H 646-SY3 SUBSTANCE, A POTENTIATOR FOR POLYENE ANTIFUNGAL ANTIBIOTIC

Sir:

We have published a new method[1] to screen anticholesterol substances produced by microbes on the basis of the antagonism[2] between polyene antifungal antibiotics and cholesterol against yeasts. Polyene antibiotics, non-polyene antibiotics, synergists for polyene antibiotics and antagonists for cholesterol can easily be differentiated from each other by the different patterns of antimicrobial zones[1]. The first *Streptomyces* cholesterol oxidase[1,3] as an antagonist for cholesterol, and H 537-SY2 substance[4] as a non-polyene antibiotic were isolated by applying the above screening method to broth cultures of *Streptomyces*.

Streptomyces H 646-SY3, isolated from a soil sample collected at Tojo-Cho, Hiroshima Prefecture and classified as belonging to *Streptomyces roseoviridis*[5], was shown to produce an antagonist for cholesterol by the above screening method. *Str.* H 646-SY3 was cultured to prepare an inoculum seed in shaking flasks containing 100 ml of an inoculation medium composed of 1.0% maltose and 0.4% yeast extract (pH 7.0) incubated at 27°C for 20 hours on a reciprocal shaker (amplitude 7 cm, 130 strokes per minute). The inoculation seed was used to inoculate shaking flasks each containing 100 ml of a production medium composed of 1.5% soluble starch, 1.0% glucose, 2.0% soyameal, 0.5% dried yeast (Ebios, Tanabe Pharmaceutical Co., Ltd.), 0.25% NaCl, 0.3% $CaCO_3$, 0.0008% $MnCl_2 \cdot 4H_2O$, 0.0007% $CuSO_4 \cdot 5H_2O$, 0.0002% $ZnSO_4 \cdot 7H_2O$ and 0.0001% $FeSO_4 \cdot 7H_2O$ (pH 7.6 before sterilization). The culture was grown at 27°C for 65 hours on the shaker as above.

Chart 1. Procedures of extraction and purification of H 646-SY3 substance

Fermentation broth

- Broth filtrate (8,000 ml, pH 6.0) — Mycelial cake
  - treated with activated carbon (24 g) for 1 hour and filtered
- Carbon cake — Filtrate
  - eluted with each 1,600 ml of acetone - H_2O - 0.5 N NH_4OH (80:20:1) for 3 times, evaporated *in vacuo* and lyophilized
- Crude substance (4.532 g; total activity, 40.4%)
  - dissolved in H_2O (20 ml) and extracted with each 10 ml of *n*-BuOH for 3 times
- *n*-BuOH layer (36 ml; total activity, 38.8%) — Aqueous layer
  - evaporated to dryness *in vacuo*
- Silica gel column (50×1.5 cm; Kieselgel 60, Merck)
  - eluted with *n*-BuOH - EtOH - H_2O (8:5:2) and collected in 15 ml fractions each
- Fractions Nos. 6~20 — Fractions Nos. 21~100
  - evaporated to dryness *in vacuo* — evaporated to dryness *in vacuo*
- Partially purified syrup (1,212 mg; total activity, 13.7 %) — Partially purified syrup (155 mg; total activity, 13.0%)
- Partially purified syrup from fractions Nos. 21~100 (72 mg)
  - dissolved in H_2O (13 ml)
- SP-Sephadex C-25 column (35×1.5 cm; H^+-type)
  - eluted with a linear gradient concentration of H_2O (200 ml)-1 M NaCl (200 ml) and collected in 10 ml fractions each
- Fractions No. 13~24
  - lyophilized and extracted with $CHCl_3$ (25 ml)
- $CHCl_3$ extract — Residual solid
  - evaporated *in vacuo*
- Purified colorless syrup (34 mg; total activity 98%)

The antagonistic activity for cholesterol was determined by the cylinder agar plate method[1] on glucose-nutrient agar using *Candida albicans* Yu 1200 as a test microbe and seed agar (5 ml) containing 0.075 ml of trichomycin solution (1 mg/ml in EtOH) and 0.025 ml of cholesterol solution (6 mg/ml in EtOH) was placed on basal agar (10 ml).

The active component occurred mainly in the culture filtrate and was extracted with *n*-BuOH at pH 8 or adsorbed on active carbon and eluted with aqueous acetone. Procedures of extraction and purification of the active substance, tentatively designated as H646-SY3 substance, are summarized in Chart 1.

The purified colorless syrup was recovered as a hydrochloride. The elemental microanalysis gave: C, 50.81%; H, 8.36%; N, 5.83% and Cl, 6.25%. $[\alpha]_D^{20} \fallingdotseq 0°$ (*c* 4.85 mg/ml, MeOH). Ultraviolet and infrared absorption spectra of H 646-SY3 substance are shown in Figs. 1 and 2. The substance was shown to be homogeneous on silica gel G thin-layer plates developed with several kinds of solvent systems. The Rf values were as follows: 0.42, *n*-BuOH-MeOH-H_2O (8 : 4 : 1); 0.37, H_2O-saturated *n*-BuOH; 0.27, H_2O-saturated *n*-BuOH-AcOEt (1 : 1); and 0.87, acetone-H_2O (4 : 1). The substance was detected by bioautography or by heating at 100°C after spraying with 40% H_2SO_4. The substance gives a positive ninhydrin reaction and decolorizes 1% $KMnO_4$ solution, while anthrone-H_2SO_4 and α-naphthol-phosphate reactions are negative. The substance is soluble in H_2O, MeOH, EtOH, *n*-BuOH or acetone, sparingly soluble in ethyl acetate and insoluble in *n*-hexane or benzene. The substance is rather labile in an alkaline solution and 85% of the activity is lost when the aqueous solution (pH 8.0) is heated at 100°C for 10 minutes, while more than 70% of the activity remains at pH 6~7 under the same conditions.

Fig. 1. Ultraviolet absorption spectra of H 646-SY3 substance

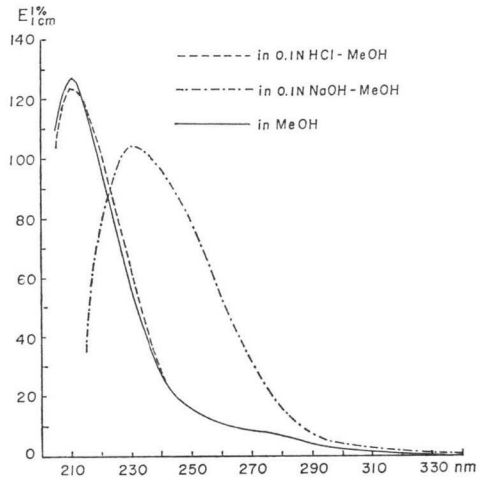

Fig. 2. Infrared absorption spectrum of H 646-SY3 substance in $CHCl_3$

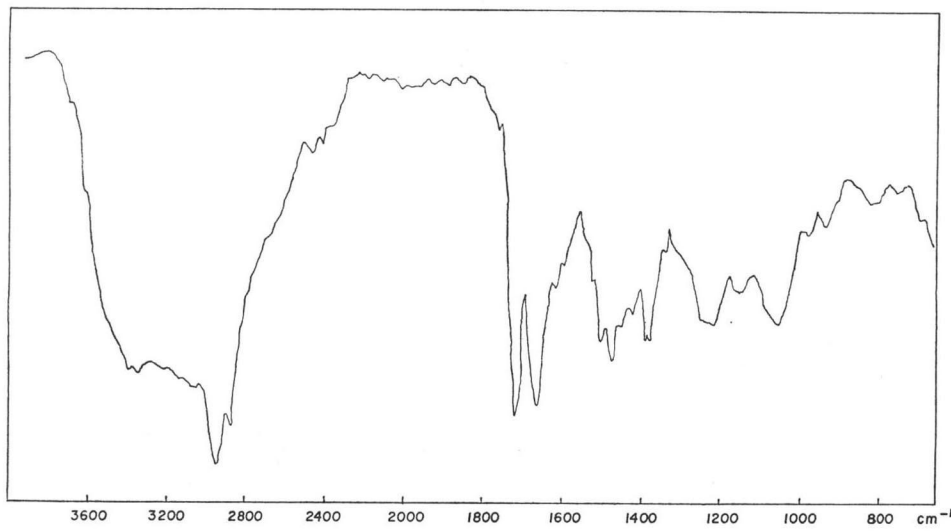

Table 1. Antimicrobial spectra of H 646-SY3 substance and trichomycin in the presence or absence of cholesterol

Test organisms	Minimum inhibitory concentration (mcg/ml)						
	I	II	III	IV	V	VI	VII
Staphylococcus aureus FDA 209P	>100						
Sarcina lutea PCI 1001	>100						
Micrococcus flavus FDA 16	>100						
Bacillus subtilis PCI 219	>100						
Mycobacterium smegmatis ATCC 607	>100						
Corynebacterium bovis 1810	>100						
Escherichia coli NIHJ	>100						
Salmonella typhi T-63	>100						
Shigella sonnei 191-66	>100						
Klebsiella pneumoniae PCI 602	>100						
Candida tropicalis NI 7495	100	0.625	10	0.156	0.039	2.5	1.25
Candida pseudotropicalis NI 7494	25	0.078	0.625	0.0095	0.0048	0.019	0.019
Candida albicans 3147	50	1.25	>10	0.156	0.156	5	0.625
Candida albicans Yu 1200	50	2.5	>10	0.156	0.156	5	2.5
Candida krusei NI 7492	>100	0.312	0.625	0.019	0.019	0.312	0.156
Saccharomyces cerevisiae	25	0.078	0.039	0.0012	<0.0006	0.019	0.0023

Minimum inhibitory concentrations were determined on glucose-nutrient agar at 37°C.
I H 646-SY3 substance
II Trichomycin
III Trichomycin+cholesterol (4 mcg/ml)
IV Trichomycin+H 646-SY3 substance (2.5 mcg/ml)
V Trichomycin+H 646-SY3 substance (5 mcg/ml)
VI Trichomycin+H 646-SY3 substance (2.5 mcg/ml)+cholesterol (4 mcg/ml)
VII Trichomycin+H 646-SY3 substance (5 mcg/ml)+cholesterol (4 mcg/ml)

H 646-SY3 substance shows very weak antiyeast activity, while the substance potentiates the antiyeast activity of trichomycin in the presence or absence of cholesterol as shown in Table 1. Intraperitoneal administration of H 646-SY3 substance (4 mg/mouse) shows no toxic effects.

Further chemical and biological studies on H 646-SY3 substance will be reported according to progress.

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