NEO-ENACTIN, A NEW ANTIFUNGAL ANTIBIOTIC POTENTIATING POLYENE ANTIFUNGAL ANTIBIOTICS. I

FERMENTATION, EXTRACTION, PURIFICATION AND PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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A new antifungal antibiotic, named neo-enactin, was isolated from the cultured mycelia of Strain H 829–MY 10, identified as a new subspecies of *Streptoverticillium* and named *Streptoverticillium olivoreticuli* subsp. *neoenacticus*. The antibiotic was produced with a tetraene antifungal antibiotic, found mainly in the cultured mycelia of Strain H 829–MY 10 and it was extracted with methanol. The antibiotic is of a basic nature and it can be extracted with ethyl acetate at alkaline pH. Purification of neo-enactin was carried out by partition chromatography on cellulose and elution with ethyl acetate bufferized with phosphate buffer (pH 8.0). Neo-enactin shows strong antifungal activity and potentiates the antifungal activity of polyene antifungal antibiotics.

H 646–SY 3 substance was previously isolated from the cultured broth of *Streptomyces roseoviridis* and observed to be a potentiator for activity of polyene antifungal antibiotics by our screening in our efforts to obtain anticholesterol substances produced by microbes[2,3]. Strain H 829–MY 10, designated as *Streptoverticillium olivoreticuli* subsp. *neoenacticus*[1], was also shown to produce a potentiator for polyene antifungal antibiotics. Although physico-chemical and biological properties of this product resembled those of H 646–SY 3 substance, the former could be extracted with ethyl acetate at pH 8.0, but the latter could not be extracted with the same solvent. Also, much higher biological activity and different chromatographic characteristics differentiated the new product from H 646–SY 3 substance. H 646–SY 3 substance was named enactin and the new antibiotic obtained from Strain H 829–MY 10 was named neo-enactin[1].

Fermentation, extraction, purification and physico-chemical and biological properties of neo-enactin are described in this paper.

Fermentation

Strain H 829–MY 10 was cultured to prepare a seed inoculum in shaker flasks, each containing 100 ml of an inoculation medium composed of 1.0% maltose, 0.2% yeast extract and 0.2% Polypeptone (pH 7.0) at 27°C for 24 hours on a reciprocal shaker (amplitude 7 cm, 130 strokes per minute). The inoculum (2 ml) was used to inoculate shaker flasks each containing 100 ml of a production medium composed of 1.5% soluble starch, 1.0% glucose, 2.0% soy bean meal, 0.5% Ebios (dried yeast, distributed by Tanabe Pharmaceutical Co., Ltd.), 0.25% NaCl and 0.3% $CaCO_3$ (pH 7.6 before sterilization). The culture was grown at 27°C for 36 hours on the same shaker.

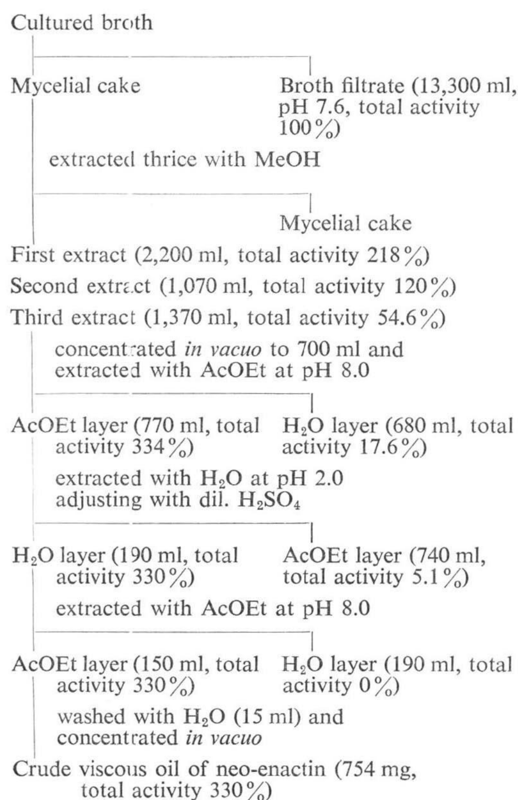

Chart 1. Extraction of neo-enactin

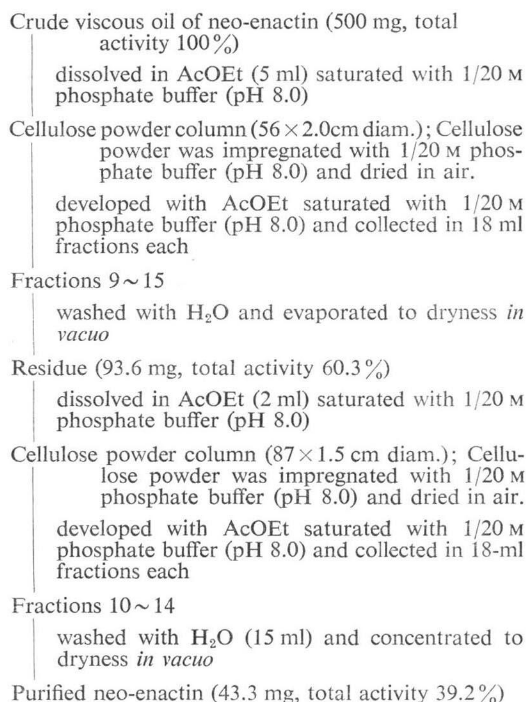

Chart 2. Purification of neo-enactin

Extraction and Purification

The antibiotic was recovered mainly from the cultured mycelia by extraction with methanol. The methanol extract was concentrated *in vacuo* to a small volume and the antibiotic was extracted with ethyl acetate from the concentrate at pH 8.0. The antibiotic in the solvent layer was transferred to an aqueous layer at pH 2.0 and again extracted with ethyl acetate at pH 8.0 as summarized in Chart 1. Purification of the antibiotic was carried out by the cellulose powder partition chromatography as shown in Chart 2. The activity of neo-enactin during fermentation, extraction and purification was determined by the cylinder agar plate method using *Candida albicans* Yu 1200 as a test microbe with the seed agar containing trichomycin and cholesterol as previously described[2,3].

Physico-chemical Properties

The purified antibiotic was obtained as a white amorphous powder, melting point 60.5~64.5°C. $[\alpha]_D^{12}$ −14.2° (*c* 3, MeOH). The elemental microanalysis gave C, 63.47; H, 10.04 and N, 7.44%, but no halogen or sulfur was observed. Neo-enactin shows the ultraviolet absorption maxima at 208 nm ($E_{1cm}^{1\%}$ 166) in MeOH, at 208 nm ($E_{1cm}^{1\%}$ 161) in 0.1 N HCl–MeOH or at 240 nm ($E_{1cm}^{1\%}$ 138) in 0.1 N NaOH–MeOH as shown in Fig. 1. The infrared absorption spectrum of neo-enactin in KBr is shown in Fig. 2. The antibiotic was shown to be homogeneous. It produced a single spot on silica gel G thin-layer plates developed with several kinds of solvent systems and assayed by bioautography, by

ninhydrin reaction or by heating at 100°C for 15 minutes after spraying 40% H_2SO_4. As indicated in Tables 1 and 2, neo-enactin can be differentiated from enactin by paper or silica gel thin-layer chromatograms developed with several kinds of solvent systems.

Neo-enactin is soluble in lower alcohols, ethyl acetate, ether or chloroform, but insoluble in H_2O, *n*-hexane or petroleum ether. Neo-enactin gives positive ninhydrin and naphthoresorcinol-phosphate (red) reactions and decolorizes 1% aqueous $KMnO_4$ solution, but it does not interact with anthrone-phosphate or α-naphthol-phosphate. Neo-enactin is somewhat labile in an alkaline solution and 80% of the activity is lost when it is kept at 100°C for 5 minutes at pH 9.0, whereas 80~90% of the activity remains when it is kept at pH 2~6 under the same time and temperature conditions. Neo-enactin (49.6 mg) was hydrolyzed with 1 N HCl (4 ml) at 110°C for 17 hours. The hydrolyzate was extracted with ethyl acetate, then the aqueous layer was evaporated to dryness, dissolved in H_2O (2 ml) and passed through a column of Amberlite IR 45 (9.5 cm×0.6 cm diam.). The eluate and washing were combined and then concentrated to a small volume *in vacuo*. An amino acid (10.7 mg) was precipitated from the concentrate by the addition of ethanol. The amino acid was identified as

Fig. 1. Ultraviolet absorption spectra of neo-enactin

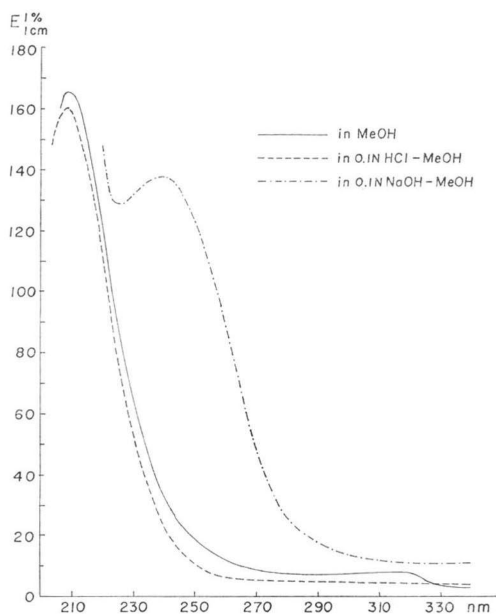

Fig. 2. Infrared absorption spectrum of neo-enactin in KBr

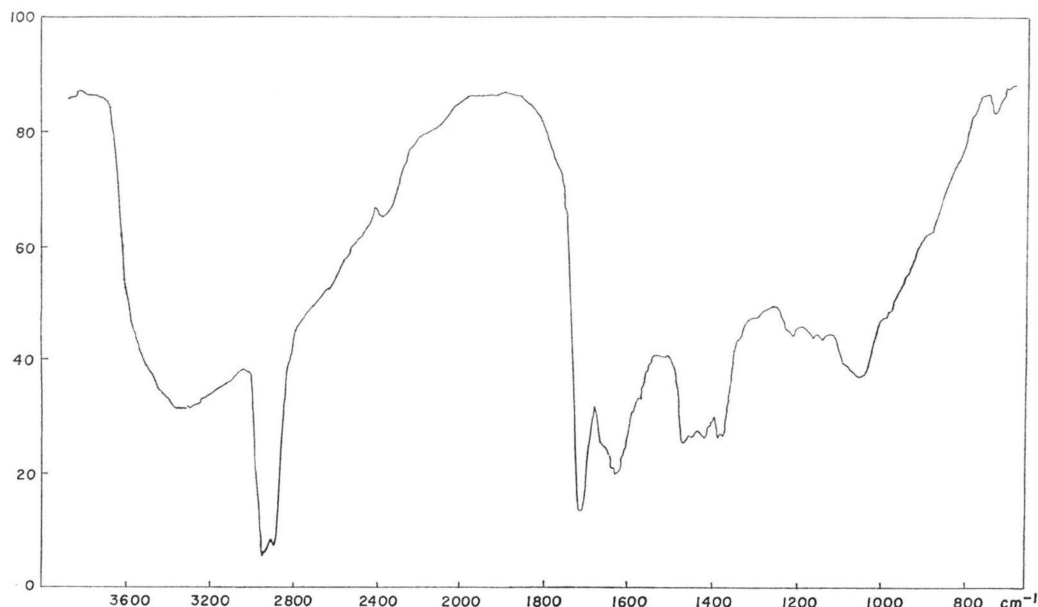

Table 1. Paper chromatography behaviors of neo-enactin and enactin

Solvent system	Rf value	
	Neo-enactin	Enactin
n-BuOH - H_2O (87: 13)	0.80	0.72
n-BuOH - AcOEt - H_2O (9: 9: 1)	0.76	0.74
AcOEt - MeOH - H_2O (10: 2: 1)	0.76	0.64
AcOEt - Acetone - H_2O (10: 3: 1)	0.73	0.64

Table 2. Silica gel thin-layer chromatography behaviors of neo-enactin and enactin

Solvent system	Rf value	
	Neo-enactin	Enactin
n-BuOH - MeOH - H_2O (8: 4: 1)	0.12	0.39
n-BuOH - H_2O (87: 13)	0.27	0.27
n-BuOH - AcOEt - H_2O (87: 100: 13)	0.07	0.26
Acetone - H_2O (4: 1)	0.38	0.89
n-BuOH - EtOH - H_2O (8: 5: 2)	0.20	0.48
$CHCl_3$ - MeOH - H_2O (65: 35: 10, lower phase)	0.31	0.45

Table 3. Antimicrobial spectra of neo-enactin and trichomycin in the presence and 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	2.5	0.313	1.25	0.156	0.078	0.625	0.313
Candida pseudotropicalis NI 7494	0.078	0.313	1.25	0.078	0.01	0.156	0.02
Candida albicans 3147	0.313	0.625	10	0.625	0.156	1.25	0.156
Candida albicans Yu 1200	0.313	0.625	10	0.313	0.313	1.25	0.313
Candida krusei NI 7492	2.5	0.313	1.25	0.156	0.156	1.25	0.625
Saccharomyces cerevisiae	0.078	0.156	0.313	0.039	0.005	0.02	0.01

Minimum inhibitory concentrations were determined on glucose nutrient agar at 37°C.
I, Neo-enactin. II, Trichomycin. III, Trichomycin+cholesterol (4 mcg/ml). IV, Trichomycin+neo-enactin (0.025 mcg/ml). V, Trichomycin+neo-enactin (0.05 mcg/ml). VI, Trichomycin+neo-enactin (0.025 mcg/ml)+cholesterol (4 mcg/ml). VII, Trichomycin+neo-enactin (0.05 mcg/ml)+cholesterol (4 mcg/ml).

L-serine by paper chromatography and the infrared absorption spectra[4].

Biological Properties

Neo-enactin shows inhibitory activity against yeasts and fungi, but not against bacteria as seen in Tables 3 and 5. Further, the antibiotic potentiates the antiyeast activity of trichomycin or amphotericin B in the presence or absence of cholesterol at concentrations of 0.025 and 0.05 mcg/ml as seen in Tables 3 and 4. No toxic symptoms were observed when neo-enactin was administrated

Table 4. Antimicrobial spectra of neo-enactin and amphotericin B in the presence and absence of cholesterol

Test organisms	Minimum inhibitory concentration (mcg/ml)					
	I	II	III	IV	V	VI
Candida tropicalis NI 7495	0.625	0.625	0.625	0.156	0.625	0.156
Candida pseudotropicalis NI 7494	0.625	0.625	0.625	0.313	0.625	0.156
Candida albicans 3147	1.25	1.25	0.625	0.313	0.625	0.156
Candida albicans Yu 1200	1.25	0.625	0.313	0.313	0.625	0.156
Candida krusei NI 7492	1.25	2.5	1.25	1.25	2.5	2.5
Saccharomyces cerevisiae	0.313	0.625	0.625	0.156	0.313	0.156

Minimum inhibitory concentrations were determined on glucose nutrient agar at 37°C.
I, Amphotericin B. II, Amphotericin B+cholesterol (4 mcg/ml). III, Amphotericin B+neo-enactin (0.025 mcg/ml). IV, Amphotericin B+neo-enactin (0.05 mcg/ml). V, Amphotericin B+neo-enactin (0.025 mcg/ml)+cholesterol (4 mcg/ml). VI, Amphotericin B+neo-enactin (0.05 mcg/ml)+cholesterol (4 mcg/ml).

to mice at a dosage of 150 mg/kg (intraperitoneal).

Discussion

The ultraviolet and infrared absorption spectra and the biological activities of neo-enactin have some similarity to those of enactin[2]. However, in contrast with neo-enactin, enactin cannot be extracted with ethyl acetate at pH 8.0. Chromatographic characteristics on paper or on silica gel thin-layer plates for neo-enactin are different from those of enactin. Also, the biological activity of neo-enactin is about 100 fold stronger than that of enactin. Consequently, neo-enactin can be considered to be a new antifungal antibiotic potentiating polyene antifungal antibiotics.

Table 5. Antimicrobial spectra of neo-enactin

Test organisms	MIC (mcg/ml)
Alternaria kikuchiana	6.25
Glomerella cingulata	12.5
Colletotrichum gloeosporioides	25
Colletotrichum lagenarium	12.5
Gloeosporium laeticolor	>100
Trichophyton asteroides 429	6.25
Trichophyton mentagrophytes	12.5
Aspergillus niger F-16	>100
Helminthosporium oryzae	0.39
Pseudomonas phaseolicola	>100
Xanthomonas oryzae N 5824	6.25

Minimum inhibitory concentration was determined on potato sucrose agar at 27°C.

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