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Further chemical studies on the Antarctic nudibranch *Austrodoris kerguelenensis*: new terpenoid acylglycerols and revision of the previous stereochemistry

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Abstract—The diterpenoid acylglycerol fraction from an extract of the mantle of a new collection of the Antarctic dorid gastropod mollusc *Austrodoris kerguelenensis* has been chemically analysed. Two novel 2-monoacylglycerols **9** and **12**, along with known 1,2-diacyl glyceryl esters **5** and **6**, now reassigned as **7** and **8**, have been isolated. The linkage of a diterpenoid moiety at C-2 of glycerol characterizes all the compounds. Because the *R* absolute stereochemistry at C-2 of glycerol has been established for the corresponding 1,3-glyceryl esters **1** and **2**, derived from **7** and **8** by acyl-migration of the terpenoid moiety from C-2 to C-3, our finding implies that 1,2-derivatives from Antarctic nudibranchs have the same *S* stereochemistry as all 1,2-*sn*-diacylglycerols from the other dorids.

1. Introduction

Terpenoid acylglycerols are an interesting class of natural bioactive molecules that peculiarly characterize the metabolite pattern of marine dorid nudibranchs belonging to the related genera *Anisodoris*, *Archidoris*, *Austrodoris*, *Doris* and *Sclerodoris*.[1–3] Because of their adaptive localization in the mantle of the mollusc and their ichthyotoxic and feeding-deterrent properties, terpenoid acylglycerols are believed to be involved in the defensive mechanisms of the animals.[4–7] In addition, these compounds display very interesting pharmacological properties, such as the activation of protein kinase C in vitro and the induction of morphogenetic effects in the regenerative test in vivo with the fresh water hydrozoan *Hydra vulgaris*.[8]

With a few exceptions, the majority of natural terpenoid acylglycerols are structurally characterized by the presence of a terpenoid acid residue esterified at C-1 of glycerol, which is further linked to an acetyl group at C-2 or C-3. The *S* stereochemistry at C-2 of glycerol has been established for many 1,2 and 1,3 glyceryl esters by different methods, including synthesis.[9–13]

A series of diterpenoid glyceryl esters, exhibiting different carbon skeletons such as *ent*-labdane (e.g. **1**),[14,15] halimane (e.g. **2**),[15,16] and isocopalane (e.g. **3**),[17] have been reported from distinct populations of the Antarctic dorid *Austrodoris kerguelenensis*, Bergh 1884. The defensive role played by these molecules in the mollusc has been recently demonstrated by ecological assays.[18] Surprisingly, the stereochemistry at C-2 of glycerol for the 1,3-derivatives **1** and **2**, isolated from different population of the mollusc, was determined by Mosher method to be *R*,[15] opposite to that of all other diacylglycerols isolated from dorids. Consequently, the structures of the corresponding 1,2 derivatives (**5** and **6**) were proposed as 2,3-*sn* by assuming too confidently that **1** and **2** derive from **5** and **6** by the acyl-migration of the acetyl group from C-2 to C-1.

This apparent anomaly has been now clarified by chemical investigation on a new collection (12 individuals) of *A. kerguelenensis*, collected off Terra Nova Bay, the Italian Station in Antarctica, in the course of the 15th Italian Expedition. Along with two new metabolites exhibiting an unprecedented *nor*-sesquiterpene carbon skeleton (e.g. austrodoral, **4**) and suggested to be produced by the animal in stress conditions,[19] the expected terpenoid glyceryl ester fractions were also found in the ether extract of the mantle of the mollusc and subsequently analysed. We report here the results of this study.

2. Results and discussion

As already described,[19] each *A. kerguelenensis* individual was carefully dissected into mantle and internal organs, which were separately extracted by acetone. A comparative chromatographic analysis of the diethyl ether soluble part from the acetone extract of both mantle and internal organs for each individual showed the presence of a series of compounds giving a pink coloration by spraying with

Keywords: marine natural products; terpenes and terpenoids; stereochemistry.

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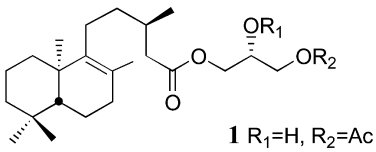
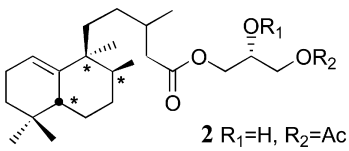
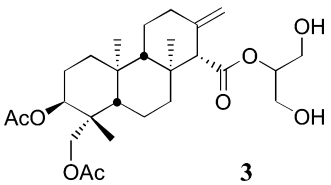


1 R_1=H, R_2=Ac
5 R_1=Ac, R_2=H



2 R_1=H, R_2=Ac
6 R_1=Ac, R_2=H

* relative stereochemistry



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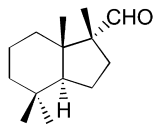

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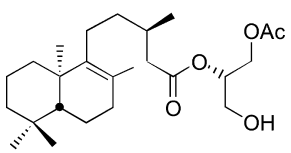
$CeSO_4$ only in the mantle extract of the animals. All compounds were present in the mantle extract of each individual, but with a different relative distribution.[19] All mantle ether extracts were combined (605 mg) and fractionated by Si-gel column (light petroleum ether/diethyl ether gradient). ^{1}H NMR analysis of fractions at R_f 0.75 and 0.35 (light petroleum/diethyl ether, 2:8) revealed that they were constituted by mixtures of bicyclic diterpene acylglycerols. In particular, the less polar fraction (41.0 mg) contained a mixture of 1,2-diacylglycerols, whereas, differently from the previous reports,[14,15] the corresponding 1,3-derivatives were not detected in this extract. However, the formation of 1,3 derivatives has been observed to occur in the mixture of 1,2-diacylglycerols during HPLC work-up. The more polar fraction (113.2 mg) contained related diterpene 2-monoacylglyceryl esters.

HPLC purification of the diacylglycerol fraction gave, along with an unresolved mixture, two main pure compounds (2.5 and 5.6 mg, respectively), the spectral data of which (^{1}H and ^{13}C NMR, $[\alpha]_D$) were identical to those reported for the previously described 1,2-diacylglycerols **5**[14] and **6**[15] from *A. kerguelenensis*. Surprisingly, a careful structural analysis of HMBC correlation experiments recorded for both molecules indicated that the diterpene moiety was linked to the secondary hydroxyl group at C-2 of glycerol and the acetyl residue esterified the primary hydroxyl group at C-1. In fact, diagnostic correlations were observed between the carboxyl of acetyl group (at δ 170.9 in **7**, 170.6 in **8**) with the CH_2 of glycerol (at δ 4.24 and 4.32 in **7**, 4.23 and 4.31 in **8**) and between the carboxyl of the terpenoidic acid (at δ 173.0 in **7**, 172.6 in **8**) with the carbinol methine of glycerol (at δ 5.10 in both **7** and **8**). This led us to assign the structures **7** and **8** to the 1,2 diacylglycerols from *A. kerguelenensis* previously incorrectly reported as **5**[14] and **6**,[15] respectively.

Bearing in mind the *R* absolute stereochemistry at C-2 of glycerol established for compounds **1** and **2**, an interesting



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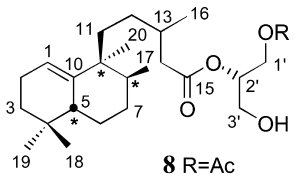
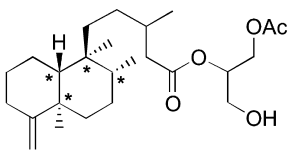


8 R=Ac
12 R=H



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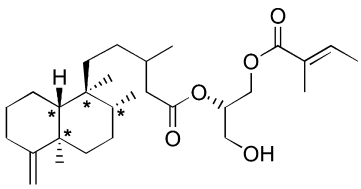

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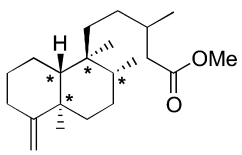
* relative stereochemistry



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consequence of this finding is that the 1,2-derivatives from Antarctic nudibranchs have the same *S* stereochemistry as all 1,2-*sn*-diacylglycerols from the other dorids. The inversion of configuration in 1,3-glyceryl esters **1** and **2**, formed by the corresponding 1,2-derivatives **7** and **8**, is due to the acyl-migration of the diterpenoid moiety from C-2 to C-3 of glycerol, being C-1 linked to acetyl residue.

^{1}H NMR analysis of the third fraction obtained by HPLC purification showed the presence of a complex mixture of isomeric bicyclic diterpenes. Every attempt to isolate the components of the fraction failed, but some considerations about their structures should be made. Analogously with **7** and **8**, HMBC spectrum of the mixture displayed diagnostic correlations between the carboxyl of acetyl groups with CH_2 protons of glycerol, indicating also for this group of diacylglycerols a substitution pattern with diterpene acids linked to the secondary hydroxyl of glycerol, further esterified at the primary hydroxyl group by an acetyl function. Careful analysis of 2D-NMR data of the mixture led us to recognise compound **9** as the major metabolite of the mixture, exhibiting the same clerodane diterpenoid residue as archidorin (**10**), a diacylglycerol previously isolated from the Atlantic nudibranch *Archidoris tuberculata*,[5] but differing in the substitution pattern at the glyceryl moiety further linked to an acetyl group in the place of the tiglic acyl residue. Methanolysis of this fraction led to the corresponding terpenoid methyl ester mixture, which was analysed by both HPLC and ^{1}H NMR. Clerodane methyl ester **11** was identified in the mixture, by comparison with a standard sample obtained from archidorin (**10**).[5]

The more polar terpenoid fraction of the extract was also submitted to HPLC purification and pure compound **12** (16.1 mg) was obtained, together with an inseparable and complex mixture of bicyclic diterpene 2-monoacylglycerols, related to the above clerodane diacylglycerol fraction. NMR data of compound **12** clearly indicated that it was closely structurally related to the co-occurring diacylglycerol **8**, being a diterpene 2-glyceryl ester exhibiting the same halimane skeleton. In fact, all ^{1}H and ^{13}C NMR resonance of the diterpenoid part of compound **12** matched those of diacylglycerol **8**, whereas the difference was in the absence of the acetyl group esterified to glycerol. Therefore, compound **12** was the deacetyl derivative of **8**. All NMR values were assigned, as reported in Table 1, by mono- and bi-dimensional experiments.

This study has clarified an apparent stereochemical anomaly of natural diterpenoid glyceryl esters from Antarctic *A. kerguelenensis*,[15] showing that 1,2-derivatives are characterized by the linkage of diterpenoid moiety at C-2 of glycerol, whereas the acetyl residue is linked to C-1 and therefore they are 1,2-*sn* diacylglycerols. Migration of the terpenoid acyl group from C-2 to C-3 occurs during work-up conditions and consequently leads to the corresponding 1,3-derivatives with the *R* absolute stereochemistry.

In addition, the finding of co-occurring biogenetically related bicyclic diterpenes exhibiting labdane skeleton as well as the rearranged halimane and clerodane framework has been reported for both marine (e.g. agelasins from the sponge *Agelas* sp.[20]) and terrestrial sources (e.g. diterpenes from the plant *Polyalthia longifolia*[21]). In particular, the halimane skeleton is believed to arise from an intermediate in the rearrangement of labdanes to clerodanes. On these bases, because the absolute stereochemistry of diterpenoid moiety of compounds **1** and **7** has been determined to be that of *ent*-labdane,[14] the *ent*-halimane and clerodane absolute stereochemistry should be suggested, by biogenetic considerations, for the co-occurring diterpenoids **2**, **8** and **12** and **9**, respectively.

Table 1. NMR data of compound **12**

C	δ ^{13}C[a]	m[b]	δ ^{1}H[c]	m, *J* (Hz)	HMBC correlations[d]
1	116.8	d	5.30	m	
2	23.2	t	2.05	m	H-3a
			1.95	m	
3	31.2	t	1.35	m	H-5, H_3-18, H_3-19
			1.05	m	
4	31.3	s	–	m	H-3a, H-5, H_3-18, H_3-19
5	43.6	d	1.50	m	H_3-18, H_3-19
6	30.2	t	1.75	m	
			1.05	m	
7	31.4	t	1.50	m	H-5
			1.45	m	
8	44.6	d	1.25	m	H-6a, H-7a, H_3-17, H_3-20
9	42.6	s	–		H-11a, H_3-17, H_3-20
10	146.2	s	–		H-5, H_2-6, H-11b, H_3-20 H_3-20
11	28.2	t	1.65	m	H_3-20
			1.00	m	
12	30.6	t	1.05	m	H_2-14,H_3-16
			0.85	m	
13	31.3	d	1.82	m	H_2-14,H_3-16
14	42.1	t	2.37	dd, 15,6	H_3-16
			2.15	dd, 15,9	
15	173.7	s	–		H_2-14, H-2′
16	20.0	q	0.93	d,7	H-12a, H_2-14
17	16.4	q	0.83	d,7	H-7a, H-8
18	28.0	q	0.85	s	H_3-19
19	27.6	q	0.85	s	H-3a, H_3-18
20	23.2	q	0.97	s	H_2-11
1′	62.6	t	3.83	d,5	H-2′,H_2-3′
2′	76.2	d	4.93	quint, 5	H_2-1′,H_2-3′
3′	62.6	t	3.83	d,5	H_2-1′,H-2′

Bruker 300, 400 and 500 MHz; $CDCl_3$; chemical shifts (ppm) referred to C*H*Cl_3 (δ 7.26) for proton and to *C*DCl_3 (δ 77.0) for carbon.
[a] Assignments by HMQC and HMBC experiments.
[b] By DEPT sequence.
[c] Assignments by ^{1}H–^{1}H COSY experiment.
[d] *J*=10 Hz.

3. Experimental

3.1. General experimental procedures

Si-gel chromatography was performed using precoated Merck F_{254} plates and Merck Kieselgel 60 powder. HPLC purifications were carried out on a Waters liquid chromatograph equipped with a differential refractometer as detector. Optical rotations were measured on a Jasco DIP 370 digital polarimeter. The IR spectra were taken on a Bio-Rad FTS 7 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a WM 500, AVANCE 400 and a DPX 300 MHz Bruker spectrometers in $CDCl_3$; chemical shifts are reported in ppm referred to $CHCl_3$ as internal standard (7.26 for proton and 77.0 for carbon). EIMS and HREIMS spectra were

measured on a TRIO 2000 VG Carlo Erba and on a Kratos MS50 instruments, respectively.

3.2. Animal material

A. kerguelenensis, Bergh 1884, (12 individuals, average size 6 cm) were collected by SCUBA at depths of 10–20 meters, off Terra Nova Bay, Antarctica, during the Austral Summer 1999–2000, in the course of the 15th Italian Expedition. Seven specimens were immediately refrigerated at −20°C, whereas the remaining five animals were kept in an aquarium for 15 days, before being frozen. The animals were then transferred to Italy and chemically analysed. A voucher specimen is stored for inspection at ICB (BTN 30).

3.3. Extraction and isolation

Each specimen was carefully dissected into mantle and internal organs, which were separately extracted by acetone (20 ml×3). Each acetone extract was partitioned between Et_2O and H_2O. The Et_2O soluble part of both mantle and internal organs obtained for each individual was analysed by TLC chromatography. Then, all mantle extracts were combined to give 605 mg of crude extract, which was chromatographed by Si-gel column (light petroleum ether/diethyl ether gradient) to give a diacylglycerol containing fraction (41.0 mg) at R_f 0.75 (light petroleum ether/diethyl ether, 2:8) and a monoacylglycerol containing fraction (113.2 mg) at R_f 0.35 (light petroleum ether/diethyl ether, 2:8). Diacylglycerol mixture was submitted to *n*-phase HPLC (Spherisorb S5W, 3.9 mm (ID)×30 cm, *n*-hexane/ethyl acetate, 9:1; flow rate 1.8 ml/min) affording compounds **7** (2.5 mg), **8** (5.6 mg) and a mixture containing **9** (1.9 mg). Monoacylglycerol fraction was also purified by *n*-phase HPLC (Spherisorb S5W, 3.9 mm (ID)×30 cm, *n*-hexane/iso-propanol, 95:5; flow rate 0.8 ml/min) to give compound **12** (16.1 mg) and a mixture (5.9 mg) not further purified.

3.3.1. Compound 7.[14] Amorphous white solid. $[\alpha]_D^{25}=-59.9$ (*c* 0.2, $CHCl_3$); ^1H NMR: δ_H (400 MHz, $CDCl_3$) 5.30 (1H, m, H-1), 5.10 (1H, quintet, J=5.2 Hz, H-2′), 4.32 (1H, dd, J=11.9, 4.5 Hz, H_2-1′a), 4.24 (1H, dd, J=11.9, 5.8 Hz, H_2-1′b), 3.74 (2H, m, H_2-3′), 2.41 (1H, dd, J=14.7, 5.7 Hz, H-14a), 2.17 (1H, dd, J=14.7, 8.5 Hz, H-14b), 2.08 (3H, s, OAc), 1.54 (3H, bs, H_3-17), 0.99 (3H, d, J=6.6 Hz, H_3-16), 0.93 (3H, s, H_3-20), 0.88 (3H, s, H_3-18), 0.83 (3H, s, H_3-19); ^{13}C NMR: δ_C (75.5 MHz, $CDCl_3$) values reassigned by HMBC experiment (J=10 Hz): 173.0 (C-15), 170.9 (OAc), 140.2 (C-9), 125.6 (C-8), 71.9 (C-2′), 62.2 (C-1′), 61.4 (C-3′), 51.7 (C-5), 41.6 (C-3), 41.3 (C-14), 39.0 (C-10), 37.3 (C-12), 36.9 (C-1), 33.6 (C-7), 33.4 (C-4 and C-18), 31.3 (C-13), 25.5 (C-11), 21.7 (C-19), 20.6 (OAc), 20.1 (C-20), 19.6 (C-16), 19.5 (C-17), 19.1 (C-2 and C-6).

3.3.2. Compound 8.[15] Amorphous white solid. $[\alpha]_D^{25}=+29.1$ (*c* 0.1, $CHCl_3$); ^1H NMR: δ_H (400 MHz, $CDCl_3$) 5.30 (1H, m, H-1), 5.10 (1H, quintet, J=5.4 Hz, H-2′), 4.31 (1H, dd, J=12.0, 4.5 Hz, H_2-1′a), 4.23 (1H, dd, J=12.0, 5.9 Hz, H_2-1′b), 3.73 (2H, m, H_2-3′), 2.35 (1H, dd, J=14.7, 5.6 Hz, H-14a), 2.12 (1H, dd, J=14.7, 8.6 Hz, H-14b), 2.08 (3H, s, OAc), 0.97 (3H, s, H_3-20), 0.93 (3H, d, J=6.6 Hz, H_3-16), 0.85 (6H, s, H_3-18 and H_3-19), 0.83 (3H, d, J=7.1 Hz, H_3-17); ^{13}C NMR: δ_C (75.5 MHz, $CDCl_3$) 172.6 (C-15), 170.6 (OAc), 146.2 (C-10), 116.8 (C-1), 72.0 (C-2′), 62.3 (C-1′), 61.6 (C-3′), 44.6 (C-8), 43.6 (C-5), 42.5 (C-9), 41.9 (C-14), 31.3 (C-4, C-7 and C-13), 31.2 (C-3), 30.6 (C-12), 30.2 (C-6), 28.2 (C-11), 28.0 (C-18 or C-19), 27.6 (C-19 or C-18), 23.2 (C-2 and C-20), 20.7 (OAc), 19.8 (C-16), 16.4 (C-17).

3.3.3. Compound 9. NMR spectra were recorded on the mixture of diacylglycerols, being compound **9** the major metabolite. Assignments were made by both analysing HSQC and HMBC spectra and comparing NMR data with those of a standard sample of archidorin (**10**). ^1H NMR: δ_H (400 MHz, $CDCl_3$) 5.09 (1H, m, H-2′), 4.50 (2H, s, H_2-18), 4.31 (1H, dd, J=12.0, 4.2 Hz, H_2-1′a), 4.23 (1H, dd, J=12.0, 5.7 Hz, H_2-1′b), 3.74 (2H, m, H_2-3′), 2.37 (1H, m, H-14a), 2.13 (1H, m, H-14b), 2.08 (3H, s, OAc), 1.03 (3H, s, H_3-19), 0.94 (3H, d, overlapped, H_3-16), 0.78 (3H, d, J=6.2 Hz, H_3-17), 0.72 (3H, s, H_3-20); ^{13}C NMR: δ_C (75.5 MHz, $CDCl_3$) 172.9 (C-15), 171.0 (OAc), 160.8 (C-4), 102.2 (C-18), 71.8 (C-2′), 62.3 (C-1′), 61.6 (C-3′), 48.5 (C-10), 41.8 (C-14), 40.0 (C-9), 38.9 (C-9), 37.4 (C-6), 36.7 (C-8), 35.4 (C-11), 32.9 (C-3), 31.2 (C-13), 29.7 (C-12), 28.7 (C-2), 27.4 (C-7), 21.6 (C-1), 20.8 (OAc and C-19), 19.8 (C-16), 18.2 (C-20), 16.0 (C-17).

3.3.4. Methanolysis of the mixture of diacylglycerols. The above mixture of diacylglycerols containing **9** (1.9 mg) was dissolved in anhydrous MeOH (1 ml) and an excess of Na_2CO_3 was added. The solution was stirred at room temperature overnight, filtered and the solvent evaporated. The residue was purified by a Si-gel column (light petroleum ether/diethyl ether gradient), obtaining the mixture of diterpene methyl esters (0.9 mg), which was analysed by *n*-phase HPLC (Kromasil S5W, 3.9 mm (ID)×30 cm, *n*-hexane, flow rate 1 ml/min) and by ^1H NMR. Clerodane methyl ester **11** was identified by comparison with a standard.

3.3.5. Compound 12. Amorphous white solid. $[\alpha]_D^{25}=+24.2$ (*c* 0.2, $CHCl_3$); IR ν_{max} (liquid film) 1735, 2865, 2926, 3420 cm^{-1}; ^1H and ^{13}C NMR in Table 1. EIMS *m/z* (%) 380 (M^+, 3), 365 (3), 349 (3), 289 (6), 191 (100), 135 (25), 121 (20), 95 (20); HREIMS: 380.2919, calcd for $C_{23}H_{40}O_4$ 380.2926.

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