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GAS CHROMATOGRAPHY–MASS SPECTROMETRY STUDY OF STEROLS FROM *PINUS ELLIOTTI* TISSUES*

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Key Word Index—*Pinus elliottii*; Pinaceae; slash pine; sterols; tissue culture.

Abstract—A comparative study of the sterol components of slash pine (*Pinus elliottii*) callus tissue cultures, seeds, and seedlings was carried out using GC–MS techniques. Cholesterol, desmosterol, campesterol, stigmasterol, sitosterol and cycloeucalenol were identified in all tissues while lophenol and 24-methylenelophenol were identified in only the seed and seedlings. 24-Ethylidenelophenol was detected in trace concentrations in only the seedlings. Sitosterol was the predominant sterol component, i.e. 80·8, 38·1 and 47·8% of the tissue culture, seed and seedling sterols, respectively.

INTRODUCTION

GERM-FREE callus tissues grown in synthetic media have proven useful in the study of certain biochemical and physiological processes in higher plants. In our previous studies, we have compared the lipid composition of tissue cultures with that of the intact plants and found that, although they are generally similar, certain qualitative and quantitative differences are present.[1–3] As part of our continuing studies to characterize the lipid components of higher plant tissue cultures, we have identified and compared the sterol components of slash pine tissue cultures, seeds and seedlings.

RESULTS AND DISCUSSION

The eleven sterols identified (as acetates and/or TMS derivatives) in the pine tissues of this study had GLC relative retention times similar to the corresponding authentic standards and those reported by Patterson.[4] Compound I is identified as cholesterol. The mass spectrum of its trimethylsilyl ether compared closely with those of authentic standards and those reported by Brooks *et al.*[5] Compound II appears to be desmosterol. The base peak is *m/e* 366 for desmosteryl acetate which corresponds to the loss of the acetate moiety from the parent molecule. The silyl ether derivative of the same compound gave a base peak of *m/e* 129 with a major fragment (68% relative intensity) at *m/e* 343 which can be interpreted as the loss of the side-chain and two protons from the parent compound. Other sterol derivatives

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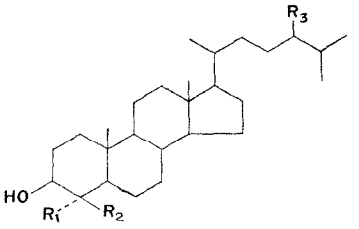
[2] LASETER, J. L., LAWLER, G. C., WALKINSHAW, C. H. and WEETE, J. D. (1973) *Phytochemistry* **12**, 817.

[3] WEETE, J., VENKETESWARAN, S. and LASETER, J. L. (1971) *Phytochemistry* **10**, 939.

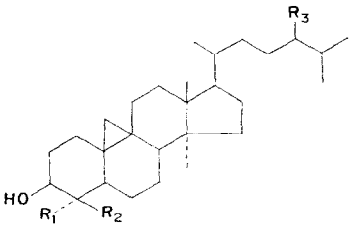
[4] PATTERSON, G. W. (1971) *Anal. Chem.* **43**, 1165.

[5] BROOKS, C. H. W., HORNING, E. C. and YOUNG, J. S., (1968) *Lipids* **3**, 391.

of the same MW and similar retention values such as zymosterol and 7-dehydrocholesterol can be eliminated on the basis of no major fragments at *m/e* 456 in the case of zymosteryl and *m/e* 351 for 7-dehydrocholesteryl trimethylsilyl derivatives.



(I)	Cholesterol	Δ^5, $R_1 = R_2 = R_3 = H$
(II)	Desmosterol	$\Delta^{5,24}$, $R_1 = R_2 = R_3 = H$
(III)	Lophenol	Δ^7, $R_1 = Me$; $R_2 = R_3 = H$
(IV)	Compesterol	Δ^5, $R_1 = R_2 = H$; $R_3 = Me$
(V)	Stigmasterol	$\Delta^{5,22}$, $R_1 = R_2 = H$; $R_3 = Et$
(VII)	24-Methylenelophenol	$\Delta^{7,24(28)}$, $R_1 = Me$; $R_2 = H$; $R_3 = CH_2$
(VIII)	Sitosterol	Δ^5, $R_1 = R_2 = H$; $R_3 = Et$
(XII)	24-Ethylidenelophenol (citrostadienol)	$\Delta^{7,24(28)}$, $R_1 = Me$; $R_2 = H$; $R_3 = CHMe$



(IX)	Cycloeucalenol	$\Delta^{24(28)}$, $R_1 = Me$; $R_2 = R_3 = H$
(X)	Cycloartenol	Δ^{24}, $R_1 = R_2 = Me$; $R_3 = H$
(XI)	24-Methylenecycloartanol	$\Delta^{24(28)}$, $R_1 = R_2 = Me$; $R_3 = CH_2$

FIG. 1. STEROL STRUCTURES IDENTIFIED FROM SLASH PINE TISSUE CULTURES, SEEDS, AND SEEDLINGS. COMPOUNDS ARE LISTED IN THEIR ORDER OF ELUTION FROM THE 1% SE 30 CHROMATOGRAPHIC COLUMN.

Compound III is identified as lophenol (4α-methylcholest-Δ^7-en-3β-ol). The acetate produced a small molecular ion with a base peak at *m/e* 442 and major fragments at *m/e* 427 (M-15), 269, and 227; the spectrum is in good agreement with that reported previously.[6] The MS of silyl derivatives of Compounds IV and V are identical to authentic standards of campesteryl (M^+472) and stigmasteryl (M^+486) trimethylsilyl ether, respectively. A compound which was only partially resolved and possessed a slightly longer retention time than stigmasterol was present in this fraction (Compound VI). The identity of this compound is not known, but the present evidence suggests that it may be a 4-methylsterol. Additional unresolved compounds are present in this peak and interfere with the spectral interpretation. The mass spectrum of Compound VII had a small molecular ion at *m/e* 454 with a base peak at *m/e* 327 and major fragments at *m/e* 439, 370, 269, 267 and 227; this is in general agreement with that published by Knights[6] for 24-methylenelophenol.

The major sterol in each of the tissues in this study was sitosterol (VIII). The spectrum of this compound was the same as that obtained for the acetate and silyl ether derivatives of authentic sitosterol.[5,6] Another compound is associated chromatographically with sitosterol and there is some evidence that it may be 28-isofucosterol (Δ^5-avenasterol) or a similar compound. In several cases, a peak at *m/e* 394 was observed which corresponds to the M-60 peak of a diunsaturated isomer of sitosterol. Further evidence for the 24-ethylidenecholesteryl acetate structure is suggested by the presence of a peak at *m/e* 296 which is the base peak for this compound.[7]

Since the remaining sterols were found in low relative concentrations, it was necessary to use trapping techniques to isolate them as they were eluted from the chromatographic column, using a trap made of glass capillary tubing packed with 1% OV 17 on Chromosorb P.

[6] KNIGHTS, B. A. (1967) *J. Gas. Chromatog.* 273.
[7] KNIGHTS, B. A. and BROOKS, C. J. W. (1969) *Phytochemistry* **8**, 463.

IX was collected repeatedly in this manner as the free sterol and analyzed using the solid injection probe of the mass spectrometer. A parent ion of the free sterol is *m/e* 426 with other prominent peaks at *m/e* 411, 408, 393, 353, 343, 300 and 283. The MS of this compound is in general agreement with that reported for cycloeucalenol.[8] X is characterized by a molecular ion at *m/e* 468 for the acetate derivative with peaks at 453, 408, 393, 365, 339, 297 and 286. The mass spectrum of this compound is in accord with those reported previously for cycloartenyl acetate.[9,10]

TABLE 1. RELATIVE STEROL CONCENTRATIONS OF THE FREELY EXTRACTABLE LIPIDS OF *Pinus elliotti* TISSUE CULTURES, SEEDS AND SEEDLINGS

	Sterol	Relative %*		
		Tissue culture	Seeds	Seedlings
I	Cholesterol	0·34	5·25	2·90
II	Desmosterol	0·15	0·66	1·74
III	Lophenol	—	2·64	0·67
IV	Campesterol	5·56	7·90	6·95
V	Stigmasterol	6·69	1·31	8·70
VI	Unknown	—	0·52	6·90
VII	24-Methylenelophenol	tr	31·85	16·13
VIII	Sitosterol	80·78	38·07	47·83
IX	Cycloeucalenol	2·01	6·58	4·63
X	Cycloartenol	4·47	3·96	2·83
XI	24-Methylenecycloartanol	tr	1·26	0·72
XII	24-Ethylidenelophenol (citrostadienol)	—	—	tr

* Expressed as relative per cent (%) of the sterols from gas chromatographic data.
tr = less than 0·1%.

The MS of the acetate derivative of XI had a molecular ion at M^+482. Additional peaks were observed at *m/e* 467, 422, 407, 379, 300 and 297 which corresponds to the fragmentation pattern of 24-methylenecyloartanyl acetate.[9,10] The acetate co-chromatographed with an authentic standard. This compound was also collected as the free sterol by the methods described above and analyzed by the direct probe of the mass spectrometer. The MS contains a significant peak at *m/e* 440 with major ion fragments at *m/e* 425, 422, 407, 379, 353, 300 and 175 with a characteristic base peak at *m/e* 107. These findings are in agreement with those reported by Aplin and Hornby[11] for 9,19-cyclosterols like 24-methylenecycloartanol. Both cycloartenol and 24-methylenecycloartanol have been recently confirmed by NMR[12] in the tall oil from southern pine.

XII, 24-ethylidenelophenol was present in sufficient quantities to obtain an identifiable spectrum only in the seedlings. A molcular ion was observed at *m/e* 468 as the acetate derivative with the base peak at *m/e* 327 and significant fragments at *m/e* 370 and 227. The fragmentation pattern was in agreement with reported spectra.[6,9] The presence of this compound has recently been verified in tall oil from southern pine by Rowe using NMR.[12]

[8] KNAPP, F. F. and NICHOLAS, H. J. (1969) *Phytochemistry* **8**, 207.
[9] BENVENISTE, P., HIRTH, L. and OURISSON, G. (1966) *Phytochemistry* **5**, 31.
[10] AUDIER, H. E., BEUGELMANS, R. and DAS, B. C. (1966) *Tetrahedron Letters* **36**, 4341.
[11] APLIN, R. T. and HORNBY, G. M. (1966) *J. Chem. Soc.* **B**, 1078.
[12] ROWE, J. W. (1972) personal communication, Madison, Wisconsin.

In a review on phytosterols, Bean[13] reported that approximately 44 sterols have been isolated and identified from plant tissues. Sitosterol has been identified from extracts of pollen from *P. sylvestris*,[14] whereas both sitosterol and campesterol were identified from the bark of *P. monticola*.[15] This report represents the most detailed investigation of the sterols of a member of the Pinaceae, but with the exception of cholesterol, desmosterol, stigmasterol and lophenol the sterols reported in this study have also been identified for the leaves of the deciduous conifer *Larix decidua*.[16] The sterols identified in the slash pine tissues represent potential intermediates in the biosynthesis of predominant phytosterols such as sitosterol.[9,16,17]

With the exception of the lophenol compounds, the identified sterols are qualitatively the same in each of the tissues studied (Table 1). Lophenol was found in the seed and seedling tissues while 24-ethylidenelophenol was detected in only the seedlings. Sitosterol is the predominant sterol in each of these tissue types, i.e. 80·78, 38·07 and 47·83% for the tissue cultures, seeds, and seedlings respectively. Perhaps the most significant difference in sterol distributions and a possible explanation for the differences in sitosterol concentrations is that 24-methylenelophenol is found in only trace concentrations (<0·1%) in the tissue cultures while it represents a significant portion of the seed and seedling sterols (31·85 and 16·13%, respectively). 24-Methylenelophenol appears to be an intermediate in the formation of 4-desmethyl phytosterols such as sitosterol and may be more readily converted enzymatically in the tissue cultures.

EXPERIMENTAL

The slash pine tissues employed in this study were grown and treated accroding to the methods described by Laseter *et al.*[3] and Brown and Lawrence.[18] The lipids were extracted and prepared for analysis by the methods previously described.[3]

Sterol separation and identification. Chromatographic separation was achieved by use of a Hewlett–Packard Model 5750 gas chromatograph equipped with a 3 m × 2 mm i.d. glass column with 1% SE 30 (GE) on 80/100 mesh Gas-chrom Q. operating isothermally at 270°. The injector and hydrogen flame detectors were maintained at 290°. Approximately 90% of the chromatographic effluent was allowed to simultaneously enter a du Pont 21–491 double focusing mass spectrometer by means of a jet-type separator. Separator and transfer lines were held at 260°. The ion source was at 210° with a filament current of 350 μA. All spectra were obtained at 70 eV with scan speeds of 4 sec/decade. Trimethylsilyl ether derivatives were prepared by treating the sterols (0·1–1 mg) at 50° with 30–40 μl of *N,O*-bis-(trimethylsilyl)-acetamide (Pierce Chemical Company, Rockford, Illinois)in dry pyridine. Sterol acetates were prepared by heating the sterols with Ac_2O for 30 min at reflux temp. In addition to GC–MS analyses of the intact sterol fraction, each fraction was further fractionated into the 4,4-dimethyl, 4-monomethyl, and 4-desmethyl sterols by the method of Goad and Goodwin[16] prior to forming the derivatives. Most chromatographic standards were obtained from Applied Science Laboratories (College Park, Pennsylvania). Other standards have been graciously supplied by Drs. L. J. Goad and Gonzales Gonzales.

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