Journal of Natural Products
Vol. 48, No. 1, pp. 17-21, Jan-Feb 1985

VALEPOTRIATE PRODUCTION OF NORMAL AND COLCHICINE-TREATED CELL SUSPENSION CULTURES OF *VALERIANA WALLICHII*[1]

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ABSTRACT.—Colchicine-treated suspension cultures of *Valeriana wallichii* produce higher amounts of valepotriates than did the respective untreated cultures. The ability to produce valepotriates in the treated culture remains in the absence of colchicine even if the chromosome status returns to normal. When the colchicine treatment is repeated, a further increase in valepotriate production can be obtained. Besides known valepotriates, a series of fourteen new compounds, hitherto not described for the parent plant, were isolated from the cell suspension culture. Eight of them are also found in plant parts in minor amounts, but six seem to be present only in tissue cultures of *V. wallichii*.

Valeriana wallichii DC roots are commercially used as a sedative in Germany. According to pharmacological investigations (1), this effect is due to a group of iridoid substances referred to by Thies as valepotriates (2). Because of a shortage of plant material, we tried to produce valepotriates by a tissue culture method. In previous publications we have reported that tissue cultures of *V. wallichii* produce substantial amounts of these natural products (3, 4). As in fermentation by microorganisms, commercial use of plant tissue cultures is possible only if highly productive strains can be obtained by effective alteration of the genetic potential and subsequent selection of mutants or variants. Thus, from a hypothetical linkage between leucine and terpenes (valepotriates are iridoid monoterpenes), we isolated cell lines resistant to a leucine analogue. This cell line accumulated up to a thirty-sevenfold amount of the amino acid as compared to the original culture (5). Unfortunately, the high leucine pool did not result in a higher pool of valepotriates. In the present paper, we describe the influence of colchicine treatment on valepotriate production in suspension cultures of *V. wallichii*. These investigations are based on observations that polyploid plants often exhibit a higher amount of secondary products than do the corresponding diploids (6). As shown by Neczypor (7), polyploids may also show altered relative amounts of the secondary metabolites. In plants, however, such positive effects are often counteracted by the fact that polyploids grow less vigorously or are more sensitive to pests.

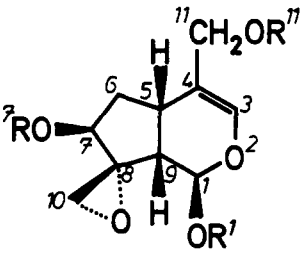
RESULTS AND DISCUSSION

To conduct these investigations, a suspension culture of *V. wallichii*, which had been grown for one year on a Murashige and Skoog's liquid medium (8) with the addition of 2 mg/liter naphthalene acetic acid and 1 mg/liter kinetin, was employed. The total valepotriate yield for fourteen subsequent cultures varied from less than 0.001% on a dry weight basis to 0.034% and averaged 0.012%. The parent plant had 0.7% valepotriates in the leaf lamina, 0.2% in the petiole, 0.05% in the stem, and 2.8% in the roots. The treatment with colchicine was conducted in three concentrations (0.05%, 0.2%, and 0.5%) for 30 days each. While 0.5% colchicine killed all cells, we obtained growing cutures with 0.05% colchicine and 0.2% colchicine. These cultures were transferred to colchicine-free medium and subcultured every 14 days. They showed the same growth characteristics as the untreated cultures (9). The subcultures produced substantial amounts of valepotriates as seen on tlc. Besides spots for valepotriates

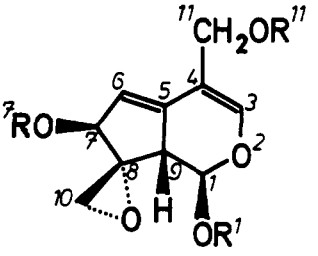
[1]Part VII in a series of tissue cultures of Valerianaceae. For part VI, see Chavadej and Becker (9).

known from the intact plant, additional compounds appeared on tlc, which gave a positive color reaction for valepotriates with dinitrophenylhydrazine reagent (10). Mean-

Valepotriates



Monoene-type



Diene-type

	R^1	R^7	R^{11}	R^5
Didrovaltrate	iVal	Ac	iVal	H
Isovaleroxyhydroxy-didrovaltrate (IVHD-Valtrate)	iVal	Ac	(α-O-iVal) iVal	OH
M	iVal	Ac	iVal	OH

	R^1	R^7	R^{11}
Homovaltrate	(β-Me)-Val	iVal	Ac
Isovaltrate	iVal	Ac	iVal
Valtrate	iVal	iVal	Ac
V_1 (Homoisovaltr.)	(β-Me)-Val	Ac	iVal
V_2 (1,7 Dihomov.)	(β-Me)-Val	(β-Me)-Val	Ac
A (1-α-Acevaltr.)	(α-OAc)-iVal	iVal	Ac
B (DIA-Valtr.)	iVal	Ac	Ac
C (Homo-A)	(α-OAc)-iVal	(β-Me)-Val	Ac
D (Homo-B)	(β-Me)-Val	Ac	Ac
Acevaltrate	iVal	(β-OAc)-iVal	Ac
E	(α-OAc) iVal	(β-OAc)-iVal	Ac
X	n.d.[a]	n.d.	n.d.
Y (Homo-Z)	iVal	(β-Me) Val	(β-OH)-iVal
Z	iVal	iVal	(β-OH)-iVal
N	iVal	Ac	(β-OH)-iVal
O	(α-OAc)-iVal	(β-OH)-iVal	Ac
F	n.d.	n.d.	n.d.

[a]n.d. = not determined.

acidic part (R^1, R^7, R^{11})	*symbol*
acetic acid	Ac
isovaleric acid	iVal
β-methylvaleric acid	(β-Me)-Val
α-isovaleroxyisovaleric acid	(α-O-iVal)-iVal
α-acetoxyisovaleric acid	(α-OAc)-iVal
β-acetoxyisovaleric acid	(β-OAc)-iVal
β-acetoxy-β-methylvaleric acid	(β-OAc) (β-Me)-Val
β-hydroxyisovaleric acid	(β-OH)-iVal

FIGURE 1. Structures of valepotriates in intact plant and cell cultures of *Valeriana wallichii*.

while, isolation of these compounds by a combination of tlc and hplc and structure elucidation by ^{13}C nmr and cims was performed (11, 12). The known structures together with fourteen newly identified compounds are summarized in Figure 1. After carefully checking all parts of the intact plant, we could detect eight of these fourteen compounds in minor amounts. Six valepotriates are found only in the cell culture.

To be sure that there is no direct influence of the colchicine on valepotriate production, we measured valeopotriates more than one year after the treatment. The ability to produce high amounts of valepotriates remained. In fourteen subsequent subcultures, the total valepotriate yield of the cultures treated with 0.05% colchicine averaged 0.8%, thus producing a sixty-sixfold increase as compared with the untreated culture during the same growth period (Table 1). The treatment with 0.2% colchicine also resulted in an increase of valepotriates (Table 1). The average amount (0.65%) was less than with 0.05% colchicine, but still much higher (fiftyfold) than the controls. When the cultures were treated a second time with the same colchicine concentrations, the average valepotriate content was increased to 1.21% when using 0.05% colchicine and to 0.80% with 0.2% colchicine.

The valepotriate composition of the cultures did not resemble that of the original plant. As already mentioned, new valepotriates occurred in the cultures. Isovaleroxyhydroxydidrovaltrate (IVHD), although present in all plant parts, could not be detected in the cultures (Table 2). Within one cell strain the valepotriate content varied considerably during consecutive passages. The reason for this instability remains unclear. It could not be caused by variations in temperature, light, or medium composition. All cultures were transferred to fresh medium prepared on the same day, and the cultures were maintained in the same rotary shaker under the same light and temperature conditions. Nevertheless, for one growth period under these standardized conditions, the 0.05% colchicine-treated cultures showed a low of 0.2% valepotriates, whereas the 0.2% colchicine-treated cultures had valepotriates above average.

The increase in valepotriates cannot be explained by a higher ploidy level. We observed a shift in ploidy from mainly diploid cells (76%) to highly polyploid cells (76%) in the first passage after the treatment (e.g., in 0.2% colchicine-treated cells). After six passages of colchicine-free cultivation the treated cultures had almost returned to their normal chromosome status (70% of diploids) (9). Whether the increase in valepotriate production is due to a selection of high-producing cells by colchicine, a gene amplification, or another mechanism necessitates further investigations.

EXPERIMENTAL

CULTURE CONDITIONS.—The culture was derived from stem cuttings of *V. wallichii* plants grown in the botanical garden of the University of Heidelberg. They were maintained on a Murashige and Skoog (8) liquid medium supplemented with 2 mg/liter-naphthalene acetic acid and 1 mg/liter kinetin. Tissue (7 g fresh weight) was transferred to 90 ml of medium in 200-ml Erlenmeyer flasks every 14 days. The cultures were maintained on a rotary shaker (New Brunswick G 53) at 110 rpm in continuous light of about 1000 Lux with a mixed white and Osram "Fluora" light. Colchicine, at concentrations of 0.05%, 0.2%, and 0.5%, was added prior to sterilization. Respective tests showed that colchicine was stable under these conditions.

Preparation of extracts: Freeze-dried tissue (1 g) was ground and extracted for 30 min with 2 × 50 ml CH_2Cl_2 using an Ultra-Turrax, filtered, and the filtrate evaporated to dryness under vacuum at 30°. The residue was dissolved in 1-10 ml of uv spectral MeOH (Merck) and used directly for analysis.

Thin-layer chromatography: Tlc was on precoated tlc plates Kieselgel 60 F (Merck) using the solvent system CH_2Cl_2-EtOAc-Me_2CO (48:1:1 v/v).

Detection: Uv 254 nm and dinitrophenylhydrazine spray reagent (10).

Hplc-procedure: Equipment—pump, Waters Model M-6000 A (Waters Assoc., Milford, MA); Waters Solvent Programmer M 660, Waters injector Model U 6 K, (4 mm i.d. × 25 cm), Lichrosorb RP 18,7 μ Merck photometer (Schoeffel-instrument SF 770). Chemicals—uv spectral MeOH (Merck) and

TABLE 2. Percentage of Valepotriates in Different Parts of Intact Plant and Different Cell Lines of Colchicine-Treated Cell suspension Cultures of *Valeriana wallichii*

Valeriana wallichii		% Valepotriate																			
		Diene type[a]																Monoene type[a]			
		Homo-valtr.	Iso-valtr.	Valtr.	V_1	V_2	A	B	C	D	Ace-valtr.	E	X	Y	Z	N	O	F	Didro-valtr.	IVHD-valtr.	M
Part of plant (bot. garden, Heidelb.)	Leaf lamina	11.0	15.8	22.8	tr.	—	0.7	tr.	—	—	3.9	—	—	—	—	—	—	—	29.8	16.0	—
	Petiole	12.8	5.2	37.6	—	—	2.9	0.2	—	—	6.2	—	—	tr.	tr.	—	—	—	31.5	3.5	—
	Stem	28.4	tr.	35.6	tr.	—	—	—	—	—	tr.	—	—	—	—	—	—	14.3	21.3	—	
	Rhizome + root	1.5	6.0	6.0	2.1	1.0	0.5	0.1	—	—	6.8	—	—	4.6	11.3	—	—	0.7	58.0	1.4	—
Part of plant (imported)	Rhizome + root	4.5	6.7	8.0	2.3	0.8	tr.	0.9	—	—	8.9	—	2.1	4.8	2.0	—	—	—	50.5	8.5	
Colchicine-treated cell suspension cultures	0.05% (1×)	5.1	9.8	8.5	2.0	0.8	16.2	9.2	1.0	2.7	2.0	5.0	tr.	1.4	4.7	6.0	1.0	1.8	23.4	—	+
	0.05% (2×)	3.0	7.6	14.8	2.1	0.8	16.3	10.4	1.8	3.2	2.9	4.1	tr.	1.5	4.5	4.1	1.2	3.0	18.9	—	+
	0.20% (1×)	5.1	9.0	14.7	2.6	1.1	16.3	9.6	2.3	3.4	tr.	1.0	tr.	1.3	4.3	1.0	0.5	1.1	26.7	—	+
	0.20% (2×)	3.6	7.4	11.3	2.9	1.0	13.8	9.1	2.7	5.2	tr.	1.4	1.3	3.5	7.8	2.6	1.0	6.8	19.4	—	+

[a]See figure 1 for explanation of the chemistry.

TABLE 1. Total Valepotriates in colchicine-treated cell suspension cultures of *Valeriana wallichii*[a]

		% valepotriates dry weight	standard deviation
Control		0.012[b]	0.02
colchicine-treated	0.05% (1 ×)	0.83	0.25
	0.05% (2 ×)	1.21	0.36
	0.2% (1 ×)	0.65	0.38
	0.2% (2 ×)	0.80	0.41

[a]All cultures were maintained under the same conditions during a cultivating period from September 1981 to April 1982.

[b]Values are means of fourteen successive passages.

freshly double distilled H_2O. Solvent mixtures filtered and degassed prior to use. Operating conditions—gradient elution with pump A MeOH-H_2O (40:60 v/v) and Pump B MeOH-H_2O (90:10 v/v).

Programm conditions: Initial 60% B final 95%, flow rate 1.0 ml/min, time 20 min, PSI 2000 temperature 25°, detection uv 254 nm for diene valepotriates and 208 nm for monoene valepotriates. Inject 1-5 μl/10 ml of extract. Quantification—pentylbenzene was used as internal standard.

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Received 9 March 1984