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Scalarane Metabolites of the Nudibranch *Glossodoris rufomarginata* and Its Dietary Sponge from the South China Sea

Margherita Gavagnin,*,† Ernesto Mollo,† Teresa Docimo,† Yue-Wei Guo,*,‡ and Guido Cimino†

Istituto di Chimica Biomolecolare, CNR, Via Campi Flegrei 34, I 80078-Pozzuoli (Na), Italy, and State Key Laboratory of Drug Research, Institute of Materia Medica, Shanghai, Institutes for Biological Sciences, Chinese Academy of Sciences, Taiyuan Road, 294, 200031, Shanghai, People's Republic of China

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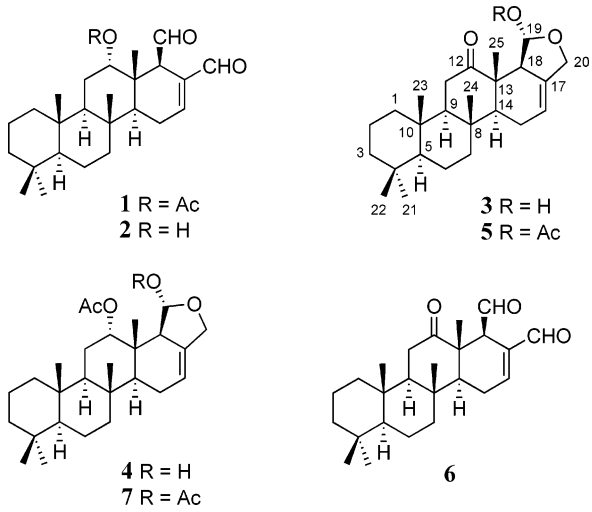
Chemical investigation of the nudibranch *Glossodoris rufomarginata* and its sponge prey provided additional evidence of the trophic relationship between *Glossodoris* nudibranchs and scalarane-containing sponges. Scalaradial (**1**) and its deacetyl derivative **2** were found to be the main components of the extract of the sponge, whereas a series of related scalaranes (**3**−**7**), probably derived from dietary scalaradial, were isolated from the mollusk. Two new scalarane sesterterpenes (**5** and **6**), exhibiting a keto functionalization at C-12, were characterized by spectroscopic methods and chemical correlations.

Chromodorids are a large group of brilliantly colored nudibranch mollusks. They are widespread in all tropical seas and extend into subtropical and temperate waters. A characteristic of chromodorids is their ability to sequester feeding-deterrent chemicals from their dietary sponges for use as a chemical defense.[1] A very specialized diet among the three genera *Hypselodoris*, *Chromodoris,* and *Glossodoris* has been generally observed. In fact, each group of animals appears to eat only sponges accumulating specific secondary metabolites, which are generally furanosesquiterpenes for *Hypselodoris*, diterpenes for *Chromodoris*, and sesterterpenes for *Glossodoris*.[2] In particular, even though conflicting chemical results have been published for the latter genus deriving from a dispute in nudibranch taxonomy, well-described associations link *Glossodoris* nudibranchs to scalarane-containing sponges.[3−6]

Scalarane compounds, the first member of which was well-known scalaradial (**1**),[7] show a series of very interesting pharmacological properties (cytotoxicity, antimicrobial, and anti-inflammatory activity, and platelet aggregation inhibition).[8] An involvement of these sesterterpenoids in the defensive mechanisms of sponges and mollusks has also been suggested by different authors, even though biological studies on feeding-deterrence of scalaradial (**1**) and related compounds have indicated that these molecules may be deterrent toward some but not all potential predators.[3]

In this paper, the chemical analysis of a population of the chromodorid nudibranch *Glossodoris rufomarginata* (Bergh, 1890) from the South China Sea and of the sponge, not yet identified, on which the mollusk was caught is described. The ether extract of the sponge was found to be mainly constituted by scalaradial (**1**) and its 12-deacetyl derivative **2**, whereas a series of related scalaranes (**3**−**7**), most likely obtained by transformation of dietary scalaradial (**1**), were isolated from the mantle and/or the internal glands of the mollusk. The already reported metabolites **1**−**4** and **7** were identified mainly by NMR, whereas the structures of the new compounds **5** and **6** were determined by both spectroscopic methods and chemical correlation with related known molecules.

G. rufomarginata (three specimens) was collected upon an unidentified sponge off Hainan Island in the South China Sea in January 2002, at a depth of 20 m. The mollusk and the sponge were immediately frozen, then they were transferred to our ICB laboratories and kept at −20 °C until being extracted. A freeze-dried sample of the sponge was ground in a pestle and extracted exhaustively with acetone. Each *G. rufomarginata* individual was carefully dissected into mantle and internal organs, which were separately extracted by acetone using ultrasound vibrations. A comparative TLC chromatographic analysis of the diethyl ether-soluble part from the acetone extracts of the sponge, mantle, and internal organs showed significant differences in the secondary metabolite pattern of both organisms and also a different distribution of such metabolites between the two anatomical sections of the nudibranch. The sponge extract was characterized by two main UV-sensitive spots more polar than sterols, whereas more complex patterns were observed for the extracts of both parts of the mollusk (a series of spots at R_f between 0.80 and 0.15 in $CHCl_3$−MeOH, 98:2). Thus, the ether extracts were separately fractionated by subsequent chromatographic steps as reported in the Experimental Section, and seven pure metabolites (**1**−**7**) were obtained. Five known scalaranes were identified by comparison of spectral data with those reported in the literature as the following: scalaradial (**1**),[7] which was the most abundant metabolite of the sponge, whereas it was completely absent in the mollusk; 12-deacetylscalaradial (**2**),[9] isolated from



* To whom correspondence should be addressed. Tel: + 39 081 8675094. Fax: + 39 081 8041770. E-mail: mgavagnin@icmib.na.cnr.it.
† Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche.
‡ Institute of Materia Medica, Chinese Academy of Sciences.

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Table 1. NMR Data of Compound **5**[a]

C	δ ^{13}C[b]	m[c]	δ ^{1}H[d]	m, *J* (Hz)	HMBC correlations[e]
1	39.4	CH_2	1.60	m	H-3a, H_3-23
			0.80	m	
2	18.0[f]	CH_2	1.42	m	
			1.60	m	
3	41.9	CH_2	1.40	m	H-2b, H_3-21
			1.15	m	
4	33.3	C			H_3-22
5	56.5	CH	0.81	m	H-6a, H-7a, H_3-21, H_3-22
6	18.4[f]	CH_2	1.42	m	H_3-21
			1.60	m	
7	41.3	CH_2	1.81	m	H_3-24
			1.00	m	
8	37.6	C			H_2-11, H-14, H_3-24
9	59.7	CH	1.22	m	H-7a, H_2-11, H_3-23, H_3-24
10	38.0	C			H_3-23
11	35.5	CH_2	2.59	t, 14	
			2.31	dd, 14, 2	
12	215.0	C			H_2-11, H_3-25
13	48.4	C			H-11a, H-14, H_3-25
14	55.4	CH	1.55	m	H_3-24, H_3-25
15	22.7	CH_2	2.15	m	H-14
16	117.5	CH	5.59	m	H_2-20
17	135.6	C			H-19, H_2-20
18	53.0	CH	2.81	m	H-20a, H_3-25
19	98.6	CH	6.35	d, 3	H-18, H-20b
20	69.5	CH_2	4.34	bd, 11	H-16, H-19
			4.19	d, 11	
21	33.3	CH_3	0.85	s	H_3-22
22	21.4	CH_3	0.82	s	H_3-21
23	15.7	CH_3	0.87	s	H-1a
24	15.7	CH_3	1.11	s	
25	13.2	CH_3	1.02	s	H-14
CH_3CO	170.2	C			H-19, −OAc
CH_3CO	21.4	CH_3	2.14	s	

[a] Bruker 300, 400, and 500 MHz; $CDCl_3$; chemical shifts (ppm) refer to C*H*Cl_3 (δ 7.26) for proton and to *C*DCl_3 (δ 77.0) for carbon. [b] Assignments by HMQC and HMBC experiments. [c] By DEPT sequence. [d] Assignments by ^{1}H−^{1}H COSY experiment. [e] *J* = 10 Hz. [f] Interchangeable values.

either the sponge or the nudibranch mantle; 12-deacetoxy-12-oxo-deoxoscalarin (**3**)[5] and 12α-deoxoscalarin (**4**),[10] both present in the mantle and the internal parts of the nudibranch; and 19-acetyl-12α-deoxoscalarin (**7**),[11] found only in the external part of the mollusk. The new compounds, 19-acetyl-12-deacetoxy-12-oxo-deoxoscalarin (**5**), isolated from both anatomical sections of *G. rufomarginata*, and 12-deacetoxy-12-oxo-scalaradial (**6**), which was exclusively present in the mantle, were characterized as follows.

The molecular formula of 19-acetyl-12-deacetoxy-12-oxo-deoxoscalarin (**5**) was deduced as $C_{27}H_{40}O_4$ by analyzing both the EIMS and ^{13}C NMR spectra. The mass spectrum showed the highest peak at *m/z* 428, along with a significant intense peak at *m/z* 368, due to loss of AcOH from molecular ion, while in the ^{13}C NMR spectrum, 23 sp^3 carbon signals (six CH_3, eight CH_2, five CH, and four C, as deduced by the DEPT sequence), two sp^2 carbon signals (δ 117.5, C-16; 135.6, C-17), and two CO signals (δ 215.0, C-12; 170.2, OAc-19) were observed. The presence of both ketone and ester functionalities was also indicated by diagnostic IR absorptions at 1706 and 1744 cm^{-1}. Taking into account the presence of these functionalities and a double bond, the remaining five unsaturation degrees indicated by the molecular formula were therefore attributed to five rings, according to a scalarane skeleton. In fact, analysis of the ^{1}H NMR spectrum (Table 1) showed the presence of five methyl singlets at δ 0.82 (H_3-22), 0.85 (H_3-21), 0.87 (H_3-23), 1.02 (H_3-25), and 1.11 (H_3-24), along with an acetyl methyl resonating at δ 2.14. A doublet at δ 6.35, attributed to an acetal proton (H-19), an olefinic sharp multiplet at δ 5.59 (H-16), and an AB system at δ 4.34 (bd, *J* = 11 Hz) and 4.19 (d, *J* = 11 Hz), due to a methylene (H_2-20) linked to an oxygen atom, suggested the presence of a partial structure (rings D and E) the same as 19-acetyl-deoxoscalarin (**7**). The keto function was positioned at C-12 by analysis of the resonance of H_3-25 (δ 1.02) and H_2-11 [δ 2.59 (t, *J* = 14 Hz) and 2.31 (dd, *J* = 14, 2 Hz)], further coupled with the H-9 angular methine (δ 1.22). This assignment was confirmed by diagnostic HMBC correlations between C-12 (δ 215.0) and both H_2-11 and H_3-25. These data indicated that **5** is the acetyl derivative of 12-deacetoxy-12-oxo-deoxoscalarin (**3**).[5] The structure characterization, including the relative stereochemistry of chiral centers, was completed by 2D NMR experiments that allowed all resonances to be assigned (Table 1). Finally, the proposed structure was confirmed by comparing the physical and spectral data ($[\alpha]_D$, ^{1}H and ^{13}C NMR) of **5** with those of a sample obtained by acetylation of 12-deacetoxy-12-oxo-deoxoscalarin (**3**).

Compound **6**, 12-deacetoxy-12-oxo-scalaradial, showed the molecular formula $C_{25}H_{36}O_3$, as indicated by its CIMS and ^{13}C NMR spectrum. In the mass spectrum, the highest peak was observed at *m/z* 385 [M + 1], whereas in the ^{13}C NMR spectrum, 25 signals were present: three carbonyl carbons resonating at δ 214.0 (C-12), 201.4 (C-19), and 192.1 (C-20), two olefinic signals at δ 150.5 (C-16) and 141.2 (C-17), and the remaining 20 signals between δ 59.1 and 15.2, attributable to sp^3 carbons. On this basis, the eight unsaturation degrees of the molecular formula were assigned to a ketone function, two aldehydic groups, one double bond, and four rings. The ^{1}H NMR spectrum (Table 2) displayed five methyl singlets at δ 0.86 (H_3-22), 0.88 (H_3-21), 0.91 (H_3-23), 1.10 (H_3-25), and 1.18 (H_3-24), along with two aldehyde signals, one of which at δ 10.22 (H-19, d, *J* = 2 Hz) coupled with a proton at δ 3.78 (m, H-18), and the other one α,β-unsaturated at δ 9.34 (H-20, s), which was long-range coupled to an olefinic proton at δ 6.98 (m, H-16), linked to a methylene at δ 2.45 (H_2-15), which was in turn coupled with an angular methine at δ 1.57 (m, H-14). These data clearly suggested for compound **6** a functionalized scalarane skeleton, in particular indicating a structure related to scalaradial (**1**). By analogy with compounds **3** and **5**, the keto group was placed at C-12, as suggested by the downfield shifted values of both H_3-25 (δ 1.10) and H_2-11 [δ 2.59 (t, *J* = 14 Hz) and 2.45 (m)] and further confirmed by diagnostic long-range correlations between C-12 (δ 214.0) and either H_3-25 or H_2-11 in the HMBC spectrum. All NMR resonances were assigned as reported in Table 2 by 2D NMR experiments (^{1}H−^{1}H COSY, HMQC, HMBC). The proposed structure was confirmed by comparing spectral data (CD, ^{1}H and ^{13}C NMR) of **6** with those of the compound obtained by oxidation of a sample of 12-deacetylscalaradial (**2**). Identical NMR spectra and CD profiles were observed in both molecules.

Chemical analysis of the nudibranch *G. rufomarginata* and its sponge prey provided additional evidence of the association of a *Glossodoris* nudibranch with a scalarane-containing sponge, also suggesting for this latter organism a taxonomic collocation in the families Spongiidae and Irciniidae. The presence of 12-deacetylscalaradial (**2**) in both the sponge and mollusk supported the trophic relationship between the two organisms. Furthermore, the absence of scalaradial (**1**) in the nudibranch containing a series of scalaranes derived from **1** suggested the ability of this mollusk to immediately transform dietary toxic scalaradial (**1**) into a series of related molecules, most likely

by a detoxification enzymatic process, the same as that suggested for the Mediterranean *Hypselodoris orsini*.[12]

The finding of scalarane sesterterpenes (compounds **3**, **5**, and **6**) exhibiting the keto function at C-12 is particularly interesting. This structural feature is quite common in the homoscalarane series but unusual in the scalaranes, and in particular it has been encountered only in nudibranch metabolites, indicating an ability of these mollusks to oxidize the hydroxyl function at C-12 of dietary scalaranes. In fact, in addition to 12-deacetoxy-12-oxo-deoxoscalarin (**3**), previously isolated from Indian *Glossodoris atromarginata*,[5] only one 12-keto-scalarane, 12-deacetoxy-12-oxo-18-*epi*-scalaradial (**8**), from Pacific nudibranch *Chromodoris youngbleuthi*,[13] has been previously reported.

Biological activities of scalarane metabolites have been evaluated by assaying their ichthyotoxicity in the *Gambusia affinis* test.[14,15] Compounds **1**, **2**, and **6** were very toxic at 10 ppm, and compounds **3**, **4**, and **5** were toxic at the same concentration, whereas compound **7** was inactive. According to these results and by analogy with data reported for other *Glossodoris* nudibranchs,[3] an involvement of scalarane metabolites in the defensive mechanisms of *G. rufomarginata* can be suggested.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP 370 digital polarimeter, and CD curves were recorded on a JASCO 710 spectropolarimeter. The IR spectra were taken on a Bio-Rad FTS 7 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on WM 500, AVANCE 400, and DPX 300 MHz Bruker spectrometers in $CDCl_3$, with chemical shifts reported in ppm referred to $CHCl_3$ as internal standard (δ 7.26 for proton and δ 77.0 for carbon). EIMS and HREIMS were measured on a TRIO 2000 VG Carlo Erba and on a Kratos MS50 instrument, respectively. Silica gel chromatography was performed using precoated Merck F_{254} plates and Merck Kieselgel 60 powder.

Animal Material. *Glossodoris rufomarginata* (three specimens) was collected by scuba diving at a depth of 20 m along the coast of Hainan Island, in the South China Sea, during January 2002. A sample of the sponge on which the nudibranchs were observed feeding was also collected. This is probably a keratose sponge belonging to the order Dictyoceratida and appeared to be tough, rubbery, and gray in color on the surface. The biological material was immediately frozen, then transferred to ICB in Naples, where it was kept at −80 °C until extraction. A voucher specimen for both animals is stored for inspection at ICB (HN-43 for the nudibranch and HN-33 for the sponge).

Extraction and Isolation. Frozen *G. rufomarginata* (three specimens) was dissected in order to separate internal organs (digestive gland, reproductive apparatus, digestive duct, etc.) from the mantle. Dissected parts from the nudibranch and fragments of the sponge on which the mollusk was collected were separately extracted with Me_2CO. After removing the organic solvent, the aqueous residue was partitioned with Et_2O (3 × 15 mL). The soluble Et_2O fractions were concentrated at reduced pressure to yield 153 mg of Et_2O extract from the internal organs, 143 mg from the mantle, and 516 mg from the sponge. TLC of the extracts was carried out in petroleum ether−Et_2O and $CHCl_3$−MeOH, and the metabolites were visualized by ceric sulfate and Ehrlich's reagent. Aliquots of the Et_2O extracts from the internal organs (56 mg), mantle (69 mg), and sponge (150 mg) were fractionated separately on a silica gel column using a petroleum ether/$CHCl_3$ gradient as eluent. The extract from the internal organs yielded fraction 2 (10.1 mg, crude **5**), fraction 3 (9.0 mg, crude **3**), and fraction 4 (28.9 mg, crude **4**). The mantle extract yielded fraction 1 (30.2 mg, crude **2**, **5**, **6**, and **7**), fraction 2 (3.1 mg, crude **3**), and fraction 4 (4.9 mg, pure **4**). Fraction 1 was further fractionated on a silica gel column using a petroleum ether/Et_2O gradient as eluent to give fraction B (4.0 mg, crude **5** and **7**), fraction C (5.8 mg, crude **2** and **5**), and fraction G (5.7 mg, crude **3**). The sponge extract yielded fraction 2 (22.1 mg, pure **2**) and fraction 4 (62.6 mg, pure **1**), in order of increasing polarity.

Table 2. NMR Data of Compound **6**[a]

C	δ ^{13}C[b]	m[c]	δ ^{1}H[d]	m, *J* (Hz)	HMBC correlations[e]
1	39.3	CH_2	1.68	m	H_3-23
			0.86	m	
2	17.9	CH_2	1.40	m	
			1.30	m	
3	41.8	CH_2	1.42	m	H_3-21, H_3-22
			1.17	m	
4	33.3	C			
5	56.4	CH	0.86	m	H_3-21, H_3-22, H_3-23
6	18.3	CH_2	1.65	m	
			1.45	m	
7	41.5	CH_2	1.90	m	H-14, H_3-24
			1.05	m	
8	37.5	C			H_2-11, H-14, H_3-24
9	59.1	CH	1.27	m	H_2-11, H_3-23, H_3-24
10	38.0	C			H_2-11, H_3-23
11	35.2	CH_2	2.59	t, 14	
			2.45	m	
12	214.0	C			H_2-11, H_3-25
13	53.3	C			H-14, H-15a, H-19, H_3-25
14	55.2	CH	1.57	m	H_3-24, H_3-25
15	23.4	CH_2	2.45	m	H-14, H-16
16	150.5	CH	6.98	m	
17	141.2	C			H-20
18	53.6	CH	3.78	m	H-14, H-19, H-20, H_3-25
19	201.4	CH	10.22	d, 2	
20	192.1	CH	9.34	s	
21	33.2	CH_3	0.88	s	H_3-22
22	21.3	CH_3	0.86	s	H_3-21
23	15.7	CH_3	0.91	s	
24	16.6	CH_3	1.18	s	H-14
25	15.2	CH_3	1.10	s	H-14

[a] Bruker 300, 400, and 500 MHz; $CDCl_3$; chemical shifts (ppm) refer to $CHCl_3$ (δ 7.26) for proton and to $CDCl_3$ (δ 77.0) for carbon. [b] Assignments by HMQC and HMBC experiments. [c] By DEPT sequence. [d] Assignments by ^{1}H−^{1}H COSY experiment. [e] J = 10 Hz.

Purification of Compounds 2, 3, and 5−7. Fractions 2 and G from the mantle extract were combined and purified on normal-phase HPLC (Kromasil 5 μm silica gel column; detector RI R401; flow rate 1 mL/min), using *n*-hexane−2-propanol (98:2) as mobile phase, to give pure **3** (2.0 mg). Fraction B from the mantle extract was further purified on normal-phase HPLC (Spherisorb 5 μm silica gel column; detector RI R401; flow rate 1 mL/min), using *n*-hexane−2-propanol (99:1), to afford pure **7** (0.8 mg) and **5** (1.1 mg). Fraction C from the mantle extract was purified on normal-phase HPLC (Spherisorb 5 μm silica gel column; detector RI R401; flow rate 1 mL/min), using *n*-hexane−2-propanol (99:1), to yield pure **2** (1.0 mg) and **5** (1.1 mg). Fraction E from the mantle extract was subjected to normal-phase HPLC (Spherisorb 5 μm silica column; detector RI R401; flow rate 1 mL/min), using EtOAc−*n*-hexane (1:3), to give pure **6** (1.0 mg). The $[\alpha]_D$ values and ^{1}H and ^{13}C NMR data of the known compounds **1**−**4** and **7** were identical with those reported in the literature.

19-Acetyl-12-deacetoxy-12-oxo-deoxoscalarin (5): oil; $[\alpha]_D$ 17.8° (*c* 0.5, $CHCl_3$); CD $[\theta]_{295}$ (*n*-hexane) 4000; IR (liquid film) ν_{max} 1705, 1744 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 428, 368, 340, 207, 191, 163; HREIMS *m/z* 368.2722 ([M$^+$ − AcOH], calcd for $C_{25}H_{36}O_2$ 368.2715).

12-Deacetoxy-12-oxo-scalaradial (6): oil; $[\alpha]_D$ −118.2° (*c* 0.1, $CHCl_3$); CD $[\theta]_{215}$ (*n*-hexane) −28,740; IR (liquid film) ν_{max} 1708 cm^{-1} (broad); ^{1}H and ^{13}C NMR, see Table 2; CIMS *m/z* 385 [M + H]$^+$.

Acetylation of 3. Compound **3** (3.3 mg) was treated with Ac_2O (0.3 mL) in dry pyridine (2.0 mL) at room temperature overnight. The mixture was dried under reduced pressure. After the usual workup, the reaction mixture was purified by

a silica gel Pasteur-pipet chromatographic step to give 3.1 mg of the acetyl derivative, which was identical in all respects ($[\alpha]_D$, ^1H and ^{13}C NMR) with 19-acetyl-12-deacetoxy-12-oxodeoxoscalarin (**5**).

Oxidation of 2. Compound **2** (5.5 mg, 0.014 mmol) was dissolved in dichloromethane (2 mL) and oxidized with pyridinium dichromate (10.0 mg, 0.027 mmol) at 0 °C, stirring for 4 h. After evaporation of the solvent, the mixture was partitioned between Et_2O and water, then the soluble Et_2O fraction was concentrated at reduced pressure and submitted to silica gel chromatography to obtain a pure oxidized compound (4.5 mg), which showed ^1H and ^{13}C NMR spectroscopic data and an $[\alpha]_D$ value identical with 12-deacetoxy-12-oxoscalaradial (**6**).

Biological Assays. Ichthyotoxicity tests against the mosquito fish, *Gambusia affinis* (Baird & Girard, 1853), were conducted according to literature procedures.[14,15] Compounds **1**−**7** were assayed at 10 ppm. The toxicity ranking was defined as in Coll et al.[15]

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