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New Hemiketal Steroid from the Introduced Soft Coral *Chromonephthea braziliensis* is a Chemical Defense against Predatory Fishes

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Abstract Recent studies show that chemical defenses in the exotic soft coral *Chromonephthea braziliensis* Ofwegen (Nephtheidae, Alcyonacea) can be one of the reasons for the success of this introduced species. We report for the first time the detailed composition of the monohydroxylated sterol fraction and a new hemiketal steroid, 23-keto-cladiellin-A, isolated from the unpalatable hexane extract from *C. braziliensis*. Bioassay-guided fractionation of this extract revealed that this hemiketal steroid exhibits potent feeding deterrent properties against a natural assemblage of fishes at the natural concentration. The major sterol fraction, containing the monohydroxylated sterols, was inactive in the bioassay. The results suggest that this active molecule may be driving the observed success of the invasion of this soft coral along the Brazilian Atlantic coast.

Keywords *Chromonephthea braziliensis* · Nephtheidae · Alcyonacea · Soft coral · Exotic species · Steroid · Antifeeding · Chemical defense · Marine chemical ecology

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Introduction

Biological invasions in marine environments are one of the lesser understood aspects of global change (Vitousek et al. 1996). They represent a serious ecological and economic threat leading to decreased biodiversity, unbalanced ecosystems, and fishery and tourism impairment (Reise et al. 2006; Occhipinti-Ambrogi 2007; Olenin et al. 2007).

Biological invasions have been considered to be rapid evolutionary changes (Lee 2002), but little is known about their patterns and processes in marine ecosystems (Sax et al. 2007). Recent studies, however, have demonstrated that chemical defensive strategies may facilitate the invasion of the Indo-Pacific exotic soft coral *Chromonephthea braziliensis* Ofwegen (2005) in the Brazilian Atlantic coast (Lages et al. 2006).

Soft corals are among the major benthic invertebrates that compete for space on tropical reefs, particularly in the Indo-Pacific Ocean (Coll 1992). Their evolutionary success in areas of high levels of predation has been attributed to their production of significant amounts of complementary (secondary) metabolites (Sammarco and Coll 1997; Blunt et al. 2007) in their tissues, which may function as predator-deterrents and serve other ecological functions (Coll 1992; Sammarco and Coll 1992; Mizobuchi et al. 1996; Slattery et al. 1999; McClintock and Baker 2001; Fleury et al. 2004, 2006; Paul et al. 2006).

The ecological and evolutionary consequences of complementary metabolites have been considered only recently, and their effects on marine biodiversity are now recognized (Hay and Fenical 1996). There is currently little evidence, however, of the adaptive response of marine invasive species under selective pressure from a new environment.

As part of our continuing interest in the complementary metabolites responsible for the invasive success of *C.*

braziliensis, we conducted a bioassay-guided fractionation of the components of the organic extract of this exotic coral. The results indicate an active molecule that may be driving the observed success. We report the isolation and identification of a new hemiketal steroid, 23-keto-cladiellin-A, that acts as a chemical defense against a natural assemblage of fishes in the natural environment.

Methods and Materials

Coral Sampling Colonies of the exotic soft coral *C. braziliensis* Ofwegen 2005 (Nephtheidae, Alcyonacea) (identified before as *Stereonephthya* aff. *curvata*, Ferreira 2003) were collected in May 2004 in a sheltered rocky shore, by SCUBA diving at about 8-m depth at Saco dos Cardeiros, Arraial do Cabo region (23° 44′ S–42° 02′ W), state of Rio de Janeiro, southeastern coast of Brazil. This region is a marine harvest reserve and sustains unique reef systems characterized by different hydrodynamic regimes. It exhibits a rich benthic community and associated ichthyofauna (over 150 fish species) (Ferreira et al. 2001). Colonies of *C. braziliensis* were kept frozen (−25°) until analysis in the laboratory.

Field Assay Field assays were performed at Pedra Vermelha, Arraial do Cabo, Brazil. Crude extract, fractions, and pure compound were incorporated at their natural volumetrically concentrations reconstituted into a matrix of carrageenan-based artificial diet at the same concentration as occurred in the fresh soft coral tissue (Lages et al. 2006). Carrageenan (Sigma C-1013 type 1) food strips were prepared by an established methodology (Fenical and Pawlik 1991; Pawlik and Fenical 1992), involving combination of 3.75 g carrageenan, 60 ml distilled water, and 30 ml commercial tunafish purée packed in water (Pawlik and Fenical 1992; Epifanio et al. 1999, 2007). The extract, fractions, or pure compound dissolved in methanol were added to this mixture. For each experiment, 20 treated and 20 control food strips (1.0×0.6×5.0 cm each) were prepared and arranged in pairs and attached to 20 ropes that were randomly deployed at Pedra Vermelha coast. During the experiments, several common tropical fishes were observed in the studied area (Chaetodontidae, Haemulidae, Labridae, and Pomacentridae families) (Ferreira et al. 2001). After 4 to 6 h, the ropes were retrieved, and the amount of each strip eaten was measured. The *Wilcoxon paired-sample test* (one-tail) was used to analyze the results (Zar 1996).

Bioassay-Guided Fractionation Freeze-dried colonies of *C. braziliensis* (286 g dry wt) were cut into small pieces and exhaustively extracted with *n*-hexane under ultrasonication at room temperature. After removal of the solvent under reduced pressure, 9.2 g of a brownish gum was obtained and used for assays of antifeeding activity. Part of this organic extract (4.26 g) was fractioned by silica gel chromatography, employing *n*-hexane with increasing concentrations of ethyl acetate. The fractions with similar chemical compositions were combined after thin-layer chromatography (TLC) analysis to yield five fractions (F1 to F5). Fraction 2 (F2; 1.81 g) was further purified by repeated flash chromatography, yielding in order of elution, fractions A and B, which were both crystallized from methanol to give two different white solids, identified by spectroscopic methods [gas chromatography (GC)/mass spectrometry (MS), ultraviolet (UV), infrared (IR), MS, ^{1}H and ^{13}C and 2D nuclear magnetic resonance (NMR)], and comparison with literature data (Fleury et al. 1994; Zhang et al. 2005). Procedures for structure elucidation of fraction 1 and compound 1 are described below.

Analytical Procedures The Fourier transform IR spectrum was recorded on a Nicolet-Magna 760 spectrophotometer in KBr pellets. The UV spectrum was determined with a UV-visible Cary 3E Varian spectrophotometer. Electron spectroscopic imaging (ESI)–MS was obtained in positive ion mode on a Q-TOF mass spectrometer (Micromass, Manchester, UK). GC–MS analysis was carried out on an HP Model 5973 with electron impact ionization at 70 eV. The monohydroxylated sterol fraction was submitted to GC/MS analysis on capillary DB-5 column (i.d.: 0.25 mm; length: 30 m; thickness: 0.33 μm), in program mode from 200 to 290°C, 10°min^{-1}, and 10 min at the upper limit (split injection 1:20). Sterol mixture compositions were calculated (%) from the GC peak areas and identified by comparison with literature data (Fleury et al. 1994) and Wiley 275 Mass Library. The NMR spectrum was performed on a Bruker AVANCE-300 spectrometer operating at 300 and 75 MHz, for ^{1}H and ^{13}C, respectively (c=10 mg/0.6 mL in $CDCl_3$+ 0.05% TMS using a 5-mm tube, at 298 K). Melting point was measured by using a Melt Temp® apparatus and is uncorrected. Isolation procedures were monitored by employing TLC on pre-coated silica gel plates (Merck, Whitehouse Station, NJ, USA, Kieselgel 60 F-254) and UV inspection, and with ceric sulfate/heat pretreatment.

Results

Field Assays Initial field assays performed with the *n*-hexane extract revealed that it deterred a natural assemblage of fish consumers (P<0.05), as previously found (Lages et al. 2006). This assay was a guide to subsequent purification

of the active compound. Five fractions from this crude extract also were incorporated into artificial diets at their natural volumetric concentrations, and tested in situ, against fish consumers. The results showed significant inhibitory activity for F2 ($P<0.05$, Fig. 1).

Similar bioassay-guided separation of the active component F2, via repeated silica gel chromatography purification afforded two fractions A and B. These were further purified by crystallization using methanol, yielding, respectively, the monohydroxylated sterol mixture as fraction 1 (535 mg, 0.20% dry wt soft coral) (see Table 1) and the pure compound, a new hemiketal steroid, 23-keto-cladiellin-A (**1**) (357 mg, 0.12% of dry wt soft coral) (Fig. 2). The major fraction had no significant inhibition on feeding relative to controls ($P>0.05$; Fig. 3). In fact, we observed fish consumers feeding on several test strips. On the other hand, feeding deterrent properties were restricted to the more polar compound (1), which significantly reduced consumption of food strips by fishes relative to controls ($P<0.01$; Fig. 3).

Monohydroxylated Sterols Fraction The mixture of nine monohydroxylated sterols showed four dominant compounds: cholesterol, campesterol, epibrassicasterol, and 22-dehydrocholesterol. Table 1 reports the molecular ion and identification of the sterol fraction by GC/MS in comparison with literature data (Fleury et al. 1994) and Wiley 275 Mass Library. Diagnostic fragment ions at *m/z* 255, 213, 145, and 105 indicated the presence of a δ^{5-}3β-ol structure (Budzikiewicz et al. 1964) substituted with different side chains.

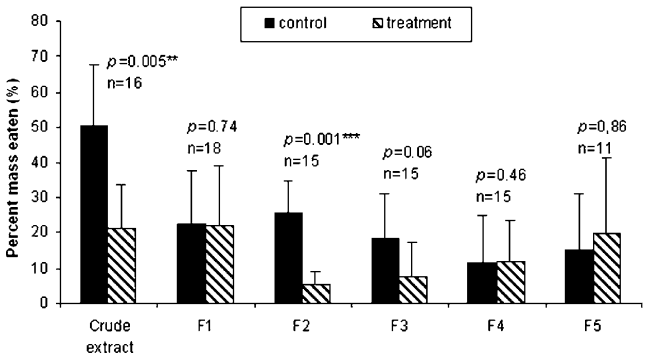

Fig. 1 Consumption of fishes on paired bait-strips with (treated) and without (control) hexane extract (crude extract), and five different fractions (F1 to F5) from this bioactive extract of *C. braziliensis*. *Vertical bars*, ±95% confidence limits. *n*, Number of pairs retrieved of 20 deployed. *P*, probability calculated by the Wilcoxon paired-sample test, one-tailed. *Asterisks*, statistically significant ($P<0.05$) reductions in feeding relative to palatable control

Structural Elucidation of the 23-keto-cladiellin-A Compound 1 (Fig. 2) had a molecular formula $C_{27}H_{38}O_4$, as determined by HRMS-ESI (nine degrees of unsaturation). Carbon-13 and DEPT NMR analysis showed the presence of four methyl groups, eight methylenes (all sp^3), nine methines (three sp^2 and six sp^3), and six quaternary carbon atoms (three sp^2 and three sp^3). The UV maximum at 242 nm was typical of a cross-conjugated cyclohexadienone functionality, which was substantiated by an IR absorption at 1,660 cm^{-1} and $^{13}C/^{1}H$ NMR signals at δ 186.4 (C-3), 123.9 (C-4), and 6.09 (H-4, d, 1.7 Hz), 127.6 (C-2) and 6.26 (H-2, dd, 10.2 and 1.7 Hz), 155.7 (C-1) and 7.09 (H-1, d, 10.2 Hz). The IR spectrum also showed an absorption at 1,716 cm^{-1}, characteristic of a ketonic carbonyl group, supported by ^{13}C NMR chemical shift at δ 208.3 (C-23). The remaining degrees of unsaturation of compound 1 could be accommodated by a steroid nucleus (3) and an additional ring (1). From the HMBC spectrum (Table 2), correlations between H-19/C-1, C-5, C-9, C-10 and H-4/C-2, C-6, C-10 were observed, elucidating rings A and B, as well as correlations between H-8/C-11, H-15β/C-8, C-13 constituting the C and D rings typical of the cholesta-1,4-dien-3-one steroidal skeleton. In this spectrum, two methyl group signals at δ 0.95 (H-26, d, 6.7 Hz) and 0.94 (H-27, d, 6.7 Hz) were correlated with C-25 (δ 23.9) and C-24 (δ 43.6), and H-24α,β was correlated with C-23 (δ 208.3). This framework was confirmed in the mass spectrum by the presence of the base peak at *m/z* 341 (^{+}O = C-CH_2-isopropyl, or M^{+}-85). The unusual quaternary carbon signal at C-22 (δ 98.8) indicated the presence of two oxygen atoms linked to it. Correlations between H-18α/C-12 and C-22, H-18β/C-12, C-13, C17, and the remaining degree of unsaturation demanded by the molecular formula, suggested the presence of a ring, part of an ether bridge. A hemiketal seemed most likely. Correlations in the NOESY spectrum observed between H-19 with H-6β and H-11β and H-18β with H-8β were in agreement with the relative stereochemistry of a normal 5α-cholestane skeleton (8β, 9α, 10β, 13β, 14α, 17β) (Mellado et al. 2005). The β-orientation of H-20 was confirmed by the correlation with H-17. Thus, the NOE correlation between H-21 and the hydrogen from the hydroxyl group on C-22 justify the β position of both. This was also supported by the correlation between H-18α,β and the hydrogen from the hydroxyl group. These assignments enabled us to conclude that the proposed structure represents a new marine steroid (Fig. 2). It was named as 23-keto-cladiellin-A because it is closely related to cladiellin A isolated from the soft coral *Cladiella* sp. (Zhang et al. 2005). However, cladiellin A is unstable in both pyridine-d_6 and $CDCl_3$ solutions yielding dehydration products, but this is not the case of the present 23-keto-cladiellin-A, which is stable even in DMSO-d_6.

Table 1 Monohydroxylated sterols composition of exotic soft coral *C. braziliensis* determined by GC/MS analysis

M	Molecular formula	Area (%)	Compounds[a]
384	$C_{27}H_{44}O$	14.0	22-Dehydrocholesterol = 22(*E*)-cholesta-5,22-dien-3 β-ol
386	$C_{27}H_{46}O$	23.4	Cholesterol = cholesta-5-en-3 β-ol
398	$C_{28}H_{46}O$	16.0	Epibrassicasterol = 22(*E*), 24 (*S*)-Ergosta-5,22–3β-ol
398	$C_{28}H_{46}O$	9.1	Brassicasterol = 22(*E*)-24(*S*)-24-Methylcholesta-5,22-dien-3β-ol
400	$C_{28}H_{48}O$	18.4	Campesterol = 24(*R*)-24-Methylcholesta-5,24-dien-3β-ol
412	$C_{29}H_{48}O$	3.5	28-Isofucosterol = 24(*Z*)-24-Ethylcholesta-5–24(28)-dien-3β-ol
412	$C_{29}H_{48}O$	8.8	28(*E*)-Ethylcholesta-5,22-dien-3β-ol
414	$C_{29}H_{50}O$	5.0	Sitosterol = 24Ethylcholesterol(24ξ)-Ethylcholesta-5–3β-ol
426	$C_{30}H_{50}O$	1.8	24(*E*)-propylcholesta-5,24-dien-3β-ol or 24(*E*)-isopropylcholesta-5,24-dien-3β-ol

DB5-capillary column
M, molecular weight
[a] Wiley 275 Mass Library and Fleury et al. 1994

Discussion

Octocorals provide an interesting model system to test for the presence of defensive interactions because they express physical and chemical defenses (Van Alstyne et al. 1994; Kelman et al. 1999; Koh et al. 2000). Generally, the physical protection afforded by a more highly calcified polypary in Nephtheidae family (Alcyonacea) appears to be associated with the lack of chemical defense (Sammarco et al. 1987). In fact, some Nephtheidae species commonly use spicules as physical defense (Coll 1992), although there are more known examples of chemical defenses in this family (Coll 1992; Lages et al. 2006).

The secondary metabolites from marine invertebrates that play a defensive role against predation usually are structurally complex and frequently present in high concentrations in animals that lack obvious physical defenses (Paul 1992; Pawlik 1993). We found the major sterol fraction, isolated from the exotic soft coral *C. braziliensis*, that contained the monohydroxylated sterols to be inactive in the field assay. A minor component, however, the steroid 23-keto-cladiellin-A, appears to have an antipredatory function. This new compound joins with a small number of metabolites from octocorals with proven antifeedant properties (e.g., Harvell et al. 1988; Wylie and Paul 1989; Fenical and Pawlik 1991; Pawlik and Fenical 1992; Gerhart and Coll 1993; Cronin et al. 1995; Epifanio et al. 1999, 2007; Maia et al. 1999). There are no clear correlations between the active and inactive metabolites. It is still early to make any generalizations about structure–function relationships and chemical defenses. A minor change in the stereochemistry, structure, or functionality of a deterrent compound may render it inactive (Pawlik 1993).

The field assay reported in this work was done under ecologically realistic conditions, in Brazilian waters, far from the natural habitat of *C. braziliensis*. Although the results reported herein were seen to be relevant, further studies, both in the laboratory and in the field, under more

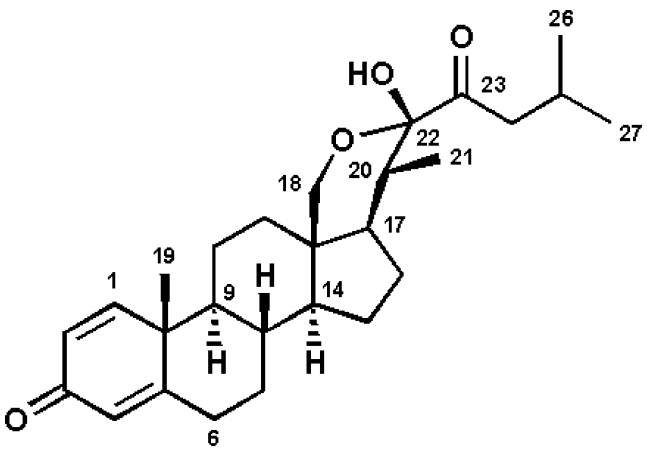

Fig. 2 Compound 1 (23-keto-cladiellin-A)

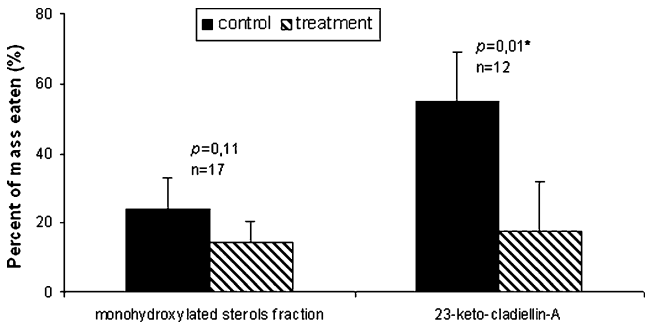

Fig. 3 Consumption of fishes on paired bait-strips with (treated) and without (control) different fractions from the bioactive F2 of *C. braziliensis*: The major fraction, monohydroxylated sterols fraction and compound 1 (23-keto-cladiellin-A). *Vertical bars*, ±95% confidence limits. *n*, Number of pairs retrieved of 20 deployed. *P*, probability calculated by the Wilcoxon paired-sample test, one-tailed. *Asterisk* statistically significant ($P<0.05$) reductions in feeding relative to palatable control

Table 2 NMR data for compound 1 (in $CDCl_3$)

Carbon	δ_C[a]	δ_H(*J*/Hz)	HMBC correlations	NOESY correlations
1	155.7 (d)	7.09 d (10.2)	C-3, C-5, C-9, C-10, C-21	H-2, H-21
2	127.6 (d)	6.26 dd (10.2, 1.7)	C-4, C-10	H-1
3	186.4 (s)	–		
4	123.9 (d)	6.09 d (1.7)	C-2, C-6, C-10	H-6β
5	169.0 (s)	–		
6α	32.7 (t)	2.46 dt (13.8, 13.8, 4.8)	C-7	H-21
6β		2.36 m		H-4
7α	33.8 (t)	1.97 m	C-9	
7β		1.03 m		
8	35.4 (d)	1.42 m	C-11	H-20α, H-20β
9	52.5 (d)	1.15 m	C-8	H-11β
10	43.7 (s)	–		H-1, 2, 4, 19
11α	22.5 (t)	1.86 m	C-9, C-12, C-13	H-8, H-21
11β		1.67 m	C-8	H-9
12α	32.1 (t)	2.37 m		H-17
12β		1.00 m		
13	42.2 (s)	–		
14	54.3 (d)	1.12 m		H-17
15α	24.3 (t)	1.71 m		H-22
15β		1.41 m		
16α	22.6 (t)	2.32 m		H-20α, H-22
16β		1.87 m	C-8, C-13	
17	48.3 (d)	1.63 m		H-12α, H-14, H-18
18α	58.6 (t)	3.80 d (11.4)	C-12, C-19	H-8, H-15α, 19-OH
18β		3.61 d (11.4)	C-12, C-13, C-17, C-19	H-8, H-11α
19	18.7 (q)	1.22 s	C-1, C-5, C-9, C-10	H-1, H-6α, H-11α
20	31.6 (d)	2.20 m	C-17	H-17
21	13.4 (q)	0.73 d (7.1)	C-17, C-18, C-19	H-15α, H-16α, 19-OH
22	98.8 (s)	–		
23	208.3 (s)	–		2H-24
24α	43.6 (t)	2.68 dd (17.8, 7.0)	C-23, C-25, C-26, C-27	H-26, 27
24β		2.38 m	C-23, C-25, C-26, C-27	H-26, 27
25	23.9 (d)	2.21 m	C-26, C-27	
26[b]	22.7 (q)	0.95 d (6.7)	C-24, C-25	H-24α, H-24β
27[b]	22.4 (q)	0.94 d (6.7)	C-24, C-25	H-24α, H-24β
19-OH	–	4.34 s	C-18, C-19, C-23	H-22, H-20α

[a] Multiplicities were determined by Dept-135 and Dept-90 experiments
[b] Signals can be interchanged

controlled conditions, with selected predator fish species are planned.

Recent studies of invasive marine species have highlighted the utility of using exotic species as model organisms (Sax et al. 2007), and, therefore, they outline a conceptual framework uniting the various mechanisms by which exotic species promote evolutionary diversification (Vellend et al. 2007). The absence of coevolved specialist enemies and the preferential consumption of native species by native generalists putatively generally give exotic species a competitive advantage over their native counterparts (Keany and Crawley 2002; Shea and Chesson 2002; Siemann and Rogers 2003). Further research on differential grazing between predators and co-occurring prey species is necessary to yield information that might support the enemy release hypothesis (Keany and Crawley 2002; Colautti et al. 2004). This theory may explain the effectiveness of the invasive species expansion in the new habitat.

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