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Biotransformation of a dietary sesterterpenoid in the Mediterranean nudibranch *Hypselodoris orsini*

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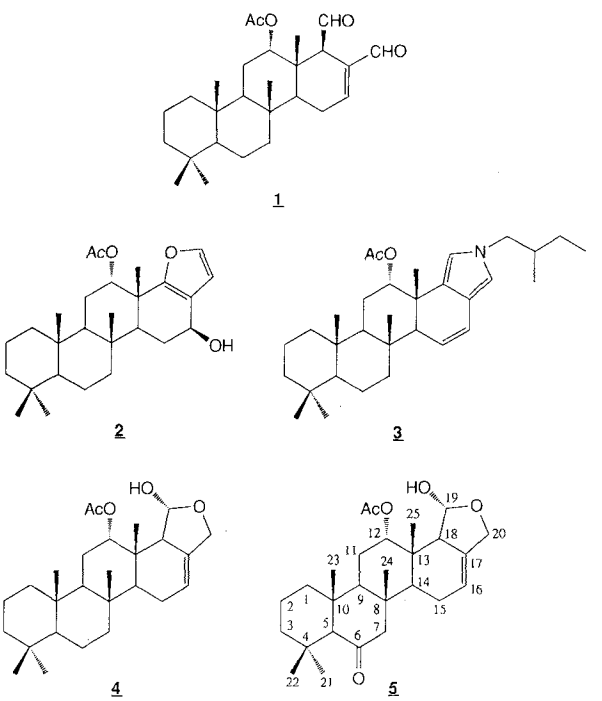
Abstract. The metabolic relationship between the marine mollusc *Hypselodoris orsini* and the sponge *Cacospongia mollior* has been reinvestigated. The predator-prey association has been confirmed even though the metabolic patterns of the two invertebrates are substantially different. Most probably the nudibranch converts the main sponge metabolite, the sesterterpenoid scalaradial (**1**), into a less oxygenated related metabolite, deoxoscalarin (**4**), followed by a second chemical transformation leading to a new sesterterpenoid, 6-keto-deoxoscalarin (**5**) which is selectively compartmentalized into some dorsal glands, mantle dermal formations (MDFs), strategically distributed near the gills. 6-keto-deoxoscalarin (**5**) has been characterized by 1D and 2D NMR methods. Finally, the unusual association of some Chromodorididae molluscs with sponges containing sesterterpenoids suggests a further analysis of their taxonomical collocation is required.

Key words. Nudibranch; sponge; *Cacospongia mollior*; *Hypselodoris orsini*; sesterterpenoids.

Nudibranchs are marine molluscs completely devoid of shell which have elaborated a series of alternative defensive strategies[2,3] either morphological, behavioural or chemical. Continuing our research on Mediterranean opisthobranchs[4-6] we have recently reinvestigated the predator-prey pair: *Hypselodoris orsini*[7] (=*Glossodoris tricolor*[8] = *Hypselodoris coelestis*) and *Cacospongia mollior*. *H. orsini* is a small blue nudibranch which lives in the Gulf of Naples closely associated with the massive black sponge *Cacospongia mollior*. Chemical studies on *C. mollior* previously led to the characterization of some sesterterpenoids with the scalarane skeleton: scalaradial[9] (**1**) and furoscalarol[10,11] (**2**) along with some metabolites [e.g. molliorin-A[12-15] (**3**)] which could derive from the condensation of **1** with biological amines.

The metabolic pattern of *H. orsini* contained, analogously with *C. mollior*, **2** as a minor component. Scalaradial (**1**) was completely absent, substituted by another scalarane sesterterpenoid, deoxoscalarin (**4**) previously found in *Spongia officinalis*[16] and in trace levels in *C. mollior*[8]. The latter finding could be due to the presence of some specimens of *H. orsini* hidden in the sponge oscula when the sponge was extracted. *H. orsini* may therefore be able either to modify the dietary metabolites, according to our hypothesis[8], or to selectively accumulate minor dietary metabolites, as suggested by other authors[2]. In this work we aim: 1) to determine the absence or presence of **4** in *C. mollior* by carefully selecting sponge samples devoid of *H. orsini* individuals in the inner part of oscula; 2) to characterize *H. orsini* metabolites further; 3) to elucidate the anatomical distribution of the chemicals in *H. orsini*.



Material and methods

Instruments. IR spectra were recorded on a BIO-RAD FTS-7 spectrophotometer. Optical activity was measured by JASCO DIP-370 digital polarimeter. ^{1}H- and ^{13}C-NMR spectra were performed by a Bruker NMR spectrometer AMX-500 equipped with a X32 computer. The samples were dissolved in $CDCl_3$. Chemical shifts are expressed in δ values relative to $\underline{C}HCl_3$ ($\delta = 7.26$)

and to $\underline{C}DCl_3$ ($\delta = 77.00$), respectively. UV spectra were recorded on a Varian DMS 90 spectrophotometer. Mass spectra were recorded with a AEI MS 30 spectrometer.

Collection of biological materials. The sponge *Cacospongia mollior* and the nudibranchs *Hypselodoris orsini* were collected in the Gulf of Naples (Sorrento – Napoli) during the summer of 1992. The nudibranch was identified by Prof. J. Ortea (University of Oviedo, Spain)[7].

Extraction of Cacospongia mollior. A thin strip of sponge was cleaned and directly extracted with 5 ml of $CDCl_3$. The organic solution was clarified by Na_2SO_4 and filtered through paper. The solution obtained was reduced under a stream of nitrogen and directly analyzed by ^{1}H-NMR (fig. 1A).

Extraction of Hypselodoris orsini sections. Anatomical dissection was made by observing the animal through a Swift Microscope (20-40 X). 120 Specimens of *H. orsini* were dissected into four parts: dermal formations (MDFs), rest of the mantle and foot, digestive gland, and rest of the viscera (reproductive organs, digestive duct, etc.). The MDFs from 120 specimens were treated with acetone. The organic solution was evaporated at reduced pressure and, then the residue (1.5 mg) was dissolved in $CDCl_3$ and submitted to NMR analysis (fig. 1C; table 1). The remaining parts of 10 animals were extracted with acetone. The organic solvent was evaporated at reduced pressure to obtain an aqueous residue that was extracted three times with diethyl ether to yield 8.0 mg of yellow oil, which was directly analyzed by NMR (fig. 1B; table 1).

Extraction of mucous secretion of H. orsini. The mucous secretion of 30 individuals was frozen at -77 °C and then lyophilized to obtain a white powder that was dissolved with acetone and analyzed by TLC.

Chromatographic analysis. The analysis of crude extracts of *H. orsini* sections were performed by SiO_2-TLC (Merck silica gel 60 UV 254, 0.25 mm precoated plates) in toluene/petroleum ether/diethyl ether 3:3:8 and visualized by ceric sulfate.

Chemical data. Deoxoscalarin (**4**): $C_{27}H_{42}O_4$; EIMS (*m/z*): 412 (M^+-H_2O, 65%), 370 (M^+-CH_3CO_2H, 5%), 205 (33%), 191 (50%), 95 (100%); IR ($CHCl_3$, c = 0.15 g/100 mL) ν_{max} 3580, 2925, 1720 cm^{-1}; $[\alpha]_D = +36.6°$ (c = 0.15, $CHCl_3$); ^{13}C- and ^{1}H-NMR data are reported in table 1. ^{1}H-NMR selected coupling constants: H-1a (ddd, $J = 4.0$, 13.4 and 13.4 Hz), H-3a (bt, $J = 13.5$ Hz), H-7a (ddd, $J = 3.6$, 12.6, 12.6 Hz), H-9 (bd, $J = 13.0$ Hz), H-12 (t, $J = 2.9$ Hz), H-19 (t, $J = 3.7$ Hz), H-20a (bd, $J = 11.2$ Hz), H-20b (bd, $J = 11.2$ Hz). 6-Keto-deoxoscalarin (**5**): $C_{27}H_{40}O_5$; EIMS (*m/z*): 426 (M^+-H_2O, 88%), 386 (M^+-CH_3CO_2H, 34%), 219 (5%), 123 (100%); IR ($CHCl_3$, c = 0.28 g/100 mL) 1712, 1728 cm^{-1}; $[\alpha]_D = +12.6°$ (c = 0.15, $CHCl_3$); ^{13}C- and ^{1}H-NMR data are reported in table 1. ^{1}H-NMR selected coupling constants: H-1b (bd, $J = 11.8$ Hz), H-3a (ddd, $J = 13.5$, 13.5 and 3.6 Hz), H-3b (bt, $J = 13.4$ Hz), H-12 (t, $J = 2.7$ Hz), H-19 (t, $J = 3.5$ Hz), H-20a (bd, $J = 11.4$ Hz), H-20b (bd, $J = 11.4$ Hz).

Discussion

The predator prey relationship between *H. orsini* (= *G. tricolor* = Hypselodoris tricolor) and *C. mollior* is well documented[8,17]. *H. orsini*, a very small (1 cm) brightly coloured mollusc with an intense blue background marked by longitudinal yellow-white lines, lives in the Gulf of Naples in close symbiosis with the very conspicuous black sponge *C. mollior*[17]. The mollusc, which is clearly visible on the surface of the sponge, is also very often hidden in the inner part of the sponge oscula. First, we tried to ascertain the metabolic relationship between *C. mollior* and *H. orsini* by a comparative analysis of the acetone extracts from the two invertebrates. A small fragment of sponge was dissected and carefully cleaned of all hidden molluscs. The $CDCl_3$ extract was directly submitted to ^{1}H-NMR analysis without any chromatographic purification. The ^{1}H-NMR spectrum (fig. 1A) revealed the presence of almost pure scalaradial (**1**) together with traces of furoscalarol (**2**) in an approximate ratio of 100/1. Moreover, the analysis of the spectrum excluded the presence of deoxoscalarin (**4**).

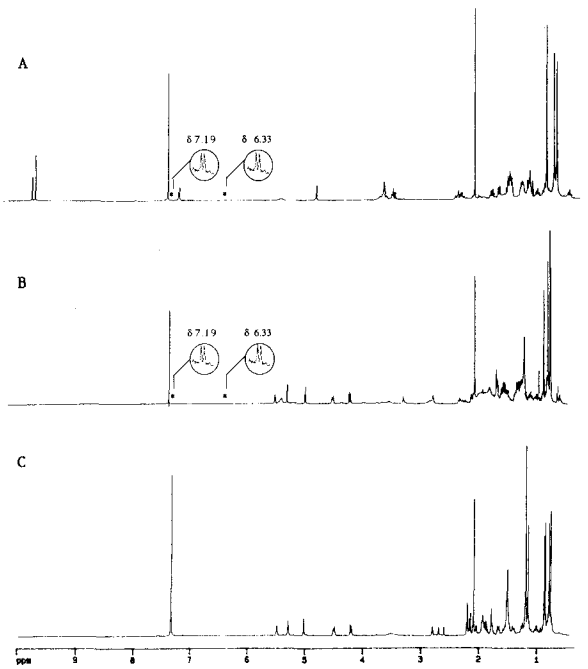


Figure 1. ^{1}H-NMR spectra in $CDCl_3$ of crude extracts from *C. mollior* (A, main metabolite: 1), *H. orsini* without MDF's (B, main metabolite: 4), and from MDF's of *H. orsini* (C, main metabolite: 5).

The chromatographic study of this fraction (SiO_2–TLC in toluene/petroleum ether/diethyl ether, 3:3:8), in comparison with the acetone extracts of *H. orsini*, confirmed this distribution in the sponge, whereas the mollusc extract displayed deoxoscalarin (**4**) (Rf = 0.4) as a main metabolite along with minor amounts of furoscalarol (**2**) and also a third more polar (Rf = 0.3) minor component.

Many *Hypselodoris* molluscs are characterized[18] by a series of dorsal glands near gills and rhinophores. Recent studies have demonstrated[5,19–21] that some chemicals are specifically accumulated in the glands. *H. orsini*, like other *Hypselodoris* species, displayed 4-5 MDFs (with a diameter of about 0.2–0.4 mm) near the gills (fig. 2).

The MDFs were carefully dissected out from 120 specimens. The rest of the mollusc body was further dissected, separating mantle from digestive glands. MDFs, mantle and digestive glands were separately extracted with acetone. The TLC analysis of the extracts revealed different chromatographic patterns: **2** (traces) and **4** were concentrated in the digestive glands whereas the more polar compound **5** was the only component of the MDFs. No characteristic metabolite was detected in the rest of the mantle. The chromatographic pattern of mucous extract was identical to that of digestive gland. The crude acetone extract of MDFs (from 120 specimens) and the lipid extract of whole animals without MDFs (10 specimens) were submitted to ^{1}H-NMR analysis. Deoxoscalarin (**4**), along with traces of furoscalarol (**2**), was immediately identified in the extract of *H. orsini* without MDFs (fig. 1B). On the other hand, the ^{1}H-NMR spectrum of MDFs (fig. 1C) revealed strong similarities to the spectrum of **4** but also some diagnostic differences, including the presence of a downfield methyl singlet at δ 1.22. Because of this, both compounds were extensively analyzed by one- and two-dimensional (^{1}H-^{1}H COSY, HOHAHA, ^{1}H-^{13}C HETCOR) NMR experiments which led to a full assignment of all ^{1}H and ^{13}C resonances (table 1).

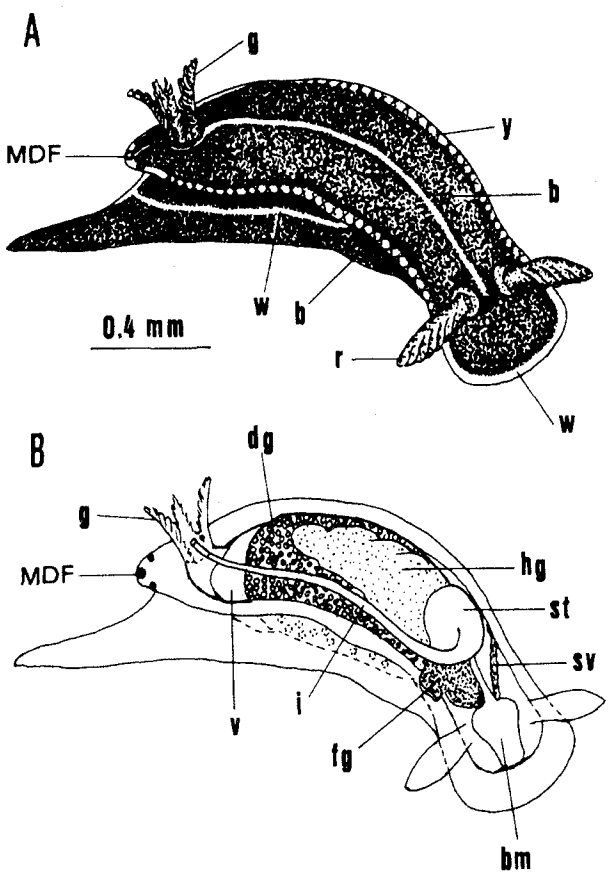


Figure 2. *Hypselodoris orsini* (Conca Azzurra, Naples, Italy). *A* Dorso-lateral aspect of the living opistobranch: b, blue; g, gills; r, rhinophore; y, yellow; MDF, mantle dermal formation; w, white. *B* Gross internal anatomy: bm, bucal mass; i, intestine; dg, digestive gland; g, gills; fg, female gland; hg, hermaphrodite gland; MDF, mantle dermal formation; st, stomach; sv, salival gland; v, ventricle.

It is noteworthy that even though the structure of **4** was suggested almost twenty years ago[16], ^{13}C-NMR assignments are completely lacking[22]. However, the ^{13}C-NMR data of **4**, when they were compared to those of 12-*epi*-deoxoscalarin[23], were in full agreement with those expected from the effects of the axial orientation of the acetoxy group at C-12 on the carbons 9 and 14. The ^{13}C-NMR spectrum of **5** was similar to the spectrum of **4**, but strong downfield shifts of C-5 (δ 66.83) and C-7 (δ 52.94) were attributed to the presence of a carbonyl group at C-6 (δ 211.53) according to the structure **5**. EIMS, along with ^{1}H- and ^{13}C-NMR data, assigned the elementary composition $C_{27}H_{40}O_5$ to **5**, while the IR spectrum displayed two carbonyl bands at 1712 and 1728 cm^{-1}. To the best of our knowledge[3,22] this is the first report of a scalarane sesterterpenoid displaying a ketone moiety at C-6.

In conclusion, this work has demonstrated that *H. orsini* is able to modify dietary metabolites. The main sponge metabolite, scalaradial (**1**), is first converted by selective reduction of the aldehyde at C-17 to deoxoscalarin (**4**), completely absent in *C. mollior*. A second chemical transformation oxidizes **4** at C-6 selectively, yielding **5** which is specifically accumulated in the glands of the mantle (MDFs). There are only a few examples in the literature of the ability of marine molluscs to modify dietary metabolites. Recently this feature has been supported by chemical studies on some ascoglossan and nudibranch molluscs[24–26].

A recent paper on three Pacific *Glossodoris* species[26] substantially supports the ability of these molluscs to modify dietary metabolites. Even though the authors did not exclude a selective accumulation of dietary metabolites, they demonstrated by ecologically relevant experiments that the sponge extracts were more toxic than those from nudibranchs. Most likely, the mollusc is well protected against predators by the sponge

Table 1. ^{13}C- and ^{1}H-NMR data for deoxoscalarin (**4**) and 6-keto-deoxoscalarin (**5**). Numbering is according to previous paper[23]

C	**4**				**5**			
	δ_{13C} [a]	(*m*)[b]	δ_1H[a]	(*m*)	δ_{13C} [a]	(*m*)[b]	δ_1H[a]	(*m*)
1	39.64	(*t*)	0.64	(*ddd*)	40.37	(*t*)	0.88	(*m*)
			1.58	(*m*)			1.71	(*bd*)
2	18.40	(*t*)	1.39	(*m*)	18.18	(*t*)	1.46	(*m*)
			1.53	(*m*)			1.60	(*m*)
3	41.97	(*t*)	1.13	(*bt*)	42.46	(*t*)	1.07	(*ddd*)
			1.39	(*m*)			1.31	(*bt*)
4	33.22	(*s*)			31.93	(*s*)		
5	56.40	(*d*)	0.86	(*m*)	66.83	(*d*)	2.23	(*m*)
6	17.97	(*t*)	1.34	(*m*)	211.53	(*s*)		
			1.60	(*m*)				
7	41.36	(*t*)	1.04	(*ddd*)	57.94	(*t*)	2.18	(*m*)
			1.69	(*m*)			2.24	(*m*)
8	36.83	(*s*)			43.74	(*s*)[c]		
9	52.50	(*d*)	1.36	(*bd*)	52.73	(*d*)	1.92	(*m*)
10	37.69	(*s*)			44.05	(*s*)[c]		
11	22.59	(*t*)	1.69	(*m*)	23.43	(*t*)	1.83	(*m*)
12	74.85	(*d*)	4.94	(*t*)	74.24	(*d*)	5.00	(*t*)
13	36.79	(*s*)			37.39	(*s*)		
14	50.23	(*d*)	1.62	(*m*)	49.85	(*d*)	1.90	(*m*)
15	22.87	(*t*)	1.92	(*m*)	22.98	(*t*)	1.98	(*m*)
			2.09	(*m*)				
16	115.62	(*d*)	5.45	(*bs*)	115.18	(*d*)	5.46	(*bs*)
17	136.44	(*s*)			136.66	(*s*)		
18	53.39	(*d*)	2.78	(*bs*)	53.45	(*d*)	2.83	(*bs*)
19	98.49	(*d*)	5.25	(*t*)	98.48	(*d*)	5.27	(*t*)
20	68.88	(*t*)	4.18	(*bd*)	68.98	(*t*)	4.20	(*bd*)
			4.47	(*bd*)			4.49	(*bd*)
21	33.22	(*q*)	0.86	(*s*)	32.33	(*q*)	0.93	(*s*)
22	21.31	(*q*)	0.80	(*s*)	21.51	(*q*)	1.22	(*s*)
23	16.21	(*q*)[d]	0.82	(*s*)	16.10	(*q*)[e]	0.95	(*s*)
24	15.86	(*q*)[d]	0.92	(*s*)	17.20	(*q*)[e]	0.86	(*s*)
25	14.73	(*q*)	0.82	(*s*)	14.55	(*q*)	0.86	(*s*)
$\underline{C}H_3CO$-	21.43	(*q*)	2.08	(*s*)	21.41	(*q*)	2.13	(*s*)
$CH_3\underline{C}O$-	170.97	(*s*)			170.62	(*s*)		

[a]Assigned by ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR. [b]Determined by DEPT sequence. [c]Values for carbons 8 and 10 are interchangeable. [d]Values for carbons 23 and 24 are interchangeable. [e]Values for carbons 23 and 24 are interchangeable.

metabolites and the chemical transformation of the main sponge products could be a detoxification process. Most likely, the same strategy is adopted by *H. orsini*, but the low amounts of 6-keto-deoxoscalarin prevented an investigation of its potential biological activities.

Deoxoscalarin (**4**) has been previously found in Tyrrhenian *Spongia officinalis*[16], whereas its acetate has been found in an Adriatic *S. officinalis* along with its epimer at C-12, erroneously reported as *ent*-12-epideoxoscalarin[27]. It is possible that the presence of deoxoscalarin (**4**) in *H. orsini* may be due to feeding on other Dyctioceratida sponges, but we exclude this possibility because *C. mollior* is widely present in the habitat where *H. orsini* has been found, and the specific predator-prey relationship between *C. mollior* and *H. orsini* is supported by the small amounts of furoscalarol (**2**) present in both the invertebrates. In addition, the chromatographic analysis of other sponges collected in the same area where *C. mollior* lives did not show related sesterterpenoids. Finally, observations in aquaria demonstrated the specific relationship of *H. orsini* with *C. mollior*; the mollusc lives in close association with this sponge and does not try to reach other sponges.

It is interesting to note that Mediterranean *Hypselodoris* generally prey on encrusting sponges, like *Dysidea fragilis*, which contains large amounts of ichthyodeterrent furanosesquiterpenoids[5]. On the other hand, Mediterranean *Chromodoris* molluscs, belonging to the same Chromodorididae family, selectively prey on other sponges, like *Spongionella gracilis*, which are characterized by diterpenoid metabolites with carbon skeletons derived from a spongiane precursor[28]. Conversely, *H. orsini* lives in close association with sponges containing large amounts of sesterterpenoids. Recent studies have demonstrated the presence of sesterterpenoids in other nudibranchs, such as *Chromodoris youngbleuthi*[29], *Chromodoris sedna*[30], *Glossodoris pallida*, *Glossodoris hikeurensis*, *Glossodoris cincta*[26] and *Chromodoris funerea*[31]. In particular, a keto derivative of deoxoscalarin, with the keto moiety tentatively placed at C-1 or at C-3, was found in *C. funerea*[31]. Most likely, all these species, and also *H. orsini*, could belong to a new distinct Chromodorididae genus.

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