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CHEMICAL VARIATION IN THE TROPICAL SEAWEED *STYPOPODIUM ZONALE* (DICTYOTACEAE)

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Key Word Index—*Stypopodium zonale*; Dictyotaceae; Dictyotales; Phaeophyta; chemotaxonomy; chemical variation; marine terpenoids.

Abstract—Five separate collections of the tropical seaweed *Stypopodium zonale* were analysed for ten secondary metabolites using a combination of high performance liquid chromatography and proton nuclear magnetic resonance spectrometry. Shallow water populations from the Caribbean were found to possess similar metabolite profiles from year to year and from widely diverse locations. *Stypopodium zonale* from the South Pacific (Palau) contained similar structure types; however, the profile was qualitatively and quantitatively dissimilar to the Caribbean algae. A deep water Caribbean form of *S. zonale* was found to contain two metabolites, epitaondiol and 6a-desmethyl-6-acetylatomaric acid, not observed in the other extracts. This latter population is morphologically and anatomically distinct from the other *S. zonale* Caribbean populations.

INTRODUCTION

As a class, terpenoid metabolites are useful chemotaxonomic markers because of their structural complexity and unique biogenetic origins. While numerous terpenoid variation studies have been described with terrestrial plants, only a few investigations of terpenoid variation in marine plants have been reported. Analyses of the haloterpenoids from red algae of the genera *Laurencia* [1–3] and *Plocamium* [4, 5] have been reported using TLC, HPLC, GC and GC/MS methods. Further, a chemotaxonomic investigation of the diterpenoids from *Cystoseira elegans* using GC/IR has been recently reported [6].

Over the past 3 years we have been investigating the unique natural products chemistry of the brown seaweed *Stypopodium zonale* (Lamouroux) Papenfuss, an abundant tropical alga of the family Dictyotaceae. The mixed biogenesis metabolites of *S. zonale* (**1–10**), which comprise the majority of the lipid extract, are naturally excreted into seawater [7, 8] and, collectively, they show considerable activity in our fish toxicity assay. As a consequence of our numerous collections of this seaweed from different locations and years, we have observed morphological and chemical variations to exist between different populations. To more rigorously ascertain the nature of these differences and to further explore the concept of chemical variation in the marine environment, we have employed the combined techniques of light microscopy, TLC, HPLC and high resolution ^{1}H NMR spectrometry.

RESULTS AND DISCUSSION

Chromatography of the crude extracts of *S. zonale* using normal phase silica HPLC provides a rapid method for the analysis of the numerous metabolites (**1–10**) in these collections. A two-fold identification procedure, HPLC retention time (μ-Porasil silica gel) and high resolution ^{1}H NMR analysis of the collected fractions, provided unambiguous identifications of these metabolites when compared with our earlier spectrochemical work [7, 8]. Further, recovery of the HPLC separated compounds provided material for ichthyotoxicity testing and the structure assignments of the new compounds **4** and **7** [8]. Several specific comparisons of the qualitative and quantitative variations in *S. zonale* chemistry are made below.

Belize samples (*B*-77, *B*-79-52)

Two Belizean samples collected from the same shallow water location in 1977 and 1979 contain the same metabolites and in roughly the same proportions (Figs 2a, 2b). Stypoldione (**10**), an oxidation product of the major metabolite stypotriol (**9**), appears minor in these HPLC traces due to its instability in the presence of activated silica gel. However, by TLC, roughly equivalent proportions of **10** were observed in these two collections. Therefore, secondary metabolite production is a relatively constant feature from year to year within this shallow water population of *S. zonale*.

Florida Keys, Belize and south Pacific samples (*F*-80, *B*-79-52, *P*-79-50)

The HPLC traces of the Florida collection and shallow water 1979 Belize collection are nearly superimposable (Figs 2b, 2c). That these distant populations (700 miles apart) of *S. zonale* possess the same secondary metabolite chemistry in both qualitative and quantitative senses suggests that this feature is constant within shallow water Caribbean populations. By contrast, the 1979 Palau

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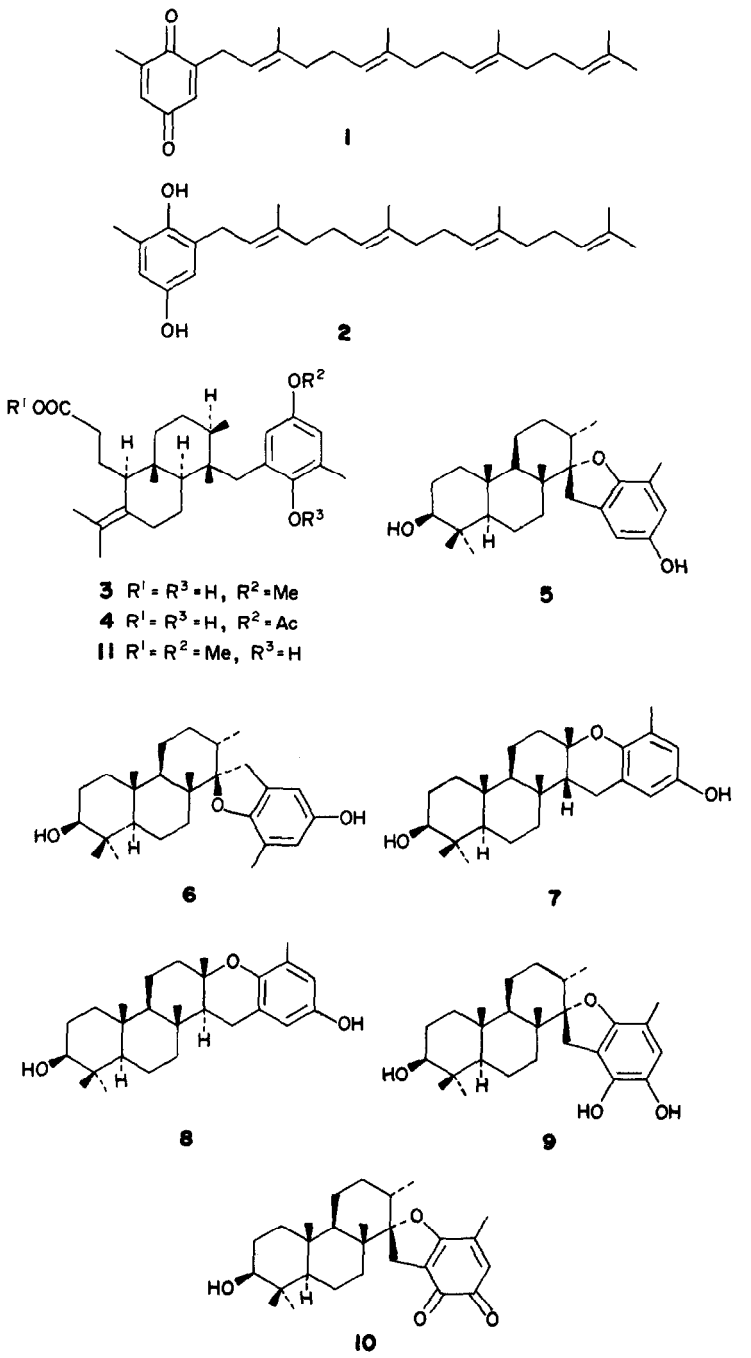

Fig. 1. Structures of *S. zonale* metabolites **1–10**.

collection is both qualitatively and quantitatively dissimilar to any of the Caribbean collections, yet still possesses the mixed biosynthetic type of chemistry (Figs 2b–2d). This latter feature strongly supports previous taxonomic evaluations of this south Pacific seaweed [9].

Shallow and deep water Caribbean samples (B-79-52, B-79-53)

The deep water form of *S. zonale* from Belize contains a higher proportion of taondiol (**8**) than the shallow water form. Additionally, it contains two natural products, **4** and **7**, which were not present in the extract of the shallow water form. Compound **7**, epitaondiol, was reported in our earlier work [8] whereas metabolite **4**, 6a-desmethyl-6-acetylatomaric acid, is tentatively assigned here as a new metabolite of *S. zonale*.

The ^{1}H NMR spectrum of chromatography peak **4** from the deep water Belizean collection (B-79-53) indicated this to be a new metabolite closely related to the known compound atomaric acid (**3**) [10]. The key difference was the absence of a 3H singlet at δ3.72 assigned to the aromatic methoxyl group in a derivative (**11**) of **3**

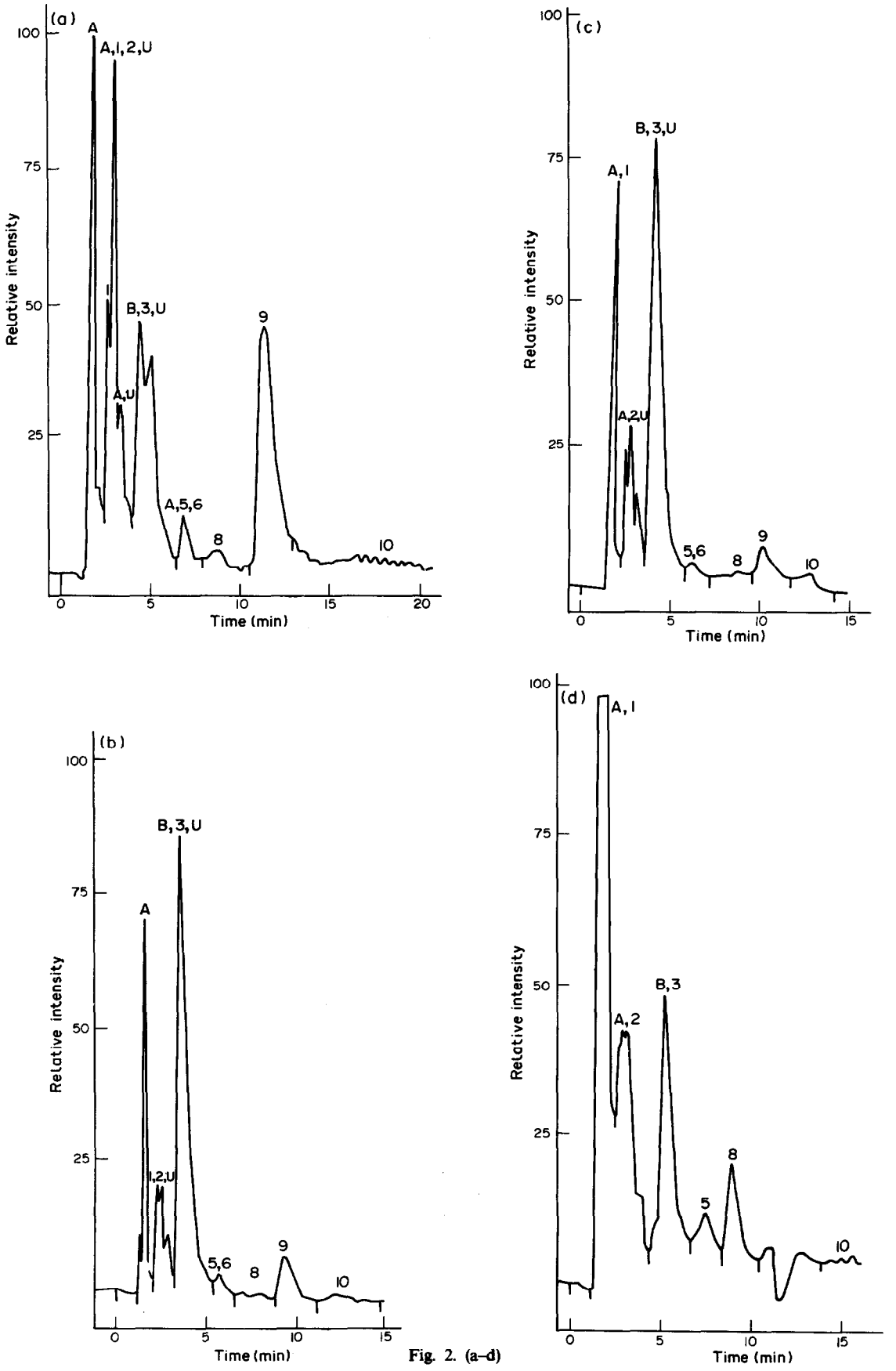
(a)
A
A,I,2,U
B,3,U
9
A,U
A,5,6
8
10
Relative intensity
Time (min)
(c)
B,3,U
A,I
A,2,U
5,6
8
9
10
Relative intensity
Time (min)
(b)
B,3,U
A
I,2,U
5,6
8
9
10
Relative intensity
Time (min)
(d)
A,I
B,3
A,2
8
5
10
Relative intensity
Time (min)

Fig. 2. (a–d)

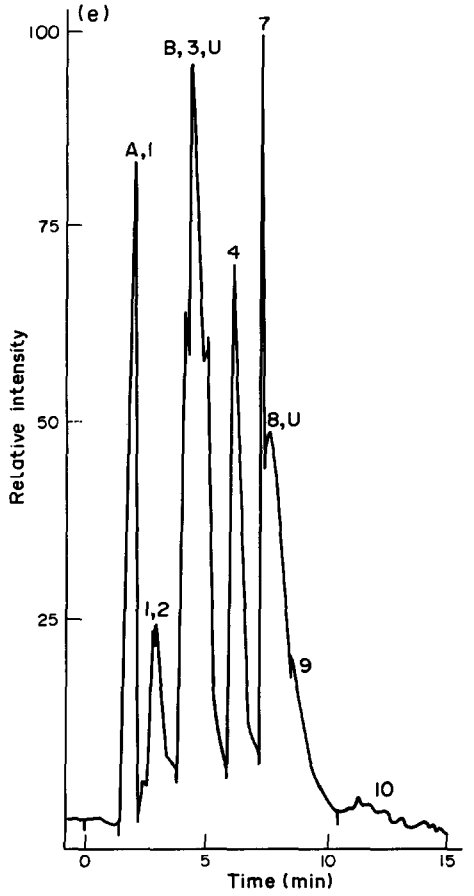

Fig. 2. HPLC traces of different collections of *S. zonale*; (a) Belize shallow water 1977 (B-77); (b) Belize shallow water 1979 (B-79-52); (c) Florida Keys 1980 (F-80); (d) Palau, Micronesia 1979 (P-79-50); (e) Belize deep water 1979 (B-79-53); for conditions see Experimental.

[10]. This singlet was replaced in **4** by a 3H singlet at δ2.26, assigned to an aromatic acetate methyl group. This conclusion was confirmed by the presence of an additional carbonyl absorption in the IR spectrum of **4** ($\nu_{C=O}$ 1710 cm^{-1}). The position of the aromatic acetate was evident from a consistent downfield shift in the two aromatic protons [δ6.85 (1H, *d*, J = 2 Hz) and 6.78 (1H, *d*, J = 2 Hz)] relative to atomaric acid [δ6.68 (1H, *d*, J = 3 Hz) and 6.43 (1H, *d*, J = 3 Hz)]. These data indicated the acetoxyl substituent to be placed at C-6 [11]. Based on these data, the structure of **4** is suggested as 6a-desmethyl-6-acetylatomaric acid.

The significant variations in secondary metabolites between shallow and deep water Belizean populations of *S. zonale* prompted a more detailed morphological and anatomical investigation of our *S. zonale* collections [12]. Belizean plants from deep water (B-79-53) were much thinner and the thalli smaller in overall size (up to 5 cm), while those from shallow water (B-77, B-79-52) were thicker, larger in overall size (up to 20 cm) and more highly iridescent. Examination of these two in transverse and longitudinal cross-section revealed that the shallow water form is consistently thicker (up to 480 μm) with a higher cortical cell–medullary cell ratio (4:1) than the deep form (up to 165 μm, 2–4:1 ratio). Shallow water plants from Florida (F-80) were 7–10 cm tall and were 200–350 μm thick, while those from Palau (P-79-50) were 5–7 cm tall and 200–375 μm thick. Thus, the deep water Belizean collections are chemically and anatomically distinct from our other Atlantic and Pacific collections. Taxonomic conclusions must be limited, however, until comparisons of reproductive material from these shallow and deep water populations can be made.

Table 1. Ichthyotoxicity of metabolites **1–10** to beau gregory damsel fish (*E. leucostictus*) and goldfish (*C. auratus*)

Compound	*Eupomacentrus leucostictus**	*Carassius auratus*†
1	N	N
2	N	—
3	N	NE
4	—	NE
5	H	H
6‡	T	—
7		H
8	N	N
9‡	T	—
10‡	T	T

*All compounds at 10 μg/ml seawater.

†All compounds at 100 μg/ml fresh water.

‡See text for minimum lethal doses for death within 1 hr.

N, Narcosis; H, hyperactivity; T, toxic; NE, no effect; blank, not tested.

The combined spectral and chromatographic methods reported here clearly show that all *S. zonale* collections possess metabolites of the same mixed biosynthetic origin. However, quantitative and qualitative variations were commonly observed. Stypoldione (**10**), the oxidation product of the major metabolite (**9**), was observed by TLC in all collections, although it appeared more major in B-77, B-79-52 and F-80 relative to the B-79-53 and P-79-50 collections.

Samples of the purified metabolites **1–10**, provided from our earlier investigations [7, 8] and obtained by chromatography here, were tested for ichthyotoxicity using beau gregory damsel fish (*Eupomacentrus leucostictus*) and common goldfish (*Carassius auratus*, Table 1). It was our goal to consider these chemical results in the light of the ecological principles of chemical defence in tropical marine environments. Therefore, it is noteworthy that the three strongly ichthyotoxic metabolites (**6**, LD $<$ 10 μg/ml; **9**, LD $\leqslant$ 0.2 μg/ml; **10**, LD $\leqslant$ 1.0 μg/ml) form roughly 6% of the crude extract. This is equivalent to 0.5% (8 g/1.4 kg) [8] of the dry wt or roughly 0.08% of the wet wt (assuming dry wt is 30% hydrated and fresh alga is 90% water) of the alga. Therefore, 1 g of fresh algal tissue contains *ca* 800 μg of these ichthyotoxins, a potentially lethal dosage to grazing fish. These results, combined with our earlier field observations that this seaweed grows luxuriantly in herbivore-rich environments, suggest that these compounds function in nature to deter herbivory [13].

EXPERIMENTAL

General. The algae were collected by hand using scuba equipment and stored in *iso*-PrOH at cool temps (0°–15°).

Material for light microscopic investigations was stored in 5% formalin in sea water and voucher material is deposited in the National Herbarium at the Smithsonian Institution.

Three separate collections of *S. zonale* were made from near Carry Bow Cay, Belize, one in April 1977 (B-77) and two in April 1979 (B-79-52 and B-79-53). The B-77 and B-79-52 collections were made from the 'North Channel' in 3–8 m water depth, while sample B-79-53 was obtained from the 'outer drop-off' in 16–25 m water depth. Sample P-79-50 was collected from the 'Sar Passage' in Palau, Micronesia in Sept. 1979. The water depth at the site of collection was between 6 and 10 m. Sample F-80 was obtained from Looe Key in the western Florida Keys at a water depth of 9–13 m in July 1980.

All collections were extracted in an identical fashion. Filtration of the storage solvent, *iso*-PrOH, yielded a nearly dehydrated algal cake which was homogenized and steeped in $CHCl_3$–MeOH (1:1). The filtered $CHCl_3$–MeOH extract was added to the previously decanted *iso*-PrOH and the organic solvents removed *in vacuo*. The resultant wet extract was partitioned between $CHCl_3$ and saturated brine and the aq. layer discarded. The $CHCl_3$ was dried over dry $MgSO_4$, filtered, and then removed *in vacuo* to yield in each case a dark green tar. The dried organic extract, dissolved in Et_2O, was stored at $-10°$.

TLC was carried out using 100% Et_2O as the developing solvent and Merck pre-coated TLC sheets (silica gel $60F_{254}$, layer thickness 0.2 mm). HPLC separations were carried out with a Waters M-6000 chromatography pump and R401 differential refractometer using two 25 cm × 3.9 mm μ-Porasil columns (Waters) in series with a 5 cm × 3.9 mm guard column packed with Merck Li Chrosorb Si 60 (mean particle size 30 μm). Samples were prepared by *in vacuo* removal of the storage solvent (Et_2O) and re-dissolution in warm 20% EtOAc in *iso*-octane. Samples were entered into the HPLC system by means of a syringe loading Waters U6K universal liquid chromatographic injector. After each injection the guard column was washed with 100% EtOAc after it was attached in the reverse direction and the μ-Porasil columns detached. This procedure removed any polar material trapped in the guard column or particulates on the 15 μm fritted filter disc. The chromatography solvent for separation of the crude material was 20% EtOAc in *iso*-octane. In cases where subsequent analyses of the collected fractions showed them to be uninterpretable complex mixtures, they were re-injected using a less polar solvent mixture, normally 15% EtOAc in *iso*-octane.

High resolution ^1H NMR spectra were obtained at 360 MHz using a home-built spectrometer (Oxford Magnet, Nicolet computer). Spectra were compared with those obtained for the previously reported pure metabolites of *S. zonale* [7, 8]. All spectra were obtained with samples dissolved in $CDCl_3$ containing 0.5% TMS ($\delta 0$) as an int. ref.

6a-*Desmethyl*-6-*acetylatomaric acid* (**4**). HPLC, as indicated in the text, gave pure **4** as a crystalline solid: ^1H NMR (360 MHz, $CDCl_3$): δ6.85 (1H, *d*, J = 2 Hz), 6.78 (1H, *d*, J = 2 Hz), 3.87 (1H, *d*, J = 13 Hz), 2.26 (3H, *s*), 2.21 (3H, *s*), 1.0–2.48 (15H, *m*), 1.67 (3H, *s*), 1.65 (3H, *s*), 1.12 (3H, *d*, J = 7 Hz), 1.01 (3H, *s*), 0.92 (3H, *s*); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450, 2950, 1740, 1710, 1460, 1380, 1190, 1040, 910.

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