BIOSYNTHESIS OF THE MACROLIDE ANTIBIOTIC PATULOLIDES BY *PENICILLIUM URTICAE* S11R59: IDENTIFICATION OF THE ORIGIN OF CARBON ATOMS BY ^{13}C NMR SPECTROSCOPY

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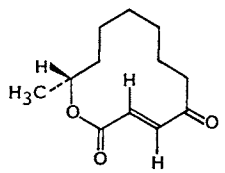
Patulolides are 12-membered macrolides produced by *Penicillium urticae* S11R59, and they are the simplest macrolide antibiotics. All carbon signals, including six methylene signals of patulolides A (**1**), B (**2**), and C (**3**), were completely assigned by use of the ^{13}C two-dimensional INADEQUATE. The biosynthesis of patulolides was investigated with ^{13}C labeled acetate. Feeding of [1-^{13}C]acetate to a culture of *P. urticae* S11R59 gave patulolides A (**1**), B (**2**), and C (**3**), each of which showed enrichment at carbons 1, 3, 5, 7, 9, and 11; enrichment at carbons 2, 4, 6, 8, 10, and 12 was observed upon feeding of [2-^{13}C]acetate. These results showed that patulolides A (**1**), B (**2**), and C (**3**) are pure acetogenic hexaketides derived from six acetate units coupled in head-to-tail fashion.

In the course of studying the biosynthetic pathway of the well-known tetraketide patulin from *Penicillium urticae* NRRL 2159A, various patulin-minus mutants were obtained[1,2]. *P. urticae* S11R59, one of these mutants, produces new macrolides that we named patulolides A (**1**), B (**2**), and C (**3**)[3,4] (Fig. 1). They are 12 membered macrolides with one double bond flanked with either two carbonyl groups or one carbonyl and one hydroxyl group. Patulolides have moderate antimicrobial activity against several species of fungi, yeasts, and bacteria[4]. They also inhibit growth in plants, as do cladosporides A (**4**) and B (**5**) produced by *Cladosporium cladosporioides*[5] (Fig. 1).

Fig. 1. Structures of patulolides and related macrolides.



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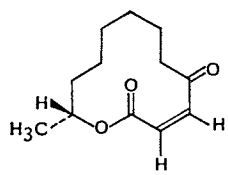

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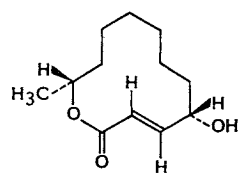

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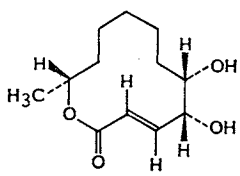

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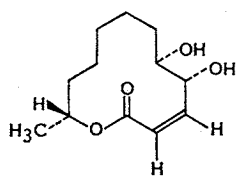

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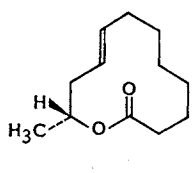

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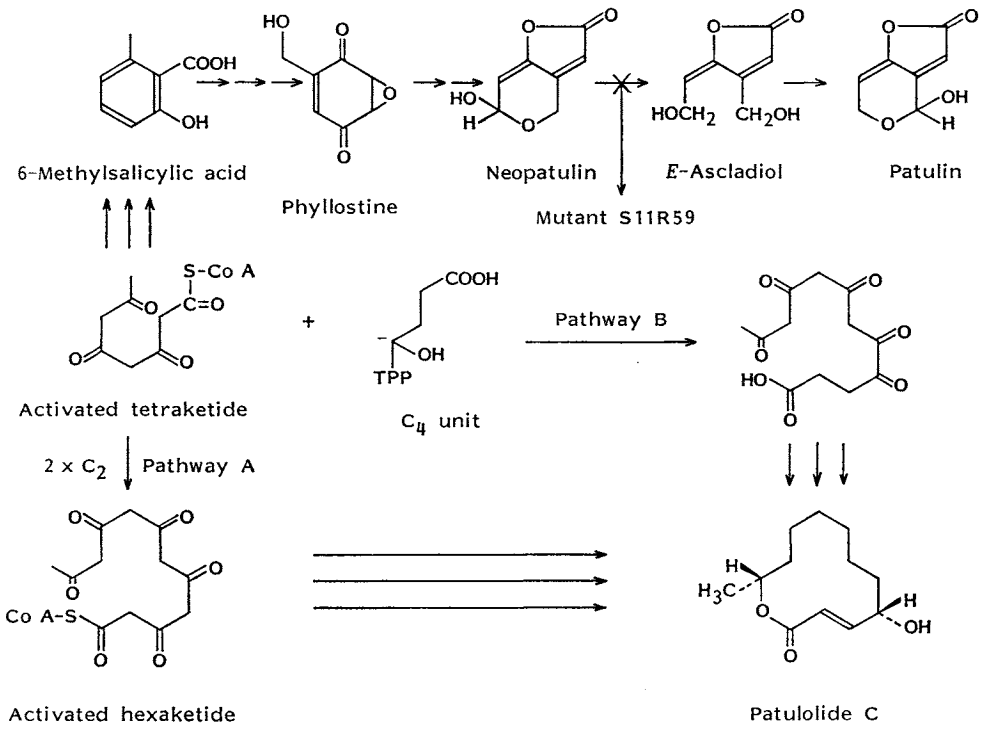
Fig. 2. Possible pathway for patulolide biosynthesis.



Among the macrolide antibiotics that can be classified into the groups of 12-membered, 14-membered, or 16-membered macrolides, the 12-membered macrolides are relatively rare. So far, except patulolides A (**1**), B (**2**), and C (**3**), only cladospolides A (**4**) and B (**5**) from *C. cladosporioides*, recifiolide (**6**) from *Cephalosporium recifei*[6,7] (Fig. 1), and methymycin and neomethymycin from *Streptomyces venezuelae*[8] are known. Most studies on the biosynthesis of macrolides have been done on 14- or 16-membered macrolide antibiotics, such as erythromycin[9], leucomyin[10] or tylosin[11], so little is known about the biosynthesis of 12-membered macrolides. The structure of patulolides is simple compared to other known macrolides, thus biosynthetic knowledge of patulolides would provide a easily accessible model for the step of macrocyclic lactone formation of other complicated macrolides[12,13].

Methymycin and neomethymycin seem to be synthesized from five molecules of propionic acid and one molecule of acetic acid[14], and other 12-membered macrolides are thought to be synthesized from acetate, propionate, butyrate or malonate from their structures[15~17], but concrete evidence about their biosynthesis is lacking. The producer of patulolides, *P. urticae* S11R59, is a blocked mutant that produces patulolides instead of patulin. The block is between neopatulin and ascladiol, which is a late step of patulin biosynthesis[1,2]. Accordingly, at first we suspected the presence of common intermediates or relationship between the biosynthetic pathways for patulin and patulolide. Therefore, it seems unsuitable to assume only the acetogenic pathway for patulolide biosynthesis from their structures but two pathways are possible (Fig. 2). In the first, patulolides are produced *via* the coupling of tetraketide with C_4 or C_5 compounds (pathway B) similar to that demonstrated in juglone biosynthesis[18]. In the other, patulolides are produced *via* pure hexaketide (pathway A). To clarify the biosynthetic pathway of patulolides, we first concentrated on confirming the building blocks of patulo-

lide skeletons. As the first step, all carbon signals of patulolides including the six methylene carbons (C-5 – C-10) were assigned unambiguously by the technique of two-dimensional (2D) INADEQUATE[19~22]. A feeding regime of sodium [^{13}C]acetate into a culture of *P. urticae* S11R59 was established, allowing the incorporation of ^{13}C labeled acetate into patulolides. The enrichment patterns observed indicated that the pure hexaketide pathway A is involved in the biosynthesis of patulolides A (**1**), B (**2**), and C (**3**).

Experimental

Materials

Sodium [1,2-$^{14}C_2$]acetate (specific radioactivity 50 mCi/mmol) was purchased from ICN Radiochemicals, Inc., CA, U.S.A. Sodium [1-^{13}C]acetate (99.5% atom %) and sodium [2-^{13}C]acetate (99.8% atom %) were obtained from MSD Isotopes, Division of Merck and Frosst Canada, Inc., Montreal, Canada. All other reagents and solvents were of reagent grade and were used without further purification.

General Procedure

^{1}H NMR spectra were recorded on either a Hitachi R-24B (60 MHz) or a Joel PS-100 (100 MHz) spectrometer. ^{13}C NMR spectra were obtained at 60 MHz on a Joel model SX-605 spectrometer. 2D INADEQUATE spectra were recorded on a Joel GX-270 spectrometer at 67.9 MHz. Chemical shifts were recorded in ppm from internal TMS in $CDCl_3$.

Radioactivity was measured with a Beckman LS 7500 liquid scintillator in the presence of toluene scintillant containing 8% (w/v) 2,5-diphenyloxazole. Analytical TLC was done on coated Silica gel 60 F_{254} aluminum plate 0.2 mm thick manufactured by E. Merck. Analytical HPLC was done with a Jasco Trirotar VI with a Cosmosil silica gel column (Nakarai Chemicals, Ltd., Kyoto). Preparative HPLC was done with a Jasco Trirotar V with a Nucleosil 100-10 silica gel column (Wako Pure Chemical Industries, Ltd.). Cultivation was done on a reciprocating incubator shaker at 28°C. Evaporation *in vacuo* refers to solvent removal on a rotary evaporator at aspirator pressure at 45°C.

Organism and Standard Culture Condition

P. urticae S11R59 was used for all of the experiments mentioned here, and was maintained as a spore suspension at −80°C in a medium consisting (per liter) of glucose 40 g, peptone 10 g, yeast extract 5 g, $MgSO_4 \cdot 7H_2O$ 2 g, KH_2PO_4 5 g, $CaCl_2 \cdot 2H_2O$ 2 g. The initial pH was adjusted to 6.5 before sterilization. For seed culture, we used a medium consisting (per liter) of glucose 50 g, yeast extract 5 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 1.9 mg, $ZnSO_4 \cdot 7H_2O$ 4.5 mg, $MnSO_4 \cdot H_2O$ 0.23 mg, and $CuSO_4 \cdot 5H_2O$ 0.15 mg, pH 6.5 (glucose - yeast extract medium[1]). For the inoculum preparation, 250 ml of glucose - yeast extract medium in a 1-liter Erlenmeyer flask was inoculated with the spore suspension to yield a final concentration of 5.4×10^5 spores/ml, and incubated at 28°C on a reciprocating shaker (100 strokes/minute) for 48 hours. The main cultivation was done with 250-ml protions of the glucose - yeast extract medium containing 1.25% glucose in 1-liter Erlenmeyer flasks that were inoculated with 12.5 ml of the 48-hour inoculum and incubated at 28°C on a reciprocating shaker (100 strokes/minute) for 48 hours. For precursor feeding, the labeled acetate was added under sterile conditions after 36 hours, corresponding approximately to the onset of patulolide production.

Incorporation of Sodium [1,2-$^{14}C_2$]Acetate in Patulolides

To optimize the culture conditions for the incorporation of sodium [^{13}C]acetate by *P. urticae* S11R59, sodium [1,2-$^{14}C_2$]acetate was used as a model for sodium [^{13}C]acetate. Incorporation of radioactive acetate into patulolides was measured. Ten μCi of sodium [1,2-$^{14}C_2$]acetate (specific activity 50 mCi/mmol) was mixed with an equal amount of nonlabeled sodium acetate. The mixture was added aseptically to 250 ml of glucose - yeast extract medium containing 5% glucose to study the incorporation profile of radioactive acetate into patulolides A (**1**), B (**2**), and C (**3**). Glucose - yeast extract medium containing 1.25, 1.50, 1.75, and 2.00% glucose was used to study the effects of glucose concentra-

tion on the incorporation of radioactive acetate into patulolide C (3). The addition was done 36 hours after inoculation, which was estimated to be the onset of detectable patulolides. At 2, 4, 6, 8, 10, 13, and 16 hours after the addition, a 3-ml portion of each culture broth was withdrawn and extracted twice with an equal volume of EtOAc. After evaporation *in vacuo*, crude extracts were spotted on the TLC plate and developed in benzene - *n*-hexane - $CHCl_3$ - EtOAc (6 : 4 : 1 : 1). By comparison with spots of authentic patulolides A (1), B (2), and C (3) (detected by a UV lamp), ^{14}C labeled patulolides A (1), B (2), and C (3) were scraped from the TLC plate and put into 10 ml of scintillation cocktail (8% 2,5-diphenyloxazole in toluene) and the radioactivity in each patulolide was measured.

Feeding of Sodium [1-^{13}C]- or [2-^{13}C]Acetate and Isolation of Patulolides A (1), B (2), and C (3)

To a 250-ml portion of glucose - yeast extract medium containing 1.25% glucose, 4 mg of either sodium [1-^{13}C]- or [2-^{13}C]acetate was added aseptically and periodically with an intervals of 10 minutes from 36 to 42 hours cultivation. At 43 hours of cultivation, the culture broth was filtered through Whatman No. 1 paper, and the culture filtrate was extracted twice with dichloromethane at pH 4.5. After being dried over Na_2SO_4, the combined extracts were concentrated to dryness *in vacuo*. One liter of the broth usually gave 400~500 mg of the crude material. A portion of the crude material was dissolved in a minimal amount of 2-propanol, and was analyzed by analytical HPLC with *n*-hexane - 2-propanol (9.5 : 0.5) as the eluting solvent with detection at 220 nm. Amounts of patulolides A (1), B (2), and C (3) were calculated by comparison to the peak area of the authentic solution. After purification of the crude material by silica gel chromatography (70~230 mesh, Merck) with *n*-hexane - 2-propanol (9.5 : 0.5) as a solvent, fractions containing patulolides A (1), B (2), and C (3) were combined and further purified on preparative HPLC with *n*-hexane - 2-propanol (9.6 : 0.4) as an eluting solvent.

2D ^{13}C NMR INADEQUATE of Sodium [2-^{13}C]Acetate Labeled Patulolide C (3)

A 2D INADEQUATE pulse sequence experiment was done on patulolide C (3) derived from sodium [2-^{13}C]acetate. Labeled patulolide C (3) was dissolved in $CDCl_3$ (1.9 M in a 5-mm tube) and the operation followed the procedure in the literature[19,20] with an 8-second delay time and spectral width of 168 ppm.

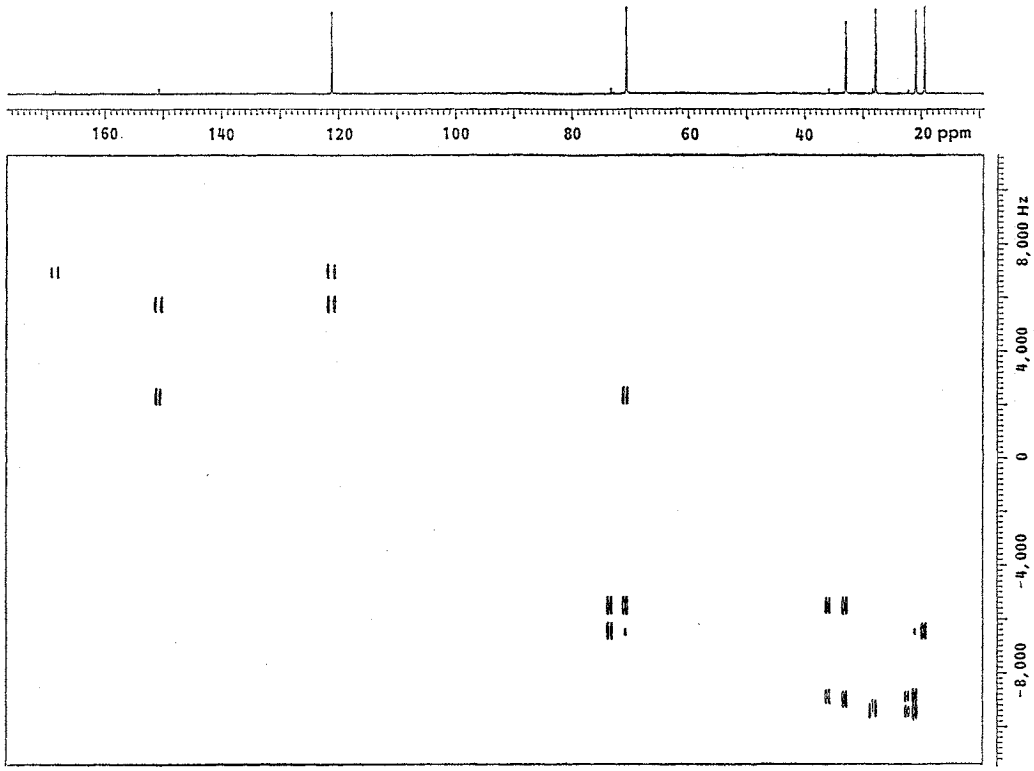
Results and Discussion

Assignment of ^{13}C NMR Spectra of Patulolides

Although we partially assigned the ^{13}C NMR spectra of patulolides A (1), B (2), and C (3) in our previous study[4], signals of six methylene carbons (C-5 to C-10) remained unassigned. Because complete identification of the ^{13}C NMR spectrum is a prerequisite for study of the incorporation pattern of ^{13}C labeled precursors, we have unambiguously assigned all of the carbon atoms of the patulolide skeletons by applying a 2D INADEQUATE pulse sequence. Using ^{13}C labeled patulolide C (3) derived from [2-^{13}C]acetate as a representative of patulolides, we have identified the connection of the carbon atoms in the patulolide C (3) skeleton (Figs. 3 and 4). Starting from the carbonyl carbon (C-1, 168.4 ppm), the connection from C-1 to C-5 (Fig. 3) as well as those among the six methylene carbons (C-5 to C-10), the C-11 methine carbon and the C-12 methyl carbon (Fig. 4) became completely clear by this technique. This allowed complete assignment of each carbon signal in patulolide C (3). ^{13}C NMR spectra of patulolides A (1) and B (2) were analyzed according to the assignment in patulolide C (3), considering that patulolide C (3) has the same skeletal origin as those of patulolides A (1) and B (2) and also on the basis of results obtained from the incorporation of [1-^{13}C]- and [2-^{13}C]acetate in patulolides A (1) and B (2)†. The signals from all carbon atoms of patulolides A (1) and B (2) were completely assigned as shown in Table 1.

† We have prepared cell-free enzyme mixture that can convert patulolide C (3) to patulolides A (1) and B (2).

Fig. 3. 2D INADEQUATE ^{13}C NMR spectrum of patulolide C (3) labeled with sodium [2-^{13}C]acetate.



Incorporation of ^{14}C Labeled Acetate

Experiments were first done with sodium [1,2-$^{14}C_2$]acetate to investigate the optimum conditions for incorporation efficiency. In our previous study, patulolides were first detected at 36 hours of cultivation when the producing strain was cultivated in a glucose - yeast extract medium containing 5% glucose. This period seems to be the onset of patulolide production in *P. urticae* S11R59. Therefore, [1,2-$^{14}C_2$]acetate was added to the culture at 36 hours and the production profile of radioactive patulolides was investigated (Fig. 5A). Patulolide C (**3**) showed the highest incorporation among all patulolides 4 hours after the addition, with an maximum incorporation of 5.3% of the total radioactivity added. The amount of radioactive patulolide C (**3**) declined sharply after 6 hours and reached a plateau after 48 hours. Incorporation of radioactive acetate into patulolides A (**1**) and B (**2**), was low (A, 1.2%; B, 0.5% incorporation), but also started instantaneously after the addition of [^{14}C]acetate and reached maxima about 4 hours after the addition. Higher concentrations of glucose may cause lower incorporation of acetate because of dilution, so we varied the initial glucose concentration in the medium (Fig. 5B). Actually, glucose concentrations below 5% gave higher incorporation in patulolide C (**3**) and incorporation became the highest (11.6%) at a glucose concentration of 1.25%, with incorporation about twice that with 5% glucose. Because we planned to add labeled acetate intermittently until about 44 hours of cultivation, enough glucose had to be supplied to maintain the mycelial activity until the end of the feeding experiment. With 1.25% glucose, only 0.03% glucose remained at 44 hours of cultivation, and this concentration gave the highest incorporation; therefore, we selected glucose -

Fig. 4. Methyl, methylene, and methine signals of sodium [2-^{13}C]acetate labeled patulolide C (**3**) in 2D ^{13}C NMR spectroscopy.

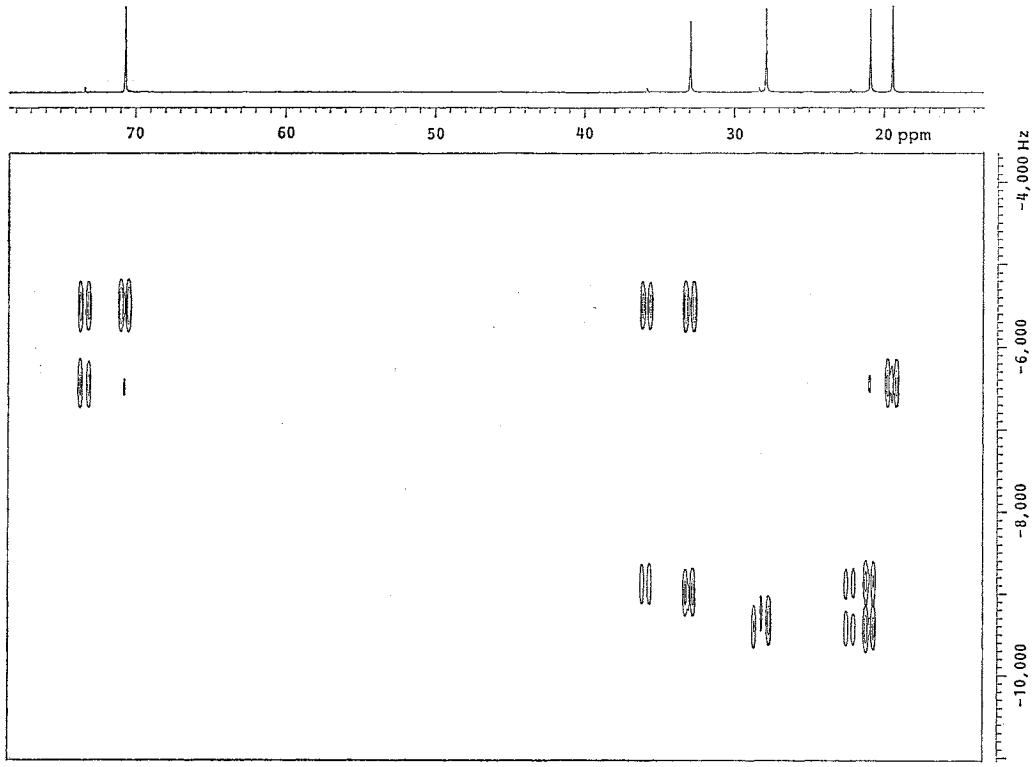
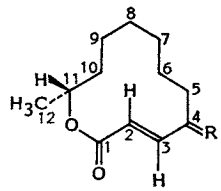


Table 1. Chemical shifts of ^{13}C NMR spectra of patulolides A (**1**), B (**2**), and C (**3**).



Patulolide A (*trans*)
Patulolide B (*cis*) — R = O
Patulolide C — R = 

Carbon No.	Functional groups	Chemical shifts (ppm)		
		Patulolide A	Patulolide B	Patulolide C
C-1	C=O	166.51	165.27	168.28
C-2	C=C	129.77	125.85	121.38
C-3	C=C	141.77	139.62	150.32
C-4	C=O (C–OH)	202.44	202.87	(70.78)
C-5	CH_2	38.83	40.17	35.82
C-6	CH_2	24.47	20.38	20.80
C-7	CH_2	25.71	24.20	28.29
C-8	CH_2	25.60	24.85	27.80
C-9	CH_2	22.32	23.34	22.21
C-10	CH_2	34.74	31.78	32.91
C-11	CHO	74.92	74.59	73.30
C-12	CH_3	20.11	19.63	19.41

Fig. 5. Incorporation of sodium [^{14}C]acetate in patulolides.

(A) Time course of [^{14}C]acetate incorporation into patulolides. ● Patulolide A, ▲ patulolide B, ■ patulolide C.

(B) Effects of glucose concentration on the incorporation of [^{14}C]acetate into patulolide C (3). Glucose: ▽ 1.25%, △ 1.50%, □ 1.75%, ○ 2.00%.

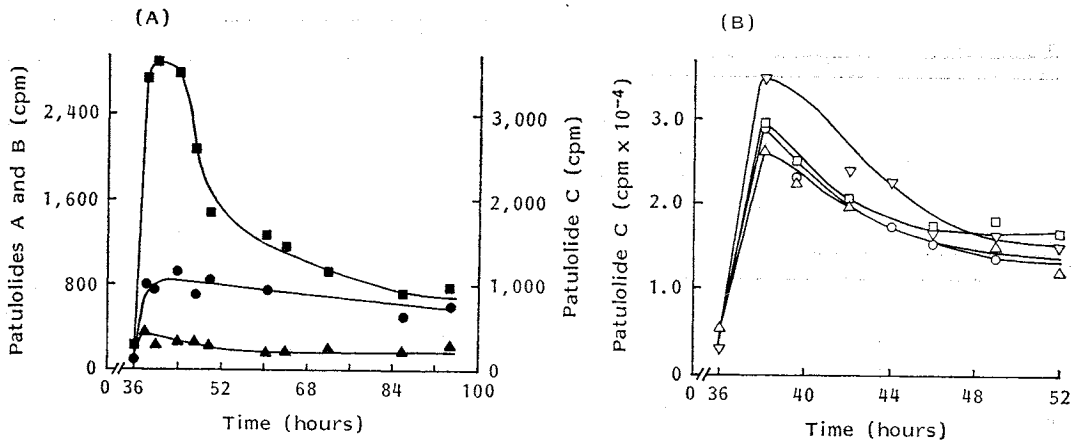
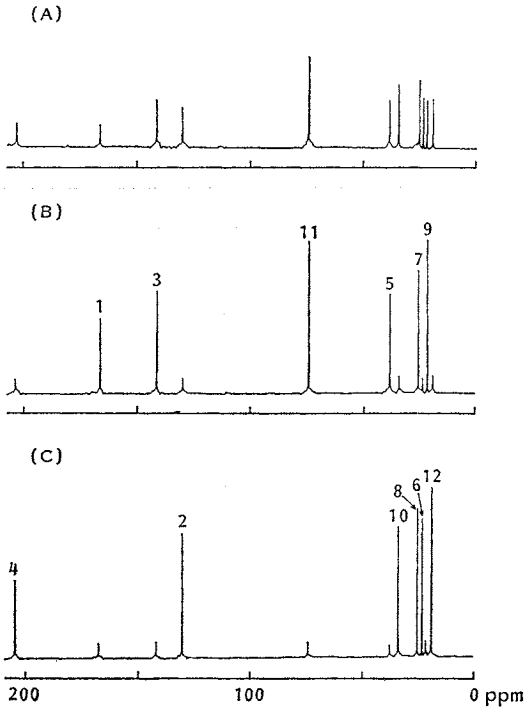


Fig. 6. ^{13}C NMR spectra of patulolide A (1) unlabeled (A), labeled with sodium [1-^{13}C]- (B) and [2-^{13}C]acetate (C).



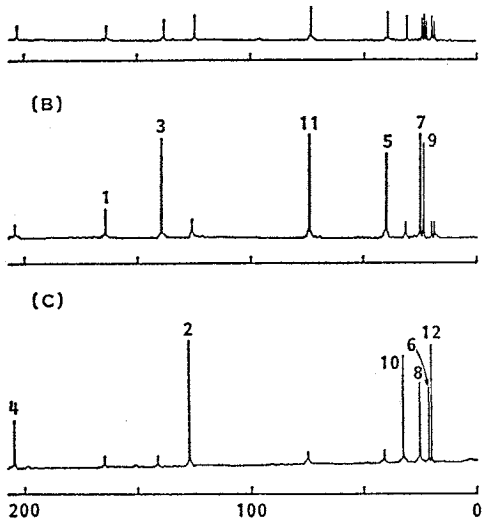
Carbon	Chemical shift	Enrichment (fold)	
		[1-^{13}C]	[2-^{13}C]
4	202.5		6.63
1	166.5	8.05	
3	141.7	13.98	
2	129.9		10.59
11	74.9	17.80	
5	38.7	11.74	
10	34.7		11.34
7	25.7	16.45	
8	25.4		12.53
6	24.3		12.06
9	22.4	18.53	
12	20.2		14.49

yeast extract medium containing 1.25% glucose for the feeding experiment.

Incorporation of [^{13}C]Acetate and Enrichment Pattern of Carbon Signals in ^{13}C Labeled Patulolides Fed with Sodium [1-^{13}C]- and [2-^{13}C]Acetate

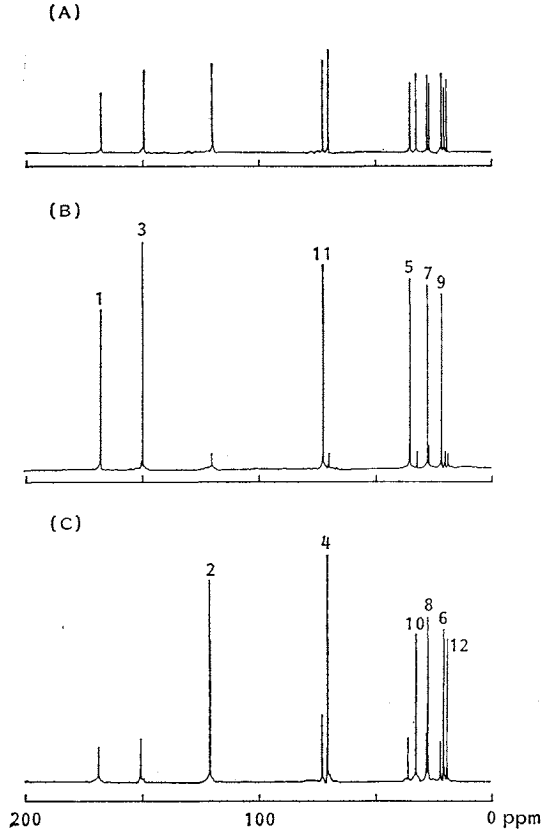
From our preliminary experiments, a high concentration of sodium acetate (1 mg/ml) was found

Fig. 7. ^{13}C NMR spectra of patulolide B (**2**) unlabeled (A), labeled with sodium [1-^{13}C]- (B) and [2-^{13}C]acetate (C).



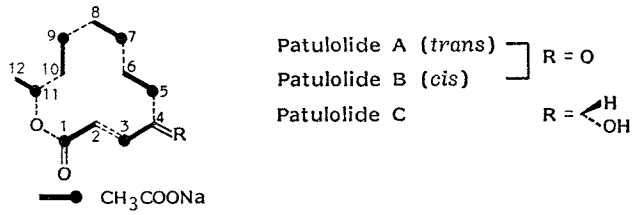
Carbon	Chemical shift	Enrichment (fold)	
		[1-^{13}C]	[2-^{13}C]
4	202.9		3.22
1	165.3	1.87	
3	139.6	6.66	
2	125.9		2.90
11	74.6	6.96	
5	40.2	5.54	
10	31.8		7.49
8	24.9		5.22
7	24.2	6.93	
9	23.3	6.37	
6	20.4		5.18
12	19.6		7.76

Fig. 8. ^{13}C NMR spectra of patulolide C (**3**) unlabeled (A), labeled with sodium [1-^{13}C]- (B) and [2-^{13}C]acetate (C).



Carbon	Chemical shift	Enrichment (fold)	
		[1-^{13}C]	[2-^{13}C]
1	168.4	15.07	
3	150.5	21.88	
2	121.1		16.42
11	73.3	19.39	
4	70.7		17.49
5	35.8	18.22	
10	32.9		12.80
7	28.2	17.57	
8	27.7		13.85
9	22.2	16.75	
6	20.8		13.00
12	19.4		12.77

Fig. 9. Distribution of acetate units in patulolides.



to inhibit patulolide biosynthesis strongly. Therefore, to overcome this problem, periodic feeding of small amounts of either sodium [1-^{13}C]- or [2-^{13}C]acetate was done. Starting at 36 hours of cultivation, 4 mg of labeled sodium acetate was added to the culture every 10 minutes for a period of 6 hours; the resulting total amount of sodium acetate was about 576 mg/liter. After extraction with dichloromethane, purification by silica gel chromatography, and preparative HPLC, 38.4 mg of patulolide A (**1**), 6.67 mg of patulolide B (**2**), and 146.7 mg patulolide C (**3**) were obtained from one liter of broth fed with [1-^{13}C]acetate. A similar yield, 37.3 mg of patulolide A (**1**), 6.67 mg of patulolide B (**2**), and 160 mg of patulolide C (**3**), was obtained from one liter of broth fed with [2-^{13}C]acetate.

The enrichment pattern of carbon signals in ^{13}C NMR spectra of the labeled patulolides showed that both [1-^{13}C]- and [2-^{13}C]acetate were incorporated into the skeleton of patulolides A (**1**), B (**2**), and C (**3**). The enrichment ratio for each of the labeled carbons in patulolides A (**1**), B (**2**), and C (**3**) were 7- to 18-fold, 2- to 8-fold, and 13- to 22-fold, respectively (Figs. 6~8). It was clear that in all patulolides, the carbons at positions 1, 3, 5, 7, 9, and 11 were enriched by sodium [1-^{13}C]acetate and the carbons at positions 2, 4, 6, 8, 10, and 12 were enriched by sodium [2-^{13}C]acetate. From the results, acetate was confirmed to be the building block of patulolides. The incorporation of both sodium [1-^{13}C]- and [2-^{13}C]acetate in alternate carbons demonstrated the head-to-tail arrangement of six acetate units without the addition of another type of precursor (Fig. 9), ruling out the idea that patulolides are hybrid products of tetraketide and some C_4 or C_5 compounds (pathway B). Therefore, we concluded that patulolides are pure acetogenic hexaketides that were probably produced *via* elongation of acetate units as in fatty acid synthesis, according to the Birch-Collie hypothesis[15]. Finally lactonisation takes place to form the final patulolides.

Acknowledgments

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