

# Terrein and Butyrolacton VII, Antibiotically Active Compounds from Symbiotic Fungus *Aspergillus Terreus* Isolated from Ascidian *Didemnum Molle*





Robert A Bara\*<sup>1</sup>, Calvyn Sondak<sup>1</sup>, Fitje Losung<sup>1</sup>, Remmy E Mangindaan<sup>1</sup>, Jimmy Posangi<sup>2</sup>, Stefan Kehraus<sup>3</sup>, Alexander Bogdanov<sup>3</sup>, Ekaterina Egorova<sup>3</sup> and Gabrielle Koenig<sup>3</sup>

<sup>1</sup>Faculty of Fisheries and Marine Science, Sam Ratulangi University, Indonesia

<sup>2</sup>Faculty of Medicine, Sam Ratulangi University Jl. Kampus Bahu, Indonesia

<sup>3</sup>Institute for Pharmaceutical Biology, University of Bonn, Germany

Received:  September 17, 2018; Published:  October 05, 2018

\*Corresponding author: Robert A Bara, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Indonesia

## Abstract

We performed the study of secondary metabolites from endophytic fungus *Aspergillus terreus*. The fungus was isolated from the ascidian *Didemnum molle* which grow in the Malalayang waters, North Sulawesi. Chemical investigation of extracts from Biomaltagar found compounds from lactone groups namely Terrein and Butyrolacton VII. The structure of the above compounds was characterized based on spectroscopic data including NMR, UV, IR and MS. Terrein and Butyrolacton VII showed a narrow spectrum antibacterial activity against Gram positive bacteria *Bacillus megaterium* DSM32T. So far there was no reports about the antibacterial activity of Butyrolacton VII.

## Introduction

The problem in controlling microorganism is increased spread of drug-resistant microorganisms. The resistance can be due to routinely use of certain antibiotics. The discovery of newly antibiotic is the essential step in order to keep the sustainability of effective antimicrobial medicine. Secondary metabolites proved to be interesting for drug development. In their natural habitat, these compounds are important for the marine organism itself. They play a crucial ecological role, shielding against potential invaders, predators, other competitors or prevent diseases caused by microorganisms. Accumulation of toxic secondary metabolites is the best strategy to fight against potential predators. These compounds show pronounced pharmacological activities and are interesting candidates for new drugs primarily in the area of cancer treatment and antibiotics.

Several natural products from the marine organisms show striking structural similarities to known metabolites of microbial origin, suggesting that microorganisms are at least involved in their biosynthesis or are in fact the true sources of these respective metabolites or host-symbiont modification Proksch et al. [1]. For example, arenastatin compound derived from *Dysidea* sponge share similarity structure to chryptophycin-1 which is a cyanobacterial metabolite Kobayashi et al. [2,3]. Recently, several new bioactive products were isolated and identified with unique

core structures and potent biological activities Bara et al. [4-9]. As a part of our ongoing search for new bioactive secondary metabolites from terrestrial to marine endosymbiotic fungi, we investigated *Aspergillus terreus* that isolated from ascidians *Didemnum molle* growing in Bunaken National Park, Manado, Indonesia. The present study represents the first investigation of natural products produced by *Aspergillus terreus* isolated from marine ascidians *D. molle*.

## Experimental Sections

### Material

The Ascidians *Didemnum molle* (Figure 1) was collected from Pangalisan Water at Bunaken National Park Manado, Indonesia (1°35'58.11"N; 124°46'59.56"E) (Figure 2). The symbiotic fungus was isolated from inner tissue of related organism following the procedure describes below. A section of a part of *D. molle* was immersed in 70% of ethanol for 30 seconds and sliced under sterile condition. The section then was positioned on the surface of malt agar in a petridish. Chloramphenicol 200mg/L was added to the medium to avoid bacterial contamination. Negative control was applied to cross check the growth of epibiont fungi from external parts of the slices. The fungus started to grow from the section after 3-14 days of incubation at room temperature (Figure 3).



Figure 1: Sample *Didemnum molle*.

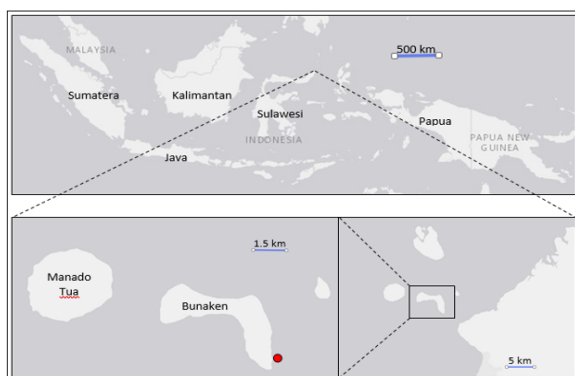


Figure 2: Sampling site of Ascidians *Didemnum molle*, Bunaken National Park, Manado, Indonesia. Red dot = sampling point.



Figure 3: Fungal strain *Aspergillus terreus* in malt agar.

### DNA Extraction

A piece of fungal mycelium (0.5cm<sup>2</sup>) slashed from the agar dish and lyophilized in an Eppendorf (2mL) followed by storing in thermafrost freezer (-80°C) and freeze-dryer for 2 hours and overnight respectively. Total DNA extracted with the Mo Bio Ultraclean DNA extraction kit (Qiagen, Hilden, Germany) from the samples. Briefly, 0.5ml of sodium pyrophosphate solution was added to the sample and tungsten carbide beads (Qiagen, Hilden, Germany) were added to the microtubes and cells were lysed by beating for 60s in a cell disrupter (Hybaid Ribolyser, Hybaid, Middlesex, United Kingdom). DNA was purified according to the protocol provided by manufacturer (Qiagen, Hilden, Germany).

### DNA Amplification and Sequencing

DNA amplification by PCR was then performed using Hot Star Taq Master Mix Taq polymerase (Qiagen) and the primer pair ITS1(F) and ITS4(R) ordered from Invitrogen White [10] in an iCycler thermocycler. In general, thermal condition followed by 15

mins at 95°C, followed by 30 cycles of 95°C for 1min, 30s at the annealing temperature (56°C) and 72°C for 1min followed with final extension (72°C; 10min). The specificity of amplicons was confirmed by running the samples on 1.2% agarose gel. The length of the amplicon was confirmed by reported articles and followed by purification of particular band. DNA sequencing is performed in order to identify the fungus strain based on its genomic content. The purified amplicon was submitted for sequencing to pt. Genetika, Jakarta, followed by BLAST search against EMBL database.

### Antibacterial Assays

For antibacterial assays, all isolated compounds were tested their antibiotic property towards *Bacillus megaterium* DSM 32Tand *Escherichia coli* DSM 498. Both isolates were originally from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. The strains were refresh grown in 10 ml slope nutrient agar in tube at 37°C temperature for overnight. The cultures were harvested and measured their density. The initial OD600 was set to 1 to reach concentration of 8x10<sup>8</sup> cells/ml. The cultures were diluted until 8x10<sup>6</sup> cells/ml prior used, 1 ml of the bacterial culture was subjected and seeded into the Luria Betani Agar (LB Agar) and after solidification, 20µl of the ethanolic extracts of each sample were spotted onto the plates. Chloramphenicol (1mg/ml) and 96% ethanol served as positive and negative controls, respectively. Plates were incubated at 37°C for 18h and checked for inhibition zones.

### Result and Discussion



Figure 4: Large scale cultures of *Aspergillus terreus*.

The fungus (strain designation N.18 Rhi) is kept in the authors' laboratory (G.K.). (Figure 3) Furthermore, fungal culture was identified according to a molecular biological protocol by DNA amplification and sequencing of the ITS region as described previously. BLAST search revealed 98 % similarity to *Aspergillus terreus* (MG669192.1). The sequence data has been submitted to GenBank with accession number MH910675. The related fungus was static cultivated in large scale following method described previously (Figure 4). Mycelia in rice and malt agar cultures of *A. terreus* were homogenized using an automatic shaker blender and exhaustively extracted with EtOAc. The crude extract of *A. terreus* was subjected to liquid-liquid fractionation using n-hexane: methanol and water to obtain n-hexane, methanol and water fractions. The methanol fractions were further injected into column chromatography using RP Grace instrumenttm chromatography system and continued by injected into HPLC to obtain compounds Terrein (1) and Butyrolacton VII (2) and furthermore the two non-

saturated fatty acids e.g. C-14 Tetradec-4-enoic acid (3) and C-18 Octadec-4-enoic acid (4) (Figure 5) were purified from n-hexane fraction subjected to NP Grace instrument flash chromatography.

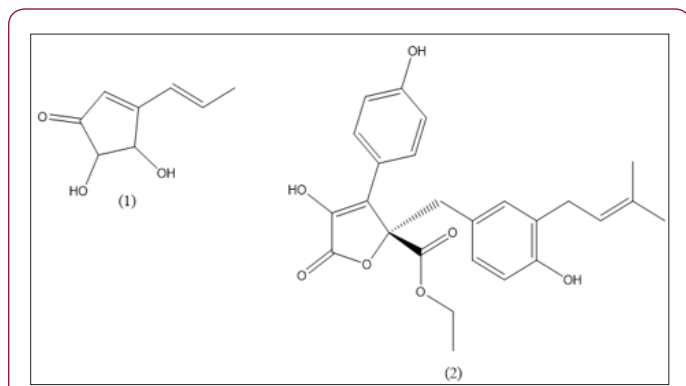


Figure 5: Isolated compound from symbiotic fungus *A. terreus*.

Compounds (1) and (2) were identified on the basis of the MS and NMR data as Terrein, Butyrolacton VII [11-13] tetradec-4-enoic acid and octadec-4-enoic acid were previously isolated from *A. terreus*. The positive optical rotations of 1<sub>D</sub> 27 +54 (c<sub>0.08</sub>, MeOH) was consistent with the literature data. <sup>1</sup>H NMR of Compound (1) (300 MHz, MeOD d-4), showed a doublet which integrated to three protons at δ 1.89 (d, J = 6.4, 3H), evidenced the presence of a methyl group. Appearance of one broad singlet at δ 4.64 (s, 1H) and one doublet at δ 4.05 (d, J = 2.4, 1H) integrated to one proton each showed the presence of two hydroxyl hydrogens. One singlet at δ 5.96 (s, 1H), showed the presence of methylene protons at C1 carbon (adjacent to carbonyl group), one doublet at δ 6.39 (d, J = 15.8, 1H) and one doublet of quintet at δ 6.79 (dq, J = 13.6, 6.7, 1H) with integration of one proton each, which corresponds to the two double bond-CH protons adjacent to methyl group. All above details of proton environment can be collectively analyzed by (Figure 6A). Furthermore, <sup>13</sup>C NMR (75 MHz, Acetone) δ 205.7, 170.8, 142.0, 126.5, 126.0, 82.4, 78.1, 19.6 ppm (Figure 6B).

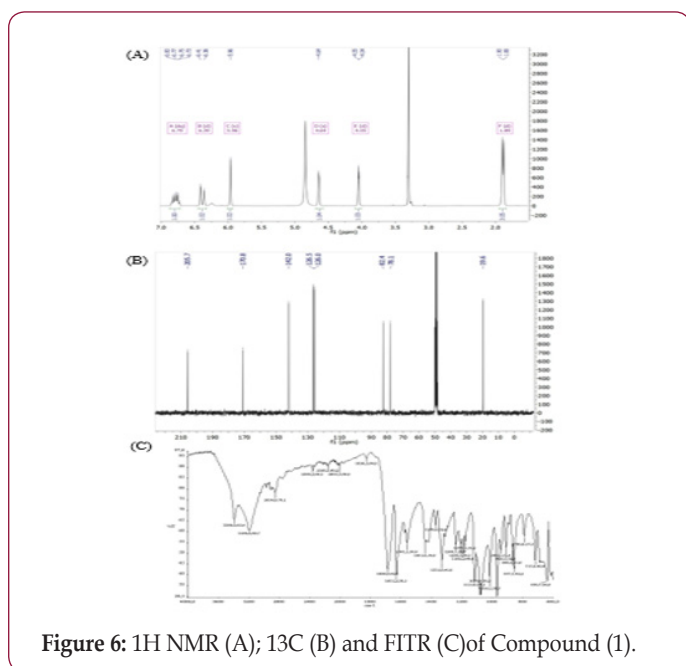


Figure 6: <sup>1</sup>H NMR (A); <sup>13</sup>C (B) and FTIR (C) of Compound (1).

Comparison of <sup>13</sup>C NMR shows perfectly match with available literature [11,13,14]. IR (KBr) max: 3,390; 3,194; 2,854; 1,686 (C=O); 1,631; 1,561; 1,441; 1,331, 1,115; 1,016 cm<sup>-1</sup> (Figure 6C). Compound (1) was identified as 4, 5-Dihydroxy-3-(1-propenyl)-2-cyclopenten-1-one and the trade name of this compound is Terrein. The isolated strain *A. terreus* itself is commonly endophytic in nature and frequently isolated from terrestrial sources (He et al. 2004) and marine environments Damare et al. [15]. In addition, the compound (1) that has been isolated from various strains of fungi such as *Microspora* *Grove* *Penicillium phoma* *Dunn*. *A. terreus* produces a variety of natural products, most notably the medically important HMG-CoA reductase inhibitor lovastatin Alberts et al. [16]. Additionally, a range of metabolites has been extracted from *A. terreus* cultures, among them terrein, citreoveridin, and butyrolactones [17,12,13]. <sup>1</sup>H NMR (300 MHz, MeOD) of Compound (2) showed a triplet resonating at δ 1.20 ppm (t, J = 7.1, 3H), two singlets resonating at δ 1.56 (s, 3H) and δ 1.65 (s, 3H) ppm which integrated to three protons, evidenced the presence of three methyl groups. Appearance of three methylene's resonating at δ 3.06 (d, J = 7.0, 2H), δ 3.41 (d, J = 3.6, 2H) and δ 4.24 (q, J = 7.1, 2H), one aliphatic methine resonating at δ 5.05 (t, J = 7.3, 1H) and seven aromatic with six overlapping methines resonating at δ 6.40 (d, J = 1.6, 1H), δ 6.51 (dt, J = 14.0, 5.0, 2H; overlapping), δ 6.86 (d, J = 8.8, 2H; overlapping), and δ 7.59 ppm (d, J = 8.8, 2H; overlapping).

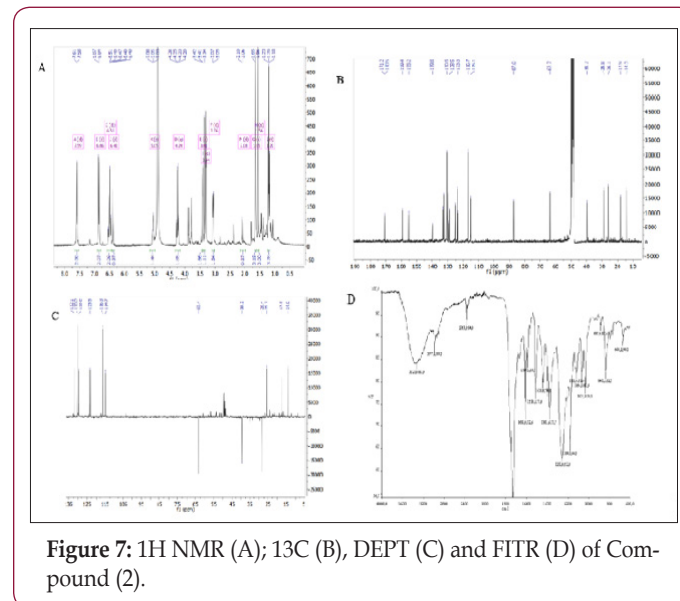


Figure 7: <sup>1</sup>H NMR (A); <sup>13</sup>C (B), DEPT (C) and FTIR (D) of Compound (2).

Analyzing of the coupling constant shows that methyl group resonating at δ 1.20 ppm is adjacent to methylene group resonating at δ 4.24 ppm that binds to ester group. The aliphatic methine resonating at δ 3.06 ppm is adjacent to methylene group resonating at δ 5.05 ppm (Figure 7A). <sup>13</sup>C NMR (75 MHz, Acetone) shows compound (2) has 23 atom carbons resonating at δ 171.2, 170.5, 159.4, 155.2, 139.7, 133.1, 132.5, 130.5, 129.9, 129.4, 128.5, 125.2, 123.7, 123.3, 116.7, 115.1, 87.0, 63.7, 39.7, 28.8, 26.1, 17.9, 14.3 ppm (Figure 7B). Comparison of <sup>13</sup>C NMR shows perfectly match with available literatures of Butyrolacton VII [15,12]. DEPT data confirm that the Compound (2) has three methyls, three methylene's and eight methines (Figure 7C). The IR (KBr) max: 3323; 2977; 2363; 1702; 1609 (C=O); 1586; 1518; 1438; 1383 (C-



O); 1252 (CH<sub>3</sub>-CH<sub>2</sub>-O-); 1180; 1115; 1064; 1035; 880; 840; 668 cm<sup>-1</sup>. (Figure 7D). Marine resources are known for the potential novel bioactive compounds. Since past decades, antimicrobial properties have been reported from different marine resources such as marine plants, animals and microbes. The marine environment is a remarkable source for discovery of potential antibiotics and bioactive compounds to cure several human diseases.

Especially, marine based fungi are considered as a drug source in the marine environment and being reported as a good source of antimicrobial lactones, anthracenes and peptides. The antibacterial test of isolated compounds shows compound (1; terrein) and (2; butyrolacton VII) have a narrow spectrum antibacterial activity against Gram positive bacteria *Bacillus megaterium* DSM32T. Terrein has been known as antibacterial and antifungal activities [18,19]. However, although Butyrolacton VII has been isolated as an *Aspergillus* natural product [12,20], so far there were no reports about the antibacterial activity of the compound, hence we reported its narrow antibacterial activity against Gram positive *B. megaterium*. No activity array is recorded from isolated compounds against Gram negative *E. coli*. So far Gram-negative infection control is often become a major problem in modern medicine, this is due to the characteristics of the particular group e.g. their peptidoglycan layer which is dense and compact thereby inhibits internalization process for antibiotics to be able to affect the cellular mechanisms within bacteria. In addition, it has "efflux-pump mechanism", a compound removal mechanism that are not needed in their cellular biotransformation processes through their secretion system [21,22].

## Conclusion

- Terrein and Butyrolacton VII were isolated from symbiotic fungus *Aspergillus terreus* isolated from inner part of ascidians *Didemnum molle* growing in Pangalisan Water, Bunaken National Park, Manado Indonesia.
- Both isolated compounds show narrow antibacterial array against *Bacillus megaterium* DSM 32T. No activity was recorded against *Escherichia coli* DSM 498.
- For the first time we reported the antibacterial properties of butyrolacton VII in this paper

## References

- Proksch P, Edrada R, Ebel R (2002) Drugs from the seas - current status and microbiological implications. *Applied Microbiology and Biotechnology* 59(2): 125.
- Kobayash M, Aoki S, Ohyabu N, Kurosu M, Wang W, et al. (1994) Arenastatin A, a potent cytotoxic depsipeptide from the okinawan marine sponge *Dysidea arenaria*. *Tetrahedron Letters* 35(43): 7969-7972.
- Eibler S, Bogner T, Nahrwold M, Sewald N (2009) Efficient Synthesis of Cryptophycin-52 and Novel para-Alkoxyethyl Unit Analogues. *Chemistry-A European Journal* 15(42): 11273-11287.
- Bara R, Aly AH, Pretsch A, Wray V, Wang B, et al. (2013) Antibiotically active metabolites from *Talaromyces wortmannii*, an endophyte of *Aloe vera*. *The Journal of Antibiotics*.
- Bara R, Zerfass I, Aly AH, Goldbach Gecke H, Raghavan V, et al. (2013) Atropisomeric Dihydroanthracenones as Inhibitors of Multiresistant *Staphylococcus aureus*. *Journal of Medicinal Chemistry* 56(8): 3257-3272.
- Bara RA, Ramadhan FA, Posangi J (2016) Antibiotic Analysis of Endophytic Fungi from *Avicennia marina* Mangrove Growing in North Sulawesi Coastal Waters. *Prosiding 1<sup>st</sup> International Seminar on Tropical Aquatic Resources Science & Management, Manado (Poster)*.
- Cheney KL, White A, Mudianta IW, Winters AE, Quezada M, et al. (2016) Choose Your Weaponry: Selective Storage of a Single Toxic Compound, Latrunculin A, by Closely Related Nudibranch Molluscs. *PLoS ONE* 11(1): e0145134.
- Supaphon P, Phongpaichit S, Rukachaisirikul V, Sakayaroj J (2013) Antimicrobial Potential of Endophytic Fungi Derived from Three Seagrass Species: *Cymodocea serrulata*, *Halophila ovalis* and *Thalassia hemprichii*. *PLOS ONE* 8(8): e72520.
- Fisch KM, Hertzner C, B Ähringer N, Wuisan ZG, Schillo D, et al. (2017) The Potential of Indonesian Heterobranchs Found around Bunaken Island for the Production of Bioactive Compounds. *Marine Drugs* 15(12): 384.
- White TJ (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols : a Guide to Methods and Applications* 315-322.
- Barton DHR, Miller E (1955) The constitution and stereochemistry of terrein. *Journal of the Chemical Society (Resumed)*(0): 1028-1029.
- Haritakun R, Rachtawee P, Chanthaket R, Boonyuen N, Isaka M (2010) Butyrolactones from the Fungus *Aspergillus terreus* BCC 4651. *Chemical and Pharmaceutical Bulletin* 58(11): 1545-1548.
- Zaehle C, Gressler M, Shelest E, Geib E, Hertweck C, et al. (2014) Terrein Biosynthesis in *Aspergillus terreus* and Its Impact on Phytotoxicity. *Chemistry & Biology* 21(6): 719-731.
- Goutam J, Sharma G, Tiwari VK, Mishra A, Kharwar RN, et al. (2017) Isolation and Characterization of Terrein an Antimicrobial and Antitumor Compound from Endophytic Fungus *Aspergillus terreus* (JAS-2) Associated from *Achyranthus aspera* Varanasi, India. *Frontiers in Microbiology* 8(1334).
- Damare S, Raghukumar C, Raghukumar S (2006) Fungi in deep-sea sediments of the Central Indian Basin. *Deep Sea Research Part I: Oceanographic Research Papers* 53(1): 14-27.
- Alberts AW, Chen J, Kuron G, Hunt V, Huff J, et al. (1980) Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proceedings of the National Academy of Sciences* 77(7): 3957-3961.
- Frisvad JC, Filtenborg O, Samson RA, Stolk AC (1990) Chemotaxonomy of the genus *Talaromyces*. *Antonie van Leeuwenhoek* 57(3): 179-189.
- Miyamoto T, Sakamoto K, Arao K, Komori T, Higuchi R, et al. (1996) Dorisenones, cytotoxic spongian diterpenoids, from the nudibranch *Chromodoris obsoleta*. *Tetrahedron* 52(24): 8187-8198.
- San Martin A, Roviroso J, Vaca I, Vergara K, Acevedo L, et al. (2011) NEW BUTYROLACTONE FROM A MARINE-DERIVED FUNGUS *ASPERGILLUS* SP. *Journal of the Chilean Chemical Society* 56: 625-627.
- He J, Wijeratne EM K, Bashyal BP, Zhan J, Seliga CJ, et al. (2004) Cytotoxic and Other Metabolites of *Aspergillus* Inhabiting the Rhizosphere of Sonoran Desert Plants. *Journal of Natural Products* 67(12): 1985-1991.
- Poole K (2007) Efflux pumps as antimicrobial resistance mechanisms. *Annals of Medicine* 39(3): 162-176.
- Posangi J, Bara R (2014) Analisis Aktivitas Dari Jamur Endofit Yang Terdapat Dalam Tumbuhan Bakau *Avicennia Marina* di Tasik Ria Minahasa. *Jurnal Pesisir Dan Laut Tropis* 1(1): 30-38.

ISSN: 2574-1241

DOI: [10.26717/BJSTR.2018.09.001834](https://doi.org/10.26717/BJSTR.2018.09.001834)

Robert A Bara. Biomed J Sci & Tech Res



This work is licensed under Creative Commons Attribution 4.0 License

Submission Link: <https://biomedres.us/submit-manuscript.php>



#### Assets of Publishing with us

- Global archiving of articles
- Immediate, unrestricted online access
- Rigorous Peer Review Process
- Authors Retain Copyrights
- Unique DOI for all articles

<https://biomedres.us/>