

1 Introduction

1.1 Significance of the study

1.1.1 Prey-predator relationship of phyllidiids and sponges

Phyllidiids (Opisthobranchia: Nudibranchia) are among the most frequently encountered nudibranch species in the coral reefs of the Thousand Islands National Park, Indonesia (Shintosari, 2001; Yasman, 2001). They are small (0.5-8.6 cm), shell-less soft bodied, diurnal organisms, and colorful. Even though phyllidiid species seem to be very vulnerable to suffer predation, they have rarely been reported to be attacked by predators (Karuso, 1987; Brunckhorst, 1993).

Phyllidiid species are predators and specialized on sponges (Karuso, 1987; Brunckhorst, 1993) which are well known to possess bioactive metabolites (Faulkner, 2002). Apparently, phyllidiids do not only subsist on a sponge but they also seem to sequester and accumulate secondary metabolites from their diets (sponges). Due to their unconcealed shell-less soft body, it has been hypothesized that phyllidiid species use the secondary metabolites of dietary origin to protect themselves from any potential predators (Faulkner & Ghiselin, 1983; Karuso, 1987; Cimino & Ghiselin, 1999).

The biology of feeding by phyllidiids has been very well described by Brunckhorst in 1993. Phyllidiid species evert their pharyngeal bulb while feeding upon a sponge (Brunckhorst, 1993; personal observation). Furthermore, Brunckhorst found that the phyllidiid *Phyllidia* spp. everted their pharyngeal bulb onto the sponges rather than into the sponges as shown by *Phyllidiella* spp. Through Brunckhorst's long field feeding observation, he reported ten species of phyllidiids's sponge-preys of which seven sponge species belong to the order Halichondrida while the rest belong to another group of sponges: *Smenospongia* sp. (Fam. Thorectidae: Dictyoceratida), *Gellius* sp. (Fam. Haliconidae: Haplosclerida), *Aplysina* sp. (Aplysiniidae: Verongida). Regarding the mode of sequestration and accumulation of dietary secondary metabolites, it is interesting to note that isocyanide/isothiocyanate/-thiocyanate metabolites have been mainly isolated from the sponge of order Halichondrida (Garson & Simpson, 2004 and all literatures

therein; Mitome *et al.*, 2004); meanwhile the later three sponge species which were predated by *Phyllidiella zeylanica*, *Phyllidiopsis phiphiensis*, and *Reticulidia halgerda* do not contain any terpene cyanides [MarinLit, version September 2004]. Logically, phyllidiid species which fed on sponges of order Halichondrida will metabolize terpene cyanides while those three phyllidiid species which fed on sponge containing no terpene cyanides should not contain any terpene cyanides. Although up to date there is no study on the chemical properties of the phyllidiid *Phyllidiella zeylanica*, *Phyllidiopsis phiphiensis*, and *Reticulidia halgerda*, but interestingly Brunckhorst (1993) used the assumption of the presence of terpene cyanides in the Phyllidiidae family as one of qualitative taxonomic characteristic to split the Family Phyllidiidae with other two dorid nudibranchs (Family Chromodoridae and Dendrodoridae). This assumption is actually acceptable since phyllidiid species have been reported to consistently metabolize terpenes isocyanide (Burreson *et al.*, 1975a; Hagadone *et al.*, 1979; Cimino *et al.*, 1982; Gulavita *et al.*, 1986; Karuso, 1987; Fusetani *et al.*, 1990-1992; Kassuehlke *et al.*, 1991; Okino *et al.*, 1996a; Simpson *et al.*, 1997; Hirota *et al.*, 1998; Yasman *et al.*, 2003; Manzo *et al.*, 2004). It remains a question how phyllidiids feeding upon sponges containing no terpene cyanides metabolize cyanide-containing compounds. Here, it is obvious that a biological feeding observation supported without a good chemical study will not be enough to assess the prey-predator relationships of phyllidiid species and their sponges.

Instead of a direct evidence of predation in a field feeding observation, any sponges could be assumed as a potential prey if phyllidiid species are found on or near any of the sponges (Swennen, 1961; Bloom, 1976). Chemically, the dietary link suggested by this assumption was confirmed when both phyllidiid species and sponges have the same set of secondary metabolites (Cimino & Sodano, 1994; Garson, 2000; Garson 2004, personal communication). The group of Scheuer who isolated 9- and 2-isocyanopupukeanane from phyllidiid *Phyllidia varicosa* and its sponge-prey *Ciocalypta* sp. (as *Hymeniacidon* sp.) has proved this assumption and further more established the selective accumulation of a chemical defense agent in the phyllidiids diet (Burreson *et al.*, 1975a; Hagadone *et al.*, 1979). By using the same approach, additional predator-prey pairs of phyllidiids and sponges have been proposed by other research groups (see Cimino *et al.*, 1982; Fusetani *et al.*, 1992; Dumdei *et al.*, 1997; Simpson *et al.*, 1997; Wright, 2003).

From this chemical point of view, phyllidiid species have been reported to feed mainly on sponges belonging to the Order Halichondrida: *Ciocalypta* sp. (as *Hymeniacidon* sp.), *Axinyssa* spp., *Halichondria* spp., *Axinella* sp., *Acanthella cavernosa*, and *Phakellia carduus* (Burreson *et al.*, 1975b; Cimino *et al.*, 1982; Fusetani *et al.*, 1992; Dumdei *et al.*, 1997; Simpson *et al.*, 1997; Wright, 2003, respectively). Many sponges of order Halichondrida have been reported to contain isocyanide/isothiocyanate/-thiocyanate metabolites (Garson & Simpson, 2004 and all literatures therein; Mitome *et al.*, 2004). But it is interesting to note that the sponges of the order Halichondrida are not the only sponge group that has been reported to contain terpene cyanides. The Okinawan sponge *Theonella* cf. *swinhoei* from the order Lithistida: Fam. Theonellidae has also been reported to contain terpene cyanide (Nakamura *et al.*, 1984). Chemically, this sponge should be also a potential prey for phyllidiid species. But up to date there is no report of phyllidiid predation on the sponge *T. swinhoei*. The group of Fusetani has isolated 3-isocyanotheonellin from Japanese phyllidiid *P. pustulosa* which is biosynthetically related to 3-formamidotheonellin and 3-isothiocyanatotheonellin isolated from the Japanese sponge *Theonella* cf. *swinhoei* (Fusetani *et al.*, 1991; Okino *et al.*, 1996a, Nakamura *et al.*, 1984). The Japanese phyllidiid *P. pustulosa* seems to metabolize 3-isothiocyanatotheonellin into 3-isocyanotheonellin which was absent in the sponge *Theonella* cf. *swinhoei*. To date there is no direct evidence of predation in the field which should be an alternative for confirming the above assumption.

1.1.2 Role of cyanide terpenes in host organisms

It is believed that organic compounds are involved in mediating a diverse array of inter- and intraspecific interactions including predation, competition, mutualism, and reproductive processes, as well as interaction between marine organisms and their physical environment (Stachowicz, 2001). This phenomenon is another point of interest for marine natural product researchers who try to assess the natural function of the secondary metabolites that they study, the so called marine chemical ecology (de Vries and Beart, 1995; Faulkner, 2000).

The bulk of research on chemical-mediated interactions of marine organisms has focused on prey-predator interaction. In most cases, predator stress has become the center issue of effort for searching some biological active metabolites from

marine organisms. This assumption is supported by the fact that more than 85% of the total marine natural products were isolated from sessile organisms (e.g. sponges, coelenterates) (Blunt *et al.*, 2003). An ecological study of toxicity in marine sponges from different latitudes on the North American continent suggested that biosynthesis of secondary metabolites may be influenced by the presence of marine fishes as natural predators (Green, 1977). These phenomena are logically clearly understood as sessile organisms have no possibility to escape from any potential predators and for that reason. Hence it has been hypothesized that they must not just rely on their mechanic defense but also have to perfect their chemical defenses (Faulkner, 2002). Even there is a tendency that sponges seem to have shifted away from spicules as a primary mechanic defense strategy, secondary metabolites are their auxiliary defense mechanism (Faulkner and Ghiselin, 1983). Soft-bodied and slow moving marine invertebrates that seem to be devoid of morphological defense structures such as spines or protective shells are also prime candidates to possess bioactive metabolites (Faulkner, 2002). Most chemical studies of those slug organisms revealed that their secondary metabolites are of dietary origin (Schupp *et al.*, 1999a; Thoms *et al.*, 2003; Yasman *et al.*, 2003).

As phyllidiids are sponge specialist predators and have been reported to always have dietary metabolites containing isocyanide functionality, it has been hypothesized that isocyanide compounds play an important role as defensive agents in both marine organisms. Up to present, hundreds of terpene isocyanides have been isolated from marine sponges and phyllidiid species [see Alvi, 1995; Chang, 2000; Garson *et al.*, 2000; Garson & Simpson, 2004]. Despite the pronounced cytotoxicity and antibiotic activity of many isolated terpene cyanides and related metabolites, there are limited ecological meaningful experiments of those bioactive metabolites (Garson & Simpson, 2004). When tested toward coral reef fishes, the sesquiterpene fraction of extract of the sponge *Acanthella cavernosa* was an effective feeding deterrent (Garson *et al.*, 2000). The isocyanide mixture was also an effective feeding deterrent against freshwater goldfish at 10 µg/mL, in contrast it was not the case for the isothiocyanate mixture (Thompson *et al.*, 1982). The major sponge metabolites axisonitrile-1 and 5-isothiocyanatopupukeanane were also ineffective as a feeding deterrent even in high concentrations (Cimino *et al.*, 1982; Marcus *et al.*, 1989). More recently, Rojer and Paul (1991) have just commented that isocyanide metabolites seem to not show any antifeedant activity. Even though isocyanide compounds seem

to show no antifeedant activity, all the isolated isocyanide compounds were toxic to fish and brine shrimps (Thompson *et al.*, 1982; Cimino *et al.*, 1982; Braekman *et al.*, 1987; Fusetani *et al.*, 1990; Fusetani *et al.*, 1991; Yasman *et al.*, 2003).

1.1.3 Biological activity of isolated cyanide metabolites

The aims of several research groups whose main interest is in marine chemical ecology might in parallel support their study based on the pharmacological activity of the secondary metabolites. Ecological phenomenon can be used for the detection of pharmacological active natural products (Paul, 1988; Proksch *et al.*, 2003). Although this approach may over estimate and not be feasible for the pharmaceutical industry, at least the result of ecological observations during collection and experimentation can be used as a first indication for the presence of biological active compounds and as one possibility to conduct a more systematic biological screening of marine organisms (Schupp *et al.*, 1999a,b; Schupp, 2000).

Many isolated isocyanide compounds from phyllidiid species and sponges have been reported to show interesting biological activity. The group of Fusetani reported that sesquiterpenes containing isocyano, isothiocyano, and thiocyano functionalities inhibited settlement and metamorphosis of cyprid larvae of *Balanus amphitrite* (Okino *et al.*, 1996a; Fusetani *et al.*, 1996; Fusetani, 1997; Hirota *et al.*, 1998). Another group has evaluated the sesquiterpene 3-isocyanotheonellin and sesquiterpene formamide which showed high antifouling activity at concentration at which it has no significant toxicity (Kitano *et al.*, 2002; Nagota *et al.*, 2003). Among the isolated diterpene isocyanides, kalihinol A and kalihinene also showed antifouling activity (Okino *et al.*, 1995; Fusetani *et al.*, 1996; Okino *et al.*, 1996b; Fusetani, 1997).

Isolated isocyanide metabolites have been reported to possess antimicrobial, antimalarial, and antihelmintic activity as well as cytotoxicity. Fusetani *et al.*, (1992) reported three sesquiterpene cyanides which showed strong antifungal activity. Low molecular weight volatile methyl isocyanide and methyl isothiocyano were also reported to be responsible for the antimicrobial activity of the sponge extract of *Ircinia felix* (Duque *et al.*, 2001). The group of König found that marine isocyanide compounds were good candidates for antimalarial compounds (Wright *et al.*, 1996; König & Wright, 1997; König *et al.*, 2000). *In vitro* antimalarial activity was

significantly demonstrated by a sesquiterpene isocyanide (Angerhofer *et al.*, 1992; Koenig *et al.*, 1992; Simpson *et al.*, 1997) and 14 diterpene isocyanides isolated from tropical marine sponge *Cymbastela hooperi* (Koenig *et al.*, 1996); whereas some polyfunctional diterpene isonitriles have reported to be biologically active in an antihelmintic assay (Alvi *et al.*, 1991). Cytotoxicity of marine isocyanide metabolites has also been reported by Fusetani *et al.* (1989).

1.2 Statement of the objective

It is apparent that both biological and chemical studies of phyllidiids and their sponge-preys are considerably needed in order to assess their prey-predator relationships. Chemical studies on both phyllidiids and probable sponge-preys without any predation evidence in the field and *vice versa* may give an ambiguous explanation of prey-predators relationships of phyllidiid species and their sponges. Through field feeding observations during sample collection, together with a predation-guided sponge collection methodology, it is possible to get some new bioactive isocyanide compounds. Feeding experiments of isolated compounds are still needed to get probable ecological roles of isolated compounds containing the isocyanide functionality. It may be needed also to test the isolated compounds for alternative ecological roles, e.g. as antifouling organisms, as suggested by Garson & Simpson (2004). Some other biological test, e.g. brine shrimps toxicity assay and antimicrobial assay, are also needed in order to get a basic information of their biological activity.

The coral reefs of the Thousand Islands, Indonesia are a prime candidate location to conduct the project. It has already been known that there are at least four genera, consisting of fifteen phyllidiid species, existing in that water area (Shintosari, 2001; Yasman, 2001). This study is chiefly aimed at conducting field feeding observations of some phyllidiid species and elucidating compounds containing isocyanide functionality present in phyllidiids and/or their sponge-preys.