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Conversion of dechlorodauricumine into chlorinated alkaloids in *Menispermum dauricum* root culture

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Abstract

^{15}N-Labeled dechlorodauricumine and dechloroacutumine were isolated from *Menispermum dauricum* roots cultured in a chloride-deficient medium, in which nitrogen-containing macro-components $K^{14}NO_3$ and $(^{14}NH_4)_2SO_4$ were replaced by $K^{15}NO_3$ and $(^{15}NH_4)_2SO_4$, respectively. These ^{15}N-labeled substrates were supplied independently to the roots cultured in a chloride-enriched medium. LC-ESI-MS analysis of alkaloids extracted from the roots, harvested 5 and 10 days after administering the ^{15}N-labeled substrates, revealed that the ^{15}N derived from dechlorodauricumine was much more effectively incorporated into chlorinated alkaloids than that derived from dechloroacutumine. These findings suggest that dechlorodauricumine is the principal precursor of the chlorinated alkaloids produced by *M. dauricum* roots.

Keywords: *Menispermum dauricum*; Menispermaceae; Chlorinated alkaloid; Biosynthesis; ^{15}N Tracer

1. Introduction

A large number of halogenated organic compounds have been isolated from bacteria, fungi, marine algae, lichens, higher plants, insects and mammals. However, not much is known about the mechanisms by which the halogen atoms are incorporated into organic compounds. Metal-dependent haloperoxidases were once proposed to be responsible for the incorporation of halogen atoms into organic molecules (van Pée, 2001). However, such halogenation reactions lack regioselectivity and substrate specificity. Recently, a different class of halogenases have been found to be involved in halometabolism; $FADH_2$-dependent halogenases were reported to be involved in the biosynthesis of several halometabolites including the antibiotics 7-chlorotetracyclin (Dairi et al., 1995) and pyrrolnitrin (Dong et al., 2005), as well as the antitumor agent rebeccamycin (Yeh et al., 2005). It is likely that regioselective halogenation reactions in bacteria are predominately catalyzed by flavin-dependent halogenases. Terrestrial plants are relatively poor in halogenated metabolites in comparison with other organisms such as fungi, lichens, bacteria, sponges and marine plants. Notable exceptions with high biological activity in plants include the growth hormone 4-chloroindole-3-acetic acid in legumes (Marumo et al., 1968) and the tumor inhibitor maytansine in *Maytenus* sp. (Kupchan et al., 1972). However, information about the enzymes catalyzing incorporation of halogen atoms during biosynthesis of these compounds is lacking.

Cultured roots of *Menispermum dauricum*, the Asian vine, produce four structurally related chlorinated isoquinoline alkaloids; dauricumine (**2**), dauricumidine (**3**), acutumine (**5**) and acutumidine (**6**) (Sugimoto et al., 2001). Additionally *M. dauricum* roots produce their dechloro-derivatives dechlorodauricumine (DCD, **1**) (Sugimoto et al., 2005) and dechloroacutumine (DCA, **4**) (Sugimoto et al., 1998). The absolute configuration of C-10, to which a chlorine atom is attached to, is exclusively *S*. Therefore,

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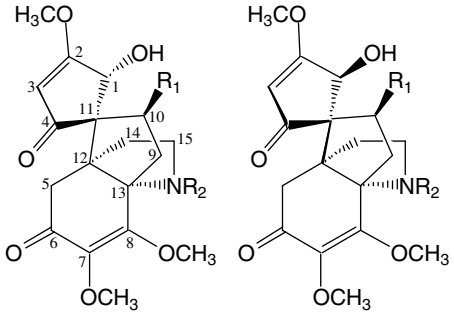
an enzyme with not only regioselectivity but also stereospecificity should be involved in introducing a chlorine atom into their dechloro-precursor(s). Since these halogenated metabolites are produced by complex biosynthetic pathways, identification of the substrate is essential to probe the halogenating enzyme(s). DCA (**4**), the dechloro-derivative of acutumine (**5**), was found to be poorly converted to acutumine (**5**) (Babiker et al., 1999b). Administering the roots with ^{36}Cl-labeled alkaloids demonstrated mutual conversion by *N*-methylation and *N*-demethylation, between dauricumine (**2**) and dauricumidine (**3**), and between acutumine (**5**) and acutumidine (**6**), respectively. Dauricumine (**2**) was converted to acutumine (**5**) and acutumidine (**6**). However, conversion of acutumine (**5**) to dauricumine (**2**) was not observed. Epimerization of acutumidine (**6**) to dauricumidine (**3**) or vice versa was not observed either (Sugimoto et al., 2001). These findings suggest that DCD (**1**) is a precursor of these chlorinated alkaloids and may be the substrate for a stereospecific chlorination enzyme. Previously, we have reported the production of DCD (**1**) in cultured roots of *M. dauricum* (Sugimoto et al., 2005). In the present study we report the efficient conversion of DCD (**1**) into the chlorinated alkaloids using ^{15}N as a tracer. Effects of culture period and chloride ion concentration in culture medium on production of the chlorinated alkaloids dauricumine (**2**), dauricumidine (**3**), acutumine (**5**) and acutumidine (**6**) and their dechloro-derivatives DCD (**1**) and DCA (**4**) are also described.



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

4 R_1 = H, R_2 = CH_3
5 R_1 = Cl, R_2 = CH_3
6 R_1 = Cl, R_2 = H

2. Results and discussion

^{15}N-Labeling facilitates investigation of the bioconversion of DCD (**1**) and DCA (**4**). Since each of these alkaloids has a nitrogen atom in the molecule, their molecular masses are detected with a high sensitivity, as the nitrogen atom is able to be protonated by mass spectrometry in the electron-spray ionization (ESI) method.

The contents of DCD (**1**) and DCA (**4**) in the *M. dauricum* roots are low, ca. 0.02% and 0.1% dry weight, respectively (Sugimoto et al., 2005). To facilitate preparation of ^{15}N-labeled DCD (**1**) and DCA (**4**), the effects of chloride ion in culture medium on root growth and alkaloid production were studied. Thus, roots were repeatedly sub-cultured in a chloride-deficient medium, in which chloride ion concentration was as low as 35 μM, and both growth and alkaloid production were evaluated (Fig. 1). As reported previously (Babiker et al., 1999a), the production of chlorinated alkaloids drastically decreased in the chloride-deficient medium. After successive sub-culturing for four generations, the production of the most abundant chlorinated alkaloid acutumine (**5**) decreased to 20% of that produced by the roots cultured in the control medium, in which the chloride ion concentration was 2 mM. By contrast, the production of DCD (**1**) and DCA (**4**) increased, peaking at the second generation of sub-culturing. Further sub-culturing in the chloride-deficient medium did not, however, increase the production of DCD (**1**) or DCA (**4**). No significant changes in root growth were observed throughout sub-culturing, irrespective of the chloride ion concentration in the medium. Accordingly the highest production of DCD (**1**) and DCA (**4**) was achieved in roots cultured for two generations in the chloride-deficient medium. The time course of growth and alkaloid production for roots cultured in the control medium (A) and roots cultured in the chloride-deficient medium (B) is shown in Fig. 2. DCA (**4**) was the most abundant alkaloid that accumulated in the roots cultured in the chloride-deficient medium. DCD (**1**) and DCA (**4**) production reached more than 0.5 and 1.6 mg/flask, respectively, in 55 days.

Based on the results described above, the roots were cultured in the control medium for three generations and then in the chloride-deficient medium for two generations. In both media, nitrogen-containing macro-components $K^{14}NO_3$ and $(^{14}NH_4)_2SO_4$ were replaced by $K^{15}NO_3$ and $(^{15}NH_4)_2SO_4$, respectively. Alkaloids were extracted from freeze-dried roots and separated by silica gel column chromatography followed by preparative HPLC. DCD (**1**) and

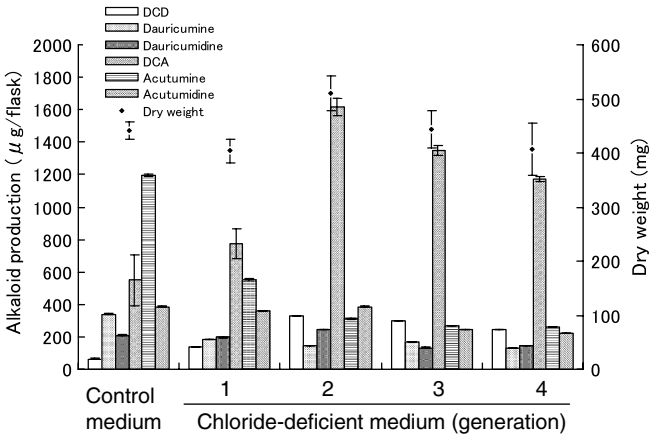


Fig. 1. Effects of chloride ion on *M. dauricum* root growth and production of alkaloids. Roots maintained in the control medium were excised and sub-cultured repeatedly in the chloride-deficient medium. After 55 days of culturing, roots were harvested and the alkaloids were extracted and analyzed. Bars indicate standard error ($n = 3$).

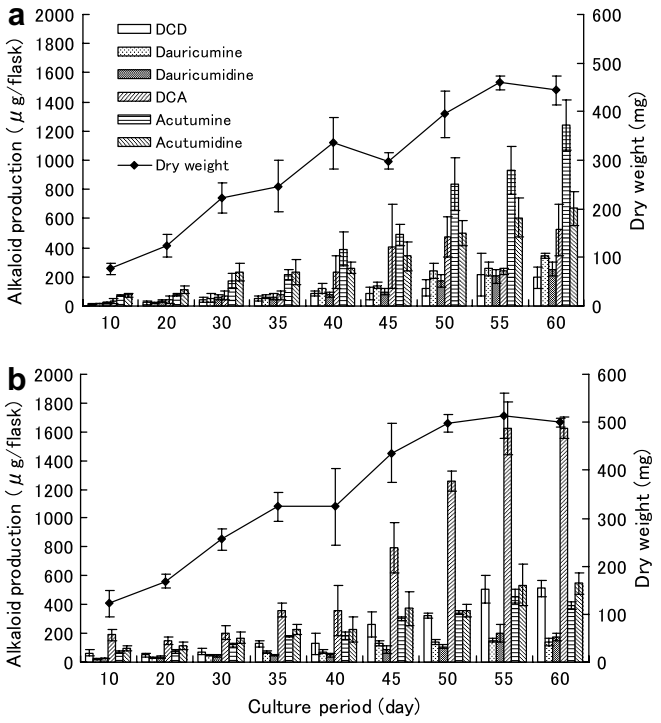


Fig. 2. Time course of *M. dauricum* root growth and production of alkaloids. Roots were cultured in the control medium (a) or in the chloride-deficient medium (b). Bars indicate standard error ($n = 3$).

DCA (**4**) were isolated and subjected to LC-ESI-MS analysis. The relative intensity of *m/z* 364 ($[M+H]^+$), 365, 366 in unlabeled DCD (**1**) and DCA (**4**) were 100:22:3.7 and 100:21:3.0, respectively, which is consistent with their theoretical values of 100:22:3.5. The relative intensity of *m/z* 364, 365, 366, 367 in the ^{15}N-enriched DCD (**1**) was 8:100:22:3.6 and that in the ^{15}N-enriched DCA (**4**) was 9:100:21:2.2. Therefore, ^{15}N-enrichments in these alkaloids were calculated as 92%, which is satisfactory to monitor their conversion into other alkaloids. It should be noted that the enrichment of ^{15}N in $K^{15}NO_3$ and $(^{15}NH_4)_2SO_4$ used in the present work was 98% and ^{14}N, in micro-components of the media, accounted for 0.7% of the total nitrogen.

Preliminary experiments showed that DCA (**4**), at less than 1.5 mg in 25-ml medium, did not reduce cell growth or alkaloid biosynthesis (data not shown). In the chloride-enriched medium, the chloride ion concentration was adjusted to 6 mM by supplementing with KCl. Such a medium was reported to be optimal for acutumine (**5**) production (Babiker et al., 1999a). ^{15}N-Labeled DCD (**1**) and DCA (**4**) at 1.2 mg each, were supplied independently to 35-day-old root cultures. Half of the roots were harvested 5 days after applying the ^{15}N-labeled substrates. The rest of the roots were incubated for an additional 5 days until harvest. Crude alkaloid extracts made 5 days after administration of the ^{15}N-labeled substrates showed a significant increase in DCD (**1**) and DCA (**4**) contents in the roots (Table 1). This finding demonstrates that these substrates

Table 1
Alkaloid contents in *M. dauricum* roots administered ^{15}N-labeled DCD (**1**) and DCA (**4**)

	Culture period (days)	Alkaloid content (μg/mg root dry weight)					
		1	**2**	**3**	**4**	**5**	**6**
Control[a]	40	0.30	0.34	0.23	0.61	1.10	0.78
	45	0.29	0.46	0.32	1.28	1.62	1.12
+ DCD (**1**)	40	1.22	0.20	0.38	0.44	0.58	0.81
	45	1.28	0.09	0.33	0.33	0.45	0.72
+ DCA (**4**)	40	0.19	0.09	0.34	1.44	0.48	0.74
	45	0.17	0.05	0.36	1.44	0.32	0.74

Roots were cultured in the chloride-enriched medium supplemented with ^{15}N-labeled DCD (**1**) or DCA (**4**).
[a] Data were from Fig. 2a.

were efficiently taken up. The alkaloid contents in the roots administered ^{15}N substrates did not increase from 5 to 10 days although a consistent increase in alkaloid contents was observed in roots cultured in the control medium (Table 1, Fig. 2a). Manipulation of roots during incubation may affect metabolism or promote alkaloid excretion, although the amount of alkaloids accumulated in the medium was less than 20% of those in the roots (Babiker et al., 1999a).

The isotopic pattern in each of the alkaloids is shown in Fig. 3, in which the molecular masses of $[M+H]^+$ in non-labeled compounds are indicated. The alkaloids obtained from roots cultured without ^{15}N showed isotopic patterns similar to their theoretical ones, respectively (Fig. 3a). No significant changes in isotopic patterns were observed in the alkaloids extracted from roots harvested 5 days after administrating ^{15}N-labeled DCA (**4**) (data not shown). Even 10 days after ^{15}N-labeled DCA (**4**) administration, slight changes in the isotopic pattern were detected only in acutumine (**5**). This is consistent with a previous finding that only 5% of radioactivity derived from DCA (**4**) was incorporated into acutumine (**5**) when ^{3}H-labeled DCA (**4**) was administered to the roots (Babiker et al., 1999b). On the other hand, the ^{15}N derived from DCD (**1**) was much more effectively incorporated into the chlorinated alkaloids. ^{15}N-Enrichment in dauricumine (**2**) was ca. 19% and obvious changes in the isotopic pattern of dauricumidine (**3**) were observed 5 days after administration. Increasing the incubation period to 10 days further increased ^{15}N-enrichment in these alkaloids to 25% and 13%, respectively.

Based on the results obtained in the present and previous work (Babiker et al., 1999b; Sugimoto et al., 2001), a biosynthetic scheme for the chlorinated alkaloids and their dechloro-derivatives is proposed (Fig. 4). Dauricumine (**2**) is the first chlorinated alkaloid formed in the roots of *M. dauricum* and DCD (**1**) is its precursor. Dauricumine (**2**) is converted to the three other chlorinated alkaloids by epimerization at C-1 and *N*-demethylation. Acutumine (**5**) is also formed from DCA (**4**). Definition of the enzymatic conversion of DCD (**1**) by cell free root extracts is presently in progress.

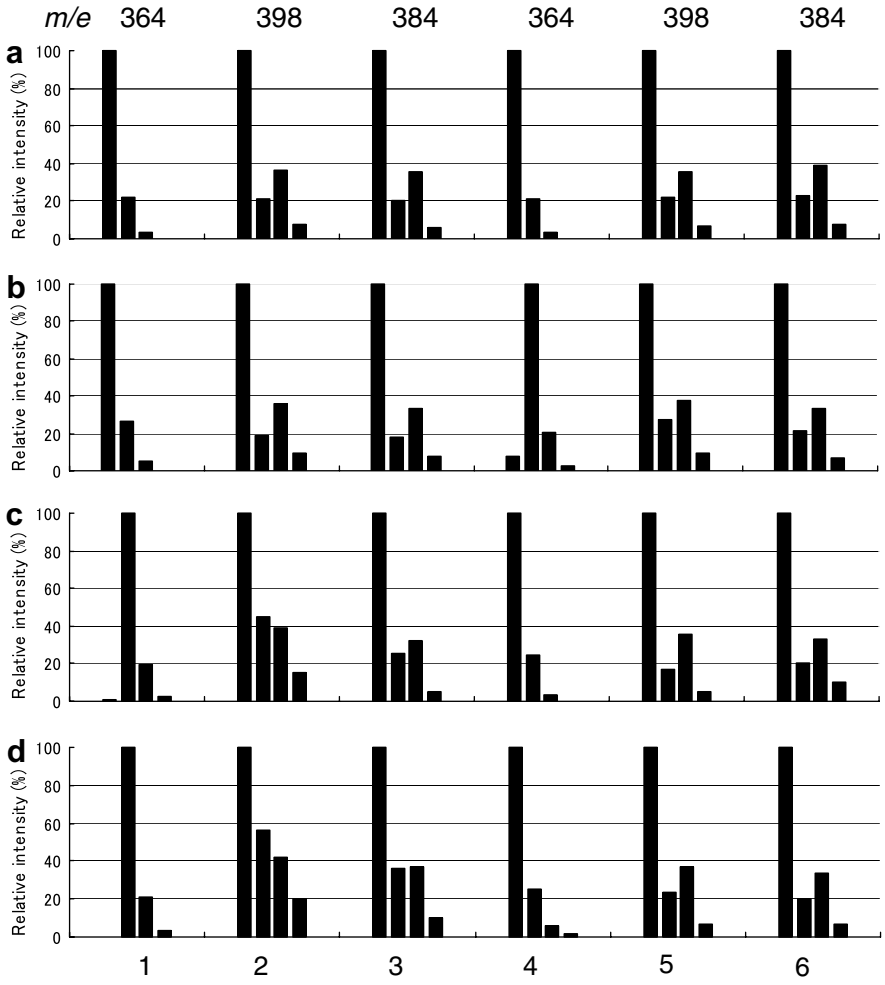


Fig. 3. Incorporation of ^{15}N-labeled DCD (**1**) and DCA (**4**) into alkaloids. Alkaloids were extracted from roots cultured for 45 days (a), roots administered ^{15}N-labeled DCA (**4**) 35 days after sub-culturing and incubated for additional 10 days (b), and roots administered with ^{15}N-labeled DCD (**1**) 35 days after sub-culturing and incubated for an additional 5 (c) and 10 (d) days.

3. Experimental

3.1. Chemicals

$K^{15}NO_3$ and $(^{15}NH_4)_2SO_4$ (98% atom%) were purchased from ISOTEC, Miamisburg, OH, USA.

3.2. Plant materials and culture conditions

M. dauricum roots were obtained from established cultures (Sugimoto et al., 1994). Excised *M. dauricum* roots were grown in a modified B5 medium containing 3% sucrose and 1 μM NAA, which is referred to as the control medium. For DCD (**1**) and DCA (**4**) production, a further modified B5 medium was employed, in which $CaCl_2$ was replaced by $Ca(NO_3)_2$. The medium is referred to as the chloride-deficient medium (Babiker et al., 1999a). For administration of ^{15}N-labeled DCD (**1**) and DCA (**4**), another modified B5 medium was used, in which the chloride ion concentration was adjusted to 6 mM with KCl. The medium is referred to as the chloride-enriched medium (Babiker et al., 1999a). The roots, placed in 200-ml flasks containing 50 ml of either of the culture media, were, unless stated otherwise, maintained in the dark at 27 °C on a rotary shaker (70 rpm) until harvest.

3.3. Extraction and analysis of alkaloids

Harvested *M. dauricum* roots were freeze-dried and powdered and the powder (25 mg) was soaked in MeOH (3 ml) overnight and filtered. Soaking in methanol (3 ml) was repeated twice and the combined filtrates were evaporated to dryness in vacuo at 40°C. The residue was dissolved in 3% citric acid, made alkaline (pH 10) with NH_4OH, and then loaded onto an Extrelut column (Merck Art. 15372). The column was allowed to stand for 10 min prior to elution with $CHCl_3$ (3.5 ml × 2). The combined $CHCl_3$ eluates were evaporated to dryness in vacuo at 30°C. The residue was then dissolved in MeOH and analyzed by HPLC using an ODS-UG-3 column (4.6 × 150 mm) eluted with MeOH–H_2O (1:1) containing 0.2% NH_4OH at a flow rate of 0.3 ml/min. A short pre-column (4.6 × 10 mm) was placed

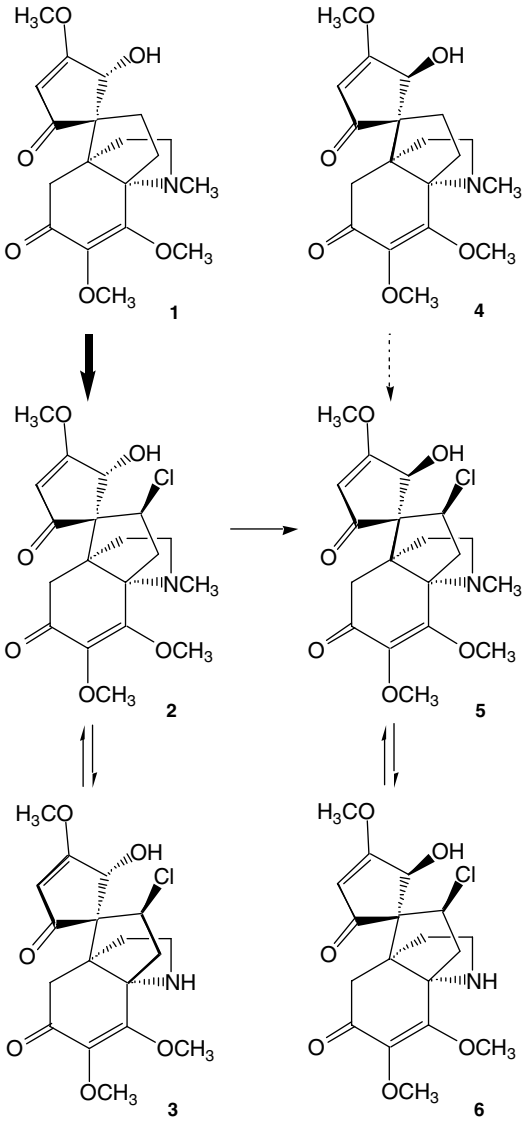


Fig. 4. Proposed biosynthetic relationship among chlorinated alkaloids dauricumine (**2**), dauricumidine (**3**), acutumine (**5**) and acutumidine (**6**), and their dechloro-derivatives DCD (**1**) and DCA (**4**). The bold arrow represents the conversion confirmed by the present work. Solid arrows represent conversions demonstrated by tracer experiments (Sugimoto et al., 2001), and the dashed arrow represents limited conversion suggested by tracer experiments (Babiker et al., 1999b).

between the injector and the separation column. The column effluents were divided into two, with one portion subjected to UV detection at 245 nm and the other to mass spectrometric detection in the ESI mode. R_ts of DCD (**1**), dauricumine (**2**), dauricumidine (**3**), DCA (**4**), acutumine (**5**) and acutumidine (**6**) were 15.4, 27.7. 10.7, 11.6, 16.5 and 8.2 min, respectively.

3.4. Isolation of ^{15}N-enriched DCD (*1*) and DCA (*4*)

Roots were sub-cultured for three generations each for 45 days in the control medium, sub-cultured in the chloride-deficient medium for 45 days, and cultured in the chloride-deficient medium for 55 days until harvest. In both media, nitrogen-containing macro-components $K^{14}NO_3$ and $(^{14}NH_4)_2SO_4$ were replaced by $K^{15}NO_3$ and $(^{15}NH_4)_2SO_4$, respectively. Harvested roots were freeze-dried and powdered. The root powder (9.28 g) was treated as described above. The basic residue (27.8 mg) was separated by silica gel cc using $CHCl_3$–MeOH as eluant as reported previously (Sugimoto et al., 2005). Further separation by semi-preparative HPLC yielded DCD (**1**) (2.1 mg) and DCA (**4**) (6.1 mg) as powders. The column used was a Capcell-pack C18 (20 × 250 mm) and the solvent was MeOH–H_2O (1:1) containing 0.2% NH_4OH at a flow rate of 4.0 ml/min. A short pre-column (4.6 × 10 mm) was placed between the injector and the separation column. R_ts of DCD (**1**) and DCA (**4**) were 18.6 and 16.5 min, respectively.

3.5. Mass spectrometry

MS spectra were obtained on a JEOL JMS-700 mass spectrometer in the ESI mode. ^{15}N-Enrichments were calculated on the basis of relative intensity of signals $(M+H+1)^+$ and $(M+H)^+$, taking theoretical intensities of isotope peaks into consideration.

3.6. Labeled substrates administration procedure

M. dauricum roots were cultured in 50-ml flasks containing 25 ml of the chloride-enriched medium for 35 days. ^{15}N-Labeled DCD (**1**) or DCA (**4**), in 58 μl MeOH each, was applied to the vigorously growing roots. Approximately half of the roots were harvested 5 days after applying the ^{15}N-labeled substrates. The rest of the roots were incubated in the same flasks for additional 5 days until harvest. The harvested roots were freeze-dried, powdered and the alkaloids were extracted and analyzed as described above.

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References

Babiker, H.A.A., Sugimoto, Y., Saisho, T., Inanaga, S., 1999a. Effects of chloride ion on acutumine and dechloroacutumine production by *Menispermum dauricum* root culture. Phytochemistry 50, 775–779.

Babiker, H.A.A., Sugimoto, Y., Saisho, T., Inanaga, S., Hashimoto, M., Isogai, A., 1999b. Biosynthetic relationship between acutumine and dechloroacutumine in *Menispermum dauricum* root cultures. Biosci. Biotechnol. Biochem. 63, 515–518.

Dairi, T., Nakano, T., Aisaka, K., Katsumata, R., Hasegawa, M., 1995. Cloning and nucleotide sequence of the gene responsible for

chlorination of tetracycline. Biosci. Biotechnol. Biochem. 59, 1099–1106.

Dong, C., Flecks, S., Unversucht, S., Haupt, C., van Pée, K.-H., Naismith, J.H., 2005. Tryptophan 7-halogenase (PrnA) structure suggests a mechanism for regioselective chlorination. Science 309, 2216–2219.

Kupchan, S.M., Komada, Y., Court, W.A., Thomas, G.J., Smith, R.M., Karim, A., Gilmore, C.J., Haltiwanger, R.C., Bryan, R.F., 1972. Maytansine, a novel antileukemic ansa macrolide from *Maytenus ovatus*. J. Am. Chem. Soc. 94, 1354–1356.

Marumo, S., Hattori, H., Abe, H., Munakata, K., 1968. Isolation of 4-chloroindolyl-3-acetic acid from immature seeds of *Pisum sativum*. Nature 219, 959–960.

Sugimoto, Y., Yoshida, A., Uchida, S., Inanaga, S., Yamada, Y., 1994. Dauricine production in cultured roots of *Menispermum daruricum*. Phytochemistry 36, 679–683.

Sugimoto, Y., Inanaga, S., Kato, M., Shimizu, T., Hakoshima, T., Isogai, A., 1998. Dechloroacutumine from cultured roots of *Menispermum daruricum*. Phytochemistry 49, 1293–1297.

Sugimoto, Y., Babiker, H.A.A., Saisho, T., Furumoto, T., Inanaga, S., Kato, M., 2001. Chlorinated alkaloids in *Menispermum dauricum* DC. root culture. J. Org. Chem. 66, 3299–3302.

Sugimoto, Y., Matsui, M., Takikawa, H., Sasaki, M., Kato, M., 2005. Dechlorodauricumine from cultured roots of *Menispermum dauricum*. Phytochemistry 66, 2627–2631.

van Pée, K.-H., 2001. Microbial biosynthesis of halometabolites. Arch. Microbiol. 175, 250–258.

Yeh, E., Garneau, S., Walsh, C.T., 2005. Robust in vitro activity of RebF and RebH, a two-component reductase/halogenase, generating 7-chlorotryptophan during rebeccamycin biosynthesis. Proc. Natl. Acad. Sci. USA 102, 3960–3965.