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THE CHEMICAL DEFENSE OF NUDIBRANCH MOLLUSCS

STRUCTURE, BIOSYNTHETIC ORIGIN AND DEFENSIVE PROPERTIES OF TERPENOIDS FROM THE DORID NUDIBRANCH *DENDRODORIS GRANDIFLORA*

G. CIMINO, S. DE ROSA, S. DE STEFANO, R. MORRONE and G. SODANO*
Istituto per la Chimica di Molecole di Interesse Biologico del C.N.R., Via Toiano n. 6, 80072, Arco Felice, Napoli, Italy

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Abstract - Nudibranch molluscs, apparently defenseless against potential predators, possess secondary metabolites localized on the body wall which help them to escape from predation. These metabolites are largely of dietary origin; in one case the biosynthetic ability of a nudibranch to elaborate its own chemical defense has been shown.

From the mantle extracts of *Dendrodoris grandiflora* polygodial (**1**) and 6β-acethoxyolepupuane (**4**), a new sesquiterpene triacetate, was isolated. These two drimane sesquiterpenoids, both endowed with antifeedant properties, are biosynthesized *de novo* by the nudibranch.

Extracts from the digestive glands of the same nudibranch yielded the previously known sesquiterpene esters **3**, microcionin-1, -2, -3, -4 (**7–10**), fasciculatin (**11**), furospongin-1 acetate (**12**), a new C-21 furanoterpene **14** and a mixture of new prenylated chromanols **17**. All these compounds, with the exception of **3**, appear to be of dietary origin.

The structures of the new compounds were determined by spectral and chemical means.

INTRODUCTION

The nudibranchs are molluscs belonging to the subclass Opistobranchia, which is characterized by the evolutionary loss of the shell.[1] In spite of the absence of the most characteristic moluscan organ of mechanical defense, few predators of these molluscs are known.[2] Many explanations,[3] including structural factors, defensive behavior and chemical secretions, have been given to account for the recognized ability of these molluscs to escape from predators.

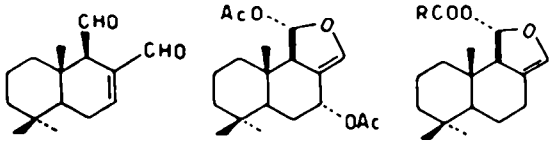
The presence of chemical secretions, stored in skin glands and released when the animal is molested, is of particular interest since it could be related to the excellent taste capability of fish[4] to explain why opistobranchs fed to hungry fishes are almost invariably refused.[5]

Thompson[6] established the occurrence of strong inorganic acid; fluids in several opistobranch molluscs and presented strong circumstantial evidence that these acid secretions function as a deterrent to predators.

More recently, several organic natural products possessing toxic or antifeedant properties have been isolated from opistobranch molluscs, especially from nudibranchs belonging to the suborder *Doridacea*.[7-14] The term "antifeedant" refers usually to the fact, shown by the appropriate bioassay, that marine and freshwater fish refuse food treated with these substances, which presumably taste extremely unpleasant.

Working with Mediterranean nudibranchs[13] we were able to find both toxic and antifeedant compounds. Of special interest is the finding of polygodial (**1**) in the dorid nudibranch *Dendrodoris limbata*, since it is known that this compound tastes very hot to the human tongue[15] and affects also the taste sense of insects.[16] Therefore, polygodial or similar substances should be responsible for the "peppery" taste to the human tongue exhibited by several secretions[17] and, accordingly, may act similarly on the taste sense of fish. A tentative explanation for the mechanism of action of polygodial *in vivo* has also been reported.[18]

Later, polygodial has been detected[14] in other porostome nudibranchs, in which cooccurs with the related sesquiterpene olepupuane (**2**).



1 2 3

Polygodial is localized in the mantle of *Dendrodoris limbata*, while it is absent in the extracts of digestive glands of the same animal, from which only a mixture of the biologically inactive esters **3** was isolated. This finding indicates that compounds useful for a defensive strategy should be present in the areas of the skin, which would be first encountered by an inquisitive predator.

Many attempts have been made in order to localize the defensive substances. Nudibranchs of the genus *Phyllidia* release a voluminous mucus, in which toxic isocyanosesquiterpenes of dietary origin are

present.[12,13] When the secretion is not evident and the nudibranchs are large enough, the mantles can be easily separated from the digestive glands and extracted separately.[8,13] Otherwise, small nudibranchs were immersed whole in an appropriate solvent: it is assumed[19] that the major metabolites obtained in this fashion constitute the major components of a defensive secretion.

Among the many fascinating questions which still remain after localization, isolation and identification of the defensive substances, is the question of the orign of the toxin or repellent. The dietary origin of many metabolites found in nudibranchs has already been demonstrated.[8,9,11-13,19-23] Nudibranchs feed mainly on sessile organisms, some of which, especially sponges, are said to be repellent to most other animals. Nudibranchs are capable of sequestering from their prey toxic or antifeedant metabolites and of using them as defense allomones. However, in the case of *Dendrodoris limbata* it has been demonstrated[24] that the nudibranch itself is capable of *de novo* biosynthesis of polygodial (**1**), its terpenoid antifeedant, thus opening the possibility of performing biosynthetic labelling experiments on some of the interesting dorid metabolites for which no dietary origin has yet been found.

Studies of Dendrodoris grandiflora

Dendrodoris grandiflora is a large nudibranch (3 × 5 cm average) belonging to the family Dendrodoridae of the suborder *Doridacea*. Specimens of *D. grandiflora* were collected in the bay of Naples in May 1981 (23 animals), April 1982 (27 animals) and May 1983 (7 animals). The 1983 collection was used for the incorporation experiments.

We reported earlier,[23] working with two animals only, that the digestive glands of the nudibranch contained fasciculatin (**11**) as the sole component. Examination of the 1981-83 collections allowed a more detailed analysis, showing that more products were present, their relative ratio being almost the same in each collection.

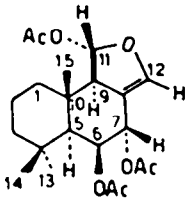
The animals were carefully dissected and the mantles and the hepatopancreas extracted separately. The extracts were chromatographed on silica gel.

Mantle extracts. The mantle extracts contained predominantly polygodial (**1**; *ca* 0.15 mg/animal), the new compound 6β-acethoxyolepupuane (**4**; *ca* 0.5 mg/animal), fats, sterols and trace amounts of the compounds present in the digestive glands.

The structure of **4** was determined as follows. The mass spectrum did not show a molecular ion; the peak at highest mass occurred at *m/z* 334 ($M^+ - CH_3COOH$); from this ion two successive losses of acetyl groups, as ketene and acetic acid, occur (*m/z* 292, 274, 250, 232). Compound **4** is a triacetate since in the ^{1}H-NMR spectrum (C_5D_5N) three acetyl methyls are present at δ 1.96, 2.01 and 2.03.

Comparison of ^{1}H- and ^{13}C-NMR spectral data of **4** with those of olepupuane (**2**)[14] suggests that **4** differs from **2** in having an additional acetyl group. Spin decoupling experiments suggested partial structure **a** (■ denotes quaternary carbons): irradiation of the triplet at δ 5.63 (H-6; J = 2.2 Hz) caused the doublets at δ 1.67 (H-5; J = 2.2 Hz) and 5.77 (H-7; J = 2.2 Hz) to become singlets. The decoupling experiments also indicated that the methine proton at δ 2.93 (H-9, m) was coupled with two doublets at δ 6.89 (J = 2.0 Hz) and 6.87 (J = 1.7 Hz), which were assigned to the olefinic and acetal protons (H-11 and H-12).

Therefore it can be deduced that the gross structure of **4** is 6-acethoxyolepupuane. $LiAlH_4$ reduction of **4** afforded a mixture of products, while $NaBH_4$ reduction slowly yielded a single product, whose ^{1}H-NMR spectrum, compared with the spectra of similar compounds,[25] suggests structure **5** for the new diol, without stereochemical implications. In particular, the 8-CH_2OH group resonates as an AB quartet (J = 12.5 Hz) with signals centered at δ 4.39 and 4.03, while the 9-CH_2OH protons resonate as the AB part of an ABX system with signals centered at δ 3.94 (dd, J = 2.5 and 11 Hz) and 3.78 (dd, J = 8 and 11 Hz). Olepupuane on reduction with $LiAlH_4$ behaves similarly,[14] affording diol **6**.



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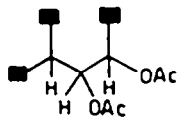

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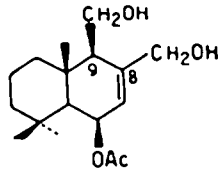

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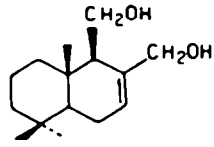

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Table 1. Nuclear Overhauser enhancements observed in the ^{1}H-NMR spectrum of 6β-acethoxyolepupuane (**4**; C_5D_5N; NOE difference spectra)

Atom	shift (δ)	NOE
H-5	1.67	←
H-9	2.93	←
H-7	5.77	←
H-12	6.89	←
H-11	6.87	←
CH_3-10	1.16	←

As far as the stereochemistry of **4** is concerned, a *trans* AB ring junction was suggested by the chemical shifts of the appropriate carbons in the ^{13}C-NMR spectrum, particularly of the C-10 methyl group[26] (δ 16.3).

Furthermore, several positive nuclear Overhauser enhancements were observed (Table 1) allowing the determination of the relative stereochemistry at C-7, C-9 and C-11 as depicted in **4**. Finally, the 6β-acethoxy configuration was deduced from the observation that H-6 resonates in the ^{1}H-NMR spectrum as a triplet having the same small coupling constant (J = 2.2 Hz) with both H-5 and H-7: these values are compatible with those calculated by means of the Karplus equation[27] only when H-6 is in an equatorial configuration.

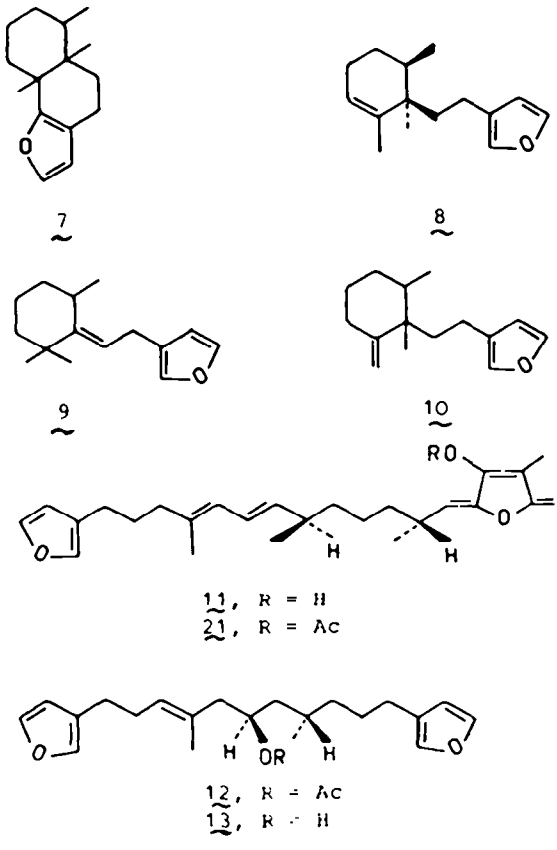
The combined evidence led to the relative stereochemistry of **4** as 6β-acethoxyolepupuane and, accordingly, to the relative stereochemistry of its reduction product **5**.

In a feeding inhibition bioassay,[13] 6β-acethoxyolepupuane (**4**) is active at 40 μg/cm^2 of food pellets, inducing in the freshwater fish *Carassius auratus* the immediate rejection of the treated food. The antifeedant properties of polygodial, which were more marked, have already been reported.[13]

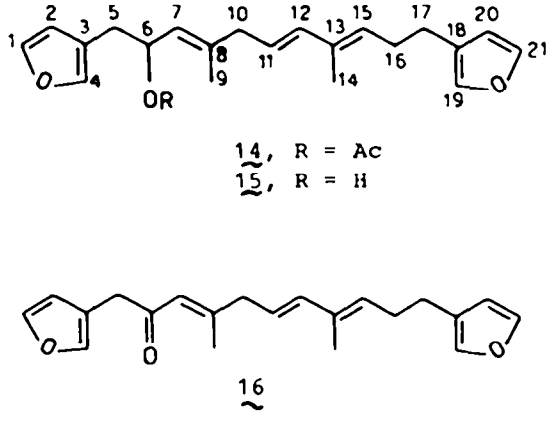
Hepatopancreas extracts. The extracts of the digestive glands yielded the known sponge metabolites microcionin-1[28] (**7**; *ca* 0.25 mg/animal), microcionin-2 (**8**; *ca* 1 mg/animal), microcionin-3 (**9**; *ca* 2.3 mg/animal), microcionin-4 (**10**; *ca* 1 mg/animal) and fasciculatin[29] (**11**; *ca* 2.5 mg/animal), the esters **3** (*ca* 5 mg/animal) previously found in the digestive gland of *Dendrodoris limbata*[30] and in other dorid nudibranchs,[14] and, in addition, three new compounds: the acetyl derivative **12** (*ca* 0.3 mg/animal) of the sponge metabolite furospongin-1 (**13**),[31] the C-21 furanoterpene **14** (*ca* 0.9 mg/animal), and the mixture of prenylated chromanols **17** (*ca* 6 mg/animal).

The structures of **3**, **7**, **8**, **9**, **10**, **11** and **12** were determined by comparison of their spectral properties (^{1}H-NMR and MS) with those previously reported.

The new C-21 furanoterpene **14** has a molecular composition of $C_{23}H_{28}O_4$ as determined by HRMS. The presence of an acetyl group in the molecule was suggested by the prominent loss of acetic acid in the mass spectrum (*m/z* 308), by IR (ν_{max} 1730 cm^{-1}) and ^{1}H-NMR (δ 1.94, 3H, s) data. The ^{1}H-NMR spectrum suggested the presence in the molecule of two β-monosubstituted furan rings (singlets at δ 7.36, 7.34, 7.33, 7.19, 6.48 and 6.25). Compound **14** contains a diene chromophore (UV λ_{max} 231, ϵ 14,500) whose presence is confirmed by the ^{1}H-NMR spectrum: an isolated ABX_2 system, with signals centered at δ 6.22 (d, 1H, J = 15.5 Hz), 5.84 (dt, 1H, J = 15.5 and 6.5 Hz) and 2.80 (d, 2H, J = 6.5 Hz), which were assigned to the protons on C-12, C-11 and C-10, respectively. The chemical shifts of H-11 and H-12 suggesting that this double bond is part of the diene chromophore. The chemical shift and the multiplicity of the protons of C-10 require that C-8 should be quaternary and a double bond between C-7 and C-8 should be present. Two 3H singlets at δ 1.64 and 1.72 were assigned to the vinyl methyls on C-8 and C-13, while the protons on C-7 and C-15 resonate as an unresolved multiplet at δ 5.22. The remaining features of the ^{1}H-NMR spectrum include an A_2B_2 system with resonances centered at δ 2.43 and 2.30 and a doublet at δ 2.20 coupled with the CHOAc proton (δ 5.65; apparent q J = *ca* 7.5 Hz).



7 **8** **9** **10** **11**, R = H; **21**, R = Ac **12**, R = Ac; **13**, R = H



14, R = Ac; **15**, R = H

16

All these data allowed assignment of **14** to the new C-21 furanoterpene. With the position of the remaining –OAc group yet to be determined.

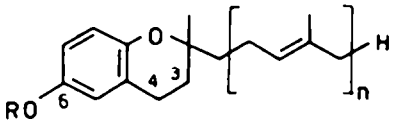
Hydrolysis of **14** yielded the free alcohol **15**, M$^+$ 326. In the ^{1}H-NMR spectrum of **15** (500 MHz) the H-7 and H-15 protons resonate as well separated signals: a doublet at δ 5.23 (J = 7.8 Hz) and a triplet at δ 5.27 (J = 6.8 Hz). Decoupling experiments showed that the doublet at δ 5.23 was coupled with the CHOH proton at δ 4.44 (apparent q J = *ca* 7.5 Hz) and, in addition, long-range coupled with the

protons at δ 2.82 (C-10 protons; irradiation of this signal caused a distinct sharpening of the doublet at δ 5.23). Thus the oxygenated function should be at C-6 in **14** and **15**.

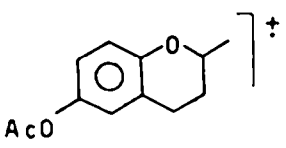
Confirmation was achieved by oxidation of **15** to a ketone **16**, whose UV spectrum lacks absorption maxima around 275 nm, the expected value if the CO group was at C-16.

Furanoterpenes containing 21 C atoms are typical sponge metabolites,[32] **14** representing a further variant of those previously described.

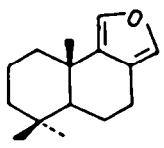
The mixture of chromanols **17**, isolated as an oil giving a positive Pauly reagent test (blue color), has a UV absorption of 297 nm bathochromically shifted by addition of alkali to 312 nm (*cf* chromazonarol).[35]



17, R = H; n = 1-6
18, R = Ac; n = 1-6



19, m/z 205



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The ^{1}H-NMR spectrum showed three low field protons at δ 6.66 (d, J = 8.8 Hz), 6.57 (dd, J = 8.8 and 2.6 Hz) and 6.54 (d, J = 2.6 Hz, long range coupled with the benzylic methylene) whose *ortho* and *meta* couplings clearly established the position of the substituents on the aromatic ring.

The remaining features of the spectrum closely resemble those of all-*trans*-tocotrienols:[33] a triplet (J = 6.8 Hz) at δ 2.70 was assigned to the benzylic methylene; a 3H singlet at δ 1.69 to the *cis* Me group of the last isoprene unit; a singlet at δ 1.60 accounted for the *trans* vinyl methyls and the 3H singlet at δ 1.28 was assigned to the methyl group linked to the C bearing the O of the pyran ring.

These data suggest that **17** possess a prenylated 2-methyl-3,4-dihydro-2H-1-benzopyran-6-ol structure.

Acetylation of **17** afforded a mixture of monoacetates **18**. Gas-chromatography/mass spectrometry of this mixture revealed that it had six components of molecular weights of 628, 560, 492, 424, 356 and 288, in the approximate ratio of 1:14:2:8:24:26.

The individual components showed the same fragmentation pattern: loss of the acetyl group as 42 m.u., loss of the first prenyl unit as 69 m.u. and loss of the successive prenyl units as 68 m.u.; the base peak occurs in all spectra at m/z 205 (**19**).

Prenylated hydroquinones have been reported from sponges,[34] but their cyclized 3,4-dihydro-2H-1-benzopyran derivatives (chromanols) were not previously encountered, a part from some chroman-sesquiterpenoids.[35,36] Prenylated chromanols, carrying methyl substituents on the aromatic ring, have been isolated from corals[37] and from *Sargassum tortile* (alga), where they acted as a symbiont attractant.[38]

Table 2. Radioactivity found in the metabolites of *Dendrodoris grandiflora* and in their derivatives after each purification step (see Experimental section for purification procedures). A total of 14 μCi (30.8 × 10^6 dpm) of [2-^{14}C] mevalonic acid–dibenzylethylene-diamine salt was injected into seven specimens.

Compound	1st purification		2nd purification		3rd purification	
	mg	dpm/mg	mg	dpm/mg	mg	dpm/mg
polygodial (1)	ca 1	68 0[a]	7	[illegible][a]	4	650[a,b]
6β-acethoxyolepupuane (4)	8	3025	12	2305[c]	8	2415[c,d]
esters (2)	68	1897	54	1720		
				furan from 2 (20)	22	1496[e,f]
			fatty acids from 2		25	372[e,f]
microcionins (7-10)	25[g]		2.5[h]	43		
fasciculatin (11)	42	132	25	55[i]		
C-21 furanoterpene (18)	9	844	4	156[j]		
chromanols (17)	87	3230	82	115	71	64[k]

[a] Polygodyal (1) was diluted to 10 mg with unlabelled material; the reported values of radioactivity are calculated taking into account the original weight.

[b] Diol 6.

[c] 6β-acethoxyolepupuane (4) was diluted to 20 mg with unlabelled material; the reported values of radioactivity (dpm/mg) are calculated taking into account the original weight.

[d] Diol 5.

[e] The unresolved mixture of the four microcionins (7-10) was isolated after the first purification step.

[f] Microcionin-1 (7).

[g] Acetate 21.

[h] Free alcohol 15.

[i] Acetates 18.

Origin of the metabolites found in Dendrodoris gradiflora

D. grandiflora represents an ideal case for checking the origin of the metabolites since it contains drimane sesquiterpenes, for which a biosynthetic ability can be suspected in analogy with the related findings with *D. limbata*,[24] along with several terpenoids for which a dietary origin seems likely, because some were previously found in sponges (7–11) and others (12, 14, 17) represent minor variants of sponge metabolites.

Therefore it could be predicted that incorporation experiments with an appropriate labelled precursor would result in the recovery of radioactive 1, 3 and 4, while the remaining metabolites should be devoid of radioactivity.

The chosen precursor, [2-^{14}C] mevalonic acid–dibenzylethylenediamine salt, was injected into the hepatopancreas of 7 specimens (1983 collection; *ca* 2μCi/animal) and after 24 hr the metabolites were extracted, purified and transformed to their derivatives as reported in the Experimental section. The minor metabolites were diluted with unlabelled material prior to subsequent purifications in order to achieve constant specific radioactivity (dpm/mg). Unlabelled furospongin-1 acetate (12) was not available and therefore this metabolite, isolated impure in only minute amount (2.5 mg) from the incorporation experiment, was not further investigated.

Radioactivity of Ke compounds was measured after each purification step; the results are reported in Table 2.

As expected, the drimane sesquiterpenes (1, 3, 4) were found substantially labelled, indicating that *D. grandiflora* is capable of biosynthesizing these compounds.

Conversely, the radioactivity associated with the other terpenoids (7–10, 11, 14, 17) dropped considerably through the purification steps reaching values slightly above background (30–40 dpm). Therefore the radioactivity associated with these compounds after the first purification must be due to impurities.

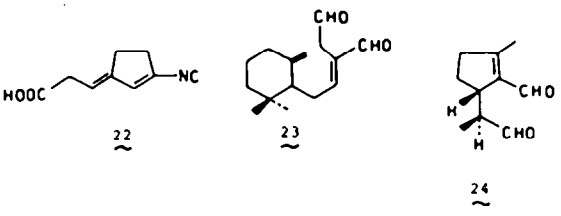
These results support the view that *D. grandiflora* sequester the terpenoids found in the digestive glands from sponge preys, with the exception of 3, which may be considered a product of further metabolism of polygodial (1).[24]

CONCLUSION

The secondary metabolites so far isolated from nudibranchs, for which toxic or antifeedant properties were established, fall roughly into three groups: isocyanosesquiterpenes,[8,12,13] furanoterpenes[8,9,11,13] and sesquiterpenes with a drimane skeleton.[9,13,14] The first two groups appear to be of dietary origin (sponges), while the drimane sesquiterpenoids seem to be biosynthesized *de novo* by the nudibranchs.

Dendrodoris grandiflora constitutes a paradigmatic case since this nudibranch contains dietary furanoterpenes, which could be potentially distasteful, only in the digestive gland, while drimane sesquiterpenes, which constitute the actual defense, have been found in the dorsum and are biosynthesized by the animal.

Finally, it appears that compounds of the three groups above, or compounds having a structural similarity, may display antifeedant properties towards organisms other than fish. The isonitrile acid 22 has recently been isolated from cultures of the fungus *Trichoderma hamatum* and is reported to be responsible for poor ruminant growth in permanent pasture.[41]



22 23 24

Polygodial (1) and related dialdehydes possess antifeedant activity against African army worms.[16] Interestingly, other dialdehydes, such as 23[42] and 24[43] are widespread in the defensive secretions of many insects, suggesting that similar defensive strategies are operating among opistobranch molluscs and insects.

EXPERIMENTAL

Mass spectra were determined on AEI MS-30 and AEI MS-902 spectrometers. NMR spectra were recorded on Varian XL-100, Bruker WH-270 and Bruker WM-500 SB instruments. IR spectra were recorded on a Perkin–Elmer 137 E spectrophotometer. UV spectra were measured with a Bausch & Lomb Spectronic instrument. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. Glc's were performed with a Carlo Erba Fractovap model GV instrument and with a Carlo Erba Fractovap model 2900 instrument equipped with capillary columns.

Radioactive counts were determined in a Tri Carb liquid scintillation counter (Packard PRIAS, model PLD) equipped with an absolute radioactivity analyzer; the quenching was corrected by external standardization. The samples (0.5–2 mg) were dissolved in Insta-Fluor II (Packard) scintillation counting fluid.

Extraction and isolation procedures. Three collections of *D. grandiflora* were made in the bay of Naples: May 1981 (23 animals), April 1982 (27 animals), May 1983 (7 animals). The first two collections were used for identification of the metabolites; the third for the incorporation experiments with [2-^{14}C] mevalonic acid.

The isolation procedure of the 1982 collection (27 animals) is reported. The animals were dissected and the mantles and the digestive glands were extracted separately.

Mantle extracts. The mantles were extracted with acetone (100 ml × 3) at room temp for one day; after concentration *in vacuo* the aqueous residue was extracted with diethyl ether (3 × 50 ml). The combined ethereal extracts were taken to dryness to give 305 mg of an oil which was chromatographed on a silica gel column (50 × 1.5 cm) using light petroleum and increasing amounts of diethyl ether. The fractions (47 mg) containing polygodial (1) and 6β-acethoxyolepupuane (4) were rechromatographed on a shorter column of silica gel (40 × 1 cm dia.) eluted with benzene–diethyl ether 9:1 to give polygodial (1; 4 mg) identified by comparison of its physical properties with those previously reported,[40,25] and 6β-acethoxyolepupuane (4; 13 mg).

6β-acethoxyolepupuane (4): $[\alpha]_D$ − 118.7° (*c* 1.3, $CHCl_3$); IR (liquid film) 2920, 1740, 1360, 1220 cm^{-1}; ^{1}H-NMR (C_5D_5N) δ 6.89 (d, 1H, J = 2.0 Hz), 6.87 (d, 1H, J = 1.7 Hz), 5.77 (d, 1H, J = 2.2 Hz), 5.63 (t, 1H, J = 2.2 Hz), 2.93 (m, 1H), 2.03 (s, 3H), 2.01 (s, 3H), 1.96 (s, 3H), 1.67 (d, 1H, J = 2.2 Hz), 1.16 (s, 3H), 1.00 (s, 3H), 0.97 (s, 3H); ($CDCl_3$) δ 6.49 (d, 1H, J = 1.8 Hz), 6.45 (d, 1H, J = 2.1 Hz), 5.30 (d + t, 2H), 2.57 (m, 1H), 2.10 (s, 6H), 2.05 (s, 3H), 1.48 (d, 1H, J = 2.2 Hz), 1.10 (s, 3H), 1.00 (s, 3H),

0.98 (s, 3H); ^{13}C-NMR ($CDCl_3$) δ 169.6, 169.5, 169.2 (acetyl carbonyls), 141.9 (C-12), 109.6 (C-8), 98.4 (C-11), 70.4 (C-6), 66.4 (C-7), 60.0 (C-9), 50.2 (C-5), 44.1 and 42.0 (C-1 and C-3), 37.5 (C-10), 33.5 (C-4), 33.3 (C-13) 23.6 (C-14), 21.2 (acetyl methyls), 18.5 (C-2), 16.3 (C-15); mass spectrum *m/z* 334 (M^+–AcOH, 5%), 292 (M^+–AcOH–CH_2CO, 4%), 274 (M^+–2AcOH, 13%), 259 (11%), 250 (M^+–AcOH–$2CH_2CO$, 14%), 232 (M^+–2AcOH–CH_2CO, 100%), 217 (M^+–2AcOH–CH_2CO–CH_3, 69%).

Reduction of 6β-acethoxyolepupuane (**4**) *with* $NaBH_4$. $NaBH_4$ (10 mg) was added to a soln of **4** (4 mg) in MeOH (3 ml). After 3 hr at r.t. the soln was acidified with AcOH and partitioned between water and diethyl ether (3 × 10 ml). The ethereal extracts were evaporated and the crude product purified by preparative TLC on SiO_2 plate (benzene–diethyl ether 4:6) to yield, the diol **5** (2 mg); ^{1}H-NMR ($CDCl_3$) δ 5.90 (m, 1H), 5.59 (m, 1H), 4.39 (d, 1H, J = 12.5 Hz), 4.03 (d, 1H, J = 12.5 Hz), 3.94 (dd, 1H, J = 2.5, 11 Hz), 3.78 (dd, 1H, J = 8, 11 Hz), 2.04 (s, 3H), 1.09 (s, 3H), 1.04 (s, 3H), 0.99 (s, 3H); mass spectrum *m/z* 218 (M^+–CH_3COOH–H_2O, 100%).

Hepatopancreas extracts. The digestive glands were extracted with acetone (150 ml × 3) at room temp for one day; after concentration *in vacuo* the aqueous residue was extracted with diethyl ether (3 × 100 ml). The combined ethereal extracts were taken to dryness to give 2.183 g of an oil which was chromatographed on a silica gel column (80 × 2.5 cm) using light petroleum and increasing amounts of diethyl ether. The fractions eluted with petrol, containing the microcionins (**7**–**10**; 140 mg), were rechromatographed on $AgNO_3$-treated silica gel as previously described[28] to yield **7**, (7 mg), **8** (27 mg), **9**, (62 mg) and **10** (28 mg) identified on the basis of their mass and ^{1}H-NMR spectra. Material eluted with 5% diethyl ether in petrol was rechromatographed on a SiO_2 column using n-hexane–diethyl ether 95:5 as eluant to obtain the esters **3** (137 mg), whose ^{1}H-NMR spectrum is identical with those reported for other esters having the same sesquiterpenoidic moiety.[14,30]

Further elution with 5% diethyl ether in petrol yielded fractions containing **12** and the new **14**. Fractions containing **12** were rechromatographed on a SiO_2 Pasteur pipette (petrol–benzene 6:4) to yield 7 mg of pure material, $[\alpha]_D$ + 9.1, identified by comparison of its ^{1}H-NMR spectrum with the spectrum of the acetate of the naturally occurring furospongin-1.[31]

Fractions containing the new C-21 furanoterpene were rechromatographed on two SiO_2 plates (petrol–benzene 1:1) yielding 24 mg of **14**.

Material eluted with 40% diethyl ether in petrol was rechromatographed on a SiO_2 column using benzene diethyl ether 95:5 to obtain the mixture of chromanols **17** (161 mg).

The material eluted with petrol–diethyl ether 3:7 contained pure **11** (72 mg) identified by comparison of its spectral properties with those previously reported.[29]

Thermolysis of the sesquiterpene esters **3**[30]

Compound **3** (50 mg) was dissolved in n-hexane (5 ml) and absorbed on silica gel (100 mg). The mixture was heated on a steam bath for 10 min; after cooling, the silica gel was eluted with n-hexane to give **20** (19 mg; current name: euryfuran), identical in all respects with an authentic sample, and then with diethyl ether to give a mixture of fatty acids purified on a silica gel column (light petroleum–diethyl ether 7:3; 23 mg yield). The fatty acids were methylated with CH_2N_2 and the resulting methyl esters chromatographed on a SiO_2–$AgNO_3$ (12%) column (petrol–diethyl ether from 9:1 to 1:1) to obtain saturated (7 mg), mono-unsaturated (4 mg) and di-unsaturated (1 mg) fatty acids. The single fractions were analyzed by glc on a 20 mt. DEGS capillary column at 122°. Saturated: i-13, 3.7%; i-14, 2.9%; a-14, 3.7%; n-14, 2.7%; i-15, 12.9%; a-15, 9.9%; n-15, 3.4%; i-16, 3.8%; a-16, 8.7%; n-16, 23.6%; i-17, 3.9%; a-17, 4.8%; n-17, 5.6%; n-18, 10.3%. Mono-unsaturated: 12:1, 13.7%; unk. 7.8%; 16:1, 23.2%; unk. 13.7%; 17:1, 3.6%; unk. 8.4%; unk. 10.3%; 18:1, 19.2%. Di-unsaturated: 13:2, 43.1%; unk. 11.5%; 17:2, 7.9%; 18:2, 4.7%; unknowns 15.4%; 18.2%; 9.9%; 7.3%; 6.1%; 20:2, 8.5%; unk. 6.6%; 22:2, 1.9%.

C-21 *Furanoterpene* **14**. $[\alpha]_D$ + 12.8 (*c* 2.4, $CHCl_3$); IR ($CHCl_3$) 1730 cm^{-1}; UV λ_{max}(MeOH) 231 (s, ε 14,500) and 223 nm (ε 15,400); ^{1}H-NMR ($CDCl_3$) δ 7.36 (s, 1H), 7.34 (s, 1H), 7.33 (s, 1H), 7.19 (s, 1H), 6.48 (s, 1H), 6.25 (s, 1H), 6.22 (d, 1H, J = 15.5 Hz), 5.84 (dt, 1H, J = 15.5 and 6.5 Hz), 5.65 (apparent q, 1H, J = *ca* 7.5 Hz), 5.22 (m, 2H), 2.80 (d, 2H, J = 6.5 Hz), 2.43 (t, 2H, J = 7.5 Hz), 2.30 (m, 2H), 2.20 (d, 2H, J = 7.8 Hz), 1.94 (s, 3H), 1.72 (s, 3H), 1.64 (s, 3H); mass spectrum *m/z* 368 (M^+, 1%), 308 (M^+–CH_3COOH, 38%), 159 (100%), 97 (55%), 81 (83%); HRMS, obsd. *m/z* 368.1972, $C_{23}H_{28}O_4$ requires 368.1987.

Hydrolysis of **14**

Compound **14** (10 mg) was dissolved in a 5% soln (1 ml) of KOH in MeOH. After 5 hr at room temp the soln was diluted with water (20 ml) and extracted with diethyl ether (15 ml × 3). The ethereal extracts were washed with distilled water (10 ml × 2), dried over Na_2SO_4 and evaporated *in vacuo*. The residue was chromatographed on a SiO_2 Pasteur pipette with petrol–diethyl ether 7:3 to give 7 mg of **15**; ^{1}H-NMR ($CDCl_3$; 500 MHz) δ 7.36 (s, 1H), 7.34 (s, 2H), 7.21 (s, 1H), 6.30 (s, 1H), 6.26 (s, 1H), 6.24 (d, 1H, J = 15.5 Hz), 5.89 (dt, 1H, J = 15.5 and 7.0 Hz), 5.27 (t, 1H, J = 6.8 Hz), 5.23 (d, 1H, J = 7.8 Hz), 4.44 (apparent q, 1H, J = *ca* 7.5 Hz), 2.82 (d, 2H, J = 6.8 Hz), 2.47 (t, 2H, J = 7.5 Hz), 2.30 (m, 2H), 2.16 (d, 2H, J = 6.6 Hz), 1.70 (s, 3H), 1.65 (s, 3H); ^{1}H-NMR (CD_3OD; 270 MHz) δ 7.40 (s, 1H), 7.35 (s, 1H), 7.33 (s, 1H), 7.20 (s, 1H), 6.52 (s, 1H), 6.30 (s, 1H), 6.28 (d, 1H, J = 15.6 Hz), 5.90 (dt, 1H, J = 15.6 and 7.2 Hz), 5.18 (m, 2H), 4.44 (apparent q, J = *ca* 7.3 Hz), 2.83 (d, 2H, J = 7.5 Hz), 2.43 (t, 2H, J = 7.3 Hz), 2.24 (m, 3H), 2.10 (d, 2H, J = 6.7 Hz), 1.68 (s, 3H), 1.63 (s, 3H); mass spectrum *m/z* 326 (M^+, 1.5%), 309 (M^+–OH, 35%), 308 (M^+ H_2O, 9.5%), 176 (22%), 150 (40%), 97 (68%), 81 (100%).

Oxidation of **15**

Compound **15** (3 mg) was dissolved in dry CH_2Cl_2 (1 ml) and 2 mg of pyridinium dichromate were added; the mixture was stirred for 18 hr at r.t. and then diluted with diethyl ether (3 ml), filtered through a SiO_2 Pasteur pipette and evaporated. The residue was purified by preparative TLC (petrol–diethyl ether 7:3) to afford 0.7 mg of the ketone **16**. Mass spectrum, *m/z* 324 (M^+, 11%), 175 (100%), 147 (44%); UV (MeOH), continuous absorption between 300 and 220 nm.

Chromanols **17**. UV λ_{max} (MeOH) 235, 297 nm; λ_{max} (MeOH–NaOH) 312 nm; ^{1}H-NMR ($CDCl_3$) δ 6.66 (d, 1H, J = 8.8 Hz), 6.57 (dd, 1H, J = 8.8 and 2.6 Hz), 6.54 (d, 1H, J = 2.6 Hz), 5.11 (m), 2.70 (t, 2H, J = 6.8 Hz), 1.69 (s, 3H), 1.60 (s, 1.28 (s, 3H).

Acetylation of **17**

The mixture of chromanols (**17**, 40 mg) was dissolved in Ac_2O and two drops of pyridine were added. After 16 hr at r.t. the solvents were removed *in vacuo* and the residue was chromatographed on a SiO_2 column (petrol–diethyl ether 95:5) yielding 35 mg of the mixture **18**. UV λ_{max} (CH_3OH) 288 (s), 282, 227 nm. ^{1}H-NMR ($CDCl_3$) 6.67 (m, 3H), 5.09 (m), 2.76 (t, 2H, J = 6.8 Hz), 2.26 (s, 3H), 1.70 (s, 3H), 1.61 (s), 1.29 (s, 3H). Glc (SE-30 1% on chromosorb W, 2 m, 310°)–mass spectrometry of the mixture **18** showed six peaks M^+'s 628, 560, 492, 424, 356 and 288 in the ratio 1:14:2:8:24:26. The fragmentation pattern was the same for all components; the mass spectrum for n = 5 is reported, as an example: *m/z* 560 (M^+, 17%), 518 (M^+–42, 10%), 491 (M^+–42–69, 12%), 423 (M^+–42–69–68, 16%), 355

(M⁺–42–69–2 × 68, 8%), 287 (M⁺–42–69–3 × 68, 6%), 205 (19, 100%).

Antifeedant bioassays

The feeding inhibition bioassays were performed on 6β-acethoxyolepupuane (4) as previously reported,[13] using *Carassius auratus* as test fish. Used concentrations of 4 on the food pellets were: 200 μg/cm², 100 μg/cm², 40 μg/cm², 15 μg/cm². The minimum active concentration observed was 40 μg/cm².

Incubation with radioactive mevalonic acid

Seven specimens of *D. grandiflora* were placed in aerated seawater (6 L), and 2 μCi of [2-¹⁴C] mevalonic acid–dibenzylethylendiamine salt (New England Nuclear, 50.1 mCi/mmol) in distilled water soln (0.05 ml) was injected into the hepatopancreas of each animal by means of a syringe. After 24 hr the animals were killed by freezing and dissected. Usual work-up yielded 151 mg of ethereal extract from mantles and 970 mg of ethereal extract from digestive glands.

The first purification was achieved using column chromatography as for the corresponding unlabelled extracts. The individual compounds were further purified similarly to the unlabelled material (see above) with the following summarized procedures. After each purification step an adequate amount (0.5–2 mg) of material was used for counting.

Polygodial (1). About 1 mg of 1 was obtained after the first purification step. The total amount was raised to 10 mg by addition of unlabelled material. The compound was further purified (2nd purification) by preparative TLC (benzene–diethyl ether 95:5) to yield 7 mg. Reduction of this material with $NaBH_4$[24] yielded the diol 6 which was purified (3rd purification) by chromatography on a SiO_2 Pasteur pipette (benzene–diethyl ether 1:1; 4 mg yield).

6β-Acethoxyolepupuane (4). Compound 4 (8 mg) was obtained after the first purification step. The total amount was raised to 20 mg by addition of unlabelled material. Column chromatography (2nd purification; benzene–diethyl ether 9:1) afforded 12 mg of pure 4 which was subsequently reduced with $NaBH_4$ to yield the diol 5, again purified (3rd purification) by column chromatography (benzene–diethyl ether 1:1; 8 mg yield).

Sesquiterpene esters (3). Mixed esters (68 mg) were obtained after the first purification. This material was rechromatographed on a SiO_2 column using n-hexane–diethyl ether 98:2 as eluant (2nd purification) to yield 54 mg of esters which were converted by thermolysis in the furan 20 and in the mixture of the corresponding fatty acids. The furan 20 was purified (3rd purification) by column chromatography (n-hexane) to yield 22 mg while the fatty acids were purified by column chromatography using petrol-diethyl ether (7:3) as eluant (25 mg yield).

Microcionins (7–10). After the first purification, the mixture of microcionins 7–10 was recovered. Rechromatography of this mixture on SiO_2-$AgNO_3$ (2nd purification) resulted in the recovery of pure 7 (2.5 mg), while the other 8–10 were obtained slightly impure.

Faciculatin (10). The material obtained after the first purification (42 mg) was acetylated with Ac_2O–pyridine and the acetyl derivative 21[29] purified (2nd purification) by column chromatography (benzene, 25 mg yield).

C-21 *Furanoterpene* (14). The material obtained after the first purification (9 mg) was hydrolyzed with KOH in MeOH and the free alcohol (15) was chromatographed (2nd purification) on a SiO_2 Pasteur pipette (petrol–diethyl ether 7:3; 4 mg yield).

Chromanols (17). The material recovered after the first purification (87 mg) was rechromatographed on a SiO_2 column (2nd purification), using benzene–diethyl ether (95:5) as eluant, to give 82 mg. The purified material was acetylated and the acetyl derivatives 18 chromatographed (3rd purification) on a SiO_2 column (petrol–diethyl ether, 95:5; 71 mg yield).

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REFERENCES AND NOTES

[1]T. E. Thomspon, *Biology of Opisthobranch Molluscs*. I. The Royal Society, London (1976).
[2]T. E. Thompson, *J. Mar. Biol. Ass. U.K.* **39**, 123 (1960).
[3]J. D. Ros, *Oecologia aquatica* **2**, 41 (1976).
[4]J. E. Bardach and J. Atema, The sense of taste in fishes. In *Handbook of Sensory Physiology—IV. Chemical Senses*-2 (Edited by L. M. Beidler), p. 293. Springer-Verlag, New York (1971).
[5]Ref. 1, p. 48.
[6]T. E. Thompson, *J. Mar. Biol. Ass. U.K.* **39**, 115 (1960); T. E. Thompson, *Comp. Biochem. Physiol.* **74A**, 615 (1983).
[7]G. R. Schulte and P. J. Scheuer, *Tetrahedron* **38**, 1857 (1982).
[8]J. E. Thompson, R. P. Walker, S. J. Wrattan and D. J. Faulkner, *Tetrahedron* **38**, 1865 (1982).
[9]J. Hellou, R. J. Andersen and J. E. Thompson, *Tetrahedron* **38**, 1875 (1982).
[10]S. W. Ayer and R. J. Andersen, *Tetrahedron Letters* **23**, 1039 (1982).
[11]G. Schulte, P. J. Scheuer and O. J. McConnell, *Helv. Chim. Acta* **63**, 2159 (1980).
[12]M. R. Hagadone, B. J. Burreson, P. J. Scheuer, J. S. Finer and J. Clardy, *Helv. Chim. Acta* **62**, 2484 (1979).
[13]G. Cimino, S. De Rosa, S. De Stefano and G. Sodano, *Comput. Biochem. Physiol.* **73B**, 471 (1982).
[14]R. K. Okuda, P. J. Scheuer, J. E. Hochlowski, R. P. Walker and D. J. Faulkner, *J. Org. Chem.* **48**, 1866 (1983); we thank Dr. D. J. Faulkner for a preprint of this paper.
[15]I. Kubo and I. Ganjian, *Experientia* **37**, 1063 (1981).
[16]K. Nakanishi and I. Kubo, *Israel J. Chem.* **16**, 28 (1977).
[17]Ref. 1, p. 52.
[18]M. D'Ischia, G. Prota and G. Sodano, *Tetrahedron Letters* 3295 (1982).
[19]J. E. Hochlowski, R. P. Walker, C. Ireland and D. J. Faulkner, *J. Org. Chem.* **47**, 88 (1982).
[20]Y. H. Kim, R. J. Nachman, L. Pavelka, H. S. Mosher, F. A. Fuhrman and G. J. Fuhrman, *J. Nat. Prod.* **44**, 206 (1981).
[21]D. Castiello, G. Cimino, S. De Rosa, S. De Stefano and G. Sodano, *Tetrahedron Letters* 5047 (1980).
[22]G. Cimino, S. De Rosa, S. De Stefano and G. Sodano, *Tetrahedron Letters* 3303 (1980).
[23]G. Cimino, S. De Stefano, S. De Rosa, G. Sodano and G. Villani, *Bull. Soc. Chim. Belg.* **89**, 1069 (1980).
[24]G. Cimino, S. De Rosa, S. De Stefano, G. Sodano and G. Villani, *Science* **219**, 1237 (1983).
[25]A. A. Aasen, T. Nishida, C. R. Enzell and H. H. Appel, *Acta Chem. Scand. B* **31**, 51 (1977).
[26]J. L. Gough, J. P. Guthrie and J. B. Stothers, *J. Chem. Soc. Chem. Commun.* 979 (1972).
[27]N. S. Bhacca and D. H. Williams, *Applications of NMR Spectroscopy in Organic Chemistry*. Holden-Day, San Francisco (1964).
[28]G. Cimino, S. De Stefano, A. Guerriero and L. Minale, *Tetrahedron Letters* 3723 (1975).
[29]F. Cafieri, E. Fattorusso, C. Santacroce and L. Minale, *Tetrahedron* **28**, 1579 (1972).
[30]G. Cimino, S. De Rosa, S. De Stefano and G. Sodano, *Tetrahedron Letters* 1271 (1981).
[31]G. Cimino, S. De Stefano, L. Minale and E. Fattorusso, *Tetrahedron* **27**, 4673 (1971).
[32]L. Minale, Terpenoids from marine sponges. In *Marine Natural Products* (Edited by I. P. J. Scheuer), p. 175. Academic Press, New York (1978).

[33]W. H. Sebrell and R. S. Harris, *The Vitamins*—V, p. 196. Academic Press, New York (1972).
[34]G. Cimino, S. De Stefano and L. Minale, *Tetrahedron* **28**, 1315 (1972); G. Cimino, S. De Stefano and L. Minale, *Experientia* **28**, 1401 (1972).
[35]G. Cimino, S. De Stefano and L. Minale, *Experientia* **31**, 1117 (1975).
[36]P. Djura, D. B. Stierle, B. Sullivan, D. J. Faulkner, E. Arnold and J. Clardy, *J. Org. Chem.* **45**, 1435 (1980).
[37]B. F. Bowden and J. C. Coll, *Austral. J. Chem.* **34**, 2677 (1981).
[38]T. Kato, A. S. Kumanireng, I. Ichinose, Y. Kitahara, Y. Kakinuma, M. Nishihira and M. Kato, *Experientia* **31**, 433 (1975).
[39]The distribution of the radioactivity between the furan **20** and the fatty acids is, as expected, major in **20**. However part of the radioactivity found in the esters **3** after the 2nd purification must be due to a persistent radioactive contamination, since the specific radioactivity (dpm/mg) is too high to account for the value subsequently found in the furan **20**.
[40]C. S. Barnes and J. W. Loder, *Austral. J. Chem.* **15**, 322 (1962).
[41]D. Brewer and A. Taylor, *J. Chem. Soc. Chem. Commun.* 1061 (1979).
[42]R. Baker, P. H. Briner and D. A. Evans, *J. Chem. Soc. Chem. Commun.* 411 (1978).
[43]J. Meinwald, T. H. Jones, T. Eisner and K. Hicks, *Proc. Nat. Acad. Sci. U.S.A.* **74**, 2189 (1977).