Physicochemical Characterization and Cytotoxic Screening of Diterpene acid

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The diterpene acid (1) was tested for its growth inhibitory effects against tumor cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Full assignments of the 1D/2D-NMR data of the compound (1) are reported. These results suggest that the diterpene acid (1) possessed a cytotoxic agent.

Key words : (MTT) assay, diterpene acid, cytotoxic agent

Introduction

The diterpene acid has also been identified in two other species of *Anisotome*[1]. These species are *A. lyallii* Hook. (the only lowland species of Anisotome) and *A. haastii* Hook., both of which show high levels of 1 or its derivatives in their ethanolic extracts. It is interesting to note that no evidence of 1 was found in *A. aromatica* Hook., even though *A. flexuosa* was once regarded as a variant of this species[2]. The diterpene acid 1 (which has been named anisotomenoic acid) has also been identified in two other species of *Anisotome*[3]. It is interesting to note that no evidence of 1 was found in *A. aromatica* Hook., even though *A. flexuosa* was once regarded as a variant of this species[2]. GC tracts of an essential oil distilled from *A. flexuosa* indicated the presence of other, possibly related compounds, in the diterpene region. In this study, the structure characterization and the cytotoxic activity of anisotomenoic acid 1 against tumor cell lines are reported in this paper.

Experimental

1. Chemicals and Instruments

All solvents were distilled before use. Removal of solvents from chromtography fractions were removed by rotary evaporation at temperature up to 40℃. Initial fractionation of crude plant extract using reversed-phase flash chromatography was performed with Octadecyl-functionalized silica gel (C-18 Aldrich) as the adsorbent. Further column fractionation was performed using Davisil silica 60 Å(35 - 70 μm, silica gel, Allth) as the adsorbent. TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F254 visualized first with a UV lamp, then by dipping in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH) followed by heating. MS, UV and IR spectra were recorded on Kratos MS-80, Shimadzu UV 240, and Perkin-Elmer 1600 FT-IR instruments respectively. NMR spectra of $CDCl_3$ solutions at 25℃ were recorded at 300 MHz and 500 MHz for ^1H and 125 MHz for ^{13}C on a Varian VXR-500 spectrometer. Chemical shifts are given in ppm on the scale referenced to the solvent peaks of $CDCl_3$, ^1H-NMR referenced to 7.25 ppm, ^{13}C-NMR referenced to 77.08 ppm. DEPT, HSQC, NOESY and CIGAR experiments were run at 45℃.

2. Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide, fetal bovine serum (FBS), streptomycin and penicillin were obtained from Sigma Chemical Co. Ltd. (St. Louis, USA). Tumor cells were obtained from Korean Cell Line Bank in the Seoul National University. All other chemicals were of reagent grade.

3. Cell culture

B16/F10, A549, MDA-MB-231 and SNU-C4 cells were grown at 37℃ in RPMI or DMEM medium supplemented with 10% FBS penicillin (100 units/mL) and streptomycin (100 μ

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g/mL). The cells were grown in a humidified atmosphere of 95% air / 5% CO_2. Cells were dissociated with 0.25% trypsin and were counted using a Hemacytomer just before transferring them for the experiment.

4. Plant Material

The *Anisotome lyallii* was collected in the Dunedin Botanical Garden in December 2001. Plant was identified by a botanist and voucher specimens deposited in the PERU herbarium.

5. Extraction

A bulk extract of fresh plant material (195.1 g, collection code 011215) was prepared by blending with EtOH (1 x 500 mL, 1 x 400 mL), and then with $CHCl_3$ (300 mL). The solvent was removed from the extracts and subsamples analysed by ^{1}H-NMR, the spectra of both extracts were similar so these were combined to give a dark yellow gum (10.58 g).

6. Isolation of anisotomenoic acid

The crude extract was subjected to C-18 flash chromatography (1.0 g pre-coated onto 2.0 g C-18, loaded onto a 10 g C-18 column), developed with H_2O, H_2O : CH_3CN (1 : 1, 1 : 3, 1 : 9), CH_3CN, CH_3CN : $CHCl_3$ (1 : 1), $CHCl_3$, hexane and ethanol. Fractions were combined on the basis of TLC results and assayed. Part of this fractions (300 mg) was dried on to 600 ㎎ Si-gel and subjected to further flash chromatography on Si-gel (3.0 g). The column was developed with 5 - 50% ethyl acetate / hexane and ethyl acetate. Fractions were combined on the basis of TLC and ^{1}H-NMR results. This fraction (130 mg) was dried on to 260 mg Si-gel and subjected to flash chromatography on Si-gel (1.3 g). The column was developed with 5 - 40% ethyl acetate / hexane and ethyl acetate. Fractions were combined on the basis of TLC. Fractions were combined (22 mg) to yield an anisotomenoic acid - pale yellow oil; Si-gel TLC (hexane : EtOAc, 3 : 1), R_f 0.333, plus blue/green with vanillin; ^{1}H and ^{13}C-NMR, DEPT, HSQC, CIGAR, NOESY and COSY data are presented in Table 1. Anisotomenoic acid 1 thus isolated was identified by comparison of its spectral data with those published or by direct comparison with an authentic sample[3].

7. 4,5-Dimethylthiazol-2-yl-2,5-diphenyl-tetrazoliumbromide (MTT) assay

The assay is dependent on the cellular reduction of water-soluble MTT (Sigma Chemical Co. St. Louis, M.O.) by mitochondrial dehydrogenase of vial cells to a blue water-insoluble formazan crystal product which can be measured spectrophotometrically[4,5]. Tumor cell lines were cultured in RPMI-1640 medium (Gubco Laboratories) containing 10% fetal bovine serum. Exponentially growing tumor cells (5×10^5) were cultured for 48 hrs at 37℃ in a humidified 5% CO_2 incubator in the presence or absence of sample.

8. Evaluation of toxicity

In order to determine the cytotoxicity mediated by 1, the colorimetric assay was used. These compounds were serially diluted in EMEM (Eagle's minimum essential medium) with 10% FBS and mixed with equal volume of NIH 3T3 fibroblasts (5×10^4 cells/mL). After one hour, fresh culture medium was supplied to a total volume of 1~100 μM. On the third day of incubation at 37℃ an incubator MTT terazolium dye (5 mg/mL; 20 μL/well ; polyscience, Inc. Warrigton, PA) was added to the cells. After 3 hr, the absorbance was measured at 540 nm using ELISA reader. All experimental data were expressed as the mean ± S.D. of triplicate experiments.

9. Statistical analysis

All values, expressed as the mean ± S.D., were statistically analyzed through analysis of Student's t-test. The P value less than 0.05 was considered as significant.

Results and Discussion

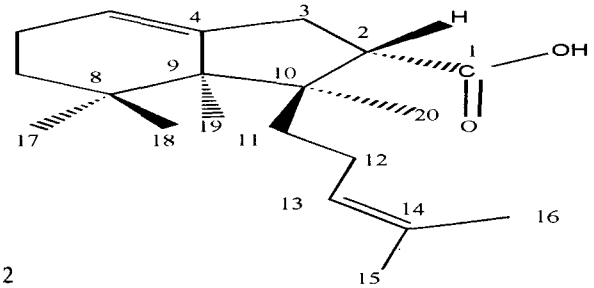
An extract (1.0 g) of *A. lyallii*, showing cytotoxicity against BSC cells[6], was subjected to reverse-phase (C-18) flash chromatography which concentrated the bioactivity into fractions that elulted with 1 : 1, 1 : 3, 1 : 9 H_2O : CH_3CN , CH_3CN, 1 : 1 CH_3CN : $CHCl_3$, $CHCl_3$ and n-hexane. The active fraction (0.3 g) was further fractionated by silica-gel column chromatography two times. The major component 1 was obtained in the fractions eluted with 5 : 95 / EtOAC : hexane. The results from chemical screening of the original crude extract of *A. lyallii*, indicated that the compounds responsible for the BSC-cell cytotoxicity of the crude extract were low to medium polarity[6]. The extract of *A. lyallii* also showed mild antibacterial activity against *B. substilis* and against the fungus *T. mentagrophtytes*. With this information a bioactivity directed isolation of the cytotoxic compounds in the crude extract was initiated. Through a series of fractionations using C-18 and then Si-gel, we were able to isolate an anisotomenoic acid (Table 1). This compound was a major component of *A. lyallii* and its signals were clearly visible in the ^{1}H-NMR spectrum of the crude extract. The ^{13}C-NMR spectrum showed 20 carbon signals including five sp^2 signals with a quaternary signal at

180.3 ppm, indicative of an acid group. This was confirmed by IR analysis showing a carbonyl stretch at 1699 cm^{-1}. DEPT analysis indicated 6 x CH_3, 5 x CH_2, 3 x CH and 6 quaternary carbons. With two carbon-carbon double bonds and a carbonyl present, the two remaining double-bond equivalents were accounted for by a biallylic structure. The NMR spectra were initially run in $CDCl_3$. There were a number of distinuishing features of the ^{1}H-NMR spectrum: 4 methyl singlets, two methyl doulblets, two single olefinic proton signals at 5.41 and 5.06 ppm, a triplet at 2.68 ppm, plus a range of multiplets between 1 and 3 ppm. The COSY spectrum showed that the signal at 5.06 ppm was coupled to two allylic geminal methyl signals, and to α-CH_2-group (2.05 ppm, brm).

This defined the 3-methyl butenyl substructure $(CH_3)_2C$=CH-CH_2-C, which was combined with a requirement for two or more rings. A HSQC analysis revealed one bond correlations between the protons experiment showed the two and three bond correlations allowing connectivities to be made. The 3-methylbutenyl substructured was confirmed, and extended to substructure 2. The COSY spectrum showed a proton at 2.68 ppm coupled equally (J=8.5 Hz) to on methylene proton (2.17 ppm).

The CIGAR correlations of these protons allowed us to extend substructure 3, including both the carboxylic acid and the other carbon-carbon double bond. Two of the three remaining methyl groups were shown to constitute a geminal pair by the mutual correlations in the CIGAR spectrum, flanked by a methylene group was also a singlets, so this was allocated to the same quaterary carbon. This information was used to propose the structure 1.

1



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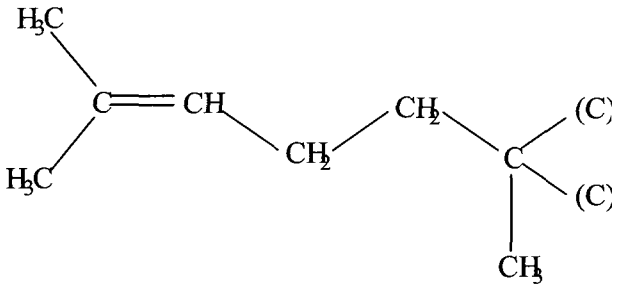

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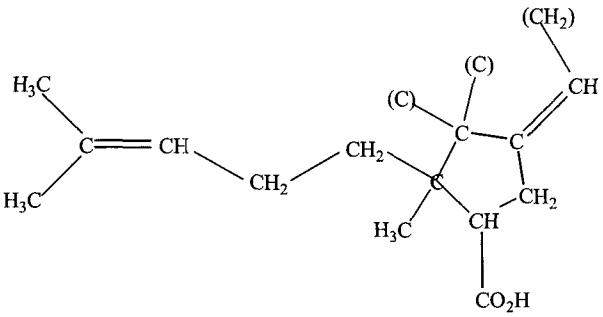

Fig. 1. The structure of anisotomenoic acid (1)

This molecule contains three chiral centres (positions 2, 9 and 10, Fig. 2). We demonstrated the relative stereochemistry (arbitrarily setting C19 as α) at the chiral centres C_2 and C_{10} by comparing NOESY interactions with results of conformational searching and molecular modellings.

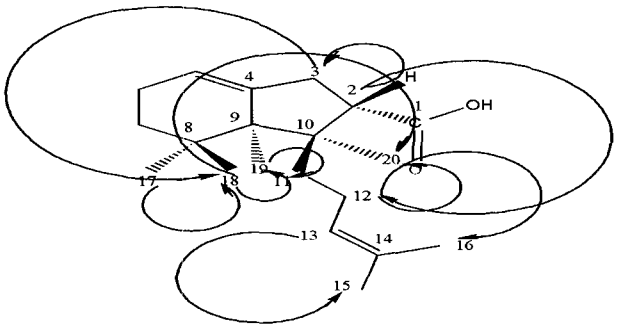

Fig. 2. Selected NOESY correlations in anisotomenoic acid 1

Irradiation of the H-2 signal gave enhancements of the signals of one H-3 (2.17 ppm), and H-12. No NOESY interaction was observed between H_3-19 and H-2, but there was a definite interaction between H_3-18 and the other H-3 (2.78 ppm). Irradiation of H_2-11 signal gave enhancements of the signal of H_3-19. Irradiation of H_2-12 signal gave enhancements of the signals of H_3-20 and H_3-16. Therefore, anisotomenoic acid (1) has H-2 cis to C-11 and trans to C-19.

Table 1 shows the potent cytotoxic activity of anisotomenoic acid (1) against cancer tumor cell lines. In general, the cytotoxic activity of this compound was in a dose-dependent manners, and the susceptibility of the cancer cell lines to anisotomenoic acid (1) was quite sensitive. The values of MTT_{50} were determined at 29.18 μg/mL against B16/F10 skin melanoma cell line. A colorimetric assay was used to detect the in vitro cytotoxicity mediated by anisotomenoic acid (1). As shown in Fig. 3, anitotomenoic acid (1) mediated cytotoxicity rapidly increased in the MTT assay against B16/F10 skin melanoma cell line when its concentration was increased from control to 100 μg/mL.

However, this compound showed a little changeable in the MTT assay against the other cancer cell lines when its concentration was increased from control to 25 μg/mL. A comparison of IC_{50} values of this compound in cancer cell lines showed that their susceptibility to this compound decreased in the following order: B16/F10 > SNU-C4 > MDA-MB-231 > A549 by the MTT assay. Anisotomenoic acid (1) was the most effective growth inhibitor of B16/F10 skin melanoma cell line, producing approximately 29 μg/mL of IC_{50} in the MTT assay.

Table 1. Inhibition of tumor cell proliferation by anisotomenoic acid (1) from *A. lyallii* by the MTT assay

Anisotomenoic acida	IC_{50} (μg/mL)[b]			
	A549	B16/F10	MDA-MB-231	SNU-C4
	55.97	29.18	45.08	36.50

[a]It was examined in three concentrations in triplicate experiments. [b]IC_{50} value of anisotomentoic acid (1) against each cancer cell lines, defined as the concentration that caused 50% inhibition of cell proliferation *in vitro*.

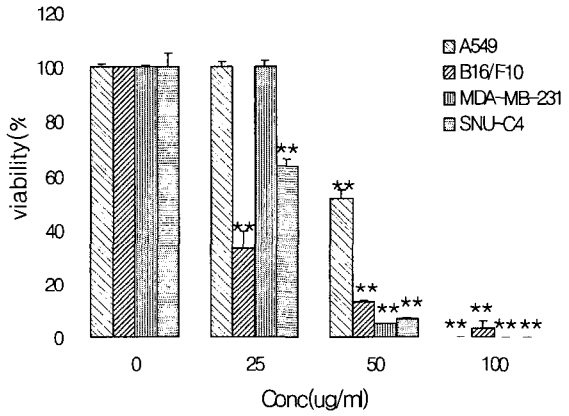

Fig. 3. *In vitro* cytotoxicity of anisotomenoic acid (1) by the MTT assay. This compound was serially diluted in RPMI-1640 or DMEM with 10% FBS. The colorimetric assay was performed as described in the materials and methods section. Data are mean values of results obtained from three sets of experiments. *Significantly different from the control value: **P<0.01 (Student's-test).

In conclusion, full assignments of the 1D/2D-NMR data of the compound (1) are elucidated. The diterpene acid (1) was tested for its growth inhibitory effects against tumor cell lines using the 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A comparison of IC_{50} values of this compound in cancer cell lines showed that their susceptibility to this compound (1) decreased in the following order: B16/F10 > SNU-C4 > MDA-MB-231 > A549 by the MTT assay. Anisotomenoic acid (1) was the most effective growth inhibitor of B16/F10 skin melanoma cell line, producing approximately 29 μg/mL of IC_{50} in the MTT assay.

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