YS-822A, A NEW POLYENE MACROLIDE ANTIBIOTIC

I. PRODUCTION, ISOLATION, CHARACTERIZATION AND BIOLOGICAL PROPERTIES

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A new polyene macrolide antibiotic, YS-822A was isolated from the culture filtrate of a mutant strain H-8 of *Streptoverticillium eurocidicum* var. *asterocidicus* S-822. Whereas the original S-822 strain produced not only YS-822 substances but also teleocidin as by-product which is well-known as a strong carcinogenic promotor, the mutagenized H-8 strain produced the antibiotic with only a trace amount of teleocidin. Chemical and biological characterizations of the antibiotic revealed that YS-822A (molecular formula: $C_{37}H_{59}NO_{14}$) is a new polyene macrolide with a wide antifungal spectrum and a low acute toxicity.

In the course of screening for new antifungal antibiotics, *Streptoverticillium eurocidicum* var. *asterocidicus* S-822[1] was found to produce a few antifungal antibiotics. One of them named YS-822A (Fig. 1) was isolated from culture filtrate of a mutant strain H-8 and characterized as a new tetraene macrolide antibiotic from its physico-chemical properties. To reduce the production of teleocidin[2], the S-822 strain was mutagenized by UV irradiation, nitrosoguanidine treatment, and regeneration of protoplast.

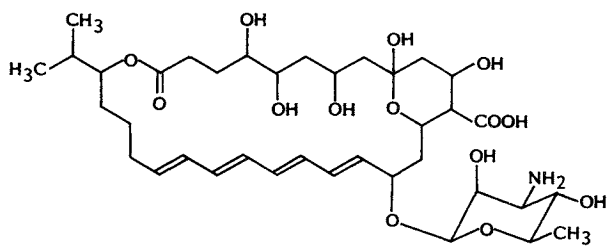
This paper deals with the isolation of the producing strain, fermentation, purification and physico-chemical and biological properties of YS-822A.

Materials and Methods

Bacterial Strain

S. eurocidicum var. *asterocidicus* S-822[1] was used as a parental strain.

Fig. 1. Structure of YS-822A.



Mutation

Spore suspension was prepared by the usual method and mutagenized by UV irradiation, nitrosoguanidine treatment and/or protoplast regeneration[3,4].

Production Media and Culture

A seed culture was maintained with Tryptone-yeast extract modified medium (Tryptone 1.0%, yeast extract 0.4%, NaCl 0.1%, pH 7.4) at 30°C for 2 days by shaking. The seed culture was transferred at the rate of 1% in a production medium (glucose 3.0%, soybean meal 2.0%, Polypeptone 0.5%, NaCl 0.5%, $CaCO_3$ 0.2%, pH 7.0) and cultivation was carried out for 5 days at 30°C.

Assay

After completing the fermentation, 10 ml of the culture broth was centrifuged at 3,000 rpm for 10 minutes. Supernatant was assayed for YS-822A content. Mycelium was extracted with 10 ml of methanol at room temperature for 30 minutes. After centrifugation, supernatant was assayed for teleocidin content.

1) Antifungal Activity: The test strain, *Saccharomyces cerevisiae* association No. 6 which cultivated with YM liquid medium at 27°C for 24 hours was inoculated at the rate of 0.2% in the 10 ml of YM agar medium. The activity of YS-822A was assayed by pulp-disk agar diffusion method.

2) Teleocidin Content Assay[5]: The extract was diluted at 2-fold with methanol and the diluted sample of 0.3 ml was added to 30 ml of dechlolinated tap water containing 3 fishes (*Oryzias latipes*; Japanese killi-fish, Himedaka) in 55 mm petri dish. Teleocidin content was calculated from maximum dilution rate by observation of life or death within 2 hours. Minimum lethal dose of teleocidin against *O. latipes* was 0.01 μg/ml.

Chemicals

The reagents employed as control, nystatin and amphotericin B were purchased from Sigma Chemical Company. Amphotericin B was used Fungizon from Squibb Japan Inc. as control for the acute toxicity. Chemicals used for isolation and purification are as follows; silica gel (70~230 mesh), TLC-plate silica gel 60 F_{254} and TLC-plate cellulose F from E. Merck, Darmdstadt, FRG and packed column of ODS from Nihon Waters Ltd.

MIC Assay

In vitro antifungal activity of the antibiotics as MICs was measured by an agar dilution method employing Sabouraud-Dextrose (SD) medium according to the method of Japan Society of Chemotherapy[6]. A well grown colony of yeast on a SD agar slant was inoculated one loop in a SD broth and cultivated at 27°C for 48 hours. A completed culture broth was diluted 100 times with the same broth. Spores harvested from fungi on each SD agar slant at 27°C for 14 days was suspended with 5 ml of saline containing 0.01% Tween 80. Each spore suspension was filtered with gauze and diluted 10 times with SD broth. Nystatin and amphotericin B were used as reference reagents.

Acute Toxicity

For solubilization in water, YS-822A was mixed with sodium deoxycholate at a rate of 1:0.3 and lyophilized. Female ICR mice (5 weeks old; weighing 27.8 ± 1.11 g) were purchased from the Shizuoka Laboratory Animal Center, Japan. Groups consisted of five mice were subjected iv injection of 0.2 ml of sterilized distilled water with different doses of YS-822A or amphotericin B. After 2 weeks of observation period, the value of LD_{50} was calculated.

Results

Isolation of Producing Strains

Mutant strains were prepared by UV irradiation of the parental strain S-822, followed by treatment of nitrosoguanidine and regeneration of protoplast as shown in Fig.2. Contents of YS-822A and teleocidin

Fig. 2. Mutation schedule of *Streptoverticillium eurocidicum* var. *asterocidicus*.

UV: UV irradiation, PR: protoplast regeneration, NTG: nitrosoguanidine treatment.

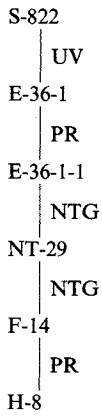


Table 1. Productivity of YS-822A and teleocidin by various mutant strains.

Strains	Aerial mycelium formation	YS-822A[a] (μg/ml)	Teleocidin[b] (μg/ml)
S-822	±	330	500
E-36-1	+	115	500
E-36-1-1	+	125	100
NT-29	+	460	50
F-14	+	295	10
H-8	−	175	<2

[a] Potency against *Saccharomyces cerevisiae* as a test organism.

[b] Teleocidin content assay: See Materials and Methods.

produced from mutant strains are summarized in Table 1. The original strain S-822 contains 500 μg/ml of teleocidin in a mycelium. Its content could not be reduced by UV irradiation, but be reduced to less than 2 μg/ml by nitrosoguanidine treatment. Extract from mycelium was contained YS-822A and teleocidin. YS-822A was toxic to fish at the concentration of 2 μg/ml and methanol in extract was also toxic more than 2% of final concentration. Because of above mentioned, teleocidin content less than 2 μg/ml in mycelium extract could not be detected by this assay method. YS-822A production was reduced on UV irradiated strain (E-36-1) and protoplast regenerated strain (E-36-1-1), but was restored by post-treatment by nitrosoguanidine to those strains.

Fermentation

S. eurocidicum var. *asterocidicus* H-8 was precultured in 1-liter of Erlenmeyer flask containing 300 ml of a seed medium at 30°C for 2 days on a reciprocal shaker. Precultured H-8 strain was transferred into fermenter (Model, MSJ-N2, B. E. Marubishi) of 50-liter capacity with 20 liters of the production medium. The fermentation was carried out at 30°C under agitation at 150 rpm and air flow of 30 liters/minute. YS-822A production was monitored by antifungal assay using agar diffusion method, and cell growth was evaluated as packed cell volume by centrifuging the fermentation broth in the graduated tube at 3,000 rpm for 10 minutes. A typical time course for the production of the antibiotic is shown in Fig. 3. Maximum antibiotic production was reached after 96 hours of fermentation. At that time YS-822A production was about 450 μg/ml and the pH was 5.5.

Isolation and Purification

The procedure for isolation and purification of YS-822A and B is summarized in Fig. 4. After 96 hours fermentation, the culture broth (50 liters) was filtered with an aid of Celite (Hyflo Super-Cel). After filtration followed by removal of oily substance with 20 liters of ethyl acetate, the aqueous layer was extracted with 30 liters of butanol. After washing with water, the butanol layer was concentrated *in vacuo* and the residue was lyophilized to yield 16 g of crude substance containing YS-822A and B. The crude substance was dissolved in a small amount of methanol and applied to silica gel column (500 g) packed with chloroform-methanol (10:1). The column was eluted with chloroform-methanol-water (6:5:1) after washing with chloroform-methanol (10:1). The eluate was monitored by TLC on silica gel

Fig. 3. Time course of YS-822A production.

● YS-822A, ○ packed cell volume, △ pH.

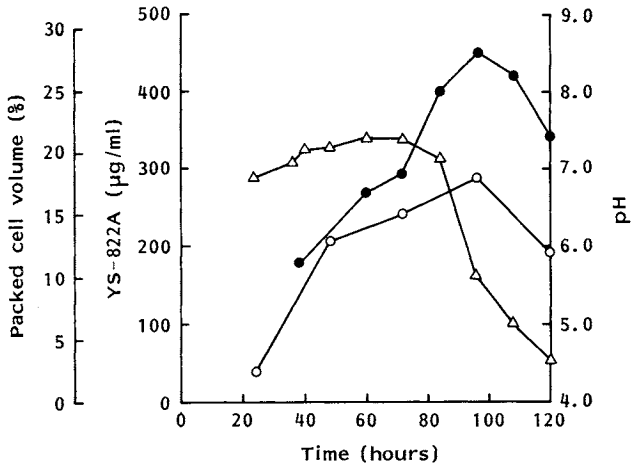
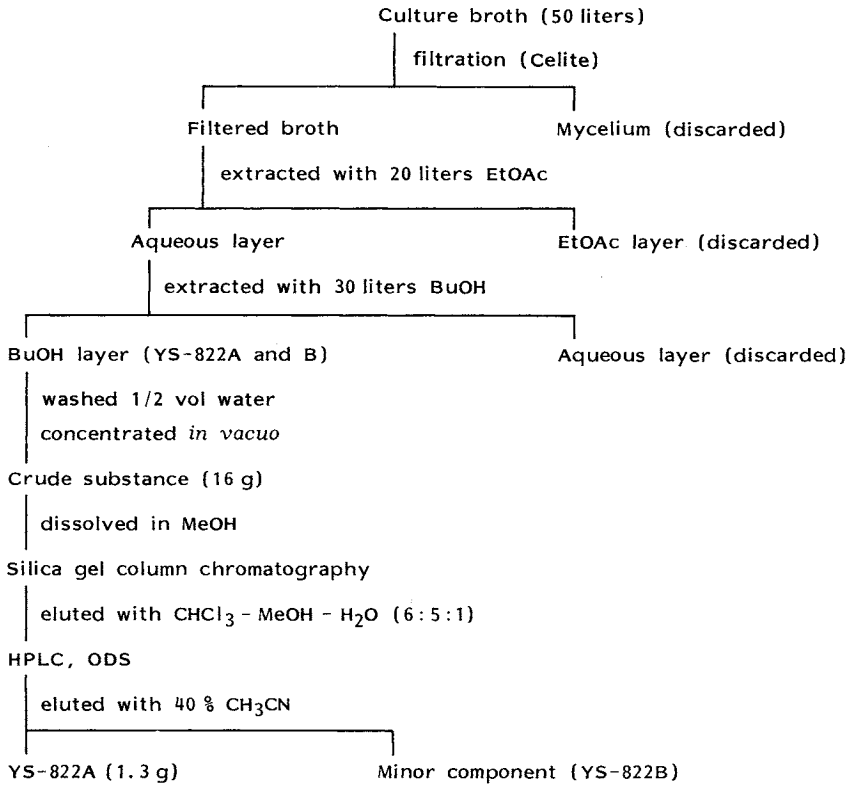


Fig. 4. Isolation and purification of YS-822A.



(chloroform-methanol-water, 6:5:1) and antifungal activity against *S. cerevisiae*. The active fractions were collected and concentrated *in vacuo* to yield a light brown residue (10 g). For the separation of YS-822A and B, the residue was further chromatographed on HPLC (RP-18, 57 mm × 30 cm) using 40% CH_3CN as eluant. Fractions containing YS-822A were collected and evaporated to remove solvent, then

lyophilized to yield light yellow powder (1.3 g). YS-822B was estimated as a tetraene macrolide antibiotic from characteristic UV spectrum, but its structure could not be identified because of deficiency of the sample amount.

Physico-chemical Properties

YS-822A is soluble in dimethyl sulfoxide and pyridine, and moderately soluble in methanol, ethanol and water, but practically insoluble in benzene, ethyl acetate, chloroform and acetone. It shows positive responses to Molisch, ninhydrin, $KMnO_4$ and bromine tests, and negative responses to Fehling and orcinol tests. The molecular formula of YS-822A was determined to be $C_{37}H_{59}NO_{14}$ by elementary analysis C 53.26, H 7.38, N 2.02, FAB-MS showing peak at m/z 742 $(M+H)^+$ and ^1H, ^{13}C NMR

Fig. 5. UV spectra in MeOH.
Nystatin (---), YS-822A (——).

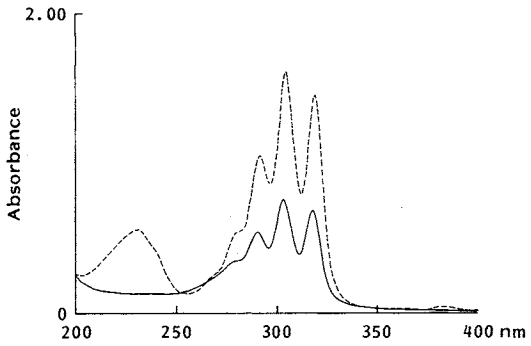


Table 2. TLC behavior of YS-822A and nystatin.

Solvent system	Rf value	
	YS-822A	Nystatin
$CHCl_3$ - MeOH - 0.05 M borate buffer pH 8.3 (2:2:1) lower layer	0.23	0.15
BuOH - AcOH - H_2O (4:1:5) upper layer	0.43	0.38

Kieselgel 60 F_{254}, E. Merck.
Detection: I_2 and UV.

Table 3. TLC behavior of aminosugar obtained by acid hydrolysis of YS-822A and nystatin.

Solvent system	Rf value	
	Aminosugar of	
	YS-822A	Nystatin
BuOH - AcOH - H_2O (4:1:5) upper layer	0.18	0.18
Pyridine - EtOAc - AcOH - H_2O (8:8:1:3)	0.53	0.53

Cellulose F, E. Merck.
Detection: Ninhydrin.

Fig. 6. IR spectrum of YS-822A.

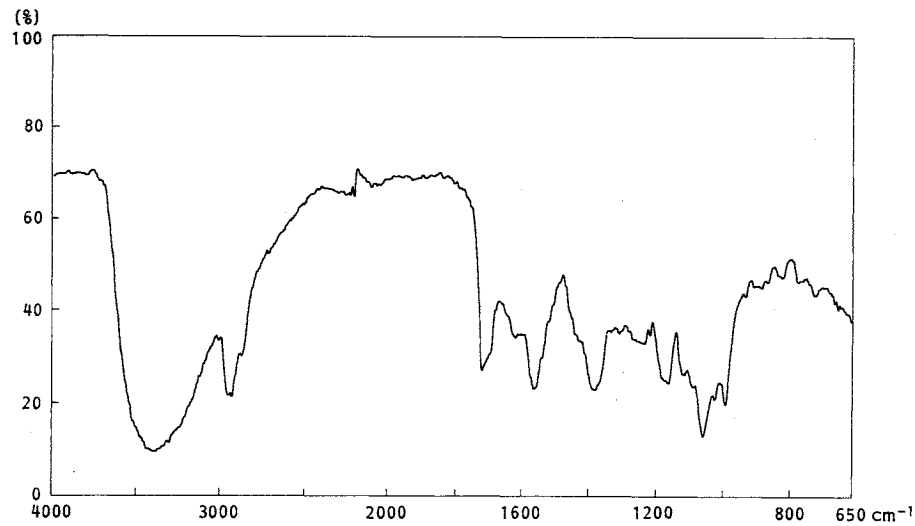


Fig. 7. Stability of YS-822A in methanolic solutions.

(A) At various pH values stored in the dark at room temperature. ○ pH 7, △ pH 4, □ pH 10.
(B) In contact with UV at pH 7.0, stored at room temperature.

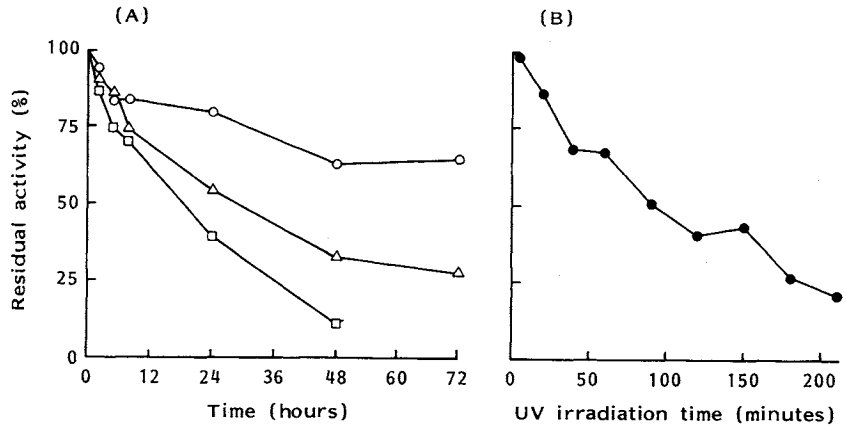


Table 4. Physico-chemical properties of tetraene antifungal antibiotics.

Name	MW	Formula	$[\alpha]_D$	
Nystatin A_1	926.1	$C_{47}H_{75}NO_{17}$	+21°	(Pyridine)
Rimocidin	767.93	$C_{39}H_{61}NO_{14}$	+75.2°	(MeOH)
Tetrin A	681.8	$C_{34}H_{51}NO_{13}$	+8.3°	(Pyridine)
Pimaricin	665.7	$C_{33}H_{47}NO_{13}$	+180°	(DMSO)
Lucensomycin	707.7	$C_{36}H_{53}NO_{13}$	+296°	(Pyridine)
PA-166	712	$C_{35}H_{53}NO_{14}$	+275°	(Pyridine)
Tetramycin	699	$C_{34}H_{53}NO_{14}$	+9.5°	(Pyridine)
YS-822A	741	$C_{37}H_{59}NO_{14}$	+21.3°	(Pyridine)

spectra. It decomposed at 265°C, and the specific rotation was $[\alpha]_D^{23}$ +21.3° (*c* 0.1, pyridine). The UV spectrum in methanol shows a characteristic polyene spectrum very similar to that of nystatin (Fig. 5) with maxima at 290 ($E_{1\,cm}^{1\%}$ 370), 303 ($E_{1\,cm}^{1\%}$ 538) and 318 nm ($E_{1\,cm}^{1\%}$ 487) corresponding to a tetraene antibiotic. The IR spectrum measured in a potassium bromide tablet is shown in Fig. 6, showing the characteristic absorptions of polyene macrolide antibiotics, *e.g.* several hydroxyl units at 3400 and 1060 cm^{-1}, a lactone unit at 1710 cm^{-1}, and an ionized carboxyl unit at 1570 cm^{-1}.

Rf value of YS-822A differs from that of nystatin by TLC on silica gel (Table 2). YS-822A has an aminosugar moiety which is identical to that of nystatin. It was determined to be mycosamine by TLC, compared with the aminosugar of nystatin after acid hydrolysis of these antibiotics by usual method[7]. TLC was visualized by spraying the plate with 0.2% ninhydrin followed by heating at 100°C. The result of Rf value comparison of the aminosugar of YS-822A with that of nystatin is summarized in Table 3.

YS-822A in methanol is stable under neutral condition at room temperature but unstable to UV light (Fig. 7).

From the comparison of these physico-chemical properties with the published tetraene antibiotics (Table 4), YS-822A was determined as a new tetraene macrolide antibiotic.

Biological Properties

Antifungal activities of YS-822A are shown in Table 5. YS-822A shows no activity against

Table 5. Antifungal activity of YS-822A and other polyene macrolides.

Strains	MIC (μg/ml)		
	YS-822A	AMPH-B	NYS
Saccharomyces cerevisiae No. 6	3.12	0.78	0.78
Candida lypolytica ATCC 8862	12.5	0.78	3.12
C. pseudotropicalis IMC-F2	3.12	0.78	0.78
C. albicans IFO 1594	6.25	0.2	0.78
C. albicans IFO 1385	6.25	0.2	0.78
C. tropicalis IFO 0006	12.5	3.12	3.12
C. tropicalis TIMM 0315	12.5	1.56	3.12
Rhodotorula minuta	6.25	12.5	6.25
Hansenula anomala IFO 0138	6.25	0.2	0.78
Aspergillus niger	3.12	3.12	6.25
Fusarium roseum IAM 5010	6.25	3.12	3.12
Gibberella fujikuroi IFO 6349	25	100	25
Alternaria kikuchiana F2	3.12	1.56	1.56
Sporothrix schenckii IFO 8158	50	50	25
Trichophyton mentagrophytes IFO 5811	50	3.12	6.25
T. rubrum IFO 5808	50	1.56	1.56
Epidermophyton floccosum IFO 9045	1.56	0.2	0.39

AMPH-B: Amphotericin B, NYS: nystatin.

Gram-positive or Gram-negative bacteria and anaerobic bacteria at 100 μg/ml over all of MIC (data not shown). MIC value of YS-822A shows as follows, 3.12~12.5 μg/ml against yeast which means YS-822A shows lower activity than amphotericin B or nystatin, 50 μg/ml against dermatopathogenic fungi such as *Sporothrix, Trichophyton* and 3.12~25 μg/ml against phytopathogenic fungi such as *Fusarium, Gibberella, Alternaria* similar to that of amphotericin B or nystatin.

The LD_{50} value of YS-822A when administered intravenously to mice is 119.7 mg/kg, when that of amphotericin B is 6.1 mg/kg.

Discussion

YS-822A is a new antifungal tetraene macrolide antibiotic isolated from the culture filtrate of *S. eurocidicum* var. *asterocidicus*. Because of the production of teleocidin in this mycelium, this strain was treated by mutagenic agents and could obtain a teleocidin-free mutant H-8 strain. YS-822A has a tetraene macrolide aglycon and mycosamine from the UV, IR and NMR spectra. There are nystatin, rimocidin, tetrin A, pimaricin, lucensomycin, PA-166 and tetramycin as antibiotics[8~14] with the resemble functional groups[15]. YS-822A is not identical with any of these antibiotics by the comparison with those MW's, molecular formulas and optical rotations. The planar structure elucidation of YS-822A will be published elsewhere. YS-822A shows *in vitro* antifungal activity but somewhat lower than that of amphotericin B or nystatin. An acute toxicity of YS-822A against mice (iv) is weaker than amphotericin B. These results might be expected the therapeutic effect against systemic mycosis.

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