Terpentecin and UCT4B, New Family of Topoisomerase II Targeting Antitumor Antibiotics Produced by *Streptomyces*: Producing Organism, Fermentation and Large Scale Purification

SHO-ZOU KAWADA[†], YOSHINORI YAMASHITA[††,*], KEIKO OCHIAI[††], KATSUHIKO ANDO[††], TOSHIAKI IWASAKI[†††], TOSHIFUMI TAKIGUCHI[†††] and HIROFUMI NAKANO[††]

Kyowa Hakko Kogyo Co., Ltd.,
[†]Technical Research Laboratories, Kyowa-machi, Houfu, Yamaguchi, Japan
[††]Tokyo Research Laboratories, 3-6-6 Asahimachi, Machida, Tokyo, Japan
[†††]Pharmaceutical Research Laboratories, Nagaizumi-cho, Shizuoka, Japan

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Terpentecin and UCT4B are new family of antitumor antibiotics with topoisomerase II mediated DNA cleavage activity. Based on the taxonomic studies, the producing strain S-464 was identified as *Streptomyces* sp. This strain is different from *Kitasatosporia griseola* which had been identified as the terpentecin-producing strain in 1988. Fermentation studies showed that natural nitrogen sources supported higher titer of terpentecin, and the synthetic medium with inorganic nitrogen sources supported selective production of UCT4B. An improved isolation method was developed for the large scale purification of terpentecin.

DNA topoisomerase II is a nuclear enzyme that alters DNA conformation through a concerted breaking and rejoining of DNA strands, thereby controlling the topological state of DNA[1]. Topoisomerase II has been shown to be the primary cellular target for a number of clinically important antitumor agents with diverse and unrelated chemical structures[2,3]. These antitumor drugs, referred to as "topoisomerase II poisons", trap the enzyme in an intermediate reversible complex with DNA, termed cleavable complex which prevents the final rejoining step of the reaction and results in increased DNA strands breaks[2,3]. Now it is believed that the ability to form the cleavable complex with topoisomerase II is responsible for the antitumor activity of these drugs.

In the course of a screening program for specific new topoisomerase II poisons, we have found that the diterpenoid antitumor antibiotics, terpentecin and clerocidin are potent inducers of topoisomerase II mediated DNA cleavage[4], and have isolated a novel compound UCT4B, from the culture broth of an actinomycete strain which produces terpentecin[5,6] (Fig. 1). Terpentecin was originally isolated by TAMAMURA *et al.* from the culture broth of *Kitasatosporia griseola* in 1988, but the production of UCT4B was not reported for this strain[7].

Recently MCCULLOUGH *et al.* reported that clerocidin inhibited bacterial type II topoisomerase (DNA gyrase)[8]. We reported previously that clerocidin, terpentecin and UCT4B formed a cleavable complex with mammalian topoisomerase II with same properties[4,5], and terpentecin and UCT4B showed the same antibacterial activity as clerocidin. These results suggested that terpentecin and UCT4B also form a cleavable complex with DNA gyrase, which might be the cause for their significant antibacterial activity.

In this paper, we report the taxonomy of the strain producing both terpentecin and UCT4B, the selective production of each compound by fermentation, and the large scale purification of terpentecin.

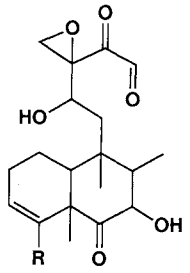
Materials and Methods

Microorganism

The producing strain S-464 was isolated from a soil sample collected in Houfu city, Yamaguchi prefecture, Japan.

This strain has been deposited at the National Institute of Bioscience and Human-Technology, Agency of

Fig. 1. Structure of terpentecin and UCT4B.



Terpentecin: R=CH_3
UCT4B: R=CH_2OH

Industrial Science and Technology, Tsukubashi, Ibaragi, Japan, as *Streptomyces* sp. S-464 with the accession number FERM BP-3036 under the Budapest treaty.

Taxonomic Studies

Growth characteristics and carbohydrate utilization were determined by the methods of the International Streptomyces Project[9] (ISP). Color codes were assigned to the substrate and aerial mass pigments according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago). The spores and mycelia of the strain were observed by scanning electron microscopy (scanning microscope model S-570, Hitachi Co., Ltd.).

Analysis of diaminopimelic acid was performed on the hydrolysate of filamentous mycelia grown in a liquid medium SRIII (glucose 5 g, soluble starch 25 g, yeast extract 1 g, peptone-A 10 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 1 g per liter of water, pH 7.2), aerial and vegetative mycelia grown on the solid medium ISP4 (soluble starch 10 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, $CaCO_3$ 2 g, NaCl 1 g, $(NH_4)_2SO_4$ 2 g, trace salts solution 1 ml per liter of water, pH 7.0~7.4).

Culture Conditions

The effect of carbon, nitrogen sources and other ingredients in the fermentation medium was investigated in 250-ml Sakaguchi flasks. The details of fermentation media and culture conditions are described in the results.

For the fermentation in 30-liter jar fermenter, a 300-ml Erlenmeyer flask containing 50 ml of SRIII medium was inoculated with a loopful of vegetative mycelium on agar slant and was incubated at 28°C on a rotary shaker for 48 hours. 10 ml of the seed culture was transferred to a 2-liter Erlenmeyer flask containing the same medium. Following 24 hours of incubation at 28°C, this second stage seed culture (0.9 liter) was used as inoculum to initiate the fermentation in 30-liter jar fermenters bathed with 15 liters of media. The fermentation was carried out at 28°C with 1.8 liters of air per minute and agitation at 300 rpm.

Culture growth was evaluated by centrifuging fermentation broth in 10-ml conical tubes at $1200 \times g$ for 10 minutes. The packed cell solids were reported as percent of total broth volume.

Analysis of Antibiotic Production

Antibiotic production was monitored as antibacterial activity during the fermentation by the paper disc method on nutrient agar using *Bacillus subtilis* as the test organism[10]. Terpentecin and UCT4B show different sensitivity against a wild type and a supersensitive mutant strain of *Bacillus subtilis*, which is deficient in enzyme for recombination (rec^-). Therefore, the following method in combination with wild type and mutant strain was used to distinguish terpentecin and UCT4B.

Melted agar medium prewarmed at 60°C with spores suspensions of the wild type strain (rec^+) at 10^3 spores/ml and the supersensitive mutant strain (rec^-) at 10^6 spores/ml was poured into plates on the level surface. After solidification, paper discs (8 mm diameter) impregnated with samples were applied on agar plates, and the plates incubated at 37°C.

Results and Discussion

Taxonomy of the Producing Strain

The cultural characteristics of strain S-464 on various media are shown in Table 1. The vegetative mycelia grew well on the various ISP agar media but grew only moderately on sucrose-nitrate agar. Strain S-464 produced a reddish brown soluble pigment on yeast extract-malt extract and oatmeal agar, a pale gold soluble pigment on nutrient agar and peptone-yeast extract-iron agar, a brown soluble pigment on tyrosine

Table 1. Cultural characteristics of strain S-464.

Medium	Cultural characteristics	
Sucrose-nitrate agar	G:	Moderate
	AM:	None
	SM:	Pearl shell tint (3ba)
	P:	None
Glucose-asparagine agar	G:	Good
	AM:	White (a)
	SM:	Flesh pink (4ca)
	P:	None
Glycerol-asparagine agar	G:	Good
	AM:	None
	SM:	Nude tan (4gc)
	P:	Pale rose
Inorganic salts-starch agar	G:	Good
	AM:	Pussywillow gray (5dc)
	SM:	Light fawn (4ge)
	P:	Pale brown
Tyrosine agar	G:	Good
	AM:	None
	SM:	Cocoa brown (5ni)
	P:	Brown
Nutrient agar	G:	Good
	AM:	White (a)
	SM:	Melon yellow (3ga)
	P:	pale gold
Yeast extract-malt extract-agar	G:	Good
	AM:	Sand (3cb)
	SM:	Deep brown (5pl)
	P:	Reddish brown
Oatmeal agar	G:	Good
	AM:	Pussywillow gray (5dc)
	SM:	Mauve wine (71/2ni)
	P:	Rose brown
Peptone-yeast extract-iron agar	G:	Good
	AM:	White (a)
	SM:	Light brown (3lg)
	P:	Pale gold

Abbreviations; G: degree of growth , AM: color of aerial mycelium, SM: color of substrate mycelium, P: color of soluble pigment.

Fig. 2. Scanning electron micrograph of the spore chains of strain S-464 grown on inorganic salts-starch agar medium (×6500).

Bar represent 3.8 μm.

agar, and a pale rose soluble pigment on glycerol-asparagine agar. No fragmentation of substrate mycelia was observed, and no sclerotia, sporangia, or flagellated spores were formed. The aerial mass was white or gray on some agar media but was not observed on sucrose-nitrate agar, glycerol-asparagine agar and tyrosine agar after incubation at 28°C for 14 days. The aerial mycelia were moderately short and formed spiral chains of spores, whose surface was spiny (Fig. 2). The arrangement of the spore chains was in the section *Spirales*. Each spore chain consisted of 10 or more ellipsoidal spores of 0.5 to 0.6 μm by 0.6 to 0.8 μm.

Physiological characteristics and the pattern of utilization of carbon sources are shown in Tables 2 and 3. Analysis of whole-cell hydrolysate by TLC revealed that filamentous submerged mycelia, aerial and vegetative mycelia grown on an agar medium contained L,L-diaminopimelic acid but no detectable *meso*-diaminopimelic acid. Thus, strain S-464 has chemotype I cell walls, indicating that strain S-464 belongs to the genus *Streptomyces*. Terpentecin was originally isolated from a culture broth of *Kitasatosporia griseola* whose vegetative mycelia contained mainly *meso*-diaminopimelic acid[7]. Therefore, strain S-464 presented here is different from the previously reported terpentecin-producing strain *Kitasatosporia griseola*.

In addition to terpentecin and UCT4B, other structurally related diterpenoid antibiotics have been reported; spirocardins A and B are produced by *Nocardia* sp.[11], and clerocidin by the fungus *Oidiodendron truncatum*[12]. Thus closely related diterpenoid antibiotics are produced by three different genera of actinomycetes as well as a fungus.

Table 2. Physiological properties of strain S-464.

Liquefaction of gelatin	Negative
Coagulation of milk	Negative
Peptonization of milk	Positive
Decomposition of cellulose	Positive
Hydrolysis of starch	Positive
Formation of melanoid pigment	Positive
Optimum growth temperature	28~32°C
Optimum growth pH	6.8~7.5

Table 3. Carbon utilization by strain S-464.

Carbon source	Utilization
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
D-Mannitol	+
Sucrose	+
m-Inositol	+
Raffinose	+
L-Rhamnose	+
Salicin	−

+: utilized, −: not utilized.

Activity of Terpentecin and UCT4B against a Mutant and a Wild Type Strain of *Bacillus subtilis*

The antibacterial activities against these strains are described as the zone of growth inhibition surrounding the test disc on the assay plates. As shown in Fig. 3, both terpentecin and UCT4B inhibited the growth of the rec$^-$ strain more potently than that of rec$^+$ strain. Since recombination deficient mutants have been known to show higher sensitivity against antibiotics which can initiate DNA damage, this suggests that terpentecin and UCT4B induce the DNA damage, through cleavable complex formation with the DNA gyrase. Selectivity was indicated by the increased difference in antibacterial activities against the rec$^-$ and rec$^+$ strains. UCT4B inhibits the growth of the rec$^-$ strain more selectively than terpentecin. Since both terpentecin and UCT4B have a complex tautomeric nature[6], a chemical analysis with TLC or HPLC is not available to distinguish between terpentecin and UCT4B in fermentation broths. Therefore, the assay based on the difference in their sensitivity against the rec$^+$ and rec$^-$ strain could be used to monitor which antibiotic, terpentecin or UCT4B, is the main product during the fermentation.

Fig. 3. Antibacterial activities of terpentecin and UCT4B against *Bacillus subtilis* wild type strain (rec$^+$; ●) and mutant strain (rec$^-$; ○).

A; terpentecin, B; UCT4B. Activity is expressed as diameter of growth inhibition zone surrounding test discs on the assay plate.

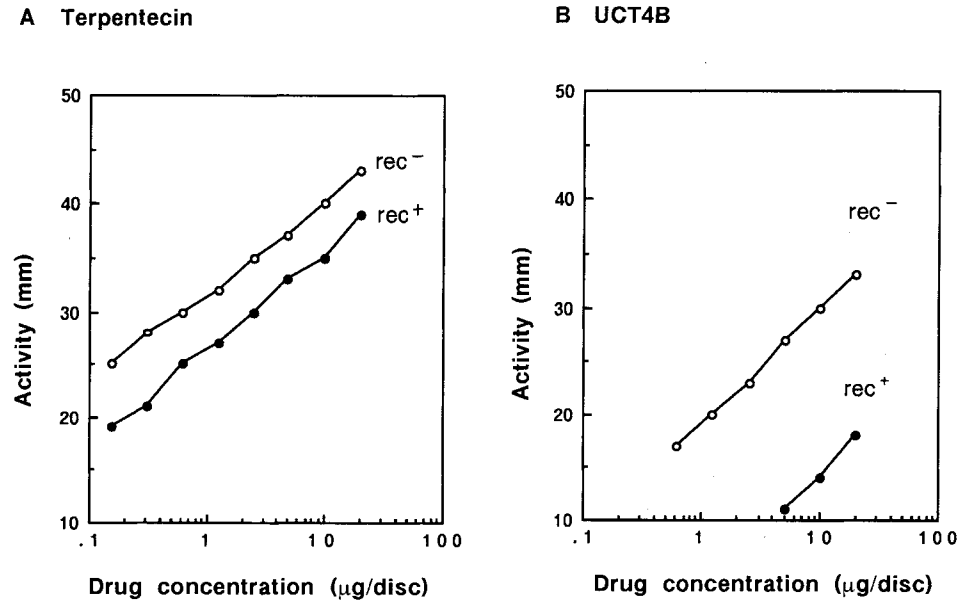
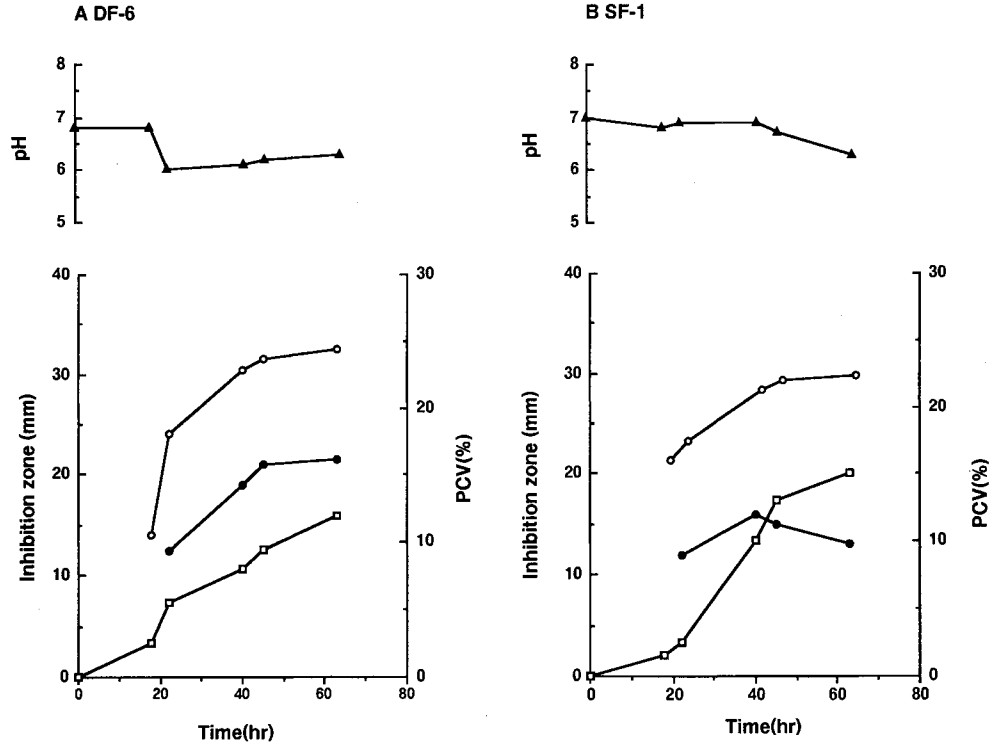


Fig. 4. Time course of fermentation in a 30-liter fermenter.

Packed cell volume (□), activity against rec$^+$ (●) and rec$^-$ (○) strain, and pH (▲).



Panel A and B show the results of fermentation with DF-6 and SF-1 medium, respectively.

Table 4. The effects of carbon and nitrogen sources on antibiotic production.

Medium	Carbon source	Nitrogen source	Activities (zone of growth inhibition; mm)		
			rec^-	rec^+	$rec^- - rec^+$
SF-1	Soluble starch	KNO_3	30	18	12
SF-2	Glycerol, glucose	KNO_3	30	20	10
SF-3	Sucrose	KNO_3	28	17	11
SF-4	Soluble starch	$(NH_4)_2SO_4$	23	10	13
SF-5	Glycerol, glucose	$(NH_4)_2SO_4$	25	12	13
SF-6	Sucrose	$(NH_4)_2SO_4$	26	15	11
DF-6	Glycerol, glucose	CSL	30	23	7

Each medium contains a 5% carbon source and an 1% inorganic nitrogen source except for DF-6 which contains 3% CSL.

Selective Production of Terpentecin and UCT4B by Fermentation

Strain S-464 grows well in liquid media containing various carbon sources . However, the maximum titer of antibiotics depends on the carbon and nitrogen sources. As a result of a systematic study on carbon and nitrogen sources, glucose and glycerol, and corn steep liquor were good carbon and nitrogen source for antibiotics production. In addition, studies with shake flasks culture indicated that the addition of 1% of KH_2PO_4 stimulates of antibiotics production. Fermentation broths with these media showed antibacterial activity with low selectivity against rec^- strain, suggesting that terpentecin was the main product. Consistent with this, purification of active material confirmed that terpentecin was preferably produced, UCT4B was being present only in a small quantity. Following these results, DF-6 (glucose 2.5%, glycerol 2.5%, corn steep liquor 3.0%, KH_2PO_4 2.0%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.5%, pH 7.0) was used for large scale production of terpentecin . Panel A in Fig. 4 shows a typical time course for production of terpentecin in a 30-liter jar fermenter under optimum culture conditions. Microbial cell growth reached a maximum at about 68 hours and the maximum potency was achieved at 60 hours.

Since the production of UCT4B was limited to a small amount under the above culture conditions, we tried to optimize the media for UCT4B production and found that synthetic media with inorganic nitrogen sources supported higher titer of UCT4B. As indicated by the increased difference in antibacterial activities against rec^- and rec^+ strains (Table 4), the media containing KNO_3 or $(NH_4)_2SO_4$ were better for selective production of UCT4B than DF-6 medium. The purification of active material confirmed that UCT4B was the main product in the culture broth with synthetic media (data not shown). From these results, SF-1 medium (soluble starch 5%, KNO_3 1%, KH_2PO_4 2.0%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.5%, pH 7.0) was selected for UCT4B production. Panel B in Fig. 4 shows a typical time course for UCT4B production in a 30-liter jar fermenter under optimum conditions. While antibiotic production, which was monitored by the activity against rec^- strain, reached a maximum at 68 hours, activity against rec^+ strain reached a maximum at 40 hours and then decreased gradually, indicating accumulation of UCT4B. These results suggest that terpentecin might be produced in the early phase of fermentation and then gradually converted to UCT4B.

Large Scale Purification of Terpentecin

The following improved procedure was established to purify a large amount of terpentecin from culture broth of a 2,000 liters fermenter. The filtrate of the culture broth (1,100 liters) was applied to a column of Diaion HP-20 (Mitsubishi Chemical Industries Limited. 50 liters), the column washed with 50% MeOH, and eluted with 60% MeOH and 80% MeOH. The active eluate (80% MeOH fraction) was concentrated and applied to Diaion HP-20SS (25 liters), washed with 70% MeOH and eluted with 80% MeOH. The active eluate was applied to a Silica gel (10 liters) column and chromatographed using $CHCl_3$-MeOH (100:1) as the eluent. The active fractions were further purified by successive HPLC using the packed column YMC R-354-10 (mobile phase, 70% MeOH; flow rate, 30 ml/minute; detection , UV 254 nm) and Lop CN 45S (mobile phase , 40% CH_3CN; flow rate, 9 ml/minute; detection, UV 254 nm) to yield 900 mg of terpentecin.

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