α-Pyrone and decalin derivatives from the marine-derived fungus Trichoderma harzianum PSU-MF79

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ABSTRACT
Two new compounds, one α-pyrone (trichoharzianone) and one decalin (trichoharzianin), along with eight known compounds including three decahins, two δ-lactones, two carboxylic acids and one isochroman were isolated from the marine-derived fungus Trichoderma harzianum PSU-MF79. The structures were determined by spectroscopic methods. The relative configuration of trichoharzianin was assigned based on NOEDIFF data and coupling constants whereas the absolute configurations were established by comparison of electronic circular dichroism data with those of the co-metabolites. Known (-)-massoia lactone exhibited mild antifungal activity against Cryptococcus neoformans ATCC90113 flucytosine-resistant, Candida albicans ATCC90028 and C. albicans NCPF3153 with MIC values of 128, 200 and 200 μg/mL, respectively, and weak cytotoxic activity against HCT-116 and MCF-7 cell lines with the respective IC50 values of 17 and 32 μM. In addition, it was noncytotoxic against noncancerous Vero cells with an IC50 value of >100 μM.
1. Introduction

*Trichoderma* spp. have received considerable attention for their production of a wide range of secondary metabolites (Javaid and Ali 2011; Faedda et al. 2012; Ahluwalia et al. 2015; Han et al. 2019; Harwoko et al. 2021). Marine-derived fungi are recognized as rich sources of new secondary metabolites with pharmaceutical potential (Blunt et al. 2018). Although the marine-derived fungus *T. harzianum* was first studied in 1993 (Kobayashi et al. 1993), chemical investigation of secondary metabolites from the marine isolates of this fungus was limited. Up to the present, more than 60 compounds with diverse structures and interesting biological activities have been isolated from the marine-derived *T. harzianum* (Shi et al. 2020; Yu et al. 2021). These included antimicroalgal diterpenes and sesquiterpenes (Song et al. 2018), antibacterial sesquiterpenes (Fang et al. 2019), cytotoxic decalins (Suzue et al. 2016; Yamada et al. 2015) and diterpenes (Yamada et al. 2017; Liang et al. 2020), and a \( \beta \)-amyloid fibrillization inhibitory cyclopentenone (Fang et al. 2017). In our ongoing search for bioactive secondary metabolites from marine-derived fungi, *T. harzianum* PSU-MF79 was isolated from an unidentified tunicate collected from Phuket Province, Thailand. The crude broth extract of the fungus PSU-MF79 displayed antifungal activity against *Cryprococcus neoformans* ATCC90113 flucytosine-resistant with an MIC value of 128 \( \mu \)g/mL. Herein, the isolation, structure elucidation, and antimicrobial as well as cytotoxic activities of some isolated compounds which were obtained in sufficient amount are described.

2. Results and discussion

Purification of the crude extracts using various chromatographic techniques led to the isolation of 10 compounds including one new \( \alpha \)-pyrone (trichoharzianone, 1) and one new decalin (trichoharzianin, 2) together with three known decalin derivatives, methyltrichoharzin (3) (Yu et al. 2021), trichoharzin (4) (Kobayashi et al. 1993) and eujavanicol A (5) (Nakadate et al. 2007), as well as five known compounds, (-)-massoia lactone (6) (Romeyke et al. 1991), (3,5R)-3-hydroxydecan-5-olide (7) (Dang et al. 2014), (3,5R)-3,5-dihydroxydecanoic acid (8) (Garg and Singh 2009), (R)-3-hydroxystearic acid (9) (Hiramoto et al. 1971) and dichlorodiaportin (10) (Larsen and Breinholt 1999) (Figure 1). The structures of all metabolites were elucidated based on spectroscopic analysis. The structures of known compounds were also confirmed based on their spectroscopic data and specific rotations (Table S3) which were in good agreement with those previously reported in the literature.

Trichoharzianone (1) was obtained as a colorless gum with the molecular formula C\(_{11}\)H\(_{14}\)O\(_{5}\) determined by the HRESIMS peak at \( m/z \) 249.0723 [M + Na]\(^+\). The UV spectrum showed an absorption band at 267 nm for a conjugated carbonyl chromophore (Zhao et al. 2017). The IR spectrum displayed absorption bands for hydroxy and two carbonyl functional groups at 3367, 1752 and 1617 cm\(^{-1}\), respectively. The \( ^1 \)H NMR spectroscopic data (Table S1) contained signals for one olefinic proton of a trisubstituted alkene (\( \delta_H \) 5.56, s, 1H), four sets of equivalent methylene protons (\( \delta_H \) 2.12 (t, \( J = 7.2 \) Hz, 2H), 1.49 (quint, \( J = 7.2 \) Hz, 2H), 1.34 (m, 2H) and 1.28 (m, 2H)) and one methyl group (\( \delta_H \) 0.88, t, \( J = 6.6 \) Hz, 3H). The \( ^{13} \)C NMR spectrum (Table S1) displayed signals for two carbonyl (\( \delta_C \) 170.5 and 157.6), three quaternary (\( \delta_C \) 171.7, 170.7 and
one olefinic methine (δC 105.2), four methylene (δC 32.4, 28.6, 23.1 and 22.0) and one methyl (δC 14.4) carbons. The 1H-1H COSY correlations of H2-8 (δH 1.49)/H2-7 (δH 2.12) and H2-9 (δH 1.28), and that of H2-10 (δH 1.34)/H3-11 (δH 0.88) as well as the HMBC correlations (Table S1) of H3-11 with C-9 (δC 32.4) and C-10 (δC 23.1) constructed a n-pentyl unit. In addition, the HMBC correlations of H-3 (δH 5.56) with C-2 (δC 157.6), C-4 (δC 170.7) and C-5 (δC 99.2) and those of H2-7 of the n-pentyl unit with C-5 and C-6 (δC 171.7) as well as the chemical shifts of C-2, C-4 and C-6 established an α-pyrone ring with a hydroxy group and the n-pentyl unit at C-4 and C-6, respectively. The substituent at C-5 was assigned as a carboxyl group based on the molecular formula and the chemical shift of 5-CO2H (δC 170.5). Therefore, trichoharzianone had the structure 1.

Trichoharzianin (2) was obtained as a colorless gum with the molecular formula C24H38O5 determined by the HRESIMS peak at m/z 429.2612 [M + Na]+. The UV spectrum showed an absorption band at 216 nm for a conjugated carbonyl chromophore (Kobayashi et al. 1993). The IR spectrum was similar to those of 5 with an additional absorption band at 1655 cm\(^{-1}\) for an unsaturated carbonyl functionality (Kobayashi et al. 1993). The 1H and 13C NMR spectroscopic data (Table S2) were similar to those of 5 except for additional signals for a 3-methylcrotonic acid unit [δH \((J = 1.2\ Hz, 1H)\), 2.18 (d, \(J = 1.2\ Hz, 3H\)) and 1.92 (d, \(J = 1.2\ Hz, 3H\)) and δC 167.2, 158.2, 116.1, 27.6 and 20.5] in 2. This unit was established on the basis of the HMBC correlations (Table S2) of H-2′ (δH 5.75) with C-1′ (δC 167.2), C-4′ (δC 27.6) and C-5′ (δC 20.5) and those of H3-4′ (δH 1.92) and H3-5′ (δH 2.18) with C-2′ (δC 116.1) and C-3′ (δC 158.2) as well as the chemical shift of C-1′. It was attached at C-2 (δC 72.4) on the basis of the HMBC cross peak of H-2 (δH 5.24) with C-1′. The NOEDIT data (Table S2; Figure S1) of 5-Me (δH 1.26)/H-4 (δH 1.64), H-6 (δH 1.95), H-8a (δH 2.12) and H\(_{ab}\)-2′ (δH 2.86 and 2.67), H-4a (δH 1.98)/H-1 (δH 3.55), and H-1/H-2 (δH 5.24) (Nakadate et al. 2007; Li
et al. 2017) indicated that 2 and 5 had an identical relative configuration of the decalin moiety. This assigned relative configuration was confirmed based on these vicinal coupling constants ($J_{1,8a} = J_{4a,8a} = J_{4,4a} = 10.8$ Hz, $J_{1,2} = J_{2,3a} = J_{2,3} = 3.3$ Hz and $J_{6,7} = 4.5$ Hz). In addition, the relative configuration at C-1′′ in 2 was identical to that of 5 based on the NOEDIFF data of H-4a/H$_2$-2′′ ($\delta_{H} 1.47$) and 1′′-Me ($\delta_{H} 0.93$)/H-7 ($\delta_{H} 5.70$) (Nakadate et al. 2007; Li et al. 2017). The absolute configurations of 2 were assigned as 1′′S, 2′′R, 4′′R, 4$a$S, 5′′S, 6′′S, 8$a$R and 1′′′R, identical to those of the co-metabolites 3–5, based on biogenesis consideration and the ECD spectrum of 2 which displayed the same negative and positive Cotton effects at 247 and 301 nm, respectively, as those of 3–5 (Figure S30). In addition, 2 showed the same sign of specific rotation as those of 3–5 (Table S3). Therefore, 2 was identified as a 3-methylcrotonate derivative of 5.

Compounds 1 and 3–8 were evaluated for antibacterial activity against Staphylococcus aureus ATCC25923, methicillin-resistant S. aureus SK1, Pseudomonas aeruginosa ATCC27853, Escherichia coli ATCC25922, Acinetobacter baumannii NPRC005 and A. baubannii NPRC007 as well as antifungal activity against Candida albicans ATCC90028, C. albicans NCPF3153, C. neoformans ATCC90112 and C. neoformans ATCC90113 flucytosine-resistant. In addition, compounds 3–8 were evaluated for cytotoxicity against HCT-116, MCF-7, Calu-3 and Vero cells. None of the tested compounds displayed antibacterial activity at the concentration of 200 µg/mL (Table S4). Only compound 6 showed antifungal activity against C. neoformans ATCC90113 flucytosine-resistant and both strains of C. albicans with MIC values of 128, 200 and 200 µg/mL (Table S4), respectively, and cytotoxicity against HCT-116 and MCF-7 cell lines with IC$_{50}$ values of 17 and 32 µM (Table S5), respectively. Furthermore, it was inactive against noncancerous Vero cells with the IC$_{50}$ value of >100 µM (Table S5).

In conclusion, one new α-pyrene (1) and one new decalin (2) together with eight known compounds (3–10) were obtained from the marine-derived fungus T. harzia-num PSU-MF79. These decalin-type analogues were produced by fungi in the genera Eupenicillium (Nakadate et al. 2007; Li et al. 2014, 2017) and Paecilomyces (Rahbaek et al. 1998). In addition, the known compounds 6–9 were isolated from this fungus for the first time. This is also the first report on antifungal activity against C. neoformans ATCC90113 flucytosine-resistant and cytotoxicity against HCT-116 cell lines of 6.

3. Experimental
3.1. General experimental procedures

The infrared (IR) spectra were recorded neat using a Perkin-Elmer spectrum BX FT-IR spectrometer. The ultraviolet (UV) absorption spectra were measured in MeOH on a Shimadzu UV-2600 UV-Vis spectrophotometer. Electronic Circular Dichroism (ECD) spectra were recorded on a JASCO J-815 polarimeter. The $^1$H and $^{13}$C NMR spectra were recorded on a 300 or a 500 MHz Bruker FTNMR Ultra Shield™ spectrometer using tetramethylsilane (TMS) as an internal standard. The 1D and 2D NMR spectra were recorded using standard Bruker pulse sequences. The specific rotations were recorded on a JASCO P-2000 polarimeter. Mass spectra were obtained on a Bruker MicrOTOF mass spectrometer and Liquid chromatograph-mass spectrometer (2090, LCT, water, micromass). Thin-layer chromatography (TLC) was performed on silica gel 60 GF$_{254}$
Column chromatography (CC) was carried out on Sephadex LH-20, silica gel (Merck) type 60 (70-230 mesh ASTM), or on reverse phase C\textsubscript{18} silica gel.

### 3.2. Fungal material

The marine-derived fungus PSU-MF79 was isolated from an unidentified tunicate collected from Phuket Province, Thailand. This fungus was deposited as BCC83157 at BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The fungus PSU-MF79 was identified based on its morphological and molecular characteristics. Colonies on potato dextrose agar (PDA) at 25°C grew very fast, reaching 4.5 cm in 3 days. The molecular analysis of the internal transcribed spacers (ITS) ribosomal DNA revealed that PSU-MF79 closely matched with several ITS nucleotides of *Trichoderma harzianum* strains comprising *T. harzianum* KY225642, KY764866, KY764853 and KY750449 (100% nucleotide similarity). The GenBank accession numbers of ITS and large subunit (LSU) rDNA are KY397993 and KY398004, respectively. Therefore, this fungus was identified as *Trichoderma harzianum*.

### 3.3. Extraction and isolation

The crude broth (1.80 g, a dark brown gum), mycelial EtOAc (1.14 g, a dark brown gum) and mycelial hexane (737.1 mg, a dark brown gum) extracts of the fungus PSU-MF79 were prepared using the same procedure as described previously (Putra et al. 2019). The crude broth extract was separated by CC over Sephadex LH-20 using 50% MeOH/CH\textsubscript{2}Cl\textsubscript{2} to obtain five fractions (AA-AE). Fraction AC was dissolved with chloroform to give chloroform-soluble (AC1) and chloroform-insoluble (AC2) subfractions. Subfraction AC1 (734.3 mg) was separated by CC over Sephadex LH-20 using 50% MeOH/CH\textsubscript{2}Cl\textsubscript{2} to obtain six subfractions (AC11-AC16). Subfraction AC13 (104.8 mg) was further purified by CC over reverse phase silica gel using 70% MeOH/H\textsubscript{2}O to give five subfractions (AC13A-AC13E). Subfraction AC13C afforded 3 (25.5 mg). Subfraction AC13D (10.3 mg) was separated by CC over Sephadex LH-20 using 50% MeOH/CH\textsubscript{2}Cl\textsubscript{2} followed by CC over silica gel using 1% MeOH/CH\textsubscript{2}Cl\textsubscript{2} to afford 2 (4.0 mg). Subfraction AC14 (388.7 mg) was separated by CC over reverse phase silica gel using 65% MeOH/H\textsubscript{2}O to obtain four subfractions (AC14A-AC14D). Subfraction AC14B (29.3 mg) was subjected to CC over reverse phase silica gel using 60% MeOH/H\textsubscript{2}O to give five subfractions (AC14B1-AC14B5). Subfraction AC14B2 contained 4 (4.5 mg). Subfraction AC14B4 (13.0 mg) was purified by CC over silica gel using 4% MeOH/CH\textsubscript{2}Cl\textsubscript{2} to yield 5 (5.1 mg). Fraction AE (8.9 mg) was separated by dissolving with chloroform to give 1 (5.4 mg). The crude mycelial EtOAc extract was subjected to CC over Sephadex LH-20 using 50% MeOH/CH\textsubscript{2}Cl\textsubscript{2} to afford five fractions (BA-BE). Fraction BB (150.5 mg) was separated by CC over silica gel using 3% MeOH/CH\textsubscript{2}Cl\textsubscript{2} to obtain four subfractions (BB1-BB4). Subfraction BB1 (17.9 mg) was further purified by CC over silica gel using 10% EtOAc/hexane to yield 6 (12.9 mg). Subfraction BB2 (64.4 mg) was subjected to CC over silica gel using 20% acetone/hexane to give 7 (40.0 mg). Subfraction BB4 (27.2 mg) was separated by CC over reverse phase silica gel using 50% MeOH/H\textsubscript{2}O to
give 8 (5.0 mg). The crude mycelial hexane extract was subjected to CC over Sephadex LH-20 using 50% MeOH/CH₂Cl₂ to obtain four fractions (CA-CD). Fraction CB (405.2 mg) was purified by CC over silica gel using 100% CH₂Cl₂ to afford six subfractions (CB1-CB6). Subfraction CB6 (84.6 mg) was subjected to CC over silica gel using 30% EtOAc/CH₂Cl₂ to obtain four subfractions (CB61-CB64). Subfraction CB63 (45.2 mg) was further purified by CC over reverse phase silica gel using 50% MeOH/H₂O followed by CC over silica gel with a mixture of CH₂Cl₂/EtOAc/MeOH in a ratio of 8:1:1 to afford 9 (8.8 mg). Fraction CC (34.7 mg) was separated by CC over silica gel using 100% CH₂Cl₂ to obtain three subfractions (CC1-CC3). Subfraction CC2 (4.9 mg) was further purified by CC over silica gel using 3% EtOAc/CH₂Cl₂ to give 10 (1.2 mg).

Trichoharzianone (1): colorless gum; UV (MeOH) λ_max nm (log ε): 267 (4.42); IR (neat) ν_max cm⁻¹: 3367, 1752, 1617; 1H and 13C NMR, Table S1; HRESIMS m/z [M + Na]⁺ 249.0723 (calcd for C₁₁H₁₄O₅Na, 249.0733)

Trichoharzianin (2): colorless gum; [α]D²⁶ +22.7 (c 0.10, MeOH); UV (MeOH) λ_max nm (log ε): 216 (3.72); ECD (MeOH, c 0.0013 M) λ_max (Δε) 247 (-2.36), 301 (+2.38) nm; IR (neat) ν_max cm⁻¹: 3345, 1690, 1655; 1H and 13C NMR, Table S2; HRESIMS m/z [M + Na]⁺ 429.2612 (calcd for C₂₄H₃₈O₅Na, 429.2611)

3.4. Bioassays

3.4.1. Antimicrobial assays

The activity was conducted using the procedure described by the Clinical and Laboratory Standards Institute (Phongpaichit et al. 2006). MIC values of positive controls are shown in Table S4.

3.4.2. Cytotoxic assays

Human colorectal cancer (HCT-116), human breast cancer (MCF-7), human lung cancer (Calu-3), and African green monkey kidney fibroblast (Vero) cells were acquired from American Type Culture Collection (ATCC; Manasas, Virginia, USA). HCT-116 and MCF-7 cells were cultured in high glucose Dulbecco’s Modified Eagle Medium (DMEM) while Calu-3 and Vero cells were grown in DMEM and Ham’s F-12 mixture (1:1), and Minimum Essential Medium Alpha (MEM-α), respectively. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37 °C under humidified atmosphere of 5% CO₂ and 95% O₂ with cultured media being replaced on alternate day. All cultured media and their supplements were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Cell viability was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays (Sigma-Aldrich Co., St. Louis, USA). In brief, HCT-116, MCF-7, Calu-3 and Vero cells were seeded on 96-well plates (Corning, NY, USA) at a density of 2×10⁴ cells/well and cultured until confluence. The cells were then treated with 50 μM of test compounds (for screening), 50 μM of afatinib (a positive control for Calu-3 cells, MedChemExpress, NJ, USA) or 50 μM of ellipticine (a positive control for HCT-116 cells and MCF-7 cells, Sigma-Aldrich Co., St. Louis, USA). After 24 h (for HCT-116, MCF-7, and Vero cells) and 72 h (for Calu-3 cells) of treatment, the cells were incubated with cultured media containing MTT reagent (0.5 mg/mL) for 4 h at 37 °C.
Following the removal of supernatant, DMSO was added to dissolve the remaining crystals with their absorbance being quantified at 570 nm using a Synergy Neo2 Multi-Mode Microplate Reader (BioTek Instruments, VT, USA). IC₅₀ value of afatinib in Calu-3 cells was 30 μM whereas IC₅₀ values of ellipticine in HCT-116 and MCF-7 cells were 10 and 5 μM, respectively.

Acknowledgments

V.R. thanks the NSTDA Chair Professor grant (the Fourth Grant) of the Crown Property Bureau and the National Science and Technology Development Agency. C.M. acknowledges the grant DBG6180029 from Thailand Research Fund (presently Thailand Science Research and Innovation) and National Research Council of Thailand. S.N. acknowledges the NSTDA Chair Professor grant for a scholarship and to the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation, Thailand, and the Graduate School and Faculty of Science, Prince of Songkla University for partial support.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the NSTDA Chair Professor grant (the Fourth Grant) of the Crown Property Bureau, the grant DBG6180029 from Thailand Research Fund (presently Thailand Science Research and Innovation) and National Research Council of Thailand, and the Graduate School and Faculty of Science, Prince of Songkla University.

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