# 63. Hydrolytic Breakdown of the Euplotins, Highly Strained, Adaptive, Hemiacetal Esters of the Marine Ciliate *Euplotes crassus*: A Mimic of Degradative Pathways in Nature and a Trick for the Assignment of the Absolute Configuration[1])

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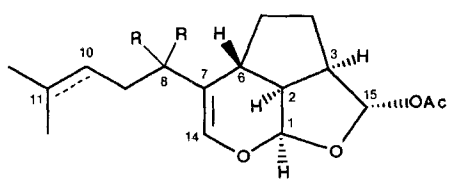
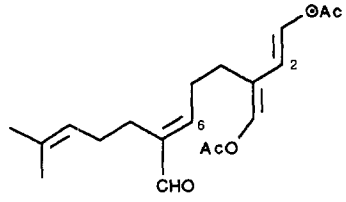
Euplotin C ((+)-**3**), the most abundant of the niche-exploitation terpenoids of the marine ciliate morphospecies *Euplotes crassus*, was found to undergo degradation in mildly basic $H_2O$/MeOH by initial hydrolysis of the acetate group, followed by, in turn, hydrolytic ring-A and ring-C opening and ring-A reclosure with indiscriminate C(1)/C(15) methanol trapping to give four diastereoisomeric aldehydic hemiacetals **5**–**8** in similar proportions; **7**, as a model for its congeners, proved biologically inactive. From these, the absolute configuration was assigned *via Mosher*'s ester methodology. These processes may be assumed to mimic inactivation of the euplotins in sea water. Degradation of (+)-**3** in either stronger base or acidic medium was also examined.

**1. Introduction.** – Highly strained sesquiterpene hemiacetal esters, euplotins A ((+)-**1**), B ((+)-**2**) [1], and C ((+)-**3**) [2], as well as their putative biogenetic precursor, preuplotin (**4**) [2], are present, in various proportions, in all strains of the ciliated morphospecies *Euplotes crassus* (DUJARDIN, 1841) so far examined. We attributed to (+)-**1** and (+)-**2** an adjuvant role in niche-exploitation by *E. crassus* on the basis that these metabolites inhibit cell division of, or kill, other marine ciliates, in cell-to-cell contacts [1], which affords a competitive advantage to euplotin-containing cells [1–3]. Similar evidence for euplotin C ((+)-**3**) is obtained here.




(+)-**1** R,R = O
(+)-**2** R,R = O, $\Delta^{10,11}$
(+)-**3** R = H, $\Delta^{10,11}$

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The mode of action and specificity of the euplotins, and other terpenoids isolated from different morphospecies of *Euplotes* [3], imply mediation by receptors. This solicits the assignment of the absolute configuration of these terpenoids as a prerequisite to unravelling the recognition processes in which they are involved. What also warrants

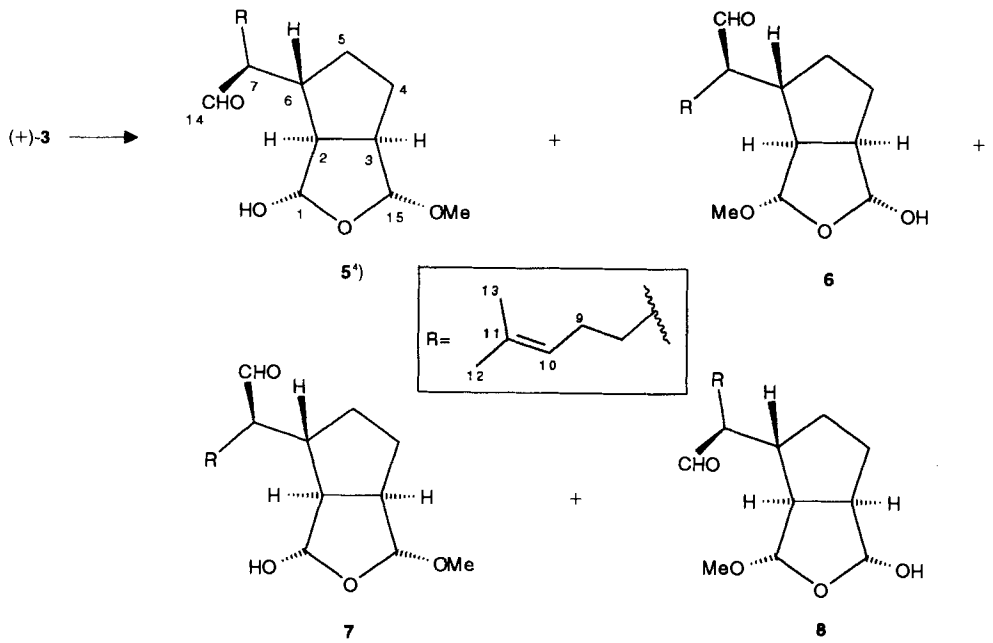
[1]) Presented in part by *G.G.* at 'XXI Convegno Nazionale della Divisione di Chimica Organica', Terrasini (Palermo), 28 September–2 October 1993.

attention is the fate of the euplotins, which undergo degradation resulting in biologically inactive mixtures [1] [2], perhaps as a strategy to end chemical signals.

We report here on the successful solution of these problems: the cytotoxicity of euplotin C ((+)-**3**) was evaluated and its degradation unravelled, which was instrumental to defining the absolute configuration.

**2. Results and Discussion.** – 2.1. *Hydrolysis of Euplotin C* ((+)-**3**) in *Mildly Basic Medium.* For euplotin C ((+)-**3**), the most abundant of the three euplotins [1] [2], MeOH/$H_2O$ 4:1 containing $K_2CO_3$ proved to be a suitable hydrolytic medium[2]), combining solubility for organic and inorganic compounds with rapidity and cleanness of hydrolysis. In this medium, (+)-**3** was observed to disappear at room temperature in a few minutes giving four diastereoisomeric products, as two couples characterized each by similar CHO chemical shifts and $J$(CHO,7) (9.68 ppm/2.8–2.9 Hz and 9.56–9.57 ppm/3.9 Hz). Extensive HPLC allowed us to obtain the two compounds **7** and **8** in pure form at the expense, however, of great losses (*Scheme 1*). Therefore, given the limited amount of material, the other two compounds **5** and **6** were used in mixture to prepare derivatives, as shown below.

Scheme 1. *Hydrolysis of Euplotin C* ((+)-**3**) *under Mildly Basic Conditions*




Acetal and hemiacetal protons of **5**–**8** showed similarly small $J$ values ($\leq$ 1 Hz), indicating *cis*-configuration at the 1,2 and 3,15 positions, corresponding to dihedral angles H–C(1)–C(2)–H and H–C(3)–C(15)–H close to 90°[3]). Thus, within each couple, these compounds must only differ as to the position of MeO, at either C(1) or

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[2]) Euplotin C ((+)-**3**) remained unaltered for at least 24 h at room temperature in either 5% aqueous $K_2CO_3$ solution (presumably because of insolubility in this medium) or in the biphasic medium hexane/$H_2O$, containing $(Bu_4)HSO_4$. On addition of MeOH, immediate degradation of (+)-**3** was observed, as above.

[3]) Arbitrary C-atom numbering; for systematic numbering and names, see *Exper. Part.*

C(15), which could be assigned from $^1$H- and $^{13}$C-NMR spectra (*Exper. Part*). The observation of $J(7,6) = 9$ Hz for all four compounds did not allow to specify the configuration at C(7). The latter could be assigned for **8**, however, from NOE enhancement between CHO and H–C(1). Similarity of $\delta$ and $J$ values for **8** and **5** allowed us assigning the same relative configuration at C(7), thus establishing the entire configuration as shown in the *Formulae*. These attributions were confirmed on MTPA esters (see below).

2.2. *Biological Assays of Euplotin C* ((+)-**3**) *and Degradation Products.* To bypass problems of solubility, the biological activity of euplotin C ((+)-**3**) was evaluated by conveying it into sea water from a DMSO solution, thus in mixtures containing 0.012–2% DMSO (*Exper. Part*), which was well tolerated by the tested ciliates. The data in the *Table*, which include euplotin A ((+)-**1**) and B ((+)-**2**) and preuplotin (**4**) of *E. crassus*, show that (+)-**3** is even more active than the other sesquiterpenoids, thus warranting selection of it, as the more abundant of the sesquiterpenoids, for degradation studies.

Table. *Biological Assays of Euplotins A* ((+)-**1**), *B* ((+)-**2**), *and C* ((+)-**3**), *and of Preuplotin* (**4**) *towards Marine Ciliates in DMSO*

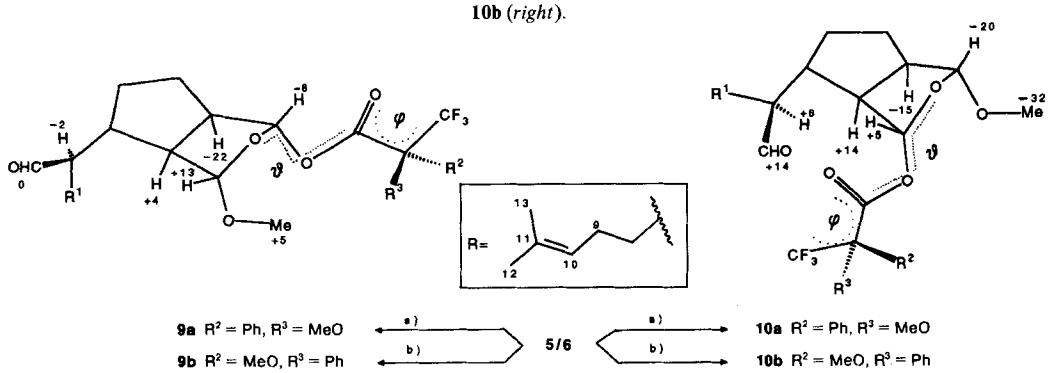
| Strain | Taxon | Origin | $LD_{100}$[a]), $ED_{100}$[b]) | | | | |
|---|---|---|---|---|---|---|---|
| | | | (+)-**1** | (+)-**2** | (+)-**3** | **4** | **1**/**2**/**3**/**4**[c]) |
| TB6 | *Euplotes vannus* (MÜLLER, 1786) | Tanabe, Japan, July 1983 | < 2.5, < 1.2 | < 2.5, < 1.2 | < 2.5, < 1.2 | 10, 5 | < 2.5, < 1.2 |
| SSt52 | *E. crassus* | Sciacca-Stazzone, Sicily, December 1986 | 20, 15 | > 20[d]), > 20 | 20, 10 | > 20, 20 | 20, 10 |
| SR2 | *Euplotes minuta* YOCUM, 1930 | San Rossore, Pisa, Italy, March 1991 | 10, 5 | 20, 5 | 10, 2.5 | 20, 10 | 10, 5 |
| SicAA | *Euplotes rariseta* CURDS, WEST and DORAHY, 1974 | Milazzo, Sicily | 2.5, < 1.2 | 5, < 2.5 | < 2.5, < 1.2 | 10, 5 | < 2.5, < 1.2 |

[a]) Lowest concentration [μg/ml] for 100% kills.
[b]) Lowest concentration [μg/ml] eliciting a fission rate delay in 100% of tested cells.
[c]) Relative [**1**]/[**2**]/[**3**]/[**4**] = 153:65:268:1, as found in strain MAL1 of *E. crassus* from Malindi coast, Kenya, October 1994.
[d]) Less than 100% kills observed at the highest attainable concentration (*ca.* 20 μg/ml) of the terpenoid in the medium used for bioassays.

Included in the *Table* are also bioassay data for a mixture of euplotins and preuplotin in the same relative concentration ratio as found in strain MAL1 of *E. crassus*: lack of synergism is revealed for these terpenoids. Degradation product **7** proved inactive towards all tested ciliates.

2.3. *Absolute Configuration.* The new functionalities generated on hydrolysis of euplotin C (*Scheme 1*) allowed us to apply *Mosher*'s NMR methodology. First, the couples of esters **9a**/**10a** and **9b**/**10b** were prepared by treating the mixture **5**/**6** with (+)-(*R*)-MTPA-Cl or (–)-(*S*)-MTPA-Cl, respectively (*Scheme 2*; MTPA = α-methoxy-α-(trifluoromethyl)phenylacetic acid = 3,3,3-trifluoro-2-methoxy-2-phenylpropanoic acid). Although these couples of esters could not be efficiently separated into pure products. $^1$H-NMR spectra of their mixture allowed us to extract $\delta(S) - \delta(R)$ data for **9a** with

Scheme 2. *Synthesis of MTPA Esters from* **5**/**6**. *a*) (−)-(*R*)-MTPA-Cl and DMPA in dry pyridine. *b*) (+)-(*S*)-MTPA-Cl in place of (−)-(*R*)-MTPA-Cl. Values represent the difference [Hz] between the corresponding resonances of the (*S*)-MTPA ester **9a** and (*R*)-MTPA ester **9b** (*left*) and the (*S*)-MTPA ester **10a** and (*R*)-MTPA ester **10b** (*right*).




respect to **9b** and for **10a** with respect to **10b**, reported as $\Delta\delta$ in *Scheme 2*. Downfield shifts of the front-side protons for **9a** with respect to **9b** and opposite trend along the other side (*Scheme 2*) indicate the (*R*)-configuration at C(15) of **5**/**6**. Conversely, (*S*)-configuration could be assigned to C(1) of **5**/**6** from examination of the $\Delta\delta$ data for **10a**/**10b** (*Scheme 2*), thus arriving – by virtue of the elucidated hydrolytic pathway – at the absolute configuration of (+)-**3**.

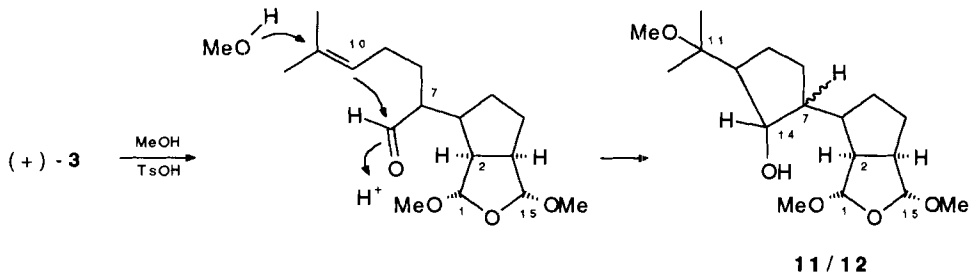
Although it seems safe to apply *Mosher*'s method when $\Delta\delta$ values vary consistently as in the above cases, in view of deviations of ±5 units from the ideal $\Delta\delta = 0$ for the 'carbonyl' H–C(1) or H–C(15) [4] (*Scheme 2*), the origin of such a consistent $\Delta\delta$ trend was examined. With the aid of molecular-mechanics calculations, it became clear – though not detailed here for sake of brevity – that *Mosher*'s, method gave the correct answer because of compensation of opposite deviations in dihedral angles $\vartheta$ and $\varphi$.

2.4. *Hydrolysis of Euplotin A* (**1**) *and, in Other Media, of Euplotin C* ((+)-**3**). The mixture of hydrolysis of (+)-**1**, carried out similarly to that of (+)-**3** above, revealed HPLC peaks for at least six products, which were examined *in situ* by $^1$H-NMR. Signals for the 8-keto analogues of the products of mild hydrolysis of (+)-**3** were observed, besides two very deshielded signals, $\delta$(H) 15.50 and 15.48, as *d*, $J$ = 6.7 Hz, for coupling with *d*'s at $\delta$(H) 8.08 and 8.10, respectively, attributable to the corresponding enol forms. No details are given here for sake of brevity.

In $CDCl_3$/KOH containing a trace of MeOH, at room temperature, euplotin C ((+)-**3**) disappeared in a few hours to give products of the type **5**–**8**, incorporating a D-atom at C(7), while the $^1$H-NMR residual $CHCl_3$ signal increased with reaction progress. The evidence is: the aldehyde signals showed up as *t* with small coupling ($J$ = 1.6 Hz), while the mass spectrum revealed complex clusters for all high-mass signals, and no aldehyde C-signals could be seen in the $^{13}$C-NMR spectra. After 15 h, products **6** and **7** were found to predominate over **5** and **8**.

In MeOH/KOH at room temperature, euplotin C ((+)-**3**) disappeared rapidly to give a more complex mixture of products of epimerization at both C(1) of **5** (and/or **7**) and C(15) of **6** (and/or **8**): the $\beta$ (pseudoequatorial) position of the O-atom in these epimers may account for $J$(1,2) (or $J$(15,3)) being higher than for **5**–**8** (0–1.3 Hz).

Scheme 3. *Hydrolysis of Euplotin C ((+)-3) under Acidic Conditions*

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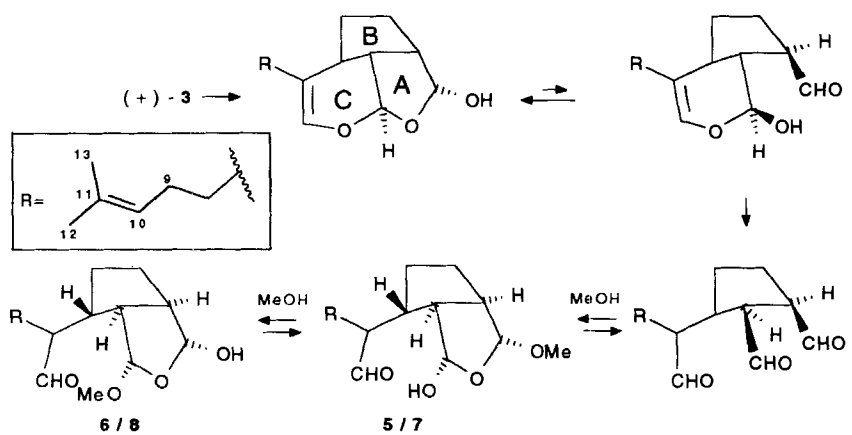
In TsOH-containing MeOH, euplotin C ((+)-**3**) also disappeared rapidly to give a complex mixture of products, the two more abundant and more polar diverging from the structural characteristics described above, however. The NMR data (*Exper. Part*) suggest acetals **11** and **12** as products, although it could not be specified which is which (*Scheme 3*).

Finally, $^1$H-NMR monitoring in $CD_3OD$/(i-Pr)$NH_2$ revealed that signals for euplotin C ((+)-**3**) are rapidly replaced by two br. *d* ($J$ = 7.5 Hz), $\delta$(H) 7.45 and 7.37, compatible with the imine proton of a C*H*=N(i-Pr) group.

**3. Conclusions.** – In mildly basic medium, degradation of euplotin C ((+)-**3**) can be rationalized as depicted in *Scheme 4*. The new functional groups generated in these processes helped us, from the intact *trans*-arrangement at C(2)–C(6), to confirm the original assignment [1] of the relative configuration of the euplotins. Furthermore, stability of these hydrolytic products confirms, by impeding reclosure involving the C(14) aldehyde group, our initial proposal [1] that such *trans*-fusion is attended by great strain.

To what extent the hydrolysis products described here mimic natural events is open to discussion, however. Use of an organic solvent was imposed by solubility problems of the hydrophobic euplotins in order to have sufficient isolable material for NMR study in nonaqueous solvents. That, however, products **5**–**8** may be considered as MeO analogues

Scheme 4. *Degradation of Euplotin C ((+)-3) in Mildly Basic Medium*




**6 / 8** **5 / 7**

of euplotin-C degradation products in sea water seems to be a sound conclusion: great complexity of mixtures under strongly basic conditions, and cleaner mixtures in mild base, warrant assuming that degradation mixtures in sea water (pH 8–9) are no more complex, if not simpler, than in laboratory treatment with mild base. To this concern, it is relevant that **7** proved biologically inactive on those ciliate strains where euplotins have ravaging effects.

Admittedly, our biological assays, by conveying into sea water the euplotins through an organic solvent, is an oversimplification dictated by present unavailability of methodologies to assay these substances under the lipophilic conditions of cell-to-cell contacts, under which they seem to act [1]. However, competition experiments between ciliate strains fully support the ecological role of their products suggested by *in vitro* tests [3].

Perhaps surprising is lack of synergism for the euplotins (*Table*, last column). This raises questions as to the maintenance of three products of virtually identical function. The close structural similarity among the euplotins supports a membership to the same metabolic path as successive steps [2]. If so, the euplotins behave as a 'unit' with respect to selection; either all three are preserved, or they disappear all together.

## Experimental Part

1. *General.* Evaporations were carried out at reduced pressure at r.t. Yields are given on reacted substrates. Flash chromatography (FC): *Merck* silica gel *Si-60* (15–25 μm). HPLC: *Merck LiChrosorb Si60* (7 μm). Reversed-phase HPLC: *Merck LiChrosorb RP-18* (7 μm). TLC: *Merck* silica gel *60 $F_{254}$* plates. NMR: *Varian XL-300* ($^1$H at 299.94 MHz, $^{13}$C at 75.43 MHz), $\delta$ in ppm rel. to internal $Me_4Si$ ( = 0 ppm) and $J$ in Hz; C-multiplicity assignments by DEPT [5]; $^{13}$C,$^1$H correlation by inverse-detection shift-correlation experiments [6]. Differential NOE: 5-s preirradiation; given as 'irradiated proton→NOE on the observed proton(s) (%)'. EI-MS (*m/z* (%)): *Kratos MS80*, with home-built computerized acquisition system.

2. *Cytotoxicity Assays.* Methodological protocols used to assess cytotoxic effects of terpenoids in strains of the selected ciliate morphospecies have been carried out according to described methodology [1] [2] [3a], except for substituting EtOH with DMSO in conveying terpenoids into sea water. The concentration of DMSO in sea water ranged from 0.012 to 2%, at the lowest and highest terpenoid dosages, respectively, in the set of consecutive steps in concentration used to cover the whole range of terpenoids' cytotoxic effects (*Table*). These were assessed microscopically. Controls were included for DMSO-treated as well as untreated ciliate cells, running them simultaneously with the terpenoid-treated individuals.

Preuplotin and euplotins A–C were extracted from *ca.* $2.4 \times 10^8$ cells, harvested by mild centrifugation from large mass cultures of strain MAL1 of the ciliated protist *E. crassus*, collected from the sandy shore of Malindi, Kenya, in Oktober 1994, and grown on the green microalga *Dunaliella tertiolecta* BUTCHER, 1959, used as food organism. The ciliate strains used as test organisms (*Table*) were cultured and handled as reported before [2].

3. *Hydrolysis of Euplotin C* ((+)-**3**) *in Basic Media.* 3.1. *In MeOH/$H_2O$/$K_2CO_3$.* Euplotin C (**3**; 9 mg) was added to 1 ml of 0.32M $K_2CO_3$ in MeOH/$H_2O$ 4:1, and the mixture was stirred for 15 min at r.t. (TLC (hexane/$Et_2O$ 1:1): no (+)-**3** left, spots at lower $R_f$). To the mixture were then added 1 ml of sat. aq. $NaHCO_3$ soln. and 3 ml of hexane/AcOEt 9:1, and the org. phase was separated on *Whatman RP* filters and evaporated to give 7 mg of crude material whose $^1$H-NMR revealed signals for the four main products **5**–**8** in relative molar ratios 25:20:32:23, which could be partially separated by HPLC with hexane/i-PrOH 92:8 (monitoring a $\lambda$ 215 nm): **7** ($t_R$ 7.8 min, 1.6 mg), **5**/**6** (38:62 according to $^1$H-NMR; $t_R$ 8.9 min, 2.9 mg), and **8** ($t_R$ 10.3 min, 1.2 mg). A portion of **5**/**6** was subjected to the same HPLC procedure as above giving 85% pure **5** and **6**. Compound **8** was used in MS experiments, so that only **7** remained available for biological assays.

*(αS,1R,3S,3aR,4S,6aR)-3,3a,4,5,6,6a-Hexahydro-3-hydroxy-1-methoxy-α-(4-methylpent-3-enyl)-1*H*-cyclopenta[c]furan-4-acetaldehyde* (**5**): $^1$H-NMR ($CDCl_3$)[3]: 5.22 (*d*, $J$(1,OH) = 9.4, H–C(1)); 2.44 (br. *dd*, $J$(2,6) = 9.1, $J$(2,3) = 8.0, $J$(2,1) ≤ 1, H–C(2)); 2.76 (br. *q*, $J$(3,2) = $J$(3,4a) = $J$(3,4b) = 8.0, $J$(3,15) ≤ 1,

H–C(3)); 2.00, 1.34 (2*m*, 2 H–C(4)); 1.25 (*m*, 2 H–C(5)); 1.93 ('*q*', $J(6,2) \approx J(6,5a) \approx J(6,7) = 9.1$, H–C(6)); 2.30 (*ddt*, $J(7,6) = 9.1$, $J(7,8a) = 8.0$, $J(7,8b) \approx J(7,14) = 3.0$, H–C(7)); 1.57, 1.22 (2*m*, 2 H–C(8)); 1.96 (*m*, 2 H–C(9)); 5.04 (br. *t*, $J(10,9) = 7.1$, H–C(10)); 1.67 (br. *s*, 3 H–C(12)); 1.56 (br. *s*, 3 H–C(13)); 9.68 (*d*, $J(14,7) = 2.9$, H–C(14)); 4.82 (br. *s*, H–C(15)); 2.90 ($J$(OH, 1) = 9.4, OH); 3.39 (*s*, MeO). $^{13}$C-NMR ($CDCl_3$)[3]: 106.34 (*d*, C(1)); 55.33 (*d*, C(2)); 50.43 (*d*, C(3)); 31.03 (*t*, C(4)); 27.64 (*t*, C(5)); 44.83 (*d*, C(6)); 55.92 (*d*, C(7)); 29.69 (*t*, C(8)); 25.74 (*t*, C(9)); 123.21 (*d*, C(10)); 133.02 (*s*, C(11)); 25.67 (*q*, C(12)); 17.75 (*q*, C(13)); 204.57 (*d*, C(14)); 111.76 (*d*, C(15)); 55.09 (*q*, MeO). MS: 264 (1, $[M - H_2O]^+$), 251 (3, $[M - MeO]^+$), 250 (3, $[M - MeOH]^+$), 233 (3, $[264 - MeO]^+$), 232 (4, $[264 - MeOH]^+$), 221 (5), 215 (3), 204 (4), 82 (100). MS: 264.1735 ± 0.005 ($C_{16}H_{24}O_3^+$, calc. 264.1725), 251.1630 ± 0.005 ($C_{15}H_{23}O_3^+$, calc. 251.1647), 250.1565 ± 0.005 ($C_{15}H_{22}O_3^+$, calc. 250.1569), 233.1525 ± 0.005 ($C_{15}H_{21}O_2^+$, calc. 233.1541), 232.1456 ± 0.005 ($C_{15}H_{20}O_2^+$, calc. 232.1463).

(α*R*,1*R*,3*S*,3a*R*,4*S*,6a*R*)-3,3a,4,5,6,6a-Hexahydro-1-hydroxy-3-methoxy-α-(4-methylpent-3-enyl)-1H-cyclopenta[c]furan-4-acetaldehyde (**6**): $^1$H-NMR ($CDCl_3$)[3]: 4.83 (br. *s*, H–C(1)); 2.52 (br. *dd*, $J(2,6) = 9.1$, $J(2,3) = 8.0$, $J(2,1) \leq 1$, H–C(2)); 2.72 (br. *q*, $J(3,2) \approx J(3,4a) \approx J(3,4b) = 8.0$, $J(3,15) \leq 1$, H–C(3)); 2.00, 1.28 (2*m*, 2 H–C(4)); 1.25 (*m*, 2 H–C(5)); 1.87 ('*q*', $J(6,2) \approx J(6,5a) \approx J(6,7) = 9.1$, H–C(6)); 2.22 (*ddt*, $J(7,6) = 9.1$, $J(7,8a) = 8.0$, $J(7,8b) \approx J(7,14) = 3.0$, H–C(7)); 1.57, 1.22 (2*m*, 2 H–C(8)); 1.96 (*m*, 2 H–C(9)); 5.04 (br. *t*, $J(10,9) = 7.1$, H–C(10)); 1.67 (br. *s*, 3 H–C(12)); 1.56 (br. *s*, 3 H–C(13)); 9.57 (*d*, $J(14,7) = 3.9$, H–C(14)); 5.18 (*d*, $J(15,OH) = 9.4$, H–C(15)); 2.91 (*d*, $J(OH,15) = 9.4$, OH); 3.41 (*s*, MeO). $^{13}$C-NMR ($CDCl_3$)[3]: 112.26 (*d*, C(1)); 54.19 (*d*, C(12)); 51.76 (*d*, C(3)); 30.40 (*t*, C(4)); 28.36 (*t*, C(5)); 44.60 (*d*, C(6)); 55.88 (*d*, C(7)); 31.48 (*t*,C(8)); 25.84 (*t*, C(9)); 123.05 (*d*, C(10)); 133.07 (*s*, C(11)); 25.69 (*q*, C(12)); 17.75 (*q*, C(13)); 204.37 (*d*, C(14)); 105.98 (*d*, C(15)); 55.69 (*q*, MeO). MS: practically superimposable to that of **5**.

(α*R*,1*R*,3*S*,3a*R*,4*S*,6a*R*)-3,3a,4,5,6,6a-Hexahydro-3-hydroxy-1-methoxy-α-(4-methylpent-3-enyl)-1H-cyclopenta[c]furan-4-acetaldehyde (**7**): $^1$H-NMR ($CDCl_3$)[3]: 5.19 (*d*, $J(1,OH) = 9.4$, H–C(1)); 2.46 (br. *dd*, $J(2,6) = 9.1$, $J(2,3) = 8.0$, $J(2,1) \leq 1$, H–C(2)); 2.76 (br. *q*, $J(3,2) \approx J(3,4a) \approx J(3,4b) = 8.0$, $J(3,15) \leq 1$), H–C(3)); 2.00, 1.34 (2*m*, 2 H–C(4)); 1.25 (*m*, 2 H–C(5)); 1.87 ('*q*', $J(6,2) \approx J(6,5a) \approx J(6,7) = 9.1$, H–C(6)); 2.21 (*ddt*, $J(7,6) = 9.1$, $J(7,8a) = 8.0$, $J(7,8b) \approx J(7,14) = 3.0$, H–C(7)); 1.57, 1.22 (*m*, 2 H–C(8)); 1.96 (*m*, 2 H–C(9)); 5.04 (br. *t*, $J(10,9) = 7.1$, H–C(10)); 1.67 (br. *s*, 3 H–C(12)); 1.56 (br. *s*, 3 H–C(13)); 9.56 (*d*, $J(14,7) = 3.9$, H–C(14)); 4.82 (br. *s*, H–C(15)); 2.90 (*d*, $J(OH,1) = 9.4$, OH); 3.40 (*s*, MeO). $^{13}$C-NMR ($CDCl_3$)[3]: 106.52 (*d*, C(1)); 56.19 (*d*, C(2)); 50.12 (*d*, C(3)); 31.57 (*t*, C(4)); 25.88 (*t*, C(5)); 45.34 (*d*, C(6)); 56.25 (*d*, C(7)); 30.40 (*t*, C(8)); 28.66 (*t*, C(9)); 123.07 (*d*, C(10)); 132.97 (*s*, C(11)); 25.69 (*q*, C(12)); 17.75 (*q*, C(13)); 204.45 (*d*, C(14)); 111.65 (*d*, C(15)); 55.11 (*q*, MeO). MS: practically superimposable to that of **5**.

(α*S*,1*R*,3*S*,3a*R*,4*S*,6a*R*)-3,3a,4,5,6,6a-Hexahydro-1-hydroxy-3-methoxy-α-(4-methylpent-3-enyl)-1H-cyclopenta[c]furan-4-acetaldehyde (**8**): $^1$H-NMR ($CDCl_3$)[3]: 4.87 (br. *s*, H–C(1)); 2.48 (br. *dd*, $J(2,6) = 9.1$, $J(2,3) = 8.0$, $J(2,1) \leq 1$, H–C(2)); 2.72 (br. *q*, $J(3,2) \approx J(3,4a) \approx J(3,4b) = 8.0$, $J(3,15) \leq 1$, H–C(3)); 2.02, 1.30 (2*m*, 2 H–C(4)); 1.25 (*m*, 2 H–C(5)); 1.93 ('*q*', $J(6,2) \approx J(6,5a) \approx J(6,7) = 9.1$, H–C(6)); 2.30 (*ddt*, $J(7,6) = 9.1$, $J(7,8a) = 8.0$, $J(7,8b) \approx J(7,14) = 3.0$, H–C(7)); 1.57, 1.22 (2*m*, 2 H–C(8)); 2.00 (*m*, 2 H–C(9)); 5.04 (br. *t*, $J(10,9) = 7.1$, H–C(10)); 1.66 (br. *s*, 3 H–C(12)); 1.56 (br. *s*, 3 H–C(13)); 9.68 (*d*, $J(14,7) = 2.8$, H–C(14)); 5.17 (*d*, $J(15,OH) = 9.4$, H–C(15)); 2.91 (*d*, $J(OH,15) = 9.4$, OH); 3.40 (*s*, MeO). NOE: H–C(1)→CHO (+6%), H–C(6) (+5%), MeO (+8%). $^{13}$C-NMR ($CDCl_3$)[3]: 112.09 (*d*, C(1)); 54.28 (*d*, C(2)); 52.12 (*d*, C(3)); 30.16 (*t*, C(4)); 27.70 (*t*, C(5)); 44.04 (*d*, C(6)); 55.93 (*d*, C(7)); 31.13 (*t*, C(8)); 25.76 (*t*, C(9)); 123.19 (*d*, C(10)); 132.98 (*s*, C(11)); 25.64 (*q*, C(12)); 17.75 (*q*, C(13)); 204.51 (*d*, C(14)); 106.09 (*d*, C(15)); 55.30 (*q*, MeO). MS: practically superimposable to that of **5**.

3.2. *In $CDCl_3$/MeOH/KOH.* To a soln. of (+)-**3** (3 mg) in $CDCl_3$ (0.5 ml) in a NMR tube at probe temp. (20°) were added 25 µl of 0.2M KOH in MeOH. After a few hours, the $^1$H-NMR signals for (+)-**3** were replaced by those for deuterated forms of **5**–**8**: 4 *t* ($J = 1.6$) at δ(H) 9.66, 9.61, 9.58, and 9.56.

3.3. *In MeOH/KOH.* A soln. of (+)-**3** (3 mg) in 0.5 ml of 0.2M KOH in MeOH was stirred for 0.5 h at r.t. and then evaporated. The $CDCl_3$ extract of the residue revealed $^1$H-NMR signals for **5**–**8** as well as for their epimers at about the same concentrations, as shown above.

4. *MTPA Esters of* **5** *and* **6**. A portion (0.9 mg) of **5**/**6** was treated with 3 mol-equiv. of (+)-(*S*)-MTPA-Cl (*Fluka*) and 0.4 mg of 4-(dimethylamino)pyridine in dry pyridine (0.5 ml). A parallel process was carried out with (–)-(*R*)-MTPA-Cl. Each mixture was quenched after 5 h with sat. aq. $CuSO_4$ soln. (1 ml). Then hexane/AcOEt 9:1 (4 ml) was added, followed by phase separation on *Whatman* filter, obtaining crude samples of 4 and 5.5 mg, resp. The 5.5-mg sample was freed from excess acid chloride and little unreacted **5**/**6** by FC (*Si-60*, 3 hexane fractions, then hexane/AcOEt gradient elution). *Fractions 4* and *5* contained the desired esters. Thus, (+)-(*S*)-MTPA-Cl gave the (*R*)-esters **9b** and **10b** (1.0 mg each after FC) and (–)-(*R*)-MTPA-Cl the (*S*)-esters **9a** and **10a** (0.9 mg after FC), which were examined by MS and $^1$H-NMR ($CDCl_3$) in mixture.

*Data of* **9a**: $^1$H-NMR ($CDCl_3$; only the most significant, assigned resonances)[3]): 4.93 (*d*, $J(1,2) = 1.4$, H–C(1)); 2.50 (br. *dd*, $J(2,6) = 9.1$, $J(2,3) = 8.0$, $J(2,1) = 1.4$, H–C(2)); 2.81 (br. *q*, $J(3,2) \approx J(3,4a) \approx J(3,4b) = 8.7$, $J(3,15) \leq 1$, H–C(3)); 2.20 (*dddd*, $J(7,6) = 9.1$, $J(7,8a) = 8.0$, $J(7,8b) = 3.0$, $J(7,14) = 3.8$, H–C(7)); 9.57 (*d*, $J(14,7) = 3.8$, H–C(14)); 6.22 (br. *s*, H–C(15)); 3.30 (*s*, MeO–C(1)); 3.56 (*s*, MeO); 7.20–7.60 (series of *m*, arom. H). MS of **9a**/**10a**: 265 (27, $[M - MTPAO]^+$), 264 (9, $[M - MTPAOH]^+$), 233 (49, $[265 - MeOH]^+$), 232 (18, $[264 - MeOH]^+$), 189 (100), 139 (32), 105 (31), 82 (37), 69 (38).

*Data of* **9b**: $^1$H-NMR ($CDCl_3$)[3]): 4.89 (*d*, $J(1,2) = 1.0$, H–C(1)); 2.49 (br. *dd*, $J(2,6) = 9.1$, $J(2,3) = 8.0$, $J(2,1) = 1.4$, H–C(2)); 2.89 (br. *q*, $J(3,2) \approx J(3,4a) \approx J(3,4b) = 8.7$, $J(3,15) \leq 1$, H–C(3)); 2.21 (*dddd*, $J(7,6) = 9.1$, $J(7,8a) = 8.0$, $J(7,8b) = 3.0$, $J(7,14) = 3.8$, H–C(7)); 9.57 (*d*, $J(14,7) = 3.8$, H–C(14)); 6.24 (br. *s*, H–C(15)); 3.28 (*s*, MeO–C(1)); 3.53 (*s*, MeO); 7.20–7.60 (series of *m*, arom. H). MS of **9b**/**10b**: 265 (19, $[M - MTPAO]^+$), 264 (6, $[M - MTPAOH]^+$), 233 (27, $[265 - MeOH]^+$), 232 (15, $[264 - MeOH]^+$), 189 (94), 139 (42), 82 (100).

*Data of* **10a**: $^1$H-NMR ($CDCl_3$)[3]): 6.35 (*d*, $J(1,2) = 1.0$, H–C(1)); 2.59 (br. *dd*, $J(2,6) = 9.1$, $J(2,3) = 8.0$, $J(2,1) = 1.0$, H–C(2)); 2.69 (br. *q*, $J(3,2) \approx J(3,4a) \approx J(3,4b) = 8.0$, $J(3,15) \leq 1$, H–C(3)); 2.35 (*ddt*, $J(7,6) = 9.1$, $J(7,8a) = 8.0$, $J(7,8b) \approx J(7,14) = 3.0$, H–C(7)); 9.65 (*d*, $J(14,7) = 2.6$, H–C(14)); 4.86 (br. *s*, H–C(15)); 3.00 (*s*, MeO); 3.51 (*s*, MeO); 7.20–7.60 (series of *m*, arom. H).

*Data of* **10b**: $^1$H-NMR ($CDCl_3$)[3]): 6.33 (*d*, $J(1,2) = 1.2$, H–C(1)); 2.54 (br. *dd*, $J(2,6) = 9.1$, $J(2,3) = 8.0$, $J(2,1) = 1.2$, H–C(2)); 2.72 (br. *q*, $J(3,2) \approx J(3,4a) \approx J(3,4b) = 8.0$, $J(3,15) \leq 1$, H–C(3)); 2.33 (*tdd*, $J(7,6) = 9.1$, $J(7,8a) = 8.0$, $J(7,8b) \approx J(7,14) = 3.0$, H–C(7)); 9.65 (*d*, $J(14,7) = 2.6$, H–C(14)); 4.86 (br. *s*, H–C(15)); 3.10 (*s*, MeO); 3.53 (*s*, MeO); 7.20–7.60 (series of *m*, arom. H).

5. *Hydrolysis of Euplotin C* ((+)-**3**) *in Acidic Media*: *2-(3,3a,4,5,6,6a-Hexahydro-1,3-dimethoxy-1*H*-cyclopenta[*c*]furan-4-yl)-5-(1-methoxy-1-methylethyl)cyclopentanal* (**11**/**12**). A soln. of (+)-**3** (5 mg in 1 ml of MeOH containing 1% TsOH) was stirred at r.t. for 0.5 h. The solvent was then evaporated and the residue subjected to FC (hexane/AcOEt gradient elution, 5-ml fractions). *Fraction 5* was evaporated to leave 8 mg of residue that was subjected to HPLC (hexane/i-PrOH 9:1): **11** ($t_R$ 7.5 min; 1 mg) and **12** ($t_R$ 8.7 min, 1.3 mg) (we do not know which is which).

*Data of* **11**: $^1$H-NMR ($CDCl_3$)[3]): 4.88 (*d*, $J(1,2) = 1.3$, H–C(1)); 2.42 (br. *dd*, $J(2,6) = 5.7$, $J(2,3) = 8.7$, H–C(2)); 2.67 (br. *q*, $J(3,2) \approx J(3,4a) \approx J(3,4b) = 8.7$, H–C(3)); 1.95, 1.30 (*m*, 2 H–C(4)); 1.25 (*m*, 2 H–C(5)); 1.81 (*m*, H–C(6)); 1.83 (*m*, H–C(7)); 1.20–1.35 (series of *m*, 2 H–C(8), 2 H–C(9)); 1.99 (*m*, H–C(10)); 1.12 (br. *s*, 3 H–C(12), 3 H–C(13)); 3.70 (*t*, $J(14,7) = J(14,10) = 8.0$, H–C(14)); 4.82 (br. *s*, H–C(15)); 3.39 (*s*, MeO–C(15)); 3.41 (*s*, MeO–C(1)); 3.21 (*s*, MeO–C(11)). $^{13}$C-NMR ($CDCl_3$)[3]): 112.12 (*d*, C(1)); 56.19 (*d*, C(2)); 50.68 (*d*, C(3)); 30.50 (*t*, C(4)); 25.27 (*t*, C(5)); 48.35 (*d*, C(6)); 53.84 (*d*, C(7)); 29.98 (*t*, C(8)); 23.69 (*t*, C(9)); 49.37 (*d*, C(10)); 24.15 (*q*, C(12)); 18.39 (*q*, C(13)); 78.37 (*d*, C(14)); 113.48 (*d*, C(15)); 55.58, 55.24, 55.04 (3*q*, MeO). MS: 297 (2, $[M - OMe]^+$), 281 (1.4, $[M - OMe - H_2O]^+$), 265 (4, $[297 - MeOH]^+$), 264 (3), 247 (3, $[297 - H_2O - MeOH]^+$), 236 (7), 233 (4, $[265 - MeOH]^+$), 204 (5), 110 (20), 73 (100).

*Data of* **12**: $^1$H-NMR ($CDCl_3$)[3]): 4.94 (*d*, $J(1,2) = 1.1$, H–C(1)); 2.57 (br. *t*, $J(2,6) \approx J(2,3) = 8.7$, H–C(2)); 2.71 (br. *q*, $J(3,2) \approx J(3,4a) \approx J(3,4b) = 8.6$, H–C(3)); 1.95, 1.30 (*m*, 2 H–C(4)); 1.25 (*m*, 2 H–C(5)); 1.77 (*m*, H–C(6)); 1.83 (*m*, H–C(7)); 1.20–1.35 (series of *m*, 2 H–C(8), 2 H–C(9)); 1.96 (*m*, H–C(10)); 1.12 (br. *s*, 3 H–C(12), 3 H–C(13)); 3.70 (*t*, $J(14,7) = J(14,10) = 8.0$, H–C(14)); 4.81 (*d*, $J(15,3) = 1.1$, H–C(15)); 3.40 (*s*, MeO–C(15)); 3.41 (*s*, MeO–C(1)); 3.20 (*s*, MeO–C(11)). $^{13}$C-NMR ($CDCl_3$)[3]): 112.58 (*d*, C(1)); 56.19 (*d*, C(2)); 50.42 (*d*, C(3)); 32.10 (*t*, C(4)); 25.19 (*t*, C(5)); 47.61 (*d*, C(6)); 54.22 (*d*, C(7)); 30.58 (*t*, C(8)); 23.58 (*t*, C(9)); 49.37 (*d*, C(10)); 24.01 (*q*, C(12)); 19.07 (*q*, C(13)); 77.92 (*d*, C(14)); 112.97 (*d*, C(15)); 55.24 (3*q*, MeO). MS: practically superimposable to that of **11**.

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