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Isolation of acetylated bile acids from the sponge *Siphonochalina fortis* and DNA damage evaluation by the comet assay

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ABSTRACT

From the organic extracts of the sponge *Siphonochalina fortis*, collected at Bahía Bustamante, Chubut, Argentina, three major compounds were isolated and identified as deoxycholic acid 3, 12-diacetate (**1**), cholic acid 3, 7, 12-triacetate (**2**) and cholic acid, 3, 7, 12-triacetate. (**3**). This is the first report of acetylated bile acids in sponges and the first isolation of compound **3** as a natural product. The potential induction of DNA lesions by the isolated compounds was investigated using the comet assay in lymphocytes of human peripheral blood as *in vitro* model. The results showed that the administration of the bile acid derivatives would not induce DNA damages, indicating that acetylated bile acids are nontoxic metabolites at the tested concentrations. Since the free bile acids were not detected, it is unlikely that the acetylated compounds may be part of the sponge cells detoxification mechanisms. These results may suggest a possible role of acetylated bile acids as a chemical defense mechanism, product of a symbiotic relationship with microorganisms, which would explain their seasonal and geographical variation, and their influence on the previously observed genotoxicity of the organic extract of *S. fortis*.

1. Introduction

Marine sponges have been the focus of interest in the last three decades in the field of natural products, since they are a rich source of biologically active secondary metabolites. The production of bioactive compounds has also been related in many cases to highly specific host-symbiont associations [1]; which may explain geographical and seasonal variations in the composition and concentration of these secondary metabolites.

Since sponges are simple and sessile organisms, during evolution they have developed potent chemical defensive mechanisms to protect themselves from competitors and predators as well as infectious microorganisms and also to compete for substrates and for the control of epibionts.

Previous studies show that secondary metabolites in sponges and their symbionts play a crucial role for their survival in the marine ecosystem [2]. Many of these unique compounds have shown antiviral, antitumor, antimicrobial and general cytotoxic activities, which make them promising leads in biomedical and pharmacological research [3]. The fact that many of these substances have been biosynthesized by sponges or their symbionts in order to play a role in the control of biofouling, has promoted their use as model compounds for the development of nontoxic antifouling agents with potential use in environmentally-friendly marine coatings [4]. This is of particular importance since currently used antifouling agents have been found to be toxic to many non-target marine organisms, triggering the search for ecologically safer options [5].

In a previous article, our group reported the genotoxicity screening of a series of sponge extracts from the Patagonian region [6]. The non polar extract of one of these samples, *Callyspongia fortis* (syn *Siphonochalina fortis* Ridley, 1881), showed significant difference in sister chromatid exchange (SCE) frequency when compared with control cultures. A great variation of this activity was observed in two samples collected from different locations, which was attributed to a difference in environmental conditions affecting the production of bioactive substances. Due to the small sample available at that time no attempts were made to identify the bioactive substances. During a recent field trip to Bahía Bustamante (Chubut, Argentina), specimens of *S. fortis* Ridley, 1881 were recollected, and, quite surprisingly, the main components of this

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sponge were identified as acetylated bile acids, a rare finding in marine invertebrates.

Bile acids, which are the end products of cholesterol catabolism in mammals, are also powerful biological detergents that have very important roles as emulsifiers in the intestine, where they form mixed micelles with lecithin and cholesterol *in vivo*, an essential role for lipid transportation, the digestion of fats and the regulation of cholesterol in bile. In mammals, C_{24} bile acids are formed from cholesterol in the liver after a complex catabolic sequence, and are major components of bile, while in fish and reptiles, bile alcohols (generally C_{27}) are formed instead. On the other hand, bile acids and alcohols are not biosynthesized by invertebrates. For example, in the digestive juices of crustaceans such as crabs and crayfish, they are replaced by other polar substances that can act as emulsifiers, such as fatty acylsarcosyltaurines and peptides. In more primitive invertebrates such as sponges, there are no digestive juices and hence no need for these substances.

Marine invertebrates, especially sponges and soft corals, display an amazing array of structurally diverse steroids, many of them unique to the marine environment. In accordance to their typical biological roles, bile acids are not among the usual classes of steroids found in marine invertebrates. There have been a few reports of bile acids and derivatives in marine invertebrates, mainly from octocorals [7–9] and sponges [10]. However, there are several taxa of marine bacteria, some of them associated with sponges, which have been reported to produce bile acids [11]. For these reasons, the sparse findings of bile acid derivatives in marine invertebrates are considered to be of symbiotic microbial origin.

In the present work, three acetylated bile acids were isolated from *C. fortis*: deoxycholic acid 3, 12-diacetate (**1**), cholic acid 3, 7, 12-triacetate (**2**) and cholic acid 3, 7- diacetate (**3**). To the best of our knowledge, this is the first report of acetylated bile acids in sponges, and the first isolation of compound **3** as a natural product.

Given the relevance of this finding and since it is known that marine invertebrates express qualitatively similar types of induced damage to that found in higher organisms in DNA structure and/or chromosomal levels, [12], we developed a preliminary study to determine the impact of acetylated bile acids on cell viability and its genotoxic potential effect, using the comet assay in peripheral blood lymphocytes, as an *in vitro* model.

2. Experimental

2.1. General

Solvents were distilled for chromatography; CH_2Cl_2 was distilled from phosphorous pentoxide. NMR spectra were recorded on Bruker AC-200 (200.13 MHz) and Bruker Avance II (500.13 MHz) spectrometers, using the signals of residual non-deuterated solvents as internal reference. All 2D NMR experiments (COSY, DEPT-HSQC, HMBC, NOESY) were performed using standard pulse sequences. HRMS were acquired on a Bruker micrOTOF-Q II spectrometer. UV spectra were obtained on a Hewlett Packard 8453 spectrophotometer and IR spectra were obtained on an FT-IR Nicolet Magna 550 instrument. TLC was carried out on Merck Sílicagel 60 F_{254} plates. TLC plates were sprayed with 2% vainillin in concentrated H_2SO_4. Merck Silicagel (230–400 mesh) was used for column chromatography. Solid phase extraction was performed with reversed-phase silica gel SPE cartridges (Thermo Scientific).

2.2. Biological material

Specimens of the pink sponge *S. fortis* Ridley, 1881 were collected from tidal pools at Bahía Bustamante, Chubut, Argentina. The samples were frozen until used. The sponge was identified by one of us (L.S.). A voucher specimen is preserved at Laboratorio de Bentos, INIDEP, Mar del Plata, Argentina[1].

Taxonomy: Phylum: Porifera, Class: Demospongiae, Order: Haplosclerida, Suborder: Haplosclerina, Family: Callyspongiidae Genus: *Siphonochalina, S. fortis* (synonymous: *C. fortis*).

2.3. Extraction and isolation

The frozen sponge sample (80 g) was macerated with 95% ethanol (500 mL). The extract was filtered and concentrated under reduced pressure, and the residue was partitioned between (90% MeOH: H_2O)/cyclohexane (100 mL each), to yield 0.294 and 0.033 g of polar and lipophilic subextracts, respectively. The lipophilic subextract was subjected to vacuum flash chromatography on silicagel (5 cm height, 4 cm i.d. syntherized glass funnel, packed with TLC grade silicagel, 2.5 cm height silica bed) and eluted sequentially using a cyclohexane/CH_2Cl_2 gradient (100 mL/fraction). Fraction 1 (8 mg) was purified by solid phase extraction on silicagel, eluting with cyclohexane to yield compound **1** (6.3 mg).

The polar subextract was subjected to a column chromatography (15 cm height, 4 cm i.d., packed with 60 g of silicagel) using a cyclohexane/EtOAc gradient to yield 4 fractions. Fraction 4 was further chromatographed (column: 15 cm height, 4 cm i.d., packed with 60 g of silicagel) using a CH_2Cl_2/EtOAc (1:1 v/v) gradient to yield 5 sub-fractions. Compound **2** (5.8 mg) was obtained by preparative TLC of sub-fraction 3 with CH_2Cl_2/MeOH (9:1 v/v). Compound **3** (3.2 mg) was purified from sub-fraction 4 by preparative TLC using cyclohexane/EtOAc (4:6 v/v) as eluant. The ^1H and ^{13}C NMR data of **1**–**3** agree well with literature data [13,14] **Supplementary Data**.

2.3.1. Deoxycholic acid 3, 12-diacetate (*1*)

White solid, mp 92–93 °C (lit. [15] mp 97 °C); ^1H NMR ($CDCl_3$, 500 MHz) δ 5.08 (1H, t, J = 2.8 Hz, H-12), 4.70 (1H, ddd, J = 11.4, 4.70 Hz, H-3), 2.40 (1H, m, H-23b), 2.25 (1H, m, H-23a), 2.10 (3H, s, H-28), 2.04 (3H, s, H-26), 0.91 (3H, s, H-19), 0.82 (3H, d, J = 6.4 Hz, H-21), 0.73 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 177.4 (C-24), 170.6 (C-27), 170.5 (C-25), 75.9 (C-12), 74.2 (C-3), 49.4 (C-14), 47.6 (C-17), 45.1 (C-13), 41.8 (C-5), 35.7 (C-8), 34.7 (C-1), 34.6 (C-20), 34.4 (C-9), 34.1 (C-10), 32.3 (C-4), 30.7 (C-23), 30.6 (C-22), 27.3 (C-6), 26.9 (C-2), 26.6 (C-16), 25.8 (C-11), 25.6 (C-7), 23.4 (C-15), 23.1 (C-19), 21.5 (C-26), 21.4 (C-28), 17.5 (C-21), 12.4 (C-18).

2.3.2. Cholic acid 3, 7, 12-triacetate (*2*)

White solid, mp 69–70 °C (lit. [8] mp 70 °C); ^1H NMR ($CDCl_3$, 500 MHz) δ 5.09 (1H, t, J = 2.8 Hz, H-12), 4.90 (1H, dd, J = 2.8 Hz, H-3), 4.60 (1H, ddd, J = 11.3, 4.4 Hz, H-7), 2.40 (1H, m, H-23b), 2.25 (1H, m, H-23a), 2.14 (3H, s, H-28), 2.10 (3H, s, H-30), 2.05 (3H, s, H-26), 0.92 (3H, s, H-19), 0.83 (3H, d, J = 6.5 Hz, H-21), 0.73 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 177.4 (C-24), 170.6 (C-29), 170.5 (C-25), 170.4 (C-27), 75.4 (C-12), 74.1 (C-7), 70.7 (C-3), 47.4 (C-17), 45.1 (C-13), 43.4 (C-14), 40.9 (C-5), 37.8 (C-8), 34.7 (C-1), 34.6 (C-6), 34.5 (C-20), 34.3 (C-10), 31.3 (C-4), 30.6 (C-22), 30.4 (C-23), 28.9 (C-9), 27.2 (C-2), 26.9 (C-11), 25.6

[1] Citation: van Soest, R. (2010). Siphonochalina fortis Ridley, 1881. In: Van Soest, R.W.M, Boury-Esnault, N., Hooper, J.N.A., Rützler, K, de Voogd, N.J., Alvarez de Glasby, B., Hajdu, E., Pisera, A.B., Manconi, R., Schoenberg, C., Janussen, D., Tabachnick, K.R., Klautau, M., Picton, B., Kelly, M. (2010) World Porifera database. Accessed through: Van Soest, R.W.M, Boury-Esnault, N., Hooper, J.N.A., Rützler, K, de Voogd, N.J., Alvarez de Glasby, B., Hajdu, E., Pisera, A.B., Manconi, R., Schoenberg, C., Janussen, D., Tabachnick, K.R., Klautau, M., Picton, B., Kelly, M. (2010) World Porifera database at http://www.marinespecies.org/porifera/porifera.php?p=taxdetails&id=193459 on 2011-07-12.

(C-16), 22.8 (C-15), 22.6 (C-19), 21.6 (C-30), 21.5 (C-26), 21.4 (C-28), 17.5 (C-21), 12.3 (C-18).

*2.3.3. Cholic acid 3, 7-diacetate (**3**)*

White solid, mp 263–264 °C (CH_3OH, lit [16] mp 261–262 °C); ^1H NMR ($CDCl_3$, 500 MHz) δ 4.90 (1H, dd, J = 2.7 Hz, H-3), 4.60 (1H, ddd, J = 11.3, 4.40 Hz, H-7), 4.00 (1H, t, J = 2.6 Hz, H-12), 2.40 (1H, m, H-23b), 2.30 (1H, m, H-23a), 2.21 (1H, dt, J = 6.2, 2.80 Hz, H-9), 2.07 (3H, s, H-26), 2.03 (3H, s, H-28), 1.81 (1H, m, H-22a), 1.40 (1H, m, H-22b), 0.99 (3H, d, J = 6.4 Hz, H-21), 0.92 (3H, s, H-19), 0.69 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 177.2 (C-24), 170.7 (C-27), 170.5 (C-25), 74.1 (C-7), 72.8 (C-12), 70.9 (C-3), 47.2 (C-17), 46.6 (C-13), 42.1 (C-14), 40.9 (C-5), 38.1 (C-8), 34.9 (C-20), 34.8 (C-6), 34.5 (C-1), 34.4 (C-10), 31.3 (C-4), 30.6 (C-22), 30.4 (C-23), 28.6 (C-11), 28.2 (C-9), 27.3 (C-2), 26.7 (C-16), 22.9 (C-15), 22.5 (C-19), 21.7 (C-26), 21.5 (C-28), 17.4 (C-21), 12.5 (C-18).

2.4. Single cell gel electrophoresis

2.4.1. Preparation of samples

The experimental design included the use of DMSO (dimethylsulfoxide) as co-solvent for the preparation of bile acid derivatives solutions, in a final concentration less than 1%. Considering the total amount of sponge extract (327 mg), the approximate quantification of the bile acid derivatives in the extract gave the following results: compound 1: 12.6 mg; compound 2:11.6 mg, and compound 3: 6.4 mg.

The tested doses of each bile acid derivative were calculated considering our previous results [6], in which a 6 μg/ml dose was determined as non cytotoxic for peripheral blood lymphocytes, based on mitotic index evaluation. In this way, the chosen biomarkers such as sister chromatid exchanges, cell proliferation kinetics and mitotic index could be analysed with an adequate response. For the evaluation of DNA damage by the comet assay, the initial dose was increased 2.5-fold and 5-fold as follows:

Compound 1 (deoxycholic acid 3,12-diacetate): dose 1: 0.23 μg/mL; dose 2: 0.58 μg/mL; dose 3: 1.15 μg/mL.

Compound 2 (cholic acid 3, 7, 12-triacetate): dose 4: 0.21 μg/mL; dose 5: 0.53 μg/mL; dose 6: 1.06 μg/mL.

Compound 3 (cholic acid 3, 7-diacetate): dose 7: 0.11 μg/mL; dose 8: 0.29 μg/mL; dose 9: 0.58 μg/mL.

2.4.2. Blood sampling

Peripheral blood heparinized samples obtained from three healthy volunteers donors with no history of exposure to any potential genotoxic agent were used as source of lymphocytes.

2.4.3. Cell viability

Cell viability was determined using the ethidium bromide/acridine orange assay described by Mc Gahon [17]. Briefly, 4 μL of dye-mix solution (100 μg/mL ethidium bromide and 100 μg/mL acridine orange) was added to 100 μL of cell suspension. Cells were observed with a 40× objective using a fluorescent microscope. At least, 200 cells were counted and results were expressed as percentage of viable and nonviable cells.

2.4.4. Alkaline comet assay

The procedure described by Singh et al., 1988 [18], was used with modifications. Each sample was processed in duplicate including negative and positive (H_2O_2 50 μM) controls. Compound samples were added at the beginning of the experiment in the above mentioned dose range. Fresh cell suspension (50 μL) was added to 950 μL RPMI (Roswell Park Memorial Institute) 1640, incubated for 1 h at 37 °C and then centrifuged at 1000 rpm for 5 min. Pellets were mixed with 200 μL of 1% low-melting point agarose solution at 37 °C and were spread onto slides precoated with 1% normal melting point agarose.

The lysis solution (2.5 M NaCl, 100 mM Na_2EDTA (Ethylenediaminetetraacetic acid), 10 mM Trizma, 1% Triton X-100 and DMSO 10%, pH 10) allows the rupture of cellular and nuclear membranes of embedded cells. The slides were submerged in cold, freshly prepared solution and left overnight at 4 °C. Afterwards, they were placed in a cold electrophoresis alkaline buffer (10 N NaOH, 200 mM Na_2EDTA, pH > 13) and the embedded cells were exposed for 20 min to allow DNA unwinding. Electrophoresis was performed in the same buffer at 25 V and 300 mA (0.75 V/cm) for 20 min at 4 °C. The slides were washed with neutralization buffer (Tris 0.4 M, pH 7,5) and DNA was stained with 50 μL of ethidium bromide (0.02 mg/ μL) and observed using a fluorescent microscope at 40×.

All the procedure was done in darkness to avoid additional DNA damage. Randomly selected cells (100) were analyzed visually on a scale of 0–4 depending of grade of damage. Damage Index Comet assay (DICA) was calculated.

2.5. Statistical analysis

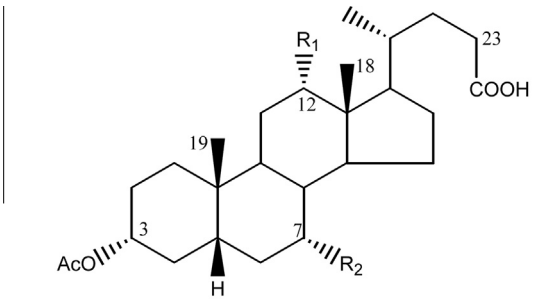
Statistical analysis was performed using one-way analysis of variance (ANOVA) test using Sigma Stat 9.0 software. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Extraction and isolation

Extracts from specimens of *S. fortis* collected at Bahía Bustamante, Chubut, Argentina, were analyzed and three major compounds were isolated and identified as deoxycholic acid 3, 12-diacetate (**1**), cholic acid 3, 7, 12-triacetate (**2**) and cholic acid 3, 7-diacetate (**3**) (Fig. 1). Compounds **1** and **2** were identified by comparison of their NMR spectra with reported data and authentic samples [13,14].

Compound **3** was isolated as an amorphous solid. The NMR spectra of **3** indicated the presence of three oxidized positions in the steroid nucleus, however only two acetates were observable. Two of the downfield multiplets (δ: 4.90 and δ: 4.59) were almost identical to the signals of H-3 and H-7 in compound **2**, however the signal corresponding to H-12 (δ: 4.01 t) showed a pronounced upfield shift, consistent with a free hydroxyl group at C-12. The acetate methyl at δ: 2.07 showed an HMBC correlation to C-7 (δ: 70.9) while the methyl at δ: 2.03 correlated to C-3 (δ: 74.1). On the other hand, Me-18 showed an HMBC correlation to the carbon at δ: 72.8,



1. R_1= OAc, R_2= H (deoxycholic acid 3, 12-diacetate)
2. R_1= OAc, R_2= OAc (cholic acid 3, 7, 12-triacetate)
3. R_1= OH, R_2= OAc (cholic acid 3, 7-diacetate)

Fig. 1. Structures of compounds 1–3 isolated from the marine sponge *Siphonocalina fortis*.

Table 1
Damage index in comet assay (DICA) in peripheral blood lymphocytes (from three donors individuals) exposed to deoxycholic acid 3, 12-diacetate (DCAd), cholic acid 3, 7, 12-triacetate (CAt) and cholic acid, 3, 7-diacetate (CAd) in three final concentrations in culture.

Damage index in comet assay							
Ind.	Doses μg/ml	Co(−)	Co(+)/H_2O_2	Co/DMSO	1-DCAd	2-CAt	3-CAd
1	1	136	196[a]	184	187.5	176	184.5
	2			184	186.5	168.5	199.5
	3			195	186.5	188	200
2	4	153	198[a]	188	182.5	183	187
	5			183	191.5	196	189
	6			191	181.5	185	198
3	7	145	175[a]	169	172.5	170	164.5
	8			174	172	175.5	170
	9			170	174	181.5	186.5

Ind.: individual; Co: control; 1 -(deoxycholic acid 3, 12-diacetate): dose 1: 0,231 μg/ml; dose 2: 0.58 μg/ml; dose 3: 1.15 μg/ml; 2- (cholic acid 3, 7, 12-triacetate): dose 4: 0.21 μg/ml; dose 5: 0.53 μg/ml; dose 6: 1.06 μg/ml; 3- (cholic acid, 3, 7-diacetate): dose 7: 0.11 μg/ml; dose 8: 0.29 μg/ml; dose 9: 0.58 μg/ml.
[a] Significant differences were found between the negative and positive control ($p < 0.05$) for all derivatives and donors individuals evaluated.

thus confirming that **3** was a 3,7-diacetate of cholic acid. All the remaining signals were assigned by careful interpretation of a complete set of 2D NMR experiments. Both cholic and deoxycholic acids were not detected in free form in the organic extract of *S. fortis* .

3.2. Cell viability and alkaline comet assay

A cell viability greater than 90% was observed in negative controls, solvent controls and treated cells. The results of the comet analysis in peripheral blood lymphocytes are shown in Table 1. All the groups exhibited a high proportion of type I and II comets whereas types III and IV were less frequent, except in the positive control which was characterized by type II, III and IV comets.

No significant differences were found between solvent control (DMSO) and treated cells with bile acid derivatives ($p > 0.05$), whereas significant differences were found between the negative and positive control ($p < 0.05$) for all derivatives and donors individuals evaluated (Table 1).

4. Discussion

Marine sponges have yielded a large number of novel metabolites of interest in various fields of scientific research. Thakur et al., [19], suggested that marine animals and their symbiotic microorganisms (bacteria and fungi) can produce an array of bioactive compounds for defensive purposes. Other secondary metabolites produced by sponges or their associated microorganisms play a dual role: they are involved in defense as well as in the activation of crucial pathways for self-defense [20].

The results of recent ecological studies indicate that in addition to the simple storage of chemical weapons, sponges have evolved mechanisms to increase the efficiency of their chemical defense and to save metabolic energy invested in this mechanism, as well as to protect themselves from cell damage caused by their own bioactive defense compounds [21].

Several parameters related to the biotransformation of mutagens/carcinogens showed that the metabolic machinery of marine sponge medulla cells is mainly oriented towards detoxification, producing high amounts of GSH [22], translating genes coding for P-glycoprotein P170, also known as a multidrug-resistance gene [23] and storing toxic metabolites as precursors which may be activated by enzymes [21].

Biosynthesis of bile acids has been reported in a large variety of marine bacterial strains that are widely distributed in seawater around the world, which are capable of producing at least three kinds of bile acids [24]. Regarding these compounds, few reports are known in marine sponges. Such is the case of an epidioxy sterol as an antifouling substance from *Lendenfeldia chondrodes* [25], the isolation of deoxycholic acid from *Ircinia* sp. [26] and bile acid derivatives from a sponge-associated bacterium *Psychrobacter* sp. that showed antibacterial activity [27]. In the last study, only one compound showed mild cytotoxicity against five human solid tumor cell lines.

Following the isolation and characterization, acetylated bile acids were subjected to analysis through the comet assay in order to evaluate its possible impact on DNA molecule (Table 1).

We developed our screening protocol taking into account that concentrations reported with an inhibitory effect in literature, turned out to be strongly cytotoxic in our system, as was established in our previous report [6]. Therefore, we tested three concentrations for each bile acid derivative; the zero dose was calculated taking into account the amount of isolated compound and relating it to the chosen dose in our previous experimental design.

Besides traditional cytogenetic methods, the single cell gel electrophoresis or comet assay allows evaluation of DNA fragmentation resulting from a variety of DNA damages (single and double strands breaks and alkali labile site including abasic sites). The Comet assay has become increasingly popular in the last 10 years because it is fast, inexpensive, and requires little biological material.

The analysis of this biomarker shows that the administration of the bile acid derivatives *per se* did not produce any statistically significant change in the parameter when compared to the solvent control, although some inter-individual differences were observed.

In this sense, it is relevant to consider the individual susceptibility factor since the subjects could be affected by other exposures, including those associated with diet, lifestyle and health. These interactions increase in those individuals exposed in the past or simultaneously to other hazardous toxins, consequently, a first exposure without immediate toxicity can cause a decrease of body's defences, an effect that alters the response to a second exposure or to another environmental agent [28].

5. Conclusions

Marine sponges have displayed evolutionarily the greatest number and diversity of natural molecules. Current knowledge of secondary metabolites as chemical defenses against large and small predators and competitors for space and resources is the most extensive of all the marine invertebrates.

In this work, an interdisciplinary approach to understand *S. fortis* chemical ecology allowed us, on one side, to extract and isolate three acetylated bile acids from this species. This is the first report

of acetylated bile acids in sponges, and the first isolation of compound **3** as a natural product. Compound **1** was previously isolated from the gorgonian *Junceela fragilis* [8], while compound **2** was isolated from a Formosan soft coral *Alcyonium* sp. [9]. Given the widely reported close relationship of sponges and their symbionts, as well as the previous reports of bile acid production by marine bacteria, a microbial origin of these metabolites is likely.

Since none of the free bile acids were detected in the extract, we can infer that the acetylated derivatives are probably not involved in a detoxification pathway. Moreover, our findings indicate that acetylated derivatives of both cholic and deoxycholic acids would not induce DNA damages *in vitro*, as single and double strands breaks and alkali labile sites, allowing us to consider acetylated bile acids as nontoxic metabolites at the tested concentrations.

The fact that most of the few examples of bile acids detected in marine invertebrates are acetylated derivatives may indicate a possible ecological role. An ecological interaction at the surface of the invertebrates mediated by a secondary metabolite requires that the released active compound presents low water solubility allowing a high surface concentration by lowering the diffusion in the water column as an effective defense strategy. In the case of a true symbiotic relationship, a bacterial strain may produce the bile acids which then can be acetylated by the sponge or the microorganism itself, to provide chemical defense for the host sponge, an interesting hypothesis that yet has to be proved.

Further studies are necessary to evaluate the possible antifouling action of these compounds. Field observations indicated that the surface of *S. fortis* is free from fouling organisms, a fact that may favor the previous hypothesis. Another aspect that will require further study is the possible qualitative or quantitative variation of these metabolites in different populations of *S. fortis*, taking account of seasonal and geographical aspects. A possible change in the production of the acetylated bile acids may explain the variable genotoxicity observed by us in samples collected from different locations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2013.05.020.

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