(+)-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 antymycobacterial 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 antymycobacterial activities of this compound.

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

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 beavercin from Fusarium proliferatum as anticancer,6 myrotheic I isolated from Paramyrothecium roridum showed strong cytotoxicity towards cell lines,8 asperphenalenone D obtained from Aspergillus sp. CPCC 400735 showed anti-HIV.9 Caspicaene as antituberular 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,2 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 *Rf* 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 *Rf* 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 *Rf* 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\(^{-1}\).

\( ^1H - \text{and} \ ^{13}C\)-NMR

TMS was used as an internal standard. The chemical shift values (ppm) relative to TMS, with solvent of acetone-\(d_6\) and C\(_2\)OD, using spectrometer Agilent and JEOL JNM-Lambda with frequencies of 500 and 125 of \( ^1H - \text{and} \ ^{13}C\)-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). Antimicrobial substances are performed in the serial concentration of 0.25 - 32 \( \mu \)g/mL for antibiotics, and 0.39 - 50 \( \mu \)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 iodonitrotetrazolium 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 \( \mu \)g/mL. Antimycobacterial test (\( Mycobacterium smegmatis \) ATCC 14468) with MIC test in broth medium (7H9) using a 96-well microplate containing medium (7H9), antymycobacterial agent and bacterial inoculants 5x10\(^5\) CFU/mL, also control media (broth), 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 \( \mu \)l 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 \( Rf \) 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: CH\(_2\)Cl\(_2\): EtOAc (2:1:1.5:0:1), 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 \( Rf \) 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 \( Rf \) 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 \([M-33]^+\) or \([M+H-2OH]^+\) 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 \([2M-33]^+\) or \([2M+H-2OH]^+\) (calc. 312.08432 - 32.99762 = 279.08670), \( m/z \) is 433.11171, namely \([3M-35]^+\) or \([3M+H-2H2O]^+\) (calc. 468.12648 - 35.01326 = 433.

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so compound is the same as the optical rotation of (+)-epoxydone, rotation measured at 24 °C with a 589 nm sodium D light source i.e., [α]D 2210 = +98.08 (c 1.0, EtOH) based on previous studies18, so compound I is confirmed as (+)-epoxydone. While optical rotation of (+)-epiepoxydone is [α]D 2213 +250
(+)-EPOXYDONE: THE MAJOR SECONDARY METABOLITE

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.crispa 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, sphaeropsadin, 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, 2214

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

| No | δC (ppm, mult.) (125 MHz, in CD3OD) | δC (ppm, mult.) (125 MHz, in Aseton-d6) | δC (ppm, mult.) (75 MHz, in Aseton-d6) | δC (ppm, mult.) (125 MHz, in Aseton-d6) | δC (ppm, mult.) (75 MHz, in Aseton-d6) |
|----|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| 1  | 54.50 (d)                        | 53.96 (d)                        | 54.0 (d)                         | 54.10 (d)                        | 54.1 (d)                         |
| 6  | 55.37 (d)                        | 55.01 (d)                        | 55.0 (d)                         | 58.79 (d)                        | 58.8 (d)                         |
| 7  | 59.57 (t)                        | 59.05 (t)                        | 59.1 (t)                         | 59.00 (t)                        | 59.1 (d)                         |
| 5  | 65.95 (d)                        | 65.43 (d)                        | 65.5 (d)                         | 63.25 (d)                        | 63.3 (d)                         |
| 3  | 135.78 (s)                       | 135.21 (s)                       | 135.2 (d)                        | 137.07 (d)                       | 137.1 (d)                        |
| 4  | 142.06 (d)                       | 141.49 (d)                       | 141.4 (d)                        | 139.30 (d)                       | 139.3 (d)                        |
| 2  | 195.28 (s)                       | 194.55 (s)                       | 194.5 (s)                        | 194.35 (s)                       | 194.4 (s)                        |

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

| No | δH (ppm, mult., Hz) (125 MHz, in CD3OD) | δH (ppm, mult., Hz) (125 MHz, in Aseton-d6) | δH (ppm, mult., Hz) (75 MHz, in Aseton-d6) | δH (ppm, mult., Hz) (125 MHz, in Aseton-d6) | δH (ppm, mult., Hz) (75 MHz, in Aseton-d6) |
|----|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| 1  | 3.39 (d, 4.0, CH)                | 3.35 (d, 4.1, CH)                | 3.34 (d, 4.2, CH)                | 3.40 (d, 3.6, CH)                | 3.40 (dd, 3.7, 0.6, CH)          |
| 6  | 3.78 (m, 3.0; 4.0, CH)           | 3.81 (m, 2.75; 4.1, CH)          | 3.80 (dd, 3.0, 6.6, CH)          | 3.78–3.76 (m, CH)                | 3.78–3.79 (m, 0.6, CH)           |
| 7  | 4.14 (dd, 2.0; 2.5; 15.0, C-H7)  | 4.12 (m, 3.4; 6.0; 15.0, C-H7)   | 4.06–4.24 (overlap, m, C-H7)     | 4.10–4.30 (overlap, m, C-H7)     | 4.16 (d, 15.6, C-H7)             |
| 7′ | 4.21 (dd, 2.0; 2.5; 15.0, C-H7′) | 4.22 (m, 3.4; 6.0; 13.2, C-H7′)  | 4.06–4.24 (overlap, m, C-H7′)    | 4.10–4.30 (overlap, m, C-H7′)    | 4.27 (d, 15.6, C-H7′)            |
| 5  | 4.71 (m, 3.0; 3.0, C-H7′)        | 4.79 (d, 2.65, CH)               | 4.77–4.80 (m, CH)                | 4.66–4.63 (m, CH)                | 4.66 (d, 4.3 Hz, CH)             |
| 4  | 6.49 (m, 2.0; 3.0, CH)           | 6.52 (t, 2.25, CH)               | 6.50 (d, 1.8, CH)                | 6.72–6.69 (m, CH)                | 6.71 (ddd, 1.8; 4.2; 1.4, CH)    |
| 7- OH | -                               | 3.14 (brs, OH)                    | 4.06–4.24 (overlap, m, OH)       | 4.10–4.30 (overlap, m, OH)       | 4.11 (brs, OH)                   |
| 5- OH | -                               | 5.01 (d, 7.25, OH)               | 4.91 (d, 7.5, OH)                | 4.92 (d, 7.5, OH)                | 4.88 (brs, OH)                   |


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 \( \delta \)-D-gluconolactone to produce (+)-epoxydone with a yield of 18.04 \% through several steps of chemical reactions.\footnote{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 \textit{E.coli} InaCC B5 (MIC value of 25 \( \mu \)g/mL) and \textit{S.aureus} InaCC B4 (MIC value of > 50 \( \mu \)g/mL). While this compound also performed as antimycobacterial against \textit{Mycobacterium smegmatis} ATCC 14468 with 40 \( \mu \)g/mL of compound 1 was able to inhibit 79.33 \% of the growth of mycobacteria, and the MIC value of partial 50 \( \mu \)g/mL (Table-4). (+)-epoxydone was less effective than the commercial drugs amoxicillin, erythromycin, and vancomycin against \textit{S.aureus} (MIC value of all commercial drugs < 0.5 \( \mu \)g/mL). While the MIC value of several antibiotics against Gram-negative bacteria, \textit{E.coli} are 8; 16, and > 32 \( \mu \)g/mL for amoxicillin, erythromycin, and vancomycin, respectively.\footnote{15}
Table-3: Bioproduction capability of (+)-epoxydone from several fungi

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

A previous study revealed (+)-epoxydone isolated from the marine-derived fungus, *Aspergillus* sp. showed potential 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). 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 *S. aureus* (μg/ml) | MIC value against *E. coli* (μg/ml) | Percentage of growth inhibition of *M. smegmatis* (%) | MIC value against *M. smegmatis* (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 (+)-epoxydone with a production capacity of 189.430 mg/L. (+)-epoxydone 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, (+)-epoxydone 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 (+)-epoxydone 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.

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²¹ A previous study revealed (+)-epoxydone 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). 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.
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