ANTIMICROBIAL ACTIVITY OF NATURAL COMPOUNDS FROM SPONGE – DERIVED FUNGUS ASPERGILLUS FLOCCULOSUS 01NT.1.1.5

Phan Thi Hoai Trinh¹*, Tran Thi Thanh Van¹, Bui Minh Ly¹, Byeoung Kyu Choi², Hee Jae Shin², Jong Seok Lee³, Hyi Seung Lee³, Phi Quyet Tien³

¹Nhatrang Institute of Technology Research and Application, Vietnam Academy of Science and Technology
²Korea Institute of Ocean Science and Technology, Busan, Korea
³Institute of Biotechnology, Vietnam Academy of Science and Technology

To whom correspondence should be addressed. E-mail: phanhoaitrinh84@gmail.com

Received: 28.6.2018
Accepted: 20.12.2018

SUMMARY

The Aspergillus fungi have been an important source of natural products that are useful for exploration in medicine, agriculture and industry. In our continuous investigation to search for new antimicrobial agents from marine-derived fungi, one new phomaligol A2 (1), together with three known compounds, wasabidienone E (2), aspertetranone D (3) and mactanamide (4), were obtained from the EtOAc extract of the culture medium of the marine-derived fungus Aspergillus flocculosus (A. flocculosus) 01NT.1.1.5 isolated from the sponge Stylissa sp. at Nhatrang Bay, Vietnam. Their chemical structures were elucidated by analysis of 1D and 2D NMR and mass spectroscopic data, as well as by comparison of the corresponding data to those previously reported in the literature. Furthermore, the aim of this study was also to evaluate the antimicrobial activity of these compounds against pathogenic microbes including Escherichia coli (E. coli) ATCC 25922, Pseudomonas aeruginosa (P. aeruginosa) ATCC 27853, Staphylococcus aureus (S. aureus) ATCC 25923, Bacillus cereus (B. cereus) ATCC 11778, Streptococcus faecalis (S. faecalis) ATCC 19433, Listeria monocytogenes (L. monocytogenes) ATCC 19111, and Candida albicans (C. albicans) ATCC 10231. Among the compounds, 1-3 were inhibitory on the growth of the yeast C. albicans with minimum inhibitory concentration (MIC) value of 16 µg/mL, which was more potent than amoxicillin and cefotaxime (MIC > 256 µg/mL), antimicrobial drugs as positive references. Moreover, compounds 1-4 were also found to be active against other pathogens including P. aeruginosa and S. faecalis with MIC values of 16 µg/mL and 32 µg/mL, respectively. Compound 4 had no inhibitory activity against L. monocytogenes, whereas compounds 1-3 had ability to against this strain with MICs of 32 to 64 µg/mL. Four of tested compounds exhibited antibacterial activity against B. cereus and E. coli with MIC values of 64-128 µg/mL. This is the first report about these compounds with antimicrobial activity obtained from marine fungus A. flocculosus isolated in Vietnam.

Keywords: Aspergillus flocculosus, antimicrobial activity, aspertetranone D, mactanamide, phomaligol A2, wasabidienone E.

INTRODUCTION

Nowadays, in spite of the advance in human drugs, infectious diseases related to the emergence of pathogens, are still major issues in public healthy worldwide, especially in developing countries. The widespread of antimicrobial resistance microbes has been reported over the world that demands more effective antimicrobial compounds. Despite the impressive advance in producing antimicrobial substances by chemical and bio-engineered synthesis, nature particularly marine environment has still considered as the richest source for new antimicrobial compounds (Blunt et al., 2010). Novel antimicrobial compounds from marine microbes have been increasingly discovered in recent years (Du et al., 2014; Habbu et al., 2016; Handayani et al., 2015). So far, a great number of antimicrobial compounds have been found in a handful of the one million different microbial species (Brown et al., 2014). Natural metabolites from marine fungi are considered an important source for novel
antimicrobial compounds because of their abundant fungal species diversity, their rich secondary metabolites and the improvements in their genetic breeding and fermentation processes (Li et al., 2014; Du et al., 2014).

The *Aspergillus* genus has more than one hundred species, and belongs to the Ascomycota division, Deuteromycotina subdivision, Hyphomycetes class, Moniliales order, Moniliaceae family. The species are widely found in nature and diverse in marine ecosystems, are well known for producing antimicrobial and anticancer compounds, bio-surfactants, etc. (Li, 2010). Thus, the *Aspergillus* fungi have been an important source of natural products useful for exploration in medicine, agriculture and industry (Petersen et al., 2015).

As part of a continuing study to evaluate the drug potential of marine-derived fungi from Vietnam, we isolated and screened 100 fungal strains from various marine habitats at Nha Trang Bay for antimicrobial activity. Among them, the strain *Aspergillus flocculosus* (*A. flocculosus*) 01NT.1.1.5 was isolated from the sponge *Stylissa* sp. at Nha Trang Bay, Vietnam, in February 2016. The fungus was identified according to its gene sequence of 28S rDNA (GenBank accession number MG972941.1). A BLAST search results indicated that the sequence was similar 100% to the sequence of *A. flocculosus* NRRL 5224. The strain was named as *A. flocculosus* 01NT.1.1.5 and currently preserved in the Marine Microorganism Collection, Nhatrang Institute of Technology Research and Application (NITRA).

**MATERIALS AND METHODS**

**General experimental procedures**

1D and 2D spectroscopic data were recorded on a Varian Unity 500 NMR spectrometer (MCKinley, Sparta, NJ). ESI-MS data were obtained on a Shimadzu hybrid ion-trap time-of-flight mass spectrometer (Shimadzu, Kyoto, Japan). HPLC was conducted on a column 250 mm x 10 mm i.d., S-5 µm, 12 nm, YMC-Pack-ODS-A, with a PrimeLine Binary pump with RI-101 Shodex, RI detector (Shoko Scientific Co., Yokohama, Japan).

**Fungal material**

The fungus *A. flocculosus* 01NT.1.1.5 was originally isolated from the sponge *Stylissa* sp. at Nhatrang Bay, Vietnam, in February 2016. The fungus was identified according to its gene sequence of 28S rDNA (GenBank accession number MG972941.1). A BLAST search results indicated that the sequence was similar 100% to the sequence of *A. flocculosus* NRRL 5224. The strain was named as *A. flocculosus* 01NT.1.1.5 and currently preserved in the Marine Microorganism Collection, Nhatrang Institute of Technology Research and Application (NITRA).

![Figure 1](image1.png)  
*Figure 1.* Sponge *Stylissa* sp. (A) and fungus *A. flocculosus* 01NT.1.1.5 (B).
Fermentation, extraction and isolation

The fungal strain was grown stationary at 28°C for 20 days in 45 Erlenmeyer flasks (500 mL), each flask containing 20 g of rice, 20 mg of yeast extract, 10 mg of KH₂PO₄, and 40 mL of natural seawater (Sobolevskaya et al., 2016).

At the end of the incubation period, mycelia and media were homogenized and extracted with EtOAc. The extract of the fungus was concentrated to dry using rotary evaporators at 40°C. The residual suspension (10 g) obtained from the culture of the fungal strain was subjected to ODS open column (200 mm x 50 mm i.d., C18) chromatography followed by stepwise gradient elution with MeOH in H₂O (v/v) (20%, 40%, 60%, 80%, 100%), 2 L each as the eluent.

The fraction(2,9),(994,995) eluted with MeOH in H₂O 40%-1 was utilized to purify compounds by analytical ODS HPLC (column YMC-Pack-ODS-A, 250 mm x 10 mm i.d., 5 µm, flow rate 3 mL/min; RI detector) using isocratic program with 15% ACN in H₂O to yield compounds 1 (9.7 mg) and 2 (45.9 mg).

The fraction eluted with MeOH in H₂O 40%-3 was further purified by a preparative HPLC (column YMC-Pack-ODS-A, 250 mm x 10 mm i.d., 5 µm, flow rate 3 mL/min; RI detector) using isocratic program with 22% ACN in H₂O to yield compounds 3 (30.8 mg) and 4 (4.9 mg).

New phomaligol A2 (1): Yellow brown oil. ESI-MS m/z 308.08 [M + H]⁺, calcd. for C₁₄H₂₀O₆. The ¹H and ¹³C-NMR (CD₂OD) was presented in Table 1.

Wasabidienone E (2): Yellow oil. ESI-MS m/z 312.02 [M + H]⁺, calcd. for C₁₄H₂₀O₆N.

¹H-NMR (500 MHz, CD₂OD) δH, J (Hz): 5.29 (1H, s, H-4), 2.50 (1H, m, H-8), 1.45, 1.72 (2H, m, H-9), 3.26 (2H, t, J = 8.5 Hz, H-10), 3.67 (2H, H-11), 1.52 (3H, s, H, 2-Me), 3.94 (3H, s, 3-MeO), 1.69 (3H, s, 6-Me), 1.18 (3H, d, J = 10 Hz, 8-Me), 0.93 (3H, t, J = 15 Hz, 9-Me); ¹³C-NMR (125 MHz, CD₂OD) δC: 192.4 (C-1), 99.9 (C-2), 174.8 (C-3), 78.6 (C-4), 164.0 (C-5), 78.1 (C-6), 175.1 (C-7), 40.1 (C-8), 26.0 (C-9), 44.4 (C-10), 58.8 (C-11), 26.8 (2-Me), 55.2 (3-MeO), 5.9 (6-Me), 15.5 (8-Me), 10.5 (9-Me). These spectroscopic data were suitable with the ones in the literature (Soga et al., 1987).

Aspertetranone D (3): Cream solid. ESI-MS m/z 435.11 [M - H]⁻, calcd. for C₂₁H₂₅O₇.

¹H-NMR (500 MHz, CD₂OD) δH, J (Hz): 4.34 (1H, s, H-6), 2.72, 2.80 (2H, d, J = 17, 18 Hz, H-10), 2.04 (1H, m, H-11), 2.34 (1H, dd, J = 9, 9.5 Hz, H-11a), 4.58 (1H, d, J = 9 Hz, H-12), 2.25 (3H, s, 3-Me), 1.94 (3H, s, 4-Me), 1.40 (3H, s, 5a-Me), 1.33 (3H, s, 8-Me), 1.37 (3H, s, 8-Me), 1.25 (3H, d, J = 7, 11-Me); ¹³C-NMR (125 MHz, CD₂OD) δC: 164.5 (C-1), 157.7 (C-3), 107.7 (C-4), 163.6 (C-4a), 84.1 (C-5a), 73.3 (C-6), 75.5 (C-6a), 208.3 (C-7), 54.9 (C-8), 211.1 (C-9), 45.2 (C-10), 75.4 (C-10a), 39.2 (C-11), 39.8 (C-11a), 63.2 (C-12), 101.9 (C-12a), 15.8 (3-Me), 8.0 (4-Me), 16.8 (5a-Me), 24.4 (8-Me), 22.6 (8-Me), 9.9 (11-Me). These spectroscopic data were suitable with the ones in the literature (Wang et al., 2015).

Maftanamide (4): White powder. ESI-MS m/z 339.05 [M - H]⁻, calcd. for C₁₀H₁₄O₂N₂.

The minimum inhibitory concentrations (MICs) of active compounds against seven pathogens were determined by a dilution method (CLSI, 2016). First, 100 µL of Mueller Hinton Broth medium (MHB) was dispensed into all wells of a microtiter plate. Two-fold dilutions of the compounds in the range of 256-0.125 µg/mL were prepared in the plates. Amoxicillin and cefotaxime were used as positive controls. The turbidity of the microbial suspensions was measured at 600 nm wavelength, and adjusted to match the 0.5 McFarland standard (10⁶ colony forming units/mL). Subsequently, 5 µL of bacterial culture was dispensed into each well 96-well plates. Finally, the plates were incubated at 37°C for 18-36 hours, and the MIC values were inspected as the lowest concentrations in which no growth could be observed. Antimicrobial assay was performed at least triplicate.
RESULTS AND DISCUSSION

The fungus was cultured for 20 days on rice medium. The EtOAc extract of the culture was purified by a combination of C18 gel column chromatography and reversed-phase HPLC to yield four individual compounds including one new phomaligol A2 (1), together with three known compounds, wasabidienone E (2), aspertetranone D (3) and mactanamide (4).

Compound 1 was obtained as a yellow brown oil. The ESI-MS spectrum showed a quasimolecular ion peak at \( m/z \) 300.88 [M+H]⁺, corresponding to the molecular formula of C_{14}H_{20}O_{7}.

The \(^1\)H NMR spectrum of 1 (Table 1) exhibited signals for two methyl groups of cyclohexen ring (δ\(_H\) 1.55/H-13; 1.66/H-14), a methoxyl group (δ\(_H\) 3.89/H-12), and an aromatic proton (δ\(_H\) 5.62/H-4). Two olefinic carbons (δ\(_C\) 173.7/C-3 and 99.2/C-4), three ketone carbons (δ\(_C\) 202.2/C-1, 192.6/C-5 and 175.6/C-7), two oxygenated carbons (δ\(_C\) 72.7/C-2 and 82.2/C-6), and a methoxy carbon (δ\(_C\) 56.3/C-12) were observed in the \(^{13}\)C NMR data of 1 (Table 1).

The \(^1\)H NMR spectrum of 1 also showed additional signals of two methyl groups (δ\(_H\) 1.22/H-10 and 1.23/H-11), two methine protons (δ\(_H\) 2.46/H-8 and 3.84/H-9). The remaining six carbon signals were attributed to a sec-butyl (δ\(_C\) 20.2/C-10, 12.1/C-11, 68.4/C-9, and 46.5/C-8) and two methyl groups (δ\(_C\) 22.7/C-14, 20.7/C-13).

![Figure 2. Structures of compounds 1 - 4.](image)

The COSY spectrum showed coupling between terminal methyl protons (δ\(_H\) 1.22/H-10) and methine proton (δ\(_H\) 3.84/H-9) which were coupled with a methine proton at δ\(_H\) 2.46 (H-8), which in turn coupled with a secondary methyl group (δ\(_H\) 1.23/H-11) (Figure 3).
In the HMBC spectrum, correlations were observed from the methyl protons H-10 (δ_H 1.22) and H-11 (δ_H 1.23) to carbons C-8 (δ_C 46.5) and at C-9 (δ_C 68.4). Furthermore, HMBC correlations from H_β-13 (δ_H 1.55) to C-1 (δ_C 201.1) and C-2 (δ_C 72.7), from H_β-14 (δ_H 1.66) to C-1 (δ_C 201.1), C-5 (δ_C 192.6) and C-6 (δ_C 82.2), and from H_β-12 (δ_H 3.89) to C-3 (δ_C 173.7). Methine protons at δ_H 2.46 (H-8) showed HMBC correlations to the carbonyl carbon at δ_C 68.4 (C-9) and δ_C 12.1 (C-11). Methine protons at δ_H 3.84 (H-9) had HMBC correlations with C-11 (δ_C 12.1). Besides, HMBC spectrum also indicated the correlations from an aromatic proton H-4 (δ_H 5.62) to carbons C-2 (δ_C 72.7), C-3 (δ_C 173.7), C-5 (δ_C 192.6) and C-6 (δ_C 82.2) (Figure 3).

Table 1. NMR data of compound 1.

| Pos. | δ_H, J (Hz) | δ_C | δ_H, J (Hz) | δ_C |
|------|-------------|-----|-------------|-----|
| 1    | 201.1       |     | 202.5       |     |
| 2    | 72.7        |     | 73.5        |     |
| 3    | 173.7       |     | 173.0       |     |
| 4    | 5.62 (1H, s)| 99.2| 5.56 (1H, s)| 99.9|
| 5    | 192.6       |     | 191.7       |     |
| 6    | 82.2        |     | 81.1        |     |
| 7    | 175.6       |     | 175.8       |     |
| 8    | 2.46 (1H, m)| 46.5| 2.50 (1H, m)| 39.8|
| 9    | 3.84 (1H, m)| 68.4| 1.73 (1H, m)| 26.6|
| 10   | 1.22 (3H, d, 6 Hz)| 20.2| 0.96 (3H, t, 7.5 Hz)| 11.3|
| 11   | 1.23 (3H, d, 7 Hz)| 12.1| 1.17 (3H, d, 7 Hz)| 16.1|
| 12   | 3.89 (3H, s)| 56.3| 3.87 (3H, s)| 56.8|
| 13   | 1.55 (3H, s)| 20.7| 1.65 (3H, s)| 24.1|
| 14   | 1.66 (3H, s)| 22.7| 1.70 (3H, s)| 23.4|
| 2-OH| 3.65 (1H, s)| 2.80 (1H, br s)|     |     |
| 9-OH| 3.58 (1H, s)|     |     |     |

The ¹H and ¹³C NMR spectrum of 1 were nearly similar to that of phomaligol A, isolated from the sponge-derived fungus *Paecilomyces lilacinus*, except for the additional hydroxyl group located at C-9 (δ_H 3.58/OH-9, δ_C 68.4/C-9) (Elbandy et al., 2009). Thus, the compound 1 was assigned as a new compound and named phomaligol A2.

Compounds 1–4 showed antimicrobial activity on *E. coli*, *P. aeruginosa*, *S. aureus*, *B. cereus*, *S. faecalis*, *L. monocytogenes*, and *C. albicans* with various values of minimum inhibitory concentration (MIC) (Table 2). Among these compounds, 1-3 exhibited antibiotic activities towards *C. albicans* with MIC of 16 µg/mL, whereas compound 4 showed antifungal activity against *C. albicans* with the MIC of 32 µg/mL. Similar report on the compound macatanamide isolated from marine-derived fungus *Aspergillus* sp., also demonstrated antibiotic activity towards *C. albicans* (Lorenz et al., 1998).
Table 2. Minimum inhibitory concentration (MIC, µg/mL) of compounds 1–4 against seven pathogens.

| Compounds | E. coli | P. aeruginosa | S. aureus | L. monocytogenes | B. cereus | S. faecalis | C. albicans |
|-----------|--------|--------------|----------|------------------|----------|------------|------------|
| 1         | 64     | 16           | 128      | 32               | 128      | 32         | 16         |
| 2         | 64     | 16           | 64       | 64               | 128      | 32         | 16         |
| 3         | 64     | 16           | 64       | 32               | 64       | 32         | 16         |
| 4         | 128    | 16           | 64       | >256             | 64       | 32         | 32         |
| Amoxicillin | 8     | 128          | 0.25     | 0.25             | >256     | >256       | 256        |
| Cefotaxime | 0.25   | 4            | 0.5      | 64               | 64       | 4          | >256       |

Note: Amoxicillin and cefotaxime were positive drugs.

Compounds 1–4 also demonstrated prominent antibacterial activity against *P. aeruginosa* (MIC 16 µg/mL), which were higher than that of the positive control amoxicillin (with MIC value of 128 µg/mL). Compound 4 did not illustrate antibacterial activity against *L. monocytogenes*. In conclusion, sponge-derived fungus *A. flocculosus* 01NT.1.1.5 might produce antibacterial secondary metabolites towards different microbes. It is believed that searching for natural products synthesized by marine fungi could be a promising way to combat the emerging of pathogens.

**CONCLUSION**

From the ethyl acetate extract of culture medium of a fungus *A. flocculosus* 01NT.1.1.5 isolated from the sponge *Styliissa* sp. at Nhatrang Bay, we obtained one new phomaligol A2 (1), together with three known compounds, wasabidienone E (2), asperteretanone D (3) and mactanamide (4). All of these compounds showed antimicrobial activity towards microorganisms with various values of MICs. The results indicated that marine fungus *A. flocculosus* 01NT.1.1.5 could produce natural compounds against pathogens. The remaining fractions and other bioactivities study of these compounds are conducting in advance.

**Acknowledgment:** This study was supported by the project grant from No.3 branch component of the Project 47 (VAST.DA47.12/16-19).

**REFERENCES**

Blunt JW, Copp BR, Munro MH, Northcote PT, Prinsep MR (2010) Marine natural products. *Nat Prod Rep* 27(2): 165-237.
HOẠT TÍNH KHÁNG SỮA CỦA HỘP CHẤT TỪ NHIỄM TỤ CHỨNG ASPERGILLUS FLOCCULOSUS 01NT.1.1.5 PHÁN LẬP TỪ BỘT BIỆN
Phan Thị Hoài Trinh1,4, Trần Thị Thanh Vân1,4, Bùi Minh Lý1,4, Byeoung Kyu Choi2, Hee Jae Shin2, Jong Seok Lee2, Hyi Seung Lee2, Phí Quốc Tiến1,4

1Viện Nghiên cứu và Ứng dụng Công nghệ Nha Trang, Viện Hàn lâm Khoa học và Công nghệ Việt Nam
2Viện Khoa học và Công nghệ Hải dương Hà Nội Quốc, Busan, Hàn Quốc
3Viện Công nghệ Sinh học, Viện Hàn lâm Khoa học và Công nghệ Việt Nam
4Học viện Khoa học và Công nghệ, Viện Hàn lâm Khoa học và Công nghệ Việt Nam

Tóm tắt

Các loại vi nấm Aspergillus được xem là nguồn quan trọng trong cho các hợp chất tự nhiên ức chế trong y được, nông nghiệp và công nghiệp. Trong tiến trình nghiên cứu của chúng tôi về các hợp chất kháng sinh mới từ vi nấm biển, một hợp chất mới phomaligol A2 (1), cùng với ba hợp chất dâ biệt wasabidienone E (2), aspertetranone D (3) và mactanamide (4) được thuần nhất từ dịch etyl acetate môi trường men chưng vi nấm Aspergillus flocculosus (A. flocculosus) 01NT.1.1.5 phân lập từ loài bò biển Styliola sp. thuộc vùng Nha Trang, Việt Nam. Cấu trúc hoá học của các hợp chất này được xác định bởi phân tích dã tử vi commissioners của các công trình nghiên cứu trước đây. Bên cạnh đó, nghiên cứu cũng tiến hành đánh giá hoạt tính kháng sinh của các hợp chất thu được đối với các chủng vi sinh gây bệnh bao gồm Escherichia coli (E. coli) ATCC 25922, Pseudomonas aeruginosa (P. aeruginosa) ATCC 27853, Staphylococcus aureus (S. aureus) ATCC 25923, Bacillus cereus (B. cereus) ATCC 11778, Streptococcus faecalis (S. faecalis) ATCC 19433, Listeria monocytogenes (L. monocytogenes) ATCC 19111, and Candida albicans (C. albicans) ATCC 10231. Trong số các hợp chất này, hợp chất 1-3 ức chế sự sinh trưởng của men C. albicans với nồng độ ức chế tối thiểu (MIC) là 16 µg/mL. Hoạt tính của các hợp chất này hiệu quả hơn hơn khi so sánh với amoxicillin và cefotaxime (MIC > 256 µg/mL), thuốc kháng sinh được sử dụng làm đối thủ chống. Bên cạnh đó, các hợp chất 1-4 cũng thể hiện hoạt tính kháng các chủng vi sinh gây bệnh khác bao gồm P. aeruginosa và S. faecalis với MIC lần lượt 16 µg/mL và 32 µg/mL. Hợp chất 4 không có hoạt tính ức chế đối với chủng L. monocytogenes, trong khi các hợp chất 1-3 có khả năng kháng chủng này với MIC từ 32 đến 64 µg/mL. Bớn hợp chất thử nghiệm đầu tiên thể hiện hoạt tính kháng khuẩn đối với chủng B. cereus và E. coli với các giá trị MIC 64-128 µg/mL. Đây là bao cáo đầu tiên về các hợp chất có hoạt tính kháng sinh thu được từ vi nấm biển A. flocculosus phân lập từ Việt Nam.

Keywords: Aspergillus flocculosus, aspertetranone D, hoạt tính kháng sinh, mactanamide, phomaligol A2, wasabidienone E