Natural Product Sciences
26(4) : 340-344 (2020)
https://doi.org/10.20307/nps.2020.26.4.340

Molecular Networking-based De-replication Strategy Leads to the Isolation of a New Chromone from *Pleosporales* sp.

Haeun Kwon[1], Jun Gu Kim[2], Jeong-Joo Oh[3], Jae-Jin Kim[3], Gyu-Hyeok Kim[3], Bang Yeon Hwang[2], Joung Han Yim[4], and Dongho Lee[1,*]

[1]*Department of Plant Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea*
[2]*College of Pharmacy, Chungbuk National University, Cheongju 28160, Republic of Korea*
[3]*Division of Environmental Science and Ecological Engineering, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea*
[4]*Korea Polar Research Institute, Incheon 21990, Republic of Korea*

Abstract – A new chromone analogue (**1**) was isolated from an EtOAc-extract of *Pleosporales* sp. culture medium, together with five known chromones (**2** – **6**). The isolation workflow was guided by a Molecular Networking-based dereplication strategy. The chemical structure of the new compound was elucidated using NMR and MS spectroscopy, and the absolute configuration was established by the Mosher's method. All isolated compounds were evaluated for their inhibitory effects on lipopolysaccharide-induced nitirc oxide production in RAW 264.7 macrophages. Compound **1** showed marginal inhibitory activity with an IC_{50} value of 118.7 μM.
Keywords – *Pleosporales* sp., Chromone, Molecular Networking, Mosher's method, Nitric oxide production

Introduction

The genus *Pleosporales* is the largest order in the Dothideomycetes, accounting for a quarter of the class.[1] This species is present in various habitats, and can be epiphytes, endophytes or parasites on living plants.[1] They produce various biologically active compounds, including depsidones,[2] sesquiterpenoids[3] and anthraquinones,[4] which possess cytotoxic,[2] anti-inflammatory[3] and antibacterial activities.[4]

As part of an ongoing project to discover bioactive metabolites from fungi, *Pleosporales* sp. was studied. Molecular Networking analysis of the EtOAc-extract of *Pleosporales* sp., which analyses chemical similarity between the metabolites based on their MS/MS spectral data, led to the isolation of a new chromone (**1**), along with five known chromones (**2** – **6**)[6,7] (Fig. 1). The structures were established by spectroscopic and chemical methods. Herein we describe the isolation, structural determination and biological evaluation of the isolated compounds on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.[9]

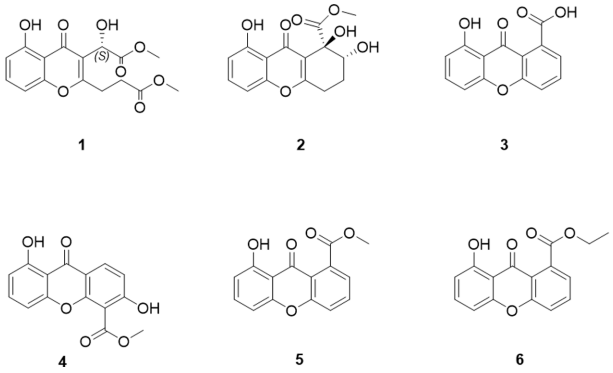


Fig. 1. The structures of **1** – **6** isolated from *Pleosporales* sp.

Experimental

General experimental procedures – Optical rotations were recorded using a Jasco P-2000 (JASCO, Tokyo, Japan) digital polarimeter. UV spectra were obtained using an Optizen POP UV-Vis spectrophotometer (Mecasys, Daejeon, Korea). NMR spectra were recorded on a Varian 500 MHz NMR spectrometer (Palo Alto, CA, USA) with

*Author for correspondence
Dongho Lee, Department of Plant Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea
Tel: +82-2-3290-3017; E-mail: dongholee@korea.ac.kr

tetramethylsilane as an internal standard. ESIMS data were acquired on Thermo LCQ ion trap mass spectrometer (Waltham, MA, USA). HRMS spectra was measured using a UPLC-q/TOF mass spectrometer (SYNAPT G2-Si, Waters) (Korea Basic Science Institute, Seoul Center, Korea). MPLC was performed using Biotage Isolera One system (Uppsala, Sweden) with Merck silica gel (230 – 400 mesh) in SNAP cartridges (25 g). HPLC was performed on a Waters system comprising a 515 pump and a 2996 PDA detector with a YMC-Pack ODS-A column (5 μm, 250 × 20 mm i.d). Column chromatography was performed using Diaion HP-20. TLC was performed using plates precoated with silica gel (0.25 mm, Merk, MA, USA).

Fungal materials – *Pleosporales* sp. was gained in January 2016 from Gaeun Mountain, Seoul, Republic of Korea and authenticated by Professor Gyu-Hyeok Kim. A voucher specimen (Korea University Culture Collection, kk1709) was deposited at the Division of Environmental Science and Ecological Engineering, College of Life Sciences and Biotechnology, Korea University, Seoul, Korea. *Pleosporales* sp. was cultivated on potato dextrose agar medium on Petri dishes (150 mm × 2 cm × 150 plates) at 25 °C for 30 days.

Molecular Networking – An EtOAc-extract of *Pleosporales* sp. culture medium was analyzed using UPLC-MS/MS equipped with Thermo Scientific™ LCQ Fleet™ Ion Trap Mass spectrometer. H_2O (A) and MeCN (B) were eluted at the flow rate of 0.3 mL/min with a linear gradient of 20 – 100% B (0 – 7 min). The data file was exported in .mzXML format by the application of MS-convert software, which is a part of the ProteoWizard package. The molecular networks were generated using Global Natural Products Social Molecular Networking (http://gnps.ucsd.edu). The parameters were set as follows[10]: product ion tolerance of 0.5 Da, the precursor ion mass tolerance of 2.0 Da, and fragment ions below 6 counts were removed from the MS/MS spectra. Molecular networks were generated using four minimum matched peaks and a cosine score of 0.65. The results were visualized by the application of Cytoscape 3.7.2 software. Annotation of known chromones was performed by manual interpretation of MS/MS spectra in comparison with our in-house fungal-derived MS/MS compound library. The MS/MS molecular network is accessible at the GNPS Web site with the following link: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=07aad0f035fe46ee8aeace4e32a9144a

Extraction and isolation – The fungal materials were harvested after 30 days and extracted with MeOH three times at room temperature. The MeOH extract was evaporated, followed by suspension in H_2O (1.5 L) and partitioned with EtOAc (4 × 2.5 L) to obtain an EtOAc layer (1.8 g). The EtOAc layer (1.8 g) was loaded onto a Diaion HP-20 column (7 × 36 cm) with H_2O–MeOH–acetone (1:0:0 to 0:0:1) to obtain eight fractions, (Fr. 1–Fr. 8). Fr. 4 (961.5 mg) was loaded onto a silica gel column (15 × 20 cm) with hexane–EtOAc gradient (1:0 to 1:1) to obtain seven sub-fractions (Fr. 4.1–Fr. 4.7). Fr. 4.6 (100.0 mg) was purified using preparative-HPLC (YMC Pack ODS-A, 5 μm, 250 × 20 mm I.D., flow rate: 5 mL/min, MeCN–H_2O, 2:3 to 1:1) to obtain compound **1** (3.6 mg, t_R = 21 min). Fr. 4.5 (113.1 mg) was purified using preparative-HPLC (MeCN–H_2O, 33:67 to 11:9) to obtain 3,4-dihydroflobosuxanthane (**2**, 18.8 mg, t_R = 22 min). Fr. 2 (85.3 mg) was purified using preparative-HPLC (MeCN–H_2O, 1:19 to 7:3) to obtain globosuxanthone D (**3**, 4.0 mg, t_R = 63 min). Fr. 6 (94.5 mg) was purified using preparative-HPLC (MeCN–H_2O, 2:3 to 4:1) to obtain 1,6-dihydroxy-7-methyl-9-oxoxanthene-5-carboxylic acid methyl ester (**4**, 29.4 mg, t_R = 32 min). Fr. 7.4 (158.9 mg) was load onto a silica gel column (15 × 20 cm) with hexane–acetone gradient (1:0 to 20:1) to obtain seven sub-fractions (Fr. 7.4.1–Fr. 7.4.7). Fr. 7.4.4 (24.2 mg) was purified using preparative-HPLC (ACN–H_2O, 7:3 to 0:1) to obtain vertixanthone (**5**, 10.0 mg, t_R = 20 min) and ethyl-8-hydroxy-9-oxo-9*H*-xanthene-1-carboxylate (**6**, 1.0 mg, t_R = 23 min).

Demethyloxalicumone C (1) – White amorphous solid; $[\alpha]^{25}_D$ +24.0 (*c* 0.1, CH_3CN); UV (MeOH) λ_{max} (log ε) 225 (3.07), 235 (3.08), 260 (3.05) nm; IR ν_{max} (ATR) 3331, 3110, 1737, 1649, 1471 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ_H 12.08 (s, 5-OH), 7.52 (1H, t, *J* = 8.3 Hz, H-7), 6.87 (1H, d, *J* = 8.3 Hz, H-8), 6.79 (1H, d, *J* = 8.3 Hz, H-6), 5.26 (1H, br d, *J* = 3.5 Hz, H-1"), 4.01 (s, 1"-OH), 3.81 (3H, s, OMe-4"), 3.74 (3H, s, OMe-5'), 3.16 (2H, td, *J* = 7.3, 3.6 Hz, H-1'), 2.86 (2H, t, *J* = 7.3 Hz, H-2'); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 181.9 (C-4), 173.0 (C-2"), 171.9 (C-3'), 167.4 (C-2), 160.6 (C-5), 156.0 (C-8a), 135.8 (C-7), 118.4 (C-3), 111.5 (C-8), 110.0 (C-4a), 106.7 (C-6), 66.1 (C-1"), 53.1 (Me-4"), 52.2 (Me-5'), 31.0 (C-2'), 26.8 (C-1'); HRMS *m/z* 359.0759 $[M + Na]^+$ (calcd. for $C_{16}H_{16}O_8Na$, 359.0743).

3,4-Dihydroflobosuxanthane (2) – Yellowish amorphous solid; $[\alpha]^{25}_D$ +26.5 (*c* 0.1, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ_H 12.08 (s, 8-OH), 7.53 (1H, t, *J* = 8.3 Hz, H-6), 6.89 (1H, d, *J* = 8.3 Hz, H-5), 6.78 (1H, d, *J* = 8.2 Hz, H-7), 4.73 (s, 1-OH), 4.09 (2H, dd, *J* = 10.3, 3.4 Hz, H-12), 4.01 (s, 15-OH), 3.86 (3H, s, OMe-13), 2.89 (1H, m, H-4), 2.30 (1H, m, H-3a), 2.17 (1H, m, H-3b); ESI-MS *m/z*

307.1 $[M + H]^+$.

Globosuxanthone D (3) – Yellowish amorphous solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 12.30 (s, 1-OH), 7.89 (1H, d, J = 7.5 Hz, H-3), 7.74 (1H, t, J = 8.3 Hz, H-6), 7.64 (1H, d, J = 7.6 Hz, H-4), 7.31 (1H, d, J = 6.5 Hz, H-2), 7.08 (1H, d, J = 8.3 Hz, H-5), 6.81 (1H, d, J = 8.2 Hz, H-7); ESI-MS m/z 257.1 $[M + H]^+$.

1,6-Dihydroxy-9-oxoanthene-5-carboxylic acid methyl ester (4) – Yellowish amorphous solid; ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 12.23 (s, 1-OH), 7.70 (1H, t, J = 8.3 Hz, H-3), 7.61 (1H, d, J = 9.1 Hz, H-8), 7.49 (1H, d, J = 9.1 Hz, H-7), 7.05 (1H, d, J = 8.3 Hz, H-4), 6.79 (1H, d, J = 8.3 Hz, H-2), 3.85 (3H, s, OMe); ESI-MS m/z 287.0 $[M + H]^+$.

Verixanthone (5) – Yellowish amorphous solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 12.23 (s, 8-OH), 7.76 (1H, t, J = 7.8 Hz, H-3), 7.61 (1H, d, J = 8.2 Hz, H-6), 7.56 (1H, d, J = 8.4 Hz, H-2), 7.33 (1H, d, J = 7.2 Hz, H-4), 6.94 (1H, d, J = 8.3 Hz, H-5), 6.81 (1H, d, J = 8.2 Hz, H-7), 4.04 (3H, s, OMe-12); ESI-MS m/z 271.0 $[M + H]^+$.

Ethyl 8-hydroxy-9-oxo-9*H*-xanthene-1-carboxylate (6) – Yellowish amorphous solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 12.24 (s, 8-OH), 7.76 (1H, t, J = 7.8 Hz, H-3), 7.61 (1H, d, J = 8.2 Hz, H-6), 7.56 (1H, d, J = 8.4 Hz, H-2), 7.33 (1H, d, J = 7.2 Hz, H-4), 6.94 (1H, d, J = 8.3 Hz, H-5), 6.81 (1H, d, J = 8.2 Hz, H-7), 4.52 (2H, q, J = 7.2, 7.2 Hz, H-12), 1.40 (3H, t, J = 7.2 Hz, H-13); ESI-MS m/z 285.0 $[M + H]^+$.

Preparation of (*S*)- and (*R*)-MTPA ester derivatives of demethyloxalicumone C (1) – 1.3 mg of compound **1** in 900 μL of pyridine-d_5 were divided into two parts and transferred into clean NMR tubes. (*R*)-(-)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride (20 μL) (Sigma-Aldrich, St. Louis, MO, USA) and (*S*)-(+)-α-MTPA chloride (20 μL) were added into the each of the NMR tubes immediately under a N_2 gas stream with a slight excess of 4-dimethylaminopyridine. The solutions were then carefully mixed. The tubes were left at 50 °C for 12 h, and the reactions were completed to yield the mono (*S*)-MTPA ester derivative of **1** (**1a**). The (*R*)-MTPA ester derivative of **1** (**1b**) was obtained from the treatment of (*S*)-(+)-α-MTPA chloride.

(*S*)-MTPA ester of 1 (1a) – ^{1}H NMR (500 MHz, pyridine-d_5) δ_H 7.91 (1H, overlapped, H-6), 4.94 (1H, s, H-1"), 3.77 (3H, s, 4"-OMe), 3.65 (3H, s, 5'-OMe); ESIMS m/z 553.1 $[M + H]^+$.

(*R*)-MTPA ester of 1 (1b) – ^{1}H NMR (500 MHz, pyridine-d_5) δ_H 7.96 (1H, overlapped, H-6), 4.96 (1H, s, H-1"), 3.75 (3H, s, 4"-OMe), 3.64 (3H, s, 5'-OMe); ESIMS m/z 553.1 $[M + H]^+$.

Measurement of LPS-induced NO production and cell viability – Raw 264.7 cells were seeded into 96-well culture plates at 2×10^6 cells/mL, and stimulated with 1 μg/mL of LPS in the presence or absence of compounds. After incubation at 37 °C for 24 h, nitrite concentration in culture medium was measured with the Griess reagent [containing equal volumes of 2% (w/v) sulfanilamide in 5% (w/v) phosphoric acid and 0.2% (w/v) of N-(1-naphthyl) ethylenediamine solution] as an indicator of NO production. The absorbance was measured at 550 nm against a calibration curve with sodium nitrite standards. Cell viability of the remaining cells was determined by MTT (Sigma Chemical Co., St. Louis, MO)-based colorimetric assay in accordance with previous reports.[9]

Result and Discussion

The EtOAc extract of the *Pleosporales* sp. culture medium was analyzed using UPLC-Iontrap-MS/MS and the generated MS/MS data were uploaded to the Global Natural Product Social Molecular Networking platform (GNPS, www.gnps.ucsd.edu).[5] A comprehensive examination of the biggest cluster allowed the annotation of the node at m/z 277.4 as an alternariol monomethyl ester and m/z 141.9 as a 6-hydroxy-2-methylchroman-4-one by an in-house fungal-derived compound library, while the spectral node in the cluster could not be annotated in the library and GNPS platfrom. Hence, it could be predicted that the molecular family forming the cluster is chromone analogues (Fig. 2). This led to the isolation of the new chromone (**1**) from an EtOAc extract of *Pleosporales* sp., together with five known chromones (**2** – **6**).

Compound **1** was obtained as a white amorphous solid and a molecular formula was determined to be $C_{16}H_{16}O_8$ by analysis of HRMS spectrum, suggesting 9 degrees of unsaturation. The ^{1}H NMR data displayed three aromatic protons [δ_H 6.79 (1H, d, J = 8.3 Hz, H-6), 7.52 (1H, t, J = 8.3 Hz, H-7) and 6.87 (1H, d, J = 8.3 Hz, H-8)], two methyl ester protons [δ_H 3.74 (3H, s, OMe-5') and 3.81 (3H, s, OMe-4")], two methylene protons [δ_H 3.16 (2H, td, J = 7.3, 3.6 Hz, H-1') and 2.86 (2H, t, J = 7.3 Hz, H-2')], one methine proton [δ_H 5.26 (1H, br d, J = 3.6, H-1")], and two exchangeable protons [δ_H 12.08 (s, 5-OH), 4.01 (s, 1"-OH)]. The ^{13}C NMR data revealed 16 carbon signals comprising two methyl, two methylene, five methine, three carbonyl, and four quaternary carbons. The ^{1}H and ^{13}C NMR data obtained for **1** were similar to those for oxalicumone C,[6] a chromone derivative, except for the absence of a methyl signal, as evidenced by the COSY cross peak of H-6/H-7 and H-7/H-8 and the HMBC

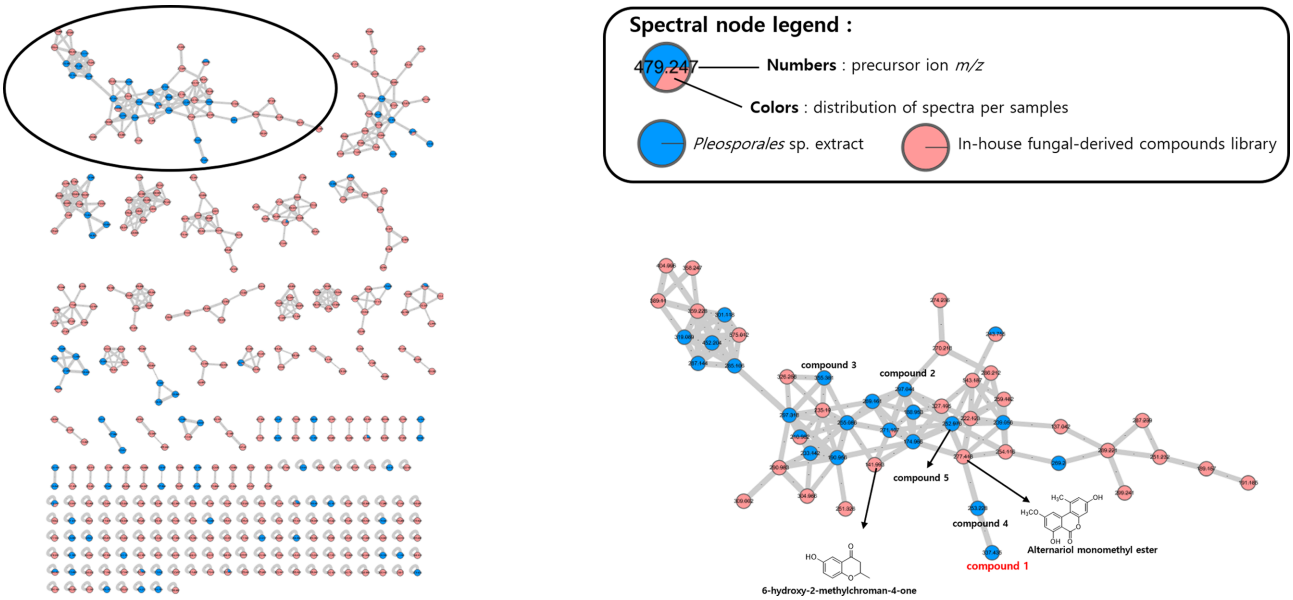
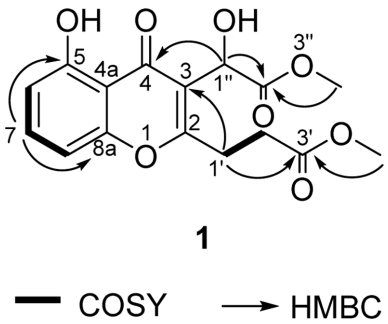


Fig. 2. Molecular Networking of EtOAc extract of *Pleosporales* sp. culture medium.



1

COSY HMBC

Fig. 3. Key HMBC (→) and COSY (—) correlations of compound **1**.

correlations of H-7/C-5 and H-7/C-8a. The gross structure of **1** was assigned by detailed analysis of 2D NMR data (Fig. 3). The absolute configuration at C-1" was assigned to be *S* by the modified Mosher's method with COSY and ^{1}H NMR. Accordingly, the new compound **1**, demethyl-oxalicumone C, was elucidated as shown.

Additionally, the five previously reported compounds were identified as 3,4-dihydroglobosuxanthane A (**2**),[6] globosuxanthone D (**3**),[6] 1,6-dihydroxy-9-oxoanthene-5-carboxylic acid methyl ester (**4**), verixanthone (**5**),[7] and ethyl 8-hydroxy-9-oxo-9*H*-xanthene-1-carboxylate (**6**). All isolates were evaluated for their ability to inhibit LPS-induced NO production in RAW 264.7 cells with amino-guanidine as the positive control (IC_{50} value, 20.4 μM).[9] None of the compounds showed obvious cytotoxicity at the concentration of 200 μM in the MTT assay (data not shown). Compound **1** exhibited marginal inhibitory activity with an IC_{50} value of 118.7 μM. However, the other compounds did not show activity (IC_{50} >200 μM).

In conclusion, a new chromone (**1**) along with five known chromones (**2** – **6**) were isolated from *Pleosporales* sp., guided by Molecular Networking-based dereplication strategy.

Acknowledgments

This research was supported by the Korea University and the National Research Foundation of Korea (NRF-2019R1A2C1006226 and NRF-2019R1A4A1020626) and Korea Polar Research Institute (Grant: PE20010).

References

(1) Zhang, Y.; Crous, P. W.; Schoch, C. L.; Hyde, K. D. *Fungal Divers.* **2012**, *53*, 1-221.
(2) Pittayakhajonwut, P.; Dramae, A.; Madla, S.; Lartpornmatulee, N.; Boonyuen, N.; Tanticharoen, M. *J. Nat. Prod.* **2006**, *69*, 1361-1363.
(3) Yasuta, E.; Terahata, T.; Nakayama, M.; Abe, H.; Takatsuto, S.; Yokota, T. *Biosci. Biotech. Biochem.* **1995**, *59*, 2156-2158.
(4) Zhang, C.; Ondeyka, J. G.; Zink, D. L.; Basilio, A.; Vicente, F.; Collado, J.; Platas, G.; Bills, G.; Huber, J.; Dorso, K.; Motyl, M.; Byrne, K.; Singh, S. B. *J. Nat. Prod.* **2008**, *71*, 1304-1307.
(5) Yang, J. Y.; Sanchez, L. M.; Rath, C. M.; Liu, X.; Boudreau, P. D.; Bruns, N.; Glukhov, E.; Wodtke, A.; de Felicio, R.; Fenner, A.; Wong, W. R.; Linington, R. G.; Zhang, L.; Debonsi, H. M.; Gerwick, W. H.; Dorrestein, P. C. *J. Nat. Prod.* **2013**, *76*, 1686-1699.
(6) Wijeratne, E. M. K.; Turbyville, T. J.; Fritz, A.; Whitesell, L.; Gunatilaka, A. A. L. *Bioorg. Med. Chem.* **2006**, *14*, 7917-7923.
(7) Miao, C.; Wang, J.; Huang, R.; Liu, S.; Zheng, K.; Liu, C.; Lu, Y.; Wu, S. *Folia Microbiol.* **2020**, *65*, 567-572.
(8) Carroll, A. R.; Nash, B. D.; Duffy, S.; Avery, V. M. *J. Nat. Prod.* **2012**, *75*, 1206-1209.

(9) Kim, J. G.; Le, T. P. L.; Hong, H. R.; Han, J. S.; Ko, J. H.; Lee, S. H.; Lee, M. K.; Hwang, B. Y. *Nat. Prod. Sci.* **2019**, *25*, 76-80.
(10) Paz, W. H. P.; de Oliveira, R. N.; Heerdt, G.; Angolini, C. F. F.; de Medeiros, L. S.; Silva, V. R.; Santos, L. S.; Soares, M. B. P.; Bezerra, D. P.; Morgon, N. H.; Almeida, J. R. G. S.; da Silva, F. M. A.; Costa, E. V.; Koolen, H. H. F. *J. Nat. Prod.* **2019**, *82*, 2220-2228.

Received December 21, 2020
Revised December 29, 2020
Accepted December 29, 2020