# Preuplotin, a Putative Biogenetic Precursor of the Euplotins, Bioactive Sesquiterpenoids of the Marine Ciliated Protist *Euplotes crassus*

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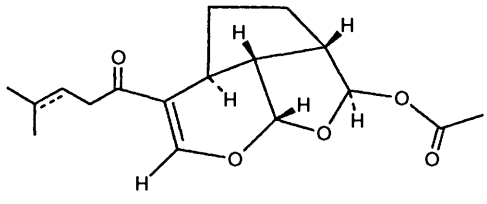
Strains of the marine ciliated protist *Euplotes crassus* typically contain preuplotin {(all-*E*)-2-[6-acetoxy-4-(acetoxymethylene)hex-5-enylidene]-6-methylhept-5-enal} **4**. This represents the C-12 deprenyl analogue of udoteal, the feeding-deterrent of tropical seaweeds of the genus *Udotea*. *Euplotes crassus* typically contains also the *O*-cyclized form euplotin C **3** which may be thought to descend biogenetically from preuplotin together with euplotins A and B previously isolated from highly-productive strains of this ciliate as likely adjuvant factors of its adaptive radiation.

We have reported in preliminary form on unique sesquiterpenes carrying aldehyde groups in protected, acetalic or vinyl ether forms, euplotin A **1** and euplotin B **2**.† These compounds can be viewed as being derived from a hypothetical oxidized farnesol unit *via* an unusual *trans*-2,6 cyclization.[1] They showed powerful biological activity, delaying the fission rate of all strains of the marine ciliates tested, except the typical, highly-productive, strains of *Euplotes crassus*, at dosages as low as 5–0.5 μg $cm^{-3}$; higher dosages produced 100% mortality.[1] Interspecific competition experiments suggested an ecological role for euplotin A and euplotin B, mediated by cell-to-cell contacts, as a mechanism supporting the adaptive radiation of *E. crassus*.[1]

The euplotins add to the scanty list of secondary metabolites found in ciliated protists, all isolated from freshwater species: (1) the photoactive phenanthroperylenequinone of *Stentor coeruleus*[2a] and *Blepharisma*,[2b] (2) the tryptophan-derived blepharismin, a mating pheromone of *Blepharisma intermedium*[2c] and (3) polycyclic triterpenes of *Tetrahymena*.[2d] In view both of this and the recognized importance of ciliated protists in the marine food-web,[3] we have looked for valuable clues about the euplotins' biogenetic pathway. This has resulted in the discovery of a putative biogenetic precursor and a new euplotin which are described here.



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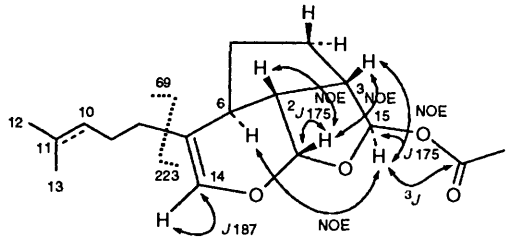

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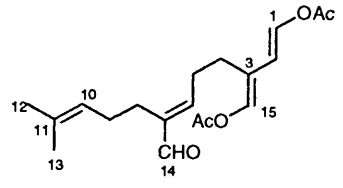


**4**

## Results and Discussion

*Structural Elucidation of Euplotin C* **3**.—Only a scanty discussion was offered about euplotins A and B in our preliminary presentation,[1] whilst their stereochemical assignment was by no means straightforward. Details are therefore given here about the structurally correlated euplotin C which, being more abundant, permitted a deeper insight, fully confirming previous structural assignment of its congeners.[1] Additional spectral data about euplotin A and euplotin B, and details about the biological assays of these substances, are also reported in the Experimental section. $^{13}C$ NMR (Table 1) and mass spectral data (Experimental section) indicated the composition $C_{17}H_{24}O_4$ for euplotin C. The IR spectrum revealed the presence of an acetate group (1735 $cm^{-1}$) whereas high resolution mass measurements on fragments *m/z* 232 and 163 suggested the loss of AcOH from the molecular ion to give $C_{15}H_{20}O_2$, followed by the loss of $C_5H_9^{\bullet}$ to give $C_{10}H_{11}O_2$. Alternatively, or in parallel, loss of $C_5H_9^{\bullet}$ from the molecular ion was observed to give $C_{12}H_{15}O_4$, followed by the loss of ketene to give $C_{10}H_{13}O_3$ and $H_2O$, converging to $C_{10}H_{11}O_2$ which represents the base peak. This showed that euplotin C has six unsaturations which are accounted for by an acetate C=O, two trisubstituted C=C (from a comparative $^1H$ and $^{13}C$ NMR analysis) and three cycles.

The signals at $\delta_C$ 103.57 and 104.82 (d), showing $^1J_{CH}$ larger by *ca.* 30 Hz than ordinary couplings, could be attributed to acetalic carbons, whereas similar information from $\delta_C$ 138.79 (*d*) suggested a vinyl ether carbon. MS data (Experimental section), coupled with the characteristic $\delta_C$ 131.66 (s), 124.41 (d), 25.76 (q) and 17.74 (q), pointed to a $C_6$ isoprenic side chain.

† No absolute configuration meaning is attached to any of the structural formulae in this work. IUPAC nomenclature and numbering is only used for retrieval purposes (see structure **11** and Experimental section). Spectroscopic data is assigned using the numbering system shown in structure **3**.

**Table 1** $^1H$ and $^{13}C$ NMR data for euplotin C **3** and preuplotin **4**[a]

| | Compound | | | |
|---|---|---|---|---|
| | **3** | | **4** | |
| Atom position | $^{13}C(CDCl_3)$ | $^1H(C_6D_6)$ | $^1H(CDCl_3)$ | $^1H(C_6D_6)$ |
| 1 | 103.57 (d), 175 | 5.82 (d, *J* 3.6) | 7.43 (dd, *J* 12.6, 0.8) | 7.60 (dd, *J* 12.7, 0.7) |
| 2 | 51.10 (d) | 1.45 (ddd, *J* 11.6, 7.1, 3.6) | 5.95 (dd, *J* 12.6, 0.8) | 5.79 (dd, *J* 12.7, 0.6) |
| 3 | 46.76 (d) | 2.29 (dddd, *J* 9.1, 7.1, 3.6, 2.7) | — | — |
| | | β: 1.84 (ddd, *J* 14.0, 9.1, 8.4) | 2.50 (m) | 2.50–1.95 (m) |
| 4 | 30.93 (t) | | | |
| | | α: 1.49 (dddd, *J* 14.0, 10.1, 6.9, 3.7) | 2.50 (m) | 2.50–1.95 (m) |
| | | β: 0.99 (dddd, *J* 13.4, 12.0, 10.1, 8.4) | 2.50 (m) | 2.50–1.95 (m) |
| 5 | 27.38 (t) | | | |
| | | α: 1.59 (ddd, *J* 12.0, 6.9, 4.9) | 2.50 (m) | 2.50–1.95 (m) |
| 6 | 39.17 (t) | 1.88 (dddd, *J* 13.4, 11.6, 4.9, 2.5) | 6.45 (t, *J* 7.2) | 5.88 (t, *J* 6.9) |
| 7 | 120.11 (s) | — | — | — |
| 8 | 29.57 (d) | 1.95 (dt, *J* 7.5, 1.2) | 2.26 (t, *J* 7.0) | 2.50–1.95 (m) |
| 9 | 27.98 (t) | 2.03 (m) | 2.04 (br q, *J* 7.0) | 2.50–1.95 (m) |
| 10 | 124.41 (d) | 5.17 (tsept, *J* 7.1, 1.5) | 5.07 (tsept, *J* 6.9, 1.3) | 5.13 (tsept, *J* 6.9, 1.3) |
| 11 | 131.66 (s) | — | — | — |
| 12 | 25.76 (q) | 1.66 (d, *J* 1.2) | 1.65 (d, *J* 1.3) | 1.62 (d, *J* 1.3) |
| 13 | 17.74 (q) | 1.54 (br s) | 1.56 (br s) | 1.53 (br s) |
| 14 | 138.79 (d), 187 | 6.18 (td, *J* 2.7, 1.2) | 9.34 (s) | 9.29 (s) |
| 15 | 104.82 (d), 175 | 6.15 (d, *J* 2.6) | 7.25 (br s) | 7.10 (br s) |
| 15-$CH_3CO$ | 169.31 (s) | — | — | — |
| 15-$CH_3CO$ | 20.77 (q) | 1.63 (s) | 2.16 (s) | 1.65 (s)[b] |
| 1-$CH_3CO$ | — | — | 2.16 (s) | 1.57 (s)[b] |

[a] *J* values are given in Hz. [b] These two signals can be interchanged.

**Table 2** $^1H$ NMR coupling constants for euplotin C **3**, compared with calculated values for the 2,6-*trans* and the hypothetical 2,6-*cis* configuration, and γ-lactone **11**[a]

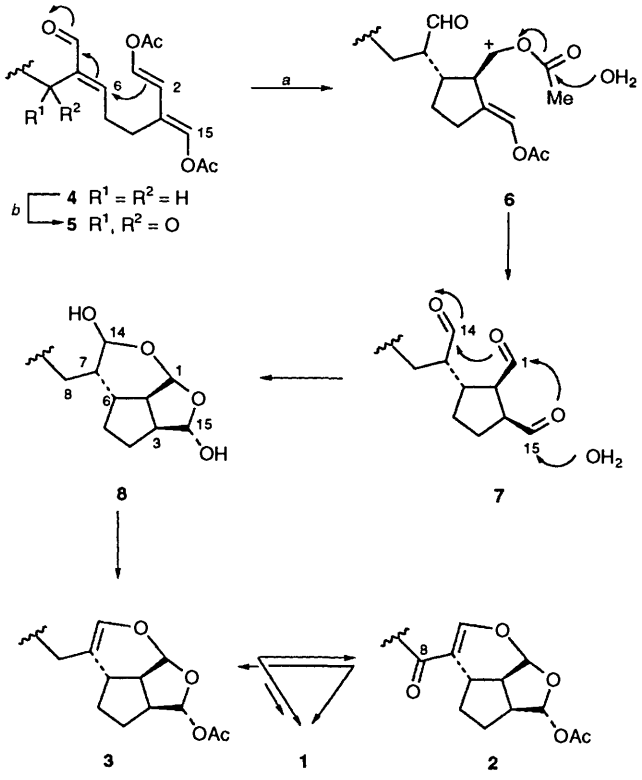
| | **3** | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2,6-*trans* | | 2,6-*cis* | | **11** | |
| Coupling between | Obs. *J* | Calc. *J*[b] | Corresp. torsional angle[c] (°) | Calc. *J*[b] | Corresp. torsional angle[c] (°) | Obs. *J*[d] | Calc. *J*[b] |
| 1, 2 | 3.6 | 4.2 | 43 | 5.0 | 36 | 5.0 | 4.9 |
| 2, 3 | 7.1 | 8.5 | 30 | 11.2 | 10 | 8.0 | 9.1 |
| 2, 6 | 11.6 | 12.7 | 176 | 9.4 | 22 | 10.0 | 10.9 |
| 3, 15 | 2.7 | 4.2 | 127 | 1.9 | 108 | — | — |
| 3, 4β | 9.1 | 10.4 | 0 | 6.6 | 37 | 8.0 | 5.1 |
| 3, 4α | 3.7 | 4.1 | 119 | 1.0 | 82 | < 1.0 | 1.0 |
| 4β, 5β | 8.4 | 8.2 | 33 | 5.6 | 46 | 8.0 | 6.0 |
| 4β, 5α | 0.8 | 0.3 | 88 | 12.8 | 167 | 13.0 | 12.5 |
| 4α, 5α | 6.9 | 8.9 | 29 | 5.6 | 46 | 6.0 | 6.4 |
| 4α, 5β | 10.1 | 10.7 | 152 | 1.0 | 73 | < 1.0 | 0.7 |
| 5β, 6 | 13.4 | 12.3 | 173 | 6.2 | 41 | 7.0 | 7.8 |
| 5α, 6 | 4.9 | 4.6 | 50 | 11.5 | 163 | 11.0 | 10.2 |
| 6, 7 | — | — | — | — | — | 9.0 | 11.5 |
| 7, 8 | — | — | — | — | — | 1.0 | 1.6 |

[a] *J* values are given in Hz. [b] Calculated through Haasnoot *et al.*'s[7] modified Karplus equation. [c] From molecular-mechanics[6] calculated minimum-energy conformers. [d] From ref. 10(*a*).

These fragments could be combined together on the basis of COSY experiments on euplotin C which furnished a complete network of $^1H$–$^1H$ and $^1H$–$^{13}C$ correlations, both one-bond and long-range (HMQC and HMBC by inverse-detection[4]). In particular, this allowed us to locate the acetyl group, and to correlate C-1 with 14-H and 15-H, C-2 with 1-H, 3-H and 6-H, C-7 with 14-H and 8-$H_2$, C-10 with 12-Me and 13-Me and C-15 with 1-H, 3-H and 4-$H_2$, thus establishing connectivities as in structure **3**.

Detailed analysis of coupling patterns, extracted from difference spin decoupling,[5] *J* resolved and one- and two-dimensional NOE experiments, allowed us to assign the relative configurations in euplotin C. Thus, the observation that $^3J$ is small (3.6 Hz) between 2-H and 1-H and large (11.6 Hz) between 2-H and 6-H indicated that 2-H is *cis*-related to 1-H and *trans*-related to 6-H. Assuming the α position for 6-H, the NMR analysis allowed us to assign both δ and *J* for the adjacent protons; in particular, it was shown that 3-H is coupled with 2-H (7.1 Hz), 4-$H_\alpha$ (3.7 Hz), 4-$H_\beta$ (9.1 Hz) and 15-H (2.6 Hz). These assignments were unequivocally confirmed by strong NOE of 3-H with both 2-H and 4-$H_\beta$ and of 15-H with both 6-H and 4-$H_\alpha$, whereas no NOE enhancement between 6-H and 2-H could be observed.

Torsional angles for euplotin C, as a system of two fused five-membered rings, could not be derived directly from the *J* values reported in Table 1. Recourse was made to molecular mechanics



**Scheme 1** Biogenetic hypothesis correlating the structure of euplotin A **1** and euplotin C **3** with preuplotin **4**

(MM) calculations[6] searching for the lowest-energy conformer for both the 2,6-*trans* **3** and the hypothetical 2,6-*cis* configuration which best fit *J* values calculated by a modified Karplus equation.[7] It is seen from Table 2 that a good fitting was obtained for the strained 2,6-*trans* arrangement of **3**, whilst for the 2,6-*cis* arrangement experimental *J* values disagreed sharply from calculated ones.

This analysis has shown that euplotin C **3** is structurally closely related to both euplotin A **1** and euplotin B **2**[1] although, lacking the enone chromophore of the latter, **3** could be less easily detected by UV-monitored HPLC.

*Structural Elucidation of Preuplotin* **4**.—The composition $C_{19}H_{26}O_5$ for preuplotin, an optically inactive material, was established by high resolution mass spectral measurements on several fragment ions (Experimental section) in agreement with $^1$H NMR spectral analysis (also for literature compounds);[8] this compensated for the unavailability of $^{13}$C NMR spectra due to shortage of material. The $^1$H NMR spectra revealed three units: (a) the 1,4-diacetoxybuta-1,3-diene moiety, characterized, in $CDCl_3$, by vinyl signals at $\delta_H$ 7.43 (dd), 7.25 (br s, overlapped by the solvent signal) and 5.95 (dd), and coincident ($\delta_H$ 2.16) acetates [which appeared as separate signals ($\delta_H$ 1.65 and 1.57) in $C_6D_6$, Table 1], (b) the α,β-unsaturated aldehyde functionality and (c) the terminal *gem*-dimethylalkene [$\delta_H$ 1.65 (br d), 1.56 (br s)]. These three units could be combined together as indicated in formula **4** on the basis of $^1$H–$^1$H COSY maps. Perfect matching of the $^1$H NMR data with all pertinent values for udoteal **10**[8] established the 6,7-*E* configuration for preuplotin too.

Molecular mechanics calculations[6] indicated that the s-*trans* conformation (as in formula **4**) of the conjugated diene moiety is more stable than the s-*cis* conformation by 1 kcal mol$^{-1}$,* corresponding to a 85 : 15 s-*trans* : s-*cis* ratio of conformer populations. On the other hand, facile Diels–Alder cycloaddition of the diene system with dihydro-1,2,4-triazole-3,5-dione[8a] suggests that the s-*trans*–s-*cis* conformational barrier must be low; this barrier was in fact calculated by MM through the dihedral angle driver[6] to be only 3 kcal mol$^{-1}$ over the s-*cis* ground state.

*Biogenetic Hypothesis*.—A biogenetic hypothesis correlating the structures of the euplotins with that of preuplotin **4** is sketched in Scheme 1 along two alternative pathways, *a* and *b*. Along path *a*, following 2,6-cyclization of preuplotin **4**, the acetate groups undergo deprotection by water attack, as indicated for the C-1 centre in the hypothetical intermediate **6**. This triggers a cascade of events starting from water attack on **7** at the aldehyde C-15 group, which in turn attacks the aldehyde C-1 group, followed by attack of the latter at C-14, thus establishing the tricyclic moiety **8** that on dehydration finally affords euplotin C **3**. This may give euplotin A **1** by reduction at C-10=C-11 and oxidation at C-8 and euplotin B **2** by oxidation at C-8. Along path *b* preuplotin **4** is envisaged to be oxidized to give the hypothetical 8-oxopreuplotin **5** which, following 2,6-cyclization and a series of deprotection–acetalization steps similar to those in pathway *a*, gives euplotin B **2**, from which euplotin A **1** and euplotin C **3** may be formed by reduction at, respectively, C-10=C-11 or C-8=O.

Under the reasonable hypothesis that ciliated protists did not evolve enzymes all at the same time but along evolutionary steps, preuplotin **4** may represent the most primitive of the sesquiterpenoids so far known for *E. crassus*. From this follows that euplotin C **3** represents the next more evolved of the sesquiterpenoids if pathway *a* is followed. In contrast, if pathway *b* is followed, euplotin C may represent the final product of the evolutionary process dealing with the sesquiterpenoids of ciliated protists, as presently known.

*Biological Activity*.—The susceptibilities of ciliated protists to preuplotin, in terms of the lowest concentration for 100% kills ($LD_{100}$) or lowest dose eliciting a fission rate delay in 100% of tested cells ($ED_{100}$), are listed in Table 3. Limited experimentation with preuplotin **4** was due to shortage of material (see Experimental section). It is apparent that there is an inter- and intra-specific variability, as already noted in our preliminary publication for euplotins A and B[1] whose cytotoxicity data are also listed in Table 3 for comparison. In general, the specific cytotoxicity of preuplotin was lower than for both euplotin A and euplotin B; combined with its low abundance in *E. crassus* cells, this makes the cytotoxic role of preuplotin in nature far less important in general than for euplotin A and euplotin B.

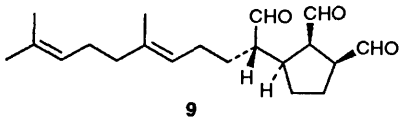
In contrast to the marked cytotoxicity exhibited by euplotin A and euplotin B, euplotin C, using the same methodology—that is, conveying the terpenoids into seawater with a small amount of ethanol, 0.05–2%, so as to be tolerated by ciliated protists[1]—proved inefficacious. This does not necessarily mean lack of cytotoxicity by euplotin C in nature, but simply failure of our assay methodology for a compound which is not appreciably soluble in seawater containing a small amount of ethanol. In fact, during the above assays euplotin C could be observed at the dissecting microscope to separate out from the medium in droplets which could not be brought into contact with the ciliated cells. Actually, if euplotins A and B act in cell-to-cell contacts,[1] then the lipophilic euplotin C might be expected to be an even more powerful cytotoxic agent than its congeners. In line with this, ongoing extensive cytotoxicity assays to be reported elsewhere further clarify previous work[1] by indicating that the presence of euplotin C, combined with overall high productivity, confer resistance. The sensitive strain

* 1 cal = 4.184 J.

**Table 3** Biological assays of euplotin A **1**, euplotin B **2** and preuplotin **4** with marine ciliates

| Ciliates | | | $LD_{100}$,[a] $ED_{100}$[b] | | |
|---|---|---|---|---|---|
| Strain | Taxon | Origin | **1**[c] | **2**[c] | **4** |
| EC1 | *E. crassus* | Porto Vecchio, Corsica, August 1986 | > 20,[d] 20 | > 20,[d] 20 | 20,[e] 10[e] |
| SL2[f] | *E. crassus* | Bentota, Sri Lanka, August 1990 | 1, 0.5 | 5, 3 | 10,[e] 5[e] |
| SR1 | *E. minuta* | San Rossore, Pisa, Italy, March 1991 | 10, 5 | 20, 5 | 15, 5 |
| TB6 | *E. vannus* | Tanabe, Japan, July 1983 | 3, 2 | 5, 3 | 10,[e] 5[e] |
| PR5 | *D. oligothrix* | Porto Recanati, Italy, June 1979 | 3, 2 | 5, 1 | 5, 1 |

[a] Lowest concentration for 100% kills. [b] Lowest dose eliciting a fission rate delay in 100% of tested cells. [c] Data from Ref. 1. [d] Less than 100% kills observed at the highest attainable concentration (*ca.* 20 μg $cm^{-3}$) of the terpenoid in the medium used for bioassays. [e] Data from limited number of cells (see Experimental section). [f] The lowest productive strain of *E. crassus*.

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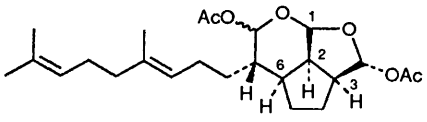


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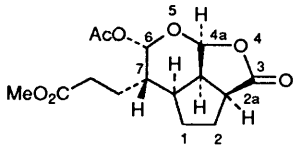
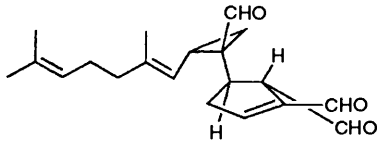



**11**

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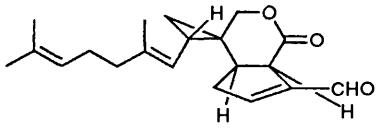


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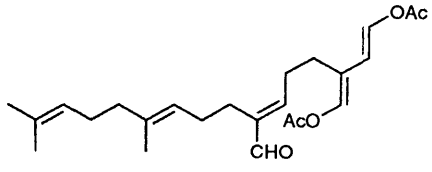


**14**

SL2 (ref. 1 and Table 3) failed to show detectable amounts of euplotin C.

The above results suggest two sets of experiments to distinguish between routes *a* and *b* in the biogenetic hypothesis indicated in Scheme 1. One relies on the distribution of preuplotin and the euplotins in ciliated protists, searching for the commonest and the rarest of such compounds, corresponding to the first and the last to appear, respectively, in the life history of ciliates. The other set of experiments relies on the relative level of cytotoxicity of these terpenoids when examined for a wide variety of species and strains of ciliates; the expectation is that the most recently evolved natural products are the most active on extant species. Both possibilities are being examined in our laboratories; we are also looking for alternative methodologies for cytotoxicity screening in order to evaluate euplotin C. Circumstantial evidence suggests that preuplotin, as the first evolved of the sesquiterpenoids of *Euplotes* so far known, no longer represents an efficient weapon for *E. crassus*, being substituted in this purpose by the euplotins; preuplotin persists in the cell merely as a biogenetic intermediate.

*Comparison with other Natural Cyclic Acetals.*—Compounds of various complexity having cycloacetal centres occur widely in nature. They comprise insect pheromones, like the brevicomins,[9] and distasteful or toxic compounds contained in tropical green seaweeds in the order Caulerpales. These include trialdehyde **9** of *Udotea flabellum*, which exists in nature as the hydrated, cyclized form, whilst it was actually isolated as the epimeric diacetates **10**.[10]

The tricyclic moiety of **10** has the 2,6-*cis* configuration* that we have ruled out for the euplotins in favour of the 2,6-*trans* configuration. That the latter is difficult to achieve is shown by the fact that when the three aldehyde groups are not *cis* spatially related, intramolecular acetalization could not be obtained in the laboratory.[11] The same difficulty, with the exception of *E. crassus*, occurs in nature, as indicated by two lines of evidence. The first one concerns the existence in nature of non-hydrated trialdehyde forms, such as halimedatrial **12**, isolated from various species of tropical Caulerpales of the genus *Halimeda*.[11] The second line of evidence is that spatial proximity of two of the three aldehyde groups offers the opportunity for enzymes of the Caulerpales to perform intramolecular cyclization involving only the two proximate aldehyde groups, such as in halimedalactone **13**, isolated from *Halimeda scabra*.[11] Thus, the euplotins emerge as uniquely strained cyclic acetals, probably selected by evolution for their high reactivity in cell-to-cell contacts.[1]

Similarity of terpenoids of the Caulerpales to those of Ciliophora of the genus *Euplotes* is not limited to the tricyclic acetal arrangement of hydrated udoteatrial **10** and the euplotins **1**–**3**. Udoteal **14**, isolated from various species of *Udotea*,[8] has just the structure of C-12 prenylated preuplotin. This close similarity of structures is remarkable for taxonomically unrelated taxa as the Chlorophyta and Ciliophora. This formally recalls the similarity of phenanthroperylenequinone pigments

* γ-Lactone **11** (deriving from pyridinium chlorochromate oxidation of **10**, and for which, in contrast with the latter, no detailed NMR data have been reported[10a]) proved to conform nicely to the NMR description for the hypothetical 2,6-*cis* conformer of the euplotins, as deduced from molecular mechanics calculations (Table 2).[6] Thus, the **11** conformer with C-5 up and C-4 down with respect to the C-3–C-2–C-6 mean plane, deriving from pseudorotation of the cyclopentane ring, is favoured; in contrast, whilst this conformation is not allowed—or is very scarely populated—for the euplotins. In this conformation, the hypothetical 2,6-*cis* epimer of euplotin C was calculated to be more stable than the 2,6-*trans* epimer by *ca.* 8 kcal $mol^{-1}$.

in ciliates of the genera *Stentor* and *Blepharisma*[2] and a fossil[12] or a 'living fossil'[13] sea lily.

These results give a hint that preuplotin and the euplotins might have taxonomic value.

## Experimental

TLC was performed on Merck Kieselgel 60 $PF_{254}$ plates, flash-chromatography (FC) on Merck Si-60, 15–25 μm and reversed-phase flash chromatography on Merck LiChrosorb RP18 (20–50 μm). HPLC was carried out on Merck-LiChrosorb Si-60 (7 μm), CN-HPLC on Merck LiChrosorb CN (7 μm) and reversed-phase HPLC on Merck LiChrosorb RP18 (7 μm); in all cases 25 × 1 cm columns and solvent flux of 5 $cm^3$ $min^{-1}$ were used. The limits of HPLC detection under normal, one scan, conditions of examination, proved to be 10 ng/injection for euplotin A, euplotin B, 5 ng/injection for preuplotin, and 30 ng/injection for euplotin C. Provided repeated HPLC scans are carried out and ground noise is accounted for, the limits may be appreciably lower. UV spectra ($\lambda_{max}$/nm, $\varepsilon$/$mol^{-1}$ $dm^3$ $cm^{-1}$) were measured on a Perkin-Elmer Lambda-3 spectrophotometer. Polarimetric data were taken with JASCO-DIP-181 polarimeter. CD spectra were recorded with a Jasco J-40AS dichrograph ($\lambda_{max}$/nm, $\varepsilon$/$mol^{-1}$ $dm^3$ $cm^{-1}$). $[\alpha]_D$ Values are given in units of $10^{-1}$ deg $cm^2$ $g^{-1}$. NMR [$\delta$ values in ppm relative to internal $Me_4Si$ (=0 ppm) and $J$ values in Hz] were taken with a Varian XL-300 spectrometer ($^1H$ at 299.94 MHz, $^{13}C$ at 75.43 MHz); multiplicities from DEPT experiments[14] Inverse-detection experiments[4] were carried out with a dedicated probe.[15] Selective, differential NOE (obtained with 5 s preirradiation): irradiated proton ⟶ % NOE on the observed proton(s). Mass spectra (EI) were taken with a Kratos MS80 mass spectrometer with home-built computerized data system.

*Culture of Ciliated Strains and Work up Procedure.*—*Euplotes crassus*, strain SSt22, collected at Sciacca-Stazzone, Italy, in December 1986, was grown in culture with *Dunaliella salina* Teodoresco (Chlorophyceae, Dunaliellales) as food organism. About 9 × $10^7$ cells were obtained by centrifugation (pellet) of mass cultures. The culture fluid was discarded since it did not contain any euplotin and the cell pellet was re-suspended in a small volume of absolute EtOH. (This led to thorough extraction of the euplotins, since breaking of cell walls by sonication failed to afford more of the euplotins.) The solution of euplotins in ethanol was separated by filtration and the filtrate was evaporated and the residue was extracted with light petroleum. On evaporation, a residue (0.2 g) was left, which was subjected to FC with hexane–$Et_2O$ gradient elution, followed by AcOEt–MeOH gradient elution to give 34 fractions of *ca.* 30 $cm^3$ each. Fractions 8–15 were subjected to CN-HPLC with hexane–isopropyl alcohol 98:2, monitoring by UV at $\lambda$ 220 nm, to yield euplotin C **3** ($t_R$ 10.3 min, 15 mg). Fractions 20–24 were similarly processed with hexane–isopropyl alcohol 95:5, monitoring at $\lambda$ 254 nm, to yield euplotin A **1** ($t_R$ 9.8 min, 2.5 mg) and euplotin B **2** ($t_R$ 11.5 min, 1 mg).

Cultures of *E. crassus D35*, collected in the Bay of Naples in 1961, were similarly carried out to give a 1.2 $cm^3$ pellet which was re-suspended in a small amount of absolute EtOH. This slurry was filtered, the cells were washed with AcOEt and the organic layers were combined and then evaporated. The residue was taken in 9:1 hexane–AcOEt (giving 0.050 g products after evaporation) leaving a EtOH-soluble residue (0.024 g) which did not contain either preuplotin or any of the euplotins. The above non-polar 0.050 g of products were subjected to FC with hexane–$Et_2O$ gradient elution, 21 fractions of 10 $cm^3$ each were collected. Fractions 6–9 proved to contain euplotin C, which was purified by HPLC (9.2 mg). Fractions 10–12 proved to contain preuplotin, which was purified by HPLC with 98:2 hexane–isopropyl alcohol, monitoring by UV at $\lambda$ 246 nm (0.30 mg). Fractions 13 and 14 proved to contain euplotins A and B in trace amounts.

*Biological Assays.*—The marine species of the unicellular ciliated protists, and the comprising strains selected for the cytotoxicity screen of the terpenoids, are reported in Table 3. All cells of each strain used for tests on a given day were members of a clone and were grown in excess food at the time they were placed in the test solution. For each strain and terpenoid tested a set of consecutive steps in concentration was used to cover the whole range of the terpenoids' toxic effect characterized by means of the lowest terpenoid concentration in μg $cm^{-3}$ for 100% kills (lethal dose, $LD_{100}$) and for eliciting a fission rate delay in 100% of test ciliates (effective dose, $ED_{100}$). These two parameters were chosen because it seemed likely that they would cover the range of the most useful measures for investigations in toxicology, biochemistry and mutagenesis. The cytotoxic effects of the terpenoids were assessed microscopically as the number of fission products of a single cell per time unit, and then as fission rate in fissions $day^{-1}$ ($ED_{100}$) or as cell's complete loss of motility ($LD_{100}$). Each strain was run at least two times on successive days. Except when otherwise indicated in Table 3, cytotoxic effects were assessed in six single cells for each terpenoid concentration at each run for each strain. In any case, effects were measured after 16 h exposure of single cells to solutions of the terpenoids in defined, artificial seawater, Allen's formula,[16] at 23 ± 1 °C. Since the terpenoids were conveyed into seawater in EtOH solution, resulting in 0.02–0.5% EtOH, controls were included for solvent-treated as well as untreated cells and they were run simultaneously with terpenoid-treated cells. New test solutions were prepared just before the series of strains were run through a toxicity bioassay. Because of scarcity of material, a more spaced series of consecutive steps in concentration was used to cover the whole range of preuplotin cytotoxic effects. Furthermore, for the same reason, for each preuplotin concentration we used less than the 18 single cells usually assayed.

*Euplotin A* **1** [(+)-(2a*S**,3*R**,4a*S**,7a*S**,7b*R**)-7-(4-*Methyl*-1-*oxopentyl*)-1,2,2a,4a,7a,7b-*hexahydro*-4,5-*dioxacyclopent*-[cd]*inden*-3-*yl Acetate*].— See Ref. 1. CD(MeOH) $\Delta\varepsilon_{max}$(247 nm) +12; *m/z* 308 (5%, $M^{\bullet+}$), 248 [55, $(M - AcOH)^{\bullet+}$], 237 [5, $(M - Me_2CHCH_2CH_2)^+$], 219 (70), 192 (35), 164 (33), 124 (44) and 43 (100) (Found: $M^+$, 248.1410 ± 0.0015. $C_{15}H_{20}O_3$ requires, *M*, 248.1412).

*Euplotin B* **2** [(+)-(2a*S**,3*R**,4a*S**,7a*S**,7b*R**)-7-(4-*Methyl*-1-*oxopen*-3-*enyl*)-1,2,2a,4a,7a,7b-*hexahydro*-4,5-*dioxacyclopent*[cd]*inden*-3-*yl Acetate*].—See Ref. 1. CD(MeOH) $\Delta\varepsilon_{max}$(249 nm) +11; $\delta_C(C_6D_6)$ 104.00 (d, C-1), 50.34 (d, C-2), 46.11 (d, C-3), 30.69 (t, C-4), 29.60 (t, C-5), 36.38 (d, C-6, 122.31 (s, C-7), 196.54 (s, C-8), 37.91 (t, C-9), 118.06 (d, C-10), 134.12 (s, C-11), 25.69 (q, C-12), 18.00 (q, C-13), 154.96 (d, C-14), 104.74 (d, C-15), 169.00 (s, *C*OCH$_3$) and 20.65 (q, CO*C*H$_3$); $\delta_H(C_6D_6)$ 5.65 (d, $J_{1,2}$ 3.5, 1-H), 1.33 (ddd, $J_{2,6}$ 11.7, $J_{2,3}$ 7.1, $J_{2,1}$ 3.5 2-H), 2.18 (dddd, $J_{3,4\beta}$ 9.2, $J_{3,2}$ 7.1, $J_{3,4\alpha}$ 3.4, $J_{3,15}$ 2.8, 3-H), 1.78 (dddd, $J_{gem}$ 12.8, $J_{4\beta,3}$ 9.2, $J_{4\beta,5\beta}$ 7.1, $J_{4\beta,5\alpha}$ 0.9, 4-H$_\beta$, 1.49 (m, 4-H$_\alpha$), 1.40 (m, 5-H$_\beta$), 2.47 (br ddd, $J_{gem}$ 11.8, $J_{5\alpha,4\beta}$ 7.1, $J_{5\alpha,6}$ 4.6, 5-H$_\alpha$), 1.66 (dddd, $J_{6,5\beta}$ 13.1, $J_{6,2}$ 11.7, $J_{6,5\alpha}$ 4.6, $J_{6,14}$ 2.5, 6-H), 3.00 (br d, *J* 7.0, 9-H$_2$), 5.48 (tq, *J* 7.0, 1.4, 10-H), 1.62 (d, $J_{12,11}$ 1.4, 12-Me), 1.49 (br s, 13-Me), 7.06 (d, $J_{14,6}$ 2.5, 14-H), 6.03 (d, $J_{15,3}$ 2.8, 15-H) and 1.61 (s, $CH_3CO$); *m/z* 246 [11%, $(M - AcOH)^{\bullet+}$], 237 [11, $(M - Me_2C{=}CHCH_2)^+$], 217 (10), 195 [60, $(237 - CH_2{=}CO)^{\bullet+}$], 177 [53, $(M - AcOH - Me_2C{=}CHCH_2)^+$], 149 (38), 107 (22), 69 (23) and 43 (100)

(Found: $M^+$, 246.1254 ± 0.0016. $C_{15}H_{18}O_3$ requires, *M*, 246.1254).

*Euplotin C* **3** [(+)-(2aS*,3R*,4aS*,7aS*,7bR*)-7-(*Methylpent-3-enyl*)-1,2,2a,4a,7a,7b-*hexahydro*-4,5-*dioxacyclopent*[cd]-*inden*-3-*yl Acetate*].—Colourless oil. $[\alpha]_D^{20}$ + 18, $[\alpha]_{546}^{20}$ +24 and $[\alpha]_{435}^{20}$ + 42 (*c* 0.4 in $CHCl_3$); CD(MeOH) $\Delta\varepsilon_{max}$ (210 nm) − 1.4; $\nu_{max}/cm^{-1}$ 1735s and 1635w; NOE($C_6D_6$) 6.15 ⟶ 6% on 1.88 and 5% on 1.49, 1.88 ⟶ 5% on 6.15, 2.29 ⟶ 2% on 5.82 and 6% on 1.45: *m*/*z* 292 (3%, $M^{\bullet+}$), 232 [19, $(M - AcOH)^{\bullet+}$], 223 [10, $(M - Me_2C{=}CHCH_2)^+$], 181 [48, $(223 - CH_2{=}CO)^{\bullet+}$], 163 [100, $(232 - Me_2C{=}CHCH_2)^+$], 135 (30), 69 (33), 43 (95) [Found: $M^+$, 232.1464 ± 0.0012. $C_{15}H_{20}O_2$ requires, *M*, 232.1463; Found: $(M - CH_2{=}CO)^{\bullet+}$, 181.0864 ± 0.0016. $C_{10}H_{13}O_3$ requires, $(M - CH_2{=}CO)$, 181.0864; found, $(M - Me_2C{=}CHCH_2)^+$, 163.0760 ± 0.0016. $C_{10}H_{11}O_2$ requires, $(M - Me_2C{=}CH{-}CH_2)$, 163.0759].

*Preuplotin* **4**.—{(all-*E*)-2-[6-acetoxy-4-(acetoxymethylene)-hex-5-enylidene]-6-methylhept-5-enal}; $\lambda_{max}$/nm 246 ($\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$ 21 400); *m*/*z* 292 [0.5%, $(M - CH_2{=}CO)^{\bullet+}$], 274 [1.5, $(M - AcOH)^{\bullet+}$], 265 [1, $(M - C_5H_5)^+$], 250 [0.7, $(M - 2CH_2{=}CO)^{\bullet+}$], 232 [5, $(M - CH_2{=}CO - AcOH)^{\bullet+}$], 214 [17, $(M - 2AcOH)^{\bullet+}$], 203 (5), 163 [16, $(232 - C_5H_5)^+$], 145 [3, $(214 - C_5H_5)^+$], 109 (39), 69 (34), 43 (100) [Found: $M^+$, 292.1665 ± 0.0030. $C_{17}H_{24}O_4$ requires, *M*, 292.1674; found: $(M - AcOH)^{\bullet+}$ 274.1569 ± 0.0030. $C_{17}H_{22}O_3$ requires, $(M - AcOH)$, 274.1569; found: $(M - C_5H_5)^+$ 265.1064 ± 0.0030. $C_{14}H_{17}O_5$ requires $(M - C_5H_5)$, 265.1076; found: $(M - 2CH_2{=}CO)^{\bullet+}$ 250.1573 ± 0.0020. $C_{15}H_{22}O_3$ requires $(M - 2CH_2{=}CO)$ 250.1569; found: $(M - CH_2{=}CO - AcOH)^{\bullet+}$, 232.1457 ± 0.0015. $C_{15}H_{20}O_2$ requires $(M - CH_2{=}CO - AcOH)$, 232.1463].

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