A NEW GROUP OF ANTIBIOTICS, HYDROXAMIC ACID ANTIMYCOtic ANTIBIOTICS

II. THE STRUCTURE OF NEOENACTINS NL₁ AND NL₂ AND STRUCTURE-ACTIVITY RELATIONSHIP

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The structures of neoenactins (NEs) NL₁ and NL₂, novel antimycotic antibiotics produced by Streptoverticillium olivoreticuli in a precursor-oriented fashion, were elucidated by \(^1\)H 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\(^4,5\) are antimycotic antibiotics potentiating activities of polyene antifungal antibiotics and antitumor agents such as bleomycin and vincristine\(^6\). 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-B₁, -B₂, -M₁ (lipoxamycin) and M₂ have been reported\(^7-10\) 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-NL₁ (I) and -NL₂ (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\(^12\).

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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>X</th>
<th>Y</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
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<td>NE-NL₁ (I)</td>
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<td>H</td>
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<tr>
<td>Bis-DNP-NE-NL₁ (Ia)</td>
<td></td>
<td></td>
<td>6-5' =O</td>
<td>6-5' =O</td>
<td>H</td>
<td>H</td>
<td>H</td>
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<tr>
<td>NE-NL₂ (II)</td>
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<td>H</td>
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<td>=O</td>
<td>H</td>
<td>H</td>
<td>C₃H₅</td>
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<tr>
<td>Bis-DNP-NE-NL₂ (IIa)</td>
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<td>DNP</td>
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<td>=O</td>
<td>H</td>
<td>H</td>
<td>C₃H₅</td>
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<tr>
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<td>H</td>
<td>H</td>
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<td>=O</td>
<td>H</td>
<td>H</td>
<td>C₂H₆</td>
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<tr>
<td>Bis-DNP-NE-A (IIia)</td>
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<td>=O</td>
<td>H</td>
<td>H</td>
<td>C₂H₆</td>
</tr>
<tr>
<td>Mono-DNP-NE-A (IIIb)</td>
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<td>CH₃</td>
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<td>H</td>
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<tr>
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<td>=O</td>
<td>H</td>
<td>H</td>
<td>C₂H₆</td>
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<tr>
<td>NE-M₂</td>
<td>H</td>
<td>H</td>
<td>=O</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>C₂H₆</td>
</tr>
<tr>
<td>NE-B₁</td>
<td>H</td>
<td>H</td>
<td>=O</td>
<td>=O</td>
<td>CH₃</td>
<td>H</td>
<td>CH₃</td>
</tr>
<tr>
<td>NE-B₂</td>
<td>H</td>
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<td>=O</td>
<td>=O</td>
<td>H</td>
<td>CH₃</td>
<td>CH₃</td>
</tr>
<tr>
<td>IIIc</td>
<td>H</td>
<td>H</td>
<td>=NNHCONH₂</td>
<td>=NNHCONH₂</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
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<tr>
<td>IIId</td>
<td>H</td>
<td>H</td>
<td>=NNHCONHC₃H₅</td>
<td>=NNHCONHC₃H₅</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
</tr>
<tr>
<td>IIIe</td>
<td>H</td>
<td>H</td>
<td>=NNHCOOC₄H₆</td>
<td>=NNHCOOC₄H₆</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
</tr>
<tr>
<td>IIIf</td>
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<td>H</td>
<td>=NOH</td>
<td>=NOH</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
</tr>
<tr>
<td>IIIg</td>
<td>H</td>
<td>H</td>
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<td>H</td>
<td>H</td>
<td>CH₃</td>
</tr>
<tr>
<td>IIIb</td>
<td>H</td>
<td>H</td>
<td>=NOCH₂C₄H₄NO₂</td>
<td>=NOCH₂C₄H₄NO₂</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
</tr>
</tbody>
</table>
Candida albicans in vitro (data not shown), the free –NH₂ 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-NL₁ and -NL₂

The existence of a free hydroxamic acid-type structure in I and II was strongly suggested by the positive FeCl₃ test. Further, the recovery of L-serine from the acid hydrolysates of I and II was proven by HPLC on an enantiomeric resolution column (TSK gel Enantio L2, 4.6 x 150 mm, Tosoh Co., Ltd., Tokyo) using 0.5 mM CuSO₄ as a mobile phase. Both I and II were converted to the corresponding stable bis-DNP-derivatives according to the previous method [Ia (mp 140°C) and IIa (mp 145°C), respectively]. The molecular formula C₁₉H₃₆N₂O₅ and C₂₀H₄₈N₂O₅ were proposed for I and II, respectively, based on the results of fast atom bombardment (FAB)-MS of I and II, and ¹H and ¹³C NMR spectra of Ia and IIa. Since the molecular formula C₁₉H₃₄N₂O₅ had been reported for III, I and II can be considered to contain one more and less CH₂ group, respectively, when compared with III.

The ¹H NMR data (400 MHz, CDCl₃) 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 CH₂ 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 99.0310 (calcd for C₆H₇O⁺ : 99.0809) and m/z 71.0856 (calcd for C₆H₁₁⁺ : 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 C₈H₁₅O⁺ : 127.1123) postulated that one of the carbonyl groups is also located at 14-position in II. The ¹³C NMR data (100 MHz, CDCl₃) of Ia and IIa and their assignments are shown in Table 2 together with those of IIIa. 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 60F₂₅₄ plate; 0.25 mm thickness, E. Merck, Darmstadt) using CHCl₃ - MeOH - H₂O (12 : 6 : 1) as a developing solvent and 5% FeCl₃ 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 CHCl₃ - MeOH (9 : 1, Method A) or CHCl₃ - 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 C₁₈ (8 x 100 mm, Waters Assoc., Mass.) using MeOH - 0.05 mM KH₂PO₄ (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$_x$, and NL$_y$.

<table>
<thead>
<tr>
<th>Assignment (position)</th>
<th>IIIa (50°C)</th>
<th>Ia (27°C)</th>
<th>Ila (27°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_2$CH$_3$</td>
<td>0.87 (3H, t, $J=6.8$, 20-H)</td>
<td>0.88 (3H, t, $J=7.0$, 19-H)</td>
<td>0.87 (3H, t, $J=7.0$, 21-H)</td>
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<tr>
<td>CH$_3$</td>
<td>1.27 (10H, m, 10, 11, 17, 18, 19-H)</td>
<td>1.26 (8H, m, 10, 11, 17, 18-H)</td>
<td>1.25 (12H, m, 10, 11, 17, 18, 19, 20-H)</td>
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<tr>
<td>CH$_2$CO (9, 12, 16)</td>
<td>1.55 (6H, m)</td>
<td>1.51 (6H, m)</td>
<td>1.53 (6H, m)</td>
</tr>
<tr>
<td>CH$_2$CO (8, 13, 15)</td>
<td>2.41 (6H, m)</td>
<td>2.40 (6H, m)</td>
<td>2.40 (6H, m)</td>
</tr>
<tr>
<td>NCH$_2$CH$_2$CO (6)</td>
<td>2.83 (2H, t, $J=5.9$)</td>
<td>2.85 (2H, t, $J=5.6$)</td>
<td>2.85 (2H, t, $J=5.9$)</td>
</tr>
<tr>
<td>NCH$_2$CH$_2$CO (5)</td>
<td>4.03 (1H, dt, $J=14.8$, 5.9)</td>
<td>4.03 (1H, dt, $J=14.4$, 5.6)</td>
<td>4.01 (1H, dt, $J=15.0$, 5.9)</td>
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<tr>
<td>HOCH$_2$ (1)</td>
<td>4.13 (1H, ddd, $J=5.5$, 5.6, 11.8)</td>
<td>4.18 (1H, ddd, $J=5.5$, 5.2, 11.8)</td>
<td>4.17 (1H, ddd, $J=5.5$, 5.5, 11.0)</td>
</tr>
<tr>
<td>NHCHCO (2)</td>
<td>4.86 (1H, br m)</td>
<td>4.88 (1H, br m)</td>
<td>4.87 (1H, br m)</td>
</tr>
<tr>
<td>H-Ar (6')</td>
<td>6.88 (1H, d, $J=9.0$)</td>
<td>6.89 (1H, d, $J=9.5$)</td>
<td>6.88 (1H, d, $J=9.0$)</td>
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<tr>
<td>H-Ar (6'')</td>
<td>7.60 (1H, d, $J=9.0$)</td>
<td>7.64 (1H, d, $J=9.5$)</td>
<td>7.62 (1H, d, $J=9.0$)</td>
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<tr>
<td>H-Ar (5')</td>
<td>8.27 (1H, dd, $J=2.8$, 9.0)</td>
<td>8.24 (1H, dd, $J=2.5$, 9.5)</td>
<td>8.26 (1H, dd, $J=2.4$, 9.0)</td>
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<tr>
<td>H-Ar (5'')</td>
<td>8.52 (1H, dd, $J=2.8$, 9.0)</td>
<td>8.53 (1H, dd, $J=2.5$, 9.5)</td>
<td>8.52 (1H, dd, $J=2.4$, 9.0)</td>
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<tr>
<td>H-Ar (3')</td>
<td>9.12 (1H, d, $J=2.8$)</td>
<td>9.10 (1H, d, $J=2.5$)</td>
<td>9.07 (1H, d, $J=2.4$)</td>
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<tr>
<td>H-Ar (3'')</td>
<td>8.95 (1H, d, $J=2.8$)</td>
<td>8.94 (1H, d, $J=2.5$)</td>
<td>8.94 (1H, d, $J=2.4$)</td>
</tr>
<tr>
<td>CNHAr</td>
<td>9.18 (1H, d)</td>
<td>9.17 (1H, d)</td>
<td>9.17 (1H, d)</td>
</tr>
</tbody>
</table>

$^1$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, CDCl₃) of bis-DNP-derivatives of neoenactins A, NL₁ and NL₂.

<table>
<thead>
<tr>
<th>Position</th>
<th>Chemical shift (δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IIIa</td>
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<tr>
<td>C-1</td>
<td>62.08</td>
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<tr>
<td>C-2</td>
<td>56.17</td>
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<td>C-3</td>
<td>172.50</td>
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<td>C-5</td>
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<td>C-6</td>
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<td>C-7</td>
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<td>C-8</td>
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<td>C-6''</td>
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$^{13}$C NMR spectra were recorded on a Jeol GX 400 spectrometer. TMS (0 ppm) was used as an internal standard.

Values with identical superscript within a column may be interchanged.

Table 3. Synthesis and purification of neoenactin A derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reagent</th>
<th>Reaction time (hours)</th>
<th>Rf values on TLC (Ia)</th>
<th>Method of purification</th>
<th>Yield (%)</th>
<th>MS m/z (M+H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>H₂NNHCONH₂·HCl</td>
<td>0.32</td>
<td>0.45</td>
<td>A⁻</td>
<td>42</td>
<td>487 (FAB)</td>
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<tr>
<td>IIIc</td>
<td>H₂NNHCONHC₆H₅·HCl</td>
<td>1.5</td>
<td>0.24</td>
<td>0.37</td>
<td>C</td>
<td>59</td>
</tr>
<tr>
<td>IIId</td>
<td>H₂NNHCOOC₂H₅·HCl</td>
<td>1.5</td>
<td>0.46</td>
<td>0.53</td>
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<td>53</td>
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<td>H₂NNHCOOC₂H₅·HCl</td>
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<td>0.48</td>
<td>0.53</td>
<td>B</td>
<td>53</td>
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<tr>
<td>IIIf</td>
<td>H₂NOH·HCl</td>
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<td>0.39</td>
<td>A+HPLC</td>
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<td>IIIg</td>
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<td>0.53</td>
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<td>67</td>
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<td>0.59</td>
<td>0.61</td>
<td>A</td>
<td>20</td>
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Kieselgel 60F₂₅₄ plate; 0.25 mm thickness, E. Merck, Darmstadt, solvent: CHCl₃ - MeOH (2 : 1).
Kieselgel 60F₂₅₄ plate; 0.25 mm thickness, E. Merck, Darmstadt, solvent: CHCl₃ - MeOH - H₂O (12 : 6 : 1).
Silica gel column chromatography, solvent: CHCl₃ - MeOH (9 : 1).
Silica gel column chromatography, solvent: CHCl₃ - MeOH (19 : 1).
Repeated crystallization from MeOH.
Radial Pak Nova Pak C₁₈ Cartridge (8 x 100 mm), mobile phase: MeOH - 0.05 M KH₂PO₄ (3 : 2, pH 2.8), flow rate: 2.0 ml/minute.

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 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.
Table 4. Antimicrobial spectra of neoenactin A derivatives.

<table>
<thead>
<tr>
<th>Test organism</th>
<th>MIC (µg/ml)</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>III</td>
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<td>IIId</td>
<td>IIIe</td>
<td>IIIf</td>
<td>IIIg</td>
<td>IIIh</td>
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<td>Candida tropicalis NI 7495</td>
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<td>25</td>
<td>0.39</td>
<td>3.13</td>
<td>3.13</td>
<td>50</td>
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<tr>
<td>C. pseudotropicalis NI 7494</td>
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<td>0.095</td>
<td>1.56</td>
<td>0.095</td>
<td>0.78</td>
<td>0.78</td>
<td>12.5</td>
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<tr>
<td>C. albicans Yu 1200</td>
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<td>0.39</td>
<td>50</td>
<td>0.78</td>
<td>3.13</td>
<td>12.5</td>
<td>50</td>
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<tr>
<td>C. albicans MTU 2013</td>
<td>0.39</td>
<td>0.39</td>
<td>25</td>
<td>0.39</td>
<td>1.56</td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>0.19</td>
<td>0.095</td>
<td>12.5</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
<td>12.5</td>
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<tr>
<td>Alternaria kikuchiana</td>
<td>1.56</td>
<td>0.39</td>
<td>25</td>
<td>0.78</td>
<td>6.25</td>
<td>12.5</td>
<td>50</td>
</tr>
<tr>
<td>Glomerella cingulata</td>
<td>0.39</td>
<td>0.047</td>
<td>0.78</td>
<td>&lt;0.024</td>
<td>3.13</td>
<td>3.13</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Gloeosporium laeticolor</td>
<td>0.78</td>
<td>0.39</td>
<td>25</td>
<td>0.39</td>
<td>6.25</td>
<td>25</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Trichophyton mentagrophytes (833)</td>
<td>3.13</td>
<td>1.56</td>
<td>50</td>
<td>1.56</td>
<td>6.25</td>
<td>6.25</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Aspergillus niger F-16</td>
<td>&gt;50</td>
<td>50</td>
<td>&gt;50</td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Pyricularia oryzae</td>
<td>0.19</td>
<td>0.19</td>
<td>12.5</td>
<td>0.19</td>
<td>0.39</td>
<td>0.78</td>
<td>25</td>
</tr>
<tr>
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<td>0.19</td>
<td>0.05</td>
<td>1.56</td>
<td>0.39</td>
<td>0.78</td>
<td>3.13</td>
<td>25</td>
</tr>
</tbody>
</table>

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

Table 5. Stability of neoenactin A semicarbazone.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>MeOH</th>
<th>H2O</th>
<th>pH 2.8</th>
<th>pH 5.0</th>
<th>pH 6.0</th>
<th>pH 7.5</th>
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<tr>
<td>0</td>
<td>0/250</td>
<td>0/250</td>
<td>30/200</td>
<td>40/210</td>
<td>0/250</td>
<td>0/250</td>
</tr>
<tr>
<td>4</td>
<td>50/200</td>
<td>110/100</td>
<td>190/20</td>
<td>150/40</td>
<td>120/60</td>
<td>70/150</td>
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<tr>
<td>18</td>
<td>60/170</td>
<td>130/20</td>
<td>200/20</td>
<td>130/20</td>
<td>100/20</td>
<td>60/20</td>
</tr>
</tbody>
</table>

The semicarbazone IIIc was dissolved in MeOH, H2O 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 C18 cartridge eluted with MeOH - 0.05 m KH2PO4 (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 IIIf which was hardly soluble in water and organic solvents such as MeOH and CHCl3.

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 H2O, 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 –NH2 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-M2, 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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References