Production of the New Antibiotic Tetrahydrokalafungin by Transformants of the Kalafungin Producer *Streptomyces tanashiensis*

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The new antibiotic tetrahydrokalafungin was produced by the transformants of kalafungin producing *S. tanashiensis* and kalafungin-nonproducing mutants carrying the recombinant plasmid pKU523. This plasmid consists of pKU501 (J. Antibiotics 44: 995~1005, 1991) which contains the gene cluster for kalafungin biosynthesis, and additional 5 kb stability region of SCP2*.

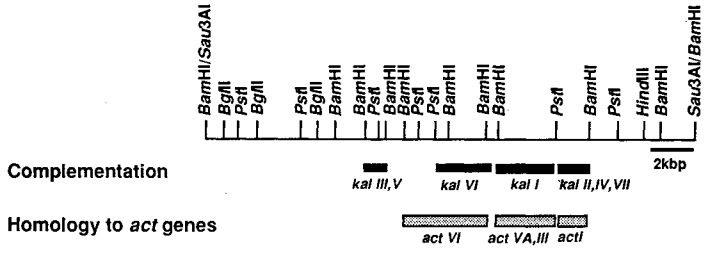
The benzoisochromanequinone antibiotic kalafungin produced by *Streptomyces tanashiensis* strain Kala[1] is biosynthesized from eight acetate units *via* a hypothetical intermediate "polyketide"[2,3]. In the study of kalafungin biosynthesis in this strain, we isolated kalafungin-nonproducing mutants and classified them into seven different phenotypes (*kal*I to VII) corresponding to the observed effects on kalafungin biosynthesis[4]. Recently, the plasmid pKU501 which contains a 28 kb DNA fragment was cloned from the genomic library of the parent strain by colony hybridization using the polyketide synthase genes *act*I and III for actinorhodin biosynthesis from *Streptomyces coelicolor* A3(2) as probes[5]. In complementation experiment with the nonproducing-mutants using subclones derived from pKU501, seven genes (*kal*I to VII) for kalafungin biosynthesis were identified[5] (Fig. 1). However, the mutations *kal*-4, 9 and 10 (regulatory mutants) and *kal*-1, 12 and 13 (these were considered to include at least two mutations) were not complemented by the subclones. Since pKU501 was barely able to replicate in *Streptomyces* sp., we modified pKU501 to allow its stable replication in *Streptomyces* sp. In this paper, we describe the modification of pKU501 and the production and structure determination of the new compound tetrahydrokalafungin produced by transformants carrying the recombinant plasmid pKU523.

Materials and Methods

Bacterial Strains and Plasmids

The kalafungin producer *S. tanashiensis* strain Kala and kalafungin-nonproducing mutants (*kal*)[4] were used in this study. The restriction-reduced mutant *S. tanashiensis* R3-5[6] was used for modification of a plasmid before transformation of *S. tanashiensis* derivatives. *S. tanashiensis* was transformed as described previously[6]. Packaging of DNA into the λ head, infection of *Escherichia coli* JM108 and selection of transductants were done as described previously[5]. The transformants of *S. tanashiensis* and transductants of *E. coli* were stored as mycelial suspensions (4 ml) containing 50% (v/v) glycerol after growing in Trypticase Soy Broth (TSB) (10 ml) supplemented with 5 μg per ml of thiostrepton at

Fig. 1. Structure of gene cluster for kalafungin biosynthesis in *S. tanashiensis*.



30°C for 16 hours. YMS agar[7] and its liquid medium (without agar) were used for production of antibiotics.

The recombinant plasmid pKU501 (Fig. 2) was isolated from *S. tanashiensis* genomic library using *E. coli*-*Streptomyces* bifunctional cosmid pKU205 as described previously[5]. Plasmid DNA was isolated from *S. tanashiensis* or *E. coli* as described by KIESER[8].

Modification of pKU501

One microgram of pKU501 was digested with *Eco*RI and treated with alkaline phosphatase. The digested pKU501 was ligated with 5 kb *Eco*RI stability region prepared from SCP2*[9]. The ligated DNA was packaged in λ head and then introduced into *E. coli* JM108. Constructed plasmid pKU523 containing 5 kb stability region was confirmed by their restriction maps. (Fig. 2)

Identification of Antibiotics Obtained in Complementation Experiments

The transformants carrying pKU523 were streaked on YMS agar (4 ml) containing 3 μg of thiostrepton per ml in a small plate and incubated at 30°C for 4 days, or inoculated in of YMS liquid medium (10 ml) and incubated at 30°C for 3 days. The products were extracted with ethyl acetate and then identified by silica gel thin-layer chromatography using bioautography and authentic samples of kalafungin and dihydrokalafungin as described previously[5].

Isolation of a New Antibiotic

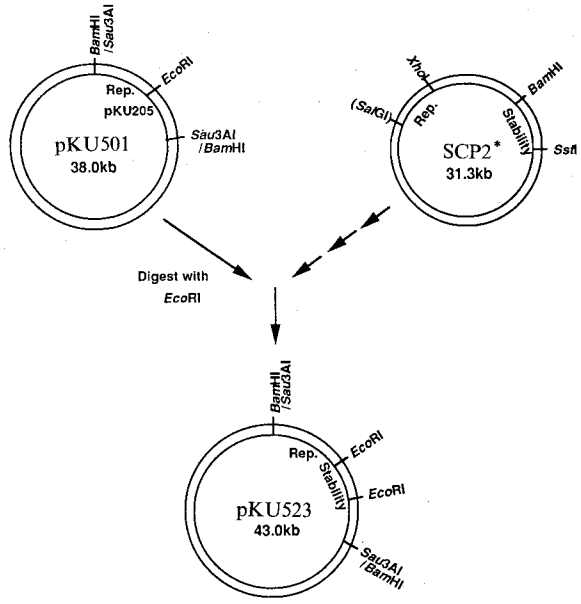
Fifty microliters of the mycelial stock suspension of *S. tanashiensis kal*-11 carrying pKU523 was inoculated in a 50-ml tube containing 10 ml of YMS liquid medium supplemented with 5 μg of thiostrepton per ml and incubated at 30°C for 20 hours on a reciprocal tube shaker to obtain a seed culture. Then 1 ml of the seed culture was transferred into a 500-ml Erlenmeyer flask containing 100 ml of YMS liquid medium supplemented with 5 μg of thiostrepton per ml and incubated at 30°C for 50 hours on a rotary shaker. The antibiotics in the cultured broth (100 ml × 20) were extracted with chloroform (2 liters × 2) after acidification with 2N HCl to pH 2. The chloroform layer was concentrated to a small volume under reduced pressure. The concentrated material was applied onto preparative silica gel TLC plate (20 × 20 cm: Merck Kieselgel 60F_{254}, 0.5 mm), and the plate was developed with $CHCl_3$-CH_3OH (25:2). A new yellow fluorescent band having an Rf value of 0.33 on the plate exhibited a positive reaction in bioassay. The Rf values of kalafungin and dihydrokalafungin under the same conditions were 0.72 and 0.36, respectively.

Results

Introduction of Stability Region into pKU501

To allow pKU501 stable replication of *Streptomyces* sp., the 5 kb stability region of SCP2* was introduced into pKU501, which contained the replication origin of SCP2* but no stability region[5], pKU523 as shown in Fig. 2 stably replicated in *E. coli* JM108 and *S. tanashiensis* derivatives in contrast to pKU501. Furthermore, complementation of kalafungin production was observed when the transformants carrying pKU523 were grown on YMS agar or in YMS liquid medium. However, in our previous paper[5], the transformants carrying subclones derived from pKU501 produced the kalafungins only when grown on YMS agar but not in the liquid medium.

Fig. 2. Construction of pKU523.



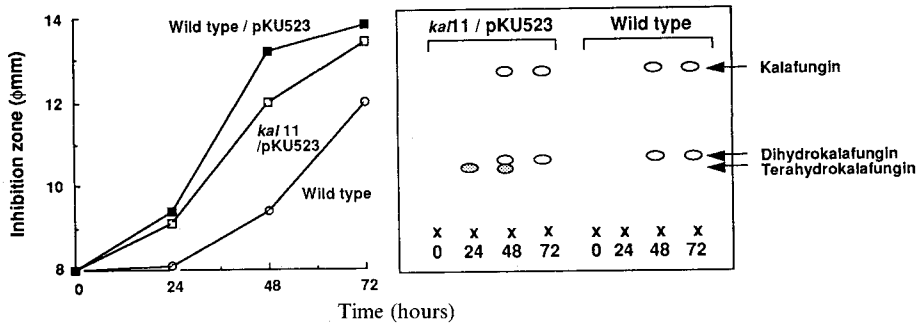
Genetic markers are abbreviated as follows. Rep., the replication region of SCP2* of *S. coelicolor* A3(2); Stability, the stability region for the maintenance of plasmid SCP2*. *Bam*HI-*Sst*I fragment of stability region of SCP2* was introduced into the *Eco*RI site by a synthetic linker.

In complementation experiments with kalafungin-nonproducing mutants, kalafungin production was not only in the mutants classified in *kal*I→VII (*kal*-2, 3, 5, 6, 8, 11, 15 and 16) but also in regulatory mutants (*kal*-4, 9 and 10) and unclassified mutants (*kal*-1, 12 and 13) introduction of pKU523. These results suggest that at least eight genes (*kal*I→VII, and regulatory genes) lie on the primary 28 kbp fragments.

Production of a New Antibiotic

Fig. 3 shows a typical time course of antibiotic production by the transformants of the *kal*-11 and wild-type strains carrying pKU523, and the wild-type strain. The production of antimicrobial substances by these transformants was observed after 24 hours but only after 48 hours by the wild-type strain. By the analysis of

Fig. 3. A typical time course of tetrahydrokalafungin production by the transformants *kal*-11 and the wild-type strain carrying pKU523 and the wild-type strain of *S. tanashiensis*.



the antimicrobial substances by silica gel TLC, three spots (kalafungin, dihydrokalafungin and a new compound) were detected. The new antibiotic was detected in the culture broth of the transformants, but not in that of the wild-type strain.

The Structure of a New Antibiotic

The physico-chemical properties of the new compound and dihydrokalafungin are summarized and compared with those of dihydrokalafungin in Table 1. The molecular formula, $C_{16}H_{16}O_6$ was assigned from its mass spectrum [*m/e* (305 M+1)]. The UV spectrum of the new compound suggested the presence of β-hydrojuglon skeleton and was sifted from 348 to 520 nm in an alkaline solution. The shifted spectrum was identical with that of dihydrokalafungin (Fig. 4) in alkaline solution. After the alkaline solution of the new compound was neutralized with 2 N HCl, the shifted spectrum was not reversed to the original one but remained identical with that of dihydrokalafungin in neutral solution. By TLC and bioautography, the product obtained by pH shift was identified as dihydrokalafungin. This result suggested that the structure of the new compound is related to dihydrokalafungin, and that it is easily converted to dihydrokalafungin by oxidation under alkaline conditions, confirming that it has β-hydrojuglon skeleton.

The structure elucidation was performed by a comparison of ^1H and ^{13}C NMR spectra ($CDCl_3$, 400 MHz) between the new compound and dihydrokalafungin. The quaternary carbons at C-4a (141.9 ppm), C-10a (146.1 ppm) of dihydrokalafungin were shifted to methine carbons 51.5(d) ppm and 42.8(d) ppm in the new compound, respectively. C-5 (182.7 ppm), C-10 (188.1 ppm) carbons of dihydrokalafungin were shifted to lower range 201.6 ppm and 196.3 ppm, confirming the presence of β-hydrojuglon skeleton in the compound. In ^1H-^{13}C 2D correlated spectroscopy (COSY), the C-4a (42.8 ppm), C-10a (51.1 ppm) carbons showed the connective with resonance of methine protons 4a-H, 10a-H at 3.24 ppm, 3.23 ppm, respectively. In the ^1H-^1H COSY, the methine proton 10a-H at 3.23 ppm coupled with methylene protons 1-H at 4.81 ppm, and 4a-H at 3.24 ppm and methylene protons 4-H at 1.58, 2.33 ppm. ORD spectrum of this compound was similar to that of dihydrokalafungin, indicating the same configuration of the both compounds. Based on these data, we propose the structure shown in Fig. 4 for the new antibiotic, name tetrahydrokalafungin.

Table 1. Physico-chemical properties of tetrahydrokalafungin and dihydrokalafungin.

	Tetrahydrokalafungin (new compound)	Dihydrokalafungin
Appearance	pale yellow	yellow
SIMS m/e	305(M+1)$^+$	303(M+1)$^+$
Formula	$C_{16}H_{16}O_6$	$C_{16}H_{14}O_6$
λ max (nm)	241, 256sh, 348 in CH_3OH	250, 274, 523 in CH_3OH
	280, 520 in 0.1N NaOH-CH_3OH	280, 520 in 0.1N NaOH-CH_3OH

Fig. 4. Structures of tetrahydrokalafungin, kalafungin and dihydrokalafungin.

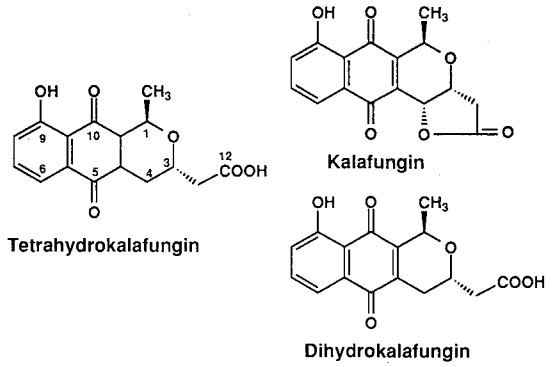


Table 2. NMR data of tetrahydrokalafungin in comparison with dihydrokalafungin (400 MHz, in $CDCl_3$).

No.	Dihydrokalafungin	Tetrahydrokalafungin		
	^{13}C-NMR	^{13}C-NMR	^{1}H-NMR	Chemical shift / Coupling
1	67.2*(d)**	68.4 (d)	1-H	4.81 dd (7.0, 4.0)
1-CH_3	19.4 (q)	13.1 (q)	1-CH_3	1.42 d (7.0)
3	63.2 (d)	65.1 (d)	3-H	4.22 m
4	27.8 (t)	31.2(t)	4-H2	1.58 m
				2.33 ddd (14.0. 4.0)
4a	141.9 (s)	42.8 (d)	4a-H	3.24 ddd (4.0)
5	182.7 (s)	196.3 (s)		
5a	131.6 (d)	137.1 (d)		
6	124.3 (d)	124.4 (d)	6-H	
7	136.1 (d)	137.3 (d)	7-H	(7.3-7.7) m
8	119.0 (d)	124.1 (d)	8-H	
9	161.4 (s)	161.8 (s)	9-OH	12.6 br s
9a	114.6 (s)	117.9 (s)		
10	188.1 (s)	201.6 (s)		
10a	146.1 (s)	51.1 (d)	10a-H	3.23 t (4.0)
11	40.3 (t)	41.0 (t)	11-H	2.57 m
12	175.7 (s)	174.0 (s)		

* δ relative to TMS. ** Multiplicity.

Discussion

Introduction of a stability region of SCP2* into a primary recombinant plasmid pKU501 allowed this plasmid to stably replicate in *S. tanashiensis*. The production of kalafungin by the transformants carrying pKU523 was observed under a liquid culture condition. The plasmid pKU523 complemented all kalafungin-nonproducing mutants isolated, indicating that the primary cloned fragment contains not only seven genes (*kal*I→VII) in kalafungin biosynthetic pathway reported previously[5] but also a regulatory gene for kalafungin biosynthesis.

Furthermore, the transformants carrying pKU523 produced a new kalafungin analog, tetrahydrokalafungin. COLE *et al.*, reported that a yellow pigment[10] and kalafungin are the intermediates of actinorhodin biosynthesis.[10] It is of interest for a further understanding of kalafungin biosynthesis whether tetrahydrokalafungin is an intermediate, a shunt product or a final product in the kalafungin biosynthetic pathway.

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