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Synthesis, Characterization and Biological Evaluation of Halolitoralin B - A Natural Cyclic Peptide

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A new potent bioactive cyclic tetrapeptide halolitoralin B (**7**) has been synthesized by solution phase technique which was previously isolated from the marine sediment-derived bacterial strain *Halobacillus litoralis* YS3106. The structure of the peptide was characterized by IR, ^{1}H NMR, ^{13}C NMR, FAB MS spectral data as well as elemental analysis. Synthesized cyclopeptide was also screened for its antimicrobial and anthelmintic activities and found to exhibit potent antifungal activity against pathogenic fungi *Candida albicans* along with potent anthelmintic activity against earthworms *Megascoplex konkanensis* and *Eudrilus sp.* In addition, peptide was also found to exhibit moderate antibacterial activity against gram negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli*. Gram positive bacteria were found to be resistant towards the newly synthesized peptide.

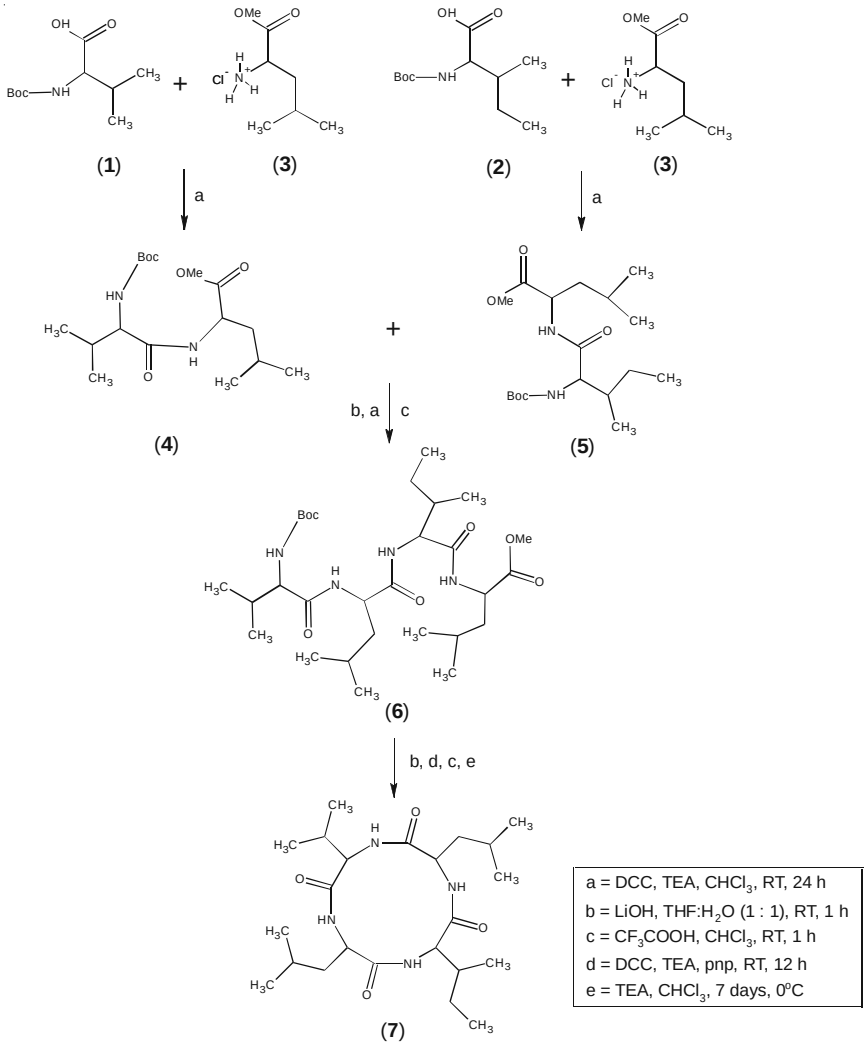
Key Words: Halolitoralin B, Cyclic tetrapeptide, Anthelmintic activity, Antimicrobial activity.

INTRODUCTION

Cyclic peptides[1-5] have played a crucial role in the pharmaceutical research as biomedically useful agents or as lead compounds for drug development. Halolitoralins are natural cyclic peptides isolated from bacterial strain *Halobacillus litoralis* YS3106 of marine origin[6]. These cyclic congeners are associated with diverse biological activities especially antifungal activity. Only minute quantities of cyclopeptides obtained from natural resources[7] restricted researchers to investigate their biological profiles in detail. Considering the wide spectrum of bioactivities associated with these natural congeners and in order to obtain a potent bioactive compound in good yields. An attempt has been made to synthesize cyclic tetrapeptide halolitoralin B (**7**) by solution phase technique in a convenient and economic manner.

In order to carry out the synthesis of halolitoralin B (**7**), two Boc-protected amino acid units Boc-Val (**1**) and Boc-Ile (**2**) were coupled with

an amino acid methyl ester hydrochloride unit Leu-OMe.HCl (**3**). Boc-Val (**1**) was coupled with Leu-OMe.HCl (**3**) to get dipeptide unit Boc-Val-Leu-OMe (**4**). Similarly, Boc-Ile (**2**) was coupled with (**3**) to obtain another dipeptide unit Boc-Ile-Leu-OMe (**5**). Then, the ester group of (**4**) was removed using LiOH and Boc group of (**5**) was removed using CF_3COOH. Both deprotected units were now coupled using DCC and TEA to get linear tetrapeptide Boc-Val-Leu-Ile-Leu-OMe (**6**) which was finally cyclized by keeping the whole contents at 0°C for 7 days in alkaline conditions to get halolitoralin B (7) (**Scheme-1**).



Scheme-1

Structure of the newly synthesized cyclopeptide as well as intermediates di/tetrapeptides were confirmed by IR, ^{1}H NMR as well as elemental analysis. ^{13}C NMR and Mass spectra were recorded for cyclic peptide only.

The newly synthesized cyclic peptide halolitoralin B was screened for anthelmintic activity against earthworms *Megascoplex konkanensis* and *Eudrilus sp*. using Garg method[8] (Table-1) and for *in vitro* antimicrobial activity against gram negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli*, gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, cutaneous fungi *Microsporum audouinii* and *Trichophyton mentagrophytes* and diamorphic fungi *Candida albicans* and plant pathogenic fungus *Ganoderma sp*. using modified Kirby-Bauer disk diffusion method[9] (Table-2).

TABLE-1
ANTHELMINTIC ACTIVITY AT 100 mg LEVEL

Compound	*Eudrilus sp*.		*M. konkanensis*	
	Mean paralyzing time(min)±S.E.	Mean death time(min)±S.E.	Mean paralyzing time(min)±S.E.	Mean death time(min)±S.E.
Halolitoralin B	10.22 ± 0.78	13.07 ± 0.86	11.26 ± 0.71	13.14 ± 0.35
Piperazine Citrate	12.48 ± 0.18	13.53 ± 0.21	12.48 ± 0.37	13.56 ± 0.22
Mebendazole	11.57 ± 0.39	13.46 ± 0.72	12.19 ± 0.93	14.05 ± 0.84

*S.E. – Standard Error

TABLE-2
ANTIMICROBIAL ACTIVITY DATA AT 50 µg/mL CONC. (ZONE FORMATION IN mm)

Compound	***B. subtilis***	***E. coli***	***S. aureus***	***P. aeruginosa***	***C. albicans***	***G. species***	***M. audouinii***	***T. mentagrophytes***
Halolitoralin B	–	14	–	17	24	Nil	9	8
Clotrimazole	–	–	–	–	16	14	17	18
Ciprofloxacin	18	16	19	20	–	–	–	–

TABLE-3
VARIOUS STERIC AND LIPOPHILICITY PARAMETERS FOR HALOLITORALIN-B

Parameter	Calculated Value
Molar Refractivity (MR^{20})	120.27 ± 0.3 cm^3
Molar Volume (MV^{20})	445.6 ± 3.0 cm^3
Parachor (P_r)	1039.2 ± 6.0 cm^3
Index of Refraction (n^{20})	1.452 ± 0.02
Surface Tension (γ^{20})	29.5 ± 3.0 dyne/cm
Density (d^{20})	0.984 ± 0.06 g/cm^3
Polarizability (α)	47.67 ± 0.5 10^{-24} cm^3
Partition Coefficient (log P)	0.14 ± 0.53

Different steric and lipophilicity parameters of cyclic tetrapeptide (7) were calculated which are needed to describe the intermolecular forces of drug receptor interaction as well as transport and distribution of drugs in a quantitative manner (Table-3). As per IUPAC rules, halolitoralin B may be named as 3-(*sec*-butyl)-6,12-diisobutyl-9-isopropyl-1,4,7,10-tetraazacyclododecane-2,5,8,11-tetraone.

EXPERIMENTAL

All the reactions requiring anhydrous conditions were conducted in flame dried apparatus. Melting points were determined by open capillary method and is uncorrected. DCC (dicyclohexylcarbodiimide), *p*-nitrophenol (pnp), TEA (triethylamine) and trifluoroacetic acid were obtained from Spectrochem Limited, Mumbai, India. Boc (*tert*-butyloxycarbonyl) protected amino acids were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. IR spectra were recorded on Shimadzu 8700 fourier transform infrared spectrophotometer using a thin film supported on KBr pellets for cyclic peptide and $CHCl_3$ as solvent for intermediate semisolids. ^1H NMR and ^{13}C NMR spectra were recorded on Bruker AC NMR spectrometer (300 MHz) using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. Mass spectra was recorded on Jeol JMS DX 303 Mass spectrometer operating at 70 eV. Elemental analysis of all the compounds were performed on Elementar vario EL III. Purity of all the compounds was checked by TLC on precoated silica gel G plates.

Preparation of peptides

Amino acid methyl ester hydrochloride (1.82 g, 10 mmol)/peptide methyl ester (2.58 g, 10 mmol) was dissolved in $CHCl_3$ (20 mL). To this, TEA (2.9 mL, 21 mmol) was added at 0°C and the reaction mixture was stirred for 15 min. Boc-amino acid [Boc-Val (2.17 g, 10 mmol)/Boc-Ile (2.31 g, 10 mmol)] / Boc-peptide [Boc-Val-Leu (3.3 g, 10 mmol)] in $CHCl_3$ (20 mL) and DCC (2.1 g, 10 mmol) were added with stirring. After 24 h, 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% $NaHCO_3$ and saturated NaCI solutions. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum. The crude product was recrystallized from a mixture of chloroform and petroleum ether followed by cooling at 0°C.

The carboxyl group of L-leucine was protected by esterification with methanol using $SOCl_2$. Peptides were prepared by Bodanszky method with certain modifications and cyclization of the linear segment was done by *p*-nitrophenyl ester method[10]. Furthermore, CF_3COOH was used for the removal of Boc group and ester group was removed by alkaline hydrolysis with lithium hydroxide.

***tert*-Butyloxycarbonyl-valyl-leucine methyl ester (4)** (Semi-solid mass, yield 4.54 g, 75.8%)

IR ν_{max}, cm^{-1} ($CHCl_3$): 3328, 3310 (s, -NH str, amide), 2927 (m, -CH str, asym, aliph. CH_2), 2849 (m, -CH str, sym, aliph. CH_2), 2896, 2888 (m, -CH str, >CH-), 2825 (m, -CH str, OCH_3), 1752 (s, -C=O str, ester), 1647, 1638 (s, -C=O str, 2° amide), 1544, 1539 (m, -NH bend, 2° amide), 1390, 1368 (m, -CH bend, ^{t}Butyl group), 1379, 1366 (s, C-H bend, isopropyl group), 1210 (s, C-O str, ester), 931, 920 (w, CH_3 rocking, ^{t}Butyl and isopropyl group), 816, 802 (w, C-C str, aliph.).

^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.88 (1H, br. s, -NH), 6.43 (1H, br. s, -NH), 4.30-4.26 (1H, t, α-H of Val), 3.96-3.86 (1H, m, α-H of Leu), 3.60 (3H, s, OCH_3), 1.90-1.76 (1H, m, β-H of Val), 1.55 (9H, s, *tert*-Butyl group), 1.53-1.21 (3H, m, β- and γ-protons of Leu), 1.06-1.04 (6H, d, J = 6.2 Hz, γ-protons of Val), 0.95-0.93 (6H, d, J = 6.3 Hz, δ-protons of Leu) ppm. [Found: C, 59.27; H, 9.36; N, 8.10; $C_{17}H_{32}N_2O_5$ requires C, 59.28; H, 9.36; N, 8.13].

***tert*-Butyloxycarbonyl-isoleucyl-leucine methyl ester (5)**
(Semi-solid mass, yield 4.87 g, 73.5%)

IR ν_{max}, cm^{-1} ($CHCl_3$): 3325, 3195 (s, -NH str, amide), 2967, 2935, 2926 (m, -CH str, asym, aliph. CH_3 and CH_2), 2877, 2855 (m, -CH str, sym, aliph. CH_3 and CH_2), 2895, 2889 (m, -CH str, >CH-), 2828 (m, -CH str, OCH_3), 1748 (s, -C=O str, ester), 1649, 1635 (s, -C=O str, 2° amide), 1540, 1534 (m, -NH bend, 2° amide), 1466, 1448 (m, -CH bend, aliph. CH_2 and CH_3), 1392, 1365 (m, -CH bend, *tert*-Butyl group), 1378, 1367 (s, C-H bend, isopropyl group), 1207 (s, C-O str, ester), 931 (w, CH_3 rocking, *tert*-Butyl group), 495, 472(m, C-C bend, aliph.).

^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.74 (1H, br. s, -NH), 5.68 (1H, br. s, -NH), 4.35-4.31 (1H, t, α-H of Ile), 4.16-4.09 (1H, m, α-H of Leu), 3.61 (3H, s, OCH_3), 1.85-1.72 (1H, m, β-H of Ile), 1.65-1.21 (5H, m, γ-protons of Ile and Leu, β-protons of Leu), 1.55 (9H, s, *tert*-Butyl group), 1.05-1.03 (3H, d, J = 6.3 Hz, γ'-protons of Ile), 1.00-0.97 (3H, t, δ-protons of Ile), 0.95-0.93 (6H, d, J = 6.2 Hz, δ-protons of Leu) ppm. [Found: C, 60.28; H, 9.55; N, 7.83; $C_{18}H_{34}N_2O_5$ requires C, 60.31; H, 9.56; N, 7.81].

***tert*-Butyloxycarbonyl-valyl-leucyl-isoleucyl-leucine methyl ester (6)**
(Semi-solid mass, yield 8.2 g, 69.5%)

IR ν_{max}, cm^{-1} ($CHCl_3$) : 3318, 3309, 3188 (s, -NH str, amide), 2965, 2959, 2934, 2930 (m, -CH str, asym, aliph. CH_3 and CH_2), 2876, 2870, 2852 (m, -CH str, sym, aliph. CH_3 and CH_2), 2896, 2885 (m, -CH str, >CH-), 2825 (m, -CH str, OCH_3), 1746 (s, -C=O str, ester), 1648-1637 (s, -C=O str, 2° amide), 1547-1535 (m, -NH bend, 2° amide), 1465, 1460, 1446, 1440 (m, -CH bend, aliph. CH_2 and CH_3), 1393, 1370 (m, -CH bend, *tert*-Butyl group), 1381, 1366 (s, C-H bend, isopropyl group), 1208 (s, C-O str, ester), 934, 922 (w, CH_3 rocking, *tert*-Butyl and isopropyl group), 811, 805

(w, C-C str, aliph.), 490-485 (m, C-C bend, aliph.).

^{1}H NMR ($CDCl_3$, 300 MHz) : δ 7.59 (1H, br. s, -NH), 7.42 (1H, br. s, -NH), 7.05 (1H, br. s, -NH), 6.43 (1H, br. s, -NH), 4.25-4.15 (3H, m, α-protons of Leu[1], Val and Ile), 3.60 (3H, s, OCH_3), 3.56-3.50 (1H, m, α-H of Leu[2]), 2.10-1.95 (1H, m, β-H of Ile), 1.89-1.57 (5H, m, β-protons of Val and Leu[1], γ-protons of Ile), 1.55 (9H, s, *tert*-Butyl group), 1.52-1.20 (4H, m, γ-protons of Leu[1], Leu[2] and β-protons of Leu[2]), 1.06-1.04 (6H, d, J = 6.2 Hz, γ-protons of Val), 1.03-1.01 (3H, d, J = 6.3 Hz, γ'-protons of Ile), 1.00-0.92 (15H, m, δ-protons of Leu[1], Ile and Leu[2]) ppm. [Found: C, 61.05; H, 9.52; N, 9.80; $C_{29}H_{54}N_4O_7$ requires C, 61.03; H, 9.54; N, 9.82].

Cyclo (valyl-leucyl-isoleucyl-leucyl) (7): (Brownish crystals, m.p. 199-200°C, $[\alpha]_D$ -114.7°, yield 5.46 g, 80.2%)

IR ν_{max}, cm^{-1} (KBr) : 3320, 3304 (s, -NH str, amide), 2965-2932 (m, -CH str, asym, aliph. CH_3 and CH_2), 2876, 2869, 2850 (m, -CH str, sym, aliph. CH_3 and CH_2), 2897, 2885 (m, -CH str, >CH-), 1645-1634 (s, -C=O str, 2° amide), 1544-1531 (m, -NH bend, 2° amide), 1468, 1459, 1448 (m, -CH bend, aliph. CH_2 and CH_3), 1379, 1368 (s, C-H bend, isopropyl group), 920 (w, CH_3 rocking, isopropyl group), 810 (w, C-C str, aliph.), 488, 476 (m, C-C bend, aliph.).

^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.64 (1H, br. s, -NH), 9.21 (1H, br. s, -NH), 7.81 (1H, br. s, -NH), 7.64 (1H, br. s, -NH), 6.10-6.05 (1H, t, α-H of Val), 5.94-5.85 (2H, m, α-protons of Leu[2] and Leu[1]), 5.30-5.25 (1H, t, α-H of Ile), 1.90-1.31 (8H, m, β-protons of Val, Leu[1], Leu[2], Ile and γ-protons of Ile), 1.16-1.14 (6H, d, J = 6.2 Hz, γ-protons of Val), 1.01-0.95 (18H, m, δ-protons of Leu[1], Leu[2] and Ile, γ'-protons of Ile), 0.89-0.72 (2H, m, γ-protons of Leu[1] and Leu[2]) ppm.

^{13}C NMR ($CDCl_3$, 300 MHz): 173.7, 172.5 (C=O, Ile and Leu[1]), 170.4 (C=O, Val), 169.2 (C=O, Leu[2]), 60.4 (α-C, Val), 58.5 (α-C, Ile), 55.8 (α-C's, Leu[1] and Leu[2]), 44.7 (β-C's, Leu[1] and Leu[2]), 35.3 (β-C, Ile), 29.9 (β-C, Val), 27.3 (γ-C's, Leu[1] and Leu[2]), 24.7 (γ-C, Ile), 21.4 (δ-C's, Leu[1] and Leu[2]), 18.6 (γ-C's, Val), 17.2 (γ'-C, Ile), 10.4 (δ-C, Ile) ppm.

FAB MS : m/z 439 $(M + 1)^+$, 411 $(439\text{-}CO)^+$, 326 $(Val\text{-}Leu\text{-}Ile)^+$, 298 $(326\text{-}CO)^+$, 213 $(Val\text{-}Leu)^+$, 185 $(213\text{-}CO)^+$, 100 $(Val)^+$, 72 $(100\text{-}CO)^+$. [Found: C, 62.96; H, 9.68; N, 12.74; $C_{23}H_{42}N_4O_4$ requires C, 62.98; H, 9.65; N, 12.77].

RESULTS AND DISCUSSION

Synthesis of halolitoralin B was carried out successfully with good yields and its structure was confirmed by elemental as well as spectral analysis. IR spectra of synthesized linear and cyclic peptides showed characteristic amide I and amide II bands of the -CO-NH- moieties. ^{1}H NMR spectra of cyclized product clearly indicated the presence of all the

four amino acids and MASS spectra showed $(M + 1)^+$ peak at m/z 439 which was in consistent with the molecular formula $C_{23}H_{42}N_4O_4$. Synthesized peptide was found to exhibit potent anthelmintic activity against earthworms *Eudrilus sp*. and *M. konkanensis* at 100 mg level and potent antifungal activity against pathogenic fungi *C. albicans*. In addition, halolitoralin B was found to exhibit moderate antibacterial activity against pathogenic bacteria *E. coli* and *P. aeruginosa* at 50 µg/mL level. But gram positive bacteria were found to be resistant. On passing toxicity tests, this compound may prove good candidate for clinical studies and can be new anthelmintic and antifungal drug of tomorrow.

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