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Targeting bacterial biofilms: Design of a terpenoid-like library as non-toxic anti-biofilm compounds

Cheikh Sall, Linda Dombrowsky, Olivier Bottzeck, Annie Praud-Tabariès, Yves Blache *

Laboratoire MAPIEM, EA 4323, UFR Sciences et Techniques, Université du Sud Toulon-Var, 83957 La Garde cedex, France

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ABSTRACT

A new class of anti-biofilm compounds possessing 1,4-disubstituted-(1*H*)-1,2,3-triazolic cores was designed. Their efficient synthesis was performed by means of click chemistry through 1,3-dipolar cycloadditions. Two compounds were found to act as specific anti-biofilm agents against a gram negative species.

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Biofilms, which present problems in many different areas, result from the adherence of bacteria to surfaces. Although bacteria grow very poorly when floating in water or biological fluids, they grow extremely well on surfaces. Bacteria produce these biofilms, in part, to help them attach to surfaces and bind to one another.[1] Bacterial biofilms cause problems in medical health care since they colonize implants such as artificial joints or catheters,[2] while in marine environment, formation of biofilms on immersed substrata leads to major economic problems which conducted to the use of toxic biocides to eradicate these communities.[3,4] Although eradication of planktonic bacteria communities have been largely controlled, it has been estimated that bacteria within a biofilm can display up to 1000-fold increased resistance to antibiotic or biocide treatment. In this context, design of original compounds which can limit formation of bacterial biofilms is of great need in the directive of the rational use of antibiotics and/or biocides. In this way, we initiated a programme aimed to allow the discovery of new potential leads from marine organisms which could be optimized by the preparation of analogues. Linear diterpenes eleganolone and eleganediol were identified as major compounds from the brown alga *Bifurcaria bifurcata* which crude extracts exhibited antibacterial activities[5] while more recently, meroditerpenes (derivatives from geranylgeranyltoluquinol) were isolated from brown alga *Halidrys siliquosa* and showed antibacterial properties against different strain of gram negative bacteria (Fig. 1).[6] Furthermore, another terpene namely 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid isolated from *Acronychia baueri* Schott was reported for its capacity to inhibit the formation of *Porphyromonas gingivalis* biofilm.[7] For our purpose 'click chemistry' methodologies retained our attention as an interesting process for the preparation of analogues by mean of bioconjugations of a natural moiety (terpenoid) and a synthetic part (related to the aromatic ring of meroditerpenes) through a triazole linker. A first polyprenyl-type library containing 1,4-disubstituted triazoles was designed and reported to exhibit anti-biofilm activities against a strain of *Pseudoalteromonas* sp.[8] However, since these compounds were obtained as *Z*/*E* mixtures from azido derivatives of terpenes, severe limitations of this methodology resided in the difficulty to separate isomers and in the final yield of pure *E*-isomers (27–60%). In continuation of our investigations aimed to explore the chemical diversity around such terpenic skeletons to probe anti-biofilm structure–activity relationships, we felt that the relatively simple structure of library A invited the synthesis of new analogues. In this way, a new polyprenyl-type library B in which triazoles are obtained as pure *E*-isomers was designed (Fig. 1).

In order to highlight analogies between the two series (A and B), a molecular modeling study was performed with the 4-methoxy derivatives (geranyl tail, R = OCH_3) of each library.[9] Results are summarized in Figure 2. Although conformations of the two compounds were similar, slight differences arised from these calculations. Considering properties of library B versus library A, the size of the triazolic linker between the terpenic unit and the aromatic pharmacophore is longer of 1.76 Å, *c* Log(*P*) is lower and an additional H-bonding site is located on the oxygen atom. Examination of electrostatic potential isosurface also highlighted differences between the two series. In the two cases, two electron-poor domains were highlighted: a large one constituted of the terpenic chain and a smaller one constituted of the aromatic ring, while an electron-rich domain is located of the triazole ring.

* Corresponding author.
E-mail address: blache@univ-tln.fr (Y. Blache).

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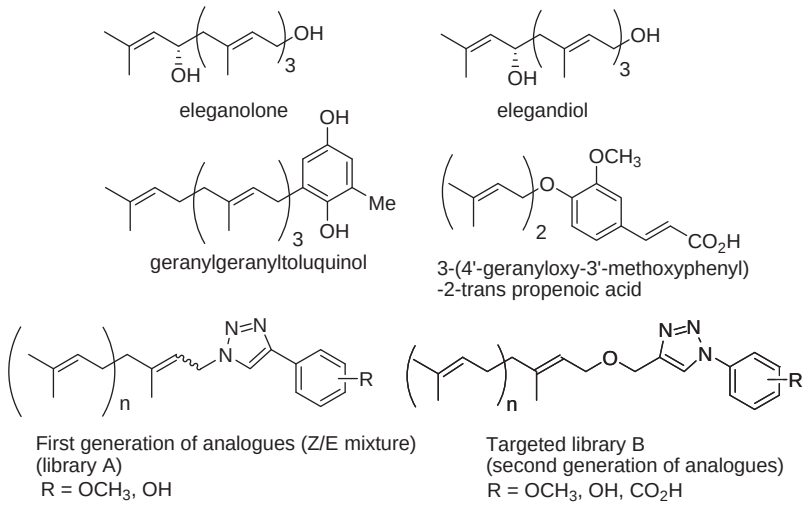


Figure 1. Structure of targeted library B, natural diterpenoids and previously studied anti-biofilm agents (library A).

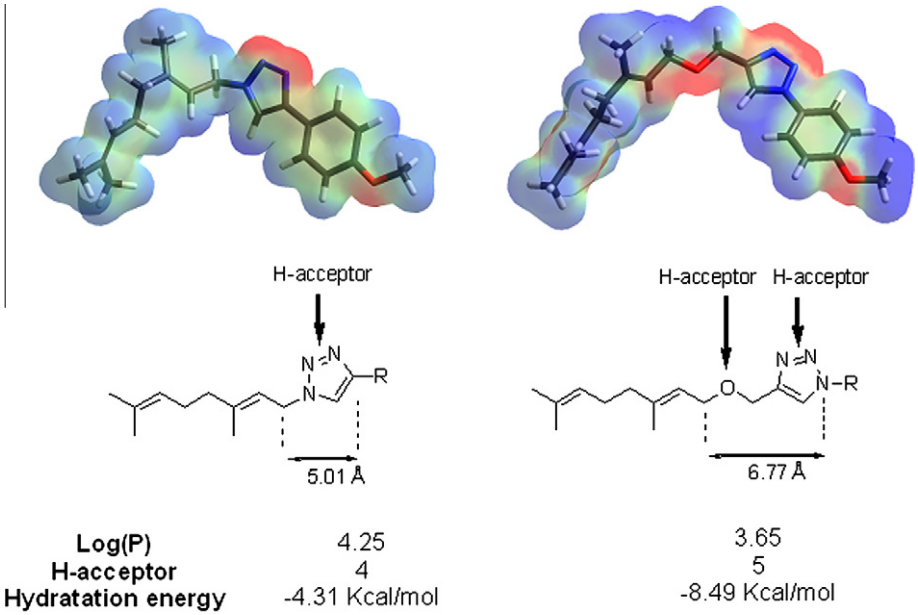
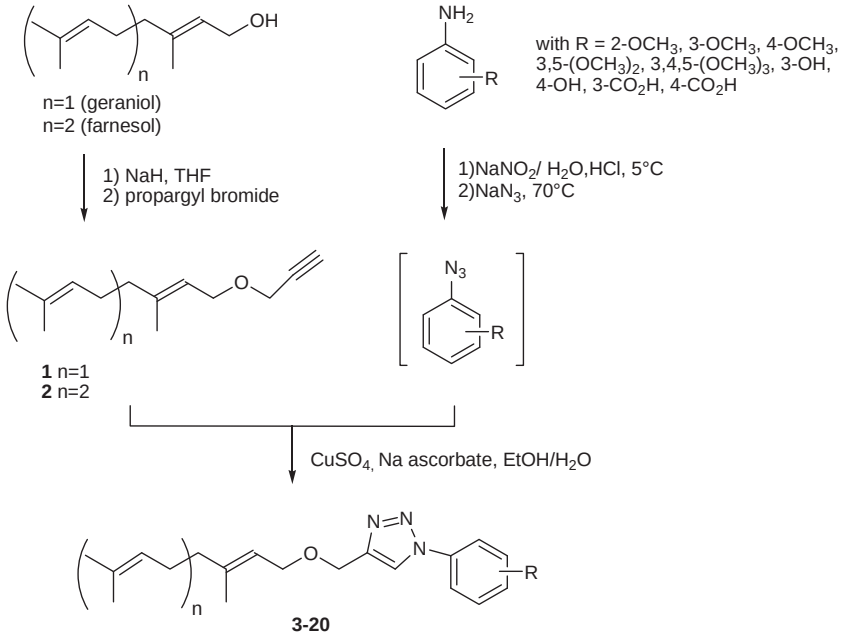


Figure 2. Conformations and properties of the two samples of library A (left) and B (right): electrostatic potential surfaces (up), the values are color-coded onto the total electron density surface, with colors toward red indicating electron rich regions of the molecule.

However, in the case of series B, this electron-poor area is extended to the terpenic chain by overlapping the ether function. As these elements should have their importance when considering interactions with biological systems, we finally believe that designing this new library should give some relevant informations in terms of SAR in regards with anti-biofilm activities of such compounds.

The practical interest of this second library resides in the facility of preparation of aromatic azides from aromatic amines in situ, and in the fact that resulting triazoles are obtained as pure *E*-isomers. Synthesis of the targeted library was achieved by performing the copper(I)-catalyzed 1,3-dipolar cycloaddition of organic azides and alkynes resulting in the formation of 1,2,3-triazoles.[10] In general, these reactions usually proceed to completion in 6–36 h at ambient temperature in water with a variety of organic co-solvents, such as *tert*-butanol, ethanol, DMF, DMSO, THF, or CH_3CN, and this reaction is useful for large classes of azides and alkynes.[11–15] Preparation of alkyne **1** and **2** was achieved as previously described.[16,17] The reactions were investigated in a one pot process without isolation of azides.[18] In practice, all aromatic azides were obtained from the corresponding amines by treatment with sodium nitrite in acidic media for 2 h (HCl/H_2O), followed by addition of sodium azide (NaN_3). Reactions were monitored by TLC and after disappearance of the starting amine, a simple extraction with dichloromethane led to the resulting azides to which a solution of appropriate alkyne, $CuSO_4$/sodium ascorbate in water/ethanol mixture (50:50) was added. Choice of ethanol rather than DMF, similar to our previous work, allowed an easier workup and a better purity of products. All reactions were performed in good to excellent yields at room temperature excepted for the 2-methoxy derivatives **3**, **12**, which were obtained only in 5% and 7% respectively. These poor yields can be explained by a probable steric effect due to the methoxy group as well as by the nature of azides which could be stabilized through a conjugation with the 2-methoxy group (*ortho effect*).[19] These two derivatives were finally obtained in good yield at 40 °C (Scheme 1 and Table 1).[20,21] Structures of all compounds were in good agreement with the spectroscopic data.



Scheme 1. Synthesis of compounds **3**–**20**.

Table 1
Selected 1,2,3-triazoles[a] and biological screening against *Pseudoalteromonas* sp. D41 biofilm

Compounds	*n*	R	Yield (%)[b]	(%) of adhesion[d] (brackets)[e]		EC_{50} (μM)
3	1	2-OCH_3	80[c]	29 ± 9	(>90)	166 ± 14
4	1	3-OCH_3	72	19 ± 6	(>90)	103 ± 10
5	1	4-OCH_3	92	20 ± 8	(3 ± 8)	276 ± 132
6	1	3,5-$(OCH_3)_2$	63	76 ± 1	(>90)	—
7	1	3,4,5-$(OCH_3)_3$	96	36 ± 5	—	—
8	1	3-OH	95	55 ± 24	(30 ± 6)	—
9	1	4-OH	75	>90	—	—
10	1	3-CO_2H	87	32 ± 5	—	74 ± 8
11	1	4-CO_2H	82	39 ± 9	—	—
12	2	2-OCH_3	75[c]	35 ± 7	(25 ± 7)	—
13	2	3-OCH_3	67	25 ± 4	(15 ± 39)	198 ± 33
14	2	4-OCH_3	81	24 ± 6	(46 ± 7)	117 ± 13
15	2	3,5-$(OCH_3)_2$	57	76 ± 5	(0 ± 7)	—
16	2	3,4,5-$(OCH_3)3$	91	50 ± 28	—	—
17	2	3-OH	90	>90	(34 ± 2)	—
18	2	4-OH	70	73 ± 29	—	—
19	2	3-CO_2H	92	>90	—	—
20	2	4-CO_2H	76	>90	—	—
Eleganolone	3	—	—	17 ± 11	—	174 ± 56
Eleganediol	3	—	—	14 ± 6	—	224 ± 65

[a] All experiments were achieved in the same conditions of concentrations of reactants, catalyst, and volume of solvents.
[b] Yield calculated from crude azides.
[c] Yield obtained at 40 °C.
[d] Percentage of adhesion at a concentration of 500 μM (% of adhesion).
[e] Values reported in brackets are those obtained for the corresponding analogues of library A.[8]

In order to assess anti-biofilm activity of these compounds as well as the natural leads eleganolone and eleganediol against a gram negative bacterial biofilm, a *Pseudoalteromonas* sp., strain was chosen.[22,23] All compounds were first screened at a concentration of 500 μM for their capacity to inhibit biofilm formation. At this concentration, eleganolone and eleganediol showed the best effect. Concerning the substituent effects on the aromatic ring, mono-methoxy derivatives exhibit better activities than di or tri-methoxy derivatives in the geranyl series ($n = 1$) as well as in the farnesyl series ($n = 2$). Replacement of the methoxy group by a hydroxy group decreases the efficiency, and activities of the carboxylic acids are modulated by the length of the terpenic chain. When comparing the effect of derivatives of the previously described library A and those of the present library B, further considerations of structure–activity relationships can be highlighted. Although it is difficult to affirm that the modification of the linker enhance the activity, we can reasonably consider that in the case of geranyl derivatives, the structure of the linker has an impact on the activities of the methoxy derivatives since the second generation of compounds are generally more active (except for the 4-methoxy and 3-hydroxy derivatives), while in the case of the farnesyl derivatives, the impact is moderate except for the 3,5-dimethoxy derivative, which was the most potent compound of library A (EC_{50} of *E*-isomer = 71 μM).

The more potent compounds **3**, **4**, **5**, **10**, **13**, **14**, as well as eleganolone and elegandiol were selected to evaluate their EC_{50} (expressed as the effective concentration to inhibit 50% of the bacterial adhesion). This evaluation showed a dose-dependent response, since the most potent candidate at 500 μM was not the best candidate of the selection. Three compounds **3**, **5**, 13 and the two natural compounds were shown be finally inactive with EC_{50} >150 μM. Two of them **4**, 14 exhibited mild activities (respectively 103 and 117 μM), and finally compound **10** was active at a concentration of 74 μM. These value is in the range of the most active derivative of library A bearing a farnesyl tail and 3,5-dimethoxy substituents on aromatic ring.

The further step was to investigate if these compounds exhibited a specific anti-biofilm activity or if this observation was simply related to a general toxic effect on the bacteria. In this way, the two more active compounds **4** and **10** were tested for their capacity to inhibit the growth of *Pseudoalteromonas* sp. (D41) using the antibiotic norfloxacin as a positive antibacterial reference.[24,25] Experiments were performed at the concentrations of 1, 2.5, 5, 10, 25, 80, 100 μM for each compound.[26] Results showed that when compared to norfloxacin, the two compounds **4**, 10 exhibit no effect on the bacterial growth even at high concentrations, indicating that the anti-biofilm activity was not connected to antibacterial effect (Fig. 3).

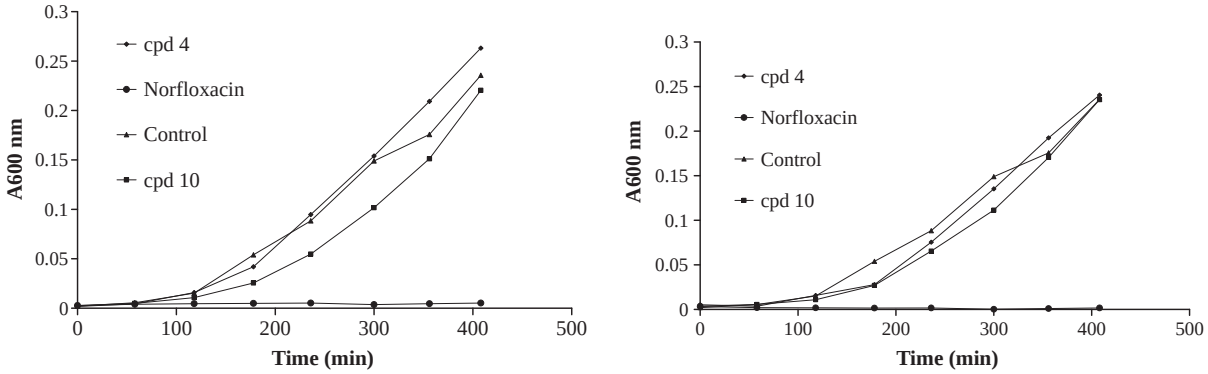


Figure 3. Effect of compounds **4**, **10** and norfloxacin on bacterial growth at concentrations of 10 μM (left) and 100 μM (right).

In summary, from natural diterpenic frameworks, we have designed biological active analogues in an efficient way allowing further developments. Through the generation of this library, the principal several relevant point when concerning SAR data in the field of specific anti-biofilm activity is that, as hypothesized from semi-empirical calculations which showed the similarities of the two series, the nature of the linker modulates the activity but does not generate fundamental changes in the biological response. However, further studies are needed in order to highlight precisely the effect of the terpenic chain. Finally, the low toxicity of the derivatives allows us to focuses our interest in the development of these molecules as non-toxic anti-biofilm compounds for potential use as non toxic co-biocides or co-antibiotics in view of rational eradication of persistent biofilms. In this way, further studies are actually in course in order to optimize the nature of the linker as well as to define the mechanism of action of this class of compounds.

Acknowledgements

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9. *Computational methods*: The geometry of a selected molecule was optimized to an rms (root mean square) gradient of 0.01 in vacuo (Polak–Ribière method). A periodic box, 15–15–15 Å around the drug was then set up, containing 112 water molecules. The system was optimized in MM+ using switched cut-offs (outer 10 and inner 14 Å) to an rms gradient of 0.5. Then a molecular dynamics program was run for 1 ps, with 0.001 ps steps, relaxation time 0.1 ps, to a 30 simulation temperature of 300 K. This was followed by MM+ geometry optimization to an rms gradient of 0.2. The molecular dynamics run was repeated and a further MM+ protocol was carried out to a gradient of rms 0.2 on the selected drug. Finally, the geometries were optimized using the semiempirical AM1 programme in singly excited configuration interaction to a gradient of rms 0.01. (RHF [Restricted Hartree–Fock], charge 0, spin multiplicity 1, lowest state, orbital criterion, five occupied and five unoccupied orbitals.). Properties (*c* Log *P*, hydratation energies) were obtained from these semiempirical calculations with the QSAR package implemented in HyperChem Release 8.05 pro for Windows (Hypercube Inc. Gainesville, Florida.)
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18. Formation of azide was monitored by TLC. Trying to purify azides by chromatography did not improve yields of the following steps, and in addition some of them were obtained after with low yields purification (probably due to their degradation under the conditions of purification).
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20. Typical method for preparation of compounds 3-20: To a stirred solution of aniline (1 g) in H_2O/HCl (50/50, 15 mL/15 mL) was added $NaNO_2$ (0.8 g, 11.55 mmol). The whole was stirred at 0 °C for 2 h, and then NaN_3 was added (0.78 g, 11.55 mmol). The resulting solution was refluxed for 3 h. After extraction with dichloromethane, the resulting crude azide (100 mg) was dissolved in a solution of H_2O/EtOH (50/50 1.5 mL/1.5 mL) containing $CuSO_4{\cdot}5H_2O$ (0.3 equiv), alkyne (1.5 equiv) and sodium ascorbate (0.4 equiv). The resulting mixture was stirred 12 h at room temperature. A saturated solution of Na_2CO_3 was added and the resulting solution extracted three times with ethyl acetate. Organic layers were then dried over Na_2SO_4 and evaporated to give the crude triazoles which were purified by flash chromatography eluted with hexane/ethyl acetate.
21. Spectral data for selected compounds: 4-[((*E*)-3,7-dimethylocta-2,6-dienyloxy)methyl]-1-(2-methoxyphenyl)-1*H*-1,2,3-triazole (**3**); (ESI, *m/z*) 364.35 ($M+Na^+$), 342.38 ($M+H^+$). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.29 (s, 3H, CH_3), 1.37 (s, 3H), 1.38 (s, 3H), 1.73-1.84 (m, 4H), 3.50 (s, 3H), 3.83 (d, 2H, *J* = 6.7 Hz), 4.39 (s, 2H), 4.88 (t, 1H, *J* = 6.8 Hz), 5.11 (t, 1H, *J* = 6.7 Hz), 6.37 (m, 2H), 7;05 (dt, 1H, *J* = 8.3 and 1.6 Hz), 7.43 (dd, 1H, *J* = 7.7 and 1.3 Hz), 7,95 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.7, 16.8, 24.9, 25.6, 38.8, 55.0, 62.4, 66.0, 111.5, 119.9, 120.2, 120.4, 123.2, 124.3, 124.9, 125.5, 129.3, 130.6, 139.7, 150.2. 4-[((*E*)-3,7-dimethylocta-2,6-dienyloxy)methyl]-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole (**4**); (ESI, *m/z*) 364.33 ($M+Na^+$), 342.37 ($M+H^+$). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.26 (s, 1H), 1.33 (s, 1H), 1.35 (s, 1H), 1.69–1.80 (m, 4H), 3.50 (s, 3H), 3.82 (d, 2H, *J* = 7.1 Hz,), 4.35 (s, 2H), 4.77 (t, 1H, *J* = 5.5 Hz), 5.07 (t, 1H, *J* = 6.7 Hz), 6,60 (m, 1H), 6.96 (m, 1H), 7.10–7.14(m, 2H), 7.90 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.3, 17.5, 25.5, 26.3, 39.5, 55.3, 60.2, 66.9, 106.0, 112.1, 114.2, 120.5, 123.9, 124.0, 130.3, 130.4, 131.3, 138.0, 140.5, 160.5, 170.7. 4-[((*E*)-3,7-dimethylocta-2,6-dienyloxy)methyl]-1-(4-methoxyphenyl)-1*H*-1,2,3-triazole (**5**); (ESI, *m/z*) 364.25 ($M+Na^+$), 342.28 ($M+H^+$), ^1H NMR ($CDCl_3$, 400 MHz) δ 1.59 (s, 3H); 1.66 (brs, 6H); 2.02–2.09 (m, 4H), 3.86 (s, 3H), 4.08 (d, 2H, *J* = 7.0 Hz·), 4.12 (s, 2H), 5.08 (t, 1H, *J* = 5.6 Hz), 5.32 (t, 1H, *J* = 6 Hz), 7.01 (d, 2H, *J* = 7.0 Hz), 7.61 (d, 2H, *J* = 7.0 Hz), 7.91 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.4, 17.8, 25.8, 26.5, 39.7, 55.7, 63.5, 67.2, 114.9, 120.4, 122.3, 123.6 (2C), 124.1 (2C), 130.6, 131.8, 139.4, 141.2, 159.9. 3-(4-(((*E*)-3,7-dimethylocta-2,6-dienyloxy)methyl)-1*H*-1,2,3-triazol-1-yl)benzoic acid (**10**); (ESI, *m/z*) 377.94 ($M+Na^+$). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.49 (s, 3H), 1.55 (s, 3H), 1.60 (s, 3H), 1.90–2.10 (m, 4H), 4.07 (d, *J* = 6.8 Hz, 2H), 4.67 (s, 2H), 4.99 (m, 1H), 5.31 (t, *J* = 6.7 Hz, 1H), 7.55 (d, 2H, *J* = 7.8 Hz), 7.98 (d, 1H, *J* = 8.0 Hz), 8,06 (m, 2H), 8.32 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.8, 17.9, 25.9, 26.5, 39.8, 63.4, 67.4, 120.1, 120.6, 121.8, 124.1, 125.7, 130.3, 130.5, 131.4, 132.0, 137.4, 141.6, 146.8, 170.7. 4-[((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trienyloxy)methyl]-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole (**13**); (ESI, *m/z*) 410.42 ($M+H^+$). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.40 (s, 3H), 1.41 (s, 3H), 1.47 (s, 3H), 1.50 (s, 3H), 1.77–1.97 (m, 8H), 3.67 (s, 3H), 3.95 (d, 2H, *J* = 6.8 Hz), 4.51 (s, 2H), 4.91 (m, 2H), 5.22 (t, 1H, *J* = 5.6 Hz), 6.74 (m, 1H), 7.05 (m, 1H), 7.13–7.19 (m, 2H), 7.84 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.1,

16.6, 17.8, 25.8, 26.4, 26.8, 39.7, 39.8, 55.6, 63.3, 67.2, 106.2, 112.3, 114.5, 120.5, 121.0, 123.9, 124.4, 130.5, 130.6, 131.2, 135.3, 138.1, 141.0, 160.6. 4-[((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trienyloxy)methyl]-1-(4-methoxyphenyl)-1*H*-1,2,3-triazole (**14**); (ESI, *m/z*) 432.34 ($M+Na^+$), 410.34 ($M+H^+$). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.60 (s, 6H), 1.66 (d, 3H), 1.96–2.10 (m, 8H), 3.86 (s, 3H), 4.08 (d, 2H, *J* = 7.2 Hz), 4.12 (s, 2H), 5.07 (m, 2H), 5.33 (t, 1H, *J* = 5.6 Hz), 7.01 (d, 2H, *J* = 6.4 Hz), 7.61 (d, 2H, *J* = 7,0 Hz); 7,98 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.1, 16.6, 17.8, 25.8, 26.4, 26.8, 39.7, 39.9, 55.7, 63.5, 67.9, 114.9, 120.0, 120.4, 122.2, 123.9, 123.9, 124.4, 131.4,135.4, 141.1, 141.8, 159.9.

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23. Typical procedure for bacterial adhesion assays (adapted from Leroy et al.[13]). *Pseudoalteromonas* sp. D41 was grown on VNSS (Vaatanen Nine Salt solution) at 20 °C and sampled in the stationary phase. After centrifugation, cells were suspended in sterile artificial sea water (ASW) until an optical density of 0.2–0.4 at 600 nm was achieved. 200 μL of ASW was inoculated on the border-row wells of the 96-well microtiter plates (sterile black polystyrene NUNC), and 100 μL of the bacterial suspension on other wells using an eight-channel pipette. 100 μL of diluted standard biocide (Seanine) and purified molecules were added in the latter wells. All the concentrations were tested in triplicates. 100 μL of ASW was added in six wells to constitute the bacterial adhesion control. After 15 h at 20 °C, the non-adhered bacteria were eliminated by three successive hand washings (36 g/L of sterile NaCl solution). The adhered bacteria were fixed for 90 min at 4 °C with sterile NaCl solution (36 g/L) containing 2% formaldehyde, then bacteria were stained by adding 200 μL of 4 μg/mL DAPI (4′,6-diamidino-2-phenylindole). The excess stain was removed by three hand washings (36 g/L NaCl solution). The DAPI was then solubilized in 200 μL of a 95% ethanol solution. Fluorescence was measured (λ_{exc} = 380 nm, λ_{em} = 495 nm) using an Infinit 200 micro-plate fluorescence reader (TECAN, Lyon, France). The dose-response curves fitting and the determination of the EC_{50} for each molecule were achieved using GraphPad Prism software.
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26. *Typical procedure for antibacterial assays*: The antibacterial assay was performed on *Pseudoalteromonas* sp. strain D41. The strains were grown on VNSS at 20 °C under shaking (120 rpm) and sampled in the exponential phase. The culture was subculture at an OD_{600} of 0.1 in VNSS. 200 μL of VNSS was inoculated on border-rowwells of the 96-well PS microtiter plates (steril,Greiner Bio-one Cellstar®). The 6th column was inoculated with 180 μL of VNSS and 20 μL of the bacterial subculture as a growth control. The 10 wells of lines A and G were filed with 180 μL of VNSS and 20 μL of Norfloxacin (standard biocides) or natural products at varied concentrations. The others wells were filled with 160 μL of VNSS, 20 μL of the bacterial subculture and 20 μL of Norfloxacin or tested products (**4** and **10**) at varied concentrations were added. All the concentrations were tested in triplicates. The maximum percentage of solvent (dimethylsulfoxide or methanol) used for the dilution of biocides was also tested as additional control in triplicate. The plate was incubated at 20 °C under shaking (120 rpm). The OD_{600} was taken all the hour by using an Infinit 200 microplate reader (TECAN, Lyon, France).