A NEW GROUP OF ANTIBIOTICS, HYDROXAMIC ACID ANTIMYCOTIC ANTIBIOTICS

II. THE STRUCTURE OF NEOENACTINS NL_1 AND NL_2 AND STRUCTURE-ACTIVITY RELATIONSHIP

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The structures of neoenactins (NEs) NL_1 and NL_2, novel antimycotic antibiotics produced by *Streptoverticillium olivoreticuli* in a precursor-oriented fashion, were elucidated by ^1H and ^{13}C NMR and mass spectroscopic studies. The structures of both antibiotics are closely related to that of NE-A, the major component of NE congeners, being classified in the group of hydroxamic acid antimycotic antibiotics in which L-serine and a diketo amine form a hydroxamic acid structure.

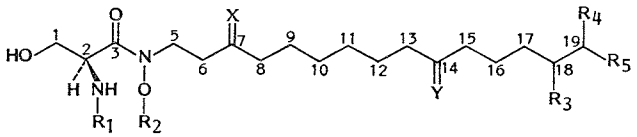
To study the role of the carbonyl groups in the biological activities of the hydroxamic acid antimycotic antibiotics, NE-A was modified by reaction with various carbonyl reagents. In terms of antimycotic activity, the derivatives are classified into two distinct groups; the first ones are fairly comparable to but not exceeding and the second ones are less active than NE-A depending on their tendency to revert to NE-A by hydrolysis. In general, the biological activities of the derivatives are inversely proportional to their stabilities to hydrolysis.

Enactins (ENs) produced by *Streptomyces roseoviridis*[1] and neoenactins (NEs) produced by *Streptoverticillium olivoreticuli* subsp. *neoenacticus*[2,3] are antimycotic antibiotics potentiating activities of polyene antifungal antibiotics and antitumor agents such as bleomycin and vincristine[4]. NEs are composed of several congeners which share similar physico-chemical and biological properties. The isolation and structural elucidation of NE-A (**III**), the main component, as well as those of NE-B_1, -B_2, -M_1 (lipoxamycin) and M_2 have been reported[5~9] and their structures are summarized in Fig. 1. In common, they contain L-serine as part of a hydroxamic acid moiety. Similar structural properties have been observed with the congeners of ENs. Therefore, we proposed the group name hydroxamic acid antimycotic antibiotics (HAAA) for ENs and NEs[10].

Recently, we isolated novel members of HAAA, named NE-NL_1 (**I**) and -NL_2 (**II**), during a study of the biosynthesis of NEs[11]. The effect of supplementation of individual amino acids at a final concentration of 0.1% to the basal medium, from which nitrogen source was reduced by 90%, on the production of NEs was studied quantitatively and qualitatively. When L-norleucine was used as an amino acid supplement, **I** and **II** were produced in rather high yields and their preliminary physico-chemical and biological properties have been reported[11].

Since the mono and bis-2,4-dinitrophenyl (DNP)-derivatives (Fig. 1) were inactive against

Fig. 1. Structures of neoenactin congeners and neoenactin A derivatives.



Compound	R_1	R_2	X	Y	R_3	R_4	R_5
NE-NL_1 **(I)**	H	H	=O	=O	H	H	H
Bis-DNP-NE-NL_1 **(Ia)**	(DNP)	(DNP)	=O	=O	H	H	H
NE-NL_2 **(II)**	H	H	=O	=O	H	H	C_2H_5
Bis-DNP-NE-NL_2 **(IIa)**	DNP	DNP	=O	=O	H	H	C_2H_5
NE-A **(III)**	H	H	=O	=O	H	H	CH_3
Bis-DNP-NE-A **(IIIa)**	DNP	DNP	=O	=O	H	H	CH_3
Mono-DNP-NE-A **(IIIb)**	DNP	H	=O	=O	H	H	CH_3
NE-M_1	H	H	=O	=O	CH_3	H	H
NE-M_2	H	H	=O	<OH H	H	H	CH_3
NE-B_1	H	H	=O	=O	CH_3	H	CH_3
NE-B_2	H	H	=O	=O	H	CH_3	CH_3
IIIc	H	H	$=NNHCONH_2$	$=NNHCONH_2$	H	H	CH_3
IIId	H	H	$=NNHCONHC_6H_5$	$=NNHCONHC_6H_5$	H	H	CH_3
IIIe	H	H	$=NNHCOOC_2H_5$	$=NNHCOOC_2H_5$	H	H	CH_3
IIIf	H	H	=NOH	=NOH	H	H	CH_3
IIIg	H	H	$=NOCH_3$	$=NOCH_3$	H	H	CH_3
IIIh	H	H	$=NOCH_2C_6H_4NO_2$	$=NOCH_2C_6H_4NO_2$	H	H	CH_3

Candida albicans in vitro (data not shown), the free $-NH_2$ and $>NOH$ groups appeared to be essential to the biological activity. In attempts to study structure-activity relationships, two carbonyl groups at 7- and 14-positions of **III** were simultaneously modified with a series of carbonyl reagents.

In this report, the structural elucidation of **I** and **II**, and the comparative study on the biological activities of the NE-A derivatives are concerned.

Structural Elucidation of NE-NL_1 and -NL_2

The existence of a free hydroxamic acid-type structure in **I** and **II** was strongly suggested by the positive $FeCl_3$ test. Further, the recovery of L-serine from the acid hydrolysates of **I** and **II** was proven by HPLC on a enantiomeric resolution column (TSK gel Enantio L2, 4.6×150 mm, Tosoh Co., Ltd., Tokyo) using 0.5 mM $CuSO_4$ as a mobile phase. Both **I** and **II** were converted to the corresponding stable bis-DNP-derivatives according to the previous method[6] [**Ia** (mp 140°C) and **IIa** (mp 145°C), respectively]. The molecular formula $C_{18}H_{34}N_2O_5$ and $C_{20}H_{38}N_2O_5$ were proposed for **I** and **II**, respectively, based on the results of fast atom bombardment (FAB)-MS of **I** and **II**, and ^1H and ^{13}C NMR spectra of **Ia** and **IIa**[11]. Since the molecular formula $C_{19}H_{36}N_2O_5$ had been reported for **III**[5], **I** and **II** can be considered to contain one more and less CH_2 group, respectively, when compared with **III**.

The ^1H NMR data (400 MHz, $CDCl_3$) of **Ia** and **IIa** and their assignments are presented in Table 1 in comparison with those of **IIIa**. The data of **Ia**, **IIa** and **IIIa** are almost identical except that eight, twelve and ten methylene proton signals are observed for **Ia**, **IIa** and **IIIa**, respectively, in the methylene proton region (δ 1.25~1.27). The existence of only one methyl group adjacent to CH_2 in **Ia**, **IIa** and **IIIa** is also shown by the chemical shift (3H, t, J=6.8~7.0 Hz), whereas the existence of any other methyl groups on tertiary or quaternary carbon atoms can be excluded. One of the carbonyl groups of **I** can be located at the 14-position by the fragment peaks at m/z 99.0810 (calcd for $C_6H_{11}O^+$: 99.0809) and m/z 71.0856 (calcd for $C_5H_{11}^+$: 71.0861) in high-resolution electron impact (HREI)-MS spectrum of **Ia**. Further, the fragment peak of **IIa** at m/z 127.1124 (calcd for $C_8H_{15}O^+$: 127.1123) postulated that one of the carbonyl groups is also located at 14-position in **II**. The ^{13}C NMR data (100 MHz, $CDCl_3$) of **Ia** and **IIa** and their assignments are shown in Table 2 together with those of **IIIa**[7]. Thus, the structures of **I** and **II** are elucidated as shown in Fig. 1.

Synthesis of NE-A Derivatives (**IIIc**~**IIIh**)

A mixture of **III** (20 mg, 0.052 mmol) in dry MeOH (1 ml) and a carbonyl reagent (0.11 mmol) was kept at room temperature for 0.6~2 hours. The reaction was monitored intermittently by Silica gel TLC (Kieselgel $60F_{254}$ plate; 0.25 mm thickness, E. Merck, Darmstadt) using $CHCl_3$ - MeOH - H_2O (12:6:1) as a developing solvent and 5% $FeCl_3$ aqueous solution for detection until the spot of **III** disappeared. The pH of the reaction mixture was adjusted to 3 by adding 5% HCl - MeOH in the case of ethyl carbazate. The summary of the purification of the various analogues is shown in Table 3. The products were purified by silica gel column chromatography developed with $CHCl_3$ - MeOH (9:1, Method A) or $CHCl_3$ - MeOH (19:1, Method B), or repeated recrystallization from MeOH (Method C).

The purity of NE-A derivatives was examined by silica gel TLC or HPLC on Radial Pak Nova Pak C_{18} (8×100 mm, Waters Assoc., Mass.) using MeOH - 0.05 M KH_2PO_4 (3:2, pH 2.8) as a mobile phase; the latter system was also used for the determination of **III** and **IIIc**.

Based on the MS data shown in Table 3, both of the carbonyl groups at 7- and 14-positions were

Table 1. ^{1}H NMR data (400 MHz, $CDCl_3$) of bis-DNP-derivatives of neoenactins A, NL_1 and NL_2.

Assignment (position)	Chemical shift (δ)		
	IIIa (50°C)	**Ia** (27°C)	**IIa** (27°C)
CH_2CH_3	0.87 (3H, t, J=6.8, 20-H)	0.88 (3H, t, J=7.0, 19-H)	0.87 (3H, t, J=7.0, 21-H)
CH_2	1.27 (10H, m, 10, 11, 17, 18, 19-H)	1.26 (8H, m, 10, 11, 17, 18-H)	1.25 (12H, m, 10, 11, 17, 18, 19, 20-H)
CH_2CH_2CO (9, 12, 16)	1.55 (6H, m)	1.51 (6H, m)	1.53 (6H, m)
CH_2CO (8, 13, 15)	2.41 (6H, m)	2.40 (6H, m)	2.40 (6H, m)
NCH_2CH_2CO (6)	2.83 (2H, t, J=5.9)	2.85 (2H, t, J=5.6)	2.85 (2H, t, J=5.9)
NCH_2CH_2CO (5)	4.03 (1H, dt, J=14.8, 5.9), 4.24 (1H, dt, J=14.8, 5.9)	4.03 (1H, dt, J=14.4, 5.6), 4.29 (1H, dt, J=14.4, 5.6)	4.01 (1H, dt, J=15.0, 5.9), 4.28 (1H, dt, J=15.0, 5.9)
$HOCH_2$ (1)	4.09 (1H, ddd, J=5.5, 5.6, 11.8), 4.13 (1H, ddd, J=5.5, 5.6, 11.8)	4.09 (1H, ddd, J=5.2, 5.2, 11.8), 4.18 (1H, ddd, J=5.2, 5.2, 11.8)	4.09 (1H, ddd, J=5.5, 5.5, 11.0), 4.17 (1H, ddd, J=5.5, 5.5, 11.0)
NHCHCO (2)	4.86 (1H, br m)	4.88 (1H, br m)	4.87 (1H, br m)
H-Ar (6′)	6.88 (1H, d, J=9.0)	6.89 (1H, d, J=9.5)	6.88 (1H, d, J=9.0)
H-Ar (6″)	7.60 (1H, d, J=9.0)	7.64 (1H, d, J=9.5)	7.62 (1H, d, J=9.0)
H-Ar (5′)	8.27 (1H, dd, J=2.8, 9.0)	8.24 (1H, dd, J=2.5, 9.5)	8.26 (1H, dd, J=2.4, 9.0)
H-Ar (5″)	8.52 (1H, dd, J=2.8, 9.0)	8.53 (1H, dd, J=2.5, 9.5)	8.52 (1H, dd, J=2.4, 9.0)
H-Ar (3′)	9.12 (1H, d, J=2.8)	9.10 (1H, d, J=2.5)	9.07 (1H, d, J=2.4)
H-Ar (3″)	8.95 (1H, d, J=2.8)	8.94 (1H, d, J=2.5)	8.94 (1H, d, J=2.4)
CNHAr	9.18[a](1H, d)	9.17[a](1H, d)	9.17[a](1H, d)

^{1}H NMR spectra were recorded on a Jeol GX 400 spectrometer. TMS (0 ppm) was used as an internal standard. Number of protons, multiplicity, coupling constants in Hz and position of protons where necessary are indicated in parenthesis.

[a] Temperature dependent.

Table 2. ^{13}C NMR data (100 MHz, 50°C, $CDCl_3$) of bis-DNP-derivatives of neoenactins A, NL_1 and NL_2.

Position	Chemical shift (δ)			Position	Chemical shift (δ)		
	IIIa	**Ia**	**IIa**		**IIIa**	**Ia**	**IIa**
C-1	62.08	62.20	62.30	C-18	31.58	22.37	29.03
C-2	56.17	56.33	56.30	C-19	22.47	13.77	31.70
C-3	172.50	172.44	172.57	C-20	13.99		22.57
C-5	43.50	43.81	43.83	C-21			13.95
C-6	38.76	38.76	38.80	C-1′	146.63[c]	146.72[c]	146.68[c]
C-7	208.37	208.05	208.07	C-2′	131.53[c]	131.70[c]	131.83[c]
C-8	42.86[a]	42.85[a]	42.94[a]	C-3′	124.22	124.07	124.16
C-9	23.36[b]	23.45[b]	23.47[b]	C-4′	137.10[c]	137.26[c]	137.44[c]
C-10	28.72	28.78	28.78	C-5′	130.48	130.38	130.45
C-11	28.72	28.78	28.78	C-6′	113.97	114.09	113.98
C-12	23.19[b]	23.28[b]	23.30[b]	C-1″	154.85	154.91	154.87
C-13	42.53[a]	42.50[a]	42.53[a]	C-2″	137.28[c]	137.40[c]	137.44[c]
C-14	212.08	211.60	211.63	C-3″	122.81	122.65	122.71
C-15	42.92	42.85	42.94	C-4″	143.01[c]	143.15[c]	143.22[c]
C-16	23.84	23.58	23.98	C-5″	129.92	129.76	129.76
C-17	28.91	31.43	29.26	C-6″	115.43	115.56	115.47

^{13}C NMR spectra were recorded on a Jeol GX 400 spectrometer.
TMS (0 ppm) was used as an internal standard.
[a~c] Values with identical superscript within a column may be interchanged.

Table 3. Synthesis and purification of neoenactin A derivatives.

Compound	Reagent	Reaction time (hours)	Rf values on TLC		Method of purification	Yield (%)	MS m/z (M+H)
			I[a]	II[b]			
III			0.32	0.45			
IIIc	$H_2NNHCONH_2 \cdot HCl$	1.5	0.24	0.37	A[c]	42	487 (FAB)
IIId	$H_2NNHCONHC_6H_5 \cdot HCl$	1.5	0.46	0.53	C[e]	59	639 (FAB, FD)
IIIe	$H_2NNHCOOC_2H_5$	2.0	0.48	0.53	B[d]	53	545 (FAB)
IIIf	$H_2NOH \cdot HCl$	0.6	0.26	0.39	A+HPLC[f]	36	403 (FAB, FD)
IIIg	$H_2NOCH_3 \cdot HCl$	2.8	0.49	0.53	B	67	431 (FAB)
IIIh	$H_2NOCH_2C_6H_4NO_2 \cdot HCl$	2.0	0.59	0.61	A	20	673 (FAB)

[a] Kieselgel $60F_{254}$ plate; 0.25 mm thickness, E. Merck, Darmstadt, solvent: $CHCl_3$ - MeOH (2 : 1).
[b] Kieselgel $60F_{254}$ plate; 0.25 mm thickness, E. Merck, Darmstadt, solvent: $CHCl_3$ - MeOH - H_2O (12 : 6 : 1).
[c] Silica gel column chromatography, solvent: $CHCl_3$ - MeOH (9 : 1).
[d] Silica gel column chromatography, solvent: $CHCl_3$ - MeOH (19 : 1).
[e] Repeated crystallization from MeOH.
[f] Radial Pak Nova Pak C_{18} Cartridge (8×100 mm), mobile phase: MeOH - 0.05 M KH_2PO_4 (3 : 2, pH 2.8), flow rate: 2.0 ml/minute.

confirmed to be substituted with individual carbonyl reagents in all the cases, and the structures shown in Fig. 1 were proposed, though the geometrical structures at 7- and 14-positions still remained to be solved.

Antimicrobial Activity of NE-A Derivatives

The antimicrobial activities of the NE-A derivatives were studied in comparison with that of **III** and the results are summarized in Table 4. The derivatives **IIIc** and **IIIe** were as potent as **III**, whereas the antimicrobial activities of **IIId**, **IIIf**, **IIIg** and **IIIh** were negligible. In general, the hydrazone type

Table 4. Antimicrobial spectra of neoenactin A derivatives.

Test organism	MIC (μg/ml)						
	III	**IIIc**	**IIId**	**IIIe**	**IIIf**	**IIIg**	**IIIh**
Candida tropicalis NI 7495	0.05	0.39	25	0.39	3.13	3.13	50
C. pseudotropicalis NI 7494	0.095	0.095	1.56	0.095	0.78	0.78	12.5
C. albicans Yu 1200	0.78	0.39	50	0.78	3.13	12.5	50
C. albicans MTU 12013	0.39	0.39	25	0.78	1.56	12.5	25
Saccharomyces cerevisiae	0.19	0.095	12.5	0.19	0.39	0.39	12.5
Alternaria kikuchiana	1.56	0.39	25	0.78	6.25	12.5	50
Glomerella cingulata	0.39	0.047	0.78	<0.024	3.13	3.13	>50
Gloeosporium laeticolor	0.78	0.39	25	0.39	6.25	25	>50
Trichophyton mentagrophytes (833)	3.13	1.56	50	1.56	6.25	6.25	>50
Aspergillus niger F-16	>50	50	>50	50	50	25	50
Pyricularia oryzae	0.19	0.19	12.5	0.19	0.39	0.78	25
Helminthosporium oryzae	0.19	0.05	1.56	0.095	0.78	3.13	25

MICs were determined by the agar dilution method on glucose nutrient agar.

Table 5. Stability of neoenactin A semicarbazone.

Time (hours)	**III** (μg/ml)/**IIIc** (μg/ml)					
	MeOH	H_2O	pH 2.8	pH 5.0	pH 6.0	pH 7.5
0	0/250	0/250	30/200	40/210	0/250	0/250
4	50/200	110/100	190/ 20	150/ 40	120/ 60	70/150
18	60/170	130/ 20	200/ 20	130/ 20	100/ 20	60/ 20

The semicarbazone **IIIc** was dissolved in MeOH, H_2O or 1/30 M phosphate buffer of different pH's at the concentration of 250 μg/ml and kept at room temperature for the period indicated. The concentrations of **III** and **IIIc** in the solution was determined by HPLC on a Radial Pak Nova Pak C_{18} cartridge eluted with MeOH - 0.05 M KH_2PO_4 (3 : 2, pH 2.8) at a flow rate of 2.7 ml/minute. The retention times of **III** and **IIIc** were 9.1 and 7.3 minutes, respectively.

derivatives (**IIIc**~**IIIe**) were more active than the oxime type derivatives (**IIIf**~**IIIh**) except for **IIId** which was hardly soluble in water and organic solvents such as MeOH and $CHCl_3$.

Stability of NE-A Derivatives

The stability of **IIIc** in various solvents is shown in Table 5. The semicarbazone **IIIc** proved to be stable in MeOH but not in H_2O, especially at acidic pH, being almost completely converted to **III** at room temperature within 4 hours. The loss in a combined amount of **III** and **IIIc** from the original one of **IIIc** at neutral and alkaline pH's might result from easy decomposition of **III** at pH's in this range. In contrast, the conversion to **III** from **IIIg**, a representative of the oxime type derivatives, was less than 1% in 1/30 M phosphate buffer (pH 6.0) even after 18 hours (data not shown). Based on these observations and the results for the other derivatives, the antimicrobial activities of the NE-A derivatives seemingly depend on their tendency to be hydrolyzed to **III** which, in turn, inhibits the growth of *C. albicans*.

Discussion

As described above, the existence of free $-NH_2$ and $>NOH$ groups appears to be essential for the biological activity of **III**. L-Serine hydroxamic acid was also inactive at concentrations as high as 100 μg/ml, implying the important role of the diketo amine moiety. In the present paper, it was confirmed that the carbonyl groups at the 7- and 14-positions were required for **III** to exhibit antimycotic

activity. Because NE-M_2, in which the carbonyl group at the 14-position is reduced to the hydroxyl group, is as potent as **III**, the function of the carbonyl group at the 14-position still remains to be elucidated.

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