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Chemical diversity of *Sarcophyton* soft corals in Okinawa

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Abstract: A total of 279 specimens belonging to nine species of soft corals of the genus *Sarcophyton* were subjected to chemical study for the diversity of cembrane diterpenes. Following morphological identification, each specimen was examined for metabolites using gradient HPLC, NMR, and other tools. The *S. glaucum* and *S. cinereum* species complex was found to be the most abundant and to contain the most diverse metabolites in the Ryukyu Archipelago, Japan. *S. trocheliophorum* and *S. ehrenbergi* were moderately abundant and diverse in metabolites, while other all species seemed to be scarce and restricted in metabolites. During this research we encountered two new compounds (**7**, **12**), whose structures are described in this report.

Key words: *Sarcophyton*, soft corals, cembrane, diterpene, NMR, Ryukyu Archipelago

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INTRODUCTION

Octocorals have been targets in the search for new secondary metabolites over the last three to four decades (Blunt et al. 2005). A large number of bioactive novel metabolites possessing both biological activities and unique chemical structures has been reported, such as: cytotoxic prostanoid punaglandins from the soft coral *Paratelesto riisei* (Baker et al. 1985), antiinflammatory agents pseudopterosins from the gorgonian *Pseudopterogorgia elisabethae* (Look et al. 1986), and antitumoral drug leads, eleutherobins, from the soft coral *Eluetherobia albiflora* (Lindel et al. 1997). Soft corals of the genus *Sarcophyton* have been known as a rich source of cembrane-class diterpenes, among which sarcophytol A (**1**) has attracted attention due to its antitumor-promoting activity (Kobayashi et al. 1979; Fujiki et al. 1989). Contributing to our interest in this bioactive metabolite as a resource in Okinawa, is the fact that no comprehensive report exists regarding chemotypes of the *Sarcophyton* soft corals that are known to be abundant on the reefs of the Ryukyu Archipelago (Benayahu 1995; 2002). Consequently, we initiated this study in order to investigate the natural products of various species of this genus and also to determine possible intraspecific variation in their constituents within the region.

MATERIALS AND METHODS

Collection and extraction of specimens

A total of 279 specimens of *Sarcophyton* were collected by hand using SCUBA between 1998-2001. The number of specimens from each collection site was as follows: 25 (Yonaguni), 39 (Ishigaki), 19 (Miyako), 35 (Kerama), 36 (Okinawa), 38 (Yoron), 17 (Okinoerabu), and 70 (Amami Island) (see Figure 1). The fresh specimens were kept chilled or frozen during transfer to a laboratory in Okinawa, where they were steeped in 80% ethanol. A portion of the ethanol solution was then filtered and concentrated under

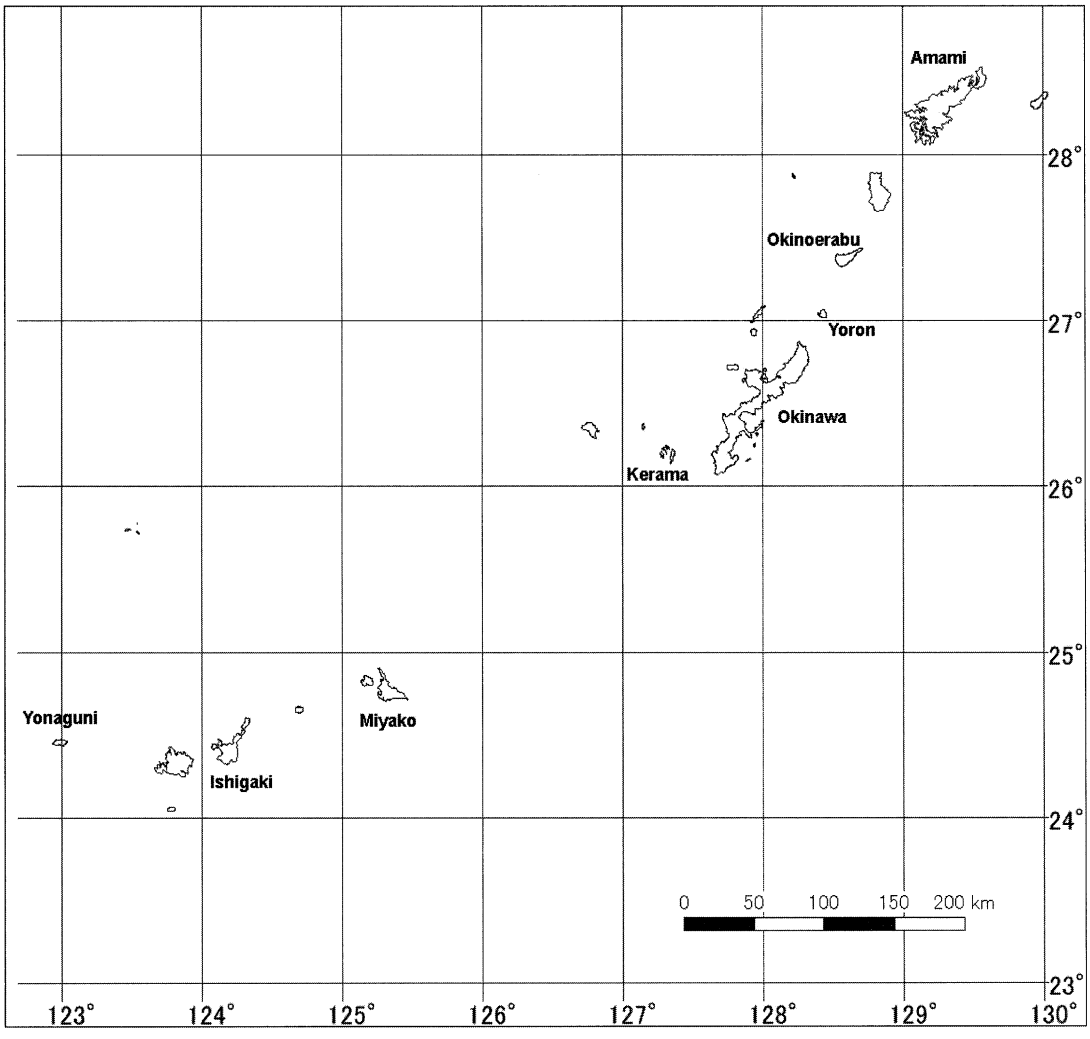

Fig. 1. Collection sites in Ryukyu Archipelago.

vacuum. The aqueous residue was extracted with dichloromethane to give a lipophilic extract for chemical analysis.

Species identification

Each specimen was kept in 70% ethanol for taxonomic identification. Sclerites were obtained by dissolving the tissue in 10% sodium hypochlorite. The specimens were first identified by JT and then confirmed by TY with reference to the taxonomic revision of the genus (Verseveldt 1982). Reconfirmation of the major part of the collection was later carried out by YB, facilitated by comparisons with permanent sclerite-preparations from type material kept at the Zoological Museum, Department of Zoology, Tel Aviv University, Israel.

General methods of chemical analyses

Each dichloromethane extract was subjected first for thin layer chromatography (TLC) and ^{1}H Nuclear Magnetic Resonance (NMR) analyses to examine whether a dominant (approximately >5 %) "marker" cembrane existed. Then, the presence of major cembranoid constituent was confirmed qualitatively by gradient High Performance Liquid Chromatography (HPLC) system equipped with a photodiode array detector using an ODS column with linear gradient elution profile in 25 minutes from 100 % water to 100% methanol. ^{1}H and ^{13}C NMR spectra were taken on a JEOL A-500 by dissolving extracts or pure compounds in $CDCl_3$ using tetramethylsilane as an internal standard. Infrared (IR) spectra were taken on a JASCO FTIR-300, MS spectra

on a Hitachi M-2500 instrument, and ultraviolet (UV) spectra on a JASCO UVIDEC 610.

Structure identification of cembrane diterpenes 1-6 and 8-11

Compounds **1-6** and **8-11** were identified by obtaining pure material separated by column, TLC, or HPLC, followed by NMR spectral com parison with those previously published (Kobayashi et al. 1979; Bowden et al. 1979; Bowden & Coll 1989; Coll et al. 1977; Toth et al. 1980; Bowden et al. 1982; Tursch et al. 1974; Ravi & Faulkner 1978; Bowden et al. 1980).

RESULTS

Cembrane diterpenes of each species

Since we encountered difficulty in differentiating between *S. glaucum* and *S. cinereum* due to lack of distinct morphological features, we considered them in this study as one complex. Of the 279 specimens collected between the Yonaguni and Amami areas, the number of samples of each species was as follows: *S. glaucum/cinereum* (187), *S. trocheliophorum* (33), *S. ehrenbergi* (19), *S. elegans* (18), *S. crassocaule* (3), *S. digitatum* (4), *S. infundibuliforme* (3), *S. pulchellum* (2), *S. cherbonnieri* (3), and unidentified (7 specimens). In the following section, we present the chemotypes revealed for each species with respect to their geographic variation.

S. glaucum/cinereum was collected in abundance throughout the Ryukyu Archipelago, indicating it as dominant species in this area (Table 1). Of the 187 specimens, *S. glaucum/cinereum* was divided into more than nine chemotypes (Table 2, Fig. 2). The cembranes found from this spe-

Table 1. Number of collected *Sarcophyton* specimens at each collection site.

Species \ Island	Yonaguni	Ishigaki*	Miyako	Kerama	Okinawa	Yoron	Okino-erabu	Amami	Total
S. glaucum/cinereum	20	19	10	16	24	36	13	49	187
S. trocheliophorum	5	3	2	11	4		1	7	33
S. ehrenbergi		2	4	1		2	3	7	19
S. elegans		9	1	1	4			3	18
S. crassocaule		3							3
S. digitatum		3						1	4
S. infundibuliforme				1	1			1	3
S. pulchellum			1	1					2
S. cherbonnieri			1		2				3
Sarcophyton spp.				4	1			2	7
Total	25	39	19	35	36	38	17	70	279

*includes Kohama Island

Table 2. Number of collected *Sarcophyton* specimens with respect to cembranes and species.

Species \ compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	*
S. glaucum/cinereum	2	10	5	–	75	20	2	9	33	8	–	–	23
S. trocheliophorum	3	28	1	1	–	–	–	–	–	–	–	–	–
S. ehrenbergi	2	2	2	–	–	–	–	–	–	–	8	–	5
S. elegans	–	3	–	–	–	–	–	–	–	–	–	–	15
S. crassocaule	3	–	–	–	–	–	–	–	–	–	–	–	–
S. digitatum	3	–	–	–	–	–	–	–	–	–	–	–	1
S. infundibuliforme	–	1	–	–	–	–	–	–	–	–	–	–	2
S. pulchellum	–	2	–	–	–	–	–	–	–	–	–	–	–
S. cherbonnieri	–	2	1	–	–	–	–	–	–	–	–	–	–
Sarcophyton spp.	–	3	1	–	–	–	–	–	–	–	–	–	1

*No "marker" cembrane was present.

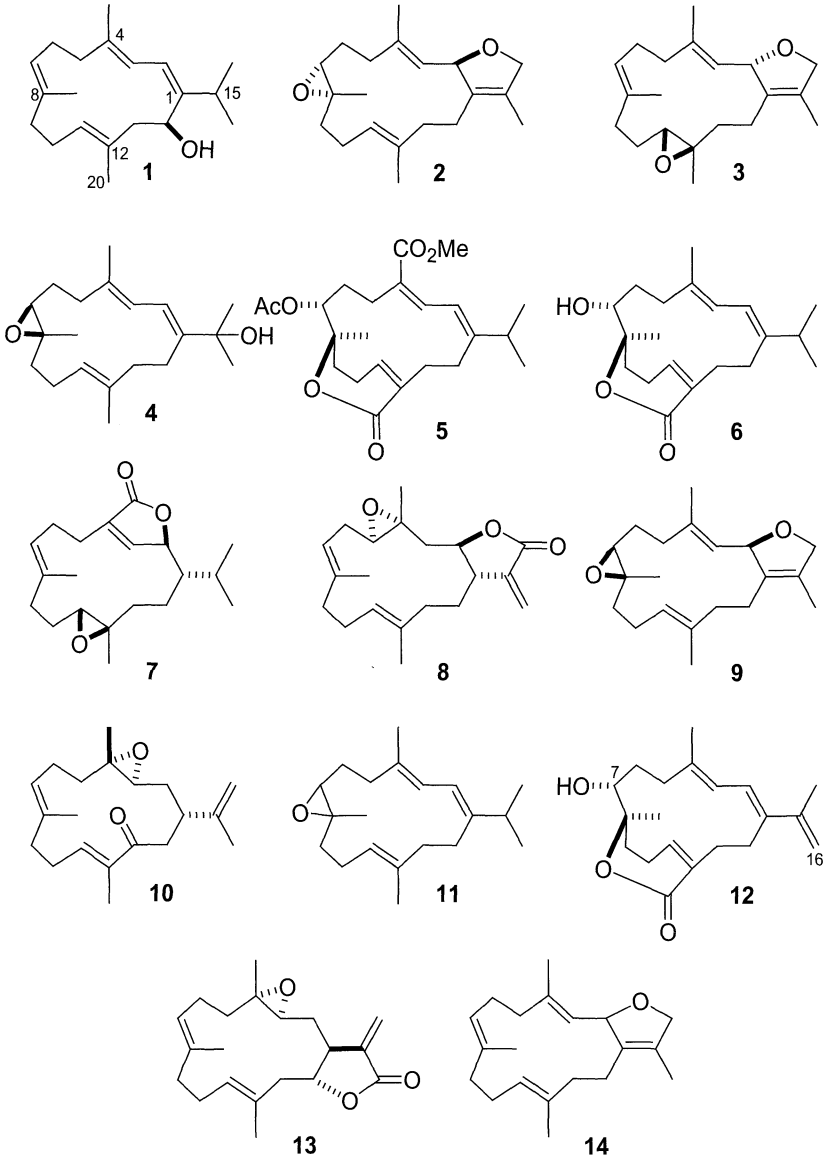

Fig. 2. Structures of cembranes from soft corals of the genus *Sarcophyton*.

cies were: emblide (**5**, 75 specimens), 2*R*,7*S*,8*S*-sarcophytoxide (**9**, 33 specimens), compound **6** (20 specimens), deoxosarcophine (= 2*R*,7*R*,8*R*-sarcophytoxide, **2**, 10 specimens, Bowden et al. 1987), lobophytolide (**8**, 9 specimens), compound **10** (8 specimens), isosarcophytoxide (**3**, 5 specimens), sarcophytol A (**1**, 2 specimens), new compound **7** (2 specimens), and those without marker cembranes (23 specimens). Abundant chemotypes containing compounds **5**, **6**, or **9** were found throughout the region. Among these, the number of specimens containing **5** was as follows: Yonaguni (12), Ishigaki (1), Miyako (5), Kerama (10), Okinawa (7), Yoron (19), Okinoerabu (6), and Amami (15 specimens). Chemotypes of **2** or **7** were found from the Ishigaki and Yonaguni regions, and chemotypes of **3** or **10** from Kerama and other eastern islands.

Among the metabolites, the structure of a new compound, **7**, was determined by spectroscopic analysis together with X-ray diffraction (Fig. 3). A few extracts containing compound **6** as a major cembrane also contained a minor constituent, **12**, whose structure the spectral data

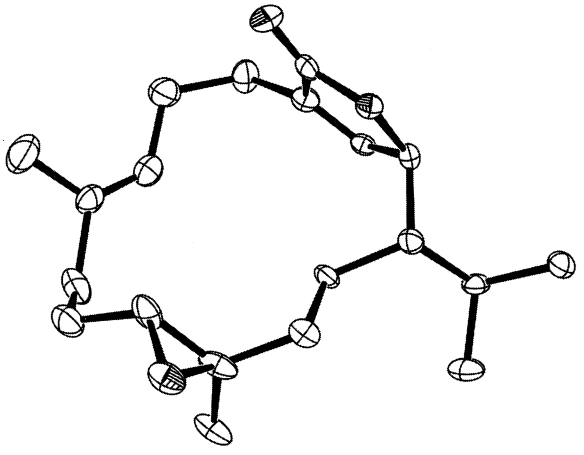

Fig. 3. Computer-generated ORTEP drawing of compound **7** (hydrogens are omitted for clarity).

revealed as a new cembrane.

S. trocheliophorum is widely distributed in the Ryukyu Archipelago (Table 1). The chemotypes of this species are divided into four groups: 2*R*,7*R*,8*R*-sarcophytoxide (**2**, 28 specimens), sarcophytol A (**1**, 3 specimens), and compounds **3** and **4** (1 specimen each). The chemotype of compound **2** was found throughout the islands, while that of compound **1** was found only in Yonaguni.

A total of 19 specimens of *S. ehrenbergi* were collected between Ishigaki and Amami. Their chemotypes are divided into four: **11** (7,8-epoxy-1,3,11-cembratriene, 8 specimens), compounds **1**, **2**, and **3** (2 specimens each), and five specimens without any definite cembrane. The major chemotype with compound **11** was found only in Okinoerabu and Amami.

Eighteen specimens of *S. elegans* were collected between Ishigaki and Amami. Of these, most (15 specimens) lacked a marker cembrane, while the remaining three contained compound **2**.

Colonies of *S. crassocaule* were found only in the Ishigaki and Kohama areas. All three specimens contained sarcophytol A (**1**) as a major metabolite.

A total of four specimens were collected for *S. digitatum*: the three collected at Ishigaki contained sarcophytol A (**1**), while the fourth did not contain a marker cembrane.

The remaining three species: *S. infundibuliforme*, *S. pulchellum*, *S. cherbonnieri*, as well as *Sarcophyton* spp. were collected between Miyako and Amami. Both *S. infundibuliforme* and *S. pulchellum* consisted of compound **2** chemotype or no marker cembrane, while *S. cherbonnieri* and *Sarcophyton* spp. consisted of chemotypes of compound **2**, or **3**, or no characteristic cembrane.

A new compound 7

Compound **7** was found only in two specimens of *S. glaucum/cinereum*, collected at Yonaguni Island. After chromatographic separation of one extract (173 mg) of the two specimens on silica gel followed by reversed phase HPLC, we obtained compound **7** (6.7 % from the extract).

Compound **7** gave the following data: mp 102-105°C (hexane-acetone); $[\alpha]_{D^{25}}$ +97.8° (*c* 0.415, $CHCl_3$); FTIR (neat) 2946, 1747, 1083 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.90 (1H, m, H-14a), 1.01 (1H, m, H-14b), 1.02 (1H, m, H-13a), 1.02 (3H, d, *J* = 7 Hz, H-16), 1.06 (3H, d, *J* = 7 Hz, H-17), 1.21 (3H, s, H-20), 1.28 (1H, m, H-10a), 1.60 (3H, brd, *J* = 1 Hz, H-19), 1.66 (1H, m, H-1), 1.69 (1H, m, H-15), 1.85 (1H, dd, *J* = 8, 13 Hz, H-13b), 2.04 (1H, m, H-9a), 2.05 (1H, m, H-10b), 2.17 (1H, m, H-6a), 2.26 (1H, brd, *J* = 10 Hz, H-9b), 2.43 (1H, ddd, *J* = 4, 8, 13 Hz, H-5a), 2.48 (1H, dd, *J* = 3, 9 Hz, H-11), 2.56 (1H, m, H-5b), 2.73 (1H, m, H-6b), 5.07 (1H, brs, H-2), 5.10 (1H, brt, *J* = 6 Hz, H-7), 7.00 (1H, q, *J* = 1 Hz, H-3); ^{13}C NMR ($CDCl_3$) δ 14.7 q (C-19), 16.4 q (C-20), 20.0 q (C-16), 21.2 q (C-17), 23.9 t (C-14), 24.3 t (C-10), 24.8 t (C-5), 25.6 t (C-6), 30.2 d (C-15), 36.6 t (C-9), 40.0 t (C-13), 46.4 d (C-1), 61.0 s (C-12), 61.4 d (C-11), 82.5 d (C-2), 124.6 d (C-7), 135.1 s (C-8), 135.8 s (C-4), 147.3 d (C-3), 173.1 s (C-18); EIMS *m/z* 318 (M^+, 50), 153 (52 rel %); HREIMS *m/z* 318.2211 (calcd for $C_{20}H_{30}O_3$ 318.2193).

Xray diffraction of compound 7

Suitable colorless crystals of compound **7** were obtained by recrystallization (hexane-acetone). The crystal (0.10 x 0.10 x 0.32 mm) belongs to the monoclinic system, space group $P2_1$, with *a* = 5.6992(3) Å, *b* = 13.949(1) Å, *c* = 11.3974(5) Å, β = 91.8004(8)°, V = 897.69(8) $Å^3$, *Z* = 2, *D* calcd = 1.186 g/cm^3, F(000) = 352, λ (MoKα) = 0.71069 Å. Intensity data were measured on a Rigaku RAXIS-RAPID diffractometer up to 2θ of 55°. A total of 2129 reflections were collected. The structure was solved by direct methods (SIR 92) and refined by a full matrix least squares procedure. The non-hydrogen atoms were given aniosotropic thermal parameters. The refinement converged to final R = 0.067, R_w = 0.034 for 1672 observed reflections [$I > 3.00\ \sigma(I)$] and 207 variable parameters. The final ORTEP figure is shown in Figure 3.

A new compound 12

A lipophilic extract (288 mg) of a specimen of *S. glaucum/cinereum*, collected at Amami Island, was separated on silica gel and following reversed phase HPLC gave two compounds: **6** (14.9 %) and **12** (2.7 % from the extract).

Compound **12** showed the following spectral data: $[\alpha]_{D^{24}}$ +130° (*c* 0.39, $CHCl_3$); FTIR (neat) 3421, 1668, 1064 cm^{-1}; UV (MeOH) λ_{max} 280 nm (loge 3.7); ^1H NMR ($CDCl_3$) δ 1.39 (3H, s, H-19), 1.56 (1H, m, H-6a), 1.84 (3H, s, H-18), 1.89 (1H, ddd, *J* = 5, 7, 13.5 Hz, H-13a), 1.97 (3H, s, H-17), 2.06 (1H, ddd, *J* = 4.5, 13.0, 15.5 Hz, H-9a), 2.18 (1H, m, H-5a), 2.23 (1H, m, H-9b), 2.29 (1H, m, H-6b), 2.40 (1H, m, H-10a), 2.46 (1H, m, H-5b), 2.54 (2H, m, H-14ab), 2.56 (1H, m, H-10b), 3.20 (1H, m, H-13b), 4.27 (1H, d, *J* = 10 Hz, H-7), 4.98 (1H, s, H-16a), 5.07 (1H, s, H-16b), 5.73 (1H, d, *J* = 11.5 Hz, H-3), 6.08 (1H, brs, H-11), 6.49 (1H, d, *J* = 11.5 Hz, H-2); ^{13}C NMR ($CDCl_3$) δ 19.4 q (C-18), 21.3 q (C-17), 22.0 q (C-19), 24.9 t (C-14), 26.5 t (C-6), 27.4 t (C-10), 31.8 t (C-5), 34.5 t (C-9), 37.5 t (C-13), 66.4 d (C-7), 83.4 s (C-8), 112.2 t (C-16), 121.7 d (C-3), 122.8 d (C-2), 133.6 s (C-12), 136.4 s (C-4), 137.9 s (C-1), 140.3 d (C-11), 143.1 s (C-15), 167.0 s (C-20); EIMS *m/z* 316 (M^+, 25), 133 (100 rel %); HREIMS *m/z* 316.2044 (calcd for $C_{20}H_{28}O_3$ 316.2037).

DISCUSSION

Structures of Compounds 7 and 12

The molecular formula of compound **7** was determined to be $C_{20}H_{30}O_3$ by mass measurements. NMR data indicated the presence of a lactone (1747 cm^{-1}, δ 173.1 s), an epoxide (δ 61.0 s, 61.4 d), two trisubstituted double bonds (δ 124.6 d, 135.1 s, 135.8 s, 147.3 d), and four methyls (δ 1.02 d, 1.06 d, 1.21 s, 1.60 brd). After the planar structure was elucidated by 2D NMR analysis (COSY, HMBC), the final structure with relative stereochemistry was determined by X-ray crystallographic work (Figs. 2, 3).

Compound **12** was isolated as a minor constituent together with **6** from a specimen of *S. glaucum/cinereum*. In addition to its similar NMR data for the portion of C-4 to C-12, findings such as its molecular formula $C_{20}H_{28}O_3$, UV absorption maximum at 280 nm, and two terminal methylene protons support the presence of an extended triene system in compound **12**. Final structure was elucidated as 7-hydroxy-1,3,11,15-cembratetraene-20,8-carbolactone by interpreting 2D NMR spectra (COSY, HMQC, and HMBC), which allowed us to assign all the ^1H and ^{13}C NMR signals. The stereochemistry of **12** was elucidated as depicted due to similar NMR signals (δ 83.4 s, 66.4 d; δ 4.27 d) to those of **6** (δ 83.5 s, 66.2 d; δ 4.30 d) for chiral centers at C-7 and C-8 and the same sign of optical rotation value as that of **6**.

Chemical diversity of soft corals of the genus *Sarcophyton*

Of the 279 *Sarcophyton* specimens collected, belonging to nine species, more than half (67%) belonged to the *S. glaucum/cinereum* species complex. Chemical analysis revealed the relation between species and cembrane diterpenes (Table 2), showing that each *Sarcophyton* species may have a distinct pattern of chemotypes. A large diversity of chemical contents was found for *S. glaucum/cinereum*, compared to a moderate diversity for *S. trocheliphorum* and *S. ehrenbergi* and only a small diversity for the remaining species.

Emblide (**5**), compound **6**, and 2*R*,7*S*,8*S*-sarcophytoxide (**9**) accounted for the major chemotypes (68%) in the *S. glaucum/cinereum* complex, while compounds **1-3**, **7**, **8**, and **10** constituted minor chemotypes. Examination of these molecules revealed that they are also diverse in the oxidation process after formation of a cembrane skeleton. It will be interesting in the future to determine how this intraspecific chemical diversity occurs biosynthetically and genetically in this species complex, together with its relation to morphological taxonomy. Cembranes from *S. glaucum* have been noted in more than a dozen papers, among which the Kobayashi group reported sarcophytol A (**1**) and related molecules as minor constituents from Ishigaki specimens (Kobayashi et al. 1979, Kobayashi et al. 1989B, Kobayashi & Osabe 1989A, Nakagawa 1981), suggesting diverse metabolites in minor quantities.

Compound **2** was found in most *S. trocheliophorum*, while four specimens from Yonaguni contained either **1** or **2**. In our previous study on the metabolites of large colonies (>30 cm diameter) of *S. trocheliophorum* from Yonaguni in the early 1990s, all the specimens were rich in sarcophytol A (**1**). Therefore, it is possible that the metabolite pattern may have changed with colony size in Yonaguni in addition to the geographic variation between Yonaguni and other Okinawan islands.

Compound **11** (7,8-epoxy-1,3,11-cembratriene)

was detected only in specimens of *S. ehrenbergi* from Okinoerabu and Amami, while compounds **1**-**3** were found in specimens from Ishigaki and Miyako. This may indicate a geographic boundary for metabolites in this species. A future examination of the chemical diversity of this species from Kerama to Yoron may provide further evidence regarding this boundary.

All the specimens of *S. crassocaule* from the Ishigaki area contained sarcophytol A (**1**). Interestingly, in our separate study of Indonesian specimens collected from Sulawesi, Sumatra, and Flores Islands, all the samples of *S. crassocaule* contained the same compound **1** (Tanaka, unpublished results). The cembranoid content of this species thus seems to be quite uniform. However, our result contradicts the finding of the metabolites from an Australian (Bowden et al. 1980) and a Taiwanese specimen (Duh et al. 2000), in which the former contained 2*R*,7*R*,8*R*-sarcophytoxide (**2**) and four other cembranes, while the latter contained sarcocrassolide (**13**) and three cembranes as cytotoxic principles.

Absence of marker cembrane diterpenes was a characteristic of most specimens (83%) of *S. elegans*. One specimen of *S. elegans*, collected near Kagoshima further north of the current study area, gave emblide-like metabolites and cembranes with oxygenation at C-10 (Uchio et al. 1983A; Uchio et al. 1983B). Since the collected numbers of specimens were quite small for the remaining species: *S. digitatum*, *S. infundibuliforme*, *S. pulchellum*, and *S. cherbonnieri*, it is not easy to generalize for these species. Sarcophytol A (**1**) was found for most specimens of *S. digitatum*, while 2*R*,7*R*,8*R*-sarcophytoxide (**2**) was found in the remaining three species.

Similar to the report by Coll (1992) that cembrane diterpenes were the only terpene constituents in *Sarcophyton*, we too detected cembranes **1**-**12** from nine species. Extracts of *Sarcophyton* soft corals have been shown to have consistently high toxicity when compared with those from other soft corals of *Anthelia* and *Cladiella*. Our results may therefore be in accord with the expected toxicity due to the presence of toxic sarcophytoxides (**2**, **9**) (Sammarco and Coll 1990), although we still need to test the toxicity of the remaining compounds in order to prove this hypothesis.

Concerning the possible ecological roles of the cembranes, there have been many studies and theories, such as defense against predators, space competition with soft and stony corals, antifouling effects, etc (Sammarco and Coll 1990; Aceret et al. 1995). Each molecule should also be examined for its biological activity such as feeding deterrence and toxicity against larvae of fouling organisms.

Koh et al. (2000) reported the distribution of cembranes in seven specimens of *Sarcophyton* soft corals consisting of three *S. glaucum*, two *S. infundibuliforme*, and one each of *S. trocheliophorum* and *S. crassocaule* collected from the Ishigaki area. Their results are somewhat different from ours: i.e., sarcophytol A (**1**) in one of *S. infundibuliforme*, absence of compound **1** in *S. crassocaule*, sarcophytonin-A (**14**) in one each of *S. glaucum* and *S. infundibuliforme*. These few examples suggest that additional chemotypes exist in the soft corals and remained to be determined.

To the best of our knowledge (for reviews on *Sarcophyton* metabolites: Anjaneyulu and Rao 1997), the present study appears to be the first comprehensive report on the intraspecific variation of cembranes from *Sarcophyton* soft corals. In light of the seasonal variation on the amount of a cembrane in *S. glaucum* (Kashman et al. 1974) and the occurrence of the same molecule in different species or even different families (Blackman et al. 1982), the possibility of chemical taxonomy has been discussed (Coll 1992). However, our findings of compound **2** in most of the studied species and of the high diversity existing in *S. glaucum/cinereum* indicate chemical taxonomy as an unreliable method. Possible reasons for this diversity include seasonal changes, reproductive stage, genetic differences, hybridization between species, induction of certain biosynthetic pathways by environmental factors including surrounding organisms, genetic differences of symbiotic algae, etc. Of these factors, symbiotic algae may be eliminated as in *Lobophytum compactum* (Michalek-Wagner et al. 2001). We hope to extend our study in the future to include the diverse soft coral genera *Sinularia* and *Lobophytum* and thus, to reveal the diversity at a genetic level.

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Sarcophyton 属ソフトコーラルの含有成分の多様性

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琉球列島で収集した *Sarcophyton* 属のソフトコーラル9種279個体に含まれるセンブレン型ジテルペンの多様性を調査した。形態的な種の同定の後に、各個体の主成分を HPLC, NMR 等を使用して検討した。形態的に区別困難な *S. glaucum* および *S. cinereum* は最も出現頻度が高く、含有成分においても最も多様であった。*S. trocheliophorum* と *S. ehrenbergi* は出現個体数および成分の多様性の点から中程度で、残りの種は出現頻度が低く含有成分も限られているようであった。今回の調査の過程で見出した新規化合物(**7**, **12**)についてその構造を報告する。