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VARIABILITY OF TERPENE CONTENT IN THE SOFT CORAL *Sinularia flexibilis* (COELENTERATA: OCTOCORALLIA), AND ITS ECOLOGICAL IMPLICATIONS

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Abstract—Colonies of the soft coral *Sinularia flexibilis* (Quoy & Gaimard) (Coelenterata, Octocorallia) were collected at Lizard Island (14°40′S and 145°28′E) Research Station. Extraction of the corals and quantitative chemical analysis for the three major diterpene components, flexibilide, dihydroflexibilide, and sinulariolide, afforded average ratios of 4:3:1 respectively. Colonies, sized on the basis of the sterile stalk circumference, were analyzed for possible correlations between size and chemical composition. The major metabolite, flexibilide, was inversely correlated with colony size, while sinulariolide concentration showed a direct correlation. The concentration of dihydroflexibilide was independent of colony size. Samples were further analyzed with respect to site of collection. Colonies were collected at three distinct reefal sites. One was characterized by large monospecific stands of *Porites cylindrica*, a second was a sandy bottom site with a mixed community of soft corals and occasional scleractinians, while the third site was a very diverse reef community with many species of scleractinian corals. *Sinularia flexibilis* was well represented at each site, and the concentration of flexibilide and sinulariolide varied significantly among sites. The concentration of flexibilide was significantly higher at the third, highly competitive site, while the concentration of sinulariolide was highest at the *Porites*-dominated site. Dihydroflexibilide levels were independent of site. It seems likely that concentrations of flexibilide, a highly cytotoxic molecule involved in interference competition, and sinulariolide, a known algicide probably responsible for colony maintenance, may be influenced by their environments.

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INTRODUCTION

Sinularia flexibilis is a common alcyonacean soft coral that makes up a substantial proportion of living cover on some Indo-Pacific reefs (Dinesen, 1983). Like many soft corals, *Sinularia flexibilis* produces a range of secondary metabolites that participate in a variety of chemical strategies (Sammarco and Coll, 1988). These secondary metabolites, mainly diterpenes, function in defense against predation (Alino, 1989), serve as antifouling agents (see Coll, 1992), and mediate allelopathic interactions (Sammarco et al., 1983).

In terrestrial chemical ecology, the role of secondary metabolites in plant ecology has been the subject of interest for a long time (Rice, 1984). Secondary metabolites in plants are also thought to have several ecological functions, serving as defense against herbivores and pathogens, as attractants for pollinators and fruit-dispersing animals, and as allelopathic agents (Gershenzon and Croteau, 1991).

Another aspect that has been extensively studied in terrestrial chemical ecology is the intraspecific variability in the production and composition of secondary metabolites. Intraspecific variability of secondary metabolites occurs at several levels. It is observed between populations of specific plants, between specimens of a given population, and also among organs of individual plants (Gershenzon and Croteau, 1992). This has been explained on the basis of genetic differences between populations and between individual plants (Lincoln and Langenhein, 1981), the physiological condition of the plant (Rice, 1984), plant age (Bowers and Stamp, 1992), environmental characteristics (Mihaliak et al., 1989) and ecological interactions (Lincoln and Langenhein, 1979; Louda and Rodman, 1983).

In the marine environment, the chemical composition of the soft coral *Sinularia flexibilis* has been extensively studied (see Coll, 1992 for review). Little is known, however, about the variability in composition and concentration of secondary metabolites between individual colonies within this species.

For an organism such as *Sinularia flexibilis*, for which chemical defenses play a major role in its survival and colonization success, individual variability in the composition and concentration of secondary metabolites would have significant implications for the efficiency of these defensive mechanisms.

In this paper, we report the results of a survey of the chemical composition of a number of colonies of *Sinularia flexibilis* collected from three distinct reefs at Lizard Island, Great Barrier Reef, in which we measured the concentration of three major diterpenes—flexibilide, dihydroflexibilide, and sinulariolide—by

^{1}H NMR spectroscopy. The results allowed an assessment of the variability in the production of secondary metabolites by *Sinularia flexibilis*. A discussion of the likely ecological functions of these metabolites is also offered.

METHODS AND MATERIALS

Collection Sites. Colonies of *Sinularia flexibilis* were sampled from three reefs in the Blue Lagoon, Lizard Island, Great Barrier Reef (14°40′S and 145°28′E). The first site (site 1) was the lagoonal reef in front of One Coconut Tree Beach, which faces the eastern entrance of the lagoon. This reef is characterized by extensive monospecific stands of the coral *Porites cylindrica* and a few species of both scleractinian and alcyonarian corals. Site 2 was Loomis reef, which is located towards the western entrance of the lagoon and is characterized by an intermediate abundance of scleractinian and alcyonacean corals. Site 3 was the eastern side of Vicki's reef, which is immediately at the western end of the Blue Lagoon. This reef was sampled because of its very diverse coral community, represented by a large number of scleractinian coral species.

Most of the *Sinularia flexibilis* colonies that could be found at depths of 2–5 m were sampled. *S. flexibilis* normally presents a patchy distribution on the reefs, mainly because of their mode of asexual reproduction by colony division. Our collection was restricted to only one colony per patch, in order to avoid the collection of genetic clones. When a colony was selected, the circumference of the colony sterile stalk about 5 cm from its base was measured. After measurement, sampling involved the cutting with scissors in situ of three branches of the polyp-rich tissues (approximately 2–4 g dry weight of tissue) from each colony, and storing these portions in separate plastic bags. Samples were then frozen at −20°C until they were freeze-dried.

In addition to the sampling of the *Sinularia* colonies, small portions of the scleractinian corals that were in contact or in close proximity (5 cm) to each of the soft corals, were collected. The small scleractinian samples where then bleached in sodium hypochlorite solution for subsequent taxonomic identification, based on the taxonomic guides by Veron and Pichon (1976, 1979, 1982), Veron and Wijsman-Best (1977), Veron and Wallace (1984), and Veron (1986).

Sample Extraction and Preanalysis Fractionation. The freeze-dried coral tissue was extracted with dichloromethane (DCM, 10 ml/g dry weight of tissue) by soaking the ground colonies in sealed vials for two 24-hr periods and decanting the solvent from the samples after each extraction. A portion of the combined extract (1 ml) from each sample was chromatographed on a small silica gel column (Merck Si gel, type 60 for TLC; 4-cm bed packed on a cotton wool plug in a Pasteur pipet). The solvent was forced through the column using compressed air until the column was free of DCM. Second and third fractions

were obtained by elution with a mixture of acetonitrile–DCM (3:2, 1 ml each). The third fraction obtained in this way from each chromatography contained a mixture of the three major diterpenes present in each extract. This fraction was compared between samples by ^{1}H NMR spectroscopy.

^{1}H NMR Analysis. The total diterpene fraction was evaporated to dryness, an accurately known amount of 2,4-dinitrobenzene (~5 mg) added as internal standard, and deuterated chloroform (~0.5 ml) used as solvent. The ^{1}H NMR spectrum of each sample was recorded on a Bruker AM300 NMR spectrometer using a pulse delay of ~5 sec to ensure complete relaxation of the 2,4-dinitrobenzene; 64 scans of each sample were recorded. Spectra were Fourier transformed using zero line broadening and appropriate signals integrated. Signals measured for 2,4-dinitrobenzene resonated at δ7.81, 8.57, and 9.08; signals used for flexibilide resonated at δ6.46 and 3.97; sinulariolide was estimated on the basis of its lactonic methine proton at δ4.07; dihydroflexibilide was estimated on the basis of its lactonic methine signal (δ4.01), which overlapped with the same signal from flexibilide (δ3.97). The quantity of flexibilide was derived from the δ6.46 signal, and the quantity of dihydroflexibilide was derived from the two-proton signal complex near δ4.0. Integration of each spectrum was carried out in triplicate and the means used to estimate the absolute amount of each compound (in milligrams).

Statistical Analyses. Data were analyzed with standard parametric procedures. One-way analysis of variance including post-hoc tests were used for the comparison of the absolute concentrations of the compounds. Linear regression models were used to describe the relationship between the compounds and between compounds and colony size. As colony division in *Sinularia flexibilis* occurs when the basal size of the colony (as measured by its circumference) reaches about 40–50 cm, the analysis of the correlation between basal size and diterpene concentration was therefore restricted to sizes below 45 cm.

For the comparison of the diterpenoid profiles of colonies from the different reefs, we converted the concentration of each compound to a proportion of the total terpene for each colony and applied arcsin transformation to accommodate the ANOVA assumptions.

The Shannon-Wienner diversity index was calculated for the species of scleractinian corals in vicinity of the soft corals from the three reefs sampled. Diversity indexes from these reefs were then pairwise compared using Hutcheson t test. Statistical tests followed routines described by Sokal and Rohlf (1981) and Zar (1984).

RESULTS

Terpene Concentrations. Major differences were found between the concentrations of each of the three diterpenes in the soft coral tissues (one-way ANOVA, $P < 0.0001$). Flexibilide occurred at the highest concentration, with

an average of 8.25 mg/g (dry weight). Dihydroflexibilide appeared at an average concentration of 6.25 mg/g (dry weight) and sinulariolide at an average of 2.00 mg/g (dry weight) (Figure 1).

For the 73 colonies analyzed, the concentration of flexibilide was found to be negatively correlated with the concentration of sinulariolide ($r = 0.524$, $P < 0.0001$) and positively correlated with the concentration of dihydroflexibilide ($r = 0.43$, $P < 0.0001$). There was no correlation between the concentration of sinulariolide and dihydroflexibilide ($r = 0.125$, $P > 0.1$).

Variability between individuals for both the total terpene concentration and the concentration of each of the three terpenes was high. Total terpene concentrations ranged from 7.6 mg/g to 31.6 mg/g (dry weight).

The concentration of flexibilide was found to be negatively correlated with basal size ($r = 0.441$, $P < 0.001$) and, as expected, concentrations of sinulariolide were positively correlated with basal size ($r = 0.268$, $P < 0.05$). That is, as the soft coral increases in size, the concentration of sinulariolide in its tissues increases, while the concentration of flexibilide decreases. No significant correlation was found between dihydroflexibilide and basal size ($r = 0.035$, $P > 0.5$) (Table 1).

Variability Among Reefs. To reduce the size-related variability of diterpene concentrations and to include a size range well represented in all reefs, only soft corals with basal sizes between 25 and 45 cm were included in these analyses.

The average of the total terpene concentration was consistent among soft corals from the different sites (one-way ANOVA, $P > 0.1$), being 17.9 for site 1, 15.8 for site 2, and 16.3 for site 3 (mg/g dry weight).

Although total terpene content was similar at all three reefs, the proportions of the three compounds were different at each reef. Colonies of *Sinularia flex-*

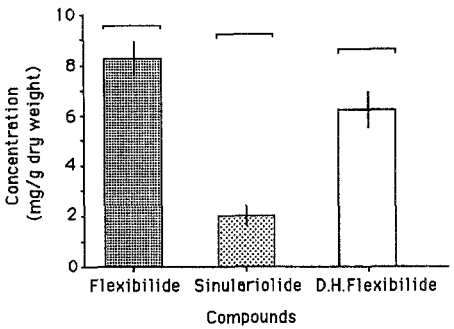

FIG. 1. Average concentration of the three major diterpene compounds from *Sinularia flexibilis*, ANOVA, $P < 0.001$, $N = 73$ for each compound. Error bars show 95% confidence intervals. Horizontal bars show similarities between groups (Tukey test, $P < 0.05$).

ibilis from site 3 contained an average of 54% of flexibilide, significantly higher than the soft corals from site 1 (average 39%). No significant difference was found between the soft corals from site 2 and site 3 (Tukey test, $P > 0.05$) or site 1 and site 2 (Tukey test, $P > 0.05$) (Figure 2).

The proportions of sinulariolide were also significantly different between reefs. This result was expected, because the concentrations of flexibilide were negatively correlated with the concentration of sinulariolide. Soft corals from site 3 contained the lowest proportion of sinulariolide, averaging 5.9% (one-way ANOVA, $P < 0.01$), significantly different from the soft corals from sites

TABLE I. REGRESSION ANALYSIS BETWEEN BASAL SIZE OF *Sinularia flexibilis* AND CONCENTRATION OF ITS THREE MAJOR DITERPENE COMPOUNDS.

	N	*r*	*F*	*P*	Significance[a]
Flexibilide	55	0.441	12.77	<0.001	**
Sinulariolide	55	0.268	4.09	<0.05	*
Dihydroflexibilide	55	0.036	0.06	>0.5	NS

[a] **, highly significant; *, significant; NS, not significant.

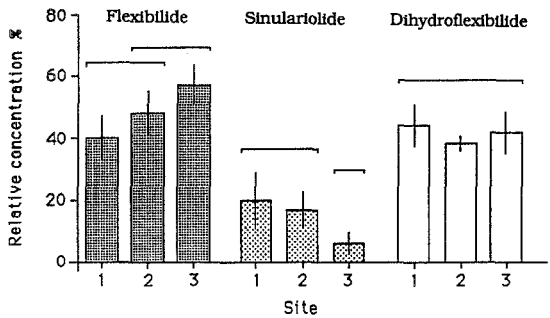

FIG. 2. Relative concentration of the three major diterpenes compounds from *Sinularia flexibilis* according to the site of collection at Lizard Island, showing a significant difference in proportion of flexibilide among sites (ANOVA, $P < 0.01$), a significant difference in proportion of sinulariolide among sites (ANOVA, $P < 0.01$), and no difference in proportion of dihydroflexibilide among sites (ANOVA, $P > 0.1$). Site 1: One Tree Coconut Reef, $N = 14$; Site 2: Loomis Reef, $N = 11$; Site 3: Vicki's Reef, $N = 16$. Proportion data were arcsine transformed to comply with test assumptions. Error bars show 95% confidence intervals. Horizontal bars show similarity between groups (Tukey-Kramer test, $P < 0.05$). Horizontal bars for dihydroflexibilide presented for comparative purposes only.

2 and 3 (Tukey test, $P < 0.05$). There was no significant variation of dihydroflexibilide between sites (one-way ANOVA, $P > 0.1$) (Figure 2).

Taxonomic identification of the scleractinian corals around the soft corals sampled indicated that *Sinularia flexibilis* colonies at site 1 were in interaction with 10 different species of scleractinian corals; at site 2 with 13 species, and at site 3 with 28 species (Table 2). The diversity of scleractinian corals species in interaction with *Sinularia flexibilis* calculated by the Shannon-Wienner index were significantly different between reefs. Site 1 presented the lowest diversity ($H' = 0.82$, $J' = 0.51$); site 2 an intermediate diversity ($H' = 1.06$, $J' = 0.66$), and site 3 presented the highest diversity scleractinian corals interacting with *Sinularia* colonies ($H' = 1.360$, $J' = 0.85$), $P < 0.001$ for all pairs (Hutcheson test) (Table 3).

DISCUSSION

Sinularia flexibilis may well be one of the most successful soft corals on tropical Indo-Pacific reefs. It certainly is the most studied in relation to its chemical composition and to the range of bioactivities associated with this chemistry. Figure 3 gives an indication of some of the chemical constituents reported from this species and suggests structural relationships between key compounds, particularly flexibilide, dihydroflexibilide, and sinulariolide. In structural terms, dihydroflexibilide appears to be something of a "dead-end" compound, in that the reactivity of the α, β-unsaturated lactone system present in sinulariolide and flexibilide has been lost by reduction. As such, it may serve as a storage compound with lower activity and greater stability than flexibilide. This is not inconsistent with the finding that levels of dihydroflexibilide are directly correlated with flexibilide concentrations.

Ecological functions have been attributed to a number of compounds shown in Figure 3, based largely on laboratory bioassays. Thus 11,12-deoxyflexibilide has been shown to be ichthyotoxic towards killifish at <1 ppm, whereas sinulariolide and flexibilide were relatively innocuous at >20 ppm in similar assays using *Gambusia affinis* (see Coll, 1992 for review). Flexibilide and, to a lesser extent, dihydroflexibilide (sinularin and dihydrosinularin) are cytotoxic to cancer cell lines (Weinheimer et al., 1977). They interfere with coral photosynthesis and respiration at <10 ppm (Webb and Coll, 1983), and eventually cause death in scleractinian corals (Coll and Sammarao, 1983). Sublethal levels (1–5 ppm) also cause zooxanthellae expulsion, nematocyst loss, and decrease in polypal activity in scleractinian corals prior to demise (Aceret et al., 1991). Flexibilide and dihydroflexibilide have been detected in the seawater around *S. flexibilis* colonies in the field at concentrations of 1–5 ppm (Coll et al., 1982). This paper reports the concentrations at which the diterpenes are present in the tissues of

TABLE 2. FREQUENCY OF SCLERACTINIAN CORAL SPECIES IN CONTACT WITH *Sinularia flexibilis* AT THREE SAMPLED REEFS

Coral species	Site 1	Site 2	Site 3
Acanthastrea hillae	—	—	1
Acropora cytherea	—	—	1
Acropora divaricata	—	—	1
Acropora humilis	—	—	1
Acropora longicyanthus	—	1	1
Acropora microphthalma	—	2	5
Acropora nasuta	—	—	1
Acropora nobilis	—	—	2
Acropora palifera	—	—	1
Acropora secale	—	2	—
Acropora selago	2	—	2
Acropora tenuis	—	3	—
Acropora valida	—	1	2
Agaricidae	—	—	1
Echinopora horrida	—	1	1
Echinopora lamellosa	2	—	2
Favidae	—	—	1
Galaxea astreata	1	—	—
Goniopora columna	—	1	—
Goniopora pandoraensis	—	1	—
Merulina ampliata	—	—	1
Montastrea curta	—	1	3
Montipora grisea	—	—	4
Montipora tuberculosa	—	—	1
Montipora verrucosa	—	—	1
Pachyseris rugosa	—	—	1
Pavona cactus	1	—	—
Pavona venosa	1	—	—
Platygyra daedalea	—	—	1
Platygyra pini	—	—	1
Pocillopora damicornis	—	1	2
Porites annae	2	—	—
Porites cylindrica	9	—	1
Porites lutea	—	1	4
Porites lychen	1	—	—
Porites sp.	—	—	1
Psammocora contigua	1	—	—
Seriatopora hystrix	—	4	—
Stylophora pistillata	1	—	1
Tubinaria raniformis	—	1	—

TABLE 3. PAIRWISE COMPARISON OF SHANNON-WIENNER DIVERSITY INDEX FOR SCLERACTINIAN CORAL SPECIES AROUND COLONIES OF *Sinularia flexibilis* SAMPLED AT THREE COLLECTION SITES (HUTCHENSON TEST)

Comparison (diversity index)	*df*	*t*	*P*	Significance
Site 1 (H' = 0.82) × site 2	112	4.33	<0.001	**
Site 2 (H' = 1.06) × site 3	123	7.56	<0.001	**
Site 3 (H' = 1.36) × site 1	100	10.55	<0.001	**

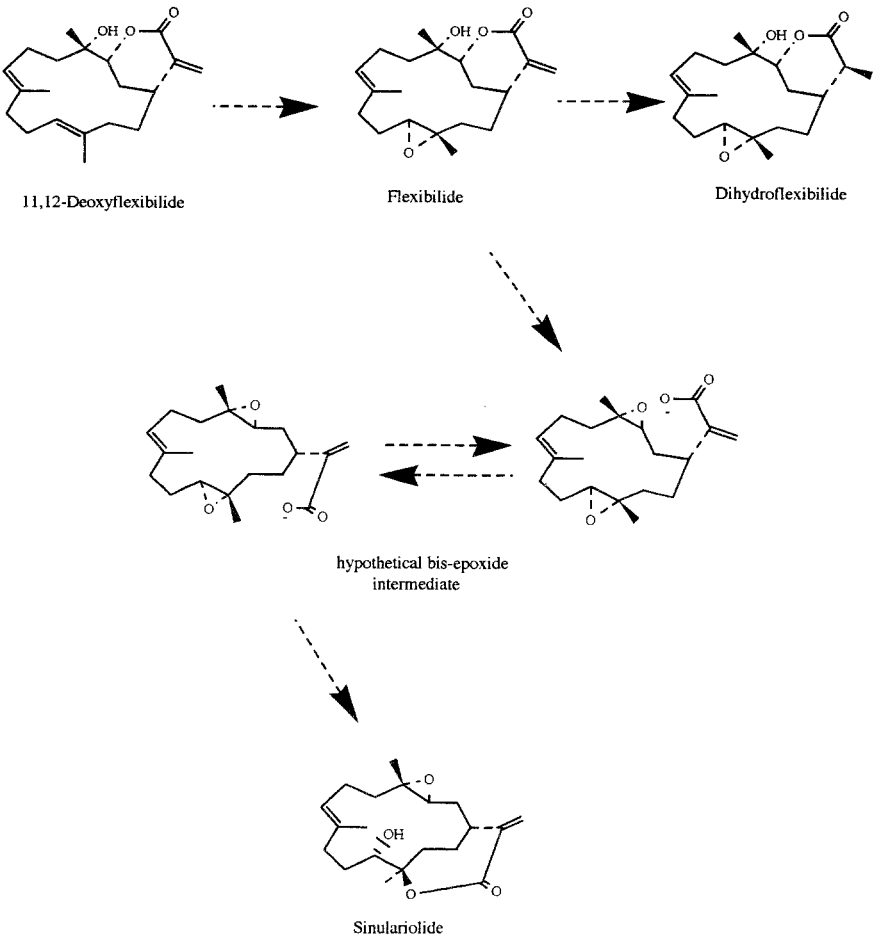

FIG. 3. Cembranolide diterpene metabolites derived from *Sinularia flexibilis* and possible biosynthetic pathways linking them.

S. flexibilis. Flexibilide kills scleractinian corals at 5–10 ppm and is strongly implicated in allelopathic effects on neighboring organisms (Sammarco et al., 1983). The two diterpenes, and particularly flexibilide, have thus been shown to be the major vectors of interference competition for *S. flexibilis*. By contrast, sinulariolide does not possess any of these properties when assayed in similar tests, although it is reported to be an effective algicide (Tursch et al., 1978; Maida, 1993). It thus seems likely that sinulariolide makes a major contribution to the prevention of fouling by algae (Coll, 1992).

Sinularia flexibilis releases secondary metabolites into the surrounding water that are capable of acting as allelopathic agents in competitive interactions (Coll et al., 1982). In a field experiment, Sammarco et al. (1983) observed that all scleractinian corals that were near or in contact with allelopathic soft corals suffered tissue necrosis and growth inhibition. In subsequent field experiments, Sammarco et al. (1985) observed that the effects of *Sinularia flexibilis* on specific neighboring scleractinian corals are variable. That is, while some colonies of a given species of scleractinian suffer deleterious effects when interacting with *Sinularia flexibilis*, other colonies in the same situation might not be affected. Although this variability of effects can be explained by an individual resistance of the scleractinian coral to allelochemicals, it may also be due to the allelopathic potential of a given *Sinularia* colony, i.e., the allelochemical content of the soft coral involved in the interaction.

Sinularia flexibilis is a long-lived soft coral, and the concretion of packed spicules in the lower, older parts of the colonies may guarantee survival, even in the face of severe competition or predation. As colonies grow older, the need for antipredator or anticompetitor compounds might thus decrease. If indeed flexibilide functions as the major vector for *Sinularia flexibilis* in competitive interactions, it might be expected that smaller colonies should have a greater need for this compound than larger colonies. This assumption is supported by the fact that the level of flexibilide was inversely proportional to colony size. The fact that the concentration of sinulariolide increased with colony size may be an indication of the need for antifouling, rather than anticompetitor compounds as colonies age, because larger size offers larger areas for fouling.

Because of the structural relationships outlined in Figure 3, it is possible that sinulariolide and flexibilide are not entirely independent products of biosynthesis. Indeed, the results show a negative correlation between the two compounds. The question then arises as to whether environmental factors play any role in the relative amounts of these metabolites in *Sinularia flexibilis* colonies.

Results from the three different reefs at Lizard Island provide an opportunity to consider this question. Two of the reefs provided the extremes in a spectrum of competitive complexity. Site 1 was dominated by *Porites cylindrica*; interactions there were mainly between *S. flexibilis* and *P. cylindrica* (9/21) and to a lesser extent with nine other species of scleractinian corals. Site 3 was a more

complex community; 45 interactions were studied between *S. flexibilis* and 28 scleractinian coral species. No single scleractinian species dominated these interactions.

Comparisons between the levels of flexibilide and sinulariolide at each of the sites is most revealing. Levels of flexibilide in colonies at the most diverse site 3 were significantly higher than those at site 1. By contrast, levels of sinulariolide at the less diverse site 1 were almost double those at site 3. The total terpene content of the three metabolites (in milligrams per gram of tissue) was independent of site. The variation occurred in the proportions of metabolites at each site. It appears that at the site with the greatest species diversity, colonies produce higher levels of flexibilide (and dihydroflexibilide) at the expense of sinulariolide biosynthesis.

The question then remains as to whether the variability in the concentration of metabolites in *Sinularia flexibilis* is due to an active biosynthetic switch that can trigger the production of one or other compound according to the ecological needs or to the selection of populations that possess specific secondary metabolite profiles that guarantee survival in a particular environment. In the case of sinulariolide and flexibilide, the former protocol is certainly a possibility.

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