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Biosynthetic studies on blazeispirane and protoblazeispirane derivatives from the cultured mycelia of the fungus *Agaricus blazei*

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Abstract—The biosynthesis of blazeispirol A, an unprecedented skeleton, was investigated by feeding ^{13}C-labeled acetates, propionate and methionine to the growing cultures of *Agaricus blazei* (Agaricaceae). ^{13}C NMR spectral analysis demonstrated the mevalonate origin of the spiroacetal moiety and the dihydronaphthalene moiety. The *O*-methyl and 28-methyl groups of blazeispirol A were labeled by feeding L-[methyl-^{13}C] methionine. The biosynthetic origin of blazeispirol A derived from ergosterol was directly demonstrated by the feeding experiment using the biosynthetically produced ^{14}C labeled ergosterol. Furthermore, the hypothetical biogenesis of the blazeispirane and protoblazeispirane derivatives is discussed.

1. Introduction

In our previous paper, we reported the isolation and structure elucidation of many blazeispirol derivatives, an unprecedented skeleton from the cultured mycelia of *Agaricus blazei* (Fig. 1).[1–5] The blazeispirol derivatives isolated from the cultured mycelium of *A. blazei* were comprised two major structural types, represented by blazeispirol A (blazeispirane type) and blazeispirol U (protoblazeispirane type) (Fig. 2). Both structure types have a 14β,22: 22,25-diepoxy structure moiety as a side chain. In addition, the blazeispirane type compounds such as blazeispirol A had a unique structure which was the first demonstration of the ring A-lost steroid in a living organism. In a preliminary paper, we reported the investigation of the biosynthesis of blazeispirol A by a feeding experiment using ^{13}C labeled acetate, propionate and methionine.[6] In this paper, we describe the investigation of the incorporation of the ^{13}C labeled compound. Furthermore, to obtain direct evidence for the biosynthetic origin, we carried out the incorporation experiment using biosynthetically produced ^{14}C labeled ergosterol. In addition, the hypothetical biogenesis of blazeispirane and protoblazeispirane derivatives was discussed.

2. Results and discussion

As shown in Fig. 3, the production of blazeispirol A started at about 2 weeks and reached a maximum after 4 weeks with a concentration of 60 mg l^{-1} medium blazeispirol A in the mycelium. Up to two weeks, the main product of the mycelia is ergosterol, and the production of blazeispirol A is observed at the same time as the disappearance of ergosterol. After both 2 and 3 weeks of culture, ^{13}C labeled compounds were administered to each flask and the cultured mycelia were harvested after one more week. The mycelia were extracted and the enriched blazeispirol A was isolated using a silica gel column and HPLC according to a previous paper.[4] Enriched blazeispirol A was completely characterized by verifying all the spectroscopic data. In particular, the NMR signals were unambiguously assigned by the 2D-NMR measurements (HMQC, HMBC, ^{1}H–^{1}H COSY). The feedings of the sodium [1-^{13}C] and [2-^{13}C] acetates resulted in signal enhancements of blazeispirol A. Ten of the 25 carbon atoms were predominantly labeled from the carboxylic group of the acetate and the other 13 carbon atoms were mainly labeled from the methyl group of the acetate as shown in Table 1 and Fig. 4. Two contiguous pairs of ^{13}C atoms at C-11/C-12 and C-8/C-14 are present in blazeispirol A from the [1-^{13}C] acetate. One pair at C-24/C-28 and a triplet of ^{13}C atoms at C-13 are present in the compound from the [2-^{13}C] acetate. The reciprocal labeling patterns in blazeispirol A observed upon feeding singly ^{13}C-labeled acetates suggested the possibility of the mevalonate pathway. In order to obtain further information on the acetate metabolite which should be the immediate biosynthetic precursor of blazeispirol A, feeding studies of the [1,2-$^{13}C_2$] acetate were carried out. The result of a sodium [1,2-$^{13}C_2$] acetate feeding experiment revealed the incorporation of eight intact acetate units in blazeispirol A by strong coupling of the following pairs: C-5/C-6, C-9/C-11, C-19/C-10, C-13/C-12, C-17/C-16, C-21/C-20, C-24/

Keywords: biosynthesis; fungi; *Agaricus blazei*; blazeispirol A; natural products; ^{13}C NMR.

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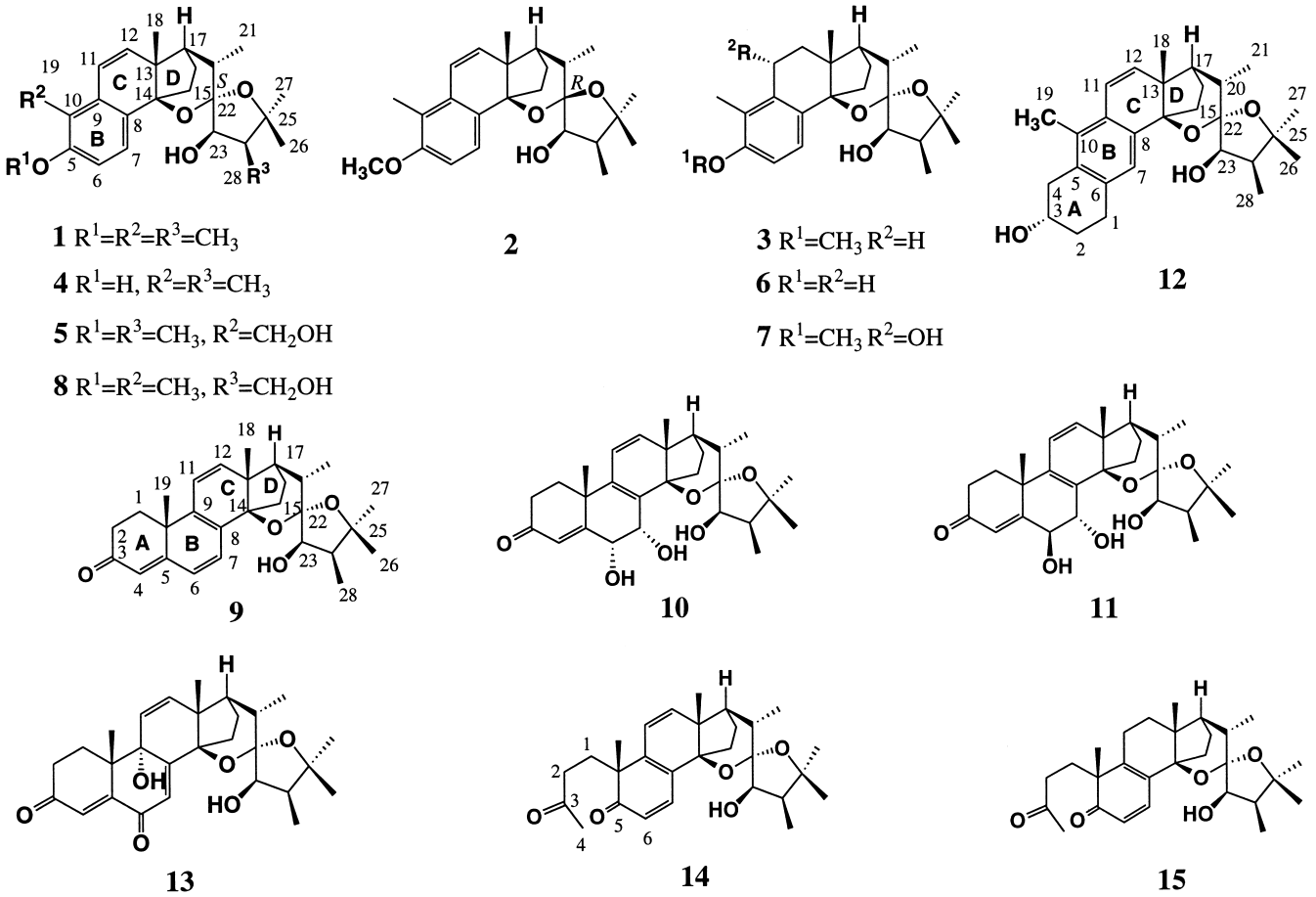

Figure 1. Structures of blazeispirols A(**1**), B(**2**), C(**3**), D(**4**), E(**5**), F(**6**), G(**7**), I(**8**), U(**9**), V(**10**), V_1(**11**), X(**12**), Y(**13**), Z(**14**) and Z_1(**15**).

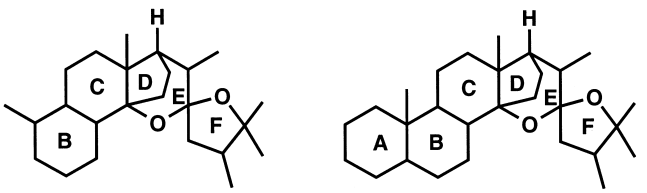

Figure 2. Blazeispirane and protoblazeispirane skeletons.

C-23 and C-26/C-25. Furthermore, in order to establish the direct origin of the *C*- and *O*-methyl groups, which were slightly enriched upon administration of the [2-^{13}C] acetate, feeding experiments were performed using sodium [1-^{13}C] propionate and L-[methyl-^{13}C] methionine. No label at all in the first case and high enrichment of the 28-methyl group in the second indicated the *C*-methylation of blazeispirol A. The result was suggested to be identical with the C-24 methylation in the ergosterol biosynthesis previously

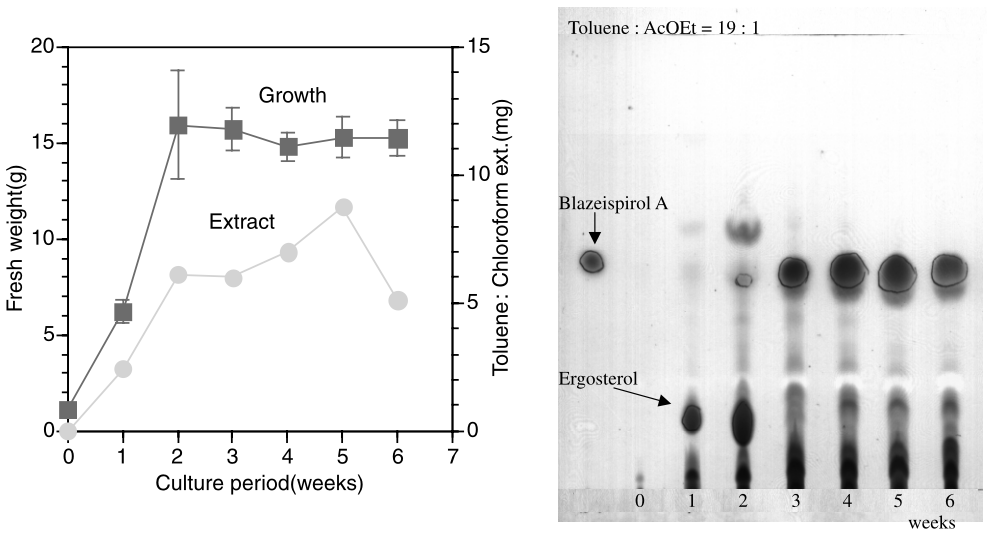

Figure 3. Time course of blazeispirol derivatives production by *A. blazei*.

Table 1. ^{13}C NMR signals of blazeispirol A in $CDCl_3$ together with specific incorporations and coupling constants after feeding with [1-^{13}C] acetate (I), [2-^{13}C] acetate (II), [1,2-$^{13}C_2$] acetate (III) and L-[methyl-^{13}C] methionine (IV)

C	δ_C (ppm)	(I)[a]	(II)[a]	(III)($^1J_{cc}$/Hz)	(IV)[a]
5	156.4	1.0	36.6	67.8	4.4
6	108.6	54.3	1.6	67.8	2.1
7	121.4	1.5	37.3	–	2.8
8	132.0	57.0	1.2	–	2.1
9	130.3	1.8	36.0	53.4	3.0
10	122.5	48.3	1.0	44.3	3.5
11	122.4	57.7	2.4	53.4	3.0
12	139.1	58.4	1.8	39.7	2.7
13	47.0	1.2	34.9	39.7	3.8
14	84.0	60.8	1.0	–	3.5
15	37.1	2.0	37.4	–	2.7
16	25.0	54.4	2.0	32.8	2.4
17	50.7	2.2	37.8	32.8	2.0
18	15.7	2.4	40.1	–	3.0
19	10.8	2.2	36.9	44.3	2.8
20	33.5	56.4	1.6	37.4	2.8
21	16.4	2.3	39.1	37.4	2.3
22	107.4	1.4	37.1	–	3.8
23	85.0	54.9	1.7	35.1	2.0
24	44.1	1.8	37.3	35.1	1.0
25	84.1	50.5	1.8	40.4	3.1
26	25.7	2.0	39.3	40.4	2.2
27	30.7	2.0	38.0	–	2.8
28	8.7	1.9	11.2	–	13.4
OCH_3	55.6	1.9	11.1	–	13.3

[a] Relative enrichments were normalized to the peak intensity of the smallest signal.

reported.[7] Additionally, the *O*-methyl group of blazeispirol A was also labeled from L-[methyl-^{13}C] methionine (Table 1). The incorporation of [2-^{13}C] acetate to the C-28 methyl and *O*-methyl groups observed at the intensity of approximately one-fourth in comparison with the other incorporated carbons was considered to be due to the ^{13}C-labeled methyl group of methionine derived from the [2-^{13}C] acetate (Table 1). The labeling patterns of **1** derived from the singly and doubly ^{13}C-labeled acetates were consistent with that of ergosterol previously reported (Fig. 4)[8–10] except for the A-ring labeling patterns. In order to obtain direct evidence for the biosynthetic origin of blazeispirol A, the incorporation experiment of [^{14}C] ergosterol was carried out. After 2–3 weeks of culture, a solution of radioactive ergosterol in ethanol was administered to each flask and the cultured mycelia were harvested after one more week of culture. After extraction of the cultured mycelia, pure labeled blazeispirol A was isolated by HPLC. Since blazeispirol A was unable to crystallize, the normal dilution analysis was unavailable for this compound. Therefore, a portion of radioactive blazeispirol A isolated from the cultured mycelia was diluted with suitable amounts of radioinactive carrier and HPLC was repeatedly performed until constant specific radioactivities were obtained. The result of the dilution analysis demonstrated that ergosterol was a direct precursor for blazeispirol A (Table 2).

Therefore, it can be assumed that blazeispirol A is biosynthesized from ergosterol by the cleavage of the C-4, C-5 and C-1, C-10 bonds upon retro aldol condensation and Michael reaction via intermediates such as A, B and C as shown in Fig. 4. This was supported by our recent report which showed the presence of intermediate B.[3] As ring A-lost steroidal compounds such as blazeispirol A have not been reported in living organisms, it seems likely that they are formed by diagenetic degradation of a steroid.[11,12] This degradation could proceed only by the pathway proposed for the 3-oxygenated triterpenoids such as β-amyrin, lupane, oleanane and ursane.[13–16] However, these speculations have not yet been demonstrated. It was very interesting that blazeispirol Z as an actual intermediate of blazeispirol A was isolated from the same mycelia.

As blazeispirol A has a 14β-oxygenated group, the 14β hydroxylation of ergosterol may occur during an early stage of the biosynthesis of blazeispirol A. Quite recently, Akihisa et al. reported the structure of triticusterol which was the first example of a naturally occurring compound with a 14β (H)-steroid skeleton and they suggested that it was formed by the stereoselective reduction of the 14, 15 double bond in an intermediate.[17] In the case of blazeispirol A, the 14β hydroxy group in blazeispirol A may be introduced through the 14β, 15β-epoxide produced by the epoxidation of the 14, 15 double bond in the ergosterol derivatives, followed by reduction of the epoxide. With respect to introduction of a 14β hydroxyl group, the possibility of addition of the water to 14, 15 double bond is also considered.

A large number of steroids, which have the ergostane skeleton, have been isolated from fungi.[18] *A. blazei*[19] and a certain mushroom[20–22] include many sterol derivatives which are oxidized at many positions in the ergostane skeleton. However, blazeispirane type compounds such as blazeispirol A are the first examples of naturally occurring des-*A*-ergostane type steroids including a spiroacetal structure moiety which should be formed from a highly oxygenated side chain.

The biosynthetic pathway of the blazeispirol A which is the major compound in cultured mycelia was demonstrated by tracer experiments using ^{13}C and ^{14}C labeled precursors as shown in Fig. 4. We have isolated thirteen compounds, blazeispirane and protoblazeispirane derivatives having different oxidation stages, from the cultured mycelia of *A. blazei* (Fig. 1). Taking into account of the result of the biosynthetic experiment of blazeispirol A and the oxidation stages of these isolated compounds, the hypothetical biogenesis of the compounds was presumed as shown in Fig. 5. Six ergosterol derivatives have been isolated from the fruit body of *A. blazei*.[19] All of them are compounds which are produced by oxidation in the ergostane skeleton. The blazeispirol and ergosterol derivatives isolated from *A. blazei* were comprised two major structural types, representing the mycelia type and fruit bodies type produced via route A and B, respectively (Fig. 5). In our recent paper, we reported the isolation of blazeispirol Z_1.[5] As a biosynthetic route of blazeispirol C, a route via blazeispirol Z_1 can be considered in addition to a route via the reduction of blazeispirol A as shown in Fig. 5. We suppose that blazeispirols Z_1 and C are biosynthesized via an epoxide intermediate. Although the possibility of not epoxide but peroxide is considered as a biosynthetic intermediate of blazeispirol A, it is supposed that blazeispirol A is biosynthesized via epoxide intermediates on the basis of

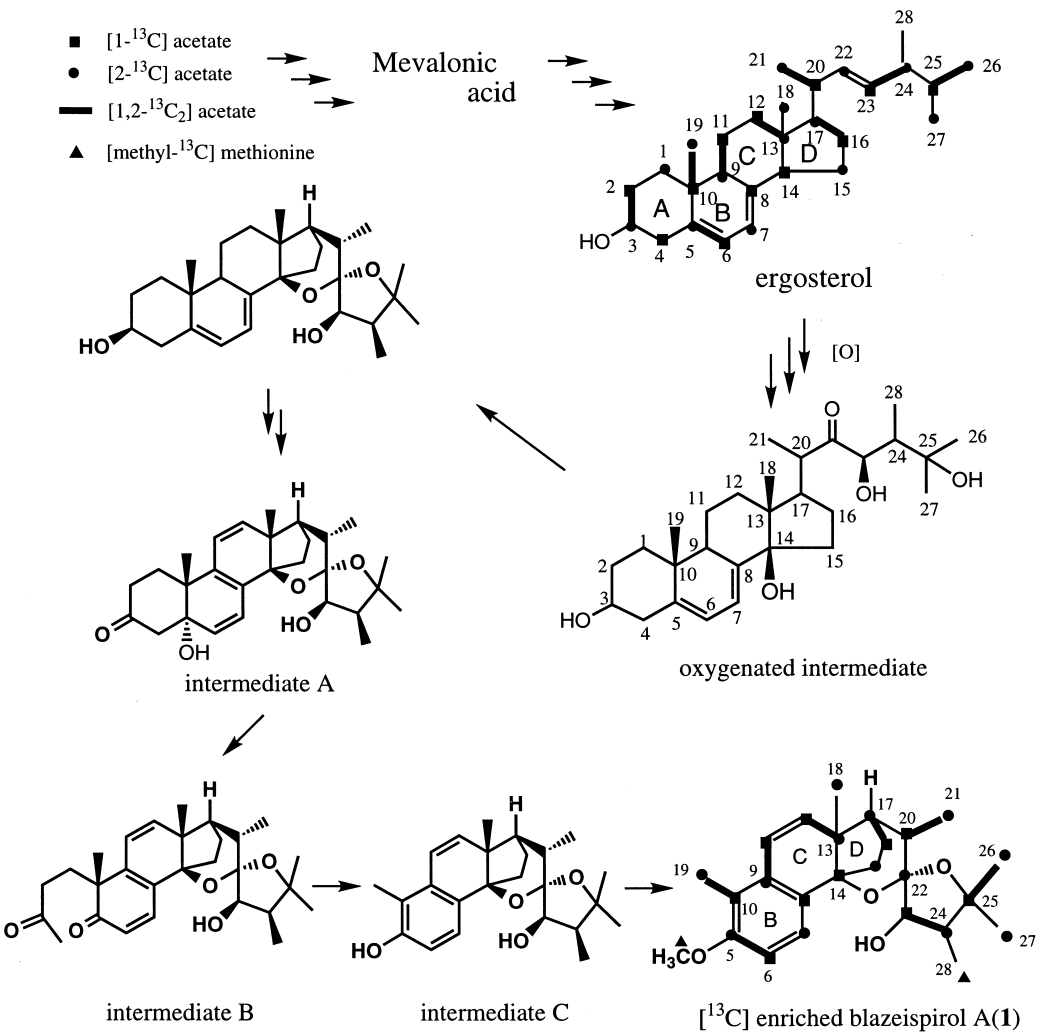

Figure 4. Proposed intermediates of the blazeispirol A biosynthesis.

the isolation of blazeispirols C and Z_1 which may be produced via monoepoxide intermediate. In the biosynthesis of blazeispirol derivatives, 3-oxo-5α,6α-epoxide derivatives which give 5α-hydroxy-3-oxo derivatives are considered to be important intermediates.

As only two anthrasteroid derivatives have been reported as natural products, blazeispirol X is a very unique compound. One of them was a sterol with an aromatized B ring isolated from the stromata of *Epichloe typhina* growing on *Phleum pratense* and the structure was established as 1(10⟶6) abeo-ergosta-5,7,9,22-tetraen-3α-ol.[23] The other was also a sterol with an aromatized B ring, citreoanthrasteroid, isolated from the mycelium of a hybrid strain KO 0011 derived from *Penicillium citreo-viride* B. IFO 6200 and 4692.[24] In a recent paper, Nakata and Yamamura reported the isolation and structure determination of citreoanthrasteroids A and B from the mycelia of a hybrid strain, KO 0231, prepared by a cell fusion technique using *P. citreo-viride* IFO 6200 and 4692.[25] They proposed that ergosterol would be oxygenated to give a peroxide, which is subjected to double 1,2-shifts to afford anthrastroid as a plausible biosynthetic pathway of the citreoanthrasteroids in the same paper.[25] This idea was agreed with the result of the organic synthesis of anthrasteroid reported by Whalley et al.[26,27] We now propose the hypothetical biogenesis of blazeispirol X, an anthrasteroid derivative via the 5α,6α epoxide formed from the assumed intermediate, polyoxygenated ergosterol, as shown in Fig. 5.

Table 2. Dilution analysis of [^{14}C] blazeispirol A (**1**) formed from [^{14}C] ergosterol

Analysis of HPLC	Dpm m mol^{-1}
Isolated blazeispirol A[a]	15.27×10^4
First dilution analysis[b]	4.05×10^4
Second[b]	3.55×10^4
Third[b]	3.77×10^4

[a] Specific radioactivity of isolated blazeispirol A.
[b] Specific radioactivity of purified blazeispirol A by HPLC after dilution with cold blazeispirol A as the carrier.

Ergosterol derivatives which have been isolated from the fruit body of *A. blazei*[19] may be biosynthesized via an epoxide intermediate like the biosynthesis of blazeispirol derivatives as shown in route B.

The isolation of plausible biosynthetic intermediates using oxidation enzyme inhibitors such as P-450 inhibitors and

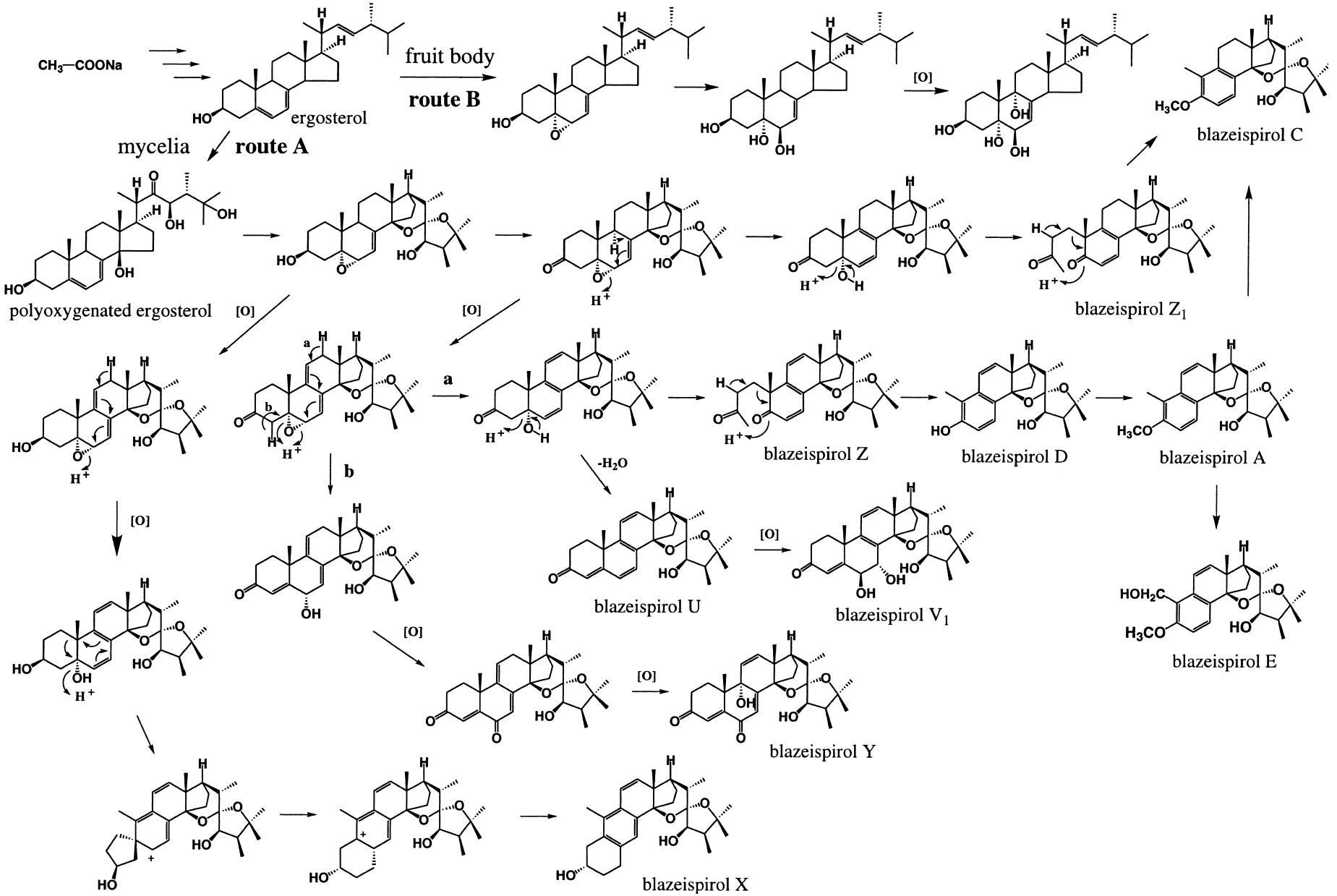

Figure 5. A hypothetical biogenesis of blazeispirane, protoblazeispirane and ergostane derivatives in *A. blazei*.

Table 3. The amounts of the administered [^{13}C] enriched compounds, $CHCl_3$ ext. and isolated blazeispirol A

Administered compound	[1-^{13}C] acetate (99 at.%)	[2-^{13}C] acetate (99 at.%)	[1,2-$^{13}C_2$] acetate (1:15 mixture of 99 at.% enriched and unlabeled acetate)	[1-^{13}C] propionate (99 at.%)	L-[methyl-^{13}C] methionine (99 at.%)
Administration method	25 mg/flask in total 250 mg two times	25 mg/flask in total 250 mg two times	25 mg/flask in total 250 mg two times	25 mg/flask in total 250 mg two times	25 mg/flask in total 250 mg two times
$CHCl_3$ ext.	205.6 mg (MyC[a]+MC[b])	245.5 mg (MyC+MC)	105.3 mg (MyC)	150.0 mg (MyC)	52.9 mg (MyC)
Isolated blazeispirol A	19.4 mg	21.5 mg	11.4 mg	16.0 mg	7.8 mg

[a] MyC: Mycelium $CHCl_3$ ext.
[b] MC: Medium $CHCl_3$ ext.

cloning of biosynthetic genes of blazeispirols are now in progress.

3. Experimental

3.1. General methods

TLC: 0.25 mm precoated silica-gel plates (MERCK silica-gel 60F254); detection by spraying the plates with 10% (v/v) H_2SO_4 solution followed by heating at 120°C.

Column chromatography (CC): Silica gel 60 (Cica-reagent, 40–100 μm). Isolation and detection of ergosterol and blazeispirol A by HPLC were performed using a Senshu pak ODS column (10×300 mm) coupled to a UV detector and a differential refractometer.

^{13}C NMR: Varian UNITY 400 (100 MHz in $CDCl_3$).

3.2. Culture conditions

The mycelium used for this experiment was isolated from the pileus under sterile conditions and subcultured on potato dextrose agar medium. The time course experiment was performed by harvesting the mycelia from five 500-ml Erlenmeyer flasks each week and the amount of mycelia and the $CHCl_3$ and toluene extract of the dried mycelia were measured in the usual manner. Feeding experiments were performed on MYS medium containing 30 g malt ext., 5 g yeast ext. and 10 g sucrose in 1 l H_2O. Flasks were inoculated with five of 10 mm plugs cut from pre-cultured agar and were then maintained in a rotary shaker at 63 rpm and 25°C in the dark. [1-^{13}C], [2-^{13}C], [1,2-$^{13}C_2$] sodium acetate (99 at.% ^{13}C; Sigma-Aldrich), L-[methyl-^{13}C] methionine (99 at.% ^{13}C; Isotec, Inc.) and [1-^{13}C] propionate (99 at.% ^{13}C; Isotec, Inc.) were purchased. [1,2-^{14}C] sodium acetate (1.85 GBq mmol^{-1}) was from NEN Life Science Products Inc. [^{14}C] ergosterol (0.43 MBq mmol^{-1}: total activity 2.5 KBq) was biosynthetically produced.

3.3. Feeding experiment with ^{13}C enriched compounds

The administration experiments were achieved as follows. After both 2 and 3 weeks of shaking the cultures of *A. blazei*, filter-sterilized solutions of the ^{13}C enriched compounds [sodium [1-^{13}C] and [2-^{13}C] acetate, [methyl-^{13}C] methionine, sodium[1-^{13}C] propionate(200 mg l^{-1}, 99 at.% enriched) and sodium[1,2-$^{13}C_2$] acetate (200 mg l^{-1}, 1:15 mixture of 99 at.% enriched and unlabeled acetate)] were administered and the cultured mycelia were harvested after one more week of culture. The mycelia were homogenized with MeOH using a Waring blender and allowed to stand for 1 week at room temperature. The homogenate was filtered and the residue was re-extracted with the same solvent. The filtrates were combined and the organic solvent was removed under reduced pressure. The residue was extracted with $CHCl_3$, dried and evaporated to dryness. The $CHCl_3$ extract was subjected to chromatography over silica gel. Further purification of the fraction containing the enriched blazeispirol A was achieved by HPLC. The amounts of the administered compounds, the $CHCl_3$ extract and the isolated

labeled blazeispirol A are summarized in Table 3. Each enriched pure blazeispirol A was recorded using ^{13}C NMR.

3.4. Isolation and purification of [^{14}C] ergosterol and isolation of blazeispirol A(1)

The administration experiments were achieved as follows. After 10 days of shaking the cultures of *A. blazei*, filter-sterilized [1,2-^{14}C] sodium acetate (1.92 Gbq mmol^{-1}; in total 1.41 MBq) was administered and the cultured mycelia were harvested after one more week of culture. After the cultures (three flasks), the mycelia and culture broth were separated using nylon mesh and the $CHCl_3$ fraction (17.8 mg) of the MeOH extract of the cultured mycelia was obtained according to a previous paper.[4] Further purification of the [^{14}C] ergosterol was achieved by HPLC (Senshu Pak ODS, ϕ 10×300 mm, 100% MeOH, 3 ml min^{-1} flow rate, 20.6 min). The pure [^{14}C] ergosterol (specific activity 0.43 MBq mmol^{-1}, total activity 2.5 KBq) was administered to the myceli according to that described above for the administration of the ^{13}C enriched compounds.

3.5. Dilution analysis of ^{14}C labeled blazeispirol A(1)

Since blazeispirol A was unable to crystallize, normal dilution analysis was unavailable for this compound. Therefore, the portion of blazeispirol A isolated from the cultured mycelia were diluted with suitable amounts (17 mg) of radioinactive carrier and HPLC was repeatedly performed until constant specific radioactivities were obtained (Table 2).

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