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REVIEW ARTICLE

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Bioactive molecules from ciliates: Structure, activity, and applicative potential

Claudio Alimenti[1] | **Federico Buonanno**[2] | **Graziano Di Giuseppe**[3,4] | **Graziano Guella**[5] | **Pierangelo Luporini**[1] | **Claudio Ortenzi**[2] | **Adriana Vallesi**[1]

[1]Laboratory of Eukaryotic Microbiology and Animal Biology, School of Biosciences and Veterinary Medicine, University of Camerino, Camerino, Italy

[2]Laboratory of Protistology and Biology Education, Department of Education, Cultural Heritage, and Tourism (ECHT), Università degli Studi di Macerata, Macerata, Italy

[3]Unit of Protistology, Department of Biology, University of Pisa, Pisa, Italy

[4]MARinePHARMA Center, University of Pisa, Pisa, Italy

[5]Bioorganic Chemistry Lab, Department of Physics, University of Trento, Trento, Italy

Correspondence
Adriana Vallesi, School of Biosciences and Veterinary Medicine, via Gentile III da Varano, 62032 Camerino (MC) Italy.
Email: adriana.vallesi@unicam.it

Funding information
UniversitÃ degli Studi di Camerino

Abstract

Ciliates are a rich source of molecules synthesized to socialize, compete ecologically, and interact with prey and predators. Their isolation from laboratory cultures is often straightforward, permitting the study of their mechanisms of action and their assessment for applied research. This review focuses on three classes of these bioactive molecules: (i) water-borne, cysteine-rich proteins that are used as signaling pheromones in self/nonself recognition phenomena; (ii) cell membrane-associated lipophilic terpenoids that are used in interspecies competitions for habitat colonization; (iii) cortical granule-associated molecules of various chemical nature that primarily serve offence/defense functions.

KEYWORDS
climacostol, euplotins, pheromones, resorcinol, signaling proteins, terpenoids

BIOACTIVE molecules are generally recognize as those natural products—organic compounds that are synthesized by living systems—which common wisdom suggests to be structurally 'optimized' and selected for by evolution in order to confer their source organisms with a competitive, ecological or evolutionary advantage (Hanson, 2003). Bioactive molecules are commonly regarded as playing no direct role in the essential processes of the life. This role is distinctive of primary metabolites (Thirumurugan et al., 2018). Nevertheless, they govern and shape the reproductive success of their source organisms by driving defense/offence mechanisms, interactions with the environment and phenomena of self/nonself discrimination. This wide range of activities overall account well for the strong interest that bioactive molecules arouse both in basic research and bioprospecting for drug discovery and design in the fields of medical chemistry and pharmacognosy (Khazir et al., 2014; Newman & Cragg, 2014).

The vast array of effective sources of bioactive molecules is historically dominated by species from prokaryotes (Javed et al., 2011; Penesyan et al., 2010), plants (Chitra et al., 2019), (micro-)fungi and filter-feeding animals, in particular porifera, bryozoa and tunicates (Haefner, 2003; Kellner Filho et al., 2019; Molinski et al., 2009; Zhang et al., 2005). Species from the vast polyphyletic confederation of protists are largely relegated on the margins. However, research on easily cultivable diatoms, algae and ciliates provide evidence that also these microeukaryotes are a mine of natural products that deserve strong interest for biotechnological applications.

[Correction added on 12 May 2022, after first online publication: CRUI funding statement has been added.]

This article provides an overview of ciliate bioactive molecules that have been characterized to different extents for their chemical structures, analyzed for their activities and assessed for their potential applications. They are represented by (i) water-borne signaling proteins collectively designated as 'pheromones', (ii) membrane lipid-borne terpenoids collectively designated as 'euplotins' and (iii) cortical vesicle-associated metabolites of more heterogeneous chemical nature (alkaloids, lactones and polyketides) variously named mostly with reference to their source ciliate.

WATER-BORNE PHEROMONES

Ciliate pheromones are diffusible molecules that distinguish chemically conspecific cells, which are otherwise morphologically identical. In relation with their functional association with genetically determined systems of so-called 'mating types'—two or multiple within a species and questionably regarded as 'sexes'—they regulate self/nonself recognition phenomena underlying the cell decision to shift from the vegetative (growth) life stage to the sexual stage manifested as cell–cell unions in mating pairs (Luporini & Miceli, 1986).

Pheromones have a long story, lately reviewed (Luporini, Alimenti, et al., 2016; Luporini, Pedrini, et al., 2016), in experimental ciliatology. Their original identification in the supernatant of *Euplotes patella* cultures via mating induction assays dates back nearly one century (Kimball, 1942). However, their isolation and chemical characterization were to be pioneered only decades later by Akio Miyake and collaborators in *Blepharisma japonicum* (Miyake, 1981). The two pheromones isolated from *B. japonicum* are unique in that their chemical nature differs strikingly. One, designated 'gamone-1' (or 'blepharmone'), is a highly unstable glycoprotein of 272 amino acids and six covalently linked sugars (Sugiura & Harumoto, 2001). The second, designated 'gamone-2 (or 'blepharismone'), was instead determined to be a very stable tryptophan derivative, namely a calcium-3-(2-formylamino-5-hydroxybenzoil) lactate (Miyake, 1981). In addition to differing in their chemical nature, gamone-1 and gamone-2 also differ in their spectrum of specificity, only the former being species-specific and the latter, instead, common to multiple species of *Blepharisma* (Kobayashi et al., 2015). This lack of species-specificity, together with the eccentricity of the chemical nature have thus suggested that gamone-2 may act not as a genuine pheromone, but rather as a hapten-like molecule that binds to, and activates the glycoprotein gamone-1 (Luporini & Miceli, 1986; Nanney, 1980). Support for this view derives from observing that *B. japonicum*, alike heterotriches in general, regularly practices self-mating, which clearly implies that both gamones 1 and 2 (yet isolated from transiently not self-mating cultures of two supposed distinct cell types, I and II) must be co-released by the same cells.

Differently from *Blepharisma* pheromones, those isolated from *Dileptus anser* and various species of *Euplotes*, namely *E. raikovi*, *E. octocarinatus*, *E. nobilii*, *E. petzi* and *E. crassus*, all are high-stable proteins that maintain long-lasting integrity and biological activity in their natural environment. For the *D. anser* pheromones (three isolated from interbreeding cultures), the characterization is narrowed to a molecular mass in the range of 3–4.5 kDa and a strong propensity to associate into unstable oligomers (Parfenova et al., 1989; Uspenskaya & Yudin, 2016). Far more characterized are *Euplotes* pheromones, known to be encoded by multiple series of codominant alleles at the *mat*-locus of the micronuclear genome (Vallesi et al., 2014). Their largely variable amino acid sequences, determined in significant numbers from each species, range in length from 32 amino acid residues in *E. petzi* (Pedrini et al., 2017) to 109 in *E. octocarinatus* (Brünen-Nieweler et al., 1998), and limit an extensive intraspecific and interspecific conservation only to the cysteine residues that are regularly paired into intrachain disulfide bonds (Figure 1). Their variability contrasts with the remarkable extent of intraspecific and interspecific identity of the signal/prepeptide and pro-segment sequences that are proteolytically cleaved from the immature cytoplasmic pheromone precursors (pre-pro-pheromones) before the secretion of the active pheromone (Ricci et al., 2019). Variations in the pheromone amino acid sequences may also closely reflect deep interspecies differences in ecological adaptation. Instructive is the case of the cold-adapted pheromones from the bipolar species, *E. nobilii*, which unfold in the range 55−−70°C, as compared to their homologs from the closely related temperate-water species, *E. raikovi*, that unfold only over 95°C (Cazzolli et al., 2013; Geralt et al., 2013). Like psychrophilic proteins, *E. nobilii* pheromones are significantly richer than *E. raikovi* pheromones in polar amino acids (44% vs. 30%), in particular threonine (12% vs. 6%) and asparagine (8% vs. 4%), and poorer (44% vs. 57%) in hydrophobic ones, in particular leucine (1% vs. 7%) and isoleucine (2% vs. 6%). These chemical features are overall consistent with stronger molecular interactions with the solvent and weaker stabilizing forces of the protein core (Alimenti et al., 2009).

By applying NMR spectroscopy and/or X-ray crystallography to native protein preparations, a variety of *Euplotes* pheromone molecular structures have been resolved: E*r*-1, E*r*-2, E*r*-10, E*r*-11, E*r*-13, E*r*-22 and E*r*-23 from *E. raikovi*; E*n*-1, E*n*-2 and E*n*-6 from *E. nobilii*; and E*p*-1 from for *E. petzi*. Comparative analyses of the specificities of these pheromone structures (the only known for water-borne proteins from protists) were object of previous reviews (Luporini, Alimenti, et al., 2016; Luporini, Pedrini, et al., 2016). In this context, one most relevant concept is worth being reiterated

(A)

E. crassus

$XCX_2CX_4CX_2CX_{11}CX_6CXCCX_2CX_5CX$

(Ec-1, Ec-2, Ec-3, Ec-4)

DDHCPTDVLMTCGYLQGRYNQGNYEEVGGLCNMSAEFCHCCSACDEPEVSPYSNCE
1 10 20 30 40 50 56

(Ec-α)

E. octocarinatus

$X_{2\text{-}4}CX_{8\text{-}12}CX_{5\text{-}6}CX_{5\text{-}6}CX_{9\text{-}11}CX_{16\text{-}25}CX_{5\text{-}6}CX_{16}CXCX_{13\text{-}15}CX_{3\text{-}5}$

(Phr1, Phr1*, Phr2, Phr2*, Phr3, Phr3*, $Phr5^{-1}$, $Phr5^{-2}$)

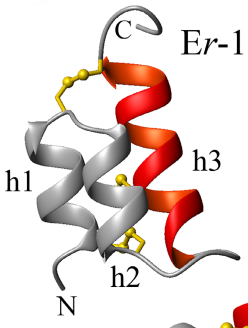
YTYGCPQTNTPTQQDCYDAMYYTFMAMCDLYPDPEHPMFPSYD
1 10 20 30 40

SCQEESDSADEFYTNQCGCGGYGMAAAHDQVCLLALGVCIPE
(Phr4) 50 60 70 80 85

E. raikovi

$X_{2\text{-}6}CX_6CX_4CX_{2\text{-}3}CX_{5\text{-}8}CX_{7\text{-}9}CX_{1\text{-}5}$

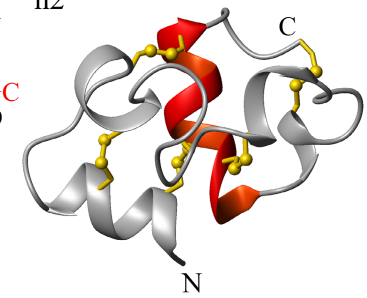
(Er-1, Er-2, Er-4, Er-5, Er-6, Er-7, Er-8, Er-10, Er-11, Er-13, Er-20, Er,21, Er-22)



Er-23

GECEQCFSDGGDCTTCFNNGTGPCANCLAGYPAGCSNSDCTAFLSQCYGGC
1 10 20 30 40 50

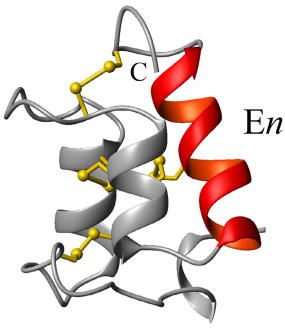
(Er-23)



E. nobilii

$X_{10\text{-}13}CX_{10\text{-}12}CX_{3\text{-}8}CX_{2\text{-}3}CCX_2CX_{8\text{-}10}CX_{5\text{-}10}CX_{0\text{-}3}$

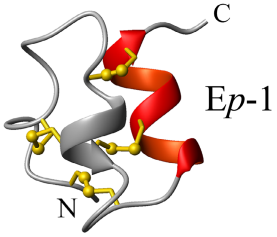
(En-1, En-2, En-6, En-A1, En-A2, En-A3, En-A4)



E. petzi

$XCX_3CX_5CX_2CCX_3CX_4CX_3CX_3$

(Ep-1, Ep-2, Ep-3, Ep-4))



(B)

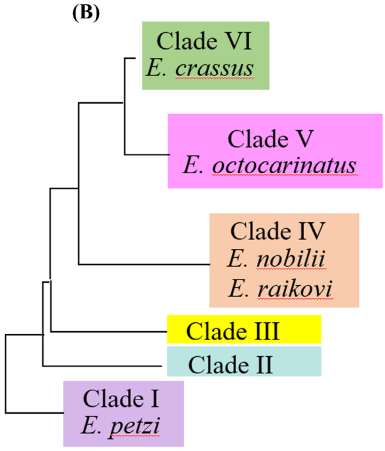


FIGURE 1 Amino acid sequences and NMR solution structures of *Euplotes* pheromones. (A) For each species-specific pheromone family, represented by the members listed between brackets, only the consensus sequence is reported. The conserved cysteines are highlighted in red, and X stands for any amino acid. The sequence of the *E. crassus* pheromone Ec-α, the only one known from a sub-family distinct from that which includes the other pheromone sequences, is reported individually and written in the single letter code. Analogously, the sequences of the *E. octocarinatus* pheromone Phr4 and *E. raikovi* pheromone Er-23 are reported individually, as they represent structurally deviant family members. The molecular structures of *E. raikovi* pheromones Er-1 (PDB code, 1ERC) and Er-23 (PDB code, 1HA8), *E. nobilii* pheromone En-6 (PDB code, 2JMS), and *E. petzi* pheromone Ep-1 (PDB code, 2N2S) are shown (in ribbon diagrams) as representative of each pheromone family. The disulfide bonds stabilizing the molecular structures are shown as yellow ball-and-stick diagrams. The helix labeled h3 in the Er-1 pheromone structure is highlighted in red to indicate the tight intraspecific and interspecific conservation of the structural backbone. N and C identify the molecule amino and carboxyl termini, respectively. (B) Very simplified version of the *Euplotes* phylogenetic tree, articulated into six clades (boxes), showing the positions of the five species analyzed for the pheromone structures

in relation to the pheromone mechanism of action. Despite considerable variations in the amino acid sequences, *Euplotes* pheromones form species-specific families of structurally homologous proteins that closely mimic one another in their molecular structures dictated by a common up-down-up three-helix fold tightly stabilized by densely spaced disulfide bonds. In close functional analogy with animal cytokines and protein growth factors, they can thus elicit different and context-dependent cell growth, or mating responses by competitively binding in autocrine (self), or heterologous (nonself) fashion to their receptors on the surface of target cells.

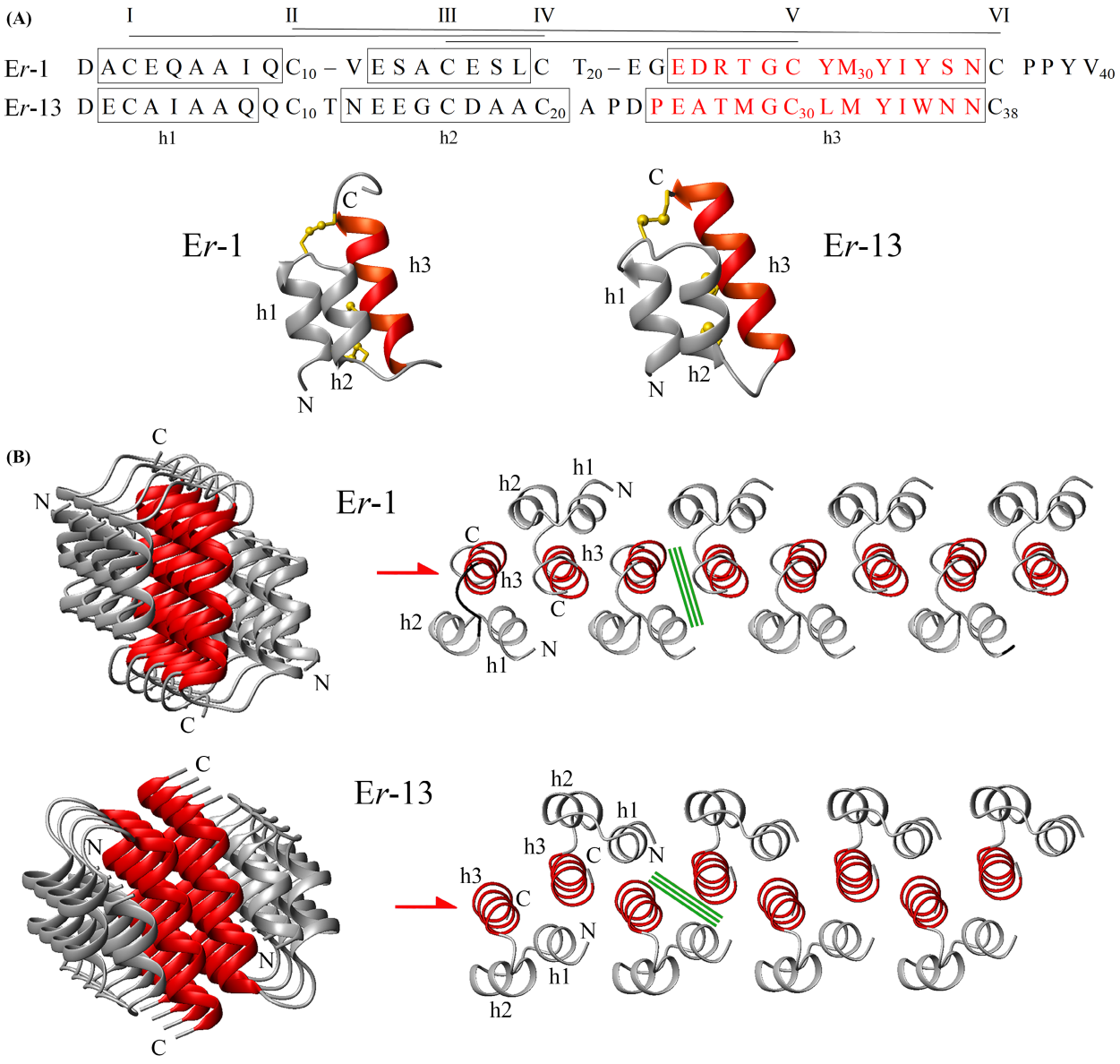


FIGURE 2 Crystal structures of *E. raikovi* pheromones Er-1 and Er-13. (A) Amino acid sequences and molecular structures of the two pheromones. In the amino acid sequences, the three helical segments (h1, h2 and h3) are boxed, and the six cysteine residues are indicated by progressive Roman numerals and connected according to their disulfide pairings represented by yellow ball-and-stick diagrams. Helix 3 is highlighted in red to indicate the central role played in establishing the contacts that pack molecules into crystals. (B) Perspective view (on the left) and top view (on the right) of the one-dimensional linear chains, propagating along a two-fold screw-symmetry rotation axis (red symbol), that Er-1 and Er-13 molecules equally form in the crystal taking rigorously alternating up and down orientations, as indicated by the positions of the amino (N) and carboxyl (C) termini. The Er-1 and Er-13 chains include a common protein–protein contact interface (green bars), that is equally derived from burying amino acid side-chains mostly residing on h3. However, they differ in the geometrical arrangement of the molecules, as denoted by observing that Er-1 molecules expose both h1 and h2 parallel to the chain sides, while Er-13 molecules expose only h2. In the Er-1 crystal (PDBs, 1ERL, 2ERL and 6E6O), this difference reflects in a further side-by-side chain association into bidimensional layers (not shown) and in two-fold symmetries specific of the space group *C*2. In the Er-13 crystal (PDB, 6E6N), it reflects in a mutual arrangement of the chains at 90° between one another (not shown), which prevents the layer formation and is consistent with four-fold screw symmetries distinctive of the space group $P4_3$

Insights into the molecular mechanisms underlying these competitive pheromone/receptor binding interactions derive from the study of the protein–protein interactions that arrange *E. raikovi* pheromone molecules into crystals (Pedrini et al., 2021; Weiss et al., 1995). The rationale for this study resides in the identification of the pheromone receptors with membrane-bound pheromone isoforms (represented by type-II transmembrane proteins oriented with the N- and C-terminus on the cell inside and outside, respectively), that are specified through an intron-splicing mechanism by the same macronuclear genes which encode the soluble pheromones (Ortenzi et al., 2000; Ricci et al., 2019, 2021). Due to this common genetic determination, in each cell type the extracellular domain of the pheromone isoforms comes to be structurally identical with the soluble pheromone, and the pheromone/ligand-binding capability of this domain is supported by mass-spectrometry evidence that native pheromone molecules can dimerize (or possibly form higher aggregates) with the subunits unstably associated by noncovalent forces (Bradshaw et al., 1990).

Two lines of evidence emerge by comparing the crystal structures of the *E. raikovi* pheromones E*r*-1 and E*r*-13 (Pedrini et al., 2021; Weiss et al., 1995), that are specific of strongly mating compatible cell types (I and XIII, respectively) and show largely overlapping backbone molecular structures (Figure 2). First, E*r*-1 and E*r*-13 molecules interact similarly in a cooperative fashion to tightly associate into linear chains that propagate along a two-fold screw-symmetry rotation axis and include subunits that are rigorously ordered with mutually opposite orientations as expected to be the case between pheromone and receptor moieties interacting on a cell surface. Second, although E*r*-1 and E*r*-13 molecules take different intrachain arrangements, which reflect a marked difference in the symmetry space group of the E*r*-1 and E*r*-13 crystals (C_2 and $P4_3$ groups, respectively), both mostly use residue side chains lying on the third one of the three helices of the basic pheromone molecular scaffold to establish the direct contacts which are responsible for the widest intrachain contact interface.

Helix-3 is thus identified as the key functional player in the pheromone/receptor interactions, and this view is strongly supported by biological and phylogenetic observations. On biological grounds, the most significant observation is relevant to a change in the pheromone activity that is observed in aging *E. raikovi* cultures and caused by the oxidation of a Met-residue lying exposed on the helix-3 of the pheromone E*r*-1 (Alimenti et al., 2012). Oxidized E*r*-1 molecules bind to their source type-I cells no longer as an autocrine growth-promoting signal, but as a heterologous (nonself) signal that induces homotypic (self) mating pair formation between their source cells. On phylogenetic grounds, the overall comparison of the *E. raikovi*, *E. nobilii* and *E. petzi* pheromone molecular structures clearly indicates that helix-3 is much more tightly conserved than the two other helices in relation to the backbone trace, extension and regular alpha-conformation. At intraspecific level, its conservation also extends to the structurally eccentric *E. raikovi* pheromone E*r*-23, in which the three-helix bundle is substantially changed into a five-helix bundle (see Figure 1) by the accommodation of two supplementary helical turns in consequence of the incorporation of a new segment of 11 residues (for a total of 51) and four new cysteine residues (for a total of 10; Zahn et al., 2001). At interspecific level, it finds a close counterpart with the largely predominant helix of *E. petzi* pheromones (see Figure 1), that may be considered as the evolutionary forerunners of all the other *Euplotes* pheromones in the light of the basal branch that the *E. petzi* clade forms in the *Euplotes* phylogenetic tree (Di Giuseppe et al., 2014). Lastly, the likely general helix-3 conservation among *Euplotes* pheromones may well explain the numerous cases of pheromone-mediated cross-mating reactions that are observed by mixing heterospecific cell cultures (Kuhlmann & Sato, 1993; Nobili et al., 1978).

In studying functional similarities of *Euplotes* pheromones with mammalian protein growth factors, the immune-stimulatory cytokine interleukin-2 (IL-2) was serendipitously found (along with epidermal growth factor and other signaling proteins) to act as a very effective competitor of pheromone/receptor binding reactions (Ortenzi et al., 1990). The significance of this finding is reinforced by observing that the *E. raikovi* pheromone E*r*-1 binds with different affinities to the α and β chains of the trimeric IL-2 receptor on the IL-2-dependent mouse T lymphocyte CTLL-2 cells, and increases (at nanomolar concentrations) DNA synthesis, proliferation and viability of human lymphoid Jurkat T-cells (Vallesi et al., 1998). In these cells, it also triggers the mitogen-activated protein kinase ERK1/2 pathway, increases the expression of an array of cytokines (including interferon-γ, tumor necrosis factor-α, IL-1β, IL-2 and IL-13), and induces the synthesis and release of factors inhibiting the cell cycle progression of human glioma U-373 cells (Cervia et al., 2013).

MEMBRANE-ASSOCIATED EUPLOTINS

The term ‘euplotin’ should strictly (and correctly) be applied to the earliest set of sesquiterpenoids that have been characterized from *Euplotes crassus* strains (Guella et al., 1994). It is here used in the plural form to collectively designate all the terpenoids that mainly localize into the membrane lipid bilayer of marine species of *Euplotes*, and have then been purified from large-scale cultures by liquid chromatography and structurally resolved by advanced analytical techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy.

The euplotin terpenoid structures vary remarkably between species, and may to some extent be credited as species-specific chemo-taxonomic markers. Despite their wide chemo-diversity, all originate from two distinct enantiomeric biogenetic precursors, farnesyl or geranyl-geraniol pyrophosphates, through biosynthetic pathways leading to sesquiterpenoid and diterpenoid structures, respectively, which have mostly been named in relation to their source species and grouped into four distinct classes in relation to their structural backbone (Figure 3).

The first class, built on an unsaturated dioxa-tricyclic backbone, includes three *E. crassus*-specific sesquiterpenoid euplotins named 'euplotin A', 'euplotin B' and 'euplotin C' (which is systematically detected from all the strains as the most abundant compound) plus the common biogenetic acyclic precursor, 'preuplotin'. The proposed biosynthetic route relies on the oxidation of the methyl groups of the farnesyl pyrophosphate that leads to the acyclic precursor preuplotin followed by an unexpected internal cyclization leading to the euplotin skeleton (Guella et al., 1994).

The second class includes two terpenoid euplotins containing a polycyclic structure built on a novel C-30 backbone, isolated from some (tropical) *E. vannus* strains and named 'vannusal A' and 'vannusal B', plus three sesquiterpenoid euplotins isolated from other (temperate-water) conspecific strains and named 'hemivannusal', 'prevannusadial A' and 'prevannusadial B' in relation to their likely role as vannusal A and B precursors (Guella et al., 2007).

The third class includes sesquiterpenoid euplotins isolated from *E. rariseta* strains (Guella et al., 1996a) and diterpenoid euplotins isolated from *E. focardii* (Guella et al., 1996b) that are similarly built on an octahydroazulene ring system. In the *E. rariseta* case, strains representing two morphologically and genetically distinct populations synthesize two epimeric forms of which one, named 'rarisetenolide', is isolated together with its epoxide derivative, 'epoxyrarisetenolide', and the second, named 'epirarisetenolide', is the corresponding C(10) epimer. The two epimeric forms may equally be derived from alternative enzymatic biosynthetic pathways, or two distinct enantiomeric biogenetic precursors. In any case, the enzymatic steps resulting in the construction of the octahydroazulene ring system of rarisetenolide and epirarisetenolide appears to be a specific feature of the ciliate secondary metabolism. In the case of *E. focardii* (represented by a single strain endemic to Antarctic waters), two diterpenoid euplotins, named '1-S-focardin' and '1-R-focardin', are isolated as an equilibrating mixture of two diasteroisomeric hemiacetals. They are in equilibrium with the unstable ring-opened aldehyde intermediate 'prefocardin', and accompany with 'epoxyfocardin' as a minor metabolite.

The last class is represented by a sesquiterpenoid euplotin built on a bicyclo[3.2.0]heptane ring system, that is specific to *E. raikovi* and named 'raikovenal' (Guella et al., 1995). As in *E. rariseta*, different *E. raikovi* populations differ in the production of C(10)-epimeric forms. Some strains synthesize raikovenal together with its putative biogenetic precursor, 'preraikovenal', while others synthesize the C(10) epimer, 'epiraikovenal', together with its seco-analog, 'secoepiraikovenal'. Considering that a metabolite and its epimer require different biogenetic pathways, the production of functional alternatives embedded in the same bicyclo[3.2.0]heptane skeleton may be viewed as an ecological adaptation strategy.

In the natural environment, a major euplotin function is most likely played in habitat colonization by inhibiting via cell-to-cell contacts the growth of competitors for space and energy resources (Dini et al., 1993). Experimental support to this view was principally derived from using native preparations of euplotin C on cultures of one *E. vannus* strain, chosen as an ecological competitor to *E. crassus* populations and selected as one of the poorest synthesizers of vannusal A and B. At a concentration of 10 μg/ml, euplotin C is lethal in short time, while at minor sublethal concentrations it alters the competitor's cell cycle, ciliary motility and the body shape by compromising the electrical properties of the membranes. A few minutes of exposure are sufficient to impair the competitor ion channel pumps and cause a rapid increase in intracellular concentrations of both Ca^{2+} and Na^{+}, which in turn threaten the cell motility (Trielli et al., 2008). Additionally, euplotin C decreases mitochondrial functions activating a caspase-dependent type of apoptosis, enhances lysosomal pH, and reduces lysosomal membrane stability. Overall, these events result in the dismantling ciliary structures and microtubules, the development of aberrant cytoplasmic vacuoles, and a drop in phagocytic activity.

On other cell systems, the noxious effects of euplotin C extend from pathogenic microorganisms (such as *Leishmania major*, *Candida albicans* and several strains of *Streptococcus* and *Burkholderia* spp.) (Savoia et al., 2004), to various mammalian tumor-derived cell lines. In corticotropic mouse pituitary tumor cells and rat pheochromocytoma cells, euplotin C induces a rapid activation of ryanodine receptors (a class of intracellular Ca^{2+} channels), a depletion of Ca^{2+} stores in the endoplasmic reticulum, the release of cytochrome c from mitochondria, and the caspase-12/caspase-3 activation which, in turn, triggers the apoptotic cell death pathway (Cervia et al., 2006, 2007). Assayed in comparison with cisplatin (a common chemotherapeutic agent used on many cancer types) on human melanoma cell lines, euplotin C similarly shows a good tolerability profile in significantly decreasing the melanoma cell viability and inhibiting the mitogen-activated protein kinase ERK1/2 and protein kinase B/Akt signaling pathways, which control many aspects of the melanoma aggressiveness and cell migration (Carpi et al., 2015, 2018).

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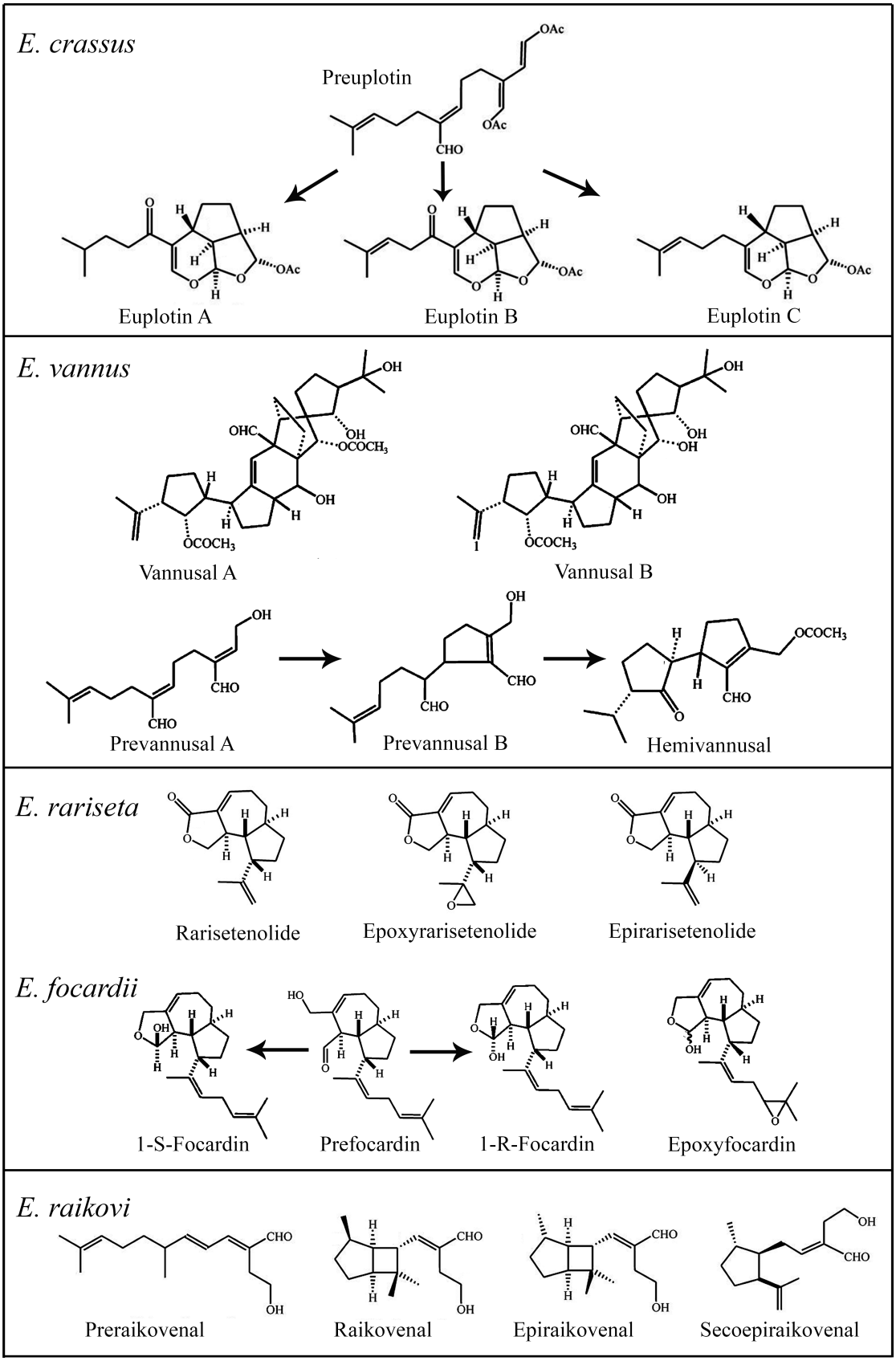

FIGURE 3 The four distinct classes of chemical structures of lipophilic terpenoids, designated euplotins, characterized from different marine species of *Euplotes*. Arrows indicate biosynthetic derivatives from precursor forms

CORTICAL GRANULE-ASSOCIATED BIOACTIVE MOLECULES

To interact with prey and predators, ciliates rely on very effective offense/defense mechanisms operating by means of an ample array of structurally diverse molecules that localize into extrusomes, or other nonextrusive (either pigmented or colorless) membrane-bounded cortical organelles. These molecules, noxious to prey and/or predators, are mostly derived from various biogenetic precursors (acetate, shikimic acid, mevalonic acid and amino acids) of the primary metabolism, and have motivated particular interest from an applied perspective for chemical structures readier to be synthesized.

Previously studied for a function primarily played only in photoreception (Checcucci et al., 1997; Maeda et al., 1997; Tao et al., 1993), 'blepharismins' and 'stentorin'—the pigment molecules that reside in the cortical granules of species of *Blepharisma* and *Stentor* and are structurally closely related to the naphthodianthrone hypericin native to the flowering plant, *Hypericum perforatum*—were later discovered to be in fact multifunctional (Lobban et al., 2007) and active in prey/predator interactions (Harumoto et al., 1998; Miyake et al., 2001). They were also utilized as antibiotics against the methicillin-resistant Gram-positive *Staphylococcus aureus* and the Gram-negative *Bacillus subtilis* and *B. cereus*, and as cytostatic compounds on HeLa cells (Cavaleiro et al., 2020; Pant et al., 1997).

In the wake of these findings, new metabolites associated with pigmented or colorless cortical granules have been characterized for their defense/offense activities and chemical structures. Among them: 'keronopsins', 'kerenopsamides' and 'erythrolactones' (represented by three active nonsulfonated forms, A2, B2 and C2, laying in the cortical granules, and the respective inactive sulfonate esters in the cell cytoplasm) from *Pseudokeronopsis rubra*, *P. riccii* and *P. erythrina*, respectively (Anesi et al., 2016; Buonanno et al., 2017; Guella, Frassanito, et al., 2010; Höfle et al., 1994); 'spirostomin' and 'mono-prenyl hydroquinone' from *Spirostomum teres* and *S. amibiguum*, respectively (Buonanno et al., 2012); and 'climacostol' from *Climacostomum virens* (Masaki et al., 1999; Miyake et al., 2003). The chemical structures of these molecules serving primary defense/offense functions are reported in Figure 4. Keronopsamides are alkaloids with a fully planar structure where the molecular skeleton is formally derived from a peptide-like condensation of a 3-pyrrolepropenoic acid with an unsaturated bromotyramine, while it is suggested that keronopsins and

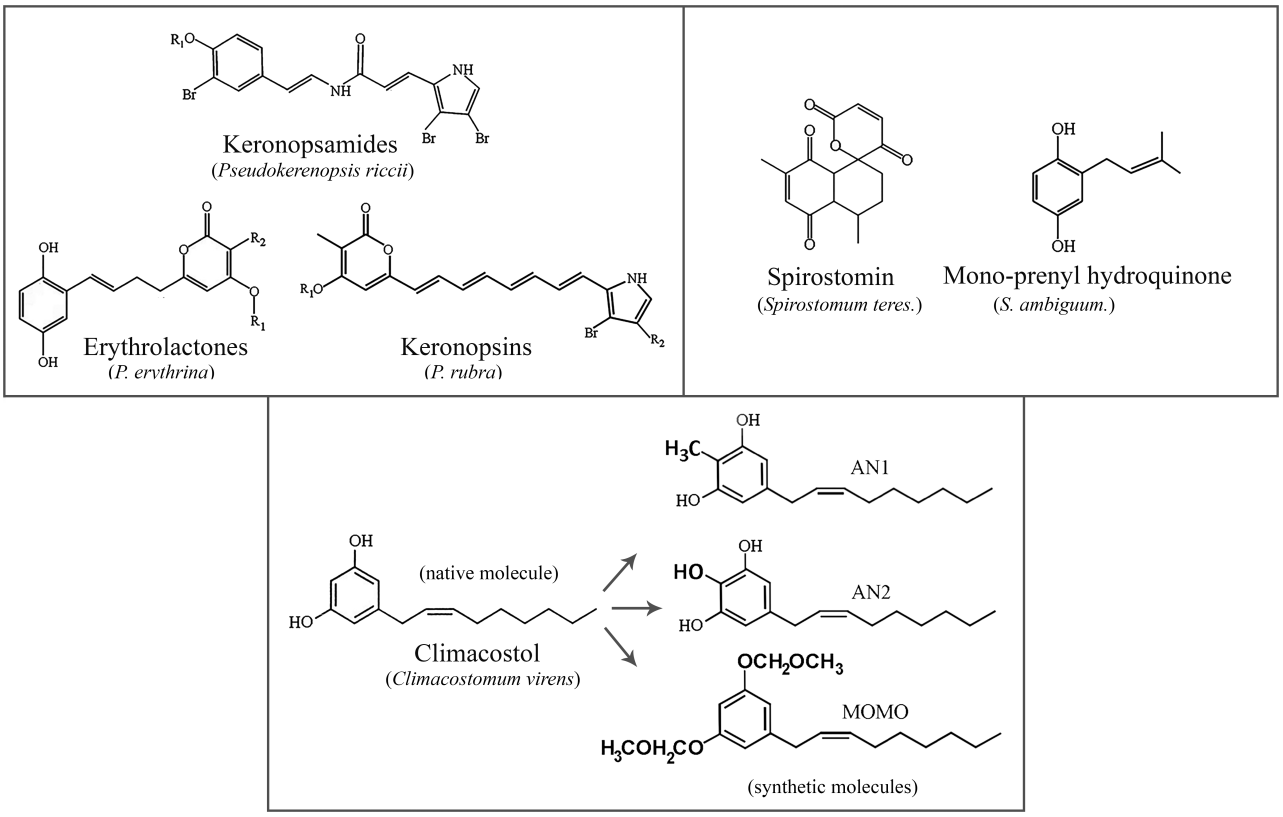

FIGURE 4 Bioactive molecules of different chemical nature from cortical granules of species of *Pseudokeronopsis*, *Spirostomum* and *Climacostomum*. In the three lactone molecules, different side chains attached to a same carbon skeleton are indicated by R_1 and R_2. In the three synthetic derivatives (indicated by arrows and named AN1, AN2 and MOMO) of native climacostol, the side chain modifications are in bold

erythrolactones derive from the metabolism of fatty acid (Guella, Skropeta, et al., 2010). In the former, a β-bromide-substituted pyrrole is linked to a sulfate pyrrole through a conjugated acyl chain; in the latter, the 4-hydroxy-unsaturated d-lactone ring is 2,6-dialkyl substituted with saturated alkyl chain at carbon-2 and a butenyl-hydroquinone group at carbon-5. More marked is the interspecific structural divergence between spirostomin and mono-prenyl hydroquinone isolated from the two *Spirostomum* species (Buonanno et al., 2012), the former being derived from intramolecular cyclization of a C14 linear polyketide backbone and the latter being a prenylated-hydroquinone derivative, a rather common metabolite among both unicellular and multicellular organisms. Lastly, climacostol is characterized by a resorcinol core to which an unsaturated C9-alkenyl chain is attached (Masaki et al., 1999).

In an applied perspective, climacostol has attracted much more interest than the other molecules in relation to two major experimental reasons: (i) the availability of substantial amounts of native preparations that have initially greatly facilitated antimicrobial and cytotoxic bioassays on a large spectrum of cell systems; and (ii) the concrete possibility to synthesize climacostol in both the native and modified more bioactive structures. Among these, one, characterized by a Z-configuration of the C=C double bond at the 2′-position of the carbon chain (Fiorini et al., 2010; Masaki et al., 1999), is also sold as apoptotic agent, available either as 'cold' or 'deuterium-labeled' molecule (Santa Cruz Biotechnology, Dallas, USA; Advanced Technology & Industrial Co., Hong Kong, China).

The activity of climacostol as pharmacological drug has mostly been assayed on human lines of melanoma, promyelocytic leukemia and tumor squamous carcinoma cells. More reliable effects are recorded on melanoma cells in which climacostol rapidly causes (at micromolar concentrations) dysfunctional autophagy, nuclear and mitochondrial DNA damage with consequent activation of the caspase apoptotic pathway. The significance of these observations is further supported by in vivo experiments, in which the development of mouse melanoma allografts is halted at various extents of effectiveness by intratumor climacostol injections (Buonanno et al., 2020; Catalani et al., 2016).

The addition of a methyl or hydroxyl group to the aromatic ring of climacostol results into synthetic analogues capable to effectively modulate both potency and mechanism of action of the native molecule (Buonanno et al., 2019), while adding a methoxymethyl ether protective group to the aromatic ring results in the synthesis of an inactive prodrug, which changes to active climacostol only at pH < 7 (Catalani et al., 2019). Delivered to pathological cell systems, this prodrug has shown to be particularly effective on melanoma cells grown in mild acidic culture medium (pH 6.3), and appears to be promising against *Leishmania* and *Plasmodium*. In the former case, by acting in the acidic environment of the parasitophorous vacuole in the host's macrophages, in the latter, by acting in the acidic extracellular medium surrounding the infected red blood cells.

CONCLUDING REMARKS

Ciliates count a few equals among eukaryotic microorganisms in outcompeting for habitat colonization and energetic resources, and the utilization of a huge reserve of structurally and functionally diverse bioactive molecules accounts well for their success in facing natural selection. Although these molecules have been isolated and studied for their native and applied activities only from a few, more easily collected species, the observations surveyed here provide good evidence for deserving strong research interest. In basic science, to trace the evolutionary forerunners of the structures and mechanisms of action of the molecules that regulate the multicellular life; in applied science, to discover new lead, bio-friendly compounds that, either in the native or a derived structure, may serve the fields of medicine and pharmacology.

In addition to plants (Lautié et al., 2020), marine fauna has also significantly contributed at the discovery of very effective chemo-therapeutic agents (Khazir et al., 2014; Newman & Cragg, 2014). Common examples are cytarabine and halichondrin (the former used in various forms of leukemia and the latter in breast cancer) from species of the sponges *Cryptotheca* and *Halichondria*, respectively, or trabectedin (used in the soft-tissue sarcoma and ovarian cancer treatment) from species of the tunicate *Ecteinacidia* (D'Incalci et al., 2014; Schwartsmann et al., 2001; Sipkema et al., 2005). However, the exploitation of the marine animal biodiversity as a direct source of pharmacologically utilizable molecules is often challenged by low yields and complex chemical structures, and may turn out to be detrimental to the ecological niche of a species and, in the longer term, result in ecosystem unbalance. By exploiting ciliates and other micro-eukaryotes as well, the environmental impact is neutralized by the real possibility to grow large-scale cultures of many species in captivity, with greatly reduced costs, no legal restrictions and no waste.

Euplotes species certainly figure among those, which are more easily collected and cultivated. In addition, they are unique for unmatched levels of interspecific and intraspecific genetic polymorphism arising from the evolution of high-multiple mating-type systems and a consequent acquisition of strongly outbreeding strategies (Nobili et al., 1978). In the case of euplotins, this polymorphism reflects as a wide diversification of skeletal structures requiring enzymatic pathways unorthodox with respect to the pathway common to the linear terpenoid precursors;

in the case of pheromones, it results in the generation of an unrestricted number of physical–chemical specificities that superimpose on, and make unique a substantially common three-helix protein fold. It also may provide practical advantages in identifying species and strains that are better able to experimentally satisfy quantitative needs in assaying the euplotin and pheromone activities. Among euplotins, up to 1 mg of euplotin C can be extracted from 1 ml of a cell pellet of certain *E. crassus* strains (Savoia et al., 2004); among pheromones, up to 300 μg of pure E*r*-1 protein can be purified from 1 L of supernatant of the *E. raikovi* type-I cell cultures (Raffioni et al., 1992). At the opposite end of the *Euplotes* species, *Climacostomum virens*, source of climacostol, is rarely collected and difficult to cultivate. In a sense, it compensates by producing a bioactive molecule that, in addition to looking as most promising in an applied perspective, has shown to be the best-predisposed to structurally engineering and synthesis.

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ORCID

Adriana Vallesi https://orcid.org/0000-0002-4127-090X

REFERENCES

Alimenti, C., Vallesi, A., Pedrini, B., Wüthrich, K. & Luporini, P. (2009) Molecular cold-adaptation, comparative analysis of two homologous families of psychrophilic and mesophilic signal proteins of the protozoan ciliate, *Euplotes*. *IUBMB Life*, 61, 838–845.

Alimenti, C., Vallesi, A., Luporini, P., Buonanno, F. & Ortenzi, C. (2012) Cell aging-induced methionine oxidation causes an autocrine to paracrine shift of the pheromone activity in the protozoan ciliate, Euplotes raikovi. *Experimental Cell Research*, 318, 144–151.

Anesi, A., Buonanno, F., Di Giuseppe, G., Ortenzi, C. & Guella, G. (2016) Metabolites from the Euryhaline Ciliate *Pseudokeronopsis erythrina*. *European Journal of Organic Chemistry*, 2016, 1330–1336.

Bradshaw, R.A., Raffioni, S., Luporini, P., Chait, B.T., Lee, T. & Shively, J. (1990) Amino acid sequence-mass spectrometric analyses of mating pheromones of the ciliate *Euplotes raikovi*. In: Horvath, C. & Nikelly, J.G. (Eds.) *Analytical biotechnology*. Washington, D.C.: American Chemical Society, pp. 143–161.

Brünen-Nieweler, C., Weiligmann, J.C., Hansen, B., Kuhlmann, H.W., Möllenbeck, M. & Heckmann, K. (1998) The pheromones and pheromone genes of new stocks of the *Euplotes octocarinatus* species complex. *European Journal of Protistology*, 34, 124–132.

Buonanno, F., Anesi, A., Di Giuseppe, G., Guella, G. & Ortenzi, C. (2017) Chemical defense by erythrolactones in the euryhaline ciliated protist, *Pseudokeronopsis erythrina*. *Zoological Science*, 34, 42–51.

Buonanno, F., Catalani, E., Cervia, D., Cimarelli, C., Marcantoni, E. & Ortenzi, C. (2020) Natural function and structural modification of climacostol, a ciliate secondary metabolite. *Microorganisms*, 8, 809.

Buonanno, F., Catalani, E., Cervia, D., Proietti Serafini, F., Picchietti, S., Fausto, A.M. et al. (2019) Bioactivity and structural properties of novel synthetic analogues of the protozoan toxin Climacostol. *Toxins*, 11(1), 1–22.

Buonanno, F., Guella, G., Strim, C. & Ortenzi, C. (2012) Chemical defence by mono-prenyl hydroquinone in a freshwater ciliate, *Spirostomum ambiguum*. *Hydrobiologia*, 684, 97–107.

Carpi, S., Fogli, S., Romanini, A., Pellegrino, M., Adinolfi, B., Podestà, A. et al. (2015) AM251 induces apoptosis and G2/M cell cycle arrest in A375 human melanoma cells. *Anti-Cancer Drugs*, 26, 754–762.

Carpi, S., Polini, B., Poli, G., Alcantara Barata, G., Fogli, S., Romanini, A. et al. (2018) Anticancer activity of euplotin c, isolated from the marine ciliate *Euplotes crassus*, against human melanoma cells. *Marine Drugs*, 16, 166–180.

Catalani, E., Buonanno, F., Lupidi, G., Bongiorni, S., Belardi, R., Zecchini, S. et al. (2019) The natural compound climacostol as a prodrug strategy based on pH activation for efficient delivery of cytotoxic small agents. *Frontiers in Chemistry*, 7, 463–484.

Catalani, E., Proietti Serafini, F., Zecchini, S., Picchietti, S., Fausto, A.M., Marcantoni, E. et al. (2016) Natural products from aquatic eukaryotic microorganisms for cancer therapy: perspectives on anti-tumour properties of ciliate bioactive molecules. *Pharmaceutical Research*, 113(A), 409–420.

Cavaleiro, J., Oliveira, N.B., Ribeiro, T.A., Guimarães, L.F., Fernandes, N.M., da Silva-Neto, I.D. et al. (2020) Distinguishing activities in the photodynamic arsenals of the pigmented ciliates *Blepharisma sinuosum* Sawaya, 1940 and *Blepharisma japonicum* Suzuki, 1954 (Ciliophora: Heterotrichea). *Photochemistry and Photobiology*, 96, 1251–1226.

Cazzolli, G., Skrbić, T., Guella, G. & Faccioli, P. (2013) Unfolding thermodynamics of cysteine-rich proteins and molecular thermal-adaptation of marine ciliates. *Biomolecules*, 18, 967–985.

Cervia, D., Catalani, E., Belardinelli, M.C., Perrotta, C., Picchietti, S., Alimenti, C. et al. (2013) The protein pheromone E*r*-1 of the ciliate *Euplotes raikovi* stimulates human T-cell activity: involvement of interleukin-2 system. *Experimental Cell Research*, 319, 56–67.

Cervia, D., Garcia-Gil, M., Simonetti, E., Di Giuseppe, G., Guella, G., Bagnoli, P. et al. (2007) Molecular mechanisms of euplotin C-induced apoptosis: involvement of mitochondrial dysfunction, oxidative stress and proteases. *Apoptosis*, 12, 1349–1363.

Cervia, D., Martini, D., Garcia-Gil, M., Di Giuseppe, G., Guella, G., Dini, F. et al. (2006) Cytotoxic effects and apoptotic signalling mechanisms of the sesquiterpenoid euplotin C, a secondary metabolite of the marine ciliate *Euplotes crassus*, in tumour cells. *Apoptosis*, 11, 829–843.

Checcucci, G., Shoemaker, R.S., Bini, E., Cerny, R., Tao, N., Hyon, J.S. et al. (1997) The chemical structure of blepharismin, the photosensor pigment for *Blepharisma japonicum*. *Journal of the American Chemical Society*, 119, 5762–5763.
Chitra, J., Khatana, S. & Vijayvergia, R. (2019) Bioactivity of secondary metabolites of various plants: a review. *International Journal of Pharmaceutical Sciences and Research*, 10, 494–504.
D'Incalci, M., Badri, N., Galmarini, C. & Allavena, P. (2014) Trabectedin, a drug acting on both cancer cells and the tumour microenvironment. *British Journal of Cancer*, 111, 646–650.
Di Giuseppe, G., Erra, F., Frontini, F.P., Dini, F., Vallesi, A. & Luporini, P. (2014) Improved description of the bipolar ciliate, *Euplotes petzi*, and definition of its basal position in the *Euplotes* phylogenetic tree. *European Journal of Protistology*, 50, 402–411.
Dini, F., Guella, G., Giubbilini, I., Mancini, I. & Pietra, F. (1993) Control of interspecific relationships in marine ciliate protists by most evolved natural products. *Naturwissenschaften*, 80, 84–86.
Fiorini, D., Giuli, S., Marcantoni, E., Quassinti, L., Bramucci, M., Amantini, C. et al. (2010) A Straightforward diastereoselective synthesis and evaluation of climacostol, a natural product with anticancer activities. *Synthesis*, 9, 1550–1556.
Geralt, M., Alimenti, C., Vallesi, A., Luporini, P. & Wüthrich, K. (2013) Thermodynamic stability of psychrophilic and mesophilic pheromones of the protozoan ciliate *Euplotes*. *Biology*, 2, 142–150.
Guella, G., Callone, E., Di Giuseppe, G., Frassanito, R., Frontini, F.P., Mancini, I. et al. (2007) Hemivannusal and prevannusadials—new sesquiterpenoids from the marine ciliate protist *Euplotes vannus*: the putative biogenetic precursors of dimeric terpenoid vannusals. *European Journal of Organic Chemistry*, 31, 5226–5234.
Guella, G., Dini, F., Tomei, A. & Pietra, F. (1994) Preuplotin, a putative biogenetic precursor of the euplotins, bioactive sesquiterpenoids of the marine ciliated protist *Euplotes crassus*. *Journal of the Chemical Society, Perkin Transactions 1*, 2, 161–166.
Guella, G., Dini, F. & Pietra, F. (1995) From epiraikovenal, an instrumental niche-exploitation sesquiterpenoid of some strains of the marine ciliated protist *Euplotes raikovi*, to an unusual intramolecular tele-dienone-olefin [2+2] photocycloaddition. *Helvetica Chimica Acta*, 78, 1747–1754.
Guella, G., Dini, F. & Pietra, F. (1996a) Rarisetenolide, epoxyrarisetenolide, and epirarisetenolide, new-skeleton sesquiterpene lactones as taxonomic markers and defensive agents of the marine ciliated morphospecies *Euplotes rariseta*. *Helvetica Chimica Acta*, 79, 2180–2189.
Guella, G., Dini, F. & Pietra, F. (1996b) Epoxyfocardin and its putative biogenetic precursor, focardin, bioactive, new-skeleton diterpenoids of the marine ciliate *Euplotes focardii* from Antarctica. *Helvetica Chimica Acta*, 79, 439–448.
Guella, G., Frassanito, R., Mancini, I., Sandron, T., Modeo, L., Verni, F. et al. (2010) Keronopsamides, a new class of pigments from marine Ciliates. *European Journal of Organic Chemistry*, 2010, 427–434.
Guella, G., Skropeta, D., Di Giuseppe, G. & Dini, F. (2010) Structures, biological activities and phylogenetic relationships of terpenoids from marine ciliates of the genus *Euplotes*. *Marine Drugs*, 2010(8), 2080–2116.
Haefner, B. (2003) Drugs from the deep: marine natural products as drug candidates. *Drug Discovery Today*, 8, 536–544.
Hanson, J.R. (2003) *Natural products: the secondary metabolites*. Cambridge UK: Royal Society of Chemistry.
Harumoto, T., Miyake, A., Ishikawa, N., Sugibayashi, R., Zenfuku, K. & Iio, H. (1998) Chemical defense by means of pigmented extrusomes in the ciliate *Blepharisma japonicum*. *European Journal of Protistology*, 34, 458–470.
Höfle, G., Pohlan, S., Uhlig, G., Kabbe, K. & Schumacher, D. (1994) Keronopsin-A and keronopsin-B, chemical defense substances of the marine ciliate *Pseudokeronopsis* rubra (Protozoa)—identification by ex-vivo HPLC. *Angewandte Chemie International Edition*, 33, 1495–1497.
Javed, F., Qadir, M.I., Janbaz, K.H. & Ali, M. (2011) Novel drugs from marine microorganisms. *Critical Reviews in Microbiology*, 37, 245–249.
Kellner Filho, L.C., Picão, B.W., Silva, M., Cunha, W.R., Pauletti, P.M., Dias, G.M. et al. (2019) Bioactive aliphatic sulfates from marine invertebrates. *Marine Drugs*, 17, 527.
Khazir, J., Riley, D.L., Pilcher, L.A., De-Maayer, P. & Mir, B.A. (2014) Anticancer agents from diverse natural sources. *Natural Products Communications*, 9, 1655–1669.
Kimball, R.F. (1942) The nature and inheritance of mating types in *Euplotes patella*. *Genetics*, 27, 269–285.
Kobayashi, M., Miura, M., Takusagawa, M., Sugiura, M. & Harumoto, T. (2015) Two possible barriers blocking conjugation between different megakaryotypes of *Blepharisma*. *Zoological Science*, 32, 53–61.
Kuhlmann, H.W. & Sato, K. (1993) Interspecific mating reactions between *Euplotes octocarinatus* and *Euplotes patella* syngen 2. *European Journal of Protistology*, 29, 24–31.
Lautié, E., Russo, O., Ducrot, P. & Boutin, J.A. (2020) Unraveling plant natural chemical diversity for drug discovery purposes. *Frontiers in Pharmacology*, 11, 397.
Lobban, C.S., Hallam, S.J., Mukherjee, P. & Petrich, J.W. (2007) Photophysics and multifunctionality of hypericin-like pigments in heterotrich ciliates: a phylogenetic perspective. *Photochemistry and Photobiology*, 83, 1074–1094.
Luporini, P., Alimenti, C., Pedrini, B. & Vallesi, A. (2016) Ciliate communication via water-borne pheromones. In: Witzany, G. & Nowacki, M. (Eds.) *Biocommunication of ciliates*. Berlin: Springer, pp. 159–174.
Luporini, P. & Miceli, C. (1986) Mating pheromones. In: Gall, J.G. (Ed.) *The molecular biology of ciliated protozoa*. New York: Academic Press, pp. 263–299.
Luporini, P., Pedrini, B., Alimenti, C. & Vallesi, A. (2016) Revisiting fifty years of research on pheromone signaling in ciliates. *European Journal of Protistology*, 55, 26–38.
Maeda, M., Naoki, H., Matsuoka, T., Kato, Y., Kotsuki, H., Utsumi, K. et al. (1997) Blepharismin 1–5, novel photoreceptor from the unicellular organism *Blepharisma japonicum*. *Tetrahedron Letters*, 38, 7411–7414.
Masaki, M.E., Harumoto, T., Terazima, M.N., Miyake, A., Usuki, Y. & Iio, H. (1999) Climacostol, a defense toxin of the heterotrich ciliate *Climacostomum virens* against predators. *Tetrahedron Letters*, 40, 8227–8229.
Miyake, A. (1981) Cell interaction by gamones in *Blepharisma*. In: O'Day, D.H. & Horgen, P.A. (Eds.) *Sexual interaction in eukaryotic microbes*. New York: Academic Press, pp. 95–129.
Miyake, A., Buonanno, F., Saltalamacchia, P., Masaki, M.E. & Iio, H. (2003) Chemical defence by means of extrusive cortical granules in the heterotrich ciliate *Climacostomum virens*. *European Journal of Protistology*, 39, 25–36.
Miyake, A., Harumoto, T. & Iio, H. (2001) Defensive function of pigment granules in *Stentor coeruleus*. *European Journal of Protistology*, 37, 77–88.
Molinski, T.F., Dalisay, D.S., Lievens, S.L. & Saludes, J.P. (2009) Drug development from marine natural products. *Nature Reviews Drug Discovery*, 8, 69–85.

Nanney, D.L. (1980) *Experimental ciliatology*. New York: Wiley.
Newman, D.J. & Cragg, G.M. (2014) Marine-sourced anti-cancer and cancer pain control agents in clinical and late preclinical development. *Marine Drugs*, 12, 255–278.
Nobili, R., Luporini, P. & Dini, F. (1978) Breeding systems, species relationships and evolutionary trends in some marine species of Euplotidae (Hypotrichida Ciliata). In: Battaglia, B. & Beardmore, L.A. (Eds.) *Marine organisms: genetics, ecology, and evolution*. New York: Plenum Press, pp. 591–616.
Ortenzi, C., Alimenti, C., Vallesi, A., Di Pretoro, B., La Terza, A. & Luporini, P. (2000) The autocrine mitogenic loop of the ciliate *Euplotes raikovi*, the pheromone membrane-bound forms are the cell binding sites and potential signalling receptors of soluble pheromones. *Molecular Biology of the Cell*, 11, 1445–1455.
Ortenzi, C., Miceli, C., Bradshaw, R.A. & Luporini, P. (1990) Identification and initial characterization of an autocrine pheromone receptor in the protozoan ciliate *Euplotes raikovi*. *Journal of Cell Biology*, 111, 607–614.
Pant, B., Kato, Y., Kumagai, T., Matsuoka, T. & Sugiyama, M. (1997) Blepharismin produced by a protozoan *Blepharisma* functions as an antibiotic against methicillin-resistant *Staphylococcus aureus*. *FEMS Microbiology Letters*, 155, 67–71.
Parfenova, E.V., Afonkin, S.Y., Yudin, A.L. & Etingof, R.N. (1989) Characterization and partial purification of mating pheromone excreted by mating type II cells of the ciliate *Dileptus anser*. *Acta Protozoologica*, 28, 11–21.
Pedrini, B., Finke, A.D., Marsh, M., Luporini, P., Vallesi, A. & Alimenti, C. (2021) Crystal structure of the pheromone E*r*-13 from the ciliate *Euplotes raikovi*, with implications for a protein-protein association model in pheromone/receptor interactions. *Journal of Structural Biology*, 214(1), 107812.
Pedrini, B., Suter-Stahel, T., Vallesi, A., Alimenti, C. & Luporini, P. (2017) Molecular structures and coding genes of the water-borne protein pheromones of *Euplotes petzi*, an early diverging polar species of *Euplotes*. *Journal of Eukaryotic Microbiology*, 64, 164–172.
Penesyan, A., Kjelleberg, S. & Egan, S. (2010) Development of novel drugs from marine surface associated microorganisms. *Marine Drugs*, 8, 438–459.
Raffioni, S., Miceli, C., Vallesi, A., Chowdhury, S.K., Chait, B.T., Luporini, P. et al. (1992) Primary structure of *Euplotes raikovi* pheromones: comparison of five sequences of pheromones from cells with variable mating interactions. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 2071–2075.
Ricci, F., Candelori, A., Brandi, A., Alimenti, C., Luporini, P. & Vallesi, A. (2019) The sub-chromosomic macronuclear pheromone genes of the ciliate *Euplotes raikovi*: comparative structural analysis and insights into the mechanism of expression. *Journal of Eukaryotic Microbiology*, 66, 376–384.
Ricci, F., Luporini, P., Alimenti, C. & Vallesi, A. (2021) Functional chimeric genes in ciliates: an instructive case from *Euplotes raikovi*. *Gene*, 767, 145186.
Savoia, D., Avanzini, C., Allice, T., Callone, E., Guella, G. & Dini, F. (2004) Antimicrobial activity of euplotin C, the sesquiterpene taxonomic marker from the marine ciliate *Euplotes crassus*. *Antimicrobial Agents and Chemotherapy*, 48, 3828–3833.
Schwartsmann, G., Brondani da Rocha, A., Berlinck, R.G. & Jimeno, J. (2001) Marine organisms as a source of new anticancer agents. *The Lancet Oncology*, 2, 221–225.
Sipkema, D., Osinga, R., Schatton, W., Mendola, D., Tramper, J. & Wijffels, R.H. (2005) Large-scale production of pharmaceuticals by marine sponges: sea, cell, or synthesis? *Biotechnology and Bioengineering*, 20, 201–222.
Sugiura, M. & Harumoto, T. (2001) Identification, characterization, and complete amino acid sequence of the conjugation-inducing glycoprotein (blepharmone) in the ciliate *Blepharisma japonicum*. *Proceedings of the National Academy of Sciences*, 98, 14446–14451.
Tao, N., Orlando, M., Hyon, J.-S., Gross, M. & Song, P.S. (1993) A new photoreceptor molecule from *Stentor coeruleus*. *Journal of the American Chemical Society*, 115, 2526–2528.
Thirumurugan, D., Cholarajan, A., Raja, S.S.S. & Vijayakumar, R. (2018) An introductory chapter: secondary metabolites. In: Vijayakumar, R. & Raja, S. (Eds.) *Secondary metabolites—sources and applications*. London: IntechOpen, pp. 1–20.
Trielli, F., Cervia, D., Di Giuseppe, G., Ristori, C., Kruppel, T., Burlando, B. et al. (2008) Action mechanisms of the secondary metabolite euplotin C: signaling and functional role in *Euplotes*. *Journal of Eukaryotic Microbiology*, 55, 365–373.
Uspenskaya, Z.I. & Yudin, A.L. (2016) Fifty years of research on serotypes and mating types in *Dileptus anser*: a review. *European Journal of Protistology*, 53, 31–44.
Vallesi, A., Alimenti, C., Federici, S., Di Giuseppe, G., Dini, F., Guella, G. et al. (2014) Evidence for gene duplication and allelic codominance (not hierarchical dominance) at the mating-type locus of the ciliate, *Euplotes crassus*. *Journal of Eukaryotic Microbiology*, 61, 620–629.
Vallesi, A., Giuli, G., Ghiara, P., Scapigliati, G. & Luporini, P. (1998) Structure–function relationships of pheromones of the ciliate *Euplotes raikovi* with mammalian growth factors: cross-reactivity between E*r*-1 and interleukin-2 systems. *Experimental Cell Research*, 241, 253–259.
Weiss, M.S., Anderson, D.H., Raffioni, S., Bradshaw, R.A., Ortenzi, C., Luporini, P. et al. (1995) A cooperative model for receptor recognition and cell adhesion: evidence from the molecular packing in the 1.6-Å crystal structure of the pheromone E*r*-1 from the ciliated protozoan *Euplotes raikovi*. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 10172–10176.
Zahn, R., Damberger, F., Ortenzi, C., Luporini, P. & Wüthrich, K. (2001) NMR structure of the *Euplotes raikovi* pheromone E*r*-23 and identification of its five disulfide bonds. *Journal of Molecular Biology*, 313, 923–931.
Zhang, L., An, R., Wang, J., Sun, N., Zhang, S., Hu, J. et al. (2005) Exploring novel bioactive compounds from marine microbes. *Current Opinion in Microbiology*, 8, 276–281.

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