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Dechlorodauricumine from cultured roots of *Menispermum dauricum*

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Abstract

Dechlorodauricumine, a possible organic substrate for biochlorination, was isolated from cultured roots of *Menispermum dauricum*, a rich source of chlorinated alkaloids. Its structure was established by spectroscopic and chemical methods.

Keywords: *Menispermum dauricum*; Menispermaceae; Dechlorodauricumine

1. Introduction

More than 3800 naturally occurring organohalogens have been identified which are formed by bacteria, fungi, marine algae, lichens, higher plants, mammals and insects, and by abiotic processes such as volcano eruptions, biomass burning and degradation of rotten plant material (Gribble, 2003). However, not much is known about the mechanisms by which halogen atoms are incorporated into organic molecules. Almost all research groups working on halogenating enzymes have used monochlorodimedone as the substrate. Using this unnatural substrate led to the selection of unspecific enzymes, haloperoxidases and perhydrolases, that accept a wide variety of substrates and therefore lack substrate specificity (Van Pée and Unversucht, 2003). The recent discovery of $FADH_2$-dependent halogenases demonstrated that enzymes with substrate specificity and regioselectivity are involved in the halogenation reactions taking place in living organisms (Keller et al., 2000). Therefore, it is necessary to know the structure of natural substrates for biohalogenation in order to isolate specific halogenating enzymes.

In a previous paper (Sugimoto et al., 2001), we reported the isolation of four chlorinated alkaloids dauricumine (**1**), dauricumidine (**2**), acutumine (**3**) and acutumidine (**4**) from cultured roots of *Menispermum dauricum*. Tracer experiments using each of the alkaloids labeled with ^{36}Cl demonstrated mutual conversion between **1** and **2**, and between **3** and **4**. Conversion of **2**–**4** or vice versa was not observed. Dauricumine (**1**) was converted to **2**, **3** and **4** while **2** was not epimerized to **1**. These findings suggest that **1** is the first chlorinated alkaloid formed in the roots, and, therefore, that dechlorodauricumine (**5**) is the precursor for biochlorination taking place in the roots (Fig. 1). This hypothesis is consistent with a previous observation that only a small part of ^{3}H-labeled dechloroacutumine (**6**) was converted to acutumine (**3**) in the *Menispermum* roots (Babiker et al., 1999). Here, we report the isolation and structure determination of dechlorodauricumine (**5**) from cultured roots of *M. dauricum*.

2. Results and discussion

M. dauricum roots were cultured in B5 medium for 60 days. Alkaloids were extracted from freeze-dried roots and separated by analytical HPLC. The column effluents were monitored by UV (245 nm) and by mass

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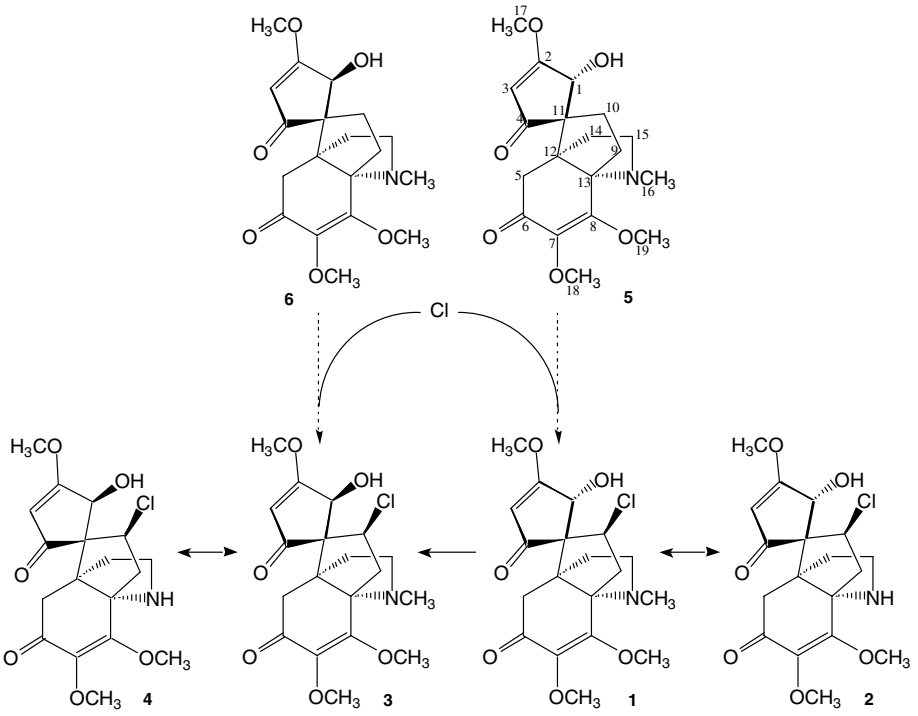


Fig. 1. Biosynthetic relationships among acutumine-type alkaloids. Solid arrows represent conversions demonstrated by tracer experiments (Sugimoto et al., 2001), and dashed arrows represent possible chlorination steps.

spectrometric analysis in the ESI mode. The mass chromatogram at *m*/*z* 364 ($[M + H]^+$) showed two peaks at *Rt*s 11.0 and 14.1 min, with the former identified as dechloroacutumine (**6**) based on the comparison of its chromatographic behavior, UV and mass spectra with those of an authentic compound (Sugimoto et al., 1998). The other component, eluted at 14.1 min, was isolated as an amorphous powder, in a separate experiment, after purification using silica-gel chromatography and preparative HPLC.

Its molecular formula was found to be $C_{19}H_{25}O_6N$, the same as that of dechloroacutumine (**6**), by high resolution mass spectrometry (*m*/*z* 363.1682 for $[M]^+$). Its EI mass spectral fragmentation pattern and UV absorption were quite similar to those of **6**. Detailed NMR spectroscopic studies, including ^1H–^1H COSY, ^{13}C–^1H COSY, NOESY, DEPT, HMBC and HMQC, were also carried out. The ^1H NMR spectrum of the base exhibited one *N*-methyl (δ 2.40) and three *O*-methyl signals (δ 3.64, 3.78 and 4.04), a one-proton singlet at δ 5.33, two mutually coupled, one-proton doublets at δ 2.98 and 3.59, and a one-proton doublet at δ 4.65 coupled with a one-proton broad doublet at δ 8.00 (Table 1). Comparing the spectrum with that of dauricumine (**1**) (Sugimoto et al., 2001), two additional protons were observed in the region of the aliphatic protons at δ 1.70 and 2.31, and splitting patterns of the upfield proton signals became more complex in this base while a one-proton quartet at δ 4.81 (H-10) of **1** disappeared. Two ^{13}C NMR signals at δ 40.8 (C-9) and 61.9 (C-10) of **1** shifted to δ 32.1 and 34.9 in this base (Table 1). The ^1H and ^{13}C NMR spectra were thus similar to those of dechloroacutumine (**6**) as well (Table 1). ^{13}C–^1H COSY and HMQC experiments revealed correlation between the carbon at δ 34.9 (C-10) and the protons at δ 1.70 and 2.31 (H-10). From the HMBC spectrum, connectivity patterns were observed between the proton at δ 4.65 (H-1) and the carbons at δ 34.9 (C-10) and δ 206.7 (C-4), and the proton at δ 5.33 (H-3) and the carbons at δ 66.7 (c-11) and 76.8 (C-1). Furthermore the NOESY spectrum revealed a correlation between H1 (δ 4.65) and H10 (δ 1.70). These observations strongly suggested that this base differed from dauricumine (**1**) only in the absence of a chlorine moiety at C-10, and that the base is the epimer of dechloroacutumine (**6**) at C-1. The NMR spectroscopic signals of this new base were assigned as shown in Table 1. It is noteworthy that relatively marked differences in δ values between this base and **6** were observed in C-1, and C-5, C-10 and C-14, which are spatially close to C-1.

The new base had a negative Cotton effect near 314 nm and a positive Cotton effect near 256 nm with a shoulder near 275 nm. As reported previously (Sugimoto et al., 1998), the CD spectral pattern of acutumine (**3**) is quite similar to that of dechloroacutumine (**6**). Both alkaloids have negative Cotton effects near 320 and 240 nm as well as a positive Cotton effect near 265 nm. On the other hand, the CD spectral pattern of dauricumine (**1**), having a negative Cotton effect near 315 nm and a positive Cotton effect near 250 nm with a shoulder near 275 nm, is remarkably different from those of **3** and **6** (Sugimoto et al., 2001). These results indicate that influence of stereochemistry at C-1 on the CD spectral pattern of acutumine-type molecule

Table 1
NMR spectroscopic data of dechloroacutumine (**6**), dechlorodauricumine (**5**) and 1-epidechloroacutumine

No.	**6**		**5**		1-Epidechloroacutumine[a]	
	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C	δ_H *J* (Hz)	δ_C
1	4.95, *d*, 6.5	72.0, *t*	4.65, *d*, 5.2	76.8, *t*	4.54, *s*	77.6
2		188.2, *q*		188.2, *q*		188.3
3	5.40, *s*	103.5, *t*	5.33, *s*	102.2, *t*	5.20, *s*	102.5
4		205.2, *q*		206.7, *q*		199.4
5	2.48, *d*, 15.9	46.8, *d*	2.98, *d*, 15.8	49.4, *d*	2.10, *d*, 15.1	47.1
	3.06, *d*, 15.9		3.59, *d*, 15.8		2.87, *d*, 15.1	
6		194.0, *q*		195.3, *q*		193.9
7		138.9, *q*		139.0, *q*		138.9
8		161.4, *q*		160.7, *q*		158.9
9	2.49, *dd*, 4.2, 12.3	31.6, *d*	1.93, *dd*, 6.0, 12.8	32.1, *d*	1.23, *m*	31.4
	2.59, *dd*, 6.8, 12.3		2.47, *dd*, 6.4, 12.8		2.29, *m*	
10	1.53, *dd*, 4.2, 10.7	29.5, *d*	1.70, *dd*, 6.4, 12.2	34.9, *d*	1.80, *m*	34.4
	2.69, *dd*, 6.8, 10.7		2.31, *dd*, 6.0, 12.2		2.57, *m*	
11		66.0, *q*		66.7, *q*		67.4
12		54.5, *q*		53.2, *q*		56.4
13		76.4, *q*		77.0, *q*		53.8
14	1.92, *dd*, 7.1, 13.0	37.3, *d*	2.20, *dd*, 4.3, 12.3	39.8, *d*	1.97, *m*	31.8
	2.10, *dd*, 6.1, 13.0		2.34, *dd*, 6.2, 12.3		2.18, *m*	
15	2.65, *dd*, 7.1, 12.4	52.5, *d*	2.50, *dd*, 4.3, 9.3	52.3, *d*	2.41, *m*	53.8
	2.83, *ddd*, 6.1, 7.1, 12.4		2.68, *dd*, 6.2, 9.3		2.93, *m*	
16	2.42, *s*	36.6, *s*	2.40, *s*	36.3, *s*	2.33, *s*	35.5
17	3.79, *s*	58.7, *s*	3.64, *s*	58.5, *s*	3.85, *s*	60.6
18	3.69, *s*	60.3, *s*	3.78, *s*	60.2, *s*	3.63, *s*	59.1
19	4.03, *s*	60.4, *s*	4.04, *s*	60.4, *s*	4.02, *s*	60.8
OH	7.85, *d*, 6.5		8.00, *d*, 5.2			

[a] Literature values (Yu et al., 2002).

is significant while that of the Cl atom at C-10 is relatively small. These facts strongly suggest that the new base is dechlorodauricumine (**5**), which was also supported by the behavior of the new base on HPLC. So far, we have identified five analogous compounds in the roots, acutumidine (**4**), dauricumidine (1-epiacutumidine) (**2**), acutumine (**3**), dauricumine (1-epiacutumine) (**1**) and dechloroacutumine (**6**). Their respective *Rt*s on analytical HPLC under the conditions described in section 3 are 7.9, 10.1, 15.1, 24.6, and 11.0 min. Under the same conditions, the new base is eluted at 14.1 min. Thus, the new base was identified as dechlorodauricumine (**5**).

Isolation of 1-epidechloroacutumine, the same compound as **5**, had been reported by Yu et al. (2002). However, the physicochemical parameters reported for the compound were inconsistent with those we obtained for dechlorodauricumine (**5**); the NMR data reported for 1-epidechloroacutumine are cited in Table 1. The inconsistencies are not caused by faults of assignment. The CD spectrum of 1-epidechloroacutumine was reported to have a positive Cotton effect near 271 nm and a negative Cotton effect near 227 nm, which is similar to that of dechloroacutumine (**6**) but exhibits significant difference from that of dechlorodauricumine (**5**). This similarity of the CD spectra was employed as a proof to support the structure of 1-epidechloroacutumine. Inconsistency was also observed in the UV spectrum and optical rotatory power. These discrepancies strongly suggest that **5** and 1-epidechloroacutumine are not the same. Therefore, further work was conducted to confirm the structure of **5**.

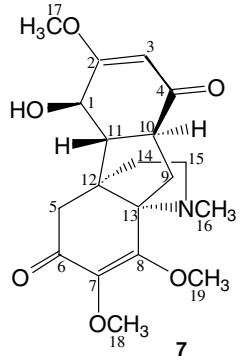
Acutumine (**3**) and dauricumine (**1**) were chemically dechlorinated by *n*-Bu_3SnH/AIBN, and the products were compared directly with dechloroacutumine (**6**) and dechlorodauricumine (**5**), respectively. Acutumine (**3**) afforded only one product, whose physicochemical properties including mass, NMR, UV and CD spectra, matched those of **6**, demonstrating that dechlorination of **3** proceeds under these conditions retaining its stereochemical configuration. Then, dauricumine (**1**) was subjected to the same reaction, resulting in two products separable by HPLC. Mass spectra indicated that both of the products had the molecular mass of *m*/*z* 363 without the isotopic pattern characteristic of Cl-containing compounds. Each product was isolated after separation by preparative HPLC. Chromatographic behavior, mass, NMR, UV and CD spectra of one of the products were identical to those of **5**. Accordingly, we conclude that the new base isolated in this work is dechlorodauricumine (**5**).

The DEPT spectrum of another dechlorinated product (**7**) of dauricumine (**1**) revealed that it has two additional methine groups at δ 48.4 and 59.2 with the loss of a C-10 methylene group at δ 34.9 and a C-11 quaternary carbon at δ 66.7, compared with 5. The HMBC spectrum revealed correlations of the methine carbon at δ 48.4 with H-3 (δ 5.50), and of the methine carbon at δ 59.2 with H-5 (δ 3.03, 3.18), H-9 (δ 2.60) and H-14 (δ 2.23). These facts

suggest that the unsaturated five-membered ring of the dauricumine molecule was rearranged to a six-membered ring though such a side reaction did not take place in the process of dechlorination of acutumine (**3**). Detailed NMR studies led to assignment of the signals of the byproduct (**7**) as shown in Table 2. The large coupling constants involving H-11 ($J_{1,11} = 10.4$ Hz and $J_{10,11} = 13.6$ Hz) indicate that H1, H10 and H11 are axial. Furthermore, a correlation between H10 (δ 2.86) and H15 (δ 2.60) was revealed from the NOESY spectrum. Therefore, the structure of **7** is proposed as depicted in Fig. 2. Dechlorination by *n*-Bu_3SnH/AIBN proceeds under radical conditions. Since C-1 and C-10 of acutumine (**3**) and dauricumine (**1**) are spatially close to each other, orientation of the hydroxyl group at C-1 may affect the stability of the carbon radical generated at C-10 in the dechlorination process.

Table 2
NMR spectroscopic data of alkaloid **7**

No.	δ_H *J* (Hz)	δ_C
1	4.75, *dd*, 5.1, 10.4	69.5, *t*
2		179.5, *q*
3	5.50, *s*	101.8, *t*
4		198.8, *q*
5	3.03, *d*, 14.8	52.6, *d*
	3.18, *d*, 14.8	
6		194.3, *q*
7		138.2, *q*
8		160.1, *q*
9	1.92, *dd*, 11.6, 13.3	32.9, *d*
	2.60, *dd*, 6.1, 13.3	
10	2.86, *ddd*, 6.1, 11.6, 13.6	48.4, *t*
11	2.35, *dd*, 10.4, 13.6	59.2, *t*
12		51.5, *q*
13		75.6, *q*
14	1.64, *m*	33.7, *d*
	2.23, *m*	
15	2.49, *m*	52.8, *d*
	2.60, *m*	
16	2.34, *s*	36.4, *s*
17	3.58, *s*	56.4, *s*
18	3.71, *s*	60.3, *s*
19	3.92, *s*	60.3, *s*
OH	7.55, *d*, 5.1	



7

Fig. 2. Proposed structure of byproduct **7**.

The present work verifies the presence of dechlorodauricumine (**5**) in *M. dauricum* roots. Taking the previous findings (Babiker et al., 1999; Sugimoto et al., 2001) into consideration, this new compound could be the precursor for biochlorination taking place in the roots. Contents of dechlorodauricumine (**5**) and dechloroacutumine (**6**) in the *Menispermum* roots were ca. 0.02% and 0.1% dry weight, respectively, suggesting that the pool size of **5** is smaller than that of **6** and/or the metabolic turnover of **5** proceeds more rapidly than that of **6**. It should be noted that the previous identification of 1-epidechloroacutumine (Yu et al., 2002), the same compound as **5**, was proven to be incorrect. Physicochemical parameters reported for the compound are those for an unidentified compound.

3. Experimental

3.1. General

^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded in pyridine-d_5 on a JEOL Lambda 400 MHz spectrometer. Chemical shifts were referenced to the solvent (δ_H 8.7 and δ_C 149.8). NMR experiments included ^1H–^1H COSY, ^{13}C–^1H COSY, NOESY, DEPT, HMBC and HMQC. MS spectra were obtained in the EI or ESI mode. IR spectra were obtained as KBr disks. UV and CD spectra were recorded in MeOH. Optical rotatory power was measured in MeOH.

3.2. Plant materials and culture conditions

Excised *M. dauricum* roots from established cultures were shaken in B5 medium containing 3% sucrose and 1 μM NAA on a rotary shaker at 70 rpm in the dark at 27 °C (Sugimoto et al., 1994).

3.3. Isolation and identification of alkaloids

Roots were cultured for 60 days. Freeze-dried roots (25 mg) were soaked overnight in MeOH and filtered. This procedure was repeated twice and the combined filtrates were evaporated to dryness at 40 °C. The dry residue was dissolved in 3% citric acid, filtered through paper into a glass tube and rendered alkaline with NH_4OH. This suspension was put onto an Extrelut column. After 10 min, $CHCl_3$ was passed through the column twice, the extracts were combined, then evaporated to dryness at 30 °C. The dry residue was dissolved in MeOH and analyzed by HPLC. The stationary phase was Develosil ODS-UG-3 (4.6 × 150 mm) and the solvent was 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 × 30 mm) was placed between the injector and the separation column. The column effluents were divided into two, one portion subjected to UV detection and the other to mass spectrometric detection in the ESI mode. *Rt*s of dechloroacutumine (**6**), dechlorodauricumine

(**5**), acutumine (**3**) and dauricumine (**1**) were 11.0, 14.1, 15.1 and 24.6 min, respectively.

Freeze-dried roots (50 g) were soaked overnight in MeOH and filtered. Methanol extraction was repeated four times and the combined filtrates were evaporated to dryness at 40 °C. The residue (12 g) was dissolved in 3% citric acid, adjusted to pH 10 with NH_4OH and extracted four times with $CHCl_3$. The combined $CHCl_3$ extracts were dried (Na_2SO_4) and the solvent was evaporated. The residue (0.77 g) was separated by silica gel chromatography using $CHCl_3$–MeOH. The proportion of MeOH in the solvent system was increased stepwise from 0% to 5%. The alkaloids were eluted from the silica gel column in the following order of solvent polarity, dauricumine (**1**) (50:1), dechlorodauricumine (**5**) (40:1), acutumine (**3**) (30:1) and dechloroacutumine (**6**) (20:1). These alkaloids were further purified on a semi-preparative HPLC. The column was a Capcell-pack C_{18} (20×250 mm) and the solvent was MeOH–H_2O (6:4) containing 0.2% NH_4OH. The flow rate was 4.0 ml/min. A short pre-column (4.6×10 mm) was placed between the injector and the separation column. The alkaloids were eluted in the same order with the analytical HPLC and their *Rt*s were 16.5, 18.6, 19.3 and 24.5 min, respectively.

3.4. Dechlorodauricumine (*5*)

Amorphous powder (6.9 mg): $[\alpha]_D^{25} + 20.7°$ (*c* 0.10, MeOH); CD $\Delta\varepsilon_{315}$ −12.5, $\Delta\varepsilon_{269sh}$ +9.6, $\Delta\varepsilon_{251}$ +11.9 (MeOH, 5.5×10^{-5} M); HREIMS: *m*/*z* $[M]^+$ 363.1682 [$C_{19}H_{25}NO_6$ requires 363.1682]; EI-MS: *m*/*z* (rel. int.) 363 (95.6), 335 (40.1), 320 (100), 292 (46.5), 220 (26.9), 209 (82.9), 208 (76.9), 180 (21.5), 166 (33.3), 150 (20.8); IR ν_{max} cm^{-1}: 1663, 1610, 1456, 1340, 1162; UV λ_{max} nm (log ε): 241.8 (4.16); for ^1H and ^{13}C NMR spectroscopic data, see Table 1.

3.5. Dechlorination of acutumine (*3*) and dauricumine (*1*)

A solution of **3** (15 mg) in dry toluene (15 ml) containing *n*-Bu_3SnH (0.25 ml) and a catalytic amount of AIBN was heated under reflux and then maintained for 5 h. Aqueous KF (35%) was added to the reaction solution after cooling to decompose excess *n*-Bu_3SnH. The solution was basified with NH_4OH and extracted with $CHCl_3$. The $CHCl_3$ residue was applied to a silica gel column eluted with $CHCl_3$–MeOH (20:1) to give a white powder (9.0 mg), whose UV, CD, MS and NMR spectra were identical with those of an authentic sample of dechloroacutumine (**6**). Dauricumine (**1**) (10 mg) was subjected to the same procedures as described above. Two dechlorinated products were separated from unreacted **1** by silica-gel chromatography, with each of the products collected after separation by preparative HPLC performed under the same conditions as described above. A base eluted at 18.6 min was isolated as an amorphous powder (3.4 mg). Its chromatographic behavior, UV, CD, MS and NMR spectra matched those of dechlorodauricumine (**5**).

Another base (**7**) eluted at 10.9 min was isolated as an amorphous powder (2.6 mg). CD $\Delta\varepsilon_{321}$ −12.5, $\Delta\varepsilon_{280}$ +2.7, $\Delta\varepsilon_{267}$ −2.5, $\Delta\varepsilon_{248}$ +6.1 (MeOH, 4.4×10^{-5} M); HREIMS: *m*/*z* $[M]^+$ 363.1672 [$C_{19}H_{25}NO_6$ requires 363.1682]; EI-MS: *m*/*z* (rel. int.) 363 (59.6), 335 (54.8), 320 (100), 292 (31.6), 208 (32.0), 166 (28.8), 150 (18.3); IR ν_{max} cm^{-1}: 1660, 1637, 1589, 1449, 1361, 1313; UV λ_{max} nm (log ε): 250.0 (4.13); for ^1H and ^{13}C NMR spectroscopic data, see Table 2.

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