The SSAO purified 447-fold is strongly sensitive to the action of the carbonyl reagent semicarbazide, which according to Yamada and Yasunobu[13] is sufficient evidence for considering pyridoxal (phosphate) as a cofactor of the enzyme. It is known that amine oxidases which have pyridoxal (phosphate) as a cofactor generally attack only terminal primary amino groups of amines[14], and that as a result of the reaction aldehydes are produced.

Experiments to purify the SSAO to homogeneity and to identify the cofactor of the enzyme by mass spectrometry are still in progress in our laboratory.

The SSAO obtained by our method was found to be useful in the enzymatic determination of the total free polyamines in blood and in assays for diagnosis and the monitoring of therapy in cancer patients[15].

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Oxytoxins, bioactive molecules produced by the marine opisthobranch mollusc *Oxynoe olivacea* from a diet-derived precursor

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Summary. The ethereal extract of the mucous secretion from the opisthobranch mollusc *Oxynoe olivacea* was examined and found to contain two novel ichthyotoxic compounds, named oxytoxin 1 and 2 (**1**, **2**). The structures of **1** and **2** are closely related to the metabolites previously isolated from the alga *Caulerpa prolifera*. The activity of the most stable compound was studied in order to investigate the possibility of a further biological role for these metabolites, which represent an uncommon example of bioactive molecules produced in vivo from a dietary precursor.

Key words. Opisthobranch molluscs; defense mechanisms; marine toxins; dialdehydes.

The opisthobranch molluscs of the genus *Oxynoe* utilize a series of defensive mechanisms to avoid predation[1]. First, they live camouflaged on the green algae of the *Caulerpa* genus on which they feed. When disturbed, these ascoglossan molluscs secrete an extremely toxic mucus capable of deterring various predators, including fish. Finally, if molested further, these molluscs also exhibit a defensive mechanism known as autotomy, which consists of the spontaneous detachment of the tail, which then continues to twitch for several minutes, thus distracting the predator and allowing the mollusc to escape to safety. The missing part is usually regenerated within a few days[2,3].

It has been suggested[4] that the mollusc mucous secretion owes its toxicity to substances of dietary origin, probably derived from the algae of the genus *Caulerpa* on which it feeds. However, no chemical characterization of these metabolites, and therefore no definite proof of this hypothesis, has yet been reported. In this paper we describe the results of experiments aimed at isolating and characterizing the substances responsible for its toxicity from the mucus of an *Oxynoe* species living in the Mediterranean Sea, *O. olivacea* (Rafinesque, 1819), and investigating the origin and other possible biological roles of the toxic substances by examining their gross distribution within the organism and their biological activity.

Oxynoe olivacea is a common species in littoral sheltered and shallow habitats, either marine or lagoon, where it is abundant and is always found associated with *Caulerpa prolifera*[5] ((Forsskal) Lamoureux, 1809). The animals studied were caught in the Bay of Naples.

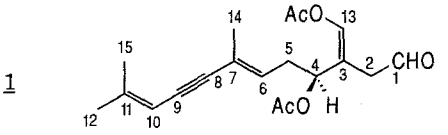
Silica gel TLC analysis of the ethereal extract of fresh mucous secretion showed the presence of two spots visible in UV light (Rf = 0.45 and 0.55, C_6H_6 : Et_2O 8:2). A

Table 1. ^{1}H-(500 MHz) and ^{13}C-(125.8 MHz) NMR data for oxytoxin-1 and -2 (**1** and **2**)

C	Oxytoxin-1 (**1**) $^{13}C^a$	^1H at $C^{a,c}$	$^{13}C^b$	(m)[d]	^1H at $C^{b,c}$	(m; J in Hz)	Oxytoxin-2 (**2**) $^{13}C^b$	(m)[d]	^1H at $C^{b,c}$	(m; J in Hz)
1	198.9	9.54	197.8	(d)	9.12	(t; 2.2)	195.6	(d)	9.12	(m)
2	42.5	3.13	42.6	(t)	2.62	(ddd; 16.5, 3.3 and 1.1)	38.9	(t)	2.84	(s)
		2.98			2.32	(bd; 16.5)				
3	122.7		122.2	(s)			137.1	(s)		
4	69.0	5.89	69.2	(d)	6.04	(t; 7.2)	152.8	(d)	5.79	(t; 7.4)
5	31.8	2.54	32.1	(t)	2.43	(m)	28.4	(t)	2.46	(t; 7.4)
		2.34			2.18	(m)				
6	129.1	5.64	129.9	(d)	5.84	(dt; 7.5 and 1.2)	130.3	(d)	5.60	(dt; 7.4 and 1.3)
7	114.7		115.1	(s)			115.1◆	(s)		
8	93.9		94.7	(s)			94.4	(s)		
9	85.3		86.0	(s)			86.4	(s)		
10	105.2	5.33	106.2	(d)	5.40	(s)	106.1	(d)	5.46	(s)
11	148.2		147.6	(s)			147.9	(s)		
12	24.8	1.80	24.5	(d)	1.46	(s)	24.5◆	(d)	1.49	(s)
13	135.3	7.17	135.6	(d)	7.02	(s)	192.5	(d)	9.00	(s)
14	17.7	1.82	17.8	(d)	1.78	(s)	17.6	(d)	1.66	(s)
15	20.9	1.87	20.9	(d)	1.80	(s)	20.9◆	(d)	1.85	(s)
13-OCOMe	167.0		166.6	(s)						
14-OCOMe	169.7		169.1	(s)						
13OCO*Me*	20.6@	2.19§	19.7^	(d)	1.56*	(s)				
14-OCO*Me*	20.8@	1.99§	20.3^	(d)	1.58*	(s)				

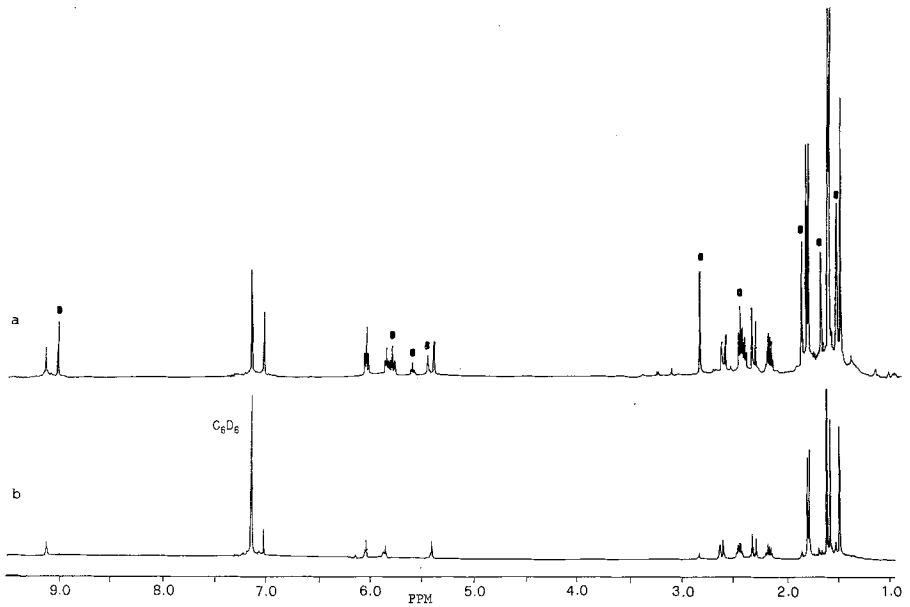
[a] $CDCl_3$; δ ^{1}H and δ ^{13}C are referred to $CHCl_3$ ($\delta = 7.26$) and to the central peak of $CDCl_3$ ($\delta = 77.0$); [b] C_6D_6, δ^1H and δ^{13}C are referred to C_6H_6 ($\delta = 7.15$) and to the central peak of C_6D_6 ($\delta = 128.0$); [c] assignments made by 2D-homo-(COSY-45) and heterocorrelations (HETCOR) experiments; @§^ ^*: assignments with the same symbol can be interchanged; ◆: superimposed to signals of oxytoxin-1; [d] determined by DEPT sequence.

comparison with the previously described metabolites from *Caulerpa prolifera*[6–8], the mollusc's food source in the Bay of Naples, showed that these two components were completely absent from the extract of *C. prolifera*. If the mucus was kept frozen for a few days, only the more polar component could be recovered from the extract, which indicated that the less polar compound was highly unstable. Moreover, attempts to purify the latter component, even from fresh mucus, were not successful. To begin with, we therefore focussed our attention on the more stable and more polar compound, named oxytoxin-1 (**1**). SiO_2 column chromatography (eluant C_6H_6 : Et_2O 95:5) of the extract from the mucus of 80 animals yielded 60 mg of pure compound. Spectral data of **1** were: UV (methanol) λ_{max} at 270 nm, $\varepsilon = 21\,600$ and 283 nm, $\varepsilon = 16\,500$, with a little shoulder at 268 nm; IR (chloroform) γ_{max} at 2740, 2184, 1763, 1745 and 1740 cm^{-1}; EIMS fragment ions at m/z 262 (40 %) and 234 (35 %); $[a]_D^{25}$ −70.6° (c, 2 %; $CHCl_3$) and −48.4° (c, 2 %; CH_3OH). NMR data for **1** are reported in table 1 and are almost identical to those reported for caulerpenyn[4,5], the most striking difference being the ^{1}H-NMR signal at δ 9.54 (t, J = 2.2 Hz) and the ^{13}C-NMR resonance at δ 198.9 which showed the presence of an aldehydic group instead of an enol acetate. This was confirmed by the HREIMS molecular ion peak found at 332.1618 (the molecular formula $C_{19}H_{24}O_5$ requires 332.1624), by the IR band at 2740 cm^{-1} corresponding to the Fermi resonance for aldehydes, and by the strong mass fragments at m/z 262 and m/z 234 (loss of acetic acid and CO). The aldehydic group was linked to a methylene (δ 2.98, d, J = 16.8 Hz, and δ 3.13, dd, J = 16.8 and 2.7 Hz, in the proton NMR spectrum) as deduced from the correlations observed in the COSY spectrum. This also showed a correlation between the olefinic proton at δ 7.17 (H-13, s) and the above methylene, leading to structure **1**:



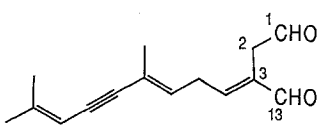
A n.O.e. experiment performed by irradiation at the frequency of the proton at C-13 (δ 7.17) showed a n.O.e. enhancement on the proton at δ 2.98, thus confirming the stereochemistry Z at the double bond between C-3 and C-13. As for the configuration at C-4, we assumed that this was identical to that previously reported for caulerpenyn[4].

The compound was tested for ichthyotoxic activity on *Gambusia affinis*[9], and was found to be active at 10 µg/ml. This toxicity was somewhat disappointing considering that when either the ethereal extract of fresh mucus or the mucous secretion itself were tested with the same bioassay the activity found was one order of magnitude higher. This suggested that, unless a synergism between the two components was occurring, which was considered unlikely, the less polar compound, named oxytoxin-2 (**2**), was responsible for most of the mucus toxicity. The extreme chemical simplicity of the mucus ethereal extract, together with the high instability of oxytoxin-2, prompted us to attempt a structural characterization of this metabolite by examining mono-and bi-dimensional NMR spectra of crude extracts of the mucus from *O. olivacea*.

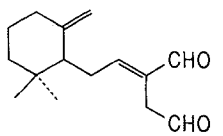


^{1}H-NMR spectra (C_6D_6, 500 MHz) of extracts from the mucus of *Oxynoe olivacea* (a) and from the intact frozen mollusc (b).

The mucus obtained from 20 specimens was treated with C_6D_6 (5 ml). After treatment with anhydrous Na_2SO_4 and concentration by N_2 flow, the extract was immediately submitted to the NMR analysis, which revealed a mixture of oxytoxin-1 and -2 at an approximate ratio of 6:4 (see fig. a, where dots over peaks indicate signals due to oxytoxin-2). Analogous extracts of intact frozen animals showed the presence of mainly the NMR peaks due to oxytoxin-1 (see fig. b). Subtraction of the signals due to the latter from the spectra of the mixture allowed a preliminary structure characterization of oxytoxin-2 (table 1). The diagnostic ^{1}H-NMR singlet at δ 9.00 (^{13}C-NMR at δ 192.5) indicated the presence of a second aldehyde function. The ^{1}H-NMR triplet at δ 2.46 (H-5, J = 7.4 Hz) coupled to the two vinylic protons at δ 5.60 (H-6, dt, J = 7.4 and 1.3 Hz) and 5.79 (H-4, t, J = 7.4 Hz) suggested the hydrolysis of the enolic acetate group with subsequent formation, by elimination of acetic acid, of a double bond conjugated to a carbonyl group. The E geometry of the C-3 double bond was determined by means of a n.O.e. experiment where after irradiation at the frequency of the proton on C-4 (δ 5.79) a n.O.e. enhancement was observed on the aldehydic proton on C-13 (δ 9.00). All these data agree well with the suggested structure **2**:



Oxytoxin-2 (**2**) shows structural analogies with a series of previously described compounds[10,11] and, in particular, with the aldehyde in Paul and Fenical[11] (**3**). The ^{1}H-NMR spectra of the $CDCl_3$ extract of the mucus, recorded for a comparison with these model compounds, definitively confirmed the suggested structure **2** (diagnostic peaks were at δ 9.62 (H-1, 1H, s), 9.44 (H-13, 1H, s), 6.72 (H-4, 1H, t), 5.74 (H-6, 1H, bt), 3.42 (H-2, 1H, s), 3.14 (H-5, 1H, t).



Separate extraction of *Oxynoe olivacea* mantle, mucus and digestive gland showed that oxytoxins are completely absent in the latter and that, while oxytoxin-1 is the predominant metabolite in the mantle (as already suggested by the spectrum in fig. b), oxytoxin-2 is mainly present in the mucous secretion. This would suggest that: 1) *O. olivacea* immediately transforms the dietary *C. prolifera* metabolites into oxytoxin-1 and transfers this substance to the mantle; 2) oxytoxin-1 might serve as a precursor to produce the more active dialdehyde oxytoxin-2 through hydrolysis of the enol acetate and elimination of acetic acid either during or immediately after the secretion of the mucus. Oxytoxin-1 is therefore the 'semiprotected' form of the dialdehyde oxytoxin-2, a case similar to that previously reported[12] of olepupuane which is the protected form of polygodial in *Dendrodoris* species. Like polygodial, the more stable oxytoxin-1 was tested for antifeedant activity on starved red fish *(Carassius carassius)*, by adsorbing various concentrations of the compound on fish food. We found that oxytoxin 1 was active in this bioassay at concentrations of 50 µg/cm^{-2} fish food or higher, whereas polygodial was active

Table 2. Biological activity of oxytoxin-1

On mosquito fish *(G. affinis)*	On red fish *(C. carassius)*	On brine shrimp *(A. salina)*	On *Hydra vulgaris*
Toxic at 10 μg/ml	Antifeedant at conc. ≥ 50 μg/cm^{-2} food	Cytotoxic $LD_{50} = 14.9$ μg/ml	Toxic at concs. higher than 15 μM 56 % increase of head regeneration speed at 10 μM 5-fold increase of the bud number at 5 μM Aberrant at 10 μM
Effect observed within 15 min of the treatment	Effect observed after a 30-s chewing by fish		The effect on head regeneration could be observed only in the long term (6 days)

at concentrations ≥ 30 μg/cm^{-2} fish food. As for olepupuane, the antifeedant effect was not instantaneous; it took about 30 s for the fish to reject the food treated with oxytoxin-1.

Several other examples of 'semiprotected' dialdehydes structurally related to oxytoxin-1 have been reported to intervene in the defensive mechanisms of opisthobranch molluscs[10]. However, it is likely that the true chemical weapons used by these marine invertebrates are the corresponding 'deprotected' compounds which are usually too unstable to be observed.

In *O. olivacea* the regenerative process following the autotomy occurring during the defensive mechanism requires the presence of chemical signals. Oxytoxin-1, being the most abundant metabolite in the ethereal extract of the mantle, might also play a role in this context. We studied the cytotoxic activity and the regenerative effect of this compound using respectively the brine shrimp (*Artemia salina*)[13] and the *Hydra vulgaris*[14] bioassays. The activities found are reported in table 2. Briefly, the cytotoxic activity was once again not very marked but significant. In the *Hydra* regeneration assay, an enhancement of the regeneration speed was observed in the long term, and also a significant effect on the number of buds. Also, aberration occurred at concentrations as low as 10 μM. However, further studies are required to ascertain whether oxytoxin-1 possesses a further biological role. In any event, oxytoxins represent an uncommon example of bioactive molecules derived from the transfer of algal secondary metabolites in marine food chains.

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