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Lipase-mediated production of defensive toxins in the marine mollusc *Oxynoe olivacea*

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Metabolites related to caulerpenyne (**1**), a toxic sesquiterpene featured by two enol-acetate residues, play a major role in the chemical defence of both algae of the genus *Caulerpa* and a few molluscs of the order Sacoglossa. Here we report the direct evidence that cell-free preparations of *Oxynoe olivacea*, a Mediterranean sacoglossan, transform efficiently the algal metabolite **1** to oxytoxin-2 (**3**), the main defensive metabolite of the mollusc. The process implies two distinct hydrolytic activities, here named LIP-1 and LIP-2, able to operate either hydrolysis of the acetyl residue at C-1 or concerted elimination of the acetyl groups at C-4 and C-13. Incubation experiments with tissue homogenates of *O. olivacea* or with commercially available lipases suggest a two-step mechanism that involves, *in vitro*, an unstable metabolite characterized as preoxytoxin-2 (**4**). The course of the entire process can be easily monitored by reverse phase HPLC/ESI-MS, as well as by NMR measurements, which provides direct evidence of the enzymatic mechanism leading to the formation of this last compound (**4**). In agreement with the literature, both fresh and frozen tissues of Mediterranean *Caulerpa prolifera* also have the capability to transform **1** into aldehydic derivatives, namely oxytoxin-1 (**2**) and oxytoxin-2 (**3**), through hydrolysis of the acetyl groups. However, differently from experiments with mollusc homogenates, the conversion is not complete and caulerpenyne (**1**) can be detected in the algal suspension for a few hours. HPLC/ESI-MS monitoring of this transformation suggests that the hydrolytic route involves different activities in the mollusc and seaweed.

Introduction

Caulerpenyne (**1**),[1] a common metabolite of algae of the *Caulerpaceae* family, is a toxic sesquiterpene showing an interesting panel of biological activities.[2] In particular, the metabolite (**1**) is supposed to play a role as a chemical deterrent in defence against herbivores and within interspecific competition, although it does not defend the algae from herbivorous fish.[3] Among Mediterranean species, the molecule is a major constituent of the extracts of both endemic *Caulerpa prolifera*[1] (Lamour) and invasive *Caulerpa taxifolia* (Vahl) C. Agardh and *Caulerpa racemosa* (Forsskål),[3c,4] two tropical species that have quickly colonized large areas of the new habitat.[5]

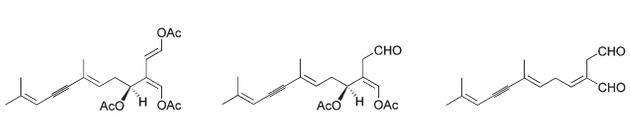
The highly unsaturated structure of caulerpenyne (**1**) is the site of spontaneous and enzymatic reactions that, recently, Guerriero and D'Ambrosio have clustered in three groups with respect to (a) hydrolysis of the acetate substituents at C-1, C-4 and C-13, (b) oxidation of C-6–C-7 and C-10–C-11 double bonds, (c) degradation of the terpene skeleton at C-5 and C-7.[6] Such transformations are suggested to have a major role in the ecological success of Caulerpales, since they contribute to the formation of a plethora of products involved in the wound-activated algal defence.[7] However, the ecological implications of these processes have not been proven so far in *Caulerpa*,[4b,7b] whereas the deterrent properties of caulerpenyne-related compounds are well documented in a group of herbivore molluscs (*e.g.*, *Oxynoe olivacea*, *Lobiger serradifalci*, *Ascobulla fragilis*, *Oxynoe antillarum*, *Volvatella* sp., *Elysia subornata* and *Elysia patina*) that live in strict association with these seaweeds.[8] Although never explored by committed experiments, it is a general opinion that the defensive substances found in these invertebrates, such as oxytoxin-1 (**2**) and oxytoxin-2 (**3**), derive from active transformation of the algal pool of caulerpenyne (**1**). These molluscs therefore represent ideal models for studying the biochemical aspects concerning the enzymatic modification of the algal sesquiterpene.

In this paper we examine the hydrolysis of caulerpenyne catalysed by cell-free preparations of *C. prolifera* and the related herbivore, the sacoglossan mollusc *O. olivacea* (Rafinesque). We show that the invertebrate is able to obtain the defensive allomones, namely oxytoxin-1 (**2**) and oxytoxin-2 (**3**),[9] by enzymatic hydrolysis of the algal metabolite. This manuscript also details the molecular mechanism leading to oxytoxins and demonstrates that production of these metabolites requires a two-step synthesis involving specific lipase activities.

Results and discussion

The Mediterranean mollusc *O. olivacea* lives in strict association with algae of the genus *Caulerpa* from which it obtains nourishment and protection from potential predators.[9] Although the capability of this mollusc to feed upon the alga is very well documented, chemical studies showed that no caulerpenyne (**1**) is detectable in the body of the invertebrate.[9] Instead, the extracts of *O. olivacea* contain two aldehydes, named oxytoxin-1 (**2**) and oxytoxin-2 (**3**), which are manifestly derived from **1** although the process has never been demonstrated by committed experiments. Both compounds are more potent than caulerpenyne (**1**) in ecological assays[10] and their presence in the molluscs has been suggested to be the result of an active transformation of the algal metabolite in relation to the development of chemical defence in the opisthobranch.[8]

In agreement with the literature, LCMS analysis revealed significant levels of oxytoxin-1 (**2**) and oxytoxin-2 (**3**) in extracts of both the mucus and the body of *O. olivacea* (data not shown). Under the analytical conditions (reverse phase, MeOH : H_2O, 75 : 25; to MeOH : H_2O, 90 : 10 in 15 min), oxytoxin-1 (**2**) appeared as a double peak (T_R 9.12 and 9.33 minutes) probably owing to the equilibrium of two cyclic species derived by intramolecular hydrogen bonding between



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the hydrated aldehyde and the acetoxy groups. To study the supposed conversion of caulerpenyne (**1**) to the aldehydic derivatives **2** and **3** in *O. olivacea*, we used mucus and tissue homogenates obtained by blending frozen sections of this mollusc in 20 mM phosphate buffer (pH = 7.0) at 4 °C. The resulting suspensions (1 mL) were incubated with 2.7 mM caulerpenyne, monitoring the enzymatic course of the reaction by LCMS on a reverse phase column. As shown in Fig. 1, the addition of caulerpenyne to mollusc homogenates led to an immediate (time = 0) appearance of oxytoxin-2 (**3**) together with an unknown product, hereafter named preoxytoxin-2 (POXY-2, **4**), eluted at 13.1 minutes. The chemical constitution of this metabolite was not investigated at that time, although the UV (272 and 212 nm) and MS [311 ($M + K^+$) and 295 ($M + Na^+$)] data suggested a strict correlation with caulerpenyne.[1] The chromatographic profile of homogenates of *O. olivacea* did not reveal any presence of oxytoxin-1 (**2**), whereas formation of caulerpenyne derivatives **3** and **4** was totally inhibited in boiled homogenates of the mollusc. The conversion of the algal sesquiterpene was quick and complete as proved by the almost total disappearance of the relative peak only a few seconds after the beginning of the experiment. Contrary to this, the levels of both oxytoxin-2 (**2**) and compound **4** slowly decreased during the time of the experiment, suggesting a gradual degradation, probably due to a cross-reaction with nucleophiles present in the homogenates. Similar results were also obtained by incubating caulerpenyne (**1**) with cell-free preparations of mollusc sections (data not shown). In fact, except for the mucus, homogenates of gland, mantle and tail were all able to promote the formation of oxytoxin-2 (**3**) and preoxytoxin-2 (**4**).

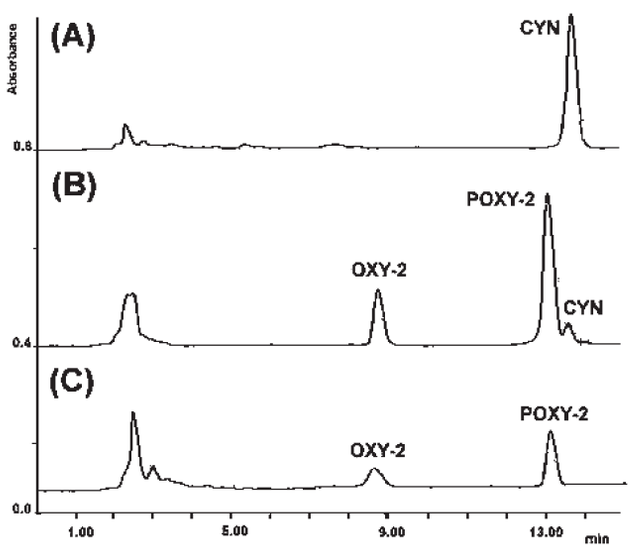


Fig. 1 Hydrolysis of caulerpenyne by homogenates of *O. olivacea*. Reverse phase LCMS profile of: (A) caulerpenyne, (B) mollusc homogenate immediately after the addition of caulerpenyne, (C) mollusc homogenate 15 minutes after the addition of caulerpenyne. Control experiments were carried out by adding caulerpenyne to boiled preparations. CYN = caulerpenyne (**1**); OXY-2 = oxytoxin-2 (**3**); POXY-2 = preoxytoxin-2 (**4**).

To gather further information on the hydrolytic process leading to **2** and **4**, a number of commercially available lipases were tested for their ability to remove the acetyl groups from caulerpenyne. The reactions were carried out under the same conditions as for the mollusc homogenates, using a suspension of lipase (1.5 mg) in 1 mL of 20 mM potassium phosphate buffer (pH = 7.0). Over six different lipases (*Aspergillus niger*, *Aspergillus oryzae*, *Mucor miehei*, *Penicillium chamemberti*, *Candida rugosa* and *Pseudomonas cepacea*), only the enzyme from *P. cepacea* was capable of hydrolyzing efficiently the enol-acetate groups of the algal sesquiterpene (Table 1). The reaction course underlined a path very similar to the

Table 1 Lipase-mediated hydrolysis of caulerpenyne (**1**)

Source organism	Reaction time/min	Conversion (%)
Aspergillus niger	90	80
Aspergillus oryzae	345	15
Mucor miehei	120	80
Penicillium chamemberti	720	No reaction
Candida rugosa	90	95
Pseudomonas cepacea	30	100

hydrolytic process previously observed with homogenates of *Oxynoe* tissues (Fig. 2). In fact, after the addition of 2.7 mM caulerpenyne, production of oxytoxin-2 (**3**) and preoxytoxin-2 (**4**) was immediate whereas no presence of oxytoxin-1 (**2**) was detectable. In 15 minutes, the incubation with *P. cepacea* lipase produced a complete conversion of **1** into **3** that was visible in the solution for several minutes. Contrary to this, the occurrence of preoxytoxin-2 (**4**) was transient as this compound reached a maximum level at the beginning of the experiment and, afterwards, faded away gradually (Fig. 2). Like the mollusc homogenates, this is consistent with a biochemical mechanism leading to oxytoxin-2 (**3**) from caulerpenyne (**1**) through preoxytoxin-2 (**4**). Unfortunately, any attempt to isolate **4** by HPLC failed, apparently because of the instability of the product. However, convincing evidence on the process was obtained by NMR monitoring of the lipase-catalysed bioconversion of caulerpenyne *in situ* (Fig. 3). The experiment was carried out by adding *P. cepacea* lipase (1.2 mg) dissolved in 100 µl of D_2O to a 0.5 mL solution (d_6-acetone : D_2O, 1 : 1) of 2 mg caulerpenyne (**1**) in a NMR tube. NMR spectra (0–12 ppm) were recorded regularly over a period of 14 hours. As expected, the signals due to the algal metabolite decreased, while the resonances of oxytoxin-2 (**3**) and preoxytoxin-2 (**4**) increased as a function of time (Fig. 3). After 10 minutes, the NMR analysis revealed two main sets of signals attributable to caulerpenyne (**1**) and preoxytoxin-2 (**4**) (Fig. 3). This latter compound was featured by an aldehydic proton at δ 9.18 (H-13), the signals of the "enol" residue at δ 7.96 (H-1) and 5.89 (H-2), the proton of a conjugated double bond at δ 6.34 (H-4) and the bis-allylic methylene moiety at δ 3.01 (H_2-5). The remaining resonances were roughly unaffected with respect to caulerpenyne (**1**) except for the region of the methyl groups where there was an evident reduction of the number of acetyl residues. Considering the formula $C_{17}H_{20}O_3$, calculated from the pseudomolecular ion $M + Na^+$ at *m/z* 295, the NMR data are in agreement with the structure of preoxytoxin-2 (**4**) depicted in Fig. 3.

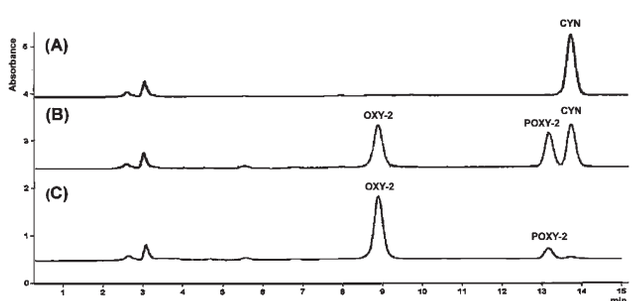


Fig. 2 Caulerpenyne hydrolysis catalysed by *P. cepacea* lipase. Reverse phase LCMS profile of: (A) caulerpenyne, (B) enzyme suspension immediately after the addition of caulerpenyne, (C) enzyme suspension 15 minutes after the addition of caulerpenyne. CYN = caulerpenyne (**1**); OXY-2 = oxytoxin-2 (**3**); POXY-2 = preoxytoxin-2 (**4**).

The molecular similarities between the hydrolytic mechanism triggered by *P. cepacea* lipase and *Oxynoe* homogenates led us to conclude that the molluscs possess lipase activity capable of promoting the simultaneous loss of two acetate units from a molecule of caulerpenyne (Scheme 1). Aptitude to promote hydrolysis of enol-acetate groups has been also reported in

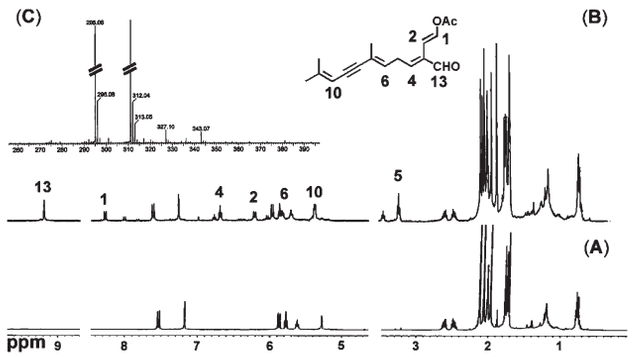


Fig. 3 Formation of preoxytoxin-2 (**4**) by hydrolysis of caulerpenyne (**1**) with *P. cepacea* lipase. ^{1}H-NMR spectra of caulerpenyne before (A) and 10 minutes after (B) the addition of the enzyme. The slight difference in the chemical shifts in the two spectra was due to the presence of the protein. The insert (C) shows the ESI-MS^{+} spectra of **4** obtained by LCMS analysis of the reaction mixture after 10 minutes.

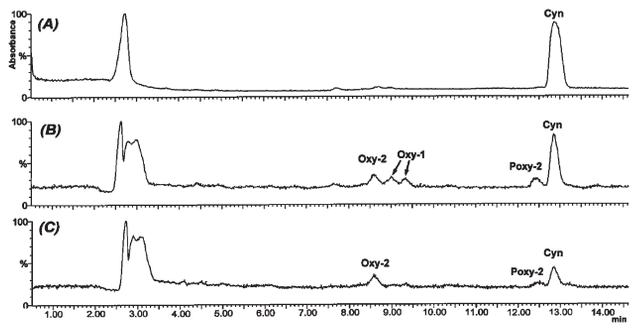
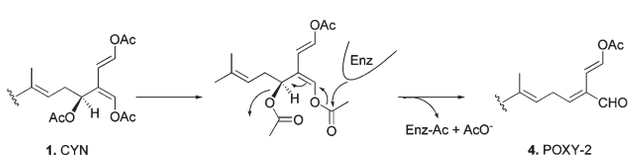


Fig. 4 Hydrolysis of caulerpenyne in ground tissues of *C. prolifera*. Reverse phase LCMS profile of: (A) caulerpenyne, (B) suspension of the algal material 30 s after grinding, (C) suspension of the algal material 15 min after grinding. Control experiments were carried out by adding caulerpenyne to boiled preparations of the alga. CYN = caulerpenyne (**1**); OXY-1 = oxytoxin-1 (**2**); OXY-2 = oxytoxin-2 (**3**); POXY-2 = preoxytoxin-2 (**4**).

preparations of algae of the genera *Caulerpa* and *Halimeda* upon mechanical damage of the plant tissue. In these seaweeds the process, that has been related to wound-activated defence, is believed to be due to aspecific esterase activities that enable release of aldehydes immediately after the tissue damage.[7] We verified that formation of both oxytoxins (**2** and **3**) was elicited by grinding frozen leaves of *C. prolifera* (2 g wet weight) in 1.5 mL of 20 mM phosphate buffer (pH = 7.0) at 4 °C (Fig. 4). However, the process differs from the mechanism leading to the synthesis of aldehydic sesquiterpenes in *O. olivacea*. In fact, LCMS analysis of aliquots (20 μL) of algal homogenate showed the immediate appearance of peaks due to aldehydes **2** and **3** together with preoxytoxin-2 (**4**). The levels of these compounds changed during the experiment. After 15 minutes, oxytoxin-1 (**2**) was not detectable any longer, whereas the concentration of both caulerpenyne (**1**), preoxytoxin-2 (**4**) and oxytoxin-2 (**3**) was drastically reduced (Fig. 4C). Afterwards the system seemed to reach a sort of equilibrium (data not shown) featured by a gradual disappearance of the peaks due to oxytoxin-1 (**3**) and preoxytoxin-2 (**4**), whereas caulerpenyne (**1**) was still traceable even after a few hours (data not shown). In analogy with the experiments with *Oxynoe*, the incubation of caulerpenyne (**1**) with algal preparations led to the appearance of HPLC peaks at low retention times. The MS analysis of this material revealed the presence of a complex mixture that was not possible to characterize.

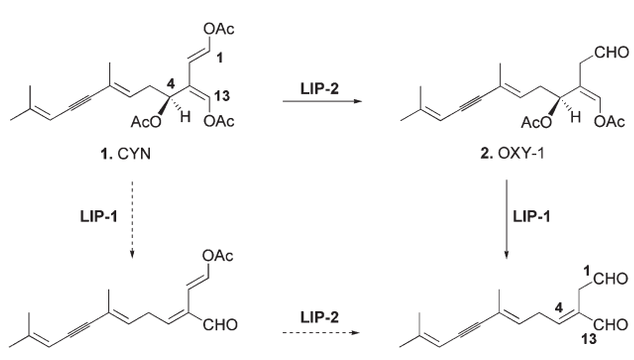


Scheme 1 Supposed enzymatic mechanism leading to preoxytoxin-2 (**4**) from caulerpenyne (**1**) through concerted loss of two acetyl groups.

Conclusions

We have shown that the mollusc *O. olivacea* possesses a specific lipase activity to produce its major defensive allomone, namely oxytoxin-2 (**3**), by hydrolysis of the algal metabolite caulerpenyne (**1**). The suspicion[11] that the mollusc is incapable of converting actively the algal caulerpenyne is proved unfounded. In fact, *in vitro* experiments with tissue homogenates of the mollusc suggest a two-step mechanism that requires conversion of sesquiterpene **1** to dialdehyde **3** through the unstable intermediate preoxytoxin-2 (**4**) (Scheme 2). It is therefore likely that the process involves two distinct hydrolytic activities, here named LIP-1 and LIP-2, able to operate the observed sequence of transformations. Considering the well-documented presence of oxytoxin-1 (**2**) and oxytoxin-2 (**3**) in *O. olivacea*,[9] the enzymatic steps may invert *in vivo*, leading to a pathway that operates through (*a*) the hydrolysis of the acetyl residue at C-1 of caulerpenyne (**1**) to give oxytoxin-1 (**2**) and (*b*) the concerted elimination of the acetyl groups at C-4 and C-13 of this latter compound to form oxytoxin-2 (**3**) (Scheme 2). In agreement with this view, *Oxynoe* homogenates, from both mantle and gland tissue, showed high efficiency in transforming oxytoxin-1 (**2**) into oxytoxin-2 (**3**) (data not shown). This reaction is apparently immediate since we did not detect any trace of the aldehyde **2** after addition to cell-free preparations of the mollusc. In our opinion, this is consistent with the suggestion that oxytoxin-1 (**2**) is the physiological substrate of the hydrolytic activity of LIP-1 (Scheme 2). Contrary to this, *in vitro* formation of preoxytoxin-2 (**4**) is a consequence of the aspecific activity of this enzyme on caulerpenyne (**1**). Considering the absence of protease inhibitors in our experiments, the incomplete conversion of POXY-2 (**4**) in the preparations of *Oxynoe* is presumably due to the partial degradation of the lipase (LIP-2) that catalyses the concerted elimination of the acetyl residues. In this line of reasoning, the apparent absence of oxytoxin-1 (**2**) in cell-free preparations of *O. olivacea* is due to the fast transformation of this compound under the artificial conditions occurring in these experiments. Incubation of caulerpenyne with *P. cepacea* lipase in a NMR tube provided direct evidence about the enzymatic mechanism leading to the concerted loss of the acetyl groups at C-4 and C-13 (Scheme 2). The total or partial ineffectiveness of other lipases in hydrolysing caulerpenyne (Table 1) may be due by the inhibitory properties of **1**.[12]

Finally, in agreement with the literature,[7] homogenates of fresh or frozen tissues of *C. prolifera* are able to promote formation of both oxytoxin-1 (**2**) and oxytoxin-2 (**3**), as well as POXY-2 (**4**). The process is, however, not complete since caulerpenyne (**1**) could be detected in the algal suspension for



Scheme 2 Proposed synthesis of oxytoxins by *O. olivacea*. Plain and dotted arrows indicate the conversion of caulerpenyne (**1**) to oxytoxin-2 (**3**) *in vivo* and *in vitro*, respectively. LIP-1 denotes the lipase activity capable of performing the concerted elimination of the acetyl group at C-4 and C-13. LIP-2 denotes the lipase activity capable of removing the acetyl group at C-1.

several minutes. On these grounds, it is evident that hydrolytic activities in the alga and in the mollusc differ significantly. Given the inherent instability of the enol-acetate groups, hydrolysis of **1** in algal damaged tissues is an unsurprising phenomenon that, unlike the transformations catalysed by *O. olivacea*, might be due to aspecific processes. In our opinion, molecular studies are required to prove the ecological role of the conversion of caulerpenyne in *C. prolifera.*

Experimental

General

Silica gel chromatography was performed using pre-coated Merck F254 plates and Merck Kieselgel 60 powder. HPLC purifications were carried out on a Waters chromatograph equipped with a 490E UV multiwavelength detector. LC-MS data were obtained using a MicroMass Micro*Q-tof* ESI^+ mass spectrometer coupled with a Waters *Alliance* HPLC equipped with a diode array UV detector. NMR spectra were recorded on Bruker AMX 500 and Bruker Avance WMX 400 spectrometers. Solvents were purchased from Carlo Erba (Milan, Italy) and distilled prior to use. Except where noted, all other chemicals were obtained from Aldrich.

Collection and dissection of molluscs and algae

Oxynoe olivacea (9 specimens) and *Caulerpa prolifera* (250 g dry weight) were collected by hand using SCUBA diving at depths of 2–12 m at Capo Miseno (Gulf of Naples, Italy) during June 2002. A second collection of algae and molluscs (6 specimens) was carried out in June 2003. Molluscs and algae were immediately frozen in liquid nitrogen and stored at −20 °C until the day of the analysis. For anatomical sections of *O. olivacea*, the frozen animals were dissected under an optical microscope in order to separate the mantle, the tail and the inner organs. Mucus was collected using a Pasteur pipette by disturbing the living sacoglossans with a glass stick. This material was frozen and stored at −20 °C after freeze-drying.

Purification of the sesquiterpenes

For the isolation of caulerpenyne (**1**), frozen leaves (55 g) of *C. prolifera* were ground in acetone. The solutions were filtered on paper and the clear filtrates were partially evaporated at a reduced pressure. The residues were diluted with distilled water (35 mL) and extracted with diethyl ether (2 × 30 mL). Caulerpenyne (121 mg) was obtained by silica gel purification of this extract, followed by HPLC on a reverse phase column (Phenomenex RP-18) with $MeOH:H_2O$, 80:20 at a flow of 1 mL min^{-1}. Compound identity was ascertained by comparison of the NMR data in $CDCl_3$ with those reported in the literature. Purity (99%) was determined by HPLC. Part of the fraction containing the sesquiterpene was divided into several aliquots of 1.0 mg each and evaporated at a reduced pressure. Bulky sample and aliquots of caulerpenyne were stored, dried, under an argon atmosphere at −80 °C. A sample of oxytoxin-1 (**2**) was prepared by freshly-released mucus of *O. olivacea* (6 specimens). Given the inherent instability, the product was directly purified on reverse phase HPLC and stored in aliquots (1 mg) under an argon atmosphere at −80 °C.

Enzymatic preparation of oxytoxins

Biological samples (mollusc sections, mucus and algae) were ground in a mortar and then homogenised in 1 mL of 20 mM potassium phosphate buffer (pH = 7.0) at 4 °C. The resulting suspensions were incubated at 4 °C with 2.7 mM caulerpenyne or 2.5 mM oxytoxin-1. An aliquot of these solutions (20 µL) was taken every 14 minutes and reactions were terminated by the addition of 15 µl MeOH. Insoluble material was removed by centrifugation (10000 rpm for 10 s). The supernatant was directly analysed by HPLC or LC-MS using a reverse phase Phenomenex RP-18 column with an elution gradient from $MeOH:H_2O$, 75:25 up to 90:10 in 15 min (flow 1 ml min^{-1}) by monitoring the elution of the metabolites with an UV detector at 254 nm. The reactions were terminated after 1 h (and the remaining solutions were processed as described above). The white powder obtained after lyophilization of the mucus was directly suspended in 1 mL of buffer and incubated as described above for the tissue preparations. For control measurements, purified caulerpenyne (2.7 mM) was kept in potassium phosphate buffer for the duration of the experiment at 4 °C. Analyses of the control and blank (potassium phosphate buffer) were carried out prior to and after each incubation.

In vitro lipase activity

Commercially available lipases from *Aspergillus niger*, *Aspergillus oryzae*, *Mucor miehei*, *Penicillium chamemberti*, *Candida rugosa* and *Pseudomonas cepacea* were tested for the conversion of caulerpenyne (**1**) into oxytoxin-2 (**3**). In a typical procedure, purified caulerpenyne (2.7 mM) was incubated with 1.5 mg of commercially available enzyme in 1 mL of 20 mM potassium phosphate buffer (pH = 7.0). The resulting suspension was kept under stirring at 37 °C and the reaction course was monitored by chromatographic analysis as described above.

^{1}H-NMR detection of oxytoxin-2 (3) after incubation of caulerpenyne (1) with lipase of *P. cepacea*

Purified caulerpenyne (2 mg) was dissolved in 250 µl of d_6-acetone. The clear solution was transferred to a narrow-bore NMR tube and diluted with 250 µl of D_2O. A ^{1}H-NMR spectrum (32 scans, 64 Kbyte) of this sample was recorded between 12 and 0 ppm at 21 °C. The NMR tube was then taken out from the NMR probe and 1.2 mg lipase of *P. cepacea* in 100 µl of D_2O was added to the solution. The sample was carefully shaken to obtain a homogeneous suspension and then placed in the NMR probe again. After quick shimming, ^{1}H-NMR spectra were recorded every ten minutes for the first 2 hours and every hour over the following 14 hours. Spectra (32 scans, 64 Kbyte) were recorded between 12 and 0 ppm at 21 °C.

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