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LC–NMR and LC–MS analysis of 2,3,10,11-oxygenated protoberberine metabolites in *Corydalis* cell cultures

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Abstract

The metabolism of 2,3,10,11-oxygenated protoberberine alkaloids was studied in cell cultures of *Corydalis* species. Without prior isolation, the structures of the metabolites were determined by LC–MS and LC–NMR analyses. Tetrahydropseudocoptisine α-*N*-metho salt, pseudoprotopine, and pseudomuramine were identified for the first time, and preliminary evidence for metabolic pathways to the formation of these alkaloids were obtained.

Keywords: *Corydalis platycarpa*; *Corydalis ochotensis* var. *raddeana*; Fumariaceae; Cell cultures; Biotransformation; LC–MS; LC–NMR; Secondary metabolism; Protoberberine alkaloids

1. Introduction

Protoberberine alkaloids differ from each other in the number and placement of various oxygen functions on the aromatic rings. The two oxygenation patterns most frequently are oxygen atoms at carbons 2,3,9,10 and 2,3,10,11. The former is the most commonly occurring type, while the latter has been labeled "pseudoprotoberberine", and is not as widespread in its occurrence (Preininger, 1986). Some representatives of the 2,3,10,11-oxygenated alkaloids display higher activity in some biological tests (e.g., antimalarial activity) than the corresponding 2,3,9,10-substituted alkaloids (Iwasa et al., 1999). While the biosynthetic conversion of the 2,3,9,10-oxygenated protoberberines into other alkaloidal types, such as the protopines, benzophenanthridines, rhoeadines, benzindanoazepines, and spirobenzylisoquinolines has been demonstrated (Zenk, 1994; Iwasa, 1995), no studies on the biosynthesis of 2,3,10,11-oxygenated protoberberines have been presented, in spite of their occurrence in some plant species including *Corydalis* species (Preininger, 1986). This paper describes the structural analysis of the metabolites obtained from cell cultures of *Corydalis* species administered with 2,3,10,11-oxygenated protoberberines (pseudoprotoberberine). Metabolites were identified by LC–MS and LC–NMR (Wolfender et al., 2001). These complimentary techniques enabled the rapid determination of their structures without the isolation of individual metabolites. This analysis is valuable to detect compounds with interesting structural features and to target their isolation (by preparative HPLC). There is no application thus far of LC–NMR to biosynthetic studies. On the other hand, application of LC–NMR to drug metabolism, natural products identification and characterization of isomeric mixtures produced by chemical reactions have been reviewed (Hostettmann et al., 1997; Wolfender et al., 1998, 2001).

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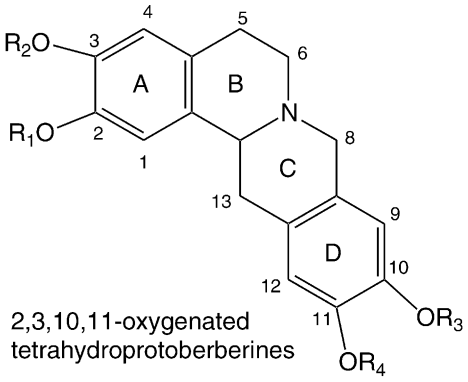

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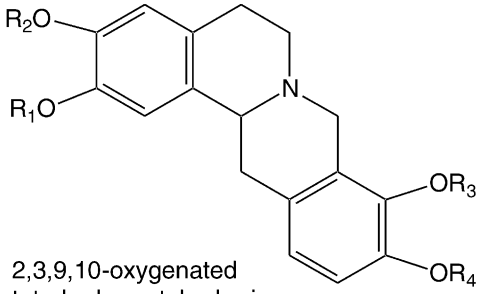

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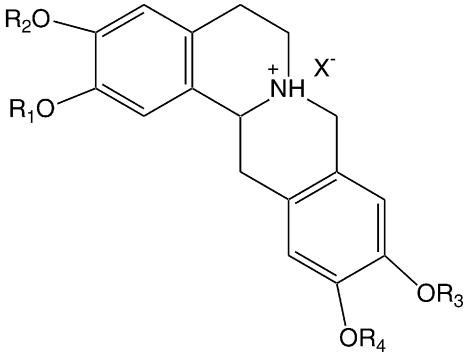
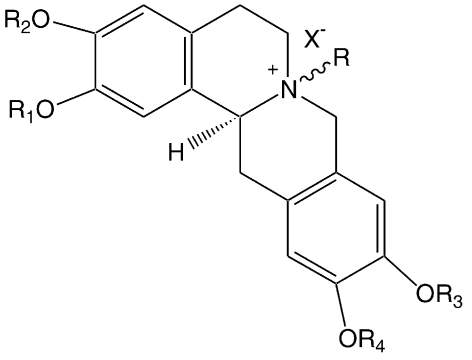
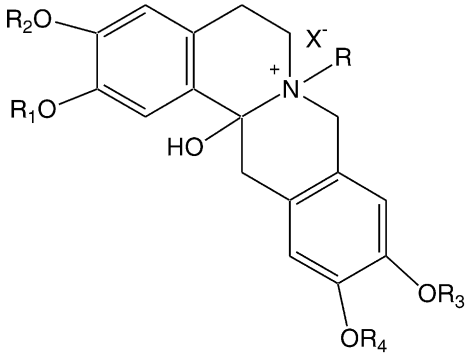


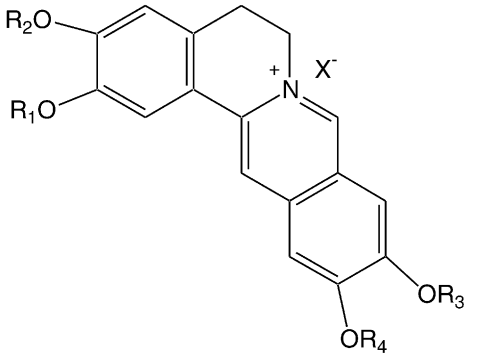
1 R_1+ R_2 = R_3+ R_4 = CH_2
2 R_1 = R_2 = R_3 = R_4 = CH_3



3a R_1+ R_2 = R_3+ R_4 = CH_2 R = ····· CH_3
3aD R_1+ R_2 = R_3+ R_4 = CH_2 R = ····· CD_3
3b R_1+ R_2 = R_3+ R_4 = CH_2 R = ▬ CH_3
3bD R_1+ R_2 = R_3+ R_4 = CH_2 R = ▬ CD_3
4a R_1 = R_2 = R_3 = R_4 = CH_3 R = ····· CH_3
4aD R_1 = R_2 = R_3 = R_4 = CH_3 R = ····· CD_3
4b R_1 = R_2 = R_3 = R_4 = CH_3 R = ▬ CH_3
4bD R_1 = R_2 = R_3 = R_4 = CH_3 R = ▬ CD_3



5 R_1+ R_2 = R_3+ R_4 = CH_2 ; R = CH_3
5D R_1+ R_2 = R_3+ R_4 = CH_2 ; R = CD_3
8 R_1 = R_2 = R_3 = R_4 = CH_3 ; R = CH_3
8D R_1 = R_2 = R_3 = R_4 = CH_3 ; R = CD_3



6 R_1+ R_2 = R_3+ R_4 = CH_2
7 R_1 = R_2 = R_3 = R_4 = CH_3

2. Results and discussion

2.1. Synthesis

The pseudoprotoberberine precursor, tetrahydropseudocoptisine **1** (Lenz, 1977) was synthesized from 2,3,10,11-tetrademethyltetrahydropseudopalmatine. The latter was prepared from tetrahydropseudopalmatine (another pseudoprotoberberine precursor) **2** (Lenz, 1977), which, in turn, was derived from tetrahydropapaverine. The α-*N*-metho salts **3a** and **4a** were obtained by treating **1** and **2** with methyl iodide, in addition to the β-*N*-metho salts **3b** and **4b**. Pseudoprotoberberines, **6** and **7** (Lenz, 1977) were prepared by dehydrogenation of **1** and **2** with Pd/C, respectively. The structures of synthetic compounds were determined by analysis MS, ^{1}H NMR, and NOESY spectral data. The deuterated *N*-metho salts (**3aD** and **4aD**) were prepared by treating **1** and **2** with deuterated methyl iodide. The ^{1}H NMR and MS spectral data of the *N*-metho salts are summarized in Tables 1 and 2, respectively.

2.2. LC–MS and LC–NMR

The LC-APCI/MS were measured with SIM (selected ion monitoring) and TIM (total ion monitoring) in the positive ion mode. Molecular weight information was obtained on the basis of a protonated ion $[M+H]^+$, or a cluster ion $[M+HCF_3]^+$ recorded in the LC–APCI/MS. The LC–NMR spectra were measured by the stopped-flow mode (Smallcombe et al., 1995).

2.3. Precursor administration

Callus tissues of *Corydalis platycarpa* Makino or *Corydalis ochotensis* var. *raddeana* were incubated at 25 °C on an agar medium for **1** and **2** or in a liquid medium for **3aD** and **4aD** containing the substrate for an appropriate time (Table 3). Following incubation, media and cells were individually extracted according to the procedure shown in Fig. 1. The alkaloid fractions, E-1, E-2, C-1, and C-2 (Fig. 1), which are organic soluble, were subjected to LC–MS and LC–NMR.

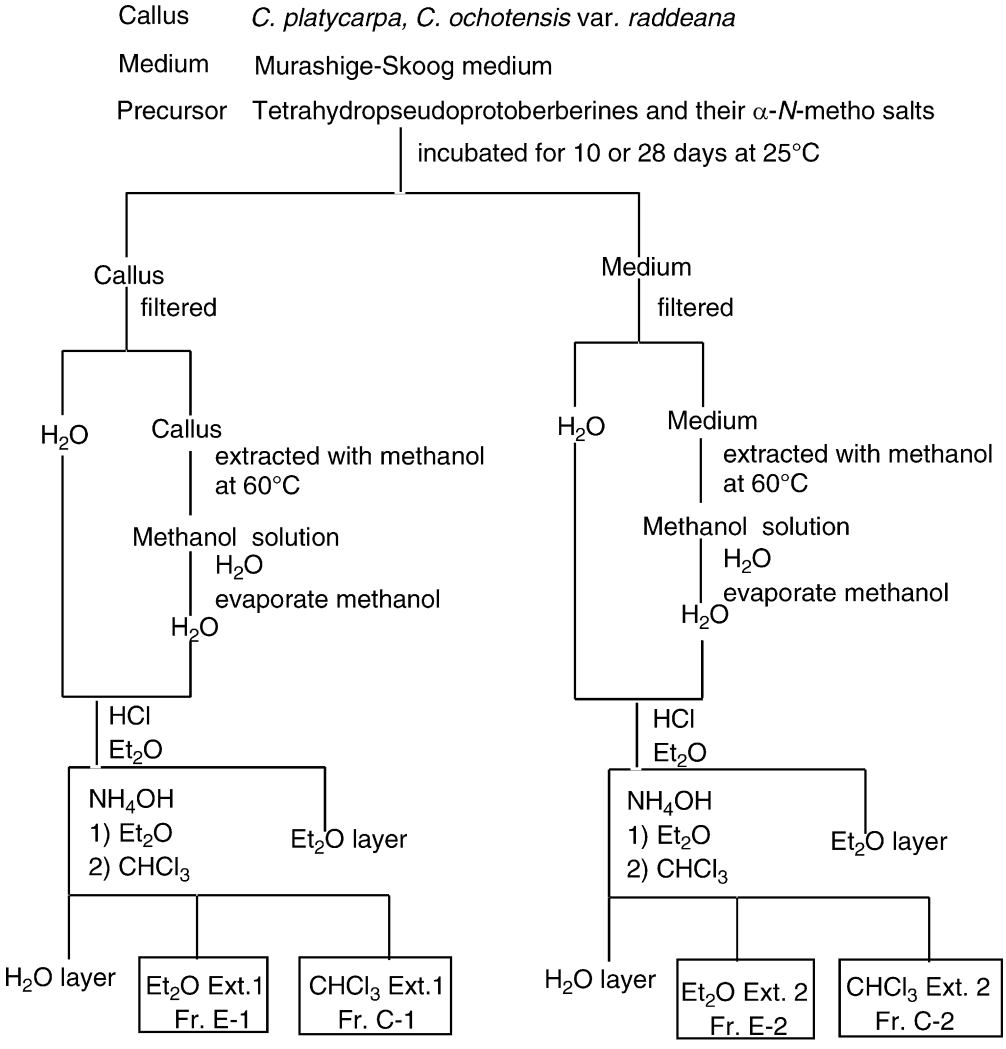
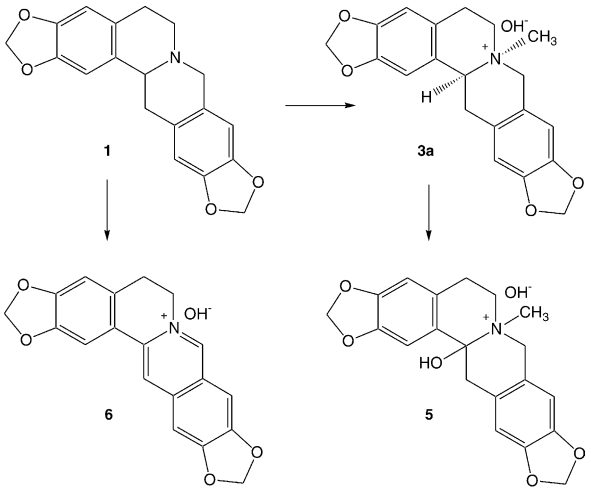
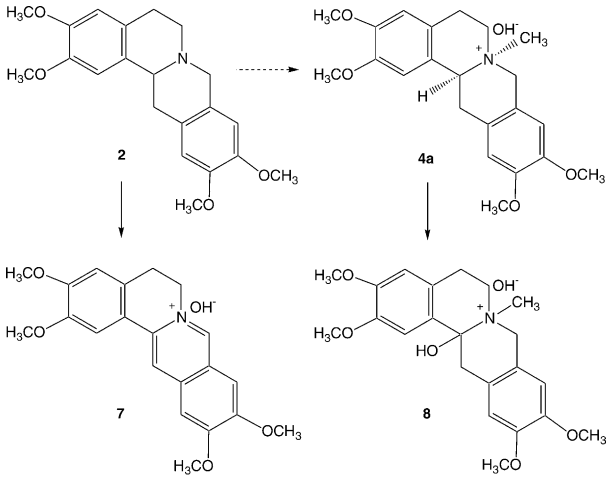


Fig. 1. Preparation of samples for LC–NMR and LC–MS measurements.



Scheme 1.



Scheme 2.

Table 1
^{1}H NMR spectral data[a] of synthetic pseudoprotoberberine alkaloids (CD_3OD; δ ppm, 500 MHz)

	1-H	4-H	9-H	12-H	OCH_2O	OMe	5-H	6-H	8-H	13-H	H-13a	N-Me
3a	6.79	6.80	6.70	6.72	5.99 5.97		3.28 *m* 3.24 *m*	3.83 *m* 3.52 *m*	4.77[b] 4.61[b]	3.44 *dd* (18; 5.5) 3.09 *dd* (18; 10)	4.74 *dd* (10; 5.5)	3.23
3b	6.98	6.80	6.73	6.89	6.00 5.99		3.39 *m* 3.14 *m*	3.91 *m* 3.84 *m*	4.74[b] 4.58[b]	3.91 *m* 3.03 *dd* (18; 12)	5.0 *dd* (12; 5.5)	2.94
4a	6.88	6.90	6.80	6.82		3.851, 3.847 3.83, 3.82	3.24–3.4 (3H)	*m* 3.53 *m*	[c] 4.65[b]	3.50 *dd* (18; 6) 3.12 *dd* (18; 10.5)	4.77 *dd* (10.5; 6)	3.24
4b	7.03	6.91	6.84	6.98		3.90, 3.88 3.87, 3.86	3.43 *ddd* (17; 12, 6) 3.18 *dm* (17)	3.97 *dd* (12; 6) 3.91 *dd* (12; 5)	4.80[b] 4.64[b]	4.06 *dd* (17.5; 5) 3.09 *dd* (17.5; 12)	5.07 *dd* (12; 5)	2.96

[a] Coupling constant (Hz, in parentheses).
[b] Doublet, $J = 14.5$–15.5 Hz.
[c] Overlapped with H_2O.

2.3.1. Administration of tetrahydropseudocoptisine **1**

Tetrahydropseudocoptisine **1** was administered to the cultured cells of *C. platycarpa* and *C. ochotensis* var. *raddeana*. Fr. E-2 (see Fig. 1) from each culture both afforded peaks 1, 2, and 3 in the LC which displayed a protonated ion, or a corresponding cluster ion, at *m/z* 354 ($[M+H]^+$), 390 ($[H+HCF_3]^+$), and 324 ($[M+H]^+$), respectively, in the APCI/MS.

The stopped-flow ^{1}H NMR spectra of peak 1 showed two *N*-methyl groups at δ 2.91 and 3.00 (ratio ca. 2:1) revealing *N*-protonation, two methylendioxy groups at δ 5.97 and 5.94, and four aromatic protons at δ 7.04 and 7.08 (1H), 6.80 and 6.75 (2H), and 6.71 and 6.66 (1H each) (Fig. 2). Peak 1 had a protonated ion at *m/z* 354 in the APCI/MS. On the basis of this evidence, peak 1 was recognized to be the trifluoroacetate of pseudoprotopine **5**.

The stopped-flow ^{1}H NMR spectra of peak 2 displayed six aromatic protons as singlets at δ 9.10, 8.36, 7.51, 7.47, 7.41, and 6.92, two methylenedioxy groups at δ6.25 and 6.05, and two methylene protons as triplets at δ 4.65 and 3.13. These data indicate that the structure represented by peak 2 is the trifluoroacetate of pseudocoptisine **6**. This assignment was supported by the APCI/MS data which showed a cluster ion at *m/z* 390 $[M+HCF_3]^+$. Comparison of the stopped-flow ^{1}H

Table 2
Mass spectral data of synthetic pseudoprotoberberine alkaloids

	formula	LSIMS[a] *m/z* [M−X]+	HR-LSIMS	
			Calc.	Found
3a	$C_{20}H_{20}NO_4$	338	338.1391	338.1387
3aD	$C_{20}H_{17}D_3NO_4$	341	341.1580	341.1574
3b	$C_{20}H_{20}NO_4$	338	338.1391	338.1372
3bD	$C_{20}H_{17}D_3NO_4$	341	341.1580	341.1582
4a	$C_{22}H_{28}NO_4$	370	370.2014	370.2020
4aD	$C_{22}H_{25}D_3NO_4$	373	373.2205	373.2213
4b	$C_{22}H_{28}NO_4$	370	370.2014	370.2031
4bD	$C_{22}H_{25}D_3NO_4$	373	373.2206	373.2208

[a] X: Cl^- ; I^- ; CF_3COO^-.

NMR spectral data with those of synthetic **6** measured under the same conditions confirmed the identification.

The stopped-flow ^{1}H NMR spectra of peak 3 exhibited four aromatic protons, two methylenedioxy protons, and nine aliphatic protons in the region of δ 3.7 and 2.7. The APCI/MS analysis of peak 3 showed a protonated ion at *m*/*z* 324. These data identify peak 3 as the trifluoroacetate of tetrahydropseudocoptisine **1**.

Fr. C-1 (see Fig. 1) obtained from each culture both afforded peaks 4 and 5 in the LC, which displayed a protonated ion and a cluster ion at *m*/*z* 338 and 390, respectively, in the APCI/MS. The stopped-flow ^{1}H NMR spectrum of peak 4 show four aromatic protons at δ 6.78, 6.75, 6.70, and 6.67, two methylenedioxy protons at δ 5.95 and 5.93, one methyl signal at δ 3.13, and nine aliphatic protons between δ 4.8 and 2.9 (Fig. 3). The structure of peak 4 was deduced to be the α *N*-metho salt of tetrahydropseudocoptisine **1**. Its stopped-flow ^{1}H NMR spectrum was identical to that of tetrahydropseudocoptisine α-*N*-metho salt **3a** and not that of

Table 3
Administration of tetrahydropseudoprotoberberines **1**, **2**, **3aD**, and **4aD** to cell cultures of *Corydalis platycarpa* and *Corydalis ochotensis* var. *raddeana*

Substrates (mg)		Callus[a]	Weight of dry cells (g)	Medium (ml)	Incubation time (days)	Weight of fractions (mg)			
						Fr. E-1	Fr. E-2	Fr. C-1	Fr. C-2
1	60[d]	A[b]	1.58	200	10	9.6	14.6	5.4	9.0
1	60[d]	B[b]	2.16	200	10	7.2	/	6.7	3.4
3aD	48	A[c]	5.02	800	28	22.2	14.1	8.0	4.5
3aD	48	B[c]	5.70	800	28	6.5	3.8	4.6	2.0
2	60[d]	A[b]	2.51	200	10	12.1	/	6.7	18.1
2	60[d]	B[b]	2.04	200	10	10.8	/	6.4	8.8
4aD	30	A[c]	4.19	800	28	20.4	8.1	15.5	12.0
4aD	30	B[c]	4.76	800	28	13.6	12.7	11.9	9.1

[a] Callus-derived plants A: *C.platycarpa*; B: *C. ochotensis* var. *raddeana*.
[b] Suspension cultures.
[c] Static cultures.
[d] Addition of *S*-adenosyl-L-methionine (60 mg).

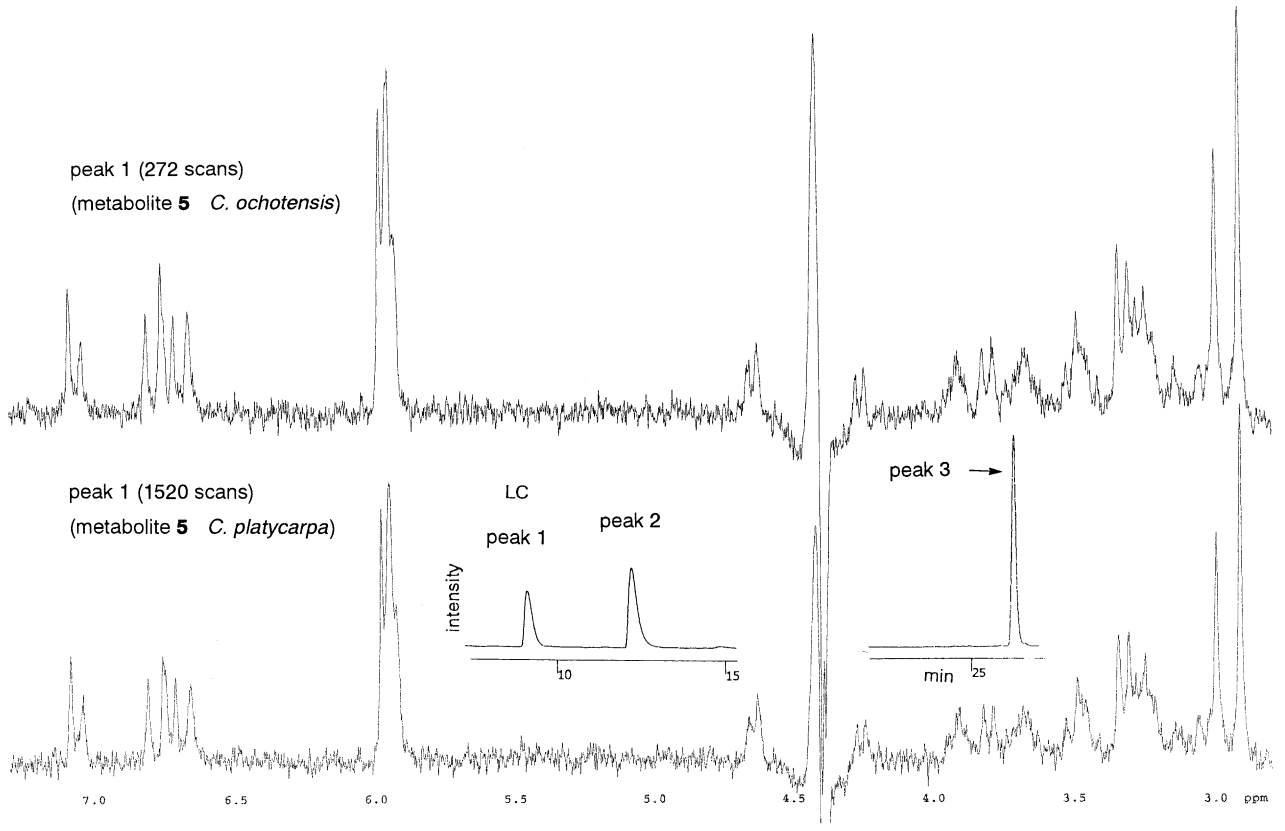


Fig. 2. Stopped-flow ^{1}H NMR spectra of peak 1 of Fr. E-2 obtained from feeding experiment of tetrahydropseudocoptisine **1**.

β-*N*-metho salt **3b**. Finally, peak 5 was found to represent the trifluoroacetate of pseudocoptisine **6** by LC–NMR and LC–MS analyses.

As a result of feeding experiments using tetrahydropseudocoptisine **1** as a precursor, in cultured cells of *C. platycarpa* and *C. ochotensis* var. *raddeana* it was determined that **1** was oxidized to produce pseudocoptisine **6** and was stereospecifically *N*-methylated to give rise to the α-*N*-metho salt **3a**, which could be oxidized to afford pseudoprotopine **5** (Scheme 1).

2.3.2. Biotransformation of 3a to 5

Administration experiments using [*N*-CD_3]-tetrahydropseudocoptisine α-*N*-metho salt **3aD** were conducted using cultured cells of *C. platycarpa* and *C. ochotensis* var. *raddeana* in order to clarify stereospecific conversion of **3a** to **5** (Table 3). An agar medium was employed in these administration experiments in order to extend the incubation period from 10 to 28 days. Fr. C-1 (see Fig. 1) obtained from each culture both afforded peaks 6 and 7 in the LC, which displayed protonated ions at *m/z* 357 and 341, respectively, in the APCI/MS. It was, therefore, likely that the ions at *m/z* 357 and 341 were due to deuterated pseudoprotopine **5D** and tetrahydropseudocoptisine α-*N*-metho salt **3aD**, respectively. The stopped-flow ^{1}H NMR spectrum corresponding to peak 6 was identical with that of protonated pseudoprotopine **5** (Section 2.3.1), except for the *N*-methyl group. The stopped-flow ^{1}H-NMRspectrum of peak 7 was identical with that of the precursor **3aD**. Alkaloid **3aD** therefore underwent oxidation to produce [*N*-CD_3]-pseudoprotopine **5D**.The metabolic conversions **1**→**3a**→**5** and **1**→**6** were thus demonstrated in the cultured cells of *C. platycarpa* and *C. ochotensis* var. *raddeana* (Scheme 1).

2.3.3. Administration of tetrahydropseudopalmatine 2

Administration of tetrahydropseudopalmatine **2** afforded peaks 8 and 9 in the LC of Fr. E-1 from each culture. Peaks 8 and 9 yielded a cluster ion and a protonated ion at *m/z* 422 and 356, respectively, in the APCI/MS. The stopped-flow ^{1}H NMR spectra corresponding to peak 8 displayed six aromatic protons as singlets at δ 9.17, 8.48, 7.58, 7.55, 7.48, and 7.04, four methoxyl groups at δ 4.06, 3.99, 3.93, and 3.88, and two methylene protons as triplets at δ 4.70 and 3.18. These data suggested that the structure of the metabolite in peak 8 was pseudopalmatine **7**. This suggestion was supported by the APCI/MS data (a cluster ion at *m/z* 422 $[M+HCF_3]^+$), and was confirmed by comparison of the stopped-flow ^{1}H NMR data with that of synthetic **7** measured under the same conditions. The stopped-flow ^{1}H NMR spectra derived from peak 9 in the LC exhibited four aromatic protons, four methoxyl groups, and nine aliphatic protons. APCIMS of peak 9 showed a protonated ion at *m/z* 356. Thus, peak 9 proved to be

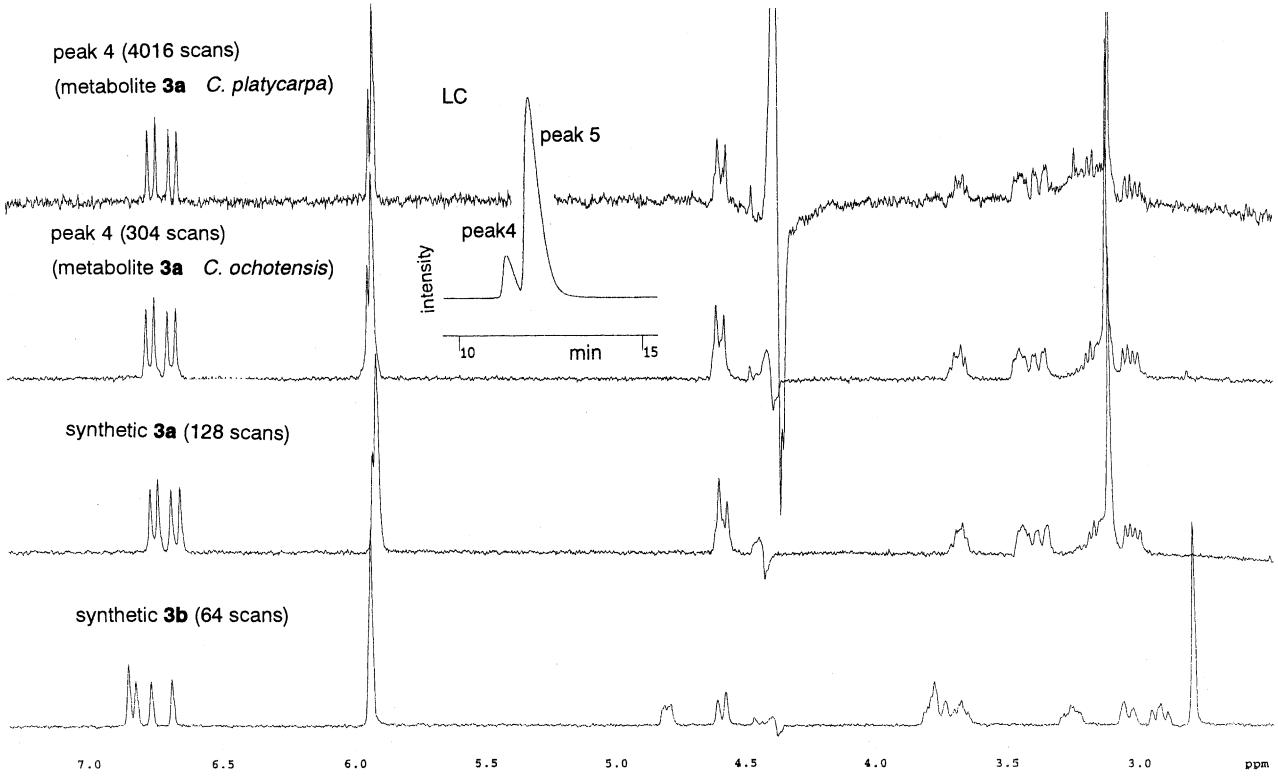


Fig. 3. Stopped-flow ^{1}H NMR spectra of peak 4 of Fr. C-1 obtained from feeding experiment of tetrahydropseudocoptisine **1** and synthetic α- and β-*N*-metho salts of **1** (**3a** and **3b**).

tetrahydropseudopalmatine **2**. Interestingly, *N*-methylated derivatives and protopine-type alkaloids were not detected in these experiments.

2.3.4. Biotransformation of **4a**

Administration of [*N*-CD_3]-tetrahydropseudopalmatine α-*N*-metho salt **4aD** was undertaken analogously as in Section 2.3.2. In the APCI/MS, peaks 10 and 11 in the LC of Fr C-1 displayed protonated ions at *m*/*z* 389 and 373, respectively. The stopped-flow ^{1}H NMR spectra of peak 10 in the LC showed a set of signals indicating the formation of two salts as in the case of pseudoprotopine **5**.

The structure related to peak 10 was recognized to be [*N*-CD_3]-pseudomuramine **8D** on the basis of the stopped-flow ^{1}H NMR (Fig. 4) and APCI/MS (*m*/*z* 389) data. The stopped-flow ^{1}H NMR spectra associated with peak 11 in the LC exhibited four aromatic protons at δ 6.87, 6.81, 6.79, and 6.74, four methoxyl groups at δ 3.75 (6H) and 3.74 (6H), and nine aliphatic protons at δ 3.0 and 4.7.

From these stopped-flow ^{1}H NMR and APCI/MS (*m*/*z* 373) data, the structure responsible for peak 11 was deduced to be [*N*-CD_3]-tetrahydropseudopalmatine α-*N*-metho salt **4aD**.

As a result of these administration experiments, the oxidation of **2** to pseudopalmatine **7** and of **4a** to pseudomuramine **8** were demonstrated (Scheme 2). However, *N*-methylation, as found for tetrahydropseudocoptisine **1** was not detected for tetrahydropseudopalmatine **2**. This may be due to the high substrate specificity of the *N*-methyltransferase for pseudo-type protoberberines.

3. Concluding remarks

A metabolic pathway to 2,3,10,11-oxygenated tetrahydroprotoberberines in cultured cells of *Corydalis* species was demonstrated for the first time by application of LC–MS and LC–NMR techniques. Tetrahydropseudoprotoberberines (**1** and **2**) were oxidized to pseudoprotoberberines (**6** and **7**) and were also *N*-methylated to stereospecifically afford the α-*N*-metho salts. The α-*N*-metho salts (**3a** and **4a**) were oxidized to produce pseudoprotopine-type alkaloids (**5** and **8**). These transformations are similar to those of 2,3,9,10-oxygenated protoberberines both in cultured cells and in living whole plants of *Corydalis* species (Iwasa, 1995). Tetrahydropseudocoptisine α-*N*-metho salt **3a**, pseudoprotopine **5**, and psedomuramine **8** are new alkaloids, first identified in these biotransformation studies. The ability of *Corydalis* cultures to biotransform pseudoprotoberberines suggests the existence of metabolic pathways leading to the formation of new pseudo-

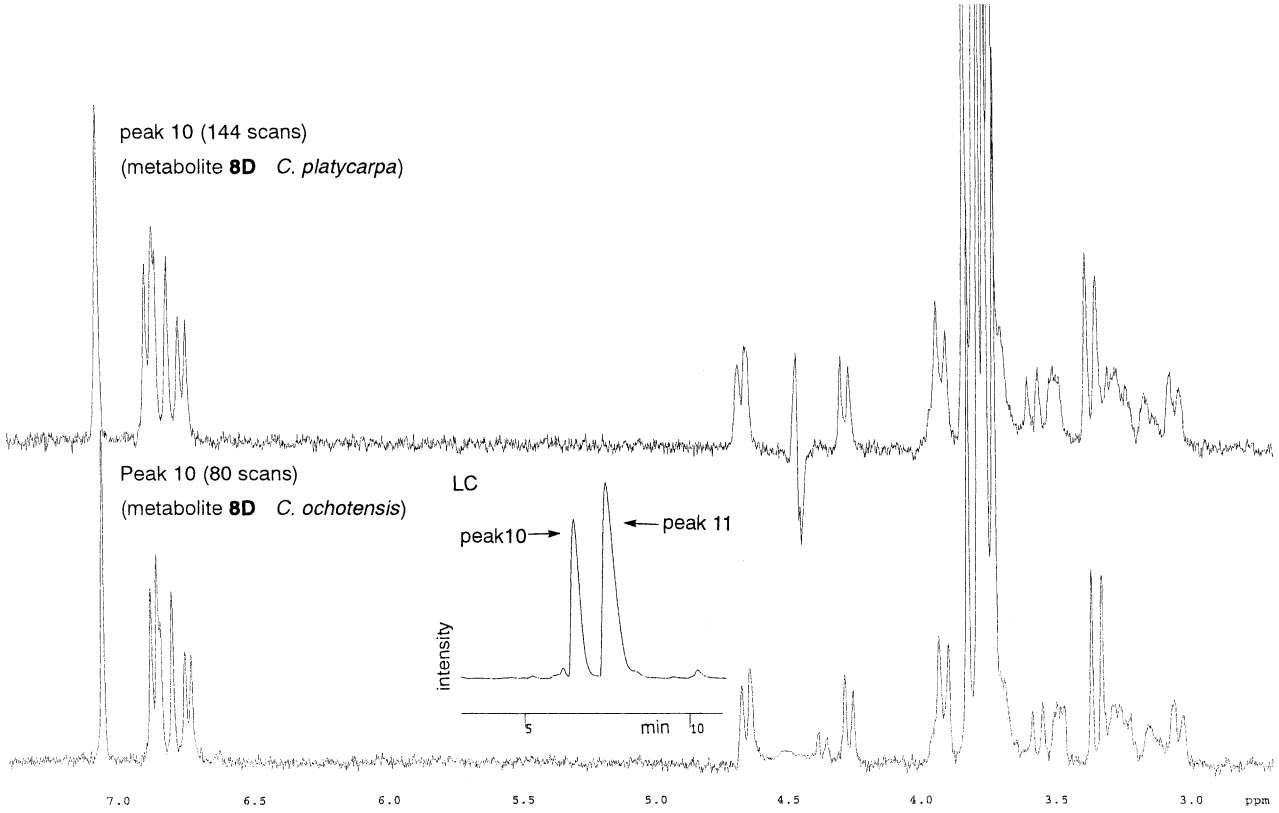


Fig. 4. Stopped-flow ^{1}H NMR spectra of peak 10 of Fr. C-1 obtained from feeding experiment of the α-*N*-metho salt of tetrahydropseudopalmatine (**4aD**).

protoberberines in some plant species, since several known pseudoprotoberberines occur in some plant species including *Corydalis* species (Preininger, 1986). The combination of HPLC with MS and NMR spectroscopy provides a powerful tool for the identification of metabolites in biotransformation in cultured cells of *Corydalis* species, and illustrates a useful approach for the structural analysis of metabolites in crude extracts of culture media and cells.

4. Experimental

4.1. General

Conventional ^{1}H NMR and NOESY spectra were obtained on a Varian VXR-500S spectrometer (^{1}H: 500 MHz) in CD_3OD. Mass spectra were determined on a Hitachi M 80 instrument at 75 eV.

4.2. Materials

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

4.3. LC–APCI/MS

LC–APCI/MS was carried out using a Hitachi M-1000H connected to a Hitachi L-6200 intelligent pump and a Hitachi L-4000 UV detector. LC was performed on a Cosmosil 5 C_{18}-AR (4.6 i.d. × 150 mm) reversed phase column. The mobile phase was 0.1 M NH_4OAc (0.05% TFA, A), to which MeCN (B) was added by a linear gradient: initial 20% of B, 10 min, 30% of B, 20 min, 30% of B, 30 min, 100% of B. The flow rate was 1 ml/min (detection: 280 nm). APCI/MS conditions: nebulizer and vaporizer temperatures were 320 and 399 °C, respectively. The drift voltage was 20 V. The quasi-molecular ions were monitored using the SIM method.

4.4. LC–NMR

LC–NMR data were acquired using a Varian UNITY-INOVA-500 spectrometer (^{1}H: 500 MHz) equipped with a PFG indirect-detection LC–NMR probe with a 60 μl flow-cell (active volume). ^{1}H–^{1}D NMR spectra were obtained in stopped-flow mode. Varian WET solvent suppression (Smallcombe et al., 1995) and related sequences were used to suppress the peaks of CH_3CN, its C-13 satellites, and the residual HOD in D_2O. FIDs were collected with 16 K data points, a spectral width, 9000 Hz, a 3 ms 90° pulse, a 1.82 s acquisition time, and a 0.08 s pulse delay depending on the sample concentrations, 24–4016 scans were accumulated (1 min–2 h and 15 min). Prior to Fourier transformation, an exponential appotization function was applied to the FID corresponding to a line broadening of 1 Hz. The HPLC system consisted of Varian Pro Star model-230 solvent delivery system and Varian Pro Star Model-310 variable-wavelength UV–vis detector. The outlet of the UV detector was connected to the LC–NMR probe via a sampling unit (Rheodyne). The separation 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 in D_2O (0.05% TFA, A), to which MeCN (B) was added by a linear gradient: initial 20% of B, 10 min, 30% of B, 20 min, 30% of B, 30 min, 100% of B. The flow rate was 1 ml/min (detection: 280 nm).

4.5. Syntheses

4.5.1. Tetrahydropseudopalmatine **2**

To a solution of tetrahydropapaverine hydrochloride (Sigma) (9.5 g) in hot H_2O (80 ml) was added dropwise 37% formaldehyde (20 ml) for 20 min. After further refluxing for 30 min, the mixture was cooled, basified with 10% KOH, and the liberated pale yellow powder was crystallized from EtOH to yield **2** (8.2 g), mp 155–157 °C. The free base was dissolved in methanolic HCl solution and the solvent was evaporated to give **2** hydrochloride, mp 195–197 °C (dec).

4.5.2. 2,3,10,11-Tetrademethyltetrahydropseudopalmatine

A solution of **2** (2 g) in 47% HBr (20 ml) was heated until reflux began, this being continued for 7 h. Following which the hydrobromic acid was evaporated in vacuo. Water was added to the residue and the crystalline product was collected by filtration to afford 2,3,10,11-tetrademethyltetrahydropseudopalmatine hydrobromide (1.85 g), mp 262–268 °C (dec). ^{1}H NMR (CD_3OD) δ 6.78 (1H, *s*, 1-H), 6.70 (1H, *s*, 12-H), 6.64 (1H, *s*, 4-H), 6.61 (1H, *s*, 9-H), 4.65 (1H, *dd*, J = 12.0, 4.5 Hz, 13a-H), 4.43 (2H,br s, 8-H), 3.77 (1H, br s, 6-H), 3.61 (1H, *br d*, J = 17.0 Hz, 13-H), 3.47 (1H, *td*, J = 12.0, 4.5 Hz, 6-H), 3.18 (1H, *m*, 5-H), 2.99 (1H, *dd*, J = 17.0, 12.0 Hz, 13-H), 2.93 (1H, *m*, 5-H).

4.5.3. Tetrahydropseudocoptisine **1**

To a solution of 2,3,10,11-tetrademethyltetrahydropseudopalmatine (200 mg) in DMSO (2 ml), were added NaOH (250 mg) and CH_2Cl_2 (20 ml). The mixture was refluxed under N_2 atmosphere for 4.5 h and was allowed to stand overnight under N_2 atmosphere. The residue

was triturated with a mixture of H_2O and CH_2Cl_2 and then extracted with dilute HCl. The acidic solution was basified with NH_4OH and extracted with CH_2Cl_2. The combined organic extracts were dried and evaporated to furnish crude **1** which was purified by prep. HPLC [0.1 M NH_4OAc (0.05% TFA)–MeOH (0.05% TFA)]. The eluent was evaporated and the residue was further purified by HPLC [H_2O (0.05% TFA)–MeOH (0.05%TFA)] to give the trifluoroacetate of **1** (70 mg), mp 171–174 °C (dec.).

4.5.4. Pseudocoptisine 6

Tetrahydropseudocoptisine (**1**) (200 mg) was refluxed with HOAc (50 ml) containing 5% Pd/C (80 mg) for 10 h. The mixture was then filtered through Celite 545. The filtrate was basified with NH_4OH, extracted with $CHCl_3$, and the organic solubles were evaporated in vacuo to dryness. The residue was purified by prep. HPLC [0.1 M NH_4OAc (0.05% TFA)–MeOH (0.05% TFA)]. The eluent was evaporated and the residue was further purified by HPLC [H_2O (0.05% TFA)–MeOH (0.05% TFA)] to give the trifluoroacetate of **6** (90 mg), mp 215–218 °C (dec.).

4.5.5. Tetrahydropseudocoptisine α- and β-N-metho salts 3a and 3b

CH_3I (1 ml) was added to a solution of tetrahydropseudocoptisine **1** (50 mg) in a mixture of Me_2CO (4 ml) and $CHCl_3$ (2 ml). After standing at room temperature for 1 h, the resulting precipitates (57 mg) were collected by decantation to afford a mixture of the α- and β-*N*-methyl iodides (16:84), which were separated by recrystallization from Me_2CO/MeOH. The β-*N*-methyl iodide was treated with AgCl in MeOH to convert it into the β-*N*-methyl chloride **3b**, mp 245–248 °C (dec.). For ^{1}H NMR and MS data, see Tables 1 and 2.

The mother liquor of the iodide mixture (57 mg) was allowed to stand at room temperature overnight, the solution was evaporated to yield crystals (21 mg), which consisted of a mixture of the α- and β-*N*-methyl iodides (88:12). This mixture was separated by prep. HPLC [0.1 M NH_4OAc (0.05% TFA)–MeOH (0.05% TFA)] and further purified by HPLC [H_2O (0.05% TFA)–MeOH (0.05% TFA)] to give the amorphous α-*N*-methyl trifluoroacetate (**3a**). For ^{1}H NMR and MS data, see Tables 1 and 2.

4.5.6. [N-CD3]-Tetrahydropseudocoptisine α- and β-N-metho salts 3aD and 3bD

CD_3I (1 g, Aldrich) in Me_2CO (5 ml) was added to a solution of **1** (340 mg) in a mixture of Me_2CO (50 ml) and $CHCl_3$ (50 ml). After standing at room temp., the resulting crystals were collected by decantation to afford a mixture of the α- and β-*N*-methyl-D_3 iodides (250 mg, 1:4). After several recrystallizations from MeOH, the iodides were further purified by prep. HPLC [0.1 M NH_4OAc (0.05% TFA)-MeOH (0.05% TFA)]. The eluent was evaporated and the residue was further purified by HPLC [H_2O (0.05% TFA)-MeOH (0.05% TFA)] to give the β-*N*-methyl-D_3 trifluoroacetate **3bD**, mp 230–231 °C (dec.).

The mother liquor of the iodide mixture (250 mg) was evaporated and after several recrystallizations in MeOH, the methiodide was further purified by prep. HPLC [0.1 M NH_4OAc (0.05% TFA)–MeOH (0.05% TFA)] and the eluent was purified with HPLC [H_2O (0.05% TFA)–MeOH (0.05% TFA)] to yield amorphous α-*N*-methyl-$\mathbf{D}_3$ trifluoroacetate **3aD** (100 mg). ^{1}H NMR data of **3aD** and **3bD** were identical with those of **3a** and **3b**, respectively, except for the *N*-methyl group.

4.5.7. Tetrahydropseudopalmatine α- and β-N-metho salts 4a and 4b

Tetrahydropseudopalmatine **2** (110 mg) was dissolved in Me_2CO (5 ml) and $CHCl_3$ (1 ml). CH_3I (1 ml) was added and the reaction mixture was allowed to stand at room temp. for 1 h. The resulting crystals were filtered to produce the β-*N*-methyl iodide **4b** (122 mg), mp 251–252 °C (dec.). The filtrate was evaporated to afford a mixture of the α- and β-*N*-methyl iodides (40 mg, 48:43). After several recrystallizations, the iodides were further purified by prep. HPLC [0.1 M NH_4OAc (0.05% TFA)–MeOH (0.05% TFA)] and subsequently with HPLC [H_2O (0.05% TFA)–MeOH (0.05% TFA)] to yield the amorphous α-*N*-methyl trifluoroacetate **4a**. For ^{1}H NMR and MS data, see Tables 1 and 2.

4.5.8. [N-CD3]-Tetrahydropseudopalmatine α- and β-N-metho salts 4aD and 4bD

CD_3I (1 g) in Me_2CO (2 ml) was added to **2** (500 mg) in Me_2CO (20 ml) and $CHCl_3$ (4 ml). The mixture was allowed to stand at room temp. for 2.5 h, the resulting crystals were collected by decantation to give the β-*N*-methyl-D_3 iodide **4bD** (490 mg), mp 259-260 °C (dec.).

The mother liquor was evaporated, and the crystals were recrystallized from MeOH to give α-*N*-methyl-D_3 iodide **4aD** (71 mg, mp 228–230°), which was converted to the α-*N*-methyl chloride, mp 218–223 °C. For MS data, see Table 2. ^{1}H NMR data of **4aD** and **4bD** were identical with those of **4a** and **4b**, respectively, except for the *N*-methyl group.

4.5.9. Pseudopalmatine 7

Tetrahydropseudopalmatine **2** hydrochloride (520 mg) in HOAc (100 ml) containing 5% Pd/C (180 mg) was heated until reflux began, this being maintained for 28 h. After addition of 5% Pd/C (100 mg), the mixture was then reflux maintained at temperature for additional 20 h. The mixture was filtered through Celite 545, and the HOAc was evaporated in vacuo. The residue was dissolved in EtOH, NaI was added, and the precipitated iodide (426 mg) was collected by

filtration. This iodide was treated with AgCl in MeOH to convert it into the **7** chloride, mp 214–217 °C (dec.).

4.6. Precursor administration

Administration experiments were carried out as follows: 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 an appropriate time (see Table 3). Cells and/or medium were separated and extracted with MeOH at 60 °C. Extracts were worked-up as described in Fig. 1.

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