Biotransformation of Phenolic Tetrahydroprotoberberines in Plant Cell Cultures Followed by LC-NMR, LC-MS, and LC-CD

Kinuko Iwasa,*[,†] Wenhua Cui,[†] Teturo Takahashi,[†] Yumi Nishiyama,[†] Miyoko Kamigauchi,[†] Junko Koyama,[†] Atsuko Takeuchi,[†] Masataka Moriyasu,[†] and Kazuyoshi Takeda[‡]

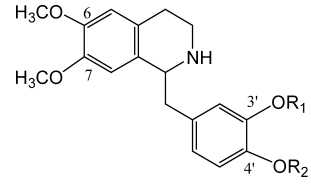
Kobe Pharmaceutical University, 4-19-1 Motoyamakita, Higashinada-ku, Kobe-shi 658-8558, Japan, and Yokohama College of Pharmacy, 601 Matanocyo, Hodogayaku, Yokohama-shi 245-0066, Japan

Received July 27, 2009

A metabolic pathway of 2,3,10,11-oxygenated tetrahydroprotoberberines having the OH group on ring D was demonstrated. Metabolism of ^{13}C- or D_2-labeled precursors was studied in cell cultures of *Macleaya*, *Corydalis*, and *Nandina* species. The structures of alkaloid metabolites obtained from feeding experiments were determined by application of combined LC-NMR, LC-MS/MS, and LC-CD techniques. (*S*)-Tetrahydropseudoprotoberberine (**5**) was stereospecifically *O*-methylated to the *S*-isomer (**12**) in cell cultures of three plant species. This *S*-isomer was further *N*-methylated to the (*S*)-α-*N*-methyl salt (**15**), which was oxidized to produce the pseudoprotopine-type alkaloid (**10**) in cell cultures of *Macleaya* and *Corydalis* species. These transformations were the same as those of 2,3,9,10-oxygenated protoberberines. The tetrahydropseudoprotoberberines (**5**, **6**, and **12**) were dehydrogenated to pseudoprotoberberines (**13**, **16**, and **14**), respectively. Both the *R*- and *S*-enantiomers of **5** were dehydrogenated in *Macleaya cordata* different from the case of 2,3,9,10-oxygenated protoberberines. Precursor **7**, with OH groups at C-10 and C-11, was *O*-methylated at C-10 in *M. cordata* and *C. ochotensis* var. *raddeana*, which was distinct from *O*-methylation in *N. domestica*, in which **7** was *O*-methylated at both C-11 and C-10. Stereoselective *O*-demethylation [(*S*)-**5** to (*S*)-**18**] occurred in *N. domestica*.

We previously investigated the biosynthesis of isoquinoline alkaloids in Papaveraceae and Fumariaceae plants and in their cell cultures, and also evaluated the metabolites for various types of biological activity.[1–5] Numerous compounds with antimicrobial, antimalarial, anti-HIV, and anticancer activities were revealed.[2–5]

Although protoberberine alkaloids differ in the number and position of oxygen functions on the aromatic rings A and D, two oxygenation patterns most frequently encountered were at carbons 2,3,9,10 and 2,3,10,11. The former group occurs most commonly, while the latter has been labeled "pseudoprotoberberine" and is not as widespread.[6] Some 2,3,10,11-oxygenated alkaloids display higher activity in biological assays (e.g., antimalarial activity) than the corresponding 2,3,9,10-substituted analogues.[3] The biosynthetic conversion of the 2,3,9,10-oxygenated protoberberines to protopines, benzophenanthridines, rhoeadines, benzindanoazepines, and spirobenzylisoquinolines has been demonstrated.[1,7] Earlier we found that 2,3,10,11-oxygenated protoberberines (tetrahydropseudocoptisine and tetrahydropseudopalmatine) underwent bioconversion into pseudoprotopine-type alkaloids via their α-*N*-methyl salts.[8] Phenolic pseudoprotoberberine-type alkaloids with an OH group on aromatic rings A or D occur in some plants,[6,9] including *Corydalis* species. Except for our studies,[10–12] the metabolic pathway leading to the formation of these alkaloids has not been investigated. Results from prior feeding experiments of 1-benzyltetrahydroisoquinolines with one (e.g., **1** and **2**) or two OH groups (e.g., **3**) at C-3′, C-4′, C-6, or C-7 have demonstrated that the presence of an OH group at C-3′ is essential for the biotransformation of 1-benzyltetrahydroisoquinolines into 2,3,9,10- and 2,3,10,11-oxygenated tetrahydroprotoberberines.[10–12] Tetrahydropalmatrubine (**4**)[13] is derived from norlaudanine (**1**, C-3′ OH) and its *N*-methyl derivative. Corytenchine (**5**)[14] is formed from **1** and its *N*-methyl derivative and also from the *N*-methyl derivative of **3** (C-3′ and -4′ OH). However, 10-demethylxylopinine (**6**)[15] and spinosine (**7**)[16] are not produced from compounds **2** (C-4′ OH) and **3** (C-3′ and -4′ OH), respectively. In our current investigation, the metabolism of **5**, **6**, and **7** (2,3,10,11-oxygenated tetrahydroprotoberberines), together with **4** (a 2,3,9,10-oxygenated tetrahydroprotoberberine as a comparison), was studied in cell cultures of *Corydalis ochotensis* var. *raddeana* (Fumariaceae), *Macleaya cordata* (Papaveraceae), and *Nandina domestica* Thumb. (Berberidaceae). Without prior isolation, the structures of the metabolites were determined by LC-NMR, LC-MS, and LC-CD analyses.



1 $R_1 = H$, $R_2 = CH_3$
2 $R_1 = CH_3$, $R_2 = H$
3 $R_1 = R_2 = H$

Results and Discussion

Synthesis. [8-^{13}C]-Tetrahydroprotoberberines [(±)-[8-^{13}C]-**4**−**7**] and (±)-[8-D_2]-**7** were prepared by the Mannich reaction of ^{13}C- or D_2-labeled 37% formaldehyde with 1-benzyltetrahydroisoquinolines (**1**, **2**, and **3**) (Supporting Information, Scheme S1), which were prepared from 3,4-dimethoxyphenylethylamine and appropriate 3,4-dioxygenated phenylacetic acids, according to reported procedures.[17,18] Structures of the synthetic compounds ([8-D_2]-**7**, [8-^{13}C]-**4**−**7**) were confirmed by analysis of their MS, HRMS, ^1H NMR, and NOESY spectra.

Feeding Experiments. Callus tissues of *Macleaya cordata*, *Corydalis ochotensis* var. *raddeana*, and *Nandina domestica* were incubated at 25 °C (*N. domestica* 27 °C) on an agar medium containing the substrate for four weeks (Table 1). Following incubation, media and cells were separated and extracted according to the procedure described previously.[11] The ether and $CHCl_3$-soluble alkaloid fractions (E-1, E-2 and C-1, C-2) (Supporting Information, Figure S1) were subjected to LC-NMR, LC-MS, and LC-CD.

***Corydalis* and *Macleaya* Species.** The metabolism of [8-^{13}C]-tetrahydroprotoberberines [(±)-[8-^{13}C]-**4**−**6**] and [(±)-8-D_2]-spinosine [(±)-[8-D_2]-**7**] (Scheme 1), which possess OH groups on the aromatic ring D, were examined in cultured cells of *C. ochotensis* var. *raddeana* and *M. cordata* (Table 1). Initially,

* To whom correspondence should be addressed. E-mail: k-iwasa@kobepharma-u.ac.jp. Tel: 081-78-453-0031. Fax: 081-78-435-2080.
† Kobe Pharmaceutical University.
‡ Yokohama College of Pharmacy.

10.1021/np900440d
Published on Web 01/19/2010

Table 1. Administration of Tetrahydroprotoberberines (**4**−**7**) to Cultured Cells of *Macleaya cordata, Corydalis ochotensis* var. *raddeana*, and *Nandina domestica*[a]

no.	callus[b]	substrate (mg)		wt of dry cells (g)
1	A	[8-^{13}C]-**4**	5	2.7
2	A	(*S*)-[8-^{13}C]-**4**	4	2.5
3	A	(*R*)-[8-^{13}C]-**4**	7	4.9
4	A	[8-^{13}C]-**5**	10	2.0
5	B	[8-^{13}C]-**5**	10	1.9
6	A	(*S*)-[8-^{13}C]-**5**	5	2.5
7	A	(*R*)-[8-^{13}C]-**5**	5	2.3
8	A	[8-^{13}C]-**6**	10	3.4
9	B	[8-^{13}C]-**6**	10	5.0
10	A	[8-D_2]-**7**	10	1.3
11	B	[8-D_2]-**7**	10	1.3
12	C	[8-^{13}C]-**4**	10	2.0
13	C	[8-^{13}C]-**5**	10	2.0
14	C	[8-^{13}C]-**6**	10	1.9
15	C	[8-^{13}C]-**7**	10	2.2
16	C	[8-D_2]-**7**	10	1.7

[a] MS medium: 400 mL; incubation period: 4 weeks. [b] A: *Macleaya cordata*; B: *Corydalis ochotensis* var. *raddeana*; C: *Nandina domestica*.

precursor [(±)-8-^{13}C]-tetrahydropalmatrubine [(±)-[8-^{13}C]-**4**] was administered to cells of *M. cordata* (Table 1, no. 1). Fraction E-1 showed four LC peaks, a_1−d_1 (I, Figure 1). In LC-APCI-MS, peaks a_1−d_1 showed $[M + H]^+$ ions at *m/z* 371, 354, 343, and 341, respectively. From LC-NMR and LC-MS data, peaks b_1 and c_1 were attributed to protopine (a component of *M. cordata*) and precursor **4**, respectively. Peak a_1 showed product ions at *m/z* 222, 205, and 204 and was identified as [8-^{13}C]-cryptopine ([8-^{13}C]-**8**) (Scheme 1) by comparison of its LC-NMR and LC-MS data with those of an unlabeled authentic sample.[10] Peak d_1 showed a product ion at *m/z* 192 in LC-MS/MS and corresponded to [8-^{13}C]-tetrahydroepiberberine ([8-^{13}C]-**9**) (Scheme 1) by comparison of the LC-MS with that of an unlabeled authentic sample.[11] Thus, the feeding of (±)-[8-^{13}C]-**4** to *M. cordata* resulted in two metabolites, [8-^{13}C]-**8** and -**9**. As shown in Scheme 1, **4** was converted to **9** (the methylenedioxy group of **9** being formed from the 9-hydroxy and 10-methoxy groups on ring D of **4**), which was further converted to **8**. This metabolic pathway has been demonstrated previously.[1,7]

Next, [(±)-8-^{13}C]-corytenchine ((±)-[8-^{13}C]-**5**) was administered to cultured cells of *M. cordata* (Table 1, no. 4). Peaks a_2−f_2 (II, Figure 1) in fractions E-1 and C-2 showed $[M + H]^+$ or $[M]^+$ ions at *m/z* 387, 371, 343, 357, 339, and 353, respectively, in the LC-MS. Peaks a_2 and b_2 also showed product ions at *m/z* 222, 205, and 204 and were identified as [8-^{13}C]-pseudomuramine ([8-^{13}C]-**10**) and [8-^{13}C]-pseudocryptopine ([8-^{13}C]-**11**) (Scheme 1) by comparison of their LC-MS data with those of protopine-type alkaloids.[10] From LC-MS/MS data, peak c_2 was deduced to be precursor [8-^{13}C]-**5**. Peak d_2 displayed a $[M + H]^+$ ion at *m/z* 357 and product ion at *m/z* 192. The ^{1}H NMR spectrum of peak d_2 showed four *O*-CH_3 groups at δ 3.76, 3.78 (6H), and 3.79 and four aromatic protons at δ 6.78 (1H, d, $J = 3.0$ Hz, ^{13}C−H long-range coupling), 6.82 (2H, s), and 6.86 (1H, s). These data indicated that peak d_2 was [8-^{13}C]-tetrahydropseudopalmatine ([8-^{13}C]-**12**) (Scheme 1). This structure was confirmed by comparison of the ^{1}H NMR data with that of authentic unlabeled sample[8] measured under the same conditions (Supporting Information, Figure S2). The signal pattern for H-8 in the ^{1}H NMR spectrum of [8-^{13}C]-**12** was different from that of unlabeled **12**, because of the ^{13}C−H coupling. Peak e_2 showed a $[M]^+$ ion at *m/z* 339 and product ion at *m/z* 323 in the LC-MS/MS. The ^{1}H NMR spectrum of peak e_2 showed three *O*-CH_3 groups at δ 3.84 and 3.89 (6H), aromatic proton singlets at δ 6.95, 6.97, 7.47, and 8.05, and aromatic proton doublets at δ 7.30 (1H, d, $J = 2.5$ Hz, ^{13}C−H long-range coupling) and 8.74 (1H, d, $J =$ 181 Hz, ^{13}C−H coupling). These data indicated that peak e_2 was [8-^{13}C]-dehydrocorytenchine ([8-^{13}C]-**13**) (Scheme 1). This structure was confirmed by comparison of the ^{1}H NMR data with that of authentic unlabeled sample[11] (Supporting Information, Figure S3). Peak f_2 displayed a $[M]^+$ ion at *m/z* 353 and product ion at *m/z* 337. The ^{1}H NMR spectrum of peak f_2 showed four *O*-CH_3 groups (δ 3.86, 3.91, 3.97, and 4.04), four aromatic proton singlets (δ 7.01, 7.46, 7.53, and 8.47), and two aromatic proton doublets at δ 7.56 (1H, d, $J = 2.5$ Hz, ^{13}C−H long-range coupling) and 9.14 (1H, d, $J = 188$ Hz, ^{13}C−H coupling). These data indicated that the compound represented by peak f_2 was [8-^{13}C]-pseudopalmatine ([8-^{13}C]-**14**) (Scheme 1). This structure was confirmed by comparison of the ^{1}H NMR data with that of authentic unlabeled sample[8] (Supporting Information, Figure S3).

The metabolites obtained from feeding (±)-[8-^{13}C]-**5** to *C. ochotensis* var. *raddeana* (Table 1, no. 5) were found to be **10**, **11**, **12**, and **13**. As shown in Scheme 1, **5** was likely converted to **11** and **12**, and the latter further converted to **10**. Compound **11** could be produced by oxidation at C-13a after formation of the methylendioxy group from the OH and OCH_3 groups on ring D, a metabolic pathway comparable to the conversion of **4** to **9** and then to **8**. Compounds **5** and **12** could also be dehydrogenated to give **13** and **14**, respectively (Scheme 1).

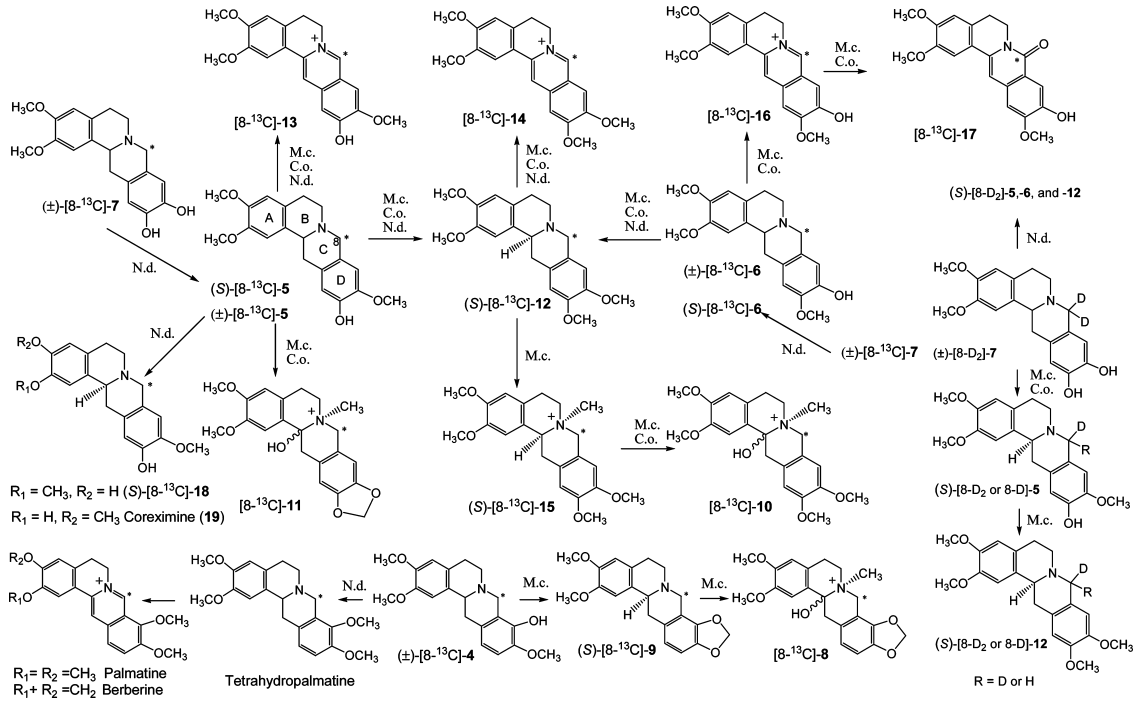
In a similar fashion, the metabolic path of (±)-[8-^{13}C]-10-demethylxylopinine [(±)-[8-^{13}C]-**6**] was examined in *M. cordata* and *C. ochotensis* var. *raddeana*. Precursor [8-^{13}C]-**6** was administered to cultured cells of *M. cordata* (Table 1, no. 8). The resulting fraction E-2 showed LC peaks a_3−h_3 (Figure 2), which produced $[M + H]^+$ or $[M]^+$ ions at *m/z* 387, 371, 369, 343, 339, 353, 357, and 355, respectively. The compounds associated with peaks a_3, d_3, f_3, and g_3 were identified as [8-^{13}C]-**10**, -**6**, -**14**, and -**12** (Scheme 1), respectively. The amount of [8-^{13}C]-**12** was smaller than that obtained from feeding of [(±)-[8-^{13}C]-**5**]. Thus, **5** is a better precursor of **12** than of **6** in *M. cordata*. LC peak b_3 showed a $[M + H]^+$ ion at *m/z* 371 and a product ion at *m/z* 206, corresponding to [8-^{13}C]-*cis*-*N*-methyltetrahydropseudopalmatinium salt ([8-^{13}C]-**15**) (Scheme 1). The structure of **15** was confirmed by comparison of its LC-MS/MS data with that of authentic **15** ($[M]^+$ ion at *m/z* 370 and a product ion at *m/z* 206).[8] Peak e_3 displayed a $[M]^+$ ion at *m/z* 339 and a product ion at *m/z* 323 in the LC-MS/MS. The ^{1}H NMR spectrum (A, Supporting Information, Figure S4) of the metabolite showed three OCH_3 groups (δ 3.86, 3.91, and 4.04), four aromatic singlets (δ 7.01, 7.44, 7.52, and 8.43), an aromatic doublet (δ 9.06, $J = 143$ Hz, ^{13}C−H coupling), and two methylene multiplets (δ 3.14 and 4.65). The structure of the metabolite was deduced to be ^{13}C-labeled 10-demethyldehydroxylopinine ([8-^{13}C]-**16**) (Scheme 1). Peak h_3 showed a $[M + H]^+$ ion at *m/z* 355 and a product ion at *m/z* 339. The ^{1}H NMR spectrum of peak h_3 (B, Supporting Information, Figure S4) showed four aromatic singlets (δ 6.92, 7.13, 7.18, and 7.36), an aromatic doublet (δ 7.58, $J =$ 3.5 Hz, ^{13}C−H long-range coupling), three OCH_3 groups (δ 3.83, 3.87, and 3.93), and two methylene multiplets (δ 2.90 and 3.24). The structure of the metabolite associated with peak h_3 was determined to be ^{13}C-labeled 8-oxo-10-demethyldehydroxylopinine ([8-^{13}C]-**17**) (Scheme 1).

The metabolites obtained from feeding [8-^{13}C]-**6** to *C. ochotensis* (Table 1, no. 9) were deduced to be [8-^{13}C]-**10**, -**12**, -**14**, -**16**, and -**17**, by analysis of ^{1}H NMR and LC-MS/MS data. These metabolites were identical to those obtained from the feeding experiment of [8-^{13}C]-**6** to *M. cordata*.

In our proposed biosynthetic pathway, **6** was *O*-methylated to give **12**, which was converted via its *N*-methyl derivative (**15**) into **10**. In addition, **12** can be dehydrogenated to **14**. Precursor **6** could also be dehydrogenated to give **16**, which was oxidized to the 8-oxo derivative **17** (Scheme 1). Although **11** was obtained from **5**, it was not obtained from **6**. This result suggested that a methylenedioxy group may not be formed from an OH at C-10 and an OCH_3 at C-11.

In the next stage of our study, the metabolism of [(±)-8-D_2]-spinosine ([(±)-[8-D_2]-**7**]), which has two OH groups on the aromatic D ring, was examined in *M. cordata* and *C. ochotensis* (Table 1, no. 10 and 11, respectively). From the LC-MS/MS data,

Scheme 1. Metabolism of (±)-[8-^{13}C]-Tetrahydropalmatrubine, -Corytenchine, -10-Demethylxylopinine, and -Spinosine, (±)-[8-D$_2$]-Spinosine [(±)-[8-^{13}C]-**4**, -**5**, -**6**, -**7**, and (±)-[8-D$_2$]-**7**] in *M. cordata* (M.c.), *C. ochotensis* var. *raddeana* (C.o.), and *Nandina domestica* (N.d.)



[8-D$_2$]-**5** ([M + H]$^+$ ion at *m*/*z* 344 and product ion at *m*/*z* 192) and [8-D$_2$]-**12** ([M + H]$^+$ ion at *m*/*z* 358 and product ion at *m*/*z* 192) were identified as the metabolites in *M. cordata*. The only metabolite from *C. ochotensis* was identified as [8-D$_2$]-**5** from LC-MS/MS data. Compound **7** was *O*-methylated selectively at C-10 to give **5**, which in turn was *O*-methylated on ring D to afford **12** (Scheme 1).

Chiral purities of the tetrahydroprotoberberine metabolites were determined by online LC-CD and LC-MS/MS analyses. Enantiomeric separations were achieved for (±)-corytenchine (pseudotype protoberberine) and (±)-tetrahydropalmatrubine using a Chiralcel OJ-RH column with 0.1 M NH_4OAc−MeCN (0.05% TFA) as eluent. The relative amounts were determined by the ratio of the peak areas in the MS and CD chromatograms. Both compounds showed a positive Cotton effect for the faster eluting peak and negative Cotton effect for the slower peak in the LC-CD spectra at 236 nm (Figure 3). The absolute configurations at C-13a for the enantiomers were previously established as *R* and *S* for the faster and slower peaks, respectively.[10]

The CD spectrum of the [8-^{13}C]-tetrahydropalmatrubine metabolite, **9**, showed a negative Cotton effect that matched that of the *S*-enantiomer. Thus, (*S*)-**4** was bioconverted via (*S*)-**9** to **8**. Metabolite **12** obtained from (±)-[8-^{13}C]-**5** and (±)-[8-^{13}C]-**6** was determined to be the *S*-enantiomer by LC-CD analysis. The metabolite of (±)-[8-^{13}C]-**6**, **15**, showed a negative Cotton effect in its CD spectra; thus, it is the *S*-enantiomer. Corytenchine (**5**) and **12**, metabolites of (±)-[8-D$_2$]-**7**, displayed negative Cotton effects in the LC/CD. Both **5** and **12** were *S*-enantiomers.

Metabolites **5** and **12** showed protonated molecular ions [M + H]$^+$ at *m*/*z* 344 and 358 ([8-D$_2$]-**5** and -**12**), respectively, as well as [M + H]$^+$ − 1 ions at *m*/*z* 343 and 357 ([8-D]-**5** and -**12**), respectively, in *M. cordata*. The ratios of 344:343 and 358:357 were ca. 3:1. The [M + H]$^+$ − 1 ion was also observed in metabolite **5** in *C. ochotensis*. The ratio of 344:343 was ca. 95:5. Pseudoprotoberberines [8-D]-**13** and [8-D]-**14** formed from [8-D$_2$]-**5** and -**12**, respectively, may be stereoselectively reduced to (*S*)-[8-D]-**5** and -**12**.

The results obtained from feeding experiments with [8-^{13}C]-tetrahydroprotoberberines [(±)-[8-^{13}C]-**4**−**6**] and (±)-[8-D$_2$]-**7** clarified the metabolic pathways of these compounds as shown in Scheme 1. The metabolic pathways for tetrahydropseudoprotoberberines (**5**−**7**) with one or two OH group on ring D were demonstrated for the first time, except for conversion of **12** into **14**.[8]

Metabolism of (*R*/*S*)-[8-^{13}C]-Tetrahydroprotoberberines ([8-^{13}C]-4 and -5) in *Macleaya cordata*. Because the metabolism of tetrahydroprotoberberines likely involves stereospecific bioconversions, the metabolism of (*R*/*S*)-[8-^{13}C]-tetrahydropalmatrubine and -corytenchine [(*R*/*S*)-[8-^{13}C]-**4** and -**5**)] was examined in *Macleaya cordata*. These precursors, (*R*/*S*)-[8-^{13}C]-**4** and -**5**, were resolved by preparative HPLC on a chiral column (OJ-RH).

Fraction E-1 (I, Figure 4) obtained from the feeding (Table 1, no. 2) of (*S*)-[8-^{13}C]-**4** to *M. cordata* showed three peaks, a_4−c_4, in the LC (I, Figure 4). In the LC-APCI-MS, peaks a_4−c_4 showed [M + H]$^+$ ions at *m*/*z* 371, 354, and 341, respectively, and product ions at *m*/*z* 204 and 190, 188, and 189 and 192, respectively. From LC-NMR and LC-MS data, peaks a_4, b_4, and c_4 were attributed to [8-^{13}C]-**8**, protopine (a component of *M. cordata*), and [8-^{13}C]-**9**, respectively (Scheme 2). Metabolite [8-^{13}C]-**9** showed a negative Cotton effect indicating the *S*-configuration at C-13a. Metabolites [8-^{13}C]-**8** and -**9** were not detected from the feeding (Table 1, no. 3) of (*R*)-[8-^{13}C]-**4**. Thus, (*S*)-**4** was likely to be converted to (*S*)-**9**, which was further converted to **8** as shown in Scheme 2.

(*S*)-[8-^{13}C]-Corytenchine [(*S*)-[8-^{13}C]-**5**] was also administered to cultured cells of *M. cordata* (Table 1, no. 6). The resulting fractions E-1 and C-1 showed peaks a_5−d_5 in the LC (II, Figure 4), with [M + H]$^+$ or [M]$^+$ ion at *m*/*z* 343, 353, 357, and 339, respectively, and product ions at *m*/*z* 204 and 192, 337, 192, and 323, respectively. The compounds associated with peaks a_5, b_5, c_5, and d_5 were identified as precursor ([8-^{13}C]-**5**), [8-^{13}C]-**14**, -**12**, and -**13** (Scheme 2), respectively, by analysis of ^{1}H NMR and LC-MS/MS data. Metabolite [8-^{13}C]-**12** displayed a negative Cotton effect that corresponds to the *S*-configuration at C-13a. Compound [8-^{13}C]-**13** was produced in the feeding (Table 1, no. 7) of (*R*)-[8-^{13}C]-**5**; however, [8-^{13}C]-**12** and -**14** were not detected.

Thus, (*S*)-**4** was transformed to (*S*)-**9**, and the *R*-isomer did not serve as a substrate. The enzyme responsible for formation of the methylenedioxy bridge was highly specific for the *S*-configurated

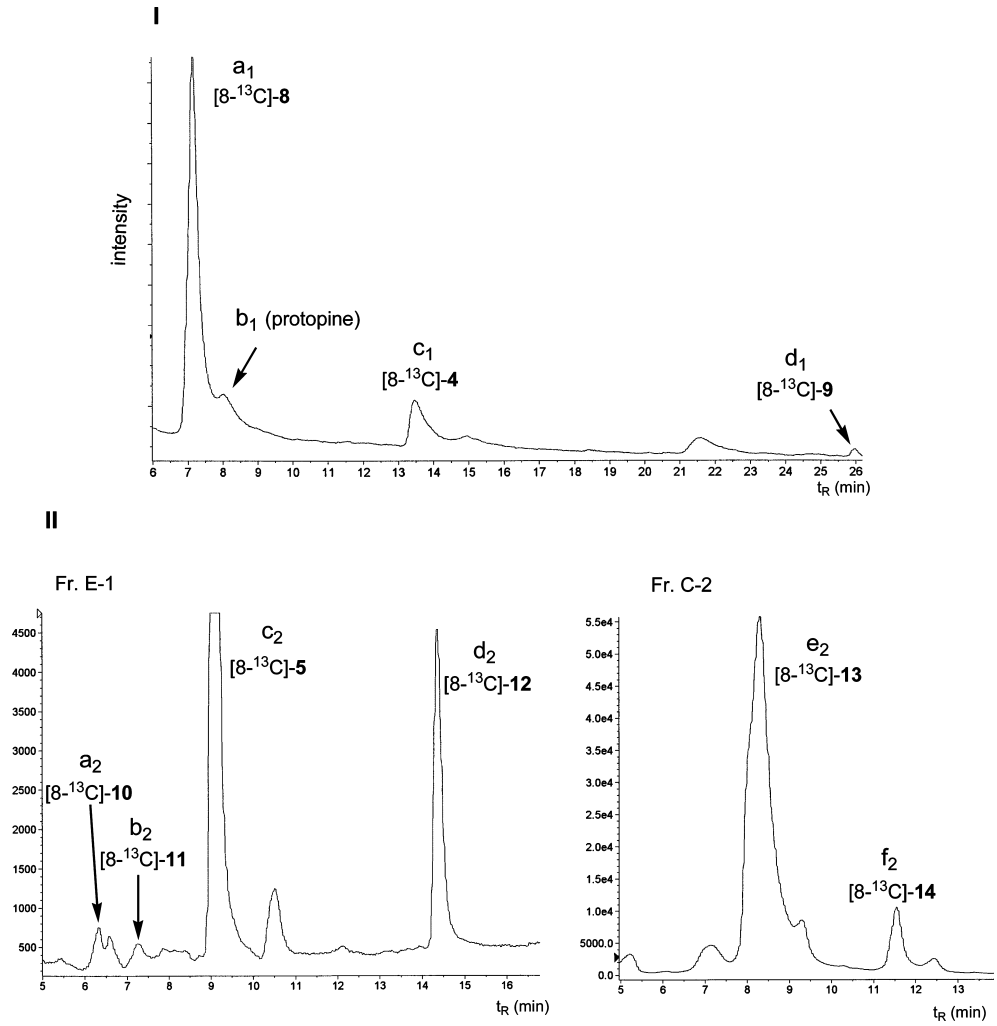


Figure 1. LC data of the alkaloid fractions obtained from the feedings of (±)-[8-^{13}C]-tetrahydropalmatrubine ([8-^{13}C]-**4**) (I) and (±)-[8-^{13}C]-corytenchine ([8-^{13}C]-**5**) (II) to *M. cordata*. Eluent: A, 0.1 M NH_4OAc (0.05% TFA); B, MeCN (0.05% TFA). Gradient A/B: initial 80:20, 25 min 55:45, 26 min 0:100. Flow rate: 1 mL/min. UV detection: 280 nm.

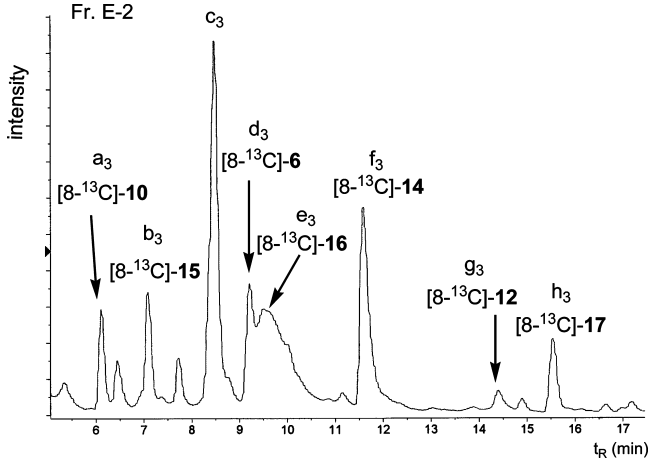


Figure 2. LC data of the alkaloid fraction (E-2) obtained from the feeding of (±)-[8-^{13}C]-10-demethylxylopinine ([8-^{13}C]-**6**) to *M. cordata*. Eluent: A, 0.1 M NH_4OAc (0.05% TFA); B, MeCN (0.05% TFA). Gradient A/B: initial 80:20, 25 min 55:45. Flow rate: 1 mL/min. UV detection: 280 nm.

isomer. (*S*)-Corytenchine was converted to (*S*)-**12** by a highly stereoselective *O*-methylation at C-11, while the *R*-isomer did not serve as a substrate. Both (*R*)- and (*S*)-**5** were converted to **13** by dehydrogenation of the C ring. This enzyme did not show any stereoselectivity. The pathway shown in Scheme 2 was demonstrated for the first time. (*S*)-Tetrahydroprotoberberine oxidase discovered in *Berberis wilsoniae* suspension cultures exhibits strict specificity for the *S*-enantiomer of tetrahydroprotoberberines.[19] (*S*)-Canadine was stereospecifically dehydrogenated to berberine by (*S*)-canadine oxidase in *Coptis japonica* cell cultures.[20] These results obtained with 2,3,9,10-oxygenated tetrahydroprotoberberines differ from those with 2,3,10,11-oxygenated tetrahydroprotoberberines in *M. cordata*.

Nandina domestica. Trace amounts of the *O*-methylated metabolite (tetrahydropalmatine) and its dehydro derivative (palmatine) were obtained from the feeding (Table 1, no. 12) of (±)-[8-^{13}C]-**4**. The enzyme responsible for the formation of the methylenedioxy bridge may be inactive in *N. domestica*, in contrast to *M. cordata* (Scheme 1).

The precursor [(±)-8-^{13}C]-corytenchine ([(±)-[8-^{13}C]-**5**]) was administered to cultured cells of *N. domestica* (Table 1, no. 13). The resulting fractions E-1 and C-1 showed peaks a_6−g_6 (Figure 5). The protonated molecular ions ($[M + H]^+$ or $[M]^+$ ion) and product ions are included in Figure 5. Peak a_6 displayed a $[M + H]^+$ion at *m*/*z* 329, 14 mass units less than the precursor, and a product ion at *m*/*z* 178. The ^{1}H NMR spectrum of peak a_6 (A, Supporting Information, Figure S5) showed OCH_3 groups at δ 3.78 and 3.80 and aromatic signals at δ 6.72 (1H, s), 6.74 (1H, s), 6.76 (1H, d, $J = 4.0$ Hz, ^{13}C−H long-range coupling), and 6.79 (1H, s). These data suggested that the compound represented by peak a_6

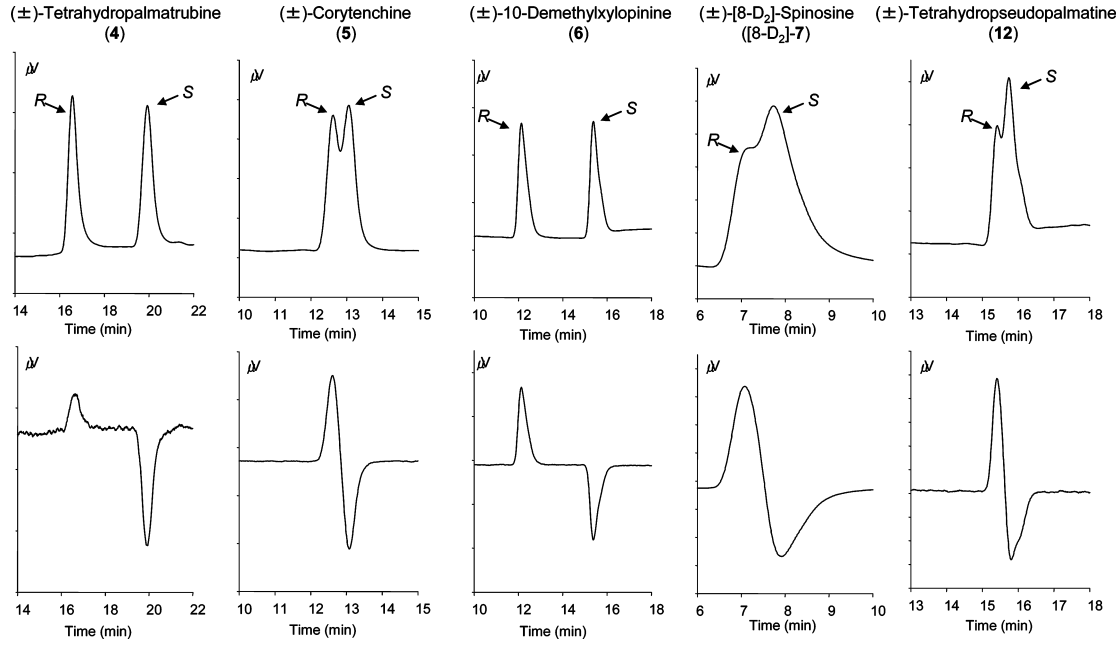


Figure 3. UV and CD chromatograms from the LC-CD of (±)-tetrahydropalmatrubine (**4**), (±)-corytenchine (**5**), (±)-10-demethylxylopinine (**6**), (±)-[8-D_2]-spinosine ([8-D_2]-**7**), and (±)-tetrahydropseudopalmatine (**12**). Column: Chiralcel OJ-RH (4.6 × 150 mm). Eluent: A, 0.1 M NH_4OAc (0.05% TFA); B, MeCN (0.05% TFA). Gradient A/B: initial 80:20, 10 min 60:40, 20 min 60:40, 30 min 0:100. Flow rate: 1 mL/min. UV detection: 236 nm.

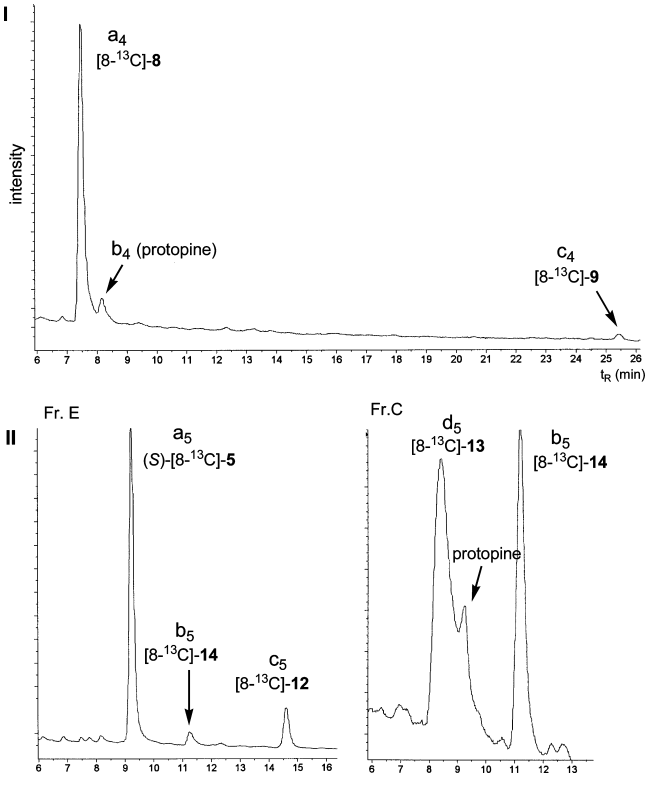


Figure 4. LC data of the alkaloid fractions obtained from the feedings of (*S*)-[8-^{13}C]-tetrahydropalmatrubine ([8-^{13}C]-**4**) (I) and (*S*)-[8-^{13}C]-corytenchine ([8-^{13}C]-**5**) (II) to *M. cordata*. Eluent: A, 0.1 M NH_4OAc (0.05% TFA); B. MeCN (0.05% TFA). Gradient A/B: initial 80:20, 25 min 55:45, 26 min 0:100. Flow rate: 1 mL/min. UV detection: 280 nm.

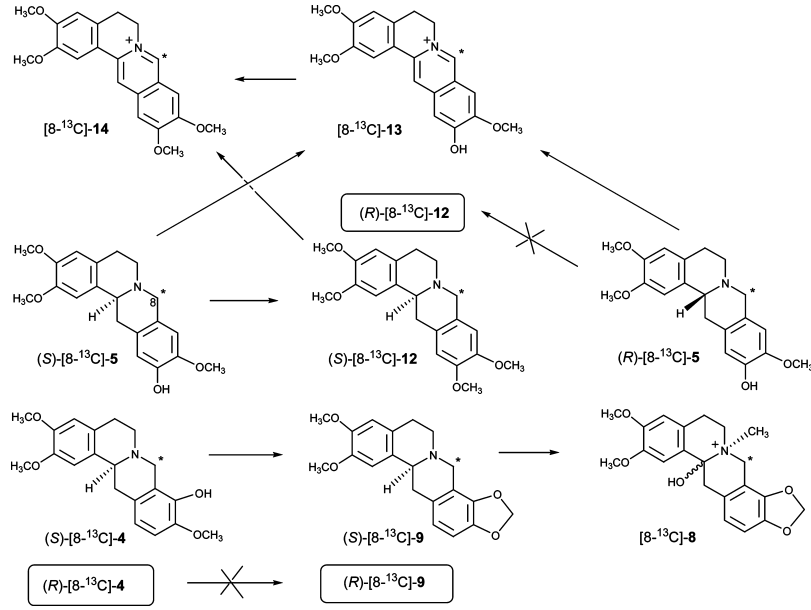
was [8-^{13}C]-isocoreximine or -coreximine ([8-^{13}C]-**18** or -**19**) (Scheme 1). The structure was confirmed to be [8-^{13}C]-**18** by comparison of the ^{1}H NMR data (A, Supporting Information, Figure S5) those (B and C, Supporting Information, Figure S5) of authentic unlabeled samples (**18** and **19**). The compound associated with peak b_6 was identified as [8-^{13}C]-**5** by analysis of ^{1}H NMR and LC-MS/MS data. Peak c_6 showed a $[M + H]^+$ ion at *m/z* 357, 14 mass units more than the precursor, and a product ion at *m/z* 192. The ^{1}H NMR spectrum of peak c_6 displayed four OCH_3 groups and four aromatic protons including a proton with $^{13}C-H$ long-range coupling. These data indicated that peak c_6 was [8-^{13}C]-**12**. This structure was confirmed by comparison of the ^{1}H NMR data with that of an authentic unlabeled sample. Metabolites, [8-^{13}C]-**18** and -**12** displayed negative CD bands (I, Supporting Information, Figure S6), corresponding to the *S*-configuration at C-13a. Peaks d_6 and e_6 (Figure 5) in the $CHCl_3$-soluble alkaloid fraction (fraction C-1) showed $[M + H]^+$ or $[M]^+$ and product ions at *m/z* 339 and 323 and at *m/z* 353 and 337, respectively, in the LC-MS/MS. These data indicated that the compounds represented by peaks d_6 and e_6 were [8-^{13}C]-**13** and -**14**, respectively. Peaks f_6 and g_6 were identified as berberine and palmatine, which are components of the callus[21] (Scheme 1).

Thus, as shown in Scheme 1, (*S*)-**5** was converted to (*S*)-**12** by *O*-methylation at C-11, which was then dehydrogenated to **14**. In addition, **5** was converted to **13** by the dehydrogenation of the C ring. This metabolism is the same as that in *Corydalis* and *Macleaya* species. However, in *N. domestica*, (*S*)-**5** was also demethylated at C-3 to give (*S*)-**18**, and this metabolism was not observed in *Corydalis* and *Macleaya* species.

In a similar fashion, the metabolism of (±)-[8-^{13}C]-10-demethylxylopinine ([8-^{13}C]-**6**) was examined in *N. domestica* (Table 1, no 14). Two metabolites showing $[M + H]^+$ ions at *m/z* 357 and 329 and product ions at *m/z* 192 and 178, respectively, were detected in the LC-MS/MS of the Et_2O-soluble alkaloid fraction (fraction E-1). The former was deduced to be [8-^{13}C]-**12** by comparison with an authentic sample using LC-MS. The latter was suggested to be the ^{13}C-labeled metabolite obtained from *O*-demethylation of ring A [329 (343−14), 178 (192−14)]. The amounts of both metabolites were less than those obtained in the feeding of (±)-[8-^{13}C]-**5**. Therefore, **5** is a better precursor than **6** for **12** in *N. domestica*. This result was identical to that of *M. cordata*.

In the next stage of our study, the metabolism of [(±)-8-^{13}C]-spinosine ([(±)-[8-^{13}C]-**7**]) was examined in *N. domestica* (Table

Scheme 2. Metabolism of (*S*)- and/or (*R*)-[8-^{13}C]-Tetrahydropalmatrubine and -Corytenchine (**4** and **5**) in *M. cordata*

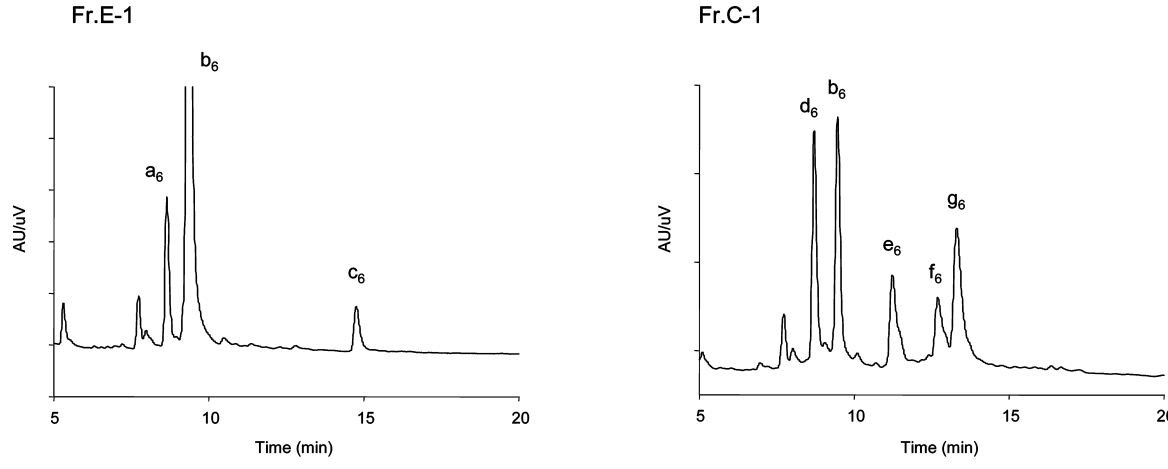


1, no. 15). By comparison of the LC-MS/MS with authentic samples, (*S*)-[8-^{13}C]-**12** ([M + H]$^+$ion at *m*/*z* 357 and product ion at *m*/*z* 192), (*S*)-[8-^{13}C]-**5**, and (*S*)-[8-^{13}C]-**6** ([M + H]$^+$ ion at *m*/*z* 343 and product ion at *m*/*z* 192) were identified as the metabolites.

As shown in Scheme 1, three (*S*)-tetrahydroprotoberberines (**5**, **6**, and **12**) were obtained from **7**. (*S*)-Spinosine (**7**) was *O*-methylated at C-10 and C-11 to give (*S*)-**5** and (*S*)-**6**, respectively. This result was different from that in *Corydalis* and *Macleaya* species, where the metabolite **5**, *O*-methylated at C-10, was obtained. Both (*S*)-**5** and -**6** could be further *O*-methylated to give (*S*)-**12**.

[(±)-8-D$_2$]-Spinosine ([(±)-[8-D$_2$]-**7**]) was administered to *N. domestica* (Table 1, no. 16) in order to examine whether a redox process shown in *M. cordata* was present in *N. domestica*. [8-D$_2$]-Tetrahydropseudopalmatine ([8-D$_2$]-**12**) ([M + H]$^+$ ion at *m*/*z* 358 and product ion at *m*/*z* 192), [8-D$_2$]-**5**, and [8-D$_2$]-**6** ([M + H]$^+$ion at *m*/*z* 344 and product ion at *m*/*z* 192) were identified as the metabolites. It was demonstrated that these alkaloids are *S*-configured at C-13a by LC-MS/MS and LC-CD analyses (II, Supporting Information, Figure S6). Protonated molecular ions at *m*/*z* 343 or 342 corresponding to [8-D]-**5** and -**6** or **5** and **6** and those at *m*/*z* 357 or 356 corresponding to [8-D]-**12** and **12** were not detected. Thus, a redox process was not present in *N. domestica* as opposed to *Corydalis* species.

The metabolic pathway was clarified by the results obtained from feeding experiments of (±)-[8-^{13}C]-**4**−**7** and (±)-[8-D$_2$]-**7** as shown in Scheme 1. Metabolism in *N. domestica* (Berberidaceae) differed from that in *M. cordata* (Papaveraceae) and *C. ochotensis* var. *raddeana* (Fumariaceae), which are closely related families in some pathways. Therefore it can be concluded that in cell cultures of *Macleaya*, *Corydalis*, and *Nandina* species *S*-configured 2,3,10,11-oxygenated tetrahydropseudoprotoberberine (e.g., **5**) was *O*-methylated stereospecifically to afford the *S*-isomer (e.g., **12**), which was *N*-methylated to the (*S*)-α-*N*-methyl salt (e.g., **15**). The (*S*)-α-*N*-methyl salt was oxidized to produce the pseudoprotopine-type alkaloid (e.g., **10**). These transformations were similar to those of



	[M+H]$^+$/Product ion
a_6: (*S*)-[8-^{13}C]-Isocoreximine (**18**)	*m*/*z* 329/178
b_6: [8-^{13}C]-Corytenchine (**5**)	*m*/*z* 343/192
c_6: (*S*)-[8-^{13}C]-Tetrahydropseudopalmatine (**12**)	*m*/*z* 357/192

	[M]$^+$/Product ion
d_6: [8-^{13}C]-Dehydrocorytenchine (**13**)	*m*/*z* 339/323
b_6: [8-^{13}C]-Corytenchine (**5**)	*m*/*z* 343/192
e_6: [8-^{13}C]-Pseudopalmatine (**14**)	*m*/*z* 353/337
f_6: Palmatine	*m*/*z* 352/336
g_6: Berberine	*m*/*z* 336/320

Figure 5. LC and LC-MS/MS data of the alkaloid fractions (E-1 and C-1) obtained from the feeding of (±)-[8-^{13}C]-corytenchine ([8-^{13}C]-**5**) to *Nandina domestica*.

2,3,9,10-oxygenated protoberberines. The tetrahydropseudoprotoberberines (**5**, **6**, and **12**) were dehydrogenated to the pseudoprotoberberines (**13**, **16**, and **14**), respectively. Both the *R*- and *S*-enantiomers of 2,3,10,11-oxygenated protoberberine (**5**) were dehydrogenated in *M. cordata,* which differed from 2,3,9,10-oxygenated protoberberines. The tetrahydropseudoprotoberberine **7**, with OH groups at C-10 and C-11, was *O*-methylated at C-10 in *M. cordata* and *C. ochotensis*, as distinct from *O*-methylation in *N. domestica*, in which **7** was *O*-methylated at C-11 as well as C-10. Stereoselective *O*-demethylation was also observed in *N. domestica*. *O*-Demethylation [from (*S*)-**5** to (*S*)-**18**] occurred in the metabolic pathway after formation of the tetrahydroprotoberberines in *N. domestica*, which differed from that in *M. cordata* and *C. ochotensis*. In conclusion, a metabolic pathway of 2,3,10,11-oxygenated tetrahydroprotoberberines having one or two OH groups on ring D was demonstrated for the first time by application of LC-MS, LC-NMR, and LC-CD techniques.

Experimental Section

General Experimental Procedures. Conventional ^{1}H NMR and NOESY spectra were obtained on a Varian VXR-500S spectrometer (^{1}H: 499.99 MHz) in CD_3OD. Mass spectra were determined on a Hitachi M-4100 instrument at 75 eV. Secondary ion mass spectra (SIMS) were measured using glycerol as matrix. HPLC and PHPLC analyses were performed using a Hitachi M-6200 intelligent pump (1 mL/ min) or Hitachi M-6250 intelligent pump (6 mL/min), respectively, with a Hitachi L-4000 UV detector (280 nm). Cosmosil 5C_{18}-AR reversed-phase columns (4.6 i.d. × 150 mm and 20 i.d. × 250 mm) were used for HPLC and PHPLC, respectively. Analyses with a Hitachi HPLC system were made using a solvent system, (A) 0.1 M NH_4OAc (0.05% TFA)/(B) MeOH (0.05% TFA) under the following gradient conditions: A/B, initial (80:20), 25 min (55:45), 26 min (0:100) for LC with a Hitachi HPLC system or initial (80:20), 10 min (60:40), 20 min (60:40), 30 min (0:100) for LC-MS and LC-CD or initial (80:20), 6 min (0:100) for PHPLC (flow rate: 60 mL/min, detection: 280 nm). PHPLC purifications were performed using a solvent system, (A) H_2O (0.05% TFA)/(B) MeOH (0.05% TFA) under the following gradient conditions: A/B 50:50 to 0:100, 30 min. The chiral analytical separation was carried out on a chiral OJ-RH column (4.6 i.d. × 150 mm, Daicel Chemical Ltd.) at rt for LC/MS and 40 °C for LC-CD. The flow rate was 0.5 mL/min (detection: LC-MS 280 nm, LC-CD 236 nm).

Materials. The calli of *M. cordata*, *C. ochotensis* var. *raddeana*, and *N. domestica* were derived in 1974, 1981, and 2003 from stems of wild plants grown in Kobe (Japan) on Murashige and Skoog's medium containing 2,4-dichlorophenoxyacetic acid (1 mg/L), kinetin (0.1 mg/ L), yeast extract (0.1%), and agar (1%). The callus tissues were subcultured every 4 or 5 weeks on the same fresh medium at 25 °C in the dark. 3,4-Dimethoxyphenylethylamine, 3-hydroxy-4-methoxyphenylacetic acid, homovanillic acid, 3,4-dihydroxyphenylacetic acid, DCDO (99 atom % D), CD_3I (99.5 atom % D), and H^{13}CHO (99 atom % ^{13}C) were obtained from commercial suppliers. (*S*)-Coreximine and (*S*)-isocoreximine were obtained from *Monathotaxis fornicata* (Annonaceae).

LC-APCI-MS, LC-NMR, and LC-CD Methods. LC-APCI-MS (/MS), LC-NMR, and LC-CD were measured using procedures described previously.[11]

Preparation of (±)-[8-^{13}C]-Corytenchine [(±)-[8-^{13}C]-5] and (±)-[8-^{13}C]-Tetrahydropalmatrubine [(±)-[8-^{13}C]-4]. A mixture of 3,4-dimethoxyphenethylamine (1.0 g) and 3-hydroxy-4-methoxyphenylacetic acid (1.0 g) was heated in an oil-bath at 180−190 °C for 1 h. Crystallization of the product from $CHCl_3$−C_6H_6 afforded the amide. To a solution of the phenolic amide and triethylamine (1 mL) in anhydrous $CHCl_3$ (50 mL) was added dropwise ethyl chloroformate (2 mL) in C_6H_6 (10 mL) with stirring at 5−10 °C. Stirring was continued for 30 min at rt, solvent was removed, and the residue was dissolved in $CHCl_3$. The $CHCl_3$ layer was washed with $NaHCO_3$ solution, dried over Na_2SO_4, and evaporated to give the *O*-protected amide. A mixture of the amide, $POCl_3$ (2 mL), and dry benzene (20 mL) was refluxed for 2.5 h. The solvent was removed, the residue was washed with Et_2O and dissolved in MeOH (70 mL), and $NaBH_4$ (700 mg) was then added in small portions with stirring at rt. The mixture was refluxed for 4.5 h, and H_2O (30 mL) was added and concentrated. The mixture was extracted with $CHCl_3$, the $CHCl_3$ extract dried, and the residue dissolved in Me_2CO (30 mL)−concd HCl (0.5 mL). The solvent was evaporated and the residue was crystallized from EtOAc−Me_2CO (2:1) to give the hydrochloride of **1**[10] (550 mg, yield 27%, mp 213−214 °C). This product (250 mg) in H_2O (25 mL) was adjusted to pH 5 with dilute HCl, and aqueous 37% H^{13}CHO (100 mg) was added. After standing overnight at rt, the resulting crystals were filtered to give (±)-[8-^{13}C]-**5** (62 mg, mp 171 °C).

Crystals of (±)-[8-^{13}C]-**5**/(±)-[8-^{13}C]-**4**, 1:1 (71 mg), which precipitated in the filtrate were collected. The mother liquid [(±)-[8-^{13}C]-**5**/(±)-[8-^{13}C]-**4**], 3:1, was separated by PHPLC. The eluents were purified by HPLC [H_2O (0.05% TFA)/MeOH (0.05% TFA)] to give (±)-[8-^{13}C]-**5** (40 mg) and (±)-[8-^{13}C]-**4** (15 mg) as trifluoroacetates. (±)-[8-^{13}C]-**5**: ^{1}H NMR data were identical with those of the unlabeled compound[10] except for H-8 (δ 4.45, d, J = 143.5 Hz, ^{13}C−H coupling) and H-9 (δ 6.78, d, J = 5.5 Hz, ^{13}C−H long-range coupling). SIMS m/z 343 $[M + H]^+$ (100), 192 (53), HR-SIMS m/z 343.1749 (calcd for $C_{19}{}^{13}CH_{24}NO_4$: 343.1738). (±)-[8-^{13}C]-**4**: ^{1}H NMR data were identical to those of the unlabeled compound,[10] except for H-8 (δ 4.46, d, J = 143 Hz, ^{13}C−H coupling). SIMS m/z 343 $[M + H]^+$ (100), 192 (27), HR-SIMS m/z 343.1753 (calcd for $C_{19}{}^{13}CH_{24}NO_4$: 343.1738).

Preparation of (±)-[8-^{13}C]-10-Demethylxylopinine [(±)-[8-^{13}C]-6]. A mixture of 3,4-dimethoxyphenethylamine (1.06 g) and homovanillic acid (1.04 g) was heated in an oil-bath at 180−190 °C for 30 min. The hydrochloride of **2** was prepared as described above (for **1**) from the *O*-protected 1-benzyltetrahydroisoquinoline (818 mg). A solution of its hydrochloride (110 mg) in H_2O (11 mL) was adjusted to pH 3 with dilute HCl, and aqueous 37% H^{13}CHO (20 mg) was added. After standing overnight at rt, the resulting white powder was filtered to give a mixture of (±)-[8-^{13}C]-**6** and **2** (85:15), which was separated and purified by PHPLC to give (±)-[8-^{13}C]-**6** (82 mg) and **2** (16.7 mg) as trifluoroacetates. [(±)-[8-^{13}C]-**6**: ^{1}H NMR (500 MHz, CD_3OD) δ 6.95 (1H, s, H-1), 6.87 (1H, s, H-12), 6.83 (1H, s, H-4), 6.66 (1H, d, J = 5.0 Hz, H-9, ^{13}C−H long-range coupling), 4.64 (1H, brd, J = 10 Hz), 4.42 (2H, d, J = 143 Hz, H-8, ^{13}C−H coupling), 3.87 (6H, s, 2- and 11-OMe), 3.84 (3H, s, 3-OMe), 3.77 and 3.03 (each 1H, m, H-13), 3.74 and 3.42 (each 1H, m, H-6), 3.24 and 3.04 (each 1H, m, H-5); ^{13}C NMR (125 MHz, CD_3OD) δ 26.90 (C-5), 34.40 (C-13), 51.44 (C-6, br), 56.48, 56.51, 56.78 (C-2, 3-, and 11-OMe], 56.63 (^{13}C-8), 61.48 (C-13a, br), 110.07 (C-1), 112.57 (C-12, d, J = 3.25 Hz), 112.85 (C-4), 113.45 (C-9, d, J = 6.0 Hz), 121.70 (C-12a), 123.47 (C-8a), 124.89 (C-4a), 125.85 (C-1a), 147.29 (C-10, d J = 5.63 Hz), 149.47 (C-11), 150.15 (C-2), 150.65 (C-3); SIMS m/z 343 $[M + H]^+$ (100), 192 (45); HR-SIMS m/z 343.1751 (calcd for $C_{19}{}^{13}CH_{24}NO_4$, 343.1738).

Preparation of (±)-[8-^{13}C]-Spinosine [(±)-[8-^{13}C]-7]. A mixture of 3,4-dimethoxyphenethylamine (1.4 g) and 3,4-dihydroxyphenylacetic acid (1.45 g) was heated in an oil-bath at 180−190 °C for 30 min. The product was dissolved in anhydrous EtOH (30 mL), and anhydrous K_2CO_3 (4.0 g) and benzyl chloride (4.0 g) were added. The mixture was refluxed for 2.5 h and filtered using Celite 545. After cooling, the resulting crystals were collected to give the di-*O*-benzyl derivative (2.49 g, yield 63%, mp 125−126 °C). This product (3.88 g) was dissolved in anhydrous $C_6H_5CH_3$ (100 mL), and C_6H_6 (50 mL) and $POCl_3$ (8 mL) were added. The mixture was refluxed for 30 min, solvent removed, and the residue washed with Et_2O to give the 3,4-dihydroisoquinoline, which was dissolved in Me_2CO−MeOH and dilute HCl (0.5 mL). Solvent was evaporated, and the residue was crystallized from Me_2CO to give the hydrochloride (2.5 g). The hydrochloride (12.4 g) was dissolved in MeOH (70 mL), and $NaBH_4$ (2 g) was added in small portions with stirring at rt. The mixture was then refluxed for 25 min, and H_2O (100 mL) was added and concentrated. The mixture was extracted with $CHCl_3$; then the extract was dried and evaporated. The residue was dissolved in EtOH, and concentrated HCl (10 mL) was added and refluxed for 2 h. Solvent was removed and the residue crystallized from Me_2CO to give the hydrochloride of **3** (1.1 g). A solution of this hydrochloride (250 mg) in H_2O (30 mL) was adjusted to pH 3 with dilute HCl, and aqueous 37% H^{13}CHO (100 mg) was added. After standing overnight at rt, the resulting white powder was filtered to give (±)-[8-^{13}C]-**7** (128 mg). (±)-[8-^{13}C]-**7**: ^{1}H NMR (500 MHz, CD_3OD) δ 6.94 (1H, s, H-1), 6.82 (1H, s, H-4), 6.72 (1H, s, H-12), 6.62 (1H, d, J = 5.0 Hz, H-9, ^{13}C−H long-range coupling), 4.64 (1H, d, J = 10 Hz), 4.40 (2H, d, J = 143 Hz, H-8, ^{13}C−H coupling), 3.86 (3H, s, 2-OMe), 3.84 (3H, s, 3-OMe), 3.74 and 3.43 (each 1H, m, H-6), 3.69 and 2.98 (each 1H, m, H-13), 3.24 and 3.04 (each 1H, m, H-5); ^{13}C NMR (125 MHz, CD_3OD) δ 26.86 (C-5), 34.20 (C-13), 51.40 (C-6, br), 56.51 (C-2 and 3-OMe], 56.76 (^{13}C-8), 61.53

(C-13a, br), 110.05 (C-1), 112.82 (C-4), 113.49 (C-9, d, $J = 5.0$ Hz), 116.10 (C-12, d, $J = 2.75$ Hz), 119.70 (C-12a), 123.47 (C-8a), 124.82 (C-4a), 125.84 (C-1a), 146.24 (C-10, d $J = 5.63$ Hz), 147.02 (C-11), 150.14 (C-2), 150.62 (C-3); SIMS *m/z* 329 $[M + H]^+$ (100), 192 (15); HR-SIMS *m/z* 329.1598 (calcd for $C_{18}{}^{13}CH_{22}NO_4$, 329.1582).

Preparation of (±)-[8-D_2]-Spinosine [(±)-[8-D_2]-7]. A solution of the hydrochloride of **3** (200 mg) in D_2O (20 mL) was adjusted to pH 3 with dilute 20% DCl (11 μL), and aqueous 37% DCDO (100 mg) was added. After standing overnight at rt, the resulting white powder was filtered to give (±)-[8-D_2]-**7** (133 mg, mp 212−222 °C). (±)-[8-D_2]-**7**: ^{1}H NMR data were identical to those of the unlabeled compound, except for disappearance of H-8. SIMS *m/z* 330 $[M + H]^+$ (100), 192 (45); HR-SIMS *m/z* 330.1668 (calcd for $C_{19}H_{20}D_2NO_4$, 330.1673).

Resolutions of (*R*)-and (*S*)-[8-^{13}C]-Corytenchine [(*R*)- and (*S*)-[8-^{13}C]-5] and (*R*)- and (*S*)-[8-^{13}C]-Tetrahydropalmatrubine [(*R*)- and (*S*)-[8-^{13}C]-4]. Chiral separation of (±)-[8-^{13}C]-**5** (50 mg) was performed using a chiral OJ-RH reversed-phase column (6.0 i.d. × 150 mm), a Jasco 880-PU intelligent pump, and a Jasco 875-UV detector. Solvents (A) 0.1 M NH_4OAc (0.05% TFA) and (B) MeCN (0.05% TFA) were used for nonlinear gradient: initial 20% of B, 10 min 40% of B, and 20 min 40% of B; 6 mL/min (detection: 280 nm). Eluents obtained from the peaks corresponding to (*R*)-**5** ($t_R = 14.5$ min) and (*S*)-**5** ($t_R = 15.1$ min) were evaporated, and the residues were further purified by HPLC [H_2O (0.05% TFA)−MeOH (0.05% TFA)] to give (*R*)-**5** (5.5 mg) and (*S*)-**5** (2.0 mg) as trifluoroacetates.

Chiral separation of (±)-[8-^{13}C]-**4** (30 mg) was undertaken in the same manner. Eluents obtained from the peaks corresponding to (*R*)-**4** ($t_R = 19.3$ min) and (*S*)-**4** ($t_R = 22.5$ min) were evaporated and the residue further purified by HPLC [H_2O (0.05% TFA)−MeOH (0.05%TFA)] to give (*R*)-**4** (7.9 mg) and (*S*)-**4** (12 mg) as trifluoroacetates. Optical purity of each enantiomer was demonstrated by LC-CD.[10]

Feeding Experiments. Substrates were dissolved in H_2O (2−4 mL) and introduced through a sterile bacterial filter into 100 mL conical flasks to which 40 mL of autoclaved MS medium (as that employed in the subculture) was added. Calli (ca. 4−5 g) were transferred to each conical flask and incubated at 25 °C (*C. ochotensis* and *M. cordata*) and 27 °C (*N. domestica*) in the dark for four weeks. After incubation, calli and medium were freeze-dried. Water was separated, and callus and medium were extracted with hot MeOH. The H_2O and MeOH extracts were concentrated and, after acidification, were washed with Et_2O, made basic with NH_4OH, and extracted with Et_2O and then $CHCl_3$ to give fractions E-1 and C-1 (callus) and fractions E-2 and C-2 (medium), which were subjected to LC-NMR, LC-MS, and LC-CD measurements.

Supporting Information Available: Scheme 1S and Figures S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Iwasa, K. In *The Alkaloids*; Cordell G. A., Ed.; Academic Press: New York, 1995; Vol *46*, pp 273−346.
(2) Iwasa, K.; Nanba, H.; Lee, D.-U.; Kang, S.-I. *Planta Med.* **1997**, *64*, 748–751.
(3) Iwasa, K.; Nishiyama, Y.; Ichimaru, M.; Moriyasu, M.; Kim, H.-S.; Wataya, Y.; Yamori, T.; Turuo, T.; Lee, D.-U. *Eur. J. Med. Chem.* **1999**, *34*, 1077–1083.
(4) Iwasa, K.; Moriyasu, M.; Yamori, T.; Turuo, T.; Lee, D.-U.; Wiegrebe, W. *J. Nat. Prod.* **2001**, *64*, 896–898.
(5) Iwasa, K.; Moriyasu, M.; Tachibana, Y.; Kim, H.-S.; Wataya, Y.; Wiegrebe, W.; Bastow, K. F.; Cosentino, L. M.; Kozuka, M.; Lee, K-H. *Bioorg. Med. Chem.* **2001**, *9*, 2871–2884.
(6) Preininger, V. In *The Alkaloids*; Brossi, A., Ed.; Academic Press: New York, 1986; Vol *29*, pp 1−98.
(7) Zenk, M. H. *Pure Appl. Chem.* **1994**, *66*, 2023–2028.
(8) Iwasa, K.; Kuribayashi, A.; Sugiura, M.; Moriyasu, M.; Lee, D.-U.; Wiegrebe, W. *Phytochemisty* **2003**, *64*, 1229–1238.
(9) Ohiri, F. C.; Verpoorte, R.; Svendsen, A. B. *Planta Med.* **1983**, *49*, 162–164.
(10) Iwasa, K.; Cui, W. H.; Sugiura, M.; Takeuchi, A.; Moriyasu, M.; Takeda, K. *J. Nat. Prod.* **2005**, *68*, 992–1000.
(11) Cui, W. H.; Iwasa, K.; Sugiura, M.; Takeuchi, A.; Tode, C.; Nishiyama, Y.; Moriyasu, M.; Tokuda, H.; Takeda, K. *J. Nat. Prod.* **2007**, *70*, 1771–1778.
(12) Cui, W. H. Doctoral Thesis, Kobe Pharmaceutical University, 2006.
(13) Patra, A.; Montgomery, C. T.; Freyer, A. J.; Guinaudeau, H.; Shamma, M.; Tantisewie, B.; Pharadai, K. *Phytochemistry* **1987**, *26*, 547–549.
(14) Lu, S.-T.; Su, T.-L.; Kametani, T.; Ujiie, A.; Ihara, M.; Fukumoto, K. *J. Chem. Soc., Perkin Trans. 1* **1976**, 63–68.
(15) Roblot, F.; Hocquemiller, R.; Jacquemin, H.; Cave, A. *Plantes Med. Phytother.* **1978**, *12*, 259–266.
(16) Queiroz, E. F.; Roblot, F.; Cave, A.; Paulo, M. Q.; Fournet, A. *J. Nat. Prod.* **1996**, *59*, 438–440.
(17) Brossi, A.; Teitel, S. *J. Org. Chem.* **1970**, *35*, 1684–1687.
(18) Ishiwata, S.; Itakura, K. *Chem. Pharm. Bull.* **1970**, *18*, 896–900.
(19) Amann, M.; Nagakura, N.; Zenk, M. H. *Eur. J. Biochem.* **1988**, *175*, 17–25.
(20) Galneder, E.; Rueffer, M.; Wanner, G.; Tabata, M.; Zenk, M. H. *Plant Cell Rep.* **1988**, *7*, 1–4.
(21) Iwasa, K.; Takahashi, T.; Nishiyama, Y.; Moriyasu, M.; Sugiura, M.; Takeuchi, A.; Tode, C.; Tokuda, H.; Takeda, K. *J. Nat. Prod.* **2008**, *71*, 1376–1385.

NP900440D