

(+)-EPOXYDONE: A MAJOR SECONDARY METABOLITE AS ANTIBACTERIAL AGENT FROM *Phomopsis* sp. TcBt1Bo-6 ISOLATED FROM STEM OF BROTOWALI PLANT (*Tinospora crispa*)

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ABSTRACT

Isolation, characterization, and bioproduction capacity of (+)-epoxydone produced by *Phomopsis* sp. TcBt1Bo-6 was performed in this present report. The fungus was cultured in potatoes dextrose broth (PDB) medium. Isolation and purification of (+)-epoxydone using chromatography methods. The metabolite structure elucidation was carried out by using UV, FTIR, 1D/2D-NMR, MS, and optical rotation, and compared with other reports. This fungus has the bioproduction ability of (+)-epoxydone of 189.43 mg/L. The metabolite of (+)-epoxydone has potent as antibacterial and antimycobacterial against *E.coli*, *S.aureus*, and *M.smegmatis* with MIC values of 25, > 50, and 50 (partial) µg/mL, respectively. This study is the new report on the bioproduction of bioactive metabolite (+)-epoxydone as well as the antibacterial and antimycobacterial activities of this compound.

Keywords: Isolation, Characterization, Bioproduction, (+)-Epoxydone, *Phomopsis* sp. TcBt1Bo-6.

RASAYAN J. Chem., Vol.15, No.4, 2022

INTRODUCTION

Endophytic fungi are considered to be rich sources of diverse bioactive metabolites.¹ Several bioactive metabolites produced by endophytic fungi exhibited biological activities such as anti-inflammatory, antioxidant, anticancer, and antibacterial.²⁻⁶ Some of those bioactive substances are cytochalasin D, antibacterial and chemotherapeutic agents produced by *Xylaria* sp. DAP KRI-5,⁷ cytochalasin H, an antibacterial substance isolated from *Diaporthe amygdali*,⁵ beauvericin from *Fusarium proliferatum* as anticancer,⁶ myrothecine I isolated from *Paramyrothecium roridum* showed strong cytotoxicity towards cell lines,⁸ asperphenalenone D obtained from *Aspergillus* sp. CPCC 400735 showed anti-HIV.⁹ Caspiciene as antitubercular agent obtained from *Aspergillus* sp.¹⁰ Monoterpene lactone derivative, an antifungal metabolite isolated from *estalotiopsis foedan*.¹¹ Neofusnaphthoquinone B was isolated from *Neofusicoccum australe* that performed as a moderate antibacterial against methicillin-resistant *S.aureus*,¹² kojic acid isolated from fungi *Aspergillus flavus* inhibited the growth of *S.aureus* and *E.coli*,⁴ and some volatile compounds such as naphthalene derivative and acetoin from *Aspergillus* sp.¹³ A previous study reported that eighty isolates of the endophytic fungi from *T.crispa* were successfully isolated and one fungus, *Phomopsis* sp. TcBt1Bo-6 has potent antibacterial activity against *Escherichia coli*.^{14,15} The main bioactive compound of this fungus after being characterized was (+)-epoxydone (unpublished data). While the metabolite isolation, chemical structure elucidation, and capacity of bioproduction of (+)-epoxydone from *Phomopsis* sp. TcBt1Bo-6 has not been studied. Based on our best knowledge, this *Phomopsis* sp. TcBt1Bo-6 from *T.crispa* is capable to produce a greater amount of (+)-epoxydone compared to other fungi. The purposes of this research are the isolation of this major metabolite, determination of the chemical

structure of the pure compound, and assessment of the production capacity of (+)-epoxydone from the endophytic fungus *Phomopsis* sp. TcBt1Bo-6.

EXPERIMENTAL

Fungal Growth

The endophytic fungus TcBt1Bo-6 from the stem of the *T.crispa* plant was obtained from Bogor, West Java, Indonesia. Identification of this fungus strain of TcBt1Bo-6 is *Phomopsis* sp., then stored at the low temperature of -80 °C in the Indonesian Culture Collection. These fungi were scaled up using PDB medium at room temperature, static, and in the dark for 3 weeks. Then the fermentation was extracted 3 times using ethyl acetate (EtOAc) solvent. The EtOAc was removed by a rotational vacuum evaporator. The extract was obtained and stored at -20 °C before being used. From 4.0 L of PDB media, 3.8 g of extract was obtained.

Extraction and Isolation of (+)-Epoxydone

The column chromatography (CC) was used for (+)-epoxydone isolation, with silica gel 60 with 230 - 400 mesh as stationary phase and the eluent used several gradients of solvents such as CH₂Cl₂: EtOAc (1:0→10:1→1:3→0:1), MeOH, followed by H₂O. From the results of the column chromatography obtained 23 fractions (F1 - F23), with F15-F18 showed identical spot patterns. The main fraction (F15) was further separated with the stationary phase of silica gel 60, and several eluent systems, namely CH₂Cl₂: EtOAc (3:1→1:3→0:1), EtOAc: MeOH (1:1) followed by methanol. From the results of the CC obtained 16 fractions. Several fractions with single and identical compound spots, namely in the F15.8 - F15.12 fractions. Fractions F15.8 - F15.12 because the *R_f* and spot pattern are identical, so they are combined into F15.8 (537.4 mg) as a pure compound 1.

Pure compound 1 was also obtained from the separation and purification of the other fractions namely F16 - F18. Based on TLC data, fractions F16 - F18 have identical spots, so they were combined as F16 and purified using CC with stationary phase: silica gel 60, eluents: CH₂Cl₂: EtOAc (2:1→1:5→0:1), EtOAc: MeOH (1:1), followed by methanol and it resulted in 22 fractions. Based on TLC data, compounds F16.6 - F16.9 also have the same *R_f* and identical spot patterns, so they are combined into F16.6 (201.7 mg). The TLC data of the F16.6 result is consistent with a single spot (pure compound). The *R_f* and the spot pattern are identical to compound 1, and then it will be characterized by their molecular structure using UV-Vis, IR, MS, and NMR spectroscopy (1D and 2D), and optical rotation analysis and evaluation as an antibacterial agent.

Elucidation of Chemical Structure

MS Analysis

MS analysis of compound 1 using the UPLC-qTOF-MS/MS System (Waters) with 0.300 mL/min as flow rate; the temperature of 40 °C, Formic Acid (FA, 0.1 %) in H₂O (solvent A); FA (0.1 %) in acetonitrile (solvent B) and column of C18, 1.7 μm, 2.1x50 mm (Acquity UPLC BEH). The isocratic elution system was run at 0 - 1.0 min with an A: B ratio of 95:5; then the linear gradient increased for solvent B from 5 % to 40 % (from 1.0 to 8.0 min); the linear gradient of increasing solvent B from 40 % to 100 % (from 8.0 to 11.00 min); the isocratic elution system was run at 11.0–13.0 min with 100 % eluent B; continued the linear gradient for solvent B was from 100 % to 5 % (from 13 to 16 min), injection volume: 1 μL, both solvent (A, B) were buffered with 1 % FA. For MS/MS analysis with a source temperature of 120 °C, a capillary voltage of 2 kV; a desolvation temperature of 500 °C; the gas flow of desolvation of 1000 L/h, and the gas flow of conical 50 L/h. ESI interface was used with positive mode.

UV-Vis Spectrophotometry

UV-Vis Spectrophotometer by using UV-1700 series instrument. The recording process is carried out by making a solution of the isolated compound using ethanol p.a as a solvent with a concentration of 0.429 mM. Then pipette 1.5 ml of ethanol solvent p.a. (as blank) and an autozero process is carried out on the instrument. Scanning is carried out from 190 - 800 nm.

FTIR Spectroscopy

Fourier transform infrared spectroscopy (FTIR) for compound 1 using Shimadzu IR Prestige 21, and DRS-

KBr. Scanning is carried out with wavenumber ranges of 500 - 4000 cm^{-1} .

¹H- and ¹³C-NMR

TMS was used as an internal standard. The chemical shift values (ppm) relative to TMS, with solvent of acetone-*d*₆ and C₃OD, using spectrometer Agilent and JEOL JNM-Lambda with frequencies of 500 and 125 of ¹H- and ¹³C-NMR.

Optical Rotation Analysis

Optical rotation analysis by using polarimeter ATAGO® SAC-i instrument. The recording process is carried out by making a solution of compound **1** using ethanol p.a as a solvent with a concentration of 0,084 %. The value of specific rotation measured at 24 °C with a 589 nm sodium D light source i.e., $[\alpha]_D^{24} +97.98$ (c, 0.084; EtOH).

Antibacterial Assays

Bacteria tests (*S.aureus*-InaCC B4 and *E.coli* InaCC B5) were cultured in MHB liquid media at 37 °C, for 24 h. Compound **1** and antibiotics were dissolved in 2.5 % DMSO (sterile). Antibacterial substances are performed in the serial concentration of 0.25 - 32 $\mu\text{g/mL}$ for antibiotics, and 0.39 - 50 $\mu\text{g/mL}$ for compound **1**. The bacteria were diluted to 5.10^5 CFU/mL. Media control (culture broth media) and growth control (broth culture and bacteria). After incubation at 37 °C, overnight then was added twenty microliters of iodinitotetrazolium chloride aqueous solution (INT, Sigma-Aldrich) (4 mg/mL). The MIC value is the minimum concentration of samples that inhibit the bacteria growth that appears in clear wells or in the absence of purple color.¹⁶ The result is expressed in $\mu\text{g/mL}$. Antimycobacterial test (*Mycobacterium smegmatis* ATCC 14468) with MIC test in broth medium (7H9) using a 96-well microplate containing medium (7H9), antimycobacterial agent and bacterial inoculants 5×10^5 CFU/mL, also control media (broth media), growth control (broth media and mycobacterial inoculants). Compound **1** and antibiotic were dissolved in 2.0 % DMSO (sterile). After the incubation at 37 °C for 24 hours was completed, 20 μL of resazurin solution 0.1 % (in distilled water and tween-80 = 1:1) was added and left in the incubator for 24 hours until it turned orange (turned to resorufin) due to the mitochondrial dehydrogenase enzymatic activity of viable cell of mycobacteria, and the MIC values were recorded as the lowest concentration of non-growing mycobacteria (appears unchanged to orange or remains blue, the initial color of resazurin).

RESULTS AND DISCUSSION

The extraction process of endophytic fungus *Phomopsis* sp. TcBt1Bo-6 yielded 3.8 g. This fungus from *T.crispa* was collected from Bogor, West Java, Indonesia. The media culture to scale up was 4.0 L of Potatoe Dextrose Broth (PDB) in a dark room, at 25 °C, and in static condition. The compounds of EtOAc extract were separated by column chromatography (CC) and resulted in F1 - F23 (with F15 - F18 showed identical spot patterns, and their's weights are 641.9; 243.5; 161.9, and 117.1 mg, respectively). The main fraction (F15) was performed further fractionation obtained 16 fractions (F15.1 - F15.16). The biggest weight fraction (F15.8) and several fractions (F15.9 - F15.12) with single and identical compound spots. Due to the *R_f* and spot pattern of F15.8 - F15.12 being identical, they are combined into F15.8 (537.4 mg) as a pure compound (**1**). Pure compound **1** was also obtained from the separation and purification of fractions F16 - F18. Based on TLC data, fractions F16 - F18 were identical, so they were combined as F16. This fraction was separated using stationary silica gel 60 (230-400 mesh) in the column chromatography, and the eluents with gradient systems: CH₂Cl₂: EtOAc (2:1 $\rightarrow$ 1:5 $\rightarrow$ 0:1), EtOAc: MeOH (1:1), and followed by MeOH. From the results of the chromatographic column obtained 22 fractions (F16.1 - F16.22). Based on TLC data, compounds F16.6 - F16.9 have the same *R_f* and identical spot patterns, so they are combined into F16.6 (201.7 mg). the TLC data of the F16.6 result is consistent with a single spot (pure compound). The *R_f* and the spot pattern are identical with compound **1**. Further analysis for the chemical structure elucidation of Compound **1** using FTIR, MS, UV-Vis, rotational optic analysis, proton and carbon NMR, DEPT-135, HMBC, HMQC, and ¹H-¹H COSY, and microdilution for antibacterial activity testing. Data of MS spectrometry of the compound **1** were *m/z* as base peak, i.e., 123.04376 (100 % abundance) is $[\text{M}-33]^+$ or $[\text{M}+\text{H}-2\text{OH}]^+$ i.e., the release of 2 hydroxyl groups, and the addition of 1 proton (calc. 156.04216 - 32.99762 = 123.04454), *m/z* of 279.09230, namely $[\text{2M}-33]^+$ or $[\text{2M}+\text{H}-2\text{OH}]^+$ (calc. 312.08432 - 32.99762 = 279.08670), *m/z* is 433.11171, namely $[\text{3M}-35]^+$ or $[\text{3M}+\text{H}-2\text{H}_2\text{O}]^+$ (calc. 468.12648 - 35.01326 = 433.

11322), m/z of M^+ of 156.04138 (calc. 156.04216, Δ -0.78 mmu) showed the formula of chemical compound of $C_7H_8O_4$. From the IR spectrophotometer measurement data of compound **1**, there is a stretching vibration of the O-H group with a band in wavenumber of 3281 cm^{-1} , and the presence of methine and methylene in the form of C-H stretching bonds for sp^3 carbon in wavenumber of 2930 cm^{-1} , C=O bonds stretching in wavenumber of 1679 cm^{-1} , the C=C stretching bond for sp^2 carbon in wavenumber of 1616 cm^{-1} , the C-H bending bond in the wavenumber of 1457 cm^{-1} , OH bending (in-plane) in the wavenumber of 1386 cm^{-1} , the epoxide bond C-O-C asymmetric and symmetric stretching in the wavenumber of 1212 and 1016 cm^{-1} , respectively. In the UV-Vis spectrum, enone compounds have a specific absorption band in the K band in the 215-250 nm (obs. 237 nm, $\log \epsilon$ 3.80) region of the $\pi - \pi^*$ transition and a weak band at the range of > 300 nm (R band, obs. 338 nm, $\log \epsilon$ 3.26) of the $n - \pi^*$ transition for ketone containing double bonds unsaturated α , and β . The electronic transition with a value of $10^3 - 10^6$ in the UV-Vis spectrophotometer measurement above is considered appropriate and allowed in the electronic transition process.¹⁷ From the data in Table-1 and Table-2, information is obtained that there are 1 methylene (CH_2), 4 methines (CH), and 2 quaternary C or a total of 7 carbon atoms and there are 6 or more protons because of the use of CD_3OD and H_2O causes protons of the hydroxyl group does not appear because of the hydrogen bonding with the solvent. While, due to the use of acetone- d_6 or $(CD_3)_2CO$, two protons of the hydroxyl group have a chemical shift at 3.14 and 5.01 ppm. The proton of hydroxyl (5-OH) with a chemical shift of 5.01 ppm has a coupling constant value of $^3J_{5-OH, 5} = 7.25\text{ Hz}$ due to the interaction with a methine proton (H5). One methylene (CH_2) may bind to oxygen ($\delta C = 59.57\text{ ppm}$) with 2 protons having different chemical shifts, namely δH 4.14 and 4.21 ppm which indicates asymmetry or electronic differences in their neighbors. Four methines consist of 3 methines ($CH\ sp^3$) bound to oxygen ($\delta C = 54.50 - 65.95\text{ ppm}$, $\delta H = 3.78 - 4.71\text{ ppm}$) and 1 methine ($CH\ sp^2$) with δC 142.06 ppm and δH 6.49 ppm. Two quaternary C in the form of unsaturated/alkene C 135.78 ppm and one quaternary carbon of carbonyl C with δC 195.28 ppm. Two vicinal protons on the epoxide ring show a typical coupling constant value of $^3J_{1,6} = 4\text{ Hz}$, and 2 geminal protons also show a typical coupling constant value of $^2J_{7,7'} = 15.0\text{ Hz}$. The long-range coupling also occurs between methylene protons and the value of the coupling constant of two methine protons are 2.5 and 2.0 Hz. Another coupling constant of methine protons are $^3J_{4,5} = ^3J_{5,6} = 3.0\text{ Hz}$. The HMBC data in Fig.-1 showed two proton methylene (δH 4.14 and 4.21 ppm) which bind to the hydroxyl group and correlate with the carbons both carbonyl (δC 195.28 ppm) and methine sp^2 (δC 135.78 ppm). The methylene linked carbonyl and methine alkene through quaternary C alkene. Two protons of epoxide correlate with carbonyl C (δH 3.39 ppm $\rightarrow$ δC 135.78 ppm) and methine that binds hydroxyl (δH 3.78 ppm $\rightarrow$ δC 142.06 ppm). While, the proton of a hydroxyl group (5-OH) correlates with the carbons of C6, C5, and C4 (δH 5.01 ppm $\rightarrow$ δC 55.01; 65.43; and 141.49 ppm), which shows the hydroxyl group was bound by C5. The data in 1H - 1H COSY (Fig.-1) describes the relationship between 2 geminal protons, namely H7 (4.14 ppm) and H7' (4.21 ppm), and between protons and their neighboring protons, namely H1 (3.39 ppm) and H6 (3.78 ppm); H6 with H1 and H5 (4.71 ppm); H5 with H6 and H4 (6.49 ppm), and H4 with H5. So, the structure of compound **1** was confirmed as epoxydone. Based on previous research, epoxydone and epiepoxydone have been structurally characterized using NMR using an acetone- d_6 solvent. Table-1 and Table-2 show no difference in the chemical shifts of carbons and protons in a compound **1** and (+)-epoxydone. However, the chemical shift of carbon and protons in compound **1** is different when compared to the chemistry of carbon and protons in the compound (+)-epiepoxydone. This may be related to the different stereochemical structures, including the position of the vicinal protons at C5 and C6, which are axial-equatorial at (+)-epoxydone while axial-axial at (+)-epiepoxydone. This also causes the position of the hydroxyl group of C5 and the epoxide ring in the *cis*-coplanar position on (+)-epoxydone and the *trans*-coplanar position on the (+)-epiepoxydone. In the methine proton that binds to the hydroxyl group at C5, the methine proton at C1 and the methine proton (C6) which are two epoxide carbons at compound **1** show $^3J < 10\text{ ppm}$ which indicates the position of two vicinal protons at the equatorial-equatorial position ($^3J_{eq-eq} = 3 - 4\text{ Hz}$). Related to the data of coupling constant, it revealed the compound **1** is (+)-epoxydone. This metabolite is different from (+)-epiepoxydone due to they are two diastereomers. The value of specific rotation measured at $24\text{ }^\circ\text{C}$ with a 589 nm sodium D light source *i.e.*, $[\alpha]_D^{24} +97.98$ (*c* 0.084; EtOH) which is the same as the optical rotation of (+)-epoxydone, $[\alpha]_D^{25} +98.0$ (*c* 1.0, EtOH) based on previous studies¹⁸, so compound **1** is confirmed as (+)-epoxydone. While optical rotation of (+)-epiepoxydone is $[\alpha]_D^{25} +250$

(*c* 1.40, EtOH).¹⁸

Table-1: ¹³C-NMR Data of Compound **1** Compared with (+)-epoxydone and (+)-epiepoxydone

No	δC (ppm, mult.) (125 MHz, in CD ₃ OD)	δC (ppm, mult.) (125 MHz, in Aseton- <i>d</i> ₆)	δC (ppm, mult.) (75 MHz, in Aseton- <i>d</i> ₆)	δC (ppm, mult.) (75 MHz, in Aseton- <i>d</i> ₆)	δC (ppm, mult.) (125 MHz, in Aseton- <i>d</i> ₆)
	Compound 1 (This study)	Compound 1 (This study)	(+)-epoxydone ¹⁸	(+)- epiepoxydone ¹⁸	(+)- epiepoxydone ¹⁹
1	54.50 (<i>d</i>)	53.96 (<i>d</i>)	54.0 (<i>d</i>)	54.10 (<i>d</i>)	54.1 (<i>d</i>)
6	55.37 (<i>d</i>)	55.01 (<i>d</i>)	55.0 (<i>d</i>)	58.79 (<i>d</i>)	58.8 (<i>d</i>)
7	59.57 (<i>t</i>)	59.05 (<i>t</i>)	59.1 (<i>t</i>)	59.00 (<i>t</i>)	59.1 (<i>d</i>)
5	65.95 (<i>d</i>)	65.43 (<i>d</i>)	65.5 (<i>d</i>)	63.25 (<i>d</i>)	63.3 (<i>d</i>)
3	135.78 (<i>s</i>)	135.21 (<i>s</i>)	135.2 (<i>d</i>)	137.07 (<i>d</i>)	137.1 (<i>d</i>)
4	142.06 (<i>d</i>)	141.49 (<i>d</i>)	141.4 (<i>d</i>)	139.30 (<i>d</i>)	139.3 (<i>d</i>)
2	195.28 (<i>s</i>)	194.55 (<i>s</i>)	194.5 (<i>s</i>)	194.35 (<i>s</i>)	194.4 (<i>s</i>)

Table-2: ¹H-NMR Data of Compound **1** Compared with (+)-epoxydone and (+)-epiepoxydone

No	δH (ppm, mult., Hz) (125 MHz, in CD ₃ OD)	δH (ppm, mult., Hz) (125 MHz, in Aseton- <i>d</i> ₆)	δH (ppm, mult., Hz) (75 MHz, in Aseton- <i>d</i> ₆)	δH (ppm, mult., Hz) (75 MHz, in Aseton- <i>d</i> ₆)	δH (ppm, mult., Hz) (125 MHz, in Aseton- <i>d</i> ₆)
	Compound 1 (This study)	Compound 1 (This study)	(+)-epoxydone ¹⁸	(+)- epiepoxydone ¹⁸	(+)- epiepoxydone ¹⁹
1	3.39 (<i>d</i> , 4.0, CH)	3.35 (<i>d</i> , 4.1, CH)	3.34 (<i>d</i> , 4.2, CH)	3.40 (<i>d</i> , 3.6, CH)	3.40 (<i>dd</i> , 3.7, 0.6, CH)
6	3.78 (<i>m</i> , 3.0; 4.0, CH)	3.81 (<i>m</i> , 2.75; 4.1, CH)	3.80 (<i>dd</i> , 3.0, 6.6, CH)	3.78–3.76 (<i>m</i> , CH)	3.78-3.79 (<i>m</i> , CH)
7	4.14 (<i>dd</i> , 2.0; 2.5; 15.0, C-H7)	4.12 (<i>m</i> , 3.4; 6.0; 15.0, C-H7)	4.06–4.24 (overlap, <i>m</i> , C-H7)	4.10-4.30 (overlap, <i>m</i> , C- H7)	4.16 (<i>d</i> , 15.6, C-H7)
7'	4.21 (<i>dd</i> , 2.0; 2.5; 15.0, C- H7')	4.22 (<i>m</i> , 3.4; 6.0; 13.2, C-H7')	4.06–4.24 (overlap, <i>m</i> , C-H7')	4.10-4.30 (overlap, <i>m</i> , C- H7')	4.27 (<i>d</i> , 15.6, C-H7')
5	4.71 (<i>m</i> , 2.5; 3.0, CH)	4.79 (<i>d</i> , 2.65, CH)	4.77–4.80 (<i>m</i> , CH)	4.66–4.63 (<i>m</i> , CH)	4.66 (<i>d</i> , 4.3 Hz, CH)
4	6.49 (<i>m</i> , 2.0; 3.0, CH)	6.52 (<i>t</i> , 2.25, CH)	6.50 (<i>d</i> , 1.8, CH)	6.72–6.69 (<i>m</i> , CH)	6.71 (<i>ddd</i> , 1.8, 4.2; 1.4, CH)
7-OH	-	3.14 (brs, OH)	4.06–4.24 (overlap, <i>m</i> , OH)	4.10-4.30 (overlap, <i>m</i> , OH)	4.11 (brs, OH)
5-OH	-	5.01 (<i>d</i> , 7.25, OH)	4.91 (<i>d</i> , 7.5, OH)	4.92 (<i>d</i> , 7.5, OH)	4.88 (brs, OH)

In this research, (+)-epoxydone, as a main bioactive metabolite was successfully obtained from *Phomopsis* sp. TcBt1Bo-6 using chromatography method. This fungus *Phomopsis* sp. TcBt1Bo-6 was obtained from *T. crista* plant. The main metabolite was chemically characterized using spectroscopy and optical rotation analysis. The isolated compound, based on the data of UV, FTIR, MS, 1D, and 2D-NMR, optical rotation analysis, and compared with other studies. The major compound from *Phomopsis* sp. TcBt1Bo-6 was confirmed to be (+)-epoxydone (Fig.-2). It was also known as epoxydon, phyllosinol, epoxydone, sphaeropsodin, antibiotic 212T2, etc.²⁰

In other studies, (+)-epoxydone was also produced by *Phoma herbarum* and *Aspergillus* sp. which is associated with marine red alga *Hypnea saidana*.^{21,22} These fungi were cultured in the SWS medium containing soluble starch (1.0 %), soytone (0.1 %), and seawater (100 %).²¹ Another study, (+)-epoxydone isolated from fungus *Nigrospora* sp. PSU-F5 was cultured that associated with a sea fan, *Annella* sp.²³ The bioproduction capacity of (+)-epoxydone was reported from a liquid culture media of *Phoma herbarum*,

Nigrospora sp. PSU-F5, *Aspergillus* sp. and *Phoma* sp. 8889: 0.500, 0.450; 0.500; 0.908 mg/L, respectively.²¹⁻²⁴ In addition, (+)-epoxydone is also produced by several other fungi, such as *Phoma* sp., *Phyllostica* sp., *Mycosphaerella ligulicola*, *Phoma sorghina*, *Ophiosphaerella herpotricha*, *Phoma glomerata* JCM997241, *Aspergillus parasiticus*, and plant of *Diospyros kaki* L.²⁵

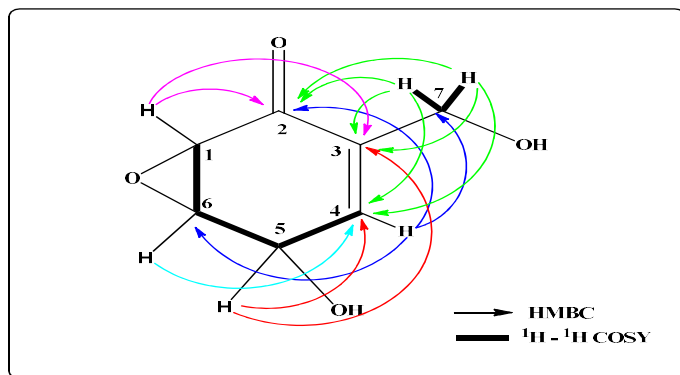


Fig.-1: Structure of Compound 1 Based on the Data of HMBC and ¹H-¹H COSY

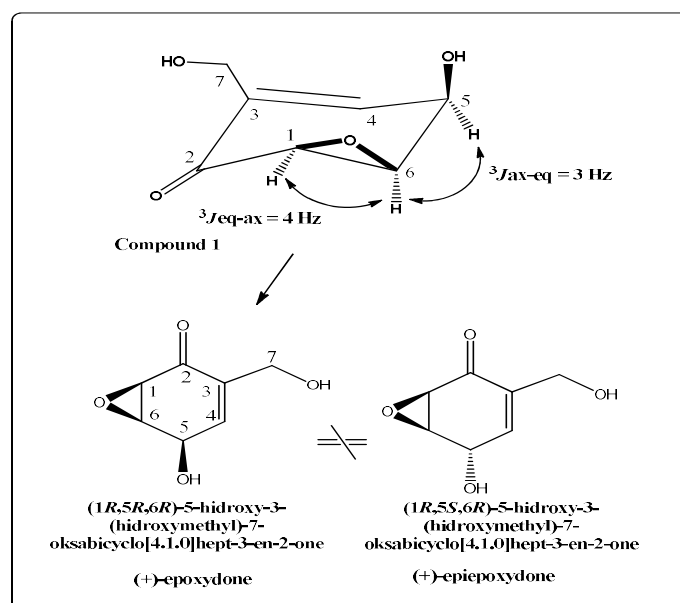


Fig.-2: Stereochemistry of the Compound 1 was Compared with (+)-epiepoxydione

In this current research, *Phomopsis* sp. TcBt1Bo-6 can produce (+)-epoxydone with a bioproduction ability of 189.430 mg/L (Table-3). The bioproduction ability of *Phomopsis* sp. TcBt1Bo-6 is more than the bioproduction ability of *Phoma herbarum*, and *Nigrospora* sp. PSU-F5. While another study reported the latest chemical synthesis method using δ -D-gluconolactone to produce (+)-epoxydone with a yield of 18.04 % through several steps of chemical reactions.²⁶ This fungus *Phomopsis* sp. TcBt1Bo-6 can be used as a natural source to obtain (+)-epoxydone derived from endophytic fungi through cultured and scale-up in a liquid medium. Compound 1 and antibiotics have been performed as antibacterial (Table-4). In this present study, (+)-epoxydone was antibacterial against *E.coli* InaCC B5 (MIC value of 25 μ g/mL) and *S.aureus* InaCC B4 (MIC value of > 50 μ g/mL). While this compound also performed as antimycobacterial against *Mycobacterium smegmatis* ATCC 14468 with 40 μ g/mL of compound 1 was able to inhibit 79.33 % of the growth of mycobacteria, and the MIC value of partial 50 μ g/mL (Table-4). (+)-epoxydone was less effective than the commercial drugs amoxicillin, erythromycin, and vancomycin against *S.aureus* (MIC value of all commercial drugs < 0.5 μ g/mL). While the MIC value of several antibiotics against Gram-negative bacteria, *E.coli* are 8; 16, and >32 μ g/mL for amoxicillin, erythromycin, and vancomycin, respectively.¹⁵

Table-3: Bioproduction capability of (+)-epoxydione from several fungi

No	Metabolite Source	Host plant	Growth Medium	The bioproduction capability of (+)-epoxydione (mg/L)	Literature
1	<i>P.herbarum</i>	<i>H.saidana</i>	SWS medium containing soluble starch (1.0 %), soytone (0.1 %), and seawater (100 %)	0.500	²¹
2	<i>Nigrospora</i> sp. PSU-F5	<i>Annella</i> sp.	PDB	0.450	²³
3	<i>Aspergillus</i> sp.	<i>H.saidana</i>	SWS medium containing soluble seawater-starch-soytone	0.500	²²
4	<i>Phoma</i> sp. 8889	<i>S.oppostifoli</i>	a barley-malt extract-agar medium	0.908	²⁴
5	<i>Phomopsis</i> sp. TcBt1Bo-6	<i>T.crispa</i>	PDB	189.430	This study

A previous study revealed (+)-epoxydione isolated from the marine-derived fungus, *Aspergillus* sp. showed potent antioxidants as DPPH radical free scavenging with IC₅₀ value of 6.0 µM and antibacterial toward resistant of *S.aureus* (MIC value of 12.5 µg/mL).²² In other studies, this compound has been performed as an anticancer by suppressing EGFR (IC₅₀ value of 0.6 µg/mL), thus inhibiting cell growth and induction of apoptosis *in vitro* and inhibiting HeLa cell proliferation up to 20 % at a concentration of 25 µg/mL.²¹

Table-4: Antibacterial Activity of Compound 1 and Some Commercial Antibiotics

Compound	MIC value against <i>S.aureus</i> (µg/ml)	MIC value against <i>E.coli</i> (µg/ml)	Percentage of growth inhibition of <i>M.smegmatis</i> *(%)	MIC value against <i>M.smegmatis</i> (µg/ml)
1	>50	25	79.33	50P
amoxicillin	< 0.5	8	NT	NT
erythromycin	< 0.5	16	NT	NT
vancomycin	< 0.5	>32	NT	NT
rifampicin	NT	NT	NT	0.4

Remark: *: The test used a concentration of the test compound of 40 (µg/ml) with 3 replications, NT: no tested, P: partial, the antibacterial activity of commercial antibiotic based on our previous study,¹⁵ the antimycobacterial activity of commercial antibiotic based on our unpublished data.

CONCLUSION

The fungal endophyte *Phomopsis* sp. TcBt1Bo-6 cultured in a liquid PDB medium that is isolated from the *T.crispa* plant can be a novel source of (+)-epoxydione with a production capacity of 189.430 mg/L. (+)-epoxydione has moderate as an antibacterial against *E.coli* but it is less potent than several commercial antibiotics. This study is the new report on the bioproduction of bioactive metabolite, (+)-epoxydione as antibacterial and antimycobacterial activities as well as the major compound isolated from the fungus *Phomopsis* sp. TcBt1Bo-6 is associated with *T.crispa* plant. This study still has limitations with the use of only PDB medium as liquid culture medium, and static condition for growing the fungus *Phomopsis* sp. TcBt1Bo-6. However, this study demonstrated the potential of bioproduction of (+)-epoxydione as the major compound from *Phomopsis* sp. TcBt1Bo-6. Further research is needed using other medium and agitates conditions for scaling up this fungus. In addition, the next research is needed to obtain pure compounds of the minor compounds and to perform the antibacterial test.

ACKNOWLEDGMENT

All authors would like to express thanks to Saintek-BRIN Scholarship for giving the Doctoral scholarship to the first author and Kemendikbudristek for the PDD research grant 2022 for funding this research and publication. The authors also thank the scientific and technical support, and also facilities of Advanced Characterization Lab., Research Center for Chemistry, Serpong, through e-Layanan Sains (Elsa), National Research and Innovation Agency (BRIN). All authors equally contributed to this work as main contributors.

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