Antifouling Compounds from Two Red Sea Organisms: a *Hyrtios* sp. Sponge and an *Okeania* sp. Cyanobacterium

Julie G. PETITBOIS

Introduction

Biofouling is defined as the accumulation of organisms on submerged structures such as ships' hulls, underwater pipelines, oil rigs, piers, buoys etc. It causes large economic loss and serious ecological problems worldwide. For instance, in naval industry, not removing biofouling leads to corrosion, resistance in the water and increase of fuel consumption. This increase results in a raise of contaminant gases, harmful particles and so of global warming. To avoid all these troubles and keep our planet livable, antifouling strategies are needed. One of them is to copy Nature by mimic sessile organisms which produce chemical defenses to be free of fouling and avoid predation. Several marine natural compounds have already been isolated from marine organisms, mainly from cnidaria, sponges and algae.

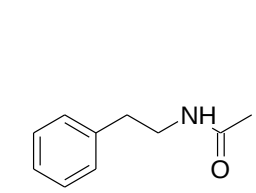
For this study, about eighty extracts of different marine organisms from the Red Sea were tested on barnacle larvae (*Amphibalanus amphitrite*) which are hard-to-remove macrofoulers and found worldwide. The Red Sea is a special ecosystem in the sense that it is partially isolated from the open ocean and, because of its location between deserts, evaporation occurs, making this sea the warmest and most saline one in the world, which allows high density of organisms so high competition among them. Because of these special conditions, Red Sea organisms probably produce unique compounds, including antifoulants, to adapt and live within the unique flora and fauna this sea offers. Therefore, the Red Sea is not the only reservoir of antifouling compounds but is probably one of the most legitimate area to search of such compounds. Among the tested extracts, about 18% were very active (active at 1 µg/mL) and 31% were moderately active (active at 10 µg/mL). Among the active extracts, two were selected because of their large amount of material: a *Hyrtios* sp. sponge and an *Okeania* sp. cyanobacterium. Sponge are prolific producers of antifouling compounds while cyanobacteria are not yet well studied for such compounds although they can be cultured in large scale to afford large amounts of active compounds for the industries.

Chapter 1: *N*-phenethylacetamide and fatty acid methyl esters from the sponge *Hyrtios* sp.

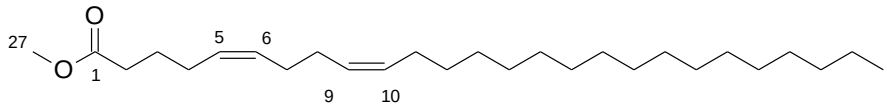
I. 1. Materials and Methods

I. 1. a. Isolation

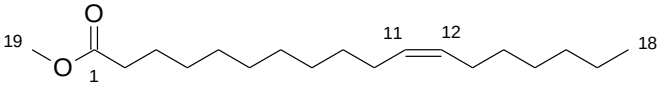
The *Hyrtios* sp. methanol extract was partitioned between EtOAc and H_2O (1:1, *v/v*). The H_2O fraction was further purified using *n*-BuOH (1:1, *v/v*). As the BuOH fraction exhibited antifouling activity on barnacle larvae, fractionation by ODS open column chromatography was performed. Semi-preparative reverse-phase (RP) HPLC fractionation of the 6:4 MeOH/H_2O and the 100% MeOH fractions from *Hyrtios* sp. afforded *N*-phenethylacetamide (**1**) (4.7 mg) and two fatty acid methyl esters (FAMEs), respectively. The two FAMEs were identified by NMR and GC-MS-MS techniques (described below) as methyl-(5*Z*,9*Z*)-hexacosa-5,9-dienoate (**2**) (3.6 mg) and methyl-(*Z*)-octadec-11-enoate (**3**) (2.5 mg).



N-Phenethylacetamide (**1**)



Methyl-(5*Z*,9*Z*)-hexacosa-5,9-dienoate (**2**)



Methyl-(*Z*)-octadec-11-enoate (**3**)

I. 1. b. Structure elucidation

The position of the double bonds of both FAMEs **2** and **3** was determined by DMDS derivatization and study of the derivatives fragments by GC-MS-MS, while their configuration was ascertained by study of the chemical shifts of the allylic carbons and the coupling constants of the olefinic protons via ^{13}C non-decoupled HSQC with ^{1}H homodecoupling technique.

I. 1. c. Antifouling assay

Antifouling activity was studied on *Amphibanalus amphitrite* barnacle larvae. Six healthy larvae were put in each well and exposed to different concentrations. After 48 hours, the number of settled and floating larvae were counted while dead larvae were counted after 120 hours. EC_{50} values were calculated by probit analysis from the settlement rate after 48 hour-exposure.

I. 2. Results.

I. 2. a. Structure of the fatty acid methyl esters

The position of the double bonds of **2** was determined by 2D NMR technique but was further confirmed by DMDS derivatization followed by study of GC-MS/MS fragmentation. The key fragments were observed at *m/z* 161 for $[C_7H_{13}O_2S]^+$ and *m/z* 339 for $[C_{22}H_{43}S]^+$, showing a cleavage between C-5 and C-6, and at *m/z* 215 for $[C_{11}H_{19}O_2S]^+$ and *m/z* 285 for $[C_{18}H_{37}S]^+$, showing a cleavage between C-9 and C-10. For **3**, fragmentation of the DMDS adduct gave two major peaks at *m/z* 245 corresponding to the fragment $[C_{13}H_{25}O_2S]^+$ and at *m/z* 145 corresponding to $[C_8H_{17}S]^+$. This shows that a cleavage occurred between C-11 and C-12 so that the position of the double bond is at C-11. The configuration of the double bonds was assessed by ^{13}C non-decoupled HSQC with ^1H homodecoupling technique and confirmed via study of allylic carbon chemical shifts. The coupling constants were between 11 and 12 Hz for **2** and **3**, close to the typical value for *cis* configuration (10 Hz instead of 16 Hz for *trans* configuration). The chemical shifts of the allylic carbons gave stronger evidence. Indeed, for **2** and **3**, these carbons have chemical shifts between 26 and 27 ppm, which is in the range of shifts for *cis* configuration (25-28 ppm, instead of 32-36 ppm for *trans* configuration). Both compounds have double bonds with a *cis* configuration.

I. 2. b. Antifouling activity

Compound **1** was the least active, with a EC_{50} of 9.8 µg/mL. Both fatty acid methyl esters **2** and **3** showed potent activity with EC_{50} values below 1 µg/mL. However, after 120 hour-exposure, some floating larvae could settle. The two FAMEs can slow down the settlement but not inhibit it completely.

I. 3. Discussion

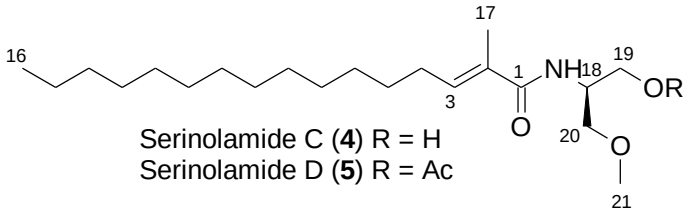
Three known compounds, **1-3**, were isolated from a *Hyrtios* sp. sponge. Compound **1** has a structure close to dopamine, which is a known antifoulant. It can act in the same way. Several fatty acid methyl esters have already been isolated from marine sponges. Because of their structure, they could act as biosurfactant so could inhibit the settlement of barnacle larvae by creating a slippery surface.

Chapter II: New fatty acid amides from the cyanobacterium *Okeania* sp.: serinolamides C and D

II. 1. Materials and Methods

II. 1. a. Isolation

The *Okeania* sp. methanol extract was partitioned between EtOAc and H_2O (1:1, *v/v*). The H_2O fraction was further purified using *n*-BuOH (1:1, *v/v*). As the EtOAc fraction exhibited antifouling activity on barnacle larvae, fractionation by silica gel open column chromatography was performed. The 6:4 Hex/EtOAc fraction gave serinolamides C (**4**) (11.7 mg) and D (**5**) (1.6 mg).



Serinolamide C (**4**) R = H
Serinolamide D (**5**) R = Ac

II. 1. b. Structure elucidation

The configuration of the double bonds of the fatty acid amides **4** and **5** was elucidated by study of the chemical shifts of the olefinic methyl. Partial synthesis and Marfey's analysis of the synthetic (*R*)- and (*S*)-*O*-methyl serinol and the hydrolysate of **4** and **5** allowed the elucidation of the configuration at C-18. The (*R*)- and (*S*)-*O*-methyl serinol were synthetized from L- and D-serine, respectively. To a solution of D-serine (1 g, 9.52 mmol) in CH_3OH (47.6 mL) was dropwise added acetyl chloride (2 mL, 28.6 mmol). The reaction mixture was then stirred overnight at 80 °C. Solvent was evaporated to quench the reaction and the D-serine methyl ester hydrochloride salt was obtained as a white solid. It was dissolved in a 1:5 *v/v* saturated solution of $NaHCO_3$ in MeOH (60 mL) and a solution of Boc_2O (2.62 mL, 11.42 mmol). The reaction mixture was stirred overnight at room temperature. Dichloromethane (100 mL) was added and washed with HCl (1 N, 3 x 50 mL) and brine (50 mL). The organic layer was dried over anhydrous sodium sulfate. After filtration, the solvent was evaporated to obtain (*R*)-methyl 2-(*tert*-butoxycarbonyl)-3-hydroxypropanoate as a white oil (820 mg, 82%). The residue was used without further purification. To a solution of (*R*)-methyl 2-(*tert*-butoxycarbonyl)-3-hydroxypropanoate (200 mg, 0.912 mmol) in CH_3CN (7.9 mL) was added Ag_2O (1.08 g, 4.65 mmol) and CH_3I (130 µL, 0.115 mmol). The reaction mixture was protected from the light, stirred and heated overnight. The mixture was then brought to room temperature

and filtered through a pad of Celite. After evaporation of the solvent, the residue was purified by flash chromatography on silica gel (5:95 EtOAc/Hex) to afford (*R*)-methyl 2-(*tert*-butoxycarbonyl)-3-methoxypropan. To (*R*)-methyl 2-(*tert*-butoxycarbonyl)-3-methoxypropan (62 mg, 0.226 mmol) in CH_3OH/H_2O (1:1, 532 µL) was added $NaBH_4$ (20.1 mg, 0.532 mmol) in four portions at 0 °C. The mixture was then stirred overnight at room temperature. It was then extracted with EtOAc (3 x 15 mL) and the organic layer was washed with brine (15 mL), dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by flash chromatography on silica gel (1:2 EtOAc/Hex) to afford (*S*)-tert-butyl-1-hydroxy-3-methoxypropan-2-ylcarbamate. To a solution of (*S*)-tert-butyl-1-hydroxy-3-methoxypropan-2-ylcarbamate (40.4 mg, 0.2 mmol) in CH_2Cl_2 (3.57 mL) was added TFA (1.43 mL) dropwise and stirred for one hour at room temperature. The reaction was then quenched by evaporation of the solvent and the obtained (*S*)-*O*-methyl serinol (or (*S*)-2-amino-3-methoxypropan-1-ol, 40.2 mg). The same synthesis was performed with L-serine to obtain the (*R*)-*O*-methyl serinol (13.2 mg). The two serinol derivatives were used as they were for Marfey's analysis. A small aliquot of **4** (0.1 mg) was hydrolyzed using 6 M HCl at 110 °C for 16 h while **5** was hydrolyzed in a similar manner using 12 M HCl at 120 °C to convert its acetate group to an alcohol. The hydrolysates were dried *in vacuo* and subjected to Marfey's analysis. The hydrolysates and the two synthesized *O*-methylserinol enantiomers were analyzed by RP-HPLC (Cosmosil 5C18-AR-II 4.6 × 250 mm, 5 µm, 1 mL/min, UV detection at 340 nm, gradient 0–45 min, 10%–50% MeCN containing 0.05% (*v/v*) trifluoroacetic acid). A comparison of the retention times (t_R) of the standard (21 and 31 min for (*R*)- and (*S*)-*O*-methylserinol, respectively) and the hydrolysates (31 min for **4** and **5**) led to the assignment of the chiral centers in compounds **4** and **5** as *S* and *R*, respectively.

II. 1. c. Antifouling assay

The same method as in Chapter I was used.

II. 2. Results

II. 2. a. Structure elucidation

1D NMR data of **4** and **5** showed characteristic signals of an unsaturated acyl chain composed of eight methylene groups, a terminal methyl group, one carbonyl, one trisubstituted olefinic methyl and one amide proton, forming fatty acid amides with *O*-methyl serinol. Compound **5** was similar to **4** except for an additional acetyl group, giving a *O*-acetyl serinol

moiety. The configuration of the double bond of **4** and **5** was determined based on the characteristic chemical shifts of the methyl group at C-17 (δ_C 12.6 ppm). The absolute configuration was determined by Marfey's analysis. The retention time of the hydrolysates of both fatty acid amides matched with that of the synthetic (*S*)-*O*-methylserinol (31 min instead of 27 min for the (*R*)-*O*-methylserinol), leading to the assignment of C-18 in **4** as the *S*-configuration and *R*-configuration for **5** because of the extra acetyl group.

II. 2. b. Antifouling activity

Both fatty acid amides **4** and **5** showed potent activity. However, after 120 hour-exposure, some floating larvae could settle. The two compounds can slow down the settlement but not inhibit it completely. Compound **5** was more active than **4** (EC_{50} of 0.04 and 0.88 µg/mL, respectively). The extra acetyl group of **5** probably increases the antifouling activity.

II. 3. Discussion

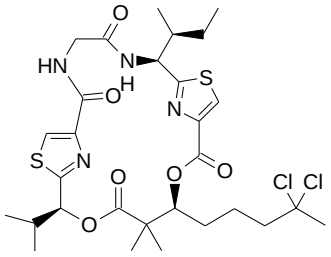
The two fatty acid amides **4** and **5** showed good antifouling activity, probably due to their biosurfactant activity. Indeed, as for **2** and **3** in Chapter I, due to their amphiphilic characteristics, such compounds can reduce surface tension and, in marine antifouling paints, prevent organisms such as barnacle larvae from settling Two fatty acid amides, oleamide and erucamide, are already used as antifoulants.

Chapter III: Isolation of lyngbyabellins and the known antifouling compound dolastatin 16 from *Okeania* sp.

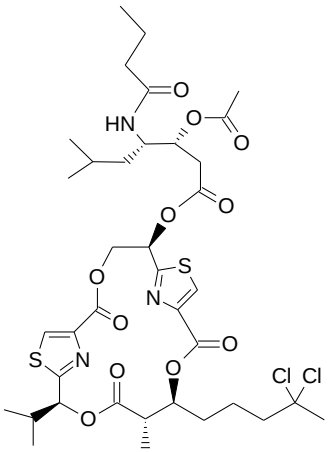
III. 1. Materials and Method

III. 1. a. Isolation

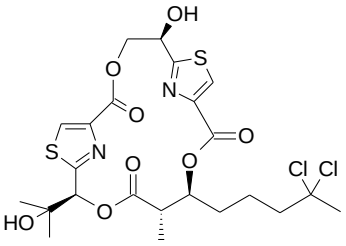
Same extraction, liquid partition and silica gel open column chromatography as in Chapter II was performed. The 4:6 Hex/EtOAc silica gel fraction offered 27-deoxylyngbyabellin A (**6**) (0.5 mg) and lyngbyabellin H (**7**) (0.5 mg), the 100% EtOAC fraction provided extra amount of **4** (12 mg), lyngbyabellins G (**8**) (3.3 mg), O (**9**) (0.8 mg) and P (**10**) (1.9 mg) and dolastatin 16 (**11**), and the 75:25 EtOAc/MeOH fraction afforded lyngbyabellin F (**12**) (1.1 mg) and extra amount of **7** (1.1 mg) and **8** (1.6 mg).



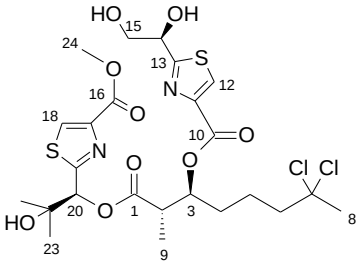
27-Deoxylyngbyabellin A (**6**)



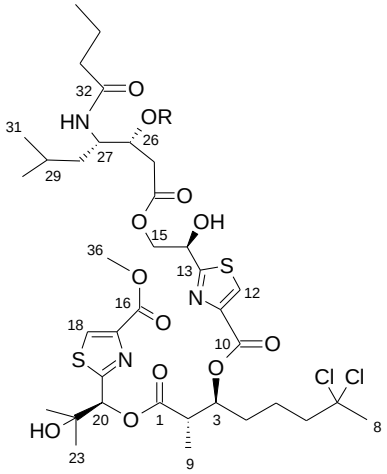
Lyngbyabellin H (**7**)



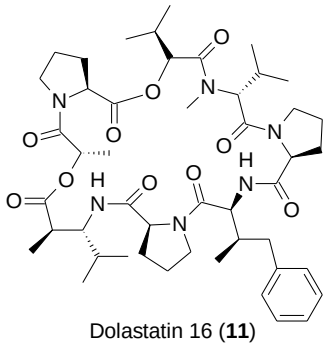
Lyngbyabellin G (**8**)



Lyngbyabellin O (**9**)



Lyngbyabellin P (**10**) R = H
Lyngbyabellin F (**12**) R = Ac



Dolastatin 16 (**11**)

III. 1. b. Structure elucidation of lyngbyabellins O and P

The planar structures of the two new compounds **9** and **10**, were elucidated by MS and NMR techniques. The absolute configuration at C-14 and C-20 of **9** was determined by comparison of degradation products (glyceric acid and 2,3-dihydroxyisovaleric acid methyl ester) and standard enantiomers. Compound **9** was dissolved in MeOH, ozonolyzed (- 78 °C, 25 min) and then base hydrolysis (2 N NaOH, 90 °C, 7 h) was performed to obtain the desired degradation products. Chiral LC-MS (Chirobiotic TAG 25 cm x 2.1 mm, 1:1 0.1% aqueous TFA/1% NH4OAC in MeOH, 0.2 °C mL/min, oven: 20 °C) was then used with the commercially available D- and L-glyceric acid standards to determine the configuration of **9** at C-14. To elucidate the configuration at C-20, the (*R*)- and (*S*)-2,3-dihydroxyisovaleric acid methyl ester standards were synthesized by Sharpless asymmetric dihydroxylation of methyl 3,3-dimethylacrylate using AD-mix-α and AD-mix-β, respectively. As these enantiomers were

not separable by chiral chromatography, MTPA derivatization was performed (CH_2Cl_2, (*R*)-MTPA, 70 °C, 1 h), followed by RP-HPLC (Cosmosil 5C18-MS-II 4.6 x 250 mm, 0-30 min: 20-30% MeOH, 254 nm, 1 mL/min). As **8** and **9** share a very similar structure, **8** being the cyclic version of **9**, methanolysis (0.1 M HCl, 85 °C, 45 min) was conducted on **8** to create a regioselective ester cleavage at C-16. The configuration of **10** at C-26 and C-27 was elucidated indirectly by deacetylation of **12** (0.1 M $NaOCH_3$, room temperature, 15 min). Indeed, the only difference between **10** and **12** is an extra acetyl for **12** at C-26. Both reactions were checked by ESI-LC-MS and mass and retention time of products compared with isolated **9** and **10**.

III. 1. c. Cytotoxicity assay

Cytotoxicity of lyngbyabellins was studied via MTT assay towards MCF7 breast cancer cells. Cisplatin was used as positive control.

III. 1. d. Antifouling activity

The same method as in Chapter I was used.

III. 2. Results

III. 2. a. Structure elucidation of lyngbyabellins O and P

The planar structure of **9** and **10** were elucidated by 1D and 2D NMR techniques. While no correlation was observed between 5 fragments, careful study of the chemical shifts allowed the determination of the structure. Both compounds contain two thiazole rings and an unusual *gem*-dichloro group, similar to reported lyngbyabellins, dolabellin and hectochlorins. Comparison of chemical shifts with these known compounds confirmed the structure of **9** and **10**. Configuration of **9** at C-14 and C-20 was determined by study of degradation products. Compound **9** was ozonolysed and hydrolysed to obtain its glyceric acid and 2,3-dihydroxyisovaleric (DHIV) acid methyl ester residues which were then studied by chiral HPLC. The hydrolysate of **9** has the same retention time as the D-glyceric acid, giving a *R* configuration at C-14. As the synthetic DHIV acid methyl ester enantiomers were not separable by HPLC, derivatization by MTPA was used. HPLC analysis showed that the hydrolysate of **9** has the same retention time as the (*S*)-DHIV acid methyl ester, giving a (*S*) configuration at C-20. Methanolysis and ESI-TOF-MS analysis showed that **8**, which is the cyclic version of **9**, can be converted into **9**, revealing that both compounds have a 2*S*, 3*S* configuration. Compound **10** slowly degraded into **9**, meaning that they both have the same 2*S*, 3*S*, 14*R*, 20*S* configuration.

ESI-TOF-MS analysis showed that deacetylation at C-26 of **12** led to **10**, showing that both compounds have a 26*R*, 27*S* configuration. As methanol was used for this deacetylation, methanolysis also occurred, converting **12** into **9**. All these conversion experiments showed a relation between **8**, **9**, **10** and **12**. Methanolysis of **8** resulted in a regioselective ester cleavage at C-16 leading to the opening of the cyclic structure and giving **9**, while methanolysis of **10** and **12** resulted in another regioselective ester cleavage at C-14 leading to the loss of the side chain and giving also **9**. Compounds **9** and **10** can be considered as artifacts but, in regard to their antifouling activity, they are still of great interest.

III. 2. b. Cytotoxicity

The IC_{50} values of **8**, **9** and **10** toward the MCF7 cells were 120 ± 2.1, > 160 and 9 ± 0.9 µM, respectively (n = 3). The IC_{50} values of **6** and **7** were 0.31 ± 0.05 and 0.07 ± 0.03 µM, respectively. These differences in the cytotoxicity could most likely be attributed to the differences in their structures. Cyclic lyngbyabellin with side chain **7** is the most active while an acyclic form or a lack of side chain decreases the cytotoxicity.

III. 2. c. Antifouling activity

A relation between the structure and the activity of lyngbyabellins was observed, as for the cytotoxicity. However, for antifouling activity, the acyclic structure without side chain (**9**) was the most active (EC_{50} of 0.24 µg/mL). The addition of side chain (**10**) decreases slightly the activity (EC_{50} of 0.62 µg/mL). The most important feature for activity is the acyclic structure, as cyclic structures were the least active (EC_{50} of < 10 and 4.4µg/mL for **7** and **8**, respectively). Compound **6** was the only toxic lyngbyabellin. The known **11** exhibited high inhibition activity (EC_{50} 0.08 µg/mL) as previously reported.

III. 3. Discussion

Two new lyngbyabellins, **9** and **10**, were isolated. Conversion experiments showed that they can be artifacts as mild methanolysis of **8**, **10** and **12** led to **9**, and deacetylation of **12** led to **10**. Moreover, studies reported the biological pathway of lyngbyabellin A and hectochlorin. Both of them show macrocyclization, meaning that cyclic compounds are natural but that acyclic compounds may be artifacts. No cluster for incorporation of side chain of observed in both pathways. Structures without side chain are probably precursors and other clusters are used to build and add the side chain. However, even if they are artifacts, **9** and **10**

were the most potent non-toxic antifouling lyngbyabellins thanks to their acyclic structure which seems to be the most important feature to inhibit the barnacle larvae settlement. The side chain of **7** and **10** decreased slightly the activity compared to **8** and **9**, respectively. Cytotoxicity assay on MCF7 breast cancer cells also showed the importance of the structure of lyngbyabellins, with the cyclic structure with a side chain (**7**) being the most active. Further studies should be done to establish a complete structure/activity relationship.

General Discussion

Three known compounds, **1**-**3**, from a *Hyrtios* sp. sponge, and eight compounds, **4**-**12**, including two new, **9** and **10**, from an *Okeania* sp. cyanobacterium were isolated. The fatty acid methyl esters **2** and **3**, and the two fatty acid amides **4** and **5**, may act as biosurfactants, creating a slippery layer on substratum. As a result, barnacle larvae cannot settle.

A relation between structure and cytotoxicity of lyngbyabellins has already been reported but it is the first time that antifouling activity of such compounds has been studied. Acyclic structure seems to be the most important feature for antisettlement activity while addition of side chain slightly decreases the activity. None of these lyngbyabellins, except **11**, showed toxicity towards barnacle larvae, which makes them good candidates for further antifouling research, especially **9** which is the most active and has the simplest structure compared to the other isolated lyngbyabellins in this study.

Red Sea organisms are well studied but mainly for pharmaceutical compounds. This study revealed that Red Sea organisms can also be good sources of antifouling compounds. Up to now, only one paper reported such compounds, and three papers reported active extracts. Moreover, cyanobacteria should be more studied as large-scale cultures can lead to large amounts of active compounds.