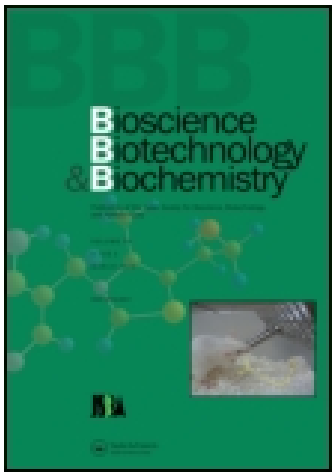

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Ansamycin Antibiotics as Free Radical Scavengers Isolated from Streptomyces by Using the Bactericidal Action of the Hydroxyl Radical

Yasujiro Morimitsu[a] & Akira Hirota[a]

[a] Laboratory of Applied Microbiology, School of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422, Japan

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Note

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

Yasujiro MORIMITSU[†,††] and Akira HIROTA

Laboratory of Applied Microbiology, School of Food and Nutritional Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422, Japan

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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 lipoxygenases.

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,[1] atherosclerosis,[2] ischemia-reperfusion injury,[3] and carcinogenesis.[4] 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.[5–7] 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.[8] Considered from a different point of view, this bactericidal action makes it possible to obtain cytoprotective 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.[9]

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 lipoxygenases 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 (H_2O_2), and/or *t*-butylhydroperoxide 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–H_2O_2). 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 rotatory 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**,[10,11] **2**,[10,11] **3**,[12,13] and **4**,[14] respectively. The identification of **1**–**4** was further confirmed by HSQC and HMBC experiments (data not shown).

The inhibitory activity of four compounds on the bactericidal action of the Fenton reaction was examined (Table I). 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–H_2O_2, 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 IC_{50} values

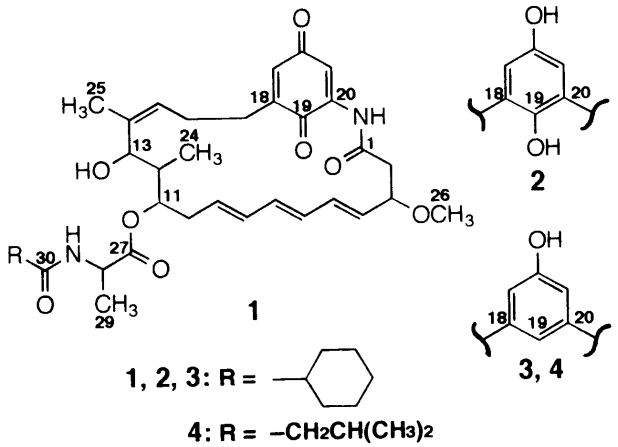

Fig. Structures of Mycotrienin I (**1**), Mycotrienin II (**2**), Trienomycin A (**3**), and Trienomycin B (**4**).

† Author to whom correspondence should be addressed.

†† Present address: *Laboratory of Food and Biodynamics, Nagoya University, School of Agriculture, Nagoya 464-01, Japan.*

Abbreviations: HSQC, ^{1}H-detected single-bond heteronuclear multiple quantum coherence; HMBC, ^{1}H-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(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–H_2O_2–Ascorbate

	Added amount of sample (μg/disk)						
	200	100	50	25	12.5	6.25	3.125
1	*[a]	*	*	+[c]	+	−	−
2	*	*	+	+	−	−	−
3	+ +[b]	+	−	−	−	−	−
4	+ +	+	+	−	−	−	−
BHT	+ +	+	+	−	−	−	−

B) Induced by Ascorbate and Pre-included $FeSO_4$

	Added amount of sample (μg/disk)						
	200	100	50	25	12.5	6.25	3.125
1	*[a]	*	+ +[b]	+ +	+[c]	−	−
2	*	*	+	+	+	+	−
3	+ +	+ +	+	+	−	−	−
4	+ +	+ +	+	+	−	−	−
BHT	+ +	+	−	−	−	−	−

[a] The sample showed antimicrobial activity at the tested concentration.
[b] The sample clearly inhibited the bactericidal action.
[c] The sample changed the shape of the bactericidal zone.

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

	DPPH radical	Soybean LO[a]	Human 5-LO
	ED_{50}[b] (μM)	IC_{50}[c] (μM)	IC_{50} (μM)
1	>100	>500	83.3
2	16.5	230	9.8
3	>100	>500	76.3
4	>100	>500	71.3
BHT	37.0	—	—
α-Tocopherol	25.0	—	—
AA861	—	—	0.4

[a] Lipoxygenase.
[b] The ED_{50} values were determined as the concentration of the compound that gave a 50% decrease in the absorbance from the blank test.
[c] The IC_{50} values were determined as the concentration of the compound that gave a 50% inhibition of lipoxygenase 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 lipoxygenase and human 5-lipoxygenase when measured by the methods indicated in our previous report (Table II).[15] 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.[16,17] These results suggest that the reducing activity is related to the lipoxygenase 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 lipoxygenase inhibitory activities.

Experimental

Spectroscopic 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-J3 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 (90 mm 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 mM), H_2O_2 (500 mM), and $FeSO_4$/EDTA (100 μM) to a paper disc. The other was by adding 20 μl of ascorbate (500 mM) to a paper disc on the L-broth agar medium containing $FeSO_4$ (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. Strain USF-319 was inoculated into 800 ml of a modified Bennet 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 rotatory 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 oily 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 ($CHCl_3$) 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 ($CHCl_3$–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 ($CHCl_3$–2-PrOH = 95 : 5) to obtain white powders of **3** (67.2 mg) and **4** (16.8 mg).

Mycotrienin I (**1**). Yellow powder; mp 115°C (117°C)[10]; FABMS *m/z*: 637 $(M+H)^+$; ^{13}C-NMR δ_C ($CDCl_3$): 9.6, 17.4, 20.5, 25.5, 25.5, 25.6, 25.6, 29.3, 29.4, 29.4, 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, 133.7, 133.7, 137.9, 139.9, 145.4, 169.7, 172.9, 176.6, 182.5, 188.2.

Mycotrienin II (**2**). White powder; mp 150°C (151°C)[10]; FABMS *m/z*:

639 $(M+H)^+$; ^{13}C-NMR (δ_C ($CDCl_3$): 9.6, 17.7, 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)[14]; HRMS *m/z* $(M+H)^+$: Calcd. for $C_{36}H_{51}O_7N_2$; 623.3615. Found: 623.3620; ^{13}C-NMR δ_C ($CDCl_3$): 9.9, 17.7, 20.4, 25.6, 25.6, 25.7, 29.4, 29.5, 29.5, 33.2, 36.3, 39.4, 43.6, 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.

Trienomycin B (**4**). White powder; mp 126–128 °C (124–126 °C)[14]; FABMS *m/z*: 597 $(M+H)^+$; ^{13}C-NMR δ_C ($CDCl_3$): 9.9, 17.7, 20.4, 22.4, 22.4, 26.2, 29.5, 33.2, 36.3, 39.5, 43.6, 45.5, 48.7, 56.8, 68.4, 75.5, 78.7, 105.8, 110.8, 112.2, 124.8, 129.3, 129.5, 130.6, 133.3, 134.2, 134.2, 138.2, 138.5, 144.1, 157.3, 168.8, 172.9, 173.2.

Measurement of lipid peroxidation inhibitory activity. Lipid peroxidation was measured by the formation of thiobarbituric acid-reactive substances (TBARS).[18] Linoleate (11.7 mg/ml) was incubated in a 12.5 mM phosphate buffer (pH 7.0) containing 25% 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 $1000 \times g$ 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.[19] An EtOH solution of a sample (200 μl) was added to a mixture of a 500 μM 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 ED_{50} 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] A MeOH 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 Na_2SO_4 and evaporated under reduced pressure to give **2** as a white powder (1.8 mg).

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