Antifungal Activity of β-Pinene-Based Hydronoperyl Quaternary Ammonium Ammonium Salts Against Phytopathogenic Fungi

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Abstract

β-Pinene can be used as a cheap source to synthesize a large number of high value-added derivatives. In this study, a series of β-pinene derivatives was prepared, and the antifungal activities of the compounds were assessed against phytopathogenic fungi. Eight N-alkyl hydronoperyl diethyl ammonium halide salts were synthesized by the reaction of hydronopetyl diethyl ammonium halide with 8 halogenated alkanes. The structures of the synthesized products were characterized by Fourier-transform infrared spectroscopy and nuclear magnetic resonance spectroscopy and mass spectrometry. The antifungal activities of these derivatives were tested against 11 plant pathogens, and the preliminary structure-activity relationship is discussed. Some derivatives exhibited moderate to significant antifungal activity due to the fusion of the hydronopetyl, a long-chain alkyl, bromine, and iodine anionic groups. In contrast to the structure-activity relationship of compounds 2a, 2b, and 2c, iodine ions in 2f, 2g, and 2f had a significant effect on enhancing the antifungal activity against Colletotrichum gloeosporioides, Sclerotinia sclerotiorum, Phytophthora capsici, Phomopsis, Sphaeropsis sapinea, Glomerella cingulata, and Fusarium oxysporum. A higher molecular weight could increase the antifungal activity against Fusarium proliferatum, Alternaria kikuchiana, Sclerotinia sclerotiorum, P. capsici, Phomopsis, and S. sapinea. Compounds 2d and 2e exhibited broad-spectrum antifungal activity against the tested strains. These derivatives are expected to be used as precursor molecules for novel pesticide development in further research.

Keywords

hydronoperyl, quaternary ammonium salt, synthesis, antifungal activity

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About 10 000 genera and 120 000 species of fungi have been described. In agriculture, forestry, and animal husbandry, 70%-80% of plant diseases are due to phytopathogenic fungi, resulting in huge economic losses. Plant pathogens not only endanger the normal growth of crops but also cause a series of foodborne diseases and seriously threaten the life and health of human beings and animals. Traditional chemical bacteriostatic agents, which have been frequently used, have played a critical role in protecting human health, increasing crop production, and enhancing food preservation. However, their long-term use or improper use can easily lead to drug resistance and eco-environmental problems. The continuous development of new antifungal agents is still an active demand, and the development of novel antifungal agents has also been a hot spot in pesticide research.

β-Pinene is a natural compound with antifungal activity that can participate in many chemical reactions. A large number of β-pinene derivatives can be synthesized by chemical modification, and some of these derivatives have been shown to exhibit good antifungal activity. For example, Gavrilov et al. synthesized 6 derivatives based on β-pinene and tested their antifungal activities against 10 kinds of fungi. The results showed that sulfides-sulfoxide III displayed high activity against Penicillium tatum and moderate activity against Penicillium chrysogenum, Epidermophyton floccosum, and Aspergillus fumigatus. Therefore, β-pinene is regarded as a good precursor for the

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Inhibitory Activity of N-Alkyl Ammonium Salts against Phytopathogenic Fungi

**Results and Analysis**

**Chemistry**

Eight N-alkyl hydronopyl diethyl alkyl ammonium halides were successfully synthesized and characterized (Figure 1).

The Fourier-transform infrared spectroscopy (FT-IR) spectrum of 2b showed an absorption band at 2910-2980 cm\(^{-1}\) due to the CH2 and CH3 groups, and the characteristic stretching vibration of the CH2 group attached to the N group was observed at δ 266.3 (M+ − I) and 534.1 (M+ + I).

**Discussion**

The results of the antifungal activity test showed that the inhibitory rates of 2d and 2e were higher than chlorothalonil against C. gloeosporioides, F. proliferatum, A. kikuchiana, S. sclerotiorum, P. capsici, C. phylostachydis, Phomopsis, S. sapinea, G. cingulata, and F. aesculi, and they exhibited broad-spectrum antifungal activity against the tested strains.

**Table 1. Inhibitory Rates of Compounds for the Mycelium Growth of 11 Phytopathogenic Fungi.**

|        | A (%) | B (%) | C (%) | D (%) | E (%) | F (%) | G (%) | H (%) | I (%) | J (%) | K (%) |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 2a     | 9.4 ± 0.3 | 23.6 ± 0.1 | 11.8 ± 0.2 | 30.4 ± 0.2 | 42.6 ± 0.1 | 70.0 ± 0.0 | 12.4 ± 0.2 | 4.2 ± 0.2 | 30.7 ± 0.1 | 6.5 ± 0.1 | 18.2 ± 0.2 |
| 2b     | 43.8 ± 0.3 | 84.0 ± 0.1 | 69.6 ± 0.5 | 100.0 ± 0.0 | 43.4 ± 0.3 | 99.2 ± 0.1 | 64.8 ± 0.3 | 59.1 ± 0.1 | 90.1 ± 0.5 | 22.3 ± 0.9 | 69.1 ± 0.6 |
| 2c     | 46.5 ± 0.1 | 81.0 ± 0.2 | 85.0 ± 0.0 | 92.1 ± 0.1 | 96.4 ± 0.1 | 85.4 ± 0.0 | 73.5 ± 0.1 | 85.4 ± 0.0 | 69.1 ± 0.0 | 28.1 ± 0.1 | 67.0 ± 0.9 |
| 2d     | 78.0 ± 0.1 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 95.4 ± 0.0 | 98.7 ± 0.0 |
| 2e     | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 |
| 2f     | 49.6 ± 0.4 | 68.8 ± 0.2 | 53.9 ± 0.1 | 93.8 ± 0.2 | 97.1 ± 0.1 | 94.0 ± 0.2 | 48.8 ± 0.1 | 54.8 ± 0.2 | 83.0 ± 0.1 | 78.0 ± 0.2 | 62.7 ± 0.3 |
| 2g     | 57.2 ± 0.3 | 94.5 ± 0.1 | 70.5 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 97.6 ± 0.0 | 78.9 ± 0.3 | 50.9 ± 0.3 | 100.0 ± 0.0 | 57.5 ± 0.2 | 79.4 ± 0.7 |
| 2h     | 56.8 ± 0.3 | 78.1 ± 0.2 | 83.8 ± 0.3 | 100.0 ± 0.0 | 100.0 ± 0.0 | 98.9 ± 0.0 | 82.8 ± 0.2 | 65.5 ± 0.1 | 100.0 ± 0.0 | 71.4 ± 0.5 | 88.0 ± 0.0 |
| 3      | 77.4 ± 0.1 | 58.1 ± 0.4 | 43.7 ± 0.1 | 93.7 ± 0.0 | 72.8 ± 0.2 | 79.4 ± 1.2 | 80.2 ± 0.1 | 86.4 ± 0.1 | 96.0 ± 0.5 | 83.5 ± 0.2 | 69.7 ± 0.1 |

Notes: 3: Chlorothalonil; A: Colletotrichum gloeosporioides; B: Fusarium proliferatum; C: Alternaria kikuchiana; D: Sclerotinia sclerotiorum; E: Phytophthora capsici; F: Ceratosphaeria phylostachydis; G: Phomopsis; H: Colletotrichum acutatum; I: Sphaeropsis sapinea; J: Glomerella cingulata; K: Fusarium aesculi.
For C. gloeosporioides, A. kikuchiana, P. capsici, Phomopsis, and G. cingulata, for 5 compounds with alkyl chains of different lengths, the inhibition rate could be ordered as follows: 2c > 2d > 2c > 3b > 2a. For A. kikuchiana, S. sclerotiorum, P. capsici, C. phyllostachydis, Phomopsis, S. sapinea, and F. aesculi, for 3 compounds with iodine ions, the inhibition rate could be ordered as follows: 2h > 2g > 2f. For C. gloeosporioides, F. proliferatum, A. kikuchiana, S. sclerotiorum, P. capsici with a bromide ion, C. phyllostachydis, Phomopsis, C. acutatum, S. sapinea, G. cingulata, and F. aesculi, for 2 compounds with a hydrophilic N⁺ group linking 3 ethyl groups, the inhibition rate could be ordered as follows: 2f > 2a.

Comparing the antifungal activity of quaternary ammonium salts containing 2 methyl groups described in our previous report and quaternary ammonium salts with iodine ions, the inhibition rate when the 2 ethyl groups were linked on a hydrophilic N⁺ group was stronger than that of 2 methyl groups for F. proliferatum, A. kikuchiana, S. sclerotiorum, P. capsici, C. phyllostachydis, Phomopsis, S. sapinea, G. cingulata, and F. aesculi. The introduction of either a long alkyl chain or an iodine ion on the hydrophilic N⁺ group of the derivatives could also improve the antifungal activity against the above-mentioned fungi. Badawy et al reported that the antifungal activity of N,N,N-(dimethylpentyl) chitosan and N,N,N-(dimethyloctyl) chitosan against Botrytis cinerea and Fusarium oxysporum increased with an increase in the alkyl substituent chain length. This is consistent with Guo et al, who found that quaternized chitosan with a high molecular weight exhibited stronger antifungal activity.

The positively charged amino group of high molecular weight quaternary ammonium salts bind to the negatively charged substance on the cell wall to form a polymer membrane on the cell surface, which can kill a variety of microbes, including yeasts, by destroying the integrity of the cell membrane. If the quaternary ammonium salt has a long alkyl chain, it can pass through the cell wall, extend the active part into the cell body, bind to the necessary components such as protein, and destroy its metabolic balance, so as to inhibit the growth of the cell.

Materials and Instruments

General

(1R,2R,5R)-Hydronopyl diethyl amine (I) (>95%) was prepared by the Institute of Chemical Processing in Forest Products, Jiangxi Agricultural University. Light petroleum, ethyl bromide, n-propyl bromide, butyl bromide, pentyl bromide, decyl bromide, iodoethane, n-propyl iodide, and n-butyl iodide were obtained from Aladdin. All reagents used were of analytical grade.

Plant pathogenic fungi (C. gloeosporioides, F. proliferatum, A. kikuchiana, S. sclerotiorum, P. capsici, C. phyllostachydis, Phomopsis, Colletotrichum acutatum, S. sapinea, G. cingulata, and F. aesculi) were provided by the Forest Protection Department of Forestry College, Jiangxi Agricultural University.

For C. gloeosporioides, A. kikuchiana, P. capsici, Phomopsis, and G. cingulata, for 5 compounds with alkyl chains of different lengths, the inhibition rate could be ordered as follows: 2c > 2d > 2c > 3b > 2a. For A. kikuchiana, S. sclerotiorum, P. capsici, C. phyllostachydis, Phomopsis, S. sapinea, and F. aesculi, for 3 compounds with iodine ions, the inhibition rate could be ordered as follows: 2h > 2g > 2f. For C. gloeosporioides, F. proliferatum, A. kikuchiana, S. sclerotiorum, P. capsici with a bromide ion, C. phyllostachydis, Phomopsis, C. acutatum, S. sapinea, G. cingulata, and F. aesculi, for 2 compounds with a hydrophilic N⁺ group linking 3 ethyl groups, the inhibition rate could be ordered as follows: 2f > 2a.

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Fuli GC 9790 (China Wenling Fuli Analytical Instrument Co., Ltd.); ZNCL-TS 500 Intelligent Magnetic Stirrer (China Shanghai Sile Instrument Co., Ltd.); SHB-3 Circulating Water Multipurpose Vacuum Pump (China Zhengzhou Dufu Instrument Factory); Nicolet IS10 FT-IR spectrometer (USA); Bruker Aman SL Mass Spectrometer (German, Bruker); Bruker AVANCE 400 NMR spectrometer (Germany); Melting Point Meter (China Shanghai Optical Instrument Factory 1); LDZX-50KBS Vertical Pressure Steam Sterilizer (Shanghai Shen’an Medical Instrument Factory); SW-CJ-ID Sterile Super Clean Workbench (China Suzhou Purification Equipment Co., Ltd.); and GHP-250 Intelligent incubator (China, Shanghai Sanfa Scientific Instrument Co., Ltd.).

The Synthesis of Quaternary Ammonium Salt

A total of 0.05 mol of (1R,2R,5R)-hydronopyl diethyl amine (I), 0.1 mol of alkyl halide, and 30 mL of light petroleum (60-90°C) were added to a conical flask with a magnetic stirrer, and reflux condensation tubes were then installed. The mixture was stirred and heated to 50°C for about 11 hours. After cooling, the crystals were separated using a suction filter and washed with cold light petroleum (30-60°C). Finally, the crystals were vacuum dried and weighed. The IR spectra of the compounds were recorded on a Nicolet IS10 FT-IR spectrometer, and the ¹H-NMR and ¹³C-NMR spectra on a Bruker AVANCE 400 NMR spectrometer using deuterated chloroform as a solvent and trimethylsilane as the internal standard. Electrospray ionization mass spectrometry was recorded on a Bruker aman SL Mass Spectrometer. The characterization data are shown in Table 2.

Biological Activity Evaluation

The inhibitory effects of 8 compounds on 11 plant pathogenic fungi were determined by the mycelium growth rate method. Potato glucose agar (PDA) without any compound was used as a negative control, and the PDA culture medium with chlorothalonil was used as a positive control. The quaternary ammonium salt was mixed with sterile water to prepare a solution with a concentration of 5 g/L. The solution was added to the PDA in a certain proportion. The final mass concentration of the quaternary ammonium salt was 500 mg/L, and 3 replicate dishes were used for each strain. After the pathogens were inoculated, they were placed in a constant-temperature incubator at 25 °C for several days. When the diameter of the culture medium in the negative control group was about 6 cm (the diameter of the culture medium was 9 cm), the diameter of the fungi was measured by the crossing method. The inhibition rate was measured by the diameter of the fungi. The inhibition rate was expressed as follows:

\[ \text{Corrected diameter} = \frac{\text{average diameter of colonies}}{\text{diameter of fungus cake}} \] (1)
Brown fine-grained crystals; purity 92%; m.p. 94.0-96.3 °C; FT-IR

White crystals; purity 94.5%; m.p. 153.8-155.0 °C; FT-IR r (cm⁻¹): 2984-2854 (C-H, Br), 1168 (C-N). MS: m/z 252.3 (M⁺ - Br), 585.4 (2M⁺ - Br), 412.1 (M⁺ + Br), 743.2 (2M⁺ + Br), 815.1 (Br).

H NMR (300 MHz, CDCl₃) δ: 3.3 (6H, m, 3α-CH₂), 3.1 (2H, m, 11-CH₂), 2.4 (1H, m, 2-CH), 2.0-1.8 (6H, m, 7-CH, 10-CH₂, 5-CH, 1-CH, 3-CH), 1.7 (2H, m, 4-CH₂), 1.3 (9H, m, 9-CH₃), 1.2 (3H, m, 3-CH₂), 1.0 (3H, m, 9-CH₃), 0.9 (1H, d, J = 9.6 Hz, 7-CH).

1³C NMR (75 MHz CDCl₃) δ: 58.5 (7-CH), 55.8 (C-11), 52.5 (2 C-α), 45.5 (C-5), 38.2 (C-6, C-1), 32.9 (C-10), 28.5 (C-7), 27.6 (C-9), 25.9 (C-4), 22.7 (C-8), 21.8 (C-3), 8.4 (2 C-β).

Pale white fine-grained crystals; purity 91%; m.p. 73.0-76.7 °C; FT-IR r (cm⁻¹): 2980-2910 (C-H, Br), 1246 (C-N). MS: m/z 266.3 (M⁺ - Br), 426.1 (M⁺ + Br), 815.1 (Br).

H NMR (300 MHz, CDCl₃) δ: 3.4 (4H, m, 2 α-CH₂), 3.2 (4H, m, 11-CH₂, 12-CH₂), 2.2 (1H, m, 2-CH), 2.0 (2H, m, 13-CH₂), 1.8-1.6 (8H, m, 7-CH, 10-CH₂, 5-CH, 1-CH, 4-CH₂, 3-CH), 1.3 (7H, m, 3-CH, 2 β-CH₃, 14-CH₂), 1.1 (3H, s, 9-CH₃), 0.9 (6H, m, 14-CH₂, 8-CH₃), 0.8 (1H, d, J = 9.2 Hz, 7-CH).

1³C NMR (75 MHz CDCl₃) δ: 59.4 (C-12), 56.9 (C-11), 54.0 (2 C-α), 45.8 (C-8), 41.0 (C-5), 38.5 (C-3), 38.3 (C-1), 33.3 (C-10), 29.3 (C-7), 28.0 (C-9), 26.0 (C-4), 23.3 (C-8), 22.3 (C-3), 15.7 (C-13), 10.9 (C-14), 8.1 (2 C-β).

Yellow crystals; purity 91.2%; m.p. 56.0-57.9 °C; FT-IR r (cm⁻¹): 2933-2867 (C-H, Br), 1044 (C-N). MS: m/z 280.3 (M⁺ - Br), 440.1 (M⁺ + Br), 815.1 (Br).

H NMR (300 MHz, CDCl₃) δ: 3.4 (4H, m, 2 α-CH₂), 3.2 (4H, m, 11-CH₂, 12-CH₂), 2.2 (1H, m, 2-CH), 2.3 (2H, m, 13-CH₂), 1.9-1.8 (8H, m, 7-CH, 10-CH₂, 5-CH, 1-CH, 3-CH), 1.7 (4H, m, 4-CH₂, 14-CH₂), 1.4 (9H, m, 3-CH, 15-CH₂, 2 β-CH₃, 14-CH₂), 1.2 (3H, s, 9-CH₃), 1.0 (3H, s, 8-CH₃), 0.9 (4H, m, 21-CH₂, 7-CH).

1³C NMR (75 MHz CDCl₃) δ: 57.7 (C-12), 56.8 (C-11), 58.8 (2 C-α), 45.7 (C-8), 40.8 (C-3), 38.4 (C-6), 38.2 (C-1), 33.1 (C-10), 29.2 (C-7), 28.2 (C-13), 27.8 (C-9), 25.9 (C-3), 23.1 (C-8), 22.2 (C-3), 22.0 (C-14), 21.6 (C-15), 13.6 (C-16), 7.9 (2 C-β).

Yellow crystals; purity 94.5%; m.p. 150.0-152.6 °C; FT-IR r (cm⁻¹): 2975-2864 (C-H, Br), 1025 (C-N). MS: m/z 252.3 (M⁺ - Br), 631.3 (2M⁺ - Br), 506.1 (M⁺ + Br), 885.1 (2M⁺ + Br), 127.1 (I).

H NMR (300 MHz, CDCl₃) δ: 3.3 (6H, q, J = 6.8 Hz, 2 α-CH₂), 3.1 (2H, m, 2-CH), 2.1 (2H, m, 13-CH₂), 1.9-1.7 (10H, m, 7-CH, 10-CH₂, 5-CH, 1-CH, 3-CH, 4-CH₂, 14-CH₂), 1.4 (7H, m, 3-CH, 15-CH₂, 16-CH₃, 17-CH₂), 1.2 (12H, m, 18-CH₂, 19-CH₂, 20-CH₂, 2 β-CH₃), 1.2 (9H, m, 9-CH₃), 1.0 (3H, s, 8-CH₃), 0.9 (4H, m, 21-CH₂, 7-CH).

1³C NMR (75 MHz CDCl₃) δ: 58.7 (C-12), 56.8 (C-11), 58.8 (2 C-α), 45.7 (C-8), 40.8 (C-3), 38.4 (C-6), 38.2 (C-1), 33.1 (C-10), 31.6 (C-13), 29.6 (C-7, C-14), 29.0 (C-15, C-16), 27.8 (C-9), 26.2 (C-17, C-18), 25.0 (C-4), 23.2 (C-8), 22.4 (C-3), 22.2 (C-19), 21.9 (C-20), 13.9 (C-21), 7.4 (2 C-β).

Brown fine-grained crystals; purity 92%; m.p. 94.0-96.3 °C; FT-IR r (cm⁻¹): 2978-2869 (C-H, Br), 1238 (C-N). MS: m/z 266.3 (M⁺ - I), 534.1 (M⁺ + I), 127.1 (I).

H NMR (300 MHz, CDCl₃) δ: 3.5 (4H, q, J = 7.2 Hz, 2 α-CH₂), 3.2 (4H, m, 11-CH₂, 12-CH₂), 2.3 (1H, m, 2-CH), 2.2 (2H, m, 13-CH₂), 1.9-1.7 (8H, m, 7-CH, 10-CH₂, 5-CH, 1-CH, 4-CH₂, 3-CH), 1.4 (1H, m, 3-CH₂), 1.3 (6H, t, J = 7.2 Hz, 2 β-CH₃), 1.1 (3H, m, 9-CH₃), 1.0 (3H, m, 8-CH₃), 0.8 (3H, t, J = 7.2 Hz, 14-CH₂), 1.0 (3H, s, 8-CH₃), 0.8 (3H, d, J = 9.6 Hz, 7-CH).

1³C NMR (75 MHz CDCl₃) δ: 58.6 (C-12), 57.2 (C-11), 54.3 (2 C-α), 45.9 (C-2), 41.3 (C-5), 38.6 (C-6), 38.4 (C-1), 33.0 (C-10), 29.4 (C-7), 28.0 (C-9), 26.1 (C-4), 23.4 (C-8), 22.4 (C-3), 15.9 (C-13), 10.9 (C-14), 8.4 (2 C-β).

(Continued)
Inhibition rate (%) = (control corrected diameter — treatment corrected diameter)/control corrected diameter × 100%.

Conclusion

In this study, a series of hydronopyl quaternary ammonium salts were synthesized using molecular structure design and organic synthesis methods. The mycelial growth rate method was used to determine the inhibitory effect of the β-pinene-based derivatives on C. gloeosporioides, F. proliferatum, A. kikuchiana, Sclerotinia sclerotiorum, Phomopsis, C. acutatum, S. sapinea, and F. aesculi. The results show that the β-pinene-based derivatives that were synthesized by the blend of hydronopyl and either amyl or decyl groups exerted good antifungal activity against the plant pathogens, exhibiting good and broad-spectrum antifungal activity. Some derivatives exhibited moderate to significant antifungal activity. The structure-activity relationship analysis showed that 2 ethyl groups on the hydrophilic N+ group of the ammonium iodide quaternary ammonium salts have a significant influence on the improvement of the antifungal activity against F. proliferatum, A. kikuchiana, S. sclerotiorum, P. sapinia, C. phyllostachydis, Phomopsis, S. sapinea, and F. aesculi. The introduction of free iodine ions of the triethylammonium halide obviously improved the antifungal activity against S. sclerotiorum, P. sapinia, and C. phyllostachydis. These derivatives can be used as precursor molecules for further pesticide development.

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Declaration of Conflicting Interests

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