UWS Academic Portal

Anti-HCV protease of diketopiperazines produced by the Red Sea sponge-associated fungus *Aspergillus versicolor*

Ahmed, Eman; Rateb, Mostafa; El-Kassem, L.T. Abou ; Hawas, Usama

Published in:
Applied Biochemistry and Microbiology

DOI:
[10.1134/S0003683817010021](https://doi.org/10.1134/S0003683817010021)

Published: 01/01/2017

Document Version
Peer reviewed version

[Link to publication on the UWS Academic Portal](#)

Citation for published version (APA):

Ahmed, E., Rateb, M., El-Kassem, L. T. A., & Hawas, U. (2017). Anti-HCV protease of diketopiperazines produced by the Red Sea sponge-associated fungus *Aspergillus versicolor*. *Applied Biochemistry and Microbiology*, 53(1), 101-106. <https://doi.org/10.1134/S0003683817010021>

General rights

Copyright and moral rights for the publications made accessible in the UWS Academic Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

If you believe that this document breaches copyright please contact pure@uws.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Anti-HCV Protease of Diketopiperazines Produced by the Red Sea Sponge-Associated Fungus *Aspergillus versicolor*

Eman F. Ahmed^a, Mostafa E. Rateb^{b,c}, Lamia T. A. El-Kassem^d, Usama W. Hawas^{e,*}

^aNatural and Microbial Products Chemistry Department, National Research Centre, Dokki, Cairo, ^bDepartment of Pharmacognosy, College of Pharmacy, Beni-Suef University, Beni-Suef, ^cSchool of Science & Sport, University of the West of Scotland, Paisley, UK. ^dPharmacognosy Department, and ^ePhytochemistry and Plant Systematic Department; National Research Centre, Dokki, Cairo, Egypt.

Abstract

Hepatitis C virus (HCV) infection is a global problem due to the difficulties in developing a protective vaccine. In this work, we demonstrated that the ethyl acetate extract of the endophytic fungus *Aspergillus versicolor* exhibited significant activity against HCV NS3-NS4A protease with IC₅₀ value of 30 µg/ml. The fungus was isolated from the black sponge *Spongia officinalis* and identified by its morphology and 18S rDNA. Large-scale fermentation of the fungus followed by chromatographic purification of the active extract from the liquid medium allowed the isolation of known metabolites related to cyclodipeptides or the so called diketopiperazines (DKPs). The diketopiperazine, cyclo(L-Tyr-L-Pro) displayed strong inhibitory effect as anti-HCV protease with IC₅₀ value of 8.2 µg/ml. A computational docking study of the isolated cyclo(L-Tyr-L-Pro) against HCV protease was used to formulate a hypothetical mechanism for the inhibitory activity of the active compound on the tested enzyme.

* e-mail: uhawas@kau.edu.sa

Key words: Red sea, black sponge, *Spongia officinalis*, *Aspergillus versicolor*, HCV Protease.

Introduction

Hepatitis C virus (HCV) infection is a major cause of human liver disease throughout the world, leading to cirrhosis, carcinoma and liver failure [1]. Recently, HCV NS3/4A protease inhibitors have emerged

as a promising potential treatment for HCV infection [2]. Unlike interferons, which work by stimulating the immune system's response to viral infection, NS3 protease inhibitors target the virus directly by inhibiting NS3/4A serine protease, a key enzyme involved in HCV replication [3].

In recent years, numerous metabolites possessing uncommon structures and potent bioactivities have been isolated from strains of fungi collected from diverse environments, such as soils, animals, plants, and sediments [4]. As ubiquitous fungus *Aspergillus versicolor* has been ascertained to afford excellent biologically active products, such as sesquiterpenoids [5,6], diterpenes [7], cyclopeptides [8], diketopiperazines [9], anthraquinone [10], isocoumarins [11], alkaloids [12,13] and diphenyl ethers [14,15]. Many of these metabolites produced by *A. versicolor* exhibit antioxidants, cytotoxic properties, antibacterial, fungicidal, insecticidal, and lipid lowering effects [16-21].

Motivated by a search for new bioactive metabolites from the fermentation products of endophytic fungi, *A. versicolor* was isolated from the inner tissues of the Red Sea black sponge *Spongia officinalis*. This paper deals with the isolation and structure elucidation of eight secondary metabolites, their antimicrobial activity, and their inhibitory effect on HCV NS3-NS4A protease using a SensoLyte™ 520 HCV protease assay kit.

Materials and Methods

General Experimental Procedures

Optical rotations were measured in methanol on a Perkin-Elmer 241 instrument at the sodium D line (589 nm). The UV spectra were acquired in methanol using Thermo Scientific NanoDrop 2000C UV-Vis spectrophotometer. IR spectra were measured using JASCOFT/IR-300E spectrophotometer. ¹H and ¹³C NMR spectra were recorded at 25°C with a Varian Inova-NMR 600 MHz NMR spectrometer. High-resolution mass spectra were acquired with a Thermo scientific LTQ/XL Orbitrap, specifications; analyzer: FTMS, mass range: normal full ms 100-2000, resolution: 30,000. HPLC was performed on Agilent 1260 Infinity preparative HPLC system with an Agilent Eclipse XDB-C18 column (5μ, 10 x 250 mm, Agilent technologies, USA). All chemical reagents were purchased from Sigma-Aldrich and used without further purification.

Enzymes and chemicals

The Sensolyte™ 520 HCV protease assay kit Fluorimetric (Lot# AK71145-1020), HCV NS3-NS4A protease, HCV NS3- NS4A protease inhibitor 2 (cat# 25346), and Sensolyte™ Green protease assay kit Fluorimetric (Lot# AK71124-1011) were purchased from AnaSpec (San Jose, CA, USA). Soybean trypsin-chymotrypsin inhibitor was purchased from Sigma Aldrich (St. Louis, MO, USA). Falcon™ Microtest™ 384-well 120 µl black assay plates, non-sterile, no lid, were purchased from Becton Dickinson (Tokyo, Japan).

Fungal isolation and culture conditions

The black sponge *Spongia officinalis* was collected from the Egyptian Red Sea at a depth of 20 m off the coast of Rass Mohamed (South Sinai, Egypt) in March 2010. The sample was selected solely on the basis of a clean and healthy exterior, and brought to the laboratory in ice. In the laboratory, specimens were washed with sterile water and processed immediately. The sample was identified by the Coral Reef Ecology and Biology Group, National Institute of Oceanography and Fisheries, Suez, Egypt.

After proper washing, samples were cut into 0.5- to 1-cm pieces, surface-sterilized with 70% ethanol for 1 min, and rinsed three times with sterile distilled water. The algal pieces were blotted on sterile blotting paper. Each piece was placed in a Petri dish on the surface of solid potato dextrose agar (PDA) medium containing (g/l) potato (200), glucose (10), and agar (15) at pH 7.5 prepared in 50% sea water supplemented with penicillin benzyl sodium salt (0.02 g/l) to avoid any bacterial growth.

Morphological and molecular identification of the endophytic isolate

After 10 days of incubation in (PDA) medium, white colonies were developed and changed to green. It is characterized by biserial aspergilla and conidia in shade of green. These morphological features are typically *Aspergillus versicolor* colonies. 18S rRNA (Ribonucleic acid) sequencing technique was employed to identify the fungal isolates on a molecular level. The universal primers 18SF149:5'-GGAAGGG(G/A)TGTATTATTAG 3' and 18SR 701: 5'-GTAAAAGTCCTGGTTCCC-3', were used

to amplify a partial fragment (522bp) of the 18SrRNA from fungal strain isolate PCR. The PCR mixture contained 25 pmol of each primer, 10 ng of chromosomal DNA, 200 mM dNTPs and 2.5 U of Taq polymerase in 50 µl of polymerase buffer. The PCR thermocycler (Eppendorf) was programmed as follow: 95°C for 5 min for initial denaturation, 30 cycles 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. and a final extension of at 72°C for 10 min. Five microliters of the obtained PCR product were analyzed on 1% agarose gel electrophoresis [13] and were visualized on UV transilluminator. The PCR product was purified using QIAquick PCR purification reagent (Qiagen). The amplified 18S rRNA fragment (552 bp) was sequenced in both directions. BLAST (www.ncbi.nlm.nih.gov/blast) sequence analysis was carried out to affiliate the strain isolate. Multiple sequence alignment and molecular phylogeny was performed using BioEdit [21].

According to sequencing similarities and multiple alignment the fungus was found to be in a close relation to *Aspergillus versicolor* strain F21 **CBS 583.65** (gb:JN252119.1) with 97% identity. DNA sequencing was carried out by Sequencer (ABI 3730XLs, Macrogen Inc.), Scientific Bourg El-Arab, Alexandria, Egypt.

Extraction and isolation of compounds

The isolated fungus *Aspergillus versicolor* was cultivated on Czapek's peptone liquid medium containing (g/l) glucose (30), yeast (2), peptone (10), NaNO₃ (3), KH₂PO₄ (0.5), KCl (0.5), in 50% sea water at room temperature. Twenty one-day-old fermentation broth (12 l) was separated from the fungal mat by filtration. The fungal mycelia were suspended in distilled water for easy blending by a Ultra Turrax model 25 at 8000 min⁻¹ (rpm) and the homogenate was extracted by ethyl acetate. The resultant extract was evaporated to dryness using a rotavapor at 40°C, followed by de-fatting with *n*-hexane.

The total extract was suspended in 50% aqueous methanol and partitioned using a modified Kupchan's scheme and then concentrated under reduced pressure. The CH₂Cl₂ fraction (2.5 g) was further fractionated on vacuum liquid chromatography column loaded with silica gel using aCH₂Cl₂-MeOH gradient to give four fractions. NMR-guided analysis revealed fraction 2 of interest. Then, Fr 2 was further purified on Agilent 1200 Infinity semi-preparative HPLC equipped with reversed phase column (C18, 250 × 10 mm, Lxi.d., Phenomenex Luna) using MeCN in H₂O (30-100%) for 30 min

followed by 100% MeCN for 10 min at 2.0 mL/min flow rate. These conditions afforded 8 compounds **1** (1.4 mg), **2** (2.2 mg), **3** (1.5 mg), **4** (1.6 mg), **5** (2.2 mg), **6** (2.7 mg), **7** (1.8 mg), and **8** (1.3 mg).

(-)-*Curvularin* (**1**): Colorless powder; $[\alpha]_{20}^D$ -33.5 (c, 0.1, MeOH); ^1H NMR (CD_3OD , 600 MHz) δ (ppm): 6.22 (1H, d, J = 2.5 Hz, H-15), 6.15 (1H, d, J = 2.5 Hz, H-13), 4.89 (1H, m, H-8), 3.83/3.59 (2H, each, d, J = 5 Hz, H-11), 3.17/2.71 (2H, each, m, H-3), 1.71-1.25 (8H, m, H-4,5,6,7), 1.09 (3H, d, J = 6 Hz, 8- CH_3); ^{13}C NMR (CD_3OD , 150 MHz) δ (ppm): 209.7 (C-2), 172.8 (C-10), 161.2 (C-14), 159.5 (C-16), 137.2 (C-12), 127.2 (C-1), 112.2 (C-13), 102.7 (C-15), 73.8 (C-8), 44.7 (C-3), 40.5 (C-11), 33.0 (C-7), 27.7 (C-5), 24.9 (C-6), 23.9 (C-4), 20.5 (8- CH_3). HRESIMS: m/z 292.1311, indicating the molecular formula $\text{C}_{16}\text{H}_{20}\text{O}_5$ (calculated $[\text{M}+\text{Na}]^+$ ion for $\text{C}_{16}\text{H}_{20}\text{O}_5\text{Na}$ at m/z 315.1205).

Cyclo(L-Pro-L-Ile) (**2**): White amorphous powder; $[\alpha]_{20}^D$ -78.3 (c, 1.0, MeOH); ^1H NMR (CD_3OD , 600 MHz) δ : 4.20 (1H, t J = 8 Hz, Pro-H), 4.08 (1H, dd J = 4.4 Hz Ile-H), 3.56–3.52 (2H, m, H-9), 2.32/2.02 (2H, m, H-7), 2.17/1.92 (2H, m, H-8), 1.93 (1H, m, H-10), 1.45/1.32 (2H, m, H-11), 1.07 (3H, d, J = 6.5 Hz, 14- CH_3), 0.93 (3H, d, J = 6.4 Hz 12- CH_3). ^{13}C NMR (CDCl_3 , 150 MHz) δ : 169.3 (C-5), 165.2 (C-2), 63 (C-3), 58.4 (C-6), 45.7 (C-9), 39.7 (C-10), 29.4 (C-7), 24.5 (C-11), 22 (C-8), 15.3 (14- CH_3), 11.3 (12- CH_3). HRESI-MS (m/z 211.1440 $[\text{M}+\text{H}]$), calcd. for $\text{C}_{11}\text{H}_{19}\text{N}_2\text{O}_2$, m/z 211.14410.

Cyclo(L-Tyr-L-Pro) (**3**): $[\alpha]_{20}^D$ -69.3 (c, 0.5, MeOH); ^1H NMR (CD_3OD , 600 MHz) δ : 7.01 (2H, d, J = 8 Hz, H-2'/6'), 6.67 (2H, d, J = 8 Hz, H-3'/5'), 3.99 (1H, m, H-3), 3.51 (1H, m, H-6), 3.02 (2H, d, J = 6 Hz, H-10), 2.41/2.05 (2H, m, H-9), 1.74 (2H, m, H-7), 1.21 (2H, m, H-8). ^{13}C NMR (CD_3OD , 150 MHz) δ : 170.8 (C-5), 166.9 (C-2), 157.6 (C-4'), 132 (C-2'/6'), 127.7 (C-1'), 116.2 (C-3'/5'), 60.0 (C-3), 57.7 (C-6), 49.8 (C-9), 37.5 (C-10), 29.3 (C-7), 22.7 (C-8). HRESI-MS (m/z 261.1237 $[\text{M}+\text{H}]$), calcd. for $\text{C}_{14}\text{H}_{17}\text{N}_2\text{O}_3$, m/z 261.2960.

Assay for determination of HCV protease inhibitory activity

Samples of 2 μL of a compound dissolved in dimethyl sulfoxide (DMSO) were placed in each well of a 384-well microplate, then 8 μL of recHCV PR (0.5 $\mu\text{g}/\text{mL}$) were added, and the plate was briefly agitated. Finally, 10 μL of the freshly prepared substrate [Ac-Asp-Glu-Dap (QXLTM520)-Glu-Glu-Abu-COO-Ala-Ser-Cys(5-FAMsp)-NH₂] were added with sequential rotational shaking. The reaction mixture was

incubated for 30 min at 37°C. The fluorimetric analyses were performed on an automated TECAN GENios plate reader (Männedorf, Switzerland) with excitation wavelength at 485 nm and emission at 530 nm. Each compound was tested in triplicate. HCV PR inhibition (%) was calculated using the following equation [20]:

$$\% \text{ inhibition} = (F_{\text{substrate}} - F_{\text{test compound}}) \times 100 / F_{\text{substrate}}$$

Where $F_{\text{substrate}}$ is the fluorescence of the substrate and enzyme without test compounds, and $F_{\text{test compound}}$ is the fluorescence of the assay mixture with the added compound.

Green protease assay

The organic extract and active HCV-PR isolated compounds **1-3** were dissolved in DMSO (2.5 µl; final content, 10% w/v) and placed in the wells of a 384-well microplate. Then 17.5 µl of assay buffer and 2.5 µl of trypsin (0.1 U/µl) were added and the plate was briefly agitated. Finally, 2.5 µl of the freshly diluted protease substrate HiLyte Fuor TM 488-labeled casein were added under sequential rotary shaking and the mixture incubated at 37°C for 30 min. The positive control was the soybean trypsin-chymotrypsin inhibitor. Inhibition was calculated as for HCV.

Docking study

Crystal structure of protease enzyme has been downloaded from protein Data bank (PDB ID: 3SV6) [22], all water molecules have been removed and native ligand has been deleted for make it ready for molecular modelling. Protein has been prepared and compounds has been designed as 3D by VEGA ZZ 3.0.5 software, it used also for adjusting the force field of the molecule as AMBER, and atomic charge which applied as Gasteiger and energy minimization of them using MOPAC and finally outputted as PDBQT. Autodock vina 1.0.3 has been applied for flexible-rigid docking of all molecules and Pymol for visualization and studying of hydrogen bonds interaction.

Results and Discussion

The isolated fungus from Red Sea sponge was identified as *Aspergillus versicolor* based on its morphology and authenticated by the molecular analysis of the ITS region of rDNA containing ITS1, and ITS4, and the intervening 5.8S rDNA gene. The fungus was grown in a static Czapek's peptone liquid medium. The culture broth was extracted with ethyl acetate, and the organic extract was subjected to liquid-liquid partitioning and HPLC to yield eight known microbial secondary metabolites (Fig.1) related to polyketides as in case of (-)-curvularin (**1**) and cyclodipeptides or the so called diketopiperazines (DKPs) as compounds **2-7**.

Compound **1** to be (-)-curvularin which was first reported from *Penicillium* sp. [23]. On the other hand, the DKPs **2-7** were previously isolated from different microbial sources and identified as cyclo-(L-Pro-L-Ile), cyclo(L-Tyr-L-Pro), cyclo(L-Phe-L-Pro), cyclo(L-Phe-L-4-hydroxyPro), and cyclo(L-Try-L-Phe) [24-27]. Compound **7** was deducted based on the HRESI-MS analysis using Antimarin database indicated the cyclic tetrapeptidecyclo-[Phenylalanyl-prolyl-leucyl-prolyl] which was previously isolated from *Pseudoalteromonas* sp.[28]. Compound **8** identified as 2-methyl-L-arginine. The isolated compounds **1-8** were identified based on their HRESI-MS data and comparison of their NMR spectroscopic data and optical rotation data with the reported data.

Biological evaluation

The ethyl acetate extract of *A. versicolor* and the isolated compounds **1-8** were evaluated for their *in vitro* antimicrobial activity and anti-HCV protease inhibition. The organic extract and the isolated compounds didn't exhibit any antagonistic activities towards the examined microbial pathogens of some selected Gram-positive and Gram-negative bacteria as well as *Candida albicans* and *Aspergillus niger* (fungi). The antimicrobial activity was carried out using the agar diffusion method with 6-mm paper disks loaded with 400 and 100 µg of the extract and each pure compound, respectively [29].

HCV NS3-NS4A protease inhibition

The ethyl acetate extract of *A. versicolor* along with compounds **1** - **8** isolated from this extract were tested for their inhibitory activity against HCV PR using HCV NS3 protease inhibitor 2 as a positive control (Table I). The ethyl acetate extract showed potent activity against HCV NS3-NS4A protease with an IC₅₀ value of 30 µg/ml. Isolated diketopiperazines, cyclo-(L-Pro-L-Ile) (**2**) and cyclo(L-Tyr-L-Pro) (**3**) were strongly inhibitory with IC₅₀ values of 13.4 and 8.2 µg/ml, respectively, while (-)-curvularin (**1**) was mild inhibitory with an IC₅₀ value of 37.4 µg/ml. The selectivity of the ethyl acetate extract and the active compounds **1** - **4** for HCV PR was tested by comparison with their ability to inhibit human trypsin. Trypsin is a serine protease similar to HCV PR [30,31]. Cyclo(L-Tyr-L-Pro) (**3**) was more selective as inhibitor of HCV PR as compared to human trypsin with an IC₅₀ value of 37.9 µg/ml (Table I). These findings suggested that the diketopiperazine **3** selectively inhibited HCV PR with highly inhibitory activity and may moderately interfere with human physiological processes requiring trypsin activity. It is noteworthy that this is the first report on the inhibition of HCV PR by diketopiperazines which warrants further investigation of other members of this widely distributed class of compounds.

Docking study

In order to interpret the results obtained from biological study, docking simulation has been employed to give a theoretical imitation of the new discovered compounds binding mode within the protease enzyme. This simulation established well the interactions between the HCV PR active site and conventional electrophiles such as aldehydes, ketones, α -ketoacids, and α -ketoamides, followed by trapping of the resulting covalently bound intermediate by the active site triad, which would provide effective inhibition. Additionally, it has been reported that the fitting of ligand functional groups to the shallow, solvent-exposed active site of the protease through other forces such as van der Waals, aromatic, hydrogen-bonding, and hydrophobic interactions plays an important role in the inhibition of the HCV PR [32].

Co-crystallized native ligand (Telaprevir) has been used to determine the spatial dimension of grid box to include the interactive amino acids; the size has been adjusted relatively larger than the binding site. 3D structure of the isolated compound cyclo(L-Tyr-L-Pro) (**3**) was constructed using VEGA ZZ 3.0.5 software [33]. Autodock vina 1.0.3 [34] has been used for docking procedures. Pymol software [35] has been employed for visualization of mode of interaction. To evaluate the docking tools, native ligand has been extracted and re-docked within the enzyme, simulated mode of orientation has shown good similarity with co-crystallized one (Fig. 2). Same parameter of re-docking has been applied for the docking of the compound. The binding mode of cyclo(L-Tyr-L-Pro) has showed interesting orientation; the small molecule **3** has been docked in the deepest part of the exposed binding site. In spite of the smaller surface of the cyclo(L-Tyr-L-Pro) compound in comparison with the native ligand, it showed surplus of hydrogen bonds interactions with the amino acids backbone of the binding site with 6 Hydrogen bonds (Fig. 3).

Conclusion

The ethyl acetate extract from the endophytic fungus *Aspergillus versicolor* has been established here as a source of natural compounds with diverse biological activities, such as anti-HCV-Protease effects. The main constituents of the extract were diketopiprazines, which are a group of widely distributed natural constituents with diverse pharmacological activities. Thus, we introduce diketopiprazines as promising lead HCV PR inhibitors for further semi-synthetic modification aiming at increasing their potency and selectivity.

REFERENCES

1. Zoulim, F.; Chevallier, M.; Maynard, M.; Trepo, C. Rev. Med. Virol. 2003, 13, 57.
2. Tsantrizos Y. S. (2008), Peptidomimetic therapeutic agents targeting the protease enzyme of the human immunodeficiency virus and hepatitis C virus. Acc. Chem. Res. 41, 1252-1263.
3. Njoroge, F. G.; Chen, K. X.; Shih, N.-Y.; Piwinski, J. J. Acc. Chem. Res. 2008, 41, 50
4. Laatsch H. (2006), Marine bacterial metabolites. In: Frontiers in Marine Biotechnology (Proksch P. and Muller W. E. G., eds.). Horizon Bioscience, Norfolk, UK, pp. 225 – 288
5. Belofsky, G. N.; Jensen, P. R.; Renner, M. K.; Fenical, W. Tetrahedron 1998, 54, 1715–1724
6. Liu, X. H.; Miao, F. P.; Li, X. D.; Yin, X. L.; Ji, N. Y. Nat. Prod. Commun. 2012, 7, 819–820.
7. Nakanishi K, Doi M, Usami Y, Amagata T, Minoura K, Tanaka R, Numata A, Yamada T. Anthcolorins A-F, novel cytotoxic metabolites from a sea urchin-derived *Aspergillus versicolor*. Tetrahedron 69 (2013) 4617-4623.
8. Zhuang Y, Teng X, Wang Y, Liu P, Wang H, Li J, Li G, Zhu W. Cyclopeptides and polyketides from coral-associated fungus, *Aspergillus versicolor* LCJ-5-4. Tetrahedron 67 (2011) 7085-7089.
9. Song, F.; Liu, X.; Guo, H.; Ren, B.; Chen, C.; Piggott, A. M.; Yu, K.; Gao, H.; Wang, Q.; Liu, M.; Liu, X.; Dai, H.; Zhang, L.; Capon, R. J. Org. Lett. 2012, 14, 4770–4773.
10. Zhang, Y.; Li, X. M.; Wang, B. G. Biosci. Biotechnol. Biochem. 2012, 76, 1774–1776.
11. Ye Y-q, Xia C-F, Yang J-X, Qin Y, Zhou M, Gao X-M, Du G, Yang H-Y, Li X-M, Hu Q-F. Isocoumarins from the fermentation products of an endophytic fungus of *Aspergillus versicolor*. Phytochemistry Letters 10 (2014) 215–218
12. He, F.; Sun, Y. L.; Liu, K. S.; Zhang, X. Y.; Qian, P. Y.; Wang, Y. F.; Qi, S. H. J. Antibiot. (Tokyo) 2012, 65, 109–111.
13. Ji NY, Liu XH, Miao FP, Qiao MF. Aspeverin, a new alkaloid from an algicolous strain of *Aspergillus versicolor*. Org Lett. 2013; 15(10):2327-9.

14. Gao H, Zhou L, Cai S, Zhang G, Zhu T, Gu Q, Li D. Diorcinols B-E, new prenylated diphenyl ethers from the marine-derived fungus *Aspergillus versicolor* ZLN-60. J Antibiot. 2013; 66(9):539-42.
15. Hu S, Jiang N, Wang X, Chen C, Fan J, Wurin G, Ge H, Tan R, Jiao R. Prenylated diphenyl ethers from the mantis-associated fungus *Aspergillus versicolor* GH-2. Tetrahedron Letters 56 (2015) 3894–3897
16. Chen Y, Mao W, Yang Y, Teng X, Zhu W, Qi X, Chen Y, Zhao C, Hou Y, Wang C, Li N. Structure and antioxidant activity of an extracellular polysaccharide from coral-associated fungus, *Aspergillus versicolor* LCJ-5-4. Carbohydrate Polymers 87 (2012) 218– 226
17. Lee, Y.M., Kim, M.J., Li, H., Zhang, P., Bao, B.Q., Lee, K.J., Jung, J.H., 2013. Marine-derived *Aspergillus* species as a source of bioactive secondary metabolites. Mar. Biotechnol. 15, 499– 519.
18. Li XB, Zhou YH, Zhu RX, Chang WQ, Yuan HQ, Gao W, Zhang LL, Zhao ZT, Lou HX. Identification and biological evaluation of secondary metabolites from the endolichenic fungus *Aspergillus versicolor*. Chem Biodivers. 2015; 12(4): 575-92
19. Wang X, Mou Y, Hu J, Wang N, Zhao L, Liu L, Wang S, Meng D. Cytotoxic polyphenols from a sponge-associated fungus *Aspergillus versicolor* Hmp-48. Chem Biodivers. 2014; 11(1):133-9.
20. Hawas UW, El-Beih AA, El-Halawany AM. Bioactive anthraquinones from endophytic fungus *Aspergillus versicolor* isolated from red sea algae. Arch Pharm Res. 2012; 35(10):1749-56.
21. Hall T. A. (1999), BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. **41**, 95-98.
22. Romano KP, Ali A, Aydin C, Soumana D, Özen A, Deveau LM, et al. (2012) The Molecular Basis of Drug Resistance against Hepatitis C Virus NS3/4A Protease Inhibitors. PLoS Pathog 8(7): e1002832. doi:10.1371/journal.ppat.1002832

23. Kobayashi, A.; Hino, T.; Yata, S.; Itoh, T.J.; Sato, H.; Kawazu, K. "Unique Spindle Poisons, Curvularin and Its Derivatives, Isolated from *Penicillium* Species", *Agricul. Biol. Chem.*, **1988**, 52, 3119-3123.
24. Kimura, Y.; Tani, K.; Kojima, A.; Sotoma, G.; Shimada, A.; Okada, K. "Cyclo-(L-tryptophyl-L-phenylalanyl), a plant growth regulator produced by the fungus *penicillium* sp." *Phytochemistry*, **1996**, 41, 665-669.
25. Ström, K.; Sjögren, J.; Broberg, A.; Schnürer, J. "Lactobacillus plantarum MiLAB 393 Produces the Antifungal Cyclic Dipeptides Cyclo(L-Phe-L-Pro) and Cyclo(L-Phe-trans-4-OH-L-Pro) and 3-Phenyllactic Acid" *Appl. Environ. Microbiol.*, **2002**, 68, 4322-4327.
26. Jayatilake, G.S.; Thornton, M.P.; Leonard, A.C.; Grimwade, J.E.; Baker, B.J. "Metabolites from an Antarctic Sponge-Associated Bacterium, *Pseudomonas aeruginosa*" *J. Nat. Prod.* **1996**, 59, 293-296.
27. Maehr, H.; Yarmchuk, L.; Pruess, D. L.; Kellett, M.; Palleroni, N. J.; Prosser, B.T.; Demny, T.C. "Antimetabolites Produced By Microorganisms. XIV: 2-Methyl-L-Arginine, A New Amino Acid with Antibiotic Properties" *J. Antibiot.* **1976**, 29, 213-220.
28. Rungprom, W.; Siwu, E.R.O.; Lambert, L.K.; Dechsakulwatana, C.; Barden, M.C.; Kokpol, U.; Blanchfield, J.T.; Kita, M.; Garson, M.J. "Cyclic tetrapeptides from marine bacteria associated with the seaweed *Digenea* sp. and the sponge *Halisarca ectofibrosa*" *Tetrahedron*, **2008**, 64, 3147-3152.
29. Cosentino S., Tuberoso C. I. G., Pisano B., Satta M., Mascia V., Arzedi E., and Palmas F. (1999), *In-vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. *Lett. Appl. Microbiol.* **29**, 130-135.
30. Love R. L., Parge H. E., Wickersham J. A., Hoastomsky Z., Habiuka N., Moomaw E. W., Adachi T., and Hostomska Z. (1996), The crystal structure of hepatitis C virus NS3 protease reveals a trypsin-like fold and a structural zinc binding site. *Cell* **87**, 331–342.

31. Wei Y., Ma C. M., and Hattori M. (2009), Synthesis of dammarane type triterpene derivatives and their ability to inhibit HIV and HCV proteases. *Bioorg. Med. Chem.* **17**, 3003-3010.
- 32 Njoroge F. G., Chen K. X., Shih N. Y., and Piwinski J. J. (2008), Challenges in modern drug discovery: a case study of boceprevir, an HCV protease inhibitor for the treatment of hepatitis C virus infection. *Acc. Chem. Res.* **41**, 50–59.
- [33] Dipartimento di Scienze Farmaceutiche "Pietro Pratesi ", Facoltà di Farmacia, Università degli Studi di Milano, Via Luigi Mangiagalli, 25 I-20133 - Milano (Italy).
- [34] Trott O. and Olson A.J. "AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading" *Journal of Computational Chemistry* 31 (2) (2010): 455-461.
- [35] The PyMOL Molecular Graphics system Version 1.1.6, DeLano Scientific LLC.
- Ausubel, F. M.; Brent, R.; Kingston, R. E.; Moore, D.D.; Seidam, J. G.; Smith, J. A.; Struhl, K. (eds) (1999), Short protocols in *Molecular Biology*. John Wiley and Sons, Inc. NY.

Fig.1

Fig. 1: Isolated compounds of *A. versicolour*

Fig. 2

Fig 2: Co-crystallized native ligand in grey color, and re-docked native ligand in green color.

Fig. 3

Fig 3: Co-crystallized native ligand in grey color and docked small molecule in white and rose color.

Table 1. Inhibition of HCV NS3-NS4A protease and human trypsin by compounds from *A. versicolor*

Sample	HCV protease inhibitory activity (IC ₅₀)	Trypsin inhibitory activity (IC ₅₀)
Ethyl acetate extract	30.00±6.2	>1000
(1)	37.49±3.6	>1000
(2)	13.66±3.3	> 1000
(3)	8.20±1.7	37.9±3.8
(4)	88.75±4.5	Nt
(7)	95.32±2.7	Nt
HCV-I₂	1.5 ± 0.5 (1.64 µM)	Nt
T-I	Nt	0.01 ± 0.4 (0.5 µM)

HCV-I₂, HCV NS3/4A protease inhibitor 2 (positive control for HCV PR); **T-I**, soybean trypsin-chymotrypsin inhibitor (positive control for trypsin); Nt, not tested.