39. Epoxyfocardin and Its Putative Biogenetic Precursor, Focardin, Bioactive, New-Skeleton Diterpenoids of the Marine Ciliate *Euplotes focardii* from Antarctica

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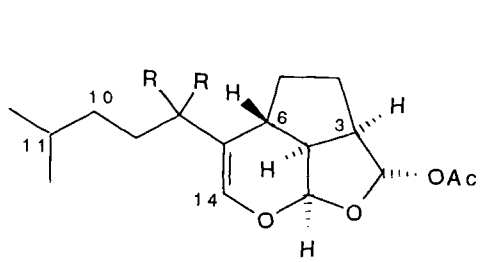
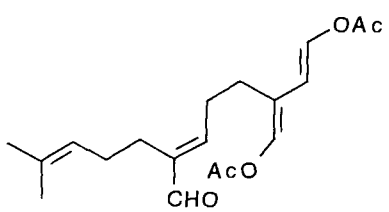
From the ciliate *Euplotes focardii*, collected from Ross Sea coastal waters, Antarctica, a new-skeleton diterpenoid, epoxyfocardin, was isolated as a 85:15 mixture of hemiacetals **8a** and **8b**. The absolute configuration of **8a**/**8b** was determined from *Mosher*'s esters **11a**/**11b** and **12a**/**12b**. Focardin **9a**/**9b**, most likely a biogenetic precursor of **8a**/**8b**, was also isolated as a minor component. Focardin, and particularly epoxyfocardin, proved to be toxic towards representatives of ciliate communities from Antarctic, temperate, tropical, and equatorial environments, constituting the first example of ecologically relevant metabolites from ciliate species that inhabit polar ecosystems.

1. Introduction. – Unicellular ciliated protists represent an important component of the grazing marine food web, thus playing a key, though scarcely investigated, role in the marine ecosystem. Ciliates comprising the family Euplotidae from temperate and tropical marine environments have been recently shown to contain new cytotoxic sesquiterpenoids to which the role of ecological niche broadening factors has been attributed. This is the case of highly strained acetylated sesquiterpene hemiacetals, euplotin A (**1**), B (**2**), and C (**3**) [1a], and their putative biogenetic precursor preuplotin (**4**) [1b], isolated from cultures of the cosmopolitan morphospecies *Euplotes crassus* (DUJARDIN, 1841). These terpenoids, which cause highly toxic effects in cell-to-cell contacts with representatives of most other ciliate species tested, have since been recognized as a highly conserved trait of *E. crassus* morphospecies [2]. *Euplotes raikovi* AMAGALIEV, 1966 contains another family of sesquiterpenoids in place of the euplotins, albeit with a variable distribution among



1 R,R = O
2 R,R = O, $\Delta^{10,11}$
3 R = H, $\Delta^{10,11}$



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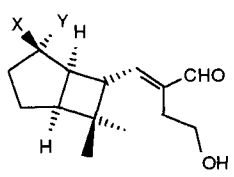
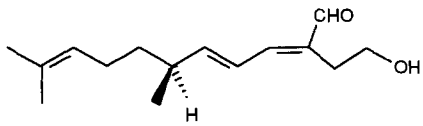


5a X=Me, Y=H
5b X=H, Y=Me



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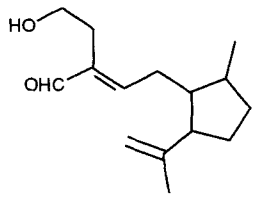

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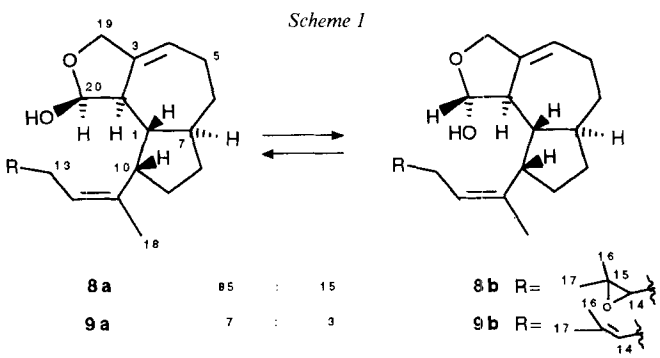
populations within the morphospecies: strain Morl, from Casablanca sea shore (eastern central Atlantic Ocean), gave raikovenal (**5a**) and its likely biogenetic precursor, preraikovenal (**6**) [3], while strains 39W from Porto Recanati (Adriatic Sea), SB8 from Santa Barbara (California, eastern central Pacific Ocean), and GA8 from Golfo Aranci (Sardinia, Thyrrhenian Sea) gave epiraikovenal (**5b**), probably deriving biogenetically from *ent*-**6** [4]. The latter two strains also afforded secoepiraikovenal **7** [4].

We show here that a ciliate from Antarctic waters, *Euplotes focardii* VALBONESI and LUPORINI, 1990, contains diterpenoids of a new skeleton, which behave as harmful, selective factors towards other ciliates.

2. Results and Discussion. – 2.1. *The Gross Structures and Relative Configurations.* The composition $C_{20}H_{30}O_3$ for epoxyfocardin was based on HR-MS data, implying 6 unsaturations and/or cycles. Two sets of ^{1}H-NMR signals indicated the presence of epimers **8a** and **8b** in a ratio of *ca.* 85:15 (*Scheme 1*). The hemiacetal H of the major component **8a** was identified at δ(H) 5.70 (*t*), heterocorrelated to the δ(C) 98.91 (*d*). The two trisubstituted olefinic bonds for **8a** were based on NMR data, implying the presence of four cycles. DDS, COSY, HMQC, HMBC, and NOE-NMR experiments were employed to determine the structure of **8a** with relative configurations of the chiral centres.

Scheme 1



8a 85 : 15 **8b**
9a 7 : 3 **9b**

Thus, H–C(2) displays a large coupling (11.7 Hz) with H–C(1) and a 4.5-Hz coupling with H–C(20), other than NOE with H–C(20) only[1]). The relative configurations at C(7) and C(10) rest on large couplings of H–C(1) with both H–C(7) (11.7 Hz; H–C(7) at δ(H) 1.78 (*m*), heterocorrelated with the δ(C) 48.71 (*d*) and H–C(10) (9.4 Hz)), while an NOE was only observed between H–C(1) and H–C(10). These assignments were confirmed by NOE between H–C(7) and H–C(2). *trans*-Junction at C(1) and C(2) was confirmed by a homoallylic '*cisoid*'-coupling (2.8 Hz) between H–C(2) and H_α–C(5). The (*Z*)-configuration for C(11)=C(12)

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

Table 1. *NMR Data* ($CDCl_3$) *for Epimer* **8a** *of Epoxyfocardin*[1])[a]). δ in ppm rel. to $SiMe_4$, J in Hz.

	^{13}C-NMR	^{1}H-NMR
H–C(1)	48.49 (*d*)	1.70 (*dt*, $J(1,10) = 9.4$, $J(1,2) \approx J(1,7) = 11.7$)
H–C(2)	49.24 (*d*)	2.70 (*qdd*, $J(2,19\alpha) \approx J(2,19\beta) \approx J(2,4) \approx J(2,5\alpha) = 2.8$, $J(2,20) = 4.5$, $J(2,1) = 11.7$)
C(3)	142.07 (*s*)	–
H–C(4)	120.06 (*d*)	5.68 (*qdd*, $J(4,19\alpha) \approx J(4,19\beta) \approx J(4,2) = 2.5$, $J(4,5\alpha) = 3.2$, $J(4,5\beta) = 9.0$)
CH_2(5)	27.3 (*t*)	α: 1.96 (*sext.*, $J(5\alpha,19\alpha) \approx J(5\alpha,19\beta) \approx J(5\alpha,6\alpha) \approx J(5\alpha,4) \approx J(5\alpha,2) = 2.8$, $J(5\alpha,6\beta) \approx J_{gem} = 13.0$) β: 2.19 (*dddd*, $J(5\beta,6\beta) = 2.8$, $J(5\beta,6\alpha) = 5.2$, $J(5\beta,4) = 9.0$, $J_{gem} = 13.0$)
CH_2(6)	35.10 (*t*)	α: 1.85 (*m*) β: 1.05 (*ddt*, $J(6\beta,5\beta) = 2.8$, $J(6\beta,7) = 10.2$, $J(6\beta,5\alpha) \approx J_{gem} = 13.0$)
H–C(7)	48.71 (*d*)	1.78 (*tddd*, $J(7,6\alpha) = 1.6$, $J(7,8\alpha) = 3.7$, $J(7,8\beta) = 9.2$, $J(7,6\beta) = 10.2$, $J(7,1) = 11.7$)
CH_2(8)	33.86 (*t*)	α: 1.95 (*m*) β: 1.26 (*dddd*, $J(8\beta,9\beta) = 7.0$, $J(8\beta,7) = 9.2$, $J(8\beta,9\alpha) = 12.0$, $J_{gem} = 13.5$)
CH_2(9)	28.39 (*t*)	α: 1.84 (*ddd*, $J(9\alpha,10) = 5.9$, $J(9\alpha,8\alpha) = 8.6$, $J(9\alpha,8\beta) = 12.0$, $J_{gem} = 13.5$) β: 1.44 (*dddd*, $J(9\beta,8\alpha) = 4.0$, $J(9\beta,8\beta) = 7.0$, $J(9\beta,10) = 9.4$, $J_{gem} = 13.5$)
H–C(10)	41.33 (*d*)	3.54 (*dt*, $J(10,9\alpha) = 5.9$, $J(10,9\beta) \approx J(10,1) = 9.4$)
C(11)	141.03 (*s*)	–
H–C(12)	122.60 (*d*)	5.21 (*qdd*, $J(12,Me) = 1.3$, $J(12,13a) = 5.7$, $J(12,13b) = 9.1$)
CH_2(13)	27.85 (*t*)	a: 2.33 (*ddd*, $J(13a,12) = 5.7$, $J(13a,14) = 7.7$, $J_{gem} = 15.3$) b: 2.41 (*ddd*, $J(13b,14) = 4.0$, $J(13b,12) = 9.1$, $J_{gem} = 15.3$)
H–C(14)	65.07 (*d*)	2.85 (*dd*, $J(14,13b) = 4.0$, $J(14,13a) = 7.7$)
C(15)	59.37 (*s*)	–
Me(16)	24.76 (*q*)	1.32 (*s*)
Me(17)	18.76 (*q*)	1.32 (*s*)
Me(18)	22.19 (*q*)	1.81 (*d*, $J(12,Me) = 1.3$)
CH_2(19)	69.76 (*t*)	α: 4.51 (*td*, $J(19\alpha,2) \approx J(19\alpha,5\alpha) = 2.5$, $J_{gem} = 12.2$) β: 4.25 (*qd*, $J(19\beta,2) \approx J(19\beta,4) \approx J(19\beta,5\beta) = 2.4$, $J_{gem} = 12.2$)
H–C(20)	98.91 (*d*)	5.70 (*t*, $J(20,2) \approx J(20,19\alpha) = 4.5$)
OH	–	4.52 (*d*, $J(OH,20) = 4.5$)

[a]) α and β refer to protons lying below and above, respectively, the plane of the paper on which structure **8a** is drawn.

rests on both strong NOE enhancement between H–C(12) and 3 H–C(18) and deshielding of C(18). Appearance of the OH group in $CDCl_3$ solution as a sharp *d* for coupling with H–C(20), which was observed as a pseudo-*t* (*Table 1*), suggests preferential folding of the side chain towards the hemiacetal group due to H-bonding of the oxirane O-atom and the OH group. Such H-bonding was also revealed by ^{1}H-NMR spectra in C_6D_6, where H–C(20) showed up as a pseudo-*t*, $J(OH,20) \approx J(2,20) = 4.5$, while OH appeared at δ 4.51 as a *d* ($J(OH,20) = 4.5$).

Molecular-mechanics calculations (MM) were carried out on a simplified model of **8a** (*Table 2*), to bypass the problem of the configuration at the oxirane moiety. The minimum requirement for the side chain proved to be a Me group at C(13) in place of the (dimethyloxiranyl)methyl moiety (*Fig.*). These MM calculations nicely emulated the coupling pattern of **8a**, indicating that the fused hemiacetal and seven-membered cycles form a rigid unit, while the five-membered carbocycle is allowed to flip rapidly between two puckered forms, the half-chair conformer (HC-**8a**) and the envelope conformer (E-**8a**) (*Fig.*). It must also be pointed out that, independently from the configuration at C(14), MM calculations for **8a** pointed to a similar folding of the side chain.

Due to its scarce abundance, **8b** allowed us to obtain less detailed spectral data than **8a**. It is relevant, however, that differences in δ(C) for **8b** with respect to **8a** are limited to the region around the hemiacetal substructure, H–C(20) in **8b** being only slightly coupled

Table 2. *MM-Calculated* J *Values for the Half-Chair* (HC-**8a**) *and Envelope* (E-**8a**) *Conformers of the Alkenyl-Chain Analogue of* **8a** (*Fig.*)

Vicinal Protons	HC-**8a**[a])	E-**8a**[a])	Averaged calculated J[b])	Observed J
1,2	12.4	12.6	12.5	11.7
1,7	11.5	11.8	11.7	11.7
1,10	9.9	7.3	8.7	9.4
2,20	4.9	4.9	4.9	4.5
4,5α	3.3	3.5	3.4	3.2
4,5β	6.5	6.5	6.5	9.0
5α,6α	1.8	1.6	1.7	2.8
5α,6β	13.1	13.1	13.1	13.0
5β,6α	5.1	5.4	5.2	5.2
5β,6β	1.8	1.6	1.7	2.8
6α,7	2.0	2.3	2.1	1.6
6β,7	12.2	12.3	12.2	10.2
7,8α	4.6	6.3	5.4	3.7
7,8β	12.5	11.2	11.8	9.2
8α,9α	7.0	11.1	9.1	8.6
8α,9β	0.7	1.0	0.8	≈ 0.0
8β,9α	12.1	6.8	9.4	9.2
8β,9β	6.2	10.9	8.5	7.0
9α,10	9.5	3.2	6.3	5.9
9β,10	8.4	10.3	9.3	9.4

[a]) Calculated trough a modified *Karplus* equation (C.A.G.Haasnoot, F.A.A.M. De Leeuw, C. Altona, *Tetrahedron* **1980**, *36*, 2783).

[b]) Calculated as $J_{av} = x_{\text{half-chair}} J_{\text{half-chair}} + x_{\text{envelope}} J_{\text{envelope}}$, where the molar fraction $x_{\text{half-chair}} = (1 + \exp(-\Delta E/RT))^{-1}$ *with* $\Delta E = 0.03$ kcal/mol and $T = 298$ K; ΔE is the difference of strain energies between the envelope and the half-chair conformers.

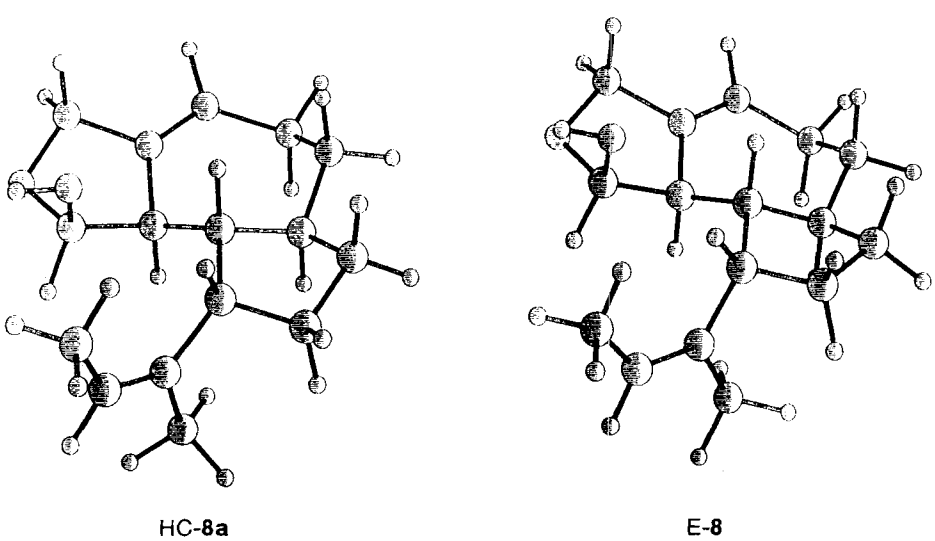


Figure. *Envelope* (E-**8a**) *and half-chair* (HC-**8a**) *conformers of the Me-Chain Analogue of* **8a**

(< 1 Hz) with H–C(2), corresponding to a H–C(20)–C(2)–H dihedral angle close to 90°. In this case, too, there is evidence for folding of the side chain around the hemiacetal group.

A similar situation was noticed for focardin (**9**), albeit with a slightly different epimeric composition, **9a**/**9b** 7:3 (*Scheme 1*). The presence of an isopropylidene group in place of the dimethyloxirane group is fully supported by HR-MS and NMR data (*Exper. Part*)[2]).

Although it is conceivable that the presence of two epimers reflects an equilibration process through a ring-opened free aldehyde, no proof for the equilibrium was obtained.

2.2. *The Absolute Configuration.* Esterification of the mixture **8a**/**8b** with (+)-(*S*)-MTPA-Cl (= (+)-(*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride = (+)-(*S*)-3,3,3-trifluoro-2-methoxy-2-phenylpropanoyl chloride) led to a mixture of the (*R*)-esters **11a** and **11b** of a composition very similar (4:1, as determined by ^{1}H-NMR) to that of the original epimeric acetals **8a**/**8b**. In contrast, using (–)-(*R*)-MTPA-Cl, a mixture of (*S*)-esters **12a** and **12b** was formed of opposite epimeric composition (3:7, as determined by ^{1}H-NMR) with respect to the starting acetals **8a**/**8b**. Very likely this results from the kinetic effect of differential interactions at the rate-limiting transition state for esterification. This is also reflected in the hydrolysis of **12a** in MeOH saturated with K_2CO_3, which gave the methoxyacetal corresponding to **8a**/**8b** in 3:7 ratio of abundance. Correspondingly, the methoxyacetal ratio was 85:15 starting from **12b.**

As indicated in *Scheme 3*, ($\delta(S) - \delta(R)$) values for the **11a**/**12a** couple, separated by HPLC, follow a uniform trend of positive values to the right of the dotted line (drawn in *Mosher*'s plane) and negative values on the left part. This allows assigning the (20*R*)-configuration to the ester, and thus the (20*S*)-configuration to **8a**. The ($\delta(S) - \delta(R)$) data for the **11b**/**12b** couple are specularly arranged with respect to those for **11a**/**12a**, indicating for **8b** the (20*R*)-configuration. This furnishes an internal control of validity for the

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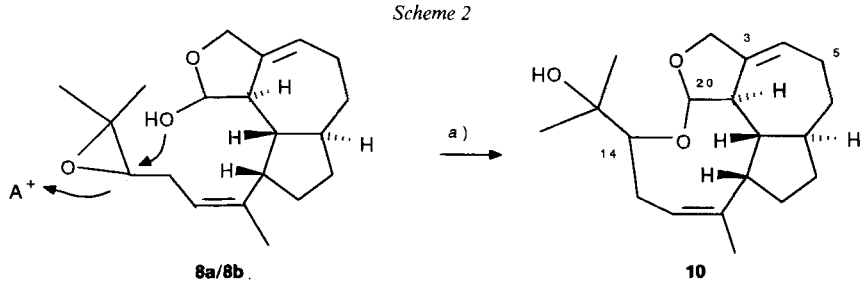
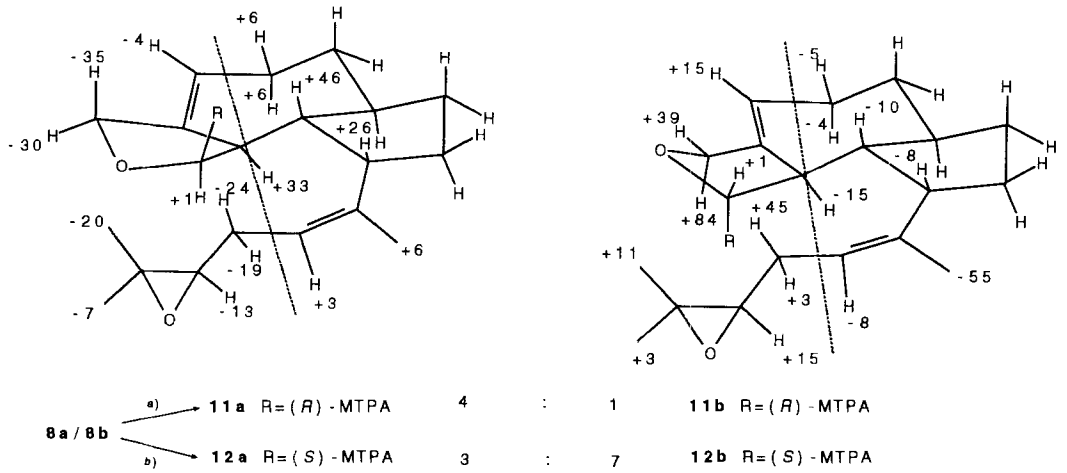
[2]) In C_6D_6, the mixture **8a**/**8b** tended to disappear, slowly at room temperature (*ca.* 20% in a few hours) or more rapidly at 40° (100% in 30 min). By HPLC, compound **10** was isolated as the major product; the structure is fully supported by ^{1}H-NMR spectra revealing an acetal moiety (largely deshielded H–C(14)) in place of the epoxide group (*Scheme 2*). The rigidity introduced by such further annelation is reflected in clear coupling patterns from the C(10)–C(14) portion. The base peak at *m/z* 230 in MS can be interpreted to derive from the elusive molecular ion by loss of $Me_2OHCCHO$, resulting from allylic C(13)–C(14) and C(20)–O bond cleavage. Conceivable as a general acid A^+-catalyzed nucleophilic opening, this cyclization was not observed in subsequent handling, however.

Scheme 2



a) In C_6D_6 solution.

Scheme 3



a) (+)-(*S*)-MTPA-Cl, 4-(dimethylamino)pyridine, pyridine. *b*) (−)-(*R*)-MTPA-Cl, 4-(dimethylamino)pyridine, pyridine. ^{1}H-NMR Resonance differences [Hz] between the (*S*)- and (*R*)-MTPA esters.

configurational assignments. It should also be noticed that the trend of $(\delta(S) - \delta(R))$ values holds also for the side chain, which gives further support to the above conformational conclusions.

2.3. *Biological Assays.* The unicellular nature, the state of autonomous individuals, the high degree of evolutionary divergence among taxa, and the membership to the grazing marine food web as a fundamental component make ciliated protists profitable model systems to assay the potential for harmful effects of chemicals on living beings, while providing at the same time the most thorough cytologic and ecological information. Cytotoxicity towards ciliates was thus used as a general screening to assess the activity of *E. focardii* terpenoids. Quantitative effects upon cell vitality in the strains are reported in *Table 3*, where selected strains represent an unbiased sample of the marine interstitial ciliate diversity and the latitudinal environments. The cytotoxicity of these diterpenoids towards representative ciliated species does not depend on the latitudinal gradient (*Table 3*), an environmentally conditioned specificity of the secondary metabolism in ciliates does not occur.

Of the two diterpenoids, focardin (**9a**/**9b**) is regularly the lowest cytotoxic. Combining such a low effectiveness with its low abundance in *E. focardii*, a minor role of focardin in nature can be guessed.

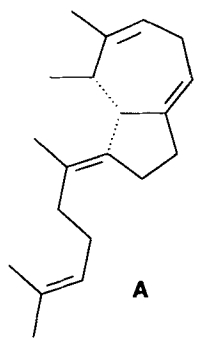
From a closer scrutiny of the data in *Table 3* an interspecific variability in susceptibility of the ciliates emerges, characterized by a striking sensitivity of the strain Li of *Litonotus lamella* (O.F. MÜLLER, 1773) and ELB2 of *Litonotus cygnus* (O.F. MÜLLER, 1773). The predacious life style that distinguishes these ciliates from all other ones listed in *Table 3* may not be casual, and a defensive role for epoxyfocardin (**8a**/**8b**) may be guessed. Moreover, the 'idiosyncratic' behavior of strains Li and ELB2, together with strain MLD2 of *Diophrys* sp. DINI and PIETRA, 1995, reveals specificity of mechanism(s) of action of **8a**/**8b**, which must entail finely tuned recognition processes.

Table 3. *Strain Designation and Latitudinal Origin of Various Ciliates and Their Susceptibility to Focardin* (**9a**/**9b**) *and Epoxyfocardin* (**8a**/**8b**), *Metabolites of the Ciliate* E. focardii

Strain	Morphospecies	Latitudinal gradient	Lowest conc. [μg/ml] for 100% kills	
			9a/**9b**	**8a**/**8b**
TN1, line A	*Euplotes focardii*	Antarctic	> 20	20
TN1, line B	*E. focardii*	Antarctic	> 20	20
AC3	*Euplotes nobilii* VALBONESI and LUPORINI, 1990	Antarctic	> 20	20
PD16	*Euplotes rariseta* CURDS, WEST and DORAHY, 1974	tropical	> 20	20
MLD2	*Diophrys* sp.	equatorial	10	10
TB6	*Euplotes vannus* (MÜLLER, 1786)	temperate	> 20	20
SSt52	*Euplotes crassus*	temperate	> 20	20
SR2	*Euplotes minuta* YOCUM, 1930	temperate	> 20	20
ELB2	*Litonotus cygnus*	temperate	10	5
Li	*Litonotus lamella*	temperate	10	5

3. Concluding Remarks. – Studies of unusual bioactive secondary metabolites from ciliates are of recent date. Besides the variety of terpenoid aldehydes mentioned here, we are only aware of toxic pyrrolic compounds – keronopsins – isolated from *Pseudokeronopsis rubra* [5]; these follow more specialized lines of study concerning pigments, however [6].

The diterpenoids reported here – which can be imagined, as shown by **A**, to arise from a geranylgeranyol precursor – may have a double bearing on these problems. First, the nature of terpenoid aldehydes structurally relates these *E. focardii* products to both *E. crassus* euplotins [1] and *E. raikovi* raikovenal [3] notwithstanding such vastly different conditions as found in isolated Antarctica and temperate, tropical, and equatorial seas. Second, although lack at present of suitable test organisms prevented exploring thoughroughly the ecological role of focardin (**9a**/**9b**)/epoxyfocardin (**8a**/**8b**), the high bioactivity of the latter against predacious ciliates such as *L. lamella* and *L. cygnus* suggests a defensive role of these substances and, therefore, that ciliates are in strong competition even in Antarctica. This finds a parallel in recent reports about defensive compounds of opisthobranch molluscs and their octocoral preys [7].



A

Substantial production of epoxyfocardin (**8a**/**8b**) weakens the hypothesis of a simple vestigial trait inherited from ancestors of shorter latitudes. It may thus be argued that the severe environmental conditions characterizing Antarctic habitats do not diminish interspecific competition among eukaryotic microorganisms of the littoral zone.

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Experimental Part

1. *General.* See [4]. Moreover: Flash chromatography (FC): *Merck Si-60*, 15–25 μm. Reversed-phase FC: *Merck LiChrosorb RP18*, 20–50 μm. HPLC: *Merck LiChrosorb Si-60* (7 μm) with hexane/i-PrOH or *Merck LiChrospher 100 RP18* (5 μm) with MeCN/H_2O; for prep. HPLC, 25 × 1 cm column, solvent flux 5 ml min^{-1}, and UV monitoring at λ 220 nm. Polarimetric data: *JASCO-DIP-181* polarimeter; $[\alpha]_D$ in dm^{-1} deg ml g^{-1}. NMR: multiplicities from DEPT [8]; for **8a**/**8b**, ^1H,^1H [9]. HMBC: heteronuclear multiple-quantum coherence pulse sequence [10a], using a dedicated probe [10b]. Selective differential NOE (obtained with 4 s preirradiation): irradiated proton → % NOE on the observed proton(s). EI-MS (*m/z*; %): *Kratos-MS80* mass spectrometer with home-built data system. Molecular-mechanics calculations (MM) were carried out by PCMODEL 4.0 (MMX force field) by *Serena Software*, Bloomington, Indiana.

2. *Culture and Isolation.* The two lines of the ciliate *E. focardii* for this study, line A and line B, were derived from strain TN1 collected from a cove, east of the Italian Antarctic Base at Terra Nova Bay (Ross Sea, 74°42′ S, 164°06′ E). Recorded environmental physical and chemical parameters [11] of the collecting location were: temperature −1.8°; pH 8.1–8.2, salinity 35%. These conditions were mimicked in the laboratory in producing large mass cultures of the two ciliate lines, except that the temperature was raised to 5° to speed up cell fission rate. In pursuing a close standardization of the cell culturing techniques, the culture medium used was a defined, artificial seawater prepared according to *Allen*'s formula [12]. The marine green microalga *Dunaliella tertiolecta* BUTCHER, 1959, was used as food organism. Culturing of this microalga [4] was modified by storing cultures at 5° for 24 h before use. Such acclimatization caused a selection among microalgal cells, producing a mat precipitate on the bottom of the *Erlenmeyer* flasks. Therefore, dead microalgae were removed before feeding ciliate cultures. From mass cultures of 21 and 24 l of line A and line B, 3.5 and 4.5 ml of tightly packed *E. focardii* cells, respectively (for a total of $1.62 \cdot 10^8$ cells), were harvested and promptly resuspended with 15 and 17 ml, respectively, of abs. EtOH and stored at −80°. Being identical under any respect, the two collections were combined and filtered on a glass frit and thoroughly washed with fresh EtOH. The combined filtrates were evaporated and partitioned between hexane/AcOEt 9:1 and H_2O, collecting the org. phase that was then evaporated to give a residue (220 mg) of mostly fats, sterols, and algal chlorophylls, besides the new terpenoids. The aq. phase contained mainly nucleosides. The 220-mg residue was subjected to FC on *RP18* (1 g), eluting the first three fractions with MeCN and the last two with MeOH and CH_2Cl_2. The former were combined and subjected to reversed-phase HPLC (*RP18 Lichrospher*, MeCN/H_2O 8:2, 6.0 ml/min solvent flux, monitoring at λ 220 nm): **8a**/**8b** (t_R 9.0 min) and **9a**/**9b** (t_R 13.5 min). Further purification was achieved by HPLC (*Si60*, hexane/i-PrOH 97:3 for and 98:2, resp.): **8a**/**8b** (4 mg), t_R 10.0, and **9a**/**9b** (1.3 mg), t_R 7.0 min. The cyclization product, **10**, from **8a**/**8b** (2 mg), obtained as described a *Footnote 2*, was purified by *Si60* HPLC with hexane/i-PrOH 98:2:1 mg (t_R 6.8 min). *L. lamella* ELB2 was collected along the beach of Marina di Campo, Elba island, on May 1995.

Epoxyfocardin (**8a**/**8b**): $[\alpha]_D^{20} = +29$ ($c = 0.2$, EtOH). EI-MS: 318 (6, M^+), 300 (13, $[M - H_2O]^{+\cdot}$), 285 (3), 272 (6), 257 (7), 201 (32), 185 (48), 173 (41), 145 (69), 131 (68), 105 (75), 91 (94), 79 (100). HR-EI-MS: 318.2192 ± 0.001 ($C_{20}H_{30}O_3^+$, calc. 318.2195).

(1R,6aS,9S,9aR,9bR)-9-[(Z)-3-(3,3-Dimethyloxiran-3-yl)-1-methylprop-1-enyl]-1,3,5,6,6a,7,8,9,9a,9b-decahydroazuleno[4,5-c]furan-1-ol (**8b**): Only NMR signals distinct from those of **8a** (see *Table 1*) are reported. ^{1}H-NMR ($CDCl_3$)[1]): 2.64 (br. *d*, $J(2,1) = 10.9$, H–C(2)); 3.33 (*dt*, $J(10,9\alpha) = 3.8$, $J(10,1) = J(10,9\beta) = 9.1$, H–C(10)); 5.20 (br. *t*, $J(12,13) = 6.8$, H–C(12)); 4.58 (*tdd*, $J(19\alpha,2) \approx J(19\alpha,5\alpha) = 2.0$, $J(19\alpha,4) = 3.2$, $J_{gem} = 12.2$, H_α–C(19)); 4.23 (*td*, $J(19\beta,2) \approx J(19\beta,4) = 2.7$, $J_{gem} = 12.2$, H_β–C(19)); 5.32 (br. *d*, $J(20,OH) = 2.9$, H–C(20)); 3.92 (*d*, $J(20,OH) = 2.9$, OH). ^{13}C-NMR ($CDCl_3$)[1]): 49.47 (*d*, C(1)); 51.66 (*d*, C(2)); 120.74 (*d*, C(4)); 27.00 (*t*, C(5)); 34.36 (*t*, C(6)); 48.82 (*d*, C(7)); 33.68 (*t*, C(8)); 41.84 (*d*, C(10)); 122.34 (*d*, C(12)); 27.85 (*t*, C(13)); 64.43 (*d*, C(14)); 24.76 (*q*, C(16)); 18.99 (*q*, C(17)); 22.96 (*q*, C(18)); 70.58 (*t*, C(19)); 102.82 (*d*, C(20)).

Focardin (**9a**/**9b**): EI-MS: 302 (20, $M^{+\cdot}$), 284 (38, $[M - H_2O]^{+\cdot}$), 269 (12, $[M - H_2O - Me]^{+}$), 215 (28), 201 (11), 190 (36), 109 (54), 91 (51), 79 (42), 67 (40), 55 (56), 41 (100). HR-EI-MS: 302.2254 ± 0.005 ($C_{20}H_{30}O_2^{+\cdot}$, calc. 302. 2246); 284.2137 ± 0.001 ($C_{20}H_{28}O^{+\cdot}$, calc. 284.2140).

(1S,6aS,9S,9aR,9bR)-9-[(Z)-1,5-Dimethylhexa-1,4-dienyl]-1,3,5,6,6a,7,8,9,9a,9b-decahydroazuleno[4,5-c]furan-1-ol (**9a**): ^{1}H-NMR ($CDCl_3$)[1]: 1.50 (*dt*, $J(1,10) = 8.7$, $J(1,2) \approx J(1,7) = 10.9$, H–C(1)); 2.51 (*qdd*, $J(2,19\alpha) \approx J(2,19\beta) \approx J(2,4) \approx J(2,5\alpha) = 2.4$, $J(2,20) = 6.2$, $J(2,1) = 10.9$, H–C(2)); 5.71 (*td*, $J(4,19\beta) \approx J(4,5\alpha) = 2.3$, $J(4,5\beta) = 8.2$, H–C(4)); 2.19 (*m*, H_β–C(5)); 1.96 (*m*, H_α–C(5)); 1.14 (*ddt*, $J(6\beta,5\beta) = 2.5$, $J(6\beta,7) = 10.2$, $J(6\beta,5\alpha) \approx J_{gem} = 13.0$, H_β–C(6)); 1.80 (*m*, H_α–C(6)); 1.25–1.60 (series of *m*, H–C(7), 2 H–C(8)); 1.63 (*m*, 2 H–C(9)); 2.99 (*dt*, $J(10,9\alpha) = 4.2$, $J(10,1) = J(10,9\beta) = 8.7$, H–C(10)); 5.20 (br. *t*, $J(12,13) = 6.8$, H–C(12)); 2.65 (*t*, $J(13,12) = J(13,14) = 6.8$, 2 H–C(13)); 5.07 (br. *t*, $J(14,13) = 6.8$, H–C(14)); 1.68 (br. *s*, 3 H–C(16)); 1.61 (br. *s*, 3 H–C(17)); 1.66 (br. *s*, 3 H–C(18)); 4,51 (*tdd*, $J(19\alpha,2) \approx J(19\alpha,5\alpha) = 2.0$, $J(19\alpha,20) = 3.2$, $J_{gem} = 12.2$, H_α–C(19)); 4.32 (*td*, $J(19\beta,2) \approx J(19\beta,4) = 2.7$, $J_{gem} = 12.2$, H_β–C(19)); 5.62 (*m*, H–C(20)). ^{13}C-NMR ($CDCl_3$)[1]): 49.66 (*d*, C(1)); 51.97 (*d*, C(2)); 140.40 (*s*, C(3)); 120.80 (*d*, C(4)); 27.07 (*t*, C(5)); 34.49 (*t*, C(6)); 48.63 (*d*, C(7)); 34.24 (*t*, C(8)); 28.60 (*t*, C(9)); 47.38 (*d*, C(10)); 140.40 (*s*, C(11)); 122.77 (*d*, C(12)); 27.71 (*t*, C(13)); 126.25 (*d*, C(14)); 137.87 (*s*, C(15)); 25.70 (*q*, C(16)); 70.44 (*t*, C(19)); 99.62 (*d*, C(20)).

Data of **9b**. Only NMR signals distinct from those of **9a** (see above) are reported. ^{1}H-NMR ($CDCl_3$)[1]): 2.55 (br. *d*, $J(2,1) = 10.9$, H–C(2)); 2.95 (*dt*, $J(10,9\alpha) = 4.6$, $J(10,1) = J(10,9\beta) = 9.0$, H–C(10)); 4.58 (*tdd*, $J(19\alpha,2) \approx J(19\alpha,5\alpha) = 2.0$, $J(19\alpha,20) = 3.2$, $J_{gem} = 12.2$, H_α–C(19)); 4.25 (*td*, $J(19\beta,2) \approx J(19\beta,4) = 2.7$, $J_{gem} = 12.2$, H_β–C(19); 5.60 (*s*, H–C(20)). ^{13}C-NMR ($CDCl_3$)[1]): 49.84 (*d*, C(1)); 51.74 (*d*, C(2)); 121.01 (*d*, C(4)); 70.75 (*t*, C(19)).

2,4,5,5a,6,7,7a,10,11,12a,12b,12c-Dodeca-α,α,8-trimethyl-1,12-dioxaazuleno[7,8,1-cde]cyclopentacyclononene-11-methanol (**10**). ^{1}H-NMR ($CDCl_3$)[1]): 1.35 (*dt*, $J(1,10) = 8.7$, $J(1,2) \approx J(1,7) = 10.9$, H–C(1)); 2.74 (*qdd*, $J(2,19\alpha) \approx J(2,19\beta) \approx J(2,4) \approx J(2,5\alpha) = 2.4$, $J(2,20) = 6.2$, $J(2,1) = 10.9$, H–C(2)); 5.68 (*td*, $J(4,19\beta) \approx J(4,5\alpha) = 2.3$, $J(4,5\beta) = 8.2$, H–C(4)); 2.20 (*dddd*, $J(5\beta,6\beta) = 2.4$, $J(5\beta,6\alpha) = 6.2$, $J(5\beta,4) = 8.2$, $J_{gem} = 15.9$, H_β–C(5)); 1.90 (*m*, H_α–C(5)); 0.98 (*ddt*, $J(6\beta,5\beta) = 2.5$, $J(6\beta,7) = 10.2$, $J(6\beta,5\alpha) \approx J_{gem} = 13.0$, H_β–C(6)); 1.80 (*m*, H_α–C(6)); 1.25–1.60 (series of *m*, H–C(7), 2 H–C(8)); 1.63 (*m*, 2 H–C(9)); 3.60 (*dt*, $J(10,9\alpha) \approx J(10,1) = 8.7$, $J(10,9\beta) = 10.5$, H–C(10)); 5.35 (*qdd*, $J(12,Me) = 1.5$, $J(12,13a) = 6.9$, $J(12,13b) = 9.0$, H–C(12)); 1.97 (*ddd*, $J(13a,12) = 6.9$, $J(13a,14) = 2.4$, $J_{gem} = 13.3$, H_a–C(13)); 2.54 (*ddd*, $J(13b,12) = 9.0$, $J(13b,14) = 11.9$, $J_{gem} = 13.3$, H_b–C(13)); 3.31 (*dd*, $J(14,13a) = 2.4$, $J(14,13b) = 11.9$, H–C(14)); 1.20 (*s*, 3 H–C(16)); 1.17 (*s*, 3 H–C(17)); 1.82 (br. *s*, 3 H–C(18)); 4.37 (*tdd*, $J(19\alpha,2) \approx J(19\alpha,5\alpha) = 2.0$, $J(19\alpha,20) = 3.2$, $J_{gem} = 12.2$, H_α–C(19)); 4.16 (*td*, $J(19\beta,2) \approx J(19\beta,4) = 2.7$, $J_{gem} = 12.2$, H_β–C(19)); 5.59 (*d*, $J(20,2) = 6.2$, H–C(20)). EI-MS: 318 (2, $M^{+\cdot}$), 300 (1, $[M - H_2O]^{+\cdot}$), 230 (100, $[M - C_4H_8O_2]^{+\cdot}$), 215 (14), 201 (11), 188 (16), 145 (15), 131 (12), 105 (16), 91 (20), 79 (17), 67 (13), 55 (17), 43 (20). HR-EI-MS: 318.2190 ± 0.001 ($C_{20}H_{30}O_3^{+\cdot}$, calc. 318.2195); 230.1669 ± 0.001 ($C_{16}H_{22}O^{+\cdot}$, calc. 230.1670).

3. *Toxicity Assays*. Cells of each ciliate strain used for the biological assays (*Table 3*) were members of a clone grown in excess food at the time they were placed in the test solns. All strains were run in parallel. Because of their insolubility in H_2O, focardin (**9a**/**9b**) and epoxyfocardin (**8a**/**8b**) were assayed in H_2O/EtOH mixtures: preserved in abs. EtOH stock soln. at a concentration of 1 mg/ml, they were taken as an aliquot of the stock soln. and stirred into sterile artificial seawater to prepare solns. at the highest concentration to be tested (20 μg/ml, *Table 3*). From this, a series of solns. at consecutive (1/2) steps in concentration were prepared. In any case, freshly prepared solns. were used for bioassays. Four concentration levels of **8a**/**8b** and **9a**/**9b** (from 5 μg/ml to 20 μg/ml) easily spanned the range from 100% survival to 100% mortality in the whole set of selected strains. Accordingly, the EtOH concentration in the test solns. ranged from 0.5 to 2%. Assays entailed treatment of six single cells for each terpenoid dosage for each strain. Solvent (at the highest concentration of 2% EtOH occurring in the test solns.) and seawater controls were run simultaneously. After 16 ± 1 h, cytotoxicity was assessed microscopically as complete loss of cell motility, and the lowest dosage (expressed in μg/ml) for 100% kills was scored (*Table 3*).

4. *Synthesis of MTPA Esters*. In dry pyridine (0.5 ml), 1mg of the **8a**/**8b** mixture was treated with 3 equiv. of (+)-(*S*)-MTPA-Cl and 0.5 mg of 4-(dimethylamino)pyridine. The same procedure was adopted for reaction with (−)-(*R*)-MTPA-Cl. In each case, the mixture was quenched after 3 h with 1 ml of sat. aq. $CuSO_4$ soln. followed by 4 ml of Et_2O and filtration on a *Whatman* phase-separation filter. The org. phase was evaporated and analyzed by ^{1}H-NMR, detecting two diastereoisomers in each case, which were separated by HPLC (*Si60*; hexane/i-PrOH 98:2). Esterification with (+)-(*S*)-MTPA-Cl and HPLC separation led to **11a** (t_R 5.5 min) and **11b** (t_R 7.0 min) in 4:1 ratio, while esterification with (−)-(*R*)-MTPA-Cl and HPLC separation led to **12a** (t_R 6.8 min) and **12b** (t_R 6.3 min) in 3:7 ratio.

Data of **11a**. ^{1}H-NMR ($CDCl_3$)[1]): 1.35 (*dt*, $J(1,10) = 8.8$, $J(1,2) \approx J(1,7) = 10.9$, H–C(1)); 2.74 (br. *d*, $J(2,1) = 10.9$, H–C(2)); 5.67 (*td*, $J(4,19\beta) \approx J(4,5\alpha) = 2.5$, $J(4,5\beta) = 8.3$, H–C(4)); 2.20 (*m*, H_β–C(5)); 1.94 (*m*,

H_α–C(5)); 1.25–1.85 (series of *m*, 2 H–C(6), H–C(7), 2 H–C(8)); 1.74 (*m*, H_α–C(9)); 1.36 (*m*, H_β–C(9)); 2.98 (*dt*, $J(10,9\alpha) = 3.7$, $J(10,9\beta) \approx J(10,1) = 8.8$, H–C(10)); 5.24 (*qdd*, $J(12,Me) = 1.5$, $J(12,13a) = 5.3$, $J(12,13b) = 9.1$, H–C(12)); 2.15 (*ddd*, $J(13a,12) = 5.3$, $J(13a,14) = 7.1$, $J_{gem} = 14.9$, H_a–C(13)); 2.28 (*ddd*, $J(13b,14) = 5.4$, $J(13b,12) = 9.1$, $J_{gem} = 14.9$, H_b–C(13)); 2.71 (*dd*, $J(14,13b) = 5.4$, $J(14,13a) = 7.1$, H–C(14)); 1.31 (*s*, 3 H–C(16)); 1.28 (*s*, 3 H–C(17)); 1.74 (br. *s*, 3 H–C(18)); 4.51 (*tdd*, $J(19\alpha,2) \approx J(19\alpha,5\alpha) = 2.0$, $J(19\alpha,20) = 3.4$, $J_{gem} = 12.2$, H_α–C(19)); 4.40 (*qd*, $J(19\beta,2) \approx J(19\beta,4) \approx J(19\beta,5\beta) = 2.7$, $J_{gem} = 12.2$, H_β–C(19)); 6.38 (*d*, $J(20,2) = 4.5$, H–C(20)); 3.54 (*s*, MeO); 7.30–7.50 (*m*, arom. H). EI-MS: 534 (1, $M^{+\cdot}$), 300 (35, $[M - MTPA]^{+}$), 282 (22, $[M - MTPA - H_2O]^{+\cdot}$), 265 (11), 228 (51), 214 (31), 211 (31), 197 (43), 189 (61), 105 (70), 91 (58), 79 (52), 43 (100).

Data of **11b**. ^{1}H-NMR ($CDCl_3$)[1]): 1.50 (*dt*, $J(1,10) = 9.0$, $J(1,2) \approx J(1,7) = 11.8$, H–C(1)); 2.90 (br. *d*, $J(2,1) = 11.8$, H–C(2)); 5.72 (*tdd*, $J(4,19\beta) \approx J(4,19\alpha) = 1.9$, $J(4,5\alpha) = 4.3$, $J(4,5\beta) = 9.0$, H–C(4)); 2.17 (*m*, H_β–C(5)); 1.94 (*m*, H_α–C(5)); 1.25–1.85 (series of *m*, 2 H–C(6), H–C(7), 2 H–C(8)); 1.87 (*m*, H_α–C(9)); 1.32 (*m*, H_β–C(9)); 3.47 (*dt*, $J(10,9\alpha) = 4.4$, $J(10,9\beta) \approx J(10,1) = 9.0$, H–C(10)); 5.14 (*qdd*, $J(12,Me) = 1.5$, $J(12,13a) = 6.0$, $J(12,13b) = 8.9$, H–C(12)); 2.22 (*ddd*, $J(13a,12) = 6.0$, $J(13a,14) = 9.1$, $J_{gem} = 14.9$, H_a–C(13)); 2.35 (*ddd*, $J(13b,14) = 5.4$, $J(13b,12) = 8.9$, $J_{gem} = 14.9$, H_b–C(13)); 2.72 (*dd*, $J(14,13b) = 5.4$, $J(14,13a) = 7.1$, H–C(14)); 1.28 (*s*, 3 H–C(16)); 1.27 (*s*, 3 H–C(17)); 1.76 (br. *s*, 3 H–C(18)); 4.31 (*tdd*, $J(19\alpha,2) \approx J(19\alpha,5\alpha) = 1.8$, $J(19\alpha,20) = 3.0$, $J_{gem} = 12.2$, H_α–C(19)); 4.16 (*qd*, $J(19\beta,2) \approx J(19\beta,4) \approx J(19\beta,5\beta) = 2.5$, $J_{gem} = 12.2$, H_β–C(19)); 6.35 (*s*, H–C(20)); 3.55 (*s*, MeO); 7.30–7.50 (*m*, arom. H). EI-MS: 534 (0.7, $M^{+\cdot}$), 300 (29, $[M - MTPA]^{+}$), 282 (16, $[M - MTPA - H_2O]^{+\cdot}$), 265 (9), 228 (38), 214 (34), 211 (32), 197 (37), 189 (75), 105 (70), 91 (53), 79 (42), 43 (100).

Data of **12a**. ^{1}H-NMR ($CDCl_3$)[1]): 1.47 (*dt*, $J(1,10) = 9.0$, $J(1,2) \approx J(1,7) = 11.8$, H–C(1)); 2.85 (br. *d*, $J(2,1) = 11.8$ H–C(2)); 5.65 (*tdd*, $J(4,19\beta) \approx J(4,19\alpha) = 1.9$, $J(4,5\alpha) = 4.3$, $J(4,5\beta) = 9.0$, H–C(4)); 2.22 (*m*, H_β–C(5)); 1.96 (*m*, H_α–C(5)); 1.25–1.85 (series of *m*, 2 H–C(6), H–C(7), 2 H–C(8)); 1.81 (*m*, H_a–C(9)); 1.50 (*m*, H_β–C(9)); 3.07 (*dt*, $J(10,9\alpha) = 3.7$, $J(10,9\beta) \approx J(10,1) = 8.8$, H–C(10)); 5.25 (*qdd*, $J(12,\ Me) = 1.5$, $J(12,13a) = 9.1$, $J(12,13b) = 5.3$, H–C(12)); 2.07 (*ddd*, $J(13a,14) = 5.3$, $J(13a,12) = 9.1$, $J_{gem} = 14.9$, H_a–C(13)); 2.21 (*ddd*, $J(13b,14) = 7.5$, $J(13b,12) = 5.3$, $J_{gem} = 14.9$, H_b–C(13)); 2.66 (*dd*, $J(14,13b) = 7.5$, $J(14,13a) = 5.3$, H–C(14)); 1.28 (*s*, 3 H–C(16)); 1.22 (*s*, 3 H–C(17)); 1.76 (br. *s*, 3 H–C(18)); 4.41 (*m*, H_α–C(19)); 4.27 (*m*, H_β–C(19)); 6.39 (*d*, $J(20,2) = 4.4$, H–C(20)); 3.49 (*s*, MeO); 7.30–7.50 (*m*, arom. H). EI-MS: Practically superimposable to that of **11a**.

Data of **12b**. ^{1}H-NMR ($CDCl_3$)[1]): 1.47 (*dt*, $J(1,10) = 9.2$, $J(1,2) \approx J(1,7) = 11.8$, H–C(1)); 2.85 (br. *d*, $J(2,1) = 11.8$, H–C(2)); 5.78 (*tdd*, $J(4,19\beta) \approx J(4,19\alpha) = 1.9$, $J(4,5\alpha) = 4.3$, $J(4,5\beta) = 9.0$, H–C(4)); 2.15 (*m*, H_α–C(5)); 1.93 (*m*, H_β–C(5)); 1.25–1.85 (series of *m*, 2 H–C(6), H–C(7), 2 H–C(8)); 1.84 (*m*, H_α–C(9)); 1.28 (*m*, H_β–C(9)); 3.43 (*dt*, $J(10,9\alpha) = 4.7$, $J(10,9\beta) \approx J(10,1) = 9.2$, H–C(10)); 5.11 (*qt*, $J(12,Me) = 1.5$, $J(12,13) = 6.8$, H–C(12)); 2.37 (*t*, $J(13,12) = J(13,14) = 6.8$, 2 H–C(13)); 2.77 (*t*, $J(14,13) = 6.8$, H–C(14)); 1.31 (*s*, 3 H–C(16)); 1.29 (*s*, 3 H–C(17)); 1.58 (br. *s*, 3 H–C(18)); 4.44 (*m*, 2 H–C(19)); 6.35 (*s*, H–C(20)); 3.52 (*s*, MeO); 7.30–7.50 (*m*, arom. H). EI-MS: Practically superimposable to that of **11b**.

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