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Identification of the New Cytotoxic Pyrrole-Terpene Molliorin F from the Mediterranean Sponge *Cacospongia Mollior*

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Abstract

Sponges are considered the most promising marine organisms as a source of natural products for applications in biotechnology and pharmacological, nutraceutical and cosmeceutical fields. Here we focused our attention on the species *Cacospongia mollior*, previously collected in the Gulf of Naples and explored for its abundance in associated microorganisms. Several natural compounds were previously isolated from this species with cytotoxic activity against solid tumors but never on melanoma, considered the most aggressive and deadly type of skin cancer. Bioassay-guided fractionation was applied, leading to an enriched fraction of *C. mollior* able to induce cell death in human melanoma epithelial cell line (A2058) with an IC_{50} of 0.4 µg mL^{-1}. Through ^{1}H-NMR and mass spectrometry, we identified and characterized the new cytotoxic pyrrole-terpene molliorin F. Overall, our findings provide the first evaluation of this new anti-cancer compound from the marine sponge *C. mollior*, as new potential drug candidate for the melanoma treatment.

Keywords: Biotechnology; Cytotoxicity; Marine natural products; Melanoma; Molliorin; Sesterterpenoids

Introduction

Cancer is the second leading cause of death among humans and for this reason has drawn much attention from worldwide researchers which are all focusing on one objective: understanding action mechanism of the disease and to discover drugs that can be used in its treatment [1,2]. Melanoma is one of the most aggressive and deadly type of skin cancer, known for its poor prognosis as soon as metastasis occurs [3]. Melanoma develops due to sun exposure-induced DNA damage, which triggers the malignant transformation of melanocytes. Even if early-stage melanoma is considered to be curable with wide local excision, melanoma is fatal when metastasis occurs because of its potential to invade the dermis within only a few months [4]. In fact, one-third of patients with advanced melanoma have already developed lung, liver or brain metastasis by the time they receive a diagnosis. Consequently, the 5-year survival rate reaches 99% for patients with localized melanoma but strongly decreases for those with distant metastasis, decreasing to 27.3%. In this context, marine-derived natural drugs are becoming increasingly important due to their antitumor properties [5]. Hence, they are being tested for their activity to be validated for their use in clinical trials [6]. Specifically, secondary metabolites of marine sponges are considered a gold mine for naturally derived compounds [7].

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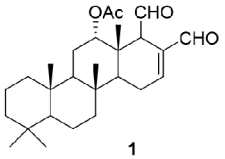
During our ongoing search for bioactive products from marine sponges [8, 9], we have re-analyzed specimens of the genus *Cacospongia* and *Scalarispongia* from the Gulf of Naples, from which many bioactive natural compounds have been previously reported [10, 11]. From the isolation of scalarin from *Scalarispongia scalaris* in 1972 [12], this genus of sponges yielded numerous and diverse cyclic sesterterpenes, collectively named scalaranes, characterized by a trans-fused 6/6/6/6 carbocyclic skeleton [13]. Among the wide structural variations, several scalaranes contain a heterocyclic five-membered rings, such as lactone, furan, lactam or pyrrole. This last group of scalarin-like pyrrolterpenes, molliorin A-E, is present in *C. mollior* [14-16] and derives by condensation of scalaradial with biogenic amine. Recently we also recorded a high microbial abundance (HMA) species in *C. mollior*, with most abundant bacterial phyla belonging at the genus *Dadabacteriales*, *Nitrospira*, *Poribacteria* and *Caldilineacea*, with high biotechnological potential [17]. In this work we deepened these studies to evaluate possible biotechnological application of the sponge *C. mollior*, collected in the Gulf of Naples, with the aim to search new cytotoxic compounds against human melanoma. Herein we discovery, along with the known cytotoxic scalaradial (**1**), a new pyrrole-terpene derivative **2**, which we named molliorin F, by a cytotoxicity-guided fractionation of the sponge on human melanoma cell line.



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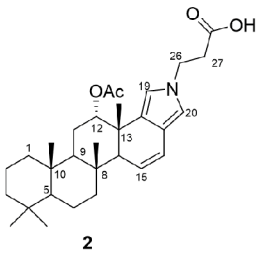

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Materials and Methods

Chemical extraction and fractionation

The sponge specimen was collected in the Gulf of Naples, Porto Paone (40°47′N, 14°9′E) at depths ranging from 15 to 17 meters [17]. *C. mollior* (35 g wet weight) was lyophilized to give 6.40 g of brown powder (dry weight). This material was extracted with methanol (Merk Life Science S.r.l., Milan, Italy) using a tissue homogenizer Precellys Evolution equipped with a cooling system Cryolys Evolution (Bertin Italia, Genoa, Italy). The protocol of extraction consisted of a run at 6200 rpm (3 cycles × 30 s), at the temperature of 16 °C to prevent degradation, followed by centrifugation of the sample at 3600 rpm for 10 min at 4 °C. The organic extract was dried in a rotatory evaporator using a maximum temperature of 24 °C. This raw sample was weighted and aliquoted using methanol-dichloromethane 2:1 (v/v) and was dried under a nitrogen flow, to obtain 1.03 g of crude extract and kept at −80 °C until further use. About 112 mg of raw extract was subjected to SPE on a GX-271 ASPEC Gilson apparatus (Gilson Italy, Cinisello, Milan, Italy) by using CHROMABOND® HRX cartridges (6 mL/500 mg, Macherey-Nagel, Düren, Germany) to obtain 5 enriched fractions: A (35.25 mg), B (3.43 mg), C (3.18 mg), D (6.49 mg) and E (6.69 mg), eluted with H_2O, CH_3OH/H_2O 1:1, CH_3CN/H_2O 7:3, CH_3CN, and CH_2Cl_2/CH_3OH 9:1, respectively [5, 18].

These fractions were tested, except fraction A that was mainly enriched in salt. The distribution of metabolites in the SPE fractions was monitored by thin layer chromatography (TLC) and ^{1}H-NMR.

On the basis of the cytotoxic tests, fraction D (6.49 mg) was subjected to another fractionation on a hydrophilic interaction liquid chromatography (HILIC) cartridge column (CHROMABOND® HILIC, Macherey-Nagel, Düren, Germany) by the automated GX-271 ASPEC apparatus (Gilson Italy, Cinisello, Italy) [5, 9]. The chromatographic process gave five fractions (A-E) that were eluted with 6 ml of THF/n-hexane 50:50 v/v (2.89 mg), THF 100% (0.55 mg), THF/MeOH 80:20 v/v (0.57 mg), THF/MeOH 50:50 v/v (0.46 mg), MeOH 100% (0.53 mg). These samples were tested again for cytotoxicity and the most active fraction C (0.57 mg), containing molliorin F, was then analyzed in CD_3OD (δ values 3.34 and 49.0 ppm) by NMR on Bruker DRX 600 MHz spectrometer equipped with a TXI CryoProbe (Bruker Bio-Spin, Fällanden, Swiss), and HRESI-MS / MSMS on a Q-Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometser (Thermo Scientific, Waltham, MA, USA) for structure elucidation (Figure S3-S8).

Cell maintenance and treatments

Extract and fractions were tested on human cell lines somatic prostate epithelium immortalized with SV40 (PNT2) and human melanoma epithelial cell line (A2058), grown in RPMI 1640 and medium Dulbecco's modified Eagle's medium (DMEM), respectively. 10% (v/v) of fetal bovine serum (FBS) was added to enrich the media together with 2 mM of L-glutamine and 100 units/mL penicillin and streptomycin. For the experiments, cells were seeded in 96-well plates at the concentration of 2’500 cells/well and kept overnight in thermostatic chamber in a 5 % CO_2 atmosphere at 37 °C to guarantee their correct attachment to the plate. For viability assays, the total extracts and fractions were suspended in dimethyl sulfoxide (DMSO) at a final concentration of 1% (v/v). Three concentrations were used for the analysis: 1, 10 and 100 µg/mL. The concentrations in the case of subfractions were 0.1, 1 and 10 µg/mL.

Cytotoxicity assay

The cytotoxicity of the crude extract and the fractions and

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subfractions was evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay (Applichem A2231). 48-hours post-treatment, 10 µL of MTT (5 mg/mL) was added in each well and the plate was incubated in the dark at 37 °C. After 3 hours the media was removed and 100 µL of isopropanol was added to the wells and incubated under agitation for 1 hour to make purple formazan crystals dissolve. The absorbance was measured with microplate reader at a wavelength of 570 nm (TECAN, Life Sciences). All experiments were performed in triplicate and the anti-proliferative activity was calculated as percentage of cell viability from the ratio of the mean absorbance of each sample and mean absorbance of controls.

Results

Bioassay-guided fractionation of the active fraction

The crude extract of the sponge and its HRX-SPE fractions [9, 18] were screened at three different concentrations (1, 10 and 100 µg/mL) against human melanoma epithelial cell line (A2058) and normal human prostate cell line (PNT2) as a control. In particular, the fraction D showed a promising cytotoxic activity with a percentage of cell viability of 65% (**Figure 1A-B**) at 10 µg/mL, a concentration at which it was not toxic to normal cells.

Chromatographic analysis (TLC) and ^{1}H NMR spectrum (Figure S1) of fraction D suggested the presence of a mixture of metabolites including the known scalaradial and other minor scalaranes. In order to purify the active metabolites, the fraction D was further processed on a second SPE column, applying an orthogonal chromatography fractionation based on a hydrophilic resin (HILIC-SPE) [5, 9]. The five new fractions obtained by this second chromatographic step were then tested on A2058 cells again (Figure 1C). The most active samples D-B and D-C were both analysed by ^{1}H NMR (Figure S2). Chemical analysis indicated the enrichment of a single metabolite which was used to define the IC_{50} of 0.4 µg/mL (Figure 1D).

Chemical elucidation of molliorin F

By ^{1}H-NMR, the active fraction D-C was clearly composed of a single molecule showing the presence of several methyl group typical of a scalarane skeleton. HR-ESIMS spectrum showed a major peak at *m/z* = 480.3137 for $[M-H]^-$ in agreement with the molecular formula $C_{30}H_{43}NO_4$ (calcd. for *m/z* 481.3192), together with the adduct $[2M-H]^-$ at *m/z* = 961.6343 (Figure S8). NMR data of this product revealed the diagnostic signals of a pyrrole ring at δ_H 6.52 (1H, d, *J* = 1.9 Hz CH-19) and 6.11 ppm (1H, d, *J* = 1.9 Hz CH-20). The ^{1}H-NMR spectra also contained two olefinic signals at

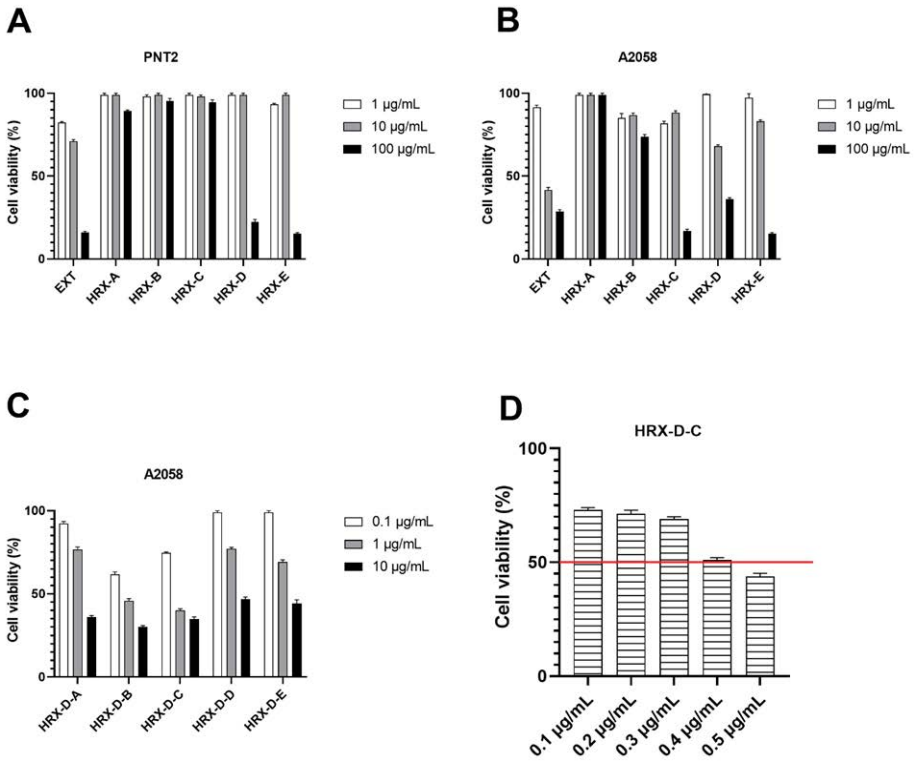

Figure 1: MTT assay of marine sponge extract and fractions on human melanoma cell lines. (A) Percentage cell viability of PNT2 and B) A2058 cells treated with total extract (EXT) and HRX-B, C, D, and E fractions from *C. mollior* at concentrations of 1, 10, and 100 µg mL^{-1}. (C) Percentage cell viability of A2058 cancer cells treated with subfractions from fractionation of HRX-D (HRX-D-A, HRX-D-B, HRX-D-C, HRX-D-D, HRX-D-E) of *C. mollior* at concentrations of 0.1, 1, and 10 µg mL^{-1}. (C) IC_{50} of HRX-D-C fraction.

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6.38 (1H, dd, J = 9.8, 2.9 Hz CH-16) and 5.54 (1H, dd, J = 9.8, 2.9 Hz, CH-15), five methyl singlet (δ_H 1.05, 1.08, 0.93, 0.88, 0.88), one acetoxy group (δ_H 2.03), a methine at δ_H 5.37 ppm correlating with an acetylic group, and two methylenes at δ_H 4.05 (2H, t, J = 14.5, 7.3 Hz CH_2-26) and 2.54 (2H, t, J = 14.5, 7.3 Hz CH_2-27) which correlated to each other in the COSY experiment. Hetero-correlations in 2D NMR spectra indicated the presence of a propionic substituent on the ring. Indeed, the protons 2.54 ppm (H_2-27) correlated with the carboxylic group (179.5 ppm) that was long-range correlated also with the protons at 4.05 ppm (H_2-26), showing also a cross peak with the carbon signals at 119.5 (C-19) and 108.6 (C-20) of the pyrrole ring (Figure 2). These data were fully supported by the infrared bands (IR) at 1567.64 and 1581.34 cm^{-1} for the acetyl and carboxylic group. The diagnostic MSMS fragment at m/z 408.2963 due to the neutral loss of the propionic acid also confirmed the designed structure.

As reported in Table 1, the other NMR signals were in complete agreement with a scalarane skeleton, thus leading to the assignment of the structure as **2**, named molliorin F in consideration of the clear similarities with the analogs previously reported. According to this, the absolute configuration of molliorin F (**2**) is proposed to be 4aS,4bR ,6S,6aS,7R,10aS,10bR,12aS, in analogy with scalaradial from which **2** very likely derive (Figure 3). Unlike others pyrrolterpenes that has been found so far, molliorin F is characterized by the presence of a propionic substituent on the pyrrolic nitrogen probably deriving by β-alanine (Figure 3).

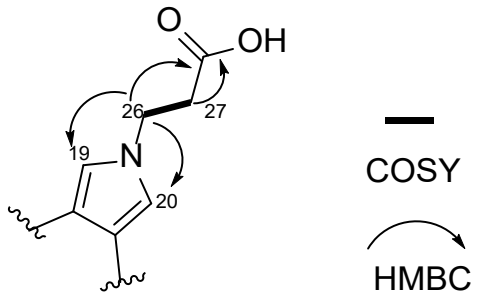

Figure 2: key HMBC and COSY correlations of 1.

Table 1: NMR Data[a] of Molliorin F in CD_3OD at 600 MHz.

C	Type	δ_c	δ_H, m (*J* in Hz)
1	CH_2	31.7	1.30, m
2	CH_2	21	1.64, m
			1.41, m
3	CH_2	43.8	1.4, m
			1.19, m
4	C	54.1	-
5	CH	58	0.90, bt
6	CH_2	41.8	1.64, m
7	CH_2	20.7	1.50, m
			1.30, m
8	C	139.2	
9	CH	54.2	1.42, m
10	C	149.4	
11	CH_2	24.7	1.84, m
12	CH	76.8	5.37, m
13	C	129.8	
14	CH	55.1	2.48, bd
15	CH	119.9	6.38, dd (9.8, 2.9)
16	CH	120.6	5.54, dd (9.8, 2.9)
17	C	120.4	
18	C	129.7	
19	CH	119.5	6.52, d (1.9)
20	CH	108.6	6.11, d (1.9)
21	CH_3	25.6	1.05, s
22	CH_3	20.2	1.08, s
23	CH_3	17.6	0.93, s
24	CH_3	34.3	0.88, s
25	CH_3	22.3	0.88, s
26	CH_2	48.8	4.05, t (14.5, 7.3)
27	CH_2	42.1	2.54, t (14.5, 7.3)
28	C=O	179.5	
OAc	CH_3	22.3	2.03, s
OAc	C=O	174.4	

[a] The structure assignment was obtained by ^1H-^1H COSY, TOCSY, HSQCedited and HMBC correlations.

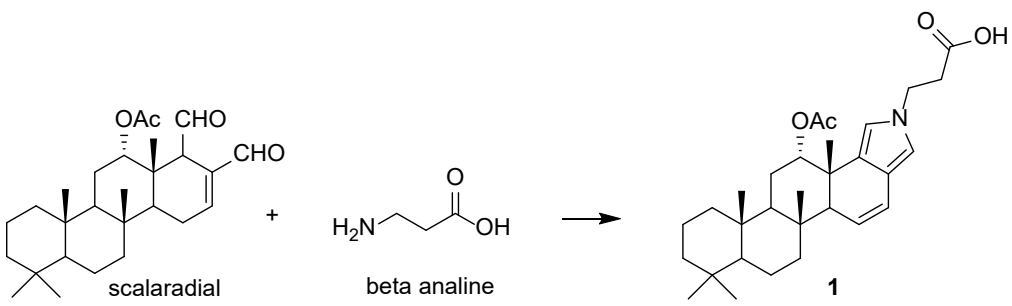

Figure 3: Suggested biogenesis of molliorin F (**2**).

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Discussion

Molliorins are pyrrole scalaranes that were first described several years ago from the Mediterranean sponge *C. mollior*. Despite their interesting structures and the large number of scalarane sesterterpenes (over two hundred) that have been reported in literature, the number of scalaranes with nitrogen-containing moieties is extremely small. After a long period following the initial isolation of pyrrole scalaranes in the 1970's [14, 16], scalaranes containing lactams or cyclic imides have been identified. Hyatelactam was the first example of a lactam-bearing scalarane [19] followed by the discover of 39 derivatives purified from the sponges of genus *Hyattella*, *Hyrtios*, *Petrosaspongia* and *Spongia* [20-25]. In particular, the latest report about this class of compounds of Shing et al. [25] describe the isolation of the first β-alanine-bearing scalarane named scalimides A–L [25]. Thus, scalarane alkaloids can be divided in different group based on the E rings general [25]. Between those containing lactams, hyatelactam and hyrtioscalarin G have been tested as anticancer compounds against several cancer cell lines (human colorectal adenocarcinoma cell line, adenocarcinoma human alveolar basal epithelial cell, human prostate) showing an IC_{50} values between 0.6 µM and 8.1 M [25]. Most of the glycine-bearing scalarane derivatives have been also tested against various human cancer cell lines by Kwon et al., but weak to no antiproliferation activity were observed [24].

By bioassay guided fractionation of the mediterranean sponge *C. mollior,* with a combination of consecutive hydrophobic and hydrophilic fractionations, we identify a new cytotoxic scalarane compound bearing the β-alanine moiety, molliorin F. The enrichment of the product was evident by comparing spectroscopic data of the HRX reference fraction and the HILIC active samples (Supplementary Figure S2). Molliorin F (**2**) represent the first cytotoxic pyrrole-terpene showing anticancer activity at submolar concentrations on human melanoma cells. Further studies to in deep investigate the mechanism of action of the active natural product are undergoing.

Mollioirn F (**2**) is not the first sesterterpene with cytotoxic activity. In previous studies on *C. mollior*, the sesterterpene cacospongionolide [25] was found responsible of apoptotic activity on human epidermoid carcinoma (A431), human breast cancer (T47D), human cervical cancer (HeLa) and colon carcinoma (HCT116) cells. Interesting results were obtained for HCT116 and HeLa cells [26, 27]. Another potent cytotoxic activity (ED_{50} = 0.58 µg/mL) against Lymphocytic Leukemia cell line (L1210) has been reported for desacetylscalaradial, a sesterterpene of *Scalarispongia scalaris*. This secondary metabolite was isolated together with scalaradial and heteronemin that did not display any cytotoxic activity [28]. Several bioactive sesterpenes named lintenolides A-G have been reported from another sponge in the genus *Scalarispongia* (*Cacospongia* cf. *linteiformis*) [29]. Among these, lintenolides F and G showed anti-proliferative activity on the murine leukemia cell line (P388) and the bovine endothelial cell line (GM7373), respectively. Lintenolide A also exhibited broad cytotoxicity on several cell lines, including murine fibrosarcoma (WEHI 164), murine monocyte/macrophage (J774), murine leukemia (P388), and bovine endothelial (GM7373) cells [29].

In addition to *C. mollior*, the number and variety of compounds extracted from the genera *Cacospongia* and *Scalarispongia* indicates that sponges belonging to this taxonomic group are a significant source of a wide variety of still unknown compounds with promising anticancer agents.

Acknowledgments

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Competing Interests

The authors declare that they have no competing interests.

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