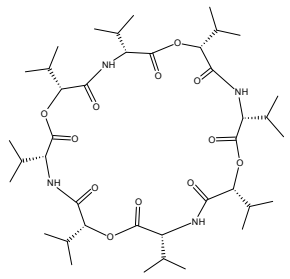


Figure 1: Structure of Amidomyycin

Amidomycin, a cyclic depsipeptide, was produced by a *Streptomyces* species. It was first isolated and its structure was elucidated by Vining L.C. et al.[8] Synthesis of amidomycin was carried out by Shemyakin M. M. et al.[9] It is an antibiotic, primarily active against yeasts.

The structure of amidomycin consists of eight units of D-valine joined through alternate amide and ester linkages.

However, no further studies were carried out on Amidomycin owing to the complexity of the synthesis of the alternate amide and ester linkages and the high cost involved in the usage of D-amino acid. Hence simple analogs were designed with only amide linkages, thus making synthesis easier and cost effective by incorporating L-amino acids.

In order to carry out the synthesis, the two cyclic octapeptides were 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 dicyclohexylcarbodiimide (DCC) 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 octapeptides.

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. 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. 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 ν_{max} (cm^{-1}). ^1H 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 ($\delta = 0$) 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.[10-12]

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 DCC (10 mmol) were added with stirring. After 36 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-L-Val-OMe and Boc-D-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-Val]-OMe and Boc-D-Val-L-[Val-Val-Val]-OMe were synthesized in this manner.

Preparation of linear octapeptides:

The Boc-group of the tetrapeptides Boc-L-[Val-Val-Val-Val]-OMe and Boc-D-Val-L-[Val-Val-Val]-OMe was removed and the ester group of the tetrapeptide Boc-L-[Val-Val-Val-Val]-OMe was deprotected. Both the deprotected units were coupled to get the two linear octapeptides.

Preparation of Cyclic octapeptide (1) and (2): 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 hrs in $CHCl_3$ with p-nitrophenol at 0°C. The reaction mixture was washed several times with saturated $NaHCO_3$ 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°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 recrystallized from $CHCl_3$/n-hexane (Scheme 1).[11,12]

Determination of Minimum Inhibitory Concentration (MIC):

The MIC of the two cyclic peptides was determined by the serial tube dilution technique [13-15] 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*). 4mg 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°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.

RESULTS AND DISCUSSION

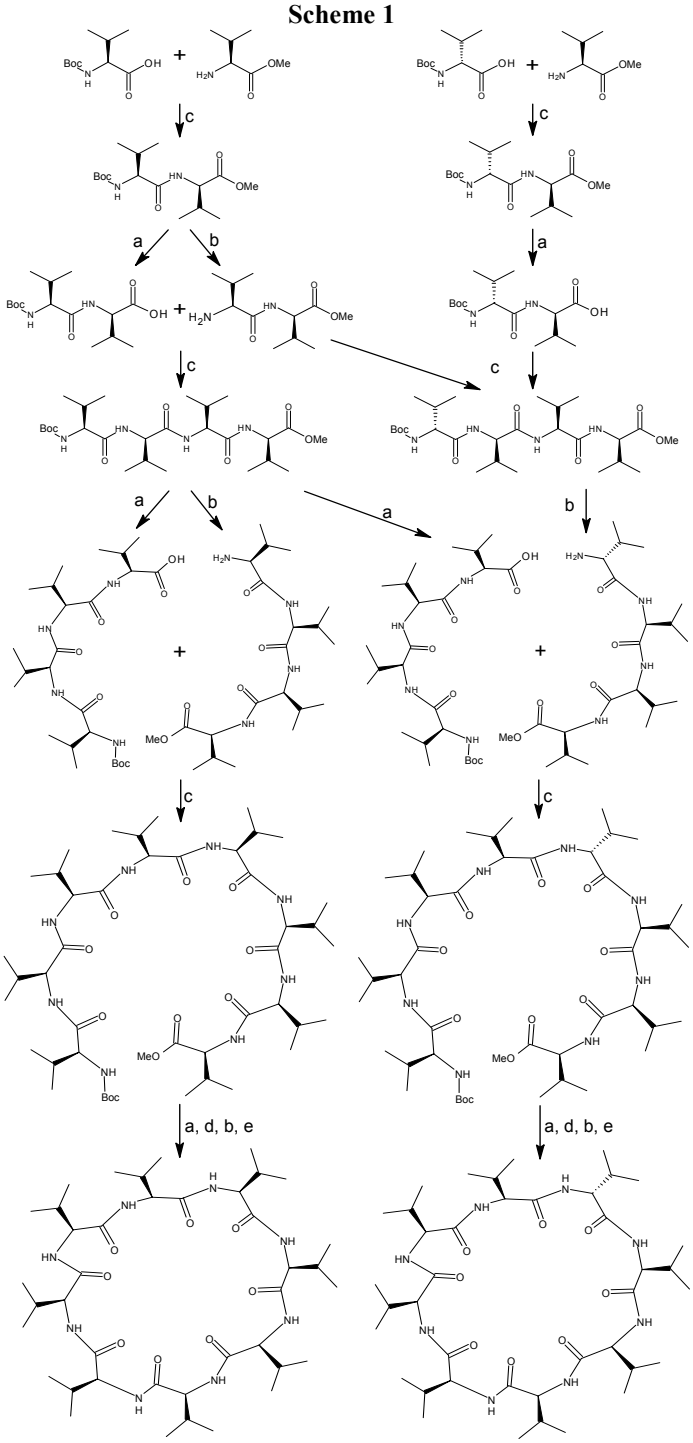
Physical Data and Spectral Analysis:

Cyclic Peptide 1: Cyclo-L-[Val-Val-Val-Val-Val-Val-Val-Val]: Yield 60.53%; light brown semisolid; IR spectrum (ν/cm^{-1}): 3290.5 cm^{-1} (br. s, -NH Stretch), 2932 cm^{-1} (s, -CH stretch), 1646.1 cm^{-1} (s, C=O stretch), 1555 cm^{-1} (s, -NH bend), 1452 cm^{-1} (s, NH bend); ^1H NMR spectrum (δ, ppm): 7.7 (1H, br. s, -NH), 7.3 (1H, br. s, -NH), 5.8 (3H, br.s, -NH), 5.2 (3H, br. s, -NH), 4.75 (1H, m, α-H), 4.4 (2H, m, α-H), 4.1 (2H, m, α-H), 3.9 (3H, m, α-H), 1.5-1.0 (8H, m, β-H), 1.0-0.8 (48H, m, $-CH_3$); ^{13}C NMR: (75.467MHz, $CDCl_3$): 170.4 (C=O of Val), 59.07 (α-C), 53.3 (α-C), 50.11 (α-C), 49.04 (α-C), 48.14 (α-C), 33.91 (β-C), 33.06 (β-C), 32.4 (β-C), 31.51 (β-C), 31.45 (β-C), 30.93 (β-C), 30.27 (β-C), 29.65 (β-C), 25.92 (CH_3), 25.81 (CH_3), 25.59 (CH_3), 25.45 (CH_3), 25.32 (CH_3), 25.13 (CH_3), 24.91 (CH_3), 24.73 (CH_3), 24.57 (CH_3), 24.35 (CH_3), 19.24 (CH_3), 17.90 (CH_3), 15.56 (CH_3); FABMASS: m/z $(M + 1)^+ = 796$; Elemental Analysis: M. F. = $C_{40}H_{72}N_8O_8$, M. W. = 795**,** Found (Cal) %C: 63.71 (64.11), %N: 10.14 (11.47).

Cyclic Peptide 2: Cyclo-D-Val-L-[Val-Val-Val-Val-Val-Val-Val]: Yield 62.13%; light brown semisolid; IR spectrum (ν/cm^{-1}): 3295.5 cm^{-1} (br. s, -NH Stretch), 2932.6 cm^{-1} (s, -CH

stretch), 1645.7 cm^{-1} (s, C=O stretch), 1560 cm^{-1} (s, -NH bend), 1453 cm^{-1} (s, NH bend); ^{1}H NMR spectrum (δ, ppm): 8.0 (1H, br. s, -NH), 7.6(1H, br. s, -NH), 7.4(3H, br.s, -NH), 6.9(1H, br. s, -NH), 6.3(2H, br. s, -NH), 4.6(2H, m, α-H), 4.4(2H, m, α-H), 4.2(2H, m, α-H), 4.0(2H, m, α-H), 1.5-1.2(8H, m, β-H), 1.0-0.85(48H, m, $-CH_3$); ^{13}C NMR: (75.467MHz, $CDCl_3$): 170.3 (C=O of Val), 60.03 (α-C), 53.17 (α-C), 48.09 (α-C), 48.01 (α-C), 33.9 (β-C), 33.57 (β-C), 32.05 (β-C), 32.87 (β-C), 30.88 (β-C), 29.65 (β-C), 25.60 (CH_3), 25.44 (CH_3), 25.17 (CH_3), 24.99 (CH_3), 24.93 (CH_3), 24.74 (CH_3), 19.24 (CH_3), 17.89 (CH_3); FABMASS: m/z $(M + 1)^+$ = 796; Elemental Analysis: M. F. = $C_{40}H_{72}N_8O_8$, M. W. = 795, Found (Cal) %C: 65.79 (64.11), %N: 10.60 (11.47).

Scheme 1



a = LiOH, THF:H_2O (1:1), RT, 1h
b = TFA, $CHCl_3$, RT, 1h
c = DCC, TEA, $CHCl_3$, RT, 24 h
d = pnp, $CHCl_3$, RT, 12 h
e = TEA, $CHCl_3$, 7 days, 0

Cyclo-L-[Val-Val-Val-Val-Val-Val-Val-Val]
Cyclic peptide 1

Cyclo-D-Val-L-[Val- Val-Val-Val-Val-Val-Val]
Cyclic peptide 2

Concentration of the solutions of the Test Compounds:
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

Minimum Inhibitory Concentration (MIC):
The synthesized cyclic peptides were evaluated for antibacterial and antifungal activities (MIC) from 200µg to 6.25µg. Cyclic peptide 1 showed better MIC activity than the cyclic peptide 2. Cyclic peptide 1 showed activity from 25µg to 200µg against all bacterial and fungal strains, whereas cyclic peptide 2 showed activity from 50µg to 200µg. 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-1	–	–	+	+	+	+	–	+	+	+	+	+	–	–	+	+	+	+	–	–	+	+	+	+
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-1	–	–	+	+	+	+	–	–	+	+	+	+	–	–	+	+	+	+
CP-2	–	–	–	+	+	+	–	–	–	+	+	+	–	–	–	+	+	+

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

CONCLUSION

The two cyclic octapeptides could be conveniently and efficiently synthesized by the prescribed scheme with good yields. Their structures were 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 bacteria from 25-100µg and against fungi from 50-200µg.

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