Natural Product Sciences
20(3) : 202-205 (2014)

Antimicrobial Constituents from the *Bacillus megaterium* LC Isolated from Marine Sponge *Haliclona oculata*

Pham Viet Cuong[1,2,*], Nguyen Thi Kim Cuc[1], Vu Thi Quyen[1], Pham Thanh Binh[1], Phan Van Kiem[1], Nguyen Hoai Nam[1], and Nguyen Tien Dat[1,*]

[1]*Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam*
[2]*Mientrung Institute of Scientific Research, VAST, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam*

Abstract – Three compounds including 7,7-bis(3-indolyl)-*p*-cresol (**1**), cyclo-(*S*-Pro-*R*-Leu) (**2**) and cyclo-(*S*-Pro-*R*-Val) (**3**) were isolated from the strain of *Bacillus megaterium* LC derived from the marine sponge *Haliclona oculata*. All the isolated compounds showed antimicrobial activity at MIC values ranging from 0.005 to 5 μg/mL against Gram-negative bacteria *Vibrio vulnificus* and *V. parahaemolyticus,* gram-positive bacteria *Bacillus cereus* and *Micrococcus luteus*, and the dermatophyte *Trichophyton mentagrophytes*. The results suggested that these compounds might have potential to be developed as agents treating dermatosis and controlling vibriosis in aquaculture.

Keywords – *Bacillus megaterium*, *Haliclona oculata*, sponge-associated bacteria, antimicrobial

Introduction

Marine sponges of the genus *Haliclona* have been known to display broad spectrum biological activities. However, very few studies on the chemical compositions and biological activities of *Haliclona oculata* have been reported except for several sterols and antifilarial effect.[1-3] Sponge-associated bacterial community has shown great potential as a source of antibiotics. Recently, Phelan et al. identified lantibiotic subtilomycin from *Bacillus subtilis strain* isolated from sponge *Haliclona simulans*.[4] Several fungi isolated from *Haliclona simulans* showed strong antimicrobial inhibition against *Escherichia coli*, *Bacillus* sp., *Staphylococcus aureus*, and *Candida glabrata*.[5] In our search for antibacterial agents from marine organisms, the strain of *Bacillus megaterium* LC isolated from *H. oculata* was found to exhibit strong antimicrobial activity. A chemical investigation of the cultured *Bacillus megaterium* LC strains led to the isolation of three antimicrobial compounds **1** - **3** (Fig. 1).

*Author for correspondence
Pham Viet Cuong, Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam
Tel: +84-4-37568261; E-mail: cuongwg@yahoo.com

Nguyen Tien Dat, Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam
Tel: +84-4-37917053; E-mail: ngtiend@imbc.vast.vn

Experimental

General experimental procedures – Optical rotation values were recorded a JASCO P-2000 digital polarimeter (JASCO, Tokyo, Japan). NMR experiments were carried out on a Bruker AM500 FT-NMR spectrometer (Bruker, Rheinstetten, Germany) using tetramethylsilane (TMS) as internal standard. The MS data were recorded on an

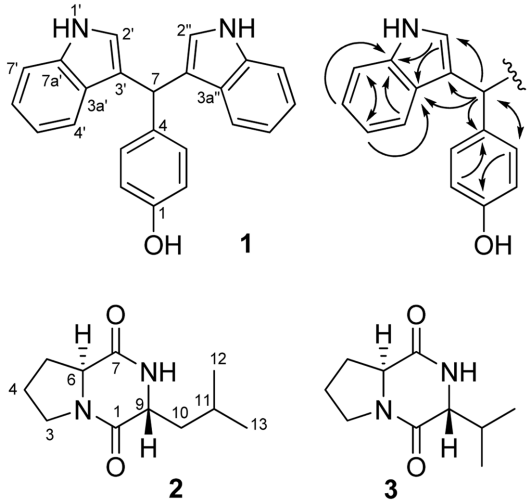

Fig. 1. The structures of **1** - **3** and key HMBC correlations of **1** (→).

Agilent 1260 Series Single Quadrupole LC/MS Systems (Agilent Technology, USA).

Biological material – The sample of *Haliclona oculata* was collected in Son Cha peninsula, Da Nang city, Vietnam and identified by Prof. Do Cong Thung, Institute of Marine Resources and Environment (VAST). A voucher specimen was deposited at the Institute of Marine Biochemistry, VAST.

A sponge specimen after washing by artificial sea water was thoroughly grounded in porcelain mortar with artificial sea water. Spread 100 µl of grounded solution on Marine agar 2216. Incubation at appropriate temperature and checking for bacterial growth after 24 hours. The obtained strain *Bacillus megaterium* LC was identified by using 16S rRNA gene sequencing method.[6] The universal primers including forward primer, 5'- AGA GTT TGA TCA TGG CTC A-3', and reverse primer, 5' - AAG GAG GTG ATC CAG CC-3', were used for amplifying nearly full length of 16S rRNA gene sequence (about 1500 bp.). The obtained sequence was analyzed by comparing with bacterial 16S rRNA sequences in GenBank by Blastn, which showed 100% similarity with *Bacillus megaterium* UF07 (GenBank Accession No. KF717520.1).

Culture, extraction and isolation – *B. megaterium* LC3CS2 strain was cultured in 250 ml flasks at 30 ºC for 24 hours with shaking at 150 rpm. Fermentation was carried out in 100 L fermenter with 50 L medium 2216 and 10% bacterial inoculum at 30 ºC for 52 hours. Neutral pH was maintained automatically by NaOH or HCl 1N. The obtained culture broth (50 L) was extracted with ethyl acetate (25 L × 3 times). The combined organic solutions were then decanted, filtered and concentrated under reduced pressure to yield 5.2 g of crude extract which was chromatographed on a silica gel column using a gradient of 1 - 100% acetone in hexane to afford nine fractions F1-9. Compound **3** (49.8 mg) was purified from F9 on a reverse phase C18 column eluted by methanol-water 1 : 1 (v/v). Similar chromatographic condition applied for F8 to obtain **2** (117.8 mg) and subfractions F8.1-2. F8.1 was then filtered through a silica gel column using chloroform-methanol 30 : 1 (v/v) to give compound **1** (6.2 mg).

7,7-Bis(3-indolyl)-*p*-cresol (**1**) – Yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.75 (s, NH), 7.32 (2H, d, J = 8.0 Hz, H-7', 7"), 7.25 (2H, d, J = 8.0 Hz, H-4', 4"), 7.12 (2H, d, J = 8.5 Hz, H-3, 5), 7.01 (2H, t, J = 8.0 Hz, H-6', 6"), 6.84 (2H, t, J = 8.0 Hz, H-5', 5"), 6.76 (2H, d, J = 2.0 Hz, H-2', 2"), 6.64 (2H, d, J = 8.5 Hz, H-2, 6), 5.69 (1H, s, H-7); ^{13}C NMR (125 MHz, DMSO-d_6): δ 155.1 (C-1), 114.7 (C-2, 6), 129.0 (C-3, 5), 135.2 (C-4), 38.9 (C-7), 123.3 (C-2', 2"), 118.6 (C-3', 3"), 126.8 (C-3a', 3a"), 119.1 (C-4', 4"), 118.0 (C-5', 5"), 120.7 (C-7', 7"), 136.5 (C-7a', 7a")); ESI-MS (positive) *m/z*: 338.4 $[M + H]^+$.

Cyclo-(*S*-Pro-*R*-Leu) (**2**) – Colorless powder. $[\alpha]_D^{24}$: –13.7 (*c* 0.1, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 4.28 (1H, t, J = 6.5 Hz, H-6), 4.14 (1H, m, H-9), 3.53 (2H, m, H-3), 2.33 (1H, m, H-5b), 2.05 (1H, m, H-5a), 2.03 (1H, m, H-4b), 1.96 (1H, m, H-4a), 1.94 (1H, m, H-10b), 1.91 (1H, m, H-11), 1.55 (1H, m, H-10a), 0.98 (3H, br d, J = 6.5 Hz, H-12), 0.97 (3H, br d, J = 6.5 Hz, H-13); ^{13}C NMR (125 MHz, CD_3OD): δ 168.9 (C-1), 46.4 (C-3), 23.6 (C-4), 29.0 (C-5), 60.2 (C-6), 172.7 (C-7), 54.6 (C-9), 39.4 (C-10), 25.7 (C-11), 23.2 (C-12), 22.2 (C-13); APCI-MS (positive) *m/z*: 210.6 $[M + H]^+$.

Cyclo-(*S*-Pro-*R*-Val) (**3**) – Colorless powder. $[\alpha]_D^{24}$: –16.8 (*c* 0.1, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 4.22 (3H, m, H-6), 4.05 (1H, d, J = 2.0 Hz, H-9), 3.58 (1H, m, H-3b), 3.53 (1H, m, H-3a), 2.50 (1H, m, H-10), 2.35-2.32 (1H, m, H-5a), 2.12-2.01 (1H, m, H-5b), 2.02-1.98 (1H, m, H-4a), 1.97-1.92 (1H, m, H-4b), 1.12 (3H, d, J = 7.5 Hz, H-12), 0.96 (1H, d, J = 7.5 Hz, H-11); ^{13}C NMR (125 MHz, CD_3OD): δ (170.2 (C-1), 45.0 (C-3), 22.2 (C-4), 28.4 (C-5), 60.3 (C-6), 164.9 (C-7), 58.7 (C-9), 28.4 (C-10), 19.0 (C-11), 15.9 (C-12); APCI-MS (positive) *m/z*: 196.9 $[M + H]^+$.

Antimicrobial assay – The antimicrobial activity were tested on Gram-negative bacteria *V. vulnificus* and *V. parahaemolyticus,* Gram-positive bacteria *B. cereus* and *M. luteus*, and the dermatophyte *T. mentagrophytes*. Briefly, 100 µL of new bacterial suspension were spread over solidified agar plates (LB medium for *M. luteus* and *B. cereus*; Sabouraud medium for *T. mentagrophytes* and TCBS agar for *Vibrio* sp.) with the help of a sterilized spreader. Five wells of 8 mm diameter were made in each agar plate by sterile cork borer. The wells were then filled with 100 µL of the respective test compound prepared in DMSO at different concentrations and allowed to diffuse at room temperature for 30 min in aseptic condition. The plates were incubated at 30 ºC for 24 hours (for bacteria) and 3 days for *T. mentagrophyte*. The MIC values were interpreted as the lowest concentration of the compounds, which showed clear inhibition zone.[7]

Results and discussion

Compound **1** was obtained as a yellow solid and its ESI-MS spectrum revealed the peak at *m/z* 339.1 $[M + H]^+$.The ^{1}H NMR spectrum of **1** showed an aromatic AA'BB' spin system at δ_H 7.12 and 6.64 (each 2H, d, J = 8.0 Hz), an aromatic ABMX spin at δ_H 7.32 and 7.25

Table 1. Antimicrobial activity (MIC values in μg/mL) of the isolated compounds **1** - **3**

Bacteria	1	2	3	Positive controls
V. parahaemolyticus	5.0	0.5	0.05	0.05 (Ampicillin)
V. vulnificus	0.05	5.0	5.0	0.05 (Ampicillin)
M. luteus	0.005	ND	5.0	1.0 (Ampicillin)
B. cereus	0.5	0.05	ND	0.2 (Kanamycin)
T. mentagrophytes	0.05	0.05	0.05	25 (Miconazole)

(each 2H, d, J = 8.5 Hz), and 7.01 and 6.84 (each 2H, t, J = 8.5 Hz). A downfield shifted proton signal at δ_H 10.75 was also recognized. The ^{13}C NMR and HSQC spectra of **1** indicated the presence of eight methine and five quaternary carbon signals. The proton signal at δ_H 10.75 was not attached to any carbon atom confirming the presence of the OH or NH group. Extensive analysis of the HMBC spectrum of **1** allowed to identify two 3-indolyl units which symmetrically attached to the *p*-cresol moiety (Fig. 1). Thus compound **1** was identified to be 7,7-bis(3-indolyl)-*p*-cresol, a compound previously isolated from *Vibrio* sp. bacteria derived from the marine sponge *Hyatella* sp.[8] Two proline-containing dipeptides, cyclo-(*S*-Pro-*R*-Leu) (**2**) and cyclo-(*S*-Pro-*R*-Val) (**3**) were identified by comparing their NMR data and optical rotations with literatures.[9]

The isolated compounds were tested for their antimicrobial activity against Gram-negative bacteria *Vibrio vulnificus* and *V. parahaemolyticus,* Gram-positive bacteria *Bacillus cereus* and *Micrococcus luteus*, and the dermatophyte *Trichophyton mentagrophytes*. As shown in Table 1, compound **1** showed inhibitory effect to all tested microorganisms, while **2** and **3** was not active against *M. luteus* and *B. cereus*, respectively. Several species of *Vibrio* are pathogens to human and aquatic animals. *Vibrio vulnificus* and *V. parahaemolyticus* have been known to cause severe food poisoning, wound infections as well as vibriosis in aquaculture. All compounds **1** - **3** inhibited the growth of these two bacteria with the MIC values ranging from 0.05 to 5.0 μg/mL. The fungus *T. mentagrophytes* belongs to dermatophyte group that causes a variety of cutaneous infections in humans and animals. Interestingly, our result showed that *T. mentagrophytes* was inhibited by all tested compounds at minimum concentration of 0.05 μg/mL, 500-fold lower than that of miconazole. Thus the obtained results suggested that the compounds **1** - **3** might be effective in dermatosis treatment and vibriosis control in aquaculture.

7,7-Bis(3-indolyl)-*p*-cresol (**1**) was previously isolated from the sponge associated bacteria *Vibrio* sp. and only showed weak inhibitory effect against *Bacillus subtilis* with a MIC of 70 μg/mL but did not affect the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and *Aspergillus niger*. In the present study, we reported potent antimicrobial activity of **1** against other microorganisms. Interestingly, synthetic product of **1** (named DIM-C-pPhOH) exhibited promising anticancer property. It deactivated orphan nuclear receptor TR3, induced apoptosis and also inhibited growth and induced apoptosis in lung cancer cells and lung tumors in murine orthotopic and metastatic models, and this was accompanied by decreased expression of survivin and inhibition of mTORC1 signaling.[10,11] Cyclicdipeptides have been widely isolated from marine organism and display broad spectrum of antibacterial activity.[12] Cyclo-(*S*-Pro-*R*-Leu) (**2**) exhibited good antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa*.[13] It prevented the growth of fungi *Aspergillus flavus* and *Aspergillus niger* on stored cereal grains. Cyclo-(*S*-Pro-*R*-Val) (**3**) has showed to be a specific inhibitor of β-glucosidase.[14] Both compounds also showed strong antibiotic effect against *Vibrio anguillarum*.[15] Consistently, cyclo-(*S*-Pro-*R*-Leu) (**2**) and cyclo-(*S*-Pro-*R*-Val) (**3**) produced from *Bacillus megaterium* isolated from marine sponge *H. oculata* significant inhibited the growth of different microorganisms.

Acknowledgments

This work is supported by a grant from the Vietnam Academy of Science and Technology (VAST06.04/13-14). We thank the Institute of Chemistry (VAST) for the NMR measurements.

References

(1) Findlay, J. A.; Patil, A. D. *Can. J. Chem*. **1985**, *63*, 2406-2410.
(2) Yu, S.; Deng, Z.; Proksch, P.; Lin, W. *J. Nat. Prod*. **2006**, *69*, 1330-1334.
(3) Gupta, J.; Misra, S.; Mishra, S. K.; Srivastava, S.; Srivastava, M. N.; Lakshmi, V.; Misra-Bhattacharya, S. *Exp. Parasitol*. **2012**, *130*, 449-455.
(4) Phelan, R. W.; Barret, M.; Cotter, P. D.; O'Connor, P. M.; Chen, R.; Morrissey, J. P.; Dobson, A. D.; O'Gara, F.; Barbosa, T. M. *Mar. Drugs* **2013**, *11*, 1878-1898.
(5) Baker, P. W.; Kennedy, J.; Dobson, A. D.; Marchesi, J. R. *Mar. Biotechnol*. **2009**, *11*, 540-547.
(6) Theves, C.; Senescau, A.; Vanin, S.; Keyser, C.; Ricaut, F. X.; Alekseev, A. N.; Dabernat, H.; Ludes, B.; Fabre, R.; Crubezy, E. *PLoS ONE* **2011**, *6*, e21733.
(7) Naz, S.; Jabeen, S.; Ilyas, S.; Manzoor, F.; Aslam, F.; Ali, A. *Pak. J. Bot*. **2010**, *42*, 455-462.
(8) Oclarit, J. M.; Ohta, S.; Kamimura, K.; Yamaoka, Y.; Shimizu, T.; Ikegami, S. *Nat. Prod. Lett*. **1994**, *4*, 309-312.

(9) Adamczeski, M.; Reed, A. R.; Crews, P. *J. Nat. Prod*. **1995***, 58*, 201-208.
(10) Lee, S. O.; Abdelrahim, M.; Yoon, K.; Chintharlapalli, S.; Papineni, S.; Kim, K.; Wang, H.; Safe, S. *Cancer Res*. **2010**, *70*, 6824-6836.
(11) Lee, S. O.; Andey, T.; Jin, U. H.; Kim, K.; Singh, M.; Safe, S. *Oncogene* **2012**, *31*, 3265-3276.
(12) Huang, R.; Zhou, X.; Xu, T.; Yang, X.; Liu, Y. *Chem. Biodivers*. **2010**, *7*, 2809-2829.
(13) Nishanth Kumar, S.; Mohandas, C.; Siji, J. V.; Rajasekharan, K. N.; Nambisan, B. *J. Appl. Microbiol*. **2012**, *113*, 914-924.
(14) Kwon, O. S.; Park, S. H.; Yun, B. S.; Pyun, Y. R.; Kim, C. J. *J. Antibiot*. **2001**, *54*, 179-181.
(15) Fdhila, F.; Vazquez, V.; Sanchez, J. L.; Riguera, R. *J. Nat. Prod*. **2003**, *66*, 1299-1301.

Received April 2, 2014
Revised July 4, 2014
Accepted July 4, 2014