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13-HYDROXYLATION OF TETRAHYDROBERBERINE IN CELL SUSPENSION CULTURES OF SOME *CORYDALIS* SPECIES

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Key Word Index—*Corydalis ophiocarpa*; *C. ochotensis* var. *raddeana*; Fumariaceae; tissue cultures; biotransformation; hydroxylation; oxidation; N- and C-methylations.

Abstract—Liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry was applied to biotransformation experiments in cultured cells of *Corydalis ophiocarpa* as well as *C. ochotensis* var. *raddeana*. Hydroxylation at C-13 of tetrahydroberberine was shown to take place in cell cultures as well as in *C. ophiocarpa* plants. N-Methylation of tetrahydroberberine occurred to form the α-*N*-metho salt incorporating the B/C-*cis*-quinolizidine system. Introduction of the C-13 methyl with berberine as substrate was confirmed to provide 13-methylberberine. In addition, the reversible oxidation–reduction of the C ring of protoberberines was demonstrated.

INTRODUCTION

Of the numerous protoberberines which have been isolated from natural sources, ophiocarpine (**1**) and corycarpine (**2**), isolated from *Corydalis ophiocarpa* and other *Corydalis* species [1], are the only 13-hydroxylated tetrahydroprotoberberine alkaloids. The routes to the benzindanoazepine- and/or spirobenzylisoquinoline-type alkaloids from the α-*N*-metho salts of (14*S*)-ophiocarpine (**1**) and (14*S*)-epiophiocarpine (**3**), in which the hydrogens at C-13 and C-14 are in a *cis* and *trans* relationship, respectively, have been demonstrated in cell cultures of several *Corydalis* species [2–5]. The α-*N*-metho salts of the *cis*- and *trans*-13-hydroxytetrahydroprotoberberines (**1** and **3**) were bioconverted into 13-oxoprotopine (**4**), which was in turn transformed into benzindanoazepine (**5**) and spirobenzylisoquinoline (**6**) (Scheme 1).

It has been established that hydorxylation of (14*S*)-tetrahydroberberine (**7**) to **1** in *C. ophiocarpa* proceeds with retention of configuration, and involves the removal of the pro-*R* hydrogen atom from the C-13 position of the precursor (**7**) [6] (Scheme 1).

One of the purposes of this study was to examine the bioconversion of tetrahydroberberine (**7**) into **1** in cell cultures of *C. ophiocarpa* as well as *C. ochotensis* var. *raddeana*. Interconversions of tetrahydroprotoberberines (e.g. **7**) and protoberberinium salts (e.g. **8**), N-methylation of tetrahydroberberines to form the α-*N*-metho salts (e.g. **11**), and methylation at C-13 of protoberberinium salts to furnish 13-methylprotoberberinium salts (e.g. **9**), have been demonstrated in cell cultures of *C. pallida* var. *tenuis* and *C. incisa* [7] (Scheme 1). Another purpose was to demonstrate C- and N-methylations in cell cultures of *C. ophiocarpa* and *C. ochotensis* var. *raddeana*.

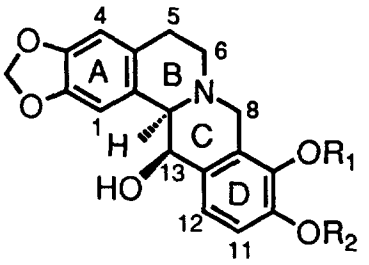
RESULTS AND DISCUSSION

Synthesis of labelled precursors

Pyrolysis of berberine (**8**) gave rise to berberrubine (**10**) by selective O-demethylation of the C-9 methoxyl (Scheme 2). Introduction of the deuterium label was accomplished by methylation of berberrubine with CD_3I to generate [9-OCD_3]berberine (**8D**) (Scheme 2). The mass spectrum (SIMS) showed a base peak at *m/z* 339 $[M - Cl]^+$. The ^1H NMR spectrum exhibited a methoxyl group at δ4.11. [9-OCD_3]Tetrahydroberberine (**7D**) was prepared by reduction of [9-OCD_3]berberine with sodium borohydride in methanol (Scheme 2). The mass spectrum of the product displayed a molecular peak at *m/z* 342, and a fragment peak at *m/z* 167 assignable to ion A, arising by way of retro-Dields–Alder cleavage of ring C.

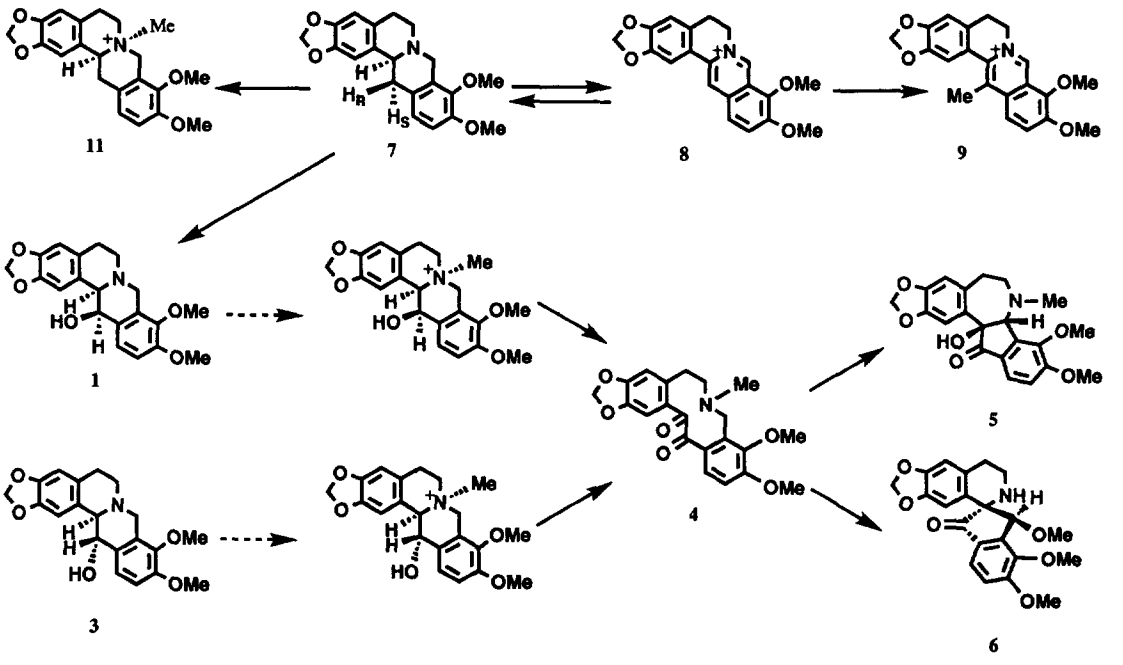
Biosynthetic experiments

Callus tissues of *C. ophiocarpa* or *C. ochotensis* var. *raddeana* were incubated in a liquid medium containing [9-OCD_3]tetrahydroberberine (**7D**) or [9-OCD_3]berberine (**8D**) at 25° for 10 days. Following incubation, the medium and cells were extracted according to the procedure outlined in Experimental. The alkaloid fractions (Frs I and II) soluble in organic solvents were subjected

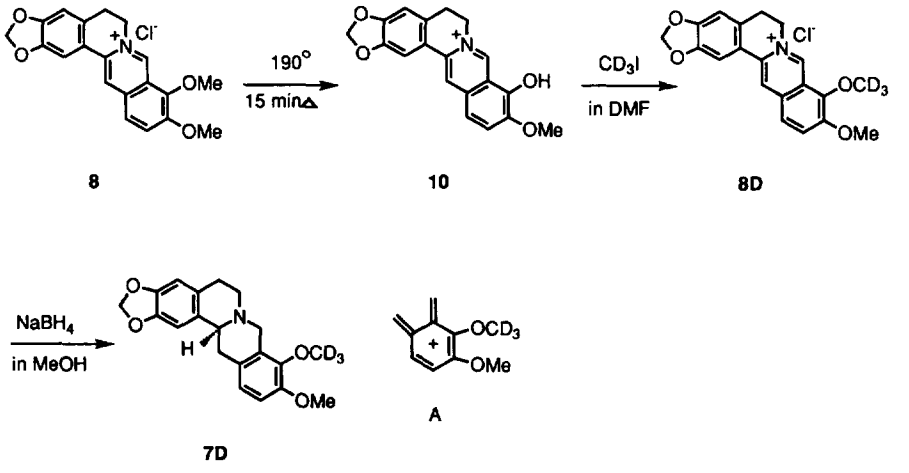


1 $R_1=R_2=Me$
2 $R_1+R_2=CH_2$

to liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry [LC/APCI-MS (SIM method)] with a mixture of H_2O and methanol [0.05% trifluoroacetic acid (TFA)] as the mobile phase. Metabolites were identified by quasi-molecular ions, molecular ions or cluster ions as described previously [tetrahydroberberine (**7**, *m/z* 340 $[M + H]^+$), berberine (**8**, *m/z* 406 $[M + CF_3]^+$), 13-methylberberine (**9**, *m/z* 420 $[M + CF_3]^+$), tetrahydroberberine-α-*N*-metho salt (**11**, *m/z* 354 $[M]^+$), allocryptopine (**12**, *m/z* 370 $[M + H]^+$), chelerythrine (**13**, *m/z* 348 $[M]^+$) [7]. Deuterated berberine (**8D**, *m/z* 409 $[M + CF_3]^+$), 13-methylberberine (**9D**, *m/z* 423 $[M + CF_3]^+$), tetrahydroberberine α-*N*-



Scheme 1.



Scheme 2.

Table 1. Administration of [9-OCD_3]tetrahydroberberine (**7D**) and [9-OCD_3]berberine (**8D**) to cell cultures of *C. ophiocarpa* and *C. ochotensis* var. *raddeana*

Wt of dry cells (g)	Medium (ml)	Substrates (mg)	Incubation time (days)	Wt of fraction (mg)		Metabolites detected (observed ions, *m/z*)						
				I	II	**1D** (359 $[M+H]^+$)	**7D** (343 $[M+H]^+$)	**8D** (409 $[M+CF_3]^+$)	**9D** (423 $[M+CF_3]^+$)	**11D** (357 $[M]^+$)	**12D** (373 $[M+H]^+$)	**13D** (351 $[M]^+$)
C. ophiocarpa												
2.40	200	**7D** 25	10	24.8		*	*†	*†	*†		*†	*†
					16.2	*	*†	*†	*†	*†	*†	*†
3.15	200	**8D** 25	10	8.0		*	*†	*†	*†		*	
					4.0	*	*†	*†	*†		*†	
C. ochotensis var. *raddeana*												
2.68	200	**7D** 25	10	22.0		*	*†	*†	*		*†	*†
					12.7	*	*†	*†	*†	*†	*†	*†
2.39	200	**8D** 25	10	21.0		*	*†	*†	*†		*†	*†
					6.0	*	*†	*†	*†			

*Metabolite was detected by LC/APCI-MS with 0.1M NH_4OAc–MeOH (0.05% TFA) as mobile phase.
†Metabolite was detected by LC/APCI-MS with H_2O–MeOH (0.05% TFA) as mobile phase.

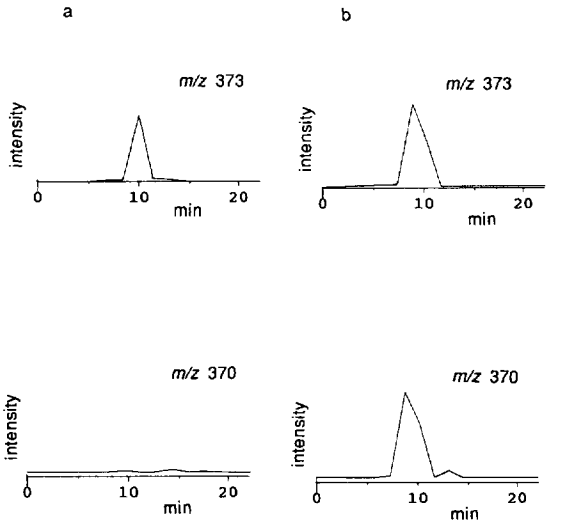


Fig. 1. Mass chromatogram (a) of Fr. I from the experiment in which [9-OCD_3]tetrahydroberberine was administered to *C. ophiocarpa* and (b) of the mixture of fr. I and allocryptopine. The quasi-molecular ions of allocrypotopine (*m/z* 370) and [9-OCD_3]allocryptopine (*m/z* 373) were monitored by a selected ion monitoring method in LC/APCI-MS (nebulizer and vaporizer temperatures: 340° and 399°; drift voltage 20 V). H_2O–MeOH (0.05% TFA) was used as the mobile phase in LC.

metho salt (**11D**, *m/z* 357 $[M]^+$), allocryptopine (**12D**, *m/z* 373 $[M+H]^+$), and chelerythrine (**13D**, *m/z* 351 $[M]^+$) were detected in experiments in which (**7D**) was administered to cultured cells of *C. ophiocarpa* and *C. ochotensis* var. *raddeana* (Table 1). Deuterated tetrahydroberberine (**7D**, *m/z* 343 $[M+H]^+$), **9D** and **12D** were detected in experiments in which [9-OCD_3]berberine (**8D**) was administered to both sets of cultured cells (Table 1). Identification of each metabolite obtained from Frs I and II was confirmed by comparison of its mass chromatogram with that of the mixture with authentic samples. An example is shown in Fig. 1.

The mass chromatogram monitored by *m/z* 359 ([9-OCD_3]ophiocarpine) was poorly resolved by LC/APCI-MS using mixtures of H_2O and methanol (0.05% TFA) as the mobile phase in LC. In Frs I and II obtained from feeding experiments in which **7D** or **8D** were fed *C. ophiocarpa* and *C. ochotensis* var. *raddeana*, [9-OCD_3]-ophiocarpine (**1D**, *m/z* 359 $[M+1]^+$) was detectable by LC/APCI-MS when mixtures of 0.1 M ammonium acetate and methanol (0.05% TFA) were used as the mobile phase (Table 1) (Fig. 2). All of the metabolites described above were also detected (Table 1).

In conclusion, the metabolic conversions, a (**7D** → **8D**), b (**8D** → **7D**), c (**8D** → **9D**) and d (**7D** → **11D**), described previously in *C. incisa* and *C. pallida* var. *tenuis* [7] have now been demonstrated in cultured cells of *C. ophiocarpa* and *C. ochotensis* var. *raddeana* (Scheme 3). The known metabolic conversions [8], e (**11** → **12**) and f (**12** → **13**), have also been confirmed in feeding experiments of the compounds labelled with deuterium. The metabolic

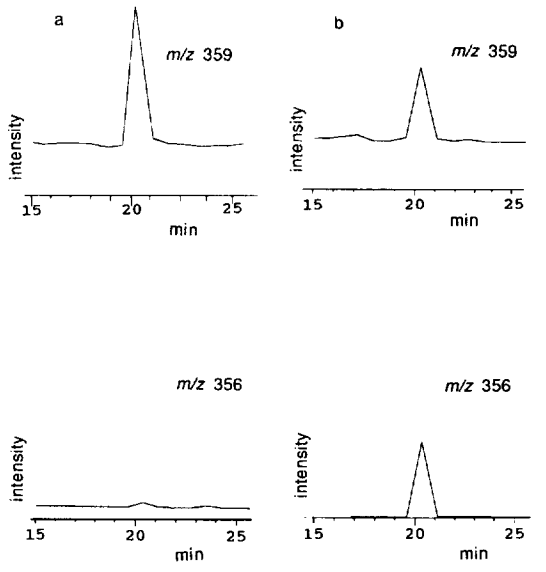


Fig. 2. Mass chromatogram (a) of fr. II from the experiment in which [9-OCD_3]tetrahydroberberine was administered to *C. ochotensis* var. *raddeana* and (b) of the mixture of fr. II and ophiocarpine. The quasi-molecular ions of ophiocarpine (*m/z* 356) and [9-OCD_3]ophiocarpine (*m/z* 359) were monitored by a selected ion monitoring method in LC/APCI-MS (nebulizer and vaporizer temperatures: 340° and 399°; drift voltage 30 V). 0.1 M NH_4OAc–MeOH (0.05% TFA) was used as the mobile phase in LC.

pathway g (**7D** → **1D**) has been demonstrated for the first time.

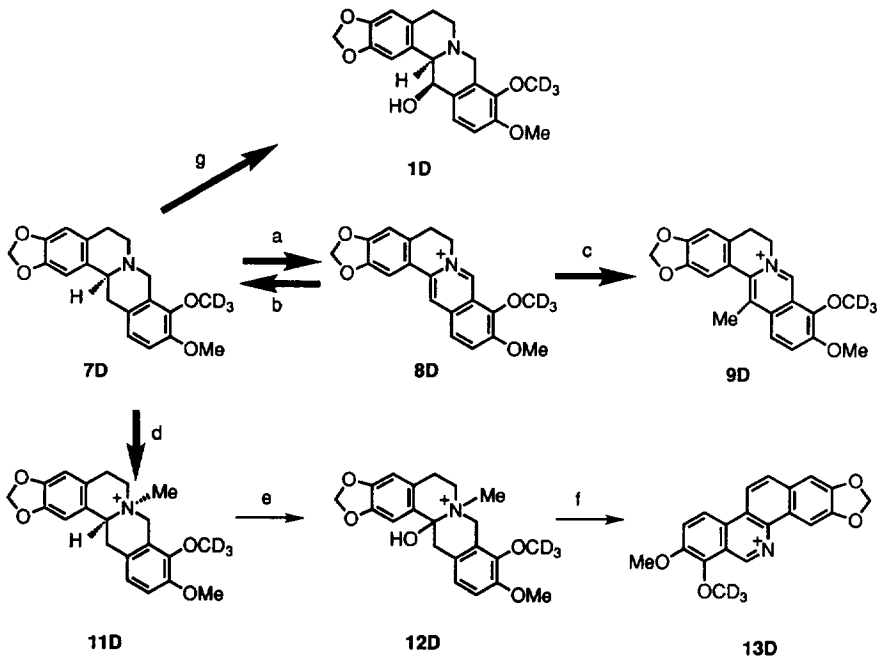
The interconversion, via redox reaction, of **7** and **8** has been established. The α-*N*-metho salt (**11**), incorporating the B/C-*cis* quinolizidine system, was produced from **7**. C-Methylation at C-13 of **8** has been confirmed to generate **9**.

Finally, hydroxylation at C-13 of **7** to give rise to **1** has been proved.

EXPERIMENTAL

Mps: uncorr.; ^1H NMR: Varian VXR-500S (500 MHz) or Varian Gemini 300 (300 MHz), in $CDCl_3$ or CD_3OD soln, TMS as int. standard; prep. TLC or TLC: Silica gel $60F_{254}$ plates (Merck); EIMS, CIMS (CH_4), SIMS (glycerol as matrix): Hitachi M-4100; HPLC: Hitachi 6300 apparatus equipped with a UV-detector (L-4000), Cosmosil $5C_{18}$-AR (4.6 mm i.d. × 150), eluents 0.1M NH_4OAc (0.05% TFA, A)–MeOH (0.05% TFA, B) mixts or H_2O (0.05% TFA, A)–MeOH (0.05% TFA, B) mixts, linear gradient–initial 25% B, 10 min 50% B, 20 min 80% B, 30 min 80% B, flow rate 1 ml min^{-1}, UV (280 nm): LC/APCI-MS: Hitachi M-1000H connected to a Hitachi L-6200 intelligent pump and a Hitachi L-4000 UV detector; nebulizer and vaporizer temps 340° and 399°, drift voltage 20–30 V. The quasi-molecular ions were monitored by the SIM method.

Preparation of berberrubine (**10**). Berberine (1 g) was heated at 190° in a dry oven under vacuum (20–30 mmHg) for 15 min. The crude product was recrystallized from EtOH–MeOH to provide **10** (660 mg), mp 270–285° (dec.). SIMS *m/z* (rel. int.): 322 $[M-Cl]^+$ (100). ^1H NMR (300 MHz, CD_3OD): δ 3.23 (2H, *t*, *J* = 6.3 Hz, H-5), 4.06 (3H, *s*, OMe), 4.86 (2H, *t*, *J* = 6.3 Hz, H-6), 6.09 (2H, *s*, OCH_2O), 6.94, 7.62 (1H each, *s*, H-1, H-4), 7.68, 7.97 (1H each, *d*, *J* = 9.0 Hz, H-11, H-12), 8.56 (1H, *s*, H-13), 9.74 (1H, *s*, H-8).



Scheme 3.

Preparation of [9-OCD_3]*berberine* (**8D**). Berberrubine (500 mg) was dissolved in dry DMF (50 ml) and then CD_3I (1 ml) was added. The reaction mixt. was allowed to stand overnight. The resulting crystals were filtered and recrystallized from MeOH to give the iodide [348 mg, mp 234–241° (dec.)]. A part of the iodide was treated with AgCl in MeOH to convert it into the chloride, mp 206–210° (dec.). SIMS *m/z* (rel. int.): 339 $[M - Cl]^+$ (100). ^1H NMR (500 MHz, CD_3OD): δ3.25 (2H, *t*, H-5), 4.11 (3H, *s*, OMe), 4.95 (2H, *t*, H-6), 6.11 (2H, *s*, OCH_2O), 6.99, 7.66 (1H each, *s*, H-1, H-4), 8.0, 8.12 (1H each, *d*, J = 9.0 Hz, H-11, H-12), 8.71 (1H, *s*, H-13), 9.77 (1H, *s*, H-8).

Preparation of [9-OCD_3]*tetrahydroberberine* (**7D**). To a soln of [9-OCD_3]berberine chloride (300 mg) in MeOH (200 ml), $NaBH_4$ (1 g) was added, and the reaction mixt. was stirred at room temp. overnight. Water was added and the soln was concd and extracted with $CHCl_3$. The organic layer was dried and evapd. The residue was crystallized from $CHCl_3$–MeOH to give **7D** (91 mg). The mother liquid was subjected to prep. TLC (C_6H_6–Et_2O, 4:1) to afford **7D** (67 mg), mp 167–169°, EIMS *m/z* (rel. int.): 342 $[M]^+$ (100), 167 (55). ^1H NMR (500 MHz, $CDCl_3$): δ3.05, 3.25, 3.55, 3.85 (1H each, *m*, H-5, H-6), 3.06 (1H, *dd*, J = 17.0, 12.0 Hz, H-13), 3.73 (1H, *dd*, J = 17.0, 4.5 Hz, H-13), 3.87 (3H, *s*, OMe), 4.42, 4.73 (1H each, *d*, J = 16.0 Hz, H-8), 4.67 (1H, *dd*, J = 12.0, 4.5 Hz, H-14), 5.98 (2H, *d*, J = 1.5 Hz, OCH_2O), 6.75, 6.94 (1H each, *s*, H-1, H-4), 7.06, 7.07 (1H each, *d*, J = 8.5 Hz, H-11, H-12).

Cell lines. The calli of *C. ophiocarpa* and *C. ochotensis* var. *raddeana* were derived from the stem of wild grown plants in Japan on Murashige and Skoog's (MS) medium containing 2,4-D (1 mg l^{-1}), kinetin (0.1 mg l^{-1}), yeast extract (0.1%) and agar (1%), several years previously. The callus tissues were subcultured every 3 or 4 weeks on the same fresh medium at 25° in the dark.

Biotransformation experiments. Feeding experiments were carried out as described below. Substrates (25 mg) were dissolved in H_2O (2–4 ml) and introduced into conical flasks (100 ml × 5) containing 40 ml autoclaved MS medium, which is the same as that employed for subculture (without agar), through a sterile bacterial filter. Calli (*ca* 4–5 g) were transferred to each conical flask and incubated at 25° in the dark for 10 days. The medium and cells were then sepd by centrifugation and the cells extracted with H_2O–MeOH. The extracts were concd and combined with the medium (final vol. 300 ml). The soln was acidified and washed with Et_2O. The aq. soln was adjusted to pH *ca* 10 (with NH_4OH) and extracted with Et_2O and then $CHCl_3$ to give the alkaloid frs (frs I and II) soluble in Et_2O and $CHCl_3$, respectively.

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