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EFFECTS OF PYROGALLOL ON *O*-METHYLATION OF DOPAMINE AND SALSOLINOL

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Key Word Index —*Corydalis pallida* var. *tenuis*; Papaveraceae; *Cynanchum vincetoxicum*; Asclepiadaceae; dopamine; salsolinol; pyrogallol; *O*-methylation; metabolism.

Abstract—In the cultured cells of *Corydalis pallida* var. *tenuis*, the formation of 6-*O*-methylated metabolites (**6**-D_4 and **6**-D_7) from salsolinol-D_4 (**2**-D_4) was reduced by pyrogallol. The production of 3-*O*-methylated derivatives (**10** and **10**-D_3) from dopamine was almost not affected by pyrogallol. Similar results were obtained in intact plants, though the effect of pyrogallol on methylation of C-6-OH of salsolinol in plants is smaller than that in the cultured cells. These results show that the effects of pyrogallol on the methylation of C-6-OH of salsolinol and that on the methylation of C-3-OH of dopamine are different, suggesting that the *O*-methylating enzymes of salsolinol and dopamine are different in *C. pallida* var. *tenuis*. The production of 3-*O*-methyldopamine in the presence of pyrogallol was reduced in intact plants of *Cynanchum vincetoxicum*, but not in the cultured cells. The effect of pyrogallol on the methylation of salsolinol was uncertain in *Cyn. vincetoxicum*. Both 6- and 7-*O*-methylations of salsolinol occur in *Cyn. vincetoxicum*, while only 6-*O*-methylation occurs in *C. pallida* var. *tenuis*. This could reveal that the *O*-methylating enzymes at C-6 and C-7 are different.

INTRODUCTION

The metabolism of 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolines (TIQs) is of interest because the heterocyclic substances may be formed in mammals normally or during certain diseases e.g. alcoholism or parkinsonism by condensation of catecholamines (CAs) with aldehydes or α-keto acids. Dopamine (**1**, DA) and related 3,4-dihydroxyphenylethylamines are *O*-monomethylated, primarily on the meta hydroxyl group, by catechol-*O*-methyltransferase (COMT) in liver, brain and other tissues. However, in contrast to CAs, the *para*- rather than the *meta*-hydroxyl (referring to the β-aminoethyl side chain) is extensively *O*-methylated (7-*O*-methylation) in 6,7-dihydroxy-TIQs, e.g. salsolinol (SAL) [(**2**, a derivative of DA and acetaldehyde)] and SAL-1 carboxylic acid (**3**, a condensation product of DA and pyruvic acid), which are also substrates for COMT [1, 2], while a stereospecific product distribution has been seen for the two other TIQs [2].

The *para*/*meta* or 6/7 isomer distribution in rat brain was 60/40 for 4-hydroxy-1-demethylSAL (**4**, a TIQ derivative from norepinephrine and formaldehyde), and 55/45 for the SAL-3-carboxylic acid (**5**, the dopa/acetaldehyde-derived TIQ). An *O*-methylation pattern has been considered to appear dependent on the substituents on the heterocyclic ring from these results [2]. However, both 6- and 7-*O*-monomethylated SAL (**6** and **7**) are produced in an *in vitro* feeding experiment of **2** in a ratio (36:64) using slices of rat liver [3], in contrast to the result obtained by Origitano and Collins [1] in which the 7-*O*-methylSAL (**7**) constitutes 94–98% of the two *O*-monomethylated metabolites of administered **2** in rat brain.

The metabolic pathway of **1** or **2** has been established in several Papaveraceae plants and their tissue cultures (Scheme 1) [4, 5]. 6-*O*-Monomethylated and 6,7-*O*-dimethylated SAL (**6** and **8**) have been formed from **2**. These different results between animals and plants, and even among animals, seem to be caused by different *O*-methylating enzymes of TIQs and/or CAs. In this report, we examined and compared the effects of a known COMT inhibitor, pyrogallol, on the formation of the *O*-methylated metabolites of **1** and **2** in intact plants of *Corydalis pallida* var. *tenuis* and *Cynanchum vincetoxicum*, and their tissue cultures in order to provide knowledge on the nature of COMT. The *O*-methylated derivatives of **1** and **2** are highly polar and difficult to isolate. In the LC/APCI-MS (Liquid Chromatography/Atmospheric Pressure Chemical Ionization-Mass Spectrometry), protonated quasi-molecular ions, which are very useful for identifying nonvolatile compounds, are observed. LC/APCI-MS was used to detect the structures of the *O*-methyl derivatives

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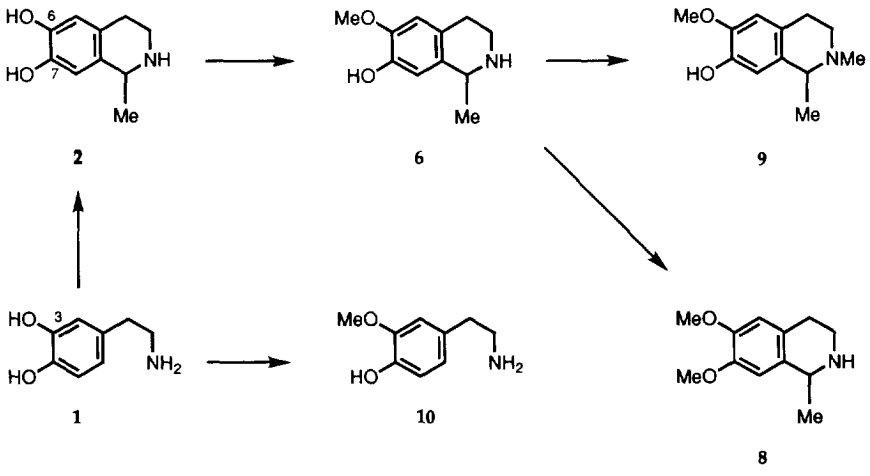
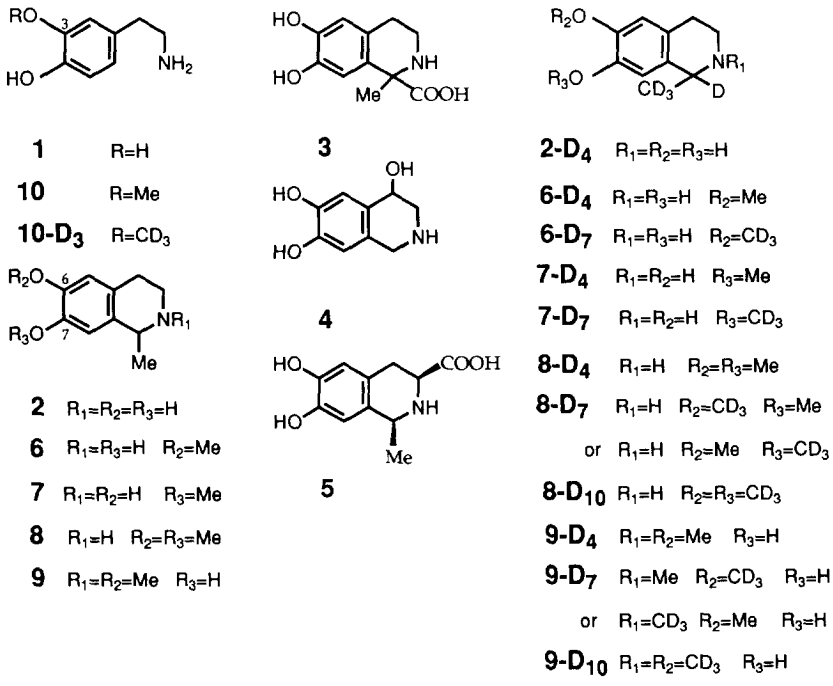


Scheme 1.



by simultaneous analysis. The APCI-MS was carried out by the SIM (Selected Ion Monitoring) or TIM (Total Ion Monitoring) method.

RESULTS AND DISCUSSION

Prior to feeding experiments, the mixture of the authentic DA or SAL derivatives was subjected to LC/APCI-MS. The LC/TIC (Total Ion Chromatogram) obtained for standard samples of DA (**1**), SAL (**2**), 6-OMe-SAL (**6**), 7-OMe-SAL (**7**), 6,7-diOMe-SAL (**8**), 7-OMe-SAL-NMe (**9**) and 3-OMe-DA (**10**) are shown in Fig. 1. Protonated quasi-molecular ions, each with a different R_t value, were observed for all the compounds tested in MC (Mass Chromatogram) obtained by SIM (Table 1). The LC obtained by UV detection (280 nm) is very similar to the TIC showing that the correspondence between the two chromatograms is very good (Fig. 1).

Feeding experiments in Corydalis pallida *var.* tenuis

Feeding experiments (Table 2) of SAL or DA with pyrogallol to the cultured cells of *C. pallida* var. *tenuis* were carried out with control experiments without pyrogallol. D-Labelled substrates were used to detect *O*-methylated metabolites easily in MS. Callus tissues were incubated on an agar medium containing SAL-D_4 (**2**-D_4) and L-[Me-D_3]methionine (Met, 99%-D) with pyrogallol at 25° for three weeks (Table 2, Expt. 1). After incubation, the medium and cells were extracted for alkaloids (Chart 1). The fraction of water–methanol extracts (**1**) after removal of neutral and acidic substances, and pretreat-

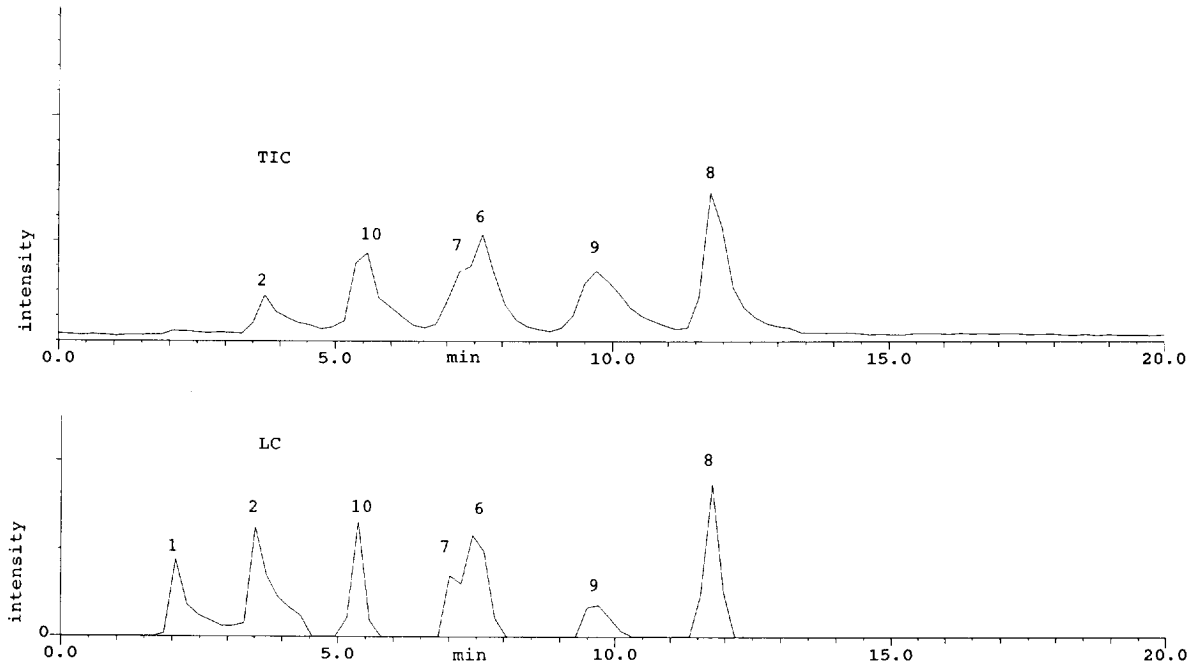


Fig. 1. Liquid chromatogram and total ion chromatogram (LC and TLC) in LC/APCI-MS of the mixture of the authentic dopamine or salsolinol derivatives (gradient 1).

Table 1. Protonated quasi-molecular ions and retention times in LC/APCI-MS of the mixture of authentic dopamine or salsolinol derivatives

Samples	R_t (min)	$[M+H]^+$ m/z
Dopamine (**1**)	2.1	*
Salsolinol (**2**)	3.7	180
3-*O*-Methyldopamine (**10**)	5.6	168
Salsoline (**7**)	7.3	194
Isosalsoline (**6**)	7.7	194
N-Methylisosalsoline (**9**)	9.8	208
Salsolidine (**8**)	11.8	208

*Protonated quasi-molecular ion was detected when the drift voltage or nebulizer temperature were changed.

ment with a centrifugal filter unit (millipore, ultrafree C-3) was subjected to LC/APCI-MS. 6-OMe-SAL-D_4 and -D_7 (**6**-D_4 and **6**-D_7, 6,7-diOMe-SAL-D_4,-D_7 and -D_{10} (**8**-D_4, **8**-D_7 and **8**-D_{10}), and 6-OMe-SAL-NMe-D_4, -D_7 and -D_{10} (**9**-D_4, **9**-D_7 and **9**-D_{10}), were found in fractions 1 obtained from Expt. 1, as well as in the control experiment (Fig. 2). The deuterated metabolites of **8** and **9** (**8**-D_{10} and **9**-D_{10}) arose in trace amounts in Expt. 1 (Fig. 2). Each deuterated metabolite was identified by the quasi-molecular ion and R_t. SAL-D_4 (**2**-D_4) recovered from Expt. 1 is *ca* 3.22-fold of that recovered from the control experiment. This indicates that metabolism of SAL is depressed by pyrogallol. The peak area in MC of each deuterated metabolite of fraction 1 obtained from Expt. 1 or the control experiment was measured. The ratio of the peak area of each metabolite obtained from the pyrogallol-experiment (Expt. 1) to the peak area of that obtained from the control experiments was shown in Table 2. So, the effect of pyrogallol on the quantities of the metabolites could be seen from the ratios. The amounts of the deuterated metabolites of **6**, **8**, and **9** were reduced under the influence of pyrogallol. It seems that *O*-methylation of C-6-OH of SAL is depressed by pyrogallol. The reduced amounts of the deuterated products of **8** and **9** may arise from the decrease of the deuterated metabolites of **6**.

An identical feeding experiment (Table 2, Expt. 2) was undertaken with cell suspension cultures (short culture period) and the results were compared with those in static cell cultures. The deuterated products of **6** were identified, but those of **8** and **9** were not detected in fraction 1 from Expt. 2. In the control experiment, the deuterated metabolites of **6**, **8** and **9** were identified. SAL-D_4 (**2**-D_4) was recovered from fraction 1 of Expt. 2 and the control experiment. The ratios (Table 2) of deuterated metabolites of fraction 1 indicate that the amounts of the deuterated products of **6**, **8** and **9** are reduced under the influence of pyrogallol. It appears that *O*-methylation of C-6-OH of SAL (**2**) is depressed by pyrogallol. The decrease of the deuterated metabolites of **8** and **9** may be due to the decrease of **6**-D_4 and **6**-D_7. This result agrees with that obtained from feeding experiments in static cell cultures.

Callus tissues from *C. pallida* var. *tenuis* were incubated on an agar medium containing DA (**1**) and L-[Me-D_3]Met with pyrogallol at 25° for three weeks (Table 2, Expt. 3). 3-OMe-DA (**10**), a trace amount of **10**-D_3 and

Table 2. Administration of dopamine (**1**) or salsolinol (**2**) to cell cultures of *Corydalis pallida* var. *tenuis*

Expt no.	Dry wt of cells (g)	Medium (ml)	**2**-D_4	L-[Me-D_3]Met	**1**	Substrates (mg) CD_3CDO	Pyrogallol	Culture period (days)	*m/z*	**6**-D_4 198	**6**-D_7 201	**8**-D_4 212	**8**-D_7 215	**8**-D_{10} 218	**9**-D_4 212	**9**-D_7 215	**9**-D_{10} 218	**2**-D_4 184	**10**-D_{10} 171
										Metabolites (ratios)*									
1	0.40	400	100	250			70	21		0.11	0.15	0.13	0.23	0.08	0.07	0.11	0.03	3.22	n.o.
2	0.38	40	10	25			3.5	10		0.20	0.37	†	†	†	†	†	†	1.19	n.o.
3	3.01	400		41	50		33	21		n.o.‡		n.o.‡			n.o.‡			1.0§	0.93¶
4	0.30	40		30	10	20	8	10		*	0.26	n.o.	*	*	n.o.	*	*	11.1	0.88

*The ratio of the peak area of each metabolite obtained from experiment under addition of pyrogallol to that obtained from control experiment.
†Metabolite in experiment with pyrogallol was not present within the detection limits of the APCI-MS utilized.
‡Corresponding undeuterated metabolite was not detected.
§*m/z* 180.
¶*m/z* 168 (ratio: 1.17).

Table 3. Administration of dopamine (**1**) or salsolinol (**2**) to plants of *Corydalis pallida* var. *tenuis*

Expt no.	Dry wt of cells (g)	**2**-D_4	L-[Me-D_3]Met	**1**	Substrates (mg) CD_3CDO	Pyrogallol	Culture period (days)	*m/z*	**6**-D_4 198	**6**-D_7 201	**8**-D_4 212	**8**-D_7 215	**8**-D_{10} 218	**9**-D_4 212	**9**-D_7 215	**9**-D_{10} 218	**2**-D_4 184	**10**-D_3 171
									Metabolites (ratios)*									
5	3.47	25	60			20	7		0.77	1.02	0.55	0.73	1.08	1.10	1.26	1.49	2.75	—
									(0.91)†			(0.53)†			(1.03)†			
6	3.63		60	25	40	20	7		0.79	0.88	0.76	0.74	0.74	1.04	1.03	1.09	2.98	0.98
									(0.81)†			(0.67)†			(0.97)†			

*The ratio of the peak area of each metabolite obtained from experiment under addition of pyrogallol to that obtained from control experiment.
†Metabolite in experiment with pyrogallol was not present within the detection limits of the APCI-MS utilized.

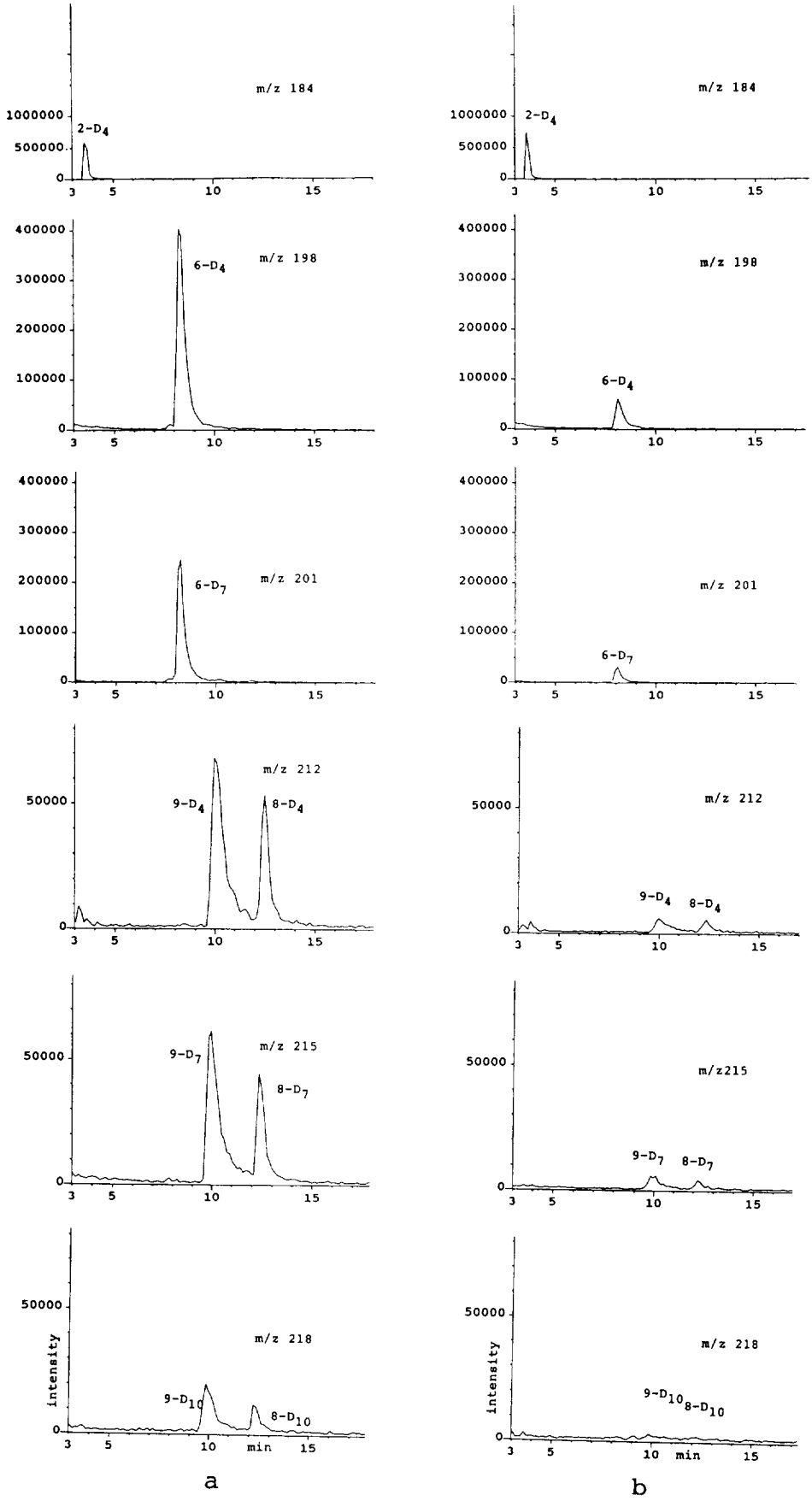


Fig. 2. Mass chromatogram of fraction 1 (gradient 2). (a) MC obtained from expt 1. (b) MC obtained from control experiment.

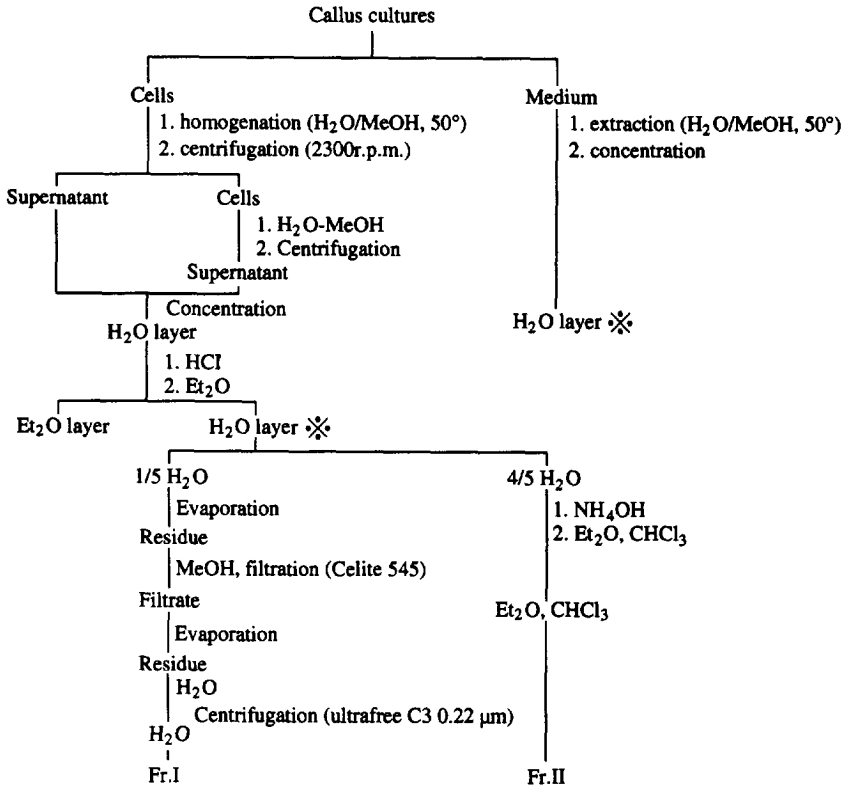


Chart 1.

Table 4. Administration of dopamine (**1**) or salsolinol (**2**) to cell cultures of *Cynanchum vincetoxicum*

Expt no.	Dry wt of cells (g)	Medium (ml)	Substrates (mg) 2-D_4	L-[Me-D_3]Met	**1**	CD_3CDO	Pyrogallol	Culture period (days)
7	2.23	400	100	250			70	21
8	1.34	400		41	50		33	21
9	0.47	40		30	10	20	8	10

SAL (**2**) were identified in fraction 1 from Expt. 3 and control experiment. 6-OMe-SAL (**6**), 6,7-diOMe-SAL (**8**) and 6-OMe-SAL-NMe (**9**) were not detected in Expt. 3. The ratios (Table 2) of **10** and **10**-D_3 suggest that *O*-methylation of C-3-OH of DA (**1**) was not depressed by pyrogallol.

The feeding experiment (Table 2, Expt. 4) was carried out in cell suspension cultures of *C. pallida* var. *tenuis* containing DA (**1**), CD_3CDO and L-[Me-D_3]Met with pyrogallol. In fraction 1 of the control experiment, **2**-D_4, **6**-D_4, **6**-D_7, a slight amount of deuterated 6,7-diOMe-SAL and 6-OMe-SAL-NMe (**8**-D_7, **8**-D_{10}, **9**-D_7 and **9**-D_{10}), and **10**-D_3 were identified. Fraction 1 from Expt. 4 contained **2**-D_4, **6**-D_7 and **10**-D_3. SAL-D_4 recovered from Expt. 4 is *ca* 11-fold of that obtained from control experiment. This shows that metabolism of SAL is inhibited by pyrogallol. The amounts of the deuterated metabolites of **6**, **8** and **9** are reduced, but that of **10**-D_3 was not reduced by pyrogallol. The ratios (Table 2) of **6**-D_4 and **6**-D_7 and that of **10**-D_3 suggest that *O*-methylation of C-6-OH of SAL is depressed by pyrogallol and that of C-3-OH of DA is not. The decrease of the deuterated metabolites of **8** and **9** may be a result of the decrease of **6**-D_4 and **6**-D_7. The results obtained from Expts 1–4 indicate that the effect of pyrogallol on the methylation at C-6 of SAL and on that at C-3 of DA are different.

Next, feeding experiments (Table 3, Expts 5 and 6) of SAL or DA with pyrogallol to intact plants of *C. pallida* var. *tenuis* were undertaken in order to compare the effect of pyrogallol on the metabolism of SAL or DA with that in the cultured cells. SAL-D_4 (**2**-D_4) and L-[Me-D_3]Met with pyrogallol (Table 3, Expt. 5) were fed to intact plants which were cultivated for one week, as well as to the control experiment. In fraction 1 obtained from Expt. 5 and from the control experiment, **6**-D_4, **6**-D_7, **8**-D_4, **8**-D_7, **8**-D_{10}, **9**-D_4, **9**-D_7 and **9**-D_{10} were identified. SAL-D_4 (**2**-

D_4) recovered from Expt. 5 is 2.75-fold of that obtained from the control experiment. This indicates that metabolism of SAL is depressed by pyrogallol. The amounts of the deuterated metabolites of **6** and **8** were reduced by pyrogallol, but the deuterated metabolites of **9** were not decreased. The ratios (Table 3) of **6** suggest that *O*-methylation of C-6-OH of SAL is depressed by pyrogallol. This result is in accord with that in the cultured cells, but the effect of pyrogallol on the methylation at C-6 of SAL in plants is smaller than that in the cultured cells.

DA, CD_3CDO and L-[Me-D_3]Met were administered to intact plants with pyrogallol (Table 3, Expt. 6). The deuterated metabolites of **6**, **8**, **9** and **10** were detected in fractions 1 from Expt. 6 and from the control experiment. SAL-D_4 (**2**-D_4) of 2.98-fold concentration of the control experiment was found in fraction 1. This shows that metabolism of SAL is inhibited by pyrogallol. The quantities of **6**-D_4, **6**-D_7, **8**-D_4, **8**-D_7 and **8**-D_{10} were diminished by pyrogallol, but **9**-D_4, **9**-D_7 and **9**-D_{10} were not. The amount of **10**-D_3 was not influenced by pyrogallol. The ratios (Table 3) of **6** and **10** indicate that *O*-methylation of C-6-OH of SAL is depressed by pyrogallol, but that of C-3-OH of DA is not.

The results obtained from Expts 5 and 6 reveal that the effect of pyrogallol on the methylation of C-6-OH of SAL and on that of C-3-OH of DA is different. This result is in agreement with that obtained in the cultured cells. This points towards the fact that the same *O*-methylating enzymes exist in intact plants and in their tissue-cultured cells. The results obtained from Expts 1–6 may suggest that the *O*-methylating enzymes of SAL and DA are different in *C. pallida* var. *tenuis*.

Feeding experiments in Cynanchum vincetoxicum

Feeding experiments (Table 4, Expts 7–9) of SAL or DA with pyrogallol to *Cyn. vincetoxicum* were undertaken with the control experiments. Callus tissues were incubated on an agar medium containing SAL-D_4 and L-[Me-D_3]-Met with pyrogallol at 25° for three weeks (Table 4, Expt. 7). No metabolite was detected in fraction 1 from Expt. 7 and the control experiment. The amount of **2**-D_4 recovered from Expt. 7 was 2.7 times that obtained from the control experiment. From fraction 2 of Expt. 7, as well as from the control experiment a trace amount of deuterated 6-OMe-SAL (**6**-D_4, **6**-D_7) and a slight amount of a metabolite, which has the same quasi-molecular ions (*m/z* 198 and 201), but a R_f only close to that of 6-OMe-SAL, were identified. The latter was identified as 7-OMe-SAL by LC/MC comparison with an authentic sample. The ratios of 7-OMe-SAL/6-OMe-SAL were 2.2 and 1.7, respectively, as calculated from MC at *m/z* 198 and 201. *O*-Methylated metabolites which have not been detected in previous studies [5] were identified. The different amount of SAL recovered from Expt. 7 and the control experiment suggests that metabolism of SAL is depressed by pyrogallol in *Cyn. vincetoxicum*. The feeding experiment (Table 4, Expt. 8) was carried out in the cultured cells containing DA and L-[Me-D_3]Met with pyrogallol. Only 3-OMe-DA-D_3 (**10**-D_3) was identified in fraction 1

Table 5. Administration of dopamine (**1**) or salsolinol (**2**) to plants of *Cynanchum vincetoxicum*

Expt no.	Dry wt of plants (g)	**2**-D_4	L-[Me-D_3]Met	**1**	Substrates (mg) CD_3CDO	Pyrogallol	Culture period (days)	Metabolites (ratios)* *m/z*	**6**-D_4 198	**6**-D_7 201	**7**-D_4 198	**7**-D_7 201	**2**-D_4 184	**10**-D_3 171
10	19.9	50	120			40	7		1.7	1.0	0.51	0.31	0.96	—
									(1.8)†		(0.85)‡		(0.94)†	
11	9.2		100	50	80	40	7		0.76	1.25	0.62	1.8	1.03	0.32

*The ratio of the peak area of each metabolite obtained from experiment under addition of pyrogallol to that obtained from control experiment.
†This value was calculated from the peak area of total ion chromatogram of each deuterated metabolite.

from Expt. 8 and from the control experiment. The ratio (0.92) for **10**-D_3 indicates that *O*-methylation of C-3-OH of **1** is not depressed by the presence of pyrogallol.

The cell suspension cultures containing DA, L-[Me-D_3]Met and CD_3CDO with pyrogallol were incubated for 10 days (Table 4, Expt. 9). Only SAL-D_4 (**2**-D_4) was detected in fraction 1 from Expt. 9 and the control experiment. The ratio (2.82) for **2**-D_4 reveals that also here metabolism of SAL is depressed by pyrogallol.

SAL-D_4 and L-[Me-D_3]Met with pyrogallol (Table 5, Expt. 10) were fed to intact plants. In fraction 1 from Expt. 10 and the control experiment, **6**-D_4, **6**-D_7, **7**-D_4, and **7**-D_7 were identified, and **2**-D_4 was recovered. The ratios of 7-OMe-SAL/6-OMe-SAL were 0.62 and 1.0 at *m/z* 198 and 201, respectively. The ratio for **7**-D_4 or **7**-D_7 (Table 5) reveals that *O*-methylation of C-7-OH of **7** is depressed by pyrogallol, but an influence of pyrogallol on *O*-methylation of C-6-OH of **6** was not deduced from the ratio for **6**-D_4 or **6**-D_7.

DA, CD_3CDO and L-[Me-D_3]Met with pyrogallol (Table 5, Expt. 11) were administered to intact plants. SAL-D_4 (**2**-D_4), D_4- and D_7-derivatives of **6** and **7**, and **10**-D_3 were identified in fraction 1 of Expt. 11 and in that of the control experiment. The ratio (0.32) [Table 5] for **10**-D_3 indicates that *O*-methylation of the C-3-OH of DA (**1**) is depressed by pyrogallol. An effect of pyrogallol on formation of 6-OMe-SAL (**6**) and 7-OMe-SAL (**7**) can not be deduced from the ratios for the deuterated metabolites of **6** and **7**.

The effect of pyrogallol on the formation of 3-OMe-DA is different between the cultured cells and intact plants of *Cyn. vincetoxicum*. It is a remarkable difference between *C. pallida* var. *tenuis* and *Cyn. vincetoxicum* that both 6- and 7-*O*-methylations occur in the latter, and only 6-*O*-methylation occurs in the former. This indicates that *O*-methylating enzymes for C-6-OH and C-7-OH of SAL are different.

EXPERIMENTAL

Materials. The callus of *Corydalis pallida* var. *tenuis* was derived in 1988 from the stems of wild growth plants in Kobe (Japan) on Murashige-Skoog's (MS) medium containing (2,4-dichlorophenoxy)-acetic acid (1 mg l^{-1}), kinetin (0.1 mg l^{-1}), yeast extract (0.1%) and agar (1%). The callus of *Cynanchum vincetoxicum* was derived from the stems of plants harvested at the Regensburg University botanical garden (Germany) in 1983. The callus tissues were subcultured every 3 weeks on fresh MS medium at 25° in the dark. [1-D, Me-D_3]-Salsolinol (**2**-D_4) was prepd as described in ref. [6]. L-[Me-D_3]Methionine (99%) and CD_3CDO (99%) were purchased from Aldrich and pyrogallol from nakarai tesque (Japan).

Feeding experiments in tissue-cultured cells. Feeding experiments (Tables 2 and 4) were performed as described in ref. [6]. The suspension cultures (Expts 2, 4 and 9) were grown in the MS medium without agar for 10 days. Cells and medium were extracted according to Chart 1. Frs 1 and 2 in H_2O and MeOH–$CHCl_3$, respectively, were subjected to LC/APCI-MS (SIM method).

Feeding experiments in plants. Feeding experiments (Tables 3 and 5) were performed as described in ref. [4]. The plants were extracted as described for cells of callus cultures. Frs 1 and 2 were subjected to LC/APCI-MS.

LC/APCI-MS method. LC/APCI-MS was carried out with a Hitachi M-1000H connected to a Hitachi L-6200 pump and a Hitachi L-4000 UV detector. LC was performed using a Cosmosil 5C_{18}-AR (4.6 i.d. × 150 mm) reversed phase column. The mobile phase was 0.1 M NH_4OAc (0.05% TFA, A) to which MeOH (B) was added under the linear gradient. Gradient 1: initial 7% of B, 5 min 14% of B, 10 min 30% of B, 20 min 50% of B; Gradient 2: initial 7% of B, 5 min 14% of B, 15 min 50% of B. The flow rate was 1 ml min^{-1}. UV 280 nm. APCI-MS conditions: nebulizer and vaporizer temps were 275 and 399°, respectively. Drift voltage was 20 V. The quasi-molecular ions were monitored by SIM method.

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