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

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

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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 analysis 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 gene knock-outs is on-going.

Keywords: *Hypoxylon rickii*, botryane, terpene synthase

Abstrak

10-oksodehidrobotridial (**1**) dan 4β-Asetoksi-9β,10β,15α-trihidroksiprobotridial (**2**) telah dipencilkan dari ekstrak miselia *Hypoxylon rickii*. Sebatian **2** dijangkakan sebagai sebatian perantara dalam pembentukkan botridial 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 gen kelompok *Botrytis cinerea*, *BcBOT*. Perbandingan antara dengan cadangan laluan biosintesis 4β-Asetoksi-9β,10β,15α-trihidroksiprobotridial (**2**) dengan *BcBOT* menunjukkan persamaan fungsi – fungsi gen. Pengesahan lanjut menggunakan RNA (mendiamkan gen) sedang dijalankan.

Kata kunci: *Hypoxylon rickii*, botrien, terpene 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 cinerea* 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 leasions 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 belong 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]. *Hypoxylon 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).

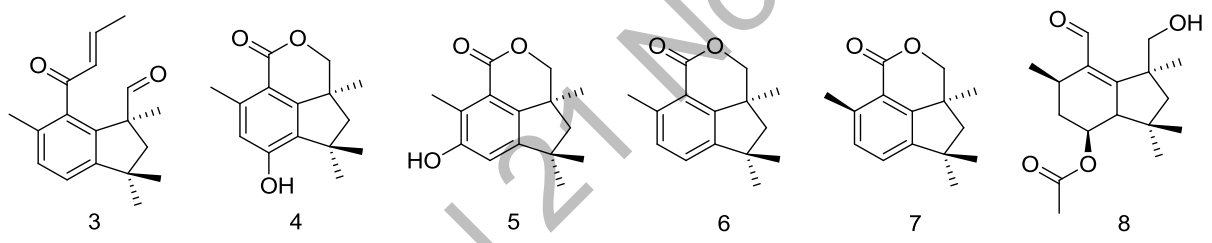


Figure 1. Botryanes isolated from *Hypoxylon rickii*

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

Hypoxylon rickii was grown in the yeast malt glucose (YMG) liquid medium at 25 ^{o}C 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 ^{o}C, 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 minutes (90% A), 10 – 13 minutes (ramped to 90% B), 13 – 15 minutes (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 minutes (90% A), 4 – 8 minutes (reduced to 70% A), 8 – 17 minutes (ramped to 95% B), 17 – 18 minutes (ramped to 90% A), 18 – 20 minutes (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 ^{o}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).

Results and Discussion

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

4β-Acetoxy-9β,10β,15α-trihydroxyprobotrydial (**1**)
Yellow oil; Yield 4.3 mg; Compound **1** 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 1.

Table 1. NMR Spectroscopic data of 10-Oxodehydrodihydrobotrydial

Position	δ_C (ppm)		δ_H (*m*, *J* in Hz, ΣH)		HMBC
	1	1*	δ_H (1)	δ_H (1*)	
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

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

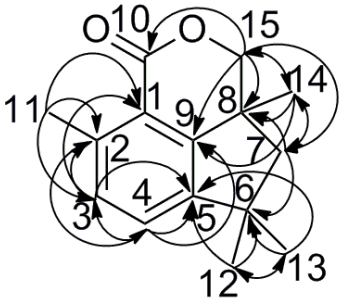


Figure 2. HMBC correlation of 10-Oxodehydrodihydrobotrydial

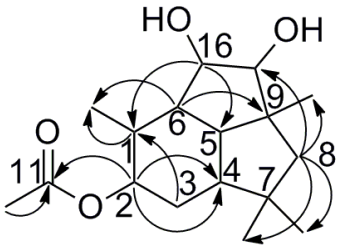


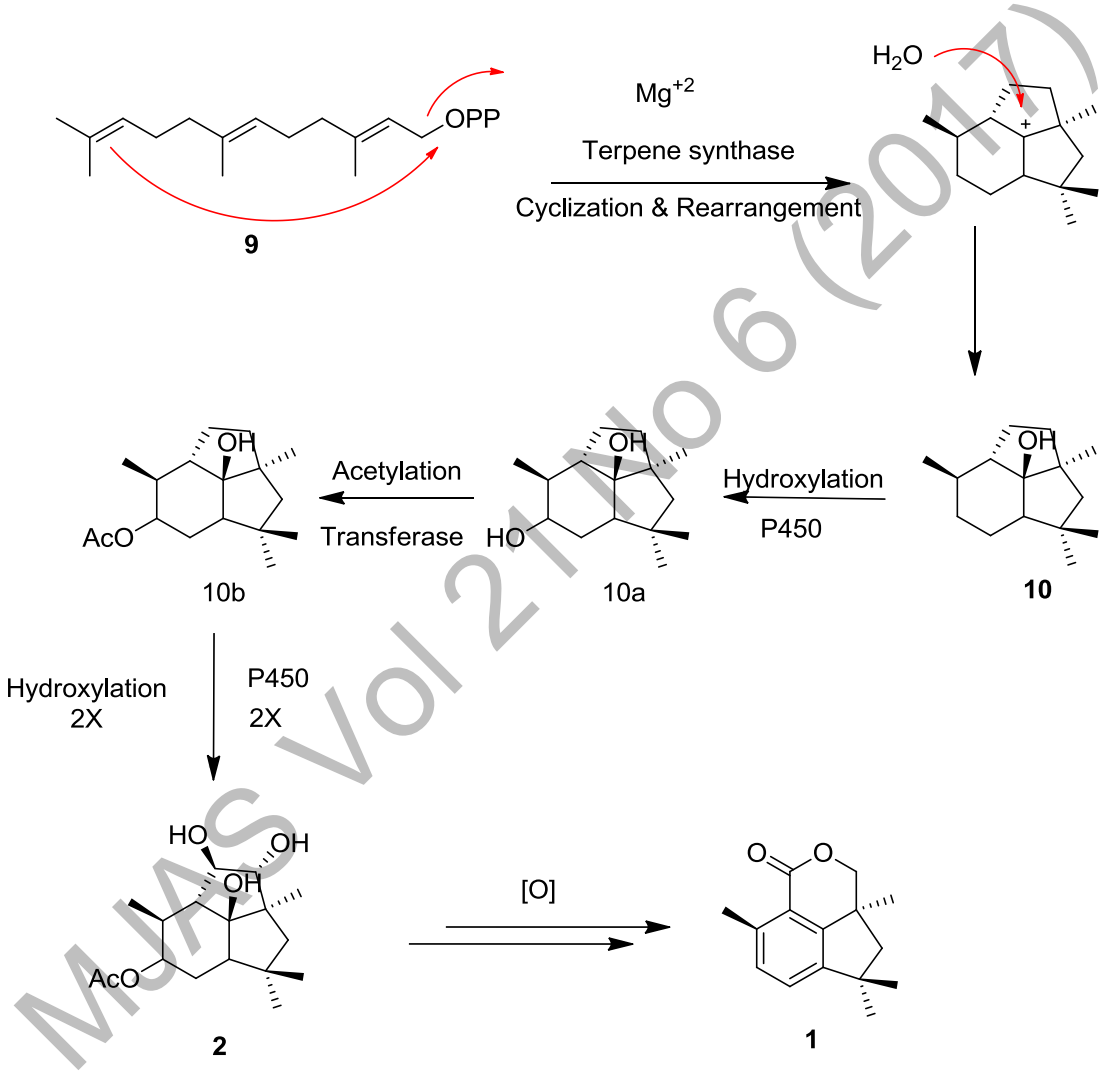
Figure 3. HMBC correlation of 4β-Acetoxy-9β,10β,15α-trihydroxyprobotrydial

Table 2. NMR Spectroscopic data of 4β-Acetoxy-9β,10β,15α trihydroxyprobotrydial in Methanol-*d*

Position	***δ_C (ppm)***	***δ_H (m, J in Hz, ΣH)***	**HMBC**
	2	**2**	
1	33.8	1.70 (m, 1H)	21, 4
2	73.1	5.11 (td, J = 10.9, 3.9 Hz, 1H)	1, 4, 11
3	39.5	1.19 – 1.14 (m, 1H)	2, 1
		1.83 (dt, J = 12.3, 3.4 Hz, 1H)	4, 2, 1
4	57.6	1.74 (d, J = 10.5 Hz, 1H)	22, 7, 9, 5, 2
5	94.1	-	
6	59.0	1.31 – 1.27 (m, 1H)	21, 1, 9, 16, 5
7	46.0	-	
8	48.4	1.07 (m, 1H)	14, 15, 9, 8
		2.06 – 2.00 (m, 1H)	14, 15, 22, 7, 9, 17
9	56.9	-	
10	0	-	
11	171.0	-	
12	19.9	1.99 (d, J = 1.7 Hz, 3H)	11
13	0	-	
14	21.5	1.12 (s, 3H)	8, 9, 17, 5
15	26.7	1.13 (s, 3H	22, 8, 3, 2
16	87.8	4.01 (dd, J = 5.7, 3.0 Hz, 1H)	1, 6, 5, 17
17	82.6	4.37 (d, J = 5.6 Hz, 1H)	14, 8, 9, 16
18	0	-	
19	0	-	
20	0	-	
21	19.9	1.01 (d, J = 6.3 Hz, 3H)	1, 6, 3

Pathway

It is proposed that both compounds derived from the botryane biosynthetic pathway. The proposed biosynthesis of **2** is shown on Scheme 1. 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].



Farnesyl diphosphate, FPP **9** catalyzed by terpene synthase will undergo multiplestep cyclization to produce tricyclic sesquiterpene alcohol, presilphiperfolan-8β-ol **10**. Trihydroxylbotrydial **1**, is converted to botrydial by the action of cytochrome P450s as well as by an acetyl transferase. As for 10-oxodehydrobotrydial **2**, their oxidative steps are yet to be determined.

Scheme 1. Proposed biosynthetic pathways of botryane

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 4).

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

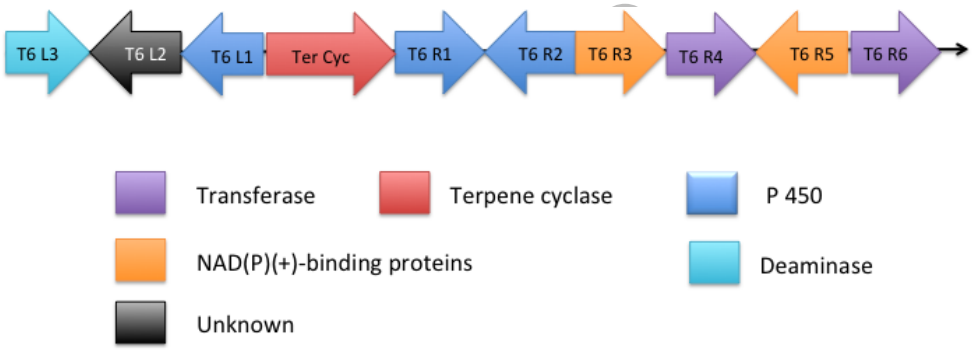
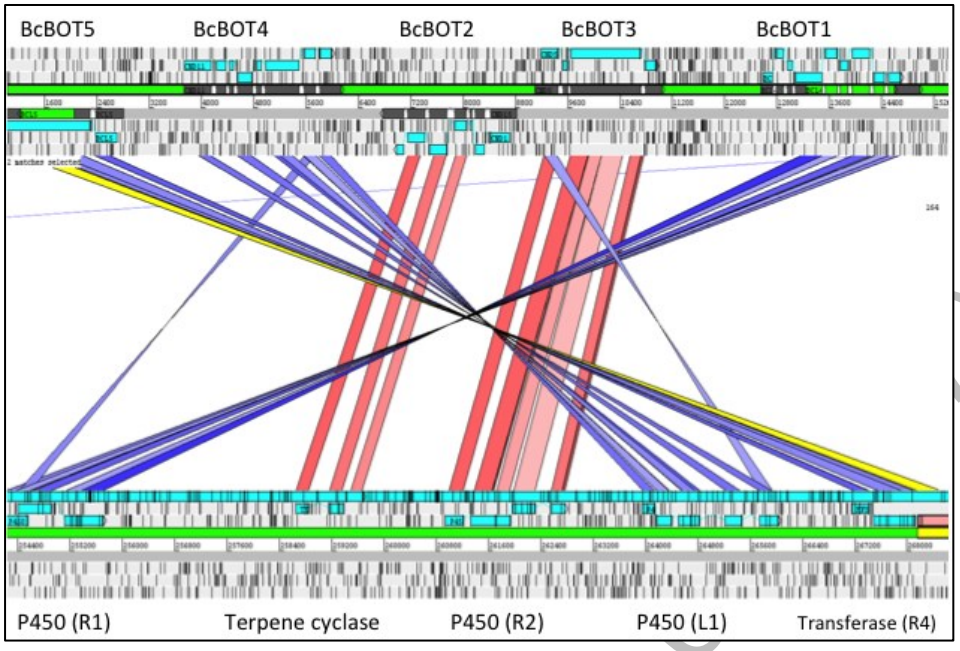


Figure 4. *HRT6* gene cluster of *Hypoxylon rickii*

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 5). All genes showed more than 50% similarities between all conserved regions except for transferases (Table 4).



*Comparison based on ARTEMIS ACT software. Red lines indicate similarities of gene sequence from 5' to 3' and blue lines 3' to 5' respectively

Figure 5. Comparison of gene sequence of *BcBOT* gene cluster with *HRT6* gene cluster

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. [14] 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 metabolites. Isotope labeling study also confirmed the backbone arrangement pathway [14].

Recent publication by Moraga et al. [15] have identified successfully the function of all 5 gene in *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 Moraga et al. [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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References

1. Breitmaier, E. (2006). General Structure: The isoprene rule. In *Terpenes :* Importance, general structure, and biosynthesis. Wiley-VCH Verlag GmbH & Co, Weinheim: pp. 1 – 9.
2. George, D. T., Kuenstner, E. J. and Pronin, S. V. (2015). A concise approach to paxilline indole diterpenes. *Journal of the American Chemical Society*, 137(49): 15410 – 15413.
3. Kuca, K., Dohnal, V., Jezkova, A. and Jun, D. (2008). Metabolic pathways of T-2 toxin. *Current Drug Metabolism*, 9(1): 77 – 82.
4. Colmenares, A. J., Dura, R. M. and Collado, I. G. (2002). Four new lactones from *Botrytis cinerea,. Journal of Natural Products*, 65(11): 1724 – 1726.
5. Yuan, Y., Feng, Y., Ren, F., Niu, S., Liu, X. and Che, Y. (2013). A botryane metabolite with a new hexacyclic skeleton from an entomogenous fungus *hypocrea* sp. *Organic Letters*, 15(23): 6050 – 6053.
6. Collado, I. G., Sánchez, A. J. M. and Hanson, J. R. (2007). Fungal terpene metabolites: Biosynthetic relationships and the control of the phytopathogenic fungus Botrytis cinerea. *Natural Product Review*, 24(4): 674 – 686.
7. Ju, Y. M., & Rogers, J. D. (1996). A revision of the genus *Hypoxylon*. *The Mycological Society of America Mycologia Memoir*, 20: 174.
8. Stadler, M., Quang, D. N., Tomita, A., Hashimoto, T. and Asakawa, Y. (2006). Changes in secondary metabolism during stromatal ontogeny of *Hypoxylon fragiforme*. *Mycological Research*, 110(7): 811 –820.
9. Quang, D. N., Hashimoto, T., Stadler, M. and Asakawa Y. (2005). Dimeric azaphilones from the xylariaceous ascomycete *Hypoxylon rutilum. Tetrahedron,* 61(35): 8451 – 8455.
10. Surup, F., Kuhnert, E., Liscinskij, E. and Stadler, M. (2015). Silphiperfolene-type terpenoids and other metabolites from cultures of the tropical ascomycete *Hypoxylon rickii* (Xylariaceae). *Natural Products and Bioprospecting*, 5(3): 167 – 173.
11. Kuhnert, E., Surup, F., Wiebach, V., Bernecker, S. and Stadler, M. 2015. Botryane, noreudesmane and abietane terpenoids from the ascomycete *Hypoxylon rickii. Phytochemistry*, 117: 116 – 122.
12. Brakhage, A. A. (2013). Regulation of fungal secondary metabolism. *Nature Reviews Microbiology*, 11(1): 21 – 32.
13. Smith, D. J., Burnham, M. K., Bull, J. H., Hodgson, J. E., Ward, J. M., Browne, P., Brown, J., Barton, B., Earl, A. J. and Turner, G. (1990). Beta-lactam antibiotic biosynthetic genes have been conserved in clusters in prokaryotes and eukaryotes. *The EMBO Journal*, 9(3): 741 – 747.
14. Pinedo, C., Wang, C. M., Pradier, J. M., Dalmais, B., Choquer, M., Le Pêcheur, P., Morgant, G., Collado, I. G., Cane, D. E. and Viaud, M. (2008). Sesquiterpene synthase from the botrydial biosynthetic gene cluster of the phytopathogen Botrytis cinerea. *ACS Chemical Biology*, 3(12), 791 – 801.

15. Moraga, J., Dalmais, B., Izquierdo-Bueno, I., Aleu, J., Hanson, J. R., Hernández-Galán, R., Viaud, M., and Collado, I. G. (2016). Genetic and molecular basis of botrydial biosynthesis: Connecting cytochrome P450-encoding genes to biosynthetic intermediates. *ACS Chemical Biology*, 11(10): 2838 – 2846.