

Reptilian chemistry: Characterization of a family of dianeackerone-related steroidal esters from a crocodile secretion

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The African dwarf crocodile, *Osteolaemus tetraspis* (Crocodylidae, Reptilia), possesses a pair of skin glands, the paracloacal glands, the secretion of which is thought to be used to mark nest sites or attract mates. Ten aromatic steroidal esters were isolated from this secretion and characterized on the basis of NMR spectroscopic investigations, electrospray ionization-MS analyses, and chemical degradation. These esters, which account for more than 90% of the paracloacal glandular secretion, are derived from either cholesterol or cholestanol, esterified with a C-20 or C-22 acid closely related to dianeackerone, the only significant volatile compound found in this secretion.

Osteolaemus tetraspis | paracloacal gland | cholesterol | biosynthesis

The paracloacal glandular secretion of crocodylians, which is generally thought to mark nest sites or attract mates (1), consists of a variety of lipids (2–7). In a recent publication, we characterized a 19-carbon aromatic ketone, dianeackerone (**1**), as the major volatile constituent in the paracloacal glandular secretion of the African dwarf crocodile, *Osteolaemus tetraspis* (7). We have observed, however, that dianeackerone constitutes only about 1% of the total glandular product. Investigation of the nonvolatile portion of this secretion reveals that over 90% of the material consists of a group of 10 esters (2–11), closely related to **1** (Fig. 1). We here report the isolation and characterization of these constituents.

Experimental Procedures

The Crocodile and Secretion. *O. tetraspis* inhabits swamps and slow-moving streams in the tropical rain forests of West and Central Africa. Samples of paracloacal gland secretion were collected from six adult males and five adult females, ranging from eight to thirty-five years old. Subjects were maintained at the Crocodile Conservation Services, Plant City, FL; Ellen Trout Zoo, Lufkin, TX; Fort Worth Zoo, Fort Worth, TX; Jacksonville Zoo, Jacksonville, FL; Memphis Zoo, Memphis, TN; North Carolina Zoological Park, Ashboro, NC; Pittsburgh Zoo, Pittsburgh, PA; St. Augustine Alligator Farm, St. Augustine, FL; and one private collection. Secretion was collected by restraining subjects and manually palpating their paracloacal glands while the mouth of a glass vial was held near the gland-duct openings. A few milliliters of dichloromethane was added to each vial before the samples were stored at -10°C . The extracts were filtered through an Acrodisc CR PTFE 0.2- μm filter (Gelman Sciences, Ann Arbor, MI) and evaporated *in vacuo*, yielding 10–90 mg of residue. Because our analytical goal was to characterize chemically as many of the constituents of this material as possible, no effort was made to obtain a complete analytical profile of any individual sample.

Isolation and Analysis. Initial chromatographic separation was carried out on flash silica gel columns (ethyl acetate/hexane: 5/95) to give five fractions, designated I–V in order of increasing polarity. Fraction I was subjected to preparative HPLC by using a silica gel column [Supelcosil (Supelco) LC-Si, 25.0 cm x 4.5 mm

x 5 μm] at a flow rate of 1.0 ml/min (CH_2Cl_2 /hexanes: 18/82) with UV detection at 262 nm, to give subfractions Ia, Ib, and Ic (Fig. 2).

GC-MS analyses were performed on a Hewlett-Packard 5890 gas chromatograph with a DB-5 coated 30 m x 0.25 mm (J & W Scientific, Folsom, CA) fused silica column, coupled to an HP-5970 Mass Selective Detector (Hewlett-Packard). Oven temperature was kept at 60°C for 4 min, increased to 270°C at 15°C/min, and maintained at 270°C for 40 min. Electrospray ionization (ESI)-MS analyses were carried out by using a Micromass (Manchester, U.K.) Quattro I mass spectrometer operated in both positive and negative ESI modes. High-resolution (HR) mass spectra were recorded on a Micromass Autospec instrument operated in electron ionization (EI) (70 eV) (1 eV = 1.602×10^{-19} J) or chemical ionization (CI) (methane as the reagent gas) mode. NMR spectra were obtained on a Varian Unity 500 spectrometer, by using CDCl_3 or C_6D_6 as solvent for gradient-echo heteronuclear multiple quantum correlation (gHMQC), gradient-echo heteronuclear multiple bond correlation (gHMBC), double quantum filtered correlated spectroscopy (dqCOSY), and nuclear Overhauser effect spectroscopy experiments. ^1H and ^{13}C NMR spectra were recorded on a Varian XL 400 instrument by using CDCl_3 or C_6D_6 as solvent.

Chemical Degradation Procedures. To chromatographic fraction II (containing **2** and **3**, 11 mg) in tetrahydrofuran (THF) and methanol (2 ml, 1:3), a solution of sodium hydroxide (10%, 0.2 ml) was added. The reaction mixture was stirred at 60°C for 2 h and then cooled to room temperature (RT). Hydrochloric acid (10%) was added until the solution was acidic (pH < 1). The mixture was extracted with ether (3 x 10 ml), and the combined organic layer was dried with anhydrous magnesium sulfate and concentrated *in vacuo*. The residue was subjected to flash column chromatography (ethyl acetate/hexane: 1/4) to give dianeackerone (**1**) (4.1 mg, 93%) and a mixture of cholesterol (**12**) and cholestanol (**13**) (5.9 mg, 95%), all of which were identified by direct GC-MS and ^1H NMR comparison with the authentic samples. The mixture of cholesterol and cholestanol (*ca.* 4:1 on the basis of GC-MS analysis) was further purified by recrystallization from methanol to yield pure cholesterol (2 mg). The supernatant from the recrystallization was evaporated, and the residue was dissolved in dichloromethane (0.5 ml) and treated with acetic anhydride (5 μl) and pyridine (10 μl) at RT. After the mixture was stirred at RT for 2 h, the reaction was quenched by adding 5% aqueous sodium bicarbonate. The organic layer was separated, and the aqueous layer was extracted

Abbreviations: dqCOSY, double quantum filtered correlated spectroscopy; gHMQC, gradient-echo heteronuclear multiple quantum correlation; gHMBC, gradient-echo heteronuclear multiple bond correlation; EI, electron ionization; CI, chemical ionization; ESI, electrospray ionization; HR, high resolution; THF, tetrahydrofuran; RT, room temperature.

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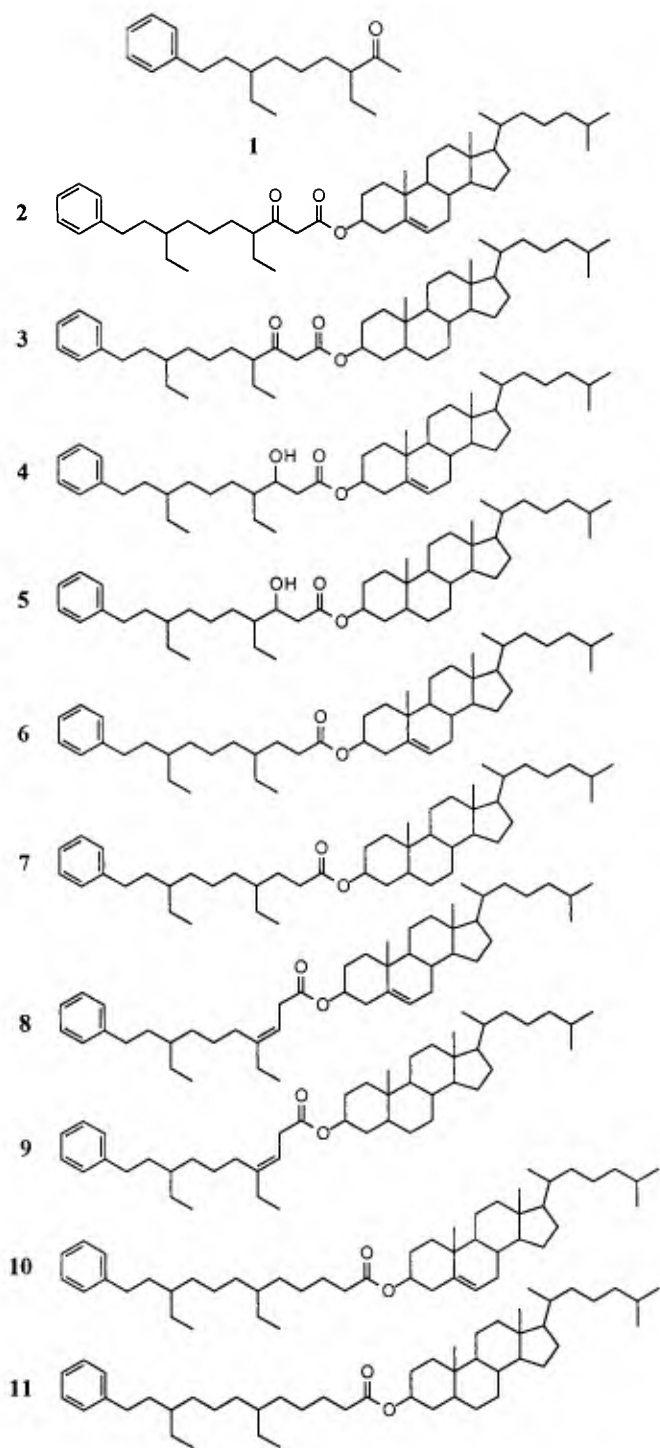


Fig. 1. Constituents of *O. tetraspis* paraoccal glandular secretion.

with dichloromethane (2 × 5 ml). The combined organic layer was washed with brine (1 ml), dried over MgSO₄, and concentrated *in vacuo*. The residue was purified by flash column chromatography (ethyl acetate/hexanes: 5/95) to give cholesterol acetate (**14**) (2 mg, mixed with some **15**) and cholestanol acetate (**15**) (1 mg, mixed with some **14**). Both GC-MS data and ¹H NMR data of **14** and **15** matched those of authentic samples of cholesterol acetate and cholestanol acetate.

Similarly, alkaline hydrolysis of fraction Ia followed by acid-

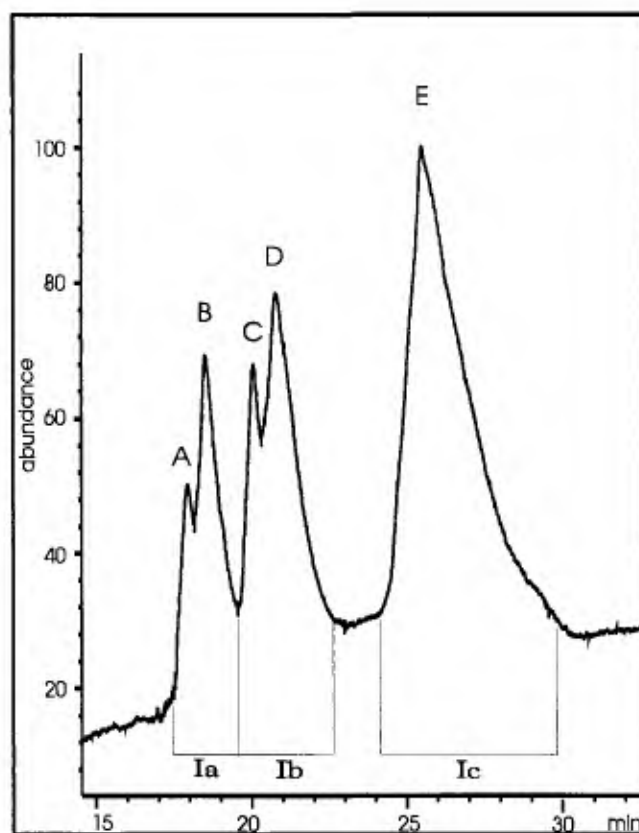


Fig. 2. An HPLC of fraction I.

ification and methylation with diazomethane yielded a single methyl ester, **16**, together with cholesterol and cholestanol in excellent yields. Other ester fractions (Ib and Ic) were characterized by the same procedure.

Analytical Data. 2. ESI-MS (positive): m/z 687 [(M + H)⁺], 429, 413, 369; HRMS (CI): m/z 687.5718 [calculated for C₄₇H₇₅O₃ (M + H), 687.5716]; ¹H NMR (CDCl₃): δ 7.17–7.30 (m, 5H), 5.39 (d, J = 4.8 Hz, 1H), 4.68 (m, 1H), 3.43 (s, 2H), 2.57 (dd, J = 9.6, 6.4 Hz, 2H), 2.52 (m, 1H), 2.36 (m, 2H), 1.80–1.91 (m, 2H) 1.92–2.04 (m, 2H), 1.06–1.62 (m, 35H), 1.01 (s, 3H), 0.92 (d, J = 6.4 Hz, 3H), 0.88 (t, J = 8.0 Hz, 3H), 0.88 (d, J = 6.4 Hz, 3H), 0.87 (d, J = 6.4 Hz, 3H), 0.86 (t, J = 8.4 Hz, 3H), 0.68 (s, 3H). ¹³C NMR (CDCl₃): δ 206.5, 181.2, 166.7, 143.1, 139.6, 128.3 (2C), 128.2 (2C), 125.5, 122.8, 75.0, 56.7, 56.1, 54.0, 49.9, 48.7, 42.3, 39.7, 39.5, 38.4, 38.0, 36.9, 36.6, 36.2, 35.8, 35.1, 33.2, 33.1, 33.0, 31.9, 31.8, 31.1, 28.0, 27.7, 25.6, 24.4, 24.3, 24.1, 23.9, 22.8, 22.6, 21.0, 19.3, 18.7, 11.9, 11.6, 10.7. **4.** ESI-MS (positive): m/z 689 [(M + H)⁺]; HRMS (CI): m/z 689.5864 [calculated for C₄₇H₇₇O₃ (M + H), 687.5873]; ¹H NMR (CDCl₃): δ 7.16–7.31 (m, 5H), 5.39 (d, J = 4.8 Hz, 1H), 4.67 (m, 1H), 4.03 (m, 1H), 2.90 (br. 1H), 2.58 (dd, J = 10, 6.4 Hz, 2H), 2.43 (t, J = 6.0 Hz, 2H), 2.34 (d, J = 7.2 Hz, 2H), 1.95–2.04 (m, 2H), 1.19–1.90 (m, 4H), 1.10–1.60 (m, 34H), 1.02 (s, 3H), 0.92 (d, J = 6.4 Hz, 3H), 0.88 (t, J = 6.8 Hz, 3H), 0.87 (d, J = 6.8 Hz, 3H), 0.87 (d, J = 6.8 Hz, 3H), 0.86 (t, J = 6.8 Hz, 3H), 0.68 (s, 3H); ¹³C NMR (CDCl₃): δ 173.1, 143.2, 139.4, 128.3 (2C), 128.2 (2C), 125.5, 122.8, 74.4, 69.4, 56.6, 56.1, 50.0, 44.5, 42.3, 39.7, 39.5, 38.5, 38.1, 36.9, 36.5, 36.1, 35.8, 35.2, 33.4, 33.2, 31.9, 31.8, 29.6, 28.2, 28.0, 27.8, 25.7, 24.4, 24.3, 23.8, 22.8, 22.5, 22.4, 21.9, 21.0, 19.3, 18.7, 11.8, 11.6, 10.7. **6.** ESI-MS (positive): m/z 673 [(M + H)⁺]; HRMS (CI): m/z 673.5917 [calculated for C₄₇H₇₇O₂ (M + H), 673.5923]; ¹H NMR (CDCl₃): δ 7.16–7.30 (m, 5H), 5.38 (d, J =

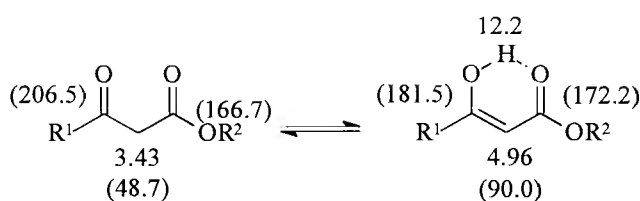


Fig. 3. Diagnostic ^1H NMR spectroscopic data (δ , ppm) and ^{13}C NMR spectroscopic data (in parentheses; δ , ppm) for β -keto ester (2) and its enol form.

4.8 Hz, 1H), 4.62 (m, 1H), 2.58 (dd, $J = 10.0$, 6.4 Hz, 2H), 2.32 (d, $J = 8.0$ Hz, 2H), 2.26 (t, $J = 8.0$ Hz, 2H), 1.92–2.04 (m, 2H), 1.80–1.88 (m, 4H), 1.10–1.62 (m, 36H), 1.02 (s, 3H), 0.92 (d, 6.8 Hz, 3H), 0.88 (t, $J = 6.8$ Hz, 3H), 0.87 (d, $J = 6.8$ Hz, 3H), 0.87 (d, $J = 6.8$ Hz, 3H), 0.86 (t, $J = 6.8$ Hz, 3H), 0.68 (s, 3H); ^{13}C NMR (CDCl_3): δ 173.6, 143.3, 139.7, 128.3 (2C), 128.2 (2C), 125.5, 122.6, 73.7, 56.7, 56.1, 50.0, 42.3, 39.7, 39.5, 38.6, 38.4, 38.2, 37.0, 36.6, 36.2, 35.8, 35.2, 33.4, 33.3, 33.2, 32.2, 31.9, 31.8, 28.3, 28.2, 28.0, 27.8, 25.7, 24.3, 23.8, 23.6, 22.8, 22.5, 21.0, 19.3, 18.7, 11.8, 10.8, 10.7. **8.** ESI-MS (positive): m/z 671 [($\text{M} + \text{H}$) $^+$]; HRMS (CI): m/z 671.5748 [calculated for $\text{C}_{47}\text{H}_{75}\text{O}_2$ ($\text{M} + \text{H}$), 671.5767]; ^1H NMR (CDCl_3): δ 7.16–7.30 (m, 5H), 5.37 (d, $J = 4.8$ Hz, 1H), 5.33 (t, $J = 6.8$ Hz, 1H), 4.63 (m, 1H), 3.03 (d, $J = 6.8$ Hz, 2H), 2.58 (dd, $J = 9.6$, 7.0 Hz, 2H), 3.32 (d, $J = 6.4$ Hz, 2H), 1.99–2.08 (m, 4H), 1.82–1.89 (m, 2H), 1.09–1.60 (m, 33H), 1.02 (s, 3H), 1.02 (t, $J = 7.6$, 7.2 Hz, 3H), 0.92 (d, $J = 6.4$ Hz, 3H), 0.88 (d, $J = 6.8$ Hz, 3H), 0.87 (d, $J = 6.8$ Hz, 3H), 0.87 (t, $J = 6.8$ Hz, 3H), 0.68 (s, 3H); ^{13}C NMR (CDCl_3): δ 172.0, 144.8, 143.2, 139.7, 128.3 (2C), 128.2 (2C), 125.5, 122.6, 114.8, 74.0, 56.7,

56.1, 50.0, 42.3, 39.7, 39.5, 38.5, 38.1, 36.9, 36.6, 36.2, 35.8, 35.2, 33.8, 33.2, 33.0, 31.9, 31.8, 30.9, 29.5, 28.2, 28.0, 27.8, 25.7, 25.2, 24.3, 23.8, 22.8, 22.5, 21.0, 19.3, 18.7, 12.7, 11.8, 10.7. **10.** CI-MS: m/z 701 ($\text{M} + \text{H}$) $^+$, 652, 624, 397, 383, 369, 355, 315; HRMS (CI): m/z 701.6220 [calculated for $\text{C}_{49}\text{H}_{81}\text{O}_2$ ($\text{M} + \text{H}$), 701.6236]; ^1H NMR (CDCl_3): δ 7.16–7.30 (m, 5H), 5.38 (d, $J = 4.8$ Hz, 1H), 4.63 (m, 1H), 2.58 (t, $J = 8.0$ Hz, 2H), 3.32 (d, $J = 7.6$ Hz, 2H), 2.26 (t, $J = 8.0$ Hz, 2H), 1.92–2.02 (m, 2H), 1.79–1.89 (m, 4H), 1.08–1.60 (m, 40H), 1.02 (s, 3H), 0.92 (d, $J = 5.6$ Hz, 3H), 0.88 (t, $J = 6.8$ Hz, 3H), 0.87 (d, $J = 6.8$ Hz, 3H), 0.87 (d, $J = 6.8$ Hz, 3H), 0.86 (t, $J = 6.8$ Hz, 3H), 0.68 (s, 3H); ^{13}C NMR (CDCl_3): δ 173.7, 143.3, 139.7, 128.3 (2C), 128.2 (2C), 125.5, 122.6, 73.7, 56.7, 56.1, 50.0, 42.3, 39.7, 39.5, 38.6, 38.4, 38.2, 37.0, 36.6, 36.2, 35.8, 35.2, 33.2, 33.0, 32.8, 32.2, 31.9, 31.8, 30.4, 29.7, 28.3, 28.2, 28.0, 27.8, 26.6, 25.7, 25.5, 24.3, 23.8, 22.8, 22.6, 21.0, 19.3, 18.7, 11.8, 10.8, 10.8. **16.** GC-MS (EI): m/z 318 (M^+), 286, 257, 245, 227, 195, 177, 145, 126, 105, 91, 55. HRMS (EI): m/z 318.2561 [calculated for $\text{C}_{21}\text{H}_{34}\text{O}_2$, 318.2559]. **17.** GC/MS (EI): m/z 316 (M^+), 284, 266, 255, 237, 193, 159, 145, 117, 104, 91, 67, 55; HRMS (EI): m/z 316.2407 [calculated for $\text{C}_{21}\text{H}_{32}\text{O}_2$, 316.2402]; ^1H NMR (CDCl_3): δ 7.16–7.30 (m, 5H), 5.32 (t, $J = 7.2$ Hz, 1H), 3.68 (s, 3H), 3.07 (d, $J = 7.2$ Hz, 2H), 2.58 (dd, $J = 10.0$, 6.4 Hz, 2H), 2.06 (q, $J = 7.6$ Hz, 2H), 2.01 (t, 8.0 Hz, 2H), 1.56 (m, 1H), 1.26–1.38 (m, 8H), 1.02 (t, $J = 7.6$ Hz, 3H), 0.87 (t, $J = 7.2$ Hz, 3H); ^{13}C NMR (CDCl_3): δ 173.0, 145.0, 143.2, 128.3 (2C), 128.2 (2C), 125.5, 114.4, 51.7, 38.4, 35.1, 33.3, 33.1, 33.0, 30.8, 29.5, 25.6, 25.2, 12.6, 10.7. **18.** GC-MS (EI): m/z 346 (M^+), 314, 285, 255, 223, 205, 145, 105, 92, 91, 69, 55; HRMS (EI): m/z 346.2874 [calculated for $\text{C}_{23}\text{H}_{38}\text{O}_2$, 346.2872].

Results

A sample of extract was subjected to preliminary separation by column chromatography into five fractions (I–V), numbered in

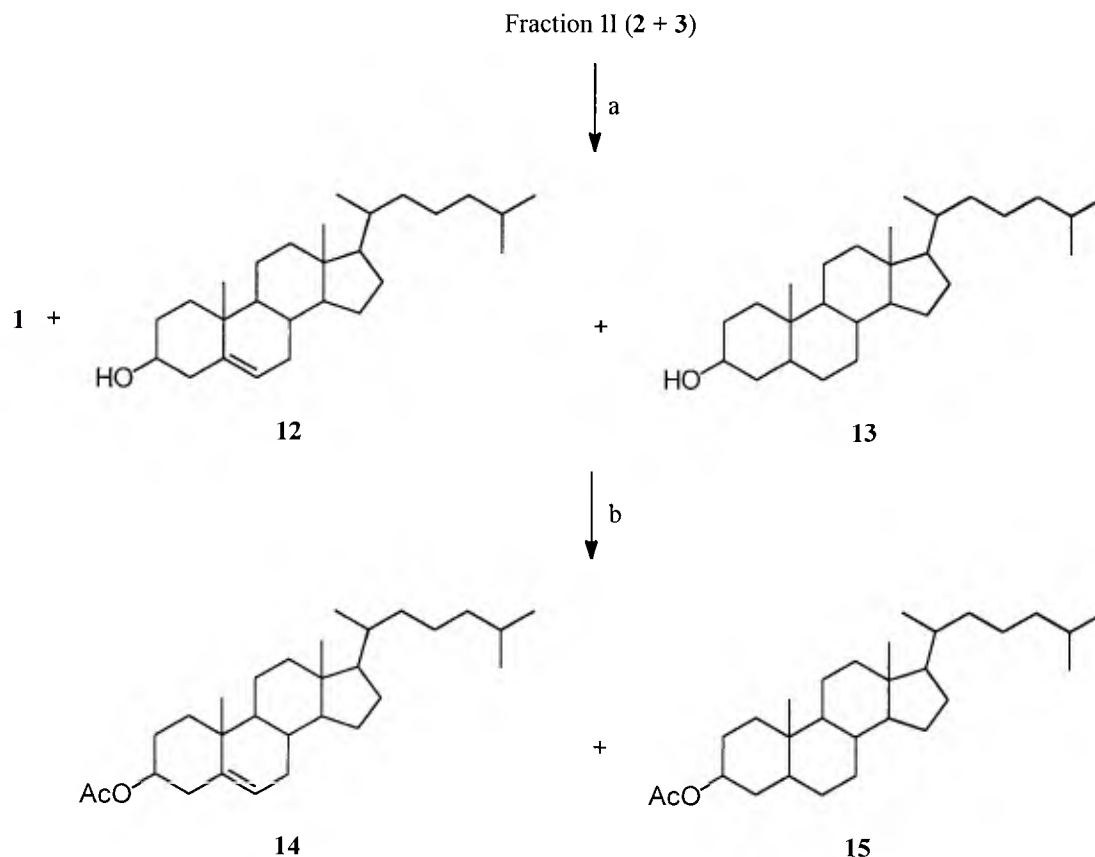


Fig. 4. Hydrolysis of 2 and 3. (a) 10% NaOH, THF/MeOH (1:3), 60°C, 2 h, and then 10% HCl; (b) Ac_2O /pyridine, CH_2Cl_2 , RT, 4 h.

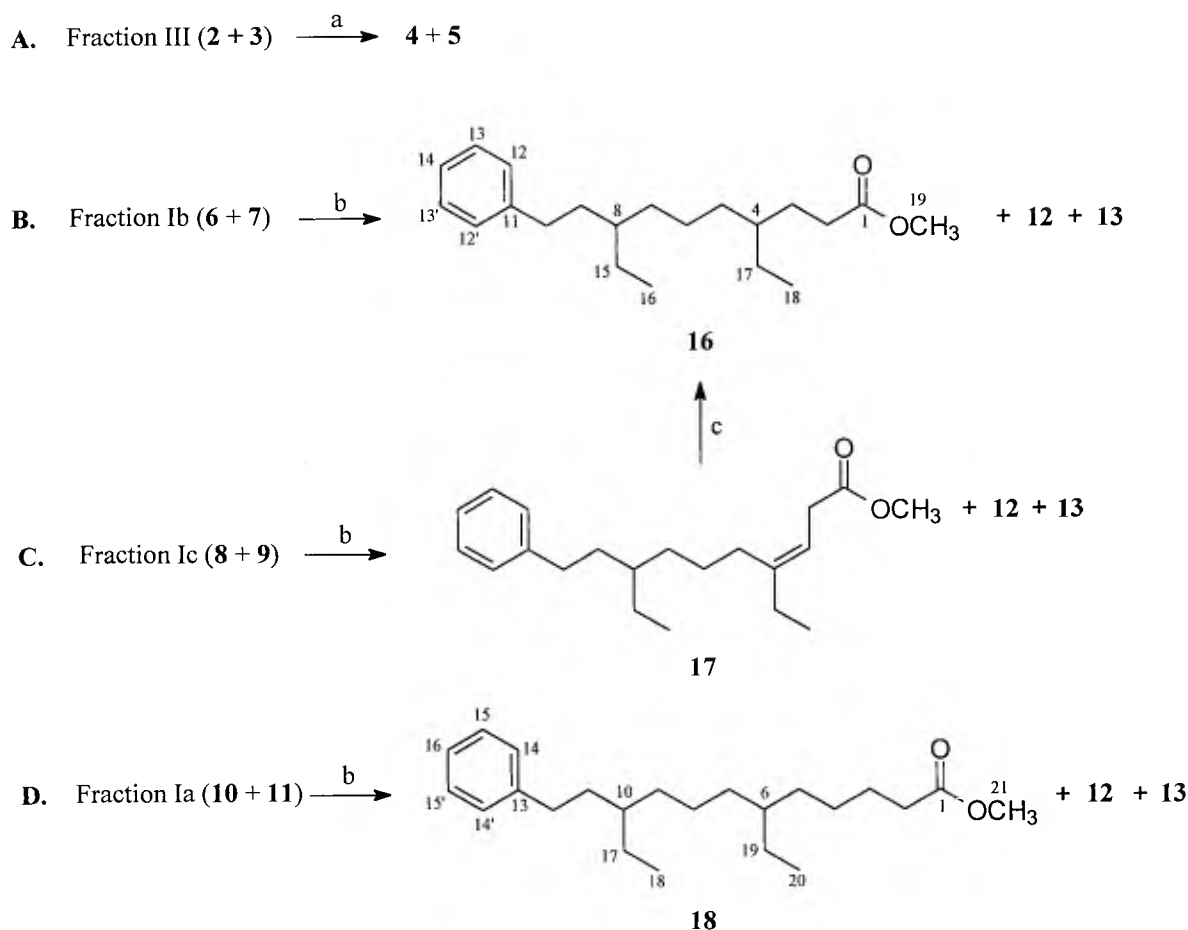


Fig. 5. Transformation and derivatization studies. (A) NaBH_4 , THF, methanol, $0^\circ\text{C} \rightarrow \text{RT}$, 100%. (B) (i) 10% NaOH, THF, MeOH, 60°C , 2 h; (ii) 10% HCl; (iii) CH_2N_2 , ether, 0°C . c: H_2 , Pd-C, MeOH, RT, 1 h.

order of increasing polarity. Fraction IV (*ca.* 1%) was recognized as the previously-identified dianeackerone (**1**), and fraction V (*ca.* 8%), obtained by washing the column with ethyl acetate, consisted of a mixture of cholesterol, cholestanol, and some free fatty acids. Fractions I and II, taken together, accounted for *ca.* 90% of the total secretion, whereas fraction III represented only an additional 1%. In total, 10 compounds were characterized from these three fractions (I-III), all of them esters derived from either cholesterol or cholestanol and a dianeackerone-related acid moiety.

^1H and ^{13}C NMR spectroscopic studies of fraction II (*ca.* 30% of the total glandular secretion) suggested that this fraction consisted of two closely related β -keto esters (ratio *ca.* 4:1), each as a mixture of keto-enol tautomers (Fig. 3). ESI-MS analysis established a molecular mass of 686 daltons [$(\text{M} + \text{H})^+$ observed at m/z 687] for the major component of this fraction, and its accurate mass, obtained from its HR CI mass spectrum, suggested a molecular formula of $\text{C}_{47}\text{H}_{74}\text{O}_3$. Although isotope peaks from this constituent made the observation of the minor constituent's quasimolecular ion in CI-MS difficult, an EI-MS experiment clearly revealed a molecular ion at m/z 688 for the minor component, because EI did not yield a molecular ion for the major component. The molecular formula of the minor compound was established as $\text{C}_{47}\text{H}_{76}\text{O}_3$ by its HR-EI mass spectrum (observed m/z 688.5799; calculated for $\text{C}_{47}\text{H}_{76}\text{O}_3$, 688.5794).

Because the NMR spectra of these β -keto esters were complicated by the presence of tautomeric forms, a simple chemical degradation was carried out to confirm their structures and complete the identification of these esters. Alkaline hydrolysis of

fraction II followed by acidification provided excellent yields of dianeackerone (**1**) (resulting from the anticipated decarboxylation of the β -keto-acid moiety), along with two steroids, cholesterol (**12**) and cholestanol (**13**) (*ca.* 4:1 based on electron-ionization GC-MS analysis) (Fig. 4). Acetylation of the mixture of **12** and **13** generated the corresponding steroidal acetates, **14** and **15**. The results provide unambiguous evidence for the assignment of structures **2** and **3** to the major and minor constituents of fraction II.

The ^1H and ^{13}C NMR spectra of fraction III showed that it too consisted of a mixture of cholesterol and cholestanol esters, again in a ratio of about 4:1. The molecular mass of the major component was determined to be 688 daltons by positive-ion ESI- and CI-MS [$(\text{M} + \text{H})^+$ observed at m/z 689], and HR-CI MS established the molecular formula as $\text{C}_{47}\text{H}_{76}\text{O}_3$. CI-MS analysis of fraction III showed an additional peak at m/z 691 [CI-HRMS m/z 691.6021; calculated for $\text{C}_{47}\text{H}_{79}\text{O}_3$, 691.6029] attributable to the minor component from the CI-MS. Moreover, an EI-MS experiment clearly showed a molecular mass of 690 daltons for the minor component, the molecular formula of which was shown to be $\text{C}_{47}\text{H}_{78}\text{O}_3$ by its HR mass spectrum (observed m/z 690.5965; calculated for $\text{C}_{47}\text{H}_{78}\text{O}_3$, 690.5951). The similarity of the NMR spectra of this mixture and that of the β -keto esters **2** and **3** suggested that these new components might be the analogous β -hydroxy-esters, **4** and **5**. This suggestion was confirmed by a partial synthesis; sodium borohydride reduction of a sample of the naturally occurring β -keto esters gave a pair of β -hydroxy esters, **4** and **5**, indistinguishable from the fraction

Table 1. ^1H (500-MHz) and ^{13}C (100-MHz) Data for **16** in C_6D_6

^{13}C data		^1H data			gHMBC correlations, carbon no.
Carbon no.	δ , ppm	Proton no.	δ , ppm	J , Hz	
C-1	174.0				
C-2	32.0	2-H	2.17	$J_{2,3} = 10.0$	C-1, 3, 4
C-3	28.9	3-H	1.62	$J_{2,3} = 10.0, J_{3,4} = 6.0$	C-4
C-4	39.0	4-H	1.19	$J_{3,4} = 6.0, J_{4,5} = 7.5, J_{4,15} = 1.0$	C-17
C-5	33.9	5-H	1.16	$J_{4,5} = 7.5, J_{5,6} = 7.0$	C-4
C-6	24.2	6-H	1.20	$J_{5,6} = J_{6,7} = 7.0$	C-5
C-7	34.1	7-H	1.20	$J_{6,7} = 7.0, J_{7,8} = 6.5$	C-8
C-8	39.1	8-H	1.25	$J_{7,8} = 6.5, J_{8,9} = 6.5, J_{8,15} = 1.0$	C-7
C-9	35.9	9-H	1.56	$J_{8,9} = 6.5, J_{9,10} = 10.0$	C-8,10
C-10	33.9	10-H	2.53	$J_{9,10} = 10.0$	C-8, 9, 10, 11
C-11	143.7				
C-12	129.1	12-H	7.13	$J_{12,13} = 9.0$	C-11, 13
C-12'	129.1	12'-H	7.13	$J_{12',13'} = 9.0$	C-11, 13
C-13	129.0	13-H	7.20	$J_{12,14} = 9.0$	C-12
C-13'	129.0	13'-H	7.20	$J_{12',14} = 9.0$	C-12'
C-14	126.3	14-H	7.09	$J_{13,14} = J_{13',14} = 9.0$	C-13, 13'
C-15	26.4	15-H	1.31	$J_{15,16} = 9.0, J_{8,15} = 1.0$	C-7, 8
C-16	11.3	16-H	0.86	$J_{15,16} = 9.0$	C-8, 15
C-17	26.2	17-H	1.18	$J_{17,18} = 9.0, J_{4,17} = 1.0$	C-4
C-18	11.2	18-H	0.80	$J_{17,18} = 9.0$	C-4, 17
C-19	51.3	19-H	3.37		C-1

III components on the basis of ESI-MS and NMR (^1H and ^{13}C) comparisons (Fig. 5A).

Fraction I (ca. 60% of the total secretion) proved to contain six additional steroidal esters. Preparative HPLC of this fraction resulted in a partial separation of these components into three fractions, Ia (peaks A + B), Ib (peaks C + D), and Ic (peak E, an unresolved mixture) (Fig. 2). ^1H and ^{13}C NMR studies of fraction Ib (C + D) confirmed the presence of two compounds (as indicated by analytical HPLC) with closely related structures, in a ratio of ca. 4:1. A quasimolecular ion from the major component (peak D) was observed at m/z 673 by positive-ion ESI-MS, and HR-CI MS established a molecular formula of $\text{C}_{47}\text{H}_{76}\text{O}_2$. The molecular mass of the minor component (peak C) was determined to be 674 daltons by EI-MS, and its molecular formula was established as $\text{C}_{47}\text{H}_{78}\text{O}_2$ by its HR mass spectrum (observed m/z , 674.5994; calculated for $\text{C}_{47}\text{H}_{78}\text{O}_2$, 674.6001). To identify the major and minor components of this fraction, we performed an alkaline hydrolysis of the mixture, followed by methylation of the acidic product with diazomethane. This yielded a single methyl ester, along with cholesterol and cholestanol (ca. 4:1), in excellent yields (Fig. 5B). The assignment of structure **16** to this ester is based on ^1H , ^{13}C NMR, dqCOSY, gHMOC, and gHMBC evidence (Table 1). It follows that the steroidal esters corresponding to peaks D and C (Fig. 2) are represented by structures **6** and **7**, respectively.

^1H and ^{13}C NMR spectra of fraction Ic showed that it also consisted of two components (ca. 4:1), again incorporating cholesterol and cholestanol moieties. ESI-MS analysis established a molecular mass of 670 daltons [$(\text{M} + \text{H})^+$ m/z 671] for the major component. The accurate mass of the protonated form of this compound, ascertained by its HR-CI mass spectrum, established the molecular formula as $\text{C}_{47}\text{H}_{74}\text{O}_2$ suggesting an unsaturated analog of **6**. The molecular ion of the minor component was observed at m/z 672 by EI-MS, and its molecular formula was established to be $\text{C}_{47}\text{H}_{76}\text{O}_2$ by its HR mass spectrum (observed m/z 672.5858; calculated for $\text{C}_{47}\text{H}_{76}\text{O}_2$, 672.5845). Hydrolysis of this mixture with sodium hydroxide, followed by acidification and diazomethane treatment again gave a single unsaturated methyl ester, in addition to cholesterol and cholestanol (ca. 4:1). The carbon skeleton of this ester was established by catalytic hydroge-

nation (Pd-C), which yielded the previously characterized methyl ester **16** (Fig. 5C). The β , γ -position of the double bond in this ester, as represented in formula **17**, was established by a dqCOSY experiment, which showed characteristic coupling between alkenyl proton *a* and methylene group *b* (Fig. 6).

A nuclear Overhauser effect spectrum of **17** established the stereochemistry of the carbon-carbon double bond. This experiment (see Fig. 6), which showed the alkenyl proton *a* (5.32 ppm, t, $J = 7.2$ Hz) to be in close proximity to methylene group *b* (3.07 ppm, d, $J = 7.2$ Hz), methylene group *c* (2.06 ppm, q, $J = 7.6$ Hz), and methyl group *d* (1.02 ppm, t, $J = 7.6$ Hz), but distant from methylene group *e* (2.01 ppm, t, $J = 8.0$ Hz), requires a *cis*-double bond (Fig. 6) and establishes the structures of the two steroidal esters making up chromatographic fraction Ic as **8** and **9**.

Finally, the least polar chromatographic peaks (A + B), which had been collected by preparative HPLC as one fraction (Ia) (Fig. 2), were examined. Although the ^1H NMR spectrum of this mixture is very similar to that of the mixture of **6** and **7**, its ^{13}C NMR spectrum showed the presence of two additional carbon atoms. The molecular mass of the major component, corresponding to peak B (Fig. 2), was found to be 700 daltons by CI-MS analysis [$(\text{M} + \text{H})^+$, m/z 701], and its HR mass spectrum established a molecular formula of $\text{C}_{49}\text{H}_{80}\text{O}_2$. The molecular mass of the minor component was shown to be 702 daltons from EI-MS data, and its molecular formula was shown to be $\text{C}_{49}\text{H}_{82}\text{O}_2$ by its HR mass spectrum (observed m/z 702.6317;

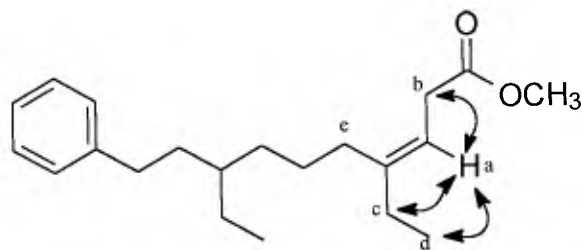


Fig. 6. Some key ^1H - ^1H correlations observed from the nuclear Overhauser effect data for **17**.

Table 2. ¹H (500-MHz) and ¹³C (100-MHz) Data for **18 in CDCl₃**

¹³ C data		¹ H data			gHMBC correlations, carbon no.
Carbon no.	δ, ppm	Proton no.	δ, ppm	J, Hz	
C-1	174.3				
C-2	32.8	2-H	2.29	J _{2,3} = 8.0	C-1, 3
C-3	28.2	3-H	1.56	J _{2,3} = 8.0, J _{3,4} = 8.5	C-2, 4
C-4	25.7	4-H	1.24	J _{3,4} = 8.5, J _{4,5} = 7.0	C-5
C-5	30.5	5-H	1.22	J _{4,5} = 7.0, J _{5,6} = 7.5	C-4, 6
C-6	38.4	6-H	1.31	J _{5,6} = J _{6,7} = 7.5, J _{6,19} = 1.0	C-7, 19
C-7	31.6	7-H	1.20	J _{6,7} = 7.5, J _{7,8} = 6.5	C-8
C-8	25.5	8-H	1.21	J _{7,8} = 6.5, J _{8,9} = 7.0	C-9, 10
C-9	33.0	9-H	1.24	J _{8,9} = 7.0, J _{9,10} = 8.0	C-10, 17
C-10	38.6	10-H	1.29	J _{9,10} = 8.0, J _{10,11} = 6.5, J _{10,17} = 1.0	C-11, 17
C-11	35.2	11-H	1.54	J _{10,11} = 6.5, J _{11,12} = 8.5	C-12
C-12	33.2	12-H	2.58	J _{11,12} = 8.5	C-11, 13
C-13	143.3				
C-14	128.3	14-H	7.20	J _{14,15} = 7.5	C-13, 15
C-14'	128.3	14'-H	7.20	J _{14',15'} = 7.5	C-13, 15'
C-15	128.2	15-H	7.30	J _{14,15} = J _{15,16} = 7.5	C-14
C-15'	128.2	15'-H	7.30	J _{14',15'} = J _{15',16} = 7.5	C-14'
C-16	125.5	16-H	7.18	J _{15,16} = J _{15',16} = 7.5	C-15, 15'
C-17	26.6	17-H	1.36	J _{17,18} = 7.5, J _{10,17} = 1.0	C-10
C-18	10.8	18-H	0.87	J _{17,18} = 7.5	C-10, 17
C-19	26.6	19-H	1.28	J _{19,20} = 7.5, J _{6,19} = 1.0	C-6
C-20	10.8	20-H	0.86	J _{19,20} = 7.5	C-6, 19
C-21	51.4	21-H	3.67		C-1

calculated for C₄₉H₈₂O₂, 702.6314). Alkaline hydrolysis of fraction Ia (Fig. 5D), followed by acidification and methylation of the resulting acid moiety yielded one methyl ester, in addition to cholesterol and cholestanol (ca. 4:1). The ¹H NMR spectra of this new methyl ester and the previously characterized **16** are very similar, but their ¹³C NMR spectra confirmed the presence of two additional carbons in the Ia-derived ester. The structure of this homolog was established as **18** on the basis of dqCOSY, gHMOC, and gHMBC experiments (Table 2). It follows that the fraction Ia steroidal esters themselves are simply the higher homologs, **10** and **11**, of **6** and **7**, incorporating two additional methylene groups into their carboxylic acid moieties.

Discussion

Notably, eight of the ten cholesterol and cholestanol esters (**2–9**) that constitute the bulk of the paracloacal glandular secretion of *O. tetraspis* appear to represent a series of biosynthetic precursors of dianeackerone (**1**). As we have demonstrated, dianeackerone is actually produced in good yield by hydrolysis of β-keto esters **2** and **3**. These β-keto esters could well be the oxidation products of **4** and **5**, or the hydration and oxidation products of the unsaturated esters **8** and **9**. The saturated esters **6** and **7** are plausible precursors of their hydroxylated and unsaturated congeners. The higher homologs, **10** and **11**, might be expected to give rise to a bis-homolog of **1** via the corresponding β-hydroxy and β-keto esters, but these products would be trace components at best.

In our initial study, we found that *O. tetraspis* produces two diastereomers (3*S*,7*S*-**1** and 3*S*,7*R*-**1**) of dianeackerone (**7**). The absolute configurations of these stereoisomers were determined by comparing the ¹³C-NMR spectra and optical rotations of

natural and synthetic dianeackerone samples (**7**). We also discovered that individuals produce different ratios of these diastereomers, although the biological significance of this stereochemical variability remains enigmatic (**7**). Nevertheless, from a biosynthetic viewpoint, it would be interesting to determine whether *O. tetraspis* also produces a pair of stereoisomers for each of the eight esters, **2–9**, in ratios matching those of the corresponding dianeackerones. The answer to this question may not be easy to come by, because the distinction even between the 3*S*,7*S* and 3*S*,7*R* forms of dianeackerone is experimentally difficult.

Dieneackerone stands out from an analytical viewpoint because of its volatility. Nonetheless, it is really the array of steroidal esters derived from five dianeackerone-related C-20 and C-22 acids that makes up the bulk of adult *O. tetraspis* paracloacal gland secretion. With this chemistry well defined, the stage is set for a study of the function(s) served by these structurally unprecedented, high molecular weight lipophilic esters and the C-19 ketone that appears to be their ultimate metabolite.

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