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Bioconversion studies in cultured cells of *Corydalis* species

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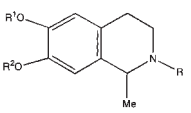
Structural analysis of the metabolites of dopamine and salsolinol in cultured cells of *Corydalis* species was carried out using the combination of LC-MS and LC-NMR techniques. Metabolic pathways were clarified without the need to isolate the individual metabolites.

1. Introduction

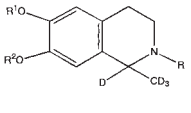
The use of LC-NMR in combination with LC-MS enables a rapid initial screening of natural products in complex mixtures such as crude plant extracts. The application of LC-NMR to drug metabolism, identification of natural products in crude plant extracts, and the characterization of isomeric mixtures prepared by chemical reactions have been reviewed (Hostettmann et al. 1977; Wolfender et al. 2001). To the best of our knowledge, there is no applications of LC-NMR to biosynthetic studies.

The bioconversions of isoquinoline alkaloids by plants and their cell cultures were investigated in our laboratory in combination with assays for biological activities (Iwasa 1995; Iwasa et al. 2001). These studies resulted in the discovery of numerous metabolites with antimicrobial, antimalarial, anti HIV, and anti cancer activities (Iwasa et al. 2001).

The simple isoquinoline salsolinol (**1**) has been found to be biotransformed into *O*- and/or *N*-methylated salsolinol by several Papaveraceae plants and their *in vitro* cultured cells (Iwasa et al. 1991, 1992). *O*-Methylated derivatives of **1** were found in the urine of healthy human subjects and in rat brain after intraventricular administration of **1** (Brossi 1993). In human and rat brains, *R*-salsolinol, enantio-selectively synthesized from dopamine and acetaldehyde, is *N*-methylated to give parkinsonism-inducing *R*-*N*-methylsalsolinol (**2**) which is not produced by plants and tissue-cultured cells (Naoi et al. 1996; Maruyama et al. 1997).



	R^1	R^2	R
1	H	H	H
2	H	H	Me
3	Me	Me	Me
4	H	Me	Me
5	Me	H	Me
6	H	Me	H
7	Me	H	H
8	Me	Me	H



	R^1	R^2	R
1-D$_4$	H	H	H
4-D$_4$	H	Me	Me
5-D$_4$	Me	H	Me
6-D$_4$	H	Me	H
7-D$_4$	Me	H	H
8-D$_4$	Me	Me	H

In the present study, LC-MS and LC-NMR are applied to define structures of isoquinoline metabolites in biotransformation studies by cell cultures of *Corydalis* species thus eliminating the need to isolate metabolites from a crude extract. Particular attention is called to the detection of *N*-methylsalsolinol (**2**) as a metabolite.

2. Investigations, results and discussion

2.1. Identification of N-methylsalsolinol derivatives

An acid-catalyzed ether cleavage of carnegine (**3**) was carried out in order to obtain the phenolic *N*-methyltetrahydroisoquinolines as reference compounds. The mixture of the phenolic products was subjected to LC-APCI/MS and LC-NMR. The APCI-MS data of four chromatography peaks (R-1, R-2, R-3, and R-4 in the LC-1) (Fig. 1) show $[M + H]^+$ at m/z 194, 208, 208, and 222, respectively. The ^{1}H NMR spectra of R-1, R-2, R-3, and R-4 comprise a set of *N*-methyl signals (ca. 2 : 1), indicating the formation of two types of salts by *N*-protonation (Table). The ^{1}H NMR spectra of R-4 and R-1 show two methoxyl signals and no methoxyl signal, respectively, indicating that R-4 was carnegine (**3**) and R-1 was *N*-methylsalsolinol (**2**), while R-2 and R-3 possess one methoxyl group. The position of the methoxyl group was determined by stopped-flow WET-NOESY data. In the NOESY spectrum of R-2, clear NOE's are shown between 4-H and 5-H and also between 8-H and a methoxyl-methyl at C-7, confirming the methoxyl group to be located at C-7 (Table). In the NOESY spectrum of R-3, NOE's between 4-H and 5-H, 5-H and a methoxy methyl at C-6, and 8-H and methyl at C-1, respectively, were observed. Therefore, the methoxyl group in R-3 is placed at C-6. Thus, R-2 and R-3 were identified as *N*-methylsalsoline (**4**) and *N*-methylisosalsoline (**5**), respectively.

2.2. Feeding of deuterated salsolinol (1-D$_4$)

[1-D,1-CD_3]-salsolinol (**1-D$_4$**) was first fed to cultured cells of *C. ochotensis* var. *reddeana* and *C. platycarpa*. Peaks 1, 2, and 3 in the LC-2 (Fig. 1) of Fr. E-1 (Chart) display $[M + H]^+$ at m/z 198, 212, and 212 in their APCI-MS.

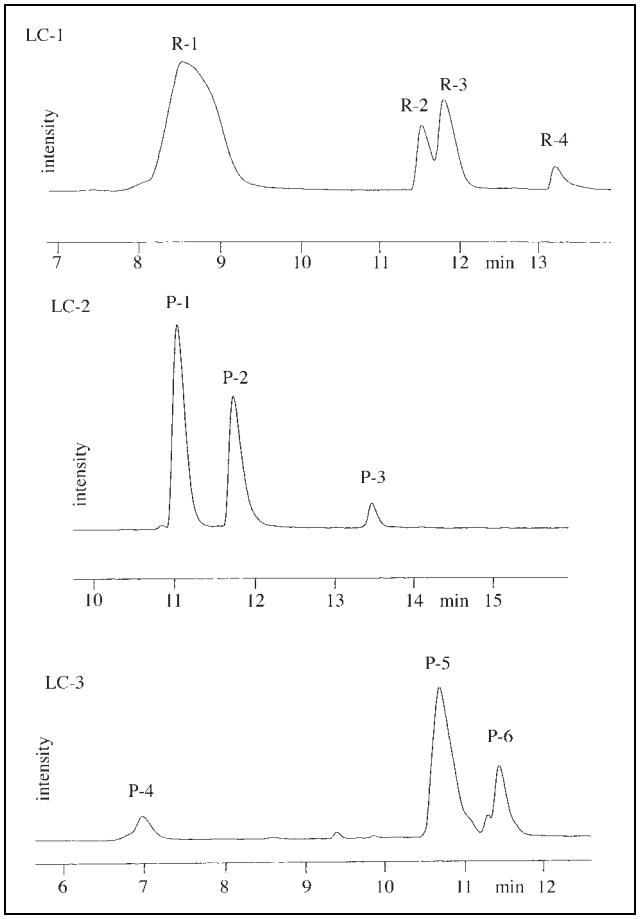


Fig. 1: LC data of the alkaloidal fractions obtained from feeding experiments (LCs 2–3) and acid-catalyzed ether cleavage products of carnegine (LC-1). LC-2: Adminstration of **1-D$_4$** in *C. platycarpa*; LC-3: Administration of dopamine and CD_3CDO in *C. platycarpa*

The ^{1}H NMR spectrum of peak 1 in LC 2 exhibits two aromatic protons as singlets and one methoxyl group. These data, together with $[M + H]^+$, m/z 198 of peak 1 suggest that peak 1 represents [1-D,1-CD_3]-salsoline or -isosalsoline (**6-D$_4$** or **7-D$_4$**). In the WET-NOESY spectrum of peak 1, the irradiation of an aromatic proton at δ 6.79 gave an enhancement of the protons at δ 2.93 as well as that of the methoxyl group at δ 3.75. Thus, the attachment of the methoxyl group at C-6 was confirmed. The structure was finally ascertained to be deuterated isosalsoline (**7-D$_4$**) by comparison of the ^{1}H NMR data with those of the references salsoline (**6**) and isosalsoline (**7**) (Table).

The ^{1}H NMR spectra of peak 2 in LC 2 (Fig. 1) show two aromatic protons as singlets, one methoxyl group, and one *N*-methyl group. The ^{1}H NMR and MS data $[(M + H)^+$, m/z 212] suggest that the structure represented by peak 2 is [1-D,1-CD_3]-*N*-methylsalsoline (**4-D$_4$**) or -*N*-methylisosalsoline (**5-D$_4$**). In the NOESY spectrum of peak 2, a correlation between aromatic proton at δ 6.81 and protons at δ 2.99 and a correlation of the same aromatic proton and the methoxyl-methyl at δ 3.77 were observed. These findings have led to the assignment of the aromatic proton to C-5. Therefore, the attachment of the methoxyl group at C-6 was confirmed. The structure was thus established to be [1-D,1-CD_3]-*N*-methylisosalsoline (**5-D$_4$**). The ^{1}H NMR spectra of the metabolite represented by peak 2 were compared with those of *N*-methylsalsoline (**4**) and *N*-methylisosalsoline (**5**) and found to be identical with that of **5** (Table).

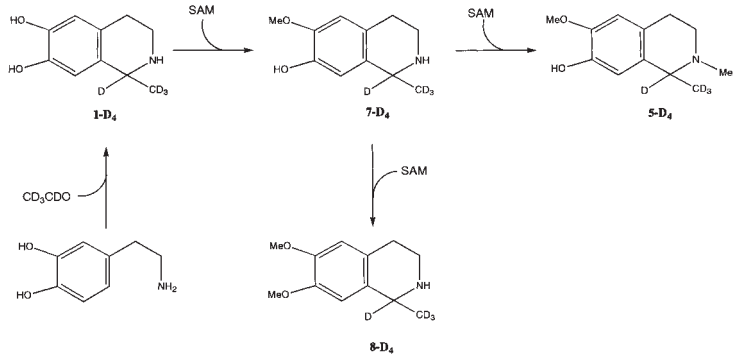
The ^{1}H NMR spectra of peak 3 in LC 2 (Fig. 1) show two aromatic protons as singlets and two methoxyl groups. APCI-MS of peak 3 shows $[M + H]^+$ at m/z 212. From these NMR and MS data it was concluded that the structure indicated by peak 3 is [1-D,1-CD_3]-salsolidine (**8-D$_4$**). This structure was confirmed to be **8-D$_4$** by comparison of its ^{1}H NMR data with those of authentic salsolidine (**8**) (Table). Deuterated *N*-methylisosalsoline, isosalsoline, and salsolidine (**5-D$_4$**, **7-D$_4$**, and **8-D$_4$**) were also obtained from feeding of **1-D$_4$** in *C. platycarpa*. *N*-Methylisosalsoline (**5**), Isosalsoline (**7**), and salsolidine (**8**) were identified as metabolites obtained by feeding experiments of salsolinol in cultured cells of *Corydalis* species. This finding confirmed previous results (Iwasa et al. 1991, 1992). *N*-methylsalsolinol (**2**) was not detected in these feeding experiments as before.

Table: Stopped-flow ^{1}H NMR data* of isoquinolines [0.1 M NH_4OAc (D_2O)-MeCN (0.05% TFA), δ, 500 MHz]**

IQ***	1-H	1-Me	2-N-Me	3-H	4-H	5-H	6-OMe	7-OMe	8-H
1	4.42 1 H br	1.51 3 H br		3.27 1 H m 3.43 1 H m	2.70 2 H m	6.65 1 H s			6.68 1 H s
2	4.38 1 H m	1.52 3 H	2.83 1.8 H s 2.92 1.2 H s	3.41 1 H m 3.61 1 H m	2.91 1 H m 3.26 1 H m	6.66 1 H s			6.64 1 H s
3	4.41 1 H br	1.55 3 H br	2.84 1.8 H s 2.99 1.2 H s	3.32 1 H m 3.57 1 H m	2.99 1 H m 3.32 1 H m	6.81 1 H s	3.75 6 H s		6.76 1 H s
4	4.40 1 H br	1.56 3 H br	2.85 1.8 H s 2.95 1.2 H s	3.30 1 H m 3.58 1 H m	2.95 1 H m 3.30 1 H m	6.70 1 H s	3.77 3 H s		6.77 1 H s
5	4.40 1 H br	1.53 3 H br	2.84 1.8 H s 2.99 1.2 H s	3.30 1 H m 3.58 1 H m	2.98 1 H m 3.30 1 H m	6.81 1 H s	3.77 3 H s		6.66 1 H s
6	4.47 1 H m	1.56 3 H br		3.29 1 H m 3.45 1 H m	2.90 2 H m	6.67 1 H s		3.76 3 H s	6.79 1 H s
7	4.43 1 H q (6.5)	1.52 3 H d (6.5)		3.28 1 H m 3.45 1 H m	2.92 2 H m	6.78 1 H s	3.75 3 H s		6.69 1 H s
8	–	1.55 3 H d (6.5)		3.30 1 H m 3.45 1 H m	2.94 2 H m	6.79 1 H s	3.75 6 H s		6.78 1 H s

* J values (in Hz) in parentheses; ^{1}H-^{1}H correlations 4-H ↔ 5-H; 5-H ↔ C(6)–OMe 8-H ↔ C(7)–OMe 8-H ↔ C(1)-Me
** 0.1 M NH_4OAc (D_2O)/MeCM (5 min 95/5, 10 min 85/15, 15 min 85/15, 20 min 65/35); –: disappeared by H_2O irrdiation
*** IQ (peak number in LC): **1** [P-4 (**1-D$_4$**): 1-H (0.6 H), 1-Me (1.8 H); **2** (R-1); **3** ((R-4); **4** (R-2); **5** [R-3, P-2 (**5-D$_4$**): 1-H (0.03 H), 1-Me (0.1 H)]
7 [P-1 (**7-D$_4$**): 1-H (0.03 H), 1-Me (0.1 H)]; **8** [P-3 (**8-D$_4$**): 1-H (0.03 H), 1-Me (0.1 H)]

Scheme



2.3. Feeding of dopamine

In the next phase, dopamine, CD_3CDO and *S*-adenosyl-L-methionine were administered simultaneously to cultured cells of *C. platycarpa*, since salsolinol (**1**) and *N*-methylsalsolinol (**2**) were biosynthesized from dopamine and acetaldehyde in human and rat brains (Naoi et al. 1996; Maruyama et al. 1997). Peak 4 in the LC 3 (Fig. 1) of Fr. E-1 (Fig. 2) displays two $[M + H]^+$ at m/z 184 and 180 (ratio of 1.4 : 1) in the APCI MS, indicating a mixture of deuterated and non-deuterated metabolites. The ^{1}H NMR spectrum of peak 4 exhibits two aromatic protons as singlets and a C-methyl signal (ca. 1.8 H) as a doublet. These data, together with the MS data [$(M + H)^+$, m/z 184 and 180], suggest that the metabolite represented by peak 4 is a mixture of [1-D,1-CD_3]-salsolinol (**1-D$_4$**) and salsolinol (**1**). Its ^{1}H NMR spectrum is identical with that of authentic salsolinol (Table).

Peak 5 in the LC-3 displays two $[M + H]^+$ at m/z 198 and 194 (ratio of ca. 1.4 : 1). The ^{1}H NMR spectrum of peak 5 showing two aromatic protons as singlets and a methoxyl group is identical with that of isosalsoline (**7**) (Table). Thus, peak 5 is a mixture of **7-D$_4$** and **7**.

The APCI-MS of peak 6 in the LC exhibits two $[M + 1]^+$ at m/z 212 and 208 (ratio ca. 1.4 : 1). The ^{1}H NMR spectrum of this peak 6 shows two aromatic protons as singlets, one methoxyl group, and a *N*-methyl group and is in accordance with that of *N*-methylisosalsoline (**5**) (Table). Thus, peak 6 is a mixture of **5-D$_4$** and **5**.

Salsolinol (**1**), *N*-methylisosalsoline (**5**), and isosalsoline (**7**) with CD_3 and CH_3 at C-1 arising from exogenous CD_3CDO and the natural source, respectively, were identified as metabolites obtained from feeding of dopamine in cultured cells of *C. platycarpa*. *N*-Methylsalsolinol (**2**) was not detected as in the feeding experiment of salsolinol.

There seems to be a difference in the biotransformations between plant cell cultures and brain cells in which *N*-methylation occurs to give **2**. The *N*-methylation process in brain cells might be important with respect to induction of parkinsonism.

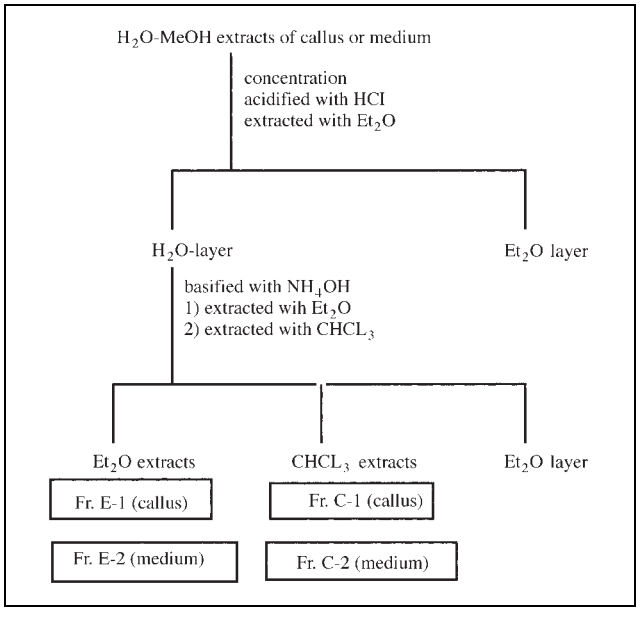


Fig. 2: Preparation of samples for LC-NMR and LC-MS

3. Experimental

3.1. Cell cultures and chemicals

Calli of *C. platycarpa* Makino and *C. ochotensis* var. *raddeana* were derived from the stems of wild plants grown in Kobe (Japan) on Murashige and Skoog's (MS) medium containing 2,4-dichlorophenoxyacetic acid (1 mg/l), kinetin (0.1 mg/l), yeast extract (0.1%), and agar (1%), in 1989 and 1981, respectively. The callus tissues were subcultured every 3 or 4 weeks on the same fresh medium at 25 °C in the dark. *S*-Adenosyl-L-methionine and CD_3CDO (99%) were purchased from Sigma (USA), and Aldrich (USA), respectively.

3.2. LC-APCI/MS

LC-MS was carried out using a Hitachi M-1000H connected to a Hitachi L-6200 intelligent pump and a Hitachi L-4000 UV detector. LC was performed on a Cosmosil 5 C_{18}-AR (4.6 i.d. X 150 mm) reversed phase column. Mobile phase: 0.1 M NH_4OAc (0.05% TFA, A), to which MeCN (0.05% TFA, B) was added by a linear gradient: (initial 100% of B, 5 min 5% of B, 15 min 25% of B, 25 min 25% of B). Flow rate: 1 ml/min (detection: 280 nm). APCI-MS conditions: nebulizer and vaporizer temperatures were 290° and 399°, respectively. Drift voltage: 20 V. The mass spectrometer was operated with SIM [selected ion monitoring] and TIM [total ion monitoring] in the positive ion mode.

3.3. LC-NMR

Data were acquired using a Varian UNITY-INOVA-500 spectrometer (H-1: 500 MHz) equipped with a PFG indirect-detection LC-NMR probe with a 60 µL flow-cell (active volume). ^{1}H NMR spectra were obtained in stopped-flow mode. Varian WET solvent suppression (Smallcombe et al. 1995) and related sequences were used to suppress the peaks of CH_3CN, its C-13 satellites, and the residual HOD in D_2O. FIDs were collected with 16 K data points, a spectral width 9000 Hz, a 3 µs 90° pulse, a 1.82 s acquisition time, and a 0.08 s pulse delay. Typically, 16–1640 scans were accumulated (1 min–1 h). Prior to Fourier transformation, an exponential apodization function was applied to the FID corresponding to a line broadening of 1 Hz. HPLC system: Varian Pro Star Model-230 solvent delivery system and Varian Pro Star Model-310 variable-wavelength UV-Vis detector. The outlet of the UV detector was connected via a sampling unit to the LC-NMR probe. NOESY spectra were obtained using a WET-NOESY pulse sequence, in which the WET element was incorporated into the 700 ms mixing time. A total of 200 hypercomplex t_1 increments with 12–

32 transients depending on the sample concentrations and 2 K data points were acquired with a spectral width in both dimensions of 3700 Hz with an acquisition time of 0.14 s, giving a total experimental time of about 1 h and 43 min−7 h and 42 min. The data were Gaussian weighted in f_2 and f_1 and zero filled in f_1 to 2 K × 2 K. Separation was performed on a Cosmosil 5 C_{18}-AR (4.6 i.d. X 150 mm) reversed phase column. The mobile phase was 0.1 M NH_4OAc in D_2O (0.05% TFA, A), to which MeCN (0.05% TFA, B) was added by a linear gradient: initial 100% of B, 5 min 5% of B, 10 min 15% of B, 15 min 15% of B, 20 min 35% of B. Flow rate and detection (see above).

3.4. Preparations of reference compounds

Salsolinol (**1**), [1-D,1-CD_3]-salsolinol (**1-D$_4$**), salsoline (**6**), isosalsoline (**7**), and salsolidine (**8**) were prepared previously (Iwasa et al. 1991).

Acid-catalyzed ether cleavage of carnegine (**3**): A solution of **3** (100 mg) in 47% HBr (1 ml) was refluxed for 10 min and evaporated *in vacuo* to give a crystalline mixture, which shows four peaks, R-1 (60%), R-2 (10%), R-3 (25%), and R-4 (5%) (see LC). A solution of the products (20 mg) in DMSO-d_6 (200 μl) was subjected to LC-APCI/MS and LC-NMR (Table).

3.5. Feeding experiments

Deuterated salsolinol (**1-D$_4$**) 400 mg was administered to *C. ochotensis* var *raddeana* and *C. platycarpa*. Dopamine 220 mg, *S*-adenosyl-L-methionine 20 mg, and CD_3CDO 330 mg were administered to *C. platycarpa*. Each substrate was dissolved in H_2O (2−4 mL) and introduced through a sterilized bacterial filter into 100 ml conical flasks containing 40 ml of autoclaved Murashige and Skoog (MS) medium (total MS 2400 and 800 ml for **1-D$_4$** and dopamine, respectively), identical with that employed in the subculture. Calli (ca. 4−5 g) were transferred to each conical flask and incubated at 25 °C in the dark for 21 days (**1-D$_4$**) and 25 days (dopamine). Cells and medium were separated and extracted with MeOH−H_2O at 60 °C, respectively. Extracts were worked-up as described in Fig. 2. The alkaloidal fractions E-1, E-2, C-1, and C-2 (Fig. 2), which are soluble in organic solvents, were subjected to LC-MS and LC-NMR.

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