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Isolation of ecdysterone from a sea anemone (Cnidaria, Actiniaria) collected off Dakar (Senegal) and in vitro cytotoxic evaluation against head-and-neck and renal carcinoma cell lines

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Abstract

Sea anemones (Cnidaria, Anthozoa, Actiniaria) are still poorly explored as a source of secondary metabolites, although some of them have shown cytotoxic and antitumour activity. A specimen collected off Soumbédioum (Dakar, Senegal) was extracted with ethanol and then with dichloromethane/methanol (1:1). The ethanolic extract, which gave the better HPLC profile, was fractionated by solid-phase extraction, partition with *n*-butanol and vacuum liquid chromatography (VLC) on RP-C18 silica gel, and a major polyhydroxylated sterol, SB1 (8.4 mg; 0.0021% of the wet weight), was obtained. HRMS ($[M+H]^+$ at *m*/*z* 481.3152, $C_{27}H_{44}O_7$; $\Delta = -1.6$ ppm) and 1D/2D NMR data (COSY, HSQC, HMBC) identified SB1 as ecdysterone (20-hydroxyecdysone), and analytical HPLC showed a single main peak. Cytotoxicity was tested by the MTT assay after 72 h of exposure (6.25–50 µM) on two head-and-neck squamous cell carcinoma lines (CAL33, CAL27) and one renal cell carcinoma line (786-O). Viability fell with concentration in all three lines. CAL33 and CAL27 were more sensitive (apparent IC_{50} ≈ 10.4 and 10.6 µM) than 786-O (≈ 31.1 µM), whose viability stayed close to 100% up to 12.5 µM. No non-cancerous cell line was tested, so these results show a difference in sensitivity between cancer lines and say nothing about selectivity for tumour cells. To our knowledge, this is the first report of ecdysterone from a sea anemone collected off the West African coast, together with a preliminary assessment of its *in vitro* cytotoxicity towards head-and-neck and renal carcinoma cells.

1. Introduction

Cnidarians are mostly marine animals found at all latitudes and depths. More than 13,000 species have been described [1], from a few millimetres in colonial forms to over a metre in solitary ones. The phylum includes hydrozoans, scyphozoans, cubozoans, staurozoans and anthozoans. The anthozoans, which comprise the reef-

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building corals and the sea anemones, account for about 6,100 species in the subclasses Hexacorallia and Octocorallia [1,2]. Sea anemones (order Actiniaria) are solitary polyps. They are regarded as a promising but little-studied source of bioactive secondary metabolites [3].

Studies on Actiniaria have mainly described polypeptides and cytolysins, but also carotenoids, indole alkaloids, anthraquinones, diterpenoids and steroids, some of which show antitumour, antimicrobial or enzyme-inhibitory activity [3–5]. Among the steroids, ecdysteroids are polyhydroxylated compounds first identified as moulting hormones of arthropods and later found in many plants (phytoecdysteroids) [6]. Ecdysterone (20-hydroxyecdysone) is the most widespread of them. In marine organisms, ecdysteroids have been reported mainly from zoantharians (Cnidaria, Hexacorallia: Zoantharia). 20-Hydroxyecdysone was isolated from a *Zoanthus* sp. from the Gulf of Thailand [7] and from *Antipathozoanthus hickmani* off Ecuador [8], and other ecdysteroids were described from *Palythoa australiae* [9] and *Parazoanthus* sp. [10]. We found no report of ecdysterone from an Actiniaria species.

Ecdysterone is known for its anabolic and adaptogenic effects and has also been tested in cancer models, although the data are few and varied. Ecdysteroids sensitised multidrug-resistant and non-resistant cancer cell lines to doxorubicin, paclitaxel and vincristine, but tended to protect them from cisplatin [11]. In breast cancer cells, ecdysterone slowed proliferation, lowered metabolic activity, induced autophagy and acted synergistically with doxorubicin, at concentrations up to 750 μM [12]. To our knowledge, there are few reports on head-and-neck or renal carcinoma cells. Both tumour types are hard to treat once they are metastatic or relapsed, which justifies the search for new chemotypes [13]. The CAL33, CAL27 and 786-O lines are well-established models and have recently been used to benchmark synthetic anticancer candidates [13,14], so they provide useful potency comparators.

This work is part of a programme on the marine biodiversity of the Senegalese coast. We isolated ecdysterone from a sea anemone collected off Soumbédioum (Dakar), characterised it by HRMS and NMR, checked its purity by analytical HPLC and tested its cytotoxicity on CAL33, CAL27 and 786-O cells with the MTT assay. The study is preliminary: it records a known ecdysteroid in a West African sea anemone, gives first *in vitro* cytotoxicity data for this compound on head-and-neck and renal carcinoma cell lines, and discusses the limits of these data.

2. Materials and Methods

2.1. Biological material

A specimen of sea anemone (Cnidaria, Anthozoa, Actiniaria) was hand-collected by scuba diving off Soumbédioum, Dakar (Senegal), in September 2016, at a depth of 3 m, and immediately frozen at −20 °C until extraction.

2.2. Extraction, isolation and structural identification of ecdysterone

The frozen wet specimen (405 g, wet weight) was extracted by ultrasound-assisted maceration, first three times with ethanol (3 × 300 mL), and then twice with dichloromethane/methanol (1:1, 2 × 200 mL), giving 7.67 g and 2.03 g of crude extract, respectively. Analytical HPLC comparison of the two extracts showed a more promising chemical profile for the ethanolic extract, which was selected for further study. After evaporation, the ethanolic extract was subjected to solid-phase extraction (SPE, RP-C18), followed by liquid–liquid partition

with *n*-butanol, affording 2.16 g of crude extract. This material was fractionated by VLC on RP-C18 silica gel using a decreasing-polarity gradient of H_2O/MeOH (100:0 to 0:100) followed by CH_2Cl_2/MeOH (50:50 to 100:0). The combined H_2O/MeOH 50:50 and 25:75 fractions were subjected to semi-preparative HPLC-DAD using an H_2O:MeCN gradient, affording three secondary metabolites, among which a major sterol (8.4 mg; 0.0021% of the wet weight), obtained as a colourless crystalline solid and hereafter referred to as SB1, was the main constituent.

High-resolution mass spectra were recorded in positive electrospray ionisation mode (ESI-HRMS) on a Thermo LCQ Advantage mass spectrometer. NMR spectra (^1H at 500 MHz, ^{13}C at 125 MHz), including 2D experiments (COSY, HSQC, HMBC), were acquired in CD_3OD on a Bruker Avance 500 MHz spectrometer at 298 K. HPLC-PDA-ELSD analyses were performed with a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA) coupled with a Waters 996 photodiode array detector and a Sedex 55 evaporative light-scattering detector (SEDERE, France), using a bifunctional Macherey-Nagel NUCLEODUR® Sphinx RP column (250 × 4.6 mm, 5 µm) consisting of a balanced ratio of propylphenyl and C18 ligands. The mobile phase was composed of H_2O (plus 0.1% formic acid) and acetonitrile (MeCN plus 0.1% formic acid), and the following gradient was used: H_2O:MeCN 90:10 for 5 min, 90:10 to 0:100 for 30 min, 0:100 for 5 min, and 0:100 to 90:10 for 15 min (flow rate: 1.0 mL/min; injection volume: 20 µL). Chromatograms were extracted at detection wavelengths of 214, 254 and 280 nm for visual inspection.

2.3. Purity of the test compound and preparation of stock solutions

The purity of SB1 was checked by analytical HPLC-PDA before the biological assays (Figure 2; Section 3.2). Stock solutions of SB1 were prepared in DMSO and diluted in complete culture medium, so that the final DMSO concentration was below 0.1% (v/v) in all wells. Vehicle control wells (0 nM) received the same amount of DMSO without compound.

2.4. Cancer cell lines and culture conditions

CAL33 and CAL27 (head-and-neck squamous cell carcinoma, tongue) and 786-O (clear-cell renal cell carcinoma) cells were used. The CAL lines were established at the Centre Antoine Lacassagne (Nice, France) [15], which supplied them. The 786-O line was provided by the Institute for Research on Cancer and Aging, Nice (IRCAN). CAL27 and CAL33 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and 786-O cells in RPMI-1640 medium, each with 10% (v/v) foetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin, at 37 °C in a humidified atmosphere with 5% CO2 [13,16]. Cells were used at low passage numbers (p+5 to p+20) and tested negative for mycoplasma on routine controls.

2.5. In vitro cytotoxicity assay (MTT test)

Cytotoxicity was measured with the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [17,18]. Cells were seeded in 96-well plates at 5 × 103 cells per well in 100 µL of complete medium and left to adhere for 24 h. They were then exposed for 72 h to SB1 at 6250, 12500, 25000 and 50000 nM (6.25–50 µM) or to the vehicle alone (0 nM; < 0.1% DMSO, Section 2.3), each condition in triplicate wells. MTT solution (0.5 mg/mL final concentration) was then added to each well and the plates were incubated for 2–4 h at 37 °C. The medium was removed, the formazan crystals were dissolved in DMSO and the absorbance was read at 570 nm with a microplate reader. Viability was expressed as a percentage of the vehicle control (100%). The whole experiment was repeated three times independently.

2.6. Data analysis

Viability is given as mean ± standard deviation (SD) of triplicate wells from three independent experiments. Each concentration was compared with the vehicle control by one-way ANOVA followed by Dunnett's test, with $p < 0.05$ taken as significant. Apparent IC_{50} values were estimated for each cell line from the mean dose–response curve by linear interpolation between the two concentrations on either side of 50% viability:

$$IC_{50} = C_1 + [(50 - V_1) / (V_2 - V_1)] \times (C_2 - C_1),$$

where C_1 and C_2 are the concentrations below and above 50% viability and V_1 and V_2 the corresponding mean viabilities (%). The calculation was also done on a $\log_{10}$ concentration scale and back-transformed. A four-parameter logistic fit was not possible because only two concentrations lie in the transition range of each curve [19]. The IC_{50} values are therefore approximate and have no confidence interval, and the cell lines are compared only through the ratio of their IC_{50} values, without a statistical test. Concentrations in µM were converted to µg/mL with M = 480.6 g/mol.

3. Results and Discussion

3.1. Structural identification of SB1 as ecdysterone

SB1 was obtained as a colourless crystalline solid (8.4 mg). The HRMS spectrum showed a protonated molecule at *m*/*z* 481.3152 ($[M+H]^+$), in agreement with the molecular formula $C_{27}H_{44}O_7$ (calcd. for $C_{27}H_{45}O_7^+$, 481.3160; $\Delta = -1.6$ ppm), which corresponds to six degrees of unsaturation. These are accounted for by the four rings of a steroid nucleus, one C=C bond and one C=O group. The ^{13}C NMR data (Table 1) display the diagnostic signals of an ecdysteroid: an α,β-unsaturated ketone (δ_C 206.3, C-6; 122.2, C-7; 167.8, C-8), a 14-hydroxylated quaternary carbon (δ_C 85.3, C-14), and a polyhydroxylated framework bearing two oxymethines on ring A (δ_C 68.7, C-2; 68.5, C-3) and three oxygenated carbons on the side chain (δ_C 78.4, C-20; 78.3, C-22; 71.0, C-25). The olefinic proton H-7 resonates as a singlet at δ_H 5.81 and shows HMBC correlations with C-9 and C-14, which defines the 7-en-6-one chromophore. The angular methyl singlets H_3-18 (δ_H 0.89) and H_3-19 (δ_H 0.97) and the side-chain methyl singlets H_3-21 (δ_H 1.20), H_3-26 (δ_H 1.19) and H_3-27 (δ_H 1.21) are also characteristic. HMBC correlations of H_3-18 with C-12, C-13, C-14 and C-17, of H_3-21 with C-17, C-20 and C-22, and of H_3-26/H_3-27 with C-24 and C-25 establish the connectivity of the C/D rings and of the 20,22,25-trihydroxylated side chain.

The chemical shifts of C-13 to C-17 and C-20 to C-27 differ by at most 0.5 ppm from those reported in CD_3OD for the C/D rings and side chain of the 20-hydroxyecdysone-type ecdysonelactones from the zoantharian *Antipathozoanthus hickmani* [8], and H-22 resonates at the same shift (δ_H 3.32). Together with the HRMS data, this supports the identification of SB1 as ecdysterone (20-hydroxyecdysone). The identification rests on comparison with literature data. The relative configuration was not established independently (for example by NOESY), and comparison with an authentic commercial standard (co-injection, optical rotation) would give further confirmation. The complete NMR data are given in Table 1.

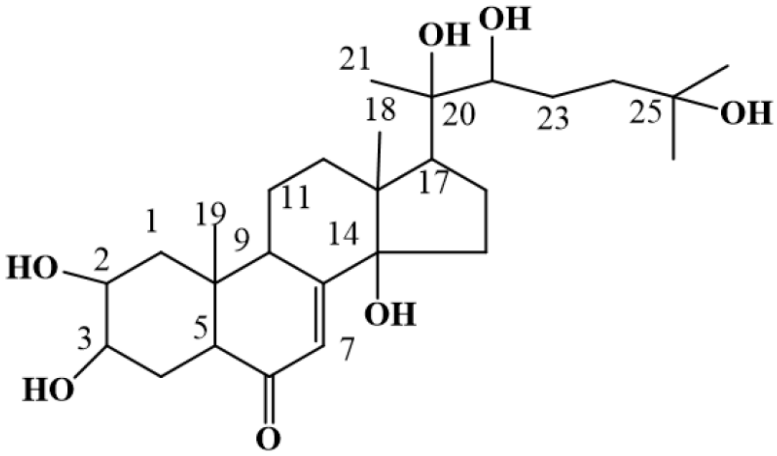


Figure 1. Chemical structure of ecdysterone (20-hydroxyecdysone).

3.2. Purity of the isolated compound

Because the cytotoxic activity reported below is attributed to SB1, its purity matters. The analytical HPLC profile of the isolated compound (Figure 2; conditions in Section 2.2) shows one main peak at a retention time of about 13 min, and no significant additional peak over the 60 min run on any detection channel. A pure sample is needed to attribute the biological effect to ecdysterone and not to a minor co-eluting contaminant, although trace impurities below the detection limit of the method cannot be excluded.

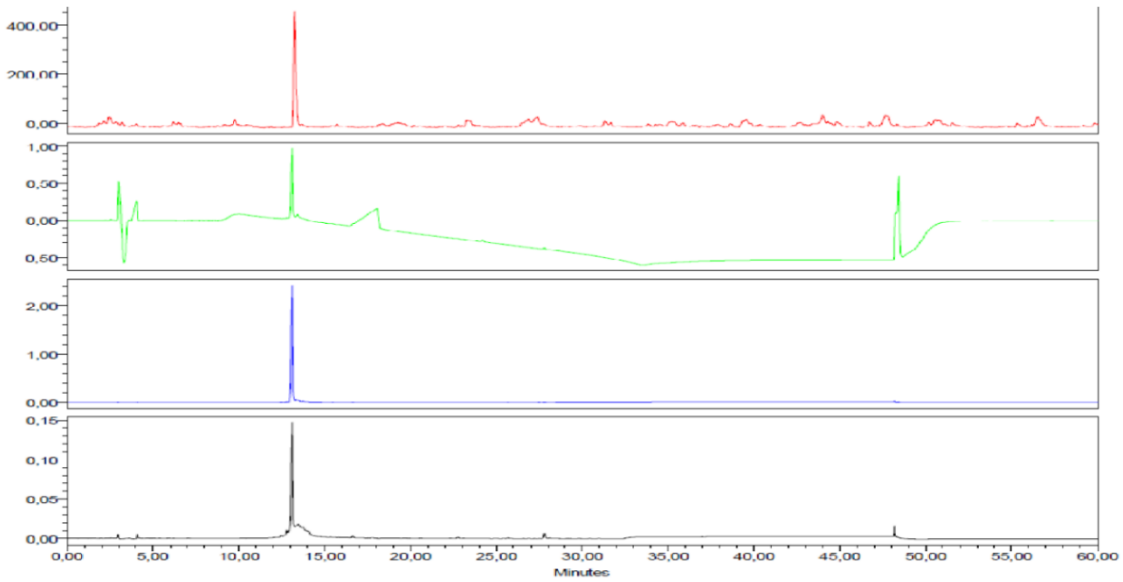


Figure 2. Analytical HPLC profile of the isolated ecdysterone, showing a single major peak at $t_R \approx 13$ min (time axis in minutes; the four traces correspond to the detection channels recorded).

3.3. Cytotoxic activity of ecdysterone against CAL33, CAL27 and 786-O cells

Figure 3 shows the effect of SB1 on cell viability after 72 h of continuous exposure (0–50 µM), and Table 2 gives the apparent IC_{50} values. Viability decreased with concentration in all three cell lines (means ± SD of

three independent experiments, each in triplicate wells). In CAL33 and CAL27, viability was 92–95% at 6.25 µM, about 30% at 12.5 µM and close to zero from 25 µM, which gave apparent IC_{50} values of about 10.4 µM (CAL33) and 10.6 µM (CAL27). The 786-O cells were hardly affected up to 12.5 µM (viability close to 100%) and responded only between 25 and 50 µM, with an apparent IC_{50} of about 31.1 µM, roughly three times higher than in the head-and-neck lines. Interpolation on a logarithmic concentration scale gave slightly lower values (9.8, 10.1 and 29.7 µM, respectively) and the same ranking (Table 2). Compared with the vehicle control (one-way ANOVA followed by Dunnett's test), viability was not significantly reduced at 6.25 µM in any line ($p > 0.05$), nor at 12.5 µM in 786-O. The reduction became significant at 12.5 µM in CAL33 (viability ≈ 28%) and CAL27 (≈ 30%) ($p < 0.001$) and at 25 µM in 786-O (viability ≈ 66%; $p < 0.01$). At 25 and 50 µM the decrease was highly significant in both head-and-neck lines ($p < 0.001$), as it was in 786-O at 50 µM (viability ≈ 1%; $p < 0.001$).

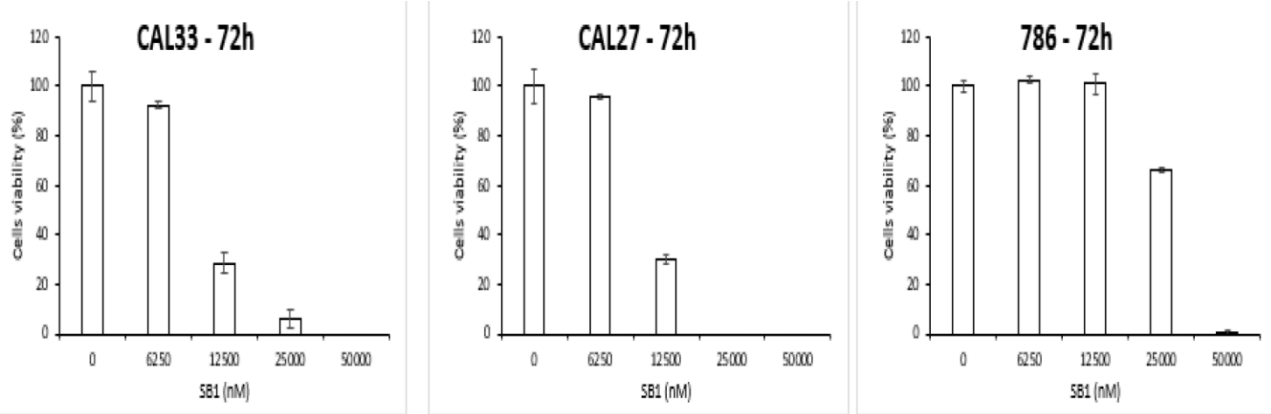


Figure 3. Effect of ecdysterone on the viability (%, mean ± SD of three independent experiments, each in triplicate wells) of CAL33 and CAL27 (head-and-neck squamous cell carcinoma) and 786-O (renal cell carcinoma) cells after 72 h of exposure, as a function of concentration (nM; 6250–50,000 nM = 6.25–50 µM). Viability is expressed relative to the vehicle-treated control (< 0.1% DMSO; 100%); MTT assay. Asterisks indicate statistically significant differences compared with the vehicle control (one-way ANOVA followed by Dunnett's test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 2. Apparent 72-h IC_{50} values of ecdysterone in the three cancer cell lines (MTT assay). Estimated from the mean dose–response curves by interpolation between the two concentrations bracketing 50% viability.

Cell line	Tumour type	Apparent IC_{50} (µM), linear interpolation	Apparent IC_{50} (µM), log-linear interpolation	IC_{50} (µg/mL)[a]	Ratio vs CAL33
CAL33	Head-and-neck (tongue) squamous cell carcinoma	10.4	9.8	5.0	1.0
CAL27	Head-and-neck (tongue) squamous cell carcinoma	10.6	10.1	5.1	1.0
786-O	Clear-cell renal cell carcinoma	31.1	29.7	15.0	3.0

[a] Calculated from the linear-interpolation IC_{50} with M = 480.6 g/mol.

3.4. Discussion

Using the usual criterion for pure compounds in cytotoxicity screening (IC_{50} below about 4 µg/mL) [20], ecdysterone is only moderately active: its apparent IC_{50} in CAL33 and CAL27 (about 5 µg/mL) is just above the threshold, and that in 786-O (about 15 µg/mL) is well above it. For comparison, optimised synthetic CXCR1/CXCR2 inhibitors gave IC_{50} values of 2–7 µM in the same three lines in a 48-h XTT assay [13]. Because the assay, the exposure time and the type of compound differ, this comparison is only indicative.

Previous anticancer work on ecdysterone concerned breast cancer cells and multidrug-resistant models, in which the compound acted mainly as a chemosensitiser or lowered metabolic activity at concentrations up to 750 µM [11,12]. Ecdysteroids are generally considered to have low toxicity towards mammalian cells and organisms [21], so an apparent IC_{50} of about 10 µM in head-and-neck cells is surprisingly low. Ecdysonelactones and other ecdysteroids from a zoantharian were not significantly cytotoxic to five human cancer cell lines [8]. The difference could come from the cell type, from the assay format and exposure time, or from a minor but very potent contaminant in our sample, or it could be an artefact. Testing a commercial ecdysterone sample alongside SB1 would settle the question. Ecdysteroids also tended to protect cancer cells from cisplatin [11], the reference drug for head-and-neck carcinoma, so any combination with this drug would need specific study.

Ecdysteroids, including 20-hydroxyecdysone, have so far been reported in marine cnidarians mainly from zoantharians [7–10], while the chemistry of sea anemones is dominated by polypeptides and cytolysins [3–5]. As far as we know, ecdysterone had not been described from an Actiniaria species. This work reports ecdysterone from a sea anemone for the first time, from a specimen collected off West Africa, and gives the first *in vitro* cytotoxicity data for this compound on CAL33, CAL27 and 786-O cells. Since ecdysterone is a well-known compound, its isolation is mainly a chemotaxonomic and ecological finding rather than a new chemotype, and the biological data are preliminary. Its presence in the anemone does not prove that the animal makes it. Sea anemones are mostly carnivorous and many harbour symbiotic dinoflagellates, so a dietary origin (arthropod prey are rich in ecdysteroids) or a symbiont origin is possible; we did not investigate this.

The head-and-neck lines were about three times more sensitive than the renal line, but this is a difference between cancer cell lines. As no non-cancerous cell line was tested, our results say nothing about selectivity for tumour cells. The selectivity index (IC_{50} in a normal cell line divided by IC_{50} in the cancer cell line) [13,22] should be determined in non-tumourigenic cells, such as primary renal epithelial cells or normal keratinocytes; earlier work with these cell lines used primary normal cells in this way [13].

This study has several limitations. The MTT assay measures mitochondrial reductase activity rather than cell death, and ecdysterone has been reported to reduce glycolytic and respiratory activity in some cancer cells [12], so MTT reduction may overestimate cytotoxicity. Other assays (for example sulforhodamine B [23] or direct cell counting, with apoptosis markers) are needed. Only one exposure time (72 h) and four concentrations were used, so the IC_{50} values are approximate. No reference cytotoxic drug was included and the mechanism of action was not studied. The specimen was identified only to order level. Finally, all data are in vitro, and nothing can be concluded on efficacy or safety in vivo.

4. Conclusion

Ecdysterone (20-hydroxyecdysone) was isolated from a sea anemone collected off Dakar (Senegal) and identified by HRMS and 1D/2D NMR, and analytical HPLC showed a single main peak. In the MTT assay it

reduced the viability of the three human cancer cell lines in a concentration-dependent way. The head-and-neck lines CAL33 and CAL27 (apparent 72-h $IC_{50} \approx 10–11$ µM) were more sensitive than the renal line 786-O (≈ 31 µM). The cytotoxic activity is moderate and depends on the cell line; selectivity for cancer cells has not been shown. Before the potential of ecdysterone and related marine ecdysteroids as anticancer leads can be judged, further work should include non-cancerous control cells, a commercial ecdysterone standard and reference cytotoxic drugs, other viability and apoptosis assays, and mechanistic studies.

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Appendix

Table 1. NMR data (500 MHz, CD3OD) of ecdysterone (SB1).

C#	δC (ppm)	Mult.	δH(ppm)	J(Hz)	COSY	HMBC
1	37.17	CH_2	1.44 /1.80	14.4/–	1b,2/1a,2	2,9,10
2	68.67	CH	3.95	–	1,3	–
3	68.51	CH	3.84	–	4,2	–
4	42.17	CH_2	1.43 /1.79	14.1/–	4b,3/ 4a,3,5	2,3,6,9,10
5	50.38	CH	2.39	–	4	6,7,9,10,19
6	206.25	qC	–	–	–	–
7	122.17	CH	5.81	s	–	9,14,15
8	167.82	qC	–	–	–	–
9	34.76	CH	3.15	–	7,11	8,11
10	39.23	qC	–	–	–	–
11	20.90	CH_2	1.73 /1.80	–	9,12	8,10,12,17
12	32.54	CH_2	1.74	–	–	11
13	48.86	qC	–	–	–	–
14	85.31	qC	–	–	–	–
15	32.41	CH_2	1.88 /2.13	–	15b,16/ 15a,16	13,16
16	21.22	CH_2	1.60 /1.98	10.2/–	16b,15,17/ 16a,15,17	15,17
17	50.38	CH	2.39	13.2;7.7	16	15,16,18
18	24.35	CH_3	0.89	s	–	12,13,14,17
19	17.96	CH_3	0.97	s	–	1,2,5,9
20	78.42	qC	–	–	–	–
21	20.94	CH_3	1.20	s	–	5,17,20,22
22	78.32	CH	3.32	–	23	20,21,23,24
23	27.22	CH_2	1.29 /1.67	13.3/–	22/ –	22
24	42.17	CH_2	1.44 /1.80	14.1/–	–/–	22,25
25	71.03	qC	–	–	–	–
26	28.92	CH_3	1.19	s	–	24,25,27
27	29.42	CH_3	1.21	s	–	24,25,26

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