Biotransformation of Phenolic 1-Benzyl-*N*-methyltetrahydroisoquinolines in Plant Cell Cultures Followed by LC/NMR, LC/MS, and LC/CD

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(±)-1-Benzyl-*N*-methyltetrahydroisoquinolines **7**–**10** and **11**–**14** with one and two hydroxy groups on the aromatic rings, respectively, were fed individually to cultured cells of *Corydalis* and *Macleaya* species, respectively. The structures of the metabolites were determined by using combinatorial techniques, including LC/NMR, LC/MS-MS, and LC/CD. The enantiomeric excesses of the metabolites were derived from LC/CD and LC/MS-MS analyses. In cell cultures of *Corydalis* and *Macleaya* species, laudanine (**7**), with a hydroxy group at C-3′, can form the berberine bridge at C-2′ and C-6′ to produce *S*- and *R*-enantiomers of 2,3,9,10- and 2,3,10,11-oxygenated protoberberines (**20** and **21**), respectively, whereas reticuline (**11**) and protosinomenine (**12**), incoporating a hydroxy group at C-3′, form the berberine bridge at C-2′ to furnish the *S*-enantiomer of 2,3,9,10-oxygenated protoberberines (**23** and **21**), respectively.

Within the biogenetic focus for the isoquinoline alkaloids, it has been well documented that tetrahydrobenzylisoquinolines will lead to protoberberines, which may undergo further transformations to furnish an array of additional isoquinoline alkaloids.[1–9]

Studies on the biosynthesis of 2,3,10,11-oxygenated protoberberines have been reported,[10,11] including *Corydalis* species[12,13] and others.

Our present focus was on establishing some of the structural parameters for the eight 1-benzyl-*N*-methyltetrahydroisoquinolines **7**–**10** and **11**–**14**, which possess one and two hydroxy groups, respectively. To this end, we concentrated on the analysis of the metabolites from the cell cultures of *Corydalis* (Fumariaceae) and *Macleaya* (Papaveraceae) species.

Our conclusions show that the norlaudanosine analogue **7**, possessing a 3′-hydroxy group, can serve as a precursor of 2,3,9,10- and 2,3,10,11-oxygenated protoberberines, whereas the remaining three isomers, **8**–**10**, were not metabolized to protoberberines. Among species **11**–**14**, with two hydroxy groups, **11** and **12**, with a 3′-hydroxy function, can form the berberine bridge to produce 2,3,9,10-oxygenated protoberberines.

By enzyme-catalyzed reaction, the structural requirements for the formation of the berberine bridge to produce 2,3,9,10- and 1,2,9,10-oxygenated protoberberines from the tetrahydrobenzylisoquinoline alkaloids are as follows:[14] the *N*-methyltetrahydrobenzylisoquinoline nucleus must have the *S*-configuration at C-1, and the aromatic carbon ortho to C-2′ must carry a hydroxy group.

Results and Discussion

Syntheses. *N*-Methyltetrahydropapaverine (**6**) and [*N*-CD_3]-**6** were obtained by reduction of *N*-methylpapaverinium salts **5** and [*N*-CD_3]-**5**, which were prepared by the *N*-methylation of commercially available papaverine with CH_3I and CD_3I, respectively. The LC/APCI-MS (Figure 1) of the products obtained from the acid-catalyzed ether cleavage of **6** was measured in the positive ion mode and showed the presence of several products bearing one or more phenolic hydroxy groups as well as the presence of starting material. The 1-benzyl-*N*-methyltetrahydroisoquinolines show two product ions representing the isoquinoline (ion A) and benzyl (ion B) moieties as shown in Scheme 1. The structures of the compounds obtained from peaks a−g and i (Figure 1) were determined by ^{1}H NMR (Table 1) and MS analyses (Table 2 and Scheme 1) to be **7**–**13** and **14**, respectively. The structures of the deuterated derivatives [*N*-CD_3]-**7** to [*N*-CD_3]-**14** were determined from ^{1}H NMR and MS data (Table 2, Scheme 1) and were identical to those of the unlabeled compounds. 1-Benzyl-*N*-methyltetrahydroisoquinolines **7**–**14** and [*N*-CD_3]-**7**–**14** were used in the feeding experiments.

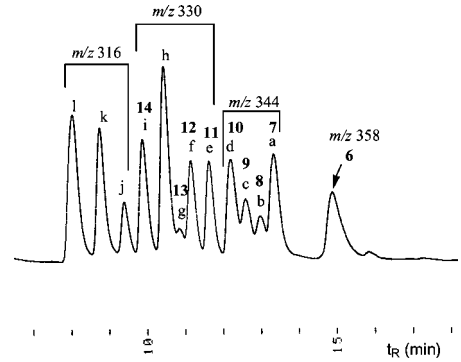

Figure 1. LC data of the crystalline products obtained by refluxing *N*-methyltetrahydropapaverine (**6**) with 47% HBr for 13 min. Pump: Hitachi L-6200; column: Cosmosil 5 C18ARII (4.6 i.d. × 150 mm); gradient: 0.1 M NH_4OAc (0.05% TFA)/CH_3OH (0.05% TFA), initial 80/20, 30 min 0/100; flow rate: 1 mL/min; UV detection: 280 nm (preparative HPLC: initial 80/20, 80 min 0/100).

Feeding Experiments. Callus tissues of *Macleaya cordata*, *Corydalis platycarpa* Makino, and *C. ochotensis* var. *raddeana* were incubated at 25 °C on an agar medium containing the substrate for four weeks (Table 3). Following incubation, media and cells were separated and extracted according to the procedure shown in Figure 2. Alkaloid fractions, E-1, E-2, C-1, and C-2 (Figure 2), were subjected to LC/NMR, LC/MS, and LC/CD.

Precursors 7–10. The metabolism of 1-benzyl-*N*-methyltetrahydroisoquinolines **7**–**10**, possessing one hydroxy and three methoxy groups on the aromatic A and D rings, respectively, was examined (Table 3) in cultured cells of *C. platycarpa* or *C. ochotensis* var. *raddeana* and *M. cordata*.

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Scheme 1

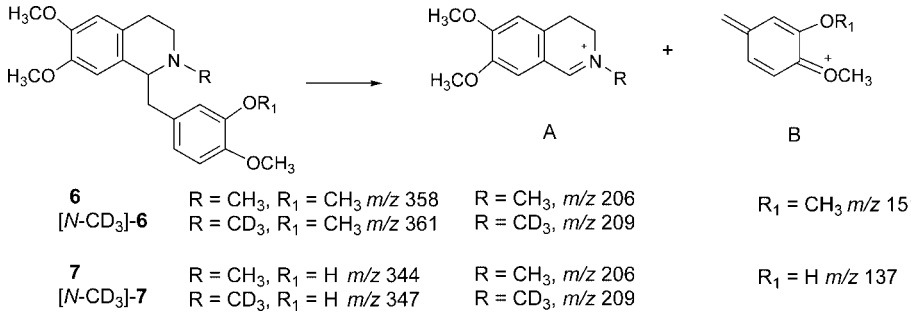


Table 1. ^{1}H NMR Data[a] of 1-Benzyl-*N*-methyltetrahydroisoquinolines (**7**−**14**)

	compound								
proton	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	NOESYcorrelation
1-H	4.52[b]	4.50[e]	3.67[g]	4.56[h]	4.53[i]	4.50[h]	4.51[i]	4.54[h]	2-NMe, 8-H 2′-H, 6′-H
2-NMe	2.96	2.92	2.53	2.98	2.92	2.95	2.93	2.95	1-H
3-H, 4-H and 9-H	3.0−3.77	3.01−3.65	2.56−3.17	3.05−3.78	3.02−3.70	2.98−3.75	2.95−3.71	2.99−3.71	
5-H	6.81	6.79	6.62	6.82	6.79	6.66	6.65	6.67	6-OMe
6-OMe	3.81	3.85		3.81	3.85				5-H
7-OMe	3.50		3.57	3.50		3.52		3.53	8-H
8-H	6.03	6.18	5.99	6.03	6.24	5.99	6.16	6.00	1-H, 7-OMe
2′-H	6.64[c]	6.69[c]	6.58[c]	6.66[c]	6.68[c]	6.64[c]	6.68[c]	6.64[c]	3′-OMe, 1-H
3′-OMe		3.75	3.79	3.76			3.75	3.76	2′-H
4′-OMe	3.84	3.82	3.85		3.85	3.84	3.82		5′-H
5′-H	6.89[d]	6.91[d]	6.77[d]	6.77[d]	6.89[d]	6.90[d]	6.91[d]	6.77[d]	4′-OMe, 6′-H
6′-H	6.60[f]	6.74[f]	6.65[f]	6.63[f]	6.62[f]	6.61[f]	6.75[f]	6.63[f]	5′-H, 1-H 9-H

[a] CD_3OD; δ ppm, 500 MHz. [b] m. [c] d, J = 2.0 Hz. [d] d, J = 8.0 Hz. [e] t, J = 6.0 Hz. [f] dd, J = 8.0, 2.0 Hz. [g] dd, J = 8.5, 5.0 Hz. [h] dd, J = 9.0, 5.0 Hz. [i] t, J = 6.5 Hz. [j] t, J = 7.0 Hz.

Table 2. MS Data of 1-Benzyl-*N*-methyltetrahydroisoquinolines (**7**–**14**) and [*N*-CD_3]-l-Benzyltetrahydroisoquinolines ([*N*-CD_3]-**7**−[*N*-CD_3]-**14**

			HRSIMS		product ions[a] *m/z*	
compound	SIMS *m/z*	formula	calcd	found	A	B
7	344	$C_{20}H_{26}NO_4$	344.1860	344.1862	206	137
[*N*-CD_3]-**7**	347	$C_{20}H_{23}D_3NO_4$	347.2049	347.2052	209	137
8	344	$C_{20}H_{26}NO_4$	344.1860	344.1873	192	151
[*N*-CD_3]-**8**	347	$C_{20}H_{23}D_3NO_4$	347.2049	347.2066	195	151
9	344	$C_{20}H_{26}NO_4$	344.1860	344.1863	192	151
[*N*-CD_3]-**9**	347	$C_{20}H_{23}D_3NO_4$	347.2049	347.2059	195	151
10	344	$C_{20}H_{26}NO_4$	344.1860	344.1875	206	137
[*N*-CD_3]-**10**	347	$C_{20}H_{23}D_3NO_4$	347.2049	347.2056	209	137
11	330	$C_{19}H_{24}NO_4$	330.1704	330.1702	192	137
[*N*-CD_3]-**11**	333	$C_{19}H_{21}D_3NO_4$	333.1892	333.1702	195	137
12	330	$C_{19}H_{24}NO_4$	330.1704	330.1707	192	137
[*N*-CD_3]-**12**	333	$C_{19}H_{21}D_3NO_4$	333.1892	333.1904	195	137
13	330	$C_{19}H_{24}NO_4$	330.1704	330.1714	178	151
[*N*-CD_3]-**13**	333	$C_{19}H_{21}D_3NO_4$	333.1892	333.1910	181	151
14	330	$C_{19}H_{24}NO_4$	330.1704	330.1726	192	137
[*N*-CD_3]-**14**	333	$C_{19}H_{21}D_3NO_4$	333.1892	333.1908	195	137

[a] These product ions were derived by APCI-MS/MS spectra.

First, precursor **7** was administered to cultured cells of *C. platycarpa* (Table 3, no. 1). Fraction E-2 (Figure 2) showed nine peaks, a_1−i_1, in LC 1 (Figure 3). From the LC/NMR and LC/MS-MS data, peaks a_1 ($[M + H]^+$: *m/z* 344, product ion: *m/z* 206), b_1 ($[M]^+$: *m/z* 322, product ion: *m/z* 307), d_1 ($[M + H]^+$: *m/z* 354, product ions: *m/z* 206, 189, and 188), and h_1 ($[M + H]^+$: *m/z* 326, product ion: *m/z* 178) were attributed to the precursor **7**, dehydrocheilanthifoline (**15**), protopine (**16**), and cheilanthifoline (**17**), respectively. Dehydrocheilanthifoline (**15**), protopine (**16**), and cheilanthifoline (**17**) were identified by comparison of their LC/NMR data with those of authentic samples from *C. platycarpa* and *C. ochotensis* var. *raddeana,* and **16** was also identified as a component of *M. cordata*.

Similarly, peak c_1 (Figure 3) corresponded to cryptopine (**18**), peak e_1 (t_R = 9.54 min) to epiberberine (**19**), peak f_1 (t_R = 9.82 min) to corytenchine (**20**), and peak g_1 to tetrahydropalmatrubine (**21**). Compounds **18**–**21** have also been identified in the feeding experiment of **1**.[11] Likewise, the stopped-flow ^{1}H NMR spectrum of peak i_1 is due to tetrahydroepiberberine **22**. Laudanine (**7**) and epiberberine (**19**) were also found in fraction C-2 (Figure 3).

Furthermore, the metabolites obtained from the feeding of **7** to *C. ochotensis* var. *raddeana* (Table 3, no. 2) were found to be cryptopine (**18**), corytenchine (**20**), tetrahydropalmatrubine (**21**), and tetrahydroepiberberine (**22**). Thus, compound **7** was bioconverted into corytenchine (**20**) and tetrahydropalmatrubine (**21**), which was further converted via tetrahydroepiberberine (**22**) to cryptopine (**18**) (Scheme 2). Tetrahydroepiberberine (**22**) might simply arise from the reduction of epiberberine (**19**). These conversions were confirmed by the feeding of [*N*-CD_3]-**7** to *C. platycarpa* (Table 3, no. 13). The deuterated metabolites cryptopine (*m/z* 372), corytenchine (*m/z* 344), tetrahydropalmatrubine (*m/z* 344), and tetrahydroepiberberine (*m/z* 342), and unlabeled epiberberine (*m/z* 336), were

Table 3. Administration of 1-Benzyl-*N*-methyltetrahydroisoquinolines to Cultured Cells of *Macleaya* and *Corydalis* Species

no.	callus[a]	substrate (mg)	medium (mL)	wt of dry cells (g)
1	A	**7** (38)	800	4.0
2	B	**7** (19)	400	2.1
3	A	**8** (42)	800	5.2
4	B	**8** (38)	800	4.0
5	A	**9** (39)	800	4.8
6	B	**9** (39)	800	6.0
7	A	**10** (35)	800	6.3
8	B	**10** (35)	800	4.3
9	A	**11** (34)	800	4.7
10	A	**12** (20)	800	7.2
11	A	**13** (39)	400	3.3
12	A	**14** (34)	800	4.8
13	A	[*N*-CD_3]-**7** (29)	800	5.4
14	C	[*N*-CD_3]-**7** (26)	800	5.9
15	A	[*N*-CD_3]-**8** (18)	400	3.5
16	B	[*N*-CD_3]-**8** (18)	400	2.6
17	C	[*N*-CD_3]-**8** (18)	400	2.3
18	A	[*N*-CD_3]-**9** (17)	400	1.9
19	B	[*N*-CD_3]-**9** (16)	400	7.3
20	A	[*N*-CD_3]-**10** (17)	400	2.8
21	B	[*N*-CD_3]-**10** (19)	400	2.1
22	C	[*N*-CD_3]-**10** (18)	400	2.4
23	A	[*N*-CD_3]-**11** (19)	800	3.7
24	A	[*N*-CD_3]-**12** (19)	800	5.0
25	A	[*N*-CD_3]-**13** (6)	400	2.2
26	A	[*N*-CD_3]-**14** (35)	800	3.7

[a] A: *Corydalis platycarpa* Makino. B: *Corydalis ochotensis* var. *raddeana*. C: *Macleaya cordata*.

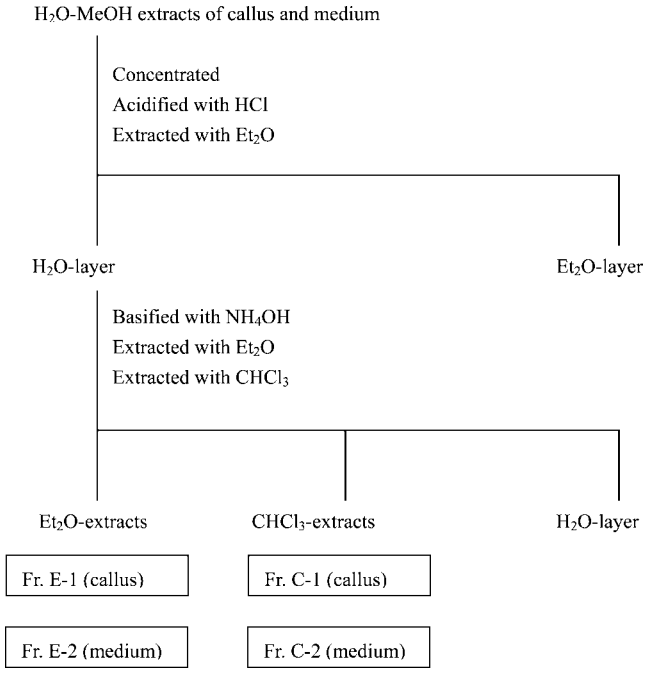
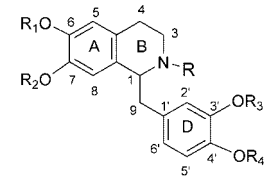


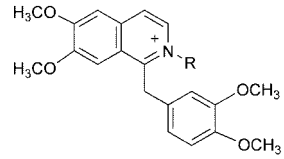
Figure 2. Preparation of samples for LC/NMR, LC/MS, and LC/CD measurements.

identified from the LC/MS-MS of fraction E-2. Deuterated tetrahydroepiberberine likely underwent dehydrogenation with the loss of deuterium to give unlabeled epiberberine. A trace amount of deuterated *N*-methyltetrahydropapaverine ([*N*-CD_3]-**6**) was also confirmed by LC/MS-MS ([M + H]$^+$ at *m*/*z* 361, product ions at *m*/*z* 209 and 151) (Scheme 1).

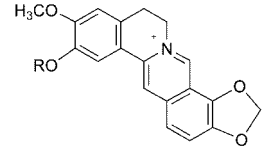
Finally, in cultured cells of *M. cordata* (Table 3, no 14), deuterated **7** was metabolized to deuterated cryptopine (*m*/*z* 372), corytenchine (*m*/*z* 344), tetrahydropalmatrubine (*m*/*z* 344), *N*-methyltetrahydropapaverine (*m*/*z* 361), and tetrahydroepiberberine (*m*/*z* 342).



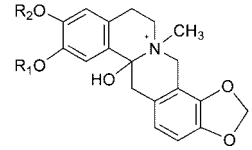
1 R = H R_1 = R_2 = R_4 = CH_3 R_3 = H
2 R = H R_1 = R_2 = R_3 = CH_3 R_4 = H
3 R = H R_1 = R_3 = R_4 = CH_3 R_2 = H
4 R = H R_1 = H R_2 = R_3 = R_4 = CH_3
6 R = CH_3 R_1 = R_2 = R_3 = R_4 = CH_3
7 R = CH_3 R_1 = R_2 = R_4 = CH_3 R_3 = H
8 R = CH_3 R_1 = R_3 = R_4 = CH_3 R_2 = H
9 R = CH_3 R_1 = H R_2 = R_3 = R_4 = CH_3
10 R = CH_3 R_1 = R_2 = R_3 = CH_3 R_4 = H
11 R = CH_3 R_1 = R_4 = CH_3 R_2 = R_3 = H
12 R = CH_3 R_1 = R_3 = H R_2 = R4 = CH_3
13 R = CH_3 R_1 = R_2 = H R_3 = R_4 = CH_3
14 R = CH_3 R_1 = R_4 = H R_2 = R_3 = CH_3



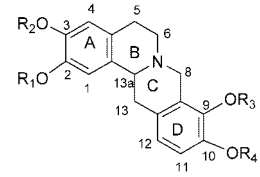
5 R = CH_3
[*N*-CD_3]-**5** R = CD_3



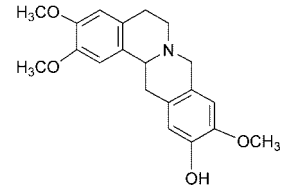
15 R = H
19 R = CH_3



16 R_1 + R_2 = CH_2
18 R_1 = R_2 = CH_3



17 R_1 = H, R_2 = CH_3, R_3 + R_4 = CH_2
21 R_1 = R_2 = R_4 = CH_3, R_3 = H
22 R_1 = R_2 = CH_3, R_3 + R_4 = CH_2
23 R_1 = R_3 = H, R_2 = R_4 = CH_3



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Just as with benzylisoquinoline **7**, precursors **8**–**10** were also administered to cultured cells of *C. platycarpa* and *C. ochotensis* var. *raddeana* (Table 3, no. 3–8). The major metabolite obtained from the feeding of **9** and **10** was identified as *N*-methyltetrahydropapaverine (**6**) by analysis of LC/NMR and LC/MS-MS data. A trace amount of **6** was also found as a metabolite of **8**. The bioconversion of **8**–**10** into **6** was confirmed by the feeding of [*N*-CD_3]-**8**, -**9**, and -**10** (Table 3, no. 15–22) (Scheme 2). Significantly, compounds **8**–**10** were not converted into tetrahydroprotoberberine- or pseudotetrahydroprotoberberine-type compounds.

Scheme 2 summarizes the metabolic transformations of 1-benzyl-*N*-methyltetrahydroisoquinolines **7**–**10** in cultured cells of *Macleaya* and *Corydalis* species. Among the four isomers (3′-, 4′-, 7-, and 6-hydroxy, respectively), only the 3′-hydroxy isomer **7** was converted into 2,3,10,11- and 2,3,9,10-oxygenated tetrahydroprotoberberines (**20** and **21**). This observation suggests that the presence of the hydroxy group at C-3′ is essential for the biotransformation of 1-benzyl-*N*-methyltetrahydroisoquinolines into 2,3,10,11- and 2,3,9,10-oxygenated tetrahydroprotoberberines. Thus, the enzyme responsible for the conversion likely shows substrate specificity. This behavior has also been found in the bioconversion of phenolic 1-benzyltetrahydroisoquinoline **1** into 2,3,10,11- and 2,3,9,10-oxygenated tetrahydroprotoberberines.[11] The structural requirement for the formation of the berberine bridge to produce 2,3,9,10- and 1,2,9,10-oxygenated protoberberines has been studied using enzyme-

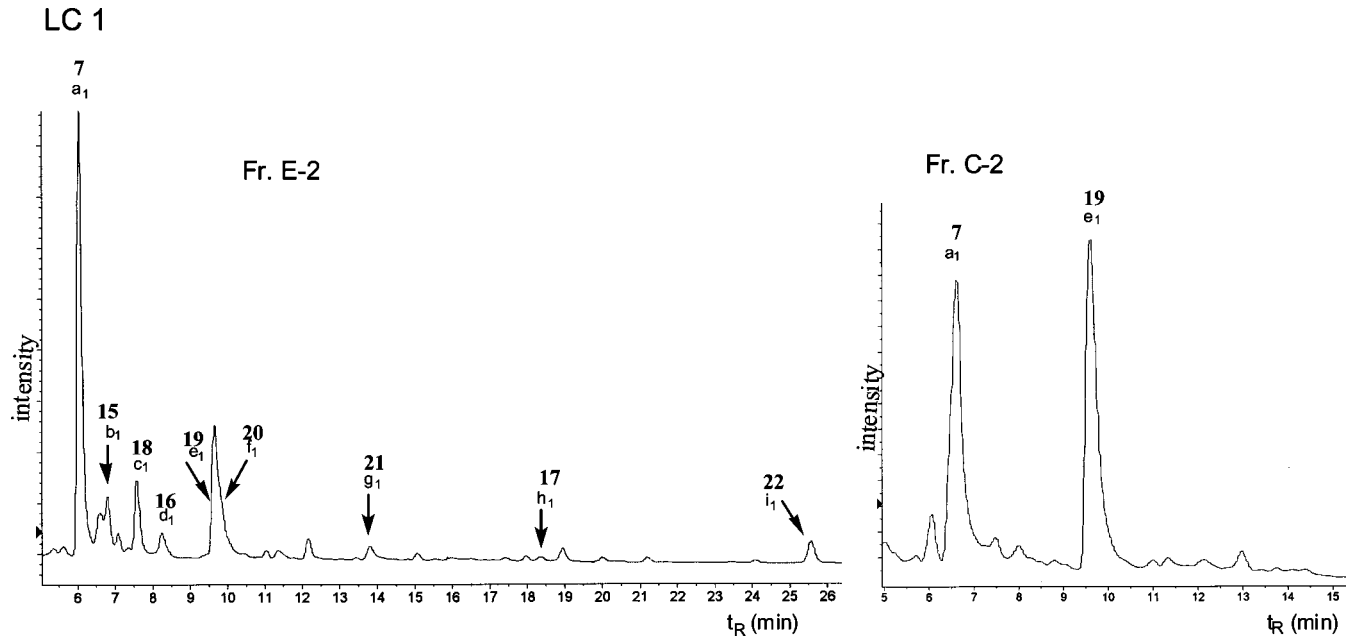
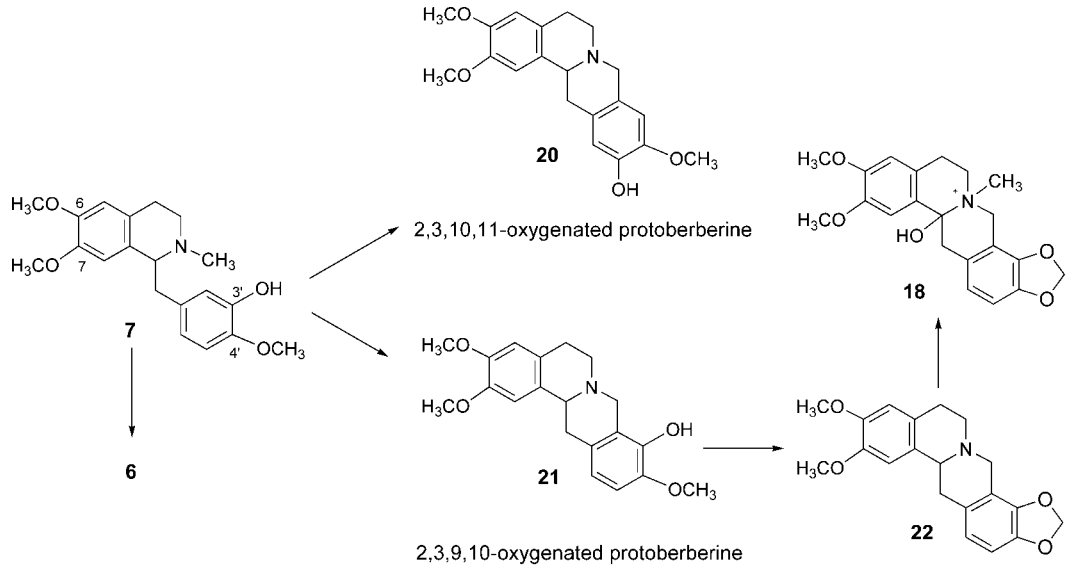
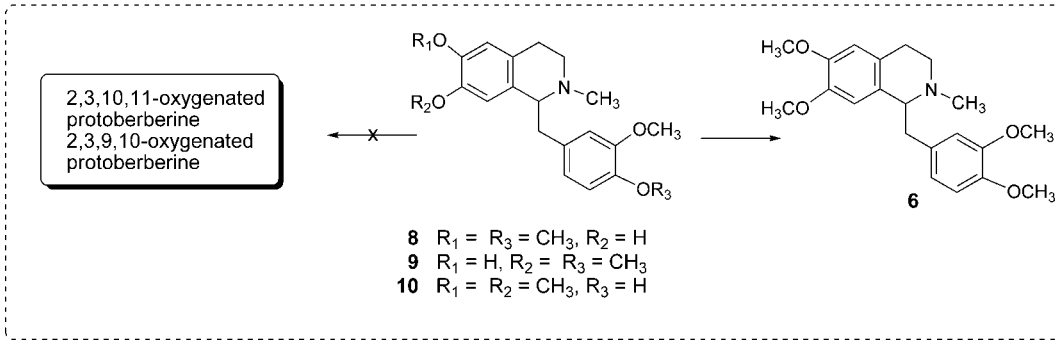


Figure 3. LC data of the alkaloid fractions (E-2 and C-2) obtained from the feeding of **7** to *C. platycarpa*. Pump: Shimadzu LC-10ADvp; column: Cosmosil 5 C18ARII (4.6 i.d. × 150 mm); gradient: A 0.1 M NH_4OAc (0.05% TFA)/B CH_3CN (0.05% TFA) A/B: initial 80/20, 25 min 55/45, 30 min 0/100; flow rate: 1 mL/min; UV detection: 280 nm.

Scheme 2





catalyzed reactions.[14] Metabolite **21** was further biotransformed via tetrahydroepiberberine (**22**) into cryptopine (**18**). All four 1-benzyltetrahydroisoquinolines **7**–**10** were *O*-methylated to give *N*-methyltetrahydropapaverine (**6**). However, with **9** and **10**, *N*-methyltetrahydropapaverine (**6**) was the major metabolite, suggesting that the hydroxy groups at C-6 and C-4′ in **9** and **10**, respectively, might be *O*-methylated more readily than those at C-7 and C-3′ in **8** and **7**, respectively.

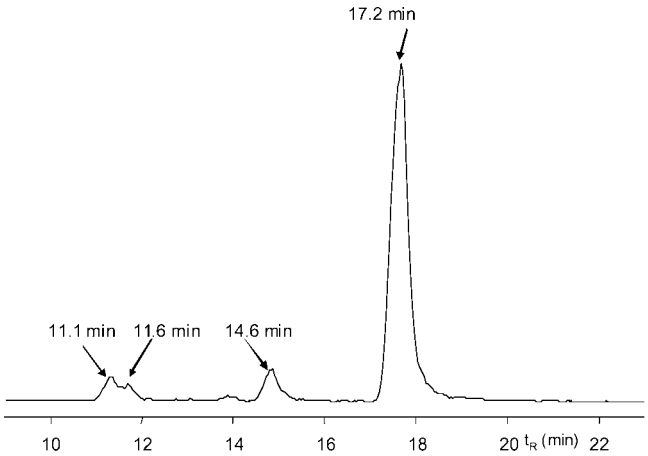


Figure 4. Mass chromatogram of the selected ions *m/z* 344 in the LC/APCI-MS of the alkaloid fraction (E-2) obtained from the feeding of [*N*-CD_3]-**7** to *C. platycarpa*. Pump: Shimadzu LC-10ADvp; column: Chiralcel OJ-RH (4.6 i.d. × 150 mm); gradient: A 0.1 M NH_4OAc (0.05% TFA)/B CH_3CN (0.05% TFA) A/B: initial 80/20, 10 min 60/40, 20 min 60/40, 30 min 0/100; flow rate: 0.5 mL/min; UV detection: 280 nm.

Table 4. Enantiomeric Excess (%) of [8-D_2]-Corytenchine ([8-D_2]-**20**) and [8-D_2]-Tetrahydropalmatrubine ([8-D_2]-**21**) Obtained from the LC/MS-MS Data

			selected ions $[M + H]^+$	
precursor	callus[a]	alkaloid fraction[b]	*m/z* 344 ([8-D_2]-**20**) *R*	*m/z* 344 ([8-D_2]-**21**) *S*
7	A	E-1	52	80
	A	E-2	30	84
	B	E-1	nd	nd
	B	E-2	46	80

[a] A: *Corydalis platycarpa* Makino. B: *Macleaya cordata*. [b] See Figure 2. nd: not determined.

Figure 4 shows the MS chromatogram of selected ions (*m/z* 344) in the LC/APCI-MS of the alkaloid fraction E-2 obtained from the feeding of [*N*-CD_3]-**7** to *C. platycarpa*. The peaks at 11.1 and 11.6 min were assigned to enantiomers of [8-D_2]-**20**, with the *R*- and *S*-configuration, respectively, at C-13a on the basis of reported data.[11] Likewise, the peaks at 14.6 and 17.2 min were assigned as enantiomers of [8-D_2]-**21**, with *R*- and *S*-configuration, respectively, at C-13a.[11] The enantiomeric excesses of [8-D_2]-**20** and -**21** were determined from the ratio of the peak areas of the MS chromatograms of the selected ion (*m/z* 344). The results summarized in Table 4 indicate that metabolites **20** and **21** contain different amounts of the *S*- and *R*-isomers. Metabolite **21** showed a high predominance of the *S*-isomer, while metabolite **20** showed a lower predominance of the *R*-isomer. Consequently, it is suggested that a stereoselective bioconversion occurs in the metabolic processes in cultured cells of *Macleaya* and *Corydalis* species.

In the next phase of our study, the metabolism of 1-benzyl-*N*-methyltetrahydroisoquinolines **11**–**14** and their [*N*-CD_3]-derivatives ([*N*-CD_3]-**11**−[*N*-CD_3]-**14**), which have two hydroxy and two methoxy groups on the aromatic A and D rings, respectively, were examined in *C. platycarpa*.

First, 7,3′-dihydroxylated **11** (reticuline) was administered to cultured cells of *C. platycarpa* (Table 3, no. 9). Trace amounts of laudanine (**7**) and scoulerine (**23**) were identified. In order to verify this result, [*N*-CD_3]-**11** was administered to *C. platycarpa* (Table 3, no. 23), and the resulting fraction E-2 showed peaks a_2−e_2 in LC 2 (Figure 5). The compounds associated with peaks a_2, b_2, and c_2 were deduced as [*N*-CD_3]-reticuline ([*N*-CD_3]-**11**), [*N*-CD_3]-laudanine ([*N*-CD_3]-**7**), and protopine (**16**), respectively, and peak d_2 corresponded to dehydrocheilanthifoline (**15**).

Peak e_2 in the LC 2 (Figure 5) showed $[M + H]^+$ at *m/z* 330 and a product ion at *m/z* 178 in the LC/MS-MS, thus corresponding to [8-D_2]-scoulerine ([8-D_2]-**23**). This structure was confirmed by comparison of the stopped-flow ^1H NMR data with those of authentic (*S*)-scoulerine measured under the same conditions. Chiral phase LC/CD (A and B in Figure 6) was also performed on fraction E-2 obtained from the feeding of [*N*-CD_3]-reticuline ([*N*-CD_3]-**11**), with authentic *S*-scoulerine used as a reference. The CD of the [8-D_2]-scoulerine metabolite at 20 min in the LC/CD (Figure 6, A) showed a negative Cotton effect at 236 nm, which matched that of *S*-scoulerine (Figure 6, B).

Precursor **12** (protosinomenine) and the deuterated precursor [*N*-CD_3]-**12**, which have the two hydroxy groups at C-6 and C-3′, was also administered to cultured cells of *C. platycarpa* (Table 3, nos. 10 and 24). Fraction E-2 obtained from *C. platycarpa* showed peaks a_3−h_3 in the LC 3 (Figure 7). From comparison of individual LC/MS-MS data with those of the corresponding alkaloids obtained from the feeding of [*N*-CD_3]-**7**, compounds associated with peaks a_3, b_3, d_3, and g_3 were determined to be [*N*-CD_3]-protosinomenine ([*N*-CD_3]-**12**), [*N*-CD_3]-laudanine ([*N*-CD_3]-**7**), [8-D_2]-cryptopine ([8-D_2]-**18**), and [8-D_2]-tetrahydropalmatrubine ([8-D_2]-**21**), respectively. [*N*-CD_3]-Laudanine ([*N*-CD_3]-**7**), [8-D_2]-cryptopine ([8-D_2]-**18**), and [8-D_2]-tetrahydropalmatrubine ([8-D_2]-**21**) are metabolites of [*N*-CD_3]-protosinomenine ([*N*-CD_3]-**12**). The isomer of scoulerine (**23**) predicted to be formed by ring closure of **12** was not detected. This may be due to readily occurring *O*-methylation at C-6 to furnish **7**. The peaks at 15.3 and 18.2 min in the MS of the selected ion at *m/z* 342 have been assigned as *R*- and *S*-enantiomers of (±)-tetrahydropalmatrubine (**21**), respectively.[11] The MS of the selected ion at *m/z* 344 ([8-D_2]-**21**) of 18.2 min was identical with that of (*S*)-tetrahydropalmatrubine (Figure 8), thus indicating that the [8-D_2]-tetrahydropalmatrubine metabolite was the *S*-enantiomer. From the LC/MS-MS analysis, the compound associated with peak f_3 was found to be epiberberine (**19**), which may be a metabolite of [*N*-CD_3]-**12**. The compounds associated with peaks c_3, e_3, and h_3 were deduced as dehydrocheilanthifoline (**15**), protopine (**16**), and cheilanthifoline (**17**), respectively, which are components of the callus.

In a similar fashion, 6,7-dihydroxylated **13** and [*N*-CD_3]-**13** were administered to cultured cells of *C. platycarpa* (Table 3, nos. 11 and 25). Codamine (**8**) and [*N*-CD_3]-**8** were identified (Table 2). In the same way, isoorientaline (**14**) and [*N*-CD_3]-**14**, with the two hydroxy groups at C-6 and C-4′, were administered to *C. platycarpa* (Table 3, nos. 12 and 26). Pseudocodamine (**10**) and *N*-methyltetrahydropapaverine (**6**) were characterized as the metabolites of **14**; [*N*-CD_3]-**10** and [*N*-CD_3]-**6**, as the metabolites of [*N*-CD_3]-**14**.

Scheme 3 summarizes the biotransformations demonstrated by the results of feeding experiments of 1-benzyl-*N*-methyltetrahydroisoquinolines **11**–**14** to cultured cells of *Macleaya* and *Corydalis* species.

Among the four 1-benzyl-*N*-methyltetrahydroisoquinolines with two hydroxy and two methoxy groups on the aromatic A and D rings (**11**–**14**), respectively, compounds **11** and **12** were converted into the *S*-enantiomer of 2,3,9,10-oxygenated tetrahydroprotoberberines **23** and **21**, respectively, while **13** and **14** were not biotransformed to tetrahydroprotoberberines. These observations with dihydroxy-substituted compounds confirm that the presence of a hydroxy group at C-3′ is essential for the biotransformation of 1-benzyl-*N*-methyltetrahydroisoquinolines into 2,3,9,10-oxygenated tetrahydroprotoberberines. The product formed by ring closure at C-6′ was not detected. This is identical with the result reported by Kutchan et al.[14]

The following sequences of *O*-methylation were thus identified in the present study. Reticuline (**11**) was biotransformed to (*S*)-scoulerine (**23**), since the hydroxy group at C-7 was not *O*-methylated readily. However, protosinomenine (**12**) was readily *O*-methylated at C-6 to provide laudanine (**7**), which in turn was bioconverted into (*S*)-tetrahydropalmatrubine (**21**). Eventually, (*S*)-tetrahydropalmatrubine

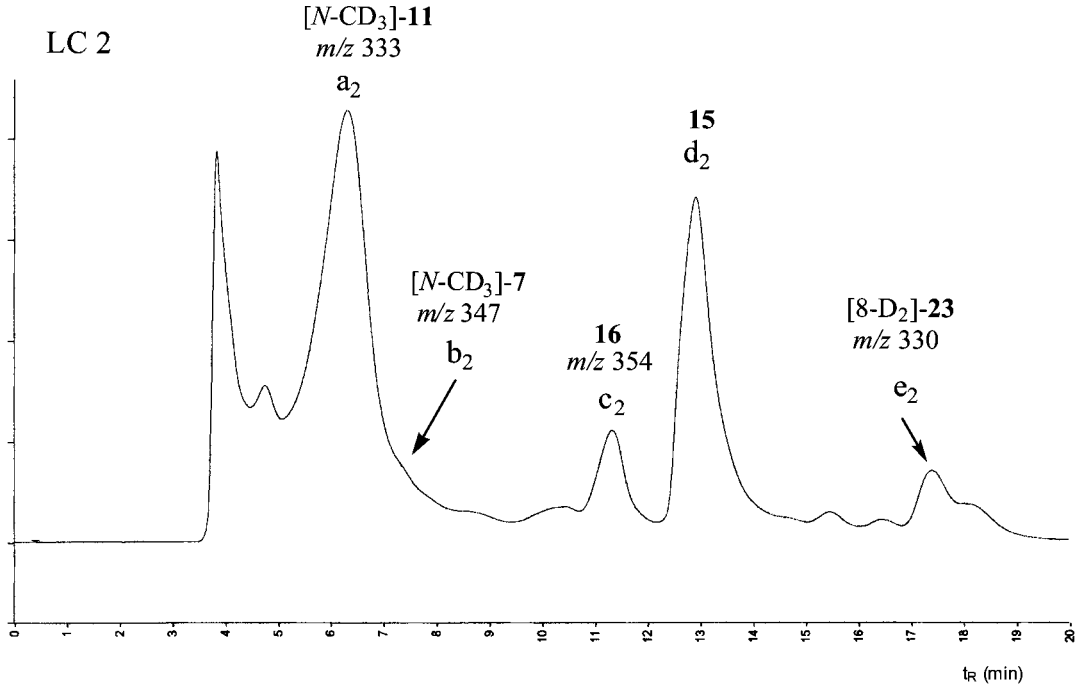


Figure 5. LC data of the alkaloid fraction (E-2) obtained from the feeding of [*N*-CD_3]-reticuline ([*N*-CD_3]-**11**) to *C. platycarpa*. Pump: Varian Prostar model 230; column: Chiralcel OJ-RH (4.6 i.d. × 150 mm); gradient: A 0.1 M NH_4OAc/D_2O (0.05% TFA)/B CH_3CN (0.05% TFA) A/B: initial 80/20, 10 min 60/40, 20 min 60/40; flow rate: 0.5 mL/min; UV detection: 280 nm.

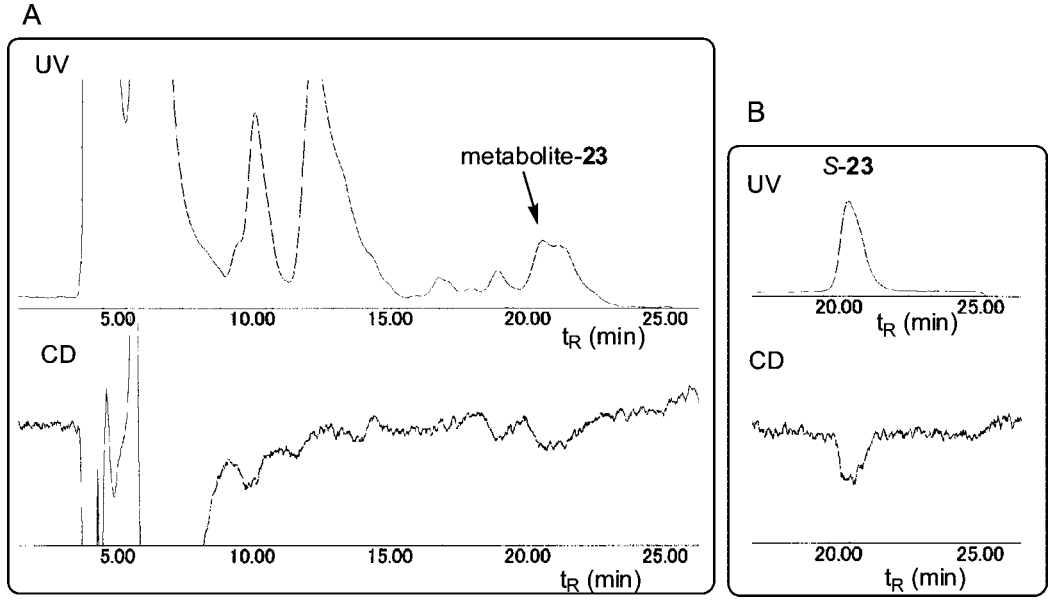


Figure 6. UV and CD chromatograms from the LC/CD of (*S*)-scoulerine (*S*-**23**) and the alkaloid fraction (E-2) obtained from the feeding of [*N*-CD_3]-reticuline ([*N*-CD_3]-**11**) to *C. platycarpa*. A: UV and CD chromatograms of the alkaloid fraction (E-2). B: UV and CD chromatograms of authentic *S*-**23**.

(**21**) was converted to cryptopine (**18**). Compound **13** was *O*-methylated at C-6 to give codamine (**8**), indicating that the hydroxy group at C-6 is more readily *O*-methylated than that at C-7. Finally, isoorientaline (**14**) underwent *O*-methylation at C-6 to produce pseudocodamine (**10**), which was in turn *O*-methylated at C-4′ to yield *N*-methyltetrahydropapaverine (**6**).

It can, therefore, be concluded that in cell cultures of *Corydalis* and *Macleaya* species, laudanine (**7**), with a hydroxy group at C-3′, can form the berberine bridge at both positions C-2′ and C-6′ to produce *S*- and *R*-enantiomers of 2,3,9,10- and 2,3,10,11-oxygenated protoberberines (**20** and **21**), respectively, whereas reticuline (**11**) and protosinomenine (**12**), incorporating a hydroxy group at C-3′, form the berberine bridge at C-2′ to furnish the *S*-enantiomers of 2,3,9,10-oxygenated protoberberines (**23** and **21**), respectively. There are differences in the position of ring closure between laudanine (**7**) and reticuline (**11**) or protosinomenine (**12**) and in the configuration at C-13a of the protoberberine metabolites obtained from **7** and **11** or **12**. These may be due to difference in the berberine bridge-forming enzyme. Formation of the protoberberines from reticuline (**11**) and protosinomenine (**12**) parallel the results demonstrated by Kutchan et al.[14]

Experimental Section

Materials. In 1974, 1989, and 1981, the calli of *M. cordata*, *C. platycarpa* Makino, and *C. ochotensis* var. *raddeana*, respectively, were derived from the stems of wild plants grown in Kobe (Japan) on

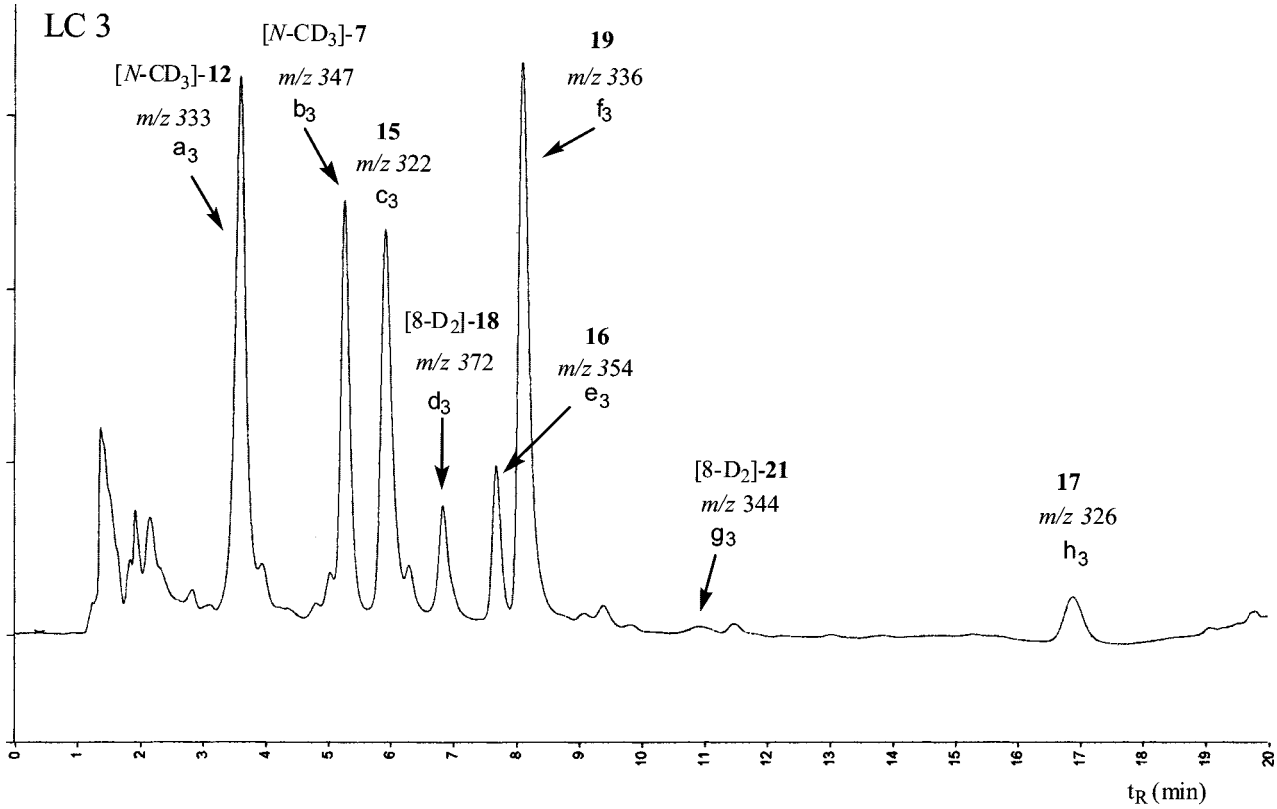


Figure 7. LC data of the alkaloid fraction (E-2) obtained from the feeding of [*N*-CD_3]-protosinomenine ([*N*-CD_3]-**12**) to *C. platycarpa*. Pump: Varian Prostar model 230; column: Cosmosil 5 C18ARII (4.6 i.d. × 150 mm); gradient: A 0.1 M NH_4OAc/D_2O (0.05% TFA)/B CH_3CN (0.05% TFA) A/B: initial 80/20, 25 min 55/45, 26 min 0/100; flow rate: 1 mL/min; UV detection: 280 nm.

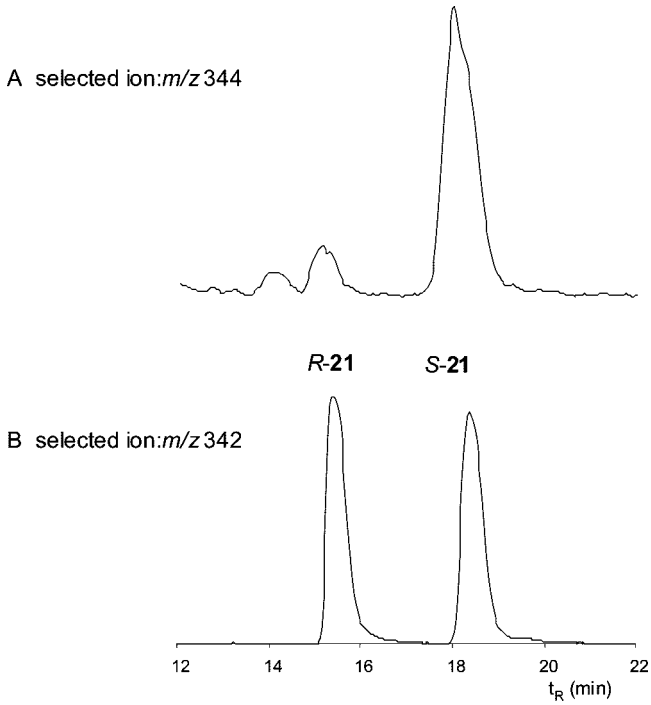


Figure 8. Mass chromatograms of the selected ions. A: alkaloid fraction (E-2) obtained from the feeding of [*N*-CD_3]-**12** to *C. platycarpa*. B: Authentic (±)-tetrahydropalmatrubine (**21**). Pump: Shimadzu LC-10ADvp; column: Chiralcel OJ-RH (4.6 i.d. × 150 mm); gradient: A 0.1 M NH_4OAc (0.05% TFA)/B CH_3CN (0.05% TFA) A/B: initial 80/20, 10 min 60/40, 20 min 60/40, 30 min 0/100; flow rate: 0.5 mL/min; UV detection: 280 nm.

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 three or four weeks on fresh medium at 25 °C in the dark.

The natural product (*S*)-scoulerine (**23**) {$[\alpha]_D$ −270 (*c* 0.12, CH_3OH)} was obtained from *C. platycarpa* Makino. Papaverine hydrochloride and CD_3I were purchased from Sigma Chemical Co.

HPLC Parameters for LC/NMR, LC/MS, and LC/CD. Chromatographic preparations were performed using a Cosmosil 5 C_{18}-AR (4.6 i.d. × 150 mm) reversed-phase column. The mobile phases [(A) 0.1 M NH_4OAc (0.05% TFA, D_2O for LC/NMR) and (B) CH_3CN (0.05% TFA) or CH_3OH (0.05% TFA)] were used for linear or nonlinear gradient elution.

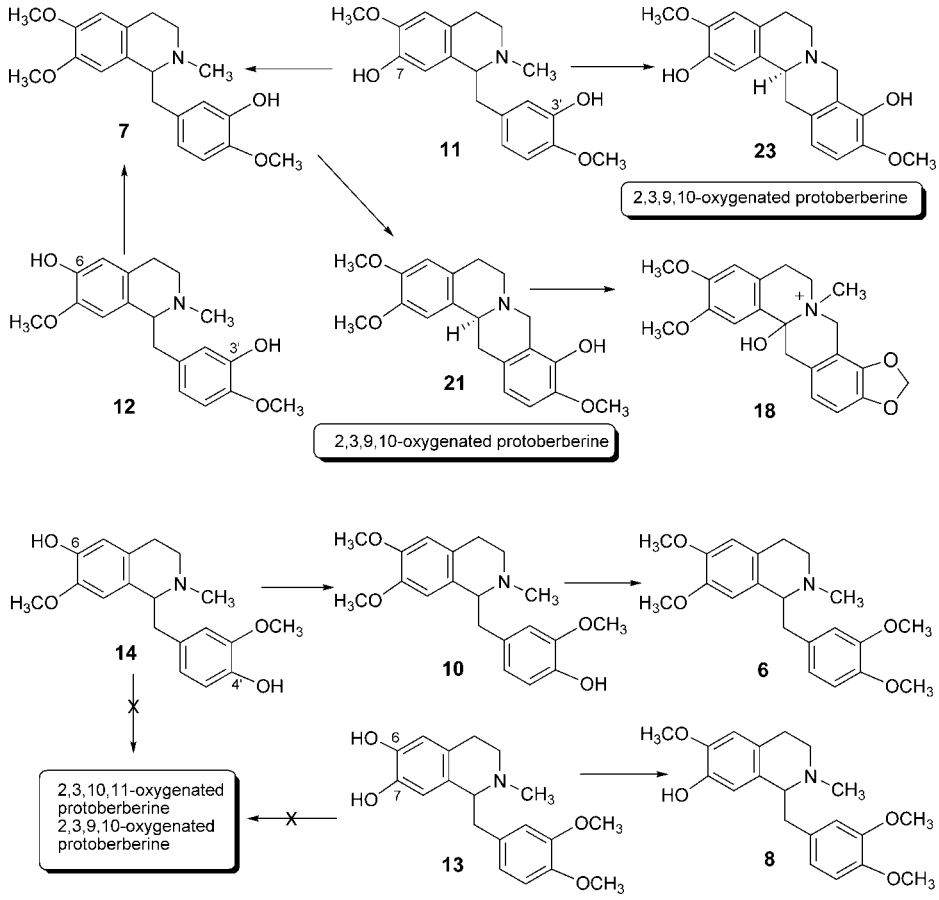
The flow rate was 1 mL/min (detection: 280 nm). The chiral analytical separation was carried out on a chiral OJ-RH column (4.6 i.d. × 150 mm, Daicel Chemical Ltd.) at room temperature for LC/MS and 40 °C for LC/CD. The flow rate was 0.5 mL/min (detection: LC/MS 280 nm, LC/CD 236 nm).

LC/APCI-MS Method. The LC/APCI-MS (/MS) spectra were measured with Q3 and product ion scans. Molecular weight information was obtained through the protonated molecular ions $[M + H]^+$, and product ions were recorded by LC/MS-MS. LC/APCI-MS for acid-cleavage products of *N*-methyltetrahydropapaverine (**6**) was carried out using a Hitachi M-1000H connected to a Hitachi L-6200 intelligent pump and a Hitachi L-4000 UV detector. APCI-MS conditions: nebulizer and vaporizer temperatures were 320 and 399 °C, respectively. The drift voltage was 40 V. The quasi-molecular ions were monitored in the SIM method. LC was performed on a Cosmosil 5 C_{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 CH_3OH (0.05% TFA, B) was added by a linear gradient (initial 20% B, 30 min 100% B). The flow rate was 1 mL/min (detection: 280 nm).

LC/APCI-MS (/MS) was measured on an Applied Biosystems API 3000 triple quadrapole mass spectrometer (MS/MS) with a heated nebulizer interface as described in the previous paper.[11]

LC/NMR Method. The LC/NMR spectra were measured in the stopped-flow mode. LC-NMR data were acquired using a Varian UNITY-INOVA-500 spectrometer (^1H: 499.83 MHz) equipped with a 60 μL triple-resonance microflow NMR probe. 1D ^1H NMR spectra were obtained in stopped-flow mode as described in the previous paper.[11]

Scheme 3



LC/CD Method. The LC/CD analysis was carried out on a chiral reversed-phase column at 236 nm. Chromatographic separations were performed using a Jasco PU-2080Plus intelligent pump with a column oven (Jasco 860-CO), a Jasco Browin NT, HSS-2000 data processor, and a Jasco CD-2095Plus CD chiral detector (Hg-Xe lamp), simultaneously monitoring the CD and UV signals at one specific wavelength (range 220–420 nm). A nonlinear gradient (initial 20% B, 10 min 40% B, 20 min 40% B, 30 min 100% B) was programmed. The flow rate was 0.5 mL/min (detection: 236 nm).

Preparation of 1-Benzyl-*N*-methyltetrahydroisoquinolines 7–14. *N*-Methylpapaverinium salt (**5**) and *N*-methyltetrahydropapaverine (**6**) were prepared according to the procedure described before.[15] A solution of racemic *N*-methyltetrahydropapaverine (**6**) (500 mg) in 47% HBr (1 mL) was refluxed for 13 min. Hydrobromic acid was evaporated *in vacuo*. The residue was dissolved in DMSO (0.5 mL) and was separated by preparative HPLC (Hitachi L-6250 intelligent pump and Hitachi L-4000 UV detector), which was performed on a Cosmosil 5 C_{18}-AR (20 i.d. × 250 mm) reversed-phase column. As a mobile phase, (A) 0.1 M NH_4OAc (0.05% TFA) and (B) CH_3OH (0.05% TFA) were used in a linear gradient (initial 20% B, 80 min 100% B). The flow rate was 6 mL/min (detection: 280 nm). The eluent obtained from the peaks a−l in the LC (Figure 1) was evaporated, and the residue was further purified by HPLC [H_2O (0.05% TFA)−CH_3OH (0.05% TFA)] to give the trifluoroacetates of peaks a (**7**, 42 mg), b (**8**, 24 mg), c (**9**, 23 mg), d (**10**, 66 mg), e (**11**, 35 mg), f (**12**, 20 mg), g (**13**, 10 mg), h (71 mg), i (**14**, 34 mg), j (35 mg), k (20 mg), and l (10 mg). For ^{1}H NMR and MS data, see Tables 1 and 2.

Preparation of [*N*-CD_3]-1-Benzyltetrahydroisoquinolines [*N*-CD_3]-7−[*N*-CD_3]-14. [*N*-CD_3]-**5** was prepared by treatment of papaverine with CD_3I according to the method used for the preparation of **5**.[15] [*N*-CD_3]-**6** was prepared from [*N*-CD_3]-**5** according to the method used for the preparation of **6**.[15] [*N*-CD_3]-**6** (1 g) in 47% HBr (1 mL) was refluxed for 9.5 min. Hydrobromic acid was evaporated *in vacuo*. The residue was dissolved in DMSO (0.5 mL) and was separated by preparative HPLC to give the trifluoroacetates of [*N*-CD_3]-**7** (80 mg), [*N*-CD_3]-**8** (48 mg), [*N*-CD_3]-**9** (38 mg), [*N*-CD_3]-**10** (86 mg), [*N*-CD_3]-**11** (19 mg), [*N*-CD_3]-**12** (19 mg), [*N*-CD_3]-**13** (6 mg), and [*N*-CD_3]-**14** (35 mg). ^{1}H NMR data were identical with those of the unlabeled derivatives, except for the *N*-methyl group. For MS data, see Table 2.

Feeding Experiments. Substrates were dissolved in H_2O (2–4 mL) and introduced through a sterile bacterial filter into 100 mL conical flasks containing 40 mL of autoclaved MS medium, 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 four weeks (Table 3). Cells and medium were separated and extracted with CH_3OH at 60 °C. Extracts were worked up as described in Figure 2.

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