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New birth-control aldehydes from the marine diatom *Skeletonema costatum*: characterization and biogenesis

Giuliana d'Ippolito,[a] Giovanna Romano,[b] Olimpia Iadicicco,[a] Antonio Miralto,[b] Adrianna Ianora,[b] Guido Cimino[a] and Angelo Fontana[a,]*

[a]*Istituto per la Chimica Biomolecolare del CNR, Via Campi Flegrei 34, 80078 Pozzuoli, Napoli, Italy*
[b]*Stazione Zoologica 'A. Dohrn', Villa Comunale, 80121 Napoli, Italy*

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Abstract—The paper describes the identification of short-chain aldehydes (**4**–**7**) from the marine diatom *Skeletonema costatum*, and their effects on copepod egg viability and sea urchin cell division. Compounds **4**–**7** were isolated as carboethoxyethylidene (CET) derivatives and their characterization was performed by NMR and GCMS analyses. Evidence is presented to support the defensive role and biosynthetic origin of these compounds in *S. costatum*. © 2002 Published by Elsevier Science Ltd.

Diatoms are small microscopic algae that are abundant in most aquatic habitats. Traditionally, they have been considered at the base of the marine food chain and as providing the bulk of the food sustaining top consumers and fisheries. There is accumulating evidence, however, that has challenged this view based on laboratory results, demonstrating that diatoms reduce egg viability on average by 80% in predatory copepods, the principal component of the zooplankton that are known to prey heavily on diatom cells.[1] This biological model is new and has no equivalent in marine plant–herbivore systems since most prey–predator chemical defense interactions are based on repellency or poisoning, but never on reproductive failure.[2] The compounds responsible for abortive and teratogenic effects in copepods were identified in the diatom *Thalassiosira rotula* as a mixture of polyunsaturated C_{10} aldehydes (**1**–**3**). Similar activity was also described for two other marine microalgae, *Skeletonema costatum* and *Pseudo-nitzschia delicatissima*, that were responsible for major diatom bloom events which caused a crash in egg hatching viability in situ copepod populations.[3] We compare here the biological activity of the diatoms *T. rotula* and *S. costatum* and present evidence that *S. costatum* produces aldehydes other than the ones already described, which are responsible for the dramatic effects

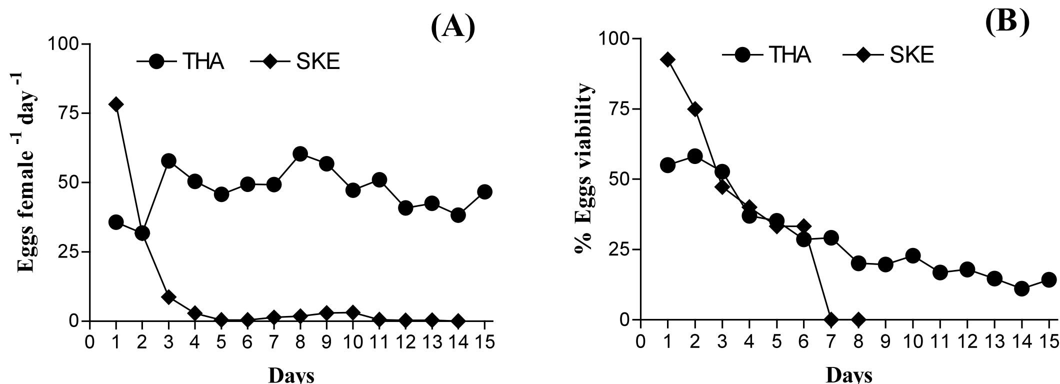

Figure 1. Laboratory experiments testing the effects of two diatom diets THA (*T. rotula*) and SKE (*S. costatum*) on egg production rates (A) and hatching viability (B) in the copepod *T. stylifera*.

* Corresponding author. Tel.: +39-081-8675096; fax: +39-081-8041770; e-mail: afontana@icmib.na.cnr.it

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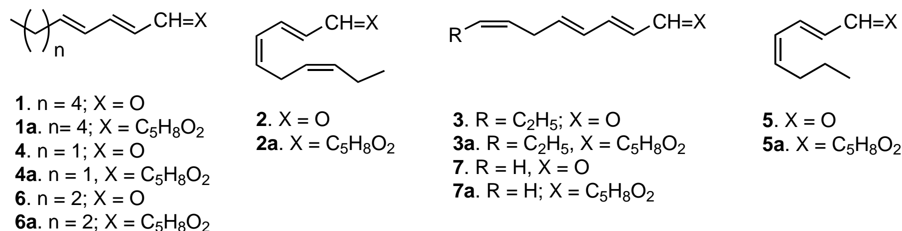


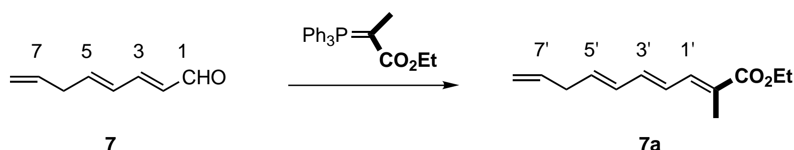
of this species on the reproductive success of the copepod *Temora stylifera*. Detection of aldehydes is based on their conversion into carboxyethylethylidene (CET) derivatives in agreement with a novel methodology recently developed in our laboratories.[4]

Egg production rates (Fig. 1A) and hatching viability (Fig. 1B) in the copepod *T. stylifera* were strongly affected by diet. With *S. costatum*, fecundity diminished to 3.7% of initial values within only 4 days and to 0% hatching success within 7 days. This effect was less intense with a diet of *T. rotula* that did not modify egg production rates and diminished hatching success to 10% within 15 days.

To identify the molecules responsible for these biological effects, we prepared an extract from an axenic culture (about 7.5 g wet weight) of *S. costatum*, as previously reported by Miralto et al.[3] The organic extracts were fractionated on SiO_2 column using sea urchin embryos as a bioassay to screen for active products.[5] The active fraction was a mixture of polyunsaturated aldehydes that still showed most of the initial activity of the extract. Treatment of this fraction with ethy-2(triphenylphosphoranylidene) propionate (Scheme 1)[4] quantitatively gave the corresponding mixture of CET derivatives (**4a**–**7a**) that was resolved by GCMS in *trans*,*trans*-2,4-heptadienal (**4**), *trans*,*cis*-2,4-octadienal (**5**), *trans*,*trans*-2,4-octadienal (**6**), and 2-*trans*-4-*trans*-2,4,7-octatrienal (**7**), **6** being the main component.[6] The mixture of CET derivatives also contained a variety of saturated and monounsaturated compounds, including tridecanal, 8-pentadecenal and pentadecanal. Except for octatrienal (**7**), all of these compounds were already known as products of lipid oxidation. Their structures were readily determined by comparison with commercial and synthetic standards. On the other hand, the structure of octatrienal (**7**) was unambiguously identified by NMR spectroscopy after partial purification. In fact, reversed phase HPLC of the CET derivatives gave a mixture of derivatized heptadienal (**4**) and octatrienal (**7**), the NMR signals of which were clearly discernible by 2D spectroscopy.[7] In particular, ^1H–^1H COSY spectrum revealed a bis-allylic methylene group (δ 2.89) coupled to the vinyl protons at δ 5.92 and 5.74; this latter signal was in turn correlated to the methylene protons at δ 5.07 (H-8a, bd, $J=17.1$ Hz) and 4.99 (H-8a, bt, $J=10.1$ Hz). The remaining part of the molecule, as well as the configuration of the double bonds in 2 and 4, were assigned in analogy with a synthetic sample of the CET derivative of *trans*,*trans*-2,4-octadienal (**6a**). GCMS and NMR data also supported the presence of trace components that were tentatively identified as the CET derivatives of 2-*trans*-4-*cis*-2,4,7-octatrienal and 2-*trans*-4-*cis*-heptadienal. However, the very low level of these products did not allow characterization to be certain.

Under the experimental conditions described above, both GCMS and ^1H NMR analyses revealed that *trans*,*trans*-octadienal (**6**) was present at significantly higher levels than the *trans*,*cis* isomer (**2**). On the other hand, it is well known that *cis*/*trans* isomerization of similar substrates easily occurs under a wide range of conditions,[8] making it thus possible that the *trans*,*trans* isomer was generated from the transient *cis*,*trans* intermediate during extraction or silica gel purification.

In conclusion, a number of linear aldehydes are detectable in diatom cells under the present analytical conditions. In particular, two series of compounds are clearly discernible: aldehydes featured by a conjugated polyunsaturated chain and aldehydes with a saturated or monounsaturated alkyl tail. Both compounds may derive by fatty acid oxidation, although two distinct mechanisms seem to be involved. Pentadecanal, 8-pentadecenal and tridecanal may be derived by α-oxidation of palmitic acid (C16:0), palmitoleic acid (C16:1) and myristic acid (C14:0). This is inferred also on the basis that the aldehydes occur in the same ratio as the corresponding acids. Conversely, it has been recently reported that diatom polyunsaturated aldehydes derive from lipoxygenase–hydroperoxide lyase oxidation of C_{20} fatty acids.[9] In agreement with this view, **4**–**7** are very likely formed by enzymatic oxidation of polyunsaturated substrates, although the lipid profile of *S. costatum* showed a predominance of eicosapentenoic acid (20:5 ω-3), whereas neither ω-1 or ω-4 C_{20} fatty



Scheme 1. Formation of the CET derivative of 2-*trans*-4-*trans*-2,4,6-octatrienal (**7a**).

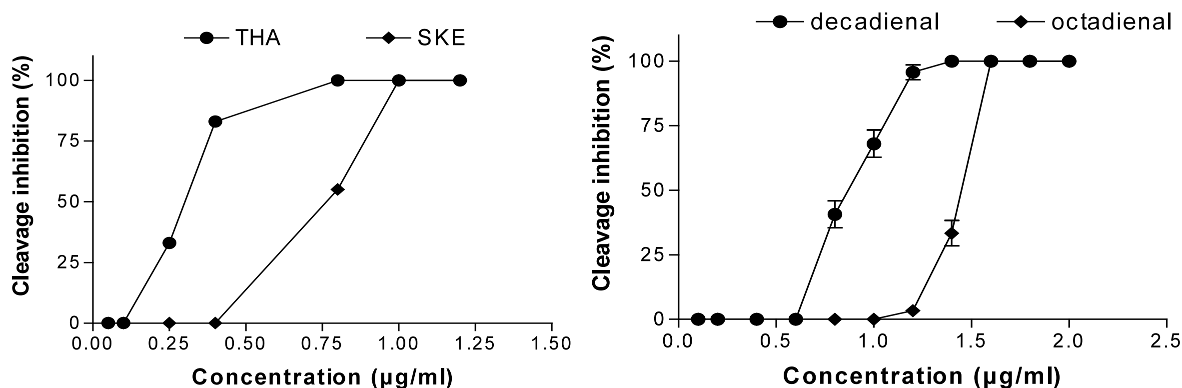


Figure 2. Effect of the aldehyde mixture obtained from *S. costatum* (SKE) and *T. rotula* (THA) on the first division of sea urchin eggs. Both aldehyde mixtures inhibited cell division in a dose-dependent manner.

acids were detectable. These findings question the direct formation of **5**–**7** from eicosanoids and suggest that other pathways may also be involved in the origin of these toxic aldehydes. To this regard, as already reported by other authors,[10] *S. costatum* lipids contained high levels of C_{16} compounds (almost 65% of the whole fatty acid content) that, on the basis of structural analogies, may account for the formation of **5**–**7**. In particular, we found significant amounts of 6,9,12-hexadecatrienoic (16:3ω-4, almost 26% of C_{16} content) and 6,9,12,15-hexadecatetraenoic acids (16:4ω-1, almost 12% of C_{16} content), which may be related to the production of octadienals (**5** and **6**) and octatrienal (**7**), respectively. It is worth noting that no other fatty acid with a terminal double bond was detected in the *S. costatum* extract, and that the structure of 6,9,12,15-hexadecatetraenoic acid was rigorously determined by NMR after purification of the product on reversed phase HPLC.[11]

The pure and raw aldehydes purified from *S. costatum* blocked division in sea urchin embryos in much the same way as the corresponding unsaturated decanals (**1**–**3**) isolated from *T. rotula* (Fig. 2). This strongly supports the involvement of **4**–**7** in the chemical defense of *S. costatum* at Sea (manuscript in preparation). The results of the present work have important ecological implications on diatom–copepod interactions since *S. costatum* is a major diatom blooming species in many areas of the world's oceans. Field studies are in progress to assay aldehyde production during various phases of diatom blooms involving *S. costatum* to better understand the impact of these aldehydes in the population dynamics of predatory copepods.

Acknowledgements

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5. For egg development tests, specimens of the sea urchin *Paracentrotus lividus* were collected in the bay of Naples. Gamete ejection was induced by injecting 2 ml of 0.5 M KCl solution into the perivisceral cavity. Eggs spawned were allowed to settle and washed three times with 0.22 µm filtered sea water (FSW), then diluted to a final concentration of 1000 eggs/ml. Concentrated sperm were collected with a Pasteur pipette and diluted immediately prior to fertilization (20 of µl sperm in 5 ml FSW). An aliquot of 20 µl of sperm suspension was added to the egg suspension (50 ml). Five minutes after fertilization, 200 µl of egg suspension was added to each of 12-well plates containing increasing concentrations of the sample to be tested, ranging from 0.1 to 2.5 µg/ml. Samples were diluted in methanol and <10 µl of methanol/ml of FSW was added to assays, a concentration that had no effect on developing embryos. A control sample was incubated in the same conditions in FSW and another control was performed in the presence of 10 µl/ml of methanol. Eggs were incubated in a final volume of 2 ml at constant room temperature (20°C). After 90 min, 100 eggs or embryos were counted for each concentration, to obtain the percentage of cleavage inhibition. Interestingly, *S. costatum* had somewhat weaker effects on cell division (total blockage at 1.0 µg/ml) than *T. rotula* (total blockage at 0.7 µg/ml). The same was also true for pure decadienal (**1**) and octadienal (**6**).

6. A cell pellet (6 g wet weight) was sonicated in distilled water (6 ml) for 1 min and left on the bench for 30 min. Acetone (6 ml) was then added and the resulting suspension was centrifuged three times at 4000 rpm for 10 min. The supernatant was transferred to a separatory funnel and then extracted with CH_2Cl_2 three times (about 15 ml of solvent). The organic layers were combined, dried over dry Na_2SO_4 and then evaporated at reduced pressure. The resulting oil was then fractionated on silica gel column by a polarity gradient (Et_2O in light petroleum), following the elution by TLC. The combined fractions were tested for the effect on developing embryos of sea urchins and the active fractions (on the whole, 8.6 mg, petroleum ether/Et_2O 90:10 v/v) were selected. The active fractions were combined, dissolved in CH_2Cl_2, and, under stirring, the solution was treated with 1.5 equiv. of ethyl-2-(triphenylphosphoranylidene) propionate. The reaction mixture was stirred at rt for 18 h and then purified by silica gel column (petroleum ether/Et_2O 95:5 v/v) to give the mixture of CET derivatives (**4a**–**7a**) that was directly analyzed by a GCMS (HP 5890 Series II Plus GC, HP 5989B MS) equipped with a HP 5MS column. Samples were dissolved in *n*-hexane or CH_2Cl_2 and analyzed by a temperature gradient from 130 to 220°C at 3°C/min (injector temperature 240°C, detector temperature 260°C, nitrogen flow 1 ml/min). Electron voltage was set at 70 eV.
7. NMR spectra (^1H, COSY, TOCSY and HSQC) were run on Bruker Avance 400 MHz equipped with a 2.5 mm microprobe. Spectra were referenced to $CHCl_3$ (δ 7.26). CET derivative of octatrienal (**7a**) δ_{1H} ($CDCl_3$) 7.20 (d, $J=11.6$ Hz, H-1′), 6.41 (dd, $J=13.7$ and 11.2 Hz, H-3′), 6.49 (m, H-4′), 6.19 (dd, $J=13.7$ and 11.6 Hz, H-2′), 5.92 (m, H-5′), 2.89 (bt, H-6′), 5.74 (m, H-7′), 5.07 (d, $J=17.1$ Hz, H-8a′) and 4.99 (d, $J=10.1$ Hz, H-8a″).
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11. 48 mg of *S. costatum* were extracted in agreement to the Modified Folch Method. The organic material was then fractionated on silica gel column to give with light petroleum/Et_2O 85:25 (v/v) a clean fraction of free fatty acids. Reversed phase HPLC [column KR 100-5 C18; flow 1 ml/min; isocratic elution with $MeOH/H_2O/TFA$ 85:15:03 (v/v/v)] of this fraction gave pure 6,9,12,15-hexadecatetraenoic acid (2.1 mg; CIMS *m*/*z* 249 [*M*+H+]) together with the other fatty acids.