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Identification of New Lactone Derivatives Isolated from *Trichoderma* sp., An Endophytic Fungus of Brotowali (*Tinaspora crispa*)

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Endophytic fungi is a rich source of novel organic compounds with interesting biological activities and a high level of structural diversity. As a part of our systematic search for new bioactive lead structures and specific profiles from endophytic fungi, an endophytic fungus was isolated from roots of brotowali (*Tinaspora crispa*), an important medicinal plant. Colonial morphological trait and microscopic observation revealed that the endophytic fungus was *Trichoderma* sp. The pure fungal strain was cultivated on 7 L Potatos Dextose Broth (PDB) medium under room temperature (no shaking) for 8 weeks. The ethyl acetate were added to cultur medium and left overnight to stop cell growth. The culture filtrates were collected and extracted with EtOAc and then taken to evaporation. Two new lactone derivatives, 5-hydroxy-4-hydroxymethyl-2H-pyran-2-one (1) and (5-hydroxy-2-oxo-2H pyran-4-yl) methyl acetate (2) were obtained from the EtOAc extracts of *Trichoderma* sp. Their structures were determined on the basic of spectroscopic methods including UV, IR, 1H-NMR, 13C-NMR, HMQC, and HMBC.

Keywords: endophytic fungus, *Trichoderma* sp., *Tinaspora crispa*, lactones

INTRODUCTION

Endophytic microorganisms are bacteria or fungi that live inside plant tissues at any moment of their life cycle, without causing damage or disease symptoms to their hosts (deSouza *et al*. 2011; Sunkar & Nachiyar 2011). They have been found in every plant species examined to date and recognized as the potential products for exploitation in medicine, agriculture and industry. Endophytes are ubiquitous with rich biodiversity. It is noteworthy that of nearly 300.000 plant species that existed on the earth, each individual plant is the host to one or more endophytes. The opportunity to find new and targeting natural products from interesting endophytes microorganisms among myriads of plants in different niches and ecosystem is remarkable (Strobel *et al*. 2005; Anitha *et al*. 2011).

The plant *Tinaspora crispa* (Family Menispermaceae) occupies a very important place in the field of medicinal plants and is widely used as a traditional medicine. *Tinaspora crispa* (locally named "brotowali" in Indonesia) is a small herb which grows widely in temperate and tropical part of Asia. More specifically, the plant is widely found in tropical and subtropical Philippines, Indonesia, Malaysia, Thailand, India, China, and Vietnam. The plant is also known by its numerous synonyms, viz., *Menispermum crispum* Linn., *Tinaspora cordifolia* F. Vill., *Tinaspora tuberculata*, and *Tinaspora rumphii* (Mohammed *et al*. 2012; Elfita *et al*. 2013).

It is widely used in the traditional medicinal practice of peoples living in Malaysia, Indonesia, and Thailand to treat ailments like fever, jaundice, hyperglycemia, wounds, intestinal worms, and skin infections. Moreover, *T. crispa* is also used to treat tooth and stomach ache, coughs, asthma, and pleurisy. In several studies, *T. crispa* has been demonstrated to possess antibacterial, antifilarial, antipyretic, antihyperglycaemic antiproliferative, and antimalarial activities and also has a mild cardiotonic effects (Al-alusi *et al*. 2010; Tungpradit *et al*. 2010), and the use of its stem to various therapeutic purposes such as treatment for diabetes, hypertension, stimulation of appetite and protection from mosquito bites (Zulkhairi *et al*. 2008).

Endophytic fungi are natural products which have specific ability to produce compounds having strong biological activities. Research on isolation of secondary metabolites from endophytic fungi of selected plants begin with chemical profiles revealed

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of secondary metabolites of its species. This fact became strong theoretical foundation to build a method of selecting bioactive compounds and extend its utilization and synthesis. Initial steps involved are selection, screening, dereplication, isolation, and structure elucidation process using spectroscopic methods combined with chemoinformatic analysis (Butler 2004; Larsen *et al*. 2005; Hassan 2007).

As part of a phytochemical study of our research, we have previously reported two alkaloid compounds which is active as antimalarial from endophytic fungus which was isolated from the stem and leave of brotowali (Elfita 2011a). The purpose of the present study was to isolate and determine two lactones from *Trichoderma* sp. from roots of brotowali to get the profile of organic compounds produced by endophytic fungi of brotowali.

MATERIALS AND METHODS

Materials. The root of brotowali were collected on April 2012 from the Indralaya, Ogan Ilir, South Sumatra. Medium for isolation of endophytic fungi: nutrient agar (NA), nutrient broth (NB), potato dextrose broth (PDB), potato dextrose agar (PDA), a series of organics solvent with technical grade and distilled before use, analytical thin layer chromatography (TLC) using Merck (Art.5554) silica gel 60 F_{254}, and column chromatography using Si gel 60 (70-230 mesh).

Instrumentations. The apparatus in the research were colony counter, autoclave, incubator, water bath, microscope, magnetic hotplate, UV lamp, column chromatography, and general apparatus in organic and microbiology laboratory, melting point, FTIR-Perkin Elmer-Spectrum One, UV-Cary Varian 100 Conc I-UV Visible Spectrometer, and NMR – Agilent DD2 500 MHz (^{1}H) and 125 MHz (^{13}C).

Isolation and Purification of Fungal Strains. Isolation of endophytic fungi was done according to the method described by Khan *et al*. (2010). The plants samples were rinsed gently in running water to remove dust and debris. Roots samples were cut into 0.5-1.0 cm segments. Each sample was disinfected with 75% ethanol for 1 min followed by immersion in Sodium hypochlorite (NaOCl 1-13% for 3-10 min, depending on the type of samples) and then once again in 75% ethanol for 30 sec. The segments were then rinsed three times in sterile distilled water and blotted-dry on sterile blotting paper. About 5-6 segments were placed on Potato dextrose agar (PDA) supplemented with penicillin G (100 units mL^{-1}) and streptomycin (100 μg mL^{-1}). The dishes were sealed with parafilm and incubated at 27 ± 2 °C for 4-6 weeks. Most of the fungal growth was initiated within 10 days of inoculation. The fungi that grew out from the segments were periodically isolated and identified by transferring the hyphal tips to fresh PDA plates without antibiotics. Fungi were grown on specified media under specified culture condition for identification. The fungi were identified on the basis of their colonial morphological trait and microscopic observation.

Cultivation of Pure Fungal Strain. Mass growth of pure fungi for isolation and identification of secondary metabolites was carried out by transferring fresh fungal cultures into 15 flasks (1 L each) containing 500 mL of PDB medium. The cultures were then incubated at room temperature (no shaking) for 8 weeks (Elfita *et al*. 2011b; Elfita *et al*. 2012a,b).

Extraction, Exploration, and Structure Elucidation. The culture broth was filtered to remove mycelia. The culture media was then extracted in 5 L ethyl acetate (2 times), followed by filtration and then taken to evaporation. Evaporation of the extract gave a dry extract which was chromatographed over a silica gel column with n-hexane:ethyl acetate as solvent (gradient elution). Based on detection by TLC (SiO gel F254, Merck, Darmstadt, Germany) using n-hexane:EtOAc as a solvent system, collected fractions were combined, and subjected to recolumn over a silica gel with n-hexane: ethyl acetate to obtain pure compounds. Identification of the molecular structure is done by spectroscopic methods including UV, IR, ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC (NMR spectra were recorded at 500 MHz (^{1}H) and 125 MHz (^{13}C) on JEOL JNM ECA-500 spectrometer).

RESULTS

Isolation, Purification, and Extraction of Fungal Strains. Two fungi were isolated from the roots of brotowali the strains were identified as *Trichoderma* sp. and *Aspergillus* sp. by the Sekolah Ilmu dan Teknologi Hayati, Institut Teknologi Bandung. The broth culture was filtered to separate the filtrate and mycelia and extracted twice by partition with an equal volume of ethyl acetate. The organic solvent was evaporated to dry under vacuum to yield 6.2 g of crude extract. All comparative thin layer chomatograpic (TLC) analyses, developed in the following solvents: n-hexane-EtOAc (5:5 v/v); n-hexane-acetone (6:4); and n-hexane-chloroform (3:7), showed two major compounds. The isolation

of these two lactones from ethyl acetate extract of *Trichoderma* sp. from brotowali is described in Figure 1.

Exploration and Structure Elucidation. A portion (1 g) of the total crude extract was subjected to silica gel column chromatography using n-hexane-EtOAc gradient elution to yield five fractions (F1-F5). Fractions (F5) showed a major compound and further separation by column chromatography using n-hexane-EtOAc (6:4-0:10) to yield four subfractions (F5.1-F5.4). Fractions (F5.4) was further purified by elution with n-hexane-EtOAc (5:5) to yield compound 1 (320 mg). Subfractions (F2) showed another major compound and further separation and purification by column chromatography using n-hexane-EtOAc (10:0-6:4 and 8:2, respectively) to yield compound 2 (23 mg).

Compound 1 (5-hydroxy-4-hydroxymethyl-2H-pyran-2-one) was obtained as a white crystal, mp. 155-156 °C; UV (MeOH) λ_{max} nm: 269; UV (MeOH + NaOH) λ_{max} nm: 313; IR (KBr)$_{vmax}$ cm^{-1}: 3361.9 (OH), 3097.7 (CH-aromatic), 2922.2 (CH-aliphatic), 1668.8 (C=O esther), 1610.6, 1581.6 (C = C aromatic), 1226.7 (C = O esther), 1072.4 (C-O alcohol): ^{1}H NMR (DPPH, 500 MHz) δ_H ppm: see Figure 2 and Table 1; ^{13}C NMR (DPPH, 125 MHz) δ_H ppm: see Table 1.

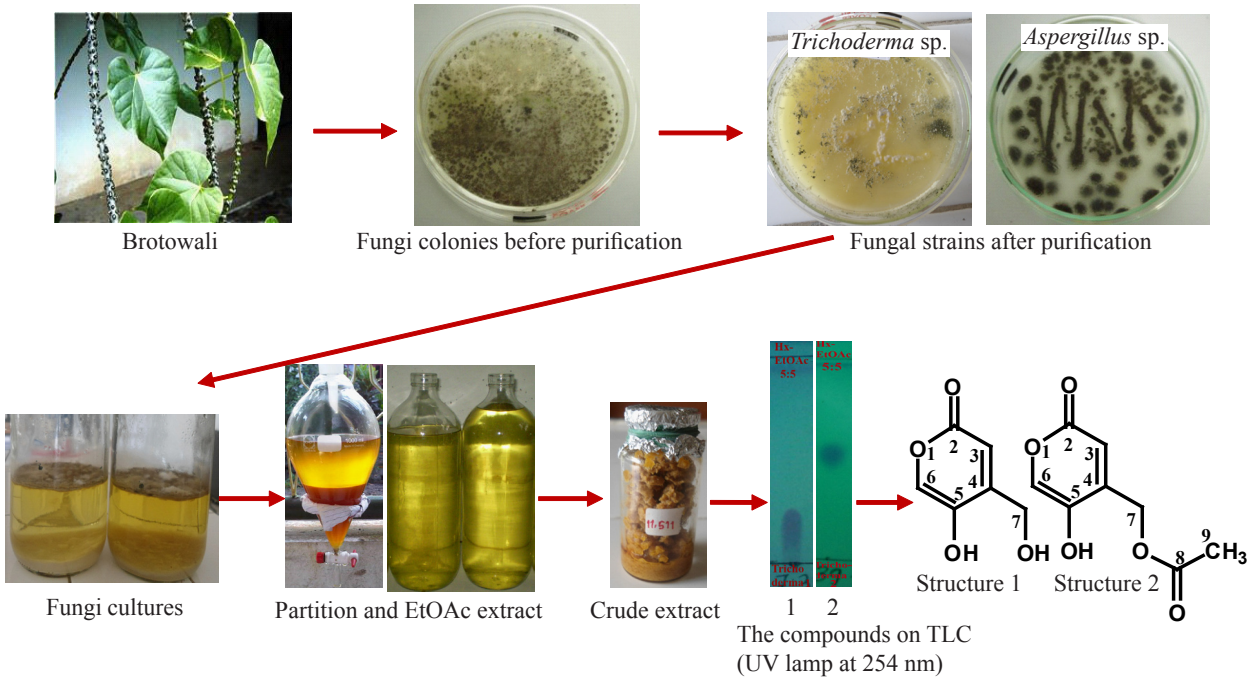

Figure 1. Brief isolation procedures of two lactones from ethyl acetate extract of *Trichoderma* sp. from brotowali.

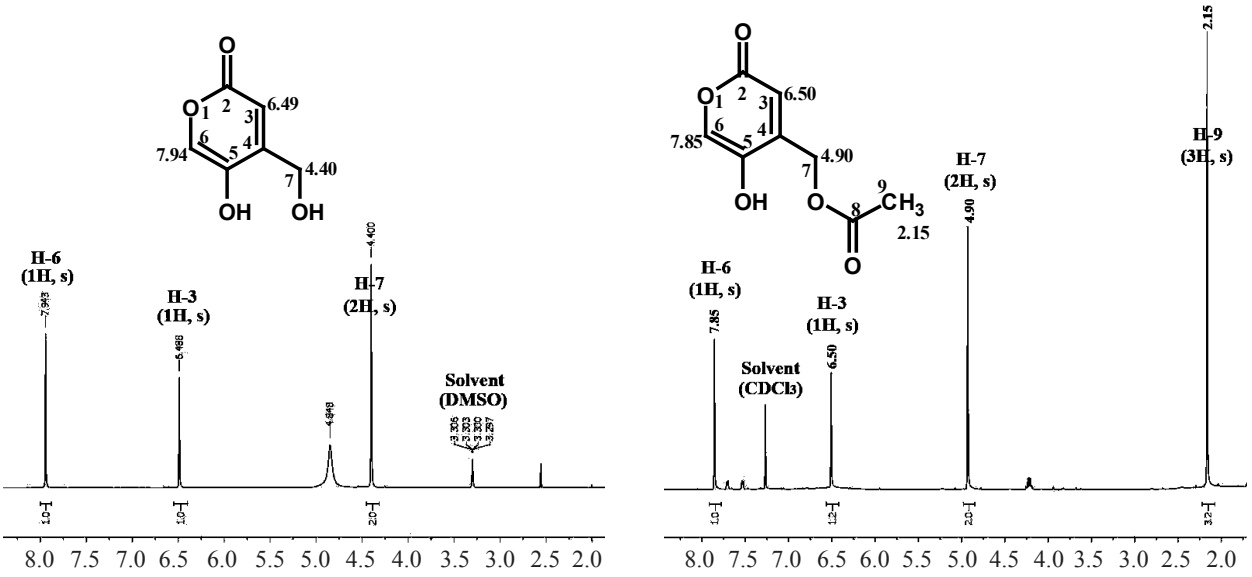

Figure 2. The ^{1}H-NMR spectrum of two lactones from ethyl acetate extract of *Trichoderma* sp.

Tabel 1. The NMR data of compound 1 and 2, recorded at ^{1}H-500MHz; ^{13}C-125 MHz in DMSO and $CDCl_3$

No. C	δ_C (ppm)		δ_H (ppm), ΣH, multiplicity, *J* (Hz)		HMBC	
	1	2	1	2	1	2
2	176.8	173.9				
3	110.7	111.2	6.49 (1H; s)	6.50 (1H; s)	61.2; 147.3; 170.4	145.8; 162.9
4	147.3	145.8				
5	170.4	162.9				
6	141.0	137.9	7.94 (1H; s)	7.85 (1H; s)	147.3; 170.4; 176.8	145.8; 162.9; 173.9
7	61.2	61.4	4.40 (2H; s)	4.90 (2H; s)	110.7; 170.4	111.2; 162.9; 169.8
8		169.8				
9		20.6		2.15 (3H; s)		169.8

Compound 2 ((5-hydroxy-2-oxo-2H pyran-4-yl) methyl acetate) was obtained as a white crystal, mp. 88-89 ^{o}C; UV (MeOH) λ_{max} nm: 269; UV (MeOH + NaOH) λ_{max} nm: 312; IR (KBr)$_{vmax}$ cm^{-1}: 3363.8 (OH), 3080.0 (CH-aromatic), 2960.7-2877.8 (CH-aliphatic), 1730.2, 1656.8 (C = O esther), 1626.0 (C = C aromatic), 1215.2 (C = O esther); ^{1}H NMR (DPPH, 500 MHz) δ_H ppm: see Figure 2 and Table 1; 13C NMR (DPPH, 125 MHz) δ_H ppm: see Table 1.

DISCUSSION

Previous studies have obtained eight endophytic fungi from leaves and stems of brotowali, but have not found the *Trichoderma* sp.; while the *Aspergillus* sp. which were found in this study have also been found in previous studies (Elfita *et al*. 2011a).

5-hydroxy-4-hydroxymethyl-2H-pyran-2-one (Compound 1) was isolated from the EtOAc extract of liquid cultures of *Trichoderma* sp. as white crystal (320 mg). It displayed UV absorbances at λ_{max} (MeOH) 269 nm indicating the presence of aromatic chromophore. These bands gave a batochromic shift with NaOH to 313 nm indicating the presence of hydroxyl group on aromatic ring. The IR spectral analysis showed the characteristic stretching vibrations at 3361.9; 3097.7; and 2922.2 nm due to the -OH, -CH-aromatic, and CH-aliphatic stretching, and C = O stretching vibrations was observed as sharp peak at 1668.8 nm and bending vibrations at 1226.7 nm confirms the presence of lactone ring in the compound. The ^{1}H and ^{13}C NMR spectra (Table 1) indicated the presence of one aromatic methylene group at δ_H 4.40 (2H; s) and δ_C 61.2 ppm, two aromatic proton singlet at δ_H 6.49 and δ_C 110.7 (H-3) as well as δ_H 7.94 and δ_C 141.0 (H-6). Furthermore, the ^{13}C NMR spectrum showed a quaternary carbon at δ_C 147.3 corresponding to C-4, one oxygenated quaternary carbon at δ_C 170.4 and one oxygenated methyne carbon at δ_C 141.0, corresponding to C-5 and C-6, respectively. The signal at δ_C 176.8 indicated the presence of a conjugated lactone (C-2). The HMBC correlations from H-3 to C-7 (δ_C 61.2) and C-5 (δ_C 170.4) and from H-7 to C-3 (δ_C 110.7) and C-5 (δ_C 170.4) revealed the connection from H-3 and H-7 to C-5. The correlations from H-6 to C-2 (δ_C 176.8) indicated the connections of C-6 and C-2 through an oxygen atom. The attachment of the methylene hydroxyl group at C-4 was confirmed by the observed HMBC correlation of C-3 and C-5 (Table 1).

(5-hydroxy-2-oxo-2H pyran-4-yl)methyl acetate (Compound 2) was isolated from the EtOAc extract of liquid cultures of *Trichoderma* sp. as white crystal (23 mg). It displayed UV absorbances at λ_{max} (MeOH) 269 nm, showing high similarity to the UV spectrum of compound 1. These bands gave a batochromic shift with NaOH to 312 nm. The IR spectral analysis showed the characteristic stretching vibrations at 3363.8; 3080.0; and 2960.7-2877.8 nm due to the -OH, -CH-aromatic, and CH-aliphatic stretching, and C = O stretching vibrations was observed as sharp peak at 1730.2 and 1656.8 nm, and bending vibrations at 1226.7 nm confirms the presence of lactone ring in the compound and another C = O ester. The ^{1}H and ^{13}C NMR spectra (Table 1) indicated the presence of one aromatic methylene group at δ_H 4.90 (2H; s) and δ_C 61.4 ppm, two aromatic proton singlet at δ_H 6.50 and δ_C 111.2 (H-3) as well as δ_H 7.85 and δ_C 137.9 (H-6). Furthermore, the ^{13}C NMR spectrum showed a quaternary carbon at δ_C 145.8 corresponding to C-4, one oxygenated quaternary carbon at δ_C 162.9 and one oxygenated methyne carbon at δ_C 137.9, corresponding to C-5 and C-6, respectively. The signal at δ_C 173.9 indicated the presence of a conjugated lactone (C-2). The HMBC correlations from H-3 to C-5 (δ_C 162.9) and from H-7 to C-5 (δ_C 162.9) revealed the connection from H-3 and H-7 to C-5. The correlations from H-6 to C-2 (δ_C 173.9) indicated the connections of C-6 and C-2 through an oxygen atom. The attachment of the methylene (alkyl) esther group at C-4 was confirmed by the observed HMBC correlation of C-3, C-5, and C-8 (C = O esther). Esterification produces acetyl group

occurred on 4-CH_2O- (not on 5-C-O-). That was indicated by the correlation of the triple bond of the methylene protons H-7 to C-8 (C = O ester) in the HMBC spectrum (Table 1). It was also appeared from the UV spectrum which gave a batochromic shift with NaOH to 312 nm indicating the presence of the free hydroxyl group at C-5. The HMBC correlation of compounds is showed in Figure 3.

Base on Dictionary Natural Products data base, these lactones are new compounds. Compound 2 is the result of esterification of compound 1 according to the mode of enzymatic esterification. Previously, similar lactone compounds have been found which are 4-hydroxy-3,6-dimethyl-2H-pyran-2-on and its ester 6-(2-hydroxypropy[]-3-methyl-2-oxo-2H-pyran-4-yl acetate from *Ampelomyces* sp. of *Urospermum picroides* (Hassan 2007). Compound 1 is disubstituted lactone (hydroxyl group at C-5 and hydroxymethyl group at C-4), while that 4-hydroxy-3,6-dimethyl-2H-pyran-2-on is trisubstituted lactone (methyl group at C-3 and C-6, and hydroxyl group at C-4). Compound 2 has esterification on hydroxymethyl group of compound 1, while esterification of 6-(2-hydroxypropy[]-3-methyl-2-oxo-2H-pyran-4-yl acetate found at hydroxyl group on lactone ring. Compound 1 also has similarities with the 6-hydroxy-2-hydroxymethyl-1,4-piron (isokojic acid), ie. have the same disubstituted (hydroxyl and hydroxymethyl group) on ring. The difference is that compound 1 is 1.2-pyrone, while for isokojic acid is 1,4-pyrone.

The fungal *Trichoderma* species inhabiting healthy tissues of host plants as endophytic fungi. Wu *et al*. (2011) investigated the AcOEt extract of the culture broth of *Trichoderma* sp. PR-35 isolated from the healthy stem of *Paeonia delavayi* which yield five sesquiterpene: trichoderic acid, 2b-hydroxytrichoacorenol, cyclonerodiol, cyclonerodiol oxide, and sorbicillin. Other compounds that have been found from *Trichoderma* sp. was taxol isolated from *Taxus chinensis* (Liu *et al*. 2009).

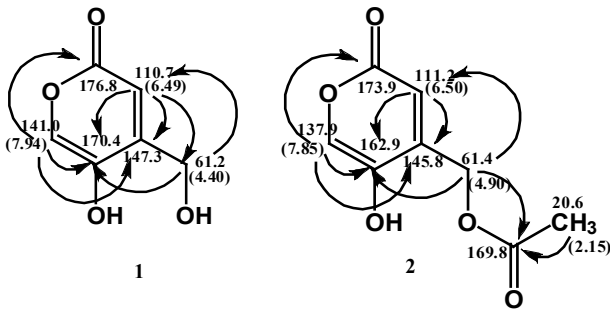

Figure 3. The HMBC correlation of compound 1 and 2.

Exploration of two lactone compounds has increased the types of secondary metabolites from endophytic fungi of brotowali. Exploration of secondary metabolites needs to be done in order to get the profile of organic compounds produced by endophytic fungi of brotowali.

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