รายงานวิจัยฉบับสมบูรณ์

โครงการ: การศึกษาชีวสังเคราะห์และการค้นหายีนที่เกี่ยวข้องกับชีวสังเคราะห์ของสาร menisporopsin A จากเชื้อรา *Menisporopsis theobromae* BCC 4162 โดยใช้เทคนิคทางการติดฉลากด้วย ^{13}C และเทคนิคทางอณูชีววิทยา

โดย นายปกรณ์ วรรธนะอมร และคณะ

สิงหาคม 2556

สัญญาเลขที่ MRG5480223

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สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

สารประกอบโพลีคีไทด์เป็นสารทุติยภูมิที่มีการออกฤทธิ์ทางชีวภาพอย่างหลากหลาย โดยสารเมทาบอไลท์กลุ่มนี้สามารถพบได้ในสิ่งมีชีวิตต่าง ๆ รวมทั้งเชื้อรา เนื่องด้วยได้มีงานวิจัยอยู่อย่างต่อเนื่องในการค้นหาสารออกฤทธิ์ทางชีวภาพจากแหล่งสิ่งมีชีวิตที่พบในประเทศไทย จึงทำให้เชื้อรานั้นเป็นสิ่งมีชีวิตที่เป็นเป้าหมายในการค้นหาสารออกฤทธิ์ดังกล่าว โดยที่เร็วๆ นี้ กลุ่มวิจัยจาก BIOTEC ได้ทำการแยกสาร menisporopsin A ที่อยู่ในกลุ่ม macrocyclic polylactone และสาร menisporopsin B ที่เป็นสารในกลุ่ม linear polyester ซึ่งผลิตโดยเชื้อรา *Menisporopsis theobromae* BCC 4162 โดยสารเหล่านี้มีการออกฤทธิ์ทางชีวภาพอยู่อย่างหลากหลาย เช่น เป็นสารยับยั้งมาลาเรียจาก *Plasmodium falciparum* K1 โดยสารในกลุ่มนี้ยังพบได้ในเชื้อราประเภทอื่น เช่น เชื้อรา *Penicillium verruculosum Hypoxylon oceanicum Periconia byssoides* และ *Aplysia kurodai* โดย ณ ปัจจุบันยังไม่มีงานวิจัยทางชีวสังเค ราะห์ของสารในกลุ่มนี้ จึงเป็น ประโยชน์อย่างยิ่งที่จะทำการศึกษาเพื่อเข้าใจกระบวนการสร้างสารในกลุ่มดังกล่าว การศึกษาชีวสังเคราะห์สามารถทำได้โดยวิธีการติดฉลากด้วย ^{13}C และการค้นหายีนที่เกี่ยวข้องกับชีวสังเคราะห์ ซึ่งการศึกษาดังกล่า วจะสามารถทำให้เราเข้าใจชีวสังเคราะห์ของสารที่มีโครงสร้างซับซ้อนเหล่านี้ได้ นอกจากนั้น ความรู้ดังกล่าวจะนำไปสู่การดัดแปลงโครงสร้างของสารออกฤทธิ์ที่มีอยู่ให้มีประสิทธิภาพในการออกฤทธิ์มากยิ่งขึ้น โดย คณะผู้วิจัยได้รายงานผลงานวิจัยซึ่งเป็นครั้งแรกที่ได้มีการศึกษาการนำ ^{13}C ไปใช้ในชีวสังเคราะห์ของแต่ละคาร์บอนบนโครงสร้างของ menisporopsin A โดยใช้ sodium acetate ที่ติดฉลากด้วย ^{13}C ที่คาร์บอนตำแหน่งที่ 1 และ 2 ตามลำดับ จาก การทดลองดังกล่าวทำให้เราสรุปได้ว่า แต่ละหน่วยที่ประกอบขึ้นเป็นสาร pentalactone menisporopsin A นั้น สังเคราะห์มาจาก polyketide synthase (PKS) โดยที่ทางคณะผู้วิจัยยังพบยีน PKS ทั้งแบบ reducing และ non-reducing ในช่วงที่เชื้อราทำการผลิต menisporopsin A อีกด้วย

คำสำคัญ: menisporopsin A, การติดฉลากด้วย ^{13}C, สารประกอบโพลีคีไทด์

Abstract

Polyketides are a large group of secondary metabolites exhibiting a wide range of biological activities. Metabolites in this family are found in different organisms including fungi. Due to the ongoing research in discovery of biologically active secondary metabolites from Thai bioresouces, this makes fungi are one of the promising target organisms for this purpose. Recently, research group from BIOTEC isolated the macrocyclic polylactone, menisporopsin A, and linear polyester, menisporopsin B, from the seed fungus *Menisporopsis theobromae* BCC 4162. These compounds exhibit a wide range of biological activities including antimalarial activity against *Plasmodium falciparum* K1. Similar compounds were also found in other fungal species such as *Penicillium verruculosum*, *Hypoxylon oceanicum*, *Periconia byssoides* and *Aplysia kurodai*. So far there is no biosynthetic study of these compounds. Such a unique structure of menisporopsin A, this would be invaluable to understand how the complex structure of this compound is assembled. ^{13}C-labelling studies and search for the gene cluster which is responsible for its biosynthesis will lead to a better understanding of its biosynthetic mechanism. Furthermore, we could genetically manipulate the formation of a variety of compounds using its gene cluster to improve the biological activities. Here, we report the first ^{13}C incorporation at individual carbons of menisporopsin A using sodium [1-^{13}C] and [2-^{13}C] acetate. This result indicates that each of the subunits of the pentalactone menisporopsin A is assembled by a polyketide synthase. Also, two polyketide synthase (PKS) genes, reducing and non-reducing PKSs, were identified during the production phase of menisporopsin A.

Keywords: menisporopsin A, ^{13}C- labelling, polyketides

Executive summary

Polyketides are a large group of secondary metabolites exhibiting a wide range of biological activities. Metabolites in this family are found in different organisms including fungi. There is a great number of ongoing research involved in screening for novel biologically active compounds from several organisms including the unexplored fungus *Menisporopsis theobromae* BCC4162. The crude extract from this fungus showed a potent antimalarial activity and this led to the isolation of two novel polyketides *i.e.* the macrocyclic polylactone, menisporopsin A[1], and linear polyester, menisporopsin B[2] (Figure 1). Similar compounds were also found in other fungal species such as *Penicillium verruculosum*, *Hypoxylon oceanicum*, *Periconia byssoides* and *Aplysia kurodai*. These compounds belong to the polyketide family. .So far there is no biosynthetic study of these compounds. Such a unique structure of menisporopsin A, this would be invaluable to understand how the complex structure of this compound is assembled. Isotope labelling studies and identification of the genes responsible for its biosynthesis will lead to a better understanding of its biosynthetic mechanism. Furthermore, we could genetically manipulate the formation of a variety of compounds using its gene cluster to improve the biological activities.

From the structure of menisporopsin A, we believe that this compound has a polyketide biosynthetic pathway as shown in Figure 2. The biosynthesis of menisporopsin A may be involved with two polyketide synthases (PKSs). The first one is reducing PKS responsible for the biosynthesis of the first 4-6 carbons and another is non-reducing PKS catalysing the formation of aromatic moieties (Figure 3). This is possibly similar to the PKS system in the biosynthesis of zearalenone and other resorcylic acid lactones such as radicicol (Figure 4).

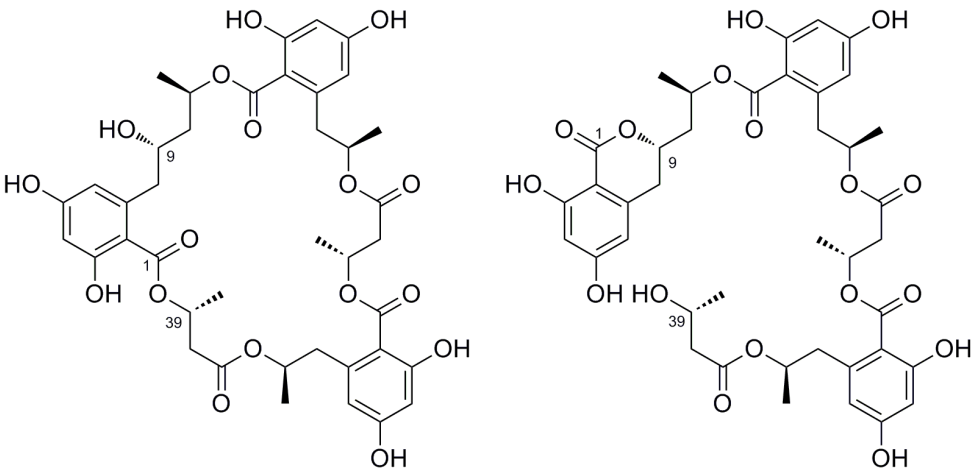

Figure 1. Chemical structures of menisporopsin A and B

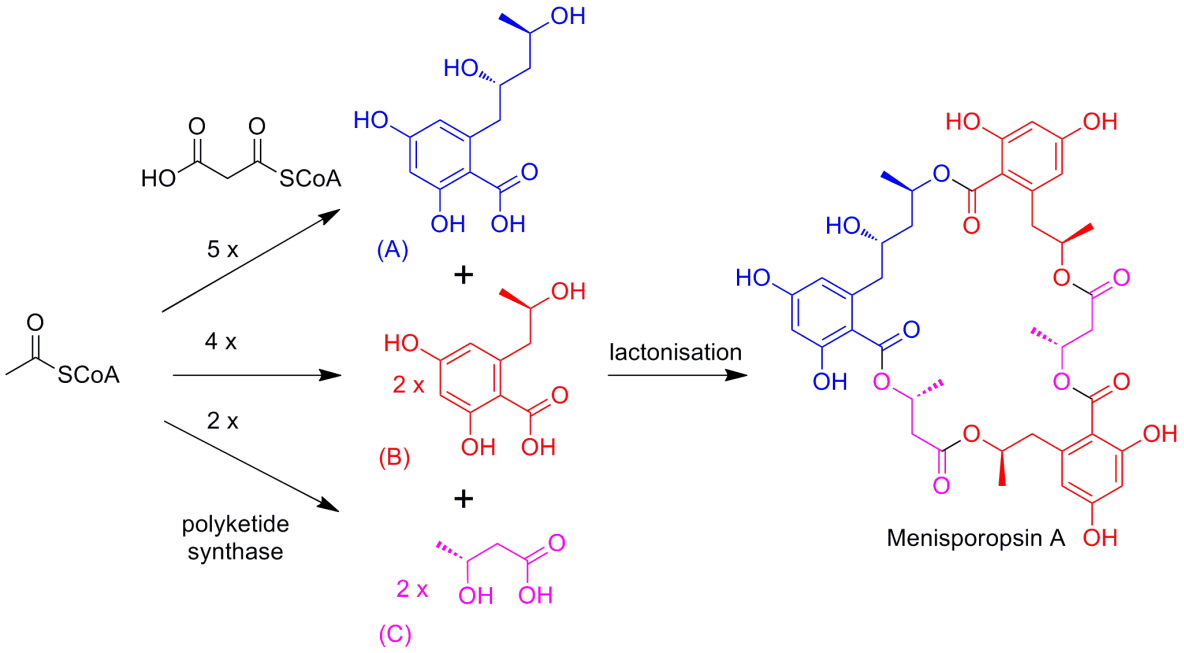

Figure 2. Proposed biosynthestic pathway of menisporopsin A. The menisporopsin A is assembled by lactonisation between (A) 2,4-dihydroxy-6-(2,4-dihydroxypentyl)benzoic acid , (B) 2,4-dihydroxy-6-(2-hydroxypropyl)benzoic acid and (C) 3-hydroxybutanoic acid.

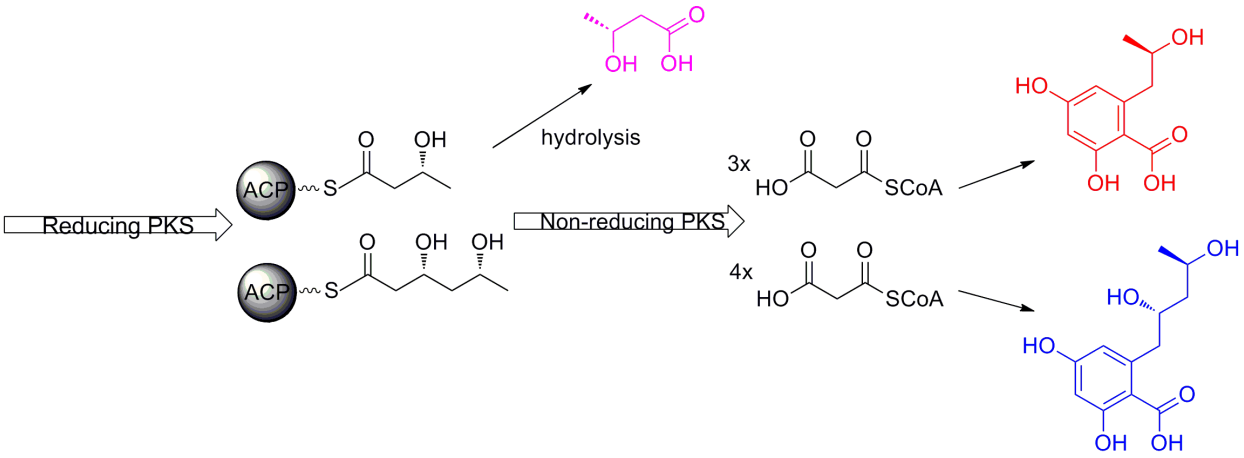

Figure 3. Possible PKS system involves in the biosynthesis of menisporopsin A.

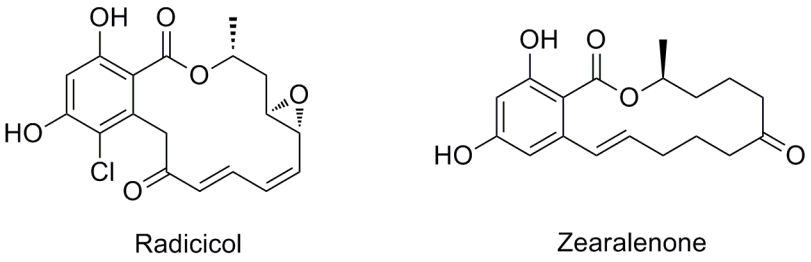

Figure 4. Structures of radicicol and zearalenone

Research objectives

1. To study the biosynthetic pathway of menisporopsin A produced by *Menisporopsis theobromae* BCC 4162 by feeding with ^{13}C-labelled precursors
2. To search genes responsible for the biosynthesis of menisporopsin A

Materials and methods

1) Production of *M. theobromae* BCC 4162 cultures[3]

1.1 The seed fungus *M. theobromae* BCC 4162 was obtained from the BIOTEC Culture Collection and activated on potato dextrose agar at 25 °C for 7-14 days.

1.2 The fungus was then transferred into 25 ml potato dextrose broth in 250 ml Erlenmeyer flask and incubated at 25 °C with shaking at 200 rev min^{-1} for 7 days.

1.3 The seed culture was transferred into fructose meat extract salt medium (FMSM) and further incubated at 25 °C with shaking at 200 rev min^{-1} for 4 days.

2) Extraction and Isolation of menisporopsin A from *M. theobromae* BCC 4162

2.1 The culture was then harvested by filtration to separate the fungal mycelia from the broth and macerated in methanol for 2 days at room temperature.

2.2 The methanol extract was then concentrated and dissolved in 100 ml distilled water and the aqueous solution was extracted with 2 x 100 ml of hexane followed by 2 x 100 ml of ethyl acetate. The ethyl acetate layer was then evaporated to dryness.

2.3 The crude ethyl acetate extract was further purified using a Sephadex LH-20 column, yielding menisporopsin A.

3) Feeding experiments with sodium [1-^{13}C] and [2-^{13}C] acetate

Acetate is a building block for the polyketide metabolites. Therefore we used sodium [1-^{13}C] and [2-^{13}C] acetate in the feeding studies. The sodium [1-^{13}C] acetate was added when the seed culture was transferred into fructose meat extract salt medium (FMSM) at a final concentration of 100 mg/L. The culture was further grown in the same condition as previously described. After 4 days, the culture was harvested and the menisporopsin A was isolated from the 1-^{13}C acetate culture. The same experiment was performed with the feeding of [2-^{13}C] acetate

4) cDNA synthesis and probing of KS gene

In order to obtain information on the gene responsible for biosynthesis of menisporopsin A, RNA was isolated from *M. theobromae* grown during the production phase (4 days) in FMSM using a Totally RNA Mini kit (Geneaid). The cDNA was synthesized from mRNA containing in the RNA extract using ProtoScript AMV LongAmp Taq RT-PCR kit. In order to probe the gene involved in the biosynthesis of menisporopsin A, first we used the degenerate primers designed from the ketosynthase domain. The ketosynthase domain is the most highly conserved domain among the fungal polyketide synthase (PKS). The degenerate primers are designed based on the amino acid sequence identity from several ketosynthase (KS) domains from a variety of fungal species[4,5]. The fungal PKSs are categorised into three classes i.e. non-reducing, partially reducing and highly reducing PKSs. The degenerate primers used for probing fungal KS genes are shown below.

1. Degenerate primer for non-reducing PKS

LC1 (forward primer) 5'-GAT CCI AGI TTT TTT AAT ATG-3'

LC2c (reverse primer) 5'-GT ICC IGT ICC GTG CAT TTC-3'

2. Degenerate primer for partially reducing PKS

LC3 (forward primer) 5'-GCI GAA CAA ATG GAT CCI CA-3'

LC5c (reverse primer) 5'-GT IGA IGT IGC GTG IGC TTC-3'

3. Degenerate primer for highly reducing PKS

KS3 (forward primer) 5'- TTY GAY GCI GCI TTY TTY AA -3'

KS4c (reverse primer) 5'- RTG RTT IGG CAT IGT IAT ICC -3'

To amplify the KS gene involved in the biosynthesis of menisporopsin A, polymerase chain reaction (PCR) was performed using the series of degenerate primers as shown above and synthesized cDNA obtained from mRNA during the production phase of menisporopsin A as a template with LongAmp *Taq* polymerase (NEB) in the buffer supplied by the manufacturer. In

25 µl reaction, all components were assembled as described by Lazarus C.M., *et al.*[4] and Cox R.J., *et al.*[5] The obtained PCR products were further purified and cloned into pGEM-T Easy vector for DNA sequencing.

5) Rapid amplification of cDNA ends (RACE) method

In order to complete the full length of PKS genes, RACE techniques was performed using FirstChoice RLM-RACE (Invitrogen) and SMARTer RACE cDNA Amplification (Clontech) kits.

Results and discussion

1) Structure confirmation of menisporopsin A

The isolated menisporopsin A was confirmed by MS, ^{1}H and ^{13}C NMR spectra as shown in Appendix. The LC/ESIMS spectrum of isolated compound gave an accurate mass of m/z 821.3 $[M+Na]^{+}$. The ^{1}H spectrum of menisporopsin A revealed the five methyl groups (δ_H 1.20-1.48), six methylene protons (δ_H 1.94-3.60), six methine protons in which five with ester linkages (δ_H 5.13-5.58) and one attached to carbon bearing –OH group (δ_H 3.96), protons on three resorcylic acid derivatives (δ_H 6.24-6.37) and broad peaks of phenolic protons (δ_H 9.25-11.71). The ^{13}C spectrum showed five ester carbonyl carbons (δ_C 171.14-172.67), three aromatic carbons bearing –OH at *ortho* position to ester carbonyl (δ_C 166.94-167.54), three aromatic carbons bearing –OH at *para* position to ester carbonyl (δ_C 164.06-164.25), three aromatic carbons at *ortho* position to ester carbonyl (δ_C 144.39-146.14), six aromatic methine carbons and three aromatic quaternary carbons (δ_C 103.30-115.42), six quaternary carbon (δ_C 70.47-73.84), six methylene carbons (δ_C 42.11-46.93) and five methyl carbons (δ_C 20.75-21.12).

^{1}H NMR (400 MHz, acetone-d_6): δ_H 6.37 (1H, d, *J* = 1.9 Hz, H-6), 6.35 (1H, d, *J* = 2.1 Hz, H-34), 6.33 (1H, d, *J* = 2.3 Hz, H-20), 6.29 (1H, d, *J* = 2.2 Hz, H-4), 6.25 (1H, d, *J* = 2.0 Hz, H-18), 6.25 (1H, d, *J* = 2.0 Hz, H-32), 5.57 (1H, m, H-39), 5.56 (1H, m, H-27), 5.54 (1H, m, H-13), 5.24 (1H, m, H-9), 5.16 (1H, m, H-23), 3.95 (1H, brt, *J* = 9.3 Hz, H-37), 3.57 (2H, dd, *J* = 7.7, 13.9 Hz, H-22b), 3.43 (2H, dd, *J* = 7.4, 13.0 Hz, H-8b), 3.33 (2H, dd, *J* = 9.7, 13.0 Hz, H-36b), 3.11 (2H, dd, *J* = 7.0, 13.0 Hz, H-8a), 2.87 (2H, m, H-26), 2.85 (2H, m, H-22a), 2.83 (2H, m, H-12), 2.61 (2H, dd, *J* = 9.7, 12.8 Hz, H-36a), 2.14 (2H, ddd, *J* = 4.3, 11.2, 11.8 Hz, H-38b), 1.97 (2H, ddd, *J* = 4.3, 11.5, 11.5 Hz,

H-38a), 1.48 (3H, d, *J* = 5.6 Hz, H-28), 1.46 (3H, d, *J* = 5.6 Hz, H-40), 1.41 (3H, d, *J* = 6.2 Hz, H-14), 1.23 (3H, d, *J* = 7.3 Hz, H-10), 12.1 (3H, d, *J* = 6.7 Hz, H-24) ^{13}C NMR (100 MHZ, acetone-d_6): δ_C 171.7 (C-29), 171.6 (C-15), 171.3 (C-1), 170.3 (C-25), 170.2 (C11), 166.5 (C-31), 166.2 (C-17), 166.0 (C-3), 163.3 (C-33), 163.0 C-19), 163.0 (C-5), 145.2 (C-35), 143.4 (C-7), 143.4 (C-21), 114.3 (C-34), 112.9 (C-6), 112.0 (C-20), 106.1 (C-2), 105.9 (C-16), 105.0 (C-30), 102.6 (C-4), 102.6 (C-18), 102.3 (C-32), 72.8 (C-9), 72.6 (C-23), 72.1 (C-39), 70.1 (C-13), 69.7 (C-27), 69.5 (C-37), 45.9 (C-36), 44.0 (C-38), 42.0 (C-8), 41.6 (C22), 41.3 (C-12), 41.1 (C-26), 20.1 (C-14), 20.0 (C-28), 19.9 (C-40), 19.9 (C-10), 19.8 (C-24).

2) ^{13}C-Incorporation studies

So far, the isolated menisporopsin A from sodium [1-^{13}C] and [2-^{13}C] acetate feeding experiments can be obtained. From the ^{13}C-NMR spectra of unlabelled, [1-^{13}C] and [2-^{13}C] acetate-derived samples of menisporopsin A as shown in Figure 5, the ^{13}C enrichments at individual positions were calculated as described by Simpson and co-workers.[6] The percentages of ^{13}C-enrichment at individual carbon position on menisporopsin A are in the range of 0.5-6 % as shown in Table 1. This result is in agreement with the proposed labelling pattern of menisporopsin A (Figure 6). When the [1-^{13}C] acetate was introduced in the labelling experiment, the signal intensities of the carbon at the odd positions are stronger compared to the unlabelled carbons at the even positions. *Vice versa*, this can be observed when the [2-^{13}C] acetate was used.

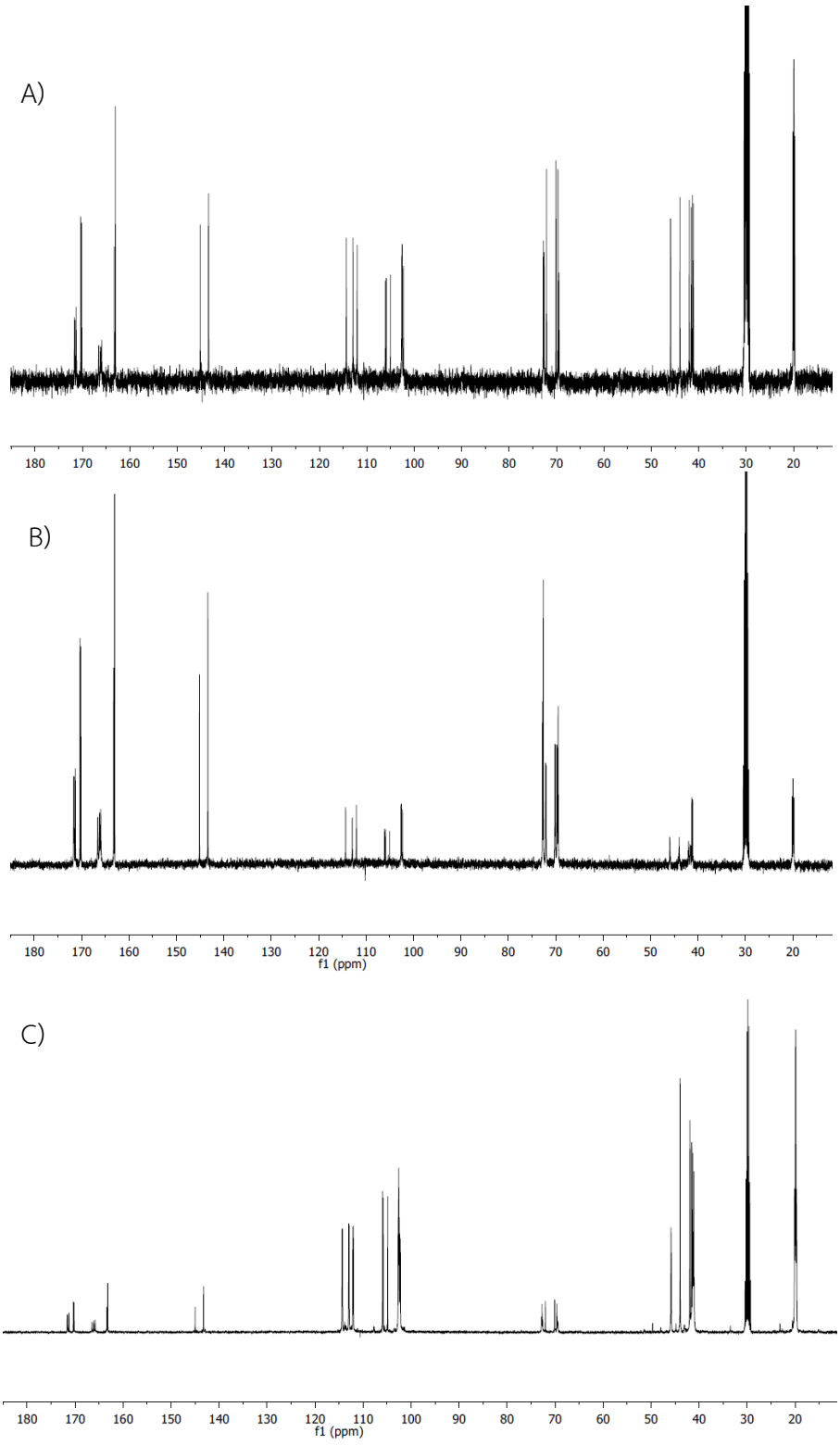

Figure 5. ^{13}C-NMR spectra of (A) unlabelled, (B) [1-^{13}C] and (C) [2-^{13}C] acetate labelled menisporopsin A.

Position of carbon	δ_c (ppm)	Observed intensities			% ^{13}C-enrichment	
		unlabelled	[1-^{13}C]	[2-^{13}C]	[1-^{13}C]	[2-^{13}C]
1	171.3	96.096	51.740	57.876	3.21	-
2	106.1	134.197	19.151	421.166	-	5.59
3	166.0	52.910	29.618	36.805	3.38	-
4	102.6	162.067	31.806	343.619	-	3.42
5	163.0	360.169	202.603	148.141	3.40	-
6	113.0	185.972	25.145	323.824	-	2.61
7	143.4	243.879	147.381	137.210	3.73	-
8	42.0	235.387	12.436	633.835	-	4.64
9	72.8	182.331	85.368	46.806	2.65	-
10	19.9	352.622	24.778	585.456	-	2.44
11	170.2	208.660	117.888	89.229	3.42	-
12	41.3	241.579	36.057	534.677	-	3.62
13	70.1	286.649	64.827	97.176	0.71	-
14	20.1	324.153	36.693	429.207	-	1.72
15	171.6	79.110	46.998	50.048	3.65	-
16	105.9	138.793	18.688	401.450	-	5.07
17	166.2	43.113	27.828	34.457	4.06	-
18	102.6	177.509	32.652	342.763	-	3.02
19	163.0	360.169	202.603	148.141	3.40	-
20	112.0	176.570	31.811	316.733	-	2.72
21	143.4	227.512	140.880	137.210	3.85	-
22	41.6	226.070	10.685	567.591	-	4.25
23	72.6	167.425	153.600	38.706	6.24	-
24	19.8	321.312	35.940	406.040	-	1.59
25	170.3	216.440	122.909	92.101	3.44	-
26	41.1	231.232	34.959	480.397	-	3.33
27	69.7	287.245	63.505	84.516	0.73	-
28	20.0	398.210	46.146	560.286	-	1.90
29	171.7	82.240	47.569	52.638	3.53	-
30	105.0	139.264	18.071	412.575	-	5.22
31	166.5	45.976	25.368	30.394	3.31	-
32	102.3	149.330	29.444	286.591	-	2.99
33	163.3	176.426	107.162	75.232	3.76	-
34	114.3	186.176	30.606	309.202	-	2.44
35	145.2	203.168	102.508	75.316	2.94	-
36	45.9	210.872	14.650	304.917	-	1.98
37	69.5	140.622	85.102	33.496	3.74	-
38	44.0	240.144	14.704	759.389	-	5.64
39	72.1	278.541	54.010	93.041	0.45	-
40	19.9	419.425	36.354	884.964	-	3.40

Table 1. Intensities of individual signals in the ^{13}C- NMR spectra of menisporopsin A enriched from [1-^{13}C] and [2-^{13}C] acetates

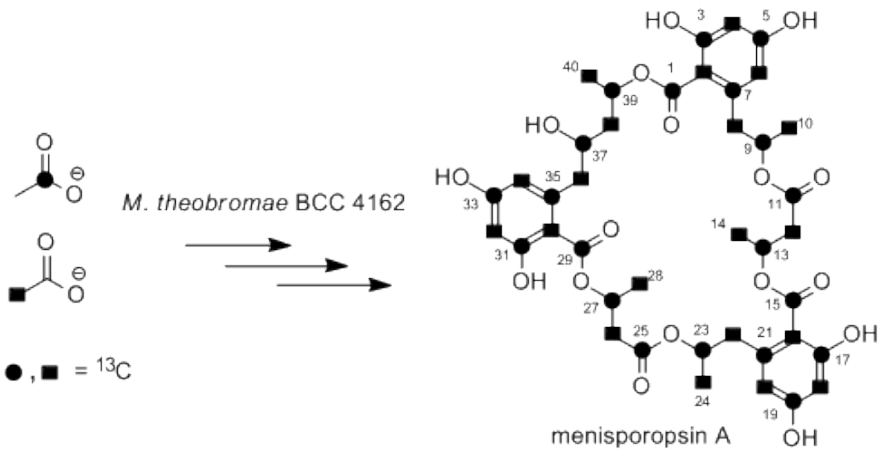

Figure 6. ^{13}C labelling pattern of menisporopsin A using 20 units of building block acetate.

3) Reducing and non-reducing polyketide synthase genes during the production phase of menisporopsin A

3.1) Identification of reducing polyketide synthase gene

The degenerate primers based on ketosynthase domain from partially (LC3/LC5c) and highly reducing (KS3/KS4c) PKSs were used to amplify the KS domain of reducing PKS during the production of menisporopsin A. The PCR products of approximately 600 bp in length (Figure 7) were obtained only from KS3/KS4c primer pair. This result is in agreement with our hypothesis as all the carbonyl group of the nascent polyketide intermediate for the first 4-6 carbons are reduced (Figure 3). For the amplified PCR products using KS3/KS4c primer pair shows high similarity with the KS gene from reducing PKS from several fungi as shown in Figure 8.

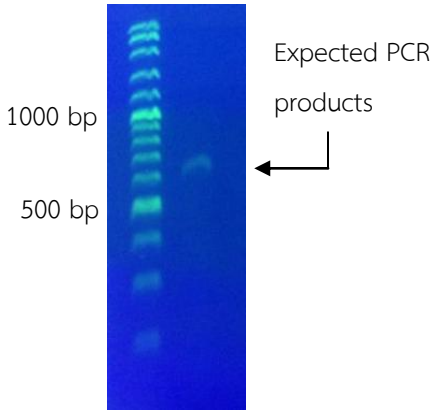

Figure 7. Amplified PCR products from KS3/KS4c primer pair.

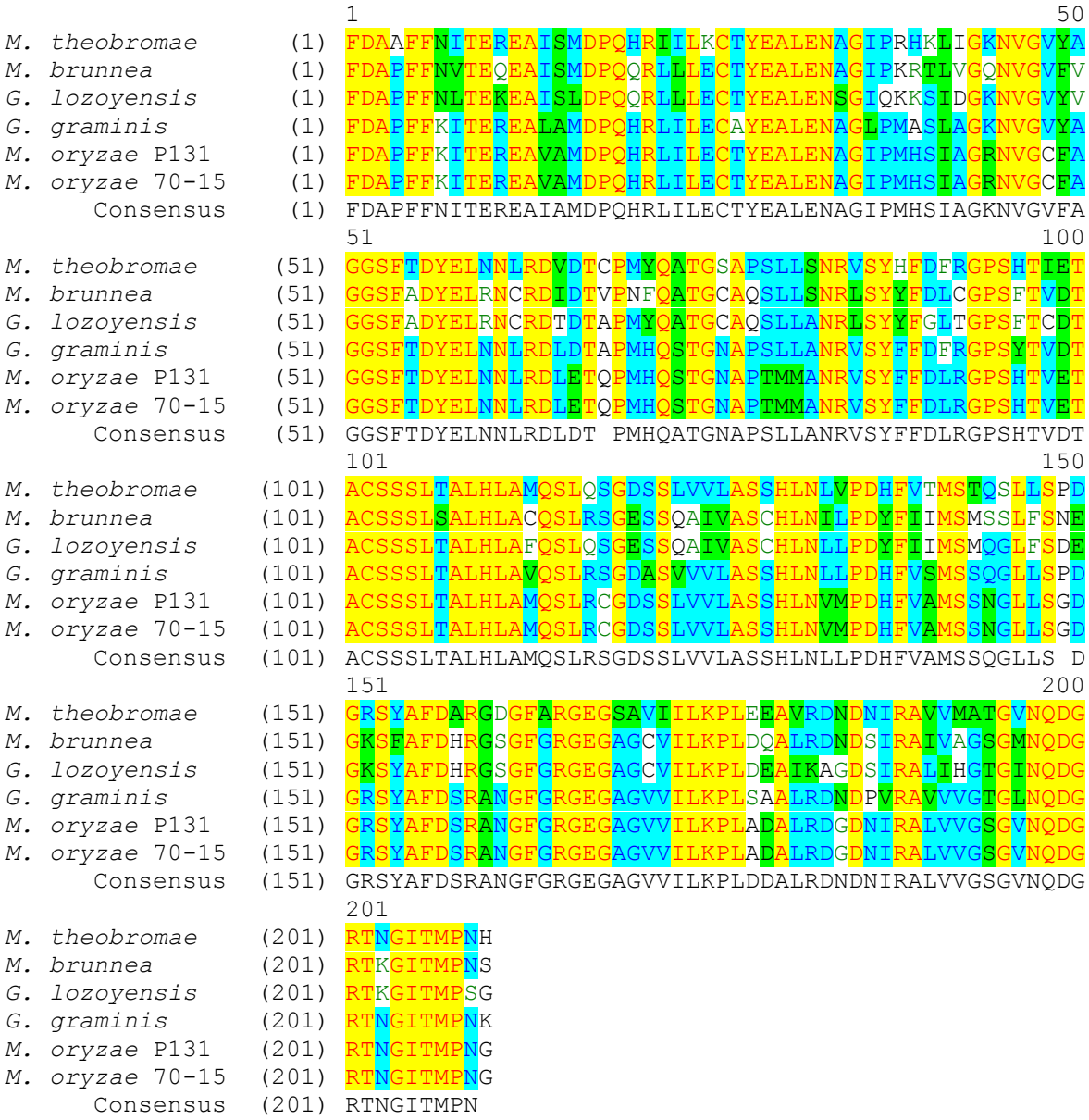

Figure 8. Amino acid sequence alignment between the KS domain from reducing PKS amplified from *M. theobromae* and other fungi which have high similarity in amino acid sequences such as *Marssonina brunnea* (EKD12633.1), *Glarea lozoyensis* (EPE30306.1), *Gaeumannomyces graminis* (EJT77326.1), *Magnaporthe oryzae* P131 (ELQ62326.1) and *Magnaporthe oryzae* 70-15 (XP 003720137.1) (Number indicates GenPept accession number)

The 5'-RACE were performed using SMARTer RACE cDNA amplification kit to complete the N-terminal of KS domain as shown in Figure 9.

```
  1    HGNITEREAN GTSHTNGVGA SSHGPKFSPV AIVGMSCRLP GSVSDPEEFY
 51    LMCSRARNGW SEMPEDRFSK KGYYHPNPDK LGSFNPVGGF FLEEDISLFD
101    APFFNITERE AISMDPQHRI ILKCTYEALE NAGIPRHKLI GKNVGVYAGG
151    SFTDYELNNL RDVDTCPMYQ ATGSAPSLLS NRVSYHFDFR GPSHTIETAC
201    SSSLTALHLA MQSLQSGDSS LVVLASSHLN LVPDHFVTMS TQSLLSPDGR
251    SYAFDARGDG FARGEGSAVI ILKPLEEAVR DNDNIRAVVM ATGVNQDGRT
301    NGITMPNH
```

Figure 9. N-terminal sequence of KS domain from reducing PKS

3.2) Identification of non-reducing polyketide synthase gene

The degenerate primers based on ketosynthase domain from non-reducing PKS were used to identify the PKS genes. The PCR products of approximately 600 bp were obtained with the pair of LC1/LC2c primers (Figure 10). The amplified KS gene from *M. theobromae* using LC1/LC2c primer shows high similarity in amino acid sequence to the KS domain from non-sporulating fungal endophytes of *Vaccinium macrocarpon* and other fungi such as *Neosartorya fischeri*, *Myceliophthora thermophila* and *Penicillium marneffei* (Figure 11). All these KS domains are from non-reducing PKS. This is in agreement of the use of LC1/LC2c primer pair. This allowed us to use the PKS from these fungi to design the new degenerate primers for other domains such as acyltransferase (AT).

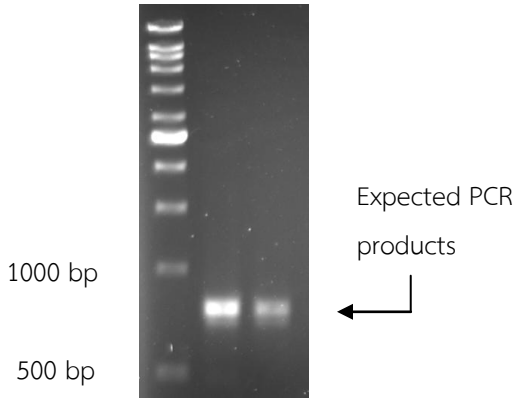

Figure 10. Amplified PCR products from LC1/LC2c primer pair.

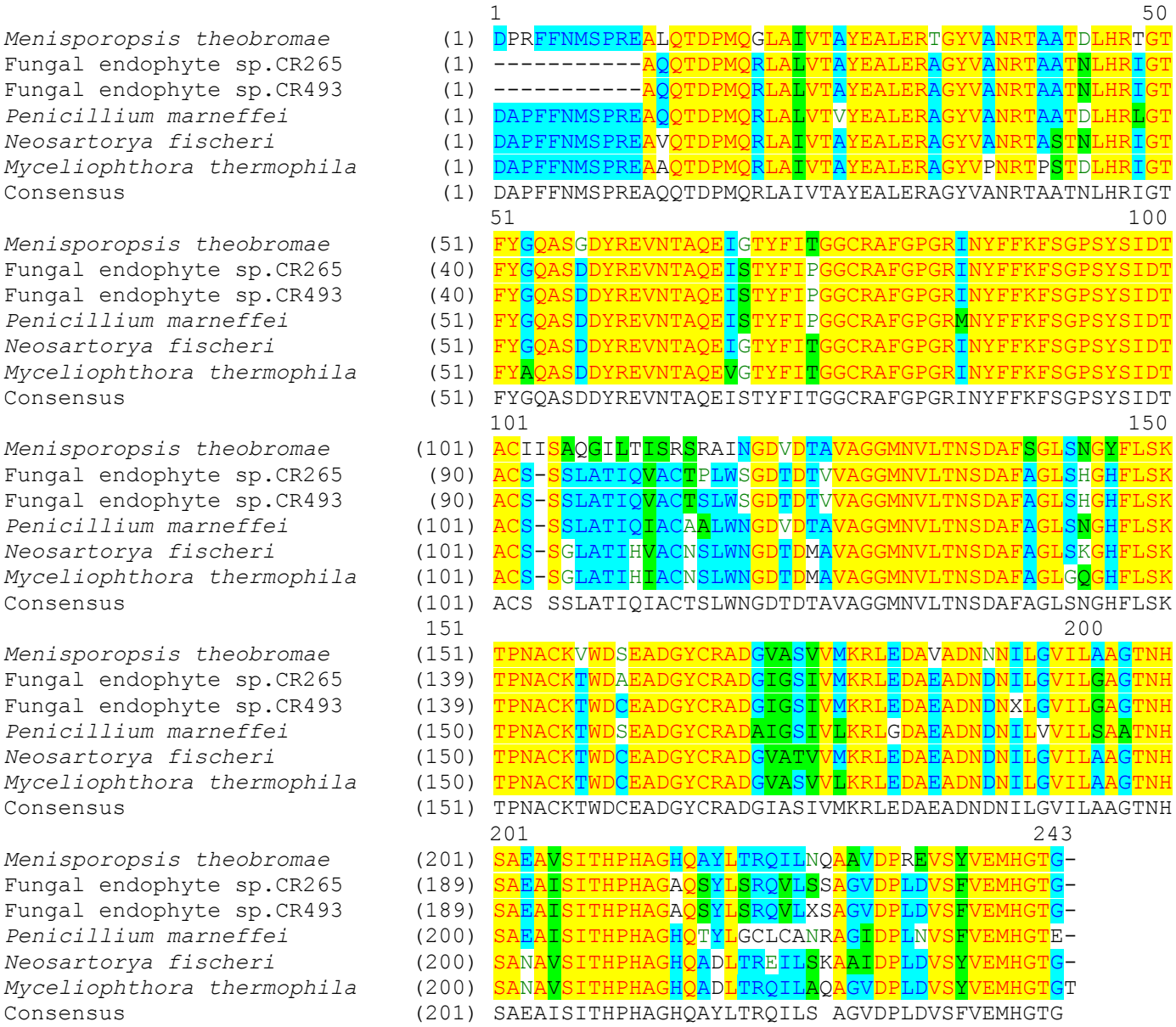

Figure 11. Amino acid sequence alignment between the KS doamin amplified from *M. theobromae* and other fungi which have high similarity in amino acid sequences such as fungal endophyte of *Vaccinium macrocarpon* (AAP79127.1 and AAP79128.1), *P. marneffei* (XP002149615.1), *N. fischeri* (XP 001266579.1), *M. thermophila* (XP003663601.1). (Number indicates GenPept accession number)

The degenerate primers for AT domains were designed based on the fungal PKS genes as mentioned above. The degenerate primer pair as shown below was used to amplify the AT domain from non-reducing PKS gene. The expected PCR products using this primer pair can be seen at 700 bp (Figure 12). This was further purified and cloned into plasmid vector for DNA sequencing. The sequencing result revealed that the design primer pair had amplified the gene in AT family and have high similarity to the AT domain from other non-reducing fungal PKSs as shown in Figure 13.

Degenerate primer for AT domain
AT (forward primer) 5'-CAY AGY YTD GGB GAR TWY GC-3'

AT (reverse primer) 5'-ACN GGR TGB GGN CCR ATY TC-3'

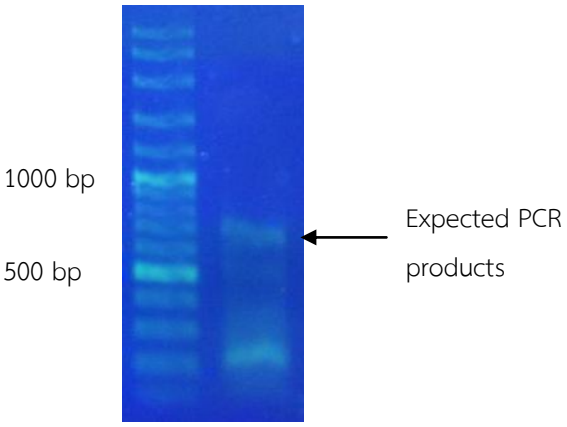

Figure 12. Amplified PCR products using design degenerate primers for AT domain.

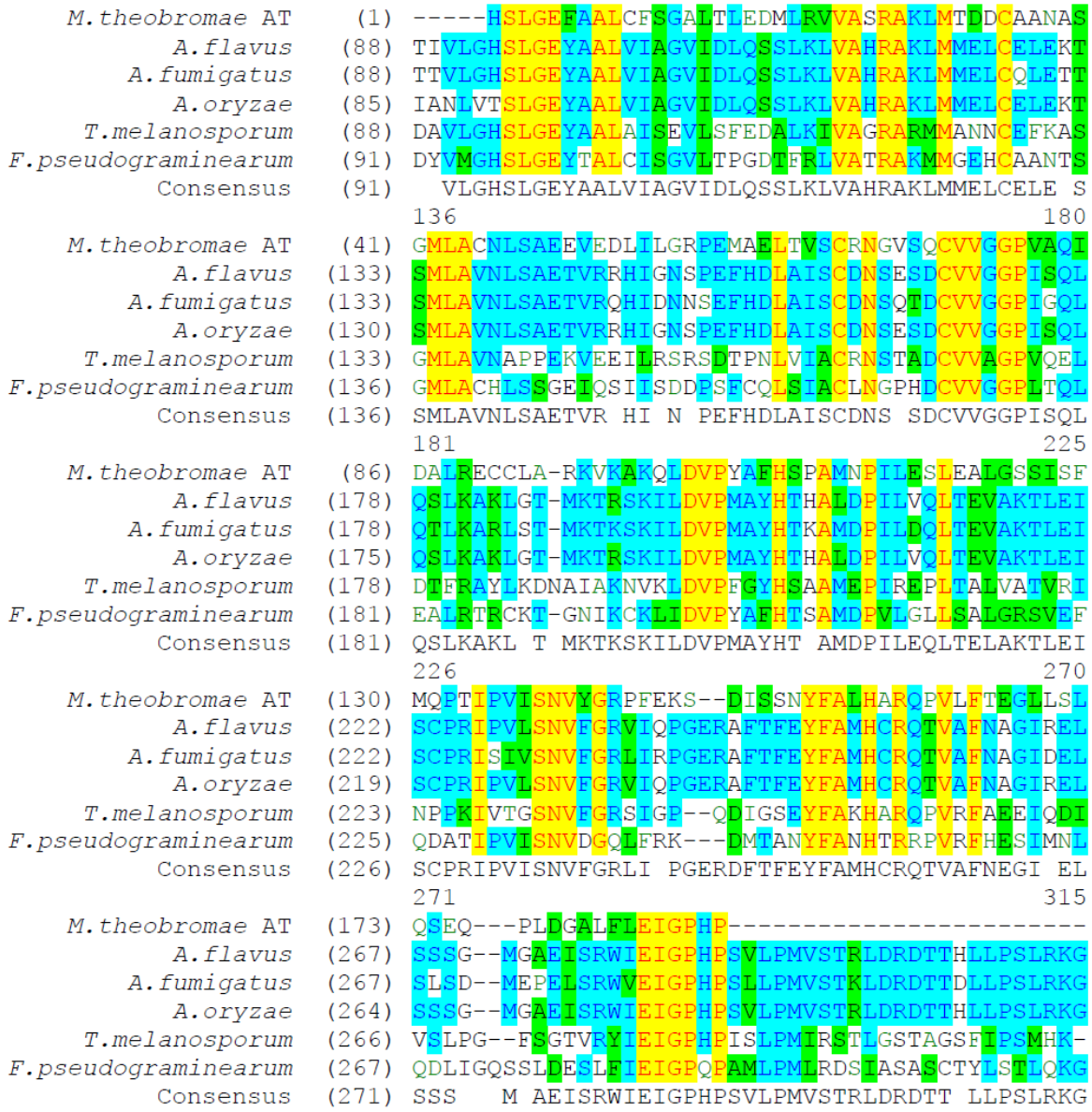

Figure 13. Amino acid sequence alignment between the AT domain amplified from *M. theobromae* and other fungi which have high similarity in amino acid sequences such as *Fusarium pseudograminearum* (EKJ74560.1), *Tuber melanosporum* (XP_002837130.1), *Aspergillus flavus* (XP_002377153.1), *Aspergillus fumigatus* (EDP49937.1), *Aspergillus oryzae* (EIT78482.1) (Number indicates GenPept accession number)

The 5'and 3'-RACE was also performed to complete the non-reducing PKS gene using primers based on nucleotide sequence of amplified KS gene. The full length of non-reducing PKS gene was identified as shown in Figure 14.

```
   1  CGCGGATCCG AACACTGCGT TTGCTGGCTT TGATGATACA CGCTACGGAG GTCAGTGCCA GCTAGTCCAT TACCCCTAAT
      ACAATCTCCC ACTTCCCTCT GGAGACTATA ATACCAGCTG ACACATTCTT TGGTAAACTT CAGAATCTTG CGGGCCCGAA
      CAAAGTCTTT ATCAGCGCAC TCGGTCGAAC GTCTGTGACC GTTAGCGGGC CGCCTGCCCG GCTCAAGCAC ATTTTTCAGG
      TGTCCGACTT CTTCCGTGAC TGCAAGTTTG TTAGCTTGCC GGTCTATGGA GGACTATGTC ACGCATCGCA CATTTATAGC
      CGAGAGCATG TGGATCGAGT AGTCCAAACC GGATCTTTGA TCTCCAAGTT CGTTCCGCGC GTCCATCTAT TTTCGACGAG
 401  CACCGGAAGG CCTTTCCCAG CTTCAGACGC CAGAGGGCTG CTTCACAACG TCGTCGAAGA GCTTCTTACC CAGGCCATAC
      AGTGGGACAA CGTCATTCGG GGCGTGATAC AACGAGCCAA AGGTGCCGAG GAGTCGCAAT GCGAGGTTCT CATGTTTCGT
      GCCTCCCTGC CTATTCGGGA TCTTATAGAG GCCCTCAACT CGGAGCTAGA GCCCTTCAAG ACGACGACGA AGGATGTGGT
      CTCGTGGATT GCAAAGCCCG AGGCGCCCCC CCAAGGCCCC CGAGGTACGC AGCAATCCTA GATCGCGATC GTGGGCATGT
      CATGCCGCAT GCCTGGGGGA GCGGCAGACA CGGAAACGTT CTGGGAGATT CTCAACTCCG GCCTCGATGT TCACAGAGAG
 801  ATCCCTCCCG ACAGATTTGA TGTCGAGACC CACTGTGACC CAACCGGCAA GCGCGTTAAC ACCAGCCACA CCCCATACGG
      ATGTTTTATT GACGAGCCTG GCCTATTTGA TGCTCCCTTT TTCAACATGT CCCCACGCGA GGCCTTGCAG ACAGATCCTA
      TGCAGGGGCT CGCCATTGTC ACGGCATATG AGGCTCTTGA GCGAACCGGA TATGTCGCGA ATCGCACCGC GGCAACCGAT
      CTGCACCGTA CCGGAACCTT CTATGGCCAG GCGAGCGGTG ACTACCGTGA AGTGAACACG GCTCAGGAGA TTGGCACGTA
      CTTCATCACC GGCGGCTGCC GCGCCTTCGG TCCGGGGCGC ATCAACTACT TTTTCAAGTT CTCTGGCCCT AGCTACAGCA
1201  TCGATACGGC TTGCTGCAGC ATCATCTCTG CCCTCCAGAT CGCGTGCAAT TCCTTATGGA ACGGTGATGT TGACACTGCC
      GTCGCCGGCG GCATGAACGT TCTGACGAAC TCCGACGCCT TTTCCGGCCT TAGCAACGGC TACTTCCTGT CCAAGACGCC
      CAACGCGTGC AAAGTCTGGG ACAGCGAGGC CGATGGGTAC TGCCGCGCGG ATGGCGTCGC TTCGGTGGTC ATGAAGCGAC
      TGGAAGATGC CGTGGCTGAC AATAACAATA TCCTCGGCGT CATCCTCGCC GCTGGCACAA ACCACTCGGC AGAGGCTGTC
      TCGATCACCC ATCCACACGC AGGCCACCAG GCTTATCTAA CGAGGCAAAT TCTGAACCAA GCCGCTGTAG ATCCTCGGGA
1601  GGTCAGCTAC GTGGAAATGC ACGGCACGGG CACGCAGGCC GGGGACGGCC AGGAGATCCA GTCGGTCACC GATGTCTTCG
      CCCCGAACGC GGGTCCAAGG CGCCGCAACG CCAAGCAGCC GCCGCACATC GGCGCGGTGA AAGCCAACGT GGGCCACGGG
      GAAGCCGTAG CGGGTACTAC GGCCCTGCTC AAGGTGTTGC TCATGTTCGA GAAGAACATG ATCCCGCCGC ATGTCGGCAT
      CAAGGGGACC ATCAACCCGG CGGTTCCGAA GGACTTGGAG AAAAGGAATC TCCACATTTC CTTACAAACC CACCCCGTGG
      CCGAGGACGC CGGAATAGGA AGCGCATTGT TGTTATCAAC AACTTCAGTG CGGCTGGCGG CAACACGAGC CTTGTCATCG
2001  AGGAGGGTCC CGTGCCGGCG CCGATCGGCA CCGCGGATCC TCGCTCCGCC CATGTCGTGG CCGTGTCAGC CAAGAGCAAG
      GTATCCCTGA AAGGGAACTT GGAGCGCCTA GTTGCTTACC TGGAGACGCA CCCCAACGTG TCGTTGGCAC ACCTGGCACA
      TACGACCACG GCGCGCCGGT ACCACCACAA CCACCGGGTC GCGGTCCACA CGTCAAGTGT CAACCACTTG AAGAAGCAGC
      TCGGCTCGTA TATCCAGTCA GCGGATGCTC ATAAGCCCAC GCCGCCGACG GGCCCCCCGC CGGTCGTGTT TTCGTTTACC
      GGCCAAGGAG CCTCGTACCG GTCCATGAAT TTGGAACTGT ACCGCGATTC GCCCTACTTC AAGTCTCAGC TGCTGCACCT
2401  GGATTCCCTT TCCCAGGGCC AAGGATTCCC GTCCTTTATC CCCGCGGTGG ATGGCACCTA CCCTAAGGAT TACGCCCATC
      CGCCCACCGT CACCCAGCTG GCCCTGGTCT GCACGGAGAT TGCGCTCGCC AGATATTGGG AGTCTCTCGG GGTGCGACCT
      GATGTTGTTA TCGGCCACAG CCTAGGCGAA TATGCGGCTC TGCACATTGC CGGGGTGCTA TCGGCCACCG ATACCATCTC
      GCTGGTGAGC CAGCGTGCCA GCATGATCGA AAGGAAGTGT CTCATTGGCA GCCATAAAAT GCTGGCAAAA AAAAAAAAAA
      AAAAAAAAAA AAAAAGTACT CTGCGTTGAT ACCACTGCTT
```

Figure 14. A nucleotide sequence of non-reducing PKS identified during the production phase of menisporopsin A. KS and AT domains are shown in green and blue respectively.

Interestingly, the non-reducing PKS encodes only KS and partial AT domain unlike other non-reducing fungal PKSs containing at least KS, AT and ACP domains. The AT domain is also not identical to our previous data. This may be a result of non-specific binding of primers and also indicates that there are more PKSs to be discovered from *M. theobromae*. From these results, 3'-RACE experiment has to be repeated to confirm the full nucleotide sequence of non-reducing PKS from the biosynthesis of menisporopsin A.

Summary

Here, we report the first biosynthetic study of menisporopsin A and also the partial genes encoding both reducing and non-reducing PKSs from *M. theobromae* BCC4162. This will give us an opportunity to study further on the biosynthesis of menisporopsin A and genetically manipulate the formation of the novel biologically active compounds based on its biosynthesis.

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Output

A part of the results from this project has been published in the Journal of Natural Products as described below.

Wattana-Amorn, P., Juthaphan, P., Sirikamonsil, M., Sriboonlert, A., Simpson, T.J., Kongkathip, N. (2013) Biosynthetic Origins of Menisporopsin A. *J. Nat. Prod. 76*, 1235-37.

JOURNAL OF NATURAL PRODUCTS

Communication

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Biosynthetic Origins of Menisporopsin A

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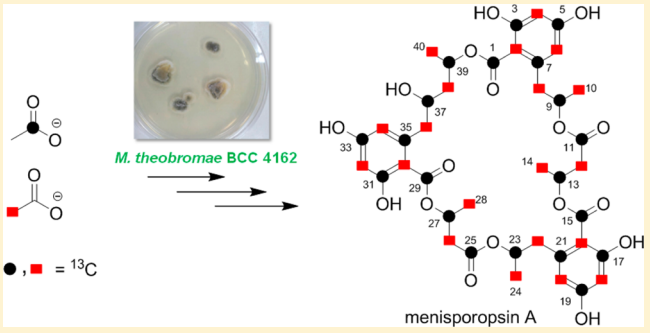
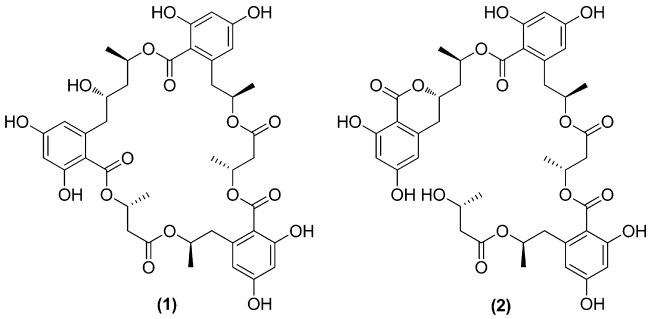
Supporting Information

ABSTRACT: Menisporopsin A, produced by *Menisporopsis theobromae,* shows antimalarial, antimycobacterial, and cytotoxic activities. Here, we report the first ^{13}C incorporations at individual carbons of menisporopsin A using sodium [1-^{13}C] and [2-^{13}C] acetate. This result indicates that each of the subunits of the pentalactone menisporopsin A is assembled by a polyketide synthase.



Polyketides are a class of secondary metabolites exhibiting a broad spectrum of biological activities,[1,2] and this class of metabolites has attracted attention for many decades as an important source for the discovery of new drug candidates. Our current research involves screening for novel biologically active compounds from micro-organisms including the fungus *Menisporopsis theobromae* BCC4162. The crude extract from this organism showed potent antimalarial activity, and this led to the isolation of two novel polyketides, menisporopsin A (**1**)[3] and menisporopsin B (**2**).[4]



Menisporopsin A was found to exhibit antimalarial and antimycobacterial activities as well as cytotoxicity against the breast cancer (BC-1) and nasopharyngeal carcinoma (KB) cell lines. Menisporopsin B shows similar biological activities to those of menisporopsin A. Menisporopsin A belongs to the macrocyclic polylactone family, whereas menisporopsin B is a linear polyester, indicating that it may be the immediate precursor for the intramolecular cyclization to close the macrocyclic polylactone ring. Nevertheless, it is also possible that the linear system is formed by intramolecular hydrolysis of the macrocycle. The structure of menisporopsin A is similar to other naturally occurring macrocyclic polylactones such as the macrosphelides from *Periconia byssoides,*[5] NG-012 from *Penicillium verruculosum,*[6] and 15G256 from *Hypoxylon oceanicum* (Figure 1).[7] To date, there has been no research into the biosynthetic pathway of these metabolites. Due to the unique structure of menisporopsin A, it would be invaluable to understand how the complex structure of this compound is assembled.

The biosynthetic pathway of menisporopsin A is believed to derive from the polyketide pathway using acetate as building blocks. The polyketides are biosynthesized by a common mode of decarboxylative condensation and reduction similar to that of fatty acids. Menisporopsin A consists of three subunits, which might be synthesized by two polyketide synthases (PKSs), i.e., highly reducing (HR) and nonreducing (NR) PKSs,[8] as shown in Scheme 1. The HR PKS is possibly responsible for the biosynthesis of the first 4−6 carbon unit followed by the extension of the tripolyketide backbone with a NR PKS to complete the aromatic moieties. This is similar to the PKS genes involved in the biosynthesis of hypothemycin and other resorcylic acid lactones such as radicicol.[9] However, it is possible that the multiple lactonizations are catalyzed by an enzyme similar to a nonribosomal peptide synthase.

In our efforts to understand the biosynthesis of menisporopsin A, a ^{13}C-labeling approach was used to determine the incorporation of individual carbons into menisporopsin A. The

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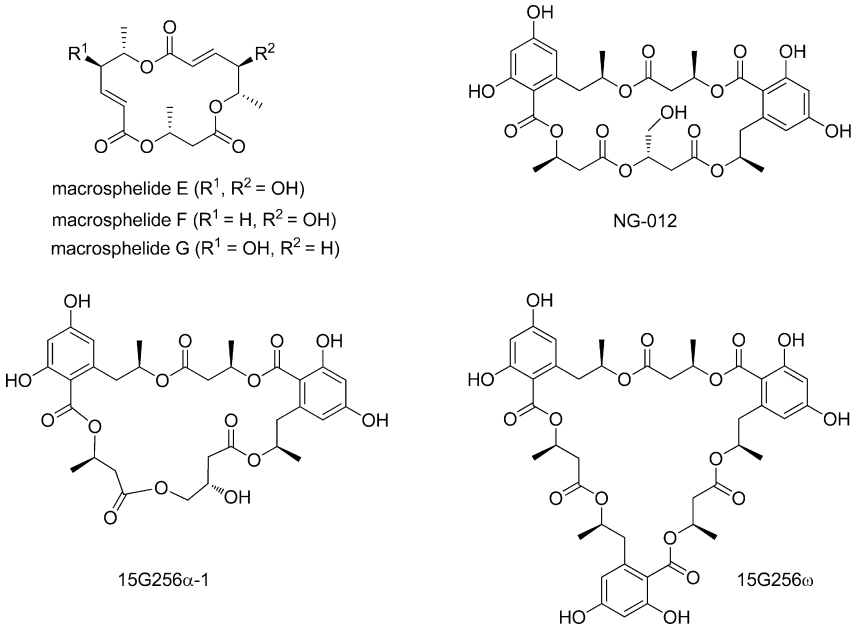
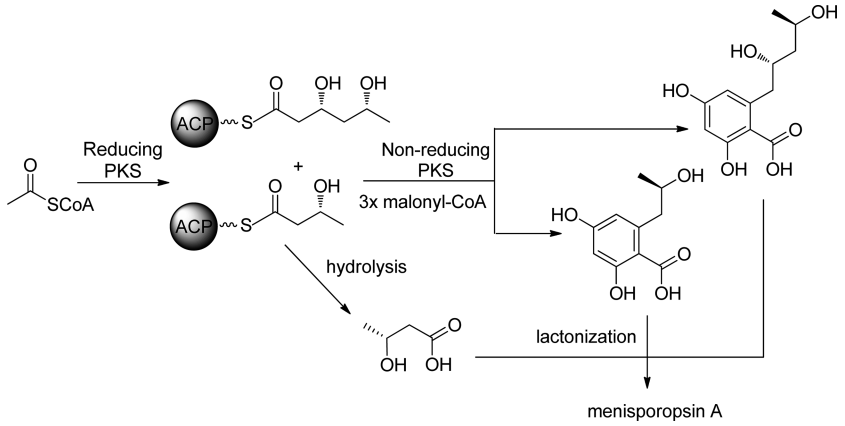


Figure 1. Structures of macrocyclic polylactone secondary metabolites.

Scheme 1. Proposed Biosynthetic Pathway of Menisporopsin A



seed fungus of *M. theobromae* BCC4162 was grown in production medium[10] and supplemented with sodium [1-^{13}C]- and [2-^{13}C]-acetate at a final concentration of 100 mg/L. The labeled menisporopsin A was purified and further analyzed by ^{13}C NMR (see Supporting Information). From the ^{13}C NMR spectra of both [1-^{13}C]- and [2-^{13}C]-labeled menisporopsin A, the change in the signal intensities between labeled and unlabeled carbons was significant. When the [1-^{13}C]-acetate was introduced into the culture, despite the substantial overlap of signals from carbons in near identical environments, it was evident that enrichment from the labeled acetate was observed at the odd-numbered positions, whereas the signal intensities of even-numbered carbons are very low. Enrichment of carbons at even positions was observed when [2-^{13}C]-acetate was used. The ^{13}C enrichments at individual carbons of menisporopsin A were calculated to be in the range 0.5−6%, as shown in Table 1.

^{13}C-Labeling shows the pattern of ^{13}C acetate incorporation into menisporopsin A. This result confirms our hypothesis that the biosynthetic pathway of menisporopsin A is polyketide involving 20 units of acetate as the building blocks (Figure 2). Identification of the genes responsible for the biosynthesis of this compound is in progress. The complete gene cluster will lead to a better understanding of the formation of these complex macrocyclic polylactone compounds, which have shown several biological activities. For example, menisporopsin B, which is a linear polyester, shows a better antimalarial activity compared to menisporopsin A. Although the activity of both compounds is less than that of artemisinin, modifications introduced via the biosynthetic machinery may help to improve their antimalarial activity.

EXPERIMENTAL SECTION

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA400 spectrometer at 400 and 100 MHz, respectively.

Production of ^{13}C-Labeled Menisporopsin A. The seed fungus of *M. theobromae* BCC 4162 was initially activated on potato dextrose agar at 25 °C for 7 days. The fungus was then transferred into potato dextrose broth and incubated at 25 °C with shaking at 200 rev min^{-1} for 7 days to produce the seed culture. The production medium, fructose meat extract salt medium (FMSM) containing of 1% fructose, 2.5% meat extract, 0.0025% KH_2PO_4, and 0.0025% $MgSO_4{\cdot}7H_2O$, was used to

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Table 1. Intensities of Individual Signals in the ^{13}C NMR Spectra of Menisporopsin A Enriched with [1-^{13}C]- and [2-^{13}C]-Acetates with the Calculated Values of ^{13}C Enrichment at Individual Carbon Positions of Menisporopsin A

		observed intensities			% ^{13}C-enrichment	
position of carbon	δ_C (ppm)	unlabeled	[1-^{13}C]	[2-^{13}C]	[1-^{13}C]	[2-^{13}C]
1	171.3	96.096	51.740	57.876	3.21	
2	106.1	134.197	19.151	421.166		5.59
3	166.0	52.910	29.618	36.805	3.38	
4	102.6	162.067	31.806	343.619		3.42
5	163.0	360.169	202.603	148.141	3.40	
6	113.0	185.972	25.145	323.824		2.61
7	143.4	243.879	147.381	137.210	3.73	
8	42.0	235.387	12.436	633.835		4.64
9	72.8	182.331	85.368	46.806	2.65	
10	19.9	352.622	24.778	585.456		2.44
11	170.2	208.660	117.888	89.229	3.42	
12	41.3	241.579	36.057	534.677		3.62
13	70.1	286.649	64.827	97.176	0.71	
14	20.1	324.153	36.693	429.207		1.72
15	171.6	79.110	46.998	50.048	3.65	
16	105.9	138.793	18.688	401.450		5.07
17	166.2	43.113	27.828	34.457	4.06	
18	102.6	177.509	32.652	342.763		3.02
19	163.0	360.169	202.603	148.141	3.40	
20	112.0	176.570	31.811	316.733		2.72
21	143.4	227.512	140.880	137.210	3.85	
22	41.6	226.070	10.685	567.591		4.25
23	72.6	167.425	153.600	38.706	6.24	
24	19.8	321.312	35.940	406.040		1.59
25	170.3	216.440	122.909	92.101	3.44	
26	41.1	231.232	34.959	480.397		3.33
27	69.7	287.245	63.505	84.516	0.73	
28	20.0	398.210	46.146	560.286		1.90
29	171.7	82.240	47.569	52.638	3.53	
30	105.0	139.264	18.071	412.575		5.22
31	166.5	45.976	25.368	30.394	3.31	
32	102.3	149.330	29.444	286.591		2.99
33	163.3	176.426	107.162	75.232	3.76	
34	114.3	186.176	30.606	309.202		2.44
35	145.2	203.168	102.508	75.316	2.94	
36	45.9	210.872	14.650	304.917		1.98
37	69.5	140.622	85.102	33.496	3.74	
38	44.0	240.144	14.704	759.389		5.64
39	72.1	278.541	54.010	93.041	0.45	
40	19.9	419.425	36.354	884.964		3.40

obtain high yields of menisporopsin A as described by Madla et al.[10] Briefly, the seed culture (5% v/v) was transferred into 1 L of FMSM supplemented with 100 mg of sodium [1-^{13}C]- or [2-^{13}C]-acetate for the production of ^{13}C-labeled menisporopsin A and further incubated at 25 °C with shaking at 200 rev min^{-1} for 4 days. The culture was harvested by filtration and subsequently macerated with methanol for 2 days at room temperature. Extraction and isolation of labeled menisporopsin A used the same approach as that of unlabeled menisporopsin A described by Chinworrungsee et al.[3]

^{13}C-Enrichment Calculation. The calculation method for ^{13}C-incorporation is described by Holker et al.[11]

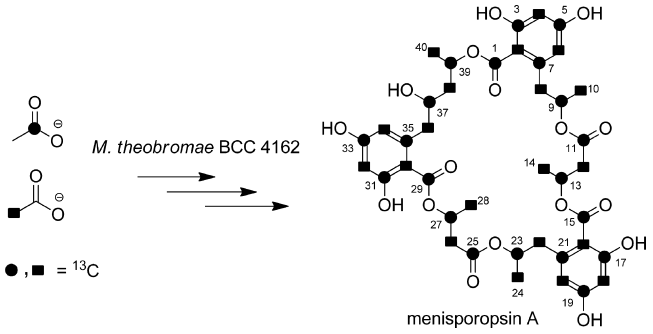

Figure 2. ^{13}C-labeling pattern of menisporopsin A.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{13}C NMR spectra of unlabeled and [1-^{13}C]- and [2-^{13}C]-acetate-labeled menisporopsin A are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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