TALLYSOMYCIN*, A NEW ANTITUMOR ANTIBIOTIC COMPLEX RELATED TO BLEOMYCIN I. PRODUCTION, ISOLATION AND PROPERTIES

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The unusual actinomycetes strain No. E465–94 produced a complex of new glycopeptide antibiotics tallysomycin, which was separated by CM-Sephadex chromatography into two major components, A ($C_{68}H_{107}N_{21}O_{27}S_2$) and B ($C_{62}H_{95}N_{19}O_{26}S_2$). They were isolated first in a copper-chelated form and showed physico-chemical properties similar to those of the bleomycin-group of antibiotics. Tallysomycin exhibited broad antibacterial and antifungal activity, and was highly active *in vivo* against bacterial infections in mice. Tallysomycins A and B demonstrated potent activity in the prophage induction of lysogenic bacteria.

In the course of our search for new antibiotics produced by rare actinomycetes strains, an organism designated as strain No. E465–94 in our culture collection was isolated from an Indian soil sample. It showed *Streptomyces*-like morphology but had an unusual type of cell-wall composition. Shaking or stir-jar fermentation of strain E465–94 produced a new water-soluble basic antibiotic complex named tallysomycin, which was extracted from the broth filtrate by an ion-exchange resin process and separated into two major components, tallysomycins A and B. They were first isolated in copper-chelated form and degradation studies indicated a glycopeptide structure for both components. As reported in a companion paper[1], tallysomycins A and B have the same chromophore as bleomycin but differ from the latter in the amino acid composition and the presence of an additional unique sugar moiety, 4-amino-4,6-dideoxy-L-talose. Tallysomycin inhibited the growth of various bacteria and fungi and showed strong induction activity in lysogenic bacteria. Strain E465–94 also produced, along with tallysomycin components, an aminoglycoside antibiotic complex which was identified as nebramycin factors[5].

This paper reports the production, isolation, physico-chemical and biological properties of tallysomycins A and B. Detailed descriptions of the producing organism[2] and the antitumor activity[3,4] of tallysomycins A and B will be reported separately.

Producing Organism

The actinomycetes strain No. E465–94 produced tufts of spore chains as well as single spore chains in the aerial mycelium. The spores showed smooth surface structure and were oval to short cylindrical in shape. In addition, small sporangium-like bodies were occasionally observed. The spore chain morphology of strain E465–94 is very similar to that reported for some *Streptomyces* species (*e.g. S.*

* This antibiotic was originally designated Bu–2231.

massasporeus, S. ramulosus, S. catenulae and *S. antimycoticus*). However, the cell-wall of strain E465–94 was found to contain *meso*-α,ε-diaminopimelic acid (*meso*-DAP) instead of LL-diaminopimelic acid which has been thought to be a key component in the cell wall of *Streptomyces* and related genera. In addition, the cell wall of strain E465–94 contained galactose, mannose and rhamnose as diagnostic carbohydrate components. Detailed descriptions of the morphological, cultural and physiological characteristics, cell-wall composition and taxonomy will be published elsewhere[2].

Antibiotic Production

A well-grown agar slant of the actinomycetes strain E465–94 was used to inoculate vegetative medium containing 1.5% glucose, 0.2% yeast extract, 0.5% Polypeptone, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.5% $CaCO_3$, the pH being adjusted to 7.2 before sterilization. The seed culture was incubated at 33°C for 48 hours on a rotary shaker (250 rpm), and 5 ml of the growth was transferred to a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium composed of 2% glycerol, 1% Pharmamedia, 1% cornsteep liquor, 0.3% $(NH_4)_2SO_4$, 0.003% $ZnSO_4 \cdot 7H_2O$ and 0.4% $CaCO_3$. Antibiotic activity in the fermentation broth was determined by paper disc agar-diffusion assay using *Bacillus subtilis* PCI 219 and *Mycobacterium smegmatis* strain M6–3* as test organisms. Tallysomycin components and the co-produced nebramycin factors were all active against *B. subtilis* but only tallysomycin components showed activity against the mycobacterial strain M6–3.

A mutant strain No. AC–10570 which was obtained by N-methyl-N′-nitro-N-nitrosoguanidine (NTG) treatment of the actinomycetes strain E465–94 showed much improved productivity over the original strain. A fermentation medium composed of 2.5% sucrose, 0.5% glucose, 3% Pharmamedia, 3% distillers soluble, 0.3% $(NH_4)_2SO_4$, 0.003% $ZnSO_4 \cdot 7H_2O$, 0.01% $CuSO_4 \cdot 5H_2O$ and 0.4% $CaCO_3$ was found suitable for the production of tallysomycin components by the mutant strain yielding nearly a 20-fold increase of the antibiotic potency in shake flask fermentations (900~1,000 mcg/ml).

Fermentation studies were also carried out in tank fermentors. In one of the experimental fermentations, a seed culture was shaken for 3 days in Erlenmeyer flasks and inoculated to 130 liters of the germination medium composed of 1.5% glucose, 0.5% peptone, 0.25% dry yeast, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.3% $CaCO_3$ in a 200-liter seed tank which was stirred at 200 rpm at 28°C for 32 hours. The seed culture was then inoculated to 3,000 liters of the fermentation medium containing 3.0% distillers soluble, 2.5% sucrose, 0.5% glucose, 3.0% fish meal, 0.3% $(NH_4)_2SO_4$, 0.003% $ZnSO_4 \cdot 7H_2O$, 0.4% $CaCO_3$ and 0.01% $CuSO_4 \cdot 5H_2O$. The production tank was operated at 28°C at 190 rpm with an aeration rate of 2,000 liters/min. The broth pH gradually rose with the progress of fermentation and reached 7.5~7.7 after 120~130 hours when a peak antibiotic potency of 300 mcg/ml was obtained.

Isolation and Purification

The harvested broth was filtered with filter aid and the bioactivity in the filtrate (65 liters, pH 7) was adsorbed on a column of Amberlite IRC–50 (NH_4^+ form, 7 liters). The column was washed with water (30 liters) and 0.25 N NH_4OH solution (5 liters) to elute the co-produced nebramycin factors. Tallysomycin complex was then eluted with aqueous 1 N HCl solution. The active fractions were combined, neutralized with aqueous ammonia and adsorbed on activated charcoal (300 g). The charcoal was washed with water (5 liters) and eluted with four 3-liter portions of a 1 : 1 mixture of dil.

* Laboratory-developed aminoglycoside-resistant mutant derived from *Mycobacterium smegmatis* ATCC 607

HCl solution (pH 2) and *n*-butanol. The butanol layer was separated, and the aqueous layer was neutralized with Amberlite IR-45 (OH^- form) and concentrated *in vacuo*. The crude solid thus obtained (14.6 g) was dissolved in aqueous 5% $CuCl_2$ solution (20 ml) and applied to a column of Diaion HP-20 (1 liter) which was then developed with water. The active fractions were combined, concentrated *in vacuo* and lyophilized to give a blue powder of tallysomycin complex (2.3 g) in copper-chelated form.

Tallysomycin complex contained two major components A and B along with two minor components C and D, which were separated by CM-Sephadex C-25 column chromatography with gradient elution by aqueous ammonium formate. The copper-chelating tallysomycin complex (3 g) was dissolved in a small amount of 1% ammonium formate solution and charged on a column of CM-Sephadex C-25 (500 ml) which was pretreated with the same solvent. The column was eluted with aqueous ammonium formate with a concentration gradient from 1 to 7%. The fractions were monitored by bioassay on *Bacillus subtilis* plate and also by the optical density at 290 nm. Component C eluted first followed by component B which was eluted at 3% concentration of ammonium formate solution. Tallysomycin A was eluted next at 5% concentration and component D came out finally with 7% formate solution. The active eluate fractions were passed over a column of high-porous adsorption resin (Diaion HP-20) for desalting. The column was washed with water and then developed with aqueous acidic methanol (adjusted to pH 2 by dil.HCl). Active fractions were neutralized by Amberlite IR-45 (OH^- form), concentrated *in vacuo* and lyophilized to give a blue powder of the hydrochloride of tallysomycin components in a copper-chelated form (Yield: component A 1.3 g, B 0.2 g, C 0.01 g, D 0.05 g). A copper-free preparation was obtained by treating the methanolic solution of the copper-complex of a tallysomycin component with hydrogen sulfide followed by chromatographic purification over a Diaion HP-20 column.

Table 1. Physico-chemical properties of tallysomycins A and B

		Tallysomycin A		Tallysomycin B	
		Cu-chelated	Cu-free	Cu-chelated	Cu-free
Microanalysis	Found C:	42.55	45.30	40.96	43.01
	H:	6.17	6.33	5.61	6.22
	N:	14.81	15.46	14.78	14.81
	S:	3.06	3.77	3.39	3.61
	Cu:	3.02	—	2.98	—
	Calc'd for	$C_{68}H_{107}N_{21}O_{27}S_2$-Cu·4HCOOH ·$3H_2O$	$C_{68}H_{107}N_{21}O_{27}S_2$ ·4HCOOH	$C_{62}H_{95}N_{19}O_{26}S_2$-Cu·3HCOOH ·$5H_2O$	$C_{62}H_{95}N_{19}O_{26}S_2$ ·3HCOOH ·$4H_2O$
	C:	42.88	45.53	41.56	43.45
	H:	6.05	6.10	5.96	6.11
	N:	14.59	15.49	14.17	14.81
	S:	3.18	3.38	3.41	3.57
	Cu:	3.15	—	3.38	—
Optical rotation	$[\alpha]_D^{23}$ (*c* 0.5, H_2O)	+50°	−21°	+76°	−19°
UV spectrum	$\lambda_{max}^{H_2O}$ in nm ($E_{1cm}^{1\%}$)	243 (125)	235 (sh)	243 (143)	235 (sh)
		291 (98)	290 (78)	291 (109)	290 (87)
	Absorbancy ratio at 240 nm/290 nm	1.28	—	1.31	—

Physico-chemical Properties

The copper-chelated form of tallysomycin A or B was obtained as an amorphous light blue powder, while they were white when copper-free. Both forms of tallysomycins A and B did not show any definite melting point but gradually decomposed above 210°C. They are readily soluble in water, methanol and dimethyl formamide, slightly soluble in ethanol and practically insoluble in other organic solvents. Both copper-chelated and copper-free preparations of tallysomycins A and B gave positive reactions

Fig. 1. IR spectrum of tallysomycin A (hydrochloride).

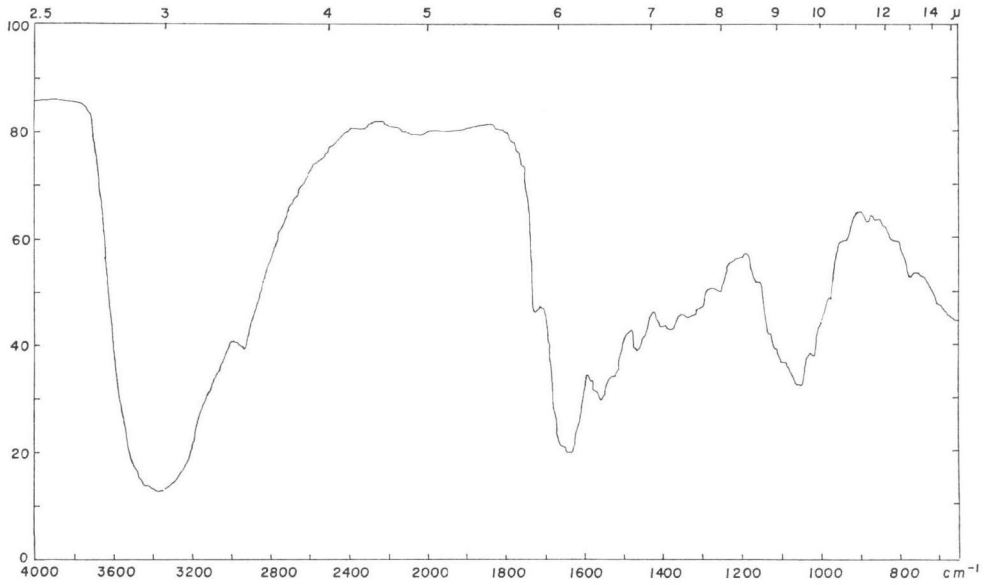
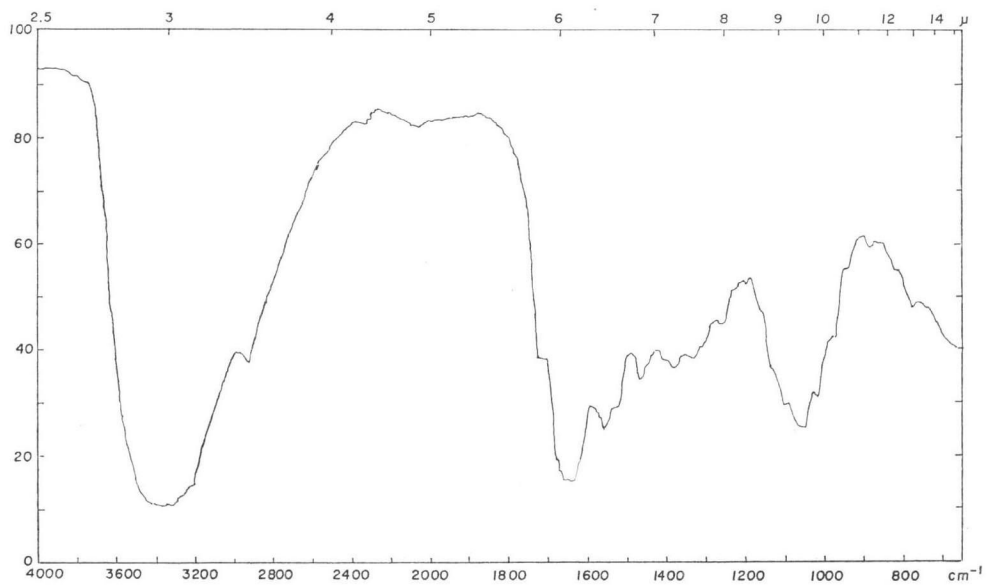


Fig. 2. IR spectrum of tallysomycin B (hydrochloride).



with ninhydrin, anthrone and RIMINI[6] reagents but were negative to FEHLING, TOLLENS and SAKAGUCHI reactions. The copper-chelated form of tallysomycin components yielded a green precipitate with rubeanic acid reagent.[7]

Fig. 3. NMR spectrum of tallysomycin A (60 MHz, in D_2O).

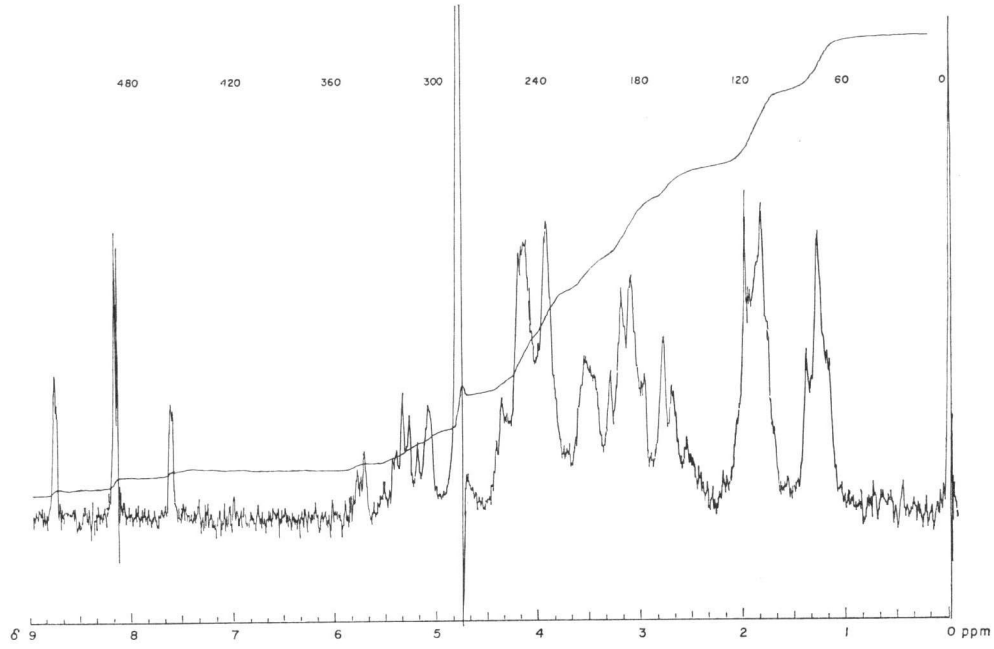


Fig. 4. NMR spectrum of tallysomycin B (60 MHz, in D_2O).

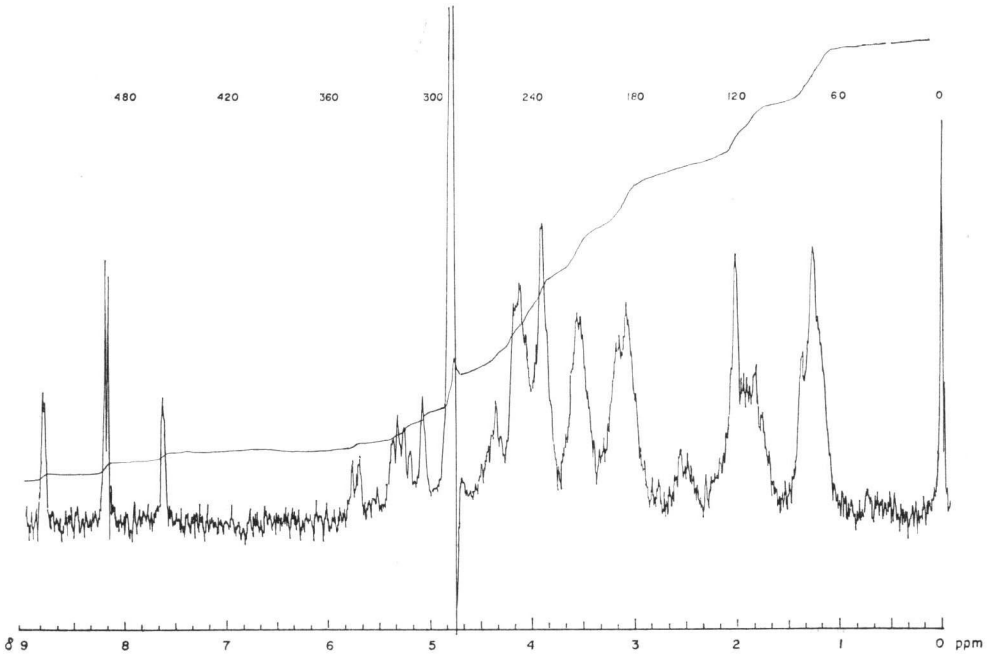


Table 2. TLC comparison of tallysomycin components

	Rf values in silica gel TLC*			
	System S–102**		System S–123	
	Cu-chelated	Cu-free	Cu-chelated	Cu-free
Tallysomycin A	0.22	0.16	0.05	0.04
〃 B	0.41	0.31	0.11	0.09
〃 C	0.58	—	0.49	—
〃 D	0.10	—	0.02	—

* Detection by ninhydrin reagent and UV light.
** S–102 : MeOH - 10% NH_4OAc (1 : 1).
S–123 : MeOH - 10% NH_4OAc - 10% NH_4OH (10 : 9 : 1).

As shown in Table 1, the analytical data for tallysomycins A and B, both analyzed as formates, agreed with the molecular formula of $C_{68}H_{107}N_{21}O_{27}S_2$ and $C_{62}H_{95}N_{19}O_{26}S_2$, respectively, indicating a difference of six-carbons between the two components, which however was firmly established only after the elucidation of the structures.[1] The specific rotation and UV spectral data of tallysomycins A and B are also shown in Table 1. The ratio of absorbancies at 240 nm and 290 nm for components A and B (1.28 and 1.31, respectively) suggested that tallysomycins A and B belong to the bleomycin group of antibiotics rather than the phleomycin group. Figs. 1 and 2 show the IR spectra of the copper-chelated HCl salt of tallysomycins A and B measured in a KBr tablet. Absorptions are observed at 3,400~3,200 cm^{-1} for OH/NH and at 1,720 (carbonyl), 1,650 and 1,550 (amide carbonyl), and in the region of 1,120~1,020 cm^{-1} (hydroxyl). The NMR spectra of the copper-free hydrochloride of tallysomycins A and B (Figs. 3 and 4) indicated four aromatic protons (δ 7.5~8.7 ppm) and seven protons in the ano-

Table 3. Antibacterial activity of tallysomycins A and B

Test organism	MIC (mcg/ml)		
	Tallysomycin A	Tallysomycin B	Bleomycin
Staphylococcus aureus 209P A9497	0.05	0.2	6.3
〃 Smith A9537	0.1	0.4	6.3
〃 D133	0.2	0.4	6.3
〃 #52–34	0.2	0.8	25
〃 A20239	0.2	0.8	25
Sarcina lutea PCI 1001	0.05	0.4	50
Micrococcus flavus D12	0.05	0.1	12.5
Bacillus subtilis PCI 219	<0.003	0.006	0.4
Escherichia coli NIHJ	0.013	0.025	0.2
〃 Juhl	0.05	0.2	0.8
〃 K12, JR35/C600 A20665	0.013	0.025	0.8
Klebsiella pneumoniae D11	0.013	0.05	0.4
〃 Type 22, #3038, A20680	3.1	6.3	>50
Pseudomonas aeruginosa D15	1.6	3.1	>50
〃 A9930	0.1	0.4	>50
Proteus vulgaris A9436	0.4	0.2	12.5
Proteus mirabilis A9554	0.4	0.2	>50
Proteus morganii A9553	0.4	1.6	50
Mycobacterium 607	0.2	0.2	1.6
Mycobacterium phlei	0.1	0.1	0.8

meric proton region (δ 4.8~5.6 ppm).

The copper-chelated preparations of tallysomycins A and B, as well as those of minor components C and D, were clearly differentiated from each other by the TLC systems S–102 and S–123 as shown in Table 2. System S–102 was found useful to separate by TLC the copper-free tallysomycin components from the corresponding copper-chelated forms.

Biological Properties

Tallysomycins A and B showed potent antimicrobial activity against various types of bacteria and fungi. The minimum inhibitory concentrations (MIC) of tallysomycins A and B against gram-positive, gram-negative and acid-fast bacteria were determined in nutrient agar by the two-fold agar dilution method using a STEERS' multi-inoculating apparatus.[8] Bleomycin complex consisting mainly of bleomycin A_2 component was used as a reference in these *in vitro* tests. As shown in Table 3, the antibacterial activity of tallysomycin A was in general 2~4 times greater than that of tallysomycin B, except for acid-fast bacteria which showed the same susceptibility to both components. Tallysomycins A and B were significantly more active than bleomycin and some bacterial strains which were not sensitive to bleomycin, such as *Pseudomonas aeruginosa* and *Proteus morganii*, were inhibited by tallysomycin components. Anaerobic bacteria were not inhibited by tallysomycins A and B (MIC: >100 mcg/ml) when tested by agar dilution method on GAM medium (Nissui, Tokyo).

The antifungal activities of tallysomycins A and B were determined by the two-fold agar dilution method in SABOURAUD agar medium against a variety of yeasts and fungi. The results are shown in Table 4. In contrast to the antibacterial activity, the antifungal activity of tallysomycin B was about 2~4 times greater than that of tallysomycin A. Bleomycin showed rather weak and limited activity against yeasts and fungi.

Table 4. Antifungal activity of tallysomycins A and B

Test organism	MIC (mcg/ml)		
	Tallysomycin A	Tallysomycin B	Bleomycin
Candida albicans IAM 4888	12.5	6.3	>100
〃 D82	0.8	0.8	12.5
Candida krusei IAM 4489	6.3	1.6	6.3
Candida tropicalis IAM 4157	3.1	1.6	50
〃 #125 A15051	6.3	3.1	>100
Cryptococcus neoformans D49	0.8	0.4	3.1
〃 IAM 4514	1.6	0.8	12.5
Saccharomyces cerevisiae ATCC 9763	3.1	0.8	3.1
Aspergillus fumigatus IAM 2530	1.6	0.8	1.6
Aspergillus niger var. Tieghem	1.6	3.1	>100
Blastomyces dermatidis IFO 8144	6.3	3.1	6.3
Blastomyces brasiliensis IFO 8145	3.1	3.1	6.3
Fusarium moniliforme NRRL A2284	3.1	1.6	>100
Penicillium citrinum IAM 7008	1.6	0.8	1.6
Piricularia oryzae D91	1.6	1.6	6.3
Microsporum canis D51	50	6.3	>100
Trichophyton mentagrophytes D155	12.5	3.1	>100
Trichophyton rubrum D55	25	6.3	>100
Trichophyton asteroides #429	12.5	6.3	>100

Table 5. *In vivo* antibacterial activity of tallysomycins A and B

Infective organism	PD_{50} (mg/kg)		
	Tallysomycin A	Tallysomycin B	Bleomycin
S. aureus Smith	0.06	0.12	3.0
E. coli Juhl	0.04	0.15	2.6
P. aeruginosa A9843	5.6	>16	>30

Table 6. Lysogenic induction activity of tallysomycins A and B

Antibiotic	MIC* (mcg/ml)
Tallysomycin A	0.0025
Tallysomycin B	0.02
Bleomycin	0.08

* minimum inducible concentration

The antibacterial activity of tallysomycin was also demonstrated *in vivo* against experimental infections in mice. The pathogenic bacteria used for the *in vivo* test were *S. aureus* Smith, *E. coli* Juhl and *P. aeruginosa* A9843. Mice were challenged with a $100 \times LD_{50}$ dose of the pathogens in a 5% suspension of hog gastric mucin (American Laboratories, Omaha, Neb.) and a single subcutaneous treatment with the antibiotic was made immediately after the bacterial challenge. A group of 5 mice was used for each dosage level and the animals were observed for 5 days to determine the median protective dose (PD_{50}). As shown in Table 5, tallysomycins A and B demonstrated very potent *in vivo* activity against the bacterial infections.

The activity of prophage induction in lysogenic bacterium, *E. coli* W1709 (λ), was determined for tallysomycins A and B according to the method of LEIN *et al.*[17] The plaque count was made on agar plates containing test material (T) and control plate (C). A T/C ratio of the plaque counts of greater than 3 was considered significant and the lysogenic induction activity (ILB activity) was expressed by the minimum inducing concentration of the test compound. As shown in Table 6, the ILB activity of tallysomycin A was about 8 times higher than that of tallysomycin B which was in turn 4 times more potent than bleomycin.

Tallysomycins A and B showed antitumor activity in experimental animal tumor systems including lymphocytic leukemia P-388, sarcoma 180, LEWIS lung carcinoma, WALKER carcinosarcoma 256 and melanoma B-16. The antitumor activity of tallysomycins will be reported in detail separately.[3,4] The acute toxicity of tallysomycins A and B was determined in mice by a single administration by

Table 7. Acute toxicity of tallysomycins A and B

	LD_{50} *(mg/kg/dose)		
	Intraperitoneal		Intravenous
	Single	Multiple**	Single
Tallysomycin A	19	4.4	17
Tallysomycin B	46	6.8	30
Bleomycin	77	18	53

* determined after 30 days' observation period.
** administered once a day for 9 days.

the intraperitoneal (ip) and intravenous (iv) routes. The multiple dose LD_{50} was also obtained by an intraperitoneal *qd* 1 → 9 schedule (given once daily for 9 days). The results are shown in Table 7. In terms of LD_{50} values, tallysomycin A was about twice more toxic than tallysomycin B by a single ip or iv administration but less so in the multiple ip dosing schedule. Bleomycin was significantly less toxic than tallysomycins A and B.

Discussion

The physico-chemical and biological properties of tallysomycins A and B described in the present paper indicated close similarities to those of the phleomycin[9] and bleomycin[10] families of antibiotics which include zorbamycin,[11] zorbonomycins B and C,[11] YA-56 X and Y,[12] victomycin,[13] and platomycins A and B.[14] The ratio of absorbancies at 240 nm and 290 nm for tallysomycins A and B (*ca.* 1.3) suggested the presence of a bleomycin-type chromophore in tallysomycin components and hence indicated a similarity to zorbonomycin B, victomycin and platomycins A and B. Victomycin and platomycins were differentiated from tallysomycins A and B because of the negative SAKAGUCHI reaction of tallysomycin components. Although no direct comparison was made with zorbonomycin B, it appears to be different from tallysomycins A or B from the TLC data reported in the literature [11,15] as summarized in Table 8. A number of bleomycin components[10,16] reported to date are different from tallysomycins A and B in the glycopeptide skeleton as described in a companion paper[1].

Table 8. TLC comparison with related antibiotics

Antibiotics*	Rf values in silica gel TLC**	
	System S–102	System S–123
Tallysomycin A	0.22	0.05
Tallysomycin B	0.41	0.11
Platomycin A	0.77 (0.73)***	0.61 (0.62)
Platomycin B	0.64 (0.60)	0.45 (0.47)
Victomycin	(0.78)	(0.40)
Zorbonomycin B	(0.76)	(0.84)
Bleomycin A_2	0.38	0.42
Bleomycin A_5	0.47	0.18

* copper-chelated form.
** for TLC system, see footnote of Table 2.
*** Rf values in parentheses are cited from ref. 15.

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