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Novel Verrucosins from the Skin of the Mediterranean Nudibranch *Doris verrucosa*

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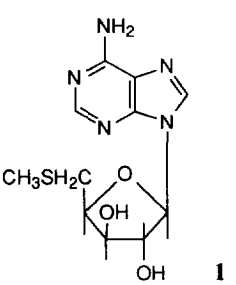
Abstract: Nine new diterpenoid acid glycerides (**4-12**) have been isolated, together with the previously reported **13** and **14**, from the skin extract of the Mediterranean nudibranch *Doris verrucosa*. The structure and the relative stereochemistry of the new metabolites were established by interpretation of spectral data. Absolute stereochemistry of the diterpenoid part was suggested by biogenetic considerations as well as by comparison of CD profiles of **4-12** with those of verrucosin-A (**2**) and -B (**3**), previously isolated from the same mollusc. The *S* absolute configuration of C-2' of the glyceryl moiety was determined by chemical methods for verrucosin-3 (**6**), as already reported for **2** and **3**, and suggested to be the same for all other verrucosins.

Doris verrucosa is a Mediterranean dorid nudibranch widely occurring in the Bay of Naples. Previous studies[3-6] of this mollusc resulted in the isolation of an analog of methyltioadenosine (**1**) and of two isomeric diterpenoid acid glycerides, verrucosin-A (**2**) and -B (**3**). These metabolites were compartmentalized in different anatomical parts of the mollusc. The purine **1** was found mainly in both the hermaphrodite gland and the egg-mass, whereas **2** and **3**, which are the most abundant components of a complex mixture of ichthyotoxic diacylglycerols, were only present in the skin extract of the animal. Due to the bioactivity exhibited by **2** and **3** in the fish toxicity bioassay and to their location, it has been proposed that these two metabolites could be involved in the chemical defensive strategy of the mollusc. Biosynthetic experiments with radiolabelled mevalonic acid and glycerol were also performed, aiming at understanding the origin of the *D. verrucosa* metabolites. However, the results were not conclusive. In fact, poor levels of radioactivity were found associated to verrucosin-A (**2**) and -B (**3**) in a series of incorporation experiments.[6] Verrucosin-A and -B possess very interesting biological activities. In fact, both diacylglycerols, **2** and **3**, display *in vivo* PKC-mediated morphogenetic properties in the *Hydra* tentacle regeneration assay.[7]



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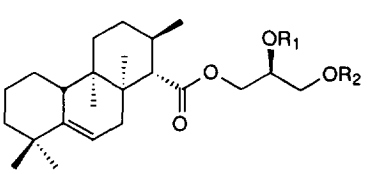
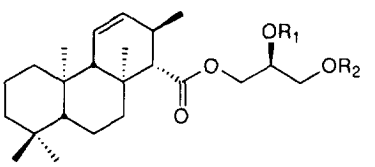


2 : R_1 = H ; R_2 = Ac
3 : R_1 = Ac ; R_2 = H

The promising bioactivity of verrucosins as well as the interest to clarify their origin prompted us to further study the skin metabolites of *D. verrucosa*, aimed at characterizing the minor components of diacylglycerol mixture. In this paper, we report the structure determination of nine new diterpenoid glycerides, verrucosins 1-9 (**4**-**12**), which were isolated along with the known glyceryl esters **13** and **14**, previously found[8] in the Western Atlantic dorid nudibranch *Archidoris carvi*.

Specimens of *D. verrucosa* (385 individuals) were collected by SCUBA divers during June 1992 in the Bay of Naples and immediately frozen at -20°C. After some days, the animals were carefully dissected. Mantles and digestive glands were exhaustively extracted with acetone. The acetone mantle extract contained the mixture of verrucosins that were completely absent in the hepathopancreas extract. The mantle extract partitioned between diethyl ether and water and the ethereal portion (1.61 g) was chromatographed on a silica gel column by using an elution gradient from light petroleum ether to diethyl ether.



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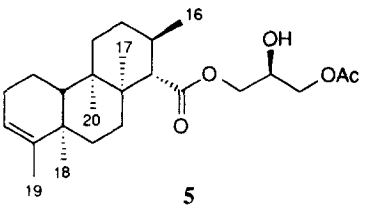

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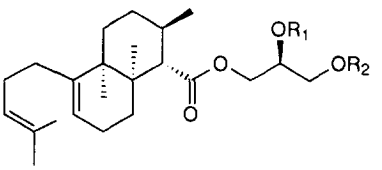

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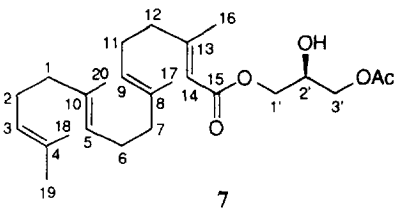

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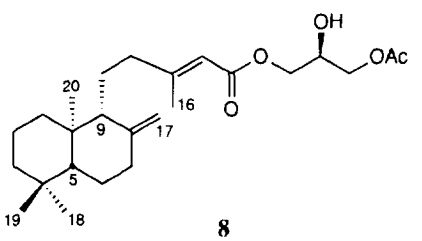

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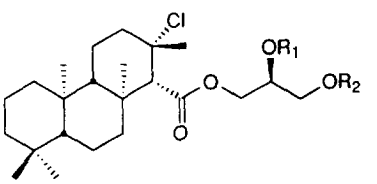
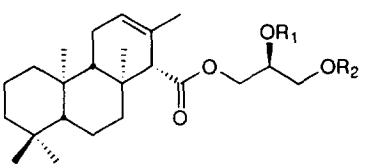


10 : R_1 = H ; R_2 = Ac
12 : R_1 = Ac ; R_2 = H

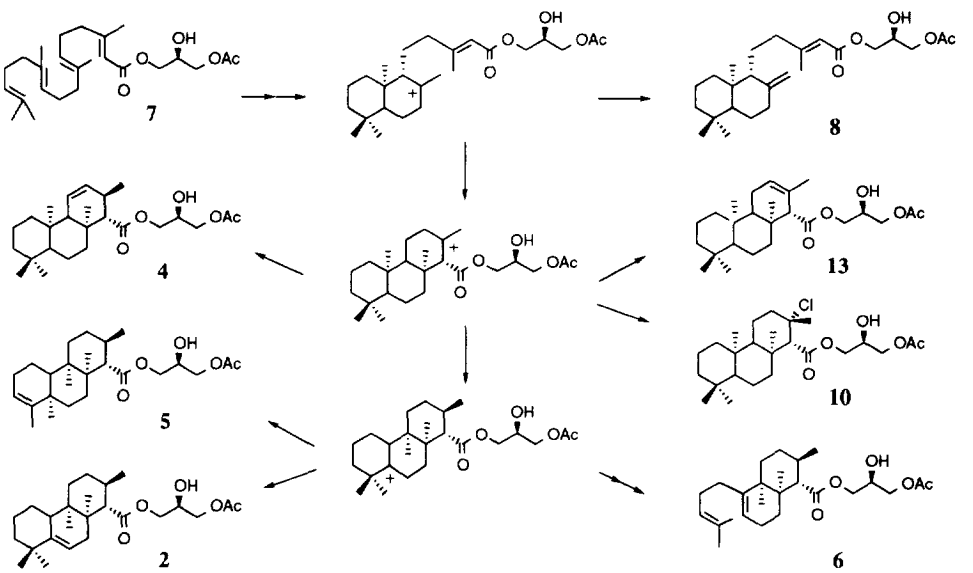


13 : R_1 = H ; R_2 = Ac
14 : R_1 = Ac ; R_2 = H

Some combined fractions (light petroleum ether/diethyl ether, 1:1) were purified by normal phase HPLC giving, along with the already described[4] major metabolites verrucosin-A (**2**, 64.0 mg) and -B (**3**, 16.6 mg), a pure compound, verrucosin-1 (**4**, 7.0 mg), and two complex mixtures (A, 52.0 mg; B, 7.1 mg). Reverse phase HPLC purification afforded five pure metabolites: verrucosin-2 (**5**, 7.5 mg), verrucosin-3 (**6**, 24.0 mg) and the 3'-acetyl-glyceryl isocopalate (**13**, 14.4 mg),[8] from mixture A; verrucosin-4 (**7**, 3.5 mg) and verrucosin-5 (**8**, 2.7 mg) from mixture B.

Other fractions (light petroleum ether/diethyl ether, 3:7) were analogously subjected to normal phase HPLC to give verrucosin-6 (**9**, 1.1 mg), verrucosin-7 (**10**, 3.5 mg), verrucosin-8 (**11**, 0.8 mg) and verrucosin-9 (**12**, 1.0 mg), together with the 1,2-diacyl glyceride (**14**, 1.0 mg),[8] isomer of **13**.

The structure elucidation of the new compounds is reported starting from verrucosin-4 (**7**), which could be formally considered the precursor of all verrucosins. Other diacylglycerols are described according to the proposed biosynthetic correlation (Scheme 1).

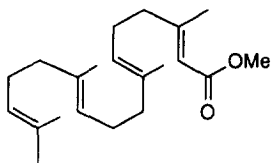


Scheme 1. Hypothetical biogenetic correlation of verrucosins.

All the verrucosins showed a diacyl glycerol structure. In particular, diagnostic signals in both ^{1}H-NMR (Table 1) and ^{13}C-NMR (Table 2) spectra indicated the presence of a monoacetylated glyceryl fragment, further linked to a diterpenoid acid. Furthermore, with the exception of verrucosin-7 (**10**) and -9 (**12**), all compounds exhibited the same molecular formula, $C_{25}H_{40}O_5$, deduced by or HREIMS either EIMS and ^{13}C-NMR data. All ^{1}H- and ^{13}C-NMR resonances of the new verrucosins were completely assigned (Tables 1 and 2) by mono- and bi-dimensional NMR experiments (^{1}H-^{1}H COSY, ^{1}H-^{13}C HETCOR, HMBC, etc.).

Verrucosin-4 (**7**)[9] was isolated as a colourless oil. A series of signals in the ^{1}H-NMR spectrum at δ 4.15 (4H, m) and 4.10 (1H, m) and in the ^{13}C-NMR spectrum at δ 64.50 (t), 68.45 (d) and 65.36 (t) immediately revealed a 1,3-diacyl glycerol structure. In addition, the ^{1}H-NMR spectrum showed signals attributable to five vinyl methyls [δ 1.60 (9H), 1.68 and 2.17], a singlet at δ 5.70 (1H) and a multiplet at δ 5.10 (3 H), suggesting the presence of a linear diterpenoid exhibiting four trisubstituted double bonds, one of which was conjugated with a carboxyl group. Furthermore, the ^{13}C-NMR values of the vinyl methyls at δ 16.00 (2C), 17.68, 19.05

and 25.70 allowed the assignment of the *E* geometry of three double bonds, the fourth being a terminal *gem*-dimethyl double bond. The presence of an α,β unsaturated ester moiety was confirmed by the IR (1718 cm^{-1}) and the ^{13}C-NMR (δ 166.72) data. The strong resemblance between the ^{1}H-NMR spectrum of **7** and that of (all *E*)-methyl geranylgeranoate (**15**)[10] indicated that (all *E*)-geranylgeranoic acid was the acyl component of the ester.



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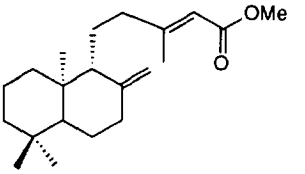

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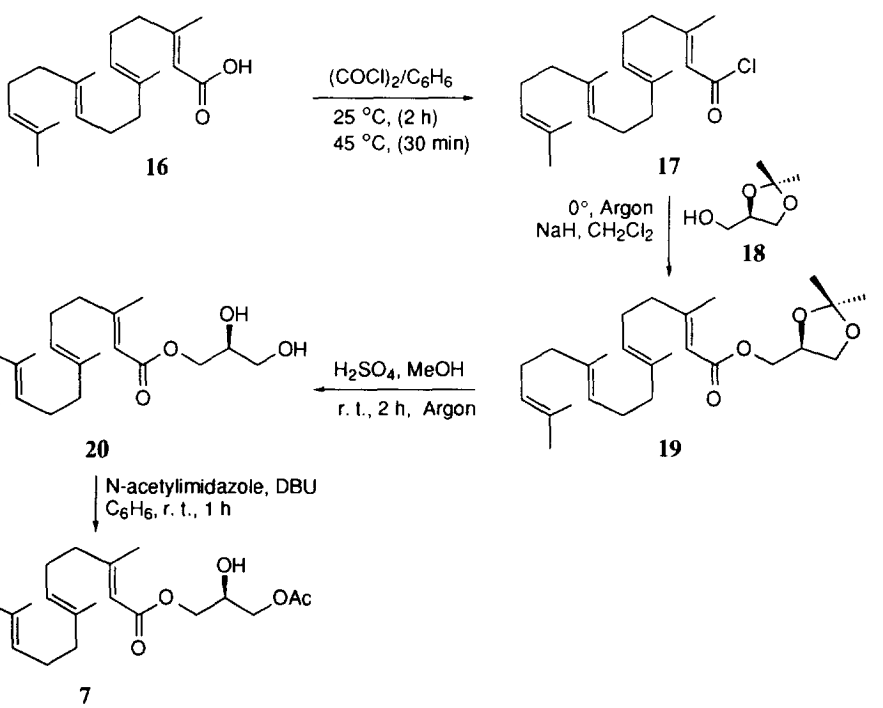
To further confirm this identity, the synthesis of **7** was performed (Scheme 2). (*E,E,E*)-geranylgeranoic acid (**16**, 150.0 mg), obtained from (*E,E*)-farnesylacetone,[10,11] was converted into the corresponding chloride **17**, by treatment with $(COCl)_2$ in dry C_6H_6.[12] Following a procedure previously performed to synthesize *ent*-isocopalic acid glycerides,[13] the crude reaction product (155.0 mg) was immediately coupled, without purification, with (-)-2,3-O-isopropylidene-*sn*-glycerol (**18**), by NaH in CH_2Cl_2, affording, after chromatographic column (Si-gel, light petroleum ether/diethyl ether, 93:7), the acetonide **19** (178.8 mg, yield 86.7% from **16**). Deprotection of **19** (100.0 mg) by 0.006 M solution of H_2SO_4 in CH_3OH gave, after purification on silica-gel column (petroleum ether with increasing amounts of diethyl ether), the glyceryl geranylgeranoate **20** (77.3 mg). Acetylation of **20** by N-acetyl imidazole (DBU, C_6H_6) afforded the 3'-acetyl derivative, which was identical in all respects with natural **7**.



Scheme 2. Synthesis of verrucosin-4 (**7**).

Table 1. ^{1}H-NMR Data [a,b] of Verrucosins 1,3 derivatives

	δ ^{1}H (m, *J*)					
Proton	**4**	**5**	**6**	**7**	**8**	**10**
1	0.82 (m)	1.42 (m)	1.91 (m)	2.00 (m)	1.01 (ddd, 12.9, 13.0, 3.9)	0.80 (m)
	1.76 (m)	1.58 (m)			1.75 (m)	1.70 (m)
2	1.40 (m)	2.00 (m)	2.08 (m)	2.10 (m)	1.55 (m)	1.42 (m)
	1.60 (m)	2.06 (m)	2.13 (m)			1.60 (m)
3	1.15 (ddd, 13.3, 12.9, 3.8)	5.22 (bs)	5.14 (m)	5.10 (m)	1.18 (ddd, 13.4, 13.5, 4.0)	1.10 (ddd, 13.4, 13.2, 3.8)
	1.39 (m)				1.39 (m)	1.37 (m)
4	-	-	-	-	-	-
5	0.84 (m)	-	5.41 (bs)	5.10 (m)	1.09 (dd, 12.6, 2.7)	0.84 (m)
6	1.40 (m)	1.48 (m)	1.98 (m)	2.10 (m)	1.32 (dddd, 12.8, 12.9, 13.0, 4.2)	1.43 (m)
	1.60 (m)	1.70 (m)	2.32 (m)		1.75 (m)	1.50 (m)
7	1.38 (m)	1.09 (m)	1.11 (bdd, 14.2, 6.7)	2.00 (m)	2.39 (m)	1.19 (m)
	1.42 (m)	1.70 (m)	1.68 (m)		1.97 (m)	1.50 (m)
8	-	-	-	-	-	-
9	1.73 (m)	-	-	5.10 (m)	1.57 (m)	0.85 (m)
10	-	1.90 (bd, 10.6)	-	-	-	-
11	5.59 (bd, 10.2)	1.32 (m)	1.46 (m)	2.15 (m)	1.50 (m)	1.60 (m)
		1.68 (m)	1.67 (m)		1.67 (m)	1.72 (m)
12	5.52 (ddd, 10.2, 2.5, 2.6)	1.07 (m)	0.83 (m)	2.15 (m)	1.95 (m)	1.62 (m)
		1.42 (m)	1.50 (m)		2.31 (m)	2.17 (m)
13	2.55 (m)	1.90 (m)	1.89 (m)	-	-	-
14	2.13 (d, 11.0)	2.90 (d, 11.8)	2.46 (d, 11.5)	5.70 (bs)	5.68 (bd, 1)	2.32 (s)
15	-	-	-	-	-	-
16	0.93 (d, 6.9)	0.85 (d, 6.3)	0.79 (d, 6.3)	2.17 (bs)	2.17 (d, 1)	1.52 (s)
17	1.03 (s)	0.98 (s)	1.02 (s)	1.60 (bs)	4.49 (bs)	1.33 (s)
					4.85 (d, 1.2)	
18	0.81 (s)	1.02 (s)	1.62 (bs)	1.60 (bs)	0.80 (s)	0.80 (s)
19	0.86 (s)	1.62 (bd, 1.4)	1.69 (bs)	1.68 (bs)	0.87 (s)	0.84 (s)
20	0.84 (s)	0.82 (s)	0.92 (s)	1.60 (bs)	0.69 (s)	0.86 (s)
1'	4.10 - 4.24 (m)	4.11 - 4.21 (m)	4.10 - 4.20 (m)	4.15 (m)	4.13 - 4.23	4.13 - 4.23 (m)
2'	4.15 (m)	4.09 (m)	4.10 (m)	4.10 (m)	4.11 (m)	4.12 (m)
3'	4.18 (m)	4.15 (m)	4.17 (m)	4.15 (m)	4.18 (m)	4.18 (m)
-OAc	2.10 (s)	2.11 (s)	2.10 (s)	2.10 (s)	2.10 (s)	2.11 (s)
-OH	2.45 (d, 4.2)	2.43 (d, 4.9)	2.52 (d, 4.1)	-	2.51 (d, 4.9)	2.47 (d, 4.5)

[a]Bruker AMX 500 MHz, $CDCl_3$, chemical shifts (ppm) referred to $C\underline{H}Cl_3$ (δ 7.26). [b]Assignments made by ^{1}H-^{1}H COSY, HMQC and ^{1}H-^{1}H homodecoupling experiments.

Table 2. ^{13}C-NMR Data [a,b] of Verrucosins 1,3 derivatives

	δ ^{13}C *m*[c]					
Carbon	**4**	**5**	**6**	**7**	**8**	**10**
1	39.19 *t*	18.09 *t*	30.25 *t*	39.69 *t*	39.07 *t*	39.91 *t*
2	18.40[d] *t*	27.13 *t*	26.56 *t*	26.74[e] *t*	19.36 *t*	18.49 *t*
3	42.13 *t*	120.56 *d*	124.68 *d*	124.35 *d*	42.10 *t*	42.05 *t*
4	33.23 *s*	144.44 *s*	131.42 *s*	131.30 *s*	33.59 *s*	33.29 *s*
5	56.20 *d*	38.33 *s*	120.37 *d*	124.04 *d*	55.48 *d*	56.75 *d*
6	18.24[d] *t*	31.04 *t*	22.11 *t*	26.57[e] *t*	24.42 *t*	17.82 *t*
7	39.11 *t*	30.65 *t*	30.04 *t*	39.69 *t*	38.28 *t*	41.46 *t*
8	37.39 *s*	39.18 *t*	37.87 *s*	136.34[f] *s*	148.29 *s*	38.95 *s*
9	58.59 *d*	38.77 *s*	41.14 *s*	122.71 *d*	56.23 *d*	60.08 *d*
10	37.16 *s*	40.70 *d*	141.12 *s*	135.08[f] *s*	39.71 *s*	37.54 *s*
11	125.49 *d*	33.05 *t*	30.77 *t*	25.96 *t*	21.51 *t*	17.77 *t*
12	132.17 *d*	29.27 *t*	31.66 *t*	41.07 *t*	39.96 *t*	45.16 *t*
13	32.14 *d*	30.61 *d*	30.04 *d*	161.99 *s*	163.04 *s*	67.73 *s*
14	61.96 *d*	51.90 *d*	53.04 *d*	114.62 *d*	114.26 *d*	66.41 *d*
15	174.36 *s*	175.18 *s*	175.32 *s*	166.72 *s*	168.85 *s*	170.64 *s*
16	19.83 *q*	21.00 *q*	20.94 *q*	19.05 *q*	19.13 *q*	34.16 *q*
17	14.78 *q*	18.99 *q*	18.56 *q*	16.00 *q*	106.34 *t*	16.79 *q*
18	21.15 *q*	20.74 *q*	17.70 *q*	17.68 *q*	21.70 *q*	21.30 *q*
19	33.16 *q*	17.94 *q*	25.70 *q*	25.70 *q*	33.59 *q*	33.29 *q*
20	16.46 *q*	21.30 *q*	24.61 *q*	16.00 *q*	14.46 *q*	16.31 *q*
1'	64.75 *t*	64.56 *t*	64.57 *t*	64.50 *t*	64.48 *t*	64.84 *t*
2'	68.41 *d*	68.37 *d*	68.36 *d*	68.45 *d*	68.47 *d*	68.23 *d*
3'	65.27 *t*	65.27 *t*	65.35 *t*	65.36 *t*	65.38 *t*	65.16 *t*
-O<u>C</u>OCH$_3$	170.97 *s*	170.98 *s*	171.03 *s*	171.08 *s*	171.09 *s*	171.01 *s*
-OCO<u>CH</u>$_3$	20.74 *q*	20.74 *q*	20.77 *q*	20.81 *q*	20.80 *q*	20.79 *q*

[a]Bruker AMX 500 MHz, $CDCl_3$, chemical shifts (ppm) referred to <u>C</u>DCl_3 (δ 77.00). [b]Assignments made by HMQC and HMBC (J = 6 and 10 Hz). [c]By DEPT sequence. [d-f]Values with identical superscript may be reversed.

Verrucosin-5 (**8**), obtained as a colourless oil, showed, analogously with **7**, a 1,3-diacyl glycerol structure and an α,β unsaturated ester functionality, as indicated by an infrared band at 1725 cm^{-1} and by the ^{13}C-NMR resonance of the carboxyl group (δ 168.85). The presence in the ^{1}H-NMR spectrum of three methyl singlets at δ 0.69, 0.80 and 0.87, a vinyl methyl at δ 2.17, an olefinic proton at δ 5.68 and two methine singlets at δ 4.85 and 4.49 suggested a bicyclic diterpenoid skeleton exhibiting an exomethylene and a trisubstituted double bond. In particular, the ^{1}H-NMR resonances (Table 1) of **8** exhibited strong analogies with those of the methyl ester of copalic acid (**21**), a constituent of the exudate from *Hymenaea courbaril*.[14,15] To prove this structural hypothesis, **8** was treated with MeOH an./Na_2CO_3, obtaining the methyl ester derivative (**21**), which was identical in all respects with an authentic sample.[16]

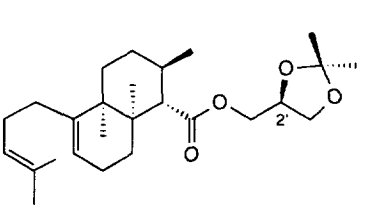
Verrucosin-7 (**10**) exhibited the molecular formula $C_{25}H_{41}O_5Cl$, deduced by HREIMS on the molecular ion at *m/z* 456.2642 (M^+, Δ 17.8 mmu). The 1,3-diacylation pattern of the glyceryl fragment was indicated by the diagnostic signals in the ^{1}H-NMR spectrum at δ 4.18 (m, 4 H) and δ 4.12 (m, 1 H). Analysis of ^{1}H- and ^{13}C-NMR spectra (Tables 1 and 2) revealed some analogies with those of compounds **13** and **14**, suggesting an isocopalane skeleton for the diterpenoid part of the molecule. In particular, the ^{1}H-NMR spectrum displayed five singlet methyls at δ 0.80, 0.84 (6H), 0.86, 1.33 and 1.52, supporting a chlorine-containing saturated tricyclic structure, which could be formally derived from **13** by addition of chloridric acid to the double bond. The position of the chlorine substituent at C-13 was indicated by the deshielded chemical shift values of both the methyl at C-13 (δ 1.52) and of H_2-12 (δ 1.62 and 2.17) and further confirmed by diagnostic correlations, in the HMBC experiment, between C-13 (δ 67.73) and H-12a (δ 2.17), H_3-16 (δ 1.52) and H_3-17 (δ 1.33). The equatorial orientation of the methyl at C-13 was suggested by comparison of the diagnostic ^{13}C-NMR value of C-16 (δ 34.16) with those of model compounds.[17] In fact, very different carbon chemical shifts have been reported for equatorial or axial C-16, resonating at about 30 ppm and 24 ppm, respectively, in isocopalane diterpenes exhibiting an hydroxy group at C-13.[17] The relative stereochemistry at both C-13 and C-14 was determined by a strong n.O.e. effect between H-14 and H_3-16. Furthermore, the irradiation of H-14 also resulted in the enhancement of H-9, establishing a *cis*-relationship between H-9 and H-14 and consequently the relative stereochemistry of all chiral centers. The absolute stereochemistry of the diterpenoid part of verrucosin-7 (**10**) should be the same as **13** on the basis of biogenetic considerations. The CD profile of **10**, identical to those of **2** and **13**,[8] confirmed this suggestion.

Verrucosin-9 (**12**) resulted to be the 2-acetyl isomer of **10**, as immediately revealed by analysis of the ^{1}H-NMR spectrum, showing strong similarities with that of **10** and differing only in the chemical shift values of H-2' (δ 5.10) and H_2-3' (δ 3.78) of the glyceryl moiety. All NMR resonances were assigned by comparison with those of **10** (see Experimental).

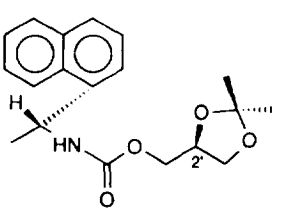
Verrucosin-1 (**4**) exhibited a 1,3 diacyl glyceryl structure and displayed, in the ^{1}H-NMR spectrum, signals for five methyls [δ 0.81 (s), δ 0.84 (s), δ 0.86 (s), δ 1.03 (s), δ 0.93 (d, *J*=6.9 Hz)], showing the presence of an isocopalane skeleton, analogously with verrucosin-7 (**10**) and **13**. In particular, the spectrum was quite similar to that of **13**,[8] differing in the presence of a secondary methyl (doublet at δ 0.93), which replaced the vinyl methyl at C-13 (broad singlet at δ 1.60), and also for the substitution pattern of the double bond, which in **4** is disubstituted [δ ^{1}H 5.52 (ddd, *J*=10.2, 2.6 and 2.5 Hz) and 5.59 (bd, *J*=10.2 Hz), δ ^{13}C 132.17 (d) and 125.49 (d)]. The two olefinic protons were coupled with both methines H-9 (δ 1.73) and H-13 (δ 2.55). These data led to location of the double bond in the ring C between C-11 and C-12. The *cis*-relationship between the methyl at C-13 and H-14 was inferred by the large coupling constant value of H-14 ($J_{13\text{-}14}$=11.0 Hz), whereas a series of n.O.e. difference experiments provided the relative stereochemistry at C-5, C-8 and C-9. In fact, the irradiation of H-13 (δ 2.55) induced a diagnostic positive enhancement of the methyl signal at δ 1.03 (H_3-17) along with the expected effects on the methyl at δ 0.93 (H_3-16) and the vinyl proton at δ 5.52 (H-12), while the irradiation of H-9 (δ 1.73) resulted in the increasing of the methine signals at δ 2.13 (H-14), δ 0.84 (H-5) and δ 5.59 (H-11), also confirming the assignments made (Table 1). Analogously with verrucosin-7 (**10**), the absolute stereochemistry of the diterpenoid portion of verrucosin-1 (**4**) was suggested to be the same as **13**. In fact, the CD profile of the dihydro derivative of verrucosin-1 was comparable with those of related verrucosins (**2**, **10**, **13**).

Verrucosin-6 (**9**) showed close spectral similarities with **4**, revealing that the only difference was the position of the acetyl group at C-2' of the glyceryl fragment and leading to structure **9**. All spectral data, closely related to those of **4**, are reported in the Experimental.

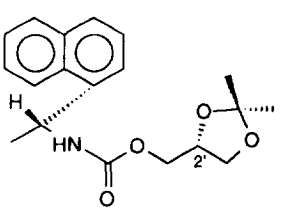
Verrucosin-3 (**6**) was obtained as an amorphous solid and showed a 1,3-diacyl structure. Analysis of both ^{1}H- and ^{13}C-NMR spectra revealed the presence of two trisubstituted double bonds which, together with the two ester functionalities, account for four of the six degrees of unsaturation required by the molecular formula ($C_{25}H_{40}O_5$), the remaining unsaturations being due to two rings. The ^{1}H-NMR spectrum displayed signals for two vinyl methyls at δ 1.62 and δ 1.69, two singlet methyls at δ 0.92 and δ 1.02, and a doublet methyl at δ 0.79. The olefinic proton at δ 5.14 showed in the ^{1}H-^{1}H COSY spectrum cross-peaks with both vinyl methyls and with a methylene at δ 2.08 and δ 2.13, which was in turn coupled to a methylene at δ 1.91, supporting the presence of an isolated 2-methyl-2-pentenyl moiety linked to a bicyclic system. In addition, the olefinic proton at δ 5.41 was connected to a methylene at δ 2.32 and 1.98, further linked to another methylene at δ 1.68 and 1.11, and allylically coupled with the methylene at δ 1.91 of the 2-methyl-2-pentenyl fragment, supporting the location of the second double bond in the ring linked to the chain. Some analogies with the spectral data of verrucosin-A (**2**) were also observed in the ^{1}H- and ^{13}C-NMR spectra of **6** (Tables 1 and 2). In particular, they suggested the presence of the same *cis*-junction between the rings B and C of both metabolites. All spectral data were consistent with the proposed structure **6**, exhibiting a new diterpenoid carbon skeleton that could derive from the same precursor leading to **2** and **5**, through the opening of the ring A and the subsequent transfer of the positive charge to C-4. The relative stereochemistry, suggested as indicated on the basis of biogenetic considerations, has been confirmed by a series of n.O.e. difference experiments. Particularly, diagnostic steric interactions were observed between H-13 (δ 1.89) and the methyls at C-8 (δ 1.02) and C-9 (δ 0.92), clearly indicating that they have the same orientation and also confirming the *cis*-junction between the ring B and C. The irradiation of H-14 (δ 2.46) induced a n.O.e. effect on H_3-16, supporting the *trans*-relationship between H-13 and H-14, also deduced by the large coupling constant value (J=11.5 Hz). Analogously with the other verrucosins, the absolute configuration of verrucosin-3 (**6**) was suggested to be the same of verrucosin-A (**2**), also supported by the CD profile, similar to that of **2**. The *S* stereochemistry at C-2' of the glyceryl moiety was established by applying a chemical method, previously performed on verrucosin-A (**2**).[5] Following the literature procedure, **6** was treated with 2,2-dimethoxypropane, in the presence of a crystal of p-TsOH, giving the acetonide derivative **22** as the main product. After purification by silica-gel chromatography, **22** was reduced with $LiAlH_4$ (THF, reflux) and treated *in situ* with (*R*)-(-)-1-(1-naphtyl)-ethyl isocyanate, affording the urethane **23**, identified by comparative HPLC with a standard compound.[5] Small amounts of the diastereoisomeric urethane **24** were also detected in the reaction mixture, most likely due to the natural co-occurence of the main 1,3-*sn*- diacylglycerol with the minor C-2' epimer.



22



23



24

Verrucosin-8 (**11**) resulted to be the 1,2-diacyl isomer of **6**, as revealed by analysis of ^{1}H-NMR spectrum, which closely resembled that of **6**, differing only for the resonances of H-2' (δ 5.09) and H_2-3' (δ 3.76) of the glyceryl residue. All NMR values were assigned also by comparison with those of **6** (see Experimental).

Finally, verrucosin-2 (**5**) was isolated as an amorphous solid compound and showed a 1,3-diacylated structure. The ^{1}H-NMR spectrum displayed one olefinic proton at δ 5.22 and five methyls: three singlets at δ 0.82, 0.98 and 1.02, a doublet at δ 0.85 and a vinyl singlet at δ 1.62, supporting a tricyclic diterpenoid skeleton containing a trisubstituted double bond. Analysis of both ^{1}H- and ^{13}C-NMR spectra revealed however some analogies with spectral data of verrucosin-A (**2**) and verrucosin-3 (**6**), particularly indicating the presence of the same ring C. The olefinic proton at δ 5.22 (H-3) was directly coupled with two geminal protons at δ 2.00 and δ 2.06, which showed in the ^{1}H-^{1}H COSY experiment cross-peaks with another methylene resonating at δ 1.42 and δ 1.58, further linked to an angular methine at δ 1.90. According to these data and bearing in mind the structure of both verrucosin-A (**2**) and verrucosin-3 (**6**), the double bond was located in the first ring between C-3 and C-4 and consequentially one of the two geminal methyls C-18 and C-19 shifted from C-4 to C-5. All NMR data were in full agreement with the proposed structure **5**, which displays a new carbon skeleton, containing a clerodane-like substructure. Some analogies with spectral data reported in the literature for bicyclic clerodane diterpenes[18] were also observed. However, the upshifted ^{13}C-NMR value of C-6 (δ 31.04) was consistent with the axial substituent at C-8. The large coupling constant value (J=11.8 Hz) between H-13 and H-14 inferred the relative stereochemistry of these two chiral centers as for verrucosin-A (**2**) and verrucosin-3 (**6**). The *trans*-fusion of the rings A and B was suggested by the diagnostic[19] chemical shift of the angular methyl at C-5 (δ 20.74), while the *cis*-junction of the rings B and C was deduced by a series of n.O.e. difference experiments. In fact, strong n.O.e. effects were observed between the methyls at C-5, C-8 and C-9, supporting their same orientation. The irradiation of H_3-18 (δ 1.02) and H_3-17 (δ 0.98) resulted in the increase of the signal at δ 0.82 (H_3-20), whereas the irradiation of H_3-20 induced an enhancement on both the methyls H_3-17 and H_3-20. In addition, diagnostic steric interactions were also observed between the two spatially close protons H-10 (δ 1.90) and H-14 (δ 2.90). Similarly with the other verrucosins, the absolute configuration of verrucosin-2 (**5**), exibiting a positive CD curve similar to those of the other verrucosins, was suggested to be the same of verrucosin-A (**2**).

The absolute configuration of the diterpenoid part of the new verrucosins, with the exception of **7** and **8**, was suggested to be the same as verrucosin-A (**2**) and -B (**3**), on the basis of obvious biogenetic considerations. However, recording the CD curves of all compounds, we observed a substantial similarity of the CD profiles of verrucosins, except for verrucosin-1 (**4**) and verrucosin-6 (**2**), both displaying a more complex curve. Anyway, the hydrogenated derivative of **4** gave a CD profile similar to all other verrucosins.

The *S* absolute configuration at C-2' of the glyceryl moiety of verrucosin-3 (**6**), identical to those of verrucosin-A (**2**) and verrucosin-B (**3**),[5] suggests the same stereochemistry for all the other verrucosins.

The finding of acyclic, bicyclic and tricyclic related diterpenoid moieties in the series of verrucosins suggests a hypothetical biogenetic correlation, which starting from **7** connects all the other compounds (Scheme 1).

Pharmacological activities of the novel verrucosins have also been investigated.[20] In particular, the 1,3-*sn*-diacyl glycerols verrucosin-1 (**4**), verrucosin-3 (**6**) and verrucosin-7 (**10**), together with the corresponding 1,2-*sn*- derivatives verrucosin-6 (**9**), verrucosin-8 (**11**) and verrucosin-9 (**12**) have been tested in the *Hydra* tentacle regeneration *in vivo* assay and in the protein kinase C activation *in vitro* assay. As already reported for

verrucosin-A (**2**) and verrucosin-B (**3**),[7] the novel verrucosins also displayed potent PKC stimulatory effects *in vitro* and PKC-mediated morphogenetic properties *in vivo* in the *Hydra* test, suggesting the use of these compounds as useful molecular probes for cell development studies.

EXPERIMENTAL

General procedures. Si-gel chromatography was performed using precoated Merck F_{254} plates and Merck Kieselgel 60 powder. HPLC purifications were carried out on a Waters liquid chromatograph equipped with a differential refractometer as detector. Optical rotations were measured on a Jasco DIP 370 digital polarimeter and CD curves were recorded on Jasco 710 spectropolarimeter. The IR spectra were taken on a Bio-Rad FTS 7 spectrophotometer. The UV spectra were obtained on a Varian DMS 90 spectrophotometer. ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker WM 500 MHz spectrometer in $CDCl_3$; chemical shifts are reported in ppm referred to $CHCl_3$ as internal standard (δ 7.26 for proton and δ 77.00 for carbon). EIMS and HREIMS spectra were measured on a TRIO 2000 VG Carlo Erba and on a Kratos MS50 instruments, respectively.

Biological material. Doris verrucosa (385 individuals, average lenght 2.5 cm) was collected in the Bay of Naples by hand using SCUBA, during June 1992 at a depth of 5 meters. A specimen is available at Istituto per la Chimica di Molecole di Interesse Biologico, for inspection (n° T107).

Extraction and isolation procedure. Doris verrucosa specimens (dry weight 87.1 g) were carefully dissected in mantle and digestive gland. The mantles were extracted by acetone at room temperature four times (250 ml x 4). The acetone extract was evaporated at reduced pressure and the residual water was extracted with diethyl ether. The ethereal part was concentrated to give 1.61 g of crude material, which was chromatographed on a Si-gel column, using light petroleum ether with increasing amounts of diethyl ether. The fractions eluted by light petroleum ether/diethyl ether, 1:1, containing verrucosins, were submitted to HPLC purification [Spherisorb S5W, 7.8 mm (ID) x 30 cm; *n*-hexane/ethyl acetate, 85:15; flow rate 3.8 ml/min], giving, in order of *Rt*, verrucosin-A (**2**, 64.0 mg), verrucosin-1 (**4**, 7.0 mg), the mixture A (52.0 mg), the mixture B (7.1 mg) and finally verrucosin-B (**3**, 16.6 mg). The mixture A was further chromatographed by HPLC [Spherisorb ODS2-5W, 7.8 mm (ID) x 30 cm; CH_3CN/H_2O, 75:25; flow rate 4.0 ml/min] affording verrucosin-2 (**5**, 7.5 mg), verrucosin-3 (**6**, 24.0 mg) and the known compound **13** (14.4 mg). The mixture B (7.1 mg) was analogously submitted to HPLC purification [Spherisorb ODS2-5W, 3.9 mm (ID) x 30 cm; CH_3CN/H_2O, 8:2; flow rate 1.0 ml/min], obtaining verrucosin-4 (**7**, 3.5 mg) and verrucosin-5 (**8**, 2.7 mg). Also the fractions eluted by light petroleum ether/diethyl ether, 3:7, containing verrucosins, were subjected to HPLC chromatography [Spherisorb S5W, 7.8 mm (ID) x 30 cm; *n*-hexane/ethyl acetate, 8:2; flow rate 3.8 ml/min], obtaining, in order of *Rt*, verrucosin-6 (**9**, 1.1 mg), verrucosin-7 (**10**, 3.5 mg), verrucosin-8 (**11**, 0.8 mg), the known compound **14** (1.0 mg) and verrucosin-9 (**12**, 1.0 mg).

Verrucosin-1 (4). 7.0 mg; $[\alpha]_D$ -48.7° ($CHCl_3$, 0.7); CD (EtOH) $[\theta]_{213}$ -3,665, $[\theta]_{232}$ +1,212; IR (liquid film) ν_{max} 3477, 1739, 1238 cm^{-1}; EIMS, *m/z* (%): 420 (M^+, 0.4), 402 (4), 286 (8), 258 (73), 190

(41), 120 (100); HREIMS, *m/z* 286.2285 ($C_{20}H_{30}O$, M^+- acylglycerol requires 286.2297); ^{1}H-NMR data in Table 1; ^{13}C-NMR data in Table 2.

Verrucosin-2 (5). 7.5 mg; $[\alpha]_D$ -4.8° ($CHCl_3$, 0.75); CD $[\theta]_{215}$ (EtOH) +5,951; IR (liquid film) ν_{max} 3482, 1733, 1233 cm^{-1}; EIMS, *m/z* (%): 420 (M^+, 4), 402 (3), 286 (11), 258 (7), 243 (10), 189 (20), 117 (100); HREIMS, *m/z* 286.2230 ($C_{20}H_{30}O$, M^+- acylglycerol requires 286.2297); ^{1}H-NMR data in Table 1; ^{13}C-NMR data in Table 2.

Verrucosin-3 (6). 24.0 mg; $[\alpha]_D$ +46.8° ($CHCl_3$, 2.4); CD $[\theta]_{223}$ (EtOH) +3,065; IR (liquid film) ν_{max} 3460, 1728, 1244 cm^{-1}; EIMS, *m/z* (%): 420 (M^+, 2), 377 (3), 286 (7), 258 (6), 243 (11), 215 (14), 189 (62), 117 (100); HREIMS, *m/z* 286.2251 ($C_{20}H_{30}O$, M^+- acylglycerol requires 286.2297); ^{1}H-NMR data in Table 1; ^{13}C-NMR data in Table 2.

Verrucosin-4 (7). 3.5 mg; $[\alpha]_D$ -9.7° ($CHCl_3$, 0.35); IR (liquid film) ν_{max} 3482, 1750, 1718, 1223 cm^{-1}; UV (MeOH) λ_{max} 223 (ε=8,930) nm; EIMS, *m/z* (%): 420 (M^+, 9), 286 (10), 217 (7), 198 (10), 149 (15), 136 (35), 117 (45), 81 (65), 69 (100); ^{1}H-NMR data in Table 1; ^{13}C-NMR data in Table 2.

Verrucosin-5 (8). 2.7 mg; $[\alpha]_D$ -15.9° ($CHCl_3$, 0.27); IR (liquid film) ν_{max} 3457, 1745, 1725, 1225 cm^{-1}; UV (MeOH) λ_{max} 224 (ε=5,320) nm; EIMS, *m/z* (%): 420 (M^+, 3), 405 (13), 347 (8), 305 (9), 286 (8), 271 (13), 244 (15), 198 (30), 117 (100); ^{1}H-NMR data in Table 1; ^{13}C-NMR data in Table 2.

Verrucosin-6 (9). 1.1 mg; $[\alpha]_D$ -37.0° ($CHCl_3$, 0.1); CD (EtOH) $[\theta]_{214}$ -2,612, $[\theta]_{232}$ +1,266; IR (liquid film) ν_{max} 3477, 1734, 1238 cm^{-1}; EIMS, *m/z* (%): 420 (M^+, 0.5), 402 (1), 360 (0.5), 286 (12), 258 (34), 243 (10), 190 (20), 117 (60), 69 (100); ^{1}H-NMR: δ 5.58 (1H, bd, *J*=10.3 Hz, H-11), 5.52 (1H, bd, *J*=10.3 Hz, H-12), 5.10 (1H, m, H-2'), 4.36 (1H, dd, *J*=12.0 and 4.5 Hz, H-1'a), 4.20 (1H, dd, *J*=12.0 and 5.7 Hz, H-1'b), 3.76 (2H, m, H_2-3'), 2.55 (1H, m, H-13), 2.10 (1H, d, *J*=10.8 Hz, H-14), 2.10 (3H, s, Ac), 1.73 (1H, m, H-9), 1.01 (3H, s, H_3-17), 0.92 (3H, d, *J*=6.9 Hz, H_3-16), 0.85 (3H, s, H_3-19), 0.83 (3H, s, H_3-20), 0.80 (3H, s, H_3-18); ^{13}C-NMR: δ 132.20 (C-12), 125.17 (C-11), 72.42 (C-2'), 61.91 (C-14), 61.77 (C-1'), 61.61 (C-3'), 58.56 (C-9), 56.19 (C-5), 42.10 (C-3), 39.16 (C-1), 39.10 (C-7), 33.16 (C-19), 32.13 (C-13), 21.16 (C-18), 20.99 (Ac), 19.82 (C-16), 18.44 (C-2 or C-6), 18.25 (C-6 or C-2), 16.47 (C-20), 14.79 (C-17).

Verrucosin-7 (10). 3.5 mg; $[\alpha]_D$ +19.1° ($CHCl_3$, 0.35); CD $[\theta]_{222}$ (EtOH) +3,663; IR (liquid film) ν_{max} 3411, 1732, 1705, 1269 cm^{-1}; EIMS, *m/z* (%): 456 (M^+, 2), 441 (8), 420 (3), 403 (5), 364 (4), 347 (4), 287 (46), 286 (43), 259 (55), 244 (20), 191 (100), 117 (95); HREIMS, *m/z* 456.2464 ($C_{25}H_{41}O_5Cl$ requires 456.2642); ^{1}H-NMR data in Table 1; ^{13}C-NMR data in Table 2.

Verrucosin-8 (11). 0.8 mg; $[\alpha]_D$ +41.5° ($CHCl_3$, 0.05); CD $[\theta]_{223}$ (EtOH) +1,454; IR (liquid film) ν_{max} 3471, 1738, 1242 cm^{-1}; EIMS, *m/z* (%): 420 (M^+, 2), 377 (4), 347 (2), 335 (4), 286 (45), 258 (17), 243 (44), 215 (37), 189 (100), 117 (99); ^{1}H-NMR: δ 5.42 (1H, bs, H-5), 5.15 (1H, m, H-3), 5.09 (1H, m, H-2'), 4.32 (1H, dd, *J*=12.0 and 4.3 Hz, H-1'a), 4.24 (1H, dd, *J*=12.0 and 5.7 Hz, H-1'b), 3.76 (2H, m, H_2-3'), 2.45 (1H, d, *J*=11.6 Hz, H-14), 2.10 (3H, s, Ac), 1.69 (3H, bs, H_3-19), 1.63 (3H, bs, H_3-18), 1.02 (3H, s, H_3-17), 0.93 (3H, s, H_3-20), 0.79 (3H, d, *J*=6.3 Hz, H_3-16); ^{13}C-NMR: δ 175.06 (C-15), 170.62 (Ac),

141.13 (C-10), 131.44 (C-4), 124.70 (C-3), 120.44 (C-5), 72.57 (C-2'), 61.74 (C-1'), 61.53 (C-3'), 53.08 (C-14), 41.18 (C-9), 37.87 (C-8), 31.67 (C-12), 30.81 (C-11), 30.27 (C-1), 30.04 (2 C, C-7 and C-13), 26.61 (C-2), 25.70 (C-19), 24.62 (C-20), 22.06 (C-6), 20.92 (C-16), 20.77 (Ac), 18.59 (C-17), 17.71 (C-18).

*Verrucosin-9 (**12**).* 1.0 mg; $[\alpha]_D$ +25.0° ($CHCl_3$, 0.04); CD $[\theta]_{222}$ (EtOH) +3,096; IR (liquid film) ν_{max} 3412, 1733, 1707, 1265 cm^{-1}; EIMS, *m/z* (%): 456 (M^+, 1), 441 (3), 420 (1), 403 (2), 364 (3), 347 (2), 287 (17), 286 (20), 259 (23), 244 (10), 204 (14), 191 (100), 117 (97); ^{1}H-NMR: δ 5.10 (1H, m, H-2'), 4.39 (1H, dd, *J*=12.0 and 4.3 Hz, H-1'a), 4.24 (1H, dd, *J*=12.0 and 5.3 Hz, H-1'b), 3.78 (2H, m, H_2-3'), 2.30 (1H, s, H-14), 1.52 (3H, s, H_3-16), 1.34 (3H, s, H_3-17), 0.88 (3H, s, H_3-20), 0.84 (3H, s, H_3-19), 0.81 (3H, s, H_3-18); ^{13}C-NMR: δ 72.34 (C-2'), 66.18 (C-14), 61.77 (C-1' or C-3'), 61.67 (C-3' or C-1'), 60.04 (C-9), 56.79 (C-5), 45.13 (C-12), 42.07 (C-3), 41.43 (C-7), 39.92 (C-1), 34.14 (C-16), 33.27 (C-19), 21.23 (C-18), 21.00 (Ac), 18.51 (C-2), 17.87 (C-6), 17.77 (C-11), 16.69 (C-17), 16.31 (C-20).

*Synthesis of verrucosin-4 (**7**).*

a) (*E,E,E*)-geranylgeranoyl chloride (**17**). A solution of $(COCl)_2$ (0.22 ml) in dry C_6H_6 (1.5 ml) was added to a solution of geranylgeranoic acid (150.0 mg) in dry C_6H_6 (1.5 ml). The mixture was stirred, under Ar atmosphere, at r.t. for 2 hr and then at 45°C for 30 min. At the end of this period, the reaction was stopped and the solvent was removed *in vacuo* to give 155.0 mg of crude **17**, which was used in the next step without purification.

b) 1-(*E,E,E*)-geranylgeranoyl-2,3-O-isopropylidene-*sn*-glycerol (**19**). A suspension of NaH (56.1 mg, 80% in oil) and 7 drops of dry pyridine were added to a solution of (-)-2,3-O-isopropylidene-*sn*-glycerol (**18**, 233.0 mg) in CH_2Cl_2 (5.6 ml), at 0° under Ar atmosphere. The reaction mixture was stirred for 10 min and then crude **17** (150.0 mg) in CH_2Cl_2 (5 ml) was added. After stirring for 20 min, the reaction was monitored by TLC and then stopped. Usual work-up afforded 198.5 mg of a crude reaction product, which was purified by Si-gel chromatographic column (light petroleum ether/diethyl ether, 93:7) obtaining 178.8 mg of pure acetonide **19**. $[\alpha]_D$ 2.2° ($CHCl_3$, c=0.62); IR (liquid film) ν_{max} 1718, 1217 cm^{-1}; ^{1}H-NMR:[21] δ 5.71 (1H, s, H-14), 5.09 (3H, H-3, H-5 and H-9 overlapped), 4.33 (1H, m, H-2'), 4.18 (1H, dd, *J*=11.5 and 4.8 Hz, H-1'a), 4.09 (1H, dd, *J*=11.5 and 6.0 Hz, H-1'b), 4.07 (1H, m, H-3'a), 3.75 (1H, dd, *J*=8.3 and 6.4 Hz, H-3'b), 2.16 (3H, bs, H_3-16), 1.67 (3H, bs, H_3-19), 1.59 (9H, bs, H_3-17, H_3-18 and H_3-20), 1.43 (3H, s, CH_3 acetonide), 1.37 (3H, s, CH_3 acetonide); ^{13}C-NMR:[21] 166.34 (C-15), 161.17 (C-13), 136.20 (C-8), 135.00 (C-10), 131.22 (C-4), 124.32 (C-3), 124.03 (C-5), 122.74 (C-9), 114.91 (C-14), 109.73 (C quaternary acetonide), 73.76 (C-2'), 66.45 (C-1' or C-3'), 63.89 (C-3' or C-1'), 40.98 (C-12), 39.66 (C-1 and C-7), 26.70 (C-2 or C-6), 26.54 (C-6 or C-2), 25.92 (C-11), 25.66 (C-19), 25.38 (2C, CH_3 acetonide), 18.93 (C-16), 17.64 (C-18), 15.99 (C-17 and C-20).

c) 1-(*E,E,E*)-geranylgeranoyl-*sn*-glycerol (**20**). 100.0 mg of **19** were dissolved in 0.5 ml of CH_3OH and then 4.3 ml of H_2SO_4/CH_3OH (0.006M) were added. The mixture was stirred at r.t. for 2 hr, under Ar atmosphere. Usual work-up gave 89.7 mg of a crude reaction product, which was chromatographed on a silica-gel column (light petroleum ether with increasing amounts of diethyl ether), to afford 77.3 mg (85.5 % from **19**) of pure **20**. $[\alpha]_D$ -0.3° ($CHCl_3$, c=0.58); IR (liquid film) ν_{max} 3423, 1728, 1647, 1217 cm^{-1}; ^{1}H-NMR:[21] δ 5.70 (1H, s, H-14), 5.08 (3H, H-3, H-5 and H-9 overlapped), 4.21 (1H, dd 11.7 and 4.8 Hz, H-1'a), 4.17 (1H, dd, 11.7 and 6.0 Hz, H-1'b), 3.93 (1H, m, H-2'), 3.68 (1H, dd, 11.5 and 4.0 Hz, H-3'a), 3.59 (1H, dd, 11.5

and 5.8 Hz, H-3'b, 2.17 (3H, bs, H_3-16), 1.67 (3H, bs, H_3-19), 1.59 (9H, bs, H_3-17, H_3-18 and H_3-20); ^{13}C-NMR:[21] 167.11 (C-15), 162.04 (C-13), 136.34 (C-8), 135.08 (C-10), 131.28 (C-4), 124.33 (C-3), 124.02 (C-5), 122.68 (C-9), 114.66 (C-14), 70.41 (C-2'), 64.49 (C-1' or C-3'), 63.32 (C-3' or C-1'), 41.05 (C-12), 39.63 (C-1 and C-7), 26.73 (C-2 or C-6), 26.56 (C-6 or C-2), 25.93 (C-11), 25.66 (C-19), 19.04 (C-16), 17.65 (C-18), 15.97 (C-17 and C-20).

d) 1-(*E,E,E*)-geranylgeranoyl-3-acetyl-*sn*-glycerol (**7**). 2.7 mg of DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) and 8.3 mg of N-acetyl-imidazole were added to 26.0 mg of compound **20**, dissolved in 0.5 ml of dry C_6H_6. The mixture was stirred at r.t. for 1 hr, then the reaction was stopped and, after usual work-up, 24.0 mg of crude product were obtained. Si-gel cromatography (light petroleum ether with increasing amounts of Et_2O) of the crude reaction mixture afforded 15.2 mg (52.7 % from **20**) of pure **7**, identical with natural product. ($[\alpha]_D$, ^{1}H-NMR).

Methanolysis of verrucosin-5 (8). 1 mg of verrucosin-5 (**8**) was dissolved in 0.5 ml of CH_3OH an. and Na_2CO_3 an. was added. The solution was stirred at r.t. for 24 hrs. After usual work-up, 0.5 mg of methyl ester derivative **21** was obtained. $[\alpha]_D$ -26.1° ($CHCl_3$, c=0.05); $[\alpha]_D$ lit. -11.4°.

Isolation of methyl isocopate (21). 110 g of commercial "Copaiva Balsam", constituted by a complex mixture of isomeric diterpenoid acids and lipids, were dissolved in 700 ml of Et_2O and extracted with 3% NaOH/H_2O (400 ml x 3 times). After removing the water, the crude acid portion of the mixture (46.1 g) was methylated with 600 ml of an Et_2O solution of diazomethane and submitted to chromatographic column (Si-gel, light petroleum ether/Et_2O gradient) obtaining 39.2 g of methyl ester derivatives of the diterpenoid acids. Hydrolysis with 5% NaOH/EtOH of the methyl ester mixture gave a saponified fraction (5.6 g), which, after separation from the remaining methyl esters not hydrolized in these conditions, was again methylated by adding 100 ml of CH_2N_2/Et_2O and further purified on a chromatographic column (Si-gel, light petroleum ether/Et_2O gradient) affording 4.9 g of methyl ester derivatives containing methyl isocopalate (**21**). This mixture was dissolved in 10 ml of Et_2O and treated with 30 ml of Et_2O solution of monoperphtalic acid, under stirring for 3 hrs. The reaction mixture was then washed with 2% NaOH/H_2O solution and extracted with H_2O; the organic solvent was removed giving 5.1 g of crude material. Chromatographic separation on a Si-gel column, using light petroleum ether/diethyl ether gradient, afforded two main fractions, one of which (2.16 g), containing **21**, was further submitted to MPLC purification, (RP-18 column, MeOH/H_2O, 9:1), giving 398.1 mg of pure methyl isocopalate (**21**). $[\alpha]_D$ -14.6° ($CHCl_3$, c=1.4).

Reaction of 6 with 2,2-dimethoxypropane. 4.1 mg of **6** were dissolved in 0.5 ml of 2,2-dimethoxypropane, and a catalytic amount of *p*-TsOH was added.The reaction mixture was stirred at r.t. for 72 hr., then the solvent was partially removed under N_2 flow. The residual solution was chromatographed on a SiO_2 column (light petroleum ether/diethyl ether gradient) affording 2.2 mg of the acetonide **22**.

Reduction of 22 and reaction with (R)-(-)-1-(1-naphtyl)-ethyl isocyanate. **22** (2.2 mg) was dissolved in an. THF (100 µl) and 100 µl of a $LiAlH_4$, THF 0.1 M solution were added. The reaction mixture was stirred at 70°C for 3.5 hr., then MeOH (some drops) was added to destroy $LiAlH_4$ excess. The mixture was treated with (*R*)-(-)-1-(1-naphtyl)-ethyl isocyanate (150 µl) and kept at 76°C for 4 days. At the end of this period, the reaction was stopped. The mixture was then purified by SiO_2 chromatographic column (light petroleum

ether/diethyl ether gradient) to give the main urethane **23** and small amounts of the diastereoisomer **24**, which were compared with standard compounds by HPLC (two Spherisorb 5 Sil columns connected in series, using as eluent *n*-hexane/i-PrOH, 99:1). Standards were prepared as previously described.[5]

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