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Enantiomeric separation of racemic 1-benzyl-*N*-methyltetrahydroisoquinolines on chiral columns and chiral purity determinations of the *O*-methylated metabolites in plant cell cultures by HPLC-CD on-line coupling in combination with HPLC-MS

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ABSTRACT

Effective enantiomeric separations of 1-benzyl-*N*-methyltetrahydroisoquinolines were achieved using commercially available Chiralcel OD-H and OJ-H columns. Online LC-CD analysis allowed for the establishment of a correlation between the absolute configuration of the separated enantiomers and their characteristic CD transitions. LC-MS combined with LC-CD analysis permitted chiral purity determinations of *O*-methylated metabolites of nine phenolic 1-benzyl-*N*-methyltetrahydroisoquinolines in cell cultures of *Corydalis*, *Macleaya*, and *Nandina* species.

1. Introduction

Benzylisoquinoline alkaloids are important secondary metabolites found in several related plant families including the Papaveraceae, Fumariaceae, Berberidaceae, Ranunculaceae, and Menispermaceae (Facchini, 2001). Benzylisoquinoline-derived alkaloids also show various pharmacological effects, including analgetic (morphine), antitussive (noscapine), and muscle relaxant [(+)-tubocurarine] properties. These alkaloids are formed biosynthetically from the common key branch-point intermediate, (*S*)-reticuline (benzylisoquinoline-type). For example, the morphine precursor salutaridine is formed from (*R*)-reticuline (Gerardy and Zenk, 1993). (+)-Tubocurarine is produced by coupling of one each of (*R*)- and (*S*)-*N*-methylcoclaurine (reticuline precursor) (Stadler and Zenk, 1993). There are stereoselective and non-stereoselective enzymes in the biosynthetic pathway to the benzylisoquinolines (Frenzel and Zenk, 1990). The chiroptical properties of benzylisoquinolines are typically characterized by their optical rotations and CD or ORD spectroscopic methods. However, these methods cannot be applied to the extract matrix without any isolation step. An online LC-CD analysis has been used to establish a correlation between the absolute configuration of separated enantiomers and their characteristic CD transitions. This method has been applied for phytochemical analysis of natural products in crude plant extracts (Bringmann et al., 1999).

Herein, we describe the enantiomeric separations of 1-benzyl-*N*-methyltetrahydroisoquinolines achieved by chiral phase HPLC, in conjunction with CD and MS spectroscopic analyses. The described method was used to determine enantiomeric purity of biosynthetic metabolites in the extract matrix obtained from administration of benzylisoquinolines to cell cultures of *Corydalis*, *Macleaya*, and *Nandina* species. The deuterated precursors (**1D**–**9D**) were employed to exclude undesired interferences.

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2. Results and discussion

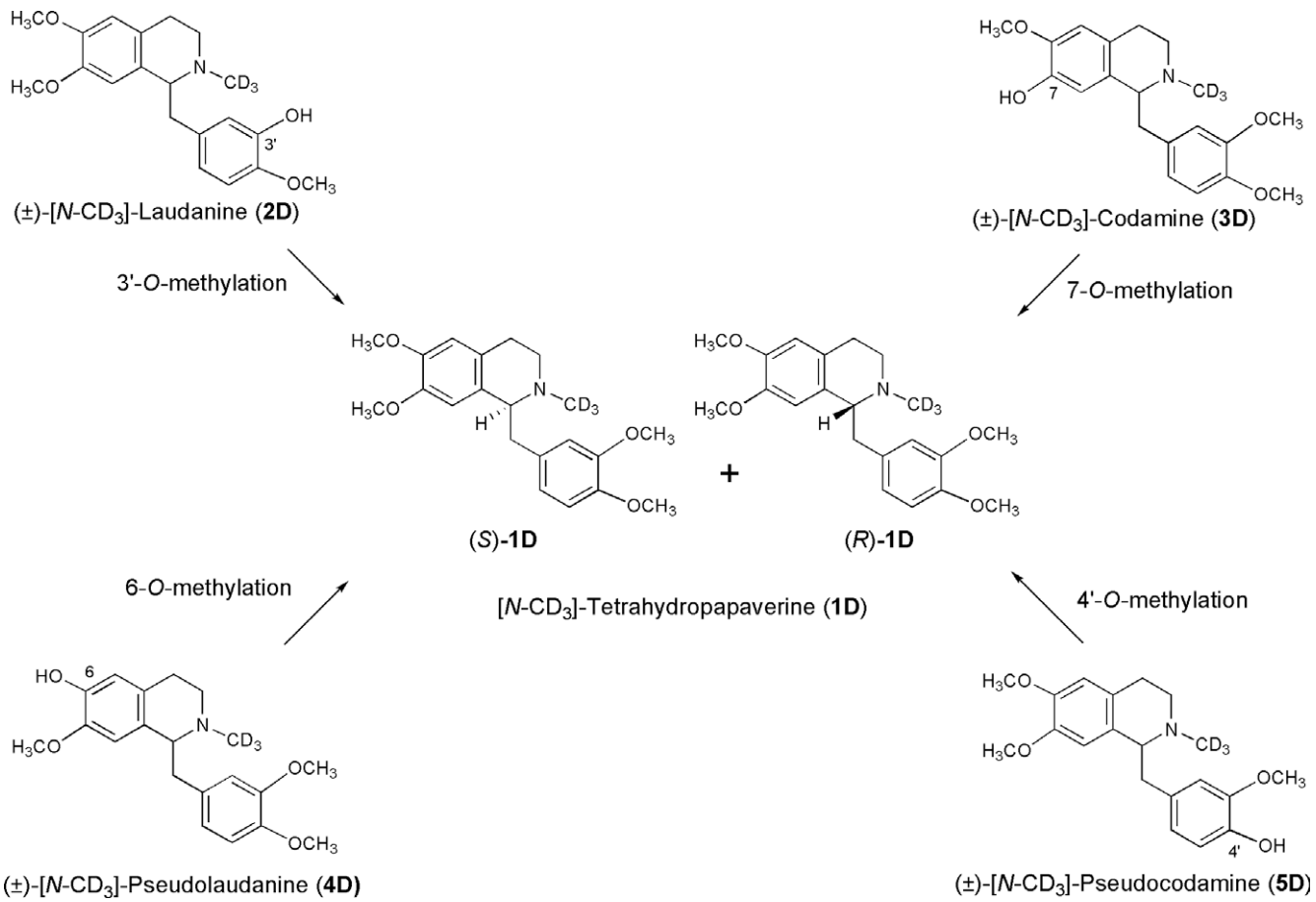
2.1. Precision comparison of (*R*/*S*) ratio of the enantiomers between LC-CD and LC-MS methods

Online LC-CD analysis was carried out to examine the enantiomeric separation of nine synthetic [*N*-CD_3]-1-benzyltetrahydroisoquinolines (**1D**–**9D**, see Schemes 1 and 2 for structures) on chiral columns. Initially, CD spectra of (±)-reticuline [(±)-**6**] and (+)-reticuline [(±)-**6**] (Brochmann-Hanssen and Nielsen, 1965) were recorded in the scan range of 220–420 nm to find a suitable wavelength for the measurement of LC-CD spectra; the flow was stopped at the maxima of the CD signals (Fig. 1). Consequently, a wavelength of 236 nm was used in measurement of LC-CD spectra. The absolute configuration at C-1 was determined by comparison of the CD spectrum of (±)-reticuline [(±)-**6**] with that of (+)-reticuline [(±)-**6**] having the (*S*) configuration at C-1. Peak A, which eluted at 11.3 min, had a positive CD band and peak B, eluted at 14.5 min, had a negative CD band. These peaks corresponded to the (*S*) and (*R*) configurations at C-1, respectively. The LC-CD spectra of (±)-[*N*-CD_3]-tetrahydropapaverine (**1D**), -laudanine (**2D**), -codamine (**3D**), -pseudolaudanine (**4D**), pseudocodamine (**5D**), -reticuline (**6D**), -pseudosinomenine (**7D**), 6-*O*-demethylcodamine (**8D**), and -isoorientaline (**9D**) (Cui et al., 2007) were measured using three chiral columns (Chiralcel OJ-H, Chiralcel OD-H, and Chiralpak IB) and two solvent systems (*N*-hexane/isopropanol/diethylamine and *N*-hexane/ethanol/diethylamine) under isocratic conditions (Table 1). Enantiomeric separations were achieved for all compounds using both Chiralcel OD-H and OJ-H columns with *N*-hexane/ethanol/diethylamine as eluent. All compounds showed a positive LC-CD signal for the faster eluting peak and a negative signal for the slower peak (Table 1). Thus, the absolute configurations at C-1 for the enantiomers were established as (*S*) and (*R*) for the faster and slower peaks, respectively, in analogy to (*S*)- and (*R*)-reticuline (**6**).

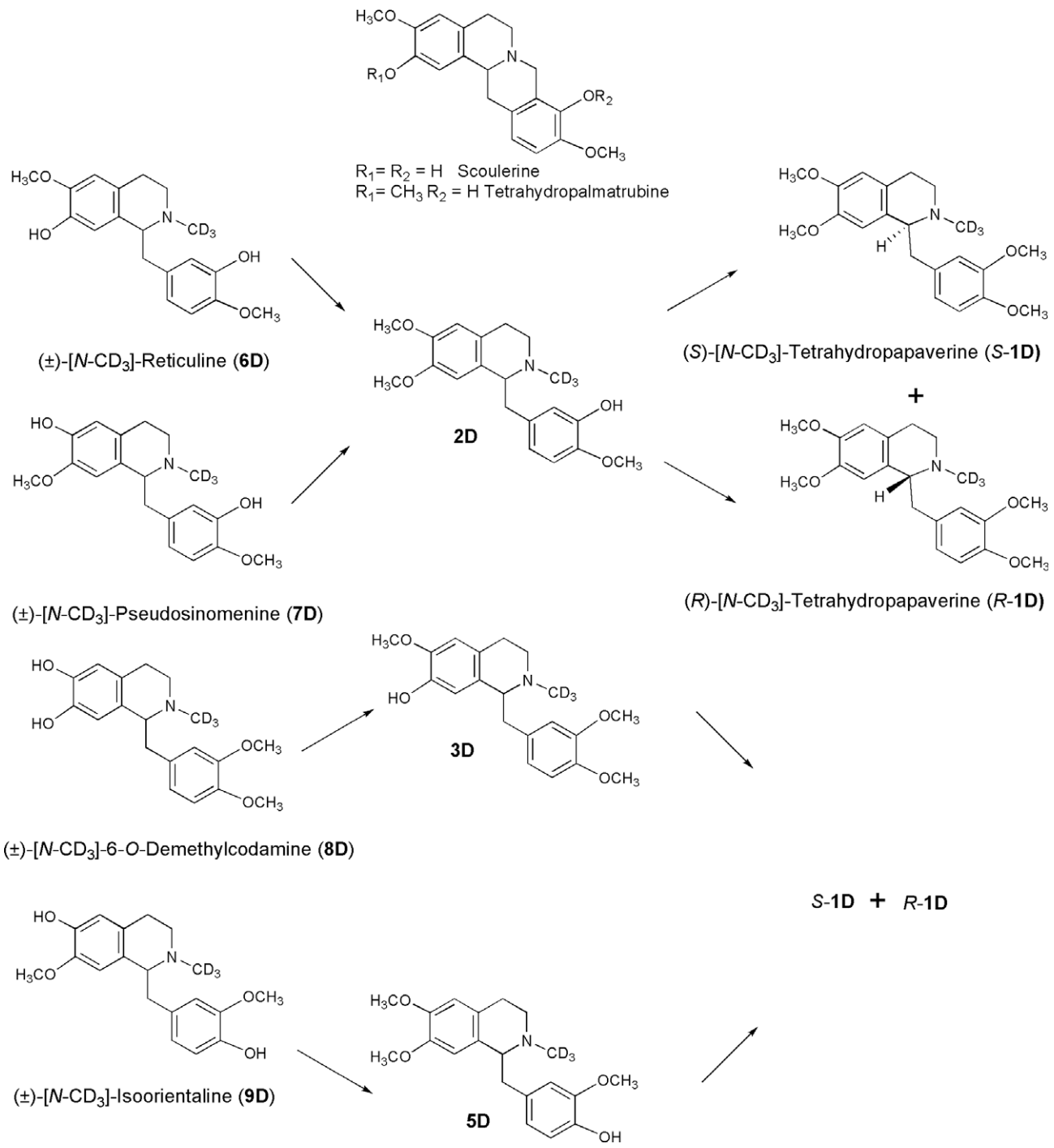
We also compared the detection precision of LC-CD and LC-MS methods. Table 2 lists the (*S*) and (*R*) enantiomeric ratios of compounds (**1D**–**9D**) detected by the two methods. The faster moving enantiomer (*S*) of **1D**–**9D** always had a much lower intensity compared to the (*R*) enantiomer in the CD chromatogram (Table 2), while the intensities of the two were approximately the same by LC-MS analysis (Table 2). The (*R*/*S*) ratio discrepancy between UV and CD may be due to a lack of precision of the CD detection. Thus, the (*R*/*S*) enantiomeric ratios of respective 1-benzyl-*N*-methyltetrahydroisoquinolines are best determined by LC-MS analysis of the protonated molecular ion.

2.2. Application of LC-MS methods to chiral purity determination of *O*-methylated metabolites of 1-benzyl-*N*-methyltetrahydroisoquinolines

Compounds **2D**–**9D** were administered to cell cultures of *Corydalis platycarpa* and *Macleaya cordata* (Cui et al., 2007) and the ether-soluble alkaloid fractions (Fig. 2, Fr. E) from these experiments were analyzed by LC-MS (Table 3, Experiment Nos. 1–11).



Scheme 1. *O*-Methylations of (±)-**2D**–(±)-**5D**. Biotransformations of (±)-**2D**, (±)-**3D**, (±)-**4D**, and (±)-**5D** into (*R*)- and/or (*S*)-**1D** were determined in cell cultures of *Corydalis platycarpa*. Biotransformations of (±)-**2D**, (±)-**3D**, and (±)-**5D** into (*R*)- and/or (*S*)-**1D** were determined in cell cultures of *Macleaya cordata*. Biotransformations of (±)-**2D** and (±)-**5D** into (*R*)- and/or (*S*)-**1D** were determined in cell cultures of *Nandina domestica*.



Scheme 2. *O*-Methylations of (±)-**6D**–(±)-**9D**. Biotransformations of (±)-**6D**, (±)-**7D**, (±)-**8D**, and (±)-**9D** into (*R*)- and/or (*S*)-**1D** were determined in cell cultures of *Corydalis platycarpa*. Biotransformations of (±)-**6D** and (±)-**7D** into (*R*)- and/or (*S*)-**1D** were determined in cell cultures of *Nandina domestica*.

(±)-[*N*-CD_3]-Laudanine (**2D**), -codamine (**3D**), -pseudolaudanine (**4D**), and -pseudocodamine (**5D**) were *O*-methylated directly to produce deuterated *N*-methyltetrahydropapaverine (**1D**) (Scheme 1). (±)-[*N*-CD_3]-Reticuline (**6D**), -pseudosinomenine (**7D**), 6-*O*-demethylcodamine (**8D**), and -isoorientaline (**9D**) were metabolized to deuterated *N*-methyltetrahydropapaverine (**1D**) in two steps (Scheme 2). [*N*-CD_3]-Laudanine (**2D**) was detected as an intermediate metabolite of both (±)-[*N*-CD_3]-reticuline and -pseudosinomenine (**6D** and **7D**), while [*N*-CD_3]-codamine and -pseudocodamine (**3D** and **5D**) were detected as intermediate metabolites of (±)-[*N*-CD_3]-6-*O*-demethylcodamine and -isoorientaline (**8D** and **9D**), respectively.

The (*R*/*S*) ratio of **1D** (protonated molecular ion: *m*/*z* 361) in Experiment Nos. 1–4 and 9–11 are shown in Table 3. The (*R*/*S*) ratios of **1D** from (±)-[*N*-CD_3]-laudanine (**2D**), -codamine (**3D**), -pseudolaudanine (**4D**), and -pseudocodamine (**5D**) in *C. platycarpa* were ca. 1/1, 1/1, 3/1, and 1/9, respectively. The (*R*/*S*) ratios of 3/1 observed in formation of **1D** from (±)-[*N*-CD_3]-pseudolaudanine (**4D**) (Fig. 3) may result from an higher enantioselectivity of the metabolizing enzyme for the (*R*) enantiomer compared with the

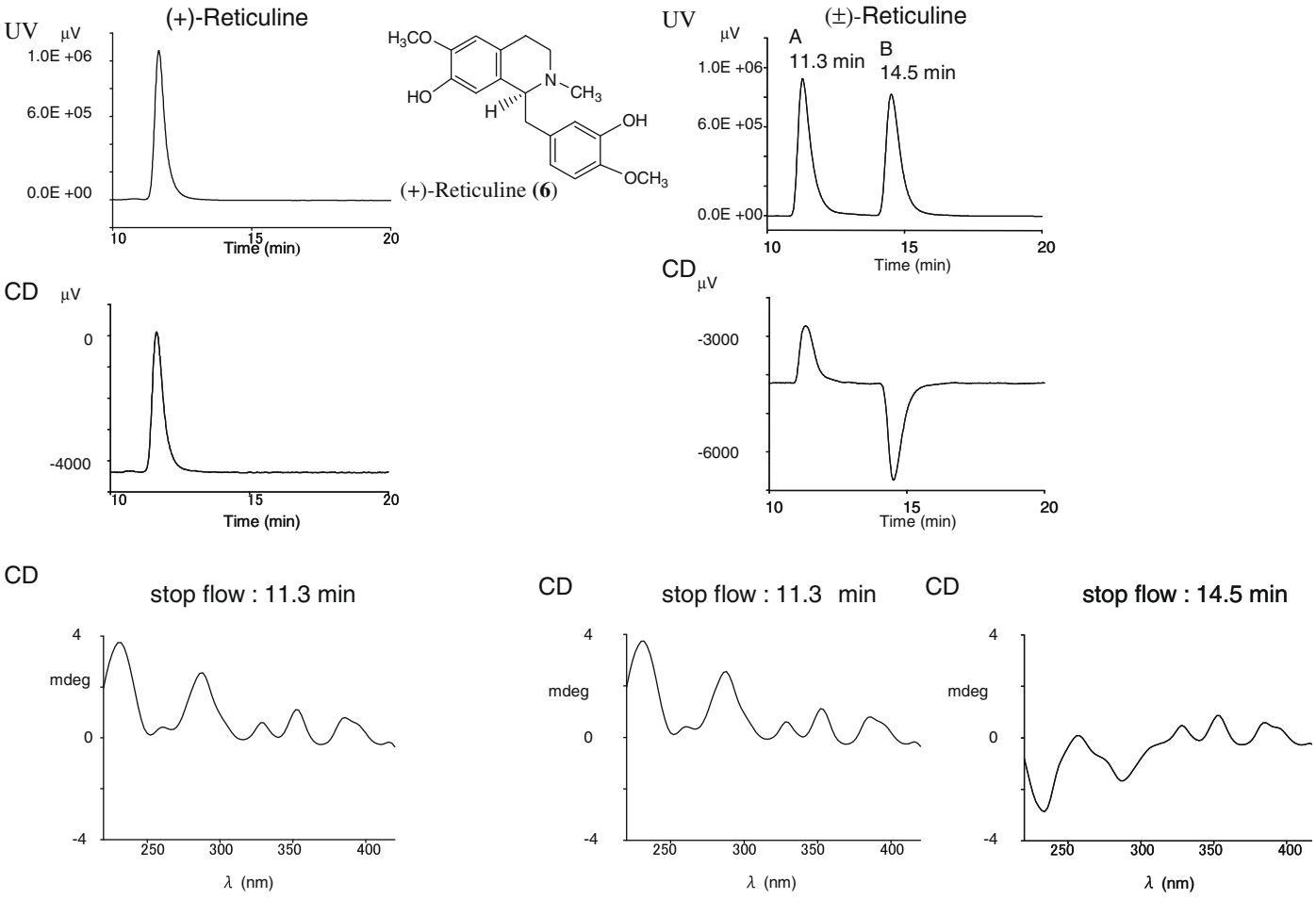


Fig. 1. LC-CD data and stop-flow LC-CD of (+)-reticuline [(+)-**6**] and (±)-reticuline [(±)-**6D**]. CD detector: Jasco CD-2095Plus; pump: Jasco PU-2SOOPlus; column: chiralcel OJ-H (4.6 × 250 mm); fluent: *N*-hexane/ethanol/diethylamine: 50/50/0.1; flow rate :0.5 mL/min. LC/CD detection: 236 nm; sample injection: 5 μg.

Table 1
LC-CD[a] data of (±)-[*N*-CD_3]-1-benzyltetrahydroisoquinolines [(±)-**1D**–(±)-**9D**].

Compounds[b]	CD bands[c]	Chiralcel OJ-H[d]		Chiralcel OD-H[d]		Chiralpak IB[d]	
		n-He/IPA/DEA[e] 50/50/0.1	n-He/Et/DEA[f] 50/50/0.1	n-He/IPA/DEA[f] 50/50/0.1	n-He/Et/DEA[e] 50/50/0.1	n-He/IPA/DEA[f] 50/50/0.1	n-He/Et/DEA[e] 50/50/0.1
		Retention time (t_R: min)[g]					
(±)-[*N*-CD_3]-Tetrahydropapaverine (**1D**)	p	4.93	9.26	9.33	7.57	5.32[e]	4.50[e]
	n	6.31	11.73	13.32	12.87	6.98[e]	5.35[e]
(±)-[*N*-CD_3]-Laudanine (**2D**)	p	5.08	9.30	4.83[e]	10.77	5.84[e]	4.54[e]
	n	7.23	11.83	5.90[e]	14.59	6.75[e]	4.88[e]
(±)-[*N*-CD_3]-Codamine (**3D**)	p	6.26	11.40	7.33	10.44	9.64	9.64
	n	7.23	13.21	7.71	13.21	10.27	10.27
(±)-([*N*-CD_3]-Pseudolaudanine (**4D**)	p	6.50	10.47	7.67	8.73	9.28	9.27
	n	7.02	11.48	8.02	11.60	11.66	11.66
(+)-([*N*-CD_3]-Pseudocodamine (**5D**)	p	4.73	8.90	n.d.	8.79	9.53	9.52
	n	6.53	11.14		15.88	10.18	10.17
(+)([*N*-CD_3]-Reticuline (**6D**)	p	7.33	11.43	n.s.	15.23	9.50	9.50
	n	10.58	14.67		17.69	10.49	10.41
(+)-([*N*-CD_3]-Pseudosinomenine (**7D**)	p	7.28	11.63	n.s.	13.60	n.s.	n.s.
	n	8.28	11.98		15.08		
(+)-([*N*-CD_3]-6-*O*-Demethylcodamine (**8D**)	p	n.d.	9.46	n.d.	14.59	n.d.	n.d.
	n		10.06		16.66		
(+)-([*N*-CD_3]-Isoorientaline (**9D**)	p	n.d.	9.73	n.d.	12.22	n.d.	n.d.
	n		11.42		16.65		

[a] LC-CD analyses were carried out on a chiral phase column at 236 nm. Chromatographic separations were performed using a Jasco PU-2080Plus intelligent pump with a column oven (Jasco 860-CO), Jasco Browin NT, HSS-2000 data processor, and Jasco CD-2095Plus CD chiral detector, sample injection: 5 μg.
[b] [*N*-CD_3]-1-Benzyltetrahydroisoquinolines **(1D–9D)** were prepared previously (Cui et al., 2007).
[c] Detection: 236 nm; p: positive; n: negative.
[d] n-He: *N*-hexane; IPA: isopropanol; Et: ethanol; DEA: diethylamine.
[e] Flow rate: 1.0 mL/min.
[f] Flow rate: 0.5 mL/min.
[g] n.d.: not detected; n.s.: not separated.

Table 2
(*R*/*S*) Ratios of enantiomers of (+)-[*N*-CD_3]-1-benzyltetrahydroisoquinolines [(±)-**1D**–(±)-**9D**].

Compounds[a]	(*R*/*S*) Ratios[b]			
	LC/CD[c]		LC/MS[d]	
	From UV	From CD	From TIC[e]	From selected ions[f]
(±)-[*N*-CD_3]-Tetrahydropapaverine (**1D**)	49/51	42/58	54/46	55/45
(±)-[*N*-CD_3]-Laudanine (**2D**)	51/49	43/57	52/48	54/46
(±)-[*N*-CD_3]-Codamine (**3D**)	50/50	40/60	49/51	50/50
(±)-[*N*-CD_3]-Pseudolaudanine (**4D**)	50/50	43/57	53/47	52/48
(±)-[*N*-CD_3]-Pseudocodamine (**5D**)	50/50	45/55	53/47	54/46
(±)-[*N*-CD_3]-Reticuline (**6D**)	51/49	42/58	49/51	50/50
(±)-[*N*-CD_3]-Pseudosinomenine (**7D**)	n.d.	43/57	n.d.	n.d.
(±)-[*N*-CD_3]-6-*O*-Demethylcodamine (**8D**)	n.d.	33/67	n.d.	n.d.
(±)-[*N*-CD_3]-Isoorientaline (**9D**)	50/50	44/56	52/48	53/47

[a] These compounds have been prepared (Cui et al., 2007).
[b] Column: chiralcel OJ-H; *N*-hexane/EtOH/DEA 50/50/0.1; n.d.: not detected.
[c] Flow rate: 0.5 mL/min 236 nm.
[d] LC/MS: Applied Biosystems API 3000; pump: shimazu LC-10ADvp; column: chiralcel OJ-H (4.6 × 250 mm); fluent: *N*-hexane/ethanol/diethylamine: 50/50/0.1; flow rate: 1 mL/min; detection: 254 nm:1.0 mL/min 254 nm.
[e] TIC: total ion chromatogram.
[f] Protonated molecular ion of **(±)-1D–**(±)-**9D**.

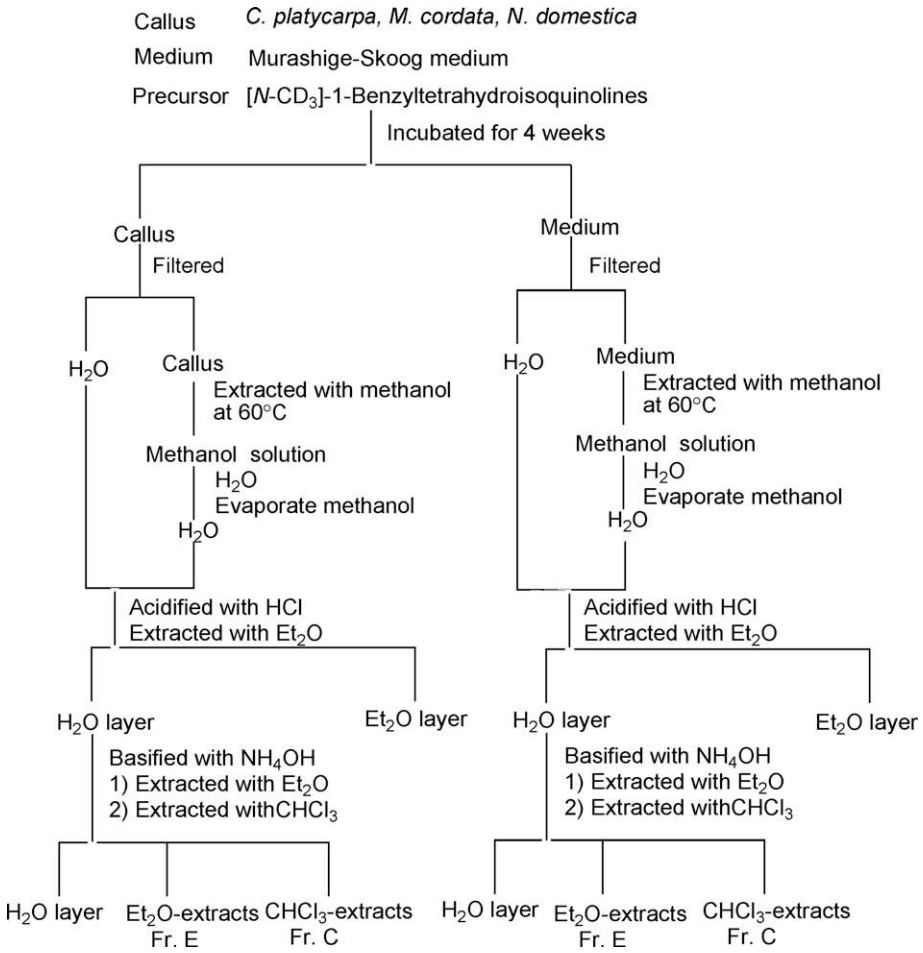


Fig. 2. Preparation of samples for LC/MS measurement.

(*S*) enantiomer of **4D**. The (*R*/*S*) ratios of 1/9 observed in the *O*-methylation of (±)-[*N*-CD_3]-pseudocodamine (**5D**) suggests that (*S*)-**5D** was predominantly *O*-methylated to produce (*S*)-**1D**.

The (*R*/*S*) ratios of the intermediate metabolite (*m*/*z* 347) as well as those of **1D** (*m*/*z* 361) were measured (Table 3, Experiment Nos. 5–8). In *C. platycarpa*, the (*R*/*S*) ratios of the mono-*O*-methylated metabolites (**2D**, **2D**, **3D**, and **5D**) of (±)-[*N*-CD_3]-reticuline, -pseudosinomenine, 6-*O*-demethylcodamine, and -isoorientaline (**6D**–**9D**) were ca. 3/1, 9/1, 1/5, and 1/1, and the (*R*/*S*) ratios of **1D** were ca. 1/1, 1/1, 1/1, and 1/4, respectively.

It has been demonstrated that (±)-reticuline (**6**) and (±)-pseudosinomenine (**7**) were bioconverted to (*S*)-scoulerine and (*S*)-

Table 3
(*R*/*S*) Ratios of metabolites obtained from intensity of peaks of selected ion in LC-MS of ether-soluble alkaloid fraction in feeding experiments of (±)-**2D**–(±)-**9D**.

Experiment No.	Precursors	Cultured cells[a]		Metabolites[b]	
				2D or **3D** or **5D** (*R*/*S*): *m*/*z* 347	**1D** (*R*/*S*): *m*/*z* 361
Corydalis platycarpa					
1	(±)-[*N*-CD_3]-Laudanine (**2D**)	A	m		48/52
		A	c		56/44
2	(±)-[*N*-CD_3]-Codamine (**3D**)	A	m		54/46
		A	c		68/32
3	(±)-([*N*-CD_3]-Pseudolaudanine (**4D**)	A	m		77/23
		A	c		75/25
4	(±)-([*N*-CD_3]-Pseudocodamine (**5D**)	A	m		7/93
		A	c		11/89
5	(±)-([*N*-CD_3]-Reticuline (**6D**)	A	m	82/18[c]	48/52
		A	c	71/29[c]	54/46
6	(±)-([*N*-CD_3]-Pseudosinomenine (**7D**)	A	m	90/10[c]	43/57
		A	c	97/3[c]	51/49
7	(±)-([*N*-CD_3]-6-*O*-Demethylcodamine (**8D**)	A	m	11/89[d]	44/56
		A	c	21/79[d]	50/50
8	(±)-([*N*-CD_3]-Isoorientaline (**9D**)	A	m	45/55[e]	15/85
		A	c	45/55[e]	19/81
Macleaya cordata					
9	(±)-[*N*-CD_3]-Laudanine (**2D**)	B	m		51/49
		B	c		52/48
10	(±)-[*N*-CD_3]-Codamine (**3D**)	B	m		49/51
		B	c		47/53
11	(±)-([*N*-CD_3]-Pseudocodamine (**5D**)	B	m		3/97
		B	c		6/94
Nandina domestica					
12	(±)-[*N*-CD_3]-Laudanine (**2D**)	C	c;m		44/56
13	(±)-([*N*-CD_3]-Pseudocodamine (**5D**)	C	c;m		2/98
14	(±)-([*N*-CD_3]-Reticuline (**6D**)	C	c;m	60/40[c]	48/52
15	(±)-([*N*-CD_3]-Pseudosinomenine (**7D**)	C	c;m	45/55[c]	41/59

[a] A: *Corydalis platycarpa*; B: *Macleaya cordata*; C: *Nandina domestica*; m: medium; c: callus; c;m: callus;medium.
[b] LC/MS conditions: Applied Biosystems API 3000; pump: shimazu LC-10Advp; column: chiralcel OJ-H (4.6 × 250 mm); fluent: *N*-hexane/ethanol/diethylamine 50/50/0.1; flow rate: 1 mL/min; detection: 254 nm.
[c] (*R*/*S*) ratio of **2D**.
[d] (*R*/*S*) ratio of **3D**.
[e] (*R*/*S*) ratio of **5D**.

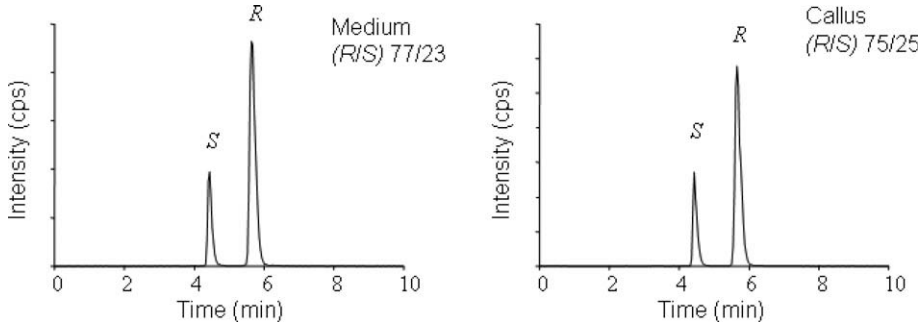


Fig. 3. (*R*/*S*) Ratios in mass chromatograms of the selected ion at *m*/*z* 361([*N*-CD_3]-tetrahydropapaverine, **ID**) in LC-MS of ether-soluble alkaloid fractions (Fr. E) obtained from callus and medium, respectively in feeding experiment of (±)-[*N*-CD_3]-pseudolaudanine (**4D**) to cell cultures of *Corydalis platycarpa*. LC/MS: Applied Biosystems API 3000; pump: shimazu LC-lOADvp; column: chiralcel OJ-H (4.6 × 250 mm) fluent: *N*-hexane/ethanol/diethylamine: 50/50/0.1; flow rate: 1 mL/min; detection: 254 nm.

tetrahydropalmatrubine (Cui et al., 2007). (±)-Reticuline (**6**) and (±)-pseudosinomenine (**7**) may be biotransformed via (*S*)-laudanine (**2**) to (*S*)-tetrahydroprotoberberines. The (*R*/*S*) ratios observed in the formation of **2D** from (±)-**6D** and (±)-**7D** may result from bioconversion of (*S*)-**2D** into (*S*)-tetrahydroprotoberberines. The (*R*/*S*) ratios of **3D** obtained from administration of (±)-**8D** cannot be explained until the *O*-methylation of (*R*)- and (*S*)-**3D** is clarified. The 6-*O*-methylated metabolite (**5D**) with (*R*/*S*) ratios of ca. 1/1 was produced from (±)-**9D** and (*S*)-**5D** was exclusively converted to (*S*)-**1D** as shown in Table 6. As the result, **1D** with (*R*/*S*) ratios of ca. 1/4 was obtained.

Experiments with (±)-[*N*-CD_3]-laudanine, -codamine, and -pseudocodamine (**2D**, **3D** and **5D**) in *M. cordata* gave (*R*/*S*) ratios of **1D** (Table 3, Experiment Nos. 9–11) that was almost identical with those obtained in *C. platycarpa* (Table 3).

Administration experiments (Table 4) of (±)-[*N*-CD_3]-laudanine (**2D**), -pseudocodamine (**5D**), -reticuline (**6D**), and -pseudosinomenine (**7D**) were carried out in *Nandina domestica* and the ether-soluble alkaloid fractions from these experiments were analyzed by LC-MS. The mass chromatograms at the selected ion of *m*/*z* 361(**1D**) in LC-MS of ether-soluble alkaloid fractions (Fr. E) obtained from administration of **2D** and **5D**–**7D** are shown in Fig. 4. The (*R*/*S*) ratios of **1D** (Table 3, Experiment Nos. 12–15) in experiments using **2D** and **5D**–**7D** in *N. domestica* were almost identical except for data for the mono-*O*-methylated metabolite (**2D**) with those obtained in *M. cordata* and *C. platycarpa* (Table 3). The differ-

Table 4
Feeding experiments of (±)-[*N*-CD_3]-laudanine, -pseudolaudanine, -reticuline, and -protosinomenine (**2D** and **5D**–**7D**) to cell cultures of *Nandina domestica* Thumb.

Experiment No.	Substrates[a] (mg)		Wt. of dry cells[b] (g)	Medium (mL)	Incubation period (week)	Alkaloid fractions (mg)[c]	
						Fr. E	Fr. C
12	(±)-[*N*-CD_3]-Laudanine (**2D**)	20	9.73	800	4	11.4	21.1
13	(±)-([*N*-CD_3]-Pseudocodamine (**5D**)	20	7.22	800	4	10.3	23.3
14	(±)-([*N*-CD_3]-Reticuline (**6D**)	10	2.77	400	4	12.7	20.8
15	(±)-([*N*-CD_3]-Pseudosinomenine (**7D**)	10	2.19	400	4	9.6	14.2

[a] Concentration of substrate: (±)-**2D** and (±)-**5D** 72 μM, (±)-**6D** and (±)-**7D** 75 μM.
[b] Calli of 80–100 g were used in experiments 1 and 2. Calli of 40–50 g were used in Experiment Nos. 3 and 4.
[c] Fr. E: ether-soluble alkaloid fraction; Fr. C: chloroform-soluble alkaloid fraction.

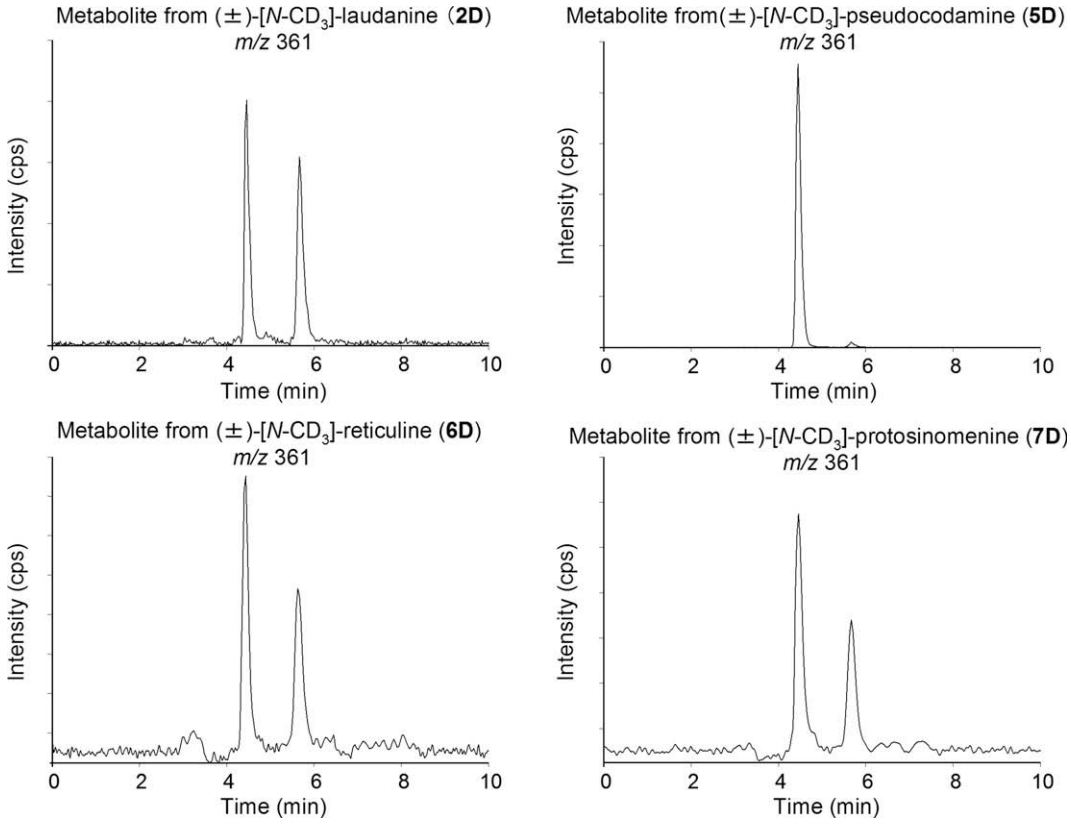


Fig. 4. LC/MS data of metabolite (**1D**) obtained from administration of (±)-[*N*-CD_3]-1-benzyltetrahydroisoquinoline-type alkaloids ([*N*-CD_3]-**2**, [*N*-CD_3]-**5**, [*N*-CD_3]-**6** and [*N*-CD_3]-**7**) to cell cultures of *Nandina domestica*. Column: chiralcel OJ-H (4.6 × 250 mm); eluent: *N*-hexane/ethanol/diethylamine: 50:50:0.1; flow rate: 1 mL/min; detection: 254 nm.

ence in the (*R*/*S*) ratios of **2D** between *N. domestica* and *C. platycarpa* may result from differences in the bioconversions of **6D** and **7D** into the protoberberines.

The above results indicate that both (*R*) and (*S*) enantiomers of **2D**–**9D** were utilized as substrates in cell cultures of *C. platycarpa*, *M. cordata*, and *N. domestica* to produce *O*-methylated metabolites, although the enantioselectivities of the corresponding *O*-methyltransferases differ.

*2.3. O-Methylation of (R)- or (S)-enantiomers of [N-CD3]-laudanine, -pseudolaudanine, and -pseudocodamine (**2D**, **4D**, and **5D**) in cell cultures of C. platycarpa*

LC-MS analyses were carried out on ether-soluble alkaloid fractions (Fig. 2, Fr. E) obtained from administration of deuterated (*R*)- and (*S*)-laudanine (**2D**), -pseudolaudanine (**4D**), and -pseudocodamine (**5D**) to cell cultures of *C. platycarpa* (Table 5). The enantioselectivity of the *O*-methyltransferases was determined from the (*R*/*S*) ratios and intensities of the selected ion (protonated molecular ion: *m*/*z* 361) of **1D** in the LC-MS which are shown in Table 6.

The ratio of 3′-*O*-methylated metabolite [(*R*)-**1D**/(*S*)-**1D**] was ca. 1/1 for both precursors (*S*)- and (*R*) -[*N*-CD_3]-laudanine (**2D**). The optical purities of recovered **2D** precursors were essentially unchanged compared with those before precursor uptake. These results suggest that inversion of configuration at C-1 may occur in (*R*)- and (*S*)-**2D**. Then 3′-*O*-methyltransferase catalyzes metabolism of both (*R*)- and (*S*)- forms of **2D** to produce almost identical amounts of *O*-methylated metabolites with (*R*) and (*S*) configurations, respectively.

(*S*)-**1D** (95%) and (*R*)-**1D** (97%) were produced exclusively from separate administration experiments of (*S*)-**4D** (95% optical purity) and (*R*)-**4D** (89% optical purity), respectively (Table 6). This result suggests that both (*R*)- and (*S*)-[*N*-CD_3]-pseudolaudanine (**4D**) are good substrates for the 6-*O*-methyltransferase.

Likewise, (*S*)-configured **1D** (99% optical purity) exclusively resulted from (*S*)-[*N*-CD_3]-pseudocodamine (**5D**) (Table 6), whereas

Table 5
Administration of (*R*) and (*S*) enantiomers of [*N*-CD_3]-laudanine, -pseudolaudanine, and -pseudocodamine (**2D**, **4D**, and **5D**) to cell cultures of *Corydalis platycarpa*.

Substrates[a] (mg)		Wt. of dry cells[b] (g)	Medium (mL)	Incubation period (week)	Alkaloid fractions (mg)[c]	
					Fr. E	Fr. C
(*S*)-**2D** (optical purity 91%)	3.4	2.03	400	4	7.4	9.2
(*R*)-**2D** (optical purity 91%)	3.4	1.64	400	4	5.9	9.0
(*S*)-**4D** (optical purity 95%)	1.6	2.13	400	4	11.1	9.6
(*R*)-**4D** (optical purity 89%)	1.6	3.28	400	4	7.7	6.2
(*S*)-**5D** (optical purity 99%)	5.5	2.76	400	4	16.8	15.1
(*R*)-**5D** (optical purity 93%)	5.5	2.44	400	4	14.8	16.6

[a] Concentration of substrate: (*S*) or (*R*)-**2D** 25 μM, (*S*) or (*R*)-**4D** 11 μM, (*S*) or (*R*)-**5D** 40 μM.
[b] Calli of 40–50 g were used in each experiment.
[c] Fr. E: ether-soluble alkaloid fraction; Fr. C: chloroform-soluble alkaloid fraction.

Table 6
R/*S* ratio of metabolite (**1D**) obtained from intensity of peaks of selected ion (*m*/*z* 361) in LC-MS of ether-soluble alkaloid fraction in feeding experiments of (*R*) and (*S*) enantiomers of [*N*-CD_3]-laudanine, -pseudolaudanine, and -pseudocodamine (**2D**, **4D**, and **5D**) to cell cultures of *Corydalis platycarpa*.

Precursors	Metabolite[a]	
	R/*S* (*m*/*z* 361)	Intensity (cps)[b]
(*S*)-**2D**	47/53	9.70E+6/1.11E+7[c]
(*R*)-**2D**	50/50	9.21E+6/9.20E+6[c]
(*S*)-**4D**	5/95	2.31E+6/4.87E+7[d]
(*R*)-**4D**	97/3	6.93E+7/2.06E+6[d]
(*S*)-**5D**	1/99	2.74E+6/4.25E+8[d]
(*R*)-**5D**	51/49	1.66E+7/1.76E+7[c]

[a] [*N*-CD_3]-Tetrahydropapaverine (**1D**).
[b] cps: (count of ions) per second.
[c] Intensity on ether-soluble alkaloid fraction (1000 ng).
[d] Intensity on ether-soluble alkaloid fraction (500 ng).

racemic **1D** was detected with (*R*)-**5D** (93% optical purity). The production of (*S*)-**1D** from (*S*)-**5D** was about 50 times larger than that of (*R*)-**1D** from (*R*)-**5D**, as determined from the intensity of the selected ion in the LC-MS. These results suggest highly enantioselective and efficient production (4′-*O*-methylation) of (*S*)-**1D** from (*S*)-**5D**, although conversion of (*R*)-**5D** to (*R*)-**1D** may also occur.

3. Conclusion

Enantiomers of 1-benzyl-*N*-methyltetrahydroisoquinolines were separated using Chiralcel OD-H and OJ-H columns. Online LC-CD analyses allowed determination of the absolute configurations of the separated 1-benzyl-*N*-methyltetrahydroisoquinolines. Chiral purity determination using an LC-MS method was, as or more accurate than that using the LC-CD method. The LC-MS method combined with the LC-CD analyses permitted determination of the enantiomeric purities of the *O*-methylated metabolites of phenolic 1-benzyl-*N*-methyltetrahydroisoquinolines administered to cell cultures of *Corydalis*, *Macleaya*, and *Nandina* species. These methods were developed to determine the enantioselectivity of the methyltransferases involved in the biosynthesis of these alkaloids. This technique can be used to predict the absolute configuration of natural 1-benzyl-*N*-methyltetrahydroisoquinolines in the extract matrix solution.

4. Experimental

4.1. Materials

In 1974, 1989, and 2003, calli of *M. cordata*, *C. platycarpa* Makino, and *N. domestica*, respectively, were derived from the 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 three or four weeks on fresh medium at 25 (*M. cordata* and *C. platycarpa*) or 27 °C (*N. domestica*) in the dark. [*N*-CD_3]-1-Benzyltetrahydroisoquinolines (**1D**–**9D**) were prepared previously (Cui et al., 2007). The natural product (*S*)-reticuline (**6**) {$[\alpha]_D$ +57 (*c* 1.2, $CHCl_3$)} was obtained from *Uvaria lucida* (Ichimaru et al., 1997).

4.2. HPLC parameters for LC-MS, and LC-CD

Chiral analytical separations were carried out using Chiralcel OD-H and OJ-H and Chiralpak IB (4.6 i.d. × 250 mm, Daicel Chemical Ltd.) columns at 40 °C for LC-MS and LC-CD. The mobile phases [*N*-hexane/EtOH/Et_2NH or *N*-hexane/iPrOH/Et_2NH: 50/50/0.1] were used for isocratic elution. The flow rate was 0.5 or 1 mL/min (detection: LC-MS 254 nm, LC-CD 236 nm).

4.3. LC/APCI-MS method

LC/APCI-MS (/MS) spectra were measured using an Applied Biosystems API 3000 Triple Quadrapole mass spectrometer (MS/MS) with a heated nebulizer interface as described previously (Iwasa et al., 2005).

4.4. LC-CD method

LC-CD analyses were carried out on a chiral phase column at 236 nm. Chromatographic separations were performed using a Jasco PU-2080Plus intelligent pump with a column oven (Jasco 860-CO), Jasco Browin NT, HSS-2000 data processor, and Jasco CD-2095Plus CD chiral detector (Hg–Xe lamp), simultaneously monitoring the CD and UV signals at one specific wavelength (range 220–420 nm).

4.5. 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 Murashige & Skoog's medium, identical with that employed in the subculture. Calli (ca. 4–5 g) were transferred to each conical flask and incubated at either 25 or 27 °C in the dark for four weeks (Tables 4 and 5). Cells and medium were extracted with CH_3OH at 60 °C. Extracts were worked-up as described in Fig. 2 (Iwasa et al., 2003). Administration of (±)-[*N*-CD_3]-1-benzyl-*N*-methyltetrahydroisoquinolines (**2D–9D**) to cell cultures of *Corydalis platycarpa* and *M. cordata* were described previously (Cui et al., 2007).

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