**Note**

**Ansamycin Antibiotics as Free Radical Scavengers Isolated from *Streptomyces* by Using the Bactericidal Action of the Hydroxyl Radical**

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Ansamycin antibiotics (1–4) were isolated from the cultured broth of *Streptomyces* sp. USF-319 strain as a result of our screening for free radical scavengers. They inhibited the bactericidal effect of the Fenton reagent toward *Bacillus subtilis* by their radical scavenging activity. Some of them also showed inhibitory activity against lipid peroxidation and lipooxygenases.

**Key words:** radical scavenger; Fenton reaction; bactericidal action; *Streptomyces*; ansamycin antibiotics

Free radicals have been reported to affect the initiation and progression of various diseases, including inflammatory disorders, atherosclerosis, ischemia-reperfusion injury, and carcinogenesis. Free radical scavengers are consequently expected to be applicable as therapeutic agents for these diseases. The hydroxyl radical is one of the highly reactive free radicals in vitro and can damage the biological molecules of living organisms. An accumulation of oxidative injury to membrane lipids, tissue proteins, enzymes, and DNA results in cell death. The hydroxyl radical generated from the iron(II)-ascorbate complex has been shown to have a killing effect on various bacteria. Considered from a different point of view, this bactericidal action makes it possible to obtain cytotoxic radical scavengers. To put it another way, the cytotoxic activity of the hydroxyl radical on bacteria is applicable to screening for free radical scavengers. One good attempt has recently been reported in which the bactericidal action of the alkyl peroxyl radical from heme-iron and organic peroxide was applied to determine the radical scavenging activity in food.

In this report, we describe the development and employment of a simple screening method for free radical scavengers. In the course of our screening, four ansamycin antibiotics (1–4) were isolated from the culture filtrate of *Streptomyces* USF-319 strain, which itself had been isolated from a soil sample collected in Yubari-city, Hokkaido, Japan. The isolation, chemical structures, and radical scavenging activity of 1–4 are also described. In addition, the inhibitory activity of 1–4 on lipid peroxidation and lipooxygenases was examined.

The bactericidal action of free radicals on an agar plate was investigated. Two Gram-positive and two Gram-negative bacteria were exposed to a cytotoxic treatment with iron(II), bovine hemoglobin, ascorbate, hydrogen peroxide (H₂O₂), and t-butyldihydroperoxide under many different conditions (data not shown). The Gram-positive bacteria, *Staphylococcus aureus* and *B. subtilis*, were more sensitive to the bactericidal effect of the hydroxyl radical generated from the Fenton reagent (iron-ascorbate–H₂O₂). Finally, *B. subtilis* was chosen as the indicator organism for this assay because of the appearance of a clear bactericidal zone on agar.

*Streptomyces* USF-319 strain was cultivated for 4 days at 30°C in a modified Bennet medium on a rotary shaker. The culture filtrate was extracted with ethyl acetate (EtOAc), and the extract was repeatedly separated in a silica gel column. Finally, four radical scavengers were isolated and identified as known ansamycin antibiotics, mycotrienin I (1), mycotrienin II (2), trienomycin A (3), and trienomycin B (4), by spectroscopic analyses (Fig.). The spectral data for 1–4 are identical with the reported data for 1, 2, 3, 11, 13, and 14 respectively. The identification of 1–4 was further confirmed by HSCC and HMBC experiments (data not shown).

The inhibitory activity of four compounds on the bactericidal action of the Fenton reaction was examined (Table 1). The inhibitory activity of both 1 and 2 was superior to that of known antioxidant BHT by 2–16 times with both induction methods. On the other hand, 3 and 4 were almost equivalent to BHT with induction by iron(II)EDTA-ascorbate–H₂O₂, but 4 times as potent as BHT with induction by ascorbate. These results suggest that the induction by ascorbate was more moderate for producing the hydroxyl radical than with the other method.

For a further investigation, the antioxidative and DPPH radical scavenging activities of 1–4 were measured. Compounds 2, 3, and 4, as well as BHT, had mild activity for suppressing lipid peroxidation (percentage inhibition of TBARS formation at 100 μM: 2, 46%; 3, 47%; 4, 42%; and BHT, 75%). Because of the poor solubility of 1–4 in this assay solution, their IC₅₀ values

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**Fig.** Structures of Mycotrienin I (1), Mycotrienin II (2), Trienomycin A (3), and Trienomycin B (4).

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**Abbreviations:** HSCC, 1H-detected single-bond heteronuclear multiple quantum coherence; HMBC, 1H-detected heteronuclear multiple bond connectivity; EDTA, ethylene diamine tetraacetic acid; BHA, 3(2)-t-butyl-4-hydroxyanisole; BHT, 2,6-di-t-butyl-4-methylphenol(t-butylated hydroxytoluene); DPPH, 1,1-diphenyl-2-picrylhydrazyl.
Table I. Inhibitory Activities of 1, 2, 3, and 4 on the Bactericidal Action of the Fenton Reaction

A) Induced by Iron(II)/EDTA- H₂O₂ - Ascorbate

<table>
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<tr>
<th>Added amount of sample (µg/disk)</th>
<th>200</th>
<th>100</th>
<th>50</th>
<th>25</th>
<th>12.5</th>
<th>6.25</th>
<th>3.125</th>
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</tr>
<tr>
<td>BHT</td>
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</table>

B) Induced by Ascorbate and Pre-included FeSO₄

<table>
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<th>Added amount of sample (µg/disk)</th>
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<th>50</th>
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<th>12.5</th>
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</table>

* The sample showed antimicrobial activity at the tested concentration.
* The sample clearly inhibited the bactericidal action.
* The sample changed the shape of the bactericidal zone.

Table II. DPPH Radical Scavenging and Lipoygenase Inhibitory Activities of 1, 2, 3, and 4

<table>
<thead>
<tr>
<th>DPPH radical (µM)</th>
<th>Soybean LO* (µM)</th>
<th>Human 5-LO (µM)</th>
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</thead>
<tbody>
<tr>
<td>ED₁₀₀²µg</td>
<td>IC₅₀₂µg</td>
<td>IC₅₀₂µg</td>
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<td>&gt;100</td>
<td>&gt;500</td>
</tr>
<tr>
<td>2</td>
<td>16.5</td>
<td>230</td>
</tr>
<tr>
<td>3</td>
<td>&gt;100</td>
<td>&gt;500</td>
</tr>
<tr>
<td>4</td>
<td>&gt;100</td>
<td>&gt;500</td>
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<tr>
<td>BHT</td>
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</tr>
<tr>
<td>α-Tocopherol</td>
<td>25.0</td>
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* Lipoygenase.
* The ED₁₀₀ values were determined as the concentration of the compound that gave a 50% decrease in the absorbance from the blank test.
* The IC₅₀ values were determined as the concentration of the compound that gave a 50% inhibition of lipoygenase activity compared with the control test.

could not be determined. Needless to say, these compounds had more potent inhibitory activity when lipid peroxidation was induced by the Fenton reagent. These results suggest that 2, 3, and 4 could efficiently scavenge the hydroxyl radical generated from the Fenton reaction. In this regard, the DPPH radical scavenging activity of 1-4 was also measured (Table II). The DPPH radical is looked upon as a model compound of a lipophilic radical. The auto-oxidation of a lipid progresses the chain reaction of lipophilic radicals. However, only 2 effectively scavenged the DPPH radical when compared with BHT and α-tocopherol, indicating that their DPPH radical scavenging activity may make little contribution to the inhibition of lipid peroxidation. Further experiments are necessary to confirm this, but their phenol and amide groups may be associated with the antioxidative activity. Compound 1 was isolated by screening for free radical scavengers, but was virtually inactive for linoleate peroxidation. It is thought that this contradiction is attributable to the reducing power of ascorbate in the Fenton reagent; by reduction with ascorbate, 1 was easily converted to 2. Only when the quinone moiety is reduced to the hydroquinone will the radical scavenging activity of 1 be revealed.

In addition, 2 showed inhibitory activity against soybean lipoygenase and human 5-lipoygenase when measured by the methods indicated in our previous report (Table II).15,16 Compared with a positive control AA-861, 2 seemed to have moderate activity. The other three compounds had weak or no activity. This tendency is similar to their DPPH radical scavenging activity. In general, the reduction potential of hydroquinone is higher than that of monophenol.17-19 These results suggest that the reducing activity is related to the lipoygenase inhibition and DPPH radical scavenging activity.

Thus, this study demonstrated that free radical scavengers can be obtained by using the bactericidal action of the hydroxyl radical as a simple and convenient screening method. This paper is the first to note that certain ansamycin antibiotics have radical scavenging, antioxidative, and lipoygenase inhibitory activities.

Experimental

Spectrophotometric measurements were taken with the following instruments: JEOL Alpha-400 (NMR) and JEOL JMS-DX705L or JMS-AX505W (MS). Melting point (mp) data were determined on the microscope hot plate of a Yanagimoto Co. MP-33 instrument.

Assay for radical scavenging activity by using the bactericidal action of the Fenton reagent. B. subtilis IFO 12210 was cultured overnight in a test tube with an L-broth medium (pH 7.2, 5 ml) at 30°C. After the incubation, 1 ml of the cultured broth was inoculated into an L-broth agar medium (100 ml), before the medium was poured into 10 Petri dishes (90mm i.d.). After drying the agar surface, a paper disc (6 mm in diameter) soaked with a sample solution was placed on the agar plate at a distance of 5 mm from a paper disc containing the Fenton reagent. The Fenton reaction was started under the following two conditions: One was by adding 10 µl each of ascorbate (500 µM), H₂O₂ (500 µM), and FeSO₄/EDTA (100 µM) to a paper disc. The other was by adding 20 µl of ascorbate (500 µM) to a paper disc on the L-broth agar medium containing FeSO₄ (1 mg/ml). Known radical scavengers tended to change the shape of the bactericidal zone, and some potent radical scavengers obviously reversed the bactericidal action of the Fenton reaction (e.g., BHA and BHT).

Isolation of ansamycin antibiotics from Streptomyces USF-319 strain. Streptomyces USF-319 was inoculated into 300 ml of a modified Bennett medium (pH 7.2) consisting of 1% glucose, 0.2% polypepton, 0.1% yeast extract, and 0.1% beef extract, and cultivated for 4 days at 30°C on a rotary shaker (130 rpm). The fermented broth was filtered, and the filtrate (30 liters) was extracted with EtOAc at pH 3.0. The EtOAc layer was evaporated in vacuo to give an oil residue (3.4 g). This extract was applied to a silica gel column by eluting stepwise with hexane EtOAc, and then respectively with acetone and methanol (MeOH). The eluates from hexane-EtOAc=20:80 and EtOAc alone showed radical scavenging activity. These eluates were then concentrated and passed through a silica gel column, eluting with chloroform (CHCl₃) containing 10% 2-propanol (2-PrOH), connected with a fraction collector (Advantec SF-160). One of the active fractions was evaporated to dryness to yield a yellow powder of 1 (10.8 mg). The other active fractions were further subjected to the silica gel column treatment (CHCl₃, 2-PrOH=95:5) to afford a crude powder of 2 and a mixture of 3 and 4. The EtOAc solution of 2 was precipitated by adding hexane to give a white powder (183.8 mg). The mixture of 3 and 4 was finally separated in the silica gel column (CHCl₃, 2-PrOH=95:5) to obtain white powders of 3 (67.2 mg) and 4 (16.8 mg).

**Myxotericin I (1).** Yellow powder; mp 115°C (117°C)±10; FABMS m/z: 637 (M+H)²⁺; 1³C-NMR δ (CDC₁₃) ppb: 9.6, 17.4, 20.5, 25.5, 25.5, 25.6, 25.9, 29.4, 29.4, 32.9, 33.0, 39.9, 44.8, 44.9, 48.5, 56.6, 68.0, 75.2, 79.2, 114.5, 122.5, 129.3, 129.5, 131.3, 133.1, 133.2, 137.7, 137.9, 139.9, 145.4, 169.7, 172.9, 176.6, 182.5, 188.2.

**Myxotericin II (2).** White powder; mp 150°C (151°C)±10; FABMS m/z:
639 (M + H)+; 13C-NMR (6 (CDCl3): 9.6, 17.1, 20.4, 25.5, 25.6, 25.6, 26.4, 29.4, 29.5, 31.7, 33.7, 38.9, 43.0, 45.0, 48.7, 56.7, 68.6, 75.7, 79.5, 107.4, 115.9, 124.0, 125.3, 129.1, 129.5, 129.5, 132.8, 133.8, 134.4, 134.9, 137.8, 141.3, 149.0, 169.6, 173.3, 176.8.

Trienomycin A (3). White powder; mp 134-136 C (128-132 C)144; HRMS m/z (M + H)+: Calc. for C20H15O3N: 365.1295; Found: 365.1301. 

Trienomycin B (4). White powder; mp 126-128 C (124-126 C)144; FABMS m/z: 597 (M + H)+; 13C-NMR 6 (CDCl3): 9.9, 17.7, 20.4, 25.6, 25.6, 25.7, 29.4, 29.5, 32.2, 36.3, 39.4, 43.0, 45.0, 48.6, 56.8, 68.4, 75.4, 78.8, 105.8, 110.8, 112.2, 124.7, 129.3, 129.6, 130.7, 133.3, 133.7, 134.2, 138.2, 138.6, 144.0, 157.4, 168.8, 173.0, 176.8.

Measurement of lipid peroxidation inhibitors activity. Lipid peroxidation was measured by the formation of thiobarbituric acid-reactive substances (TBARS).138 Linoleate (11.7 mg ml) was incubated in a 12.5 mM phosphate buffer (pH 7.0) containing 5% ethanol (EtOH) at 37 C for 2 days. After the incubation, the reaction solution (1 ml) was mixed with 20% (w/v) trichloroacetic acid (0.5 ml) and 0.67% (w/v) 2-thiobarbituric acid (1 ml). The reaction mixture was then heated in boiling water for 10 min, and centrifuged at 1000 g for 10 min. The TBARS value of the supernatant was measured by the absorbance at 532 nm. The percentage inhibition was calculated by comparison with the control test on the mean values of duplicates.

Measurement of DPPH radical scavenging activity. An EtOH solution of a sample (200 ml) was added to a mixture of a 500 mM DPPH EtOH solution (1 ml), EtOH (2 ml), and 0.1 M acetate buffer (pH 5.5, 2 ml). After Vortex mixing, the mixture was stored at room temperature for 30 min, before the absorbance at 517 nm was measured. The ED50 values were determined as the concentration of the compound that gave a 50% decrease in the absorbance from a blank test.

Reduction from 1 to 2.10 Methanol solution (2 ml) of 1 (3 mg) and ascorbate (2 mg) were mixed and stirred for 30 min at room temperature. The reaction solution was diluted with EtOAc (20 ml) and washed with water. The resulting solution was dried over Na2SO4 and evaporated under reduced pressure to give 2 as a white powder (1.8 mg).

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References