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Gas-Liquid Chromatograms of Sesquiterpenes as Finger Prints for Soft-Coral Identification

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Abstract

A possible additional means for aiding in the identification of soft corals based on their sesquiterpene composition, as determined by gas-liquid chromatography (GLC), is discussed. The use of this method for several species of *Sinularia* and *Sarcophyton* is illustrated. Several sesquiterpenes were identified, some of them for the first time from marine origin. Preliminary tests indicate that the sesquiterpene composition in the tested soft corals remained quite constant during different seasons of the year. It is suggested that such "finger prints" are produced by the corals themselves and not by the zooxanthellae, and that they are species-specific.

Introduction

Octocorals consist of 5 orders: Stolonifera, Gorgonacea, Alcyonacea, Teletacea and Pennatulacea. The present work is concerned only with soft corals, the Alcyonacea. Ecological studies dealing with Alcyonacea are scarce, and usually constitute a minor part in general descriptions of coral-reef surveys. Benayahu and Loya (1977) studied the community structure of soft corals in the northern Gulf of Eilat (Red Sea) and provided quantitative analysis of space partitioning among soft corals, stony corals and algae in these reefs.

Intensive taxonomic study that has been carried out during the last decade shows that a very diverse and rich fauna of soft corals (approximately 150 species) inhabits the coral reefs of the Red Sea, around the Sinai Peninsula (Verseveldt, 1969, 1970, 1974; Verseveldt and Cohen, 1971).

The soft corals are very common, especially in shallow waters (0 to 3 m). Among the most abundant genera are *Sinularia* and *Sarcophyton*, which tend to form large monospecific "carpets" of several square meters. Thus, *Sinularia leptoclados*, *S. polydactyla* and *Sarcophyton decaryi* often constitute 80 to 90% of the total living coverage of the alcyonarian community (Benayahu and Loya, 1977).

A recent study describes 5 new species and 14 recorded for the first time from the Red Sea (Verseveldt and Benayahu, 1978). Out of this list, 2 new species, *Sarcophyton pauciplicatum* and *S. gemmatum* and 3 species recorded for the first time in this area (*Sinularia minima*, *S. notanda*, and *Sarcophyton decaryi*) were examined for the presence of sesquiterpenes in the present study. They contained varying amounts of the latter compounds (0 to 3% dry weight of the soft coral).

Classical taxonomy of soft corals involves careful examination of anatomical features (such as form, size and structure of spicules) and morphological features (such as growth forms). Quite often the identification of a given specimen is extremely difficult, and opinions of different specialists may vary. Any additional criteria that can aid in the systematic description of these soft corals is, therefore, of great advantage. The present paper provides for the first time a possible additional feature for the identification of soft corals based on their sesquiterpene gas-liquid chromatograms which serve as "finger-prints". Indeed, the origin of the sesquiterpenes is intriguing (Ciereszko and Karns, 1972), as most of the soft corals live in symbiotic relationship with unicellular algae, the zooxanthella (Muscatine,

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1974). However, this need not essentially diminish the value of the suggested analysis.

Materials and Methods

All soft corals (8 species of *Sinularia* and 7 species of *Sarcophyton*) were collected in the Gulf of Eilat (Red Sea) at a depth of 5 to 20 m, and were immediately frozen (dry ice). Freeze-drying of the soft corals (ca. 100 to 1000 g wet samples) took 24 to 48 h, after which the corals were left for an additional 24 h in the freeze-dryer to ensure exhaustive evaporation of the hydrocarbon fraction. The aqueous emulsion collected during the whole drying process was extracted 3 times with $CHCl_3$ or hexane (50 ml to 500 ml of water-emulsion). The combined organic phase yielded, after drying (Na_2SO_4) and careful evaporation, the crude oil fraction (0 to 3% dry weight). GLC was performed using a Hewlett Packard 5803-A chromatograph equipped with a flame-ionization detector (FID); 6 ft (2 m), 1/8" (3 mm) inner diameter, coiled-glass column containing 5% SE-30 (a silicone gum rubber) on gas-Chrom Q; temperature, 160°; carrier gas, N_2. The pure compounds were obtained by a combination of $AgNO_3/SiO_2$ and preparative GLC columns, and were identified according to their boiling point (bp), optical rotation [$(\alpha)_D$], infrared (IR), ultraviolet (UV), nuclear magnetic resonance (NMR) and mass spectra, which were compared with the data of the known compounds or their enantiomers.

IR spectra were recorded on a Perkin-Elmer Infracord Model 257, and UV spectra on a Perkin-Elmer 137 UV. NMR spectra were recorded on Jeol JNM-C-60HL and Bruker WH-90 spectrometers using 5 to 10% solution of $CDCl_3$ with tetramethyl silane (TMS) as an internal standard. Mass spectra were recorded with a DuPont 21-491B instrument. Optical rotations were taken on a Bellingham and Stanley polarimeter in $CHCl_3$ solutions. Packard 871 and Hewlett Packard 5803-A instruments were used for gas-liquid chromatography (FID) with glass columns.

Results

Gas-liquid chromatograms of the studied alcyonarians revealed that most of the tested species [excluding 2 *Sinularia* species (*S. macrodactyla* and an unidentified sp.) and 1 unidentified *Sarcophyton* species which lacked volatile material under the described experimental conditions] possess a unique hydrocarbon combination as seen from their GL chromatograms (Figs. 1 and 2). The variation in the hydrocarbon contents are given in parentheses for the *Sinularia* species in Fig. 1 (0.01 to 3.0%) (even a few milligrams suffice to obtain the GL chromatograms). The hydrocarbon fractions isolated as described in "Materials and Methods" constituted over 90% of the total volatile fraction (only minute amounts of additional material, <50 mg per 100 g sample, were distinguishable in a petrol-ether extract of the dried soft corals). Several of the main sesquiterpenes have been isolated by chromatography and were fully characterized; among them are 6 new natural compounds: isozonarene (an isomer of zonarene: Fenical *et al.*, 1972), $\Delta^{9(15)}$-africanene, african-7-ene, seco-africanene (Kashman *et al.*, in preparation), (+)-alloaromadendrene (Dolejs *et al.*, 1961) and ledene (Cheer *et al.*, 1976) — which is reported for the first time from a natural source (Fig. 3). Other fully characterized sesquiterpenes are: germacrene-C (Morikawa and Hirose, 1969) together with its Cope-rearrangement product δ-elemene (Ganter and Keller-Wojtkiewicz, 1971); (+)-α-muurolene (Weinheimer *et al.*, 1968; Kashman *et al.*, 1978), (+)-β-cubebene (Vlahov *et al.*, 1967); (+)-alloaromadendrene (Dolejs *et al.*, 1961) and (+)-α-gurjunene (Palmade *et al.*, 1963) (Fig. 3). Germacrene-C, (+)-β-cubebene and (+)-α-gurjunene are reported (Fig. 3), to the best of our knowledge, for the first time from a marine origin; and the last two of these are enantiomers of those isolated from plants. (Germacrene-C was also isolated by us from two other soft corals: *Lithophyton arboreum* and *Steronephthya cundabiluensis*; this compound is unstable and turns blue very rapidly after purification). Another similar pair of germacrene (A) and elemene (β) were isolated by Weinheimer *et al.* (1968, 1970) from the gorgonian *Eunicea mammosa*.

Preliminary tests have shown that the sesquiterpene composition in several of the tested soft corals remains quite constant during the year, although the total amount of hydrocarbons changes. Most of this work was conducted on *Sarcophyton glaucum* in which we found the most significant changes of the diterpenoid components (Bernstein *et al.*, 1974; Kashman *et al.*, 1974).

In addition to hydrocarbons, several more polar compounds determined by GLC and TLC (thin-layer chromatography) were also isolated. Some of these compounds have already been identified by us, e.g. (+)-palustrol (Cheer *et al.*, 1976) and 7-acetoxy-α-muurolene (Kashman *et al.*, 1978), both isolated from *Sinularia erecta*;

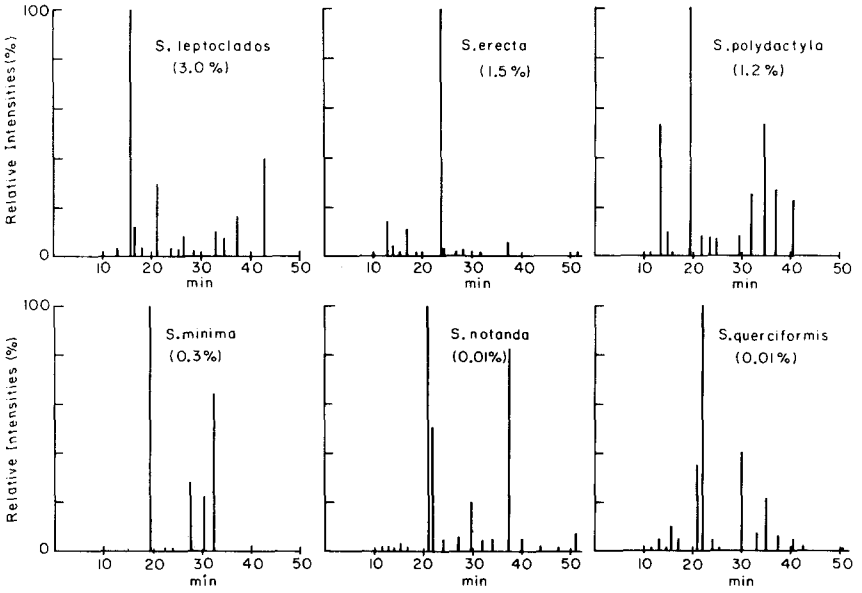

Fig. 1. *Sinularia* spp. Gas-liquid chromatograms of sesquiterpenes in 6 species. Total percentage of the sesquiterpenes in each soft coral (% of dry animal weight) is shown in parentheses

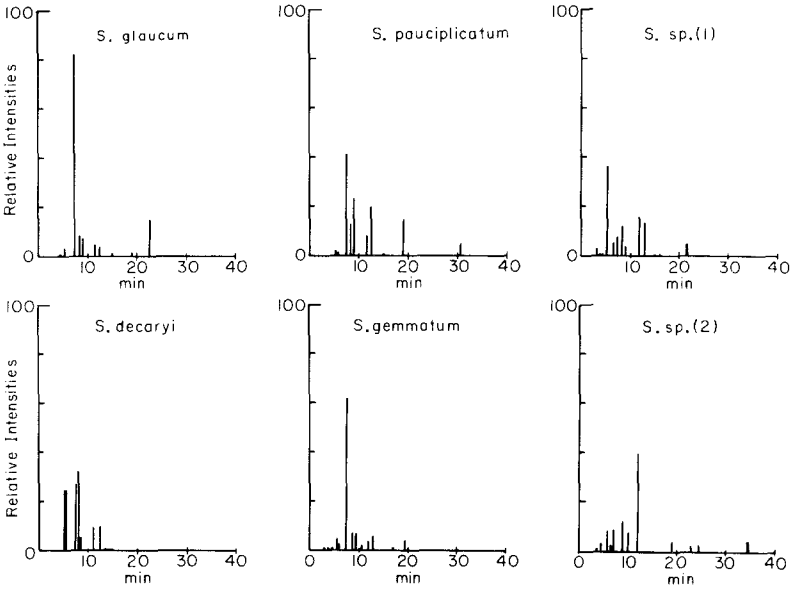

Fig. 2. *Sarcophyton* spp. Gas-liquid chromatograms of sesquiterpenes in 6 species. S. sp (*1*) and S. sp (*2*) are two unidentified *Sarcophyton* species

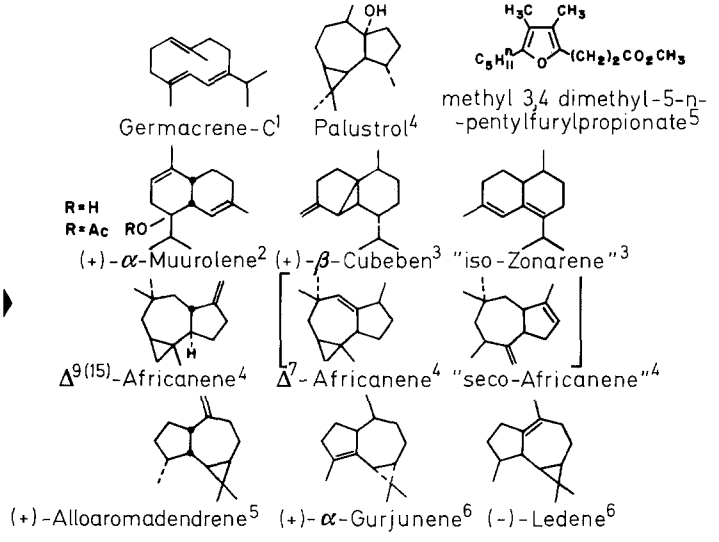

Fig. 3. Sesquiterpenes isolated from: *1*, *Sinularia polydactyla* (isolated also from *Lithophyton arboreum* and *Stereonephthya cundabiluensis*); *2*, 7-α-acetoxy-α-muurolene isolated from *Sinularia erecta* (together with the corresponding alcohol and the parent hydrocarbon, also isolated from *Heteroxenia fuscescens*); *3*, *Sinularia minima*; *4*, *Sinularia erecta*; *5*, *Sarcophyton glaucum* and *S. gemmatum*; *6*, *Sarcophyton decaryi*

and a new furanoid fatty acid (similar to acids recently isolated from fish liver by Glass *et al.*, 1975, and references therein) isolated from *Sarcophyton glaucum* and *S. gemmatum* (Groweiss and Kashman, 1978) (see Fig. 3 of present paper). For practical reasons, we did not include the more polar compounds in the identification chromatogram.

Discussion

Clearly not all soft corals contain sesquiterpenes. The occurrence or lack of sesquiterpenes may serve also as a typical characteristic in the systematic identity of soft corals. (Other natural products might also serve as markers for species identification.)

A question still remains: to what extent does the chemical composition of soft corals vary in different seasons of the year or, during different physiological conditions (e.g. during the reproductive period). Preliminary tests have shown that the reproducibility of the individual sesquiterpene composition in the tested soft corals during different seasons of the year remained quite constant, although the total amount of hydrocarbons varied.

Such finger prints may serve as a useful and rapid complementary tool for identification of soft corals, especially in cases where doubt exists due to variability in growth forms; although, of course, not as a substitute for classical taxonomy. Environmental conditions dictate to a large degree growth forms of soft corals. Closely related species are very hard to identify, even for the specialist using all classical taxonomic methods. For example, in the Red Sea, our present state of knowledge indicates that the genus *Sinularia* consists of 25 species and the genus *Sarcophyton* consists of 12 species (Benayahu, in preparation). The GLC-finger prints of these species may largely contribute to solving difficulties of identification of closely related species (see Figs. 1 and 2). Furthermore, this method may distinguish new species, not previously described, with greater ease than classical taxonomy.

A difficulty arises in the interpretation of the GL-chromatograms, due to the existence of zooxanthellae in the endodermal cells of the soft corals. It may be argued that the observed finger prints are the product of the soft corals themselves, the zooxanthellae, or a combination of the specific soft coral and its associated zooxanthellae population (Tursch, 1976). However, the reproducibility of typical finger prints, within colonies of the same species, even in different seasons of the year, supports the assertion that these finger prints are produced by the corals themselves and that they are species-specific. Our present knowledge of the identity of zooxanthellae in soft corals is very poor. Under the light microscope, zooxanthellae from different cnidarians bear strong superficial resemblance to each other, and it is now evident that electron microscopy and observation of motile forms in culture are required for specific identification (Muscatine, 1974). Nevertheless, from reported studies on zooxanthellae associated with soft corals in the Red Sea (Svoboda, 1978), it is most likely that we are dealing with one species only, namely *Gymnodinium microadriaticum* (Freudenthal).

The possibility that several species of zooxanthellae exist and that they are the ones who produce the observed sesquiterpene finger prints is less likely, although not impossible. In such an event, it would be necessary to have a different zooxanthellae population within each coral species as suggested from the specific finger prints shown in Figs. 1 and 2. Our present knowledge on the taxonomy and nature of association between zooxanthellae and marine cnidarians makes the latter possibility very unlikely (see Muscatine, 1974). In conclusion, the variability observed in the GL-chromatograms of different soft coral species supports the view that the origin of the sesquiterpenes is from the soft corals themselves, and that it is species-specific.

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