

Four Antibacterial Monoterpenoid Derivatives from the Herba of *Senecio cannabinifolius* Less

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Fractionation of a butanol extract of *Senecio cannabinifolius* Less. led to the isolation of two novel monoterpenoid derivatives, named cannabicide D and cannabicide E, whose structures were determined by spectroscopic analyses as 1-(2-hydroxy-2,6,6-trimethyl-4-β-D-glucosyloxy-cyclohexylidene)-butane-2, 3-dione, 6-Hydroxy-3-(3-O-β-D-glucopyranosyl-but-1-enyl)-2, 4,4-trimethyl-cyclohex-2-enone, along with two known glycosides. All the compounds had antibacterial activity, showing particularly potent activity against *Staphylococcus aureus* IFO 3060 and *Bacillus subtilis*.

The plant *Senecio cannabinifolius* Less. is a member of the family Compositae and distributes mainly in the Northeast and Hebei Province of China, Korea, Japan, and far east of pre-Soviet Union. It is used as a traditional remedy for treating virus influenza, enteritis, and pneumonia in China^{1,2}. Plants belonging to the genus are notable for producing a wide variety of pyrrolizidine alkaloids and furoeromophilanes. The large, very diverse genus *Senecio* has already been studied extensively for its secondary chemicals³. In this paper, we report the isolation and structure elucidation of the four monoterpenoid derivatives isolated from the butanol extract of the herba of *Senecio cannabinifolius* Less., along with their antibacterial activity.

MATERIALS AND METHODS

¹H- and ¹³C-NMR, DEPT, COSY, HMQC and HMBC spectra were measured using JNM-LA400 (JEOL Datum Ltd., Japan). Chemical shifts are shown in δ (ppm) with tetramethylsilane (TMS) used as an internal reference. UV spectra were recorded on an UV-VIS recording spectrophotometer (Shimadzu UV-2201), and IR spectra on a Bruker IFS 55 (Switzerland). HR-ESI-MS (positive ion-mode) was obtained with Finnigan LCQ (USA) using a heated capillary temperature of 180°, a capillary volt of 13 V, sheath and auxiliary nitrogen gas velocities of 50

a.u. and 10 a.u. in total-scanning manner. TLC was carried out on precoated Si gel 60 F₂₅₄ plates (Merck) and RP-18 F_{254s} plates (Merck). Spots were detected under UV (254 nm and 365 nm) before and after spraying with 10% H₂SO₄ ethanol solution, followed by heating the plate at 110° for 5 min. Column chromatography was carried out on wakogel C-100 (Wako Pure Chemical Industries Ltd.). The HPLC separation was performed on NPHPLC and RPHPLC; the former consisted of the following parts: a Merck-Hitachi system (L-7100 pumps, L-7400 UV detector, L-7490 RID, L-7300 column heater, L-7200 autosampler and D-7000 interface) and a Aquasil SS-4251 (60) column (10 mm×250 mm); the latter was composed by the following parts: a Shimadzu system (LC-6AD pump, SPD-10Avp, UV detector, RID-10A detector, CTO-10ACvp column heater, SCL-10Avp system controller and SCL-10Advp auto injector) and a Cosmosil 5C₁₈-AR-□ Waters column (10 mm×250 mm, Japan Nacalai Tesque).

Plant material:

The plant material was supplied by Jilin Hua Kang Pharm. Co. Ltd., China, in May 2001 and has been identified to be the herb of *S. cannabinifolius* L. in July 2001 by the researcher Dr. Zhong-Gai Yan (Changchun Research Institute of Traditional Chinese Materia Medica, Jilin Province, China).

Extraction and isolation:

The plant material was dipped into water overnight (1:10) and then extracted for three times after the material had

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been boiled for 2 h. Filtration and rotary evaporation of the resulting extract gave a residue (780 g), which was dissolved in water and extracted with chloroform, ethyl acetate, and n-butanol. The n-butanol layer was passed through a lowbar ODS column (1.873 g) eluted with MeOH-H₂O (5:95, 1:9 v/v) and by HPLC with 15% MeOH-H₂O-1% THF to furnish 1 (31.5 mg, fig. 1), 2 (16.3 mg, fig. 2), 3 (11.6 mg, fig. 3), and 4 (11.5 mg, fig. 4).

Compound 1:

Elution of the column with MeOH-H₂O (5:95) afforded yellow liquid 1. IR ν_{\max} (KBr): 3396, 2928, 1710, 1669, 1365, 1242, 1157, 1077 cm⁻¹. HRESIMS (positive) m/z : 403.1959[M+H]⁺ (calcd. for C₁₉H₃₁O₉, 403.1963). ¹H-NMR (400 MHz, DMSO-*d*₆): δ 5.76 (1H, s, H-1), 2.11 (3H, s, CH₃-4), 2.25 (1H, dd, J=2.0; 12.8 Hz, H₂-3'a), 1.26 (1H, m, H₂-3'b), 4.16 (1H, m, H-4), 1.97 (1H, dd, J=2.4; 12.4 Hz, H₂-5a), 1.35 (1H, m, H₂-5b), 1.28 (3H, s, CH₃-7), 1.31 (3H, s, CH₃-8), 1.07 (3H, s, CH₃-9), 5.09 (1H, s, OH-2), 4.24 (1H, d, J=8.0 Hz, H-1'), 2.89 (1H, dt, J=8.0; 4.4 Hz, H-2'), 3.07 (1H, m, H-3'), 3.13 (1H, m, H-4'), 3.10 (1H, m, H-5'), 3.66 (1H, ddd, J=2.0; 5.2; 10.4 Hz, H₂-6'a), 3.45 (1H, ddd, J=5.2; 5.2; 10.4 Hz, H₂-6'b), 4.88 (1H, d, J=4.4 Hz, OH-2'), 4.88 (1H, m, OH-3'), 4.91 (1H, m, OH-4'), 4.46 (1H, t, J=5.2 Hz, OH-6'). ¹³C-NMR (100 MHz, DMSO-*d*₆): δ 99.6 (C-1), 208.9 (C-2), 197.6 (C-3), 26.2 (C-4), 118.4 (C-1'), 70.4 (C-2'), 46.0 (C-3'), 70.6 (C-4'), 46.9 (C-5'), 35.5 (C-6'), 30.3 (C-7'), 28.7 (C-8'), 31.6 (C-9'), 101.2 (C-1''), 73.4 (C-2''), 76.7 (C-3''), 70.0 (C-4''), 76.7 (C-5''), 61.0 (C-6'').

Compound 2:

Elution of the column with MeOH-H₂O (1:9) afforded

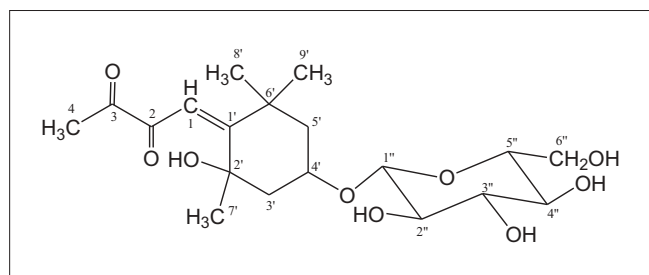


Fig. 1: Compound 1.

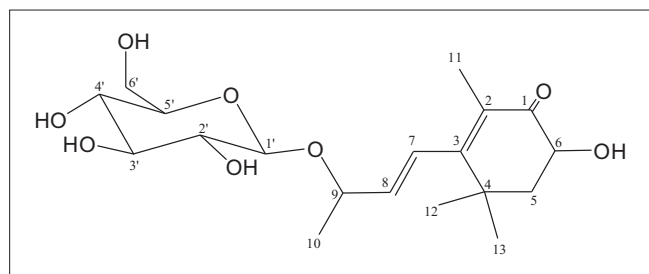


Fig. 2: Compound 2.

yellow liquid 2. IR ν_{\max} (KBr): 3423, 2927, 1668, 1374, 1076 cm⁻¹. HRESIMS (positive mode) m/z : 387.2011[M+H]⁺ (calcd. for C₁₉H₃₁O₈, 387.2013). ¹H-NMR (400 MHz, DMSO-*d*₆): δ 2.00 (1H, dd, J=5.6; 12.8 Hz, H₂-5a), 1.75 (1H, t, J=12.8 Hz, H₂-5b), 4.25 (1H, m, H-6), 6.30 (1H, d, J=16.0 Hz, H-7), 5.80 (1H, dd, J=6.4; 16.0 Hz, H-8), 4.45 (1H, p, J=6.4 Hz, H-9), 1.32 (3H, d, J=6.4 Hz, CH₃-9), 1.81 (3H, s, CH₃-2), 1.13 (3H, s, CH₃-4), 1.30 (3H, s, CH₃-4), 5.12 (1H, br.d, J=3.6 Hz, OH-6), 4.29 (1H, d, J=7.6 Hz, H-1'), 3.01 (1H, m, H-2'), 3.10 (1H, m, H-3'), 3.14 (1H, m, H-4'), 3.20 (1H, m, H-5'), 3.66 (1H, ddd, J=1.6; 6.0; 12.0 Hz, H₂-6'a), 3.51 (1H, ddd, J=6.0; 6.0; 12.0 Hz, H₂-6'b), 5.06 (1H, d, J=4.4 Hz, OH-2'), 4.95 (1H, d, J=2.4 Hz, OH-3'), 5.00 (1H, br.s, OH-4'), 4.45 (1H, br.d, J=6.0 Hz, OH-6'). ¹³C-NMR (100 MHz, DMSO-*d*₆): δ 199.9 (C-1), 126.9 (C-2), 159.3 (C-3), 36.3 (C-4), 45.7 (C-5), 68.5 (C-6), 125.4 (C-7), 139.5 (C-8), 75.0 (C-9), 20.7 (C-10), 13.4 (C-11), 30.0 (C-12), 25.5 (C-13), 101.1 (C-1'), 73.7 (C-2'), 76.8 (C-3'), 69.7 (C-4'), 76.7 (C-5'), 60.8 (C-6').

Compound 3:

Elution of the column with MeOH-H₂O (1:9) afforded pale yellow oil 3. $[\alpha]_D^{20} +101.2^\circ$ (c 0.12, MeOH). UV λ_{\max} (MeOH): 236 nm. IR ν_{\max} (KBr): 3420, 2924, 1653, 1373, 1040 cm⁻¹. HRESIMS (positive) m/z : 387.2010[M+H]⁺ (calcd. for C₁₉H₃₁O₈, 387.2013). ¹H-NMR (400 MHz, DMSO-*d*₆): δ 2.04 (1H, d, J=16.4 Hz, H₂-2a), 2.56 (1H, d, J=16.4 Hz, H₂-2b), 5.75 (1H, s, H-4), 5.94 (1H, d, J=15.2 Hz, H-7), 5.63 (1H, dd, J=6.4; 15.2 Hz, H-8), 4.42 (1H, p, J=6.4 Hz, H-9), 1.18 (3H, d, J=6.4 Hz, CH₃-9), 0.92 (3H, s, CH₃-1), 0.91 (3H, s, CH₃-1), 1.81 (3H, s, CH₃-5), 4.99 (1H, s, OH-6), 4.08 (1H, d, J=7.6 Hz, H-1'), 2.95 (1H, m, H-2'),

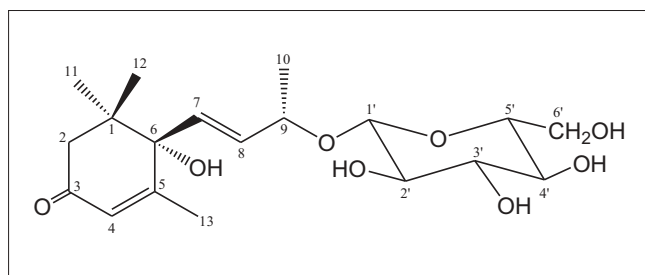


Fig. 3: Compound 3.

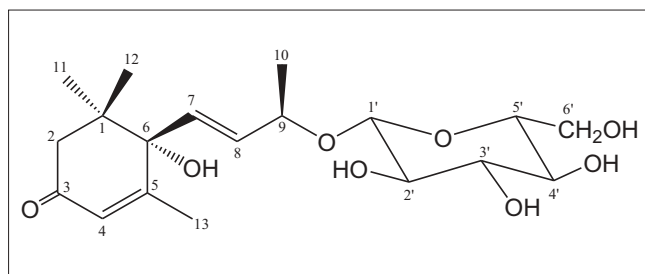


Fig. 4: Compound 4.

2.93 (1H, m, H-3'), 3.02 (1H, m, H-4'), 3.04 (1H, m, H-5'), 3.63 (1H, p, J=6.0 Hz, H₂-6'a), 3.40 (1H, m, H₂-6'b), 5.11 (1H, d, J=4.4 Hz, OH-2'), 4.96 (1H, d, J=3.6 Hz, OH-3'), 4.86 (1H, d, J=4.4 Hz, OH-4'), 4.47 (1H, t, J=6.0 Hz, OH-6'). ¹³C-NMR (100 MHz, DMSO-*d*₆): δ40.9 (C-1), 49.3 (C-2), 197.1 (C-3), 125.4 (C-4), 163.6 (C-5), 77.8 (C-6), 131.5 (C-7), 131.3 (C-8), 71.9 (C-9), 22.1 (C-10), 23.1 (C-11), 24.1 (C-12), 18.6 (C-13), 99.8 (C-1'), 73.2 (C-2'), 77.1 (C-3'), 70.0 (C-4'), 76.9 (C-5'), 61.0 (C-6').

Compound 4:

Elution of the column with MeOH-H₂O (1:9) afforded pale yellow oil 4. $[\alpha]_D^{20}$ -24.5° (c 0.11, MeOH). UV λ_{max} (MeOH): 238 nm. IR ν_{max} (KBr): 3418, 2927, 1654, 1373, 1075 cm⁻¹. HRESIMS (positive) *m/z*: 387.2009[M+H]⁺ (calcd. for C₁₉H₃₁O₈, 387.2013). ¹³C-NMR data (100 MHz, DMSO-*d*₆): δ40.3 (C-1), 49.3 (C-2), 197.1 (C-3), 125.5 (C-4), 163.8 (C-5), 77.7 (C-6), 130.2 (C-7), 133.1 (C-8), 74.5 (C-9), 20.8 (C-10), 23.0 (C-11), 24.0 (C-12), 18.9 (C-13), 100.7 (C-1'), 73.6 (C-2'), 76.7 (C-3'), 69.9 (C-4'), 76.3 (C-5'), 61.0 (C-6').

Antibacterial bioassay:

Three microbial cultures – *Staphylococcus aureus* IFO 3060, *Bacillus subtilis* and *Escherichia coli* – were supplied by Faculty of Agriculture, Kochi University, microbial laboratory culture collection. Got proper amount of each fraction to form the solution of the crude drug (100 g/ml) with 80% ethanol and diluted it with sterile distilled water before the experiment. Transferred 100 µl of sample solution into the test tube 1 and diluted it with 900 µl of distilled water in order to change the concentration to be 10 g/ml. Drew another 100 µl of solution from tube 1, then repeated the procedure as mentioned before to obtain the sample solution in tube 2 with concentration of 1 g/ml.

The qualitative antibacterial bioassay of each extract employed was the agar diffusion assay. LB liquid medium was prepared by mixing 10 g of peptone, 5 g of yeast, and 5 g of NaCl solution together in 1000 ml distilled water, adjusting pH value to be 7.2 by 2 N NaOH solution. Then added 15 g of agar to LB liquid medium to obtain LB agar solid medium. The bacteria were incubated in three test tubes with 3 ml of LB liquid medium in under shaking at 37° for 18 h. The inocula of target strains were injected into the LB agar solid medium sterilized at 121° for 15 min (100 µl of each inoculum). Then dispensed the agar medium into a sterilized culture dish with seven stainless steel wells (interior diameter: 6

mm, external diameter: 8 mm and height: 10 mm). After the medium was solidified, got rid of the wells, added 100 µl of sample solution to each well, and repeated with every diluted concentration (10 g/ml and 1 g/ml). Finally, sealed the culture dish. The diameters of the inhibition zones were measured using a hand-held digital calliper after incubation for 24 h at 30°. The mean values count (two times).

MIC values of purified compounds were determined by the turbidimetry. Just the right amount of the purified compound was dissolved in DMSO to achieve the solution with concentration of 10 mg/ml. Drew 500 µl of solution by a micropipette before transferring to a graduated centrifuge tube, diluted the solution (1:1) with sterile distilled water, and mixed under shaking by the mini-oscillator to change the concentration to be 5 mg/ml. Then drew another 500 µl of solution and transferred to next centrifuge tube before diluting it with 500 µl of distilled water. Repeated the operation as mentioned above. As a result, a series of the sample solution in decreasing concentration was obtained by a ratio of 0.5 (final concentration: 10 mg/ml to 9.78 µg/ml). Drew 100 µl of solution from each sample of different concentration and added the solutions into the test tubes that have already been prepared by pouring 900 µl of LB liquid medium into them. After the inocula of target strains were uniformly added (20 µl), put on the lid of tubes and incubated them at 30° under shaking for 48 h. Growth was followed by subjecting to UV spectrophotometry (Shimadzu UV-1200) at 600 nm using DMSO as control to measure the absorbance. The MIC value was defined as the lowest concentration to inhibit visible growth. DMSO, at used concentration in the test, did not interfere with the microbial growth.

RESULTS AND DISCUSSION

Compound 1 was isolated as a yellow liquid. The Molish's reaction was positive, indicating that this compound was glycoside. The molecular formula of 1 was established as C₁₉H₃₀O₉ on the basis of the protonated molecular ion peak [M+H]⁺ at *m/z* 403.1959 and the NMR data, allowing five degrees of unsaturation. The ¹³C-NMR spectrum showed 18 carbon signals, of which 6 carbon signals were assigned to a sugar portion, but the O-bearing carbon signal at δ76.7 (C-3'' and C-5'') was extremely strong, implying the degeneration of the two carbon signals, which was further supported by the correlations from three protons at δ3.01-3.15 (3H, m) to two methine

carbons (δ 70.0 and 76.7). Considering the result of subjecting the hydrolyzate of 1 and authentic sample of glucose to PPC and the anomeric proton resonance at δ 4.24 (1H, d, $J=8.0$ Hz, H-1"), the sugar was identified as β -D-glucose. Correlation was observed from the lone proton at δ 5.76 (1H, s) to δ 28.7 (C-8'), 35.5 (C-6'), 70.4 (C-2'), 118.4 (C-1'), 197.6 (C-3) and 208.9 (C-2). Two three-proton singlets of four methyl groups at δ 1.07 (3H, s) and 1.31 (3H, s) were meta-methyl moieties attached to a quaternary carbon (δ_c 35.5). The HMBC of 1 showed connectivities from H-7' to C-3', C-2' and C-1'. In the ^1H NMR spectra, five exchangeable proton resonances at δ 5.09 (1H, s), 4.91(1H, m), 4.88 (2H, m), 4.46 (1H, t, $J=5.2$ Hz) were assigned to OH-2', OH-4", OH-3", OH-2" and OH-6".

The HMBC connectivities from the anomeric proton at δ 4.24 to C-4' (δ 70.6) allowed the glucose to attach at C-4'. The novel compound was thus assigned the structure 1-(2-hydroxy- 2, 6, 6-trimethyl-4- β -D-glucosyloxy-cyclohexylidene)-butane-2,3-dione, which was reported for the first time and named cannabicide D.

Compound 2, obtained as a pale yellow oil, was found to have a molecular formula of $\text{C}_{19}\text{H}_{30}\text{O}_8$ as determined by HRESIM, implying five elements of unsaturation. The Molish's reaction was positive, indicating that this compound was glycoside. From the coupling constant of anomeric proton at δ 4.29 (1H, d, $J=7.6$ Hz) and the hydrolyzate, compound 2 was identified as β -D-glucopyranoside. ^1H -NMR contained five exchangeable proton resonances at δ 5.12 (1H, br.d, $J=3.6$ Hz), 5.06 (1H, br.d, $J=4.4$ Hz), 5.00 (1H, br.s), 4.95 (1H, d, $J=2.4$ Hz) and 4.45 (1H, d, $J=6.0$ Hz), characteristic of hydroxyl functions, four of which must locate at the glucose, and the proton (δ 5.12) should bond to C-6 (δ 68.5) because of long-range connectivities observed from the hydroxyl proton at δ 5.12 to carbonyl carbon (δ 199.9), methine carbon (δ 68.5), and methylene carbon (δ 45.7). There was also discerned correlation from methyl protons at δ 1.81 (3H, s) to carbonyl carbon (δ 199.9) and sp^2 hybridized quaternary carbon (δ 159.3 and 126.9). A series of proton signals at δ 6.30 (1H, d, $J=16.0$ Hz) and 5.80 (1H, dd, $J=16.0$; 6.4 Hz) observed in alkenyl proton region of the ^1H -NMR spectrum were affirmed to be trans-alkenyl protons coupling with each other and assigned to methine protons at δ 125.4 and 139.5 by the DEPT 135° spectrum. Carbons (δ 139.5 and 125.4) were correlated with methine proton at δ 4.45 (1H, p, $J=6.4$ Hz) and long-range connectivities were observed from methyl protons at δ 1.32 (3H, d, $J=6.4$ Hz) to C-8 (δ 139.5) and C-9 (δ 75.0).

In NMR, spectra indicated two lone methyl groups at δ 30.0 [1.13 (3H, s)] and 25.5 [1.30 (3H, s)], between which, marked correlation was displayed in HMBC. Strong long-range connectivities were observed from methyl protons at δ 1.13 (3H, s) and 1.30 (3H, s) to C-3 (δ 159.3), C-4 (δ 36.3), and C-5 (δ 45.7); from alkenyl proton at δ 6.30 (1H, d, $J=16.0$ Hz) to C-2 (δ 126.9); and from alkenyl proton at δ 5.80 (1H, dd, $J=6.4$; 16.0Hz) to C-3 (δ 159.3).

The anomeric proton at δ 4.29 (1H, d, $J=7.6$ Hz) was long-range connected to C-9 (δ 75.0) in HMBC, suggesting the glycoside was formed between the glucose and OH-9. Thus by the analysis of above evidence, compound 2 was determined to be 6-Hydroxy-3-(3-O- β -D-glucopyranosyl- but-enyl)-2,4,4-trimethyl-cyclohex-2-enone, which has not been reported before and named cannabicide E.

Compound 3 was yielded as a pale yellow oil and analyzed for $\text{C}_{19}\text{H}_{30}\text{O}_8$ by HREMIS. The positive Molish's reaction and acid hydrolysis of 3 indicated it to be β -D-glucopyranoside in accordance with the coupling constant of anomeric proton at δ 4.08 (1H, d, $J=7.6$ Hz). Of the five exchangeable protons at δ 5.11 (1H, d, $J=4.4$ Hz), 4.96 (1H, d, $J=3.6$ Hz), 4.86 (1H, d, $J=4.4$ Hz), 4.47 (1H, t, $J=6.0$ Hz) and 4.99 (1H, s), the last one was a lone active proton linked with a quaternary carbon, and the other protons were resided at the glucose. The alkenyl proton region in ^1H NMR spectrum contained a series of proton signals at δ 5.94 (1H, d, $J=15.2$ Hz) and 5.63 (1H, dd, $J=15.2$; 6.4Hz) whose chemical shifts and large coupling constants implied them to be coupling trans-alkenyl protons in the DEPT 135° spectrum; therefore, the isolated natural product was confirmed to be 9-O- β -D-glucopyranosyloxy-6-hydroxy-3-oxo- α -ionol. Consulting the NMR data of the known compound (6S, 9S)-roseoside^{4,5}, the compound was identified as (6S, 9S)-roseoside, which was isolated for the first time in genus *Senecio*.

Compound 4 was obtained as a pale yellow oil with the positive Molish's reaction, indicating it to be glycoside. Comparing its ^{13}C NMR spectrum with that of compound 3, the two spectra are almost the same, except for the methine carbon (δ 74.5). As a result, compound 3 and 4 were determined to be epimers. The identification of their absolute stereo-configuration is under consideration.

By the antibacterial (screening) bioassay *in vitro*, the CHCl_3 , EtOAc and n-BuOH soluble fractions, except for the water layer, showed the potential antibacterial

TABLE 1: ANTIBACTERIAL ACTIVITY OF COMPOUNDS 1-4

Comp.	Minimum inhibition concentration (MIC)		
	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>
1	3.9	15.6	>1000
2	125.0	250.0	>1000
3	31.2	62.5	>1000
4	31.2	31.2	>1000

MIC in µg/ml

activities. The minimum inhibitory concentrations (MICs) of all tested substances toward several pathogenic bacteria are shown in Table 1.

Compounds 1-4 were all active at concentrations less than 250.0 µg/ml against two gram-positive organisms (*Staphylococcus aureus* IFO 3060, *Bacillus subtilis*), but none were active against the gram-negative organism (*Escherichia coli*), being MIC values for the strain greater than 1000 µg/ml by employing turbidimetry.

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