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BIOTRANSFORMATIONS OF PROTOBERBERINES IN CELL CULTURES OF *DICENTRA SPECTABLIS*

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Key Word Index—*Dicentra spectablis*; Fumariaceae; cell culture; biotransformation; protoberberines.

Abstract—A liquid chromatography-atmospheric pressure chemical ionization mass spectrometry procedure was applied to biotransformation experiments in cultured cells of *Dicentra spectablis*. Interconversions of tetrahydroberberine and berberine and those of *cis*- and *trans*-13-methyltetrahydroprotoberberines and 13-methylberberine were demonstrated. The corresponding α-*N*-metho salts having the B/C-*cis* ring-junction were produced from *cis*- and *trans*-13-methyltetrahydroprotoberberines. These findings show that the metabolism of protoberberines in *D. spectablis* proceeds analogously to that found in *Cordyalis* species. © 1997 Elsevier Science Ltd

INTRODUCTION

The biogenetic pathways leading from nonphenolic protoberberines to the other skeletal classes of alkaloids have been established through feeding experiments with some whole plants and their tissue cultures of species of the Fumariaceae and Papaveraceae [1]. Feeding experiments of protoberberine alkaloids were carried out in a suspension cell culture of *D. spectablis*. LC-APCIMS (liquid chromatography-atmospheric pressure chemical-ionization mass spectrometry) [2] was applied in order to identify the metabolites without performing isolation procedures. Results obtained indicate that the bioconversion of protoberberines in *D. spectablis* closely parallels that found in *Corydalis species*.

RESULTS AND DISCUSSION

[9-OCD_3]Tetrahydroberberine (**1D**) and [9-OCD_3]berberine (**2D**) have been prepared [3]. Pyrolysis of 13-methylberberine (**3**) gave rise to 13-methylberberrubine (**4**) by selective *O*-demethylation of the C-9-methoxy (Scheme 1). Introduction of the deuterium label was accomplished by methylation of **4** with CD_3I to produce [9-OCD_3]13-methylberberine (**3D**). The mass spectrum (SIMS) showed a base peak at *m/z* 353 $[M\text{-}Cl]^+$. The ^1H NMR spectrum exhibited a methoxyl group at δ 4.12. This indicates that the *O*-methyl group at C-9 was completely labelled with deuterium. [9-OCD_3]Thalictricavine (**5D**) and [9-OCD_3]mesothalictricavine (**6D**) were prepared by reduction of **3D** with zinc powder in acidic methanol. Both mass spectra of **5D** and **6D** displayed a $[M]^+$ at *m/z* 356 and a fragment peak at *m/z* 181 assignable to ion A, arising by way of retro-Dields–Alder cleavage of ring C (Scheme 1). The ^1H NMR spectra of **5D** and **6D** showed a methoxyl group at δ 3.86 and 3.84, respectively.

Callus tissues of *D. spectablis* were incubated in liquid media containing, in the first experiment, deuterium-labelled compounds **1D** and **2D**. The starting materials in the second experiment were **3D**, **5D** and **6D**. In all cases, the runs were made at 25° for 10 and 14 days in the former and in the latter, respectively (Table 1).

Following incubation, extraction was carried out according to the procedure outlined in the Experimental section. The alkaloid fractions soluble in ether and chloroform (Frs I and II) were subjected to LC-APCIMS. Metabolites were detected on the mass chromatograms monitored by either quasi-molecular ions or $[M]^+$ or cluster ions characteristic of individual alkaloids as described previously [4]. Identification of each metabolite was confirmed by comparison of the mass chromatogram of fraction I or II with that of a mixture of fraction I or II and each authentic sample.

Deuterated berberine (**2D**, *m/z* 409 $[M+CF_3]^-$), allocryptopine (**7D**, *m/z* 373 $[M+H]^+$ and chelerythrine (**8D**, *m/z* 351 $[M]^+$) were detected in an experi-

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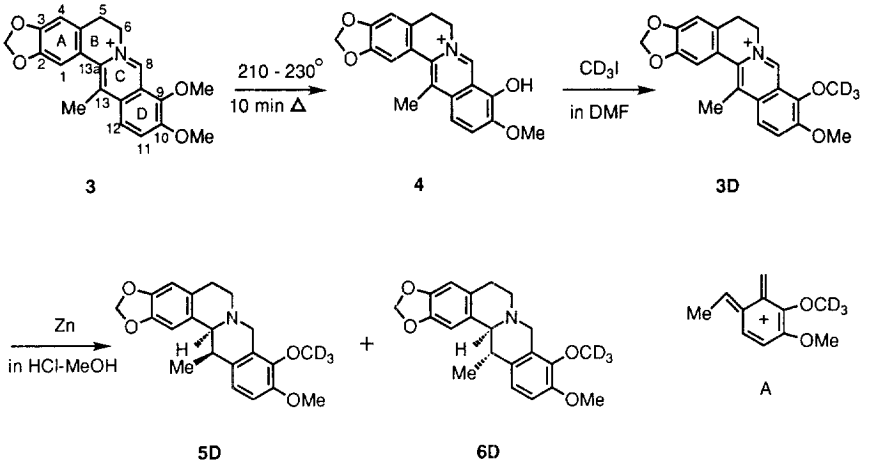
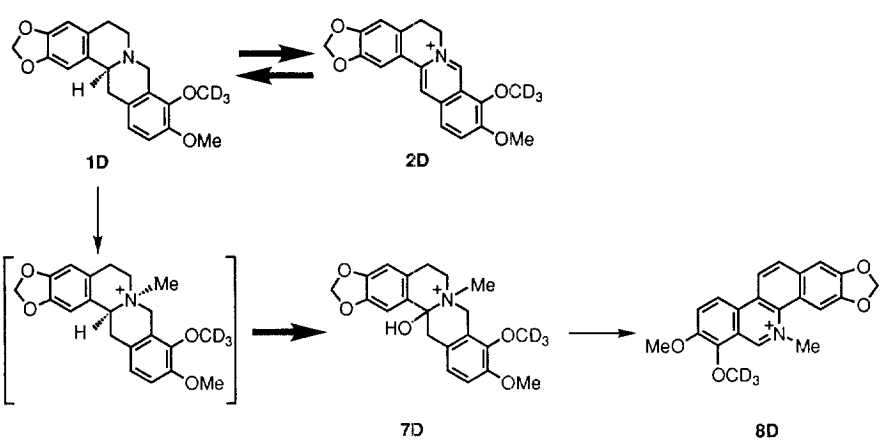


Scheme 1. Preparations of [9-OCD_3]13-methylberberine (**3D**), [9-OCD_3]thalictricavine (**5D**), and [9-OCD_3]mesothalictricavine (**6D**).

Table 1. Administration of deuterated protoberberine alkaloids (**1D**–**3D**, **5D** and **6D**) to cell cultures of *Dicentra spectablis*

Wt of dry cells (g)	Medium (ml)	Substrate (mg)	Incubation time (days)	Wt of fractions (mg) I*	II†	Metabolites detected [observed ions (*m/z*)]
1.38	200	**1D** 25	10	16.5	7.0	**2D** (409), **7D** (373), **8D** (351)
1.54	200	**2D** 25	10	12.4	7.0	**1D** (343), **7D** (373)
1.79	200	**3D** 25	14	26.0	7.7	**5D** (357), **6D** (357), **9D** (387)
1.95	200	**5D** 25	14	20.0	8.4	**3D** (423), **6D** (357), **9D** (387), **10D** (371)
2.10	200	**6D** 25	14	11.0	11.0	**3D** (423), **5D** (357), **9D** (387), **11D** (371)

* Ether extracts.
† Chloroform extracts.



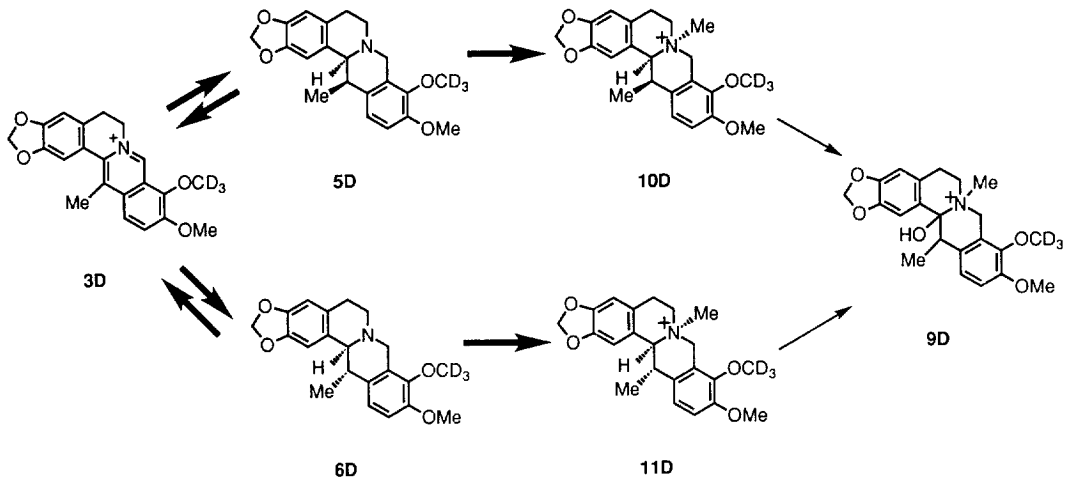
Scheme 2. Metabolic transformations of deuterated tetrahydroberberine (**1D**) and berberine (**2D**) in cultured cells of *Dicentra spectablis*. Thin arrow indicates that this route was demonstrated indirectly.

ment in which [9-OCD_3] tetrahydroberberine (**1D**) was administered (Table 1, Scheme 2).

In a variation on the theme, deuterated tetrahydroberberine (**1D**, *m/z* 343 $[M+H]^+$) and allocryptopine (**7D**) were detected in an experiment in which [9-OCD_3]berberine (**2D**) was fed (Table 1, Scheme 2). In this case, no **8D** was detected. Precursor **2D** has to be converted to **1D** and then **1D** may be bioconverted *via* **7D** into **8D**. A longer incubation period may be needed in order to form **8D** from **2D**.

Deuterated 13-methylberberine (**3D**, *m/z* 423 $[M+CF_3]^+$), mesothalictricavine (**6D**, *m/z* 357 $[M+H]^+$), 13-methylallocryptopine (**9D**, *m/z* 387 $[M+H]^+$) and thalictricavine α-*N*-metho salt (**10D**, *m/z* 371 $[M]^+$) were identified in the second experiment, in which [9-OCD_3]thalictricavine (**5D**) was utilized (Table 1, Scheme 3).

Additionally, deuterated 13-methylberberine (**3D**), thalictricavine (**5D**, *m/z* 357 $[M+H]^+$), 13-methylallocryptopine (**9D**) and mesothalictricavine α-*N*-



Scheme 3. Metabolic transformations of deuterated 13-methylberberine (**3D**) and *cis*- and *trans*-13-methyltetrahydroprotoberberines (**5D** and **6D**) in cultured cells of *Dicentra spectablis*. Thin arrows indicate that these routes were demonstrated indirectly.

metho salt (**11D**, *m/z* 371 $[M]^+$) were detected in an experiment in which [9-OCD_3]mesothalictricavine (**6D**) was administered (Table 1, Scheme 3).

No **11D** and **10D** were detected in the feeding experiments with **5D** and **6D**, respectively. Precursors **5D** and **6D** have to be converted to **6D** and **5D**, respectively, *via* **3D**, in order to form **11D** from **5D** and **10D** from **6D**.

Finally, in the last experiment, deuterated thalictricavine (**5D**), mesothalictricavine (**6D**) and 13-methylallocryptopine (**9D**) were identified in an experiment in which [9-OCD_3]13-methylberberine (**3D**) was fed (Table 1, Scheme 3). In this case, there is undetectable **10D** or **11D**, because **10D** or **11D** formed from **3D** may be easily bioconverted into **9D**.

Each deuterated metabolite was identified by comparison of its mass chromatogram with that of each authentic sample as illustrated in Figs 1 and 2. Mass chromatograms (Fig. 1) of a, b and c were detected by LC-APCIMS of the mixture of fraction I or II obtained from the feeding experiment with [9-OCD_3] tetrahydroberberine (**1D**) and each authentic sample [a: allocryptopine (**7**) (*m/z* 370), b: berberine (**2**) (*m/z* 406), c: chelerythrine (**8**) (*m/z* 348)]. Mass chromatograms arising from **2D**, **7D** and **8D** without **2**, **7** and **8** were observed in the LC-APCIMS of fraction I or II. Therefore, all metabolites are completely labelled with deuterium in the *O*-methyl group at C-9.

Mass chromatograms (Fig. 2) of a, b, c and d were detected in the LC-APCIMS of the mixture of fraction I or II obtained from the feeding experiment with [9-OCD_3]thalictricavine (**5D**) and each authentic sample [a; thalictricavine α-*N*-metho salt (**10**) (*m/z* 368), b: 13-methylberberine (**3**) (*m/z* 420), c: [*N*-$^{13}CH_3$]13-methylallocryptopine (**9**) (*m/z* 385), d: methothalictricavine (**6**) (*m/z* 354)]. Mass chromatograms resulting from **3D**, **6D**, **9D** and **10D** without **3**, **6**, **9** and **10** were observed in LC-APCIMS of fraction I or II. This indicates that the *O*-methyl group at C-9 of all metabolites are labelled with deuterium.

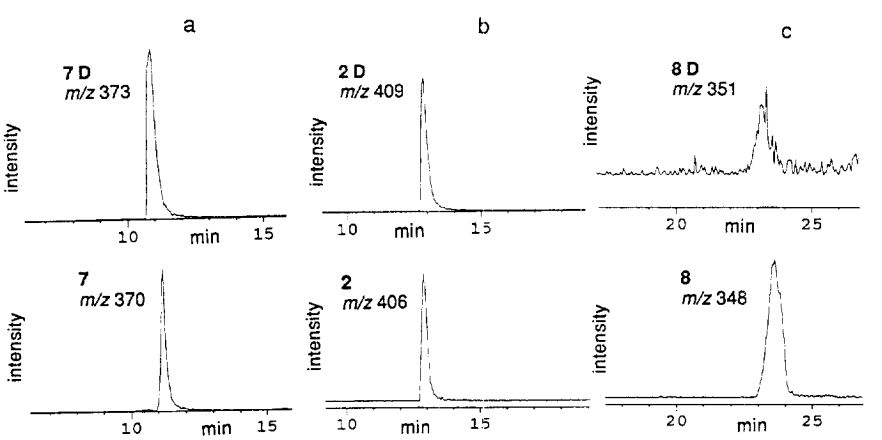


Fig. 1. Identification of metabolites [**2D** (*m/z* 409), **7D** (*m/z* 373) and **8D** (*m/z* 351)] in Fr I or II obtained from feeding experiment with [9-OCD_3]tetrahydroberberine (**1D**). Authentic samples [**2**: berberine (*m/z* 406), **7**: allocryptopine (*m/z* 370), **8**: chelerythrine (*m/z* 348)]. All ions were monitored by SIM after LC-APCIMS.

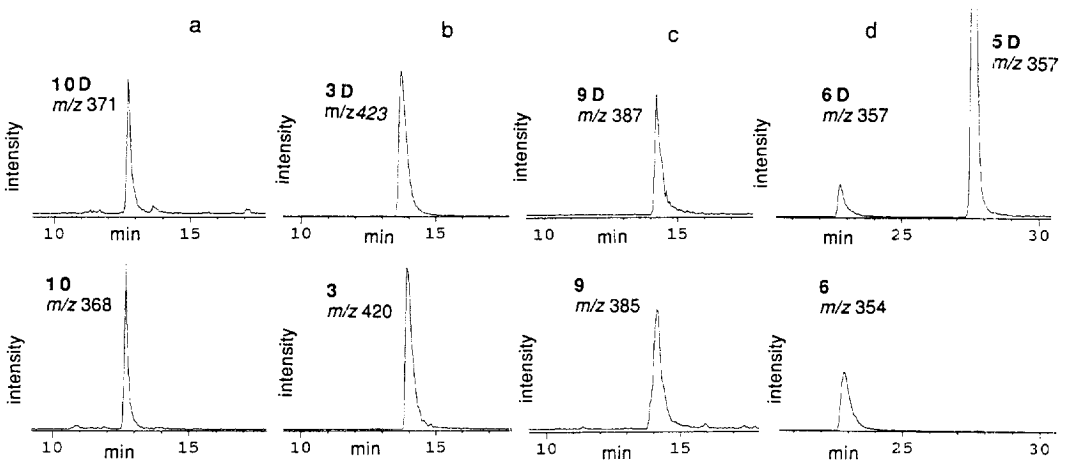


Fig. 2. Identification of metabolites [**1D** (*m/z* 423), **3D** (*m/z* 357), **4D** (*m/z* 371) and **6D** (*m/z* 387)] in Fr I or II obtained from feeding experiment with [9-OCD_3]thalictricavine (**2D**). Authentic samples [**1**: 13-methylberberine (*m/z* 420), **3**: meso-thalictricavine (*m/z* 354), **4**: thalictricavine α-*N*-metho salt (*m/z* 368), **6**: [*N*-$^{13}CH_3$]13-methylallocryptopine (*m/z* 385)]. All ions were monitored by SIM after LC-APCIMS.

We can, therefore conclude that the metabolic conversions **1D** → **2D**, **2D** → **1D**, **3D** → **5D**, **5D** → **3D**, **3D** → **6D** and **6D** → **3D** were also demonstrated in cultured cells of *D. spectablis* as previously demonstrated in some *Corydalis* species [3, 4] (Schemes 2 and 3). In other words, the interconversions, *via* redox reactions, of **1D** and **2D** or **3D** and **5D** or **3D** and **6D** were established. The interconversions between **5D** and **6D** occur *via* **3D**. The metabolic pathways **1D** → **7D** → **8D**, **5D** → **10D** → **9D** and **6D** → **11D** → **9D** were also proved in cultured cells of *D. spectablis*, as indicated previously in some *Corydalis* species [3, 4] (Schemes 2 and 3). The bioconversion of tetrahydroberberine-type (**1D**), *via* protopine-type (**7D**), into benzophenanthridine-type (**8D**) could take place. Biotransformation of the α-*N*-metho salt (**10D** or **11D**) into 13-methylprotopine (**9D**) also takes place. These findings show that the metabolism of protoberberines in *D. spectablis* closely parallels that found in *Corydalis* species.

EXPERIMENTAL

General. Mps: uncorr. ^1H NMR were obtained on a Varian VXR-500S 500 MHz spectrometer using $CDCl_3$ or CD_3OD soln with TMS as int. standard. EIMS, CIMS (CH_4) and SIMS (glycerol). Sil gel $60F_{254}$ plates (Merck) were used for prep. TLC (C_6H_6–Et_2O, 4:1).

LC-APCIMS. LC was performed on a Cosmosil 5 C_{18}-AR (4.6 mm i.d. × 150 mm) reversed-phase column. The mobile phase was aq. 0.1 M NH_4OAc (0.05% TFA, A), to which MeOH (0.05% TFA, B) was added by a linear gradient: initial, 25% of B–10 min, 50% of B–20 min, then 80% of B. The flow rate was 1 ml min^{-1}. UV detection was at 280 nm. Frs I and II were dissolved in a mixt. of $CHCl_3$ (200 μl) and MeOH (300 μl) and 5 μl of the soln was injected. APCIMS conditions: nebulizer and vaporizer temps were 320 and 399°, respectively, with drift voltage at 20 V. The quasi-molecular ions were monitored by SIM.

Plant material. Roots of *D. spectablis* purchased from Takii Seeds Company, Kyoto, Japan, were grown at the botanical garden of Kobe Pharmaceutical University and the whole plant was identified by the staff. A voucher specimen (*KPU* 1990 *D.S.*) is deposited in the botanical garden. The callus was derived from the stem on Murashige and Skoog's medium [5] containing 2,4-D (1 mg l^{-1}), kinetin (0.1 mg l^{-1}), yeast extract (0.1%), and agar (1%), in 1991. The callus tissues were subcultured every 3 or 4 weeks on the same fr. medium at 25° in the dark.

Preparation of 13-methylberberrubine (**4**). 13-Methylberberine (1.72 g) [4] was heated at 210–230° in a dry oven under vacuum (20–30 mm Hg) for 10 min to give crude 13-methylberberrubine (1.7 g, HPLC 97% pure). The crude product (300 mg) was recrystallized from EtOH–MeOH to provide **4** (210 mg), mp 293–295° (dec). ^1H NMR (CD_3OD): δ 2.72 (3H, *s*, Me-13), 3.04 (2H, *t*, *J* = 6.0 Hz, H_2-5), 3.89 (3H, *s*, OMe), 4.51 (2H, *t*, *J* = 6.0 Hz, H_2-6), 6.05 (2H, *s* OCH_2O), 6.93 (1H, *s*, H-4), 6.95 and 7.55 (1H each, *d*, *J* = 8.5 Hz, H-12 and H-11), 7.20 (1H, *s*, H-1), 9.31 (1H, *s*, H-8). ^{13}C NMR (CD_3OD): δ 17.7 (Me-13), 30.0 (C-5), 56.6 (OMe), 57.0 (C-6), 103.3 (OCH_2O), 105.9 (C-12), 109.0 (C-4), 111.7 (C-4), 120.9 (C-13), 123.1 (C-1a), 123.2 (C-11), 129.0 (C-8a), 133.8 (C-12a), 134.2 (C-4a or C-13a), 134.2 (C-4a or C-13a), 145.9 (C-8), 148.1 (C-2), 150.2 (C-3), 151.1 (C-10), 164.9 (C-9). SIMS *m/z* (rel. int.): 336 $[M-Cl]^+$ (100).

Preparation of [9-OCD_3]*13-methylberberine* (**3D**). Crude 13-methylberberrubine (HPLC 95% pure) prepd from 13-methylberberine (1.08 g) was dissolved in dry DMF (100 ml) and then CD_3I (1 ml) in DMF (1 ml) added. The reaction mixt. placed in a glass-

stoppered bottle was allowed to stand at room temp. for 4 days. After additional CD_3I (0.5 ml) was added, the mixt. was left at room temp. overnight. Et_2O was added to the mixt. and the resulting ppts collected (1.2 g) and recrystallized from MeOH to give **3D** iodide (770 mg). A part of the iodide (330 mg) was treated with AgCl in MeOH to convert it into the chloride **3D** (231 mg), mp 195–199° (dec); ^1H NMR data was identical with lit. [4], except for the disappearance of a methoxyl group at δ 4.21. SIMS *m/z* (rel. int.): 353 $[M\text{-}Cl]^+$(100).

Preparation of [9-OCD_3]*thalictricavine* (**5D**) *and* [9-OCD_3]*mesothalictricavine* (**6D**). [9-OCD_3]13-Methylberberine (**3D**) (300 mg) was dissolved in MeOH (100 ml) and conc. HCl (10 ml) and, after addition of Zn dust (3 g), the mixt. was stirred overnight at room temp. The Zn dust was filtered off and the filtrate diluted with H_2O and concd to a small vol. under red. pres. After cooling, the soln was made alkaline with NH_4OH and extracted with $CHCl_3$. The dried $CHCl_3$ soln was evapd and the residue subjected to prep. TLC (C_6H_6–Et_2O 4:1) to give **5D** (66 mg) and **6D** (36 mg). **5D**. ^1H NMR ($CDCl_3$): δ 0.95 (3H, *d*, *J* = 7.0 Hz, Me-13), 2.57 (2H, *m*, H_2-5), 3.06 (1H, *m*, H-13), 3.17 (2H, *m*, H_2-6), 3.49 and 4.19 (1H each, *d*, *J* = 16.0 Hz, H_2-8), 3.66 (1H, *br s*, H-13a), 3.86 (3H, *s*, OMe), 5.91 and 5.93 (1H each, *d*, *J* = 1.5 Hz, OCH_2O), 6.58 (1H, *s*, H-4), 6.68 (1H, *s*, H-1), 6.82 and 6.88 (1H each, *d*, *J* = 8.5 Hz, H-11 and H-12). EIMS *m/z* (rel. int.) 356 $[M]^+$ (67), 181 (100). **6D**: ^1H NMR ($CDCl_3$): δ 1.44 (3H, *d*, *J* = 7.0 Hz, Me-13), 2.79 (1H, *m*, H-13), 2.90 (2H, *m*, H_2-5), 3.05 (2H, *m*, H_2-6), 3.58 (1H, *d*, *J* = 8.0 Hz, H-13a), 3.84 (3H, *s*, OMe), 3.96 and 4.08 (1H each, *d*, *J* = 16.0 Hz, H_2-8), 5.89 and 5.90 (1H each, *d*, *J* = 1.5 Hz, OCH_2O), 6.59 (1H, *s*, H-4), 6.72 (1H, *s*, H-1), 6.80 and 6.94 (1H each, *d*, *J* = 9.0 Hz, H-11 and H-12). EIMS *m/z* (rel. int.) 356 $[M]^+$ (60), 181 (100).

Feeding experiments. The chloride or hydrochloride of each substrate (25 mg) was dissolved in H_2O (2–4 ml) and introduced into conical flasks (100 ml × 5) containing 40 ml autoclaved MS medium, which is the same as that employed for subculture (without agar), through a sterile bacterial filter. Calli (*ca* 4–5 g) were transferred to each flask and incubated at 25° in the dark for 10 and 14 days in feeding experiments with **1D** and **2D** and those with **3D**–**5D**, respectively (see Table 1).

Extraction and identification of alkaloids from callus. After incubation, the callus was homogenized and then sepd by centrifugation. Cells were extracted with H_2O–MeOH several times, then the extracts were concd and combined with the supernatant liquid sepd by centrifugation. The aq. soln was adjusted to pH *ca* 10 (with NH_4OH) and extracted with Et_2O (Fr I) and then $CHCl_3$ (Fr II) to give the alkaloid frs. The obtained frs I (11–26 mg) and II (7–11 mg) were subjected to LC-APCIMS [2]. Frs I or II mixed with each authentic sample was also subjected to LC-APCIMS. Metabolites were detected by quasi-molecular ions, $[M]^+$, or cluster ions as described previously [3, 4] (see Table 1). Identification of each metabolite was confirmed by comparison of its mass chromatogram with that of the corresponding authentic sample (Figs 1 and 2).

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