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Chemical Studies on a Curcumin Analogue Produced by Endophytic Fungal Transformation

[1,3]P. Simanjuntak, [1]T.K. Prana, [1]D. Wulandari, [2]A. Dharmawan,
[3]E. Sumitro and [1,3]M.R. Hendriyanto
[1]Research Center for Biotechnology, Indonesian Institute of Sciences,
Jalan Raya Bogor km 46, Bogor 16911, Indonesia
[2]Research Center for Chemistry, Indonesian Institute of Sciences,
Kawasan Puspitek, Serpong 15314, Indonesia
[3]Faculty of Pharmacy, Pancasila University, Jalan Srengseng Sawah,
Jagakarsa, Jakarta 12460, Indonesia

Abstract: With the aim of obtaining more potent curcumin analogues as well as to study the promise of curcuma endophytes in biotransformation process, fungal transformation of curcumin using an endophytic fungus was investigated. Endopyhtic fungi, existing as non-pathogenic microbes in the tissues of plants, have a capacity to transform certain chemical substances into their more potential analogues. Previous study showed that an endophytic fungus with code CL-Bel-5F, isolated from the rhizome of *Curcuma longa* L., was able to transform curcumin (1) into its analogue, however, its chemical structure has not been established yet. The purpose of current study, therefore, was to obtain the chemical structure of curcumin analogue produced by endophytic-fungal transformation. The result showed that biotransformation using endophytic fungus CL-Bel-5F, was successfully able to transform curcumin (1) to hexahydrocurcumin (5-hydroxy-1,7-bis(4-hydroxy-3-metoxyphenyl)-hepta-3-one) (2), whose structure was established based upon extensive spectroscopical analyses particularly 1D and 2D-NMR studies.

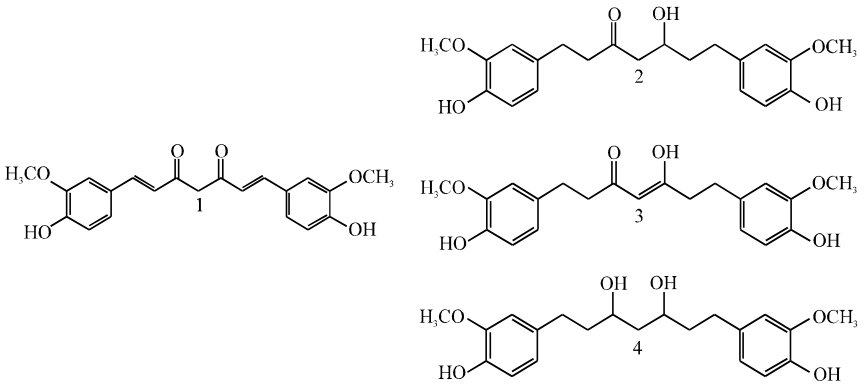
Key words: Curcumin, endophytes, fungal transformation, spectroscopical analysis

INTRODUCTION

Curcumin [1,7-bis(4-hydroxy-3-metoxyphenyl)-1,6-heptadiena-3,5-dione)] (1), a natural yellow pigment compound isolated from many *Curcuma* species, is widely used as an innovative therapeutic agent since, it exhibits many important pharmacological activities such as anti-oxidant, anti-inflammatory and anti-carcinogenic activities (Lin and Lin-Shia, 2001). Many studies found that many analogues of 1 exhibit more potent pharmacological activities. For example, its hydrogenated analogues such as, hexahydrocurcumin (2), tetrahydrocurcumin (3) and octahydrocurcumin (4), exhibit more potent antioxidant activity (Morales *et al*., 2007). Other synthetic analogues of 1 also demonstrate more potent anti-carcinogenic activity and no toxicities *in vivo* (Ohori *et al*., 2006).

Corresponding Author: Mario Rovani Hendriyanto, Research Center for Biotechnology, Indonesian Institute of Sciences, Jalan Raya Bogor km. 46, Bogor 16911, Indonesia Tel: (+62)-21-8754587 Fax: (+62)-21-8754588

However, most of these analogues are produced by chemical synthesis, except for 2-4 which occur as metabolites of 1 in human and rat hepatocytes (Ireson *et al*., 2001). To our knowledge, no microbial transformation studies of 1 particularly using plant-associated microorganisms (endophytes) have been published thus far, although many studies found that endophytic microorganisms are obviously potential to modify the structures of certain natural compounds into their analogues (Zikmundová *et al*., 2002; Shibuya *et al*., 2003; Agusta *et al*., 2005; Bastos *et al*., 2007).



It was decided to carry out fungal transformation using an endophytic fungus isolated from *Curcuma longa* L. to convert 1 into its analogue. Our previous study has been done to examine 45 endophytic fungi isolated from the rhizome of *Curcuma longa* L. which was taken from different locations in Java, Indonesia. Of these, a fungal strain with code CL-BL-5F was consistently found to perform biotransformation of 1 along with other 3 fungal strains (Prana *et al*., 2009). The main goal of the current study was to obtain the chemical structure of the biotransformed product by endophytic fungus CL-Bel-5F which could be advantageous to understand the biotransformation pathway of 1 by the fungus. Furthermore, it may give a significant contribution for both chemical synthetic and bioindustrial process, since, fungi are reliable source of interesting biosynthetic genes and enzymes. The current study found that endophytic fungus CL-Bel-5F was capable of transforming 1 into hexahydrocurcumin [5-hydroxy-1,7-bis(4-hydroxy-3-metoxyphenyl-hepta-3-one)] (2). The chemical structure of this biotransformed compound was fully established based on extensive spectroscopical studies particularly 1D and 2D-NMR.

MATERIALS AND METHODS

General Experimental Procedures

The current study was conducted in 2008. Both ^{1}H-NMR spectra and ^{13}C-NMR spectra were recorded on a JEOL α400 spectrometer in $CDCl_3$ at 500 MHz using TMS as standard. The UV spectra were recorded on a Shimadzu UV 160 instrument, while the IR spectra were obtained on a Shimadzu FT-IR 8400 spectrophotometer. The MS spectra were taken on Liquid Chromatography-Mass Spectrometry Mariner Biospectrometry. The HPLC was performed on Shimadzu FRC-10A with conditions as follows: Capcell Pak C_{18} (250/6 mm); flow rate (1 mL min^{-1}); UV detector (290 nm); asetonitril:H_2O (1:1). TLC experiments were carried out on silica gel GF_{254} precoated plates (Merck). The fermentation media (PDA and PDB) were obtained from DIFCO. Curcumin as standard was purchased from Merck.

Fungal Transformation of Curcumin by the Endophytic Fungus

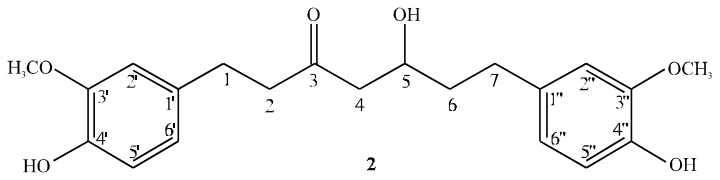
Pure isolate of endophytic fungus with code CL-Bel-5F, previously isolated from fresh and healthy *Curcuma longa* L. taken from Serang, Indonesia (Prana *et al*., 2009) was used to perform biotransformation. Preculture isolate (5 mL) of endophytic fungus with code CL-Bel-5F was inoculated into potato dextrose broth medium (1000 mL) and cultivated for 3 days under shaking at 120 rpm at 25°C. Fungus in the medium without any additions served as controls. A solution of curcumin (1, 100 mg) in MeOH (50 mL) was added to the cultivation medium and shaking continued for 14 days. The stability of the substrate in the medium was analyzed in separate uninoculated flask. The reaction mixture was then filtrated to remove the fungus bodies. The filtrate was extracted with EtOAc (1:1) and concentrated in vacuo to give crude extract, which was then purified by Silica gel column chromatography (SiO_2, gradient eluation : n-hexane; n-hexane : EtOAc = 20:1, 5:1, 2:1; $CHCl_3$: MeOH = 1:1) and preparative TLC on Si gel precoated plates by using n-hexane-EtOAc (1:1) as the solvent system to yield 2 (10,7 mg, 2.14%). The purity of 2 was then confirmed by HPLC. Subsequently, the chemical structure of 2 was established by spectroscopy methods particularly one dimensional (^{1}H-NMR, ^{13}C-NMR and DEPT) and two dimensional NMR studies (^{1}H-^{1}H COSY, HMQC and HMBC).

RESULTS

TLC analysis of biotransformed product: The endophytic-fungal transformation of 1 produced more polar biotransformed compound as shown on TLC (Fig. 1). These compound showed different Rf value compared to 1 and controls.

Extraction and Purification

Extraction on filtrate with EtOAc (1:1) gave crude extract (385.3 mg) which was then fractionated by silica gel column chromatography to yield 23 fractions. The desired compound existed in fraction 14-22 based on TLC. The appropriate fractions were combined and purified using preparative TLC to yield pure compound (10.7 mg, 2.14%). The HPLC analysis gave single peak with Rt 6.8 min, assuming that the biotransformed compound was pure:



2

Structure Elucidation of Biotransformed Product (2)

Chemical structure of compound 2 was fully established using spectroscopical studies. The NMR spectroscopical analysis then confirmed that biotransformed compound is hexahydrocurcumin.

The Spectroscopical Data of Compound 2

The UV (MeOH) λ max 288 nm; IR (KBR) cm^{-1}: 3416.66, 1705.92. LC-MS m/z: 375 (M^+). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.56, 1.76 (CH_2); δ 2.54 (CH_2); δ 2.57, 2.72 (CH_2); δ 2.71, 2.81 (CH_2); δ 3.85 (OCH_3); δ 3.86, 4.03 (-CH-); δ 6.65 (=CH-): δ 6.66 (=CH-); δ 6.69 (=CH-); δ 6.80 (=CH-); δ 6.82 (=CH-). ^{13}C-NMR ($CDCl_3$, 500 MHz): δ 29.43 (t, C-1); δ 45.57 (t, C-2); δ 211.66 (s, C-3); δ 49.51 (t, C-4); δ 67.03 (d, C-5); δ 38.49 (t, C-6); δ 31.58 (t, C-7); δ 133.85 (s, C-1');

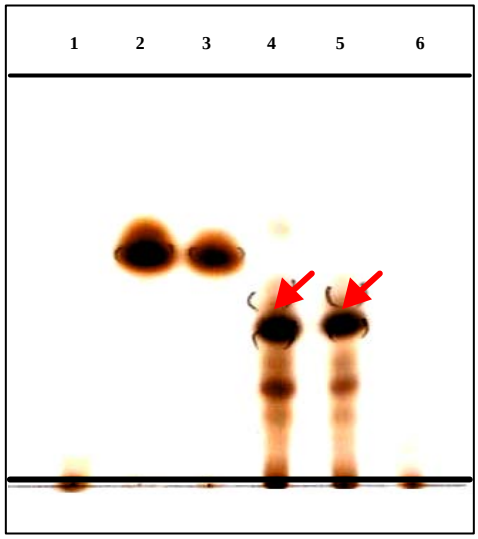


Fig. 1: TLC profile of biotransformed product (Note: Lane 1: fermentation medium; Lane 2: curcumin; Lane 3: curcumin with medium; Lane 4~5: biotransformed compound and Lane 6: fungus with medium). Arrows indicate the position of biotransformed compound

δ 111.12(d, C-2'); δ 146.56 (s, C-3'); δ 143.86 (s, C-4'); δ 114.56 (d, C-5'); δ 121.07 (d, C-6'); δ 132.70 (s, C-1"); δ 111.22 (d, C-2"); 146.61 (s, C-3"); 144.13 (s, C-4"); δ 114.42 (d, C-5"); 120.88 (d, C-6"); δ 56.03 (q, OCH_3). Comprehensive review on these spectroscopical data was discussed below.

DISCUSSION

Compound 1 was transformed into 5-hydroxy-1,7-bis(4-hydroxy-3-metoxyphenyl-hepta-3-one (2), which showed the lack of ketone at C-5 and is substituted by a hydroxyl. Its IR spectrum displayed intense absorptions at 1602, 1515, 1452 (Aromatic C=C), 1705 (C=O) and 3416 (OH) cm^{-1}. The LC-MS analysis gave a quasimolecular ion (M^+) at m/z: 375, which is 7 amu more than that of 1 (m/z: 368 [M^+]) and could correspond to its hydrogenated analogue, hexahydrocurcumin as those reported by other researchers (Yamagashi *et al*., 1972; Morales *et al*., 2007). The ^{1}H-NMR spectrum featured a proton singlet at δ 5.29 due to the aromatic hydroxyl group. The methoxyl group at both aromatic rings was confirmed by a proton singlet at δ 3.86 which is the same as that of 1. Proton multiplet centered between δ 6.65~6.82 was assigned to the aromatic methylen groups, while another proton multiplet at δ 4.03 was due to the hydroxyl at C-5, which is distinctive for compound 2. This result is nearly similar with those reported by other researcher, suggesting that compound 2 is hexahydrocurcumin (Yamagashi *et al*., 1972).

The ^{13}C-NMR spectrum of 2 exhibited resonances for all twenty-one carbons. The multiplicity of each carbon was determined by DEPT experiments. It confirmed the presence of five CH_2, seven CH, and six single carbons. The ^{13}C-NMR spectrum showed singlet at δ 211.66 due to a carbonyl at C-3. The aromatic methoxyl signals resonated at δ 56.03. Signal

Table 1: NMR spectral data of compound 2

Compound position	$^{13}C^{a)}$	Connected $^{1}H^{b)}$	HMBC correlations	COSY correlations
1	29,43 (t)	2,81; 2,71	C-1/H-2, H-2'	H-1/H-2
2	45,57 (t)	2,71; 2,81	C-2/H-1	H-2/H-1
3	211,66	-	C-3/H-1, H-2, H-4	-
4	49,51 (t)	2,54	-	H-4/H-5
5	67,03 (d)	4,03	C-5/H-4, H-6, H-7	H-5/H-4, H-6
6	38,49 (t)	1,59; 1,76	C-6/H-4, H-7	H-6/H-5, H-7
7	31,58 (t)	2,57; 2,72	C-7/ H-5, H-6, H-2''	H-7, H-6
1'	133,85 (s)	-	C-1'/H-1, H-5'	-
2'	111,12 (s)	6,66	C-2'/H-1, H-6'	H-2'/H-5', H-6'
3'	146,56 (s)	-	C-3'/H-2', H-5'	-
4'	143,86 (s)	-	C-4'/H-2', H-5', H-6'	-
5'	114,56 (s)	6,82	C-5'/H-6'	H-5'/H-2'
6'	121,07 (s)	6,65	C-6'/H-1	H-6'/H-2'
3'-OMe	56,03 (q)	3,85	C-3'/OMe	-
1''	132,70 (s)	-	C-1''/H-7, H-5''	-
2''	111,22 (s)	6,66	C-2''/H-7, H-6''	-
3''	146,56 (s)	-	C-3''/H-2'', H-5''	-
4''	144,13 (s)	-	C-4''/H-2'', H-5'', H-6''	-
5''	114,42 (s)	6,80	C-5''/H-6''	H-5''/H-6''
6''	120,88 (s)	6,65	C-6''/H-7	H-6''/H-5''
3''-OMe	56,03 (q)	3,85	C-3''/OMe	-

(a) Multiplicities were determined by DEPT, (b) Connections were determined by HMQC

at δ 67.03 confirmed the presence of hydroxyl at C-5. The presence of aromatic rings were confirmed by doublets ranging between δ 111.12~146.61. The ^{1}H and ^{13}C NMR spectrum of 2 are nearly similar with that of 1, however the distinctive hydroxylated methine signals at δC 67.03 and δH 4.03 of 2 exhibited useful distinctiveness between the two.

Two-dimensional NMR experiments (HMQC, COSY, and HMBC) are very informative (Silverstein *et al*., 2005). The HMQC experiment helped to confirm the ^{1}H- and ^{13}C-NMR chemical shift assignments. The HMQC spectrum of 2 exhibited correlations of hydroxyl proton (δ 4.03) to δ 67.03 (C-5) in the 4-hydroxy-hepta-3-one moiety. The aromatic protons at δ 6.65 ~ 6.82 showed direct connectivity with aromatic carbon at δ 111.12 ~ 121,07. Similarly, proton signal at δ 3.85 showed a one-bond correlation with the methoxylated C-4' (δ 56.03). The $^{1}H/^{13}C$ one-bond correlations are showed in Table 1. The ^{1}H-^{1}H COSY analysis (Table 1) gave correlation between proton H-2' (δ 6.66) and H-6' (δ 6.65); H-1 (δ 2.81) and H-2 (δ 2.71); H-5 (δ 4.03) and H-4/H-6 (δ 2.54/δ 1.76). The HMBC analysis (Table 1) gave correlations of C-1 (δ 29,43) to H-2 (δ 2,71); C-6 (δ 38,49) to H-7 (δ 2,57); C-5 (δ 67,03) to H-4 (δ 2,54); C-2' (δ 111,22) to H-6' (δ 6,65); C-5" (δ 114,42) to H-6" (δ 6,65); C-1" (δ 132,70) to H-7 (δ 2,72); C-1' (δ 133,85) to H-5' (δ 6,82); C-3' (δ 146,56) to H-2' (δ 6,66); the C-3 carbonyl (δ 211,66) to H-1; H-2; H-4 respectively (δ 2,71; 2,81; 2,54).

Based on these spectroscopy analysis, the biotransformed compound (2) is confirmed as hydrogenated curcumin analogue, hexahydrocurcumin which is naturally present as metabolized compound of 1 in human and rat hepatocytes (Ireson *et al*., 2001). The fungus is capable of hydrogenating at both 1,6-diena and one carbonyl in heptadiena moiety of 1 as shown in the biotransformation pathway in Fig. 2. This fact corresponds to the hypothesis that endophytic microorganisms are potential to perform biotransformation of certain natural compounds. Further biotransformation studies using other substrate instead of 1 should be performed in order to confirm the substrate specificity of both hydrogenation and oxidation enzymes of the endophytic fungus. Investigations of anticancer and hepatoprotector activities of compound 2 as well as the classification of endophytic fungus CL-Bel-5F are still in progress.

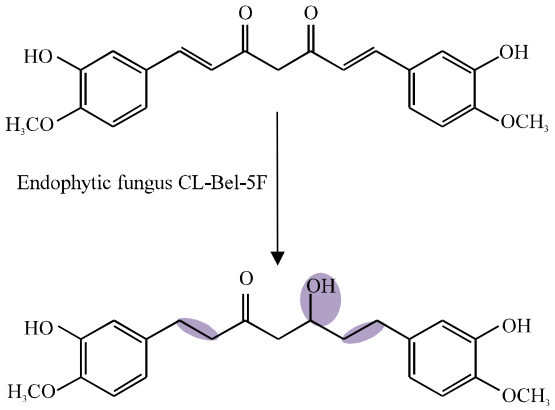


Fig. 2: The biotranformation pathway of 1 by endophytic fungus CL-Bel-5F

The result obtained showed that the utilization of endophytic fungi isolated from the same source as the substrate could be potential to obtain new analogues of certain natural compounds. The utilization of endophytic fungi in this research could, thus, be a possible method for obtaining analogues of 1. The analog obtained comes from hydrogenation reactions, which normally take place during mammalian metabolism. Nevertheless, this analogue might be useful in order to investigate the metabolism of 1 in mammals.

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