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SESQUITERPENES AND PHYTOSTEROLS IN THE TISSUE CULTURES OF *LINDERA STRYCHNIFOLIA**

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Abstract—Components of the tissue cultures derived from *Lindara strychnifolia*, Vill. were investigated. Lindenenol, lindenenol acetate, linderane, linderalacton, lindesterene and caryophyllene were detected by gas chromatography and TLC, and the ratios of the sesquiterpene contents in the tissue cultures were compared with the ratios of those in the original plant. Moreover, campesterol, stigmasterol and β-sitosterol were detected by gas chromatography.

INTRODUCTION

TISSUE cultures derived from a number of higher plants have shown the ability to synthesize secondary metabolites. For example, in tissue cultures the formation of nicotine by *Nicotiana*,[1] tropane alkaloids by *Datura*,[2] *Vinca* alkaloids by *Catharanthus*,[3] reserpine by *Alstonia*,[4] indol alkaloids by *Ipomea*,[5] visnagine by *Ammi*,[6] formononetin by *Cicer*,[7] solasonine by *Solanum*,[8] diosgenin by *Dioscorea*[9] and several sterols and triternenes by *Nicotiana*[10] and various other species[11] have been observed. However, the formation of sesquiterpenes in tissue culture has not yet been reported.

Lindera strychnifolia Vill. (Lauraceae) has long been used as a "Chinese drug" in China and Japan and the components have previously been studied by Takeda *et al.* They isolated many sesquiterpenes having a furan ring and established their structures. We report here on the presence of lindenenol†[12] (I), linderane[13] (III), lindenenol acetate†[14] (II), linderalacton[15] (IV), lindestrene[16] (V) and caryophyllene (VI) in the tissue cultures derived from

* Part I in a projected series "Chemical Studies on Plant Tissue Cultures".

† The names, linderene and linderene acetate have been changed to lindenenol and lindenenol acetate, respectively: K. TAKEDA *et al.*, in press.

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[2] W. N. CHAN and E. J. STABA, *Lloydia* **28**, 55 (1965).

[3] L. A. HARRIS, H. B. NYLUND and D. P. CAREW, *Lloydia* **27**, 322 (1964); G. B. BODER, M. GORMAN, S. JOHNSON and P. J. SIMPSON, *Lloydia* **27**, 328 (1964); I. K. PITCHER, K. STOLLE, D. GROGER and M. MOTHES, *Naturwissenschaften* **52**, 305 (1965).

[4] D. P. CAREW, *Nature* **207**, 89 (1965).

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[10] P. BENVENISTE, L. HIRTH and G. OURISSON, *Phytochem.* **5**, 31 (1966).

[11] J. D. EHRHARDT, L. HIRTH and H. OURISSON, *Phytochem.* **6**, 815 (1967)

[12] K. TAKEDA and M. IKUTA, *Tetrahedron Letters* No. 6, 277 (1964).

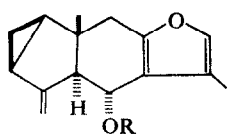
[13] K. TAKEDA, H. MINATO and H. HORIBE, *Tetrahedron* **19**, 2307 (1963).

[14] K. TAKEDA, H. MINATO, M. ISHIKAWA and M. MIYAWAKI, *Tetrahedron* **20**, 2655 (1965).

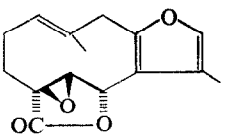
[15] K. TAKEDA, H. MINATO and M. ISHIKAWA, *J. Chem. Soc.* 4578 (1964).

[16] K. TAKEDA, H. MINATO, M. ISHIKAWA and M. MIYAWAKI, *Tetrahedron* **20**, 2655 (1964).

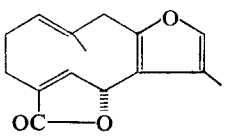
shoots of *L. strychnifolia*. Moreover, β-sitosterol, campesterol and stigmasterol were detected by gas chromatography.



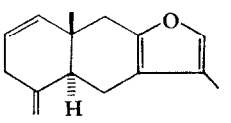
Lindenenol (I) R = H
Lindenenol Acetate (II) R = $COCH_3$



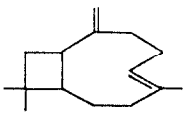
Linderane (III)



Linderalacton (IV)



Lindestrene (V)



Caryophyllene (VI)

RESULTS AND DISCUSSIONS

The callus of *Lindera strychnifolia* was derived from young defoliated shoots and green fruits on Linsmeier-Skoog agar medium fortified with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin. The combinations and concentrations of growth regulators added to the medium are shown in Table 1.

TABLE 1. EFFECTS OF S,4,-D AND KINETIN ON THE CALLUS FORMATION FROM EXCISED FRUITS AND SHOOTS OF *Lindera strychnifolia*

2,4-D conc. (M)	Organ	Concentrations of kinetin (ppm) 0	0·02	0·2	2·0
0	Fruit	−	−	−	−
10^{-7}	Fruit	−	−	−	−
10^{-6}	Fruit	±	±	±	+
	Shoot	+	+	++	+
10^{-5}	Fruit	++	++	++	++
	Shoot	++	++	++	++

Culture period: 29 days.
−: No induction. ±: Poor induction. +: Good induction. ++: Excellent induction.

As shown in Table 1, the callus was formed at 10^{-5} M and 10^{-6} M of 2,4-D, regardless of the kinetin concentration. This callus derived from shoots was subcultured on the Linsmeier-Skoog medium for 1 yr and established as a stable tissue culture. In subculturing of the callus, 2,4-D was essential and it could not be replaced with indolacetic acid. Moreover, the addition of 0·2% yeast extracts, 0·2% peptone and 0·2% casein hydrolysate to the medium was not effective in furthering the growth of the tissue cultures.

The ether extracts of the tissue cultures were assayed chromatographically for sesquiterpenes. TLC of ether extracts was carried out on silica gel using benzene–ethyl acetate

(9:1), and lindenenol, lindenenol acetate, linderane, lindestrene were detected by spraying with Ehrich's reagent. These sesquiterpenes each showed a characteristic colour reaction and also gave R_fs identical to those of authentic samples. A mixture of phytosterols was isolated by preparative TLC, and β-sitosterol, campesterol and small amounts of stigmasterol were detected by gas chromatography (Table 2). These results show that the callus retains the ability to synthesize these sesquiterpenes and phytosterols even after being subcultured for 1 yr.

TABLE 2. THE QUANTITATIVE ANALYSIS OF PHYTOSTEROLS IN THE TISSUE CULTURES OF *Lindera strychnifolia* BY GAS CHROMATOGRAPHY

Compound	Relative retention time*		Ratio of sterol contents
	Alcohol	Trimethylsilyl ether	
Campesterol	2·18	2·56	6
Stigmasterol	2·32	2·73	1
β-Sitosterol	2·64	3·16	53

* Retention times relative to chloestane.

It was, however, anticipated that the ratios of the sesquiterpene contents in the tissue cultures would differ from the ratios of those in the original plants. Since these differences are an important concern when considering the utilization of the tissue cultures for manufacture of these compounds, a quantitative analysis of the sesquiterpenes was carried out by gas chromatography. As shown in Table 3, a main component of the sesquiterpenes found in the roots is lindenenol acetate and it is present in amounts 3 to 5 times higher than other components. Leaves contain mainly lindenenol acetate and the other four components are present only in trace amounts. On the other hand, the tissue cultures produce nearly equal amounts of lindenenol, lindenenol acetate, linderane and linderalacton, respectively. Thus, the characteristic compositions of sesquiterpenes in the differentiated tissues of the plant were lost in undifferentiated tissue cultures of *L. strychnifolia.*

TABLE 3. THE QUANTITATIVE ANALYSIS OF SESQUITERPENES IN THE TISSUE CULTURES OF *Lindera strychnifolia* BY THIN-LAYER AND GAS CHROMATOGRAPHY

Compound	R_f* (TLC)	Retention time (GLC) (min)	Ratio of contents		
			Root	Leaf	Tissue cultures
Lindenenol acetate	0·61	2·5	3	Main	1·6
Lindenenol	0·37	2·8	0·6	Trace	1
Linderalacton	0·34	6·4	1	Trace	2
Linderane	0·50	10·9	0·8	Trace	2
Linderene	0·69	—	1	Trace	Trace
Caryophyllene	—	5·2	—	—	Trace

* Solvent system: Benzene–ethylacetate (9:1).

EXPERIMENTAL

Source of Plant Material

The young shoots of *Lindera strychnifolia* used were collected in June from the Owase area of Mie prefecture, Japan.

Tissue Culture and Preparation of Media

The young defoliated shoots were sterilized in 70% alcohol followed by immersion in 0·2% $HgCl_2$ for 20 min and were then washed three times with sterilized water. The shoots were cut to an appropriate size (0·7–1·0 cm). The explants were placed under sterile conditions on the Linsmeier-Skoog medium containing 0·8% agar, 0–2·0 ppm 2,4-dichlorophenoxyacetic acid and 0–2·0 ppm kinetin (Table 1). After 2–4 weeks, greyish-white callus was formed from those explants inoculated on the medium containing 10^{-5}–10^{-6} M 2,4-D. This callus was subcultured on the Linsmeier–Skoog medium supplemented with 10^{-5} M 2,4-D and 0·02 ppm kinetin every 4 weeks.

Extraction of Sesquiterpenes

The callus (fresh wt. 24·39 g) was extracted three times with acetone (3 × 100 ml) at room temp for 48 hr. The solvent was evaporated and the residue was re-extracted with ether. The ether extract was evaporated to dryness (30·7 mg) and examined using thin-layer and gas chromatography.

Gas Chromatography of Sesquiterpenes

(i) Gas chromatography was run on a Shimadzu Gas Chromatograph GC-4A (PF) instrument fitted with a hydrogen flame ionization detector. A glass U-column (1·5 m × 4 mm o.d.) packed with 1% Carbowax (20 M) on Gas-Chrom Q (100–120 mesh) was operated under the following conditions. Temperatures of the injection port, detector block and column oven were 191°, 241°, and 206°, respectively. The carrier gas was N_2, with a flow rate of 60 ml/min.

The ether extract (30·7 mg) obtained from the tissue cultures was dissolved in 50 μl of CH_2Cl_2 and examined quantitatively for sesquiterpenes (Table 3).

(ii) Caryophyllene was detected by a gas chromatograph with a heat transfer detector. An aluminium coil column (10 ft × $\frac{3}{8}$ in. o.d.) packed with 5% diethylene glycol succinate on Chromosorb W (45–60 mesh) was operated at 130°. The carrier gas was He, with a flow rate of 100 ml/min.

Extraction of Phytosterols

The fresh callus (*ca.* 167 g) was extracted three times for 4 hrs with boiling methanol. The methanol extracts were combined and evaporated to dryness. After the residue was saponified with 10% ethanolic KOH for 2 hr at the b.p., the solution was extracted three times with petroleum ether. The petroleum ether extracts were evaporated to dryness. The residue (10·1 mg) was examined for phytosterols as follows.

Thin-layer and Gas Chromatography of Phytosterols

(i) The residue described above was applied to silica gel plates (0·3 mm) and developed with *n*-hexane–$CHCl_3$–EtOAc (4:1:1). To visualize the mixture of phytosterols, the plates were sprayed with water. A mixture of phytosterols appeared on the plate as a white zone (R_f 0·27) and extracted three times with $CHCl_3$. These extracts were evaporated to dryness and the residue (3·6 mg) was analysed quantitatively by gas chromatography.

(ii) For the quantitative analysis of β-sitosterol, stigmasterol and campesterol, a gas chromatograph fitted with a hydrogen flame ionization detector was used. A glass U-column (3 m × 4 mm o.d.) packed with 1% OV-101 on Gas-Chrom Q (100–120 mesh) was operated under the following conditions. The temperatures of the injection port, detector block and column oven were 260°, 282°, and 265°, respectively. The carrier gas was N_2, with a flow rate of 60 ml/min.

Gas chromatography of the trimethylsilyl ethers of the sterols was carried out under the same conditions.

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