

## Cembrene A and a Congeneric Ketone Isolated from the Paracloacal Glands of the Chinese Alligator (*Alligator sinensis*)

Daniell L. Mattern,<sup>†</sup> William D. Scott,<sup>†</sup> Clarence A. McDaniel,<sup>‡</sup> Paul J. Weldon,<sup>§</sup> and David E. Graves<sup>\*†</sup>

Department of Chemistry, University of Mississippi, University, Mississippi 38677, Southern Forest Experiment Station, USDA-FS, P.O. Box 2008 GMF, Gulfport, Mississippi 39505, and Conservation and Research Center, Smithsonian Institution, 1500 Remount Road, Front Royal, Virginia 22630

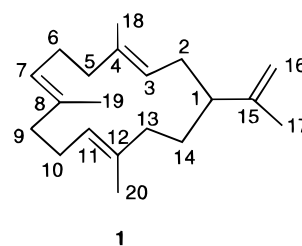
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A diterpene hydrocarbon and diterpene ketone isolated from the paracloacal gland secretions of the Chinese alligator (*Alligator sinensis*) were examined by GC–MS and <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, and IR spectroscopy, and identified as 4,8,12-trimethyl-1-(1-methylethenyl)-3,7,11-cyclotetradecatriene (cembrene A, **1**) and its congeneric ketone, 4,8,12-trimethyl-1-(1-methylethenyl)-3,7-cyclotetradecadien-10-one (**2**), respectively. This is the first report of cembrene A from a vertebrate; the ketone has not been described previously.

Analysis by GC–MS of the paracloacal gland secretions of alligators and caimans (Alligatoridae) has demonstrated the presence of acetates, free fatty acids, alcohols, and other lipids.<sup>1–5</sup> Analytical TLC has suggested hydrocarbons in the glandular exudates of a number of crocodylians,<sup>6,7</sup> but the structures of these compounds are known only for the smooth-fronted caiman (*Paleosuchus trigonatus*), both sexes of which contain high concentrations of (*E,E*)-7,11,15-trimethyl-3-methylenehexadeca-1,6,10,14-tetraene ( $\beta$ -springene) and trace amounts of  $\beta$ -farnesene.<sup>8</sup>

A previous analysis by GC–MS, TLC, and FT-IR spectroscopy indicated a diterpene hydrocarbon and a diterpene ketone from the paracloacal glands of adult male, but not female, Chinese alligators, *Alligator sinensis* Fauvel (Crocodylia, Reptilia).<sup>1</sup> The molecular weight of the diterpene hydrocarbon (272 Da) was the same as that of  $\beta$ -springene, but the mass spectra of these compounds differed. FT-IR data suggested that the diterpene hydrocarbon and diterpene ketone were related, but their structures were not resolved. We report here on the identity of the diterpene hydrocarbon and diterpene ketone from the paracloacal glands of this species.

Samples of the diterpene hydrocarbon and diterpene ketone were obtained by chromatographic fractionation of extracts of male *A. sinensis* paracloacal gland secretions. Mass spectra of the diterpene hydrocarbon and a sample subjected to exhaustive hydrogenation were compared. Hydrogenation resulted in an increase of 8 mass units in the parent ion, indicating the presence of four  $\pi$  bonds in the starting diterpene. A search of the Wiley/NIH mass spectrum library resulted in a 99% match of the hydrogenated material with 1,7,11-trimethyl-4-(1-methylethyl)cyclotetradecane; this strongly suggested that the starting material was a cembrene. A match of the <sup>13</sup>C-NMR data with literature values<sup>9</sup> confirmed the identity of this compound as 4,8,12-trimethyl-1-(1-methylethenyl)-3,7,11-cyclotetradecatriene (cembrene A, **1**).



The <sup>13</sup>C-NMR spectrum of the diterpene ketone (**2**) revealed the presence of 20 carbons (Table 1). Chemical shifts and DEPT experiments classified the carbons as one carbonyl, six vinylics, two methines, seven methylenes, and four methyls; the presence of the carbonyl (210.1 ppm) was confirmed by a band at 1715 cm<sup>-1</sup> in the FT-IR spectrum. The DEPT results gave a proton count of 32, consistent with a molecular formula of C<sub>20</sub>H<sub>32</sub>O, corresponding to a molecular ion at 288 Da in the mass spectrum. These data suggested that **2** was a ketone in the cembrene family.

Proton–carbon connectivities were determined from HETCOR and HMQC experiments and are indicated in Table 1. <sup>1</sup>H multiplicities from the <sup>1</sup>H-NMR spectrum are also given in Table 1. A COSY spectrum revealed couplings between vicinal (and allylic) protons, and the HMBC spectrum revealed two- and three-bonded couplings between protons and carbons. (These spectra are available as Supporting Information.) This information allowed deduction of the structure of compound **2** as follows.

The presence of two geminal, vinyl protons (H-16a and H-16b) suggested an isopropenyl group. The HMBC spectrum demonstrated coupling between both H-16's and the methylene carbon C-17; thus, the allylic methyl of the isopropenyl group was assigned as C-17. The HMBC spectrum also showed the vinyl H-16's to be coupled either to methine C-1 or methylene C-11. The coupling cannot be to C-11 because proton H-11a showed HMBC coupling to four other carbons (C-10, C-12, C-13, C-20), which is not possible if C-11 devotes a valence to an isopropenyl carbon. The isopropenyl group is, therefore, bonded to the methine C-1. From these results, we infer the structure labeled fragment I (Figure 1).

The COSY spectrum showed that methylene H-6 was coupled to methylene H-5 and to vinyl triplet H-7; this

\* To whom correspondence should be addressed. Phone: (601)232-4799. FAX: (601)232-7300. E-mail: graves@graves.chem.olemiss.edu.

<sup>†</sup> University of Mississippi.

<sup>‡</sup> Southern Forest Experiment Station.

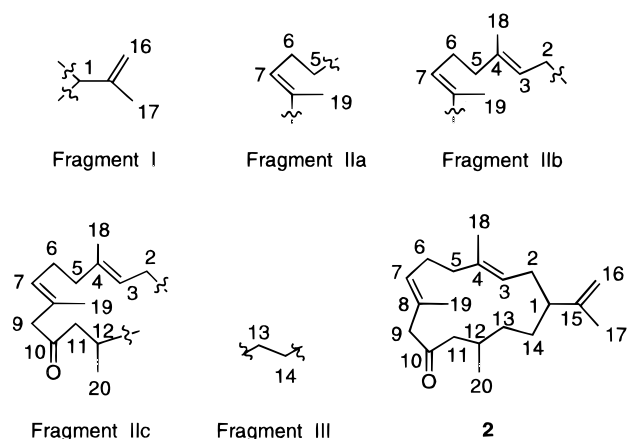
<sup>§</sup> Smithsonian Institution.

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**Table 1.** NMR Data for Dihydrocembrenone (**2**)

position	<sup>13</sup> C		<sup>1</sup> H		
	δ (ppm)	multiplicity <sup>a</sup>	position <sup>b</sup>	δ (ppm)	multiplicity
C-10	210.1	C=O			
C-15	148.5	C			
C-4	134.8	C			
C-8	129.8	C			
C-7	129.3	CH	H-7	5.19	t, <i>J</i> = 6 Hz
C-3	124.5	CH	H-3	5.16	t, <i>J</i> = 7 Hz
C-16	110.4	CH <sub>2</sub>	H-16a	4.77	s
			H-16b	4.71	s
C-9	54.2	CH <sub>2</sub>	H-9	2.95	s
C-1	46.4	CH	H-1	1.89	m
C-11	45.4	CH <sub>2</sub>	H-11a	2.80	dd, <i>J</i> = 8, 20 Hz
			H-11b	1.92	m
C-5	38.7	CH <sub>2</sub>	H-5	2.17	m
C-2	33.2	CH <sub>2</sub>	H-2	1.98	m
C-13	32.1	CH <sub>2</sub>	H-13	1.28	m
C-12	26.7	CH	H-12	1.95	m
C-14	25.7	CH <sub>2</sub>	H-14a	1.36	m
			H-14b	1.11	m
C-6	24.9	CH <sub>2</sub>	H-6	2.24	m
C-20	21.1	CH <sub>3</sub>	H-20	0.89	d, <i>J</i> = 7 Hz
C-17	19.1	CH <sub>3</sub>	H-17	1.64	s
C-19	16.7	CH <sub>3</sub>	H-19	1.64	s
C-18	15.7	CH <sub>3</sub>	H-18	1.57	s

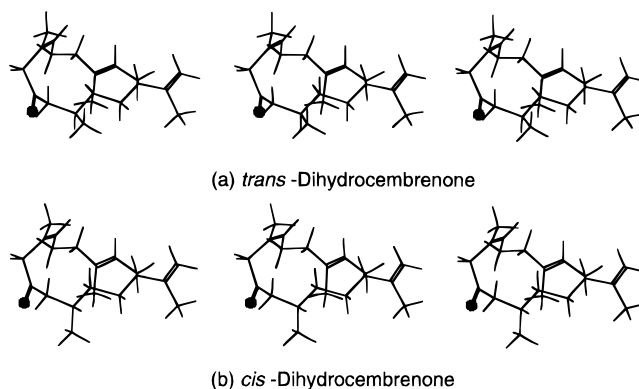
<sup>a</sup> From the DEPT spectrum. <sup>b</sup> HETCOR/HMOC correlations.

**Figure 1.** Fragments leading to determination of the structure of dihydrocembrenone (**2**).

requires the pattern C-5, C-6, C-7. The COSY spectrum also showed H-7 to be weakly coupled either to H-17 or H-19. Because H-17 is part of fragment I, the allylic methyl on the C-7 double bond must be C-19. These connections are represented as fragment IIa (Figure 1).

The H-3 resonance appeared as a vinyl triplet coupled to a region containing H-1, H-2, H-11b, and H-12. The coupling is to H-2 because it is the only methylene in the group; therefore, C-3 is bonded to C-2. H-3 also weakly coupled to H-18, thus identifying the allylic methyl on the C-3 double bond as C-18. The CH<sub>3</sub>-18 signal was HMBC-coupled to C-3, C-4, and C-5. This identifies C-3's double-bond partner as C-4 and shows that the C-3–C-4 double bond is attached to C-5 of fragment IIa, as in fragment IIb.

Vinyl H-7 exhibited weak HMBC coupling to C-9, showing that C-9 is attached to the C-7 double bond. Furthermore, H-9 exhibited coupling to C-19 or C-18. Because C-18 is isolated in fragment IIb, the H-9 coupling must be to C-19, identifying C-19 as a methyl on the C-7 double bond. The chemical shift of H-9 (δ 2.95 ppm) indicated that there are two adjacent π bonds.

**Figure 2.** Stereoviews of molecular mechanics calculated conformations of the *trans* and *cis* diastereomers of dihydrocembrenone (**2**).

The only remaining π bond is the carbonyl C-10, so C-9 is connected to C-10; this connection was confirmed by HMBC coupling between H-9 and C-10. C-10 was HMBC-coupled to H-11a, and the low chemical shift of H-11a (2.80 ppm) indicated that C-11 is bonded to the C-10 carbonyl. H-11a was also coupled to C-20; H-20 appeared as a methyl doublet and therefore is adjacent to a methine. Methine C-1 is accounted for in fragment I, so C-20 is bonded to the remaining methine, C-12. These relationships are shown in fragment IIc.

Unaccounted for thus far are the two coupled methylenes, H-13 and H-14 (fragment III). The H-11a resonance was HMBC-coupled to C-13, which requires that C-12 be bonded to C-13, thus giving the sequence C-11, C-12, C-13, and C-14.

Vinyl C-15 was HMBC-coupled to the H-14's and to either H-17 or H-19. Because H-19 is isolated in fragment IIc, C-15 must be part of the fragment I isopropenyl group, which contains H-17. (The remaining unassigned vinyl carbon, C-8, must, therefore, be part of the C-7 double bond.) Because C-15 was also coupled to H-14a and H-14b, fragment I must connect C-14 and C-2, leading to the proposed structure of the cembrene ketone, 4,8,12-trimethyl-1-(1-methylethenyl)-3,7-cyclotetradecadien-10-one (**2**).

To address the question of stereochemistry of **2**, we performed molecular mechanics calculations using Insight II (Biosym/MSI, San Diego, CA) on the diastereomer with the isopropenyl/Me-20 groups *cis* (that is, 1*R*,12*S*/1*S*,12*R*) and the diastereomer with those groups *trans* (1*R*,12*R*/1*S*,12*S*). Insight II uses the constant valence force field (CVFF); minimal energy structures were obtained by convergence to an rms value < 0.001 using the conjugate gradient minimization method. Stereoviews of the calculated structures are shown in Figure 2. Molecular dynamics simulations (1000 equilibrium iterations followed by 5000 dynamic iterations, simulated at 400 K) were performed on each isomer, using the Discover module of Insight II, to search for a global minimal energy structure. One-tenth of the conformations were sampled, yielding a statistical set of more than 400 structures. Families of lowest-energy structures with nearly identical energies and structures were obtained for both isomers; the *trans* conformer family was lower in energy than the *cis* family by about 1 kcal/mol.

The torsional angles between the two protons on C-11 and the single proton on C-12 are distinct in the two isomers. For the *trans* isomer, the molecular mechanics

calculation predicted H-11, C-11, C-12, H-12 angles of 66.5° and 178.5°, corresponding (through the Karplus relationship)<sup>10</sup> to coupling constants of 1 and 10 Hz, respectively. For the *cis* isomer, the angles were 53.2° and 59.8°, corresponding to coupling constants of 3 and 2 Hz. The proton spectrum revealed that H-11a is coupled to two protons. According to our analysis above, one is the geminal H-11b ( $J = 20$  Hz), and the other is H-12 ( $J = 8$  Hz). This large coupling to H-12 is consistent with the torsional angles calculated for the *trans* isomer; we therefore tentatively identify **2** as *trans*-11,12-dihydrocembren-10-one.

Several trivial names have been used to denote the diterpene hydrocarbon (**1**) identified from *A. sinensis*, including neocembrene, cembrene A, and neocembrene-A; we adopted cembrene A following Vanderah *et al.*<sup>11</sup> As Patil and co-workers<sup>12</sup> note, this compound is among the most elementary tetraenes derived from the cyclization of geranyl-geranyl pyrophosphate, a normal precursor in the synthesis of some diterpenoids. Isomers of this compound have been found in plant resins,<sup>12,13</sup> the extracts of soft corals,<sup>11,14,15</sup> and the glandular secretions of insects, including several termites<sup>9,16,17</sup> and the Pharaoh ant (*Monomorium pharaonis*).<sup>18</sup> In termites, cembrene A acts as a trail pheromone. In *M. pharaonis*, it is hypothesized to act as a queen-recognition pheromone. The pheromonal roles of cembrene A (**1**) for these insects are the only known functions for this compound.

Our discovery of cembrene A (**1**) in the glandular secretions of *A. sinensis* constitutes the first identification of this compound in a vertebrate.  $\beta$ -Springene and  $\beta$ -farnesene from the smooth-fronted caiman (*Paleosuchus trigonatus*) are the only other hydrocarbons known from the paracloacal glands;<sup>8</sup> several other diterpenes were observed in *P. trigonatus*, but their structures were not resolved.

The congeneric ketone of cembrene (**2**) identified from the glandular secretions of *A. sinensis* has not been described previously from nature, although similar naturally occurring compounds are known. For example, several isomers of 1,3,11-cembratrien-6-one and other related ketones have been reported from a Caribbean sea whip (*Eunicea calyculata*).<sup>19</sup> Biological activity has not been determined for these compounds.

## Experimental Section

**General Experimental Procedures.** GC-MS was performed on a Hewlett-Packard 5890 gas chromatograph with a 30 m  $\times$  0.25 mm DB-1 (J&W Scientific, Fulsom, CA) fused silica column, chromatography-grade helium at a head pressure of 105 kPa as the carrier gas, and the following operating temperatures: injection port, 250 °C; transfer line, 280 °C; oven, 50 °C programmed to 150 °C at 25 °C/min, then to 325 °C at 4 °C/min. Mass spectra were obtained at an ionizing voltage of 70 eV using a Hewlett-Packard 5970 mass selective detector. <sup>1</sup>H- and <sup>13</sup>C-NMR spectra were obtained using a Bruker DRX-500 spectrometer at 500.1 and 125.8 MHz, respectively, with CDCl<sub>3</sub> as solvent. Optical activity was measured using a Perkin-Elmer 241MC polarimeter with a 1 dm cell. FT-IR spectra were obtained on a Perkin-Elmer 1600 spectrophotometer.

**Animal Material.** Paracloacal gland secretions were obtained from eight adult male *A. sinensis*. Alligators

were restrained while their paracloacal glands were manually palpated and compressed. Secretions discharged through the gland duct openings on both sides of the vent were collected in vials containing 2–3 mL of CH<sub>2</sub>Cl<sub>2</sub>. The vials were stored at –10 °C.

**Extraction and Isolation.** Secretion extracts were evaporated under a stream of N<sub>2</sub> and redissolved in hexane. Lipids were separated on a Si gel column eluted with hexane followed by 5% EtOAc in hexane (v/v), 10% EtOAc in hexane, and 25% EtOAc in hexane. Cembrene A (**1**) was observed in the first hexane fraction. The dihydrocembrenone (**2**) was observed primarily in the 5% EtOAc fraction, with a small amount in the 10% EtOAc fraction. The ketone was further separated from co-elutents by TLC on Si gel plates developed with 5% EtOAc in hexane. We have previously reported<sup>1</sup> that **1** represents 2% and **2** represents 15% of the extract's neutral lipids.

**Hydrogenation.** The isolated hydrocarbon (**1**) was hydrogenated in an ether solution in a 5-mL Reacti-Vial (Wheaton Scientific) using PtO<sub>2</sub> as catalyst. Hydrogen gas was bubbled into the solution at approximately 0.5 mL/min for 30 min. (Attempts at microhydrogenation using PdCl<sub>2</sub> on diatomaceous earth in a capillary tube resulted in the formation of an intractable mass and the loss of all starting material.) GC-MS of the hydrogenation product revealed the presence of four isomers, all with similar mass spectra:  $m/z$  280 [M]<sup>+</sup> (**1**), 236 (**29**), 97 (**52**), 83 (**48**), 69 (**76**), 68 (**9**), 67 (**12**), 57 (**75**), 55 (**100**).

**Cembrene A (1):** oil; IR  $\nu_{\max}$  3075, 2930 (s), 2860, 1787, 1642, 1446, 1383, 891 cm<sup>-1</sup>; <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) d 149.2 (s, C-15), 134.7 (s, C-8), 133.8 (s, C-4), 133.4 (s, C-12), 125.9 (d, C-11), 124.0 (d, C-7), 121.7 (d, C-3), 110.1 (t, C-16), 45.9 (d, C-1), 39.4 (t, C-9), 38.9 (t, C-5), 33.9 (t, C-13), 32.4 (t, C-2), 28.1 (t, C-14), 24.8 (t, C-6), 23.7 (t, C-10), 19.2 (q, C-17), 18.0 (q, C-18), 15.5 (q, C-19), 15.2 (q, C-20); EIMS  $m/z$  272 [M]<sup>+</sup> (**4**), 257 (**12**), 107 (**45**), 93 (**67**), 81 (**56**), 79 (**42**), 69 (**23**), 68 (**100**), 67 (**82**), 55 (**44**), 53 (**45**), 41 (**77**).

**4,8,12-Trimethyl-1-(1-methylethenyl)-3,7-cyclotetradecadien-10-one (2):** colorless oil,  $[\alpha]_D^{20} +32.8^\circ$  (c 0.057, CHCl<sub>3</sub>); IR  $\nu_{\max}$  3075, 2931 (s), 1721 (s), 1644, 1447, 1382, 892 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) and <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz), see Table 1; EIMS  $m/z$  288 [M]<sup>+</sup> (**12**), 270 (**2**), 123 (**47**), 109 (**69**), 93 (**58**), 81 (**45**), 69 (**42**), 68 (**62**), 67 (**82**), 55 (**49**), 53 (**40**), 41 (**100**).

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**Supporting Information Available:** <sup>1</sup>H, <sup>13</sup>C, COSY, HMQC, and HMBC NMR spectra for **2** (5 pages). Ordering information is given on any current masthead page.

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