A Lichen Substance as an Antiproliferative Compound against HL-60 Human Leukemia Cells: 16-O-Acetyl-leucotylic Acid Isolated from Myelochroa aurulenta

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Received June 16, 2009; Accepted July 21, 2009; Online Publication, November 7, 2009
[doi:10.1271/bbb.90419]

The lichen substance, 16-O-acetyl-leucotylic acid (1), was isolated from an acetone extract of Myelochroa aurulenta and found to exhibit antiproliferative activity against HL-60 human leukemia cells. This is the first report on its anti-leukemia activity (EC50 = 21 μM) which is greater than that of leucotylic acid (2) and the structurally related anti-tumor agent, betulinic acid (4).

Key words: lichen; triterpene; 16-O-acetyl-leucotylic acid; HL-60; antiproliferation

Lichens are symbiotic organisms composed of algae and fungi. They are known to be edible for animals and are a potential source of variable secondary metabolites derived from the polyketide, shikimate and mevalonate pathways. Lichen metabolites, including polyaromatic compounds and steroids, have been reported from a large number of lichen species as being antimicrobial, antifungal, herbicidal, antifeedant and antitumor compounds.1–3) In order to find new bioactive substances in lichens, we have examined a chromatographic separation process for lichen extracts guided by the antiproliferative effect on HL-60 human leukemia cells. In this paper, we describe the first report of the isolation of 16-O-acetyl-leucotylic acid (1) from Myelochroa aurulenta as an antiproliferative compound against HL-60 cells, and we compare it with the structurally related anticancer lupane triterpenes.

It was found that the acetone extract of lichen M. aurulenta had an antiproliferative effect on HL-60 cells. Separation of the extract by silica gel column chromatography gave an active white solid that was eluted with hexane/EtOAc. After PTLC purification, the triterpenoid, whose content was 3.8 mg/g of the lichen body, was obtained and found to have antiproliferative activity against HL-60 cells.4) H- and 13C-NMR analyses indicated that the isolated compound was 16-O-acetyl-leucotylic acid (1) (Fig. 1). The EI-MS analysis showed a fragment peak of m/z 456 (M–AcOH), its [α]D value being +50° in CHCl3. Although compound 1 together with leucotylic acid (2) had previously been reported as lichen constituents by Yosioka et al.,4–6) the reported data were those of its methyl ester, an authentic sample of which we did not have. Therefore, we confirmed its structure by a full assignment of 1H- and 13C-NMR data based on extensive 2D NMR analyses, including COSY, NOESY, HSQC, and HMBC (Fig. 2).

This is the first report of the isolation of compound 1 from M. aurulenta and of its antiproliferative effect on HL-60 cells. We next evaluated the activities of 1 and 2 by comparing them with the closely related lupane-triterpene, commercially available betulin (3) and betulinic acid (4), shown in Fig. 1, which have been isolated from the bark of Betulaceae trees (the white birch) as well as many other plants. Betulinic acid 4 has been found to selectively inhibit human melanoma cells,7,8) and its derivatives reported to have an inhibitory effect on several other tumors and HIV9–11) Leucotylic acid (2), which was subsequently tested in the assay, was obtained by alkaline hydrolysis of 1 with K2CO3 in MeOH. The 1H- and 13C-NMR data for compound 2 were in agreement with those of previously isolated leucotylic acid,12) but its [α]D value (+36°) was much smaller than the reported value (+330°). Since the [α]D values of methyl 16-O-acetylleucotylate and methyl leucotylate in the literature were +95° and +29°, respectively, our result seems to be appropriate.

The EC50 values for these tested compounds were determined from plots of the cellular proliferation (%) versus log concentration of the compounds (Table 1). In the cell proliferation assay, 3 and 4 inhibited the HL-60 proliferation at concentrations from 20 to 100 μM. The value of EC50 for these compounds seemed to range from 50 to 80 μM, but this could not be accurately determined. The reason for this is that the compounds were less soluble in the cell culture medium than 1 and 2, and the medium containing 3 or 4 became a suspension at a concentration greater than 50 μM. Lichen substance 1 exhibited a greater antiproliferative effect (EC50 = 21 μM) than 3 and 4. Decacylation of 1, which afforded 2, increased the solubility in the medium and decreased the activity to EC50 = 72 μM.

A modification of betulinic acid (4) has recently been examined to develop a more potent active agent. The introduction of hydroxyl groups on the carbon framework and their acylation were investigated and found to enhance the bioactivity.9,12–15) In our results, 2 and 4, which were structurally analogous, showed similar antiproliferative activity against HL-60 cells. We consider that the conversion of isopropenyl to tertiary

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hydroxyl groups on the lupane framework might not affect the anti-HL-60 cell activity, while increasing the solubility in water. Acetylation of the 16-hydroxyl group of 2 could increase the activity; thus, modification of the 16-hydroxyl is suggested to lead to an improvement of its bioactivity. It is proposed that compound 1 and its derivatives have the potential to be useful as anti-leukemia agents comparable with the closely related lupane-triterpene, betulinic acid (4).

In conclusion, we isolated 16-O-acetyl-leucotylic acid (1) for the first time from Myelochroa aurulenta as an antiproliferative compound against HL-60 cells. We found that 1 was more active than leucotylic acid (2) and betulinic acid (4). We are currently exploring other bioactive substances in lichen.

Experimental

General. All commercially supplied reagents were used as received. Optical rotation values were recorded by a Jasco P-1020 digital polarimeter. NMR spectra were obtained with Jeol ECP-400 and Bruker DRX-500 spectrometers for solutions in CDCl3 and CD3OD. 1H and 13C chemical shifts were referenced to the solvent signals of 7.26 and 77.0 ppm for CDCl3, and 7.15 and 128.0 ppm for CD3OD. Mass spectra were recorded with Jeol JMS-SX102A (EI) and JMS-T100CS (ESI) mass spectrometers.

Lichen sample. Myelochroa aurulenta (collection no. Yamamoto 10020806) was collected in Kyoto City, Japan in 2000 and identified by judging from its apparent and chemical characteristics by comparison with authentic materials. It was stored at ~30 °C in a freezer in the Plant Creation System Laboratory of Akita Prefectural University before extraction. The plant body (12.53 g) was soaked in acetone for 24 h and filtered, and the filtrate was concentrated in vacuo below 30 °C. The plant body was re-extracted three times in the same manner, and the extracts were combined to give 810 mg of a crude solid which was dissolved in dimethylsulfoxide (DMSO) to prepare a 12.5 mg/ml solution for the HL-60 proliferation assay.

Cells. HL-60 cells were obtained from the Riken Gene Bank (Tsukuba, Japan) and were maintained in an RPMI1640 medium supplemented with 10% fetal bovine serum (FBS). HL-60 cells in the log phase (approximately 1.0 × 107 cells/ml) were diluted to 1.0 × 105 cells/ml and used for the assay.

Cell proliferation assay. The level of proliferation was measured for HL-60 cells grown in 96-well microtiter plates by using alamar BlueTM (Biosource International, Lewisville, TX, USA), an oxidation-reduction indicator. Triplicate experiments were performed for treated and untreated cells with test samples. To each well, 1.0 × 105 cells/100μl of an HL-60 cell suspension was added and then mixed with 100μl of the medium containing serial dilutions of the samples to be assayed. Usually, 6.4-μl of a DMSO or EtOH solution from the extract was mixed with 800μl of the culture medium, and a 4-fold serial dilution was made in microtiter plates so that the final DMSO or EtOH concentration did not exceed 0.4% (v/v). After 3 d of incubation, 20μl of alamar BlueTM was aseptically added to each well, before incubating for 24 h. The absorbance at 570 and 600 nm was measured with a micro plate reader (BioRad), and the cellular proliferation, as a percentage of the untreated control cells (6.0 × 105 cells/ml), was calculated based on the alamar BlueTM reduced form, according to the manufacturer’s instructions.

Isolation of 16-O-acetyl-leucotylic acid (1). The crude acetone extract (76 mg) from the lichen was separated twice by column chromatography (SiO2, hexane/EtOAc), giving the active fraction (30 mg) eluted with 50–80% EtOAc. This fraction was further purified by PTLC (Merck F254 silica gel 60, hexane/EtOAc= 1:1) giving 16-O-acetyl-leucotylic acid (1, 4.5 mg) as a white solid; [α]D+20° +73° (c 0.22, CHCl3); 1H-NMR (500 MHz, CDCl3) δ 5.40 (1H, ddd, J = 3.9, 9.6, 12.2 Hz, H-16), 2.41 (1H, dt, J = 10.0, 9.4 Hz, H-21), 1.95 (1H, dd, J = 3.9, 12.2 Hz, H-15), 1.91 (1H, H-3), 1.93–1.56 (3H, 3H, H-17, H-20), 1.49–1.33 (6H, H-1, H-2, H-6, H-7a, H-11, H-19), 1.30–1.28 (18S, H-2, H-6, H-12 = x2, H-20), 1.26 (3H, 3H, H-4, H-5), 1.23–1.20 (3H, 3H, H-9, H-13, H-15p), 1.18 (3H, 3H, H-22, H-4), 1.16 (3H, 3H, H-24), 1.15 (1H, H-11), 0.99 (3H, 3H, H-14, H-16), 0.90 (1H, H, H-7), 0.82 (3H, 3H, H-8, 0.81 (1H, H-19), 0.75 (1H, H-1), 0.71 (3H, 3H, H-10e), 0.70 (3H, 3H, H-10c); 13C-NMR (125 MHz, CDCl3) δ 185.1 (CO2H), 167.0 (COCH3), 159.4 (C-16), 72.87 (C-22), 57.4 (C-17), 52.0 (C-21), 50.5 (C-5), 50.1 (C-9), 49.4 (C-13), 47.9 (C-4), 46.7 (C-18), 44.3 (C-8), 42.5 (C-14), 41.8 (C-19), 41.5 (C-15), 39.6 (C-1), 37.3 (C-2), 36.8 (C-10), 33.0 (C-7), 31.0 (22-Me-a), 28.0 (22-Me-b), 23.7 (C-12), 21.9 (C-6), 21.3 (COCH3), 20.8 (C-11), 18.1 (14-Me), 18.0 (C-2), 17.0 (18-Me), 16.8 (M-8e), 16.5 (4-Me), 16.2 (10-Me); HR-EI-MS (positive): calcd. for C30H45O4 (M+AcOH, 456.3603; found, m/z 456.3573. The 1H-NMR signals of H-3e, H-11 (1.15 ppm), H-1 (0.75 ppm) were overlapped with the neighboring methyl groups, preventing their multiplicity to be determined.

Preparation of leucotylic acid (2). To a solution of 1 (3.2 mg, 6.2 mmol) in MeOH (0.3 ml), excess K2CO3 (6.4 mg) was added. After stirring at 25 °C for 1 h, the reaction mixture was acidified with formic acid and concentrated in vacuo. The residue was purified by silicon gel column chromatography (hexane/CHCl3 = 1/1, i-PrOH = 0–10%), affording leucotylic acid (2, 1.9 mg, 85%) as a white solid; [α]D+24° +36° (c 0.17, CDCl3); 1H-NMR (400 MHz, CDCl3) δ 4.13 (1H, br, H-16), 2.49 (1H, dt, J = 10.3, 9.6 Hz, H-21), 1.26 (3H, s), 1.17 (3H, s), 1.12 (3H, s), 1.03 (3H, s), 0.98 (3H, s), 0.85 (3H, s), 0.77 (3H, s); 13C-NMR (100 MHz, CDCl3) δ 182.9, 74.6, 67.0, 60.4, 50.8, 49.9, 49.0, 47.4, 45.8, 44.0, 43.4, 42.3, 41.6, 39.5, 36.8, 36.3, 32.6, 31.1, 27.7, 26.8, 23.6, 21.5, 20.7, 18.3, 17.8, 17.2, 16.7, 16.3, 16.1; HR-ESI-MS (positive); calcd. for C19H19NaO4 (M+Na, 497.3606); found, m/z 497.3578.

Table I. Antiproliferative Activity (EC50) of 1, 2, and Lupane Triterpenes

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (μM)</th>
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<tbody>
<tr>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
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<tr>
<td>3</td>
<td>&gt;50</td>
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Fig. 1. CO2H

Fig. 2. COSY, HMBC, and NOE Correlations for 1.
References