Biosynthetic Evidence Supporting the Generation of Terpene Chemodiversity in Marine Mollusks of the Genus *Doriopsilla*

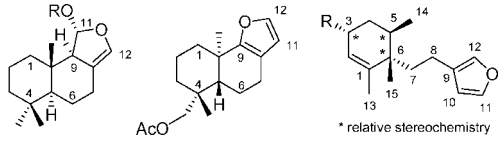
Helena Gaspar,[†] Adele Cutignano,[‡] Teodora Ferreira,[†] Gonçalo Calado,[§] Guido Cimino,[‡] and Angelo Fontana*[,‡]

Instituto Nacional de Engenharia, Tecnologia e Inovação (INETI), Estrada do Paço do Lumiar, Edifício F, 1649-038 Lisboa, Portugal, CNR−Istituto di Chimica Biomolecolare (ICB), Via Campi Flegrei 34, 80078 Pozzuoli (Naples), Italy, and Centro de Modelação Ecológica IMAR, FCT/UNL, Quinta da Torre, 2825-114 Monte da Caparica, Portugal

Received July 17, 2008

This paper reports the study of terpene biosynthesis in the marine nudibranch *Doriopsilla pelseneeri. In vivo* feeding experiments with ^{13}C-glucose proved the *de novo* origin of the terpene metabolites via a mevalonate pathway. Characterization of the stereochemical relationship of the new acetyl pelseneeriols (**7** and **8**) suggests a two-step mechanism for the sesquiterpene cyclization, leading to a slight refinement of terpene tailoring in this family of marine invertebrates.

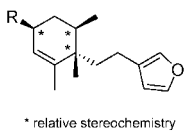
Doriopsilla pelseneeri d'Oliveria 1985 (family Dendrodoridae) is an endemic porostome nudibranch occurring off the Iberian coastal waters (Atlantic and Mediterranean). Previous study of this mollusk showed that its secondary metabolites are characterized by a mixture of sesquiterpenes (e.g., **1**−**4**) falling into three different structural classes having both monocyclic and bicyclic terpene skeletons.[1] Interestingly, the major products, prototyped by **1** and **2**, have opposite absolute stereochemistry of the carbons at the decalin ring junction. These stereochemical characteristics are not unusual, being described in the same terpene skeletons from a Pacific *Dysidea* sponge[2] and from *Doriopsilla areolata*,[3] a *D. pelseneeri* congener, which biosynthesizes *de novo* **1** and **2** through a process that we have previously proposed to stem from the protonation-induced one-step cyclization of farnesyl-pyrophosphate (FPP) or *β-E*-farnesene.[4] In this paper, we address the biogenesis of the three cyclic classes of sesquiterpenes described in *D. pelseneeri*. These studies are based on feeding experiments with [1-^{13}C]-glucose and were aimed at gathering further clues on the terpene cyclization mechanism operating in this group of marine mollusks.



1. R= fatty acyl
5. R= Ac

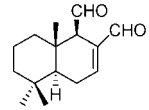
2.

3. R= 3 OH
7. R= 3 OAc



* relative stereochemistry

4. R= 3 OH
8. R= 3 OAc



6.

D. pelseneeri (19 specimens) was collected by scuba off the coast of Portugal in May 2006. After a few hours of acclimatization in an aquarium, 11 mollusks were injected five times with [1-^{13}C]-glucose (6 mg/60 μL^{-1} per mollusk) every other day. A control sample (8 specimens) was frozen without any treatment. The two groups of animals were extracted and their metabolites purified on silica.[1] Major terpenes were recovered and dissolved in $CDCl_3$ for ^{13}C NMR analysis. Drimane esters (**1**) and 15-acetoxy-*ent*-pallescensin A (**2**) showed a specific labeling pattern with an average increase of 30−40% for the ^{13}C NMR signals of products isolated from the treated animals (Table 1). Except for C-12 of **2**, both terpene skeletons were enriched at positions corresponding to carbons 2, 4, and 5 of the isoprene units, which, following Rohmer's arguments,[5] proves the mevalonate origin of **1** and **2** in *D. pelseneeri*.

Table 1. ^{13}C Enrichments of **1** and **2** after *in Vivo* Feeding Experiments with [1-^{13}C]-Glucose

	1		**2**	
	δ_C	% increase[a]	δ_C	% increase[a]
1	39.6	33.2	35.5	38.3
2	18.6	2.2	18.2	0.0
3	42.3	20.3	36.5	74.9
4	33.3	0.0	36.7	3.5
5	53.2	27.9	52.8	40.0
6	22.6	0.0	19.9	0.0
7	23.3	28.0	23.0	69.3
8	114.3	0.0	113.7	0.0
9	64.5	24.3	159.1	70.6
10	37.1	0.0	36.4	0.0
11	98.3	3.4	110.0	77.6
12	134.6	24.4	140.3	49.8
13	14.0	30.4	21.6	58.2
14	21.8	28.4	27.5	81.5
15	33.7	30.6	67.2	79.6
CO	172.9	n.a.	171.3	0.0
alkyl	30.3	n.a.		
CH_3	14.1	n.a.	21.0	102.6

[a] Increase was obtained by the comparison of peak areas of products from treated and control animals. Spectra were normalized to C-4 for **1** and C-10 for **2**. n.a. = not analyzed.

The mantle extracts of the mollusks also contained pelseneeriols (**3** and **4**), at a concentration significantly lower than that previously reported[1] (less than 0.02 mg per individual), and four other terpenes (**5**−**8**) undisclosed in the previous study. Compounds **5** and **6** were easily identified by NMR as 7-deacetoxyolepupuane[6] and polygodial,[7] respectively. These two metabolites have already been reported in other marine mollusks,[8] where they are suggested to be part of a defense strategy based on conversion of **5** into the toxic dialdehyde **6**.[9]

Compounds **7** and **8** were characterized as acetyl derivatives of **3** and **4**. For these compounds, HRESIMS showed pseudomolecular ions $[M + Na]^+$ at *m/z* 299.1633 and 299.1611, in total agreement with the molecular formula $C_{17}H_{24}O_3Na^+$ (calcd 299.1623) expected

* Corresponding author. Tel: +39 0818675096. Fax: +39 0818041770. E-mail: afontana@icb.cnr.it.
[†] INETI.
[‡] CNR-ICB.
[§] Centro de Modelação Ecológica IMAR.

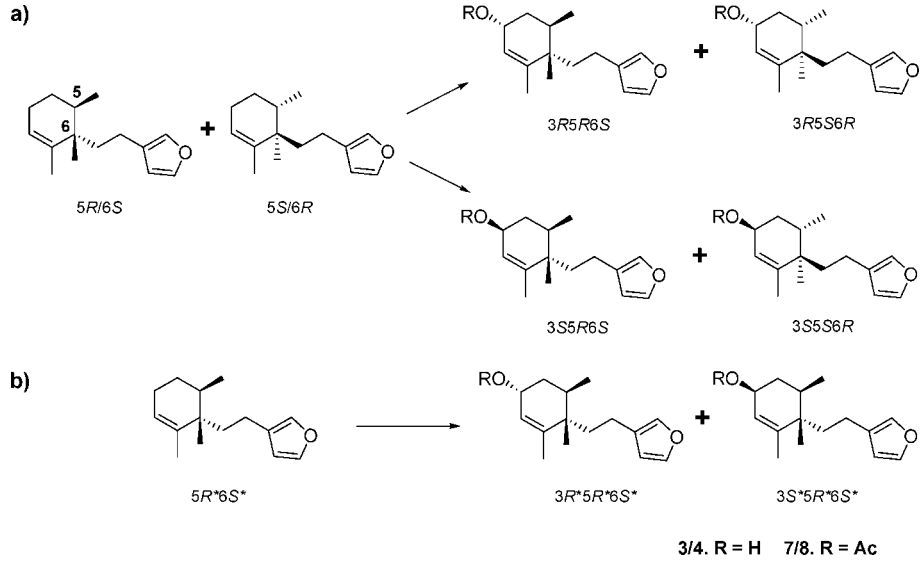
10.1021/np8004387 CCC: $40.75
Published on Web 11/18/2008

Table 2. NMR Data ($CDCl_3$) of Acetylpelseneeriol-1 (**7**), Acetylpelseneeriol-2 (**8**), and Keto-Derivative **9**[a]

	7			**8**			**9**	
	δ_C	δ_H	m, *J* (Hz)	δ_C	δ_H	m, *J* (Hz)	δ_H	m, *J* (Hz)
1	147.4			144.3				
2	122.1	5.60	d, 4.8	124.8	5.42	bs	5.90	s
3	67.5	5.12	bs (H_{eq})	70.8	5.31	bt, 7 (H_{ax})		
4	32.7	1.70	bm (H_{eq})	31.8	1.92	bm, (H_{eq})	2.36	m[b]
		1.62	bd, 14 (H_{ax})		1.51	m, (H_{ax})	1.65	m
5	28.0	2.08	bm	32.4	1.87	bm	2.32	m[b]
6	40.6			41.0				
7	35.6	1.74	m	36.0	1.68	ddd, 14, 9, 4	1.90	m
		1.68	m		1.62	ddd, 14, 13, 5		
8	19.3	2.41	ddd, 14, 12, 6	19.7	2.35	ddd, 15, 13, 5	2.45	m
		2.09	m		2.01	ddd, 15, 9, 4	2.13	ddd, 14, 10, 4
9	125.6			125.3				
10	110.8	6.26	s	110.9	6.25	s	6.25	s
11	142.6	7.35	s	142.8	7.34	s	7.34	s
12	138.3	7.21	s	138.6	7.20	s	7.20	s
13	19.0	1.72	s	19.0	1.70	s	1.70	s
14	15.2	0.9	d, 7	15.9	0.92	d, 7	0.92	d, 6
15	19.5	0.86	s	20.0	0.94	s	0.94	s
Ac	21.2	2.05	s	21.1	2.06	s	2.06	s
	170.7			171.0				

[a] Assignments are supported by 2D-NMR experiments: COSY, TOCSY, HSQC, and HMBC (optimized for either 7 and 10 Hz). [b] Overlapped signals.

Scheme 1. Putative Biogenetic Pathways Leading to Pelseneeriols (**3** and **4**) and Acetyl Pelseneeriols (**7** and **8**) by (a) Stereospecific Hydroxylation of a Racemic Sesquiterpene Mixture or (b) Racemic Hydroxylation of an Enantiopure Terpene Precursor



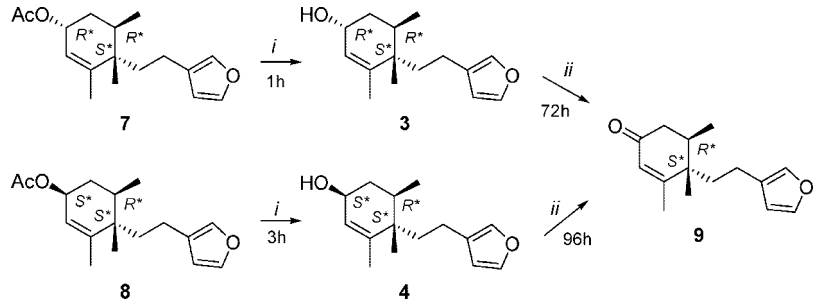
for acetyl pelseneeriols. The new compounds had NMR spectra very similar to those of the parent pelseneeriols **3** and **4** (Table 2) except for the downfield shift induced by acetylation. In particular, compound **7** showed a singlet at δ 2.05 for the acetyl group (δ_C 170.7 and 21.2) and a broad singlet at δ 5.12 for the equatorial H-3 (δ_C 67.5). On the contrary, compound **8** was characterized by the acetate (δ_C 171.0 and 21.1) at δ 2.06 and a broad triplet (J = *7.5* Hz) at δ 5.31 (δ_C 70.8) for the axial-oriented H-3. These resonances together with the values of the chemical shifts of C-1, C-2, and C-4 allowed unambiguous identification of **7** as the acetyl derivative of pelseneeriol-1 (**3**) and **8** as the acetyl derivative of pelseneeriol-2 (**4**).

Like for the pelseneeriols (**3** and **4**), the purified amounts of **7** and **8** (0.03 mg per individual) were insufficient to allow us to determine the ^{13}C-labeling pattern of the monocyclic terpene skeleton. However, their presence in the extracts of *D. pelseneeri* has biogenetic significance since a monocycle farnesane-type carbocation is a putative intermediate in the synthesis of the bicyclic structures of **1** and **2**. Pelseneeriols (**3** and **4**) and their acetyl derivatives (**7** and **8**) are diasteromeric mixtures of hydroxylated terpenes with three chiral carbons. Of the four possible diasteromeric pairs, only two (3*R5R*6S/3*S5R6S* and 3*S5S6R*/3*R5S6R*) are in agreement with the *cis* stereochemistry of the methyl groups at C-5 and C-6. Biogenesis of these diastereomeric skeletons implies two different routes (Scheme 1) with either stereospecific hydroxylation of a racemic pair of terpenes or racemic hydroxylation of an enantiomerically pure terpene (Scheme 1).

To determine the pathway leading to the diasteromeric skeletons of pelseneeriols, compounds **7** and **8** were transformed into **9** through enzymatic hydrolysis with the esterase from hog liver[10] followed by oxidation with MnO_2 in *n*-hexane (Scheme 2). With either starting compound, the reactions led to a single enantiomer, as proven by CD analysis of the final products ($[\theta]_{219} = +26\,400$ and $[\theta]_{228} = -11\,100$ for **9** from **7**; $[\theta]_{219} = +21\,100$ and $[\theta]_{228} = -10\,900$ for **9** from **8**). This outcome indicated that acetyl pelseeneriols (**7** and **8**) are epimeric at C-3, thus suggesting that their biosynthesis involves racemic hydroxylation of an enatiomerically pure monocycle-farnesane analogue (route b in Scheme 1). Due to the very low amount, the absolute stereochemistry of this precursor of acetyl pelseneeriols remains undetermined.

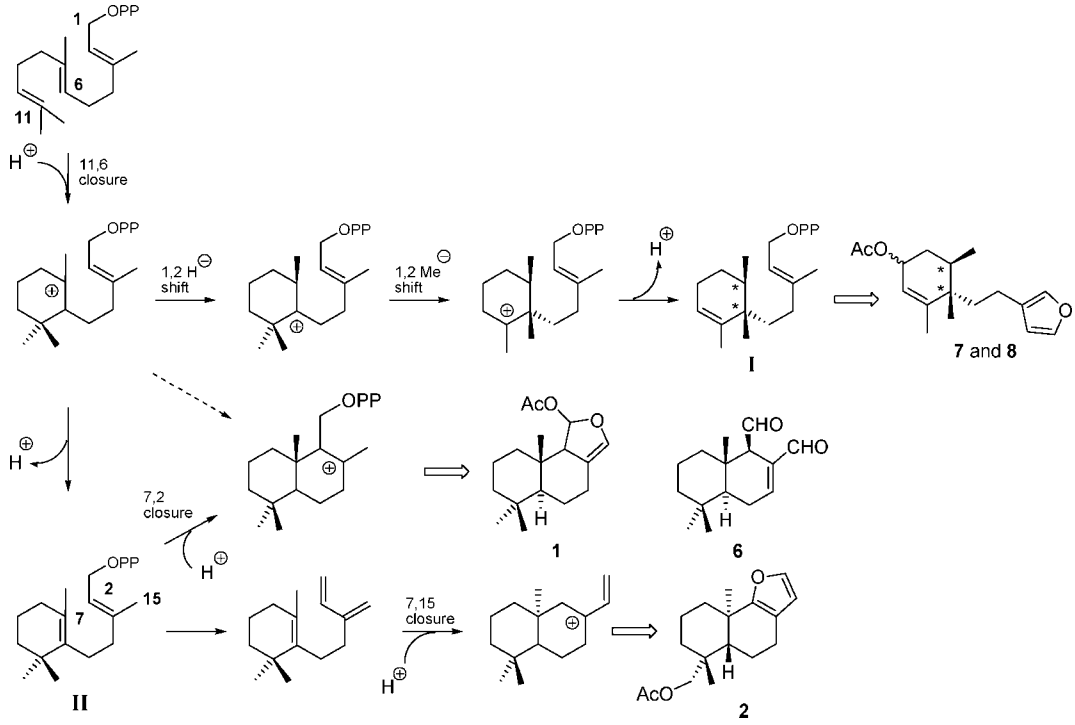
In conclusion, like other dorid nudibranchs, *D. pelseneeri* biosynthesizes *de novo* the terpenoids that serve as defensive compounds (e.g., **6**) to deter predation. Labeling of the major compounds **1** and **2** proves unambiguously the origin of these

Scheme 2. Conversion of Acetyl Pelseneeriols into Compound **9**[a]



[a] Conditions: (i) esterase from hog liver in acetone/phosphate buffer, 1:10, rt, (ii) MnO_2 in *n*-hexane, rt.

Scheme 3. Proposed Biogenesis of Sesquiterpene Skeletons in *D. pelseneeri* (*relative stereochemistry)



molecules through the mevalonate pathway, confirming the current knowledge on terpene biosynthesis in marine opisthobranch mollusks.[11]

The diversity of sesquiterpene skeletal types in the extract of the mollusk suggests a cyclization mechanism starting by protonation-mediated C-11/C-6 closure followed by a second cyclization to give the enatiomeric ring closure of drimane and *ent*-pallescensin skeletons (Scheme 3). This stepwise process, which has been described for a few diterpene cyclases,[12] is in agreement with the synthesis of pelseneeriols by racemic hydroxylation of the allylic position at C-3 of the monocyclofarnesyl derivative **I**. In this view, the carbon skeletons of **1** and **2** should arise from reprotonation of the monocyclic pyrophosphate **II**, followed by alternate C-7/C-2 or C-7/C-15 ring closures according to the mechanisms previously suggested.[4] As shown in Scheme 3, the presence of pelseneeriols opens the possibility (dashed line) of synthesis of the drimane skeleton in **1** and **6** from direct cyclization of the carbocation initially generated by protonation-mediated C-11/C-6 ring closure. With respect to our previous proposal on biosynthesis of drimane and *ent*-pallescensin skeletons in marine nudibranchs,[4] the present data provide further details on terpene tailoring and allow a refined knowledge of skeleton transformations leading to the terpene diversity in this group of organisms.

Experimental Section

General Experimental Procedures. Circular dichrosim measurements were obtained by a Jasco J-710 spectropolarimeter at room temperature. NMR spectra were acquired in $CDCl_3$ (δ values are reported referred to $\underline{C}HCl_3$ at 7.26 ppm and to $CHCl_3$ at 77.0 ppm) on a Bruker DRX-600 operating at 600 MHz for proton, using an inverse TCI CryoProbe fitted with a gradient along the Z-axis. Mass spectra were performed on a Micromass Q-TOF Micro equipped with an ESI source and a Lock-Spray apparatus for accurate mass measurements. Silica gel chromatography was performed using precoated Merck F_{254} plates and Merck Kieselgel 60 powder. HPLC purification was carried out on a Shimazu liquid chromatograph equipped with a Shimazu UV detector. Esterase from hog liver (EC 3.1.1.1) was purchased from Sigma-Aldrich. All solvents were distilled prior to use.

Biological Material. *D. pelseneeri* (average size 2.5 cm) specimens were collected off Arflor (38°30′24″ N; 08°55′09″ W), Setúbal, along the Western coast of Portugal, on April 24 (5 specimens) and May 26 (14 specimens), 2006, at a depth of 8−10 m. The taxonomic identification of *D. pelseneeri* was made by one of us (G.C.). A voucher specimen (preserved in absolute EtOH) is deposited at "Instituto Português de Malacologia", Portugal, reference number IPM.MO.100.

Feeding Experiments. Some of the mollusks (8 specimens, dry weight 3.0 g) were treated as controls and not subject to any experiment. The remaining animals (11 specimens, dry weight 3.5 g) were kept alive in aquaria for 12 days. On days 1, 3, 5, 7, and 9, the animals were injected into the hepatopancreas with [1-^{13}C]-glucose (6 mg/

specimen in 60 μL of distilled water). On day 12 all the animals were frozen at −80 °C and stored at −20 °C until extraction. Each group of mollusks was processed separately following the same protocol.

Isolation of the Metabolites. Terpenes of *D. pelseneeri* were obtained in agreement with ref 1. Extracts of inner organs gave drimane esters **1** (control 21.1 mg, treated 19.5 mg), whereas mantle extracts were chromatographed on a silica gel column by using increasing amounts of diethyl ether (EE) in light petroleum (EP). Fractions containing compounds **2**, **5**, **7**, and **8**, eluted with EP/EE (99.5/0.5), were purified by normal-phase HPLC (column Phenomenex-Kromasil Silica 5 μm, 100A, 250 × 4.60 mm) with *n*-hexane/isopropanol, 99.92/0.08 (1 mL/min). This purification gave **2** (control 0.8 mg, treated 1.0 mg), **8** (control 0.5 mg, treated 0.2 mg), **7** (control 0.3 mg, treated 0.1 mg), and **5** (control 0.3 mg, treated <0.1 mg) in order of increasing retention time. Fractions eluting from silica with EP/EE (80/20) contained compounds **3**, **4**, and **6**, which were further purified by HPLC according to ref 1, affording pure **3** (control 0.1 mg, treated <0.1 mg), **4** (control 0.2 mg, treated 0.1 mg), and **6** (control 0.4 mg, treated 0.3 mg).

3-Acetylpelseneeriol-1 (7): colorless oil; CD (*n*-hexane) $[\theta]_{219} = +26\,400$, $[\theta]_{228} = -11\,100$; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS$^+$ *m/z* 299.1633 $[M + Na]^+$ (calcd for $C_{17}H_{24}O_3Na$, 299.1623).

3-Acetylpelseneeriol-2 (8): colorless oil; CD (*n*-hexane) $[\theta]_{219} = +21\,100$, $[\theta]_{228} = -10\,900$; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS$^+$ *m/z* 299.1611 $[M + Na]^+$ (calcd for $C_{17}H_{24}O_3Na$, 299.1623).

Transformation of Compounds 7 and 8 to 9. Hog liver esterase (2.1 mg of protein, 165 U/mg) was added to a solution of each pure acetyl pelseneeriol (**7**, 0.4 mg; **8**, 0.7 mg) in phosphate buffer (50 mM, pH = 7, 500 μL containing 10% acetone). The mixture was stirred at room temperature for 1−3 h. The resulting aqueous solution was extracted with EtOAc (4 × 2 mL). The organic layers were combined, dried (Na_2SO_4), filtered, and concentrated to yield the corresponding alcohols (**3** from **7**, and **4** from **8**) as proven by comparison with the chromatographic comparison with authentic pelseneeriols **3** and **4**. *n*-Hexane (1.5 mL) and MnO_2 (1 mg) were added to each obtained pelseneeriol, and the resulting solution was stirred at room temperature for 72−96 h, after which TLC (petroleum ether/diethyl ether, 1:1) indicated complete consumption of the starting material and the formation of one major Ehrlich positive product at R_f 0.45. The mixture was then filtered and evaporated to give compound **9** from both pelseneeriols (0.1 mg from **7** and 0.2 mg from **8**).

Compound 9: colorless oil; UV (MeOH) λ_{max} 224 (ε 8900,) nm; ^{1}H NMR (600 MHz, C_6D_6) δ *J* (Hz) 7.16 (H-11), 6.96 (H-12), 5.98 (H-10), 5.91 (H-2, s), 2.22 (H-4), 2.02 (H-4), 1.85 (H-5), 1.62 (H_2-7), 1.36 (H_3-13), 0.53 (H_3-14, d, 6.1), 0.54 (H_3-15, s); ^{13}C NMR (150 MHz, C_6D_6, data were obtained by HSQC and HMBC experiments) δ 197.3 (C-3), 165.2 (C-1), 140.2 (C-11), 138.2 (C-12), 128.7 (C-2), 110.6 (C-10), 41.6 (C-4), 34.1 (C-6), 33.5 (C-5), 26.2 (C-13), 14.3 (C-14), 14.5 (C-15); HRESIMS$^+$ *m/z* 255.1372 $[M + Na]^+$ (calcd for $C_{15}H_{20}O_2Na$, 255.1361).

Acknowledgment. We thank M. Zampa for HRESIMS, C. Iodice for CD analysis, and A. Maiello for technical assistance. This project was carried out in the frame of the bilateral project "Characterization of Terpene Biosynthetic Enzymes of Biotechnological Interest from Marine Invertebrates". We thank GRICES, RSTL-CNR "Intermedi e regolazione del pathway non-mevalonico in microrganismi", and the European Network of Excellence on "Marine Biodiversity and Functioning" for financial support.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Gaspar, H.; Gavagnin, M.; Calado, G.; Castelluccio, F.; Mollo, E.; Cimino, G. *Tetrahedron* **2005**, *61*, 11032–11037.
(2) Butler, M. S.; Capon, R. J. *Aust. J. Chem.* **1993**, *46*, 1255–1267.
(3) (a) Gavagnin, M.; Mollo, E.; Calado, G.; Fahey, S.; Ghiselin, M.; Ortea, J.; Cimino, G. *Chemoecology* **2001**, *11*, 131–136. (b) Gavagnin, M.; Mollo, E.; Castelluccio, F.; Ghiselin, M. T.; Calado, G.; Cimino, G. *Tetrahedron* **2001**, *57*, 8913–8916.
(4) Fontana, A.; Tramice, A.; Cutignano, A.; d'Ippolito, G.; Gavagnin, M.; Cimino, G. *J. Org. Chem.* **2003**, *68*, 2405–2409.
(5) Rohmer M. In *Natural Product Chemistry: Isoprenoids Including Carotenoids and Steroids*; Cane, D. E., Ed.; Elsevier: Oxford, 1999; Vol. 2, pp 45−67.
(6) Avila, C.; Cimino, G.; Crispino, A.; Spinella, A. *Experientia* **1991**, *47*, 306–310.
(7) (a) Barnes, C. S.; Loder, J. W. *Aust. J. Chem.* **1962**, *15*, 322–327. (b) Jansen, B. J. M.; Groote, Ae. *Nat. Prod. Rep.* **2004**, *21*, 449–477.
(8) Blunt, J. W.; Copp, B. R.; Hu, W.-P.; Munro, M. H. G.; Northcote, P. T.; Prinsep, M. R. *Nat. Prod. Rep.* **2008**, *25*, 35–94, and previous articles in this series.
(9) (a) Long, J. D.; Hay, M. E. *Mar. Ecol.: Prog. Ser.* **2006**, *307*, 199–208. (b) Cimino, G.; Ciavatta, M. L.; Fontana, A.; Gavagnin, M. In *Bioactive Natural Products: Isolation, Structure Elucidation and Biology Properties*; Tringali, C., Ed.; Taylor and Francis, London, 2001; pp 577−637.
(10) Mori, K.; Puapoomchareon, P. *Liebigs Ann. Chem.* **1991**, 1053–1056.
(11) (a) Kashman, Y.; Rudi, A. *Phytochem. Rev.* **2004**, *3*, 309–323. (b) Fontana, A. *Prog. Mol. Subcell. Biol.* **2006**, *43*, 303–332. (c) Moore, B. S. *Nat. Prod. Rep.* **2006**, *23*, 615–629. (d) Kubanek, J.; Graziani, E.; Andersen, R. J. *J. Org. Chem.* **1997**, *62*, 7239–7246. (e) Graziani, E.; Andersen, R. J. *J. Am. Chem. Soc.* **1996**, *118*, 4701–4702.
(12) (a) Oikawa, H.; Nakamura, K.; Toshima, H.; Toyomasu, T.; Sassa, T. *J. Am. Chem. Soc.* **2002**, *124*, 9145–9153. (b) DaviesE. M.; CroteauR. In *Biosynthesis*; LeeperF. J., Vederas, J. C., Eds; Springer-Verlag: Berlin, 2000; Vol. 209, pp 53−95.

NP8004387