ABSTRACT
The different biomineralization processes of the two "living fossils" Astrosclera willeyana and "Acanthochaetaetes" wellsii were studied by epifluorescence microscopy, SEM, TEM, and biochemical analyses. The basal skeleton of A. willeyana is made of aragonitic spherulites. These spherulites are built in the dermal layer in large vesicle cells (LVC) in the early stages. After a releasing event the spherulites fuse together by epitaxial growth. In "A." wellsii the basal skeleton is made of high Mg-calcite. It is constructed of bundles of elongated, tangentially orientated crystals. The biomineralization takes place in 3 different places in the skeleton, the active zones are localized in the uppermost parts of the skeleton. In both cases the mineralization is associated with highly acidic mucus substances, rich in aspartic and glutamic acid, which control the biomineralization processes. The understanding of the biomineralization processes in these two cases gives insight into the formation of the skeleton of ancient reef constructors like the stromatoporoids and the chaetetids.

INTRODUCTION
Calcified sponges were dominant reef building organisms at the beginning of the Phanerozoic. The archaeocyaths are thought to be the oldest representatives. Later the very widely developed and diversified stromatoporoids and chaetetids occurred. Since the late Paleozoic they developed a large variety of skeletal morphologies and microstructures. Their role was again very important at different periods of the Triassic, Jurassic, and the Cretaceous. Replaced in their reef-building function by scleractinian corals in modern reefs, the living relatives of these calcified sponges ("coralline sponges") are living in cryptic habitats of almost all Recent coral reefs. Back in the Cambrian sponges were the first metazoans producing a carbonate skeleton. Their microstructural features have remained nearly unchanged over the very long period of time. The biomineralization processes are extremely conservative and still present in extant calcified sponges.

Both studied coralline sponges are ultraconservative taxa with very slow phylogenetic alterations. The oldest representatives of the agelasid taxon Astrosclerida are known from middle to late Permian reefs of Tunisia and Sicily. The diversity of this type of coralline sponge with intracellurally formed spherulites was very high in the late Triassic. Fossil remains of Astro sclera are rare during younger earth periods. The extant genus Astroasclera is the only relic taxon which occurred first in Cenomanian cryptic reef environments of the Tethyan realm (Reitner 1989). The taxon "Acanthochaetaetes" occurred first in the Lower Creta ceous. The oldest types are known from the Lower Aptian of northern Spain and the Mural Limestone in Arizona (USA). This type of sponge has close phylogenetic affinities to the soft sponge Spirastrella (Vacelet 1985; Reitner 1992) and is restricted to dark cryptic niches of tropical reefs of the Tethyan realm. (Reitner 1989; Reitner and Engeser 1987).

Within this group of organisms very little is known about the modalities of skeletal formation. The basal carbonate skeleton is produced independently from the spicular skeleton and combines a mineral phase of calcite and aragonite, and a complex organic matrix. The latter reveals two major compounds when extracted during decalcification using EDTA or acetic acid (pH 4): the so called "soluble organic matrix" (SOM) is mobilized with calcium salts while the "insoluble organic matrix" (IOM) can be easily isolated by centrifugation. The organic matrix plays an important role for the nucleation of initial seed crystals as well as for the further growth of the skeleton (Simkiss 1986; Mann et al. 1989).

Biominerlization events have been investigated in two different taxa of coralline sponges from the Great Barrier Reef (Australia). The secondary calcareous skeleton of the agelasid stalkmorph Astroasclera is made of aragonite spherulites (Lister 1900; Ayliffe 1982; Reitner 1992), the hadromerid chaetetid "Acanthochaetaetes" produces a high Mg-calcite skeleton beside the spicular skeleton (Hartman and Goreau 1975; Reitner and Engeser 1987). The present study is an attempt to understand the processes of biomineralization in two different coralline sponge taxa and to locate the different sites of mineralization during skeletal formation.

MATERIAL AND METHODS
The investigated specimens of Astrosclera and "Acanthochaetaetes" were collected by SCUBA diving in shallow water reef caves around Lizard Island in the northern part of the Great Barrier Reef (Australia) (Fig. 1).

Fig. 1: Location of investigated reef caves at Lizard Island (northern Great Barrier Reef, Australia)
For histological, SEM, and TEM studies some specimens were fixed in a sodium cacodylate-buffered 4% glutaraldehyde solution in seawater immediately after collecting. They were stored at 8-4°C in the refrigerator for 1-2 days according to their size, then washed in running sea water. Postfixation was carried out in 2% osmium tetroxide solution in filtered sea water for 1-3 hours depending on the amount of organic matter. After fixation, the specimens were washed in 30% ethanol, moved in ascending ethanol concentrations and stored at 70%. For TEM, the specimens were first decalcified with EDTA and then desiccated in 5% HF. Samples for SEM were not demineralized, but dehydrated with 100% ethanol, and treated with PELEDI II (Plano), then shock frozen with liquid nitrogen and immediately broken.

Some specimens were selected for in vivo investigations using staining techniques. These were stained with Ca<sup>2+</sup>-chelating fluorochromes (Calcinein, Na<sup>+</sup>-Calcinein, Achromycin-HCl, and Chlorotetracycline-HCl) which allow one to study growth rates and the loci of calcification fronts. The sponge was in situ covered with a plastic bag filled with fluorescent dye. Because of the toxicity of the tetracyclines (antibiotics) to the symbiotic bacteria of the sponge, we used highly concentrated solutions in seawater and stained the specimens between 15-30 minutes only. Using short staining times with very high concentrations was successful and not lethal for the sponge (Reitter 1993; Gautret et al. 1996).

For examination they were embedded in epoxy resins (araldite and LR White) and cut with a LIEIT2 hardpart microtome. Thin sections were studied with an epifluorescence microscope ZEISS Axiophot using the UV high-performance narrow-band pass filter 01 (BP 365/12 nm, LP 397 nm, and wide-band pass filters 05 blue violet (BP 395-440, LP 470), 09 blue (BP 450-490, LP 520), and 17 green (BP 485-515, LP 565).

Also non-fluorescent histological staining dyes with the basic red fuchsin, toluidine blue 0, and methylene blue were applied to fixed specimens for histological studies using optical microscopy (more details in Reitter 1993).

Freeze-dried specimens were used for biochemical analysis of the intraskeletal organic phase (amino acid and monosaccharide composition). For the biochemical analyses, the intraskeletal organic matrices were extracted by a soft decalcification with acetic acid (pH 4, automatically controlled by a titrimer) and separated from calcium salts by a desalting gel filter chromatography with a Sephadex 25 column after a centrifugation aiming to remove insoluble compounds such as collagen fibrils. Half of the purified soluble matrix material was used for the determination of amino acid contents, the other half for the monosaccharide composition.

The amino acid composition was determined after 6N HCl hydrolysis (24 hrs, 110°C, in free air sealed tubes) and phenylisothiocyanate (PITC) derivatisation, by reverse phase chromatography using a HPLC-BECKMAN system (Na<sup>+</sup>-phosphate buffer with a nonlinear gradient of ace tonitrile) and a 5μm C18 NUCLEOSIL column.

Glucidemic compounds were hydrolyzed with 2N TFA (4 hrs, 1 110°C). Neutral and amino monosaccharide chromatographic separation was performed with an HPAE (High performance anion exchange)-DIONEX system coupled to a pulsed amperometric detection (PAD) with a sodium hydroxide gradient (10 to 100 mM).

RESULTS

Astrosclera willeyana Lister 1900
The soft tissue organization of Astrosclera willeyana exhibits an easily recognizable architecture in a dermal zone (Fig. 2), a choanosomal part, and a prominent exhalant system (cf. Lister 1900, PI. XLVI, XLVII). The soft tissue itself occupies only a few millimeters of the youngest portion of the basal skeleton. The inner choanosomal layer is characterized by a more or less dense mesohyle with numerous bacteria (mostly Vibrio-type) and small choanocyte chambers (about 10-15 μm). The bacteria may represent 30-50% of the biomass within this zone. The prominent exhalant canal system ends in astorhizate patterns often located on superficial mamelons.

The biocalcification processes in Astrosclera can be divided into two main processes:

1. The dermal layer and related mesohyle is free of bacteria and enriched in motile cells. Most of them have an archaeocyte character.

![Fig. 2: Light microscope photograph showing the upper part of the basal skeleton (dermal area) of A. willeyana with newly formed spherulites (arrows). Scale Bar: 50 μm.](image)

The mesohyle is formed by a dense network of EDTA insoluble fibers where the mobile cells are moving. Within the studied specimens up to 60% of the mobile cells are spherulite forming (large vesicle cells - LVC). They increase in size as they grow. The smallest of the LVC have a size of 3-5 μm and the largest of about 20 μm. The external shape of the LVC is more or less round to egg-shaped. They possess a large nucleus with a nucleolus, abundant mitochondria, and a lot of small vacuoles with reserve granules and/or phagocytized bacteria. The granules are extremely electron dense (amphophilic) and therefore enriched in lipids. Early ontogenetic stages of the LVC are characterized by one large vacuole which includes a minimum of 50% of the total volume of the entire cell. In mature cells, shortly before the release of the spherulite from the cell, the volume of the vacuole is more than three times larger than that of the remaining cell (Fig. 3). The vacuole is primarily filled up with a three dimensional network of fibers and sheets, which are probably formed under control of the electron dense reserve bodies. Sheets and fibers form small containers (30-50 nm) in which the first seed crystals are formed. The entire vacuole is filled up with Ca<sup>2+</sup>-binding glycoproteic mucus which exhibits a strong tetracycline and/or calcine induced fluorescence.

The seed crystals are euhedral and in the early, 2-3 μm stage, randomly orientated. In the later stages of development (3-10 μm) the seed crystals become more orientated in the direction of the c-axis of an aragonite crystal. All observed aragonite fiber crystals of the spherulites are compounds of 30-50 nm sized seed crystals (Reitter 1992). The aragonite fiber crystals are now more or less radially orientated and form aster shaped spherulites with a large remaining space filled with acidic organic mucus. The aster spherulites hatch normally in the 15μm stage. At this stage...
stage the LVC is lysing, the membranes are broken and the aragonite asters are free in the mesohyle. In some sections an enrichment of small amoebocytes was observed which probably transport the asters to certain places.

The amino acid and monosaccharide composition were studied on an approximate 400-year-old specimen from Ribbon Reef No. 10 (Lizard Island Section, Great Barrier Reef, Australia) (detailed data in Wörheide et al. in press). Amino acid and monosaccharide composition of the insoluble intracrystalline matrix are very stable in all portions of the skeleton. No strong diagenetic effect on the insoluble organic matrix (IOM) is visible due to the stable composition. The IOM is dominated by proteins and is represented by the intravacuole fibers and sheets forming the containers for the seed crystals. Collagen was not detected in the IOM.

The soluble organic matrix (SOM) is characterized by acidic glycoproteins, high amounts of proline, which is needed for the synthesis of glutamic acid, and high amounts of aminosugars. The glucids are the dominant fraction of the SOM. The character of the SOM is very typical for Ca²⁺-binding mucus substances. A strong diagenetic effect is visible in the SOM, both in composition of amino acids and monosaccharides and in the quantity (cf. Wörheide et al. in press).

"Acanthochaetetes" wellsi Hartman and Goreau 1975

Only the youngest part of the calicles (0.5-1 mm thick) of the chaetetid-type basal skeleton is occupied by the living soft tissue. Soft tissue and basal skeleton exhibit a vertical anatomy divided in five major zones (Fig. 5).

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Fig. 3: Large vesicle cell (LVC) of A. willeyana with a large vacuole containing a newly formed spherulite. The vacuole is totally filled with the insoluble organic matrix (IOM) of the spherulite, now visible after decalcification. RG = reserve granule. TEM section, decalcified with EDTA. Scale Bar: 1 µm.

Fig. 4: SEM photograph of A. willeyana, showing an aster-shaped spherulite. The aragonite fibers of the spherulite interperse with fibers of other spherulites, already forming the basal skeleton (arrows). Scale Bar: 20 µm.

Fig. 5: Vertical section of "A." wellsi showing the 5 major zones of the living part of the skeleton: I. outer dermal area with layer of spiraster microscleres, II. internal dermal area with LCC's, III. choanosome, IV. enrichment of LCC's upon horizontal tabula, V. closed space between tabulae containing storage cells (Archeocytes). Scale Bar: 600 µm.
At the uppermost dermal area settles a thick crust layer of spiraster microscleres (Zone I) and tylostyle megascleres which are arranged in clear plumose bundles suggesting a close phylogenetic relationship to Spirastrella. Below the outer dermal area, the internal dermal area (Zone II) is formed by mesohyle tissue. It is enriched in mobile cells and devoid of choanocyte chambers. Large inhalant chambers (lacenae) and canals cross this zone, serving the choanosome with water filtered through the ostia. The mesohyle is characterized by large cells (ca. 10 μm) containing numerous inclusions (LCG: large cells with granules) (Reitner 1992). These cells are mobile. LCG cells are not typical spherulous cells as known from Vaceletia crypta. Their shape varies often and normally they exhibit a triangular and flat shape. Only in rare cases do they show a spherulous shape.

The biocalcification process can be divided into three main locations and processes:

1. LCG’s are enriched in the upper part of the tubes at the top of the walls. The LCG’s are responsible for the secretion of collagen fibrils (cf. Reitner and Gautret 1996: Pl. 50, Fig. 1) and they probably derive from lophocytes. Collagen fibrils form strong bundles which cross through the basal pinacocyte layer, and anchor into the rigid skeleton (Vacelet and Garrone 1985; Reitner and Gautret 1996) (Fig. 6).

Fig. 6: "A." wellsi: LCG’s secreting collagen anchor fibers (CAF), anchoring in the basal skeleton (arrows). SEM photograph. Scale Bar: 5 μm.

The space between the basopinacoderm and the calcareous wall is filled up with acidic mucus substances, probably derived from metabolic processes during the synthesis of the collagen fibrils. This mucus and the aquatic fluids are enriched in Ca"-ions, detected by a strong epifluorescence behavior in calcin and chloro-tetracycline stained specimens. The aquatic fluids exhibit an increased carbonate alkalinity. The source of the carbon is not related to the sponge metabolism but to the ambient seawater (Reitner 1992, 1993). The mucus is acting as the soluble organic matrix and is forming molecular monolayers in the form of "sheet structures (cf. Simkias 1986) on the above described collagen fibrils. The collagen fibrils are acting as the insoluble organic matrix. The first nucleation of irregular Mg-calcite seed crystals takes place on the "sheet. The mature high Mg-calcite crystals formed by this process have a size of 0,5-1μm and exhibit often an anhedral shape (Fig. 7).

Fig. 7: "A." wellsi: TEM photograph of anhedral high Mg-calcite seed crystals (arrows). Decalcified with EDTA. Visible are the remains of the organic matrix (10M). Scale Bar: 250 nm.

2. A second type of collagen fibrils is present. These fibrils are produced by lophocytes which are widely distributed in the intercellular mesohyle. "Acantho- chaetetes" bears only few small-sized bacteria, located between these fibrils. At the top of the walls the fibrils become organized into a weak frame-building matrix. Remains of this matrix are entrapped inside skeletal structures after calcification. The main area of calcification is located in the very narrow space between the basopinacoderm and the mineralized surface of basal skeleton. This space is filled up with acidic mucus substances. The basopinacocytes produce soft folded organic strings (Fig. 8) ("cooked spaghetti" sensu Reitner and Gautret 1996), which are templates for the acicular high Mg-calcite crystals. The mucus substances become organized on these templates in a molecular monolayer ("sheet, BOM, see above). Mineralization starts on the templates in the form of very small seed crystals (50-100 nm). They grow together epitaxially (c-axis orientated) and during this process the folded templates become stretched. Newly formed high Mg-calcite crystals exhibit therefore a strong "knobby" structure (cf. Reitner and Gautret 1996, Pl. 51, Fig. 7). Due to further epitaxial crystal growth the irregular biocrystals become flat as known from the mature basal skeleton. The mature high Mg-calcite crystals have an elongated, accicular shape, an average length of 2-5μm, and a diameter of 200-500 nm (Fig. 9).

Fig. 8: TEM photograph of "A." wellsi showing the soft folded organic strings (arrows), produced by basopinacocytes in the narrow space between the basopinacoderm and the basal skeleton. These organic strings are templates for seed-crystal nucleation. Scale Bar: 500 nm.
Fig. 9: SEM photograph of "A." wellsi showing the soft folded organic strings in a later stage of development. Compare Fig. 8. Scale Bar: 2 μm.

3. The third area where biominalization consists of the horizontal tabulae which are dividing the calicete tubes of the chaetetid skeleton (zone IV). These are formed by the basopinacoderm also, first as a thin organic phragma or sheet. Below the chanoonosomal zone, LCG cells become sometimes enriched and cause the mineralization of the organic sheet (Fig. 10). Continuous upward moving basopinacoderm is forming a space filled with Ca⁺-binding and mineralizing organic mucus as known from the upper portions. This mineralizing process happens only when LCG’s are present (Reitner 1992).

Fig. 10: Light microscope photograph of "A." wellsi showing an enrichment of LCG’s directly above a calcified tabula (CT). Scale Bar: 50 μm.

The mineralization of the skeleton in all above described situations is only happening when the LCG’s are present and physiologically active.

The closed spaces between tabulae contain accumulations of modified archaeocytes with numerous storage granules (thecocyte-like cells) and few spiraster microscleres (zone V). These cells play a role in regeneration processes (Vacelet 1985, 1990) enabling the sponge to grow again when it has been drastically damaged.

The soluble matrix extracted from the superficial part of the skeleton contains high amounts of glycine, proline and hydroxyproline-rich compounds (collagenic affinity). Amino sugars are enriched in this zone. The presence of highly concentrated materials with collagenic or glucidic affinities results in the fact that relative amounts of acidic amino acids (Asp and Glu) appear less represented here than in the immediately underlying older part. However, absolute quantities of these two amino acids should be at least 3 to 5 times higher in the uppermost part of the skeleton (Gautret et al. 1996). The transformation starting from the area immediately below the active mineralizing zone exhibits a regular tendency with decreasing acidic amino acids as the most obvious feature, whereas aromatic amino acids (Tyr and Phe), serine and amino sugars also decrease. The increasing constituents are basic and aliphatic amino acids (mainly glycine).

The insoluble matrices exhibit quite similar, collageneic amino acid compositions in all parts of the skeleton. Only the quantity of insoluble matrix changes in an important way, decreasing considerably from the surface to the base. This matrix completely differs from soluble compounds, with much less acidic amino acids, less serine and threonine and almost no amino sugars. It is strongly enriched in all aliphatics (Gly, Ala, Val, Leu), aromatics (Phe, Tyr), proline and hydroxyproline (for detailed data see Reitner and Gautret 1996).

DISCUSSION

Two different modi of formation of the rigid calcareous basal skeleton in two different taxa of coralline sponges were described. Leading to a comparable endproduct, a rigid calcareous skeleton, each taxon forms the basal skeleton in its highly specialized way using different modifications of calcium carbonate (Astrosclera = aragonite; "Acanthochaetetes" = High Mg-calcite). In both cases modified lophocytes play an important role in the initiation of the mineralization. In Astrosclera, the LVC’s form a template of 3-dimensional fibers inside a large vacuole. The vacuole is filled with highly acidic mucus rich in Asp and Glu. The fibers and the mucus act as the organic matrix for seed crystallization. In "Acanthochaetetes", the lophocytes secrete collagen fibrils which act somewhat as a framebuilding matrix for the soluble highly acidic organic matrix which initiates the nucleation of calcite seed crystals. At a later stage, modified basopinacocytes control the mineralization process. In Astrosclera, basopinacocytes cover the released and fused spherulites. The epitaxial growth of the spherulites is controlled by a highly acidic mucus between the crystal surface and the basopinacocytes. This mucus is EDTA soluble and exhibits a strong calcine induced epifluorescence. In "Acanthochaetetes", the epitaxial crystal growth is also controlled by acidic mucus substances, filling up the space between the basopinacocytes and the mature basal skeleton or the tabulae.

Most important in both cases is the presence of an organic matrix, forming a template for crystal nucleation (Simkiss 1986; Mann et al. 1989). The presence of a soluble organic mucus rich in Asp and Glu, is controlling the crystal growth in the earliest and latest stages of mineralization. The mineral preference, aragonite or Mg-calcite, is controlled by the structure of the organic macromolecules, forming molecular monolayers (β-sheets) on an EDTA insoluble template (ICM) and acting as attractors for divalent cations (Ca⁺). The distances of the free carboxyl-groups of the macro-molecules control the crystallographic base plane of the initial calcite or aragonite crystal (Mann et al. 1989; Reitner 1993).

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