Studies of the Biogenesis of Verrucosins, Toxic Diterpenoid Glycerides of the Mediterranean Mollusc *Doris verrucosa*

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Dedicated to the memory of Prof. Guido Sodano

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The biogenesis of verrucosins, diterpenoid glycerides of the marine mollusc *Doris verrucosa*, has been investigated by feeding experiments with labelled precursors. Incorporation of radioactive D-[U-^{14}C]-glucose into the diterpenes proved the de novo origin of verrucosins in the mollusc. Biogenesis of glycerol and terpenoid substructures has been investigated by feeding experiment with ^{13}C-labelled precursors.

The shell-less molluscs of the order Nudibranchia have developed a complex strategy of defence that includes the use of substances noxious to potential predators.[1] Most of the defence products are derived from the diet, but de novo biosynthesis has also been claimed to explain the origin of some of these metabolites.[1] In particular, such an ability has been repeatedly demonstrated in molluscs of the families Dendrodoridídae and Doridídae.[2]

Verrucosins **1**−**13** are toxic esters of *sn*-glycerol isolated from the mantle of the Mediterranean mollusc *Doris verrucosa* (Family Doridídae).[3] The group of glycerides is composed of eight different diterpenoid skeletons. Two series of verrucosins are clearly distinguishable: 1-diterpenoyl-2-acetyl-*sn*-glycerol and 1-diterpenoyl-3-acetyl-*sn*-glycerol. Migration of the acetyl from C(2)−OH to C(3)−OH occurs even under very mild acidic conditions, thus 1,3-diacyl-*sn*-glycerides are probably formed during the chemical workup of the extracts. Verrucosin A (**1**) and B (**2**) are nearly 80% by weight of the entire fraction and, apart from the position of the acetyl group, their molecular arrangement might represent the target of the biosynthetic process. The glycerides **1**−**13** have relevant biological activities, including a well-documented role as defensive compounds[3] and activators of the protein kinase C (PKC).[4]

A few pieces of evidence have been collected in support of the de novo biosynthesis of verrucosins in *D. verrucosa*: (a) searching for **1**−**13** or their precursors in the potential preys of *D. verrucosa* (e.g. *Hymeniacidon sanguinea*) has been fruitless; (b) most of the verrucosins **1**−**13** have unprecedented terpenoid skeletons; (c) these metabolites are selectively present only in the mantle extract of the nudibranch. *D. verrucosa* is able to metabolise exogenous precursors, since injection of radioactive mevalonate gives incorporation into the sterol pool.[3d] However, earlier experiments with radioactive mevalonate or glycerol failed to give significant levels of incorporation in **1**−**13**, although similar methodologies succeeded in labelling the diterpenoyl glycerides **14**−**17** of other dorid nudibranchs.[5]

Here we report for the first time direct evidence of the biosynthesis de novo of verrucosins in *D. verrucosa*. Furthermore, the paper also describes the results of feeding experiments with ^{13}C-labelled glucose and pyruvate, with interesting implications for the origin of the glycerides in the mollusc.

Results and Discussion

Mantle sections of *D. verrucosa* (six specimens) fed with [U-^{14}C]glucose were extracted as reported in the Exp. Section. Column separation of the Et_2O-soluble material (33.5 mg; 881 dpm/mg) gave clear labelling of fractions containing verrucosins (6.6 mg; 1196 dpm/mg). Further HPLC purification of this fraction on normal phase HPLC (*n*-hexane/ethyl acetate, 85:15) led to verrucosin A (**1**, 0.8 mg, 2016 dpm/mg), verrucosin-1 (**5**, 0.2 mg, 6720 dpm/mg), a mixture of verrucosin-2 (**7**) and verrucosin-3 (**8**) (0.3 mg, 1440 dpm/mg), verrucosin B (**2**, 1.2 mg, 3500 dpm/mg), and a mixture of the remaining glycerides (0.6 mg, 1400 dpm/mg). Compound identities were ascertained by ^{1}H NMR

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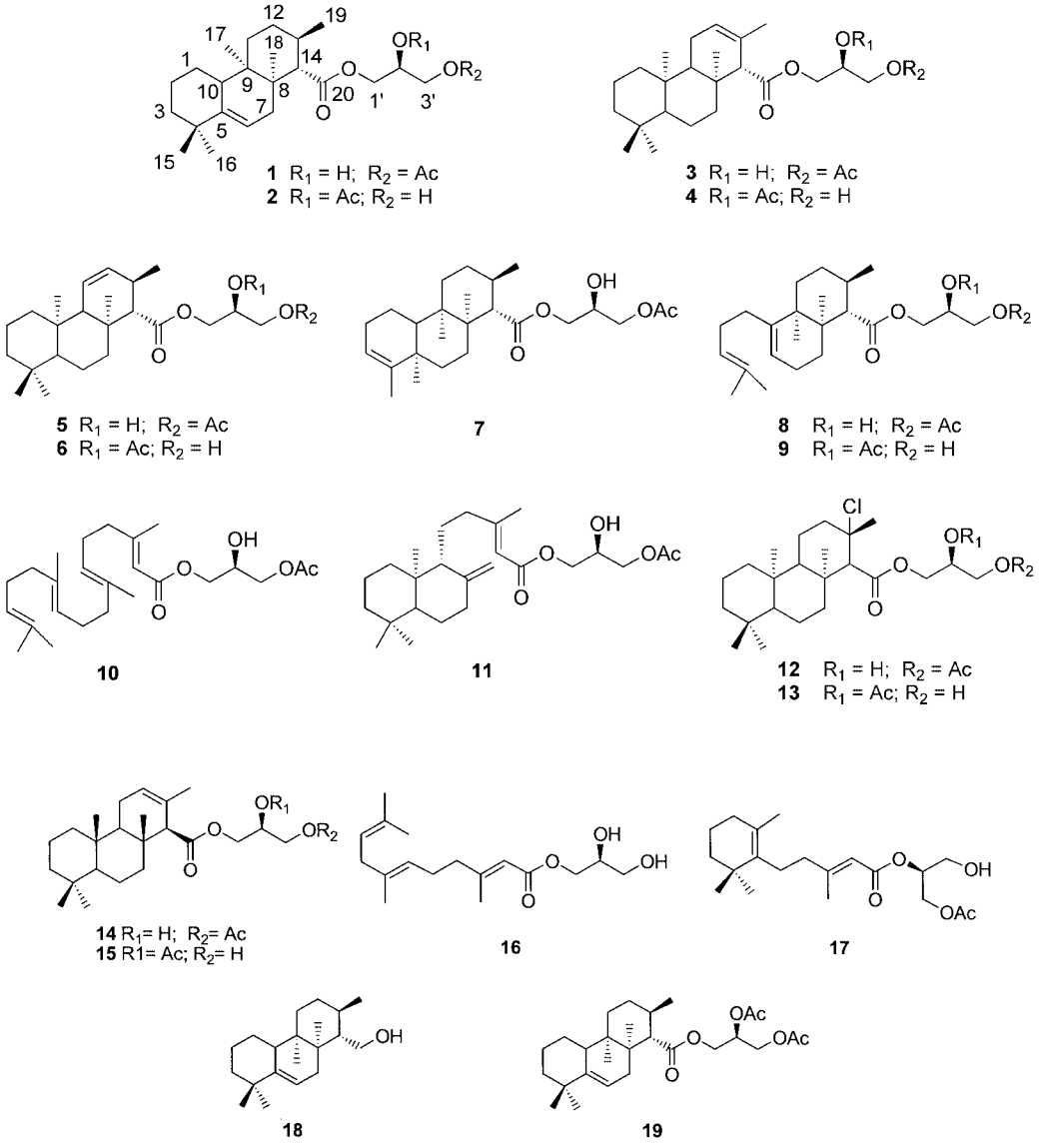


spectra and HPLC co-elution with cold material. To confirm the incorporation of radioactivity in the terpenoid part of the molecules, verrucosin B (**2**) was reduced with $LiAlH_4$ in THF (refluxed for 12 h).[3a] After quenching, the reaction mixture was purified to give a major component (0.4 mg) that was identified as **18** on the basis of co-elution with cold material obtained by reduction of a mixture of natural verrucosins A and B.[3a] Radioactivity counting of this product showed a low, but significant, level of incorporation (3110 dpm/mg, less than 0.1% incorporation). The remaining verrucosins were not further fractionated, although the HPLC peaks that contained them showed satisfactory levels of radioactivity. These data (Table 1) provided the first experimental evidence for de novo biosynthesis of verrucosins in *D. verrucosa*. Surprisingly, no trace of labelling was found in the crude sterol fraction (38 dpm/mg), thus giving results totally different from those recorded using radioactive mevalonate that had led to high incorporation into the sterols.[3]

Table 1. Levels of radioactivity found in verrucosin and verrucosin-derived compounds

1st purification[a]		2nd purification[b]		3rd purification[c]	
Sterol	38 dpm/mg	**1**	2016 dpm/mg	**18**	3110 dpm/mg[d]
Fatty acid	21 dpm/mg	**2**	3500 dpm/mg	blank	23 dpm
1−**13**	1196 dpm/mg	**5**	6720 dpm/mg		
blank	23 dpm	blank	19 dpm		

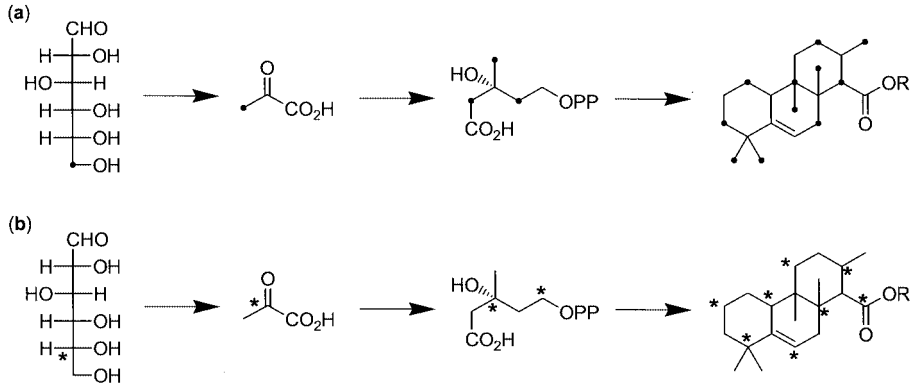
[a] After fractionation of the extract. [b] After HPLC. [c] After reduction. [d] The slight decrease of the specific radioactivity was probably due to the loss of labelled glycerol. The specific radioactivity was established after two purifications.

Prompted by these results, we started investigating verrucosin biosynthesis by using ^{13}C-labelled precursors. Like the other opisthobranchs, *D. verrucosa* is a seasonal organism, with an adult stage in July. Experiments with ^{13}C-labelled tracers were performed using different feeding procedures

Table 2. Apparent ^{13}C incorporation for acetyl verrucosin A (**19**) and verrucosin A (**1**). Signal enhancement is based on feeding experiments with [6-^{13}C]glucose for **19** and with [5-^{13}C]glucose for **1**

	19[a]		**1**[b]			**19**		**1**	
	ppm	Enhancement %	ppm	Enhancement%		ppm	Enhancement %	ppm	Enhancement %
1	28.4	2.0	28.4	3.3[c]	**15**	29.5	9.0	29.5	n.a.[d]
2	22.9	7.0	22.8	8.7	**16**	28.1	2.0	28.3	3.3[c]
3	40.7	17.0	40.7	0.0	**17**	18.7	14.0	18.7	0.0
4	37.8	0.0[b]	37.9	6.5	**18**	18.0	17.0	18.0	0.0
5	144.7	1.0	145.0	0.0	**19**	21.3	7.0	21.4	5.3
6	113.9	3.0	113.8	5.3	**20**	174.7	0.0	175.3	22.9
7	34.9	8.0	34.9	0.0	**C1′**	61.3	1.0	65.2	0.0
8	36.0	1.0	36.0	8.8	**C2′**	69.2	2.0	68.3	14.3
9	37.2	11.0	37.2	0.0	**C3′**	62.4	16.0	64.3	0.0
10	36.3	2.0	36.3	18.4	$\mathbf{Ac_{CO}}$	170.4	3.0	170.9	13.9
11	29.6	17.0[e]	29.7	25.9[f]	$\mathbf{Ac_{Me}}$	20.9	3.0	20.7	0.0[b]
12	29.6	17.0[e]	29.7	25.9[f]	$\mathbf{Ac_{CO}}$	170.0	19.0		
13	29.0	2.0	28.9	3.7	$\mathbf{Ac_{Me}}$	20.7	1.0		
14	51.6	9.0	51.9	0.0					

[a] ^{13}C assignments were based on 1D and 2D NMR experiments. [b] Prior to the comparison to unlabelled material, spectra were normalised to C4 for acetyl verrucosin A (**19**) and the methyl group of the acetyl residue for verrucosin A (**1**). [c] Enrichment not calculable for signal overlapping. [d] n.a. = not attributable. [e] Enrichment not calculable for signal overlapping. [f] Enrichment not calculable for signal overlapping.



Scheme 1. Expected labelling of the diterpenoid skeleton of verrucosin A (**1**) from feeding experiment with [6-^{13}C]glucose (**a**) and [5-^{13}C]glucose (**b**) via acetate/mevalonate path

on nudibranchs collected for three summers in succession. Analyses of the first set of experiments ([6-^{13}C]glucose and [2-^{13}C]pyruvate) were carried out on acetyl verrucosin A (**19**) which was easily obtained through the acetylation of the raw mixture of verrucosins. In a subsequent experiment, incorporation of [5-^{13}C]glucose was directly monitored by the effect on the ^{13}C NMR spectrum of verrucosin A (**1**). Despite our efforts, we were not able to increase the levels of isotopic enrichment, which, whatever the experimental conditions, never gave signal enhancement higher than 20%. It should be noted that the ester carbonyl (C20) of **1** was originally misassigned to the resonance at δ = 170.9 ppm.[3a] Re-examination of the HMBC spectrum of **1** showed correlations from a proton at δ = 2.32 (14-H) to the carbon at δ = 174.9 ppm (C20) and from the methyl at δ = 2.08 (acetyl residue) to 170.9 ppm, thus leading to a reversal in the original assignment.[3a]

Table 2 summarises the ^{13}C-enrichment of **1** and **19** isolated from molluscs provided with [6-^{13}C]glucose and [5-^{13}C]glucose. The poor incorporation did not allow us to

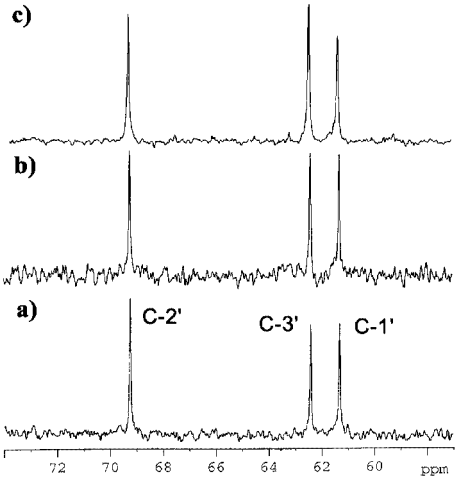
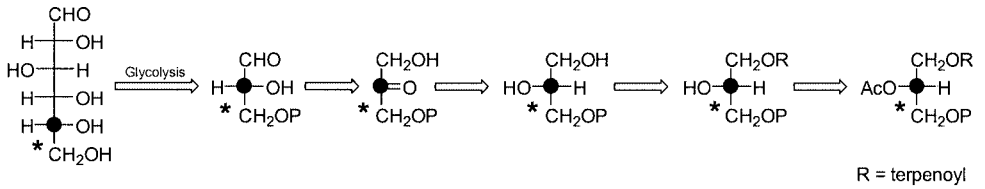


Figure 1. Labelling of glycerol carbons of **19** from control (**a**), [2-^{13}C]pyruvate (**b**) and [6-^{13}C]glucose (**c**) experiments



Scheme 2. Proposal for the verrucosin-producing condensation reactions. The labelling pattern of glycerol is deduced from feeding experiments with [6-^{13}C]glucose (*) and [5-^{13}C]glucose (●)

draw a definite conclusion for the biosynthesis of the verrucosin's isoprene part, although the labelling patterns in both experiments are consistent with an acetate/mevalonate origin (Scheme 1).[6] In fact, according to this hypothesis, experiments with [6-^{13}C]glucose led to incorporation of the methyl groups and of C3 (17%), C9 (11%), and C14 (9%). Significantly, no enhancement was observed for these carbons in the experiment with [5-^{13}C]glucose, which, in agreement with the expected labelling (Scheme 1), gave selective incorporation of C4 (6.5%), C8 (8.8%), C10 (18.4%), and C20 (22.9%).

Furthermore, in both experiments, the conversion of glucose to acetate was also proved by the specific enrichment of C1 (from [5-^{13}C]glucose) and C2 (from [6-^{13}C]glucose) of the acetyl groups linked to glycerol.

Unlike the terpenoid part, the experiments with labelled precursors were useful to determine unambiguously the biosynthesis of verrucosin's glycerol. As illustrated in Figure 1, molluscs injected with [6-^{13}C]glucose showed clear enrichment of C3′ (δ = 62.4, 16.0±3.1% signal enhancement) (Figure 1, c), whereas we recorded simultaneous incorporation at C1′ (δ = 61.3, 14.4±3.3% signal enhancement) and C3′ (δ = 62.4, 14.2±2.7% signal enhancement) in the experiment with [2-^{13}C]pyruvate (Figure 1, b). In the same way, analysis of samples of verrucosin A (**1**) were selectively labelled at C2′ (δ = 68.3, 14.3±2.4% signal enhancement) in experiments with [5-^{13}C]glucose,[7] thus confirming the origin of *sn*-glycerol from glycolysis-derived D-glyceraldehyde 3-phosphate (GAP) (Scheme 2). However there is no simple explanation for the labelling pattern recorded from the feeding experiment with [2-^{13}C]pyruvate. The simultaneous enrichment of C1′ and C3′, in fact, is not consistent with the direct conversion of pyruvate to GAP via gluconeogenesis or the citrate cycle, since such paths should lead to the specific labelling of C2′. It is interesting to note that symmetric labelling at both ends of glycerol has also been reported in feeding experiment with [1,2-^{13}C$_2$]acetate that gave simultaneous incorporation of C$_2$-units at C1′/C2′ and C2′/C3′ in the terpenoid glycerides **14**−**16**.[5b]

Conclusion

In conclusion, this work demonstrates the de novo biosynthesis of verrucosins **1**−**13** in the Mediterranean nudibranch *D. verrucosa*. As depicted in Scheme 2, the labelling pattern of glycerol is consistent with the stereoselective esterification of GAP-derived *sn*-glycerol 3-phosphate. From this line of reasoning, the production of verrucosins would follow a biosynthetic pathway very similar to the condensation process that leads to L-phosphatidate in the biosynthesis of phospholipids and triglycerides. On the contrary, we are not able to rationalise the labelling pattern of glycerol from experiment with [2-^{13}C]pyruvate. Analogies with the results of feeding experiments reported by Andersen and co-workers, as well as the results with [5-^{13}C]glucose, seem to suggest that C$_2$-units may be involved in the process. Finally, the feeding experiments, including the replicates with [6-^{13}C]glucose and the use of [5-^{13}C]glucose, suggest that the isoprenoid part of verrucosins should derive from mevalonate. Because of the poor level of enrichment, further studies are required before reaching a definitive conclusion on this point. It is worth noting that many factors can affect the assimilation of the precursors and the incorporation rate. Most of these are related to cell metabolism, others depend on kinetic aspects, such as membrane permeability or distribution of the metabolite in the mollusc body. Similar factors may also explain the rather low enrichment recorded in the above experiments, as well as the different incorporation into the sterol pool by feeding radioactive mevalonate and radioactive glucose to *D. verrucosa*.

Experimental Section

General Methods: 1D and 2D NMR spectra were recorded on Bruker AMX 500 and Bruker Avance DPX 300 instruments. The $CHCl_3$ resonances at δ = 7.26 and 77.0 ppm were used as internal references for ^{1}H and ^{13}C spectra, respectively. Silica-gel chromatography was performed using precoated Merck F254 plates and Merck Kieselgel 60 powder. HPLC purifications were carried out on a Waters chromatograph equipped with an R401 differential refractometer and a 490E UV multiwavelength detector. ^{13}C NMR (ns, 13000; time delay 3.5 s) spectra were acquired in $CDCl_3$ (75 MHz) with 16 K data points and 4.0 Hz line broadening. ^{14}C-Incorporation was monitored by a Packard 1600TR liquid scintillation analyser. MS data were obtained by a Hewlett & Packard 5989B mass spectrometer equipped with a 5890 Series II Plus gas chromatograph.

Biological Material: Three collections of *Doris verrucosa* were carried out by hand using SCUBA at depths of 3−10 m at Castellammare in the Gulf of Naples (Italy) during July 1999 (35 specimens), July 2000 (31 specimens), and July 2001 (100 specimens). Some of the molluscs (21 specimens) was treated as controls and not subjected to any experiment. These animals were extracted with acetone and worked up as outlined below. The remaining animals were kept alive in aquaria and treated for the feeding experiments as described below.

Feeding Experiments: The labelled precursors were dissolved in sterile marine water (100−150 μL) and injected into the digestive gland through the mollusc foot. Nudibranchs, unless otherwise stated, were kept in an aquarium filled with 5 L of cooled sea water (18−19 °C).

[U-^{14}C]Glucose: Six specimens of *D. verrucosa* collected in July 1999 were treated with 3 μCi/animal of [U-^{14}C]glucose in a 2.5-L aquarium. The animals were kept alive for 12 days and then immediately frozen at −80 °C. Aquarium seawater was changed every four days.

[6-^{13}C]Glucose: Eight specimens (July 1999) of *D. verrucosa* were injected with 5 mg/ animal of [6-^{13}C]glucose dissolved in 125 μL of distilled water. The animals were kept alive in the aquarium for another six days and then immediately frozen. A second experiment was carried out with 20 specimens of the July 2000 collection, feeding the precursor (1.5 mg/specimen of [6-^{13}C]glucose) every other day for 11 days. Four days after the last injection, the molluscs were frozen and stored at −80 °C. A third experiment was carried out with *D. verrucosa* (20 specimens) of the 2001 collection. The molluscs were maintained in an aquarium for ten days and the tracer (10 mg/animal) was injected on days 1 and 3. In each experiment, the seawater was changed every three days.

[5-^{13}C]Glucose: Twelve specimens of *D. verrucosa* collected in July 2001 were treated with [5-^{13}C]glucose (5 mg /specimen in 40 μL of distilled water) on days 1, 3, and 5. Three days after the last injection, the molluscs were frozen and stored at −80 °C.

[2-^{13}C$_1$]Pyruvate: Twenty specimens of *D. verrucosa* collected in July 2001 were treated with sodium [2-^{13}C]pyruvate (5.0 mg /specimen in 100 μL of distilled water) on days 1 and 3. One week after the last injection, the molluscs were frozen and stored at −80 °C.

Extraction and Fractionation: In a typical preparation, the inner organs and mantle of the frozen molluscs (treated and control animals) were separated by anatomical dissection. Mantle sections were immersed in acetone (three times) and the release of secondary metabolites promoted by using an ultrasound treatment for 2 min. The acetone fractions were combined, reduced in vacuo, and partitioned between Et_2O and water (three times). The diethyl ether layers were dried with anhydrous sodium sulfate and the solvents evaporated to yield, on average, 8 mg of yellow oil (mantle extract) per mollusc. A raw mixture of verrucosins (**1**−**13**, approximately, 1.8 mg per specimen) was obtained by silica-gel column chromatography using an increasing polarity gradient of eluents (Et_2O in petroleum ether). The final purification of the glyceryl diterpenoids was carried out by normal-phase HPLC (*n*-hexane/ethyl acetate, 85:15; Kromasil KR100-5, 250 × 4.6 mm) that led, in order of elution, to verrucosin A (**1**, 0.6 mg/specimen), verrucosin-1 (**5**, 0.1 mg/specimen), a mixture (0.2 mg/specimen) of verrucosin-2 (**7**) and verrucosin-3 (**8**), verrucosin B (**2**, 0.6 mg/specimen), and a mixture of the remaining glycerides (0.3 mg/specimen). Compound **19** was prepared by purification on reversed-phase HPLC (CH_3CN/H_2O, 95:5; Kromasil KR100-5C18, 250 × 4.6 mm) after peracetylation (acetic anhydride in dry pyridine at room temperature for 14 h) of the verrucosin mixture. The structure of the verrucosins was determined by both NMR assignments and comparison with standard compounds. Radioactive compounds were identified by co-elution with cold material.

Reduction of Radioactive Verrucosin B (2): Verrucosin B (**2**, 0.7 mg, 0.0017 mmol) was dissolved in dry THF (0.8 mL) and reacted under argon with a large excess (4 equiv.) of $LiAlH_4$ (1 M in THF). The reaction was stirred under reflux for 12 h and then neutralised with 10% H_2SO_4. The grey suspension was diluted with water and extracted three times with Et_2O. The diethyl ether layers were dried with Na_2SO_4, filtered through cotton, and the solvents evaporated at reduced pressure to give a residue (1.2 mg) that was finally purified on a silica column with $CHCl_3$/MeOH (98:2) to yield **18**[3a] (0.3 mg). EIMS: *m/z* (%) = 290 (5) [M^+], 275 (10) [$M - CH_3]^+$, 257 (6) [$M - CH_3 - H_2O]^+$, 189 (8), 154 (50), 123 (100).

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