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NEW DITERPENOIC ACID GLYCERIDES FROM THE ANTARCTIC NUDIBRANCH *AUSTRODORIS KERGUELENSIS*

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Abstract: A single specimen of the Antarctic nudibranch *Austrodoris kerguelensis* contained two major glyceride esters, 2'-acetoxyglyceryl (5R,10R,13R)-labda-8-en-15-oate (**1**) and 3'-acetoxyglyceryl (5R,10R,13R)-labda-8-en-15-oate (**4**), the diketones **6** and **7** that arise from oxidation of the 8,9-olefinic bond, and glyceryl (5R,10R,13R)-7-ketolabda-8-en-15-oate (**8**). The probable source of these metabolites is discussed.

Nudibranchs are delicate shell-less marine molluscs that are often brightly colored. Despite their high visibility and lack of physical defenses against fish, crabs, and other predators, reports of predation are virtually non-existent. Nudibranchs appear to employ secretions containing defensive chemicals to discourage predation.[1,2] Although most nudibranchs obtain their defensive chemicals from sponges and other invertebrates on which they feed,[3] there is a small group of nudibranchs that can synthesize their own defensive chemicals. Among these are *Archidoris montereyensis* and *A. odhneri*, both of which synthesize glyceryl esters of diterpene and sesquiterpene acids.[4,5] In this paper we report the isolation and identification of five diterpene glycerides from a specimen of *Austrodoris kerguelensis* (Bergh) (= *A. mcmurdensis*)[6] that was collected in Antarctica by McClintock *et al.*, who have reported the defensive properties of the mantle tissue of the nudibranch.[7]

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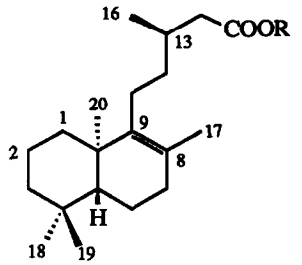
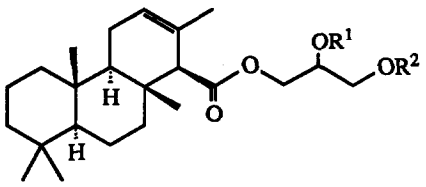
The acetone extract of a single specimen of *Austrodoris kerguelensis* (*ca* 100 g wet wt.) was concentrated and partitioned between ethyl acetate and water. Silica gel flash chromatography of the ethyl acetate soluble portion gave several crude fractions that were examined by ^{1}H NMR spectroscopy. Selected fractions were further purified by HPLC on both normal and reverse phase columns to obtain five new diterpene glycerides, 2'-acetoxyglyceryl (5R,10R,13R)-labda-8-en-15-oate (**1**, 12.1 mg), 3'-acetoxyglyceryl (5R,10R,13R)-labda-8-en-15-oate (**4**, 3.5 mg), the diketones **6** (2.6 mg) and **7** (1.8 mg), and glyceryl (5R,10R,13R)-7-ketolabda-8-en-15-oate (**8**, 1.1 mg).

The major metabolite, 2'-acetoxyglyceryl (5R,10R,13R)-labda-8-en-15-oate (**1**), was isolated as a colorless oil with a molecular formula of $C_{25}H_{42}O_5$ that was established from HREIMS data. The IR spectrum of **1** showed hydroxyl (3700–3300 cm^{-1}) and ester (1750, 1735 cm^{-1}) absorption bands. A series of signals in the ^{1}H NMR spectrum of **1** at δ 3.71 (d, 2 H, J = 5 Hz), 4.17 (dd, 1 H, J = 6, 11 Hz), 4.29 (dd, 1 H, J = 4.5, 12 Hz) and 5.07 (p, 1 H, J = 5 Hz) are in accordance with values published for the 1'-acyloxy-2'-acetoxy-3'-hydroxypropane moiety of compound (**2**) that was previously isolated from the dorid nudibranch *Archidoris montereyenis*.[4] The monoacetylated glyceryl ester fragment therefore accounts for five of the twenty-five carbon atoms in **1**. The four methyl resonances at δ 0.79 (s, 3 H), 0.85 (s, 3 H) 0.90 (s, 3 H) and 1.02 (d, 3 H, J = 6.5 Hz) and the vinylic Me resonance at 1.51 (s, 3 H) suggested a diterpenoid origin for the remaining twenty carbon atoms. Signals at δ 125.6 (s) and 140.4 (s) in the ^{13}C NMR spectrum were assigned to a $\Delta^{8,9}$ olefin in a labdane skeleton. The single olefinic functionality, the two ester carbonyls and the two rings in the proposed structure of **1** account for the five degrees of unsaturation required by the molecular formula.

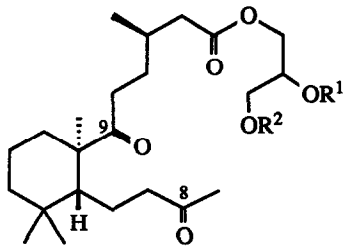
The absolute stereochemistry of the diterpene portion of the molecule was established by chemical interconversion of **1** with the known compound methyl (5R,10R,13R) labda-8-en-15-oate (**3**).[8] Saponification of the glyceride ester **1** with methanolic potassium carbonate and subsequent methylation of the acidic product with diazomethane gave the known methyl ester (**3**) as an optically-active colorless oil ($[\alpha]_D$ = – 49°, $CHCl_3$; lit.[8] – 48°). The IR and ^{1}H NMR spectral data for the methyl ester **3**, prepared as shown above, were virtually identical to those reported for a (5*S*,10*S*) isomer of **3** that has $[\alpha]_D$ = +63°.[9]



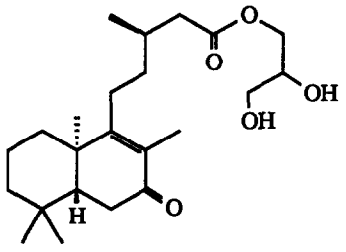
1 R = -CH_2-CH(OAc)-CH_2OH
3 R = Me
4 R = -CH_2-CH(OH)-CH_2OAc



2 R^1 = Ac R^2 = H
5 R^1 = H R^2 = Ac
9 R^1 = R^2 = H



6 R^1 = Ac R^2 = H
7 R^1 = H R^2 = Ac



8

The *ent*-labdane stereochemistry of the ring junction and the 13R configuration in the side chain of **1** were thus established. Both the *ent*-labdane ester **3** and its parent acid are rare in nature and neither have previously been isolated from a marine source. The stereochemistry at C-2' in the glyceryl ester moeity remains undefined.

A second glyceride ester, 3'-acetoxyglyceryl (5R,10R,13R)-labda-8-en-15-oate (**4**), was isolated as a colorless oil and was shown by HREIMS data to be isomeric with glyceride **1**. The ^1H and ^{13}C NMR spectra of compounds **1** and **4** differ only in the chemical shifts of the proton and carbon resonances assigned to the glyceryl ester fragment. The five proton multiplet at δ 4.08-4.15 in the ^1H NMR of **4** indicates that the single acetoxy group is at the 3' position on the glyceryl ester. These data are similar to those reported for the 1'-acyloxy-2'-hydroxy-3'-acetoxypropane residue of compound **5**.[4] The absolute

sterochemistry of the diterpene fragment of **4** is assumed, on the basis of the optical rotation and biosynthetic arguments, to be the same as that of glyceride **1**.

Diketones (**6**) and (**7**) both have the same molecular formula, $C_{25}H_{42}O_7$, obtained from HRCIMS data. The ^1H NMR spectra of these two isomers indicated that they were two different monoacetylated glyceryl esters of the same diterpenoic acid. The ^1H NMR signals assigned to the 2'-acetoxyglyceryl and 3'-acetoxyglyceryl ester moeities in compounds **1** and **4** were identical with those observed in the spectra of diketones **6** and **7** respectively. The carbonyl bands in the IR spectrum of compound **6** at 1715 and 1695 cm^{-1} and the two carbonyl signals at δ 209.7 (s) and 217.0 (s) in the ^{13}C spectrum suggested that the diterpene skeleton contained two ketones. Since the molecular formula of **6** requires five degrees of unsaturation, the diterpene skeleton must be monocyclic.

Oxidative cleavage of the $\Delta^{8,9}$ bond in ring B of compound **1** would yield a monocyclic diketone (**6**). This proposed structure was supported by interpretation of the ^1H and ^{13}C NMR spectra. The downfield chemical shift of the Me-17 signal at δ 2.07 (s, 3 H) is typical of a methyl ketone. The deshielded positions of the Me-20 signal at δ 1.20 (s, 3 H) and the C-10 signal at δ 53.2 (s) in the ^{13}C spectrum of **6** placed the other keto group at C-9. The structure of diketone **6** (and, by analogy, diketone **7**) were confirmed by ozonolysis of compound **1** in 1:1 dichloromethane-methanol at -40°C followed by reductive work-up of the ozonolysis products with trimethyl phosphite and purification by HPLC to obtain a colorless oil ($[\alpha]_D$ = -9°) that was identical by TLC, ^1H NMR and EIMS with diketone **6**.

Glyceryl (5R,10R,13R)-7-ketolabda-8-en-15-oate (**8**) was isolated in very low yield after extensive HPLC purification. The ^1H NMR spectrum of ketone **8** indicated that this compound was not acetylated and the series of signals at δ 3.58 (dd, 1 H, J = 11, 6 Hz), 3.70 (dd, 1 H, J = 13, 4 Hz), 3.89 (m, 1 H), 4.13 (dd, 1 H, J = 12, 5Hz) and 4.22 (dd, 1 H, J = 12, 5 Hz) were virtually identical to those reported for the 1'-acyloxy-2',3'-dihydroxypropane fragment in compound (**9**) isolated from *A. montreyensis*.[4] The five methyl signals at δ 0.85 (s, 3 H), 0.88 (s, 3 H), 0.99 (d, 3 H, J = 6.5 Hz), 1.04 (s, 3 H), and 1.71 (s, 3 H) suggested that the diterpene carbon skeleton was the same as that in glyceride **1**. High resolution mass spectrometry revealed a molecular formula of $C_{23}H_{38}O_5$ for **8** and the IR spectrum showed hydroxyl (3700-

3300 cm^{-1}), ester (1735 cm^{-1}), and α,β–unsaturated carbonyl (1645, 1610 cm^{-1}) bands. An absorption at 249 nm in the UV spectrum of **8** is in accordance with the value calculated for an endocyclic α,β–unsaturated ketone with one α and two β alkyl substituents, as expected for a 7–ketolabda–8–ene derivative. The stereochemistry of **8** was again assumed, on the basis of biosynthetic arguments, to be the same as that in glyceride **1**.

The structural similarities between the metabolites of *Austrodoris kerguelensis* and those of *Archidoris* spp. led us to examine the possibility that *A. kerguelensis* was obtaining the glycerides by *de novo* biosynthesis. There is no experimental evidence to support this hypothesis. However, observations by Dayton *et al.*[10] suggest that *A. kerguelensis* feeds almost exclusively on sponges of the Order Hexactinellida (glass sponges), which are renowned for their paucity of organic biomass and lack of secondary metabolites. With no obvious dietary source for the glycerides, *de novo* biosynthesis is an attractive alternative.

Experimental Section

^1H and ^{13}C NMR spectra were recorded on a Bruker WP200–SY spectrometer. Low resolution mass spectra were recorded on a HP 5988 mass spectrometer and IR spectra were recorded on a Perkin Elmer model 1600 FT–IR spectrometer. Optical rotations were measured on a Perkin Elmer 141 polarimeter using a 1 dm cell. Merck Silica Gel 230–400 mesh was used for flash chromatography and Dynamax C–18 and Whatman Magnum 9–Partisil 10 columns were used for preparative HPLC.

Collection, extraction and chromatography: A single specimen of *Austrodoris kerguelensis* (*ca.* 100 g wet wt.) was collected by hand using SCUBA (25–35 m depth) at Cape Armitage, McMurdo Sound, Antarctica and was stored in acetone (1 L) for a year. The acetone extract was concentrated under reduced pressure and the aqueous concentrate extracted with EtOAc (3 × 100 mL). The combined EtOAc extracts were dried over anhydrous Na_2SO_4 and evaporated to yield a red–brown oil (1.0 g). Flash chromatography of this oil on silica gel (30 g) using solvents of increasing polarity from hexane to EtOAc gave eight fractions. The acetylated glycerides **1** and **4**, and **6** and **7** were eluted with 3:1 and 2:1 hexane–EtOAc solutions respectively, and the non–acetylated glyceride **8** was eluted with a 1:1 hexane–EtOAc solution. Final separation of compounds **1** and **4**, was achieved by using reversed phase HPLC (MeOH–H_2O, 95:5) followed by normal phase HPLC (hexane–EtOAc, 65:35). The same reversed phase HPLC conditions were used for the initial purification of compounds **6–8**, but the final normal phase HPLC separations required

elution with more polar (35:65 and 25:75) hexane–EtOAc solvent systems.

2'-Acetoxyglyceryl (5R,10R,13R)-labda-8-en-15-oate (1): oil; $[\alpha]_D$ = – 48° (c = 0.66, $CHCl_3$); IR (neat) 3650–3300, 2940, 1750, 1735, 1240, 1050 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.79 (s, 3 H), 0.85 (s, 3 H), 0.90 (s, 3 H), 1.02 (d, 3 H, *J* = 6.5 Hz), 1.51 (s, 3 H), 2.06 (s, 3 H), 3.71 (d, 2 H, *J* = 5 Hz), 4.17 (dd, 1 H, *J* = 6, 11 Hz), 4.29 (dd, 1 H, *J* = 4.5, 12 Hz), 5.07 (p, 1 H, *J* = 5 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 19.1 (t, C-2), 19.1 (t, C-6), 19.5 (q, C-16), 19.5 (q, C-20), 20.1 (q, C-17), 20.8 (q, Ac), 21.7 (q, C-19), 25.4 (t, C-11), 31.4 (d, C-13), 33.3 (q, C-18), 33.3 (s, C-4), 33.6 (t, C-7), 37.0 (t, C-1), 37.3 (t, C-14), 39.0 (s, C-10), 41.5 (t, C-12), 41.8 (t, C-3), 51.9 (d, C-5), 61.5 (t, C-3'), 62.3 (t, C-1'), 72.0 (d, C-2'), 125.6 (s, C-8), 140.4 (s, C-9), 172.9 (s, C-15), 172.9 (s, Ac); EIMS, *m/z* (intensity, %) 422 (15), 407 (20), 191 (100), 121 (21), 117 (33), 95 (28); HREIMS, obsd. *m/z* = 422.3018, $C_{25}H_{42}O_5$ requires 422.3032.

3'-Acetoxyglyceryl (5R,10R,13R)-labda-8-en-15-oate (4): oil; $[\alpha]_D$ = -53° (c = 0.34, $CHCl_3$); IR (neat) 3650–3300, 2930, 1740, 1240, 1050 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.80 (s, 3 H), 0.85 (s, 3 H), 0.90 (s, 3 H), 0.95 (d, 3 H, *J* = 6.5 Hz), 1.51 (s, 3 H), 2.08 (s, 3H), 4.08–4.15 (m, 5 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 19.1 (t, C-2), 19.1 (t, C-6), 19.5 (q, C-16), 19.6 (q, C-20), 20.1 (q, C-17), 20.8 (q, Ac), 21.7 (q, C-19), 25.4 (t, C-11), 31.4 (d, C-13), 33.3 (q, C-18), 33.3 (s, C-4), 33.6 (t, C-7), 37.0 (t, C-1), 37.3 (t, C-14), 39.0 (s, C-10), 41.4 (t, C-12), 41.8 (t, C-3), 51.9 (d, C-5), 65.0 (t, C-3'), 65.2 (t, C-1'), 68.3 (d, C-2'), 125.6 (s, C-8), 140.4 (s, C-9), 173.4 (s, C-15), 173.4 (s, Ac); EIMS, *m/z* (intensity, %) 422 (14), 407 (22), 191 (100), 121 (21), 117 (27), 95 (29); HREIMS, obsd. *m/z* = 422.3005, $C_{25}H_{42}O_5$ requires 422.3032.

Diketone 6: oil; $[\alpha]_D$ = -9° (c = 0.24, $CHCl_3$); IR (neat) 3650–3300, 2955, 1740, 1715, 1695, 1460, 1235, 1050 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.88 (s, 3 H), 0.89 (s, 3 H), 0.93 (d, 3 H, *J* = 6.6 Hz), 1.20 (s, 3 H), 2.05 (s, 3 H), 2.07 (s, 3 H), 3.72 (d, 2 H, *J* = 5 Hz), 4.19 (dd, 1 H, *J* = 6, 11 Hz), 4.30 (dd, 1 H, *J* = 4.5, 12 Hz), 5.08 (p, 1 H, *J* = 5 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 17.2 (q, C-20), 18.2 (t, C-2), 19.6 (q, C-16), 20.8 (q, Ac), 22.4 (t, C-6), 22.5 (q, C-19), 29.9 (q, C-17), 29.9 (d, C-13), 30.5 (t, C-11), 33.5 (q, C-18), 34.3 (s, C-4), 34.7 (t, C-1), 37.1 (t, C-7), 41.1 (t, C-3), 41.5 (t, C-14), 45.8 (t, C-12), 47.6 (d, C-5), 53.2 (s, C-10), 61.5 (t, C-3'), 62.4 (t, C-1'), 72.3 (d, C-2'), 170.5 (s, Ac), 172.5 (s, C-15), 209.5 (s, C-8), 217.0 (s, C-9); EIMS, *m/z* (intensity, %) 321 (1), 303 (2), 259 (16), 177 (21), 125 (22), 117 (31), 43 (100); HRCIMS, obsd. *m/z* = 455.3008, $C_{25}H_{43}O_7$ $(MH)^+$ requires 455.3009.

Diketone 7: oil; $[\alpha]_D$ = -7° (c = 0.17, $CHCl_3$); IR (neat) 3650–3300, 2955, 1740, 1715, 1695, 1460, 1235, 1050 cm^{-1}; ^1H-NMR (200 MHz, $CDCl_3$) δ 0.88 (s, 3 H), 0.89 (s, 3 H), 0.93 (d, 3 H, *J* = 6.6 Hz), 1.20 (s, 3 H), 2.07 (s, 3 H), 2.08 (s, 3 H), 4.02–4.30 (m, 5 H); EIMS, *m/z* (intensity, %) 259 (7), 177 (6), 117 (7), 69 (21), 43 (100); HRCIMS, obsd. *m/z* = 455.3007, $C_{25}H_{43}O_7$ $(MH)^+$ requires 455.3009.

Glyceryl (5R,10R,13R)-7-ketolabda-8-en-15-oate (8); oil; $[\alpha]_D$ = -61° (c = 0.09, $CHCl_3$); IR (neat) 3650–3300, 2930, 1730, 1645, 1610, 1460, 1160, 1055 cm^{-1}; UV (MeOH) 249 (ε 10700); ^1H NMR (200

MHz, $CDCl_3$) δ 0.85 (s, 3 H), 0.88 (s, 3 H), 0.99 (d, 3 H, *J* = 6.5 Hz), 1.04 (s, 3 H), 1.71 (s, 3 H), 3.58 (dd, 1 H, *J* = 11, 6 Hz), 3.70 (dd, 1 H, *J* = 13, 4 Hz), 3.89 (m, 1 H), 4.13 (dd, 1 H, *J* = 12, 5 Hz) and 4.22 (dd, 1 H, *J* = 12, 5 Hz); EIMS, *m/z* (intensity, %) 394 (8), 233 (12) 205 (18) 135 (31), 109 (19), 69 (50), 43 (100); HREIMS, obsd. *m/z* = 394.2701, $C_{23}H_{38}O_5$ requires 394.2719.

Saponification and methylation of glyceride 1. Glyceride **1** (3.9 mg) was added to a solution of K_2CO_3 (30 mg) in MeOH (1.5 mL) and that was then stirred overnight at room temperature. The methanolic solution was cooled, diluted with H_2O (1 mL) and acidified with 6N HCl (0.1 mL). The acidic solution was extracted with Et_2O (3 × 5 mL) and the combined Et_2O extracts were washed with H_2O (2 × 5 mL) and dried over Na_2SO_4. The solvent was evaporated to yield a pale yellow oil (2.9 mg). Methylation of the oil with excess diazomethane followed by normal phase HPLC (80:20 hexane–EtOAc) of the product gave methyl ester **3** as a colorless oil: 2.3 mg; $[\alpha]_D$ = −49° (c = 0.21, $CHCl_3$, Lit[8] = −48°); IR (neat) 2930, 1740, 1460, 1375, 1260, 1155, 1010 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.80 (s, 3 H), 0.85 (s, 3 H), 0.90 (s, 3 H), 0.95 (d, 3 H, *J* = 6.5 Hz), 1.51 (s, 3 H), 3.64 (s, 3 H); EIMS, *m/z* (intensity, %) 305 (7), 191 (52), 121 (34), 107 (34), 91 (46), 41 (100).

Reductive ozonolysis of glyceride 1: A stream of ozone in O_2 was bubbled through a cold (−40°C) solution of glyceride **1** (3.5 mg) in a 1:1 mixture of CH_2Cl_2–MeOH (6 mL) for 15 minutes. The excess ozone was removed in a stream of N_2 and the solution was treated with trimethyl phosphite (0.2 mL) at room temperature for 1 h. The solvent was evaporated and excess trimethyl phosphite was removed azeotropically with toluene (3 × 2 mL). Chromatography of the ozonolysis product on normal phase HPLC (35:65 hexane–EtOAc) gave a colorless oil [1.4 mg, $[\alpha]_D$ = −9° (c = 0.14, $CHCl_3$)] that was identical by TLC, ^1H NMR and EIMS with diketone **6**.

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