Lipid Constituents of Callus Tissues of *Mentha spicata*

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A callus tissue culture was established from leaves and stems of *Mentha spicata* L. Lipid constituents isolated from callus tissues were composed of fatty acids, fatty acid methyl esters, triglycerides, squalene, stigmasterol, sitosterol, oleanolic acid (1), ursolic acid (2) and pomolic acid (3). These liquid constituents did not contain monoterpenoids.

Staba *et al.*^{1~3)} have reported that callus tissues induced from the stems and the seedlings of *M. spicata* produce carotenoids and xanthophylls. However, constituents other than these compounds have not been investigated yet. In connection with our studies of the biosynthesis of isoprenoids in higher plants,⁴⁾ we have conducted callus tissue cultures of *M. spicata* and investigated lipid constituents produced by the callus tissues.

Induction and growth of callus tissues

In order to determine the optimum nutrition conditions for initiating the callus formation, excised segments of leaves and stems of *M. spicata* were cultured, separately, in the dark at 25°C in the B5 medium^{5,6)} containing either coconut milk (CM) or casein hydrolyzate (CH) as a supplement and 2,4-dichlorophenoxyacetic acid (2,4-D) as an auxin, as shown in Table I. The callus proliferation was facilitated in the media supplemented with 2 ppm and 6 ppm of 2,4-D but became difficult with more than 10 ppm of 2,4-D. Addition of CH to the B5 medium facilitated the initiation of the leaf callus proliferation, whereas supplying CH or CM was not effective for the initiation of the callus proliferation from the stems.

Effects of the supplements and the auxin on the growth of the leaf callus tissues were examined. Addition of 2 ppm of 2,4-D and 0.2% of CH to the B5 medium was found to

TABLE I. EFFECTS OF THE SUPPLEMENTS AND THE AUXIN ON THE INITIATION OF CALLUS PROLIFERATION OF *M. spicata* IN THE B5 BASAL MEDIUM

Exp. No.	Supplement	2,4-D (ppm)	Original organs ^c	
			Stem	Leaf
1	—	2	++	+
2	—	6	+++	+
3	CH ^a	0	—	—
4	CH	2	+	+++
5	CH	6	++	++
6	CH	10	—	+
7	CM ^b	2	+	++
8	CM	6	+	—

^a 0.2% (w/v) casein hydrolyzate (CH).

^b 15% (v/v) coconut milk (CM).

^c + + +, + +, +, —; the relative degree of callus proliferation.

greatly facilitate the growth of the callus, and the growth attained the maximum after a period of 4 weeks, as shown in Fig. 1.

Lipid constituents of the callus tissues

These callus tissues derived from the leaves and the stems of the plant were, separately, treated with methanol to give a methanol extract, which was then extracted with ether. GLC and TLC of the ether soluble fraction from the stem callus were superimposable on those of the ether soluble fraction from the leaf callus. This indicated that the ether soluble

FIG. 1. Effects of the Addition of CH and 2,4-D on the Growth of Leaf Callus Tissues of *M. spicata* in Various Media.

(a), the B5 medium; (b), the B5 medium with 2 ppm of 2,4-D; (c), the B5 medium with 0.2% (w/v) of CH; (d), the B5 medium with 0.2% (w/v) of CH and 2 ppm of 2,4-D.

w/w_0 denotes the ratio of the weight of callus tissues grown during the periods shown to the initial weight of callus tissues used.

fraction of the leaf callus had the same constituents as the ether soluble fraction of the stem callus. Therefore, only the leaf callus fraction was used for identification of constituents thereafter. The ether soluble fraction was chromatographed on a silica gel column to give fatty acid methyl esters, triglycerides, squalene, stigmasterol, sitosterol, fatty acids, oleanolic acid (1), ursolic acid (2) and pomolic acid (3).^{7,8)} The quantities of these constituents

are shown in Table II. The presence of a large amount of fatty acid methyl esters suggested that the esters might be due to the use of

TABLE II. QUANTITIES OF THE LIPID CONSTITUENTS IN AN ETHER-SOLUBLE FRACTION OF THE METHANOL EXTRACT FROM THE CALLUS TISSUES OF *M. spicata*

Constituents	Quantities of the constituents (%) ^a
Fatty acids	0.002
Fatty acid methyl esters	0.041
Triglycerides	0.017
Squalene	<0.0001
Phytosterols	0.008
Triterpenoids	0.026

^a Expressed as the weight percent of the constituents for the weight of the fresh callus tissues.

MeOH for the extraction of lipid constituents. However, when the callus was extracted with acetone, in place of MeOH, TLC and GLC analyses of the ether soluble fraction showed the same results as above.

As shown in Table III, palmitic and stearic acids were major fatty acids in the free fatty acids, the fatty acid methyl esters and the triglycerides. Compositions of the phytosterols and the triterpenoids are shown in Table IV. None of the monoterpenoids characteristic of an essential oil of the intact plants of *M. spicata* were found in the ether soluble fraction obtained from the leaf callus tissues. Similar observations have been reported by several workers.⁹⁻¹¹⁾ These results are incompatible with a communication showing that GLC of volatile constituents from cultured cells of *Perilla frutescens* indicated the formation of some monoterpenoids.¹²⁾

The lack of the formation of such monoterpenoids may be attributable to the defect of an oil gland in these callus tissues, as Becker¹¹⁾ has described that no callus tissues formed an oil gland even when the tissues are induced from an essential oil-producing plant. In addition, it is well known that the re-differentiation ability of callus tissues decreases on subculturing the tissues for a long time. In such a case, a change in the ability to produce monoterpenoids may be expected. However, further investigations are necessary.

TABLE III. FATTY ACID COMPOSITIONS OF FREE FATTY ACIDS, FATTY ACID METHYL ESTERS AND TRIGLYCERIDES PRODUCED BY THE CALLUS TISSUES OF *M. spicata*

Constituents	Composition of fatty acids (%)					
	16:0	18:0	18:1	18:2	18:3	Others
Free fatty acids	47.8	37.3	5.9	0	9.0	trace
Fatty acid methyl esters	63.1	20.2	1.6	0	3.9	11.2
Triglycerides	43.7	14.9	12.1	0	0	29.3

TABLE IV. COMPOSITIONS OF PHYTOSTEROLS AND TRITERPENOIDS FROM THE CALLUS TISSUES OF *M. spicata*

Constituents	Compounds	Composition (%)
Phytosterols	Sitosterol	82.6
	Stigmasterol	17.4
Triterpenoids	Oleanolic acid (1)	21.5
	Ursolic acid (2)	72.0
	Pomolic acid (3)	6.5

EXPERIMENTAL

Mps were determined using a hot plate and are uncorrected. GLC analyses were performed with an instrument with FID and a column (2 m × 3 mm) packed with 10% DEGS on Celite (60~80 mesh), 2% SE-30 on Celite (60~80 mesh) or 3% OV-17 on Chromosorb W (80~100 mesh). TLC analyses were carried out with silica gel (Merck GF₂₅₄; 0.25 mm thick) using three different solvent systems [(i) EtOAc/hexane = 3/7, (ii) EtOAc/hexane = 1/9 and (iii) MeOH/CHCl₃ = 1/19] for each case.

Tissue cultures from M. spicata. The stems and the leaves were sterilized by soaking in 70% EtOH for 5 min, followed by immersing in a 10% bleaching powder solution for 10 min, and then by washing three times with sterilized water. Segments of the stems and the leaves were placed, separately, under sterile conditions in eight kinds of media, as shown in Table I. The cultures were maintained at 25°C in the dark. After one to three weeks, a yellowish-white callus was induced from the plant tissues. The callus growing on the B5 basal medium supplemented with 0.2% of CH and 2 ppm of 2,4-D was subcultured every four to six weeks for over two years prior to use.

Lipid constituents of the callus tissues. The leaf callus (fresh wt. 1 kg) was extracted with MeOH for 36 hr in a Soxhlet apparatus, through the condenser of which was circulated chilled water. The MeOH solution was carefully

concentrated under slightly reduced pressure. The MeOH extract of the leaf callus was carefully subjected to TLC and GLC analyses under the conditions shown above and directly compared with the monoterpenoid constituents isolated from intact plants of *M. spicata*. However, none of the monoterpenoid constituents were found.

Then, the MeOH extract was repeatedly extracted with ether. Careful removal of the solvent from the ether solution at atmospheric pressure gave an ether soluble fraction (4.0 g), which was subjected to TLC and GLC analyses in the same manner as for the MeOH extract. However, none of the monoterpenoids were found. The ether soluble fraction was then chromatographed on a silica gel column with hexane-EtOAc to give fatty acid methyl esters (406 mg), triglycerides (167 mg), phytosterols (83 mg) and a mixture of fatty acids and triterpenic acids (245 mg).

On the other hand, the stem callus (fresh wt. 20 g) was extracted in the same manner as above to give an ether soluble fraction (65 mg). GLC of the ether soluble fraction obtained from the stem callus tissues was superimposable on that of the ether soluble fraction obtained from the leaf callus. TLC of both the fractions also showed completely the same patterns under the conditions given above.

Fatty acid methyl esters and squalene. A 19:1 hexane-EtOAc eluate gave an oily substance (406 mg), which was identified with known samples of fatty acid methyl esters by means of co-GLC. The composition of these fatty acid esters were determined by the peak areas on the GLC chromatogram (Table III). A tiny peak of squalene appeared among these ester peaks. The identity of the hydrocarbon was established by means of co-GLC with an authentic sample.

Triglycerides. A 9:1 hexane-EtOAc eluate afforded a viscous oil: IR $\nu_{\max}^{\text{liq}}$ cm⁻¹: 1740 and 1160 (ester); NMR (60 MHz, CDCl₃) δ : 5.34 (>CH-O- or -CH=CH-), 4.22 (-CH₂-O-) and 1.53 (straight CH₂ chain). This oil was saponified with 5% KOH in MeOH to five the acidic components, which were methylated with CH₂N₂ and identified with respective authentic samples by means of co-GLC (Table III). An aqueous solution separated from the acidic components was concentrated to a small volume and subjected to GLC analysis to identify glycerol.

Phytosterols. A 7:3 hexane-EtOAc eluate gave white crystals: mp 139~140°C; IR $\nu_{\max}^{\text{KBr}}$ cm^{-1} : 3280 (OH), 1640 and 1050 ($\text{C}=\text{C}$); NMR (CDCl_3) δ : 5.33 (1H, m, $-\text{CH}=\text{C}<$), 1.00, 0.96, 0.90, 0.78 and 0.70 (CH_3). GLC analysis indicated the crystals to be composed of a 5:1 mixture of sitosterol and stigmasterol, based on the peak areas. The identity of these compounds was established by means of co-GLC (2% SE-30; 250°C) with authentic samples.

Fatty acids and triterpenic acids. A 1:1 hexane-EtOAc eluate gave white needles (245 mg), a part of which was methylated with CH_2N_2 and subjected to TLC and GLC (10% DEGS; 180°C) analyses. The needles were found to be composed of the fatty acids shown in Table III and three triterpenic acids. The fatty acids were identified by means of co-GLC of their methyl esters. The triterpenic acids were isolated and identified to be as described below.

The white needles (240 mg) were chromatographed on a silica gel plate developed continuously with hexane-EtOAc (85:15) to give oleanolic acid (1) (48 mg), ursolic acid (2) (161 mg) and pomolic acid (3) (15 mg). These showed the following physical data: a) oleanolic acid (1), mp 300~305°, MS m/e (rel. int.): 456 (M^+ , 6) 262 (69) and 203 (100). The methyl ester of its acetate, mp 220~222° (lit.¹³) mp 219~220°; $^1\text{H-NMR}$ (CDCl_3) δ : 5.28 (1H, m, $-\text{CH}=\text{C}<$), 4.50 (1H, t, $J=8.0$ Hz, $>\text{CH}-\text{OCOCH}_3$), 3.63 (3H, s, COOCH_3), 2.04 (3H, s, $\text{O}-\text{COCH}_3$), 1.13 (3H, s, $\text{C}(27)-\text{H}_3$), 0.93~0.86 (15H, $5 \times \text{CH}_3$) and 0.73 (3H, s, $\text{C}(26)-\text{H}_3$); b) ursolic acid (2), mp 235~239°; MS m/e (rel. int.): 456 (M^+ , 3), 248 (100) and 203 (68). The methyl ester of its acetate, mp 241~242° (lit.¹⁴) mp 248~250°; $^1\text{H-NMR}$ (CDCl_3) δ : 5.20 (1H, m, $-\text{CH}=\text{C}<$), 4.60 (1H, t, $J=8.0$ Hz, $>\text{CH}-\text{OCOCH}_3$), 3.60 (3H, s, COOCH_3), 2.03 (3H, s, $\text{O}-\text{COCH}_3$), 1.07 (3H, s, $\text{C}(27)-\text{H}_3$), 0.94~0.86 (15H, methyl signals) and 0.75 (3H, s, $\text{C}(26)-\text{H}_3$) and c) pomolic acid (3), mp 281~285°. The methyl ester of its acetate, mp 250~251° (lit.⁷) mp 248~249°; MS m/e (rel. int.): 528 (M^+ , 2), 468 (8), 179 (34) and 43 (100); $^1\text{H-NMR}$ (CDCl_3) δ : 5.38 (1H, m, $-\text{CH}=\text{C}<$), 4.53 (1H, t, $J=8.0$ Hz, $>\text{CH}-\text{OCOCH}_3$), 3.62 (3H, s, COOCH_3), 2.60 (1H, s, $\text{C}(18)-\text{H}$), 2.05 (3H, s, $\text{O}-\text{COCH}_3$), 1.28 (3H, s, $\text{C}(29)-\text{H}_3$), 1.23 (3H, s, $\text{C}(27)-\text{H}_3$), 0.95~0.92 (12H, $4 \times \text{CH}_3$) and 0.70 (3H, s, $\text{C}(26)-\text{H}_3$). The identity of each

compound, as indicated by the physical data, was established by comparison (mixed mp, co-TLC, IR and NMR) with the reported data and/or respective samples.^{7,8,13~15}

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