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# ALKALOID PRODUCTION IN CULTURED CELLS OF *DIOSCOREOPHYLLUM CUMMINSII**

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**Key Word Index**—*Dioscoreophyllum cumminsii*; Menispermaceae; cell culture; alkaloid production; jatrorrhizine; magnoflorine; palmatine.

**Abstract**—Jatrorrhizine and magnoflorine were isolated from cultured cells of *Dioscoreophyllum cumminsii*. The jatrorrhizine content in cultured cells was 40–100 times higher than that of the intact plant, but columbamine, which is a minor component in the original plant, was not detected. Moreover, it was observed that the addition of IAA or NAA to the growth medium increases the alkaloid content as compared with 2,4-D.

## INTRODUCTION

*Dioscoreophyllum cumminsii* (Stapf) Diels (Menispermaceae) is a climbing plant grown in the forest areas of tropical west Africa. The tubers, stalks and leaves are used in indigenous medicine for a variety of purposes [1, 2]. Phytochemical studies on sweet protein [3], diterpenoid bitter principles [4] and fatty acids [5] have been carried out. Recently, S. K. Adesina *et al.* [6] have reported the isolation of two protoberberine alkaloids, jatrorrhizine (**1**) and columbamine (**3**), and an aporphine alkaloid, magnoflorine (**2**). Cultured cells of *D. cumminsii*, which were first derived from the stem, have a yellow or red-yellow color and the media are also colored yellow. Therefore, it is suggested that the cultured cells produce quaternary protoberberine alkaloids.

In this paper, we wish to report the isolation of two quaternary alkaloids from cultured cells. The quantitative determination of the alkaloids was also undertaken by HPLC to examine the effect of plant hormones on alkaloid production and to compare the alkaloid composition with that of the living plant.

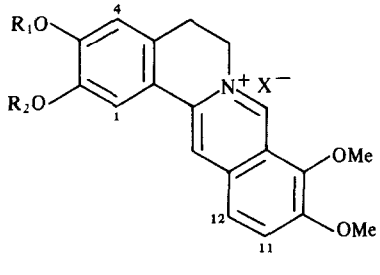
*Part 41 in the series "Studies on Plant Tissue Culture". For Part 40, see Furuya, T., Yoshikawa, T., Orihara, Y. and Oda, H. (1983) *J. Nat. Prod.* (in press).

## RESULTS

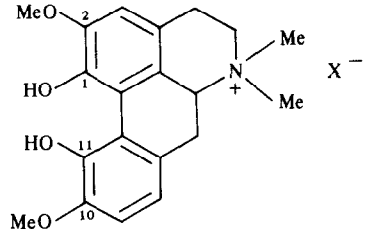
### *Identification of alkaloids*

Base I, which was crystallized as the iodide, mp 205.5–206.0° (decomp.), had a molecular formula $C_{20}H_{20}NO_4I \cdot 1/2H_2O$. The UV spectrum ($\lambda_{max}^{EtOH}$ nm: 225, 265, 350, 410–434) was characteristic of quaternary protoberberine alkaloids and following the addition of sodium hydroxide or sodium bicarbonate a bathochromic shift was observed [7]. The IR spectrum showed the presence of a hydroxy group. The mass spectrum showed *m/z* 338 (27.8%) as $M^+$ and important ions were observed at *m/z* 353 (4.3%), 339 (16.9%), 323 (100%) and 308 (22.6%). This fragmentation pattern appeared to be caused by disproportion analogous to that of berberine hydroxide [8]. The $^1H$ NMR spectrum of base I showed singlets for three methoxy groups on the aromatic ring at $\delta$ 3.92, 4.03 and 4.08. The AB quartet at $\delta$ 7.97 and 8.11 ($J$ = 8.6 Hz) was ascribed to the two aromatic protons at C-11 and C-12, and the singlets at 6.86 and 7.66 were ascribed to C-1 and C-4. The spectral data of the tetrahydro derivative of base I was the same as that of authentic tetrahydrojatrorrhizine [9]. Therefore, base I was identified as jatrorrhizine (**1**) by mmp and comparison of IR with that of an authentic sample.

Base III, crystallized as the iodide, mp 249° (decomp.),




**1** $R_1 = H$, $R_2 = Me$
**3** $R_1 = Me$, $R_2 = H$
**4** $R_1 = R_2 = Me$




**2**

had the formula $C_{20}H_{24}NO_4I$. The UV spectrum ($\lambda_{max}^{MeOH}$ nm: 226, 269, 306–310.5) was characteristic of aporphine alkaloids substituted at C-1, C-2, C-10, and C-11 [10]. The IR spectrum showed the presence of a hydroxy group. The mass spectrum showed as major fragments *m/z* 342 $[M]^+$ (0.8%), 327 (2.3%), 284 (1.0%) and 58 (100%) which suggested a quaternary aporphine base containing a hydroxy and a methoxy group [11]. The $^1H$ NMR spectrum showed a singlet of two *N*-methyl groups at $\delta$ 2.99 and methoxy groups at 3.87. The CD spectrum showed a similar Cotton effect to (+)-magnoflorine [12]. Therefore, base III was identified as (+)-magnoflorine (**2**) by comparison of IR with that of an authentic sample.

Palmatine (**4**) was not isolated in our expts, although its presence was indicated by TLC and HPLC.

*Quantitative analysis of the alkaloids in the cultured cells and the original plant by HPLC*

The chromatographic conditions were studied using the Toyo soda LS-170 column and $H_2O$–MeCN–$Et_3N$–HOAc (75:25:0.8:0.3) as eluant [13]. The $R_t$s of jatrorrhizine (**1**), magnoflorine (**2**), columbamine (**3**) and palmatine (**4**) were 20.0, 17.5, 28.6 and 35.2 min, respectively. The calibration curves for the four alkaloids were all linear and the alkaloid contents were calculated by the peak height method. The results are listed in Table 1.

In all cultured cells, **1**, **2** and **4** were produced, but not **3**, which was confirmed in the original plant by co-chromatography with a reference sample. In cultured cells the alkaloid content was 0.3–0.9% dry wt, but in the plant it was 0.02% in roots and 0.03% in aerial parts. All cultured cells contained **1** as the major component, 0.2–0.5% dry wt, up to 50% in total alkaloids. Moreover, it was shown that IAA and NAA increased the alkaloid content by 2.3 and 2.0 times, respectively, as compared with 2,4-D. However, the growth rate was the highest with 2,4-D.

## DISCUSSION

In plant tissue culture, it was observed that production of useful secondary products is lower than that in the original plants or is negligible. However, cultured cells of *D. cumminsii* had a higher alkaloid content than that of the living plant. The alkaloid content of cultured cells per g fr. wt was almost the same as that of the plant, except for RIC and RNC cells (see Experimental), but per dry wt was 15–45 times higher than that of the plant. In particular, the jatrorrhizine content in cultured cells was 40–100 times more per dry wt as compared with that in the original plant. Moreover, it is interesting that in the biosynthesis of the alkaloids the cultured cells produced **1**, **2** and **4**, but not **3** which is produced in the intact plant.

The cultured cells of *D. cumminsii* required for growth a coconut milk or casamino acid as a natural additive in the medium. Both IAA and NAA increased the alkaloid content in comparison with 2,4-D, whereas 2,4-D facilitated growth. It is known that IAA facilitates production of nicotine in cultured cells of *Nicotiana tabacum* [14], coumestrol in *Phaseolus aureus* [15] and digitolutein in *Digitalis lanata* [16], whereas 2,4-D inhibits production of nicotine [14, 17], polyphenol [18] and anthocyanin [19]. In *D. cumminsii* the production of protoberberine and aporphine alkaloids is not completely inhibited by 2,4-D, but is depressed somewhat. 4-PU [*N*-phenyl-*N'*-(4-pyridyl) urea] inhibited alkaloid production as compared with kinetin.

The details of these hormone effect on alkaloid production are being investigated using a cell suspension culture.

## EXPERIMENTAL

Mps are uncorr. NMR spectra were recorded with TMS as int. standard.

Table 1. Alkaloid content and growth of plant and cell cultures of *D. cumminsii*

| Callus | Medium* | Growth ratio | Alkaloid content (mg/100 g fr. wt) | | | | | Alkaloid content (Jat. content) (dry wt %) |
|---|---|---|---|---|---|---|---|---|
| | | | Jat.† | Mag. | Pal. | Col. | Total | |
| CM | MS, D, K, CM | 3.2 | 3.3 | 1.5 | 2.6 | — | 7.4 | 0.4 (0.2) |
| RCM | RT, D, K, CM | 3.3 | 4.9 | 1.1 | 2.3 | | 8.3 | 0.5 (0.3) |
| RDC | RT, D, K, CA | 4.7 | 3.7 | 1.8 | 1.2 | — | 6.7 | 0.4 (0.2) |
| RIC | RT, IAA, K, CA | 2.7 | 12.3 | 10.6 | 3.1 | — | 26.0 | 0.9 (0.4) |
| RNC | RT, NAA, K, CA | 2.8 | 7.9 | 2.8 | 2.3 | — | 13.0 | 0.8 (0.5) |
| RNP | RT, NAA, P, CA | 2.5 | 4.6 | 1.5 | 1.5 | — | 7.6 | 0.3 (0.2) |
| Roots | — | — | 0.6 | 4.4 | 0.3 | 0.04 | 5.34 | 0.02 (0.002) |
| Aerial parts | — | — | 1.3 | 4.9 | 0.9 | 0.17 | 7.27 | 0.03 (0.005) |

*MS, Murashige and Skoog's basal medium, RT, Revised Tobacco medium; D, 2,4-dichlorophenoxyacetic acid, 1 ppm; NAA, 1-naphthaleneacetic acid, 1 ppm; IAA, 3-indoleacetic acid, 1 ppm; K, kinetin, 0.1 ppm; P, (*N-phenyl-N'*-(4-pyridyl) urea, 0.1 ppm; CM, coconut milk, 7%; CA, casamino acid (Difco), 0.1%.

†Jat. Jatrorrhizine; Mag., magnoflorine; Pal., palmatine; Col., columbamine.

*Materials. D. cumminsii* (Stapf) Diels plants used for the induction of the callus were cultivated at the Izu Experiment Station of Medicinal Plants, National Institute of Hygenic Sciences. Plants used for the determination of alkaloids by HPLC were cultivated at the Medicinal Plant Garden of our University. The callus was derived from the stem of *D. cumminsii* in 1977 on Murashige and Skoog's (MS) medium containing IAA, 1 ppm, and kinetin, 0.1 ppm. After 5 months (5th generation) the callus was subcultured onto MS medium containing 2,4-D. 1 ppm, kinetin 0.1 ppm and 7% coconut milk (CM medium).

*Culture conditions.* CM callus cultured on CM medium was subcultured every 4 weeks at 26° in the dark and then transferred to Revised Tobacco (RT) medium [20] containing various growth regulators as described in the following. RDC medium: 2,4-D 1 ppm, kinetin 0.1 ppm, 0.1% casamino acid (CA); RCM: 2,4-D 1 ppm, kinetin 0.1 ppm, 7% coconut milk; RNC: NAA 1 ppm, kinetin 0.1 ppm, 0.1% CA; RIC: IAA 1 ppm, kinetin 0.1 ppm, 0.1% CA; RNP: NAA 1 ppm, 4-PU 0.1 ppm, 0.1% CA. Each callus was subcultured every 4 weeks under the conditions described above.

*Extraction and isolation of alkaloids.* Callus (8 kg fr. wt) cultured on RDC medium for 4 weeks was homogenized with cold MeOH in a Waring blender and refluxed for 3 hr with MeOH. The extracted soln was evaporated to dryness *in vacuo* and the resulting residue dissolved in 0.5 l. 1 N HCl and extracted with 2 l. $CHCl_3$. The aq. acid soln was adjusted to pH 9–10 with 25% $NH_4OH$ and extracted with 2 l. $CHCl_3$ to remove the tertiary alkaloids. The aq. soln was acidified with 6 N HCl to pH 3–4 and treated with excess Mayer's reagent. The ppted quaternary base was separated by centrifugation and the $HgI_2$ complex decomposed by dissolving the ppt in $Me_2CO$–MeOH–$H_2O$ (6:2:1), filtering the soln and then eluting through an anion exchanger (Amberlite IRA 400, $Cl^-$ form) according to ref. [6]. The column was thoroughly washed with 50% aq. $Me_2CO$ and the combined eluates concd to dryness to give 4.78 g of a dark-brown residue. This residue was extracted with EtOH–*n*-hexane and the supernatant evaporated to give 1.65 g of a red-yellow residue (SeQ I). The insoluble material was fractionated over an $Al_2O_3$ column with MeOH and $H_2O$. The eluates were combined and evaporated to give 2.16 g of a dark-brown residue (SeQ II).

*Chromatography of quaternary alkaloids.* The crude residue SeQ I was fractionated on a Si gel column (600 g) with MeOH–25% $NH_4OH$–$H_2O$ (8:1:1). The eluate was collected in 50 ml fractions which were combined into similar fractions after monitoring by TLC. After pre-eluting with 1 l. of solvent, the red-yellow materials were obtained from fractions 1–35 and gave a crystalline iodide salt as base I (589 mg). The crude residue (SeQ II) was also fractionated as above. The residue from fractions 40–65, (227 mg), further purified by prep. TLC with the same solvent system as above gave a crystalline iodide salt as base III (2.8 mg).

*Base I* (*jatrorrhizine*). Recrystallization from EtOH–*n*-hexane gave red-yellow needles, mp 205.5–206° (decomp.). (Found: C, 50.56; H, 4.14; N, 2.96. $C_{20}H_{20}NO_4I \cdot 1/2H_2O$ requires: C, 50.65; H, 4.46; N, 2.95%.) UV $\lambda_{max}^{EtOH}$ nm (log ε): 225 (4.6), 265 (4.5), 370 (4.6), 410–434 (3.9); + NaOH: 220 (4.6), 248 (4.5), 400 (4.6). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3400 (OH). MS *m/z* (rel. int.): 353 (4.3), 339 (16.9), 338 $[M]^+$ (27.8), 323 (100), 308 (22.6). $^1H$ NMR (DMSO-$d_6$): δ 3.14 (2H, *m*), 4.92 (2H, *m*), 3.95, 4.06, 4.10 (3H each, all *s*), 6.85, 7.70 (1H each, both *s*), 8.04, 8.18 (1H each, AB*q*, $J = 8.6$ Hz), 9.00, 9.86 (1H each, both *s*).

*Base III* (*magnoflorine*). Recrystallization from EtOH–*n*-hexane gave colorless needles, mp 249° (decomp.). (Found: $M^+$ 342.170. $C_{20}H_{24}NO_4$ requires: 342.171.) UV $\lambda_{max}^{MeOH}$ nm (log ε): 226 (4.7), 269 (4.1), 310.5–306 (3.9). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3400 (OH). MS *m/z* (rel. int.): 342 $[M]^+$ (0.8), 341 (2.7), 327 (2.3), 284 (1.0), 270 (3.9), 128 (19.2), 58 (100). $^1H$ NMR ($CD_3OD$): δ 2.99 (6H, *s*), 3.87 (6H, *s*), 6.77 (1H, *s*), 6.83 (2H, *s*). CD (MeOH; *c* $4.54 \times 10^{-4}$) Δε (nm): + 83.34 (232.6), − 19.02 (268.6), + 3.73 (315.0).

*Quantitative analysis of alkaloids by HPLC.* Fresh callus tissues (100 g fr. wt harvested from 10–20 flasks) and plants were homogenized in cold MeOH in a Waring blender and refluxed with MeOH. The extracts were evaporated *in vacuo* and the resulting residue dissolved in 1 N HCl. The aq. acid soln was adjusted to pH 9–10 with 25% $NH_4OH$ and treated with Amberlite XAD-2. The resin was throughly washed with $H_2O$ and eluted with 1% HCl in MeOH. The eluates were evaporated to dryness and the residue chromatographed on Sephadex LH-20 with EtOH. The eluate was evaporated to dryness, the residue dissolved in MeOH and the alkaloid content determined by HPLC. HPLC was carried out using a LS-170 column (60 cm × 4 mm i.d., Toyo Soda) to determine the amount of each component, monitoring the UV absorption at 254 nm. The eluent was $H_2O$–MeCN–HOAc–$Et_3N$ (75:25:0.3:0.8).

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