Pergamon

PII: S0031–9422(98)00118–6

Phytochemistry, Vol. 49, No. 5, pp. 1293–1297, 1998

Printed in Great Britain
0031–9422/98/$—see front matter

DECHLOROACUTUMINE FROM CULTURED ROOTS OF *MENISPERMUM DAURICUM*

YUKIHIRO SUGIMOTO,* SHINOBU INANAGA, MASAKO KATO,† TOSHIYUKI SHIMIZU,‡ TOSHIO HAKOSHIMA‡ and AKIRA ISOGAI‡

Arid Land Research Center, Tottori University, 1390 Hamasaka, Tottori 680-0001, Japan; †Department of Chemistry, Nara Women's University, Kita-uoya-nishi machi, Nara 630-8263, Japan; ‡Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama-cho, Ikoma 630-0101, Japan

(*Received in revised form* 9 *December* 1997)

Key Word Index—*Menispermum dauricum*; Menispermaceae; root culture; alkaloid; dechloroacutumine; acutumine.

Abstract—A novel alkaloid, dechloroacutumine, was isolated from *Menispermum dauricum* roots, a rich source of the chlorine-containing alkaloid acutumine, cultured in chlorine-deficient medium. Its structure was elucidated by spectral and crystallographic analysis. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Natural chlorine-containing compounds of higher plants are of increasing interest due to their frequent occurrence, although it was thought ten years ago that they were not very common [1]. The number of natural organochlorine compounds has grown to more than 1500, most of which are produced by living organisms, such as marine and terrestrial plants, bacteria, fungi, lichen, insects, marine animals and some mammals [2]. However, limited knowledge is available about the biosynthetic pathways of chlorinated metabolites, especially that of the chlorination step. Chloroperoxidases, enzymes catalyzing the formation of carbon chlorine bonds in the presence of hydrogen peroxide and chlorine ion, have been obtained from algae, bacteria and lower fungi [3]. These enzymes exhibit a very broad specificity with respect to substrates and types of reactions catalyzed. Their presence in higher plants has yet to be established.

Acutumine is a chlorine-containing alkaloid found in *Sinomenium acutum* [4], *Menispermum canadense* [5] and *M. dauricum* [6]. Acutumidine, an analogous chlorine-containing alkaloid, was isolated from *M. canadense* [5] and *M. dauricum* [6]. Furthermore, another analogue, acutuminine, was found in *M. dauricum* [7]. Recently, we found that cultured roots of *M. dauricum* produced acutumine as one of major constituents [8]. Chlorine sources in the artificial culture medium are defined and, therefore, can be manipulated.

In the present work, we report the isolation and structural elucidation of a new alkaloid, named dechloroacutumine, which accumulates in *M. dauricum* roots cultured in a chlorine-deficient medium.

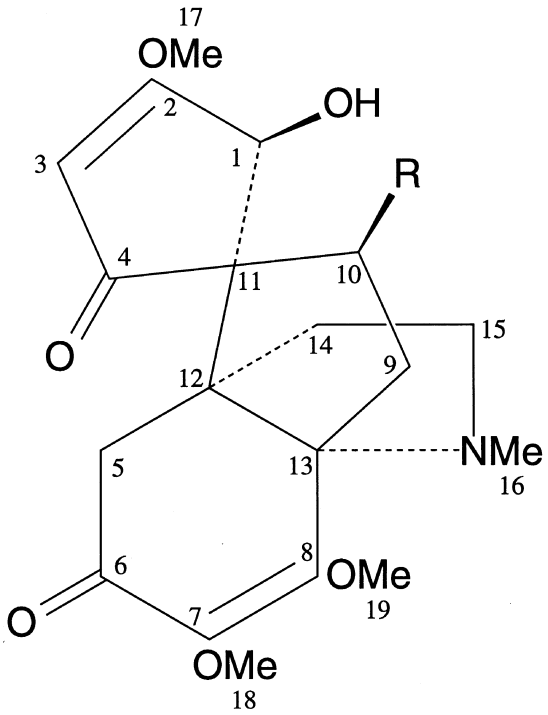
RESULTS AND DISCUSSION

Roots were cultured in a chlorine-deficient medium, which contained only 3.4% chlorine compared with the standard B5 medium. Root growth was not affected by chlorine deficiency. After 55 days of culture, roots were harvested and dried. Alkaloids were extracted from the dried roots and analyzed by HPLC. This showed that the acutumine (**2**) content decreased from *ca* 0.15% to 0.1% of the dry wt and that an unknown base accumulated. This base was obtained as colourless crystals after purification as described in the Experimental and its structure was elucidated as described below.

Mass spectral ions of the unknown base did not show isotopic patterns for chlorine atoms. The molecular formula was found to be $C_{19}H_{25}O_6N$ by high resolution mass spectrometry (m/z 363.1678 for $[M]^+$). The EI mass spectral fragmentation pattern and UV absorption, which were similar to those of **2**, as well as the molecular formula, suggested that this base was a dechlorinated analogue of **2**.

The ^{1}H NMR spectrum of the base exhibited one *N*-methyl and three *O*-methyl signals, two one-proton singlets at δ 4.57 and 5.28, and two mutually coupled one-proton doublets at δ 2.26 and 2.49. Comparing with the spectrum of **2**, a one-proton quartet at δ 5.16 disappeared and two additional protons were

* Author to whom correspondence should be addressed.



observed in the region of the aliphatic protons. Splitting patterns of the upfield proton signals became more complex in the unknown base. The ^{13}C NMR spectrum of the base was similar to that of **2**, except for two signals. Carbons at δ 41.5 (*t*) and 57.9 (*d*) of **2** shifted to δ 29.7 (*t*) and 32.0 (*t*) in the unknown base. HMQC experiments revealed a correlation between the carbon at δ 57.9 and the proton at δ 5.16. These observations strongly suggested that the unknown base differed from acutumine (**2**) only in the absence of the chlorine atom at C-10.

Recrystallization of the base from hexane-EtOAc provided crystals suitable for single-crystal X-ray diffraction analysis, which unambiguously established the molecular structure and relative stereochemistry of the base (Figure 1). The atomic coordinates are given in Table. 1. The absolute configuration of **2** has been determined by X-ray analysis using the anomalous dispersion of the chlorine atom [9]. For the determination of the absolute configuration of the new base, its CD spectrum was compared with that of **2**. The CD spectral patterns of both compounds were quite similar and have negative Cotton effects near 320 and 240 nm as well as a positive Cotton effect near 265 nm. Therefore, the absolute stereochemistry of the base was confirmed as that shown in structural formula **1**; the new compound is named as dechloroacutumine.

^{1}H and ^{13}C NMR assignments for **1** and **2** were based on DEPT, COSY, HMQC and HMBC experiments (Table 2). The ^{1}H NMR spectrum of compound **1** displayed an *N*-methyl signal at δ 2.40 and its attached carbon signal was located at δ 37.3 in the ^{13}C NMR spectrum. From the HMBC spectrum, connectivity patterns were observed between the *N*-methyl proton and the C-15 carbon at δ 53.6, as well as the C-13 carbon at δ 77.3. Connectivities of C-12 with H-15 (δ 2.51 and 2.74) and H-1 (δ 4.57) were also observed. H-1 was one of the two downfield one-proton singlets and another was assigned as H-3 (δ 5.28). Further connectivities were observed for H-3 with C-4 (δ 207.6) and C-11 (δ 66.8); C-2 (δ 189.9) with H-1 and H-17 (δ 3.88); C-4 with H-10 (δ 1.51 and 2.24). Thus, ^{1}H and ^{13}C signals of the five-membered α, β-unsaturated ketone system were assigned. HMBC experiments also revealed correlation of C-6 (δ 196.4)

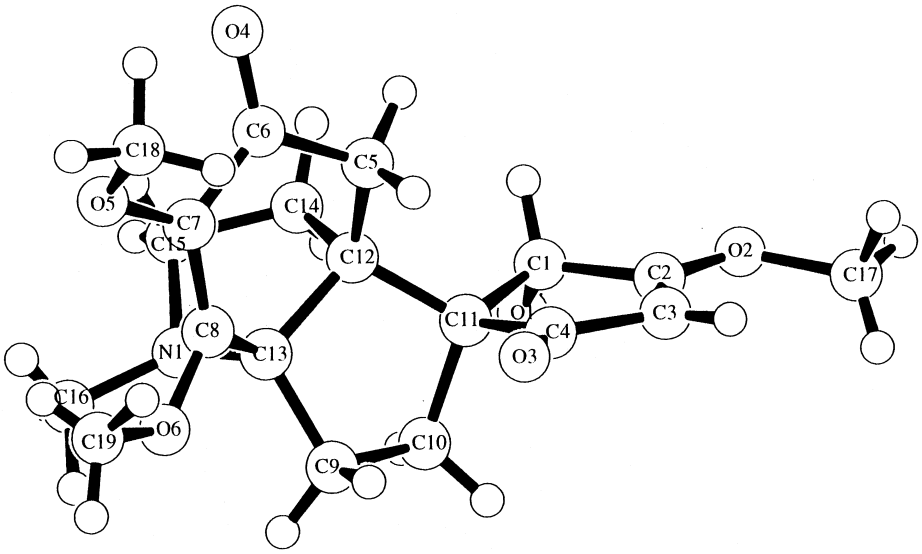


Fig. 1. Crystal structure of dechloroacutumine (**1**).

Table 1. Atomic coordinates and equivalent isotropic displacement parameters for compound **1** (estimated standard deviations in parentheses)

Atom	x	y	z	B(eq)
O(1)	−0.1693(1)	−0.3349(1)	0.0638(3)	3.14(4)
O(2)	−0.0349(1)	−0.3526(1)	−0.2213(3)	3.72(4)
O(3)	−0.3375(2)	−0.3328(1)	−0.5012(3)	4.28(5)
O(4)	−0.4839(1)	−0.5839(1)	−0.2814(3)	3.71(4)
O(5)	−0.6470(1)	−0.4958(1)	−0.3058(3)	4.04(4)
O(6)	−0.6133(1)	−0.3331(1)	−0.2440(4)	4.72(5)
N(1)	−0.4906(2)	−0.3656(1)	0.0916(3)	3.16(4)
C(1)	−0.1923(2)	−0.3722(1)	−0.1046(4)	2.51(5)
C(2)	−0.1280(2)	−0.3505(1)	−0.2627(4)	2.86(5)
C(3)	−0.1738(2)	−0.3341(2)	−0.4197(4)	3.07(5)
C(4)	−0.2754(2)	−0.3389(1)	−0.3852(4)	2.92(5)
C(5)	−0.3808(2)	−0.4751(1)	−0.2634(4)	2.96(5)
C(6)	−0.4768(2)	−0.5143(1)	−0.2646(3)	2.77(5)
C(7)	−0.5595(2)	−0.4641(2)	−0.2646(4)	3.08(5)
C(8)	−0.5488(2)	−0.3889(2)	−0.2219(4)	3.15(5)
C(9)	−0.4374(2)	−0.2769(2)	−0.1517(4)	3.24(6)
C(10)	−0.3318(2)	−0.2744(1)	−0.0994(4)	3.03(5)
C(11)	−0.2926(2)	−0.3512(1)	−0.1752(3)	2.40(5)
C(12)	−0.3727(2)	−0.4089(1)	−0.1247(3)	2.44(4)
C(13)	−0.4661(2)	−0.3592(1)	−0.1079(4)	2.65(5)
C(14)	−0.3584(2)	−0.4420(1)	0.0737(4)	2.92(5)
C(15)	−0.4572(2)	−0.4386(2)	0.1578(3)	3.10(5)
C(16)	−0.5867(3)	−0.3461(2)	0.1504(5)	4.62(7)
C(17)	0.0291(2)	−0.3245(3)	−0.3633(5)	4.86(8)
C(18)	−0.6552(3)	−0.5322(3)	−0.4830(7)	5.91(9)
C(19)	−0.7026(2)	−0.3429(2)	−0.3322(6)	4.65(8)

with H-5 (δ2.26 and 2.49), of C-5 (δ 46.5) with H-14 (δ 2.30), of H-5 with C-14 (δ 38.3) and C-7 (δ139.6), and of C-7 with H-18 (δ 3.61). The remaining carbon at δ 164.5 was assigned as C-8 and correlation of C-8 with H-19 (δ 4.08) and H-9 (δ 2.25) was observed by HMBC.

The ^{1}H and ^{13}C signals of the known compound acutumine (**2**) were also assigned, because there had been no reports on its NMR assignment since its structure was established by X-ray diffraction analysis 30 years ago [9]. The ^{1}H NMR spectrum of acutumine (**2**) was simpler than that of **1**, because a proton atom attached to C-10 of dechloroacutumine (**1**) was replaced by a chlorine atom. The ^{1}H and ^{13}C signals were assigned unambiguously as shown in Table 2, in a similar manner to that employed for **1**.

Barton *et al*. [10] deduced that **2** was a type of benzylisoquinoline alkaloid, and proposed a complicated biosynthetic pathway from a simple benzylisoquinoline. The order of the steps they presented was arbitrary and there are obvious alternative steps as they mentioned. They showed that the chlorine was introduced not by a working-up process, but by a true process of biosynthesis, on the basis of radiochemical methods. Recently, the proposed pathway was in part verified by Sugimoto and coworkers [8], where they demonstrated that tyrosine was a building block for **2**. However, it is still remained obscure at what stage of the biosynthesis chlorination takes place. Depending on the structure of dechloroacutumine, it can be converted to acutumine by chlorination at C-10. The biosynthetic relationship between **1** and **2** is now under investigation.

EXPERIMENTAL

General.

^{1}H and ^{13}C NMR spectra were recorded in CD_3OD and pyridine-d_5 for dechloroacutumine (**1**) and acutumine (**2**), respectively, on a JEOL Lambda 400 MHz spectrometer. NMR experiments included ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY, DEPT, HMBC and HMQC. IR spectra were obtained from KBr disks.

Plant material.

Menispermum dauricum roots were obtained from established cultures as described previously [11].

Root culture.

There are four chlorine-containing components in Gamborg's B5 medium [12], $CaCl_2{\cdot}2H_2O$ (1.0 mM), thiamine · HCl (30 μM), pyridoxine · HCl (5 μM) and $CoCl_2{\cdot}6H_2O$ (0.2 μM). Roots were cultured in a modi-

Table 2. NMR data of dechloroacutumine (**1**) and acutumine (**2**)

	1 (CD_3OD)						**2** (C_5D_5N)				
	^{13}C		^{1}H				^{13}C		^{1}H		
Position	δ (ppm)	m*	δ (ppm)	m	J(Hz)	Position	δ (ppm)	m*	δ (ppm)	m	J(Hz)
1	72.7	*d*	4.57	*s*		1	70.7	*d*	5.00	*s*	
2	189.9	*s*	—			2	189.0	*s*	—		
3	103.9	*d*	5.28	*s*		3	105.6	*d*	5.58	*s*	
4	207.6	*s*	—			4	201.4	*s*	—		
5	46.5	*t*	2.26	*d*	17	5	47.3	*t*	2.50	*d*	15
			2.49	*d*	17				3.02	*d*	15
6	196.4	*s*	—			6	192.9	*s*	—		
7	139.6	*s*	—			7	139.0	*s*	—		
8	164.5	*s*	—			8	159.8	*s*	—		
9	32.0	*t*	1.92	*m*		9	41.5	*t*	2.64	*dd*	7, 12
			2.25	*m*					3.14	*dd*	12, 12
10	29.7	*t*	1.51	*m*		10	57.9	*d*	5.16	*dd*	7, 12
			2.24	*m*							
11	66.8	*s*	—			11	68.4	*s*	—		
12	55.3	*s*	—			12	53.3	*s*	—		
13	77.3	*s*	—			13	73.0	*s*	—		
14	38.3	*t*	1.53	*m*		14	38.6	*t*	1.61	*m*	
			2.30	*m*					2.65	*m*	
15	53.6	*t*	2.51	*m*		15	51.8	*t*	2.43	*m*	
			2.74	*m*					2.65	*m*	
16	37.3	*q*	2.40	*s*		16	36.4	*q*	2.38	*s*	
17	59.7	*q*	3.88	*s*		17	58.9	*q*	3.71	*s*	
18	60.8	*q*	3.61	*s*		18	60.2	*q*	3.78	*s*	
19	61.1	*q*	4.08	*s*		19	60.5	*q*	4.03	*s*	

*Peak multiplicities confirmed by DEPT spectra.

fied B5 medium, in which $CaCl_2$ was replaced by $Ca(NO_3)_2$. The medium, which was also supplemented with 3% sucrose and 7.5 μM NAA, is referred to as the chlorine-deficient medium. Roots (0.1 g fr. wt) were cultured in 100 ml flasks containing 25 ml of the chlorine-deficient medium in the dark at 27° on a rotary shaker (70 rpm).

Extraction and isolation.

After 55 days of culture, roots were harvested and freeze-dried. A sample (16.6 g) was powdered and sonicated for 30 min in MeOH. Roots were then filtered from MeOH through filter paper. Treatment with MeOH was repeated twice. Thereafter, the combined MeOH extracts were dried by evapn. The dry residue (4 g) was dissolved in 200 ml of 3% citric acid, then the acidic aq. soln was passed through filter paper. The filtrate was made alkaline with aq. NH_3 and extracted $\times 4$ with $CHCl_3$. The $CHCl_3$ extracts were dried by evapn. The basic residue (0.32 g) was chromatographed over silica gel (50 g) with $CHCl_3$-MeOH. The proportion of MeOH in the solvent system was increased stepwise. Fras eluted with $CHCl_3$-MeOH (20:1) were concd (44 mg) and further purified by semi-prep HPLC. The column was Capcell Pak C18 (250×20 mm) with a solvent of 60% MeOH containing 0.2% aq. NH_3 at a flow rate of 7 ml/min^{-1}. A short pre-column (10×4.6 mm) was placed between the injector and separation column. Dechloroacutumine (**1**), Rt 9.2 min, was detected by UV absorption at 245 nm. The collected fras yielded a white powder (19 mg). Recrystalization of this from EtOAc-Hexane gave **1** as colourless crystals, mp 178.0–178.5°. $[\alpha]_D^{25.0} -54°$ (MeOH, *c* 0.1). CD $\Delta\epsilon_{319.8} -8.8$, $\Delta\epsilon_{262.4} +17.5$, $\Delta\epsilon_{238.0} -18.7$ (MeOH, 7.59×10^{-5} M). UV λ_{max} nm (log ϵ): 241 (4.27), 268 (3.99). IR ν_{max} cm^{-1}: 1679, 1638, 1611, 1579, 1349. ^1H and ^{13}C NMR: Tab. 2. EIMS (70 eV) *m/z* (rel. int) 363 [M$^+$] (100), 335 (41), 320 (56), 220 (26), 209 (47), 208 (51), 181 (25), 166 (11), 150 (7). HRMS m/z 363.1678 [M$^+$] ($C_{19}H_{25}O_6N$ requires 363.1682).

Acutumine was obtained as colourless crystals from roots cultured in a B5 medium with 7.5 μM NAA and 3% sucrose as described previously [8]. CD $\Delta\epsilon_{321.2} -10.1$, $\Delta\epsilon_{265.4} +19.5$, $\Delta\epsilon_{239.6} -20.0$ (MeOH, 3.51×10^{-5} M). ^1H and ^{13}C NMR: Tab. 2.

*X-ray crystallographic analysis of dechloroacutumine (**1**).*

A colourless prismatic crystal of $C_{19}H_{25}O_6N$ having approximate dimensions of $0.6 \times 0.4 \times 0.4$ mm, *Mr* 363.17, orthorhombic, $a = 14.05$ (1), $b = 17.47$ (1), $c = 7.219$ (4) Å, $V = 1771$ (1) Å^3, space group $P2_12_12_1$ (#19), $Z = 4$, $Dc = 1.363$ g cm^{-3}, μ (Cu$K\alpha$) = 8.43 cm^{-1},

F (000) = 776. All measurements were made on a Rigaku AFC7R diffractometer with graphite monochromated Cu$K\alpha$ radiation and a rotation anode generator. A total of 1555 unique reflections were collected and the intensities of three representative reflections were measured after every 150 reflections. The structure was solved by direct methods (SIR92) [13] and expanded using Fourier techniques [14]. The final cycle of full-matrix least-squares refinement was based on 1482 observed reflections ($I > 3\sigma(I)$) and 336 variable parameters, and converged with unweighted and weighted agreement factors of $R = 0.042$ and $Rw = 0.057$. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.23 and $-0.23\,e\text{Å}^{-3}$, respectively. All calculation were performed using the teXsan crystallographic software package of Molecular Structure Corporation. The non-hydrogen atoms and hydrogen atoms were refined anisotropically and isotropically, respectively. All data have been deposited at the Cambridge Crystallographic Data Centre.

Acknowledgements—This work was supported, in part, by Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science, Sports and Culture to Y. S. and by a grant from the Plant Cell Culture Research Association to A. I.

REFERENCES

1. Engvild, K. C., *Phytochemistry*, 1986, **25**, 781.
2. Gribble, G. W., *Pure and Applied Chemistry*, 1996, **68**, 1699.
3. Cohen, P. A. and Towers, G. H. N., *Phytochemistry*, 1997, **44**, 271.
4. Goto, K. and Sudzuki, H., *Bulletin of the Chemical Society of Japan*, 1929, **4**, 220.
5. Doskotch, R. W. and Knapp, J. E., *Lloydia*, 1971, **34**, 292.
6. Tomita, M., Okamoto, Y., Kikuchi, T., Osaki, K., Nishikawa, M., Kamiya, K., Sasaki, Y. and Matoba, K., Goto, K., *Tetrahedron Letters*, 1967, 2421.
7. Okamoto, Y., Yuge, E., Nagai, Y., Katsuta, R., Kishimoto, A., Kobayashi, Y., Kikuchi, T. and Tomita, M., *Tetrahedron Letters*, 1969, 1933.
8. Sugimoto, Y., Uchida, S., Inanaga, S., Kimura, Y., Hashimoto, M. and Isogai, A., *Bioscience, Biotechnology and Biochemistry*, 1996, **60**, 503.
9. Nishikawa, M., Kamiya, K., Tomita, M., Okamoto, Y., Kikuchi, T., Osaki, K., Tomiie, Y. and Nitta, I., Goto, K., *Journal of the Chemical Society* (*B*), 1968, 652.
10. Barton, D. H. R., Kirby, A. J. and Kirby, G. W., *Journal of the Chemical Society* (*C*), 1968, 929.
11. Sugimoto, Y., Yoshida, A., Uchida, S., Inanaga, S. and Yamada, Y., *Phytochemistry*, 1994, **36**, 679.
12. Gamborg, O. L., Miller, R. A. and Ojima, K., *Experimental Cell Research*, 1968, **50**, 151.
13. Altomare, A., Cascarano, G., Giacovazzo, C., Guagliardi, A., Burla, M. C., Polidori, G. and Camalli, M., *Journal of Applied Crystallography*, 1994, **27**, 435.
14. Beurskens, P. T., Admiraal, G., Benurskens, G., Bosman, W. P., de Gelder, R., Israel, R. & Smits, J. M. M., The DIRDIF-94 program system, Crystallography Laboratory, University of Nijmegen, The Netherlands, 1994.