Lsolation of Diterpene acid from *Anisotome Lyallii*

Jin A Lim, Eun Young Choi[1], In Kio Oh[2], Hyung Min Kim[3], Young Ok Kim[4], Nigel B Perry[5], Seung Hwa Baek*

*1 : Department of Skin and Care, Kimcheon Science College, 2:Department of Environmental Science, Wonkwang Health Science College, *:Department of Herbal Resources, Professional Graduate School of Oriental Medicine and Institute of Basic Natural Sciences, Wonkwang University, 3:Department of Pharmacology, School of Oriental Medicine, Kyunghee University, 4:Department of Clinical Pathology, Jeonju Kijeon Women,s College, 5:Plant Extracts Researvh Unit, New Zealand Institute for Crop & Food Research Limited, Department of Chemistry, Otago University, P.O B56 Dunedin, New Zealand*

The diterpene acid 1 was isolated from the roots of *Anisotome lyallii* (Apiaceae/Umbelliferae). The structure of the compound was elucidated as anisotomenoic acid 1 on the basis of spectroscopic methods. This compound was evaluated against P388 murine leukaemia and B 16/F10 melanoma cells.

Key words : *Anisotome lyallii*, anisotomenoic acid, P388 murine leukaemia, B 16/F10 melanoma cells

Introdution

Several species of Anisotome have been investigated as part of this bioacitivity-directed isolation work[1] and as part of the Essential oils programme.[2] The steam-distilled essential oils of two of the sub-Antarctic Anisotome species have been analysed. *A. antipoda* Hook. f. oil was found to contain mainly common monoterpenes such as γ-terpinene (35%) and α-terpinolene (34%).[2] In this work, no biologically active compounds were isolated from this species. Several compounds with anti-fungal activity were isolated from *A. latifolia* Hook. f. oil. The anti-fungal compounds were identified as the well-known phenylpropanoids apiole (9%) and elemicin (34%).[2] β-Sesquiphellandrene (13%) was also found in the oil of *A. latifolia* along with a number of other sesquiterpene hydrocarbones.[2] β-Sesquiphellandrene has been found co-occurring with apiole and elemicin previously in a species of Ligusticum.[3] The rare sesquiterpene α-trans-bergamoten-10-one was also identified at levels of up to 25% in *A. latifolia* essential oils. The diterpene acid has also been identified in two other species of Anisotome.[4] 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.[5] The chemistry of *A. flexuosa* was investigated after screening of the crude ethanol extract showed whole-well cytotoxicity aganinst BSC-cells.[1,5] The initial investigation also indicated that the compounds responsible for this cytotoxicity were of low to medium polarity. C-18 and silica gel column chromatography gave the main cytotoxic compound whose signals were clearly visible in the ^{1}H-NMR spectrum of the crude extract. The diterpene acid 1 (which has been named anisotomenoic acid) has also been identified in two other species of Anisotome.[6] 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.[5] GC tracts of an essential oil distilled from *A. flexuosa* indicated the presence of other, possibly related compounds, in the diterpene region. One of these was tentatively identified as the diterpene acetate derivative, but was not obtained pure, so full characterisation was not possible.[5] The diterpene acid 1 and its derivative form a new class of irregular diterpenes. Their unusual structures and their presences in several Anisotome species raises many questions about the biosynthesis of these compounds and their functions in the plant. There does not seem to be any obvious biosynthetic pathway to the formation of 1, which makes it an interesting candidate for further study.

* To whom correspondence should be addressed at : Seung Hwa Baek, Department of Herbal Resources, Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan 570-749, Korea.

· E-mail : shbaek@wonkwang.ac.kr · Tel : 063-850-6225

· Received : 2003/04/22 · Revised : 2003/05/31 · Accepted : 2003/07/28

Experimental

1. Chemicals and Instruments

All solvents were distilled before use. Removal of solvents from chromatography fractions were removed by

solvents from chromatography fractions were removed by rotary evaporation at temperature up to 40℃. Initial fractionation of crude plant extract using reverse phase column 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 ㎛ 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. Gas chromatography (GC) was performed on a Perkin-Elmer Autosystem gas chromatograph (under the control of PE-Omega software) equipped with a 10 mJ & WDB-1 column with a 0.25 mm ID and 0.25 ㎛ film. The temperature was programmed (50 ℃ - 250 ℃ at 15 ℃ min^{-1}) and the carrier gas used was hydrogen with a flow rate of 50 cm s^{-1}. 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.

2. Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium-bromide, 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

B 16/F10, P388 and NIH 3T3 cells were grown at 37℃ in RPMI medium supplemented with 10% FBS penicillin (100 units/ml) and streptomycin (100 ㎍/㎖). 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 ㎖, 1 x 400 ㎖), and then with $CHCl_3$ (300 ㎖). The solvent was removed from the extracts and subsamples analysed by ^1H-NMR, the spectra of both extracts were similar so these were combined to give a dark yellow gum (10.58 g).

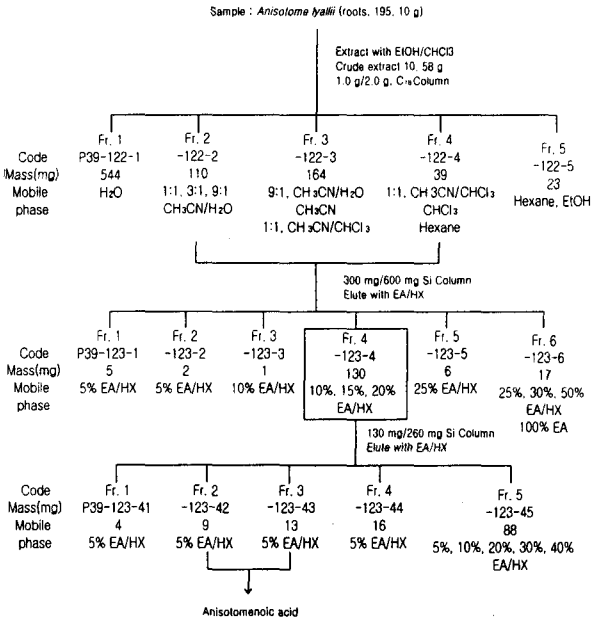
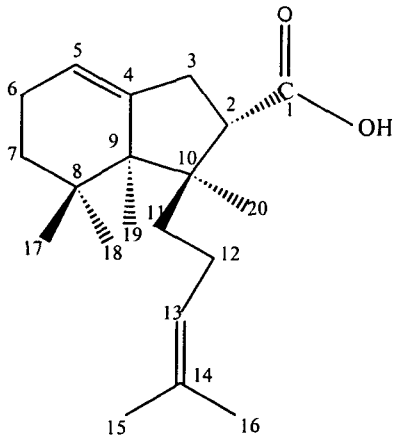


Fig. 1. Isolation of anisotomenoic aicd 1 from *A. lyallii*

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 ㎎) 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 (p39-124-4) were combined on the basis of TLC and ^1H-NMR results. This fraction (130 ㎎) was dried on to 260 ㎎ 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 p39-123-42 to p39-123-43 were combined (22 ㎎) to yield an anisotomenoic acid - pale yellow oil; Si-gel TLC (hexane : EtOAc, 3 : 1), R_f 0.333, plus blue/green with vanillin. ^1H-NMR ($CDCl_3$) δ 2.68 (1H, t, J=8.5 Hz, H-2), 2.78 (1H, brm, 2-H), 2.17 (2H, dd, J=9.0, 14.5 Hz, H-3), 5.41 (1H, t, J=3.0 Hz, H-5), 1.94 (1H, brm, H-6), 1.88 (2H, m, H-6), 1.59 (2H, brm, hidden, H-7), 1.23 (2H, brm, H-7), 1.60 (2H, m, H-11), 1.31 (2H, m, H-11), 2.05 (2H, brm, H-12), 5.06 (1H, dq, J=1.5, 7.0

Hz, H-13), 1.67 (3H, d, J=1.0 Hz, H-15), 1.61 (3H, d, J=0.5 Hz, H-16), 0.93 (3H, s, H-17), 1.01 (3H, s, H-18), 1.05 (3H, s, H-19), 1.13 (3H, s, H-20); ^{13}C-NMR ($CDCl_3$) δ 180.3 (C-1), 49.3 (C-2), 32.5 (C-3), 145.0 (C-4), 117.4 (C-5), 22.0 (C-6), 36.3 (C-7), 36.8 (C-8), 50.9 (C-9), 53.4 (C-10), 41.6 (C-11), 23.7 (C-12), 125.1 (C-13), 131.2 (C-14), 25.7 (C-15), 17.7 (C-16), 28.6 (C-17), 26.0 (C-18), 20.0 (C-19), 17.6 (C-20). Anisotomenoic acid 1 thus isolated was identified by comparison of its spectral data with those published or by direct comparison with an authentic sample (Fig. 2).[6]



1

Fig. 2. The structure of anisotomenoic acid 1

7. 4,5-Dimethylthiazol-2-yl-2,5-diphenyl-2H-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.[7,8] P388 murine leukaemia and B 16/F10 melanoma 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. The 50% cytotoxic dose (CD_{50}) was calculated using the computer program.

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

1. Analysis of anisotomenoic acid 1 by gas chromatography

In New Zealand, the Apiaceae are predominatly plants of subalpine areas.[9] The only reported chemistry of the genus Anisotome is our work on two subantarctic Anisotome species, the essential oils of which yielded a number of known monoterpenes, sesquiterpenes phenolic compounds.[10] An extract (1.0 g) of *A. lyallii*, showing cytotoxicity against BSC cells1, 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 (Fig. 1). 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. 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. Anisotomenoic acid 1 was sufficiently volatile to be analysed by capillary gas cheromatography (GC). Acid 1 was the major compound detected by GC analysis of a crude ethanol extract of *A. lyallii*. By contrast, 1 was only a minor component from the simultaneous hydro-distillation /solvent extraction (SDE) of *A. flexuosa*.[10] This could be due to low volatility of 1, or to reactions in boiling water. It would be interesting to indentify the volatile components in *A. lyallii*, by GC-MS initially. The volatile oils[11] and crude ethanol extrats of *A. aromatica*, *A. imbricata* and *A. haastii* were analysed by GC. The crude ethanol extracts were also analysed by 1H-NMR spectroscopy of $CHCl_3$ solubles. The volatiles *A. lyallii* contained the deterpene acid 1 at a level of about 4% each. There were several other peaks in the same region and 1 was the major component.

2. lsolation of anisotomenoic acid 1

Through a series of fractionations using C-18 and then Si-gel we were able to isolate an anisotomenoic acid (Figs. 1 and 2). 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. The NMR spectra were initially run in $CDCl_3$. There were a number of distinuishing features of the 1H-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. Anisotomenoic acid 1 shows the potent cytotoxic activity. The cytotoxicity was a dose-dependent. It was cytotoxic to B 16/F10 melanoma cells (IC_{50}, 30.13 μg/ml) and to P388 murine leukaemia cells (IC_{50}, > 25.00 μg/ml).

Acknowledgements

This research was supported by Wonkwang Health Science College and Brain Korea 21 Project.

References

1. Lorimer, S. D., Barns, G., Evans, A. C., Foster, L. M., May, B. C. H., Perry, N. B. and Tangney, R. S. : Cytotoxicity and antimicrobial activity of plants from New Zealand's subantarctic islands. Phytomed. 2 (4), 327-333, 1996.
2. Van Klink, J. W. and Perry, N. B. : Essential Oils of *Anisotome antipoda* and *Anisotome latifolia* from New Zealand's Subantarctic Islands. J. Essent. Oil Res.(accepted).
3. Brandt, J. J. and Schultze, W. : Composition of the essential oils of *Ligusticum mutellina* (L.) Crantz (Apiaceae). J. Essent. Oil Res. 7, 231-235, 1995.
4. Bandeen, P., Perry, N. B. and Weavers, R. T. Unpublished results. PERU report 1998.
5. Van Klink, J. W. and Perry, N. B. : Diterpenes from *Anisotome flexuosa.* PERU Report, 1997.
6. Van Klink, J. W., Barlow, A. J., Perry, N. B. and Weavers, R. T.: A new irregular diterpene skeleton from *Anisotome flexuosa.* Tetrahedron Lett., 40, 1409-1412, 1999.
7. Carmichael, J., Degraff, W. G., Gazear, A. F., Minna, J. D. and Mitchell, J. B. : Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. Cancer Res. 47: 936-942, 1987.
8. Mosmann, T. : Rapid colorimetric assays for cellular growth and survival: application to proliferation and cytotoxicity assays. Immunol. Methods, 65: 55-63, 1983.
9. Dawson, J. W. and Webb, C. J. : Generic problems in Australasian Apioideae (Umbelliferae). Actes du 2eme Symposium International sur les Ombelliferes (Perpignan, 1977). "Contributions pluridisciplinaires a la Systematique" (Paru 1978:), 21-32.
10. Mark, A. F. and Adams, N. M. : New Zealand Alpine Plants, Godwit, Auckland 1995.
11. Van Klink, J. W. and Perry, N. B. : Analyses of native Apiaceae extracts: Anisotome and Gingidia. PERU Report, 1996