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Lytic Activity of *l*-Menthol Derivatives against the Snow Blight Disease Fungus, *Micronectriella nivalis*

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Lytic activity of *l*-menthol (**1**) derivatives [(−)-(1*S*,3*R*,4*S*,6*S*)-6-hydroxymenthol (**2**), (−)-(1*S*,3*R*,4*S*)-1-hydroxymenthol (**3**), and (+)-(1*S*,3*R*,4*R*,6*S*)-6,8-dihydroxymenthol (**4**)] against the snow blight disease fungus, *Micronectriella nivalis* was investigated. Compounds **2**, **3**, and **4** had 85.0, 63.9, and 81.9% lytic activity, respectively, at a concentration of 0.2 mg/mL. The activity of each of the three compounds increased in a dose−response manner. To study the structure−activity relationship, acetyl esters of **1**−**4** [(−)-menthyl acetate (**1Ac**), (−)-6-hydroxymenthyl diacetate (**2Ac**), (−)-1-hydroxymenthyl 3-monoacetate (**3Ac**), and (−)-6,8-dihydroxymenthyl 3,6-diacetate (**4Ac**)] were synthesized with yields of 80.2−99.8% and were also assayed. The acetyl esters of **1Ac**, **2Ac**, **3Ac**, and **4Ac** had 51.2, 91.5, 66.0, and 95.2% lytic activity, respectively, at a concentration of 0.2 mg/mL, and these compounds showed further high lytic activity compared with the alcohols of **1**−**4**. These acetyl esters also showed higher lytic activity as their concentration was increased. Of particular interest is the fact that **2Ac** and **4Ac** both had higher lytic activity at 0.05−0.2 mg/mL compared with copper 8-hydroxyquinolate, a standard chemical widely used to control snow blight. This is the first report on lytic activity of *l*-menthol derivatives.

KEYWORDS: ***Rhizoctonia solani*; *l*-menthol derivatives; *Micronectriella nivalis* MAFF 305031; lytic activity; copper 8-hydoxyquinolate**

INTRODUCTION

Snow blight brings about significant disease damage on gramineous plants such as wheat, barley, and oat and on leguminous plants in cold snowy districts. This disease is characterized by damage on the plants under the fallen snow, whereby the damaged plant may, after the thawing of the snow, be dead from rotting of the foliage and blighted upon drying or may suffer from retarded growth. Such damage due to snow blight disease is well-known for lawn grass. Because disease occurs beneath the fallen snow, a prophylaxis or therapy for the disease is difficult. To control the disease, organic copper compounds, such as copper 8-hydroxyquinolate, have hitherto been used as an effective component. However, these compounds provoke the problem (*1*, *2*) of environmental pollution in watering canals by rainfall or an accidental flooding. Therefore, it is necessary to find safe control methods alternative to the organic copper compounds. Using a lytic activity of beneficial microorganisms against the pathogen is one such method (*3*).

Our laboratory has studied the biotransformation (*4*) and biological activity (*5*) of terpenoids. Terpenoids are known as raw materials for biologically active substances and are easily available in large amounts from plants or chemical synthesis. A great majority of biologically active terpenoids are produced as plant secondary metabolites, and these terpenoids have been shown to have biological activity against plants, microorganisms, and insects. On the other hand, they serve as flavors and fragrances or can be useful as chiral synthons for chemical synthesis. As the properties of these compounds depend on specific configurations, modifications demand reactions with high stereo- and enantioselectivity. Therefore, biotransformation of terpenoids presents a helpful support in this field. Various attempts have been made to search for new biologically active terpenoids. Biotransformation is one way to produce biologically active terpenoids. For example, biotransformation of terpenoids has been investigated not only in many kinds of microorganisms such as bacteria, yeasts, and fungi (*6*) but also in mammals (*7*) and insects (*8*, *9*).

We previously reported the isolation and structure elucidation of (−)-(1*S*,3*R*,4*S*,6*S*)-6-hydroxymenthol (**2**) as a metabolite of *l*-menthol, in incubation with the soil-borne plant pathogenic fungus *Rhizoctonia solani* (*10*). From the same source, two other compounds, (−)-(1*S*,3*R*,4*S*)-1-hydroxymenthol

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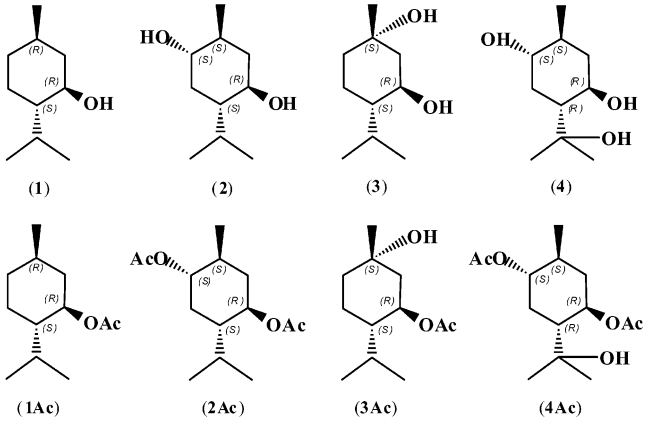

Figure 1. Compounds tested for lytic activity.

(**3**) and (+)-(1*S*,3*R*,4*R*,6*S*)-6,8-dihydroxymenthol (**4**), were isolated. However, there are no reports of the lytic activity of these metabolites.

In this study, the lytic activity of *l*-menthol (**1**) derivatives [(−)-(1*S*,3*R*,4*S*,6*S*)-6-hydroxymenthol (**2**), (−)-(1*S*,3*R*,4*S*)-1-hydroxymenthol (**3**), and (+)-(1*S*,3*R*,4*R*,6*S*)-6,8-dihydroxymenthol (**4**)] against the snow blight disease fungus, *Micronectriella nivalis* was investigated at concentrations of 0.025, 0.05, 0.1, and 0.2 mg/mL, respectively. To study the structure−activity relationship, acetyl esters of **1**−**4** (**Figure 1**) were synthesized and were also assayed. Finally, to compare levels of lytic activity, copper 8-hydroxyquinolate (as a standard chemical used to control snow blight) was also tested, and we discuss the possibility of whether these compounds could be used alternative to the organic compound, copper 8-hydroxyquinolate.

MATERIALS AND METHODS

Materials. *l*-Menthol was purchased from Wako Pure Chemical Industries, Ltd. Acetyl chloride and copper 8-hydroxyquinolate were purchased from Tokyo Kasei Kohgyo Co., Ltd.

Microorganisms. *Micronectriella nivalis* MAFF 305031 isolated from diseased bent grass was used.

General Procedure. Thin-layer chromatography (TLC) was carried out on a 0.25 mm thick silica gel plate (Merck silica gel 60 GF_{254}) in EtOAc/hexane (7:3). For gas chromatography (GC), a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector, a DB-5 capillary column (30 m length, 0.25 mm i.d.), and a split injection of 20:1 were used. Helium at a flow rate of 0.6 cm^3 min^{-1} was used as a carrier gas. The oven temperature was programmed from 80 to 240 °C at 4 °C min^{-1}. The injection temperature was 270 °C, and the detector temperature was 280 °C. The peak area was integrated with a Hewlett-Packard HP3396 series 2 integrator. For gas chromatography−mass spectrometry (GC-MS), a Hewlett-Packard 5890A gas chromatograph equipped with a split injector was combined by direct coupling to a Hewlett-Packard 5972A mass spectrometer; an HP-5MS capillary column (30 m length, 0.25 mm i.d.), and a split injection of 20:1 were used. Helium at 0.6 cm^3 min^{-1} was used as a carrier gas. The temperature of the ion source was 180 °C, and the electron energy was 70 eV. The electron impact (EI) mode was used.

High-resolution mass spectrometry (HR-MS) was carried out with a JEOL-HX100 (with a JEOL JAM-DA 5000 mass data system) instrument. The infrared (IR) spectra were obtained with a Perkin-Elmer 1760X spectrometer. Chloroform was used as a solvent. Nuclear magnetic resonance (NMR) spectra were obtained with a JEOL FX-500 [500 MHz (^{1}H) or 125.65 MHz (^{13}C), chloroform/deuterium with tetramethylsilane (^{1}H) or chloroform (^{13}C) as internal standard] spectrometer.

Lytic Activity Test. *M. nivalis* MAFF 305031 isolated form diseased bent grass was previously grown on potato dextrose agar (PDA) medium at 25 °C for 5 days. Mycelial plugs (diameter = 3 mm) from colonies growing on PDA medium were placed on autoclaved cellophane (7 mm × 7 mm) on water agar media [2% (w/v) agar and 1% (w/v) glucose] and then incubated at 25 °C for 48 h. After 48 h of incubation at 25 °C, the cellophanes were removed from the media and placed on the 300 μL solution [sterilized distilled water and test compound dissolved in DMSO (concentration = 0.025, 0.05, 0.1, and 0.2 mg/mL)] in the well (inside diameter = 10 mm; depth = 15 mm) of 24 well multidish cases and then incubated at 3 °C for 48 h. After 48 h of incubation at 3 °C, the cellophanes were removed from the case and placed on microscopic slides. The hyphal area was stained with 5% cotton blue. The existence ratio of stained and nonstained hyphe was counted using the microscope (×40). The existence ratio was necessary for evaluation.

(−)-(1S,3R,4S,6S)-6-Hydroxymenthol (2): colorless needlelike crystal; mp 143 °C; $[\alpha]^{25}_D$ −42.0° ($CHCl_3$; *c* 0.4); HR-MS, *m*/*z* 172.1465; EI-MS, *m*/*z* (rel int) 154 (15), 139 (48), 121 (13), 111 (15), 97 (40), 95 (17), 83 (33), 69 (37), 67 (10), 55 (67), 43 (100); IR, υ_{max} cm^{-1} 3264, 1450, 1343, 1029; ^{13}C NMR δ 15.9 (C-9), 18.1 (C-10), 20.9 (C-8), 25.7 (C-7), 32.4 (C-5), 38.3 (C-1), 42.5 (C-2), 48.5 (C-4), 70.6 (C-3), 75.9 (C-6); ^{1}H NMR (500.00 MHz, in $CDCl_3$, TMS as internal standard) δ 0.82 (3H, d, *J* = 7.0 Hz, H-7), 0.99 (3H, d, *J* = 7.1 Hz, H-9), 1.03 (3H, d, *J* = 7.2 Hz, H-10), 2.15 (1H, dd, *J* = 7.2, 7.1 Hz, H-8), 3.19 (1H, ddd, *J* = 11.2, 11.0, 3.9 Hz, H-6), 3.76 (1H, m, H-3).

(−)-(1S,3R,4S)-1-Hydroxymenthol (3): colorless needlelike crystal; mp 164 °C; $[\alpha]^{25}_D$ −38.6° ($CHCl_3$; *c* 1.0); HR-MS, *m*/*z* 172.1463; EI-MS, *m*/*z* (rel int) 154 (8), 139 (62), 121 (12), 111 (20), 97 (13), 87 (76), 81 (15), 71 (48), 67 (8), 55 (34), 43 (100); IR, υ_{max} cm^{-1} 3342, 1463, 1348, 1051; ^{13}C NMR δ 16.4 (C-9), 19.3 (C-5), 20.9 (C-10), 26.1 (C-8), 31.6 (C-8), 38.6 (C-6), 48.4 (C-4), 50.2 (C-2), 68.6 (C-3), 71.4 (C-1); ^{1}H NMR (500.00 MHz, in $CDCl_3$, TMS as internal standard) δ 0.85 (3H, d, *J* = 7.0 Hz, H-9), 0.95 (3H, d, *J* = 7.0 Hz, H-10), 1.24 (3H, s, H-7), 3.76 (1H, ddd, *J* = 11.0, 11.1, 4.0 Hz, H-3).

(+)-(1S,3R,4R,6S)-6,8-Dihydroxymenthol (4): colorless oil; $[\alpha]^{25}_D$ +5.2° ($CHCl_3$; *c* 1.0); HR-MS, *m*/*z* 188.1413; EI-MS, *m*/*z* (rel int) 173 (1), 155 (1), 137 (3), 116 (22), 94 (80), 83 (10), 79 (30), 70 (11), 58 (100), 53 (4), 43 (47); IR, υ_{max} cm^{-1} 3332, 1369, 1159, 1022; ^{13}C NMR δ 17.9 (C-7), 23.7 (C-9), 30.1 (C-10), 36.1 (C-5), 38.1 (C-1), 42.2 (C-2), 51.8 (C-4), 72.0 (C-3), 74.8 (C-8), 75.6 (C-6); ^{1}H NMR (500.00 MHz, in $CDCl_3$, TMS as internal standard) δ 1.02 (3H, d, *J* = 7.0 Hz, H-7), 1.22 (3H, s, H-9), 1.24 (3H, s, H-10), 3.21 (1H, ddd, *J* = 10.5, 10.2, 4.3 Hz, H-6), 3.77 (1H, ddd, *J* = 10.3, 10.1, 4.1 Hz, H-3).

Synthesis of Acetyl Esters. Acetyl chloride (1.2 equiv mol) was added dropwise with stirring to *l*-menthol (**1**) (20.0 mg, 1.0 equiv mol), (−)-6-hydroxymenthol (**2**) (20.0 mg, 0.5 equiv mol), (−)-1-hydroxymenthol (**3**) (20.0 mg, 1.0 equiv mol), and (+)-6,8-dihydroxymenthol (**4**) (20.0 mg, 0.5 equiv mol), respectively, and pyridine (0.6−1.2 equiv mol) in an organic solvent ($CHCl_3$) (30 mL). Then, the solution was heated at 25 °C for 1 h. After cooling, the mixture was poured into water (100 mL) and the aqueous phase was extracted thoroughly with $CHCl_3$. The combined $CHCl_3$ extracts were washed successively three times with 5% HCl (100 mL each time), three times with 5% $NaHCO_3$ (100 mL each time), and then with water (100 mL), dried with Na_2SO_4, and evaporated to give a colorless oil. This oil was chromatographed on silica gel (eluent: hexane/EtOAc) to give each acetate (**1Ac**, **2Ac**, **3Ac**, and **4Ac**) of compounds **1**−**4**.

(−)-(1S,3R,4S)-Menthyl monoacetate (1Ac): colorless oil; $[\alpha]^{25}_D$ −118.9° ($CHCl_3$; *c* 1.0); HR-MS, *m*/*z* 198.1624; EI-MS, *m*/*z* (rel int) 183 (1), 165 (1), 138 (40), 123 (31), 109 (8), 95 (87), 82 (24), 81 (56), 67 (19), 55 (24), 43 (100); IR, υ_{max} cm^{-1} 2956, 1732, 1462, 1372, 1241, 1051; ^{13}C NMR δ 16.3 (C-9), 20.7 (C-10), 21.3 ($\underline{C}H_3CO$), 22.0 (C-7), 23.5 (C-5), 26.3 (C-8), 31.4 (C-1), 34.3 (C-6), 40.9 (C-2), 47.2 (C-4), 74.2 (C-3), 170.7 ($CH_3\underline{C}O$); ^{1}H NMR (500.00 MHz, in $CDCl_3$, TMS as internal standard) δ 4.68 (1H, ddd, *J* = 11.0, 10.9, 4.0 Hz, H-3).

(−)-(1S,3R,4S,6S)-6-Hydroxymenthyl diacetate (2Ac): colorless oil; $[\alpha]^{25}_D$ −88.2° ($CHCl_3$; *c* 1.0); HR-MS, *m*/*z* 256.1678; EI-MS, *m*/*z* (rel int) 154 (3), 136 (30), 121 (21), 107 (5), 93 (40), 81 (8), 69 (8), 55 (10), 43 (100); IR, υ_{max} cm^{-1} 2960, 1735, 1456, 1370, 1241, 1025; ^{13}C NMR δ 16.2 (C-9), 17.8 (C-10), 20.5 (C-7), 21.1 and 21.2 ($\underline{C}H_3CO$), 26.2 (C-8), 29.0 (C-5), 35.4 (C-1), 38.1 (C-2), 45.2 (C-4), 72.4 (C-3), 77.1 (C-6), 170.6 and 170.8 ($CH_3\underline{C}O$); ^{1}H NMR (500.00 MHz, in

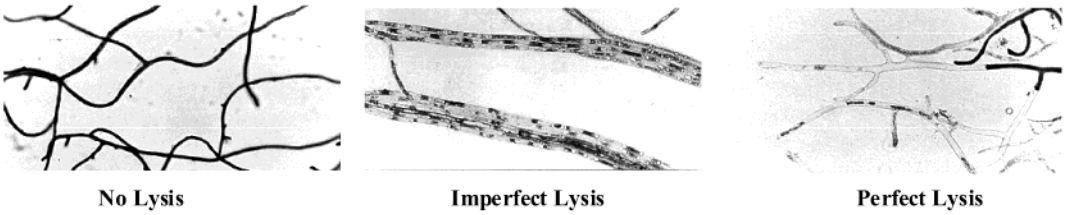

Figure 2. Typical photographs of hyphal lysis stained with 5% cotton blue.

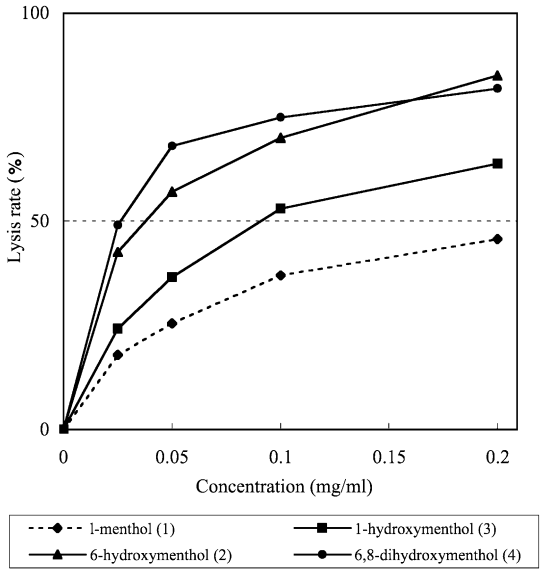

Figure 3. Lytic activity of **1**–**4** against *M. nivalis* MAFF 305031.

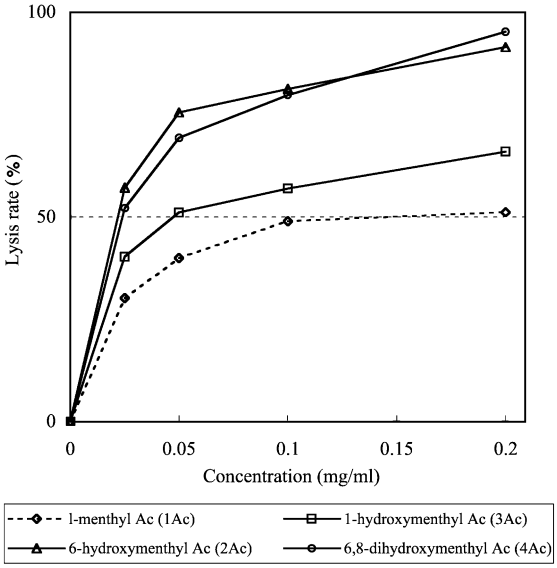

Figure 4. Lytic activity of **1Ac**, **2Ac**, **3Ac**, and **4Ac** against *M. nivalis* MAFF 305031.

$CDCl_3$, TMS as internal standard) δ 4.45(1H, ddd, J = 11.0, 10.7, 3.9 Hz, H-6), 4.68 (1H, ddd, J = 11.2, 10.9, 3.9 Hz, H-3).

*(−)-(1S,3R,4S)-1-Hydroxymenthyl 3-monoacetate (**3Ac**):* colorless oil; $[\alpha]^{25}_D$ −97.8° ($CHCl_3$; *c* 1.0); HR-MS, *m*/*z* 214.1562; EI-MS, *m*/*z* (rel int) 154 (7), 139 (13), 136 (13), 121 (9), 111 (17), 97 (10), 87 (10), 81 (9), 69 (14), 55 (13), 43 (100); IR, υ_{max} cm^{-1} 3450, 2959, 1714, 1460, 1371, 1244, 1026; ^{13}C NMR δ 16.5 (C-9), 19.4 (C-5), 21.0 (C-10), 26.3 (C-8), 31.4 (C-7), 37.8 (C-6), 44.6 (C-4), 47.0 (C-2), 71.0 (C-3), 71.9 (C-1); ^{13}C NMR δ 16.5 (C-9), 19.4 (C-5), 20.7 (C-10), 21.3 ($\underline{C}H_3CO$), 26.3 (C-8), 31.4 (C-7), 37.8 (C-6), 44.6 (C-4), 47.0 (C-2), 71.0 (C-3), 77.9 (C-1), 170.6 ($CH_3\underline{C}O$); ^1H NMR (500.00 MHz, in $CDCl_3$, TMS as internal standard) δ 5.00 (1H, ddd, J = 10.0, 10.1, 4.0 Hz, H-3).

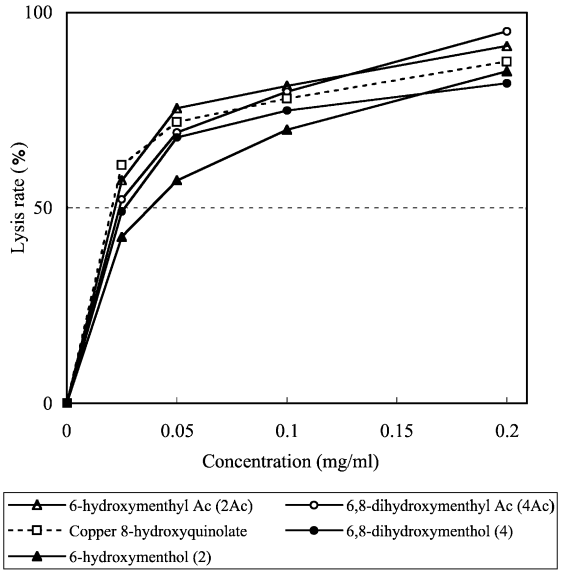

Figure 5. Lytic activity of **2**, **4**, **2Ac**, **4Ac**, and copper 8-hydroxyquinolate against *M. nivalis* MAFF 305031.

*(−)-(1S,3R,4R,6S)-6,8-Dihydroxymenthyl 3,6-diacetate (**4Ac**):* colorless oil; $[\alpha]^{25}_D$ −35.8° ($CHCl_3$; *c* 1.0); HR-MS, *m*/*z* 272.1621; EI-MS, *m*/*z* (rel int) 197 (1), 152 (2), 138 (1), 134 (6), 109 (3), 100 (2), 94 (77), 79 (29), 69 (3), 59 (21), 55 (7), 43 (100); IR, υ_{max} cm^{-1} 3472, 2928, 1736, 1456, 1371, 1241, 1025; ^{13}C NMR δ 17.7 (C-7), 21.1 and 21.5 ($\underline{C}H_3CO$), 22.7 (C-9), 28.3 (C-10), 35.3 (C-1), 36.1 (C-5), 38.1 (C-2), 49.6 (C-4), 72.5 (C-3), 74.1 (C-8), 76.5 (C-6), 170.0 and 170.7 ($CH_3\underline{C}O$); ^1H NMR (500.00 MHz, in $CDCl_3$, TMS as internal standard) δ 4.45 (1H, ddd, J = 10.2, 10.3, 4.1 Hz, H-6), 4.80 (1H, ddd, J = 10.0, 10.1, 4.0 Hz, H-3).

RESULTS AND DISCUSSION

The existence ratio of stained hyphae and no stain was counted using the microscope (**Figure 2**). Compounds **2**, **3**, and **4** had 85.0, 63.9, and 81.9% lytic activity, respectively, at the concentration of 0.2 mg/mL. On the other hand, the lytic activity of **1** was 45.7% at 0.2 mg/mL. These compounds each showed higher lytic activity as their concentration was increased (**Figure 3**). Especially, compounds **2** and **4** had 57.1 and 68.1% lytic activities at 0.05 mg/mL.

Acetyl esters **1Ac**, **2Ac**, **3Ac**, and **4Ac** showed high lytic activity against *M. nivalis*. Compounds **1Ac**, **2Ac**, **3Ac**, and **4Ac** had 51.2, 91.5, 66.0, and 95.2% lytic activities at a concentration of 0.2 mg/mL and showed higher lytic activity compared with compounds **1**, **2**, **3**, and **4**. Each of these acetyl esters showed higher lytic activity as their concentration was increased (**Figure 4**). In addition, copper 8-hydroxyquinolate (as a standard snow blight control agent) was tested and also compared with the high lytic activity compounds **2**, **2Ac**, **4**, and **4Ac** (**Figure 5**). Copper 8-hydroxyquinolate had 87.5% activity at 0.2 mg/mL. However, **2Ac** had a higher lytic activity compared with copper 8-hydroxyquinolate at 0.05−0.2 mg/mL, and **4Ac** had a higher activity at 0.1−0.2 mg/mL.

From the above results, compounds **2**−**4** showed high lytic activity compared with **1**, and **2** and **4** showed high lytic activity compared with **3**. These results indicated that the increasing hydroxyl group at **1** and hydroxylation at the C-6 position are important factors for lytic activity against *M. nivalis*. On the other hand, the acetyl esters **1Ac**, **2Ac**, **3Ac**, and **4Ac** showed further high lytic activity compared with the alcohols **1**−**4**. These results also indicated that the acetylation of the hydroxyl group is an important factor for lytic activity. We considered that hydroxylation at the C-6 position and acetylation of the C-6 position hydroxyl group are the most important factors for lytic activity. Finally, **2Ac** and **4Ac** had equal levels of lytic activity compared with copper 8-hydroxyquinolate. On the basis of these findings, **2Ac** and **4Ac** could be potential alternative methods to the organic copper compound (copper 8-hydroxyquinolate) for the control of snow blight disease.

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