Efficient isolation of anthraquinone-derivatives from Trichoderma harzianum ETS 323

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Abstract

Anthraquinone-derivatives, chrysophanol and pachybasin, were purified by a silica column chromatography with two different solvent systems from Trichoderma harzianum ETS 323. The fungus was incubated in sugarcane bagasse solid medium at room temperature without rotation. Structure of chrysophanol was solved by X-ray diffraction and pachybasin by NMR spectra. About 233 ± 13 mg of pure chrysophanol and 773 ± 40 mg of pure pachybasin were recovered per kg of solid cultural medium, with yields 1.7 ± 0.2% and 5.6 ± 0.5%, respectively.

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1. Introduction

Anthraquinones exist in many Chinese herbal plants such as Cassia acutifolia [1], Polygonum cuspidatum (Hu Zhang), Rheum palmatum (Du Huang), Scutellaria baicalensis (Huang Qin), Polygonum multiforum (He Shou Wu), Gänoderma tuidum (Ling Zhi) and Cassia occidentalis (Wang Jiang Nan) [2]. Some microorganisms were reported producing anthraquinones: a few examples are Penicillium islandiicum, Trichoderma polysporum, Aspergillus crystallinus, Phoma foveata, Trichoderma viride, Ascocchyta pisi, and Digitalis spp. [3].

Anthraquinones exhibit many biological functions, such as serving as laxatives [4], diuretics [5], in vivo inhibitory effects towards P388 leukemia in mice [6], phytoestrogens [7], plus various anti-platelet [8], antifungal [9,10], antiviral [11,12], and anticancer activities [2]. They were reported containing the photoprotease activities [13]. They are also used in industry as textile dyes, food colourants [14], and bugs repellents.

Chrysophanol and pachybasin have been purified from some medicinal plants, such as Rheum palaestinum [8] and Rheum emodi [9]. The organic synthesis of chrysophanol has been presented [15] but not pachybasin. T. viride had been reported to produce chrysophanol in liquid medium [3].

Trichoderma species are well-known biocontrol agents which inhibited the growth of soil-borne phytopathogens such as Rhizoctonia and Botritis species [16]. Two anthraquinone-derivatives from a sugarcane bagasse culture of Trichoderma harzianum ETS 323 were isolated and physically characterized as 1,8-dihydroxy-3-methyl anthraquinone (chrysophanol, Fig. 3A), and pachybasin (1-hydroxy-3-methylanthraquinone, Fig. 3B). Although both chrysophanol and pachybasin are known compounds, this is the first time they had been isolated and purified from the T. harzianum. In scientific point views, (1) our preliminary results suggested that chrysophanol and pachybasin involved directly in biocontrol function of T. harzianum against Rhizoctonia solani; (2) our results and a report [13] indicated that anthraquinone derivatives, chrysophanol and pachybasin, in our investigation, with UV illumination can generate radicals cleaving proteins. The latter imply that these free radicals might...
also involve in the antifungal activity. Also, we reported here an economic cost-efficient and high-yield purification method to obtain these two compounds.

2. Materials and methods

2.1. Chemicals

CaCl₂, KH₂PO₄, Na₂HPO₄, MgSO₄·7H₂O, Tween-20, K₂CO₃, FeCl₃, ZnSO₄·7H₂O, (NH₄)₂SO₄, KMnO₄, NaOH, Triton-100, ethyl acetate, hexane, and dichloromethane were purchased from Sigma-Aldrich (St. Louis, USA). Nonidet P 40 substitute was obtained from Fluka (Buchs, Switzerland). Silica gel (Kieselgel 60, 70–230 mesh) and silica plate (DC-Fertigplatten DURASIL-25 UV₂₅₄) were obtained from Macherey-Nagel (Düren, Germany).

2.2. Fungi and culture media

T. harzianum ETS 323 was isolated in Nantou, Taiwan from Dr. Lo’s Laboratory in National Formosa University. T. harzianum ETS 323 was grown on potato dextrose agar (PDA, Difco, MD, USA). Solid medium was prepared. Briefly, sugarcane bagasse was obtained from the local market and dried in the oven at 60 °C for 48 h. Then, it was ground for 5 s with a single speed blender (1000 rpm, ShangTai, Taiwan). About 15 g of the grounded sugarcane bagasse was soaked with 100 ml media (KH₂PO₄, 7 g/l, Na₂HPO₄, 2 g/l, MgSO₄·7H₂O, 1.5 g/l, FeCl₃, 2 mg/l, (NH₄)₂SO₄, 0.5 g/l, glucose, 30 g/l, CaCl₂, 0.1 g/l, ZnSO₄·7H₂O, 1 mg/l). The soaked solid medium was placed in a 500-ml flask. All media were autoclaved (121 °C, 15 lb/in.²) prior to use.

2.3. Isolation of chrysophanol and pachybasin

Conidia of T. harzianum ETS 323 grown on PDA for 6 days were collected by sterile deionized water. About 5 ml containing 10⁶ conidia/ml was added to the soaked solid medium flask and incubated for 8 days at 25 °C. Ethyl acetate (400 ml) was added and dried under reduced pressure at 25 °C. The dry material was redissolved in dichloromethane and applied to a silica column eluted with ethyl acetate and hexane mixture (1:9), with a flow rate of 60 ml/min. The fractions with yellowish color were...
collected and dried under reduced pressure at 25 °C. This material was dissolved in dichloromethane and applied to the silica column (pretreated with NH₃) with a flow rate of 60 ml/min and eluted with a mixture of ethyl acetate and hexane (1:19). The compound 1 and the compound 2 fractions were collected accordingly. Each pool was concentrated under reduced pressure. Both were kept at 4 °C until later use.

2.4. Analytical methods

To run the thin layer chromatography, 1 mg of purified material was added to the silica plate, pretreated with ammonia gas, and developed with a mixture of ethyl acetate and hexane (1:9 or 1:19). UV spectrum of the compound 1 (10 μg/ml in ethyl acetate) was scanned by a DU-640i spectrophotometer (Beckman, USA). The molecular mass of the compound 1 was determined by GC–Mass (Trans Mass) with a column Rtx®-5MS (0.25 mm×30 m, Restek, USA), 70 eV, temperature began at 50 °C and went to 300 °C with increments of 20 °C/min and eluted with helium gas (99.999%) at 1 ml/s. The crystal was formed from a saturated concentration of the compound 1 (chrysophanol) (6 mg/ml) in ethyl acetate. The orange crystal with a dimension of 0.30×0.15×0.10 mm³ was formed under the over-saturated solution at room temperature. The structure was determined by a single-crystal diffractometer (Siemens Smart CCD, Germany) at the National Tsing Hua University Instrument Center (Hsinchu, Taiwan). The structure of the compound 2 was determined by 1D and 2D NMR (500 MHz NMR, Varian UNITYINOVA) at the National Tsing Hua University Instrument Center (Hsinchu, Taiwan). The compound was tested by ¹H NMR, ¹³C NMR, DEPT, HSQC, HMBC, ¹H, and ¹H COSY.

3. Results

3.1. Isolation of compounds

In this report, T. harzianum ETS 323 was grown on an improved solid sugarcane bagasse medium for 8 days. Two molecules, namely compound 1 and compound 2, were purified by silica column chromatography with two different solvent polarities, mixtures of ethyl acetate and hexane, with a ratio (v/v) of 1:9 and 1:19, respectively, after the culture medium was

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Production of pachybasin and chrysophanol from different sources</th>
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<td>Compound/</td>
<td>R. emodi (Kg)</td>
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<tr>
<td>Source</td>
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<tr>
<td>Chrysophanol</td>
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</table>

* a Chrysophanol (mg) per kg root of R. emodi.
* b Pachybasin and chrysophanol (mg) per kg of solid sugarcane bagasse medium.
* c Pachybasin and chrysophanol (mg) per kg of liquid medium.
* d Total mass is higher than the starting material.
* e No datum.

<table>
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<th>Table 2</th>
<th>Yield of pachybasin and chrysophanol from different sources</th>
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<tbody>
<tr>
<td>Compound/</td>
<td>R. emodi</td>
</tr>
<tr>
<td>Source</td>
<td></td>
</tr>
<tr>
<td>Pachybasin</td>
<td>ND</td>
</tr>
<tr>
<td>Chrysophanol</td>
<td>2.23%</td>
</tr>
</tbody>
</table>
* Yield greater than 100.
* a No datum.
extracted with ethyl acetate. Compounds 1 and 2 were tested on the TLC and they are homogeneous (Fig. 1).

3.2. Identification of compounds

3.2.1. Chrysophanol

Compound 1 was isolated as fine needles via flash chromatography and recrystallization from ethyl acetate. A crystal of sufficient quality of compound 1 (Fig. 2) was obtained for X-ray structural analysis. The structural information were C15H10O4, M = 254.23, monoclinic, space group, C2/c, α = 10.739(5) Å, β = 9.810(5) Å, γ = 21.505(11) Å, V = 2264.5(18) Å³, Z = 8, T = 295(2) K, Dc = 1.491 g cm⁻³, F(000) = 1056, μ = 0.109 mm⁻¹, R₁ = 0.135, Rn = 0.2047 for 2828 independent reflections which, upon completion, led to the solution of the structure of 1,8-dihydroxy-3-methyl-anthraquinone (chrysophanol, Fig. 3A), with formula C15H10O4, molecular mass 254 Da, and absorption peaks at 436 and 256 nm. NMR studies on this natural product confirmed the X-ray analysis to be chrysophanol. Signals in the ¹H spectrum indicated the presence of a methyl (δH 2.69), five aromatic protons (δH 7.11, 7.28, 7.66, 7.85, 5H), and two hydroxyl protons (δH 12.04, 12.15, 2H).

3.2.2. Pachybasin

Compound 2 was subjected to 1D and 2D NMR studies. The ¹³C NMR spectrum of compound 2 revealed at 187.9 (C-9), 161.0 (C-10), 153.5 (C-11), 134.1 (C-12), 133.5 (C-9a), 127.3 (C-5, 8, 12), 126.1 (C-5, 8, 12), 122.1 (C-2, d), 120.7 (C-4, d), 114.0 (C-3, s), and 22.2 (C-11, q) ppm, indicated the presence of two ketone carbonyls and six carbon–carbon double bonds. Because the unsaturation number is 11, compound 2 must, therefore, be a tricyclic compound. The ¹H NMR spectrum of compound 2 revealed an aromatic proton at 8.23 (2H, t, J = 6.0 Hz), 7.75 (2H, d, J = 5.0 Hz), 7.57 (1H, s), and 7.04 (1H, s). A methyl signal at 2.41 (3H, s) established a methyl group connected with sp² carbon. The hydroxyl signal at 12.50 (1H, s) was a phenolic proton. The protonated carbons of compound 2 were all assigned via a heteronuclear, single quantum correlation (HSQC) experiment. A combination of ¹H-¹H COSY and ¹H-detected heteronuclear multiple-bond correlation (HMBC) experiments allowed for the partial structure of compound 2. A similar compound to compound 2 was identified by X-ray diffraction and was assigned as chrysophanol. Compound 2 differed from chrysophanol in losing a hydroxyl group at C-8, and was accordingly dubbed pachybasin (1-hydroxy-3-methyl-anthraquinone), with formula C15H10O4 (Fig. 3B), molecular mass being 238 Da established by GC–MS (Fig. 4) and absorption peaks at 402 and 254 nm. Chrysophanol has one more OH group than pachybasin, located at C-8, making the difference in absorption peaks of chrysophanol and pachybasin at 430 and 402 nm, accordingly. Different solvents such as Triton-100, Tween-20, DMSO, acetic acid, ethyl acetate, and NP40 had been used to try to dissolve both compounds. Up to 6 mg ml⁻¹ of each compound dissolved in DMSO, respectively.

3.3. Productions and yields

The productions were 233±13 mg for chrysophanol, and 773±40 mg for pachybasin per kg of solid medium (Table 1). The yield was calculated based on the dry weight of purified compound divided by dry weight of the extracted material that generated in 1 kg of solid sugarcane medium. So, they are 1.7±0.2% and 5.6±0.5% for chrysophanol and pachybasin, respectively (Table 2).

4. Discussion

The isolation of chrysophanol was reported from Rheum emodi [9], and T. viride [17]. The organic synthesis of chrysophanol was also reported [15]. The pulverized roots were extracted with methanol and applied to silica gel column chromatography. The primary elution solvents were mixtures of hexane and chloroform with ratios (v/v) of 3:1, 2:1, 1:2 and 1:4; and changed solvents to mixtures of chloroform and methanol with ratios (v/v) of 9:1, 8:2, 7:3, and 1:1. The fractions containing chrysophanol were collected and concentrated by reduced pressure. Then, the concentrated material was applied to a silica gel column with secondary elution solvents, mixtures of hexane and chloroform, with ratios of 3:1, 2:1, 1:1, 1:3 and chloroform. Through these long procedures, plus many years of growth period, 147.4 mg chrysophanol per kg roots of R. emodi was obtained (Table 1) with a yield of 2.23% (Table 2) at a tremendous cost. Both compounds were obtained from T. viride. T. viride was incubated in liquid PDB media for 14 days with shaking. The culture media were then applied to a silica column eluted with a mixture of petroleum ether and benzene, with a ratio (v/v) 3:1 and 1:1, respectively, and finally eluted with a mixture of benzene and chloroform. Although T. viride produced 714 mg of pachybasin and 247 mg chrysophanol in one liter of PDB (Table 1), the yields we calculated exceeded 100% (Table 2). The organic synthesis of chrysophanol was also reported with a yield of 14.7% [9] (Table 2). Pure chrysophanol could be harvested after six synthesized steps and six purifications procedures. However, pachybasin has not been reported by organic synthesis.

To produce chrysophanol and pachybasin, T. harzianum ETS 323 was incubated in sugarcane bagasse obtained free of charge from the local street vendors to whom it was a waste. Solvents volume used to extract compound were constrained to minimum because of solid sugarcane bagasse medium. During incubation, T. harzianum ETS 323 was incubated at room temperature without shaking (compared to T. viride which was incubated in liquid medium with shaking). However, instead of multiple steps of extraction and purification from other resources, only one silica column and two solvent systems applications could obtain both compounds with much better production and yield from T. harzianum ETS 323. The productions were 233±13 mg for chrysophanol, and 773±40 mg for pachybasin per kg of solid medium (Table 1). The yield was 1.7±0.2% and 5.6±0.5% for chrysophanol and pachybasin, respectively (Table 2). Cost efficiency, environment friendliness, and energy saving were met in this investigation.
The structure and function relationship of anthraquinone derivatives had been reported and shown to be highly diverse. The alkylating functionalities in the molecules maximize the anticancer activity by binding tightly with DNA to disrupt the DNA function [18]. Also, the inhibition of protein kinase CK2 by anthraquinone derivatives was proposed as an anticancer function [19]. The hydroxyanthraquinones with unchelated hydroxyl group exhibited phytoestrogen functions with an affinity to human estrogen receptors [7]. Hydroxyanthraquinones are therefore treated as common scaffolds to develop inhibitors of protein kinase CK2 [19]. Both compounds in this research have methyl group on the C-3 indicating the possible anticancer function. Further investigation will be required to discern the biochemical mechanism of anthraquinone derivatives.

Other than the high yield, to obtain these two compounds from \textit{T. harzianum} will present several advantages over the other resources: (1) growth period of this microorganism is much shorter than that of plants which always require year-long cultivation; (2) the production of these compounds by \textit{T. harzianum} is much more consistent than that from the root of plants which usually varied under different environmental conditions; and (3) solid sugarcane bagasse cultivation save more energy and solvent than the liquid cultivation of \textit{T. viride} or organic synthesis process. Here we have established an efficient approach concerning microorganism resources of chrysophanol and pachybasin from \textit{T. harzianum} ETS 323, and it is a cost-efficient method to obtain these two common scaffolds.

5. Simplified description of the method and its application

In this report, chrysophanol and pachybasin were purified from \textit{T. harzianum} ETS 323 with one silica gel chromatography column with two combinations of ethyl acetate/hexane solvent systems after the fungus grown on sugarcane bagasse had been saturated with media for 8 days at room temperature without shaking. The cost-efficient methodology is obviously superior to others and the common scaffolds for anthraquinone derivatives are the applications.

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