A NEW GROUP OF ANTIBIOTICS, HYDROXAMIC ACID ANTIMYCOTIC ANTIBIOTICS

I. PRECURSOR-INITIATED CHANGES IN PRODUCTIVITY AND BIOSYNTHESIS OF NEOENACTINS NL_1 AND NL_2

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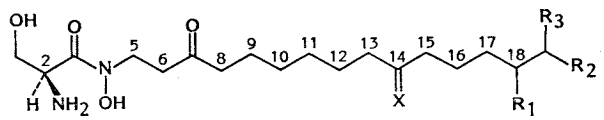
Neoenactins (NEs) are L-serine-containing antifungal antibiotics produced by *Streptoverticillium olivoreticuli*. The effect of supplementation of individual amino acids on the production of NEs by this organism was examined from both quantitative and qualitative view points by using a nitrogen source-restricted medium. L-Alanine, L-arginine, L-glutamine, L-histidine, L-lysine and L-proline increased significantly the total productivity of NEs without changing the production ratio of the congeners. The supplementation of L-norvaline, L-isoleucine, L-leucine and L-valine to the culture medium resulted in selective enhancement of the production of NEs A, B_1, B_2, and M_1, respectively, while significant change was not observed in terms of overall production of NEs. When L-norleucine was employed as an amino acid supplement, new NE congeners, named NEs NL_1 and NL_2, were preferentially produced; but the amount of NEs produced was not markedly affected.

Neoenactins (NEs), are novel antifungal antibiotics produced by *Streptoverticillium olivoreticuli* subsp. *neoenacticus*[1,2], potentiating candicidal activities of polyene antifungal antibiotics and tumoricidal activities of the antitumor agents including bleomycin and vincristine[3,4]. NEs have been separated into several congeners and the structures of NE-A, the main component, $-B_1$, $-B_2$, $-M_1$, and $-M_2$ have been elucidated in the preceding papers[5,6] (Fig. 1). NE-M_1 was, however, found to be identical with lipoxamycin reported by WHALEY *et al.*[7] and WHALEY[8]. These antibiotics are commonly composed of L-serine and an aliphatic amine containing one or two carbonyl groups (AAC) to form the hydroxamic acid structure. Enactins (ENs), produced by *Streptomyces roseoviridis* and formerly designated as antibiotic H 646-SY3[9], have similar biological properties and chemical structures.

Fig. 1. Structures of neoenactin (NE) congeners.



NE-A	R_1=H	R_2=CH_3	R_3=H	X=O
NE-M_1	R_1=CH_3	R_2=R_3=H		X=O
NE-M_2	R_1=H	R_2=CH_3	R_3=H	X= 
NE-B_1	R_1=R_2=CH_3		R_3=H	X=O
NE-B_2	R_1=H	R_2=R_3=CH_3		X=O
NE-NL_1	R_1=R_2=R_3=H			X=O
NE-NL_2	R_1=H	R_2=C_2H_5	R_3=H	X=O

However, the antimycotic activities of ENs are much less active than NEs (<0.01). Therefore, we proposed the group name "Hydroxamic Acid Antimycotic Antibiotics (HAAA)" for NEs and ENs[10]. In this paper, we describe effects of the addition of amino acids to the culture medium on productivity of NEs and on the structures of resulting NEs.

Materials and Methods

Fermentation

The culture medium consisted of soluble starch 1.5%, glucose 1.0%, soy bean meal 0.2%, Ebios (dried yeast, distributed by Tanabe Seiyaku Co., Ltd.) 0.05%, $CaCO_3$ 0.3% and NaCl 0.25% (pH was adjusted to 7.6 before sterilization). This medium is referred to as the nitrogen source-restricted (NSR) medium. The seed culture (2 ml), prepared as described previously[1], was inoculated into a 500-ml Sakaguchi flask containing 100 ml of NSR medium supplemented with 100 mg of each amino acid. The flasks were incubated at 27°C for 30 hours on a reciprocal shaker (amplitude 7 cm, 130 strokes per minutes).

Effect of Amino Acid on the Total Productivity of NEs

The culture broth (100 ml) was centrifuged at 4°C for 10 minutes at 3,000 rpm and the mycelium was washed twice with each 10 ml of distilled water and lyophilized. After measurement of the dry cell weight, NEs were extracted with 10 ml of MeOH. The extract was concentrated *in vacuo* to *ca.* 0.5 ml and diluted with 9.5 ml of 0.05 M phosphate buffer (pH 7.0). The productivities of NEs in the culture filtrate and the mycelial extract were determined by the cylinder agar plate method on glucose nutrient agar using *Candida albicans* Yu 1200 as the test microorganism and NE-A as a standard sample.

Quantitative and Qualitative Analyses of NEs in the Cultured Broth

The culture filtrate (30 ml) was percolated through a C_{18} Sep-Pak cartridge (8×10 mm, Waters Assoc., Mass., U.S.A.). The mycelial MeOH extract (10 ml) was concentrated *in vacuo* to *ca.* 0.5 ml and diluted with distilled water (9.5 ml) to prepare the test sample for the cartridge. NEs were eluted with 10 ml of 70% aqueous MeOH from the cartridge after being washed with each 10 ml of distilled water and 30% aqueous MeOH in this order.

Quantitative and qualitative analyses of NEs thus obtained were determined by HPLC on a Radial Pak Nova Pak C_{18} cartridge (8×100 mm, Waters Assoc., Mass., U.S.A.) monitored at 214 nm, using MeOH - 0.05 M KH_2PO_4 (3 : 2, pH 2.8) as a mobile phase and 2.0 ml/minute flow rate.

Results

Effect of Amino Acid on the Production of NEs

As reported previously[1], NEs were recovered mainly from the mycelium when the nitrogen source-enriched production (NSEP) medium containing soy bean meal 2% and Ebios 0.5% was used. In contrast, NEs were recovered from the culture filtrate when NSR medium was used, as shown in Table 1, with the productivity of NEs being 20% of that recovered from the mycelium grown on NSEP medium. The addition of 0.1% of L-alanine, L-arginine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-lysine or L-proline to NSR medium increased 5~14 times the total yield of NEs in both the culture filtrate and mycelial cake without increasing the dry cell weight. L-Serine, the constituent of HAAA, was expected to increase significantly HAAA productivity. Nevertheless, the addition of L-serine showed rather weak increasing effect on the productivity of NEs.

Those amino acids described above did not show significant change in the production ratio of NE congeners. The addition of L-valine to NSR medium stimulated preferential production of NE-M_1 in the culture filtrate as shown in Fig. 2b. Likewise, when L-norvaline, L-isoleucine and L-leucine

Table 1. Effect of the addition of amino acid on the productivity of neoenactins (NEs).

Amino acid	pH	Dry cell weight (mg/ml)	NE productivity[a] (μg/ml)	
			Culture filtrate	Mycelia
None	5.6	3.2	1	0.2
NSEP medium[b]	5.8	4.6	4	1.0
L-Alanine	5.2	2.6	11	1.5
β-Alanine	5.6	3.5	5	0.7
L-Arginine-HCl	5.6	3.2	11	1.8
L-Asparagine	5.2	3.2	6	0.9
L-Aspartic acid	5.2	2.6	3	0.4
L-Cysteine	5.6	2.3	2	0.1
L-Glutamic acid	5.4	2.9	7	1.1
L-Glutamine	5.6	3.2	14	2.0
Glycine	5.0	3.2	8	1.0
L-Histidine	5.6	3.5	13	2.0
L-Hydroxyproline	5.2	2.6	2	0.2
L-Isoleucine	5.6	3.2	1	0.1
L-Leucine	5.4	2.3	2	0.3
L-Lysine-HCl	5.6	3.5	13	1.4
L-Methionine	5.2	2.9	1	0.1
L-Norleucine	6.0	3.2	2	0.3
L-Norvaline	6.0	2.2	4	0.3
L-Ornithine	5.8	3.2	6	0.9
L-Phenylalanine	5.6	3.9	2	0.2
L-Proline	5.4	2.9	13	1.3
L-Serine	6.0	3.5	5	0.6
L-Threonine	5.2	2.6	1	0.1
L-Tryptophan	5.4	2.3	1	0.1
L-Tyrosine	5.6	3.5	2	0.3
L-Valine	5.2	1.9	2	0.3

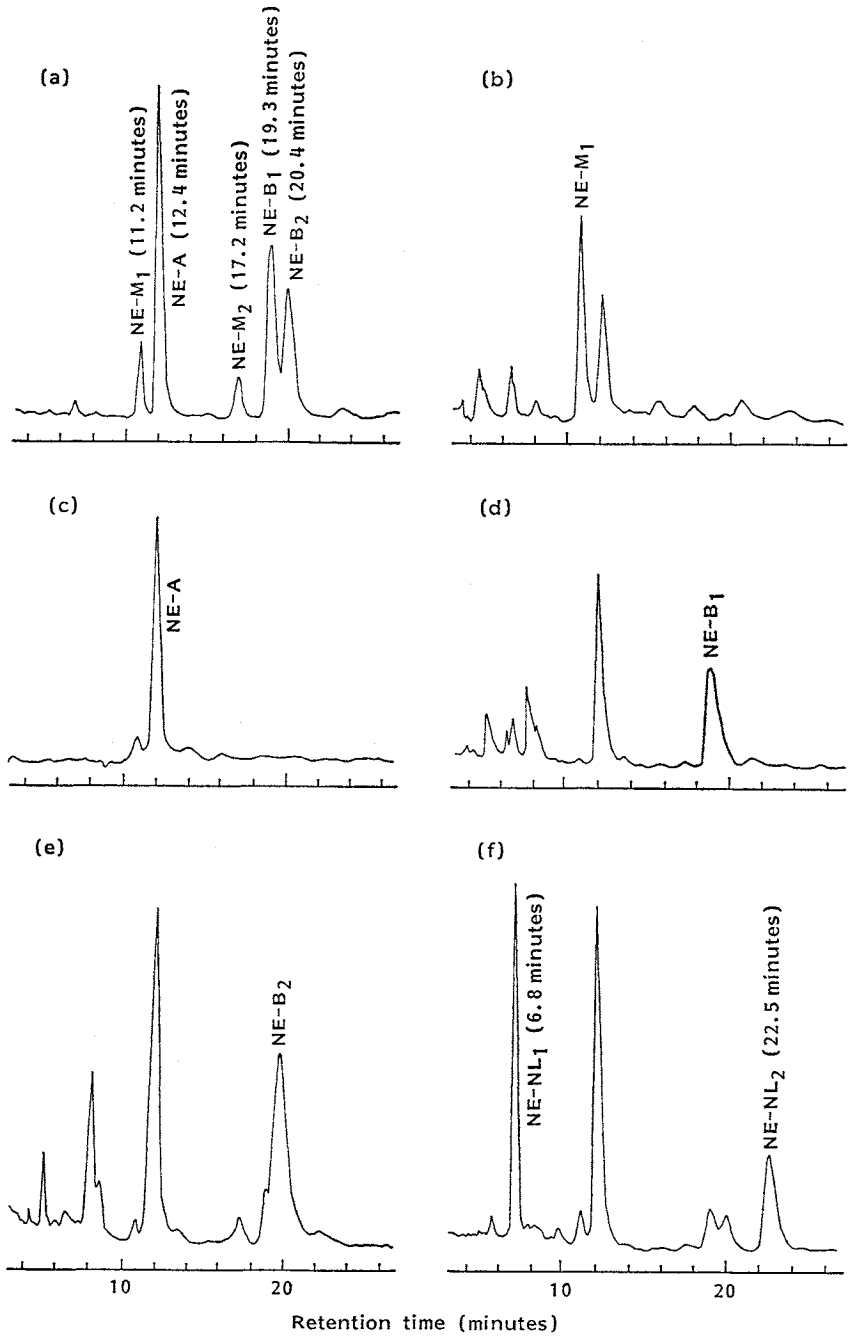
[a] Productivity of NEs was shown as the amount of NE-A.
[b] The nitrogen source-enriched production medium was used as control.

were used, preferential production of NE-A, -B_1, and -B_2 in culture filtrate was enhanced respectively (Fig. 2c~2e). Moreover, the addition of L-norleucine to NSR medium induced production of novel NEs, named NE-NL_1 and -NL_2, in the culture filtrate as can be seen in Fig. 2f.

Isolation and Purification of NE-NL_1 and -NL_2

The culture filtrate (17 liters, pH 6.0) from the culture on NSR medium supplemented with 0.1% of L-norleucine was stirred with Amberlite XAD-2 (600 ml) for 1 hour at room temperature. The resin was washed with distilled water (2.5 liters) and the active compounds were eluted batchwisely from the resin 5 times with each 700 ml of 80% aqueous acetone. The eluates were combined and concentrated *in vacuo* to 900 ml. The concentrate was adjusted to pH 8.0 then extracted 3 times with each 300 ml of methyl isobutyl ketone (MIBK). The active compounds were transferred from MIBK into water (900 ml, adjusted to pH 2.0 with dilute HCl). The aqueous phase was concentrated *in vacuo* after adjusting to pH 5 and lyophilized to give the crude NE mixture (205.4 mg). NE-NL_1 and -NL_2 were purified by HPLC on a Radial Pak Nova Pak C_{18} cartridge using MeOH - 0.05 M KH_2PO_4 (1 : 1 for NE-NL_1 and 3 : 2 for NE-NL_2, pH 2.8) as mobile phases. The free bases of NE-NL_1 and -NL_2 were converted to the sulfates and recrystallization from MeOH gave white needles, respectively.

Fig. 2. Effect of amino acid on the production of neoenactins (NEs).
(a) None, (b) L-valine, (c) L-norvaline, (d) L-isoleucine, (e) L-leucine, (f) L-norleucine.



Physico-chemical Properties of NE-NL_1 and -NL_2 Sulfates

Physico-chemical properties of NE-NL_1 and -NL_2 sulfates are summarized in Table 2. The fast atom bombardment (FAB)-MS data of NE-NL_1 and -NL_2 sulfates indicated the molecular ion peak of those free forms. The structures of NE-NL_1 and -NL_2 were determined as shown in Fig. 1

Table 2. Physico-chemical properties of neoenactins (NEs) NL_1 and NL_2 (sulfates).

	NE-NL_1	NE-NL_2
Molecular formula	$C_{18}H_{34}N_2O_5 \cdot \frac{1}{2}H_2SO_4$	$C_{20}H_{38}N_2O_5 \cdot \frac{1}{2}H_2SO_4$
FAB-MS[a] (m/z M[b]+H)	359	387
MP (°C, dec)	145	145
Rf on silica gel TLC[c]	0.28	0.32
Absorption UV λ_{max}^{MeOH} nm (ε)	211 (5,200)	211 (5,200)

[a] Mass spectra were measured with a Jeol MS-HX110 mass spectrometer.
[b] M indicated the molecular ion peak of free form.
[c] Kieselgel 60 F_{254} (Merck); solvent: $CHCl_3$ - MeOH (25 : 1).

Table 3. Antimicrobial spectra of neoenactins (NEs) NL_1 and NL_2 (sulfates).

Test organisms	MIC (μg/ml)		
	NE-A	NE-NL_1	NE-NL_2
Candida tropicalis NI 7495	0.78	6.25	1.56
C. pseudotropicalis NI 7494	0.024	0.78	0.024
C. albicans Yu 1200	0.78	6.25	1.56
C. albicans MTU 12013	0.78	6.25	1.56
Saccharomyces cerevisiae	0.024	0.39	0.047
Alternaria kikuchiana	0.78	3.13	3.13
Glomerella cingulata	0.095	3.13	0.047
Gloeosporium laeticolor	0.78	6.25	1.56
Trichophyton mentagrophytes (833)	1.56	6.25	3.13
Aspergillus niger F-16	50	>50	50

MIC was determined by the agar dilution method on glucose nutrient agar.

and the details of structure elucidation will be described in a separate paper[11].

Biological Properties of NE-NL_1 and -NL_2

Table 3 shows the antimicrobial spectra of NE-NL_1 and -NL_2 against various strains of yeasts and fungi in comparison with that of NE-A.

Discussion

WHALEY *et al.* reported that production of lipoxamycin (NE-M_1) by *Streptomyces virginiae* var. *lipoxae* was increased by the addition of L-alanine, L-arginine, L-glutamic acid or L-histidine[7]. Production of NEs by *S. olivoreticuli* is also increased by the addition of the same amino acids.

Although these amino acids did not change the ratio of the NE congeners, the addition of L-norvaline, L-isoleucine, L-leucine and L-valine stimulates preferentially the production of NE-A, -B_1, -B_2, and -M_1, respectively. These results suggested the possibility of production of novel NE congeners by the addition of an alternate amino acid which might be incorporated into the terminal of NE structure as shown in Fig. 1. Actually, the addition of L-norleucine induced two new compounds, namely NE-NL_1 and -NL_2.

Acknowledgments

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