Chlorinated Alkaloids in *Menispermum dauricum* DC. Root Culture

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Feeding experiments using ^{36}Cl showed that *Menispermum dauricum* root culture produces four alkaloids containing chlorine. They included the novel alkaloids dauricumine and dauricumidine as well as the known alkaloids acutumine and acutumidine. The structures of novel alkaloids were established by spectroscopic, crystallographic, and chemical methods. These four alkaloids were labeled with ^{36}Cl, isolated, and fed independently to root cultures. Mutual conversion between acutumine and acutumidine, and between dauricumine and dauricumidine by *N*-methylation and *N*-demethylation, was demonstrated. Moreover, dauricumine was converted to acutumine and acutumidine. Epimerization of acutumidine to dauricumidine or vice versa was not observed. These results suggest that dauricumine is the first chlorinated alkaloid formed in cultured *M. dauricum* roots. Skewed distribution of radioactivity derived from labeled dauricumine is proof that epimerization at C-1 proceeds at a lower rate than *N*-demethylation.

Introduction

Naturally occurring organohalogen compounds are produced by marine and terrestrial plants, bacteria, fungi, lichens, insects, marine animals, some higher animals, and a few mammals.[1] The involvement of haloperoxidases in the biosynthesis of these halometabolites is generally accepted. These enzymes catalyze the oxidation of halides (X^-) in the presence of hydrogen peroxide to the corresponding hypohalous acids (HOX). If a convenient nucleophilic acceptor is present, a reaction will occur with HOX to form a diversity of halogenated products.[2] This reaction mechanism is consistent with the fact that all isolated bacterial and eukaryotic haloperoxidases have very low or no substrate specificity.[3] Natural compounds containing halogens in higher plants are rare,[4] and presence of haloperoxidases in higher plants has yet to be fully established. Chloroperoxidase may be involved in the chlorination of orcinol derivatives in diseased bulbs of *Lilium maximowiczii*.[5]

We established *Menispermum dauricum* DC. root cultures that produce acutumine (**3**) and its dechloro derivative dechloroacutumine (**5**).[6] In vitro plant cultures offer suitable systems for studying the biosynthesis of plant metabolites.[7] Hence, *M. dauricum* root cultures are useful tools with which to study the chlorination mechanism. The production of compounds containing chlorine is affected by the Cl^- ion concentration in culture medium.[8] Dechloroacutumine is derived from the same biosynthetic pathway as acutumine. Moreover, dechloroacutumine (**5**) is a biosynthetic precursor of acutumine (**3**). However, only 5% of the radioactivity derived from the former was transferred into the latter during 25 days culture when ^3H-labeled dechloroacutumine was fed to the roots.[9] The low conversion rate suggested that dechloroacutumine (**5**) might not be the immediate precursor of acutumine (**3**) and that more important biosynthetic pathways could be involved in the production of acutumine (**3**). We therefore investigated other chlorinated alkaloids produced in this culture. Here we describe the identification of such alkaloids based on ^{36}Cl incorporation. These alkaloids were isolated in a separate experiment and their structures were elucidated. Moreover, biosynthetic relationships among four compounds containing chlorine were studied by feeding respective ^{36}Cl-labeled compounds to cultured roots.

Results and Discussion

Menispermum dauricum roots were cultured in B5 medium in which ^{35}Cl and ^{37}Cl were substituted with radioactive ^{36}Cl (1.5×10^7 dpm). After a 50 day incuba-

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Table 1. Uptake and Incorporation of ^{36}Cl into Cultured Roots and Chlorinated Alkaloids

	incorporation (%)				
uptake[a] (%)	crude alkaloids	acutumidine (**1**)	dauricumidine (**2**)	acutumine (**3**)	dauricumine (**4**)
29.8	5.33	2.49	1.42	1.21	0.21

[a] Calculated on the basis of radioactivity remaining in the culture medium.

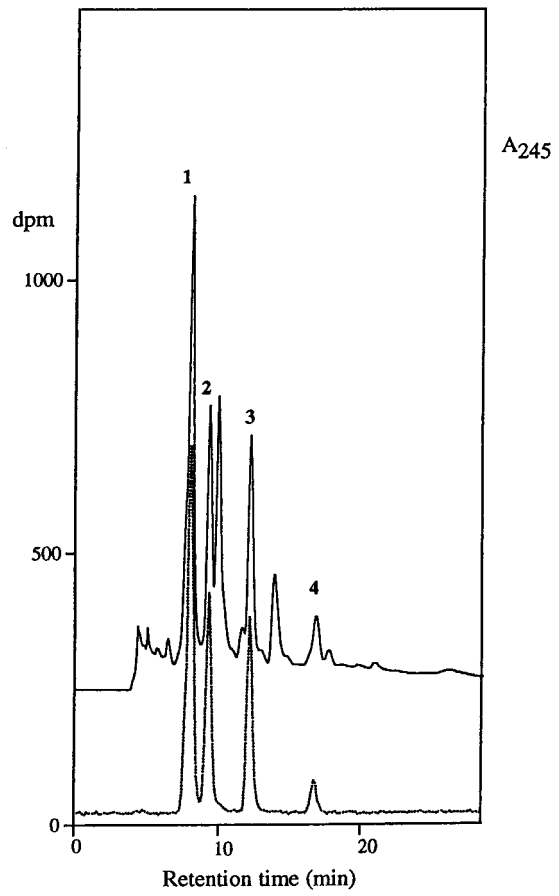

Figure 1. HPLC analysis of the basic fraction extracted from *M. dauricum* roots cultured with $Na^{36}Cl$. (.....) Radioactivity of the fractions; (_____) absorbance at 245 nm.

tion, the roots were harvested, lyophilized, and extracted with methanol to yield crude alkaloids. On the basis of the radioactivity remaining in the medium, 30% of the ^{36}Cl was incorporated into the roots, 18% of which was recovered in the crude alkaloid preparation (Table 1). Analysis by HPLC showed four peaks (t_Rs 7.9, 9.2, 12.1, and 16.8 min) labeled with ^{36}Cl. These four peaks were tentatively named compounds **1**−**4** in the order of elution shown in Figure 1. The amount of radioactivity detected in these four peaks accounted for the total injected for analysis (Table 1). These alkaloids were isolated in another experiment as described in the Experimental Section and structurally analyzed.

Compound **3** eluted at t_R 12.1 min was identified as acutumine on the basis of a direct comparison of chromatographic behavior on HPLC, as well as mass, CD, and NMR spectra with authentic acutumine. The mass spectra of the others were also identical to those of compounds having a chlorine atom. HREIMS of compounds **1** (*m*/*z* 383.1134 for $[M]^+$) and **2** (*m*/*z* 383.1137 for $[M]^+$) revealed that the formulas of both were $C_{18}H_{22}NO_6Cl$, which corresponds to the loss of one carbon and two hydrogens from acutumine (**3**). The molecular formula for compound **4** was $C_{19}H_{24}NO_6Cl$, which was the same as that of acutumine (**3**) determined by HREIMS (*m*/*z* 397.1293 for $[M]^+$). The EI mass spectral fragmentation patterns of compounds **1** and **2** were identical. Most of the values for the fragment ions of both compounds were 14 less than that of acutumine. The EI mass spectra of compound **4** and acutumine were identical. Moreover, UV absorption of the three compounds was quite similar to that of acutumine. The above data suggested that compounds **1**, **2** and **4** are analogous to acutumine (**3**).

Nineteen ^{13}C NMR resonances of compound **4** were almost completely superimposable upon those of acutumine (**3**), and DEPT spectra exhibited four methyl, four methylene, three methine, and eight quaternary carbon signals. Furthermore, the ^1H NMR spectrum of **4** showed one *N*-methyl and three *O*-methyl signals. These observations suggest that compound **4** is a diastereomer of acutumine. The ^1H and ^{13}C NMR signals of **4** were unambiguously assigned as shown in Tables 2 and 3, respectively, on the basis of $^1H-^1H$ COSY, HMQC, and HMBC data. Recrystallization of **4** from MeOH yielded colorless prismatic crystals suitable for X-ray crystallographic analysis and its relative configuration was determined as (1*R**,10*S**,11*R**,12*S**,13*S**). The absolute configuration of acutumine is (1*S*,10*S*,11*R*,12*S*,13*S*) according to X-ray analysis.[10] Moreover, the configurations at C-12 and C-13 of acutumine (**3**) have been confirmed by CD spectral data showing a negative Cotton effect near 320 nm.[11] To determine the absolute configuration of **4**, its CD spectrum was compared with that of **3**. The CD spectrum of compound **4** showed a negative Cotton effect around 315 nm, indicating that the configurations at C-12 and C-13 of **4** are consistent with those of **3**. Therefore, the absolute stereochemistry of compound **4** was (1*R*,10*S*,11*R*,12*S*,13*S*), an epimer of acutumine (**3**) differing only in its stereochemistry at C-1. The new compound was named dauricumine (**4**).

No *N*-methyl signals were observed in the ^1H and ^{13}C NMR spectra of compounds **1** and **2**. Taking mass spectral data into consideration, these data indicate that **1** and **2** are *N*-demethyl derivatives of acutumine (**3**) and dauricumine (**4**), respectively. To confirm the deduced structures, compounds **1** and **2** were exposed to formalin and formic acid. Chromatographic behavior, and NMR and mass spectra of the *N*-methylated products of **1** and **2** matched those of **3** and **4**, respectively. Moreover, positive and negative Cotton effects in CD spectra of the *N*-methylated products were consistent with those of their corresponding alklaoids. Therefore compound **1** was identified as acutumidine, a chlorinated alkaloid found in *S. acutum*, *M. canadense*, and *M. dauricum*.[12] Com-

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Table 2. ^{1}H NMR Data[a] for Acutumidine (1), Dauricumidine (2), Acutumine (3), and Dauricumine (4)

position	acutumidine (**1**)	dauricumidine (**2**)	acutumine (**3**)[b]	dauricumine (**4**)
1	5.00[c]	4.90 s	5.00 s	4.89 d (6.0)
3	5.49 s	5.53 s	5.58 s	5.61 s
5	2.42 d (17.3)	2.66 d (17.7)	2.50 d (15)	2.99 d (16.3)
	2.75 d (17.3)	3.44 d (17.7)	3.02 d (15)	3.39 d (16.3)
9	2.78 dd (7.0, 12.3)	2.62 dd (6.7, 12.1)	2.64 dd (7, 12)	2.45 dd (6.7, 12.0)
	3.17 dd (12.3, 12.3)	3.08 dd (12.1, 12.1)	3.14 dd (12, 12)	3.03 dd (12.0, 12.0)
10	5.23 dd (7.0, 12.3)	4.87 dd (6.7, 12.1)	5.16 dd (7, 12)	4.81 dd (6.7, 12.0)
14	1.60 dd (3.9, 11.6)	2.32 ddd (6.2, 11.3, 12.7)	1.61 m	2.28 m
	2.59 ddd (6.3, 11.6, 11.6)	2.40 dd (4.5, 12.7)	2.65 m	2.42[c]
15	2.81 ddd (3.9, 9.6, 11.6)	2.86 ddd (4.5, 9.6, 11.3)	2.43 m	2.45[c]
	2.92 dd (6.3, 9.6)	2.98 dd (6.2, 9.6)	2.65 m	2.62 m
16			2.38 s	2.35 s
17	3.64 s	3.64 s	3.71 s	3.68 s
18	3.88 s	3.89 s	3.78 s	3.77 s
19	4.05 s	4.06 s	4.03 s	4.07 s
OH	8.37 br d (5.5)	8.15 br s	8.30 br s	8.20 d (6.0)

[a] NMR chemical shifts were referenced to pyridine-d_5 (δ_H 8.7). [b] Literature values (ref 6b). [c] Overlapping NMR signals.

Table 3. ^{13}C NMR Data[a] for Acutumidine (1), Dauricumidine (2), Acutumine (3) and Dauricumine (4)

position	acutumidine (**1**)	dauricumidine (**2**)	acutumine (**3**)[b]	dauricumine (**4**)
1	71.0	75.4	70.7	75.0
2	188.9	189.4	189.0	189.6
3	105.6	106.0	105.6	105.9
4	200.5	202.3	201.4	203.3
5	45.4	47.4	47.3	50.0
6	191.3	192.4	192.9	193.9
7	136.2	136.4	139.0	139.0
8	161.6	161.2	159.8	160.3
9	48.1	47.6	41.5	40.8
10	56.7	61.0	57.9	61.9
11	68.5	68.5	68.4	68.8
12	52.5	51.9	53.3	52.8
13	70.2	70.8	73.0	73.5
14	41.0	43.3	38.6	41.1
15	43.8	44.2	51.8	52.0
16			36.4	36.0
17	58.7	58.7	58.9	58.8
18	60.2	60.2	60.2	60.0
19	60.8	60.8	60.5	60.6

[a] NMR chemical shifts were referenced to pyridine-d_5 (δ_C 149.8). [b] Literature values (ref 6b).

pound **2** was named dauricumidine. The ^{1}H and ^{13}C NMR signals of acutumidine (**1**) and dauricumidine (**2**) were assigned as shown in Tables 2 and 3, respectively.

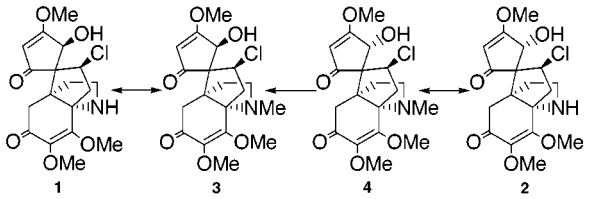
Crude alkaloid preparation was labeled with ^{36}Cl as mentioned above (Figure 1) and loaded on a semi-preparative HPLC, and the four alkaloids were separated. Their purity and specific radioactivity was determined by analytical HPLC. ^{36}Cl-labeled acutumidine (**1**, 0.348 mg, 2.3×10^5 dpm, 2.6×10^8 dpm/mmol), dauricumidine (**2**, 0.150 mg, 1.0×10^5 dpm, 2.6×10^8 dpm/mmol), acutumine (**3**, 0.106 mg, 7.7×10^4 dpm, 2.9×10^8 dpm/mmol), and dauricumine (**4**, 0.007 mg, 7.0×10^3 dpm, 3.8×10^8 dpm/mmol) were used as tracers with which to study the biosynthesis of these alkaloids. Each labeled substrate was dissolved in 50 μL of MeOH and then applied to 18-day-old *Menispermum* roots cultured in chloride-deficient medium.[8] Over 25 days, each substrate was taken up by the roots very efficiently; 81.7% for dauricumine (**4**) and 94% or more for the others (Table 4). HPLC analyses of crude alkaloids obtained from roots exposed to either of radiolabeled substrates, showed that the exogenous precursors were metabolized in vivo to other chlorinated alkaloids. *Menispermum* roots converted acutumidine (**1**) to acutumine (**3**) (17.9%), and dauricumidine (**2**) to dauricumine (**4**) (20.9%). Recovery

Table 4. Uptake and Conversion of ^{36}Cl-labeled Acutumidine (1), Dauricumidine (2), Acutumine (3), and Dauricumine (4)

			product and conversion rate (%)			
substrate	uptake (%)[a]	recovery (%)	**1**	**2**	**3**	**4**
acutumidine (**1**)	94.1	31.3	–	nd[b]	17.9	nd
dauricumidine (**2**)	95.2	24.5	nd	–	nd	20.9
acutumine (**3**)	97.0	21.7	13.6	nd	–	nd
dauricumine (**4**)	81.7	16.9	1.8	22.6	5.2	–

[a] Calculated on the basis of remaining radioactivity in culture media. [b] Not detected.

Scheme 1



of the substrates was 31.3% and 24.5% for acutumidine and dauricumidine, respectively (Table 4). Conversion of acutumidine to dauricumidine or vice versa was not observed. Labeled acutumine (**3**) and labeled dauricumine (**4**) were *N*-demethylated to acutumidine (**1**) (13.6%) and dauricumidine (**2**) (22.6%), respectively. Recovery of the substrates was 21.7% and 16.9%, respectively (Table 4). Acutumine (**3**) was not epimerized to dauricumine (**4**). However, two additional radioactive peaks, corresponding to acutumine (5.2%) and acutumidine (1.8%), were detected when ^{36}Cl-labeled dauricumine was given to roots (Table 4). This finding is evidence that dauricumine (**4**) is epimerized in vivo to acutumine (**3**) and the latter is subsequently *N*-demethylated to acutumidine (**1**). The higher levels of radioactivity detected in dauricumidine (**2**) than those in acutumidine (**1**) and acutumine (**3**) (Table 4) suggest that less epimerization occurs at C-1 than *N*-demethylation. The possible biosynthetic interrelationship among these compounds is summarized in Scheme 1. Acutumine (**3**), acutumidine (**1**), and dauricumidine (**2**) are derived from dauricumine (**4**), suggesting that **4** is the first alkaloid formed in the roots that contains chlorine.

Experimental Section

General. Mps are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded in pyridine-d_5. NMR experiments included ^{1}H–

^{1}H COSY, 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 pyridine.

Plant Materials and Culture Conditions. Excised *Menispermum dauricum* roots from established cultures[13] 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.

Feeding Experiments. Na^{36}Cl (483 kBq/mg Cl) was purchased from Amersham International plc, England. Roots (0.15 g fresh wt.) were cultured for 50 days in 25 mL of modified B5 medium supplemented with 3% sucrose and 1 μM NAA, which included 1.5×10^7 dpm of ^{36}Cl. Harvested roots were freeze-dried (75 mg), 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 (pH 10) with NH_4OH. This suspension was put onto Extrelut column. After 10 min, $CHCl_3$ was passed through the column twice, and the extracts were combined and then evaporated to dryness at 30 °C. The dry residue was dissolved in MeOH and analyzed by HPLC. The stationary phase was Develosil ODS-3 (4.6 × 150 mm), and the solvent was 60% MeOH containing 0.2% NH_4OH. The flow rate was 0.3 mL/min. A short precolumn (4.6 × 30 mm) was placed between the injector and the separation column. Fractions of about 150 μL of column effluent were collected in scintillation vials containing a xylene-based scintillation cocktail. Radioactivity of each fraction was measured using a scintillation counter. Four peaks of radioactivity eluted with t_Rs of 7.9, 9.2, 12.1, and 16.8 min.

The radioactive alkaloids were purified on a semipreparative HPLC. The column was Capcell-pack C_{18} (20 × 250 mm), and the solvent was 60% MeOH containing 0.2% NH_4OH. The flow rate was 4.0 mL/min. A short precolumn (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 t_Rs were 18.6, 21.1, 26.0, and 33.7 min, respectively. The quality and quantity of the purified alkaloids were determined by analytical HPLC.

Acutumidine (**1**), dauricumidine (**2**), acutumine (**3**), and dauricumine (**4**) labeled with ^{36}Cl were dissolved in 50 μL of MeOH and fed to 18-day-old *M. dauricum* roots grown in 50-mL flasks containing 12 mL of chloride-deficient medium.[8] After 25 days, alkaloids were extracted from the roots and separated by analytical HPLC, and the amount of radioactivity in the fractions was measured as described above.

Isolation and Identification of Chlorinated Alkaloids. Roots were cultured for 55 days in B5 medium containing 3% sucrose and 1 μM NAA, harvested, and lyophilized, and then 50 g was soaked overnight in MeOH and filtered. Methanol extraction was repeated three times, and the combined filtrates were evaporated to dryness at 40 °C. The residue (8.99 g) was dissolved in 3% citric acid, rendered alkaline with NH_4OH, and extracted four times with $CHCl_3$. The combined $CHCl_3$ extracts were evaporated. The residue (0.89 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 the solvent polarity, dauricumine (**4**) (50:1), acutumine (**3**) (30:1), dauricumidine (**2**) (20:1), and acutumidine (**1**) (20:1). These alkaloids were further purified on a semipreparative HPLC as described above.

Acutumidine (1): white powder (8.4 mg), mp 210 °C (decomp.); $[\alpha]^{25}_D$ −24° (*c* 0.1, pyridine); CD $\Delta\epsilon_{265}$ +17.6, $\Delta\epsilon_{240}$ −25.5 (MeOH, 5.2×10^{-5} M); HREIMS: *m/z* $[M]^+$ 383.1134 [$C_{18}H_{22}NO_6Cl$ requires 383.1136]; EIMS: *m/z* (rel int.) 385 (6.1), 383 (18.0), 348 (46.5), 320 (14.5), 195 (100), 194 (20.5), 152 (37.2); IR ν_{max} cm^{-1}: 1609, 1455, 1351, 1322, 1092, 981; UV λ_{max} nm (log ϵ): 242.4 (4.07); ^{1}H and ^{13}C NMR data are shown in Tables 2 and 3, respectively.

Dauricumidine (2): white powder (9.1 mg), mp 180 °C (decomp.); $[\alpha]^{23}_D$ +43° (*c* 0.1, pyridine); CD $\Delta\epsilon_{316}$ −2.8, $\Delta\epsilon_{261}$ +17.2, $\Delta\epsilon_{238}$ −9.3 (MeOH, 5.2×10^{-5} M); HREIMS: *m/z* $[M]^+$ 383.1137 [$C_{18}H_{22}NO_6Cl$ requires 383.1136]; EIMS: *m/z* (rel int.) 385 (6.3), 383 (17.8), 348 (54.5), 320 (13.4), 195 (100), 194 (21.2), 152 (30.3); IR ν_{max} cm^{-1}: 1687, 1674, 1606, 1357, 1322, 1101, 998; UV λ_{max} nm (log ϵ): 244.2 (4.11); ^{1}H and ^{13}C NMR data are shown in Tables 2 and 3, respectively.

Dauricumine (4): white powder (9.8 mg), recrystallization of this alkaloid from MeOH gave **4** as colorless crystals, mp 205 °C (decomp.); $[\alpha]^{25}_D$ −42° (*c* 0.1, pyridine); CD $\Delta\epsilon_{315}$ −16.9, $\Delta\epsilon_{276sh}$ +9.6, $\Delta\epsilon_{250}$ +16.8 (MeOH, 5.0×10^{-5} M); HREIMS: *m/z* $[M]^+$ 397.1293 [$C_{19}H_{24}NO_6Cl$ requires 397.1292]; EIMS: *m/z* (rel int.) 399 (7.0), 397 (21.2), 362 (59.9), 334 (16.1), 209 (100), 208 (23.5), 166 (27.0), 150 (15.9), 108 (14.6); IR ν_{max} cm^{-1}: 1698, 1654, 1620, 1608, 1348, 1235; UV λ_{max} nm (log ϵ): 243.8 (4.16); ^{1}H and ^{13}C NMR data are shown in Tables 2 and 3, respectively.

X-ray Crystallographic Analysis of Dauricumine (4). Crystal data for **4**: A colorless prismatic crystal of $C_{19}H_{24}NO_6Cl$ having appropriate dimensions of 0.60 × 0.60 × 0.36 mm, Mr 397.13, orthorhombic, a = 12.539 (3), b = 16.976 (4), c = 8.736 (4) Å, V = 1859 (1) Å^3, space group $P2_12_12_1$ (No. 19), Z = 4, D_c = 1.421 g cm^{-3}, μ(Mo Kα) = 2.42 cm^{-1}, F_{000} = 840.00. The data were collected on a Rigaku AFC-7R four-circle diffractometer with graphite monochromated Mo Kα radiation and a 18 kW rotating anode generator. The structure was solved by direct methods (SIR92)[14] and expanded using Fourier techniques.[15] Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final cycle of full-matrix least-squares refinement[16] was based on 2665 observed reflections ($I > 2\sigma(I)$) and 249 variable parameters and converged with R1 = 0.032 and wR = 0.116 (all data). All calculations were performed using the teXsan crystallographic software package developed by Molecular Structure Corporation.

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Note Added after ASAP Posting

The version of this paper posted on April 14, 2001, had incorrect compound numbering in Scheme 1 and the TOC graphic. The corrected version was posted on May 11, 2001.

Supporting Information Available: ^{1}H and ^{13}C NMR spectra for dauricumidine and dauricumine and X-ray crystallographic analysis data of dauricumine. This material is available free of charge via the Internet at http://pubs.acs.org.

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