Structure and Stereochemistry of Aplyolides A−E, Lactonized Dihydroxy Fatty Acids from the Skin of the Marine Mollusk *Aplysia depilans*

Aldo Spinella,*[,1] Eva Zubía,[2] Eugenia Martînez,[3] Jesus Ortea,[3] and Guido Cimino

Istituto per la Chimica di Molecole di Interesse Biologico, CNR,[4] Via Toiano 6, 80072 Arco Felice (NA), Italy

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The opisthobranch *Aplysia depilans* contains in its dorsum five unprecedented C-16 and C-18 fatty acid lactones (**2**−**6**). Their structures were assigned on the bases of chemical and spectral methods. Lactone **2** is derived by cyclization of 15(*S*)-hydroxyhexadeca-4(*Z*),7(*Z*),10(*Z*),13(*Z*)-tetraenoic acid. The absolute stereochemistry at C-15 was determined by Mosher's method after opening of the lactone ring. Two other lactones (**3** and **5**) result from the cyclization either at C-15 or C-16 of 15,16-dihydroxyoctadeca-9(*Z*),12(*Z*)-dienoic acid. They differ from the remaining pair (**4** and **6**) by the absence of an additional double bond at C-6. *S* absolute stereochemistry of the free carbinols in **3**−**6** was suggested by applying Mosher's method. The same absolute stereochemistry was assigned at all lactonized carbinol centers by isomerization of the lactones by ring opening and subsequent enzymatic cyclization.

Introduction

Opisthobranchs mollusks are soft-bodied marine invertebrates without physical protection.[5] Many chemical studies[6,7] have proved that these mollusks are often chemically protected against their potential predators by small organic molecules. Members of the order Anaspidea (sea hares) have long been the targets of natural product research because they tend to be large and conspicuous in shallow water habitats. Virtually all previous workers have focused on whole animal or digestive gland metabolites, which have shown convincing links to the animals algal diet.[8] If the dietary metabolites are used as defensive allomones, it is necessary to move them from the digestive glands to the surrounding environment either by expelling them into mucus secretions or by transferring them to the most exposed anatomical parts of the animal. However, little is known about the release mechanism used to deter potential predators. Recently, defensive allomones have been isolated from the skin of the Mediterranean *Aplysia fasciata*.[9a] Structurally they are closely related to some unusual degraded steroids found in the skin of the Pacific *Aplysia korodai*.[9b]

In our work with Mediterranean and Atlantic anaspideans we have followed a different approach by carefully dissecting the animal and tracing individual compounds to specific body parts. By using this approach with *Aplysia depilans* we succeeded in isolating five unprecedented macrolactones, aplyolides A−E (**2**−**6**), from the external body parts of the animal. We have further shown that the aplyolides are ichthyotoxic. It is remarkable that lactones of fatty acids possess significant bioactivity. It is worth noting that diterpenoids, e.g., **1**, were isolated from the digestive glands of *A. depilans* which were feeding on the brown alga *Dictyota dicotoma* in the Gulf of Naples,[10,11] while animals from Spain were feeding on the green alga *Ulva lactuca*.

Results and Discussion

Specimens of *A. depilans* were collected in the Atlantic (Asturias, North Spain; Cadiz, South Spain) and Mediterranean (Naples, South Italy) coasts. The mollusks were carefully dissected, separating the internal organs (digestive gland, hermaphrodite gland) from the external parts (mantle, parapodial border). The materials after dissection were extracted with acetone. Evaporation of the solvent under vacuum gave an aqueous suspension which was extracted with diethyl ether. Chromatographic comparison of the extracts from both internal and external parts of the animals showed different patterns. TLC (SiO_2) analysis revealed that some components were exclusively in the extract from the external tissues of the mollusk. The same chromatographic pattern was observed for all analyzed specimens without any influence of the collection site. Purification by HPLC (two SiO_2 Spherisorb columns in series; *n*-hexane/2-propanol 99.5:0.5) yielded five products named aplyolides A (**2**), B (**3**), C (**4**), D (**5**), and E (**6**) (Chart 1).

Aplyolide A (**2**), $[\alpha]^{25}_D = -57.9°$ ($c = 0.4$, $CHCl_3$), showed an intense carbonyl absorption at 1724 cm^{-1} in the IR spectrum. The HREIMS indicated a molecular formula of $C_{16}H_{22}O_2$. ^1H and ^{13}C NMR spectra showed the presence of four unconjugated double bonds with

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(1) Dipartimento di Chimica, Facoltà di Scienze, Università di Salerno, 84081 Baronissi (SA), Italy.

(2) Departamento de Quìmica Orgànica, Facultad de Ciencias del Mar, Universidad de Càdiz, Apdo. 40, 11510 Puerto Real, Cadiz, Spain.

(3) Departamento de Biologia de Organismos y Sistemas, Universidad de Oviedo, C/J. Arias de Velasco, Oviedo, Spain.

(4) Associated with the National Institute for the Chemistry of Biological Systems (CNR).

(5) Thompson, T. E. *Biology of Opisthobranch Molluscs*, Vol. 1; The Ray Society: London, 1976.

(6) Karuso P. In *Bioorganic Marine Chemistry*, Vol. 1; Scheuer, P. J., Ed.; Springer Verlag: Heidelberg, 1987; pp 31−60.

(7) Cimino, G.; Sodano, G. *Chem. Scr.* **1989**, 29, 389.

(8) Carefoot, T. H. *Oceanogr. Mar. Biol. Ann. Rev.* **1987**, 25, 167. Faulkner D. J. In *Ecological Roles of Marine Natural Products*; Paul, V. J., Ed.; Cornell University Press: Ithaca, 1992; pp 120−129.

(9) (a) Spinella, A.; Gavagnin, M.; Crispino, A.; Cimino G.; Martinez E.; Ortea J.; Sodano G. *J. Nat. Prod.* **1992**, 55, 989. (b) Miyamoto, T.; Higuchi, R.; Komori, T.; Fujioka, T.; Mihashi, K. *Tetrahedron Lett.* **1986**, *27*, 1153.

(10) Minale, L.; Riccio, R. *Tetrahedron Lett.* **1976**, 2711.

(11) Fattorusso, E.; Magno,S.; Mayol, L.; Santacroce, C.; Sica, D.; Amico, V.; Oriente, G.; Piattelli, M.; Tringali, C. *J. Chem. Soc., Chem. Commun.* **1976**, 575.

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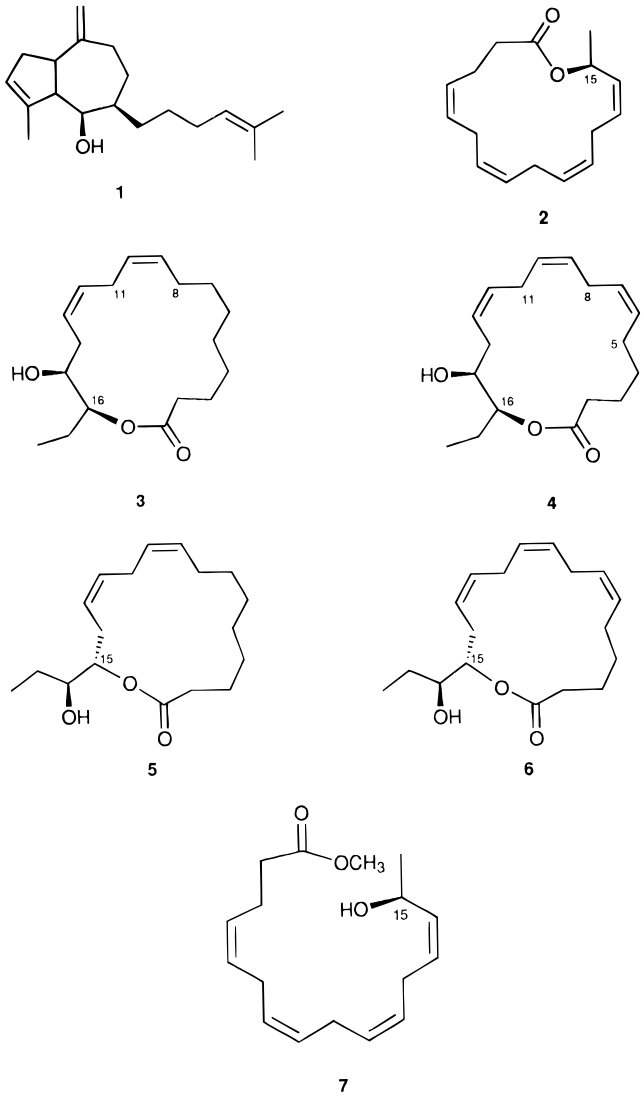
Chart 1



eight olefinic protons resonating from δ 5.36 to 5.45. The ^{1}H NMR spectrum also contained signals attributable to two vicinal methylenes (δ 2.33, H_2-2; δ 2.54, 2.31, H_2-3) and to a methine (δ 5.63, H-15) coupled to a methyl (δ 1.30, H_3-16). The HMQC 2D experiments connected all these protons with carbons resonating at δ 34.7 (C-2), 23.8 (C-3), 67.0 (C-15), and 20.7 (C-16), respectively. Furthermore, the ^{1}H NMR spectrum showed coupled signals at δ 2.97, 2.74, at δ 2.66, 3.02, and at δ 2.69, 3.21 due to methylene protons between two double bonds. All these protons were correctly assigned by some NOE experiments. In fact, irradiation of the signal at δ 3.21 caused enhancement at δ 5.63 (H-15) and 5.45 (H-13), allowing the assignment of the signals at δ 3.21, 2.69 to position 12. Another NOE experiment was performed by irradiating at δ 2.54. In this case clear enhancement at δ 5.36 (H-4), at 2.97 (H-6), and at 2.33 (H-2) was observed. The remaining two signals (δ 3.02, 2.66) were assigned to C-9. An ester function was suggested by the ^{13}C NMR resonance at 172.8 ppm and the IR absorption at 1724 cm^{-1}, linking C-2 to the carbonyl and C-15 to the oxygen. Since the presence of an ester carbonyl failed to complete six unsaturations calculated from the molecular formula, a lactone moiety was postulated. The ^{13}C NMR chemical shifts of the doubly-allylic methylene carbons (δ 25.4, 25.8, 26.2) clearly indicated *Z* stereochemistry of all double bonds.[12] In order to confirm the suggested structure the lactone was opened with Na_2CO_3 in anhydrous methanol resulting in methyl ester **7**.

Aplyolide B (**3**), $[\alpha]^{25}_D = -42.8°$ ($c = 0.2$, $CHCl_3$), had the molecular formula $C_{18}H_{30}O_3$. The IR spectrum showed absorptions due to hydroxyl (3600−3176 cm^{-1}, br) and ester (1722 cm^{-1}) functions. The presence of a secondary hydroxyl group was indicated by a ^{1}H NMR signal at δ 3.71 (H-15) coupled in the HMQC spectrum to the carbon signal at δ 71.3 (C-15). The presence of an ester group was confirmed by the NMR data: δ_c 173.4 (C-1), δ_c 76.7 (C-16), δ_H 4.83 (H-16). The ^{13}C NMR spectrum contained four olefinic signals at δ 124.9 (C-13), 127.7, 130.4, and 131.8 (C-12) coupled in the C,H COSY spectrum with protons at δ 5.41 (1H, m), 5.37 (2H, m), and 5.62 (1H, m). These two double bonds were separated by a methylene whose δ value in the ^{13}C NMR spectrum (26.0 ppm) allowed assignment of *Z* geometry to the double bonds.[12] The ^{1}H−^{1}H COSY spectrum easily connected all protons in the chain from C-8 to C-18. In fact, H-16 was easily linked both to a terminal ethyl (H_2-17, δ 1.76; H_3-18, δ 0.92) and to the oxymethine proton at δ 3.71 (H-15), which in turn was coupled to the allylic protons (H_2-14, δ 2.31, 2.18) near the above described diene system, further coupled to H_2-8 (δ 2.04, 1.97). In addition to the above signals, ^{1}H and ^{13}C NMR spectra showed signals due to six methylenes in a linear alkyl chain. Consequently, aplyolide B (**3**) was a lactonized dihydroxy fatty acid.

Aplyolide C (**4**), $[\alpha]^{25}_D = -26.7$ ($c = 0.7$, $CHCl_3$), had a molecular formula of $C_{18}H_{28}O_3$ which was determined by HREIMS and NMR data (Table 1). Again, the presence of hydroxy and ester groups was shown by IR bands at 3600−3158 cm^{-1} (br) and 1717 cm^{-1}. The ^{13}C NMR spectrum includes one carbonyl signal (δ 173.1, s) assigned to the ester moiety, and after a DEPT experiment, six sp^2 methines, two oxymethines, eight sp^3 methylenes, and one methyl group were clearly detected. Detailed analyses of the ^{1}H−^{1}H and ^{1}H−^{13}C COSY 2D NMR spectra allowed us to construct structure **4** which differs from **3** only in the presence of an additional double bond between C-6 and C-7. The stereochemistry of the double bonds was defined on the basis of the chemical shift of the two bis-allylic methylene carbons.

The other two compounds, aplyolides D (**5**) and E (**6**), were eluted after aplyolide C (**4**). They showed a strong resemblance to aplyolides B (**3**) and C (**4**). In particular, aplyolide D (**5**), $[\alpha]^{25}_D = +28$ ($c = 0.1$, $CHCl_3$), had identical elemental composition ($C_{18}H_{30}O_3$, HREIMS) to that of **3**. The broad IR band centered at 3440 cm^{-1} and a band at 1733 cm^{-1} indicated the presence of hydroxy and ester functionalities. Connectivities were secured by ^{1}H−^{1}H and ^{1}H−^{13}C COSY 2D NMR experiments. Complete NMR data are provided in Table 1. In particular, the oxymethine proton at δ 3.57 (H-16) was coupled to methylene protons at C-17 (δ 1.50), which were further coupled to the terminal methyl group (H_3-18, δ 0.99), while the oxymethine proton H-15, coupled to the allylic methylene protons (δ 2.51, 2.43, H-14), showed a lower field chemical shift (δ 4.96) due to acylation. Therefore, aplyolide D is a 16-membered lactone with C-1 carbonyl linked to C-15 oxymethine.

Aplyolide E (**6**) ($C_{18}H_{28}O_3$, HREIMS; $[\alpha]^{25}_D = +46.3$ ($c = 0.3$, $CHCl_3$); IR 3500 (br), 1729 cm^{-1}) was clearly

(12) Wenkert, E.; Buckwalter, B. L.; Burfitt, I. R.; Gasic, M. J.; Gottlieb, H. E.; Hagaman, E. W.; Schell, F. M.; Wovkulich, P. M. In *Topics in Carbon-13 NMR Spectroscopy*; Levy, G. C., Ed.; Wiley-Interscience: New York, Vol. 2, Chapter 2.

Table 1. NMR Data for Aplyolides

	aplyolide A (**2**)		aplyolide B (**3**)		aplyolide C (**4**)		aplyolide D (**5**)		aplyolide E (**6**)	
position	^{1}H δ, m	^{13}C δ, m	^{1}H δ, m	^{13}C δ, m	^{1}H δ, m	^{13}C δ, m	^{1}H δ, m	^{13}C δ, m	^{1}H δ, m	^{13}C δ, m
1		172.8 s		173.4 s		173.1 s		173.4 s		173.2 s
2	2.33 m	34.7 t	2.47 ddd	34.1 t	2.48 ddd	34.3 t	2.31 ddd	33.8 t	2.41 ddd	33.8 t
			2.38 ddd		2.34 ddd		2.38 ddd		2.32 ddd	
3	2.54 m	23.8 t	1.77 m	24.9 t	1.67 m	24.8 t	1.73 m	24.5 t	1.73 m	24.2 t
	2.31 m		1.65 m		1.80 m		1.62 m		1.63 m	
4	5.36 m	130.3[a] d	1.37 m	27.3 t	1.46 m	28.7 t	1.34 m	28.0[a] t	1.42 m	28.0 t
5	5.36 m	127.7[a] d	1.37 m	27.4 t	2.01 m	26.9 t	1.34 m	27.1[a] t	2.20 m	26.7 t
					2.16 m				1.98 m	
6	2.97 m	25.4 t	1.37 m	28.3 t	5.34 m	128.1[a] d	1.34 m	27.2[a] t	5.33 m	128.5[a] d
	2.74 m									
7	5.41 m	128.6[b] d	1.37 m	27.7 t	5.34 m	128.1[a] d	1.34 m	27.5[a] t	5.33 m	128.5[a] d
8	5.45 m	129.4[c] d	2.04 m	26.2 t	2.86 m	25.9[b] t	2.15 m	25.9 t	3.01 ddd	25.7 t
			1.97 m		2.75 m		1.95 m		2.60 m	
9	3.02 m	25.8 t	5.37 m	127.7[a] d	5.50 m	128.4[a] d	5.38 m	129.9[b] d	5.56 m	128.1[a] d
	2.66 m									
10	5.45 m	126.9[c] d	5.37 m	130.4[a] d	5.37 m	128.8[a] d	5.38 m	127.6[b] d	5.47 m	128.5[a] d
11	5.45 m	127.8[c] d	2.85 ddd	26.0 t	2.82 m	26.0[b] t	2.90 m	25.7 t	2.89 m	25.5 t
			2.78 ddd				2.76 m		2.77 m	
12	3.21 ddd	26.2 t	5.62 m	131.8 d	5.64 m	131.7 d	5.51 m	131.5 d	5.39 m	131.7[b] d
	2.69 m									
13	5.45 m	132.3[c] d	5.41 m	124.9 d	5.39 m	124.9 d	5.38 m	124.6[b] d	5.39 m	125.0[b] d
14	5.41 m	128.3[b] d	2.18 ddd	32.5 t	2.32 m	32.5 t	2.51 ddd	29.5 t	2.58 m	29.7 t
			2.31 ddd		2.16 m		2.43 m		2.29 m	
15	5.63 dq	67.0 d	3.71 m	71.3 d	3.72 m	71.1 d	4.96 m	75.4 d	4.96 m	75.4 d
16	1.30 d	20.7 q	4.83 m	76.7 d	4.80 m	76.6 d	3.57 m	73.6 d	3.57 m	74.2 d
17			1.76 m	24.0 t	1.74 m	23.9 t	1.50 m	26.9 t[a]	1.45 m	26.5 t
18			0.92 t	10.0 q	0.92 t	10.0 q	0.99 t	10.0 q	1.00 t	9.9 q

[a−c] Values with the same superscript in the same column may be interchanged.

Table 2. Selected ^{1}H NMR Data for *R* and *S* MTPA Esters Derived from Aplyolides A−E (2−6)[a]

	2			**3**			**4**			**5**			**6**		
	(*S* MTPA ester)	(*R* MTPA ester)	$\Delta\delta$ ($\delta_S - \delta_R$)	(*S* MTPA ester)	(*R* MTPA ester)	$\Delta\delta$ ($\delta_S - \delta_R$)	(*S* MTPA ester)	(*R* MTPA ester)	$\Delta\delta$ ($\delta_S - \delta_R$)	(*S* MTPA ester)	(*R* MTPA ester)	$\Delta\delta$ ($\delta_S - \delta_R$)	(*S* MTPA ester)	(*R* MTPA ester)	$\Delta\delta$ ($\delta_S - \delta_R$)
H-10										5.38	5.36	+0.02			
H-11	5.38	5.36	+0.02	2.51	2.67	−0.16	2.54	2.70	−0.16	2.68	2.57	+0.11			
				2.71	2.77	−0.06	2.67	2.70	−0.03	2.76	2.72	+0.04			
H-12	2.98	2.96	+0.02	5.45	5.55	−0.10	5.44	5.55	−0.11	5.49	5.46	+0.03			
H-13	5.55	5.51	+0.04	5.27	5.36	−0.09	5.29	5.39	−0.10	5.35	5.30	+0.05			
H-14	5.46	5.37	+0.09	2.27	2.34	−0.07	2.46	2.53	−0.07	2.41	2.32	+0.09	2.39	2.33	+0.06
				2.47	2.55	−0.08	2.26	2.31	−0.05	2.25	2.14	+0.11	2.24	2.11	+0.13
H-15													5.19	5.16	+0.03
H-16	1.33	1.40	−0.07	5.00	4.97	+0.03	4.98	4.93	+0.05						
H-17				1.57	1.43	+0.14	1.58	1.45	+0.13	1.67	1.71	−0.04	1.66	1.70	−0.04
							1.53	1.36	+0.17						
H-18				0.90	0.84	+0.06	0.89	0.82	+0.07	0.86	0.94	−0.08	0.86	0.94	−0.08

[a] Assignments aided by ^{1}H−^{1}H COSY 2D experiments.

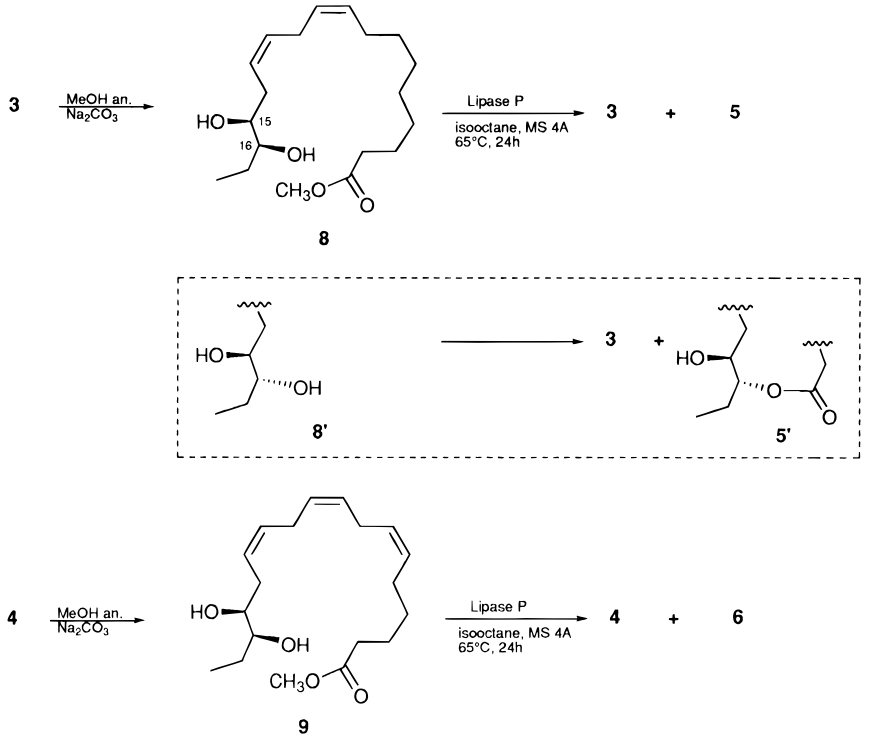
related to compound **4** because both ^{1}H and ^{13}C NMR spectra were quite similar. Analysis of one- and two-dimensional NMR experiments together with the bands observed in the IR spectrum showed the presence of the same functional groups (hydroxy and ester) and three isolated double bonds. The difference was based on the oxymethine linked to the C-1 carbonyl, making aplyolide E (**6**) a 16-membered lactone, the same as compound **5**.

The absolute stereochemistry of the only asymmetric center (C-15) of aplyolide A (**2**) was assigned by the modified Mosher method[13] on the hydroxy acid methyl ester (**7**) obtained after methanolysis of compound **2**. The alcohol was treated with (*S*) and (*R*) 2-methoxy-2-phenyl-2-(triflouromethyl)acetic acid (MTPA) chlorides to give (*R*) and (*S*) MTPA ester derivatives, respectively. ^{1}H NMR signals of both esters were assigned by ^{1}H−^{1}H COSY experiments, and $\Delta\delta$ ($= \delta_S - \delta_R$) values were calculated (Table 2). Satisfactory results were obtained, and thus the absolute configuration of C-15 was assigned as *S*. The absolute stereochemistry of the carbinol asymmetric centers of **3**, **4**, **5**, and **6** were next examined by applying the same procedure. Each compound was treated with (*S*) and (*R*) MTPA chlorides obtaining (*R*) and (*S*) MTPA esters. After having assigned the most significant signals in the NMR spectra of all derivatives, $\Delta\delta$ were calculated. The absolute configuration at C-15 in both aplyolide B (**3**) and aplyolide C (**4**) was *S*. Analogously, the *S* absolute stereochemistry was assigned at C-16 in both aplyolide D (**5**) and aplyolide E (**6**). However, each compound contains another asymmetric center. It is likely that both, aplyolides B (**3**) and D (**5**), derive from the same (15*S*,16*S*) dihydroxy acid. To test this hypothesis we subjected the dihydroxy ester (**8** or **8′**), obtained after methanolysis of aplyolide B (**3**), to enzymatic cyclization conditions[14] (Scheme 1). The reaction furnished a mixture of aplyolide B and another

(13) Ohtani, I.; Kusumi, T.; Kashman,Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092.

(14) Yamada, H.; Ohsawa, S.; Sugai, T.; Ohta, H.; Yoshikawa, S. *Chem. Lett.* **1989**, 1775.

Scheme 1. Opening of Aplyolides B and C Followed by Enzymatic Cyclization of the Corresponding Diols



compound which showed the same HPLC retention time and the same ^{1}H NMR spectrum as aplyolide D (**5**). The paucity of the reaction products prevented us from measuring optical rotation. However, knowing the absolute stereochemistry (*S*) at C-15 of the starting material (aplyolide B), the second compound could exhibit either 15*S*,16*S* (**5**) or 15*S*,16*R* (**5′**) stereochemistry. On the other hand, aplyolide D has the 16*S* stereochemistry; therefore the second compound, obtained after enzymatic cyclization, could be just aplyolide D, or in the case of 15*S*,16*R* stereochemistry, its enantiomer. In order to eliminate any doubt, we subjected aplyolides B (**3**) and D (**5**) to methanolysis conditions. Both the dihydroxy esters obtained had identical optical rotations, showing unambiguously that compounds **3** and **5**, giving the same derivative, have the same absolute stereochemistry. In the same way **4** and **6** were shown to have identical absolute stereochemistry.

It is interesting to note that the aplyolides are ichthyotoxic to the mosquito fish *Gambusia affinis*[15] at 10 ppm whereas the corresponding methyl esters are completely inactive.

The aplyolides belong to a small group of hydroxy fatty acid lactones isolated from marine organisms.[16] Many of these compounds show interesting biological activities, but the paucity of material precluded further investigation. However the anatomical localization of the aplyolides suggests their potential biological role as defensive allomones.

From a biogenetic point of view marine oxylipins such as the aplyolides are very unusual. In fact, the common biogenetic pathway should lead, from octadecapolyenoic acids by the action of a lipoxygenase, to allylic carbinols, whereas aplyolides are obtained by an attack on an isolated double bond of an epoxygenase. However, in this latter case the identical stereochemistry of both the pairs of lactones excludes the attack of the carboxylate at either C15 or C16 of the same epoxide. Therefore, the biogenesis must involve some additional steps. Perhaps, enzyme-directed epoxide opening by H_2O produces a vicinal 15*S*,16*S* diol and this is then converted to both lactones. Even though it has been impossible to perform biosynthetic experiments, it is likely that the aplyolides are synthetized *de novo* by the mollusks. This hypothesis has been indirectly supported by finding the same mixture of aplyolides, with similar HPLC relative ratios, in the extracts of *A. depilans* populations from distant geographical areas (Atlantic, Asturias from North Spain; Cadiz from South Spain; Mediterranean; Naples from South Italy), even though the metabolite patterns in their digestive glands are completely different.

Experimental Section

General Methods. NMR spectra were measured at 500 and 400 MHz for ^{1}H and 125 and 100 MHz for ^{13}C. The signals of $CDCl_3$ were taken as reference (the singlet at 7.26 ppm for ^{1}H NMR and the triplet at 77.0 ppm for ^{13}C NMR data). Two-dimensional NMR experiments were performed by using standard Bruker software. Mass spectra were run in the electron impact mode (70 eV). HREIMS were obtained on a Kratos MS80RFA spectrometer. Column chromatography was done on silica gel Merck (60−200 μm). HPLC analyses were performed on a chromatograph equipped with a UV−vis detector. Optical rotations were measured using a 10 cm cell.

Collection, Extraction, and Isolation Procedures. Specimens of *A. depilans* were collected in the east part of Cabo Peñas, Asturias, in August 1991 (13 animals), from Cadiz in November 1994 (25 animals), and from the Bay of Naples in June 1992 (35 animals). Extraction and the isolation procedure for the August 1991 collection follows. The specimens were carefully dissected into external parts (parapodial lobes, mantle) and internal organs. All sections were separately extracted with acetone by sonication at room temperature. The

(15) Coll, J. C.; La Barre, S.; Sammarco, P. W.; Williams, W. T.; Bakus, G. J. *Mar. Ecol. Prog. Ser.* **1982**, 8, 271. Gunthorpe, L.; Cameron, A. M. *Mar. Biol.* **1987**, *94*, 39.

(16) Gerwick, W. H. *Chem. Rev.* **1993**, *93*, 1807 and references therein cited.

filtered acetone solutions were concentrated and then, after dilution with water, extracted with diethyl ether. Preliminary TLC (SiO_2) analysis of the extracts (Merck silica gel 60 UV 254, 0.25 mm precoated plates; visualization 2% $CeSO_4$ in H_2SO_4; eluant petroleum ether:diethyl ether 1/1) showed the presence of products **2**, **3**, **4**, **5**, and **6** only in the extracts from the external parts of the animals. Column chromatography of the external organs extracts (silica gel; petroleum ether with increasing amounts of diethyl ether) afforded fractions containing aplyolide A (**2**) (15 mg) and mixtures of the other aplyolides. HPLC (Spherisorb column; *n*-hexane:ethyl acetate 9/1) allowed isolation of aplyolides B (**3**) and C (**4**), but still yielded mixtures of these compounds with their isomers **5** and **6**. Complete separation of all products was obtained using two columns in series which yielded 15 mg of **3**, 43.5 mg of **4**, 5 mg of **5**, and 13 mg of **6**. The extracts always contained the same compounds, although the relative ratios were similar, but they were not identical. For example, the extracts from the June 1992 collection yielded 6.5 mg of **3**, 30 mg of **4**, 2 mg of **5**, and 4.7 mg of **6**.

Aplyolide A (2): oil; $[\alpha]^{25}_D = -57.9°$ ($c = 0.4$, $CHCl_3$); IR ($CHCl_3$) 1724 cm^{-1}; EIMS (70 eV) *m/z* (relative intensity) 246 (3), 191 (17), 152 (22), 131 (49), 117 (34), 105 (57), 91 (82), 79(100); HREIMS obsd 246.1623, calcd for $C_{16}H_{22}O_2$ 246.16197. Table 1 for NMR spectral data. J_{H-H} selected values (Hz): 16.8, 7.0, 7.8 (δ 3.21, ddd, H-12); 6.4, 8.0 (δ 5.63, dq, H-15); 6.4 (δ 1.30, d, H_3-16).

Aplyolide B (3): oil; $[\alpha]^{25}_D = -42.8°$ ($c = 0.2$, $CHCl_3$); IR ($CHCl_3$) 3600−3176 (br), 1722 cm^{-1}; EIMS (70 eV) *m/z* (relative intensity) 294 (2), 276 (5), 247 (4), 236 (3), 222 (15), 208 (7), 149 (11), 132 (2), 121 (22), 107 (30), 93 (65), 79 (100); HREIMS obsd 294.2190, calcd for $C_{18}H_{30}O_3$ 294.21948. Table 1 for NMR spectral data. J_{H-H} selected values (Hz): 15.3, 9.0, 4.6 (δ 2.47, ddd, H-2); 15.3, 7.7, 4.7 (δ 2.38, ddd, H-2); 16.0, 7.0, 7.0 (δ 2.85, ddd, H-11); 16.0, 6.2, 6.2 (δ 2.78, ddd, H-11); 14.7, 5.2, 5.2 (δ 2.18, ddd, H-14); 14.7, 9.0, 9.0 (δ 2.31, ddd, H-14); 7.5 (δ 0.92, t, H_3-18).

Aplyolide C (4): oil; $[\alpha]^{25}_D = -26.7$ ($c = 0.7$, $CHCl_3$); IR ($CHCl_3$) 3600−3158 (br), 1717 cm^{-1}; EIMS (70 eV) *m/z* (relative intensity) 292 (5), 274 (11), 245 (2), 220 (5), 206 (10), 147 (9), 132 (16), 119 (37), 105 (59), 91 (81), 79 (100); HREIMS obsd 292.2046, calcd for $C_{18}H_{28}O_3$ 292.20383. Table 1 for NMR spectral data. J_{H-H} selected values (Hz): 15.0, 7.5, 7.5 (δ 2.48, ddd, H-2); 15.0, 7.3, 7.3 (δ 2.34, ddd, H-2); 7.5 (δ 0.92, t, H_3-18).

Aplyolide D (5): oil; $[\alpha]^{25}_D = +28$ ($c = 0.1$, $CHCl_3$); IR ($CHCl_3$) 3440 (br), 1733 cm^{-1}; EIMS (70 eV) *m/z* (relative intensity) 294 (5), 276 (74), 247 (46), 236 (34), 222 (35), 147 (19), 133 (34), 121 (64), 107 (94), 93 (98), 79 (100); HREIMS obsd 294.2194, calcd for $C_{18}H_{30}O_3$ 294.21948. Table 1 for NMR spectral data. J_{H-H} selected values (Hz): 15.1, 8.3, 4.0 (δ 2.31, ddd, H-2); 15.1, 9.0, 4.1 (δ 2.38, ddd, H-2); 14.7, 8.4, 8.4 (δ 2.51, ddd, H-14); 7.5 (δ 0.99, t, H3−18).

Aplyolide E (6): oil; $[\alpha]^{25}_D = +46.3$ ($c = 0.3$, $CHCl_3$); IR ($CHCl_3$) 3500 (br), 1729 cm^{-1}; EIMS (70 eV) *m/z* (relative intensity) 292 (2), 274 (3), 235 (2), 220 (2), 206 (2), 149 (5), 133 (13), 119 (34), 105 (51), 91 (89), 79 (100); HREIMS obsd 292.2039, calcd for $C_{18}H_{28}O_3$ 292.20383. See Table 1 for NMR spectral data. J_{H-H} selected values (Hz): 15.4, 7.3, 7.5 (δ 2.41, ddd, H-2); 15.4, 7.0, 6.6 (δ 2.32, ddd, H-2); 15.6, 7.9, 7.5 (δ 3.01, ddd, H-8); 7.4 (δ 1.00, t, H_3-18).

Methanolysis. Aplyolide (2 mg) was dissolved in 1 mL of anhydrous MeOH, and 10 mg of solid Na_2CO_3 was added. After 12 h at room temperature, the MeOH solution was filtered.

Hydroxy ester 7: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.46−5.34 (m, 8H), 4.69 (dq, $J = 7.2$ and 6.4 Hz, 1H), 3.67 (s, 3H), 2.88 (m, 2H), 2.84 (m, 4H), 2.38 (m, 4H), 1.26 (d, 6.3 Hz, 3H).

Dihydroxy ester 8: $[\alpha]^{25}_D = -15.7$ ($c = 0.14$, CH_3OH); ^{1}H NMR (500 MHz, $CDCl_3$; assignment aided by COSY experiment) δ 5.56 (m, 1H, H-12), 5.46 (m, 1H, H-13), 5.40 (m, 1H, H-9), 5.33 (m, 1H, H-10), 3.67 (s, 3H, OCH_3), 3.51 (m, 1H, H-15), 3.40 (m, 1H, H-16), 2.82 (bt, 7.2 Hz, 2H, H_2-11), 2.32 (m, 2H, H_2-14), 2.30 (t, 7.5 Hz, 2H, H_2-2), 2.06 (m, 2H, H_2-8), 1.60 (m, 2H, H_2-3), 1.50 (m, 2H, H_2-17), 1.36 (m, 2H, H_2-7), 1.30 (m, 6H, H_2-4, H_2-5, H_2-6), 1.00 (t, 7.5 Hz, 3H, H_3-18).

Dihydroxy ester 9: $[\alpha]^{25}_D = -8.3$ ($c = 0.12$, CH_3OH); ^{1}H NMR (500 MHz, $CDCl_3$; assignment aided by comparison with NMR data of compound **8**) δ 5.57 (m, 1H, H-12), 5.47 (m, 1H, H-9), 5.37 (m, 4H, H-6, H-7, H-10, H-13), 3.67 (s, 3H, OCH3), 3.51 (m, 1H, H-15), 3.40 (ddd 13.2, 4.8, 4.7 Hz, 1H, H-16), 2.85 (bt, 7.2 Hz, 2H, H_2-8), 2.81 (m, 2H, H_2-11), 2.32 (4H, H_2-2 and H_2-14), 2.07 (m, 2H, H_2-5), 1.64 (m, 2H, H_2-3), 1.49 (m, 2H, H_2-17), 1.39 (m, 2H, H_2-4), 1.00 (t, 7.4 Hz, 3H, H_3-18).

Preparation of Mosher Esters. Each compound (**3**, **4**, **5**, **6**, **7**), dissolved in anhydrous pyridine, was separately treated with an excess of *S* and *R* 2-methoxy-2-(trifluoromethyl)phenylacetyl chloride. The solvent was evaporated under N_2 and the residue purified by chromatography on silica gel (petroleum ether−diethyl ether 9:1) yielding the *R* and *S* Mosher esters of each compound. ^{1}H NMR data of Mosher esters obtained from aplyolides are reported in Table 2.

Enzymatic Cyclization Conditions. Dihydroxy methyl ester **8** (2 mg) was dissolved in 2 mL of anhydrous isooctane; then lipase from *Pseudomonas* sp. (10 mg) and 4A molecular sieves (20 mg) were added. The suspension was stirred at 65 °C for 24 h. The products **3** (0.1 mg) and **5** (0.5 mg) were then isolated and purified by HPLC. The same procedure was applyed to dihydroxy methyl ester **9**. In this case **6** was more abundant than **4** in the resulting lactone mixture.

Ichthyotoxicity Test. Ichthyotoxicity assays were conducted on the mosquito fish Gambusia affinis as previously described.[15]

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of aplyolides (10 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from ACS; see any current masthead page for ordering information.

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