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MINI-REVIEW

Natural products as antifouling compounds: recent progress and future perspectives

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Since early 2008, an increasing number of countries have ratified an international treaty to ban the application of antifouling (AF) coatings based on organotin compounds (eg tributyltin (TBT) and triphenyltin). As a result, the demand for environmentally friendly, non-toxic or low-toxicity AF compounds and technologies (green AF agents) has become an urgent reality. Marine coatings based on Cu_2O and various other biocides have a negative impact on the environment and they must eventually be replaced by new, effective, and environmentally friendly AF compounds. This mini-review describes important AF compounds discovered from a variety of organisms from 2004 until mid 2009, and discusses recent and general trends in the discovery of AF compounds. Finally, a perspective on the future of AF compound development is presented. The discussion is aimed at updating scientists and engineers on the current challenges facing AF research.

Keywords: antifouling; biofouling; marine natural products; antifouling compounds; microbial metabolites

Introduction

A variety of toxic materials (eg copper, lead, mercury, arsenic) were used to control fouling organisms until organotins such as tributyltin (TBT) were introduced in the 1960s. Organotins were the most effective antifouling (AF) agents known, but also among the most toxic biocides ever introduced because they are not readily degraded in the natural environment and because they act on both target and non-target organisms. This led the International Maritime Organization (IMO) to prohibit their application to ships, effective from17 September 2008 (IMO 2007).

Alternative biocide-based AF paints, containing compounds such as Irgarol 1051, diuron, Sea-Nine 211, chlorothalonil, dichlofluanid, and zinc pyrithione are the most frequently used booster biocides worldwide, and some of these have also been found to accumulate in coastal waters at levels that are deleterious for marine organisms (Omae 2003b; Konstantinou and Albanis 2004; Bellas 2006; Thomas and Brooks 2010). A more environmentally friendly AF strategy has focused on the characterization and development of products that are based on the chemical defenses of sessile marine organisms that maintain their body surfaces free of fouling. Many AF substances have been extracted from seaweeds and sessile marine invertebrates and characterized (cited references in Fusetani 2004) and the application of some of those bioactive compounds in AF paints has been exploited (Fusetani and Clare 2006; Hellio and Yebra 2009). However, most marine AF compounds are difficult to obtain in large quantities, thereby preventing the development of natural-product-based AF paints. To overcome the supply issue, more diverse sources (eg marine bacteria, fungi, cyanobacteria, aquatic plants, and terrestrial plants) have been explored in recent years (Omae 2003a; Fusetani 2004; Dahms et al. 2006; Dobretsov et al. 2006).

Mechanism-oriented AF strategies form the basis of another non-toxic AF approach, although the molecular mechanism underlying biofouling is poorly understood (Fusetani 2004). A number of antagonists and agonists for receptors, cellular and neural signal transduction systems, channels, and enzymes have been tested for their AF activity, with some promising results and these have been included in this review.

To select candidate compounds for further development as AF products, the LC_{50}/EC_{50} ratio, often referred to as the therapeutic ratio has been commonly used as a yardstick of the potential of a compound. (EC_{50} refers to the effective concentration that inhibits 50% of the biological activities of the test organism while LC_{50} refers to the concentration that kills 50% of the test organisms in comparison with the control.) Although a compound with a LC_{50}/EC_{50} ratio >15 is often considered as a non-toxic AF compound, a much higher LC_{50}/EC_{50} ratio is highly recommended when selecting candidate compounds. Based on the authors'

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experience, it is suggested that only small molecules with a LC_{50}/EC_{50} ratio >50 and an EC_{50} <5 $\mu g\ ml^{-1}$ against both hard and soft foulers should be considered. However, it is important to keep in mind that LC_{50} and EC_{50} values as well as the LC_{50}/EC_{50} ratio of candidate compounds will be dependent on the properties of the AF substances, eg their solubility and stability. A relatively toxic compound with a low LC_{50}/EC_{50} ratio may still be considered if it can be easily degraded in the natural environment.

This review describes important AF compounds discovered from a variety of organisms from 2004 to mid 2009 (see Fusetani (2004) for a summary of compounds as potential AF product candidates prior to 2004). The compounds are sorted according to their sources and classification with respect to recent strategies for AF compound discovery. Potentials and challenges are discussed with respect to the development of environmentally friendly AF paints based on natural compounds.

Compounds from marine microorganisms (Figure 1)

Microorganisms, particularly when present in biofilms, play an important role in macrofouling, both in terms of its facilitation and its inhibition (Wieczorek and Todd 1997; Callow and Callow 2002; Dobretsov et al. 2006; Qian et al. 2007). The dearth of AF inhibitors isolated from marine bacteria and fungi is surprising (Fusetani 2004), and is in stark contrast to the active exploration of medicinally important natural product metabolites. This is also true for cyanobacteria: to date, only maculalactone A (**10**) (Brown et al. 2004) is known to inhibit macrofouling, although cyanobacteria have been suggested as a potential source of AF agents (Dahms et al. 2006; Volk and Furkert 2006; Gademann 2007). The lack of AF compounds from microorganisms is primarily due to the fact that (1) it is much easier to collect sponges, corals, seaweeds, and other macroorganisms from the natural environment than to isolate and culture microbes such as bacteria and fungi, and (2) it is more convenient to harvest a large amount of biomass from macroorganisms than to generate an equivalent amount of biomass from microbes. However, microbes offer more promising sources of bioactive compounds. New AF compounds isolated from marine microbes in recent years are listed in Figure 1.

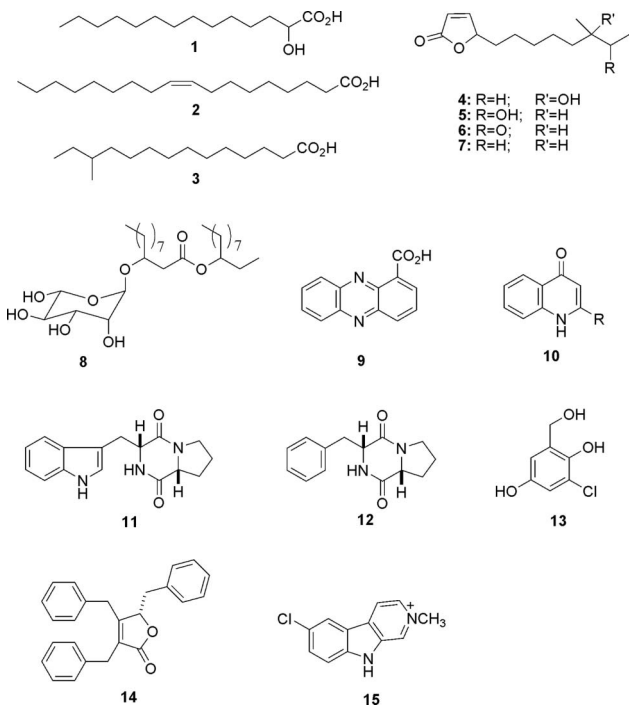


Figure 1. AF compounds isolated from marine microorganisms.

1-hydroxymyristic acid (**1**) and 9-*Z*-oleic acid (**2**), isolated from the marine bacterium *Shwanella oneidensis*, completely inhibited germination of spores of *Ulva pertusa* at 10 $\mu g\ ml^{-1}$ (Bhattarai et al. 2006). More importantly, panels coated with paints containing 10% fatty acids (dry weight), which had been immersed in the ocean for 1.5 years, were essentially free of both micro- and macrofoulers (Bhattarai et al. 2006). Similarly, 12-methylmyristic acid (**3**), obtained from a *Streptomyces* sp. isolated from sea sediments collected at a depth of 5774 m, inhibited larval settlement of the polychaete *Hydroides elegans* with an EC_{50} of 0.6 $\mu g\ ml^{-1}$ and an LC_{50} of 80 $\mu g\ ml^{-1}$ (Xu et al. 2009b). The therapeutic ratio for this compound is >133, indicating it is a very potent and non-toxic AF compound against this tube worm. Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of larvae treated with **3** revealed downregulation of the guanosine-5′-triphosphatase(GTPase)-activating protein gene and upregulation of the adenosine-triphosphate (ATP) synthase gene. Nevertheless, the mechanism of the AF activity of these fatty acids remains an interesting and unsolved problem.

Similar to the activity of brominated furanones derived from seaweed (de Nys et al. 2006), alkylated butenolides (**4**–**6**) isolated from a deep sea *Streptomyces* sp. exhibited potent AF activity against the larvae of important fouling organisms, but their toxicity was weak. Studies of structure–activity relationships have revealed that the 2-furanone ring is essential for activity, and that lipophilicity significantly influences AF activity (Xu 2009a). Based on these findings, a simple alkyl butenolide **7** was synthesized, and its AF activity was evaluated. This compound exhibited promising AF activity; more importantly,

panels coated with paints containing 5% of **7** were free of fouling for 3 months in a field experiment.

Pyolipic acid (**8**), phenazine-1-carboxylic acid (**9**), and 2-alkylquinol-4-ones (**10**) have been identified as antibacterial constituents from a culture extract of a *Pseudomonas* sp. isolated from nudibranchs. The extract incorporated into paints showed promising AF activity in both laboratory and field experiments (Burgess et al. 2003; Eguia and Trueba 2007). The AF activity of these compounds against macrofoulers has yet to be examined.

Diketopiperazines (DKPs), a common class of bacterial and fungal metabolites, have been isolated as AF compounds from a bacterium and a fungus. Five DKPs (eg *cyclo*(L-Trp-L-Pro) (**11**)) were obtained from *Streptomyces fungicidicus* isolated from marine sediments collected at a depth of 5000 m (Li et al. 2006), and *cyclo*(D-Pro-D-Phe) (**12**) was isolated from the sponge-associated fungus *Letendraea helminthicola* (Yang et al. 2007). The AF activity of these cyclopeptides was not remarkable ($EC_{50} = 0.1$–0.28 mM against cypris larva settlement), but their toxicity was low ($LD_{50} = 0.47$–0.75 mM). Quorum sensing (QS) inhibitors often exhibit AF activity through an unknown mechanism (de Nys et al. 2006; Dobretsov et al. 2007a; 2009). DKPs are an example of such compounds, because they are known to inhibit bacterial QS (Holden et al. 1999).

The fungus *Ampelomyces* sp., isolated from natural biofilms, produced 3-chloro-2,5-dihydroxybenzyl alcohol (**13**) that inhibited settlement of larvae of *Balanus amphitrite* ($LC_{50} = 3.19$–3.81 $\mu g\ ml^{-1}$; $LC_{50} = 266.7$ $\mu g\ ml^{-1}$) and of *H. elegans* ($EC_{50} = 0.67$–0.78 $\mu g\ ml^{-1}$; $LC_{50} = 2.64$ $\mu g\ ml^{-1}$), thereby establishing it as a promising candidate as a non-toxic antifoulant (Kwong et al. 2006). Maculalactone A (**14**), a tribenzylbutyrolactone isolated from the marine cyanobacterium *Kyrtuthrix maculans*, was lethal to the naupliar larvae of several barnacles, with LC_{50} values of 1.1–5.2 $\mu g\ ml^{-1}$ (Brown et al. 2004). Preliminary field tests with synthetic maculalactone A-containing paints showed AF activity specific to bivalves. Nostocarboline (**15**), isolated from a freshwater cyanobacterium *Nostoc* sp., and its synthetic analogs showed remarkable algicidal and antimicrobial activities, but their AF activity against macrofoulers has yet to be examined (Blom et al. 2006).

The bacterium *Pseudoalteromonas issachenkonii*, isolated from sea sediments collected at a depth of 3283 m, produced a novel protease that inhibited larval settlement of the bryozoan *Bugula neritina*, with an EC_{50} of 0.5 ng ml^{-1}, while a field assay with the enzyme incorporated into water-based paints demonstrated significant inhibition of settlement of *B. amphitrite* and *B. neritina* (Dobretsov et al. 2007b). The enzyme was a cold-adapted, neutral, halophilic metalloprotease of 32 kDa that remained active in seawater for 14 days as well as in acetone (Xiong et al. 2007). These promising studies raise the possibility of large-scale production of the enzyme for further assays.

Compounds from seaweeds and aquatic plants (Figure 2)

Spurred on by the discovery of potent AF compounds such as brominated furanones and elatol in seaweeds (Fusetani 2004), this group of plants has been actively explored for AF compounds (Nylund and Pavia 2003; Bhadury and Wright 2004; Marechal et al. 2004; Nylund et al. 2005; Dworjanyn et al. 2006; Brock et al. 2007; da Gama et al. 2008). Seagrasses and mangrove plants have also been screened for AF activity, and the development of applications containing zosteric acid, a seagrass metabolite, as a non-toxic antifoulant has been attempted (Xu et al. 2005). The AF compounds recently identified from seaweeds and aquatic plants are listed in Figure 2.

Three meroditerpenoids **16**–**18** isolated from the brown alga *Halidrys siliquosa* inhibited settlement of cyprids of *B. amphitrite* with IC_{50} values of 1, 5, and 5 $\mu g\ ml^{-1}$, respectively (Culioli et al. 2008).

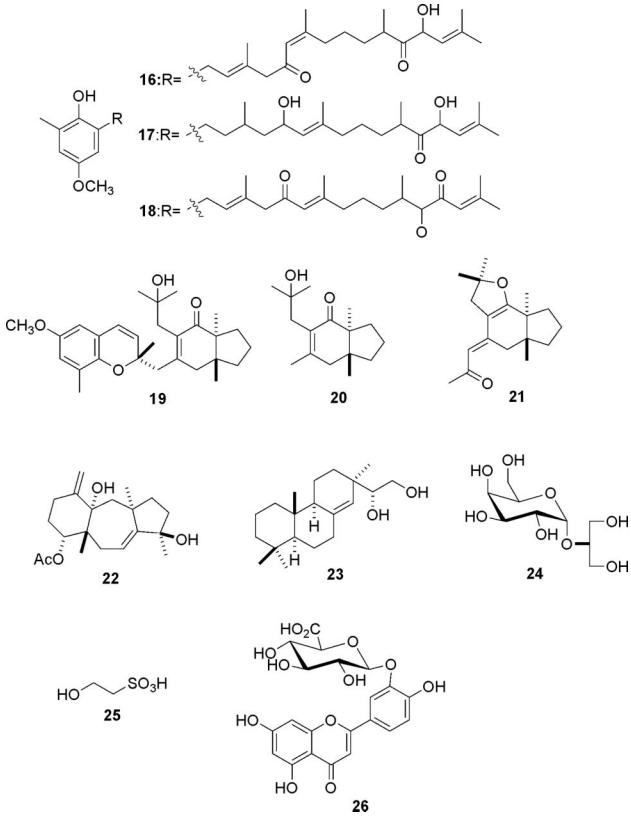


Figure 2. AF compounds isolated from seaweeds and aquatic plants.

Interestingly, compound **18** was non-toxic to nauplii ($LD_{50} > 100$ μg ml^{-1}), whereas the other two compounds exhibited marked toxicity ($LD_{50} = 5$ μg ml^{-1}). These compounds inhibited attachment of four marine bacteria at low concentrations. Another merditerpenoid **19** and its derivatives **20** and **21** derived from the brown alga *Cystoseira baccata* inhibited the growth of two macroalgae and the activity of mussel phenoloxidase, with **20** being the most active ($IC_{50} = 1$ μg ml^{-1} for all assays) (Mokrini et al. 2008). These compounds were not toxic to sea urchin and oyster larvae. Three dolastane diterpenoids isolated from the brown alga *Canistrocarpus cervicornis* inhibited mussel byssal formation (**22** was most active but no IC_{50} value was provided) (Bianco et al. 2009).

A primarene diterpene **23** isolated from the mangrove plant *Ceriops tagal* was a highly effective AF agent against settlement of cyprids of *Balanus albicostatus*, with an IC_{50} value of 0.04 μg cm^{-2}, but its toxicity was low ($LC_{50} > 10$ μg cm^{-2}) (Chen et al. 2008). Interestingly, its 12-keto derivative was much less active.

Floridoside (**24**), isolated from the red alga *Grateloupia turuturu*, showed potent anti-barnacle activity ($EC_{50} < 1$ μg ml^{-1}) but was non-toxic to nauplii at 10 μg ml^{-1}, whereas co-occurring isethionic acid (**25**) was less active, but more toxic (Hellio et al. 2004). Interestingly, a mixture of **20** and **21** inhibited *N*-octanoyl homoserine lactone-mediated QS, although neither showed inhibitory activity alone (Liu et al. 2008). Luteolin-4′-glucuronide (**26**), isolated from the seagrass *Enhalus acoroides*, was a potent inhibitor of settlement of *B. neritina* larvae, with an EC_{50} value of 0.52 μg ml^{-1}, although its toxicity was very low (Qi et al. 2008a).

Compounds from marine invertebrates (Figure 3)

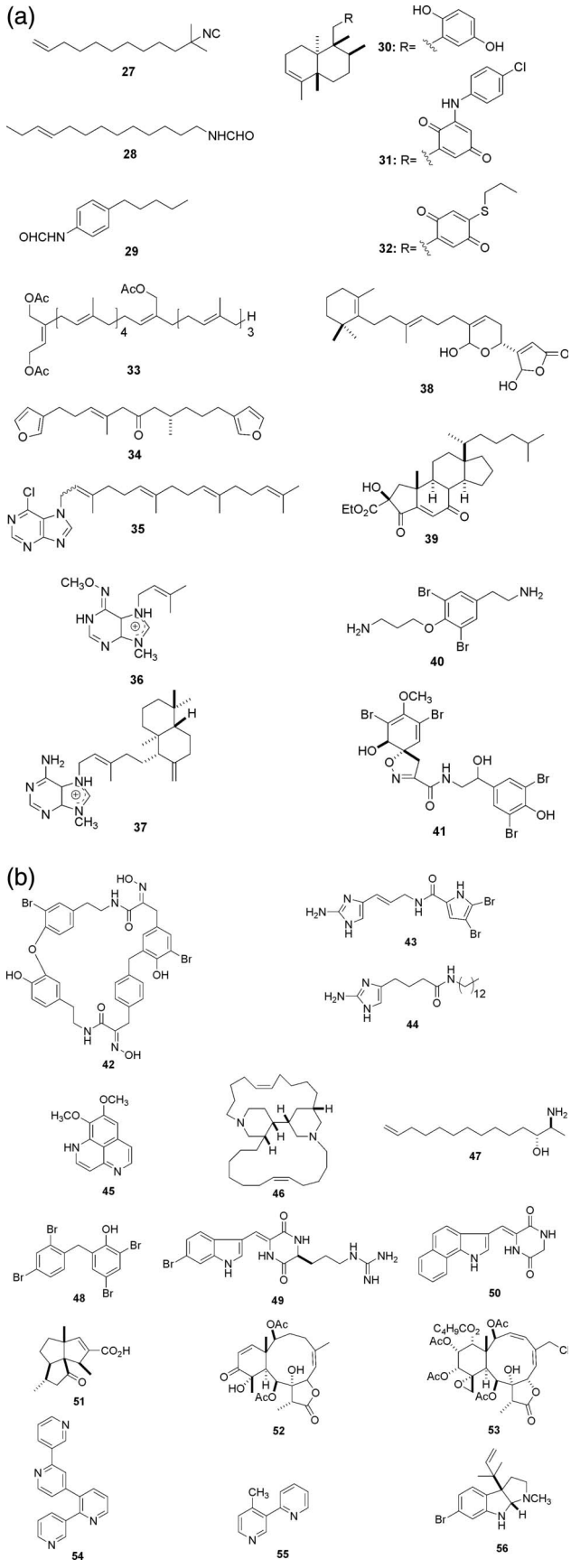
Marine invertebrates have developed chemical defense systems over the course of their evolution, and their metabolites are an excellent source of non-toxic AF compounds, as demonstrated by previous work (Fusetani 2004). Development of non-toxic AF agents has been attempted from sponge metabolites, isocyanoterpenoids (Nogata and Kitano 2006), 3-alkylpyridinium salts (Chelossi et al. 2006; Elersek et al. 2008), and from halogenated 1-methylgramines derived from a bryozoan (Kawamata et al. 2006).

Because isocyanoterpenoids isolated from sponges and nudibranchs showed significant anti-barnacle activity (Fusetani 2004; Nogata and Kitano 2006), previous studies have examined the structure–activity relationship of simple sesquiterpene isocyanides and alkyl isocyanides (Kitano et al. 2003; Nogata et al. 2004). These studies led to the discovery of an alkyl isocyanide **27**, and a preliminary field trial yielded promising results (Nogata and Kitano 2006). To study the mode of action of isocyano compounds, the AF activity of fluorescently labeled probes based on isocyanide **27** and the subsequent localization of the fluorescence in cyprids were examined. The accumulation of fluorescence was observed in oil cells (Kitano et al. 2005), a result that is difficult to interpret. Simple alkyl and phenyl formamides, a hydrolyzed form of isocyanides, were synthesized, and their AF activity against cyprids of *B. amphitrite* was evaluated. The former compounds (eg **28**) were highly active and non-toxic, whereas the latter (eg **29**) had high AF activity, but were toxic (Braekman and Daloze 2004).

Avarol (**30**), a sesquiterpenoid hydroquinone isolated from the sponge *Dysidea avara*, and six analogs showed AF activity against cyprids of *B. amphitrite* at concentrations ranging from 0.45 to 26 μg ml^{-1}. Particularly promising were 3′-(*p*-chlorophenylamino) avarone (**31**) and 4′-propylthioavarone (**32**), which showed good therapeutic ratios ($LC_{50}/EC_{50} > 100$) (Tsoukatou et al. 2007). Similarly, 14 terpenoids isolated from Mediterranean sponges were evaluated for AF and toxic activities against barnacle larvae, among which hydroquinone A acetate (**33**) and dihydrospongin II (**34**) were found to be the most promising (Hellio et al. 2005). AV1003 A (**35**) and AKB695 (**36**) were the most attractive AF agents chosen from a test group composed of agelasine D (**37**), an unusual diterpenoid of sponge origin, and 20 synthetic analogs. These agents were assessed on the basis of their therapeutic ratios (Sjögren et al. 2008). It should be noted that manoalide (**38**) and its analogs, sesterterpenoids derived from the marine sponge *Luffariella variabilis*, were found to inhibit bacterial QS at low concentrations during screening for QS inhibitors in Great Barrier Reef organisms (Skindersoe et al. 2008). The inhibition of larval settlement by these compounds is interesting. A-*nor* steroids (eg **39**), isolated from the marine sponge *Acanthera cavernosa*, showed moderate anti-barnacle activity (Qiu et al. 2008).

Bromotyrosine derivatives originating from sponges are a promising source of antifoulants, as shown previously (Fusetani 2004). Moloka'iamine (**40**) and hemifistularin-3 (**41**) were highly active in zebra mussel reattachment assays (Diers et al. 2004). Bastadins, another class of bromotyrosines, showed considerable anti-barnacle activity. Among these, bastadin-9 (**42**) was the most active, with a minimum inhibitory concentration (MIC) of 1.0 μM (Ortlepp et al. 2007).

Oroidin (**43**), a pyrroloimidazole alkaloid derived from sponges, strongly inhibited bacterial attachment (Kelly et al. 2003), which prompted the exploitation of



more active inhibitors based on oroidin, such as the highly active analog **44**, with an IC_{50} value of 0.73 μM against *Pseudomonas aeruginosa* (Ballard et al. 2008). Aptamine (**45**), a benzonaphthyridine alkaloid of sponge origin, and its analogs were highly active in zebra mussel reattachment assays (EC_{50} = 12–24 μM), but they produced a phytotoxic response at 300 μM (Diers et al. 2006). The AF activity of these compounds is probably due to inhibition of the α_2-adrenoceptor, as is the case for medetomidine (Nakamura et al. 2003). Interestingly, haliclonacyclamine A (**46**) and halaminol A (**47**), isolated from the sponge *Haliclona* sp., induced rapid settlement of ascidian larvae, but prevented metamorphosis at precisely the same life cycle stage at a concentration of 10 μg ml^{-1}. Both compounds showed similar effects on sponge, polychaete, gastropod, and bryozoan larvae, inhibiting both settlement and metamorphosis (Roper et al. 2009).

Polybrominated diphenyl ethers, metabolites of marine sponges of the genus *Dysidea*, and their synthetic analogs were evaluated for AF activity against barnacle larvae. The most potent AF compound was **48**, with an EC_{50} of 1.26 μM. Compound **48** also inhibited attachment of mussels (EC_{50} = 0.66 μM) and diatoms (EC_{50} = 0.24 μM) (Ortlepp et al. 2008). Barettin (**49**) and 8,9-dihydrobarettin, brominated DKPs isolated from the marine sponge *Geodia baretti*, exhibited high AF activity against barnacle larvae, with EC_{50} values of 0.9 and 7.8 μM, respectively. Importantly, their effects were reversible and non-toxic (Sjögren et al. 2004). Further studies of structure–activity relationships led to the discovery of benzo[*g*]dipodazine (**50**), which showed an EC_{50} value of 0.034 μM; its activity was reversible (Sjögren et al. 2006). Field tests were conducted for 8 weeks to test the AF activity of barettin incorporated into specialty polymer coatings (SPC) polymer (0.1% dry weight). The tests revealed reductions in barnacle and mussel recruitment by 89 and 81%, respectively (Sjögren et al. 2004). Since these compounds share a structural feature with serotonin that can act on settlement of barnacle larvae (Yamamoto et al. 1998), they may act on serotonin receptors (Fusetani 2004).

Subergorgic acid (**51**), isolated from a gorgonian, inhibited settlement of larvae of *B. amphitrite* and *B. neritina*, with EC_{50} values of 1.2 and 3.2 μg ml^{-1}, respectively, and LD_{50} values of >200 μg ml^{-1} (Qi et al. 2008b). As with renillafoulin (**52**), a briarane diterpenoid isolated from the sea pansy *Renilla*

Figure 3. (a),(b) AF compounds isolated from marine invertebrates.

reniformis (Fusetani 2004), some 12 briaranes isolated from the gorgonian *Junceella juncea* showed high AF activity against barnacle larvae. For example, **53** showed an EC_{50} value of 0.004 μg ml^{-1} against cyprids of *B. amphitrite* (Qi et al. 2006, 2009). However, the relevant structure–activity relationships were not determined.

Hoplonemertines are carnivorous marine worms, some of which contain pyridyl alkaloids used for predation and defense. Nemertelline (**54**) and 4′-methyl-2,3′-bipyridyl (**55**), a synthetic analog of natural bipyridyls, showed promising AF activity (Kem et al. 2003). Two bromophysostigmines (eg **56**), isolated from the bryozoan *Flustra foliacea*, inhibited bacterial QS and the growth of bacteria, suggesting the presence of potential AF compounds (Peters et al. 2003).

Compounds from terrestrial natural products and other sources (Figure 4)

In the 1980s, terrestrial plants were explored as a source of AF compounds (Omae 2003a), but were superseded by investigations on marine organisms in the 1990s. Recently, terrestrial natural products, pharmaceuticals, and enzymes have been recognized as important sources of non-toxic antifoulants.

Various compounds have been isolated in the search for AF substances against barnacle larvae. Cycloviolacin O2 (**57**), a cyclotide (a cyclic peptide), inhibited settlement of cyprids of *B. improvisus* completely at 0.25 μM. Its effects were reversible and non-toxic at 2.5 μM (Goransson et al. 2004). 2′-methoxykurarinone (**58**) and matrine (**59**), isolated from the Chinese medicinal plant *Sophora flavescens*, inhibited settlement of cyprids of *B. albicostatus*, with EC_{50} values of 2.0 and 7.1 μg ml^{-1}, respectively, while their toxicity was low ($LC_{50} > 25$ and > 250 μg ml^{-1}, respectively) (Feng et al. 2009a). Among 19 capsaicin-related compounds evaluated for inhibition of zebra mussel attachment, capsaicin (**60**) showed the most promising activity, being highly AF (EC_{50} 13.7 μM), but weakly toxic toward mussels and water fleas (Angarano et al. 2007). Xu et al. (2005) also reported its toxicity toward freshwater bacteria. Consequently, *N*-vanillylnonamide (pseudocapsaicin) (**61**) may be more promising as an environmentally friendly antifoulant. Because capsaicin is an agonist of V1 vanilloid receptors (V1R), a low-potency agonist (anandamide) and related compounds were tested for inhibition of zebra mussel byssal attachment and cumulative toxicity toward *Daphnia magna*. Anandamide (**62**) and a synthetic analog, O-2050 (**63**), showed the best overall profile among environmentally friendly antifoulants (Angarano et al. 2009).

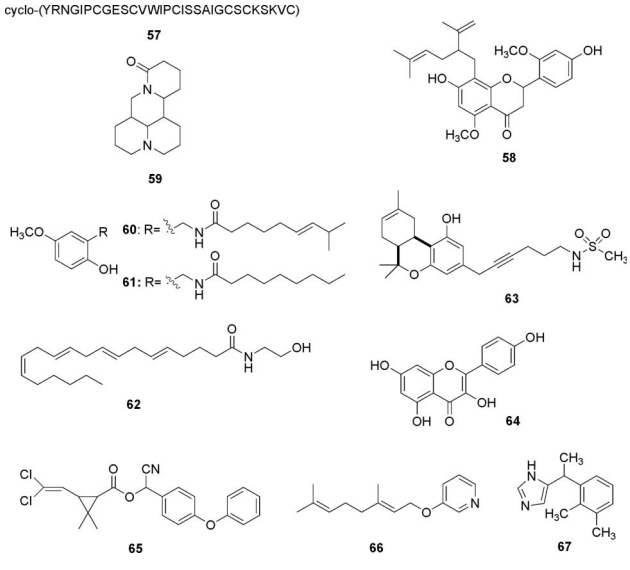


Figure 4. AF compounds isolated from terrestrial and other sources.

A total of 17 flavonoids and isoflavonoids were evaluated for AF activity against larvae of *B. amphitrite*, which led to the discovery of a highly promising non-toxic antifoulant, genistein (**64**), with an EC_{50} value of 3.0 μg ml^{-1} and a therapeutic rate of > 16.6 (Zhou et al. 2009). More significantly, **64** exhibited promising activity in a preliminary field test. Similarly, pyrethroids, which are plant-derived insecticides, showed promising activity. In particular, cypermethrin (**65**) inhibited the settlement of larvae of *B. albicostatus*, with an EC_{50} value of 0.11 μg ml^{-1} ($LC_{50} > 250$ μg ml^{-1}) (Feng et al. 2009b). Surprisingly, a field test indicated that it was as active as TBT. It should be noted that "highly active cypermethrin", one of the stereoisomers of **65**, was more active than cypermethrin. Juvenoids, analogs of insect juvenile hormones, were also highly promising as antifoulants. 3,7-dimethyloctyl 2-methyl-5-pyridyl ether (**66**), in particular, inhibited settlement, with an LC_{50} of 6 ng ml^{-1} against *Balanus balanoides* cypris larvae, and an 8-month field test resulted in no barnacle attachment (Skattebol et al. 2006).

Tannins are polyphenolic compounds, widely distributed in plants and display a variety of biological activities; three tannins were found to be narcotic to naupliar larvae of *B. amphitrite*, and a field test with soluble matrix paints prevented colonization by barnacles (Stupak et al. 2003). Quebracho tannin and aluminum tannate completely immobilized movement of appendages of barnacle and polychaete larvae at low concentrations, and reduced macrofouling significantly in 28-day field trials (Pérez et al. 2007).

A variety of commercially available enzymes have been explored as non-toxic antifoulants (Pettitt et al. 2004; Olsen et al. 2007; Kristensen et al. 2008; Leroy et al. 2008), as they have the advantages of being readily available, non-toxic, and biodegradable. However, there are some crucial issues to be solved before enzymes can be developed for AF paints (eg increased stability and control of release rates). The most promising as AF enzymes are proteases, such as the serine protease, Alcalase®, which was shown to degrade barnacle cyprid cement thereby preventing larval attachment (Aldred et al. 2008). The active ingredient of Alcalase, Subtilisin A, immobilized on a polymer matrix has been shown to reduce the attachment and adhesion strength of algal cells (Tasso et al. 2009).

Pharmaceuticals are also potential candidates for non-toxic antifoulants, some of which are readily available at low cost, and their modes of action are known (see Rittschof et al. 2003; Zhou et al. 2009). The characterization of receptors and molecules involved in larval settlement allows antagonists or agonists to be more directly engineered in the development of antifoulants (Fusetani 2004). Adrenoceptor antagonists inhibited the settlement of barnacle, bryozoan, and polychaete larvae in a concentration- and taxon-dependent manner (Dahms et al. 2004). The best-studied adrenoceptor antagonist is medetomidine (**67**), an α_2-adrenoceptor antagonist that is being exploited as a non-toxic antifoulant (Dahlstrom and Elwing 2006). The effects of medetomidine on a variety of marine organisms have been examined and the results appear to meet the criteria for an environmentally friendly antifoulant (Bellas 2006).

Obstacles and future perspectives

The discovery of effective and environmentally friendly AF compounds (ie compounds that have no or very low acute and chronic toxicity to marine organisms and can be easily and quickly degraded in the marine environment) from natural sources has been a hot topic and the ultimate goal of many research institutions and commercial laboratories over recent decades. However, progress has been rather slow due to (1) insufficient funding for tackling both scientific and technological obstacles to the discovery of green AF compounds and their development into commercial products, and (2) a lack of strong incentives for scientists to fully commit themselves to these goals. Without sufficient funding support, it is difficult to sustain research programs on a variety of AF agents. The recent ban imposed on organotin-based marine coatings and the urgent need for green AF products may force maritime countries to increase the funding available for this type of work.

The issue of supply has always been a major obstacle to the development of marine natural compounds into commercial products. In recent decades, a number of potent AF compounds have been isolated and characterized from extracts of marine macroorganisms such as sponges, corals, seaweeds, and nudibranchs (see Fusetani and Clare 2006). Additional potent, natural AF compounds from these sources are added to the list each year. None of these compounds has been developed into an AF product because it is almost impossible to produce them on a scale sufficient for commercial purposes. Theoretically, large-scale production of marine natural products can be achieved through (1) direct extraction from marine macroorganisms that can be harvested from the sea or from mariculture farms, and (2) chemical synthesis. In reality, however, most of the compounds cannot be synthesized at a price or scale that is affordable to the commercial sector. In most cases, these compounds exist at extremely low concentrations in marine macroorganisms (Dobretsov et al. 2006) and it is not sustainable to harvest target macroorganisms from the sea. Mariculture of macroorganisms for chemical compound production has also proven to be a technically challenging and expensive process (Mendola 2003).

In contrast, there are a number of reasons why microorganisms may represent a more promising source of marine natural products, allowing the obstacle of compound supply to be overcome. Firstly, several bioactive compounds have been isolated from marine microbes in recent years, and evidence suggests that microbes associated with macroorganisms may be the true producers of bioactive compounds previously isolated from macroorganisms. Unfortunately, most of these microbes are uncultured. Considering that marine microbes remain the largest untapped source of marine natural products and biological resources, the potential for microbes containing novel and bioactive compounds is immense. Secondly, the full genome of many microbes has been completely sequenced, as this task is relatively easy and inexpensive. Sequencing technologies provide a solid database for mapping out the biosynthetic pathways for bioactive compounds and identifying the gene or gene clusters responsible for compound synthesis. This approach will pave the way for engineering microbes for improved biosynthesis of bioactive compounds *in vitro* and/or *in vivo*. However, engineered biosynthesis may not be easy, especially for large-scale production. Thirdly, technological advances in microbial fermentation will enable the yield of compounds from culturable microbes to be

maximized. Considering the number of bioactive compounds extracted from marine microbes and the number of microbes screened to date, it is clear that marine microbes are a rich source of compounds with an array of biological activities. It is anticipated that increasing numbers of natural product research teams will turn their focus to marine microbes.

The second obstacle to the discovery of AF compounds is the as-yet unmet requirement for rigorous, robust, and broad-spectrum bioassay systems in research laboratories. A literature review reveals that a variety of marine organisms have been used as targets in laboratory bioassay systems (including marine bacteria, fungi, unicellular algae, spores of seaweed, settling larvae of sessile marine invertebrates, juveniles of marine bivalves, rotifers, *Daphnia* sp.), and a variety of endpoints have been selected, such as inhibition of growth, attachment, adhesion, metamorphosis, chemotaxis, swimming speed, larval development, and survival of test organisms (Briand 2009). However, there is also a large discrepancy in the duration of bioassays and testing procedures, thereby complicating comparisons of results among laboratories.

Overall, the following common practices may have hampered the discovery of AF compounds with a broad spectrum of AF activities. Firstly, many laboratories have relied heavily on bioassays with either microfoulers (bacteria, fungi, or microalgae) or macrofoulers (ie larval settlement assays), which may have led to the discovery of effective anti-microfouling compounds, which are later found to be ineffective against macrofoulers and *vice versa*. Secondly, regardless of anti-microfouling or anti-macrofouling bioassay results, only a few species are used as targets. Only a few marine bacteria or one to two species of marine invertebrate larvae or algal cells are usually used in most research laboratories, which often leads to the identification of AF compounds with a very narrow spectrum of AF activity. Thirdly, research laboratories commonly use locally available species as targets for their bioassays, which generally yield compounds that are only effective against these local species. Overall, more fouling organisms from tropical environments have been used in AF bioassays than organisms from temperate and cold waters. Finally, the scarcity of pure compounds means that laboratory bioassays and field tests are commonly conducted on a limited scale with a limited number of replicates or a limited range of fouling species before product development is initiated. To overcome these problems, it is strongly recommended that research laboratories seek to develop and adopt both anti-microfouling and anti-macrofouling bioassay systems and use as many target species as possible. A rigorous anti-macrofouling bioassay system should include at least both sessile hard foulers (eg barnacle and tube-building worms) and soft foulers (eg the bryozoan *B. neritina*) or seaweed (eg *Ulva*) that have a wide global distribution and are frequently found in fouling communities. To overcome geographic barriers, better collaboration between research laboratories and industries along a latitudinal gradient of environments should be fostered in the future.

Another major obstacle to discovery of effective AF compounds is an ignorance of the need for mechanistic studies against target fouling organisms. In the past, most AF compounds were marketed prior to knowledge of their mode of action, due to market-driven pressures. This poor understanding of the mode of action of particular AF compounds against settling larvae or spores of macrofoulers or attachment of microbes may have led to the short lifespan of a number of AF compounds or the failure to develop AF coatings, either on technical or environmental grounds. Analysis of the modes of action of bioactive AF compounds is extremely challenging because (1) genomic or proteomic information of almost all major fouling organisms remains unknown, making it difficult to identify gene or protein targets for active compounds; (2) a given AF compound may target different genes or proteins in different fouling species or have multiple targets in the same species; therefore, it may be difficult to delineate the relationship between an active compound and its target(s); (3) an analysis of how a compound functions in cells (using cell lines) may not be easily extrapolated to a whole animal system; and (4) an analysis of modes of action requires a long-term, expensive effort, commonly involving a mismatch between market demands and industrial interests. Nevertheless, to safeguard marine environments and to develop an effective, environmentally friendly AF product with a long market lifespan, the authors strongly argue for systematic studies of the modes of action of bioactive compounds before new AF compounds are introduced to the market and into the marine environment. Such studies may also help to identify molecular biomarkers for the rapid screening of marine natural products as AF compounds.

Poor understanding of the mechanisms controlling the settlement and adhesion of larvae and spores of fouling organisms at both the molecular and ecological level is also a large obstacle to the discovery of AF compounds. Without a better knowledge of how settling propagules perceive chemical signals, interact with surfaces and go through both morphological and behavioral changes before completing attachment and adhesion, it is difficult to identify effective inhibitors, to know the targets of inhibitors, and to know the short-term and long-term impact of the inhibitors.

Finally, developing a candidate natural compound into a commercial product will also necessitate compliance with the various national regulations with respect to environment safety issues. Since there is no guarantee that a natural compound with outstanding AF activity will have a lower negative environmental impact than the current suite of booster biocides, it will be necessary to determine the half-life, breakdown profile, environmental fate, toxicity, and other possible negative environmental impacts of a given compound before it is commercialized. These requirements increase the risk, cost, and challenges associated with the discovery of marine natural compounds as AF products. Nevertheless, since marine natural compounds are extracted from marine resources and often exist in the natural environment, and have higher tendency to be biodegraded, there is an increased opportunity to discover highly potent, but less harmful AF compounds, from the oceans than from other sources.

A reasonable number of active AF compounds have already been identified from marine resources; therefore, instead of investing effort in the search for additional bioactive compounds, it may be wiser to exploit existing knowledge and identify the most potent compounds with relatively simple chemical structures with a high LC_{50}/EC_{50} ratio, search for analogs, and carry out intensive analysis on structure–activity relationships (Fusetani and Clare 2006). If this approach is followed, it is highly probable that a more active form of a simple structure, which is amenable to chemical synthesis, will be found. Alkyl isocyanides are a good example of natural-product-derived AF compounds with commercial potential. By fostering closer collaborations with molecular biologists, it may also be possible to achieve a breakthrough in the study of the modes of action of AF compounds. Together, these strategies have the potential to rapidly advance the development of AF products.

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