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Biosynthesis of Sespendole

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Abstract Sespendole is the first reported fungal metabolite having an indolosesquiterpene core structure. The biosynthesis of sespendole was studied here by feeding experiments with [^{13}C]acetate, [^{15}N]anthranilic acid and [^{13}C]tryptophan. The data suggested that a farnesyl residue derived from the mevalonate pathway and an anthranilate-derived indole-3-glycerol phosphate residue are condensed, and then cyclization occurs along with rearrangement to form the indolosesquiterpene core.

Keywords sespendole, biosynthesis, fungal metabolite, indolosesquiterpene

Introduction

Sespendole (**1**), discovered as an inhibitor of lipid droplet synthesis in macrophages [1], is the first reported fungal metabolite having the indolosesquiterpene core structure (Fig. 1) [2, 3]. A number of fungal metabolites having the indoloditerpene core structure and their biosynthesis have been reported [4~6]. Fundamentally, an indole moiety and a diterpene moiety derived from geranylgeranyl pyrophosphate through the mevalonate pathway are condensed and cyclized to form the core structure. In this study, the biosynthesis of **1** was investigated by feeding experiments using [^{13}C]acetate, [^{13}C]tryptophan and [^{15}N]anthranilic acid to confirm the biosynthetic origin of the indolosesquiterpene compound.

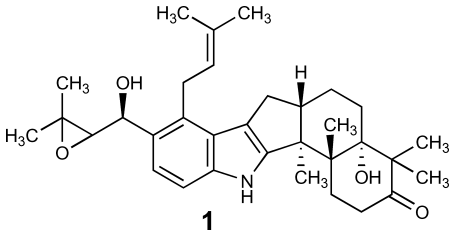

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Fig. 1 Structure of sespendole (**1**).

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Materials and Methods

Spectroscopic Measurements

The patterns and rates of incorporation of ^{13}C-labeled **1** were determined using ^{13}C NMR spectra obtained using a JEOL EX-270 (270 MHz) spectrometer and the ^{13}C–^{15}N coupling of ^{15}N-labeled **1** was determined from the ^{13}C NMR spectra obtained using a Varian Inova 600 (600 MHz).

Materials

Isotope precursors were purchased as follows: sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate and sodium [1,2-^{13}C]acetate from SIGMA, and [2-^{13}C]tryptophan and [^{15}N]anthranilic acid from Cambridge Isotope Laboratories. Sespendole was isolated from the culture broth of fungal strain *Pseudobotrytis terrestris* FKA-25 [3].

Sespendole Production

A stock culture of strain *P. terrestris* FKA-25 was grown on slants adjusted to pH 6.0 and containing 0.1% glycerol, 0.02% yeast extract (Oriental Yeast Co.), 0.02% $MgSO_4 \cdot 7H_2O$, 0.08% KH_2PO_4, 0.08% K_2HPO_4, 0.02% KCl, 0.02% $NaNO_3$ and 1.0% agar. The slants were incubated at 27°C and stored in tubes sealed with screw caps at 27°C. Prior to production of **1**, *P. terrestris* FKA-25

Table 1 Summary of ^{13}C enrichment and coupling constants resulting from incorporation of various [^{13}C]acetate into 1 (CD_3OD)

No.	Chemical shift (ppm)	[2-^{13}C]Acetate		[1-^{13}C]Acetate		[1,2-^{13}C]Acetate[a]	
		Intensity ratio[a]	$J_{13C-13C}$ (Hz)	Intensity ratio[a]	$J_{13C-13C}$ (Hz)	Intensity ratio[a]	$J_{13C-13C}$ (Hz)
2	154.6	1.0		1.0		1.0	—
3	53.9	1.0		2.0	(40.5)[b]	2.2	35.4
4	44.5	0.9		1.6	(40.5)[b]	1.9	35.0
5	29.6	2.5		1.0		2.2	(35.2)[b]
6	35.6	1.0		2.0		2.3	37.8
7	221.0	2.5		0.9		2.5	37.9
8	56.2	1.0		1.9		2.0	34.1
9	81.8	2.5		0.9		2.1	37.8
10	32.1	1.0		2.1		2.2	37.8
11	22.3	2.1	(36.2)[b]	0.8		2.1	(37.1)[b]
12	51.0	2.1	(36.2)[b]	0.7		1.8	31.5
13	30.4	1.0		1.9		2.2	31.5
14	116.8	1.0		0.9		1.1	—
15	126.6						
16	131.9	1.1		0.8		1.1	—
17	129.6	0.9		0.8		1.0	—
18	120.1	0.9		0.8		1.0	—
19	110.9	1.0		0.9		1.1	—
20	141.5	0.9		0.8		1.2	—
21	17.0	2.2		0.9		2.5	35.4
22	23.4	2.3		0.7		2.4	35.0
23	24.8	2.3		0.7		2.4	34.2
24	22.9	2.3		0.9		2.3	(36.0)[b]
25	29.7	1.0		2.2		2.5	41.3
26	126.6	2.2		0.6		2.0	41.2
27	131.2	1.0		2.1		2.3	41.5
28	18.3	2.2		0.8		2.2	(47.2)[b]
29	25.8	2.5		0.8		3.1	41.6
30	71.6	0.9		2.0		2.3	50.1
31	69.9	2.6		0.9		2.4	50.0
32	60.2	1.0		1.7		2.0	(40.0)[b]
33	25.1	2.3		0.8		3.2	43.6
34	19.7	2.2		0.8		2.6	43.7

[a] Values indicate X-fold enrichment levels as compared with natural abundance.

[b] Values in parentheses result from multiple labeling.

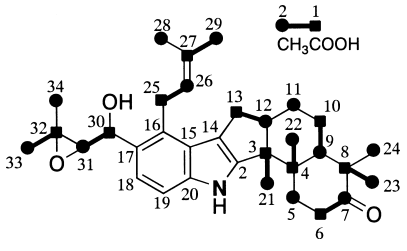

Fig. 2 Incorporation patterns of [^{13}C]acetate into sespendole.

was inoculated into a 500-ml Erlenmeyer flask with 100 ml of seed medium containing 2.0% glucose, 0.2% yeast extract (Oriental Yeast Co.), 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% Polypepton (Pharmaceutical CO., LTD.), 0.1% KH_2PO_4 and 0.1% agar, adjusted to pH 6.0 prior to sterilization and shaken with a rotary shaker at 210 rpm at 27°C for 4 days. The main culture was initiated by transferring 1 ml of the seed culture into ten 500-ml Erlenmeyer flasks with 100 ml of production medium containing 2.0% sucrose, 1.0%

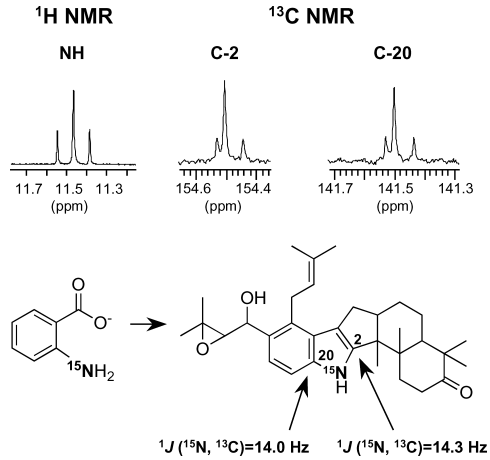

Fig. 3 Incorporation of [^{15}N]anthranilate into sespendole.

Analysis of the NMR spectra of sespendole produced in the presence of [^{15}N]anthranilate showed the NH proton and two carbon signals with distinct satellites resulting from ^{1}H–^{15}N and ^{13}C–^{15}N coupling.

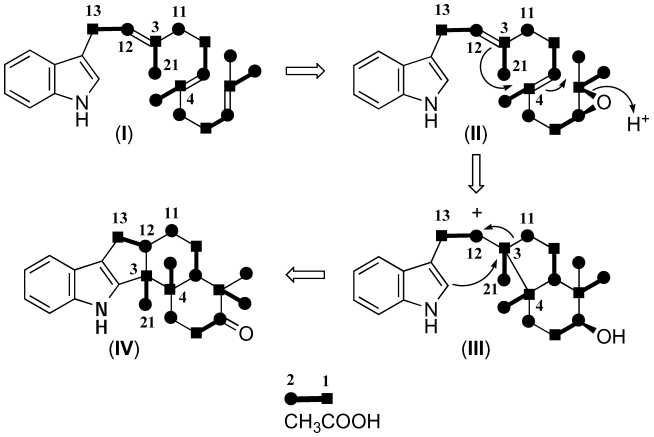

Fig. 4 A possible mechanism of cyclization and rearrangement to form the indolosesquiterpene core structure in sespendole.

glucose, 0.5% corn steep powder (Iwaki Co., Ltd.), 0.5% meat extract (Kyokuto Pharmaceutical Co. Ltd.), 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.3% $CaCO_3$ and 0.15% agar, adjusted to pH 6.0 prior to sterilization, and fermentation was carried out at 27°C with agitation at 210 rpm.

Isolation Procedures

After 4 days of fermentation, the culture broth (100 ml) was centrifuged to obtain the mycelium, which was treated with acetone. After concentration by removing acetone, the aqueous solution was extracted with ethyl acetate (30 ml). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a brown oil. The oil was washed with hexane and the residue containing **1** was purified by HPLC under the following conditions: column, Pegasil ODS (Sensyu Scientific Co., Ltd., i.d. 20× 250 mm); mobile phase, 60% aq CH_3CN; flow rate, 8 ml/minute; detection, UV 240 nm. Compound **1** was eluted as a peak with a retention time of 16 minutes. The peak was collected and the fractions were concentrated to dryness to give pure **1** as a colorless amorphous solid.

Biosynthetic ^{13}C and ^{15}N-Labeling of Sespendole

Biosynthetically ^{13}C-labeled and ^{15}N-labeled **1** were prepared by adding a labeled precursor (0.1 mg/ml of sodium [1-^{13}C], [2-^{13}C] or [1,2-^{13}C]acetate or [^{15}N]anthranilic acid solution) to the culture (100 ml) during cultivation for 40 hours. Labeled **1** was purified as described above.

Results and Discussion

[^{13}C]Acetate Incorporation

Metabolism of [1-^{13}C]acetate or [2-^{13}C]acetate through the mevalonic acid pathway should lead to ^{13}C incorporation at the 1 and 3 or the 2, 4 and 5 positions of isopentenyl pyrophosphate (IPP), respectively. [^{13}C]Sespendole was prepared biosynthetically using sodium [^{13}C]acetate. All of the ^{13}C NMR data of the ^{13}C-enriched **1** are summarized in Table 1. From the sodium [1-^{13}C]- and [2-^{13}C]acetate incorporation experiments, 10 carbons (C3, C4, C6, C8, C10, C13, C25, C27, C30 and C32) and 15 carbons (C5, C7, C9, C11, C12, C21, C22, C23, C24, C26, C28, C29, C31, C33 and C34) were enriched in [^{13}C], as illustrated in Fig. 2. Analysis of ^{13}C–^{13}C couplings of **1** prepared from the sodium [1,2-^{13}C]acetate incorporation experiments indicated 10 pairs. These data indicated that the two isopentenyl moieties attached to the indole benzene ring are derived from IPP and that the aliphatic C_{15} unit connected to the 2,14-positions of the indole ring appears to be derived from a farnesyl group, although the labeling pattern of the five carbons C3, C11, C12, C13 and C21 is unusual.

[^{15}N]Anthranilic Acid and [^{13}C]Tryptophan Incorporation

The biosynthetic origin of the indole moiety was examined. An attempt to incorporate [2-^{13}C]tryptophan into **1** was unsuccessful; no ^{13}C enrichment of any carbon in **1** was observed. This result was unexpected in view of the reported incorporation of tryptophan into the indoloditerpene penitrem A [4, 5]. Next, the incorporation of [^{15}N]anthranilic acid, a precursor of tryptophan

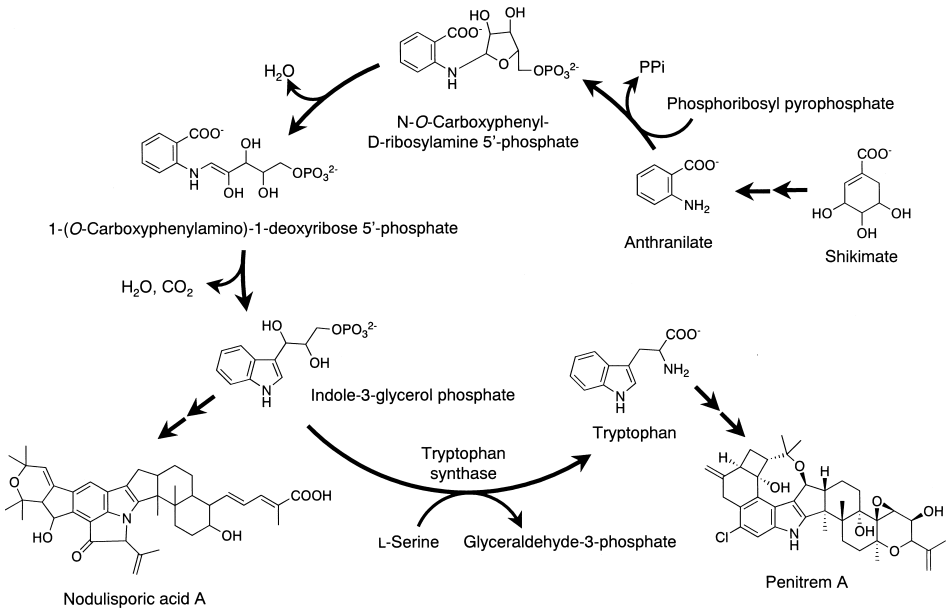

Fig. 5 Tryptophan biosynthesis and biosynthetic pathway producing penitrem A and nodulisporic acid A having indoloditerpene structures.

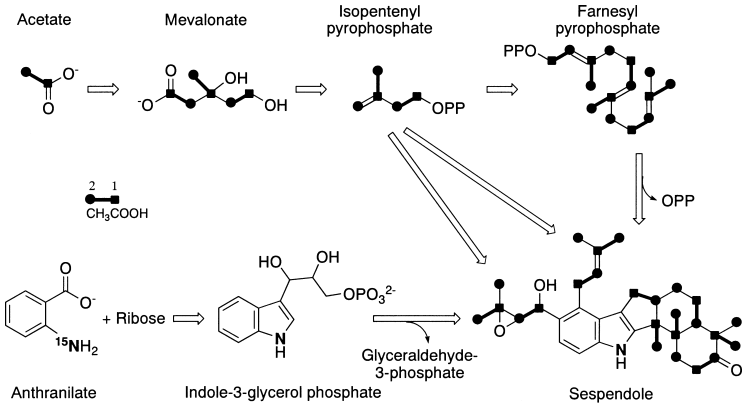

Fig. 6 Summary of the proposed biosynthetic scheme for **1**.

biosynthesis, was tested. In the ^{13}C NMR spectrum taken in CD_3OD of **1** obtained from the [^{15}N]anthranilic acid feeding experiment, distinct satellite signals due to the ^{13}C–^{15}N coupling were observed at C2 (δ 154.6 ppm) and C20 (δ 141.5 ppm) (Fig. 3). The respective values of the satellite couplings were 14.3 and 14.0 Hz, which were in agreement with the expected range of within 15 Hz [7]. Furthermore, in the ^1H NMR spectrum taken in C_5D_5N, a distinct satellite due to ^1H–^{15}N coupling was also observed for the NH proton signal (δ 11.46 ppm) (Fig. 3). Thus, it was demonstrated that anthranilic acid is incorporated in the indole moiety of **1**.

Biosynthesis of Sespendole

Labeling of **1** with sodium [^{13}C]acetate (Fig. 2) indicated that the sesquiterpene moiety and the two side chains of **1** are biosynthesized *via* the mevalonate pathway. However, the labeling pattern of the five carbons (C-3, C-11, C-12, C-13 and C-21) is unusual, suggesting that a rearrangement should occur during the condensation and cyclization of farnesyl pyrophosphate (FPP) with the indole moiety. A possible process for the cyclization and rearrangement is shown in Fig. 4; 2-(10,11-epoxyfarnesy)-indole (II) is cyclized to form an intermediate (III), which is rearranged along with 1,2 migration of the C3–C11 bond to yield an indolosesquiterpene skeleton (IV). This rearrangement was

supported by the observation that the two prominent ^{13}C–^{13}C couplings of **1** prepared from sodium [1-^{13}C]- and [2-^{13}C]acetate were 40.5 Hz between C-3 and C-4, and 36.2 Hz between C-11 and C-12, respectively. Similar rearrangements were reported in studies on the biosynthesis of the indoloditerpene skeleton in penitrem A [4, 5] and nodulisporic acid A [6].

Regarding the origin of fungal indole moieties, two biosynthetic pathways have been reported, as illustrated in Fig. 5. For most indoloditerpenes, such as penitrem A [4, 5], 3-hydroxy-3-methylbutenyl paspalinine [8] and paxilline [8], tryptophan is used as a precursor for the indole moiety, while indole-3-glycerol phosphate, a precursor of tryptophan biosynthesis, is directly incorporated into nodulisporic acid A [6]. In studies of sespendole, anthranilic acid was found to be incorporated into the molecule but tryptophan was not. These results indicated that the indole moiety of sespendole is biosynthesized from anthranilic acid *via* indole-3-glycerol. Thus, we demonstrated the biosynthetic origin of sespendole having the indolosesquiterpene core.

The biosynthetic scheme producing **1** is summarized in Fig. 6. FPP derived from the classic mevalonic acid pathway condenses with indole-3-glycerol phosphate derived from anthranilic acid and ribose, and then cyclization occurs along with rearrangement. Finally, two dimethylallyl pyrophosphate are incorporated into C-16 and C-17 to form **1**.

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