ISOLATION OF TWO BOTRYANES FROM *Hypoxylon rickii* AND IDENTIFICATION OF THE ENCODING GENES

(Pemencilan Sebatian Botrien dari *Hypoxylon rickii* dan Pengenalpastian Gen yang Mengekodkannya)

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Abstract

10-oxodehydrobotrydial (**1**) and 4β-acetoxy-9β,10β,15α-trihydroxyprobotrydial (**2**) have been isolated from *Hypoxylon rickii* mycelia extract. Compound **2** is probably an intermediate of botrydial, which is a known sesquiterpene phytotoxin. Cluster Omega and Artemis software analyses suggested that *HRT6,* a possible botrydial-like gene cluster with four genes shows more than 50% similarities compared to *Botrytis cinerea* botrydial gene cluster, *BcBOT*. A comparison between proposed 4β-Acetoxy-9β,10β,15α- trihydroxyprobotrydial (**2**) producing pathway and *BcBOT* proposed pathway shows compatible function of each gene prediction. Further confirmation using RNA (silencing gene expression) is on-going.

Keyword: *Hypoxylon rickii*, Botryane, Terpene synthase

Abstrak

10-oxodehydrobotrydial (**1**) dan trihydroxylbotrydial (**2**) telah dipencilkan dari ekstrak miselia *H. rickii*. Sebatian **2** dijangkakan sebagai sebatian perantara dalam pembentukkan botrydial iaitu sejenis fitotoksin sesquiterpena. Penggunaan dua perisian Cluster Omega dan Artemis telah berjaya mengenalpasti gen kelompok yang berkemungkinan dari *H. rickii* sebagai *HRT6* dengan empat gen menunjukkan persamaan lebih daripada 50% berbanding *Botrytis cinerea* gen kelompok, *BcBOT*. Perbandingan dengan cadangan laluan biosintesis 4β-Acetoxy-9β,10β,15α- trihydroxyprobotrydial (**2)** dengan *BcBOT* menunjukkan persamaan fungsi-fungsi gen. Pengesahan lanjut menggunakan RNA (mendiamkan gen) sedang dijalankan.

Kata kunci: *Hypoxylon rickii*, Botriena, Terpena sintes

Introduction

Terpene is a diverse class of secondary metabolite (SMs) produced notably by fungi. These compound have a cycloaliphatic basic structure built from isoprene units [1]. Most of the terpenes produced by fungi have various biological functions. Terpene include commercially fascinating compounds due to their properties, such as paxilline an indole diterpene, gibberelic acid, a pentacyclic diterpene plant growth hormone and trichothecenes a sesquiterpene mycotoxin [2, 3].

Botryane are a group of sesquiterpenes produced notably by *Botrytis cinera* which is a fungus causing gray-rot disease in economically important crops such as grapes, strawberries and many more. They are known as broad spectrum toxins with botrydiol derivatives causing lesions to the leaves [4]. Other bioactivities include fungicide and cytotoxic activities [5]. Even though sesquiterpenes form the largest group of terpenes and include commercially important compounds, they are difficult to chemically synthesize because of the complex structure that often leads to low yield [6].

Hypoxylon sp. was first identified by Ju and Rodgers in 1996 and belongs to the Xylariaceae family which has interesting characteristics in producing a variety of secondary metabolites [7]. *Hypoxylon fragiforme* is known to produce frangiformins A and B, cytochalasin H and mitorubin azaphilones [8] while *Hypoxylon rutilum* produces rutilin A and B, entonaemin A, rubiginosin A and B [9].

Hypoxylon rickii colonies grown on agar plate, are at first whitish, then becoming fulvous, velvety to felty,

azonate, with diffuse margins and a reverse dull green colour can be seen [7]. *H. rickii* has a nodulisporium-like conidiogenous structure that differentiates it from other *Hypoxylon* taxa with red to orange red granules in a wild species [7].

The research group of Prof. Marc Stadler in Helmholz Center for Infection Research Institute previously has identified various type of secondary metabolites from *H. rickii* such as Silphiperfolene-type terpenoids, diketopiperzine, botryenanol, α-ramulosin**,** α-eleostearic acid and many more [10]. Recently, they manage to isolate new botryane ((1S)-7-[(2E)-but-2-enoyl]-1,3,3,6-tetramethyl-2,3-dihydro-1H-indene-1 carbaldehyde **3**, (3aS)-6-hydroxy-3a,5,5,8-tetramethyl-3,3a,4,5-tetrahydro-1H-cyclopenta [de] isochromen1-one **4**, (3aS)-7-hydroxy-3a,5,5,8-tetramethyl-3,3a,4,5-tetrahydro-1H- cyclopenta[de]isochromen-1-one **5** with 3 known botryanes (3aS)-3a,5,5,8-tetramethyl-3,3a,4,5-tetrahydro-1H-cy-clopenta[de]isochromen-1-one **6**, (3aS,8R)-3a,5,5,8-tetramethyl-3,3a,4,5,7,8-hexahydro-1H-cy-clopenta[de]isochromen-1-one **7** and botryenanol **8** and many more [11] (Figure 1).

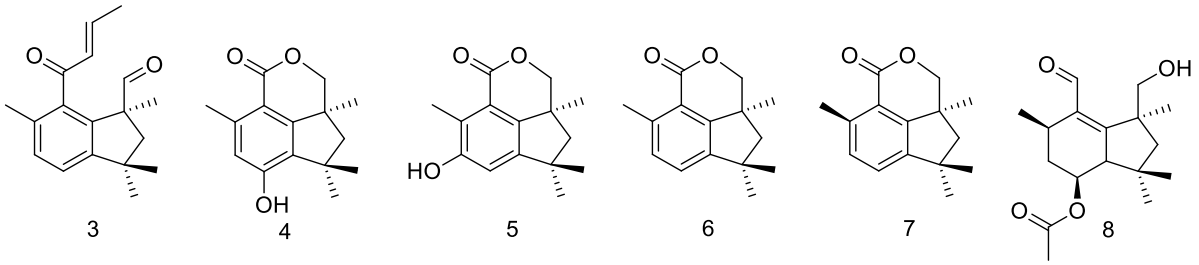

Figure 1. Botryanes isolated from *Hypoxylon rickii*

Genome mining provides information on the gene cluster responsible for producing of respective SMs. Generally, biosynthetic genes for fungal SMs are located in clusters and it can consist of more than 10,000 bases. These clusters contain one or several central biosynthetic genes encoding extremely large multidomains or multimodular enzymes belonging to the polyketide synthases (PKSs), terpene synthases (TSs), non-ribosomal peptide synthases (NRPSs) or a mix of them [12]. Each of the genes in a gene cluster has its own functions corresponding to step of the biosynthesis [13]. This knowledge enables us to manipulate and produce derivatives of the SMs tailored to our needs.

Materials and Methods

Material

Strain 53309 from Prof Marc Stadler's group, collected in Martinique, France was used. The sample has been deposited at the public culture collections (MUCL 53309, CBS 129345). It was identified based on comparing the *H. rickii* sample ITS sequence JQ009313 with deposited ITS sequences in the NCBI database under accession number AJ390408 and KC968932.

Culturing

H. rickii was grown in the yeast malt glucose (YMG) liquid medium at 25 ºC with 200 rpm rotation for 3 to 4 days except for isolation of secondary metabolites where 7 to 15 days of cultivation in malt extract medium and tomato medium were required. Each medium was inoculated with a mycelium suspension (100-500 µL, 50 % glycerol). For solid medium cultures the plate were inoculated with mycelium from a liquid culture (200 µL) or from an agar plate and incubated at 25 °C.

Extraction and Isolation

The production culture was centrifuged (10,000 rpm, 4 ºC, 25 min). The supernatant was filtered under vacuum to remove any cells that were not pelleted out and its pH adjusted to 4.0. The supernatant was extracted thrice with ethyl acetate. The organic layers were combined and dried over anhydrous magnesium sulphate (Fisher) and filtered under vacuum. Mycelium from previous extraction was extracted thrice with acetone. The organic layers were combined and dried over anhydrous magnesium sulphate (Fisher) and filtered under vacuum.

The supernatant organic extract was evaporated to leave a dark green oil which was dissolved in methanol and analysed by LCMS while the mycelium organic extract was evaporated to leave a dark reddish oil which was dissolved in methanol and analysed by LCMS.

Analytical chromatographic analysis were perform on a Waters Platform 2695 LC system comprising of a Waters 600 pump system, a Waters 2998 diode array detector (detecting between 210 and 400 nm), a Waters 2420 ELS

detector, a Waters QuatroMicro Platform LC mass spectrometer (detecting between 150 and 600 *m/z* units ESI^+) with a Kinetex 2.6 µm C_{18} (Phenomenex, 100 × 4.6 mm) reverse phase column. The mobile phase gradient program was run as follows: 0-10 min (90% A), 10-13 min (ramped to 90% B), 13-15 mins (ramped to 90% A). Solvent A: water + 0.05% TFA, solvent B: MeCN + 0.04% TFA. Flow rate: 1 mL/min. Injection volume: 20 µL. Concentration of the sample: 20 mg/mL.

Preparative chromatography was carried out on a Waters Platform 2695 LC system that include diode array detector (detecting between 210 and 400 nm), ELS detector, LC mass spectrometer (detecting between 150 and 600 *m/z* units ESI^+) with the Kinetex 5 µm C_{18} (New Coloum, 250 × 21.2 mm) reverse phase column. The program was run 0-4 min (90% A), 4-8 min (reduced to 70% A), 8-17 mins (ramped to 95% B), 17–18 mins (ramped to 90% A), 18-20 mins (90% A). Solvent A: water + 0.05% TFA, solvent B: MeCN + 0.04% TFA. Flow rate: 20 mL/min. Injection volume: 50 - 200 µL. Concentration of the sample: 20 mg/mL.

NMR spectra recorded on a Bruker 400 MHz Ascend or a Bruker 500 MHz Ultrashield with DRX console and 5 mm TCI 1H-13C/15N (Z-GRD) cryo-probe and BACS sample changer and HR-ESI-MS data were obtained using Micromass GCT.

Isolation of Fungal Genomic DNA

Cells were separated from the liquid medium culture by centrifugation (10,000 rpm, 25 mins, 4 °C) and the supernatant removed. The sample then freeze-dried for overnight. The cells (approx. 100 mg) were crushed with a pestle and mortar in liquid nitrogen. The genomic DNA was obtained by following the manufactures instructions of the GenElute Plant Genomic DNA Kit (Sigma) with 100 mg of tissue. gDNA was analysed by gel electrophoresis. Full length genome sequence were obtained using Illumina sequencing service provided by CeBITEC, University of Bielfield, Germany (later transferred to Bioinformatics & Systems biology Justus-Liebig-University Giessen, Germany).

Result and Discussion

Structural Elucidation

Structural elucidation of both compounds was carried out using several spectroscopic techniques including ^1H and ^{13}C NMR, 2D-COSY, HSQC and HMBC and compare with previous data. The isolated compounds include the following:

(3aS)-3a,5,5,8-tetramethyl-3,3a,4,5-tetrahydro-1H-cy-clopenta[de]isochromen-1-one (10-oxodehydrobotrydial) **(1):** Colourless oil; Yield 0.8 mg; ^1H NMR (500 MHz) and ^{13}C NMR (150 MHz) data were consistent with those previously reported [11] are tabulated in Table 1; HRESIMS m/z 231.1392 $[M + H]^+$ (calculated for $C_{15}H_{19}O_2$ m/z 231.1380); Rt = 12.2 min; Compound **1** have been previously isolated from *H. rickii* [11]. The structure of the compound **1** was further confirmed using 2D NMR experiments including HMBC. Correlation HMBC showed in Figure 2.

Table 1. NMR Spectroscopic data of 10-Oxodehydrodihydrobotrydial in MeOH-d_4

Position	δ_C (ppm)		δ_H (*m*, *J* in Hz, ΣH)		HMBC
	1	**1***[11]	**1**	**1*** [12]	
1	119.0	119.6	-	-	-
2	139.1	139.6	-	-	-
3	131.5	131.7	7.21 (*d*, *J* = 7.7 Hz, 1H)	7.20 (*d*, *J* = 7.8 Hz, 1H)	C-1, C-2, C-4, C-5
4	127.5	127.3	7.33 (*d*, *J* = 7.7 Hz, 1H)	7.14 (*d*, *J* = 7.8 Hz, 1H)	C-2, C-3, C-5
5	151.6	151.4	-	-	-
6	40.5	40.8	-	-	-
7	51.4	52.1	2.03 (*d*, *J* = 10.1 Hz, 1H) 1.94 (*d*, *J* = 10.1 Hz, 1H)	1.84 (*d*, *J* = 13.0 Hz, 1H) 1.96 (*d*, *J* = 13.0 Hz, 1H)	C-6, C-8, C-14, C-15
8	44.7	45.1	-	-	-
9	147.2	147.0	-	-	-
10	165.0	164.0	-	-	-
11	18.9	20.3	2.59 (s, 3H)	2.61 (s, 3H)	C-1, C_2, C-3
12	29.6	30.7	1.35 (s, 3H)	1.44 (s, 3H)	C-5, C-6, C-13
13	29.5	30.7	1.35 (s, 3H)	1.31 (s, 3H)	C-5, C-6, C-12
14	23.5	24.7	1.50 (s, 3H)	1.50 (s, 3H)	C-7, C-8, C-9, C-15
15	79.2	79.2	4.40 (*d*, *J* = 10.1 Hz, 1H) 4.17 (*d*, *J* = 10.1 Hz, 1H)	4.12 (d, *J* = 10.1 Hz, 1H) 4.35 (d, *J* = 10.1 Hz, 1H)	C-7, C-8, C-9, C-10, C-14

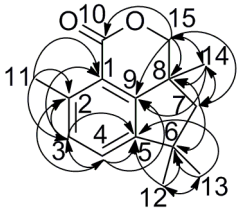

Figure 2. HMBC correlation of 10-Oxodehydrodihydrobotrydial (**1**)

***4β-Acetoxy-9β,10β,15α-trihydroxyprobotrydial* (2):** Yellow oil; Yield 4.3 mg; Compound **2** has not yet isolated from *H. rickii* but similar compound have been isolated from *Botrytis cinera* [12]. ^{1}H NMR (500 MHz) and ^{13}C NMR (150 MHz) are tabulated in Table 2. The structure of the compound **2** was further confirmed using 2D NMR experiments including HMBC. Correlation HMBC showed in Figure 3.

Table 2. NMR Spectroscopic data of 4β-Acetoxy-9β,10β,15α trihydroxyprobotrydial (**2**) in MeOH-d_4

Position	**δ_C (ppm)**	**δ_H (m, J in Hz, ΣH)**	**HMBC (^{1}H↔^{13}C)**
1	33.8	1.70 (m, 1H)	C-21, C-4
2	73.1	5.11 (td, J = 10.9, 3.9 Hz, 1H)	C-11, C-4, C-11
3	39.5	1.19 – 1.14 (m, 1H) 1.83 (dt, J = 12.3, 3.4 Hz, 1H)	C-2, C-1 C-4, C-2, C-1
4	57.6	1.74 (d, J = 10.5 Hz, 1H)	C-22, C-7, C-9, C-5, C-2
5	94.1	-	
6	59.0	1.31 – 1.27 (m, 1H)	C-21, C-1, C-9, C-16, C-5
7	46.0	-	
8	48.4	1.07 (m, 1H) 2.06 – 2.00 (m, 1H)	C-14, C-15, C-9, C-8 C-14, C-15, C-22, C-7, C-9, C-17
9	56.9	-	-
10	O	-	-
11	171.0	-	-
12	19.9	1.99 (d, J = 1.7 Hz, 3H)	C-11
13	O	-	-
14	21.5	1.12 (s, 3H)	C-8, C-9, C-17, C-5
15	26.7	1.13 (s, 3H	C-22, C-8, C-3, C-2
16	87.8	4.01 (dd, J = 5.7, 3.0 Hz, 1H)	C-1, C-6, C-5, C-17
17	82.6	4.37 (d, J = 5.6 Hz, 1H)	C-14, C-8, C-9, C-16
18	O	-	-
19	O	-	-
20	O	-	-
21	19.9	1.01 (d, J = 6.3 Hz, 3H)	1, 6, 3

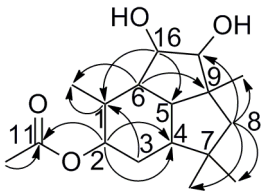

Figure 3. HMBC correlation of 4β-Acetoxy-9β,10β,15α-trihydroxyprobotrydial (**2**)

Pathway

It is proposed that both compounds derived from the botryane biosynthetic pathway. The proposed biosynthesis of **2** is shown on Figure 4. The biosynthesis starts with farnesyl pyrophosphate (FPP) **9**, a sesquiterpene**.** Terpene synthase or sesquiterpene synthase will bind to the pyrophosphate (PP) of FPP via Mg^{2+} and nucleophilic prenyl chain close concurrent to PP cleavage. A few rearrangement and cyclization of the structure produce the intermediate that eventually quenched by H_2O resulting in presilphiperfolan-8β-ol **10**. Hydroxylation at carbon 2 of presilphiperfolan-8β-ol **2** by cytochrome P450, allowed the acetylation of acetate into carbon 2 aided by transferase enzyme. Further hydroxylation via cytochrome P450 at carbon 10 and 15 might produce 4β-Acetoxy-9β,10β,15α-trihydroxyprobotrydial **2** as suggested from previous study using isotope labeling acetate experiment [6].

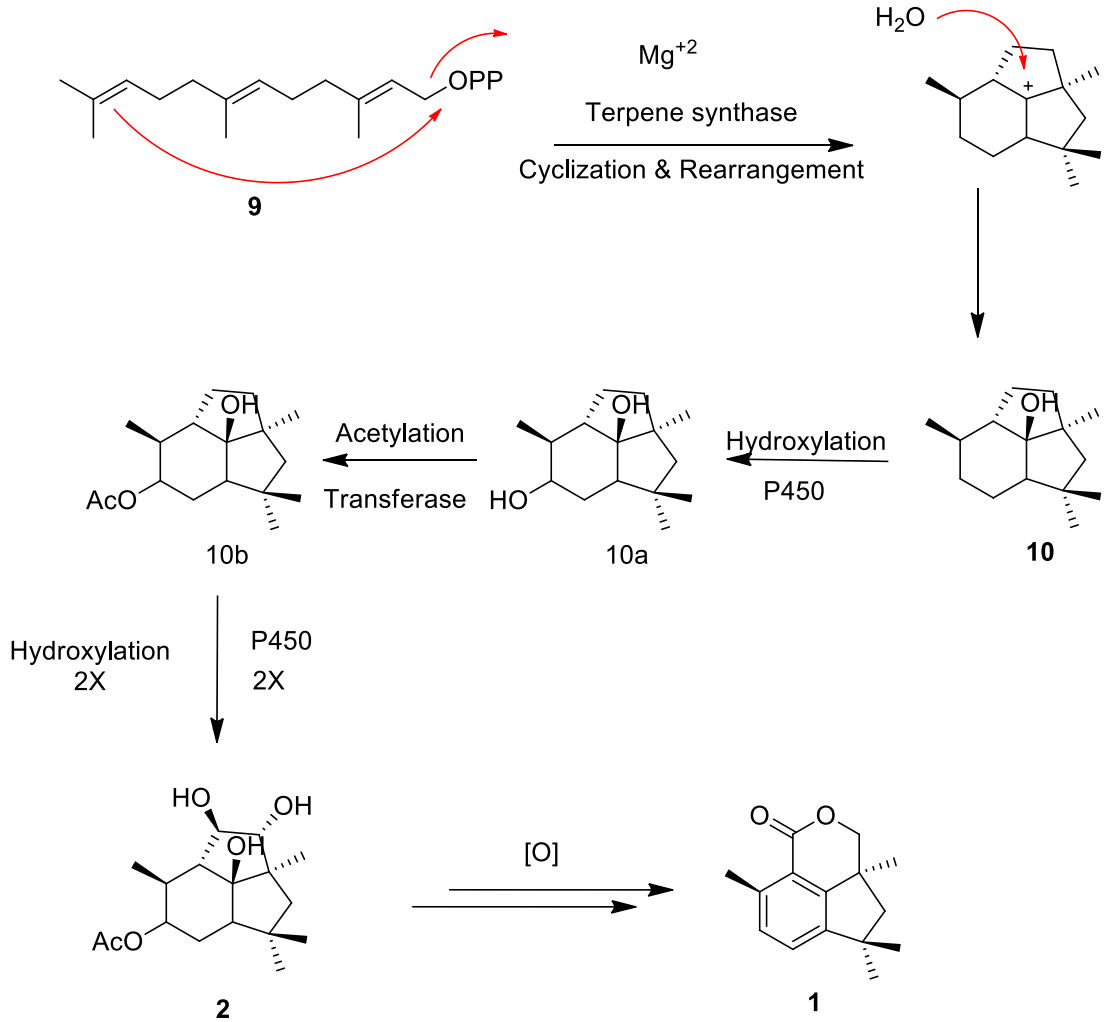

Figure 4 Proposed biosynthetic pathways of Botryane;

Farnesyl diphosphate, FPP **9** catalyzed by terpene synthase will undergo multiplestep cyclization to produce tricyclic sesquit erpene alcohol, presilphiperfolan-8β-ol **10**. Trihydroxylbotrydial **1**, is converted to botrydial by the action of cytochrome P45 0s as well as by an acetyl transferase. As for 10-oxodehydrobotrydial **2**, their oxidative steps are yet to be determined.

Gene Clustering

Genome data are stored in the GenDBE system of Bioinformatics & Systems Biology Justus-Liebig-University Giessen, Germany. *Hypoxylon rickii* genes have been annotated to have 59 secondary

metabolite producing gene clusters as in Table 3. From this data, the 6th terpene gene cluster out of 12, has been named as *HRT6* gene cluster, consist of terpene cyclase with three cytochrome P450s and two transferases (figure 6).

Table 3. Number of annotated secondary metabolite gene clusters in *Hypoxylon rickii*

SM gene cluster	No.
NRPS-PKS	4
PKS	26
NRPS	5
Terpene	12
Terpene –PKS	1
Others	11
Total	59

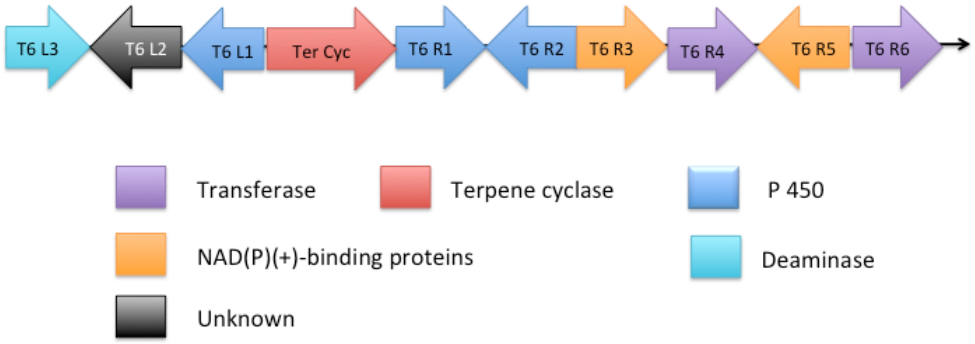

Figure 6. *HRT6* gene cluster of *Hypoxylon rickii*

Comparison of the *HRT6* have been conducted with previously studied *BcBOT* botrydial gene cluster from *Botrytis cinera* using ARTEMIS ACT software and cluster OMEGA. ARTEMIS ACT software allows comparison according to the gene sequences similarities and orientation, while cluster OMEGA allows comparison of the conserved region. Comparison between *BcBOT* and *HRT6* using ARTEMIS ACT shows similarities for five genes, namely three P450s (T6 R1, T6 R2 and T6 L1), one terpence cyclase (Ter Cyc) and one transferase (T6 R4) (Figure 7). All genes showed more than 50% similarities between all conserved regions except for transferases (Table 4).

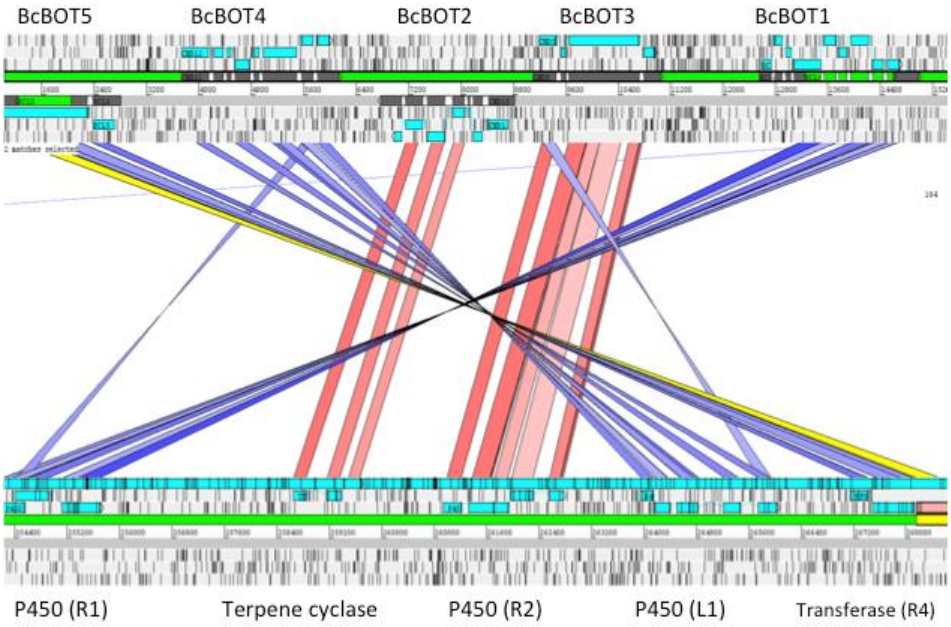

Figure 7. Comparison of gene sequence of *BcBOT* gene cluster with *HRT6* gene cluster;
*Comparison based on ARTEMIS ACT software. Red lines indicate similarities of gene sequence from 5' to 3' and blue lines 3' to 5' respectively

Table 4. Percentage comparison between *BcBOT* gene cluster and *HRT6* gene cluster

BcBOT gene cluster	**HRT6 Cluster 6 *H. rickii***	**% similarities**
BcBOT1 P450	T6 R1 (P450)	59.17
BcBOT2 (Sesquterpene synthase)	Terpene cyclase	53.70
BcBOT3 P450	T6 R2 (P450)	51.99
BcBOT4 P450	T6 L1 (P450)	62.45
BcBOT5 (acetyl Transferase)	T6 R4 (Transferase)	21.18

*Comparison based on Cluster OMEGA program.

Study by Pinedo *et al*. (2008) have examined the *BcBOT2* gene as sesquiterpene synthase by knocking it out and the mutant culture did not produce any botryanes. It is presumed that cyclization of FPP **9** to the key probotryane alcohol intermediate presilphiperfolan-8β-ol **10**, thus verifying the biosynthetic pathways of these secondary met abolites. Isotope labeling study also confirmed the backbone arrangement pathway [14].

Recent publication by Collado group in August 2016 [15] have identified successfully the function of all 5 gene i n *BcBOT* gene cluster and its as proposed in this *HRT6* gene cluster. *BcBOT4* have catalyzes the first regio- and stereospecific hydroxylation of the probotryane skeleton of presilphiperfolan-8β-ol **10** at C-3, however in *HRT6* gene the first hydroxylation was at carbon 2. For further reaction, *BcBOT1* was responsible for the regiospecific hydroxylation at carbon 16 and *BcBOT3* is involved in the regio- and stereospecific β-hydroxylation at carbon 17 [15] suggesting the same pattern of hydroxylation happen in proposed *HRT6* pathways.

As for the formation of 10-oxodehydrobotrydial **1**, the same study by Collado group [15] have suggest that *BcBOT1* also produce the key intermediate to the structure by cleavage the bond between carbon 16 and 17. *HRT6* gene cluster have an additional transferase (T6 R6) and NAD(P) binding protein that might have mechanism to remove the acetoxyl group at carbon 2 and turned it into benzene ring.

Conclusion

Hypoxylon rickii HRT6 gene cluster was annotated to be similar to *BcBOT* botrydial gene cluster isolated from *Botrytis cinera.* Four out of ten genes in the HRT6 gene cluster showed more than 50% similarities to genes from *BcBOT* botrydial gene cluster and similarities in function as well by comparing to the recent study. These findings suggested that *HRT6* highly possible into producing 4β-Acetoxy-9β,10β,15α-trihydroxyprobotrydial and it also might produce 10-oxodehydrobotrydial in right condition. An on-going project is to conduct gene knockout or gene knockdown of the terpene cyclase gene from *HRT6* to support this hypothesis.

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