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MICROWAVE ASSISTED SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF HALOLITORALIN A AND ITS N-ALKYLATED ANALOGS

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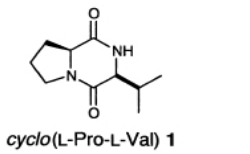
ABSTRACT
Total microwave-assisted synthesis of Halolitoralin A (a cyclic hexapeptide) isolated from the ferment broth of a marine sediment-derived *Halobacillus litoralis* YS3106 and its two N-methyl and N-ethyl analogs were described and characterized by spectral data. The synthesized compounds were screened for antibacterial activity against gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*), gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and antifungal activity against *Candida albicans* (Diamorphic fungi).
Keywords: Halolitoralin A, synthesis, antibacterial, antifungal, microwave.

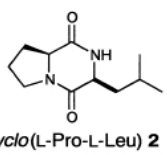
INTRODUCTION

Microwave irradiation has become very useful tool in organic synthesis and a great number of classical organic reactions have been carried out using microwave hearing technique[1]. The marine environment has been a hugely important source of structurally novel and biologically important natural products for more than thirty years now[2].

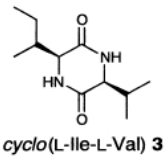
Peptides are the important class of organic compounds with potent biological activities[3-9]. Three new cyclopeptides, including Halolitoralin A (4) (a cyclic hexapeptide), halolitoralin B (5) and C (6) (two cyclic tetrapeptides), together with three known cyclic dipeptides, cyclo(Pro-Val)(1), cyclo(Pro-Leu)(2) and cyclo(Ile-Val)(3) were isolated from the ferment broth of a marine sediment-derived *Halobacillus litoralis* YS3106. The cyclopeptides show surprisingly simple architectures with highly repeated residue units, which showed moderate antifungal and weak antitumor activities in vitro[10]. An attempt is being made to synthesize Halolitoralin A and its N-methylated and N- ethylated analogs. The synthesized compounds will be evaluated for their antibacterial, antifungal activity.



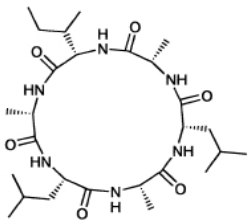
cyclo(L-Pro-L-Val) **1**



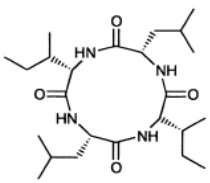
cyclo(L-Pro-L-Leu) **2**



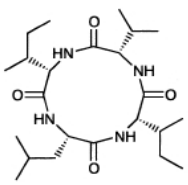
cyclo(L-Ile-L-Val) **3**



Halolitoralin A **4**



Halolitoralin B **5**



Halolitoralin C **6**

RESULTS AND DISCUSSION

The synthesis of cyclic hexapeptide-1 was carried out as follows:

The Boc-Ile (27) and Ala-OMe HCl (28) was coupled using DIPC and NMM to get the dipeptide Boc-Ile-Ala-OMe (29). In the same way the dipeptide Boc-Leu-Ala-OMe (31)was prepared. From Boc-Leu (30) and Ala-OMe HCl (28).

The ester group of dipeptide Boc-Ile-Ala-OMe (29) was removed with LiOH and the Boc-group of dipeptide Boc-Leu-Ala-OMe (31) was removed by using TFA. Both the deprotected dipeptides were coupled using DIPC and NMM to get the tetrapeptide Boc-Ile-Ala-Leu-Ala-OMe (34).

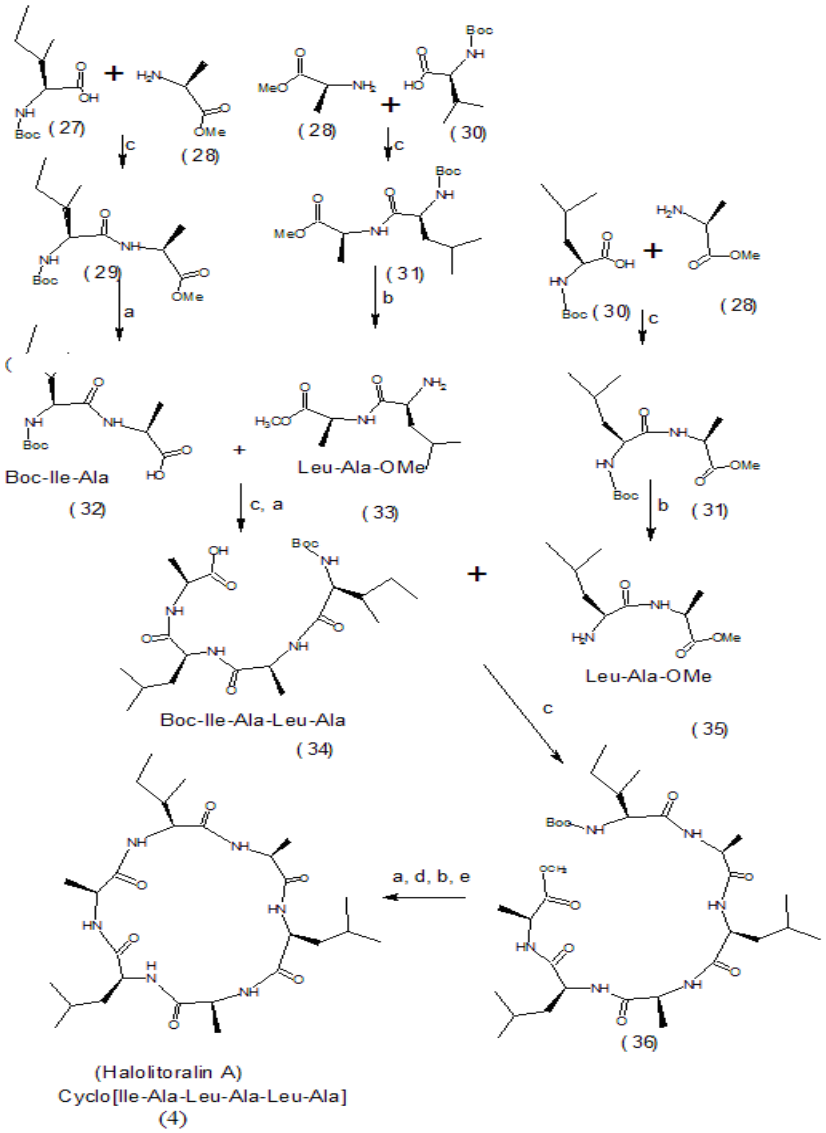
The ester group of tetrapeptide was removed with LiOH. The deprotected tetrapeptide unit was coupled with Leu-Ala-OMe (35) to obtain linear hexapeptide Boc-Ile-Ala-Leu-Ala-Leu-Ala-OMe(36).

The ester group of hexapeptide was removed with LiOH and p-nitrophenyl group was introduced by stirring at room temperature for 12 hrs with DIPC and p-nitrophenol in chloroform.

The Boc-group was removed by using TFA. And the linear fragment was cyclized by adding NMM and keeping the whole contents at 0°C for seven days to get a cyclic hexapeptide, Halolitoralin A (4).

The structure of the molecule was confirmed by IR, ^{1}H NMR, FABMASS and elemental analysis.

The above synthetic steps are shown in Scheme-(2.1)



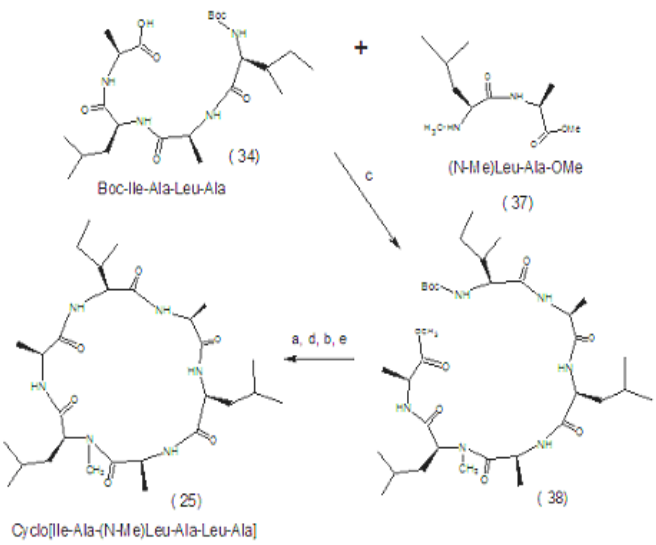
a = LiOH, THF:H_2O(1:1), MW, 180W. 3 mins
b = TFA, $CHCl_3$, 1h, RT
c = DCC, NMM, DMF, MW, 180W, 10mins
d = p-nitrophenol, DCC, 12h, RT
e = NMM, DMF, MW, 180W, 10mins

Scheme-2.1

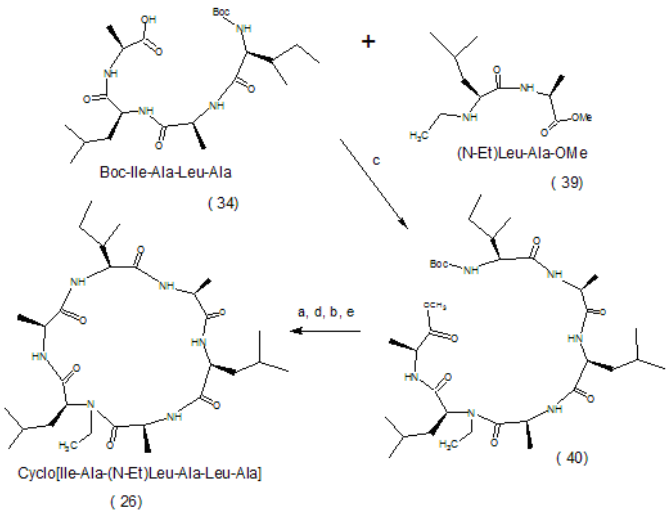
For the synthesis of N-Methyl analog (25) and N-Ethyl analog (26) the tetrapeptide(34) was coupled with (N-Me)Leu-Ala-OMe(37) and (N-Et)Leu-Ala-OMe(39) to obtain linear hexapeptide Boc-Ile-Ala-Leu-Ala-(N-Me) Leu-Ala-OMe (38) and Boc-Ile-Ala-Leu-Ala-(N-Et) Leu-Ala-OMe (40) respectively.

The ester group of hexapeptide was removed with LiOH and p-nitrophenyl group was introduced by stirring at room temperature for 12 hrs with DIPC and p-nitrophenol in chloroform.

The Boc-group was removed by using TFA. And the linear fragment was cyclised by adding NMM and keeping the whole contents at 0°C for seven days to get cyclic hexapeptides CYCLO[Ile-Ala-(N-Me) Leu-Ala-Leu-Ala] (25) **Scheme 2.2** and CYCLO[Ile-Ala-(N-Et) Leu-Ala-Leu-Ala] (26) **Scheme 2.3.**



Scheme 2.2



Scheme 2.3

EXPERIMENTAL SECTION

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, trifloroacetic 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}). ^1H NMR spectra were recorded on Bruker Avance 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. FABMASS spectra were recorded on a Jeol Sx-102 (FAB) mass spectrometer using xenon as the carrier gas. The spectra were recorded at room temperature. M-nitrobenzyl alcohol was used as the matrix.

Synthesis Of Halolitoralin A, Cyclo[Ile-Ala-Leu-Ala-Leu-Ala] (4)

Thionyl chloride (1.4ml, 20.0 mmol) was added to methanol (30ml) slowly at 0°C and the amino acid (20 mmol) was added to this solution and the reaction mixture was irradiated at 180W for 15 minutes (Model M 1739 N) to give a pasty mass of methyl ester hydrochloride which was triturated with ether at 0°C to remove excess dimethyl sulphite. The resulting solid was recrystallize from methanol and diethyl ether at 0°C.

By using above method alanine methyl ester hydrochloride (31) and leucine methyl ester was prepared with a good yield.

The amino acid (20 mmol) was dissolved in 1N NaOH (20ml) and isopropanol (20ml). Tert-butyloxycarbonyl anhydride (26 mmol, 6ml) in isopropanol (10ml) was added followed by (1N NaOH) (20ml) to the resulting solution. The solution was stirred at room temperature for 2 hours, washed with light petroleum ether (40-60^{0}C) (20ml), acidified to $_P$H 3.0 with 2N H_2SO_4 and finally extracted with chloroform (3 x 20ml). The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure to give the Boc- amino acid. The crude product was recrystallize from chloroform and petroleum ether.

Using the above method the following Boc-Leucine(30) and Boc-Isoleucine(27) were prepared.

Amino acid methyl ester hydrochloride (10 mmol) was dissolved in DMF (20 ml). To this, triethylamine (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 DMF (20 ml) and DIPC (10 mmol) were added with stirring.

The reaction mixture was irradiated at 180 W for 10 sec. (Model M 1739 N) and stirred at RT for 10-15 minutes. The above procedure was repeated, till total irradiation time is 15 minutes. After this, the reaction mixture was filtered and residue was washed with DMF (20ml) and added to the filtrate. From the filtrate excess of DMF was distilled off to give a pasty mass. So formed pasty mass, was dissolved in $CHCl_3$ (20ml) and the resultant solution was washed with 5% $NaHCO_3$ (20 ml) and saturated NaCl (20 ml) solutions. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum. To remove the traces of the dicyclohexylurea (DCU), the product was dissolved in minimum amount of chloroform and cooled to 0°C. The crystallized DCU was removed by filtration. Petroleum ether was added to the filtrate at 0°C to recrystallize the pure product.

By using above method Boc-Ile-Ala-OMe (29) and Boc-Leu-Ala-OMe (31) were prepared.

To a solution of the protected peptide Boc-Ile-Ala-OMe (29) (1.0 mmol) in THF : H2O (1:1) (36ml), LiOH (1.5 mmol) was added at 0°C. The mixture was refluxed at 55-60°C for 15 mins and then acidified to pH 3.5 with 1N H_2SO_4 . The mixture was extracted with solvent ether (3x15ml). The combined ether extracts were dried over Na_2SO_4 and concentrated under reduced - pressure to get Boc Ile-Ala(32).

The protected peptide Boc-Leu-Ala-OMe (31) (1 mmol) was dissolved in $CHCl_3$ (15ml) and treated with CF_3COOH (2mmol, 0.228 g). The solution was stirred at room temperature for 1 hour, washed with saturated NaHCO3 (5ml). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The product was purified by recrystallization from CHCl3 and petroleum ether to get Leu-Ala-OMe(33).

Boc Ile-Ala(32) and Leu-Ala-OMe(33) are coupled using procedure similar to that of dipeptide coupling to get tetrapeptide Boc-Ile-Ala-Leu-Ala-OMe (34)

The ester group of tetrapeptide was removed with LiOH. The deprotected tetrapeptide unit was coupled with Leu-Ala-OMe (33) to obtain linear hexapeptide Boc-Ile-Ala-Leu-Ala-Leu-Ala-OMe(36).

Cyclisation of the linear fragments was attempted by p-nitrophenyl ester method. The ester group of the linear segment was removed with LiOH and the *p*-nitrophenyl 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^{0}C. 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 0^{0}C for 7 days. 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.

Cyclic hexa peptide Halolitoralin A (4): yield 73.17 %, Found: C: 55.34 %, N: 9.17 %. $C_{27}H_{48}N_6O_6$ required C, 56.34% N, 10.06% ^{1}H NMR (300MH$_Z$, $CDCl_3$): 6.7 (2H, m, NH), 5.6(2H, m, NH), 4.6 (1H, s, NH), 4.3(1H, m, NH), 4.1-3.9(3H, m, α-H), 3.7-3.4 (3H, m, α-H), 1.5 (15H, m, CH_3), 1.1(9H, m, CH_3 of Ala), 0.9(12H, m, β-and ϒ–CH/CH_2/CH_3) FABMass in m/z: Molecular Ion peak was observed at m/z 575 correspondence to the molecular formula $C_{27}H_{48}N_6O_6$. IR ($CHCl_3$): 3299.2(-NH stretch.), 2965.8(aliph.-CH stretch.), 1646.4(C=O stretch of amide), 1536.3 (-NH bend), 1465.4 (-CH bend).cm^{-1}.

Synthesis OF N-Methyl analog, Cyclo[Ile-Ala-(N-Me)Leu-Ala-Leu-Ala] (25)

To a solution of Leucine methyl ester hydrochloride (20 mmol) in $CHCl_3$ (20 ml) was added triethylamine (4 mmol) followed by $(Boc)_2O$ (6 ml). The solution was stirred for 2 hours at room temperature, diluted with ether (15 ml) and washed with 10% $NaHCO_3$ (10 ml). The organic layer was separated, dried and concentrated to get the Boc-amino acid methyl ester which was recrystallized from n-hexane at 15 ^{0}C to get the title compound. Using the above procedure, the following Boc- Leucine methyl ester was prepared.

Boc-Leucine methyl ester (5.5 mmol) was dissolved in DMF (30ml), sodium hydride (0.75 gm, 16.5 mmol) was added at room temperature, followed by methyl iodide (6.3 gm, 44 mmol).The solution was stirred for four hours at room temperature then dilute with ether. The solution was washed with saturated with NH_4Cl (20 ml) 20% $Na_2S_2O_3$ (20ml). The organic layer was dried with anhydrous Na_2SO_4 and concentrated under reduced pressure to give the title compound, Boc-(N-Me)Leucine methyl ester (Bentoin method)[11] obtained as viscous oil. The ester group of Boc-(N-Me)Leucine methyl ester was removed with LiOH and coupled with Ala-OMe (28) to get dipeptide Boc-(N-Me)Leu-Ala-OMe. The Boc group of dipeptide was removed by using trifluoro acetic acid to get (N-Me)Leu-Ala-OMe (37).

By using the procedures described in synthesis of Halolitoralin A and following the scheme 2.2 the cyclic hexapeptide CYCLO[Ile-Ala-(N-Me)Leu-Ala-Leu-Ala] (25) was prepared.

Cyclic hexa peptide CYCLO[Ile-Ala-(N-Me)Leu-Ala-Leu-Ala] (25): yield 78.54 %, Found: C: 60.81 %, N: 9.69%. $C_{28}H_{50}N_6O_6$ required C, 59.04% N, 10.26% ^{1}H NMR (300MH$_Z$, $CDCl_3$): 6.3 (3H, br.m, NH), 5.3(1H, m, α-H), 5.2 (1H, s, NH), 4.7(1H, s, NH), 4.1 (3H, m, α-H), 3.9 (2H, m, α-H), 2.8 (3H, s, N-CH_3), 1.6 (9H, d, CH_3 of Ala), 1.2(12H, m, β-and ϒ–CH/CH_2/CH_3), 0.9(15H, m, CH_3) FABMass in m/z: Molecular Ion peak was observed at m/z 589 correspondence to the molecular formula $C_{28}H_{50}N_6O_6$ IR ($CHCl_3$): 3301.5(-NH stretch.), 2967.5(aliph.-CH stretch.), 1649.3(C=O stretch of amide), 1537.4 (-NH bend), 1455.6 (-CH bend). cm^{-1}.

Synthesis OF N-Ethyl analog, Cyclo[Ile-Ala-(N-Et)Leu-Ala-Leu-Ala] (26)

By using the procedures described in synthesis of Halolitoralin A and Cyclic hexa peptide CYCLO[Ile-Ala-(N-Me)Leu-Ala-Leu-Ala] (25) and following the scheme 2.3 the cyclic hexapeptide CYCLO[Ile-Ala-(N-Et)Leu-Ala-Leu-Ala] (26) was prepared.

Cyclic hexa peptide CYCLO[Ile-Ala-(N-Et)Leu-Ala-Leu-Ala] (26): yield 65.23 %, Found: C: 57.71 %, N: 9.57%. $C_{29}H_{52}N_6O_6$ required C, 58.63% N, 10.41% ^{1}H NMR (300MH$_Z$, $CDCl_3$): 6.7 (1H, br.m, NH), 6.2 (2H, br.m, NH), 5.1 (2H, br.m, NH), 4.6-4.4 (2H, br.m, α-H), 4.1-3.8 (2H, br.m, α-H), 3.7 (5H, d, N-C_2H_5), 3.6 – 3.4 (2H, br.m, α-H), 1.9-1.7 (12H, br.m, β-and ϒ–CH/CH_2/CH_3), 1.6-1.4 (9H, br.m, CH_3 of Ala), 0.9 (15H, br.m, CH_3). FABMass in m/z: Molecular Ion peak was observed at m/z 603 correspondence to the molecular formula $C_{29}H_{52}N_6O_6$ IR ($CHCl_3$): 3289.5(-NH stretch.), 2965.6(aliph.-CH stretch.), 1650.5(C=O stretch of amide), 1555.3(-NH bend), 1455.5(-CH bend) cm^{-1}.

Biological activity of synthesized compounds

The synthesized compounds were screened for antibacterial activity against gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*), gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and antifungal activity against *Candida albicans* (Diamorphic fungi). The test microorganisms were grown for 24 hr at 37^{0}C (Bacteria) and for 72 hr at 30^{0}C (Fungi). Each petri plate containing nutrient/Sabouraud's agar medium was inoculated with one bacterial/fungal culture by spreading the suspension of the organism with a sterile cotton swab. Each plate was divided into four equal portions and in each portion a cup of 6mm diameter was cut with a cork borer. Two cups were filled with 0.1ml of the sample. While other two were filled with 0.1 ml of standard drug (Positive control) and 0.1ml of solvent Dimethylformamide (DMF) (Negative control) respectively. All the plates were kept in the refrigerator for 30 minutes to allow the diffusion of sample in to the surrounding agar medium. The petri dishes were incubated at 37°C for 24 hrs. Diameter of the zone of inhibition was measured and the average diameter of each sample was calculated. The diameter obtained for the test samples were compared with that of the diameter produced by standard drug. The diameter of zone of inhibition is proportional to the antibacterial activity of the substance. According to the results obtained, all the test compounds found to be active against test microorganisms. Out of these compound 25 showed highly promising antifungal activity and weak antibacterial activity but other compounds exhibited weak antibacterial and antifungal activity. The data of antibacterial and antifungal activity of synthesized compounds has been given in **Table 1**

Table 1: Antibacterial and antifungal activity of synthesized compounds in mm

Compound	Antibacterial activity				Antifungal activity
	B. subtillus	*S. aureus*	*E. coli*	*P. aeruginosa*	*Candida albicans*
4	08	10	16	06	17
25	13	16	22	08	20
26	09	12	17	07	18
Gentamycin	14	16	21	13	NA
Clotrimazole	NA	NA	NA	NA	20
DMF	-	-	-	-	-

(-) indicates no inhibition zone (no activity) (NA) indicates not applied

CONCLUSION

In conclusion, we have carried out microwave-assisted synthesis of the Halolitoralin A (a cyclic hexapeptide) isolated from the ferment broth of a marine sediment-derived *Halobacillus litoralis* YS3106 and its two N-Methyl and N- Ethyl analogs. The synthesized compound was characterized by IR, ^{1}H NMR and FAB-Mass spectral studies and subjected to antimicrobial studies. All the test compounds found to be active against test microorganisms. Out of these compound 25 showed highly promising antifungal activity and weak antibacterial activity but other compounds exhibited weak antibacterial and antifungal activity.

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REFERENCES

1. Kamotra P, Gupta AK, Gupta R, Microwave-assisted synthesis of substituted-4-oxo-4-H-1-benzopyran-3-carboxaldehydes using Vilsemeier reagent over silica gel. Indian journal of Chemistry., 2007; 46:866.
2. McKveever B, Pattenden G, Total synthesis of the cytotoxic cyclopeptide mollamide, isolated from the sea squirt Didemnum molle, Tetrahedron., 2003; 59:2701.
3. Lee MD, Yuhong MJ, Soskis CP, Borella M, Humanmitochondrial peptide deformylase, a new anticancer target of actinonin-based antibiotics, J. Clinical Investigation., 2004; 114: 8.
4. Luke Simmons KL. McPhail B, Eduardo OB, Susan L, William HG, Belamide A, A new antimitotic tetrapeptide from a Panamanian marine cyanobacterium, Tetrahedron Letters., 2006; 47: 3387.
5. Beverly AT, Ara G, Roy H, Palombella VJ, Julian A, The Proteasome Inhibitor PS-341 in Cancer Therapy, Clinical Cancer Research., 1999; 5: 2638.
6. Katsuhirokudo, Shuichi O, Yasuyuki T, Benkeblia N, Shiomi N, Antioxidative activities of some peptides isolated from hydrolyzed potato protein extract, Journal of functional foods., 2009; 1: 170.
7. Gunnur ZD, Ginelle CG, Pakize D, Heejeong Y, Young BL, Chang HA and Jerry WS, In vivo and Invitro Effects of a HIF-1α Inhibitor, RX-0047, Journal of Cellular Biochemistry., 2008; 104: 985.
8. Dale LB, Jiacheng Z, Brian W and Paul AK, Key analogs of the Tetrapeptide Subunit of RA-VII and Deoxybouvardin, Bioorganic and Medicinal Chemistry., 1995; 3: 1579.
9. Mayera AM, Gustafson KR, Marine pharmacology in 2005–2006: Antitumor and cytotoxic compounds, European journal of Cancer., 2008; 44: 2357.
10. Ling y, Ren-xiang T, Qian W, Wei-yi H and yong-xian Y. Antifungal cyclopeptides from Halobacillus litoralis YS3106 of marine origin. Tetrahedron letters 2005 Aug;46:7069-72.
11. Bentoin NL, Akyusckli D, Chen FM, N, N-dialkoxy-carbonyl amino acid from the sodium hydroxide mediated reaction of akyl chloroformates with anhydrides of N.alkoxy carbonyl amino acids, Ind J pro protein Res., 1995; 45:446.

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