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Cytotoxic Agents of *Thujopsis dolabrata* (L. fil.) Sieb. et Zucc.

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A cytotoxic agent which inhibits the division of HeLa cells at metaphase stage was isolated from *Thujopsis dolabrata* (L. fil.) Sieb. et Zucc. and identified as desoxypodophyllotoxin. Three substances, desoxypodophillic acid, desoxypicropodophyllin and β-peltatin-B-methylether were isolated from the recrystallization mother liquor of desoxypodophyllotoxin after treatment with alkali. Desoxypodophyllotoxin was found to be almost exclusively contained in the leaf and bark of this tree and only in minute quantity in its wood. It was also shown to be contained in *T. dolabrata* var. *hondae* Makino, *Juniperus rigida* Sieb. et Zucc. and *J. chinensis* L. by thin-layer chromatography and cytotoxicity.

Thujopsis dolabrata (L. fil.) Sieb. et Zucc. (Cupressaceae, Japanese name "asunaro") is an evergreen tree usually less than 30 m tall and widely distributed in Japan. In the course of continuous search for substances toxic to cultured cells, an alcoholic extract of this plant was found to strongly inhibit the division of HeLa cells at the metaphase stage. Because the wood of this plant is much used as material for building and for lacquared ware, it has been the subject of many investigations and numerous terpenes[2] have been isolated. However none of these substances are known to have toxic activity against the cultured cells. This led us to undertake a characterization of the active principle. As preliminary fractionation of the alcoholic extract showed that the active substance was contained in the neutral portion, this portion was further purified by column and thin-layer chromatography (TLC).

Table I. Location and Time of Collection of the Plant Materials

Species	Japanese name	Collected in	Collected at
Thujopsis dolabrata (L. fil.) Sied. et Zucc.	asunaro	June	Agematsu, Nagano Pref.
T. dolabrata var. *hondae* Makino	hinoki-asunaro	July	Takisawa, Aomori Pref.
Thuja standishii (Gord.) Carr.	kurobe	June	Agematsu, Nagano Pref.
Chamaecyparis obtusa (Sieb. et Zucc.) Sieb. et Zucc.	hinoki	September	Mt. Katsuragi, Osaka Pref.
C. pisifera (Sieb. et Zucc.) Sieb et Zucc.	sawara	June	Agematsu, Nagano Pref.
Juniperus conferta Parlat	hainezu	August	Mutsuyokohama, Aomori Pref.
J. rigida Sieb. et Zucc.	nezu	May	Mt. Shakadake, Osaka Pref.
J. chinensis L.	ibuki	June	Saigasaki, Wakayama Pref.

1) Location: *Fukushima-ku, Osaka.*

2) J. Kawamura, *Bull. Govt. Forest Expt. Sta.*, **30**, 59 (1930); T. Nozoe, A. Yasue, and K. Yamano, *Proc. Japan Acad.*, **27**, 15 (1951); T. Nozoe, K. Takase, and M. Ogata, *Chem. Ind.*, **1957**, 1070; R. Hodges, *J. Chem. Soc.*, **1961**, 4247; S. Ito, K. Endo, H. Honma, and K. Ota, *Tetrahedron Letters*, **1965**, 3777; M. Ito and K. Abe, *Bull. Chem. Soc. Japan*, **38**, 324 (1965); Y. Kitahara and A. Yoshikoshi, *ibid.*, **38**, 735 (1965); W.G. Dauben and P. Oberhansli, *J. Org. Chem.*, **31**, 315 (1966); T. Yanagawa, Y. Hirose, and T. Nakatsuka, *Nippon Mokuzai Gakkaishi*, **13**, 160 (1967); T. Tada and T. Nakatsuka, *ibid.*, **14**, 344 (1968).

A population of this plant native to North Japan is treated by some botanists as a variety and named *T. dolabrata* var. *hondae* Makino ("hinoki-asunaro"). Constituents of this variety were compared with those of *T. dolabrata*, and the occurrence of the active principle of this plant in some other Japanese species of this family was investigated.

Experimental

Plant Materials——The materials used in this study are listed in Table I. After collection, the materials were air-dried and stored until the extraction.

Preliminary Extraction and Fractionation of Cytotoxic Principles from *T. dolabrata*——Fresh leaves and twigs of *T. dolabrata* (26 g) were ground in ethanol (200 ml) and allowed to stand for a week at room temperature. The filtrate through a filter paper was evaporated to dryness under reduced pressure and yielded a green tar (2.768 g). A portion of this extract (2.067 g) was treated as summarized in Fig. 1. Only the neutral fraction inhibited the division of HeLa cells at a concentration of 0.1 μg/ml.

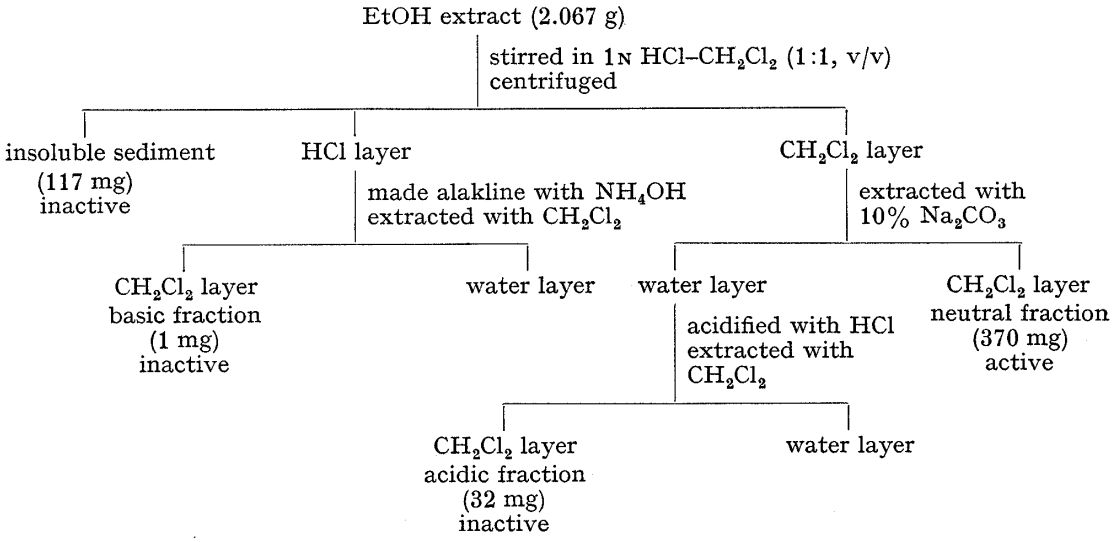


Fig. 1. Preliminary Fractionation of the Cytotoxic Agent contained in *T. dolabrata*

Isolation of Active Principles from *T. dolabrata*——Dried leaves and twigs (4.2 kg) of *T. dolabrata* were extracted with methanol (72 liters) under reflux. A residue (146 g) after evaporation of methanol was chromatographed on charcoal (200 g). A methanol eluate (12.40 g) was discarded and chloroform and benzene eluates (100.94 g) were again chromatographed on silica gel (800 g). Benzene–chloroform (1: 1), chloroform and chloroform–methanol (1: 1) eluates (28.756 g) were extracted with petroleum ether (3 liters). The insoluble portion was partitioned between ether (0.5 liter) and 10% Na_2CO_3 (0.5 liter). The ether solution was evaporated to dryness and the residue (6.880 g) was subjected to preparative TLC (Silica gel G plate, developed by a mixture of acetone and methylene chloride (1: 9 v/v)). An active portion (2.380 g) was repeatedly crystallized from absolute ethanol to yeild 166 mg of colorless prisms (I), mp 166—169°, $[\alpha]_D^{27}$ $-113.9\pm1.6°$ (c=0.985, $CHCl_3$). *Anal.* Calcd. for $C_{22}H_{22}O_7$: C, 66.32; H, 5.57; O, 28.11; OMe, 23.37. Found: C, 66.34; H, 5.39; O, 27.88; OMe, 23.66. This compound was identified as desoxypodophyllotoxin by comparison of its melting point, mobilities on thin-layer plates, infrared (IR), circular dichroism (CD), ultraviolet (UV), nuclear magnetic resonance (NMR), and mass spectra with those of an authentic sample. A residue (2.114 g) recovered from the mother liquor of the desoxypodophyllotoxin crystallization was dissolved in 80% methanol (40 ml) containing K_2CO_3 (2 g), refluxed for 2 hr, then water (200 ml) was added and the solution was acidified with conc. HCl. The precipitate which formed was filtered off, washed with chloroform (200 ml), and recrystallized from methanol to yield colorless needles (II, 343 mg), mp 164—167°. The melting point was raised to 171—172° by further recrystallization. $[\alpha]_D^{24}$ $-158.5\pm2.3°$ (c= 0.877, pyridine). *Anal.* Calcd. for $C_{22}H_{24}O_8$: C, 63.45; H, 5.81; O, 30.74; OMe, 22.36. Found: C, 63.19; H, 5.93; O, 30.94; OMe, 22.59. IR ν_{max}^{Nujol} cm^{-1}: 3564 (sh.), 3450, 3400 (OH); 3085 (sh.), 3045, 1712 (COOH), 1592 (aromatic C=C). UV λ_{max}^{EtOH} nm (ε): 293 (3.71), 235 (sh.) (4.08), 205.5 (4.76). Mass Spectrum m/e: 398 (M^+-18). This was identified as desoxypodophyllic acid by comparison of its physical properties with those of an authentic sample. The acidic filtrate was shaken with chloroform. A chloroform extract (1621 mg) was crystallized from methanol to yield colorless needles (III, 752 mg), mp 171—172°, $[\alpha]_D^{23}+34.7\pm$ 0.8° (c=1.010, chloroform). *Anal.* Calcd. for $C_{22}H_{22}O_7$: C, 66.32; H, 5.57; O, 28.11; OMe 23.37. Found: C, 66.51; H, 5.60; O, 27.84; OMe, 23.13. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1774, 1765 (γ-lactone); 1590 (aromatic C=C),

UV λ_{max}^{EtOH} nm (ε): 204 (4.80), 240 (sh. 4.13), 293 (3.39). Mass Spectrum *m/e*: 398 (M^+). This was identified as desoxypicropodophyllin by comparison of its physical properties with those of an authentic sample. The residue (859 mg) recovered from the crystallization mother liquor of desoxypicropodophyllin was crystallized from methanol to yield colorless needles (IV, 144 mg), mp 181—183°, $[\alpha]_D^{25}+12.6\pm0.6°$ ($c=0.907$, $CHCl_3$). *Anal.* Calcd. for $C_{23}H_{24}O_8$: C, 64.48; H, 5.65; O, 29.88; OMe, 28.98. Found: C, 64.48; H, 5.76; O, 29.79, OMe, 28.86. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1770 (γ-lactone), 1623, 1593 (aromatic C=C). UV λ_{max}^{EtOH} nm (ε): 210 (4.83), 235 (4.30) (sh.), 281 (3.42), 287 (3.37) (sh.). Mass Spectrum *m/e*: 428 (M^+). NMR ($CDCl_3$) τ: 3.63 (aromatic, 3H), 4.10 (methylenedioxy, 2H), 5.5—5.7 (2H), 6.00 (OMe, 3H), 6.17 (OMe, 3H) 6.22 (OMe, 6H), 6.5—7.4 (5H).

Estimation of Desoxypodophyllotoxin Concentration in Tissues of *T. dolabrata*——A tree of *T. dolabrata* (about 35 years old, 5 m height) was cut down just above the ground in June at Agematsu, separated into leaf, tip of twig, bark, sap wood, and heart wood and air-dried. A part of the root was also dug up and separated into bark and wood. Aliquots (about 3 g) of the dried materials were extracted in a Soxhlet apparatus with methanol. The methanol extracts obtained after evaporation of the solvent were again dissolved in methanol at a concentration of 1 mg/ml and subjected to serial ten-fold dilutions with the culture medium. The samples were added to the HeLa cell cultures suspended in an YLE medium containing 10% bovine serum at a concentration of 1.0×10^5 cells/ml and incubated at 37° on the third day of cultivation. After two days of incubation with the sample, the number of cells was determined using an electronic cell counter (TOA electronic Co., Ltd.). A set of three cultures was employed for each dosis of the samples. The growth inhibition rate (%) was calculated for each dose level from the following formula: $T-C_0/C-C_0\times100$, where C and T are final cell numbers in control and experimental cultures respectively and C_0 is a cell number in the control at the time of drug administration. It was assumed that the regression line between the inhibition rate and the log of the concentration of the drug is straight within defined limits of response. The effective dosis of 50% growth inhibition (ED_{50}) was determined graphically.

Distribution of Desoxypodophyllotoxin among the Cupressaceae Plants——Fresh twigs of the Japanese Cupressaceae plants summarized in Table I were extracted with methanol. Sample of the methanol extracts were spotted on thin-layer plates. After development with benzene–acetone (9: 1, v/v) or methylene chloride–acetone (9: 1, v/v), the plates were sprayed with Dragendorff reagent. Desoxypodophyllotoxin was detected as an orange red spot on a pale yellow background. Samples of the extracts were also added to the HeLa cell suspensions to examine the cytotoxicity.

Result and Discussion

The cytotoxic principles I, II and III were identified as desoxypodophyllotoxin, desoxypodophyllic acid and desoxypicropodophyllin respectively. Desoxypodophyllic acid and desoxypicropodophyllin were derived from authentic desoxypodophyllotoxin according to the literature.[3] Although the infrared spectrum of substance II was identical with that given for desoxypodophyllic acid in the literature[4] and supported a structure possessing an opened lactone ring, the peaks of its mass spectrum were identical with those of desoxypicropodophyllin, the highest peak being *m/e* 398. This indicates that elimination of water occurs promptly after injection of the substance into the mass chamber. The melting point and IR spectrum of substance IV were identical with those of β-peltatin-B-methylether.[5] Its analytical value, and mass (M^+, *m/e* 428) and NMR spectra supported this structure.[6] To further elucidate the conformation of IV, its CD curve was examined. As shown in Fig. 2a, the curve in the range 260—300 nm demonstrated the *cis* (2:3)-*trans* (3:4) arrangement of IV and its configurational identity with desoxypicropodophyllin except for the fourth methoxyl group.[7] The temperature-dependent change in the shape of the curve in this region (Fig. 2b) also supported this flexible ring system.[7] From these results, substance IV was confirmed to be β-peltatin-B-methylether. Because it is well known that podophyllotoxin and its derivatives are converted irreversiblly to their B-series isomers by treatment with alkaline reagent and B-series isomers are generally more easily crystallized than their A-

3) J.L. Hartwell, A.W. Schrecker, and J.M. Johnson, *J. Am. Chem. Soc.*, **75**, 2138 (1953).
4) A.W. Schrecker and J.L. Hartwell, *J. Am. Chem. Soc.*, **75**, 5916 (1953).
5) J.L. Hartwell, A.W. Schrecker, and Y. Greenberg, *J. Am. Chem. Soc.*, **74**, 6285 (1952).
6) A.M. Duffield, *J. Heterocyclic Chem.*, **4**, 16 (1967); A. Pelter, *J. Chem. Soc.* (*C*), **1968**, 74.
7) R.J. Swan, W. Klyne, and H. MacLean, *Can. J. Chem.*, **45**, 319 (1967); D.C. Ayres, J.A. Harris, and P.B. Hulbert, *J. Chem. Soc.* (*C*), **1971**, 1111.

series isomers,[8] it seems likely that substance IV is contained as β-peltatin-A-methylether (V) in plant bodies. Although desoxypodophyllotoxin inhibited the division of HeLa cells (ED_{50}=0.0036 μg/ml), it failed to retard the death of mice affected with lymphatic leucemia L1210 at 90 mg/kg. On the contrary, desoxypicropodophyllin inhibited the cell division only at 1 μg/ml but significantly retarded the death of mice at 50 mg/kg. As the crude alcoholic extract of *T. dolabrata* inhibited the cell division at 1 μg/ml but did not retard the death caused by L1210, this active principle is probably contained as desoxypodophyllotoxin in the plant body.

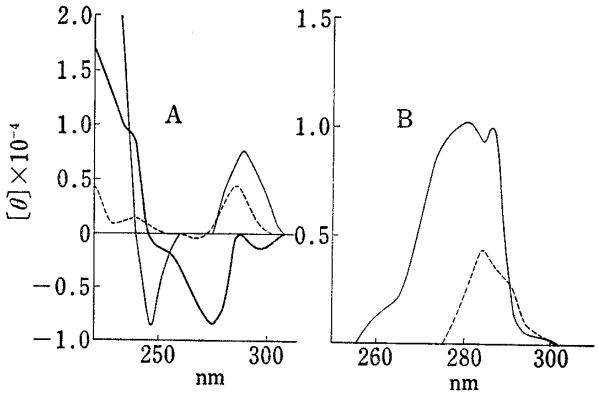
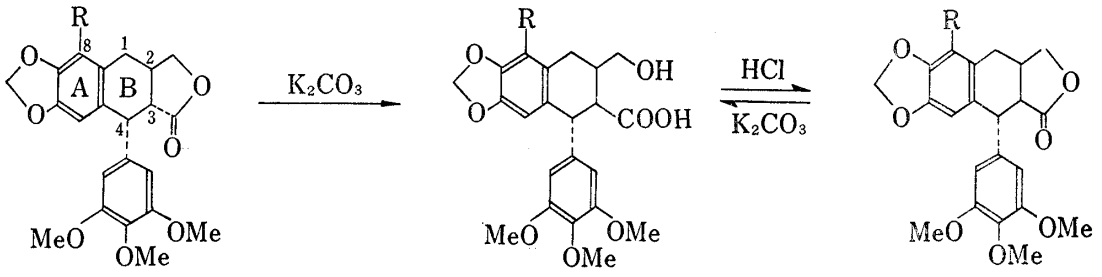


Fig. 2. CD Curves of Desoxypodophyllotoxin, Desoxypicropodophyllin and β-Peltatin-B-methylether

A: (—) desoxypodophyllotoxin: (—) desoxypicropodophyllin: (···) β-peltatin-B-peltatin-β-methylether. The compounds were dissolved in methanol. The cruves were taken at 25°.
B: Temperature-dependent section of the CD curves of β-peltatin-B-methylether: (···) 25°, (—) 190°, Diethylether–ethanol–isopentane (5: 2: 5) was used for the measurements.

The concentration of the active principles in the tissues of the plant was estimated from the inhibitory activities of the alcoholic extracts against HeLa cells. Although β-peltatin-B-methylether did not inhibit cell division, it is not impossible that β-peltatin-A-methylether contributes to some degree to the cytotoxic action of the plant extract because the latter is reported to inhibit the growth of Walker carcinama 256[9] and the



I, V II III, IV

R=H, desoxypodophyllotoxin (I)
R=OCH_3, β-peltatin-A-methylether (V)

R=H, desoxypodophyllic acid (II)

R=H, desoxypicropodophyllin (III)
R=OCH_3, β-peltatin-B-methylether (IV)

activity of podophyllotoxin derivatives is generally known to be greatly reduced when the compounds are converted to their B series isomers.[10] However, as the yeild of β-peltatin-B-methylether was lower than that of desoxypodophyllotoxin plus desoxypicropodophyllin, the toxic activity of the plant was considered to be mainly due to desoxypodophyllotoxin. This was also supported by the continuous rise in activity during the purification procedure for isolation of desoxypodophyllotoxin. We have therefore expressed the concentration of the active principles as that of desoxypodophyllotoxin. As summarized in Table II, the

8) A.W. Schrecker and J.L. Hartwell, *Helv. Chim. Acta*, **37**, 1541 (1954).
9) E. Bianchi, K. Sheth, and J.R. Cole, *Tetrahedron Letters*, **1969**, 2759.
10) M.G. Kelly, E.W. Ligon, Jr., C. Davison, and P.K. Smith, *Cancer Res.*, **9**, 555 (1949); M.G. Kelly, R.C. MacCardle, and P.K. Smith, *ibid.*, **9**, 599 (1949); M.G. Kelly, A.P. Truant, and P.K. Smith, *Fed. Proc.*, **8**, 306 (1949); M.G. Kelly, J. Leiter, A.R. Bourke, and P.K. Smith, *Cancer Res.*, **11**, 263 (1951); M.G. Kelly, J. Leiter, R.C. MacCardle, and P.K. Smith, *ibid.*, **11**, 263 (1951); M.G. Kelly, J. Leiter, O. Ghosh, and P.K. Smith, *J. Nat. Cancer Inst.*, **12**, 1177 (1952).

Table II. Distribution of the Active Principles in *T.dolabrata*

Part examined	Concentration, % dry weight
Trunk, wood	0.000022[a)]
bark	0.0042
Root, wood	0.000064
bark	0.0038
Leaf (containing tip of twig)	0.018
Twig	0.026

a) as desoxypodophyllotoxin

highest concentration was found in the leaf and tip of the twig. Although the bark of the trunk and the root contained considerable quantities of this substance, it was found only in trace in the wood.

The distribution of desoxypodophyllotixn among Japanese Cupressaceae plants was examined by the inhibitory effects against HeLa cells and thin-layer chromatograpms of their alcoholic extracts. Table III shows that, apart form *Thujopsis dolabrata*, it is contained only in *Juniperus chinensis* L. and *J. rigida* Sieb. et Zucc. A fraction having the same *Rf* value as this substance was also detected in the alcoholic extract from *J. conferta*, but it did not inhibit the cell division. As this was considered to be probably due to the low concentration of the substance, a further examination was not carried out. The constituents of *T. dolabrata* var. *hondae* were same as those of *T. dolabrata*, although differences in the concentrations of the substances were found between these two plants.

Table III. Distribution of Desoxypodophyllotoxin among Japanese Cupressaceae Plants

Species	Toxicity to Hela cells 10 μg/ml	1 μg/ml	TLC (Dragendorff)
Ch. obtusa	−	−	−
Ch. pisifera	−	−	−
J. chineniss	++	−	+
J. chinensis var. *sargentii*	−	−	−
J. conferta	−	−	+
J. rigida	++	−	+
Thuja standishii	−	−	−
Thuja dolabrata	+++	+	+
Thuja dolabrata var. *hondae*	+++	+	+

Podophyllotoxin and some of its derivatives are known to be toxic to tumor cells both *in vivo* and *in vitro*.[11)] Desoxypodophyllotoxin was isolated from several plants and reported to have a prominent inhibitory activity against sarcoma 37 in mice and cultured cells (KB) derived from human carcinoma of the nasopharynx.[12)] In the present study, this compound

11) J.L. Hartwell, and M.J. Shear, *Cancer Res.*, **7**, 716 (1947); J.L. Hartwell, *J. Am. Chem. Soc.*, **69**, 2918 (1947); J.L. Hartwell and W.E. Detty, *ibid.*, **70**, 2833 (1948); **72**, 246 (1950); J. Leiter, V. Downing, J.L. Hartwell, and M.J. Shear, *J. Nat. Cancer Inst.*, **10**, 1273 (1950); E.M. Greenspan, J. Leiter, and M.J. Shear, *ibid.*, **10**, 1295 (1950).

12) K. Noguchi and M. Kawanami, *Yakugaku Zasshi*, **60**, 629 (1940); C. Hata, *Nippon Kagaku Zasshi*, **63**, 1540 (1942); L. Marion, *Can. J. Research*, **20B**, 157 (1942); J.L. Hartwell, J.M. Johnson, D.B. Fitzgerald, and M. Belkin, *J. Am. Chem. Soc.*, **74**, 4470 (1952); H. Kofod and C. Jorgensen, *Acta Chem. Scand.*, **9**, 346 (1955); D.B. Fitzgerald, J.L. Hartwell, and J. Leiter, *J. Nat. Cancer Inst.*, **18**, 83 (1957); S.M. Kupchan, R.J. Hemingway, and J.C. Hemingway, *J. Pharm. Sci.*, **56**, 408 (1967); E. Bianchi, M.E. Caldwell, and J.R. Cole, *ibid.*, **57**, 696 (1968).

was found to be toxic to HeLa cells too. It is noteworthy that, like other podophyllotoxin derivatives,[10] it lost almost entirely its activity against cultured cells but became toxic to mouse leukemia L1210.

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