A NEW GROUP OF ANTIBIOTICS, HYDROXAMIC ACID ANTIMYCOTIC ANTIBIOTICS

III. ISOLATION AND CHARACTERIZATION OF ENACTIN CONGENERS

KATSUMI YAMAMOTO, YOSHIKAZU SHIINOKI, MAKI NISHIO, YASUCHIKA MATSUDA, YOSHIO INOUYE and SHOSHIRO NAKAMURA

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan

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A simple and multipurpose screening method was devised by taking advantage of the antagonism of cholesterol against the fungicidal activity of polyene antifungal antibiotics (PAA)[1]. Enactin (EN), formerly designated as H 646-SY3 substance[2], and neoenactin (NE)[3~5] are antimycotic antibiotics discovered by this screening as potentiators for PAA. Besides ability to potentiate the antimycotic activity of PAA, EN and NE share the common structural features, releasing L-serine by acid hydrolysis and showing positive $FeCl_3$ reaction. Later, NE was found to be composed of several congeners and the structures of the main component NE-A and the minor components NE-B_1, -B_2, -M_1 and -M_2 were elucidated as shown in Fig. 1[6~8]. These NE congeners contain L-serine to form the hydroxamic acid structure. Therefore, we proposed the group name "hydroxamic acid antimycotic antibiotics (HAAA)" for EN, NE and the related antibiotics[9~13]. Like NE, EN is a complex of several congeners which are more hydrophilic and less active against *Candida albicans* Yu 1200 as compared with the NE congeners. In this paper, the purification and physico-chemical properties of the EN congeners are concerned.

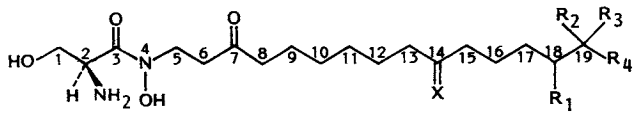
Streptomyces roseoviridis, the EN-producing microbe, was cultured to prepare an inoculum seed as described previously[2]. The seed culture (500 ml) was used to inoculate 150 liters of the production medium consisting of soluble starch 1.5%, glucose 1.0%, soybean meal 2.0%, dried yeast 0.5%, NaCl 0.25% and $CaCO_3$ 0.3% in a 200-liter tank fermenter and the fermentation was carried out at 27°C for 26 hours under agitation at 160 rpm and aeration at 130 liters per minute.

The antimycotic activity of EN was determined by a paper-disk diffusion method on glucose-nutrient agar supplemented with trichomycin 15 μg/ml and cholesterol 30 μg/ml using *C. albicans* Yu 1200 as a test microbe[2].

EN was adsorbed onto Diaion HP-20 from the broth filtrate and eluted with 80% aqueous acetone. The following procedures to obtain crude EN are summarized in Chart 1. HPLC profiles of the crude EN mixture are shown in Figs. 2 and 3.

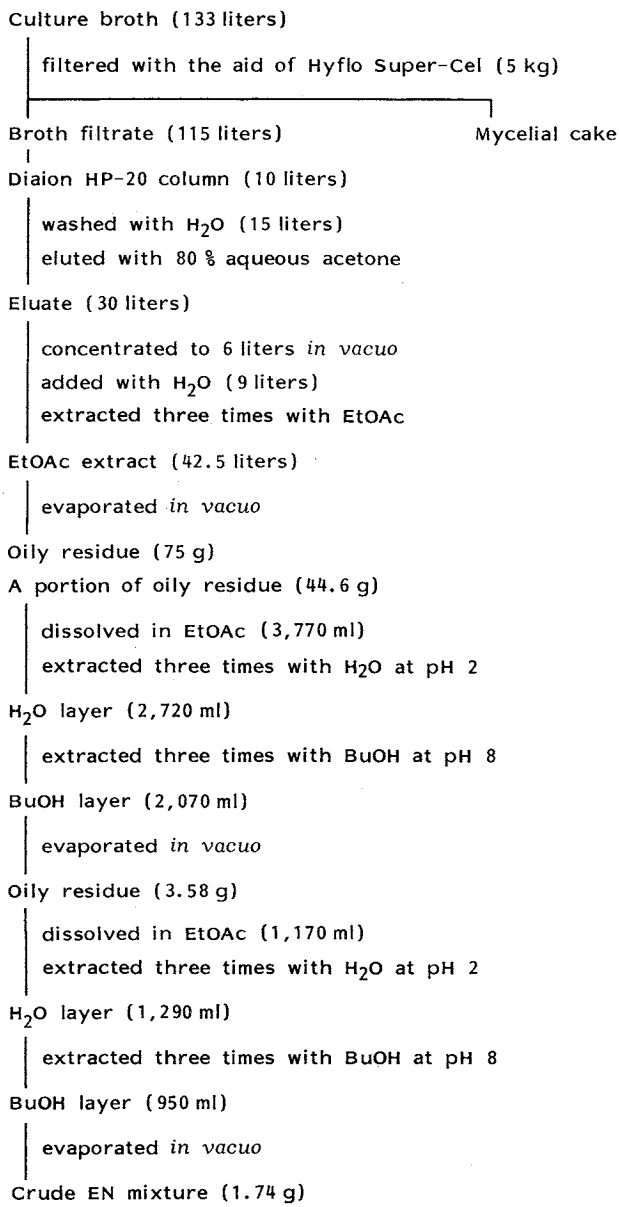
Separation and purification of the EN congeners were conducted by HPLC as illustrated in Chart 2. The crude EN mixture was applied to preparative

Fig. 1. Structures of EN and NE congeners.



Compound	X	R_1	R_2	R_3	R_4
EN-Ia	=O	H	OH	CH_3	CH_3
EN-Ib_1	=O	CH_3	H	OH	CH_3
EN-Ib_2	=O	CH_3	OH	H	CH_3
EN-IVa (=NE-M_1)	=O	CH_3	H	H	H
EN-IVb (=NE-A)	=O	H	H	H	CH_3
EN-Va	<OH H	CH_3	H	H	H
EN-Vb (NE-M_2)	<OH H	H	H	H	CH_3
EN-VIa (NE-B_1)	=O	CH_3	H	H	CH_3
EN-VIb (NE-B_2)	=O	H	H	CH_3	CH_3
NE-NL_1	=O	H	H	H	H
NE-NL_2	=O	H	H	H	C_2H_5

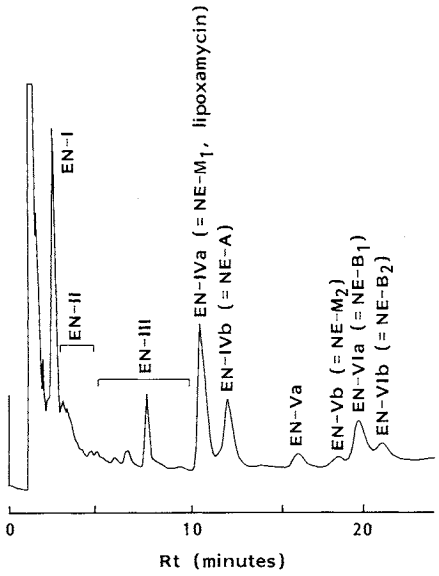
Chart 1. Extraction of crude EN mixture.



HPLC on ODS column (YMC Pack S-343 I-15, 20×250 mm, Yamamura Chem. Lab. Co., Ltd.). Partially purified EN was obtained from the individual fractions positive in the $FeCl_3$ reaction (Fractions I, II, III, IV, V and VI, in the order of elution from the column) by removal of MeOH, extraction with BuOH (Fractions I, II and III) or $CHCl_3$ (Fractions IV, V and VI) and evaporation of the organic solvent.

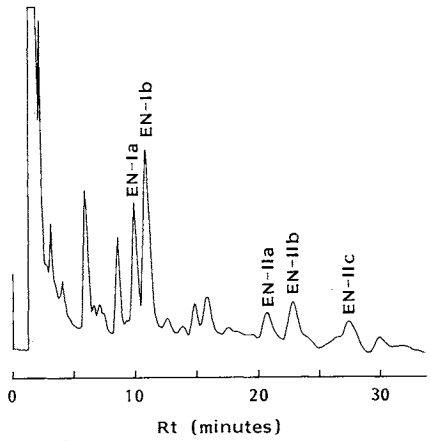
EN-Ia and -Ib were obtained by applying Fraction I to analytical HPLC using Methods B and C. EN-Ia and -Ib were retained on SEP-PAK C-18 cartridge (Waters Assoc., Mass., U.S.A.) after removal of MeOH *in vacuo* at pH 6.5. The cartridge was washed with H_2O and purified EN-Ia and -Ib were recovered by elution with MeOH. The purification procedures of the other EN congeners were basically similar to those of EN-Ia and -Ib. EN-IIa, -IIb and -IIc were isolated from Fraction II; EN-III from Fraction III; EN-IVa and -IVb from Fraction IV; EN-Va and -Vb from Fraction V; and EN-VIa and VIb from Fraction VI. EN-III

Fig. 2. HPLC profile of crude EN mixture.



Column: Radial Pak Nova Pak C_{18} (8 × 100 mm, 4 μm, Waters), mobile phase: MeOH - 0.05 M KH_2PO_4 (2 : 3, pH 2.8), flow rate: 2.0 ml/minute, detection: UV 214 nm.

Fig. 3. HPLC profile of crude EN mixture.



Column: Radial Pak Nova Pak C_{18} (8 × 100 mm, 4 μm, Waters), mobile phase: MeOH - 0.05 M KH_2PO_4 (9 : 11, pH 2.8), flow rate: 2.0 ml/minute, detection: UV 214 nm.

was still revealed to be composed of several very minor EN congeners. The summary of purification is shown in Chart 2, in which the weight of each sample obtained from 1.74 g of the crude EN mixture was given in the parenthesis.

Among the EN congeners thus isolated, EN-IVa, -IVb, -Vb, -VIa and -VIb were proved to be identical with NE-M_1, -A, -M_2, -B_1 and -B_2, respectively, on the basis of Rf values on PEI-cellulose and silica gel TLCs, the results of co-elution experiments on HPLC with the authentic standards and mass spectrometry data. The other congeners were considered to be novel compounds and the physico-chemical properties are listed in Table 1.

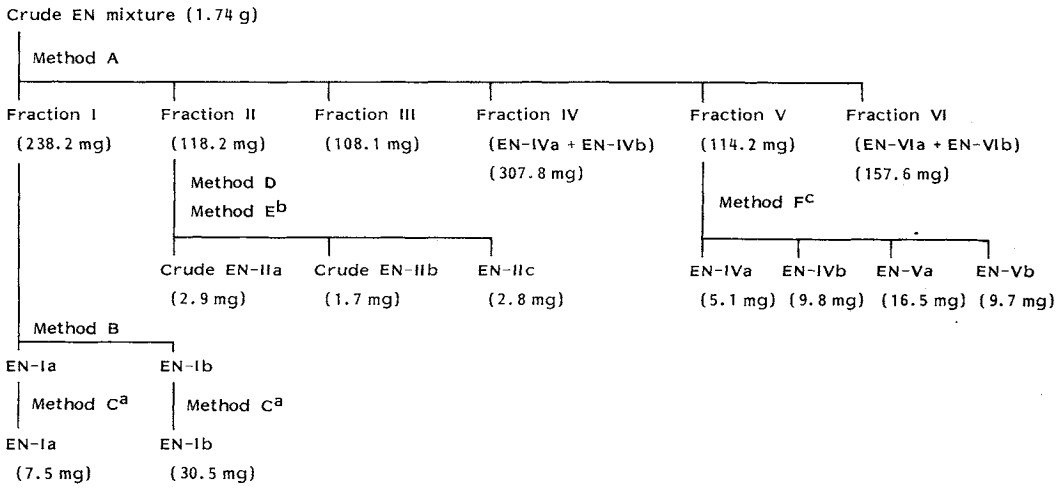
Later, EN-Ib was disclosed to consist of an almost equal amount of diastereoisomers, EN-Ib_1 and -Ib_2, by ^{1}H NMR of bis-2,4-dinitrophenyl-EN-Ib. The structures of EN-Ia, -Ib_1, -Ib_2 and -Va were elucidated by ^{1}H and ^{13}C NMR and FAB-MS, as shown in Fig. 1. The details will be published elsewhere[14]. The EN congeners which had no identical components in the NE congeners with the exception of EN-Va are more hydrophilic than the latters, implying the contribution of the 19-hydroxy group in these EN congeners to their increased hydrophilicity.

The antimicrobial activities of EN-Ia, -Ib, -IIc, and -Va are shown in Table 2 in comparison with that of NE-A. The novel EN congeners showed similar antimicrobial spectra to that of NE-A. The effects of combination of PAA and HAAA on the individual antimicrobial activities were assesed by the paper strip-cross method on glucose - nutrient agar using *C. albicans* Yu 1200 as a test microbe and the results are shown in Table 3. The potentiation was observed by any combination except for those between HAAA and pentamycin. The lack of amino sugar mycosamine in pentamycin might be responsible for its inability to potentiate the antimicrobial activity of HAAA.

The mode of action of HAAA has been studied, so as to identify the primary site of action. Most antimycotic antibiotics are classified with respect to a mode of action as follows: (1) those interfering with biosynthesis of membrane components (*e.g.*, azasterols and cerulenin); (2) those affecting membrane functions by interacting with membrane components (*e.g.*, PAA); (3) those interfering with oxidative phosphorylation (pyrrolnitrin, siccanin, antimycins, *etc.*); (4) those primarily inhibiting biosynthesis of cell wall polysaccharides (*e.g.*, polyoxins and nikkomycins); (5) those inducing dysfunction of microtubules (*e.g.*, griseofulvin); (6) inhibitors of protein synthesis (*e.g.*, cycloheximide); and (7) amino acid analogs (*e.g.*, RI-331).

The increased uptake of vincristine in the presence of NE was observed in HeLa cells when the cells were treated with sublethal concentrations of NE[5].

Chart 2. Separation and purification of EN congeners by various HPLC systems.



Method A: Column, YMC Pack S-343 (20 × 250 mm); mobile phase, MeOH - 0.05 M KH_2PO_4 (17 : 8, pH 2.8); flow rate, 9.0 ml/minute.

Method B: Column, Radial Pak Nova Pak C_{18} (8 × 100 mm); mobile phase, MeOH - 0.05 M KH_2PO_4 (1 : 1, pH 2.8); flow rate, 2.0 ml/minute.

Method C: Column, Radial Pak Nova Pak C_{18} (8 × 100 mm); mobile phase, MeOH - 0.05 M KH_2PO_4 (2 : 3, pH 2.8); flow rate, 2.0 ml/minute.

Method D: Column, YMC Pack S-343 (20 × 250 mm); mobile phase, MeOH - 0.05 M KH_2PO_4 (11 : 9, pH 2.8); flow rate, 9.0 ml/minute.

Method E: Column, Radial Pak Nova Pak C_{18} (8 × 100 mm); mobile phase, MeOH - 0.05 M KH_2PO_4 (9 : 11, pH 2.8); flow rate, 2.0 ml/minute.

Method F: Column, Radial Pak Nova Pak C_{18} (8 × 100 mm); mobile phase, MeOH - 0.05 M KH_2PO_4 (3 : 2, pH 2.8); flow rate, 2.0 ml/minute.

HPLC was monitored by absorbance at 214 nm for all methods.

[a] Rt's of EN-Ia and EN-Ib are 21.1 and 23.2 minutes, respectively.

[b] Rt's of EN-IIa, EN-IIb and EN-IIc are 20.6, 22.8 and 27.3 minutes, respectively.

[c] Rt's of EN-IVa, EN-IVb, EN-Va and EN-Vb are 11.2, 12.4, 14.8 and 17.1 minutes, respectively.

Table 1. Physio-chemical properties of EN congeners.

	EN-Ia	EN-Ib	EN-IIc	EN-Va
Molecular formula	$C_{20}H_{38}N_2O_6$	$C_{20}H_{38}N_2O_6$	$C_{19}H_{38}N_2O_6$	$C_{19}H_{38}N_2O_5$
MW	402	402	390	374
FAB-MS[a] (m/z, $(M+H)^+$)	403	403	391	375
MP (dec) of sulfate (uncorrected, °C)	134.5	128 ~ 129	ND	125.5 ~ 128.5
UV λ_{max}^{MeOH} nm (ε)	212 (4,100)	213 (4,400)	213 (4,000)	216 (3,400)
Rf of TLC[b] (a)	0.26	0.28	0.22	0.29
(b)	0.82	0.88	0.83	0.52
(c)	0.79	0.83	0.80	0.56
$FeCl_3$	Red	Red	Red	Red
Ninhydrin	Yellow	Yellow	Yellow	Yellow
I_2	Positive	Positive	Positive	Positive

ND: Not determined.

[a] Mass spectra were measured with a Jeol HX-110 mass spectrometer.

[b] TLC systems were as follows: (a) Silica gel 60 F_{254} plate (Merck) developed with $CHCl_3$ - MeOH - H_2O (12 : 6 : 1); (b) PEI-cellulose F plate (Merck) developed with 0.2 M NaCl; (c) PEI-cellulose F plate (Merck) developed with 0.1 M citrate - 0.2 M NaH_2PO_4 buffer (pH 4.0).

Table 2. Antimicrobial spectra of NE-A and EN congeners.

Test organisms	MIC (μg/ml)				
	NE-A	EN-Ia	EN-Ib	EN-IIc	EN-Va
Candida tropicalis NI 7495	1.56	100	200	50	12.5
C, pseudotropicalis NI 7494	0.10	12.5	12.5	6.25	1.56
C. albicans Yu 1200	3.13	>100	>200	>100	50
C. albicans MTU 12013	0.78	50	200	50	12.5
Saccharomyces cerevisiae	0.20	25	40	12.5	12.5
Alternaria kikuchiana	6.25	>100	200	>100	25
Glomerella cingulata	1.56	>100	>200	>100	25
Colletotrichum lindemuthianum No. 1	0.39	50	200	50	12.5
Gloeosporium laeticolor	6.25	>100	>200	>100	50
Trichophyton mentagrophytes (833)	3.13	>100	>200	>100	100
Aspergillus niger F-16	>100	>100	>200	>100	>100
Helminthosporium oryzae	0.39	50	50	25	3.13

Table 3. Synergism between EN congeners and polyene antifungal antibiotics.

PAA	EN-Ib	EN-II	EN-III	EN-IVa	EN-IVb	EN-Va	EN-Vb	EN-VIa	EN-VIb
Trichomycin	+	+	+	+	+	+	+	+	+
Amphotericin B	−	±	+	+	+	+	+	+	+
Pimaricin	−	−	±	±	+	+	+	+	+
Pentamycin	−	−	−	−	−	−	−	−	−
Nystatin	−	−	±	+	+	−	+	+	+

+: Positive, −: negative, ±: doubtful.
EN-II and EN-III are the extracts of Fractions II and III, respectively, as mixtures of minor EN congeners.

The concentrations of NE employed in that experiment, though non-toxic to HeLa cells, were at least 10-fold higher than the MIC against *C. albicans*. In a separate experiment, NE-A caused hemolysis in only a small portion of sheep erythrocytes at concentrations up to 10-fold the MIC against *C. albicans* (H. YAMAGUCHI *et al.*, unpublished data). Apparently contradictory results obtained in these experiments were due to the divergent susceptibilities of various membranes to HAAA. HAAA consist of hydrophilic and hydrophobic portions and it seems plausible that their surface-active natures are responsible for their effects on membrane integrity. However, at fungistatic concentrations (*e.g.*, 0.1 ~ 0.5 μg/ml in the case of NE-A against *C. albicans*) NE-A did not cause K^+-release. NE-A also did not affect macromolecular synthesis as detected by incorporation of radioactive precursors into acid-precipitable fractions, exogenous respiration, polysaccharide synthesis and synthesis of non-saponifiable sterols. In addition, NE-A was not able to interfere with polymerization of microtubules. NE-A affected [^{3}H]acetate incorporation into fatty acids substantially but not completely at the fungistatic concentrations. The details of these findings will be published elsewhere. Cerulenin was reported to affect primarily sterol and fatty acid biosynthesis[15] in yeast. Recently, GEORGOPAPADAKOU *et al.*[16] reported that cerulenin was specific for fatty acid synthesis in contrast to the previous findings[15]. Thus, the mode of action of HAAA is most closely related with that of cerulenin among the known antimycotic antibiotics. In a cell-free system, however, NE-A does not affect fatty acid synthesis. Therefore, the primary site of HAAA still remains to be identified.

Acknowledgments

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