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Biosynthesis of Drimane Terpenoids in Dorid Molluscs: Pivotal Role of 7-Deacetoxyolepupuane in Two Species of *Dendrodoris* Nudibranchs

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Abstract

Described in this paper is the biosynthesis of defensive drimanes in dorid nudibranchs. Two geographically distinct populations of molluscs belonging to the genus *Dendrodoris* have been studied. The *de novo* synthesis of 7-deacetoxyolepupuane (**2**) has been demonstrated by radiolabelled precursors in both species. The results of the studies are consistent with the pivotal role of **2** in the formation of the other drimanes both in the mantle and in the egg masses of the molluscs. The new drimane 6-β-acetoxypolygodial (**7**) is reported from the skin extract of the pacific *Dendrodoris arborescens*. Its structure elucidation was accomplished by NMR spectroscopy.

Keywords: Natural Products; Biosynthesis; Biologically Active Compounds; Terpene;

Introduction

Molluscs belonging to the genus *Dendrodoris* appear to employ chemicals to deter the attack of predators [1]. Most of the substances involved in their defense mechanisms are sesquiterpenes of the drimane series [2]. The biological activities associated with these compounds have prompted investigations to rationalize their biogenetic origins [3,4]. It has been suggested that most of these compounds (**1-3**) may represent inactive forms of the antifeedant metabolite polygodial (**4**), that is believed the actual deterrent product [3-5].

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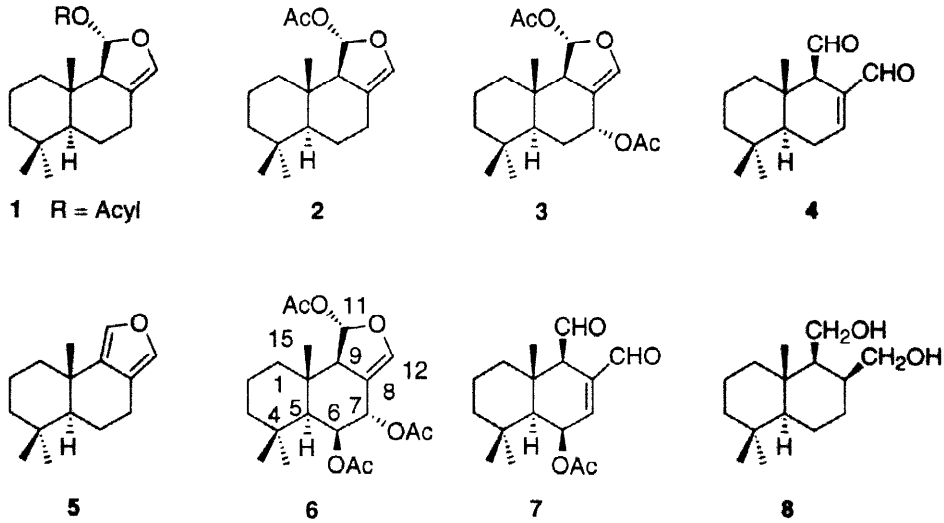


Although the *de novo* production of drimane terpenoids has been demonstrated in Mediterranean nudibranchs [3,4], to date their biosynthetic pathway in molluscs has never been fully elucidated, and the biological role of **1** and **2** has never been explained by direct evidence. Moreover, as most of these compounds have been also isolated from sponges [6-8], some authors have rised the question of the dietary origin for the drimane metabolites in dorids [6]. Particularly debatable is the role of 7-deacetoxyolepupuane (**2**) which could be the precursor of the other dorid drimanes, even though it has also been isolated in large amount from sponges of the genus *Dysidea* [6-8].

In our ongoing research on defensive chemicals from nudibranchs, we have analysed a Pacific population of *Dendrodoris arborescens* that contained drimane esters (**1**) and 7-deacetoxyolepupuane (**2**) but neither polygodial (**4**) nor olepupuane (**3**). Recently, Andersen and coworkers re-examined the dependence of allomone pattern in dorids on sponge diet, considering the geographic variability to be a consequence of a presumed dietary origin of the compounds [9]. In this content, *D. arborescens* provided the chance to re-investigate the biosynthesis of drimane compounds in dorid nudibrachs and to clarify the origin of **1** and **2**. This paper describes: (a) the incorporation of [^{14}C]-mevalonate in Mediterranean *Dendrodoris limbata* and Pacific *D. arborescens*; (b) the *de novo* biosynthesis of **2** in both dorid nudibranchs; (c) the allomone pattern of Pacific *D. arborescens*; (d) the anatomical distribution of the allomones in the Pacific nudibranch; (e) the biosynthetic pathway of drimane compounds in mollusc tissues.

Results and Discussion

It has been reported that Mediterranean *Dendrodoris* shows a tissue-dependent distribution of the drimane terpenoids [5]. In particular, studies of *D. limbata* and *Dendrodoris grandiflora* revealed that a mixture of drimane esters (**1**) is located both in the hermaphrodite gland and in the egg masses, whereas 7-deacetoxyolepupuane (**2**) is a

component of the mixture, but it is also selectively present in the gills. All these compounds are rather less active than **4** in antifeedant assays [5,10], thus suggesting other physiological functions. As a first step, we have re-investigated the incorporation of mevalonate in Mediterranean dorids. Specimens of *D. limbata* were injected with 2μCi DL-[2-^{14}C]-mevalonic acid dibenzylethylenediamine (DBED) salt dissolved in 200 μL of distilled water. The incorporation of labelled mevalonate was expressed as specific radioactivity (dpm/mg). The experiment was run for 24 h with two specimens and for 48 h with other two molluscs. After that time, the nudibranchs were frozen and then dissected in order to separate the mantle from inner organs. Fractionation of the acetone extracts (see Experimental Section) gave a labelled mixture (4.5 mg, 11630 dpm/mg) of polygodial (**4**) and olepupuane (**3**) from mantle, and labelled drimane esters (**1**) (14 mg, 14500 dpm/mg) and 7-deacetoxyolepupuane (**2**) (0.5 mg, 25560) from the gland. The incorporation in the terpenoid skeleton was tested on **1** and **2** by thermolysis on silica (Scheme 1a). Both compounds gave labelled euryfuran (**5**) (21800 dpm/mg and 28000 dpm/mg from **1** and **2**, respectively) allowing us to rule out the possible incorporation of radioactivty in the acyl groups linked to the drimane nucleus. This experiment provided conclusive evidence for the *de novo* biosynthesis of 7-deacetoxyolepupuane (**2**), but we could not demonstrate any time-dependent relationship among the drimane compounds.

With the direct proof of the *de novo* formation of **2** in hand, we could address the pathway of the drimane biosynthesis and explore the relationships among the terpenoids. *D. arborescens* (6 specimens) is a dorid nudibranch living along the coast of Koino-ura, Fukuoka (Japan). The mollusc (2 specimens) was frozen and dissected in order to separate the mantle from the inner organs. The Et_2O extracts of these sections were dissolved in $CDCl_3$ and analysed by NMR without any other purification. ^{1}H-NMR spectra revealed the presence of drimane esters in the extract from inner organs (22.3 mg), but otherwise showed aldehydic compounds in the mantle. Silica gel separation of this latter fraction gave an unresolved

Scheme 1

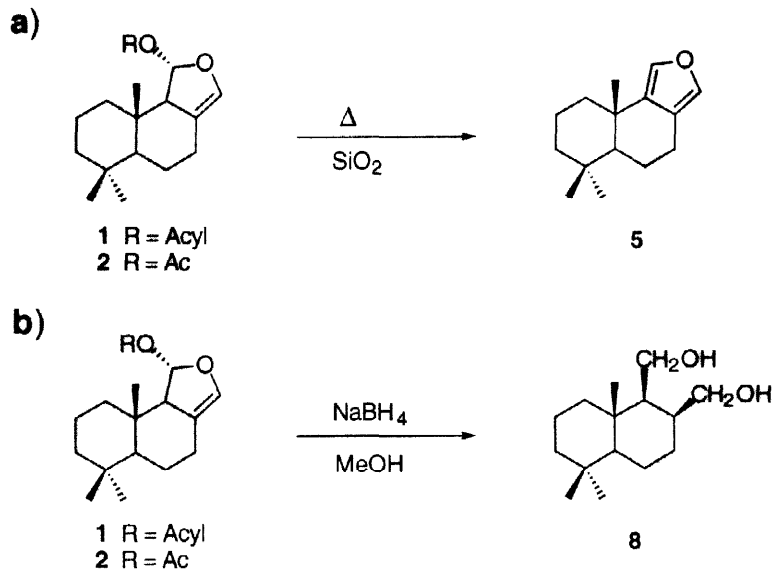


Table 1. Radioactive recoveries in *D. arborescens* at different incubation times.

Section	Time	Diethyl Ether Extract		Compound **1**		Compound **2**		Compound **6** and **7**	
		Yield	dpm/mg	Yield	dpm/mg	Yield	dpm/mg	Yield	dpm/mg
gland	2 days	185 mg	2430	4.3 mg	18040	0.5 mg	22120	-	-
gland	7 days	57.6 mg	1650	9.7 mg	1860	3.5 mg	300	-	-
mantle	2 days	20.3 mg	3100	-	-	-	-	1.4 mg	1160
mantle	7 days	20.1 mg	2920	-	-	-	-	2.1 mg	5900

mixture (1.2 mg) of drimane compounds, that were identified as **6** and **7** after further NMR investigations and by comparison with the literature data [4]. To the best of our knowledge compound **7** is new in nature. Its drimane skeleton was inferred by analysis of the mixture of **6** and **7**. In fact besides the resonances attribuitable to the known structure **6**, the ^{1}H NMR spectrum showed signals for two aldehyde moieties (δ 9.59, H-11; 9.53, H-12), one acetyl (δ 2.13) and three methyl (δ 1.21, 1.16, 1.02) groups. The olefin proton at δ 6.97 (H-7) exhibited both a long range correlation with the aldehydic signal at δ 9.53 (H-12) and a stronger coupling with the methine hydrogen at δ 5.85 (H-6), in turn coupled to the bridgehead H-5 (δ 1.39). The 6β-acethoxy configuration was deduced from the observation that H-6 resonated in the ^{1}H-NMR spectrum as a broad triplet having two small coupling constants (J = 4.6 and 4.1 Hz) due to the correlation with H-5 and H-7. The spectral data of **7** were completed by the correlation of the downshifted proton at C-9 (δ 2.83) with the aldehyde signal at δ 9.59 (H-11). GC-MS analysis of **6**-**7** mixture exhibited two peaks, of which the mass spectra were in agreement with the structures of **6** and **7** (see experimental section), thus supporting the assignment described above. The remaining individuals (4 specimens) of *D. arborescens* were maintained at 20 °C in sea water. Individual specimens were injected with 2 μCi of [^{14}C]-mevalonate under the conditions above reported for *D limbata*. Two days after the injection, two specimens of *D. arborescens* were carefully removed from the aquarium and frozen. The other two specimens were kept in the aquarium for five days more. Fortuitously, these animals deposited three egg-masses which were removed and frozen together with the animals a week after the beginning of the feeding experiment. Frozen nudibranchs, starved for either 2- or 7-days, were dissected and extracted with acetone as above described for *D. limbata*. The ether soluble portion of the acetone extracts from the 2-day starved animal mantle was fractionated by sequential SiO_2 columns to give labelled **6** and **7**, whereas the gland sections of the same animals contained only drimane esters (**1**) and 7-deacetoxyolepupuane (**2**). A similar tissue distrubution was observed in the animals kept for 7-days in the aquarium, though these animals showed a gland content of drimane compounds significantly lower than that found in the 2-day starved specimens. Table 1 reports a breakdown of the [^{14}C]-mevalonate feeding experiments in *D. arborescens*. The specific incorporation of the labelled mevalonate in the terpenoic part of the molecules was demonstrated by conversion of **1** and **2** into the labelled diol **8** (Scheme 1b). In fact,

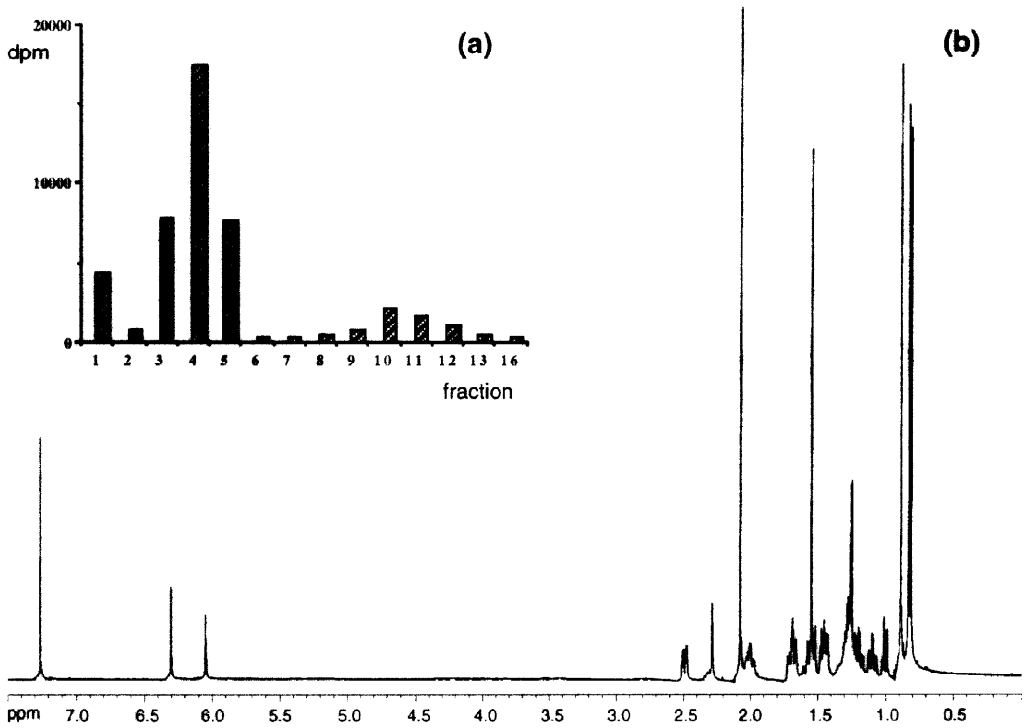


Figure 1. Fractionation of egg mass extract of *D. arborescens*. (**a**) Elution profile *vs* radioactivity (dpm) - fraction #1: euryfuran (**5**); fraction #3: drimane esters (**1**); fraction #4 and #5: 7-deacetoxyolepupuane (**2**); (**b**) ^{1}H-NMR spectrum of fraction #4 (7-deacetoxyolepupuane, **2**).

reduction of **1** (2 mg) and **2** (0.5 mg) from 2-day starved molluscs gave, respectively, 0.9 mg (23400 dpm, 26000 dpm/mg) and 0.3 mg (9450 dpm, 31500 dpm/mg) of **8**. In the same way, 2.2 mg (5630 dpm, 2560 dpm/mg) and 0.4 mg (890 dpm, 2200 dpm/mg) of **8** were obtained by treatment with $NaBH_4$ of **1** (4 mg) and **2** (3 mg) from 7-day starved molluscs. The axial orientation of the substituent at C-8 was inferred on the basis of the multiplicity of H-8 that resonated as a broad quintet (J = 6.0 Hz) at δ 2.20. This stereochemistry and the *cis* geometry between the hydroxymethylene groups at C-8 and C-9 were further confirmed by comparison of the ^{1}H-NMR data of **8** with those reported in the literature for similar compounds [7]. As described above for mantle and gland, the frozen egg masses were extracted by acetone and analysed by chromatographic methods. Fractionation of the Et_2O portion of the acetone extract gave euryfuran (**5**; 0.5 mg, 20000 dpm/mg), drimane esters (**1**; 5 mg, 2258 dpm/mg) and 7-deacetoxyolepupuane (**2**; 1.7 mg, 15000 dpm/mg) (Figure 1). Once more, the specific incorporation in the drimane skeleton of **1** (3 mg) and **2** (1 mg) was demonstrated by conversion into the diol **8** (2800 dpm/mg and 16100 dpm/mg from **1** and **2**, respectively). Furthermore, methanolysis of **1** (1.5 mg, 3400 dpm) with Na_2CO_3/ MeOH followed by silica gel purification led a mixture of methyl esters of linoleic acid (37%), oleic acid (19%), elaidic acid (22%) and stearic acid (21%). No incorporation of radioactivity was found in

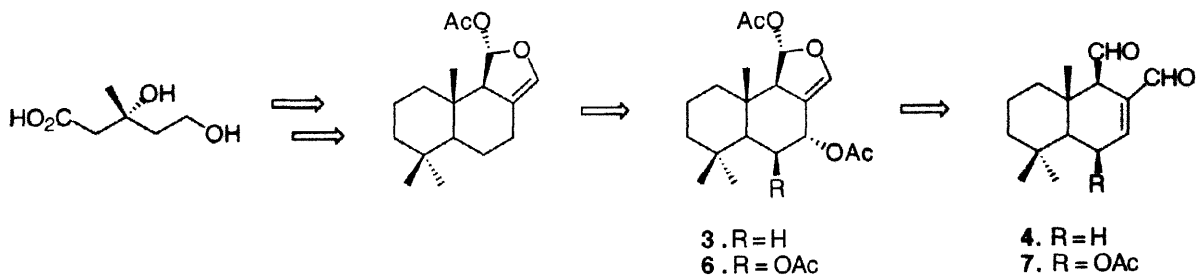
these metabolites. Interestingly, the same fatty acid composition was found by GC-MS analysis of the triglycerides present in the egg masses of the mollusc.

Conclusions

The compound 7-deacetoxyolepupuane (**2**) is a drimane sesquiterpenoid with a very simple structure. Despite this, **2** offers many intriguing starting points to investigate some aspects of the ecology of marine invertebrates. This study gave conclusive evidence of the *de novo* biosynthesis of 7-deacetoxyolepupuane (**2**) in two different species of *Dendrodoris*. Although the origin of the allomones in dorid nudibranchs is a matter of discussion, the anatomical distribution of drimane sesquitepenoids can facilitate the experimental procedures. Compound **2** appeared selectively compartmentalized in the hermaphrodite gland where it was always found in association with the mixture of fatty acid esters (**1**). On the contrary, aldehyde compounds (**4** and **7**), and their precursors (**3** and **6**), were only present in the animal skin. Feeding experiments at different times suggested that **2** could be the former precursor in the biosynthesis of drimane compounds in dorids (Scheme 2). In fact, both *D. arborescens* and *D. limbata* showed high levels of incorporation in the fractions containing **2**. This was clear in the short time feeding experiments which might support the idea of a gradual transfer of radioactivity from **2** to other drimane compounds as time goes by. In particular, the low level of hot material in the gland extracts of the 7-day starved specimens of *D. arborescens* can be explained on the basis of the allomone transfer from the hermaphrodite gland to the egg masses. This hypothesis is supported by the high recovery of labelled **1** and **2** in the egg masses. However, the presence of radioactive **2** in these extracts is further circumstantial evidence for the pivotal role of this metabolite in the drimane biosynthesis and is in agreement with the presence of **2** as the unique allomone in the juvenile forms of *D. limbata* [11].

An interesting issue in the investigation of drimane compounds is the role of **1** in the ecology of dorid molluscs. In analogy with other opisthobranchs, it has been suggested that such metabolites may have a physiological function associated with sexual cycles and egg development. In this case, one would have to suppose a specific composition of the fatty acids linked to the drimane part. However, the GC - MS analysis did not show any difference

Scheme 2. Biosynthesis of drimane terpenoids in dorid nudibranch.



between the mixture of fatty acids in **1** and in the triglycerides of the same animal. This may indicate a role of storing or of transporting for **1**.

In conclusion this paper proved that: 1) 7-deacetoxyolepupuane (**2**) is present in *Dendrodoris* molluscs from geographically distinct areas; 2) both studied populations, *D. limbata* from Italy and *D. arborescens* from Japan, are able to biosynthesize *de novo* 7-deacetoxyolepupuane (**2**); **2** is the precursor of drimane sesquiterpenoids co-occuring in the dorids studied; 4) 6-β-acetoxyolepupuane (**6**) should play the same biological role as olepupuane giving, through opening of the hemiacetal ring, compound **7**. This last metabolite displays the same conjugated dialdehyde moiety of polygodial (**4**) and, therefore, should possess related bioactivity [12].

Experimental

General Methods

- Merck kieselgel 60 (70-230 Mesh) was used for silica gel chromatogaphy and precoated kieselgel 60 F254 plates (Merck 0.25 mm precoated plates) were used for analytical TLC. NMR spectra were recorded by a Bruker WM-500 (500 MHz) and AMX 400 (400 MHz) spectrometers. Chemical shifts are reported in ppm referred to $CHCl_3$ (δ=7.26 for proton and 77.0 for carbon). Radioactivity was counted in 5-mL polyethylene tubes by a Packard 1600-TR liquid scintillation analyzer. Each labelled sample was dissolved in 5 mL of scintillation cocktail (Wallac Oy. Optiphase).

Biological Material

-The nudibranchs *D. limbata* (6 specimens) was collected by scubadiving in the Gulf of Naples (Italy) in April 1997 (Voucher specimen #DL497). *D. arborescens* (6 specimens) was collected off Koino-ura, Fukuoka (Japan) in June 1998 (Voucher specimen #1998-kym-003).

Feeding Experimental Conditions, Extraction and Fractionation

-The specimens of *D. limbata* were injected with 2 μCi/animal of DL-[2-^{14}C]-mevalonic acid DBED salt (NEN^{TM} Life Science) according to our previously reported procedure [4,5]. Two animals were kept in the aquarium for 24 h and two other individuals for 48 h. Then, the animals were separately extracted with acetone and the ether soluble material from the acetone extracts was fractionated by sequential silica gel columns [4] to give compounds **1-4**. The mollusc *D. arborescens* (6 specimens) were supplied with 2 μCi/animal of DL-[2-^{14}C]-mevalonic acid DBED salt (NEN^{TM} Life Science) as above reported for *D. limbata*. The animals were kept at 20° C in the aquarium on the basis of the following programme: 2 animals were incubated for 2 days, and 2 specimens for 7 days. Other two specimens of *D. arborescens* were frozen and kept as controls. At the end of the incubation time, each mollusc

was dissected in order to separate the inner organs from the mantle structure. Then, each section was extracted with acetone according to our usual method [5] and the diethyl ether soluble material was fractionated by sequential silica gel columns to give **1, 2, 6** and **7**. The same procedure was applied to the egg masses deposited by the 7-day incubated molluscs to afford only **1** and **2**. Compounds **1-6** were determined by comparison of spectral data and chromatographic behaviour with authentic standards [4,5,10,13].

Analysis of the mixture of 6 and 7

-colorless oil; ν_{max} (liquid film) 2935, 1736, 1716, 1680, 1360 cm^{-1}; 6β-acetoxyolepupuane (**6**) ^{1}H-NMR ($CDCl_3$) δ 6.48 (1H, d, *J* = 2.4 Hz), 6.44 (1H, d, *J* = 2.4 Hz), 5.30 (2H, m), 2.57 (1H, bs), 2.09 (6H, s, CH_3CO-), 2.05 (3H, s, CH_3CO-), 1.09 (3H, s), 0.99 (3H, s), 0.98 (3H, s); 6β-acetoxypolygodial (**7**). ^{1}H-NMR ($CDCl_3$) δ 9.59 (1H, d, *J* = 4.1 Hz, H-11), 9.53 (1H, s, H-12), 6.97 (1H, dd, *J* = 4.6 and 2.3 Hz, H-7), 5.85 (1H, bt, *J* = 4.6 and 4.1 Hz, H-6), 2.83 (1H, bs, H-9), 2.13 (3H, s, CH_3CO-), 1.65-1.25 (6H, m), 1.39 (1H, d, *J* = 4.1 Hz, H-5), 1.21 (3H, s, H_3-15), 1.16 (3H, s, H_3-14 or H_3-13), 1.02 (3H, s, H_3-13 or H_3-14); ^{1}H-NMR (C_6D_6) δ 9.53 (1H, bs, H-11), 8.98 (1H, s, H-12), 6.81 (1H, s, H-7), 6.29 (1H, bs, H-6), 2.45 (1H, s, H-9), 1.63 (3H, s, CH_3CO-), 1.50-1.10 (7H, m), 0.98 (3H, s, H_3-15), 0.80 (3H, s, H_3-14 or H_3-13), 0.74 (3H, s, H_3-13 or H_3-14). The mixture containing **6** and **7** was dissolved in diethyl ether (2 μg/μL) and directly analysed by GCMS under temperature gradient (from 200 °C to 290 °C in 10 min.) to give **6** [R_t 3.80 min, EIMS *mz* 334 (10, M-AcOH), 274 (15, M-2 AcOH), 259 (15), 232 (100)] and **7** [R_t 8.05 min, EIMS *mz* 292 (15, M-AcOH), 274 (10, M-AcOH-H_2O), 250 (15, M-AcOH-CH_2CO), 232 (100, M-2 AcOH)].

Conversion of 1 and 2 to euryfuran (5)

-According to the procedure reported in ref. 4, a small amount (ca. 0.5 mg) of **1** or **2** was absorbed on catalytic silica and heated at 100 °C for 5 min. Then, the silica was loaded onto a short column and eluted with petroleum ether (5 mL) and then with Et_2O (5 mL). The collected fractions were then chromatographied on TLC (Petroleum ether 100%) and **5** was detected by spraying with Ehrlich's reagent. The two fractions were then added to the scintillation cocktail and the residual radioactivity was counted for five minutes.

Conversion of drimane esters (1) and 7-deacetoxyolepupuane (2) into the diol 8

-The fractions containing the drimane esters (**1**) and 7-deacetoxyolepupuane (**2**) were reduced by $NaBH_4$ in MeOH. In a typical procedure, the terpene (ca 0.7 mg each) was dissolved in 0.6 mL MeOH and reacted with $NaBH_4$ (tip of spatula) at room temperature for 3 h. The excess of reagent was destroyed by 1N HCl (2 mL) and the reaction mixture was diluted with brine (2 mL) and extracted with EtOAc (3 x 2 mL). The organic solvent was removed at reduced pressure and the residue fractionated by SiO_2 column eluant (100% $CHCL_3$, 18 mL) to afford the diol **8**.

Compound 8. colorless powder, $C_{15}H_{28}O_2$; ν_{max} (liquid film) 3325 (br) cm^{-1}; $[\alpha]_D$ = + 16.6 (*c* 0.1 , MeOH); ^{1}H-NMR ($CDCl_3$) δ 3.94 (1H, dd, *J* = 10.4 and 6.2 Hz, H-12a), 3.83 (1H, dd, *J* = 10.4 and 5.4 Hz, H-11a), 3.75 (1H, t, *J* = 10.4, H-11b), 3.60 (1H, dd, *J* = 10.4 and 7.0 Hz, H-12b), 2.20 (1H, q, *J* = 6.0 Hz, H-8α), 1.91 (1H, bd, *J* = 15.1 Hz, H-7β), 1.65-150 (6H, m), 1.39 (1H, m, H-2β), 1.27 (1H, m, H-6β), 1.16 (1H, ddd, *J* = 15.4, 15.4 and 2.7, H-3α), 1.02 (1H, bddd, *J* = 15.8, 15.8 and 1.0 Hz, H-1α), 0.89 (1H, m, H-5α), 0.88 (3H, s, H_3-14), 0.85 (3H, s, H_3-13 or H_3-15), 0.80 (3H, s, H_3-15 or H_3-13).EIMS *m/z* (%) 240 (M^+,4), 222 (M-H_2O, 8), 207 (M-H_2O-CH_3, 20), 69 (100). HREIMS *m/z* 240.2094 ($C_{15}H_{28}O_2$ required 240.2089). Part of the column fractions was also added to the scintillation cocktail and the residual radioactivity was counted for five minutes.

GC-MS analysis of fatty acids

-Fatty acids linked to glyceryl or drimanic moieties were converted to fatty acids methyl esters (FAME) by a base catalysed trans-esterification with Na_2CO_3 in dry MeOH. In brief, glyceride fractions were transferred to graduated screw-top vials and treated with Na_2CO_3 (5 mg) in dry MeOH (1mL). The reaction vials were heated at 40° C for 3 h and then the organic solvent was reduced to a small volume. The solutions were transferred to a separatoring funnel and a saturated solution of NaCl (4.5 mL) was added. The mixture was extracted with Et_2O (3 x 3 mL) and the organic layer was recovered, reduced to a small volume and analysed by GC-MS under temperature gradient (from 120 °C to 260 °C in 70 min.). Part of the Et_2O soluble material from the methanolysis of **1** was added to the scintillation cocktail and the residual radioactivity was counted for five minutes.

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